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DAVID M. B[illegible]

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Control Mechanisms in Cellular Processes

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Preface

This volume on the control mechanisms in cellular processes consists of papers presented at the symposium of the Society of General Physiologists at its annual meeting in 1960. It will, we trust, prove useful as a report to the diverse mechanisms now known to act as regulatory systems.

Biologists have often felt that biochemists take a homogenized and quite unregulated view of cellular events. In fact, the feeling has been expressed that biochemists view cells simply as sacks full of enzymes. There is, of course, some truth in this statement; the elucidation of biochemical events has indeed required the use of cell-free material. Such investigations, however, have proved singularly rewarding; we now enjoy detailed knowledge of the precise mechanisms involved in energy transfer, in the biosynthetic processes concerned with the formation of substrate molecules, and in the synthesis of such macromolecules as enzymes and nucleic acids. We have come to an era in which fruitful consideration can be given not only to the integration of separate biochemical events but to the coordinate regulation of cellular biochemistry. In addition, consideration can now be given these subject areas, in part at least, at the molecular level.

Multicellular animals have characteristic developmental stages. Growth and development are intrinsic properties of biological systems; the biochemical characteristics of diverse differentiated cells are clearly different. Even in bacteria, biochemical differences have been noted at different stages in their growth cycle. These are biological facts which must be accounted for, and the answer presumably could in part be deduced from an understanding of the systems which regulate cellular biochemistry. What are the regulatory systems known at present?

A classic agent of cellular regulation is, of course, the gene. In the past decade magnificent work from many laboratories has given clear experimental proof that one action of genetic material is the

determination of enzyme structure. The "central dogma," in fact, states that the gene (DNA) carries the code of the amino acid sequence of its product enzyme and that a specific messenger RNA transfers this code from DNA to the site of enzyme formation. Thus, both DNA and RNA might be considered as regulatory elements of the cell. Discussions of regulation by the nucleic acids are presented in two chapters. It is now clear, however, that the action of all genetic material cannot be accounted for simply in these terms. Genes have been found which control quantitative aspects of enzyme formation. Enzyme repression has been known for a number of years, but it has recently been shown that repression is genetically determined by a locus distinct from the locus controlling the structural characteristics of the formed enzyme. Distinct genetic elements therefore appear to control the qualitative and quantitative characteristics of enzyme formation. Enzyme repression together with its genetic control thus represents a system of major interest in terms of regulatory mechanisms; this is treated in the second chapter.

All regulation is not directly gene-controlled. Control of biochemical reactions and biochemical sequences by feedback provides an elegant mechanism for the maintenance of metabolic balance. This topic is discussed in a separate chapter. The biochemical basis of regulation by hormones is receiving intensive attention at present, as are regulatory mechanisms in glycolysis. Two chapters deal with hormonal regulation in animals and in plants; another examines regulation in energy metabolism. Light and time as regulatory factors are additional fields of active inquiry; discussions of each are included.

As convener of this symposium, I should like to express my appreciation to the contributing authors for their cooperation in the preparation of this volume. On behalf of the Society of General Physiologists, I wish to express appreciation to the National Institutes of Health, United States Public Health Service, whose financial support made possible the symposium and this volume.

DAVID M. BONNER

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Control Mechanisms in Cellular Processes

1

Genetic Control of Enzyme Structure[1]

SIGMUND R. SUSKIND[2] and CHARLES YANOFSKY[3]

Introduction

Some of the basic concepts for our present-day understanding of the gene-enzyme relationship were formulated by Garrod in the early 1900's, on the basis of studies of human inborn errors of metabolism (Garrod, 1923). These concepts were further developed and experimentally supported by Beadle, Ephrussi, Tatum, and others in studies with eye color mutants of *Drosophila* and by other workers in studies of anthocyanin formation in plants (Wagner and Mitchell, 1955). It was because of the difficulties in performing biochemical studies with existing material that studies with the ascomycete, *Neurospora crassa*, were begun by Beadle, Tatum, and their co-workers. These studies as well as later investigations with *Escherichia coli* and other microorganisms led to the elucidation of numerous biosynthetic pathways. More important, from a genetic standpoint, they offered repeated confirmation of a basic relationship between gene, biochemical reaction, and the enzyme catalyzing the reaction. As you know, this relationship formed the basis for the "one gene and one enzyme" hypothesis which was developed and expanded by Beadle, Tatum, Bonner, Horowitz, and others (Beadle, 1959; Tatum, 1959). It is this relationship of gene to enzyme that we wish to discuss, particularly with regard to the problem of genetic control of enzyme structure.

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It is clear that a number of different types of gene control over enzyme formation and function exist. Mutations do cause qualitative changes in specific enzyme proteins. However, genetic effects on the quantitative aspects of enzyme formation are also observed. Enzyme levels in the cell and the time and rate of appearance of enzymes are often affected by gene change. In addition environmental factors come into play in interactions with the genome in the regulation of enzyme formation (Bonner, 1959; Bonner *et al.*, 1960). Some of these manifestations of gene control will be discussed by other participants in the symposium (Vogel, 1961). Furthermore, there is evidence for both intergenic and intragenic interaction at the enzyme level as seen in studies on suppressor gene action, partial reversion, and complementation (Fincham, 1959; Yanofsky and St. Lawrence, 1960).

From the studies with many gene-enzyme systems in diverse organisms, and particularly from current investigations with human hemoglobins (Ingram, 1958; Singer and Itano, 1959), a concept of the relationship between gene and protein has emerged which is providing a working basis for future explorations in molecular genetics. As a result of studies on DNA transformations in bacteria, on the biochemistry of phage infection and replication, and on the physical, chemical, and enzymatic studies of DNA structure, a model of the genetic material as a replicating complementary two-stranded structure has evolved (Crick, 1958). This structure is thought to contain, in its specific nucleotide sequence, the coded information corresponding to the amino acid sequences of specific proteins. A gene, then, defined in these terms, is a polynucleotide segment which controls the primary structure of a protein. This certainly does not exclude the possibility that genetic information also specifies secondary and tertiary structure.

On the basis of the limited experimental evidence available and considerable theory, the current interpretation of the role of the gene in protein synthesis can be summarized as follows: Base-coded information in DNA is transferred to RNA which participates with ribosomal or microsomal components in the determination of the primary structure of specific protein (Hoagland *et al.*, 1959). Amino acids are enzymatically activated and transferred to specific soluble RNAs. These amino acid-RNA complexes then enter the ribosomes and are oriented in or on the ribosomes in a sequence specific for a particular protein, thereby reflecting the "information" originally

present in the specific DNA segment or gene. When all the amino acids are present in the proper sequence, synthesis can then proceed. Hence, in theory, these are the events leading from a gene to a specific protein. In view of the complexities of genetic effects, it is remarkable that a reasonably clear-cut, though perhaps naive, picture of the relationship between gene and enzyme is available. If the DNA nucleotide sequence is responsible for the proper sequence of amino acids in a protein, then events (mutations) which exchange, delete, or add nucleotides in DNA might be expected to cause the formation of altered proteins. Such altered proteins could differ from the normal molecule in physical, chemical, or catalytic properties (Horowitz, 1956; Fincham, 1959; Yanofsky and St. Lawrence, 1960) and even in amino acid sequence. From studies on the human hemoglobins (Ingram, 1958; Singer and Itano, 1959), on several microbial enzymes (Yanofsky and St. Lawrence, 1960; Garen, 1960) and on certain viral proteins (Dreyer and Streisinger, 1960), it appears that mutations may in fact cause amino acid substitutions in a protein. These amino acid substitutions may in turn result in a change in the secondary and tertiary structure of the protein, by this means affecting the properties of the protein molecule.

Generally, two types of nucleotide substitution mutations have been considered (Crick, 1959): (1) a "mis-sense" type, which results in the specification of a wrong amino acid and (2) a "nonsense" type, in which no amino acid is specified. In the "mis-sense" type, an altered protein would be formed, while in the "non-sense" type, since no information is provided for one of the "linking" amino acids, no protein or perhaps only fragments of a protein would be formed. Attempts to detect both categories of mutational effects at the protein and amino acid level would seem to be essential for an understanding of mutation and the DNA-coding problem.

At present, the approaches to the study of mutational effects on proteins are in a somewhat more advanced state than analogous studies with DNA. However, there are many promising approaches to the problem of "decoding" DNA including studies on "incorporation" and "replication" mistakes using base analogs (Benzer and Freese, 1958; Freese, 1959), enzymatic degradation of DNA and RNA and separation of oligonucleotides (Lehman, 1960), and the use of antibody prepared against different types of DNA (Levine *et al.*, 1960; Thomas, Mobley, and Suskind, 1961).

Nevertheless at present, the most fruitful approach to the analysis of DNA alterations appears to be an indirect one—the genetic analysis of mutants which produce altered proteins (Benzer, 1957; Yanofsky and St. Lawrence, 1960). This approach should reveal whether or not there is a linear correspondence between genetic sites and amino acid sequence. Furthermore, from such studies it is hoped that evidence can be obtained relating specific genetic damage to specific modifications of an enzyme (Suskind, 1957b).

It is this approach, using the tryptophan synthetase (Tsase) system in *N. crassa* and *E. coli*, with which this paper will be concerned. The studies described have been carried out at Yale by Dr. Bonner's group, at Stanford, and at Johns Hopkins. This paper, in attempting to present an over-all picture of the tryptophan synthetase system, has drawn on the work of many investigators in these laboratories, including Drs. J. De Moss, Y. Suyama, and A. Lacy; Drs. I. Crawford, P. Lerner, B. Maling, D. Helinski, M. Rachmeler, and J. Stadler; and Drs. W. Mohler, M. Carsiotis, and Mrs. D. Ligon.

Experimental

The material we would like to discuss can best be considered in several sections: (1) A comparison of the *N. crassa* and *E. coli* tryptophan synthetase (Tsase) systems. Studies with these systems offer a unique opportunity to correlate the properties of an enzyme catalyzing the same reactions in two different microorganisms, with mutations occurring at specific sites within well-defined genetic regions. (2) Progress in studies of the effects of mutations on the coli and Neurospora enzymes at the protein and amino acid level. (3) Some of the intergenic and intragenic interactions affecting the function of genetically damaged tryptophan synthetase.

Three reactions are catalyzed by the wild type Tsase of Neurospora and coli (Yanofsky, 1960). These reactions are shown in Fig. 1–1.[4] The physiological essential reaction appears to be reaction 1.

In Neurospora these three reactions are catalyzed by a single protein, having a molecular weight of about 140,000 (Mohler and Suskind, 1960). In coli the enzyme system can be readily dissociated

[4] The following abbreviations are used in the text: B_6al PO_4 = pyridoxal phosphate; Tsase = tryptophan synthetase; CRM = cross-reactive material; In = indole; InGP = indole glycerol phosphate; Ser = L-serine; and TP = triose phosphate.

into two separate proteins, termed A and B (Crawford and Yanofsky, 1958). The coli A-protein has a molecular weight of about 29,500, while that of the B-protein is not yet known. Physical contact between the A- and B-proteins appears to be required for significant activity in any of the three reactions, although each protein alone has slight activity in one of the reactions (A in reaction 2; B in reaction 3) (Yanofsky, 1960).

Reaction	Catalyzed by
1. Indole glycerol phosphate + L-serine $\xrightarrow{B_6 al\ PO_4}$ L-tryptophan + triose phosphate	Wild-type Tsase and some CRM's
2. Indole glycerol phosphate $\rightleftharpoons$ indole + triose phosphate	Wild-type Tsase and some CRM's
3. Indole + L-serine $\xrightarrow{B_6 al\ PO_4}$ tryptophan	Wild-type Tsase and some CRM's

Fig. 1–1. The reactions catalyzed by wild-type tryptophan synthetase and by certain CRM-proteins in *N. crassa* and *E. coli.*

In both Neurospora and coli, many tryptophan-requiring mutants have been isolated which are defective in their ability to form normal Tsase (Yanofsky and Bonner, 1955a). Genetic analyses have shown that these mutants are all damaged within a small genetic region. In Neurospora this region is called the *td* locus, and in *E. coli,* the A and B genetic regions (Yanofsky, 1960).

Using rabbit neutralizing anti-enzyme, prepared against highly purified preparations of wild-type Neurospora or coli tryptophan synthetase, it is possible to scan extracts of the tryptophan-requiring mutants for the presence of defective proteins which still retain sufficient structural similarity to the normal enzyme to render them serologically cross-reactive (Suskind *et al.*, 1955; Suskind, 1957a; Lerner and Yanofsky, 1957).

In Neurospora and coli, many mutants are found which contain cross-reacting material, called "CRM," although a number of mutants do not (Suskind, 1957a; Lerner and Yanofsky, 1957). This latter class are termed "CRM-less mutants." Thus, the Tsase mutants can be divided into two different groups simply on the basis of their ability to form CRM.

Several obvious and important questions arise relating to the finding of CRM's. (1) Are the CRM's mutationally altered proteins? (2) Are the CRM's identical or do they represent a constellation of

specific mutationally altered proteins formed as a consequence of damage to different sites within the Tsase region? (3) Do the CRM-less mutants represent a class of so-called "non-sense" mutations in which information specifying a linking amino acid is absent? If so, perhaps no molecule large enough, or of sufficiently similar configuration to the enzyme, is synthesized which can be detected either by its immunogenic or its antigenic properties. (4) Is there any relationship between the type of CRM protein produced by the mutant and the site or location of specific genetic damage within the Tsase locus?

As a result of detailed study of a number of Tsase mutants in Neurospora and coli, several conclusions can be reached. (1) The CRM's do actually represent genetically altered tryptophan synthetase proteins. This conclusion is based on physical, enzymatic, and immunochemical data (Suskind, 1957a; Suskind and Kurek, 1957; Lerner and Yanofsky, 1957; Yanofsky and Stadler, 1958; De Moss and Bonner, 1959; Mohler and Suskind, 1960; Carsiotis *et al.*, 1960). (2) The CRM's found in the various mutants are distinguishable when a sufficient number of criteria are utilized for comparison (Suskind and Kurek, 1957; De Moss and Bonner, 1959; Yanofsky, 1960; Suskind and Ligon, 1960). (3) There is some suggestion that one CRM-less mutant may form some material related to Tsase. (4) It appears that the location or site of genetic damage within certain regions of the Tsase locus does determine the type of specific altered protein which is formed, and that striking similarities are found between the organization of the Tsase genetic region in *E. coli* and in *N. crassa* (Bonner *et al.*, 1960; Yanofsky, 1960).

Criteria which have been used to distinguish a number of different CRM proteins in *E. coli* and *N. crassa* Tsase mutants follow:

1. Enzymatic activity and substrate-cofactor requirements
2. Acid treatment
3. Heat treatment
4. Metal sensitivity
5. Energy of activation
6. Column chromatography and salt fractionation
7. Immunogenic and antigenic properties
8. Enzymatic activity/antigenic activity ratios
9. Suppressor gene action
10. Complementation.

The last two, suppressor gene action and complementation, will be discussed in a later section of this paper.

In the case of Neurospora, some CRM proteins do not exhibit any enzymatic activity in any of the three reactions which are catalyzed by normal Tsase and which are listed in Fig. 1–1. However, some CRM's still retain the capacity to catalyze parts of the total reaction, for example, reaction 2 or reaction 3 but not reaction 1 (Suskind and Jordan, 1959; De Moss and Bonner, 1959; Yanofsky and Stadler, 1958; Yanofsky, 1960). Among the Neurospora CRM's which catalyze reaction 2, there are three types: (1) those with no pyridoxal phosphate or serine requirement, (2) those with a pyridoxal phosphate requirement, and (3) those with a pyridoxal phosphate and a serine requirement (De Moss and Bonner, 1959; Bonner *et al.*, 1960). There is also a temperature mutant that forms a CRM which can catalyze all three reactions but is metal-sensitive (Suskind and Kurek, 1957). This enzyme has an abnormally high apparent energy of activation for tryptophan synthesis (Suskind and Ligon, 1960). Thus, in each of these instances, mutation has resulted in either a total loss of all catalytic activity, in a partial loss of activity, or in the formation of an enzyme which is fully functional only under unique circumstances. In those cases where the CRM's do retain some catalytic activity, additional substrate or cofactor requirements may be evident.

In the case of the coli mutants, both A-CRM's and B-CRM's are found (Yanofsky, 1960). Hence, one finds mutants which make normal A-protein and B-CRM (or are B-CRM-less), as well as those which form normal B-protein and A-CRM (or are A-CRM-less). It is found that in B mutants with B-CRM, the B-CRM permits full activity of the normal A-protein in reaction 2. In the A mutants which form A-CRM, the A-CRM permits normal B-protein to function maximally in reaction 3. In most cases the affinities of the normal protein or the CRM protein with its associated protein partner appear to be the same (Maling and Yanofsky, 1960). In one case, where an altered B with low affinity for A is found, the addition of serine is required for effective combination (Gibson *et al.*, 1961).

While a number of CRM types have been described and the properties of some have been examined, very little is known at the protein level about the CRM-less mutants mentioned earlier. One CRM-less mutant in *Neurospora*, strain td_1, does form some com-

ponent that behaves similarly to a component in highly purified preparations of wild-type Tsase (Carsiotis *et al.*, 1960). This component can be resolved on starch electrophoresis and by the use of antigen-antibody reactions in agar gel. The latter is shown in Fig. 1–2. Perhaps this component in the CRM-less mutant bears some structural relationship to Tsase.

Clearly, many types of mutationally altered proteins are formed by Tsase mutants, and it becomes of considerable importance to

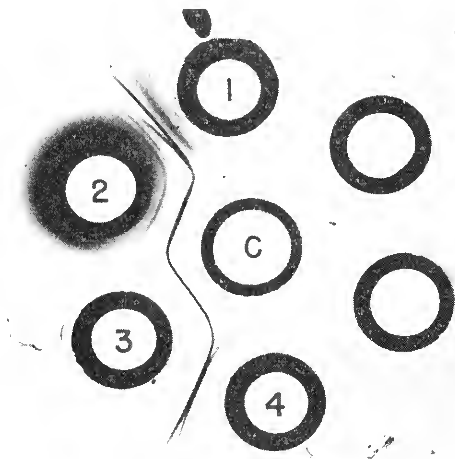

Fig. 1–2. Antigen-antibody reactions in agar gel. Reservoirs: c. anti-Tsase, partially absorbed with fractions of mutant td_1; 1. unabsorbed anti-Tsase; 2. Tsase having a specific activity of 225; 3. Tsase having a specific activity of 1940; 4. unabsorbed anti-Tsase.

establish whether mutants with similar protein damage possess defects in the same genetic region. On the basis of interallelic crosses in *N. crassa* and transduction mapping in *E. coli*, the fine structure of the gene controlling Tsase formation in both organisms is being analyzed. In agreement with the concept of mutations primarily causing substitutions in amino acid sequence, and subsequently in the conformation of the protein, it was found that CRM mutants which produce altered A-proteins with similar properties were clustered in particular genetic areas and were *not* distributed at random throughout the entire Tsase genetic region (Yanofsky and Crawford, 1959; Yanofsky, 1960). From the work of Suyama and Bonner at Yale, it appears that Neurospora CRM mutants which retain the InGP $\rightleftharpoons$ In + TP reaction are localized in one region of the genetic

map, and a mutant retaining the In + Ser → Trypt reaction is at the opposite end. It further appears that the CRM mutants having B_6al PO_4 and B_6al PO_4-serine requirements for the InGP ⇌ In + TP reaction also fall into distinct genetic regions (Bonner *et al.*, 1960).

These results are schematically summarized in Fig. 1–3, which compares the genetic maps and types of mutationally altered proteins in coli and Neurospora. From the data already available, it seems that there is a clustering of mutationally altered sites which

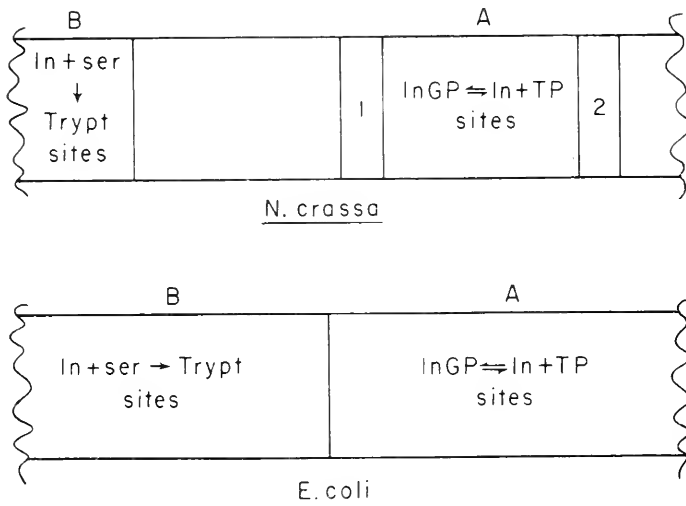

Fig. 1–3. A schematic representation of the clustering of the genetic regions (*td* locus) which control the structure of tryptophan synthetase in *N. crassa* and *E. coli*. *N. crassa*: region 1, pyridoxal phosphate site; region 2, serine site.

give rise to similar phenotypic aberrations (in terms of the type of altered protein synthesized by the organism). Furthermore, there appears to be a remarkable parallelism between the distribution of mutationally altered sites causing similar effects in the two organisms. The localization of Neurospora CRM formation into two major genetic regions is comparable in effect to the localization of the coli A and B genetic areas. In addition, as indicated in Table 1–1, the enzymatic properties of the coli A- and B-CRM proteins can be compared to the enzymatic properties of the different Neurospora CRM proteins; thus, it seems that the organization of the genetic regions controlling Tsase in coli and in Neurospora shows several striking similarities. Further study should prove most informative particu-

larly when more information is available about the detailed structure of the CRM proteins and the normal proteins in both organisms.

Studies on the structure of Tsase at the amino acid level are in progress at Stanford, using the coli A-protein in order to compare the peptide composition of normal A and of different A-CRM's.

The A-protein is a highly purified crystalline protein (Henning, 1960) which behaves as a single component in the ultracentrifuge and on electrophoresis. From the specific activity of the pure material, it can be estimated that the amount of A-protein formed in mutants grown on low levels of indole or tryptophan is of the order of 1–2 per cent of the total extractable protein of the organism

TABLE 1–1

A Comparison of the Enzymatic Properties of the CRM Proteins in *N. crassa* and *E. coli*

N. crassa CRM Types (enzymatic activity still retained)	Corresponding *E. coli* CRM Types			
	Normal A (InGP $\rightleftharpoons$ In + TP)	A-CRM (InGP $\not\rightleftharpoons$ In + TP)	Normal B (In + Ser $\rightarrow$ Trypt)	B-CRM (In + Ser $\not\rightarrow$ Trypt)
1. None		∘		∘
2. InGP $\rightleftharpoons$ In + TP	∘			∘
3. In + Ser $\rightarrow$ T		∘	∘	

(Yanofsky, 1960). As mentioned earlier, many A-CRM mutants have been isolated, and in view of the ease of isolation of the A-protein and its small size, it has received considerable attention using chromatographic and electrophoretic separation methods (the "fingerprinting" technique of Ingram, 1958) and also quantitative amino acid analysis.

A number of A-CRM's and the normal A-protein are being compared in this manner. Preliminary results obtained by Dr. Helinski at Stanford indicate that single peptide differences do exist between the normal and three mutationally altered A-proteins (Helinski and Yanofsky). One can hopefully look forward to information correlating changes in the primary structure of the A-protein with specific genetically damaged sites within the Tsase region.

Studies at the amino acid level with Neurospora Tsase have been hampered by the difficulty of obtaining pure enzyme. Through the work of Drs. Mohler and Carsiotis and Mrs. Ligon, the Neurospora enzyme can now be routinely purified to a very high degree, and a number of the physical properties of the purified protein have been

studied (Mohler and Suskind, 1960; Carsiotis *et al.*, 1960). Fine structure studies on the normal and mutant Neurospora proteins should be possible in the near future. Recently, Neurospora Tsase has been subjected to trypsin digestion in the hopes of differentially inactivating the enzymatic and antigenic activities of the molecule. It was found that wild-type Tsase enzymatic activity can be destroyed rapidly by trypsin, while antigenic activity disappears only after prolonged incubation (Garrick and Suskind, 1960). Hence it is possible to obtain preparations with no enzymatic but considerable antigenic activity. The effect of trypsin on the activity of several CRM's is being examined, and the use of other proteolytic enzymes is anticipated. Since mutations at the *td* locus are able to cause the loss of all or only part of the enzymatic activity of Tsase, it is tempting to hope that analysis of the trypsin-prepared fragments and the antigenically active residue may provide a clue as to those amino acids essential for enzymatic and antigenic activity. Perhaps this approach will also offer a more systematic means for analyzing CRM-less mutants at the structural level.

For some time it has been known that certain allelic strains of Neurospora, which are defective for a particular enzymatic activity, can regain this activity when they are in a heterocaryon—that is, a mycelium containing nuclei of unlike types. This phenomenon, called "intragenic complementation," has been employed in the Neurospora Tsase system to determine to what extent mutants which show different CRM properties will cooperate to form a functional Tsase. Studies at Yale (Lacy and Bonner, 1958; Lacy, 1959) and at Stanford (Rachmeler, 1960) have provided some information on this problem. They have found that tryptophan independent heterocaryons can be formed between four to five groups of *td* mutants. Fig. 1–4 shows the relative order of mutational sites in the *td* locus of Neurospora determined by recombination analysis, as well as a complementation map prepared on the basis of heterocaryon tests.

Several observations concerning the CRM proteins are available from these complementation studies (Bonner *et al.*, 1960). CRM formation is required for complementation, and in several instances the CRM's produced by the contributing mutants are structurally and functionally different. The fact that effective complementation requires CRM formation by *both* participating mutants suggests the existence of repair mechanisms involving specific protein–protein interactions (Woodward, 1960).

In several heterocaryons studied by Rachmeler and Yanofsky, the tryptophan-independent heterocaryons were analyzed for the type of Tsase using enzymatic and antigenic criteria. It appears that these heterocaryons form several types of Tsase-like proteins. They continue to synthesize the parental CRM type, they form enzyme having normal properties, and there is some evidence for a fourth Tsase-like protein. If this fourth protein type proves to be a doubly defective molecule, it would suggest that recombination or reconstitution of some type may also occur at the protein-forming

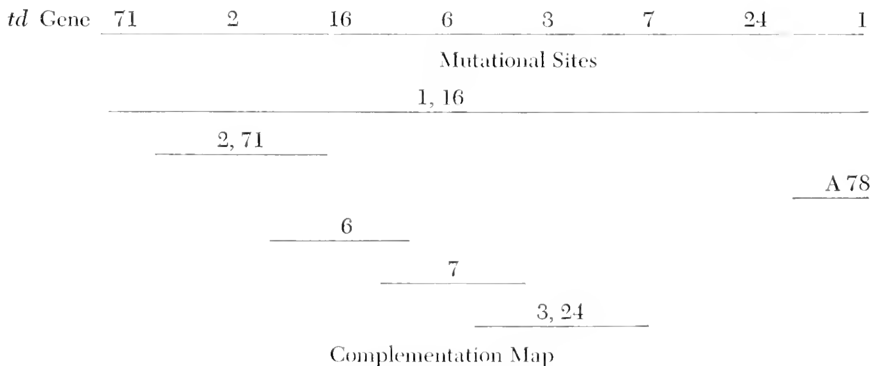

Fig. 1–4. A comparison of mutational site order and complementation maps in *N. crassa*. When two lines in the complementation map overlap, these mutants will not complement. Conversely, mutants which are not in overlapping groups will complement.

level. It should prove of great interest to determine the structure of these heterocaryon proteins and also to assess the effects of relative nuclear input, P^{32} decay, and other factors on the role of nuclear and cytoplasmic elements in complementation.

A second type of interaction can be found in the cases of partial reversion in Neurospora and coli Tsase mutants (Yanofsky and Crawford, 1959; Esser *et al.*, 1960). Reversion in a biochemical mutant is thought to be due to a change at the same genetic site as the original mutation, restoring the original genetic information or nucleotide sequence in the wild-type gene. This change allows functioning of the gene in a normal manner, and normal growth occurs in the absence of a nutritional supplement. In some instances one finds a second type of reversion in which the growth rate of the revertant is slower than the original wild type. These are called "partial reversions."

A number of partial revertants of Neurospora and coli Tsase mutants have been studied, and some have been found to differ from both the wild-type organism and the true revertant. The tryptophan synthetase formed by these strains appears to be specifically altered in terms of its relative enzymatic and antigenic properties (Yanofsky, 1960; Esser *et al.*, 1960). Furthermore, different categories or groups of partial revertants occur, suggesting that "meaningful" alternate nucleotide sequences can exist within a limited genetic region. In addition, perhaps nucleotide changes not confined to the exact site of the original mutation, but located elsewhere in the Tsase gene, can restore a functional protein. Elucidation of the nature of partial reversion is basic to understanding the possible mechanisms of genetic repair and function.

The last type of gene action we would like to discuss concerns the problem of suppressor gene action. A suppressor mutation can be defined as one which phenotypically reverses the effect of the primary mutation but which is itself located at a different genetic locus. In the case of the Tsase mutants of both Neurospora and coli, suppressor genes exert their effect by permitting mutants to form low levels of functional Tsase and so to grow slowly in the absence of exogenous tryptophan (Yanofsky and Bonner, 1955a). However, in most cases the effect is not complete, and growth is stimulated by the addition of tryptophan. Several important questions arise in considering the suppressor gene effect. The first of these is the question of the allele specificity of suppressor genes; that is, do suppressor genes which affect one *td* mutant allele affect others as well? Fig. 1–5, compiled from extensive genetic analyses, shows quite clearly that suppressor genes are able to function only in conjunction with specific *td* alleles (Yanofsky and Bonner, 1955a). It can also be seen that one allele (td_1) is not affected by any suppressor gene (Yanofsky and Bonner, 1955a). Furthermore, repeated occurrences of the suppressor-2 mutation can be isolated, and these all exhibit identical allele specificity (Yanofsky and Bonner, 1955b). Most important, these recurrent suppressor-2 genes, on allelism tests, proved to be non-allelic, indicating that a number of genetic sites are capable of reversing the effects of the td_2 mutation. Similar allele specificity is also found in coli suppressor studies (Yanofsky, 1960).

A second question bears on the requirements for genetic suppression. Here there appears to be a difference between the Neurospora and coli systems. In Neurospora, CRM-formation is required for

suppression (Suskind *et al.*, 1955). None of the CRM-less mutants are affected by any of the known suppressor genes. However, the CRM requirement is a necessary but not sufficient one for suppression, since there is at least one CRM mutant which is also non-suppressible. The coli CRM-less mutants, on the other hand, do re-

Mutational sites within *td* gene	SUPPRESSOR GENE			
	$Su_{2, 2a, 2b, 2c, 2d}$	Su_3	Su_6	Su_{24}
71 2 16 6 3 7 24 **1**	O	O	O	O
71 **2** 16 6 3 7 24 1	+	O	+	O
71 2 16 6 **3** 7 24 1	O	+	O	+
71 2 16 **6** 3 7 24 1	O	O	+	O
71 2 16 6 3 7 **24** 1	O	+	O	+

Fig. 1–5. The specificity of suppressor genes affecting mutational sites within the *td* locus of *N. crassa*. A plus sign indicates suppression. (Data adapted from Yanofsky and Bonner, 1955a.)

spond to suppressor genes (Yanofsky and Crawford, 1959). This difference may be related to the sensitivity of the methods for detecting CRM proteins. Whatever the explanation, the CRM requirement for suppression in Neurospora suggests that protein of a certain type or limiting size must be made or that the system for synthesizing the protein must be available before suppressor gene action can be effectively exerted.

In all of the suppressed Tsase mutants of Neurospora and coli, one characteristically finds very low levels of functional Tsase as compared to the wild-type enzyme level (Yanofsky and Bonner, 1955a).

When the suppressed mutants are examined for antigenic as well as enzymatic activity, it is found that exceedingly large quantities of CRM are formed even in the presence of suppressor genes (Suskind *et al.*, 1955). This would suggest either that the primary mutation is still partially or fully expressed or that the suppressed mutant enzyme has a very low turnover number. Hence, the question arises as to the nature of the suppressed mutant enzyme. Is this normal protein and has the suppressor gene provided information for the repair of the primary structure of the molecule? Or might not the suppressor gene be primarily concerned with control of the function of a still altered mutant enzyme?

In Neurospora, a temperature-sensitive mutant, strain td_{24}, was isolated which forms little or no active enzyme at 25° C and which requires tryptophan at this temperature (Yanofsky and Bonner, 1955a). Elevation of the growth temperature to 30°–33° results in slow growth of the organism on minimal medium. One would like to know whether temperature had affected the formation or the function of Tsase in this instance.

On fractionation of crude mutant extracts which initially had little or very low enzymatic activity but considerable quantities of CRM, it was found that 30–50 per cent of the wild-type enzyme level could be demonstrated (Suskind and Kurek, 1957). Further study revealed that this fractionated mutant enzyme has a number of properties which clearly distinguish it from the wild-type enzyme. These include its sensitivity to low concentrations of zinc, the release of metal inhibition by fractionation and by EDTA, sensitivity to heat and to dialysis, an extremely high apparent energy of activation for tryptophan synthesis, and immunogenic and antigenic differences (Suskind and Ligon, 1960). There appears to be a good correlation between the properties of the mutationally altered enzyme *in vitro* and in the *in vivo* conditions which permit tryptophan synthesis and subsequent growth. In this situation, therefore, environmental control permits the functioning of a genetically altered Tsase. Only two other means of "activating" the system *in vivo* are known. One of these is by complementation and the other by suppressor gene action (Lacy, 1959; Yanofsky and Bonner, 1955a).

To determine whether the enzyme formed in the suppressed mutant is of the normal or the mutant type, the properties of wild-type, td_{24}, and $td_{24}Su_{24}$Tsases were compared. The results of these experiments are summarized in Table 1-2 (Suskind and Kurek,

1959; Suskind and Ligon, 1960), and they permit several conclusions to be drawn. First, the $td_{24}Su_{24}$Tsase does not resemble wild-type Tsase but does resemble the enzyme from the unsuppressed mutant. Second, $td_{24}Su_{24}$ still forms large quantities of CRM. Third, td_2 and td_2Su_2, a pair used for control purposes, resemble wild type in those properties examined (Suskind and Ligon, 1960; Yanofsky and Bonner, 1955a). Thus, in the case of $td_{24}Su_{24}$, it appears either that a mutant enzyme is still being formed and that the suppressor

TABLE 1–2

A Comparison of the Properties of Tryptophan Synthetase from Wild-Type, Mutant, and Suppressed Mutant Strains of *N. crassa*

	Strain				
Property	Wild Type	td_{24}	$td_{24}Su_{24}$	td_2	td_2Su_2
Resistance or sensitivity to zinc (10^{-5} M)	Resistant	Sensitive	Sensitive	. . .	Resistant
Stability or liability to overnight dialysis at 4° C.	Stable	Labile	Labile	Stable	Stable
Stability or liability to 55° C for 5 min.	Stable	Labile	Labile	Stable	Stable
Apparent energy activation (calories)	9,000–10,000	30,000–50,000	30,000–50,000	. . .	9,000–10,000
Immunogenicity and antigenicity.	Standard	Abnormal	Abnormal	Similar to wild type	Similar to wild type

gene *in vivo* acts to permit this enzyme to function or that there is a very low level of wild-type-like enzyme present which has not been detected.

In coli, suppressor genes are also found which are specific for certain mutations of the A or the B genes. In one instance there is evidence that a suppressed A mutant, in addition to forming an A-CRM, does form a second A-protein which is effective in catalyzing the InGP $\leftrightarrows$ In + TP reaction. This second A-protein seems to behave like the wild-type A-protein on column chromatography, suggesting that the suppressor gene has initiated the synthesis of some normal Tsase molecules (Yanofsky, 1960).

Regardless of the mechanism of action, it is nevertheless indisputable that a specific suppressor gene can cooperate with a specific

mutationally altered gene to elicit the formation of a *functional* tryptophan synthetase.

Conclusion

The studies summarized in this paper indicate that genetic information, contained within a restricted region which controls the formation of the enzyme Tsase, is subject to considerable modification. These modifications result in specific alterations in the properties of tryptophan synthetase. These are being examined at both the protein and the amino acid level. Furthermore, the types of specific structural modifications in the protein, observed following mutation, can be correlated with damage to very specific regions within the total genetic area which controls tryptophan synthetase formation. These regions also show very specific types of intergenic and intragenic interaction.

Perhaps this paper can best be concluded by framing several questions. For example, do changes other than changes in the primary structure of a protein occur as a result of mutation? Are CRM-less mutations really mutations of the "non-sense" type? What explanations exist at the molecular level for suppressor action, partial and true reversion, and complementation? And finally, can these mutant systems provide clues to the relationship of the DNA code to protein structure? Certainly approaches are now available which may provide answers to most of these questions in the near future.

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2

Control by Repression

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Among the mechanisms controlling cellular function, there are two, namely repression and induction, that represent effects of specific small molecules on the synthesis of enzymes and possibly other proteins. These two mechanisms complement each other and, in various ways, have curiously intertwined histories. About sixty years ago, Dienert (1900) described phenomena that presumably reflect enzyme induction, and he also reported an apparent antagonism to enzyme formation that suggests enzyme repression. Our interest in this area stems from an experiment (Vogel and Davis, 1952) that was intended to bear on the possibility that even "constitutive" enzymes, such as those of arginine synthesis, require induction for their formation. The results then obtained seemed to be compatible with this notion; further work, however, pointed to a different explanation and led to the finding that arginine added to growing cultures of a strain of *Escherichia coli* represses the formation of acetylornithinase (Vogel, 1953a). This enzyme catalyzes a step in the arginine pathway, namely the conversion of acetylornithine to ornithine (Vogel, 1952; Vogel and Bonner, 1956). Interestingly enough, a number of other early instances of repression were discovered almost simultaneously in several laboratories (Table 2-1), among them, the antagonism of galactose to the synthesis of β-galactosidase

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(Monod and Cohen-Bazire, 1953a), the very enzyme which had proved so useful in the elucidation of the induction phenomenon. Repression and induction were brought into still closer juxtaposition when it was proposed (Vogel, 1957a, 1957b) that they are related not only through their function in the cell but also through the underlying molecular events (in terms of the interference with or promotion of the dissociation of template product from template). As detailed in another section, strong evidence has indeed been obtained that the induction of β-galactosidase represents an antagonism to the action of an endogenous repressor (Pardee, Jacob, and Monod, 1958, 1959).

Since 1953, repression has been observed upon the addition to growing organisms of any of a wide variety of substances, including amino acids, purine and pyrimidine compounds, carbohydrates, succinate, and phosphate (Table 2-1). The various small-molecule repressors may not function as such, but rather as "active" derivatives (see the section Mechanism of Repression). A broad range of enzymes are subject to repression, and this phenomenon appears to occur regardless of the type of catalytic activity of the enzyme involved; additionally, permease formation can be repressible (Vogel, 1960a). With respect to the organisms in which it is found, repression is also widespread: it occurs in a variety of bacteria (with which the bulk of the work on repression has been carried out) as well as in mammalian cells (DeMars, 1958; Walker, 1959, 1960) and probably in fungi (Yura and Vogel, 1959; Horowitz *et al.*, 1960).

Frequently, as Table 2-1 reveals, the repressors are "end products" of the pathways in which the respective repressible enzymes occur, and the repressors usually seem to be quite specific in their action (Monod and Cohen-Bazire, 1953b; Vogel, 1957a). These properties of repressors would appear to be of particular relevance to the control of cellular function.

Basic Picture and Kinetics of Repression

Definition of Repression. Enzyme repression has been defined as a relative decrease, resulting from the exposure of cells to a given substance, in the rate of synthesis of a particular apoenzyme (Vogel, 1957a). This definition was worded in analogy with that proposed for enzyme induction by Cohn *et al.* (1953b). The notion of repression has been extended to include enzyme-like substances such

TABLE 2–1

Selected Examples of Repression

Enzyme*	Repressor	Reference
Acetylornithine δ-transaminase (e)	Arginine	Albrecht and Vogel (1960)
Acetylornithinase (e)	Arginine	Vogel (1953a)
Ornithine transcarbamylase (e)	Arginine	Gorini and Maas (1957)
Acetylornithine permease (e)	Arginine	Vogel (1960a)
Arginine-glycine transamidinase (r)	Creatine	Walker (1959)
Glutamine synthetase (h)	Glutamine	DeMars (1958, 1960)
Imidazoleglycerol phosphate dehydrase (s)	Histidine	Ames and Garry (1959)
Imidazoleacetol phosphate transaminase (s)	Histidine	Ames and Garry (1959)
L-Histidinol phosphate phosphatase (s)	Histidine	Ames and Garry (1959)
L-Threonine deaminase (e)	Isoleucine	Umbarger and Brown (1958a)
Methionine synthetase (e)	Methionine	Cohn *et al.* (1953a); Wijesundera and Woods (1953, 1960)
Pyrroline-5-carboxylate reductase (n)	Proline	Yura and Vogel (1959)
Tryptophan synthetase (a)	Tryptophan	Monod and Cohen-Bazire (1953b)
Tryptophan synthetase, Components A and B (e)	Tryptophan	Yanofsky and Crawford (1959)
Valine transaminase (e)	Valine	Adelberg and Umbarger (1953)
Acetolactate-forming enzyme (e, a)	Valine	Umbarger and Brown (1958b); Halpern and Umbarger (1959)
Inosine 5′-phosphate dehydrogenase (a)	Guanine, 8-azaguanine	Levin and Magasanik (1959)
Inosinicase (a)	Guanine, 8-azaguanine	Levin and Magasanik (1959); Magasanik (1960)
Aspartate transcarbamylase (e)	Uracil	Yates and Pardee (1957)
Dihydroorotase (e)	Uracil	Yates and Pardee (1957)
Dihydroorotic acid dehydrogenase (e)	Uracil	Yates and Pardee (1957)
Alkaline phosphatase (e)	Phosphate	Horiuchi *et al.* (1959); Torriani (1960)
β-Galactosidase (e)	Galactose	Monod and Cohen-Bazire (1953a)
Histidase (a)	Glucose	Magasanik *et al.* (1958)
myo-Inositol dehydrogenase (a)	Glucose	Magasanik *et al.* (1958)
Glycerol dehydrogenase (a)	Glucose	Magasanik *et al.* (1958)
Isocitratase (m)	Succinate	Kornberg *et al.* (1960)

* The origin of the enzymes is indicated by the letters in parentheses: a, *Aerobacter aerogenes;* e, *Escherichia coli;* h, human (HeLa) cells; m, *Micrococcus denitrificans;* n, *Neurospora crassa;* r, rat kidney; s, *Salmonella typhimurium.* The enzyme-like acetylornithine permease has been included.

as permeases (Vogel 1960a). (For further extensions of the repression concept, see the section Genetic Aspects.)

Kinetics. The basic features of the repression phenomenon can perhaps best be illustrated in kinetic terms. Fig. 2-1 shows the formation of acetylornithinase in a single culture of strain 39A-23R3 of *E. coli*, first under conditions of repression by arginine and then under derepressed conditions, i.e., under conditions of repression release (Vogel, 1957a). The results are presented as a "differential plot" (Monod *et al.*, 1952) of amount of enzyme versus amount of total protein produced. Strain 39A-23R3, a reisolate of strain 39A-23 (cf. Vogel, 1953a), has an early block in the ornithine-arginine pathway (Vogel, 1953b) and gives a growth response to acetylornithine or to arginine (or to arginine precursors following acetylornithine in biosynthetic sequence); mixed supplements of acetylornithine and arginine, in suitable proportions, give diphasic growth (Vogel, 1953a, 1957a): arginine is used preferentially, at wild-type growth rate, in the first phase, and acetylornithine, due to restrictive uptake (Vogel, 1960a), is used at a lower exponential growth rate in the second phase. This growth behavior on appropriate mixed supplements appears to reflect conditions of intracellular arginine concentration that also underlie the kinetics of acetylornithinase formation shown in Fig. 2-1. The first growth phase (during which an excess of arginine is available) corresponds to repression, and the second growth phase (during which the intracellular arginine level is restricted) corresponds to derepression. The change in the differential rate of enzyme synthesis occurs relatively abruptly and nearly simultaneously with the sharp transition from first-phase to second-phase growth. It will be seen that under conditions of repression as well as of derepression, linear differential rates of enzyme synthesis are obtained, i.e., the enzyme is synthesized as a constant fraction of new protein produced. Under derepression, this fraction is greater than it is under repression. The relative promptness of onset of derepression is noteworthy; the onset of repression of acetylornithinase can also be shown to be very rapid.

Acetylornithinase formation by the W strain of *E. coli* (from which strain 39A-23R3 was derived) is also illustrated in Fig. 2-1; strain W was grown, without added arginine, in the same general manner as was strain 39A-23R3. Strain W can be seen to give a linear differential rate indicative of a partial repression. This partial

effect is thought to result from the particular steady-state level of arginine produced by this organism under the test conditions. Further repression is obtained upon addition of arginine to growing cultures of strain W (Vogel, 1953a).

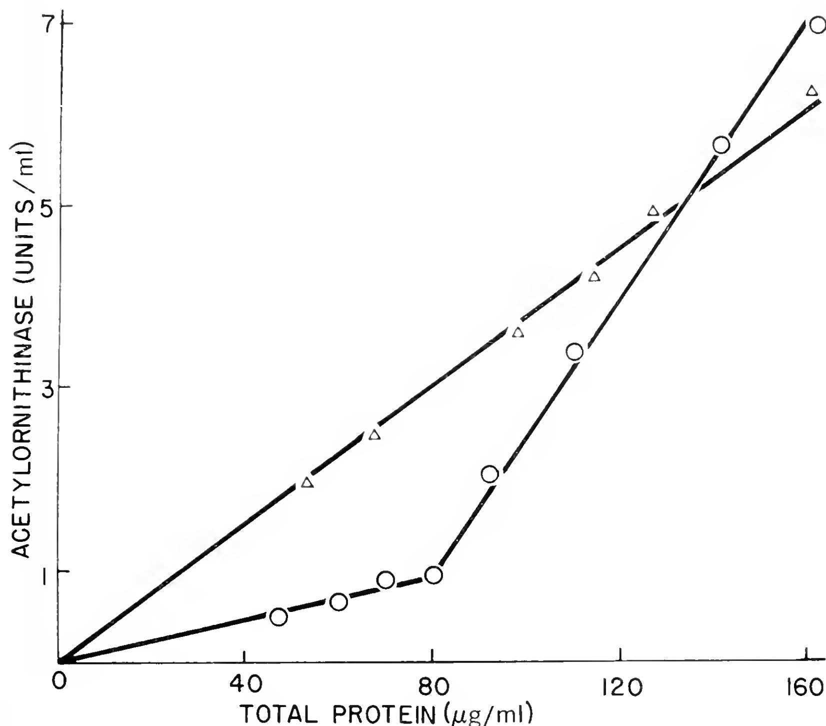

Fig. 2–1. Repressed and derepressed formation of acetylornithinase in mutant strain 39A-23R3 (circles) of *Escherichia coli,* and partially repressed formation of the enzyme in wild-type strain W (triangles) of this species. Strain 39A-23R3 was grown, under anaerobic conditions at 37° C, on a glucose-salts medium, in the presence of a supplement of L-arginine hydrochloride and N^{α}-acetyl-L-ornithine. The amount of (the preferentially utilized) arginine was such as to support growth corresponding to a "total protein" value of 80 μg per ml of culture. Acetylornithinase (per ml of culture) was determined after harvesting of organisms and their disruption (in 0.1 *M* phosphate, *p*H 7, containing 1.0 m*M* glutathione) with the aid of a Mullard Ultrasonic Disintegrator; the assay procedure and enzyme unit have been described (Vogel and Bonner, 1956). Note the abrupt change from repressed to derepressed synthesis at the beginning of the (growth rate-limiting) utilization of acetylornithine. Strain W was cultivated on unsupplemented glucose-salts medium and studied in the same general manner as was the mutant strain.

The repressed rate of acetylornithinase formation in strain 39A-23R3 cannot be further repressed by increasing the initial arginine concentration used by factors up to 100. The *derepressed* rate, however, of this strain is susceptible to variation, as discussed below.

PACE-SETTING PHENOMENON IN ENZYME DEREPRESSION. The derepression of acetylornithinase formation was studied after varying periods of repression by arginine (Vogel, 1960c). This was accom-

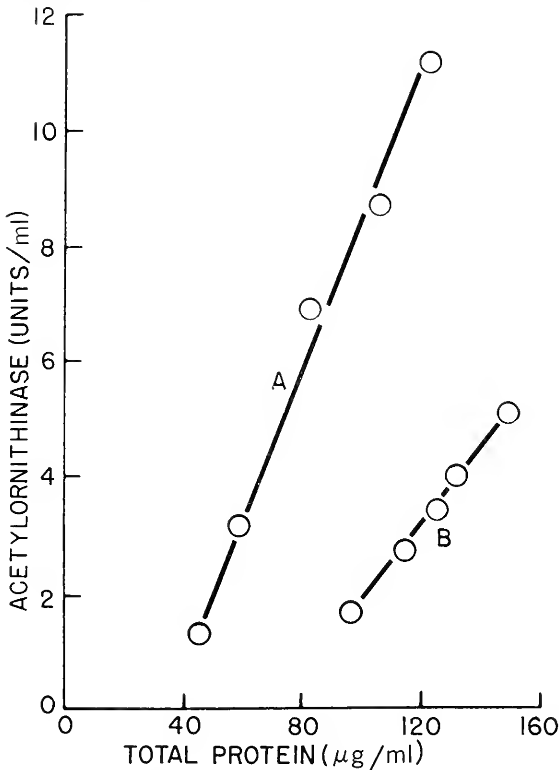

Fig. 2–2. Formation of acetylornithinase following early (A) or late (B) onset of derepression (data from Vogel, 1960c). After preliminary cultivation in a glucose-salts medium supplemented with a growth-limiting amount of arginine, strain 39A-23R3 of *Escherichia coli* was grown in a glucose-salts medium containing N^{α}-acetyl-L-ornithine, 50 μg/ml, and L-arginine hydrochloride, either 5 μg/ml (Culture A) or 15 μg/ml (Culture B). The two cultures were incubated anaerobically at 37° C and, after establishment of second-phase growth (*i.e.*, of derepressive conditions, see the text), were sampled at intervals. Organisms from the samples taken were harvested, disrupted, and assayed for acetylornithinase (see the legend for Fig. 2–1). The differential rates of synthesis of the enzyme for A and B are 0.129 and 0.063 units per μg protein, respectively.

plished with the aid of strain 39A-23R3 and the use of acetylornithine-arginine supplements, in which the initial concentration of acetylornithine was held constant and that of arginine was varied; in this manner, it was possible to effect a swift transition from repression to derepression, without interruption of cultivation, and the point of transition was easily fixed by the arginine concentration in the supplement employed. In the two cases shown in Fig. 2–2, the enzyme and the total protein are seen to be formed in a constant ratio; for Culture B, this ratio is smaller than it is for Culture A. The lower differential rate of acetylornithinase synthesis in Culture B is associated with the higher initial arginine concentration (Fig. 2–2), and consequently with the longer cultivation in the presence of added arginine and the later termination of repression. Since the constant differential rates of derepression can be shown to start, within the accuracy of the methods used, from the respective points of onset of derepression (cf. Fig. 2–1), these rates can be viewed as reflecting a "pace-setting" phenomenon. The differential rates usually maintain their constancy until shortly before growth stops (Vogel, 1960b). The pace-setting effect is interpreted in terms of a repression-like antagonism to the *formation* of a component, or components, of the acetylornithinase-forming system. This effect is of possible relevance to an understanding of the molecular basis of repression and will be discussed further in the section Mechanism of Repression.

COORDINATE REPRESSION AND DEREPRESSION. Very large quantitative variations in derepression have been observed for different enzymes. For example, in the case of aspartate transcarbamylase, the differential rate of synthesis upon derepression can exceed the repressed rate by a factor of more than 1000 (Shepherdson and Pardee, 1960a, 1960b). In contrast, the corresponding factor for acetylornithinase, depending on the point of onset of derepression, is of the order of magnitude of 10. These two enzymes, of course, occur in different biosynthetic pathways. A revealing analysis of the derepression of enzymes occurring in a single pathway has been reported by Ames and Garry (1959) and Ames *et al.* (1960). These authors studied the repression by histidine of the last four enzymes in the histidine pathway of *Salmonella typhimurium*. Unlike the acetylornithinase in strain W of *E. coli* (see above), these histidine enzymes of S. *typhimurium* are maximally repressed when the wild-

type organism grows on "minimal medium." The fact that these enzymes are repressed becomes evident under conditions of repression release. Such conditions were realized with the aid of the "leaky" histidine-requiring mutant strain *hisE-11* of *S. typhimurium*. When this strain is cultivated in the presence of histidine, repressed enzyme levels are obtained. Upon exhaustion of the added histidine, the mutant strain continues to grow, albeit more slowly, due to its leaky block, and the conditions are now derepressive with respect to the four enzymes of histidine synthesis. As shown in Fig. 2–3,

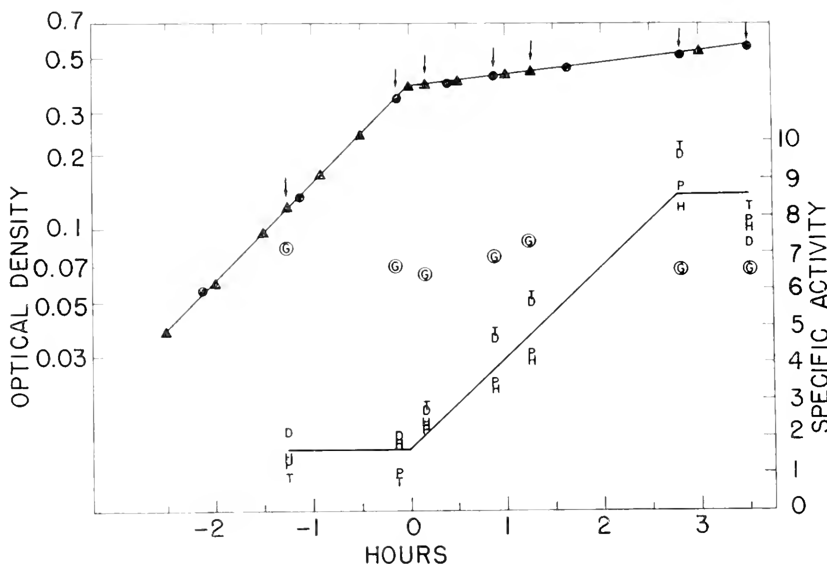

Fig. 2–3. The specific activities of four histidine biosynthetic enzymes and glutamic dehydrogenase during the growth of the "leaky" histidine-requiring mutant strain *hisE-11* of *Salmonella typhimurium*. The amount of histidine that was added (0.03 mM) was exhausted during the growth period (at zero time), and the mutant strain then grew at a lower rate that was limited by the small amount of histidine it was capable of making. A liter culture was grown in a two-liter flask, agitated at 37° C in a rotary shaker, and optical density readings were taken at various times. The solid triangles and circles on the growth curve represent two separate experiments run under identical conditions. At the times indicated by the arrows, 100-ml aliquots were harvested for protein and enzyme assays (see Ames and Garry, 1959). The specific activities, in normalized units, of imidazoleglycerol phosphate dehydrase (D), imidazoleacetol phosphate transaminase (T), histidinol phosphate phosphatase (P), and histidinol dehydrogenase (H) are presented; specific activities of glutamic dehydrogenase (circled G) are included for comparison. (*Courtesy*, B. N. Ames and B. Garry.)

the four enzymes appear to derepress jointly and at the same rate, within the accuracy of the enzyme assays used. Hence, the initial extent of repression is the same for all four enzymes, a phenomenon called "coordinate repression" by Ames and Garry (1959). Recent findings indicate that at least two, and probably all, of the earlier enzymes in this pathway are repressed coordinately with the last four (Ames, 1960; Magasanik, 1960). These results, in conjunction with those of Hartman *et al.* (1960a, 1960b), are considered again in the section Genetic Aspects. Coordination does not, however, seem to be a necessary attribute of the repression of enzymes within a given biosynthetic sequence, since, as pointed out by Ames and Garry (1959), the repression of three enzymes of pyrimidine biosynthesis (Yates and Pardee, 1957) apparently is non-coordinate.

REPRESSION AS A FUNCTION OF INTRACELLULAR REPRESSOR CONCENTRATION. As discussed above, release from repression is obtained when the supply of the appropriate repressor available to the cell is restrictive. A dependence of repression or derepression on the intracellular repressor concentration is thus indicated. In the case of acetylornithinase, restrictive supply conditions of the repressor, arginine, were realized during rate-limiting growth of an arginine-requiring mutant on acetylornithine (Vogel, 1953a, 1957a, 1960a). Slowly utilizable substrates likewise proved useful in derepression studies of enzymes of pyrimidine (Yates and Pardee, 1957; Sheperdson and Pardee, 1960b) and histidine (Ames and Garry, 1959) synthesis. A restrictive supply of repressor can also be achieved in a number of other ways: with leaky mutants (Yates and Pardee, 1957; Maas and Novick, 1958; Ames and Garry, 1959; Yanofsky, 1960; Shepherdson and Pardee, 1960b); with mutants growing in a chemostat on a limited amount of repressor (Gorini and Maas, 1957, 1958); with cells, e.g., protoplasts or mutants, having impaired concentrating mechanisms (Rogers and Novelli, 1959; Schwartz *et al.*, 1959; Maas, 1959); by interference with the synthesis of an endogenous repressor through the use of an antimetabolite (Lester and Yanofsky, 1960; Moyed, 1960) or of constraining metabolic conditions, e.g., anaerobiosis (Gorini and Maas, 1958); or by selective enrichment of repressor-free culture medium (Gorini and Maas, 1958).

In the derepression experiments with acetylornithinase and the enzymes of histidine synthesis (Fig. 2–1 and 2–3), derepressive conditions were maintained following the onset of derepression. The

employment of different conditions permitted an interesting kinetic study of the effect of intracellular repressor concentration on the formation of ornithine transcarbamylase, which is repressible by arginine (Gorini and Maas, 1957, 1958). As shown in Fig. 2–4, when cells of *E. coli*, strain W, grown in the presence of arginine are inocu-

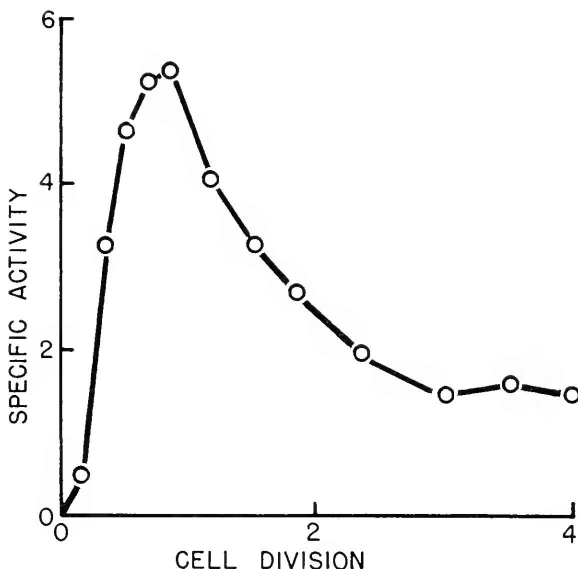

Fig. 2–4. Ornithine transcarbamylase synthesis in flask cultures of *Escherichia coli*, strain W. Washed cells from cultures growing exponentially in the presence of arginine were inoculated into an arginine-free lactate-salts medium and incubated at 37° C with shaking. For the assay procedure and enzyme unit, see Gorini and Maas (1957). The specific activity is expressed in units of enzyme per milligram of dry weight of bacteria. The cell division time was 60 minutes. (*Courtesy*, L. Gorini and W. K. Maas.)

lated into arginine-free minimal medium, there is a relatively rapid initial rise in enzyme level, followed by a drop to an approximately steady level. The initial rise is attributed to enzyme synthesis occurring under conditions of low endogenous arginine concentration, i.e., under conditions of derepression. The ornithine transcarbamylase and other enzymes in the arginine pathway thus formed are considered to allow increased synthesis, and hence an accumulation, of arginine, which in turn exerts a repressive effect.

Derepression as *de novo* Protein Synthesis. Implicit in kinetic studies, such as those presented above, is the notion that the appear-

ance of enzyme activity upon derepression represents *de novo* synthesis of the enzyme protein. More direct evidence on this point has been obtained by Yates and Pardee (1957) who showed that the aspartate transcarbamylase activity found on derepression is accompanied by the selective synthesis of new protein having the electrophoretic mobility of the transcarbamylase. Rogers and Novelli (1959) have concluded that derepression of ornithine transcarbamylase also represents the synthesis of new protein. Particularly graphic illustrations of new protein synthesis have come from recent studies of enzymes that show quantitatively very large derepression effects, such as aspartate transcarbamylase (Shepherdson and Pardee, 1960a, 1960b) and alkaline phosphatase (Horiuchi *et al.*, 1959; Torriani, 1960; Garen and Levinthal, 1960). Of relevance to *de novo* enzyme synthesis upon derepression are the well-known studies of β-galactosidase induction (Monod and Cohn, 1953; Rotman and Spiegelman, 1954; Hogness *et al.*, 1955), since the induction of this enzyme has been concluded to represent release from an endogenously produced repression (Pardee, Jacob, and Monod, 1958, 1959; see below).

General discussions of the basic features of repression can be found in the highly informative reviews by Monod (1959b), Magasanik *et al.* (1959), and Pardee (1959, 1960) and in an article by the present writer (Vogel, 1959).

Genetic Aspects

In view of the above-mentioned finding that the induction of β-galactosidase represents a release from repression, the body of genetic knowledge from this well-explored system can be considered pertinent to the genetics of repression. Additional results in this area have come from investigations of other repressible systems, including enzymes of tryptophan (Cohen and Jacob, 1959) and arginine (Gorini, 1960a; Maas, 1960) metabolism, as well as alkaline phosphatase (Echols *et al.*, 1960) and tyrosinase (Horowitz *et al.*, 1960). For these systems, and for β-galactosidase (Jacob and Monod, 1959), enzyme synthesis appears to be under the control of (a) "structural" genes, which are thought to contain information for the structure (particularly the amino acid sequence) of the enzyme involved, and of (b) regulatory genes governing the expression of the structural ones through the agency of a repressor.

Relations Between Structural and Regulatory Genes. Genetic evidence shows that the synthesis of β-galactosidase in *E. coli* is controlled by a portion of the genome which has been called the *Lac* region (cf. Lederberg *et al.*, 1951). For recent discussions of this subject, see Wollman and Jacob (1959), Pardee *et al.* (1959), Jacob (1960), and Jacob *et al.* (1960b). This (relatively small but complex) region contains at least four loci (*y*, *z*, *o*, and *i*) concerned with the metabolism of β-galactosides. The *y* locus governs the formation of a permease for β-galactosides (Rickenberg *et al.*, 1956; Cohen and Monod, 1957) and is not of immediate relevance to the synthesis of β-galactosidase. The *z* locus, on the other hand, is intimately connected with the synthesis of this enzyme, and has for some time been thought to contain the code for the latter's amino acid sequence. The *o* and *i* loci are considered to have regulatory functions in the above-defined sense. Mutations at the *z* locus, as expected for a structural gene, can result in the loss of the capacity to synthesize β-galactosidase; mutations at the *i* locus can bring about conversion from inducibility (i.e., ability to respond with enzyme formation to an inducer that can antagonize an endogenous repressor) to constitutivity (i.e., ability to form the enzyme in the absence of an added inducer); and mutations at the *o* locus can lead either to constitutivity or to simultaneous loss of the ability to synthesize the enzyme and the permease (Jacob *et al.*, 1960a). An elegant analysis of the properties of the *o* and *i* loci has been performed by Jacob *et al.* (1960a) who studied the dominance and position behavior of suitable heterozygotes (see below). The role of the *z* locus has been exhibited graphically by recent experiments of Perrin *et al.* (1959): mutations at the *z* locus can bring about the synthesis of proteins that are antigenically related to β-galactosidase but enzymatically inactive; nevertheless, these structurally altered proteins can exhibit the properties of repressibility and inducibility characteristic of β-galactosidase. The interrelations among structural and regulatory genes as established by studies with bacteria have a counterpart in results with *Neurospora* that have suggested gene functions in addition to those performed by structural-type genes (Bonner *et al.*, 1960).

Functioning of Regulatory Genes. The finding of two regulatory loci governing β-galactosidase synthesis in *E. coli* (see Jacob *et al.*, 1960a) has uncovered the existence of a complex genetic control

system whose functioning has been profitably explored through studies of dominance and position in mating experiments. In this species, conjugation involves the injection of a chromosome from a male (Hfr) into a female (F^-) cell and generally results in the formation of an incomplete zygote (merozygote); and segregation of recombinants from heteromerozygotes is sufficiently slow to allow time for experimentation (Wollman *et al.*, 1956).

A revealing analysis of the functioning of the *i* gene and the expression of inducibility in the β-galactosidase system has been presented by Pardee *et al.* (1958, 1959). In very thoughtfully designed conjugation experiments, they crossed a male of the genetic constitution z^+i^+ to a female of the genetic constitution z^-i^-. Thus, the male, but not the female, carried an intact "structural" allele for enzyme synthesis, and the male had an "inducible" allele, whereas the female had a "constitutive" one. Additionally, the male had markers for streptomycin sensitivity and T6 phage sensitivity, and the female had the corresponding "resistant" markers. It was therefore possible to use streptomycin and T6 phage to prevent induction of the male cells by any added inducer and to block remating. The results of such a cross are shown in Fig. 2–5, which illustrates the synthesis of the enzyme, in the absence or in the presence of inducer, over a period of several hours. It will be seen that, in the absence of inducer, enzyme synthesis occurs over an interval of time and then ceases. Addition of inducer at the point indicated brings about continued enzyme synthesis. Accordingly, the initially constitutive zygotes have become inducible. When z^-i^- males are crossed with z^+i^+ females in the absence of inducer, no enzyme synthesis is observed, even several hours after the mating. In other words, the allele from the male is never expressed. The "inducible" (i^+) allele is, therefore, indicated to be dominant with respect to the "constitutive" (i^-) allele. The authors concluded from the kinetics of expression of the i^+ character that the *i* locus controls the synthesis of a specific substance that represses the formation of β-galactosidase, and that the constitutive state results from loss of the capacity to make active repressor. The results of these experiments also show that there is no appreciable mixing of the male and female cytoplasms during mating; the cytoplasm of the female determines initial inducibility or constitutivity. Cohn *et al.* (1960) have studied the synthesis of β-galactosidase in *Shigella dysenteriae* fol-

lowing transfer of genetic material from *E. coli;* they found that *S. dysenteriae* appears to be genotypically i^+.

As indicated above, the *i* and *z* loci are part of the *Lac* region and are thus relatively closely linked. This situation may be contrasted with the regulatory system of tryptophan synthesis (Cohen

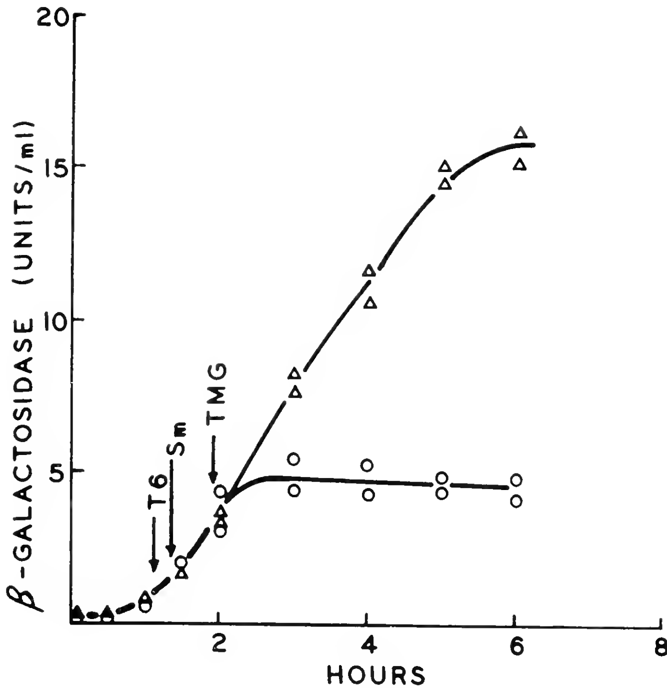

Fig. 2–5. β-Galactosidase formation in heteromerozygotes of *Escherichia coli.* Matings were performed in the absence of inducer. At times indicated, phage T6 and streptomycin (Sm) were added to all of the cultures, and the inducer, methyl-thio-β-D-galactoside (TMG), was added to two of the cultures (triangles); the other two (circles) did not receive any inducer. See Pardee *et al.* (1959) for the strains, cultivation and mating conditions, assay procedure, and enzyme unit. (*Courtesy,* A. B. Pardee, F. Jacob, and J. Monod.)

and Jacob, 1959). In this case, the regulatory locus (R_{try}) thought to control the formation of an active repressor of enzymes of the tryptophan pathway could be shown by conjugation and transduction experiments to be located in the genome at a considerable distance from (presumably structural-type) determinants governing the synthesis of these enzymes. Mutations at this regulatory locus

can abolish the repressibility of the enzymes in this pathway. The "repressible" allele is dominant with respect to the "non-repressible" one. Maas (1960) has described mutants of *E. coli* in which arginine no longer represses the formation of enzymes in its pathway, and Gorini (1960b) has isolated arginine-repressible mutants from a wild-type strain (B) of *E. coli* that is non-repressible with respect to enzymes of the arginine pathway. Thus, in the arginine system, too, control appears to be exerted by structural as well as regulatory genes, and the repressibility character can be studied in genetic-transfer experiments (Gorini, 1960a). Additional complexities of the arginine system are indicated by the finding that relatively high levels of the repressible ornithine transcarbamylase can be produced under the influence of a genetic factor distinct from the one that controls repressibility (Gorini, 1960a). Alleles controlling repression have also been explored in the alkaline phosphatase system of *E. coli* (Echols *et al.*, 1960).

Horowitz *et al.* (1960) have studied the induction of tyrosinase formation in *N. crassa* and concluded that different genes appear to control the structural and the regulatory aspects of the tyrosinase system. Interestingly enough, sulfate acts like a repressor of this system. These authors found that, in a wild-type strain, the enzyme is inducible on a high-sulfate medium but gives the appearance of being constitutive on a low-sulfate medium. This seemingly constitutive behavior is interpreted as involving self-induction. Horowitz *et al.* (1960) also isolated certain mutants which are inducible on either medium.

It was indicated above that genes considered to control the synthesis of active repressors, such as the i or R_{try} genes, may or may not exhibit close linkage with their corresponding structural genes. A different situation obtains in the case of the second regulatory locus, o, of the β-galactosidase system. Two types of mutations can occur at this locus: (a) dominant mutations (o^c) causing constitutivity that affect both β-galactosidase and the permease, provided the mutant allele is in the *cis* position with respect to the z and y genes (Jacob and Monod, 1959; Jacob *et al.*, 1960a) and (b) recessive mutations (o^o) mimicking deletions by bringing about loss of the capacity to synthesize both the enzyme and the permease. These results have been interpreted in terms of a genetic factor, the operator, corresponding to the o locus which, being adjacent to the

closely linked *z* and *y* genes, constitutes with them a unit, called the *operon*. The operator is thought to govern the activity of the genes within its operon and to be susceptible to a repressor produced under the control of a particular regulatory gene (*i*). In the presence of the repressor, the expression of the group of genes is considered to be inhibited through the intermediate agency of the operator (Jacob and Monod, 1959; Jacob *et al.*, 1960a). The expression of a structural gene can be independent of the regulatory system to which it is normally subjected, if the structural gene is linked to determinants obeying another regulatory system (Buttin *et al.*, 1960).

An operator-type gene may also be involved in the control of the formation of enzymes of the histidine pathway in *S. typhimurium*. Ames *et al.* (1960) reported that mutant *hisG-203*, which maps within the *G* locus (Hartman *et al.*, 1960b) corresponding to the first enzyme of the histidine pathway, seems to lack not only the first enzyme but also several, if not all, of the remaining enzymes of this pathway. Accordingly, the mutation carried by *hisG-203* seems to give a "switch effect" suggestive of an operator-like locus (Ames, 1960). This result is particularly interesting in view of the finding of Ames *et al.* (1960), in conjunction with the genetic work of Hartman *et al.* (1960b), that the histidine genes appear to be linked in a sequence that corresponds to the sequence of the enzymatic steps in the histidine pathway (cf. Demerec, 1956). The suggested existence of an operator gene in this system would be consistent with the aforementioned finding of coordinate repression of the enzymes of histidine synthesis (Ames and Garry, 1959). It should be stressed, though, that mutations within loci of the histidine path other than *G* apparently can give position-type effects in that they seem to bring about an impairment in the function of neighboring genes (Ames *et al.*, 1960; Hartman *et al.*, 1960b).

A contrast to the sequential arrangement of the genes of histidine synthesis is provided by the arginine case in *E. coli*. The structural genes of arginine synthesis in strains B and K-12 of this species seem, to a considerable extent, to be scattered in the genome (Gorini, 1960b) and yet are under the control of a single regulatory gene, R_{arg} (Gorini, 1960a; Maas, 1960). In *S. typhimurium*, as in *E. coli*, the genes governing arginine synthesis are not all closely linked (Demerec *et al.*, 1960). At least five, if not all, of the enzymes of arginine synthesis are repressible in strains of *E. coli* carrying the

appropriate regulatory allele (Vogel, 1953a; Gorini and Maas, 1958; Gorini, 1960c; Albrecht and Vogel, 1960). It is noteworthy that, in a derivative of the K-12 strain of *E. coli*, at least two enzymes of arginine synthesis, namely acetylornithinase and acetylornithine δ-transaminase, can exhibit approximately coordinate derepression (Vogel and Albrecht, 1960).

Genetic factors, such as the o^o allele of the β-galactosidase system (and possibly the characteristic allele of mutant strain *his*G-*203*), may be viewed as representing a switch that is always in the "off" position; the o^c type may represent a reduced affinity of the operator or switch gene, or a product thereof, for the corresponding repressor which is assumed to be produced under the control of the *i*-type gene (cf. Jacob *et al.*, 1960a).

The problem of the role of the *i*-type genes in the production of repressors (or active repressors) has been examined by Pardee and Prestidge (1959). These authors performed experiments, such as those of Pardee *et al.* (1959) illustrated in Fig. 2–5, except that protein synthesis, including β-galactosidase synthesis, in the zygotes was inhibited by 5-methyltryptophan from the moment of mating the bacteria until reversal of the inhibition by the addition of tryptophan 75 minutes later. At the end of this period, β-galactosidase was formed only when inducer was added. Conditions for inducibility were thus created under conditions of inhibition of protein synthesis. The authors concluded that the repressor probably is made directly by the gene rather than by an enzyme that itself is produced under the control of the gene and, moreover, that the repressor is not a protein. The repressor, or part of the active repressor may, however, be a ribonucleic acid. Pardee and Prestidge (1959) indicate, though, that a possible role of protein synthesis in the production of repressor as governed by the *i* gene has not been entirely ruled out. Jacob and Campbell (1959) have obtained evidence from a study of lysogenic bacteria that the formation of a repressor may occur in the absence of protein synthesis.

The repression concept has been successfully applied not only to investigations of lysogeny but has been extended to considerations of various episomic elements (Jacob *et al.*, 1960b). This concept has also been used in speculations on antibody formation (Szilard, 1960a, 1960b). Instructive analogies between β-galactosidase formation and antibody synthesis have been discussed by Monod (1959a).

Mechanism of Repression

The Regulator Hypothesis. The diversity and abundance of recent contributions—both biochemical and genetic—to the field of repression make it attractive as well as difficult to attempt some kind of integration of the results in the context of molecular mechanisms. As a point of departure, it may be helpful to consider a hypothesis of repression that was formulated in general terms in 1956, on the occasion of the Symposium on the Chemical Basis of Heredity at the McCollum-Pratt Institute (Vogel, 1957a).

It seemed reasonable to assume that repression of enzyme formation reflects an interference with the functioning of the enzyme-forming system. The hypothesis addressed itself to the question of how a repressor of low molecular weight compared to that of a protein can hamper the performance of an enzyme-forming system. It was proposed that this type of interference involved the binding of a newly synthesized enzyme molecule to the site of its formation through the agency of the repressor (or its "active" derivative). Such binding might block the further production of enzyme molecules until the enzyme-forming site is freed again. Whether this kind of binding would involve the catalytically active site of the enzyme or some other site is a question that was explicitly left open. It was pointed out that induction can also be viewed in terms of removal of an enzyme molecule from its site of synthesis (Vogel, 1957a). If repression reflects a binding of nascent protein to its site of formation, induction could reflect the neutralization of such a binding effect, and the possibility of an antagonism of inducers and repressors (collectively called regulators) of one and the same enzyme was subsequently considered; a unified hypothesis, termed regulator hypothesis, was thus proposed (Vogel, 1957b). It was also contemplated that, at least in some instances, induction could comprise a ("simple") promotion of the removal of an enzyme molecule from its site of synthesis (Vogel, 1957a, 1957b). Such a promotion effect, which would not involve an antagonism to a repressor, might be thought of as "positive induction." Cogent evidence in favor of a repressor-inducer antagonism has come from the work of Pardee *et al.* (1959), described in the preceding section. While it seems likely that this kind of mechanism is widespread in similar systems, the possibility of positive induction in some cases should perhaps be kept in mind.

It will be recalled that Pardee *et al.* (1959) concluded that in the β-galactosidase system induction represents an antagonism of an *exogenous* inducer to an *endogenous* repressor. Gorini (1960a), in carefully controlled experiments with double mutants, examined ornithine transcarbamylase formation as a function of the interaction of an *exogenous* repressor (arginine) and an *exogenous* antagonist (ornithine) of this repressor. He found that ornithine reverses the repressive effect of arginine within certain concentration ranges; however, ornithine was without effect under conditions of complete repression or derepression. Thus, ornithine can, under appropriate conditions, have an inducing effect on the formation of the transcarbamylase by antagonizing arginine repression. Various relationships between induction and repression have been the subject of well-documented reviews by Monod (1959b) and Halvorson (1960). Interesting thoughts on repressor and inducer action have been presented by Brenner (1959).

Features of Repression Relevant to Its Mechanism. At this point, it would seem advisable to list some of the biochemical and genetic data, discussed above, that have become available since 1957 and bear rather directly on the problem of mechanism. Any such considerations should, of course, be viewed against the background of current knowledge of protein synthesis which has been admirably summarized in recent reviews by Cohen and Gros (1960), Fincham (1960), and Yanofsky (1960).

Among the predominantly biochemical features are the kinetics of repression and derepression (Fig. 2–1), the pace-setting phenomenon in derepression (Fig. 2–2), coordinate (and non-coordinate) repression (Fig. 2–3), the effects of intracellular repressor concentration (Fig. 2–4), the promptness of repression and derepression, and the relatively large differential rates of derepression of some systems. Among the predominantly genetic features are structural and regulatory determinants, including operator-type genes, and their linkage and dominance relationships (cf. Fig. 2–5). Additional results of relevance to mechanism will be cited in the context of the discussions below.

"Active" Repressors. The regulator hypothesis contemplates that repressors may act as "active" derivatives (Vogel, 1957b). This thought implies that the functional repressor is a bipartite molecule made up of a small-molecule repressor (such as arginine) in combi-

nation with a moiety (of small or large molecular size) which may have the character of a type of adaptor. The principal support for the notion of active repressors comes from genetic experiments. The existence of genetic loci (such as *i*, R_{try}, or R_{arg}) that control repressibility, and may or may not be linked to the corresponding structural genes, is highly compatible with the view that such regulatory loci govern the formation of active repressors and that the small-molecule repressors (for example, arginine) cannot function until they are combined with their activating moieties. Particularly strong evidence for the activation of repressors is provided by the experiments of Pardee *et al.* (1959), taken together with those of Pardee and Prestidge (1959). As pointed out above, these authors concluded that in the β-galactosidase system (a) a specific repressor is formed and (b) the appearance of this repressor does not seem to entail protein synthesis; they suggested that the functional repressor may contain ribonucleic acid. On the basis of the studies by Monod *et al.* (1951) on the specificity of induction, it seems reasonable to assume that the functional repressor of β-galactosidase synthesis possesses a moiety that is structurally related to galactosides, although such a moiety need not be a derivative of galactose itself.

The activating moieties of repressors could be repressor-specific soluble-ribonucleate (S-RNA) molecules. Such S-RNA-type molecules may be different from the S-RNA species thought to participate in general protein synthesis, since a mutation in a regulator locus does not seem to affect the over-all functioning of the cell. It should be remembered, however, that the experiments of Pardee and Prestidge (1959) did not eliminate the possibility of a role of protein synthesis in the production of repressor, as governed by the regulator gene. A result of Nisman and Fukuhara (1960) may also be relevant in this connection: they made an *in vitro* study of β-galactosidase synthesis with a preparation derived from a constitutive strain of *E. coli* and found β-galactosidase formation to be stimulated by a nucleic acid fraction of the same strain; when this nucleic acid fraction was replaced by an analogous one derived from an inducible, but non-induced strain, an inhibition of enzyme formation resulted, which they considered to be repression, and, interestingly enough, this inhibition was not relieved when the nucleic acid fraction from the inducible strain was pretreated with ribonuclease. If this inhibition of enzyme synthesis was indeed repression, the find-

ings of Nisman and Fukuhara (1960) would argue against, but not exclude, S-RNA as a component of the active repressor.

The possibility that nucleic acid is a component of active repressors has a happy analogy with the adaptor hypothesis of Crick (1958) in reference to general protein synthesis. To date, however, there is no conclusive evidence that repressors require any activation at all for their functioning. What seems to be quite clear is that there are well defined genetic loci controlling repression, but such control need not entail compound formation between the repressor and some other substance. Various other types of interaction between repressors and regulatory genes or the latter's products are conceivable, and, in particular, a joint activity involving a repressor and a regulatory gene product without compound formation does not seem to be in conflict with the known facts. An advantage inherent in this possibility would be that prompt relief from repression, when necessary, would be achieved through mere utilization of the small-molecule repressor, without the need for removal or breakdown of an active repressor.

Site of Repressor Action. In the regulator hypothesis, it was assumed, as mentioned above, that functional repressors and inducers act on enzyme-forming systems, i.e., at the level of the presumable secondary templates (the genic templates being considered the primary ones). A substantial body of evidence supports such a view; recently, though, some authors have entertained the possibility of an interaction of primary templates with repressors. These considerations were based on the results from continuous cultivation experiments under repressive conditions (Cocito and Vogel, 1958; Vogel, 1958), on coordinate repression (Ames and Garry, 1959), on studies of immunity of lysogenic cells (Jacob, 1960), and on studies of the operator gene (Jacob *et al.*, 1960a). However, as pointed out by these authors, alternative explanations are possible, and in particular, operator-type genes could express themselves at the level of secondary templates.

As discussed in an earlier section, coordinate repression was first described for enzymes of histidine synthesis in *S. typhimurium,* and the genes corresponding to these enzymes are all closely linked. In contrast, the genes of arginine synthesis in this organism and in *E. coli* are not all closely linked, and yet the enzymes of arginine synthesis apparently can be subject to repressor action under the con-

trol of a single regulatory gene. In the arginine case, therefore, the picture of repressor action at the primary-template level is somewhat less inviting than it is in the histidine case. The enzyme-forming systems of the arginine pathway may thus each have sites sensitive to the same repressor (cf. Jacob *et al.*, 1960b). Alternatively, the possibility exists that the enzyme-forming systems, despite the lack of close linkage of all the corresponding genes, may be physically associated and jointly susceptible to repressor action. In the case of enzymes, such as tryptophan synthetase, that consist of more than one protein component, each of which is repressible (Yanofsky and Crawford, 1959; Yanofsky, 1960), it would appear especially likely that the protein-forming systems producing the two components are in close juxtaposition. Drs. Suskind and Yanofsky (Chap. 1) have reviewed their very revealing studies on the formation of tryptophan synthetase in *E. coli* and *N. crassa*.

Further arguments in favor of repressor and inducer action at the secondary-template level can be based on such findings as the constant differential rates of induction of β-galactosidase in cryptic (permease-deficient) strains of *E. coli* (Herzenberg, 1959; cf. Monod, 1958) and as the generally observed promptness of onset of repression, derepression, and induction. The virtually immediate onset of repression of acetylornithinase, for example, indicates a prompt cessation of derepressed enzyme synthesis that is compatible with an effect of the repressor on the functioning of existing secondary templates but that would seem to be less compatible with an exclusive effect on primary templates.

Repression and Nucleic Acid Metabolism. These thoughts regarding templates now lead to further considerations on the relation of repression and induction to nucleic acid metabolism. Recent general discussions of this topic have been presented by Magasanik *et al.* (1959), Neidhardt and Magasanik (1960), Chantrenne (1958a, 1958b), and Maaløe (1960). A comprehensive coverage of ribonucleic acid metabolism is provided by the thoughtful article of Dr. Zalokar (Chap. 4).

Important advances in this area have been made as the result of investigations of ribosomes in *E. coli*, which appear to contain the secondary templates of this organism. The work of McQuillen *et al.* (1959), Bolton *et al.* (1959), and Roberts (1960), for example, has shown that ribosomes are indeed active sites of protein synthesis,

which characteristically are heterogeneous and in a highly dynamic state. Other advances have come from recent studies with extracted ribosomal systems, which made it possible to obtain *in vitro* synthesis of induced (Kameyama and Novelli, 1960) and constitutive (Nisman and Fukuhara, 1960) β-galactosidase. Rogers and Novelli (1960) have adduced evidence for the *in vitro* formation of ornithine transcarbamylase. Kameyama and Novelli (1960) demonstrated an inducer-dependent synthesis of β-galactosidase that is inhibited by chloramphenicol, ribonuclease, or deoxyribonuclease. These results indicate, among other things, an involvement of ribonucleic and deoxyribonucleic acids in the synthesis of this enzyme. The intimate connection that seems to exist between β-galactosidase formation and deoxyribonucleic acid has recently been underscored by several different lines of evidence. Nisman and Fukuhara (1960) noted a stimulation of the *in vitro* formation of constitutive β-galactosidase attributable to deoxyribonucleic acid. A continuing role of deoxyribonucleic acid in β-galactosidase formation is also indicated by the fact that irradiation of bacteria with ultraviolet light (Rushizky *et al.*, 1960; Novelli, 1960) or infection of the bacteria with T2 phage (Novelli, 1960) readily stops the formation of this enzyme. A similar indication has come from P^{32} decay experiments in zygotes (Riley *et al.*, 1960). After "thymine saturation," i.e., when deoxyribonucleic acid presumably exists as fully paired strands (Maaløe and Hannawalt, 1960), both induced β-galactosidase formation (Fig. 2–6) and synthesis of deoxyribonucleic acid show a lag and then proceed hand in hand (Roberts, 1960). These various results do not, however, force the conclusion that the induction of the enzyme requires *concomitant* deoxyribonucleic acid synthesis. Roberts (1960) suggested that the critical factor may be the availability of deoxyribonucleic acid in the proper *state*. In the thymine-saturated condition, deoxyribonucleic acid, in view of the lag, could be inactive for this induction, but other interpretations of these results have not been ruled out. Significantly, cells that are non-inducible due to thymine *starvation* seem to be able to synthesize the enzyme at a level of the order of magnitude of the usual non-induced (basal) level (Roberts, 1960).

The ornithine transcarbamylase system is indicated to stand in contrast to the β-galactosidase system with respect to dependence on deoxyribonucleic acid: the derepressed synthesis of the transcarbamylase is not immediately affected by phage infection and is

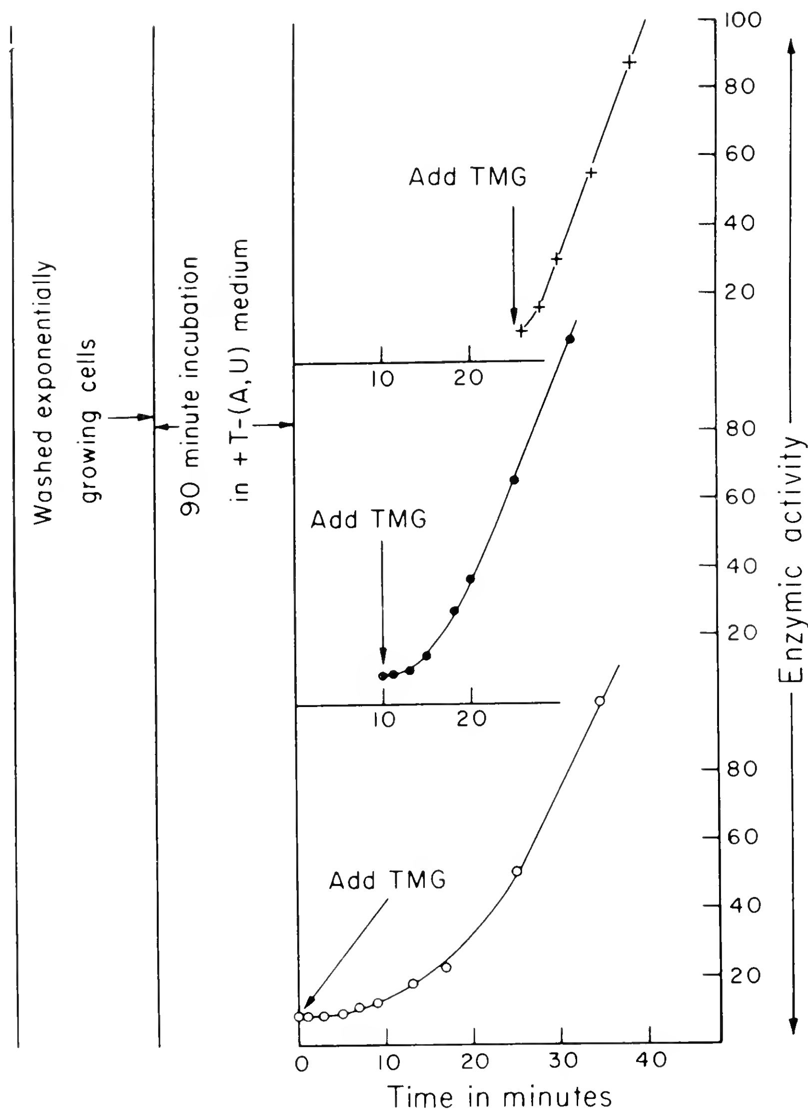

Fig. 2–6. Induction of β-galactosidase following "thymine saturation." A mutant strain of *Escherichia coli* that requires thymine (T), arginine (A), and uracil (U) was incubated in the presence of thymine, but in the absence of arginine and uracil, for the achievement of the thymine-saturated state. Arginine and uracil were added at zero time; inducer, methyl-thio-β-D-galactoside (TMG), was added at the times indicated. Enzyme activity is presented in arbitrary units. Upon addition of inducer at zero time, enzyme formation proceeds after a pronounced lag; a later addition (at 10 minutes) results in a shorter lag. If the inducer is supplied after a sufficiently long period of incubation, lag is

relatively insensitive to irradiation with ultraviolet light (Novelli, 1960).

Apparently, a concomitant formation of *ribonucleic acid* is not necessary for the synthesis of ornithine transcarbamylase: Rogers and Novelli (1959) showed that the enzyme can be produced in a mutant of *E. coli* under conditions of uracil starvation (Fig. 2–7). Such enzyme synthesis was attributed by these authors to the presence of preformed synthesizing sites. Rogers and Novelli (1959) also found that, even under conditions of uracil starvation, the synthesis of ornithine transcarbamylase is repressible by arginine (Fig. 2–7).

Yates and Pardee (1957) had previously obtained evidence for the derepressed formation of (uracil-repressible) enzymes of pyrimidine synthesis in uracil-starved *E. coli* mutants. Recently, Kennell and Magasanik (1960) investigated the derepressed synthesis of the inosine 5′-phosphate dehydrogenase of *Aerobacter aerogenes*, as a function of the concentration of ribosomes in the cell. This enzyme is repressible by guanine and shows derepressed synthesis under conditions of guanine starvation (Levin and Magasanik, 1959) and hence in the absence of any net synthesis of ribonucleic acid. Upon control of the ribosome level through magnesium deprivation, Kennell and Magasanik (1960) were able to demonstrate that the rate of derepressed synthesis of the dehydrogenase is proportional to the concentration of ribosomes in the cell. These authors concluded, among other things, that ribosomes capable of producing the dehydrogenase apparently are present in the cell even when the derepressed synthesis of the enzyme is not taking place.

These indications that repressible enzyme synthesis can occur without concomitant ribonucleic acid formation are, of course, highly relevant to considerations of the site of repressor action. Support for these indications comes, for example, from the extensive studies on general protein synthesis of Magasanik *et al.* (1959) and Neidhardt and Magasanik (1960) with *A. aerogenes*. Consistent with these studies in bacteria are the graphic results of Zalokar (1959,

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virtually abolished (top curve). Following the addition of the arginine and uracil (at zero time), deoxyribonucleic acid synthesis, as judged by thymine incorporation, proceeds with a lag similar to that of β-galactosidase formation (bottom curve); in contrast, general protein synthesis and ribonucleic acid formation are initiated promptly. (*Courtesy*, R. B. Roberts.)

1960) on the localization of newly formed proteins and ribonucleic acid in hyphae of *N. crassa*. The ingenious combination of centrifugation and autoradiography techniques used is discussed in Chapter 4 by Dr. Zalokar. Further evidence in favor of the view that protein synthesis can occur without simultaneous ribonucleic acid formation is furnished by enucleation experiments with certain protozoa. Re-

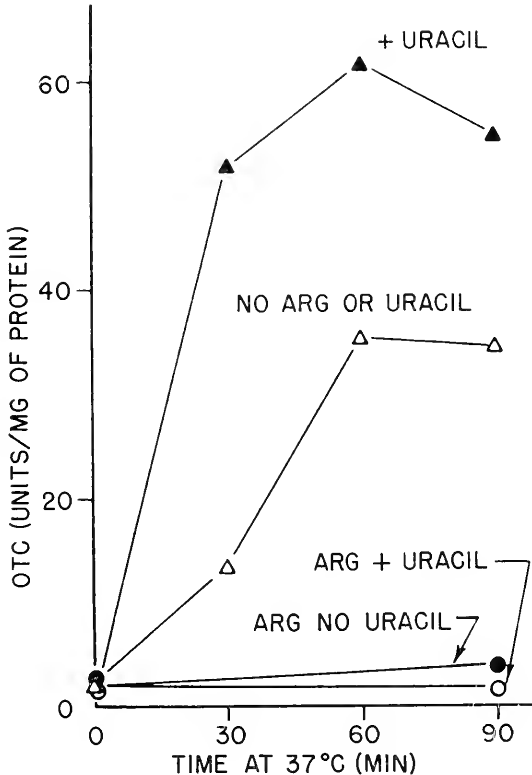

Fig. 2–7. Synthesis of ornithine transcarbamylase (OTC) under conditions of uracil starvation. Cells of a uracil-requiring mutant strain of *Escherichia coli* were grown on a glycerol-salts medium, supplemented with uracil (20 μg/ml) and arginine (200 μg/ml), and were starved in this medium modified by the omission of uracil, for 75 minutes at 37° C. The cells were then suspended in unsupplemented medium and divided into 4 portions: the first portion received uracil (solid triangles); the second, arginine (solid circles); the third, uracil and arginine (plain circles); and the fourth, no addition (plain triangles). Incubation was carried out at 37° C with shaking, and samples were removed, as indicated, for enzyme and protein determinations. See Rogers and Novelli (1959) for the procedures and the enzyme unit used. (Courtesy, P. Rogers and G. D. Novelli.)

cently, for instance, Prescott (1960b) reported that enucleated fragments of *Tetrahymena pyriformis* are able to synthesize protein, as judged by the incorporation of labeled amino acids into trichloracetic acid-insoluble material, but are completely unable to produce ribonucleic acid, as judged by the lack of incorporation of labeled cytidine into acid-insoluble material. Similar results were obtained with an *Acanthamoeba* species (Prescott, 1960a, 1960c). In the alga, *Acetabularia mediterranea*, the synthesis of ribosomal and soluble ribonucleic acid likewise seems to depend on nuclear function; the synthesis of ribonucleic acid associated with a large granule fraction consisting mainly of chloroplasts, however, apparently is more independent (Naora *et al.*, 1960).

These studies with fungal, protozoan, and algal systems thus provide evidence that in such organisms the nucleus produces the ribosomal as well as the soluble ribonucleic acids, which would include those ribonucleic acid species presumed to contain the information for enzyme synthesis. It is possible that such information-bearing ribonucleic acid is present in certain metabolically very active ribonucleic acid fractions whose base ratio pattern mimics that of the deoxyribonucleic acid of the organism of their origin. Ribonucleic acid fractions of this type have been demonstrated in the studies of Volkin and Astrachan (1957), Astrachan and Volkin (1958), and Volkin *et al.* (1958) and in the recent extensive investigations of Nomura *et al.* (1960), all on the subject of the nucleic acid metabolism of phage-infected bacteria, during certain stages of infection. Apparently similar metabolically active ribonucleic acid fractions have been exhibited in short-term tracer incorporation experiments with uninfected bacteria (Astrachan and Fischer, 1960) as well as with yeast (Yčas and Vincent, 1960).

THE SITE PROBLEM. In the light of the available knowledge of repression (and induction) in relation to nucleic acid metabolism, the question may now be examined further, whether repressors and inducers act at the level of the primary (deoxyribonucleic acid) template or of the secondary (ribonucleic acid) template (or, conceivably, of both). Action at the primary-template level would presumably be tantamount to action in the *production* of information-bearing elements of secondary templates; action at the secondary-template level might be directed (a) at the functioning of the secondary templates in enzyme synthesis or (b) at such func-

tioning in combination with a possible propagation of secondary templates.

The possible involvement in repression of a propagation-type behavior of secondary templates would be in harmony with the findings that ribosomes can be in a highly dynamic state and, in some sense, apparently are semiautonomous in that they seem either to be self-duplicating or to participate in determining the rate at which they are synthesized (Roberts, 1960; Kennell and Magasanik, 1960). Self-duplication of ribosomes would imply that they can direct the synthesis of ribonucleic acid, including information-bearing molecular species. Ribonucleic acid synthesis at the secondary-template level may be thought unlikely in view of the above-mentioned results with fungal, protozoan, and algal systems but should perhaps be kept in mind in the case of bacteria. The rate at which functional ribosomes are formed could depend, in part, on themselves, if the process of protein synthesis includes (a) a splitting of ribosomes (that may or may not involve a concomitant liberation of nascent enzyme) and (b) reutilization, for functional ribosome formation, of ribosome fragments, presumably in conjunction with information-bearing ribonucleic acids produced in the nucleus. If so, it seems reasonable to assume that the ribosome fragments cannot be reutilized indefinitely and that nuclear control extends not only to the production of such information-bearing ribonucleic acids but also, to a degree, to the production of entire functional ribosomes.

The Primary Template as a Possible Site of Repressor Action. Arguments in favor of repressor action at the deoxyribonucleic acid level would be based mainly on genetic results and on the behavior of the β-galactosidase system. Among the relevant genetic results are those on coordinate repression of enzymes whose structural genes are closely linked and those on the activity of operator-type genes. However, the repressibility of enzymes of arginine synthesis, whose genes are not all closely linked, reduces the appeal of such genetic arguments.

For the β-galactosidase system, the possibility of repression and induction at the primary-template level suggests itself in view of the close relationship that has been inferred to exist between this system (in contrast to certain other systems) and deoxyribonucleic acid. It remains to be seen, though, if this apparent difference between the β-galactosidase and other systems is fundamental. The difference

might, for example, largely be due to a requirement of the β-galactosidase-forming machinery for an *in situ* production of ribonucleic acid, under the direction of deoxyribonucleic acid, to facilitate the relatively rapid ribosome metabolism that would be necessary under conditions of induction (absence of repression). Even if so, the possibility of repressor action at the primary-template level is by no means excluded.

THE SECONDARY TEMPLATE AS A POSSIBLE SITE OF REPRESSOR ACTION. Strong arguments can be made in favor of repressor action at the secondary-template level; in fact, the weight of the evidence seems to lean heavily in this direction. Support for repressor action at this level is provided, for example, by (a) the speed of onset of repression; (b) the speed of onset of derepression or induction; (c) the constant differential rates of β-galactosidase induction in permease-deficient organisms; (d) general protein synthesis in enucleated organisms; (e) enzyme synthesis, including repressible enzyme synthesis, apparently without concomitant ribonucleic acid formation; and (f) the semiautonomous behavior of ribosomes. This support is particularly firm, if these pieces of evidence are taken in conjunction with one another. For instance, the promptness of onset of repression, taken by itself, would seem to favor the secondary template as repression site; this promptness would, however, not eliminate the primary template from consideration, since repression, at this level, could interfere with the production of a ribonucleic acid species that might be continuously required for enzyme formation. Thus, repressor action, even at the primary-template level, could express itself rapidly. However, in view of the fact that repressible enzyme synthesis apparently can proceed without concomitant ribonucleic acid formation, the promptness of onset of repression gains strength as evidence in favor of repressor action at the secondary-template level.

Accordingly, we are led to the view that enzyme repression and induction probably can occur at the level of the ribonucleic acid template, and hence of the ribosome. A possible mechanism of repression (and induction) at the level of the enzyme-forming system has been proposed in the regulator hypothesis in terms of the separation of template products from their templates (see the discussion The Regulator Hypothesis). The available evidence is consistent with such a picture, which can now be viewed in the context of ribo-

some metabolism and propagation, as discussed in The Site Problem. It may thus well be that (functional) repressors impede the separation of nascent enzymes from their templates and, thereby or independently, interfere with ribosome propagation. Inducers (in functional form) would have an opposite effect through antagonizing a repressor or conceivably through "positive" induction.

The notion of a dual effect of repressors on enzyme formation and on ribosome propagation has received a measure of support from the pace-setting phenomenon described in an earlier section and discussed below.

Pace-setting Effect and Site of Repression. Upon variation of the point of onset of acetylornithinase derepression, it was found that the later the onset, i.e., the longer the period of prior repression, the lower is the subsequent differential rate of derepressed enzyme formation (Fig. 2–2). Although several interpretations of this pace-setting effect are possible, the most adequate working hypothesis would seem to be that, in addition to repression of acetylornithinase formation by arginine, there is a repression-like antagonism, apparently also by arginine, to the formation of a component, or components, of the corresponding enzyme-forming system. It is assumed that the differential rates of acetylornithinase formation reflect the number of corresponding functional enzyme-forming sites available, per unit mass of the organisms, at the point of onset of derepression. During the period preceding the onset of derepression, while acetylornithinase is being repressed, the number of such sites per unit mass of organisms would decrease, and on subsequent derepression the pace-setting effect would become apparent.

The proposed dual effect, at the secondary-template level, on (a) the production of the enzyme molecules and (b) the production of the enzyme-forming sites can be readily visualized in terms of the possible metabolism and propagation-type behavior of ribosomes (see the discussion The Site Problem). Conceivably, the very act of binding, contemplated in the regulator hypothesis, of the nascent enzyme molecule to its template may interfere with the postulated splitting-and-reutilization behavior of the ribosomes; alternatively, the repression of enzyme formation and the interference with ribosome propagation may be somewhat less directly related. According to this general model, the repressor site (i.e., the region of the ribosome-nascent enzyme complex for which the functional repressor

is thought to have affinity) could be on the nascent enzyme, on the ribosome, or on both. A repressor site on the nascent enzyme need not be identical with the nascent dynamic (catalytically active) site of the enzyme (Vogel, 1958).

Repression in the Control of Cellular Function and of Development

Repression and Feedback Inhibition. Quite different from repression is the phenomenon of feedback inhibition, which characteristically consists in the interference with the flow of metabolites through a pathway by inhibition of an early enzymatic step of the pathway, through the agency of the "end product" involved. Feedback inhibition thus occurs at the level of enzyme *action* rather than of enzyme *formation* and can be viewed as a control mechanism that frequently, but not necessarily, acts in parallel with repression of enzyme formation. Since feedback inhibition is the subject of Dr. Umbarger's detailed, basic contribution to this volume (Chap. 3), only certain instances of such inhibition that bear rather directly on enzyme repression will be considered here.

Gorini (1958) has carried out a study of arginine repression of ornithine transcarbamylase in relation to feedback inhibition in the arginine pathway of a strain of *E. coli.* In experiments with the chemostat, at a given bacterial density, added arginine does not become repressive for the transcarbamylase, until the supply of this exogenous amino acid exceeds that necessary for an arginine-requiring mutant of this strain to attain the same density. Maximal feedback inhibition appears to take place at intracellular arginine concentrations lower than those needed to give maximal repression. These two mechanisms thus tend to cooperate in furnishing an economical and efficient control system which, within appropriate limits, permits an (endogenous) synthesis of arginine that is precisely complementary to the supply of (preferentially utilized) exogenous arginine.

With the aid of metabolite analogues, it has been shown that certain compounds (false feedback inhibitors) can mimic, under suitable conditions, the end product of a pathway in its action as a feedback inhibitor but not as a repressor. For instance, the antimetabolite 2-thiazolealanine was found to give such false feedback inhibition of histidine synthesis in *E. coli* (Moyed, 1960; Shedlovsky

and Magasanik, 1960; Magasanik, 1960). By virtue of this kind of inhibition, the antimetabolite can slow the production of histidine (which, in contrast to the antimetabolite, has repressor activity) and hence can bring about derepression of the synthesis of enzymes in the histidine pathway. Yates and Pardee (1957) have obtained derepression in the pyrimidine path of *E. coli* with 6-azauracil which, presumably as nucleotide (Handschumacher and Welch, 1960), acts as a false feedback inhibitor.

A derepression effect in the tryptophan pathway in *E. coli* has been demonstrated by Lester and Yanofsky (1960) through the use of 3-methylanthranilic acid. This compound seems to function not as an analogue of the end product, tryptophan, but rather as an analogue of a tryptophan precursor. 3-Methylanthranilic acid can curtail the synthesis of (the repressive) tryptophan, apparently by inhibition of the conversion of anthranilic deoxyribulotide to indoleglycerol phosphate, and can thereby bring about derepressed tryptophan synthetase formation.

Although false feedback inhibitors, such as 2-thiazolealanine, are not repressive, cases of "false repressor" action have been reported. Thus, the guanine analogue 8-azaguanine strongly interferes with the formation of inosinicase and of inosine 5′-phosphate dehydrogenase, both of which are repressible by guanine. This interference by 8-azaguanine is specific for these two enzymes and, under the conditions used, does not extend to the formation of enzymes in pathways other than those of purine synthesis (Levin and Magasanik, 1959; Magasanik, 1960). Recently, Tonomura and Novelli (1960) showed that the repressive action of uracil on the production of dihydroorotic acid dehydrogenase of *E. coli* can be mimicked, with a measure of specificity, by cysteine, although the latter compound does not seem to have an obvious structural similarity to uracil; cysteine apparently can have the usual type of repressive effect on enzymes of its own path of synthesis (Bourgeois *et al.*, 1960).

An instructive example of cooperative action of feedback inhibition and repression in the control of cellular function occurs in the glyoxylate cycle of *Micrococcus denitrificans*. Kornberg *et al.* (1960) have studied the change from autotrophy to heterotrophy (acetate utilization) in this organism and found that this change is accompanied by a metabolic shift from the Calvin cycle to the glyoxylate cycle; the operation of the latter cycle was concluded to be gov-

erned through a combination of feedback inhibition and repression of isocitratase by succinate, as indicated in Fig. 2–8.

A revealing case of the interplay of multiple feedback inhibitions and repressions can be seen in the purine nucleotide system described by Magasanik (1960). The regulation of the purine nucleotide interconversions (Fig. 2–9) includes (a) feedback inhibitions by guanosine 5′-phosphate and adenosine triphosphate at steps 8 and 10, respectively, and (b) repressions by guanine, as discussed above, at steps 2 (inosinicase) and 8 (inosine 5′-phosphate dehydrogenase); additionally, histidine can produce a feedback inhi-

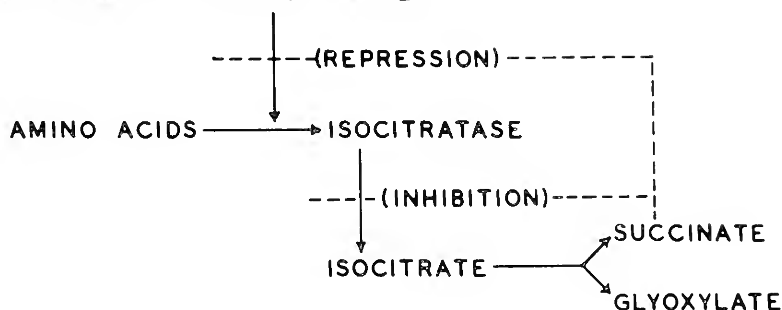

Fig. 2–8. Repression and feedback inhibition of the isocitratase of *Micrococcus denitrificans*. (Courtesy, H. L. Kornberg J. F. Collins, and D. Bigley.)

bition at step 6, as well as repression of the enzymes catalyzing the steps from adenosine triphosphate to imidazoleglycerol phosphate. Both types of regulatory mechanisms thus contribute to the great precision with which the purine nucleotide system is controlled (Magasanik, 1960).

The "Glucose Effect." Evidence is available that the formation of a number of enzymes, especially inducible ones, is antagonized by glucose or metabolic products of glucose. This type of antagonism, frequently termed "glucose effect," appears to be enzyme repression (Magasanik *et al.*, 1958, 1959; Cohn and Horibata, 1959a, 1959b; Magasanik and Bojarska, 1960; Neidhardt, 1960a, 1960b). In view of the pivotal position of glucose in metabolism, instances of this effect would seem to represent repressions of particular significance to the control of cellular function.

It has been suspected for some time that, in the glucose effect, glucose itself is not the functional repressor (Magasanik *et al.*, 1958)

but is converted to other substances that, possibly upon "activation," cause the various repressions. Support for this view has come from the discovery by Neidhardt (1960a, 1960b) of a mutant of *A. aerogenes* in which glucose fails to repress a number of enzymes whose formation is glucose-sensitive in the parent strain; gluconate, however, is equally repressive for the mutant and the parent strains. Magasanik and Bojarska (1960) have examined peculiarities in the glucose metabolism of this mutant strain and concluded that the repressive action of glucose may be exerted via gluconate.

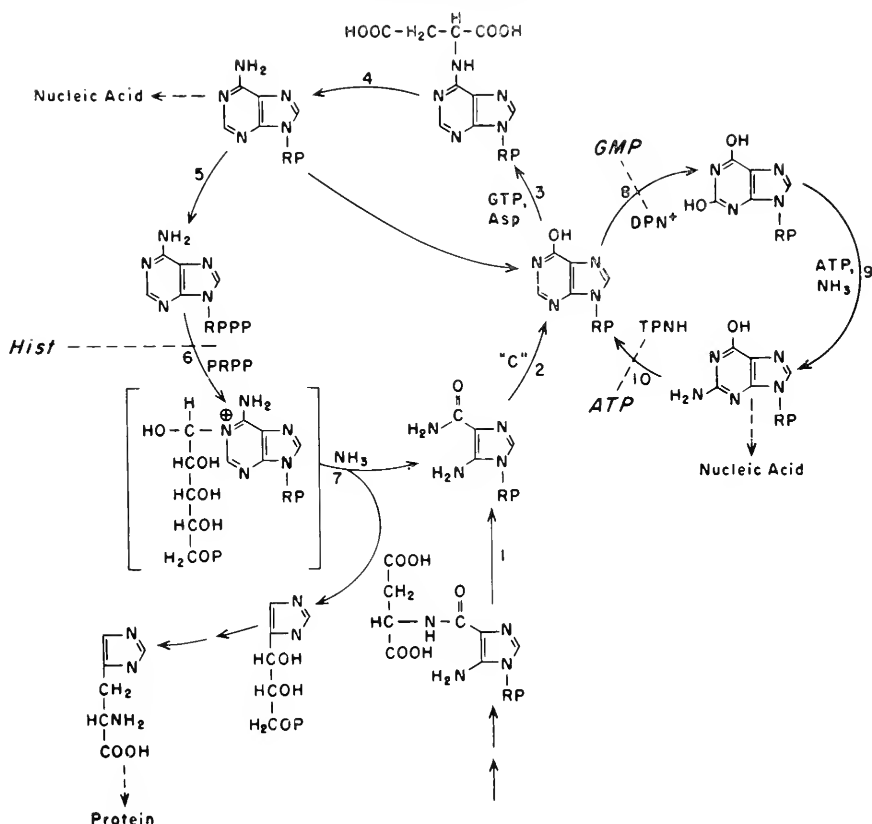

Fig. 2–9. The regulation of the cyclic interconversions of purine nucleotides. See the discussion by Magasanik and Karibian (1960). The dashed lines intersecting arrows indicate the sites of feedback inhibition by guanosine 5′-phosphate (GMP), adenosine triphosphate (ATP), or histidine (Hist). The enzymes corresponding to steps 2 and 8 are repressible by guanine; the enzymes catalyzing step 6 and the conversion of the product of this step to imidazoleglycerol phosphate are repressible by histidine. (Courtesy, B. Magasanik and D. Karibian.)

Magasanik *et al.* (1958) pointed out that repressibility by glucose seems to be characteristic of enzymes whose physiological role is to supply energy, carbon, or nitrogen by the degradation of organic compounds (e.g., β-galactosidase, "galactozymase," inositol dehydrogenase, glycerol dehydrogenase, histidase, tryptophanase, and amino acid deaminases). Repression of the formation of such "energy-supplying" enzymes by products of glucose metabolism would be comparable to the repression of an enzyme in, say, a path of amino acid synthesis by the "end product" of the path.

Recently, Neidhardt (1960b) has discussed the mechanism of the glucose effect and has reviewed available evidence in the light of the notion that this effect represents enzyme repression. Cohn and Horibata (1959b) have presented an extensive analysis of the mechanism of glucose antagonism to β-galactosidase formation; their results are consistent with an interpretation of this case of the glucose effect in terms of enzyme repression.

Comments on the General Biological Significance of Repression. It seems clear that control by repression applies to a wide variety of enzymes in a diversity of metabolic systems. In general, repression has been studied in linear or cyclic pathways capable of supporting a substantial flow of metabolites through them. It may, however, well be that certain kinds of enzymes are not subject to such control. This possibility is suggested, for example, by the finding of Ames (1960) that the histidine-activating enzyme of S. *typhimurium,* in contrast to the enzymes of histidine synthesis, is not repressible by this amino acid. Pardee (1960) has discussed the question of the control of systems, such as those of coenzyme synthesis, for example, that carry a relatively small flow of metabolites.

For the regulation of cellular function, the frequently observed cooperative action of repression and feedback inhibition seems particularly significant. Control by feedback inhibition is rapid; presumably, it makes itself felt as soon as a sufficient concentration of the inhibitor is built up in the vicinity of the susceptible enzyme. Repression, as a mechanism capable of controlling the flow of metabolites through a pathway, acts relatively slowly, despite the promptness with which the onset of repression can occur: repression decreases the rate of enzyme formation; it does not interfere with existing enzyme molecules (whose concentration in the cell can

diminish through dilution, in the course of cell multiplication, or through destruction).

As a control mechanism of enzyme formation, though, repression, as emphasized above, is rapidly acting. In this capacity, repression is effective in avoiding the production of temporarily unnecessary enzymes. Such control not only provides substantial savings of energy and materials but also tends to prevent a possible harmful overproduction of enzymes.

If the pace-setting phenomenon (Fig. 2–2) indeed reflects a regulation of the formation of enzyme-forming sites, this (probably fast-acting) phenomenon would represent another control mechanism with which the repression process can cooperate. One can thus visualize a hierarchy of regulatory mechanisms that operate at the levels of (a) the synthesis of small-molecule metabolites, (b) the synthesis of enzymes, and (c) the synthesis of enzyme-forming systems. These control devices have in common the property of being responsive to endogenous or exogenous substances of relatively low molecular weight. In this context, it is noteworthy that additional regulation can be provided by repressible permeases (Vogel, 1960a) which would be involved in the transfer of exogenous materials into the cell. The complementary nature of enzyme repression and induction as control mechanisms has been discussed elsewhere (Vogel, 1957b).

The high degree of usefulness to the cell of control by repression is attested to by the latter's broad distribution with respect to metabolic systems. It seems evident that repressibility of enzymes is a property that must have been positively selected in the course of evolution (Vogel, 1957a). Consistent with this thought is the apparently widespread occurrence of repression with respect to organisms. Because of the experimental advantages that they offer, bacteria have received more attention in this connection than have other organisms. However, in view of the possible significance of the repression and induction processes to the development and differentiation of higher forms of life (Vogel, 1958), the indicated existence of these processes, particularly of repression, in mammalian systems is of considerable interest.

DeMars (1958) has studied the antagonism of glutamine to the formation of glutamyl transferase in cultures of human cells. The general character of this antagonism was suggestive of repression. In addition to glutamyl transferase activity, the enzyme preparations

used had glutamine synthetase activity. Since extensive purification has not resulted in any separation of the two activities, one and the same enzyme seems to be involved (DeMars, 1960). Accordingly, there is every reason to believe that this antagonism by glutamine represents a case of repression entirely analogous to that exerted on the formation of bacterial enzymes by the corresponding "end products."

Walker (1959) has investigated a presumable instance of repression of enzyme formation by an end product in an intact animal. He found that the addition of creatine to the diet of rats brings about a marked lowering of the level of kidney arginine-glycine transamidinase (which catalyzes a step in creatine synthesis). He pointed out that the lowering of the transamidinase level might be related to the turnover of the enzyme in the tissue examined. Other possible instances of enzyme repression in animals have been considered by Auerbach *et al.* (1958), Auerbach and Waisman (1958), Potter and Auerbach (1959), Knox (1960), and by McFall and Magasanik (1960). In a review of protein and nucleic acid turnover in relation to biochemical differentiation, Mandelstam (1960) has discussed the possible roles of enzyme repression and induction in the context of development. The possibility that some hormones may have a repressor-like action has also received some attention (Knox, 1960; cf. Vogel, 1958).

In conclusion, it would seem fair to say that the area of repression has recently seen a great deal of progress. The interest in this area may be due, in part, to the latter's position in the interdisciplinary no man's land adjacent to various fields of chemistry and biology and, in part, to the fact that repression can serve in constructs of high precision or of broad scope. For the same reasons, the pace set is likely to be maintained or increased in the future.

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3

End-Product Inhibition of the Initial Enzyme in a Biosynthetic Sequence as a Mechanism of Feedback Control

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The pathways by which small molecule building blocks are biosynthesized have been studied in yeast, *Neurospora* and such Gram negative bacteria as *Aerobacter aerogenes, Escherichia coli,* and *Salmonella typhimurium.* These organisms are able to synthesize their entire spectrum of protoplasmic constituents and grow in a medium containing essentially glucose, ammonia, and inorganic salts. A striking feature of such organisms is not only that they contain all the enzymes to catalyze these biosynthetic steps but also that the activities of these enzymes are so integrated that there is an orderly flow of metabolites to the sites of synthesis of macromolecules such as proteins and nucleic acids.

In the case of amino acids, though there is a small but measurable pool of free amino acids within the cells, very little of any amino acid appears in the medium. Thus, the cell forms virtually only as much of each amino acid as it requires. Furthermore, if an amino acid or a freely permeable precursor had been added to the medium, the exogenous compound is used and, for some small molecule building blocks, endogenous formation of the compound stops.

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How striking is this economy of synthesis for one amino acid, isoleucine, was shown by isotope competition experiments performed by the Biophysics Group of the Carnegie Institution's Department of Terrestrial Magnetism (Roberts *et al.*, 1955). These workers showed that when *E. coli* was grown in a minimal medium containing glucose uniformly labeled with carbon-14 and unlabeled isoleucine, the isoleucine in the cells contained no more than 5 per cent of the glucose (labeled) carbon atoms. In other words, the cells had utilized the isoleucine in the medium in preference to making their own. In similar experiments, it was shown that for most amino acids there was either partial or complete suppression of endogenous synthesis. For a few amino acids, however, the cells continued synthesis of the amino acid to the same extent whether or not it had been available exogenously. Obviously, the question arises: By what mechanism did the cells respond to the exogenous amino acid?

The interest of physiologists employing mutant methodology for the elucidation of biosynthetic pathways became focused on this question as a result of observations on the kinetics of the accumulation of precursors by auxotrophic (nutritionally deficient) mutants. In the early studies, investigators were not at all surprised to find that certain auxotrophic mutants accumulated compounds that could be utilized for growth by mutants blocked earlier in the same pathway. This property of mutants was the basis for syntrophism which occurred when mutants blocked at different points in the same pathway were cultivated in the same tube or were streaked side by side on solid media (Davis, 1950). The early investigators were surprised, however, by the fact that the accumulation of precursors had a peculiar relationship to growth which is idealized in Fig. 3–1.

It was noted that if the mutant was grown in a series of flasks containing increasing amounts of the growth factor, the amount of growth obtained after, for example, a 30-hour incubation would be nearly proportional to the amount of growth factor added until some other factor, such as carbon source, became the limiting one. When such cultures were examined for precursor accumulation, however, it was observed that the point of maximal growth was not the point of maximal precursor accumulation.

Alternatively, if a kinetic experiment was performed using a single level of growth factor, it was observed that accumulation did not begin until just shortly before growth stopped, owing to growth

factor exhaustion. If growth stopped for some other reason while growth factor was still present, there was no precursor accumulation. It was therefore concluded that the addition to the medium of the end product of a biosynthetic sequence prevents further synthesis not only of that end product, as had been revealed by the isotope competition experiments, but also of very early intermediates as revealed by the study of precursor accumulations.

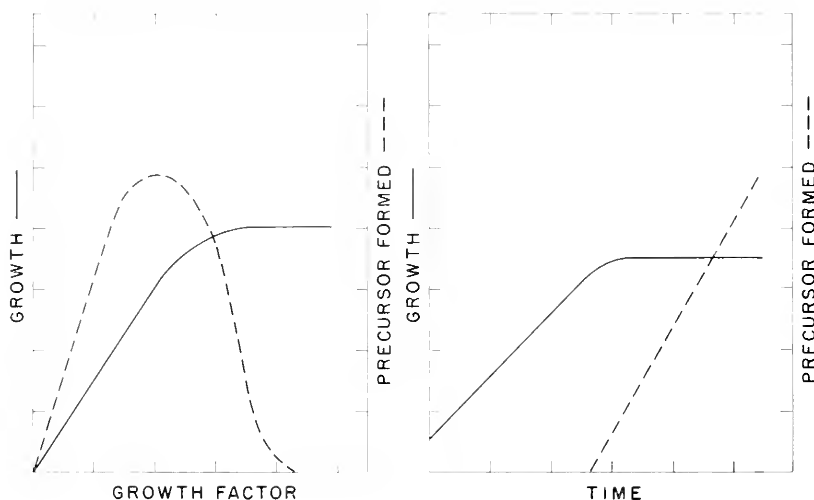


Fig. 3–1. Relationship of the accumulation of a precursor by an auxotrophic mutant to its growth.

A kinetic experiment like that represented in Fig. 3–1 does not permit one to decide which of two possible mechanisms is responsible for delayed accumulation The failure of the precursor to accumulate until the end product has disappeared could be due either to the absence of one or more enzymes in the pathway or to the failure of at least one enzyme to function. Actually by the time this inhibition of endogenous synthesis by the end product had been recognized as a general property of biosynthetic pathways in microorganisms, there was already evidence which supported both possibilities.

In support of the idea that one or more enzymes in the pathway might be absent as long as exogenous end product was present, there were the observations by Cohn *et al.* (1953) and independently by

Wijusundra and Woods (1953, 1960) on the synthesis of methionine by *E. coli.* Both groups observed essentially that *E. coli* cells harvested from a glucose–mineral salts medium could convert homocysteine to methionine in the presence of serine and *p*-amino benzoic acid. However, if methionine had been incorporated into that medium, the harvested cells were unable to perform this conversion. In another example in *E. coli,* Vogel and Davis (1952) observed that when the cells of an arginine auxotroph blocked earlier than ornithine had been previously grown in an arginine medium, growth in an ornithine or citrulline medium occurred only after a lag period. In contrast, ornithine grown cells grew in media containing ornithine, citrulline, or arginine without lag.

Although the conversion of homocysteine to methionine has recently been undergoing a vigorous enzymatic analysis (Hatch *et al.*, 1959; Szulmajster and Woods, 1960), the missing enzyme in the methionine grown cells has not been revealed. In contrast, not only has the repression of the arginine synthesizing enzymes in arginine grown cells been demonstrated (Vogel, 1956; Gorini and Maas, 1957), but, as shown in the chapter by Vogel (1961), this pathway has supplied the model par excellence for the study of enzyme repression in a biosynthetic pathway.

An example of the evidence supporting the view that the enzymes might be present but not functioning was described by Brooke *et al.* (1954). They employed a uracil auxotroph of *A. aerogenes* which was blocked after orotic acid and which accumulated this compound. Cells of this mutant did not accumulate orotic acid in a medium containing excess uracil. However, upon transfer to a uracil free medium, orotic acid accumulation began immediately. Thus, uracil had prevented the *action* of an enzymatic sequence already present in the cells. Similarly, in view of the way in which the isotope competition experiments cited above were performed, it must be concluded that the cells contained the necessary enzymes for isoleucine biosynthesis (wild-type cells harvested from minimal medium). Yet upon transfer to an unlabeled isoleucine–labeled glucose medium, it was apparent that the pathway from glucose to isoleucine was completely and immediately blocked.

An opportunity to study the mechanism of this block between glucose and isoleucine in *E. coli* presented itself while the first step leading specifically to isoleucine was being studied. This step (Re-

action I, Fig. 3–2) is the conversion of L-threonine to α-ketobutyrate.[2] It had been observed in isotope competition experiments with *E. coli* that the carbons of exogenous threonine were converted to isoleucine (Abelson, 1954). Thus, auxotrophs blocked before threonine use this amino acid not only for incorporation into protein but also for the formation of isoleucine. The requirement for the latter function could be bypassed, however, by incorporating isoleucine into the medium. When this was done, it was noted that isoleucine ex-

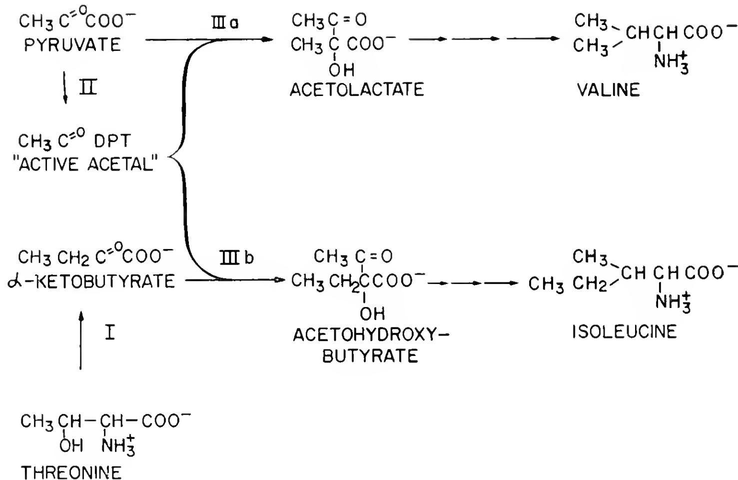


Fig. 3–2. The early steps in the biosynthetic pathways leading to isoleucine and valine.

erted a sparing effect on the threonine requirement (Umbarger, 1955). Thus, the presence of isoleucine somehow prevented threonine from being irreversibly converted to any intermediates along the pathway to isoleucine. While the inhibition of any step in the isoleucine pathway would have explained the absence of endogenously synthesized isoleucine, only the inhibition of L-threonine deamination, which is irreversible, would have explained the sparing action. While the inhibitor of L-threonine deamination might be any compound freely in equilibrium with the internal pool of isoleucine,

[2] So far as is now known, α-ketobutyrate has no other function in the cell than to condense with a two carbon fragment to yield α-aceto-α-hydroxybutyrate (Leavitt and Umbarger, 1959). The earlier steps in the pathway between glucose and isoleucine should be considered as the threonine pathway. Therefore, the pathway leading to isoleucine is restricted to those steps of the pathway which lead from the biosynthetic branch point, threonine.

the simplest situation would result if isoleucine itself were the inhibitor. Accordingly, threonine deaminase activity of *E. coli* extracts was measured in the presence and absence of isoleucine. Table 3–1 shows that isoleucine was indeed a powerful inhibitor of the L-threonine deaminase of *E. coli*, whereas the other amino acids showed little or no such activity (Umbarger, 1956). Subsequent study revealed that the interaction between threonine and isoleucine was a competitive one and that the enzyme had about a hundredfold greater affinity for the inhibitor, L-isoleucine, than for the substrate, L-threonine (Umbarger and Brown, 1958a).

TABLE 3–1

The Inhibition of Threonine Deamination in Crude Extracts of *E. coli*

Amino Acid Tested	Concentration	Inhibition (%)
L-Isoleucine	10^{-2} *M*	100
L-Isoleucine	10^{-4} *M*	52
L-Leucine	10^{-2} *M*	55
L-Aspartate	10^{-2} *M*	30
L-Valine	10^{-2} *M*	0
L-Alanine	10^{-2} *M*	0
L-Methionine	10^{-2} *M*	0
L-Homoserine	10^{-2} *M*	0

For conditions, see Umbarger (1956).
Reprinted from *Science* (Umbarger, 1956) by permission.

It is clear that the deamination of L-threonine is ideally suited to be the reaction by which isoleucine controls its own biosynthetic pathway. For example, if the utilization of α-ketobutyrate (Reactions II and IIIb, Fig. 3–2) rather than its formation (Reaction I, Fig. 3–2) had been the inhibited reaction, threonine would have been destroyed by deamination to the same extent with as without isoleucine. Also, isoleucine might have inhibited the formation of threonine rather than the deamination. However, inhibition of this reaction would have led to the inability of the cells to grow in media supplemented with isoleucine unless threonine was also present, i.e., isoleucine would have been a growth inhibitor, the action which would have been non-competitively antagonized by threonine In other words, Nature has chosen the only effective reaction for controlling isoleucine biosynthesis.

This mechanism of control can be considered as a negative or inverse feedback mechanism which explains how the oversynthesis

of isoleucine in the wild-type organism is prevented and, in the presence of a mechanism for the entry of isoleucine into the cell, accounts for the sparing effect on the threonine requirement of threonine auxotrophs, for the failure of isoleucine auxotrophs to accumulate precursor until the growth factor is exhausted, and for the preferential utilization of exogenous isoleucine.

As is emphasized elsewhere in this volume, end-product inhibition of the initial enzyme in a biosynthetic sequence is not the only mechanism for controlling biosynthesis. Indeed, while studying the accumulation of isoleucine precursors, it became clear that isoleucine not only exerted an inhibitory effect on the action of threonine deaminase but also on the formation of the enzyme (Umbarger and Brown, 1958a). However, in the strains examined, the inhibitory effect on the formation of threonine deaminase (enzyme repression[3]) was not complete, i.e., a basal level of L-threonine deaminase was assured even in the presence of excess isoleucine. This incomplete repression is in marked contrast to the findings in the arginine synthesizing enzymes of certain strains of *E. coli* (Vogel, 1961).

Thus, in the isoleucine pathway two kinds of control operate. In order that the patterns of the two may be clearly differentiated, their essential features are shown diagrammatically in Fig. 3–3. In this scheme the end product of the biosynthetic sequence, *P*, whether formed endogenously or having entered from outside the cell, takes part in a feedback loop (*II*). When its concentration is sufficiently high, *P* blocks the action of the first enzyme (*a*) in the sequence which converts *A*, an intermediate supplied by glucose catabolism, to *P*. This is the pattern revealed so strikingly in the isoleucine

[3] The term "repression" was introduced as an operational term in the phrase "repression of enzyme formation" by an end product (Vogel, 1956). It has been quite convenient, yet sufficiently explicit, to use the single word "repression" in referring to this operational concept. Gradually, the term has also come to imply the concept of the physiological significance attributed to the repression of enzyme formation as a feedback mechanism. This has been a very natural evolution and has not required the introduction of new words or the redefinition of old ones. Unfortunately, it has not been possible to derive a similarly specific, operational word for the phrase "inhibition of the activity of the initial enzyme in a biosynthetic sequence," etc. The term "feedback inhibition" is not an operational term but, rather, one that implies a functional significance. It would be useful, therefore, to employ a term which is operationally explicit and yet implies that the interaction in question is potentially important as a feedback mechanism. While not entirely satisfactory, it is proposed here that the phrase "end-product inhibition" be employed to describe the inhibition of the activity of the initial enzyme in a biosynthetic sequence by the end product of that sequence. Use of that phrase does not require proof of the physiological importance of the interaction yet signals its potential importance.

pathway. The other kind of control (repression) is shown in the diagram as feedback Loop I in which *P* or a derivative of *P* interferes with the formation of enzyme *a*. Since the analogy between feedback in a biosynthetic sequence and that in a Williamson amplifier is not so immediately obvious to most biologists, it may be more profitable to compare the biological system to more mechanical examples of feedback control such as the governor of a steam engine [4] which decreases the steam supply at excessive speed and increases it at low speeds.

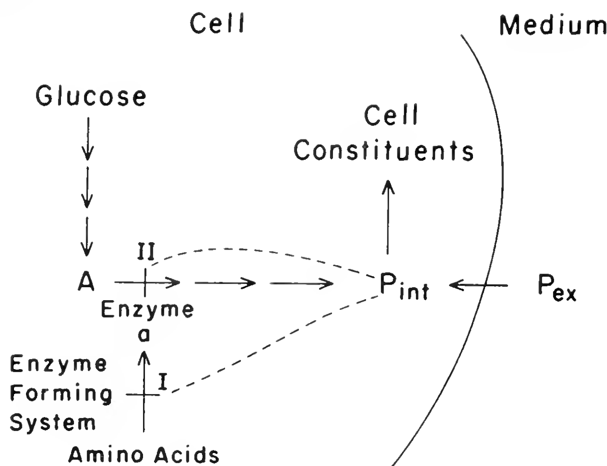


Fig. 3–3. Diagrammatic representation of feedback loops controlling (*I*) the synthesis of enzyme *a* and (*II*) the activity of enzyme *a* by the end product of the biosynthetic pathway leading from *A* to *P*. For further details, see text. (Reprinted from the *Journal of Biological Chemistry* [Umbarger and Brown, 1958a] by permission.)

The fact that the pattern of control which was observed in the isoleucine pathway was discovered at about the same time to be operating in the biosynthetic pathways leading to pyrimidines (Yates and Pardee, 1956) and to proline (Strecker, 1957) suggested that this pattern might be a general one. In each case the feedback loop involved the end product of the sequence and what presumably was the first enzyme leading specifically to that end product. Therefore,

[4] A more homely, and perhaps self-explanatory, model is seen in the float-controlled valve of a domestic tank-type toilet. The valve (enzyme) is closed (inhibited) completely when the level of water (intracellular pool of product) is sufficiently high. Carrying the analogy further, the factory or assembly line which constructs toilets is like the enzyme-forming system and will shut down or speed production depending on the need for additional units (enzymes).

it was anticipated that in other pathways in which an effective feedback control existed, it would involve the first enzyme leading irreversibly to the end product.

A subsequent study on the biosynthesis of valine offered an opportunity to test the corollary of this dogma: that an enzyme which is inhibited by the end product of the reaction sequence of which it is a part is the initial enzyme of that sequence (Umbarger and Brown, 1958b). The reaction studied was the formation of α-acetolactate (see Fig. 3–2).

Acetolactate had earlier been postulated as an intermediate in valine biosynthesis on the basis of isotopic experiments with yeast by Strassman *et al.* (1953). Proof of the obligatory nature of acetolactate formation as a step in valine biosynthesis would be rigorous only by demonstrating its presence in a wild-type organism and its absence in an appropriately blocked valine auxotroph. Unfortunately, no mutants blocked in this step were available. However, it was noted that the reaction fitted the dogma exactly as though it was the first enzyme in the pathway (Umbarger and Brown, 1958b).

Thus, as Fig. 3–4 shows, valine competitively inhibits the conversion of pyruvate to acetolactate. In addition, it was observed that the acetolactate-forming system was "derepressed" by growing valine auxotrophs under conditions of limiting valine. In other words, the formation of valine would occur maximally only when the valine supply was low. Although the evidence was not rigorous, it was concluded that the sole function of this reaction in *E. coli* was to provide acetolactate for valine biosynthesis.

Recently, Dr. Richard Leavitt (Leavitt and Umbarger, 1959), working with the corresponding reaction in the isoleucine pathway, α-aceto-α-hydroxybutyrate formation (see Fig. 3–2), had obtained evidence that this reaction is also catalyzed by the system forming acetolactate. Although fractionation studies on the acetolactate-acetohydroxybutyrate-forming system have not progressed very far, the availability of a method for studying acetohydroxbutyrate formation has made possible a further analysis of the inhibition of this system by valine.

In the competition between valine and pyruvate shown in Fig. 3–4, valine might have interfered with either the acetal donor function or the acetal acceptor function of pyruvate (Fig. 3–2, Reactions II and IIIa, respectively). In examining the other conversion (Reactions II and IIIb) catalyzed by this enzyme system, Leavitt

(Leavitt and Umbarger, 1960) has observed that valine competes with pyruvate but not with diphosphothiamine or α-ketobutyrate. He has therefore concluded that, in exerting control over valine biosynthesis, the end product competes with pyruvate in the generation of an active acetaldehyde-diphosphothiamine complex.

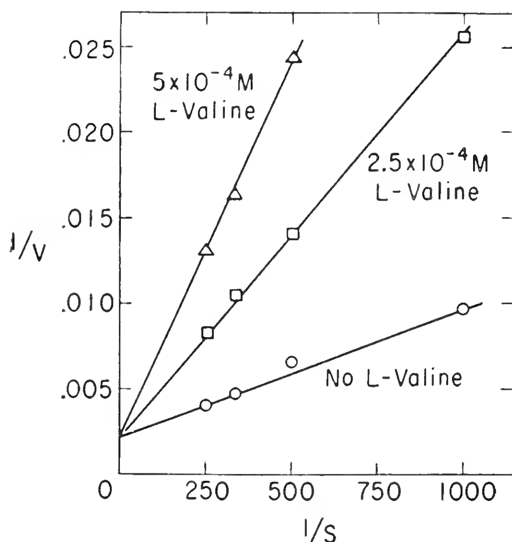


Fig. 3–4. Double reciprocal plot showing the competitive nature of the valine inhibition of acetolactate formation. 1/S = reciprocal of pyruvate concentration in moles per liter. 1/V = reciprocal of Klett reading. For other conditions, see Umbarger and Brown (1958b). (Reprinted from the *Journal of Biological Chemistry* [Umbarger and Brown, 1958b] by permission.)

By now there is an impressive list of reactions which appear to be involved in feedback control mechanisms by virtue of the fact that the enzymes catalyzing them are inhibited by the end products of the respective biosynthetic chains. There would be an even more impressive list if one were to catalog examples of indirect evidence for the operation of feedback controls. Those that have been studied *in vitro* and have come to the author's attention are listed in Table 3–2. One that is of interest is the inhibition by serine of phosphoserine phosphatase, the last enzyme in the sequence leading from glucose to serine in animals. However, the two preceding enzymes, 3-phosphoglycerate dehydrogenase and the glutamate-phosphoserine transaminase connect the serine pathway to glycolytic intermediates

TABLE 3–2

Examples of Inhibition of the Initial Enzyme in a Biosynthetic Sequence by the End Product

Reaction	Inhibitor	Organism	Reference
L-Threonine ⟶ α-ketobutyrate	Isoleucine	*E. coli*	Umbarger, 1956
Aspartate + carbamyl phosphate ⟶ ureidosuccinate	Cytidine-5′-phosphate	*E. coli*	Yates and Pardee, 1956
Glutamate ⟶ Δ^1 pyrolline-5-carboxylic acid	Proline	*E. coli*	Strecker, 1957
Pyruvate ⟶ α-acetolactate	Valine	*E. coli* *A. aerogenes*	Umbarger *et al.*, 1957
ATP + phosphoribosylpyrophosphate ⟶ 5-amino-1-ribosyl-4-imidazole carboxamide ribotide	Histidine	*E. coli*	Moyed, 1958
Inosine-5′-phosphate ⟶ xanthosine-5′-phosphate	Guanine-5′-phosphate	*A. aerogenes*	Magasanik, 1958
Guanine-5′-phosphate ⟶ inosine-5′-phosphate	ATP	*A. aerogenes*	Magasanik, 1958
Homoserine ⟶ O-phosphohomoserine	Threonine	*E. coli*	Wormser and Pardee, 1958
Phosphoserine ⟶ serine	Serine	Rat liver	Borkenhagen and Kennedy, 1958, Neuhaus and Byrne, 1959
N-Acetylglutamate ⟶ N-acetyl-γ-glutamyl phosphate	Arginine	*M. glutamicus*	Udaka and Kinoshita, 1958
Phosphoribosyl pyrophosphate + glutamine ⟶ phosphoribosylamine	Purine ribotides	Pigeon liver	Wyngaarden and Ashton, 1959
5-Phosphoshikemate ⟶ anthranilate	Tryptophan	*E. coli*	Moyed, 1960

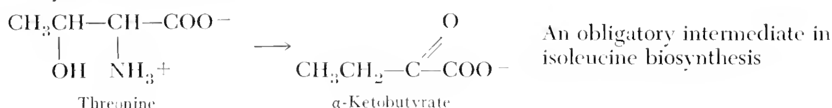
by catalyzing a pair of reversible reactions (Ichihara and Greenberg, 1957).

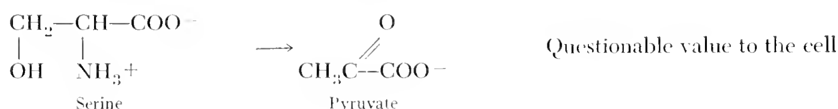
The immediate reaction of the physiologist in contemplating the delicacy of feedback control is to emphasize only its beneficial aspects. However, closer scrutiny reveals a somewhat complicating consequence owing to the fact that the formation and action of a given enzyme are so rigidly geared to a specific function. This consequence is that such an enzyme lacks the versatility expected of the reaction it catalyzes.

TABLE 3–3

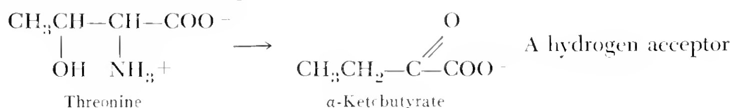
The Functions of Threonine and Serine Deamination in *E. coli*

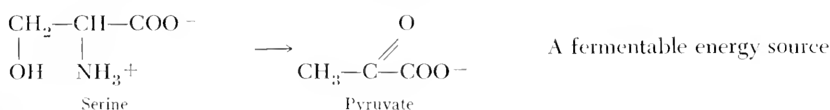
1. Biosynthesis of isoleucine





2. Anaerobic amino acid degradation





For example, in *E. coli* the L-threonine deaminase is also an L-serine deaminase. As shown in Table 3–3, these reactions can have two distinct functions. The one is in isoleucine biosynthesis. The enzyme catalyzing this reaction also deaminates L-serine *in vitro,* but it seems unlikely that it does so in the cell to endogenously formed serine. A second, distinct function is in furnishing a fermentable energy source (pyruvate) and a hydrogen acceptor (α-ketobutyrate) during anaerobic growth on a mixture of amino acids (e.g., nutrient broth) in the absence of any fermentable sugar. Clearly, the enzyme which is inhibited by isoleucine could not fulfill such a

function in the usual peptone medium which would be rich in isoleucine.

However, *E. coli* meets this lack of versatility by being able to form two distinct L-threonine–L-serine deaminases (Umbarger and Brown, 1957). As shown in Table 3–4, the different properties and the different conditions affecting their formation make the two enzymes clearly distinguishable. The enzyme having primarily a catabolic function is an adaptive enzyme studied in *E. coli* most intensively by Wood and Gunsalus (1949). It is formed only anaerobically in the absence of a fermentable energy source. For it to have a biosynthetic role would require the very special circumstances of anaerobic cultivation in an isoleucine-free mixture of amino acids. Therefore, loss of the biosynthetic enzyme results in isoleucine α-ketobutyrate auxotrophy even though the genetic apparatus for forming the second enzyme is present.

TABLE 3–4

The Threonine-Serine Deaminases of *E. coli*

Catabolic Enzyme	Biosynthetic Enzyme
Cofactors demonstrated	Cofactor demonstrated
Pyridoxal phosphate Glutathione Adenosine-5-phosphate	Pyridoxal phosphate
First order kinetics	Second order kinetics
No effect of isoleucine	Isoleucine represses formation and inhibits action
Formation prevented by aeration and fermentable sugar	Formed in minimal media
	No effect of aeration

An exactly analogous situation has been described for the first step in the pathway leading to valine, acetolactate formation (Halpern and Umbarger, 1959). It is well known to diagnostic bacteriologists that one of the properties that differentiates *E. coli* from *A. aerogenes* is the appearance of acetylmethylcarbinol in the culture fields of the latter. As the studies of Juni (1952) have shown, the formation of acetylmethylcarbinol proceeds by the way of acetolactate. It is interesting that the system forming acetolactate and the decarboxylase that accompanies it are formed and function at *p*H values of 6 or less. The fact that acetoin, a neutral product, would

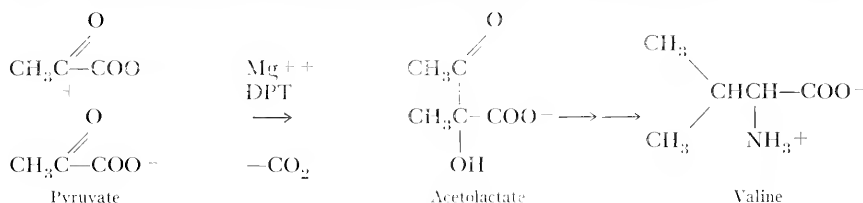
thus be formed instead of acidic products during the fermentation of glucose has been interpreted as a selective advantage in this organism.

Juni (1952) demonstrated quite clearly that this acetolactate-forming system and the decarboxylase were absent from *E. coli*—an observation that was to cast doubt on the possibility that acetolactate could be an intermediate in valine biosynthesis in *E. coli*. However, this discrepancy was clarified when it was observed that in *E. coli* the acetolactate-forming system had a *p*H optimum near 8

TABLE 3–5

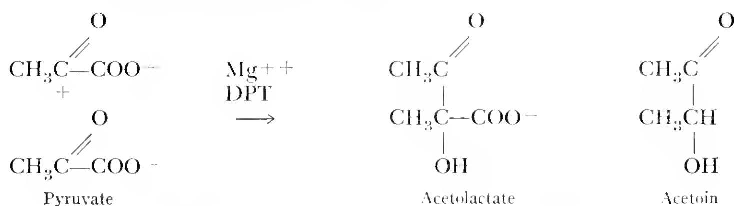
The Biological Functions of Acetolactate Formation in *A. aerogenes*

1. As an intermediate in valine biosynthesis



Pyruvate Acetolactate Valine

2. As an intermediate in acetoin formation



Pyruvate Acetolactate Acetoin

(Umbarger and Brown, 1958b). There was no decarboxylase. In addition, there were several other features of the *E. coli* system which differentiated it from the *A. aerogenes* system of Juni. The most striking of these was the sensitivity of the *E. coli* system to valine, the end product of the biosynthetic sequence.

Thus, two distinct physiological functions are known for the conversion of pyruvate to acetolactate. These are illustrated in Table 3–5. One is distinctly concerned with glucose catabolism; the other is a biosynthetic function. Assuming that *A. aerogenes* formed its valine via acetolactate, it would exhibit both of these functions. In view of the fact that *E. coli* had two enzymes for threonine deamination, it was of interest to determine whether *A. aerogenes* performed

the two functions with one or a pair of enzymes. Experiments to answer this question were performed by Dr. Y. S. Halpern, visiting this country from the Hadassah Medical School in Israel.

A summary of Dr. Halpern's results is given in Table 3–6. The strain of *A. aerogenes* (1033) examined not only had the classic acetolactate-forming system discovered by Juni but also had a system with a higher *p*H optimum that was almost identical in its properties to that found in *E. coli*.[5] The one functioning at a higher *p*H was concerned with supplying acetolactate for valine biosynthesis since it was under feedback control by valine. It might therefore be called a biosynthetic enzyme. The second enzyme was not under feedback control by valine—an observation that accounts for the formation of

TABLE 3–6

The Acetolactate Forming Systems of *A. aerogenes*

Catabolic Enzyme	Biosynthetic Enzyme
Low optimal *p*H	High optimal *p*H
High affinity for Mg^{++} and diphosphothiamine	Low affinity for Mg^{++} and diphosphothiamine
No effect of valine	Valine represses formation and inhibits action
Formed at *p*H 6 or less	Formed above *p*H 6
Absent in *E. coli*	Present in *E. coli*

acetylmethylcarbinol in media containing valine supplied in the bacteriological peptone of nutrient broth. Its most striking role is played during the fermentation of glucose. It therefore has a catabolic function. Since growth occurred at *p*H values at which the biosynthetic enzyme is absent, Dr. Halpern concluded that the usually catabolic enzyme then secondarily assumes the biosynthetic function of supplying acetolactate required for valine biosynthesis. This could be fulfilled in spite of the presence of acetolactate decarboxylase in the cell.

[5] Radhakrishnan and Snell (1960) recently reported that *E. coli* also exhibited an acetolactate-forming system which functioned at *p*H 6 in addition to the system functioning at *p*H 8. This apparent discrepancy can perhaps be resolved by recalling the observation made by Juni and Heym (1956) that most pyruvic oxidase systems (including that from *E. coli*) will form acetolactate in the presence of high concentrations of pyruvate. This acetolactate, however, is a racemic mixture and, therefore, unlike the one formed at *p*H 6 by *A. aerogenes* and at *p*H 8 by both *A. aerogenes* and *E. coli*, only half of it could be converted to valine or to acetoin by bacterial enzymes. Thus, while there is a physiological role for the function of acetolactate at either *p*H by *A. aerogenes*, it seems likely that *E. coli* exhibits a functionally significant acetolactate-forming activity only at the higher *p*H.

There are now two examples of the phenomenon where the same chemical reaction has two distinctly different physiologicalal roles, and in which the cell has a separate enzyme to perform each function. In each case the enzyme is controlled by factors that make it uniquely suited to its particular function. The experience with these systems leads to the proposal of a second principle to come from these studies. This is that whenever a chemical reaction in a cell is catalyzed by an enzyme whose formation and activity are under the rigid control of repression and end-product inhibition, a second enzyme will be needed by the cell if there is some other essential role for that reaction to play in the cell.

There are additional ways in which an effective feedback control may complicate the metabolism of a cell. One of these is illustrated again in valine and isoleucine biosynthesis. It was noted earlier that the formation of acetolactate and probably more specifically the generation of the acetal group required for acetolactate formation is under feedback control by valine. It was also noted that the same enzyme system appeared to catalyze the formation of the corresponding isoleucine precursor, acetohydroxybutyrate. The question then arises: How can isoleucine biosynthesis occur when exogenous valine is present? The answer is that, in a cell in which the feedback control by valine is quite effective, it cannot. An example of such an organism is the K-12 strain of *E. coli,* observed long ago to be inhibited by exogenous valine unless isoleucine was present (Bonner, 1946). It was later shown that any precursor of isoleucine from acetohydroxybutyrate on was able to reverse valine inhibition non-competitively (Umbarger, 1958). Recent experiments would bear out the inference that in the valine-sensitive K-12 strain, there is an extremely effective feedback control so that exogenous valine, in quenching biosynthesis along its own pathway, also prevents isoleucine formation. In contrast, *E. coli* strain W, which is not inhibited by exogenous valine, had an acetolactate-acetohydroxybutyrate-forming system that was less sensitive to inhibition by valine. Likewise, a mutant of the K-12 strain, K-12/V, selected for growth in the presence of valine, exhibited an enzyme less sensitive to inhibition by valine. These differences in the sensitivity to feedback control were reflected in the level of the free, endogenously formed valine as shown in Table 3–7. In this experiment the cells were harvested at intervals, and the extracellular valine and that liberated from the cells by boiling water were determined. It can

be seen that the K-12 strain with its very sensitive acetolactate-forming system, had a very small internal pool of valine. On the other hand, in those strains (W and K-12 V) in which acetolactate formation was more resistant to inhibition by valine, the intracellular pool of free valine was high. Toward the end of growth, when aeration became the limiting factor, valine as well as other amino acids were excreted. Until this excretion occurred, the external valine was almost undetectable. Thus, because of the unique feature in isoleucine and valine biosynthesis of a series of common enzymes for the two pathways, the cell can have either good feedback control over valine biosynthesis or an isoleucine pathway resistant to valine, but not both.

TABLE 3–7

The Regulation of Free Intracellular Valine by Its Feedback Control

Strain	Inhibition of Acetolactate Formation by Valine	Free Intracellular Pool µg Valine/mg Cell Protein	
		O.D. = 200	O.D. = 400
K-12	Strong	0.4	0.2
K-12/V	Weak	3.8	2.0
W	Weak	2.7	1.4

A closely related complication arising in a feedback mechanism has been described by Moyed and Friedman (1959b). In this example, an analog of the end product may so resemble the natural product that, although it cannot be incorporated into a macromolecule, it may prevent growth by inhibiting the enzyme that is normally subject to end-product inhibition, i.e., by exerting "false feedback." The antagonism by the end product of the action of such an inhibitor would be expected to be non-competitive. However, Moyed and Friedman (1959a) described an example in which this did not occur. 5-Methyltryptophan, a weak inhibitor of an early step in tryptophan biosynthesis, also hinders the entry of tryptophan so that a precursor of tryptophan, indole, is more effective in reversing the action of the analog than is tryptophan itself.

The experiments of Moyed have, in addition, demonstrated that one way for a cell to become resistant to an analog which exerted "false feedback" was for the sensitive enzyme to become resistant to the action of the analog. Examination showed that one such

altered enzyme was also resistant to feedback control by the end product so that in a glucose minimal medium, the end product was excreted.

While such organisms would not be expected to fare as well in non-selective environments as organisms having effective control mechanisms, they would be of value as the basis of microbial processes for the production of the excreted compound. Nevertheless, industrial screening programs have resulted in the isolation from soil of organisms which convert a substantial proportion of the carbon source to an amino acid (Udaka, 1960). Another, more rational, systematic procedure was employed by Adelberg (1958) for obtaining amino acid excretors. His method was to select organisms resistant to amino acid analogs. Undoubtedly each procedure was selecting organisms in which the initial enzyme in a biosynthetic sequence was resistant to the end product of the sequence.

Finally, a comment should be made on the relative physiological role of the kind of control mechanism emphasized here. Certainly, both repression and end-product inhibition are important regulatory mechanisms. It would be assumed a priori that in terms of the synthesis of small molecules, repression at best is a sluggish control mechanism. It would function to provide for preferential utilization of an exogenous supply of a metabolite only following a dilution of the pre-existing enzymes by growth. In contrast, one would expect end-product inhibition of the action of an enzyme to occur immediately and provide for complete preferential utilization. For the arginine pathway, this distinction has been shown by Enis and Gorini (1959). They found, superimposed upon an extremely effective control by repression (feedback Loop I in Fig. 3–3), an even more delicate feedback control that could only be explained by assuming a mechanism like that represented by feedback Loop II in Fig. 3–3. With this elegant demonstration, there would appear to be little doubt about the relative roles of the two control mechanisms.

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4

Ribonucleic Acid and the Control of Cellular Processes

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Introduction

Several recent reviews deal with the production of RNA [2] and its function in protein synthesis (Brachet, 1957; Chantrenne, 1958; Roberts *et al.*, 1959; Simkin, 1959; Woods, 1959). They demonstrate the important role that RNA plays in cell metabolism. A direct experimental approach to the problem of RNA control of cellular processes has seldom been made and any discussion of it will have to be based on few facts and many assumptions.

Our knowledge of cell physiology has advanced enormously in recent years and most important has been the recognition of the role of nucleic acids in heredity. The concept of DNA as the chemical basis of genes emerged from many experimental facts. The concept found its main support in the demonstration that DNA is the active principle in bacterial transformations (Avery *et al.*, 1944; Hotchkiss, 1951; Ephrussi-Taylor, 1951), that in bacteriophage, DNA serves in the production of new phage particles in the bacterial host (Hershey and Chase, 1952; Hershey, 1953), and that in higher organisms the DNA content of cell nuclei is constant and proportional to ploidy (Boivin and the Venderlys, 1948; Mirsky and Ris, 1949). A model

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[2] The following abbreviations are used: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

for the structure of DNA proposed by Watson and Crick (1953) helped to explain self-replication of DNA and a coding system (Gamow *et al.*, 1956) suggested a means by which DNA could carry the necessary genetic information.

It is believed today that the basic function of genes is to store and transmit information concerning the sequence of amino acids in proteins. When the amino acid sequence was determined in insulin (Sanger, 1956), this hypothesis became open to experimental verification. Indeed it has been found that the insulin molecules of different animal species differ by slight modifications of their amino acid sequence (Sanger and Smith, 1957) and that hemoglobins of genetically different strains have one amino acid substituted for another in an otherwise unchanged molecule (Ingram, 1957). Many laboratories are now engaged in the arduous task of finding direct proof that gene mutation expresses itself in a change of amino acid sequence of enzyme whose formation is controlled by the gene.

In the meantime, cytochemical observations led Caspersson (1941) and Brachet (1942) to suggest that RNA functions in protein synthesis. The idea found experimental support when protein synthesis was obtained *in vitro* with preparations containing microsomes, cell particles rich in RNA (Siekevitz, 1952; Zamecnik and Keller, 1954; Borsook, 1956). Evidence is continuously accumulating that RNA directs protein synthesis. This has become the basis for the hypothesis, gradually accepted, that RNA is the primary product of gene action and serves to transfer genic information to the cytoplasm. Tracer experiments have shown that nuclei are particularly active in RNA synthesis (Smellie, 1955), and modern autoradiography, as we shall see later, has given strong support to these observations by clearly demonstrating RNA synthesis in the nucleus.

Besides being active in protein synthesis, RNA is capable of carrying the necessary genetic information. Many plant and animal viruses are ribonucleoproteins, not containing any DNA. It has been possible to isolate RNA from some of them (Fraenkel-Conrat, 1956; Gierer and Schramm, 1956) and to infect the hosts with pure RNA. The infected cells produced new complete viruses, proving that RNA and not the protein moiety was necessary for virus reproduction. In tobacco mosaic virus, Fraenkel-Conrat (1956) went a step further and reconstituted a virus from its RNA and protein component parts. RNA could be thus combined with a protein of another strain, differing in several properties. Such a "hybrid" virus was perfectly

infective and produced progeny with the protein characteristics of the RNA donor, not of the protein donor.

All these facts together, some of which will be discussed more extensively, contributed to the growing acceptance of the hypothesis DNA → RNA → protein. I want to present in this paper some facts in favor of this hypothesis and speculations about how this hypothesis could explain the processes controlling cell function.

RNA as Gene Product

A Nuclear Origin of RNA. Modern research in biochemical genetics showed first that specific biochemical reactions are under genetic control, and later, that genes must control the production of enzymes responsible for these reactions. Since the genes are located in chromosomes, inside the nucleus, they cannot directly form proteins, which are made in the cytoplasm. The information contained in the genes must be carried to the cytoplasm by a "messenger." The substance which serves as a "messenger" must conform to certain a priori conditions. It has to be synthesized in the nucleus, where the genes are located, and it has to be transferred into the cytoplasm. There should be no other place in the cell where its synthesis is possible. In the cytoplasm the substance should direct protein synthesis. It appears now that RNA is most likely to be the substance.

Recently, evidence has accumulated for the nuclear origin of RNA. It has been possible, by the use of radioactive tracers, to detect the site of formation of new RNA and to follow the fate of RNA in the cell. Studies of incorporation of P^{32} into different cell fractions showed that the nucleus is particularly active in the incorporation of phosphorus into RNA (Marshak and Calvet, 1949; Jeener and Szarfarz, 1950; Smellie, 1955). Many such experiments have indicated that the nuclei may be the main sites of RNA synthesis, although opposite opinions have also been expressed (Barnum *et al.*, 1953). Clearer proof of nuclear origin of RNA has been obtained since by the use of autoradiography.

Goldstein and Plaut (1955) fed amoebas P^{32} which was incorporated into the nucleic acids. Extraction of the fixed material with the appropriate solvents and treatment with crystalline ribonuclease allowed the study of the incorporation into RNA, separately from the incorporation into DNA and other phosphorylated compounds.

Autoradiography showed that this incorporation occurred mainly into the nuclear RNA. Amoebae could be cut into nucleate and anucleate halves and only the former incorporated P^{32} into RNA. When a nucleus containing radioactive RNA was transplanted into a non-radioactive nucleated or enucleated amoeba, the radioactivity was transferred into the cytoplasm, where it could still be recognized as RNA. The conclusion was clear: RNA is made in the nucleus and transferred to the cytoplasm. In later work, however, Plaut and Rustad (1957, 1959) observed RNA formation in enucleated halves also, concluding that the nucleus is not the only site of RNA formation. Prescott (1959) tried to explain this later finding by a persistence of some live bacteria in the food vacuoles of experimental amoebae. By using an axenic culture of *Acanthamoeba,* Prescott could (1960a) demonstrate that there is no uptake of C^{14} cytidine by the enucleated halves, even over a 24-hour period.

Plaut's contention of RNA formation in the absence of nuclei seemed to be supported by experiments in *Acetabularia.* Brachet *et al.* (1955) reported an increase in RNA content in anucleate fragments of this alga, as measured by the tracer dilution method. Richter (1957), measuring RNA production with the UV light-absorption method, could find no such increase. Finally, Naora *et al.* (1959), in Brachet's laboratory, by using both methods, could show that there was no increase in the cytoplasmic RNA after enucleation, but rather a small decrease. However, anucleate plants were still able to incorporate C^{14} precursors into RNA. The meaning of this incorporation is not clear; it could be due to a real turnover of cytoplasmic RNA or only to exchange reactions. This points to the pitfalls of tracer work. It is not possible to distinguish between net synthesis and exchange reactions in the incorporation of radioactive precursors. Furthermore, in autoradiography, it is not easy to follow the incorporation into a specific substance, since other incorporating substances may not be readily eliminated.

In *Neurospora,* it has been possible to demonstrate that, in the actively growing mycelium, the major part, if not all, of the RNA is synthesized in the nuclear fraction (Zalokar, 1959a, 1960b). Hyphae were fed H^3 uridine for different intervals of time and then centrifuged to separate cell constituents inside still living hyphae. Centrifuged hyphae were fixed, extracted with cold trichloracetic acid and autoradiographs made. Centrifugation sedimented all ribosomes (Zalokar, 1960c, 1961) into a layer, separated from a layer

of nuclei by a mitochondrial layer. Although the ribosome layer contained most of the cellular RNA, all radioactivity newly incorporated into RNA was found in the nuclear layer for the first few minutes. After four minutes, more and more radioactivity appeared in the ribosome layer until, after one hour, the ribosome fraction was labeled much more than the nuclear fraction. Later, similar experiments were made with H^3 cytidine and C^{14} adenine, with essentially the same results. Kinetic studies of uridine and adenine uptake into the cell excluded another possible explanation, that of a delayed RNA synthesis in ribosomes. Administration of radioactive precursor for a brief time, followed by an excess of non-radioactive precursor, proved that it must have been actually RNA which migrated from the nuclear into the ribosome fraction. The amount of precursor incorporated in these experiments corresponded roughly to the amount of RNA formed in the same period, thus excluding the possibility of unspecific exchange reactions.

The nuclear origin of RNA was confirmed also in autoradiographic experiments with the salivary glands of *Drosophila* (Taylor *et al.*, 1955; Zalokar, 1960a), with mammalian tissue cultures (Goldstein and Micou, 1959a; Feinendegen *et al.*, 1960) and with plant cells (Woods, 1959; Woods and Taylor, 1959). Then came the disturbing report of Harris (1959) who obtained contrary results in tissue cultures. He fed H^3 cytidine or H^3 adenosine to rabbit macrophages and rat heart fibroblasts, freshly subcultured from an animal. The first survived in culture as undividing cells. They incorporated large quantities of adenosine in the nuclei and nucleoli, less in the cytoplasm. After transfer to a non-radioactive medium, the labeling of nuclei and nucleoli disappeared rapidly, and there was no indication that this RNA passed into the cytoplasm. Connective tissue cells, multiplying *in vitro,* incorporated adenosine rapidly also, but after the transfer to the non-radioactive medium, nuclear labeling remained unchanged for about two hours and cytoplasmic labeling continued to increase. After that time, the nuclear labeling decreased rapidly, while the cytoplasmic labeling remained at a constant level. Again, these results did not indicate that appreciable amounts of the nuclear RNA could pass intact into the cytoplasm. The author concluded that there must be a rapid turnover of RNA in the nuclei and nucleoli, but that this RNA does not enter the cytoplasm intact. Only a small proportion of the nuclear RNA could have passed into the cytoplasm without being detected.

It is difficult to reconcile these results with the hypothesis of the nuclear origin of cytoplasmic RNA. For the time being, it must be conceded that in cells as used by Harris, nuclear RNA is continuously formed and degraded, and that some of the cytoplasmic RNA is formed inside the cytoplasm, but there is still a margin allowing for some nuclear RNA to pass into the cytoplasm. The quantitative autoradiography with tritium may be less precise than the author claims. It is extremely difficult to avoid personal bias in the choice of material to be counted, since the labeling of the cells is usually highly irregular and cell sizes vary appreciably. In the case of tritium, the β-rays are absorbed in very short distances, about 90 per cent in 1.2 μ of water (Fitzgerald *et al.*, 1951), so that self absorption of preparations of uneven thickness can give erroneous quantitative estimates. The results with fibroblasts could indicate a much larger flow of RNA from nuclei into the cytoplasm, if the error was substantially higher than assumed.

Intranuclear Site of RNA Production. There may remain doubt about the exclusive synthesis of RNA in the nucleus in all cells, but it can be considered as proved that at least part of the cytoplasmic RNA does originate in the nucleus. This does not necessarily mean that RNA is produced by genes. For a long time, the nucleolus was considered by many as the RNA synthetic site since it was the part of the nucleus richest in RNA. If that had been the case, the theory of genic origin of RNA could not be maintained. Cytochemical observations showed an accumulation of RNA inside the nucleolus in cells with a high rate of protein synthesis (Caspersson, 1941). The available methods could not discern if the nucleolus were the real site of RNA formation; only the use of autoradiography with radioactive precursors could provide the refinement necessary.

Thus, Taylor *et al.* (1955) found an accumulation of P^{32}, incorporated into RNA, in the nucleoli of *Drosophila* salivary gland cells. Later, those experiments were repeated by using C^{14} adenine and H^{3} cytidine as a precursor (McMaster-Kaye and Taylor, 1959). RNA appeared in the nucleoli before it was noticeable in the chromosomes. It was found in the chromosomes at about the same time as it appeared also in the cytoplasm. New RNA was also observed to accumulate in puffs of salivary gland chromosomes of *Rhynchosciara* (Ficq *et al.*, 1958) and *Drosophila* (Rudkin and Woods, 1959). These and other similar experiments (Woods and Taylor, 1959)

using long time intervals could prove only that the nucleolus and puffs accumulated RNA. The real synthesizing site could still be elsewhere in the nucleus since the newly formed RNA could have been transposed into these structures immediately after formation.

It was shown in *Neurospora* (Zalokar, 1960b) that newly formed proteins leave the site of formation within 15 seconds after synthesis, indicating a possibility of fast movement of macromolecules inside the cell. Only experiments using time intervals of 15 seconds or less could clearly indicate the prevalence of newly synthesized proteins in the ribosome fraction. Similarly, RNA may move shortly after its

TABLE 4–1

Incorporation of H^3 Uridine into RNA of Salivary Gland Cell Nuclei of *Drosophila*

Time After Injection	Nucleus	Nucleolus	Nu/N
15 sec.	1.24	2.86	2.3
2 min.	14.0	25.6	1.8
4 min.	54.2	117.0	2.1
Area μ^2	168	72	1/2.3
Volume μ^3	2,090	405	1/5.1

Silver grains were counted over total area of nucleus (excluding nucleolus) and nucleolus and were calculated for one-day exposure of the autoradiograph. Averages of ten counts are given.

formation from the chromosomes into the nucleolus. I tried, therefore, to use extremely short time intervals in following the uptake of H^3 uridine into RNA of *Drosophila* salivary glands. With H^3 uridine of high specific activity (640 mc/mM), the highly sensitive Ilford G-5 emulsion and 4 months of exposure time, it was possible to detect the incorporation into RNA of salivary glands at 5 to 15 second intervals after injection of the precursor into the larva. Both the chromosome and the nucleolus parts of the nucleus became labeled. Silver grain counts gave an approximation of the uptake rate in both fractions. The values for nucleoli and chromosomes increased at a proportional rate for the first 4 minutes (Table 4–1). There was no indication of prior accumulation of RNA in either chromosomes or nucleolus, which would be the case if one of them supplied newly formed RNA, or a high molecular precursor for RNA, to the other. It could be concluded that RNA was formed independently in the chromosomes and in the nucleolus, and rough estimates, taking into

the account respective volumes, show that about twice as much is formed in the chromosomes as in the nucleolus. Woods (1959) came to a similar conclusion in his experiments with roots of *Vicia faba.*

The nucleolus in the salivary gland preparations used for autoradiography is not easily distinguished from the chromocenter and it may be that the high synthetic activity of the nucleolus is actually due to the heterochromatin of the chromocenter. Pelling (1959) and later Sirlin (1960) showed in different Chironomidae that the nucleolar RNA appears first in one side of the nucleolus only, the part which is closest to the supporting chromosome. No such regional RNA formation could be observed in our preparations, since in one minute the nucleolus was radioactive throughout.

A few other cells synthesize RNA fast enough to allow detection of newly formed RNA after very short time intervals. Active RNA synthesis can usually be found in ovocytes. In *Drosophila,* nurse cells take over the task of RNA synthesis and it can be shown that newly synthesized RNA accumulates quickly inside the nuclei (Zalokar, 1960a). Unfortunately, the nuclear structure of *Drosophila* nurse cells does not allow an easy distinction between the nucleolus and the chromatic material in autoradiographs.

More favorable are the panoistic ovaries of more primitive insects, as found in the order of Orthoptera. Cockroach (*Blatella germanica*) has large ovocyte nuclei with beautiful nucleoli. Egg production is, however, slower than in *Drosophila*, so that longer times are needed to detect incorporation of precursors into RNA. I wish to report here a few unpublished experiments with *Blatella.* The ovaries of cockroaches which were still carrying the egg case with the previous batch of eggs, were incubated *in vitro* in H^3 uridine-containing physiological solution. They were fixed 4 minutes, 16 minutes, 1 hour and 4 hours later (Fig. 4–1). In 4 minutes, no incorporation could be detected. In 16 minutes, the nucleoplasm, containing "lampbrush" chromosomes was labeled, the label was especially evident over the chromosomes. The nucleolus was only slightly labeled. After one hour, the nucleolus became labeled, but only on several spots of the nucleolar periphery. After four hours, total labeling of the nucleus was much higher, and the nucleolus became labeled throughout, indeed more than the surrounding nucleoplasm. These preliminary results appear to show a chromosomic synthesis of RNA and a migration of RNA from the chromosomes to the nucleoli.

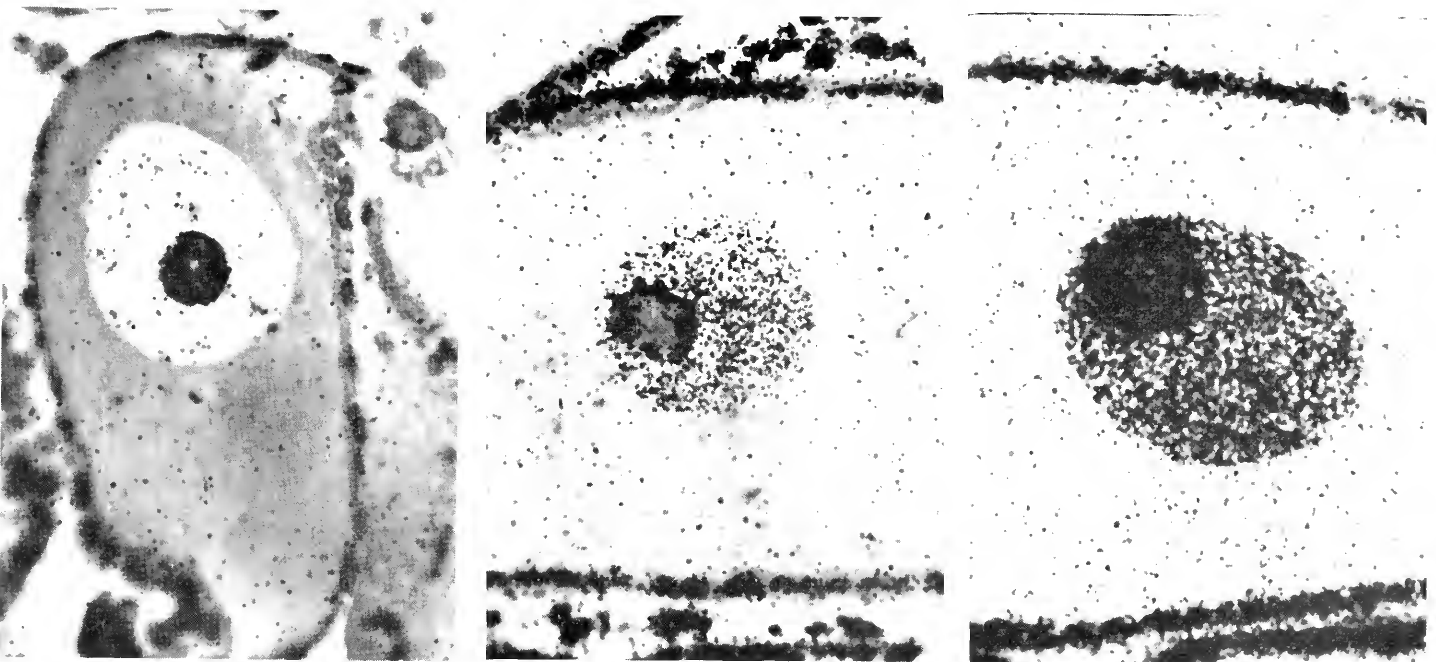

Fig. 4–1. Incorporation of H^3 uridine into RNA of ovocyte nuclei of *Blatella germanica*. Ovaries were incubated in physiological solution containing H^3 uridine (640 mc mmole), fixed after appropriate times by freeze-substitution, sectioned to 4 μ thickness and autoradiographs made. Stained with Delafield's hematoxylin. All autoradiographs were exposed for 12 days. A. Fixed after 16 min. Intense staining of nucleolus indicates high RNA content; lack of staining of nucleoplasm indicates low RNA content. B. Fixed after 1 hr. Radioactive RNA agglomerates at the periphery of nucleolus. Nuclei of follicle cells surrounding ovocytes are labeled. C. Fixed after 4 hrs. Nucleolus labeled more than nucleoplasm.

Similarly, the lampbrush chromosomes of *Triturus* ovocytes were found to incorporate C^{14} adenine into RNA (Brachet and Ficq, 1956; Gall, 1958) sooner than did nucleoli. In electron microscope pictures and in cytochemical preparations, it has even been possible to observe the organization of RNA rich nucleolar material on the chromosomes and their final movement into the nucleoli (Gall, 1958). Guyénot and Danon (1952) have indicated previously that lampbrush chromosomes express a highly active state of genes.

In tissue cultures, Goldstein and Micou (1959b) could also follow a transfer of RNA from chromatin to nucleoli. Similarly, Feinendegen *et al.* (1960) found in HeLa cells that the nucleoplasm becomes labeled earlier than the nucleoli. On the other hand, Harris (1959) maintains that the RNA turnover is equal in the nucleolus and the rest of the nucleus. Further experiments with reliable quantitative methods will be needed to establish beyond criticism the facts indicated by autoradiographic methods.

It may be premature, then, to formulate a hypothesis of RNA production, but it is difficult to resist the temptation. An inviting hypothesis was formulated by Bonner (1959): RNA is formed in the chromosomes, transferred to the nucleolus where the protein part of the nucleoprotein is made. The finished nucleoprotein particle, a ribosome, is transferred to the cytoplasm from the nucleolus. The known facts do not contradict this hypothesis; nevertheless, it cannot be fully accepted until more is known about the movements of RNA in the cell.

Chemistry of RNA. In the preceding discussion, RNA was considered as a class of substances, all with similar function and similar origin. This was an oversimplification, since various types of RNA could be distinguished by chemical analysis or by differing rates of incorporation of radioactive precursors in the cell (Siekevitz and Palade, 1959). Some of these types may correspond to differences which must exist among gene-produced RNA molecules, each built to carry the information of the corresponding gene.

Nuclear and cytoplasmic RNA differ in their base composition (Vincent, 1955; Elson *et al.*, 1955). This has been used by many as an argument against the nuclear origin of RNA. The argument is not valid, since it could be quite possible that there is one RNA, which is a constituent of the nucleus, and that the cytoplasmic RNA, also synthesized in the nucleus, is continuously removed to the cyto-

plasm so that it never reaches appreciable concentrations in the nucleus.

Experiments with *Neurospora* (Zalokar, 1960b) showed that all the RNA synthesized in the nucleus is not emptied into the cytoplasm. When H^3 uridine was added for one minute and then replaced with non-radioactive uridine, radioactivity due to RNA remained in the nuclear fraction as much as one hour later. In *Drosophila*, fed P^{32} for one hour and non-radioactive phosphorus subsequently, radioactivity did not disappear completely from the nucleus (Taylor *et al.*, 1955). On the other hand, Goldstein and Micou (1959b) could demonstrate in tissue culture cells the essentially complete disappearance of radioactivity from the nuclear fraction when a non-radioactive precursor followed a short treatment with the tracer. These differences can be explained by the fact that the nuclei in *Neurospora* are multiplying, and in *Drosophila* increasing in size, therefore continuously adding to their own substance, part of which is nuclear RNA. In tissue culture, the nuclei may have been active mainly in the production of cytoplasmic RNA and therefore would not have increased their own substance.

Although most of the nuclear RNA seems to be inside the nucleolus, careful consideration of different measurements shows that the amount of RNA in the rest of the nucleus is as large or even larger (Vincent, 1955). The nucleolar RNA obtained from isolated nucleoli was shown to be different from the cytoplasmic RNA (Vincent, 1952), and this may account for the main part of the nucleus-specific RNA. About one third of the total synthesis of RNA in the nucleus of the salivary glands of *Drosophila* appears to occur in the nucleoli, and it may well be that it is mainly the specific nucleolar RNA which is made in the nucleolus. This finding does not contradict the hypothesis of the genic origin of RNA: the nucleoli are themselves of chromosomal origin, as was clearly demonstrated by McClintock (1934) in her work on the nucleolar organizer. The nucleolar RNA must have some special function in the nucleus and is, therefore, produced in relatively large quantities, the production still depending on the continuous activity of one or a few gene loci.

Another chemically well-defined RNA is the so-called soluble RNA, of importance in the transfer of activated amino acids for protein synthesis (Hoagland *et al.*, 1957). While gene-produced RNA is expected to carry information on the sequence of many amino acids in the protein molecule, the soluble RNA molecule is specific only

for one particular amino acid, so that the cell must contain 20 different such molecules corresponding to the different amino acids (Schweet *et al.*, 1958a; Berg and Ofengand, 1958). According to present theories, the soluble RNA carries activated amino acids onto the ribosomal RNA template and functions as an "adapter" (Hoagland, 1959) for the amino acid on an appropriate site of the template. Nothing is known about the origin of this RNA and it is possible that it has been overlooked in experiments with autoradiography. In *Neurospora*, there was no indication of a soluble RNA being present free in centrifuged cells (Zalokar, 1960b), where it should not be sedimented, but it is possible that in a living cell it is loosely bound to the ribosome fraction. Recent reports (Venkataranan and Lowe, 1959) indicate, however, that an appreciable fraction of ribonucleoprotein becomes soluble in alcohol after treatment with cold trichloracetic acid. Since the preparations used in autoradiography were subjected to these chemicals, it cannot be excluded that soluble RNA was removed.

Chemistry of RNA Synthesis. All the evidence presented for the synthesis of RNA from DNA has been rather indirect, and a direct proof of such a synthesis can be obtained only by discovering the chemical mechanism of RNA synthesis. The immediate precursor for RNA is believed to be a nucleotide. The appropriate nucleotides are presumed to be assembled on a DNA template and bound into an RNA molecule. Ochoa and his collaborators (Grunberg-Manago *et al.*, 1955) discovered an enzyme which is able to build polynucleotides from nucleoside diphosphates. A primer was probably needed (Mii and Ochoa, 1957), but the polynucleotides built reflected the original proportion of nucleotides in the reaction mixture (Ochoa, 1957). It is not probable that the action of such an enzyme by itself would be sufficient to produce information-carrying molecules. But in conjunction with a DNA template, the enzyme may perform the function of linking the nucleotides into a polynucleotide chain.

It was believed for a while that RNA synthesis requires concomitant protein synthesis (Spiegelman *et al.*, 1955). Later, by using chloramphenicol to inhibit protein formation, it was demonstrated that RNA can be formed in the absence of protein formation, but a complete set of free amino acids is still necessary (Gale and Folkes, 1953; Pardee and Prestidge, 1956; Yčas and Brawerman, 1957; Gros and Gros, 1958). The RNA thus formed seemed to differ from nor-

mal RNA (Pardee *et al.*, 1957; Hahn *et al.*, 1957; Neidhardt and Gros, 1957; Horowitz *et al.*, 1958). Nothing is known about the function of amino acids in the synthesis, although several interesting models have been proposed (Raacke, 1958).

In broken cell preparations it has been shown that DNA is required for RNA synthesis. RNA synthesis was specifically stopped in homogenized preparations of bacteria after enzymatic destruction of DNA with deoxyribonuclease and reconstituted upon the addition of bacterial DNA (Gale and Folkes, 1958). Allfrey and Mirsky (1957) showed that in isolated nuclei, the incorporation of radioactive precursors into RNA was reduced after the depletion of DNA and restored with the addition of DNA. Later, however, equally good restoration was obtained with some polyanions (Allfrey and Mirsky, 1958), so that the direct role of DNA in RNA synthesis could not be demonstrated.

While intact DNA seems to be necessary for RNA formation, a simultaneous DNA replication is not necessary. When DNA replication was inhibited in bacteria with mustard gas or other chemicals, RNA synthesis continued (Harold and Ziporin, 1958; Kerridge, 1958). Mutants deficient in thymine were unable to build new DNA, but increased their RNA (Barner and Cohen, 1958; Okazaki and Okazaki, 1958). Also, in the resting cells of higher organisms, DNA is normally not replicating, while RNA synthesis proceeds continuously.

If DNA is responsible for RNA formation, it could be expected that DNA could not function when it is being replicated. In higher plant tissues, it was possible to follow by autoradiography, using P^{32} or orotic acid C^{14} as precursors, the periods when RNA and DNA are synthesized in the cell (Taylor and McMaster, 1954; Sisken, 1959). DNA was formed for short periods during the interphase, before or after mitosis, and RNA formation was low or absent during these periods. In *Tetrahymena* also, RNA synthesis was highest after the end of the period of DNA synthesis (Prescott, 1960b). In bacteria with synchronized divisions induced by temperature changes, DNA and mass synthesis were more or less in phase with cell division, whereas RNA synthesis was most intense during the absence of DNA synthesis (Lark and Maaløe, 1956). McFall and Stent (1959) believe, however, that the periodicity of DNA and RNA production is an artefact due to the synchronization procedure. In normally reproducing bacteria, it could be shown that bacteria

of all ages incorporate precursors into DNA and RNA (McFall and Stent, 1959; Abbo and Pardee, 1960). In yeast, the RNA content increased steadily, irrespective of the state of DNA reproduction (Mitchison and Walker, 1959). We can still assume that there is no RNA formation during DNA replication, but in some cases, only part of the DNA replicates at a time, so that the rest of it can still be active in RNA production. During mitotic division, however, DNA is condensed in chromosomes and the RNA synthesis is effectively prevented. It would be desirable to follow this interdependence of DNA and RNA synthesis in cells of a greater variety of organisms.

When it comes to the chemistry of DNA action, only models exist. If the Watson-Crick model is accepted, it is not easy to see how it could be used as a template (Wilkins, 1956). RNA synthesis may require a free, single-stranded DNA molecule. This possibility was recently discussed by Rich (1959, 1961), who was able to show that a single strand DNA could combine with a single strand RNA in a double helix. Helixes could be formed also with three strands, or with RNA strands only. It is then conceivable that RNA uses DNA as a template and is replicated in a manner similar to the replication of DNA, as proposed by Watson and Crick (1953).

In this case the base composition of RNA should be complementary to the base composition of DNA. This does not seem to be so, since it was found that the base composition of RNA does not vary much in different bacteria, while the DNA composition varies widely (Belozersky and Spirin, 1958). Yet, Belozersky could observe a slight correlation between the base compositions of both nucleic acids and assumed that only a small proportion of bacterial RNA has a base composition corresponding to DNA, while the major part is composed of approximately equal amounts of each base. Volkin and Astrachan (1957) could show that RNA newly synthesized after a virus infection has a base composition more closely resembling that of DNA. Yčas and Vincent (1960) found that the more active fraction of newly formed RNA in yeast has a base composition similar to yeast DNA.

These observations are intriguing: on the one hand, they give strong support to the hypothesis that DNA serves as a template for the synthesis of RNA, on the other hand, they indicate that the cell contains a large proportion of RNA which did not originate on DNA. Is it possible that this non-DNA-dependent RNA is the RNA which

many researchers claim was not synthesized in the nucleus? Or, is it a product of a few specially active synthetic sites, as in the nucleolus? Or, finally, does it represent biologically inactive RNA which has lost its specificity through exchange reactions in the cytoplasm and only awaits degradation?

Nuclear Secretion. Granted that RNA is made in the nucleus, it has to be transported into the cytoplasm, the site of its function. What is known about this transfer? It is surprising to find that this phase of the research of nuclear function has a long history and notable achievements. Many observations on nuclear secretion were, however, made more than fifty years too early, and never struck the imagination of biologists enough to become accepted in a comprehensive theory of gene action. The fact that the nucleus secretes basophilic material was observed by several workers before the end of the previous century. An excellent review of the older literature was written by Maziarski (1910), a disciple of the school of Cracovie, where this phenomenon was studied most actively. A more recent review was written by Hertl (1957). One of the reasons why nuclear secretion was not accepted as a general phenomenon is that it was observed in only a few specialized cells with active protein metabolism.

The first observations were made on the nuclei of the pancreas (Ogata, 1883), which emitted during secretion vesicles of nuclear substance, called plasmosomes or pyrenosomes. Several authors observed the nucleolar origin of these plasmosomes. Nuclear secretion can be observed also in most ovocytes. In the cockroach, *Periplaneta,* the process of nuclear growth and secretion was studied very carefully more recently (Gresson, 1931). The nucleolus increased in size with the advancing ovocyte's development, and became strongly basophil and irregular in outline. At the same time, it gave rise to emissions which passed through the nuclear membrane to the ooplasm. Later, the nucleolus became larger and vacuolated, giving origin to further extrusions. Once in the cytoplasm, nucleolar extrusions disintegrated and their material became mixed with the cytoplasmic constituents. Around the particles left by disintegration, an accumulation of newly formed vitellus was observed.

Most of the studies of nuclear secretion emphasized the chromatic nature of the secretion product and often this was considered to be part of the chromosomal chromatin. This is why Hertwig (1902)

called the secretion granules chromidia. At the time when most of this research was done, it was not yet known that all cells have two types of nucleic acids, and that the basophilia is mainly due to them. It was not possible at that time to identify the nuclear secretion products as RNA, and they were sometimes confused with expelled chromatic material (Goldschmidt, 1905). Only modern research has shown that RNA is actually present in the granules secreted by nuclei.

It would be interesting to know if ribosomes are present in the secretion product. Electron microscopy can provide a partial answer to this problem, although no positive method of identifying a ribosome in thin sections is available. Nucleoli, under the electron microscope, show fine granular structure and these granules are indistinguishable from ribosomes (Gall, 1956; Bernhard, 1959). Similar granules are also found scattered in the nucleoplasm. Nuclear secretion was studied by the electron microscope in several cells (Anderson and Beams, 1956; Gay, 1956, De Groot *et al.*, 1956). In the pancreas, Clark (1960) could identify three types of secretion material, one of them similar to the structure of nucleoplasm, another similar to nucleoli. The secretion vesicles remained enclosed in an evagination of nuclear membrane and disintegrated later. The contents became scattered in the cytoplasm and the nucleolar type of material could not be distinguished from nearby ribosomes. If all the stages of nuclear secretion could be followed under the electron microscope as carefully as was done before with the optical microscope, new important information on nuclear function could be obtained.

Although nuclear secretion seems to be a property of a few types of cells, it must be a general phenomenon, functioning as the transfer of nuclear information into the cytoplasm. It can be observed only in cells where large quantities of RNA are formed and liberated. In most cells, perhaps only very small granules, unobservable under the ordinary microscope, are continuously secreted. Individual ribosomes could leave the nuclei in this way. On the other hand, it may be significant that it is the nucleolar material which leaves the nucleus, as if ribosomes were first gathered in the nucleoli before being released in the cytoplasm.

Flies in the Ointment. The overwhelming evidence which has been presented indicates that the nucleus and DNA are implicated

in the production of RNA, but the picture is marred by the existence of "self-reproducing" RNA. The prime examples are plant and animal viruses, of which tobacco mosaic virus and recently poliomyelitis virus have been the most thoroughly studied. It was proved that the virus itself carries the necessary information for its reproduction and that RNA controls the composition of newly formed virus proteins (see above). Does the RNA inside the infected cell reproduce itself? If this is so, it may be that such a self-reproducing RNA is a special case, different from that of the usual RNA which is devoid of this ability. On the other hand, the infective RNA may convey its information to the cellular DNA, i.e., induce in the cell the production of the corresponding DNA which is used for further RNA production. Such a sequence of events could be admitted if something like Stent's scheme (Stent, 1958) of DNA reproduction is accepted. According to this scheme, DNA first produces a complementary RNA which then reproduces another DNA molecule. This model would allow not only the transfer of information from DNA to RNA, but also in the opposite direction.

Not enough is known of the cellular site of virus RNA reproduction. If the basic steps of reproduction occur inside the nucleus, as has been shown for some DNA viruses (Morgan *et al.*, 1954; Kellenberger *et al.*, 1959), and indicated for tobacco mosaic virus (Schramm and Röttger, 1959), the implication of DNA would become more probable. If, on the contrary, it could be demonstrated that the infectious virus never penetrates the nucleus, a self-reproduction of RNA should be admitted. Whichever be true, I feel that the self-reproducing RNA is a special case, occurring only in viruses and possibly in isolated instances of so-called cytoplasmic inheritance.

At the present time, the bulk of evidence indicates that the prime carrier of genetic information is DNA. If one considers the immediate function of the DNA molecule, the possibility cannot yet be entirely excluded that, instead of RNA, a protein is the first product of gene action. The gene could serve as a model for the formation of a specific protein which would act as a template for RNA. This RNA could then remake the protein in the cytoplasm. Simultaneous RNA and protein synthesis need not be necessary, since RNA synthesis could proceed with previously formed protein templates. According to this hypothesis, RNA formed in the absence of template proteins should be biologically inactive. In fact, it has been observed that RNA formed in the presence of chloramphenicol has

different properties from RNA made in normal cells (Pardee *et al.*, 1957; Hahn *et al.*, 1957; Neidhardt and Gros, 1957; Horowitz *et al.*, 1958). Chloramphenicol may have inhibited the synthesis of proteins which either function in the formation of RNA, or which are necessary to stabilize the RNA once formed. The need for a complete set of amino acids in RNA synthesis (Gale and Folkes, 1953; Pardee and Prestidge, 1956; Gros and Gros, 1958) indicates at least that a protein-like structure may be needed to adapt nucleotides to the template. Since amino acids function as catalysts, this would mean that they are released as soon as the RNA molecule is made. Wilkins (1956), however, has presented an argument against this hypothesis by pointing out that the structure of DNA does not look like a template for amino acids.

An entirely different role in inheritance was attributed to RNA by Haurowitz (1950), who assumed that RNA does not carry any information and serves only as a backbone for the protein template, keeping it in an unfolded state. Probably the strongest argument against this hypothesis is the case of tobacco mosaic virus, where RNA, not protein, carries the necessary information for virus reduplication.

Function of RNA

Localization of RNA in Cytoplasm. What is the fate of RNA when it leaves the nucleus and what is its function? RNA can be found in the cell in the portion of its cytoplasm which is basophilic. Garnier (1899), who studied the properties of this cytoplasm, named it "ergastoplasm." He visualized its nuclear origin and its role in the production of proteins. It took many years before the basophilic material was recognized as RNA (Brachet, 1941). Although a lamellar structure of the ergastoplasm was suspected by its discoverer, only the electron microscope could really reveal its fine structure (Porter, 1954; Palade, 1955, 1956). These authors named the observed structures "endoplasmic reticulum," superimposing this term on the old and very appropriate designation, ergastoplasm. The "reticulum" consists of a system of cisternae, vesicles and tubules with small, osmiophilic granules associated with them. The granules, often called Palade's granules, after their discoverer (Palade, 1955), are about 160 Å in diameter, stain dark with osmic acid and are believed to be the seat of basophilia and of RNA. In animal cells,

these granules appear normally associated with lamellae (in so-called "rough" reticulum), although lamellae can exist also without granules ("smooth" reticulum). In some young animal cells and ovocytes, most of the granules were observed free in the cytoplasm (Porter, 1954; Afzelius, 1957; Pasteels *et al.*, 1958), and in most plant cells they are not associated with reticulum (Buvat and Carasso, 1957; Sitte, 1958; Blondel and Turian, 1960). Since the term ergastoplasm means total basophilic cytoplasm, which encompasses granules and lamellae, it may still be appropriate to retain special names for the lamellar system without granules and for the granules themselves.

The proof that the granules and not the lamellae are the seat of RNA was obtained when it was possible to separate granules by centrifugation and analyze them chemically. When Claude (1946) first devised a technique of differential centrifugation of homogenized cells, he obtained a fraction which sedimented only at high centrifugal forces. The fraction consisted of granules rich in RNA called microsomes. Microsomes were not exact counterparts of structures found in a living cell, but rather fractions of ergastoplasm, appearing as vesicles of various sizes, carrying osmiophilic granules. Further treatment of this material with desoxycholate (Littlefield *et al.*, 1955) dissolved the membranes of vesicles and allowed the isolation of pure granules. The granules were also isolated from bacteria and found to vary in size, having different sedimentation constants (Tissières and Watson, 1958). The bulk of the granules have a sedimentation constant 70S; there are also 100S particles and smaller ones, 51S and 32S. The molecular weight of 70S units is 2.8×10^6, of 51S units — 1.85×10^6 and of 32S units — 0.95×10^6. The distribution into different sedimentation classes may vary in different organisms (Ts'o *et al.*, 1956; Peterman and Hamilton, 1957; Chao, 1957). Chemical analysis showed that these granules are practically pure nucleoproteins, consisting of from 40 to 60 per cent RNA; the rest is protein, plus some occasional lipids.

These granules are not an artefact of homogenization or fixation, as some would believe (Sjöstrand and Baker, 1958; Hanzon *et al.*, 1959), since it was possible to centrifuge them inside a living cell in *Neurospora* (Zalokar, 1961) and to sediment them into a basophilic, RNA rich layer near the centrifugal end of the cell. Electron microscopy revealed that this layer was composed of densely packed osmiophilic granules of around 150 Å diameter, and entirely devoid of any cytoplasmic lamellae.

Before the advent of electron microscopy, various basophilic granules of the cytoplasm were called chromidia and that was the name that many European authors adopted for the Palade granules (Monné, 1948; Weber, 1958). Most biochemists were at that time still talking of microsomes, a term which in the history of cytology has also had many different meanings. When the ribonucleoprotein nature of these granules was recognized and when they could be isolated from microsomes, the term ribosomes was introduced and, subsequently, widely accepted.

RNA and Protein Synthesis. Broken cell preparations consisting of microsomes were shown to be able to synthesize proteins (Zamecnik and Keller, 1954; Borsook, 1956). Later, the purified ribosomes were found to be the functional part of microsomes. It could be thus demonstrated that ribonucleoproteins are active in protein synthesis and the original ideas of Caspersson (1941) and Brachet (1942) bore ample fruit.

The occurrence of protein synthesis in ribosomes could also be demonstrated in a living cell. When *Neurospora* was fed H^3 leucine and centrifuged 5 to 15 seconds later, the radioactivity newly incorporated into proteins could be found mainly in the ribosome layer of the cell. The mitochondrial fraction also became labeled, but later it could be shown that probably most of this activity was due to ribosomes which filled the spaces between mitochondria (Zalokar, 1961). Radioactivity which was found in nuclei could be assigned to the synthetic function of the RNA which is a constant component of the nuclei.

The search for the chemistry of protein synthesis and the exact function of RNA is one of the most active fields of biochemistry today, and it is not my domain to report it in more detail. Several recent reviews cover the subject (Brachet, 1957; Chantrenne, 1958; Roberts *et al.*, 1959; Simkin, 1959).

It is established today that RNA functions in protein synthesis, and it is assumed that it functions as a template. How the amino acids are assembled on this template is not yet quite clear. It was found that the soluble RNA accepts activated amino acids and that there is a specific RNA for each amino acid (Schweet *et al.*, 1958a; Berg and Ofengand, 1958). Soluble RNA, carrying activated amino acids, is necessary for ribosomes to perform protein synthesis, and it is believed that the soluble RNA serves as an "adaptor" on the ribosome

template (Hoagland, 1959). It is not entirely certain yet that this is the only mechanism of protein formation, since it has been observed that ribosomes can incorporate amino acids into proteins directly, in the absence of activating enzymes or adaptors (Beljanski and Ochoa, 1958; Zalta, 1960).

Other systems of protein synthesis have been proposed and studied. Mitochondria were found to incorporate amino acids into proteins, especially into cytochromes (McLean *et al.*, 1958; Bates *et al.*, 1958). The mechanism of incorporation is not clear yet, but the role of RNA could not be excluded. All mitochondrial preparations contain small amounts of RNA which would suffice to explain the amount of protein produced. The fact that ribonuclease cannot inhibit protein synthesis does not necessarily mean that RNA is not required. The RNA may be protected and not open to ribonuclease in intact mitochondria. Indeed, Rendi (1959) could isolate, from mitochondria destroyed by desoxycholate, RNA particles which seemed to be responsible for most of mitochondrial protein synthesis and which became sensitive to the action of ribonuclease. Similar results were obtained after the destruction of mitochondria by sonication (Kalf and Simpson, 1959).

Nuclei were frequently considered as protein-producing sites even before the modern advances of cell biology. Caspersson (1941) still assumed that the important production of proteins occurred in nuclei and in their immediate surroundings. It has been possible to show since that the main protein synthesis occurs inside the cytoplasm. In insect cells this could be demonstrated directly by autoradiography. The salivary glands of *Drosophila*, the nurse cells of *Drosophila* and the silk glands of *Malacosomma americana* were treated with H^3 leucine for 5 to 15 seconds (Zalokar, 1960a, and unpublished results). After fixation and elution of unincorporated precursors, autoradiographs showed high incorporation inside the cytoplasm and practically none inside the nucleus (Fig. 4–2). The nucleus became labeled only later and certainly could not contribute to the cytoplasmic labeling. In silk glands treated with H^3 glycine, the main component of silk fibroin, the nucleus remained practically unlabeled still after one hour, while the cytoplasm and the excreted silk were heavily labeled.

There can be no doubt that proteins are made inside the nucleus also (Lang *et al.*, 1953; Allfrey and Mirsky, 1957; Allfrey *et al.*, 1957). Is this due to the same mechanism as the protein synthesis

A B

C D

inside the cytoplasm? Allfrey *et al.* (1957) believed that other systems may be involved. They were able to show protein synthesis in isolated nuclei and this synthesis was suppressed with desoxyribonuclease. The addition of DNA or its degradation products restored the synthesis. Later it was found (Allfrey and Mirsky, 1958) that even some unrelated polyanions could restore protein synthesis. Thus, it could not be proven that nuclei have a specific DNA dependent protein synthesis. Recently, it has been reported that nuclear synthesis requires activated amino acids and soluble RNA, similar to microsomal synthesis (Hopkins, 1959; Rendi, 1960).

While the nucleus probably does not contribute to the cytoplasmic protein production, part of the nuclear proteins may be synthesized in the cytoplasm. Goldstein (1958) showed that a protein rich in sulphur, labeled with S^{35}, found in the nucleus, can travel through the cytoplasm and accumulate in another nucleus. It is obvious that nucleus and cytoplasm should not be taken as two strictly separate entities, but as cell organelles with continuous interaction and no absolute barrier to prevent exchange of even the largest molecules.

Exchange Reactions. Many cases have been reported where amino acids were incorporated into proteins in the complete absence of RNA. Proteolytic enzymes are capable of building up protein molecules (Fruton and Simmonds, 1953), and in transpeptidations, individual amino acids or large fractions of protein molecules can be exchanged. It would be imprudent to confuse this kind of amino acid incorporation with the building of a specific protein, unless it could be proved that such reactions could lead to a specific sequence of amino acids in the polypeptide chain. While enzyme chemistry showed that a sequence of enzymatic reactions can lead to the formation of specific and often quite complicated molecules, it would be difficult to conceive a set of enzymes able to produce hundreds

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Fig. 4–2. Incorporation of H^3 leucine and H^3 glycine into silk glands of *Malacosomma americana*. Radioactive precursors were injected into young caterpillars, silk glands were prepared and fixed by freeze-substitution at appropriate times, sectioned to 4 μ thickness and autoradiographs made. A. H^3 leucine (3570 mc/mmole), fixed after 1 min, exposure time 30 days. B. H^3 leucine, fixed after 20 min, exposure time 3 days. C. H^3 glycine (44.2 mc/mmole), fixed after 1 min, exposure time 30 days. D. H^3 glycine, fixed after 20 min, exposure time 3 days.

of different proteins and arranging amino acids in as many different sequences. It should be expected that such a buildup would proceed stepwise, via small peptides, and then many proteins should have these peptides in common. There is, however, no evidence to support this view while the evidence of protein synthesis from free amino acids is ample.

More pertinent facts and arguments on the role of RNA in protein synthesis may be found in recent reviews written on this subject (Marshak, 1958; Roberts *et al.*, 1959; Cohen and Gros, 1960). In conclusion, all the facts support the generally accepted hypothesis DNA → RNA → protein. In the next two sections I would like to discuss how, in the light of this hypothesis, RNA could control cellular processes.

Control of RNA Production

Genic Control. Cellular processes must be somehow controlled by genes, since the nature of a cell or organism depends on its genetic constitution. Genetic control can be exerted either directly, through the quality and the rate of production of primary gene products, or indirectly, at different levels, through an intricate interplay of biochemical reactions (Strauss, 1955), directed by primary or secondary gene products. If we think of enzymes as products of gene action, then genes should determine the nature of these enzymes, the rate of their formation, and the time when they are formed in the cell life. Enzymes, once in the cytoplasm, direct innumerable biochemical reactions, and their mutual action results in orderly functioning of cell metabolism. The rates of enzymatic processes are subject to many factors (Pardee, 1959), such as the supply of precursors, the quantity of enzyme present and the functional state of enzymes, determined by their properties and environmental conditions.

The gene determines the specificity of a protein molecule by imposing a certain order of amino acids in the polypeptide chain. This it does by the intermediary of an RNA particle. The problem of the genic control of enzyme structure has been discussed elsewhere in this symposium (Suskind and Yanofsky, 1961) and in a recent book (Anfinsen, 1959). The quantity of an enzyme present in the cell and also the relative quantity of different proteins (some may be without enzymatic function), characteristic of every cell,

should be controlled by the functional state and quantity of enzyme forming sites. According to our model, this means the kind and quantity of ribonucleoprotein particles. The quantity of enzyme forming sites, i.e., quantity of specific RNA, is directly dependent on gene activity. Similarly, the time at which a certain protein appears in the cell depends on the presence of protein-forming sites, and the production of these, on the activity of genes. In this chapter I would like to discuss whether and how the genes control the production and the quantity of protein-forming sites of the cell.

The rate of RNA production by the gene will depend on a specific property of the gene, on the availability of precursors, and on environmental factors. The availability of a precursor may become a rate-limiting factor for different RNA molecules if these molecules are built of different amounts of this precursor. If for example one of the four nucleotides is in limited supply, then it is possible that the RNA molecule carrying more of that nucleotide would be produced at a lower rate than other RNA molecules. However, in all cases where the base composition of RNA was studied (Belozersky and Spirin, 1960), it was found that the four nucleotides occur in approximately equal amounts and that their ratios vary only to a small extent from organism to organism. Also, according to coding theories (Gamow *et al.*, 1956) the amount of the four nucleotides in nucleic acid cannot vary greatly, variation existing mainly in order along the chain. A limiting supply of one nucleotide can, therefore, not have an important regulatory power in the production of specific RNA molecules. On the other hand, a limiting supply could be of importance if several genes were competing for precursors. Activation of a particular gene could then reduce the production of RNA by other less active genes.

Gene Activation. From the preceding discussion it follows that all genes should produce RNA molecules at approximately similar rates, unless the genes are in different functional states. It would be highly uneconomical for a cell to produce RNA continuously when it is not needed. On the other hand, if there is need for an excessive synthesis of one protein, it would be unreasonable to accelerate total RNA production to supply the needed RNA in sufficient quantities. In such a case, the unneeded RNA should be continuously and specifically destroyed to release enough precursors for the predominant RNA. There must be, then, a mechanism which

allows some genes to be active, others inactive in different phases of cell life. This is, in essence, what T. H. Morgan (1934) proposed in his discussion of embryology and genetics.

The most dramatic illustration of differential gene activities can be found in the research by Breuer and Pavan (1955) and Beerman (1952) on the polytenic chromosomes of Diptera. These authors observed that the Balbiani rings and "puffs" of these chromosomes varied both with the developmental stage of the larva and from organ to organ. The authors interpreted this, and the fact that these structures accumulated RNA, as a sign of special gene activity. This interpretation will become absolutely convincing when it will be possible to link these formation sites with the appearance of a specific protein in the cell, and with the genetic locus responsible for the formation of such a protein.

On a purely genetic level, the control of gene activity has often been assigned to accessory genes such as the controlling elements of McClintock (1956) and the modulator gene of Brink (1954). The nature of these units is obscure; they do not seem to be integral parts of the chromosomes, since they migrate to other sites on the same or other chromosomes. McClintock's controlling elements can affect gene action at whatever locus they go to, while Brink's mutator loses most of its action when removed from the specific R locus, which it can suppress completely. These units do have genetic continuity and it would be attractive to think of them as regulators of gene activity, but it is not easy to see how they can accomplish this in a regular manner. As Brink (Van Schaik and Brink, 1959) says: "One looks in vain in the present data for evidence that the transpositions of modulators are related to gene activation and deactivation in ways that are meaningful for development of the organism." Also, in order to explain all possible gene functions, the nucleus should contain nearly as many regulator genes as genes themselves. Jacob *et al.* (1960) recently introduced a notion of a group of genes with coordinated expression, called "operon." Pardee *et al.* (1959) found in the system of β-galactosidase induction in *E. coli* that a closely linked regulator gene determines whether the enzyme will be constitutive or adaptive by synthesizing a specific repressor for the system. This repressor acts on another gene, situated next to the genes determining the structure of the enzymes, which then directs the expression of the enzyme genes. By expanding this finding into a theory, Jacob *et al.* (1960) suggested that each gene or

group of genes, which by themselves determine the amino acid sequences of functionally related enzymes, contain next to them an "operator" which determines the functional state of these genes. This suggestion, if altered slightly, could provide a real basis for the understanding of gene activation. The "operator" does not have to be a special gene, nor distinct from the amino acid determining part of a gene. The "operator" could be an integral part of the gene, a site where RNA formation is initiated, and this site could have a structure open to the activation of specific inductors. The site could at the same time determine a part of the sequence of amino acids in the protein, functionally unessential for the protein, but having its meaning in gene activation. It is well known that enzymes can still preserve their function when parts of the molecule are removed or modified (Neurath *et al.*, 1954). On a chemical level, a model explaining RNA synthesis by a process similar to DNA replication (Rich, 1961) could offer a very interesting possibility for speculation on gene activity. This model requires a partial unwinding of the double-stranded DNA during RNA production. Genes in a double-stranded condition would be inactive. They would become activated by partial unwinding—without being detached from the chromosome. A specific agent could at different places and times, activate such unwinding.

The control of gene activity, as visualized here, should conform to a certain pattern, leading to the establishment of an organic whole. This becomes important especially in the development of higher organisms, where cell differentiation must be coordinated to produce an adult organism with all its complexity. Each cell must perform at definite times the function to which it is assigned. If the function is under genic control, then the gene must somehow know at which time to act. An intrinsic activation timing would lead to highly uncoordinated results if some external factors slowed one or another function of the cell. The gene activation must, therefore, be responsive to the functional state of the cell, which means that the signal for activation must come from the cell or its environment. A gene, as it makes RNA and releases it into the cytoplasm, can have no knowledge of the state of this RNA, since the probability that RNA or its product, the enzyme, would diffuse back to the gene, is small and contrary to what we know about cells. The signal from the cytoplasm must be brought back to the gene by a carrier, which should either have special affinity for the chro-

mosome (and the specific gene) or should diffuse freely through the cell so that it would be highly probable that it would enter into contact with the gene site. Small molecular products of cell metabolism would be the probable signal carriers. The cell then could inform the gene about the functional state of RNA and the enzyme it produces by sending back either products or substrates of enzyme action.

In many cases gene activity should not depend on one particular enzymatic reaction, but rather on a state of more complex metabolic processes. An effective control in this case may demand simultaneous occurrence of several signals or of substances which are not exactly related to the enzymatic reaction controlled by the gene. Some possibilities of this kind will be discussed later.

After presenting the theoretical considerations for an hypothesis of gene activation, I would like to inquire whether there are facts to support it. First: Is it a fact that RNA is formed unequally in different phases of cell life and does RNA formation precede the formation of new proteins, when they appear in the cell? Second: What are the possible factors involved in gene activation?

Enzyme Induction and Repression. One of the best studied cases where a cell starts to produce a new protein is that of inducible enzymes. There is an excellent recent review on this subject (Pollock, 1959) and in the present volume, Vogel considers the problem of enzyme induction and repression. I shall try only to see how the established facts support the proposed hypothesis of gene action and RNA function.

The distinction between constitutive and inducible enzymes is not absolute, inducible enzymes being formed in the absence of the inducer in very small quantities. The cell, therefore, contains a gene which determines the amino acid sequence of the enzyme. The quantity of the enzyme produced is governed by the presence or absence of an inducer. Where does the inducer act? It has been well established that induction initiates enzyme formation from free amino acids. This gives us two possible sites of activation: either the RNA molecule, which is the enzyme forming site, or the gene, the specific RNA forming site.

If the induction occurs at the level of the RNA molecule, this would imply that such molecules are continuously present in the cell. Induction then should be possible in the absence of RNA

synthesis. The experiments of Kramer and Straub (1956) showed that the destruction of existing RNA by ribonuclease prior to induction did not impair inducibility, while ribonuclease applied after the inducer prevented new enzyme formation. The inhibition of RNA formation by analogs prevents induction (Spiegelman *et al.*, 1955; Creaser, 1956). If RNA formation is the primary effect of induction, DNA should be necessary. McFall *et al.* (1958) have found that when bacterial DNA is destroyed by "suicide" after incorporation of P^{32}, the ability to form induced enzymes is lost. Gale and Folkes (1955) were able to induce β-galactosidase formation in homogenized preparations of *E. coli.* The induction was effectively suppressed with desoxyribonuclease and reinstated by the addition of bacterial DNA. It is probable then, that the inducer does not act on the enzyme forming site, but rather activates the formation of new RNA.

If RNA synthesis is necessary for the synthesis of induced proteins, then an increase of the synthesis of the specific RNA should be the first effect of induction. It would be difficult to find such an increase, since only a small proportion of RNA formed is used for enzyme formation. However, if RNA must be formed first, a certain delay between the addition of the inducer and the beginning of enzyme production should be noticeable. While the synthesis of a protein molecule in the cell takes a very short time (McQuillen *et al.*, 1959; Zalokar, 1959b), it seems that the synthesis of an RNA molecule is slower. Kinetic studies of the uptake of C^{14} adenine and C^{14} uridine into *Neurospora* and incorporation into its RNA (unpublished results) indicate that a few minutes are necessary to build up an RNA molecule. A similar conclusion was reached by Yčas and Vincent (1960) for the synthesis of RNA in yeast, using P^{32} as a precursor. If this is the case, there should be a delay of at least a few minutes between induction and the onset of enzyme production. In the induction of penicillase, a latent phase of about 10 minutes was found (Pollock, 1952). No such lag seems to be observable in the induction of β-galactosidase and some other inducible enzymes (Pollock, 1959). A short lag, of a few minutes or less, could easily be overlooked or not measurable by the methods used in these studies. If the induced enzyme begins to be formed immediately after induction and proceeds at a linear rate, it would be difficult to assume prior RNA synthesis. In such a case, an activation of the preexisting enzyme-forming site should be considered.

This activation may lead only later to an increase of specific RNA synthesis, thus assuring continuous enhanced function of enzyme-forming sites.

If the first assumption is correct, the inducer should act directly on RNA production, i.e., it should activate the gene. As a result, new RNA molecules would be formed and transferred to the cytoplasm. There, enzyme production would start and the production rate would be proportional to the number of RNA molecules present. As long as the gene acts and there is no destruction of RNA, the rate should steadily increase. The rate of formation of induced enzymes is rather constant for a period of time (Pollock, 1959). This implies either that all the necessary RNA molecules are produced right at the beginning of the induction, or that the new RNA molecules are soon inactivated so that a steady state is quickly reached. The first could explain the case of penicillase production. In the initial 10 minutes of the lag period, the enzyme production rises to a constant rate and remains there for a long time. The ability to produce penicillase is retained by the cell long after the inducer is removed (Pollock, 1959). If the removal of the inducer puts a halt to RNA synthesis, the continued synthesis of the enzyme could be explained only by a long half-life of existing RNA-enzyme-forming sites. This explanation is at variance with Pollock's explanation, which is that molecules of the inducer combine somehow with the activated site and keep it active for an extended period.

The case of β-galactosidase would follow the second scheme. Newly formed RNA is soon destroyed. As soon as the inducer is removed, enzyme-forming sites are inactivated and further enzyme formation stops. The picture is complicated slightly by the fact that the enzyme, once formed, remains active in the cell for a long time (Wainwright and Pollock, 1949; Rickenberg *et al.*, 1953).

The opposite of induction and an active field of research today is enzyme repression. In the case of repression, an enzyme is produced until a product of enzyme action, be it immediate or remote, accumulates to stop its production. In a preceding paper, Vogel (1961) gave us all the facts known of the phenomenon and discussed a possible explanation of its mechanism. I would like briefly to consider repression within the framework of our hypothesis of gene action. If we consider a gene as intrinsically active, the presence of a repressor would stop its activity, through a mechanism not unlike that of induction. In some cases the repressor may compete with

the inducer for the activating site: when the inducer occupies the site, the gene functions; when the repressor occupies it this function is prevented. Again, it cannot be excluded that the repression acts at the enzyme-forming site, independent of RNA synthesis.

It is easy to visualize the value of induction and repression for the control of cell activity. The presence of a precursor starts enzyme production and its disappearance stops it. If a product of enzyme action starts to accumulate, repression stops further enzyme production. The quality of the gene itself would determine what concentration of inducer or repressor is appropriate for activation to occur or stop.

Secretory Glands. The essential fact of secretion pertinent to this discussion is that cells of secretory organs produce large quantities of proteins and that this production can be activated or lowered. Of all the possible secretory glands, let us consider the pancreas, since the cytology of pancreatic enzyme production is being extensively studied and has been summarized in recent reviews (Hirsch, 1958; Hirsch, 1960).

Several enzymes are produced by the pancreas: trypsin (in the form of trypsinogen), amylase, lipase, invertase, nuclease and others. The production and liberation of these enzymes is controlled by an intricate nervous control system, but can also be influenced by drugs, like pilocarpin. Let us consider one particular enzyme-producing cell. The cell is merocrinous, i.e., the cell accumulates the secretion product, empties it and resumes its synthesis. The enzyme-producing part of the cell seems to be ergastoplasm, long ago studied by Garnier (1899). The electron microscope has resolved ergastoplasm as a system of cytoplasmic lamellae carrying RNA particles, ribosomes. The ergastoplasm is the basophilic part of the cytoplasm and basophilia or, in modern words, RNA content, varies with the functional state of the cell. The enzymes accumulate in "zymogen" granules until they are released into the pancreatic ducts.

When the pancreatic secretion is activated with pilocarpin, the zymogen granules are released and new enzyme synthesis starts. The most important changes occur in the nuclei (Altmann, 1952). The nucleolus releases its substance into the cytoplasm (see above). Chromosomes appear to take part in the release of the nucleolar substance. Thereafter, the nucleus increases in size, and new synthesis of nucleolar substance begins. It can be seen how the sub-

stance originates on chromatic filaments to be concentrated into droplets, finally confluent into a new nucleolus. In the meantime, the cellular basophilia becomes stronger and the number of ribosome particles in the cell increases (Siekevitz and Palade, 1958). These cytological observations clearly indicate a renewal of activity in RNA production. An actual increase of RNA content and of P^{32} incorporation into RNA was detected in the activated pancreas by Russian authors (Guberniev and Iljina, 1950) while no appreciable increase of RNA has been measured by others (Daly and Mirsky, 1952; Morris and Dickman, 1960).

Despite a lack of final proof, it can be considered that the release of nucleolar RNA and further RNA synthesis precede enzyme synthesis in the activated pancreas. Pilocarpin or a nervous stimulus is the first cause of activation, but the action of these agents is primarily toward the liberation of enzymes from the cell and the new synthesis is a secondary effect. If this is so, an intracellular control system should exist, giving signal to the nucleus when enzyme reserves are depleted. Since the substrates for most of the pancreatic enzymes are found only in the digestive tract, a control similar to that of inducible enzymes cannot be considered. Also, the activation should start the production of several quite different enzymes and could not be induced by one simple substrate. Until this system of activation of enzyme synthesis is better understood, we can only say that the activation process should act simultaneously at several gene loci and that the depletion of cellular enzymes (or RNA) gives this signal.

In the salivary glands, as in the pancreas, pilocarpin stimulates saliva secretion, but it also induces a nearly immediate new synthesis of salivary amylase (Schneyer and Schneyer, 1957). The RNA content of activated salivary glands increases (Laird and Barton, 1958). In the stomach, RNA production is increased during active HCl secretion (Yakhnina, 1956). All these and other similar cases will have to be studied in more detail before the exact relationship between activation of secretion and RNA production can be determined.

Hormones. Endocrine glands can suddenly increase the production of hormones upon activation. Some of the hormones are of a proteinaceous nature, where activation would mean the induction of new protein formation. Most hormones are smaller molecules,

a big class of them belonging to steroids, and the activation of their production may be at a level quite remote from the nucleus. Still, specific enzymes are needed for production of these hormones and activation may mean new enzyme synthesis. The possibility that the nucleus, through RNA synthesis, exerts control over hormone production, will be examined.

One of the best studied examples of hormone production is that of the thyroid gland. Besides the active principle, thyroxin, the gland produces also a specific thyreoglobulin which functions in hormone accumulation and excretion in a still unknown manner. Consideration of the thyroid gland can be particularly instructive because of a mutual control system between the thyroid and the hypophysis. Pituitary thyreotropic hormone stimulates thyroxin production while thyroxin inhibits the production of thyreotropic hormone. The relation between these two glands shows a feedback system similar to that found in many biological processes, for example, inducible or repressible enzymes.

Does the thyreotropic hormone activate the thyroid gland at the nuclear level? In a resting thyroid gland, the acini have a flat epithelium surrounding large vesicles of "colloid." Upon activation, the colloid shows signs of resorption: vacuoles appear near the cellular periphery and the amount of colloid slowly diminishes (Ponse, 1951; Gross, 1957). At the same time, the epithelial cells grew cubic and finally prismatic. The nuclei become enlarged and the nucleoli more prominent (Koch, 1958; Herman, 1960). Cytoplasmic basophilia increases together with ergastoplasm, which becomes vesiculated (Dempsey and Peterson, 1955). The cytological picture gives every evidence of active RNA production. Direct measurements of RNA have confirmed this: RNA increased in thyroid glands activated with thyreotropic hormone and decreased in glands suppressed by the injection of thyroxin (Matovinovic and Vickery, 1959). There is no doubt that activated thyroid glands produce large quantities of hormones (including thyreoglobulin) and this increase in hormone production is paralleled by RNA synthesis.

As pilocarpin activates the cells in the pancreas, so does thyreotropic hormone in the thyroid gland. The exact mechanism of action of thyreotropic hormone is still unknown. The application of this hormone is followed by so many events that it is difficult to separate the primary from the secondary effects. Consideration of the exact timing of all the effects of activation, starting at very short

time intervals, may show the real first cause. Unfortunately, most endocrinological studies are concerned with effects following experimental interference after hours or even days. Many effects of activation may be too small to be detected in a shorter time, but with refined techniques, especially the use of tracers, it should be possible to study the response within minutes.

Some responses to activation are slow enough so that longer observation periods have been sufficient. Cytological changes appear first. The total uptake of P^{32} is activated immediately (Lamberg *et al.*, 1955), but it is a question whether this reflects an increase in nucleic acid synthesis. Two hours after the injection of thyreotropic hormone, the colloid content decreases and a release of iodine, as measured by I^{131}, is observed (Wolff, 1951; Reichlin and Reid, 1955). An increase in the uptake of I^{131} by the gland starts 24 to 48 hours later (Wahlberg, 1955). Activation of iodine metabolism must really mean the activation of the corresponding enzymes and it would not be impossible that activation of RNA production is the real cause.

It is estimated that several enzymes are needed in the iodine incorporation process, so that activation should act on several sites simultaneously. However, it would suffice to activate one key enzyme of the chain which would induce the others in sequence, as in sequential enzyme induction studied by Stanier (1950). Whichever the case, thyreotropic hormone can be considered as a specific activator of genic activity in the thyroid cell. It is interesting to note that this hormone is a polypeptide of relatively low molecular weight.

Thyroxin acts on the hypophysis by suppressing the production of thyreotropic hormone. It has been possible to find which cells in the hypophysis are producing thyreotropic hormone and follow their behavior under different conditions of hormone production (Purves and Griesbach, 1951). In the normal hypophysis these cells are characterized as basophil cells, rich in RNA. After suppression with thyroxin, the percentage of basophil cells diminishes and after stimulation of thyreotropic hormone production, it increases (Griesbach and Purves, 1943). Basophils of the stimulated hypophysis increase in size and their nuclei become bigger and contain several prominent nucleoli (Stevens *et al.*, 1955). This cytological picture again indicates direct involvement of RNA production in the activation of the hormone synthesis of the hypophysis.

The feedback control of hormone activity is known in several other cases involving the hypophysis. Everyone knows today about ACTH (adrenocorticotropic hormone) and cortisone. The overwhelming amount of new literature on this subject prohibits me from following all the new developments, but I am sure that a case similar to that of thyroid could be presented. A similar relationship also exists between the production of gonadotropic hormones and male and female hormones, or between luteinizing hormones and corpus luteum growth and activity.

My thesis is that in all these cases, hormones act on the genic sites for hormone production of regulated glands. The hypophysal "tropic" hormones activate the gene sites responsible for the production of enzymes functioning in hormone synthesis; while the feedback of these hormones acts by repressing the gene sites responsible for the production of "tropic" hormones. This mechanism differs from that of induction or repression, since the active molecules are in no way related to the action of the proteins produced. It can then be imagined that activating (or repressing) hormones act on several unrelated genes to influence a set of reactions. The parts of these genes susceptible to activation should then be identical and I would not be surprised if some day it will be found that parts of the protein molecules of enzymes depending on the same activator are identical.

The nature of hormone action on cell metabolism remains unknown. Many theories try to explain this action by changes in cell permeability, or by activation or inhibition of enzymes. If this is so, hormone action is very remote from the subject of our discussion. Still, I would like to suggest the possibility that in this case also, hormones act directly as gene activators, not for a particular enzyme but for a class of enzymes. To give an example, we could think of sex hormones as key substances to unlock—activate—a set of genes involved in the expression of sexual characters. The antagonistic action of male and female hormones could easily be explained similarly to the antagonistic action of enzyme inducers and repressors.

Gene Action and Cell Differentiation. Until now, I have discussed the changes in protein production, as dependent on RNA and gene activity for the lifetime of cells of one particular type. In a growing, multicellular organism, cells specialize in morphology and function, especially with respect to the kinds of proteins they

produce. One of the most puzzling and central problems of biology is the understanding of the mechanisms of this differentiation.

If differentiated cells have a complete set of genes, how is it that they perform only restricted functions? Weissman (1892) long ago formulated the theory of nuclear differentiation, a progressive loss of genic function during development by different cell strains. The problem was not approached experimentally until recent times, when Briggs and King (cf. King and Briggs, 1956) started their experiments on nuclear transplantation into enucleated eggs. These authors seem to have demonstrated that nuclear potentialities actually change irreversibly during development. The proof that this change is related to the restricted function of nuclei in differentiated cells is not absolutely convincing yet. Fischberg *et al.* (1959) have shown, on the contrary, that nuclei from differentiated cells of advanced stages of development, transplanted into eggs, were able to direct the formation of a complete organism.

Whichever is the case, irreversible nuclear differentiation is not a necessary condition for cellular differentiation. A beautiful example of this is lens regeneration in *Triturus* (Wolff, 1895). Pigmented iris epithelium, undoubtedly a highly differentiated type of cell, during lens regeneration loses its pigment, starts to divide and soon forms a lens cell able to synthesize highly specific lens proteins. Botanists can testify to many cases where an apparently differentiated cell can revert into a meristematic cell, able to produce a whole new plant. This can be seen in the formation of adventitious meristems from parenchymatous cells, normally not functioning in any further cell multiplications (Sinnott, 1946). A very instructive case can be found in peat-moss (*Sphagnum*) (Zepf, 1952). The leaves of *Sphagnum* are composed of two types of cells, small chlorophyll-bearing and large, water-storing cells. The latter, in a fully grown leaf, become dead cell walls. At the apex of the leaf bud, an apical cell divides unequally, giving one cell destined to be chlorophyll-bearing and another to be a water-storage cell. Each type of cell develops further according to its prospective potentiality. If there is any functional differentiation of nuclei, it must have occurred at the time of unequal cell division. Zepf could, however, under proper conditions, induce regeneration of a whole plant from both types of cell. If there was any nuclear differentiation it could not have been irreversible.

This evidence certainly supports the assumption that a complete set of genes is present in every cell. One of the primary points of the present discussion is that it would be uneconomical for all genes to be active in the cell at all times. Therefore, in development, gene activity must also be regulated, and this regulation must be of an orderly character, not a chance activation and inactivation entirely at the mercy of external influences. Each gene should become activated at an appropriate time in development. This can be achieved only if a signal indicating the developmental stage of the organism is sent back to the cells, and also a signal indicating a cell's position in the whole. These signals then would induce or repress appropriate gene functions. What is their nature?

Could differentiation depend on a system akin to enzyme induction? In some cases, this may be possible. As soon as a certain substance appears in the medium, the corresponding enzyme could be induced. However, it would be difficult to explain thus all the facts and intricacies of development. What about inducing the formation of proteins which are not enzymes, such as structural proteins, collagen, keratin, or silk fibroin? Enzyme induction can be found in higher organisms, but not as a regulatory mechanism in differentiation. Knox and his collaborators (1961) have found several inducible enzymes in rabbit liver. One of these, tryptophane peroxidase, is induced by tryptophane and the process of induction seems to be similar to that in bacteria. The system is more complicated, really, since cortisone highly increases inducibility and may be required for induction to occur at all. Besides, the cells can be induced only after a certain developmental stage is reached, not before the second day after the birth of the animal. At that time something must have happened to the cell to give it competence for induction. And finally, the inducer acts only on liver cells, not on other cells of the organism. Inductive enzyme formation then is a special case, not inherent in all cells with the same genetic constitution. It is the induction of competence which is important, and this is an embryonic process, which must act on different principles than plain induction.

Could embryonic induction be similar to the action of hormones? Again, it is obvious that the production of hormones is restricted to certain tissues and activation by other hormones acts on this tissue only. The cells of these tissues must have acquired, during develop-

ment, the competence to make hormones. The new competence usually rests with the cells permanently, or at least is not easily reversed, and therefore must be of a different nature than the temporary gene activation due to inducers or hormones.

Embryologists, since the ground breaking work of Spemann (1938), are in continuous pursuit of the embryonic inductor, the one which gives special competence to the cells. Many theories have been proposed and rejected and even today, we are far from understanding embryonic induction. The problem has been discussed in several recent reviews (Niu, 1956; Yamada, 1958; Tiedeman, 1959).

All classes of substances have been proposed as embryonic inductors, from glycogen and lipids to proteins and RNA. In recent times, new hope that the real inductor would be finally discovered arose with experiments of Niu (1956, 1958). If the presumptive ectoderm of an amphibian gastrula is cultured by itself, it does not differentiate. If it is cultured with an organizer, usually the mesoderm underlying the neural fold, it differentiates into neural tissue. Niu observed that gastrula ectoderm, explanted into the culture liquid in which previously an inductor was cultured, itself turns into neural tissue. Some substance with organizing properties must have been liberated by the previous culture. Various tests, especially the use of specific enzymes, indicated that the substance was RNA in *Triturus torosus*, but in *T. rivularis*, it may have been a protein. Works of other investigators (Yamada and Takata, 1955; Engländer and Johnen, 1957; Tiedeman, 1959) showed that the inductor was resistant to ribonuclease and that it behaved like a protein. Thus, it is not possible yet to ascertain the role of RNA in embryonic induction. Further discussion of the role of RNA in embryogenesis can be found in a recent book of Brachet (1960).

Embryonic induction may be a process too complicated for easy analysis. It would be advantageous to study a system where induction changes one cell type into another, differing in only a few characteristics and not in the ability to grow into an entire organ system. In the development of the eye, the eye vesicle induces lens formation in the overlaying ectoderm. Early in development, this ectoderm is still nearly omnipotent and the induction by the eye vesicle must activate sites specifically responsible for the production of lens proteins, while repressing other potencies. In the lens regeneration of newts (*Triturus*), retinal tissue induces new lens formation from already differentiated pigmented epithelium of the

iris. Induction is mediated by a substance excreted by the retina (Wachs, 1914; Zalokar, 1944), and the presence of the inducer is required during the early phases of lens growth. The nature of this substance is still entirely unknown. The important thing in this developmental process is that as an effect of induction, cells, besides their morphological changes, start to produce a new set of proteins, specific for lens tissues. Preceding this protein formation, an increase in cellular basophilia could be observed.

There are other systems in the development of organisms, where cells differentiate into new types destined to produce specific proteins. Thus, mesenchyme cells differentiate either into cartilage producing chondroblasts or muscle-fiber producing myoblasts. Either new type of cell is specialized in the production of proteins relatively well known chemically and structurally. Holtzer (1961) discusses this problem in a recent review. In the formation of red blood cells, erythroblasts differentiate into cells specialized in the production of one main protein, haemoglobin. Haemoglobin is one of the better known proteins today, with even its tertiary structure determined (Perutz *et al.*, 1960). The formation of haemoglobin has been studied extensively and the process can be obtained *in vitro* by whole cells (Borsook, 1956) or by homogenized preparations (Schweet *et al.*, 1958b). Cytochemical studies indicated the importance of RNA in erythroblast growth and production of haemoglobin (Thorell, 1947).

Both lens formation and mammalian red blood cells offer another interesting feature of importance in the study of gene action and RNA function. After the specific protein formation is initiated, the cells lose their nuclei. Protein formation can continue, but no new RNA formation is observable. Cellular RNA decreases with time and protein synthesis becomes reduced also. No new protein synthesis can be found after total RNA disappearance.

In the silkworm (*Bombyx mori*) silk-gland cells are synthesizing proteins at a rate surpassing all other known protein producing cells. The gland consists of two functionally different parts, the first producing fibroin, the main silk protein (Lucas *et al.*, 1958), the second producing sericin, the second component of silk. The characteristic of gland cells is an enormously increased nucleus and chromatin (Gilson, 1890), as if genes had to multiply in order to provide enough sites for the high synthetic activity. Needless to say, the rate of production of RNA in those cells is very high. It appears

that high protein production can be achieved in insect cells only by increasing the complement of genes, either by polyploidy or by polyteny, as in the salivary glands of Diptera. It is, therefore, even more imperative that all of the genes thus multiplied do not remain active. As was discussed previously, the gene activity can be seen directly, in salivary gland chromosomes, by the appearance of "puffs" (Beerman, 1952; Breuer and Pavan, 1955), indicating that only few loci actively produce RNA.

Control of RNA Activity

In the preceding chapter, the amount of RNA and protein synthesis in the cell was shown to depend on the rate of RNA production by the genes. It was assumed, to simplify the argument, that RNA, once formed, produces proteins without further impairment. In reality, many factors inside and outside the cell can also contribute to the control of protein production and influence the functioning of RNA.

The level of RNA present in the cytoplasm will depend on an equilibrium between the formation rate and destruction rate. If RNA once formed remained active in the cell for the rest of cell life, it may continue to produce proteins which in later cell life are unnecessary or undesirable. This would interfere with the effective control of cell activity and, therefore, RNA should have a limited life. Different rates of destruction could effectively control the amounts of various types of RNA present in the cytoplasm, independent of direct gene control. To postulate such differential rates would mean another set of regulators for RNA activity, specific for each type of RNA molecule. Such control would require a reduplication of genic controls in cytoplasm, which seems improbable. It may be, however, that the RNA particle has its own life span determined by its structure and activity. High protein production rate may accelerate the inactivation of the RNA particle, or the life of the particle may be proportional to the number of protein molecules made. It is interesting to note that ribosomes have their own ribonuclease built in the particle (Elson, 1959). This enzyme is inactive as long as the particle is intact, but becomes active after certain chemical treatments. Apart from such a possibility of differential destruction rates for different types of RNA, it may be also that all the RNA in the cytoplasm has a certain half-life, independent of its

nature, and is eventually destroyed by cellular enzymes. In the latter case, the relative amounts of RNA present would remain entirely under direct genic control.

The RNA which mediates protein synthesis in the cytoplasm does not function as long as it is still inside the nucleus, as has been shown in silk production (see above). The lack of cytoplasmic RNA activity before it leaves the nucleus cannot be due to the lack of necessary precursors, since nuclear RNA is able to synthesize proteins. RNA may have to be combined first with a protein to form a nucleoprotein particle, in order to become active. This probably happens inside the nucleolus which seems to store inactive RNA for some time. Thus, cellular activity could be controlled by the timing of the nucleolar release of RNA particles, but this release could hardly be specific for only one type of RNA. In secretory glands, such a release of nucleolar material has been actually observed during activation by pilocarpin (Altmann, 1952).

It would appear that RNA becomes functional as soon as it leaves the nucleus, as was postulated by Caspersson (1941), who observed the accumulation of RNA and protein synthesis in the nuclear periphery. However, there are cells containing high amounts of RNA and still not producing a large amount of protein. This is particularly true for "resting" reproductive cells. Fungus conidia, such as those of *Neurospora,* are rich in RNA (Minagawa *et al.*, 1959), yet, for a period of time they do not grow, their metabolism is at a low level, and certainly they do not produce any new proteins. In the ovocytes of many animals, RNA accumulates before the start of the most intensive protein production. In *Acetabularia,* morphogenesis can proceed in the absence of the nucleus and therefore, in the absence of RNA synthesis (barring the possibility of extranuclear RNA synthesis, see above), provided that nuclear morphogenetic products were previously accumulated in sufficient quantities (Haemmerling, 1953). It would be reasonable to assume that this morphogenesis requires the production of new kinds of proteins. RNA for the production of these proteins must have been present in the cell all along but must have become active only to form specific structure.

It has been suggested (Tissières and Watson, 1958) that the size of RNA particles may reflect their functional state. McQuillen *et al.* (1959) found, indeed, by isolating ribosome fractions of bacteria after feeding radioactive amino acids, that the highest incorporation

occurred in 70S particles. The activity of ribosomes may also depend on their position in the cell. When ribosomes were separated from other cell constituents by centrifugation, protein synthesis stopped (Zalokar, 1960b, 1961), even in cases where ribosomes were not completely separated from mitochondria. It appeared that ribosomes, to function normally, should not be separated from endoplasmic reticulum.

Many other factors can regulate the synthesis of proteins by RNA. The activity of RNA in the cytoplasm depends on the mass-action of precursors, co-factors and enzymes necessary in protein synthesis. Since all RNA molecules serving as templates differ probably only by the sequence of their nucleotides, it could be expected that they will all be affected equally by the above factors. The availability of precursors may affect synthesis differentially if the proportion of the precursors in the protein varies. If one protein does not contain a particular amino acid, while others do, then this protein will still be synthesized when the amino acid is absent as a precursor. If a protein is made largely of one amino acid, its synthesis rate will be affected more by a limited supply of this amino acid, than would that of other proteins. To my knowledge, no experiments have been made to demonstrate this, although it would be easy to do so with the many available amino acid-less mutants of various microorganisms, or with several amino acid analogs. Even if such control existed, it could cope only with a few general classes of proteins and would not be very significant in the exact regulation of cell functions.

The only mechanisms which could explain an orderly regulation of RNA function would be similar to the ones discussed in RNA production. There is no clear evidence that such mechanisms exist at this level and the arguments presented above were in favor of such controls at the genic level. In the case of some inducible enzymes, the inducer may act directly on RNA, since new enzyme formation starts immediately after induction (Pollock, 1959). This suggests that if DNA had a special mechanism responding to the induction, this mechanism should have been transmitted to RNA during its formation and may function as well in RNA. This would become even more probable if it was found that there is a protein (or protein-like assembly of amino acids) involved as an intermediary in the formation of RNA. The examples of inactive RNA mentioned show that there must be some mechanism to activate RNA

inside the cytoplasm, and sometimes even specifically, but the nature of this mechanism is obscure and may become evident only after further progress in biochemistry.

Conclusions

This is the picture I wanted to present. Let us step back and look at its main features. Genes, as DNA, carry information about the amino acid sequence in proteins, but also determine the rate of protein formation through the control of RNA production and the stage in the cell life when the formation should occur. While the first lies entirely in the gene, the two other qualities emerge as an interaction with the rest of the cell or the organism.

There is a flow of information from the nucleus to the cytoplasm and this flow can be explained in chemical terms. RNA is synthesized on the genes, to carry the information into the cytoplasm. It is the rate of RNA synthesis which is the main determining factor for the amount of enzyme or protein to be produced in the cell. This rate depends on gene activity—all genes are not active at all times. In order for the cell to function normally, certain information should be fed back to the nucleus. It is not probable that RNA, once incorporated in the ribosomes, could serve as a feedback directly. It must be a product of RNA action which informs the nucleus about the state of RNA in the cell. This could be the protein produced by RNA. But protein molecules themselves are large, moving through the cytoplasm sluggishly and being easily absorbed on specific structures. Then the product of enzyme action must be the feedback signal. This is usually a small molecule, present in larger amounts than either RNA or enzyme. This molecule can easily diffuse back to the nucleus and the probability that it will meet the gene is high.

But such a simple relationship is not sufficient to control all protein productions. Some proteins may be devoid of enzyme activity, others may be required to initiate new metabolic reactions. In these cases, there would be no ordinary feedback of the product of gene action, but a more complicated condition, where the nucleus is informed of a particular state of the cell or organism. This information, under appropriate conditions, would activate genes to produce RNA for building of new proteins and enzymes. Again, the signal

carrier should be expected to move from cell to cell freely, to penetrate easily into the nucleus, and to have the ability to activate or inactivate one group of genes.

Such activators may be hormones, known to play a role in many enzymatic reactions of the cell. Others must be related to embryonic inductors, elusive substances desperately sought by experimental embryologists. These latter substances may be able to confer to genes a relatively permanent competence to act in the production of RNA molecules, required for the synthesis of certain proteins at specific times and sites in development. All the above factors may act on genes together to determine their state of activity.

It is possible that this changing activity of genes was actually observed under the microscope. In the polytenic chromosomes of certain Diptera, "puffs" are formed at specific loci at specific times (Beerman, 1952; Breuer and Pavan, 1955). RNA is accumulated in these puffs (Pavan, 1958; Rudkin and Woods, 1959). One can visualize it as if a little red light flashes whenever a gene becomes activated.

RNA, once inside the cytoplasm, being part of ribosomes, is now active in building cell proteins. The rate of protein production will be a function of the quantity and of the activity of the RNA particles present. The quantity will depend on the rate of RNA production by the genes and on the rate of degradation in the cytoplasm. The activity will depend on some intrinsic properties of RNA and on a variety of factors which govern cellular metabolism. RNA particles are at the mercy of the precursor supply, of energy donors, of peptide-bond forming enzymes and a series of activators and inhibitors. Still, RNA alone carries the information of the amino acid sequence, and therefore, the nature of the enzyme it forms.

This Symposium has shown that control of cellular function can occur at many levels. It would be oversimplifying if the main controlling function was assigned to RNA and its production. The quality and the quantity of enzymes and other proteins formed in the cell starts an intricate interplay of many factors, each controlling another, and the concerted action of them all produces what we see as a living cell or organism.

Addendum

During the discussions at the Symposium, I was made aware of two pieces of research which may clarify many puzzling phenomena observed in RNA formation. First, W. S. Vincent reported that over 80 per cent of nucleolar RNA of starfish nucleoli consists of "soluble RNA." This would explain independent and high production rate of RNA in nucleoli. Second, French workers in Monod's laboratory, as a result of experiments with β-galactosidase, believe now that a major part of ribosome RNA does not carry genetic information, but serves as a "workbench" for the action of genetically determined "messenger" RNA. The total capacity of protein formation in the cell would thus depend on the amount of ribosome RNA present, while the kind of proteins formed would depend on the presence of specific "messenger" RNA. Messenger RNA has a high turnover and is supposed to function in the formation of only one or few protein molecules. The ribosomal "workbench" RNA may still be synthesized in the nucleus, or nucleolus, but if it does not carry any genetic information, its formation in the cytoplasm could be possible. Such concepts would help to explain cytoplasmic RNA synthesis, if this is proved to exist at all in a normal cell.

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5

Regulatory Mechanisms in Energy Metabolism[1, 2]

Jan van Eys[3]

Introduction

Cellular metabolism cannot be readily subdivided into neat separate sections which work independently. There are, however, sequences of reactions which are generally considered as having the primary function of donating chemical compounds which are required for anabolic processes. Thus the tricarboxylic acid cycle, while it does donate substrates for biosynthetic processes, is thought of primarily as a generator of ATP.[4] Similarly the Embden-Meyerhof pathway donates ATP under anaerobic conditions and donates the fuel for the tricarboxylic acid cycle under aerobic conditions. The hexose monophosphate shunt is now thought of as a donor of TPNH.

Since the need of ATP and TPNH will vary, it is only logical that the rate at which these substances are supplied is variable, depending on the conditions of the cell. However, in spite of the really staggering amount of work which has been devoted to the mecha-

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[4] Abbreviations used in this paper are: AMP, ADP, and ATP: adenosine monophosphate, diphosphate, and triphosphate, respectively; DPN and DPNH: oxidized and reduced diphosphopyridine nucleotide; TPN and TPNH: oxidized and reduced triphosphopyridine nucleotide.

nism by which this regulation is mediated, little that is definitive can be said about the phenomenon at the present time.

In order to discuss this assertion clearly, one should define the terms to be used. A great deal of confusion exists due to the meaning attached to the term *glycolysis*. In this paper *glycolysis* will be used as defined by Hatch and Turner (1959): "Glycolysis is the breakdown of carbohydrate to pyruvate by the Embden-Meyerhof-Parnas pathway, irrespective of the subsequent fate of pyruvic acid."

The regulatory phenomena referred to are generally known as the "Pasteur effect (reaction)" and the "Crabtree effect." The difficulty of defining the Pasteur effect has not been solved since the initial observation by Pasteur himself. Many definitions in the literature are chosen to accommodate the experimental conditions. The Pasteur effect will be defined here as "the steady state inhibition of glycolysis through the utilization of oxygen." The Crabtree effect will be defined as "the inhibition of endogenous respiration by the utilization of exogenous carbohydrates."

These definitions were chosen among the many variants available not because an explanation will be offered to fit the effects as defined, but because they allow discussion of the widest possible variety of experimental phenomena.

Pertinent Metabolic Reactions. It is inherent in any discussion of metabolic interrelationships and metabolic control mechanisms that we assume that the sequence of reactions involved are at least qualitatively known and understood. It seems worthwhile to investigate this assumption for a moment.

We are interested in the catabolism of hexose to pyruvate and lactate (or ethanol) and the further oxidation of pyruvate to CO_2 and water. On this second stage of carbohydrate metabolism, we can be confident. While variants of the tricarboxylic acid cycle do exist in nature, we know that the cycle is the main pathway and are reasonably certain that all steps in the oxidation are known. Of course we are not sure how such oxidation traps energy in the form of ATP, but we are certain that it does.

While the sequences of glucose to pyruvate are usually considered to be equally well known, these need some further discussion. The Embden-Meyerhof sequence is firmly established and so is the hexose monophosphate shunt, but there is evidence that these two do not quantitatively account for carbohydrate fermentation and oxidation.

First of all, there is the recently recognized glucuronic acid pathway (Touster, 1959; Burns, 1959). Glucose metabolized through this pathway would yield xylulose-5-phosphate, utilizing rather than generating TPNH. Furthermore, in contrast to the shunt, after glucose-6-phosphate ATP is utilized. To operate, the presence of mitochondria is required. These differences could becloud interpretation of Pasteur phenomena. However, experiments by Hiatt and Lareau (1958) indicate that very little glucose is metabolized through this pathway. It is therefore probably justified to ignore it in the present discussion.

TABLE 5–1

Accumulation of Methylglyoxal

	μmole Lactate	μmole Methylglyoxal
Liver homogenate	3.09	1.46
Brain homogenate	4.20	1.18

Homogenates were used, incubated in the system described by van Eys and Warnock (1959), with the addition of 30 μmole of 2-deoxyglucose. In the liver homogenate, 12 μmole fructose diphosphate were used; in the brain homogenate, 20 μmole fructose diphosphate were initially present. Incubation of 40 minutes. Methylglyoxal was measured by the method of Dische and Robbins (1934).

A second possible pathway poses more problems. Meyerhof (1934) dismissed methylglyoxal as an artifact. Since that time the participation of methylglyoxal as an intermediate in carbohydrate metabolism has been generally discarded as a possibility. Yet, at regular intervals the possible role of methylglyoxal in carbohydrate breakdown has been suggested. Recently, McKinney and co-workers claim such a role in leukocyte glycolysis (McKinney and Martin, 1956; McKinney and Rundles, 1956). Salem (1954, 1955) is the most recent investigator who found that methylglyoxal accumulates in thiamine-deficient rats. On the other hand, Weinhouse (Lewis *et al.*, 1959) found in tumor tissues little evidence of methylglyoxal as an intermediate in the conversion of glucose to lactic acid.

Consideration of methylglyoxal may appear to be reviving closed issues. However, the troublesome fact exists that, when one looks for methylglyoxal in tissue homogenates, which are incubated with substrates, one invariably finds its formation (Table 5–1). Even if this formation of methylglyoxal is an artifact, it still could give rise to considerable lactic acid and thus complicate the interpretation of lactic acid analyses.

A third point of question on the Embden-Meyerhof pathway is the presence of a TPNH catalyzed reduction of pyruvate. This activity exists in corneal epithelium (Kinoshita, 1957), liver (Navazio *et al.*, 1957), brain (Potter and Niemeyer, 1959), and virus infected chorioallantoic membranes (Kun *et al.*, 1960). It appears possible, especially in the latter instance, that two separate dehydrogenases are involved. Thus, this constitutes a second source of lactic acid not directly on the classic Embden-Meyerhof pathway. It is possible, however, that the "normal" lactic dehydrogenase could catalyze the reaction (Holzer and Schneider, 1958). In either case, since the observation has been made that DPN inhibits the utilization of TPNH, low DPN levels are required for the reaction to proceed (Kun *et al.*, 1960; Navazio *et al.*, 1957). Normally this is probably not the case, but such conditions could be provoked by experimental design.

Cellular Organization and Compartmentation. Many studies on the Pasteur effect, and recently on the Crabtree effect, are now performed on homogenates, rather than whole cells. It is becoming increasingly clear that cellular organization and compartmentation of enzymes and substrates play an important role in maintaining normal metabolism, even in the absence of hormonal influences. That enzymes are localized in specific fractions of the cell is well known, but recently the concept has grown that the compartmentation of metabolites is important. In fact, a variety of explanations of the Pasteur and Crabtree effects are based on this phenomenon.

The evidence for such compartmentation of metabolites should be examined. Reduced and oxidized pyridine nucleotides definitely appear to be compartmentized. Carefully prepared mitochondria from a variety of sources will not oxidize DPNH. This was first reported by Lehninger (1955) and further elaborated upon by Bücher and Klingenberg (1958). To accomplish oxidation of DPNH by mitochondria an external pathway is necessary. This can be furnished either by microsomal cytochrome-c-reductase (Lehninger, 1955) or by a coupled action of a pair of soluble and insoluble dehydrogenases. Such a pathway was first suggested by Bücher (Bücher and Klingenberg, 1958; Zebe *et al.*, 1959) and by Estabrook and Sacktor (1958). Originally this glycerol-1-phosphate cycle was proposed for insect flight muscle, but there is no reason to believe that such a cycle should be limited to insects (Zebe *et al.*, 1959). A

similar cycle was proposed by Devlin and Bedell (1960) for mammalian tissue, using the couple β-hydroxybutyrate-acetoacetate.

There is further suggestive evidence that such compartmentation exists *in vivo.* Thus the experiments by Hoberman (1958) reveal significantly less utilization for glycogen synthesis of the hydrogen of malate and β-hydroxybutyrate as compared to that of lactate. Further evidence is afforded by the observation that the ratios of DPNH/DPN and TPNH/TPN differ when the soluble fraction and mitochondria are compared (Glock and McLean, 1956; Jacobson and Kaplan, 1957).

However, direct evidence is lacking for the compartmentation of other metabolites we need to consider, such as ATP, ADP, AMP, and inorganic phosphate. The argument that the ratio of glucose-1-phoshate/inorganic phosphate is incompatible with glycogen synthesis in yeast cells (Trevelyan *et al.*, 1954), and therefore that inorganic phosphate should be unavailable, is less pertinent now that the uridine pathway of glycogen synthesis is known (Leloir *et al.*, 1959). This does not mean that such compartmentation does not exist but merely that sufficient evidence for establishing the fact is lacking.

Limiting Factors in Glycolysis. It would be logical to assume that the point of regulation of glycolysis is the enzyme which is rate-limiting. Numerous attempts to pinpoint such a site have been unsuccessful largely because the potential rate of the enzymes present exceeds by far the rate actually observed in intact cells (Wu and Racker, 1959a; Racker *et al.*, 1960). While this is not always the case (such as in human leukocytes, where glycolysis of intact cells equals glycolysis of homogenates [Beck, 1958]), it is at the present state of knowledge most likely that the rate-limiting factors are not the enzymes themselves but rather the availability of metabolites.

Pasteur Effect

We come to the heart of the question: What is the mechanism of the Pasteur effect? The classic review by Burk (1939) lists the multitude of theories and interpretations which had been given up to that date. A number of other reviews have been published (Burk, 1937; Dixon, 1937; Lipmann, 1942; Dickens, 1951; Krebs, 1957). One is forced, in reading through the literature, to make a decision on

whether there is more than one cause for a Pasteur effect or whether there is unity in Pasteur effects. It has been assumed that most of the published data are correct and that they have been interpreted with the knowledge at the time at which the work was carried out. The assumption then that there is unity in the biological world has as unavoidable corollary that some of the investigators created artifacts mimicking nature but not imitating it. There is great danger in making such decisions; nevertheless, it will be considered in the following paragraphs that there is one Pasteur reaction only.

In spite of the great progress made in our knowledge of intermediary metabolism, there are now, as there were in 1939 (Burk, 1939), still three great groups of Pasteur reaction explanations: (a) a direct oxygen inactivation of the glycolytic process; (b) the accumulation of an inhibitor of the glycolytic process due to aerobic conditions, and (c) competition of oxidative and glycolytic processes for a common intermediate. Only the most recent investigations will be dealt with in any detail.

Direct Oxygen Inactivation. Lipmann (1942) reviewed the experimental observations on oxygen inhibition of glycolysis. Special attention was given to the action of redox dyes. At that time Lipmann concluded that the most likely site of action was inhibition of triose phosphate dehydrogenase. Recently the inhibitory effect of methylene blue on aerobic glycolysis was reinvestigated (Glogner *et al.*, 1960), and the conclusion was reached that the aerobic inhibition of glycolysis was due to peroxide formation and a consequent decrease in DPN content of the cells.

It is not necessary, however, to inactivate enzymes or cofactors with redox dyes. In experiments on pea-seed extracts, Hatch and Turner (1959) pinpointed an aerobic inhibition of triose phosphate dehydrogenase. This reaction was reversible (as a Pasteur reaction should be). Later investigations showed that pea-seed extracts have indeed a mechanism by which protein-bound SH groups can be reversibly oxidized and reduced through pyridine nucleotide dependent enzymes (Hatch and Turner, 1960). The actual inactivation of triose phosphate dehydrogenase was presumably non-enzymatic. However, if this observation can be generalized for *in vivo* conditions, this non-enzymatic inactivation should be cyanide sensitive, since cyanide mimicks an anaerobic condition even in the presence of oxygen (cf. Lynen, 1957).

Similar results were obtained by Balasz (1959) in glycolyzing brain mitochondria. In *fortified* systems the glycolysis was inhibited, apparently due to inhibition or inactivation of triose phosphate dehydrogenase.

Accumulation of an Inhibitor. It has been observed that the addition of liver mitochondria to a glycolyzing brain supernatant results in a strong inhibition of glycolysis (Aisenberg *et al.*, 1957; Aisenberg and Potter, 1957). In this system competition for phosphate acceptance was probably not involved, since the effect could be seen with non-phosphorylating mitochondria (Aisenberg and Potter, 1957). The observations were explained by the maintenance of a high level of TPN and consequent accumulation of 6-phosphogluconic acid. This results in inhibition of phosphofructokinase, which in turn results in levels of glucose-6-phosphate which are inhibitory to hexokinase (Potter and Niemeyer, 1959). A similar result was obtained by the addition of liver mitochondria to ascites tumor supernatant (Tiedemann and Born, 1958).

However, while the above experiments resembled a Pasteur effect in that the mitochondrial inhibition was only present aerobically, addition of liver mitochondria to muscle supernatant inhibits anaerobically as well as aerobically (Sissakian and Pinus, 1958). Furthermore, when homologous mitochondria, i.e., mitochondria from the same tissue, are used, such inhibition is absent altogether. This is true for muscle (Sissakian and Pinus, 1958), Walker carcinoma (Aisenberg *et al.*, 1957), ascites tumor cells (Tiedemann and Born, 1958), and brain (Cremer, 1960). The latter investigator also found that liver mitochondria inhibited anaerobic CO_2 release from bicarbonate buffer; he attributed this to decreased lactic acid formation and hence a glycolysis inhibition.

Here an additional difficulty arises. The classic way of measuring glycolysis by a Q_{CO_2} (though not as prevalent nowadays) is extremely deceiving, since hexokinase is a hydrogen ion liberating reaction. The apparent discrepancy between Aisenberg's results and Cremer's result may well be non-existent. Sachsenmaier (1960) found an inhibition as described by Cremer. He, however, traced the effect to the action of mitochondrial DPN-ase, in contrast to the conclusions by Cremer who favored the ATP-ase activity as the explanation of the effect of liver mitochondria.

The lack of effect of homologous mitochondria is, however, a se-

rious discrepancy. It is hard to assume that addition of liver mitochondria to brain restores conditions that are lost by ordinary brain homogenization. Therefore the *in vivo* applicability of the system as used by Potter is doubtful indeed. The question of inhibition of glucose utilization by substrate inhibition is further complicated by the fact that yeast hexokinase is rather insensitive to substrate inhibition; yet yeast shows a normal Pasteur effect.

Competition of Respiration and Glycolysis: Phosphate Metabolism. At the present time, the key to the Pasteur effect is most generally sought in the oxidative and glycolytic phosphorylative processes. This was first proposed by Johnson (1941) and independently by Lynen (1941). Theories along these lines have the advantage of being teleologically satisfying. Lynen's experiments clearly indicate that glucose assimilation is the same aerobically and anaerobically (Lynen, 1957). Therefore, since the rate of regeneration of ATP is many times slower anaerobically than it is aerobically, given an identical need, glycolysis must be faster anaerobically than aerobically. The difficulty lies in identifying the actual pacemaker in the triad ATP, ADP, inorganic phosphate. Both ADP and inorganic phosphate can limit the rate of glycolysis. There is ample evidence that large amounts of inorganic phosphate can alleviate the Pasteur effect (Kvamme, 1958d; Wu and Racker, 1959b). However, the changes in inorganic phosphate in the transition from anaerobic to aerobic conditions are small (Lynen *et al.*, 1959). Wu and Racker (1959b) also noted that there was a discrepancy between the rate of glycolysis and the inorganic phosphate concentration. There are ways out of this difficulty: either phosphate is compartmentalized or phosphate measurements include labile phosphate esters.

An additional point which must be considered is that lack of phosphate or lack of ADP may limit glycolysis but will not necessarily limit glucose uptake if the level is sufficiently high for normal respiration. In fact, reconstructed systems, as studied by Gatt and Racker (1959a, 1959b), demonstrate this phenomenon. The addition of mitochondria to purified glycolytic enzymes blocks lactate formation but maintains glucose phosphorylation. When adenine nucleotides are limiting, this can be changed by appropriate selection of their concentrations, but this observation re-emphasizes the fact that limiting ADP alone will not impair glucose utilization.

Furthermore, to impair both glucose utilization and lactate formation by the addition of mitochondria, the phosphate level in the reconstructed system had to be very low indeed.

Thus the experiments by Lynen and by Wu and Racker require compartmentation of adenine nucleotides as well (cf. Chance and Hess, 1956; Racker, 1956). A more involved corollary exists: intramitochondrial ATP must then be available for one process and not for another. There is no doubt that ATP formed intramitochondrially can phosphorylate glucose, even *in vivo* (Lynen *et al.*, 1959). On the other hand, the behavior of phosphofructokinase as judged by glucose-6-phosphate and fructose diphosphate levels appears to indicate that it cannot utilize mitochondrial ATP. It is difficult to accept this assumption. Every other process in the cell can utilize ATP made intramitochondrially. Quastel and Bickis (1959) found little difference between aerobic and anaerobic energy-requiring processes. It is reasonable to postulate that intramitochondrial ATP, in the first few minutes after a transition from the anaerobic to aerobic state, may be formed at a rate faster than it can diffuse out of the mitochondria. This will explain the rapid changes which take place in the concentration of metabolites during such a transition. However, the final steady state which is still an inhibited Pasteur state shows no difference in ATP or ADP levels, only a lowering of the concentration of fructose diphosphate and a raising of the concentration of glucose-6-phosphate. The reversed experiment shows the reverse to happen, raising of fructose diphosphate and lowering of glucose-6-phosphate anaerobically (Lynen *et al.*, 1959). While the early transition stages shed light on the sequence of events, the final anaerobic and aerobic steady states, reached in 2-10 minutes, are the important phenomena.

Dinitrophenol is an uncoupler of oxidative phosphorylation and releases the Pasteur inhibition in the presence of respiration. It does not, however, inhibit the utilization of synthesized ATP for anabolic reactions. This can be shown by the experiment by Lynen (Lynen *et al.*, 1959) where glucose assimilation was unimpaired. Also valine incorporation is nearly unimpaired by dinitrophenol (van Eys and Neal, unpublished results) (Table 5–2). Therefore, dinitrophenol seems to result in making glycolytic ATP in contrast to mitochondrial ATP. If this is the cause of an accelerated phosphofructokinase reaction (Lynen *et al.*, 1959), phosphofructokinase must occupy a unique position in the cell. Furthermore, there is disagreement

about the fructose phosphate levels. Kvamme (1958d) found that fructose phosphates are considerably higher aerobically than anaerobically in ascites cells. This difference is accentuated at high phosphate concentrations; yet the Pasteur effect is less under these conditions, or in fact reversed. On the other hand, when cells were inhibited by malonate and made to respire fumarate, the phosphofructose levels were reversed. Kvamme concluded, however, that apart from the effect on fructose phosphate there is no fundamental difference between the two types of Pasteur reactions. He therefore limited the explanation solely to competition for inorganic phos-

TABLE 5–2

The Effect of Dinitrophenol on Valine-1-C^{14} Incorporation in Yeast

	μmole Glucose Utilized	μmole O_2 Uptake	Valine Incorporation cpm/planchet
Zero time			5
Control	17.4	14.0	4,109
+ Dinitrophenol	21.6	15.1	3,525

Each flask contained 30 μmole of glucose; 1 μmole valine-1-C^{14} (specific activity 10^6 cpm/μmole); citrate buffer, pH 6.0, 30 μmole; baker's yeast 6 mg in a final volume of 3.0 ml. To the appropriate flask, 0.15 μmole of dinitrophenol were added. At the end of the incubation time (70 min.), the yeast was centrifuged out and diluted with additional cells; the proteins were precipitated with trichloroacetic acid. After the proteins were washed free of absorbed valine, they were counted at infinite thickness.

phate. Yet he found that complete inhibition of triose phosphate dehydrogenase with iodoacetate abolished glucose uptake anaerobically but not completely aerobically, thus indicating that glucose uptake can proceed in the absence of glycolysis. This is again an argument that phosphate competition alone is not an explanation for the total Pasteur reaction. Furthermore, in homogenates Terner (1954) found that the level of inorganic phosphate was such that it could not mediate the pronounced Pasteur reaction in his preparations. And as mentioned before, Wu and Racker (1959b) found inorganic phosphate levels well above the K_m for triose phosphate dehydrogenase in ascites cells. Even under conditions where inorganic phosphate is not the obvious explanation, nitrophenols alleviate the effect (Terner, 1954, 1956; Wu and Racker, 1959b; Meyerhof and Fiala, 1950).

There are doubts whether the effects of dinitrophenol are as clear as generally assumed. Ronzoni and Ehrenfest (1936) found that

dinitrophenol stimulated lactic acid production anaerobically in frog muscle. Recently similar results were obtained in ascites cells (Slechta and Bentley, 1960). Windisch and Heumann (1960) found that levels of dinitrophenol which inhibited the Pasteur reaction stopped yeast from growing altogether. Yet all these effects cannot be explained solely by the mere activation of ATP-ase. First of all, for at least two functions ATP is readily available (cf. Table 5–2). Secondly, it is even doubtful whether ATP-ase activation by dinitrophenol actually exists *in vivo*. It seems reasonable to assert that the evidence of dinitrophenol reversal of the Pasteur effect in itself is not sufficient to claim ATP availability as the underlying cause.

Increased and lowered ADP availability could do the same as inorganic phosphate. However, again compartmentation will then have to be involved, since the ADP levels do not strikingly differ from the anaerobic to aerobic state (Lynen *et al.*, 1959). Furthermore, as has been mentioned before, with normally respiring systems, ADP lack, just as phosphate lack, would not lower glucose phosphorylation.

Competition of Respiration and Glycolysis: DPNH Formation. The suggestion by Ball (1939) of competition for reduced coenzymes between glycolysis and oxidation needs some further investigation. It is generally discredited as an explanation for the Pasteur effect. However, DPN is definitely compartmentized. In whole cells it is difficult to distinguish between non-mitochondrial and mitochondrial DPN, so that studies involving pyridine nucleotide levels require homogenization of the tissue.

In the whole animal the cytoplasmic DPN/DPNH ratio is about 5–7/1 (Glock and McLean, 1956; Jacobson and Kaplan, 1957). This is reflected in a lactate/pyruvate ratio of about 20/1 (Bücher and Klingenberg, 1958).[5] When one homogenizes liver and brain and incubates it with glucose or fructose diphosphate, the lactate/pyruvate ratio drops to about unity (van Eys and Warnock, 1959). This is also found in brain supernatant (Aisenberg *et al.*, 1957). If this pyruvate level still reflects DPN/DPNH ratios, the level of DPNH must be negligible. This is a serious departure from the normal situation. Surprisingly enough, addition of an ATP acceptor system lowers this pyruvate level to the usual *in vivo* concentration (van

[5] This is not a thermodynamic equilibrium with free DPN and DPNH. However, most pyridine nucleotides are bound to dehydrogenases, thus giving a considerably different potential to the DPN/DPNH couple.

Eys and Warnock, 1959) (Fig. 5–1). With measurable DPNH levels restored, the removal of glycolytically reduced DPN can be a rate-limiting step, which determines the rate of aerobic lactic acid production.

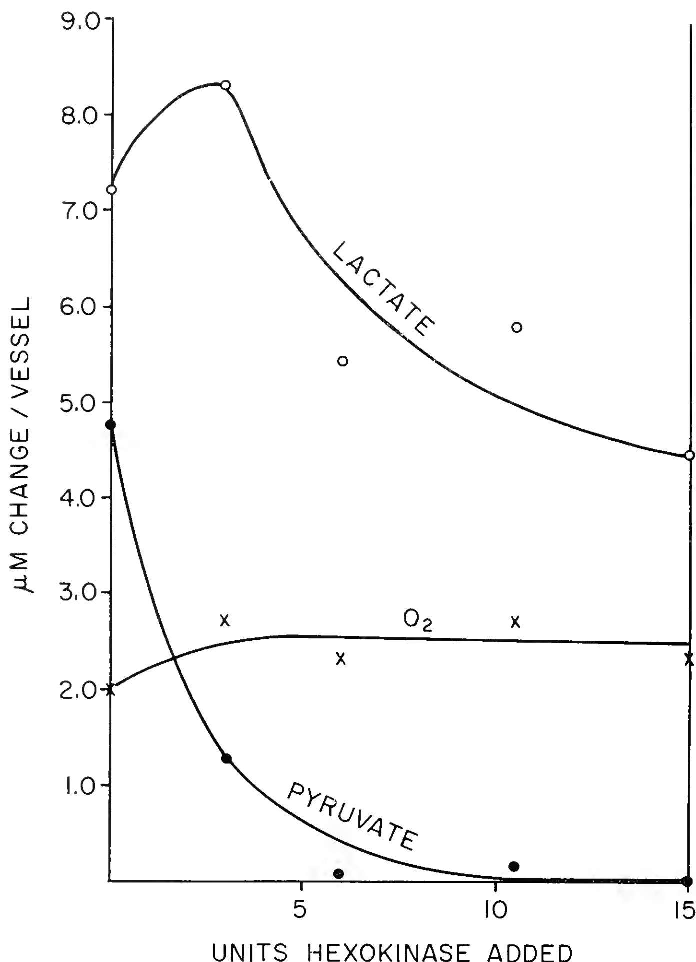

Fig. 5–1. The effect of the addition of hexokinase and 2-deoxyglucose on aerobic glycolysis of fructose diphosphate. All flasks contained 30 μmole of 2-deoxyglucose. One unit of hexokinase is that amount of enzyme which phosphorylates 1 μmole of glucose per minute.

The following experiments performed by Mr. Warnock in this laboratory illustrate this. In mouse liver homogenates the lactic acid production from fructose diphosphate can be lowered if microsomes and cytochrome c are added (Table 5–3). This effect is seen only

when pyruvate levels are lowered by the addition of hexokinase and 2-deoxyglucose or glucose. This is not necessarily an indication that microsomal cytochrome-c-reductase is the physiological mediator. However, the glycerol-1-phosphate cycle is not the mediator. In spite of the fact that DPNH can be catalytically oxidized through the cycle by liver mitochondria (Ciaccio and Keller, 1960), the rate is far too low, due to the insoluble enzyme, for the requirement imposed by the rate of glycolysis. Furthermore, the preferential utiliza-

TABLE 5–3

The Effect of Microsomes and Cytochrome c on Coupling Respiration and Glycolysis

Experiment	Conditions	O_2 Uptake (μmole)	Lactate Formation (μmole)
1	Mitochondria + supernatant	3.01	5.17
	Mitochondria + supernatant + microsomes	3.88	1.70
2	Whole homogenate, no cytochrome c	1.69	5.55
	Whole homogenate, cytochrome c added	3.57	3.39
3	Whole homogenate, no cytochrome c, no hexokinase	1.58	6.24
	Whole homogenate, no hexokinase, cytochrome c added	2.49	7.80

The incubation system described by van Eys and Warnock (1959) was used; 30 μmole 2-deoxyglucose were included. Where reconstituted homogenates were used, the amounts of tissue fractions correspond to the original homogenate.

tion of glycerol-1-phosphate hydrogen for glycogen biosynthesis in liver and kidney tends to argue against a glycerol-1-phosphate cycle in these tissues (Bloom, 1959).

This may be different in the brain, where the glycerophosphate cycle is possible, due to the high rate of insoluble α-glycerophosphate-dehydrogenase (Green, 1936; Ringler and Singer, 1959; Zebe *et al.*, 1959). It may be pertinent that the soluble α-glycerophosphate-dehydrogenase is low in tumors (Boxer and Shonk, 1960; Delbrück *et al.*, 1959) and that glycolysis in tumors fails to give rise to α-glycerophosphate (Ciaccio *et al.*, 1960). Furthermore, the insoluble α-glycerophosphate-dehydrogenase is under control of thyroxine (Lee *et al.*, 1959); thus the glycerol-1-phosphate cycle may become operational in liver in hyperthyroid states.

Such a mechanism of control would once more manifest itself as inhibition at the triose phosphate dehydrogenase level, with a consequent fluctuation of fructose diphosphate. It fails to explain the lowering of glucose uptake seen in aerobic glycolysis. The effect of subtle changes in DPNH removal may be enhanced by varying phosphate concentrations and ADP concentrations, thus supplying

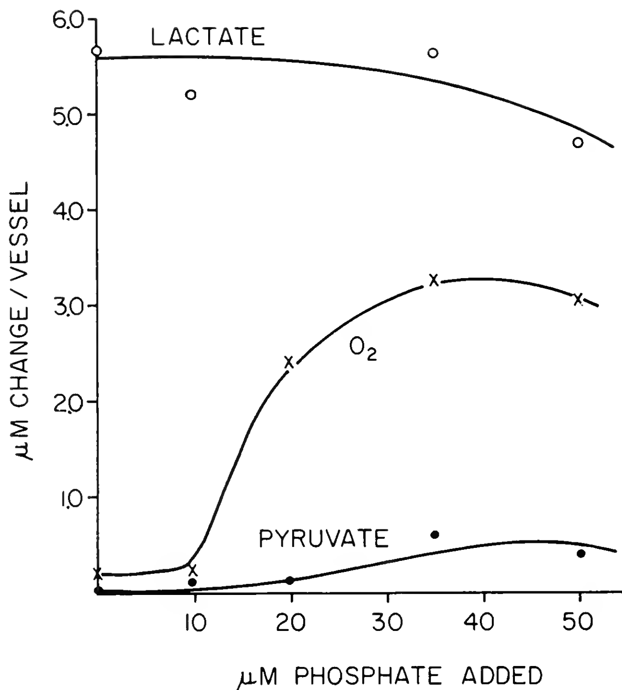

Fig. 5–2. The effect of increasing phosphate concentrations on aerobic glycolysis and respiration. All flasks contained hexokinase and 30 μmole 2-deoxyglucose. The remainder of the additions were as described previously (van Eys and Warnock, 1959). The values are corrected for changes in the absence of fructose diphosphate.

an interplay of many factors at the sensitive site. It must be noted here, however, that under conditions of low pyruvate it is respiration and not glycolysis which is sensitive to low phosphate concentrations (van Eys and Warnock, 1959). See Fig. 5–2.

It is clear that no explanation of the Pasteur effect is completely satisfying at the present time. No choice will be made, nor will any new theories be advanced at this time.

Crabtree Effect

The inhibition of endogenous respiration by glycolysis discovered by Crabtree (1929) is often called a reverse Pasteur effect. This is somewhat unfortunate in that this implies functional and mechanistic relationships. This is not necessarily true. Many hypotheses of the Crabtree effect indeed are based on interrelationships between phosphate compounds similar to those involved in the Pasteur effect. There are, however, a number of hypotheses divorced from this concept.

In recent years the Crabtree effect has received more attention even than the Pasteur effect. This is not surprising since it is a phenomenon generally associated with malignant cells. It is found in a variety of tumors (Krah, 1930; Elliot and Baker, 1935; Victor and Potter, 1935; Burk *et al.*, 1941; Kun *et al.*, 1951; McKee *et al.*, 1953). The high aerobic glycolysis exhibited by such cells is a general but not absolutely distinguishing characteristic. It is often said that such high glycolysis is evidence that the Pasteur effect is lacking in tumor cells (cf. Áes *et al.*, 1955). This is not necessarily true. Some tumor cells exhibit both a Crabtree and a Pasteur effect. Ascites tumor cells are the classic example. Also the Crabtree effect is not limited to tumor cells. It has been found in renal papillae and bovine cartilage (Rosenthal *et al.*, 1940), bull spermatozoa (Lardy and Phillips, 1941), leukocytes (McKinney *et al.*, 1953; Martin *et al.*, 1955), retinal cells (Cohen, 1957), and in *Tetrahymena* (Warnock and van Eys, unpublished observations).

The observation that both Crabtree and Pasteur effects can be present together or either one alone is sufficient indication that the two phenomena are separate problems. The Crabtree effect, just as did the Pasteur effect, elicited three main theories as to its cause: (a) competition between respiration and glycolysis for phosphate metabolites, (b) enhanced operation of the hexose monophosphate shunt due to the addition of carbohydrate, and (c) variation in hydrogen ion concentration.

Competition for Phosphorylated Intermediates. Since we are dealing in the Crabtree effect with induced variations in respiration, it is of vital importance to know what the pacemaker of respiration is. The extensive evidence accumulated on this subject deserves more than the mention which can be given here, but it is sufficient

for the present discussion to adopt the views of Chance that ADP is the "best candidate" for such control *in vivo* (cf. reviews by Slater and Hülsmann, 1959; Chance, 1959; and Lehninger *et al.*, 1959). There may, however, be factors which release the control of ADP (Lehninger *et al.*, 1959).

In view of this prominent role of ADP in respiration rates, one is tempted to seek ADP competition. However, the actual amount of ADP in the cell could be mediated through parallel ATP and inorganic phosphate levels. All three phosphate intermediates are indeed candidates for the causative agent of the Crabtree effect.

That inorganic phosphate is important may be indicated by the observation of Brin and McKee (1956) that high phosphate levels inhibit the Crabtree effect in ascites cells. However, Wu and Racker (1959b) found that such an effect was variable, while Kvamme (1958a) did not find this effect at all. All agree nevertheless that intracellular phosphate drops on the addition of glucose (Kunz and Schmid, 1957; Kvamme, 1958a; Wu and Racker, 1959b). Thus phosphate as a rate-limiting compound could be involved. However, even during a sustained Crabtree effect inorganic phosphate levels tend to rise again (Kunz and Schmid, 1957). This indicates that inorganic phosphate cannot be rate-limiting for a prolonged period (cf. Ibsen *et al.*, 1958; Seelich *et al.*, 1956).

The addition of iodoacetate does not inhibit the Crabtree effect (Kvamme, 1958b; Wu and Racker, 1959b; Seelich and Letnansky, 1960). However, iodoacetic acid results in a pronounced drop of adenine nucleotides (Kvamme, 1958b; Wu and Racker, 1959b; Wu, 1959) accompanied by a release of inosine derivatives (Wu and Racker, 1959b). The effect of dinitrophenol is less clear; Kvamme (1958b) found that it does not release the Crabtree effect, while Wu and Racker (1959b) observed only partial release. Seelich *et al.* (1956) and Ibsen *et al.* (1958) did find Crabtree effect release. On the other hand the Crabtree effect is absent after the addition of iodoacetate plus dinitrophenol or dinitrophenol at high inorganic phosphate concentrations (Kvamme, 1958b). The immediate question arises as to how glycolysis could exert its influence on respiration through inorganic phosphate in the presence of iodoacetic acid, which blocks the only phosphate-requiring enzyme in the glycolytic sequence. Furthermore, how could inorganic phosphate influence respiration in the presence of dinitrophenol? The latter difficulty could be overcome if the rate of substrate phosphorylation were the

determining factor in the rate of oxidation. Evidence of the rate-limiting function of α-ketoglutaric acid oxidation has indeed been obtained (Kvamme, 1958c). The iodoacetate dilemma could be circumvented by asserting that the adenine nucleotide drop now becomes the determining factor.

Competition for ADP between respiration and glycolysis is a more direct explanation. Gatt and Racker (1959a) found in a reconstituted system that the competition between glycolysis and respiration for ADP took place at limited adenine nucleotide concentrations. This could be imitated by creatine kinase plus creatine phosphate. Furthermore, dinitrophenol released the inhibition of respiration. However, at low phosphate concentrations a Crabtree effect could also be observed provided respiration and glycolysis were maintained through hydrolysis of the formed ATP.

Chance and Hess (1959) prefer ATP as the causative agent. They envision the events as follows: upon addition of glucose to the cell, a rapid release of ADP occurs which accelerates respiration for a brief period. When all the ADP is phosphorylated, the intramitochondrial ADP becomes exhausted, and the respiration slows down, especially due to the compartmentation of ATP. Packer and Golder (1960) sought confirmatory evidence for this hypothesis on the basis of the behavior of mitochondria inside the cell as judged by lightscattering. Electron microscopy also indicates mitochondrial changes after the addition of glucose to ascites cells (Merkel *et al.*, 1960a). The observations, which must be interpreted in the light of the theories but which pose other difficulties, are that the Crabtree effect can be elicited with sugars other than glucose. Fructose and mannose (Brin and McKee, 1956), as well as sugars which are not readily glycolyzed, such as glucosamine (Günther and Greiling, 1960), have been found to induce a Crabtree effect. Finally, 2-deoxyglucose gives the Crabtree effect (Ibsen *et al.*, 1958; Packer and Golder, 1960; Chance, 1960). This sugar cannot be metabolized at all. In fact it inhibits glycolysis from endogenous glycogen. The only reaction the sugars have in common is the phosphorylation step. In fact their relative effectiveness as Crabtree effect inducers is proportional to their relative affinity for kinase reactions (Packer and Golder, 1960). Acetyl glucosamine, which is not phosphorylated, does not give a Crabtree effect. (Günther and Greiling, 1960).

The phosphorylation of these sugars does not require inorganic phosphate; thus it is unlikely that inorganic phosphate per se is the

regulating constituent. The short-term Crabtree effect observed and described by Chance and Hess appears to indicate compartmentation of ATP. The intense inhibition of respiration after the addition of glucose is paralleled by an equally intense inhibition of glucose uptake (Chance and Hess, 1956). However, if this compartmentation is only through the rate of diffusion of ATP out of the mitochondria rather than through absolute compartmentation, it is not certain whether this mechanism is the explanation of the Crabtree effect seen in experiments of one hour or longer duration. It does not explain either the observation by Kunz and Schmidt (1957) that during the intense inhibition the total adenine nucleotide level drops to low levels. However, this was not seen by Ibsen *et al.* (1958).

An alternative explanation of such ATP and ADP compartmentation was offered by Ibsen *et al.* (1958). These workers suggested that, if the diffusion of ATP or ADP through the mitochondrial membrane requires the adenylate kinase system, the extent of unavailability of nucleotides may be dependent on the proper levels of all three compounds: AMP, ADP, and ATP.

Stimulation of the Hexose Monophosphate Shunt. Seelich and Letnansky (1960) proposed a theory, based on the stimulation of the hexose monophosphate shunt. They reason that the endogenous substrate in ascites cells is non-carbohydrate. Therefore, since it is known that the shunt stimulates lipid synthesis, a competition between lipid anabolism and catabolism arises, resulting in lowered respiration. This would explain the lack of effect of iodoacetate. In fact, in the presence of iodoacetate they found that the respiration is nearly malonate independent and thus represents mainly shunt oxidation.

They assume that the Crabtree effect of 2-deoxyglucose indicates that this sugar does result in TPNH formation.[6] Furthermore, in ascites cells glucosamine is converted rapidly to glucose-6-phosphate (Merker *et al.*, 1960b). A more serious objection that the effect is not alleviated by switching to carbohydrate substrates, such as pyruvate, is explained by the assumption that pyruvate is reduced to lactate due to the formed TPNH. Thus pyruvate would not be a substrate for respiration. That glucose does not inhibit acetate

[6] In this respect it is interesting that in brain homogenates, which glycolyze in the presence of 2-deoxyglucose, not all the utilized 2-deoxyglucose can be accounted for as 2-deoxyglucose-6-phosphate (J. van Eys and W. Hill, unpublished observations).

oxidation is explained by the fact that acetate oxidation does not require TPN, while higher fatty acids do. However, the observations by Kvamme (1958c) that α-ketoglutarate oxidation is subject to a Crabtree effect may not fit this theory. On the other hand, if ammonia were present reduction to glutamate might occur. Holzer and Witt (1960) found that the addition of ammonia to yeast cells greatly accelerated the shunt.

Another objection might be that it is very likely that TPNH is equally compartmentized, so that TPNH in the mitochondria is not TPNH in the cytoplasm. It must be mentioned, however, that isolated mitochondria are more permeable to TPNH than they are to DPNH (Birt and Bartley, 1960). However, a point for consideration is that dinitrophenol, when it releases the Crabtree effect, does not raise the TPN level (Ibsen *et al.*, 1958).

Hydrogen Ion Concentration. One of the characteristic features of the ascites tumor cell is the extremely sharp *p*H optimum exhibited by the endogenous respiration (McKee *et al.*, 1953). It is therefore obvious that the *p*H requires control, and therefore control of hydrogen ions has remained a constant worry for Crabtree investigators. An explanation of the Crabtree effect based on hydrogen ion concentration was first offered by Tiedemann (1952).

Arguments against a *p*H effect have been repeatedly brought forth. There is no need to list the large number of experiments used to indicate that extracellular *p*H is no factor in the Crabtree effect. There are indications however that the extracellular lactate is not a true reflection of intracellular lactate, so that hydrogen ion concentration as a factor in intact cells is not definitely ruled out (Dewey and Green, 1959).

Obviously the intracellular *p*H is the factor of interest. To study this effect homogenates are required. As far as is known the only study of the Crabtree effect in homogenates is that of Cereijo-Santalo (1960). Ascites tumor cell homogenates show a Crabtree effect which is largely similar to that of intact cells with two major exceptions: iodoacetic acid abolishes the effect and 2-deoxyglucose is not a strong Crabtree effect inducer. However, all other sugars, glucose, fructose, mannose, which induce a Crabtree effect in intact cells, induce the same effect in homogenates. The effect is seen whether an oxidizable substrate, such as glutamate, is present or endogenous substrates are used (Cereijo-Santalo and Park, 1960).

The fact that extra hexokinase enhanced the effect, and that glucose-6-phosphate did not elicit the Crabtree effect, pinpointed to the phosphorylation step as the point of interest. In almost all cases the Crabtree effect could be traced to *p*H effects in the homogenate. In fact, the initial *p*H of the homogenate determined whether a Crabtree effect was present.

The addition of an equivalent amount of brain mitochondria (calculated per unit homogenate) to a tumor supernatant gave a homogenate in which the effect was absent. Tumor mitochondria with brain supernatant, on the other hand, gave a preparation which does show a Crabtree effect. However, a brain homogenate could show a Crabtree effect by lowering the activity of mitochondria either by removing part of the mitochondria or by their partial inhibition with amytal (Cereijo-Santalo and Park, 1960).

This is consistent with the multitude of sugars which can provoke a Crabtree effect, and explains why tumor homogenates and homogenates with high glycolytic rates (Lugenova *et al.*, 1957) show a Crabtree effect. The major deficiency in tumors is not impaired mitochondria but fewer mitochondria per unit tissue (Schneider and Hogeboom, 1950). In fact, raising the amount of tumor mitochondria abolished the Crabtree effect in tumor homogenates. This explanation is not valid in the reconstructed system of Racker (Gatt and Racker, 1959a). Whether this explanation is correct for the whole cell remains to be seen.

The Significance of Crabtree and Pasteur Effects

It was mentioned before that the Pasteur effect is teleologically reasonable. This tends to persuade one to regard it as a true regulatory process. On the other hand, the Crabtree effect is only associated with those cells which have aerobic lactate formation. This is generally regarded as a major tumor characteristic. From the experiments of Cereijo-Santalo, it is suggested that this may be the result of a quantitative difference rather than a qualitative one. It is therefore possible that the Crabtree effect is an expression of the properties of abnormal cells; an effect which one could call a biological artifact.

The Pasteur effect may have further implications, however. There are indications that glycolysis has functions above and beyond its pyruvate-donating powers. Recent studies with oxamic

acid, which inhibits lactic dehydrogenase only in the direction of pyruvate reduction (Novoa *et al.*, 1959), show that it completely inhibits the glycolysis of ascites cells (Papaconstantinou and Colowick, 1957, 1960a) and the growth of HeLa cells (Papaconstantinou and Colowick, 1959, 1960b) and of *Tetrahymena* (Warnock and van Eys, unpublished). If the action of oxamic acid is really as limited as it appears, then lactic acid formation may be a required function of the cell. Whether this is because the lactic acid formation is an indication of a need for glycolysis at a rate in excess over pyruvate respiration, or whether TPNH reoxidation in excess over biosynthetic processes is required, remains to be seen.

Furthermore, if methylglyoxal is a possible intermediate, aerobic lactic acid formation may not reflect pyruvate reduction solely. We must be cautious, therefore, not to limit our view of glycolysis as a single metabolic process, and keep an open mind on its possible physiological role. Control of glycolysis is therefore possibly of even greater significance than a single adaptation to energy requirements.

Conclusions

It is impossible to remain completely divorced from hypotheses. Yet it is the strong feeling that no hypothesis explains the Pasteur effect theory completely satisfactorily at present. The view taken is that there is one Pasteur effect, which is fundamental but unexplained. The position taken on the Crabtree effect is that it is an expression of the properties of certain cells and constitutes no fundamental regulatory process but rather a biological artifact. Even these limited conclusions are open to criticism.

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6

Some Pyridine Nucleotide Transhydrogenase Reactions Mediated by Estrogenic Steroids

H. G. Williams-Ashman [1]

Introduction

The chemical constitution of the ovarian estrogens is well established, and the principal biochemical transformations involved in the biosynthesis and metabolism of these steroid hormones have been elucidated (Engel, 1959; Marrian, 1959). A variety of nonsteroidal substances also induce estrus in spayed animals, and much empirical information relating molecular architecture to estrogenic activity is available (Huggins, Jensen, and Cleveland, 1954; Dodds, 1957). One of the most central problems of female reproductive physiology is now the mechanism of action of estrogens at a molecular level. A fruitful approach to this problem has been to study metabolic and enzymatic changes in the uterus following the administration or deprivation of estrogens (Szego and Roberts, 1953; Mueller, 1957). Such investigations have uncovered many important biochemical concomitants of estrogen-induced tissue growth which must ultimately be explained by any comprehensive theory of estrogen action.

This paper will discuss some chemical aspects of the direct stimulation of a single isolated enzyme system by estradiol-17β and certain other steroidal estrogens. It has been shown that these hor-

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mones mediate the transfer of hydrogen between pyridine nucleotides. These transhydrogenase reactions are catalyzed by a soluble enzyme isolated from human placenta. The concentrations of estradiol-17β required to saturate this enzyme are very low and are commensurate with those at which this hormone exerts its estrogenic action. But the mechanism by which estrogenic steroids stimulate these transhydrogenase reactions must be understood in precise chemical terms before meaningful experimentation to assess their wider physiological significance can be undertaken.

The following topics are considered in this paper: experiments with crude placental extracts; transhydrogenase reaction; reaction mechanism; enzyme purification; binding and interaction of pyridine nucleotides; reversibility; steroid specificity; interconversion of hydroxy- and ketosteroids; stereospecificity of hydrogen transfer; inhibition experiments; model reactions with hepatic and bacterial hydroxysteroid dehydrogenases; and biological implications.

Experiments with Crude Placental Extracts

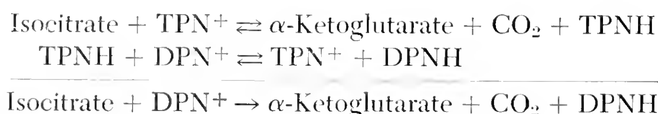
Eight years ago, Hagerman and Villee (1953; Villee and Hagerman, 1953) reported that the oxidative metabolism of glucose and pyruvate by slices of human endometrium and placenta was accelerated by the direct addition of low concentrations (4×10^{-6} M) of estradiol-17β. Villee later extended these studies to cell-free extracts of human placenta from which particulate material was removed by ultracentrifugation (Villee and Hagerman, 1953; Villee, 1955; Gordon and Villee, 1955; Villee and Gordon, 1955). The reduction of DPN [2] (but not of TPN) by isocitrate catalyzed by these extracts was stimulated profoundly by estradiol-17β, and Villee suggested that the site of action of the hormone was a soluble, DPN-specific isocitric dehydrogenase. Hagerman and Villee (1957) found that this "estrogen sensitive isocitric dehydrogenase" was very unstable, and they were unable to purify the enzyme substantially. They observed (Gordon and Villee, 1956) that estradiol-17β was

[2] The following abbreviations are used: DPN, diphosphopyridine nucleotide; DPNH, dihydrodiphosphopyridine nucleotide: TPN, triphosphopyridine nucleotide; TPNH, dihydrotriphosphopyridine nucleotide; deaminoDPN, the hypoxanthine analogue of diphosphopyridine nucleotide; acetylpyridineDPN, the 3-acetylpyridine analogue of diphosphopyridine nucleotide; acetylpyridineTPN, the 3-acetylpyridine analogue of triphosphopyridine nucleotide; pyridinealdehydeDPN, the pyridine-3-aldehyde analogue of diphosphopyridine nucleotide; thionicotinamideDPN, the thionicotinamide analogue of diphosphopyridine nucleotide.

bound very firmly to this enzyme system, with a dissociation constant of 2.6×10^{-8} M. Villee concluded from this fact that the hormone combined with an inactive form of the enzyme and thereby converted the protein into an enzymatically active form.

Transhydrogenase Reaction

The enhancement by estradiol-17β of DPNH formation in such crude placental extracts is variable and usually disappears upon fractionation of the extracts by treatment with ammonium sulfate. But with fractionated enzyme preparations, activity could be restored by the addition of *catalytic* amounts of TPN (Talalay and Williams-Ashman, 1958). This suggested that estradiol-17β did not activate a DPN-linked isocitric dehydrogenase but stimulated the transfer of hydrogen from TPNH to DPN. The apparent activation of a DPN-specific isocitric dehydrogenase could be accounted for by the following coupled reaction:



$$\text{Isocitrate} + \text{TPN}^+ \rightleftarrows \alpha\text{-Ketoglutarate} + CO_2 + \text{TPNH}$$
$$\text{TPNH} + \text{DPN}^+ \rightleftarrows \text{TPN}^+ + \text{DPNH}$$
$$\text{Isocitrate} + \text{DPN}^+ \rightarrow \alpha\text{-Ketoglutarate} + CO_2 + \text{DPNH}$$

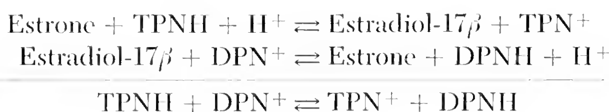
This formulation implies that the action of estradiol-17β promoted the transfer of hydrogen from one form of pyridine nucleotide to another and was in no special way connected with the oxidation of isocitrate. It was supported by the demonstration (Talalay and Williams-Ashman, 1958; Talalay, Hurlock, and Williams-Ashman, 1958) that TPN-specific dehydrogenases for either glucose-6-phosphate or acetaldehyde could replace isocitric dehydrogenase as a TPNH-generating system. Moreover, an estradiol-dependent transfer of hydrogen to DPN from TPNH added as such to the reaction mixture was demonstrable in the presence of low levels of DPN and a specific DPNH-oxidizing system (acetaldehyde and yeast alcohol dehydrogenase).

Villee and Hagerman (1958) and Hollander, Hollander, and Brown (1959a) confirmed the requirement for catalytic levels of TPN and a TPNH-reducing system for the estradiol-mediated reduction of DPN by crude placental extracts. The hypothesis that estradiol activated a DPN-specific isocitric dehydrogenase was retracted by Villee and Hagerman (1958), who accepted the conclu-

sion of Talalay and Williams-Ashman (1958) that this hormone stimulated the transfer of hydrogen from TPNH to DPN.

Reaction Mechanism

Estradiol-17β and estrone are equally effective in promoting the TPNH-DPN transhydrogenase reaction in soluble placental extracts. Partially purified enzyme preparations were found to catalyze the reduction of DPN and TPN by estradiol-17β, and the oxidation of DPNH and TPNH by estrone (Talalay and Williams-Ashman, 1958). This suggested that these steroids stimulated the transfer of hydrogen from TPNH to DPN by undergoing alternate oxidation and reduction, as follows:



$$\text{Estrone} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{Estradiol-17}\beta + \text{TPN}^+$$
$$\text{Estradiol-17}\beta + \text{DPN}^+ \rightleftharpoons \text{Estrone} + \text{DPNH} + \text{H}^+$$
$$\overline{\text{TPNH} + \text{DPN}^+ \rightleftharpoons \text{TPN}^+ + \text{DPNH}}$$

According to this reaction mechanism, these steroids transport hydrogen in a coenzyme-like manner by virtue of the change steroid alcohol $\rightleftharpoons$ steroid ketone.

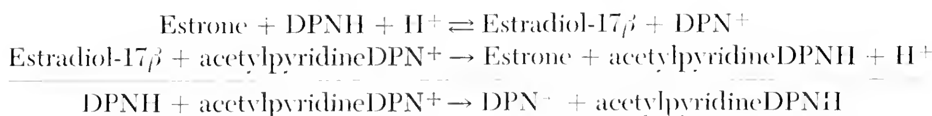
Previously, Langer and Engel (1958) characterized a placental 17β-hydroxysteroid dehydrogenase which reacted with a number of 1, 3, 5-estratriene derivatives including estrone and estradiol-17β. Both DPN and TPN served as hydrogen acceptors for the oxidation of estradiol-17β. Talalay and Williams-Ashman (1958, 1960) have provided evidence that the estradiol-mediated transhydrogenase reactions in placenta are catalyzed by an enzyme which exhibits the properties of the 17β-hydroxysteroid dehydrogenase of Langer and Engel (1958). The enzyme catalyzes both dehydrogenase and transhydrogenase reactions with a number of pyridine nucleotide analogues as well as with DPN and TPN.

Enzyme Purification

The placental enzyme was purified by Talalay, Hurlock, and Williams-Ashman (1958). The purification procedure involved removal of all particulate material by treatment with calcium chloride, heat denaturation of inert protein, and fractionation with ammonium sulfate. The enzyme is very unstable, and estradiol-17β was added

as a stabilizing agent at an early stage in the purification procedure. The enzyme prepared in this way contained bound estradiol-17β, which could not be removed completely by repeated precipitation of the protein with ammonium sulfate or with acetone.

Each fraction was tested for dehydrogenase activity with DPN, TPN, deaminoDPN, and various pyridine nucleotide analogues as hydrogen acceptors (Talalay and Williams-Ashman, 1960). Transhydrogenase assays which necessitate the addition of auxiliary enzymes for the generation of TPNH are not trustworthy. Consequently, transhydrogenase activity was determined by measurement of the estradiol-dependent reduction of acetylpyridineDPN by DPNH. Catalytic amounts of estradiol-17β or estrone mediate this reaction, apparently by the following mechanism:



$$\text{Estrone} + \text{DPNH} + \text{H}^+ \rightleftarrows \text{Estradiol-17}\beta + \text{DPN}^+$$
$$\text{Estradiol-17}\beta + \text{acetylpyridineDPN}^+ \rightarrow \text{Estrone} + \text{acetylpyridineDPNH} + \text{H}^+$$
$$\text{DPNH} + \text{acetylpyridineDPN}^+ \rightarrow \text{DPN}^+ + \text{acetylpyridineDPNH}$$

Since acetylpyridineDPNH absorbs light strongly at 400 mμ, whereas DPNH does not (Kaplan and Ciotti, 1956), this reaction can be followed spectrophotometrically at 400 mμ in the presence of high concentrations of DPNH. However, this assay system is complicated by the fact that a number of soluble flavoprotein enzymes also catalyze the DPNH-acetylpyridineDPN exchange (Weber and Kaplan, 1957). Such enzymes are highly active in crude placental extracts and contaminate the most purified preparations of the placental enzyme which catalyze estradiol-dependent transhydrogenase reactions. But these "non-specific" DPNH-acetylpyridineDPN transfers are unaffected by TPN, whereas the estradiol-dependent transhydrogenation between the latter nucleotides is virtually obliterated by low levels of TPN. Thus, the estradiol-dependent DPNH-acetylpyridineDPN exchange could be estimated if suitable controls to which TPN was added were employed. In later experiments (Talalay and Williams-Ashman, 1960), the DPNH-acetylpyridineTPN and the TPNH-acetylpyridineTPN transfer reactions were also measured at various stages of the purification procedure.

Talalay and his co-workers purified the placental enzyme more than a hundredfold with retention of a major portion of the original estradiol-DPN dehydrogenase activity. They were unable to

obtain convincing evidence for the existence of separate proteins catalyzing the oxidation of estradiol-17β by six different pyridine nucleotides, or a variety of tranhydrogenase reactions. The most purified preparations of the placental enzyme invariably reacted in both dehydrogenase and transhydrogenase systems with the same pyridine nucleotides that served as hydrogen donors or acceptors in crude placental extracts. Table 6–1 shows that of ten acceptor

TABLE 6–1

Specificity of Purified Placental Enzyme for Pyridine Nucleotides and Analogues *

Nucleotide Acceptor	Reactivity in Dehydrogenase Reaction	Reactivity in Transhydrogenase Reactions
DPN	+	+
TPN	+	—
DeaminoDPN †	0	0
3-AcetylpyridineDPN	+	+
3-Acetylpyridine deaminoDPN	0	0
Pyridine-3-aldehydeDPN	+	+
Pyridine-3-aldehyde deaminoDPN	0	0
ThionicotinamideDPN	+	+
Nicotinamide mononucleotide	0	0
Ribosyl nicotinamide	0	0

* The reactions were studied at *p*H 7.4 under conditions similar to those described by Talalay, Hurlock, and Williams-Ashman (1958) and by Talalay and Williams-Ashman (1960). Estradiol-17β was used as substrate for the dehydrogenase reactions. Hydrogen transfer from TPNH to acceptor nucleotides (transhydrogenase reactions) was studied in the presence of 4×10^{-6} *M* estradiol-17β and continuously generated TPNH at a final concentration of 3×10^{-6} *M*. The concentration of the acceptor nucleotides in both the dehydrogenase and transhydrogenase reactions was 4×10^{-4} *M*.

† DeaminoDPN is inert in both the dehydrogenase and transhydrogenase reactions at *p*H 7.4 but will function as a hydrogen acceptor in both reactions at *p*H 8.5 (Talalay and Williams-Ashman, 1960).

nucleotides tested with the purified enzyme, five were active and five were inactive in the dehydrogenase system. These nucleotides reacted in exactly the same way in the transhydrogenase reactions.

The experimental findings of the Chicago investigators (Talalay, Hurlock, and Williams-Ashman, 1958; Talalay and Williams-Ashman, 1960) are completely at variance with the report of Hagerman and Villee (1959) that an enzyme catalyzing both the TPNH-DPN and the DPNH-acetylpyridineDPN transhydrogenase reactions can be separated from *two* pyridine nucleotide-linked estradiol dehydro-

genases, specific for DPN and TPN, respectively. Hagerman and Villee (1959) described the separation of these enzymes by electrophoresis on a starch block and also by filter-paper curtain electrophoresis in barbital buffer of *p*H 8.6. The transhydrogenase and the two estradiol dehydrogenases were also separated by chromatography on DEAE cellulose (Villee, Hagerman, and Joel, 1960). The specificity of the two estradiol dehydrogenases toward various pyridine nucleotide analogues, or toward different steroids, was not reported. Hagerman and Villee (1959) found that a mixture of their placental DPN- and TPN-linked estradiol dehydrogenases did not catalyze the transfer of hydrogen from TPNH to DPN in the presence of estradiol-17β. They put forward the hypothesis that estradiol-17β stimulates the TPNH-DPN and the DPNH-acetylpyridineDPN transhydrogenase reactions by converting, in an unspecified manner, an inactive form of the placental enzyme into an active one. This implies that estradiol-17β or estrone does not undergo alternate oxidation and reduction during the course of these transhydrogenations and that the steroids cannot be regarded as hydrogen carriers in these reactions.[3]

Binding and Interactions of Pyridine Nucleotides

The affinity of the purified placental enzyme for various pyridine nucleotides in the dehydrogenase reaction with estradiol-17β and estrone as substrates has been studied extensively (Talalay, Hurlock, and Williams-Ashman, 1958; Talalay and Williams-Ashman, 1960). At *p*H 7.4, the Michaelis constant for $\text{TPNH} \leqslant \text{TPN} < \text{DPNH} < \text{DPN} <$ acetylpyridineDPN. The affinity for TPN(H) was so high that their Michaelis constants could not be measured accurately by the usual spectrophotometric procedures, and the same was true for acetylpyridineTPN. The affinity for acetylpyridineDPN and pyridinealdehydeDPN is relatively low, and the rates of oxidation of estradiol-17β by the latter nucleotides are unusually sensitive to changes in *p*H.

The reduction of DPN by estradiol-17β was inhibited almost completely by one-hundredth the molar concentration of TPN, and de-

[3] *Note added in proof.* Since this article was submitted for publication, Drs. J. Jarabak and P. Talalay in this laboratory have purified the placental hydroxysteroid dehydrogenase more than 2500-fold, and in high yield. No separation between various dehydrogenase and transhydrogenase functions was apparent at any stage of the purification procedure which involved, *inter alia,* chromatography on ion-exchange resins and electrophoresis.

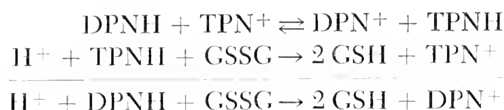
tectable inhibition of this dehydrogenase reaction occurred when the DPN/TPN ratio was 10^3. Similarly, the oxidation of estradiol-17β by acetylpyridine DPN was powerfully inhibited by TPN. But the reduction of TPN by the steroid was unaffected by DPN. If it is assumed that the different pyridine nucleotides compete for the same binding site(s) on the enzyme, then these inhibitions of various dehydrogenase reactions are in accord with the relative affinities of the nucleotides.

The affinities for different pyridine nucleotides in the dehydrogenase reaction appeared to give some insight into the nucleotide interactions in various transhydrogenase systems. The rate of the TPNH-DPN exchange was very dependent upon the relative concentrations of DPN and TPNH. At each level of DPN, there is an optimum concentration of TPNH. At *p*H 7.4, the optimum DPN/TPNH ratio is in the region of 50–200 (Talalay, Hurlock, and Williams-Ashman, 1958). The transfer of hydrogen from TPNH to DPN is inhibited at high concentrations of TPNH. Transhydrogenation between TPNH or DPNH and their corresponding 3-acetylpyridine analogues is exquisitely sensitive to the relative concentrations of nucleotides in the reaction mixture. Thus, the DPNH-acetylpyridineDPN, the DPNH-acetylpyridineTPN, and the TPNH-acetylpyridineTPN exchange reactions proceed in the presence of relatively high levels of the donor nucleotides, whereas the transfer of hydrogen from TPNH to acetylpyridineDPN is detectable only if the concentration of TPNH is kept very low. Again, transhydrogenation between DPNH and either acetylpyridineDPN or pyridinealdehydeDPN is inhibited by very low concentrations of TPN or TPNH.

Reversibility

The oxidation of estradiol-17β to estrone with DPN or TPN as hydrogen acceptor is readily reversible at *p*H 7.4. Langer and Engel (1958) reported the equilibrium constant $K = (DPNH)\ (H^+)\ (Estrone)/(DPN^+)\ (Estradiol\text{-}17\beta)$ to be $1.8 \times 10^{-8}\ M$ at 25° (cf. Talalay, 1957a). Since the oxidation reduction potentials of the DPNH-DPN and TPNH-TPN couples are the same, it might be expected that the estradiol-mediated transfer of hydrogen from TPNH to DPN would be readily reversible. But Talalay, Hurlock, and Williams-Ashman (1958) found that the placental enzyme did not catalyze the transfer of hydrogen from low levels of DPNH (generated

in situ by the action of alcohol dehydrogenase) to TPN in the absence or presence of estradiol-17β. Villee and Hagerman (1958) reported similar findings and concluded that the estradiol TPNH-DPN transfer was irreversible. However, Talalay, Hurlock, and Williams-Ashman (1958) demonstrated that this reaction was readily reversible under appropriate experimental conditions, namely, with a high initial ratio of DPNH to TPN at pH 7.4. The reaction was followed by measuring the disappearance of DPNH (1×10^{-4} M) in the presence of low levels of TPN and a specific TPNH-oxidizing system (oxidized glutathione and glutathione reductase):



$$\begin{array}{r} \text{DPNH} + \text{TPN}^+ \rightleftarrows \text{DPN}^+ + \text{TPNH} \\ \text{H}^+ + \text{TPNH} + \text{GSSG} \rightarrow 2\,\text{GSH} + \text{TPN}^+ \\ \hline \text{H}^+ + \text{DPNH} + \text{GSSG} \rightarrow 2\,\text{GSH} + \text{DPN}^+ \end{array}$$

The optimal concentration of TPN was found to be 1.6×10^{-6} M. Marked inhibition of the DPNH-TPN reaction was observed if the TPN level was raised to 5.4×10^{-6} M, and almost complete inhibition occurred when the TPN concentration was 1.6×10^{-5} M. This situation is reminiscent of the inhibition by TPN of the TPNH-DPN exchange in the forward direction and also of the transfer of hydrogen between DPNH and acetylpyridineDPN. Very recently Hosoya, Hagerman, and Villee (1960) have confirmed the reversibility of the estradiol-mediated TPNH-DPN transhydrogenase reaction.

Steroid Specificity

The specificity for steroids in both the dehydrogenase and the transhydrogenase reactions is very similar. Hollander, Nolan, and Hollander (1958) showed that of some 30 substituted 1, 3, 5-estratriene derivatives, only those compounds bearing a 17β-hydroxyl or 17-ketone group stimulated the transfer of hydrogen from TPNH to DPN by crude placental extracts. In a series of synthetic racemic *iso*estrones, only *dl*-8-*iso*estrone functioned as a mediator of the transhydrogenase reaction and as a substrate in the dehydrogenase reaction (Hollander, Hollander, and Brown, 1959b). The steroid specificity for the dehydrogenase reaction with DPN as hydrogen acceptor has been studied exhaustively by Langer, Alexander, and Engel (1959). They found only three compounds which failed to function in *both* the dehydrogenase and the transhydrogenase reac-

tions. Neither estriol nor 17α-ethynylestradiol-17β reduces DPN in the presence of the placental enzyme, yet both of these substances stimulate the TPNH-DPN exchange (Villee and Hagerman, 1958; Hollander, Nolan, and Hollander, 1958). However, the latter transhydrogenase reaction was stimulated by these two steroids only in relatively high concentration, and the activity of 17α-ethynylestradiol-17β could be accounted for if it was assumed that the sample contained 1 per cent by weight of either estradiol-17β or estrone as an impurity (cf. Talalay and Williams-Ashman, 1960). According to Hollander, Nolan, and Hollander (1958), 16-ketoestradiol-17β and 16-ketoestrone do not stimulate the transhydrogenase reaction. Yet 16-ketoestrone is hydrogenated at about one-fourth the rate of estrone (Langer, Alexander, and Engel, 1959). But strangely enough, 16-ketoestradiol-17β will not reduce DPN in the presence of the placental enzyme at *p*H 9.2. This suggests that the equilibrium for the reaction of 16-ketoestrone with DPNH is very much in favor of complete reduction of the steroid and quite different from the equilibrium for the oxidation of estradiol-17β. If this is the case, one would not expect either 16-ketoestrone or 16-ketoestradiol-17β to act as efficient mediators for the TPNH-DPN exchange via a mechanism which involved oxidoreduction of the steroids.

Interconversion of Hydroxy- and Ketosteroids

Hollander, Hollander, and Brown (1959a) found that when the placental enzyme catalyzed the TPNH-DPN transhydrogenase reaction in the presence of estradiol-17β-*16*-C^{14}, a steady state is soon achieved in which the ratio of estrone/estradiol-17β is aproximately 3. During the course of transfer of hydrogen from DPNH to acetylpyridineDPN, estradiol-17β-*6,7,t* becomes interconverted with estrone (Talalay and Williams-Ashman, 1960). These experiments suggest, but do not prove, that estradiol-17β undergoes alternate oxidation and reduction when it mediates transhydrogenase reactions catalyzed by the placental enzyme.

Stereospecificity of Hydrogen Transfer

In experiments using DPN-nicotinamide-4-*t* and TPN-nicotinamide-4-*t*, Jarabak and Talalay (1960) showed that the dehydrogenation of estradiol-17β by the placental enzyme occurs with transfer

of hydrogen to side II (β) (cf. Levy and Vennesland, 1957) of the nicotinamide moieties of TPN and DPN. Hydrogen transfer from DPNH to acetylpyridineDPN mediated by estradiol-17β likewise involves the abstraction of hydrogen from side II of the DPNH.

Inhibition Experiments

In crude placental extracts, Hollander, Hollander, and Brown (1959a) observed that the dehydrogenase and transhydrogenase functions of crude placental extracts were inhibited to about the same extent upon storage at 3° at various hydrogen ion concentrations. Hagerman and Villee (1959) reported that heating a partially purified placental preparation to 56° for 2 hours inactivated the DPN-dehydrogenase activity to a greater extent than that of the TPN-dehydrogenase; the TPNH-DPN transhydrogenase reaction was much less inhibited. But Talalay and Williams-Ashman (1960) were unable to demonstrate differences in the heat stability of the estradiol-DPN dehydrogenase reaction and of transhydrogenation between DPNH and acetylpyridineDPN.

However, differential inhibition of various dehydrogenase and transhydrogenase functions can be demonstrated under some experimental conditions. For example, the concentration of adenosine-2′-monophosphate and of 2′, 5′-adenosine diphosphate required to inhibit the oxidation of estradiol-17β by DPN is considerably greater than that needed to depress the TPNH-DPN transhydrogenase reaction (Hollander, Hollander, and Brown, 1959c). Conversely, both *p*-chloromercuriphenyl sulfonic acid and thyroxine inhibit the estradiol-DPN dehydrogenase reaction at lower concentrations than are required to inhibit either the TPNH-DPN or the DPNH-acetylpyridineDPN exchanges (Hagerman and Villee, 1959; Villee, Hagerman, and Joel, 1960). But in these inhibition experiments, dehydrogenase and transhydrogenase activities were not measured under identical experimental conditions, especially in relation to pyridine nucleotide concentration. Thus, they do not provide unequivocal evidence for separate enzymes catalyzing transhydrogenation and dehydrogenation, as Hollander, Hollander, and Brown (1959c) point out.

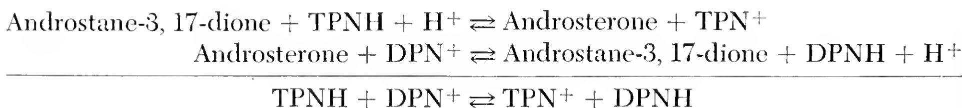
It is noteworthy that the temperature coefficients for the reduction of different pyridine nucleotides by estradiol-17β vary widely with the purified placental enzyme. Large differences in the *relative* as well as the absolute rates of various dehydrogenase and transhy-

drogenase activities are found if the reactions are studied at different temperatures (Talalay and Williams-Ashman, 1959).

Model Reactions with Hepatic and Bacterial Hydroxysteroid Dehydrogenases

It would apear at first sight that hydrogen transfer from one form of pyridine nucleotide to another should be catalyzed by any dehydrogenase which reacts with the requisite nucleotides in the presence of its substrate. However, transhydrogenation between TPNH and DPN is not catalyzed by a β-hydroxyacyl-CoA dehydrogenase which is specific for both DPN and TPN (Stern, 1958). Moreover, glutamic (cf. Colowick, Kaplan, Neufeld, and Ciotti, 1952) and lactic dehydrogenases do not promote the TPNH-DPN exchange unless the concentration of enzyme, substrate, and pyridine nucleotide is extraordinarily high (Holzer and Schneider, 1958). The liver of the guinea pig contains two testosterone dehydrogenases (Kochakian, 1959). One of these enzymes is specific for DPN and is bound to cytoplasmic particles, while the other is a soluble dehydrogenase which reacts only with TPN. According to Villee, Hagerman, and Joel (1960), a mixture of these two testosterone dehydrogenases does not catalyze the transfer of hydrogen from TPNH to DPN in the presence of testosterone. These authors also state that the TPNH-DPN transhydrogenase reaction is not catalyzed by a mixture of their DPN- and TPN-specific placental estradiol dehydrogenases upon the addition of catalytic quantities of estradiol-17β.

However, steroid-dependent hydrogen transfers between pyridine nucleotides have been shown unequivocally with a number of hydroxysteroid dehydrogenases. Hurlock and Talalay (1958) found that the soluble 3α-hydroxysteroid dehydrogenase of rat liver catalyzes an androsterone-dependent TPNH-DPN exchange according to the following equations:



$$\text{Androstane-3, 17-dione} + \text{TPNH} + \text{H}^+ \rightleftarrows \text{Androsterone} + \text{TPN}^+$$
$$\text{Androsterone} + \text{DPN}^+ \rightleftarrows \text{Androstane-3, 17-dione} + \text{DPNH} + \text{H}^+$$
$$\overline{\text{TPNH} + \text{DPN}^+ \rightleftarrows \text{TPN}^+ + \text{DPNH}}$$

This was confirmed by Koide, Chen, and Freeman (1960). This 3α-hydroxysteroid dehydrogenase also catalyzes an androsterone-

dependent transfer of hydrogen from DPNH to acetylpyridineDPN. The latter transhydrogenation is inhibited by low concentrations of TPN as is the case with the placental enzyme. Hydrogen transfer from TPNH to DPN or to acetylpyridineDPN by the hepatic soluble 3α-hydroxysteroid dehydrogenase can be demonstrated only if the concentration of TPNH is kept very low in comparison with the concentration of the acceptor nucleotides. This accounts for the fact that Stein and Kaplan (1959) were unable to demonstrate more than negligible rates of hydrogen transfer from *high* concentrations of TPNH to acetylpyridineDPN when soluble liver extracts were incubated with androsterone.

The 3α- and the (3- and 17-)β-hydroxysteroid dehydrogenases of *Pseudomonas testosteroni* (Marcus and Talalay, 1956) are two enzymes of this class which have been isolated in a high state of purity (cf. Talalay, 1957a, 1957b). Neither of these enzymes reacts with TPN, but Talalay and Adams have demonstrated that they reduce a number of pyridine nucleotide analogues in the presence of their steroid substrates (cf. Talalay and Williams-Ashman, 1960). They also promote the transfer of hydrogen from DPNH to certain analogues upon the addition of catalytic levels of appropriate steroids. Thus, Talalay and Adams found that the bacterial 3α-hydroxysteroid dehydrogenase catalyzes an androsterone-dependent transhydrogenation between DPNH and the acetylpyridine, pyridinealdehyde, and thionicotinamide analogues of DPN. The bacterial (3- and 17-)β-hydroxysteroid dehydrogenase catalyzes the reduction of pyridinealdehydeDPN by DPNH in the presence of catalytic levels of 4-androstene-3, 17-dione. In these model transhydrogenations with bacterial hydroxysteroid dehydrogenases, the rates of hydrogen transfer were most sensitive to the relative concentrations of donor and acceptor nucleotides in the reaction mixture.

It would appear that the high affinity of hydroxysteroid dehydrogenases for steroids and the favorable equilibrium constants for the oxidoreduction of steroids by pyridine nucleotides are two factors which determine the peculiar efficiency with which hydroxysteroid dehydrogenases catalyze transhydrogenations between pyridine nucleotides in the presence of very low levels of steroids (Talalay and Williams-Ashman, 1958, 1960).

Biological Implications

The TPNH-DPN transhydrogenase reaction catalyzed by the placental enzyme is mediated by concentrations of estradiol-17β as low as 10^{-9} M and by even lower levels of equilin and equilenin (Hollander, Nolan, and Hollander, 1958). This concentration of estradiol-17β may be regarded as "physiological" in the light of recent experiments of Jensen and Jacobson (1960). These workers showed that following the administration of 0.1 μg of estradiol-17β-6, 7t to immature rats, this estrogen is concentrated in the uterus and vagina. Maximal incorporation was observed from 2 to 6 hours after injection of the tritiated estrogen, at which time the concentration of estradiol-17β in the uterus (assuming equal distribution between the tissue and its intra- and extracellular water) was of the order of 10^{-8} M. Thus, activation of transhydrogenase reactions catalyzed by the placental enzyme is demonstrable with concentrations of estradiol-17β which fall within the range of those at which the hormone promotes the growth of the rodent uterus. It is of interest that Jensen and Jacobson (1960) found that from 2 to 6 hours after its administration, most of the tritiated estradiol-17β was present in the uterus in a free, ether-extractable form. Very little protein-bound, or water-soluble, radioactivity could be detected. Jensen and Jacobson (1960) also showed that, under these experimental conditions, the level of radioactive estrone in the uterus was less than 5 per cent of that of estradiol-17β.

The well-established division of metabolic labor between DPN and TPN (cf. Klingenberg and Bücher, 1960) has led to many suggestions that enzymes which catalyze the transfer of hydrogen between TPN and DPN may be of central importance in the regulation of cell metabolism. The brilliant studies of Colowick and Kaplan have shown that *Pseudomonas fluorescens* (Colowick, Kaplan, Neufeld, and Ciotti, 1952) and the mitochondria of some animal tissues (Kaplan, Colowick, and Neufeld, 1953; cf. Ball and Cooper, 1957; Humphrey, 1957) contain pyridine nucleotide transhydrogenases which catalyze the direct transfer of hydrogen between TPNH and DPN. These transhydrogenases do not require a low-molecular weight cofactor, and their action almost certainly does not involve an intermediary hydrogen carrier. The transhydrogenases of animal tissues are firmly bound to cytoplasmic particles (cf. Stein, Kaplan, and Ciotti, 1959). There is evidence that the mitochondrial pyri-

dine nucleotide transhydrogenase facilitates the capture of energy from the oxidation of TPNH by promoting the transfer of hydrogen to DPN and thus permitting the synthesis of adenosine triphosphate (Kaplan, Swartz, Frech, and Ciotti, 1956; Vignais and Vignais, 1957). But whether estradiol-dependent transhydrogenations of the type catalyzed by the placental enzyme are of regulatory significance (Talalay and Williams-Ashman, 1958) is problematical. Villee, Hagerman, and Joel (1960) have shown that the direct addition of estradiol-17β to placental slices increases the incorporation of radioactive acetate and glycine into protein, lipide, and the adenine and guanine of nucleic acids. Hosoya, Hagerman, and Villee (1960) observed a direct stimulation of fatty acid synthesis by estradiol-17β in a cell-free placental extract. However, insufficient evidence is available to relate these interesting effects of estradiol-17β *in vitro* to the stimulatory influence of this estrogen upon the transfer of hydrogen from TPNH to DPN. In placenta, the rates of this transhydrogenation catalyzed by the soluble, estradiol-stimulable enzyme are considerably slower than those catalyzed by the mitochondrial transhydrogenase, which is insensitive to estradiol-17β (Villee, Hagerman, and Joel, 1960).

A major objection to the hypothesis that transhydrogenations catalyzed by the soluble placental enzyme are related to the mechanism of estrogen action is the fact that this enzyme is not stimulated by many synthetic, non-steroidal estrogens (Villee, 1957; Talalay and Williams-Ashman, 1958). Thus, substances such as diethylstilbestrol, hexestrol, substituted triphenylethylenes, etc., do not mediate the TPNH-DPN reaction, and in substrate amounts they do not oxidize or reduce pyridine nucleotides, in the presence of the placental enzyme. This is not altogether surprising, as these compounds are devoid of either secondary alcohol or ketone groups and cannot undergo oxidoreduction in the same way as estradiol-17β. Like many other phenols, both natural and artificial *phenolic* estrogens may promote hydrogen transport in catalytic concentrations in a number of oxidations catalyzed by phenolases and peroxidases (Williams-Ashman, Cassman, and Klavins, 1959; Klebanoff, 1959; Temple, Hollander, Hollander, and Stephens, 1960; Talalay and Williams-Ashman, 1960). But only those estrogens possessing at least one free hydroxyl group transport hydrogen in these model reactions, and the ability of various phenols to stimulate these metalloprotein catalyzed oxidations bears no relationship to their estrogenic activity.

Furthermore, these reactions are not catalyzed by the purified placental enzyme. There is no evidence that synthetic estrogens such as hexestrol and diethylstilbestrol are transformed metabolically into steroid-like substances (cf. Talalay and Williams-Ashman, 1960), and it would seem improbable that the biochemical mechanism(s) responsible for the biological action of synthetic non-steroidal estrogens is completely different from that involved in the action of natural hormones such as estradiol-17β.

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7

Hormonal Regulation of Plant Cell Growth

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Regulation of the activities of plants by hormones was first discovered in the growth responses, or tropisms, of plant shoots to gravity and illumination. The growth-promoting hormone which is involved in these responses is called auxin. The natural auxin which has been detected most widely is 3-indoleacetic acid; many synthetic (not naturally occurring) compounds also show auxin activity. Auxins have been found to influence a wide variety of physiological processes in plants, and they are now believed to play a role in regulating many aspects of plant development and function, other than cell enlargement in young stems or stemlike organs, although promotion of this latter kind of growth is still considered one of the most diagnostic actions of an auxin. More recently other types of growth substances, which probably exercise a hormonal influence, have been found in plants, notably the gibberellins and kinins.

Comprehensive treatments of the various aspects of plant growth and its hormonal regulation have recently been given by Thimann (1960) and Audus (1959). Audus has brought together the many theories about the "primary action" of auxin, which attempt to explain how it promotes cell enlargement. This subject has been reviewed recently also by Galston and Purves (1960). They point out that the "primary" action, by which is meant the way in which the hormone molecule itself interacts directly with a process in the plant, is not necessarily exerted on cell growth, something which

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workers have tended to assume probably because promotion of cell enlargement is an action so characteristic of auxins. Galston and Purves rightly feel that the primary action has so far eluded certain detection. The present discussion is concerned with the nevertheless important question, what is the mechanism by which a plant cell grows, and how does auxin cause it to be promoted, whether the action of auxin be of a "primary" or "secondary" character.

To introduce this problem we shall first discuss some aspects of present thinking on the mechanism of plant cell growth, though we cannot hope to survey the bulk of literature which lies behind the various views which prevail. Our interest will be restricted to the kind of growth by cell enlargement which auxin promotes strongly, that is in stems and stemlike tissues or organs such as grass coleoptiles, not roots (nor leaves). Most cells in such situations grow either throughout their surface area, or if "elongating" in one direction, then throughout their length, rather than growing at a local area at the tip as do root hairs, pollen tubes, and several other types of plant cells. Sections of young stems, coleoptiles, and certain other similarly behaving tissues will grow quite considerably if excised from the plant and placed in a suitable simple medium containing auxin; most ideas about the mechanism of plant cell growth are based on experiments with such material.

Uptake of Water

Unlike typical growth in animals or microorganisms, plant growth does not consist primarily of protein synthesis. While cell enlargement in the intact plant is normally accompanied by vigorous synthesis of protein, this synthesis commonly is far from sufficient to keep pace with the volume increase of the cell. Excised tissues may grow, furthermore, with relatively minor synthesis of protein (see discussion by Thimann and Loos, 1957). The predominant material which is added to the plant cell is simply water, which enters and expands the cell's vacuole and makes the cell larger. The prominence of water uptake in the growth of plant cells led to a tendency, in the past decade, to expect that water uptake would be the process which auxin affects.

Plant cells have, of course, an inherent osmotic ability to absorb water, due to their osmotic potential (Π), which as the cell takes up water tends to be balanced by the development of turgor pres-

sure (P) against the elastically stretched, largely polysaccharide cell wall. The following effects on water uptake have been suggested as possible explanations for the promotive action of auxin on growth: (a) causing the Π of the cells to increase; (b) increasing the osmotic permeability; and (c) inducing active transport of water, resulting in more water uptake than would occur osmotically. The first of these (a) has been excluded pretty conclusively in the case of growing potato tissue and oat coleoptile sections, for Π may fall during growth.

A positive effect of auxin on osmotic permeability has been reported, but this could cause a significant effect on growth rate only if the growing cells were far from being in osmotic equilibrium (i.e., in a dilute medium $P << \Pi$), so that rate of osmotic water uptake is "limiting" the rate of growth. On the other hand, if active transport of water were involved, it would most likely act by increasing P above the osmotic equilibrium value (i.e., in a dilute medium $P > \Pi$) and thus "stretching" the cell.

In the last few years it has become widely accepted that growing plant tissues are so permeable to water that they must remain practically at osmotic equilibrium regardless of whether the continuously occurring water uptake is entirely osmotic or involves some tendency toward active transport, so that neither significant excess nor deficiency of P could prevail. Unfortunately there is no satisfactory method for determining P directly. Brauner and Hasman (1952) and Ordin, Applewhite, and Bonner (1956), using the conventional indirect method of determining $(\Pi - P)$ for plant tissues, reported, respectively, that growing potato tissue and oat coleoptile sections were practically in osmotic equilibrium. The conventional method would not, however, detect an "actively maintained" P with any certainty. And there is considerable doubt as to its even approximate validity for a rapidly growing tissue such as the oat coleoptile, in which possible growth during the interval of measurement is large compared with elastic changes in size, due to changes in P, which the method depends upon detecting. Convincing demonstration that active transport of water occurs in plants has not yet been obtained, and it does appear likely that slow-growing tissues such as potato are nearly at osmotic equilibrium. Levitt (1953) calculated that this should be so, but he considered only water uptake into a single cell from the medium rather than through the tissue and employed an osmotic permeability value determined for tissue

other than potato. Measurement of diffusion of labeled water (Thimann and Samuel, 1955; Ordin and Bonner, 1956) suggesed too that osmotic equilibrium should practically prevail, although this consequence was not shown explicitly, and it should be noted that diffusion of water is not identical with the process of osmosis by which it is assumed the cells are absorbing water.

In our own experiments of the type to be described below, we find that the rate of expansion of oat coleoptile sections in auxin is of the same magnitude as the rate of osmotic swelling or shrinking immediately after the section is transferred between two growth-inhibitory (but non-plasmolyzing) concentrations of mannitol 0.1 *M* apart (differing by 2.4 atm of osmotic potential). This would suggest that the growing cells could not be close to osmotic equilibrium. There is still considerable uncertainty about the actual value of P in rapidly growing cells, despite the importance of this quantity in the consideration of possible mechanisms of growth.

Regardless of whether effects on rate of water uptake are involved in auxin effects on growth rate, some explanation is still needed of the fact that water uptake into growing cells is accompanied by irreversible enlargement of their cell walls, whereas in non-growing cells the walls merely stretch elastically until an equilibrium value of P has built up, and absorption ceases. This feature of growth is of primary interest at the present time, and it would be expected to be the controlling process if, as many assume, the P of growing cells never departs far from the osmotic equilibrium value as they take up water.

Growth of the Cell Wall

The idea that expansion of the cell wall is the controlling process in growth and in the action of auxin on it goes back to the early auxin literature and was made prominent especially by Heyn (1931, 1933). He obtained evidence that growth is a passive "plastic extension" of the cell wall under the force which turgor exerts on it, and that auxin causes increase in the "plasticity," or rate of irreversible stretching under unit force. That growth seems to depend upon turgor has long been known, for a wilted plant does not grow. The inhibitory effect of mannitol on growth of stem and coleoptile sections, at osmotic concentrations approaching that of the cells, is widely accepted as affording a measure of the dependence of growth

rate on turgor pressure. That the inhibition is nearly linear with mannitol concentration has tended to suggest that growth rate is proportional to, or at least increases with, increasing turgor pressure, as a passive stretching might be expected to, although as previously indicated the actual turgor pressures are not known with any certainty.

An interesting feature of the cell wall which seems to be connected with the stresses and strains it is subject to, is the orientation of its structural elements. The cell wall contains what looks like a "meshwork" of submicroscopic "microfibrils" of α-cellulose (in higher plants largely β-1,4-D-glucosan), which appear to be embedded in an amorphous "matrix" of non-cellulosic polysaccharides. Because of the high tensile strength of α-cellulose, and other considerations, the tendency has been to ascribe to the microfibrils a determining role in the mechanical properties of the cell wall; sometimes they are likened to the reinforcing rods of concrete.

The lateral walls of typical more or less cylindrical cells which are elongating in the direction of the cylinder axis show, as a general rule (with exceptions), microfibrils oriented predominantly in the transverse direction with respect to the axis of growth. It is easy to see how this reinforcing could result in the observed longitudinal enlargement of the cell, even though the forces imposed by turgor tend to stretch it in all directions. Electron microscopists have also noticed that elongating cell walls commonly have less well oriented or even longitudinally oriented (parallel with axis of growth) microfibrils at the outer surface. This has been explained (see Roelofsen, 1959) by assuming that during growth the cell adds microfibrils in close to transverse orientation at the inner surface, next to the protoplast, while the already existing cell wall simply stretches, passively bringing about a gradual reorientation of the older, outer microfibrils to the longitudinal direction as the cell wall becomes stretched more and more in that direction.

This brings up the old and persistent question, as to whether synthesis of the cell wall, which during growth goes on usually at a rate comparable with the rate of extension of the cell, occurs at the inner surface of the cell wall ("apposition") or within and throughout the thickness of the cell wall ("intussusception"). Much of the literature is pervaded by the belief that the growing (primary) wall is synthesized by intussusception. An evident possibility for the mechanism of cell growth is that internal synthesis of the cell

wall (intussusception) expands its surface area, and water uptake naturally follows osmotically. Such a mechanism would appear to explain much better the strong dependence of growth upon respiratory metabolism, which has been widely studied, than would the idea of passive plastic stretching. Auxin treatment does promote synthesis of the cell wall, although to obtain large effects in stem or coleoptile sections, it appears to be necessary to extend experiments over many hours, whereas the effect of auxin on the growth rate develops well within one hour. Several different views hold that the promotive effect of auxin on cell wall synthesis is a secondary consequence of its effect on growth.

There are some cases in which cell wall synthesis is much less than proportional to cell enlargement, so that the cell wall appears to become thinner during growth. One example is the oat coleoptile section growing in auxin plus sugar, which was reported by Bayley and Setterfield (1957) and has been confirmed repeatedly by the author. Furthermore, when coleoptile sections grow in auxin solution without added sugar, synthesis of new cell wall material is usually negligible, even though 50 per cent elongation may occur; this was noted by Bennet-Clark (1956) and has been found in many of our own experiments. And long ago Bonner (1934) reported that coleoptile sections grow at 2° C with negligible synthesis of cell wall. It seems necessary to conclude that in this and other cases growth is not caused by gross synthesis of the cell wall, but instead results from an action on the existing cell wall, and may or may not be accompanied by further synthesis. The rate relationships between growth and synthesis appear to fit better an apposition rather than an intussusception process, which would be expected to be tied closely to the rate of growth. The observations do not, however, exclude the possibility that a minor amount of cell wall synthesis may be critical to growth.

About the actual site of wall synthesis during growth, there is as yet very little evidence. These walls are usually less than 1 μ thick, which cannot be resolved by present radioautographic methods. Setterfield and Bayley (1958) reported radioautographs of cross-sections of the 4 μ thick outer wall of the epidermis of oat coleoptile sections which had been grown in tritium-labeled sucrose, which they felt indicated incorporation throughout the thickness of the wall. Unfortunately in the critical experiment the section had more than doubled its original size (and according to our data might

nearly have doubled its total cell wall material) so that, had all synthesis occurred at the inner surface of the cell wall, label should be spread throughout the inner half of the thickness of the wall. This is exactly the impression given by the clearest part of Fig. 4, of Setterfield and Bayley (1958), representing the cellulose residue. The unextracted preparation of their Fig. 2 does seem to show label throughout the wall, which might indicate that non-cellulosic materials are introduced throughout its thickness.

By an elegant self-absorption method Green (1958) studied the site of incorporation of tritium into the cell walls, about 1 μ thick, of single growing cells of the alga *Nitella*, and obtained results consistent with the assumption that all new cell wall material was being added at the inner surface. He subsequenty (1960) showed that the orientation of the microfibrils changes, as one traverses the thickness of this cell wall, in a manner which agrees qualitatively with what would be expected from the reorientation hypothesis of Roelofsen, mentioned above. Green (1960) felt that these results indicated that the cell wall is an "inert accumulation," which simply yields passively to turgor forces rather than participating actively in growth. It is hard to reconcile this with the view, arrived at above, that the mechanism of growth must involve some action on the existing cell wall, and it seems most unlikely that growth even in *Nitella* could be nothing but a passive stretching of inert material, independent of any regulation by the cell. Conceivably growth could involve some action on the cell wall materials which would not result in incorporation of tritium, but it also should be noted that the self-absorption method was probably not precise enough to detect incorporation throughout the wall of a small proportion, say a few per cent, of the total new material synthesized. It is interesting that Roelofsen (1959) found it helpful to postulate an introduction of non-cellulosic matrix materials throughout or across the thickness of the cell wall, in order to explain certain observations, even though he held that the microfibrils behave passively and are added only or mostly at the inner surface.

The author has conducted rather extensive, as yet unpublished experiments, with oat coleoptile sections, comparing effects on growth with effects on cell wall synthesis, under various treatments. While the quantitative relation between the two processes is such as to suggest, as noted earlier, that the gross aspects of wall synthesis are not the basis of growth, yet the parallelism between effects on growth

and on synthesis in different types of experiments is truly striking. We are not yet prepared to reject the possibility that a small amount of cell wall synthesis, possibly not of a qualitatively different nature from the bulk apposition synthesis, may go on within the already formed cell wall and be involved in the mechanism of growth.

The fact that the predominant direction of orientation of the microfibrils is the direction in which the cell grows least has long suggested that the properties which determine cell enlargement should be sought in the non-cellulosic matrix of the cell wall, the hemicelluloses and pectins. Van Overbeek stated this view clearly in 1939. Roelofsen (1959) insists instead that the "connections" between cellulose microfibrils must play the critical role, but this idea would not in actuality be a different one if the microfibrils are, as seems probable, separated from one another by these other substances. Although much has been written in the belief that some kind of action on the microfibrillar system itself is the basis of growth, this view rests almost entirely on the (possibly equivocal) interpretation of electron micrographs and is not supported by direct evidence. Some reports have, however, indicated the occurrence of a chemical action on the matrix which is affected by auxin; these we shall now discuss.

The Pectin Hypothesis

The view is generally prevalent in the botanical literature that primary cell walls (the walls of growing cells) contain a large proportion of pectin (polygalacturonic acid partially esterified with methanol). Kerr (1951) has often been cited as having indicated that pectin makes up the "continuous phase" or matrix of the cell wall, although this was an assumption on his part, and he was aware that the "pectin" which is extracted from cell walls is actually a mixture containing pentosans and hexosans as well as pectin itself. Since that time, however, authors regularly refer to the non-fibrillar part of the cell wall in terms such as the "pectin matrix," and consider that this matrix is responsible for the "plasticity" of growing cell walls.

Ordin, Cleland, and Bonner (1955, 1957) and Bennet-Clark (1956) suggested that the inhibitory effect of Ca^{++} on growth of stem and coleoptile sections could be explained by the formation of Ca^{++} double salts involving two anhydrogalacturonic acid carboxyl groups,

which could cross-link galacturonic acid residues of different pectin molecules and might thus be expected to stiffen the cell wall, inhibiting growth. In pectin some of the carboxyl groups are esterified with methanol and hence cannot form salts or cross-links. These authors envisioned that the action of auxin could be to promote the metabolic "methylation" of pectin carboxyl groups and, by reducing the number of possible cross-links, increase the "plasticity" of the cell wall.

Ordin, Cleland, and Bonner (1955, 1957) reported that auxin did promote the incorporation of C^{14} from methyl-labeled methionine (a suitable methyl donor) into ester groups of the hot water soluble pectin of oat coleoptile cell walls (although it did not promote the much larger incorporation into insoluble "protopectin"). This effect has been found also in corn coleoptiles—but not in first internodes of corn seedlings, the growth of which is promoted by auxin (Cleland, 1960). These workers have felt that this effect of auxin could be directly responsible for its promotion of growth. Adamson and Adamson (1958), who gave a succinct description of the pectin hypothesis, found support for it in effects on cell wall "plasticity" caused by auxin at 3° C and in the absence of O_2, which they felt were consistent with the idea that auxin increases the methyl ester content of cell wall pectin by inhibiting the hydrolysis of methyl ester groups by pectin methylesterase.

The pectin cross-linkage hypothesis is about the only specific chemical explanation which has been advanced as to how auxin can affect the "plasticity" of the cell wall, and it attractively explains why "plasticity," hence growth, should depend upon metabolism.

Jansen *et al.* (1960) analyzed oat coleoptile cell walls in respect to uronic acid residues and methyl ester groups. They found no change in the absolute amounts of either of these in the presence of auxin (although auxin promotion of incorporation of labeled methyl into pectin ester was confirmed). Thus it seems necessary to give up the idea that an increase in plasticity could result from a decrease in the number of pectin double salt cross-links in the presence of auxin; Bennet-Clark has also reached this conclusion (see Galston and Purves, 1960). It was thought possible that increased *turnover*—increased rate of formation and removal of methyl ester groups—could be occurring. If this were to cause increased "plasticity," what must be contemplated is that auxin increases the rate of wall stretching by increasing the rate of bond breaking, with-

out changing the total number of bonds. This would mean that stretching is controlled by the breaking of bonds by a chemical process (methylation) rather than by forcible physical deformation ("pulling" bonds apart); the former process could not be considered a passive plastic stretching in the sense that the latter is. If the total number of cross-links were, as indicated, no different in the presence and absence of auxin, the physical "plasticity" of the cell wall, if it does have such a property, should be no different. The present author (Ray, 1960) has called this type of possible growth mechanism a "molecular mosaic," because it is somewhat analogous, at the molecular level, to the "mosaic growth" proposed by Frey-Wyssling and Stecher (1951), which was supposed to occur stepwise by loosening in localized areas of the cell wall, while the wall structure as a whole remained rigid. (The "mosaic growth" proposal itself has not attracted wide support, since the electron micrographs on which it was based can be interpreted quite differently.)

The pectin cross-linkage hypothesis thus calls one's attention to two biophysically different types of possible growth mechanism which there has been some tendency to confuse under the notion of "plasticity." The question of which type of mechanism, if either, is actually involved in growth appears to be a fundamental and general one irrespective of whether the pectin cross-linkage hypothesis itself is correct, and we shall return to it subsequently.

The pectin hypothesis seems to be encountering further difficulties. Jansen *et al.* (1960) reported that the hot-water-soluble pectin is almost fully esterified, whereas the residual insoluble polyuronides ("protopectin"), comprising more than 80 per cent of the total cell wall uronic acid, are only about 30 per cent esterified. So it appears that the number of possible double salt cross-links in that fraction in which IAA does promote methylation (the water-soluble) is a very minor proportion, less than 3 per cent, of the total, and it is rather hard to see how these few cross-links could be the critical ones to cell wall rigidity. Also, from the fact that the hot-water-soluble material is the most easily removed, hence least strongly bonded cell wall constituent, it would seem unlikey that bonding involving the hot-water-soluble fraction could be of critical importance in the strength of the cell wall. It seems entirely possible that the inhibition of growth by Ca^{++} is caused by an "artificial" rigidification of the cell wall through setting up double salt cross-links which, in the

absence of inhibitory concentrations of Ca^{++}, exist to only a minor extent and are not of critical importance to wall expansion.

Albersheim and Bonner (1959) recently suggested that the promotive effect of auxin on methyl incorporation does not involve methylation of existing polyuronides, but instead synthesis of new pectin. If true, this would seem to eliminate entirely the hypothesis that action on existing double salt cross-links is the cause of promotion of growth by auxin. However, besides the effect on methyl incorporation, further indication that some aspect of pectin metabolism may be affected by auxin is given by the reports of auxin effects on activity of pectin methylesterase (see reviews cited in the introduction).

Promotion of pectin *synthesis* (Albersheim and Bonner, 1959) might soften or plasticize the cell wall, since pectin is a rather hydrated and "soft" material, as has often been mentioned. With growing tobacco and potato tissue it has been reported that auxin causes larger promotions of pectin synthesis than of cellulose synthesis (Wilson and Skoog, 1954; Carlier and Buffel, 1955). The increase in pectin content in oat coleoptile sections treated with auxin was very slight compared to those noted in these other tissues. But it is conceivable that, of the various cell wall polysaccharide fractions, the "softening" effect of the hot-water-soluble fraction would be largest because, as mentioned above, it is the least strongly bonded material. Introduction of these molecules might weaken or break linkages between other components. This would make the meaning of an auxin effect specifically on it more understandable than would the supposition that it bears the critical bonding forces. Since it is a minor constituent, this would fit in with the observation that growth can occur without substantial wall synthesis. The manner in which it acts could, of course, involve either a molecular mosaic or an increase in passive plasticity. This reasoning could also apply to the large promotion by auxin of synthesis of "cold water soluble pectin" found by Albersheim and Bonner (1959), who suggested that this material might be a very weakly bonded cell wall constituent.

Bishop, Bayley, and Setterfield (1958) reported analyses of oat coleoptiles from which they concluded that the cell walls contain less than 1 per cent polyuronides or pectin. They felt that this was such a minor proportion that to explain growth, attention should be

redirected to the hemicelluloses, which comprise the bulk of the cell wall matrix. Our own analyses (Ray, 1958) also showed that the major part of both the hot-water-soluble "pectin" and the hot-dilute-mineral-acid-soluble "protopectin" fractions is pentosans and hexosans, that is hemicelluloses, rather than polyuronides, so it no longer seems reasonable to consider that these primary walls have a "pectin matrix." However, our determinations have indicated a total polyuronide content of the order of 5 per cent of the cell wall, in good agreement with the figures of Jansen *et al.* (1960). Neither the carbazole nor the CO_2-yield methods of determining uronides, which were employed, can be corrected accurately for the effects of simultaneously present sugars, especially when uronides constitute a small percentage of the material, so these data overestimate the amount of uronides. But they certainly appear to be closer to the actual amounts than the figure given by Bishop *et al.* (1958), because we have been able to recover more than 50 per cent of the amounts indicated in crude extracts, as actual uronic acids after acid hydrolysis and separation of uronic acids from sugars by anion exchange resin chromatography. The conclusion of Bishop *et al.* (1958) concerning polyuronide content seems to be erroneous, because it was based on a method (removal of sulfuric acid as $BaSO_4$) which in our experience causes large losses of uronic acids when these are present in small amount.

We have recently found that the uronic acid of oat coleoptile cell walls consists of both galacturonic and glucuronic acids, with the amount of glucuronic being equal to or greater than the amount of galacturonic in all the fractions, apparently even the hot water soluble. The composition of these polysaccharides is, therefore, that of "polyuronide hemicelluloses" rather than pectin, and we wonder to what extent any of the work discussed has really dealt with pectin.[2]

It might finally be noted that no direct evidence establishes that any of the "pectin" effects observed actually occur within the cell wall rather than in the middle lamella, where direct observation of Ruthenium Red stained sections suggests that most of the uronides

[2] Since this was written, we have found that the uronic acid which is consistently found at the same R_f as glucuronic acid, in hydrolysates of oat coleoptile cell wall polysaccharides, is *not* glucuronic acid. It appears to give galacturonic acid and xylose on further hydrolysis, and is probably an oligosaccharide derived from incomplete hydrolysis of a polyuronide hemicellulose containing both xylose and uronic acid residues. Its abundance indicates that a considerable proportion of the cell wall uronic acid may be in polyuronide hemicelluloses as opposed to pectin.

are concentrated in the case of the oat coleoptile, except that they are found throughout the outer wall of the epidermis (cf. Heyn, 1933). If the effect of IAA (indoleacetic acid) noticed were really on the middle lamella, it would seem unlikely to be able to account for enlargement of the much more rigid cell wall proper. Northcote, Goulding, and Horne (1958) did not detect any uronic acids in hydrolysates of cell walls of *Chlorella,* where there is no middle lamella.

It would seem desirable not only to investigate what part hemicelluloses might play in expansion of the cell wall but also to consider if cell wall protein might have a structural or enzymatic role. Primary cell wall preparations have generally contained some protein, but the question of whether this was adhering cytoplasm tended to obscure its significance. Lamport and Northcote (1960) reported that the protein of sycamore tissue culture cell wall preparations, unlike the bulk protein of the cells, contains a large proportion of hydroxyproline (but cf. Pollard and Steward, 1959). This raises the possibility that the primary wall may contain a distinct protein and is also interesting in view of the structural role of hydroxyproline-containing proteins (collagen) in animals. Kessler and Nickerson (1959) obtained several polysaccharide-protein "complexes" from yeast cell walls; these workers (Nickerson and Falcone, 1959) are advocating the idea that disulfide cross-links between such proteins may be structurally important and that splitting of the cross-links by reduction to —SH could cause the cell wall expansion which is involved in cell multiplication. This might be a good example of a "molecular mosaic" growth mechanism.

Cell Wall Plasticity

In the previous section we indicated some doubt that the ability of the cell wall to grow under turgor is with certainty a plastic stretching, as opposed to some biophysically distinct process of superficially similar behavior in experiments. Long before the recent revival of interest in wall plasticity as an explanation of growth, Heyn's conception that growth was nothing but a physical stretching lost favor because of the demonstrated dependence of growth upon metabolism. Some workers who attempt to measure "plasticity" now qualify the term, indicating that they think this property is not to be considered simply a passive physical deformation; for

example Cleland (1959) simply regards plasticity as the "biological process of wall deformation." Such a usage tends to obscure the interesting and fundamental question as to the immediate mechanism of plant cell growth, and the possibility that growth may in fact be a passive stretching, but involving a property of the cell wall (plasticity) which is maintained by metabolism. The language of most authors who discuss plasticity still suggests that they are indeed thinking in terms of such a passive process as the immediate mechanism of growth. Recently, for example, Jansen *et al.* (1960) stated, "it is established that auxin . . . increases the rate of cell elongation in *Avena* coleoptile sections by causing the cell walls to become more stretchable, more plastic."

Three principal methods have been employed to measure the "plasticity" of cell walls of growing tissues. The first, introduced by Heyn (1931), is to hold sections by one end in a horizontal position and to apply a weight at the other end. The angle of bending which remains after the weight is removed is considered to result from plastic stretching and to measure plasticity. Heyn and subsequent workers found that auxin increases the angle of bending. Unfortunately the method has never been made quantitative by analyzing the stresses to which the tissue is subjected in the experiment. Although it is generally felt that the measurement is made in the absence of any real growth, Heyn (1931) did record that isolated coleoptiles deprived of water grew appreciably, and we have often observed such coleoptiles to show geotropic curvature, a growth response. It cannot be assured that the "plastic" bending might not simply be a differential growth response to the different stresses on the two sides of the tissue, even if growth were occurring by a mechanism other than plastic stretching.

The second method is to study the "irreversible enlargement" of the tissue under its own turgor pressure, by comparing the lengths of totally plasmolyzed sections before and after a period of expansion in some medium in which the cells possess turgor. At best this is, of course, simply a measure of growth itself and cannot arbitrarily be called plastic stretching unless plastic stretching is defined as growth, as by Cleland (1958, 1959). Cleland and Bonner (1956) and Cleland (1958) found that auxin pretreatment in 0.25 *M* mannitol, in which coleoptile sections did not grow, would cause an increase in irreversible enlargement when the sections were subsequently transferred to water in the absence of O_2. Since in this last

step auxin would not promote if added directly, they regarded this as a "residual effect of auxin on the cell wall," of a plastic nature. This effect has the interesting peculiarity of disappearing if 3×10^{-4} *M* KCN is applied in the last (plastic stretching) step, all the odder because this step is conducted under anaerobic conditions, so the KCN effect presumably could not be simply on cell respiration. It makes one wonder whether the residual effect can in fact be a persistent physical alteration of the cell wall.

The third and most direct approach to plasticity is to apply known forces to plasmolyzed or otherwise inactivated tissues and directly measure their reversible and irreversible deformations. Heyn (1931, 1933) used it but did not explore it extensively except to measure elastic properties. The kind of difficulty encountered in trying to relate such measurements to growth can be illustrated with the results of a careful study by Brauner and Hasman (1952). They cut strips of potato tissue 3×3 mm in cross-section, followed their growth, then plasmolyzed them and subjected them to a tension, and determined the amount by which the tissue remained stretched after release of the tension (plastic stretching). Tissue which had been growing in auxin, was stretched "plastically" 1.7–1.9 per cent by a tension of 20 g acting for 5 minutes. As previously mentioned, Brauner and Hasman established also that growing tissue was practically in osmotic equilibrium with its dilute medium, so the turgor pressure of the growing cells must have been practically equal to their osmotic potential, which was determined to be about 6 atm. It can readily be computed that in the growing tissue a force of more than 500 g must have been acting on the cell walls across a 3×3 mm cross-section; this tissue actually grew 26–27 per cent of its initial weight in 24 hours, or only 0.09 per cent in 5 minutes (which includes all three dimensions, not just the direction of the cross-section considered). Thus, the apparent rate of extension in the stretching experiment was at least 20 times the rate of wall extension during growth, despite the fact that the stretching force was less than one-twentieth of the force acting during growth. It would seem that the property being measured in the stretching experiment could not have been the property which was controlling the growth rate, even had this involved plastic stretching. The stretching treatment may possibly measure the ability of a force to distort the shapes of the cells on which it acts rather than to expand the cell wall "plastically."

Our feeling is, therefore, that existing approaches to "plasticity" have not positively distinguished it from other possible mechanisms of cell growth, and we have attempted to think of means by which a plastic stretching and a molecular mosaic mechanism could be distinguished. Some experiments in this direction are described in the next section.

Experiments on the Immediate Mechanism of Growth

The essential distinction between what we have called a molecular mosaic growth mechanism and a plastic stretching can be thought of in the following terms. Plastic stretching is a forcible physical deformation depending upon a viscosity-like property (plasticity) which may be maintained by metabolism but can be said to have a particular (passive) value at any moment, whereas in a molecular mosaic it is some activity of the cell (most easily visualized as a chemical reaction), rather than physical stress, which breaks the critical or controlling bonding forces within the cell wall and leads to expansion. Either mechanism could require turgor pressure for actual displacement, hence visible growth, to occur. There is, of course, a possible middle ground involving processes of both types. But if one confines one's thinking to the simple possibility that the controlling process in growth has one or the other character, it would seem that one operational distinction between them would be a separation in time between the action of metabolism and the occurrence of growth in the case of plastic stretching, but not in the case of a molecular mosaic. We could speak of the plasticity decaying with some characteristic "half-time" if synthetic metabolism were suddenly suppressed, while growth by molecular mosaic would have to cease immediately. One presumes that the half-time for plasticity would be short in comparison with the normally used intervals of one-half hour or longer in growth measurements.

Albert W. Ruesink and the author have experimented with a method of following accurately the growth of oat coleoptile sections over periods of minutes (usual interval between measurements, one minute). Our original experiments were intended to determine how rapidly the rate of growth changes when one suddenly changes the temperature. We mount a section, about 7 mm long and conveniently hollow, horizontally on a small thermocouple junction with

which we could measure its temperature to 0.1° C. As a precaution we tie the fixed end of the section on securely with fine thread. The junction is mounted rigidly from the floor of a clear Plexiglas trough which is held on the mechanical stage of a compound microscope; the trough is so designed that from a reservoir a continuous gentle stream of solution flows in one side and out the other, at a level just covering the section, and by exchanging pinch clamps on the inlet side hose we can suddenly change from solution of one temperature (or composition) to another, getting the full temperature response within the tissue in about 5 seconds. With green light as dim as possible, we observe continuously the end wall of a particular subepidermal cell at the free end of the section, with a 4 mm objective lens, against an ocular micrometer scale with 100 divisions, each division being equivalent to 1.5 μ. We can read to 0.5 division. In auxin at 23° C the sections normally grow about 6–10 divisions per minute. When the section approaches the end of the ocular scale, we restore it to near zero by suddenly moving the mechanical stage, and immediately reread. The most important requirement for accuracy and clear vision is that the objective be immersed in the growth solution. We shall describe the details of the apparatus and technique fully elsewhere. As auxin we use a solution of 3 mg per liter indoleacetic acid in distilled water.

When the temperature of a rapidly growing section, in auxin, is suddenly lowered from 23° to 5° C, growth immediately practically ceases as far as intervals of time convenient for us to employ are concerned. Since we should like to know whether the rate changes subsequent to the initial effect, we have studied more extensively the result of transferring between 23° C and 12° C, at which growth is much depressed but not prevented. Fig. 7–1 shows such an experiment, with arrows indicating the time of transfer between temperatures. It is observed that there is a slight thermal contraction of the apparatus during the first 20 seconds after the temperature transfer. It is clear that the entire effect of temperature on the growth rate had developed within this interval and is registered in our first measurements made thereafter 15 seconds apart within the first minute.

The effect of this 11° change in temperature is approximately 3.5-fold, which is of the magnitude one might expect if the effect were due entirely to changes in rates of metabolic reactions. We should allow, however, for the possibility that the observed change

in rate is due to temperature dependence of plasticity (viscosity is temperature dependent, but not usually with as large a temperature coefficient as seen here), which would be an instantaneous effect. In this case we should expect the depression of metabolism, due to decrease in temperature, to exert itself as a further decrease in growth rate as the metabolism-dependent plasticity decays to a depressed value, and we should also see a further increase in rate

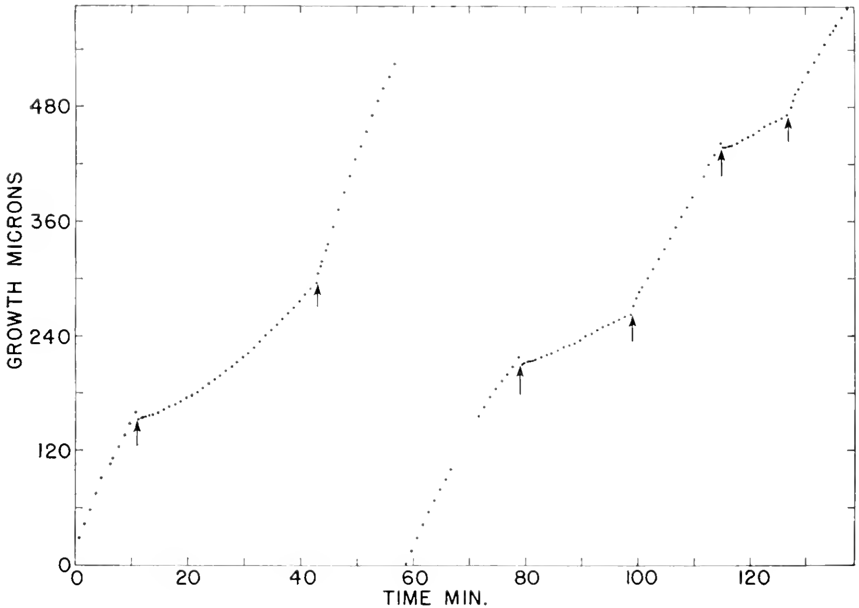

Fig. 7–1. Effect of changing the temperature from 23° to 12° C (*first arrow*) and back to 23° C (*second arrow*) on growth of an oat coleoptile section 9 mm long, in 3 mg per liter indoleacetic acid. The same changes of temperature were repeated at subsequent arrows.

after the initial effect when the temperature is raised. Subsequent to the initial change in rate there was, if any change, only a tendency for the rate to recover somewhat at the lower temperature (for example in the first 12° treatment in Fig. 7–1) or to fall slightly at the higher temperature, which effects we know were not due to corresponding changes in temperature since temperature was followed throughout.

There remains the possibility of a "deepfreeze effect," that the connection between metabolism and plasticity has such a long-time

constant that we simply did not wait long enough to detect it. We decided to study this by investigating the effect of changing the rate of metabolism, at one temperature, by using poisons or depriving the cells of O_2. The speed with which such treatments give effects is likely to be dependent upon diffusion, but at least an indication of the maximum time required for a change in metabolism to show itself in the growth rate might be obtained.

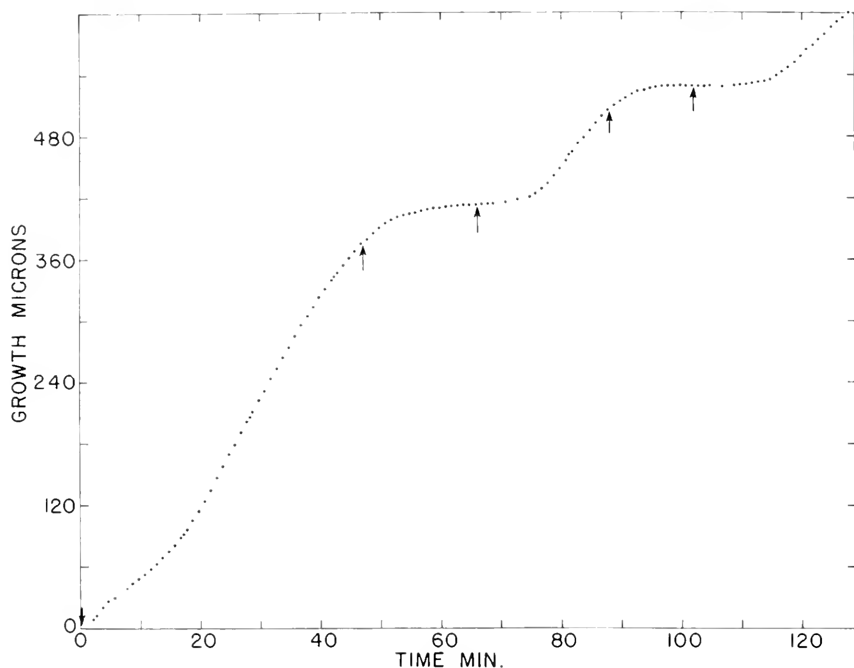

Fig. 7–2. Effect of transfer from 3 mg per liter indoleacetic acid, at 23° C, to the same plus 3×10^{-4} M KCN (*at first upward-pointing arrow*), and back to auxin alone (*second arrow*). These transfers were repeated at the third and fourth arrows. Coleoptile section 8 mm long, transferred from air to auxin solution at beginning of record.

Fig. 7–2 shows the effect of changing from auxin to auxin plus 3×10^{-4} *M* KCN, and then after inhibition was complete, back to auxin alone. A fall in the growth rate was detectable 2 minutes after KCN was added, and the half-time for approach to the final inhibited rate was generally about 5 minutes. Upon return to auxin alone, a characteristic lag of 5–10 minutes always occurred, and the rate then quickly returned to, or nearly to, the previously prevailing

rate; as seen in Fig. 7–2, this cycle could be repeated. The lag may represent the time required to deplete the tissue, by diffusion, to below a practically "saturating" concentration of KCN for the affected system, although we have not studied this extensively. The rapid action of KCN when added indicates that the growth rate can be affected by changes in rates of presumably enzymatic processes within a very few minutes. At 12° C similar results were obtained, except that the half-time for approach to the inhibited rate was slightly longer, about 7 minutes. It would seem that temperature effects on metabolic processes should have showed themselves within the period of 12° treatment in an experiment such as that of Fig. 7–1. Therefore, the change in rate of metabolism or some part of it, caused by changing the temperature, must have already exerted its effect on the growth rate within the first minute. It seems difficult to escape the conclusion that the growth rate follows changes in metabolic processes essentially immediately. If the growth rate were truly controlled by a physical "plasticity," this would seem to have such a short metabolic time constant as to be tantamount to a molecular mosaic mechanism.

Fig. 7–3 shows an experiment testing the response to depriving the coleoptile section of O_2. We mounted the section on the end of a syringe needle of appropriate size, so as to be able to gas it internally with N_2 at the time of changing from an aerated auxin solution to one gassed with prepurified ($>$ 99.996 per cent) N_2; the growth chamber was closed and was flushed all the while with N_2, so that the section had to obtain all its O_2 from the auxin solution. After transfer to O_2-free solution (and brief gassing of the section), there always followed a period of about 10 minutes before any pronounced effect on the growth rate occurred; then it rapidly fell to an inhibited value (complete inhibition did not occur). Upon return to O_2-containing medium, response was very rapid, the original rate being recovered in about 3 minutes. This behavior is the opposite of that with KCN, and it may reflect the fact that depletion and entry kinetics are just the opposite in the two cases. In any event the rapid response on return to O_2 shows clearly how rapidly the growth rate responds to change in the rate of some metabolic process and seems to reinforce the conclusion reached above.

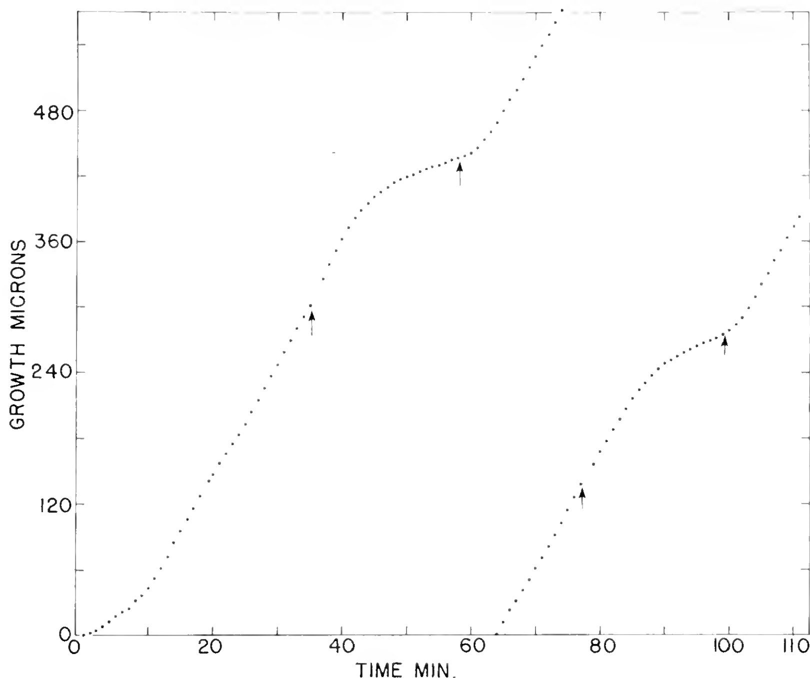

Fig. 7–3. Effect of transfer from aerated auxin solution to auxin solution gassed with prepurified N_2 (*first arrow*), and back to aerated auxin solution (*second arrow*). These transfers repeated at third and fourth arrows. Coleoptile section 9 mm long.

Regulation of the Growth Rate

This technique can be used to study other aspects of the growth process and its control by auxin. One fact which was clearly established was the time required to get a change in growth rate after auxin is added. When transfer is made between water and 3 mg per liter indoleacetic acid, the previously prevailing rate of growth (usually 2–3 divisions per minute) persists for from 10 to 15 minutes, and then a rapid increase in rate occurs, usually reaching a new steady rate, 2–4 times greater than previously, within 4 to 6 minutes.

Another point we have examined is osmotic inhibition of growth. We first follow the growth rate in water or auxin, then transfer stepwise to increasing concentrations of mannitol (or mannitol plus auxin), waiting each time until a new steady rate of growth is established. The response is usually rather rapid (3–6 minutes).

Complete inhibition is usually first obtained in 0.2 *M* mannitol, in agreement with results in the literature. This is true both with and without auxin.

It seems significant that complete inhibition of expansion occurs at a mannitol concentration substantially below that equivalent to the osmotic potential of the cells, and therefore at a positive value of turgor pressure. With each section we continue to higher mannitol concentrations above the totally inhibitory one until we observe plasmolysis in the subepidermal parenchyma cells at the end of the section; we then return to successively lower concentrations and find to within 0.1 or 0.5 *M* the one which first completely reverses plasmolysis, which sets limits to Π. We encountered values below 0.3 *M* only in occasional sections (whose growth was inhibited in 0.1 *M* mannitol). Since non-growing sections are in fact in osmotic equilibrium with the medium (unless they have additional "active" P), it is justifiable to compute P from the difference in Π inside and outside the cells, which indicates that turgor pressures of 2 atm or more still exist in the cells at the point of complete osmotic inhibition of cell enlargement.

Since mannitol inhibits cell wall synthesis (see for example Bayley and Setterfield, 1957), it seemed desirable to check this result with some chemically unrelated substance, to which the cells might be presumed to be at least as impermeable as to mannitol. We have employed Carbowax 4000, a polyethylene glycol. With it also we observe complete inhibition of the growth rate at a concentration definitely below that equivalent to the osmotic potential of the cells, and it may be noted that with either this or mannitol the completely inhibited sections feel turgid to the touch. It seems certain that the statements in the literature referring to treatments of oat coleoptile sections in 0.2 to 0.28 *M* mannitol as "isotonic" or "slightly hypertonic" are an error, possibly terminological, but they convey the impression that to inhibit growth completely it is necessary to reduce turgor pressure to zero, which seems not to be the case with the oat coleoptile section.

With this method one can also follow closely the time course of osmotic swelling and shrinking of the sections when transferred into or between non-plasmolyzing mannitol concentrations. An example of such an osmotic shrinking is shown in the first part of Fig. 7–4. The half-times of these osmotic changes are generally about 5–6 minutes, and we have noticed no striking effect of auxin on them.

As noted in the section on water uptake, above, we have attempted to compare the growth rate with the osmotic changes in an effort to estimate the turgor pressures of growing cells. However, we are not yet confident enough about the quantitative interpretation of the results to warrant any more definite statement than appears in that section.

A peculiar phenomenon which we first observed in experiments with mannitol is illustrated in Fig. 7–4. The section was initially

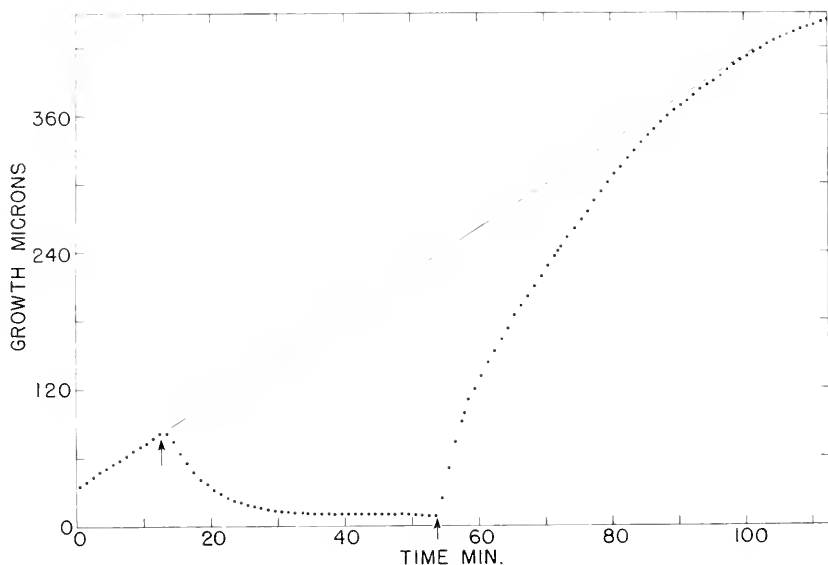

Fig. 7–4. Effect of transfer from water to 0.25 M mannitol (*at first arrow*) and back to water (*second arrow*). Coleoptile section 6 mm long. Broken line is an extrapolation of the original growth rate, which may be compared with the actual expansion which occurred after return to water, shown by the experimental points.

growing in water and was then transferred to 0.25 *M* mannitol, whereupon it shrank osmotically and did not resume growth. When it was transferred back to water, it exhibited a much greater apparent growth rate than previously (probably augmented during the first few minutes by reversal of the previous osmotic shrinkage), which continued until the section had attained approximately the length that it would have reached had the mannitol treatment not been given and the previous growth rate remained unchanged; at this point the rapid rate declined to about the previously prevailing

rate. We call this effect "stored growth," since it is as if the growth which would have occurred if the inhibitory treatment had not been given, is stored up and makes its appearance, adding onto the expected growth rate, when the inhibition is removed.

We have studied the stored growth effect more extensively using 15 per cent Carbowax 4000 (w/v), which inhibits growth nearly completely but causes relatively negligible osmotic shrinkage (for reasons we do not understand), which complicates the interpretation of the results with mannitol. The stored growth effect does not appear to be a passive osmotic swelling, because it can be inhibited by KCN, whereas osmotic swelling and shrinking is not prevented by KCN. The most clear-cut effect is obtained if a Carbowax-inhibited section is transferred to Carbowax plus 10^{-3} *M* KCN and, after several minutes to allow KCN inhibition to take effect, to KCN in water. A negligible expansion occurs. When transferred then to water, after the usual lag of several minutes for abatement of KCN inhibition, the rapid stored growth rate appears and continues until the section reaches about the length it would have attained had no inhibitory treatments been given. This indicates further that the stored growth effect is not an alteration of the cell wall which becomes lost upon KCN treatment; KCN just temporarily prevents its appearance. Furthermore, we were surprised to find that KCN treatment itself, of sections growing in water, gives rise to a comparable stored growth effect, in which case also osmotic changes could not be involved. On the other hand, we have not noticed any stored growth effect in sections treated with auxin, for example, the KCN treatments in Fig. 7–2.

There may be some relationship between the stored growth effect and the "residual effect" described by Cleland, mentioned above. He interpreted the inhibitory effect of KCN on it as indicating that there had occurred a plastic "loosening" of the cell wall which was being maintained by metabolism and became lost if metabolism was inhibited by KCN. As noted above, this does not seem to fit the behavior of the stored growth observed by us.

We were struck by the fact that the growth rate observed during the stored growth response is generally of the magnitude which would have been expected had auxin, rather than the inhibitory treatment, been given. We tend to think that this has some connection with the fact that stored growth has not conspicuously ap-

peared when we inhibit sections already treated with auxin. At the moment we are inclined toward the following tentative interpretation of the stored growth effect: the growth rate is considered to depend upon and be controlled by the rate of some chemical (enzymatic) reaction acting on the cell wall, which is sensitive to KCN and requires turgor or some effect which turgor causes (such as elastic expansion of the cell wall, or forcible juxtaposition of cell membrane and cell wall site acted upon). This reaction uses some substrate, to be called X, which is produced by general metabolism within the cell, at such a rate that, in sections untreated with auxin, the growth reaction is rate-dependent upon X. When the growth reaction is temporarily blocked, metabolism continues to produce X which accumulates so that, when inhibition is relieved, the growth reaction occurs at a more rapid rate, until the excess X has been used up. At this point the amount of growth which has occurred should be the same as if growth had continued uninterrupted, and the rate should then fall until it again becomes equal to the rate of formation of X. We suppose that the effect of auxin is to promote some reaction in the metabolic sequence leading to X; optimal auxin concentration accelerates the formation of X to such an extent that its concentration builds up to a practically saturating value for the "growth enzyme." Hence, further accumulation of X, as in inhibition of auxin-promoted growth, cannot increase the growth rate further or lead to stored growth, whereas in the absence of added auxin a period of inhibition sufficient to build up the saturating concentration of X will lead to the same rate of growth (temporarily) as if auxin were added.

We have recently tested the relation between auxin and stored growth as follows: a section growing in water at the rate of 2.7 μ per minute was transferred to 10^{-3} M KCN, which quickly gave nearly complete inhibition. After 20 minutes it was returned to water and, after a lag of 9 minutes, began to grow and attained a rate of 11.4 μ per minute, which persisted until the section somewhat overshot the length to be expected from the previous growth rate. Then its growth rate fell to 2.0 μ per minute (including a decline in control growth rate?). It was then transferred to auxin, and after 12 minutes its growth rate began to increase and quickly reached 8.4 μ per minute. It was then treated with auxin plus 10^{-3} M KCN, again giving nearly complete inhibition; 17 minutes

later it was returned to auxin alone, and after the usual lag its growth resumed and attained 7.8 μ per minute, so this time the growth did not "catch up." In this case we seem to have shown, with a single section, that stored growth is obtained previous to auxin treatment but not during auxin treatment, which is consistent with the explanation suggested above.

It may be noted that the suggested explanation of auxin promotion of growth involves an *indirect* action on the growth of the cell wall, that is, auxin gives indication of acting directly only on some aspect of metabolism within the cell which supplies the growth process with "substrate." This material, whatever its nature, may be imagined to be capable of supporting kinds of activity other than cell enlargement in cells equipped with enzymes other than the growth enzyme, and in this way the different effects of auxin on different tissues and organs might be explicable.

Recently Carr and Ng (1959) investigated the "residual effect" of auxin, using wheat coleoptile sections, and concluded that the promotive effects caused by auxins and inhibitors could best be interpreted as due to rate effects on various metabolic reactions, in directions which cause a shift in the balance of cell metabolism in favor of syntheses. A similar explanation for promotive effects of certain inhibitors had been put forward by Slocum and Little (1957). The suggestion made above may be considered an extension of these views which indicates somewhat more specifically the relationship between processes acted on by auxin and the other agents studied, and the growth process itself. We would emphasize again, however, that it is a tentative interpretation of the observations, and other possible explanations of them have by no means been excluded. Also, it undoubtedly oversimplifies growth by formally reducing it to those steps indicated by the kinetic evidence.

Summary

Current views on the mechanism of plant cell growth, with some of their experimental basis, are discussed. Experiments on oat coleoptile sections are described which suggest that the immediate mechanism of growth is not a passive plastic stretching, indirectly maintained by metabolism; instead it appears that growth rate is controlled by the rate of some metabolic reaction, most likely acting on the cell wall. Experiments on the effect of inhibitory treatments

of short duration, with and without auxin, suggest that the promotive effect of auxin is not directly on a process acting on the cell wall but is on some aspect of metabolism within the cell, which indirectly influences the rate of a process acting on the cell wall.

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8

Control by Light

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Rather than concentrate on a single small aspect of light physiology, I shall try to review two general topics and to point out areas that may have been neglected.

Let us immediately exclude from consideration both photosynthesis and vision. These have an immense literature and are relatively—though only relatively—well understood. At least all biologists, whatever their special interests, are aware that photosynthesis and vision exist, which is more than can be said for many other light effects. For similar reasons let us also exclude ultraviolet effects—wavelengths below 4000 Å—although they will enter slightly a bit later.

It has become clear to botanists only relatively recently that many actions of light, even on green plants, bear no close relationship to photosynthesis; zoologists occasionally still seem to assume that all effects of light on animals are necessarily mediated through vision, and some microbiologists seem unaware that light can affect many of their experimental organisms. This brief paper will have served its purpose if it merely emphasizes the multiplicity and widespread nature of light effects, as well as our ignorance concerning most of them.

At least two major questions are asked by the light physiologist. The first is: What is the compound absorbing the light involved in a particular process—the photoreceptor pigment or pigments? The second is: What processes intervene between light absorption and

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the final effect one seeks to explain? In practice, one tries to limit the second question to those processes following fairly immediately upon light absorption, since otherwise its answer can, and often does, involve almost every aspect of physiology. This discussion will deal largely with the first question.

A twofold approach is often used in trying to identify a photoreceptor pigment. It consists in determining an action spectrum for the process in question, and then demonstrating that a compound obtainable from the organism has an absorption spectrum similar enough to the action spectrum to justify the conclusion that it is absorbing the effective light. This procedure, of course, is rarely as straightforward as it sounds. Even action spectra alone, however, can often tell a great deal.

One general principle—though a negative one—seems to be that an effect of light on the same—or apparently the same—physiological process in different organisms will not necessarily be mediated by the same pigment. Let us trespass on the topic of the next paper for a brief example. In higher plants, the initiation and phase-control of rhythmic phenomena by light is probably (e.g., Lörcher, 1958) exerted through the red, far-red reversible system which we will discuss next. In the dinoflagellate *Gonyaulax*, however, Hastings and Sweeney (1960) have found no evidence at all for such control. Rather, the action spectrum follows the absorption spectrum of the whole cells, with prominent peaks in both the red and the blue.

A related general principle is that apparently quite different physiological processes in a given organism may be controlled through the same light reaction. This is well illustrated by the red, far-red reversible system. Since study of this system also represents a triumph, or at least an impending triumph, for the action-spectrum and extraction approach to photoreceptor identification, let us review its essential features here.

Shortly after the discovery of the photoperiodic control of flowering, it became clear that photoperiodism was a response to the duration, or timing, of light and darkness, with little dependence on total light energy during each cycle. Thus, a long-day plant can flower only on daylengths longer than a certain critical value—which is to say, only if the dark periods are sufficiently short, but flowers even with long nights if these are interrupted by a brief flash of light. Conversely, flowering in a short-day plant, requiring sufficiently long nights, will be inhibited if these nights are interrupted by light-

breaks, as these brief exposures have been called. Since only short exposures and relatively low energies are required for effective light-breaks, it became possible to obtain reasonably accurate action spectra for at least one of the light reactions in photoperiodism.

This work, done mainly at the U.S. Department of Agriculture at Beltsville by a group including Borthwick, Hendricks, and Parker as well as others, showed that red light in the region of 6400–6600 Å was the most effective for light-breaks, with no evidence of any other important peaks in the action spectrum.

A major advance took place in 1952, when the same group confirmed and extended observations made over 15 years earlier by Flint and McAlister on the germination of lettuce seeds. Under certain conditions, these require a brief light treatment in order to germinate after being returned to darkness. Red light in the photoperiodically effective region is also most active here. However, if a brief treatment with the region around 7300 Å—now generally referred to as far-red—is given right after the red, germination is prevented; if red light is given again, following the far-red, germination will take place. In short, if the seeds are alternately exposed to red and far-red light, they will germinate or not, depending on whether red or far-red came last.

Red, far-red reversibility could be demonstrated equally well even when the seeds were chilled to near 0° C during the irradiation period, although, of course, they had to be brought back to a favorable temperature for observation of the germination response. These data suggested that reversibility was a purely photochemical matter; one could thus infer the existence of a pigment that reversibly changed its absorption spectrum, depending on the wavelength absorbed last, and of which the two forms had different physiological activities. Within the next few years the Beltsville group, as well as others, was able to show that this red, far-red reversible photoreaction could control not only seed germination and the light-break effect in photoperiodism but also many other plant processes too numerous to discuss here. This pigment system is probably ubiquitous among higher plants at least, and these days the plant physiologist suspects its presence wherever low energies of red light appear to have an effect (for all the preceding, see, e.g., Borthwick *et al.*, 1956; Withrow, 1959b).

The postulated reversibility of the pigment's absorption characteristics seemed to provide a useful handle for its isolation and iden-

tification. This handle was firmly seized in 1959 by Butler, Norris, Siegelman, and Hendricks, all at Beltsville. Using special spectrophotometric techniques, they have been able to show the actual occurrence of the expected reversible change in absorption *in vivo,* and then in tissue extracts as well. Their work has already been confirmed elsewhere (Bonner, 1960), and the purification and identification of the reversible pigment itself is thus in sight, although it may be a considerable task. It is either a protein or intimately bound to protein; naturally both speculations and investigations on its enzymatic activity will be vigorously pursued. In the past, many metabolic roles for the red, far-red system have been suggested, including the possibility that it controls plant growth by affecting either auxin or gibberellin levels, or both, but such effects seem likely to be highly indirect (see, e.g., Hillman, 1959). The possibility that it may act by controlling the rate of oxidative phosphorylation has been raised (Gordon and Surrey, 1960) but is also far from being demonstrated as yet.

Let us now leave the higher plants to consider two related questions. These are whether photoperiodism in other organisms is mediated by the red, far-red reversible pigment and whether, in fact, the pigment occurs in other organisms at all. The short answer is that we don't know, as yet, but this is hardly explicit enough.

Both photoperiodism and the red, far-red system probably occur in most of the lower green plants, although there has been very little work of this kind, and particularly little with the unicellular forms, which would make valuable experimental material. It is known that certain algae fail to grow heterotrophically in darkness, even if supplied with sugars (e.g., Killam and Myers, 1956). Recently, studies with a strain of the Cyanophyte *Nostoc* have shown that low light intensities are not only required for rapid growth but also exert profound morphogenetic effects (Lazaroff, 1960). A crude action spectrum suggests the possible activity of the red, far-red system.

Among the fungi most of the relatively few light effects studied show action maxima in the blue or near ultraviolet, with red light equivalent to darkness (see Cantino and Turian, 1959; Cochrane, 1958). There appear to be exceptions, however. One is that the sporulation of certain brewer's yeasts is reported to be photoperiodically controlled, there being both long- and short-day strains. The red region of the spectrum appears to be the most effective (Oppen-

oorth, 1956, 1957). Although the data leave something to be desired, at least partly because of the many difficulties involved in controlling yeast sporulation at all, there is surely something here. Turning from yeasts, there are several reports in the literature that red light is effective in inhibiting the spore germination of several parasitic fungi, including the powdery mildew of wheat (Dillon-Weston, 1932; Pratt, 1944). Certainly both these phenomena merit more detailed investigation than they have received.

Photoperiodism has been known in animals almost as long as in the higher plants. As in plants, it may control many aspects of reproduction and development, as well as adaptations to temperature and other seasonal changes. The most detailed studies have been done on birds, but reptiles, mammals, and insects as well as certain other invertebrates have also been investigated (see Withrow, 1959b).

Identification of the photoreceptor pigment here immediately poses a problem already touched upon. Is photoreception ocular or non-ocular? If ocular, does it depend on the visual pigments or on others? This question has been studied most in birds, and there is evidence that both ocular and non-ocular photoreception can bring about a photoperiodic gonadal response. Crude action spectra indicate that red light is by far the most effective if the eyes are intact. However, this may be due largely to differential screening by the tissues. When light is given directly to the hypothalamus, yellow, green, and blue as well as red are all effective (see Farner, 1958, 1959).

Probably in most vertebrates, photoperiodically active light enters by the eye, but certainly direct photoreception in some portion of the brain is possible in forms such as birds and some reptiles, in which the skull is highly fenestrated (Bartholomew, 1959). It is also worth noting that although the photoperiodic responses of fishes have not been studied specifically with regard to photoreception (Harrington, 1959), blinded fish are known to show pigmentation responses to changes in illumination (Sumner, 1940). Although these responses are not always the same as those of normal individuals, they again suggest the presence of a non-visual control by light, and this might occur also in photoperiodism.

A further piece of evidence for non-visual photoreception in photoperiodism has been recently provided by de Wilde *et al.* (1959) working with the Colorado beetle (*Leptinotarsa*). Diapause in this,

as in many other insects, is photoperiodically controlled. An apparently complete blocking of vision has little or no effect on the response.

In summary, our questions about the photoreceptor pigments in animal photoperiodism, and the possible presence of the red, far-red system, stay unanswered. They will remain so until adequate action spectra are available and until the visual and non-visual components can be distinguished. One should note here that some of the data relating the red, far-red system to the rate of oxidative phosphorylation, mentioned previously, have been obtained with rat-liver mitochondria (Gordon and Surrey, 1960). Although a temporary suspension of judgment seems called for, at least the visual component is eliminated in this case.

Before leaving this subject, I cannot resist mentioning a recent paper (Ballard, 1958) on the phototactic response of the stable fly, *Stomoxys calcitrans*. There are several peaks in the action spectrum, including a very distinct one at about 6400 Å. In addition, light of about 7300 Å has a clearly opposite—repellent—effect and is the only region that does so. Yet, oddly enough, there is no evidence for the presence of pigments with absorption peaks at those wavelengths in the eye itself. It is barely possible that some of these results may be due to slight temperature differences, but it is also tempting, at least to those of us who see the red, far-red system under every bed, to see it here. Unfortunately, the author, who himself was puzzled by these results, was apparently unaware of the existence of the red, far-red pigment and thus did not examine the possibility further.

If some of what has been said so far sounds like a criticism of other biologists for not doing photoreceptor research as successfully as plant physiologists, the next topic could not even suggest such a tone. Perhaps the majority of non-visual, non-photosynthetic light effects on all organisms have action spectra with maxima in the blue or blue-green. It is probably safe to say that these are no better understood now than they were some 20 years ago, although we can suggest more possible mechanisms and also more possible pitfalls. Before examining these, let us list some of the phenomena which are known, or may be reasonably suspected, to be affected by blue light. These include phototropism in higher plants and in fungi (Banbury, 1959; Galston, 1959; Reinert, 1959; Went, 1956); pho-

totaxis and photo-orientations of all kinds in protista and other organisms (Bendix, 1960; Mast, 1911); pigmentation, morphogenesis, and sporulation in fungi (Bonner, 1959; Cantino and Turian, 1959; Cochrane, 1958); pigmentation in bacteria (Baker, 1938; Kreitlow, 1941); protoplasmic streaming (Haupt, 1959); and respiration (Föckler, 1938).

As with photoperiodism, study of blue-light effects in many animals is complicated by the presence of vision, although there is, again, evidence that morphologically specialized photoreceptor organs need not be involved. In a sea-anemone (North, 1957) and a starfish, for example, the skin itself appears to be light-sensitive; in the latter, the same blue-absorbing pigment occurs both in the skin as a whole and in specialized pigment bodies (Rockstein, 1956). However, work on the identification of possible blue-receptive pigments has been most intensively carried out by investigators of phototropism in the higher plants and the fungi, and it is such studies we must now consider briefly. (General references for the following discussion on phototropism are the four given above.)

For a long time, β-carotene or related carotenoids were believed to be the only possible photoreceptors in phototropism. This belief was based on action spectra, on the appropriate distribution of carotenoids in the sensitive tissues, and also on the known role of such compounds in vision. Some ten years ago, however, flavine compounds were suggested, and the situation is no longer as clear as it appeared to be.

In various published action spectra, single, double, or even triple peaks in the blue are found. In addition, a peak near 3800 Å, in the near-ultraviolet, may be evident. The presence of multiple peaks in the blue has been taken (e.g., Curry and Gruen, 1959) as evidence for the functioning of carotenoids, since the absorption of riboflavin and certain related compounds in water solution generally shows a single broad peak or a peak and a shoulder. However, this argument against flavine involvement has been weakened by demonstrations that certain flavoproteins in water, or simpler flavine compounds in various other solvents, can show multiple peaks in the blue (Asomaning, 1960; Beinert and Crane, 1956; Searls and Sanadi, 1959). On the other hand, the action-spectrum peak in the near-ultraviolet has been used to support the flavine hypothesis, since it fits the absorption of many flavine compounds very well. An ob-

jection to this is that it also fits the absorption of certain carotenoids; besides which, one cannot assume that all portions of the action spectrum necessarily depend on a single pigment.

Instead of going into the many objections that various investigators have urged against each others' interpretations, let us consider a few of the general problems of interpreting such action spectra. First of all, precision, particularly for such a complex response as phototropism, is limited by the variability of the material. Thus the relative height, position, and even existence of closely placed peaks is not easily ascertained. A second difficulty is that, since phototropism depends on an asymetric distribution of light about the responding organ, it might be affected by pigments, not active photoreceptors themselves, that screen or filter the light passing through the organ. Thus, the action spectrum could reflect the absorption characteristics of both photoreceptor and screen. Relatively few quantitative evaluations of this possibility have been made, although Delbrück and Shropshire (1960) have found that the effect is probably negligible in the fungus *Phycomyces.*

Perhaps the most disturbing question is that already implied on page 219. Once an action spectrum is established, to what absorption spectrum of a suspected pigment shall it be compared? It is evident, at least with flavines, that the absorption spectrum is affected by the solvent, and there seems to be no obvious way of deciding what solvent—be it water or any of many organic substances—will approximate the state of the pigment *in vivo.*

We have tacitly assumed throughout this discussion that both major possible photoreceptors—carotenoids and flavines—are usually present together in quantities sufficient to make a decision on the basis of relative concentration impossible. This is generally true. Some work with albino seedlings having a low carotenoid content has been done, but the results have been interpreted both ways (cf. Galston, 1959, and Went, 1956) and are quantitatively unsatisfactory. Similar work with certain fungi, in which carotenoid content can be artificially lowered, is also equivocal so far. Of course, the chance of ever obtaining flavine-free tissues is very small, considering the number of functions performed by such compounds.

This necessarily brief summary indicates that, even considering only flavines and carotenoids, we have too many candidates for the blue-light photoreceptor unless we simply assume that both classes of compounds may be active. This is, of course, quite possible. In

addition, however, one must note that even the major proponent of the flavine hypothesis, Galston (1959), taking the position that the bulk of the evidence is against carotene, points out that such evidence does not automatically favor the flavines. Other yellow compounds such as the pteridines—which have been suggested as visual photoreceptors in some organisms (Forrest and Mitchell, 1954)—have been relatively little investigated but cannot be excluded from consideration.

Compare for a moment the success of the search for the photoperiodic pigment with the failure of that for the phototropic. The approaches have been similar—wherein lies the difference? This is easily answered. Investigators of the photoperiodic pigment soon learned that they had the good fortune of dealing not with one but with two related action spectra—the red-peaked spectrum for the reaction in one direction and the far-red-peaked spectrum for its reversal. Basically it is this circumstance that has made further advances possible, and nothing like it seems to be available for students of blue action spectra. Since one cannot very well suggest that reversibility be discovered where it does not exist, there are few if any lessons for the future here. Faced with a multiplicity of yellow compounds, what is the blue-light physiologist to do?

There is still, of course, room for more action spectra, for more absorption spectra, and for careful studies of the responses correlated with varying pigment contents. Work of this kind may yet prove adequate to the task. But it is now time to recall that the light physiologist has another interest besides the identity of the absorbing pigment and that is the nature of the events immediately following light absorption. If one knew what the immediate consequences of light absorption were, one might then be able to decide which of the several possible pigments was capable of bringing them about. This approach, while not yet particularly fruitful, has had a strong influence on the study of phototropism.

It has been generally accepted for many years that phototropic bending in higher plants is due to an unequal distribution of growth hormone—auxin—in the illuminated axis. This is probably not an immediate result of light absorption. It has not been demonstrated to the satisfaction of all concerned whether it results from an unequal destruction of auxin, from a light-induced movement of auxin, from an unequal photoinhibition of auxin synthesis, or from some combination of these or other mechanisms. That auxin is involved,

however, cannot be doubted, and it is from this basis that proponents of the flavine hypothesis have proceeded.

The demonstration that riboflavin could sensitize the *in vitro* photodestruction of the auxin indoleacetic acid and its probable precursor, tryptophane, and evidence that a flavoprotein could also participate in the so-called "indoleacetic acid (IAA) oxidase" system, formed the beginning of this approach. It is now quite clear that light can affect a great many substances and reactions in the presence of riboflavin and related compounds. In contrast, the only relevant activity that has been ascribed to carotenoids is a role in the *in vitro* photoinactivation of "auxin-a-lactone," and the entire status of auxin-a is, of course, uncertain (Galston, 1959; Went, 1956). Thus, the flavine hypothesis has the general advantage that it is easy enough to see what flavines might do, and not so easy to see this for carotenoids. It is probably this, more than any other consideration, that has led a number of recent workers to favor the flavines. There are two general grounds, however, on which this line of reasoning can be criticized.

The first is that it is based primarily on *in vitro* experiments. This is true both of the riboflavin-mediated photodestruction of various compounds and of any hypothesis invoking the IAA oxidase system. There is no unambiguous evidence, at least in my opinion, that such events or systems operate in intact tissues at all, and there are some reasons to doubt it. A second major difficulty is that the flavine hypothesis provides an embarrassment of riches. At least *in vitro*, flavines in the presence of light can bring about a great many different effects. In addition, light absorption may well affect the properties of flavine compounds involved in electron transfer systems (Commoner and Lippincott, 1958). The question then becomes one of how to obtain any specificity from such action, and the same can be asked about other yellow compounds besides flavines.

The answer to the question of specificity may well come from understanding the relationship of the photoreceptor molecule, whatever its identity, to the fine structure of other molecules and macromolecules around it, a problem about which we know almost nothing as yet. In this connection, one should avoid a misleading impression that might result from the exclusion of vision and photosynthesis from this discussion. The photoreceptor molecules for these processes occur in highly ordered structures, while the locations of the photoreceptor molecules in other light-dependent proc-

esses are not nearly as well understood. However, they certainly cannot be lying around at random, dissolved in some relatively homogeneous ground substance, particularly since recent electron micrographs indicate that there is little if anything homogeneous about a living system (e.g., Whaley *et al.*, 1960). In addition, experiments with polarized light have suggested that photoreceptor molecules are probably highly oriented even in relatively simple systems (see Jaffe, 1960). A recent hypothesis (Thimann and Curry, 1960) on phototropism suggests that the pigment molecules are located in particles—proplastids, for example, in the higher plants—and that the first event following light absorption may be a reorientation of these particles. Much here remains to be demonstrated, but it is interesting particularly since the mechanism suggested essentially parallels that invoked in the "statolith" theory of geotropism and thus might provide some unifying link between the two phenomena. It will certainly be worthwhile, in view of such considerations, to see what the electron microscope might show about immediate structural changes, if any, following illumination.

It should be added parenthetically that the artificial distinction made here between red- and blue-light effects is just that—artificial. For example, there is evidence both for effects of blue light on the red, far-red system (see Borthwick *et al.*, 1956; Withrow, 1959b) and for phototropic effects of red light under certain circumstances (see Galston, 1959). These observations pose further problems of the same kind as those already discussed. A recent paper on diapause induction in the cabbage-butterfly, *Pieris brassicae*, seems even more formidable. The effect of light appears to depend strongly on the portion of the diurnal cycle in which it is given; but in addition, the shorter wavelengths—red and yellow—inhibit diapause in the phase during which blue and violet promote it, while they promote it during the phase in which blue and violet inhibit. This may be due to the presence of two separate pigment systems, both affecting diapause, but the question requires much further investigation (Bünning and Joerrens, 1960).

In terms of the topic of this symposium—control mechanisms—the basic mechanism for the control of many diverse processes by light is, of course, that many biologically active compounds are pigments. They absorb light and can thus be excited into states with properties different from their normal ground states. Whether or not this is then in some way amplified to a physiological response depends

on the circumstances. From this statement of the problem, one might be more surprised that, in a relative sense, so few light effects are known, rather than so many, in view of the many colored compounds with biological roles.

Probably the most important point so far omitted that should now be emphasized, is that many of the known effects of light occur at extremely low energies. This is evident in both the phototropic and photoperiodic literature, as well as elsewhere. In terms of intensities, the threshold for certain morphogenetic responses through the red, far-red system is about 10^{-8} μw (10^{-7} ergs/sec) per square centimeter, while the threshold for phototropic responses is about 10^{-3}. For comparison, the lower limit of rod vision in the dark-adapted eye is about 10^{-5} μw/cm^2—lying between the phototropic and photoperiodic thresholds—and the intensity of full moonlight is far higher than any of these, being more than 10^{-1} (Withrow, 1959a). For this reason, the absence of a light effect on an organism or reaction cannot be assumed unless the dark control represents absolutely uninterrupted total darkness for an extended period of time. There is the additional possibility, observed, for example, in *Amoeba* (Bendix, 1960), that light effects may be observable only within a short time following a change in intensity. The wider recognition of these simple principles could in itself result in the observation of new light effects, which might also help to explain further those we know today.

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9

Temporal Regulation in Cellular Processes

J. Woodland Hastings [1]

There are at least two distinct classes of cellular processes which may be considered to exhibit temporal regulation. The mitotic cycle may be considered as an example of the first of these. Starting arbitrarily with a daughter cell from a recent division, there occur an orderly and regulated sequence of physical and chemical events leading to the division of this cell into two cells, each being similar to the cell at the time when we started our consideration.

There are many studies concerned with the mitotic cycle (Ducoff and Ehret, 1959). Hase, Mihara, and Tamiya (1960) for example schematically represent the life cycle in *Chlorella* as being divided into four major phases which follow in sequence: growth, ripening, maturing, and division. There are not very many generalizations which we can make concerning the mechanism of the control involved here. The description of the cycle in terms of four phases suggests that certain events in one phase may trigger the onset of the subsequent phase. On the other hand, there is evidence that cell division depends upon the completion of a certain number of essentially independent or parallel requisite chemical steps. Certainly the understanding of the regulatory mechanisms involved must await better knowledge of the chemical events and their sequence.

As examples of temporal regulation, life cycles of organisms may be considered to be essentially similar to the life cycle of the cell. It is quite evident that pathways of biosynthesis change at different

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stages in the life cycle. This is spectacularly illustrated in the course of embryological development (McElroy and Glass, 1958). Here also a possible presumption is that an essential aspect of the control mechanism is that certain events A, B, C, D, E, etc., occur in a fixed sequence, but it is not known how a given event relates to the others in the series. In these cases also we are largely concerned at the present time with gaining descriptive information but may certainly hope that information concerning the nature of the chemical control will be forthcoming.

Distinctly set apart from mitotic and life cycles are the daily tidal, lunar, and annual cycles exhibited by cells and organisms. These also involve cyclical changes, but their frequencies match the corresponding cycles of the physical environment (Symposium on Biological Clocks, 1961; Brown, 1959; Bünning, 1958a; Hastings, 1959; Pittendrigh and Bruce, 1959). A possible relationship—insofar as mechanism is concerned—between these two major kinds of cycles is not yet definitely ruled out.

In the present paper, we shall limit ourselves to a consideration of daily or diurnal rhythms. The persistence of diurnal rhythms with a period of approximately 24 hours indicates that an endogenous control mechanism is responsible for the cyclically recurring processes (Pittendrigh, 1958). In this respect the nature of the mechanism may be similar to mitotic and life cycles. But in a second respect diurnal rhythms exhibit a major difference. The period of the cycle is always close to 24 hours (but rarely if ever exactly that), and it is not readily subject to change as a consequence of different environmental conditions. Whereas the period of the mitotic cycle is generally found to be shorter at higher temperatures, and vice versa, with Q_{10} values in the range of 2 or 3, the period of diurnal rhythms has been found to be relatively unchanged by temperature (Pittendrigh and Bruce, 1957).

The important implication of the temperature-independent period of biological rhythms is that physiological and biochemical processes may be regulated in time by means of a cellular or subcellular mechanism, a kind of chemical clock, with the important features necessary for relatively accurate timekeeping.

A large number of animals and plants have been shown to exhibit diurnal rhythms. In every case studied, the free-running period (i.e., the period of the rhythm under conditions of constant tem-

perature and illumination) is not greatly different at different but constant temperatures.

These include the following rhythms: leaf movement in *Phaseolus* (Bünning, 1931; Leinweber, 1956); growth rate in *Avena* (Ball and Dyke, 1954); exudation in *Helianthus* (Grossenbacher, 1939); phototaxis in *Euglena* (Bruce and Pittendrigh, 1956); sporulation in *Oedogonium* (Bühnemann, 1955a); sporulation in *Pilobolus* (Schmidle, 1951; Uebelmesser, 1954); zonation of growth in *Neurospora* (Pittendrigh *et al.*, 1959); bioluminescence and cell division in *Gonyaulax* (Hastings and Sweeney, 1957b; Sweeney and Hastings, 1958); mating in *Paramecium* (Ehret, 1959); eye pigment migration in *Cambarus* (Welsh, 1941); color change in *Uca* (Brown and Webb, 1948); the time sense in the bee (Renner, 1957; Wahl, 1932); eclosion in *Drosophila* (Pittendrigh, 1954); activity in the cockroach (Bünning, 1958b); and activity in the lizard (Hoffman, 1957).

Many possibilities have been considered to explain the phenomenon of temperature independence (Pittendrigh and Bruce, 1957). The only one which has received any substantial experimental support is that of a temperature-compensation mechanism. It is clear that an appropriately coupled physicochemical mechanism could exhibit essential temperature independence. Thus, the rate of one process could be decreasing and that of another increasing such that their net coupled output was constant.

Various other possibilities, such as feedback control via the regulation of enzyme activity, have been mentioned (Hastings and Sweeney, 1957a, 1959). Because of our scanty knowledge concerning the chemistry of the cycle, it is probably not fruitful to elaborate in detail concerning these and other possibilities.

We have been concerned with various biochemical aspects of the rhythmic mechanism in the dinoflagellate, *Gonyaulax polyedra*. The studies to be described here have been concerned with the effects of inhibitors.

In previous studies utilizing inhibitors, it had generally been found that the phase and the period were relatively insensitive to modification by chemical agents. Bühnemann (1955a), studying the rhythm of sporulation in *Oedogonium*, found essentially no effects utilizing NaCN, 2-4 dinitrophenol, NaF, Na arsenate, iodoacetic acid, quinine, copper sulfate, cocaine, β-indoleacetic acid, adenosine triphosphate, and riboflavin. Bünning (1959), studying the leaf

movement in *Phaseolus*, has reported that a few compounds—colchicine, urethane, and ethyl alcohol—show effects upon the period.

Before describing the inhibitor studies, some few details will be given concerning the nature of the rhythms we are studying and the techniques used. As shown in Fig. 9–1, we have observed several different rhythms in *Gonyaulax*. With cells maintained on an

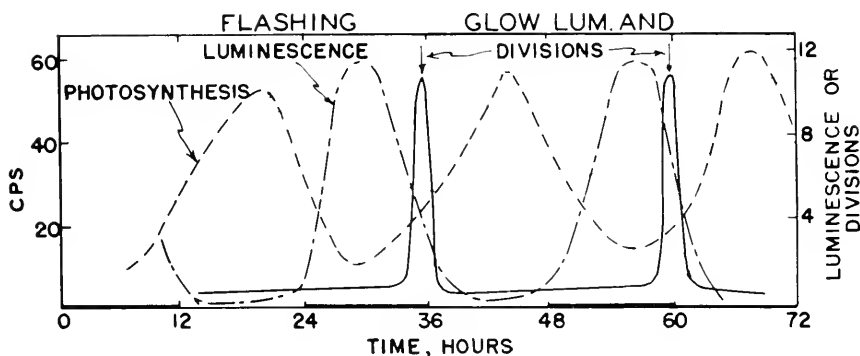


Fig. 9–1. The several rhythms and their phase relationships in *Gonyaulax*. All measurements were made with cells maintained in alternating light and dark periods of 12 hours each, the dark periods being indicated by the black bars on the abscissa. The light intensity during the light period was 960 foot candles, and temperatures varied from a maximum of 26° C during the light periods to a minimum of 23° C during the dark periods. Cell densities were uniform in all aliquots, about 11,000 cells per milliliter. Ordinates: on left, counts per second incorporated, corrected for background and controls; on right, luminescence or divisions in arbitrary units. Abscissa: time in hours. For the measurement of rate of incorporation of $C^{14}O_2$, two flasks, each containing 20 ml of cells, were removed and incubated in the light at an intensity of 960 foot candles for 15 minutes, in the presence of 12.5 microcuries of C^{14}. At the same time appropriate controls were incubated in the dark. Luminescence measurements and measurements of cell division were made with aliquots removed at the times indicated.

artificial day-night schedule of 12 hours light and 12 hours dark, the maximum in the typical bioluminescence, i.e., flashing induced by stimulation, occurs in the middle of the dark period (Sweeney and Hastings, 1957).

There exists also a rhythm in the photosynthetic capacity of the cells, shown by the curve whose maximum falls in the light period. Cells were assayed for their photosynthetic capacity under standard conditions at various times (Hastings and Astrachan, 1959). This

has been measured by both manometric techniques and by $C^{14}O_2$ incorporation measurements. Sweeney, in experiments utilizing diver techniques, has demonstrated this rhythm in single isolated cells (Sweeney, 1961). This, it is believed, is the first instance in which a diurnal rhythm has been observed from measurements of single cells.

There is, in addition, a rhythm of cell division (Sweeney and Hastings, 1958). Cells divide predominantly at the time of day indicated by the solid line, at approximately the end of the dark period. We do not find that all cells divide every day, but those cells which do divide in any given 24-hour period do so at this time. Under optimum conditions we have been able to obtain 65 per cent or more of the cells dividing during one day.

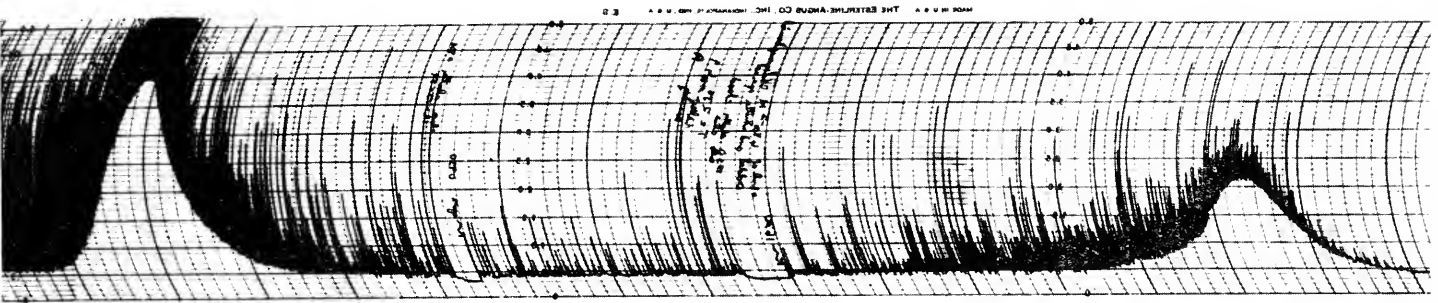
Finally, there exists also a rhythm of a steady glow, which is maximum at about the same time as is cell division (Sweeney and Hastings, 1958). In contrast to the light emitted by flashing, this steady glow is spontaneous and does not require stimulation. Its intensity is very dim and not readily visible to the dark-adapted eye.

All these rhythms continue to occur in constant conditions and all are similarly temperature-independent, showing Q_{10} values in the range of 0.85.

The glow rhythm has been used in the experiments to be described, and it may be assayed by placing a culture in front of a phototube, as shown in Fig. 9–2 (top). The increase in the baseline represents the glow; the irregular vertical lines are from the spontaneous flashing of single cells. Note that the phase of the spontaneous flashing rhythm becomes displaced from the glow rhythm.

Measuring the glow in this way requires that the culture be always kept in front of the phototube, therefore in the dark. This results in the starvation of the cells because of lack of light for photosynthesis (Sweeney *et al.*, 1959). Only three or four peaks may be measured in the dark, as is suggested by the decline in the magnitude of the second peak (see also Sweeney and Hastings, 1957).

To overcome this difficulty and to be able to assay many samples at a time, we assembled an automatic sampling apparatus, constructed from a Packard Liquid Scintillation counter. It has a capacity for 100 vials on a turntable, with light sources overhead whose location, intensity, and programing can be controlled. A vial is moved from the turntable down to a dark chamber in front of the



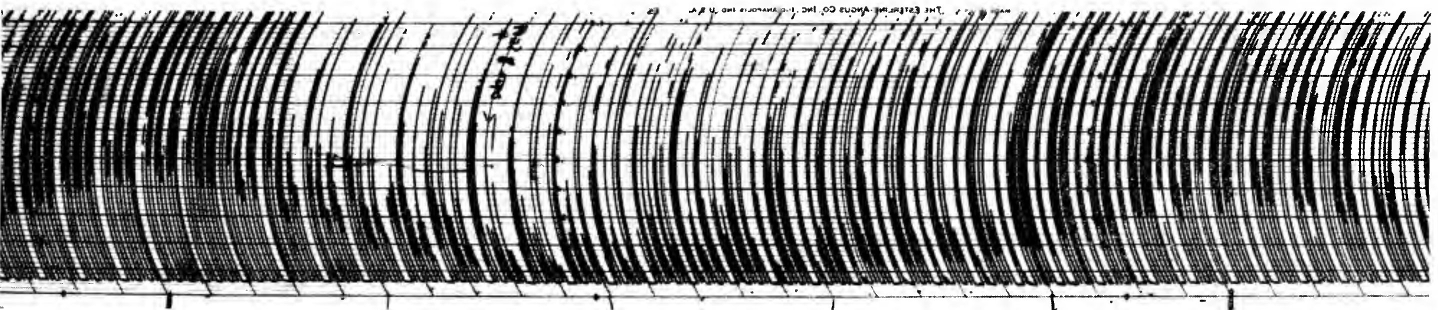


Fig. 9–2. The luminescent glow rhythm, measured from one vial of cells kept in constant darkness (*top*) and from 6 vials (*bottom*). These were kept in constant dim light, except for brief periods every hour when each vial in turn was automatically moved to the dark in a phototube chamber to measure luminescence. Ordinate: light intensity; abscissa: time, one division on lower graph being equal to one hour. Scale on other graph the same. The change in the baseline level results from the steady glow; the vertical lines are from spontaneous flashing of individual cells. Temp. 23° C.

photomultiplier tube by means of automatic elevators. There the light emission is detected and then amplified and recorded on a graphic chart (Fig. 9–2, bottom). The vial is brought back up and returned to the turntable, and the next vial is similarly measured. After all samples have been looked at, the cycle is repeated, usually being scheduled to repeat once every hour or once every 2 hours.

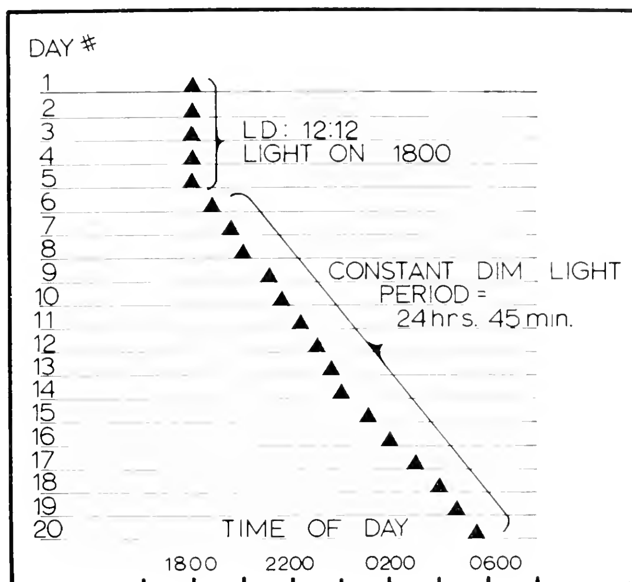


Fig. 9–3. The time of day at which the maximum in the luminescent glow occurred on each of 20 successive days. The values were obtained by measuring the luminescence from this vial approximately every two hours, similar to the fashion illustrated in Fig. 9–2, *bottom*. For the first few days the culture was kept on a daily light-dark cycle, and the peak occurred at the same time each day. Subsequently the light remained on continuously, and whereas the rhythm continued, its maximum occurred about 45 minutes later each day, indicating that the free-running period in this case was 24 hours 45 minutes. Temp. 24° C.

The chart record in Fig. 9–2 shows six replicate samples on constant dim light, assayed once every hour. Peaks of the glow are readily discernible but are evidently somewhat broader compared to those from the sample kept in the dark.

The values for each vial are tabulated and curves plotted separately, and then the time at which the peaks occur is recorded. The data may be presented as in Fig. 9–3 where we plot values for the time of maximum luminescence taken from a single vial which we

recorded for 20 days. As long as the vial was exposed to a light-dark cycle, the time of day at which the peak in luminescence occurred was exactly the same. But on constant light the peak occurred about 45 minutes later each day, so that the free-running period was about 24 hours 45 minutes.

An important observation, which the design of these experiments relies upon, is that a pulse of bright light, applied at the proper time in the cycle, causes a phase shift, which phase shift persists so long

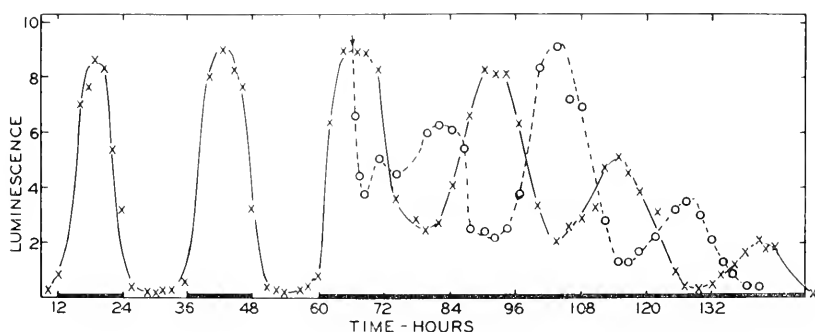


Fig. 9–4. Shifting of the phase of the rhythm of luminescence by a single exposure of the cells to light. The cells were grown with alternating light and dark periods of 12 hours each (LD) and were pipetted into test tubes at 0 hours. They were then replaced in LD conditions, and the typical rhythm of luminescence is shown. Cells remaining in the dark after 60 hours continue to show the rhythm with a period of approximately 24 hours, although the amplitude decreases (*solid line*). Cells treated similarly, except for a 3-hour exposure to light between 66 and 69 hours, also show the rhythm of luminescence (*broken line*) but with a shift in the phase or time at which the maxima occur. The number of hours by which the phase is shifted is dependent upon the intensity and color of the light used for irradiation.

as we are capable of assaying the system (Hastings and Sweeney, 1958). This is illustrated in Fig. 9–4 for the flashing rhythm. It is similar in the case of the glow rhythm, however.

Cells kept on a 12 hours light–12 hours dark schedule were transferred to the dark at 60 hours, and the controls, left in the dark thereafter, showed a free-running period of about 24 hours. The experimentals were exposed at 66 hours to a 3-hour light pulse, and the large phase shift is evident. The number of hours by which the phase is shifted is dependent upon both the intensity and color of the light used for irradiation and upon the duration of the light pulse

and the time in the cycle when it is administered (Hastings and Sweeney, 1960).

The action of such a light pulse is presumably mediated via a photochemical reaction, and our experiments were designed to attempt to mimic this photochemical reaction in one way or another. That is, instead of giving a pulse of light, might it not be possible to obtain a phase shift by exposing the cells for a relatively brief period of time to an inhibitor or metabolite? Might it not be possible to inhibit (for a short while) the processes involved in the rhythm and thereby see a phase shift? In this respect our procedure differs from previous inhibitor studies, which have been generally carried out by adding an inhibitor but never removing it.

The procedure and method of presenting the results are evident from Fig. 9–5. A single vial, such as an uncentrifuged control shown at the bottom, is traced along the horizontal. Having previously been on a 12 hours light–12 hours dark schedule, the maximum glow was just at the end of the dark period. The vial went into dim light at 6 A.M. of the first day shown, and glow peaks occurred approximately every 24 hours thereafter, as indicated by the triangles. The vertical lines are drawn through the time at which control peaks occurred, so that phase shift in other vials, shown on other horizontal lines, may be ascertained by noting the distance of the glow peaks from the lines.

(It should be noted here that we always do both centrifuged and uncentrifuged controls and always include more than are illustrated. Also, we have always continued the measurements for five or six periods, but for illustrative purposes we have not shown them all.)

Seven-hour light pulses given at different times of day are illustrated. The most effective one starts about 10 A.M. and goes to 5 P.M.; the bars on the horizontal line indicate when they were exposed. The phase shifting is evident from the glow peak. In all subsequent experiments we have included a light-induced phase-shift control.

In the experiments illustrated at the top of Fig. 9–5, we have used 5′-fluoro-3′-deoxyuridine (FUDR). As with the other compounds tested, we have given pulsed exposures; that is, we have added the compound at one time of day and 8 or 9 hours later have centrifuged and resuspended the cells in fresh medium. In order not to overlook the possibility that the cell is sensitive at one time in the cycle but not at another, we have staggered these treatments to cover the full

24 hours. Note also that we have included a vial in which FUDR was added but not removed. With some compounds this required using a considerably lower concentration than that which could be used in a pulse.

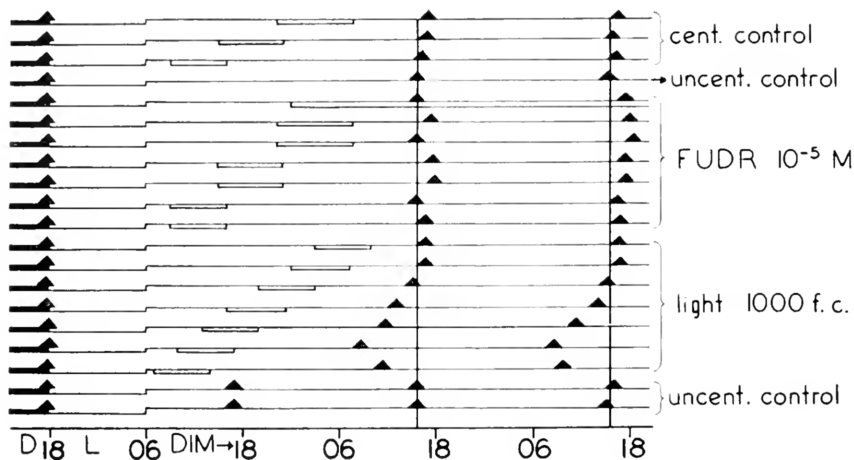


Fig. 9–5. This and the subsequent figures show many cultures in each figure, all at 24° C. Each culture is traced along a horizontal line, and the times at which maxima occurred on successive days are indicated by black triangles. All cultures were placed in constant dim light subsequent to exposure to light-dark cycles (12 hours light; 12 hours dark), which ended at last dark period (black rectangle on abscissa). In this figure are shown phase shifts resulting from exposures to bright light at different times of day. The times at which these light treatments were given are indicated by bars along the horizontal lines. Note that the phase shift is highly dependent upon the time of day when light is administered. Chemical "pulses" are indicated in a similar way, the time period of exposure being indicated by a bar on the horizontal line. Where the compound was not removed, the bar continues. The compound and its approximate final concentration is indicated to the right. Centrifuged controls are vials to which distilled water instead of the compound was added, and centrifugation and resuspension in fresh medium was carried out as usual. FUDR = 5′-fluoro-3′-deoxyuridine.

With FUDR the glow maxima occur very much as in the controls. There may be a slight shift of some 1 to 3 hours. Whether or not this is significant is not known, but we are inclined to think that it is not.

In these experiments we have attempted to characterize the actual effect that the compound has upon the biochemical processes

in the cell—as distinct from the phase-shifting measurement. We have used as a measure of the effectiveness of different compounds, their effects upon growth rate, cell division, and the incorporation of radioactive sulfate, phosphate, and carbon dioxide. We have not examined in these ways all the compounds which we have tested for phase-shifting potential.

FUDR is structurally similar to thymidine monophosphate, and one of its known effects is upon DNA synthesis, by virtue of the fact that it blocks the conversion of deoxyuridine monophosphate to thymidine monophosphate (Bosch *et al.*, 1958). With *Gonyaulax* we have not proved directly that FUDR has this effect, but we have shown that it has effects which would be expected as a consequence of this blockage. At concentrations of 10^{-5}, FUDR abruptly stops cell division and growth. Moreover, it has a marked inhibitory effect upon the rate of inorganic phosphate incorporation at a concentration of 8.5×10^{-6} *M*. However, at the same concentration an effect upon the rate of light-dependent $C^{14}O_2$ incorporation is evident only after a lag period of about 12 hours, as might be expected.

We can also stop growth and division by maintaining the light intensity at a low value. Actually, in all the experiments that we are describing, there was little or no growth. However, only when we add a specific inhibitor can we be more certain that the metabolism or turnover in a particular biochemical system is being arrested.

We undertook to investigate protein synthesis and its possible relationship to the problem of rhythmicity. In view of the fact that the activity of the enzyme luciferase varies with time of day (Hastings and Sweeney, 1957a), we hoped that by blocking or accelerating protein synthesis we might alter the phase of the clock. We have used numerous compounds: amino acids, analogs of amino acids, inhibitors of protein synthesis, and intermediates in pathways involved in protein synthesis. Many compounds are without effect. Chloramphenicol does have an effect, but it does not appear to be specifically upon protein synthesis. At a concentration of 6×10^{-4} *M* chloramphenicol, sulfate is incorporated at approximately 50 per cent of the normal rate, and cell division and growth stop sharply. CO_2 fixation drops to about 15 per cent of the control rate. No phase shift occurs (Fig. 9–6). This was repeated on two occasions.

Certain other compounds, some of which are shown here, have been found to cause very pronounced and stable phase shifts. The

interpretations that follow concerning these effects are certainly very tentative.

2, 4 Dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, had a marked effect in the case shown here. A repeat of this experiment (not illustrated) gave no effect. This kind of result was encountered many times and requires explanation. Note that the phase shift which occurs, whatever it may be, is stable from one day to the next.

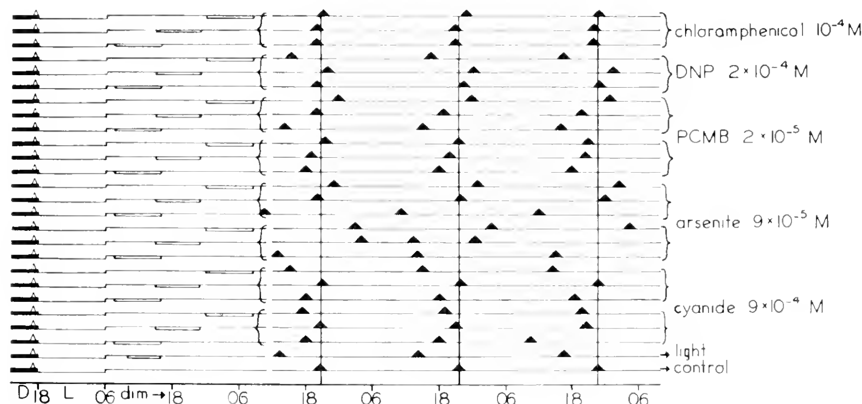


Fig. 9–6. Several of the compounds shown here have pronounced and apparently specific phase-shifting effects. Note in particular the marked effect of arsenite in this regard. (See text and legend to Fig. 9–5 for details.) DNP = 2,4 dinitrophenol; PCMB = p-chloromercuribenzoate.

With *p*-chloromercuribenzoate (PCMB), an inhibitor of sulfhydryl groups, there was a marked phase shift. In this case, however, the pattern was somewhat similar—but not exactly the same—in the second run, as illustrated. Photosynthesis was strongly inhibited by PCMB at these concentrations.

Arsenite, an inhibitor of dithiol compounds, gave similar results in four separate experiments, two of which are shown. Arsenite is by far the most effective of the compounds tried by us, and a more detailed study of this may lead to significant findings.

Cyanide has a marked effect upon the cells and upon the phase, but, as in the case of DNP, this phase-shifting effect is quite variable.

The marked variability such as we note here with DNP and cyanide was also observed in the presence of compounds such as KCl and $AgNO_3$. It seemed necessary to run a large number of controls

through all the manipulations of adding, centrifuging, resuspending, etc., without adding any inhibitors (Fig. 9–7). Note that in these controls there occur appreciable variations. There are some few cases where the phases differed by 2 hours. The fact that these occur normally must not be overlooked. But in general it is evident that the reproducibility in the controls is good; the variance is small.

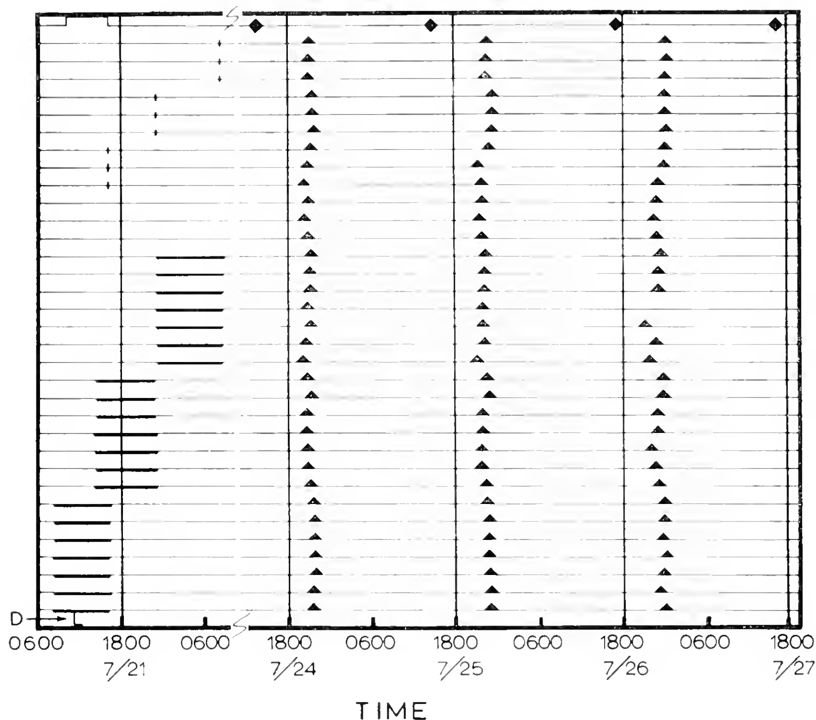


Fig. 9–7. A series of control experiments to illustrate the accuracy of the experimental procedure. (See text and legend to Fig. 9–5 for details.)

From this we have concluded that chemical or inhibitor perturbations may lead to less accuracy, i.e., to an increase in the observed variance, but that the precise effect, i.e., whether we see a phase advance or a phase delay, may differ from one experiment to the next. We would, therefore, emphasize that the assay system should be tested for variance with many controls as illustrated here and that any suspected phase shift ascribed to particular conditions should be repeated many times to assure that one is not simply looking at increased inaccuracy.

In Fig. 9–8 are illustrated experiments using several kinds of compounds. Two of the most interesting are CMU and DCMU, mono- and dichlorophenyl dimethyl urea. These are substituted urea compounds developed as herbicides by Du Pont (Bucha and Todd, 1951). Their most important property is that they specifically inhibit photosynthesis (Bishop, 1958). With *Euglena*, for example, heterotrophic growth in the dark occurs perfectly normally even in high concentrations of CMU or DCMU. But in the light, even in the presence of an organic carbon and energy source, growth is markedly inhibited.

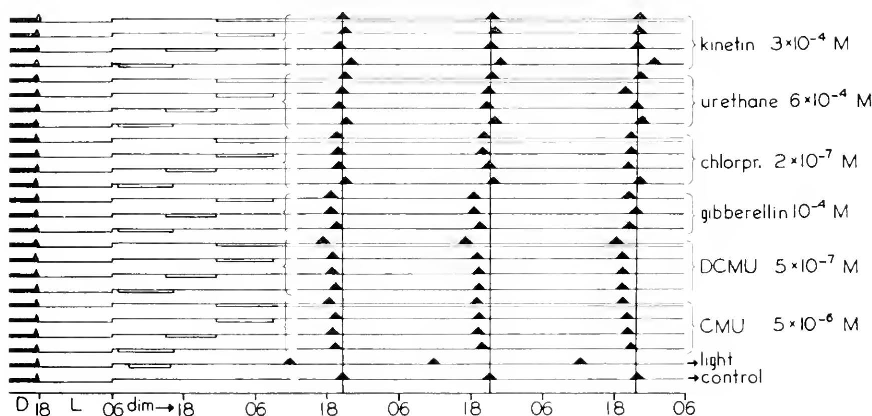


Fig. 9–8. Kinetin, urethane, chlorpromazine, gibberellin, di- and mono-chlorophenyl dimethyl urea have relatively little effect upon the phase of the rhythm. (See text and legend to Fig. 9–5 for details.)

Both of these compounds strongly inhibit light-dependent $C^{14}O_2$ uptake in *Gonyaulax;* at a concentration of 10^{-7} *M* DCMU, the rate is less than a few per cent of normal. We have not observed any specific phase-shifting effect by these compounds with either pulsed or continuous treatment. Moreover, and this is probably an even more significant result, cells inhibited with DCMU give the normal phase shift when pulsed with light.

In a photosynthetic form it is significant to exclude photosynthesis from an active role in clock function, particularly in view of two facts: (1) there is a diurnal rhythm of photosynthesis and (2) action spectra studies have indicated that chlorophyll and also carotenoids are the photosensitive pigments involved in phase shifting (Hastings and Sweeney, 1960). Thus, photosynthesis may be blocked

continuously, and the clock continues in the proper phase and with the characteristic free-running period.

Stopping photosynthesis for a while is similar to a dark period, insofar as products of photosynthesis are concerned. An actual dark pulse causes phase shift; inhibiting photosynthesis does not. Conversely, we see that photosynthesis need not be occurring during a light pulse in order for the light to cause a phase shift.

Photosynthesis, therefore, is an example of a clock-controlled process which apparently has little or no feedback to the clock. We may thus distinguish clock-controlled chemical changes from the biochemistry of the clock itself (Hastings and Sweeney, 1958).

Finally, we may note the lack of effect with a number of other compounds of interest: gibberellin, kinetin, chlorpromazine, and urethane. Azide, not shown in Fig. 9–8, is similarly ineffective at a concentration of 4×10^{-4} *M*.

Conclusions can be most readily made at this time with regard to biochemical systems which are *not* directly involved in clock function. However, we have offered unequivocal evidence for the proposition that we may observe chemically induced phase shifts. But, as in previous studies of this kind, the mechanism and specificity of the reaction is not clear. Care must be observed in such experiments with respect to design and interpretation, and with particular attention to the question of reproductibility. It is to be expected that additional studies concerning various aspects of the biochemistry of rhythmic systems will be of value.

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