BBA 12232

CHANGES IN THE INDUCIBILITY OF GALACTOKINASE AND β-GALACTOSIDASE DURING INHIBITION OF GROWTH IN *ESCHERICHIA COLI*

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(Received January 16th, 1963)

SUMMARY

The induction of galactokinase (EC 2.7.1.6) is initially inhibited in *Escherichia coli* growing in complex media. The inhibition was shown to arise from the presence in broth and casein hydrolysate of repressors of galactokinase induction which are consumed during growth; in particular leucine, valine, histidine, and serine. These amino acids have in common an inhibitory effect on the growth of *E. coli*. It is suggested that they block anabolic processes and thus cause an accumulation of the intermediary pool. The enlarged intermediary pool then represses the formation of galactokinase through catabolite repression. This interpretation was supported by the following observations: (a) a similar amino acid repression is exerted on β-galactosidase (EC 3.2.1.23) induction; (b) other growth inhibitors which block the synthesis of macromolecules also preferentially inhibit galactokinase and β-galactosidase induction; and (c) iodoacetate, which inhibits growth by blocking metabolism of the carbon source, enhances induction.

It is concluded that under normal growth conditions the total rate of macromolecular synthesis can be a primary factor in regulating the formation of enzymes feeding the intermediary pool. The physiological role of catabolite repression may therefore be expanded to include not only the activities of compounds which affect the rate of carbon entry into the pool, but also the activities of compounds which modify the rate of utilization of the intermediary pool for synthetic purposes.

INTRODUCTION

It has long been known that the addition of glucose to bacterial cultures inhibits the formation of a variety of enzymes[1,2]. More recently, it has become apparent that all metabolizable carbon sources exert this inhibition to varying degrees, and that glucose is simply considerably more effective than most[3–6]. NEIDHARDT AND MAGASANIK[7,8] have proposed that it is the pool of intermediates in carbohydrate metabolism which represses the synthesis of enzymes whose function it is to feed compounds into the pool. In this view, the degree to which any carbon source can exert a generalized repression is a function of the rate at which it can contribute intermediates to the pool. The evidence for their interpretation has recently been summarized by NEID-

HARDT[9] and MAGASANIK[10] who has rechristened the phenomenon "catabolite repression".

In connection with studies of the genetics and physiological expression of transducing variants of the bacteriophage λ which carry the galactokinase gene[11], control experiments were recently carried out on the inducibility of this enzyme under several conditions of growth in normal cells of *Escherichia coli*. The effects which were observed are most easily interpreted by assuming that the synthesis of this and other enzymes feeding the intermediary pool is regulated, under conditions of normal growth in this organism, by the rate of utilization of this pool for macromolecular synthesis, as well as by the rate of entry of carbon into the pool.

These experiments are presented here in detail since they illustrate the surprising extent to which enzyme induction is sensitive to slight changes in physiological conditions; and because they provide experimental support for the idea that the size of the intermediary pool influences the rate of formation of enzymes feeding it.

MATERIALS AND METHODS

Bacteria

The strain of bacteria used throughout these experiments was a prototrophic strain of *E. coli* K12 (W-3110) which is sensitive to phage λ.

Culture media and compounds

Tryptone broth was used at 1% concentration (pH 7.0–7.2) with the addition of 0.5% NaCl. Salts–glycerol contained per liter: 1.4 g Na_2HPO_4, 0.6 g KH_2PO_4, 0.2 g NH_4Cl, 0.5 g NaCl, 0.01 g gelatin, 10^{-3} M $MgSO_4$, $3 \cdot 10^{-6}$ M $FeCl_3$ and 1.0 g glycerol. This medium is relatively dilute; however, it supports the same rate of growth as the more concentrated M-9 medium from which it is derived. It has been used in this laboratory because the lowered concentrations of phosphate and NH_4Cl show less interference in the assay of some enzymes. The casein hydrolysate preparation was a vitamin-free hydrolysate (Difco) treated with Norit until the absorbance at 254 mμ was less than 0.02 for a 1% solution.

D(+)Fucose was purchased from K & K Laboratories and has been reported by BUTTIN[12] and YARMOLINSKY AND WIESMEYER[13] to be a non-metabolizable inducer of galactokinase. Isopropylthiogalactoside was a gift of Dr. A. B. PARDEE. [^{14}C]-Galactose was obtained from the National Bureau of Standards and diluted with glucose-free galactose (Sigma Chemical Corporation) for use. All other materials were commercial products.

Enzyme assays

For galactokinase (ATP:D-galactose 1-phosphotransferase, EC 2.7.1.6) assays the cells were lysed and the lysate was then allowed to form [^{14}C]galactose 1-phosphate from [^{14}C]galactose and ATP. The reaction product was precipitated as the lead salt whose radioactivity was determined. An aliquot of cells containing $5–50 \cdot 10^7$ cells in 0.5 ml was added to 0.25 ml of a lysis mixture containing 0.0075 M EDTA, 0.3 mg/ml lysozyme and 0.10 M Tris–acetate (pH 8.0). Lysis was complete in 5 min at 37°. To

the lysate was then added 0.25 ml of substrate mixture containing 0.02 M $MgCl_2$, 0.019 M ATP, $4 \cdot 10^{-3}$ M [1-^{14}C]galactose at a specific activity of 0.02 μC/μmole (equal to 15 000 counts/min under standard counting conditions) and 0.4 M imidazole–acetate buffer (pH 7.20). The mixture was incubated for 60 min at 37° and the reaction stopped by immersing the tubes in a boiling-water bath for 1 min. After chilling the tubes 1.0 ml of 0.05 M $Pb(NO_3)_2$ was added and the resulting precipitate collected on glass fiber disks by filtration. They were washed with 0.01 M $Pb(NO_3)_2$, ethanol, and ether and assayed for radioactivity.

The addition of carrier galactose 1-phosphate was unnecessary under ordinary assay conditions. The limiting solubility of the lead salt of galactose 1-phosphate under these conditions is approx. 0.001 μM. The pH of the final precipitation mixture must be kept above 7.1 to insure the precipitation of galactose 1-phosphate, but as the pH is raised further an increasing precipitation of galactose occurs which produces high blanks. A final pH of 7.2 has been found most useful. The yield of product was directly proportional to both time and enzyme concentration. Enzyme activity is expressed as the number of counts/min of galactose fixed in 1 h under standard conditions.

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) was assayed in appropriate aliquots by the colorimetric procedure of LEDERBERG[14] after lysing the cells under the same conditions used for galactokinase. The substrate mixture was 0.50 ml of $6 \cdot 10^{-3}$ M *o*-nitrophenyl galactoside, 0.20 M sodium phosphate (pH 6.9) and 0.01 M $MgCl_2$. After incubation for 20 min at 30°, the reaction was stopped by immersing the tubes in a boiling-water bath for 1 min; 1.0 ml of 0.2 M Na_2CO_3 containing 0.01 M EDTA was added, and the absorbancy determined at 415 mμ. The addition of $MgCl_2$ was necessary to insure maximal activity; activity in the absence of added $MgCl_2$ was less than 10% of that in its presence. COHN[15] has reported the existence of a Mg^{2+} requirement following exposure of the enzyme to EDTA. Enzyme activity is expressed as mμmoles of *o*-nitrophenol liberated per min.

Other methods

Protein was determined by the method of LOWRY *et al.*[16] using bovine serum albumin as a standard. Turbidity is reported as the apparent absorbancy of a culture at 550 mμ in a Zeiss PMQII spectrophotometer using a 1-cm light path. Cells in log phase growing in minimal salts–glycerol have approx. $6.5 \cdot 10^8$ cells/ml and 0.24 mg protein/ml/unit absorbancy.

Culture samples were placed on ice in the presence of 25–40 μg/ml of chloramphenicol prior to enzyme assay. Both enzymes were stable under this condition for at least 24 h.

The growth rate constant, k, is defined by the relation $\ln_e$ cell number $= kt$, when time is measured in hours.

RESULTS

Induction in complex media

Fig. 1 shows the kinetics of enzyme formation obtained when galactokinase was induced in a complex medium. The galactokinase content of the culture has been

plotted as a function of cell mass for a culture of cells growing exponentially in broth to which fucose, a non-metabolizable inducer of galactokinase, has been added (Curve a). Following an initial period of growth, during which the enzyme was formed at a level of 8400 units/unit absorbancy, there was an abrupt change to a new rate of enzyme formation corresponding to 19 800 units/unit absorbance. This change in the differential rate of synthesis of galactokinase occurred while the cells were still in logarithmic growth and suggested that the inducibility of this enzyme depended upon some unappreciated change in the physiology of the culture, as well as upon the specific inducer present. Identification of some of the factors involved in this transition was attempted.

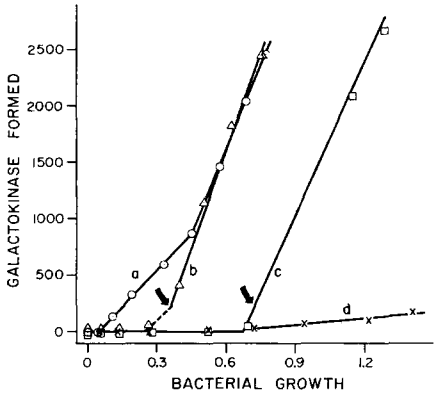

Fig. 1. The induction of galactokinase in broth culture. The enzyme content per 0.25 ml of culture is plotted as a function of the increase in absorbancy at 550 mμ exhibited by the culture. The cells were grown to approx. $1.7 \cdot 10^8$ cells per millilitre in full strength tryptone broth, harvested by centrifugation and inoculated at an initial cell concentration of $0.7 \cdot 10^8$ cells in half strength tryptone broth containing: a, none (○—○); b, 0.3 (△—△); c, 1.0 (□—□) and d, 2.8 μmoles/ml glucose (×—×). Fucose at $5 \cdot 10^{-3}$ M was added at the start of the experiment as inducer. The arrows indicate the times at which the cultures exhausted their glucose as judged from their abrupt change in growth rate.

Galactokinase is fully repressed by the presence of glucose in broth cultures containing fucose (Curve d). Similar cultures were therefore grown in the presence of limiting amounts of glucose in order to determine whether the transition from the first to the second phase of enzyme induction required the prior accumulation of a critical level of galactokinase activity. This would be the case, for example, if an endogenous product of the galactose metabolizing system were a more efficient inducer than fucose itself. In these cultures enzyme formation was fully repressed until the glucose had been completely utilized, which was indicated by an abrupt decrease in the growth rate of the culture. Following the disappearance of glucose, galactokinase was immediately formed at a rate which would be appropriate to the same growth stage of a culture in which enzyme synthesis had been occurring continuously. This is shown in Curves b and c of Fig. 1. We can therefore conclude that the prior formation of galactokinase is not required in order to enter the second phase of induction.

Further experiments have shown that the primary factor responsible for the transition to the second phase of more rapid enzyme formation was a change in the

properties of the medium. If the cells which had entered the second phase of induction in a culture were separated from the medium by centrifugation and introduced into fresh medium, they again showed a biphasic enzyme induction. However, if the old medium was re-inoculated with fresh cells, the initial rate of enzyme formation was nearly maximal. These results suggest that complex media contain substances, consumed during bacterial growth, which act as inhibitors of galactokinase induction.

Further analysis showed that similar effects could be demonstrated in a minimal salts–glycerol medium to which casein hydrolysate was added, indicating that amino acids, which are the principal constituent of broth, are a responsible factor. From the experiments summarized in Fig. 2, we can conclude that the occurrence of an initial lag period for enzyme induction depends upon the presence of amino acids in the

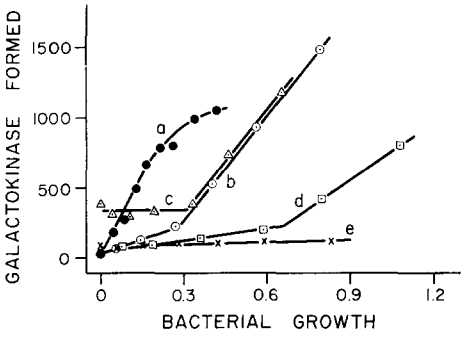

Fig. 2. Induction of galactokinase in synthetic-amino acids media. The enzyme content per 0.5 ml of culture is plotted against the increase in absorbancy of the culture as in Fig. 1. Cells were grown to $2–3 \cdot 10^8$ in salts–glycerol, harvested by centrifugation, and inoculated at $1.7 \cdot 10^8$ into fresh medium. These media were: a, salts–glycerol + $5 \cdot 10^{-3}$ M fucose (●—●), b and c, same + 3 mg/ml casein hydrolysate amino acids (○–○) and (△—△); d, same + 12 mg/ml casein hydrolysate (□—□), e, salts–glycerol alone, fucose omitted (×—×). Pre-induced cells grown for approx. five generations in salts–glycerol + $5 \cdot 10^{-3}$ M fucose were used as the inoculum in (c).

medium, and that the duration of the effect was dependent upon the quantity of amino acids added. The fact that preinduction of the cells in the absence of amino acids did not modify the response suggests that amino acids do not act by inhibiting a process preliminary to enzyme induction, such as the formation of a permease for the inducer. Rather the amino acids appear to intervene at the level of enzyme induction itself, acting as repressors of galactokinase synthesis.

Repression of galactokinase by amino acids

Tests of each of the amino acids separately, and in various combinations, indicated that several are able to exert strong repressive effects on galactokinase synthesis. In particular, valine, leucine, histidine, and serine are strong repressors. These amino acids are not known to share a common metabolic pathway, and it is difficult to imagine a common metabolic product unique to this group. They are all, however, to some degree inhibitory to the growth of *E. coli* K12. As shown in Fig. 3, their relative potency as growth inhibitors approximately parallels their ability to repress galactokinase, and the addition of combinations of amino acids (as for example valine

+ leucine + isoleucine or serine +methionine) which relieve the growth inhibitions simultaneously overcome galactokinase repression. The results obtained were independent of the duration of bacterial growth, over the tested range of 1–3 h.

It is clear that there exists a strong correlation between the rate of growth of the cells, as modified by the presence of various amino acids, and their ability to form galactokinase. This fact was confirmed by the observation that for leucine, histidine, and serine, inhibition of enzyme inducibility paralleled increasing growth inhibition as the concentration of amino acid was varied.

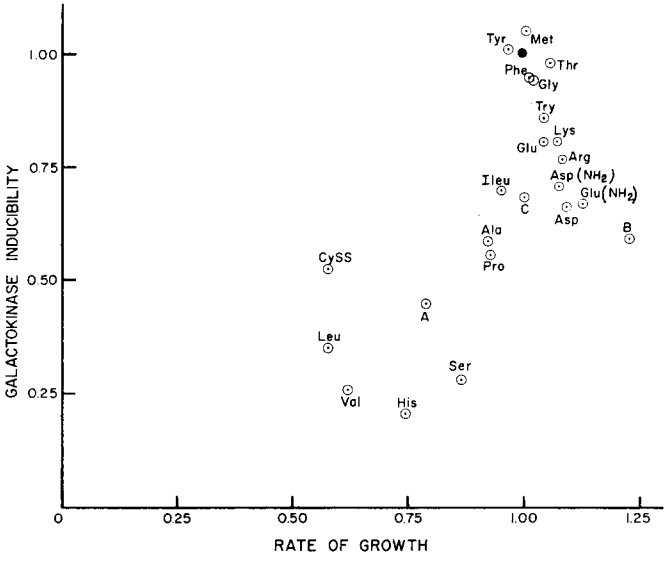

Fig. 3. The inhibition of galactokinase induction by individual amino acids. The inducibility of galactokinase (expressed as enzyme formed per unit increase in absorbancy of a culture, relative to the same quantity in the control) is plotted as a function of the growth rate of each culture (expressed as the growth rate constant of the culture relative to the same quantity in the control). The control values obtained in the absence of added amino acids are indicated by the closed circle and were 1320 units/unit absorbancy at 550 mμ and $k = 0.40$. The cells were grown to $5 \cdot 10^8$/ml in salts–glycerol, harvested by centrifugation and inoculated at $2.6 \cdot 10^8$/ml into separate cultures of fresh salts–glycerol to which the amino acids were added singly. Each amino acid was present at an initial concentration of $5 \cdot 10^{-3}$ M except for cystine, which was at $5 \cdot 10^{-4}$ M, and tyrosine and tryptophane, which were at 10^{-3} M. The cultures were shaken at 37° for 2 h. Growth was stopped by the addition of 25 μg/ml chloramphenicol and galactokinase assayed. The amino acid mixtures tested were: A, methionine + cystine; B, serine + threonine; and C, isoleucine + leucine + valine. In the case of mixtures each amino acid was at the same concentration as when used singly.

The components of the intermediary pool of metabolites apparently can repress the synthesis of enzymes feeding the pool[7,8]. The extent of this repression probably depends upon the size of the pool since the relative abilities of various carbohydrates to exert repression are correlated with the rate at which they enter the pool[4–6]. We should therefore expect that if the size of the pool is important in repression, then compounds which increase its size by decreasing the rate at which pool components are utilized for synthetic processes should also be effective in repressing the formation of enzymes leading to the pool. The ability of growth inhibiting amino acids to

repress galactokinase induction may thus be understood if those amino acids which slow the growth rate do so by interfering with various synthetic processes. In the case of valine, this hypothesis agrees with the detailed mechanism of growth inhibition. LEAVITT AND UMBARGER[17] have shown that valine exerts a feedback inhibition on the formation of acetohydroxybutyrate, which is in the pathway of isoleucine synthesis. Valine thus indirectly inhibits protein synthesis by making the cells deficient in isoleucine.

Repression of β-galactosidase

If we are correct in assuming that a process analogous to catabolite repression is responsible for the repression exerted by some amino acids, then the same pattern of amino acid repression should be exerted on the synthesis of other carbohydrate metabolizing enzymes. Fig. 4 reports an experiment, similar to that performed on galactokinase, in which the dependence of β-galactosidase induction on the bacterial

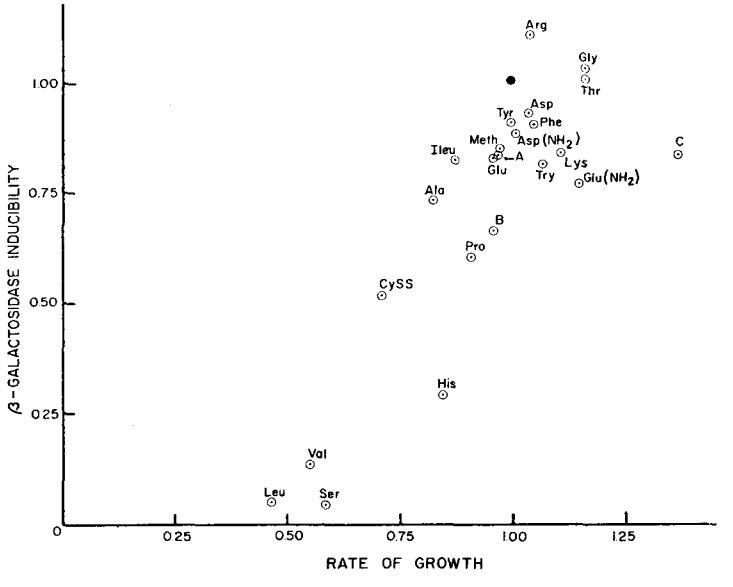

Fig. 4. The inhibition of β-galactosidase induction by individual amino acids. The inducibility of β-galactosidase is plotted as a function of the growth rate of each culture. The methods of calculation and experimental procedures were the same as those in Fig. 3, except that $5 \cdot 10^{-4}$ M isopropylthiogalactoside was used in place of fucose. The control values obtained in the absence of added amino acids are indicated by the closed circle and were 2960 units formed/unit increase in absorbancy at 550 mμ and $k = 0.34$. The amino acid mixtures tested were: A, leucine + isoleucine; B, valine + isoleucine; C, threonine + serine.

growth rate was determined. The cells were cultured in the same fashion in a salts–glycerol medium containing inducer to which individual amino acids were added singly, and the rate of synthesis of the enzyme determined during subsequent growth. It is clear that β-galactosidase shows a pattern of response similar to that of galactokinase.

A number of such amino acid growth experiments have been carried out in which either galactokinase or β-galactosidase induction was studied. (For technical reasons it is not possible to simultaneously induce both enzymes in the same culture.) In these experiments each amino acid tends to show a characteristic position on the inducibility *versus* growth plot which is independent of the enzyme induction being examined. Figs. 3 and 4 illustrate the approximate extreme range of variation observed.

The effect of chloramphenicol

If we are correct in assuming that the repressive abilities of some amino acids arise as a consequence of an increase in the size of the intermediary pool following growth inhibition, then other growth inhibitors should exert similar effects. Bacteriostatic agents which interfere with synthetic processes should inhibit the formation of catabolite repressible enzymes more strongly than they inhibit the synthesis of other proteins. Fig. 5 records the effects on galactokinase and β-galactosidase formation of sub-toxic doses of chloramphenicol. At doses just below those which produced a measurable inhibition of growth (and thus of total protein synthesis), the induction

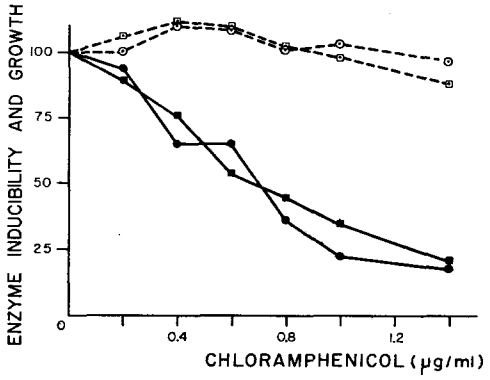

Fig. 5. The inducibility of galactokinase and β-galactosidase in the presence of chloramphenicol. Closed symbols represent the concentration of enzyme in newly formed protoplasm (expressed as in Fig. 3) plotted as a function of chloramphenicol concentration: galactokinase, ●—●; β-galactosidase, ■—■. The relative growth rates of each culture are also indicated by the open symbols for galactokinase, ○—○, and β-galactosidase, □—□. Control values obtained in the absence of added chloramphenicol were 1560 units of galactokinase and 2840 units of β-galactosidase formed per unit increase in absorbancy and a growth rate constant $k = 0.30$ for both cultures. For this experiment cultures were grown in salts–glycerol to $1.5 \cdot 10^8$, the cells harvested by centrifugation and inoculated at an initial density of $1.8 \cdot 10^8$ in fresh salts–glycerol containing either $5 \cdot 10^{-3}$ M fucose or $5 \cdot 10^{-4}$ M isopropylthiogalactoside as inducers of galactokinase and β-galactosidase respectively, plus varying concentrations of chloramphenicol. The cultures were incubated 2 h at 37°. Growth was stopped by adding 50 μg/ml chloramphenicol and chilling, and the cultures assayed for the respective systems.

of these enzymes is already appreciably inhibited. (The use of turbidity as a measurement of growth and protein synthesis was validated in parallel experiments in which protein was measured directly by the procedure of LOWRY *et al.*[16]; identical results were obtained.) Similar effects have also been demonstrated with puromycin, actinomycin-D, and mitomycin-C, indicating that the effect is a general one and does not derive from the specific site of action of chloramphenicol.

Enzyme formation and growth

All of the experiments reported thus far indicate the strong inhibition of galactokinase and β-galactosidase inducibility under conditions of partial growth inhibition. These experiments do not preclude the possibility that enzyme formation depends upon some correlate of growth other than the size of the intermediary pool. A strong indication that it is in fact the size of the pool which is the determining factor would be the finding that a growth inhibitor which reduces the size of the pool by interfering with carbohydrate utilization enhances, rather than represses, the formation of galactokinase and β-galactosidase.

Fig. 6 shows the dependence of β-galactosidase induction upon the growth rate when growth was inhibited by increasing concentrations of the glycolytic inhibitor, iodoacetate. For comparative purposes, the results obtained when growth was inhibited by 2,4-dinitrophenol are included. Growth inhibition by iodoacetate would be expected to stimulate enzyme formation by partially blocking the entrance of glycerol into the intermediary pool at the glyceraldehyde-3-phosphate dehydrogenase step;

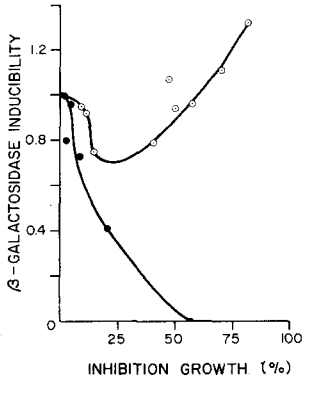

Fig. 6. The induction of β-galactosidase in the presence of iodoacetate and 2,4-dinitrophenol: The inducibility of enzyme is plotted as a function of growth inhibition. Enzyme concentration is calculated as in Fig. 3, growth is plotted as the fractional increase in culture mass compared to the control. Cells were grown in salts–glycerol, harvested by centrifugation, inoculated into fresh salts–glycerol containing $5 \cdot 10^{-4}$ M isopropylthiogalactoside, plus varying concentrations of iodoacetate (○—○) (from $1.5 \cdot 10^{-5}$ to $3 \cdot 10^{-4}$ M) and 2,4-dinitrophenol (●—●) (from $3 \cdot 10^{-6}$ to $9 \cdot 10^{-4}$M). In the case of iodoacetate the starting culture was grown to $1.6 \cdot 10^{8}$ and inoculated at $2.8 \cdot 10^{8}$, control values were 1650 units formed per unit absorbancy and $k = 0.44$; for 2,4-dinitrophenol the corresponding values were $3.2 \cdot 10^{8}$, $3.1 \cdot 10^{8}$, 2370 units enzyme per unit absorbancy and $k = 0.36$. Other experimental procedures were as described in Fig. 5.

whereas the uncoupling action of 2,4-dinitrophenol would be expected to preferentially inhibit β-galactosidase induction in a manner analogous to that of chloramphenicol. The experimental results are in agreement with these predictions at higher iodoacetate concentrations, and the finding that at these concentrations increasing growth inhibition stimulates the differential rate of β-galactosidase synthesis, is thus strong support for the idea that a form of catabolite repression is responsible for the amino acid effects observed.

The reason for the initial fall in the inducibility of β-galactosidase at low iodo-

acetate concentrations is not clear. It is possible that even at low concentrations iodoacetate may not be completely specific in its inhibition of glycolysis, or that it may not completely prevent the introduction of carbon into the pool since its point of inhibition lies after the formation of glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate from glycerol. A similar ability of iodoacetate to enhance enzyme induction was also demonstrated in glucose grown cells. (2-Deoxyglucose, which in principle would be a more useful agent than iodoacetate, unfortunately did not inhibit intact cells of *E. coli*.)

DISCUSSION

The experiments which have been reported demonstrate that a variety of conditions which produce partial growth inhibition in bacteria drastically alter the rates of synthesis of galactokinase and β-galactosidase. The common denominator which appears to link these factors is their expected effects on the rate at which the pool of intermediary compounds is utilized for the synthesis of macromolecules. This finding suggests that under normal growth conditions the overall rate of macromolecular synthesis can be a primary factor in regulating the formation of those enzymes which feed the intermediary pool.

Since the most obvious explanation for the operation of this regulatory mechanism appears to be the suggestion of NEIDHARDT AND MAGASANIK[7,8] regarding the role of the intermediary pool in enzyme repression, the present results should be viewed as experimental support for their original hypothesis. It is reasonable therefore that the concept of catabolite repression should be generalized to include not only the behavior of compounds which affect the rate at which material enters the pool, but also the behavior of compounds which affect the rate at which material leaves the pool.

The physiological significance of a regulation of this kind is apparent. It serves to hold the supply of biochemical intermediates at a constant level, compensating for changes both in the rate of utilization as well as the rate of formation of the pool.

Several observations have been recorded in the literature which demonstrate that under conditions of severe growth inhibition the presence of a metabolizable carbon source enhances repression. This has been demonstrated in *E. coli* for a pyrimidine requiring mutant in the absence of uracil[18] and for wild type cells starved of nitrogen[5] in the induction of β-galactosidase. MCFALL[19] has shown that the inhibition of β-galactosidase induction which develops as a consequence of ^{32}P suicide in *E. coli* is considerably relieved by removing the carbon source from the medium, and MCFALL AND MAGASANIK[20] have suggested that it is the failure to utilize the intermediary pool which also explains the loss of β-galactosidase inducibility that occurs during thymineless death.

In the light of these findings and the present results, an enhanced repression by the intermediary pool would almost certainly appear to be the explanation for the observation of PARDEE AND PRESTIDGE[21] that 7-aza-tryptophane completely inhibits the induction of β-galactosidase but not that of D-serine dehydratase (EC 4.2.1.14). Catabolite repression would also seem to explain the recent observations of SYPHERD *et al.*[22] that chloramphenicol and other metabolic inhibitors preferentially inhibit the induction of some degradative enzymes.

The extent to which relatively dramatic changes in the differential rate of formation of some enzymes may result from seemingly minor changes in cell physiology should be emphasized. Bacterial cells appear to be remarkably sensitive to minor changes in their environment. This sensitivity should be taken into account when interpreting the results of experiments in which the induction of an enzyme is modified, particularly when the enzyme is sensitive to catabolite repression or to repression by an end product which is a component of the pool.

ACKNOWLEDGEMENTS

The author is indebted to Mr. N. ROGGOW for his help in carrying out the experiments reported. This work was supported in part by a grant (E-3027) from the U.S. Public Health Service.

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