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Effect of Dibutyryladenosine 3′:5′-Cyclic Monophosphate on Growth and Differentiation in *Caulobacter crescentus*

(β-galactosidase/*Escherichia coli*/cyclic AMP/catabolite repression)

LUCILLE SHAPIRO, NINA AGABIAN-KESHISHIAN, ALLEN HIRSCH, AND ORA M. ROSEN

Department of Molecular Biology, Division of Biological Sciences, and Department of Medicine,
Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT ***Caulobacter crescentus* goes through a series of morphological changes during its life cycle, including the coincident expression of synthesis of flagella, pili, and receptor sites for DNA bacteriophage. Upon transfer of a mixed population of cells to medium containing lactose as the sole carbon source, these changes were blocked for about 20 hr until β-galactosidase activity became apparent. The addition of dibutyryl cyclic AMP to the blocked cultures brought about the resumption of cell differentiation, growth, and the appearance of β-galactosidase activity within 1 hr. Unlike *Escherichia coli*, the intracellular and extracellular concentrations of cyclic AMP in *C. crescentus* did not vary under several growth conditions, including catabolite repression. It would appear, therefore, that although there is an effect of cyclic AMP on the induction of β-galactosidase and differentiation in *C. crescentus*, regulation of these processes occurs without consistent changes in the cellular level of this nucleotide.**

It is becoming increasingly evident that cyclic 3′:5′-AMP (cAMP) plays a major role in regulating growth and differentiation in both eukaryotic and prokaryotic cells (1-4). This small effector molecule appears to regulate the transcription of mRNA for many inducible enzyme systems in the Enterobacteriaceae (2). Since *Caulobacter crescentus* is a eubacterium that exhibits defined morphogenesis during each division cycle (5, 6), we have begun an investigation of the role of cAMP in the expression of differentiation events and in the induction of enzymes required for the growth of this organism.

MATERIALS AND METHODS

Materials

Cyclic 3′:5′-AMP, dibutyryl cyclic 3′:5′-AMP, and 5′-AMP were purchased from Sigma Chemical Co. *o*-Nitrophenyl-β-D-galactoside and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from Mann Research Laboratories. [^{3}H]Adenosine (23.4 Ci/mmol) and [^{3}H]cyclic AMP (16.3 Ci/mmol) were purchased from Schwarz BioResearch. Omnifluor was obtained from New England Nuclear Corp. Protein kinase inhibitor protein was generously donated by Dr. Charles Rubin, and cyclic nucleotide phosphodiesterase was a gift of Dr. Elihu Goren, both of this laboratory.

Growth conditions

Caulobacter crescentus strain CB13B1a was grown at 30° in minimal medium containing 0.5 g NH_4Cl, 1.74 g Na_2HPO_4, 1.06 g KH_2PO_4, and 10.0 ml of modified Hutner's mineral base (6) per liter of deionized water in the presence of either glucose (0.2%) or lactose (0.5%) as a source of carbon. Under these conditions the generation time of *C. crescentus* was 220 min. In nutrient broth, containing 0.2% bactopeptone, 0.1% yeast extract, and 0.02% $MgSO_4 \cdot H_2O$, *C. crescentus* had a generation time of 110 min. The glucose content of this medium was less than 0.03%.

Bacteriophage φCbK was cultured and purified as described (8) in the presence of 1 mCi of [^{3}H]adenine. It has been shown that only one form of *C. crescentus* is a host for φCbK (9). For determination of the progress of the host's life cycle in relation to the number of cells sensitive to phage infection, the injection of φCbK DNA was measured as described (9). Bacterial motility was monitored with a Zeiss phase contrast microscope.

Measurement of intra- and extracellular levels of cAMP

Bacteria grown under specified conditions were centrifuged at 12,000 × *g* in a Sorval RC2-B centrifuge for 10 min, and the pellet (0.3–0.4 g wet weight) was suspended in 10 volumes of 0.05 N HCl and heated at 95° for 10 min. The sample was then centrifuged at 12,000 × *g*, and the supernatant fluid was adjusted to pH 7.0 with NaOH. An aliquot (0.15 ml) was added to a reaction mixture containing 10 μmol of Tris·HCl (pH 8.0), 1 μmol of $MgSO_4$, and either 3 μg of purified beef heart cyclic nucleotide phosphodiesterase (10) or H_2O in a final reaction volume of 0.175 ml. The reaction mixtures were incubated at 35° for 60 min, after which the reaction was terminated by heating at 95° for 5 min. A sample of each of these reaction mixtures was then assayed for cAMP by a modification of the procedure described by Gilman (11). The total reaction volume of 0.2 ml contained 0.05 ml sample, 10 mmol of sodium acetate (pH 4.0), 50 μg of protein kinase inhibitor (12), 5 pmol of [^{3}H]cyclic AMP (16.3 Ci/mmol), and 2.5 μg of cAMP binding protein. The binding protein was prepared from bovine skeletal muscle as described by Gilman (11). Reaction mixtures were incubated for 60 min in an ice bath and then diluted with 1.0 ml of 20 mM potassium phosphate (pH 6.0). The mixtures were poured onto Millipore filters and washed with a total of 15 ml of the same buffer. The filters were then dried, placed in vials containing Omnifluor (4 g Omnifluor/liter toluene), and counted in a liquid scintillation spectrometer. The content of cAMP in the original sample was calculated from the difference between the [^{3}H]cyclic AMP retained by the filter with and without pre-

Abbreviations: cAMP, cyclic 3′:5′-AMP; IPTG, isopropyl-β-D-thiogalactopyranoside.

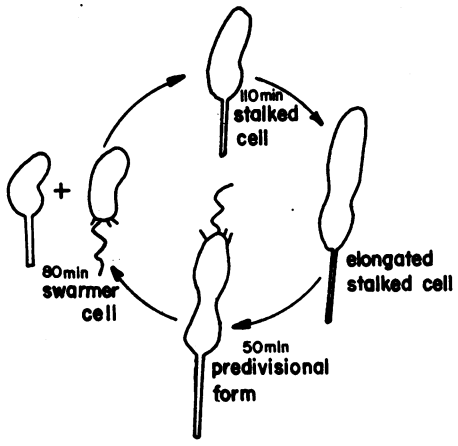

FIG. 1. Scheme of the life cycle of *Caulobacter crescentus* in nutrient broth. (see *Methods*).

treatment of the aliquot with diesterase. Final molarities for the intracellular concentrations of cAMP were calculated as described by Makman and Sutherland (13). The intracellular concentrations of cAMP in *E. coli* B grown in either nutrient broth or minimal medium plus glycerol (0.5%) were found to be the same (5×10^{-7} M).

Extracellular concentrations of cAMP were measured as above after the culture filtrates were concentrated by lyophilization and resuspended in 1/10 volume of H_2O.

RESULTS

Growth patterns of *C. crescentus* in different media

During a single life cycle of *C. crescentus* there are two distinct patterns of morphogenesis: (*a*) a *swarmer cell* becomes a *stalked cell*, and (*b*) an *elongated stalked cell* takes on swarmer cell characteristics, forming a *predivisional cell* (Fig. 1). Upon cell division, the stalked bacteria give rise to a motile swarmer cell and a sessile stalked cell. In order to begin another division cycle, the swarmer cell must differentiate into the stalked form. Among the events occurring during this differentiation are the loss of the flagellum and the synthesis of a new stalk at the precise location of the old flagellum (5). The mature stalked cell then enlarges to form an *elongated stalked cell*. A second defined series of morphological changes occurs at one pole of this cell, resulting in the transition of the *elongated cell* to the *predivisional form*. This differentiation event involves the synthesis of at least three new cellular components. These are a flagellum (Shapiro and Maizel, unpublished), pili which are receptor sites for RNA bacteriophage (14, 15), and a lipopolysaccharide component, which is a receptor site for DNA phage necessary for the injection of phage ϕCbK DNA (6, 8, 9).

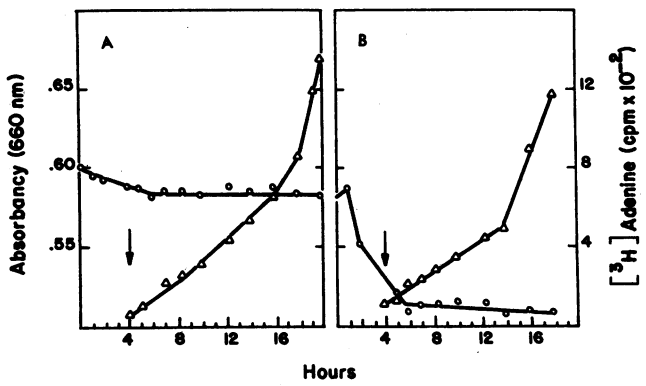

FIG. 2. The effect of dibutyryl cyclic AMP on growth and formation of a phage receptor site in *C. crescentus* growing on minimal medium plus lactose. *C. crescentus* was grown to mid-logarithmic phase in minimal medium plus glucose (0.2%); cells were collected by centrifugation at 12,000 × *g* for 10 min, and the pellet was gently resuspended in prewarmed minimal medium containing 0.5% lactose. (*A*) The absorbance at 660 nm was measured as a function of time; (*B*) the ability of the culture to accept bacteriophage ϕCbK DNA labeled with [^{3}H]adenine was measured as described in the *text*, both in the absence (O——O) and presence (Δ——Δ) of 3×10^{-3} M dibutyryl cyclic AMP. The nucleotide was added to the media 4 hr after the shift from glucose to lactose.

Growth in minimal medium plus glucose resulted in a doubling of the generation time and proportional increases in the times required for morphological changes. As in nutrient medium, flagella, pili, and phage receptor sites appeared coincidently. When cultures grown to mid-logarithmic phase in minimal medium plus glucose were shifted to minimal medium plus lactose, the cells completed a single life cycle and stopped further morphogenesis and growth at the *elongated stalked* cell form. The optical density of the culture remained constant for about 16-20 hr before growth resumed (Fig. 2*A*). Evidence that the cell culture became blocked at the *elongated stalked cell* upon transfer to minimal medium plus lactose include: (*a*) the disappearance of the cell type that acts as host for bacteriophage ϕCbK (Fig. 2*B*), (*b*) cessation of motility, which is a function dependent upon flagella, and (*c*) the appearance, microscopically, of a homogeneous population of elongated stalked cells.

The ability of the culture to accept phage ϕCbK DNA was measured as an index of the continued production of *predivisional cells* containing the phage receptor site. After the shift to minimal medium plus lactose the injection of labeled phage DNA steadily decreased until 4 hr, when it essentially stopped (Fig. 2*B*).* Since the normal growth cycle of *C. crescentus* in minimal medium takes about 220-240 min, it appears that the loss of receptor sites during a 4-hr period corresponds to the completion of the life cycle of each cell in the initially mixed population.

If a cell had just entered the predivisional form at the time of the shift to minimal medium plus lactose, it would take about 110-120 min for the loss of flagella and, consequently, motility. It was observed, microscopically, that motility ceased within the first 2 hr after the shift, and by 4 hr the entire population was blocked at the elongated stalked cell stage. After about 20 hr in this medium, however, motility was again observed; the cells resumed normal growth and synchronously entered the *predivisional* phase of the differentiation cycle.

Effect of dibutyryl 3′:5′-cyclic AMP on growth in lactose

The addition of dibutyryl cyclic AMP (3×10^{-3} M) to a 4-hr culture blocked in minimal medium plus lactose resulted in the resumption of growth, appearance of motile swarmer cells with 1 hr, and the appearance of a phage receptor site for

* In cell-free extracts bacteriophage ϕCbK injects its DNA into metabolically inert cell fragments as long as the intact LPS receptor site is available to the phage (6).

phage ϕCbK (Fig. 2). A slight effect was observed with cAMP at 5×10^{-3} M. The stimulatory effect of these cyclic nucleotides could not be replaced by the addition of 3×10^{-3} M 5'-AMP, 5'-GMP, or up to 10^{-2} M butyrate.†

When shifted from a medium containing glucose to one containing lactose, *C. crescentus* required the addition of exogenous cAMP to grow and differentiate. It appeared possible, therefore, that the enzymes required for lactose utilization and growth, such as β-galactosidase, could not be synthesized until sufficient quantities of cAMP had accumulated within the cells. The intracellular concentrations of cAMP in cells growing in minimal medium with either glucose or lactose as a sole carbon source were very low; the small differences that were detected between cells grown in glucose and those grown in lactose were not considered significant. The addition of dibutyryl cyclic AMP to cultures of cells growing in minimal medium plus lactose brought about the rapid appearance of β-galactosidase activity (Fig. 3).

Without added nucleotide, enzymic activity first appeared between 20 and 22 hr, and reached maximal activity (about 45 nmol/mg per hr) at 48 hr after the shift. If dibutyryl cyclic AMP was added to the blocked culture after a shift to minimal medium plus lactose, β-galactosidase activity was detected within 1-2 hr, and reached a maximal level at 30 hr.

There appears, therefore, to be a time period in the *Caulobacter* division cycle, just prior to the general "turning on" of the differentiated functions in the *elongated stalked cell*, that is particularly sensitive to nutritional deprivation. After a period of time the enzymes required for lactose utilization are synthesized and the cells can overcome the block and resume differentiation and growth. The addition of exogenous dibutyryl cyclic AMP enables the blocked cells to rapidly resume growth and differentiation coincident with the appearance of β-galactosidase.

Cyclic AMP concentrations under conditions of catabolite repression

Because of the correlation between intracellular concentration of cAMP and the induction of catabolic enzymes, such as β-galactosidase in *E. coli* (2), the phenomenon of catabolite repression of inducible enzymes was examined in *C. crescentus*. Lactose was used to induce β-galactosidase, since *C. crescentus* appeared to be impermeable to isopropyl-β-D-thiogalactoside (IPTG), methyl-β-D-galactoside, or methyl-β-D-thiogalactoside. Cells were grown to mid-logarithmic phase in nutrient broth and then divided into two flasks of fresh nutrient broth. To one was added lactose (0.2%), and to the other glucose (0.1%) plus lactose (0.2%). The induction of β-galactosidase was diminished in the culture of *C. crescentus* growing in the presence of lactose plus glucose (Fig. 4). β-Galactosidase activity was not detected until 3 hr under these conditions, but was detected within 1 hr in the culture containing lactose alone. The repression of β-galactosidase activity was observed in the presence of 0.1% glucose even when up to 1.0% lactose was present. Unlike the situation in *E. coli* (16), the addition of 3×10^{-3} M dibutyryl cyclic AMP to the culture containing both glucose and lactose did not relieve the transient repression of β-galactosidase (Fig. 4). During the 3-hr period of repression the intracellular levels of cAMP in the cultures containing lactose and lactose plus glucose were found to be the same, although there was a 4- to 5-fold difference in the specific activity of β-galactosidase (Table 1). An appreciable amount of cAMP was detected in the culture filtrate, but here, too, the concentration was essentially the same in both cultures. Measurements of concentrations of cAMP at earlier time-points during these conditions of repression (30 min and 60 min) showed lower concentrations of cAMP, but the concentrations in both cultures were always similar. During the period of transient catabolite repression in *E. coli* that had been grown on minimal medium plus glycerol and induced with IPTG, the intracellular level of cAMP was consistently 2- to 5-fold higher in the absence of glucose (Table 2). Under these conditions the specific activity of β-galactosidase was 4- to 5-fold higher in the absence than in the presence of glucose. It appears, therefore, that during catabolite repression

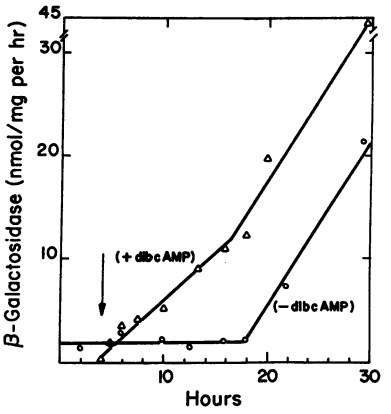

FIG. 3. The effect of dibutyryl cyclic AMP on β-galactosidase activity. *C. crescentus*, growing in minimal medium plus glucose (0.2%) were shifted to minimal medium plus lactose, as described in the legend to Fig. 2. β-Galactosidase was measured by the method of Pardee, Jacob, and Monod (18) on cultures grown in the absence (O——O) and presence (Δ——Δ) of 3×10^{-3} M dibutyryl cyclic AMP (dib CAMP).

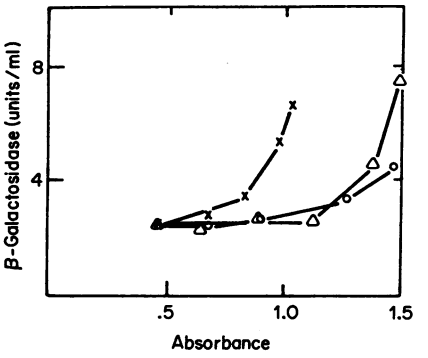

FIG. 4. Effect of glucose on lactose stimulation of β-galactosidase activity. *C. crescentus* was grown to mid-logarithmic phase in nutrient broth in the absence of added hexoses and divided into three flasks to which were added either 0.2% lactose (×——×), 0.2% lactose plus 0.1% glucose (O——O), or 0.2% lactose, 0.1% glucose, and 10^{-3} M dibutyryl cyclic AMP (Δ——Δ). Absorbance and β-galactosidase activity were measured every hour for 6 hr as described in Fig. 3. One unit of enzyme is defined as producing 1 nmol *o*-nitrophenol/hr at 28° (pH 7.0).

† Although *C. crescentus* is a gram-negative bacterium, its cell wall differs from that of *E. coli* in that it lacks the components of the lipid A portion of a normal lipopolysaccharide (6). This difference in wall structure may result in the observed preference for dibutyryl cyclic AMP.

TABLE 1. *Intra- and extracellular concentrations of cyclic AMP in C. crescentus during catabolite repression*

	Hr	Absorbancy (660 nm)	β-Galactosidase (nmol/mg per hr)	Intracellular cAMP ($M \times 10^{-7}$)	Extracellular cAMP ($M \times 10^{-9}$)
Lactose	0	0.36	0	1.0	—
	3	0.90	2.86	9.9	4.2
Lactose + glucose	0	0.39	0	1.0	—
	3	1.02	0.53	10.1	5.3

Conditions of growth are as described in the legend to Fig. 4. Cyclic AMP concentrations were measured as described in the *text*.

in *C. crescentus* a consistent change in the cellular level of cAMP was not detectable under the same assay conditions that registered changes in *E. coli*.

The first and most dramatic demonstration of the depressive effect of glucose on intracellular concentrations of cAMP was in nongrowing cultures of *E. coli* (13). Makman and Sutherland proposed that glucose suppressed the formation of cAMP and stimulated a mechanism for the rapid release of cAMP from the cell (13). In experiments modeled after those of Makman and Sutherland, *E. coli*, *Pseudomonas aeruginosa*, and *C. crescentus* were grown to mid-logarithmic phase in minimal medium plus glucose and then transferred to either potassium phosphate buffer, or potassium phosphate buffer plus glucose (Table 3). The cAMP content of *E. coli* cells incubated in potassium phosphate buffer was 80-fold greater than that of cells incubated in potassium phosphate buffer plus glucose, and in *P. aeruginosa* there was a 9-fold difference in concentrations of cAMP. In *C. crescentus*, however,

TABLE 2. *Intracellular levels of cyclic AMP in E. coli during catabolite repression*

	Hr	Absorbancy (660 nm)	β-Galactosidase (μmol/mg per hr)	Intracellular cAMP ($M \times 10^{-7}$)
Glycerol + IPTG	0	0.46	0.52	—
	30	0.59	8.55	5.3
Glycerol + IPTG + glucose	0	0.49	0.34	—
	30	0.62	0.86	1.4

E. coli B was grown in minimal medium containing 0.5% glycerol to mid-logarithmic phase. The cells were collected by centrifugation at 12,000 × *g* for 10 min and resuspended in fresh prewarmed minimal medium plus glycerol. The culture was incubated at 37° for 60 min and then 10^{-3}M IPTG was added to one aliquot and 10^{-3}M IPTG plus 1.5% glucose was added to another. Absorbancy and β-galactosidase activity were measured as described in Fig. 3. Intracellular levels of cAMP were measured as described in the *text*.

TABLE 3. *Effect of glucose on intracellular levels of cyclic AMP*

Bacteria	Medium	Cyclic AMP ($M \times 10^{-7}$)
Escherichia coli B	PO_4	757.9
	PO_4 + glucose	9.3
Pseudomonas aeruginosa	PO_4	28.7
	PO_4 + glucose	3.0
Caulobacter crescentus	PO_4	1.5
	PO_4 + glucose	1.1

E. coli, *P. aeruginosa*, and *C. crescentus* were each grown in minimal medium containing 1% glucose. The cells, treated as described by Makman and Sutherland (13), were centrifuged at 25° and resuspended in 0.125 M phosphate buffer (pH 7.0), or 0.125 M phosphate buffer (pH 7.0) containing 1% glucose and were incubated for 30 min with shaking at 37°. Intracellular levels of cAMP were measured as described in the *text*.

cAMP failed to accumulate in the absence of glucose, and no difference was observed in the cAMP content of cells incubated in the presence or absence of glucose. It may be, therefore, that *C. crescentus* lacks a mechanism for retaining cAMP.

DISCUSSION

When *C. crescentus* was forced to use lactose as a sole carbon source, the general expression of differentiation functions was temporarily blocked at a specific stage in the life cycle concomitant with a delay in the synthesis of the inducible enzyme, β-galactosidase. The cell cultures arrested at the stage of the *elongated stalked cell* just prior to the localized expression of swarmer cell characteristics that marks the *predivisional form*. Exogenous dibutyryl cyclic AMP greatly accelerated the ability of the arrested cells to induce β-galactosidase, and to both grow and differentiate. It cannot be determined at this time whether the exogenous cAMP had a primary effect on the expression of differentiated functions, such as the synthesis of flagella, pili, and phage receptor sites, or whether it facilitated the induction of enzymes needed for growth on lactose, permitting the cells to continue their growth cycle and thus differentiate. Experiments are now in progress with mutants of *C. crescentus* that are unable to use lactose unless cAMP is added to the medium, in order to further define the regulation of differentiation events in relation to the growth potential of the cells. It has been observed that the specific activity of adenylate cyclase varies during the *C. crescentus* cycle in nutrient media, consistently increasing 2- to 3-fold at the *predivisional form* (Sun, unpublished). Since cells blocked just before the appearance of the *predivisional form* can be rescued by cAMP, it appears possible that nutritionally starved cells are unable to achieve the elevated levels of adenylate cyclase needed to synthesize sufficient cAMP for this cell transition to occur.

The synthesis of the inducible catabolic enzyme, β-galactosidase, was transiently repressed by glucose in *C. crescentus*, although the intracellular and extracellular levels of cAMP in cultures exhibiting this repression were the same as in unrepressed cultures. As would be expected from these results, the repression could not be overcome by the addition of exogenous dibutyryl cyclic AMP. In the case of *E. coli*, under conditions of transient repression, the intracellular levels of

cAMP were about 2- to 5-fold lower in the presence of glucose than in its absence‡, and it has been previously established that exogenous cAMP overcomes repression by glucose (16). The manner in which glucose regulates inducible catabolic enzymes in *C. crescentus* is unknown. It may be that the concentrations of cAMP were changing in the *C. crescentus* cultures, but that the changes were too rapid or too small to be detected by the assay used. However, the specific activity of adenylate cyclase (5 nmol/mg per hr) remained constant under conditions of repression and the extracellular concentrations of cAMP were the same when the cells were growing in the presence and absence of glucose. It was also observed that under conditions in which the intracellular concentration of cAMP in *E. coli* changed dramatically, i.e., the transfer of glucose-grown cells to potassium phosphate buffer, the intracellular concentration of cAMP in *C. crescentus* was unaltered. The fact that glucose appears unable to effect stable changes in the intracellular concentrations of cAMP in *C. crescentus* may reflect an efficient cyclic nucleotide secretory mechanism functioning independently of environmental control.

Inherent in the life cycle of *C. crescentus* is the functioning of a biological clock that controls the expression of defined morphological changes occurring at specific times and at specific sites in the cell. Although cell septation and division in *E. coli* represents a similarly defined sequence of events, the possibility exists that the more elaborate changes occurring in the *Caulobacter* cell require different or additional mechanisms of control. It may be that it is necessary for the *Caulobacter* cell to maintain a generally low level of small regulatory molecules, such as cAMP, which can be rapidly and transiently modulated independent of gross environmental conditions in order for the cell to perform a defined function at a specified time.

‡ Pastan and Perlman (2) have reported a 10-fold drop in cAMP concentration when glucose was added to a culture of *E. coli* growing on glycerol. Monard, Janecek, and Rickenberg reported a 2-fold decrease in intracellular levels of cAMP in *E. coli* during catabolite repression of a culture grown in glycerol by glucose (17).

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