THE MECHANISM OF THE SYNTHESIS OF ENZYMES

II. Further Observations with Particular Reference to the Linear Nature of the Time Course of Enzyme Formation

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In a recent publication from this laboratory Koppel and his collaborators (14) described an experimental system, involving the enzyme β-galactosidase in *Escherichia coli* B, for the study of the biosynthesis of enzymes. The theoretical and practical advantages of the system used were discussed. In the present report a modification of technique is described which very greatly increases the usefulness of the methods previously employed. As a result it has been possible to demonstrate that, when factors affecting the rate of synthesis are made non-limiting, the time course of enzyme formation is linear. The implications of this finding in terms of mechanism are discussed and a new theory of enzyme formation is proposed.

Monod (21) and Koppel *et al.* (14) have reported that formation of the enzyme β-galactosidase can be induced in *E. coli* by the α-galactoside, melibiose. It was further reported by Koppel *et al.* (14) that β-methyl galactoside is able to induce formation of the enzyme α-galactosidase. This latter observation was of necessity based on measurements of oxygen uptake with whole cells since no specific method of measuring α-enzyme activity was available. In order to be in a position to study this fundamentally important question of inductor specificity, a method of synthesis of ortho-nitrophenol-α-D-galactoside (α-niphegal) corresponding to that of β-niphegal described by Seidman and Link (25) was developed. Using this specific substrate the results of the respiration studies, mentioned above, have been confirmed.

Methods

The methods used in (*a*) maintenance of *E. coli* B and *E. coli* 3400, (*b*) preparation of "standard cells" for experiment, and (*c*) determination of β-galactosidase activity were the same as those described by Koppel *et al.* (14). The modification in technique referred to earlier was a modification in the method used for enzyme induction.

Pretreatment Method of Enzyme Induction.—A 40.0 ml. volume of a standard cell suspension was mixed at room temperature in a 250 ml. centrifuge bottle with 32.0 ml. of an aqueous solution of lactose, yeast extract (Difco), sodium succinate, and chloromycetin such that the final concentrations were 0.25 M, 0.5 per cent, 0.01 M, and

16 μg./ml. respectively. After exactly 2 minutes, the suspension was centrifuged at 2400 R.P.M. for 20 minutes. The supernatant was decanted, the walls of the bottle rinsed with 10 to 15 ml. of distilled water (care being taken not to disturb the pellet of cells), and wiped free of excess water with a pad of filter paper. The cells, "pretreated" in this way, were resuspended in 40.0 ml. of M/20 potassium phosphate buffer (pH 7.05) containing yeast extract (0.02 per cent) and sodium succinate (0.01 M). The resuspended cells were immediately transferred to an L tube and shaken (50 strokes/minute) in a water bath maintained at 37°C. At intervals 5.0 ml. samples were removed and enzyme formation was stopped by mixing with 1.0 ml. of chloromycetin solution (to give a final concentration of 16 μg./ml.) in 15 ml. centrifuge tubes. These samples were centrifuged at 4300 R.P.M. for 10 minutes and, after decanting the supernatant and wiping the walls of the tubes with filter paper, the cells were resuspended in potassium phosphate buffer containing chloromycetin (16 μg./ml.). The β-galactosidase activities of the samples[1] were determined as described previously. Specific conditions are noted in the relevant experiments.

Preparation of Ortho-Nitrophenol-α-D-Galactoside (α-Niphegal).—Pentaacetyl-β-D-galactose (M.P. 142°C.: $[\alpha]_D^{20}$ +28°; concentration, 4.0, chloroform) was prepared according to the method described by Erwing and Koenigs (5). This was converted to pentaacetyl-α-D-galactose (M.P. 95.5°C.: $[\alpha]_D^{20}$ +106.1°; concentration 4.084, chloroform) by treatment with zinc chloride as described by Hudson and Parker (12).

To a melt, consisting of 32 gm. of ortho-nitrophenol and 5.0 gm. of freshly fused and ground zinc chloride, 20 gm. of pentaacetyl-α-D-galactose was added. The mixture was heated at 125–130°C. for 20 minutes with constant stirring. The resulting dark red solution was cooled and extracted with 150 ml. of benzene and 200 ml. of water. The benzene extract was washed twice with 200 ml. of water, three times with 200 ml. of 2 N NaOH, and a further three times with 200 ml. of water. It was then dried by shaking with anhydrous sodium sulfate for 5 to 10 minutes. After filtration, the pale straw-colored solution was reduced to a syrup *in vacuo*, the temperature being kept below 35°C. The last traces of benzene were removed by a strong current of warm dry air. The heavy syrup was dissolved in 25 ml. of absolute ethanol by warming on the water bath. The product crystallized out immediately on cooling. The mixture was stored at 4°C. and the crystals were separated on the following day. These were washed with a small volume of absolute ethanol and purified by repeated crystallization from this solvent. The tetraacetyl ortho-nitrophenol-α-D-galactoside had a M.P. 175–176.5°C. and $[\alpha]_D^{22}$ +178.5° (concentration, 1.0, chloroform).

The above tetraacetate was converted to the free galactoside as follows. 1 gm. of the tetraacetyl galactoside was suspended in 50 ml. of absolute methanol and cooled to 0°C. To this suspension was added 1.0 ml. 0.4 N barium methylate. The reaction mixture was stored at 4°C. for 24 hours, during which time it was shaken frequently and the tetraacetate gradually went into solution. The mixture was tested for excess barium methylate and, if the test was negative, a further 1.0 ml. barium methylate was added and the mixture refrigerated for another 24 hours. The barium was removed

[1] The β-galactosidase activities of samples so prepared were found to be completely stable at 2°C. for at least 24 hours.

by passing the solution through a cation exchange column: permutit, 60 to 80 mesh, was washed thoroughly with water, charged by washing with 2 per cent hydrochloric acid; excess acid was removed by washing with water and the water removed by thorough washing with absolute methanol. The methanol was removed from the eluate *in vacuo* at room temperature and the residue dissolved in the minimum amount of hot absolute ethanol. Crystalline ortho-nitrophenol-α-D-galactoside was obtained in good yield after storing the solution at 2°C. overnight. It was purified by repeated recrystallization from absolute ethanol and had a M.P. 145–147°C. and $[\alpha]_D^{25}$ +224.4° (concentration, 0.8, water). The specific rotation of the free galactoside appears to have a large temperature coefficient ($[\alpha]_D^{26.5}$ +221.8°, concentration, 0.8, water) as noted by Link for the β-isomer (17).[2]

RESULTS

The Relation between Enzyme-Forming Capacity and the Physiological State of the Cell.—It has been reported that bacterial cells have the maximum capacity to form an enzyme in response to an induction stimulus when in (*a*) the lag or early logarithmic phase of their growth cycle (10) or (*b*) at the end of the logarithmic or in the early stationary phase of growth (8, 14, 23). Because of this disparity and in order to be certain that the *E. coli* B cells used in this study had maximum enzyme-forming capacity (E.F.C.), the relationship between E.F.C. and the changes, during the growth cycle, in the physiological state of the cells used for induction experiments was reinvestigated.

A series of 200 ml. Erlenmeyer flasks, containing 100 ml. of tryptose medium, were inoculated with 1.0 ml. of a culture of *E. coli* B (specific conditions are noted for each group of experiments) and shaken (50 strokes/minute) in a water bath maintained at 37°C. Growth was followed by measurements of bacterial density in an Evelyn photoelectric colorimeter using a 660 mμ filter. At intervals one of the series of flasks was removed from the bath, the bacterial density and pH of the medium were determined, and the cells were harvested by centrifugation at 2400 R.P.M. for 20 minutes. These cells were washed twice with M/20 potassium phosphate buffer (pH 7.05) and finally made up in this buffer to a photometric density of 0.699 (20 per cent transmission). An aliquot of this cell suspension was subjected to a standard pretreatment induction (see Methods). The level of β-galactosidase attained was taken as a measure of the E.F.C. of the cells. It should be emphasized that E.F.C. was measured under conditions in which no appreciable cell division occurred and in which the same enzyme induction stimulus was applied in all cases.

The results of such an experiment are shown in Fig. 1. In this case, the inocula for the series of flasks were taken from a 16 hour culture. It is apparent that there is a small increase in E.F.C. during the lag phase, followed by a sharp

[2] After the above synthesis had been worked out, the description of a somewhat similar method was received from Dr. D. M. Bonner. However, this latter method was found to be not as satisfactory as the one described from the point of view of ease of manipulation, yield, and quality of product.

drop as cell division begins, and finally a rapid rise in E.F.C., foreshadowing the cessation of cell division, until a peak is reached during the stationary phase. From this experiment, it seemed reasonable to predict that, if the inoculating

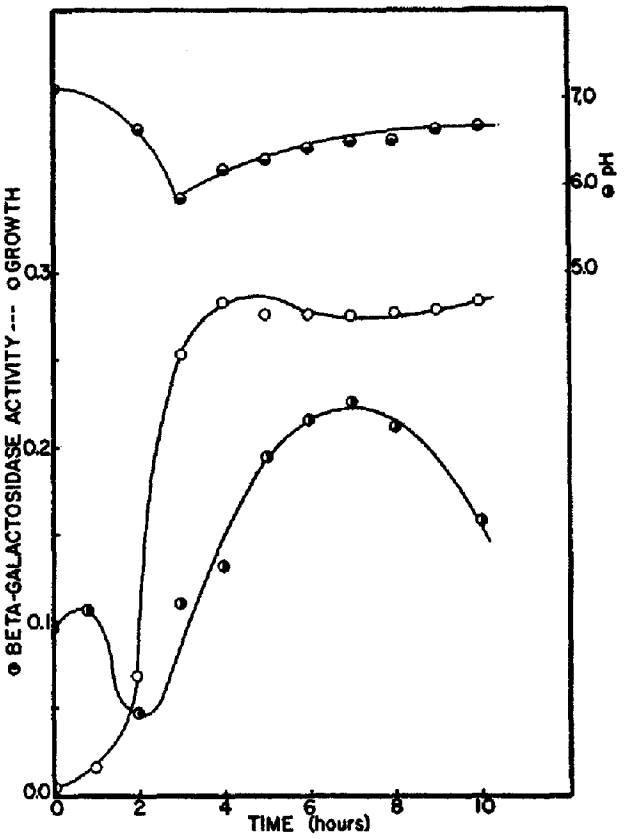

FIG. 1. The relationship between enzyme-forming capacity and the physiological state of the cell. The inoculum for the experimental culture of *E. coli* B was obtained from a culture in the stationary phase of growth (16 hours, 37°C., pH 7.05). Cells harvested at the times indicated were subjected to standard pretreatment induction and the E.F.C. measured by the level of β-galactosidase attained after a resuspension period of 10 minutes (see Methods).

cells were taken from a 2½ hour culture (*i.e.*, with low initial E.F.C.), there should be a pronounced peak during the lag phase. On the other hand, if the inocula were obtained from a 7 hour culture (*i.e.*, with high initial E.F.C.), one would not expect any lag phase peak but rather a steady decline to the low E.F.C. of the midlogarithmic phase. These predictions are borne out by the data shown in Figs. 2 and 3.

Thus it would appear that, when active cell division is taking place, the organism has a low capacity to respond to the induction stimulus, whereas cells in the lag phase and more particularly in the stationary phase of the growth

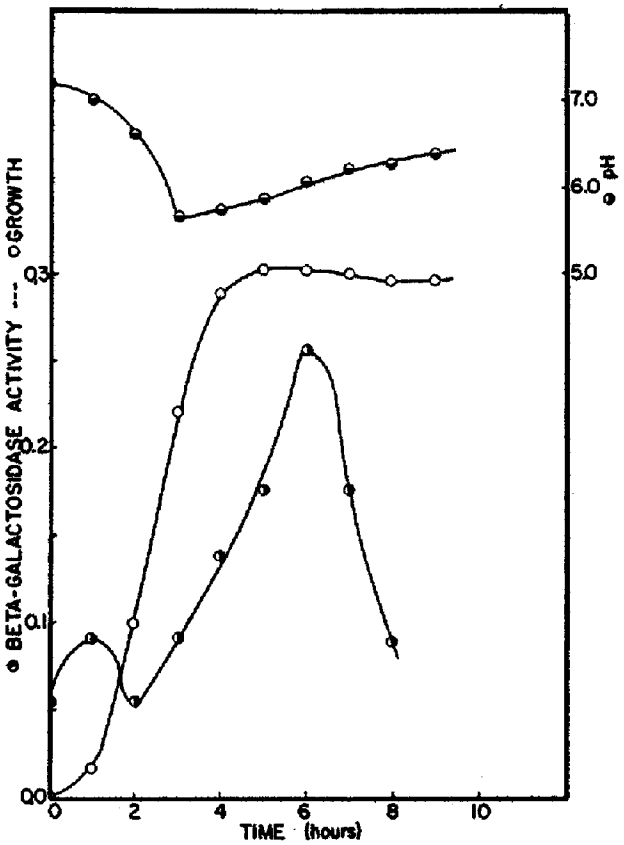

FIG. 2. The relationship between enzyme-forming capacity and the physiological state of the cell. The inoculum for the experimental culture of *E. coli* B was obtained from a culture in the logarithmic phase of growth (16/2.5 hours, 37°C., pH 7.05). Cells harvested at the times indicated were subjected to a standard pretreatment induction and the E.F.C. measured by the level of β-galactosidase attained after a resuspension period of 10 minutes (see Methods).

cycle have a much greater ability to respond to the stimulus for induced enzyme formation. It is therefore very important in enzyme induction studies to control closely the initial physiological state of the organisms.

Since the changes in growth rate are closely followed not only by the changes in E.F.C. but also by changes in the pH of the growth medium, it was necessary to determine whether the variations in E.F.C. were a consequence of, or in-

dependent of the drop and the rise in pH. The above experiments were repeated using tryptose medium buffered with M/10 potassium phosphate buffer (pH 7.05) in which case the alteration in pH did not exceed 0.1 pH unit, as against

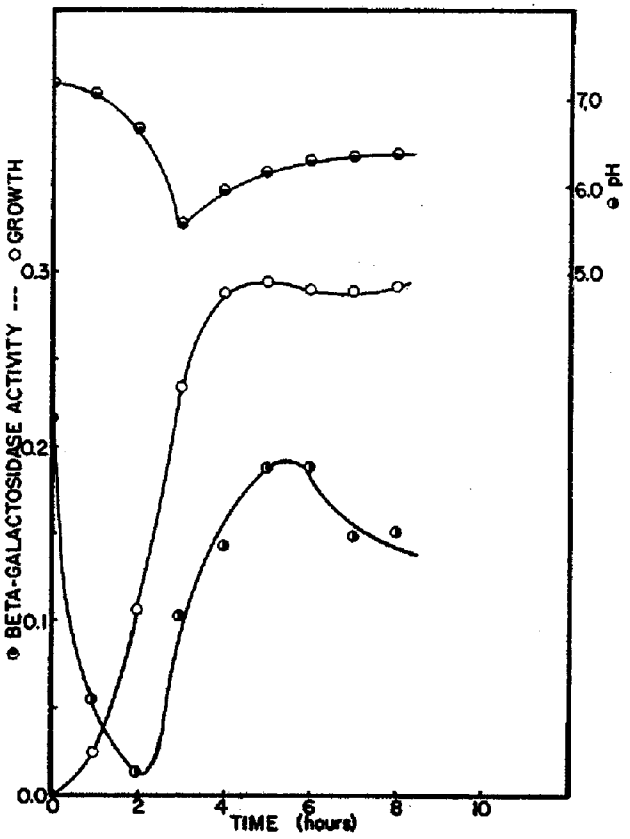

FIG. 3. The relationship between enzyme-forming capacity and the physiological state of the cell. The inoculum for the experimental culture of *E. coli* B was obtained from a culture in the early stationary phase of growth (16/7 hours, 37°C., pH 7.05). Cells harvested at the times indicated were subjected to a standard pretreatment induction and the E.F.C. measured by the level of β-galactosidase attained after a resuspension period of 10 minutes (see Methods).

a change of 1 unit or more with unbuffered media. It was observed that the E.F.C. curve maintained exactly the same relationship to the growth curve as before. It was therefore concluded that the variations in E.F.C. were not the result of changes in pH during growth.

From the data given it is evident that maximal E.F.C. was reached, under

these conditions, about 6 hours after inoculation. When the above experiments, using both buffered and unbuffered media, were carried out with incubation of the cultures at 25°C. instead of 37°C., maximal E.F.C. was reached approximately 14 hours after inoculation. The relationship between the curves for E.F.C. and for increase in bacterial density was maintained exactly as shown above. It is therefore obvious that, as the physiological state of the organism changes during the growth cycle, there is an accompanying variation in the ability of the cells to respond to a constant induction stimulus. The alterations in E.F.C. are so abrupt and of such magnitude that in studies of enzyme formation the technique for obtaining cells to be used experimentally must be very carefully standardized.

For convenience the following has been adopted as routine procedure. Tryptose medium (pH 7.05) is inoculated with the contents of a vial of lyophilized *E. coli* and incubated with shaking (50 strokes/minute) in a water bath maintained at 25°C. for 24 hours. A second volume of medium is inoculated with 1.0 ml. of the first culture and incubated 7 hours. A third culture is prepared in the same way from the second and incubated for 14 hours. Such a culture is designated by the following code: 24/7/14; 25°C.; pH 7.05.

The Nature of the Time Course of β-Galactosidase Formation in E. coli B.— On the basis of the observation that the action of chloromycetin in inhibiting β-galactosidase formation was reversible, a new and more flexible technique for the study of enzyme induction has been developed (pretreatment induction —see Methods). This technique makes it possible to separate the induction process into at least two distinct phases: (*a*) a presynthetic or assimilatory phase and (*b*) a synthetic phase during which active enzyme is formed. In phase (*a*) the cells assimilate inductor nitrogen, carbon, and energy in the presence of chloromycetin which completely inhibits enzyme formation; in phase (*b*) the cells, after centrifugation and resuspension in a chloromycetin-free medium, begin to form active enzyme. The application of this pretreatment method of induction to the problem of the nature of the time course of lactose-induced β-galactosidase formation in *E. coli* B has revealed that a variety of factors greatly influence the observed rate of synthesis. Whenever one or another of these factors is limiting, the over-all time course has the appearance expected of an autocatalytic process. When, however, these limitations do not exist, the rate of enzyme formation follows a strictly linear path up to the point at which some factor again becomes limiting. The pertinent data are shown in Figs. 4 to 9, the specific conditions being given in each case.

It is evident that in the presynthetic phase (*a*) the concentration of inductor, (*b*) the nature of the nitrogen source, and (*c*) the duration of the pretreatment are important factors in determining the rate of β-galactosidase formation observed in the subsequent synthetic phase, and that during the latter phase

oxygen tension and temperature influence the rate of formation. When limitations in respect to these factors are removed and after a short lag[3] of approxi-

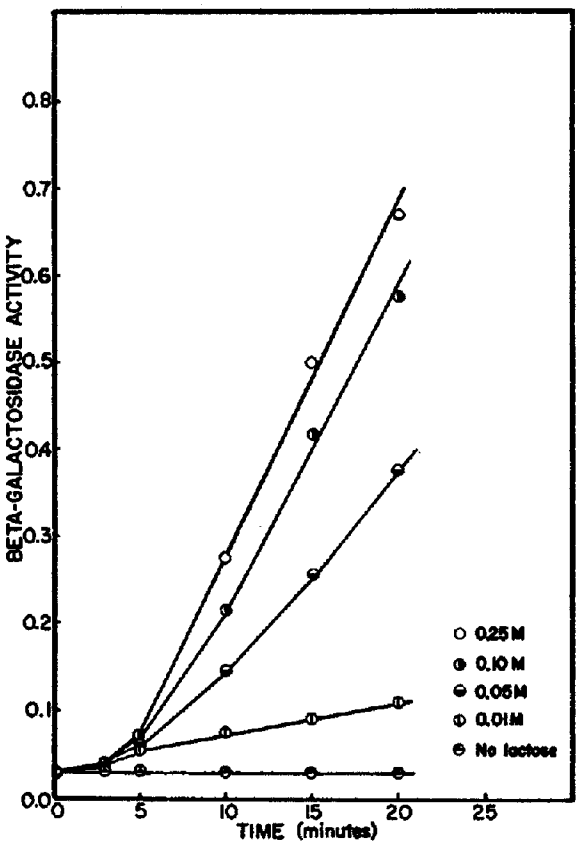

FIG. 4. The effect of inductor (lactose) concentration on the rate of β-galactosidase formation. Aliquots of a standard cell suspension, prepared from a culture of *E. coli* B (24/7/14 hours, 25°C., pH 8.2), were subjected to a pretreatment induction in the presence of the concentrations of lactose indicated. β-Galactosidase formation was followed in the manner previously described (see Methods).

mately 2 minutes during which no enzyme appears, β-galactosidase is formed at a linear rate. Thus the sigmoid type of progress curve for enzyme formation

[3] The explanation for this lag is not clear at the moment. It is a constant time over a range of chloromycetin concentrations from 16 to 200 μg./ml. and therefore does not appear to be related to the time required for the chloromycetin to diffuse out of the centrifuged and resuspended cells. The lag may represent a "metabolic lag" before the intracellular machinery is ready to form enzyme. This point is being further investigated.

most frequently reported (20, 27) would appear to be due to a limitation of the rate of synthesis imposed by some factor in the environment of the cells. The fact that, when such limitations are removed, the rate of formation follows a

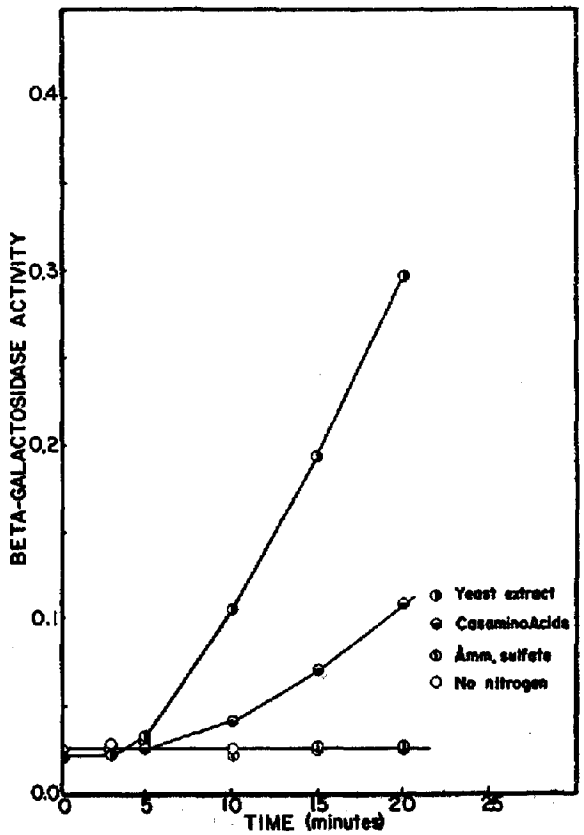

FIG. 5. The effect of various nitrogen sources on the rate of β-galactosidase formation. Aliquots of a standard cell suspension, prepared from a culture of *E. coli* B (24/7/14 hours, 25°C., pH 8.2), were subjected to a pretreatment induction in the presence of various nitrogen sources present in concentrations equivalent in terms of nitrogen to 0.5 per cent yeast extract (Difco). β-Galactosidase formation was followed in the manner previously described, after resuspension in M/20 potassium phosphate buffer, pH 7.05, not containing any nitrogen (see Methods).

strictly linear time course demonstrates conclusively that the process is not *inherently* autocatalytic.

Preliminary Experiments Using α-Niphegal.—Since no information was available in the literature regarding the physical characteristics of ortho-nitrophenol-α-D-galactoside (α-niphegal), it was necessary to establish that the

α-isomer had in fact been synthesized. On the basis of measurements of oxygen uptake it was known that *E. coli* B had the ability to form both α- and β-galactosidases (using melibiose and lactose as substrates). On the other hand,

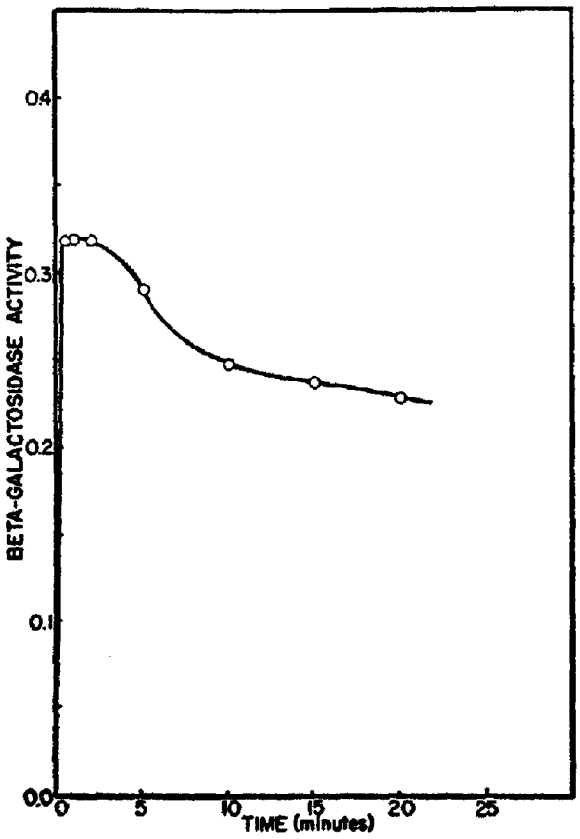

FIG. 6. The effect of the duration of pretreatment on the rate of β-galactosidase formation. Aliquots of a standard cell suspension, prepared from a culture of *E. coli* B (24/7/14 hours, 25°C., pH 8.2), were subjected to a pretreatment induction, the period before centrifugation being varied as indicated. β-Galactosidase formation was determined in the usual manner (see Methods) after 10 minutes' resuspension.

E. coli 3400[4] had the ability to form the β-enzyme but was constitutionally unable to form an α-galactosidase (21). When standard cell suspensions of these two strains were incubated in the presence of ammonium sulfate and melibiose for 60 minutes, both possessed β-galactosidase activity (with β-niphegal) but only *E. coli* B cells showed activity with the presumptive α-

[4] Made available through the kindness of Professor J. Monod.

niphegal. It was therefore concluded that the galactose derivative synthesized was, in fact, ortho-nitrophenol-α-D-galactoside and that it was not contaminated with β-niphegal.

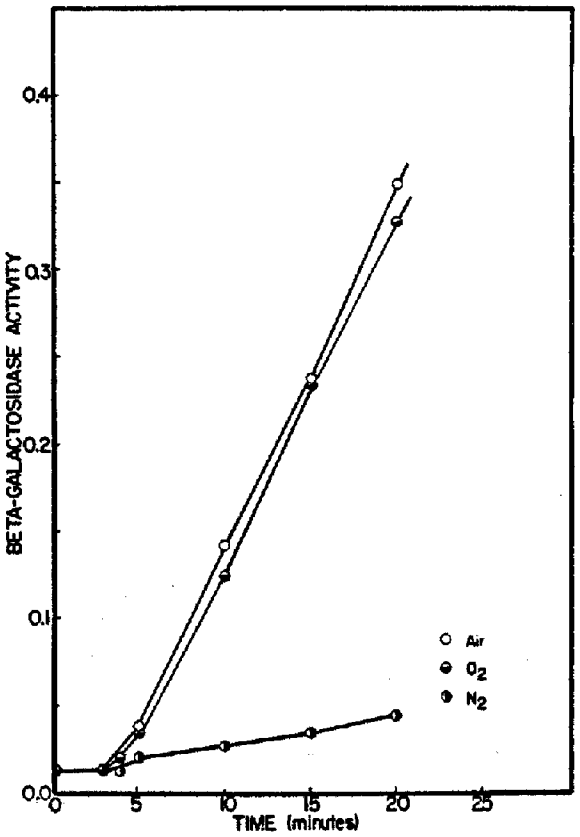

FIG. 7. The effect of oxygen tension on the rate of β-galactosidase formation. Aliquots of a standard cell suspension prepared from a culture of *E. coli* B (24/7/14 hours, 25°C., pH 7.4), were subjected to a pretreatment induction. The pretreated cells were resuspended in L tubes under an atmosphere of air, oxygen, or nitrogen as indicated. β-Galactosidase formation was followed in the manner previously described (see Methods).

Using α-niphegal as substrate, the specific determination of α-galactosidase activity could be carried out in precisely the same manner as described by Lederberg (15) for the β-enzyme using β-niphegal. Experiments with whole cell preparations of *E. coli* B have demonstrated that formation of α-galactosidase may be induced by not only the α-galactosides, melibiose and α-methyl

galactoside, but also by the β-galactosides, lactose and β-methyl galactoside. Further investigation of inductor specificity by this means is in progress.

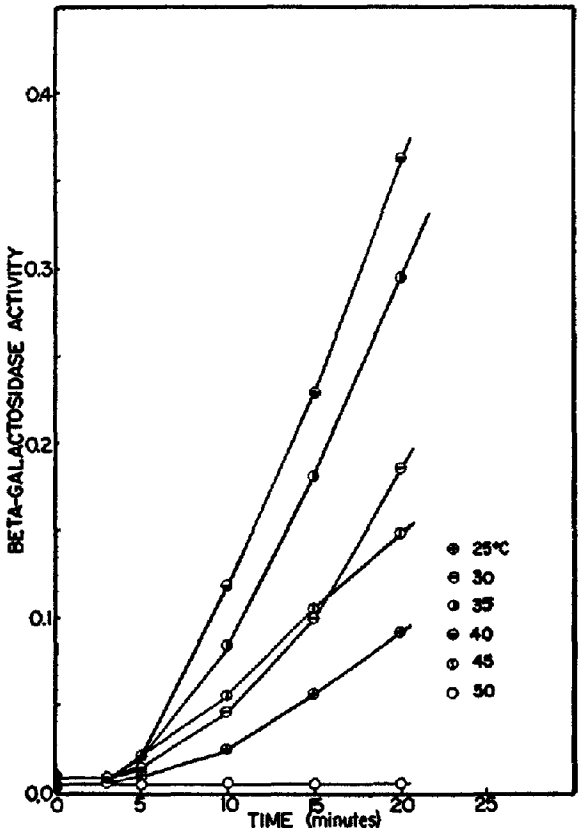

FIG. 8. The effect of temperature on the rate of β-galactosidase formation. Aliquots of a standard cell suspension, prepared from a culture of *E. coli* B (24/7/14 hours, 25°C., pH 7.4), were subjected to a pretreatment induction. The pretreated cells were resuspended in L tubes maintained at the temperatures indicated. β-Galactosidase formation was followed in the manner previously described (see Methods).

Experiments with intact cells can, however, give only a qualitative answer even for positive inductors. For quantitative estimation of α-galactosidase it is necessary to obtain "cell-free" preparations of the organism in order to obviate consideration of cell permeability to α-niphegal as a factor. Attempts were made to achieve this by treatment of melibiose-induced cells (which as intact cells exhibited α-galactosidase activity) with T_{2r+} phage or toluene or by grinding the cells with alumina. Although such procedures had been found to produce highly active β-galactosidase preparations (14), they gave,

with melibiose-induced cells, preparations completely devoid of α-enzyme activity. An intensive search for a lytic or "sieving" agent which would not

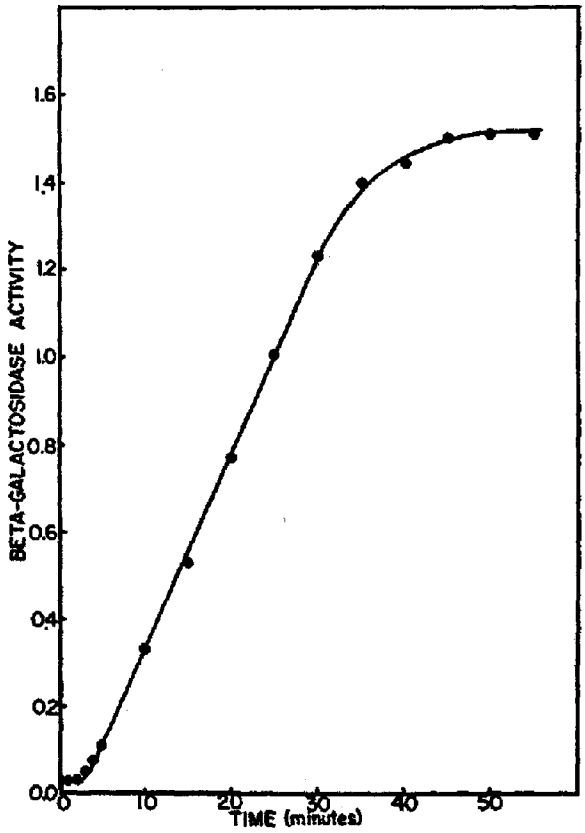

FIG. 9. The linear time course of β-galactosidase formation in the absence of limiting factors. A standard cell suspension, prepared from a culture of *E. coli* B (24/7/14 hours, 25°C., pH 8.2), was subjected to a pretreatment induction. Optimum conditions in regard to the concentration of inductor, the nature of the nitrogen source, duration of pretreatment, oxygen tension, and temperature obtained. In addition, sodium chloride (0.1 M final concentration) was added to the resuspension mixture to minimize the effect of osmotic change between pretreatment mixture and resuspension mixture (KCl or LiCl were found to do as well). β-Galactosidase formation was followed in the manner previously described (see Methods).

at the same time result in destruction of α-galactosidase activity has been made without success. Any agent, which can be shown to have an effect on the cell boundary (as tested by β-galactosidase activity), results in loss of α-galactosidase activity. This problem is being further investigated.

DISCUSSION

On the basis of a study of the induced formation of hydrogenlyase in *E. coli* and galactozymase in yeast, Yudkin (29) proposed the Mass action theory of enzyme formation. According to this hypothesis, the enzyme is formed from a postulated precursor with which it is in equilibrium. It was assumed that combination of the enzyme with its substrate (or product, inhibitor, *etc.*) disturbed this equilibrium so that more enzyme was formed from the precursor. In a recent series of papers, Mandelstam and Yudkin (18) have elaborated the original statement of the Mass action theory but the essential reaction involving the inductor remains unchanged. Spiegelman (26) pointed out that from the theory, as originally stated, one could conclude that the rate of enzyme formation should be greatest at the onset and diminish continuously as the precursor concentration decreased. As a result of a series of studies on the induced formation of galactozymase and maltozymase in yeast, he came to the conclusion that enzyme formation followed a sigmoid, rather than a hyperbolic time course and questioned the validity of Yudkin's theory. More recently Monod (21) has reported that (*a*) a substance, with no demonstrable affinity for the enzyme β-galactosidase, may nevertheless induce the formation of this enzyme in *E. coli* and (*b*) a substance with a demonstrably high affinity for β-galactosidase may have no power whatever to induce its formation. Monod concludes that affinity for the enzyme formed is unrelated to the ability of a substance to induce its formation and that Yudkin's theory cannot be valid.

Because the experimental data for enzyme formation could be fitted accurately to a curve derived on the basis that the primary rate-limiting process in induced enzyme formation is an autocatalytic one, Spiegelman (27) was led to formulate the Plasmagene theory of enzyme formation which implies that the rate of enzyme formation is a function of the amount of enzyme already present. According to this theory a genetically determined cytoplasmic factor, the plasmagene, combines with the inductor and the enzyme already formed to produce a complex (Pl.-Ind.-Enz.) which then reduplicates itself. Thus the rate of formation of enzyme would increase *autocatalytically*. In the discussion of his theory Spiegelman emphasized the very useful and important concept that while the *potentiality* of enzyme formation is genetically determined, the *actuality* in the cell at any given time is flexibly determined by the physicochemical environment of the cell.

More recently, however, Pollock (24) and Ephrussi and Slonimsky (4) have reported data which indicate a linear time course for enzyme formation. Since an unequivocal answer to the question regarding the nature of the kinetics of enzyme formation was obviously fundamental to any valid theory of the mechanism of this phenomenon, the investigations in this laboratory were directed to this problem. The data reported in this paper confirm the conclusion

of Pollock and of Ephrussi that the rate of formation follows a linear course and, further, indicate an explanation for Spiegelman's observations of an apparently autocatalytic time course (*i.e.* limiting factors).

Since neither the Mass action theory nor the Plasmagene theory appears to account for the known facts of enzyme induction, the following theory is suggested as a working hypothesis; it is consistent with what is known of the mechanism of enzyme formation and has the virtue of suggesting further experiments.

There is a small but increasing body of experimental evidence which indicates clearly that induced enzyme formation involves the synthesis of specific protein (22, 28). Thus one should regard induced enzyme formation as simply a part of the general protein metabolism of the cell, a special part only in that the specific activity of the enzyme formed offers a means of readily determining its presence and amount. The problem therefore resolves itself into an explanation of the production by the cell of a specific polypeptide, with a certain amino acid sequence and under definite environmental conditions among which are the presence of an inductor, of sources of nitrogen, and of energy.

The main difficulties in explaining the biosynthesis of a specific amino acid sequence in a polypeptide have been (*a*) that one has assumed that the nature of each of the components of a peptide bond is specifically determined at each stage in a long stepwise synthesis, and (*b*) that no enzymes of the requisite absolute specificity are known. According to this view the specific amino acid sequence is *predetermined.*

In recent years the transpeptidase type of reaction has been demonstrated by Fruton (7) and by Hanes and his collaborators (9), and it has been suggested that this type of reaction is involved in the biosynthesis of proteins. In a study of transpeptidation reactions involving the transfer of the carboxyl component of a peptide bond, Hanes has demonstrated (*a*) that the enzymes exhibit a narrow specificity for the carboxyl component transferred but a wider specificity as regards the—NH_2 (or acceptor) component, and (*b*) that a number of the transpeptidation reactions studied are reversible. A biosynthesis of this type would not specifically determine the components of a peptide bond at any stage, but rather one would expect the simultaneous synthesis of several different peptide partners at each stage in the synthesis and of numerous final polypeptide products (Figure 10).

Therefore one would picture this process as resulting in the synthesis of a large number of polypeptides with very different amino acid sequences. Some of the polypeptides formed would be pictured as corresponding to components of various cellular proteins, *i.e.* structural proteins, hormones, or enzymes, and others, as will be evident, might never be incorporated into protein at all. The pool of interconvertible polypeptides here postulated might constitute only a small fraction of the total nitrogenous compounds of the cell, but its significance

would lie in its providing a wide assortment of chain fragments presenting different amino acid sequences for subsequent incorporation into protein. A mechanism which would result in the synthesis in quantity of a particular protein would be one which drew off from this pool the fragments required for this protein and so, by *terminal selection*, would tend to "channel" the peptide-synthesizing capacity of the cell toward the formation of this particular product. If this type of mechanism were a general one, the simultaneous synthesis of a number of proteins would constitute a competitive drain on the peptide-synthesizing mechanism as a whole.

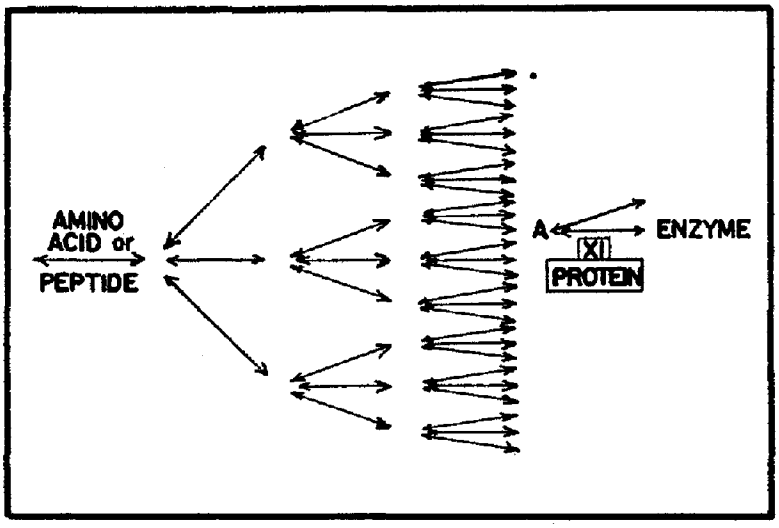

FIG. 10. The "terminal selection" of specific polypeptides from the "pool" of protein biosynthetic products.

The following theory of the mechanism of enzyme formation is based on the above concept of protein synthesis which developed in the course of discussions with Professor Hanes. It is postulated that in a cell there is present, for each enzyme, a specific substance (X) which is genetically determined. It is further postulated that, in the formation of an enzyme, substance X combines with the inductor (I) to form a complex (XI), which is the cofactor of the terminal enzyme in the chain constituting the protein biosynthetic mechanism. (According to this view, both X and I are essential parts of this cofactor.) Thus the amount of X_AI_A formed would determine the rate of synthesis of enzyme A and therefore, under the normal conditions of dynamic equilibrium in the cell, the *amount* of enzyme A synthesized.

This theory is consistent with the following experimental observations:

1. The "potentiality" of enzyme production by a cell is genetically de-

termined, while the "actuality," within these limits, is determined by environmental conditions (2, 19, 26, and others). The so called "constitutive" enzymes (13) would be those involved in normal metabolic reactions for which, through metabolism, the specific inductor is always present. The so called "adaptive" enzymes would be those involved in reactions on the "fringes" of metabolism, for which there is little, if any, inductor normally present.

2. In the production of "adaptive" enzymes, exogenous sources of nitrogen, carbon, and energy stimulate formation. This is completely understandable since protein synthesis is involved, and would be a possible explanation of some of the non-specific inductions which have been reported (3, 11, 16). In the absence of externally supplied nitrogen, because of the reversibility of the biosynthetic mechanism, one would expect that the substance of one enzyme could be used in the synthesis of another, whose formation is stimulated, as has been observed by Spiegelman (27).

3. Induced enzyme formation follows a linear time course (4, 24, the present study). Since there will be a number of "outlets" or "sinks" for the biosynthetic mechanism, these will "fill up" or come to equilibrium, depending on the relative affinities of the various *XI* enzymes for the products of transpeptidation, until a point is reached at which the material begins to flow into the enzyme *A* channel. From this point on it would be expected that production of *A* would proceed linearly with time.

4. The addition to a bacterial cell of a "foreign" DNA fraction may be followed by the appearance of increased amounts of one or more enzymes, as has been observed in "bacterial transformations" (1). This could be thought of in terms of the addition to the cytoplasm of a new "*X*," with the subsequent formation of a new *XI* complex and the formation of a completely new enzyme, not previously within the "actuality" range of the cell. That such a transformation may be a permanent and heritable characteristic (affecting the genotype) is not germane to the present discussion.

5. Evidence is presented by Koppel *et al.* (14) which indicates that structural analogues of the inductor not only inhibit enzyme formation but may do so competitively. The inhibitory *analogue* may be pictured as competing with the *inductor* for *X* and so reducing the amount of *XI* formed and hence the rate of enzyme formation.

6. The strict structural specificity required for inductive capacity (14, 21) is not only consistent with, but a fundamental requirement of the theory. The inductor (*I*) must have the necessary structural features to combine with *X* on the one hand and also to carry out its function as part of the cofactor, *XI*, on the other. In fact this theory offers a possible explanation for a very puzzling observation, namely that raffinose, which possesses a galactosidyl radical, is not only not an inductor of β-galactosidase in its own right but is actually an inhibitor of lactose-induced β-galactosidase formation. One would suggest

that raffinose possesses the necessary structural affinity for *X* but, possibly due to steric considerations, is not able to function in the cofactor.

There is no suggestion made that this theory is, in any way, a final one. However, it is consistent with what is known of enzyme formation by living cells and possesses the essential virtue of any useful hypothesis, namely of suggesting further experiments which will, in turn, increase the fund of pertinent knowledge regarding the mechanism of enzyme formation and open the way for a more satisfactory theory. The following predictions are suggested as necessary consequences of the theory as it stands:

(*a*) The inhibition by raffinose of lactose-induced β-galactosidase formation should be found to be of the competitive type.

(*b*) If, as postulated, the enzyme induced is the product of the action of an *XI* enzyme, one should be able to observe the inhibitory effect (by Mass action) of the product (*i.e.* induced enzyme) at high concentrations. Thus, if successive pretreatment inductions were carried out, eventually at some level of induced enzyme and in spite of the supply of adequate raw materials, one should find that enzyme already formed inhibits further enzyme formation.

(*c*) If, as the experiments of Pollock (24) with *B. cereus* and penicillinase induction with minute amounts of penicillin would seem to indicate, the *XI* complex is quite stable, one should find, on successive inductions with suboptimal amounts of inductor, a progressive increase in the amount of *XI* and hence successively higher rates of formation of enzyme, in spite of a constant intensity of stimulus. Such an observation has been made by Fishman (6) in the case of animal β-glucuronidase.

(*d*) On the basis of the present theory, one would predict that on successive induction with optimal concentrations of inductor, *etc.*, the rate of enzyme formation would be constant, up to the point at which formed enzyme begins to inhibit further formation.

SUMMARY

1. The pretreatment induction method of studying the formation of β-galactosidase in *E. coli* B has been described.

2. It has been found that *E. coli* B cells have their maximum capacity to form β-galactosidase, in response to a constant induction stimulus, when they are in the stationary phase of the growth cycle.

3. The concentration of inductor, the nature of the nitrogen source, the duration of the assimilatory phase, oxygen tension, and temperature are factors which affect, and may limit, the rate of β-galactosidase formation.

4. When limitations imposed by these factors were removed, the time course of induced β-galactosidase formation was strictly linear from the onset.

5. The implications of this finding were discussed and a new theory of the mechanism of enzyme formation has been proposed.

6. A very satisfactory method of synthesis of ortho-nitrophenol-α-D-galacto-

side has been described. This substance is a suitable chromogenic substrate for the specific determination of α-galactosidase activity.

7. Preliminary experiments using this substrate have confirmed the results of respiration studies and shown that in *E. coli* B α-galactosidase formation may be induced by β- as well as by α-galactosides.

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