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ASPECTS OF SYNTHESIS AND ORDER IN GROWTH

THE THIRTEENTH SYMPOSIUM OF
THE SOCIETY FOR THE STUDY OF
DEVELOPMENT AND GROWTH

Executive Committee, 1954

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Aspects of Synthesis and Order in Growth

R. Y. STANIER · SEYMOUR S. COHEN · RALPH EMERSON

DIETRICH BODENSTEIN · CLIFFORD GROBSTEIN

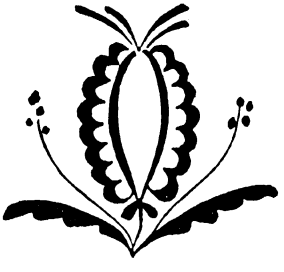
ELIZABETH S. RUSSELL · LINUS PAULING

JOSEPH S. FRUTON · JAMES D. EBERT

S. B. HENDRICKS AND H. A. BORTHWICK

NELSON T. SPRATT, JR.

EDITED BY DOROTHEA RUDNICK



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FOREWORD

De cent membres et visages qu'a chasque chose, j'en prends un, tantost à leicher seulement, tantost à efflorer, et parfois à pincer jusqu'à l'os. . . .

MONTAIGNE

THE Thirteenth Symposium of the Society for the Study of Development and Growth was held at Dartmouth College, Hanover, New Hampshire, on June 23–26, 1954. This volume consists of the written versions of the papers delivered there.

Even in the enchanted New Hampshire countryside in June it is not possible to do justice to all the aspects of the growth process that have concerned biologists during the year. The organizing committee chooses speakers for the individual interest of their work rather than its bearing on a preconceived design. The resulting document is not always predictable; the plan that emerges, as in all organic events, lies implicit in the actual material. The editor has only to allow the chapters to arrange themselves.

Analysis of growth should either begin or end with molecular events; in the present instance the two more strictly chemical chapters offer a balanced beginning. Linus Pauling's essay focuses sharply on the central critical incident of replication of specific molecules, while J. S. Fruton marshals experimental information on the steps and circumstances required to build proteins *in vitro* or *in vivo* from small units.

The five following papers exemplify different biological devices by which synthetic processes are promoted or, conversely, restrained or negated in living organisms. The convincing evidence that in some microbes, material presented to the cell from the outside can in a sense control a whole series of syntheses by sequential inductions, has not been without effect on the lively search for the mechanics of protein differentiation in multicellular organisms. The chapters by R. Y. Stanier and J. D. Ebert explore such problems. The genetic apparatus, presiding from the nucleus over growth and differentiation, can be studied, as in E. S. Russell's paper, by close analysis of developmental effects of mutated individual genes. S. S. Cohen shows how nucleoprotein from outside the cell, as virus, can also operate to control growth in microorganisms. Quite a different sort of control can be exercised from outside by light, operating on photoresponsive processes; S. B. Hendricks and H. A. Borthwick report such machinery in a wide variety of plants and animals.

The last four papers are concerned with patterns of growth and differentiation in multicellular organisms. Development on the cell-tissue-organism level continues to be a challenge to scientific ingenuity; the role of the sort of mechanism discussed in the preceding group of reports has scarcely begun to be assessed. The developmental studies progress in some cases by extension in comparative and genetic directions, as Ralph Emerson's review demonstrates; in others by approaching the transition to adult physiology (Dietrich Bodenstein). In still other cases, stimulating perspectives are opened by intensification and precision of the study of the cellular basis of circumscribed developmental events, as both N. T. Spratt and Clifford Grobstein found in their material.

It is not possible to hold or to publish a symposium of this nature without incurring more debts than can be enumerated. The scientific community at large, both within and without the Society, has been generous with advice, criticism and encouragement of the sort that traditionally remains anonymous but not unappreciated. The Society is obliged to the National Science Foundation and to the National Cancer Institute, of the National Institutes of Health, Department of Health, Education and Welfare, for financial support of the Symposium, and to Dartmouth College and its Local Committee for gracious hospitality. Finally, we thank Mr. Herbert S. Bailey, Jr., of the Princeton Press, in particular for his judicious mixture of patience and energy which has resulted in appreciable negative growth of the time interval between the Symposium and its publication.

DOROTHEA RUDNICK

Yale University

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ASPECTS OF SYNTHESIS AND ORDER IN GROWTH

I. THE DUPLICATION OF MOLECULES

BY LINUS PAULING

DURING recent years a great deal of information has been gathered about the structure of molecules and the nature of intermolecular forces. This information is so extensive, precise, and reliable that it has become possible to use it in reaching a definite conclusion about the process of duplication of molecules, which is involved in the mechanism of heredity.

After many years of thinking about this problem, I am now confident that the process of duplication of molecules is a two-step process, each step involving the action of a molecule as a template for the formation of a complementary molecule, and that the forces that operate significantly in the production of the complementary molecule are short-range forces, effective over a distance of only a few Ångströms. These forces are in the main not those involved in the formation of covalent bonds between atoms, but those that are usually classed as intermolecular forces. They include the forces between positively and negatively charged atoms and groups, the forces of hydrogen-bond formation, and van der Waals attractive and repulsive forces.

A great amount of information bearing on the problem of the manufacture of molecules with well-defined specific properties has been obtained through the study of antibodies, in particular the work initiated by Landsteiner (1945) on the preparation of antibodies to artificial antigens (usually azoproteins) containing haptenic groups of known structure, and the study of the interaction of the antibodies with precipitating azoproteins containing the same or similar haptenic groups and of the inhibition of precipitation by simple substances. A great advantage of serological systems over other systems showing biological specificity, such as enzyme systems, is the large range of variation of molecular structures for which serological properties may be measured. Thus in general an enzyme has measurable catalytic power for only a few substrates, and its action is interfered with by only a few inhibitors. It is conceivable that a series of enzymes could be found with primary specificity directed toward a series of closely similar substrate molecules, permitting comparative studies of the enzymes of the series to be made; but in fact no such series is known. On the other hand, specific antibodies may be prepared for dozens of closely related haptenic groups, as was done by Landsteiner and his collaborators, and the properties of these antibodies

in relation not only to the haptenic groups but also to many related simple substances may be investigated quantitatively. For example, azoproteins for use as antigens may be prepared with the *p*-azobenzene arsonic acid group, the *m*-azobenzene arsonic acid group, the *o*-azobenzene arsonic acid group, the *p*-azobenzoic acid group, the 4-azophthalic acid group, and many other similar groups as haptenic groups. Through the quantitative study of the cross reactions of these specific antisera with precipitating proteins involving these haptenic groups and of the formation of soluble complexes with small molecules, such as the various substituted benzene arsonic acids themselves, a great deal of information about the combining powers of haptens and haptenic groups with antibody in relation to the structure of these haptens or haptenic groups and also of the haptenic group of the injected azoprotein can be obtained.

The results of all of the many quantitative experiments of this sort that have been made are in agreement with the postulate that the bonds between the antibody molecule and the haptenic group are due to short-range intermolecular forces, operating through the minimum distance—the distance of closest approach of the atoms on the surface of the combining regions of the antibody molecules and the atoms of the haptenic groups (Pauling et al., 1940, 1942). All of the properties of serological systems can be satisfactorily accounted for in terms of short-range forces. The specificity of interaction of antigens and antibodies is due to a complementariness of surface structure that allows close contact to occur over a rather large area (of the order of 100 Å^2), permitting the weak forces of intermolecular interaction to cooperate to produce a strong bond. The complementariness between an antibody molecule and an antigen molecule is the result of a folding of the polypeptide chain of the antibody molecule in such a way that as large a portion as possible of the chain is brought into immediate juxtaposition with a portion of the surface of the antigen molecule. In addition to similarity of surface configuration, the complementariness of structure involves the juxtaposition of special combining groups, such as a negatively charged group in the antibody with a positively charged group in the antigen, or a hydrogen-bond-forming group carrying the proton with a similar group presenting an electron pair.

There is no satisfactory alternative explanation of the specificity of antibodies. There exists no significant experimental evidence in support of the concept of long-range forces with specificity approaching that of antibodies, enzymes, or other biological molecules. The apparently purposeful approach of corresponding chromosomes during fertilization may be caused by the contraction of a few long protein molecules with specific

groups at their ends; these molecules might carry out random motion in the cell, until the specific groups chance to collide with a complementary structure on a chromosome, and to become attached; then the contraction of the long molecules, by a mechanism not yet understood (perhaps similar to muscular contraction), might cause the approach of the chromosomes to one another.

The explanation of biological specificity through complementariness in structure and the resultant cooperation of weak intermolecular forces over a sufficiently large area to produce a strong bond was proposed a quarter of a century ago by Breinl and Haurowitz (1930), Stuart Mudd (1932), and Jerome Alexander (1931). Quantitative studies of the combining powers of haptens with antibodies have provided detailed support for this idea.

First, we may discuss the degree of complementariness of the haptenic group or hapten and the atoms in the combining region of the antibody. The energy of van der Waals electronic attraction of two atoms is proportional to the inverse sixth power of the distance between the centers of the atoms. In order for it to be significant, the atoms must be at nearly their minimum distance of approach. If the antibody has a well-defined structure that is complementary to a haptenic group, a larger haptenic group or a haptenic group with a significantly different shape would be expected not to combine with it, because of steric hindrance—its inability to fit itself into the combining region of the antibody. This expectation is borne out by experiment. It is found, for example, that the replacement of a hydrogen atom on the benzene ring of benzene arsonic acid by an atom or group, such as chlorine or methyl, that is about 0.8 Å larger in radius operates to decrease significantly the combining power of the hapten with the antibody homologous to the azobenzene arsonic acid group. If the larger group, on the other hand, is in the position occupied by the azo group in the injected azoprotein, the larger group increases the combining power of the substituted benzene arsonic acid, because of the increased van der Waals force of attraction over that of the hydrogen atom. This experimental result indicates that the fit of the antibody around the haptenic group is a tight one, the complementariness in structure being such that an increase in size of 0.8 Å of one atom in the group is enough to cause significant steric hindrance.

The striking fact that the replacement of a methyl group by a chlorine atom or bromine atom causes essentially no change in serological properties is also explained by the complementariness theory. The methyl group has about the same size and shape as a chlorine atom or bromine

atom (the van der Waals radius of chlorine is 1.81 Å and that of bromine 1.95 Å; the methyl group is somewhat scalloped, its average radius being about 1.9 Å).

By the measurement of the combining powers of benzene arsonic acids with substituent groups of various sizes in the ortho, meta, and para positions with antibodies homologous to *p*-azobenzene arsonic acid, *m*-azobenzene arsonic acid, and *o*-azobenzene arsonic acid it has been possible to evaluate the closeness of fit of the antibodies to the haptenic group of the injected azoprotein (Pauling and Pressman, 1945). It has been found that for each of the anti-azobenzene arsonic acid antibodies a rapid decrease in combining constant occurs when the hapten has such size and shape as to require a radial dilatation of about 1.0 Å relative to the shape of the haptenic group of the injected azoprotein. The fit of anti-*p*-azobenzoic acid antiserum is closer; it corresponds to an approximation to the homologous haptenic group to within about 0.7 Å in radius. On the other hand, a somewhat larger radial dilatation, about 1.5 Å, has been found for the anti-*p*-(*p*-azobenzene-azo)benzene arsonic acid antiserum.

When a substituent group consisting of one atom or a few atoms is present in the hapten in the position occupied by the azo group in the injected azoprotein, the combining constant with antibody is in general somewhat larger than that for benzene arsonic acid itself. The increase in combining constant has been explained as resulting from an increase in van der Waals attraction of the protein and the substituent group. The London theory of van der Waals attraction leads to the approximate equation

$$W = -\frac{38{,}000}{r_{AB}^{6}} \times R_A R_B \text{ cal/mole}$$

for the energy of interaction of two groups A and B the distance r_{AB} apart. Here R_A and R_B are the mole refractions of the groups in cm^3, and r_{AB} is in Ångströms. In the derivation of this expression the average energy differences of normal and excited states of the groups have been taken as 14 electron volts. By consideration of the amount of protein in contact with a small group, similar to the azo group, and of the mole refraction of water and of protein, the effective energy of interaction of a group A, compared with a hydrogen atom, has been calculated to be (cf. Pauling and Pressman, *op. cit.*)

$$\triangle W_A = -\frac{400{,}000}{r_{AB}^{6}} \times (R_A - R_H) \text{ cal/mole}$$

Here r_{AB} is in Ångströms, and the mole refractions are in cm^3. The values of combining groups of substituted benzene arsonic acids and benzoic acids with I, Br, Cl, OCH_3, CH_3, and F as calculated with this equation with use of the known mole refractions and radii of the atoms and groups are in good general agreement with the observed values of free energy of interaction of the substituted haptens. There seems to be little reason to doubt that the differences in combining powers of these haptens with the antibody are to be attributed to differences in the energy of van der Waals attraction, as determined by the polarizabilities (mole refractions) of the groups, and that there is essential contact of the combining region of the antibody with the hapten, extending as far as the position occupied by the azo group in the injected azoprotein.

Similar rough quantitative explanation of observed combining powers has been given for intermolecular interactions involving also hydrogen bonds and ionic forces.

The complementariness theory immediately suggests a process of formation of antibodies which is compatible with present knowledge of molecular structure and intermolecular forces (cf. Pauling et al., 1940, 1942). It is assumed that the globulin molecule that is a precursor of antibodies consists of a polypeptide chain (or perhaps of more than one such chain) containing several hundred amino-acid residues of such nature and in such order that many configurations accessible to the molecule have nearly the same energy. In the absence of an antigen molecule the newly synthesized polypeptide chain might fold up in any one of a number of ways, representing the maximum stability under the conditions then existent in the cell. If, however, a foreign molecule (antigen molecule) were present the polypeptide chain would be expected to fold up into a configuration complementary in structure to the antigen molecule. Such a configuration would give to the system greater stability (lower energy) than a non-complementary configuration. The greater stability would be the result of the interaction, over a large area (the area of contact), of the atoms in the folded chain and the atoms of the antigen molecule—interaction corresponding to ionic forces, hydrogen bonds, van der Waals attraction, and other weak intermolecular forces. While it is not expected that true equilibrium would be achieved, and that the number of molecules with complementary structures could be accurately predicted, on the basis of their lower energy than non-complementary molecules, by application of the Boltzmann factor (the equations of statistical mechanics), nevertheless it is probable that the rate of assumption of more stable configurations, the complementary configurations, would be greater than that of

the less stable configurations, and that the complementary structures would accordingly be assumed by the folding polypeptide chains.

One interesting and unexpected prediction of the theory has been borne out by experiment. It was predicted that the antisera produced by strong haptenic groups would be less highly specific than those produced by the weaker haptenic groups. The argument underlying this prediction is that the circulating antibodies, which constitute the antisera, have to be removed from the antigen molecule (that serves as a template) by the disrupting influence of thermal agitation. Inasmuch as this disrupting influence, at body temperature, is constant, the bond strength between circulating antibody and antigen might be expected to be essentially constant for different antigen-antibody systems, this being the bond strength that just permits dissociation at body temperature. However, a strong hapten will hold a closely fitting antibody with such a strong bond that dissociation will not occur; only the antibodies that fit rather poorly will dissociate into the blood stream. We can accordingly understand why the *p*-azobenzene arsonic acid haptenic group, which has the same charge as the benzoic acid group but an additional OH group, which confers additional forces of van der Waals attraction and hydrogen bond formation, produces antibodies that fit less tightly than do those produced by the benzoic acid group, and that the *p*-(*p*-azobenzene-azo) benzene arsonic acid group, with an additional azobenzene, produces antibodies that fit still less tightly, as mentioned above.

This mechanism of formation of antibodies by molding a plastic material, the polypeptide chain, to the antigen molecule, which serves as a template, is such a reasonable one for the formation of molecules with specific structure and properties that we might well expect it to be used in other biological processes. I believe that this is the only reasonable molecular mechanism that is now known that can be advanced for the synthesis of biological molecules in general.

Fourteen years ago Professor Max Delbrück and I wrote (1940) "It is our opinion that the processes of synthesis and folding of highly complex molecules in the living cell involve, in addition to covalent-bond formation, only the intermolecular interactions of van der Waals attraction and repulsion, electrostatic interactions, hydrogen-bond formation, etc., which are now rather well understood. These interactions are such as to give stability to a system of two molecules with *complementary* structures in juxtaposition, rather than of two molecules with necessarily identical structures; we accordingly feel that complementariness should

be given primary consideration in the discussion of the specific attraction between molecules and the enzymatic synthesis of molecules.

"A general argument regarding complementariness may be given. Attractive forces between molecules vary inversely with a power of the distance, and maximum stability of a complex is achieved by bringing the molecules as close together as possible, in such a way that positively charged groups are brought near to negatively charged groups, electric dipoles are brought into suitable mutual orientations, etc. The minimum distances of approach of atoms are determined by their repulsive potentials, which may be expressed in terms of van der Waals radii; in order to achieve the maximum stability, the two molecules must have complementary surfaces, like die and coin, and also a complementary distribution of active groups.

"The case might occur in which the two complementary structures happened to be identical; however, in this case also the stability of the complex of two molecules would be due to their complementariness rather than their identity. When speculating about possible mechanisms of autocatalysis it would therefore seem to be most rational from the point of view of the structural chemist to analyze the conditions under which complementariness and identity might coincide.

"From the biological side it would seem most rational to postulate the possibility of both processes: viz., formation of complementary non-identical structures and formation of complementary identical structures, and to proceed by analyzing experimental data for clear-cut evidence as to their occurrence."

In discussing this suggestion we may ask first what significance there is in attempting to differentiate between the formation of complementary non-identical structures and the formation of complementary identical structures. I now think that, to the extent that this differentiation has significance, the second alternative can be eliminated. Let us give the symbols A and A^{-1} to two mutually complementary molecules, such that A can serve as the template for the manufacture of A^{-1} and A^{-1} can serve as the template for the manufacture of A. Now let us bind A and A^{-1} together, either by a covalent bond or in some other way. If the bond between A and A^{-1} did not interfere with their serving as templates for one another, the molecule $A\text{-}A^{-1}$ would be self-complementary, and could serve as the template for the manufacture of an identical molecule. From this example we see that molecular duplication in one step might be essentially equivalent to molecular duplication in two steps.

We may imagine self-complementary molecules that can not be reasonably described as consisting of a complex of two mutually complementary halves, A and A^{-1}, bonded together. For example, let us consider a protein with the configuration of a pleated sheet. It is a flat molecule, about 10 Å thick. There exists the possibility that the large surface on one side would be complementary to the large surface on the other side. The nature of the pleated sheet, however, is such that we cannot reasonably describe one side as a structure A and the other as a structure A^{-1}; they are instead two aspects of the same polypeptide chain. It is highly unlikely that the amino-acid side chains that project to one side of the sheet would be related to those that project to the other side in such a way as to effect this complementariness of the two sides. The analogy with a thin sheet of metal that has been pressed against a die is fallacious: the complementariness of the two sides of the sheet of metal results from a constancy in thickness of the sheet, but a pleated-sheet protein is not constant in thickness—its thickness varies from 3.5 Å in a region occupied by glycine residues to 12 Å or more in a region occupied by the largest amino-acid residues. I think that we may conclude that self-complementary structures of this nature occur not at all or only very rarely in organisms.

The foregoing discussion is well illustrated by the structure proposed by Watson and Crick (1953) for deoxyribose nucleic acid. In this structure the molecule consists of two complementary polynucleotide chains, which we may call A and A^{-1}. They have their polar axes oppositely directed. Each has the configuration of a helix, and the two helixes are intertwined so as to bring nitrogen bases into positions favorable to the formation of hydrogen bonds. The hydrogen bonds can be formed only between specific pairs of nitrogen bases, and in consequence the two chains are mutually complementary. The helical molecule formed by the two chains may be described as a self-complementary molecule, AA^{-1}. Watson and Crick have described it as a complex of two mutually complementary molecules A and A^{-1}. The process of its duplication consists in the separation of the two polynucleotide chains, and the service by each as a template for the manufacture of a duplicate of the other.

Let us now ask whether it might not be possible that the two chains A and A^{-1} are really a single chain, A and A^{-1} being attached to one another at one end. I think that this can be rejected. If A and A^{-1} are separate chains, the process of duplication of AA^{-1} consists in the untwining, over the length l, of the double helix AA^{-1}, and the service of A and A^{-1} as templates for the manufacture of new double helixes AA^{-1} and $A^{-1}A$, with total length for the newly synthesized chains $2l$. If, on the other

hand, A and A^{-1} are attached together at one end, the process of duplication of AA^{-1} is more complex. First, the double helix AA^{-1} is untwined, to form the extended chain $A\text{-}A^{-1}$, with length $2l$. This then serves to produce the compound helix $\begin{matrix} A^{-1}A \\ AA^{-1} \end{matrix}$, with total length $2l$. The double helix must then be untwined, and each of the halves AA^{-1} and $A^{-1}A$ must coil to form the original complex and its duplicate. There are thus two processes of untwining that are involved, rather than a single one. Now, if the second process of untwining does not occur, we might be satisfied to accept the double chain of length $2l$ as a complex of a molecule AA^{-1} and its self-complementary duplicate, $A^{-1}A$. This process cannot, however, be accepted, because there is no mechanism for insuring the retention of complementariness of one end of the chain of length $2l$ and the other end. Mutation might occur, changing the nature of one end or of the other end, and if the chain, in the process of duplication, did not fold at some stage into the configuration of the double helix with length l, the lack of complementariness of the two ends of the chain would not interfere at all with the process of duplication. Instead, the molecule produced by use of the chain with length $2l$ as template would then be complementary to the original molecule, and would presumably itself serve as a template for the manufacture of a duplicate of the original molecule. This situation is then the one described by Watson and Crick, in which the double helix consists of two independent polynucleotide chains, A and A^{-1}.

We may also ask about another significant difference between the manufacture of antibodies and the duplication of genes. The antiserum produced in response to injection of a given antigen is highly heterogeneous—we have found that the heterogeneity may correspond to change by a factor of 10,000 in the equilibrium constant for combination of antibody and homologous antigen (Pauling, Pressman, and Grossberg, 1944), whereas the duplication of genes seems to be perfect, or nearly perfect. I think that this difference need not require a difference in mechanism, for the following reason. The manufacture of an antibody molecule is a process that occurs only once, so far as that molecule is concerned, and the molecule is not itself then used as a template. If the conditions within the cell in which the antibody molecule is being manufactured happen to be such as to favor the formation of a good molecule, with high complementariness to the antigen molecule, the combining constant will have a large value, whereas if the conditions are unfavorable the combining constant for the antibody molecule that is formed will have a small value. There is no process of trial and error, such that the system can

improve in the manufacture of antibodies against the injected antigen. The process of duplication of genes is different. Let us consider that a gene has undergone mutation—for example, that it has been damaged as a result of the absorption of a quantum of radiation. The damaged molecule, which we may call A_1, will serve as the template for the manufacture of a complementary molecule, A_1^{-1}. The latter will then serve as the template, in the next cell division, for the manufacture of a molecule complementary to it, which we may call A_2. In general we would not expect A_2 to be an exact duplicate of A_1. The process of two-stage duplication would then continue, through A_2^{-1}, A_3, A_3^{-1}, A_4, A_4^{-1}. . . . If after any number of duplications of this sort a molecule, A_n, happens to be formed that is especially well suited to serving as a template for the two-stage manufacture of a duplicate of itself, the process of duplication from then on would be a reliable one. The mechanism of heredity thus permits, in this way, the ultimate discovery of a mutated gene that permits true duplication; but the mutated gene may, and in general will, not be the original gene that has been damaged by x-radiation or produced in some other way, by a primary process, but will be a product obtained after many steps of two-stage duplication. This argument was presented briefly a few years ago (Pauling, 1949).

At the present time it is, I believe, possible to make effective use both of biological information and of information about molecular structure that has been obtained from the study of simple molecules in the discussion of the biological processes of duplication of molecules, and of biological specificity in general. The attack on biological problems with the effective use of information about molecular structure has only recently been begun. We may hope that it will be attended with success.

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II. THE BIOSYNTHESIS OF PROTEINS AND PEPTIDES

BY JOSEPH S. FRUTON[1]

One of the most challenging tasks that faces the biochemist is the elucidation of the chemical pathways in the biosynthesis of proteins from amino acids. The task is especially forbidding because of the complexity of the chemical structure of proteins, and because it is not possible as yet to study protein synthesis with cell-free preparations. So long as the *de novo* formation of proteins from amino acids is uniquely the property of the intact living cell, it will be extremely difficult, if not impossible, to unravel the mystery of the cellular apparatus responsible for protein synthesis. The best we can hope to do in the present discussion is to consider the problem in relation to some of the recent experimental findings, and to examine some of the working hypotheses that have been proposed to guide future research.

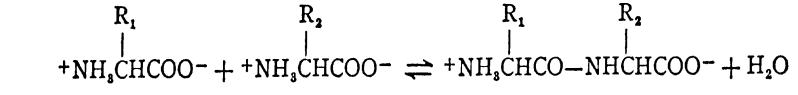
In a discussion of protein synthesis, we are forced, by the very nature of the experimental information, to consider the question from two points of view. First, we will discuss the knowledge gained from chemical studies on the energy changes in the synthesis of peptide chains from amino acids, and on the chemical specificity of known intracellular enzymes that act at peptide linkages. The purpose of studies on the energy changes in peptide synthesis is to gather information about the work that a cell may have to do in putting amino acids together to form the CO-NH bonds of a long peptide chain. The aim of the enzyme studies is to seek cellular catalysts that may be involved in the chemical pathways of protein synthesis, and whose chemical specificity is such as to make them possible agents in the remarkable process whereby a variety of amino acid residues are arranged in the complex sequence revealed by studies on the chemical degradation of proteins. In this discussion, we shall consider only the problem of the biosynthesis of the peptide chains of proteins, recognizing at the same time that the characteristic properties of proteins also depend on the integrity of bonds other than peptide linkages, and that the process of peptide synthesis may be closely linked to the formation of these accessory bonds.

The second line of experimental approach is the more biological one, involving studies on protein synthesis *in vivo,* and directed to a clearer definition of the overall process. Such research has attempted to define the

[1] Yale University.

energy and nutrient requirements of the intact cell for protein formation, and through the use of labeled precursors and of specific inhibition, to identify possible intermediates in protein synthesis. Clearly, the immediate goal of both the chemical and the biological lines of work is to bridge the wide gap that still separates the two.

Let us then consider first what we know of the energy changes in the synthesis of peptide bonds. The simplest reaction is the synthesis of a dipeptide from its component amino acids. At pH 7, the reaction may be formulated as follows:

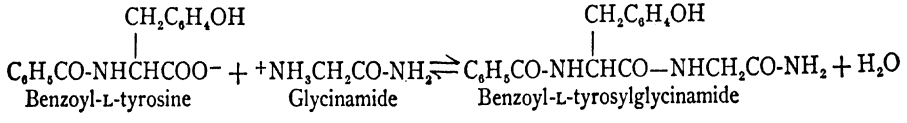


We know that the reverse of this reaction, the hydrolysis of the dipeptide, proceeds very far in the direction of the free amino acids, and we can make an estimate of the equilibrium constant of this reaction from the available data on the standard free energy of formation of each of the four components (Borsook et al., 1948, 1953). Thus, for the reaction:

$$\text{DL-leucine}^{\pm} + \text{glycine}^{\pm} \rightarrow \text{DL-leucylglycine}^{\pm} + H_2O$$

$\triangle F^{\circ}_{310}$ is about $+3.3$ kcal., corresponding to an equilibrium constant of about 0.005. Similar data are available for the synthesis of the dipeptides DL-alanylglycine and glycylglycine; here, the $\triangle F^{\circ}_{310}$ values are about $+4.1$ and $+3.6$ kcal. respectively, corresponding to equilibrium constants of about 0.001 and 0.002 (Zamecnik and Frantz, 1949). It follows, therefore, that in a homogeneous solution, work would have to be done on the system to effect any measurable synthesis of a charged dipeptide from the two dipolar amino acid ions. Thus far, it has not been possible to find an enzyme-catalyzed exergonic reaction that can be coupled to the synthesis of a dipeptide from the component amino acids.

If we proceed to the case of a peptide bond present in an uncharged molecule, the free energy change is less unfavorable for synthesis. For example, the equilibrium constant in the reaction:



is approximately 0.5, on the assumption that all the activity coefficients and the activity of water are unity. This equilibrium constant was determined in an experiment in which benzoyl-L-tyrosine was incubated at pH

7.9 and 25°C, in the presence of the proteinase chymotrypsin, with glycinamide that had been labeled with N^{15} in the glycine nitrogen. After a suitable time, the chymotrypsin was inactivated, non-isotopic benzoyl-L-tyrosylglycinamide was added as carrier, and the peptide derivative was isolated in analytically pure form and recrystallized to constant N^{15} concentration. The isotope data permitted us to calculate the concentration of isotopic benzoyl-L-tyrosylglycinamide present at equilibrium (Dobry et al., 1952). From the equilibrium constant, the value of $\triangle F°_{298}$ was calculated to be about +0.42 kcal. Calorimetric determinations of the value of $\triangle H_{298}$ for the hydrolysis of benzoyl-L-tyrosylglycinamide by chymotrypsin (the reverse of the reaction written above) showed this to be −1.55 kcal. per mole at pH 7.9. If the value of $\triangle F°_{298}$ for the hydrolytic process is taken to be −0.42 kcal., the standard entropy change ($\triangle S°_{298}$) in the hydrolysis is −3.8 cal. per mole per degree. I have cited these data because they illustrate how one may combine the isotopic method for the estimation of the equilibrium constant with the calorimetric method for the determination of the enthalpy change in hydrolysis, and thus characterize the thermodynamics of a reaction in which a peptide bond is formed in an enzyme-catalyzed condensation reaction.

The data on the energy relations in the synthesis of the central CO-NH bond of benzoyl-L-tyrosylglycinamide provide experimental evidence for the correctness of the views presented by Linderstrøm-Lang some years ago, and summarized by him recently (1952). From the available values for the free energy of formation and ionization of amino acids and peptides, he suggested that the magnitude of $\triangle F°$ in the synthesis of a peptide bond would decrease as the bond is moved farther from the charged terminal amino or carboxyl group of a peptide. Comparison of the free energy values for the synthesis of the dipeptides (*ca.* 3.5 kcal.) with that for benzoyl-L-tyrosylglycinamide (0.4 kcal.) supports this suggestion. For our present discussion, it is important to emphasize that the model experiment with benzoyl-L-tyrosylglycinamide resembles the situation that may be encountered if two long-chain peptides were joined by a peptide bond in an enzyme-catalyzed condensation reaction. In this sense, therefore, the lower free energy value appears to be a more probable estimate of the free energy change in the synthesis of central peptide bonds in the peptide chain of a protein molecule.

In a reaction that involves the condensation of two molecules, the extent of synthesis obviously depends greatly on the molar concentrations of the two reactants. For example, in the enzymic synthesis of benzoyl-L-tyrosylglycinamide from benzoyl-L-tyrosine and glycinamide, only 1.3

per cent of the ionized reactants had undergone condensation when their initial concentration was 0.025 M. It may be calculated that at an initial concentration of 0.001 M, the extent of synthesis would be reduced to about 0.05 per cent. Such negligibly small values have raised doubt whether direct condensation of carboxyl and amino groups to form peptide bonds is possible in the living cell. At this point, it may be well to reemphasize what has been said in earlier discussions (Bergmann and Fruton, 1944) of this problem. The living cell obviously cannot be considered to represent a homogeneous system in which the chemical reactants are present in equilibrium concentration. Rather, we must envisage the strong possibility that the products of a series of condensation reactions, leading to the formation of central peptide bonds, are rapidly removed by subsequent processes, some of which may be strongly exergonic. To illustrate such a mechanism, one may consider the situation when chymotrypsin catalyzes the condensation of benzoyl-L-tyrosine with glycinanilide. Here benzoyl-L-tyrosylglycinanilide crystallizes from the solution, and under conditions where about 1 per cent synthesis of benzoyl-L-tyrosylglycinamide was observed, the yield of insoluble anilide may be 65 per cent. The driving force in the formation of such large amounts of the anilide is its removal from solution, because its solubility is lower than its concentration at equilibrium. Thus, the endergonic synthesis of the peptide bond is coupled to the exergonic process of the removal of the peptide derivative from supersaturated solution. In a coupled mechanism of this kind, one may perhaps speak loosely of the peptide synthesis as being "pulled," through a mass-action effect, by later exergonic steps in the overall process. Although such a mass-action effect seems a possibility in the formation of the central peptide bonds of proteins, it is much less plausible as a driving force in peptide bond syntheses where the value of $\triangle F^\circ$ is about 3 to 4 kcal. For this reason, it has generally been assumed, by analogy with other biochemical endergonic reactions, that one of the reactants is converted to a more reactive form, containing a so-called "energy-rich" bond. In this process, the synthesis of the CO-NH bond may be thought of as being "pushed" by an exergonic reaction, such as the cleavage of a pyrophosphate bond of adenosine triphosphate.

It may well be that this distinction between "pull" and "push" has some validity for the problem of the biosynthesis of proteins from amino acids, and that both types of mechanism are involved, but at different stages of the overall process. However, in many recent discussions of the problem, emphasis has largely been placed on the latter type of

mechanism, because evidence for its biological occurrence has come from studies on the biosynthesis of substances such as hippuric acid, glutathione, and glutamine. Borsook and Huffman (1948, *cf.* also Borsook, 1953) have provided thermodynamic data for the synthesis of the hippurate ion from the benzoate ion and the glycine dipolar ion; the value of $\triangle F^{\circ}_{310}$ was calculated to be about $+2.6$ kcal. Much work has been done on the formation of hippuric acid (and of *p*-aminohippuric acid) by enzyme preparations from kidney and liver; however, the immediate significance of these studies for the understanding of the mechanism whereby peptide chains are synthesized is not readily apparent. Although it is probable that benzoyl-CoA is the acylating agent in the process of hippurate formation (Chantrenne, 1951; Schachter and Taggart, 1953), recent studies have shown that the enzyme involved is specific for glycine (Schachter and Taggart, 1954). Despite the limited scope of the hippurate-synthesizing system, it illustrates a mechanism whereby energy may be "pushed" into the synthesis of a compound with a CO-NH bond from a carboxylic acid and an amine. The synthesis of hippurate from benzoyl-CoA and glycine is an exergonic reaction, but the formation of benzoyl-CoA from benzoate and coenzyme A requires energy; this is provided by the cleavage of a pyrophosphate bond of ATP (Mahler et al., 1953). I need not remind you that the discovery of coenzyme A and of acetyl-coenzyme A stems from the studies of Lipmann (1949) on the enzymatic mechanisms in the acetylation of sulfanilamide by various cell preparations. The synthesis of the CO-NH bond in acetylsulfanilamide involves the reaction of acetyl-CoA with the amine; the formation of acetyl-CoA from acetate and CoA requires the scission of a pyrophosphate bond of adenosine triphosphate in a manner subsequently shown to apply to other acyl-CoA derivatives, including benzoyl-CoA.

Another example of the coupling of the synthesis of a CO-NH bond to the cleavage of ATP to AMP and pyrophosphate has been encountered in the study of the formation of pantothenic acid by extracts of *E. coli*. Here, coenzyme A does not appear to be involved, and it has been assumed that there is formed a reactive "enzyme-pyrophosphate" compound which reacts with pantoic acid to form a "pantoyl-enzyme" compound; this, in turn, reacts with β-alanine to give pantothenic acid. Lipmann (1954) has suggested that a mechanism of this type may be operative in the synthesis of the polypeptide chains of proteins by assuming that "amino-acid-specific activation spots are lined up on a structure in a demanded sequence," and that at each of these "spots" a pyrophosphoryl radical becomes attached by cleavage of ATP. Each of

the pyrophosphoryl groups is then replaced by the α-carbonyl group of a particular amino acid; when all the amino acids are aligned, they are thought to condense to a peptide chain, by the reaction of the carboxyl phosphate groups with adjacent α-amino groups.

Of more immediate interest for the problem of peptide bond synthesis is the work of Bloch and his associates (Snoke et al., 1953) on the formation of the tripeptide glutathione from the component amino acids by enzyme preparations from pigeon liver. This process requires the participation of ATP, but all efforts to demonstrate a role for coenzyme A appear to have been unsuccessful. The synthesis of the tripeptide proceeds in two steps, in each of which one pyrophosphate bond of ATP is cleaved:

L-Glutamic acid + L-Cysteine + ATP → γ-L-Glutamyl-L-cysteine + ADP + P_i
γ-L-Glutamyl-L-cysteine + Glycine + ATP → Glutathione + ADP + P_i

Thermodynamic data are not available for the synthesis of glutathione from the amino acid ions, but by analogy with the values for compounds of related structure it may be surmised that the synthesis of each of the two CO-NH bonds of the tripeptide is accompanied by a positive free energy change of about 4 kcal. Evidence has been provided (Snoke, 1953) for the view that in the enzymic synthesis of glutathione from γ-L-glutamyl-L-cysteine and glycine, the cleavage of ATP leads to the formation of a reactive "enzyme-phosphate" compound.

In addition to the studies on the biosynthesis of hippuric acid and of glutathione, much work has been done on the enzyme-catalyzed formation of glutamine from glutamic acid and ammonia. The studies of Speck and of Elliott (1953) have shown that the biosynthesis of glutamine requires ATP, and it would appear that as in the biosynthesis of glutathione, the synthesis of glutamine requires direct coupling with an energy-yielding reaction in which a pyrophosphate bond of ATP is cleaved. Recent studies indicate that the reaction may be written as follows:

$$\text{L-Glutamic acid} + NH_3 + \text{ATP} \rightarrow \text{L-Glutamine} + \text{ADP} + P_i$$

It has been reported (Levintow and Meister, 1954) that the equilibrium constant of this reaction is 1200. Although the $\triangle F°$ of hydrolysis of ATP is not known precisely, it is estimated to be about −9 kcal. (Burton and Krebs, 1953); from these figures, the $\triangle F°$ for the formation of glutamine from glutamic acid and ammonia may be calculated to be about +5 kcal.

In glutamine or asparagine, the amide group is at the γ-carbon of glutamic acid or the β-carbon of aspartic acid. The recent discovery (du

Vigneaud et al., 1953) that the peptide hormone oxytocin contains a terminal glycinamide group focuses attention on the possibility that α-amides of amino acids and peptides may be intermediates in metabolism. For this reason it may be worthwhile to add at this point some thermodynamic data on the hydrolysis of such α-amides. Unfortunately, no free energy values are available, but calorimetric measurements have been made on the enthalpy change in the hydrolysis of various CO-NH_2 bonds cleaved by proteolytic enzymes. For example, in the hydrolysis of benzoyl-L-tyrosinamide by chymotrypsin to benzoyl-L-tyrosine and NH_4^+, $\triangle H_{298}$ was found to be —5.8 kcal., and in the hydrolysis of glycyl-L-phenylalaninamide to glycyl-L-phenylalanine and NH_4^+, also catalyzed by chymotrypsin, $\triangle H_{298} = -6.2$ kcal. (Dobry and Sturtevant, 1952; Sturtevant, 1953). These relatively large negative values for the enthalpy change in hydrolysis lead one to expect that the free energy changes in the reverse synthetic reactions are relatively large positive numbers, and help to explain why the isotope method employed with benzoyl-L-tyrosylglycinamide was insufficiently sensitive to allow us to determine a value for $\triangle F°$ in the case of benzoyl-L-tyrosinamide. It would appear that, as among esters of phosphoric acid, so also among compounds containing a CO-NH bond, there is a wide variation in the magnitude of the enthalpy change and free energy change of hydrolysis. Although it is not suggested here that one should refer to "high-energy" CO-NH bonds, it is important to recognize that there are some amide bonds whose hydrolysis is accompanied by an enthalpy change equal to that found in the hydrolysis of pyrophosphate (Ging and Sturtevant, 1954), which is usually included among the compounds having a "high-energy" phosphate bond. Clearly, if such α-amides of peptides are intermediates in metabolism, the synthesis of the terminal CO-NH_2 group must be "pushed" by direct coupling with an energy-yielding reaction, possibly involving the cleavage of a pyrophosphate bond of adenosine triphosphate.

The view that adenosine triphosphate participates in the overall process of protein synthesis is supported by experiments on the incorporation of isotopic amino acids into the proteins of tissue preparations. Such studies (Siekevitz, 1952) have shown that the incorporation depends on energy derived from oxidative processes, and the inhibition of the incorporation by dinitrophenol has been interpreted in terms of the capacity of this reagent to "uncouple" oxidation from the synthesis of the pyrophosphate bonds of adenosine triphosphate.

After this brief review of the energy changes in the synthesis of

CO-NH bonds, I should like next to summarize some of the available information about the chemical specificity of the intracellular enzymes that can catalyze the synthesis of peptide linkages in peptide chains. Here also our knowledge is fragmentary, but all we can do is to examine the present situation and the possibilities for future experiment. Thus far, the members of only one well-defined group of enzymes have been shown to catalyze the interaction of amino acid residues to form peptide chains; these are the so-called proteolytic enzymes. We have been accustomed to think of the physiological action of these peptide-splitting enzymes in terms of what we know of the digestive proteinases pepsin, trypsin, and chymotrypsin, and the digestive peptidases (carboxypeptidase, the aminopeptidases, the dipeptidases, etc.) that attack the partial degradation products of the dietary proteins. We know a good deal about the properties of many of these digestive proteinases and peptidases because they have been obtained in purified form by crystallization (Northrop et al., 1948), and because their specificity and mode of action has been extensively studied by the use of synthetic peptides and peptide derivatives (Bergmann and Fruton, 1941; Neurath and Schwert, 1950). However, much less work has been done on the proteinases of animal tissues such as spleen, liver, or kidney, although it has been known for many decades that these tissues contain such enzymes, whose action becomes evident upon autolysis. The physiological function of these intracellular proteinases, which we term cathepsins, has not yet been established, but as we shall see shortly, they can act at CO-NH bonds not only as hydrolytic agents, but also as catalysts of peptide bond synthesis. In addition to the cathepsins of animal tissues, many plants contain active proteinases, among which the enzyme papain (from *Carica papaya*) is the best known. During the past few years, we have been studying the possible role of some of these animal and plant proteinases in the elongation of peptide chains, and I should like to discuss the relation of our results to the general problem of protein synthesis.

If one makes an aqueous extract of autolyzed beef spleen, and allows it to act on a protein as the substrate, the protein is extensively degraded to amino acids and small peptides. Upon fractionation of the spleen extract, it becomes evident that this degradation is the result of the cumulative action of a number of proteolytic enzymes of different specificity. In order to separate and purify the individual enzymes, and to study their specificity and mode of action, it is necessary to use as substrates simple peptide derivatives that contain only one sensitive CO-NH bond, in place of the complex protein which contains many peptide bonds and

is therefore attacked by a variety of proteinases. Although much more needs to be done to identify the various proteolytic enzymes present in a tissue such as beef spleen, it is already clear that a crude spleen extract contains at least three different cathepsins, and it is very probable that there are many more than this number. In addition, there are a host of peptidases. For convenience, we have designated the three spleen proteinases identified thus far as cathepsin A, B, and C respectively (Tallan et al., 1952). Because of its favorable properties, we have been able to purify cathepsin C appreciably (de la Haba et al., unpublished). In what is to follow, we shall discuss mainly our results with the animal proteinases cathepsin B and cathepsin C, and the plant proteinases papain and ficin.

In speaking of the proteolytic enzymes, it has been customary in the past to consider them solely as catalysts of hydrolysis, and to write the reaction effected at a sensitive CO-NH bond as follows:

$$RCO—NHR' + H_2O \rightarrow RCOO^- + {}^+NH_3R'$$

The work of the past few years (Fruton, 1950; Johnston et al., 1950a, b) has shown clearly that this is too restrictive a formulation, since proteolytic enzymes also catalyze replacement (or transfer) reactions of the following type:

$$RCO—NHR' + NH_2X \rightarrow RCO—NHX + NH_2R'$$

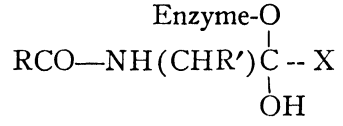
Such replacement reactions have been termed "transamidation" or "transpeptidation" reactions, and differ from the hydrolytic process only in the substitution of an amino compound for water in the forward bimolecular reaction. For our present discussion, it is a matter of some importance that the known intracellular proteinases are effective catalysts of such transamidation reactions, although this property is also exhibited to a greater or lesser degree by other proteolytic enzymes. It is by no means excluded that, under favorable conditions, the water or the amine may be replaced in the above bimolecular reactions by an alcohol or a mercaptan; some indirect evidence for this possibility has been reported (Fruton et al., 1951).

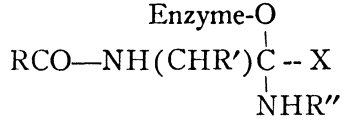
Before presenting some of the results on the catalysis of transamidation reactions by intracellular proteinases, it may be appropriate to note that since the unequivocal demonstration of the ability of proteolytic enzymes to catalyze transamidation reactions, it has become clear that many enzymes once thought to function solely as catalysts in hydrolytic reactions also catalyze replacement reactions, and the sharp distinction

often made between "hydrolases" and "transferases" is no longer valid. It has in fact been recognized by many enzyme chemists that hydrolysis is a special case of a transfer reaction, in which water is a reaction partner. To cite but two examples, it has been shown that one of the classical hydrolytic enzymes, invertase, functions as a transfructosidase (Bacon and Edelman, 1950), and that ribonuclease catalyzes replacement reactions involving pyrimidine nucleotides (Heppel et al., 1954). Results of this kind make it inadvisable to assume that, because an intracellular enzyme, after isolation, is found to catalyze a hydrolytic reaction, its physiological function is exclusively a hydrolytic one. It may well be that, under suitable conditions, its intracellular role as a catalyst of a transfer reaction may be much more important. The obverse of this possibility must also be considered, and attention may well be given to the hydrolytic action of some of the known "transferases." For example, it has been shown (Harting and Velick, 1954) that triose phosphate dehydrogenase, which catalyzes an acyl transfer, also catalyzes the hydrolysis of acetyl phosphate, and thus appears to act as a phosphatase. Results of this kind underline the importance of careful studies both of so-called "hydrolases" and of "transferases" to determine the relative extent of hydrolysis and replacement under a variety of experimental conditions (concentration of reactants, pH, temperature).

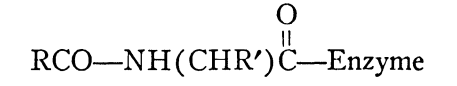
To illustrate some of the principles involved in the action of intracellular proteinases at CO-NH bonds, it may be useful to consider first the plant enzyme papain. This enzyme catalyzes the hydrolysis of central peptide bonds of protein chains and of a variety of synthetic substrates of the general type RCO—NH(CHR′)CO -- X, the broken line denoting the sensitive bond. For hydrolysis, its pH optimum is in the region of pH 5, and its activity depends on the presence of one or more SH groups in the enzyme protein. Among the synthetic substrates of papain, there are various acylamino acid amides (e.g., benzoyl-L-argininamide, carbobenzoxyglycinamide, carbobenzoxy-L-glutamic acid α-amide). Although papain appears to have a much broader "side-chain" specificity than, say, trypsin which also acts on benzoyl-L-argininamide, it is not indiscriminate in its catalytic action, since CO-NH bonds involving L-tyrosine or L-phenylalanine are not readily affected (Dekker et al., 1949). In addition, the group X in the above type formula need not be $-NH_2$, since esters corresponding to susceptible amides are hydrolyzed by the enzyme (Kimmel and Smith, 1954), in analogy to the action of trypsin on esters such as benzoyl-L-arginine methyl ester (Neurath and Schwert, *op. cit.*).

It is tempting to speculate about the mechanism of the catalytic action of papain and related intracellular proteinases. One may consider at least two possibilities. In the first hypothesis, the enzyme may be thought to combine with the oxygen atom of the carbonyl group and, in analogy to the known mechanism for the non-enzymatic hydrolysis of esters (Bender, 1951), facilitates attack at the carbon atom by nucleophilic reagents such as water, amines, or alcohols. With water or an amine (NH_2R''), the following transient intermediates may be postulated, with the subsequent elimination of the group X as HX.





An alternative hypothesis is that a reactive group (possibly an SH group) on the enzyme combines with the carbon atom of the carbonyl group, with the elimination of X as HX, and the formation of a reactive acyl-enzyme complex, as shown, which may then be cleaved by a nucleophilic reagent such as water or an amine.



To account for the stereochemical specificity of papain, and of other proteinases, it is necessary to assume the interaction of additional groups in the substrate with the enzyme. However, there are insufficient data available at present to permit one to decide whether either of these possibilities is correct.

Whatever the precise mechanism of papain action may be, it is extremely probable that the same catalytic sites on the enzyme are involved in the catalysis of hydrolysis and of transamidation. This view is supported by extensive data which show that the specificity with respect to the structure of the compound containing the sensitive CO-NH bond is the same for transamidation as for hydrolysis (Johnston et al., 1950a). In an aqueous solution containing papain, one of its substrates (e.g., benzoyl-L-argininamide, BAA), and an amine (e.g., hydroxylamine), there is a competition between water and NH_2OH for successful reaction with the papain-BAA complex. It has recently been possible to estimate the relative efficiency of NH_2OH and of H_2O in such a system, and it turns out that the amine is about 420 times more efficient as a reactant than is water (Durell and Fruton, 1954). It will not surprise organic chemists

to hear that an amine is a more effective nucleophilic reagent than is water, but it is of interest that the magnitude of the relative efficiency depends on the nature of the enzymic catalyst that is employed. Trypsin catalyzes the same reactions of benzoyl-L-argininamide as does papain, but here NH_2OH is only 30 times as effective as H_2O. This indicates that in the competition between the replacement agent and water for "activated" substrate molecules, the enzyme takes an active part. It is perhaps significant that an intracellular proteinase should be so much more effective as a catalyst of transamidation than a digestive proteinase.

In the catalysis of transamidation reactions by papain, and by other intracellular proteinases, two factors must be considered that do not enter into the hydrolytic process. The first of these is the effect of variation of the nature of the amine. Transamidation reactions have been studied in the presence of a variety of replacement agents (NH_2OH, $N^{15}H_3$, amino acid amides, dipeptides, dipeptide amides, etc.), and it has become clear that the chemical structure of the amine exerts an important influence on the rate of transamidation. This means that in considering the chemical specificity of a papain-catalyzed transamidation reaction, one must give attention not only to the structure of the compound containing the sensitive CO-NH bond, but also to the structure of the replacement agent.

The second feature of the enzyme-catalyzed transamidation reactions, which distinguishes them from the hydrolytic process, is that the amine component, in order to act as nucleophilic reagent, must be in the uncharged form. Consequently, at any given pH value, the concentration of the free amine will depend on the value for pK' of the corresponding cation. As the pH of the solution is changed, so as to decrease or increase the molar concentration of the reactive conjugate base, the extent of transamidation is correspondingly decreased or increased. We noted before that the pH optimum for the hydrolytic action of papain on its synthetic substrates is near pH 5; other intracellular proteinases exhibit similar pH optima for hydrolysis. However, at this pH, the extent of transamidation, relative to hydrolysis, is very small. As the pH is shifted into the region around pH 7, the extent of transamidation increases markedly, because the effective concentration of the free amine is increased. Thus, at equal total concentrations, hydroxylamine ($pK' = 6.0$) or glycinamide ($pK' = 7.9$) are more effective replacement agents near pH 7 than are ammonia ($pK' = 9.4$) or glycine ($pK'_2 = 9.6$). However, if instead of referring to equal total concentrations of both charged and uncharged amine, one calculates the rate of transamidation

at different pH values, but at constant concentration of uncharged amine, it becomes clear that the rate increases as one goes from pH 7 toward pH 5, thus suggesting that the true optimum for hydrolysis and transamidation may in fact be the same. Consequently, under conditions where the pK'_2 values of amino acids or peptides are shifted to a more acid pH, these substances should serve as relatively efficient replacement agents at pH values less than 7.

The fact that at pH values near pH 7, transamidation by intracellular proteinases is a quantitatively important process led us to suggest that the catalysis of replacement reactions may represent a major physiological role of these enzymes. It should be added, however, that there are some indications that the values for the intracellular pH of animal tissues may be more acid than pH 7, although the methods employed make these determinations uncertain. Also, little is known about the pH gradients in cells, and the dissociation constants of amino acids at the intracellular sites of protein formation.

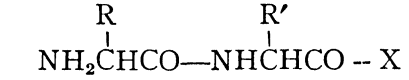
Although the pK′ of the cation corresponding to the replacement agent is an important factor in determining the extent of transamidation at a given pH, it is by no means the only determinant factor, since two replacement agents of different structure, but having the same pK′ values, may differ in their effectiveness in a transamidation reaction. To illustrate this, one may cite some experiments (Dowmont and Fruton, 1952) with papain as the enzyme, carbobenzoxyglycinamide (0.05 M) as the component having the sensitive CO-NH bond, and as the replacement agent one of the dipeptides glycylglycine, L-leucylglycine, or D-leucylglycine (0.05 M). The pK'_2 values of the three dipeptides are nearly the same (ca. 8.1), but the ratio of extent of replacement to hydrolysis at pH 7.5 was found to be 0.15, 2.0, and 0.09, respectively. It is of special interest that the L-isomer of leucylglycine was about 20 times more effective as a replacement agent than the D-isomer. Results of this kind show clearly that the efficiency of the proteinase-catalyzed transamidation reaction depends both on the structure of the sensitive substrate and the structure of the replacement agent, whereas in hydrolysis, where water is the common reactant, the specificity depends only on the structure of the sensitive substrate. Such increased selectivity of enzyme action permits the enzyme to direct preferentially one of several possible chemical reactions. For example, it has been found experimentally that if carbobenzoxyglycinamide is incubated with papain in the presence of both glycylglycine and L-leucylglycine, very little of the carbobenzoxyglycylglycylglycine appears, and the product of transamidation is almost

exclusively carbobenzoxyglycyl-L-leucylglycine (Mycek and Fruton, unpublished).

In the enzyme-catalyzed reaction between carbobenzoxyglycinamide and L-leucylglycine, there occurred the replacement of a small group ($-NH_2$) by a dipeptide unit, thus effecting the elongation of the peptide chain from the carbonyl end of the sensitive substrate. This example of the enzymic catalysis of the elongation of peptide chains is but one of many that have now been found (Fruton et al., 1951, 1953), and because of the possible importance of such reactions in the formation of the peptide chains of proteins and of naturally occurring polypeptides, they merit further discussion.

It may be useful first to mention the thermodynamic aspects of such elongation reactions involving the replacement of a $-NH_2$ group by a larger group. A reaction for which we have some of the necessary energy values is the following, catalyzed by chymotrypsin: Benzoyl-L-tyrosinamide + $^+$glycinamide → Benzoyl-L-tyrosylglycinamide + NH_4^+. The free energy change in this reaction cannot be calculated, because we do not have a value for the $\triangle F°$ of hydrolysis of benzoyl-L-tyrosinamide. However, one can calculate the enthalpy change in the replacement reaction from the $\triangle H$ values for the hydrolysis of benzoyl-L-tyrosinamide and of benzoyl-L-tyrosylglycinamide, cited earlier in this discussion. Such a calculation gives a value of about −4.3 kcal. for the $\triangle H$ of the transamidation reaction, and suggests that the value for $\triangle F°$ is probably also a large negative number. If this is so, it would follow that replacement reactions of this kind are strongly exergonic, with equilibrium positions far to the right.

The exergonic nature of the enzyme-catalyzed elongation of peptide chains was exemplified in a striking manner by results obtained with the intracellular proteinase cathepsin C, which we have purified extensively from beef spleen. The specificity of this enzyme in hydrolytic reactions is of considerable interest, since it appears that cathepsin C only acts on derivatives of dipeptides composed of two α-amino acid residues, as shown.



The broken line denotes the sensitive bond, and the group X may be either $-NH_2$ (as in an amide) or $-OCH_3$ (as in an ester). The R groups may be varied over a wide range, although cathepsin C acts preferentially on substrates in which R′ is the side chain of phenylalanine

or of tyrosine (Wiggans et al., 1954). In this respect, it resembles chymotrypsin in specificity. However, a distinctive structural requirement in substrates of cathepsin C is that there be a specified spatial separation of the terminal amino group and the sensitive carbonyl group, since the enzyme does not act on amino acid amides or esters (chymotrypsin hydrolyzes L-phenylalanine ethyl ester), or on dipeptide derivatives such as β-alanyl-L-phenylalanine ethyl ester or γ-L-glutamyl-L-phenylalaninamide (Clark-Lewis and Fruton, 1954). Suitable substrates of cathepsin C are amides or esters of dipeptides such as glycyl-L-phenylalanine, L-alanyl-L-phenylalanine, or L-seryl-L-tyrosine. Glycylglycine ethyl ester is hydrolyzed by the purified enzyme, but glycylglycinamide is split only slowly.

If cathepsin C is allowed to act on a dipeptide amide such as L-alanyl-L-phenylalaninamide at pH 5, where its hydrolytic activity is optimal, the terminal amide bond is rapidly hydrolyzed with the formation of NH_4^+ and the dipeptide. However, if the reaction is conducted at pH 7.5, ammonium ions still appear rapidly, but the extent of hydrolysis is negligible, and there separates from the solution a precipitate which, on chemical analysis, was found to be the amide of the hexapeptide L-ala-L-pha-L-ala-L-pha-L-ala-L-pha. It is a matter of some importance that the yield of this product corresponded to 86 per cent of the theory, based on the amount of NH_4^+ liberated. Clearly, what had occurred was the proteinase-catalyzed polymerization of the dipeptide amide by two successive transamidation reactions in each of which one equivalent of NH_4^+ had been released.

Other dipeptide derivatives that are hydrolyzed by cathepsin C at pH 5 also are transformed by the enzyme into polymeric products at pH 7.5. With glycyl-L-phenylalaninamide, the product is, on the average, an octapeptide; with glycyl-L-tyrosinamide, the average chain length is that of a decapeptide. Whereas the hexapeptide obtained from L-alanyl-L-phenylalaninamide appears to be chromatographically homogeneous, the other two polymers are not, and probably represent mixtures of polypeptides of varying chain lengths.

From the available information about the specificity of cathepsin C, it would seem most likely that at each step of the polymerization, a dipeptide unit is added to the amino end of the growing peptide chain, until the resulting product becomes too insoluble to remain in solution. In this respect, the action of cathepsin C resembles that of crystalline muscle phosphorylase, which catalyzes the successive addition of the glucosyl units of glucose-l-phosphate to the non-reducing end of a grow-

ing amylose chain. It is a matter of some interest that cathepsin C is tightly bound by the polymer, and when the latter separates from the solution it removes much of the enzyme with it, thus stopping the reaction before all the dipeptide amide has undergone polymerization. Further studies are needed to determine whether the enzyme becomes attached to the growing peptide chain while it is still in solution.

Although the specificity of cathepsin C is exacting in respect to the "backbone" structure of the sensitive substrate, the enzyme catalyzes transamidation reactions involving a variety of replacement agents. This is suggested by the results of the polymerization reactions, where peptides of varying chain length, as well as a second molecule of the sensitive substrate, can serve as replacement agents. In addition, cathepsin C catalyzes transamidation reactions of suitable dipeptide amides (e.g., glycyl-L-phenylalaninamide) with hydroxylamine or with amino acid amides (e.g., L-argininamide). Some of these replacement agents appear to be more effective than is glycyl-L-phenylalaninamide, and thus strongly inhibit polymer formation. For example, with L-argininamide as the amine component, the major product of the reaction could be shown chromatographically to be glycyl-L-phenylalanyl-L-argininamide.

It is a logical extension of experiments such as these to attempt to couple the reaction between glycyl-L-phenylalaninamide and L-argininamide, catalyzed by cathepsin C, with a succeeding reaction in which the carbonyl group of the arginine residue participates. For this purpose, cathepsin B would be the catalyst of choice since, like crystalline trypsin, it appears to be specific for CO-NH bonds in which the carbonyl group belongs to an L-arginine or L-lysine residue. Cathepsin B acts rapidly on acyl-L-argininamides, but does not split L-argininamide readily. Like cathepsin C, it has its pH optimum for hydrolysis near pH 5 and, at pH values near pH 7, it catalyzes transamidation reactions. It appeared desirable, therefore, to attempt a coupled transamidation reaction in which both cathepsin B and C were present as catalysts, and in which glycyl-L-phenylalaninamide, L-argininamide, and L-isoglutamine were present as reactants. If the coupled transamidation had gone as expected, there should have been formed the amide of the tetrapeptide glycyl-L-phenylalanyl-L-arginyl-L-glutamic acid. On analysis of the mixture by paper chromatography, numerous ninhydrin-positive components were identified; one of them yielded on hydrolysis the four expected amino acids. This experiment, and others that we have done like it, can only be considered as suggestive rather than conclusive. For more unequivocal demonstration of the expected coupled transamidation, we need more

purified cathepsins, and especially purified cathepsin B, and we must use better methods for the separation and identification of complex polypeptides. For the latter purpose, chromatography on 2 per cent cross-linked Dowex-50 resins has proved to be a valuable technique, and should facilitate the further exploration of the scope of coupled transamidation reactions.

The difference in the action of cathepsin B on L-argininamide and its acyl derivatives merits further comment, since it may exemplify a phenomenon of more general interest. An analogous observation was made with ficin (a plant proteinase similar to papain), which acts rapidly on acyl-L-methioninamides but does not readily attack L-methioninamide. Among the substrates of ficin is carbobenzoxy-L-isoglutamine; when the enzyme acts on this compound at pH 7.2 in the presence of L-methioninamide, there results a polymeric product that contains, on the average, 11 methionine residues per glutamic acid residue and, therefore, may be denoted as carbobenzoxy-α-L-glutamyl-(L-methionyl)$_{10}$-L-methioninamide (Fried and Fruton, unpublished). The first step in the polymerization is probably the formation of carbobenzoxy-α-L-glutamyl-L-methioninamide (an acyl methioninamide), and the peptide chain grows by successive transamidation reactions. No polymer is observed when L-methioninamide is replaced by D-methioninamide; if carbobenzoxy-α-L-glutamyl-D-methioninamide were formed, this product would not be acted upon by ficin at the methionine carbonyl group because this enzyme is stereospecific for the L-form. Also, no polymer is observed when ficin is allowed to act on L-methioninamide in the absence of carbobenzoxy-L-isoglutamine. Waelsch (1952) has suggested that the γ-glutamyl group may be a physiological acyl substituent at the α-amino group of an amino acid in the course of the biosynthesis of a peptide bond involving the carbonyl of the amino acid.

Although the various experimental results that have been described above amply document the view that the intracellular proteinases can catalyze the elongation of peptide chains in fairly specific transamidation reactions, they give no information whatever as to the actual occurrence of such transamidation reactions in the formation of proteins *in vivo*. At the present stage of our ignorance, it is perhaps a matter of taste whether one wishes to assign to the intracellular proteinases and to transpeptidation reactions any significant role in protein synthesis. I may add that although I recognize the absence of proof for the biological significance of transpeptidation reactions, I do not find much cogency in the arguments presented against it. One of these objections is that a whole series

of proteinases would be needed to make one protein, and many more for all the different proteins. There is no valid reason (other than the search for simplicity) against the participation of a series of proteinases in the synthesis of a protein, and indeed it would be more in accord with the experience gained from the study of other biochemical processes, leading to the synthesis or breakdown of molecules much simpler than proteins, to assume that a multi-enzyme system is involved. It may well be that this multi-enzyme system is an organized structure in the living cell, and that the intermediate products leading to the completed protein molecule are not released from this structure. Another objection raised against the possibility of transpeptidation reactions in protein synthesis is that so far no evidence has been found for as many intracellular proteinases as may be thought to be required. This may well be not because they are not there, but because the methods for discovering them have not been applied or are not available. As was noted earlier, the three cathepsins identified thus far certainly do not represent the sum total of the proteinases present in tissues such as spleen or liver.

In questioning the validity of these objections, I wish to reiterate that it still remains to be determined whether transpeptidation reactions catalyzed by intracellular proteinases are of biological importance in protein synthesis. However, the concept of a multi-proteinase system allows one to speculate that the reproducibility of protein synthesis may be a consequence of the additive specificity of a series of enzymes involved in the elongation of peptide chains. The coupling of specific steps in each of which preference is shown for a particular reaction may be expected to accentuate these preferences and, in a dynamic system, to favor the synthesis of a unique structure. If this speculation should find additional experimental support, it would provide to the biochemical experimenter a more satisfying alternative to hypotheses involving a "template" in the biosynthesis of proteins.

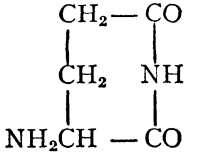
If transamidation reactions should prove to play a role in protein synthesis, it will be necessary to link such reactions with energy-yielding processes in the cell. We have seen that in each of the transamidation reactions the proteinase acts on a preformed CO-NH bond. Clearly, if a biological system is to make its proteins from free amino acids, some CO-NH bonds must be made *de novo* in endergonic reactions. From the earlier discussion of the energy relations in the hydrolysis and synthesis of CO-NH bonds, it will be obvious that the formation of the peptide bonds of a dipeptide or of glutathione, or of a $CO\text{-}NH_2$ bond, as

in glutamine, requires coupling to the exergonic cleavage of a pyrophosphate bond of adenosine triphosphate.

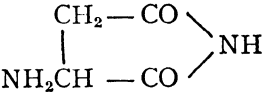
In search for possible links between proteinase-catalyzed transamidation reactions and the energy-yielding processes of the cell, the recent studies of Elliott (*op. cit.*) and of Lajtha et al., (1953) are of special importance. This work has shown that several of the enzyme preparations (from various biological sources) that catalyze the synthesis of glutamine from glutamic acid and ammonia also catalyze transamidation reactions involving the $CO\text{-}NH_2$ bond of glutamine. The possibility has been suggested that the common intermediate in both reactions is an "activated" γ-glutamyl group, probably attached to a reactive group on the enzyme. Among the possible sites of attachment of such an acyl group to the enzyme protein, one may consider the SH-group of cysteine, the imidazolyl group of histidine, and possibly even the ϵ-amino group of lysine. The formation of the "γ-glutamyl-enzyme" from free glutamic acid and the enzyme may be thought to require a stoichiometric reaction with adenosine triphosphate; after the acyl-enzyme is formed, it should be possible to transfer the γ-glutamyl group, in a reversible reaction, to ammonia. A similar series of reactions has been suggested for the enzymic synthesis of γ-L-glutamyl-L-cysteine from L-glutamic acid and L-cysteine (Webster and Varner, 1954). In a transamidation reaction involving glutamine, the "γ-glutamyl-enzyme" may then react with the replacement amine (NH_2OH, peptides, etc.), and the efficiency of the transamidation at pH 7 would depend in large part on the structure of the amine and the pK′ of its cation. Evidence has been presented for this type of transamidation by Waelsch (1952), who demonstrated the formation of several γ-glutamyl peptides (including glutathione) in replacement reactions (Fodor et al., 1953) and by Williams and Thorne (1954) who have shown that an enzyme preparation from cultures of *B. subtilis* converts L-glutamine to γ-glutamyl polypeptides.

It was with these possibilities in mind that we suggested early in 1950 that the coupling between the energy-yielding processes of the cell and the synthesis of the peptide bonds of proteins may be funnelled through a relatively small number of amides or peptides such as glutamine or glutathione. Similar hypotheses were advanced independently by Hanes et al. (1952) and by Waelsch (*op. cit.*). These investigators came to this view through their discovery of enzyme systems that catalyze replacement reactions involving glutathione (Hanes et al.) and glutamine and asparagine (Waelsch et al.). It must be added that at

present there is no clear-cut evidence for the utilization of these γ-glutamyl derivatives for the formation of the peptide bonds linking α-amino groups and α-carboxyl groups of amino acids. A possible mechanism, which deserves renewed consideration, is the intermediate formation from glutamine of derivatives of aminoglutarimide, which could undergo cleavage at the γ-carbonyl group to form derivatives of isoglutamine. A chemical analogy for such a process has recently been described by Sondheimer and Holley (1954), who showed that carbobenzoxy-L-asparagine methyl ester is converted by alkali to carbobenzoxyaminosuccinimide, which can be hydrolyzed to a mixture of carbobenzoxyasparagine and carbobenzoxyisoasparagine. The further exploration of the possible biological occurrence of such $\gamma \rightarrow \alpha$ or $\beta \rightarrow \alpha$ shifts with glutamine and asparagine seems desirable.



Aminoglutarimide



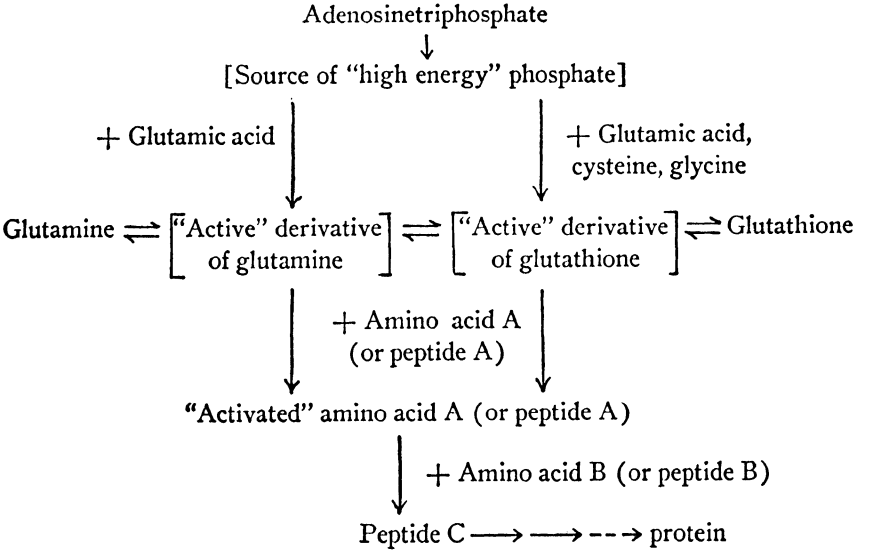
Aminosuccinimide

The biological occurrence of α-amides of amino acids is not well established, except in the case of oxytocin and vasopressin, but in view of their susceptibility to the action of intracellular proteolytic enzymes, they may not accumulate, and thus may escape detection.

We may now venture to summarize diagrammatically the present status of the chemical approach to the problem of protein synthesis by presenting on page 35 a hypothetical scheme (Fruton et al., 1951) including most of the speculations that have come from the studies of several laboratories, including our own. At best, the scheme is a statement of a working hypothesis that is currently guiding the research of some of the investigators in this field. It certainly says more about what we do not know than about what we do know in respect to the chemical pathways in the formation of the peptide chains of proteins from the component amino acids.

I said at the outset that the gap between our chemical knowledge about protein synthesis and the biological observations of this phenomenon is still unbridged. It is not possible here to review all, or even a major part, of the large body of information that has been obtained in recent years by the study of protein formation in intact cells and or-

ganisms. Before discussing some of the findings that seem to me to be the most significant, let me summarize the situation by saying that, just as the chemical studies have not provided clear answers about the nature of the chemical pathways in protein synthesis, the biological experiments have not done so either.



In recent years, much attention has been devoted to the study of the incorporation of isotopic amino acids into the proteins of intact animals. Important experiments along this line have been done by Muir et al. (1952), Askonas et al. (1954), and Simpson and Velick (1954). In all these studies, isotopic amino acids were administered to an animal, discrete proteins were isolated from the tissues, and determinations were made of the isotope concentration of the amino acids obtained upon hydrolysis. Thus, Muir et al. administered C^{14}-valine to rats, and showed that the isotope content of the valine in rat hemoglobin was the same whether the valine had been derived from the amino end of the peptide chains, or whether the valine had been located at a non-terminal position in the peptide chains. In an even more striking experiment, Simpson and Velick administered to a rabbit five isotopic amino acids, and 38 hours later isolated from the muscle two enzymes (aldolase and glyceraldehyde-3-phosphate dehydrogenase) which were recrystallized to maximal enzymic activity to ensure the purity of the two proteins. Upon analysis of eleven of the amino acids obtained after hydrolysis, Simpson and Velick found that the ratio of specific radioactivity of each of these amino acids

in aldolase to the specific activity of the same amino acid in the dehydrogenase was constant. The logical conclusion drawn from this experiment, and that of Muir et al., is that the enzyme systems involved in the synthesis of the proteins under study were drawing on a single "pool" of each of the amino acids, and that the occurrence of quantitatively significant amounts of a "pool" of peptide intermediates was unlikely. These results may be interpreted in terms of (1) a simultaneous condensation of amino acids previously aligned along a template; (2) the rapid successive formation of peptide bonds without the release from the protein-synthesizing system of intermediates that accumulate; or (3) the "pools" of intermediate peptides are very small or equilibrate rapidly with the amino acid "pools." The available information from such incorporation studies does not permit a decision whether any of these possibilities is correct, but the second two seem to make more sense from a chemical point of view than does the first. It should be added that Steinberg and Anfinsen (1952) have obtained evidence for unequal labeling in ovalbumin obtained from tissue preparations incubated with isotopic precursors. However, the results of these *in vitro* studies are not comparable with those of Muir et al., and of Simpson and Velick, and there is no necessary contradiction between the conclusions drawn from the two types of experiments. In an *in vivo* experiment, Steinberg and Anfinsen again found unequal labeling of ovalbumin. As matters stand, therefore, it is not possible to draw any definite conclusions about the chemical pathways of protein synthesis from the available reports on amino acid incorporation.

Another avenue of biological experimentation designed to elucidate the mechanism of protein synthesis has involved the use of microorganisms, and especially the study of the induced formation of "adaptive" enzymes. Among the several researches in this field, those of Monod et al. (1952), and of Halvorson and Spiegelman (1953) are of special interest, and the results of these studies have been interpreted as indicating that the synthesis of adaptive enzymes proceeds only from free amino acids, and that no peptide intermediates are involved. For example, when labeled amino acids are supplied under conditions when adaptive enzyme formation occurs, the isotope appears in the enzyme; however, if the cell proteins are labeled before adaptation, and non-isotopic amino acids are given, the enzyme is not labeled. Halvorson and Spiegelman have made the important observation that *p*-fluorophenylalanine inhibits adaptive enzyme formation, but that peptide intermediates do not accumulate in inhibited cells. The question may be raised whether in

such inhibited cells the degradative processes, suppressed during cell multiplication, are operative, thus leading to the rapid breakdown of any peptides that may have been formed. Whether this is so or not, it seems difficult to accept the interesting findings of Halvorson and Spiegelman as evidence for the "template" hypothesis, as has been suggested, so long as alternative multi-enzyme mechanisms are not excluded by experiment.

Much has been written about the role of cellular nucleic acids in protein synthesis, and the possible function of nucleic acids in serving as a "template" for the alignment of amino acids prior to peptide bond formation. I need not remind you that the early observations of Brachet (1950) and of Caspersson (1950) pointed to the high concentrations of nucleic acids in cells that produce protein rapidly. More recently, Gale (1953) has shown in a striking manner the close interdependence between nucleic acid synthesis and protein formation in Staphylococci, and has presented important data on the effects of various antibacterial agents on these processes. That there is a metabolic relation between cellular nucleic acids and cellular proteins cannot be denied, but it would be premature to interpret the available knowledge as favoring the role of nucleic acids as "templates" in protein synthesis. One of the several hypotheses that have been proposed is that of Dounce et al. (1952, 1953), who suggested that the amino acids combine with the nucleic acid after a preliminary phosphorylation of the polynucleotide, and then the amino acids are "peeled off" in the form of a polypeptide chain. There are many conceptual difficulties in accepting this idea as a working hypothesis, but these are less important than the fact that, thus far, there is no chemical evidence for the occurrence of processes such as those postulated in the scheme.

In a consideration of hypotheses about the role of nucleic acids in the formation of specific proteins, attention should also be given to the suggestion that the function of cellular ribonucleic acids in protein synthesis may be to direct the transformation of "a relatively undifferentiated 'primary' protein" (Koritz and Chantrenne, 1954) into the individual biologically active proteins. The assumption of a "pre-protein" has recurred several times during the past twenty years. In various forms, this hypothesis has been presented by Alcock (1936), Northrop (1948), and others. No decision on this question can be made at present, but the experiments of Koritz and Chantrenne led them to suggest that the incorporation of amino acids into peptide chains does not require ribonucleic acids, but that the conversion of the undifferentiated precursor to the individual proteins may involve the participation of ribonucleic acids.

It would seem that the view of most of those who have approached the problem of protein synthesis from the biological side is to favor either a "template" at which there occurs a "zipper" action performed by unknown chemical processes, or a multi-enzyme system that rapidly catalyzes a series of successive peptide syntheses without releasing peptide intermediates. The latter possibility is one that can readily be reconciled with the hypotheses based on the available chemical information about the energetics and enzymatic catalysis of peptide formation. If the term "template" is considered to represent an organized cellular assembly of enzymic catalysts plus other constituents such as nucleic acids, and if the nature of this assembly is genetically controlled, then much of the difference in opinion reduces itself to difference in language.

Let me add before concluding that, although the tide of current opinion is against them, it would be premature to count the peptide intermediates out altogether. As was pointed out by Steinberg and Anfinsen (1952), it is difficult to evaluate the kinetics of the process whereby a group of amino acids are converted into a protein molecule, and it may well be that many of the biological experiments conducted heretofore involved time periods that were too long to permit the identification of peptide intermediates. That this may perhaps be true is suggested by a preliminary report of Turba and Esser (1953), who incubated *Torulopsis utilis* with C^{14}-acetate. By an adaptation of the method used by Calvin for the identification of the intermediates in photosynthesis, they stopped the experiment after very short time intervals (10 seconds, 1 minute, 3 minutes, etc.) and examined the distribution of radioactivity in the amino acids, peptides, and proteins of the yeast. They report that C^{14} appeared first in the acidic amino acids and peptides (including glutathione), then in the neutral amino acids and peptides, then in the basic amino acids and peptides; only after about 30 minutes was radioactivity demonstrable in the cellular proteins. This problem is under study in the laboratory of Dr. McManus at Yale, and she has confirmed many of the findings reported by Turba and Esser. Obviously, much more work is needed to establish more clearly the significance of these observations for the understanding of the mechanism of protein synthesis, but their implication cannot be lightly disregarded.

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III. THE PLASTICITY OF ENZYMATIC PATTERNS IN MICROBIAL CELLS

BY R. Y. STANIER[1]

THE enzymatic constitution of a microbial cell, observed at any given point in time, is the result of an interplay between two forces, one internal and the other external: the genome and the physico-chemical environment. During the past fifteen years, extensive investigations of the biochemical genetics of microorganisms have shown that the genetic control of enzyme synthesis is both precise and sensitive, a single gene mutation sufficing to confer on or withdraw from a cell the capacity to synthesize a given enzyme. By itself, however, a suitable genotype is not always a sufficient condition for enzyme synthesis, and in many microorganisms the production of certain enzymes in physiologically significant quantities is determined by the environment. These environmentally-induced changes of enzymatic constitution which take place without a change of genotype have long been studied by microbiologists, and are commonly referred to in the microbiological literature as *enzymatic adaptations.* This designation is a somewhat misleading one, since in biological parlance the word *adaptation* carries no implication of mechanism, being applied to any change in the properties of an organism which confers increased fitness, whereas the essential and distinguishing property of enzymatic adaptation in the microbiological sense is its nongenetic causation. Furthermore, many of these environmentally-induced changes of enzymatic constitution may be wholly devoid of adaptive value. For these reasons, it was recently proposed (Cohn et al., 1953) to designate them as *induced syntheses of enzymes,* and this is the term that I shall employ in the following pages.

What is the nature of the environmental factors which cause the induction of enzyme synthesis? Studies on the induction of single enzymes, notably of β-galactosidase in the bacterium *Escherichia coli* (Monod, Cohen-Bazire, and Cohn, 1951), show that the process is a highly specific one, brought about by exposure of the cell to a particular kind of chemical substance known as an *inducer.* Substrates for the enzyme in question are commonly but not necessarily inducers of its formation; and other sterically related substances which are not themselves substrates may nonetheless possess inductive function. The separability of inducer and substrate function may be illustrated by two examples taken from the

[1] Department of Bacteriology, University of California, Berkeley.

above-mentioned studies on β-galactosidase formation by *E. coli*. Neolactose (β-D-galactosidoaltrose) is hydrolyzed by the enzyme, but cannot induce its formation; melibiose (α-D-galactosidoglucose) is almost as powerful an inducer as lactose, the natural substrate for the enzyme, but cannot be detectably metabolized by many strains of *E. coli* and is wholly incapable of combining with β-galactosidase. As the example of melibiose shows, however, even those inducers which cannot serve as substrates possess a general steric similarity to the substrates of the enzyme whose formation they evoke.

Not all the enzymes in the microbial cell are synthesized by a machinery which requires sparking by external inducers. Such enzymes, whose levels in the cell remain largely uninfluenced by changes of the external environment, are known as *constitutive* or more precisely, as *constitutively synthesized* enzymes. The mechanism of constitutivity is still obscure. There is some reason to believe, however, that no fundamental differences exist between constitutive and inducible enzymes, either in structure or in mode of synthesis. Cohen-Bazire and Jolit (1953) observed that the β-galactosidase of *E. coli,* normally in large part inducible, can become strictly constitutive as a result of mutation; and studies on isolated constitutive and induced β-galactosidases reveal no detectable functional or immunochemical difference between them (Monod and Cohn, 1952). The simplest explanation of constitutivity is the hypothesis that it is caused by *endogenous induction,* the necessary inducer being present at all times within the cell as a consequence of endogenous metabolic activities. This hypothesis possesses the attraction of economy; but conclusive evidence for its validity is still lacking, although many of the facts which will be presented in this paper are consonant with it.

In summary, then, the genome of the cell sets qualitative limits to the enzyme-forming capacities, which can be transcended only by mutation or by the introduction from external sources of new genetic material. Within the genetically determined limits, certain enzyme-forming systems (the constitutive ones) operate largely uninfluenced by the external environment, while others (the inducible ones) are brought into action only by specific inducers which enter the cell from the environment. As a consequence, the environment exerts a continuous, direct, selective influence on the enzymatic patterns of the cell, entirely distinct from its *indirect* selective action on mutational events which happen to govern enzymological properties. Particularly when both kinds of selection operate on a single enzyme system, it is by no means a simple matter

to recognize their respective roles. Ryan (1953) has made a beautiful analysis of one such system, in which the environment acted both to select for a gene mutation governing the synthesis of a particular enzyme, and to induce its specific synthesis.

The purpose of this paper is not to analyze the inner mechanism of induced enzyme synthesis, but rather to assess its physiological importance. To what extent do induced modifications of the enzymatic machinery of the cell equip microorganisms to meet the needs of a changing environment? Can we attribute a major physiological and ecological role to such inductions, or is their function subsidiary to genetic selection?

I. THE PHENOMENON OF SEQUENTIAL INDUCTION

In many cases of induction the inducer evokes the formation of a single enzyme. However, the provision of a single inducer does not necessarily have such a simple effect on the enzyme-forming machinery of the cell. Provided that the inducer is also a substrate, it will be acted upon by the enzyme whose formation it causes, and the product of this enzymatic transformation may in turn be the inducer for the formation of a second enzyme. In this fashion, exposure of a cell to one inducer-substrate may be the triggering event for an extremely complex sequence of induced enzyme syntheses within the cell, resulting from the fact that each intermediate in the metabolic chain leading from the primary inducer-substrate has a dual function, as inducer and as substrate. Apart from the initial induction by the exogenously-furnished substrate, this series of enzyme syntheses is the result of *endogenous induction,* since under normal physiological conditions the intermediate inducer-substrates never escape from the cell. These sequential inductions are largely unidirectional, as can be shown by testing the inductive effect of known intermediates in the inducible reaction-sequence when they are supplied exogenously to previously uninduced cells. Such experiments show that any given intermediate, provided that it can enter the cell, induces formation of the enzymes which act upon it and upon its metabolic successors but not, as a rule, of those which act upon its metabolic precursors. A few minor exceptions are known; occasionally an exogenously supplied intermediate will induce formation of the enzyme catalyzing the immediately preceding step in the reaction-chain, but such inductive "backings-up" seldom if ever extend beyond one step. Since the product of a particular enzymatic reaction may well possess the configuration required for induction of the enzyme concerned, one-step backings-up are readily understandable. One might imagine that the formation of the preceding enzyme

would cause the conversion of part of the exogenous inducer-substrate into its metabolic precursor, leading to further back-induction. This is certainly possible in principle; but the oxidative reaction-sequences in which sequential inductions have been studied most extensively are essentially irreversible under physiological conditions.

The existence of sequential inductions was discovered independently by Suda, Hayaishi, and Oda (1949), Karlsson and Barker (1948), and Stanier (1947). As an illustration of the role which sequential induction may play in bacterial metabolism, we may consider the work which has been done with a single organism, *Pseudomonas fluorescens*. This is a common aerobic bacterium which has served as the biological material for most of our own studies. Many years ago, it was shown by den Dooren de Jong (1924) that *P. fluorescens* can grow in a simple mineral medium supplemented with a single organic compound to furnish its carbon and energy requirements. Furthermore, he established that these requirements can be satisfied by any one of an extraordinarily wide variety of organic substances, including aliphatic mono-, di-, and tricarboxylic acids, primary and secondary alcohols, sugars, amines, amides, amino acids, isocyclic and heterocyclic compounds, and even halogen-substituted aromatic substances. In all, he found some ninety compounds which could support the growth of this bacterium, and the list could no doubt be extended by anyone with the time and patience to test additional substances. During the past decade, we have studied manometrically the oxidation of many (though by no means all) of these growth substrates, and in nearly every case it has turned out that the primary attack is an inducible one: cells grown in the absence of the compound in question show a marked lag before they begin to oxidize it at maximal rate, whereas cells grown in its presence can oxidize it immediately at maximal rate.

Present evidence suggests that the terminal respiration of *P. fluorescens* occurs via the tricarboxylic acid cycle, and that the enzymes of the cycle itself are constitutive (Barrett and Kallio, 1953).[2] Hence the inducibility of the attack on any given primary substrate reflects the induction of the enzyme or enzymes required to convert this primary substrate into a member of the terminal respiratory cycle, where constitutive control takes over. This may be a very simple operation, enzymatically speaking, if the primary substrate already possesses a molecular configuration similar to that of a cycle component. On the other

[2] The interpretation of experiments bearing on this point is exceedingly complicated, and will be considered in detail in a later section.

hand, there are many oxidizable substrates which differ very substantially in structure from the components of the cycle, and in such cases the enzymatic maneuvers necessary to break them up into metabolic fragments capable of passing into the cycle may be extremely elaborate. We may illustrate this point by considering the oxidation of L-tryptophan (Stanier and Hayaishi, 1951; Stanier, Tsuchida and Hayaishi, 1951; Hayaishi and Stanier, 1951). Fig. 1 shows grossly the inducible nature

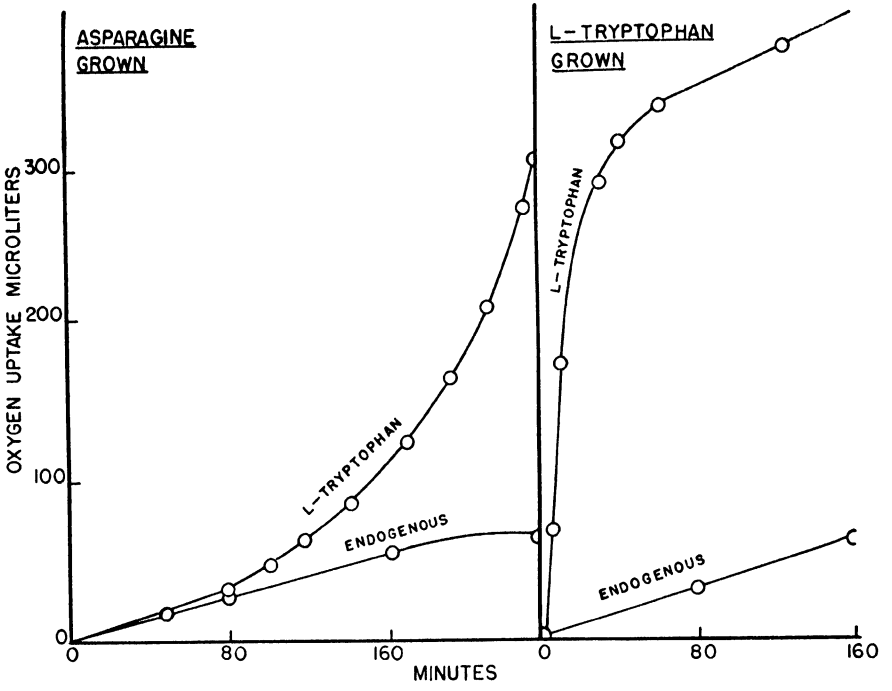

Fig. 1. The effect of conditions of cultivation upon the oxidation of L-tryptophan (2 mμ moles) by a pseudomonad.

of tryptophan oxidation by *P. fluorescens*. Cells grown at the expense of tryptophan oxidize it immediately at a high rate, whereas cells grown on asparagine begin to manifest oxygen uptake at the expense of tryptophan only after a lag of some 80 minutes. This lag masks a long series of inductive and enzymatic events. The biochemical mechanism for the dissimilation of L-tryptophan, as elucidated by enzymatic studies on cell-free extracts prepared from tryptophan-grown cells (Hayaishi and Stanier, 1951), is shown in Fig. 2. The fate of the ultimate C_6 fragment, β-ketoadipic acid, is not yet known, but there is some evidence (Kilby, 1951) to suggest that it is split, possibly after acyl activation, into succinic

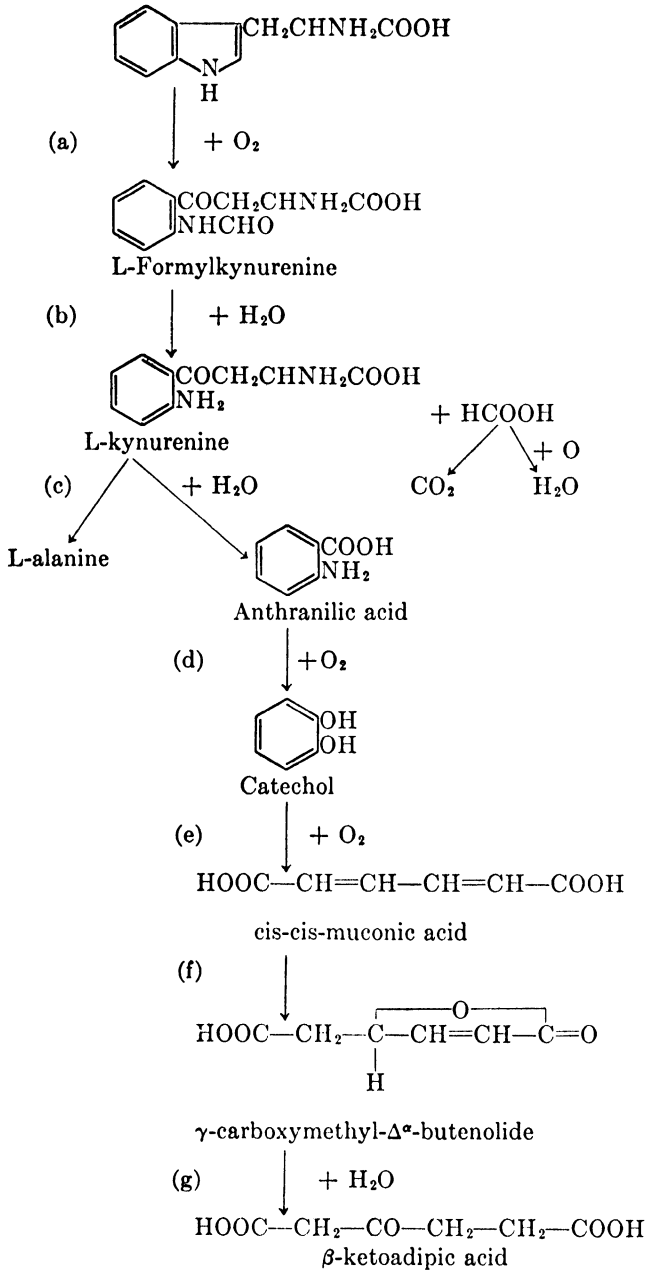

Fig. 2. The biochemical pathway for the oxidation of L-tryptophan by *Pseudomonas fluorescens*. The following inducible enzymes are involved: (a) tryptophan peroxidase + oxidase; (b) formylase; (c) kynureninase; (d) anthranilic acid oxidase (probably a multienzyme system); (e) pyrocatechase; (f) lactonizing enzyme; (g) delactonizing enzyme. The further breakdown of β-ketoadipic is catalyzed by an inducible enzyme, the decomposition of alanine is a constitutive reaction.

acid and a C_2 fragment on the acetate level of oxidation. Both these fragments, together with the pyruvate derived in a much earlier step via L-alanine from the sidechain of the tryptophan molecule, can then enter the terminal respiratory cycle. The net effect of all these enzymatic maneuvers has been to break up the primary cyclic substrate into aliphatic fragments which together contain nine of the eleven original constituent carbon atoms of tryptophan, the two remaining ones having been lost en route as CO_2. This operation has involved the action of at least eight distinct enzymes, and a more refined analysis of individual step-reactions might well double the number. In so far as we have possessed sensitive assays for specific enzymes of the sequence, we have been unable to detect appreciable activities in extracts prepared from uninduced (viz., asparagine-grown) cells; it seems probable that all the enzymes involved in the conversion of tryptophan to alanine and β-ketoadipic acid are strictly inducible. Hence in this case exposure of the cells to a single inducibly-attacked substrate evokes the synthesis of an array of enzymes at least as varied and as numerous as the enzymatic apparatus of glycolysis; with the exception of tryptophan peroxidase, they are all formed by endogenous inductions.

Other experiments (Sleeper and Stanier, 1950; Evans, Smith, Linstead, and Elvidge, 1951; Gunsalus, Gunsalus, and Stanier, 1953; Gunter, 1953; Stanier and Ingraham, 1954; Sistrom and Stanier, 1954) have shown that the oxidations of many isocyclic compounds by *P. fluorescens* merge at one point or another with the pathway for tryptophan oxidation, and Fig. 3 shows the complex network of dissimilatory processes, all eventually funnelling through β-ketoadipic acid, that has been established so far. Every step shown in Fig. 3 is inducibly controlled, and a unique inductive pattern is established in the cell by exposure to each primary substrate or intermediate. A simple calculation reveals the order of magnitude of inducible enzymes which this bacterium must be capable of synthesizing through sequential inductions in order to carry out its known dissimilatory activities. The scheme shown in Fig. 3 accounts biochemically for the oxidation of only four primary substrates: L-tryptophan, D-mandelic acid, phenol and *p*-hydroxymandelic acid. The *minimum* number of inducible enzymes required for conversion of these four compounds to components of the terminal respiratory cycle is eighteen. In the class of aromatic substrates alone, there are at least six inducibly-attacked compounds whose metabolism has not yet been studied in detail, but which are known on the basis of cursory study to be dissimilated by pathways which differ from the one well-established pathway

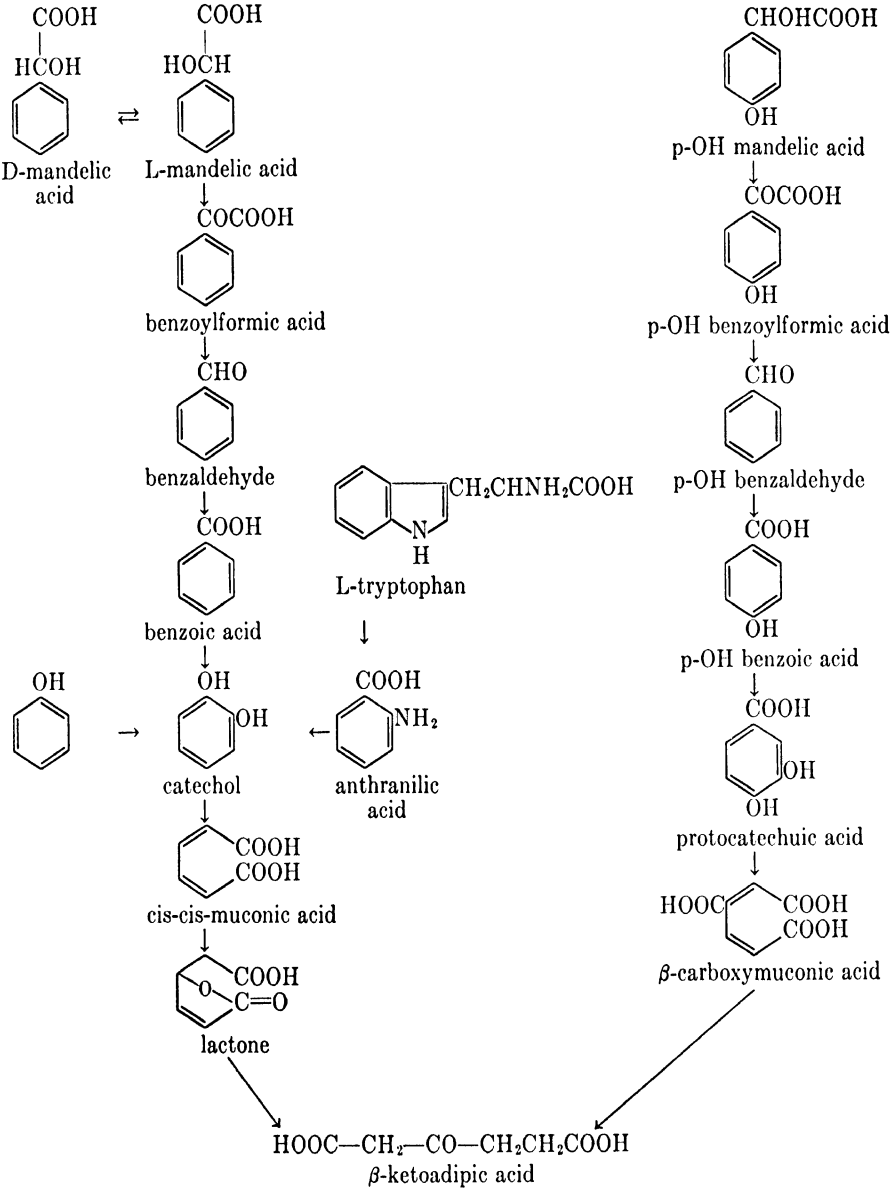

Fig. 3. The network of pathways for the oxidation of aromatic compounds by *Pseudomonas fluorescens* which leads eventually to the formation of β-ketoadipic acid. Every step shown is under strictly inducible control. Apart from the two sequences mandelic acid → benzoic acid and *p*-OH mandelic acid → *p*-OH benzoic acid, which are probably catalyzed by a common set of enzymes, each step in the various parallel pathways is catalyzed by a different inducible enzyme.

through β-ketoadipic acid. The whole group of aromatic substrates comprises, of course, only a small fraction of the array of compounds potentially oxidizable by *P. fluorescens*. If we set 50 as a reasonable figure (probably low!) for the number of primary substrates attacked by this bacterium, it follows that at least 200 inducible enzymes must participate in their dissimilation. The entire tricarboxylic acid cycle operates with about eight enzymes; hence in the oxidative metabolism of *P. fluorescens* the inducible enzymes probably outnumber the constitutive ones by about 20 to 1. Indeed, the extreme metabolic versatility manifested by *P. fluorescens* is no doubt possible only because such a large part of the enzymatic apparatus of dissimilation lies under inducible control; these enzymatic potentialities are stored away in the genome, to be simply and rapidly activated through sequential induction when a suitable primary substrate enters the environment.

II. INDUCTION OF ENZYMES CONCERNED WITH TERMINAL RESPIRATION

The shifts of enzymatic pattern so far discussed concern what one might term the peripheral enzymatic machinery of the cell, namely, the arrays of enzymes whose function it is to bring primary substrates into the central respiratory cycle. Since the enzymes of the tricarboxylic acid cycle itself are of such cardinal importance in oxidative metabolism, it might be imagined that, at least in strictly aerobic organisms, their levels would not be subject to major fluctuations; this is precisely the kind of enzyme system for which a teleologically-minded biologist would posit constitutivity. Yet paradoxically enough, Karlsson and Barker (1948) encountered sequential induction during a study on the oxidation of intermediates in the tricarboxylic acid cycle by a strictly aerobic bacterium, *Azotobacter agilis*. They found that cells grown at the expense of acetate showed marked adaptive lags in the oxidation of such cycle components as α-ketoglutarate, succinate, and fumarate. Succinate-grown cells immediately oxidized all the C_4 dicarboxylic acids of the cycle, although still showing a lag in oxygen uptake with α-ketoglutarate. Cells grown on α-ketoglutarate showed immediate oxidation of all these members of the cycle. Some typical manometric data are shown in Figs. 4 and 5. Interpreted in the light of the concept of sequential induction, these facts suggested that acetate was not oxidized via the cycle, and since this interpretation accorded with the results of tracer experiments, Karlsson and Barker concluded that in *Azotobacter agilis* the tricarboxylic acid cycle was inoperative as a general mechanism of terminal

respiration. It was clear that at least parts of the normal cyclic mechanism could be induced sequentially by exogenous provision of cycle components; but the very fact that such inductions were unidirectional (e.g. no induction of α-ketoglutarate oxidation by succinate) suggested very strongly that they were noncyclic. Further evidence for the absence of a tricarboxylic acid cycle was furnished by the discovery of a remark-

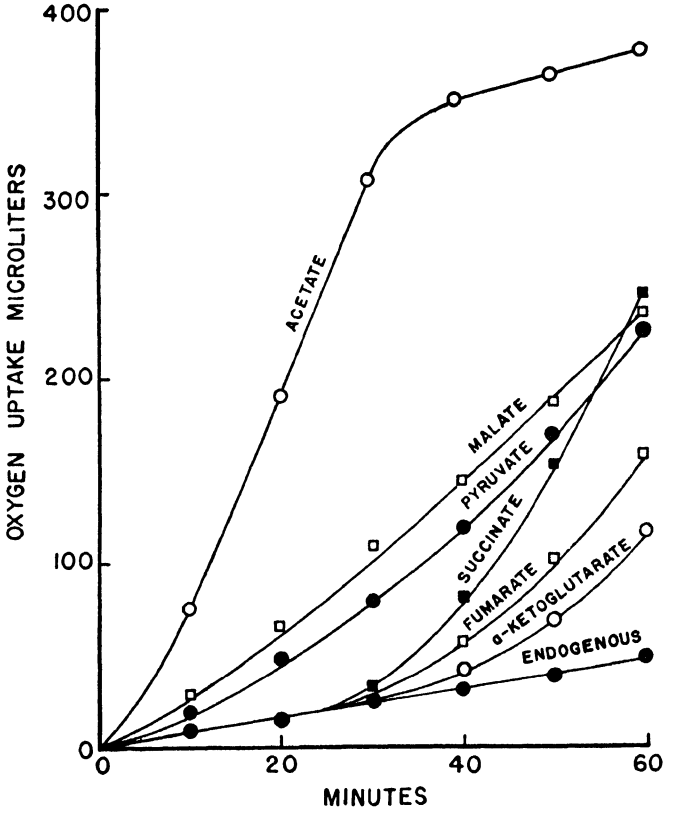

Fig. 4. Respiration curves for acetate-grown cells of *Azotobacter agilis*. Taken from Karlsson and Barker (1948a).

able biochemical mutant (Karlsson and Barker, 1948b), which had lost the ability to grow at the expense of all normal substrates for *A. agilis* (sugars, dicarboxylic acids, pyruvate) with three exceptions: ethanol, acetate, and malonate. Biochemical analysis (Karlsson, 1950) showed that this mutant has a defective ability to metabolize pyruvate and oxalacetate; the C_4 dicarboxylic acids are still oxidizable, but are quantitatively converted to a mixture of oxalacetate and pyruvate, which are

excreted into the medium. Despite this, acetate oxidation by the mutant strain is indistinguishable in rate or extent from acetate oxidation by the parent strain. Hence the evidence for the absence of a tricarboxylic acid cycle in this bacterium appeared most compelling.

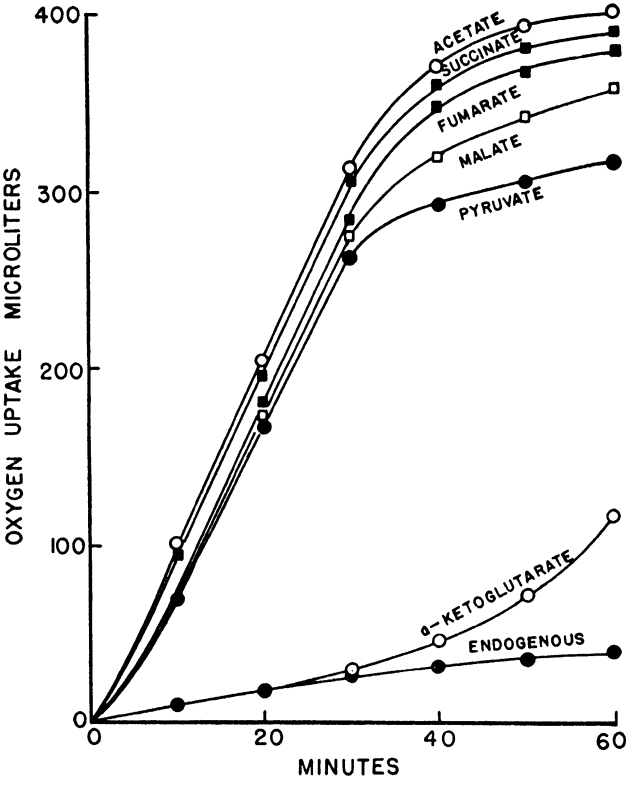

Fig. 5. Respiration curves for succinate-grown cells of *Azotobacter agilis*. Taken from Karlsson and Barker (1948b).

The experiments of Karlsson and Barker were conducted exclusively with intact cells; and when other investigators turned to the examination of cell-free extracts, some very curious discrepancies began to emerge. In 1950, Stern, Shapiro, and Ochoa demonstrated that acetate-grown cells of *A. agilis* contain substantial quantities of the condensing enzyme, which initiates the reactions of the cycle by the condensation of acetyl-CoA and oxalacetate to form citrate. Working with another species, *A. vinelandii,* Stone and Wilson (1952a, 1952b) found that crude extracts could immediately oxidize the acids of the cycle, whereas the intact cells from which these extracts were prepared showed varying lag periods

before oxidizing the same substrates. Their extracts were capable of oxidizing acetate; and acetate oxidation could be "sparked" by the presence of C_4 dicarboxylic acids and greatly stimulated by coenzyme A, both features strongly suggestive of an oxidation via the classical tricarboxylic acid cycle. In 1953, Repaske and Wilson performed similar experiments with Karlsson and Barker's strain of *A. agilis* grown on acetate, and again found the presence of a complete complement of enzymes for the cycle in extracts.

Thus a striking difference between the behavior of whole cells and of extracts was established. Judged by patterns of induction determined with whole cells, the members of the *Azotobacter* group did not appear to use the tricarboxylic acid cycle as a mechanism of terminal respiration; yet extracts from these cells contained all the enzymes of the cycle, and at reasonably high levels. When such discrepancies are encountered in biochemical work, it is customary to ascribe them to "permeability," and for the biochemist the invocation of this magic word puts an end to further thought about the matter. However, the kinetics of the oxidations of cycle components by members of the *Azotobacter* group, as exemplified by the data shown in Figs. 4 and 5, are not of the kind that one would expect to result from a permeability barrier. Typically, a permeability barrier to the entry of an oxidizable substrate into the cell is reflected by an oxidation at a *constant* rate, far less than the rate of oxidation of a precursor substance to which the cells are freely permeable; in the extreme case, the rate of oxidation is zero. A typical example of a permeability effect of this kind is shown in Fig. 6, taken from our own work. β-Ketoadipic acid is a highly polar substance, and is oxidized by intact cells at a steady rate never more than 30 per cent of the rate at which aromatic precursors are oxidized; nevertheless, enzymatic studies show beyond doubt that it is a central metabolite in the oxidation of many aromatic compounds. Hence no simple concept of a permeability barrier can permit an adequate explanation of the behavior of the *Azotobacter* group towards cycle intermediates.

Similar discrepancies between the behavior of intact cells and cell-free extracts with respect to the oxidation of cycle components have emerged in studies on other strictly aerobic bacteria. Some workers have accepted the results obtained with intact cells at face value, and have concluded that in such bacteria the classical tricarboxylic acid cycle is inoperative. Others, particularly those with an enzymological training, have taken the findings with cell-free extracts as proof of the operation of the cycle. Surprisingly, few workers have realized that the observed discrepancies re-

flect a very subtle and interesting problem in cellular physiology, which calls for closer analysis. By far the best attempt at such an analysis has been made by Barrett, Larsen, and Kallio (1953) and Barrett and Kallio (1954), who used *P. fluorescens* as their biological material. Like the members of the *Azotobacter* group, *P. fluorescens* shows pronounced lags in oxygen uptake at the expense of some cycle components after growth on acetate; but a careful study of its oxidative behavior after

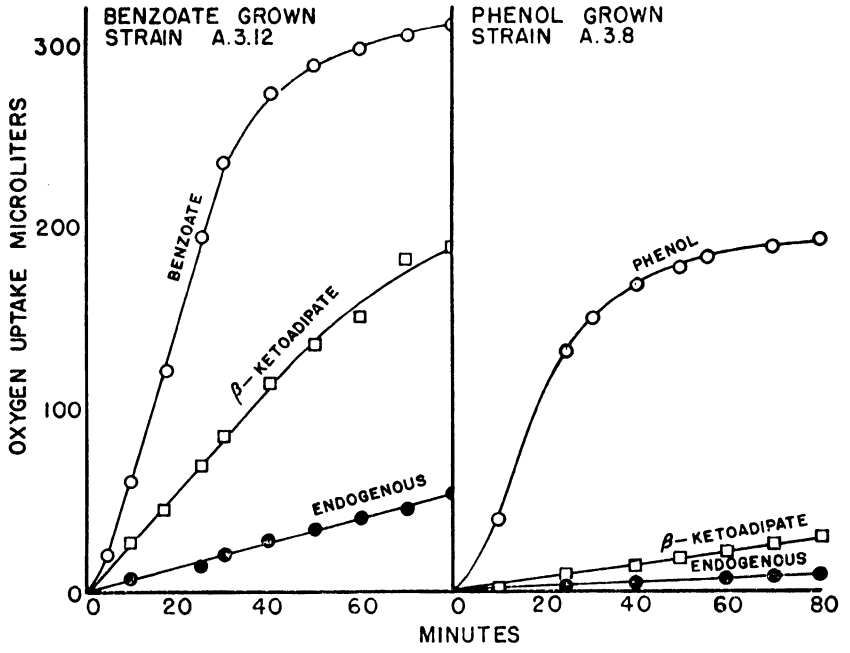

Fig. 6. The oxidation of β-ketoadipate by *Pseudomonas fluorescens*, as compared to the oxidation of aromatic precursors. Note that the rate of oxidation of β-ketoadipate is far less than the rate of oxidation of the aromatic precursors, and that it is constant. This is an example of a classical permeability effect.

growth at the expense of each main cycle component (Barrett and Kallio, 1954) failed to reveal the orderly patterns of sequential induction which Karlsson and Barker had found in *Azotobacter agilis*. Some typical data with *P. fluorescens* are shown in Table I. Barrett and Kallio then proceeded to compare the specific activities of certain key enzymes of the cycle in extracts prepared from cells grown on acetate and on citrate. In both extracts these enzymes could be readily demonstrated, and the levels were essentially identical. One is, accordingly, forced to conclude that the enzymes of the tricarboxylic acid cycle are constitutive in *P. fluores-*

TABLE I. Patterns of induction established in *Pseudomonas fluorescens* by growth at the expense of members of the tricarboxylic acid cycle.*

Growth substrate	Immediate oxidation of:	Inductive lag with:
Citrate	Succinate Pyruvate Malate Fumarate Citrate *cis*-Aconitate	Acetate α-Ketoglutarate
Succinate	Succinate Pyruvate Malate Fumarate α-Ketoglutarate	Acetate Citrate *cis*-Aconitate
Malate	Succinate Malate Pyruvate	Acetate Citrate α-Ketoglutarate Fumarate
Pyruvate	Citrate *cis*-Aconitate Malate Succinate Fumarate Pyruvate Acetate	α-Ketoglutarate
Acetate	Succinate Malate Fumarate Pyruvate Acetate	α-Ketoglutarate Citrate *cis*-Aconitate

* Data taken from Barrett and Kallio (1953).

cens. Such being the case, however, some explanation has to be furnished for the lags in oxygen uptake shown by intact cells. If such lags do in fact reflect induced enzyme syntheses, it should be possible to abolish the induction completely by the use of selective inhibitors which are known to block enzyme synthesis without affecting other pre-existing cellular activities. Two such highly selective inhibitors exist: ultraviolet irradiation, and treatment with amino acid analogs. Barrett, Larsen, and Kallio (1953) showed that either of these treatments extended indefinitely the lag in the oxidation of citrate by fumarate-grown cells; and further, that

the effect of an amino acid analog could be completely reversed by the addition of the corresponding amino acid (Fig. 7). Similar results with ultraviolet irradiation have been reported for the same bacterium by Kogut and Podoski (1953). These findings provide very powerful evidence to support the view that the observed lags in the oxidation of cycle

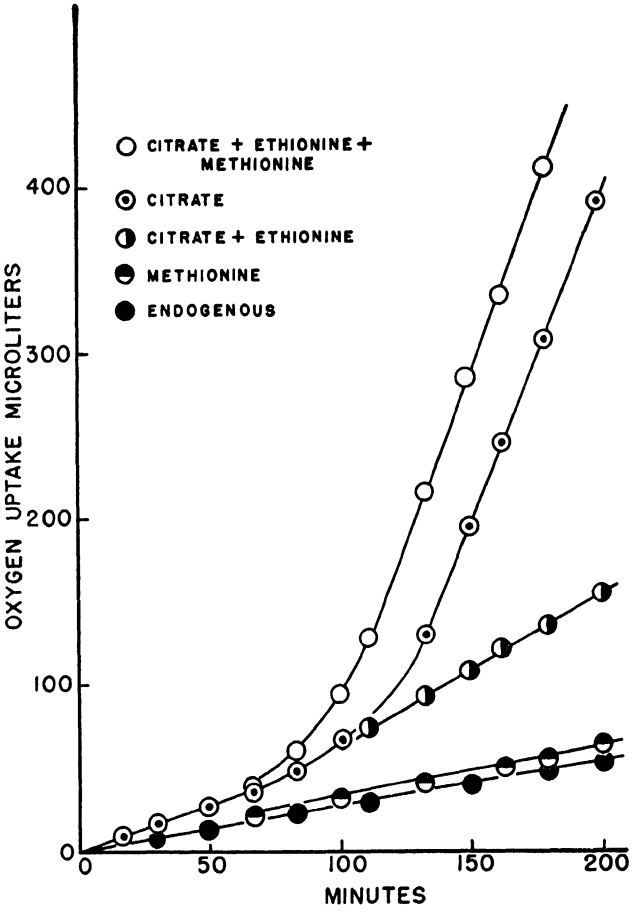

Fig. 7. The inhibitory effect of ethionine on the overcoming of the lag in citrate oxidation by fumarate-grown cells of *Pseudomonas fluorescens*, and the reversal of this effect by addition of methionine. Data taken from Barrett, Larson, and Kallis (1953).

intermediates do reflect the initial absence of enzymes whose formation is induced by exposure to the substrates in question. However, these enzymes are clearly not the ones that catalyze the reaction of the cycle proper; the best guess seems to be that they are transport enzymes. It is possible that they are not concerned with transport of cycle intermediates

across the cell membrane, but rather with the introduction of these intermediates into a normally closed cyclic system localized within the cell, which in the uninduced condition can be freely entered only at certain points, notably the initial condensing reaction (acetyl-CoA + oxalacetate → citrate). A further analysis of this fascinating problem is severely handicapped by our ignorance of the reactions catalyzed by these inducible systems, which consequently cannot be assayed *in vitro*.

Thus the net result of much work on strictly aerobic bacteria has been the confirmation of something which the naive biochemist would have predicted *a priori;* namely, that the intracellular levels of the enzymes of the terminal respiratory cycle do not change appreciably, since the cycle is the final acceptor of the carbon fragments derived from the oxidation of primary substrates. In general, substantial fluctuations in the levels of the cycle enzymes themselves might be expected to occur only in the facultative anaerobes, which can shift to fermentative processes of substrate breakdown not involving the tricarboxylic acid cycle. Indeed, we now possess very good evidence that such changes do take place in the case of two facultative anaerobes.

The demonstration that a shift from anaerobiosis to aerobiosis causes profound changes of enzymatic constitution was first provided by Slonimski (1953) for the yeast *Saccharomyces cerevisiae*. This work was an outgrowth of studies on the cytoplasmic mutation of yeast to respiratory deficiency induced by treatment with acridines. These so-called "petite colonie" mutants have lost irreversibly and nearly completely their ability to respire, as a result of the elimination of certain respiratory enzymes, notably cytochromes a and b and cytochrome oxidase. Slonimski observed that the growth of normal yeast in the absence of oxygen brings about a similar suppression of respiratory activity, likewise accompanied by changes in the cytochrome system. In contrast to acridine-induced respiratory deficiency, the respiratory deficiency induced in normal yeast by anaerobic cultivation is freely reversible; if such cells are aerated for some hours in the presence of glucose, the cytochrome spectrum gradually changes to that characteristic of aerobically-grown cells, with an accompanying restoration of respiratory activity. A more extensive analysis showed that anaerobic growth has effects on enzymatic constitution that extend far beyond the cytochrome system. The levels of virtually all enzymes operative in the tricarboxylic acid cycle are much reduced, in extreme cases thirtyfold; and the reformation of these enzymes likewise takes place when anaerobically-grown cells are exposed to oxygen in the presence of glucose.

Hence it is evident that in yeast the systems of terminal respiration and aerobic electron transport fall to physiologically nonfunctional levels during anaerobic growth, but can be resynthesized when the cell is exposed to oxygen.

A second, very similar example of such behavior has been described recently in a bacterium, *Pasteurella pestis,* by Engelsberg, Gibor, and Levy (1954) and Engelsberg, Levy, and Gibor (1954). Unlike yeast, *P. pestis* is still capable of consuming oxygen at the expense of glucose after a period of anaerobic growth; but this reaction is not cytochrome-linked and results in a very low total uptake, accompanied by the accumulation of large amounts of pyruvic acid and other organic end-products which can be further oxidized only at negligible rates, owing to the very low activities of the enzymes of the tricarboxylic acid cycle and of the aerobic electron transport system. Cells grown aerobically catalyze a far more extensive oxidation of glucose, unaccompanied by the excretion of organic end-products. Anaerobically grown cells can be converted to the pattern of aerobically grown ones by aeration for several hours in the presence of glucose and casein hydrolysate; during this time, growth is negligible. Enzymatic and spectroscopic studies have shown that this conversion is accompanied by the synthesis of cytochromes and of the enzymes of the tricarboxylic acid cycle. Just as in yeast, these increases in specific activity may be very large; the specific activities of condensing enzyme and of isocitric dehydrogenase, for example, are raised twenty-fold.

It is thus evident that in two facultative anaerobes exposure of the cells to oxygen can cause profound and complex enzymatic changes. However, the mechanism of oxygen induction is far from clear. In both yeast and *P. pestis,* the disappearance of the normal cytochrome system under anaerobic conditions is accompanied by a marked diminution of the total hemin content of the cells, and one might thus postulate that the primary site of action of oxygen is in the biosynthetic chain leading to the formation of hemins. In anaerobically grown yeast, however, where the depletion of hemins is far less marked than in *P. pestis,* there still remains a quantity of protoheme sufficient for the production of considerable amounts of cytochrome (Slonimski, 1953), and the changes in the levels of the cytochromes themselves are many times greater than the change in hemin level. Hence it seems probable, at least in the case of yeast, that oxygen acts on the last steps of cytochrome synthesis, either by inducing synthesis of the apoenzymes, or by inducing the coupling of these apoenzymes with the heme group, and that the observed effects on hemin levels

are a secondary consequence of this. If one assumes such a primary mode of action for oxygen, it becomes very tempting to consider all the other complex changes in enzyme levels which accompany the shift from anaerobiosis to aerobiosis as the consequences of a sequential induction, albeit one which differs in certain respects from the sequential inductions considered in the preceding section. In the case of the aromatic oxidations, the sequentiality is established at the substrate level and affects the chain of substrate dehydrogenations, not the transport system of the cell; in the case of respiratory induction, the sequentiality is established at the other end of the respiratory system. As Slonimski (1953) has suggested, the enzymes of respiratory electron transport have two specific substrates, a donor and an acceptor, both of which might have to be present in order for enzyme synthesis to take place. A completely satisfactory interpretation of all the known facts about respiratory induction is, however, not possible on the basis of present knowledge.

III. INDUCED CHANGES IN THE PIGMENT SYSTEM OF PHOTOSYNTHETIC BACTERIA

We have begun to explore the possibility of induced shifts in the enzymatic patterns of facultatively photosynthetic organisms, and I should like to conclude this survey by discussing some of the recent work on this problem by Dr. Germaine Cohen-Bazire and myself. So far, we have limited ourselves to a gross examination of changes in the photochemical apparatus, as reflected by changes in the levels of the photochemically active pigments. As biological material, we have used the bacterium *Rhodopseudomonas spheroides,* a photoheterotroph which can grow aerobically in the dark by oxidizing organic compounds, or anaerobically in the light by using the same organic compounds as hydrogen donors for bacterial photosynthesis. The pigment system consists of three chlorophyll-proteins with distinct infrared peaks at 805, 855 and 875 mμ, all containing the same molecular species of chlorophyll (bacteriochlorophyll), together with a mixture of yellow and red carotenoids which confer upon the cells their characteristic color. Occasional so-called "green" mutants arise in normal cultures. They differ from the wild type only in that they contain a single yellow carotenoid; the chlorophyll spectrum is unchanged, and they are still fully capable of photosynthetic development. Since the presence of a single carotenoid greatly simplifies the quantitative determination of carotenoid content, we have mostly used one of these mutants in the experiments to be described below.

The synthesis of the pigment in *R. spheroides* is controlled by two principal factors: light intensity and oxygen. The effect of oxygen is illustrated by the data in Fig. 8. In this experiment, three identical cultures were prepared from cells grown anaerobically in the light. One culture was maintained anaerobically and illuminated; one was main-

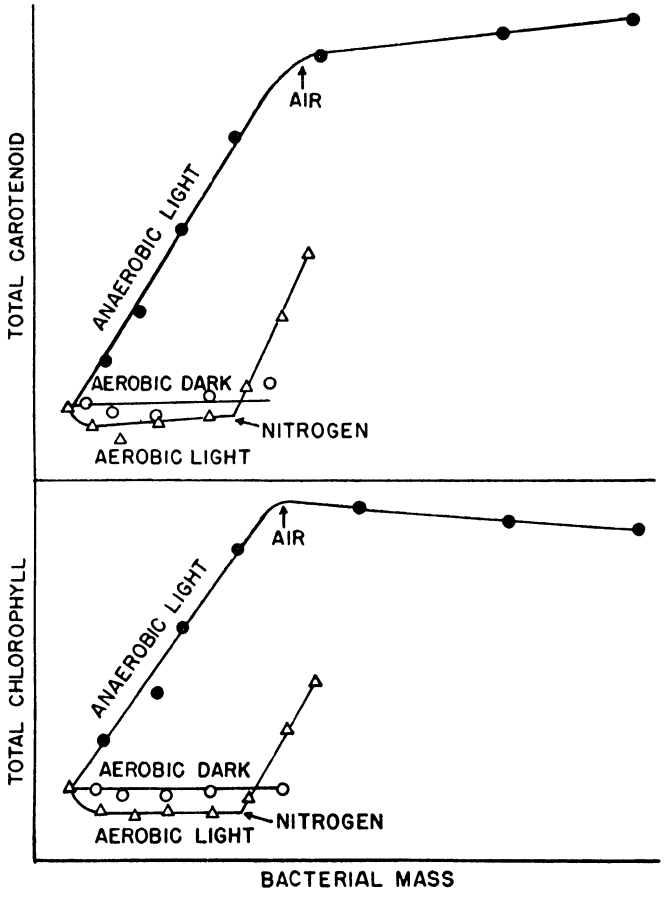

Fig. 8. The effect of oxygen on the synthesis of chlorophyll and carotenoid by growing populations of *Rhodopseudomonas spheroides.*

tained aerobically and illuminated; and one was maintained aerobically in the dark. In all cases growth was exponential from the start, and the growth rates were nearly identical. In Fig. 8 the pigment concentration of the cells from the three cultures has been plotted as a function of the total amount of cell material; in other words, the graph shows the *differential rate of pigment synthesis.* The differential rate of both

chlorophyll and carotenoid synthesis in the presence of air is essentially zero, whether in light or dark. Only the cells exposed to light under anaerobic conditions continue to synthesize pigment. After about 1.5 cell divisions had occurred, at the point indicated by an arrow on the graphs, the gas mixtures passed through the two illuminated cultures were reversed. It can be seen that this caused a more or less abrupt change in the differential rates of pigment synthesis. The culture which passed from anaerobiosis to aerobiosis ceased to synthesize both pigments, whereas the culture passed from aerobiosis to anaerobiosis began to manufacture both at high differential rates. Since time is excluded as a parameter in a plot of this kind, the effect of the change in gaseous environment on growth rates cannot be seen. In fact, the growth rate of the anaerobic culture was unaffected by the introduction of oxygen, whereas that of the aerobic culture fell to zero when anaerobic conditions were introduced, and growth was resumed only after a lag of several hours. Hence despite continuous illumination, the preceding period of aerobic cultivation had resulted in a serious derangement of the photosynthetic apparatus. This is caused partly, of course, by simple dilution of the photochemically active pigments through aerobic growth, but the magnitude of the observed effects suggests that other changes in the system may be occurring concurrently. If *Rhodopseudomonas spheroides* is passed through several subcultures in the dark under strongly aerobic conditions, the pigment content of the cells falls to very low levels. Such cells, when placed once more under strictly anaerobic conditions in the light, sometimes remain in the lag phase for as long as 30 hours, since the only source of the energy required for pigment synthesis in anaerobiosis is photosynthesis, of which the cells have become virtually incapable. During the initial stages of recovery the differential rate of pigment synthesis reaches a very high value; in fact, it takes place essentially without growth.

The effects of light intensity on pigment synthesis are also very marked. Under anaerobic conditions, the rate of both chlorophyll and carotenoid synthesis is an inverse function of light intensity. Fig. 9 shows the effect of transferring highly pigmented cells, previously grown under conditions of low light intensity, to a high light intensity. Growth is exponential from the start, whereas there is a total suppression of both chlorophyll and carotenoid synthesis for about one generation. When the pigment content of the cells has fallen through growth to the level characteristic of the new light intensity, pigment synthesis resumes, and thereafter proceeds at a rate parallel to the growth rate. Whether one

starts out with weakly pigmented or highly pigmented cells, the rate of pigment synthesis in the two growing populations eventually becomes identical at any given light intensity. In addition to the quantitative

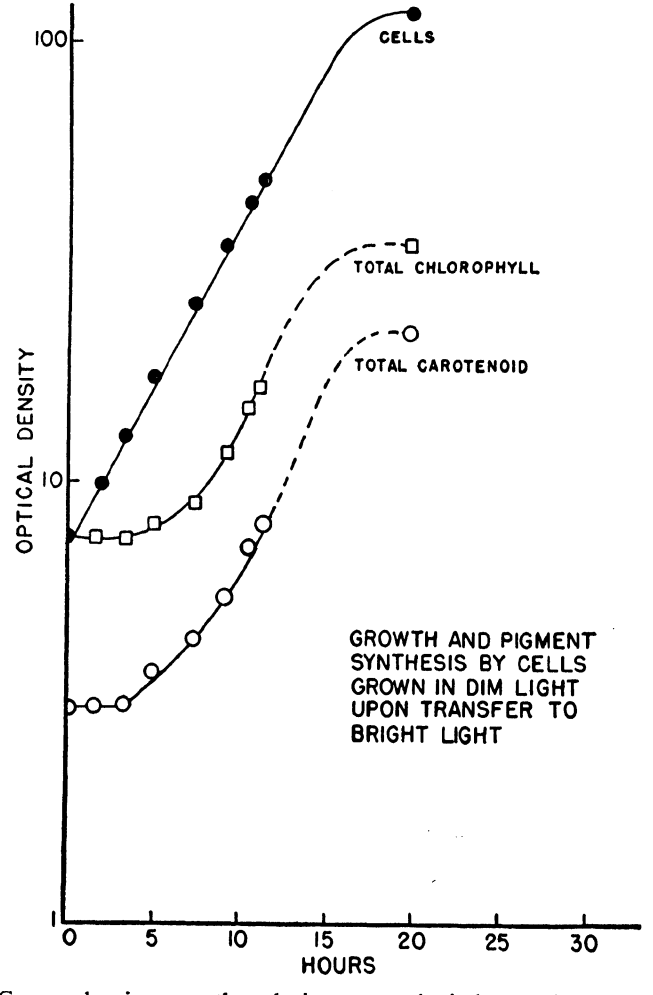

Fig. 9. Curves showing growth and pigment synthesis by a culture of *Rhodopseudomonas spheroides* cultivated in bright light after a preceding period of cultivation in dim light. Pigments determined after methanol extraction, and expressed in arbitrary units.

effects which light intensity exerts on pigment synthesis, it has an interesting qualitative effect. Under conditions of high light intensity, synthesis of the chlorophyll-protein with a peak at 875mμ is more rapid than the synthesis of the two other chlorophyll-proteins. This is illus-

trated by Fig. 10, which shows the absorption spectrum of cells measured at equivalent cell densities before and after a short period of growth under conditions of high light intensity.

In none of the experiments so far performed have we succeeded in dissociating sharply synthesis of the two classes of pigments, which show linked responses to all the environmental changes investigated. It is most

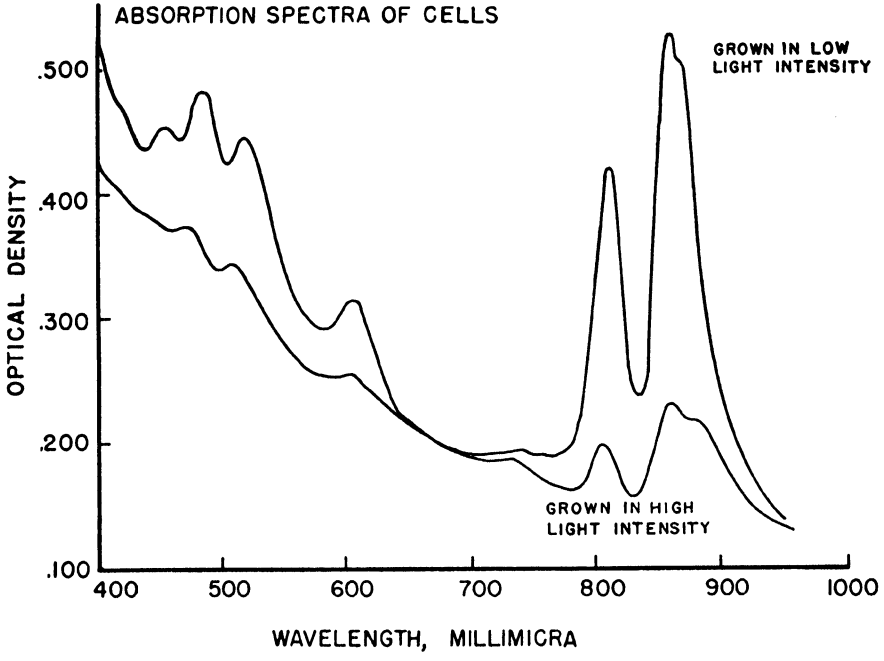

Fig. 10. Absorption spectra of whole cells, compared after growth at low and high light intensities. The two suspensions were adjusted to identical optical densities at 680 mμ, where light absorption by the pigments does not occur. Note the difference in the shape of the infrared portion of the spectrum, reflecting light effects in the ratio of the 875 mμ and 855 mμ chlorophyll proteins.

improbable on biochemical grounds that this linkage reflects a common pathway of biosynthesis. Since the two pigments are physically associated within the cell in very large submicroscopic particles, the so-called chromatophores (Schachman, Pardee, and Stanier, 1952), it is tempting to imagine that the effects of light and oxygen operate on the level of chromatophore synthesis rather than of pigment synthesis *per se,* but so far there is no evidence on this point.

IV. GENERAL CONCLUSIONS

When the occurrence of induced enzyme synthesis in microorganisms received general recognition about twenty-five years ago, the phenomenon appeared to play a relatively restricted role in the regulation of metabolism. The inductive systems originally studied were, or at least appeared to be, simple in enzymatic terms, involving the specific evocation by the inducer of a single enzyme. The discovery of this phenomenon did not, therefore, seem to call for any radical changes in general notions about enzyme synthesis: the bulk of the cell's enzymes were presumably "constitutive" and invariant in level, with inductive systems as a sort of peripheral adornment. In the past ten years, however, the discovery of sequential inductions has shown that a single inductive event may bring about profound changes of the cell's enzymatic constitution. This first became evident when complex sequential inductions by substrates were found, but is emphasized even more dramatically by the inductive effect of oxygen on the energy-yielding system of facultative anaerobes, since here it is the central cyclic reactions and mechanisms of aerobic electron transport that are affected. The effects of light and oxygen on synthesis of the photosynthetic pigment system of nonsulfur purple bacteria probably also involve major alterations in the levels of photosynthetic and respiratory enzymes, analogous to those observed in facultative anaerobes. The inductive effects of oxygen are at present admittedly difficult to interpret, although it is attractive to imagine that oxygen can also establish a series of sequential inductive events in the cell. Be that as it may, however, we can no longer consider any enzyme-synthesizing activity of a microbial cell as irrevocably fixed by the genome and beyond the direct influence of the environment. The possibility of manipulating enzyme levels experimentally by induction is limited in the main by the basic physiological adaptability of the cell: in strict aerobes, it appears that the levels of enzymes operative in the tricarboxylic acid cycle cannot be appreciably modified, for the reason that it is impossible to grow these organisms under conditions where the cycle is not necessarily in operation; in facultative anaerobes the existence of glycolysis as an alternate means of satisfying energy requirements makes it possible to bring about experimentally a major depression of the levels of the cycle enzymes.

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IV. SOME ASPECTS OF PROTEIN BIOSYNTHESIS IN DEVELOPMENT

BY JAMES D. EBERT[1]

For some years developmental biology has progressed steadily toward an ultimate goal of rational interpretation of structural and functional relationships in differentiation and growth at the molecular level. The emphasis at this symposium, in fact, its unifying theme, has been on experimental approaches directed toward the translation of our knowledge of interactions at the cell group or tissue level to the interactions of specific molecules. I propose to continue in this vein; my principal objective is to evaluate the extent to which current studies of protein biosynthesis contribute to our understanding of the mechanisms of differentiation and growth; to a lesser degree I will consider the converse question of whether developmental biology has contributed to our knowledge of the mechanism of protein biosynthesis. Attention will be centered on problems of embryonic development. I have no doubt that for the study of protein biosynthesis, the inductive (adaptive) enzyme systems of intact microorganisms (Monod and Cohn, 1953) and the protein-forming systems of disrupted cells (Gale and Folkes, 1954) may provide particularly favorable material, yet I do not believe that one must be preoccupied with the idea that differentiation is equivalent to the controlled production of enzymatic patterns (Spiegelman, 1948). My purpose is not to dispute the pragmatic value of this concept if it is stated clearly as one aspect of the differentiation process. The term 'differentiation' encompasses the totality of reactions of which a cell is capable at any given stage, and includes the sum total of changes in cell characters from one stage to the next (Weiss, 1949). Contrary to older opinion, the complement of proteins and other macromolecules of the early embryo is highly complex, as revealed by enzymologic, immunochemical, and nutritional studies (Moog, 1952; Ebert, 1952, 1953 a). Differentiation, growth and morphogenesis involve the following processes at the molecular level:

[1] Department of Zoology, Indiana University (contribution number 554). The original work from the author's laboratory has been supported by research grants from the American Heart Association, the National Science Foundation, and the National Heart Institute of the National Institutes of Health. A part of the author's work was carried out at the Medical Department, Brookhaven National Laboratory, sponsored by the Atomic Energy Commission.

I. CHANGES IN THE KINDS OF MOLECULES PRESENT

A. The addition of new molecular species. Changes in complement of proteins and other complex molecules undoubtedly accompany development; a consistent body of evidence has demonstrated changes in quantity, in association with other molecules or groups and in distribution of macromolecules in ontogeny (Ebert, 1952; Woerdeman, 1953). I use the word 'ontogeny' in the broad sense, to include postnatal development, for there are several well-founded examples of the appearance of previously undetected antigenic molecules well after birth (Briles, McGibbon, and Irwin, 1948; Irwin, 1949). While the question remains as to whether each protein is represented in the egg and its progeny in minute quantities (specimen or positive template molecules), in view of the number of complex molecules of an organism, such a rigid concept seems improbable.

B. The loss of macromolecules.

(1) From the entire organism, apparently without synthesis of related molecules to replace those lost: an example is found in the investigations of Pedersen (1944, 1947), who isolated by ammonium sulphate precipitation from the serum of the calf and fetal cow and sheep a new protein, apparently absent from adult serum. The sedimentation constant of this protein, named fetuin, was found to vary considerably with the concentration, and was different for fetal than for calf fetuin. According to Deutsch (1954), fetuin is a mucoprotein; however, its lability and tendency to aggregate in acid solution make critical immunochemical and physical studies difficult.

(2) From the entire organism, with sequential synthesis of related molecules to replace those lost: the development of the hemoglobin molecule is an excellent case in point. The hemoglobin of embryos has been shown by many methods to differ from that of the adult (Hall, 1934; Boyer, 1950). Embryonic and adult hemoglobins behave as different antigens (Darrow, Nowakovsky, and Austin, 1940), have different solubilities (Wyman, Rafferty, and Ingalls, 1944), sedimentation constants and electrophoretic mobilities (Andersch, Wilson, and Menten, 1944), and osmotic pressures (McCarthy and Popjak, 1947). While most investigators agree that it is the globin moiety which differs in the embryo from that of the adult, Riggs (1951) has proposed that, at least in the transition from larval to adult hemoglobin in the bullfrog, the difference may be due to a change in the mode of linkage between the heme and globin components. The sequential development of different

hemoglobins is illustrated best by the situation in man. Two systems of hemoglobin synthesis have been described, one leading to the formation of fetal hemoglobin, and the other replacing the first during normal development, leading to normal adult hemoglobin. The fetal and adult hemoglobins are sharply distinct as shown by immunochemical techniques (Chernoff, 1953; Goodman and Campbell, 1953) and by studies of their susceptibility to alkaline denaturation. The question is unresolved as to whether the capacity to synthesize fetal hemoglobin is ever completely lost, since "normal" adult individuals may possess traces of the fetal type molecule. Certain genetic blocks or environmentally induced anemias are characterized by the maintenance (or reactivation) of the fetal mechanism. The adult mechanism is also subject to genetic deviation, e.g., the sickle cell allele which leads to the formation of a distinctly different adult hemoglobin. Again, the synthesis of the globin, not of the heme, seems to be involved (reviewed by Neel, 1952; Horowitz and Owen, 1954).

(3) From certain regions of the embryo, but not from others, resulting in an altered spatial pattern of distribution of the macromolecule; the development of the contractile protein, myosin, in the heart-forming areas of the early chick embryo, is thought to be an excellent case in point. The evidence for this opinion will be assessed subsequently.

II. QUANTITATIVE CHANGES IN MOLECULAR POPULATION

Protein synthesis is a prominent aspect of cell maintenance, of the formation of specific cell products, e.g., glandular secretions, and of growth. Growth involves a net increase in protein, but whether it results from an increased rate of synthesis or an inhibition of protein breakdown, or both, is not clear. On the other hand progressive differentiation may involve either an increase or decrease in number of a given molecular type. It has been demonstrated convincingly (Levy and Palmer, 1940, 1943; Moog, 1952) that certain enzymes are more active in early stages than in later ones. However, a note of caution is necessary here: enzymes are generally measured by determination of their activity in tissue extracts or homogenates. Thus one cannot be certain that a change (increase or decrease) in activity results from a change in concentration of the enzyme rather than from an alteration in turnover rate.

III. STRUCTURAL TRANSFORMATIONS OF PROTEINS

In the foregoing discussion I have dealt with problems of differentiation in terms which imply that differentiation may be no more than a chain of chemical reactions of which the net result is the localized syn-

thesis of molecular species characteristic of a particular tissue. This limited view of differentiation neglects the interaction of molecular populations which lead to selective molecular groupings and structural order. I suggest that in the formation of complexes with definite spatial orientation, a factor of singular importance may be the ability of certain proteins and nucleoproteins to undergo reversible transformations from the globular to the fibrous form either by end-to-end aggregation of globular molecules or by unfolding of the molecule. On the basis of his finding that of the well-known animalizing and vegetalizing agents of the sea urchin egg the former cause a molecular transformation from the fibrous to the globular form (depolymerization), and the latter stabilize proteins in the fibrous state, Ranzi (1953) has proposed that such transformations may play an important role in normal differentiation. Other lines of evidence support the view that reversible molecular transformations of proteins may have a major role in cell activity and movement, e.g., the striking demonstration by Mazia and Dan (1952) of the fibrous protein nature of the spindle fibers which periodically disappear and are reaggregated in dividing cells, and the evidence presented by Goldacre and Lorch (1950) for the role of contractile proteins in ameboid movement (cf. Meyer and Mark, 1951). Hayashi (1953) has initiated a frontal attack on these problems; he suggests that cellular structures are formed by and composed of protein (or possibly lipoprotein) molecules, surface-spread at the interfaces of the heterophasic system within the cell. A number of facts clearly demonstrate the existence of a highly organized submicroscopic protein lattice in cytoplasm (Porter, 1954); the major problems now before us are (1) to determine the origin of preferential orientations within this lattice and (2) to ascertain the pathways by which the chemical energy of metabolites is transferred to the molecular links of the lattice.

IV. PENTOSE NUCLEIC ACID PRECURSORS, PENTOSE NUCLEOPROTEIN GRANULES AND PROTEIN SYNTHESIS

I am not unmindful of the wealth of evidence which compels the continued search for the role of pentose nucleic acid (PNA) in protein synthesis; however, this subject has been considered in detail recently (Brachet, 1952; Pollister, 1954), and progress since the publication of these comprehensive reviews does not compel a reevaluation of the field. Therefore, my remarks will be limited to those recent findings which hold promise for future study or bear directly on other topics under discussion. Comparisons with respect to PNA content and capacity for

protein synthesis in different types of cells led Caspersson (1950) and Brachet (1947) to suggest that PNA is in some way connected with protein synthesis. Attempts to proceed beyond this point have hardly been successful until recently. Indeed, Daly and Mirsky (1953) have offered evidence which indicates that Caspersson's contention that the quantity of PNA in the pancreas varies during the cycle of synthesis and secretion is incorrect. Yet Allfrey, Daly, and Mirsky (1953) have presented data which support the view that cytoplasmic pentose nucleoprotein is part of the cell's equipment for protein synthesis and that the protein component of the nucleoprotein functions as a precursor material for proteins synthesized in the cell. It appears, therefore, that while PNA may be involved in protein synthesis, an absolute correlation between PNA content and protein formation may be unnecessary. This conclusion is in agreement with findings of Monod, Pappenheimer, and Cohen-Bazire (1952), and Pardee (1954) that the PNA found in the bacterial cell is largely inert and that the *continuous formation* of PNA [but not desoxypentose nucleic acid (DNA)] is essential for protein synthesis. Monod et al. found, in testing the requirements for presence of amino acids or amino acid components for enzyme induction, that a uracil-requiring mutant of *Escherichia coli* was unable to produce beta-galactosidase in the absence of uracil. Pardee's more extensive investigation showed that strains of *Escherichia coli* which require purines, pyrimidines, or phosphate for growth also require these compounds for enzyme induction; within a few minutes after exhaustion of the compounds from the medium, induction of enzymes ceases. The data suggest that the effect is not due to blocking of amino acid synthesis or to destruction of existing enzyme. That DNA synthesis is unnecessary was shown by the fact that inhibition of DNA synthesis did not affect enzyme induction. An approach which offers considerable promise of reconciling the divergent views on the role of nucleic acids in the synthesis of proteins and in the incorporation of (labeled) amino acids into preformed protein is found in the demonstration by Gale and Folkes (*op. cit.*) that suspensions of disrupted bacterial cells provide an experimental system with which the effect of nucleic acids on protein synthesis can be studied. The major findings of the investigation thus far can be summarized briefly: (1) Either DNA or PNA can render amino acid residues in already-formed protein exchangeable. Exchange reactions take place with all amino acids tested, but to varying extent; the data available do not permit an explanation of the different degrees of incorporation obtained with different amino acids. (2) A comparison of the effectiveness of

DNA, PNA, and a purine-pyrimidine mixture in the production of three enzymes (an enzyme system producing acid from glucose, catalase, and beta-galactosidase) suggests the following functions: DNA is not capable of effecting neo-protein synthesis (although it may bring about exchange reactions), but acts in the synthesis of PNA. Once PNA is constructed, neo-protein synthesis takes place. A distinction is seen between the PNA involved in the production of stable proteins and that involved in inductive enzyme synthesis. In the latter case, the specific PNA itself is unstable in the absence of the inducer; hence the continuous formation of PNA should be expected and, as has been pointed out, does occur in the inductive synthesis of beta-galactosidase.

Brachet (1952) and Pollister (1954) have summarized skilfully a second line of evidence in favor of the involvement of PNA in protein synthesis, viz., the diverse data which suggest that the cytoplasmic microsomal fraction is the site of protein synthesis. Pollister has, in fact, suggested that the microsome may be the proteogenase system of the cell, just as the mitochondria represent the cyclophorase system. The major contributions of this concept lie in its emphasis on the interrelationships of metabolism and structural organization, a specific sequence of reactions being the result of the structural fixation of enzymes in a given pattern, and in the demonstration of the concerted action of metabolites of the microsomal and mitochondrial fractions in coupling oxidative phosphorylation and synthetic reactions. This is illustrated by the penetrating analysis of Siekevitz (1952) who showed that the incorporation of C^{14}-labeled alanine into the microsomal proteins of rat liver is the result of a combination of two events, one in mitochondria which is dependent upon oxidative phosphorylation and another in the microsomes which does not have this requirement. Further experiments demonstrated that a non-tissue-specific, soluble factor produced by mitochondria promoted the incorporation of alanine into the microsomal protein. On the whole, experiments tend to bring more and more support to the hypothesis favored by Brachet that the pentose nucleoprotein granules are the site of protein synthesis. With the exception of one brief report from Brachet's laboratory (LeClerc, 1954), there has been no further substantiation for the other biological properties attributed to the microsomes by Brachet, viz., "autoduplication" and passage from cell to cell. Earlier studies (Shaver and Brachet, 1949; Shaw and Shaver, 1953) indicated that suspensions of microsomes, when cultivated on the chorioallantoic membranes of host chick embryos, increased in mass, supporting Brachet's hypothesis that the pentose nucleoprotein granules

are capable of autoduplication. It was recognized, however, that the methods employed left considerable doubt as to the interpretation of the results (Brachet, 1952). The experiments reported by LeClerc represent a noteworthy attempt to solve the problem. She finds a progressive increase in glucose-6-phosphatase activity in suspensions of liver microsomes cultured on the chorioallantoic membrane. This quantitative approach constitutes stronger evidence than that previously presented, but cannot be regarded as conclusive since increased enzyme activity does not necessarily reflect increased enzyme concentration (*vide supra*). The problem might be solved by a combination of enzymologic and immunochemical methods (cf. Monod and Cohn, 1953).

One should bear in mind that the strongest lines of evidence concerning the role of pentose nucleoprotein granules in synthesis and morphogenesis are derived not from studies of embryonic or other developmental systems, but from studies of specialized adult tissues, e.g. liver. While one cannot help but be impressed by Brachet's arguments, it should be pointed out that since the cytoplasmic particulates of adult tissues are products of differentiation, considerable care must be exercised in assigning specific roles to embryonic particulates based on the performance of those from mature tissues (cf. Holter, 1953).

Considering the principal hypotheses discussed, together with other recent theoretical statements, one arrives at the following tentative scheme which leans heavily on the model for polypeptide synthesis suggested by Lipmann (1954) on the basis of his elucidation of the pyrophosphoryl synthesis of a peptide bond, and the observation by Chance (1952) that in electron transmission from cytochrome to cytochrome the specific sequence of electron transfers is realized through a structural fixation of the individual cytochromes, allowing a directed transfer of electrons. It is assumed that amino-acid-specific activation centers are lined up on a structure in a demanded sequence. It may be helpful to think of the activation centers as a series of enzymatically active polynucleotides (Binkley, 1952, 1954), closely associated with proteins in the microsomes. The centers each become activated by a combination with pyrophosphoryl groups transferred to the enzyme from ATP; the latter step requires the participation of the mitochondria. Next, these are exchanged with the carboxyl groups of the corresponding free amino acids, which are thus lined up with the alpha-amino group of each adjacent to the carboxyl group of the next. The polypeptide is then formed through a condensation reaction. There is no reason to assume that transpeptidation could not furnish amino acids to this sequential arrangement or

that peptides could not act as receptors for amino acids. Gale and Folkes have suggested that neo-protein synthesis takes place at a rate dependent upon the amount of specific pentose nucleic acid catalyst present, the initial organizing structure for PNA synthesis being DNA or a DNA-protein complex. The evidence accumulated by Binkley indicates that nucleic acids or derivatives of nucleic acids are involved directly as the catalysts in the formation of peptides. Although crucial evidence is lacking, there can be no doubt of the heuristic value of this idea.

V. GASTRULATION AND THE SYNTHESIS OF NUCLEIC ACIDS AND PROTEINS

Oogenesis, a period of active synthesis of proteins and other macromolecules and probably of direct transfer of proteins from the maternal blood stream to the egg (Clavert, 1953) is followed, in the activated egg, by a period of relative inactivity of the protein-synthetic mechanisms during cleavage. Cleavage, a period of intense cellular multiplication, is not characterized by over-all growth. In general, the bulk of the evidence available suggests strongly that the cleaving egg remains relatively constant in composition, the major exception reported being the active synthesis of DNA during the first half of each division cycle (Zeuthen, 1953). The extent and mechanism of this synthesis remains an open question. In addition to numerous studies on the mechanism of synthesis of nucleotides and nucleic acids from smaller precursor molecules, the following special mechanisms have been suggested: (1) Conversion of PNA into DNA. According to Steinert (1951) there is no significant change in PNA content of individual amphibian eggs during cleavage. Elson, Gustafson, and Chargaff (1954) have shown that following an initial drop at fertilization, the PNA content of the sea urchin (*Paracentrotus lividus*) egg rises slowly and levels off during the first nine hours of development. Abrams' (1951) findings show that the DNA purines synthesized during the development of the sea urchin egg have a specific activity ten times greater than those of PNA, which, therefore cannot be their precursor. It appears that Brachet's hypothesis of conversion of PNA to DNA may be incorrect. As Brachet pointed out, however, the critical experiment, viz., tagging the PNA synthesized during oocyte growth and then finding out if it is a precursor of DNA, remains to be accomplished. (2) Nuclear synthesis of DNA from quantities of "precursor" DNA stored in the cytoplasm. Hoff-Jørgensen and Zeuthen (1952) and Sze (1953) have demonstrated that the mature frog egg carries a store of cytoplasmic DNA, and Zeuthen (1953) has

suggested that nuclear synthesis of DNA might be of a limited nature, involving only small changes in the conversion of cytoplasmic DNA into the finished product. Sze has proposed an alternative possibility—that cytoplasmic DNA is a spill-over of excess DNA produced in the nucleus, under conditions in which the rate of DNA synthesis exceeds the rate of protein synthesis. No evidence has been presented which bears on this hypothesis.

Despite a rapidly increasing store of analytical data, it has not proved possible to assign to the composition of pentose nucleic acids a biological significance comparable to the specific differences in composition shown by the desoxypentose nucleic acids from different species. Elson et al. (*op. cit.*) have concluded from their extensive study that the composition of the pentose nucleic acids of the sea urchin egg during the first forty-eight hours of development does not vary appreciably and does not reflect the intense and abrupt changes in morphogenesis during that period. It is possible that the pentose nucleic acids have several functions in view of the growing evidence for the existence within the same cell of differently composed types of PNA (Elson and Chargaff, 1952; Marshak, 1951; Vincent, 1952) and that embryogenesis is accompanied by composition changes limited to a small fraction of the total PNA.

In contrast to our uncertainty with respect to the question of possible changes in composition of PNA during development, there is good reason to believe that, following the relatively constant period of cleavage, the synthesis of PNA and proteins is resumed at gastrulation. Brachet (1952) has reviewed the extensive literature pertaining to PNA; his conclusions concerning PNA increase at gastrulation are clearly supported by the findings of Elson et al. (*op. cit.*) of a significant rise in PNA content in the sea urchin egg at the onset of gastrulation.

In attacking the problems of the initiation and localization of protein-synthetic mechanisms at the onset of gastrulation, three major approaches have been employed. It is likely that a combination of these, together with experimental methods yet to be designed, will be needed to achieve some measure of understanding of the problem. Since the field is relatively new, however, and most investigations have been confined to a single approach, I will consider each approach separately.

1. *Analysis of qualitative and quantitative changes in free, non-protein and protein-bound amino acids and peptides in the developing organism.* The question may be posed as follows: To what extent can the appearance of specific functional and structural properties of a developing embryo be correlated with changes in amino acid or peptide content? Let

us examine some of the results of experiments designed to answer this question. Holtfreter, Koszalka, and Miller (1950) studied the amino acid content of eggs and embryos of several species of vertebrates and invertebrates by paper chromatography. Chromatograms of total hydrolysates indicated a great similarity in the amino acid composition of all stages investigated. The authors concluded that their findings gave no indication that new amino acids appear or others disappear in the course of embryonic and larval development. A similar conclusion was reached by Eakin, Berg, and Kutsky (1950) on the basis of preliminary studies in which no marked changes in free amino acids were observed during the early development of *Hyla regilla* and *Rana pipiens*. In a more extensive analysis, Kutsky, Eakin, Berg, and Kavanau (1953) found by microbiological assay that while minor fluctuations in quantities of several free and non-protein amino acids occurred during the development of *Rana pipiens* from the unfertilized egg to the completed neurula stage, no over-all pattern of change was apparent. With the exception of glutamic acid which reaches a maximum at the beginning of gastrulation, correlations with morphological events were not evident. Chen and Rickenbacher (1954) have made a preliminary statement that the results of a study of free amino acids in the development of *Triton palmatus* and *T. alpestris* are in general agreement with those of Kutsky et al.

In contrast to these reports, Berg (1950) found that in the development of *Strongylocentrotus purpuratus,* the quantity of at least three amino acids (alanine, glutamine, and lysine) progressively declined. From studies of the total amino acid content in the development of *Paracentrotus lividus,* Gustafson and Hjelte (1950, 1951) concluded that only slight changes occurred during cleavage, whereas important synthetic processes were initiated at gastrulation, as indicated by a drop in free, and a concomitant rise in peptide, amino acids. This approach has been continued by Kavanau (1953). In a comprehensive study, using chromatographic and microbiological assay methods, he found that the immediate post-fertilization behavior of free amino acids and peptides appeared to depend on the prior history of the eggs, leading him to postulate that unfertilized eggs draw on energy from deamination for maintenance. During subsequent development, while the free amino acids fell into several groups with respect to quantitative changes, there was no consistent pattern. One group (histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tyrosine, and valine) showed a decrease in concentration during early cleavage, followed by a rise at hatching and a sharp drop in the mesenchyme blastula stage. A continuous in-

crease was noted from the late gastrula to the pluteus larva. The second group (arginine, glutamic acid, and lysine) continuously decrease until gastrulation, after which time their behavior follows the pattern of the first group. The concentration of glycine decreases continuously with the exception of a shallow minimum in the late mesenchyme-blastula. All of the peptide-contained amino acids show a sharp rise in concentration at the onset of gastrulation and a gradual increase as gastrulation proceeds. Following gastrulation a decline to the time of hatching occurs. The protein-bound amino acids decline continuously until hatching, when a sharp drop occurs. In a comprehensive and well-documented discussion of his findings, Kavanau emphasizes their bearing on the general problem of protein metabolism, while he devotes little attention to their significance with regard to the problems of differentiation. In fact, it is an open question whether this approach, no matter how meticulously carried out, can contribute much to our understanding of developmental mechanisms. Amino acids are ubiquitous and so essential for normal life activities of all cells that determinations of their quantitative fluctuations during development of the *entire* embryo provides little more than an index of the general course of metabolism of the organism. Despite the wealth of detailed information available, there is no clear-cut support from this approach for the widely held generalization of an increase in protein synthesis at gastrulation. Nor should one expect that this approach will yield definitive evidence in this direction, for protein metabolism during this period must involve the break-down of proteins from the vitelline reserve and the synthesis, from the amino acids made available in this manner, of new specific proteins. It is interesting to note that in a recent report Kavanau (1954) has obtained evidence from similar studies in *Paracentrotus lividus* of cyclic changes in protein-bound amino acids suggestive of alternate periods of yolk degeneration and tissue protein synthesis.

2. *Studies of the incorporation of radioactively-tagged amino acids and other intermediates during development.* Brachet (1952) has reviewed some of the evidence favoring the idea that protein-synthetic mechanisms are activated at gastrulation, viz., the increased incorporation of labeled compounds reported by Hultin (summarized in 1953), who used ammonium or glycine containing N^{15}, and by Friedberg and Eakin (1949), who employed glycine labeled with C^{14}, in the eggs of the sea urchin and frog, respectively. The latter study, as well as that by Eakin, Kutsky, and Berg (1951) suggested a difference between the dorsal and ventral areas of the blastopore in terms of labeled amino acid

uptake. This conclusion is supported by Flickinger (1954), who studied the regional incorporation of C^{14} by dissected parts of *Rana pipiens* gastrulae incubated in $C^{14}O_2$.

Radioisotope methods offer several attractive possibilities for attacking questions concerned with the regional activity of embryos and the interaction of tissues, e.g., the question of the passage of materials from one cell group to another in embryonic induction. In an early attempt, Abercrombie and Causey (1950) were unable to detect any significant isotope activity in host chick embryos in which secondary neural structures were induced by neural tube tissue labeled with P^{32}. Recently, however, Ficq (1954) has described experiments which indicate the passage of labeled molecules from grafts of prospective chorda mesoderm to induced neural structures in embryos of several species of amphibians. The isolated tissues were allowed to incorporate the labeled compound (either glycine-C^{14} or orotic acid-2C^{14}-6 carboxyl) for three hours *in vitro* and then grafted to host embryos. Recovery was made after three to five days and autoradiograms of donor and host tissues were prepared. Although full details have not been published, the data presented support the conclusion of molecular transfer from donor to host tissue. Preliminary experiments in which the donor tissue is treated with ribonuclease and protein-denaturing agents before transplantation further suggest that the molecules involved may be ribose nucleoproteins.

It is difficult to assess the value of studies of the time course of incorporation of labeled amino acids during development of either the entire embryo or of isolated embryonic tissues. A considerable number of investigations have indicated that the rate of incorporation of amino acids by tissue homogenates or particulate fractions is markedly higher in embryonic than in adult tissues (Greenberg, Friedberg, Schulman, and Winnick, 1948; Rayford and Friedberg, 1954). From the viewpoint of general interest in protein metabolism, it appears that comparative studies of incorporation of labeled amino acids into specific organs, during clear-cut periods in which cell degeneration and replacement are not important factors in differentiation, might be of considerable value in reassessing the concept of the dynamic state of proteins. Monod and Cohn (1953) have emphasized that the "dynamic state" may be in fact not molecular but cellular. In general, the turnover of proteins is especially rapid in those tissues in which cell replacement is marked, and slow in those tissues which are stable from the standpoint of cell division.

3. *Immunochemical studies of changes in antigenic constitution during development.* Among the numerous biological phenomena which

can be investigated profitably with the techniques of immunochemistry are the problems of differentiation and growth. Studies of the time of origin, site of origin and the functions of specific antigens during development are basic to further analyses of synthetic mechanisms. In a recent discussion I have attempted an evaluation of our present level of understanding of the problems of recognition and mechanisms of development of tissue, individual and species differences (Ebert, 1954b); hence I will not present a detailed review of the literature here. Interest has centered, in the main, on two experimental approaches: (1) studies of the formation of well-defined proteins and other antigenic molecules, e.g., the serum proteins and other blood antigens (Cooper, 1946 et seq.; Schechtman and his collaborators, 1947 et seq.; Nace, 1953; Telfer and Williams, 1953; Telfer, 1954), the lens proteins (Ten Cate and Van Doorenmalen, 1950; Woerdeman, 1953), and the cardiac contractile proteins (Ebert, 1953b) employing as tools antibodies directed against the specific proteins of adult origin; and (2) comparisons of the antigenic properties of progressive stages in ontogeny, employing antisera prepared against complex antigenic mixtures derived from the embryonic tissues under investigation (Clayton, 1953; Flickinger and Nace, 1953; Nettleship, 1953; Perlmann, 1954; Spar, 1953). A variety of immunochemical and immunobiological procedures have been employed, frequently coupled with other methods of protein physical chemistry, e.g., electrophoresis and immunophoresis. While each of these approaches offers insight into the development of tissue specificities, the second suffers from operating too much in terms of complex entities. Instead of considering the development of a number of antigens of whole embryos, or nervous tissue or liver tissue, it should be possible to investigate the development of some single substance, to follow quantitatively the formation of a protein in normal development and under experimental conditions circumscribed by previous investigations at the cell group or tissue level. Currently there is much interest in our own laboratory in determining whether this approach has any real value. It may be of interest to examine the conclusions arising from a first approach to the problem. The technical details of the work are given elsewhere (Ebert, 1953b; Ebert, Tolman, Mun, and Albright, 1955).

Tests of developmental potencies of small pieces of the chick blastoderm through transplantation to the chorioallantoic membrane, or cultivation *in vitro* clearly show that each of the isolated pieces possesses a relatively restricted developmental capacity. Particular structures develop only from rather definite areas of the blastoderm; in short, the

organ-forming areas are localized to a considerable degree (Willier and Rawles, 1935; Rawles, 1943; Rudnick, 1944). Waddington (1952) recently has reemphasized the major objection to this approach, namely, that each of the isolated pieces is triploblastic, thus rendering interpretation and further analysis difficult. Most of the investigators who have employed the approach have been acutely aware of this limitation, however. It has been recognized that the degree of specificity of localization of the several organ-forming areas differs. For example, on the basis of his recent study of the localization of prospective neural plate in the early chick blastoderm, Spratt (1952) has concluded that both transplantation and carbon-marking methods reveal the prospective fates of different regions of the blastoderm. In the case of heart-forming areas

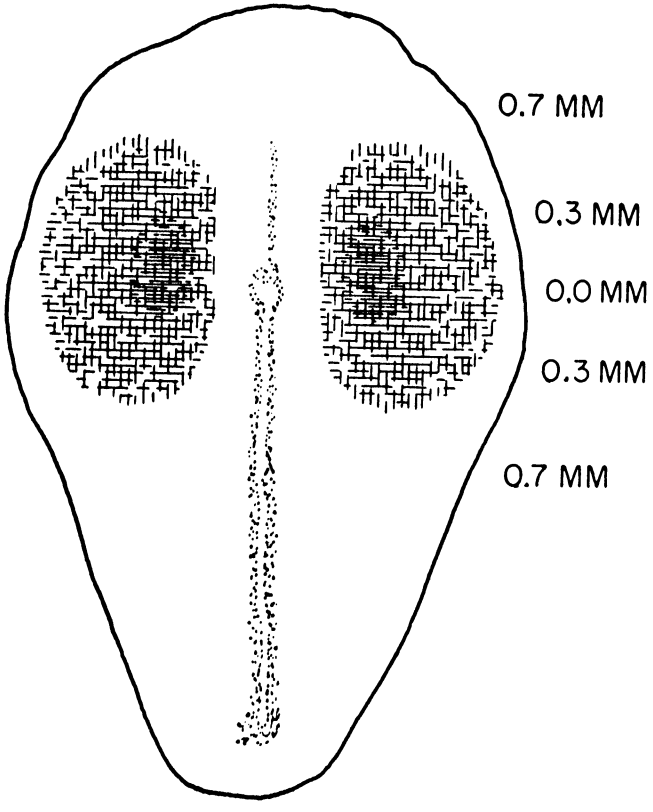

Fig. 1. Heart forming areas in the head-process blastoderm of the chick (From Rawles, 1943).

(Fig. 1), however, as Rawles and Rudnick have emphasized, the areas are amazingly large, exceeding in size the actual myocardial anlagen.

Thus it appears that the heart-forming areas are areas of prospective potency and not of prospective fate.

Implicit in earlier definitions of organ-forming areas, e.g., the heart-forming areas, has been the assumption that the cells of a region of the embryo so defined are destined to become specialized at some future time for the synthesis of the specific proteins characteristic of the definitive organ and that the establishment of the heart-forming areas, for example, is a localization of prospective myosin- (and other cardiac muscle protein-) synthesizing cells. However, recent findings in our laboratory (Ebert, 1953b) indicate that a revision of earlier concepts of the establishment of the heart-forming regions is required, for, by immunochemical methods, the progressive restriction of specific reactive groups of cardiac myosin—and not of prospective cardiac muscle cells—has been demonstrated. Cardiac myosin is synthesized in the early embryo, and can be detected in the blastoderm at the mid-streak stage (Hamburger and Hamilton, 1951; stage 3); in the embryo at stage 4 (definitive primitive streak) the antigenic reactive groups of the protein are not localized, but can be detected in all sections of the embryo analyzed, being more concentrated in the middle and posterior than in the anteriormost regions. Preliminary experiments (Ebert, unpublished data) indicate that at stages 3 and 4, at least, cardiac myosin is confined to the epiblast, being absent from all extracts of hypoblast tested. In the embryo at stage 5 (head process) and more clearly at stage 6 (head fold) cardiac myosin is restricted in distribution to two lateral regions, in good agreement with the limits of the heart-forming areas as defined by Rawles. The findings indicate that a major synthesis of cardiac myosin is initiated only after the prospective mesodermal cells have begun to move through the primitive streak. It is suggested that cardiac myosin formation is limited to the epiblast, but the evidence at hand does not permit the assignment of myosin-forming capacity more precisely. It is clear, despite the coincidence in time of the onset of myosin synthesis and cell movement through the patent streak, that cells which have not yet passed through the streak are capable of myosin formation, as shown by the widespread distribution of the protein in the stage 4 blastoderm, particularly in the most anterior levels.

The establishment of the heart-forming areas, then, clearly involves the disappearance from a large part of the embryo of an already established ability to synthesize the specific protein cardiac myosin. The information at hand does not permit an unequivocal conclusion concerning the mechanism of restriction of myosin synthesis. At least two

hypotheses are possible: First, that the localization of the protein is a sorting-out or segregation phenomenon, the result of cellular movements within the mesoderm. According to this view, the over-all synthetic ability of the embryo would not be reduced, but merely aggregated in discrete regions. While this interpretation is not supported by the facts on hand concerning morphogenetic movements within the mesoderm, it cannot be ruled out, for present knowledge of morphogenetic movements does not permit a critical evaluation. According to the second hypothesis, the myosin-synthetic mechanism would be lost in the embryo except for the heart-forming regions. Barring a concomitant increased rate of synthesis in the heart-forming areas, therefore, the total synthesis of cardiac myosin should be reduced. A quantitative immunochemical analysis of the rate of accumulation of cardiac myosin in the heart-forming regions and rate of disappearance in the adjacent parts of the embryo, coupled with a radiochemical study of the rate of incorporation of labeled amino acids as one index of rate of synthesis in the heart-forming areas, may provide the information necessary to permit a choice between these two alternatives.

It becomes of interest to examine next the development of the second major cardiac contractile protein, actin. We ask the following questions: Do cardiac myosin and actin develop simultaneously? Is the development of each protein an independent event, or does the formation of one precede that of the second and have a causal role in its synthesis? The detailed results of the investigation to date have been presented elsewhere (Ebert et al. 1955); however, for clarity, passing attention is needed to the questions of the antigenicity and antigenic specificity of cardiac actin. These remarks will be brief.

A comparative, immunochemical study of actins extracted from cardiac and skeletal (hind limb) muscle of adult chickens has shown that actin from both sources is antigenic in rabbits. Two methods have been followed in preparing purified actin, viz., the ultracentrifugation technique employed by Mommaerts (1951) following extraction of crude actin according to Straub (1943), and the method proposed by Tsao and Bailey (1953) involving butanol extraction and dialysis. Despite reports that these methods yield homogeneous actin preparations, immunochemical studies clearly show our preparations to be complex mixtures. Cardiac actin prepared by either method is a mixture of at least three antigenic molecules. It is encouraging to note that the cardiac actin preparations from the two extraction procedures are indistinguishable immunologically. Tests by the Oudin serum-agar method coupled

with reciprocal absorption studies indicate that of the three antigens present in cardiac actin, at least one is held in common with skeletal actin. It is possible through absorption methods to prepare antisera which are specific for the cardiac actin antigenic complex, by removing the antibody against the common antigenic component. These specific antisera serve as sensitive agents for the study of the time of appearance and distribution of cardiac actin in the chick embryo. It was demonstrated first that the actin (antigenic complex) present in adult heart is present in and can be isolated from the heart of the 12-day-old chick embryo. At earlier stages it is necessary to employ a crude extract of cardiac muscle, since a measureable yield of purified actin could not be obtained from the small quantities of material available. All preparations of cardiac muscle, both the purified actin complex and the crude extract, from embryos of 12 to 18 days, and 36 hours to 11 days, respectively, reacted positively with all antisera tested. Moreover, when the specific antisera are applied directly to the embryo, *in ovo,* at 48 to 60 hours, the further development of the heart is blocked, resulting in the death of the embryo. These studies demonstrate that combining groups identical with or closely related to those of adult cardiac actin are present in the embryo as early as 36 hours of incubation. However, both specific antigens of the cardiac actin complex may not be present prior to the twelfth day, for tests by the Oudin serum-agar method indicate only that at least one antigen is present.

This investigation has been extended to an examination of the formation and distribution of cardiac actin in the early blastoderm. The approach parallels that employed in the earlier study of the development of cardiac myosin. Extracts of intact mid-streak to early somite stage embryos and of surgically isolated regions of definitive primitive streak, head process, and head fold stage embryos were tested for the presence of antigens of the actin-complex by sensitive precipitin methods. These experiments, in which more than 1600 early blastoderms have been analyzed in 124 test series, have established that at least one antigen of the cardiac actin complex first can be detected by precipitin methods in the embryo at the head process stage. Further, it has been shown that when first detectable in the embryo, the specific antigen is confined to those areas previously defined as the heart-forming areas. At the head process stage, there are traces of cardiac actin in the region of the streak, but by the head fold stage, the antigen is completely restricted in distribution.

The sequence of development of the cardiac contractile proteins in the chick embryo can be summarized as follows (Fig. 2): (1) The major

synthesis of cardiac myosin is initiated during the formation of the primitive streak; the protein first can be detected with the methods employed at the mid-streak stage. (2) In the definitive primitive streak stage embryo cardiac myosin is distributed throughout the epiblast; it cannot be detected in the hypoblast. (3) Beginning at the head-process stage, cardiac myosin is restricted to the heart-forming regions of the embryo. (4) The restriction in distribution of cardiac myosin coincides with the initiation of synthesis of at least one antigen of the cardiac actin complex which is already limited in distribution to the heart-forming areas at the time it first is detected.

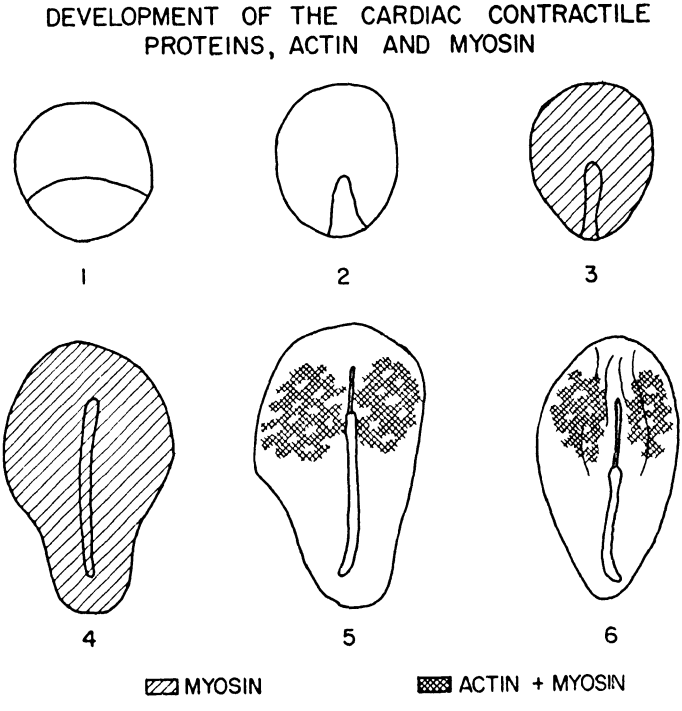

Fig. 2. Distribution of actin and myosin in the chick blastoderm at Stages 1-6 of Hamburger and Hamilton (1951).

These facts have strategic value in the sense that they help forge a link between previously unrelated lines of research and offer attractive experimental targets for further study. From the viewpoint of bridging the gap between the tissue and molecular levels the following questions are of interest: In what manner are normal patterns of synthesis modified in isolated tissues and tissues placed in an altered tissue environment? The posterior one-third of the early chick embryo isolated *in vitro* pro-

duces a pulsating heart. What factors are absent from the isolate that normally prevent the loss of synthetic activity which occurs in that region in the whole embyro? Are myosin and actin synthesized in the epiblast in the absence of the hypoblast? Of relevance in this general context are Yamada's experiments (1940) which show that undifferentiated mesoderm cells form myoblasts if located next to the notochord, but form erythroblasts if transplanted more laterally. The synthesis of the specific proteins clearly is subject to the influence of the metabolic activities of adjacent regions. At the molecular level, it would be interesting and instructive to know more in detail of possible relationships between the spatial limitation of myosin synthesis and the initial synthesis of actin. The formulation of an attack on this problem leans heavily on the kinetic analysis of cardiac myosin synthesis discussed earlier. The sequential synthesis of cardiac myosin and actin demonstrated in these experiments tends to oppose the hypothesis advanced by Kominz, Hough, Symonds, and Laki (1954) that myosin may be composed of actin plus tropomyosin. This hypothesis is based largely on the fact that the amino acid compositions of actin and tropomyosin "add up" to that of myosin. Myosin has not been synthesized from mixtures of actin and tropomyosin, nor has myosin yielded these smaller proteins upon degradation.

VI. THE CONCEPT OF ORGAN-SPECIFIC CONTROL OF GROWTH AND DIFFERENTIATION

The history of attempts to formulate a general theory of growth regulation testifies both to the need for a conceptual framework for the fundamental data which are available and to the difficulties which have barred the way. The methods available for the study of this complex problem, although still over-simple and inadequate, now permit a preliminary restatement of the problem in terms of molecular interactions. Since protein synthesis is one of the fundamental aspects of growth, the discussion of these problematic issues has particular relevance at this time. There is little advantage in delving into the long history of the conceptual schemes which have been developed; it will be clear, however, as the discussion unfolds, that most recent formulations owe a considerable debt to those of the past. Our point of departure is the theoretical statement presented by Weiss at an earlier annual symposium of this society (1945) and later published simultaneously with a somewhat similar statement by Tyler (1947). This concept, extended and supplemented in subsequent discussions (Weiss, 1950, 1952, 1953a, b), is centered on the question as to whether organ-specific compounds in-

strumental in the synthesis of a given protoplasm occur in complementary pairs, with the relative ratios of the partners determining growth rates. According to this view, "protoplasmic synthesis of a given organ yields (a) 'templates' for further reproduction and (b) accessory diffusible compounds capable of inactivating the former. As the latter, accumulating in the common humoral pool, reach critical concentration, growth ceases. Partial removal of an organ by reducing the concentration of (b) will automatically entail compensatory growth in the rest of the organ" (Weiss, 1953 a). Three lines of evidence have been mentioned in support of this idea: (1) Incorporation of organ fragments, e.g., liver or kidney, from 6-day-old chick embryos into the area vasculosa of 4-day-old embryo hosts results in an increase in weight of the homologous organ of the host. (2) The injection of embryonic kidney brei into younger embryos produces after one or two days a fifty per cent increase above normal in mitoses in the host kidney. (3) The partial destruction of the metanephros in a "prefunctional" stage resulted in a compensatory hyperplasia in the remaining kidney, marked by an increase in mitotic activity of over fifty per cent after twenty-four hours. On the other hand, the addition of an organ extract might be expected to reduce the differentiation capacity of the homologous organ. In a preliminary report, Weiss (1952) has presented the results of experiments supporting this proposal. Embryonic chick tissues were reared in tissue culture media prepared with extracts of (1) whole embryos and (2) embryos from which the homologous tissue had been removed. Of 333 heart fragments with healthy growth in full extract, only two pulsated after the fourth day, while of 349 fragments similarly reared, except for omission of heart extract from the medium, 129 pulsated, indicating progressive muscular differentiation. In a similar experiment, of 1007 embryonic kidney (metanephros) fragments in full extract, only 74 formed new tubules, against 176 of 1006 cultures grown without kidney tissue in the extract.

Related ideas are embodied in the "general theory of cellular differentiation and embryonic pattern formation based on an hierarchy of self-limiting reactions" presented by Rose (1952). It is argued that an important mechanism in progressive differentiation is the successive inhibition of one gene-initiated reaction after another by the collection of specific metabolites which inhibit in the mass action relationship. Rose further states that the established fact of compensatory restoration of lost tissue, e.g., liver growth after partial hepatectomy (Higgins and Anderson, 1931; Ferrari and Harkness, 1954) demonstrates that the

growth of differentiated tissues also is self-limiting and advances the hypothesis that growth is resumed following a reduction in concentration of inhibitory metabolites. This is, and has been for many years, an attractive hypothesis; in 1918, Jacques Loeb wrote as follows: "By measuring the influence of the mass of a leaf attached to an isolated piece of stem upon the process of regeneration in the piece, it has been possible to prove that the quantity of regeneration is in equal time and under equal conditions in direct proportion to the mass of the leaf . . . If we measure the rate of regeneration by the mass of the material regenerated in a given time, the law of regeneration becomes a special case of the law of chemical mass action. That this mass action on a bud is only possible in a piece of stem after it has been isolated, the writer explains on the assumption that the apex of an intact plant sends constantly inhibitory substances into the stem preventing the buds contained in the stem from growing and consuming the material required for growth . . . The mystifying phenomenon of an isolated piece restoring its lost organs thus turns out to be the result of two plain chemical factors: The law of mass action and the production and giving off of inhibitory substances in the growing regions of the organism." Although Rose has revived this hypothesis with respect to growth regulation, supporting evidence of a critical nature has not been presented; the experiments of Bucher, Scott, and Aub (1950) which show that partial hepatectomy of one member of a pair of parabiotic mice leads to an increase in mitosis in the liver of its partner suggest only a blood-borne mechanism for control of liver mitosis.

In applying the hypothesis to the mechanism of differentiation and pattern formation, a major assumption is that there are specific metabolites which, in sufficient concentration, will inhibit differentiation of the cell type which produced them. In support of this view, Rose has reported briefly that the differentiation of embryonic frog tissues (blood, brain, and heart) is suppressed by culturing embryos through the neurula stage in a medium containing suitable concentrations of the homologous tissues (e.g. an intact organ or an organ extract). Shaver (1954) has stated that a granular fraction of adult frog brain arrests brain differentiation. Neither the conditions nor the results of these experiments have been reported fully.

There has been considerable discussion of the ideas advanced by Weiss and Rose. The two theoretical statements agree in the general sense that differentiation and growth of a tissue may be influenced by its own products. Rose has stressed the self-limiting nature of tissue dif-

ferentiation, emphasizing that following an initial general stimulus, differentiation results from a series of specific inhibitions. The growth of differentiated tissues is considered within the same general plan. Weiss, on the other hand, has developed a more comprehensive scheme to account for the specificity in tissue growth, harmonious growth relations between different parts of the same organism and their regulation upon disturbance. Growth, the reproduction of tissue-specific molecules is proportional to the number of "template" molecules available. During the type-specific synthetic process, complementary "anti-templates" are produced. These "anti-templates" are capable of diffusion from the cell and can pass freely to the exterior, whereas the "templates" are fixed within the cell. Growth proceeds until an equilibrium is established between the intra- and extracellular populations of "anti-templates." As the number of "anti-templates" leaving the cell is reduced, the number available to react with "templates" within the cell is increased; this results in a reduction in free "templates"; hence a reduction in synthesis. If part of an organ, e.g., the liver, is removed, the number of liver "templates" and "anti-templates" will be reduced. Since the "templates" are proposed to be strictly intracellular entities, the reduction in their number might not be perceptible to the organism as a whole. As the population of extracellular "anti-templates" was reduced, "anti-templates" would diffuse from within the cells, leaving uncovered "templates," thus resulting in growth of the homologous tissue. As Weiss has remarked, this is an over-simplified model, intended to help in formulating experimental attacks on the problem. Several lines of experimentation have been initiated by Weiss and his co-workers; from experiments in which it was shown that grafts of older embryonic chick tissues made to the vascular areas of 4-day-old chick embryo hosts result in a relatively specific response, viz., an increase in mass of the homologous organ (*vide supra*), Weiss suggested that "either specifically shaped parts of liver cell proteins can act directly as nuclei for further synthesis when entering the appropriate environment, i.e., another liver cell, or they act merely as models for moulds which would turn out more of the original product."

In 1948 I began an investigation of the factors concerned in the enlargement of organs of the host chick embryo following transplantation of homologous tissues from older embryos and adult fowls in the belief that the information might be pertinent to an understanding of protein biosynthesis and the growth of the organism. At the outset of the study the major experimental target was the chick spleen. The observation that chorioallantoic grafts of adult chicken spleen elicit a striking enlarge-

ment of the spleens of host chicks was made first by Murphy (1916) and was confirmed subsequently by Danchakoff (1916, 1918), Willier (1924 and unpublished data), and Pomerat (1949). Although considerable evidence had been presented concerning the cellular nature of the response, the data available regarding the specificity of the effect and mechanism of action were contradictory and incomplete. In a first approach to the problem, therefore, the tissue- and class-specificities of the effect were determined. The findings which have been published (Ebert, 1951) clearly demonstrate that the effect is class-specific and quantitatively tissue-specific. While adult chicken spleen grafts result in a striking increase in weight of the host chick spleen, grafts of adult mouse spleen are ineffective. Of eleven adult chicken tissues tested in addition to spleen, only two, thymus and liver, affect the host spleen and in each case the effect is quantitatively less than that observed with splenic grafts. It was shown, in addition, that the ability of chicken spleen grafts to effect host spleen enlargement is correlated directly with the age of the donor, the factor or factors concerned appearing first in the embryonic chick spleen at approximately the fourteenth day of incubation and increasing thereafter until the adult level is reached. The latter finding suggested that it might be possible to correlate the progressive increase with age in the ability of spleen grafts to evoke enlargement of the host's spleen with the development, during a similar period in ontogeny, of the specific effector molecules. In appraising the evidence relative to the specificity of the effect, it was noted that the results were in agreement with immunological studies on organ-specificity which show clearly that each organ contains a large number of antigenic molecules, certain of these being organ-specific, and others being held in common with one or more organs. The lack of absolute organ-specificity might well be attributed to the presence in spleen, thymus, and liver of certain common antigenic molecules, in addition to the specific antigens characteristic of each tissue. Thus, an immunochemical approach to the problem was suggested, and subsequently undertaken. I need not recount the steps in the investigation here, for the major findings have been published *in extenso* (Ebert, 1951, 1952). It has been shown that the time of appearance of the specific factors concerned in host spleen enlargement is paralleled by the development of a group of splenic antigens. I wrote optimistically in 1951 that "these findings suggest a correlation between the progressive increase with age in the ability of spleen grafts to evoke enlargement of the host's spleen and the development of a specific group of spleen antigens." It has been possible to demonstrate by using the serum-

agar precipitin technique in combination with reciprocal absorption methods that the minimal number of specific antigens in the group which is detected first on the fourteenth day of incubation is three (Ebert, 1952). That any of these specific antigens is effective in promoting splenic enlargement has not been proved, however. Although the antigens are separated one from another by the method employed, each remains bound to its specific antibody. It has been a common experience that the antigen-antibody complex may be split to yield purified antibody and denatured antigen, the antibody being more resistant than the antigen to the agents employed. In general, efforts to split the antigen-antibody complex by techniques sufficiently mild to allow recovery of the purified antigen have not met with success. Agar bands containing the antigen-antibody complex have been isolated and implanted on the chorioallantoic membrane; no effects on the host spleen have been observed. These negative findings may not be meaningful, however, for the reactive groups of the antigen could be masked by complexed antibody and, perhaps of greater significance as will be more evident later, the concentration of antigen administered probably is below the effective level.

With this critical point unsettled, the major questions may be restated as follows: (1) Does the enlargement of the embryonic spleen following an homologous transplant reflect a true increase in protein, or is it the result of an accretion of smaller molecules? (2) If, as postulated earlier, the enlargement results from a transfer of protein or other tissue-specific molecules, what is the magnitude of the transfer? Is a small number of molecules involved (template or catalytic hypothesis) or is a larger population of molecules transferred? Recently I reported the findings from a series of experiments designed to answer these questions (Ebert, 1954a).

Analyses of the total and non-protein nitrogen content of normal and enlarged embryonic host spleens following sham operations or grafts of inactivated chicken spleen and fresh adult chicken liver and spleen, respectively, prove that the nitrogen content per milligram of tissue does not differ significantly in the several groups. The data demonstrate that the increase in weight of the stimulated spleens is paralleled by an increased nitrogen content; the mean weight of host spleens stimulated by transplants of fresh adult spleen is approximately four times that of the controls; the nitrogen content is increased to a similar extent. These findings clearly support the view that the increase in weight of host embryo spleens is not due to an accretion of smaller molecules, but reflects a true increase in protein content.

Does enlargement of the host spleen result from a transfer of tissue-specific molecules from the transplant to the homologous host tissue? If so, what is the magnitude of the transfer? In attacking this question, the basic approach involved the transplantation of adult chicken and mouse tissues labeled with sulfur-35 to the chorioallantoic membrane of host embryos. At the time of recovery, the grafts and several host tissues were analyzed to determine the radioactivity of the graft and extent of transfer and localization of radioactive sulfur in the host tissues. Only those aspects of the methodology essential to an evaluation and clear understanding of the findings will be presented here. Adult chickens and mice were injected intraperitoneally with S^{35}-labeled L-methionine according to a schedule designed to obtain a high level of radioactivity without radiation damage in the proteins of the tissues to be transplanted. At sacrifice, the following steps were carried out rapidly: (1) spleen, kidney, liver, gonad, and heart samples were fixed for histologic and autoradiographic preparations; (2) spleen, kidney, and liver samples were weighed and stored in the frozen state pending digestion for determination of specific activity; the remainder of the spleen and kidney was weighed and used in chorioallantoic transplants. After three or five days the transplants and host spleen, kidney, and liver were recovered. The specific activity of the radiosulfur in protein and non-protein fractions of the donor and host tissues was determined as counts per minute per micromole of sulfate in each fraction, sulfate being extracted by digestion of tissues with fuming nitric and perchloric acids and precipitated as benzidine sulfate on a fritted glass disc. Radioactivity was counted in a methane flow proportional counter and sulfate determined titrimetrically.

The principal object of the study was the spleen. The choice of a second, or control, tissue for transplantation lay between heart and kidney, both of which may be transplanted successfully. Adult kidney was selected because its specific activity was considerably higher than that of adult heart. The findings constitute a consistent body of evidence which shows clearly that following transplantation of labeled adult chicken spleen or kidney, there is a specific transfer of radioactivity from the graft to the homologous host tissue (Fig. 3). The transfer is selective both from spleen to spleen and from kidney to kidney. Moreover, in additional experiments, it was shown that there is no evidence of selective incorporation following grafts of labeled mouse spleen (Fig. 4).

These experiments offer striking support for the concept of class- and tissue-specificity in the effect of transplants on homologous embryonic tissues, and constitute the most direct evidence presented to date for a

transfer of tissue-specific molecules from donor to host tissues. However, the findings do not support the idea that the growth of the host tissues is the result of the catalytic action of a small number of "template" molecules. When the data are expressed as the ratio of specific activity of host tissue protein to specific activity of graft tissue protein, it is apparent that as early as the third post-operative day, as much as fifteen per cent of the homologous host tissue protein may be derived, or contain specific molecular contributions, from the proteins of the graft. To explore this possibility further, a further series of experiments was undertaken, in which S^{35}-labeled methionine was injected into the yolk sac

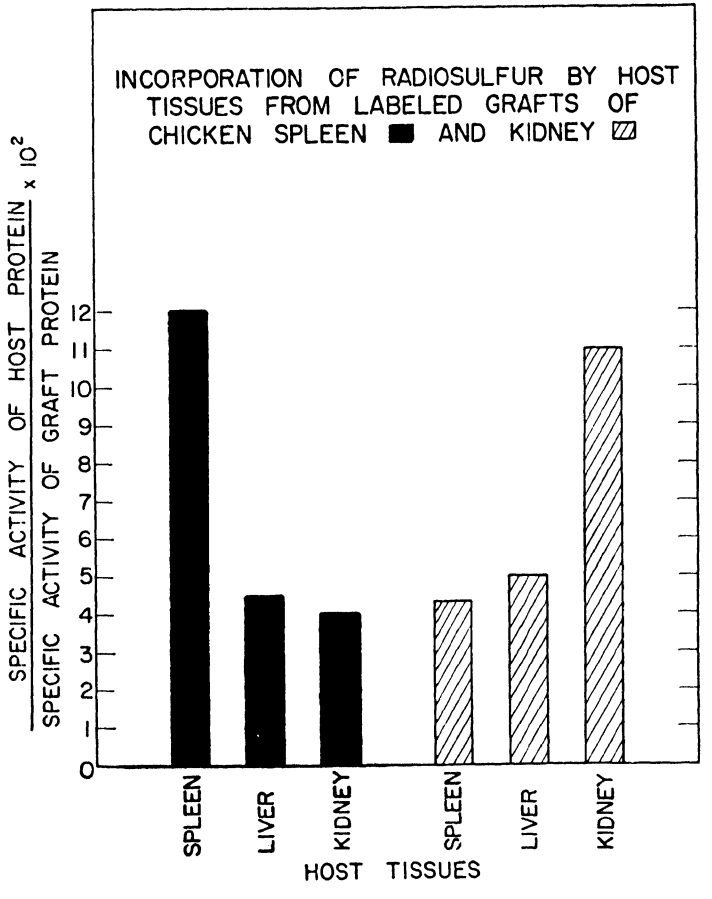

Fig. 3

of nine-day-old embryos immediately following either grafts of non-radioactive spleen or sham operations. Grafts and host tissues were re-

covered after three days. Although the host spleens were enlarged in the former group and thus total activity was greater, the specific activities (activity per micromole of sulfate) in the tissues of the two groups did not differ significantly (Fig. 5). Coupled with the previous findings, these results suggest that the growth of embryonic host organs following homologous grafts is due, at least in large part, to a selective incorporation into the proteins of the host tissue of tissue-specific components from the graft. Thus, the question is raised as to the nature of the specific components transferred.

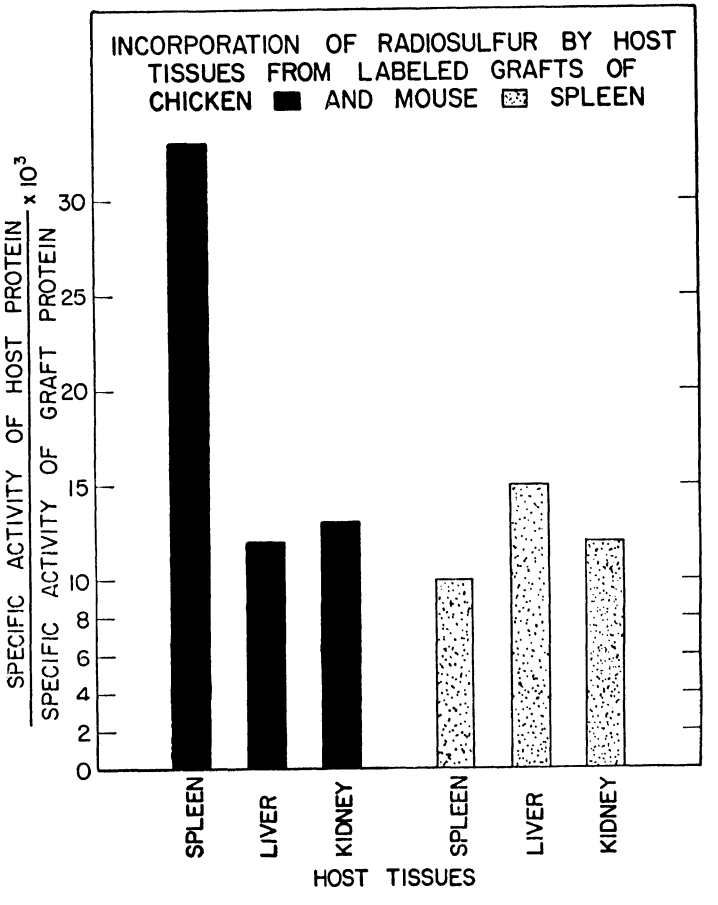

Fig. 4

In view of the observations by Weiss and Andres (1952) which suggest that dissociated embryonic cells injected into chick embryos participate in a type-specific localization in the embryonic host and are

incorporated into the normal pattern of differentiation, it seemed a distinct possibility that whole cells might be transferred from graft to host tissue. Upon critical examination, however, this view proved to be untenable; three lines of evidence have been considered: (1) Microscopic examination of histologic preparations of embryonic host kidney reveals no accumulation of cells not incorporated into the normal cellular pattern of the kidney. One might expect to find intermediate stages if such a process were involved; none have been found. At the cellular level the response in host spleens following spleen grafts made to nine-day-old embryonic hosts is a stimulation of granulopoiesis, granulocyte

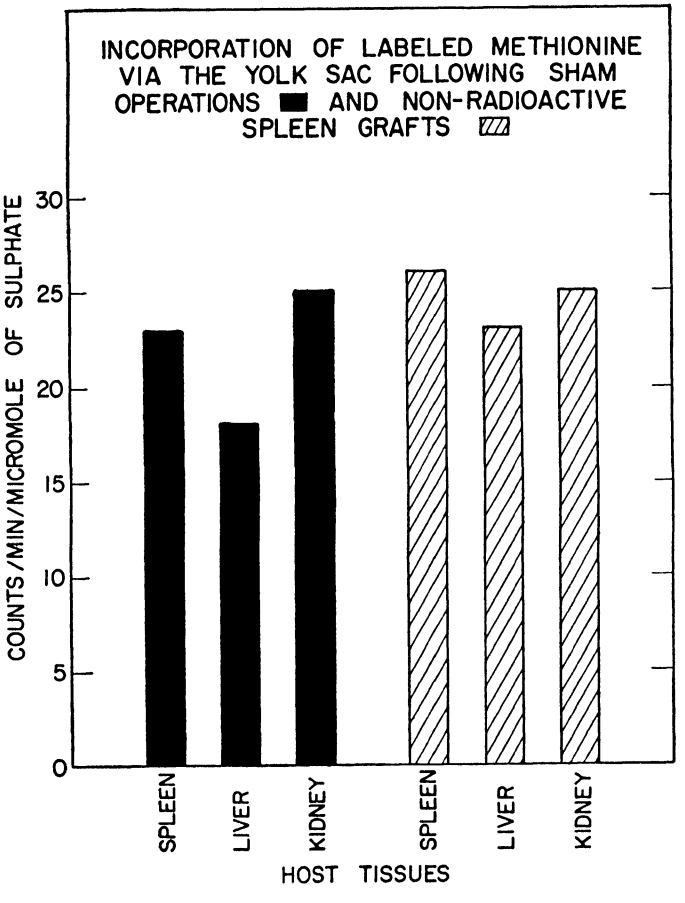

Fig. 5

formation being in progress in the spleen during the same period in normal development. (2) A study of autoradiograms of grafts and kid-

ney, spleen, and other tissues of host embryos indicates that while there is, in general, a higher level of radio-sulfur in the homologous tissues, there is no localization either to any particular region or to any specific cell type of the kidney or spleen. (3) Analyses of DNA in enlarged and normal spleens show that the DNA content of the enlarged spleens does not increase concomitantly with the increase in protein content, a finding in agreement with the transfer of tissue-specific molecular constituents rather than of whole cells.

The findings in the investigation to date strongly suggest that the growth of host tissues following homologous grafts is due to a "building block" rather than a "template" or catalytic mechanism. The latter possibility has not been completely ruled out, however, since the two hypotheses are not mutually exclusive. The "building block" hypothesis, i.e., the idea of selective incorporation from grafts into homologous host tissues of tissue-specific proteins or specific constituents larger than amino acids, permits us to focus on several clear experimental targets: (1) a detailed study of the rate of incorporation of labeled amino acids from the yolk sac into embryonic tissues under normal and graft conditions; (2) an analysis of the post-operative time required for graft substances to reach the homologous tissues of the host, together with the subsequent rate of transfer; (3) a study of the effects of tissue homogenates, e.g., homogenates of spleen, and fractions thereof. The chief obstacle to be hurdled is the difficulty encountered in determining the effects of the small quantities which can be injected into the embryonic circulation. It has not been possible to obtain splenic enlargement by injections of fresh spleen homogenates into the yolk sac, a finding which is not unexpected since the activity of the proteolytic enzymes of the yolk sac reaches a maximum at the ninth or tenth day and does not decline thereafter (Romanoff, 1952). In preliminary experiments, only a slight stimulation of splenic growth was observed after injections of 0.1 and 0.2 ml. of an homogenate of fresh adult spleen via the chorioallantoic vessels, a fact which further suggests that the effect is not catalytic, but that a constant flow of graft materials is required.

Let us consider briefly a further test of the concept of suppression of differentiation by older tissues or tissue homogenates. Recently I posed the question as to whether a graft of spleen made to the chick embryo early in the fourth day of incubation, well before the spleen has made its appearance, might suppress the development of the spleen (1954a). Such a finding might be expected if the hypothesis advanced by Rose has general validity. In an attempt to answer this question, two lines of

experimentation have been initiated: (1) the transplantation of spleen from the newly hatched chick to the coelom of sixty-six to seventy-two hour host embryos with recovery at twelve days (in collaboration with Dr. R. A. Tolman) and (2) the injection of homogenates of fresh adult chicken spleen into the coelom of the chick embryo at seventy-two and seventy-eight hours, with recovery at nine and fourteen days. I can report that in the host embryos recovered to date the spleen is quite evident. The histologic analysis of the first group has not been completed. In the second group, however, the differentiation of the spleen has progressed to the same extent in the embryos that received a total of 0.1 ml. of spleen homogenate as in the embryos that received 0.1 ml. of chick Ringer's solution.

VII. TRANSPLANTATION SPECIFICITY AND ACTIVELY ACQUIRED TOLERANCE

The question arises next as to the bearing of the findings reported here on the concept of actively acquired tolerance. Owen (1945) first demonstrated the establishment of permanent natural transplants of hematopoietic tissue exchanged between bovine twins. These observations have been confirmed and extended in later investigations of multiple births in cattle (Owen, Davis, and Morgan, 1946). The exchange of hematopoietic tissues has been linked with the freemartin condition in those cases in which the embryos have been of different sexes. Recently, Dunsford, Bowley, Hutchison, Thompson, Sanger, and Race (1953) have described a human female erythrocyte chimaera which is interpreted as illustrating the same phenomenon, but without associated sexual abnormality. Earlier experiments dealing with transplantation specificity in twin cattle by Anderson, Billingham, Lampkin, and Medawar (1951) and Billingham, Lampkin, Medawar, and Williams (1952) indicated that cells or materials other than erythrocyte primordia might be exchanged between twins. This body of evidence suggested to Billingham, Brent, and Medawar that it might be possible to prolong the life of skin homografts in adult mice by a prior exposure of the fetal mouse to foreign homologous cells. An experimental test of the proposal has indicated strongly that it may be correct (Billingham et al., 1953). The evidence presented suggests that the results are not due to either antigenic transformation of the grafts or to the competitive absorption of antibody. The effect is not of an all-or-nothing character; all degrees of success are noted. The immunological specificity is high: prior exposure of a CBA mouse in the fetal period to cells of a strain A donor induces tolerance

to A cells, but not to AU cells. There is a suggestion, however, that a general quiescence of antibody-forming cells is necessary, for if CBA mice rendered tolerant to and bearing A grafts are challenged with AU grafts, as the latter are destroyed, the A grafts undergo an immunological crisis. Thus, although embryos do not produce circulating antibody, they respond in a manner which prejudices their ability to do so in later life. Moreover, this response is highly specific. Owen, Wood, Foord, Sturgeon, and Baldwin (1954) have reported briefly that a similar tolerance acquired during the fetal period may be, in part, responsible for the observed variation in response of Rh negative persons exposed to Rh antigens.

Great interest attaches to the recognition that the mechanism through which the specificity of protein synthesis is accomplished can be modified during the fetal period. Several questions of considerable importance are raised: Weiss and Andres (*op. cit.*) have reported that donor cells can participate in a type-specific localization in an embryonic host; Ripley and Owen (cited by Horowitz and Owen, 1954) have produced persistent erythrocyte mosaicism by injecting embryonic rat cells into rat embryos of different blood types. These observations, together with the demonstration of the localization of tissue-specific proteins, or molecular constituents larger than amino acids, from grafts in host embryonic tissues, lead one to inquire as to the fate of the fetal inoculum employed by Billingham et al. Is it feasible to undertake experiments to trace the materials injected? Is there a type-specific localization or a concentration in the cells of the developing reticulo-endothelial system? Does "actively acquired tolerance" result only from the establishment of permanent lines of donor-type *cells,* or are *particulate* or *molecular* inocula effective? A great deal more work must be done before the general significance of this promising field can be properly assessed.

VIII. THE ROLE OF PEPTIDES AND PROTEINS AS SPECIFIC PRECURSORS IN PROTEIN SYNTHESIS

The evidence and interpretation just presented for the ability of embryonic tissues to incorporate type-specific cellular and molecular constituents and to respond to the inoculation of fetal tissues in such a way that one of the major protein-forming systems, i.e., the antibody-forming system, is altered in a highly specific sense, naturally raises the question as to whether other lines of evidence offer any support to such a concept.

It has been noted earlier in the discussion that recent investigations have been centered about the role of free amino acids as precursors of

proteins. An important feature of the concentrative activity of cells for amino acids undoubtedly is that it provides a reserve supply of precursors for protein synthesis. Other possible precursors representing the various amino acids have not been detected except in much lower supply. Borsook, Deasy, Haagen-Smit, Keighley, and Lowy (1949) have suggested that a peptide fraction extracted from liver may be a reserve precursor; this material has been found to be highly complex, containing at least four substances (Fels and Tiselius, 1952), but has not been assessed further. The scarcity of intermediate precursors has been observed in yeast by Halvorson and Spiegelman (1952) who reported that none of the amino acids were depleted from the cells when the utilization of one of them for growth or inductive enzyme formation was prevented by the presence of a synthetic analogue. Therefore, no appreciable quantity of intermediate precursors appeared to be involved in the synthesis of the enzyme or other proteins except for possible precursors already so complex as to require the entrance of the amino acid whose utilization was blocked. The authors concluded that already-formed proteins have no direct role in the formation of inductive enzymes without passing through the free amino acid pool, a conclusion in agreement with that reached by Monod and Cohn (1953) in their study of the synthesis of beta-galactosidase in *E. coli.*

Muir, Neuberger, and Perrone (1951) have presented data which indicate that there are no differences in specific activities of valine at terminal and non-terminal positions of hemoglobin synthesized by the rat supplied with the labeled amino acid. Additional evidence which is interpreted as favoring the idea of simultaneous incorporation of amino acids in the formation of a protein is found in a comprehensive investigation by Simpson and Velick (1954) who have studied the incorporation of a mixture of five labeled amino acids (glycine-1-C^{14}, DL-alanine-1-C^{14}, DL-lysine-2-C^{14}, DL-phenylalanine-3-C^{14}, and DL-methionine-S^{35}) into the enzymes aldolase and glyceraldehyde-3 phosphate dehydrogenase in the rabbit. The authors conclude that the two enzymes were synthesized from the same pool of precursors but at different rates. It should be pointed out, however, that the enzymes were recovered for analysis thirty-eight hours after injection of the labeled compounds. A study after much shorter intervals is indicated.

The low level of tissue peptides, aside from specialized structures, has led to considerable uncertainty as to the place of intermediates in protein synthesis. Yet it does not seem at all likely that all of the peptide bonds

of a new protein are closed simultaneously. In contrast to the foregoing discussion, several lines of evidence favor the concept of peptide intermediates. In collaboration with Steinberg, Anfinsen (1951, 1952) has reported non-uniform labeling of different residues of the same amino acid in ovalbumin. Peters (1953) has concluded from a tracer study of serum albumin synthesis that amino acids are in intermediate compounds for at least twenty minutes before the fully-formed protein can be detected. The nature of the intermediates is unknown, although they do not resemble the protein in serological properties, or electrophoretic or solubility behavior. From a study of the synthesis of globulins in pea seeds, Snellman and Danielsson (1953) have concluded that the synthesis is a stepwise phenomenon involving the formation of oligopeptide intermediates. Francis and Winnick (1953) have compared the mode of utilization by tissue cultures of cardiac muscle of free C^{14}-labeled amino acids with that of the same amino acids in protein bound form. From experiments in which relatively large concentrations of non-labeled amino acids were added to the medium as metabolic traps, it was concluded that most of the C^{14} singly labeled proteins could be transferred from a nutrient embryo extract medium without the release of free amino acids.

This conclusion recalls the long debated role of the plasma proteins in the transfer of protein precursors from cell to cell. In a recent review, Christensen (1953) concludes that "satisfactory evidence does not appear to be available for assigning a function of quantitative importance to the undegraded plasma proteins in transferring nitrogen from cell to cell." Studies by Barry (1952) indicate that the plasma proteins have no detectable part as precursors of milk proteins, a conclusion supported by Campbell and Work (1952) with respect to casein; for the whey proteins a minor but significant participation by precursors other than free amino acids seemed to be involved. Conversely, Ten Cate (1953 a, b) and Nace (1953) have pointed out that the transfer of proteins from the blood may account for some of the immunological similarities that have frequently been detected between the egg and blood proteins. It seems not unlikely that analogous mechanisms are utilized in the management of other proteins. Telfer (1954) has provided a sound body of evidence that a specific antigen is secreted into the blood of the pupa of the *cecropia* silkworm by some tissue, as yet unidentified, and that during the period of egg formation, the ovaries remove the antigen from the blood and deposit it in the yolk. The evidence for the last point

comes from a well-designed experiment in which Telfer demonstrated by immunochemical methods that the ovaries of the *polyphemus* moth incorporate *cecropia* antigen from transfused *cecropia* blood.

The most striking evidence for the transfer of protein from plasma to fixed tissues followed by transformation of the plasma protein to other kinds of intracellular protein is provided by an extensive and critical study by Humphrey and McFarlane (1954 a, b) of the fate of homologous antibody in the rabbit. Let us consider the findings and interpretations of this approach in some detail. It should be noted first that when Yuile et al. (1951, 1952) produced plasma proteins labeled with lysine-C^{14} in a dog and fed them to a second dog, the fate of the isotopic carbon was essentially the same as when free labeled lysine was fed along with a protein digest, indicating that the homologous protein probably underwent digestion. In contrast, if the labeled plasma protein was injected, as much as twenty per cent of the radioactive carbon was found after seven days in the circulating plasma protein and as much as forty per cent in the tissues. The respiratory loss of labeled CO_2 was only about one-tenth as great as after feeding the proteins, which was interpreted as indicating that the lysine had not passed through the free state in the conversion from plasma to tissue protein. Christensen (*op. cit.*) has remarked that an alternative explanation might be that the lysine gradually released from the plasma protein over a long period would be used more extensively for new synthesis than a large concentration available for a short time. Moreover, Yuile et al. did not reveal how much of the radioactivity found in the tissues was in the form of unchanged transfused protein and how much was in newly formed tissue proteins. Nor did they distinguish between labeled intracellular protein and labeled plasma protein in the lymph.

In their analysis of the problem, Humphrey and McFarlane first showed that C^{14}-labeled antibody and C^{14}-labeled normal gamma globulin disappear from the circulation of the rabbit at similar rates. Evidence was advanced to support the view first put forward by Heidelberger, Treffers, Schoenheimer, Ratner, and Rittenberg (1942) that antibody molecules in the circulation do not exchange their constituent amino acids and it was suggested that normal globulin molecules probably also do not do so. Antibody molecules may therefore be permissible tools for use in the study of the ultimate fate of plasma globulins. In fact, they offer considerable advantage for they can be recovered and identified by means of specific precipitin reactions.

In the experiments to be described, antibody was prepared for use as a tool in the following manner: A rabbit was injected with carboxy-C^{14} phenylalanine in divided doses over a period of forty-eight hours, beginning two days after the last of a series of injections of pneumococci. The rabbit was bled fifteen hours later and the serum proteins fractionated. For the purpose of these experiments a globulin fraction was employed comprising forty per cent specifically precipitable labeled antibody and sixty per cent other labeled globulins. One gram of this fraction was transfused intravenously into a rabbit. This was followed after sixteen hours by an injection of Evans Blue dye. After twenty-five additional hours the animal was sacrificed and perfused. By extracting the tissues with carrier antibody and measuring the amount of residual lymph by means of the Evans Blue dye, it was shown that practically all of the residual antibody could be accounted for in the residual lymph, suggesting that the remainder of the radioactivity present was in the form of protein which had lost its original specific antibody configuration. Transfused antibody entered cells in organs which became highly labeled, viz., liver, kidney, spleen, bone marrow, lung, and gut, and was rapidly changed into some other kind of intracellular protein. Preliminary determinations of plasma-free amino acid radioactivity showed that some part of the carbon in several organs (liver, bone marrow, lung) had a higher specific activity than the plasma free amino acid fraction and could not have arisen from the plasma-free amino acids. These latter findings are not to be regarded as critical, however, since the exact proportion of phenylalanine in the plasma free amino acid portion compared with that in the tissue proteins has not been determined. The results are compatible with the idea that some of the radioactively labeled tissues may have used transfused globulins directly for protein synthesis.

The selective incorporation of radioactivity from proteins of labeled chorioallantoic grafts of adult chicken tissues into proteins of homologous tissues of host chick embryos and the other lines of evidence cited above offer strong support for the conclusions of Francis and Winnick and Humphrey and McFarlane that embryonic and adult tissues, respectively, can utilize already-formed proteins for the neosynthesis of protein; in fact, they favor the further conclusion that the incorporation may be of a highly specific nature in that the constituents of one protein may be used preferentially in the synthesis of the homologous tissue protein. It is not necessarily implied that free amino acids were

not formed as intermediates, but only that the intracellular amino acid pool was not in immediate, complete equilibrium with the plasma-free amino acid pool.

In the above pages a few of the problems of protein synthesis in differentiation and growth have been presented. Many of these problems were formulated long ago, but in different terms; only recently have we been able to make a first approach toward their restatement in terms of molecular interactions. Imposing as these problems may be, the real challenge lies ahead, for protein biosynthesis constitutes only one of the ordered sequence of chemical and physical transformations which result in cellular differentiation and morphogenesis. Not the least of the problems which lie ahead is the definition in molecular terms of the category of tissue interactions described as "tissue affinities." The type-specific localization of cells and molecular constituents which results in their incorporation into normal patterns of differentiation and growth described above illustrates one present attack on this problem. Probably the methods now employed will prove to be over-simple and inadequate. The purpose of discussion is to reveal the problems and to suggest possibilities for their solution.

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V. REVIEW OF THE PLEIOTROPIC EFFECTS OF W-SERIES GENES ON GROWTH AND DIFFERENTIATION

BY ELIZABETH S. RUSSELL[1]

THE study of gene action in mammals is a fascinating and challenging field of investigation. It is one which can make use of a large body of existing knowledge of embryology, physiology, histology, and biochemistry, and also can make unique contributions to these fields by the analysis of natural experiments performed by genes. The types of gene effects observed in mammals are quite different in their relation to original gene action from the beautiful examples of gene-controlled biochemical processes in Neurospora. The mammalian effects are almost of necessity farther removed from the original time and place of gene action, and involve differences in inductive processes, in form and function of particular organs and tissues, and in endocrine relationships, for example. Any particular gene under investigation seems to exert its influence in a major way only in certain tissues or at certain stages. A matter of great interest is the nature of factors responsible for evoking these effects and for limiting them to definite times and sites.

In this paper I would like to draw together results of investigations and pose questions for future study concerning the nature of actions of one particular series of allelic genes, the *W*-genes of the mouse. These have major effects on quite disparate characteristics: erythrocyte level and blood formation; viability; gonad development and function; and extent and intensity of coat pigmentation. There are three alleles, *W*, W^v, and *w*, and thus six possible genotypes for this locus. A general description of the effects in three of these genotypes, the normal *ww*, the severely anemic but viable, sterile, black-eyed white W^vW^v, and the extremely anemic lethal, *WW*, is given in Fig. 1. It should be understood that other genotypes are also affected; WW^v is also very severely anemic, and W^vw slightly anemic.

In the normal *ww*, the mean erythrocyte level at the 16th day of embryonic life is 2.6×10^6RBC/mm^3, the mean in the newborn is $4.8 \times$

[1] Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. This work has been supported in part by a grant to the Jackson Memorial Laboratory from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. It has also been aided by grants to the Jackson Laboratory from the Anna Fuller Fund, Jane Coffin Childs Memorial Fund, and the National Cancer Institute, U. S. Public Health Service.

10^6RBC/mm^3, and the mean in the adult rises to approximately 9-10 $\times$ 10^6RBC/mm^3 in the strain under investigation (deAberle, 1927; Russell and Fondal, 1951). In the severely anemic but viable W^vW^v, the mean 16-day embryonic erythrocyte level is only 1.3 $\times$ 10^6RBC/mm^3, the newborn level is only 2.2 $\times$ 10^6RBC/mm^3, and the adult level rises to approximately 5-5.5 $\times$ 10^6RBC/mm^3. In the even more severely anemic *WW*, the 16-day erythrocyte level is 0.5 $\times$ 10^6RBC/mm^3, the new-

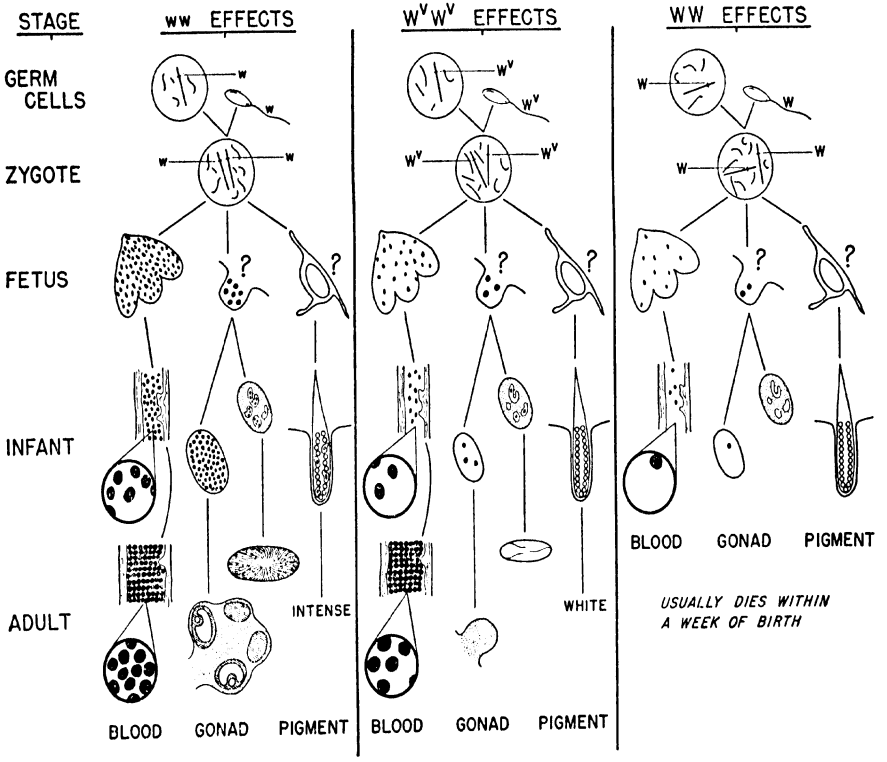

Fig. 1. Summary diagram of the effects of *ww*, W^vW^v, and *WW* substitution upon blood formation, gonad development and function, and hair pigmentation.

born level is 0.8 $\times$ 10^6RBC/mm^3, and practically all of these individuals die very shortly after birth. Blood cells are formed first in mammalian embryos in the yolk sac, later (from the 12th to the 16th day in the mouse) in the liver, and hematopoiesis in the mouse bone marrow commences at the 16th day of embryonic life. Thus blood formation at any or all of these sites or the survival of blood cells formed in any of them could be affected by *W*-gene action.

W^vW^v adults of both sexes are almost universally infertile and the

sterility definitely involves a great reduction in the number of developing germ cells (Coulombre and Russell, 1954). The adult ovary may contain a very few developing follicles as late as three months, but thereafter none have been observed, although the normal *ww* ovary contains many large follicles at one month, corpora lutea by six weeks, and continues to produce large numbers of ova which develop normally long after one year, when other ageing effects have greatly reduced the total reproductive efficiency. The adult W^vW^v testis is also almost devoid of spermatogenesis. Most of the tubules contain only Sertoli cells, and the few germ cells which are observed are almost universally spermatogonia. Since the *anemia* effect is present before birth, it would be of great interest to learn when and how the sterility effect arises. It would also be of interest to find if the gonads of *WW*'s show any signs of defect during the brief life of these individuals. The adult W^vW^v individual always has a white coat, but differs from an albino in having pigmented eyes. Occasional *WW* individuals have survived, either spontaneously or as the result of transfusion, and these also are black-eyed whites. Heterozygous *Ww* and W^vw individuals have white belly spots. In addition the coat of W^vw is somewhat less intensely colored than its *ww* or *Ww* counterpart. The beautiful work of Rawles (1947) has established that cells originating in the neural crest, and migrating posteriorly and laterally over the entire body between the eighth and twelfth days of embryonic life, are responsible for coat-color pigmentation in the normal *ww* mouse. Nothing is known as yet of possible differences in numbers, migration, or survival potentialities of these pigment-potential cells of the neural crest in differently pigmented *W*-series genotypes. It would also be very valuable to look for differences in the adult hair follicles of pigmented and non-pigmented types, and to compare the W^vW^v follicles with albino follicles.

Thus *one* set of genes clearly affects *three* quite different types of tissue. How did all of these effects result from one cause? Possible ways of attacking the problem have been mentioned during the presentation of each type of effect. Some of my questions were really rhetorical; I already know the answers and will describe the work which produced them. For others, only possible methods are known. In still other places, we are frankly awaiting suggestions of possibilities. The analyses involve either retrograde studies going back into embryonic life as far as possible to seek ultimate time and place of the origin of differences, or else application of special experimental techniques to determine the nature of metabolic differences within known affected tissues. All of this

study is an attempt to make "developmental" genetics truly "physiological."

It is perhaps best to give first a detailed discussion of work on the blood defect. The red-cell numbers already presented indicate that the anemia is present by the 16th day of embryonic life. Population studies of erythrocyte counts from 14-day embryo litters with and without potential anemics indicate that it is also present at this stage (Russell, Fondal, and Smith, 1950). Studies of the rate of increase of erythrocyte numbers, based on a value proportional to the estimated total number per individual, show that all genotypes, normal and anemic, make approximately the same relative increase during each time interval, and thus show the same relative response to the physiological stimulus of the individual's growth (Russell and Fondal, 1951). Gruneberg (1939) showed that W^vW^v anemics will respond to stress such as bleeding and low barometric pressure by producing more erythrocytes. It thus appears that although there may be great differences in activity, no genotype is completely aplastic. With this knowledge, two approaches suggested themselves: study of hematopoietic tissues, and study of chemical structure and life-span of completed erythrocytes. In the normal *ww,* the embryonic liver contains very many hematopoietic cells, while in the lethal anemic *WW* the number is much reduced (Borghese, 1951). A similar difference in cellularity has been found in the newborn (Russell, Snow, Murray, and Cormier, 1953). The marrow of the newborn *ww* is highly cellular, that of the severely anemic W^vW^v is distinctly less so, that of WW^v still less, and that of the even more severely anemic *WW* is extremely hypoplastic. Studies of sternum, tibia, and vertebrae at 2, 4, 6, and 8 weeks show the bone marrow of the normal *ww* very rapidly reaching maximum cellularity. The marrow of W^vW^v individuals also increases in cellularity, and by 4 weeks is just significantly, but certainly not obviously, less packed with cells than the normal. Almost all of the *WW* individuals (especially on the genetic background used in this study) died within a few days after birth. If 4 week old *ww* and W^vW^v individuals have such similar bone marrow, why is the erythrocyte level in one approximately 9 million, and in the other 5 million? Comparison of proportional counts of cell types in femoral marrow smears from normal and anemic animals at 2, 4, 6, and 8 weeks showed a highly significant arrest in erythroid maturation in the anemics at some stage prior to the formation of the mature erythroblast with pyknotic nucleus (Russell, Snow, Murray, and Cormier, 1953). These marrow differences prove that the W-series genes are associated with

varying degrees of macrocytic anemia through the blocking of some stage of normoblastic erythropoiesis.

Although these findings suggested that study of erythropoiesis might be the most profitable field to continue, the chemistry of the completed erythrocyte was not neglected. With the cooperation of S. J. Singer of Yale, electrophoresis patterns of hemoglobins from normal *ww*, slightly anemic $W^{v}w$, and severely anemic $W^{v}W^{v}$ individuals were compared, as well as the sedimentation constants and solubility. The results indicate that the hemoglobins of *ww*, $W^{v}w$, and $W^{v}W^{v}$ mice are very similar, if not identical (Singer and Russell, 1954). Although subtle differences not detectable by the techniques employed may exist, it seems highly probable that the physical-chemical properties of the hemoglobins are sufficiently similar to exclude these properties as primary factors in the causation of the anemias.

The final work on the blood defect which I would like to report has been presented briefly to the Biochemical Society and to the Genetic Society meetings, but this paper is its first detailed presentation for publication. It seemed to me in 1950 that certain very pertinent aspects of this anemia had not yet been investigated. Although there has never been evidence of excessive hemolysis in the anemics, no tests of their erythrocyte life-span have been reported. And while there appears to be an inhibition of hematopoiesis at least from the beginning of activity in the liver, no search has been made for specific alteration of cellular metabolism. Thus I was extremely receptive to the suggestion of cooperative experiments with Dr. Kurt Altman, Dr. Kurt Salomon, and Dr. James K. Scott, all of the Atomic Energy Commission at the University of Rochester, which seemed to offer the possibility of filling in these gaps and advancing knowledge of hematopoiesis in normal and anemic mice through tracer studies using α-C^{14} glycine.

The labelled glycine was made available in the body pool of normals and anemics for a limited period of time, during which it could be incorporated into many different substances in a wide variety of tissues. Only the incorporation into hemoglobin has been analyzed. Glycine regularly goes independently into two distinct portions of the hemoglobin molecule, small amounts being incorporated into the protoporphyrin which is a precursor of the central heme unit, much larger amounts into the four units of globin. By collecting blood from treated animals at varying intervals after the glycine injection, and preparing from this material protoporphyrin dimethyl ester and globin, it was possible to compare the level of radioactivity in the two portions of the hemoglobin

molecule of normals and anemics. From these data it was possible to deduce the relative proportions of newly formed heme and globin and the rate of formation of each of these units. Once incorporated into an erythrocyte, a particular unit of radioactivity remains in the blood stream for the life of that cell. Thus comparison of the length of the plateau of constant radioactivity readings in the different genotypes gives an index of the life-span of their erythrocytes. The animals used in these experiments were littermate groups, raised at the Jackson Laboratory, from matings of $W^v w$ parents isogenic with the C57BL/6 Jax strain. They were a somewhat selected group, since on this genetic background the severely anemic $W^v W^v$ is not completely viable in the pre-weaning period. Each $W^v W^v$ individual which survived to weaning was kept with a littermate ww and $W^v w$, of the same sex if possible. At two to four months of age, littermate groups were shipped to Rochester, where litters were combined to provide treatment units with four of five animals of the same genotype. All animals were injected intraperitoneally with 2 millicuries of C^{14} glycine per 100 g. body weight, and were sacrificed after time intervals which varied among units from two to thirty-three days. Within units, blood of animals of the same genotype was pooled. From the observed C^{14} activities of globin and protoporphyrin (Table I) it is apparent that duplicate units collected after the same time interval show excellent agreement, and between-unit error is negligible. Quantitative comparison of the levels in different genotypes is complicated by the differences in levels of pre-existing erythrocytes (approximately 10×10^6 in ww, 8.5-9 $\times 10^6$ in $W^v w$, and 5×10^6 in $W^v W^v$). To facilitate comparison of absolute rather than relative amounts of new protoporphyrin and globin, the observed levels in $W^v w$ were multiplied by $\frac{7}{8}$, and those in $W^v W^v$ by $\frac{1}{2}$ (Figure 2). Both absolute and relative methods of presentation have their value for interpretation, and it is not certain which gives the truer picture.

By either method it is clear that in all genotypes appreciable amounts of newly formed labelled globin are present in erythrocytes released to the blood stream less than 48 hours after isotope administration, and that maximal amounts appear after three to seven days and persist through most of the experiment. There is no clear relationship of globin formation to genic effect.

It is equally clear that the genotypes differ markedly in time of appearance of labelled protoporphyrin in mature erythrocytes, since normal ww's and $W^v w$ heterozygotes show appreciable amounts in two days and maximal amounts in seven days, while $W^v W^v$ homozygotes show

no activity in protoporphyrin at seven days, and maximal amounts at fourteen to thirty-three days. Interpretation of the quantitative relationships among the genotypes depends somewhat on whether absolute or relative levels give the true picture. In both, the severely anemic W^vW^v homozygote is always lower than the normal. The heterozygote

TABLE I. C^{14} activity of protoporphyrin dimethyl ester and globin prepared from blood of mice previously injected with 2 millicuries of α-C^{14} glycine per 100 g. body weight. Each value within parentheses is based on the pooled blood of four or five animals of the same genotype treated and sacrificed at the same time. The times given represent intervals between completion of C^{14} injection and sacrifice. The segregating genotypes came from groups of littermates of the same age under each heading.

Age	Genotype	C^{14} Activity*			
		Protoporphyrin dimethyl ester		Globin	
48 hrs.	ww	4.5	(4.4, 4.6)	157	(154, 160)
	W^vw	6.0	(5.8, 6.2)	107	(105, 109)
	W^vW^v	0	(0, 0)	35.4	(35.0, 35.8)
72 hrs.	ww	26.1	(26.0, 26.2)	680	(670, 690)
	W^vw	21.7	(21.7, 21.7)	306	(302, 310)
	W^vW^v	0	(0, 0)	432	(428, 436)
7 days	ww	30.1	(29.6, 30.7)	522	(517, 527)
	W^vw	25.1	(25.1)	356	(356)
	W^vW^v	0	(0, 0)	727	(720, 734)
14 days	ww	29.3	(29.0, 29.6)	460	(457, 463)
	W^vw	24.8	(24.3, 25.3)	345	(340, 350)
	W^vW^v	19.2	(19.0, 19.4)	640	(635, 645)
22 days	ww	26.8	(25.9, 27.7)	454	(450, 458)
	W^vw	25.4	(25.0, 25.8)	357	(352, 362)
	W^vW^v	20.4	(20.4)	626	(626)
33 days	ww	24.1	(23.7, 24.5)	416	(410, 422)
	W^vw	24.4	(23.9, 24.9)	396	(392, 400)
	W^vW^v	18.3	(18.1, 18.5)	279	(274, 284)

* $C^{14} \times 10^4$ = disintegrations $\times$ min.$^{-1}$ $\times$ mM^{-1}

lags behind the normal throughout the dilution-corrected absolute level curve, and until twenty-two days in the uncorrected relative level curve. Thus it appears that there is a significant difference among the genotypes in the rate of protoporphyrin formation, and that this difference is related to the erythrocyte level of these genotypes.

The nature of the gene-influenced difference in cellular metabolism may be of either of two kinds. One possibility is that the difficulty in

making protoporphyrin comes quite early in the path of gene action, and is not too far removed from original gene action. Under this assumption, the delay in erythroid maturation results directly from the delay in formation of this part of the hemoglobin molecule. Such a close connection would be a feather in the cap of mammalian physiological geneti-

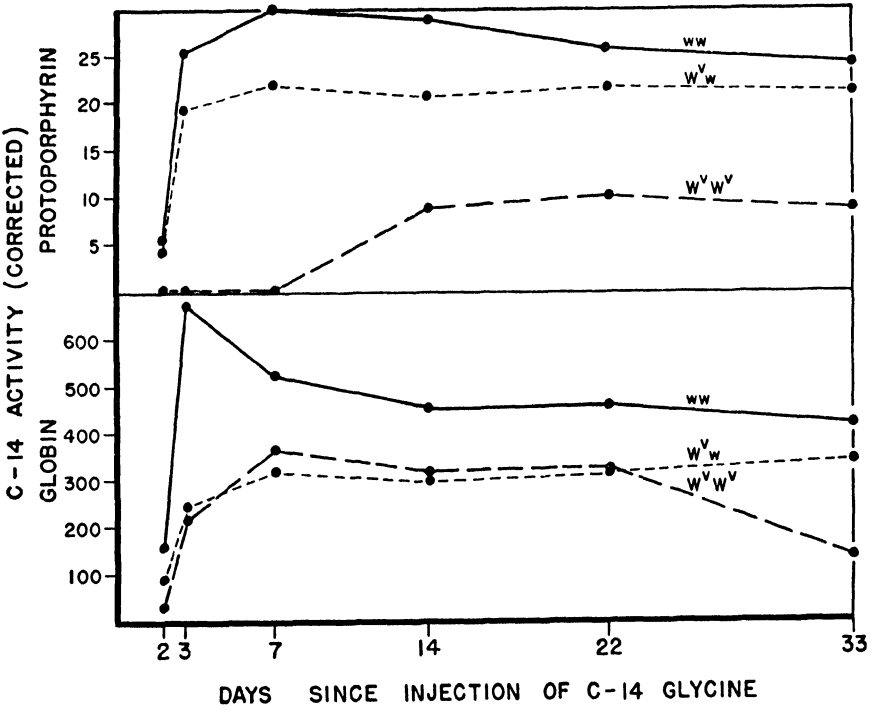

Fig. 2. Graphic presentation of the C^{14} activity in protoporphyrin dimethyl ester and in globin prepared from blood of mice previously injected with α-C^{14} glycine and sacrificed after varying time intervals. The values have been corrected for variations in dilution effect resulting from differences in total preexisting erythrocyte level characteristic of the genotypes tested, as explained in the text.

cists, but all of the investigators involved in these experiments feel that the second type of interpretation is more probable. It may be that by some mechanism as yet undiscovered the W-gene activity alters the maturation of erythroid elements at a time when their chief activity as regards hemoglobin formation is the production of protoporphyrin. At the stage when globin is formed and/or added to the central heme unit, retardation is no longer effected by these deleterious genes, and there is no genotypic difference. This leaves a much larger field for speculation as to the true basic nature of W-gene effect on blood-forming tissue, but

may give to this effect a wider area of similarity to the effects on other affected tissues, as will become apparent in the discussion of W-series effects on germ-cell development and function.

Differences in normal and potentially sterile gonads, which occur in both sexes, are already present at birth. It is well known that primitive germ cells, possibly originating in the gut entoderm and migrating to the germinal ridge, are present in embryonic gonads. These are still present in the newborn mouse. In the normal *ww* 0-day ovary, there are literally thousands of them (Coulombre and Russell, 1954) throughout the organ. They are likewise found in all of the testicular tubules of newborn *ww* males. In contrast, the ovaries and testes of both W^vW^v and WW newborns are almost devoid of these primitive germ cells.

By 28 days, the ovaries of potentially fertile types have increased greatly in size, and many follicles have reached a stage with large coalescing antra. Numerous small nests of primary follicles may be seen throughout the ovary, particularly just beneath the germinal epithelium. In the defective WW^v and W^vW^v 28-day ovaries, the number of developing follicles is always much smaller than in the normals, and they tend to be less advanced (Coulombre and Russell, 1954).

The postnatal development of testes also differs greatly between genotypes. By 28 days, the size of the individual tubules and of the entire gonad has increased greatly, and all stages of spermatogenesis may be seen. In the defective genotypes, the tubule and organ sizes have increased much less than in the normal. Each tubule consists of a single layer of undifferentiated cells whose cytoplasm forms a syncytium. Less than 4% of the tubules contain any spermatogenic cells, and these are spermatogonia only except for a very few small regions with primary spermatocytes (Coulombre and Russell, 1954). Here also the degree of development of germ cells is much less in the WW^v and W^vW^v individuals than in the normal.

"The striking difference in amount of germinal tissue, occurring in both sexes and already present at birth, supports strongly the hypothesis that the W-gene inhibition of fertility is mediated through an effect upon the germ cells themselves and is initiated early in development" (Coulombre and Russell, 1954). What is the nature of this effect? At what stage or stages does it occur? A method of approach to these questions is that of organ transplantation, so that gonads of each genotype may grow in an environment foreign to them, and demonstrate the presence or absence of capability for autonomous development at successive stages in embryonic life. This method is particularly appealing in the case of

the germ-cell deficiency in the W^vW^v, WW^v, and WW genotypes, since the extreme anemia found even during embryonic life in these same individuals might be responsible for the germ-cell failure, and the differences should in that case disappear or at least be minimized if the gonads were grown from earlier and earlier developmental stages in neutral blood-supply sites. To test this possibility, embryonic gonads have been removed from their anemic or normal embryonic environment and transplanted to the spleen of adult castrate C57BL/6 males. The results of this experiment will be reported in detail elsewhere, but a brief summary here will help in our attempted analysis of *W*-gene action. Since the transplant and host were isogenic, except for the *W*-genes, many implants, especially testes, developed successfully. A linked gene, with clear-cut embryonic expression, luxate, was used to identify genotypes of embryos from which gonads were taken. The implants were allowed to develop in the spleen to the equivalent of 14 days postnatal. Gonads of both sexes from 16-day, 14-day, and 13-day embryos, recovered from the spleen, have developed autonomously, according to their original genotype (Table II). As far as the 12-day embryo results go, they are

TABLE II. Results of embryonic gonad transfers into the spleen of adult castrate hosts. The proportion of functioning tubules is universally higher in the potentially fertile testis transplants. The number of developing follicles is higher in the transplants of potentially fertile ovaries. Thus at 13 and probably also at 12 days of embryonic life the gonads have already been determined to develop autonomously according to their own W-series genotype.

TESTES: *Proportion of functioning tubules*					
Genotype		12 day donors	No. cases	13 day donors	No. cases
Known	ww	239/274	4	256/338	8
"	W^vW^v	9/45	2	44/373	9
"	WW	—	–	10/163	4
OVARIES: *Total developing follicles*					
Known	ww	29	1	13, 15, 18, 118, 16	5
"	W^vW^v	—	–	0, 3	2
"	WW	—	–	0	1

indistinguishable from the 13-day results, and definitely support the theory of autonomy of development of germ cells according to original genotype. Thus the hypothesis of germ-cell inhibition through embryonic anemia must be cast out. All the data so far suggest that the effect may be a local one, with gene action altering the metabolism of the germ cells directly. Tracing blood and germ-cell effects back to the twelfth to

thirteenth day of embryonic life has not drawn together the branches of the "pleiotropic" tree; they are as far apart relatively as in the adult.

It is no easier to relate the pigment effects to either blood or germ-cell effects. Some such relationship undoubtedly exists. In a study of the relationship between the effects of W and W^v substitution on hair and on erythrocytes, it was shown (Russell, 1949) that one dose of the W^v gene always produces a slight macrocytic anemia, and on five different color backgrounds (full-color black, chinchilla black, full-color brown, pink-eyed sepia, and full-color yellow) such a single dose always reduces the number of pigment granules and sometimes reduces granule size. A single dose of the W-allele has no effect on pigment granule number or size in a full-color black stock whose histology was extensively studied, nor does it affect the intensity of pigmentation on any background against which it has been observed. W^vW^v, WW^v, and surviving WW individuals are all black-eyed whites except for occasional pigmentation of ear skin. Such spots of color are more prevalent in W^vW^v than in WW^v, and have never been observed in WW. "In both blood and pigment-forming cells of the hair, W has no effect in one dose, and a very severe effect in two doses, while W^v has a significant effect in one dose and a milder effect than W in two doses. This close correlation in the dominance relations of the two alleles suggests a very close connection between the W-gene action leading to blood changes and that leading to pigment-intensity changes."

In the mouse, a number of mutants other than the W-series have occurred where a tendency to white-spotting is associated with other extremely deleterious effects. Among these may be named flexed tail, Varitint Waddler, Splotch, Dominant White, and Steel. One can not help feeling that an understanding of the basic causes of failure of pigment distribution, at the level of gene action, might provide cross-information useful in understanding other gene-controlled pathology. One fact which has emerged very clearly from recent work is that the hair follicles of all white-spotted areas from seven different genotypes, and of the entire coat of W^vW^v, WW^v, WW black-eyed whites completely lack melanoblasts, whereas the hair follicles of albinos and of extreme dilute yellows, which are white, exhibit "pigmentless pigment cells" (Silvers, 1953). Do potential pigment-forming cells migrate outward from the neural crest to all parts of the body in these genotypes? How, why, and at what stage do they disappear? Since the other deleterious effects of the genes listed above include siderocyte anemia, neuromuscular defect, spina bifida and other abnormalities of the neural tube,

reduction in eye size, and still another anemia, it is improbable that all of their effects upon melanoblasts will be of exactly the same nature. However, they all produce the same end result, lack of pigment-forming cells in the hair follicles of an animal or area of an animal. It would thus be interesting to test the effects in combination of these diverse genes which have in common the one effect on pigment. Early stages of such an experiment (Russell, Coulombre, and Fekete, 1952) have been carried out, and may be pertinent to understanding of *W*-gene action on pigment. By a bilateral breeding scheme, the effects of *W* and W^v in combination with the genes for yellow (A^y) and dominant white (Mi^{wh}) were compared. In addition to the previously established difference in effect on pigment intensity, these results indicated that $W^v w$ on four different genetic color backgrounds produced more white-spotting than did *Ww*. The non-allelic genes ($W^v w$ or *Ww* with $Mi^+ Mi^{wh}$) have a synergistic effect on amount of white-spotting when they are combined. A side line of great interest for pigment geneticists is the observation of four new backgrounds where the gene A^y, which when heterozygous alters the nature of pigment from dark eumelanin to yellow phaeomelanin, and when homozygous causes death at the morula stage, tends to reduce the amount of white in a spotted coat. This field of analysis of pigment physiology offers many and various possibilities for the future, but as yet little evidence of time, place, or nature of gene action is available.

It seems most suitable to close this paper by referring again to the first figure and trying to integrate the findings in the various tissues into a "pattern of gene action." In analysis of the blood defect it has been possible to follow genotypic differences well back into embryonic life; to make deductions from the quantitative relationships in red-cell numbers among genotypes at all stages; to locate the pathology in the bone-marrow and to a certain extent in certain cells within the bone-marrow by quantitative histological study; to establish by physical-chemical methods a lack of *W*-series gene effects on hemoglobin structure; to determine the erythrocyte life-span with the use of radio-isotopes; and actually to probe within affected cells and characterize to a very considerable extent the biochemical nature of a gene-conditioned difference in cellular metabolism. In the analysis of the sterility effect it has been possible to implicate defects in the germ cells of both sexes as the immediate cause of sterility; to demonstrate that primitive and definitive germ cells are both very severely affected; to show that both numbers and degree of development of definitive germ cells are affected by *W*-gene substitution; to show that the germ cells of 13- and even 12-day embryos

develop autonomously according to their original genotype upon transplantation to a neutral site; and thus to show that the germ-cell defect is *not* dependent upon the embryonic anemia for its development. In the analysis of the pigment defect, the existence of a relationship of the gene action to that in the blood defect has been proven by quantitative comparison of the effects of W and W^v substitution upon both characters; establishment of the lack of melanoblasts in the hair follicles of white-spotted areas and throughout the coat of the black-eyed whites has traced the pigment effect back one brief step; and study of W and W^v in combination with other genes with effects on pigment-extension has suggested a profitable field for future exploration.

Although it is perfectly clear that there must be a relation between the actions of the W-series genes in these various tissues, there is nothing in any retrograde analysis presented which tends to narrow down the "pedigree of causes" toward a single tissue in which the primary action occurs. At first thought this failure would tend to argue against the principle of "unity of gene action," and suggest that this series of genes presents a real case of what Gruneberg has called "genuine" pleiotropism (1943), a phenomenon which "has never been conclusively demonstrated." However, I do not feel that this series of genes does have a multiplicity of primary gene actions. I would like to reintroduce a suggestion put forward five years ago (Russell, 1949). There may be "unity of gene action" without absolute "tissue or cell specificity." "It is logically possible . . . that the same original gene product could by its intrinsic nature be active in two (or more) types of cells, long separated in development and different in structure. The gene in question might either act on the same substrate which has been independently produced in two different localities, or, perhaps more probably, it might be general enough in its action to affect two substrates. Where (three) types as different in origin and function as erythrocytes, (germ cells, and melanoblasts) of the hair follicle are involved, it seems this category of pleiotropism must be considered. In one sense there (probably) is unity of gene action, since the gene (may) release only one product. In another sense there is real pleiotropism, as this one gene product (appears to be) doing two things directly by means of different mechanisms." To date, the closest we have come to one of these basic actions is the delay in protoporphyrin formation of W^vW^v anemics, but we have suggested that this may reflect rather than cause the erythroid maturation arrest. Although there are absolutely no data available which bear on this point, it could be that the same basic retardation occurs by local gene

action in hematopoietic cells, in germ cells, and in migrating melanoblasts.

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VI. VIRUS-INDUCED METABOLIC TRANSFORMATIONS AND OTHER STUDIES ON UNBALANCED GROWTH

BY SEYMOUR S. COHEN[1]

I SHALL really be presenting several papers in one, but fortunately all the experiments I wish to describe are on growth, and fall easily within the scope of this Symposium. In addition, the experiments are held together by a logical thread in that they derive from what seemed to be a single reasonable line of research. As we shall see, it has been my good and bad fortune within the last two years to be confronted with many new and unexpected phenomena. This paper will merely point to their existence, since there has been little time in that period to do much to clarify our findings.

I. INHIBITION OF BACTERIAL GROWTH BY VIRUS

Some years ago intensive studies were begun by a number of workers on certain bacterial viruses, designated the T set, which infect *Escherichia coli*. The chemists of this coterie, including myself, concentrated their efforts for the most part on the T even group of these viruses. The T_2, T_4, and T_6 bacteriophages are closely related in a morphological, serological, and chemical sense. All known bacterial viruses are tadpole shaped, possessing a longer or shorter tail, appear to contain deoxyribonucleic acid (DNA) as their sole nucleic acid, and lack all enzymes for which they have been tested. Infection by the T set of viruses in general and the T even group in particular results in the loss of the host's ability to multiply, and a shunting of the direction of the cell's enzymes away from cell growth to the almost exclusive production of virus (cf. Cohen, 1947). These phenomena are associated with the following sequence of events:

1. The tadpole shaped virus particle adsorbs tail first to the bacterium.
2. The contents of the head pass through the tail into the bacterium.
3. The inhibition of host deoxyribonuclease is released, and the Feulgen-reactive nuclear bodies of the bacterium are fragmented. The fragments of the host DNA become available for virus synthesis.

[1] The Children's Hospital of Philadelphia (Department of Pediatrics) and the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania. The studies described in this paper were supported by a grant from the Commonwealth Fund. Much of the data was obtained in collaboration with Miss Hazel Barner.

4. The infected cell synthesizes virus protein and nucleic acid from low molecular constituents which formerly passed to the synthesis of host material.
5. These virus specific substances are organized, multiply in a noninfectious vegetative state, and are finally entombed in mature infectious virus particles.

Until some four years ago, it was thought that the biological effects of these viruses were rather typical of the effects of all phages. As is well known, Lwoff and his collaborators have recently shown that the unusually severe pathology and sequence of events described above is but one of several types of reactions open to most systems of virus infected bacteria (cf. Lwoff, 1953). It appears to be a far more common reaction for the infecting virus particle to pass through a reductive transformation in which a non-infective prophage merges in some manner with the bacterial nucleus and multiplies in coordination with it. Under such conditions, in systems known as "lysogenic," virus infection may scarcely affect cell viability, metabolism, or reproduction at all. Various environmental conditions, or the properties of the virus or host, may prevent the reductive transformation of the virus, resulting in a more severe parasitism of the bacterium. This type of infection is manifested by a greater percentage of the bacterial metabolism being diverted to virus production. Or the prophage may be induced under rigorously specified conditions depending on host and virus to multiply in this more virulent vegetative state leading to the maturation of fully infectious virus particles. Nevertheless, in either case, in lysogenic systems, the pathology resulting from maturation leading to fully formed virus is by no means as severe as the pathology observed with the T viruses. In maturation in lysogenic systems the infected cell may still produce cell nucleic acids and proteins, adapt to new substrates, and even multiply (perhaps one more division); eventually, however, it lyses, liberating mature infectious virus particles.

We may, therefore, distinguish three types of infected cells, in two of which, existing in lysogenic systems, some considerable degree of cellular synthesis continues. In the third type, that of cells infected by the T viruses, there is a complete reorientation to virus production from the first moments of infection. I shall discuss some aspects of this system in some detail, in the first place because it is the system with which I have mainly worked, and secondly because it seems possible that the information available permits a reasonable explanation of the extreme parasitism observed in the case of cells infected by the T even phages.

II. HYDROXYMETHYLCYTOSINE IN T_2-INFECTED CELLS

The almost exclusive production of virus in T_2-infected cells is particularly evident if we consider nucleic acid metabolism (Cohen, 1951). Uninfected growing cells may make five times as much ribose nucleic acid (RNA) as DNA. On virus infection, RNA accumulation ceases, and the amount of this substance present in the cell does not change. DNA synthesis, on the other hand, occurs at a markedly increased rate and the newly synthesized DNA is virus DNA. This effect is also manifested in a shifting of alternative pathways of glucose utilization. Thus, the amount of glucose passing through the phosphogluconate pathway leading to ribose phosphate is reduced. As has been described elsewhere, this shifted balance in functioning systems of glucose utilization appears to be caused only indirectly by virus, since infection does not appear to inhibit the enzymes of the phosphogluconate pathway (Cohen and Roth, 1953). Infection and the effects on nucleic acid synthesis merely draw glucose-6-phosphate away from the phosphogluconate pathway into the Embden-Meyerhof system.

A more direct cause for the marked change in nucleic acid metabolism appeared with the discovery of 5-hydroxymethylcytosine or HMC (Wyatt and Cohen, 1953; Cohen, 1953). This pyrimidine, whose formula is given in Fig. 1, is a constituent of the DNA of T_2, T_4, and T_6, replacing cytosine in these viruses. The compound has not been found in the host DNA; the latter contains cytosine. Indeed, HMC has not been found to this date in any other virus, microorganism, plant, or animal in which it has been sought. In other members of the T set, the DNA contains cytosine. Thus, we have the first unique viral constituent of low molecular weight appearing in a very few viruses. This fact has already been of use in providing a tag for a kinetic analysis of the rate of degradation of host DNA and of the synthesis of T_2 DNA (Hershey, Dixon, and Chase, 1953).

The existence of HMC permits the following hypothesis concerning the mechanism of the extreme parasitism observed with the T even viruses: it is supposed that cells infected by these viruses can no longer make or accumulate cytosine derivatives, but are compelled to produce derivatives of HMC. Since cytosine is a constituent of host RNA and DNA, in the absence of available cytosine infected cells would then be unable to synthesize these important host polymers. This inhibition would presumably extend to those proteins and other elements in the integrated structures of the cell in which the host nucleic acids are

essential constituents. It seems likely that this would affect the synthesis of cytoplasmic particulates and chromatin, resulting in the apparent inhibition of division and of the synthesis of host proteins and enzymes.

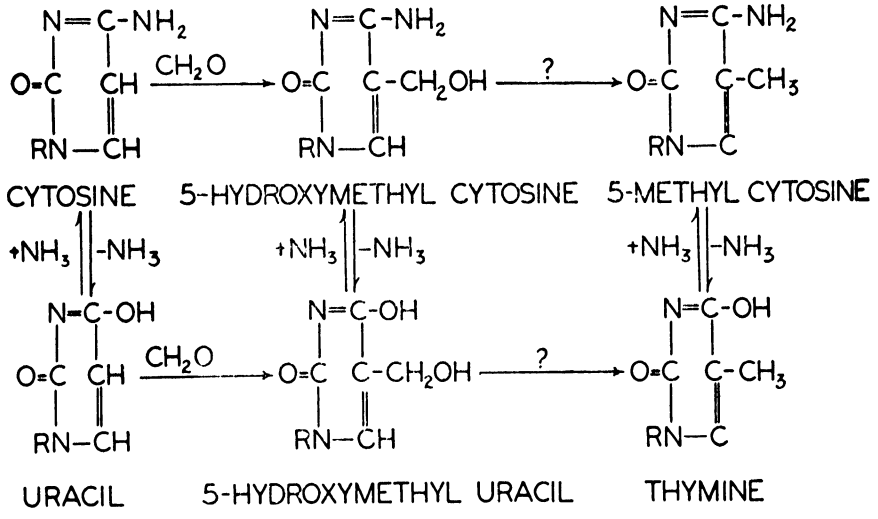

Fig. 1. Some possible interrelations of the pyrimidines.

It has been found in support of the hypothesis that:

1. The cytosine in host DNA is converted specifically to the HMC of virus (Cohen, 1953; Cohen and Weed, 1954).
2. Although cytosine and uracil may be metabolized slowly by *E. coli*, 5-hydroxymethyl pyrimidines are completely inert. (Cohen, *op. cit.*). Thus, the hydroxymethyl pyrimidines or their derivatives may accumulate as the normal pyrimidines disappear.
3. However, the metabolism of cytosine derivatives in *E. coli* appears to proceed mainly in the form of the nucleosides. *E. coli* normally contains a very active deaminase for cytosine deoxyriboside which is much less active (ca 3%) on hydroxymethylcytosine deoxyriboside. Cytosine deaminase is not normally present, but when its synthesis is induced, the enzyme is inactive on HMC (Cohen, *op. cit.*).
4. Deoxyribosides of hydroxymethylcytosine or of hydroxymethyluracil do not fulfill pyrimidine requirements in organisms which grow in the presence of deoxyribosides of cytosine or of uracil. Thus, hydroxymethylation appears to be irreversible (Cohen, *op. cit.*).
5. The deoxyriboside of hydroxymethylcytosine is not liberated in significant amounts from virus DNA by a combination of DNAase

and of phosphatase which liberates cytosine deoxyriboside from other DNA (Cohen, *op. cit.*). Thus, the linkage of the hydroxymethylated derivative in virus DNA is more stable in the face of the enzymes active in the infected cell.

The increased stability of HMC in the free state or in all of its combinations in virus DNA permits this compound to accumulate in competition with cytosine or cytosine deoxyriboside in the infected cell. It does not appear to be necessary to invoke an inhibition of cytosine synthesis to account for the disappearance of cytosine derivatives, and the accumulation of derivatives of HMC. Thus, the pathology of T_2-infected cells appears to be explained in large measure by the competitive advantage afforded by hydroxymethylation of a single pyrimidine deoxyriboside.

III. THE ORIGIN OF NEW METABOLIC ACTIVITIES

The tests providing these data also served to indicate that hydroxymethylated pyrimidines or their derivatives are probably not normal intermediates in pyrimidine metabolism, and more particularly in thymine synthesis. Despite the close similarity of hydroxymethyluracil to thymine (Fig. 1), a close examination of the former structure will reveal the difficulty of removing oxygen from a unit lacking available hydrogen atoms. Although it seems reasonable to suppose that hydroxymethyl groups are indeed converted to methyl groups, it appears unlikely that this is effected on the pyrimidine ring.

If, as has been shown, the competitive relations of the pyrimidines are such as to result in the accumulation of HMC at the expense of cytosine, why does not this situation exist in the normal cell? We have tentatively concluded that this type of hydroxymethylation does not exist in normal cells for one of two reasons:

1. The host does not contain the components essential to this reaction, and these are supplied by the virus, or
2. The hydroxymethylation mechanism is inhibited in the cell, and this inhibition is released by virus infection.

Thus, virus infection appears to have induced the appearance of a new metabolic activity in the cell. It is tempting to think that a mutation in the cell resulting in the appearance of this activity might have led inevitably to the genesis of a T even phage.

IV. INDUCTION OF THYMINE SYNTHESIS IN A THYMINE-REQUIRING MUTANT OF E. COLI

A thymine-requiring mutant of *E. coli,* strain 15_T-, was obtained for the purpose of studying the possible intermediary role of hydroxymethyluracil in thymine synthesis. The organism did not respond to the hydroxymethyl derivative. As is customary in our laboratory, the effects of T_2 infection on the bacterium were then examined. It was found that the infected cell produced virus and synthesized DNA, whether thymine was present in the medium or not (Cohen, 1953; Barner and Cohen, 1954).

In Fig. 2 are presented data on the net synthesis of various bases in T_2-infected 15_T- cells. The cell nucleic acids accumulate only those bases

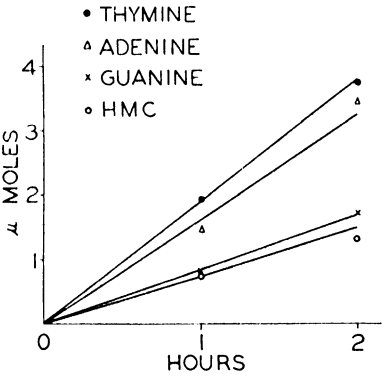

Fig. 2. The synthesis of the bases of nucleic acid in strain 15_T- infected by T_2.

which appear in T_2 virus, and indeed the amounts of the bases synthesized are approximately in the ratio found in the virus. Of special interest is the fact that the only pyrimidines synthesized and accumulated by the infected cell are thymine and HMC, bases which do not appear to be made in the uninfected cell.

The similarity of the problems involved in the induction of HMC synthesis and in the induction of thymine synthesis has led us to an intensive investigation of the properties of the thymine-requiring mutant. As a prelude to an analysis of the mechanism of induction, we began with a study of the uninfected organism.

V. LIFE AND DEATH OF THE THYMINELESS BACTERIUM

It was discovered that unlike other strains of *E. coli,* the relation of turbidity to the numbers of viable cells was not simple. Exhaustion of

thymine from the medium did not produce an abrupt cessation of turbidity increase as is frequently observed with the exhaustion of glucose or most other essential metabolites. In addition, the thymine (or thymidine) concentration determined the rate of turbidity increase. After some trial and error, a minimal thymine concentration (2 μg per ml.) was arrived at, which permitted a rate of turbidity increase and yield of viable cells and of bacterial mass, consonant with results obtained in the prototrophic parent, strain 15, on an equivalent amount of glucose (1 mg. per ml.) in the standard mineral medium. This thymine concentration is about three times that permitting a comparable total turbidity, but at the lower thymine concentration a marked death rate becomes evident.

Incubation of 15_T- in the absence of thymine results in a significant increase of turbidity. Examination of stained cells revealed a very considerable enlargement of the cell in girth and length. Estimation of the nucleic acid content of the cells suspended in thymine-free media revealed at least a doubling of their RNA content and a much smaller and variable, but seemingly significant, increment in DNA. Determination by plating of the numbers of cells capable of forming colonies showed that in this test the cells were dying at the rate of about 90% per division time (about 50 minutes).

The loss of colony-formers is dependent upon the presence of a carbon and nitrogen source, that is, the cells must be able to metabolize and grow in order to die. Curiously, cells grown in thymine excrete a substance other than thymine which appears to prevent this type of death (Barner, and Cohen, 1954). Thus, since the general picture is that of extensive cytoplasmic synthesis in the absence of nuclear synthesis, the phenomenon of thymineless death is construed to mean that at some level of cytoplasmic growth the nucleus is confronted with an unfavorable environment, even when thymine is provided, accounting for the irreversible loss of the power to multiply. We may term this "death by unbalanced growth."

We have found that the development of a thymine deficiency in other organisms also produces death. This probably accounts for the fact that until the present time this is the only known mutant with a single genetic block in thymine synthesis, other thymineless cells having been selected against during the isolation of mutants. We have induced thymine deficiency in *E. coli,* strain B, by growing the cells in the presence of sulfanilamide. Under these circumstances a growth rate approaching the normal rate requires many compounds containing one-carbon fragments. The biosynthesis of these substances normally requires folic acid. The

necessary compounds include methionine, serine, purines, pantothenate, etc., and thymine. If these compounds, other than thymine, are omitted from the medium, the cells do not die; in the presence of these compounds but in the absence of thymine, death is precipitous. Thus, sulfanilamide, generally considered to be bacteriostatic, is bactericidal under the conditions stated, which are essentially those producing unbalanced growth.

It will be recalled that penicillin is bactericidal under conditions of growth, the "killed" cells being abnormally long, and showing other signs of unbalanced growth. We have found that the presence of thymine or thymidine in the medium did not alter the rate of penicillin-induced death.

Most known mutants do not die in the absence of their requirement. A uracil-requiring strain of *E. coli,* W_c-, does not die in the absence of uracil. Since uracil appears to be a precursor for both RNA and DNA pyrimidines, a uracil deficiency would not be expected to permit unbalanced growth.

VI. THE EXTENT OF THYMINE SYNTHESIS DURING CELL MULTIPLICATION

Experiments were performed to determine the extent of thymine synthesis. The thymineless mutant was grown to a 150 fold increase in number in the mineral medium containing uniformly labelled glucose as sole carbon source at 1 mg. per ml. and unlabelled exogenous thymine at 2 μg. per ml. The nucleic acids were extracted with hot trichloroacetic acid and after removal of this acid the bases were liberated by hydrolysis in formic acid (Wyatt and Cohen, 1953). The hydrolysate was chromatographed initially in isopropanol-2N HCl; each band was extracted with H_2O and the contents were then rechromatographed in this same solvent. Each base was again eluted and chromatographed in butanol-NH_4OH. The bases were extracted in H_2O, the guanine being eluted in dilute HCl, and each solution was evaporated to dryness in vacuo. The bases were dissolved in water, and their purity and amount was estimated spectrophotometrically. The spectra of these were satisfactory with the exception of that of the thymine, which showed significant end absorption below 250 mμ. The bases were plated at infinite thinness, and their radioactivity was estimated by the usual technique with a windowless flow counter. Data are given in Table I.

The thymine preparation isolated possessed significant radioactivity and was further purified by paper electrophoresis at pH 11 in 0.1N

NaOH. The thymine band was cut into a front and back region, and each portion of the band was eluted, examined spectrophotometrically, and replated. The two portions were spectrophotometrically pure thymine, and appeared essentially identical with respect to radioactivity. The fractions were then pooled and were adsorbed on and eluted from

TABLE I. Radioactivities of the bases of strain 15_T- grown in uniformly labelled glucose-C^{14} and unlabelled exogenous thymine.

Substance	μMoles Carbon Plated	Counts/Min	Counts/Min/ μMole C
Glucose	1.5-6.2	—	259
Uracil	1.70	440	258
Cytosine	1.81	423	235
Guanine	1.90	477	251
Adenine	1.55	340	220
Thymine			
1. Chromatography	4.90	67.2	13.8
2. Electrophoresis	9.4	62.7	6.7
	6.5	50.2	7.7
3. Ion Exchange	10.6	45.9	4.4

a Dowex-1-acetate column at pH 8.9 (Friedkin and Roberts, 1954). The earliest and latest fractions of the symmetrically displaced thymine were discarded and the major fractions were pooled, plated, and counted. The activity of this material seems significant, and reveals an activity in thymine carbon of about 2% that of the glucose. Thus, it appears that the block in thymine synthesis under conditions of growth in the presence of thymine is 98% complete, and does not indicate an absolute lack of thymine synthesizing enzymes.

VII. THE EXTENT OF THYMINE SYNTHESIS DURING DEATH

Strain 15_T- was grown in the presence of unlabelled glucose and thymine, washed, and resuspended in uniformly labelled glucose (1 mg. per ml.) without exogenous thymine. In 2 hours the cells had consumed all of the glucose; in this interval viable count had fallen from 6.2×10^8 to 9.4×10^6 per ml. In 2% perchloric acid, the medium minus cells had an optical density of 0.65 at 257.5 mμ, the absorption maximum of the absorbing mixture excreted into the medium. The medium was slowly drained through a charcoal-celite column (Magasanik and Brooke, 1954) which adsorbed the ultraviolet absorbing components. The column was washed with water, and slowly eluted with ammoniacal ethanol-water mixture. The eluates which accounted for most of the

ultraviolet absorbing material present in the medium were separated in three major fractions which were taken to dryness. The fractions were chromatographed on paper in *n*-butanol-H_2O, and the separate bands were eluted, taken to dryness as crystalline white solids, and were characterized spectrophotometrically in acid, neutral, and alkaline solutions.

Six compounds were thereby isolated, of which three have been positively identified. In addition to the spectra, the compounds were characterized by R_f values, C analyses, and extinction coefficients. These compounds were uracil, orotic acid, and hypoxanthine; the uracil accounted for more than half of the ultraviolet absorbing material present in the medium, i.e. about 4 μg. per ml. Orotic acid was present in amounts sufficient for several recrystallizations of the isolated material. No evidence for the presence of a hydroxymethylated pyrimidine was found.

The remaining three unknown compounds present in small amount have not previously been reported in the literature. They do not contain diazotizable amine, and one at least contains pentose. They will be described elsewhere in greater detail than is possible here.

The advantages accruing from the use of uniformly labelled glucose in this type of experiment are numerous. Spectra and R_f values strongly suggested the nature of three of the compounds. Extinction coefficients permitted an estimation of the amount of carbon plated in each case, assuming the compounds to have been synthesized and excreted during thymineless death, rather than having been derived from autolysis of the unlabelled cell. The actual carbon contents of the preparations estimated from the radioactivities of the preparations are given in Table II.

TABLE II. Identification of compounds synthesized during thymineless death of strain 15T-.

Fraction	UV max*	Counts/ μMoles C Min.	μMoles C	μMoles C Plated Assuming ϵ_{max}
	$m\mu$			
Glucose	—	259	1	
1	258-259	134	0.52	0.57; Uracil ($\epsilon_{258} = 8.13 \times 10^3$)
2	282	226	0.87	0.89; Orotic Acid ($\epsilon_{282} = 7.35 \times 10^3$)
3	248-249	234	0.90	0.89; Hypoxanthine ($\epsilon_{250} = 10.5 \times 10^3$)

* Spectra taken in 0.1N HCl.

Thus, it can be seen that the carbon contents of the isolated material agree very well with that calculated from the extinction coefficients of the supposed compounds. Furthermore, it would appear to be proved in this manner that since the radioactivity of the carbon of the com-

pounds is identical with that of the glucose used in the medium, the compounds have been synthesized and excreted during unbalanced growth leading to death.

The bases of the nucleic acids of the "dead" cells were isolated as described above, and the thymine was subjected to the purification procedure involving two separations on paper chromatograms in different solvents, paper electrophoresis, and the ion exchange separation. It was counted only after each of the last two steps. The activities of the bases are recorded in Table III.

TABLE III. Radioactivities of the bases of strain 15_T- incubated in uniformly labelled glucose-C^{14} in the absence of exogenous thymine.

Substance	μMoles Carbon Plated	Counts/ Min.	Counts/ Min/ μMole C	% Activity of Glucose
Glucose	—	—	259	100
Uracil	2.90	368	127	49
Cytosine	2.92	335	114	44
Adenine	4.20	538	128	49
Guanine	3.98	386	97	37
Thymine				
1. Electrophoresis	5.50	78.4	12.6	4.9
2. Ion Exchange	4.45	46.7	10.6	4.1

From the uracil data it can be calculated that there is a synthesis of uracil and RNA to the extent of 96% of that originally present, whereas in the same interval there is a DNA synthesis of about 4% of the DNA originally present.

The less than absolute quality of the block in thymine synthesis is thereby confirmed. Nevertheless, despite the small DNA synthesis observed this is inadequate to prevent death by unbalanced growth. However, since some synthetic activity has been detected by this procedure, these results lend support to the hypothesis that T_2 infection removes an inhibitor from the system.

VIII. INDUCED BIOSYNTHESIS OF XYLOSE ISOMERASE IN STRAIN 15_T-

As we have seen, cells were available that lacked nuclear synthesis, as manifested by almost negligible synthesis of DNA, but continued an active cytoplasmic synthesis, as indicated by the extensive RNA formation. It was of interest to determine whether such cells were capable

of the induced biosynthesis of an enzyme in response to a new substrate (Cohen and Barner, 1955). No evidence has been previously reported concerning the site of enzyme synthesis.

We elected to study the response to D-xylose and have shown that xylose isomerase is an adaptive enzyme in 15_T-, catalysing the equilibrium: D-xylose $\rightleftharpoons$ D-xylulose. The respiration of 15_T- in media containing glucose or xylose in the presence or absence of thymine is presented in Fig. 3. The usual growth curve can be seen in the presence of glucose plus thymine. In the absence of thymine, there is a marked increase in the rate of O_2 consumption over the initial rate suggestive of an increase in the amount of respiratory enzymes. The assimilation of the glucose is only slightly impaired, as indicated by a 20% increase in O_2 consumed per mole of substrate and an essentially unchanged RQ. In the presence of xylose in the absence of thymine, the increase in rate of O_2 consumption is slower than that prevailing in growth. However, xylose does begin to be used at similar times.

In Fig. 4, it can be seen that the utilization of substrate is accompanied by death. In the presence of xylose, no death occurs until the substrate is assimilated. The time necessary for adaptation to use the substrate has thus displaced the curves for substrate uptake and death on xylose.

Analysis of the rate of enzyme synthesis, as presented in Fig. 5, indicates that enzyme begins to be elaborated at about the same time whether thymine is present or not. This occurs before an increase in turbidity becomes perceptible. After turbidity begins to increase, the extent of enzyme synthesis is roughly proportional to the turbidity increment. Thus, the induction and rate and quantity of enzyme synthesis do not appear to be significantly affected by the absence of thymine. These data may be interpreted to indicate that the induced biosynthesis of enzymes is not a function of nuclear synthesis, and to suggest that the cytoplasm is the site of this function.

It is not possible to use a uracilless mutant to show that the absence of RNA synthesis specifically prevents synthesis of enzymes. Uracil-containing nucleotides have been implicated in many areas of metabolism other than that of nucleic acid metabolism. For example, uracil deficiency results in an inhibition of respiration on glucose (Cohen and Barner, 1955).

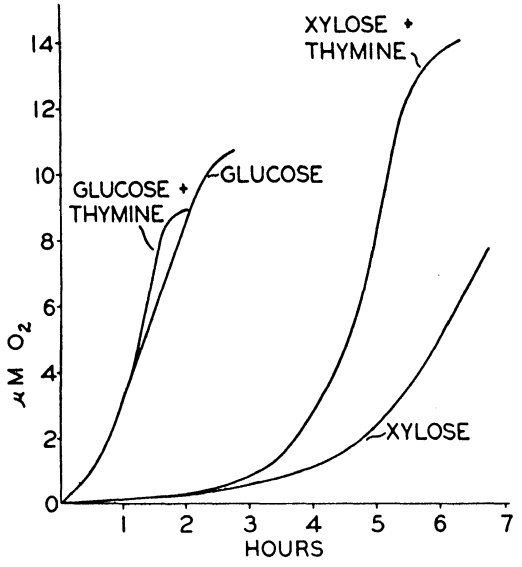

Fig. 3. The respiration of 15T- grown in glucose and thymine and suspended in media containing glucose or xylose in the presence or absence of exogenous thymine.

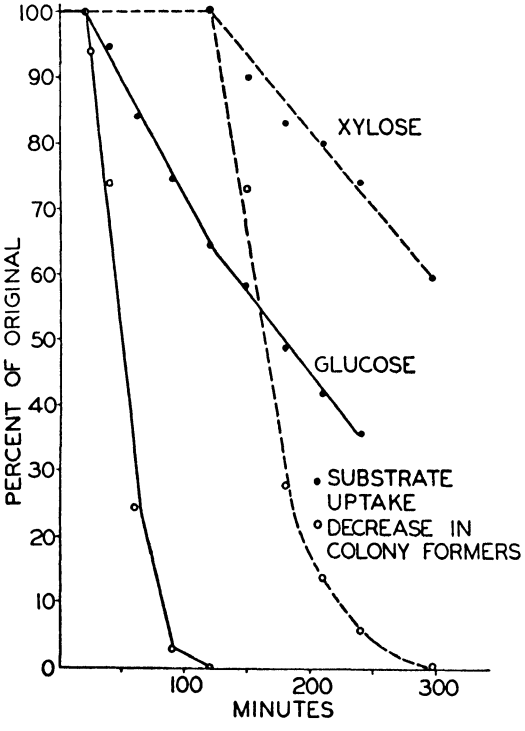

Fig. 4. The utilization of carbon sources and consequent death of strain 15T- in the absence of exogenous thymine.

IX. THE REPLACEMENT OF THYMINE BY BROMOURACIL

Bromouracil has been reported to be a competitive antagonist for thymine in lactobacilli (Hitchings, Elion, and Falco, 1950); in other microorganisms, including *E. coli*, it was found to be incorporated in the nucleic acid fraction (Weygand and Wacker, 1952). We have found that 5-bromouracil partially replaces the thymine requirement in 15_T-,

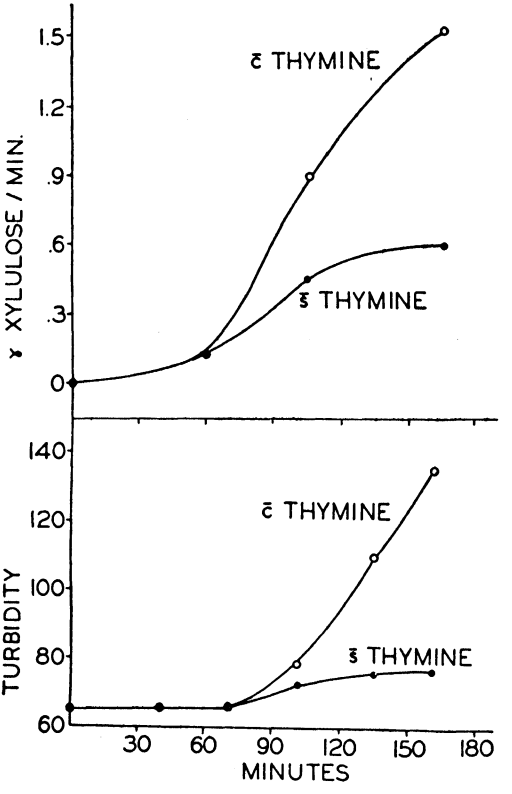

Fig. 5. The formation of xylose isomerase by strain 15_T- incubated at 37° in xylose in the presence or absence of thymine.

permitting a fivefold increment in turbidity and markedly stimulating both RNA and DNA synthesis. However, despite the increment in these functions evident in Fig. 6A and 6B, after an initial slight increase in the number of viable cells the number of cells capable of forming colonies eventually falls off markedly. Thus, most of the DNA synthesized is quite unsatisfactory to the bacterium, being inadequate to prevent death by unbalanced growth.

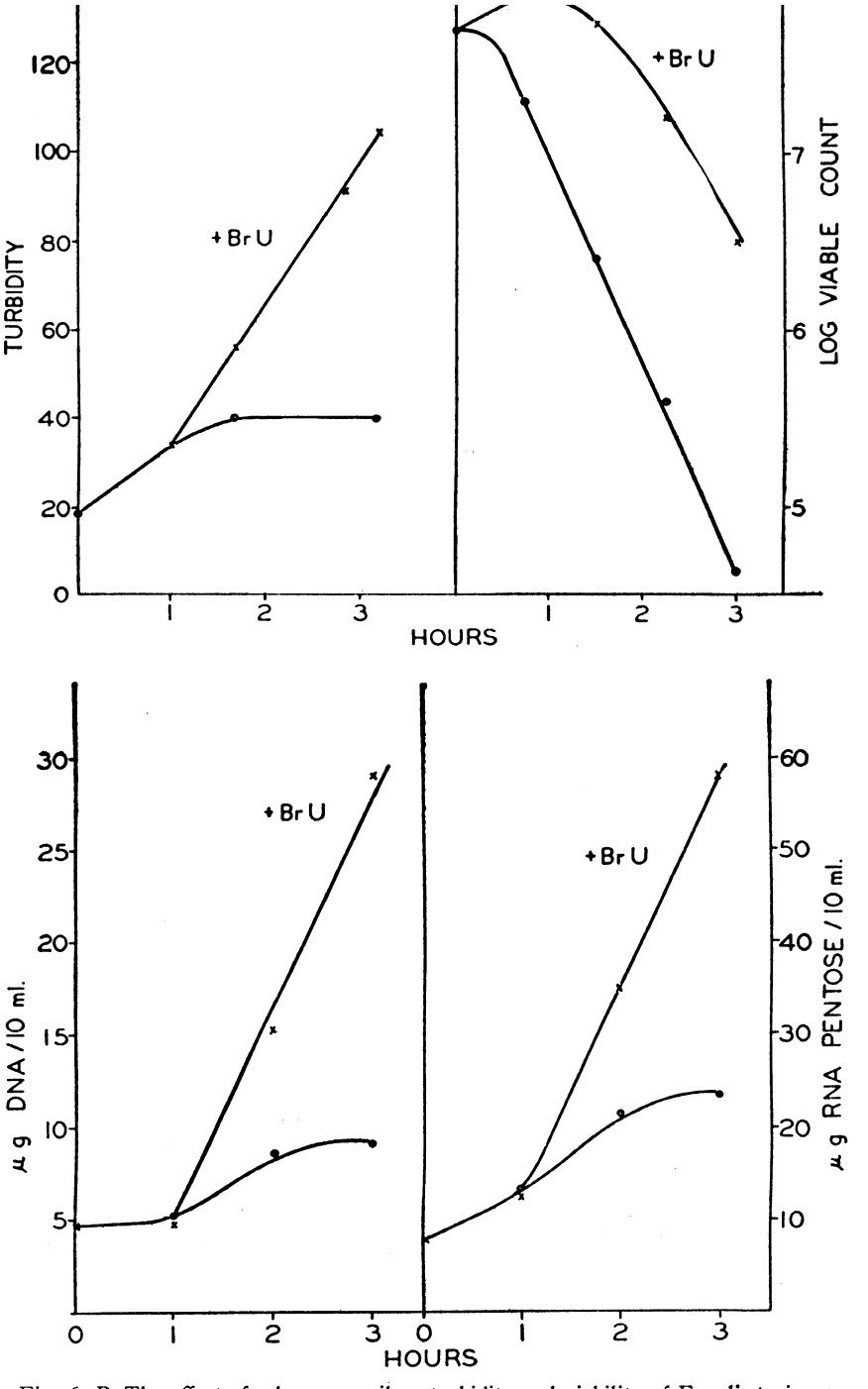

Fig. 6. B. The effect of 5-bromouracil on turbidity and viability of E. coli strain 15T-, incubated in the absence of thymine. B. The synthesis of nucleic acid by 15T- stimulated by 5-bromouracil in the absence of exogenous thymine.

X. RADIATION DAMAGE AND UNBALANCED GROWTH

Reports in recent years on various types of reactivation and hypotheses on the mechanism and site of radiation injury suggested the desirability of examining properties of ultraviolet irradiated 15_T^- in the absence of DNA synthesis. Bacteria were grown on a complete medium, washed, and resuspended in the mineral medium. The bacteria were irradiated at 10^7 per ml. with an ultraviolet lamp to give approximately 90% inactivation (about 2-3 hits), as estimated by plating on broth agar. Control cells or irradiated cells were diluted 1:5 in the appropriate medium and aerated at 38°. Diluted aliquots of these cultures were plated on broth agar at various intervals.

Let us examine the separate curves in Fig. 7. The growth and division of unirradiated cells of strain 15_T^- in the presence of thymine are presented in the uppermost curve. In the absence of thymine it is seen that the bacteria maintain their ability to multiply for 20 minutes, and then begin to die as a function of abnormal growth. When cells are irradiated and incubated in the absence of thymine, there is a rapid reactivation (restoration) to a three- to fivefold increase in viable cells in 20 minutes, followed by a loss of colony-formers paralleling the curves of thymineless death. This relation has been obtained for all levels of irradiation injury from a survival of .1 to .001. If one ignored the curve of irradiated 15_T^- incubated with thymine, it might seem as if the death curve were nothing more than thymineless death.

However, new phenomena appear in the curve of irradiated 15_T^- incubated with thymine. It can be seen that, despite the presence of thymine, there is a death of the restored cells, the curve of viable cells again increasing after 60 minutes. Extrapolation of this last increasing portion of the curve to zero time places the curve back to the number of originally inactivated cells. Thus, this exponential increment in the latter part of the curve merely represents the growth curve of the undamaged survivors of the culture. Since the death curve in this instance returns to the growth curve of the survivors, it can be seen that the restoration is entirely transitory; all irradiated cells, including the restored cells, eventually die on incubation. Furthermore, they die at a rate which is the same as that at which thymineless death occurs. It, therefore, seems reasonable to suppose that death occurs for a similar reason, that is death by abnormal growth, perhaps similarly resulting from a lesion in DNA synthesis. In this instance the lesion would be at a site other than that of thymine supply. In support of this picture, it can be seen that the restored

cells are all dead after about 60 minutes, thereby approximating the division time of the organism. Thus, cells die twice, the last death apparently being a function of division.

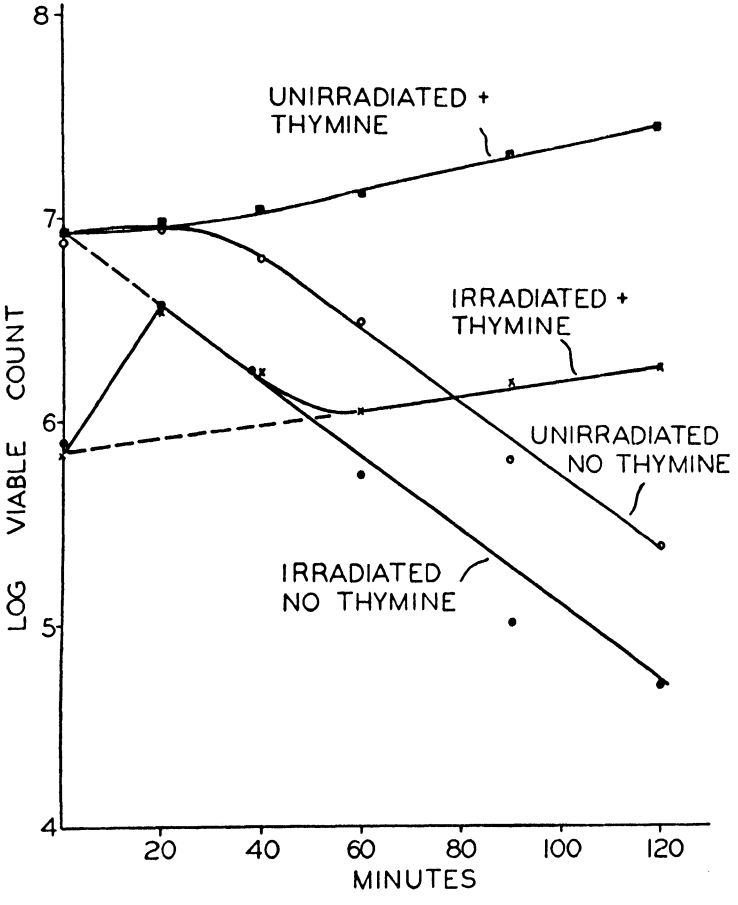

Fig. 7. The restoration and death of ultraviolet-irradiated strain 15_T- as a function of incubation in the presence or absence of thymine.

A comparable experiment with bromouracil instead of thymine reveals essentially the same result. These data can be seen in Fig. 8. In this experiment again it can be seen that bromouracil almost, but not quite, replaces thymine for 15_T-.

If restoration and death of irradiated cells are independent of the presence of thymine, it could be expected that the prototrophic parent, *E. coli,* strain 15, would behave similarly under normal conditions of growth. It does, as can be seen in Fig. 9. In view of these data, our

experiments have continued with this organism rather than with 15_T-. It must be stressed, however, that the nature of the second death would have been far more obscure if we had not the opportunity to compare it with thymineless death.

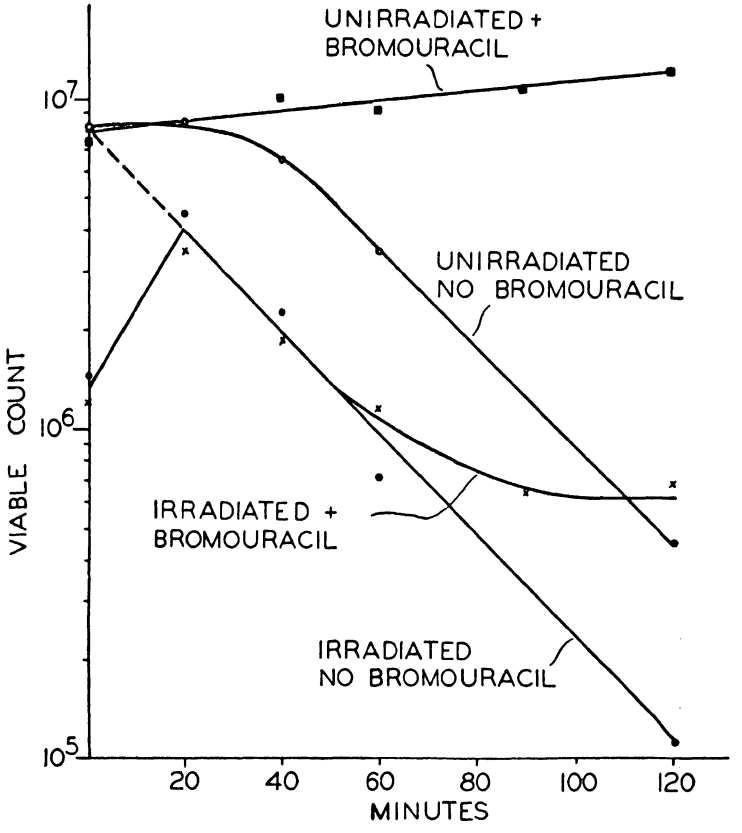

Fig. 8. The effect of 5-bromouracil on the death of ultraviolet-irradiated 15_T-.

If death is a function of growth, why do not the restored cells die on the broth agar plate? If restoration is attempted in broth, it does occur and is followed by death. If restoration is effected in the synthetic medium and broth is added at 20 minutes, there is no inhibition of death such as must occur in the plating process. The nature of the factors in the plating process which permit the fixation of restoration has not yet been clarified.

In further experiments on the relation of growth of death, bacteria were grown in synthetic media limiting with respect to glucose or

ammonium salts until these substances were exhausted and turbidimetric increase ceased. The cells were irradiated and incubated in the deficient media. Over a two-hour interval unirradiated cells showed insignificant growth. The irradiated cells were reactivated, but did not die. The data

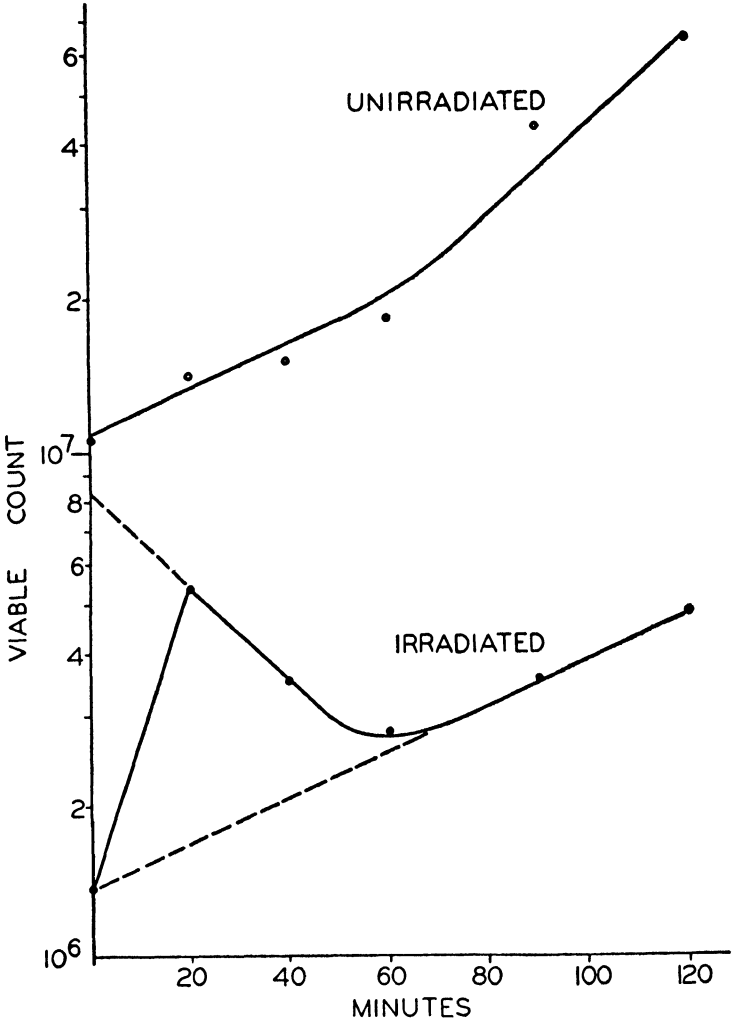

Fig. 9. The restoration and death of ultraviolet-irradiated strain 15.

from such an experiment on restoration in the absence of exogenous glucose are presented in Fig. 10. It is seen that restoration is somewhat slower and less complete than in the presence of glucose. The rate of reactivation of cells in nitrogen deficient media is not inhibited, and by

consequence restoration is more complete since the subsequent death has been prevented. Since thymineless death also requires a carbon and nitrogen source, the similarity of radiation death to thymineless death is again emphasized. The nature of restoration which does not require exogenous glucose or nitrogen is quite obscure; perhaps it may be fruitfully explored by means of inhibitors.

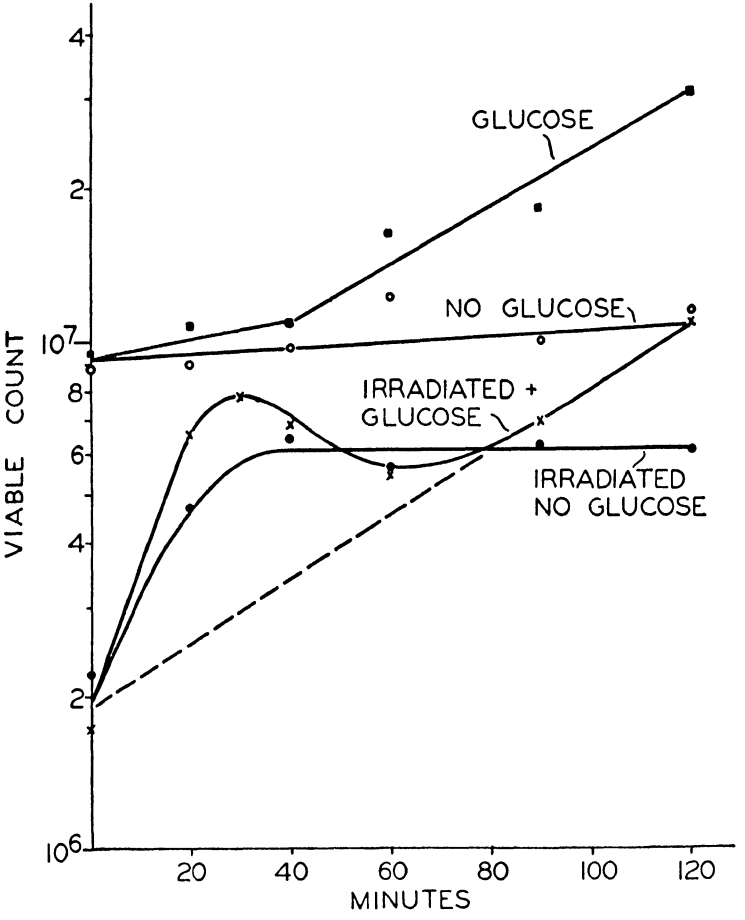

Fig. 10. The restoration and death of ultraviolet-irradiated strain 15 as a function of the presence or absence of glucose.

In the presence of adequate glucose and nitrogen, the amino acid antagonist, 5-methyltryptophan, will prevent irreversible death at concentrations at which it will completely inhibit growth (2.5×10^{-4} M). Restoration is apparently unimpaired under these conditions.

In conclusion, it should be remarked that the phenomena accompany-

ing unbalanced growth might have been predicted (although most were not). In any case, only one of the possible types of unbalanced growth would seem to be described by thymine deficiency and by radiation damage. It is now tempting to speculate concerning a situation in which cytoplasmic synthesis could be inhibited without inhibition of nuclear synthesis. Such a condition might occur if there was a block in the synthesis of cytosine riboside, but not of the deoxyriboside. In such circumstances, the nuclei would multiply within the confines of a fixed cytoplasm, and one would expect this situation to result in death by unbalanced growth. Is it possible that a mechanism such as this would account for a first step in the origin of a virulent phage such as T_5 which contains cytosine deoxyriboside, prevents the accumulation of RNA, and permits the synthesis of virus DNA?

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VII. PHOTORESPONSIVE GROWTH

BY S. B. HENDRICKS AND H. A. BORTHWICK[1]

GROWTH of most, if not all, seed plants is subject in some aspects to control by a single reaction that can be influenced by radiation. In animals certain functions of the pituitary or its functional antecedent are responsive to a photostimulus. Photoperiodism, noted in relation to reproduction of both plants and animals, represents one mode of this control. The light action is, in plants at least, far more general as a control of growth. The subject is developed here chiefly for plants. This is not a review of literature (cf. Borthwick, Hendricks, and Parker, in press; Lange, 1952; Naylor, 1953) but rather a report on the development of an explanation.

That many different growth responses in plants are subject to the same causal photocontrol can be rigorously demonstrated from the response or "action" spectra without an understanding of the reactions involved or a careful analysis of factors that might influence an action spectrum. After this exploration, quantification gives an understanding of the reversible photoreaction involved and of a coupled subsequent reaction leading to biological action. These are found to constitute the time-measuring system in plant photoperiodism; the phenomena appear ubiquitous in higher plants.

I. EQUIVALENCE OF LIGHT ACTION FOR CONTROL OF VARIOUS RESPONSES

A start can be made by considering seeds of *Lactuca sativa* in some varieties of which (e.g., Grand Rapids) germination is promoted by visible radiation (Flint, 1934). In principle, then, it is simple to measure the response spectrum for this promotion of germination in terms of incident energy (Borthwick, Parker, and Hendricks, 1948). This is done with a spectrograph of sufficient dispersion, aperture, spectral purity, and source brightness to permit short irradiations, building up of adequate populations and resolutions of fine structure if present (Parker et al., 1946). The two-prism instrument used gives a spectrum about 10 cm. broad with a dispersion of 16 Å/cm. at 5000 Å and 75 Å/cm. at 7500 Å. The spectrum is spread out over about 1.7 meters for the visible with not more than 0.05 percent radiation impurity. A carbon

[1] U. S. Department of Agriculture, Agricultural Research Service, Beltsville, Maryland.

arc operating at 150 Amp. is generally used as a source with an effective slit width of 50 Å at 5000 Å. In other words, the spectrograph will serve for irradiation in narrow spectral regions at high irradiances of objects as large as leaves of bean plants or several snails in a Stender dish.

Lettuce seeds that have imbibed water in darkness are placed along the spectrum and irradiated for various periods that might range from a few seconds to an hour according to the experimental design. They

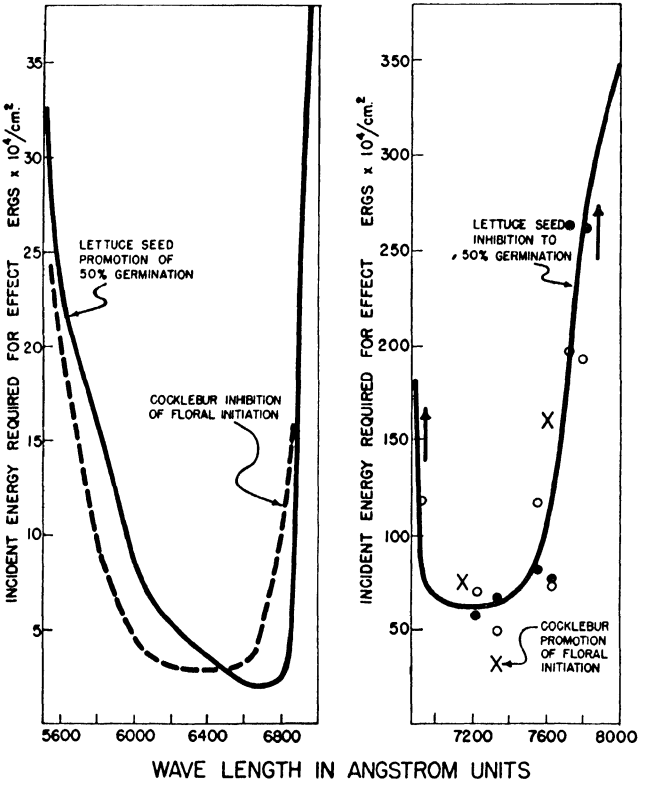

Fig. 1. Action spectra for promotion (left) and inhibition (right) of lettuce seed (*Lactuca sativa* var. Grand Rapids) germination and for inhibition (left) and promotion (right) of floral initiation of cocklebur (*Xanthium saccharatum*). From Borthwick, Hendricks, and Parker, 1952; Borthwick et al., 1954.

then are returned to darkness, at suitable temperature, where they remain for several days to germinate as they may. The response spectrum for any percent germination can be obtained from the result, after the germination response is known, as a function of energy of irradiation. That for promotion of germination to 50 percent is shown in Fig. 1. A maximum of effectiveness is found near 6500 Å.

Lettuce seeds of the variety used had been shown by Flint and McAlister (1935) to be inhibited in germination in the region of 7000 to 8000 Å. This observation was verified and the response spectrum for inhibition was measured on imbibed seeds that had been irradiated with another light source to promote germination fully just prior to irradiating on the spectrograph for inhibition. The response spectrum for inhibition of germination to 50 percent is also shown in Fig. 1 with a maximum of effectiveness near 7300 Å.

TABLE I. Photoreversal of promotion and inhibition of *Lactuca sativa* var. Grand Rapids seed germination in a series of irradiations alternating between red and far red at two temperatures. From Borthwick et al., 1954.

Number of irradiations		Final irradiation	Germination at 20°C with irradiation at indicated temperature	
Red	Far red		26°C	6-8°C
			percent	percent
1	0	Red	70	72
1	1	Far red	6	13
2	1	Red	74	74
2	2	Far red	6	8
3	2	Red	76	75
3	3	Far red	7	11
4	3	Red	81	77
4	4	Far red	7	12

The two photoresponses are the parts of a single reversible photoreaction. This can most readily be shown by results (Table I) from a succession of promoting and inhibiting irradiations at two temperatures. The germination response depends solely upon the last irradiation and is independent of temperature during the sequence of exposures. Without loss of generality this reversible photoreaction can be written as either (I) or (II).

$$\text{(I) pigment} \underset{7350}{\overset{6500}{\rightleftharpoons}} \text{isomeric pigment}$$

$$\text{(II) pigment} + \text{reactant} \underset{7350}{\overset{6500}{\rightleftharpoons}} \text{changed pigment} + \text{changed reactant}$$

The pigment necessarily exists in two forms, which are either isomeric or differ by addition or deletion of atoms or groups. The objective is to find out more about this reaction, particularly whether (I) or (II) is valid and how coupling to biological response is effected.

A simpler matter, however, is to use the fact of ready reversibility and the details of the action spectra for comparison of germination with several other responses of plants to radiation. Consider the photoperiodic control of floral initiation, the so-called length-of-day phenomenon. The influence of an adequate dark period or sequence of dark periods with alternating light periods as in a succession of days and nights can be nullified by irradiating for short times near the middle of the dark periods. The energies required for effectiveness of the interruption in their dependence on wave length give the action spectrum.

The action spectrum for floral initiation of the long-night plant *Xanthium saccharatum* (Borthwick, Hendricks, and Parker, 1952) is shown in Fig. 1. It, too, has a promotive region at wave lengths greater than 7000 Å and an inhibitive one in the region where germination of lettuce seed is promoted. Moreover, the action in the two regions can be repeatedly reversed. Most remarkable for this particular comparison of germination and floral initiation control is that the agreement is on an absolute scale for incident energy despite many modifying factors. The significance of this will be considered later.

Garner and Allard (1920) discovered not one, but several types of flowering response such as long, short, indeterminate, and intermediate "day-length" requirements. Do these arise from the same causal photoreaction? Definitely so, as shown in Fig. 2 for the action spectra on a relative scale for inhibition of floral initiation for the long-night plants, Biloxi soybean and cocklebur, and promotion in the case of the short-night plant, Wintex barley. Each of these is reversible, as also is floral initiation of the short-night plant *Hyoscyamus niger* (Parker, Hendricks, and Borthwick, 1950), with radiation in the region of 7000 to 8000 Å. So the opposite flowering responses arise from the same initial controlling photoreaction.

The stem- and leaf-lengthening responses of seedlings plants—the etiolation phenomena—also have the same action spectra as measured on barley (Borthwick, Hendricks, and Parker, 1951) and peas. In barley, moreover, the control on stem length is known for six albino types. The photoreceptive pigment system, then, is present also in plants which lack chlorophyll and the carotenoid pigments.

Even though one might not understand the reversible photoreaction, it can be used simply as a device for uncovering two further consequences of a relatively obscure phenomenon. The coloration of the tomato fruit cuticle by a flavone where genetically possible is under photocontrol. The action spectrum for coloration is shown in Fig. 3 in comparison

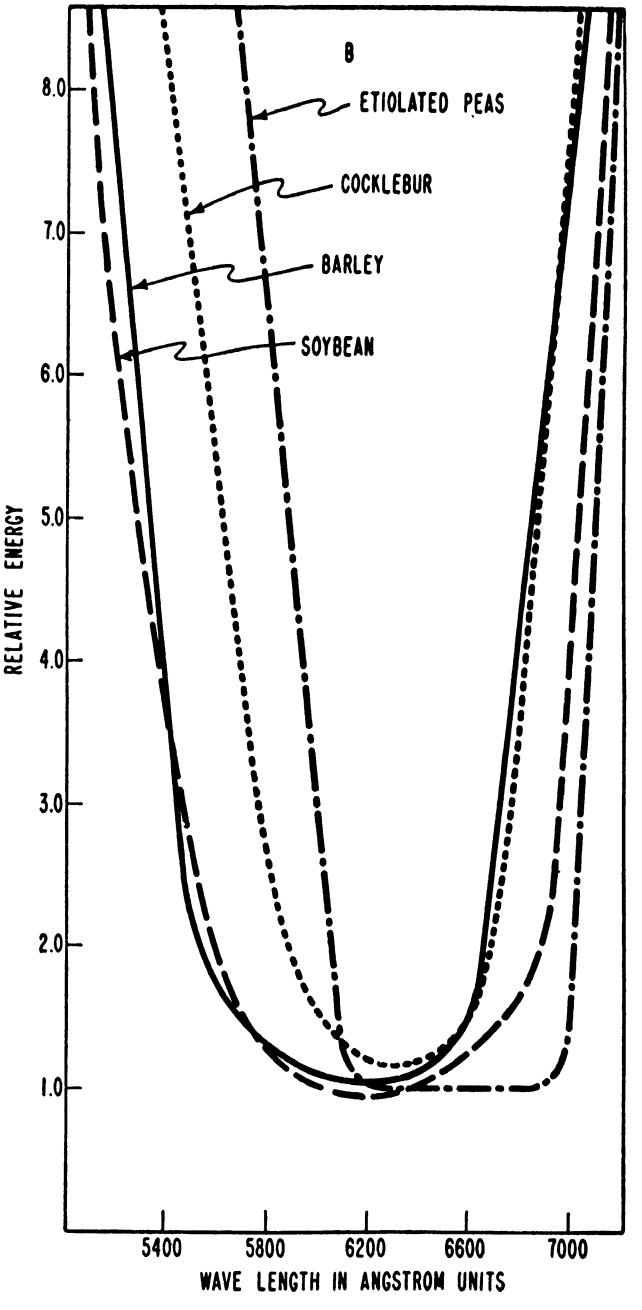

Fig. 2. Action spectra in the red portion of the spectrum for inhibition of floral initiation of soybean (*Glycine max* var. Biloxi) and cocklebur (*X. saccharatum*), for promotion of flowering of barley (*Hordeum vulgare* var. Wintex) and of leaf lengthening of etiolated pea seedlings (*Pisum sativum* var. Little Marvel). From Parker et al., 1946; Borthwick, Hendricks, and Parker, 1948; Parker et al., 1949.

with that for floral initiation of cocklebur. The cuticle coloration response, too, is photoreversible by radiation in the region of 7000 to 8000 Å. The control reaction is the same, then, as for floral initiation. The significance is that the tomato, which also shows the etiolation response, is an indeterminate plant for flowering. It has the controlling system, but flowering control is not displayed.

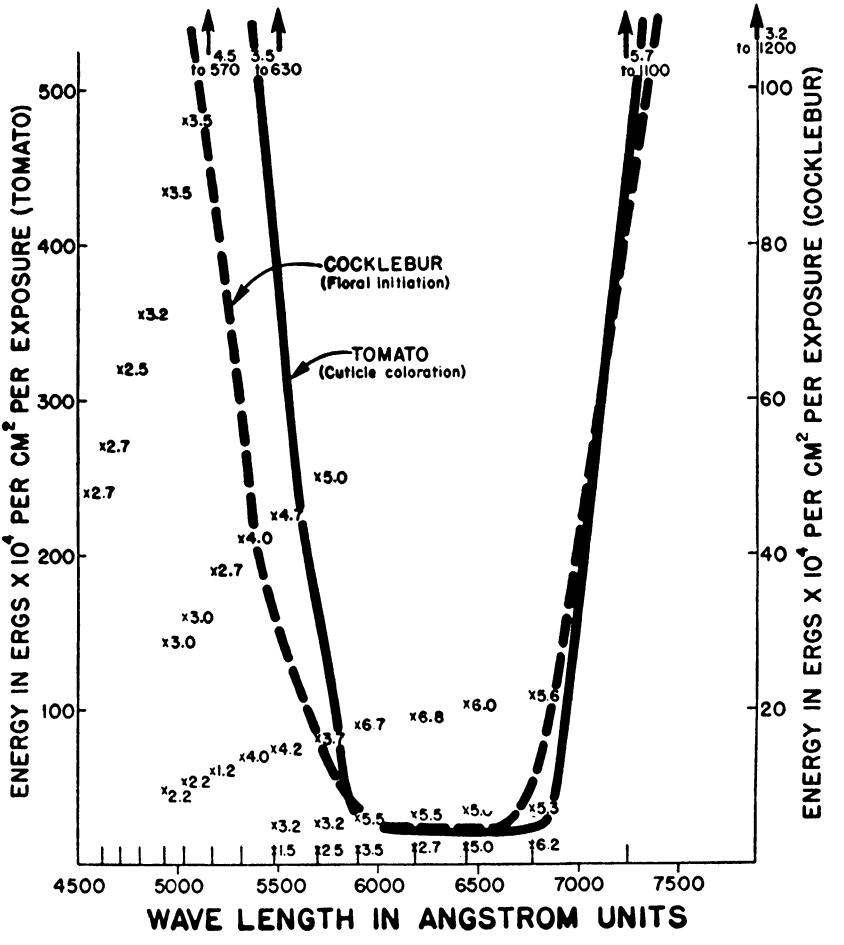

Fig. 3. Action spectra for cuticle coloration of tomato fruit (*Lycopersicon esculentum* var. Rutgers) compared with that for inhibition of flowering of cocklebur (*X. saccharatum*). From Piringer and Heinze, 1954; Parker et al., 1946.

The coloration of the tomato fruit does not extend more than about 2 mm. beyond the irradiated area according to Piringer and Heinze (1954). This response, then, has a high degree of localization, while

floral initiation at a terminal meristem is controlled, if the plant is photoperiodically responsive, by the photoreaction in a distant leaf.

The possibilities of obtaining quantitative information after this pattern for numerous other features of growth and for all species of seed plants are unlimited. Plants other than Spermatophytes are not considered in this paper because of lack of study rather than improbability of their responding. It is well to return to further consideration of the causal reactions with the realization that aspects of any of the varied phenomena might be useful bases for deductions.

II. REACTANTS IN THE REVERSIBLE PHOTOREACTION

How can a distinction be made between reaction (I) and (II) from physiological observations? Two indications were found that reaction (II) in which reactants other than the pigment are involved is correct. The sensitivity of seed germination to radiation can be influenced by several factors such as the temperature and time of imbibition and the presence of various compounds in the imbibed solutions. The effect of time of imbibition in tap water prior to irradiation on germination of *Lepidium virginicum* seeds is shown in Fig. 4. It can be seen that as sensitivity to promotion changes, that to inhibition changes in the opposite direction for periods shorter than 72 hours. This is also the case when the change is induced by the presence of nitrate or thiourea in the imbibed solution as shown by results in Table II.

TABLE II. Energies required (ergs $\times$ 10^5/cm^2) for 50 percent germination of *Lepidium virginicum* seeds by promotion at 6300-6600 Å or by inhibition at 7300-7500 Å in the presence of water or solutions. From Toole et al., (1955).

Liquid imbibed	Energy for inhibition		Energy for promotion	
	20°C	15°-25°C	20°C	15°-25°C
H_2O	0.3	0.4	14	5.8
0.2% KNO_3	0.50	0.55	5.8	1.7
0.5% $CS(NH_2)_2$	—	0.75	—	2.0

Since transition probabilities between the several states of the pigments are very unlikely to be influenced in this simple and continuous way, the implied change in quantum efficiency must arise from other molecules' being required in the reaction. It should be recalled, however, that the reversible photoreaction does not change in rate with temperature. Collision between the pigment and the reactant of varying con-

centration, then, cannot be limiting to the rate of the reaction. This situation can be realized if the pigment and reactant are closely associated as in a cage or "sticky collision" as is often the case for other photoreactions in solutions (Umberger and LaMer, 1945). The quantum efficiency

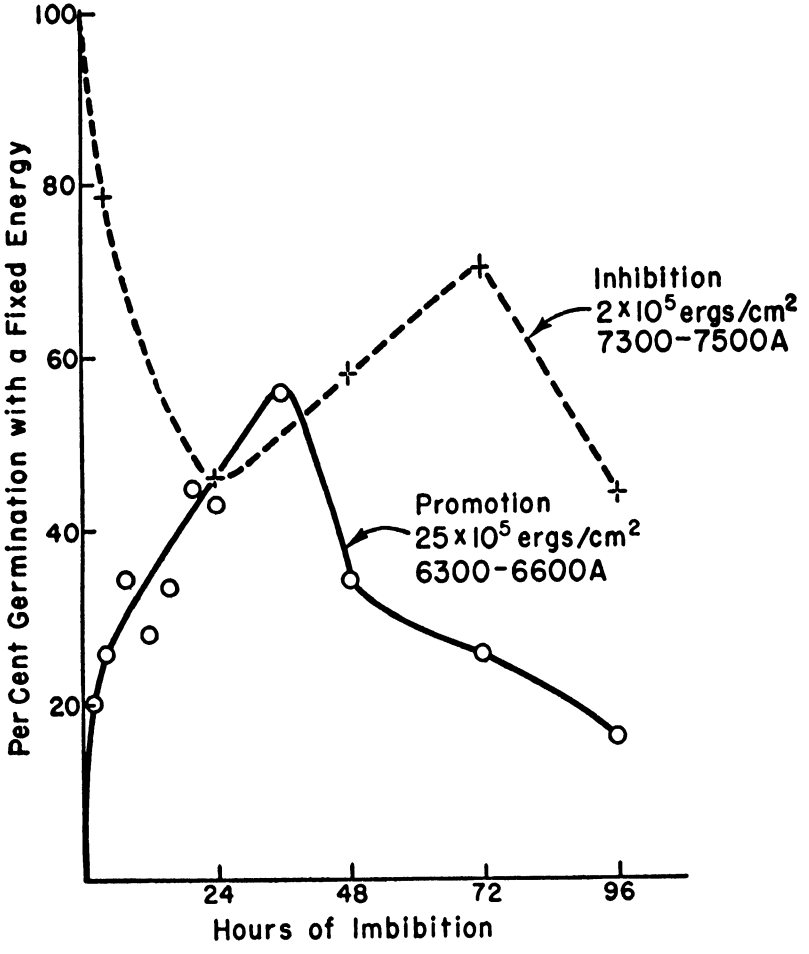

Fig. 4. Variation of sensitivity to promotion and inhibition of germination at 20°C of L. virginicum seeds imbibed in 0.2 percent KNO_3 solution with time of imbibition prior to irradiation. From Toole et al., in press.

for the photoreaction in the two directions, then, will depend upon the ratio of the reactant and changed reactant concentrations. The quantum efficiency might accordingly be expected to change over a great range of values.

The second indication that reaction (II) rather than (I) is involved

is that the variance of the population for promotion of *L. virginicum* seed germination is different from that for inhibition, while each variance is uninfluenced by changes in sensitivity caused by the presence of nitrate or thiourea or by duration of imbibition (Toole et al.). This fact is shown in Fig. 5, which is a graph of the probit-energy lines (Finney, 1952).

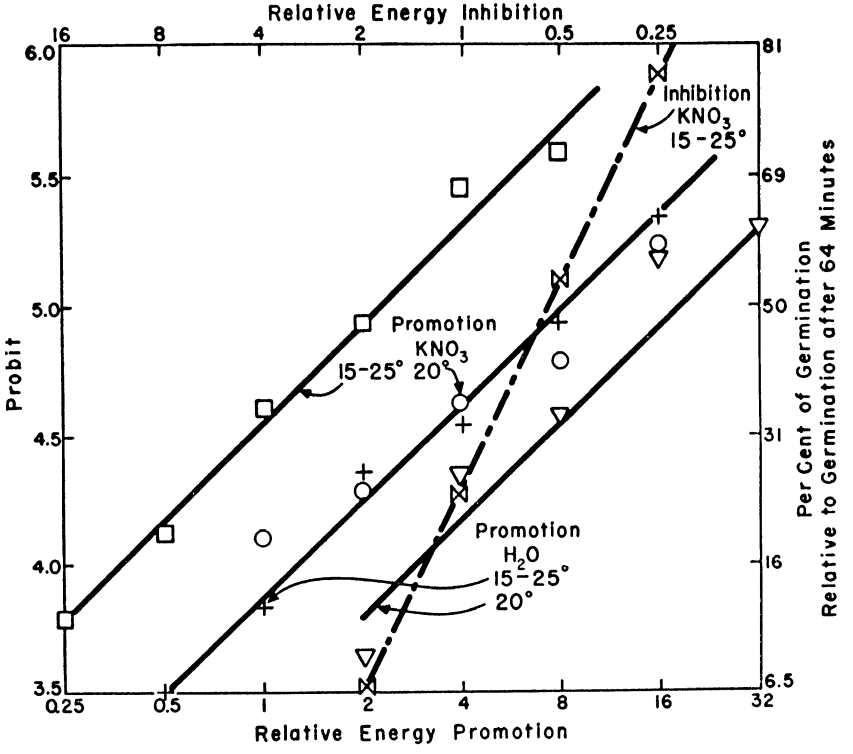

Fig. 5. Variation in germination of *Lepidium virginicum* seeds with radiant energy at a constant temperature of 20°C and with a shift at time of irradiation from 15 to 25°C in presence of H_2O or 0.2 percent KNO_3 solution. The longer broken line shows inhibition of germination at 7300-7500 Å. From Toole et al., in press.

The slope of the lines is the reciprocal of the variance which with the mean describes the normal population. Such a result would be expected if the individuals of the population vary in their ratios of reactant to changed reactant. The support for equation (II), in other words, comes from the features of both the mean values and the variances.

Variation of the photoresponse with the relative quantities of reactant can be illustrated as in Fig. 6. Each possibility has been realized; thus

germination of *L. densiflorum* and of *Lactua sativa* var. Grand Rapids seeds is described by the center graph. *L. virginicum,* on the other hand, requires about 47 fold more energy for a 50 percent response in the

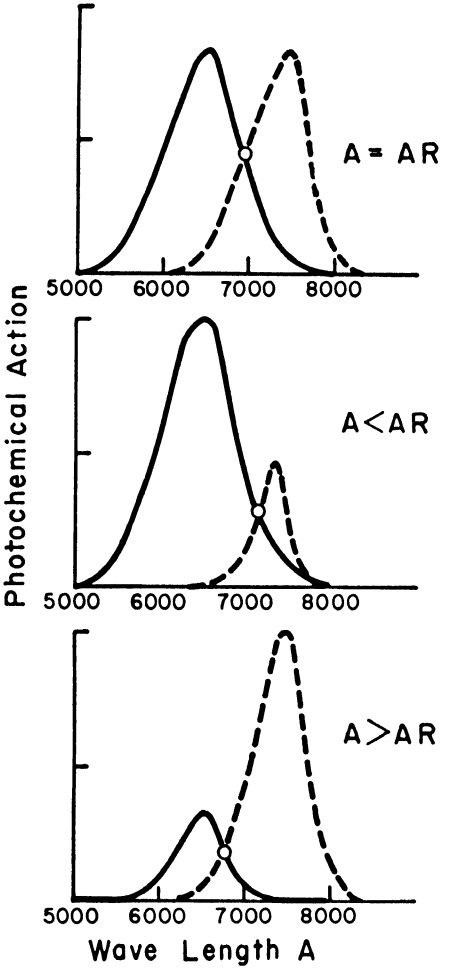

Fig. 6. Possible variation of photoresponse with concentration of the molecules A and AR that react with the pigment. From Toole et al., in press.

region near 6500 Å than is needed near 7300 Å, corresponding to the bottom graph. Moreover, the prediction that the isobastique point shifts toward longer wave lengths as the relative sensitivity to 7300 Å increases is closely followed (Toole et al., *op. cit.*).

III. REACTION SUBSEQUENT TO THE PHOTOREACTION

The information about the reversible photoreaction has been gained from a simple use of the growth response as an assay. This simple use can be extended to answer two other questions. Which side of the photoreaction is coupled with biological response? How is it coupled? The first question should be appreciated in a sense of symmetry, namely, that to a point responses can be interpreted as arising from activation by a positive factor or inhibition by a negative factor. Answers came from some further details about the several photoreversibilities.

The floral initiation of cocklebur was found by Downs (1954) to lose reversibility as a function of the time between the reversing irradiances (Table III). This loss of reversibility, moreover, had a marked tempera-

TABLE III. Effect of temperature during interval between irradiations on loss of reversibility for floral initiation of *Xanthium saccharatum*. From Downs, 1954.

Interval between red and far-red radiations	Flowering conditions * with various temperatures during the interval	
	20°C	4°C
min.		
0	7	–
20	4	6
30	2	4
40	1	4
60	0	3

* Flowering condition was estimated on a scale of eight stages of primordium development at time of dissection of four plants in each group, stage 8 being most advanced.

ture dependence, as shown in the table. The loss takes place when reaction (II) is driven to the right by the action of red radiation but not when it is forced to the left; that is, the loss in reversibility takes place when the pigment is in the form with an absorption maximum near 7300 Å. The generality of the observation is indicated by the similar behavior of floral initiation in Biloxi soybean and cuticle coloration of tomato fruit (Piringer and Heinze, *op. cit.*). The interpretation is that the right side of equation (II) is associated with biological response. This response, then, either involves depletion of the changed pigment or the changed reactant. The controlling reaction scheme in case the pigment is depleted might be

$$(II_a)\ \text{pigment} + \text{reactant} \underset{7350}{\overset{6500}{\rightleftharpoons}} \text{changed pigment} + \text{changed reactant}$$

$$(II_b)\ \text{changed pigment} + \text{molecule A} \rightarrow \text{changed pigment—molecule A}$$

In the second possibility where the changed reactant is depleted the changed pigment might be serving as an enzyme for the depletion.

There are many inviting approaches offered by the second possibility that might lead to recognition of the actual compounds involved in the reactions. These are being examined both in seed germination and control of floral initiation.

IV. TIME MEASUREMENT IN PHOTOPERIODISM

The question of the measurement of time in photoperiodic responses of plants can now be answered. The existence of this phenomenon, of course, implies that plants measure either the duration of darkness or of light or of both darkness and light. A dark reaction is surely the controlling one since numerous experiments on floral initiation have shown that the length of the dark period is determinative (Borthwick, Hendricks, and Parker, in press; Lang, 1952; Naylor, 1953).

A reaction such as II will change in darkness towards an equilibrium at a rate determined by a free energy of activation. This reaction was found for seeds of *Lactuca sativa* var. Grand Rapids to proceed toward the left, i.e., in the direction that it is driven photochemically by 7350 Å (Borthwick et al., 1954). If these seeds are irradiated near 6500 Å and then held at 20°C in darkness, germination follows. Temperatures above 30°C, however, block germination. Seeds held in darkness at 35°C following irradiation near 6500 Å will not germinate upon return to 20°C unless irradiated a second time in the promotive region. This indicates that the reaction went from right to left, that the pigment in darkness at 35°C changed to the form with an absorption maximum at 6500 Å.

The time of half return for the pigment in darkness from the right to the left of equation II for Grand Rapids lettuce seed, then, is of the order of 24 hours at 35°C. In photoperiodic responses the critical dark time, on the other hand, is near night-lengths of nature. There is, moreover, some crude temperature independence not admitted by the simple reversal of (II) with a free energy of activation of the order of 20,000 calories. Thus, while the reversal of reaction (II) proceeding from right to left is

surely involved in time measurement of photoperiodism, the way in which coupling to biological response is effected also must be involved.

V. OPPOSITE RESPONSES TO THE SAME PHOTOREACTION

Another question to be answered is how can the same causal reactions give opposite responses of producing flowering in some plants and preventing it in others? There are two parts to the answer, one describing the basic principle involved and the other giving reasons for its operation in control of flowering.

The basic principle, essentially as stated earlier (Borthwick, Parker, and Hendricks, 1948), is as follows: *The active substance must reach an adequate concentration to effect floral initiation, but it is self-inhibitory at higher concentrations.*

The operation of the same principle in action of auxin on cellular elongation was recognized by Foster, McRae, and Bonner (1952) and quantitatively developed on the basis of the Michaelis-Menten expression for first-order kinetics involving a rate-limiting activated complex. The expression developed by Foster et al. closely describes the effect of indole-3-acetic acid concentrations on growth of *Avena* coleoptile sections over a 100,000 fold change in concentration. The self-inhibition arises from attachment of the indole-3-acetic acid at two points for biological action as schematically illustrated in Fig. 7.

The second part to the answer involves the extent to which indole-3-acetic acid might be involved in the flowering response or the other phenomena controlled by the reversible photoreaction. Liverman and Bonner (1953) observed some interdependence of exogenous indole-3-acetic acid and the reversible photoreaction on elongation of *Avena* coleoptile sections. Bonner and Thurlow (1949) and von Denffer (1950) found that effectiveness of an adequately long night in promoting flowering of long-night plants such as cocklebur can be nullified by spraying with dilute indole-3-acetic acid solutions at the start of the dark period. There are other compounds, too, particularly coumarin, that seem to be associated in their action with growth control by the reversible photoreaction.

VI. UBIQUITY OF THE PHOTOREACTION

The phenomenon of growth control by the reversible photoreaction is possibly present in all seed plants. But many flowering plants, such as the tomato, are apparently not dependent upon a photoperiodic control of floral initiation, at least so far as they have been studied. The control is

often sharply expressed for one variety of a species but not for another, as in tobacco varieties. Despite the probable occurrence of the controlling reaction, seeds of most species might germinate in darkness (Crocker and Barton, 1953).

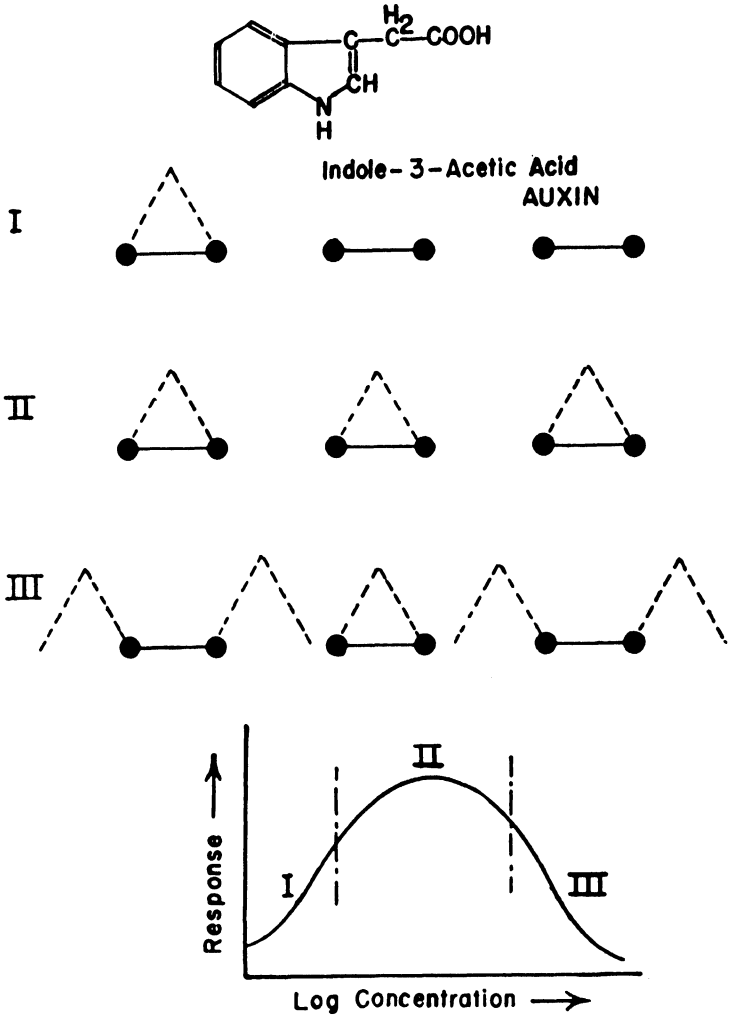

Fig. 7. A schematic diagram for self-inhibition in biological action arising from two-point attachment between two molecules where II is the active combination.

The universality of the response is best evident from control of cell enlargement in the etiolation response. This is very apparent in all higher plants. Seeds, too, that will germinate in darkness have been modified so as to come under light control. In the case of lettuce this can be done

either by holding them imbibed at a temperature higher than 30° (Borthwick, Hendricks, and Parker, 1952) or by imbibition of coumarin at concentrations greater than 10^{-5} molal (Nutile, 1945).

The question really is: With the controlling reaction present, why does it fail to control? And the answer is that control is expressed only when the concentration limits are met for the compounds involved. The situation is not unlike that of gene action that is only recognized when the gene is recessive, or a physiological response that is only pathologically evident.

The plant growth responses that are probably to be considered as under this control, interacting with other variable factors, are the following:

Seed germination
Seedling elongation
Leaf enlargement
Plumular hook unfolding
Epinasty
Leaf abscission
Bulb formation
Rhizome production
Casparian strip formation
Floral initiation
Flower development
Pigmentation
Phylloidy of bracts
Succulency
Sex expression
Root development
Response to day and night temperature

The list is not exhaustive but rather includes only those examples for which some analytical information is available.

The ecological significance of the control system and the mode of adaptation can be appreciated without detailed development. The control enters in dormancy of seeds, the persistence of seeds in soil, emergence of seedlings, light requirements in growth, temperature requirements, plant form, and reproduction. In short, the entire cycle of growth is influenced in its dependence on environment as expressed by temperature and light regimens.

VII. ACTION IN THE SPECTRAL REGION OF 4000 TO 5200 Å

We have not studied another aspect of photoresponsive growth control namely, phototropic responses of plants, and for this reason we shall leave the development of the essence of the subject to others (duBuy and Nuernbergk, 1934; Went, in press). Some comments are required, however, since the phototropic and reversible growth responses both possibly involve auxin. Phototropism has been shown to take place through an auxin-transport control (Cholodny, 1926; Went, 1928). The response

spectrum extends from 5200 Å towards shorter wave lengths with a maximum or double maximum near 4800 Å—the greatest effectiveness being in the blue rather than in the red and far red as for the reversible growth control. The effective pigment has been interpreted to be a carotenoid or riboflavin (Galston, 1950) or the first for the "tip" response and the second for the "base" response in *Avena* coleoptiles (Went, in press). Response in a similar spectral region is also possible for changes in protoplasmic viscosity and protoplasmic streaming (Virgin, 1948).

This cursory mentioning of phototropic response is to serve as a background for a further comment about the reversible photoreaction: namely, what is the response spectrum in regions below 5200 Å? Naturally this spectrum has been measured (Borthwick, Parker, and Hendricks, 1948; Borthwick et al., 1954) since possible absorption in the blue has an important bearing on the nature of the pigment. All that need be said about it is that the energy required for either of the actions in the region of 4000 to 5000 Å is much greater than near 6500 Å or 7350 Å. This could be due to overlapping of equal photochemical effectivenesses of the two forms of the pigment in this region, either with high values or low values of effectiveness compared with the most effective regions. In the case of germination of *Lactuca sativa* seeds the slow approach produced by high energies between 4000 to 5000 Å to a photochemical equilibrium value of the pigment system displaced to either extreme shows that the photochemical effectiveness is low compared with the maxima near 6500 and 7350 Å (Borthwick, Hendricks, and Parker, 1952). The small effect observed could result from operation of the phototropic control system, i.e., the action could be on the phototropic pigment system.

VIII. SCREENING OF LIGHT ACTION IN PLANTS AND ANIMALS

A reading of the critical article by Loofbourow (1948) on factors affecting action spectra, and knowledge of the elaborate studies on effectiveness of carotenoids and chlorophyll in photosynthesis (Rabinowitch, 1951), might cause one to wonder why so simple an approach as the one used here led so straightforwardly to the essential answers. Why can screening of obvious pigments in seed coats and green leaves, possible scattering, and other factors fail to obscure the regularities? Perhaps the first point to remember is that while measurements are highly quantitative for a physiological subject, the essence of the arguments depends only on orders of magnitude and on the fact of reversibility. A second point to be made is that the actions involved are in a spectral region in which chlorophylls alone among prominent plant pigments

absorb strongly; and the absorptions in the red of chlorophyll in the green plant resemble closely the action of the red-absorbing pigment. Effectiveness of chlorophyll as a screen, too, is subject to analysis if the photoreversible pigment system is in the plastid. In fact, we have been inclined to base an argument that the photoreversible system is not in the plastid on the apparent ineffectiveness of screening.

Restraint is also required in comments on photoperiodic responses of animals. The principal approach with animals has been the ecological one of evaluating "day-length" control for gonad development. Since endocrine work on animals is so advanced, its very successes have diverted attention from the immediate causal system for light action as also has predominant attention to vertebrates. The gap in knowledge is in the source of the control over the hormonal responses, i.e., in the control over the pituitary or its antecedents. A quotation from the general conclusions given in "Vertebrate sexual cycles," by Bullough (1951), a thoughtful review of the subject, is pertinent: "In an animal such as the ferret which breeds in spring the *stimulus of the increasing day length enters the eyes and passes in the form of nervous activity through the optic nerve* and the brain to the pituitary gland." This is intended as a most general conclusion avoiding controversial detail. Its purpose is to draw attention to the fact that the effective radiation enters the animal in the region of the eye and that eventually there is a response of the pituitary mediated through the nervous system. The underlined part, however, states that the stimulus is a long day where in reality it might be a short night. To pass in the form of nervous activity through the optic nerve implies that the photoconversion took place in the eye while in reality the site of action might be the hypothalamus or deep in some portion of the white matter.

A general approach is to measure an action spectrum, but here the limitations emphasized by Loofbourow (1948) become paramount in practically excluding the vertebrate with its screening blood as an experimental subject. Small invertebrates, such as mosquito larvae (Baker, 1953), aphids (Marcovitch, 1924), etc., that have definite "photoperiodic responses" in metamorphosis and are essentially transparent to visible radiation can be studied. With Dr. Charles E. Jenner and Mr. Oscar H. Paris, of the University of North Carolina, we have attempted to obtain the action spectrum for egg release from the pulmonate snail, *Lymnea palustris*. The egg release control is as precisely dependent upon night length as in the case of the turkey, a dark period shorter than 11 hours and 45 minutes being required at 27°C. The snail is a short-night

animal in contrast to the long-night sheep. But to date no conclusion has been reached on the action spectrum of a dark-period interruption other than that effectiveness in egg release is restricted to wave lengths shorter than 6500 Å.

IX. ACTION SPECTRUM FOR GAMETE RELEASE BY HYDRACTINIA

Greater success has been had with control by radiation of gamete might exist that the control is the general photoperiodic one for animals. release from the hydrozoan *Hydractinia echinata,* but in this case doubt

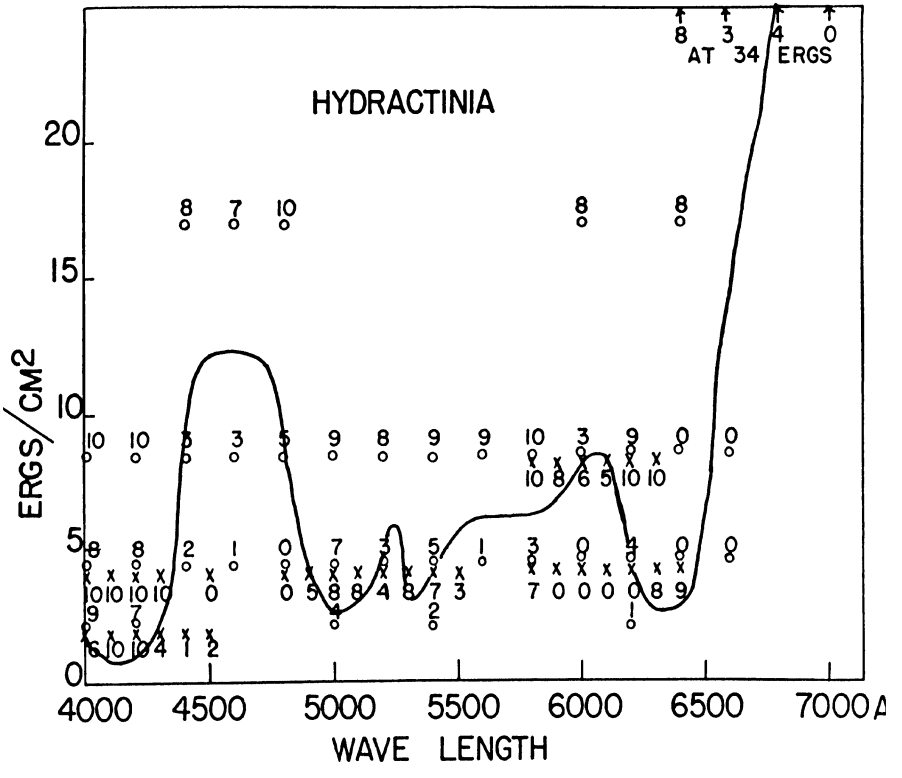

Fig. 8. Action spectrum for 50 percent gamete release from the hydrozoan *Hydractinia echinata.* The individual numbers represent the degree of release, on a scale of zero to 10 with 10 as complete release, for the indicated wave length and incident energy.

The gamete release at 22°C starts 55 minutes (Ballard, 1942) after effective irradiation of as low as 0.1 erg/cm² incident in an appropriate spectral region. The action spectrum as we have measured it with Dr. Jenner and Mr. Paris is shown in Fig. 8. This follows closely the absorption spectrum of a porphyrin, agreeing particularly well with that of

coproporphyrin III (Lemberg and Legge, 1949). MacMunn, in his early work, drew attention to porphyrins as present in Coelenterata. More recently Klüver (1944) found coproporphyrin III to be of widespread occurrence in white matter of the central nervous system of vertebrates. It is particularly abundant in the white matter of the brains of birds. Birds, too, are strikingly photoperiodically responsive for gonad development and for migration (Wolfson, 1942).

The photoperiodic phenomenon as observed in animals has the same spotty appearance among species as it does in plants. Its appearance among many animal phyla, however, is definite. This suggests that, just as among plants, the phenomenon might be very widespread even among animals in which it is not recognized.

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VIII. THE BIOLOGY OF WATER MOLDS

BY RALPH EMERSON[1]

I. INTRODUCTION

No doubt the founders of the Society for the Study of Development and Growth had quite specific original aims in mind. And yet from the broadest viewpoint one could well say that such a society would, in the long run, find itself concerned with each and every aspect of living things. A full understanding of development and growth must inevitably encompass all life's processes, and for this great field of knowledge we have a time honored name, to wit, Biology. My title, therefore, has validity here and need only be qualified by the acknowledgement that it attempts to cover too much. In directing attention to the biology of aquatic fungi, in attempting indeed to learn all one can of their development and growth, one ventures into many specialties and exposes oneself to the attendant danger of superficiality. Hopefully the loss in depth can be offset by the width of the horizon and some values may be derived from this account of our studies.

Present evidence suggests that the three major groups of true fungi are phylogenetically related in an ascending sequence from Phycomycetes to Ascomycetes to Basidiomycetes. Most primitive, most diverse, and hence most interesting and promising experimentally in many respects are the aquatic Phycomycetes at the bottom of this evolutionary series. Occurring as saprophytes and parasites in marine and fresh waters throughout the world they are commonly referred to as water molds. Although there are now well over a thousand described species, many of which have been intensively investigated by taxonomists and morphologists, only rarely have they been used for detailed and controlled experimental investigations of growth and development. Mostly they have remained in the immediate and regrettably obscure domain of the diligent monographer. We need not dwell here on the underlying reasons for this situation. It will be enough to point out that water molds are

[1] The Department of Botany, University of California, Berkeley. Acknowledgement is made here to Mr. Arthur T. Brice of Ross, Marin County, California, for his very kind permission to reproduce Fig. 2 and the photographs in Plates II–VIII. These photographs are selected frames from a film entitled *Syngamy and Alternation of Generations in Allomyces, a Watermold.* The film was produced by Mr. Brice in our laboratories and was shown at the Thirteenth Growth Symposium immediately following the present paper. I wish to thank Dr. Francis V. Ranzoni for his very able preparation of Figs. 1, 3, 4, and 5, and I am indebted also to the several kind critics who helped with the final preparation of the manuscript.

not always easy to find in nature, and the artful devices which have been worked out over the years by successful collectors deserve particular recognition (Sparrow, 1943). Methods of pure culture are, likewise, sometimes intricate and painstaking (Emerson, 1950), and many genera still remain to be isolated.

More than fifty years ago, however, a great pioneer of developmental plant physiology, the German botanist Georg Klebs (1899), made the first experimental study of growth and reproduction in an aquatic Phycomycete. Just before the turn of the century he published the account of his work on *Saprolegnia* which demonstrated conclusively that the reproductive structures of this mold are not inherently formed in a fixed order; on the contrary, their development can be inhibited or called forth at will by adjusting the environmental conditions. He showed, for example, that *Saprolegnia mixta* remained in a purely vegetative state as long as it was held continuously in fresh nutrient medium. Zoosporangia could be induced to appear quickly and in profusion whenever a vigorous mycelium was deprived of nutrient by washing and transfer to water. The distinctive sexual structures, oogonia and antheridia, were always formed when mycelium was placed in agar.

This pioneering work of Klebs on *Saprolegnia* has taken its place among the great classics of mycology. One might have supposed that further fundamental research would have developed quickly from such an imaginative and revealing beginning. True, Kauffman (1908), Pieters (1915), and others followed in Klebs' footsteps and repeated, verified, and elaborated upon his findings. *Saprolegnia* found its way into every elementary text book of botany, and freshman teachers the world over have, perhaps unknowingly, applied Klebs' findings in setting up laboratory material to show sexual and asexual reproduction in the "fish mold." The principles have been found to apply to many other genera of aquatic Phycomycetes, and mycologists everywhere know that grown mycelium transferred to fresh water will usually show copious zoospore formation whereas agar cultures are more apt to reveal oogonia and antheridia. The strange fact remains, however, that during the half century following Klebs' discoveries our basic understanding of these phenomena has not advanced appreciably, with one very important exception. John Raper's demonstration and elucidation of the hormonal mechanism that controls the formation and development of the sexual structures in heterothallic species of *Achlya,* a genus closely related to *Saprolegnia,* is a great step forward and represents one of the major contributions to modern biology. Readers are referred to the immediately

preceding Symposium Volume for a review of this brilliant work presented to the Society in 1953 (Raper, 1955). Bishop's (1940) investigations of *Sapromyces* show that sexual reproduction in that genus is almost certainly under some similar type of hormonal control, and without doubt the same basic mechanism is operative in many other water molds having comparable sexual reproductive structures.

Fascinating and important as these hormonal systems are, they provide only a partial answer to the problems raised by Klebs' observations. Among the hundreds of aquatic Phycomycetes, varying so widely in the details of their development and sexual and asexual reproduction, what can we discover about the underlying processes that lead to the formation and differentiation of sporangia, resting spores, or gametangia, or set the pattern of regularly alternating haploid and diploid phases in the life cycle? How are these developmental processes initiated and how are they integrated with nutrition, metabolism, genetic make-up, and the physical environment? These are large questions indeed, but the answers to such questions have been coming with increasing frequency from studies of small and comparatively simple organisms.

II. THE EXPERIMENTAL SUBJECTS

My interest in the processes of differentiation and reproduction in water molds was aroused some twenty years ago by Professor William H. Weston at Harvard University. He has contributed profoundly to the field of experimental mycology by his inspiring teaching, and there are many of us who owe him a deep debt of gratitude. It was he who directed my attention in 1933 to an exceptional group of saprophytic Phycomycetes which were still relatively obscure at that time but are, as we shall see, quite well known now and recognized for their intrinsic interest as well as their special suitability for various fundamental studies of growth and reproduction. They make up the small family, Blastocladiaceae, which presently includes the three genera, *Blastocladia* Reinsch (1878), *Allomyces* Butler (1911), and *Blastocladiella* Matthews (1937).

A. Allomyces. Our attention will be focussed mainly on *Allomyces* in the ensuing discussion, and it will be well to acquaint you at once with its structure and reproductive behavior. When the distinguished British mycologist, E. J. Butler, first discovered this mold in India, his broad knowledge of the fungi prompted him to select a name—*allo* (different), *myces* (fungus)—which was peculiarly prophetic, for only in later years, through the research of other investigators, has the extraordinary degree

of difference become fully apparent. During the closing months of his eminent scientific career, Hans Kniep (1929, 1930) found in *Allomyces* a type of sexuality and reproductive behavior not previously known in any fungus.

It is now evident that species of *Allomyces* are widespread throughout the tropical and warmer temperate regions of the world (Emerson, 1941). If dried mud- or soil-samples from these areas are used to inoculate water cultures "baited" with boiled hemp seeds, after several days a fringe of mycelium will usually appear, surrounding the seeds and extending out into the water to a distance of 5 mm. or more (Plate VIII, 1). The growth often proves to be *Allomyces,* and pure cultures can subsequently be obtained readily by routine microbiological techniques.

One can best appreciate the distinctive features of the vegetative thallus of *Allomyces* by following its development from a spore or zygote. The figures in Plates II, III, and IV vividly portray the processes involved. The first delicate germ tube pushes out rapidly, penetrates the substratum, and branches profusely to form the basal system of rhizoids that serve as organs of attachment and absorption. When the rhizoids have become well established, another much stouter hyphal germ tube grows from the opposite side of the spore. This is the rudiment of the main mycelium, and its origin, 180° away from the initial rhizoidal tube, gives evidence of a marked polarization which has not yet been investigated. The hyphal tube soon branches dichotomously (Plate III, 1); the branches elongate and divide again; and so, by repeated dichotomies and elongation, the arbusculate thallus continues to fan out and grow (Plate IV) until it reaches maturity and forms reproductive structures.

The mycelium appears to have cross walls at irregular intervals; all these pseudosepta, however, have wide perforations through which pass large strands of cytoplasm. Nuclei are distributed along the coenocytic hyphae and tend to be more numerous in the growing hyphal tips where the cytoplasm is more dense and granular. Reproductive structures originate terminally on the hyphae and occur singly or in short chains. Their characteristic arrangement results from repeated sympodial branching and elongation of the supporting hyphae as shown in Plate VI, 9-11.

All of the reproductive organs (whether sporangia or gametangia, see below) have a basic similarity of pattern. They are always single cells cut off from the parent hypha by a complete septum formed quite early in their ontogeny (Plate VI). They are multinucleate and produce swarmers—spores or gametes—by a process known as progressive

cleavage in which the entire protoplast is ordinarily divided up into uninucleate blocks. They release swarmers through a pore formed by the dissolution of a gelatinous plug termed a discharge papilla. Papillae are evident in Plate I, 2 and 5. The swarmers themselves also conform to a fixed structural pattern, involving certain fundamental features shown in Fig. 1, namely the thin elastic wall that permits semi-amoeboid motion (Plate VIII, 5), the single, posteriorly situated, whip-like flagellum, and the single nucleus embedded in the posterior end of a conspicuous top-

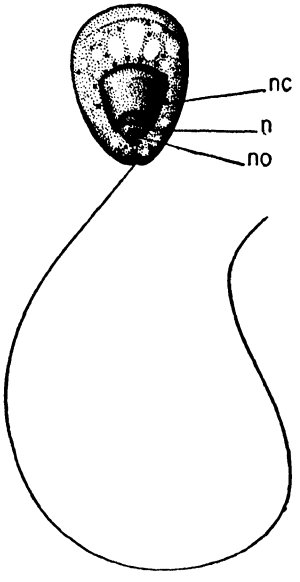

Fig. 1. A zoospore (mitospore) of *Allomyces* showing the characteristic posterior flagellum; the large "nuclear cap," nc; the nucleus, n; and the nucleolus, no (× approximately 1750). Meiospores, female gametes, and male gametes all have this same basic structure.

shaped structure known as the nuclear cap. Posteriorly uniflagellate swarmers characterize a whole major series of the aquatic Phycomycetes, but the nuclear cap is a feature distinguishing *Allomyces* and its near relatives.

The full sexual cycle of *Allomyces macrogynus* and *A. arbuscula* involves two entirely separate, "equal," and alternating generations as shown in Fig. 2. Each of the four different kinds of reproductive structures normally has a fixed place in the cycle and a specific function determined, we must assume, by its particular physiological make-up. Two kinds of sporangia are borne on the diploid sporophytic plant. The thin-walled gray ones (Plate I, 5) are called mitosporangia and the 50 or so

spores they release are called mitospores because each one contains a diploid nucleus formed by mitosis. After a period of swarming (Plate VIII, 5), mitospores develop asexually into new diploid sporophytic plants. Hence they constitute a means of rapid asexual multiplication and spread of the diploid phase with no change in chromosomal material. Aside from certain yeasts, the Blastocladiales are the only fungi with a

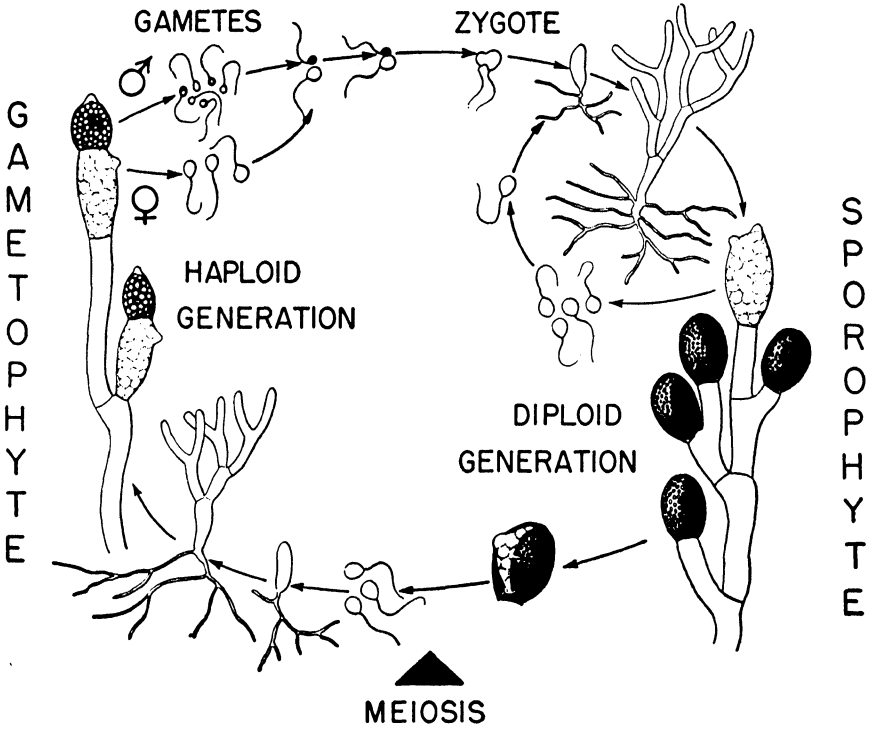

Fig. 2. Semischematic portrayal of the life cycle and reproductive structures in *Allomyces macrogynus*. Mitospores from the single mitosporangium are shown reproducing the sporophyte asexually. Meiospores, released from the dark meiosporangia, develop into gametophytic plants, and there male and female gametangia are formed in pairs. (Drawing made by Raphael Rodríguez and reproduced by permission of Arthur T. Brice.)

natural mechanism such as this which allows indefinite clonal propagation of any diploid strain without genetic change.

The other sporangia on the diploid plant are very different (Plate I, 6). They have an outer wall that is thick, conspicuously pitted, and distinctively colored by brownish melanoid pigments (Emerson and Fox, 1940). They are usually referred to as resistant sporangia (RS) because they become dormant and serve to carry *Allomyces* and the other Blasto-

cladiaceae through long adverse periods when temperatures are unfavorable or water is too scarce for vegetative growth. Under suitable environmental conditions the RS germinate—i.e., dehisce—in a characteristic manner as shown in Plate V. There is first a rapid uptake of water that results in a swelling of the contents and rupturing of the heavy outer wall. One or more discharge papillae are soon formed on the thin inner wall which protrudes through the cracks. Presently the spores are cleaved out, the exit papillae dissolve, and the spores are released.

Cytological studies of *Allomyces* (Wilson, 1952) have shown that each RS originally contains about 12 diploid nuclei. During the early stages of germination these nuclei go through two meiotic divisions in rapid succession. In the subsequent cleavage process each of the 48 spores receives one of the haploid nuclei thus produced. Hence resistant sporangia can be called meiosporangia and the spores they release are meiospores.

Aside from their slightly smaller size these meiospores are outwardly identical in appearance and behavior with the mitospores (Plate VIII, 5). Moreover they swarm, settle down, round up and grow in precisely the same way. No difference becomes apparent until reproductive organs start to develop on these new haploid thalli. At that time the first indication of a change can sometimes be detected by the occurrence of the young reproductive structures fairly regularly in terminal *pairs*. Subsequently, marked differences arise in these paired cells (Plate I, 2 or 3). One of them continues to develop into a structure which resembles in all respects a mitosporangium; it is unpigmented and releases swarmers that are structurally indistinguishable from mito- or meiospores. The other, however, besides being somewhat smaller, soon begins to reveal in its cytoplasm a bright orange color resulting from the accumulation of γ-carotene (Emerson and Fox, 1940). Furthermore, when swarmers are released they are about half as large as any of those previously described (Plate VII; Plate VIII, 2 and 3). The full significance of the differentiation that has occurred becomes apparent when each small orange swarmer pairs off and fuses with one of the larger colorless swarmers from the adjacent cell. We have here a haploid, hermaphroditic, self-fertile, gametophytic plant bearing colorless female gametangia and orange-colored male gametangia. There is still no other fungus in which this striking anisogamy of motile gametes is known to occur.

Whereas, in the great majority of Phycomycetes, syngamy leads to the formation of a resistant or resting zygote, in *Allomyces* the zygote de-

velops directly into a new thallus. At first the zygote is motile, propelled by the two closely juxtaposed flagella (Plate VIII, 4), one from the female gamete and one from the male. But after a period of swarming it settles down and develops just as mitospores and meiospores do. In this instance the single diploid fusion nucleus divides mitotically to populate the new plant with diploid nuclei. The reproductive structures that make their appearance are again mitosporangia and meiosporangia.

The life cycle of *Allomyces,* which has just been reviewed, exhibits with diagrammatic clarity what botanists speak of as the alternation of equal generations. While this type of life cycle has long been recognized in many different algae, it had never been observed in the fungi until Kniep made his investigations of *Allomyces javanicus* in 1930. The only other instance of a similar cycle was reported in the closely related genus *Blastocladiella* in 1938 by Harder and Sörgel (see below).

B. Blastocladia and Blastocladiella. By far the greater part of the experimental work done in our laboratories has involved *Allomyces,* but a few observations of unusual significance have concerned the related genera *Blastocladia* and *Blastocladiella.* Moreover, in the past five years our former student, Dr. E. C. Cantino, with his associates at the University of Pennsylvania, has been making an intensive study of growth and differentiation in a species of *Blastocladiella.* This work is so interesting and bears so directly on our own observations that reference to it, even though necessarily brief, is essential. We must, therefore, point out here a few of the significant features of *Blastocladia* and *Blastocladiella.*

So little is known about the reproductive processes of *Blastocladia*—even though the genus was first discovered nearly 80 years ago!—that few comments can be made. In all essential morphological respects the known species of *Blastocladia* correspond to sporophytes of *Allomyces* (cf. Sparrow, 1943). Their irregularly branched thalli bear thin walled sporangia, undoubtedly mitosporangia, and typical resistant sporangia that cannot be called meiosporangia because no cytological work has been done and no sexual stage has been detected in the genus. A comprehensive picture of the distribution, substrate relations, nutrition, and unusual metabolism of *Blastocladia* will be found in the papers by Emerson and Cantino (1948) and Cantino (1949) and other references cited therein.

Superficially *Blastocladiella* appears to be quite different: the thallus is unbranched and each plant bears but one single reproductive structure. On the other hand, the life cycle depicted in Fig. 3, which Harder and Sörgel (1938) described for *B. variabilis,* agrees in every important de-

tail with that of *Euallomyces*. The main difference is that each sporc phytic plant bears, necessarily, *either* a mitosporangium *or* a resistant spc rangium (meiosporangium by implication and homology with *Euallomy ces,* but not determined cytologically). Likewise, each gametophyte bear *either* a colorless female gametangium *or* an orange male gametangiun Cantino has recognized, as we shall see, that this extraordinary littl organism has certain features which make it exceptionally suitable for variety of morphogenetic studies.

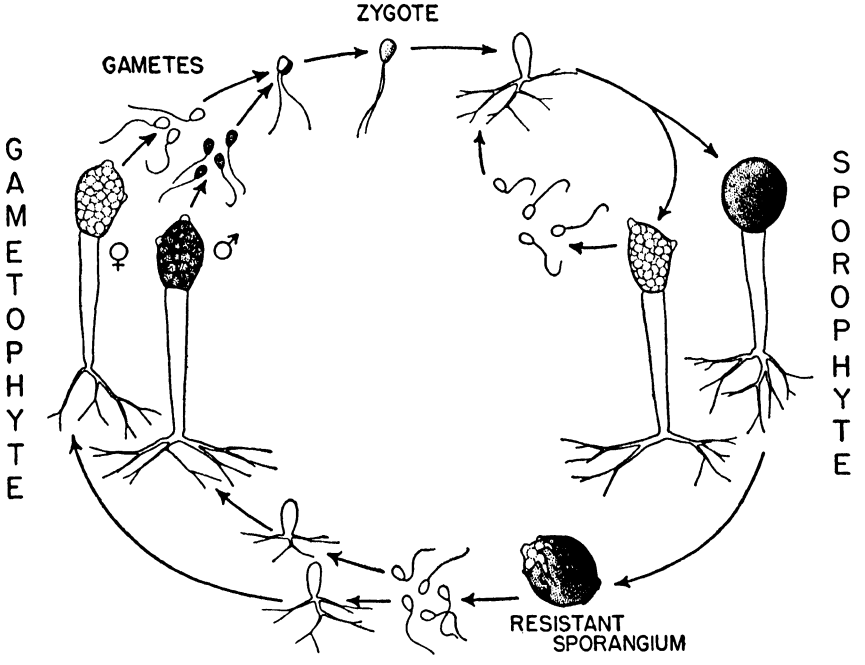

Fig. 3. The life cycle and reproductive structures in *Blastocladiella variabilis* as described by Harder and Sörgel (1938). Each plant bears only a single reproductive structure. Hence, sporophytes have either a mitosporangium (on the left) or resistant sporangium (on the right) while gametophytes have either a male (♂) or a female (♀) gametangium. The gametes are the same size but the males are distinguished by their orange color.

III. ASPECTS OF CULTURE AND MANIPULATION

Although the gametophyte and the sporophyte of *Allomyces* are obviously integral parts of a single developmental sequence, they are completely disconnected from each other physically and are entirely independent in their nutrition and growth. It is not at all surprising, therefore, that the two generations can be cultivated quite apart from each other (Emerson, 1941) and may be perpetuated virtually indefinitely

on agar media by vegetative transfer of hyphal tips. Under these conditions the reproductive structures that characterize each generation are formed in profusion near the surface of the cultures. The thousands of meiosporangia lend a characteristic brown hue to sporophytic cultures whereas the accumulated carotene in the male gametangia imparts a bright orange color to gametophytic cultures (Plate I, 1 and 4).

The formation and release of spores or gametes can usually be suppressed by preventing any accumulation of water on the agar cultures. Conversely, when mature sporangia or gametangia are placed in water, mass discharge of swarmers can be induced in a short and predictable time. Thus, by scraping the agar cultures gently with a flattened transfer wire, quantities of sporangia or gametangia can be removed uninjured and placed in water. Mitosporangia, female gametangia, and male gametangia will usually start to release swarmers within sixty minutes after transfer to water at 20-25°C. (Plate VII, 2). Meiosporangia ordinarily take somewhat longer but can be expected to discharge spores in less than two hours (Plate V). The time between suspension in water and the onset of discharge is sufficient to permit segregation of the different kinds of reproductive structures. Thus meiosporangia and mitosporangia can be separately isolated from sporophytic suspensions, while male and female gametangia can be separately isolated from gametophytic suspensions. In each instance the distinctive color difference between the two structures concerned (Plate I) facilitates positive identification at about 50× magnification under the dissecting microscope, and rapid manipulation is readily accomplished with micropipettes of suitable bore.

It should also be noted that Machlis (1953a, 1953b) has developed precise techniques for growing sporophytic mycelia of *Allomyces* under strictly defined and uniform conditions in liquid cultures. Gametophytic plants are somewhat more difficult to maintain in this environment.[2] In either instance, however, crops of reproductive structures can be initiated and brought to discharge within a predictable time. The more rigid environmental control that can be achieved in liquid cultures has played an important part in certain of the studies to be described below.

IV. SEXUAL DIFFERENTIATION AND THE ARRANGEMENT OF GAMETANGIA

In an effort to present the results of our investigations from the point of view of a specialist on growth and differentiation—if there really is

[2] Turian (1954) has recently reported that small amounts of boric acid in the medium inhibit syngamy. This discovery should facilitate the growth of strictly gametophytic material in liquid cultures.

such a one—we will focus our attention on three particular problems even though each is related to the others and no very sharp separations can be made. First, what controls the formation and differentiation of gametangia? Second, what factors affect the formation, maturation, and germination of resistant sporangia? And third, what mechanism determines sporophytism and gametophytism and keeps these two generations alternating regularly in the reproductive cycle? If we had complete answers to these three questions we would have unlocked some of the major mysteries of biology.

A. The inheritance of gametangial arrangement in Allomyces. Over the years, since Kniep's discovery (1929) of sexuality in *Allomyces,* it has become increasingly apparent that there are two clear-cut natural species which together comprise the subgenus *Euallomyces.* The basic morphological difference between them lies in the arrangement of the gametangia (Plate I, 2 and 3). In *A. arbuscula* the female gametangium is always terminal and the male is subterminal or *hypogynous;* in *A. macrogynus* the female gametangium is always subterminal and the male is terminal or *epigynous.*[3] We can express these facts by saying that *A. arbuscula* is 100% H (hypogynous) and *A. macrogynus* is 100% E (epigynous).

The absolute regularity (so far as we have observed) with which these two types of gametangial arrangement occur clearly implies some very fixed processes of development and a precise mechanism which is under genetic control. From the nature of the gametophytes of *Allomyces* we must also conclude that sexual differentiation is not determined by a simple segregation of sex chromosomes or sex-determining genes. The haploid nuclei in the gametophyte are all derived by mitosis from the single haploid nucleus of the original meiospore. Hence the nuclei in the developing male gametangium will be genetically identical to those in the young female gametangium. This type of sex determination is often spoken of as *phenotypic* in contrast to the other type which is called *genotypic.* One should not forget, of course, that phenotypic sex differentiation is probably, in the last analysis, just as genetically controlled as is genotypic.

It has seemed to us that the differentiation of gametangia in *Allomyces* presents one of the neatest, simplest, and most clearly defined problems of morphogenesis. Starting with an undifferentiated hyphal tip, in the course of about one hour the apical portion is cut off and becomes one

[3] It is particularly important to understand that these statements apply only to the primary, terminal pairs of male and female gametangia. Secondary ones are often found in longer or shorter chains below the primaries, but in these sequences the positions of male and female cells are not fixed.

sex while the subapical portion, similarly walled off, has become the opposite sex (Plate VI, 1 to 8). The position of the two gametangia is fixed and the appearance of carotenoid pigment in the male serves as a clear marker. The γ-carotene provides, in fact, a visible indicator of the different sequence of metabolic reactions that go to make a cell male rather than female in this organism.

In 1936 the place of meiosis in the life cycle of *Allomyces* was not known and we decided to see whether the approximate position of the reduction divisions could be determined by genetic experiments. The character we chose to study at that time was gametangial arrangement, simply because no other clear-cut differences had been found—nor have they even today—among all the 80 or more isolates of *Euallomyces,* in either the diploid or haploid phase. It was soon discovered that reciprocal interspecific crosses between *A. arbuscula* and *A. macrogynus* could indeed be achieved, and during the subsequent years some 150 hybrid strains have been derived from individual zygotes. Details regarding the procedures that were followed and the genetic analyses that were made have been presented fully elsewhere (Emerson and Wilson, 1954). The point of importance here is that the crosses gave considerable insight into the genetic and chromosomal mechanism whereby control is exerted over the differentiation of paired male and female gametangia.

A summary of the observations is presented in Fig. 4. The first point that became apparent immediately was that, despite what one might have expected regarding inheritance in haploids, many F1 gametophytes, derived from single meiospores, were distinctly intermediate (I). That is to say, they were neither pure E nor pure H. In some of the primary terminal pairs of gametangia, the male was terminal, while in others *on the same plant* the female was terminal. Not infrequently large numbers of paired males or paired females also occurred. When percentages of epigyny and hypogyny were determined in these hybrids it was found that a whole range of intermediacy from nearly pure E to nearly pure H appeared in the population of F1 gametophytes derived from a single hybrid sporophyte. Moreover, if enough gametophytes were analysed both parental types, pure E and pure H, appeared sooner or later.

Not only was the regular arrangement of male and female gametangia in the terminal pairs disturbed, but the normal ratio between total number of male gametangia and total number of female gametangia was also strongly modified in many instances. Whereas the parental strains have a male: female ratio of about 1, this ratio often deviated very

widely from unity in the hybrids, and occasionally a gametophyte appeared with less than 1 female per 1,000 male gametangia (Table I). Such cultures were often striking in their bright orange coloration.

These observations on the inheritance of gametangial arrangement in interspecific hybrids permitted us to draw certain basic conclusions. Firstly, hybridization has upset or deranged the very precise mechanism that determines the spatial relations of the male and female gametangia in the parental species. In other words, we have interfered with some parts of the machinery governing sexual differentiation and hence sex determination. The large shifts away from the normal sex ratio lend further emphasis to this conclusion. Secondly, since intermediates are produced in hybrid haploids, we must conclude that gametangial arrangement is not under the control of a single pair of non-duplicated allelic genes. Thirdly, it was possible to say with considerable confidence that meiosis in *Euallomyces* occurs in the resistant sporangia.

The question of the place of meiosis requires some further discussion here. It will be recalled that the hybridization studies had originally been designed to locate the site of chromosome segregation and reduction. Precise knowledge of the extent of haploidy and diploidy was also essential if we were to have a sound basis for further studies of sex determination and other differentiation processes in *Allomyces*. Previous workers (Kniep, 1930; Hatch, 1938) had come to conflicting conclusions based upon cytological observations, and the existence of an entirely independent diploid phase was open to doubt since it had never been found in any other filamentous fungus. The segregation of E-, I-, and H-type gametophytes from each hybrid sporophyte derived from a single hybrid zygote pointed clearly to the existence of diploid nuclei in the sporophytic hyphae and the probable occurrence of meiosis in the resistant sporangia. The uninucleate mitospores of *Allomyces* offered a nice means of further clarifying this point. F1 mitospores were isolated and germinated to give secondary F1 sporophytes. On germinating the RS of these plants, segregation occurred exactly as before and the population of derived gametophytes showed the full range of gametangial arrangement. The conclusion was inescapable that mitospores of *Allomyces* are diploid.

Further indirect evidence of meiosis in the RS was revealed by comparison of the viability of spores from parental and hybrid RS (Fig. 4). On the average, 63% of the former were viable while only 0.11-3.25% of the latter were capable of growth and development to maturity.

B. Chromosomes in the parental and hybrid strains of Allomyces. Most of the observations described in the foregoing section were made ten or fifteen years ago. It was not until 1949, however, that elucidation

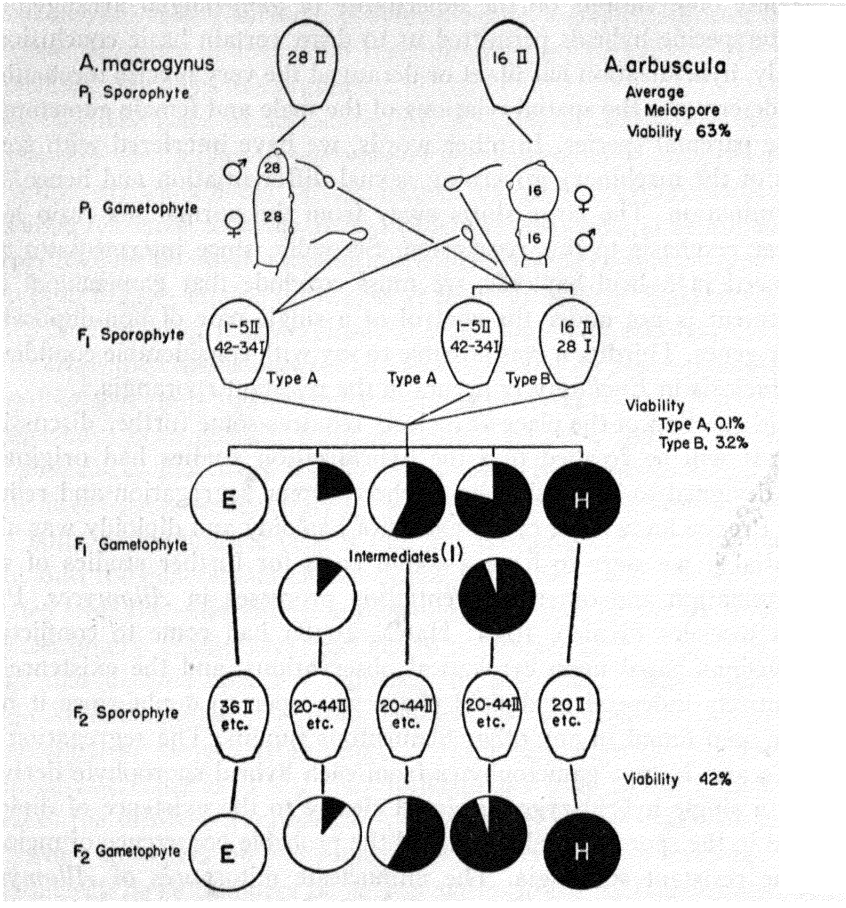

Fig. 4. Inheritance of the character "gametangial arrangement" in interspecific, reciprocal hybrids of *Allomyces*. The parental and filial generations are indicated on the left; meiospore viability is shown on the right. The numbers within the stylized outlines of resistant sporangia show the number and pairing-behavior of chromosomes at meiosis in each generation. (II stands for pair; I stands for single; see text for discussion of Types A and B.) The degree of epigyny (E) and hypogyny (H) in the F1 and F2 gametophytes is schematically represented by the black and white circles.

and extension of many of the interpretations became possible as a result of outstanding cytological investigations by Charles M. Wilson (1952). His chromosome studies of *Allomyces* constitute the first step-by-step examination of meiosis in the Phycomycetes and one of the few first class accounts of this process in the fungi. Despite the fact that the

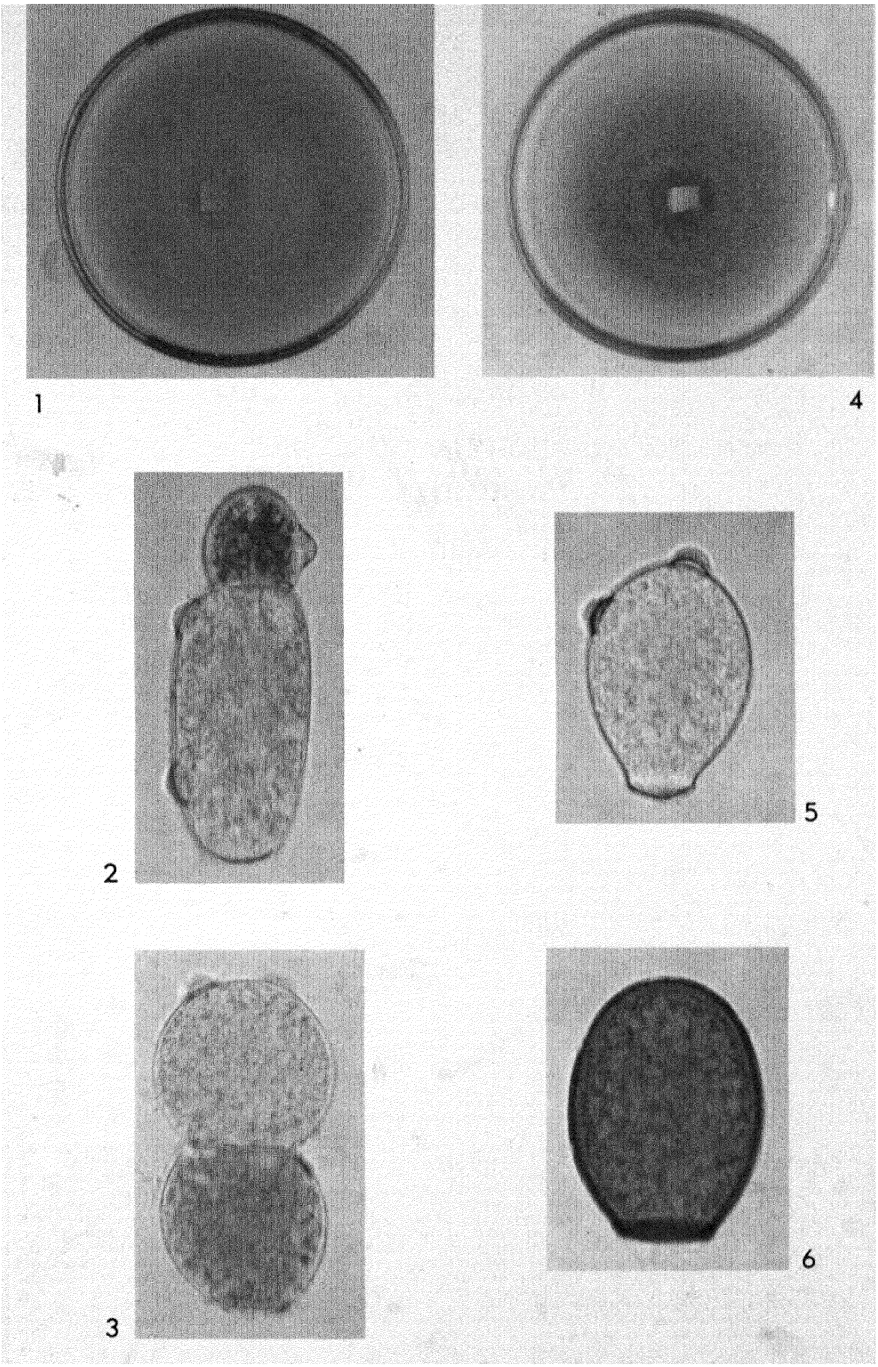

PLATE. I. 1. The gametophyte of *Allomyces* on yeast-starch agar. 2. A primary pair of gametangia of *A. macrogynus*; the orange male is epigynous. 3. A primary pair of gametangia of *A. arbuscula*; the orange male is hypogynous. 4. The sporophyte of *Allomyces* on yeast-starch agar. 5. A zoosporangium (mitosporangium) of *A. macrogynus*. 6. A resistant sporangium (meiosporangium) of *A. macrogynus*. Natural color photo-

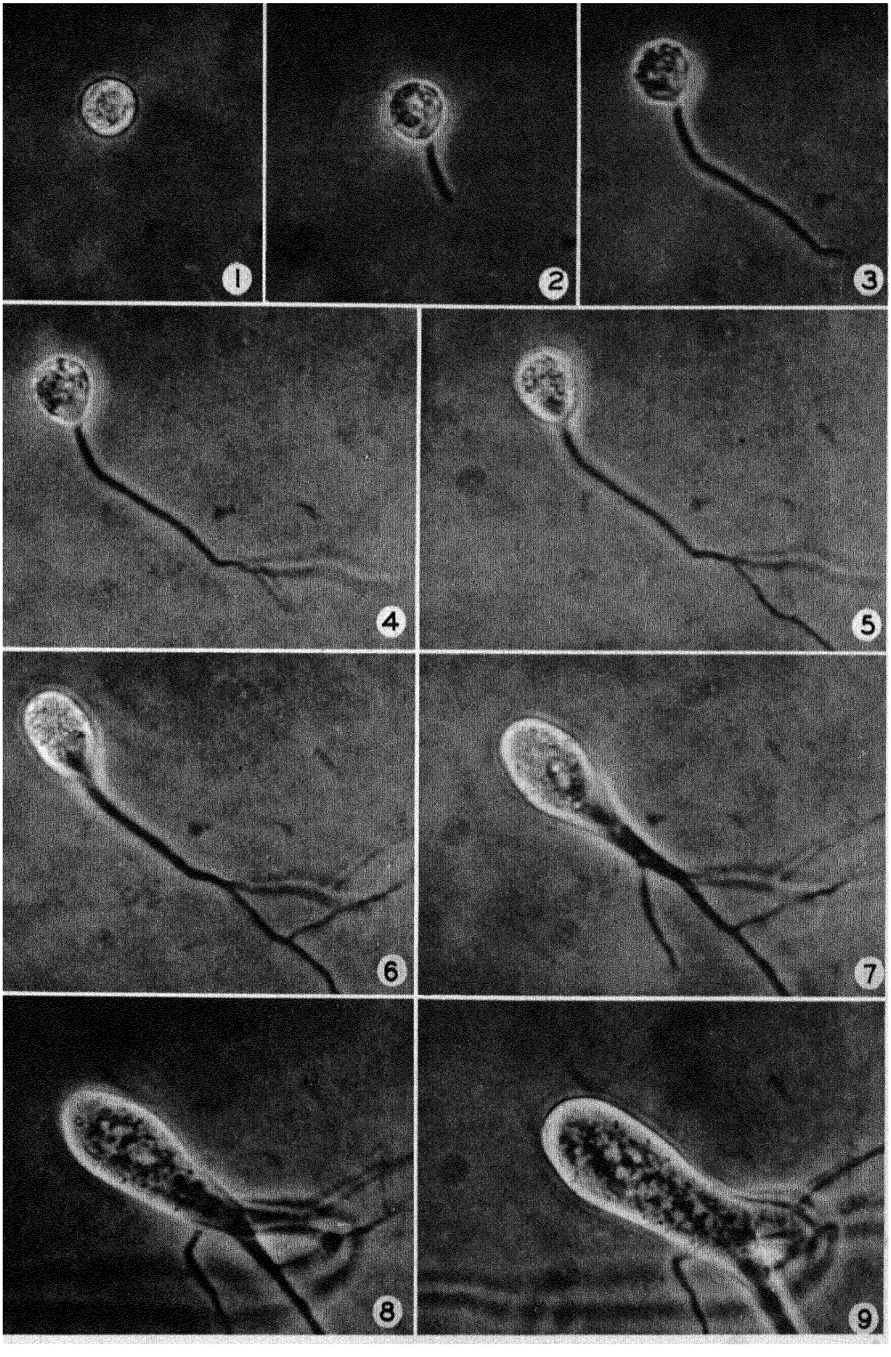

PLATE II. Early development of the zygote of *Allomyces* during the first 8 hours of growth. 1. The zygote has lost its flagellum and rounded up. 2. About 20 minutes later the first rhizoidal germ tube has appeared. 3-5. Photomicrographs at 40-minute intervals show growth and branching of the rhizoids. 6-9. The intervals here are longer, *ca.* 100 minutes; enlargement of the main body of the zygote is followed by elongation of the initial hyphal tube (8, 9). Phase contrast photomicrographs, all ×735.

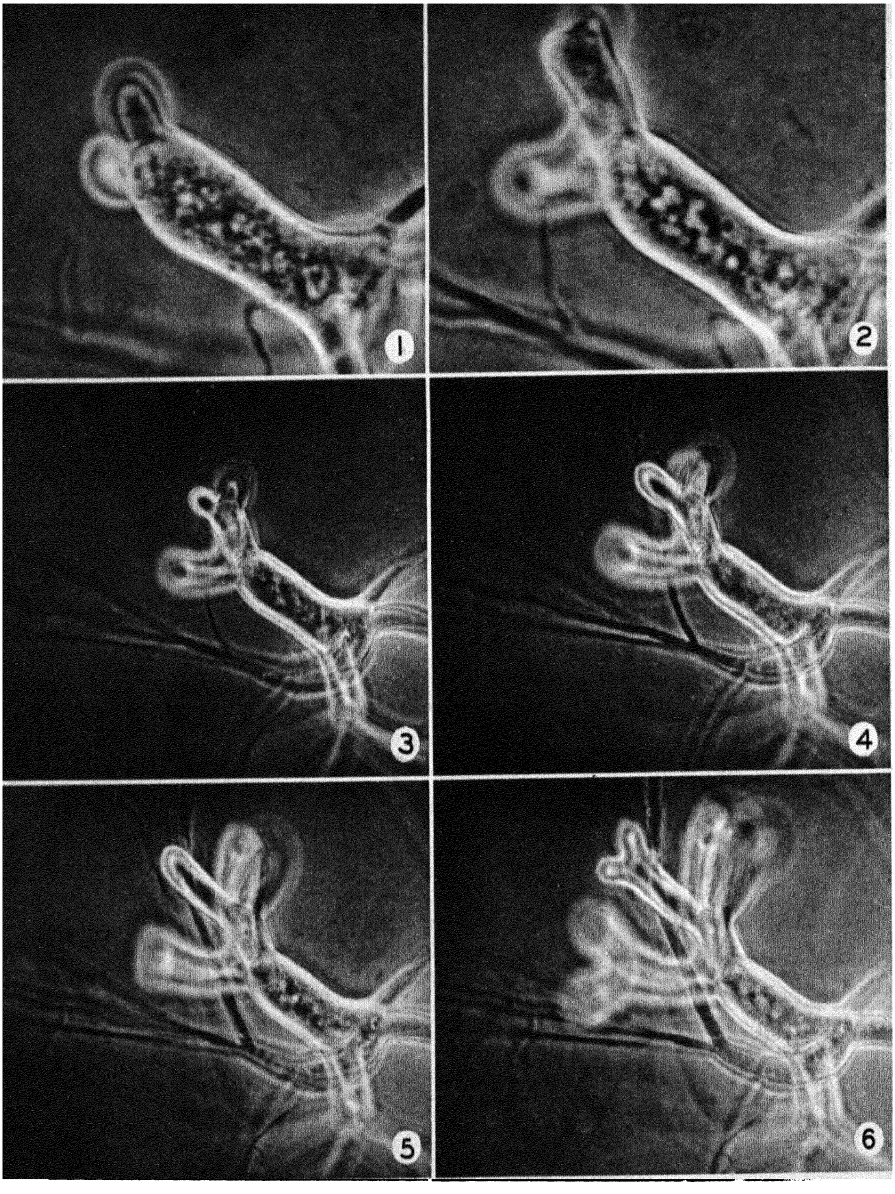

PLATE III. Development of the young sporophyte of *Allomyces* from the ninth to the fifteenth hour of growth. Intervals between pictures are approximately 60 minutes. 1. First dichotomy of the hyphal tube. 2. Second dicotomy. 6. Third dichotomy. Phase contrast photomicrographs, 1 and 2 ×735, 3-6 ×335.

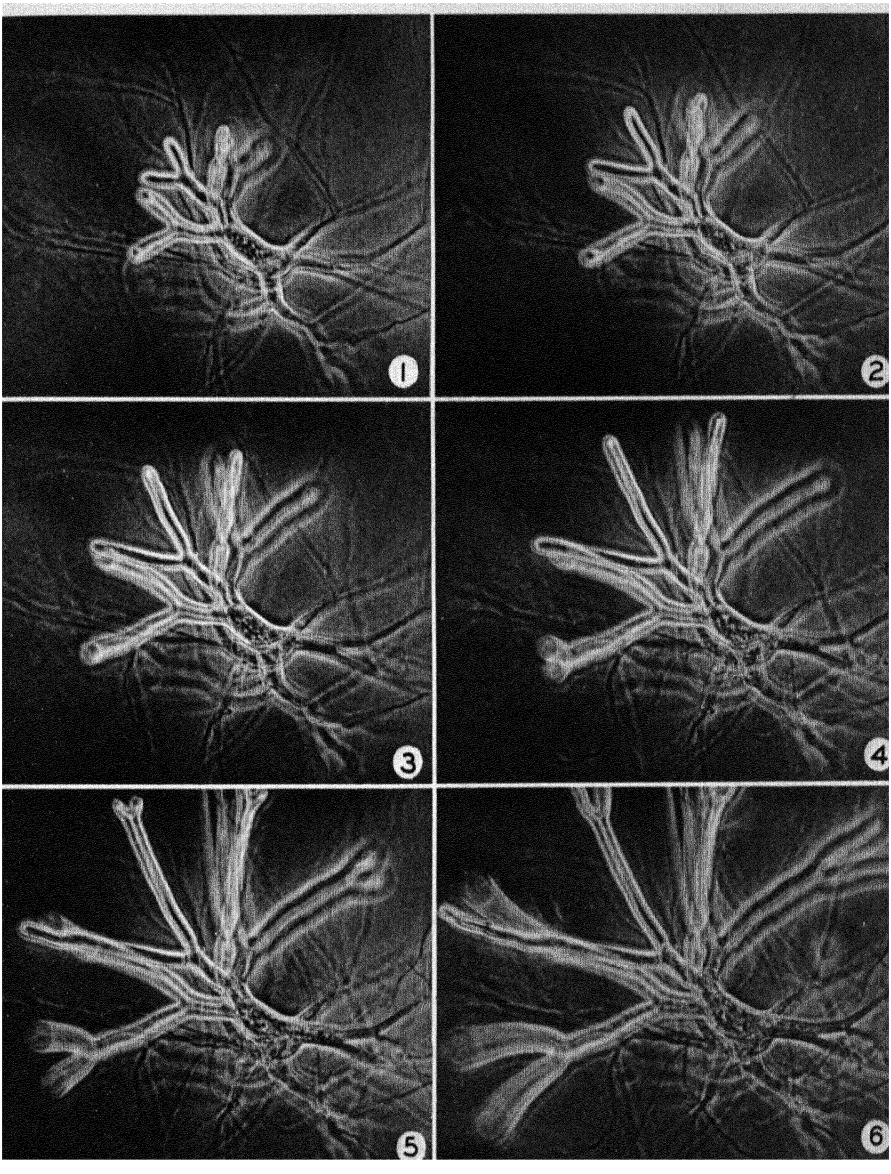

PLATE IV. Growth of the sporophyte of *Allomyces* from the sixteenth to the twentieth hour. Intervals between pictures are approximately 45 minutes. The basal rhizoidal system and the apical hyphal system become fully established. Phase contrast photomicrographs, all ×167.

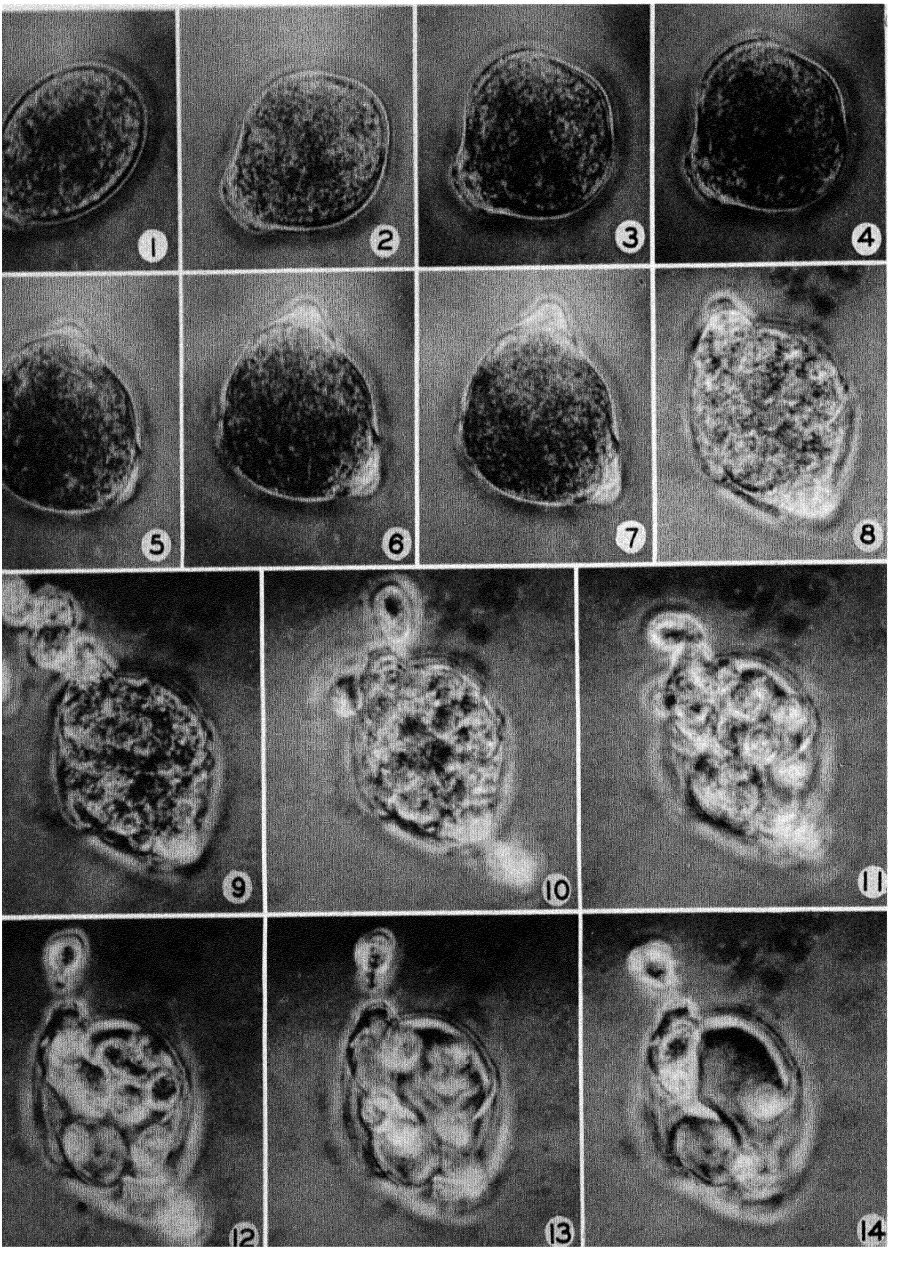

PLATE V. The germination of a resistant sporangium (meiosporangium) of *Allomyces* and release of the meiospores. 1. Mature sporangium that has been in water about 20 minutes. 2-4. Pictures at 3.2-minute intervals showing the rapid swelling of the sporangium. 5-7. Time intervals of about 10 minutes revealing the appearance and formation of the discharge papillae. About 45 minutes have elapsed between 7 and 8. 8. Cleavage has been completed and the outlines of the meiospores can be dimly seen. 9. Spore discharge has just begun. 10-14. Scenes of discharge taken at about 3-minute intervals until the last few spores are escaping and the cracks in the heavy outer sporangial wall have become clearly visible. Phase contrast photomicrographs, all ×500.

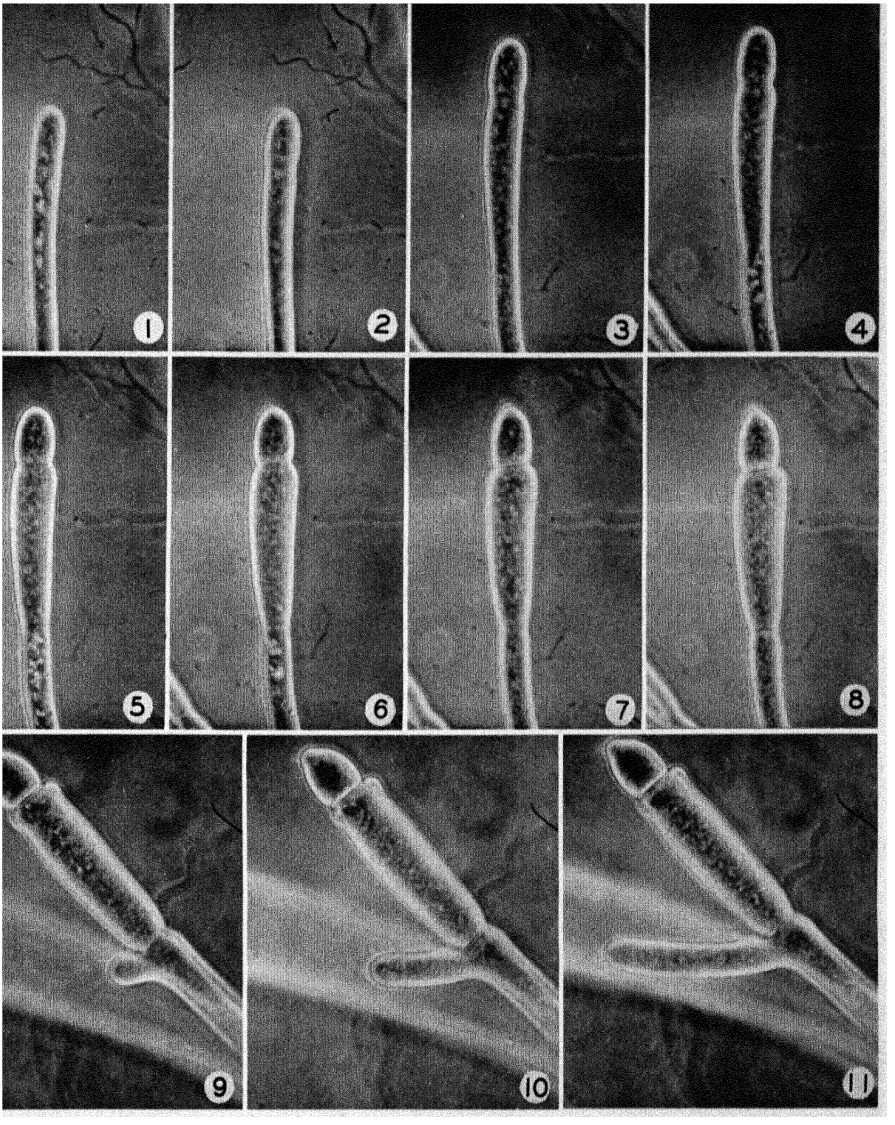

PLATE VI. Morphogenesis of male and female gametangia in *Allomyces macrogynus*. 1. An outwardly undifferentiated hyphal tip at time 0. 2-8. Pictures taken at 15-minute intervals thereafter and showing the first cross-wall delimiting the young male gametangium near the apex (2,3), the second cross-wall lower down delimiting the female (4,5), and the subsequent swelling of the paired gametangia and formation of discharge papillae (7,8). 9-11. Pictures spaced 24 minutes apart and showing how hyphae continue their growth by sympodial branching just below the reproductive structures. Phase contrast photomicrographs, all ×335. (The gametangia shown here were formed in a micro-culture chamber and hence they are considerably smaller than those shown in Plates I and VII.)

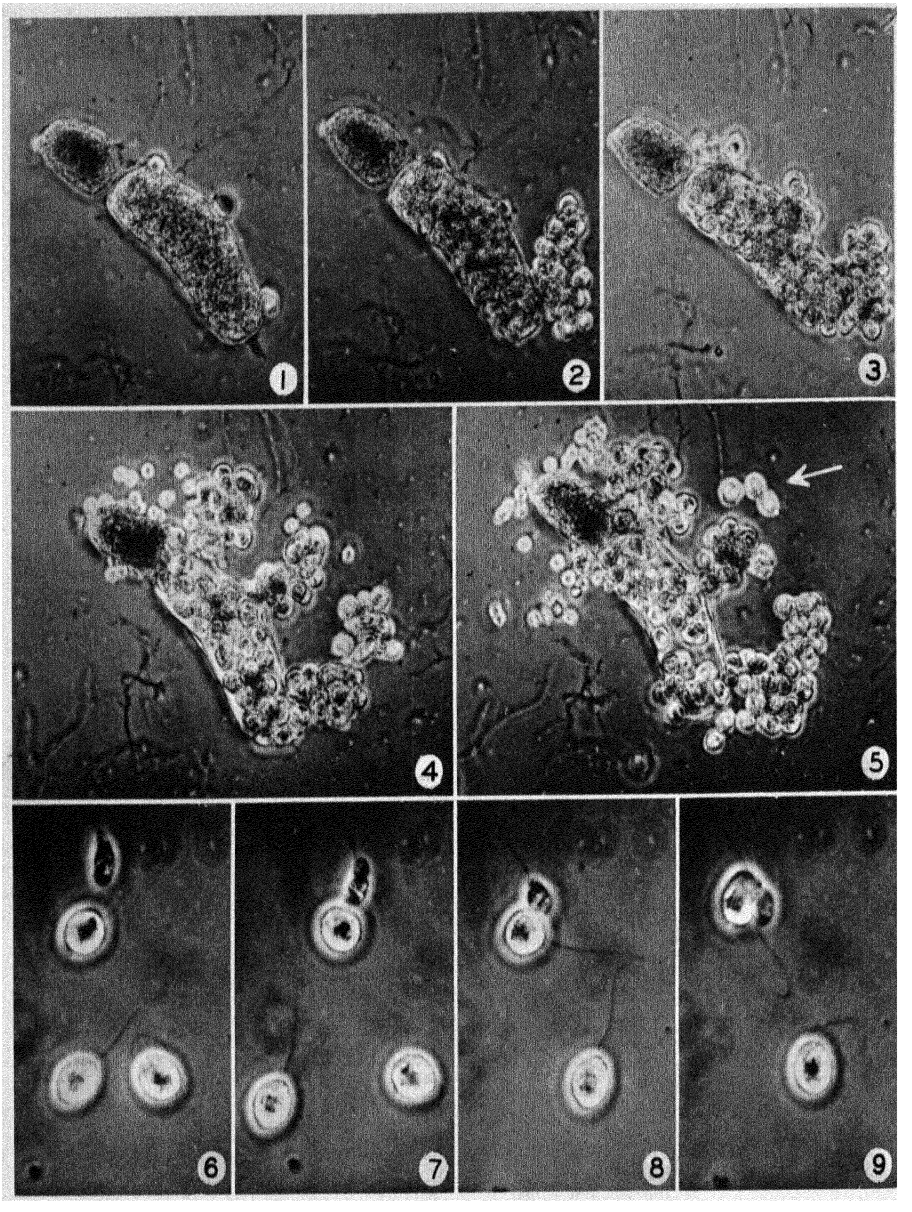

PLATE VII. 1-5. The release of gametes from gametangia of *Allomyces macrogynus*. Scenes are taken at about 1-minute intervals. 1. A pair of mature gametangia that have been in water for about 55 minutes. A small discharge papilla shows clearly at the apex of the darker epigynous male gametangium, and three larger papillae are apparent on the female. 2. Female gametes are starting to emerge from the bottom papilla. 3. Discharge is occurring from all three female papillae. 4. Male gametes are being released and can be detected by their smaller size. 5. Gametic fusions are taking place rapidly; three zygotes are indicated by the arrow. 6-9. A sequence covering 14 seconds and showing (6) a male gamete approaching three females from above, (7) making contact with one of the females, (8) undergoing copulatory movements, and (9) beginning the actual process of syngamy. Phase contrast photomicrographs, 1-5 X250, 6-9 X500.

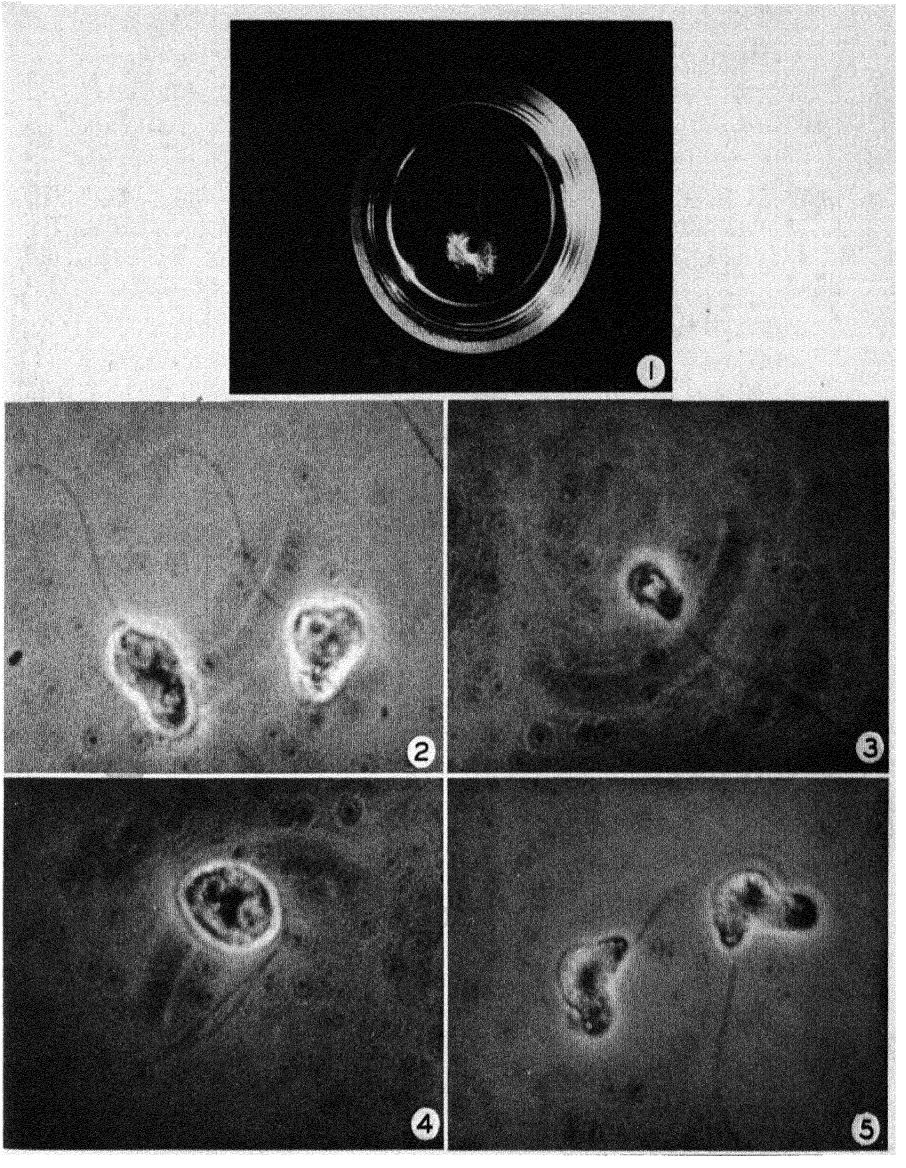

PLATE VIII. 1. A natural growth of *Allomyces* in water culture on a hemp seed, ×0.65. 2-5. Phase contrast images of two female gametes (2), a male gamete (3), a zygote (4), and two mitospores (5) of *Allomyces macrogynus*. The characteristic semi-amoeboid motion exhibited frequently by gametes and spores is well shown in 2 and 5. All ×1000.

nuclei of *Allomyces,* in their most expanded condition in Meiosis I, are seldom over 7 microns in diameter, Wilson succeeded in documenting the main stages of meiosis with excellent photomicrographs. From this work, then, it finally became absolutely clear that (1) the two meiotic divisions occur in the resistant sporangia of *Allomyces,* (2) the sporophytic plant is an independent, truly diploid generation quite unlike anything found elsewhere in the fungi outside of the yeasts, and (3) the haploid, hermaphroditic, gametophytic plant, when selfed, must give completely homozygous offspring.

Wilson's cytological findings immediately opened up a whole series of possibilities in the fields of cytogenetics and cytotaxonomy. Inevitably, the investigations that followed (cf. Emerson and Wilson, 1954) were directly related to and interwoven with the previous studies of gametangial arrangement. They are, therefore, of importance in our consideration of the mechanisms controlling sexual differentiation and can most logically be reviewed in this section of the discussion.

Early in the observations on meiosis it became apparent that we would be able to make chromosome counts of the various species and isolates with reasonable accuracy despite the rather large numbers and very small size of the chromosomes. A summary of these findings is included in Table I. Several features of particular interest are shown. The existence of polyploidy in *Euallomyces* was clearly established, with counts of 8, 16, 24?, and 32 in *Allomyces arbuscula* and 14, 28, and 56? in *A. macrogynus.* There are very few instances where polyploidy has been demonstrated by direct cytological means in the algae or fungi. Discovery that the previous genetic studies had involved crosses of *A. arbuscula* $n = 16 \times$ *A. macrogynus* $n = 28$ set us upon the trail of chromosomes in the hybrids and gave us some clues as to the mechanics whereby genes controlling gametangial arrangement were being distributed. Two quite distinct patterns of chromosome behavior were found at Meiosis I in the meiosporangia. In Type A (see Fig. 4) there are $16 + 28 = 44$ chromosomes, very few bivalents occur, and the many univalents are distributed randomly to the poles. Only about one in a thousand of the meiospores formed following such irregular chromosome distribution is viable. In Type B, on the other hand, about 55 chromosomes have been detected, approximately half of them pair, and the remainder go to the poles at random. Viability, though still low, is some thirty times higher (*ca.* 3%) than in Type A. From several correlated observations (Emerson and Wilson, 1954, pp. 413-416) we have deduced that Type B hybrids almost certainly result from a

doubling of the chromosome set contributed by *A. arbuscula* at the time of syngamy, so that there are $2 \times 16 + 28 = 60$ chromosomes present, though slightly fewer than this have actually been counted.

TABLE I. Gametangial arrangement, sex ratio, and chromosome number in natural isolates and hybrid strains of *Euallomyces*

	Name	Gametangial arrangement	♂ : ♀ Ratio	Haploid chromosome number
BASIC SPECIES	*A. arbuscula*	Pure H	0.79–1.23	8 (2 isolates) 16 (14 isolates) 24? (2 isolates) 32 (1 isolate)
	A. macrogynus	Pure E	1.13–1.33	14 (1 isolate) 28 (3 isolates) 56? (1 mutant strain)
NATURAL HYBRIDS	*A.* × *javanicus*	I (epigyny predominant)	0.61–24.0	13 (1 isolate) 14 (1 isolate) 15 (1 isolate) 17 (1 isolate) 19 (1 isolate) 21 (2 isolates)
ARTIFICIAL HYBRIDS	*A. arbuscula* × *A. macrogynus*	E, H, and I (epigyny predominant in Type A; hypogyny predominant in Type B)	0.10–1000+	20 (1 strain) 22 (1 strain) 27 (2 strains) 32 (1 strain) 36 (1 strain) 42 (2 strains) 44 (2 strains)

The wide range of sexual characters, involving gametangial arrangement as well as sex ratio, that were found in the hybrid strains can certainly be accounted for by this irregular and haphazard distribution of chromosomes at meiosis in the F1. On the other hand, we have no notion at present how many genes are concerned in determining the sexual characters of *Allomyces,* nor can we do more than guess what role polyploidy is playing in the inheritance of gametangial arrangement in our 16×28 crosses. We have found that there is a clear-cut preponderance of epigyny in the gametophytes from Type A hybrids whereas hypogyny predominates in Type B (Fig. 5). The former condition is not easy to explain but the latter seems definitely to result from doubling of the arbuscula chromosome complement which is carrying the genes for hypogyny.

Naturally the discovery that we had used polyploid parents in all of our early crosses led to the hope that a simpler, more understandable, and more meaningful pattern of inheritance might be revealed by

crosses of the apparent base numbers, *A. arbuscula* $1n = 8 \times A.$ *macrogynus* $1n = 14$. During recent months these crosses have been made in our laboratory[4] by Dr. Francis V. Ranzoni. Although the final results of genetic analyses and chromosome observations are not yet gathered together, enough information is at hand to suggest very strongly that our hopes have not been borne out. All of the evidence indicates a picture astonishingly similar in every respect to the results with the larger chromosome numbers. The same range from E through

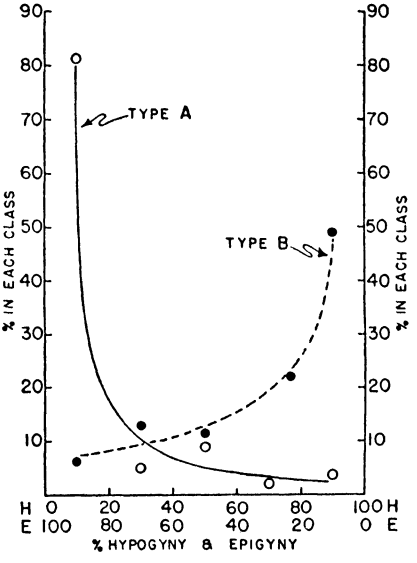

Fig. 5. A comparison of gametangial arrangement in hybrid gametophytes of *Allomyces* arising from Type A and Type B sporophytes. Epigyny is markedly predominant in the former whereas hypogyny predominates in the latter.

I to H and the same graded shifts in sex ratios have appeared. Chromosome configurations essentially like Types A and B, but with half as many chromosomes, have also been found. Apparently, whether the chromosome sets being combined are 8 and 14 or 16 and 28, enough genes are involved in the control of the sexual characters in both instances so that there is no obvious difference in the two patterns of inheritance. Possibly the numbers $1n = 8$ and $1n = 14$ are themselves polyploid, and strains of *Allomyces* with $1n = 4$ or $1n = 7$ remain to be discovered. Only future studies will tell.

Second and third filial generations have been secured by selfing the

[4] This work is being carried on with the aid of Research Grant #2149R from the National Institutes of Health.

individual F1 gametophytic strains (Fig. 4). Bearing in mind that such selfing leads to complete homozygosity in *Allomyces,* it is not surprising to find that (1) chromosome pairing is again complete and normal in the F2 and F3 meiosporangia, (2) meiospore viability returns to a comparatively high level (42%), and (3) F2 and F3 gametophytes continue to exhibit the same general features of gametangial arrangement and sex ratio that were found in the individual F1 gametophytes from which they were derived. As we had predicted from the random distribution of chromosomes in the F1 meiosis, chromosome counts made in the F2 and F3 revealed a series of numbers ranging from 20 to 44 pairs (Table I). We were unable, however, to discover any correlation between chromosome number and the arrangement of gametangia. It appears that control of maleness and femaleness must depend upon the final balance which exists in any given hybrid genotype. Or, to put it in another way, if any degree of intermediacy between pure E and pure H can be produced with as few as 20 chromosomes or as many as 40, a fairly large number of independently assorting genes must be involved in the sex-determining mechanism.

Before we leave this discussion of hybridity in *Allomyces,* one further point must be made. Intermediate strains, showing both epigyny and hypogyny, have also been produced in nature, evidently by natural hybridization. Analyses of their gametangial arrangement, sex ratios, and variable chromosome numbers, as shown in Table I, have clarified their status and permited us to group them (Emerson and Wilson, 1954) in the hybrid species *Allomyces* × *javanicus* Kniep (*arbuscula* × *macrogynus*). It is particularly interesting to recall that the first published account of sexuality in *Allomyces* was written by Kniep (1929) who, as we now know, was working with one of these hybrid strains which he had isolated from soil obtained in Java.

C. Mitochondria, carotenoids, and metabolism. Questions of sex determination and morphogenesis in *Allomyces* have intrigued quite a number of investigators ever since Kniep's papers appeared. About two decades ago Hatch (1935) published a detailed description of gametogenesis in *A. arbuscula,* and he made an interesting comment at that time regarding the possible role of mitochondria in sexual differentiation. He observed that there appeared to be a dense accumulation of mitochondria in the most apical portion of hyphal tips that were differentiating into pairs of gametangia. Thus high density of mitochondria was associated with femaleness while lower density or absence might be associated with maleness. Much more recently Ritchie and

Hazeltine (1953) have been studying the mitochondria of *Allomyces* by phase contrast microscopy and other modern techniques. So far as I know, however, no expert on mitochondria has yet availed himself of the neat opportunity afforded by *A. macrogynus:* it will be very interesting indeed to learn whether in that species there is a marked accumulation of mitochondria *just behind the hyphal tip* when gametangia are about to be formed. Our own observations sometimes suggest that the protoplasm which is differentiating to become female has a more densely granular character, but we would hesitate to say whether this results directly from differential distribution of mitochondria. In any event, the problem takes on special significance when we recall that modern biochemical studies have demonstrated the localization of tricarboxylic acid cycle enzymes in the mitochondria.

There have also been some interesting studies of carotene synthesis in *Euallomyces,* and this matter is of great interest to us because of the regular correlation between orange pigment and maleness. Turian (1952) first showed that carotenoid synthesis in the male gametangia of *Allomyces* × *javanicus* could be inhibited by the use of diphenylamine but that such inhibition interfered in no detectable manner with the subsequent differentiation, liberation, and copulation of gametes. Furthermore, the zygotes produced were entirely normal and capable of germination in the usual manner. Under these conditions visibly detectable amounts of carotene were not present but, as Turian emphasized, chemically detectable quantities certainly were. The normal functioning of male gametes from virtually colorless gametangia has long been known (Emerson and Fox, 1940), and Turian and Haxo (1954) again stressed this point following their further studies of diphenylamine inhibition of colorless as well as colored polyenes. They suggest (*op. cit.,* p. 259) that the preferential synthesis and accumulation of γ-carotene in male gametangia may merely be incidental to or a consequence of some more fundamental metabolic change more directly associated with male differentiation. They note, however, that as Emerson and Fox (1940, p. 290) pointed out, it is still quite conceivable that carotenoids in very small amounts do play some vital role in the sexual reproduction of *Allomyces.* In this connection it is significant that neither Emerson and Fox nor Turian and Haxo detected any colored polyenes in the *sporophytes* of *Euallomyces,* and the absence of colorless C_{40}-polyenes was also established by the latter investigators.

On this same matter of the occurrence and possible function of carotenoids in the sexual processes of the Blastocladiaceae, a number of

highly interesting observations have been made on *Blastocladiella.* As we have explained previously, this little organism has an unbranched hyphal system and each plant bears only one reproductive cell (Fig. 3). According to Harder and Sörgel (1938), the gametophytic plants of *B. variabilis* bear either a colorless female gametangium or an orange-colored male. Harder and Sörgel found these two kinds of plants in about equal numbers and drew the conclusion that sexuality was probably genotypically determined at meiosis. Having in mind, however, the firmly fixed hermaphroditic character of *Allomyces,* as well as the many basic points of similarity between that genus and *Blastocladiella,* I had always felt that a phenotypic sex determining system would ultimately be found to occur in *Blastocladiella.* We were able to shed some light upon this question several years ago when Cantino obtained a new isolate of *Blastocladiella* in Pennsylvania and discovered that, like *B. variabilis,* it too produced both orange, presumably male, and colorless, presumably female, plants when spores from resistant sporangia were plated out. It was possible to show (Emerson, 1950, p. 182) that swarmers from colorless plants could develop apomictically (without sexual fusion) through nine successive generations and that orange plants always appeared in the populations thus produced. These observations proved conclusively that the presence or absence of orange color was being directed not genotypically but rather phenotypically just as in *Allomyces.*

Cantino and his colleagues have pursued an intensive program of research with their *Blastocladiella* which they have named *B. emersonii.* While appreciating deeply the sentiments that engendered this nomenclature, I am forced to admit the highly irregular behavior of my namesake. Cantino and Hyatt (1953a) have reported, and I have myself repeatedly observed, the strange ineffectiveness of the orange swarmers which were presumed to be male gametes. Never have they been seen to undergo normal gametic fusions either among themselves or with the presumed female gametes from colorless plants. On the other hand Cantino and Horenstein (1954) have recently described a most unusual kind of cytoplasmic exchange occurring through temporary cytoplasmic bridges formed between colorless and orange swarmers. Under the circumstances one cannot be certain whether or not one is investigating phenomena associated with sex determination. However, Cantino and Hyatt (1953b) believe that the orange pigment in *B. emersonii* is, as in *Allomyces,* largely γ-carotene. The orange swarmers are, moreover, slightly smaller and considerably more active than the color-

less ones. Everything points to their homology with the male gametes of *Allomyces* and the orange gametes reported in *B. variabilis* by Harder and Sörgel.

Let us then, for the present, accept the idea that the genesis of carotene in *B. emersonii* is governed by a mechanism related to that which controls carotene production and maleness in *B. variabilis* and *Euallomyces*. If this is so, Cantino and his group have penetrated close to some highly fundamental aspects of sexual differentiation. The interested reader must examine their papers in detail but in its broad outlines the complex picture as they see it is this. The wild-type strain of *B. emersonii* produces only about 2% orange plants and 98% colorless plants whereas this can be shifted to a ratio of 25% orange to 75% colorless if plants are raised in a medium containing the antibiotic cycloheximide (Cantino and Hyatt, 1953a). Moreover, naturally occurring mutant strains have been found that form orange plants almost exclusively. Cell-free extracts of wild-type strains (98% colorless plants) show (Cantino and Hyatt, 1953c) marked activity of cytochrome oxidase, aconitase, isocitric dehydrogenase, α-ketoglutarate oxidase, succinoxidase, and fumarase. Mutant strains (100% orange plants) show, on the other hand, all of these enzymes except aconitase and α-ketoglutarate oxidase. Finally Cantino and Horenstein (1954, footnote, p. 153) have found many Nadi-positive granules in the wild-type plants but relatively few or none in the cytoplasm of the orange mutants. Cantino has put these various pieces of information together in a working hypothesis which shows a high degree of ingenuity and imaginativeness. It is suggested (*loc. cit.*) that: (1) the Nadi-positive particles correspond to the cytoplasmic factor gamma (first hypothesized by Cantino and Hyatt, 1953a); (2) gamma carries the α-ketoglutarate oxidase and aconitase activities but none of the other tricarboxylic acid cycle enzymes found in the wild type; (3) the relative absence of gamma in orange plants is what has caused them to differentiate toward "maleness"; (4) conversely, the presence of gamma causes "femaleness"; (5) the ratio of 98% colorless to 2% orange plants in normal wild populations derived from resistant sporangia results from random distribution of gamma particles when spores are being cleaved out; and (6) gamma particles are involved—just how is not clear—in the transference of materials across the temporary cytoplasmic bridges formed between orange and colorless swarmers. One can also infer that cycloheximide raises the percentage of orange plants in a wild population (Cantino and Hyatt, 1953a) by interfering with the normal rate of formation of gamma.

Such a scheme is highly reminiscent of the situation Ephrussi (1953) has described in yeasts. For us here, the features of similarity between *Blastocladiella* and *Euallomyces* are of special significance. The naturally occurring, highly orange mutants of *Blastocladiella* must be closely comparable to the hybrids that are 99.9% male in *Allomyces*. The shift from colorless to orange plants induced by cycloheximide treatment of *Blastocladiella* may well have a parallel in the induction of orange gametophytes by cycloheximide treatment of mitospores of *Allomyces* (see discussion of gametophytism *v.* sporophytism below). And, finally, these observations and hypotheses about cytoplasmic particles in *Blastocladiella* bring us right back to Hatch's (1935) suggestions regarding sex determination and differential distribution of mitochondria in *Allomyces arbuscula.*

Unquestionably insufficient distinction has been made between observed fact and imaginative hypothesis in many of the foregoing papers on *Blastocladiella.* The discussion and diagram (Cantino and Hyatt, 1953a, Fig. 14) of the role of meiosis in the life cycle of *B. emersonii,* for example, are based entirely upon inference since no information about meiosis in *Blastocladiella* has yet been published. Nevertheless, it would be a mistake not to recognize the inherently fundamental nature of Cantino's investigations. One of the most interesting of all biological problems is that which concerns the morphogenesis of male and female, and as the various biological studies on the genetics, metabolism, enzyme systems, pigment syntheses, and so forth, of the Blastocladiaceae continue, we can expect to come closer to a solution of this problem in clear physico-chemical terms.

V. FORMATION, MATURATION, AND GERMINATION OF RESISTANT SPORANGIA

Among the myriads of microscopic organisms that have become adapted to life in the fresh waters of the world the vast majority have evolved some mechanism for withstanding periods of climatic or environmental adversity. The protozoan forms its cyst while countless species of algae and most aquatic Phycomycetes typically undergo sexual reproduction and form a thick-walled, highly resistant zygote. So accustomed, indeed, were botanists to the concept of a sexually formed resistant stage in the water molds, that decades after the discovery of *Blastocladia* by Reinsch in 1878 students of that genus and *Allomyces* were still trying to interpret the blastocladiacious resistant sporangium as some kind of oöspore. As we have seen, however, these very characteris-

tic, brown, pitted, thick-walled structures are asexually formed in all the Blastocladiaceae and they constitute a distinctive feature of the entire order Blastocladiales. Tests have shown that they can remain alive in a dry condition for at least twenty years, and viability for considerably longer periods is very probable. Observing their highly modified structure, noting their special function, and recalling that they are the locus of the critical reduction divisions in *Allomyces,* one can immediately recognize their intrinsic interest. The information we have gained about their origin and behavior is still fragmentary but is just beginning to take shape in an integrated picture.

A. Blastocladia and the "CO_2-effect." The problem of RS formation had never concerned us particularly until we made our first pure culture isolations of *Blastocladia* (Emerson and Cantino, 1948) in 1936. *Blastocladia* is a peculiar organism in many ways. It occurs in nature growing on submerged fruits, such as rose hips, apples, grapes, etc., in very foul conditions. We were especially elated by our success in obtaining pure cultures both because they had not been previously achieved and, more particularly, because they appeared to hold the key to all further fundamental studies. Moreover, at that time no one had succeeded in germinating the RS of *Blastocladia,* nor had any sexual stage been observed in the genus. Unfortunately our hopes soon went by the board: although we had devised a standard medium and established favorable conditions for copious growth, we could never detect a single resistant sporangium in any of the pure cultures. This was all the more astonishing because *Allomyces* usually produced RS in great numbers in water cultures as well as on agar media.

For eight years the pure cultures of *Blastocladia* were a thorn in our side, requiring constant attention but yielding little information and not a single RS. We tried modifying various factors of the environment but all to no avail. Then in 1944 we hit upon the answer: *Blastocladia pringsheimii* would form RS in profusion if pure cultures were held in an atmosphere of pure CO_2 (Emerson and Cantino, 1948). This was not simply a response to anaerobic conditions because parallel cultures under tank nitrogen showed no RS at all. Moreover a high level of CO_2 is essential because no RS matured in cultures exposed to 10% CO_2 in air. Cantino (1949) subsequently showed that pH was also an important factor: in cultures exposed to a continuous flow of pure CO_2 and held at different pH values ranging from 4 to 7.6, RS were formed only between about pH 5.5 and 5.8.

By this time we had learned that *Blastocladia* is a facultative anaer-

obe, that its metabolism is the same whether it is growing in air or under nitrogen, that it forms no CO_2, and that the major products of glucose breakdown are roughly 90% lactic acid and 10% succinic acid. Because CO_2 is evolved by acids reacting with carbonates, *B. pringsheimii* would sometimes form RS when it was simply grown in the presence of calcium carbonate. Finally, it is probably also significant that the ratio of lactic acid to succinic acid was shifted from 9:1 in aerobic cultures to about 8:2 in cultures under CO_2. Cantino (1949) discussed the probability that a CO_2-fixing mechanism is involved, and later, by using radioactive CO_2, Lynch and Calvin (1952) demonstrated such a system in *B. pringsheimii* (as well as *Allomyces arbuscula*). They found most of the activity in aspartic and glutamic acids, with smaller amounts in succinic acid.

The foregoing observations were of great interest to us and appeared to point clearly to some fundamental relationship between CO_2 and the morphogenesis or initiation of RS in *Blastocladia pringsheimii*. Nevertheless, many aspects of the problem remain unsolved and we must admit that we still have not succeeded in producing and germinating a large crop of normal RS of *Blastocladia* in pure culture.

B. Blastocladiella and the biochemistry of differentiation. Further intensive work on the "CO_2-effect"—as we may call it—in the Blastocladiales has, however, been carried on by Cantino and his associates. They have used the isolate of *Blastocladiella emersonii* that we referred to previously, and again, as in the studies of sexual differentiation, they have striven to get at the biochemical foundation of morphogenesis. Like *Allomyces, Blastocladiella* ordinarily forms many RS in the usual media without any special treatment. Cantino (1951) discovered, however, that evenly scattered plants on peptone agar do not form RS whereas those in dense clusters usually do. He then observed that the same medium supplemented with 0.5 to 1.0% $CaCO_3$ or M/60 to M/45 $NaHCO_3$ induced a large majority of plants to form RS even when they were widely scattered. For reasons that are not yet clear, gaseous CO_2 was not as consistently effective: CO_2 is toxic to *Blastocladiella* and inhibits growth at levels above 5%; somewhat below this level ". . . the incidence of RS plants seldom reached 50%" (*op. cit.*, p. 332) and averaged "*ca.* 5 to 25%" (*op. cit.*, p. 350). On the other hand, extreme reduction of the atmospheric CO_2 ". . . often induced the formation of large quantities (50 to 85%) of RS plants" (*op. cit.*, p. 335) on peptone medium.

When the peptone in the medium was replaced by casein hydrolysate,

$NaHCO_3$ and $CaCO_3$ were not effective in inducing RS. The effectiveness of $CaCO_3$ as an RS inducer was regained, however, if α-ketoglutarate or citrate were provided in small amounts or if the respiratory poisons sodium arsenite or semicarbazide were added. From these and related observations Cantino (*op. cit.*, p. 361) ". . . concluded that retardation of the oxidative decarboxylation of α-ketoglutarate, and simultaneous accumulation of its precursors back to the citrate stage, is directly or indirectly related to the formation of RS."

All of the subsequent work has been directed toward elucidation and elaboration of this concept. In an interesting study reported the following year (Cantino, 1952) it was found that there are critical times at which reversals of the morphogenetic pattern cannot occur. Under the conditions employed a germling exposed to bicarbonate would develop into a plant with a mature RS in 108 hours. If, at any time prior to about 65 hours, the germling were transferred to a medium relatively free of bicarbonate, a mitosporangium would be formed, but if it were transferred at any time after 65 hours the pattern of RS formation could not be reversed. It is suggested (*op. cit.*, p. 402) that plants which are to form RS become progressively less permeable to CO_2. Thus, stimulated by bicarbonate in the medium or by metabolic CO_2 that accumulates in crowded clusters on bicarbonate-free media, the plants become less and less permeable to CO_2, the CO_2 concentration builds up, it interferes with oxidative decarboxylation of α-ketoglutarate and accelerates CO_2-fixation, and the resulting compounds finally shunt the metabolism in a direction that leads to the formation of RS.

The most recent studies (Cantino and Hyatt, 1953c) have shown that a majority of the enzymes in the tricarboxylic acid cycle, including α-ketoglutarate oxidase and aconitase, are present in cell-free extracts of *B. emersonii*. As we have noted earlier, all of these enzymes *except* α-ketoglutarate oxidase and aconitase were also found in the mutant orange strains. The point of importance here is that the mutant strains are incapable of forming RS even in the presence of bicarbonate. The conclusion is drawn (*op. cit.*, p. 719) that "the mutant has lost the enzymes which, in the normal strain, respond to the accumulation of metabolic CO_2 and thus impose a limitation on growth by bringing about differentiation," i.e., the formation of RS. Apparently carbonate did not inhibit the *in vitro* activity of α-ketoglutarate oxidase although arsenite and semicarbazide did.

A criticism that has been raised regarding Cantino's "CO_2-hypothesis" (oral comments in the symposium on "Physico-chemical Control

of Growth and Differentiation," at the meetings of the American Association for the Advancement of Science, December 28, 1954) is the apparent failure to rule out pH effects in most of the investigations of bicarbonate action. According to theory it would be impossible to alter the bicarbonate without simultaneously altering the pH if the composition of the atmosphere remains constant. Like the studies on sex determination, these analyses of RS formation in *Blastocladiella* also suffer through a failure to draw sharp lines between fact and hypothesis. It is to be hoped that by careful repetition, sound interpretation, and lucid description this pioneering and imaginative work on *Blastocladiella* can be placed on an unassailable basis.

C. Allomyces and RS cytology. As yet no biochemical work has been done on the mechanisms that lead to RS formation in *Euallomyces.* On the other hand we have very precise information about the cytology of the RS. We know, for example, that they are the site of meiosis. We have also discovered and investigated in some detail an interesting sequence of very regular and conspicuous cytoplasmic changes that take place during RS development, maturation, and final germination (Wilson, 1952).

When young resistant sporangia are first formed at the hyphal tips their protoplasm is evenly granular and similar to that of the vegetative hyphae. Within about 48 hours, however, aceto-orcein smears reveal many spherical, deeply staining bodies that have appeared quite suddenly and form a conspicuous part of the cytoplasm. They are one to several microns in diameter, and we have called them *chromospheres* because of their shape and staining properties. They persist in the resistant sporangia during the entire period of maturation, which lasts from one or two to many weeks depending upon the isolate concerned and the conditions of culture. Then, very suddenly, probably in less than 24 hours, all the chromospheres are disintegrated and disappear. Simultaneously the expanded diploid nuclei are revealed and prove to be in an advanced prophase of Meiosis I. If the RS are air-dried at this time they can remain viable for at least twenty years with meiosis arrested in prophase (Wilson, 1952). If, however, they are placed in water as soon as the chromospheres have disappeared, they will usually germinate. So regular is this cycle of chromosphere appearance, RS maturation, chromosphere disappearance, and RS germinability that aceto-orcein smears can be used to determine rapidly and quite accurately the stage of development that has been attained.

Machlis and Ossia (1953a) followed these initial investigations with

a much more detailed and precise study of the features of RS maturation. They were able to reduce the maturation time by more than two thirds by using liquid shake-cultures instead of agar media. Even more striking, they succeeded in cutting the time from three weeks to about two days in one instance. The plants were grown in synthetic broth and transferred to non-nutrient liquid; this treatment yielded a crop of RS that could be germinated 48 hours later. Machlis and Ossia also verified the general relation between chromosphere disappearance and germinability but showed that a second factor was involved. This was evidently not osmotic pressure, which remained relatively constant in liquid culture at about 39 atmospheres throughout the time when maturation was being completed and germinability was reached.

In a second paper Machlis and Ossia (1953b) presented data showing that indoleacetic acid appreciably reduces the time for chromosphere disappearance and raises the percentage of RS which become capable of germination. Indolebutyric acid and tryptophan were also active but indole was not. The suggestion is made that the growth substances affect germinability by bringing about certain alterations in the RS wall. These changes, which can sometimes be hastened by desiccation, presumably allow the available, strong, osmotic forces to become operative through sudden cracking of the heavy wall and swelling of the protoplasmic contents. The dramatic rapidity of the action is vividly shown in time-lapse cinemicrography (cf. Plate V, 1 to 4).

TABLE II. An approximate timetable of meiosis and meiosporangium (RS) germination in an isolate of *Allomyces macrogynus*

Time, minutes	*Cytology*	*Sporangial behavior*
0	Meiosis I, prophase	Dormant
20-40	——	Swelling & cracking
40-70	Meiosis I, metaphase	Papillae forming
70-100	Meiosis II	Papillae fully formed
100-130	Post-meiotic chromospheres and cleavage	Spore discharge

One of the most interesting features of the actual germination or dehiscence of RS in *Allomyces* is the exact timing of the sequence of steps that occurs (Table II). Because each RS as it reaches maturity and germinability enters a state of suspended activity at late prophase of Meiosis I, a very uniform crop of RS can be produced by growing

standard strains under well controlled conditions. The simultaneous reactivation of an entire batch of RS can then be initiated at will simply by placing them in water. Thereafter, with a little practice, one can predict quite precisely just what stage has been reached in the meiotic divisions and the subsequent cleavage of the meiospores. This predictability and ease of handling will be extremely useful in any analyses of the physiological changes that accompany meiosis or any investigations of the effects of environmental factors on this fundamental process.

During the last 20 minutes of RS dehiscence, when both meiotic divisions have been completed, but shortly before the spores are being cleaved out, aceto-orcein smears again reveal momentarily spherical masses of deeply staining material in the cytoplasm. These have been called (Wilson, 1952) post-meiotic chromospheres, to distinguish them from the pre-meiotic chromospheres that are found during the maturation period. They appear to coalesce and form the nuclear caps which are so conspicuous in the spores of the Blastocladiales (Fig. 1). We still have no knowledge of the chemical nature of pre- or post-meiotic chromospheres or of the nuclear caps. Nor can we say anything of their respective functions. The precision of their appearance and disappearance and the regularity of their association with meiosis and the motile stages of *Allomyces* mark them as distinctive cytochemical features with morphogenetic roles of undoubted significance.

In summary, it can be said that we know a good deal about the formation, maturation, and germination of RS in the Blastocladiales. But taken genus by genus our knowledge is still very spotty and we must resist strong temptations to draw too obvious analogies. Meiosis must be studied in *Blastocladiella,* the effects of bicarbonate must be investigated in *Allomyces,* and the whole reproductive cycle must be worked out in *Blastocladia.* When these advances have been made it will be time indeed for a real synthesis.

VI. SPOROPHYTISM VERSUS GAMETOPHYTISM

One final item merits careful scrutiny, namely the mechanism that controls the alternation of generations. In some respects this alternation is the most interesting of all morphogenetic phenomena in plants. Ever since the days of Hofmeister botanists have been fascinated by the problem posed by the regularly alternating haploid and diploid phases of most plant species. Virtually all of the work has been done with green plants, more particularly bryophytes and ferns (see Bower, 1935; Allen, 1935; Burgeff, 1943; Manton, 1950), and in these studies the conclu-

sion has repeatedly been reached that it is not the chromosome number *per se* that determines sporophytism and gametophytism. Darlington and Mather (1949, p. 189) have expressed the matter as follows: "The power of the cytoplasm in guiding the course of events is very well shown by the alternation of gametophyte and sporophyte in the mosses, ferns and higher plants. Normally the one is haploid and the other diploid. But this momentous difference does not decide the line which development will take in the haploid spore or the diploid egg [i.e., zygote]. For when the spore happens to be diploid (through the failure of meiosis) or the egg happens to be haploid (through the failure of fertilization) the alternation of generations follows its regular sequence undisturbed." Well and good, perhaps, for these higher plants, but there seems to be a firmer tie between ploidy and the alternation of generations in the more primitive thallophytes. We shall see shortly, for example, that a very strict relationship holds in *Allomyces* as far as we have been able to determine. Indeed the statement made by Darlington and Mather (*op. cit.*, p. 190) in the very next paragraph of their discussion fits the facts in *Allomyces* more closely even though it would seem to contradict the quotation presented just above. According to this more plausible suggestion, development is controlled by an interaction between nucleus and cytoplasm. "In each pollen grain, or embryo sac or fertilized egg a nucleus of a new type produced by meiosis or fertilization is suddenly implanted in a cytoplasm accustomed to, associated with, or generated by, a previous and different nucleus." In other words it is the occurrence of a haploid nucleus in "diploid cytoplasm" (in the meiospore for example) that ordinarily initiates gametophytism and a diploid nucleus in "haploid cytoplasm" (in the zygote) that sets off sporophytism.

In fungi, as a rule, the true diploid phase is of extremely limited duration; the reduction divisions directly follow karyogamy except in a few of the yeasts. It is not surprising, therefore, that mycological contributions to the problem were relatively insignificant until the full life cycle of *Euallomyces* had been worked out. This water mold now appears to offer some of the best possible material for experimental investigation of the mechanisms underlying the alternating gametophytic and sporophytic phases.

A. Comparative nutrition. In many plants, all seed plants for example, the morphological and physiological differences between gametophyte and sporophyte are very marked. In other instances, such as the ferns perhaps, there would appear to be more physiological similarity,

and in the isogenerate algae the haploid and diploid phases are astonishingly similar. *Allomyces* certainly falls in this last category. In working with it one is constantly impressed by the detailed similarities between the sporophyte and gametophyte. The vegetative thalli are morphologically indistinguishable from the earliest germling stage until just before maturity. Even the reproductive cells, different as they are in function and behavior, all have the same basic architecture: mitosporangia, gametangia, and meiosporangia all release swarmers by the deliquescence of identical discharge papillae; mitospores, meiospores, female gametes and male gametes all show the same design of a single posterior flagellum (Plate VIII), nuclear cap, etc.

By comparison, the differences between sporophyte and gametophyte, basic and important as they obviously are, seem very minor in degree. Perhaps the nicest outward evidence becomes expressed in the pigmentary differences, the γ-carotene we use to distinguish the gametophyte and the melanoid RS wall that marks the sporophyte (Plate I). Perhaps, too, in searching for an explanation of the alternation of phases, we are fortunate that the differences appear so clear and relatively simple. We would like to know, then, just where in the ontogeny of gametophyte and sporophyte their respective physiologies depart from a common path. One obvious approach to this question has concerned nutrition. The various main aspects of nutrition of the sporophyte have been very fully explored (Quantz, 1943; Machlis, 1953a, b, and c; Ingraham and Emerson, 1954; Sistrom and Machlis, 1955). Machlis and Crasemann (personal communication) have recently completed a parallel study of the gametophyte, and they have found that the nutritional requirements and characteristics of the gametophyte agree with those of the sporophyte in every essential. Apparently then, the entire single set of genes in the haploid chromosome complement orders the nutritional needs exactly as the entire double set of these same genes does in the diploid. It would be a mistake to assume, however, that precisely the same actual genes are necessarily involved in the two situations. That is to say, if genes A, B, and C are concerned in the thiamine requirement of the gametophyte, for example, genes MM, PP, and RR (rather than AA, BB, and CC) *may* have the same function in the sporophyte.

We also have a good preliminary understanding (Ingraham and Emerson, 1954) of glucose metabolism by sporophytic mycelia of *Allomyces*. Comparative data on the gametophyte are still to be obtained, but general observations certainly suggest that the basic metabolic ma-

chinery of the two phases will be closely similar. Somewhere along the way, of course, striking and important deviations must occur. It is the discovery and understanding of these first deviations that should provide the key to our analysis of gametophytic *v.* sporophytic differentiation.

B. Plasticity of the Euallomyces pattern. Talk as we will of precise mechanisms and rigidly controlled conditions, to many of us the abiding fascination of biology comes from its very foibles, its delightful surprises, its apparent determination to defy or negate each newly discovered physico-chemical application. The Blastocladiales are no different from most other living things in this respect, and it would be unfortunate, wholly erroneous in fact, to gain the impression that the reproductive cycle depicted in Fig. 2 is an inescapable certainty. The versatility of *Allomyces* has sometimes angered us, often humiliated us, and always challenged us.

The first of the many possible deviations from the general pattern was discovered by Kniep (1929) when he was working out the life cycle of *Allomyces*. He found that isolated female gametes of *A.* × *javanicus* would occasionally develop parthenogenetically into new hermaphroditic, gametophytic plants. Kniep was especially interested in this phenomenon because he considered it one of the most convincing examples to illustrate the latent maleness in female gametes. Kniep's observations on parthenogenesis have been repeatedly confirmed (Sörgel, 1937; Emerson, 1941), but Sörgel (*op. cit.*) first demonstrated that parthenogenetic development may also not infrequently lead to the formation of *sporophytic* plants. We have found that this type of deviation from the regular life cycle occurs readily in several strains of *A. arbuscula* (Emerson, 1941) and that it accounts for the occurrence of pure hypogyny in all the F_1 gametophytes obtained from certain putative hybrids in the genetic studies (Emerson and Wilson, 1954). Naturally, for many years we were concerned as to the chromosome behavior in such instances. Through the efforts of Dr. A. S. EL-Ani we were finally able to obtain cytological preparations of meiotic figures in RS formed on a parthenogenetically produced sporophyte (*op. cit.*, p. 426). The normal diploid chromosome complement of 16 pairs was found and we have concluded that doubling of the chromosome set probably occurs following the first chromosome division in the apomictic female gamete.

Another deviation that has long been known in the Blastocladiales (Sörgel, 1937; Blackwell, 1940; Emerson, 1941; Couch and Whiffen, 1942) involves the formation of sporophytes directly by spores from

RS. This by-passing of the gametophyte occurs very extensively in certain strains of *Allomyces,* and Emerson (1941) established a special subgenus, *Brachyallomyces,* to accommodate isolates in which the short-cycling appears to be a fixed character. We might suppose that in these instances meiosis occurs normally in the RS and is then immediately followed by chromosome doubling in the germinating meiospore (just as in parthenogenetic female gametes). Such peculiar chromosome behavior would not be particularly unusual in the fungi (cf. Olive, 1953). Wilson (1952) has found, however, that the adjustment occurs in quite another fashion: it results from failure of chromosome pairing in the RS so that the two nuclear divisions at RS germination are mitotic, rather than meiotic, and the full diploid number is maintained. Hence, in *Brachyallomyces* we can say that mitospores are produced in the RS as well as in ordinary mitosporangia.

What inhibits meiosis in the short-cycle forms? If we were able to answer this question we would know a great deal about the fundamental difference between mitosis and meiosis, and we might even find an explanation of gametophytism and sporophytism. These speculations are not entirely idle because *Allomyces* provides a full array of types ranging all the way from those that are strictly long-cycle to those that are always short-cycle. In between are some most interesting isolates which I have facetiously referred to as "schizophrenics." When the RS of these intermediate types are germinated one sometimes gets a good population of gametophytes (meiosis); at other times a good population of sporophytes (mitosis); and at still other times a very poor *mixed* population ("schizophrenia"). It seems quite clear, from unpublished work of C. M. Wilson, that the third condition results from nuclear divisions most of which are neither mitotic nor meiotic. Unmistakable evidences of erratic and incomplete pairing have been found in aceto-orcein preparations of such cultures. This type of chromosome behavior coupled with greatly reduced viability is reminiscent of meiosis in the interspecific hybrids. There, however, the poor pairing resulted from nonhomology of the two chromosome sets, whereas here it must surely be related to malfunctioning of the mechanism which comes into play when mitosis is to be replaced by meiosis and the haploid gametophyte is to begin. We are confident that it will be possible to define the environmental conditions favoring either mitosis or meiosis in these instances, and such observations may contribute to our understanding of factors operative prior to and during the all-important reductional divisions. It is interesting in this connection to note that Hatch and Jones (1944)

concluded from their experiments on *Allomyces arbuscula* that RS dried for longer periods and germinated in weak nutrient were more likely to give gametophytes, whereas those dried for short periods and germinated in more concentrated nutrients usually gave sporophytes. In most fresh-water algae and fungi the resistant stage is the site of meiosis (cf. Emerson, 1950). There appears to be some fundamental and widespread tie-in between the physiology of dormant resistant structures and the factors that induce chromosome pairing throughout this great assemblage of primitive plants.

A third deviation from the normal cycle of *Euallomyces* (Fig. 2) is one that has baffled us for more than twenty years. Not infrequently, both in liquid and agar cultures, resistant sporangia have appeared on otherwise normal gametophytes (Hatch, 1935, 1944; Sörgel, 1936; Emerson, 1941, p. 104). Hatch (1944) has stated positively that the spores from these RS give rise to gametophytes. There are, however, obvious difficulties in gathering and germinating enough RS of known gametophytic origin to make cytological studies. We do not know, therefore, whether they are haploid or diploid and whether their nuclei undergo mitosis or meiosis at germination. In any event the combination, on one thallus, of orange male gametangia—the *sine qua non* of gametophytism—and resistant sporangia—the *sine qua non* of sporophytism—certainly puts the entire problem in a nutshell. Sörgel (1936) concluded many years ago that such plants contained a mixed population of nuclei of different sizes which he believed were haploid and diploid. His observations were not supported by chromosome counts.

It is fitting that we should conclude these remarks on sporophytism and gametophytism in *Euallomyces* with a brief account of Whiffen's (1951) recent experiments with cycloheximide. She first found that small amounts of this drug, incorporated into the nutrient medium, caused sporophytic mycelia to shift over to gametophytic growth and start producing typical, paired, gametangia. Subsequently, by subjecting diploid mitospores to cycloheximide, she was able to induce a small percentage of them to develop directly into gametophytes. Now nearly everything we have said here about the alternation of generations in *Allomyces* links gametophytism with haploidy and sporophytism with diploidy. The polyploid series cast some doubt on this interpretation, and the occurrence of resistant sporangia on gametophytes is also hard to fit into the scheme. Otherwise most of the observations are in line. It is not surprising, therefore, that Whiffen (*op. cit.*) concluded her observations with the comment that cycloheximide probably exerted its

effect by causing some sort of somatic meiosis which reduced the chromosome number to the haploid condition characteristic of the gametophytic phase.

Admittedly, Whiffen's proposal looks like the most plausible one. As we have pointed out, however, the work on ferns has shown very clearly that chromosome number alone does *not* determine gametophytism there. Moreover we have a deep seated feeling that it should be possible to alter the metabolic machinery of *Allomyces* at will in such a way as to cause sporophytism or gametophytism regardless of diploidy or haploidy. So, we argued, perhaps cycloheximide is the very thing we are after: if it induces diploids to become gametophytes without a change in chromosome number, then these plants when selfed will give tetraploid sporophytes and the tetraploid number should be revealed at the next meiosis. We have repeated Whiffen's experiments, therefore, using mitospores of another strain of *A. arbuscula,* and like her we have obtained a small percentage of gametophytes in the population subjected to cycloheximide. These plants have been grown and selfed, the resulting sporophytes have produced mature RS, and the RS have been germinated. Dr. F. V. Ranzoni has recently made the first preliminary observations[5] of meiosis in these RS and they appear to have the normal diploid complement of chromosomes. It would appear, therefore, that Whiffen's hypothesis is sound and that cycloheximide exerts its effect by causing chromosome reduction. Nevertheless, we shall continue in our search for means to alter the phases of the life cycle independently of chromosome number. Eventually it should be possible to break the close tie between haploidy and gametophytism, diploidy and sporophytism in *Euallomyces.*

C. Predominant diploidy in Cystogenes. The wealth of diversity in the lower Phycomycetes is truly prodigal, for even within the genus *Allomyces* something quite different from *Euallomyces* or *Brachyallomyces* has evolved. In the subgenus *Cystogenes* (Emerson, 1938, 1941) are to be found plants so like the sporophytes of *Euallomyces* in every respect that they can scarcely be distinguished in any manner. Here again these plants are diploid and meiosis occurs in the germinating resistant sporangia (Wilson, 1952). But at that point nearly all similarity ends: there is no separate, independent, vegetative, gametophytic plant whatsoever in *Cystogenes.* Spores from the RS encyst and within about an hour each cyst forms and releases four small, colorless isogametes (McCranie, 1942; Teter, 1944). Syngamy occurs at once and

[5] See footnote 4 of this paper.

the zygote then develops into a new diploid plant like the parent. There is only one mitotic division of haploid nuclei in this entire life cycle (Wilson, 1952). If *Euallomyces* is a "truly different fungus" because it is half diploid, *Cystogenes* is still more unusual because it is virtually all diploid like the brown alga *Fucus* as well as nearly all animals.

We have little information thus far about the physiology of the cyst-forming species of *Allomyces,* but they have the same nutritional requirements as *Euallomyces* (Ingraham and Emerson, 1954) and one can predict that they will show many other close similarities despite their radical morphological deviation in the haploid phase. Even as far back as 1940 it had been shown (Emerson and Fox, 1940), for example, that *Allomyces moniliformis,* a species in the subgenus *Cystogenes,* produces carotenoid pigment. Three aspects of this observation now assume special interest: (1) the pigment was identified as γ-carotene, (2) it occurs throughout the diploid mycelium (whereas that of *Euallomyces* is in the haploid plants only and appears to be restricted to the male gametangia), and (3) its presence has not been detected at all in *Allomyces cystogenus,* the other cyst-forming species. For the present, the role of γ-carotene in *Cystogenes* remains as obscure as does its function in *Euallomyces.* In the meanwhile cystogenes-type life cycles have been discovered in other genera of the Blastocladiales including *Blastocladiella* (e.g., *B. cystogena,* Couch and Whiffen, 1942) so that a wealth of opportunity lies before us.

VII. BY WAY OF CONCLUSION

We have endeavored to show how some of the water molds grow and develop, how their sexual and non-sexual reproductive structures arise, and how, through mitosis and meiosis, haploidy and diploidy play their regulated roles. Our investigations along these lines have led us into nearly every major field of biology. We have tried many doorways and opened a few upon broad vistas. Others remain obdurately shut, and among these must be counted our repeated failures to induce clear-cut, single-gene mutations. Whether because of polyploidy or other special characteristics, *Allomyces* appears to be an exceptionally stable organism. Certainly it does not sector frequently in laboratory culture as do so many other fungi. When we have suitable mutants in *Euallomyces* we shall be able to compare gene action in the haploid and diploid phases and to investigate dominance effects in a truly diploid filamentous fungus. At some future time the interactions between genes and cytoplasm that control each feature of development and growth will be

fully revealed. Only then shall we be able to say that we know the biology of these water molds: by then they will have told us much about life itself.

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IX. STUDIES ON THE ORGANIZER CENTER OF THE EARLY CHICK EMBRYO

BY NELSON T. SPRATT, JR.[1]

ONE of the most puzzling and interesting problems with which the student of developmental physiology is confronted is that of how the diverse cell types of an adult organism arise from a single, uninucleate reproductive unit such as a fertilized egg. One rather obvious way in which the different cell types could be thought of as arising during cell division or cleavage of the reproductive unit would be through separation of preexisting, localized, cytoplasmically different regions by the planes of cleavage. Another way in which two or more presumably identical cells could be thought of as becoming diversified would be through the interaction of these cells with different environments, i.e., with agents external to them such as another group of different cells, different concentrations of their own products or those of other cells, etc.

Regardless of the particular type of cellular differentiation mechanism that might be operating (see the recent review of Spratt, 1954) the preeminent feature of the entire developmental process from egg to adult is its orderliness and its remarkable resistance to externally imposed attempts to change or disrupt this orderliness without killing the system. In other words, there seems to be some over-all control of the diversification and formative processes going on in each part of the developing organism (Whitman, 1888 and Wright, 1953). The nature and mode of operation of this coordinating or integrating principle, operating within, not externally to, the developmental system, is thus another equally important and interesting problem for the student of development.

One of the striking features of developmental systems is that cellular differentiation does not, as a rule, occur all over or throughout the system at once but seems to start at a center and, so to speak, radiate out in one or more directions through the germ. Furthermore, the center appears to *control* as well as initiate the cellular transformations. One of the most thoroughly studied examples of such a developmental center

[1] Department of Zoology, the University of Minnesota. Many of the studies reported here have been supported in part by grants from The National Institutes of Health, U. S. Public Health Service; The Office of Naval Research; and the Graduate School, University of Minnesota. This assistance is sincerely appreciated.

is the apical meristem in plants, a relatively small population of cells at the growing tip of the shoot. From this center all of the plant organs: stem, leaves, buds, flowers, etc. are progressively formed as the plant elongates. Experimental analysis of this center has revealed that it controls the differentiation and arrangement of the various tissues which arise from it while itself remaining totipotent and relatively autonomous (Wardlaw, 1952). In vertebrate embryos, there is also a somewhat comparable center located during gastrulation in the dorsal lip of the blastopore (node of primitive streak types). This is the famous "organizer" region of Spemann (1938). At later stages the developmental center is located in the tail-bud and it is through its continued activity that axial organ formation and elongation of the embryo body occur (Spratt, 1947a, Gaertner, 1949, Spratt, 1953, and Fig. 3).

Fig. 1 is designed to emphasize the diversity and wide-spread occurrence of developmental centers. Although in all the examples illustrated the centers have one common feature, that they are centers of great developmental activity, some of them probably differ greatly in their roles as centers of cellular differentiation and as "organizer" or control centers. A detailed comparison of the known activities and properties of these diverse types of centers would be interesting and, perhaps, enlightening but this is beyond the scope of the present paper. For our purposes Fig. 1 has been presented to show the relationship of the more specific problem of the nature of the node organizer center of the chick embryo to the more general problem of the role of developmental centers. It was with this background and with the general problem of the nature of cellular differentiation as a stimulus that a series of miscellaneous studies on the node center were begun about three years ago.

The actual purpose of the studies, many of which are still in progress, is to discover those properties of the node center area, and of the cells which comprise it, which distinguish it from other areas of the early blastoderm. It is hoped that in a study of its tissue organization, cytology and cytochemistry, cellular movements, regulatory and organizer (interactive) properties, nutritional and other environmental requirements, and of its enzymatic activities some of the *developmentally important properties* of the node center might be discovered and recognized as such. At least, this is the rationale behind the present program of experiments. Only the general conclusions of the numerous studies are described below—the details will be published in separate papers.

The histogenetic properties of the node center area which distinguish

it from all other areas of the early blastoderm have been demonstrated by the grafting of pieces of the blastoderm to the chorio-allantois of host embryos. Beginning with the excellent studies of Willier and Rawles (1929, 1931, 1935), Hunt (1931), and Rawles (1936), many workers have shown that the node area is clearly a center of tissue and organ

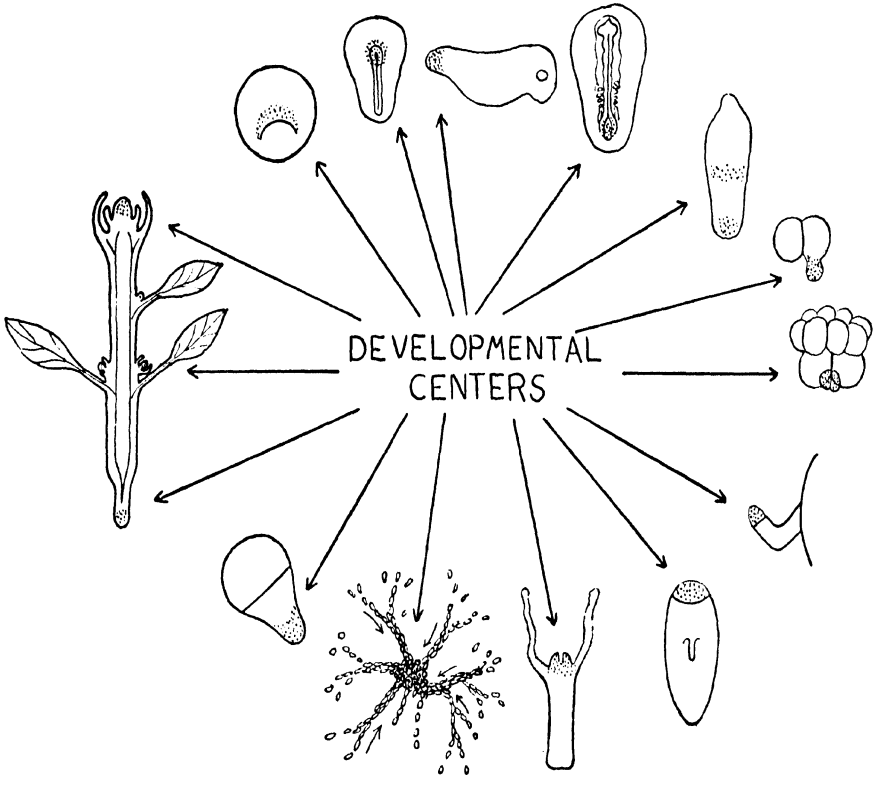

Fig. 1. Diverse types of developmental centers (stippled regions in the diagrams). Beginning at the left with the root, bud and shoot apical centers of the plant, and reading clockwise, the following centers are illustrated: dorsal blastopore lip of the amphibian embryo, node of the chick embryo, tail-bud of the amphibian embryo, tail-bud of the chick embryo, differentiation and activation (Bildungszentrum) centers of the insect egg, polar lobe of mollusc embryo, micromeres of sea urchin embryo, blastema of regenerating urodele limb, blastema of planarian, oral cone of Hydra, aggregation center of slime mold and rhizoid of Fucus egg.

formation (see the review of Rudnick, 1944). Fig. 2, a summary of the results of grafting experiments, strikingly illustrates this fact. The multiplicity of tissue forming potencies of this area is shared with no other region and, furthermore, it is from this area alone that most of these tissue types arise during the course of normal development.

I. THE NODE AS A CENTER OF AXIAL ORGAN FORMATION

The node also differs from other areas of the early embryo in being a center out of which axial organs such as the notochord, spinal cord, somites, mesonephros, gonad rudiments, etc. are progressively formed in a head to tail direction as the node moves posteriorly (Peebles, 1898, Wetzel, 1929, Hunt, 1931, Spratt, 1947a, 1952b, and 1953, etc.). The general relation of the regressing node to the elongating axial parts is

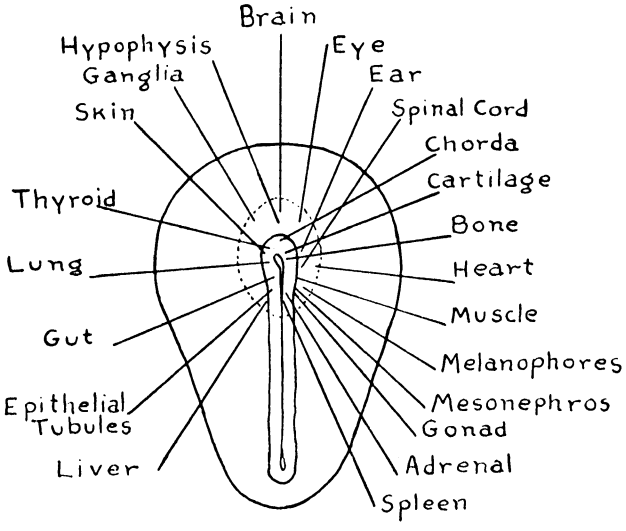

Fig. 2. Tissue and organ forming capacities of the node area of the definitive primitive streak chick blastoderm. No other region of the blastoderm possesses such a wide array of differentiation potencies and many of these are peculiar to the cells of the node center area.

shown in the diagram of Fig. 3. Note that elongation of the embryo body appears to occur between two originally very close, carbon-marked points on the surface of the epiblast. The figure poses a number of problems: Would elongation of mesodermal axial organs (e.g., chorda and somite rows) likewise occur between two correspondingly close points in the mesodermal (and endodermal) layer of the embryo? What is the source of cells that are used for axial organ formation and elongation? Is there a special center of cell division activity in the node comparable to that found in the shoot or root apices of plants? Would a study of the detailed cellular and tissue organization of the moving node area provide some clues as to its axial organ forming mechanism?

A solution to these problems was first approached through a series of

studies on the localization and general organization of the mesodermal components of the node area of definitive streak and early somite blastoderms (Spratt, 1955a). The various procedures which have been used include carbon, carmine, and vital dye marking of explanted and *in ovo* blastoderms; transverse, longitudinal and diagonal transections of explanted blastoderms; excision of small groups of cells from the node area of explanted blastoderms; explantation of the isolated node area; localization of reducing enzyme activities with neotetrazolium chloride; etc.

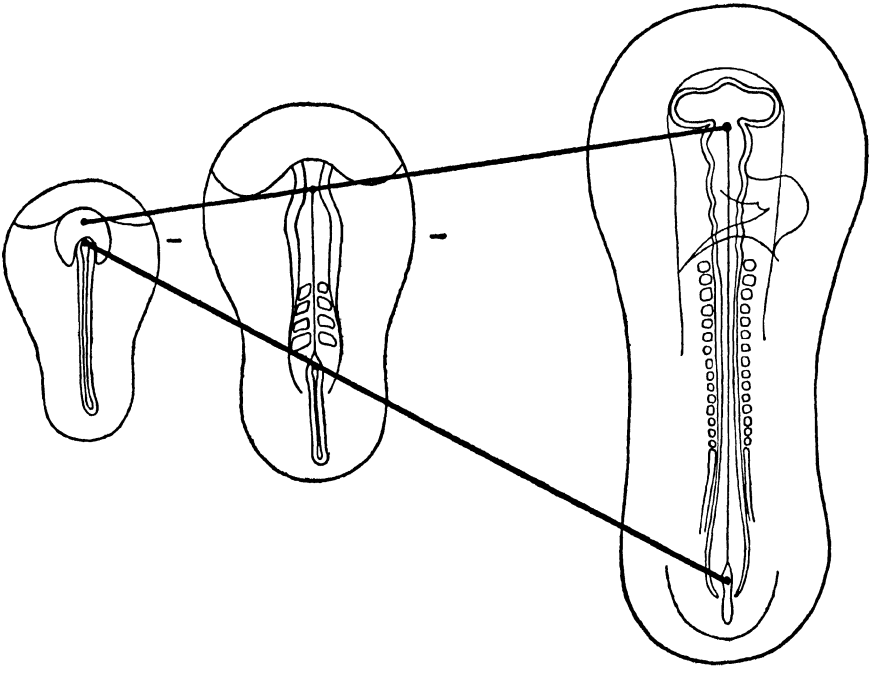

Fig. 3. Diagram showing the general relationship of the regressing node center to the elongating axial parts of the chick embryo. The heavy lines and dots illustrate the changing positions of carbon marked epiblast (ectoderm) cells. Similar movements in cells of the mesoblast and endoblast are described in the text.

The results obtained with these various methods agree in demonstrating the existence of a relatively small group of chorda center (organizer) cells localized within the node and two groups of somite center cells just postero-lateral to the primitive pit of the node as distinct from larger groups of prospective notochord and somite cells posterior to the node. This and other important distinctions between the components of the axial, mesodermal areas are shown in the provisional map of Fig. 4.

Continued formation of notochord and somites in explanted pieces of the blastoderm depends upon the presence of the centers as well as upon the corresponding prospective cells lying posterior to them (Figs. 4, 5, and 10). Complete excision of the centers results in cessation of chorda and/or somite formation (Fig. 10), but incomplete removal at these and earlier stages may be followed by complete regulation and uninterrupted axial organ development (Fig. 10).

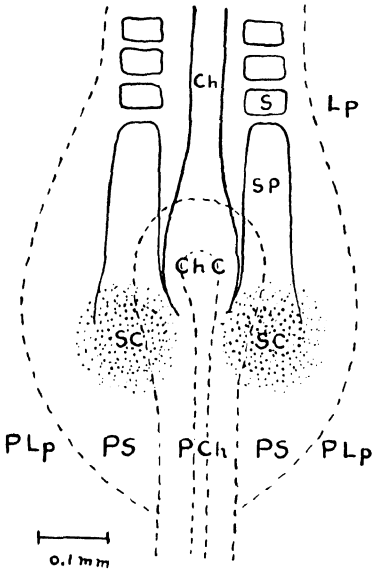

Fig. 4. Provisional diagram of the node center area of an early somite blastoderm showing primarily the organization and localization of the mesodermal components: ch = notochord, S = somite, Lp = lateral plate, SP = segmental plate, ChC = chorda forming center (chorda bulb), SC = somite forming (or segmental plate forming) center, PLp = prospective lateral plate area, PS = prospective somite area, PCh = prospective notochord cells. The approximate positions of the epiblastic portion of the primitive streak and the overlying neural plate (rhomboidal sinus) are outlined by broken lines.

The isolated node area, including the chorda and somite centers plus some of the remaining prospective axial mesoderm, shows remarkable powers of autonomous notochord, somite and neural tube formation when explanted *in vitro*. This occurs in pieces ranging in size from 0.5 × 0.5mm to 0.3 × 0.3mm (Fig. 10).

During regression, the epiblastic portion of the node, along with part of the posterior neural plate, moves posteriorly relative to the ventral and originally more posterior chorda and somite forming cells in the mesoblast. Some (most?) of the chorda and somite center cells regress

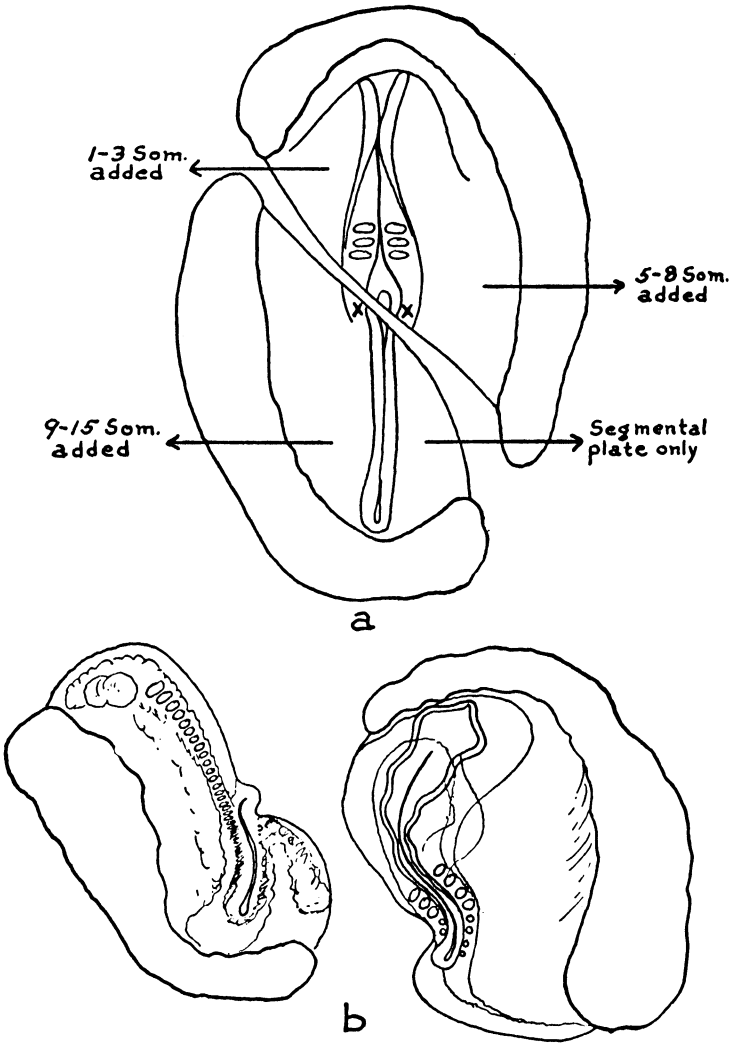

Fig. 5. Summary of results obtained by diagonal transection of the blastoderm through the node center. Positions of the somite centers are denoted by the X's. a and b are camera lucida drawings of a representative case. Note that the presence of the somite center is necessary for continued somite formation (left side of posterior isolate) but that a somite center deprived of its prospective somite cells (right side of anterior isolate) is not able to continue somite formation very long.

along with the node epiblast, leaving behind the differentiated notochord and somites. These observations which are suggestive of a mechanism of notochord and somite formation will be more fully described below.

It appears that cells of the prospective, axial mesodermal area pos-

terior to the node differ among one another in their prospective fates only by virtue of their different positions within the area and realize their prospective fates only by interacting with cells of their respective "organizer centers" in the node area. We shall presently see that this is indeed the case.

II. MODE OF AXIAL ORGAN FORMATION

In the search for an answer to the question regarding the source of cells for axial organ formation and elongation, several procedures have been employed (Spratt, 1955b). These include: (a) a cytological study of the living node area, (b) carbon and carmine marking of the node area mesoderm, (c) removal of prospective notochord and somite cells, and (d) isolation or separation of the chorda and somite centers from their differentiation products. The major findings to date are listed below:

1. Cytological studies show that the chorda center is a morphological structure having the form of a bulb-like enlargement of the proximal (nodal) end of the notochord (Fig. 4), located below the primitive pit of the node. Cells within the chorda bulb have the appearance of notochord cells but prospective chorda cells, even those just posterior to the bulb, are of a rather generalized ("undifferentiated") type indistinguishable from the remainder of the mesoderm cells posterior and postero-lateral to the node. Furthermore, the chorda bulb cells have a greater dehydrogenase activity (as revealed by the reduction of neotetrazolium chloride, Spratt, 1955a) and this drops sharply to a much lower level at the posterior border of the bulb.

2. Elongation of the notochord is accomplished to a certain extent by over-all increase in the number of notochord cells by cell division. Results of the marking and other experiments, however, have shown that the region of maximum elongation is in the notochord just anterior to the bulb and that elongation of this region is accomplished mainly by addition of cells originally posterior to the chorda bulb. Cells arising by division of the chorda bulb cells also contribute to notochord elongation but the results of the marking experiments which constitute our evidence for this latter mode of chorda formation are insufficient to establish its significance or relative role as a notochord elongation mechanism. Fig. 6 is a summary of the results of the marking experiments.

3. Apparently most, but not all, of the cells constituting the chorda bulb undergo an actual regression in space which is much faster than the general regression of the post-nodal mesoderm. This results in its

by-passing not only more lateral regions, but in its approaching, "catching up with," and then by-passing more posteriorly located median regions as well (Fig. 6).

4. Surprising as these results are, no other interpretation seems possible than that the chorda bulb consists of a special, relatively permanent cell population capable of picking up prospective (visibly unspecified) notochord cells, transforming (or organizing) these into specific noto-

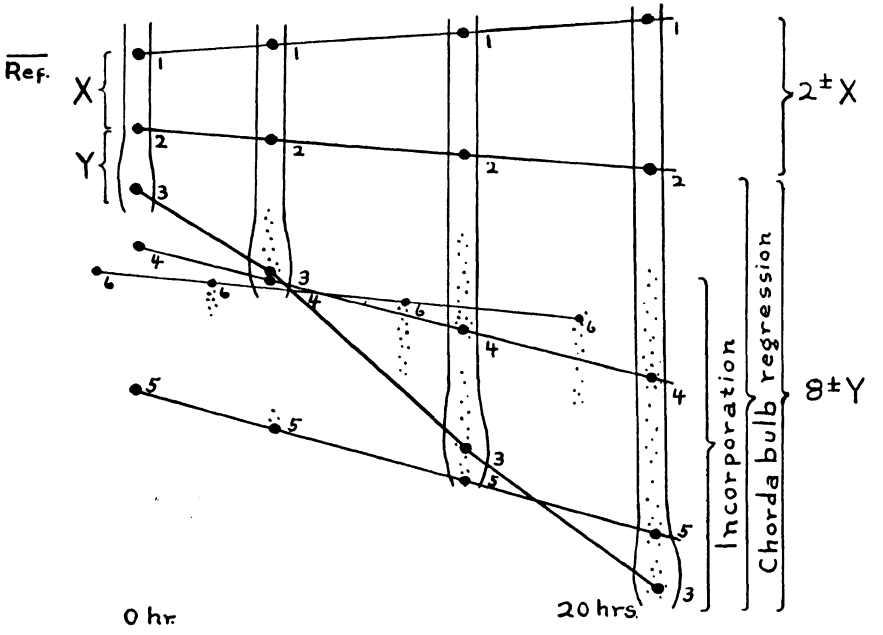

Fig. 6. Diagram summarizing the results of numerous carbon and carmine marking experiments. Certain features of the mechanism of notochord formation and elongation are illustrated. The positions of the marked, mesodermal cells are numbered 1-6. See text for further explanation.

chord cells, adding them to the notochord just anterior to the bulb and, in the process, by-passing them to pick up more cells which happen to lie in the path of the bulb.

5. The somite centers, although not morphologically definable like the chorda center, have been shown by appropriate marking, isolation and defect experiments also to undergo regression along with the chorda bulb. The somite centers appear to pick up prospective somite cells lying posterior to them, regress with these cells for a brief interval of time, and then to by-pass them (Fig. 6). Although critical evidence has not been obtained, it seems that unlike the chorda bulb there are no relatively "permanent" cell members of the somite center population.

6. Formation of mesodermal axial organs by the chorda and somite centers is independent of the products of these centers (axial notochord and segmental plate or formed somites). The continuation of regression and notochord formation by the chorda bulb isolated from the blastoderm anterior to it attests to its remarkable autonomy, a property it also shares with the somite centers (Figs. 5 and 10).

7. However, continuation of the developmental activities of the chorda and somite centers is dependent upon the number of prospective axial mesodermal cells with which the center is in continuity. That is, these more posteriorly lying cells are the principal cellular materials out of which the centers construct the axial organs. The limitation of axial organ formation by the isolated node center *in vitro* is indicated in Fig. 10. Conversely, the prospective axial mesodermal cells depend upon contact or interaction with cells of the centers for their development into axial organs. On the other hand, further development of the products of the centers (notochord histogenesis, formation of somites out of the segmental plate) is not dependent upon the continued presence of the centers (Fig. 10).

The mechanism and source of cells for spinal cord elongation is under study and may prove to be interesting in view of the fact that the spinal cord will continue its posterior elongation in the absence of any underlying notochord or chorda bulb (i.e., after excision of the chorda bulb), extending quite far posterior to the point at which chorda formation has ceased. Is there also a neural plate or spinal cord "organizer" center localized in the posterior part of the neural plate?

III. REGULATORY PROPERTIES OF THE NODE CENTER

In the belief that a revelation of the latent, regenerative or repair potentialities of the node center might provide some insight to the processes which normally occur in this region, numerous operations were done on explanted blastoderms and isolated node areas (Spratt, 1955c). A brief résumé of some of the results will illustrate these properties:

1. Although head-process and early somite blastoderms are not capable of replacing the chorda bulb or somite centers following their complete removal, they are capable of replacing the excised posterior ½ or ⅔ and the lateral ½ or ⅔ of the chorda bulb and anterior, posterior, right or left ½ of each somite center. Although the blastoderm frequently cannot replace the excised anterior ½ of the chorda bulb, the posterior half of the bulb is capable of continuing its regression and notochord forming activities.

2. Removal of the node of long and possibly of definitive primitive streak blastoderms may be followed by closure of the hole and continued notochord formation, i.e., by complete replacement of the excised tissue (Spratt, 1955a, c). It has been suggested that complete removal of the node at these stages does not accomplish the complete removal of all of the prospective chorda center cells (see also Grabowski, 1953).

3. Removal of portions of the prospective notochord and/or somite cells posterior to the node is usually followed by closure of the hole and uninterrupted regression of the chorda and somite centers through the wound area.

4. Longitudinal splitting of the streak from the posterior edge of the chorda bulb back to the edge of the blastoderm and separation of the two halves at an angle of about 90° has always been followed by a progressive "zipping together" of the halves of the streak and continued formation of a single, median notochord. When the streak and the chorda bulb are split longitudinally and the halves are prevented from healing together, the right and left halves of the bulb continue regression, functioning as well, apparently, as the whole chorda bulb (Fig. 10).

IV. ORGANIZER PROPERTIES OF THE NODE CENTER

Grafting of the node or node area to host blastoderms has revealed the general organizer properties of the node in inducing a secondary embryo (or part of an embryo) in the host (see reviews of Rudnick, 1944, and Waddington, 1952; also Grabowski, 1953). A test for the possession of mesodermal organizer properties by the mesodermal cells of the node centers was undertaken by allowing the regressing chorda bulb and somite center cells to interact with non-prospective chorda and somite cells. To obviate the technical difficulty of exchanging cell groups by grafting, two methods were used: (a) The prospective chorda cells, for example, were removed from the path of the chorda bulb by cutting out a piece of the primitive streak. As the hole closes, prospective somite or lateral plate cells are drawn into the path of the bulb. (b) The node area explant was trimmed in such a way as to remove ¾ or possibly all of the prospective somite cells on one side of the explant and all of the prospective notochord, somite and lateral plate cells on the other side (see Fig. 10). The chorda bulb is thus forced to deviate in its regression and to come into contact with non-prospective chorda cells.

Variations of these methods have given the following results (see also Spratt, 1955c):

a. Chorda bulb + prospective notochord cells → definitive notochord
b. Chorda bulb + prospective somite cells → definitive notochord
c. Chorda bulb + prospective lateral plate cells → definitive notochord
d. Somite center + prospective somite cells → somites
e. Somite center + prospective notochord cells → somites
f. Somite center + prospective lateral plate cells → somites

In other words, mesodermal cells posterior to the node may be designated prospective chorda, somite or lateral plate cells only by virtue of their positions in the blastoderm. Otherwise, they apparently differ in no other way from one another. They realize their prospective fates only by the action upon them of their respective center cells, the latter acting in the ordinary sense as organizers or inductors.

V. DIFFERENTIAL ENVIRONMENTAL REQUIREMENTS OF THE NODE CENTER AND REMAINDER OF THE BLASTODERM

It does not seem unreasonable to assume that the profound developmental activities peculiar to node center cells might be a consequence of their possession of peculiar metabolic properties. At least, the pattern of differential developmental activity which characterizes the early blastoderm could be expected to be underlain by some sort of corresponding pattern of metabolic activity (Spratt, 1952c). Evidence which supports this assumption is provided by the differential susceptibility of early blastoderms to poisons or metabolic inhibitors such as cyanide (Hyman, 1927), azide, fluoride, iodoacetate, etc. (Spratt, 1950b) and by differential staining with vital dyes (Child, 1925, Rulon, 1935, Miller, 1941). In all of these instances the node center was the region most sensitive to the inhibitor and also the region most active in reducing the vital dyes.

Further demonstration of the correspondence of developmental and metabolic activity patterns has been provided by experimentally modifying the environment of explanted early blastoderms (see review of Spratt, 1952c). The results of numerous experiments carried out by the writer are summarized in the diagram of Fig. 7. It is to be noted that, in general, the node area of the embryo where developmental activities are greater is more sensitive to adverse environmental conditions (whether they consist of anaerobiosis, presence of inhibitors, starvation, etc.) than are regions which have already passed through a period of developmental activity (e.g., hind-brain, spinal cord, noto-

chord, etc.). Of particular interest is the inhibition of axial organ formation by the node in the presence of whole embryo extract and heart and liver homogenates or slices of older embryos. Preliminary studies

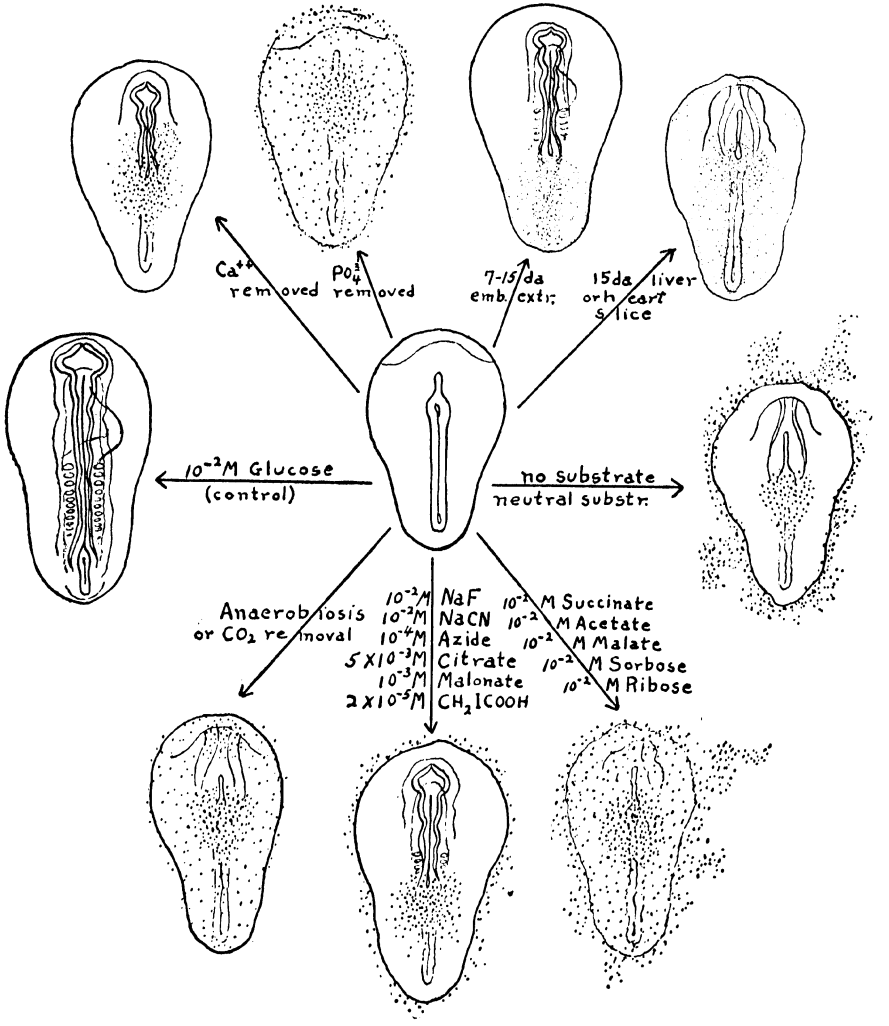

Fig. 7. Diagram summarizing the differential effects of various adverse environmental conditions (calcium removal, tissue extracts, starvation, presence of inhibitors, etc.) on the node center of explanted blastoderms.

have shown that this inhibitory effect is heat labile. The effects of these extracts, homogenates or slices of older embryo organs and whole embryos on the node area are different from those of all other adverse environmental effects observed in that there is no cell dispersal in the

node area—only an inhibition of morphogenesis (and histogenesis?) of the axial organs: spinal cord, notochord, somites, etc. An explanation of these peculiar effects must await further study.

VI. NUTRITIONAL REQUIREMENTS OF THE ISOLATED NODE CENTER

The metabolic properties characteristic of node center cells revealed by explantation of whole blastoderms to various media (Fig. 7) have been subjected to further study by explanting the excised node center area to various carbohydrate and other relatively simple nutrient media. A complete description of the results, summarized in Fig. 8, will be presented in subsequent papers, but for our purposes here a few salient

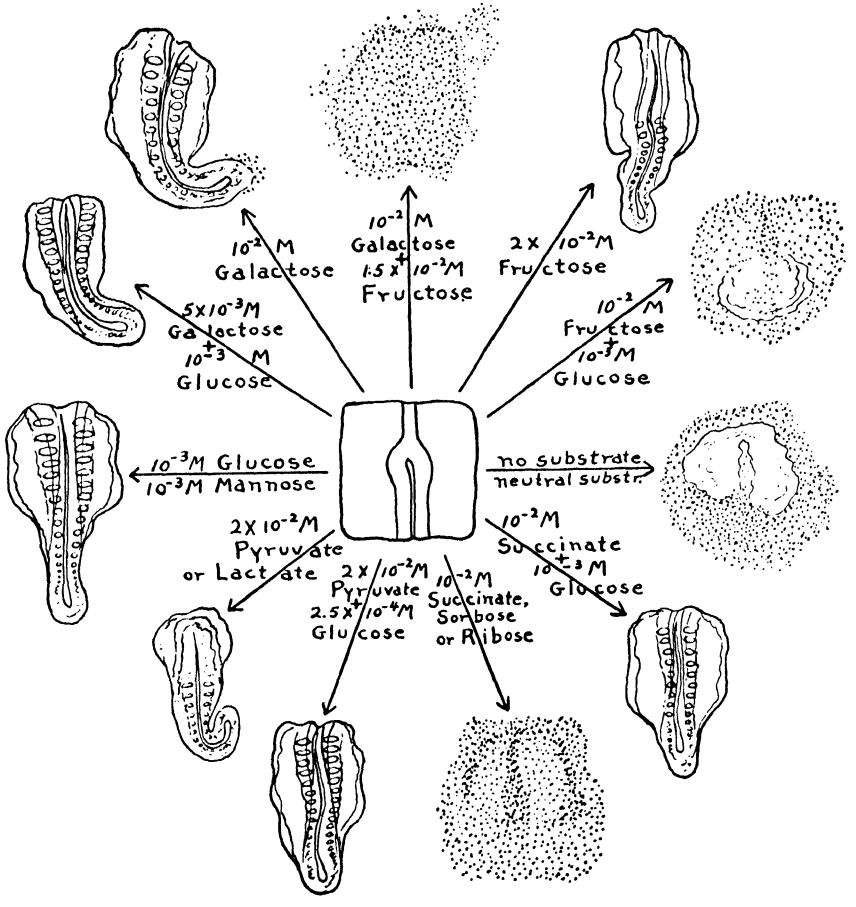

Fig. 8. Diagram summarizing the nutritional (mainly carbohydrate) requirements of the isolated, explanted, node center area (see text).

features may be mentioned. Among the substrates and combinations of substrates indicated in the figure, glucose and mannose are superior to all others, although galactose, fructose and pyruvate or lactate, in this order, are decreasingly effective in supporting continued axial organ formation by the isolated center.

When the isolate is placed on a medium containing no substrate or a neutral substrate (i.e., a substance such as sucrose, lactose, glycerol, etc. which apparently have no effect whatsoever on the cells) the center manifests no further developmental activity. On the other hand, substrates such as succinate, sorbose, ribose, etc. not only do not support development but appear to inhibit endogenous maintenance metabolism as well, with resultant cell dispersal and disintegration throughout the explant. When glucose is combined with other substrates such as galactose, succinate or pyruvate, development of the explants is similar to that of those on glucose alone, even when the molar concentration of glucose is only $\frac{1}{5}$ to $\frac{1}{100}$ that of the other substrate. In contrast to this, combination of fructose with either glucose or galactose results in extensive degeneration and dispersal of the explants. It appears that fructose, in combinations too low to support development, actively inhibits the exogenous utilization of galactose and the exogenous and endogenous utilization of glucose.

These studies together with earlier ones on the nutritional requirements of whole, explanted blastoderms (Spratt, 1947b, 1948, 1949, 1950a, b, etc.) clearly demonstrate that the cells of the node center differ from all other cells of the embryo in being more fastidious in their nutrient and other environmental requirements. It has been thought for some time that these exacting requirements of the node organizer cells are a reflection of their peculiar enzymatic properties (Spratt, 1950b).

VII. ENZYME ACTIVITIES OF THE NODE CENTER

In earlier studies it was found that in 20-30 hour blastoderms explanted to albumen or Ringer-glucose media containing the reducing enzyme indicators: triphenyl- or neotetrazolium chloride or potassium tellurite, the aerobic pattern of reduction of the colorless reagents to formazan (red or purple) and tellurium (black), respectively, coincides with the pattern of developmental activity. Thus the node center cells in early blastoderms and the tail-bud cells in older ones reduce the enzyme indicators more rapidly and more completely than do other cells of the blastoderm (Spratt, 1951).

When lower (relatively non-toxic) concentrations of tetrazole are used in albumen media, development of the explants continues for about 24 hours. Under these conditions not only the node (and sometimes the fore-brain) shows marked reducing activity (production of the red formazan), but also structures or parts (somites, chorda, neural tube) that have developed out of the node center during exposure to the indicator, in contrast to other structures formed prior to exposure which remain white (Spratt, 1951). This is exactly what one would expect from a knowledge of the role of the node center in axial organ formation (Spratt, 1947a, 1955a, b).

Node center cells also exhibit: (1) greater cytochrome oxidase (indophenol blue) activity (Moog, 1943, Spratt, 1952c); (2) greater cytoplasmic basiphilia (ribonucleic acid concentration?) when stained with toluidine blue (Gallera and Oprecht, 1948, Spratt, 1952c); and (3) greater staining with and reduction of Janus green, presumably a fairly specific indicator of mitochondrial activity (Rulon, 1935, Miller, 1941). Since the major locus of tetrazole reduction within the cells seems to be the mitochondria (Kun, 1951, Spratt, 1952d), the various studies cited above suggest that the quantitatively greater enzyme activities peculiar to node center cells may be the result of their possession of greater numbers of mitochondria or of more active mitochondria or both. Further studies in progress are directed toward the clarification of this possibly very important property distinguishing node organizer cells from other cells.

Attempts to more specifically characterize the greater reducing activity of the node center cells have been undertaken (Spratt, 1952a). Although a full account of these studies is still in preparation some of the major results are presented in summary form in Fig. 9. It is to be noted that the node center cells of explanted blastoderms are able to reduce neotetrazolium chloride to formazan in the presence of nine different substrates under aerobic conditions and eleven different substrates under anaerobic conditions—manifesting a total of twenty kinds of activity. On the other hand, cells of the brain and spinal cord reduce the tetrazole in the presence of five different substrates aerobically and four different substrates anaerobically—a total of only nine kinds of activity. Here, apparently, is an example of differentiation in reducing enzyme capacities between the node cells (a center of axial differentiation activity) and the brain and spinal cord (already partially differentiated structures). In other words, the node cells exhibit a wide array of specific (substrate controlled) reducing capacities, whereas the

brain and cord cells presumably have gained some but lost most of these in the course of their development out of the node center.

In conclusion to the above very brief outline of the results of studies of the metabolic properties of node center cells it should be recalled that

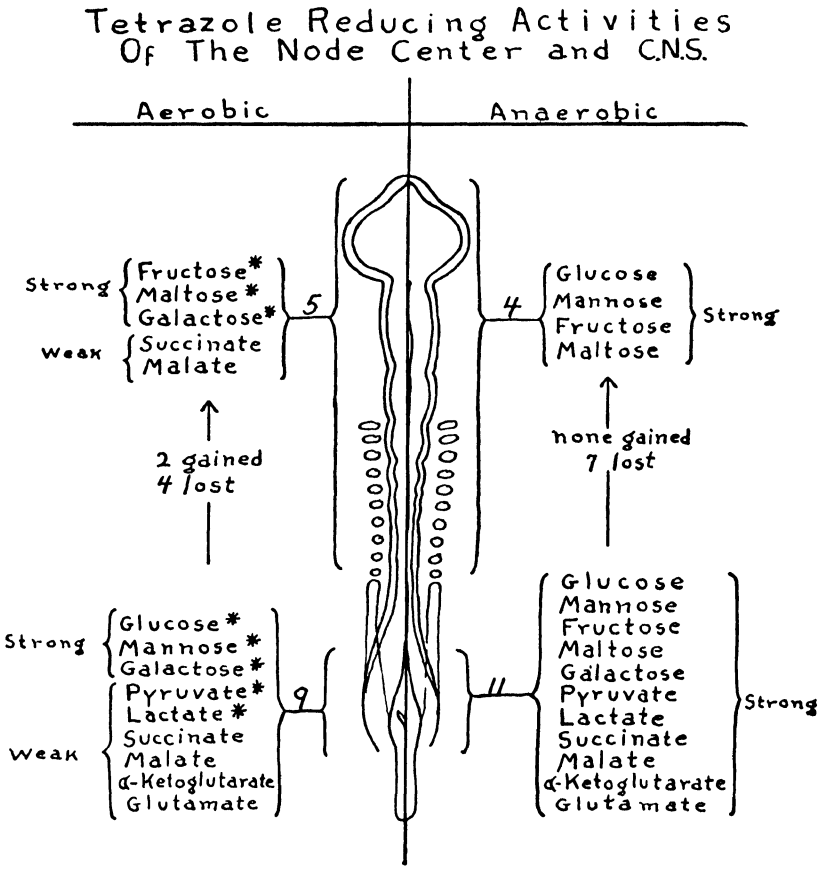

Fig. 9. Diagram summarizing the results obtained by explanting 6-10 somite blastoderms to neotetrazolium chloride media containing different substrates. Note that the cells of the brain and spinal cord differ in their reducing enzyme capacities from the cells of the node area. Only those substrates marked with an asterisk support continued development of explanted blastoderms. See text for further explanation.

these studies are being continued with the hope that some of the important metabolic properties may eventually be discovered. Perhaps all, perhaps none of the distinguishing properties so far discovered are important to our better comprehension of organizer-cell activity. Our studies may have only scratched the surface.

VIII. ORIGIN OF THE ORGANIZER CENTER

One of the most interesting questions one might raise regarding the activities and significance of developmental centers is that of their origin. For example, does the node center, with its peculiar properties, arise gradually during the course of development of the morphologically recognizable node or are these properties to be found already limited to certain localized cells long before the appearance of the primitive streak? Preliminary observations indicate that the intense, localized reducing enzyme activity of the node center does not appear until the node is almost fully formed, i.e., cells of the anterior end of the beginning or medium-broad primitive streak do not exhibit any greater tetrazole reducing activity than do the remaining cells of the blastoderm. However, in some blastoderms there is a "mosaic pattern" consisting of actively reducing cells scattered among weakly reducing cells—reminiscent of similar patterns found after staining with nile blue sulfate (Spratt, 1946). In these latter cases the actively reducing cells of the epiblast, mesoblast, and probably hypoblast are more numerous in the posterior ½ to ⅓ of the blastoderm. A definite answer to the question must await further refinement of enzyme localization methods and the use of other methods.

IX. CONCLUSIONS: COMPARISON OF THE NODE CENTER WITH THE SHOOT APEX

In our statement of the problem, toward which the varied types of procedures outlined above are directed, it was pointed out that the developmental center of the vertebrate embryo, for example the node area of the chick blastoderm, has certain features in common with the apical tip or shoot apex of the growing plant, yet in other respects it appears to be quite a different kind of developmental system. In both systems we have to deal with an apical center of cellular differentiation activity in which non-axially specified ("undifferentiated") cells become transformed into and added to the elongating axial structures (Spratt, 1954; 1955a, b). An interesting and possibly suggestive way of briefly summarizing our discussion of the node as a developmental center might be through a comparison of the results of approximately comparable experiments carried out on the shoot apex and node center. Such a comparison is diagrammatically presented in Fig. 10. The results shown for the shoot apex are based on numerous studies reviewed by Gautheret (1946), Ball (1948), Wetmore and Wardlaw

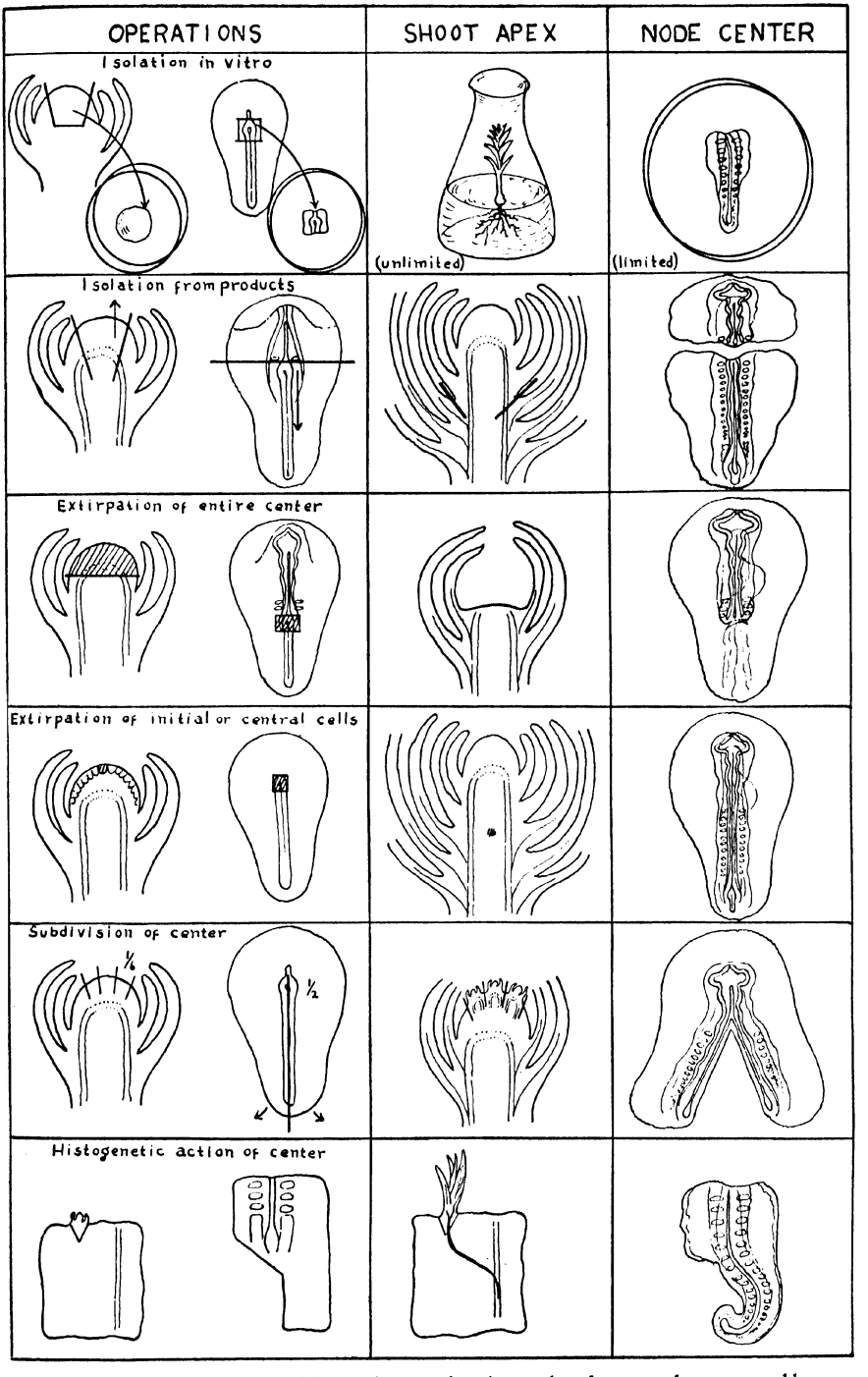

Fig. 10. Diagram summarizing and comparing the results of more or less comparable operations on the shoot apex of a plant and the node center of the chick embryo. See summary of the text for an explanation.

(1951), and Wardlaw (1952). The results shown for the node center are based on studies by the writer. Although the table is fairly self-explanatory, some interesting features of the comparison may be pointed out:

1. Both the shoot apex and the node center after isolation *in vitro* exhibit a remarkably autonomous capacity for continued axial organ formation. It is to be noted, however, that whereas the shoot apex after isolation can develop into a whole new plant (unlimited activity) the isolated node center, deprived of most of its prospective axial organ forming cells, soon ceases its axial organ forming activities (limited activity). An important difference between the source of cells for axial organ formation is thus demonstrated: the shoot apex contains a center of cell proliferation capable of providing all the cells necessary for its continued activity while the node center, not possessing such a proliferation center, depends upon cells lying in its path of movement. However, very recent marking experiments on the isolated node center suggest the possible presence of a few, rapidly dividing stem cells capable of furnishing an appreciable number of cells for axial organ formation. Differences between the mechanics of operation of the two systems may thus not be as pronounced as originally thought.

2. Both the shoot apex and node center are capable of continued activity (apparently unlimited in both cases) after isolation from the products of their activity. On the other hand, structures formed by the centers (leaf primordia, vascular strands, etc. of the plant and somites, notochord, neural tube, etc. of the embryo) continue their further differentiation independently of the centers.

3. Destruction or extirpation of the *entire* developmental center (shoot apex or node area) results in cessation of all axial organ formation—no new center being formed by the cells below the excised shoot apex or by the cells around the excised node area.

4. When only the initial cell of some plant apices or only the central cells of the node center (e.g., the node proper of long streak blastoderms) are destroyed or removed, the remaining cells of the developmental center are capable of taking over and continuing the functions of the center.

5. About ⅙ of the shoot apex and ½ of the node center are able to continue apical center activity. However, it is to be noted in the case of the node center that only the divided chorda bulb and not the neural plate shows this capacity completely, i.e., the half neural plates do not regulate to form two complete neural tubes.

6. Both the shoot apex and the node center exhibit "organizer" properties in that both have a remarkable histogenetic action on non-center cells. For example, an apical meristem grafted to a piece of root phloem can initiate the differentiation of vascular tissues in the phloem parenchyma. The chorda bulb of the node center, forced to deviate in its regression, can cause prospective somite or lateral plate cells to develop into notochord cells, presumably by interacting in some fashion with them.

It has been our purpose in this brief review of current studies on the node center to present some of the problems and to list what appear to be some of the more important properties which characterize the cells of a developmental or organizer center. Much of the work must be considered only as a prelude to a more intensive analysis which some day may provide us with a better understanding of the functioning of such a developmentally important group of cells.

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X. TISSUE INTERACTION IN THE MORPHOGENESIS OF MOUSE EMBRYONIC RUDIMENTS IN VITRO

BY CLIFFORD GROBSTEIN[1]

THREE years ago Holtfreter (1951) analyzed before this Society "Some aspects of embryonic induction." Summarizing the complexities of the literature on inductive processes in amphibian development, he deliberately gave little attention to the question of inductive mechanism *per se,* finding that the intensive efforts of the thirties to identify inductive agents had ended in "a blind alley." Holtfreter sought to remarshal the biological facts so as to correct the distortion in our conception of induction which had resulted from a number of too-simple assumptions. And he placed the burden of developing new approaches to the knotty problem squarely on the shoulders of the "analytically minded morphologist." The line of investigation which I will describe was in its beginnings three years ago, and was motivated by essentially these same considerations. The biochemical search for inductive substances was initiated by the finding that dead tissues are inductively active in the amphibian gastrula, and it was frustrated by the embarrassment of riches that was found. What Holtfreter argued was that the response of the gastrula is far more complex than was being assumed, far too complex to be explained in terms of reaction to one or two substances. He emphasized that, while dead tissues and artificial inductors lead to partial responses which resemble the normal developmental phenomenon, it is only in the interaction of living tissues that the full phenomenon is achieved. A return to the interaction of living tissues would appear indicated, if we are to find a profitable new point of departure.

In returning, however, the lessons already learned are not to be forgotten. The notion of embryonic induction arose from studies of the amphibian gastrula and, though many inductions in later development have been demonstrated and studied, theoretical considerations have been largely dominated by the early primary interaction of chordamesoderm and gastrular ectoderm. That this reaction should have proven so intriguing—and bewildering—is not surprising, since upon it depends the fundamental organization of the embryo. Yet, as we all know, the search for mechanisms does not necessarily proceed most effectively in the

[1] National Cancer Institute, National Institutes of Health, Bethesda 14, Maryland.

analysis of the most fundamental and complex. If there is a common character in the inductions which occur earlier and later in development, and we at the moment assume that there is, then it seems sound to suppose at least provisionally that in the later inductions, where much less is at stake, the mechanisms may be simpler and more easily unravelled. It seems probable, in fact, that wherever two tissues of different developmental history come together and interact, there is something to be learned about embryonic induction.

In these terms, and for present purposes, I would define embryonic induction as developmentally significant interaction between closely associated but dissimilarly derived tissue masses. "Developmentally significant" is used to imply a change in one or both masses which is progressive in terms of the life cycle of the organism, and which is sufficiently stable to persist in some degree if the two masses are separated. Under this general definition, which deliberately avoids any reference to cellular determination or differentiation, we leave our minds quite open as to mechanism, and we are free to pick cases for analysis in terms of their experimental advantages.

What advantages would we like to have? First, an unambiguous response, simple, yet clearly recognizable and characterizable. Second, a standardizable response, repeatable, predictable as to time course, and quantifiable. Third, a response accessible to interruption of the reaction at any time, to separation and recombination of interactants, to application of reagents. Fourth, an isolable response occurring under controlled conditions, free of complicating variables including interactions with the rest of the organism. Fifth, a response which can be directly observed throughout the inductive course. Sixth, a response, if the nature of the mechanisms involved does not exclude it, occurring across an intervening space or interzone within which the effective agents can be visualized, and possibly analyzed, independently of the cellular masses.

To specify these advantages is to point strongly in the direction of an *in vitro* approach. Such methods have been used extensively in studies of primary induction in the amphibian gastrula (Holtfreter, 1951) and applied much earlier to other developmental problems (see White, 1954). With mammalian material, on which the data to be presented are exclusively based, the ordinary methods of experimental embryology must, almost of necessity, give way to the *in vitro* approach pioneered by Maximow 1925, Waterman 1933, Waddington 1937, Nicholas and Rudnick 1938. Fortunately, the work of these authors and of Fell (1951) and

her associates on the culture of small rudiments has laid a foundation on which all future developments will depend.

The data that I shall present represent a continuation of these efforts, focused particularly on the question of inductive tissue interaction and its mechanisms. Concerning the latter, I shall take advantage of the earlier connotation of "Symposium" to speculate somewhat more loosely than I otherwise might. I have in mind the probability, indicated by Weiss (1950) and others, that there is no single inductive mechanism, that in different inductive interactions we must be prepared to find different mechanisms operating, and possibly more than one in each case. The objective has been to find several inductive systems that can be compared, and then to manipulate the experimental conditions in such a way as to exclude or to demonstrate the operation of one or more of the mechanisms which have been postulated.

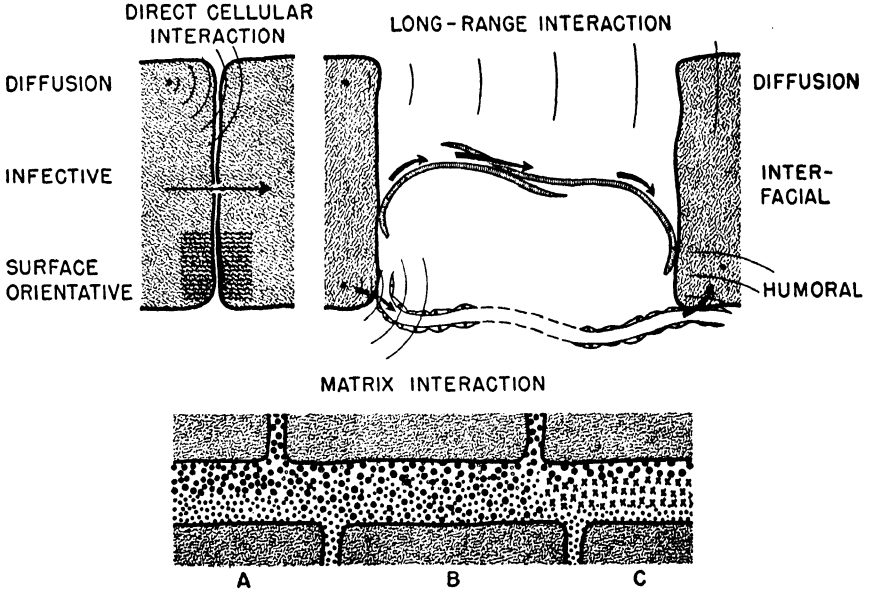


Fig. 1. Assumed or demonstrated mechanisms of tissue interaction and induction in development. A, B, C = Three hypothetical cases of matrix interaction, see text.

A rough guide to postulated or demonstrated mechanisms of tissue interaction is provided by the scheme in Fig. 1. Distinction is made between direct short-range interactions proceeding essentially cell to cell, and longe-range interactions across a considerable intervening distance. It does not seem important to specify the distance too sharply, since there

may well be intergrading cases. The main point of the distinction at the moment is operational. If interactions are of the direct type, they would be limited to, or localized in, the immediate areas of tissue contact, and the active agent would be less likely to be demonstrable in extracts or in fluids in which active tissues are cultured. If they are of the long-range type, they would be less localized and more likely to involve active agents demonstrable in extracts or culture fluids. Direct interactions can be further subdivided as involving either simple diffusion of freely dissociated molecules, infective transmission by organized particles on the viroid model (Medawar, 1947), or contact surface orientative effects such as have been postulated by Weiss (1947). The long-range interactions similarly can be subdivided as involving simple diffusion, movement oriented by interfaces such as are provided, for example, at the surfaces of fibrillar components of the interspace, or movement through channels such as are provided by vessels. In the last instance, of course, we bridge over into morphogenetic effects of hormones in later development.

There is a third conceivable class of interactions, in a sense logically intermediate between the other two, and this is indicated under the heading of matrix interaction. At the moment largely speculative, the notion is included only because results in the investigations to be described have more and more insistently drawn attention to the need for its consideration. It will be discussed further below.

From the results that have been accumulating in our studies, which I would emphasize are still very much in their early stages, I have selected facts to indicate the following points: First, procedures are available for mammalian studies which can satisfy to a considerable degree the desiderata indicated above for analysis of inductive mechanisms. Second, in the cases so far analyzed by these procedures, inductive interactions show high specificity. Third, in at least two of the reactions studied, there is strong spatial localization of effects. Fourth, inductions probably do not all proceed by the same mechanism. Fifth, the intercellular matrix deserves consideration as a possibly important factor in induction.

Before describing some of the analytical results, a few words should be said about methods. The rudiments and tissues used have come exclusively from mouse embryos derived from matings of BALB/C females with C3H males. Nine-day embryos (the morning of discovery of a vaginal plug is taken as zero time) were the standard source of somites, 11-day embryos of metanephric rudiments, and 13-day embryos of submandibular salivary rudiments.

The site of culture was always a substrate-clot interface, with a fluid supernatant nutrient medium consisting of 9-day chick embryo juice, Tyrode's solution and horse serum (1 : 2 : 2) above the clot (Fig. 2). In standard cultures, the tissues were introduced into the clotting mixture (fowl plasma plus nutrient medium, 2 : 3 or 1 : 1) and positioned on the substrate. In cultures involving filters, one tissue was placed upon a previously formed clot, the filter laid on top, and the second tissue introduced and positioned in a second clot layered over the first. Both tissues thus were at a filter-clot interface on opposite sides of the filter. Certain other procedural variations will be mentioned in connection with specific experiments below. In all cases, the supernatant nutrient medium was changed daily.

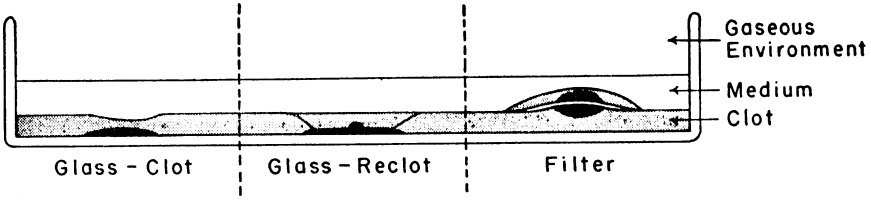


Fig. 2. Types of culture. Tissue solid black, clot gray. Standard glass-clot culture (left) varied in some instances by addition of second tissue to first followed by reclotting (center). "Filter culture" (right) involves two superimposed tissues on top of original clot with filter between the two tissues.

The culture vessels in early experiments were Carrel flasks. In later experiments these were replaced by flat, loosely covered dishes containing a small central well, within which the clot and tissues were placed. With penicillin and streptomycin added to the medium, and the dishes incubated in an atmosphere of high humidity containing 5% CO_2, the culture behavior in these vessels was virtually indistinguishable from that in Carrel flasks. The dishes have the advantage of requiring considerably less plasma than Carrel flasks, and they permit greater convenience in operative procedures.

The culture conditions described are adequate to permit characteristic morphogenesis of at least three embryonic rudiments, those of the submandibular salivary gland, the lung, and the kidney (Figs. 3, 4, 5, Plate I). It is to be noted that at the time of isolation these several rudiments have a similar structure, consisting of a central epithelial bud branching in variable degree, and a surrounding mesenchymal mass distinguishable from the general loose mesenchyme by its greater cellularity ("condensation"). In the case of the lung, there is also a peripheral

thin mesothelial layer. In all three cases the mesenchyme spreads peripherally as a cohesive sheet, and the epithelium undergoes morphogenesis centrally to produce a branching pattern which is characteristic for the particular organ, though by no means identical with that produced *in vivo*. Experimentally advantageous is the fact that the morphogenesis takes place essentially in two dimensions, permitting continuous observation and photography.

It is important for the discussion to follow that the mesenchyme of the metanephric rudiment, in reality the caudal end of the nephrogenic cord which has become associated with the ureteric bud, also undergoes a morphogenesis. While mesenchyme of lung and salivary gland spread thin and remain quite simple and homogeneous in culture, the kidney mesenchyme undergoes local condensations eventually to form S-shaped and then coiled tubules among the branches of the ureteric bud. These epithelial tubules condensed out of the nephrogenic mesenchyme clearly correspond in origin with the secretory tubules and glomeruli of the normal kidney and, like them, they secondarily hook to the collecting system resulting from the branching of the ureteric bud. Thus the major features of metanephric morphogenesis occur in this *in vitro* system, both components demonstrating characteristic behavior and the resulting products achieving normal anatomical relationships.

The experimental analysis begins with the findings that epithelio-mesenchymal rudiments of the kind described can be readily and cleanly separated into their two components, following brief exposure to trypsin solutions (Grobstein, 1953a). Such separated components are viable but do not continue morphogenesis when cultured separately. Mesenchymal components spread into a thin homogeneous mass from which, after several days in culture, individual fibroblasts migrate out into the clot or along the glass interface. Epithelial components show somewhat variable behavior depending upon the stage of development of the rudiment, but their general tendency also is to spread into a thin sheet at the glass-clot interface.

When trypsin-separated components are immediately recombined, however, their morphogenesis can continue in culture in much the same way, though usually not so strongly, as that of untreated rudiments. This suggests that there is some mutual dependence between the two components, some kind of interaction which is essential for continued development. Epithelium alone produces simple formless sheets, epithelial rudiments in the presence of their mesenchyme produce a characteristically branching set of tubules. The question may be raised whether such

influences of mesenchyme on epithelium are "inductive." This appears to be a matter of definition. A developmentally significant action of one tissue upon a dissimilarly derived one is certainly involved, and hence the case is admissible under the definition specified earlier.

These facts are illustrated, and new facts are revealed, when the separated components of salivary and kidney rudiments are cross combined, i.e., when salivary epithelium is combined with kidney mesenchyme, or kidney epithelium with salivary mesenchyme (Grobstein, 1953b). The result is particularly striking (Figs. 6-9) when both kinds of epithelium are included in one culture with each kind of mesenchyme. In the experiment illustrated, a salivary epithelial rudiment and a kidney epithelial rudiment were combined in a cluster of salivary mesenchyme fragments in one culture (Fig. 8), and the corresponding epithelial rudiments from the opposite sides of the same embryos were combined in another culture with fragments of kidney mesenchyme (Fig. 6). In the first culture the salivary epithelium underwent characteristic morphogenesis, but the kidney epithelium remained quiescent (Fig. 9). In the second culture (Fig. 7) the kidney epithelium underwent morphogenesis, but the salivary epithelium simply rounded up into a small mass. Particularly interesting was the fact that in close association with the salivary epithelium, as well as in the usual association with the developing kidney epithelium, there arose in the nephrogenic mesenchyme condensations from which coiled tubules later formed. These observations suggest, on the one hand, that epithelial morphogenesis is dependent not only upon association with mesenchyme, but upon the particular mesenchyme that envelops the epithelium in normal development. The mesenchymal contribution thus appears to be a fairly specific one. On the other hand, the formation of tubules in nephrogenic mesenchyme depends upon an inductive stimulus which seems less specific, since salivary epithelium as well as the normal inductor can supply it.

Further data have continued to support this difference in specificity between the epithelial and mesenchymal response, although it should be noted that both responses are relatively highly specific in comparison with neuralization of amphibian gastrular ectoderm. In the case of the response of salivary epithelium (Grobstein, 1953c), no mesenchyme other than that of the submandibular salivary gland has been found capable of supporting morphogenesis. Tests have been made of mesenchyme from other sites in the 13-day embryo (from which this epithelium is derived), of mesenchyme from other epithelio-mesenchymal rudiments at comparable stages of development (lung and kidney of 11-day em-

bryos), and of mesenchyme from various regions of the 8-day embryo in early somite stages. Mesenchyme from all of these sources has an effect on the epithelium, causing it to round up into a quiescent mass instead of spreading in a thin sheet as it does in isolation, but none of them promotes morphogenesis. Not yet tested, but of interest in defining the nature and preciseness of the specificity, are mesenchyme from sublingual and parotid rudiments, from older submandibular rudiments, and from submandibular rudiments of other species.

On the basis of available data, there appears to be a high degree of specificity in the mesenchymal contribution made to submandibular epithelial morphogenesis. It seems significant, though the explanation is not clear, that whatever the nature of the specific mesenchymal contribution it gradually declines when the mesenchyme is cultured by itself for varying periods and then tested by recombination with freshly isolated epithelial rudiments. Tested at the end of each of three "generations" of sub-culturing of five to seven days duration, the mesenchyme shows a gradual shift in at least two properties, the rate and "typicalness" of the epithelial morphogenesis it can induce, and the density and cohesiveness of the mesenchymal mass, a phenomenon particularly noticeable at the periphery (Fig. 10). It is not certain that these two effects are related, but the possibility is suggested that the inductive influence may depend, at least in part, on the original organization of the tissue which is gradually disrupted during culturing. The behavior is particularly suggestive because the mesenchyme, as it loses its inductive capacity, passes in its culture behavior from the cohesive type characteristic in our experience of embryonic, morphogenetically active tissues to the more dispersed type generally observed in older tissues. The question is raised whether what is involved in tissue cohesiveness is also involved in the inductive activity.

Turning to tubule induction in metanephric mesenchyme, the data suggest somewhat less, though still quite high, specificity in the response. We have noted that the epithelium of both kidney and submandibular rudiments can induce tubule formation, the former in a manner more characteristic of normal development, the latter in a ring in close juxtaposition to the epithelial surface. In addition, 11-day embryonic spinal cord is a strong inductor of kidney tubules (Fig. 14), dorsal regions more strongly than ventral ones. Activity of spinal cord as an inductor of kidney tubules has been recorded in amphibia and chick (Gruenwald, 1952). Preliminary tests also showed some activity in the medulla and cerebrum of the 11-day embryo, but apparently at lower levels than in

the cord. On the other hand, various adult non-nervous tissues, embryonic lung, gastric and pancreatic epithelia, and embryonic spinal ganglia proved negative as inductors in combination with metanephric mesenchyme. All heat-killed tissues tested have been negative, including 11-day embryonic spinal cord. Curiously enough, however, it appears that metanephric mesenchyme implanted directly into the eye forms tubules in the absence of its epithelium or any other deliberately included inductor. Nonetheless, in culture, neither iris nor cornea of the adult eye appears capable of stimulating tubule formation. Thus, the data indicate that, though there may be several alternatives to the normal inductor of kidney tubule formation, the reaction is by no means easily and nonspecifically induced. Why the salivary epithelial ingrowth should possess a common property with the ureteric bud is at the moment not easy to see. It may be noted, however, that the tubule-inducing dorsal regions of the neural tube earlier were the lateral regions of the medullary plate, and these are fairly closely associated with the early nephrogenic mesenchyme.

A high order of specificity is indicated also in another inductive system on which studies have recently been made in collaboration with Mr. George Parker and Dr. Howard Holtzer. Trypsin-isolated somites from 9-day embryos form simple, thin, homogeneous masses when explanted in clusters of six to eight somites. If such clusters also include a lateral hemisection of embryonic spinal cord, cartilage masses appear around the cord on the fourth to sixth days (Fig. 15) (Grobstein and Parker, 1954). The observation supports the notion, based on *in vivo* studies in lower vertebrates, that the spinal cord exerts an inductive influence in formation of the axial skeletal system. It turns out that in this reaction ventral cord is the active source, dorsal cord being virtually inactive (Grobstein and Holtzer, 1955). Otocyst and embryonic brain also show activity, but various living or killed adult tissues and killed cartilage are inactive.

To summarize the results on the three inductive systems tested: with one and possibly two exceptions, no inductors have been demonstrated other than the living tissues which might be expected to carry out that particular induction in the developing embryo. This is encouraging in suggesting that the reactions under investigation are occurring in ways not too different from those obtaining in the embryo, and that the mechanisms involved are in some sense "first order," without intervening regulation, injury, etc., which have made the interpretation of amphibian primary induction so complicated.

With this encouragement, we may consider a second property of these *in vitro* systems, that of "localization" of response, a phenomenon which has important bearing on questions of mechanism. The term "localization" is intended to designate simply the degree to which the induced response conforms spatially with the inductor. In the strongest localization, the induced response occurs only in the immediate vicinity of the inductor (contact). An important factor determining localization would be expected to be the range of the inductive influence.

The difference in reaction of the salivary epithelial rudiment to its own (autogenous) and other (heterogenous) mesenchyme offers an opportunity for testing localization (Grobstein, 1953c). Simultaneous exposure (Fig. 11) of an epithelial rudiment to fragments of autogenous and heterogenous mesenchyme placed on its opposite sides showed that branching tubules formed on the autogenous side, while the epithelial surface was completely quiescent on the heterogenous side (Figs. 12, 13). Moreover, the line separating the two types of epithelial pattern seemed to conform quite closely to the line of junction between the two types of mesenchyme (Fig. 12). Clearly, whatever the nature of the influences involved, under these circumstances their effects were limited to the immediate vicinity of the respective kinds of mesenchyme.

The same conclusion is suggested by another type of experiment involving interaction among the same three tissues (Fig. 16). Autogenous salivary mesenchyme was pre-grown in a thin clot in the absence of epithelium. On the second day, when the overlying clot was largely digested, a sector was cut out of the center of the thin, spread disc of tissue. Into the hole thus formed an epithelial rudiment was placed (Fig. 16, middle row, left) and a new clot made to hold it in position. Mesenchyme migrating in to fill the gap made contact with the epithelium, and recognizable morphogenesis occurred (Fig. 16, middle row, right). When, however, two small fragments of heterogenous (maxillary) mesenchyme were placed on either side of the epithelium so that in their migration they would surround it before the autogenous mesenchyme arrived, there was no sign of morphogenesis (Fig. 16, top row). When the heterogenous mesenchyme was so placed as to surround the epithelium only incompletely, tubule outgrowth occurred at precisely the point where closure was incomplete and autogenous mesenchyme was able to reach the epithelium (Fig. 16, lower row).

Similar localization seems to be involved in tubule induction in kidney mesenchyme *in vitro,* although experiments comparable to those on the salivary gland have not been done with the kidney system, and it should be noted that in scattered instances single tubule rudiments have been

seen at a distance from their presumed inductors. The overwhelming general rule, nevertheless, is that tubule rudiments first appear in intimate association with the surface of the inductive tissue (Fig. 14). And, except in special cases where other factors are involved, the tubules seem to occur in association with the entire available surface.

The situation is different in the case of the spinal-cord-cartilage system. Here, the actual cartilage does not form in contact with the surface, but nearly always with an intervening interspace (Fig. 15). Moreover, small cartilage nodules fairly frequently arise at considerable distances (as much as 2 mm or more) from the spinal cord (Grobstein and Holtzer, 1955). On the other hand, there is no evidence at the moment that cartilage can form in somite mesenchyme which is not at some point in contact with the spinal cord. The possibility that cartilage can only be formed by cells which have been close to the cord and which have migrated peripherally seems excluded by experiments in which the somites were placed initially at a distance from the cord so the cells would have to migrate toward it to make contact. Cartilage forms at a distance nonetheless, and it is difficult to believe that the cells migrate in and then back out again. For the moment, the available evidence suggests that cartilage induction involves the spread of some influence, in some manner, over considerably greater distances than in the case of epithelio-mesenchymal interaction in the salivary rudiment, or of kidney tubule induction.

In the latter two instances, as in the study of such amphibian systems as chordamesoderm-medullary plate and eye-vesicle-lens, the impression from the experiments so far described is one of interaction in such intimate association that the requirement for actual cellular contact is easily assumed. However, a further set of observations raises doubts about this assumption, though not fully excluding it. These relate to inductive effects obtained across membrane or molecular filters.

Efforts have been made by a number of investigators (Holtfreter, 1933; Brachet and Hugon de Scoeux, 1949; McKeehan, 1951) to obtain inductive effects across barriers that do not permit cellular penetration. The purpose has been to determine whether extracellular mobile materials are involved in induction, and the negative results generally obtained have bolstered the notion of a contact requirement. Only Brachet and Hugon de Scoeux appear to have obtained anything resembling an induction. They separated amphibian chorda mesoderm from gastrular ectoderm by means of a membrane (exact nature and thickness unspecified) with average pore diameter of 3.4 microns, and obtained evidence of clear but weak neuralization of the ectoderm. With membranes of

lower porosity (230 millimicrons) they saw only faint and irregular response.

The membrane used in the present studies ("Millipore," Millipore Filter Corp., Watertown, Mass.) is available in two porosity grades, "hydrosol" (HA) and "aerosol" (AA). In sheets of 150-micron thickness the AA filter is estimated to have *average* pore diameter in the vicinity of 0.7 micron, the HA filter in the vicinity of 0.3 micron. The thickness of the material actually used, however, was 30 microns or less, since at these thicknesses the sheets are translucent when wet. There is reason to believe that the porosity of the thinner sheets may be less than that of the thicker, but no reliable measurements of the average pore diameter of the thinner material are available. Some impression of the porosity of the several samples used is conveyed following silver impregnation (Fig. 17). The samples are designated as to thickness and porosity grade, e.g., 20H is a hydrosol filter of 20-micron thickness. Crude measurements of the apparent pores in these preparations, subject to several possible sources of error, give values ranging over 1 micron for 30A, up to 1 micron for 20A, and in the vicinity of 0.5 micron for 20H. These values are for the larger pores in the sample rather than the average and are given only to indicate relative orders of magnitude.

The procedure for use was briefly as follows. The filter sheets were cut into small squares 3-5 mm on a side, sterilized in 70% alcohol, and washed through changes of Tyrode and horse-serum-Tyrode. Having deposited one tissue in a minimum of fluid on the clot surface, the filter, drained of excess fluid, was placed on top. The second tissue was then positioned over the first in a second clotting mixture. Every effort was made to ensure that both tissues were in contact with the filter surface.

By these procedures trans-filter morphogenetic effects have been obtained with all three systems under discussion (Plate VI). When salivary epithelium was placed on one side of a 22H filter with autogenous, heterogenous, or no mesenchyme on the opposite side, three clearly different responses were obtained (Grobstein, 1953d). In the absence of mesenchyme the epithelium spread as a simple sheet as on glass (Fig. 18). Presence of heterogenous mesenchyme below the filter resulted in rounding of the epithelium into a mass (Fig. 19) which frequently became cystic. Autogenous mesenchyme on the under side (Fig. 20) promoted atypical and varied morphogenesis which frequently resembled but never duplicated the morphogenesis resulting when these two tissues are directly combined.

Sections of these filter-cultures (Fig. 21) showed that cells were ef-

fectively barred from entering or passing across the filter, and there were only occasional indications that cytoplasmic processes might be penetrating the filter for short distances. There was no evidence of cytoplasmic bridges across the filter, although the possibility is not excluded that bridges exist below the limit of resolution of the optical microscope.

When dorsal spinal cord was used on one side of the filter and nephrogenic mesenchyme on the other, clear evidence was obtained for a transfilter tubule-inducing effect (Fig. 23). With the 22H filter the results were variable, positives being obtained in only about half the cases. Substitution of a 15H filter, on the assumption that shorter distances were required, only further weakened the effects. When four filter samples—30A, 20A, 20H and 15H—were compared, the effect declined in this order, with the sharpest drop between 20A and 20H. This behavior corresponds well with what can be made out of the relative porosities, and suggests that it is this factor rather than thickness which controls the effect in this thickness range.

Also correlated with porosity is the frequency and depth of penetration of the filter by processes, some of which are almost certainly cytoplasmic (Fig. 17). Stainable with haematoxylin and eosin, these are particularly abundant in the 30A filter, somewhat less abundant in the 20A, infrequent as has been noted in the 20-22H, and virtually entirely absent in the 15H. The occurrence and significance of these processes, which seem similar to the basophilic hyaline filaments noted by Brachet and Hugon de Scoeux (1949) in their 3.4 micron filter, are still under study, but current impressions can be tentatively summarized as follows. Kidney tubule induction probably can take place through filters whose porosity precludes significant penetration of optically visible processes, but the frequency and reliability of the induction is greater when the porosity is sufficient to permit penetration by such processes. The processes are not limited to the interzone between two tissues in inductive interaction, but occur even where tissue is present on only one side of the filter. There is some evidence, however, that they may be more abundant in the interzone itself at particular stages of induction.

How to interpret the role of the stainable processes observed in the filter is not clear at the moment. In the higher porosity filters (AA), distinct tortuous cytoplasmic processes wind in and out of the optical plane and occasionally may be traced completely across the filter. In the lower porosity filters (HA), nothing comparable can be observed. Since the processes are visually demonstrable in the higher porosity filters, the possibility mentioned above certainly must be considered, i.e., that they

exist in lower porosity filters at dimensions below those necessary for optical resolution. Reports of long drawn-out filopodia formed by cultured cells and observed with the phase contrast microscope (Algard, 1953) and the electron microscope (Porter, Claude, and Fullam, 1945) reinforce this possibility.

It does not seem safe, therefore, to conclude from data now available that the inductions under study can occur in the total absence of contacts between cells. It does, however, seem safe to say, first, that these inductions do not require massive cellular contact in the sense of apposition of large areas of the cellular surfaces and, second, that the effects are much reduced in a barrier porosity range that would not be expected to restrict passage of even the largest freely dissociated molecules. Something intermediate between full cellular contact and free diffusion is suggested. The question remains whether this intermediate mechanism involves the processes themselves, whose frequency seems to correlate at least to some degree with intensity of the effect, or whether the processes depend upon, and thus are indicators of, some penetrating material more directly involved in the induction.

At this point, results of another recent line of investigation are relevant, although they are hardly more than suggestive to date. It is possible to demonstrate in several ways that the properties of a filter incubated for 24 hours in contact with a fragment of the dorsal half of the spinal cord are locally altered in the contact zone. On treatment by the silver impregnation procedure of Friedenwald, Buschke, and Crowell (1945), or fixation in lead acetate-formalin, the tissue area is sharply marked off as a clear spot in the blackened filter. When fixed in absolute ethyl alcohol-formalin (9: 1) the tissue, easily removable in the unfixed state, becomes tightly cemented to the filter. If the tissue is scraped off the filter with considerable difficulty, a clear, translucent, sharply outlined spot is disclosed, apparently corresponding closely to the area of adherence (Fig. 24). The greater translucency of the spot with respect to the rest of the filter suggests that some material has filled the pores in this region, thus increasing its optical homogeneity. This impression is strengthened by exposure of such spotted filters to trypsin solution, in which the spot disappears (Figs. 24-27) after 5-10 minutes at room temperature if the solution is made with crude trypsin powder (Difco 1 : 250), more slowly but just as definitely when the solution is made with twice recrystallized trypsin. The spot stains variably but sometimes strongly with the Schiff reagent following oxidation by periodic acid

(Hotchkiss procedure) (Fig. 28), and toluidin blue localizes in it fairly sharply but orthochromatically in sections (Fig. 29).

Thus, in the filter above the living spinal cord, a material appears to collect which gives at least some of the characteristic reactions for glycoprotein and behaves in most ways as would be expected of embryonic ground substance. What lends some significance to these sketchy and essentially preliminary observations is the fact that the area of origin of tubules in nephrogenic mesenchyme placed on a filter with living dorsal cord below appears to conform closely to the clear spot revealed by fixation. Mesenchyme placed on a fixed and thoroughly washed spot spreads, but shows no tendency to form tubules. To decide whether the material constituting the spot is involved in the induction, either as an essential pathway for the transit of the active agent or through its own morphogenetic activity, will require additional data.

What is the significance of the results we have been describing for understanding the mechanisms of embryonic induction? Certainly, they reemphasize that in speaking of embryonic induction we probably are referring not to a single kind of process always proceeding by the same mechanism, but to several processes each of which may involve different combinations of mechanisms. The three systems which have been partially analyzed in these investigations clearly are quite different from each other. Cartilage induction differs from the other two, because the induced tissue arises at some distance from the inductive source, and after a considerably longer time interval. The morphogenesis of salivary epithelium and kidney tubule formation occur more rapidly, and appear to depend upon a much more intimate relation with the inductive tissue. Yet, these two are different, too, in that salivary epithelial morphogenesis so far seems to be strictly specific in its requirement for salivary capsular mesenchyme, while kidney tubule morphogenesis is induced by tissues other than the normal inductor: salivary epithelium and embryonic spinal cord. Particularly interesting is the difference between the activity of dorsal and ventral embryonic spinal cord as inductors of cartilage and kidney tubules. Here the mechanisms are not only clearly different, but appear to be in some sense inversely related, the dorsal cord being a strong inductor of kidney tubules but virtually inactive for cartilage, while the ventral cord is strong for cartilage but much less active for kidney tubules.

The relatively high degree of specificity shown by these reactions is noteworthy. The term "specificity" in this context refers to the degree

to which inductive activity is limited to what is assumed to be the normally operative inductive tissue. In none of the three cases is there anything approaching the low specificity of the neuralization reaction in amphibia. Even in the kidney reaction only two abnormal inductors, i.e. salivary epithelium and embryonic spinal cord, have been found, and their activity may be based upon an undiscovered property which they share with each other and with ureteric bud epithelium, the normal inductor. In none of the three cases has morphogenesis been promoted by killed tissues. We can only speculate as to why the degree of specificity should be so high here in contrast to the puzzling and frustrating relative nonspecificity of the neuralization reaction. Certainly to be considered is the fact that these reactions occur later in development, are in fact so-called secondary and tertiary inductions. It seems in some sense reasonable, and it conforms to data in other areas, that interactions should become more specific in their requirements with rising levels of complexity, differentiation and integration.

This, however, is more rationalization than reason, and we certainly want to know more about the mechanisms involved. In this connection the conditions under which the several reactions take place may be relevant. As previously noted (Grobstein, 1952; Grobstein and Zwilling, 1953), the conditions at a glass-clot interface have strong morphogenetic consequences, cellular masses tending to flatten and spread peripherally on the substrate. The filter-clot interface has very similar effects. In contrast, the usual procedures for amphibian material, involving implants into the whole embryo or explants in fluid medium, afford considerably greater opportunity for regulative reorganization. The enforced simplicity and uniformity of behavior at strongly orientative interfaces may mean that under these circumstances we are dealing with something more closely approximating "first-order" reactions, and that specificities are then revealed which are obscured in the more complex morphogenetic reactions undergone by amphibian gastrular ectoderm in combination with test inductors. Essentially, this suggests that apparent nonspecific inductors of neuralization in amphibia may be operative not directly but indirectly, by setting up conditions within the mass which lead to specific tissue interactions essential to neuralization. This would be in consonance with observations on mouse and chick primitive streak material (*Ibid.*) cultured at interfaces, where neuralization could be inhibited by sufficient disaggregation and nonspecific inductors were without effect. Determination of the validity of such speculation must await further data.

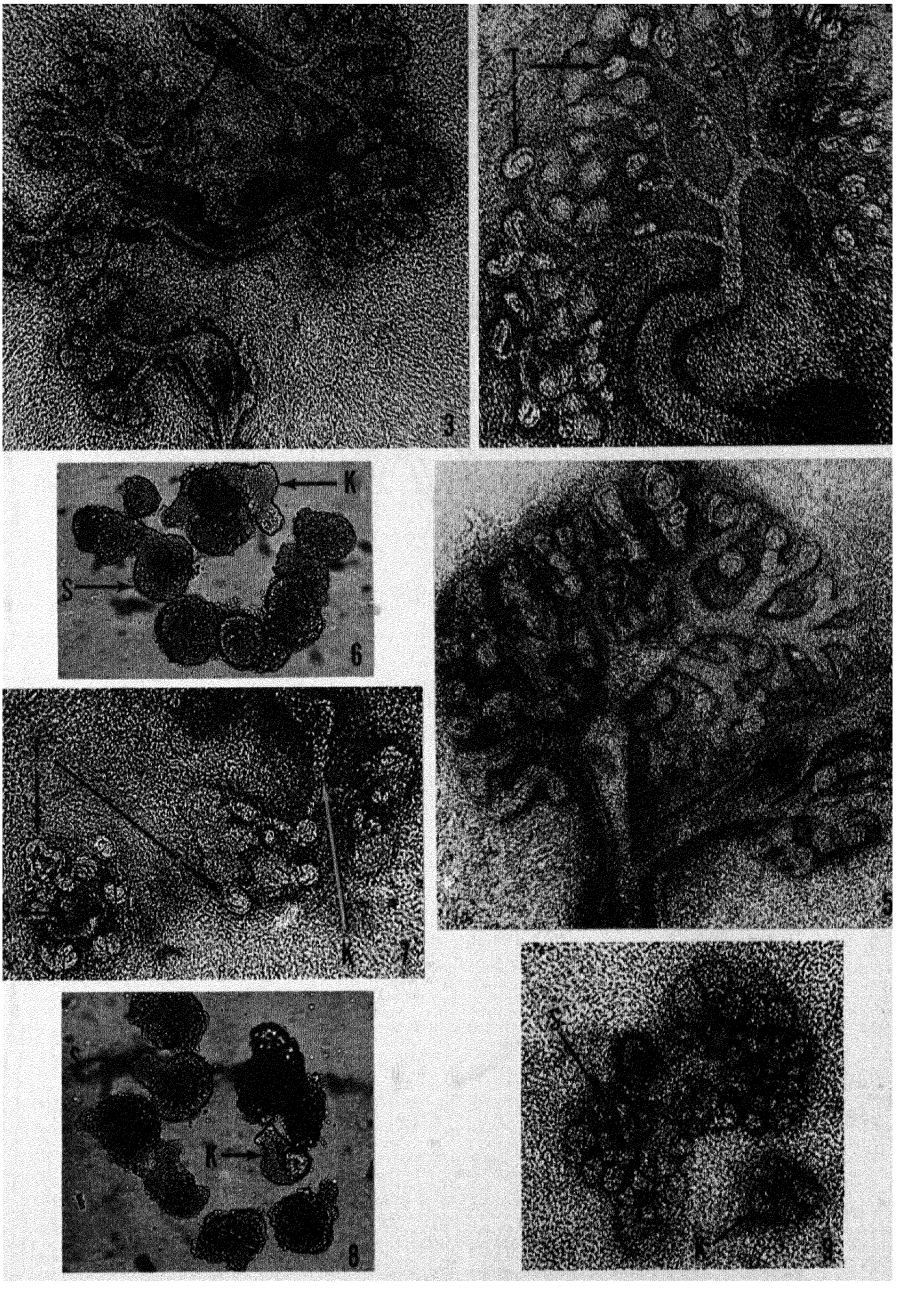


Fig. 3. Thirteen-day submandibular salivary gland rudiments (4) on third day in culture. Characteristic pattern of branching ducts and terminal adenomeres.

Fig. 4. Eleven-day metanephric rudiment on sixth day in culture. Note coiled secretory tubules (T) which form in mesenchyme and link up with collecting tubules derived by branching of ureteric bud.

Fig. 5. Eleven-day lung rudiment on fifth day in culture.

Figs. 6-9. Cross-combination of salivary and kidney epithelial and mesenchymal components. Figures 6, 7. Both types of epithelium in kidney mesenchyme at 0 and 3 days respectively. Figures 8, 9. Both types of epithelium in salivary mesenchyme at 0 and 3 days respectively. S = salivary

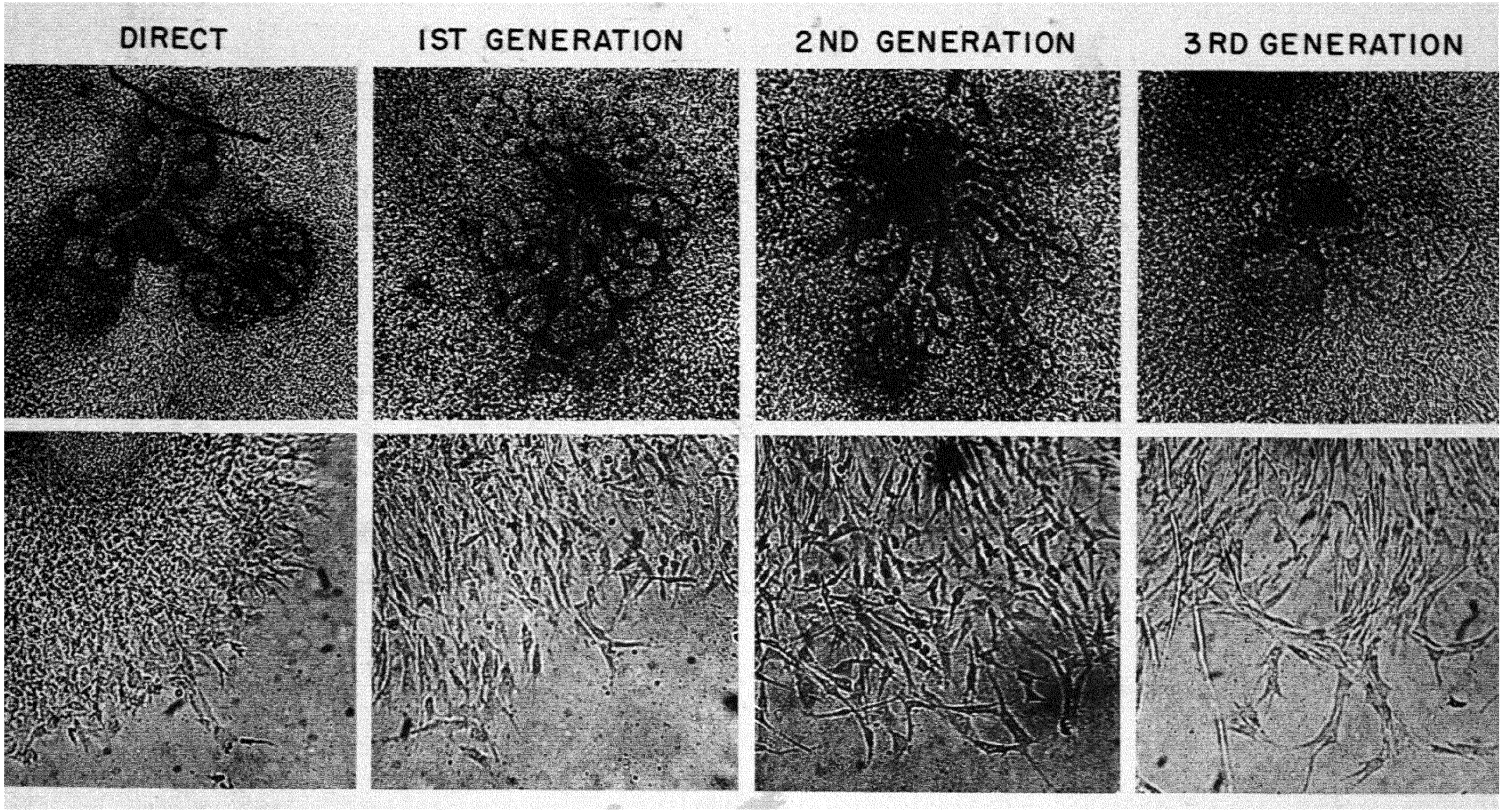
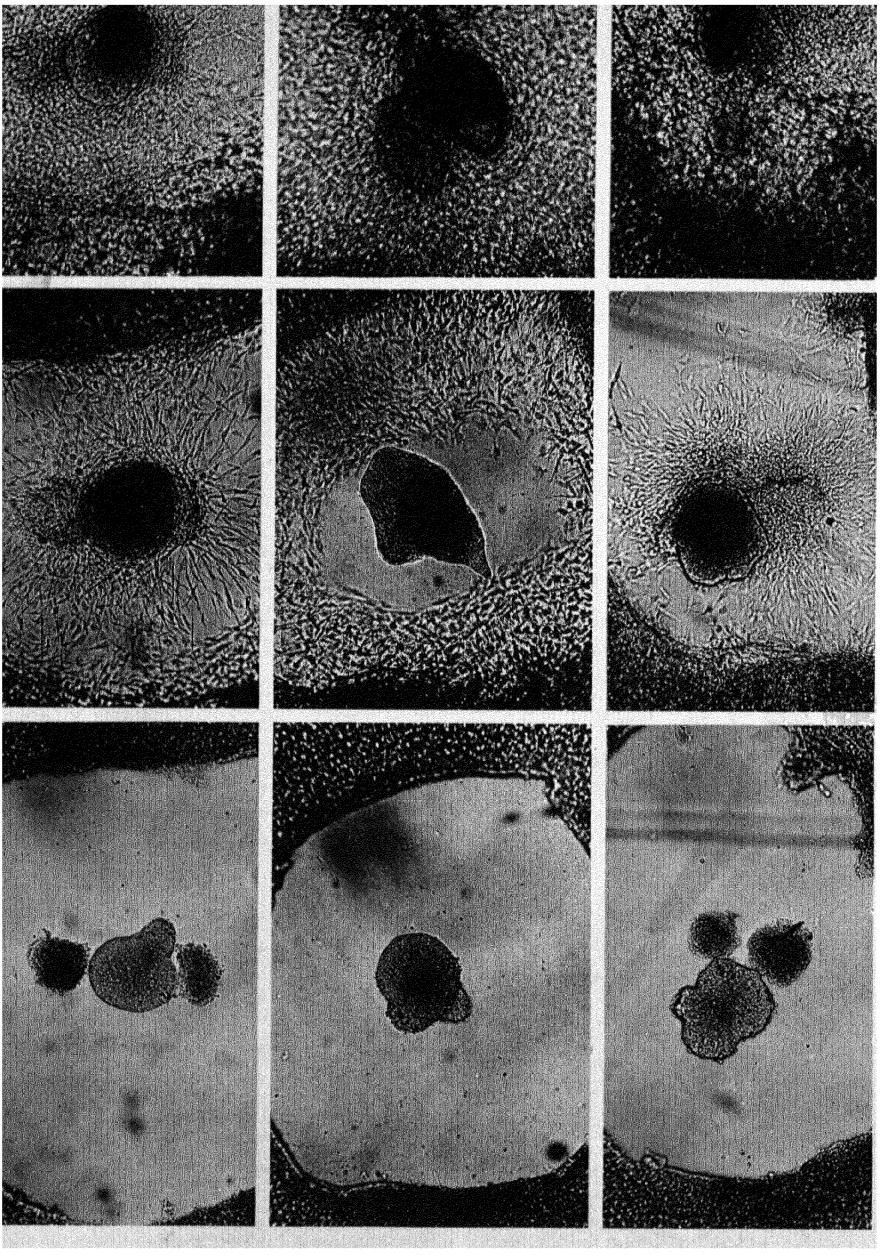


Fig. 10. Combination of freshly isolated salivary epithelium with mesenchyme freshly isolated or pre-cultured through 1-3 successive 5-6 day culture generations. Upper row, epithelial behavior 3-5 days after combination; lower row, culture periphery showing degree of mesenchymal cohesiveness.



Figs. 11-13. Simultaneous combination of salivary epithelial rudiment with its own capsular (autogenous) and maxillary (heterogenous) mesenchyme.

Fig. 11. Before incubation.

Fig. 12. After 40 hours in culture, epithelium undergoes morphogenesis on autogenous side (left), remains quiescent on heterogenous side (right). Note difference in mesenchymal outgrowth character on two sides of line of juncture.

Fig. 13. On fifth day.

Fig. 14. Tubule formation around dorsal 11-day embryo spinal cord in metanephric mesenchyme. Three days in culture.

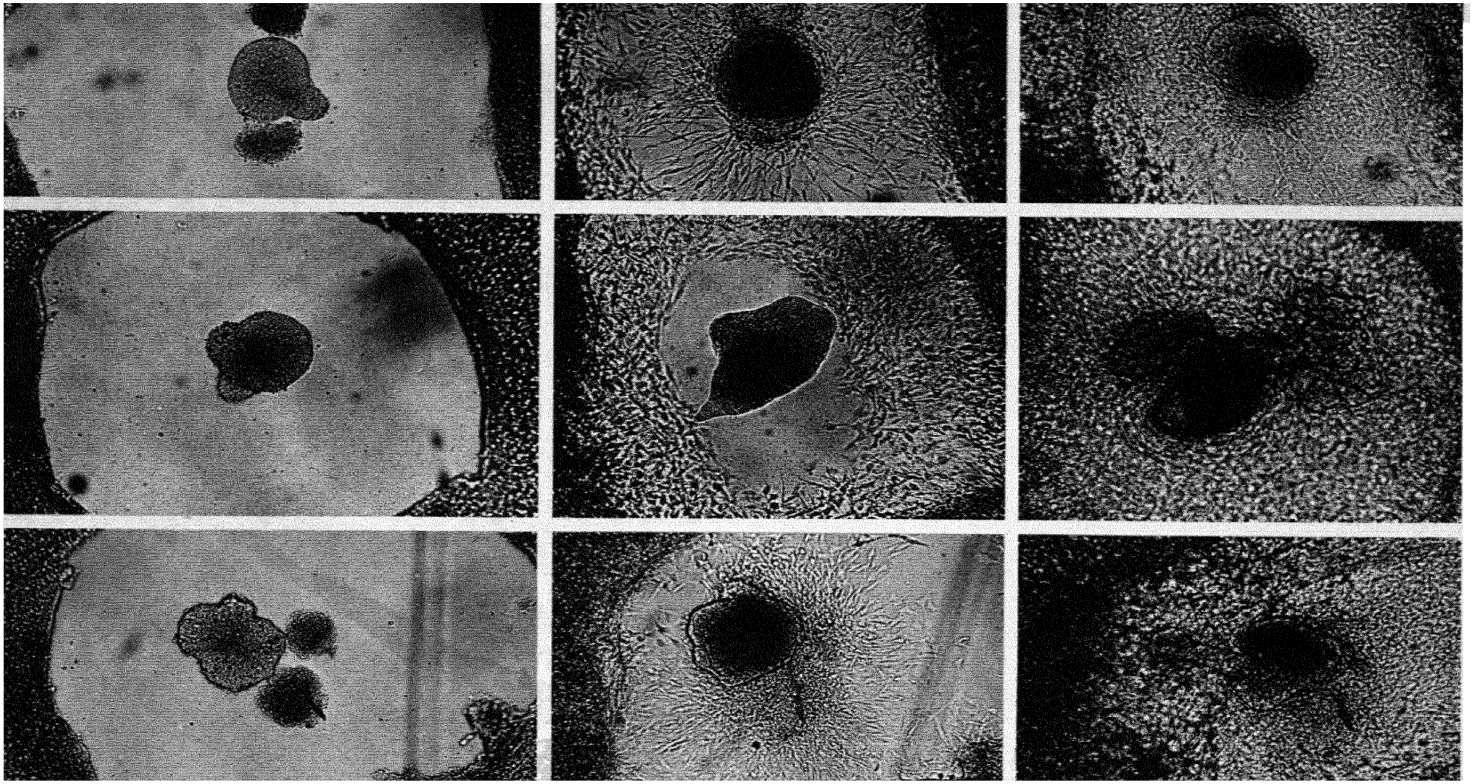


Fig. 16. Interaction of autogenous and heterogenous mesenchyme in their effects on salivary epithelial morphogenesis. Top and bottom rows, epithelium and heterogenous mesenchyme fragments in gap cut in pre-cultured homogenous mesenchyme. Middle row epithelium alone in gap. Left column, before incubation; middle column 16-24 hours of incubation; right column 42 or more hours of incubation. Further explanation in text.

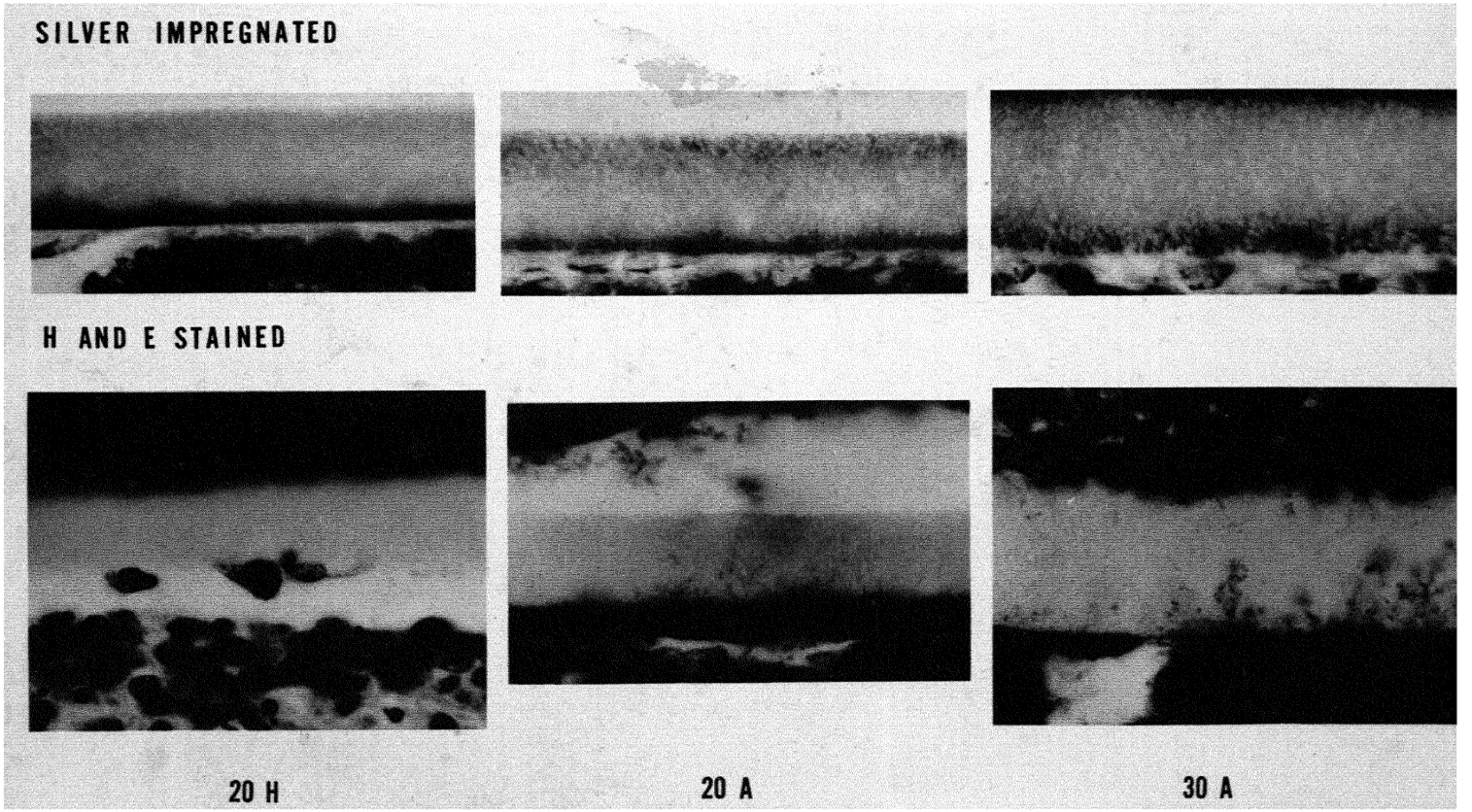
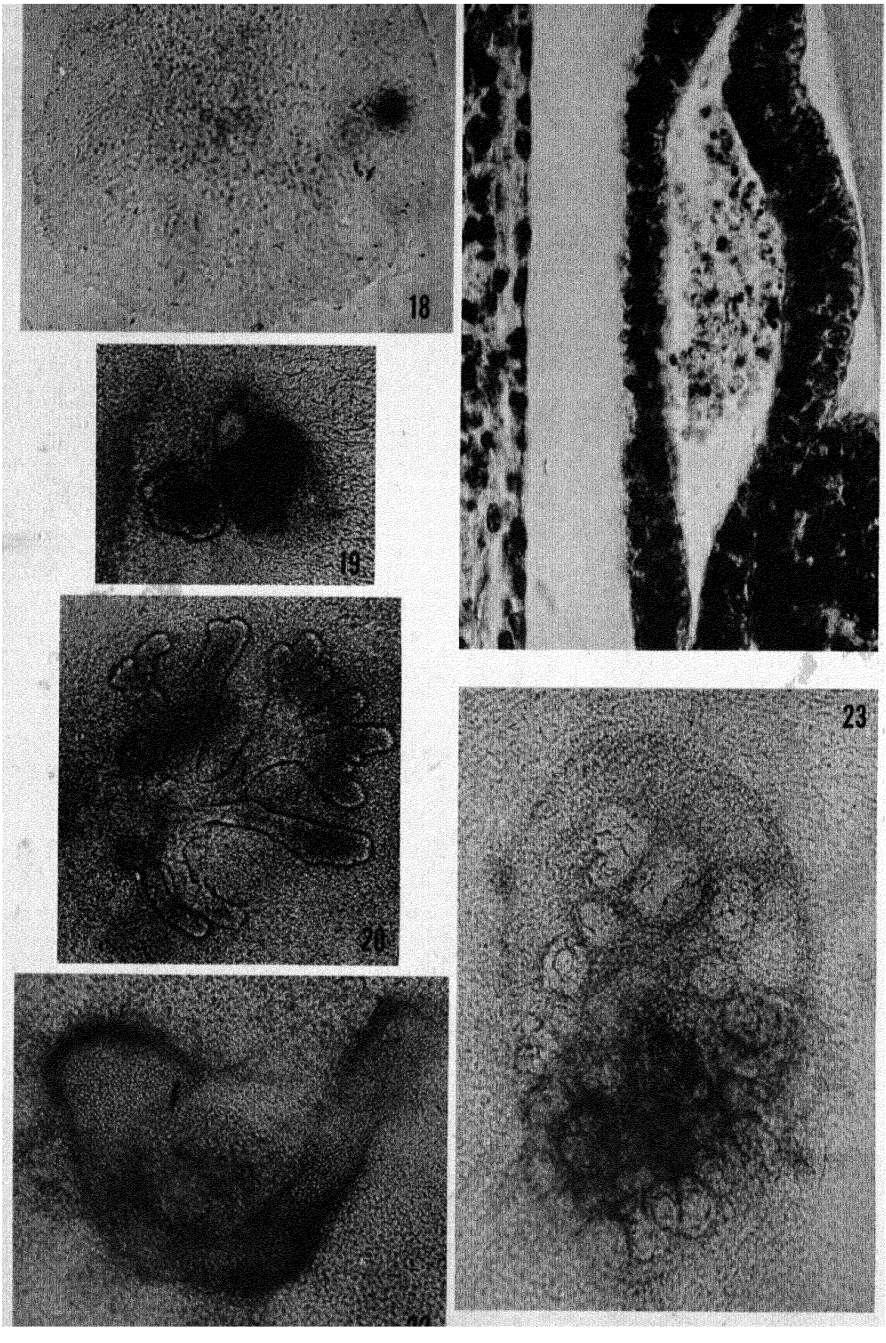


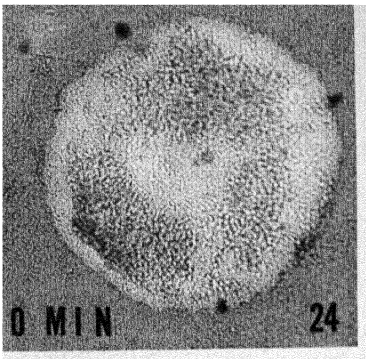
Fig. 17. Porosity and process penetration of three samples of membrane filters. 20H = hydrosol, 20 microns thick; 20A = aerosol, 20 microns thick; 30A = aerosol, 30 microns thick.

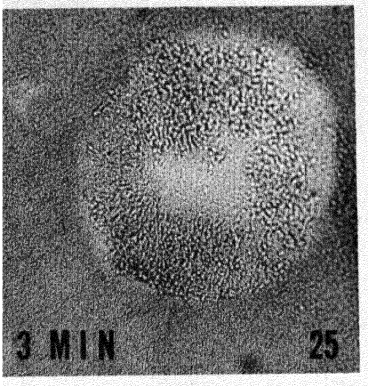


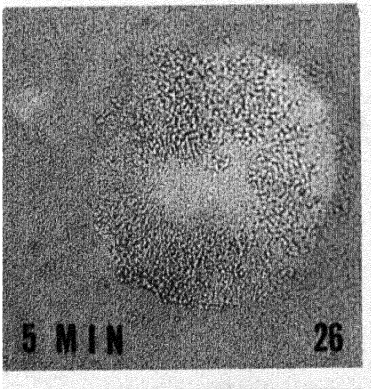
Figs. 18-20. Salivary epithelial behavior on filter with no mesenchyme (Fig. 18) heterogenous (Fig. 19) or autogenous (Fig. 20) mesenchyme on opposite side.

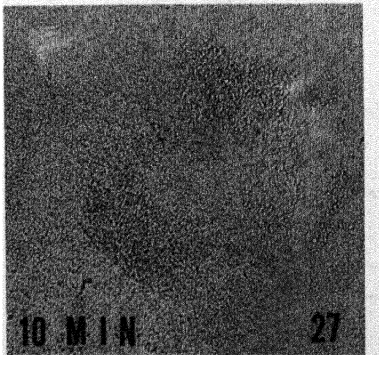
Fig. 21. Stained section through autogenous mesenchyme (left) and salivary epithelium (right) with filter (20H) between.

Fig. 22. Cartilage appearing in somite material on opposite side of filter from 11-day embryo









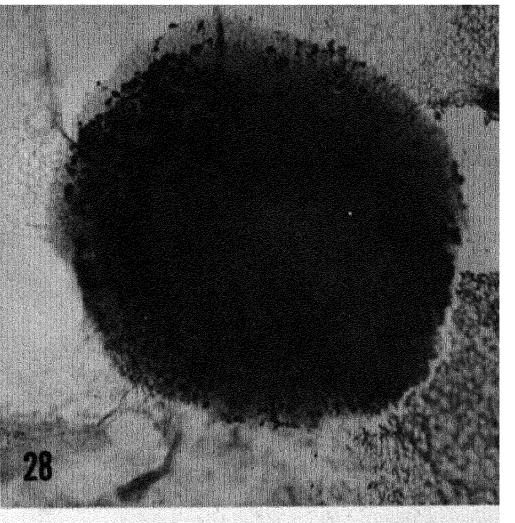


Fig. 28. "Spot" stained with periodic acid—Schiff procedure gives purplish color with adhering tissue fragments deep blue.

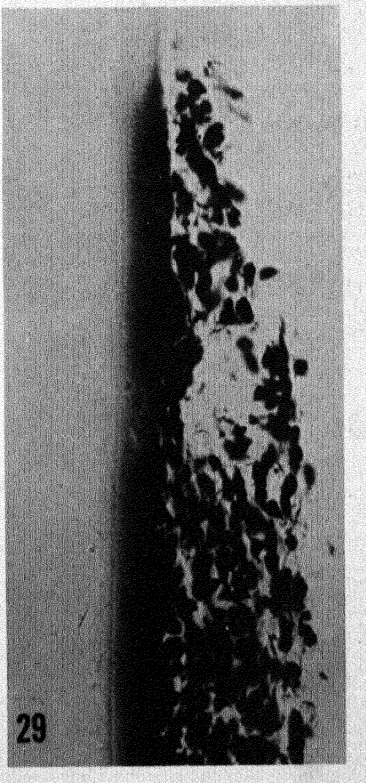


Fig. 29. Toluidin blue staining in filter to left of spinal cord disappears abruptly at edge of cord (top).

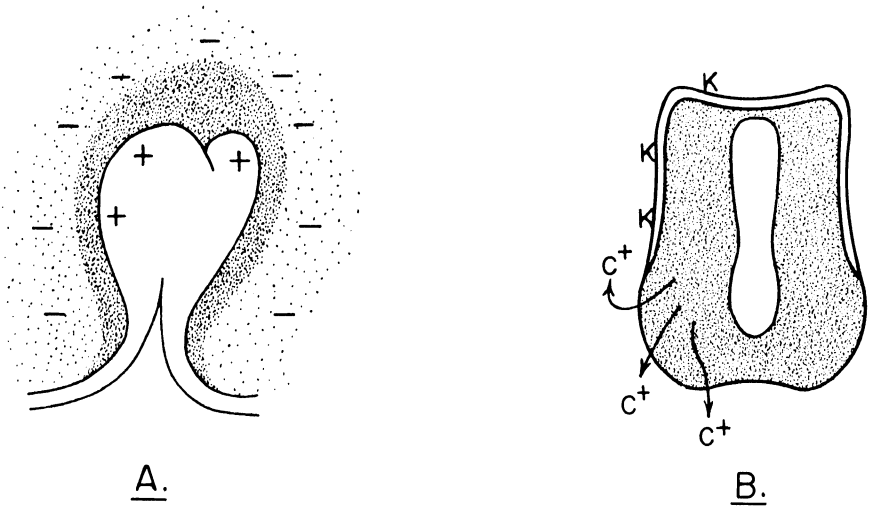


Fig. 30. Schema of possible interactions in salivary and cartilage inductions. A. Salivary epithelial morphogenetic tendency (+) insulated from inhibitory effect of general mesenchyme (—) by barrier of condensed capsular mesenchyme. B. Motile cartilage-promoting influence (C) from ventral cord is limited in distribution or effect by non-motile material on dorsal surface which can also induce kidney tubule formation (K) when in contact with metanephric mesenchyme.

What seems clear at the moment is that in all three inductive systems analyzed in these investigations, and apparently also in the chordamesoderm-gastrular ectoderm system reported by Brachet and Hugon de Scoeux (1949), at least some component of the inductive mechanism can pass filters that exclude whole cells or massive direct surface contact between them. In the case of kidney tubule induction by embryonic spinal cord, on which most though still incomplete data are available, it is difficult to see how either short-range surface orientative effects based on direct cell-to-cell contacts or long-range freely diffusing materials can be involved. In this case, as in the salivary system, any postulated mechanism must provide for sharp localization without direct massive surface-to-surface contact of cell layers. The cartilage case is different in that the occurrence of the end point, cartilage formation, at a distance from the inductor requires the assumption of considerably greater mobility in some component of the mechanism. Even here, however, an uncomplicated assumption of diffusion hardly seems satisfactory, since it fails to explain the non-cartilage-forming area immediately around the inductive source. The proviso that cartilage formation occurs only at some submaximal concentration of the agent is logically possible, but without experimental support at the moment.

The notion seems worth entertaining that what is passing across the filter is not simply solutes but a structured material involving at least proteins and complex polysaccharides. Such a notion conforms with a number of observations, though it cannot be said to be demonstrated by any of them. The evidence includes: the correlated decline of cohesiveness and inductive capacity noted in cultured salivary mesenchyme, the fixing and staining reactions of the "spot," the restrictive effect on inductive interaction exerted by porosities far greater than would be expected to limit even the largest molecules if freely dissociated, and the sharp localization of inductive effects through the filter.

The speculation, once entertained, directs attention toward the intercellular matrix or embryonic ground substance and its morphogenetic role. Such consideration is by no means new. Baitsell (1925), although not the first to call attention to the ground substance, strongly emphasized its all-pervading distribution in the embryo in early stages. He saw it as a homogeneous, gelatinous material secreted by the cells within which the various specialized fibers of the adult gradually condensed. He emphasized the guiding role of the ground substance and its fibers as a substrate for cell motility, and called attention to the early recognition of the relation of this role of the ground substance to that of the clot in the be-

havior of cells in tissue culture. Gray (1926) also attributed great significance to matrix or ground substance, noting its developmental continuity with surface layers in the egg and its stabilizing effect both on individual cells and epithelial layers.

Weiss (1933) has stressed the morphogenetic importance of the ground substance, saying that "The interior architecture of the body, as expressed by the arrangement of cells and intercellular formations, is to a certain extent determined by the ultrastructural organization of the colloidal continuum which fills the interior of the organism." He has supplied experimental evidence for the orientative role of colloidal exudates on the "outgrowth" of nerve fibers from embryonic nervous tissue in vitro (Weiss, 1945), and has suggested that comparable material, differing in composition in different organs, may be broadly operative in morphogenetic mechanisms. In particular, he has emphasized the notion that advancing cells and their processes only progress on surfaces previously wetted by their own exudates. Snessarew (1932) and Polejaev (1940) have also both given great weight to the structure of the matrix or ground substance as important in morphogenetic mechanisms and tissue architecture, and Willmer (1945) has discussed the cement substances in relation to the behavior of cells in tissue culture and pointed to their probable glycoprotein character. Holtfreter (1948) has referred to intercellular cement substances as possibly involved in the affinities and disaffinities of embryonic cell layers.

Recent developments in allied fields (Duran-Reynals, 1950) have heightened interest in the role of intercellular matrices, and the glycoproteins or mucopolysaccharides which appear to be their characteristic component. Studies of inflammation and resistance to systemic infections have emphasized the varying permeability of the adult ground substance under particular conditions and its capacity to act as a barrier to the passage of materials ranging in complexity from simple electrolytes to highly organized particulates such as bacteria. Chemical, enzymatic, and pharmacological studies have demonstrated the complexity of the glycoproteins, their high level of polymerization, and their lability under varying physiological conditions. Immunochemical and immunogenetic studies indicate antigenicity and high specificity in complex polysaccharides, as well as sensitivity of these substances to genotypic alterations (e.g., the familiar DNA-induced genetic transformation in pneumococcus involving alteration in the serological specificity of the capsular polysaccharides: Braun, 1953). Finally, there is the evidence of involvement of similar

materials in virus penetration of cells (Luria, 1953) and the mechanisms of fertilization (Runnstrom, 1949; Tyler, 1949).

A material with the properties implied by even this sketchy listing offers explanatory possibilities to the investigator of morphogenesis and induction. A labile intercellular continuum, locally alterable in penetrability and other properties by physiological shifts, endowed with a high degree of specificity, closely responsive to the genotype, able through polymerization to condense to higher levels of order thus providing boundaries and interfaces, seems to be exactly the missing piece required in many puzzles of development. Affinities and disaffinities in cellular aggregation and disaggregation, mass cellular movements such as stretchings and foldings, gradient and field phenomena, regulation, mutant developmental abnormalities, all might find a rationale at least in part in such a complex bio-physico-chemical matrix.

Against this speculative background we may look more closely at what is labelled matrix interaction in our earlier diagram of possible inductive mechanisms (Fig. 1). What is suggested by the diagram for consideration is that induction may involve interaction between the intercellular materials or matrices of cells, rather than direct interaction between cells themselves in the sense of exchange between cytoplasms or contact of their cytoplasmic boundaries. In saying this one must keep in mind that cellular boundaries are not always sharp and that the distinction between hyaloplasm and matrix in many situations may be primarily conceptual. Matrix interaction would be assumed to be a governing relationship rather than an exclusive one. Each homogeneous cellular mass is thought of as embedded in a common continuum within which there are no discontinuities but within which there may exist graded properties. The properties of the continuum, assumed to be produced by the cells, naturally would be in part dependent upon the cellular properties. But since the continuum provides the immediate environment of the cells (Duran-Reynals, 1950, has pointed out that it is the cell-significant part of the internal milieu), the properties of the cells would be in part dependent upon the properties of the continuum.

Two dissimilar cell masses making contact would be assumed to do so via their matrices (Fig. 1, "Matrix interaction"). Where these (A) were completely incompatible (non-interpenetrable), an interface would be formed, and exchange would be limited to materials which could pass through both matrices. In this situation there would be no adhesion, and the cellular masses might be expected to exert relatively little influence

on each other and might even reseparate. Where the matrices (B) were compatible (interpenetrable), adhesion would occur, and essentially a new matrix would be created between the two which might have very different properties, and which in turn would alter the properties of the interacting cell layers. Altered levels of polymerization, as a too-simple example, might lead to different tensional states on the interacting versus the non-interacting sides of a cell layer, resulting in a folding. As a third, and quite interesting, possibility (C) interactions at the molecular level might lead to formation of qualitatively new elements not represented in either of the original matrices. These interactions might be one source of the "something new" continually appearing and stabilizing in development, specifically, for example, (Baitsell, 1925) the formation of such bounding and hence stabilizing structures as basement membranes.

These three possibilities are indicated only to give some concreteness to the notion we are discussing. There are many other ways in which matrices might interact, including molecular complementariness, as has been suggested by Weiss (1947) and Tyler (1947) for cell surfaces. In fact, as with so many notions founded largely on ignorance, all sorts of subsidiary *a posteriori* assumptions can be made on the matrix model to explain nearly anything.

The central point, which does relate to what we know at the moment and seems useful in explaining the results described, is the notion that the intercellular matrix may be at least an essential intermediary in inductive processes. In the salivary and kidney inductions, explanation in terms of long-range movement of freely dissociated molecules appears unsatisfactory in view of the sharp localization, and yet, direct cellular contact over large surfaces seems to be excluded from consideration by the trans-filter activity. The matrix notion supplies a satisfactory intermediate.

The possible role of direct transmission of materials via cellular processes, considered by Brachet and Hugon de Scoeux (1949), has not been excluded. However, if Weiss' contention that migration of cellular processes only follows surface wetting by exudate (matrix) is accepted, and many of our own observations support the contention, even for processes to enter the filter a preliminary penetration by matrix would be required. It is possible then that processes in the filter, rather than being involved in the mechanism, are only indicators of the degree of activity of the cell surface and hence of the presence and character of the matrix.

From what is known of adult matrix or ground substance it seems possible that embryonic matrix may be either negatively or positively

significant in developmental interactions, i.e., it may itself in some situations *impose* morphogenetic reactions or *transmit* their effective agents or it may, in other situations, block passage of morphogenetically active materials, thus acting as an isolating mechanism.

On this basis the data which have been presented might be brought together in the following speculative scheme (Fig. 30). In the salivary system there are three differing tissues to be considered: (1) the epithelium whose tendency to form dichotomously branching tubules can be disrupted by direct exposure to a surface; (2) the "condensed" specific or autogenous capsular mesenchyme immediately surrounding the epithelial bud which at least protects and may promote its morphogenetic tendency; (3) the general mesenchyme which inhibits the morphogenetic tendency of the epithelium, possibly because of an overly strong anti-spreading effect. The relations between these three, as diagrammed in Fig. 30, immediately suggest a possible barrier role for the capsular mesenchyme, protecting the morphogenetic tendency of the epithelium from an inhibitor present in the general mesenchyme. The appearance and consistency of the capsular mesenchyme are in conformity with the supposition that it involves a different, and possibly more gelated, matrix from the surrounding general mesenchyme, but there are not as yet enough data on the salivary trans-filter effect to provide any support for the notion that it is this material which is involved.

Similarly, a diagram of the morphogenetic activity of the spinal cord suggests interesting possibilities. The reciprocal relation of chondrogenic and nephrogenic inductive capacity in the cord, plus the apparent localization of the factor involved in the former in contrast to the mobility of the latter, admits the following interpretation, although other possibilities certainly exist. Under this assumption, the glycoprotein matrix covering the more dorsal regions of the cord is involved in the kidney tubule induction but impenetrable to the passage of the mobile cartilage-inducing factor produced in the ventral regions. The tubule-inducing activity of the more dorsal regions of the 11-day cord, revealed in experimental situations, has no obvious *in vivo* consequence at this stage since no nephrogenic tissue remains in its immediate vicinity. Thus, an activity of possible significance in earlier association (with more lateral mesoderm in medullary plate stages) serves as an indicator of the presence of a material of quite different significance in its new associations. The new associations are with the massed mesenchyme of the somite and effects might be expected, under the hypothesis, in the behavior of this tissue. One of these effects may be to pattern chondrification by blocking

or otherwise inhibiting the process in regions where it would otherwise be set in motion by the mobile, cartilage-inducing influence emanating from the ventral cord. In this way it may contribute to the ventro-dorsal sequence of cartilage formation in the axial skeleton, and to the formation of a neural canal of appropriate configuration and dimensions. In this assumed behavior it would be acting in a directly analogous way to that suggested for salivary capsular mesenchyme, to limit and control the distribution of a mobile influence with morphogenetic effects. These assumptions, of course, are not to be taken too seriously as to detail. They are discussed only to indicate more concretely what is in mind in suggesting a morphogenetic role for the matrix, and how the concept may apply to the particular data we have been considering.

Since speculation has dominated what I have just been saying, I should like in summary to repeat the factually better-grounded major points. First, *in vitro* methods afford advantageous experimental approaches to at least three inductive systems in mammals. Second, in these three systems more than one kind of inductive mechanism appears to be operating. Third, the reactions studied show a high degree of specificity, i.e., with few exceptions they are called forth only by living tissues expected to be involved in them. Fourth, that though all three can be mediated through filters whose porosity excludes massive cell contact, there is evidence in two systems that the effects nevertheless are short-range and quite localized. Fifth, the evidence at the moment invites attention to the idea that embryonic ground substances or intercellular matrices play an important role in inductions. In this last lies the possibility that embryonic induction is not simply an enigmatic *event* peculiar to early development, but the beginning of a *process* which establishes and stabilizes relations among groups of cells and their matrices—relations which, becoming increasingly fixed in what we recognize as structure, persist into the adult and are important in the maintenance of its tissue architecture.

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XI. HUMORAL AGENTS IN INSECT MORPHOGENESIS

BY DIETRICH BODENSTEIN[1]

GROWTH in insects finds its external expression in molting. But molting always involves differentiation, for at each molt when the animal passes from one stage to the next many of its structures have to be formed anew. The complex cuticular elements of the larval skin, for instance, and a great part of the tracheal system, are rebuilt from instar to instar. The most profound changes always occur at the last larval stage, when with the final larval molt the immature animal metamorphoses into the imaginal form. This is true for insects that metamorphose at the last molt directly into the adult (hemimetabola), and for insects in which a pupal stage intervenes between the last larval stage and the imago (holometabola). Yet it is important to recognize that whether a larval or a metamorphosis molt occurs, morphogenetic events of great complexity always take place.

The evidence accumulated within the last two decades suggests that molting and metamorphosis in insects are controlled by hormones. Since the problem of molting and metamorphosis is manifestly one of growth and differentiation, it can be said that it is through the mediation of a humoral system that these morphogenetic happenings are triggered and regulated. The following narrative is dedicated to an analysis of these matters. Of necessity the presentation will be short and limited to a few representative examples that may help to document and clarify the various problems under discussion.

I. THE HUMORAL CYCLE IN CONTROL OF MOLTING AND METAMORPHOSIS

When after a molt the larva maintains its juvenile characteristics, the molt is called a larval (or nymphal) molt. But when the larva changes with the molt into an adult individual, either directly or through the intervention of a pupal stage, this molt is spoken of as a metamorphosis molt. In these happenings, as mentioned above, hormones play a decisive role. Not only do hormones induce molting, but specific endocrine combinations decide also whether the target material responds with a larval or a metamorphosis molt. The mechanism of action of the humoral cycle that

[1] Medical Laboratories, Army Chemical Center, Maryland.

decides which of these two alternatives is realized is illustrated in Fig. 1 and can be summarized shortly as follows.

Humoral activity is inaugurated periodically by a stimulus or stimuli about which we know rather little. In some instances this stimulus is nervous in nature (Wigglesworth, 1934). It provokes the secretion of a hormone by special neurosecretory cells found in the pars intercerebralis

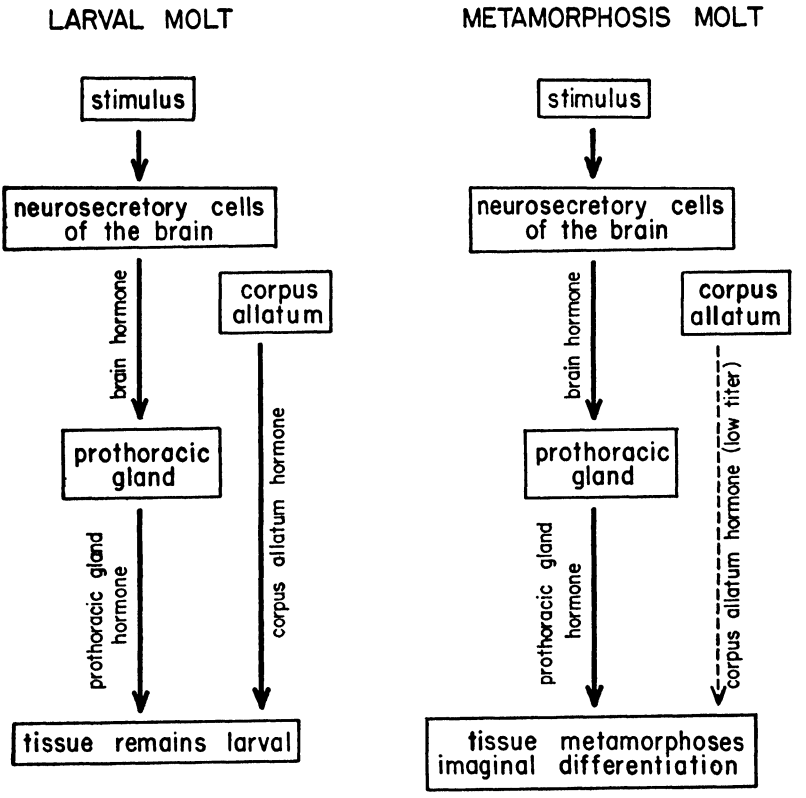

Fig. 1. Diagrammatic representation of the humoral mechanism responsible for the larval molt and the metamorphosis molt.

of the brain. This brain hormone in turn activates the prothoracic gland, an organ of internal secretion usually found, as the name implies, in the prothorax of the immature insect. The secretion of this gland acts directly on the target system and induces molting. Under the influence of the prothoracic gland hormone, there is first set into motion a wave of proliferation in the epidermal cells; the mitotic wave thus created provides the necessary condition for molting. Because of this growth promoting effect, the prothoracic gland hormone has been called a growth hormone.

Under the sustaining effect of the prothoracic gland secretion, abundant growth and finally differentiation occur. This leads finally to the deposition of a new cuticle, which is imaginal in character when the prothoracic gland hormone alone is present; the animal thus undergoes a metamorphosis molt. Evidently the prothoracic gland hormone controls imaginal differentiation as well as growth, and it has therefore been named growth and differentiation hormone. A low titer of prothoracic gland hormone apparently causes growth, while a higher titer sets the processes of differentiation into motion. However, the humoral threshold at which the target responds with differentiation depends upon the age of the reacting material; younger tissues seem to need a higher prothoracic gland hormone titer than older ones for the differentiation response.

Larval molts occur when the action of another gland of internal secretion, the corpus allatum, is added to that of the prothoracic gland. The corpus allatum modifies in some manner the action of the prothoracic gland hormone and guarantees the formation of larval characters at the ensuing molt. Thus a larval molt takes place. The participation of this gland in the molting cycle prevents metamorphosis and is responsible for the maintenance of the juvenile features of the animal. For this reason the allatum hormone is also called the juvenile hormone. But the presence of the prothoracic gland hormone is a prerequisite condition for the normal function of the corpus allatum hormone. It must be understood that the process of molting, whether it is a larval or a metamorphosis molt, is under the control of the prothoracic gland hormone. The endocrine activity of the corpus allatum gradually decreases during the last larval stage, leaving the mature larva with a low allatum hormone titer. At this time and stage, the allatum hormone is virtually ineffective. It is also possible that the activity of the prothoracic gland in relation to that of the corpus allatum is higher in the last stage than in younger stages. However this may be, there is a shift in the balance between these two hormones in the last larval stage in favor of the prothoracic gland hormone. The last stage larva, being under the predominant influence of the prothoracic gland hormone, is caused to metamorphose. Having fulfilled its function, the prothoracic gland degenerates after metamorphosis. Lacking this gland, the adult insect is unable to molt again. Other glands of internal secretion do not break down during metamorphosis. The corpora allata, for instance, together with other endocrine organs, play an important part in controlling the developmental events concerned with egg maturation.

At this place in the discussion it must be pointed out that the scheme

of humoral activity related above, though undoubtedly true in its general aspects, is an oversimplification. There is a real possibility that other glands not mentioned above may be involved in the cycle. The corpus cardiacum is one example. Its participation in the molting process has been revealed recently in experiments on cockroaches, but its precise mode of action is still quite ambiguous (Bodenstein, 1953b). One other great difficulty in evaluating accurately the role played by one gland in the cycle is posed by the fact that the various glands are apparently interdependent in their function. Although much is known, more is still obscure, and many experimental facts are difficult to reconcile with our present interpretation.

II. EXAMPLES

Nothwithstanding these complications, the scheme presented approaches rather closely the real state of affairs, as will be seen from the experiments to be reported below. Space limitation allows for only a few examples. They are selected to document the specific action of the two main glands involved—the corpus allatum and the prothoracic gland—and at the same time to demonstrate some of the operative methods used in this kind of experimentation.

A. Experiments demonstrating the role of the corpus allatum. One will recall that the corpora allata lose at the last larval stage much of their activity and that as a consequence the animal metamorphoses. By extirpation of the corpora allata of younger stages, the same situation can be created experimentally. Such allatectomized individuals may then be expected to metamorphose prematurely. When such an experiment is performed, this is exactly what happens. The removal of the corpora allata from young silkworm caterpillars at the fourth stage, i.e. one stage before the last, prevents the continuation of larval molts, and the caterpillar metamorphoses prematurely into a small but otherwise perfect moth. Very small adults can be produced when the allatectomy is carried out on caterpillars one stage younger (Fukuda, 1944). Essentially the same results can be obtained by removal of the corpora allata from cockroach nymphs younger than the last stage. Here again, instead of undergoing a larval molt, the immature animals metamorphose prematurely, giving rise to diminutive adults (Scharrer, 1946).

If, as assumed, the corpus allatum produces the juvenile hormone, the transplantation of corpora allata from young larvae into last stage larvae should prevent metamorphosis. That this actually takes place is shown convincingly by the following experiment. By transplanting the

corpora allata from young (3rd or 4th stage) nymphs of the walking-stick insect into last stage nymphs, as many as four additional nymphal molts can be produced before the animal metamorphoses. When metamorphosis finally occurs, the resulting adult may be twice as large as a normal imago (Pflugfelder, 1939). The same result can be obtained by a similar experiment on the tropical bug *Rhodnius*. When the corpora allata of a 4th instar nymph are transplanted into the abdomen of a last stage nymph, the host animal molts; but instead of becoming an adult, it retains its nymphal character and develops into a giant 6th stage nymph (Wigglesworth, 1936). The metamorphosis inhibiting effect of the corpus allatum can also be demonstrated by an entirely different experimental procedure, namely, by combining in parabiosis two cockroach nymphs. If one combines a last stage nymph with an individual one stage younger, then at the molt of this pair the corpora allata of the young partner prevent the metamorphosis of the older, which becomes a giant nymph (Bodenstein, 1953a).

B. Experiments demonstrating the role of the prothoracic gland. It has been stated above that the prothoracic gland hormone is responsible for growth (molting) and differentiation. Proof for this contention is ample and will now be discussed. The case to be taken up first concerns the imaginal discs of the fruit fly *Drosophila*. The larva of *Drosophila* carries within its body small groups of cells which represent the anlagen of the future organs, such as wings, legs, eyes, and so on, of the adult organism. These organ rudiments, or imaginal discs as they are called, are already present in the youngest larva. They grow in the course of larval life and differentiate during the pupal period; that is, they are transformed at this time into adult structures. The growth of these discs during larval life is considerable. They thus provide an excellent test object for growth responses. It is possible to remove these discs from the larva and to transplant them into the body cavity of adult flies, where bathed in the body fluid of the adult organism they maintain themselves perfectly but do not grow. One must remember here that the adult insect possesses no prothoracic gland, it having degenerated during metamorphosis. The cessation of the growth of the transplanted discs may well be the reflection of the lack of prothoracic gland hormone. If this is true, the discs should be induced to grow again if supplied anew with prothoracic gland hormone. This hormone can be provided by transplanting simultaneously with the discs a so-called ring gland. This larval structure is a compound gland and contains as part of its make-up the equivalent of the prothoracic gland. When this experiment is performed, the

transplanted imaginal disc continues to grow and if left long enough in its new adult environment develops to imaginal completion. Not only the growth of imaginal discs but also that of other larval structures, as for instance the larval salivary gland, is under the control of this hormone (Bodenstein, 1943a and b).

The effect of the prothoracic gland hormone on growth and differentiation is also well illustrated in tissue culture experiments of the germ cells of the giant silkworm *Platysamia cecropia.* When spermatogonia and spermatocytes of this species are cultured in a hanging drop of blood taken from pupating larvae—i.e. from individuals whose blood contains prothoracic gland hormone—one observes a multiplication of spermatogonia and a maturation of the sex cells into well differentiated spermatids. No growth or differentiation takes place when blood from diapausing pupae—that is, blood that contains no prothoracic gland hormone—is used for the culture medium (Schmidt and Williams, 1953).

A variation of the previously mentioned parabiosis experiments on cockroaches is the healing together of isolated body parts of insects. This procedure performed in moth pupae has been used to good advantage to demonstrate the effect of the prothoracic gland hormone on the differentiation of imaginal characters. Thus it has been shown that if the abdomen of a pupa is severed from the thorax immediately after pupation by means of a ligature, the isolated pupal abdomen is unable to develop further and remains permanently in the pupal stage. If the same operation is made on somewhat older pupae, the isolated abdominal parts continue their development to imaginal completion. Early isolated, and thus arrested, abdominal parts can be made to complete their imaginal differentiation by fusing them to a complete pupa or to an anterior pupal part (Bodenstein, 1938). An uninterrupted hormone flow for a certain length of time from the anteriorly located prothoracic gland is thus necessary for the normal completion of imaginal differentiation. Proof that the prothoracic gland is actually the initiator of these happenings has been given by transplanting this gland directly into arrested abdominal parts (Fukuda, 1941).

As far as the induction of molts by the prothoracic gland is concerned, one experiment on *Drosophila* will suffice. The anterior tip of a newly emerged *Drosophila* larva containing the mouth armature with its two conspicuous mandibular hooks was transplanted together with several ring glands into the abdomen of an adult fly. After an appropriate time the transplant was removed and studied. It was found that the trans-

planted small larval fragment had molted twice, as indicated by the presence of three complete sets of mouth parts. The three pairs of mandibular hooks were especially striking signs of the graft's molt. In the control experiment, where the larval fragment was transplanted without the ring glands, no molt had occurred; only one set of mouth hooks, namely those contained in the fragment at the time of the transplantation, were found (Bodenstein, 1944). The necessity of the ring gland for the initiation of the larval molts in *Drosophila* was thus indicated. Other experiments have since shown that the prothoracic gland part of the ring gland must be held responsible for these effects.

III. HUMORAL BALANCE

Frequently one finds in nature curious monstrosities, insects which seem to be partially larval and partially adult. Such freaks of nature have always interested the student of teratology. Not until some understanding of insect endocrinology was achieved, could any satisfactory explanation as to the causes of these abnormalities be given. Now one can produce such creatures practically at will. They occur when the normal humoral balance between corpus allatum and prothoracic gland hormone is disturbed. If at any time in the larval period of the insect the hormone balance is shifted—as for example, in favor of the prothoracic gland hormone—the animal will usually possess prematurely adult characteristics in the molt occurring after the shift. This does not necessarily mean that the entire insect will become prematurely adult. If the hormone balance has not shifted too far in the direction indicated above, the insect will still retain some of its juvenile characteristics. The more the prothoracic gland hormone is favored in the balance shift, the more pronounced the adult characteristics will be. By appropriate experimentation one can obtain a graded series of intermediates that ranges from purely larval to completely adult forms. The untimely appearance of adult structural patterns can obviously be caused either by an abnormally high prothoracic gland hormone titer or by an abnormally low corpus allatum hormone titer; the same is true, in reverse, for the juvenile pattern. It is difficult in most instances to ascertain which of these two alternatives actually has occurred; but from the stage of the larva at which the upset occurred and from the kind of the intermediate pattern produced, one can determine quite accurately in which way the humoral balance has shifted. The following examples will illustrate how the humoral balance can be disturbed experimentally in order to effect the production of intermediate forms.

The first experiment consists of transplanting corpora allata from younger stages into successively older last instar wax-moth (*Galleria*) caterpillars. The appearance of larval characters at the pupal molt was then used as a criterion for the presence of corpus allatum hormone. It was found that the earlier in the last stage the transplantation took place, the more pronounced were the juvenile characters that appeared after the ensuing molt (Piepho, 1942). The explanation for the relative ineffectiveness of the grafted corpora allata in older stages, as far as the control of juvenile characters is concerned, must be found in the changed internal environment of the older hosts. At the end of larval life, the prothoracic gland hormone has apparently reached such a high titer as to render the corpus allatum hormone impotent. In the earlier transplantations, however, the grafted glands made the allatum hormone titer abnormally high and thus shifted the endocrine balance in favor of this hormone. This resulted in a shift of the morphological pattern towards the larval side.

The second experiment was performed on silkworms. Here a functioning prothoracic gland was transplanted into young last stage caterpillars in order to make them molt precociously. Since it was known from other experiments that the corpora allata of young last stage larvae still produce juvenile hormone, it was expected that after the prematurely induced molt some larval characters would be maintained. This is exactly what happened. The operated individuals, instead of giving rise to pupae, formed larva-like creatures which possessed various organs of pupal type. Those individuals that molted less precociously showed fewer larval characteristics (Fukuda, 1944).

The most important outcome of these studies on the humoral balance has been the realization that the various hormone systems are at all times in the postembryonic life of insects in a definite balance which is stage and time specific, and that any disturbance of this balance may lead to severe structural and metabolic alterations (Bodenstein, 1953c).

IV. HUMORAL PERIODICITY

So far the impression has been given that the hormone level rises at the end of each stage to an effective titer and that after the molt it declines and remains low until it rises again as the next molt approaches. In other words, the hormone activity seems to be cyclic. The question arises: is there any time in the inter-molt period in which there is no hormone in the system? The answer is in the negative. Ap-

parently the hormones are never completely exhausted. This can be demonstrated as follows.

The adult female cockroach possesses a glandular accessory sex structure, which might be called a shell gland. It produces a proteinaceous secretion that is needed for covering of the egg capsule or oötheca. A mature female lays in the course of her life a number of such oöthecae, each of which contains many eggs. The shell gland appears during metamorphosis and is made up of a great number of finely branched tubules. In the newly metamorphosed adult, the tubules are empty; they fill with the secretion product within the first two days of adult life. The production of this material is under the control of the corpus allatum hormone. If the hormone production is cut short by cutting the newly metamorphosed insect in half by means of a ligature, the shell glands in the isolated abdominal parts remain empty. Now if nymphal or adult corpora allata are transplanted into these young isolated abdominal fragments, the secretion of the shell gland continues normally and within two days the tubules are filled (Bodenstein, unpublished). The formation of the secretion product in the tubules thus provides a good measure for the presence of corpus allatum hormone in the system. It is possible to transplant small bits of empty shell glands consisting of only a few tubules into the abdominal cavity of intact animals. When such transplantations are made, using different nymphal stages as hosts and choosing for the transplantation different times within the intermolt periods, it appears from the evidence so far collected that there is at all times enough corpus allatum hormone in the insect to sustain the formation of the secretion product in the transplanted glands (Bodenstein, unpublished). From this one must conclude that corpus allatum hormone seems to be present in the system at all times—perhaps sometimes in a low, but always in a detectable, titer.

The test for the presence of prothoracic gland hormone needs as background information some short comments on regeneration. The cockroach nymph regenerates its legs with great ease. Autotomized at the coxa-femur level, a complete and almost normal sized leg is regenerated already at the molt following the amputation. The adult cockroach, of course, is unable to regenerate its legs. One can make an adult cockroach molt again by the transplantation of prothoracic glands. If the leg of an adult individual is amputated and the animal then induced to molt by prothoracic gland transplantation, leg regeneration takes place. The processes of regeneration are therefore under the control of

the prothoracic gland hormone (Bodenstein, unpublished). The entire progress of regeneration, from its earliest beginning almost to completion, can be observed through the rather transparent organ stump (the coxa) in the living animal. When nymphal legs are amputated at successively later times within an intermolt period, the regenerate becomes visible about eight days after the amputation, regardless of the time the amputation took place. Since the initiation of regeneration is only possible in the presence of prothoracic gland hormone, it follows that a high enough titer of this hormone to ensure leg regeneration must be present in the animal throughout the entire inter-molt period. Therefore one must conclude that the insect maintains throughout postembryonic life, except in the adult stage, a certain level of prothoracic gland hormone. Other experiments have shown that the regeneration response of nymphal tissues requires a lower titer of prothoracic gland hormone than does that of adult tissues (Bodenstein, unpublished). This brings us to the problem of the responsiveness of the target material towards humoral stimuli.

V. THE REACTING MATERIAL

The premature metamorphosis of young larval stages has been discussed above. It shows that the formative material can be made precociously old, if one may state the matter thus. The question whether one can make adult material revert to its larval state, that is, make it young again, has not been answered. Can one take the ability of an adult individual to molt and regenerate again as an indication of rejuvenation? Perhaps one can, for these two attributes of the responding tissues are strictly juvenile characteristics. The problem is complex. For instance, if one transplants skin from an adult cockroach into a young nymph, the transplant can undergo in synchrony with its new host four molts before the host metamorphoses. In all these molts, however, the adult graft, though molting, remains adult—this in spite of the fact that the humoral situation in the host called for a nymphal molt (Bodenstein, 1953a). There are other instances with like implication. All the evidence shows that adult structures can be made to molt again, and in doing so repeat their own adult pattern. They are apparently unable to revert to their juvenile state. This last point might not be entirely true; it has been reported (Wigglesworth, 1940) that if the corpus allatum titer is raised experimentally much above the normal level, some tissues might change back to their juvenile state. In such instances the adult animal, after having molted, contains small patches

of nymphal cuticle. Summarizing, one can state that while it is rather easy to age larval material prematurely, only in a limited way is it possible to rejuvenate the adult structure.

Although it is true that humoral factors can produce dramatic developmental effects, it is well to remember that these effects become manifest in the target materials. Variations in the responsive capacity of the target are as important as the kind and concentration of hormones that cause these responses. The morphogenetic characteristics of the developing organism are the results of the interaction of both these factors.

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