CONTRIBUTIONS OF STUDIES ON THE β-GALACTOSIDASE OF *ESCHERICHIA COLI* TO OUR UNDERSTANDING OF ENZYME SYNTHESIS[1]

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I. INTRODUCTION

Traditionally the recipient of the Eli Lilly award reviews the field to which he has contributed, inevitably paying most attention to the work for which he has been honored. This places me at distinct disadvantage compared with my predecessors for, instead of playing the more comfortable role of a night watchman in Hamlet, I suddenly find myself giving the soliloquy. But perhaps it is just as well that it is a soliloquy since I intend to discuss the most controversial aspect of this field, its methodology.

Induced enzyme synthesis interests me because it poses in experimental terms the question of the specific factors involved in the synthesis of proteins. As we know today, a cell contains some several thousand distinct enzyme proteins which differ one from the other not in the origin and nature of their basic elements, amino acids, but in how these amino acids are linked together and assembled to give each protein its characteristic configuration. Any biologist, carrying out an experiment in which enzymatic activity appears after the addition of a given compound to a bacterial population, would be struck immediately with the elegance of the system for studies on differentiation. The specific factors controlling the formation and structure of enzymes are merely one aspect of the problem of differentiation. It need not be stressed, therefore, why "adaptive" enzyme systems have proved so useful in studying the origin of enzyme specificity, since the phenomenon is so well known and the subject has been so thoroughly reviewed (2–17).

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To give some perspective to the material I shall analyze, I should like to divide studies on induced protein synthesis into three chronological phases.

1. During the first stage lasting about 60 years until the 1940's, numerous observations were made that populations of cells could undergo a change in their ability to metabolize various compounds placed in the environment. Workers during this period struggled with the problems of the origin of this acquired ability and by 1950 they had clearly distinguished mutation and selection from "adaptation." Thus, the biological nature of the system was defined with precision for it was established: (a) that induced enzyme synthesis was under genetic control and (b) that change in phentotype could be achieved without prior change in genotype. These pioneer studies of Duclaux, Went, Dienert, Karström, Dubos, Stephenson, Yudkin, Gale, Spiegelman, and Monod are all the more remarkable when one realizes that they were carried out without the benefit of the tremendous advances in microbial genetics and biochemistry made in the last 10 years. With these foregoing problems definitely answered, workers in this field knew that they had in their hands a tool which would permit the analysis of the mechanism of enzyme synthesis. Such studies, however, necessitated the search for well defined systems with which quantitative experimentation at the biochemical level could be carried out. This led to the second phase of the development of the subject.

2. These last 10 years, the second stage, saw a redefinition of the problem as one of induced enzyme-protein synthesis, with a resultant emphasis on the characterization of the enzyme protein, on the kinetics of enzyme formation at the cellular level, on the nature of the precursor of the enzyme-protein, on the metabolism of inducers, and on the genetic factors in enzyme formation. During this period two model systems were introduced, the β-galactosidase of *Escherichia coli* and the penicillinase of *Bacillus cereus*. The workers who developed the model systems arrived at these more firmly grounded conclusions (8):

(a) Induced enzyme synthesis involves the *de novo* formation of the enzyme-protein molecule from its amino acids. There is no accumulation of a protein precursor in either the presence or absence of inducer (18, 19).

(b) The over-all pathway between amino acids and enzyme protein is virtually irreversible (18, 20).

(c) The process of enzyme induction is independent of enzyme action, *i.e.*, the functions of inducer and substrate are distinct (21, 22).

(d) The inducer acts catalytically in the sense that one molecule of inducer may activate the synthesis of more than one enzyme molecule (10, 23).

However, studies at the cellular level are only first approximations, and the whole experience of biochemistry has pointed out that the next step will necessitate the use of resolved subcellular systems. This is the third stage.

3. We are at present entering this third stage, the analysis of enzyme synthesis at the subcellular level. As yet the subject is too young for any general comments. Nevertheless, the direction this field is taking is already portended by the protoplast systems of Spiegelman (12) and the disrupted cell systems of Gale (24–27). I shall not deal with this phase here but shall confine my discussion to the second stage of development of this field. Although I have decided to emphasize the methodology rather than the results, it will become obvious that this dichotomy is too artificial to be realistic.

We might begin in 1952 with Monod's (3) redefinition of the phenomenon. The word "adaptation," because of its too broad meaning and teleological overtones, had led to serious confusion. In its place the term "induced enzyme synthesis" was proposed (3, 28) to describe the increased rate (induction) of enzyme-protein synthesis under the influence of specific substances. The essential distinctions made in early investigations between induced enzyme synthesis and adaptation of a population by mutation and selection were completely confirmed and clarified by the rapid strides in microbial genetics on the one hand and in induced enzyme synthesis on the other. This distinction, of course, was incorporated into the definition of induced enzyme synthesis.

The term, induced enzyme synthesis, implies that we have to deal with specific *enzyme proteins*. However, studies of enzyme synthesis rest almost entirely on determinations of activity which presumably measure the quantity of *enzyme protein* produced by a cell. The use of an uncharacterized enzyme leaves a great margin of uncertainty as to the validity of this assumption. Therefore, in a review (3) written in 1952, we suggested that the study of induced enzyme synthesis should begin with a careful characterization of the enzyme protein. We pointed out the dangers in interpreting investigations in which poorly identified enzymatic systems were used, especially those whose assay depended on over-

all measurements of metabolic activity, *e.g.*, galactozymase, glucozymase, or those which had never been obtained in cell-free extracts. At that time we attempted to analyze only those inducible enzymes which had at least been obtained in a cell-free state. Today, I believe that, aside from the careful characterization of an induced protein as an enzyme, extensive purification should be carried out in order to determine eventually the parameters of its catalytic activity as well as such structurally dependent characteristics as its molecular weight, electrophoretic and immunochemical behavior, amino acid composition and sequence (where possible). Such information not only makes the use of activity as a measure of enzyme protein more certain, but also provides the tools to investigate such phenomena as the nature of the precursor, the problem of the stoichiometric or catalytic role of the inducer, and the gene-enzyme relationship at the molecular level.

With the present day goals being the synthesis of protein in cell-free extracts, the study of well characterized enzyme proteins is essential. Certainly evidence for protein synthesis in cell fragments based on changes in enzymatic activity is weakened by the use of "glucozymase," or a galactosidase unobtainable in cell-free extracts (25, 26, 29), or a cursorily characterized catalase activity (30, 31).

It is not surprising therefore that the two inducible systems which have contributed so much to the recent advances of this problem involved well characterized enzymes, *e.g.*, the penicillinase of *Bacillus cereus* and the β-galactosidase of *Escherichia coli*. It would give me great pleasure to review the outstanding work of Pollack on the penicillinase system, but this is not the occasion, and I shall confine myself mainly to β-galactosidase.

II. CHARACTERIZATION OF β-GALACTOSIDASE

The *Escherichia coli* β-galactosidase, because of its peculiar properties and great stability, is particularly suited to studies on induced enzyme synthesis. In addition to the Paris group (32–34), we owe the characterization of this enzyme especially to Lederberg (35), to Kuby and Lardy (36), and to Wallenfels (37–40).

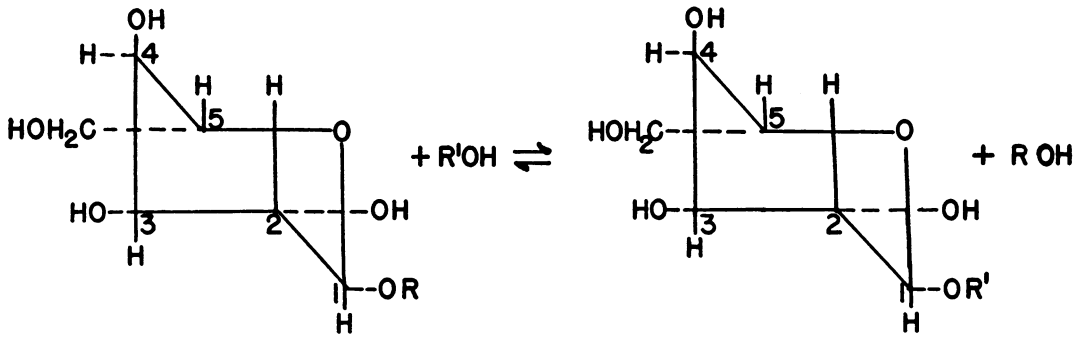
A. Purification

The purification of an enzyme is usually followed by the increase in specific activity. Alternatively one could follow purification by the decrease in other proteins provided that some assay for these substances were available. Induced enzyme systems can provide just such a situation, and purification can be followed by two independent measurements, that of the specific enzymatic activity and that of the amount of protein present other than the enzyme under investigation. As will be illustrated, the procedure involves the preparation of a crude extract in which every protein except the enzyme to be isolated is labeled with a radioactive isotope. The amount of radioactivity in the enzyme preparation at every step during its isolation is a measure of the amount of contaminating protein. The value of this method as a criterion of purity of an enzyme depends upon how good the evidence is that the enzyme under purification is the only unlabeled protein in the initial extract.

The purity of β-galactosidase has been determined during its isolation from extracts which have been prepared as follows: Single-step lactose-negative mutants which make no detectable β-galactosidase under any known conditions are assumed to differ from lactose-positive mutants by the absence of a single enzyme, β-galactosidase. Under this assumption, if an extract from a lactose-negative mutant, whose proteins are isotopically labeled, is mixed with an unlabeled extract from lactose-positive mutants which contain β-galactosidase, then presumably one has a mixture in which the only unlabeled protein is the β-galactosidase itself. From such extracts, β-galactosidase has been isolated possessing 1 to 2 per cent protein impurity as judged by the contaminating radioactivity. The specific activity of this preparation was found to be 2.1 mmoles of *ortho*-nitrophenyl β-D-galactoside hydrolyzed per min per mg protein nitrogen at 28 C and pH 7.1 in M/10 sodium phosphate buffer. The specific activity of these preparations cannot be further increased by repeated electrophoresis in starch, differential centrifugation, or fractional precipitation.

B. Substrate Specificity

One naturally occurring substrate of this enzyme is lactose and, in fact, the first quantitative assays of this enzyme involved the enzymatic determination of the glucose liberated by the hydrolysis of lactose (32, 33). It was, however, as a result of the introduction by Lederberg (35) of the chromogenic substrate *o*-nitrophenyl



β-D-Galactoside

where R'OH = water, glucose, lactose, galactose, etc.

Figure 1

β-D-galactoside, NPG, that the determination of activity became exceedingly rapid and easy, permitting accurate assays even in whole bacterial suspensions treated with toluene (21).

β-Galactosidase has been investigated most often as a hydrolase acting on β-D-galactosides to yield galactose in the β configuration [*vide infra* from (41)] and the aglycone. Since Wallenfels (37–40) and Aronson (42) have shown that during lactose hydrolysis various trisaccharides and digalactosides are formed, this enzyme should probably be considered a transglycosidase which catalyzes the transfer of the galactosidyl residue of β-D-galactosides to some acceptor, R'OH (36–40) as shown in figure 1.

This enzyme has neither activity on nor affinity for analogs of the above configuration which are modified by inversion of the hydroxyls on C2, 3, 4 or by oxidation of C6 to COOH, or by reduction of the hydroxyls on C2 or 6, or by substitution, *i.e.*, methylation, on the hydroxyls of C2, 3, 4, 6 (21). Likewise, the inversion of the β-galactosidic linkage to the α configuration causes a complete loss of activity, but a partial loss of affinity which in some complex way depends upon the nature of the activating ion, Na^+ or K^+ (21). On the other hand, β-galactosidase has a slight affinity for and activity on α-L-arabinosides, which are the derivatives of β-galactosides lacking the C6. The aglycone itself has the effect of changing the affinity and activity constants of the galactosidase. Interestingly enough, fluoro-β-D-galactoside is a good substrate which is cleaved to yield galactose and fluoride ion. The substitution of sulfur for oxygen in the glycosidic linkage destroys the activity of the compound as a substrate but leaves unimpaired the ability to combine with enzyme, *i.e.*, to act as a competitive inhibitor.

C. Metal Activation

In his study on this enzyme, Lederberg made the important observation that with NPG as substrate, β-galactosidase is activated by Na^+ ions (35). We had found that with lactose as a substrate, K^+ ion and not Na^+ ion is the best activator (33). A careful analysis of this apparent contradiction showed that the affinity for and activity on a given substrate depends on the monovalent cation with which the enzyme is complexed. The other monovalent cations, Rb^+, Cs^+, Li^+, NH_2^+, RNH_3^+, and even H^+, also play a role in determining the affinity for and activity on any substrate (33). For our studies, however, the K^+ and Na^+ activations have been the most important.

Parenthetically, it is interesting that the β-galactosidases of other organisms, *Saccharomyces* (43), *Lactobacillus*, and *Clostridium*, are also activated by monovalent cations. These enzymes also show divalent ion (Mg^{++}, Mn^{++}, and Fe^{++}) activation, which is masked in the *E. coli* enzyme until it is treated with complexing agents such as versene, after which a specific effect of Mg^{++} (and to a lesser extent of Mn^{++}) is revealed. No theory has as yet appeared which quantitatively accounts for the kinetics of these activations.

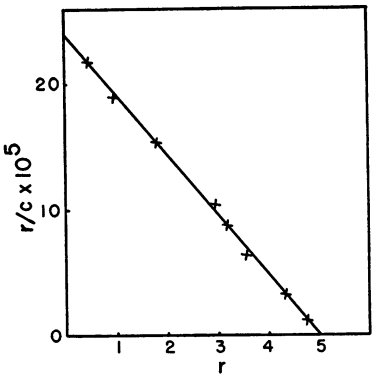


Figure 2. The binding of phenyl ethyl-β-D-thiogalactoside by β-galactosidase at 4 C (see text).

D. Physical Properties

The purified enzyme is heterogeneous in the ultracentrifuge, showing two peaks, a major monodisperse component as 80 per cent of the total and a minor polydisperse heterogeneous component. Both components contain enzymatic activity. The major component has an S_w^{20} of 13.9, while the minor component has a spread of sedimentation constants between 18 and 26. One possibility which would account for this sedimentation pattern is that the minor fraction is a polymerized product resulting from handling. Enzyme-containing fractions of lower size than the major $S_w^{20} = 13.9$ component have not been encountered even in crude extracts, and it is probable that the enzyme molecule itself is of this size. The approximate molecular weight of β-galactosidase is 700,000. Its turnover number then for NPG under standard assay conditions is 4000 moles NPG/sec/mole enzyme, which is quite high for hydrolases.

This high turnover number made us wonder whether there really was only one combining site per molecule. The technique which could answer such a question is that of equilibrium dialysis (44, 45) in which enzyme in a dialysis sac is equilibrated with a competitive inhibitor (I) and the concentration of free inhibitor outside of the sac is measured. Assuming the relationship

$$E + nI \rightleftharpoons EI_n,$$

one can derive (44, 45) that

$$r/c = nK - rK$$

where r = ratio of moles of I bound/mole enzyme, c = free concentration of inhibitor, K = association constant, n = number of combining sites per molecule of enzyme.

The plot of r/c *versus* r (figure 2) gives a straight line which intercepts the abscissa at 5 sites/molecule of enzyme if a molecular weight of 665,000 is assumed (figure 4). The turnover rate of each site is then of the order of 800 moles/sec/site under our assay conditions.

The dissociation constant measured by equilibrium dialysis is the same as the inhibition constant (K_I) determined by the competitive inhibition of the hydrolysis of three different substrates (NPG, methyl-β-D-galactoside, lactose) of β-galactosidase. These data provide evidence that (a) the site which binds the inhibitor in the equilibrium dialysis experiments is identical to the catalytically-active site involved in the hydrolytic reaction, and (b) any one site can catalyze the hydrolysis of the three different substrates.

As a further comment on this enzyme, its absorption spectrum is similar to that of most proteins with a molar extinction coefficient of 1.07×10^9 at the point of maximum absorption, 278 mμ, and a 280/260 ratio of 1.73. There is no evidence of any prosthetic group other than amino acids, and the enzyme contains no phosphorus.

E. Comparison of Enzymes from Different Sources

The use of various competitive inhibitors in the presence of different ion activators enables a precise definition of a specific enzymatic property by which one could hope to distinguish slightly altered enzyme proteins either present in mutants or as a result of treatment. In laboratory parlance, we called one such test an enzyme fingerprint (3). The fingerprint of a given enzyme preparation was made by determining the relative affinity of three competitive inhibitors of the enzyme in the presence of either K^+ or Na^+. A comparison of these values by several statistical methods showed that none of the enzymes obtained from various mutants of *E. coli* ML, or induced by several inducers, were distinguishable from an arbitrarily chosen reference enzyme. *E. coli* K12 or *Aerobacter aerogenes*, or *Shigella sonnei*, also yielded enzymes indistinguishable from the standard. On the other hand, the β-galactosidases of *Saccharomyces* or *Lactobacillus* give totally different values from that of the standard and differ quite significantly between themselves. I might add that the *E. coli*, *Aerobacter* and *Shigella*

sonnei β-galactosidases are also immunologically indistinguishable when tested with anti- *E. coli* β-galactosidase serum (3, 46), whereas this antiserum does not react with the *Saccharomyces* and *Lactobacillus* enzymes. These studies illustrate with what definition the specific combining properties of this enzyme can be mapped, not only in terms of affinity constants but also in terms of molecular activity (turnover number), the latter being defined by immunological analysis as the amount of substrate hydrolyzed per unit time per antigenic grouping (3, 46).

F. Assay of β-Galactosidase in Whole Cells

Since in our studies on the kinetics of enzyme induction, the amount of enzyme is determined with toluenized whole cells, it must be shown that this method assays the totality of the β-galactosidase present. The interpretation of the data on the kinetics of formation depends upon this demonstration which illustrates an aspect of the methodology that has been neglected by many workers with other systems. The argument that the β-galactosidase assay is valid consisted of showing that the amount of activity determined by the use of enzyme preparations made in the sonic oscillator, from an acetone powder, or from alumina ground bacteria was the same as that found with cells treated with toluene. Great care must be used in the toluene treatment for slight changes in culture conditions render cells resistant to this solvent. Weiner (unpublished observations) has introduced the use of deoxycholate plus toluene and has shown that more reproducible and accurate assays are possible than with toluene alone.

I have summarized only these properties of β-galactosidase which have proved of importance in the study of its synthesis. Whereas this enzyme is not yet as well characterized as we would like to have it for certain kinds of experiments, what is already known about its activity and physical properties has permitted experiments on the kinetics and specificity of induction and on the precursor relationships which thus far have not been possible in other systems.

III. THE PROTEIN SYNTHESIZING REACTION

A. The Appearance of β-Galactosidase Activity as a Measure of Protein Synthesis

Even with as well characterized an enzyme as β-galactosidase, the demonstration of an increase in enzyme activity does not necessarily entail a quantitatively corresponding increase in enzyme protein for, as we know already, the activity of an enzyme is a function of many variables (presence of inhibitors, parasite reactions, ionic composition of cellular interior), many of which we cannot control in crude extracts. In fact, it is from this point of view that several workers (47) were correct in criticizing all studies on enzymatic "adaptation" aimed at an analysis of protein synthesis. The answer to this difficulty would be to measure the appearance of enzyme protein by some method independent of enzymatic activity. One very specific way of doing this is by the use of immunological methods, which today have been applied thoroughly only to the penicillinase (48) and the β-galactosidase (46, 49–54) system.

In collaboration with Dr. Torriani, the following immunological analysis was carried out. Rabbit antisera were prepared against purified enzyme (activity = 1.6 mmoles/min/mg N). These antisera precipitated but did not inactivate the β-galactosidase. An equivalence between antibody and enzyme activity could be defined in terms of the units of enzyme (activity) precipitable by a given volume of antiserum. With this titrated antiserum we could compare the extracts from glucose-grown cells, which contained only traces of activity, with those from lactose-grown cells which had high enzymatic activity. It was possible to absorb this antiserum with the extract of glucose-grown cells containing no enzyme until the serum no longer reacted on further addition of the extract. However, this absorbed serum continued to precipitate the β-galactosidase from the extract of lactose-grown cells (46). Thus, we had evidence that not only is there an increase in enzymatic activity during growth on lactose, but also a quantitatively corresponding increase in an antigenic component identifiable as β-galactosidase. This observation strongly supports the hypothesis that induction of β-galactosidase activity corresponds to the synthesis of new protein.

When it became clear that induction leads to the accelerated formation of a distinct molecular structure, the question of where this protein comes from was posed in experimental terms. In other words, what is the nature of the immediate precursor?

B. The Precursor Problem

Although this aspect of enzyme synthesis has been discussed at great length in many papers and reviews (12–16, 18–20, 52, 54), I should like to restate the problem because the work on the nature of the precursor poses very important methodological questions. The direct experiment we have just described told us that induced activity is equivalent to induced antigenic determinant groups following induction. Such groups could arise during induction by rearrangement of a pre-existing protein, by the addition to or removal from a protein of some specific structures, or by complete *de novo* synthesis from amino acids. What kind of experimental approach can be used to distinguish such alternatives?

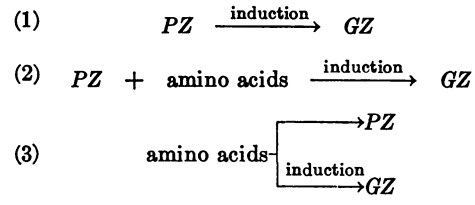
Immunology offers a general yet relatively unexploited method for studying the structural patterns of protein molecules. From the configurational similarities, the parental or filial relationships between protein molecules can be analyzed. If one protein is derived from another by differentiation or from many others by condensation or polymerization, one can expect that these proteins could have antigenic determinant groups in common, and that this relationship would be expressed by the manifestation of a serological cross reaction. With this rationale as a basis we analyzed induced and noninduced cells for the presence of proteins which cross reacted with antibody specific for β-galactosidase. The following observations (49) were made:

1. Extracts from induced cells whose enzymatic activity is very high contain two distinct antigens, designated *GZ* and *PZ*. *GZ* is the homologous antigen which precipitates all of the specific antibody. The identity of the protein *GZ* with β-galactosidase is quantitatively demonstrable. The antigen *PZ* has no galactosidase activity and gives a heterologous cross reaction in that it will precipitate only a fraction of the antigalactosidase antibody. The absorbed serum will precipitate *GZ* to the exclusion of *PZ*.

2. Extracts of noninduced cells which contain negligible amounts of enzyme possess *PZ* but no antigen *GZ*.

3. Under certain conditions, the induction of formation of *GZ* is accompanied by a small but consistent decrease in the rate of synthesis of *PZ*.

The immunological and kinetic data could be analyzed in terms of several hypotheses (50).



These hypotheses are generalizable in that we might ask whether, upon addition of a specific inducer, *PZ* or any other protein is converted to a given enzyme, either as is or by addition of cofactors.

Various kinds of indirect evidence were used in an effort to decide among the foregoing three hypotheses. These same arguments are often used today to demonstrate that the appearance of a given enzymatic activity actually involves *de novo* protein synthesis (11, 13, 14, 20, 25, 26, 54–57). Since none of these arguments is decisive, I should like briefly to review the objections.

A direct conversion of *PZ* (or any other precursor) to *GZ* not requiring energy and analogous to the zymogen-to-enzyme reaction appeared to be eliminated by the demonstration that no enzyme was formed after induction in the presence of metabolic poisons such as azide, cyanide, and dinitrophenol (11, 58). These experiments show that "energy" is required, but they do not say at what level this is needed. In fact, as we know today, all the experiments testing the effect of metabolic poisons were performed under conditions where entrance of the inducer into the cell required the presence of a transport system whose functioning was blocked by metabolic poisons (59–61). Therefore it is the induction pathway, *i.e.*, the entrance of inducer into the cell, and not the protein-synthesizing pathway, which could have been affected. The observation that β-galactosidase could not be induced in cells starved of nitrogen, sulfur, or carbon did not rule out the energy-linked direct conversion of a *PZ*-like precursor to enzyme if one assumed that the synthesis of nucleic acids or other cofactors accompanied the conversion.

These findings spoke against hypothesis 1, but did not distinguish 2 and 3. However, let us note that if the synthesis of some cellular component were necessary for the inducer to become effective in activating the $PZ \rightarrow GZ$ conversion, then even hypothesis 1 is not eliminated. The same argument holds true for the discovery that auxo-

trophs starved of required amino acids are not inducible since the synthesis of certain nonprotein cellular constituents such as ribose-nucleic acids seems to require the presence of amino acids (62–64). The blocking of enzyme induction by chloromycetin does not rule out that *PZ* is a precursor of *GZ* because it is possible the nucleic acid formed during protein blockage is not functional, and nonfunctional nucleic acid could affect inducer metabolism. The use of amino acid analogs, *e.g.*, *p*-fluorophenyl alanine, β-2-thienyl alanine, and tryptazan, must also be viewed with great caution. It was believed that the blocking of the appearance of induced enzyme activity by an amino acid analog was equivalent to starving an auxotroph of the corresponding amino acid. However, here, too, the argument was weakened by the demonstration of Munier and Cohen (65) that many of these analogs are incorporated into proteins.[2] These, of course, might be enzymatically inactive. Therefore, the lack of induction of enzyme activity does not mean that the enzyme protein has not been made, and *PZ* could have been converted by addition of the amino acid analogs to an enzymatically inactive *GZ*. These latter findings rule out hypothesis 1 only to the extent that the analogs are assumed to have no effect on the cell other than to be indistinguishable by the protein-synthesizing mechanism from naturally occurring amino acids.

Therefore, none of the above cited experiments provided a sufficient argument against any one of the proposed hypotheses. What we needed was a direct experiment in which the precursor was labeled with an isotope and the level of incorporation of this isotope into enzyme measured. The experiments designed to do this were carried out by Rotman and Spiegelman (19) and by Hogness *et al.* (18, 54).

[2] Halvorson and Spiegelman (56, 66) have shown with *p*-fluorophenyl alanine inhibition of protein synthesis in yeast that: (a) qualitatively the analog blocks the disappearance of amino acids from the pool; and (b) quantitatively the glutamic acid level in the pool remains unchanged in the presence of the analog. Provided (a) that there is no degradation of protein and resynthesis from the pool to give a steady state level, and (b) that the low-level incorporation of *p*-fluorophenyl alanine into protein (unpublished results of H. O. Halvorson) is negligible, one can conclude that these data support hypothesis 3 for maltase synthesis in yeast.

TABLE 1

Incorporation of sulfur into β-galactosidase synthesized by labeled Escherichia coli cells in nonradioactive medium (16)

Experiment	Per Cent Maximal Enzyme Level	$K = \frac{\text{Radioactivity}}{\text{Enzyme Activity}}$	Per Cent of K for Fully Labeled Enzyme*
Step I	0.06	(0.45)†	(100)†
Step II			
A	4.8	0.0050	0.1
B	32	0.0043	0.8
C	58	0.00072	0.1
Controls			
Fully labeled enzyme	100	0.45	100
Isolation control	4.8	0.0018	0.4

* Corrected for basal activity.

† Basal level assumed to be equal to fully labeled enzyme.

A noninduced culture whose proteins were labeled with radioactive S^{35} is represented in Step I. Under conditions (Step II) where the only source of radioactive sulfur was the proteins of this noninduced culture, growth in the presence of inducer was allowed to take place to various extents and samples for analysis were taken at moments A, B, and C. The β-galactosidase from each sample was isolated and its specific activity determined. The specific activities of these preparations are to be compared with two controls:

(a) the specific activity of the fully labeled enzyme from cells grown in the same medium as the noninduced cells except that inducer was added; and

(b) the specific activity of an isolation control made identical to sample A by mixing unlabeled enzyme with a labeled extract of noninduced cells.

The isolation control is a measure of the efficacy of our techniques for purifying the enzyme which, as shown above, has 0.4 per cent of the radioactivity of the fully labeled enzyme (100 per cent).

The hypothesis that these isotope experiments were designed to analyze was whether or not trichloracetic acid-insoluble protein precursors (of the *PZ* type) accumulated in the cell, either in the absence or presence of inducer (see table 1). The design of the experiment was to label with a suitable isotope [Rotman and Spiegelman

(19) used C^{14} and Hogness *et al.* (18) used S^{35}] the proteins of the noninduced cell (step I). Then in unlabeled medium, with acid-insoluble material as the only source of isotope, the cells were induced, and the incorporation of the label into the enzyme measured (step II). Clearly then it was crucial to the experiment that a way be devised of specifically labeling the proteins to the exclusion of low-molecular weight pools (amino acids, glutathione, etc.), for it was evident by this time that such substances eventually ended up in protein (67). In Rotman and Spiegelman's experiments this was accomplished by allowing a period of growth in unlabeled medium before induction, and in Hogness' experiments the cells were prestarved of sulfur.

Considering the data of Hogness *et al.* (see table 1), the amount of radioactivity associated with the enzyme synthesized in step II at three levels of induction A, B, and C was respectively 0.1, 0.8, and 0.1 per cent of that of the fully labeled enzyme. Inasmuch as there was no definite order to the value (*i.e.*, A > B > C) and because these values were within the range of reproducibility of the isolation control, it was concluded that in each sample, less than 0.8 per cent of the sulfur of the enzyme synthesized in step I was derived from cellular proteins present before induction (step I). This result, taken in conjunction with the fact that in sample A the enzyme level was only 5 per cent of that found in the fully induced bacteria, indicated that, if any protein precursor of β-galactosidase existed in noninduced cells, its level must be less than 0.04 per cent of that for β-galactosidase in fully induced bacteria.

Thus the possibility that *PZ* is a precursor of *GZ* was effectively eliminated. More generally, the results of the precursor experiment indicate that β-galactosidase is synthesized almost exclusively from the material that is assimilated after the addition of the inducer and hence proteins existing in the noninduced bacteria play no significant role as precursors. Rotman and Spiegelman's experiments (19) with carbon instead of sulfur labeling gave the same answer. These experiments provide very strong evidence that the induced appearance of β-galactosidase activity involves the complete *de novo* synthesis of a protein molecule from its amino acids. I think that the early objections (47) to the use of induced enzyme formation as a model for protein synthesis have been formally answered.

More recently Markovitz and Klein (68, 69) have carried out analogous experiments with the extracellular α-amylase of *Pseudomonas saccharophila*. This example is particularly interesting because it involves a secreted protein which might have been expected to have an inactive precursor, analogous to that described by Gorini and Lanzavecchia for proteinases (70). These workers found significant incorporation into the α-amylase of cellular materials present before induction. However, since they did not distinguish experimentally between the amino acid precursor pool and the high-molecular weight protein precursors, their result is not comparable to ours. A test of the stated hypotheses necessitates that a distinction be made between the amino acid pools and preformed protein as precursors.

The isotope incorporation experiments, then, tell us "the source of the enzyme not its route (71)." However, the further experiments described by Hogness *et al.* (18), as well as studies of the kinetics of enzyme formation (see later), show that any protein "precursor," if it exists, does not accumulate in detectable amounts either in the absence or the presence of inducer.[3] Our findings do not rule out the presence of trace amounts of precursor which remain in constant amount per cell but are synthesized and converted to enzyme, following induction, at a more rapid rate. These formulations which accompany the concept of the template have been thoroughly analyzed by Spiegelman in a recent review (12).

The results of the isotope incorporation experiments have focused new attention on two ancient problems in this field, that of protein interactions (3, 11, 16, 58) and that of protein turnover (57). Let us turn to them now.

C. The Problem of Interaction in the Synthesis of Enzymes

In the 1940's the problem of interactions in the formation of enzymes appeared to be a

[3] The demonstration by Wainwright (72) that nitratase and tetrathionase activities can be induced in nitrogen-starved *Escherichia coli* cells is preliminary evidence that there are systems in which the inducer may act on an accumulated differentiated precursor. However, we must await isolation and characterization of these enzymes before we can evaluate to what extent we are dealing with synthesis of enzyme protein and not simply activation.

key to understanding the mechanism of enzyme synthesis. All workers discussing this subject placed under this heading two kinds of findings: (a) the inhibition of induced enzyme synthesis by certain carbohydrates (73); (b) the mutual inhibition of "galactozymase," "glucozymase," and "maltozymase" by simultaneous adaptation in resting cell suspensions (11, 74).

Today, however, we need no longer consider these early experiments from the point of view of the precursor problem because: (a) the diauxic inhibition, at least for the moment, appears to involve the induction pathway and not the protein synthesizing pathway (see later); and (b) the mutual inhibition, during simultaneous adaptation in resting cells, was studied in multienzyme systems under conditions where the data are not specifically interpretable. However, the *PZ-GZ* interaction involving a defined system seemed to remain. The two relationships which bore on the precursor question were: (a) the structural relationship between *PZ* and *GZ* as revealed immunologically (49); and (b) the inhibition (and in some cases induction) of *PZ* synthesis upon induction of *GZ* (50).

The elimination of the precursor hypothesis thus left the previously mentioned hypothesis that the two proteins were related in that a common enzyme-forming system (EFS) produces *PZ* constitutively, but upon addition of an inducer the same EFS is activated to produce *GZ* to the partial exclusion of *PZ*. The interaction in synthesis then would be at the level of competition for the common enzyme-forming system which turned out both proteins, *PZ* and *GZ*.

The observation that a wide variety of mutants which were not inducible to make *GZ*, could make *PZ*, does not necessarily contradict this hypothesis, because one could always assume that it is the induction pathway and not the protein synthesizing one which is affected by the mutation. In a survey of *Enterobacteriaceae* it was shown that whereas all strains of *Escherichia coli*, *Aerobacter aerogenes* and *Shigella sonnei* (which are lactose-negative cryptics) possessed *PZ*, this protein was not present in other *Shigella* and *Salmonella* species. With the development of systems for genetic crossing and transduction between *E. coli* and *Shigella* sp. one could immediately ask whether the introduction of the ability to make β-galactosidase (*GZ*) into *PZ*-negative *Shigella* would also confer the ability to make *PZ*. The simplest interpretation of our hypothesis would predict that *PZ*- and *GZ*-making ability would go together from *E. coli* into *Shigella* sp.

Spiegelman and Lennox developed these ideas and then prepared the strains to test them (unpublished results). Strains of *Shigella dysenteriae* were rendered lactose positive either by transduction with phage grown on *E. coli* or by crossing with *E. coli*. It was surprising to find that of the several strains of *S. dysenteriae* rendered lactose positive, none contained *PZ*. Thus, the ability to make β-galactosidase could apparently be transferred without concomitant passage of capacity to synthesize *PZ*. The separation of *PZ* and *GZ* in *S. dysenteriae* does not fit the simple hypothesis that *PZ* and *GZ* are synthesized at a common site.

Now what kind of interpretation can be placed on our present information? There are two ways to account for the *PZ-GZ* relationships: (a) the above mentioned hypothesis that there is one enzyme-forming system which makes both *PZ* and *GZ*, or (b) that there are two independent enzyme-forming systems which happen to make similar molecules.

The separation of the synthesis of *PZ* from that of *GZ* in *Shigella* is strong evidence that we are dealing with independent systems. However, it does not formally rule out the hypothesis that one EFS is involved, for it can be assumed that *PZ* which is constitutively synthesized in *E. coli* is inducible in *S. dysenteriae*. The absence of *PZ* synthesis could merely mean that we do not know the inducer.

The hypothesis of independent enzyme-forming systems requires a qualification to account for the interaction. However, I do not think that these data on interaction are sufficiently established today to warrant that they be used as a critical argument in distinguishing the two hypotheses. Although usually the induction of *GZ* led to a barely detectable depression of the rate of synthesis of *PZ*, there were strains in which induction of *GZ* led to increased rates of synthesis. In any case, under the second hypothesis, inducers of *GZ* could be considered to be concomitant inhibitors of *PZ* synthesis in much the same way that many substances act to depress constitutive synthesis of enzymes or, in some mutants, to increase it (2).

In summary then, there is no established example of interaction in enzyme synthesis which

necessitates hypotheses involving competition for protein precursors.

D. The Turnover Question

The first and most important hypothesis in this field we owe to Yudkin (75), who suggested that adaptive enzymes were in equilibrium with their precursors in the cell schematized as follows:

$$\text{Precursors} \rightleftharpoons \text{enzyme} + \text{inducer} \rightleftharpoons \text{enzyme-inducer complex}$$

The equilibrium was very strongly in favor of the precursors, only traces of enzyme appearing in the cells in the absence of inducer. In the presence of inducer which formed a specific complex with enzyme, the equilibrium was displaced with the accumulation of enzyme.

The demonstration that β-galactosidase, once formed, is stable within the cells and that induction results in the initiation of an essentially irreversible process, ruled out all interpretations of this system in terms of the Yudkin hypothesis (57, 76–79). Furthermore, these findings showed not only that β-galactosidase is irreversibly synthesized, stable and static within the growing cells, but that this is true of most of the proteins of *E. coli*. Recently the extremely sensitive experiment of Koch and Levy (80) showed that the minimum half life of the *E. coli* protein is 30 days, thus making the experiments with bacteria comparable with that of animal cells. I shall not dwell upon the apparent contradiction between "static" bacterial proteins and "dynamic" animal proteins. This question is hotly disputed, and I have no new arguments to add to those which have been presented already (18, 20, 52, 54, 81).

Instead of considering the general question of turnover, let us specificially ask if there is any clear evidence that the enzyme-protein synthesizing reaction itself is reversible *in toto* or in part. We are trying to find out whether there is any evidence that the synthesis of protein from amino acids as well as the degradation of protein to amino acids *are* catalyzed by a single system. We shall not consider the question as to whether the enzyme protein can be or is degraded intracellularly to amino acids by other means such as proteinases.[4] As far as the mechanism of protein synthesis is concerned, the nonspecific degradation via proteinases is irrelevant.

A discussion such as this is difficult to document using evidence gathered with whole animals, organs or tissue slices, until it is shown that the enzyme-protein under analysis is being made in a single species of cells which are in an essentially homogeneous condition. Therefore, I shall confine my remarks mainly to bacterial systems, where in the best studied example, the synthesis of β-galactosidase, the over-all pathway from amino acids to enzyme protein is virtually irreversible.

However, this demonstration needs generalization, for the argument has been made (13) that β-galactosidase synthesis in *E. coli* might be a special case. There are after all numerous recent reports (57, 84, 85) concerning (a) intracellular degradation of protein and (b) exchange of amino acids with "proteins." Such observations might be interpreted to support reversibility and contradict any generalization of the conclusions we have drawn from one system. However, before accepting that the *E. coli* β-galactosidase represents the exceptional case, let us ask if this above-cited evidence means that we are dealing with true reversibility of the synthesizing reaction.

There are two kinds of findings which might be interpreted as showing that amino acids are derivable from enzyme protein. The first is that based (a) on an "energy-linked" destruction of enzymatic activity in the absence of inducer or (b) on the formation of induced enzyme in nitrogen-starved cells, or (c) on the replenishment of an amino acid pool under certain deficiency conditions. The second is that based on incorporation of a given amino acid into a protein fraction and its loss from that protein fraction under conditions where *increases* in protein are not detected (exchange reactions).

Consider first the data based on instability of enzyme activity during "deadaptation." It is observed that certain induced enzymes, in yeast

[4] The fact that after removal of the inducer, β-galactosidase is neither synthesized nor destroyed but is simply diluted out in the increasing bacterial protoplasm (3, 82, 83) means that the pathways of enzyme destruction, *not* via reversibility of synthesis, are negligible in *E. coli*. If the enzyme-synthesizing reaction were reversible, removal of inducer might be expected to block the forward as well as the backward reaction. In the case of β-galactosidase, however, even in the presence of inducer the synthetic reaction is not detectably reversible (18).

particularly, lose total activity rapidly on removal of the substrate (11, 86). This is to be contrasted with the β-galactosidase of *E. coli* which is simply diluted out during growth (3, 82, 83). Clearly the same criteria which apply to the interpretation of appearance of enzyme activity must also apply to its disappearance. Loss of such complex activities as "galactozymase" (11) or "maltozymase" (86) or the disappearance of an activity measured *in intact cells*, *e.g.*, the lysine decarboxylase of *E. coli* (87), could be due to innumerable factors (*e.g.*, denaturation, destruction of cofactors, formation of inhibitors, or development of impermeability to the substrate[5]), none of which involves any kind of intracellular degradation of enzyme protein to amino acids, not to mention reversal of the synthesizing pathway. As part of the same argument, the appearance of pools of amino acids during *nitrogen starvation* of cells (13, 85) provides evidence that there are internal reserves of nitrogen which can be converted to amino acids but says nothing as to what the nature of such reserves is. In yeast, Halvorson has shown that this nitrogen pool replenishment comes in part from the breakdown of nucleic acid and in part from "protein." Similarly, in *E. coli*, upon sulfur starvation, the alcohol-soluble proteins amounting to 15 per cent of the total protein, are converted to alcohol-insoluble protein, possibly via degradation to their amino acid constituents (67). The alcohol-soluble proteins might also be the source of the amino acid pool shown by Mandelstam (85) to appear in *E. coli* under certain conditions of starvation. Cells lacking the alcohol-soluble protein as a result of sulfur starvation grow normally without lag upon addition of sulfur to the medium, suggesting the possibility that these proteins do not contain enzymes essential to growth. Thus it appears that both *E. coli* and yeast can store amino acids as protein and call upon these reserves under restricted conditions. Unfortunately, we have insufficient information to decide whether the pathway of breakdown of these yeast and *E. coli* proteins is via a reversal of the synthetic reaction.

Therefore, I think that we can conclude that no evidence *as yet* gathered through investigations on deadaptation, or intracellular protein degradation, contradicts the hypothesis arrived at with the β-galactosidase system that the overall conversion of amino acid to enzyme protein follows an essentially irreversible reaction.

Let us now turn to the second line of evidence, namely that of the "exchange reaction." I shall deal here with the observations of Gale and coworkers on the incorporation and loss of single amino acids from the proteins of the staphylococci.

The staphylococci require a large number of amino acids for growth. Under conditions where protein synthesis could not proceed because of the lack of these essential amino acids, the incorporation into protein and the release from protein of only one amino acid, glutamate for example, would not be expected unless there were some kind of exchange reaction between the glutamic residues of preformed protein and free glutamate. Under conditions which I shall discuss later, the reaction whereby protein "combined" glutamate (hot trichloracetic acid insoluble glutamate) exchanges with free glutamate would require an "energy" source provided that the exchange takes place through the intermediate of an "activated" glutamate. This essentially is the basis for the experiments of Gale and his associates on the exchange reaction.

The demonstration by these workers of the exchange reaction is based largely on four observations:

1. Intact washed staphylococci or disrupted cells incorporate radioactive glutamic acid into protein in the presence of an energy source and in

[5] In fact, a little known but very important study by Terui and Okada (88) brings evidence that there are two kinds of mechanism operating in the disappearance of maltozymase of *Saccharomyces saké* during deadaptation: first, the inactivation of "enzyme" which is not reversible by the presence of substrate (probably denaturation); and second, that which is reversible on addition of substrate. In a clever analysis of the deadaptation, by correcting for irreversible loss of activity, they conclude that "the activity per cell was diluted by the newly formed maltozymase-free plasm." Recently Robertson and Halvorson (89) showed that the loss of "maltozymase" activity during the deadaptation involves a complex situation in which there are permeability factors, change of state (insoluble to soluble) of α-glucosidase and then true loss of α-glucosidase activity in extracts. This latter observation, important for our discussion, needs to be extended to answer the question of whether the loss of activity means conversion of the enzyme-protein to amino acids or simple denaturation.

the absence of added exogenous amino acids which are required for growth (condition 1) (27, 90).

2. Intact washed staphylococci or disrupted cells incorporate radioactive glutamic acid 2.5 times more rapidly if the other required amino acids are added (condition 2) (27, 90).

3. Incorporation under conditions 1 and 2 appears to have different sensitivities to antibiotics (27, 90).

4. Once incorporated, glutamic acid is released from the "*protein*" of disrupted cells specifically in the presence of adenosine triphosphate (ATP) plus hexose' diphosphate (HDP) plus glutamic acid (24).

The conclusion that these data represent an exchange reaction depends upon the extent to which other mechanisms are ruled out. Consider these two reactions as examples:

(a) Single amino acid exchange

protein-combined glutamate $\rightleftharpoons$

activated glutamate $\rightleftharpoons$ free glutamate

(b) Protein degradation, *e.g.*, proteolysis' followed by resynthesis

protein-combined glutamate $\xrightarrow{\textit{degradation}}$

free glutamate + all amino acids

resynthesis via independent synthetic pathways

Both mechanisms would account for an incorporation of a single amino acid in the absence of *added* essential amino acids under conditions of no net protein synthesis.

Gale and Folkes (62) showed that, when intact, washed staphylococci were incubated with glucose and glutamate alone (condition 1), they lost in 1 hr about 12 per cent of their total "combined" glutamate which effectively is a measure of protein (91). In whole cells, then, under condition 1 where there is protein degradation there is incorporation into protein of radioactive glutamate.

It seems to me that it is simpler to interpret these incorporation data as net protein synthesis where amino acids are supplied from traces in the pools and from protein breakdown. The principal arguments against this interpretation are: (a) that there is a difference in the sensitivity of the incorporation reaction to antibiotics depending upon whether glutamate is given alone (condition 1) or all the amino acids are present (condition 2) (90); and (b) that *p*-chlorophenylalanine blocks phenylalanine incorporation into intact cells but has no affect upon glutamate incorporation (92).

These arguments are not sufficient. In the first place, the difference in sensitivity of glutamate incorporation to a spectrum of antibiotics under condition 1 or 2 does not mean that the two processes are inherently different. The presence or absence of an externally supplied battery of amino acids might enhance or inhibit the action of an antibiotic for reasons quite removed from the question of exchange or protein synthesis.[6] This problem is all the more accented when it is realized that there is not a single antibiotic whose precise mode of action is known. Secondly, the demonstration that *p*-chlorophenylalanine blocks phenylalanine but not glutamic acid incorporation into the protein of intact cells is not an argument for exchange of glutamate with the equivalent residues of protein, for it is possible either that the *p*-chlorophenylalanine analog itself is incorporated into protein, or that glutamate is incorporated into a nonphenylalanine-containing protein fraction.[7] Now let us look more closely at the implications of the exchange reaction.

Suppose that we were dealing with nothing more than a simple reversible synthetic reaction in which

Free glutamate + X-Y $\rightleftharpoons$ glutamate-X (activated glutamate) + Y

glutamate-X (activated glutamate) + other activated amino acids or protein residue $\rightleftharpoons$ protein "combined" glutamate + X

where X-Y represents a high-energy compound such as ATP
X represents as an example the adenylic acid portion
Y represents as an example the pyrophosphate portion.

[6] This possibility is suggested by the demonstration of Gale and Folkes (90) that "there are marked differences in the sensitivity of the incorporation processes for various amino acids."

[7] The fact that glutamate incorporation in whole cells is independent of whether or not the essential amino acid, phenylalanine, is added (90) suggests this latter possibility.

The exchange reaction between protein "combined" glutamate and free glutamate requires only the presence of X and Y, illustrated as follows:

$$\text{protein "combined" glutamate} \overset{X}{\rightleftharpoons} \text{activated glutamate} \overset{Y}{\rightleftharpoons} \text{free glutamate.}$$

Under this formulation the need for an energy source, X-Y, to show an exchange between protein "combined" glutamate and free glutamate can only arise indirectly, under conditions where the X and the Y components are missing, by providing a source for them. In whole cells the blocking of incorporation of glutamic acid (under condition 1) by omission of energy source (glucose) or by addition of metabolic poisons is not a necessary consequence of an exchange reaction. The need for an energy source is a necessary consequence of *de novo* synthesis. The metabolic blocks, produced either by omission or by poisoning, might either prevent penetration of glutamic acid into the cell or else inhibit *de novo* synthesis which this incorporation seems to represent. Furthermore, this series of exchange reactions predicts that, *during synthesis*, one should get the loss of isotopically labeled glutamate from preformed protein merely by flooding the system with unlabeled free glutamate.

In the *E. coli* β-galactosidase system this type of experiment has failed to reveal any exchange. In intact staphylococci Gale and Folkes (90) point out the difficulty of carrying out this experiment by stating that, "Attempts to do this with intact cells have not been successful since the highly efficient concentration of free glutamic acid within the cells during the incorporation process has made it technically almost impossible to reverse the situation." Therefore, these workers turned to cells damaged by sonic vibration to demonstrate exchange. Under condition 1, disrupted cells cease to incorporate C^{14}-glutamic acid (even when added in high concentrations) when the amount of glutamic acid taken up corresponds to about 5 per cent of the total protein-glutamate residues in the preparation, and therefore, the great majority of glutamic residues have not been shown to be susceptible to exchange (93). As with intact cells, the disrupted cells, on incubation with an "energy" source and glutamate, actually lost about 10 per cent of the total protein while at the same time incorporating labeled glutamate (27). This loss makes unnecessary any interpretation of "exchange" as anything other than simple *de novo* protein synthesis concomitant with some degradation. In order to interpret the ATP-HDP requirement for incorporation as simple exchange of the type pictured above, it must be assumed either that the high-energy compounds are being used indirectly, not as "energy" sources for the synthesis of activated intermediates, or else that there are parasite reactions which are removing the activated intermediate which require regeneration. On the other hand, if there is simply *de novo* synthesis at a trace level, not exchange, the apparent ATP requirement is quite comprehensible.

Using disrupted staphylococci cells, Gale and Folkes (24, 27) have shown that the incorporated radioactive glutamate can be partially removed from the cell fragments by incubation specifically with an "energy source" (HDP + ATP) plus unlabeled glutamate. Removal of incorporated glutamate under the above conditions is of the order of 30 to 40 per cent of that fixed instead of 100 per cent as would be expected if a simple exchange reaction were occurring (24, 27). Only part of the incorporated glutamate of disrupted cells has been shown to be specifically removable then, and the remainder seems to be irreversibly fixed. There is no direct evidence as yet that the removable fraction is actually in peptide linkage in protein.

What are the conclusions that are suggested by this work? Since over 95 per cent of the protein-"combined" glutamate does not appear to enter into exchange reactions, it is clear that the overall synthesis is essentially irreversible. Further experimentation is needed to determine whether the remaining so-called exchangeable glutamate: (a) involves a protein fraction similar to the alcohol-soluble proteins of *E. coli*; (b) is in some way an artifact irrelevant to protein synthesis; or (c) as Gale postulates (27) is in reality some kind of nucleoprotein intermediate in the pathway of protein synthesis which has several reversible steps.

Exchange reactions are not universally or inevitably encountered in cell-free systems. Using cell-free preparations obtained from rat liver, Zamecnik (94) found that "once C^{14}-leucine or C^{14}-valine has become incorporated into microsome protein, it has not been possible to 'wash it out' with large quantities of inert leucine or

valine. The incorporation is thus a relatively irreversible step on the path to protein synthesis and differs from the exchange process described by Gale and Folkes for the incorporation of C^{14}-glutamic acid into protein (and possibly glutathione) in the fragmented *Staphylococcus aureus*." Allfrey *et al.* (95) have confirmed Zamecnik's observation using preparations of cell nuclei.

Finally, note that studies on incorporation of amino acids or their loss from "protein," defined simply by its precipitability with trichloracetic acid (or other acid precipitants), are very difficult to interpret in terms of the reversibility of the synthetic reaction because "protein" is a heterogeneous collection of materials of widely varying properties and composition, each component capable of slightly different behavior in its incorporation reaction. A clear argument for exchange reactions could be made by showing that isotopic label corresponding to one amino acid can be taken into and lost from a single isolated enzyme protein under conditions where there is no *de novo* synthesis of this protein. In other words, experiments such as those performed with β-galactosidase should be repreated with other systems to test the generality of the findings.

In summary then, the exchange reaction between free amino acids and amino acid residues in protein requires further study before it will be necessary to abandon the hypothesis that the over-all pathway from amino acids to enzyme protein is essentially irreversible. "This does not mean that all proteins in all cells have to be stable at all times (96)," but it does mean that the dynamic state is *not as yet* a "fundamental fact that must be kept in mind in any consideration of the mechanism of protein synthesis (97)."

IV. THE INDUCTION PATHWAY

I have placed the discussion of the kinetics of enzyme formation under the problem of the metabolism of inducers not only because the isotope incorporation experiments are sufficiently unambiguous to be independent of the kinetic argument, but also because our understanding of the kinetics has been greatly clarified by the discovery of Cohen, Rickenberg, Buttin, and Monod (60) of specific systems which bring the inducer into the cell. I shall rely on the papers by this group to give the background to this aspect of the discussion (8, 59, 60, 61).

A. The Kinetics of Enzyme Synthesis as a Function of Growth

It is not surprising that the kinetics of induced enzyme synthesis is the most disputed and confused aspect of this subject. Kinetic analyses are difficult to carry out, and interpretations are often dangerously ambiguous, especially when whole cells are involved. Futhermore, arguments based on kinetics must always be supported by other evidence. Clearly then, the analyses of the kinetics of enzyme formation cannot be carried out unless certain simplifications and approximations are resorted to. How can we do this?

Specifically, we should like to know what is the kinetics of the reaction whereby amino acids are converted under the action of the inducer and some cell component (or components) to induced enzyme. As a setting for this problem it should be remembered that there are two steps in the synthesis of a protein: the "nonspecific" step, common to all proteins, at which amino acids are activated[8] and thereby prepared for the specific step where the characteristic configuration is formed. In protein synthesis, only two factors which control the specific steps have been uncovered: (a) chemical factors (action of specific inducers and inhibitors of induction); (b) genetic factors (mutation and segregation of hereditary units).

The kinetic formulations concern themselves essentially with action of the chemical factors which, against a fixed hereditary background, intervene to provoke the cell to make a given enzyme.

I have tried to illustrate the problem by the following diagram of a cell which is not only supplying activated structural materials "specifically" to the enzyme system under consideration, but also "nonspecifically" to all the other protein-forming mechanisms. The problem is to isolate "specifically" the β-galactosidase-synthesizing reaction which I illustrated by the double line enclosure, from the "nonspecific" flow of building blocks to the rest of the cell (figure 3). Clearly then, if we set as our goal the study of the specific factors involved in enzyme synthesis (our common nonspecific denominator

[8] I do not want to beg the question as to whether activation of amino acids is prior to the action of or part of the enzyme-forming system. For our purposes the distinction is of no importance.

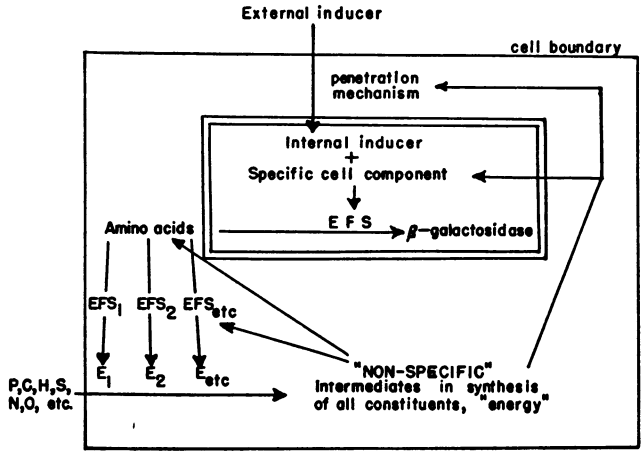


Figure 3

to general protein synthesis being the metabolic factors, *e.g.*, amino acid precursors, etc.), then we should study enzyme synthesis as a function of general protein synthesis. In this way we can distinguish the specific from the nonspecific effects of any given agent on the synthesis of the enzyme under investigation.

The course of the synthesis of a cellular constituent as a function of time is difficult to interpret in terms of the action of specific factors because it depends simultaneously on the nonspecific metabolic factors. As a first approximation these metabolic factors are eliminated when, instead of considering time-rate of synthesis (dE/dt) of a given enzyme protein (E), we consider the rate of synthesis relative to the total rate of protein synthesis (dE/dx) where x represents the total mass of protein. We have simply substituted "physiological time" (dx) for "absolute time" (dt). The relationship (dE/dx) is called the *differential rate of synthesis* (P) (20).

It should be noted that in general it is not x, the protein, which is measured but simply bacterial mass, B. This latter measurement is valid for studies provided that one is working with exponentially growing cells. However, there are many situations where x is not equivalent to B and this lack of equivalence can be misleading. Since the specific activity of β-galactosidase is known, I shall express dE/dx in terms of units of enzyme protein/unit of cellular protein.

The study of the differential rate of synthesis is not only *a priori* preferable, but it actually eliminates many difficulties and complexities of experimentation. As we shall see, its use has revealed certain simple and useful relationships which had remained masked so long as "absolute" time rates were considered.

Under conditions where the only protein being made by the cell is the induced enzyme under investigation (absolute preferential synthesis), if this protein were made *de novo*, the differential rate of synthesis (P) would equal one, and if there were conversion of precursor enzyme to enzyme, P would be infinity. Since in most cases there is concomitant protein synthesis, P is less than one, and the kinetics will not necessarily distinguish between *de novo* formation and conversion of precursors.

The suggestion that induced enzyme synthesis should, in general, be expressed in terms of overall protein synthesis is not invalidated by the finding of examples where an induced enzyme is synthesized to the exclusion of all other protein constituents (98). Such a finding only means that the differential rate of synthesis, expressed in the above units, is one or infinity. Furthermore, the eventual development of a cell-free system which makes β-galactosidase as its only protein will not invalidate this methodology. Under such *in vitro* conditions where $P = 1$, other formulations of the kinetics of formation of enzyme might become more informative.

Ideally, we should study the formation of a given enzyme in a growing single cell as a function of general protein synthesis because the problem of enzyme formation is defined at a cellular and not at a populational level. However, suitable

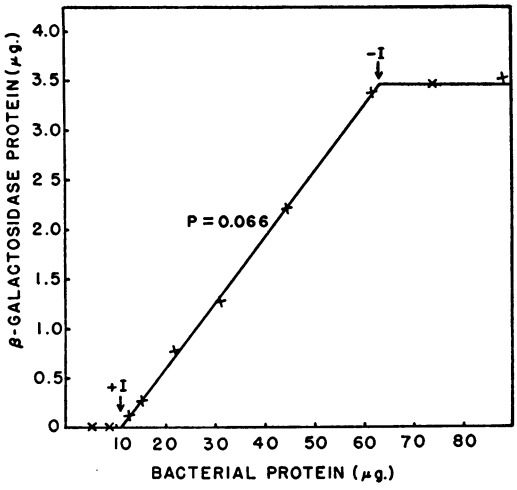


Figure 4. To a culture of ML 30 (lac +) growing exponentially on succinate-inorganic salts medium, methyl-β-D-thiogalactoside (*I*) was added at a concentration of 5×10^{-4} M. The enzyme activities measured were converted to μg of enzyme protein by using the value 3.36×10^3 μmoles/μg protein. At the point marked, $-I$, the inducer was effectively removed by adding 10^{-2} M phenyl-β-D-thiogalactoside, a competitive inhibitor of induction (8). For our purposes the addition of the inhibitor illustrates what would be seen if the inducer were removed by washing. Note that 6.6 per cent of the total bacterial protein is the induced enzyme itself.

single cell systems are not yet available (although not far away), and as an approximation, a culture growing exponentially in an essentially steady-state condition is used. The exponential steady-state growth phase is today the most reproducible and only meaningful *in vivo* system in which to study induced enzyme synthesis.[9] Such a steady-state culture can be obtained either by continuous culture (99, 100) or by free growth at low bacterial concentration in a medium which possesses all of its components in sufficient excess so that slight changes in concentration have no affect on the composition of the cell. For the *E. coli*-β-galactosidase system we often use a well aerated inorganic salts medium with succinate or maltose as only carbon source, and a bacterial density no greater than 5×10^8 cells/ml.

Consider one such experiment (figure 4). If to an exponentially growing culture, a suitable inducer (*I*) at a saturating concentration is added, the synthesis of β-galactosidase begins virtually without lag (*i.e.*, within a period of less than 5 min in a culture with a generation time of 70 min) (see page 159) and continues as long as the culture grows in the presence of inducer (20). Removal of inducer results in immediate cessation of synthesis and the already formed enzyme is simply diluted out in the exponentially increasing population (3, 82, 83). The differential rate of synthesis is a constant from the moment of addition of the inducer (20). This result is simple and enables quantitative comparisons between various inducers or inhibitors, specific or general.

The fact that P is a constant from the start of induction suggests that the synthesis of enzyme is an essentially irreversible process. Any hypothesis involving an equilibrium between β-galactosidase and its precursors leads to the prediction that the differential rate of synthesis will reach constancy only gradually (20). This conclusion was reached before the isotope incorporation experiments and as we already know, was amply confirmed by them (18, 19).

The differential rate of synthesis is determined both by the amount of active EFS per cell and by the availability of building blocks. Since in a steady-state culture this latter factor might be expected to remain unchanged, the constant differential rate of synthesis is evidence that the amount of EFS per cell remains constant on induction. As Monod (8) has discussed the evidence for this conclusion in detail, I shall simply refer to his paper.

In a publication in 1951, regrettably unknown to me until recently, Terui and Okada (88) independently formulated the differential rate of synthesis and applied it to "maltozymase" formation in yeast. They showed that induced "maltozymase" activity follows a constant differential rate of synthesis. It would be informative in yeast systems to study this relationship with a defined enzyme.

B. The Kinetics of Enzyme Formation as a Cellular Phenomenon: The Methodology of Gratuity

The first simplification then in the study of kinetics is to use the differential rate of synthesis. Having decided this, the next problem arises from the fact that enzyme synthesis is being studied in a population in which each cell behaves as an independent unit. In order that the interpretation of the kinetics of formation of enzyme

[9] Unfortunately, enzyme synthesis has not as yet been studied in synchronous cultures.

in the population (over-all kinetics) be applicable at the cellular level, we must know: (a) whether the response of all cells to induction is simultaneous and equal (homogeneity); (b) whether only a fraction respond (heterogeneity); or (c) whether all respond but at different times (heterogeneity). Clearly, the problem is not solved by elimination of the most obvious cause of heterogeneity, namely mutation and selection.

To make this methodological formulation clear let me illustrate with a concrete case. Suppose that a noninduced culture of *Escherichia coli* grown specifically to starvation on succinate is transferred to a medium containing lactose as only carbon source. In order to use lactose the cells must synthesize some of the β-galactosidase system (60). Since there is no appreciable leaking out of either enzyme or its products, each cell is a closed metabolic and induction system. Furthermore, not only is lactose metabolized exclusively via the β-galactosidase system, but the synthesis of this system requires a source of carbon. Therefore, whether and when any cell shall start synthesizing enzyme must depend on traces of enzyme already present, or upon internal metabolic reserves. Once started, the process of enzyme synthesis will be self-accelerating (autocatalytic) since the rate of enzyme synthesis will depend upon available metabolic energy which, in turn, depends upon the amount of enzyme present at any moment. Any cell that had a slight initial advantage for any reason would gain increasingly and the population would tend to become increasingly heterogeneous with respect to enzyme content. Evidently then the determination of the over-all kinetics of enzyme synthesis for the population as a whole would not represent even approximately the course of events at the cellular level. The observed over-all kinetics would be an average depending upon the rate at which cells become inducible by lactose and then are induced, and therefore over-all kinetics would not be interpretable at the cellular level. What we need is a system in which the over-all kinetics is an average of the formation of enzyme in individual cells where every cell is induced.

Conditions of induction then where the inducer is simultaneously the only metabolic source should be expected to and do give rise to spurious kinetic effects which mask the true course of induction at the cellular level (83). Until 1951 most studies on kinetics of induction were carried out under precisely these conditions (11, 17, 60) and it was to clarify this situation that Monod *et al.* (3) developed the methodology of "gratuity." This stated that the kinetics of enzyme formation should be studied under conditions where neither the presence of the enzyme itself, nor its inducer, influences the general cellular metabolism. The inducer should introduce *only one* new factor, namely a supplementary process of specific synthesis which is precisely the phenomenon under investigation.

We have illustrated previously the simplicity of the kinetics of enzyme formation under conditions of metabolic gratuity in which the inducer methyl-β-D-thiogalactoside, not metabolizable as a carbon or sulfur source, was used. That a system analogous to this one actually involves a homogeneous population, was shown by the ingenious experiments of Benzer (83), and even today the β-galactosidase system is the only one for which it has been shown by direct analysis that the over-all kinetics are a reflection of the cellular kinetics. Furthermore, Benzer showed that *metabolic nongratuity* led to the expected heterogeneity. The whole situation appeared intellectually satisfying since a constant differential rate of synthesis was the only result which could not be predicted from any likely distribution of cellular properties. I am dwelling on these obvious formulations because the rule of gratuity has taken on a more subtle form in recent times which I shall deal with later.

C. The Kinetics of Enzyme Formation in "Unresting" Cells

The methodology which I have discussed is very little used. By far the greater number of publications (29, 68, 76–79, 87, 101–107) on adaptive enzymes involve the use of washed cell suspensions, termed "resting" cells. Aside from the question of mutation *versus* adaptation, studies with such preparations were carried out with the hope that during induction, the lack of a "significant" increase in optical density or plate count meant that the interpretation of the data would be greatly simplified. Why is this not so?

If one prepared specifically starved cells,[10] *i.e.*,

[10] It was once thought that the use of specific starvation by growth to a given limit is methodologically equivalent to the use of growth inhibitor analogs of amino acids and purine and pyrimidine bases in that the analogs set up specific starva-

the growth of the cells ceases because of the exhaustion of one known component, then with the exception of thymineless mutants (108) no induced enzyme formation can be provoked (20). (See footnote 3, page 148). In all cases of specific starvation not only is no induced enzyme formed, but also during this starvation period no protein is made. With the thymineless mutant, while it is clear that induced enzyme is made, it must not be forgotten that general protein synthesis is not affected. These experiments (20) show that at the "nonspecific" level, as defined previously, induced enzyme synthesis is part and parcel of general protein synthesis. Obviously, then, when the cells are "resting" because they have been starved of a known component, then either no induced enzyme-protein formation occurs or, in unbalanced growth, enzyme formation follows a relationship describable by the differential rate of synthesis. Therefore, the use of specifically starved cultures can and does give precise data about the mechanism of enzyme synthesis (20), only because no enzyme is formed (see footnote 3, page 148). If some enzyme were formed, then increase in protein would have to be measured and one would return to investigating the differential rate of synthesis.

If, on the other hand, an overnight culture in complex medium is washed and resuspended in phosphate buffer, generally with a carbon and energy source, some induced enzyme formation can occur. Inasmuch as specifically starved cells usually do not produce induced enzyme, these washed suspensions must derive their basic building blocks either: (a) from stored up critical constituents (such as amino acids and alcohol-soluble proteins) derived from the complex growth medium from which it was washed, (b) from the resuspension medium which contains essential trace elements (metal ions, ammonia); or (c) from the lysis of some cells. Clearly the absence of obvious cell division does not mean the absence of *de novo* protein synthesis. The appearance of enzymatic activity in a cell suspension which is not increasing in optical density or in plate count, may attest only to the greater sensitivity and accuracy of the measurement of enzymatic activity. The belief that with resting cells one dissociates the growth of a new cell from enzyme synthesis, implies that the only protein being made is the one the experimenter is investigating (69).[11] Direct experimental evidence for this is needed, *e.g.*, the demonstration that radioactive amino acids are incorporated only into the enzyme protein under investigation. Furthermore, in no study with washed cell suspensions has any consideration of the heterogeneity of the system been entertained, especially important because there is every reason to believe that the individuals of a washed cell suspension would have all levels of internal reserves with a resultant wide variation in ability to make enzymes. The resultant kinetics, therefore, would not be applicable at the cellular level.

In the 1940's the use of resting-cell suspensions to settle the question of mutation *versus* adaptation was justified. Today, however, except in specific instances, the study of the time kinetics of enzyme formation with these "resting" nongratuitous systems is meaningless, and conclusions which have been derived from such studies on the kinetics of enzyme formation and on the interaction of protein-forming systems are no longer valid.

D. Autocatalysis in Induced Enzyme Synthesis: The Inducer Transport System

Now that the main elements of the methodology have been discussed, we must come to the very important question of whether there is any

tions. This, in most cases, is incorrect because it is now known that many of these analogs do not block macromolecular synthesis but are incorporated themselves to yield inactive macromolecular units. Therefore, the enzyme activity as a measurement of protein becomes meaningless since inactive enzyme protein could be formed. An amino acid starved cell differs from one blocked by certain amino acid analogs in that the former does not make DNA, RNA or protein, while the latter makes all three (62–64). The protein synthesized in the presence of an amino acid analog often possesses no enzymatic activity as a result of which one observes linear instead of exponential growth (65).

[11] This has been expressed as follows: "On the other hand enzymic adaptation can be sharply distinguished from what is ordinarily meant by bacterial growth and in some cases has been shown to occur in its absence." (106, p. 185).

"The use of growing cultures in enzymatic adaptation experiments complicates any attempts at analyzing the kinetics of the process. It is difficult under such circumstances to dissociate the growth of a new cell from the appearance of enzyme activity, and the time-activity curves obtained would be primarily determined by the growth characteristics of the culture" (11, p. 279).

evidence for autocatalysis in the induction of an enzyme. This question was introduced when it was found that under certain conditions the kinetics of formation of enzyme as a function of time could be accounted for by the assumption that the primary rate-limiting process was an autocatalytic one. This means simply that the rate of enzyme formation is a function of the amount of enzyme present. Provided that one is not dealing with a spurious kinetics, such a finding implies that upon addition of inducer the amount of active enzyme-forming system per cell increases. This could occur either by induced duplication of the EFS (109) or by induced activation of a dormant EFS (110). These important speculations have stimulated a great deal of work in this field. Today, however, we know that most of the experiments which gave the classical S-shaped curves relating enzyme activity to time are subject to the criticisms I have discussed, (a) because they were carried out under nongratuitous conditions in "resting" cells, and (b) because the enzyme systems under analysis were poorly defined.

The only data available today which indicate that the synthesis of induced enzyme follows an inherently autocatalytic kinetics are those obtained with slow-adapting populations of yeast upon exposure to galactose (110–117). Several years ago in discussion of this phenomenon in a review written in French (3), we proposed a model which, starting with the rule of gratuity, described how a heterogeneous response to an inducer arises and how this heterogeneity becomes clonally distributed. A direct translation follows:

"Let us imagine a system possessing the following properties: In a suspension of growing cells, an inducer I provokes the synthesis of an enzyme Z. The probability that any cell will synthesize at least one molecule of Z per unit time is (1) infinitesimal in the absence of I; (2) small in the presence of I for those cells which do not possess already one molecule of Z; (3) large in the presence of I for the cells which possess already one molecule of Z.

It is clear that in such a system the synthesis of the enzyme will be a phenomenon practically discontinuous at the cellular level by comparison with the over-all kinetics. However, in order to show such a discontinuity, it would be necessary to reveal the presence of enzyme in individual cells, unless, of course, the supplementary condition is added that the 'unit of time' is equal to the average time which separates two cell generations. This is equivalent to supposing that the probability of formation of a first molecule of Z within a generation time is small. Under these conditions, the discontinuity in question would not only be reflected in a given cell but further, during a certain time in the descendants. At one level the capacity to synthesize rapidly the enzyme, Z, would become a clonal property appearing like a mutation, while at the other level, the 'adaptation' of such a population in the presence of inducer would be slow and would cover a duration corresponding to a rather large number of cellular generations."

This model predicts: (a) that there will be a preinduction effect; and (b) there will be a clonal distribution of enzyme-forming cells which will arise in the population in a manner simulating a mutation. The yeast system has not as yet been analyzed in terms of this model because the kinetics of induction of a complex enzyme system "galactozymase" is inferred indirectly from growth studies. The *E. coli*-β-galactosidase system, on the other hand, has been carefully analyzed to test the predictions of this model and both a preinductive effect and a clonally distributed heterogeneity have been uncovered in this system.

In order to permit the use of general terms in the discussion of the kinetics of enzyme synthesis, I shall have to introduce a precise description of the time scale. The cultures of *E. coli* used in our studies had generation times of approximately 70 min. The sensitivity of the experiments carried out were such that enzyme formation will be said to begin without lag if the differential rate of synthesis attains constancy within 5 min after the addition of inducer. A 5-min period in a generation time of 70 min is equivalent to a 5 per cent increase in bacterial mass. A lag in formation, then, implies that the differential rate has reached constancy in a period greater than that required for a 5 per cent increase in bacterial mass.

1. Preinductive or maintenance effects. The terms *preinductive* effect and *maintenance* effect have been used by Monod (8) and by Novick (118), respectively, to describe systems in which an induced population of cells behaves differently toward an inducer than does a noninduced population.

As we have seen (figure 4), if a noninduced growing culture of a lactose-positive *E. coli* is placed in a saturating concentration of inducer, the differential rate of synthesis is constant

virtually from the start. If the concentration is lowered to nonsaturating levels (figure 5), then the differential rate shows an acceleration phase which reaches constancy only gradually, depending upon the concentration of inducer (8, 118). If a fully induced culture, which has had its inducer removed by any of several methods, is allowed to grow in the nonsaturating concentration of inducer, enzyme synthesis begins immediately at a constant (or falling) differential rate depending on inducer concentration. There is no acceleration phase.

The second example of these effects (figure 6) is derived from Monod's original observation that glucose inhibits the induced formation of β-galactosidase (73). If glucose is added simultaneously with inducer to a noninduced culture, the synthesis of β-galactosidase is entirely blocked. However, if the same experiment is carried out with an induced culture, enzyme synthesis takes place in spite of the presence of the inhibitor. Glucose, then, appears to decrease the effective concentration of the inducer.

The induced cell then has acquired something which makes it able to respond to low concentrations of inducer or to inducer in the presence of certain inhibitors such as glucose. This acquired something has the property of being inheritable as long as the cells are kept in the presence of

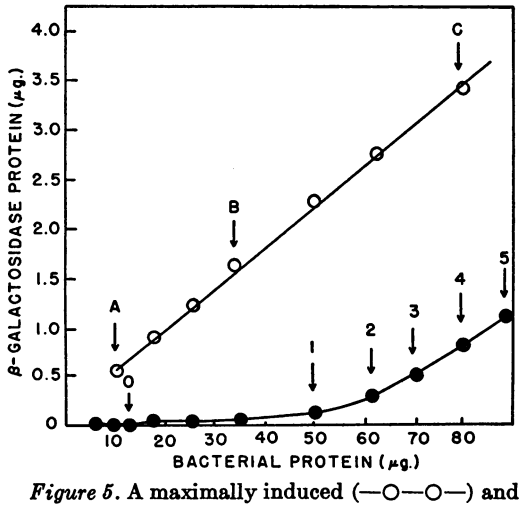


Figure 5. A maximally induced (—○—○—) and a noninduced (—●—●—) culture of ML 30 (lactose +) were placed in growth medium containing as inducer methyl-β-D-thiogalactoside at a nonsaturating concentration of 1.5×10^{-5} M. (See text for discussion and see table 3 for further analysis.)

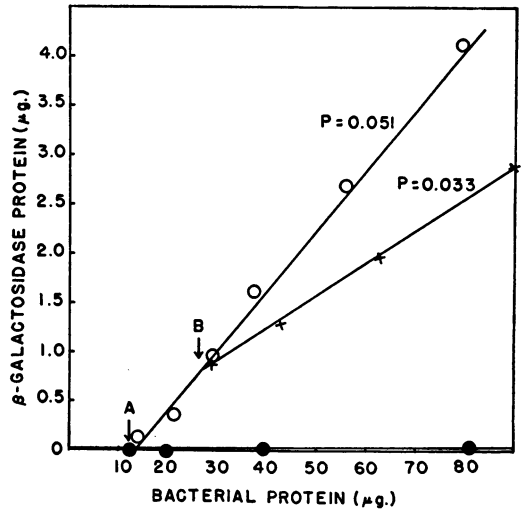


Figure 6. An exponentially growing noninduced culture of ML 30 is placed at moment labeled *A* into TMG, 10^{-4} M (—○—○—), and into TMG, 10^{-4} M, glucose 10^{-3} M (—●—●—). When the culture in TMG alone reached the state of induction indicated by *B* above, glucose, 10^{-3} M, was added to an aliquot (—*X*—*X*—). (See table 2 for data on duration of maintenance in glucose.)

inducer. Consider the example given in table 2. Fully induced and noninduced single cells are each placed in nonsaturating concentrations of inducer and into glucose plus inducer. The clone of 10^8 cells which is eventually derived from the inoculated cell either possesses enzyme or it does not, depending upon whether it was preinduced or not. This difference in ability to respond to low concentrations of inducer is maintained for 79 generations, and where the glucose system is used, a difference has been established through 135 generations. Novick and Weiner (118) have shown that under the controlled conditions of continuous culture, maintenance with low concentrations of inducer can be established for at least 180 generations, instead of the 79 generations shown in the experiment reported here. This is a demonstration that a permanent difference as regards enzyme synthesis can be established between two genotypically identical populations growing in the same medium.

If preinduced cells are grown in the absence of inducer (deadaptation) and at various times aliquots are placed into maintenance conditions, the ability to reverse the glucose inhibition or to be induced without lag by low concentrations is

TABLE 2
Duration of maintenance in Escherichia coli

Passage	Number of Gene-Rations	Mass Increase	P($E_{Protein}/B_{Protein}$) × 10^2		
			TMG 10^{-4} M	Glucose 10^{-3} M + TMG 10^{-4} M	TMG 8×10^{-6} M
I					
Induced	26.6	10^8	5.7	3.3	2.6
Noninduced			5.2	0.0091	0.022
II					
Induced	53.1	10^{16}	4.5	2.7	2.1
Noninduced			4.9	0.0033	0.039
III					
Induced	79.7	10^{24}	5.3	3.0	1.6
Noninduced			5.5	0.0052	0.065
IV					
Induced	106	10^{32}	5.8	2.4	0.20
Noninduced			5.0	0.0081	0.25
V					
Induced	135	10^{41}	4.4	3.1	0.23
Noninduced			4.7	0.0010	0.35

A fully induced and a noninduced culture of ML 30 were each diluted to one cell/ml into growth media containing the above-indicated additions of inducer, methyl-β-D-thiogalactoside (TMG), and inhibitor, glucose. When the culture had grown to the level of 10^8 cells/ml enzyme activities were determined and the culture was again rediluted as above.

lost in 4 to 6 generations as compared to the 135 generations in the presence of inducer. This means that the system acquired by preinduction is diluted out or inactivated in some way in the absence of inducer, but once started is self-perpetuated in its presence.

2. Heterogeneity and the pseudo-mutational appearance of enzyme-forming ability. I have already pointed out that, under conditions of metabolic gratuity and saturating inducer concentrations, the induction of enzyme is homogeneously distributed in the population. This demonstration by Benzer (83) so dominated thinking that his warning that "the use of low concentrations of inducer . . . might be lead to heterogeneity" was overlooked for a long time. However, Novick and Weiner (118) in a detailed study of the kinetics of β-galactosidase formation of low inducer concentration, uncovered that heterogeneity obtains at nonsaturating levels of inducer.

They used the maintenance effect as a tool to demonstrate that cultures making enzyme at less than maximum rate are composed of individual cells, some making enzyme at maximal rate and the remainder making none. Novick and Weiner (118) used maintenance at low concentrations of inducer, and we have carried out similar experiments using maintenance in the presence of a suitable glucose and inducer concentration.

Consider now the experiment described in table 3 in which the data shown in figure 5 are further analyzed. A growing culture is placed in a low concentration of inducer, and as we mentioned, the differential rate of synthesis increases constantly to an apparently steady value. However, now we can analyze the population by diluting to the level of single cells into maintenance concentrations of glucose plus inducer, and analyzing for the level of enzyme in the resultant clones. The noninduced culture uniformly yields clones with no activity. At the other extreme, using the induced culture, all of the resultant clones show enzyme, and, therefore, the response to induction by saturating concentrations of inducer is homogeneous, in confirmation of

TABLE 3
Heterogeneity during induction at low inducer concentrations

Sample	Starting Culture	Multiplicity	% Positives
A	Induced	0.79	>99
B	Induced	0.65	>99
C	Induced	0.90	>99
0	Noninduced	0.55	<1
1	Noninduced	1.0	3 ± 1.5
2	Noninduced	0.88	11 ± 6
3	Noninduced	0.81	18 ± 4
4	Noninduced	0.73	24 ± 4
5	Noninduced	0.68	31 ± 3

From the cultures described under figure 5, samples were taken at the moments indicated by arrows and numbers, and diluted into tubes containing glucose 10^{-3} M and TMG 10^{-4} M to a multiplicity indicated above. The positives were those clones which showed a differential rate of synthesis, P (see table 2) of more than 1.0, while the negatives had less than 0.02. There were no clones between these values of P.

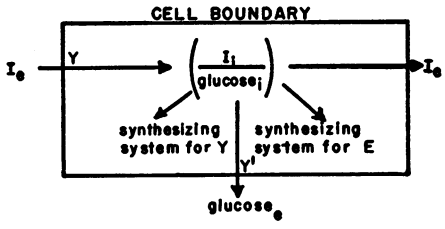


Figure 7

Benzer's findings. At low concentrations of inducer, the response of a noninduced culture is heterogeneous, and one has a population of enzyme synthesizing and nonsynthesizing cells. The number of cells induced increases with time to some steady-state value which Novick and Weiner (118) have shown to be a balance between the probability of a cell being induced and the selection against induced cells by the faster growing noninduced cells.

Under conditions of deadaptation, starting with a culture in which every cell shows maintenance, after 4 to 6 generations heterogeneity appears, *i.e.*, cells which cannot be maintained, and after 10 generations most of the population behaves as a noninduced culture. The kinetics of deadaptation have not been worked out as yet.

These two puzzling results, preinduction and heterogeneity, were clarified with the discovery by Rickenberg, Cohen, Buttin, and Monod (60) of a specific induced system responsible for bringing the galactoside inducers into the cell. Since this observation a wealth of data (60, 61, 118) has been gathered to show that both the preinduction and heterogeneity are a result of the presence of the induced permeation system (Y), I shall not go into any details on this mechanism which has been discussed by Cohen and Monod in this issue (61). The picture which has been developed is illustrated in figure 7 (119).

The externally added inducer (I_e) passes through the Y system to become internal inducer (I_i) responsible for the induction of both Y and E (β-galactosidase). Clearly, a cell which has a high level of Y can capture I_e more efficiently than a cell that does not have Y. The internal concentration of I_i depends then on both the external concentration of I_e and the level of Y so that the lower the level of I_e the greater the advantage the presence of Y is to a cell's inducibility. Glucose enters the cell by a distinctly different constitutive transport system, Y', to become the inhibitor, symbolized as glucose$_i$, which may be a derivative of glucose. It is the ratio of internal inducer to internal inhibitor (I_i/glucose$_i$) which determines the induction of both E and Y. Noninduced cells which have little or no Y will evidently have a low ratio of I_i/glucose$_i$ and consequently will be inhibited by glucose for the production of both Y and E. Induced cells with high levels of Y will have high internal concentrations of I_i, a high ratio of I_i/glucose$_i$, and consequently the glucose inhibition will be reduced with the maintenance of a steady state of induction as a result.

The Y system then accounts for the preinduction effects.[12] How does it explain the heterogeneity in induction? Here we must return to our model in which Z, the factor which generated the heterogeneity, is now identified as the induced-permeation system (Y), described by Monod's group. The Y system is responsible for the misleading over-all kinetics at low inducer concentration because we have a system which is being studied under peculiar conditions of nongratuity.

Our original formulation of the concept of gratuity, illustrated previously by the induction of β-galactosidase by lactose as sole carbon source, referred essentially to *metabolic* nongratuity. Here at low inducer concentration we have a case where the pathway of induction passes via the very system being induced so that even under conditions of metabolic gratuity one can have *induction* nongratuity leading to heterogeneity of the population. *Induction* nongratuity arises when an inducer in order to be effective must be acted upon by the very enzyme whose formation it provokes. This is the more subtle violation of the law of gratuity mentioned previously.

If this analysis is correct, we should find that under conditions of strict gratuity: (a) the preinduction or maintenance effects should disappear as well as (b) the acceleration phase due to heterogeneity of the population in response to induction. This is precisely what Herzenberg and Monod (61) discovered when they investigated the induction of β-galactosidase in a mutant (lactose-negative cryptic) which lacked the Y system. Over a range in inducer concentration from 4×10^{-5} M to saturation, equivalent

[12] I shall not deal with the mechanism of the glucose inhibition, but I should like to refer to the interesting papers of Neidhardt and Magasanik (120–123) which bear directly on this point.

to a difference of 80-fold between the minimum measurable value of the differential rate of synthesis and the maximum, a culture of this mutant responded to induction following a constant differential rate of synthesis with no evidence of an acceleration phase. A constant differential rate of synthesis from the moment of addition of inducer is evidence that the induction is proceeding homogeneously in the population. No preinduction effect was detectable either for low concentrations of inducer or for the glucose inhibition. Thus these very careful studies failed to reveal any autocatalytic step as a rate-limiting reaction in the induction of β-galactosidase. There was no indication that the amount of enzyme-forming system per cell increases. The kinetics of induced enzyme synthesis then has become quite simple. Either the differential rate of synthesis is constant from the moment of addition of inducer or else the response to induction by the population is heterogeneous.

The yeast system showing long-term adaptation (110–117) is so nearly analogous to the *E. coli* system that I feel reasonably certain that we are dealing with a general phenomenon and that eventually one mechanism will describe both findings. It is clear that the *nongratuity* model we previously proposed and the identification of *Z* as an induced permeation system explain every known aspect of the *E. coli* system. Whether this model by identifying *Z* with one of the enzymes of "galactozymase" (possibly the *Y* system) will eventually account for all of the observations with long-term adapting yeasts remains to be seen.

V. CONCLUDING REMARKS

As I warned at the start, this discussion has been almost entirely devoted to the methodology and to the very controversial aspects of this subject. However, in closing I should like to escape from this limitation. Induced enzyme synthesis is thought of by workers in this field as a model system for studies on protein synthesis, on differentiation and evolution, and on growth, normal and abnormal. Discussions of this subject always carry the analogies to other fields (2–17). We do this because we are convinced of the similarity of the basic mechanisms governing the processes whereby specific complex structures are molded from non-specific simpler ones. We know that when we shall have tied in the elements of induced enzyme synthesis, we shall have a large package which will contain unexpected gifts.

Yet, those investigators studying "protein" synthesis or "differentiation" or "normal and abnormal growth" do not appear to feel any impact of this subject on theirs. And in a sense there is no reason they should, for we have merely offered analogies between our findings and theirs.

However wary one must be of reasoning by analogy, the methodology which has been defined for induced enzyme synthesis should have repercussions in other fields. The danger in interpreting relationships at a molecular (or even cellular) level by studies in animals or tissues has already become obvious. Few microbiologists would study induced enzyme synthesis (or any process) in a sample of bacteria without isolating in pure culture the various organisms involved. Animal tissue culture is today just at the point of establishing general methods for isolating pure cell lines and these advances will certainly open up a new era in the analysis of differentiation and "abnormal" growth. However, new as the subject is, it is clear that workers with animal cells will have to develop simple, defined systems, and experiments will have to ask and answer similar questions to those we have faced. Therefore, while I agree that "embryology will ultimately have to be studied in embryos" (124, 125), investigators working on the growth and differentiation of animal cells, should, for a long time, be aided by the methodology and results of studies on enzyme synthesis in microorganisms.

VI. ACKNOWLEDGMENTS

Most of you reading this paper will realize how much I owe to Dr. Jacques Monod with whom I worked for six years at the Pasteur Institute, Paris. It is to the warm hospitality and to the guidance of both Drs. Monod and Lwoff that numerous investigators from this country, as well as myself, owe our interest and training in this and related fields. None of the work would have been possible without the devoted and skilled technical assistance of René Mazé, Madeleine Jolit, Janine Capdupuy and Raymond Barrand.

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VII. REFERENCES

1. DAVIES, R., AND GALE, E. F., *editors*. 1953 *Adaptation in micro-organisms*. Cambridge University Press, Cambridge, England.
2. COHN, M., AND MONOD, J. 1953 Specific inhibition and induction of enzyme biosynthesis. In reference 1, pp. 132–149.
3. MONOD, J., AND COHN, M. 1952 La biosyntheses induite des enzymes (adaptation enzymatique). Advances in Enzymol., **13**, 67–119.
4. MONOD, J. 1952 La synthèse de la β-galactosidase chez les Entérobacteriacees. Facteurs génétiques et facteurs chimiques. (Société Suisse de Microbiologiés, June 14–15, 1952, at Lugano.). Schweiz. allgem. Pathol. Bakteriol., **15**, 407–417.
5. MONOD, J. 1952 Inducteurs et inhibiteurs spécifiques dans la biosynthèse d'un enzyme. La β-galactosidase d'*Escherichia coli*. Bull. World Health Organization, **6**, 59–64.
6. MONOD, J. 1950 Adaptation, mutation and segregation in the formation of bacterial enzymes. Biochemical Society Symposia, No. 4, pp. 51–58. Cambridge University Press, Cambridge, England.
7. GAEBLER, O. H., *editor*. 1956 *Enzymes: Units of biological structure and function*. Academic Press, Inc., New York, N. Y.
8. MONOD, J. 1956 Remarks on the mechanism of enzyme induction. In reference 7, pp. 7–28.
9. MANDELSTAM, J. 1956 Theories of enzyme adaptation in microorganisms. Intern. Rev. Cytol., **5**, 51–85.
10. POLLOCK, M. R. 1953 Stages in enzyme adaptation. In reference 1, pp. 150–183.
11. SPIEGELMAN, S. 1950 Modern aspects of enzymatic adaptation. In *The enzymes*, Pt. 1, pp. 267–306. Vol. I. Edited by J. B. Sumner and Karl Myrbäck, Academic Press, Inc., New York, N. Y.
12. SPIEGELMAN, S. 1957 Nucleic acids and the synthesis of proteins. In *Chemical basis of heredity*, pp. 232–267. Edited by W. D. McElroy and B. Glass. The Johns Hopkins University Press, Baltimore, Md.
13. SPIEGELMAN, S., HALVORSON, H. O., AND BEN-ISHAI, RUTH. 1955 Free amino acids and the enzyme-forming mechanism. In *Amino acid metabolism*, pp. 124–170. Edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md.
14. SPIEGELMAN, S., AND HALVORSON, H. O. 1953 The nature of the precursor in the induced synthesis of enzymes. In reference 1, pp. 98–131.
15. SPIEGELMAN, S. 1956 The present status of the induced synthesis of enzymes. Proc. Congr. Biochem., 3rd Congr. Brussels, 1955, pp. 185–195.
16. SPIEGELMAN, S., AND CAMPBELL, A. M. 1956 The significance of induced enzyme formation. In *Currents in biochemical research*, pp. 115–161. Edited by D. E. Green. Interscience Publishers, Inc., New York, N. Y.
17. STANIER, R. Y. 1951 Enzymatic adaptation in bacteria. Ann. Rev. Microbiol., **5**, 35–56.
18. HOGNESS, D. S., COHN, M., AND MONOD, J. 1955 Studies on the induced synthesis of β-galactosidase in *Escherichia coli*: The kinetics and mechanism of sulfur incorporation. Biochim. et Biophys. Acta, **16**, 99–116.
19. ROTMAN, B., AND SPIEGELMAN, S. 1954 On the origin of the carbon in the induced synthesis β-galactosidase in *Escherichia coli*. J. Bacteriol., **68**, 419–429.
20. MONOD, J., PAPPENHEIMER, A. M., JR., AND COHEN-BAZIRE, G. 1952 La cinétique de la biosynthèse de la β-galactosidase chez *Escherichia coli* considérée comme fonction de la croissance. Biochim. et Biophys. Acta, **9**, 648–660.
21. MONOD, J., COHEN-BAZIRE, G., AND COHN, M. 1951 Sur la biosynthèse de la β-galactosidase (lactase) chez *Escherichia coli*. La specificite de l'induction. Biochim. et Biophys. Acta, **7**, 585–599.
22. POLLOCK, M. R. 1950 Penicillinase adaptation in *B. cereus*: Adaptive enzyme formation in the absence of free substrate. Brit. J. Exptl. Pathol., **31**, 739–753.
23. POLLOCK, M. R., TORRIANI, ANNE-MARIE, AND TRIDGELL, E. J. 1956 Crystalline bacterial penicillinase. Biochem. J., **62**, 387–391.
24. GALE, E. F. 1953 Amino-acid incorporation and protein synthesis in *Staphylococcus aureus*. In *Symposium on microbial metabolism*, pp. 109–125. 6th Intern. Congr. Microbiol., Rome, Italy.
25. GALE, E. F., AND FOLKES, JOAN P. 1954 Effect of nucleic acids on protein synthesis and amino-acid incorporation in disrupted

staphylococcal cells. Nature, **173**, 1223–1227.

26. GALE, E. F., AND FOLKES, JOAN P. 1955 The effect of nucleic acids on the development of certain enzymic activities in disrupted staphylococcal cells. Biochem. J., **59**, 675–684.
27. GALE, E. F., AND FOLKES, JOAN P. 1955 The incorporation of labelled amino acids by disrupted staphylococcal cells. Biochem. J., **59**, 661–675.
28. COHN, M., MONOD, J., POLLOCK, M. R., SPIEGELMAN, S., AND STANIER, R. Y. 1953 Terminology of enzyme formation. Nature, **172**, 1096.
29. CREASER, E. H. 1955 The induced (adaptive) biosynthesis of β-galactosidase in *Staphylococcus aureus*. J. Gen. Microbiol., **12**, 288–297.
30. THEORELL, H. 1951 The iron-containing enzymes—B. Catalases and peroxidases. "Hydroperoxidases." In *The enzymes*, Pt. 1 pp. 397–427. Vol. II. Edited by J. B. Sumner and K. Myrbäck. Academic Press, Inc., New York, N. Y.
31. CHANCE, B., AND MAEHLY, A. C. 1955 Assay of catalases and peroxidases. In *Methods in enzymology*, pp. 764–775. Vol. II. Edited by E. Colowick and N. O. Kaplan. Academic Press, Inc., New York, N. Y.
32. MONOD, J., TORRIANI, ANNE-MARIE, AND GRIBETZ, J. 1948 Sur un lactase extraite d'une souche d'*Escherichia coli* mutabile. Compt. rend., **227**, 315–316.
33. COHN, M., AND MONOD, J. 1951 Purification et propriétés de la β-galactosidase (lactase) d'*Escherichia coli*. Biochim. et Biophys. Acta, **7**, 153–174.
34. COHEN-BAZIRE, GERMAINE, AND MONOD, J. Note (presented by M. Jacques Tréfouël) 1951 La compétition entre les ions hydrogène et sodium dans l'activation de la β-D-galactosidase d'*Escherichia coli* et la notion d'antangoisme ionique. Compt. rend., **232**, 1515–1517.
35. LEDERBERG, J. 1950 The beta-D-galactosidase of *Escherichia coli*, strain K-12. J. Bacteriol., **60**, 381–392.
36. KUBY, S. A., AND LARDY, H. A. 1953 Purification and kinetics of β-D-galactosidase from *Escherichia coli* Strain K-12. J. Am. Chem. Soc., **75**, 890–896.
37. WALLENFELS, K., BERNT, E., AND LUNBERG, G. 1953 Isolierung von Lactotriose, Lactobiose und Galaktobiose aus dem enzymatischen Hydrolipat von Lactose. Ann. Chem. Liebigs, **579**, 113, 122.
38. WALLENFELS, K., AND BERNT, E. 1953 Über den Verlauf der enzymatischen Spaetung von Lactose mit β-Galaktosidase von Schimmelpilzen, *Helix pomatia*, *Escherichia coli* und Kälberdarin. Ann. der Chem., Liebigs, **584**, 63–85.
39. WALLENFELS, K. 1953 Gruppenübertragung im Bereich der Carbohydrasen. Colloquium der Gesellschaft für physiologische chemie, pp. 160–176. Springer-Verlag, Göttingen, Germany.
40. WALLENFELS, K., AND BERNT, E. 1952 Über die gruppenübertragende Wirkung von disaccharid-spaltenden Enzymen. Angew. Chem., **64**, 28–29.
41. ARMSTRONG, E. F. 1903 Study on enzyme action. I. The correlation of stereoisomeric α and β glucosides with the corresponding glucoses. J. Chem. Soc., **83**, 1305–1308.
42. ARONSON, MOSHE 1952 Transgalactosidation during lactose hydrolysis. Arch. Biochem. and Biophys., **39**, 370–378.
43. CAPUTTO, R., LELOIR, L. F., AND TRUCIO, R. E. 1948 Lactase and lactose fermentation in *Saccharomyces fragilis*. Enzymologia, **12**, 350–356.
44. KLOTZ, I. 1953 Protein interactions. In *The Proteins*, Pt. B, pp. 727–804. Vol. I. Edited by H. Neurath and K. Bailey. Academic Press, Inc., New York, N. Y.
45. SCATCHARD, G. 1949 The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci., **51**, 660–668.
46. COHN, M., AND TORRIANI, ANNE-MARIE. 1951 Étude immunochimique de la biosynthèse adaptative d'un enzyme: La β-galactosidase d'*Escherichia coli*. Compt. rend., **232**, 115–117.
47. SEVAG, M. G. 1946 Enzyme problems in relation to chemotherapy, adaptation, mutations, resistance and immunity. Advances in Enzymol., **6**, 33–121.
48. POLLOCK, M. R. 1956 An immunological study of the constitutive and the penicillin-induced penicillinases of *Bacillus cereus*, based on specific enzyme neutralization by antibody. J. Gen. Microbiol., **14**, 90–108.
49. COHN, M., AND TORRIANI, ANNE-MARIE. 1952 Immunochemical studies with the β-galactosidase and structurally related proteins of *Escherichia coli*. J. Immunol., **69**, 471–491.
50. COHN, M., AND TORRIANI, ANNE-MARIE. 1953 The relationships in biosynthesis of

the β-galactosidase- and PZ-proteins in *Escherichia coli*. Biochim. et Biophys. Acta, **10**, 280–289.

51. COHN, M. 1953 Étude immunologique de la biosynthèse d'une protéine. J. méd. Bordeaux et Sud-Ovest, **130**, 346–350.
52. COHN, M. 1954 The immunological analysis of the biosynthesis of a protein. In *Serological approaches to studies of protein structure and metabolism*, pp. 38–54. Edited by W. H. Cole. Rutgers University Press, New Brunswick, N. J.
53. COHN, M. 1953 A note on the use of the antigen excess zone to reveal the existence of certain types of cross reactions in unidentified mixtures of antigens. J. Immunol., **70**, 317–320.
54. MONOD, J., AND COHN, M. 1953 Sur le mécanisme de la synthèse d'une proteine bacterienne. La β-galactosidase d'*Escherichia coli*. In *Symposium on microbial metabolism*, pp. 42–62. 6th Intern. Congr. Microbiol. Rome, Italy.
55. HALVORSON, H., SPIEGELMAN, S., AND HINMAN, R. L. 1955 The effect of tryptophan analogs on the induced synthesis of maltase and protein synthesis in yeast. Arch. Biochem. and Biophys., **55**, 512–525.
56. HALVORSON, H. O., AND SPIEGELMAN, S. 1952 The inhibition of enzyme formation by amino acid analogues. J. Bacteriol., **64**, 207–221.
57. BORSOOK, H. 1956 The biosynthesis of peptides and proteins. In *Symposium on structure of enzymes and proteins*. J. Cellular Comp. Physiol., **47**, Supplement 1, 35–80.
58. MONOD, J. 1947 The phenomenon of enzymatic adaptation and its bearings on problems of genetics and cellular differentiation. Growth, **11**, 223–289.
59. COHEN, G. N., AND V. RICKENBERG, H. V. (Presented by M. Jacques Trefouël) 1955 Etude directe de la fixation d'un inducteur de la β-galactosidase par les cellules d'*Escherichia coli*. Compt. rend., **240**, 466–468.
60. RICKENBERG, H. V., COHEN, G. N., BUTTIN, G., AND MONOD, J. 1956 La galactoside-permease d'*Escherichia coli*. Ann. inst. Pasteur, **91**, 829–857.
61. COHEN, G. N., AND MONOD, J. 1957 Bacterial permeases. Bacteriol. Revs., **21**, 169–194.
62. GALE, E. F., AND FOLKES, JOAN P. 1953 Nucleic acid and protein synthesis in *Staphylococcus aureus*. Biochem. J., **53**, 483–492.
63. PARDEE, A. B. AND PRESTRIDGE, L. S. 1956 The dependence of nucleic acid synthesis on the presence of amino acids in *Escherichia coli*. J. Bacteriol., **71**, 677–683.
64. GROS, F., AND GROS, FRANÇOISE. 1956 Role des aminoacides dans la synthese des acides nucléiques chez *Escherichia coli*. Biochim. et Biophys. Acta, **22**, 200–201.
65. MUNIER, R., AND COHEN, G. N. 1957 Incorporation d'analogues structuraux d'aminoacides dans les proteines bacteriennes. Compt. rend., *in press*.
66. HALVORSON, H., AND SPIEGELMAN, S. 1953 Net utilization of free amino acids during the induced synthesis of maltozymase in yeast. J. Bacteriol., **65**, 601–608.
67. ROBERTS, R. B., COWIE, D. B., ABELSON, P. H., BOLTON, E. T., AND BRITTEN, R. J. 1955 Studies of biosynthesis in *Escherichia coli*. Carnegie Inst. Wash. Publ. No. 607.
68. MARKOVITZ, A., AND KLEIN, H. P. 1955 Some aspects of the induced biosynthesis of alpha-amylase of *Pseudomonas saccharophilia*. J. Bacteriol., **70**, 641–648.
69. MARKOVITZ, A., AND KLEIN, H. P. 1955 On the sources of carbon for the induced biosynthesis of alpha-amylase in *Pseudomonas saccharophila*. J. Bacteriol., **70**, 649–655.
70. GORINI, L., AND LANZAVECCHIA, G. 1954 Recherches sur le mécanisme de production d'une proteinase bactérienne. II. Mise en évidence d'un zymogène precurseur de la protéinase de *coccus p*. Biochim. et Biophys. Acta, **15**, 399–410.
71. GUNSALUS, I. C. 1955 In the *Discussion* of reference 93, pp. 219–228.
72. WAINWRIGHT, S. D., AND NEVILL, ANN. 1956 The influence of depletion of nitrogenous reserves upon the phenomenon of induced enzyme biosynthesis in cells of *Escherichia coli*. J. Gen. Microbiol., **14**, 47–56.
73. MONOD, J. 1941 Recherches sur la croissance des cultures bactériennes. Thesis at Sorbonne University, Paris. Hermann et Cie, publishers.
74. SPIEGELMAN, S., AND DUNN, R. 1947 Interaction between enzyme forming systems during adaptation. J. Gen. Physiol., **31**, 153.
75. YUDKIN, J. 1938 Enzyme variation in microorganisms. Biol. Revs. Cambridge Phil. Soc., **13**, 93–106.
76. MANDELSTAM, J., AND YUDKIN, J. 1952

Some aspects of galactozymase production by yeast in relation to the 'mass action' theory of enzyme adaptation. Biochem. J., **51**, 686–693.

77. MANDELSTAM, J. 1952 The 'mass action' theory of enzyme adaptation. Biochem. J., **51**, 674–681.
78. KOPPEL, J. L., Porter, C. J., AND CROCKER, B. F. 1953 The mechanism of the synthesis of enzymes. I. Development of a system suitable for studying this phenomenon. J. Gen. Physiol., **36**, 703–722.
79. PORTER, C. J., HOLMES, R., AND CROCKER, B. F. 1953 The mechanism of the synthesis of enzymes. II. Further observations with particular reference to the linear nature of the time course of enzyme formation. J. Gen. Physiol., **37**, 271–289.
80. KOCH, A. L., AND LEVY, R. H. 1955 Protein turnover in growing cultures of *Escherichia coli*. J. Biol. Chem., **217**, 947–957.
81. COHN, M. 1956 Comment in reference 7, pp. 96–98.
82. RICKENBERG, H. V., YANOFSKY, C., AND BONNER, D. M. 1953 Enzymatic deadaptation. J. Bacteriol., **66**, 683–687.
83. BENZER, S. 1953 Induced synthesis of enzymes in bacteria analyzed at the cellular level. Biochim. et Biophys. Acta, **11**, 383–395.
84. GALE, E. F. 1953 Assimilation of amino acids by gram-positive bacteria and some actions of antibiotics thereon. Advances in Protein Chem., **8**, 285–391.
85. MANDELSTAM, J. 1956 Turnover of protein in *Escherichia coli*. Biochem. J. **64**, 55–56P.
86. SPIEGELMAN, S., AND REINER, J. M. 1947 The formation and stabilization of an adaptive enzyme in the absence of its substrate. J. Gen. Physiol., **31**, 175–193.
87. SHER, I. H., AND MALLETTE, M. F. 1954 The adaptive nature of the formation of lysine decarboxylase in *Escherichia coli* B. Arch. Biochem. and Biophys., **52**, 331–339.
88. TERUI, G., AND OKADA, H. 1951 An inquiry into the adaptive fermentability of maltose with *Saccharomyces saké*. Osaka Univ. Tech. Repts., **1**, (No. 23), pp. 293–307.
89. ROBERTSON, J. J., AND HALVORSON, H. O. 1957 The components of maltozymase in yeast, and their behavior during deadaptation. J. Bacteriol., **73**, 186–198.
90. GALE, E. F., AND FOLKES, JOAN P. 1953 The incorporation of glutamic acid into the protein fraction of *Staphylococcus aureus*. Biochem. J., **55**, 721–729.
91. GALE, E. F. 1951 The relationship between accumulation of free glutamic acid and the formation of combined glutamic acid in *Staphylococcus aureus*. Biochem. J., **48**, 290–297.
92. GALE, E. F., AND FOLKES, JOAN P. 1953 The inhibition of phenylalanine incorporation in *Staphylococcus aureus* by chloramphenicol and *p*-chlorophenylalanine. Biochem. J., **55**, 730–735.
93. GALE, E. F. 1955 From amino acids to proteins. In *Amino acid metabolism*, pp. 171–192. Edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md.
94. ZAMECNIK, P. C., KELLER, E. B., LITTLEFIELD, J. W., HOAGLAND, M. B., AND LOFTFIELD, R. B. 1956 Mechanism of incorporation of labeled amino acids into protein. In *Symposium on structure of enzymes and proteins*. J. Cellular Comp. Physiol., **47**, Supplement 1, 81–92.
95. ALLFREY, V. G., MIRSKY, A. E., AND OSAWA, S. 1957 Protein synthesis in isolated cell nuclei. J. Gen. Physiol., **40**, 451–490.
96. MONOD, J. 1956 Comment in reference 7, p. 173.
97. CHANTRENNE, H. 1953 Problems of protein synthesis. In *The nature of virus multiplication*, pp. 1–20. Edited by P. Fildes and W. E. Heyningen. Cambridge University Press, Cambridge, England.
98. RICKENBERG, H. V., AND LESTER, G. 1955 The preferential synthesis of β-galactosidase in *Escherichia coli*. J. Gen. Microbiol., **13**, 279–284.
99. MONOD, J. 1950 La technique de culture continue. Théorie et application. Ann. inst. Pasteur, **79**, 390–409.
100. NOVICK, A., AND SZILARD, L. 1950 Description of the chemostat. Science, **112**, 715–716.
101. SPIEGELMAN, S., AND HALVORSON, H. O. 1954 On the role of the inducer in the synthesis of maltase in yeast. J. Bacteriol., **68**, 265–273.
102. CREASER, E. H. 1956 The effect of 8-azaguanine upon enzyme formation in *Staphylococcus aureus*. Biochem. J., **64**, 539–545.
103. PINSKY, M. J., AND STOKES, J. L. 1952 The influence of age on enzymatic adaptation in microorganisms. J. Bacteriol., **64**, 337–345.
104. PINSKY, M. J., AND STOKES, J. L. 1952 Requirements for formic hydrogenlyase

adaptation in nonproliferating suspensions of *Escherichia coli*. J. Bacteriol., **64**, 151–161.

105. MANDELSTAM, J. 1954 Induced biosynthesis of lysine decarboxylase in *Bacterium cadaveris*. J. Gen. Microbiol., **11**, 426–437.
106. KNOX, R. 1953 The effect of temperature on enzymic adaptation, growth and drug resistance. In reference 1, p. 185.
107. STOKES, J. L. 1952 Inhibition of microbial oxidation, assimilation and adaptive enzyme formation by methylene blue. Antonie van Leeuwenhoek, J. Microbiol. Serol., **18**, 63–81.
108. COHEN, S. S., AND BARNER, H. D. 1954 Enzymatic adaptation in a thymine requiring strain of *Escherichia coli*. J. Bacteriol., **69**, 59–66.
109. SPIEGELMAN, S. 1946 Nuclear and cytoplasmic factors controlling enzymatic constitution. Cold Spring Harbor Symposia Quant. Biol., **11**, 256–277.
110. CAMPBELL, A. M., AND SPIEGELMAN, S. 1956 The growth kinetics of elements necessary for galactozymase formation in "long term adapting" yeasts. Compt. rend. trav. lab. Carlsberg Ser. physiol., **26**, 13–30.
111. SPIEGELMAN, S., SUSSMAN, R. R., AND PINSKA, E. 1950 On the cytoplasmic nature of "long-term adaptation" in yeast. Proc. Natl. Acad. Sci. U. S., **36**, 591–606.
112. SPIEGELMAN, S., DELORENZO, W. F., AND CAMPBELL, A. M. 1951 A single-cell analysis of the transmission of enzyme-forming capacity in yeast. Proc. Natl. Acad. Sci. U. S., **37**, 513–524.
113. SPIEGELMAN, S. 1951 The particulate transmission of enzyme-forming capacity in yeast. Cold Spring Harbor Symposia Quant. Biol., **16**, 87–98.
114. SPIEGELMAN, S., AND DELORENZO, W. F. 1952 Substrate stabilization of enzyme-forming capacity during the segregation of a heterozygote. Proc. Natl. Acad. Sci. U. S., **38**, 583–592.
115. SPIEGELMAN, S. 1954 Heritable differences in enzyme synthesizing capacity amongst cells of identical genotype. Proc. 2nd Natl. Cancer Conf., Virology Panel, pp. 1345–1348.
116. CAMPBELL, A. M., AND SPIEGELMAN, S. 1953 Dissociation of enzyme forming system from functional enzyme in long term adapting yeasts. Bacteriol. Proc. (Soc. Am. Bacteriologists), pp. 91–92.
117. SPIEGELMAN, S., AND LANDMAN, O. E. 1954 Genetics of microorganisms. Ann. Rev. Microbiol., **8**, 181–236.
118. NOVICK, A., AND WEINER, M. 1957. Enzyme induction, an all or none phenomenon. Proc. Natl. Acad. Sci., *in press*.
119. COHN, M. 1956 Added comment: On the inhibition by glucose of the induced synthesis of β-galactosidase in *Escherichia coli*. In reference 7, pp. 41–48.
120. NEIDHARDT, F. C., AND MAGASANIK, B. 1956 The effect of glucose on the induced biosynthesis of bacterial enzymes in the presence and absence of inducing agents. Biochim. et Biophys. Acta, **21**, 324–334.
121. NEIDHARDT, F. C., AND MAGASANIK, B. 1956 Inhibitory effect of glucose on enzyme formation. Nature, **178**, 801–802.
122. NEIDHARDT, F. C. 1957 Reversal of the glucose inhibition of histidase biosynthesis in *Aerobacter aerogenes*. J. Bacteriol., **73**, 253–259.
123. NEIDHARDT, F. C., AND MAGASANIK, B. 1957 Effect of mixtures of substrates on the biosynthesis of inducible enzymes in *Aerobacter aerogenes*. J. Bacteriol., **73**, 260–263.
124. LEDERBERG, J. 1952 Cell genetics and hereditary symbiosis. Physiol. Revs., **32**, 403.
125. EPHRUSSI, B. 1956 Enzymes in cellular differentiation. In reference 7, pp. 29–41.