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Kinetics of the Onset of Catabolite Repression in *Escherichia coli* as Determined by *lac* Messenger Ribonucleic Acid Initiations and Intracellular Cyclic Adenosine 3',5'-Monophosphate Levels[1]

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The rates of synthesis of β-galactosidase (EC 3.2.1.23) and the intracellular levels of cyclic 3',5'-adenosine monophosphate (cAMP) soon after the addition of glucose or glycerol to exponentially growing cultures of *Escherichia coli* have been determined. Within 10 s of its addition, glucose, but not glycerol, lowered the apparent initiation frequency of *lac* messenger ribonucleic acid. The glucose-generated reduction in initiations is identified as catabolite repression by its reversibility with cAMP. The intracellular cAMP levels respond virtually identically to glucose and glycerol additions. Thus, no correlation was observed between the rate of messenger ribonucleic acid initiation and the level of cAMP.

The addition or presence of glucose or some other catabolites depresses the inducibility of many operons in *Escherichia coli* (11, 18, 21). This effect has been termed catabolite repression and is thought to be mediated via cyclic adenosine 3',5'-monophosphate (cAMP) and its receptor, cAMP receptor protein (CRP) (14, 22-24).

While almost overwhelming evidence has accumulated showing that cAMP and CRP are necessary elements for transcription of catabolite-sensitive operons (6-10, 12), the role of cAMP and CRP in the mediation of catabolite repression has not been unambiguously established. The following have linked cAMP and CRP to transcriptional control as well as catabolite repression: (i) in vitro synthesis of messenger ribonucleic acid (mRNA) of the *lac* and *ara* operons with highly purified systems (6, 16) or in vitro synthesis of *lac* and *ara* enzymes in coupled transcription-translation systems requires cAMP and CRP (2, 7, 8, 9, 12, 32); (ii) loss through mutation of either CRP (10) or adenyl cyclase (25) (EC 4.6.1.1), the enzyme necessary for the production of cAMP, mimics strong catabolite repression; and (iii) exogenously added cAMP reverses catabolite repression (22, 23).

Recent evidence has indicated that modulation of cAMP levels may not be sufficient to explain the range of catabolite repression phenomenon observed (5, 31). Although it has not been demonstrated that cyclic guanosine 3',5'-monophosphate (cGMP) is directly involved in vivo as a protagonist of cAMP action, its involvement in transcriptional control is suggested by the following observations: (i) cGMP exists in *E. coli* and its levels rise under catabolite repression conditions (4); (ii) a mutant CRP has been isolated that appears to be activated by cGMP in vivo (29); (iii) cGMP inhibits expression of catabolite-repressible operons in vivo (3) and in vitro (28); and (iv) the measurements which have been made do not show a good correlation between inducibility and cAMP levels in vivo (5, 20, 26, 31); although, as we shall show, special care must be taken in the growth of cells, preparation of samples, and assaying of cAMP in order to insure meaningful measurements.

To further explore the possibility that cAMP and CRP are only part of the catabolite repression system we have measured the very early kinetics, beginning at 5 s, of the onset of catabolite repression. This necessitated measuring the cAMP levels in this interval as well as utilizing techniques developed for the examination of induction of operons at very early times after inducer addition. Catabolite repression begins and cAMP levels fall within 10 s after the addition of glucose. However, cultures receiving glycerol instead of glucose, but otherwise treated identically, also depressed their cAMP levels while not displaying catabolite repression. We thus conclude that (i) the transition to catabolite repression after glucose ad-

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dition is very rapid and (ii) catabolite repression cannot be fully explained by a drop in cAMP level.

MATERIALS AND METHODS

(i) Strains and cell growth. *E. coli* B mutant AS19, originally isolated as actinomycin sensitive and which is highly permeable to rifampin (30), was used for all induction studies and corresponding cAMP measurements. *E. coli* K-12, #5336 (25), deficient in adenyl cyclase, was used as a zero cAMP control for the cAMP assay.

Cells were grown aerobically by vigorous shaking at 37 C and their growth was closely monitored by measurement of their turbidity at 550 nm in a Zeiss spectrophotometer. Cells were grown in M9 minimal salts medium (1) supplemented with 5×10^{-5} M $MnCl_2$, 10 μg of B_1 per ml, 1% Casamino Acids, and 0.2% glycerol. All chemicals to be added to growing cells were dissolved in medium and prewarmed to 37 C before addition with prewarmed pipettes. Experiments were performed on cells whose growth had been exponential for at least five doublings and had not exceeded $1 \times 2 \times 10^8$ cells/ml. Reproducible growth and induction of strain AS19 necessitated cleaning all glassware with dichromate cleaning solution, rinsing with sodium citrate, and thoroughly flushing with distilled water before use.

(ii) Assay of β-galactosidase. Up to 1.4 ml of cells to be assayed were centrifuged at 3,000 $\times$ *g* for 30 min and the pellets were suspended in 1 ml of minimal salts medium containing 100 μg of chloramphenicol per ml. A mixture of 0.1 ml of cells and 0.9 ml of assay mix (0.25 M sodium phosphate buffer at pH 7.0, 10^{-3} M $MgSO_4$, 0.1 M β-mercaptoethanol, and 100 μg of chloramphenicol per ml) was mixed vigorously with toluene (10 μl) to make the cells permeable. Incubation was begun after the addition of 0.7 mg of orthonitrophenyl-β-D-galactoside in 0.2 ml of assay mix. After an overnight incubation at 30 C, 1 M Na_2CO_3 (0.5 ml) was added and the absorbance was read at 420 nm in a Zeiss spectrophotometer.

(iii) Sequential addition of inducer and rifampin to growing cells. One-milliliter aliquots of exponentially growing cells (1.1×10^8 cells/ml) were transferred to rapidly shaking test tubes (15 by 150 mm) (37 C). Four minutes after transferring, timed additions were begun. Isopropyl-β-D-thiogalactoside (IPTG) and glucose or glycerol in medium (0.1 ml) were added to final concentrations of 2.8 mM inducer and 0.5% sugar at a time defined as zero. Rifampin (0.2 ml) was added at time equals t to a final concentration of 100 μg/ml. The cells were shaken at 37 C for an additional 15 min to permit completion of protein synthesis before preparation as described in section (ii). Samples for measurement of uninduced basal levels received rifampin and were either chilled immediately or shaken at 37 C for 15 min before being chilled. Enzyme levels in both cases were approximately the same.

(iv) Sequential addition of inducer and rifampin in the presence of exogenous cAMP. Cyclic AMP in medium (0.1 ml) was added to each tube before cells were transferred as described above and reached a final concentration of 5 mM after the addition of cells. Control tubes received 0.1 ml of medium only. As timed additions were begun 4 min after cells were transferred, cAMP was able to diffuse into the cells before inducer was added. Cells were induced and treated as described in section (iii).

(v) Determination of rifampin entry time. Cells were grown and transferred to tubes as described above. At t = 0, IPTG and glucose or glycerol (0.1 ml) were added; and at time = Δt seconds, rifampin (0.2 ml) was added. The concentration of the initial rifampin solution was adjusted such that the final concentration of rifampin varied from 12 to 100 μg/ml. In a given set of experiments, Δt did not vary. For simultaneous addition, Δt = 0, IPTG, glucose or glycerol, and rifampin were mixed and added in a total volume of 0.2 ml.

(vi) Purification of binding protein and inhibitor. The cAMP-dependent protein kinase and protein kinase inhibitor were purified from fresh beef muscle according to Gilman (13). The ratio of inhibitor to kinase was adjusted to give maximum cAMP binding and the two components were mixed just prior to their use in the assay.

(vii) Assay of cAMP. Samples were assayed in duplicate by the method of Gilman (13). Samples (0.1 ml) were incubated in a total volume of 0.2 ml containing 0.2 pmol of ^{3}H-labeled cAMP, 50 μg of bovine serum albumin, and 75 mM sodium acetate buffer (pH 4.0). Incubation was begun with the addition of the binding protein and inhibitor and continued for at least 6 h at 0 C to allow the binding reaction to attain equilibrium. The reaction was quenched by the addition of 0.8 ml of 20 mM potassium phosphate buffer (pH 6.0) at 4 C. After 4 min at 0 to 4 C, the samples were collected on membrane filters (25 mm, 0.45 μm, punched from Millipore HAWP 01000 sheets), rinsed with 10 ml of the cold potassium phosphate buffer, and dried under a heat lamp. The radioactivity of the dried filters was determined in Omnifluor (New England Nuclear).

This is a competition assay with cold cAMP replacing ^{3}H-labeled-cAMP on the protein. Standard curves were obtained by using known amounts of cAMP and carrying them through the entire preparation procedure. Values for unknown samples can be determined directly from the graph of radioactivity bound to the filter versus total cAMP in the assay. As the assay is inhibited about 40% by an unidentified component which eluted from the Dowex columns, it was necessary to run standards through the columns (see section [viii]). Extracts of the cAMP-deficient strain gave results which were identical, within experimental error, to the zero cAMP standards.

(viii) Collection and preparation of samples for cAMP measurements. One milliliter of medium or 1 ml of medium containing glucose or glycerol was added to 10 ml of cells (1.1×10^8 cells/ml) in a 50-ml flask at 37 C. After t seconds the cells were collected on membrane filters (47 mm, 0.45 μm, Millipore HAWP 04700) and rinsed with 10 ml of prewarmed medium of the corresponding concentration. The filters were then quickly transferred to 150-ml beakers containing 20 ml of hot water (90 to 95 C) and kept at that temperature for 10 min. The entire process of

filtering, rinsing, and transferring required about 8 s. A small quantity of ^{3}H-labeled cAMP (0.01 pmol) was added to each sample to monitor the overall yield of subsequent steps. This small quantity did not interfere in the final assay. After the 10-min heating, samples were adjusted to 0.1 N HCl and heated for an additional 5 min to aid in the release of cAMP from the cells. Samples were then adjusted to 0.2 N HCl but not heated as this lowers cAMP recoveries.

The cAMP standards and samples (20 ml) were loaded onto 3-ml Dowex columns (Dowex 50W-X8, H^+ form from Bio-Rad) in Pasteur pipettes. Columns were eluted with water and cAMP was collected in the 6- to 8-ml fraction. The cAMP-containing fractions were dried overnight under a stream of air and suspended in 0.3 ml of 50 mM sodium acetate buffer (pH 4.0). Overall recovery was determined by measuring the radioactivity of an aliquot (50 μl) in Aquasol (New England Nuclear). Overall yields averaged 50%, ranging from 30 to 70%. Intracellular cAMP levels were calculated from the measured cAMP value and the overall recovery assuming a cell volume of 10^{-15} liters.

(ix) Chemicals. Rifampin was purchased from Calbiochem, IPTG, orthonitrophenyl-β-D-galactoside, and cAMP from Sigma, and ^{3}H-labeled-cAMP from New England Nuclear.

RESULTS

(i) Inducibility after glucose or glycerol additions to growing cultures. The objective of the work reported in this paper is to measure the kinetics of the onset of catabolite repression and to measure the change in intracellular cAMP levels after glucose addition. Proper comparison of catabolite repression data necessitates comparison of mRNA initiation rates to cAMP levels. Usually, however, one measures the level of enzyme per cell, and it is necessary to correct for the effect of different growth rates by converting enzyme per cell to a rate of synthesis per unit time according to the equation:

$$\text{rate of synthesis} = (\ln 2) \times (\text{enzyme/cell})/(\text{doubling time})$$

However, this is not completely correct since cells grown at different rates possess different average numbers of operon copies. To minimize these differences our studies were made on a system in which the growth rate changes very little after the addition of glucose-minimal medium containing glycerol and Casamino Acids.

The experiments were begun with the addition of glucose to a concentration of 0.5%. As a control for the possible nonspecific, osmotic effects of glucose addition, we compared the results of these experiments to controls where additional glycerol was added in place of glucose.

The most sensitive probe for measuring the kinetics of the onset of catabolite repression would be to add glucose and measure the rate of initiation of mRNA at later times. This could be accomplished by adding inducer at the desired time, then blocking further messenger initiations after a few seconds by the addition of rifampin. By examining this induction "window" at varying times after glucose addition, a curve of the catabolite repression kinetics could be determined. This ideal experiment is not feasible for two reasons: first, the documented interference by glucose of inducer uptake (19) would prevent induction of the operon after glucose had been added, and second, glucose could alter the time required for rifampin to enter the cells and block messenger initiations. In this first section we circumvent the problem of inducer entry by adding inducer with glucose, and in the next section we show that glucose addition has no effect on the subsequent rifampin entry time.

In these experiments the amount of enzyme produced from the completed mRNA is assumed to be proportional to the number of initiations that occurred in the interval after induction and before inactivation by rifampin. By adding rifampin at varying times after the addition of inducer, the relative number of initiations that occurred in succeeding intervals was determined. Adding inducer and glucose together insures that each culture receiving glucose is induced in the same manner. As the only difference among the tubes is the time at which rifampin is added, any differences in enzyme produced can be attributed to variation in the time of rifampin addition. The results of an experiment in which the time of rifampin addition has been varied is shown in Fig. 1. In all cases, the amount of inducer added is twice that required for maximum induction under the given conditions.

The amount of enzyme produced varies linearly with the induction interval for both the glucose experiment and the glycerol control. The rate for glucose samples is about 60% that for the glycerol samples. This lowered rate of synthesis is seen in the first interval examined as well as later intervals. From the data presented it can be seen that catabolite repression begins within 10 s after the addition of glucose.

(ii) Determination of the rifampin entry time. A modification of the technique used by Hirsh and Schleif (15) was employed to determine the rifampin entry time. It has been shown that under certain conditions the rate of rifampin entry into strain AS19 is directly proportional to the concentration outside the cells. If inducer and rifampin are added simultaneously, a plot of the resulting enzyme level versus

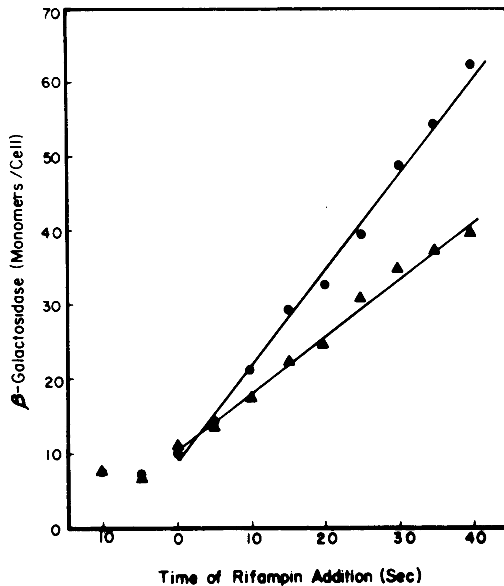

FIG. 1. *The synthesis of β-galactosidase in the presence and absence of glucose. Inducer and either glucose or glycerol were added at the start of the induction interval; rifampin (100 µg/ml) was added at the time indicated on the abscissa. Symbols: ●, 0.5% glycerol; and ▲, 0.5% glucose. The final concentration of IPTG was 2.8 mM in all cases.*

1/rifampin concentration is a straight line. Extrapolation of this line to the enzyme level corresponding to 1/[rifampin] equals zero, i.e., an infinite concentration of rifampin yields the amount of enzyme that would have been produced if rifampin had acted immediately. This approach was used to determine the rifampin entry times at 15, 25, and 40 s after the addition of glucose or glycerol. In all of these experiments, inducer was added at the same time as glucose or glycerol. Each point in Fig. 2 corresponds to the amount of enzyme produced by Δt seconds of induction, either 15, 25, or 40 s as noted, plus the amount of enzyme which results from initiations that occurred after rifampin was added to the sample but before sufficient amounts to inhibit polymerase had entered the cells. A plot of enzyme levels versus 1/rifampin concentration for a given Δt yielded a straight line which, when extrapolated to 1/[rifampin] equals zero, gave the amount of enzyme produced by Δt seconds of induction with no dependence upon the rate of entry of rifampin. A plot of the extrapolated values determined from Fig. 2 versus Δt is shown in Fig. 3. The slopes of the induction curves in Fig. 3 are the same as those in Fig. 1, indicating that glucose does not alter the rate of rifampin entry. This experiment also demonstrates that catabolite repression begins within 10 s of the addition of glucose.

(iii) Reversal of catabolite repression by exogenous cAMP. To demonstrate that the depressed inducibility of the *lac* operon after glucose addition is the result of catabolite repression, we showed that the effect is reversed by cAMP addition. cAMP at a concentration of 5 mM was added to the growing cells before the cells were induced as described in section (i); the results are shown in Fig. 4. While the addition of cAMP to the cells receiving glycerol had no effect, the addition of cAMP to the cells receiving glucose restored synthesis to the level seen in the absence of glucose. This is strong evidence that the observed repression is catabolite repression.

(iv) Measurement of in vivo cAMP levels after the addition of glucose. If decreased cAMP levels depress the rate of messenger initiations from inducible operons, the level of cAMP should be lowered in our cells within 10 s after the addition of glucose. Making meaningful cAMP measurements on this time scale necessitated the development of techniques for rapid sample collection. The previously used methods of centrifugation or filtration of large numbers of cells are wholly inappropriate for

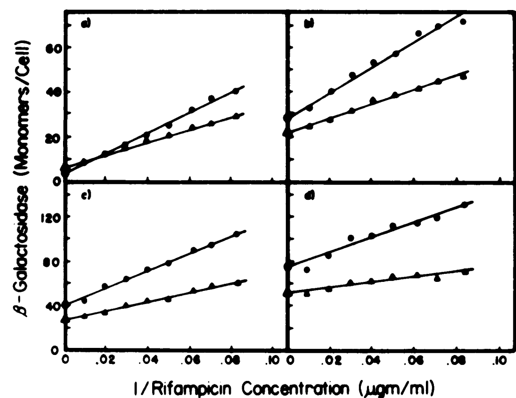

FIG. 2. *The synthesis of β-galactosidase in the presence and absence of glucose as a function of rifampin concentration. Inducer and glucose or glycerol were added at the start of the induction interval. After the appropriate time interval varying amounts of rifampin were added to different cultures. The concentrations of rifampin are given by the inverse of the values on the abscissa. Extrapolation of the least mean squares fit to zero yields the amount of enzyme that would have been produced if rifampin acted immediately upon addition to the cultures. Symbols: ●, 0.5% glycerol; and ▲, 0.5% glucose. Rifampin was added at the following times: (a) with inducer, (b) 15 s after inducer, (c) 25 s after inducer, and (d) 40 s after inducer.*

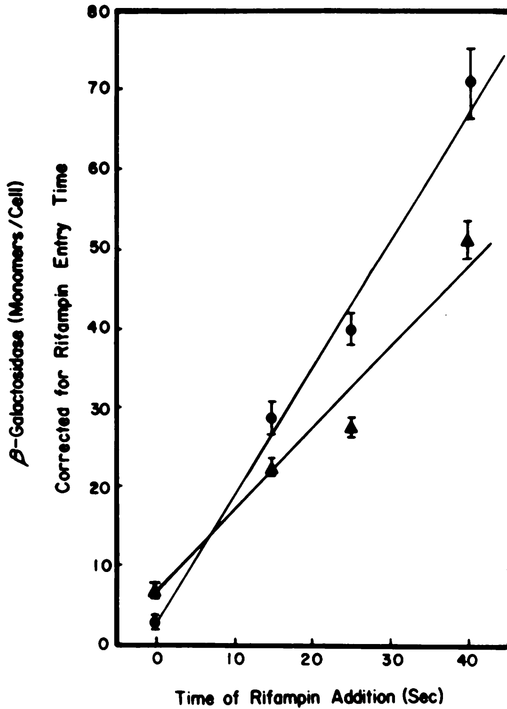

FIG. 3. *Synthesis of β-galactosidase corrected for rifampin entry time. Calculated enzyme levels corresponding to zero entry time as determined in Fig. 2. Symbols: ●, 0.5% glycerol; and ▲, 0.5% glucose. Error bars represent one standard deviation.*

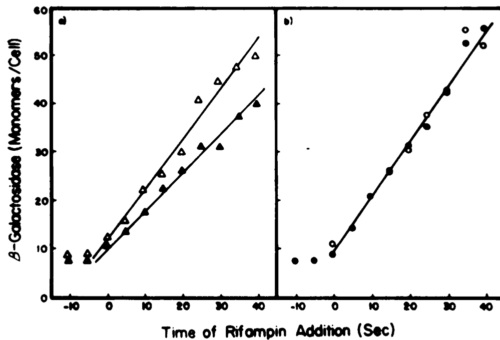

FIG. 4. *The effect of exogenous cAMP on the synthesis of β-galactosidase in the presence and absence of glucose. Cyclic AMP (5 mM) was added 4 min before induction was begun. Control cultures received medium instead of cAMP. Induction was as described in Fig. 1. Symbols: (a) ▲, 0.5% glucose; and △, 0.5% glucose plus cAMP; (b) ●, 0.5% glycerol; and ○, 0.5% glycerol plus cAMP.*

our needs. In addition, the long collection times, slowly changing temperatures, and anaerobic conditions associated with most of the commonly used cell collection methods raise the possibility that intracellular cAMP levels have changed appreciably during sample collection. An additional problem associated with sample collection is the need for complete, but nontraumatic, removal of cells from growth medium. This need arises from the fact that growing cells excrete significant quantities of cAMP, and at the time of measurement, 98% of the cAMP in the flask is extracellular (31). In order that meaningful measurements be made on the remaining intracellular 2%, the extracellular cAMP must be removed. We found that filtration of up to 1.4×10^9 cells in 10 ml of medium on 47-mm diameter membrane filters (Millipore) followed by rinsing with 10 ml of prewarmed medium provided very rapid removal of extracellular cAMP. Further metabolism, either synthesis or degradation, of cAMP was quickly halted by immersing the filter containing the cells in boiling water. Complete release of cAMP from the cells was effected by acidifying with hydrochloric acid and heating. The sample was further acidified and cAMP was separated from much cellular debris by chromatography on Dowex. The column eluate was concentrated and finally assayed for cAMP by its ability to compete with radioactive cAMP in binding to a cAMP-binding protein in the assay developed by Gilman (13; Fig. 5a). As a control for this experiment, as in the induction experiments, a parallel culture received an additional 0.5% glycerol in place of glucose. The samples were treated as described for glucose and the cAMP level determined (Fig. 5b). In order to determine cAMP levels before the addition of any sugar, a third culture was filtered and treated as above without receiving any additional sugar or medium.

It can be seen that cAMP levels do drop within 10 s. However, comparison of the results obtained on cultures having received glucose to

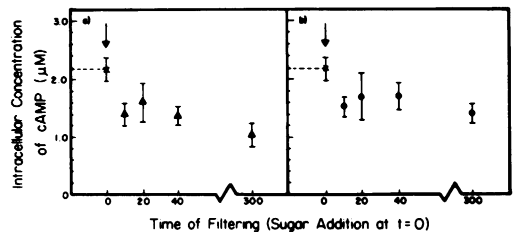

FIG. 5. *Intracellular concentration of cAMP in the presence and absence of glucose. Glycerol or glucose was added at zero time and sample collection was begun at the indicated time. Intracellular concentration is based on an accessible cell volume of 10^{-15} liters. Symbols: ×, no addition; ●, 0.5% glycerol; and ▲, 0.5% glucose. Error bars represent one standard error of the mean.*

those having received glycerol indicates that this fall in cAMP levels is the same in both cases. To test whether this lowering of cAMP levels was due to excess sugar, an amount of medium equivalent to what was added with the glucose and glycerol was added to cells, and the standard protocol was used to determine the cAMP content. The cAMP level was lowered to that observed just after the addition of glucose or glycerol. It thus appears that the decrease in cAMP is caused by a component of the medium and not the glucose. Such an effect has been reported recently (27). After longer time periods, about 5 min, the cAMP levels in the presence of glucose appear to be decreasing with respect to the level in the presence of glycerol. Nonetheless, the results do not show that cAMP levels differ between cultures receiving glycerol and cultures receiving glucose at a time when their inducibility certainly does differ. The error limits of the cAMP measurements, however, do not preclude a small difference in cAMP levels between the glucose samples and the glycerol controls.

DISCUSSION

Our results indicate that parallel treatment of growing cells (addition of glucose or glycerol) in which the intracellular cAMP levels respond similarly lead to easily detected differences in the inducibility of the *lac* operon. Within 10 s, the culture having received glucose was markedly depressed in its inducibility. This repression was identified as catabolite repression by its reversibility by the addition of cAMP to the medium. Since the intracellular cAMP levels in cells having received glucose were nearly identical to those in cells having received glycerol, we conclude—subject to the reservations to be discussed—that lowered intracellular cAMP levels are not the sole effector of catabolite repression. It can be argued that minor differences in cAMP levels, differences that are within the error limits of our measurements, could result in the observed depression in enzyme synthesis. The rate of transcriptional initiation could be sensitive to small changes in cAMP levels. However, this is unlikely in view of the in vivo experiments of Lis and Schleif (17) in which they observed a less than linear increase in enzyme level relative to an increase in exogenous cAMP.

Numerous measurements of the intracellular levels of cAMP in *E. coli* have been made in the past. However, there has been little agreement in the actual level (4, 5, 31). Some of the differences undoubtedly arise from strain differences. For example, the strain we used, AS19, does not display transient repression as do many strains. Another serious source of the non-reproducibility is the use of cells which have entered stationary phase, where they may be partially anaerobic, nutrient starved, or poisoned by excreted products. In order to have reproducible data as well as results which could be compared to the vast amount of existing physiological data, we performed all measurements on cells in stable exponential growth at densities below 2×10^8 cells/ml. In view of the rapid changes in cAMP levels that could occur, and indeed which we found do occur, we kept the time required for harvesting the cells to a minimum. While the time required, 8 s, was longer than we desired, it is an order of magnitude shorter than that used previously. Nonetheless, our results must still be subject to the reservation that substantial changes in cAMP levels could have occurred in this 8 s.

In this and all previous work, the total amount of intracellular cAMP was determined. However, since levels of cAMP-CRP complex and not cAMP are responsible for transcription initiation from catabolite repressible operons, it is possible that cells lower the amount of active cAMP-CRP complex without reducing the number of cAMP molecules in the cell. Two simple ways to accomplish this are to prevent cAMP from binding to CRP either by directly blocking its binding or by binding cAMP to some other protein. Either of these two possibilities could be feasible without consuming excessive cellular protein since the intracellular levels of cAMP, 1 to 3 μM, represent only about one thousand molecules per cell. In vitro studies have suggested the first possibility. It has been shown that cGMP inhibits transcription by binding to CRP and preventing the formation of the cAMP-CRP complex. The subsequent discovery of cGMP in *E. coli* and the observation that its levels rise after the addition of glucose suggested the involvement of cGMP in control of catabolite repression (4). However, the concentration of cGMP in vivo (4) is significantly lower than that of cAMP, while the concentration of cGMP needed to inhibit in vitro transcription is approximately equal that of cAMP (28).

We have not completely eliminated the ambiguity that could arise from a change in translational efficiency. It must be kept in mind that we are determining messenger initiations by measuring the resulting enzyme; therefore, any factor that alters the translation of messenger into protein will affect our results. However, this does not appear to be so in this work, as evidenced by the reversal of glucose inhibition

by cAMP which is known to act at the level of transcriptional initiation. Cultures which received cAMP as well as glucose yielded the same results as cultures receiving only glycerol or glycerol and cAMP. As added cAMP did not affect enzyme levels in the presence of glycerol, it appears that cAMP does not affect translation. These results also indicate that glucose acts only at the transcriptional initiation level as cAMP did increase synthesis in the presence of glucose to that seen in the presence of glycerol.

That translational efficiency is not affected by the presence of glucose or glycerol has been further tested by adding glucose or glycerol after initiations have been blocked by rifampin but before significant protein has been synthesized. Therefore, starting with equivalent amounts of mRNA, the amount of enzyme synthesized in the presence of glucose can be compared to the amount of enzyme synthesized in the presence of glycerol. Under these conditions neither glucose nor glycerol affected the amount of enzyme produced.

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