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The way it was: a commentary by

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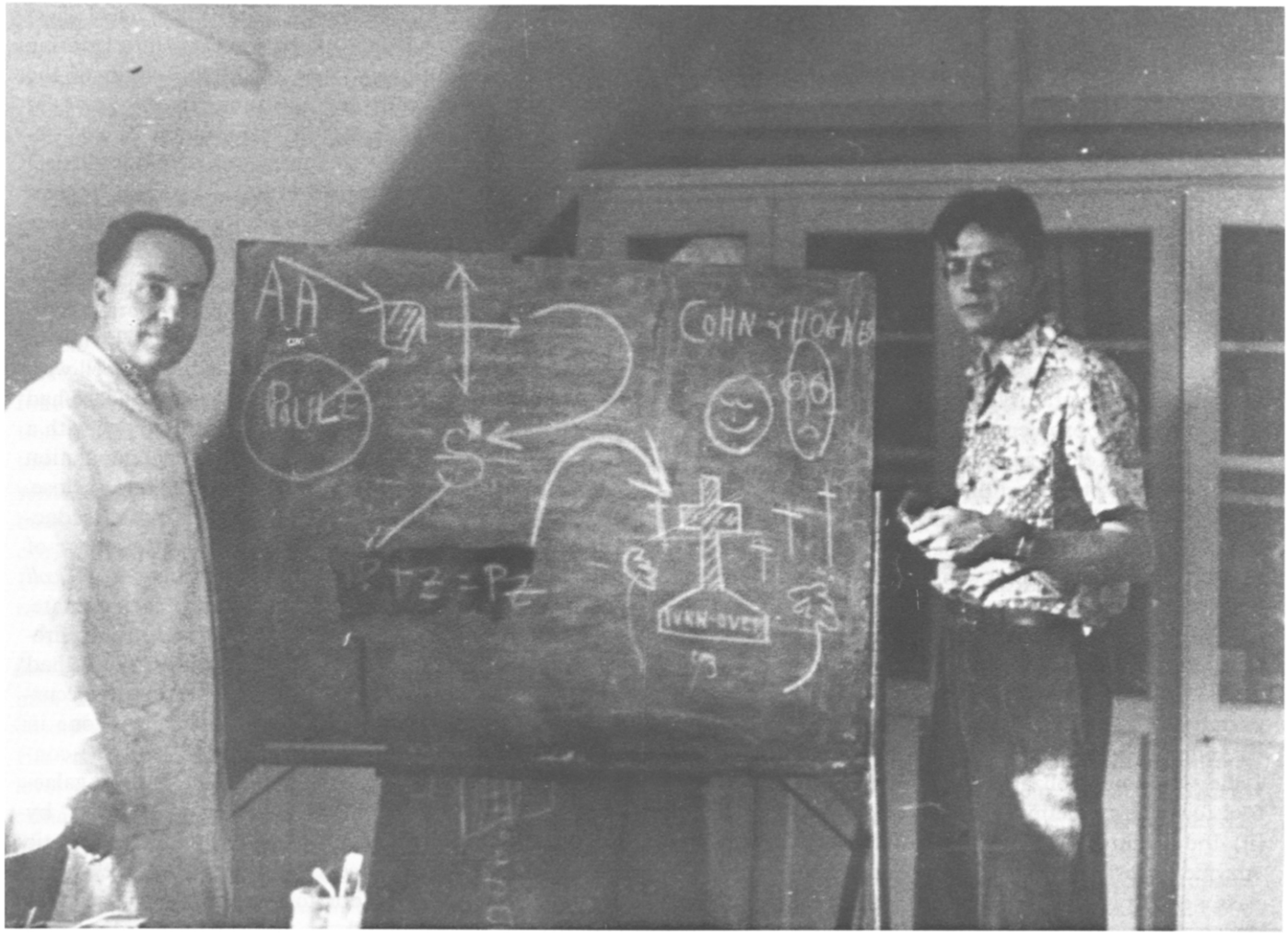
on 'Studies on the induced synthesis of β-galactosidase in *Escherichia coli*:
The kinetics and mechanism of sulfur incorporation'
by D.S. Hogness, M. Cohn and J. Monod,
Biochim. Biophys. Acta 16 (1955) 99-116
with Biochim. Biophys. Acta 7 (1951) 585–599 (summary)

I arrived in Paris in the summer of 1949 to work with Jacques Monod at the Pasteur Institute as a postdoctoral fellow. Monod proposed that I choose an 'adaptive' enzyme and study the mechanism of its induction. As the laboratory emptied out for the summer vacation, I found myself in delicious solitude with an assay for



Melvin Cohn (left) and David Hogness.

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glucose and a handful of 'adaptive' enzymes to play with, all of which yielded glucose as a product: invertase, α-galactosidase, β-galactosidase, amylomaltase, β-glucosidase and α-glucosidase. These enzymatic activities were induced in *Escherichia coli* or *Aerobacter aerogenes* in response to the addition of substrate. My idea of a first step was to prepare an antiserum specific for one of them and use it to characterize the conditions of induction of the enzyme as a protein. This required purification. That summer I made extracts containing these enzymes and explored their fractionation with ammonium sulfate. Only β-galactosidase resisted precipitation in harsh summer heat and I chose it for study. Had the laboratory possessed a cold room, much less a bucket of ice, the course of the history of the *lac* operon would have been different. When Monod returned from vacation he agreed, adding with a touch of humor, that until our cold room was built it was not an unreasonable choice.

The investigation reprinted here was a late, albeit crucial, step in establishing the β-galactosidase system as a model for the analysis of how the response to a change in the environment in regulated. Whereas, today, some of the choices of the questions we tackled may seem baroque, at the time they were prompted by a dominant theme. The subject of 'adaptation' was at the center of the then prevailing 'Lysenko-Mitchourin controversy' and leading 'Marxist' biologists like Haldane, Knight, Needham, Prenant and Bernal – not to mention the whole intellectual left of France – had tightened the screws on our views of the gene–environment interaction by arguing that we could well be missing 'heritable' changes induced by the substrate. Most of the innards of their argumentation were brought directly into the limelight by the phenomenon of 'adaptive' enzyme formation. As these charismatic scientists were frequent visitors to our laboratory, we were inevitably influenced in our choice of problem.

We began (Ref. 1; summary also reproduced here) by attempting to settle the following questions:
(i) Was the inducer of an enzyme obligatorily a substrate?
(ii) Were the best inducers substances of highest affinity for the enzyme?
(iii) Were competitive inhibitors of enzyme activity also competitive inhibitors of induction?

We synthesized a new family of galactosides which permitted us to establish the answers [1]:
(i) Inducers are not obligatorily substrates of the enzyme. Non-metabolizable inducers became a precious tool for us, e.g., methyl β-D-thiogalactoside.
(ii) Inducibility is independent of the affinity for the enzyme. Substances with high affinity for β-galactosidase, e.g., phenyl β-D-thiogalactosides, were noninducers.
(iii) Competitive inhibitors of the enzyme such as phenyl β-D-thiogalactoside were non-competitive inhibitors of induction whereas non-competitive inhibitors of the enzyme such as melibiose (α-galactoside) were inducers.

We concluded that the enzyme was not necessarily on the pathway of its induction; the inducer had to interact with a product other than the enzyme itself to initiate synthesis. We know today that this product is the *lac* repressor. These results raised two paradoxes, one philosophical, the other a detail of mechanism.

First, we had created a laboratory situation where *E. coli* growing in synthetic medium on succinate responded to methyl β-D-thiogalactoside (a non-metabolizable inducer) to synthesize an enzyme, β-galactosidase, which comprised 10% of total protein and was useless to the organism. This was hardly 'adaptive' and we referred to the laboratory construct as 'l' induction gratuite' (induction without purpose). At the time it seemed to illustrate the absurdity of the position championed by the 'Marxist' biologists who had given evolution a goal or purpose. This led us to change the name of the phenomenon we were studying from 'adaptive enzyme synthesis' to 'induced enzyme synthesis' [2,3]. By doing this we hoped to avoid a teleological polemic. In fact, we only fooled ourselves into ignoring the central evolutionary question of the origin of the regulation. This leads me to comment on going in circles versus going in helices. Given that *E. coli* responds in nature only to substrates, it is paradoxical that a naturally occurring substrate, lactose, which gives the *lac* operon its name, is not an inducer; in fact it is an anti-inducer. Lactose must be metabolized by the enzyme to allolactose, the true inducer [4–6]. Further, lactose cannot enter the cell and requires a permease to transport it internally. Seymour Benzer, in 1952 while in our Paris laboratory, had shown that an *E. coli* cell with a spontaneously high basal level of β-galactosidase had a better chance to be induced by lactose than one with a low basal level [7]. This translated at the population level into a heterogeneous rate of response to lactose, i.e., the infamous S-shaped curve of the rate of induction of β-galactosidase versus time. This was not true of the induction by alkyl β-D-thiogalactosides of *E. coli* cells growing on an unrelated carbon source, succinate. These latter inducers enter the cell without the requirement for a permease and are inducers as such. If we had analyzed as an inducer lactose, the only naturally occurring substrate known in 1950, we would have gone in circles, ending up where we had started, with the conclusion that the interaction of inducer with β-galactosidase itself was the crucial initial step. However, by analyzing a laboratory construct based on synthetic non-metabolizable inducers and special culture conditions, we opened the door to the pathway of negative control by the *lac* repressor that, in turn, led us in a

helix to a higher level of understanding of natural substrates, the selective pressure of evolution operating on the *lac* operon.

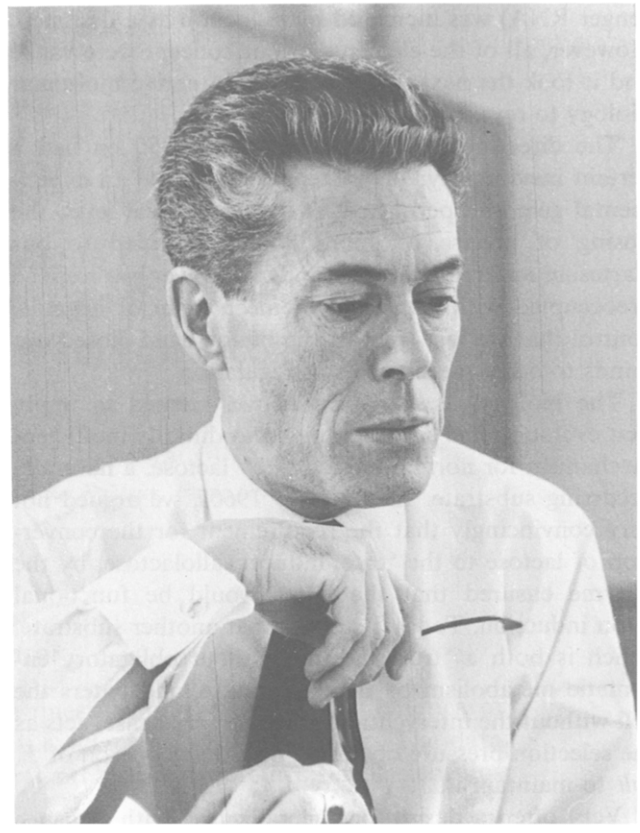
Second, we faced another paradox because the induction of enzyme activity seemed to involve the conversion of an inactive precursor to active enzyme, the analogy with the trypsinogen conversion to trypsin being evident. The immunological analysis had shown that *E. coli* expressed a protein, referred to as Pz, with very similar structure to β-galactosidase (Gz). On the one hand, all attempts to reveal a precursor-product relationship failed [8,9]. Induction required growth; activation of Pz to Gz could not be accomplished in extracts; and the level of Pz did not dramatically decrease during the induced increase in Gz. On the other hand, the postulate of a precursor permitted an explanation of our specificity studies [1], namely that the lactose analogues interacted with Pz to yield a pattern distinguishable from that of the product to which it was hypothetically converted, Gz. We needed a direct analysis of the relationship between Pz and Gz. It is this problem with which the paper [10] reprinted here deals.

David Hogness arrived in Paris in 1953, bringing the enthusiasm to introduce the use of radioactive technology which, coupled to our already sophisticated immunological purification and culture methodology, had to yield an answer to the general question, "Is the synthesis of β-galactosidase initiated from amino acids following induction?"

There was one small difficulty. We had to build a Geiger counter to do this and thanks to the expertise of Dr. Louis Siminovitch, we scoured the physics laboratories of Paris and salvaged enough electronic equipment to construct one.

The very first experiments were unambiguous [11]. The enzyme is synthesized de novo from amino acids following induction. Pz is not a precursor and its function to this day is a mystery. Further, once synthesized, the enzyme is stable. Removing the inducer stopped synthesis and the enzyme was diluted out in the growing bacterial mass. This challenged the then dominant view originating with Schoenheimer and Rittenberg in the 1940s that proteins in cells are in a dynamic state of reversible equilibrium with amino acids.

Our group owed a great deal to the visits and lively seminars of Sol Spiegelman. Although his Pasteur seminars are often likened to a 'corrida', this does not describe the importance of the interactions with him. His visits gave our group credibility at an international level as well as a chance to test our ideas against the criticism of a rapier mind at a time when it was most needed. At this stage it was not who was correct that counted. At one such seminar in 1954, Sol Spiegelman described his study with Boris Rotman on the induced synthesis of β-galactosidase. Their experiment [12] was an identical twin to ours [10] in design and, totally



Jacques Monod (photograph reproduced by kind permission of the Institut Pasteur (Musée Pasteur), Paris).

independently, we had come to the same conclusions. Up to this point our conceptualizations had been very different and this crossing of paths marked a turning point, as we were now of one mind. It also marked a change in my personal relationship with Sol, who became a lifelong friend and whose creativity, imagination and overreaching originality was a constant source of marvel and guidance to me. If this probing experiment was simultaneously and independently duplicated in an anti-world (what Urbana was to Paris), I know now that it could only have been done by Sol Spiegelman and that, in itself, is our greatest flattery.

As doesn't happen too often, in essence we understood all of the implications of our study [1,10]. The inducer interacted not with the enzyme, but with some other cell product (today's *lac* repressor) to yield a complex which led to the de novo synthesis of the enzyme from amino acids. Finally, in 1965 Suzanne Bourgeois [13] formally demonstrated by genetic means that the *lac* repressor was a protein and not RNA, as many workers, including the Paris group, believed in the early 1960s [14,15].

In 1955, there was the general feeling, expressed best by Jean Brachet, that the pathway of protein synthesis was DNA → RNA → protein. We reasonably assumed that the synthesis of RNA (the modern term is mes-

senger RNA) was increased by induction as a first step. However, all of the elements of our concept were vague and it took the next two decades of intensive molecular biology to reveal them.

The direction of the progress from 1955 on had a certain inevitability; the system had become an experimental gem and permitted, at the conceptual level, the posing of precise questions which appealed to our Cartesian and reductionist minds. However, we were so preoccupied with the detail of mechanism of negative control that we lost sight of the biology and closed our minds to other mechanisms of regulation.

The biology, as discussed above, seemed to imply that evolution had selected an extraordinarily inefficient mechanism for normal induction by lactose, a naturally occurring substrate. In the early 1960s, we argued not very convincingly that the requirement for the conversion of lactose to the 'true' inducer, allolactose, by the enzyme ensured that the latter would be functional upon induction. Today I suspect that another substrate, which is both a 'true' inducer without obligatory enzymatic metabolism by β-galactosidase, and enters the cell without the intervention of the *lac* permease, acts as the selection pressure operating on the *lac* operon of *E. coli* to maintain it.

Very often a desire for generalization with elegance plus parsimony is so compelling that other relevant great discoveries are blotted out. Having revealed negative control by the *lac* repressor, truly a spectacular achievement, the Pasteur group was resistant to any challenge to its universality. Any hint of other mechanisms of control was shrugged off on intuitive grounds, sometimes correctly, sometimes incorrectly. In particular, they were unable to think about the equally spectacular achievement of Ellis Englesberg, who by 1965 had put together an impressive case for positive regulation [16]. It is always possible to find an ambiguity in or limitation to the interpretation of any experiment. A generalization from the experiment that is mapped onto an overall picture of differentiation and development, is more difficult. This the Pasteur group did, but it has never been clear to me what the logic of evolution was that would have made regulation universally mediated by the circuitry of negative switches. Yet their resistance to acknowledging the existence of more than one mechanism was of tremendous value in unifying the field. Although the price paid is a delay in the appreciation of equally important advances, this is the only way great science evolves, alas!

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References

1 Monod, J., Cohen-Bazire, G. and Cohn, M. (1951) Biochim. Biophys. Acta 7, 585–599.
2 Monod, J. and Cohn, M. (1952) in Advances in Enzymology (Nord, F.F. ed.), Vol. XIII, pp. 67–119, Interscience Publishers, New York.
3 Cohn, M., Monod, J., Pollock, M.R., Spiegelman, S. and Stanier, R.Y. (1953) Nature 172, 1096.
4 Burstein, C., Cohn, M., Kepes, A. and Monod, J. (1965) Biochim. Biophys. Acta 95, 634–639.
5 Jobe, A. and Bourgeois, S. (1972) J. Mol. Biol. 69, 397–408.
6 Jobe, A. and Bourgeois, S. (1973) J. Mol. Biol. 75, 303–313.
7 Benzer, S. (1953) Biochim. Biophys. Acta 11, 383–395.
8 Cohn, M. and Torriani, A.-M. (1952) J. Immunol. 69, 471–491.
9 Cohn, M. and Torriani, A.-M. (1953) Biochim. Biophys. Acta 10, 280–289.
10 Hogness, D.S., Cohn, M. and Monod, J. (1955) Biochim. Biophys. Acta 16, 99–116.
11 Monod, J. and Cohn, M. (1953) Symposium on Microbiological Metabolism. VIth International Congress on Microbiology, Rome, p. 42–62.
12 Rotman, B. and Spiegelman, S. (1954) J. Bacteriol. 68, 419–429.
13 Bourgeois, S., Cohn, M. and Orgel, L.E. (1965) J. Mol. Biol. 14, 300–302.
14 Jacob, F. and Monod, J. (1961) J. Mol. Biol. 3, 318–356.
15 Monod, J. and Jacob, F. (1961) Cold Spring Harbor Symp. Quant. Biol. 26, 389–401.
16 Englesberg, E., Irr, J., Power, J. and Lee, N. (1965) J. Bacteriol. 90, 946–957.

STUDIES ON THE INDUCED SYNTHESIS OF β-GALACTOSIDASE IN *ESCHERICHIA COLI*: THE KINETICS AND MECHANISM OF SULFUR INCORPORATION*

by

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INTRODUCTION

The present paper is concerned with the question of the origin and kinetics of incorporation of the elements of an inducible (*i.e.* adaptive) enzyme-protein. The data also present some bearings on the more general problem of protein interrelationships and protein turnover within growing cells.

The inducible enzyme β-galactosidase of *E. coli* was chosen as object of these experiments because of the exceptionally convenient properties of this system[22,1,2,3].

A culture of *E. coli* growing in a medium of mineral salts with a non-galactosidic carbon source, such as succinic acid, produces only a trace of β-galactosidase. The addition of a suitable galactoside to the growing culture is immediately followed by a sharp increase of up to 5000 fold in the rate of synthesis of β-galactosidase. This high rate of synthesis is maintained as long as the bacteria grow in the presence of the inducing galactoside (inducer). However, if the inducer is removed, the rate of synthesis falls immediately to the original trace value, and any enzyme present at that time is thereafter diluted in the increasing bacterial mass. Thus we have a system in which the formation of a given protein can be initiated and stopped at will. Furthermore in this system, it is possible to use inducers which are not hydrolysed by the enzyme nor utilized as carbon and energy source by the bacteria and which apparently do not affect the synthesis of the bulk of the other protein.

The questions we have sought an answer to in the present work are:

(a) to what extent do other *proteins* of the cell contribute, either directly (as specific precursors) or indirectly (as sources of amino acids or peptides) to the synthesis of β-galactosidase;

(b) whether there is any significant turnover of β-galactosidase within growing cells.

These questions were clearly formulated, albeit only partially answered, through previous immunochemical and nutritional studies[3,4,5] the results of which should be briefly recalled here.

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** Ely-Lilly research fellow of the National Research Council, 1952–1954.

*** U.S. Public Health fellow, 1952–1954.

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Immunochemical analysis of the β-galactosidase system[4,5], has shown that:

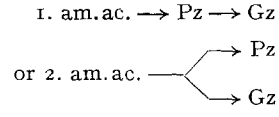
1. the induced biosynthesis of β-galactosidase (Gz) corresponds to the appearance of a new protein molecule identifiable as a distinct antigen, not detectable in non-induced bacteria;

2. the non-induced as well as the induced cells contain a structurally related protein (Pz) which cross-reacts with the antibody to the enzyme (Gz);

3. the induction of β-galactosidase synthesis is accompanied by a decrease in the overall rate of synthesis of the Pz protein;

4. among diverse species of *Enterobacteriaceae* only those which possess the Pz protein are capable of synthesizing the enzyme (Gz).

These results led to the conclusion that the mechanisms for the synthesis of the enzyme (Gz) and of the Pz protein were related and that the two proteins were either successive or twin members of the same biosynthetic pathway:



However, nutritional and kinetic studies on β-galactosidase synthesis[6] have shown that:

1. virtually all indispensable amino acids are immediately required for β-galactosidase synthesis;

2. the rate of β-galactosidase synthesis is a constant fraction of the rate of synthesis of total bacterial protein, from the time of addition of the inducer.

These results suggested that the elements of the β-galactosidase molecule are not derived from other proteins, and also that β-galactosidase synthesis is essentially irreversible, *i.e.* that the enzyme molecule is not in a "dynamic state" within the cells.

However these conclusions could be directly tested only by studying the incorporation of radioactive atoms into β-galactosidase. The present paper presents the results of such a study. Some preliminary data have already been discussed in a general report by MONOD AND COHN[7].

MATERIALS AND METHODS

Strain

The mutant (ML 324co) of *E. coli* ML[8] was used throughout these experiments.

Media

Unless otherwise specified all cultures were grown in the sulfur deficient medium 61 (13.6 g KH_2PO_4; 2.0 g NH_4Cl; 0.2 g magnesium citrate; 0.01 g $Ca(NO_3)_2$; 0.005 g $FeCl_3$; 1000 ml H_2O; and sufficient KOH to bring the pH to 7.0. The carbon source (succinic acid) and the sulfur source ($(NH_4)_2SO_4$) were added separately in the amounts specified in each experiment.

When desired this medium was made radioactive by the addition of ^{35}S "carrier-free" sulfate obtained from the Isotope Division, A.E.R.E., Harwell, England. All of the radioactivity of this material was precipitable with barium ion. The amount of sulfur thus added constituted an insignificant fraction of the total present in the medium.

Inducer

The inducer used in these experiments was methyl-β-D-thiogalactoside (MTG) synthesized by Prof. HELFERICH (Bonn, Germany)[9] to whom we should like to express our thanks. MTG is not hydrolyzed by the β-galactosidase of *E. coli*, nor is it metabolized as a carbon source by the bacteria. The kinetics of induction by MTG under the conditions used here are similar to those exhibited

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by methyl-β-D-galactoside[6] in that the increase in enzyme is directly proportional to the increase in bacterial protein from the moment the inducer is added, provided a "saturating" concentration of inducer is used ($> 10^{-4}$ *M*).

Method of culture and measurement of growth

The cultures were grown in conical flasks shaken in a 37° C water bath. The methods used for following bacterial growth have been described elsewhere[6]. Under the conditions employed here, the generation time in the exponential phase of growth is one hour.

Determination of β-galactosidase activity

β-galactosidase activity of toluenized suspensions of bacteria and of extracts was determined in $2.7 \cdot 10^{-3}$ *M* *o*-nitrophenyl-β-D-galactoside (NPG) and 0.05 *M* sodium phosphate buffer (pH = 7.0) at 28° C by previously described methods[8]. The unit of β-galactosidase activity is defined as the amount of enzyme which hydrolyzes 1 mμ mole of β-NPG in one minute under the above conditions.

Preparation of the crude extracts

Crude extracts were prepared from bacteria which had been harvested by centrifugation and washed three times with 0.05 *M* sodium phosphate buffer (pH = 7.0). The volume of culture employed for each extract was sufficiently large to contain a total of $6 \cdot 10^5$ units of β-galactosidase or approximately 2 mg of enzyme protein. The wet bacteria were then mixed with an equal weight of dry alumina and the mixture ground for five minutes in a mortar. The ground material was then taken up in an equal volume of 0.05 *M* sodium phosphate buffer (pH = 7.0), centrifuged and the supernatant saved, while the precipitate was ground for five more minutes in the same mortar and then taken up in an equal volume of buffer. This was centrifuged and the supernatant mixed with the previous one and centrifuged for one hour at 12,000 r.p.m. in a Sorvall SS-1 centrifuge. The resulting, clear supernatant constitutes the crude extract. All of the above operations were carried out in the cold room (0–3° C).

Isolation of β-galactosidase

There are four different stages in the purification procedure: 1. precipitation of nucleic acids with streptomycin; 2. fractionation with $(NH_4)_2SO_4$; 3. fractionation by means of electrophoresis in starch; and 4. specific precipitation of the enzyme with an antiserum. Unless otherwise specified all operations were carried out in the cold room (0–3° C).

1. When one volume of a 10% solution of streptomycin was added to ten volumes of crude extract a stringy precipitate formed which, after standing overnight, was separated by centrifugation and the supernatant saved. The precipitate is largely nucleic acid[10]. The ratio of the optical density at 280 mμ to that at 260 mμ changed from 0.55 in the crude extract to 1.2 in this supernatant. No β-galactosidase is precipitated by this treatment.

2. A saturated solution of $(NH_4)_2SO_4$ (700 g + 1 liter H_2O) was added to the above supernatant to a final concentration of 40% saturation and the mixture allowed to stand for 12 hours. After centrifugation the precipitate was taken up in about 2 ml of 0.02 *M* tris-(hydroxymethyl)aminomethane-HCl (TRIS) buffer (pH = 8.1). This solution was centrifuged for one hour at 12,000 r.p.m. in the Sorvall SS-1 centrifuge and the supernatant subjected to dialysis against 6 l of 0.02 *M* TRIS buffer (pH = 8.1). The overall yield of enzyme at this point was about 70%.

3. The dialysed extract was fractionated by electrophoresis in starch, a method for purifying β-galactosidase that was suggested to us by SPIEGELMAN. The electrophoresis apparatus consisted of a rectangular trough of lucite 50 cm long, 3 cm wide and 2 cm high with walls 2 mm thick and open at the top. This trough was mounted horizontally and to each end was fastened a section of glass wool which made contact with an electrode vessel containing 4 l of 0.02 *M* TRIS buffer (pH = 8.1) and a carbon electrode. Rhone-Poulenc potato starch was washed four times with twice its weight of water and then with 0.02 *M* buffer (pH = 8.1) until the pH of the wet starch was 8.1. This thixotropic, wet starch was then poured into the lucite trough to form a layer of 1.5 cm high, making contact at each end with the glass wool. The levels of the buffer in the two electrode vessels were then made equal with a siphon and the system allowed to equilibrate for 3–4 hours at 0–5° C. Approximately 1 ml of the dialysed extract was mixed with washed, dried starch to form a paste of the same consistency as that of the starch in the trough and the mixture was placed in a hole which had been cut in the starch trough 15 cm from the cathode end. The electrodes were then connected to a 450–500 volt D.C. source and a current of 6 to 7 mA passed through the starch. After 15–18 hours the position of the β-galactosidase in the trough was determined by punching the starch at 1 cm intervals along the length of the column with thin-walled glass capillary tubing (diam. = 1 mm), adding a drop of 0.01 *M* *o*-nitrophenyl-β-D-galactoside (β-NPG) in 0.25 *M* sodium phosphate (pH = 7.0) to the starch removed with the capillary tube, and observing the appearance of the yellow color resulting from the enzyme catalysed hydrolysis of the β-NPG. Those 1 cm sections of starch that contained the enzyme were then removed and each section placed in a glass tube

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(14 mm in diameter and 10 cm long) which was constricted at the bottom and had a small amount of glass wool placed in this constriction to prevent the starch from leaking out of the tube. The enzyme was then eluted from the starch by passing 2.0 ml of 0.05 *M* sodium phosphate buffer (pH = 7.0) through the starch in the tube.

The enzyme activity and radioactivity of the eluates were determined and that eluate with the lowest radioactivity to enzyme activity ratio constituted the purified enzyme extract, this generally also being the eluate with the maximum amount of enzyme. The results from two typical electrophoresis fractionations are shown in Fig. 1. The total amount of enzyme recovered in the eluates was about 70% of that added to the starch, whereas the enzyme in the eluate that contains the maximum amount of enzyme constituted about 15% of that added. Thus the overall yield from crude to purified extract was about 10%.

The purification factor, or the ratio of the specific enzyme activity (units per mg sulfur) for the purified extract to that for the crude extract, was about 20 for extracts derived from fully induced bacteria (100 enzyme units per mg bacterial N). The approximate purification factors for steps 1, 2 and 3 of the procedure was 1.6, 4 and 3 respectively. Extracts from partially induced bacteria (enzyme units per bacterial N less than 100) obviously exhibit larger purification factors.

4. The specific precipitation of the enzyme was carried out with anti-enzyme sera absorbed with Pz[4]. This technique is discussed later (Table III).

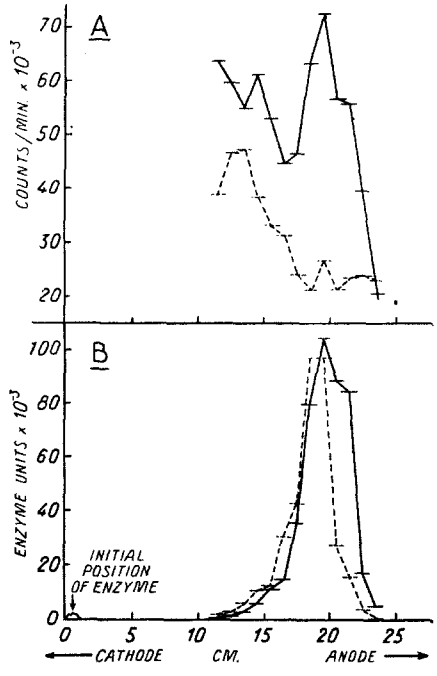

Fig. 1. Electrophoresis in starch of the fully labelled enzyme (full line) and its isolation control (dotted line). See the precursor experiment for an explanation of these extracts. A. Radioactivity in eluate versus distance along the column. B. Enzyme activity in eluate versus distance along the column. The total enzyme placed on the column was $8.0 \cdot 10^5$ units for the fully labelled enzyme and $5.2 \cdot 10^5$ units for its isolation control. The conditions under which the electrophoresis took place were as follows: voltage gradient = 9.2 volts per cm; current = 6 mA; duration of run = 16 hours for the fully labelled enzyme and 15 hours for its isolation control; temp. of column = 5–10° C.

Determination of radioactivity

The radioactivity of a given sample was determined by adding 0.30 ml of the sample to an aluminium cup (15 mm in diam. and 3 mm high) which was then placed under the infra-red lamp to dry. The dried sample was placed 12 mm under the window of a shielded Geiger Muller counter (General Electric Company, England-Type EHM2S with a mica window of 2–3 mg per cm^2 weight) and the number of counts per minute determined. The efficiency of this counter was approximately 5% and the shielded background 12 counts per min. All samples were analysed in duplicate and the total number of counts observed was always greater than 1000. Under these conditions the counts per min were proportional to the concentration of the radioactive component up to $3 \cdot 10^3$ counts per min and the reproducibility better than 5%. Consequently the samples were always diluted so that the radioactivity put on the aluminium cup was less than $2.5 \cdot 10^3$ counts per min.

EXPERIMENTAL

The specific labelling of the protein fraction of the bacteria with radioactive ^{35}S

If the synthesis of β-galactosidase were induced in a culture in which the only source of radioactivity was the bacterial proteins, then the isolation of the enzyme and the determination of its radioactivity would provide a sensitive means of evaluating what proportion of the elements of β-galactosidase is contributed by other proteins. Let us therefore first direct our attention towards the problem of specifically labelling the

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non-β-galactosidase proteins (*i.e.* those produced in the non-induced state) with a radioactive isotope.

Since *E. coli* grows well in a synthetic medium containing sulfate as the sole source of sulfur, it is convenient to use the radioactive isotope of sulfur, ^{35}S, as the labelling atom. The experiment described in Fig. 2 illustrates the manner in which the bacteria incorporate sulfur. During the logarithmic phase of growth, the incorporated sulfur consists of two fractions: the trichloracetic acid (TCA) soluble fraction (25% of incorporated sulfur) and the TCA insoluble or protein fraction (75% of incorporated sulfur). When approximately 90% of the total sulfur has been incorporated, the growth diminishes and sulfur incorporation comes to a halt. At this point the ratio between the TCA insoluble and soluble fractions begins to increase until all of the incorporated sulfur is in the TCA insoluble or protein fraction, at which point growth ceases. That growth ceases because of a lack of sulfur is evident from the fact that growth will resume immediately upon the addition of sulfate to the culture.

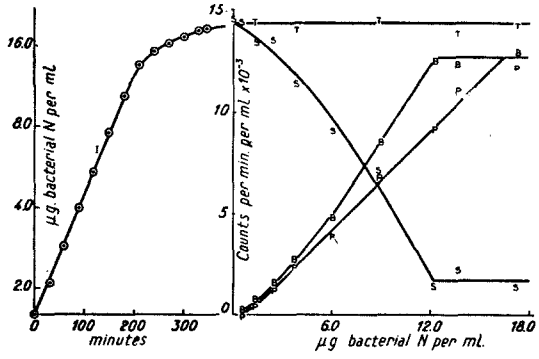

Fig. 2. Incorporation of radioactive ^{35}S by *E. coli*. ML 32400 grown in medium 61 containing a limiting amount of $(NH_4)_2SO_4$ labelled with ^{35}S. Samples taken at various times and analysed as follows. Left: total bacterial nitrogen per ml (curve I). Right: – curve T, total radioactivity per ml of culture; – curve B, radioactivity of bacteria after three washings; – curve P, radioactivity of bacteria after above washing, treatment with 5% trichloracetic acid (TCA) for 15 minutes at 0–5° C, and three washings with 5% TCA; – curve S, radioactivity of supernatant after centrifugation of culture.

These results confirm the work of COWIE, BOLTON AND SANDS[11] who found that the incorporation of ^{35}S of sulfate into the proteins of *E. coli* is proportional to the growth. Similarly ROBERTS AND BOLTON[12] found that 25% of the incorporated sulfur of *E. coli* is TCA extractable and consists primarily of glutathione, which can be incorporated into the protein fraction. That sulfur which remains in the supernatant after growth ceases has not been identified but it apparently consists of non-utilizable sulfur since it is not incorporated by bacteria growing in this supernatant supplemented with a limiting amount of non-radioactive sulfate.

From the above data, it is evident that the non-β-galactosidase proteins can be specifically labelled with the radioactive isotope ^{35}S by simply allowing the culture to grow to starvation in a non-inducing medium which contains a limiting amount of ^{35}S labelled sulfate, the other components being kept in excess. If non-radioactive sulfate and an inducer are added to such a starved culture, β-galactosidase will then be synthesized in cells whose other proteins are labelled with ^{35}S although the medium contains no utilizable ^{35}S sulfur. This is the system we desire.

The de novo synthesis of β-galactosidase. Precursor experiment

Employing the above system, the precursor experiment schematized in Fig. 3 was performed. Cells in phase I were obtained by inoculating a synthetic medium that contained a limiting amount of ^{35}S-labelled sulfate and no inducer. This culture was allowed to grow to a maximum (*i.e.* until all of the sulfate had been utilized) and was kept in this starvation state for one hour so that all of the incorporated sulfur was in the

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protein fraction. These cells in phase I were then allowed to pass into phase II by diluting the starved culture into a non-radioactive medium that contained the inducer, methyl-β-D-thiogalactoside (MTG), and the same limiting amount of sulfate as previously employed, but not labelled with ^{35}S. Immediately upon effecting this dilution, the bacteria began to grow and synthesize β-galactosidase, this growth and synthesis ceasing when the newly added sulfate was consumed. Three different dilutions were made in order that cultures could be obtained in which the specific enzyme activity varied from 5 to 58% of the maximum found in fully induced cultures. For if a part of the sulfur of β-galactosidase were derived from pre-existing proteins, then the fraction of the enzyme synthesized initially should contain the highest proportion of ^{35}S and consequently the β-galactosidase isolated from cultures of lower specific enzyme activity should have the higher ratio of radioactivity per enzyme unit. These three starved cultures were then harvested by centrifugation and the bacteria washed and ground up to make the crude extracts from which the enzyme was isolated. The details and results of this culture phase of the precursor experiment are presented in Table I.

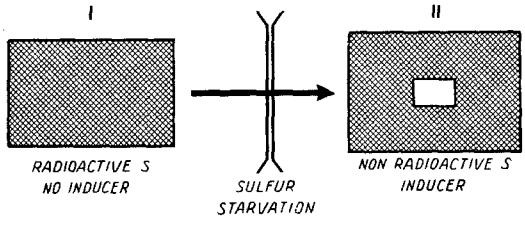

Fig. 3. Schematic of the precursor experiment. Rectangles represent total bacterial protein. Central rectangle represents β-galactosidase. Shading indicates radioactivity.

TABLE I

PRECURSOR EXPERIMENT – CULTURES

Cultures	Bacterial concentration			Enzyme concentration			
	Initial (μg N/ml)	*Final-starved (μg N/ml)*	*% Total growth occurring in phase II*	*Initial (units/ml)*	*Final-starved (units/ml)*	*100 Initial/Final*	*Final specific activity (units/μg N)*
Phase I	<0.1	118	—	0	7	—	0.06
Phase II A	106	126	16	6	630	1.0	4.8
B	89	124	28	5	3,900	0.13	32
C	67	118	43	4	6,800	0.06	58
Fully labelled Enzyme control	<0.1	112	—	0	11,200	—	100

Medium for phase I was the medium 61 plus 10 mg succinic acid per ml and 38 μg of ^{35}S labelled $(NH_4)_2SO_4$ per ml (9.2 μg S per ml). The specific radioactivity of the sulfur was $1.9 \cdot 10^4$ counts per minute per μg S. The medium added to achieve phase II was the medium 61 plus 10 mg succinic acid per ml, 38 μg non-radioactive $(NH_4)_2SO_4$ per ml, and sufficient methyl-β-D-thiogalactoside (MTG) to obtain a final concentration of $1.0 \cdot 10^{-3}$ *M*. The medium used for the fully labelled enzyme control was the same as that employed in phase I but contained in addition MTG at $5.0 \cdot 10^{-4}$ *M*.

Before considering the results obtained upon isolating the enzyme from these extracts, we must discuss the controls that were used. The first control was designed to obtain ^{35}S-labelled β-galactosidase in which the specific radioactivity of the sulfur was the same as that of the radioactive medium employed in phase I above. We shall call this the *fully labelled* enzyme. It served as a standard of comparison whereby the radioactivity of the enzyme isolated from the three extracts could be evaluated. Thus a

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medium identical with that used in phase I except that it contained the inducer MTG, was inoculated with a trace of *E. coli* (see Table I). This culture was allowed to grow to its sulfur limit, was harvested by centrifugation and the bacteria washed and ground up to provide an extract containing the fully labelled enzyme.

The other controls consisted of artificial mixtures of an extract of non-induced ^{35}S-labelled bacteria with an extract of fully induced non-radioactive bacteria. The enzyme isolated from these controls should contain no radioactivity if all the other proteins had been eliminated in the isolation procedure. Consequently these controls check the isolation technique and will be referred to as *isolation controls*. Two such controls were made: one containing the same radioactivity and enzyme activity as the fully labelled enzyme extract and the other duplicating the radioactivity and enzyme activity of extract A (phase II).

TABLE II

PRECURSOR EXPERIMENT – EXTRACTS

Extract	*Enzyme activity (units/ml)*	*Radioactivity (counts/min/ml)*	$C = \frac{\text{Radioactivity}}{\text{Enzyme activity}}$ *(counts/min/enzyme unit)*
Phase II	$\times 10^{-4}$	$\times 10^{-4}$	
A – crude	1.40	109	78
– purified	0.42	0.91	2.1
B – crude	6.8	130	19.1
– purified	2.64	1.38	0.52
C – crude	12.0	84	7.0
– purified	8.0	0.95	0.118
Controls			
1. Fully labelled enzyme – crude	10.1	110	10.9
– purified	4.2	2.78	0.66
2. Isolation control for fully labelled enzyme – crude	10.5	109	10.4
– purified	5.0	1.33	0.27
3. Isolation control for phase II-A – crude	1.37	91	66
– purified	0.60	0.45	0.76

The preparation of the crude extracts and their purification are described in MATERIALS AND METHODS.

The essential element in the isolation procedure consisted of a specific precipitation of the enzyme by anti-β-galactosidase serum. This serum was completely absorbed with an extract of non-induced bacteria, and then fractionated[4] to isolate the antibody-containing γ globulin. This preparation would not precipitate with Pz, or for that matter with any other protein in the inactive extract, but it continued to precipitate the enzyme. Since the antibody did not inactivate the enzyme it was possible to determine the enzymic activity as well as the radioactivity on the washed specific precipitate. With the purification procedure which was finally adopted the isolation control contained only 0.4% of the radioactivity of the fully labelled enzyme (Table III). Thus a technique was realized whereby β-galactosidase could be isolated from an extract containing only 2–3 mg of enzyme, representing less than 0.2% of the total material.

Bearing in mind the significance of these controls, let us now turn our attention to

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TABLE

PRECURSOR EXPERIMENT

Purified extract	*Totals*			
	Enzyme activity (units × 10⁻³)			*Radioactivity (counts/min × 10⁻³)*
	from extract	*carrier*	*total*	
Phase II				
A	6.0	26.8	32.8	12.9
B	29.0	—	29.0	15.2
C	28.0	—	28.0	3.3
Controls				
1. Fully labelled enzyme	25.4	—	25.4	16.7
2. Isolation control for fully labelled enzyme	27.5	—	27.5	7.3
3. Isolation control for state 2, I	8.3	24.0	32.3	6.3

β-galactosidase was precipitated from the extracts by adding 1.00 ml of antiserum capable of precipitating $4 \cdot 10^4$ units of enzyme (MATERIALS AND METHODS) to 2.00 ml of extract containing approximately $3 \cdot 10^4$ units of enzyme. In the case of phase II-A and its isolation control it was necessary to add carrier enzyme (partially purified non-radioactive enzyme extract) because of the small amount of enzyme recovered in the purification procedure. Immediately after mixing the antiserum and the enzyme extract, a 0.100 ml sample was withdrawn and analysed for enzyme activity and radioactivity (totals). The mixture was then allowed to stand in a 37° C bath until the precipitate

the results of the precursor experiment, summarized in the last column in Table III which gives the radioactivity per enzyme unit for the β-galactosidase in the various extracts, relative to that of the fully labelled enzyme. In considering the relative radioactivity of the enzyme in the three experimental extracts A, B and C, the amount of radioactivity corresponding to the trace amount of enzyme synthesized in the non-inducing radioactive medium employed in phase I must be taken into account. Thus in the case of sample A, 1.0% of the total enzyme extracted would be expected to be fully labelled since this percentage was synthesized in phase I (see Table I). In samples B and C only 0.13 and 0.06% of the total enzyme would be expected to be fully labelled as a result of synthesis in phase I (Table I). The relative value for the radio-activity per enzyme unit found in the specific precipitates should therefore be corrected by substracting the percentage of enzyme in the extracts that is fully labelled due to synthesis in phase I. Such a correction shows that for samples A, B and C, the amount of radioactivity associated with the enzyme synthesized in phase II is respectively 0.1, 0.8 and 0.1% of that of the fully labelled enzyme. Since there is no definite order to the values (*i.e.* A > B > C) and since these values are within the range of reproducibility of the isolation controls, we may conclude that in each sample less than 0.8% of the sulfur of the sulfur of the enzyme synthesized in phase II was derived from non-β-galactosidase proteins synthesized in phase I. This result, taken in conjunction with the fact that in sample A the enzyme level was only 5% of that found in the fully induced bacteria, indicates that if any protein precursor of β-galactosidase exists in the non-induced bacteria, its level (expressed as amount of sulfur per bacterial nitrogen) must be less than 0.04% of that for β-galactosidase in fully induced bacteria.

Thus the possibility that the Pz protein is a precursor of β-galactosidase is effectively

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III

– SPECIFIC PRECIPITATES

Supernatants		*Precipitates*			
Enzyme activity (units × 10⁻³)	*Radioactivity (counts/min × 10⁻³)*	*Enzyme activity (units × 10⁻³)*	*Radioactivity (counts/min × 10⁻³)*	$C = \frac{\text{Radioactivity}}{\text{Enzyme activity}}\left(\frac{\text{counts/min}}{\text{enzyme units}}\right)$	*Percent of C for fully labelled enzyme*
0.48	12.6	29.6 (5.4)	0.027	0.0050	1.1
1.29	14.4	26.0	0.112	0.0043	0.9
0.085	3.0	26.2	0.019	0.00072	0.16
0.093	5.4	25.9	11.6	0.45	100
0.120	7.3	27.8	0.052	0.0019	0.42
0.35	6.4	28.3 (7.2)	0.013	0.0018	0.40

had flocculated (*ca.* 2 hours). It was centrifuged and the supernatant withdrawn for analysis. The precipitate was washed three times with cold (0–5° C) 0.85% NaCl solution, suspended in 1.00 ml of 0.05 *M* sodium phosphate buffer, pH = 7.0, and the enzyme activity and radioactivity of this suspension determined.

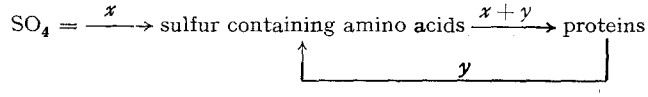
The values in the parentheses in the column for enzyme activities of the precipitate equal the amount of enzyme from the extract (*i.e.* after correction was made for carrier enzyme). The *C* values in the next to last column are calculated from these corrected enzyme activities.

eliminated. For the Pz protein level in non-induced bacteria (expressed as immunological combining units per bacterial nitrogen) is approximately 30% of the enzyme (Gz) level in fully induced bacteria[5] and consequently if the Pz protein were a precursor of the enzyme, one would be forced to the highly improbable conclusion that the amount of sulfur per unit of Pz protein would have to be less than 0.1% of that for the enzyme.

More generally, the results of the precursor experiment indicate that β-galactosidase is synthesized exclusively from material that is assimilated after the addition of the inducer and hence proteins existing in the non-induced bacteria play no significant role as precursors.

The stability of proteins in vivo

A second conclusion can be drawn from the results of the precursor experiment, namely, that non-β-galactosidase proteins are stable, not being degraded to amino acids by any mechanism. For if the state of the proteins within the cell consists of a continual synthesis from and breakdown to their constituent amino acids (*i.e.* state of "dynamic equilibrium"), then one would expect the β-galactosidase synthesized in phase II of the experiment to be labelled with ^{35}S as a result of the breakdown of the radioactive proteins. Since the amount of radioactivity found in the enzyme synthesized in phase II was less than 0.8% of that for the fully labelled enzyme, we can conclude that the rate of breakdown of the non-β-galactosidase proteins must be less than one percent of the rate of synthesis. That this is true is clear from a consideration of the following diagram:



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in which x equals the rate of net synthesis of the proteins and y equals the rate of breakdown. The specific radioactivity of the sulfur in the non-β-galactosidase proteins in sample A is 84% of that of the proteins in phase I or of that of the fully labelled enzyme (see Table I). Hence the minimum specific radioactivity of the sulfur in the amino acids resulting from any breakdown of these proteins would be 84% of that of the fully labelled enzyme. Since the sulfur-containing amino acids synthesized directly from the sulfate of the medium during phase II cannot be radioactive and since there is no appreciable amino acid pool in *E. coli*, then the specific radioactivity of the sulfur in the total amino acids which act as precursors to protein synthesis would have a minimum specific radioactivity that was $84y/(x + y)$ percent of that of the fully labelled enzyme. Thus the β-galactosidase synthesized from these amino acids would have a minimum specivc radioactivity that would also be $84/(x + y)$ percent of that of the fully labelled enzyme. Since the enzyme in sample A that was synthesized in phase II was found to contain less than 0.8% of the radioactivity of the fully labelled enzyme, then $84y/(x + y$ must be less than 0.8, or $y/(x + y)$ must be less than 0.01, *i.e.* the rate of breakdown of

TABLE IV

STABILITY OF β-GALACTOSIDASE DURING BACTERIAL GROWTH IN THE ABSENCE OF AN INDUCER

I. Cultures

Culture	*Bacterial concentration*		*Enzyme concentration (units/ml)*	
	Initial (μg N/ml)	*Final (μg N/ml)*	*Initial*	*Final*
Phase I	< 0.1	95	0	10,150
Phase II	12.0	126	1,300	1,290

Medium for phase I was the medium 61 plus 10 mg succinic acid per ml, 38 μg $(NH_4)_2SO_4$ per ml and $5 \cdot 10^{-4}$ *M* MTG. The medium for phase II was the same except that the MTG was omitted and the $(NH_4)_2SO_4$ was labelled with ^{35}S such that the specific radioactivity of the sulfur was $1.9 \cdot 10^4$ counts per min per μg S, *i.e.* the same as that employed in the precursor experiment.

II. Extracts

Extract	*Enzyme activity (units/ml × 10^{-4})*	*Radioactivity (counts/min/ml × 10^{-4})*	$C = \frac{\text{Radioactivity}}{\text{Enzyme activity}}$ *(counts/min/enzyme unit)*
Phase II – crude	1.74	126	72
– purified	1.29	0.98	0.76

See MATERIALS AND METHODS for the preparation of the crude extract and the purification procedure.

III. Specific precipitates

Purified extract	*Total*			
	Enzyme activity (units × 10^{-3})			*Radioactivity (counts/min × 10^{-3})*
	from extract	*carrier*	*total*	
Phase II	15.7	20.7	36.4	11.9

See the legend of Table III for a description of the method and an explanation of the data given in the various columns. The fully labelled enzyme control is that given in Table III since

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the non-β-galactosidase proteins must be less than one percent of the rate of synthesis.

The validity of this interpretation is dependent upon the assumption that all of the amino acids derived from the proteins are re-utilized for protein synthesis. That this assumption is reasonable is supported by the work of COWIE, BOLTON AND SANDS[11] who showed that amino acids once incorporated into the protein of *E. coli* are not released to any significant extent into the medium. This result, which we have confirmed, is also a striking demonstration of stability of the proteins of *E. coli* during growth.

This conclusion, surprising in view of the generally accepted idea of the "dynamic state" of proteins *in vivo*, led us to investigate the stability of the β-galactosidase by the very simple and sensitive experiment schematized in Fig. 4. Cells in phase I were obtained by allowing the bacteria to grow to a limit on non-radioactive sulfate in the presence of the inducer, MTG. These starved cells were washed to remove the inducer and were then placed in a non-inducing, ^{35}S-labelled sulfate medium in which a ten-fold increase in bacterial mass took place before growth stopped due to lack of sulfate (phase II). It can be seen from Table IV that the total enzyme in the culture remained constant during growth in phase II. Thus any incorporation of ^{35}S into the enzyme is a measure of both the amount of synthesis and of the amount of breakdown of the β-galatosidase in phase II, *i.e.* in bacteria growing in the absence of the inducer. From Table IV it can be seen that the amount of radioactivity in the enzyme isolated from phase II bacteria is only 0.4% of that of the fully labelled enzyme. The isolation control for sample A of the precursor experiment also applies to this experiment since the enzyme activity and radioactivity of the crude extracts are approximately the same. The fact that the phase II enzyme has the same radioactivity per enzyme unit as its isolation control makes it highly improbable that the radioactivity in the phase II enzyme is significant. In any case it must be less than 0.4% of that of the fully labelled enzyme. This means that less than 0.4% of the enzyme could have been broken down while the total bacterial mass increased by 900%. Since in the presence of an inducer such as MTG the increase in the enzyme is proportional to the increase in bacterial mass, then the rate of breakdown of the enzyme during bacterial growth in the absence of the inducer is less than 0.2% of the rate of synthesis of the enzyme in the presence of the inducer.

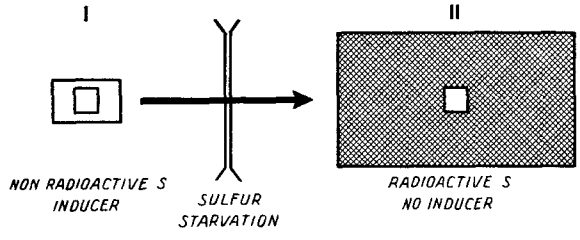

Fig. 4. Schematic of experiment for determining the stability of β-galactosidase during bacterial growth in the absence of an inducer. (Same symbolism as in Fig. 3).

Supernatant		*Precipitate*			
Enzyme activity (units × 10⁻³)	*Radioactivity (counts/min × 10⁻³)*	*Enzyme activity (units × 10⁻³)*	*Radioactivity (counts/min × 10⁻³)*	$C = \frac{\text{Radioactivity}}{\text{Enzyme activity}}\left(\frac{\text{counts/min}}{\text{enzyme units}}\right)$	*Percent of C for fully labelled enzyme*
0.44	11.8	30.3 (13.1)	0.024	0.0018	0.40

sulfur of the same specific radioactivity was used in the two experiments.

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While the above experiment indicates a very high degree of stability of β-galactosidase in bacteria in which no synthesis of the enzyme occurs (*i.e.* in the absence of the inducer), it tells us nothing of the stability of β-galactosidase in bacteria in which active β-galactosidase synthesis occurs (*i.e.* in the presence of the inducer). Since the inducer functions much like a catalyst in promoting the synthesis of the enzyme, one could imagine that the inducer also acts to catalyse the breakdown of the enzyme. It was therefore necessary to determine if any breakdown of the enzyme occurs during synthesis in the presence of the inducer. The experiment schematized in Fig. 5 was designed for this purpose. Bacteria in phase I were obtained by inoculating a radioactive medium containing the inducer MTG and allowing the bacteria to grow till starved on sulfur. The inducer was removed by washing and the bacteria were then placed in a non-radioactive medium in which a ten-fold increase in bacterial mass took place before growth stopped as a result of exhaustion of the carbon source, succinic acid (phase II). Thus the specific radioactivity of the sulfur in the non-β-galactosidase proteins of the bacteria in phase II decreased to nine percent of that in phase I whereas the specific radioactivity of the sulfur in the enzyme remained constant at the phase I value. Inducer MTG and succinic acid were then added, growth and β-galactosidase synthesis beginning immediately upon effecting this addition and ceasing when the succinic acid was again used up (phase III). Three different samples of phase III bacteria, in which the percentage of the enzyme that was synthesized in phase I varied form 13 to 68%, were obtained by the addition of different amounts of succinic acid to the starved phase II culture. The data for these cultures are given in Table V and for the extracts derived from them in Table VI, while Table VII gives the results of the specific precipitation of the enzyme from the purified extracts.

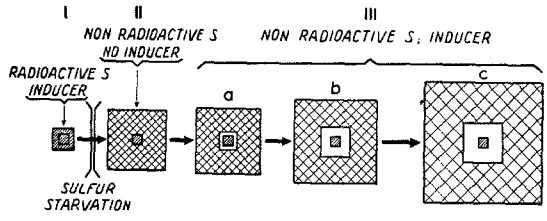

Fig. 5. Schematic of experiment for determining the stability of β-galactosidase during bacterial growth in the presence of an inducer. (Same symbolism as in Fig. 3).

TABLE V

STABILITY OF β-GALACTOSIDASE DURING BACTERIAL GROWTH IN THE PRESENCE OF AN INDUCER CULTURES

Culture	*Bacterial concentration*			*Enzyme concentration (E)*		
	Initial (μg N/ml)	*Final (μg N/ml)*	*% total growth occurring in phase I*	*Initial (units/ml)*	*Final (units/ml)*	*% total enzyme synthesized in phase I*
Phase I	< 0.1	132	100	0	13,700	100
Phase II	10.1	115	8.8	1,050	1,020	100
Phase III						
A	115	122	8.3	1,020	1,500	68
B	115	130	7.8	1,020	4,930	20.7
C	115	160	6.3	1,020	8,100	12.6

The medium used in phase I was the medium 61 plus 10 mg succinic acid per ml and 42 μg of ^{35}S labelled $(NH_4)_2SO_4$ per ml (10.2 μg S per ml) and $5 \cdot 10^{-4}$ *M* MTG. The specific radioactivity of the sulfur was $1.35 \cdot 10^4$ counts per min per μg S. The medium for phase II was medium 56(7) plus 3.5 mg succinic acid per ml. When the phase II culture had ceased to grow due to lack of succinic acid, it was divided into three parts (cultures A, B and C) and the inducer, MTG, and succinic acid were added such that the MTG concentration was $1.0 \cdot 10^{-3}$ *M* for each of the three cultures and the succinic acid concentration was 0.2, 0.5 and 1.6 mg per ml for cultures A, B and C respectively.

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TABLE VI

STABILITY OF β-GALACTOSIDASE DURING BACTERIAL GROWTH IN THE PRESENCE OF AN INDUCER EXTRACTS

Extract	Enzyme activity (units/ml × 10^{-4})	Radioactivity (counts/min/ml × 10^{-4})	$C = \frac{\text{Radioactivity}}{\text{Enzyme activity}}$ (counts/min/enzyme unit)
Phase I – crude	32	256	8.0
– purified	9.5	3.4	0.36
Phase II – crude	2.5	20.8	8.3
– purified	1.9	0.78	0.41
Phase III			
A – crude	3.8	25.4	6.7
– purified	2.2	0.66	0.30
B – crude	6.7	14.4	2.1
– purified	1.9	0.20	0.105
C – crude	10.3	13.4	1.3
– purified	6.9	0.56	0.081

See MATERIALS AND METHODS for the preparation and purification of the crude extracts.

If there were no breakdown of β-galactosidase in phase III of the experiment, then the total amount of radioactivity associated with enzyme should remain constant and hence the enzyme radioactivity per ml should be the same for cultures in phase II, and phase III, (samples A, B and C). The enzyme radioactivity per ml of these cultures is equal to the enzyme concentration in the culture (E) multiplied by the radioactivity per enzyme unit (C) found in the specific precipitate. If no breakdown took place during active enzyme synthesis, then $E.C = K$, a constant, and consequently a plot of C versus $1/E$ should yield a straight line with slope K and intercept at the origin. When the data are plotted in this fashion (Fig. 6) it is clear that they fit such a straight line. The curved dashed line in Fig. 6 is the curve that would be expected if the rate of breakdown of β-galactosidase were ten percent of the rate of net synthesis. This curve definitely lies outside the limits of experimental error associated with the determination of the C and E values, these errors being such that the lowest breakdown rate detectable would be 5% of the rate of net synthesis. Therefore the rate of breakdown of β-galactosidase during bacterial growth in the presence of the inducer must be less than 5% of the rate of net synthesis of the enzyme.

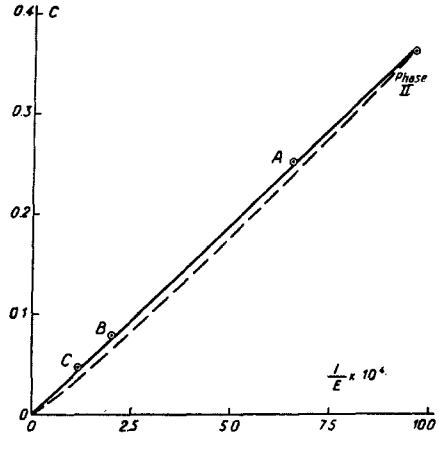

Fig. 6. Stability of β-galactosidase during bacterial growth in the presence of an inducer. Abscissa: Inverse of enzyme activity (units per ml) in the cultures during phase II and phase III (A, B, C) of the experiment schematized in Fig. 5. Ordinate: radioactivity to enzyme activity ratio of the specific precipitates given in counts per min. The dashed-line represents the expected curve if the rate of breakdown were 10% of the net rate of synthesis. The errors involved in the determination of C and E are approximately seven and five percent respectively. [5]

Although this latter experiment is less sensitive than the previous two it nevertheless indicates that β-galactosidase synthesis in

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TABLE

STABILITY OF β-GALACTOSIDASE DURING BACTERIAL GROWTH IN

Purified extract	*Totals*		*Supernatants*	
	Enzyme activity (units × 10⁻³)	*Radioactivity (counts/min × 10⁻³)*	*Enzyme activity (units × 10⁻³)*	*Radioactivity (counts/min × 10⁻³)*
Phase I	27.5	9.8	0.110	0.98
Phase II	28.0	11.7	0.120	2.25
Phase III				
A	28.0	8.5	0.160	1.78
B	28.2	3.0	0.30	0.96
C	27.5	2.25	0.078	1.09

See the legend of Table I for a description of the method and an explanation of the data given in the various columns. Note that the average *C* value for the specific precipitate of phase I and phase II (fully labelled enzyme) is 78% of that found for the fully labelled enzyme in the precursor experiments (Table III). This is due to the fact that the sulfur used in this experiment had a specific radioactivity that was 71% of that used in the precursor experiment (Tables I and V).

the presence of the inducer is an essentially irreversible process. Therefore we must conclude that the proteins of *E. coli* are extremely stable molecules *in vivo*, synthesized by essentially irreversible reactions.

DISCUSSION

The results reported above should be discussed in relation to the specific problems of enzyme induction, before considering their bearings on the more general problems of protein synthesis and turnover.

The incorporation data demonstrate conclusively that the induced synthesis of β-galactosidase involves the complete, *de novo*, formation of the protein molecule from its elements. The hypothesis that the induced enzyme is a conversion product of another protein, formed and accumulated in the absence of inducer, is eliminated. Thus the relationship of the Pz protein to β-galactosidase can not be one of a precursor to a final product. However, the remarkable structural and physiological relationships of the two proteins remain a very significant fact, which can be understood on the assumption that the two molecules are synthesized by the same mechanisms, or at the same sites. This invites certain interesting speculations concerning in particular the analogy between the Pz-Gz system and the normal and antibody globulin[13, 14] systems. However these aspects need not be considered now, since the data presented here have no specific bearings upon them.

It should be noted that our experiments involved a step of sulfur starvation during which sulfur from the TCA soluble fraction was incorporated into protein. The experiments do not exclude, therefore, the possibility that such materials might function as a source of elements for β-galactosidase. COWIE *et al.*[15] have observed that *E. coli* B normally contains a certain alcohol-soluble "protein" fraction which disappears during sulfur starvation. If such material could serve as reserve of sulfur for β-galactosidase, it would not have been detected by our experiments. However the same workers have found that under normal conditions of growth, no sulfur from this particular fraction went into other proteins.

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VII

THE PRESENCE OF AN INDUCER. SPECIFIC PRECIPITATES

Precipitates			
Enzyme activity (units × 10⁻⁸)	*Radioactivity counts/min × 10⁻⁸)*	$C = \frac{\text{Radioactivity}}{\text{Enzyme activity}}$ $\left(\frac{\text{counts/min}}{\text{enzyme unit}}\right)$	*Percent of C for phase II*
26.4	9.0	0.34	95
25.4	9.2	0.36	100
26.8	6.6	0.25	69
26.2	2.08	0.079	21.9
25.0	1.18	0.047	13.0

Thus if the C values are converted to the more absolute quantity, the number of sulfur atoms per enzyme unit, with the aid of the specific radioactivities, then the values $4.6 \cdot 10^{11}$ and $4.9 \cdot 10^{11}$ atoms S/enzyme unit are found for the precursor experiment and the above experiment respectively. These two values are within the experimental error involved in the determination of the C values (*ca* 7%) and the specific radioactivity (*ca* 10%).

It should also be remarked that our experiments were carried out with labelled sulfur and therefore the conclusions apply rigorously only to the sulfur-containing precursors. However an experiment similar to our "precursor" experiment has been carried out by SPIEGELMAN using carbon-14 as a label, with essentially similar results (personal communication).

The incorporation data also demonstrate that β-galactosidase, once formed, is stable within the cells. These observations therefore confirm the tentative conclusions drawn from previous kinetic and nutritional studies[6]. Induction in the case of β-galactosidase, results in the initiation or acceleration of an esssentially irreversible process. The enzyme is evidently not in "equilibrium" with a precursor, nor with any other protein within the cells, nor with a pool of precursors or amino acids. Therefore, all interpretations of enzyme adaptation in terms of the alteration of an "equilibrium" between proteins within the cell[16,17,18,23,24], are shown to be inadequate.

This leads us to considering the broader aspects of our findings. As we have already noted, the results demonstrate not only that β-galactosidase is irreversibly synthesized, stable, and static within the cells, but that this must be true of essentially all, or at least of the bulk of *E. coli* proteins. A simple calculation (p. 107) shows that if there is any degradation of proteins, or exchange of amino acids between proteins within the growing cells, the rate of such a process must be so low that it plays no role in fixing the net rates of protein synthesis. Nor does this process contribute appreciably to determining the relative composition and structure of the growing cells, as far as proteins are concerned. In other words, all or most proteins, within *E. coli* cells, are in a static, not in a dynamic state.

These results might therefore appear, at first, to be at variance with the classical findings of SHOENHEIMER and his school on the turnover of proteins in the body or tissues of higher organisms. Rather than concede that this inconsistency implies that the cellular state of the proteins in mammalian tissues is essentially different from that in *E. coli*, we have sought an explanation for this inconsistency in the different properties of the two systems.

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One objection that we should like to dismiss before considering the different properties of the two systems is that our experiments were of too short a duration to detect degradation rates of the order of magnitude of those found in mammalian system. It is obvious that the rate of degradation must be a significant fraction of the rates of synthesis if the concept of a "dynamic state" is to have any general physiological significance at the cellular level. Therefore it is the rate of degradation relative to that of synthesis which is of importance and not the absolute rate of degradation.

The critical question appears to be the *interpretation* of the incorporation data in the case of the mammalian systems. The interpretations of such data in terms of a "dynamic state" of the protein molecules within the cells involve some inherent ambiguities. The nature of this difficulty is evident when one considers the degree of homogeneity of the cellular populations in the two systems.

The bacterial system used in our experiments consists of a homogeneous population of cells in which there is no observable cell lysis or secretion of proteins from the cells and where all the cells are placed in an identical environment. The incorporation data are therefore interpretable in terms of the synthesis and of the state of the proteins within the cells. Any renewal would have to be interpreted as reflecting a dynamic state of the protein within the cells. However, as we see, no such renewal is detected with these homogeneous systems.

Mammalian systems on the other hand consist of heterogeneous populations of cells placed under different environmental conditions in which some cells grow and multiply, some die and lyse, others secrete large amounts of proteins while still others appear to remain very stable. Thus there are three possible pathways by which tissues of such systems may lose proteins: 1. intracellular degradation; 2. secretion; and 3. cell lysis.

Labelling experiments with mammalian systems do not by themselves give the information necessary to determine by which of the above three paths the protein is lost from the tissue. That secretion and cell lysis play a dominant role in the mechanism by which proteins are removed from the tissue is indicated by the fact that tissues with high turnover rates are those in which the mitotic rate (*i.e.* cellular replacement) is very high (intestinal mucosa) or which are known to secrete proteins actively (liver) whereas very low turnover rates are associated with tissues in which both cellular replacement and protein secretion are minimal [muscle and nerve (cf. [19])]. This suggests very strongly indeed that turnover rates measured under these conditions, express the dynamic state of the tissue, rather than the state of the protein molecules within the cells. In any case, there is, to our knowledge, no experimental evidence that the proteins within the cells of mammals are any more "dynamic" than those of *Escherichia coli*. And it should be pointed out that it is not easy even to imagine an experiment which would test intracellular turnover with mammalian tissue systems. Possibly the best illustration of these difficulties can be drawn from the beautiful experiments of VELICK and coworkers on the synthesis of the three enzymes aldolase, phosphorylase and glyceraldehyde-3-phosphate dehydrogenase in muscle tissue*[20,21]. These workers found that the extremely low rate of incorporation of several amino acids into the dehydrogenase of rabbit muscle, was significantly lower than the rate of incorporation into the two other enzymes. This might of course be interpreted as expressing a difference in the intracellular turnover of the different molecules. It might however, just as well, be interpreted

* It should be noted that these experiments were designed to study the intermediates in protein synthesis, not to test the dynamic state hypothesis.

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as resulting from differences in composition, and rates of net synthesis in different parts, or at different levels in the tissue. Wherever there may exist metabolic gradients of any sort, a heterogeneity of the cell population as regards the rates of synthesis of various proteins is to be expected. The experience gained in the study of enzyme make-up of homogeneous bacterial populations renders such an hypothesis very likely, since it is commonly observed that even slight changes in conditions (nutritional and other) may profoundly alter the relative rates of synthesis of different proteins within the cells, as well as the net rate of cell growth.

To sum up: there seems to be at present no conclusive evidence that the protein molecules within the cells of mammalian tissues are in a dynamic state. Moreover our experiments have shown that the proteins of growing *E. coli* are static. Therefore, it seems necessary to conclude that the synthesis and maintenance of proteins within growing cells is not necessarily or inherently associated with a "dynamic state".

The experimental work on this problem was begun by Dr. A. M. PAPPENHEIMER Jr. during his stay with us at the Pasteur Institute. We should like to gratefully acknowledge his early and decisive contribution.

We would like to thank Mr. RAYMOND BARRAND for his competent and enthusiastic technical assistance.

SUMMARY

A study of the kinetics of sulfur incorporation into the molecule of β-galactosidase during the induced synthesis of this enzyme in *E. coli* brings proof that the enzyme-protein is synthesized entirely *de novo* without any appreciable participation of materials coming from other cellular proteins. Furthermore, there is no measurable renewal of β-galactosidase sulfur in growing cells whether or not the enzyme is being synthesized. The induced synthesis of β-galactosidase appears as a virtually irreversible process. The bulk of the other cellular proteins in *E. coli* are equally stable and do not undergo any appreciable degradation and resynthesis during growth.

The apparent contradiction between these results and the generally accepted concepts regarding the dynamic state of intracellular proteins is discussed.

RÉSUMÉ

L'étude de l'incorporation du ^{35}S dans la molécule de β-galactosidase, au cours de sa synthèse induite chez *Escherichia coli*, apporte la preuve que la protéine enzymatique est synthétisée *de novo* à partir des éléments du milieu, et sans participation appréciable d'éléments provenant d'autres protéines cellulaires. En outre, il n'y a pas de renouvellement mesurable du soufre de la β-galactosidase intracellulaire. La synthèse induite de la β-galactosidase est un processus pratiquement irréversible. Les autres protéines cellulaires, chez *E. coli*, sont également extrêmement stables et ne subissent pas de dégradation et de renouvellement appréciable au cours de la croissance.

L'apparente contradiction entre ces résultats et les conceptions courantes sur l'état dynamique des protéines intracellulaires fait l'objet d'une discussion.

ZUSAMMENFASSUNG

Das Studium des Einbaues von ^{35}S in das β-Galaktosidase-Molekül, im Verlauf der Synthese dieses Moleküls durch *Escherichia coli*, erbringt den Beweis, dass das Enzymprotein völlig *neu* synthetisiert wird, ohne nennenswerte Beteiligung von Elementen, die aus anderen Zellproteinen stammen. Ferner ergibt sich, dass der Schwefel der intrazellulären β-Galaktosidase nicht messbar erneuert wird. Die induzierte Synthes von β-Galaktosidase ist ein praktisch irreversibeler Process. Die anderen zellulären Proteine von *E. coli* sind gleich extrem stabil. Sie werden im laufe des Wachstums weder wesentlich abgebaut noch restituiert.

Der sichtbar Widerspruch zwischen diesen Resultaten und den allgemeinen Annahmen über den dynamischen Zustand der intrazellulären Proteine wird diskutiert.

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REFERENCES

1 J. LEDERBERG, *J. Bact.*, 60 (1950) 381.
2 S. A. KIRBY AND H. A. LARDY, *J. Am. Chem. Soc.*, 75 (1953) 890.
3 J. MONOD AND M. COHN, *Adv. Enzymol.*, 13 (1952) 67.
4 M. COHN AND A. M. TORRIANI, *J. Immunol.*, 69 (1952) 471.
5 M. COHN AND A. M. TORRIANI, *Biochim. Biophys. Acta*, 10 (1953) 280.
6 J. MONOD, A. M. PAPPENHEIMER JR. AND G. COHEN-BAZIRE, *Biochim. Biophys. Acta*, 9 (1952) 648.
7 J. MONOD AND M. COHN, *Symposium on Microbial Metabolism, VIth Intern. Congress Microbiol.*, Rome (1953) 42.
8 J. MONOD, G. COHEN-BAZIRE AND M. COHN, *Biochim. Biophys. Acta*, 7 (1951) 585.
9 B. HELFERICH, H. GRUNEWALD AND F. LANGENHOFF, *Chem. Ber.*, 86 (1953) 873.
10 S. S. COHEN, *J. Biol. Chem.*, 168 (1947) 511.
11 D. B. COWIE, E. T. BOLTON AND M. K. SANDS, *J. Bacteriol.*, 60 (1950) 233; *Ibid.*, 62 (1951) 63.
12 R. B. ROBERTS AND E. T. BOLTON, *Science*, 115 (1952) 479.
13 P. GROS, J. COURSAGET AND M. MACHEBOEUF, *Bull. soc. chim. biol.*, 34 (1952) 1070.
14 H. GREEN AND H. S. ANKER, *Biochim. Biophys. Acta*, 13 (1954) 365.
15 D. B. COWIE, R. B. ROBERTS AND E. T. BOLTON, *Science*, 119 (1954) 579.
16 J. YUDKIN, *Biol. Rev.*, 13 (1938) 93.
17 S. SPIEGELMAN, *Cold Spring Harbor Symposium Quant. Biol.*, 11 (1946) 256.
18 J. MONOD, *Growth*, 11 (1947) 223.
19 D. M. GREENBERG AND T. WINNICK, *J. Biol. Chem.*, 173 (1948) 199.
20 M. V. SIMPSON AND S. F. VELICK, *J. Biol. Chem.*, 208 (1954) 61.
21 M. V. SIMPSON AND S. F. VELICK, *J. Biol. Chem.* (in print).
22 J. MONOD, A. M. TORRIANI AND J. GRIBETZ, *Compt. rend. Acad. Sci.*, 227 (1948) 315.
23 J. MANDELSTAM, *Biochem. J.*, 51 (1952) 674.
24 J. MANDELSTAM AND J. YUDKIN, *Biochem. J.*, 51 (1952) 686.

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SUR LA BIOSYNTHESE DE LA β-GALACTOSIDASE (LACTASE) CHEZ *ESCHERICHIA COLI*. LA SPECIFICITE DE L'INDUCTION*

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RÉSUMÉ

1. La formation de la β-galactosidase chez *E. coli* est induite exclusivement par des substances possédant un radical galactosidique intact. Diverses inversions ou substitutions en une ou plusieurs positions ainsi que la suppression du carbone 6 se traduisent par la disparition de la propriété inductrice.

2. Les substances douées de la propriété inductrice ne sont pas nécessairement des substrats de l'enzyme. Ainsi certains α-galactosides (mélibiose) sont inducteurs encore qu'ils ne soient pas hydrolysés par la β-galactosidase.

3. L'inductivité est, d'une manière générale, indépendante de l'affinité pour l'enzyme. Certains corps (phényl-β-thiogalactoside) doués d'une haute affinité pour la β-galactosidase *in vitro* comme *in vivo* sont dépourvus de la propriété inductrice.

4. La formation de la β-galactosidase est inhibée par le phényl-β-D-thiogalactoside, mais cette inhibition est non compétitive alors qu'elle devrait être compétitive si elle était due à l'inhibition de l'enzyme.

5. Ces observations sont incompatibles avec toute hypothèse qui suppose que l'induction est liée soit à l'activité de l'enzyme, soit à la formation d'un complexe spécifique entre l'enzyme et l'inducteur.

SUMMARY

1. The formation of β-galactosidase by *E. coli* is induced exclusively by substances possessing an intact galactosidic radical. Various inversions or substitutions at one or more positions as well as the suppression of the carbon 6 result in the disappearance of the inductive property.

2. The substances which have the inductive property are not necessarily substrates of the enzyme. Thus certain α-galactosides (melibiose) are inductors yet they are not hydrolysed by β-galactosidase.

3. The inductivity is, in general, independent of the affinity for the enzyme. Certain substances (phenyl-β-thiogalactoside) which have a high affinity for β-galactosidase *in vitro* as *in vivo* are deprived of the inductive property.

4. The formation of β-galactosidase is inhibited by phenyl-β-D-thiogalactoside, but the inhibition is not competitive, while it should be if it was due to the inhibition of the enzyme.

5. These observations are incompatible with all hypotheses which imply that the induction is connected, either with the activity of the enzyme, or with the formation of a specific complex between the enzyme and the inductor.