The Repression of Constitutive β -Galactosidase in *Escherichia coli* by Glucose and other Carbon Sources

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Experiments on the formation of induced enzymes in *Aerobacter aerogenes* led to the suggestion (see Neidhardt & Magasanik, 1956; Magasanik, Neidhardt & Levin, 1958) that glucose inhibition of synthesis of inducible enzymes is due not to glucose itself but to intermediary metabolites which act as repressors. In general, glucose repression affects only those inducible enzymes which are concerned in providing the organisms with carbon and energy. In washed suspensions of *Escherichia coli*, β -galactosidase formation was repressed not only by glucose but by any utilizable carbon source (Mandelstam, 1957, 1960, 1961), and the repression produced by metabolites such as succinate or lactate could be reversed with 2,4-dinitrophenol. These experiments raised the question (see Mandelstam, 1961) whether the repressor formed from these carbon sources is the same repressor as that postulated by Pardee, Jacob & Monod (1958, 1959). These authors concluded on the basis of genetic experiments that the property of inducibility was due to the production of a repressor: to obtain synthesis of enzyme, the action of the repressor had to be reversed by adding a suitable specific inducer. On this interpretation, the constitutive (i^-) strain was believed to produce high activities of enzyme without an inducer being present because the cells were unable to synthesize the repressor. The experiments to be described show that the constitutive mutant is nevertheless subject to carbon-source repression so that this repressor is presumably quite different from the repressor which produces the inducible character. On the assumption that repression was due to a metabolite produced from glucose (or other carbon source), the bacteria were subjected to nitrogen-starvation in a continuous-flow apparatus so that such metabolites might accumulate during the period of transition to the steady state. This was found to accentuate the repression and to provide a convenient system for varying the enzyme activity in a much more reproducible manner than is possible in flask cultures. A brief report by Brown & Monod (1961) indicates that similar observations have been made, but no details are given.

METHODS

Organism. *E. coli* ML308 (constitutive for β -galactosidase) was kindly supplied by Dr J. Monod. The medium contained the following (per l.): KH_2PO_4 , 13.6 g.; NH_4Cl , 1.0 g.; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g.; MgSO_4 , 20 mg.; $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 15.6 mg.; glucose or other carbon source, 20 g.; pH was adjusted to 7.2 with NaOH. This will be referred to as full medium. When a nitrogen-limiting medium was required, the ammonium salts were decreased to 50 mg./l. each, and in a carbon-limiting medium the carbon source was decreased to 0.5 g./l., unless stated otherwise. The C- and N-limiting media gave a bacterial density of 0.25–0.30 mg. dry wt./ml. when the cells were grown in a continuous-flow apparatus. The organisms were either grown at 35° with shaking in a flask (100 ml. in a 500 ml. conical flask) or in the continuous-flow apparatus described by Rogers (1957). The culture (100 ml.) was incubated in a rapidly rotating flask through which sterile air was drawn (1.25 l./min.). The flow rate of medium was controlled by a peristaltic pump. Samples (3–5 ml.) were obtained by collecting the effluent from the growth flask for a few minutes. The doubling time (T) of the culture is given by $T = [\text{vol. of culture (ml.)} / \text{flow rate (ml./min.)}] \times \log_2 2$.

Assay of β -galactosidase. The samples were centrifuged, the bacteria resuspended in 0.05 M-sodium phosphate buffer (pH 7.0) at a density of about 0.3 mg. dry wt./ml. and treated with toluene (2–3 drops) at 35° for 30 min. The enzyme was assayed according to Rickenberg & Lester (1955) with *o*-nitrophenyl β -D-galactoside. A unit of enzyme liberates 1 μ mole of *o*-nitrophenol/hr.

Protein analysis was done by the method of Lowry, Rosebrough, Farr & Randall (1951), serum albumin being used as standard.

RESULTS

Effect of carbon source on β -galactosidase activity of flask cultures. The bacteria were grown in flask cultures with the carbon compound at 20 g./l. The enzyme activities obtained with any carbon source were somewhat variable from day to day (the reasons for this will be discussed below). The values in Table 1 are typical, and range from 136 units with lactose to over 800 with lactate. For comparison, the doubling time of this strain of *E. coli* in flask culture is shown for each of the carbon compounds. A very rough correlation can be made: those compounds which allowed rapid growth (doubling time of 50 min. or less) produced an

enzyme activity below 300. With poorer carbon sources (i.e. when the doubling time was longer) the enzyme activity was higher. In spite of the variability of enzyme activities (e.g. values with glucose have been in the range 150–300), the activity with glucose was always less than 50 % of that obtained with the 'poor' carbon sources. This degree of inhibition is similar to that reported by Cohn & Horibata (1959).

Effect of growth rate on the repression of β -galactosidase by glucose. The effect was examined of slowing the growth rate in a nitrogen-limiting medium so that products of carbohydrate metabolism could accumulate and exert a maximum

Table 1. *Effect of carbon source on activity of constitutive β -galactosidase of flask cultures compared with doubling time of the cells*

The bacteria were grown with shaking in synthetic medium with the carbon compound at 20 g./l. They were harvested at about 1 mg. dry wt./ml. and assayed for β -galactosidase.

Carbon source	β -Galactosidase (units/mg. dry wt.)	Doubling time (min.)
Lactose	136	45
Glucose	200	48
Glucanate	260	50
Galactose	432	54
Glycerol	499	60
Succinate	600	65
Fructose	613	67
Lactate	816	65

Fig. 1. Effect of growth rate on the repression of β -galactosidase by glucose. Bacteria from a flask culture were grown in a continuous-culture apparatus with nitrogen-limiting medium, and glucose as carbon source (see Methods section). The initial doubling time was 165 min. At the point indicated by the arrow, the repression was relieved by changing the flow rate to give a doubling time of 51 min. The continuous curve is the theoretical curve representing total repression of enzyme synthesis.

repressor effect. A culture was grown to 0.25 mg. dry wt./ml. in full medium with glucose as carbon source. A portion (100 ml.) was centrifuged and the bacteria were resuspended in 100 ml. of medium containing glucose (20 g./l.) but no nitrogen. The suspension was transferred to the continuous-flow apparatus and addition of nitrogen-limiting medium (with glucose at 20 g./l.) begun at a rate of 25 ml./hr. This rate corresponds to a doubling time of 165 min. as compared with 48 min. in a flask culture. The change in β -galactosidase was followed as the system approached the steady state.

In the course of 4.5 generations (12.75 hr.) the specific activity fell from 154 to 19 units of enzyme/mg. (Fig. 1), and appeared to be reaching a steady state at about this value. Fig. 1 also shows the theoretical curve, calculated on the assumption that synthesis of β -galactosidase ceased entirely at zero time and that the existing enzyme was then 'diluted out' in the growing bacterial mass. For the first generation the experimental values were very close to theoretical; thereafter there was an increasing divergence, indicating that β -galactosidase formation had not ceased entirely but was proceeding at a low rate. A steady-state value of about 15 units/mg. would represent 90 % inhibition of enzyme synthesis in comparison with the flask culture.

The protein content of the culture after four generations was 176 μ g./ml., compared with 185 μ g./ml. at the beginning of the experiment. This difference was within the error of the method. The pH was also constant within 0.15 pH unit.

After 4.5 generations the flow-rate was altered to 1.35 ml./min., giving a doubling time for the bacteria of 51 min. At this growth rate (i.e. close to the minimum doubling time in the medium) the bacteria would be expected to be only just nitrogen-limited and, as expected, there was a rapid recovery in enzyme synthesis which, at the end of three generations, had brought the specific activity back to the normal range (see Fig. 1).

Other good carbon sources such as lactose and glucanate gave a comparable degree of repression.

Repression of β -galactosidase by other carbon sources. The effect of glycerol as a repressor was examined in a similar manner. With the same doubling time as in the previous experiment (165 min.) there was no fall in specific enzyme activity. After 1.3 generations the flow rate of medium was decreased still further to give a doubling time of 300 min. This sufficed to produce a repressor effect, and the specific activity of the enzyme fell in the ensuing two generations to 135 units/mg. (Fig. 2). There was even greater divergence than in the previous experiment between the measured values and the theoretical curve corresponding to total repression. Increasing

the flow rate so as to reduce the doubling time to 70 min. relieved the repression. After three generations the specific activity was nearly 400 units/mg. (Fig. 2), and a steady-state activity of 500 units/mg. was achieved after six generations.

Repression was also obtained with lactate as carbon source. The initial enzyme activity after growth in flask culture was 816 units/mg. After the bacteria had been grown for two generations at a doubling time of 360 min. the activity had fallen to 254 units, which apparently represented a steady-state value since no further change occurred

Fig. 2. Effect of growth rate upon the repression of β -galactosidase by glycerol. Procedure was as described for Fig. 1 but glycerol was used instead of glucose. The initial doubling time was 165 min. After 1.3 generations (left-hand arrow) this was changed to 300 min. The repression was relieved by increasing the flow rate to give a doubling time of 70 min. (right-hand arrow). The continuous curve represents the theoretical values for total repression.

Fig. 3. Effect of carbon starvation upon β -galactosidase. Bacteria from a flask culture (glucose medium) were grown in a continuous-culture apparatus with glucose-limiting medium. The doubling time was 173 min.

on further growth. Succinate, as might have been expected from flask-culture experiments, was very similar in its action. It should be noted that with both these compounds as well as with glycerol the enzyme activities after repression were still about as high as those found in flask (i.e. 'unrepressed') cultures with glucose as carbon source.

Effect of glucose starvation on β -galactosidase activity. The observation that, in flask cultures, the enzyme activity obtained with glucose was appreciably lower than with other carbon sources suggested that even when the cells were growing 'freely' there was appreciable repression. In an attempt to demonstrate this, bacteria were grown in a flask in full medium with glucose as carbon source, centrifuged, resuspended in medium with no carbon source and transferred to the continuous-flow apparatus. Glucose-limiting medium (0.5 g. of glucose/l.) was then added at 0.4 ml./min. (This gave a doubling time of 173 min.)

The ensuing state of carbon starvation caused the specific activity of the β -galactosidase to rise from 321 to 640 units/mg. in two generations (Fig. 3). With glycerol and gluconate, 'de-repression' (i.e. an increase in activity) was also found. However, with lactate or succinate, carbon starvation failed to raise the specific activity. Instead, in most of the experiments, the activity tended to fall somewhat. It therefore appears that, with these carbon sources, the enzyme is already in a state of minimum repression even in the flask culture.

Effect of methyl β -D-thiogalactoside on glucose repression. The type of repression which is postulated as the cause of inducibility of β -galactosidase in wild-type *E. coli* can be relieved by adding any of a number of galactosides which function as inducers of the enzyme. One of the more potent of these, methyl β -D-thiogalactoside, was tested to see whether it would reverse the repression produced by glucose.

Bacteria were grown, as already described, in the continuous-culture apparatus in nitrogen-limiting medium with glucose as carbon source at a doubling time of 180 min. After 4 hr., when the enzyme activity had fallen considerably, 50 mg. of inducer in water (2 ml.) was injected directly into the culture vessel. This concentration is at least ten times as great as that needed for maximum induction of the enzyme in wild-type bacteria. The addition of the thiogalactoside produced no measurable effect upon the rate of fall of enzyme activity.

It was thought that the inducer might be able to reverse the repression produced by a less-potent repressor than glucose. The experiment was therefore repeated with lactate as the carbon source and adding the thiogalactoside only after the enzyme

had fallen to its steady-state value. The lactate repression was also completely unaffected by the addition of the inducer.

DISCUSSION

These experiments make it apparent that, under appropriate physiological conditions, any compound which the bacteria can use as a source of carbon and energy may repress constitutive β -galactosidase. The specific activity of the enzyme can be varied from 20 to over 600 units/mg. by varying the conditions of growth in glucose medium.

Glucose is clearly not a unique repressor, and differs only quantitatively from compounds such as succinate and lactate. The carbon sources tested form a spectrum in which the property of being a good carbon source (i.e. producing rapid growth in flask culture) is correlated with the ability to repress the enzyme both in flask culture and in the continuous-culture apparatus. The poorer the carbon source, the higher is the enzyme activity in flask culture and the more difficult it is to depress the enzyme by growth of the bacteria under conditions of nitrogen limitation. The results support the suggestion (Magasanik *et al.* 1958; Mandelstam, 1961) that this type of repression is produced by metabolites that can be formed from a variety of carbon compounds. The amount of such repressor substances is likely to be affected by the age of the culture, and also by less controllable factors such as the degree of aeration. It is consequently not surprising that the specific enzyme activity of flask cultures can vary quite considerably from day to day.

We can now consider the bearing of these experiments on current ideas about induced enzyme formation. According to the theory of Pardee *et al.* (1958), the constitutive (i^-) strain of *E. coli* lacks the ability to form the repressor normally present in the cytoplasm of the wild-type (inducible) cells. The fact that it has proved possible to obtain repression in this mutant indicates that we are dealing with a distinct type of repression. It follows, too, that the formation of enzyme in the inducible strain is controlled by two separate repressors, one of which is specific and whose synthesis is controlled by a gene in the 'lac' region of the chromosome (Jacob & Monod, 1961). The second type, the carbon-source repressor, has not been genetically characterized but can apparently be synthesized equally by aerobic and anaerobic pathways in *E. coli* (Mandelstam, 1961). It is not known whether it is specific for β -galactosidase or whether it will cause repression of other inducible enzymes concerned with the utilization of carbon sources. The function of this repressor in

the biochemical changes involved in adaptation from growth on glucose to growth on lactose has been discussed by Mandelstam (1960).

Neither the nature nor the site of action of either type of repressor is known. Pardee & Prestidge (1959) have suggested that the specific β -galactosidase repressor may be ribonucleic acid since it is formed in the presence of agents which block protein synthesis. Jacob & Monod (1961) have proposed a scheme in which the repressor acts directly at the level of the gene by preventing the formation of the 'messenger ribonucleic acid' that conveys information to the ribosome for the assembly of the enzyme molecule.

The carbon-metabolite repressor could also be assumed to act in the same way. However, Hauge, MacQuillan, Cline & Halvorson (1961) have shown that, in the presence of glucose, yeast ribosomes contain more bound β -galactosidase than in its absence. This occurred even though the glucose caused 90% repression of the enzyme. If the ribosome-bound enzyme is, in fact, nascent enzyme and not simply enzyme adsorbed non-specifically from the cytoplasm, this result would suggest that the glucose repression prevents the separation of the finished enzyme from the site of synthesis.

SUMMARY

1. When *Escherichia coli* is grown in flask culture with various carbon sources, the activity of constitutive β -galactosidase is correlated with the doubling time of the culture; the poorer the carbon source the higher is the enzyme activity.

2. When bacteria are transferred from a flask to a continuous-culture apparatus and grown in a nitrogen-limiting medium (i.e. with excess of carbon source) the formation of the enzyme is severely repressed.

3. The effect of glucose as a repressor is not unique. Under appropriate conditions of growth all utilizable carbon sources act as repressors.

4. The repression produced by carbon compounds is not reversed by methyl β -D-thiogalactoside (an inducer of the enzyme in wild-type bacteria).

5. It is concluded that the synthesis of inducible β -galactosidase in wild-type *E. coli* is controlled by two distinct types of repressor, one of which is still able to function in the constitutive mutant.

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Factors Affecting the Primer for Deoxyribonucleic Acid Polymerase

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In previous reports from this Laboratory (Smellie, Keir & Davidson, 1959; Smellie *et al.* 1960; Gray *et al.* 1960), deoxyribonucleic acid polymerase of Ehrlich ascites-tumour cells has been described. The requirements for its action include Mg^{2+} ions, the 5'-triphosphates of deoxyadenosine, deoxycytidine, deoxyguanosine and thymidine, and primer deoxyribonucleic acid. The extent of synthesis of polydeoxyribonucleotide in a given time is dependent upon the amount of primer deoxyribonucleic acid present in the reaction mixture. This paper describes some of the experiments carried out to investigate the nature of the primer, and some of the treatments which can influence its priming capacity in the synthetic system.

EXPERIMENTAL

Abbreviations. Abbreviations used in this paper are: deoxyadenosine 5'-triphosphate, dATP; deoxycytidine 5'-triphosphate, dCTP; deoxyguanosine 5'-triphosphate, dGTP; thymidine 5'-triphosphate labelled with ^{32}P in the α -phosphate group, [^{32}P]TTP; thymidine 5'-monophosphate labelled with ^{32}P , [^{32}P]TMP; sodium salt of ethylenediaminetetra-acetic acid (pH 7.0), EDTA.

Biological material. The Landschutz-ascites carcinoma was maintained by serial transplantation in albino mice. The ascitic fluid was harvested by collection in an equal volume of 0.154M-NaCl 7 days after inoculation, and the following operations, differing slightly from the original procedure (Smellie *et al.* 1959), were carried out at 0–4°.

Preparation of soluble extract. Cells were sedimented by centrifuging at 500g for 5 min. and were washed twice by suspending in 0.154M-NaCl, followed by centrifuging at 500g for 5 min. The cells were disrupted osmotically in 13–15 vol. of water, pH 7.0, and the preparation was made

either 0.03M with KCl and 0.01M with potassium phosphate buffer, pH 7.5, or 0.01M with potassium phosphate buffer, pH 7.5. The soluble extract was obtained by centrifuging the disrupted-cell suspension at 105 000g for 60 min., the sediment being retained for the preparation of DNA. There was no difference in DNA-polymerase activity of the extracts obtained by these two procedures if allowance was made for differences in the concentration of K^+ ions. In most of the experiments to be described, the soluble extract was the source of polymerase activity, but in some studies fractions prepared from the soluble extract were used. In these cases, the polymerase preparation is denoted by the term 'polymerase fraction'.

Preparation of DNA. The method of Kay, Simmons & Dounce (1952) was followed for the preparation of DNA from the 105 000g sediment mentioned above. The DNA was dissolved in water or in 0.01M-NaCl to give a concentration of 2 mg. of DNA/ml.

A portion (200 ml.) of the solution was incubated with 400 μ g. of ribonuclease (crystalline, Nutritional Biochemicals Corp., Cleveland, Ohio) and 20 ml. of 1.0M-tris-HCl buffer, pH 7.6, for 2 hr. at 37°. An equal volume of water-saturated phenol was then added and the mixture was gently shaken for 60 min. at 20°. The supernatant aqueous layer which was obtained on centrifuging the mixture at 2000g for 20 min. was drawn off, leaving the phenol layer and a thin interfacial film of insoluble material, which was carefully removed. The phenol layer was treated twice more with an equal volume of water in the manner described above, the aqueous layers being collected. The pooled aqueous layers were extracted three times with equal volumes of ether to remove phenol. The viscous solution was then made 1.0M with respect to NaCl, and DNA was precipitated by addition of ethanol to 50% (v/v). The precipitate was washed three times with ethanol and three times with acetone, and was dried in air and stored at –10°. Before use it was dissolved in 0.01M-NaCl and dialysed for 24 hr. at 4° against four 4 l. changes of