NUCLEAR AND CYTOPLASMIC FACTORS CONTROLLING ENZYMATIC CONSTITUTION

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Modern physiological research has emphasized the predominant role of enzymes in controlling cellular processes. On the basis of overwhelming evidence we accept as established the fact that a cell can do whatever it does by virtue of the enzymes it contains. Genetics, on the other hand, has provided us with another set of units, genes, which are critical in determining cell properties. It is customary to synthesize the results of these two biological disciplines in terms of a relationship between their fundamental units. Thus, it is commonly assumed that the genotype determines the phenotype by virtue of genic control over enzyme synthesis. On this basis, a mutant would differ from the normal type because the introduction of an atypical gene would result in a modification of the cytoplasmic enzymatic constitution.

The question now naturally arises, what is really meant by such statements as "genes control enzymes" in terms of the mechanism and extent of such control. It is clear that the gene must in some manner influence one or more of the steps in the chain of reactions leading to the transformation of protein into some specific enzyme. Before one could profitably speculate, therefore, on the mechanism of gene-enzyme relationships, it would seem essential to obtain some information on how proteins are transformed into enzymes and what connections such transformations have with other metabolic processes. Some of the basic issues of the problem may be posed in terms of the following questions:

(1) How are enzymes formed in cells?
(2) What mechanisms are available for the maintenance of normal enzymatic constitution?
(3) How far and in what directions can the enzymatic make-up of a cell with a given genome be modified experimentally?
(4) How does this enzymatic variation depend on the genome?

The present paper will attempt to summarize and interpret the results of experiments with adaptive enzymes in yeast designed to obtain information on these and related problems.

Enzymatic Adaptation

The phenomenon of enzymatic adaptation may be simply stated in the following terms: a population of cells placed in contact with some substrate acquires, after the lapse of some time, the enzymes necessary to metabolize the added substrate. The removal of substrate leads to the disappearance of the enzyme system it evoked. So widespread is this phenomenon that it is now customary to follow Karström (*6*) in designating as "adaptive" those enzymes that are produced as a specific response to the presence of the homologous substrate. Such enzymes are differentiated from the "constitutive" ones, which are presumed to be synthesized by the cell at all times regardless of the presence or absence of their homologous substrates.

It has been noted previously (*19, 20*) that the reality of the qualitative difference implied by such a division of enzymes into two classes may well be questioned. This point will be returned to later and discussed briefly in the light of some more recent data. For the moment it is sufficient to point out that the existence of the phenomenon of enzymatic adaptation, by allowing for the controlled variation of enzyme content with the aid of substrate, provides a unique opportunity for an experimental analysis of some of the problems raised in the introduction.

Whether or not enzymatic adaptation can be used as a tool for the analysis of the gene-enzyme problem depends on the possibility of performing these enzyme-inducing experiments against a controlled genetic background. The realization of this possibility was afforded only recently through the fundamental work of Winge and Laustsen (*35, 36, 37*) in Denmark and of the Lindegrens (*9, 10, 11*) in this country on the genetics and life cycle of the yeasts.

Previous papers (*29, 27*) have dealt with and defined the conditions necessary for the analysis of the adaptive process against a constant genome. These results emphasized the importance of employing known diploid strains in which the haplophase could be suppressed whenever genetic constancy was desired during the course of an experiment. Haplophase cultures are uncontrollably variable with respect to both biochemical and morphological properties. Such cultures were, however, extremely useful since they permitted the demonstration (*28*) that capacity to form enzyme in response to substrate is, like other genetically controlled characters, subject to mutational changes. Mutations resulting in both the loss and gain of such characters were observed in haploid clones.

The Genetics of Enzymatic Adaptability

If a better understanding of the nature and extent of the genic control over enzymatic adaptability was to be attained, it was essential to study the

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mechanism of its transmission through crosses of various kinds. Winge and Laustsen (*37*) were the first to provide information of this nature. They used *S. validus* and *S. mandshuricus*, both of which possess the ability to ferment melibiose. Hybrids were created between these forms and others incapable of fermenting this disaccharide. Their results led them to state that "the presence of a specific enzyme is dominant to its absence in all the instances studied."

These pioneering experiments of Winge and Laustsen were of primary importance in opening up the possibility of a more direct experimental approach to the gene-enzyme problem. Usually genetic experiments study the transmission of such characters as color, shape, or the presence or absence of complete structures. These, of course, involve enzyme activity. However, they usually are the end results of many enzyme-controlled reactions. Any conclusions, drawn from such data, about the nature of the relation between gene and enzyme are necessarily indirect. Here for the first time the transmission of a well-defined enzyme, rather than the products of enzymatic activity, was being analyzed genetically. The necessity for extending these results in several directions was immediately obvious. It seemed desirable to perform experiments which would characterize the phenotype of the two haploids used in the mating, as well as to examine the segregation of the fermentative property in the haploid spores derived from the hybrid. Another point these experiments raised is the following; enzymes are cytoplasmic constituents, and hence the dominance observed could arise from the fact that the enzyme was carried into the cytoplasm of the hybrid as a result of the fusion of the two mating haploids.

Certain features of the mating method employed by Winge and Laustsen made it difficult to analyze some of these questions. It consisted of placing the two spores in contact with each other and allowing the resulting hybrid to grow. The resulting clone was then tested for the desired character. Since the two haploids are consumed in the process, examination of their phenotype is impossible.

The use of the hybridizing procedure developed by Lindegren and Lindegren (*10*) permitted the further genetic analysis of the inheritance mechanism, In this method, hybrids are produced by mixing haplophase cultures. Since only part of the culture is needed for the mating, the remainder can be used to determine the characteristics of the parent strain. Portions of the clone-culture are also available for back-crossing or mating to other clones of interest.

A genetic analysis of melibiose inheritance was made by Lindegren, Spiegelman, and Lindegren (*12*), using this hybridization procedure and employing *S. cerevisiae*, which cannot form melibiase, and *S. carlsbergensis*, which can. In all, 175 progenies of the interspecific and of related progenies were examined. Table 1 gives some typical results. All haploid segregants from *S. carlsbergensis* could form melibiase, whereas all spores derived from *S. cerevisiae* failed to do so, indicating a homozygous condition for the presence and absence of this character in the two strains.

Hybrid I, which was formed by crossing a carlsbergensis haploid with a cerevisiae one, was phenotypically positive, confirming the dominant nature of the fermentative capacity. When asci from such a mating were dissected, three different types were found. Out of 6 asci, from each of which all four

TABLE 1. FERMENTATION OF MELIBIOSE BY PROGENY OF VARIOUS CROSSES BETWEEN S. CEREVISIAE AND S. CARLSBERGENSIS

Four spores were removed from each ascus and designated arbitrarily as A, B, C, D. Plus and minus signs indicate, respectively, capacity to form melibiase and inability to do so.

Diploid	Phenotype of Copulants	Phenotype of Diploid	Phenotype of Haploid Segregants A	B	C	D
S. carlsbergensis		+	+	+	+	+
S. cerevisiae		−	−	−	−	−
Hybrid I	+×−	+	+	+	+	+
			+	+	+	−
			+	+	−	−
Hybrid IV	+×−	+	+	+	−	−

spores were recovered and tested, 3 behaved like the original *S. carlsbergensis*, yielding all four segregants as positive; 2 asci yielded 3 positives and 1 negative; and 1 ascus produced the 1:1 ratio of positives to negatives expected of a heterozygote segregating a single dominant gene. Essentially the same results are obtained from any hybrid involving the mating of one of the original carlsbergensis segregants to a negative haploid, the origin of the latter apparently not influencing the results.

When a hybrid (Hybrid IV of Table 1) is formed by mating a positive spore derived as a segregant from Hybrid I with one of the negative haploids from *S. cerevisiae*, the resulting diploid, although phenotypically positive like Hybrid I, always yields a 1:1 ratio of positives to negatives. The peculiar and unexpected distribution of phenotypes observed to occur in Hybrid I may be explained in the following three ways:

(1) Only one gene actually segregates, but the expected 1:1 Mendelian ratio is obscured by mutations of some of the negative spores to positives.

(2) Only one gene actually segregates, but the expected 1:1 Mendelian ratio is obscured by cytoplasmic components originating from the positive spore, which can form the enzyme in the absence of the gene.

(3) Two or more genes, either one of which can mediate the formation of the melibiase, are segregated.

The first explanation seems to be quite definitely ruled out by the fact that no *S. cerevisiae* haploid was ever observed to mutate to melibiose fermentation, even under conditions of long-continued and intensive selection for such mutants. As further evidence against this hypothesis we may cite the 1:1 ratios always observed with hybrids similar to IV, in the segregants of which the same tendency towards such mutations should obtain. Either one of the last two explanations would adequately explain the data. However, as will be shown in the following section, the accumulated evidence strongly supports the cyptoplasmic-factor hypothesis.

Subsequently, similar experiments were done (*7, 8*) involving another adaptive enzyme, galactozymase. For these experiments, *S. bayanus*, which cannot adapt to ferment galactose, and *S. cerevisiae*, which can, were used. These experiments were complicated by illegitimate diploidization, which made uncertain the origin of sporulating diploids recovered from the mixture of the two haplophases since they need not have resulted from a hybrid mating. Fortunately, another genetic marker was present to mitigate this situation. *S. bayanus* produces large cylindrical cells in both the haplophase and the diplophase, whereas the haploid cells of *S. cerevisiae* are small and round. The hybrid between these two was large and cylindrical, showing that the bayanus type cell is dominant. The segregation of cylindrical versus round cells from such hybrids was regularly Mendelian, thus permitting the distinction between legitimate and illegitimate diploids.

The results of the segregation of the first interspecific hybrid (analogous to Hybrid I of Table 1) again gave asci all of whose spores possessed fermentative capacity. The fact that the same spores which segregated the cell-type character in a perfectly normal Mendelian fashion did not segregate the enzyme served to emphasize the peculiarity in the inheritance of the enzymatic character. Again, as in the case of melibiase inheritance, either the cytoplasmic hypothesis or the multiple-gene hypothesis could explain the data obtained with the legitimate hybrids and their segregants.

The Extent of Gene Control of Enzyme Formation

In the introductory paragraphs it was pointed out that one of the basic issues of the gene-enzyme problem is the extent of the control exerted by a gene over the enzyme whose synthesis it is presumed to determine. It is clear from the very existence of the phenomenon of enzymatic adaptation that this control is not unlimited. It is evident, for example, that the possession by *S. cerevisiae* of the gene required for the formation of the enzyme galactozymase does not alone guarantee that this enzyme will be found in the cell. The presence of the specific substrate, galactose, is also required.

The question remains whether intervention by the appropriate gene is necessary every time a molecule of enzyme is formed. In view of the second hypothesis offered for the segregation experiments described in the previous section, the possibility clearly exists that such direct genic intervention need not be necessary.

It was noted in an earlier paper (*19*) that a careful analysis of the kinetics of adaptation could provide data which would be helpful in deciding the relative role of the gene during the formation of an enzyme.

The usual description of gene action assumes that the gene mediates directly the production of the enzyme it controls. From this point of view, every replication of every enzyme molecule would require the intervention of the appropriate gene. On this basis one would ascribe the increase in enzyme in the presence of substrate to the stabilizing influence of the substrate on the enzyme. We may picture this mechanism by the following reaction diagram.

$$\begin{array}{c} G_1 \\ \downarrow k \\ P_r \underset{k'}{\rightleftarrows} E_1 \end{array} \qquad (1)$$

Here P_r is the immediate protein precursor, whose transformation yields enzyme, the activity of which is measured. The velocity constant of the transformation from P_r to E_1 is k, and its magnitude is determined by the gene controlling the reaction. The enzyme E_1 is very unstable, however, and reverts to P_r quickly, the velocity constant of the back reaction, k′, being very much larger than that of the forward one. Under such conditions only very small amounts of enzyme would accumulate in the cell. We now assume that substrate S stabilizes E_1, and that, in the presence of excess substrate, E_1S is formed predominantly. This effectively lowers the value of k′. Reaction diagram (1) and the above assumption predict, then, that in the presence of substrate the enzyme activity should increase with time according to the following equation:

$$E = \bar{P}(1 - e^{-kt}) \qquad (2)$$

where $\bar{P}$ is the total amount of enzyme finally formed. According to (2), adaptation curves should always be concave to the time axis, and the observed rate of increase in enzyme activity should be greatest at the onset and decrease continuously until maximal activity is reached.

In the course of examining the appearance of various adaptive enzyme systems (galactozymase, maltase, melibiase, hydrogenlyase), well over 1200 adaptation curves have been obtained. In no case does the activity curve resemble the course pre-

dicted by equation (2). In all instances, the initial part of the curve is characterized by a rising rate of enzyme formation. This is then followed by a declining rate portion, when presumably the protein being transformed into enzyme becomes limiting and is finally exhausted. These facts would rule out the simple mechanism suggested by reaction diagram (1).

The increasing rate of enzyme formation with increasing amount of enzyme suggested an obvious modification of the mechanism detailed in diagram (1). Retaining all the properties ascribed to the first mechanism, we add the additional one that the enzyme is part of a cytoplasmic self-duplicating mechanism, such that, once the enzyme is formed, further formation of enzyme molecules can proceed without the intervention of the gene. A mechanism of this sort would result in an autocatalytic transformation of P into E, and would predict that enzyme activity would increase with time according to the following relation:

$$E = \frac{\overline{P}}{1 + e^{a-kt}} \qquad (3)$$

where a is a constant depending on initial conditions and the other symbols have their usual meanings. Table 2 reproduces representative results for two

TABLE 2. KINETICS OF ADAPTATION

The calculated values were determined by equation (3) on the assumption of an autocatalytic process in which the velocity of enzyme synthesis is a function of the amount of enzyme present.

Melibiase Activity			Galactozymase Activity		
Minutes	Calculated	Observed	Minutes	Calculated	Observed
60	12	8	60	4	2
90	26	28	90	11	10
120	56	51	120	26	25
150	99	98	150	48	48
180	150	149	180	87	91
240	218	216	240	152	156
300	237	238	300	181	181

enzymes when the observed values of enzyme activity in an adapting culture are compared with those calculated with the aid of equation (3). There is no doubt that the data lend support to the cytoplasmic self-duplication hypothesis.

CYTOPLASMIC DUPLICATION OF AN ENZYME IN THE ABSENCE OF THE GENE

An important and critical prediction stemming from the self-duplication mechanism is one that is explicitly stated in the second of the three explanations offered previously for the atypical segregation of enzyme activities; *viz.*, once the process of enzyme synthesis is started it should be able to proceed in the absence of the gene that initiated it. A test of this prediction is feasible only in a case where the initiating gene can be eliminated. An opportunity of performing this experiment was offered by the existence of such hybrids as IV in Table 1, which regularly segregate the potentiality for enzyme formation. Under such conditions one could be relatively certain that only two out of every four spores carried the gene responsible for the fermentative capacity.

It is evident from the discussion of the phenomenon of adaptation and the role of substrate in it that the case of exhibiting any self-duplicating cytoplasmic system involved in enzyme synthesis would be greatly facilitated by the presence of substrate. This suggested that a test of this hypothesis could be made by comparing the segregability of fermentative character in the presence and absence of substrate.

Such experiments were carried out by Spiegelman, Lindegren, and Lindegren (*30*). A hybrid

TABLE 3. SEGREGATION UNDER NORMAL CONDITIONS OF ABILITY TO FORM MELIBIASE FROM A HYBRID SIMILAR TO HYBRID IV OF TABLE 1

Ascus Number	Spores A	B	C	D
8	+	+	−	−
9	+	−	+	−
10	+	−	−	+
11	+	−	−	+
12	−	−	+	+
13	−	+	+	−
14	+	−	−	+
15	−	+	−	+
16	+	−	+	−
17	−	+	−	+

TABLE 4. SEGREGATION IN THE PRESENCE OF MELIBIOSE OF THE ABILITY TO FORM MELIBIASE

Ascus Number	Spores A	B	C	D
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	−	−

similar to IV of Table 1 from *S. cerevisiae* × *S. carlsbergensis* was employed. This hybrid was formed in the usual manner and allowed to sporulate, the asci dissected and tested for the character. The results are shown in Table 3. It is clear that the regular 1:1 Mendelian ratio is obtained, characteristic of a heterozygote segregating a dominant gene. It should be noted for later reference that, in handling asci 8 and 9, the agar in which the dissected spores were planted contained melibiose.

In these experiments, the cells came into contact with melibiose for the first time in the test for adaptability after segregation had already taken place. Using exactly the same haploids, the same cross was carried out in the presence of melibiose. Segregations were also allowed to occur in the presence of this sugar. The data on the phenotypes of the haploid segregants obtained in this manner are summarized in Table 4. With the exception of ascus 7, identical heterozygotes treated with melibiose yielded four adaptable spores from each ascus.

The segregation results obtained in the absence of substrate (Table 3) prove that only 2 spores from each tetrad in asci 1-6, inclusive, of Table 4 contain the specific gene responsible for the initiation of the adaptation towards the fermentation of melibiose. Despite this, all four spores from these tetrads produced haplophase cultures which fermented melibiose. It would thus appear that with the aid of substrate we were successful here in obscuring what is normally a simple Mendelian segregation and duplicated the abnormal segregations earlier noted with the interspecific hybrid between carlsbergensis and cerevisiae.

It might be argued that, since all steps were carried out in the presence of melibiose, selection of adaptable mutants from haploids originally unable to ferment melibiose may have occurred. Several specific facts would appear to rule out this possibility: (1) during the testing of many haploid segregants from *S. cerevisiae*, all of which are negative, no mutation to an adaptable type has ever been observed, whether melibiose is present or not; (2) the same is true of negative haploids derived from heterozygous hybrids; and (3) asci 8 and 9, whose segregants were planted on melibiose, yielded the standard 1:1 ratio.

We are thus led to suppose that the cultures from two spores of each tetrad from the first 6 asci in Table 4 lacked the gene and were able to ferment melibiose only owing to the enzyme-forming factors present in the cytoplasm. If this is correct, it might be expected that removal of the melibiose would lead not only to the disappearance of the melibiase but also, if the cytoplasmic factors were sufficiently labile, to the eventual loss of readaptability in those two out of every four spores of asci 1-6 that lacked the gene.

Such experiments were performed, and, to exclude the complications of mutation away from adaptability, nondividing cultures, suspended in M/15 KH_2PO_4 containing glucose, were used. Portions of all 24 adapted cultures originating from the first 6 asci were treated in this way in the absence of melibiose. In varying periods of time, ranging from 7 to 20 days, all these suspensions lost the ability to evolve significant amounts of CO_2 anaerobically from melibiose. Twenty-four hours after a suspension showed no significant traces of enzyme activity, a sample was removed and incubated with melibiose aerobically to test for readaptability. At the same time its ability to ferment glucose was also examined. This was done to avoid testing cells whose physiological condition was seriously impaired by the long and vigorous shaking in a relatively unfavorable environment. With cells unable to ferment glucose, the inability to ferment melibiose would be difficult to interpret. Three suspensions of the original 24 were eliminated on this basis. The data on the asci all four of whose clones survived the treatment are given in Table 5. The removal of the melibiose leads to the disappearance of the cytoplasmic factors responsible for the appearance of the enzymatic character in all four segregants and the reappearance of the expected Mendelian ratio of 1:1 on readaptation.

TABLE 5. READAPTABILITY OF SPORES OBTAINED BY MATINGS IN THE PRESENCE OF MELIBIOSE AFTER HAVING LOST ALL MELIBIASE ACTIVITY

Ascus Number	Spores A	B	C	D
1	+	−	+	−
2	−	−	+	+
4	+	+	−	−
6	+	+	−	−

This appearance of enzyme in the cytoplasm of cells apparently not carrying the gene could have been due to a passive transfer and subsequent retention of the enzyme which was induced by substrate in the cytoplasm of the parent diploid. That this was not the case was shown by experiments in which portions of all clones were allowed to fall to low enzyme-activity values ($Q^N_{CO_2}$ between 1.8 and 10.1) in the absence of melibiose. Aliquots were then removed and incubated with melibiose, and evidence for regeneration of activity followed at intervals. In all cases, including those that eventually lost their ability to adapt and therefore presumably lacked the gene, marked increases in activity were observed. Activity values greater than 100 were attained in every instance. Furthermore, all the clones were carried in standard media with melibiose and were tested at weekly intervals. At the end of three months they could all without exception ferment melibiose at rates equal to or greater than the ones they started with. This period is equivalent to over 2000 cell generations. It is evident from these results that the enzyme is actually synthesized in the cytoplasm of these cells in the absence of the genes.

In view of these experiments, the atypical segregation values of the interspecific hybrid, and the kinetics of the adaptive process, it seems difficult to avoid adopting the cytoplasmic hypothesis. All three types of results can easily be understood in these terms.

We may therefore explain the effect of melibiose on the inheritance of the corresponding enzyme as follows: because the mating is performed in the presence of melibiose, the cytoplasm of the haploid carrying the gene is packed with melibiase. Since both copulating haploids contribute cytoplasm equally to the diploid, it starts out with some enzyme and builds up more, since it has the gene also. Because sporulation occurs in the presence of melibiose and because the sporulation period is characterized by growth and considerable storage, the enzyme molecules are stabilized and possibly increased in amount. Each of the four haploid segregants derives its cytoplasm from the diploid hybrid, and it follows that each will have enzyme molecules in its cytoplasm no matter what its genetic constitution chances to be. Finally the enzyme molecules are stabilized and are duplicated in the cytoplasm of the clones derived from the spores which did not carry the gene as long as they are kept in contact with substrate.

The fact that Hybrid IV normally gives typical Mendelian segregation of the capacity to form enzyme, whereas Hybrid I does not, may be explained by the fact that the carlsbergensis cytoplasm has been twice diluted with cerevisiae cytoplasm. The cytoplasmic factors that obscure the Mendelian picture of Hybrid I have thus apparently been diluted in Hybrid IV to the point where they can no longer play critical roles in determining potentiality for melibiase formation.

The cytoplasmic hypothesis received further support from subsequent experiments (*7, 8*) involving galactozymase. Results analogous to those just described with melibiase were obtained with the *S. cerevisiae* × *S. bayanus* pedigree. Some asci which yielded four adaptable spores were shown to be heterozygous for the gene required to initiate galactozymase production, since on growing the four segregant clones in the absence of substrate two of them irreversibly lost the capacity to adapt to galactose.

Another series of experiments offers indirect support for the importance of cytoplasmic factors in the transmission of enzymatic adaptability, by making the polygenic hypothesis offered as an alternative seem very unlikely. In these experiments, the same *S. carlsbergensis* haplophase employed previously was crossed with a haplophase strain lacking the ability to adapt to either galactose or melibiose. Most of the asci obtained from this cross yielded a 1:1 ratio for the capacity to form galactozymase as well as a 1:1 ratio for the ability to form melibiase, and, furthermore, both characters segregated completely independently. However, a few of the asci obtained produced four spores, all of which exhibited the capacity to form *both* galactozymase and melibiase. This latter result would be difficult to understand on a genic basis but would be expected if in such asci the proper Mendelian ratio for the two characters was being obscured by the transfer of cytoplasmic components.

The general picture which thus emerges of enzyme formation and the relation of the gene to this process may be described as follows: enzymes may be produced by cytoplasmic components which possess the capacity for self-duplication. In such instances the sole function assignable to the gene is the initiation of the enzyme synthesis. The initiation could be effected by virtue of a low but ever-present capacity of the gene to mediate the production of a few of the cytoplasmic self-duplicating units involved in the formation of the enzyme. The presence of the gene would thus retain indefinitely for the cell the capacity to adapt by forming a large number of enzyme molecules when it comes in contact with the proper substrate.

The introduction of a cytoplasmic component as critically determining in enzyme formation naturally raises a whole host of new problems. Of primary interest are the following two questions:

(1) What is the nature of this cytoplasmic component and what relation does it have to the homologous gene and enzyme?

(2) How does it intervene in the formation of the enzyme?

Actually when the findings on melibiase were reported (*30, 19*) it was suggested that the enzyme itself was the self-duplicating system. Subsequently, Lindegren (*7*) identified the enzyme with the self-duplicating cytoplasmic unit which he called the "cytogene." It is clear, however, that the evidence thus far presented does not permit a decisive conclusion as to whether the cytoplasmic self-duplicating unit is the enzyme itself or something mediating its formation.

It soon became apparent that an extension of these results in an attempt to answer questions regarding the identity of this cytoplasmic unit and its role in enzyme formation must be preceded by the acquisition of some insight into the physiological and biochemical details of the process. Without further information of this kind on the pathway or mechanism of enzyme formation it seemed impossible to think fruitfully about these aspects of the problem.

An adequate description of the mechanism of enzyme formation must necessarily provide answers to the following questions, among others:

(1) What is the relation of enzyme formation to over-all cellular metabolism?

(2) What is the source of protein for the formation of an enzyme?

(3) What is the source of energy required for its synthesis?

The Relation of Enzyme Formation to Cellular Metabolism

Earlier workers had already established the fact that enzymatic adaptation requires physiologically

functioning cells. Attempts to obtain adaptation with nonviable cells, or with cells whose physiology has been seriously interfered with by various reagents, have uniformly met with failure. The sole exception was the report by Abderhalden (*1*) that he had obtained adaptation to galactose fermentation with dried dead yeast cells. This experiment has never been successfully repeated, and his failure to check the possibility that the adaptation may have been due to the growth of a few surviving cells throws grave doubt on the validity of his conclusions. Von Euler and Nilsson (*34*) reported that adaptation to galactose would not occur when the cells were suspended in ordinary phosphate solution containing galactose, and they maintained that the addition of "Z" factor was necessary. These observations were in direct contradiction to the earlier experiments of Dienert (*5*), who observed adaptation with washed cells suspended in ordinary phosphate.

Our own experience agrees with that of Dienert. All our adaptations were carried out with thoroughly washed cells suspended in phosphate solutions of substrate. These experiments establish the important fact that enzymes can form in yeast cells in the absence of an external source of nitrogen. It must be noted, however, that the maximal level of enzymatic activity reached under such conditions is about half or less that attained when the adaptation is carried out in the presence of an exogenous source of nitrogen.

Yeasts are cells which possess both an aerobic and an anaerobic metabolic pathway, either of which can be used for purposes of growth. The first problem that was experimentally analyzed was to see whether both of these pathways could be utilized for enzyme formation. Evidence on this question was conflicting. Stephenson and Yudkin (*32*) state that oxygen has no influence on the adaptive process. On the other hand, Schultz, Atkin, and Frey (*17*), who also investigated the adaptation of yeast to galactose fermentation, reported a very striking effect of oxygen. This question was reinvestigated (*20*) with the aid of five known diploid strains. Two of these strains were unable to initiate the formation of galactozymase if they experienced only anaerobic contact with galactose, while the others could form the enzyme anaerobically. However, the rate of anaerobic adaptation in these latter cases was only 1/40 of that attained in the presence of oxygen, and furthermore, this anaerobic adaptability could easily be abolished by dissimilation for a few hours. This same relative inefficiency of anaerobic incubation with substrate in forming the homologous enzyme was also observed with maltose and melibiose.

It might be supposed that anaerobic inadaptability could be explained by the inability of the cell to use anaerobic metabolism for enzyme synthesis. This was tested with one of the strains, which was completely incapable of anaerobic adaptation. The changes in enzyme activity under anaerobic conditions were followed subsequent to periods of aerobic incubation with galactose. It was found that if sufficient aerobic contact with the substrate was allowed, so as to attain an enzyme activity equal to or greater than a $Q^N_{CO_2}$ of 15, further enzyme was formed during a subsequent period of anaerobic contact with the substrate.

These experiments made it quite evident that the anaerobic metabolic pathway could also be used for enzyme formation. The condition that apparently had to be met here was that sufficient enzyme be formed first aerobically so that the cell would be in a position to utilize the energy content of the galactose molecule anaerobically at a rate adequate for the synthesis of more enzyme. We thus have here the interesting biological situation that the substrate not only stimulates the formation of the enzyme but in addition acts as the ultimate source of energy for the synthesis.

The critical role of an energy supply from the metabolic cycle for the adaptive process is illustrated by several other facts. It has been shown (*31*) that for these strains, in common with others (see *33*), the endogenous reserves are not fermentable and can only be used in the presence of oxygen. Such cells, when placed under anaerobic conditions in the absence of a fermentable substrate, do not metabolize. It is therefore not surprising that the capacity to form enzymes anaerobically is so poor.

The same viewpoint suggests that aerobic adaptation occurred with such ease because with oxygen present the cell could draw on the energy coming from the oxidation of the endogenous reserves for the synthetic activity. To confirm this, experiments were performed (*19*) to examine adaptation times (time required to reach a $Q^N_{CO_2}$ value of 100) when galactose was added at different levels of the endogenous respiration. It was found that little difference in adaptation times was encountered as long as the galactose was added during the early period of the endogenous material before the declining-rate phase was attained. Subsequent to this, adaptation times increased sharply. The surprising fact to emerge, however, was that adaptation, although delayed, still took place when the galactose was added after all metabolic activity had ceased as measured by oxygen uptake and CO_2 output. These experiments seem to indicate that adaptation can occur after all the oxidizable reserves have been exhausted and there is no apparent source of energy. This puzzling situation was clarified, however, by the finding (*21*) that the galactose itself is oxidized by some enzyme system other than the fermentative one, which forms later under its stimulation.

It is evident from these experiments that the presence of a functional aerobic or anaerobic metabolic system is necessary for the formation of the enzyme.

The Source of Protein for Enzyme Formation and the Interconvertibility of Enzymes

It has already been noted that the presence of an exogenous source of nitrogen raises the maximal attainable level of enzyme activity. This seemed to indicate that the cell could use externally supplied nitrogen for the purpose of increasing its activity. However, the fact that enzyme activity can appear in a nitrogen-free environment implies that there exist endogenous sources of protein, which are available for transformation into enzyme protein. It has generally been supposed that most cells contain very little "storage protein" and that at any given moment most of the protein is functional, enzymatic or otherwise. There existed the possibility, therefore, that when a cell is forced to form a new enzyme it may draw upon existent enzymes as a source of protein. Presumably an interaction of this kind would be most pronounced between unstabilized labile enzymes—i.e., between members of the so-called adaptive group of enzymes.

This possibility was tested (*23*) in the following manner: one group of cells was fully adapted to galactose and another to maltose. Then a comparison was made of the ability of galactose-adapted cells to form maltase when incubated with an equal mixture of galactose and maltose in the presence and absence of an external source of nitrogen. A similar comparison was made of the ability of maltose-adapted cells to form galactozymase. If such an interaction between enzymes of a cell exists,

Table 6. Competitive Interaction between Adaptive Enzymes

Enzyme	Maximal Activities ($Q^N_{CO_2}$)			
	Without External Nitrogen		With External Nitrogen	
	Cells adapted to galactose	Cells adapted to maltose	Cells adapted to galactose	Cells adapted to maltose
Galacto-zymase	163	101	232	227
Maltase	86	182	229	248

inducing to maximal activity one enzyme of a cell and stabilizing it with its substrate should influence the ability of another enzyme to establish itself in the same cell. The results of a typical experiment, which are given in Table 6, leave no doubt that such an interaction exists. The presence in a cell of maltase depresses considerably the attainable level of galactozymase, and vice versa. Both inhibitions are effectively abolished by the addition of a nitrogen source, which apparently permits the cell to form new enzyme without drawing upon existent proteins.

Another way of studying this phenomenon of interaction is to actually follow what is happening to the existent enzymes of a cell while a new enzyme is being formed. Thus if one starts out with a culture fully adapted to maltose ($Q^N_{CO_2}$ = 240) and adapts to galactose one finds that as the galactozymase activity increases there is a sharp drop in maltase activity so that within four hours practically all of it is gone.

A test of the generality of this type of interaction between cytoplasmic enzymes was made by seeing whether the induction of an adaptive enzyme resulted in any loss of activity in a constitutive one. For this purpose a culture was adapted to galactose,

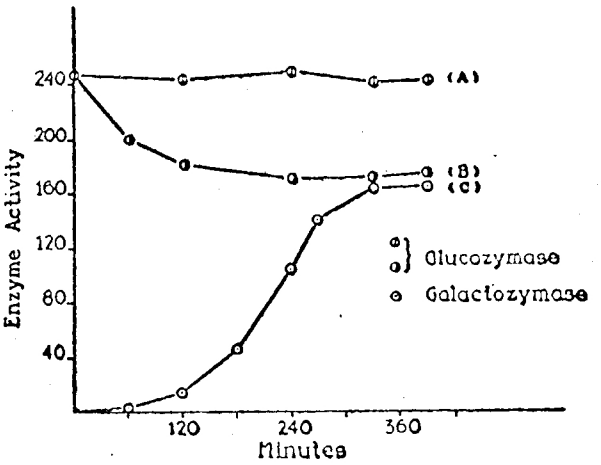

Fig. 1. Interaction between galactozymase and glucozymase during adaptation to galactose in the absence of an exogenous source of nitrogen. Curve (A) is a control, demonstrating stability of glucozymase in a nonadapting culture.

and at intervals samples were removed for simultaneous determinations of glucozymase and galactozymase activities. The results of an experiment of this type are depicted in Fig. 1. The same results are obtained here as in the case of interaction between two adaptive enzymes. To ascertain whether a question of nitrogen supply is involved here also, a control experiment was run in which the adaptation was carried out in the presence of nitrogen. Fig. 3 gives the results. It will be noted that the adaptation rate is increased and the maximal enzyme activity level attained is greater; and, even more significantly, parallel measurements in glucozymase activity during the course of the adaptation show no change.

Thus these experiments provide some information on the question of the protein source during adaptation. If an external source of nitrogen is present this will be employed. In its absence, however, a cell will use existent cellular enzymes as a source of protein to form the enzyme being induced by substrate.

The fact that a constitutive enzyme was observed to disappear during the formation of an adaptive one throws serious doubt on the basis for

the distinction made in the literature on enzyme variation between the two types of enzymes. It emphasizes once more that the only difference that exists is in degree of stability and utilization. The importance of these findings resides in the fact that

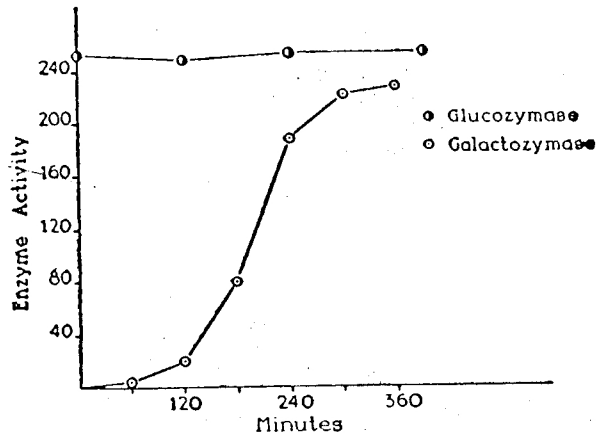

FIG. 2. Protective action of exogenous nitrogen source on glucozymase activity of an adapting culture.

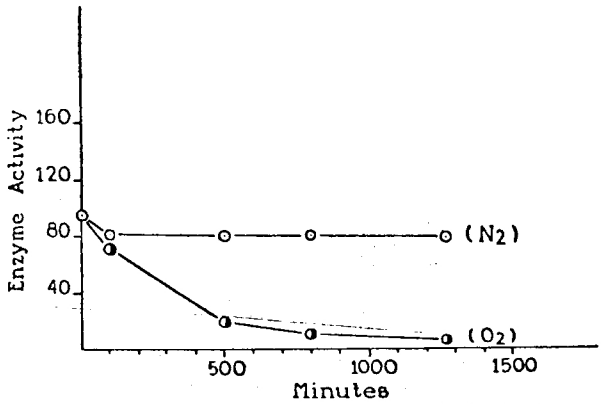

FIG. 3. Comparison of galactozymase stability in absence of substrate under aerobic and anaerobic conditions.

they naturally make it possible to extend the results obtained with adaptive enzymes to enzyme synthesis and maintenance in general.

THE RELATION OF ENZYME STABILITY TO THE SYNTHESIS OF OTHER ENZYMES

The finding that the formation of a new enzyme by a cell affects other enzymes suggested that the whole question of enzyme stability and maintenance must be looked at from a different point of view. Whether a particular enzyme survives in the cytoplasm during the course of an enzymatic change would, from this point of view, depend not only on its inherent instability but perhaps even more significantly on the ability of the units involved in its synthesis to compete with other such units for proteins.

An obvious consequence of this is that the stability of a particular enzyme at any given time would depend on the rate of enzyme turnover at that moment. Complete stabilization of enzymatic constitution could thus conceivably be attained if all metabolic activity could be stopped. This conclusion was tested with galactozymase, since it was felt that if this was true for an adaptive enzyme, the instability of which is well known, its generality would be more certain. It will be recalled that, under anaerobic conditions, no detectable metabolic activity can be observed in yeast suspension in the absence of a fermentable substrate. The experiment was therefore performed of testing the stability of galactozymase in oxygen and in nitrogen in the absence of substrate. The results are given in Fig. 3. Except for a slight drop at the beginning, complete stabilization of the enzyme was attained in the absence of metabolic activity. The same suspension in the same period of time lost almost all of its activity when allowed to metabolize its endogenous reserves.

In Fig. 4, the ability of a suspension to retain its galactozymase activity is compared during

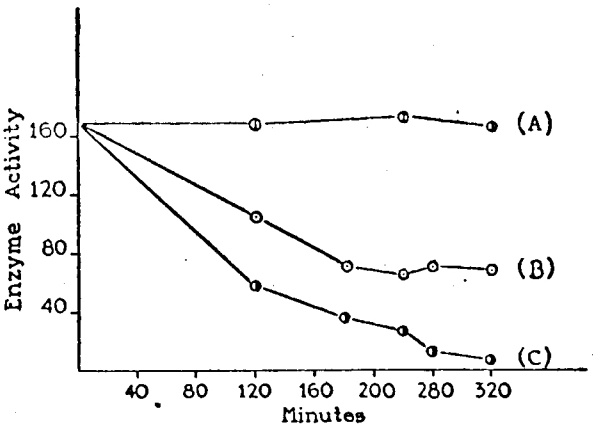

FIG. 4. Comparison of galactozymase stability in cells metabolizing (A) galactose, (B) endogenous reserves, (C) glucose.

endogenous and exogenous metabolism. It is seen that galactozymase is lost faster in the suspension metabolizing externally placed glucose. A control was run in which the same suspension was allowed to consume galactose. No enzyme activity was lost, indicating that substrate can effectively prevent such losses. Since exogenous metabolism of glucose is between two and three times as high as the maximal rate of endogenous metabolism, it is evident that these findings are consistent with the idea that the level of metabolic activity can affect enzyme stability.

Monod (*14*) in a series of extremely interesting and suggestive experiments on his "diauxie" phenomenon demonstrated a similar inhibitive action of a constitutive enzyme on a variety of adaptive sys-

tems. The fact that the same general conclusions were arrived at gains greater interest in view of the fact that this author used bacteria as his material and employed mainly growth rates as a measure of adaptation.

These results strongly support the concept that enzyme formation and disappearance are closely linked. One would predict from this that any inhibitor which could interfere with enzyme synthesis would freeze the existent enzymatic constitution independently of what substrate was being metabolized. The verification of this prediction will be presented in the next section. The experiment on stability in the absence of metabolic activity would suggest that the inability of a cell to maintain a particular enzyme while metabolizing another substrate is probably not due primarily to an "inherent instability" in this enzyme but rather to competitive interaction between enzyme-forming systems.

The Inhibition of Enzyme Formation

It has been pointed out previously that any procedure that seriously interferes with cell viability or prevents the cell from metabolizing results in a loss of enzymatic adaptability. This only tells us that adaptation, like other cellular synthetic processes, requires a properly functioning metabolic cycle to supply the energy for the proper reactions. It tells us nothing of the nature of the linkage between the adaptive process and the metabolic reactions.

One method of approaching this problem was to attempt to dissociate the process of adaptation from the over-all metabolic cycle. In such cells, where enzyme formation is prevented without affecting the over-all metabolic rate, one could hope to analyze the nature of this link. Dissociations of this kind between synthetic processes and metabolism have been accomplished with the aid of various compounds. Thus the ability of NaN_3 to prevent the utilization of metabolic energy for synthesis is quite general, having been demonstrated for such diverse processes as cell division, embryonic development, regeneration, and carbohydrate and ammonia assimilation. It was of some interest to determine whether enzymatic adaptation behaved in a manner similar to these synthetic processes with respect to inhibition by azide.

Since azide is a powerful inhibitor of aerobic metabolism, it was trivial to find that it inhibited aerobic formation of enzyme. Putting azide into the adapting medium is equivalent to establishing anaerobiosis, and we have already noted that anaerobic conditions, if instituted from the beginning, are effective inhibitors of enzyme formation. Of greater interest were the experiments (*22*) in which the effect of azide was tested on anaerobic synthesis of enzyme subsequent to a period of aerobic incubation with substrate. Fig. 5 describes such an experiment. Cells were incubated aerobically with galactose until the galactozymase activity reached a $Q^N_{CO_2}$ value of 40, after which anaerobiosis was instituted and incubation with the substrate continued. Control suspensions continued to increase their enzyme content in a manner depicted by the first three points and the dashed part of the curve. If at any point in the development of this enzyme activity azide is introduced in concentration of 5×10^{-3} M or higher, no further enzyme is formed

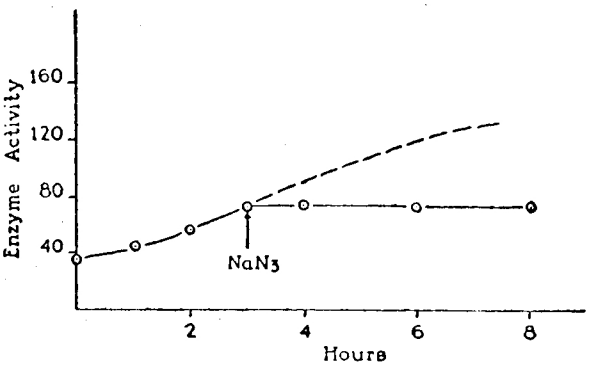

FIG. 5. Effect of NaN_3 (5×10^{-4} M) on anaerobic formation of galactozymase.

and the suspension continues to ferment the galactose at the rate attained at the time of the addition. Azide is also effective in preventing cells from utilizing the energy of other fermentable substrates for adaptive purposes. Schultz, Atkin, and Frey (*17*) have shown that adaptation to maltose fermentation can occur in the absence of oxygen providing a small amount of glucose is furnished. This utilization of the energy obtained from the glucose to form maltase is completely prevented by azide (unpublished experiments). It is also of interest to recall that assimilation of ammonia by yeast cells was shown by Winzler, Burk, and du Vigneaud (*39*) to be prevented by the same concentrations of azide.

Enzymatic adaptation thus appears to behave in a manner similar to other synthetic processes in that it is possible by the addition of azide to prevent its occurrence without affecting the measured over-all metabolism. It was pointed out previously that the ability of a cell to maintain a particular enzyme is probably closely linked with the intensity with which other enzymes are being synthesized. If azide can effectively prevent enzyme synthesis in general, it follows that this compound should prevent not only the synthesis of some enzyme, but also its disappearance, no matter what substrate the cell was metabolizing nor how fast it was doing this. Fig. 6 gives the results of an experiment testing this possibility. A galactose-adapted culture was allowed to consume glucose at maximal rate under anaerobic conditions. As shown by the broken line, the galactozymase activity disappears quite rapidly.

If at any time in this process, however, azide is added, further disappearance of this enzyme ceases immediately and the enzyme activity remains indefinitely at the level reached when the azide was added. The fact that the same compounds or conditions that inhibit enzyme formation also inhibit

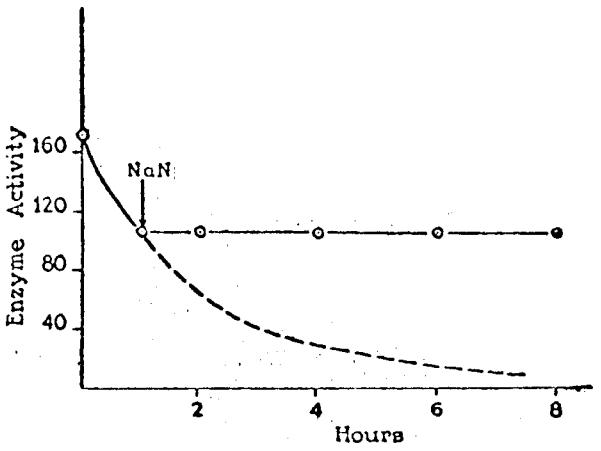

FIG. 6. Complete stabilization by NaN_3 (5×10^{-4} M) of galactozymase in cells metabolizing glucose.

enzyme loss makes it difficult to avoid accepting competitive interaction between cytoplasmic enzyme-synthesizing units as playing a critical role in determining cellular enzymatic constitution.

THE MECHANISM OF AZIDE INHIBITION OF ENZYME SYNTHESIS AND ITS RELATION TO PHOSPHORYLATION

The ability of azide to prevent the formation of enzyme without disturbing over-all metabolism obviously represented a system the analysis of which could provide a clue to the link between the metabolic energy cycle and the synthesis of enzymes. An attempt was therefore made to determine the mechanism of this inhibition.

On the assumption that P-bond energy as generated by the glycolytic system forms the primary source of energy for cell function and growth, experiments were undertaken (*26*) to examine the effect of NaN_3 on phosphorus metabolism, using radioactive phosphorus (P^{32}) as a tracer. All the experiments were done under anaerobic conditions. The procedure in these experiments was to suspend cells in an inorganic phosphate medium containing a known amount of tracer P and allow the cells to ferment glucose anaerobically, removing samples at intervals for radioactive and chemical analysis. The results of a typical experiment of this kind are given in Fig. 7. In this experiment 2×10^{-3} M NaN_3 was used. It is seen from the upper curve that the presence of this amount of azide did not disturb the ability of the suspension to metabolize, since both consumed glucose at precisely the same rate. However, whereas the control exhibited a relatively rapid uptake and exchange of inorganic phosphate, the experimental did not. Chemical analyses for total, inorganic, and organic phosphates, and radioactive examination of these fractions, confirmed these results. Azide is able to prevent the

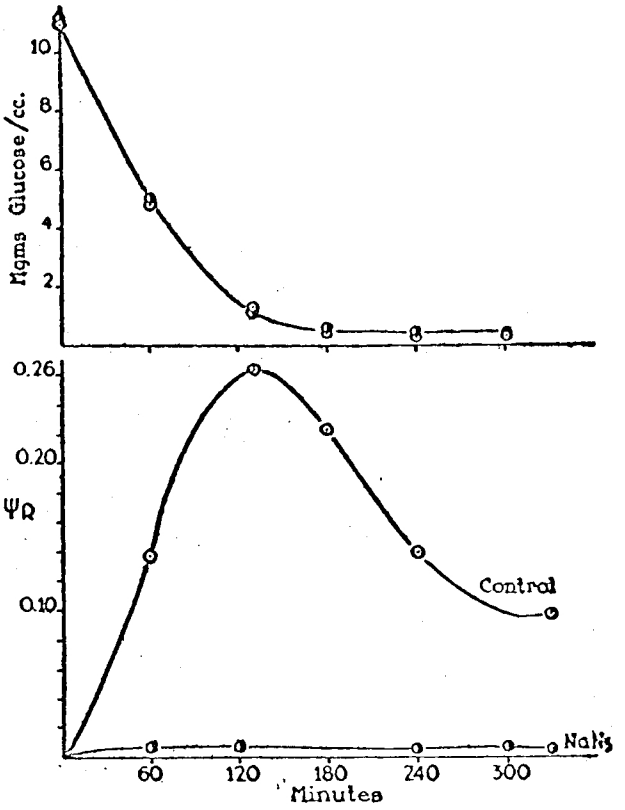

FIG. 7. Effect of NaN_3 (2.5×10^{-3} M) on P turnover during anaerobic glycolysis. Ordinate of lower curve represents ratio of specific activity of P inside the cell to that of the P outside. Upper curves describe consumption of glucose in the absence of NaN_3 (open circles) and in the presence of NaN_3 (2.5×10^{-3} M) (half-shaded circles).

accumulation and formation of organic phosphate bonds which normally accompany the metabolism of carbohydrates.

These results suggest that the capacity of NaN_3 to prevent cellular utilization of metabolic energy for enzyme synthesis (as well as other synthetic processes) resides in its ability to dissociate carbohydrate metabolism from the generation of energy-rich organic phosphate bonds.

Further experiments were performed (*24*) with the purpose of determining the site of the uncoupling of phosphorylation from carbohydrate metabolism in the presence of azide. According to the classical glycolytic mechanism, the formation of organic phosphate bonds from inorganic phosphate occurs by the entrance of inorganic P into

the carbohydrate cycle in two places: (1) phosphorolysis of glycogen; (2) the coupled oxidation and phosphorylation of glyceraldehyde phosphate to di-phosphoglyceric acid.

The involvement of the first step seemed unlikely, since no polysaccharide is synthesized by yeast cells in the presence of azide (*38*). Since it seemed most probable that the azide was affecting the second step, experiments were devised to examine the behavior of the glycolytic cycle at this level in the presence of azide. It is well known that iodoacetic acid (IAA) is a strong inhibitor of the enzyme (triose-phosphate oxidase) controlling this step. It was reasoned that if azide modified the phosphorylative mechanism at this step it might be expected that a parallel change in the sensitivity of the fermentation to IAA might appear, and such was actually found to be the case. A concentration of IAA (2×10^{-4} M) which was sufficient to inhibit fermentation completely within 10 minutes in the absence of azide took 180 minutes to bring the rate down to zero in the presence of 5×10^{-3} M NaN_3, and failed to affect the rate at all for the first 70 minutes. Lower concentrations of azide protected against IAA inhibition to a lesser extent. Higher concentrations continued to lengthen the period of protection until inhibitory concentrations of azide were reached, whereupon the protective action against IAA disappeared. It should also be noted that azide is completely unable to protect fermentation against the inhibitory action of fluoride, which poisons an enzyme controlling an entirely different step.

Nucleoprotein as a Controlling Element in Enzyme Synthesis

Any attempt at elucidating the mechanism of a biological synthesis must of necessity concern itself with the problem of the immediate donor of energy and substrate in the synthetic reaction. The experiments thus far described on the relation between phosphate metabolism and enzyme adaptation have dealt with the formation of organic phosphate bonds. They demonstrated that inhibition of the generation of these bonds is always accompanied by an inhibition of enzyme formation. This tells us only that organic phosphate bonds are required. It does not tell us which particular ones are critical nor does it tell us how they are used. The insufficiency of this knowledge is pointedly emphasized by experiments with dinitrophenol, which, as was independently shown by Monod (*15*), is also capable of preventing enzyme adaptation without inhibiting over-all oxidative metabolism. However, this compound does not interfere with organic phosphate esterification nearly so effectively as azide. Suppression of only 20% is obtained with dinitrophenol concentrations capable of preventing enzyme adaptation.

Clearly some information had to be obtained on what happened to the various organic phosphate compounds while a protein or enzyme was being synthesized. Experiments were designed (*25*) to obtain such information, again using radioactive phosphate. In these experiments the phosphate in the various fractions of the cells was tagged with tracer before the beginning of the experiment so that subsequent movement of the P from one fraction to another could be followed.

The most consistent correlation between phosphorus metabolism and protein or enzyme formation was found in the flow of phosphate *from* the nucleoprotein fraction (NP). This latter is the residue phosphate remaining after successive extractions with water, cold trichloroacetic acid, alcohol, and hot alcohol-ether (3:1).

The behavior of the phosphate in this fraction was followed under various conditions, employing P^{32}, in the following manner. Cells were grown in the usual media at 30° C. in the presence of P^{32} (activity, 5×10^5 cts./min./mg. P). This resulted in complete equilibration of the labeled phosphorus in all fractions. After 48 hours these cells were harvested, washed three times in unlabeled M/15 KH_2PO_4, resuspended in unlabeled M/15 KH_2PO_4 with 4% glucose, and allowed to ferment the carbohydrate under completely anaerobic conditions. No budding or increase in protein nitrogen is observed in such suspensions. Samples were withdrawn at intervals for activity measurements. It was found that within four hours about one-half of the total P content of the cells. The total activity was found that (except for 1 or 2%) this loss in activity could be completely accounted for in the acid-soluble fraction which forms about 50% of the total P content of the cells. The total activity (P^{32} content) as well as total P^{31} of the nucleoprotein fraction had actually increased slightly (8%) during this period, indicating flow of phosphate into this fraction. These data clearly showed that rapidly metabolizing but nondividing cells did not lose phosphate from the nucleoprotein (NP) fraction even though the major portion of the remaining phosphate was being rapidly equilibrated. Since activity of the phosphate in the acid-soluble fraction of such cells was about one-fourth that of the NP phosphate, they were favorable material for the further study of exchanges between the two fractions. Allowing such cells to ferment carbohydrate for longer periods of time (up to six hours) again left the total activity of the NP fraction unchanged, although the specific activity was decreased slightly owing to dilution by the flow of low-specific-activity phosphate from the acid-soluble fraction.

The entire behavior of the NP fraction was changed, however, when such cells were induced to form new protein either by adding ammonia or by forcing the synthesis of a new enzyme. The results obtained in a typical experiment are exemplified by

the data in Fig. 8. In this experiment cells were suspended in physiological saline containing: (a) glucose, (b) glucose + $(NH_4)_2SO_4$, (c) glucose + $(NH_4)_2SO_4$ + NaN_3, (d) glucose + $(NH_4)_2SO_4$ + dinitrophenol. The amount of $(NH_4)_2SO_4$ was equivalent in nitrogen to 50% of the nitrogen content of the yeast. The concentrations of NaN_3 and dinitrophenol were 5×10^{-3} and 5×10^{-4} respectively, sufficient to completely inhibit enzyme formation.

It will be noted that with glucose alone there was no change in activity, whereas when ammonia was present, with consequent budding, the nucleoprotein P dropped to 38% of its original total activity,

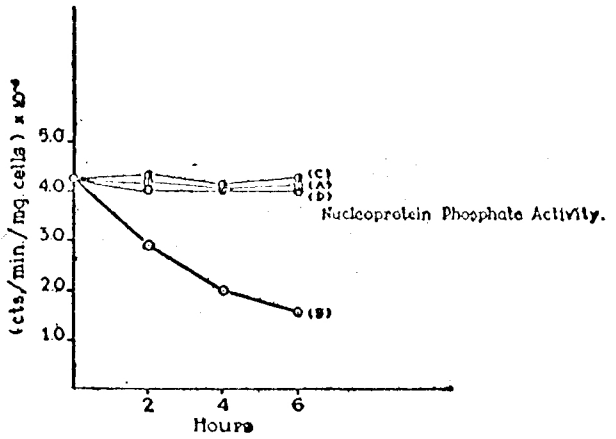

FIG. 8. Flow of nucleoprotein phosphate in cells suspended in (A) glucose, (B) glucose + $(NH_4)_2SO_4$, (C) glucose + $(NH_4)SO_4$ + NaN_3, (D) glucose + $(NH_4)SO_4$ + dinitrophenol.

indicating a flow of phosphate from this fraction. It is evident that the azide and, to only a slightly lesser extent, the dinitrophenol were able to prevent this utilization. Except for the fact that the transfer of less phosphate was involved, the same phenomenon was observed when cells were induced to form a new enzyme. Thus, in an experiment in which cells were adapted to maltose, a 34% drop in activity of the nucleoprotein phosphate was observed. Again azide and dinitrophenol in the above concentrations prevented both the formation of the enzyme and the transfer of phosphate from the nucleoprotein fraction.

These findings provide us with the following correlations between protein or enzyme syntheses and the transfer of phosphate from the nucleoprotein fraction:

(1) Rapidly metabolizing cells, which are not synthesizing new protein, do not transfer phosphate from the NP fraction.

(2) Synthesis of new protein or enzyme is always paralleled by a marked transfer of phosphate from the NP fraction.

(3) Agents that are effective in inhibiting enzyme formation and protein synthesis also prevent flow of P from the NP fraction.

To these must be added the fundamental observations of Caspersson and his collaborators on yeast (*2*), as well as on many other cells, which point to a rigid connection between nucleic-acid metabolism and protein synthesis.

A Proposed Function of Nucleoprotein in Enzyme Synthesis

The data presented in the previous section leave little doubt that nucleoprotein metabolism is essential for the synthesis of enzymes and proteins. The question that naturally arises is what role the nucleoprotein plays in these synthetic processes. Is it a matter of energy supply, or substrate, or specificity?

No definite answer is available. However, modern biochemical research (*13*), which has emphasized the role of organic phosphate bonds as sources of energy for synthetic activities, provides a foundation upon which may be based a reasonable hypothesis of nucleoprotein function in enzyme formation. Of particular value here is the mechanism of complex polysaccharide synthesis, the elucidation of which we owe to the brilliant work of the Coris (*3*) and their collaborators. Two things are required in the formation of a compound like glycogen. One is, of course, the basic hexose unit. The other is the energy necessary to form the bonds linking these units into the complex polysaccharide. The fundamental contribution of the Coris was to show that in the synthesis of a glycosidic bond, glucose-1-phosphate rather than glucose is the reactant involved. This phosphate ester of glucose (Cori ester) already contains in the (C-O-P) link the amount of energy required for the formation of the glycosidic bond. This makes it unnecessary to involve some other compound as an energy donor for the purposes of driving the reaction towards synthesis. The unique feature here is the conversion of an energy-requiring synthetic step into a spontaneous reaction by supplying the necessary energy in the *molecular structure of one of the reactants.*

There seems little doubt that this finding can profitably be accepted as a model on which may be based efforts towards the elucidation of other complex biological syntheses. Several important consequences immediately flow from the adoption of this point of view. It appears that the quantitative energy requirement for a particular synthetic reaction is not the crucial issue in determining its mechanism. There are many phosphorylated compounds (*e.g.*, di-P-glycerate, adenosinetriphosphate) which have more than sufficient energy to form a glycosidic link if there actually existed some mechanism for "feeding" it directly into the reaction. No such mechanism exists, however, and

therefore the energy content of these compounds cannot be used for this purpose. Clearly, in addition to the purely quantitative aspect, there is what may be called the "specificity" of the bond energy. Thus, the actual nature of the bond, and the compound carrying the energy, will determine its suitability for driving a particular reaction. The energy generated by the "catabolic wheel" and trapped in such energy accumulators as adenosine-triphosphate or creatine-phosphate cannot be used as such in driving all the various synthetic mechanisms of anabolism. The energy contained in such compounds must first be transferred to others, which can then act as specific energy donors for particular synthetic reactions.

This concept unifies and simplifies the problem of biological synthesis, since it avoids separating the problem of synthesis into one involving the reactants and another concerned with the source of the "coupled" driving energy. This principle tells us that the solution of one necessarily leads us to the solution of the other aspect of the problem, since they are one and the same.

From this point of view and the established importance of phosphorylated compounds in synthetic reactions, it is not surprising to find that nucleoproteins are controlling elements in enzyme synthesis. We may further plausibly suggest that these phosphorus-containing proteins are the specific energy donors which make possible reactions leading to protein and enzyme synthesis.

Effects of Nucleoprotein Fraction Extracts on Adaptation

One must be cautious in drawing any final conclusions about the precise role of nucleoprotein either from the preceding data or from the discussion. Even granting that nucleoproteins are directly involved in the synthesis of proteins, it does not follow that they necessarily intervene in the final steps leading to the conversion of such protein molecules into enzymes.

Clearly, some demonstration of *specific* influence by the nucleoprotein on enzymatic constitution would be necessary, before one could draw conclusive inferences of direct determination of enzymatic specificity by a nucleoprotein component. The most critical experiment one could offer in this direction would be one analogous to the already classical investigations of Avery and McCarty on pneumococcus transformation; i.e., the induction of a particular enzyme with a nucleoprotein component in a cell lacking the homologous gene.

No such experiments have yet been successfully concluded with yeast. However, some results of a very preliminary nature have been obtained recently, which bear on this question and warrant mention here. It was reasoned that, if the nucleoprotein fraction (NP) was specifically concerned with enzyme synthesis, it should be possible to observe specific effects of such fractions on adapting cells. Various types of fractionation were tried, and I shall here report briefly on the procedure that yielded the most active preparations.

Cells were extracted with $NaHCO_3$ at pH 9.0 for 3 hours at 30° C. with constant stirring. On removal of the cells the supernatant was adjusted to pH 3.5 and the resulting precipitate washed and redissolved. This was followed by two subsequent precipitations.

The activity of these fractions was tested in the following manner. Unadapted cells were suspended in buffer at pH 7.8, to which had been added the adapting substrate, biotin and pantothenic acid, 5γ/cc., and 1 mg. of nitrogen per cc. in the form of $(NH_4)_2SO_4$. The last three compounds were added to insure that a source of nitrogen was available and utilizable. It was experimentally found that the presence of these substances provided the optimal conditions for testing activity, since certain preparations which were inactive in their absence were extremely active when they were added.

It was found that, when cells adapting to galactose were incubated with such NP fractions prepared from galactose-adapted cells, the adaptation time could be cut down from 180 minutes to 20 minutes. Similar preparations from normal unadapted or maltose-adapted cells possessed no stimulatory activity towards galactose-adapting suspensions. In a similar manner fractions from maltose-adapted cells were only capable of stimulating adaptation to maltose, and the formation of this enzyme was not affected by the addition of extracts from galactose-adapted or normal cells.

That the enzyme itself is being transferred to the unadapted cells in this fraction seems very unlikely from several experiments. The fractions possessed no enzymatic activity by direct test. No maltase activity could be detected in the NP fraction from maltose-adapted cells, nor could the similar fraction coming from galactose-adapted cells influence added galactose. More decisive, however, is the fact that neither could confer the specific activity on active glucozymase extracts obtained from unadapted cells. Neither, therefore, contained in detectable amounts an apoenzymatic or coenzymatic component of the specific enzyme involved.

The conditions for obtaining uniformly active preparations have not yet been completely determined. Since the discovery of this phenomenon about 5 months ago, 32 separate preparations have been made. Of these, 21 have exhibited specific activity in increasing rate of enzyme synthesis. The range of activities observed in the active preparations has been large, extending from 50γ to 0.001γ per cc. for obtaining maximal stimulation. Nothing is known at present about the actual active component in this mixture.

In view of our ignorance of the nature of the

active component, it is clear that nothing can be said about identifying it with nucleoprotein. The interesting and suggestive fact to emerge from these experiments is that it is possible to extract in the nucleoprotein portion a component peculiar to adapted cells, which can specifically stimulate the formation of the same enzyme contained in the cells from which the fraction originated. Until further information is obtained on its biochemical identity we may call this active component by the neutral term "adaptin."

A Theory of Gene Action

As was pointed out in the introduction, the primary purpose of the present investigation is to acquire information that may lead to an adequate concept of gene action. It is proposed in the present section to suggest a mechanism of gene action based on the experimental findings described here, as well as on previously available information. It need hardly be emphasized that the mechanism to be described must be regarded only as a tentative working hypothesis—its usefulness to be assessed in terms of its success in unifying the diverse data on hereditary phenomena and its fruitfulness as a guide to future experiments.

We may summarize the important findings and conclusions relating to genes and enzyme formation by the following statements.

(1) Under normal conditions the transmission of characters (enzymes and the products of their activities) follows the classical Mendelian laws derived from the assumption that the controlling units are self-duplicating entities, genes, located on chromosomes in the nucleus.

(2) The existence of a particular gene in the nucleus of a cell does not guarantee that the corresponding enzyme will be found in the cytoplasm, as evidenced by such phenomena as cellular differentiation and enzymatic adaptation. Genes, therefore, have as their primary function the indefinite retention for the cell of the *potentiality* for enzyme formation. Certain recessive genes (*e.g.*, the melibiase locus in *S. cerevisiae*) do not possess the capacity for effectively performing this function.

(3) The actual formation of an enzyme in the cytoplasm is mediated directly by a cytoplasmic unit (plasmagene) which possesses the capacity for self-duplication in the presence or absence of the corresponding gene. (Following Wright, *40, 41*, the term "plasmagene" is adopted for the cytoplasmic self-duplicating entity postulated here:)

(4) The presence of the homologous substrates greatly accentuates the capacity of these self-duplicating plasmagenes to produce enzyme.

(5) Competitive interactions exist among the cytoplasmic enzyme-forming units.

(6) Nucleoproteins are involved in the synthesis of enzymes.

Obviously, the view adopted concerning the identity of the cytoplasmic self-duplicating unit will in large part determine the nature of the hypothesis devised to explain the mechanism of genic control of enzymatic constitution—particularly since the same hypothesis must afford some understanding of the relations between such units and the genes.

It would be hazardous at present to attempt to offer a definitive formulation of what we mean by a "self-duplicating" unit. However, one attribute such a unit is likely to possess is the ability to transform and accumulate energy within its own molecular structure which can be used for the synthesis of similar units. At any rate, it is relatively easy to show that the growth kinetics of such "energy accumulators" is of the self-duplicating or autocatalytic type. Of all the proteins, then, those which would be most likely to be self-duplicating are those which are involved as energy donors in protein or enzyme synthesis. Presumably these proteins could, in addition to aiding the formation of other proteins, drive protein-synthesizing reactions towards the formation of units like themselves.

In view of our interpretation of the role of nucleoprotein in protein synthesis, and of the above discussion, it seems reasonable provisionally to identify the self-duplicating plasmagenes which mediate enzyme synthesis with the nucleoproteins.

Such a hypothesis would be in harmony with the findings that all accepted self-duplicating entities have been found to be linked with nucleic-acid-containing compounds; among such entities may be mentioned genes, plastogenes, viruses, and the pneumococcus "transforming principle."

It must be emphasized that stating that these cytoplasmic self-duplicating units are nucleoprotein in nature does not imply that all nucleoproteins are capable of self-duplication.

Identifying the cytoplasmic unit with the nucleoprotein, rather than with the enzyme as had been done in earlier publications, has several theoretical and experimental consequences. It would be expected that cells not possessing the initiating gene for a particular enzyme could still retain capacity for synthesis of this enzyme, even in its absence, provided an adequate number of the appropriate nucleoprotein units were present. It will be recalled that experiments with melibiase are consistent with this point of view. In some of the clones lacking the gene, irreversible loss of potentiality for melibiase synthesis was not obtained until about 20 hours subsequent to the disappearance of all measurable enzyme activity. Thus, for a considerable period of time, these cells retained the capacity for the synthesis of this enzyme in the absence of any evidence for its presence in the cytoplasm. The experiments cited with galactozymase are also suggestive of this interpretation, since they demonstrated that cytoplasmic trans-

mission of the capacity to form enzyme can occur in the absence of any measurable enzyme activity.

From a theoretical point of view, the nucleoprotein concept of the plasmagene more or less dictates its relation to the gene. In view of the presumed similarity between the two, it seems almost necessary to conclude that the self-duplicating nucleoprotein in the cytoplasm, which mediates the formation of an enzyme, is derived from the gene. One immediate value of this conclusion resides in the fact that it provides us with an experimentally analyzable and testable entity, which can bridge the gap between the gene in the nucleus and the enzyme in the cytoplasm.

We are thus led to propose the following concept of gene action. Genes *continually* produce at various rates more or less complete replicas of themselves, which enter the cytoplasm. These replicas or plasmagenes are nucleoprotein in nature and possess

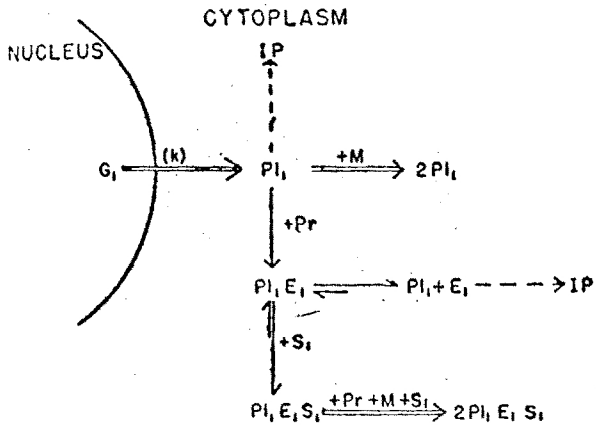

FIG. 9. A mechanism for gene control of enzyme synthesis. The symbols are G_1 for gene, Pl_1 for plasmagene, Pr for enzyme precursor, E_1 for enzyme, S_1 for substrate, and M for cytoplasmic material used in duplication of plasmagenes. The constant (k) is a reaction-velocity constant, measuring rate of production of plasmagenes from genes. All double arrows indicate a self-duplicating process; broken-line arrows denote decay to inactive protein (IP) of the elements from which the arrows point. Where forward-and-backward reactions are denoted, the longer of the two arrows indicates the major tendency of the reaction.

to varying degrees the capacity for self-duplication. Their presence in the cytoplasm controls the types and amounts of proteins and enzymes synthesized. These plasmagenes, like all self-duplicating entities, compete with each other for protein and energy, and the outcome of such competitive interactions then determines the enzymatic constitution of the cytoplasm. Inherent in this concept is the possibility of changing the ultimate result of this competition by varying the conditions (*e.g.*, substrates available) under which it takes place. The various reactions and the role of substrate in the process are detailed in Fig. 9 in terms of one gene G_1 and its corresponding enzyme E_1.

All the double arrows denote self-duplicating reactions. Gene G_1 continually produces its plasmagenes (Pl_1) at a rate denoted by k. The plasmagene by its very nature must possess heterocatalytic potentialities; i.e., in addition to being autosynthetic it must possess the capacity for catalyzing the synthesis of units (enzymes) other than itself. Consequently, once it is in the cytoplasm several things may happen to plasmagene (Pl_1). If it is successful in obtaining the proper material (M) in the cytoplasm it will duplicate itself. It may, on the other hand, combine with precursor protein (Pr) and convert it to E_1, resulting in the formation of the Pl_1E_1 complex.

Since little enzyme is found experimentally in the absence of substrate, one must assume that this complex is highly unstable and quickly breaks up into its two components. A plasmagene once formed cannot, of course, exist indefinitely, particularly in a population of other such units actively competing for the material of which it is composed. The reaction leading from Pl_1 to IP (inactive protein) in the figure describes this fact. It will be noted that in accord with the fact described in previous sections the same is assumed to be true for the E_1 formed. By IP or inactive protein we mean merely that the plasmagene has broken down to a protein unit which has lost the capacities for self-duplication and enzyme formation, and, in the case of enzyme, into a protein which no longer possesses enzyme activity.

Thus far we have described the reactions that take place in the absence of substrate. It is clear that little enzyme would be found in the cell, unless the rate of Pl_1 production by G_1 were extremely high or the stability of the enzyme or enzyme-plasmagene complex very great. Neither condition is apparently satisfied in the cases of the enzymes reported on here. When substrate S_1 is added, however, it will combine with E_1 and two things may result. It is well known that the addition of substrate to enzyme stabilizes it against inactivation. Hence the presence of substrate would decrease the rate at which free E_1 is converted to inactive protein. More critical, however, is the possibility that S_1 would combine with E_1 while the latter is still united with Pl_1, thus resulting in a $Pl_1E_1S_1$ complex. The substrate now not only would stabilize the enzyme but also could stabilize the unstable plasmagene-enzyme (Pl_1E_1) combination. Such stabilizations of unstable complexes by the addition of a third component are quite common in organic chemistry.

Inherent in the very definition of a self-duplicating entity is the concept that, should such a unit undergo a modification at any given moment, all subsequent replicas would bear this modification. Thus when Pl_1 exists alone it *duplicates* only Pl_1,

but when substrate is added and the stable $P_1E_1S_1$ combination results *this* is now duplicated, which is indicated in the diagram by the double arrow. This scheme provides, therefore, a concrete mechanism whereby substrate can modify competitive interactions between plasmagenes. What is essentially accomplished by substrate is the creation of a new self-duplicating unit, which duplicates not only the plasmagene but also the enzyme corresponding to the substrate added. It is clear that this scheme would fit both the genetic and the kinetic data discussed in this paper.

From an experimental point of view this mechanism suggests that the enzymes of a cell should be found associated with the nucleoprotein components, in agreement with the data recently accumulated on the enzymatic constitution of the nucleoprotein-containing cytoplasmic granules. More important, it offers a possible experimental solution to a very irritating dilemma which attends any effort at identifying a particular biologically active molecule from a population of chemically similar ones. This is well illustrated by the present-day situation of the pneumococcus transforming principle. Only a relatively small number (about 1 in 10,000,000) of all the molecules in an active preparation of desoxyribonucleate is actually capable of inducing the specific transformation. The biological test for the presence of the active principle is so much more sensitive than any available chemical tests that positive identification of the chemical identity of the active molecules is at present impossible. Thus, while there is little doubt that desoxyribonucleic acid is an essential component of the principle, the possibility that, for example, a protein component is associated with it has not been excluded.

It is obvious that any further efforts on our part to identify by chemical means the nature of the "adaptin" in the nucleoprotein fraction will very likely encounter precisely the same difficulties and arrive at the same impasse. The above theory suggests, however, that the biological specificity of the "adaptin" may be employed as an analytical tool by forming the PIES complex. Thus the corresponding enzyme and substrate could conceivably be used as fractionating devices in separating a particular plasmagene from a host of others, which, while they might be chemically similar, would not combine with any but their own enzymes and substrates. A further advantage which is suggested is that the various stages in the fractionation and isolation of a particular PIES complex can be followed enzymatically if one works with a well-defined and easily measured enzyme.

From a more general point of view, the unique feature of the above theory of gene action is that, while supplying a link between the gene and the enzyme, it at the same time predicts that cells with identical genomes need not possess identical enzymatic constitutions. Whether a particular character (enzyme) will be transmitted from one cell generation to another in a Mendelian fashion will thus depend on the relative rates of duplication of the controlling cytoplasmic units as compared with their rate (k of the diagram) of production from the genome. If the latter is quantitatively determining, Mendelian inheritance will be observed. If the former is determining, the Mendelian picture will be obscured to varying degrees depending on the self-duplicating capacity of the plasmagenes. It is also clear how substrate could so intensify cytoplasmic inheritance of a particular enzyme as to completely obscure the segregation of the corresponding gene.

As a tentative working hypothesis, this theory has the advantage of providing a unified point of view from which such diverse and apparently contradictory phenomena as classical Mendelian genetics, cytoplasmic inheritance, cellular differentiation, and enzymatic adaptation may be analyzed. The basic problem of cancer involves explaining the appearance of a sudden *heritable* change in somatic cells, analogous in several ways to enzyme adaptation or cellular differentiation. It is, therefore, not surprising that cancer investigators were one of the first groups of biological workers to support strongly the suggested existence of a cytoplasmic hereditary unit. An entity of this kind, by being self-duplicating, provides them with another level at which a mutation can take place and be subsequently transmitted via the cytoplasm from one cell-generation to the next.

More or less similar views have been proposed by geneticists. Wright (*40*) in particular emphasized several difficulties in trying to explain either growth or differentiation in terms of the classical Mendelian concept of the gene. Thus, the assumption that every time a new protein molecule is formed during growth the gene on the chromosome must intervene as a kind of model implies that growth must proceed linearly from a relatively minute portion of the cell. The kinetics of cell growth follow an autocatalytic law and so are not consistent with this. Wright therefore suggested that perhaps "duplicates or partial duplicates of genes reach the cytoplasm when the nuclear membrane disappears in mitosis and that these can produce duplicates in turn, and so on, permitting exponential increase." To explain the fact that cytoplasmic inheritance is rarely observed, he assumed that the self-duplicating capacity of these free genic replicas is subject to decay. Those that retain this capacity indefinitely he called "plasmagenes."

Again, in connection with cellular differentiation Wright (*41*) pointed out that the heritable stability of the differentiated state is more easily understood if we assume the existence of self-duplicating cytoplasmic components (plasmagenes), which can undergo controlled mutations. Stimulated by the fundamental observations of Sonneborn (*18*), Darlington (*4*) also postulated the existence of a

cytoplasmic self-duplicating unit, which he called the "plasmagene" and which he assumed controls heredity at the "molecular level."

It may be of importance for reasons of clarity to emphasize certain differences between the plasmagene concept developed here and those employed by Wright and Darlington. The plasmagene as defined in the present paper is a more or less complete gene-replica, which possesses to a varying extent the capacity to self-duplicate. It is not a special or unique cytoplasmic component in the sense that it is outside normal physiological processes. It is an integral part of the enzyme-synthesizing system and is the normal link by means of which genes can effect control over protein formation in the cytoplasm. Whether or not plasmagenes are "molecular" is not pertinent to their definition. It seems probable, however, that they would perform their synthesizing functions on the surfaces of relatively large particles (cytoplasmic granules), which could provide the necessary protein and energy-rich groups.

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DISCUSSION

ZAMENHOF: The Delaporte method, mentioned by Dr. Spiegelman for the obtaining of his "nucleoprotein fraction," was devised originally to obtain desoxyribose nucleic acid; the first step of this method is to get rid of ribose nucleic acid by extraction with dilute sodium bicarbonate solution; the extract contains ribose nucleic acid but no desoxyribose nucleic acid. After extraction, the metachromatic granules disappear from the cells but the latter retain their full vitality as long as their desoxyribose nucleic acid content has not been touched. Dr. Spiegelman uses in his method just this first extract and therefore the chances are that his extract contains ribose nucleic acid and no desoxyribose nucleic acid. If this proves to be correct, then his active principle will be basically different from the one involved in the transformation of pneumococci.

If his principle proves to be indeed ribose nucleic acid, then it is perhaps worth mentioning that according to the recent work of Brachet (Enzymologia, 1945) there may be two kinds of ribonucleic acid in yeast: one "granular," which is thrown down when centrifuging at 50,000 r.p.m., and one "free," which remains in supernatant. While the "granular" fraction is always quantitatively constant and may be involved in some important unknown functions, the quantity of the free fraction can be easily decreased by growing yeast on phosphate-free medium; it is to this "free" fraction that Brachet ascribes many metabolic functions which may make it similar to Dr. Spiegelman's active principle.

Dr. Spiegelman's substance is probably quite depolymerized, since he incubates it for 3 hours at 30° at pH 9.5. However, one has to be careful with such a statement. In Boivin's recent work on transformation of *E. coli*, he uses as much as 1 mg./ml. of his desoxyribose nucleic acid obtained at pH 3.5 (and therefore depolymerized). Had this publication appeared before the work of Avery and McCarty, one would think that a large amount of depolymerized substance is necessary to induce transformation; actually, as we know from the Avery and McCarty experiments, the amounts needed are of the order of 10^{-5} mg., but the substance needed must be highly polymerized; evidently in Boivin's work some very small amounts escaped depolymerization.

COHEN: Before the genetic implications of Dr. Spiegelman's exciting experiments and theory are discussed, I should like to make a plea for a more temperate use of the term "nucleoprotein." The nucleic acids exist in most organized cell structures. In actual practice, especially in one involving an isoelectric precipitation of intracellular material at pH 3.5, it becomes very difficult to obtain material which does not contain nucleic acid. But fractions of this sort contain, in addition to nucleic acid and protein, phospholipids, sterols, neutral fats, enzymes, several types of antigens, vitamins, carbohydrates, etc. Thus almost every type of substance is found in these fractions. In other words, depending on your point of view, these materials might be called lipoproteins, glycoproteins, enzymes, etc. To call the fraction which Dr. Spiegelman has obtained "nucleoprotein" is to imply a causal relationship between his phenomenon and nucleic acid for which there is no evidence at present. As a matter of fact, the data directly relating nucleic acid to specifically inheritable phenomena are very sparse in general. The reactions described by Dr. Spiegelman stand on their own feet and gain not at all in interest by allusions to substances which may or may not be involved in the curious effects described.

MONOD: Since the inspiring results and interpretations reported by Dr. Spiegelman are based on experiments with yeasts, it may be of interest to see how they agree with what is known about enzymatic adaptation in bacteria.

In the first place it should be pointed out that the interactions in the formation of different specific enzymes reported by Spiegelman agree very well with the data obtained on bacteria. In fact, it has been shown that such interactions are of very wide occurrence among carbohydrate-attacking enzymes, and that they account for the phenomenon of diauxis. The basic facts concerning this phenomenon have been briefly mentioned in Lwoff's paper. From a rather extensive study of the occurrence and mechanism of diauxis, the essential conclusion to be recalled in connection with this discussion seems to be that all the adaptive and constitutive carbohydrate-attacking enzymes in bacteria may depend on a common mechanism of synthesis, or, more precisely, on a common precursor. A similar situation, as shown by Spiegelman, appears to exist with glucozymase, galactozymase, and melibiozymase in yeast. (It should be noted, however, that the mutual-exclusion effect of different specific enzymes seems to be much more pronounced with bacteria than with yeast,

since in the latter it occurs only in the *absence* of an external supply of nitrogen. Furthermore, this is in accord with the fact that an external nitrogen source is required for the adaptation of bacteria but not for the adaptation of yeasts.)

From the results on bacteria and yeasts, the problem naturally arises of the nature and degree of specificity of the common precursor (which I have called preenzyme) and of the mechanism of its substrate-induced transformation into a variety of specific enzymes. The data on bacteria seem to show: (a) that the mutual-exclusion effect expressed in diauxis results from a competitive interaction of the *substrates* for the preenzyme; (b) that the rate of adaptation does not depend on the degree of saturation of the pre-existing enzyme. Consequently, it was concluded that the formation of the enzyme occurs as the result of a *substrate-preenzyme* combination, and does *not* depend on the *substrate-enzyme* combination.

Clearly these conclusions do not agree with Yudkin's "mass action" scheme of enzymatic adaptation, and it is quite noteworthy that Spiegelman, on the basis of entirely different experiments with another material, should also have been led to discard this theory.

The same conclusions may also appear, at first glance, to disagree with Spiegelman's scheme of substrate-induced adaptation, since here, as in Yudkin's scheme, the rate of adaptation may seem to depend on the substrate-enzyme combination. However, the main object of Spiegelman's scheme is to explain gene-controlled adaptation and the role of the plasmagene. It implies no hypothesis as to the nature or specificity of the precursor. I believe that with a little further specification Spiegelman's scheme could be made to agree well with the conclusions mentioned above. One need simply assume that the formation of the PlES complex results from the reaction Pr + Pl + S = PlES.

The rate of formation of new enzyme—that is, the rate of adaptation—would then depend on the product of the concentrations of Pr, Pl, and S, and also in part on the affinity of Pr for S. Such a scheme would agree with all the data available at the present time on enzymatic adaptation in both bacteria and yeast, and it might lead to a number of conclusions which could be experimentally tested. For instance, it can be predicted from it that the synthesis of enzymes depending on a common precursor might be affected by two types of mutation:

(a) Mutations involving a change in a "specific" plasmagene, which would affect a single enzyme of the group.

(b) Mutations involving a change in the structure of the precursor, which might affect simultaneously several or all of the enzymes of the group.

It may be stated here that a preliminary study of a number of bacterial mutations affecting carbohydrate-attacking enzymes appears to confirm that mutations of type (b) may indeed occur. However, no definite conclusions as to the validity of these views can be drawn at present from experiments with bacteria. Further confirmations will have to be sought with a material on which straight genetic tests can be performed.

REINER: As a matter of fact, it is possible to obtain direct evidence for the inhibitory effect of constitutive upon adaptive enzymes, using direct measurements of adaptation rates instead of growth experiments. For example, we have shown this for the case of glucose and galactose. If washed cells are adapted in the presence of galactose alone, the initial rate of adaptation is about twice as high as when glucose is added, measuring this rate of adaptation by the increase of anaerobic fermentation rate on galactose in a given period of time.

In view of this, it seemed likely that there should be competition between galactose and other substrates than glucose. It also seemed likely that the intensity of substrate competition should be strongest for substrates whose configurations are close to that of galactose, and become progressively weaker as less similar substrates are introduced. This possibility was tested by studying the effect of these substrates on the breakdown of the adaptive galactozymase. We found an appreciable decrease in adaptive fermentation rate when adapted cells were shaken aerobically in phosphate buffer for 4 to 8 hours, and a very much higher rate of breakdown when glucose was added during this treatment. According to our suppositions, substrates like alcohol and pyruvate should have an effect intermediate between glucose and buffer alone. It was found that the curves for alcohol and pyruvate coincided with each other, and fell just about halfway between the other two curves, in accordance with the hypothesis.

With regard to the question of rate-limiting reactions raised by Dr. Monod, I should like to direct attention to the theoretical demonstration by Burton, a number of years ago, that the over-all rate of a chain of reactions depends not only on the rate of the slowest reaction, but on the rate of other components of the chain as well. Burton indicated that the effect of a slow reaction on the over-all rate depended on the rates of the adjacent reactions and on the presence of other slow reactions in the chain. In view of this, it is conceivable that the introduction of one or more new (adaptive) reaction steps might change the characteristics of a chain without necessarily introducing a step slower than any of those already present. This point deserves more careful investigation.

We have some evidence that the adaptive steps in the case of galactozymase constitute a rather short branch close to the beginning of the Meyerhof scheme for glucose. It seems to be most economical biologically if an adaptive substrate is switched into existing metabolic channels as early as possible, in-

stead of requiring the adaptive formation of a large set of new enzymes. This appears to be the case with galactose. The experiments will bear a good deal of repetition before we are entirely satisfied with them. As far as they go, however, they indicate that galactose is first phosphorylated, and that the resulting galactose phosphate is acted upon by an isomerase, which converts it to glucose phosphate. There are some indications that even unadapted cells can carry out the phosphorylation, and that the isomerase is the adaptive enzyme. In any event, galactose would seem to enter the Meyerhof scheme after at most two steps.

In connection with Dr. Spiegelman's experiments with adaptin, it may be of interest to mention some experiments performed in my laboratory about a year ago, whose interpretation seemed rather obscure at the time. Suspensions of *S. carlsbergensis* were incubated aerobically with galactose and buffer according to our usual technique for adaptation experiments. Samples were taken at intervals, the cells removed by centrifugation or filtration, and the supernatant liquid added to fresh unadapted cells in the presence of fresh galactose. The time required for adaptation to begin in the fresh cells was determined manometrically in the usual way. It was found that supernatant obtained before 45 minutes of previous incubation had no effect, but that supernatant obtained after 45 minutes shortened the time of adaptation, the effect increasing with the length of the incubation period. Supernatant after 180 minutes cut the time down from the control value of 90 minutes to about 45 minutes.

It was supposed at the time that the supernatant might contain intermediates of galactose fermentation which would act as fermentable substrates for the unadapted cells (*e.g.*, pyruvate). However, an investigation of the compounds which could be detected in cells and supernatant during various phases of the adaptive process made this seem quite unlikely. In the light of Dr. Spiegelman's experiments, it appears possible that our active supernatants contained enough adaptin to produce the effects which were observed.

The question which was raised concerning the demonstration that adaptin itself contains no enzyme may be answered by referring to some unpublished experiments by Spiegelman. It was shown that an apozymase prepared from unadapted cells would not ferment galactose when supplemented with a cozymase preparation (boiled yeast juice) made from adapted cells. On the other hand, an apozymase from adapted cells would ferment galactose with cozymase preparations from unadapted or from adapted cells. It is clear from these results that unadapted cells already contain all the cofactors necessary for galactose fermentation, while they do not contain the enzyme. The addition of adaptin to a Lebedew juice from unadapted cells should therefore result in fermentation if any enzyme were present in the adaptin. The negative result conclusively demonstrates the absence of enzymatic activity.

SPIEGELMAN: I shall in what follows consider some of the questions raised in the preceding discussions. One can hardly fail to agree wholeheartedly with Dr. Cohen's plea for caution in using chemically defined terms for biological agents of undetermined chemical constitution. In reporting the facts of our investigations here I have spoken of the "nucleoprotein fraction," by which I meant to imply only that the active principle or process can be localized in that fraction. No conclusive chemical identification with nucleoprotein was made. It is in this spirit that such chemically neutral terms as "adaptin" and "plasmagene" were employed for the biologically active agents. No objection can be raised, however, against speculations concerning the biochemical roles and nature of these agents, as long as care is taken to distinguish theory from fact.

Our work on the chemistry of the active fraction is still in a primitive stage, especially when compared with the remarkable advances recently made in Avery's laboratory on the pneumococcus transforming principle. Under the circumstances, therefore, I cannot agree with Dr. Zamenhof that one can be relatively certain that the preparation contains no desoxyribose nucleic acid. There is little doubt that ribose nucleic is present, but it is certainly impossible to say now whether it is either the active principle itself or a component of it.

Dr. Pontecorvo raised the interesting question why more instances of cytoplasmic inheritance are not observed in the higher plants and animals if genes normally function via self-duplicating cytoplasmic units as is postulated in the theory proposed here.

I don't think it is merely a matter of coincidence that as soon as attention was focused on the mechanism of transmission of characters in the lower organisms (protozoa, rusts, yeasts) cytoplasmic components almost immediately began to exhibit their capacity to dominate the nuclear hereditary units. The "distance," from the point of view of cell generation and extent of differentiation, between an individual and its gametes is almost vanishingly small in these lower forms. Thus, for example, a diploid yeast cell goes through only two divisions, one equational and one reductional, to produce its haploid gametes. Here both types of gamete, and not merely the "female type," inherit quite directly the cytoplasm of the individual from which they arose, by a process which probably leaves its plasmagene population relatively undisturbed. If the cytoplasm possessed self-duplicating units, this type of gametogenesis would certainly tend to augment and intensify any capacity possessed by these units to overshadow the transmission of the nuclear heredity-determining units.

Compare this situation with the relatively complicated series of differentiations which accompany either spermatogenesis or oögenesis in the higher organisms. The cytoplasm of the resulting gametes is highly specialized as a result of these processes, and the final plasmagene population is quite different from the initial one. A rigid selection of the proper plasmagenes must have occurred during differentiation to form a cell so uniquely suited to perform its biological function. In addition to this, it must be remembered that the germ line is isolated as such relatively early in the embryogenesis of higher plants and animals. Consequently, any plasmagene that is to disturb the Mendelian mechanism must survive in the cytoplasm for a relatively long period extending from early in embryonic development to sexual maturity. The chances of any but the "proper" (i.e., necessary for the onset of differentiation in the zygote) plasmagenes surviving this long waiting period and then the subsequent selection accompanying differentiation must be relatively small, although not completely impossible as demonstrated by the existence of maternal cytoplasmic effects. Presumably, the survival of the "proper plasmagenes" is encouraged by providing the appropriate environmental conditions and substrates.

It is not difficult to understand why the higher plants and animals would tend to develop mechanisms leading to rather rigid control over the plasmagene populations of their gametes, and hence to suppression of the tendency for such cytoplasmic units to determine the transmission of characters. The adult, gamete-producing individuals in the higher plants and animals are the result of a long, complicated series of differentiation reactions delicately synchronized in space and time. The disturbance of any one of these steps could lead to the death of the organisms. Insuring the uniformity of the starting zygotic cytoplasm by suppressing all unnecessary, and therefore possibly harmful, cytoplasmic elements in the gametes would provide an important factor of safety for the developmental process. Obviously, where developmental processes leading to the adult form are either trivial or altogether nonexistent, as in the single-celled forms, control over gamete plasmagene population would confer relatively little selective advantage.

Dr. Stern raised the question of the relation of the gene dosage effect experiments to the results and theory of gene action presented here. Whether or not a gene dosage effect with a particular gene will be observed will depend on whether the rate of production of the plasmagene from the gene, or the rate of plasmagene self-duplication, is determining. If the latter is low, either because of an inherently poor capacity for autosynthesis or because of competitive conditions in the cytoplasm, then increasing the dosage of this gene in the nucleus will increase the number of the corresponding plasmagenes that will be found in the cytoplasm. On the other hand, no dosage effects will be observed with any gene whose plasmagene possesses a self-duplicating capacity high enough to quantitatively overwhelm the production rate of the gene, in so far as determining the number of plasmagenes in the cytoplasm is concerned.

It should be emphasized that the theory developed here is one concerned primarily with the mechanism of gene *action* and not of gene *transmission*. The present theory assumes that the latter is strictly Mendelian and is supplementary to the established rules of transmission. Inherent in the proposed mechanism of gene action is the possibility of explaining why in certain instances the transmission of a *character* may not follow that of the *gene*. We sometimes call such cases instances of non-Mendelian inheritance. Actually, since classical Mendelian theory refers only to nuclear gene transmission, such phenomena do not represent violations of Mendel's laws.