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Cyclic 3′:5′-Adenosine Monophosphate in *Escherichia coli* during Transient and Catabolite Repression

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ABSTRACT **Cyclic AMP concentrations were low for an adenylate cyclase-deficient mutant (*cya*$^-$) and abnormally high for a catabolite receptor protein-deficient mutant (*crp*$^-$). A fall in cellular cAMP concentration was always found when cells were subjected to transient repression. No consistent correlations were observed between catabolite repression and cellular cAMP levels.**

cAMP is believed (1, 2) to regulate glucose-induced transient and permanent (catabolite) repression of β-galactosidase and other enzymes in *Escherichia coli*. There are five lines of evidence linking cAMP to enzyme repression. (*a*) High concentrations of exogenous cAMP reverse transient and catabolite repression *in vivo* (3, 4). (*b*) Mutants (*cya*$^-$) deficient in adenylate cyclase activity and deficient in catabolite-sensitive enzymes can be corrected by exogenous cAMP (5). (*c*) Other mutants (*crp*$^-$) deficient in catabolite-sensitive enzymes and containing an altered cAMP-binding protein (CRP) are not corrected by cAMP (6). (*d*) cAMP (7, 8) and CRP (9–13) are required for *in vitro* transcription of the *lac* and *gal* (13) operons. (*e*) Glucose has been reported to inhibit cAMP accumulation both intracellularly (1, 14, 15) and in the medium (14).

Measurements of cAMP in growing *E. coli* are scarce (1, 15–18). We now wish to report levels of cAMP in several strains of *E. coli* growing in glucose medium during transient and catabolite repression. We find a good correlation between transient repression of β-galactosidase (β-gal EC 3.2.1.23) synthesis and reduced levels of cellular cAMP, but no consistent relation between cAMP levels and catabolite repression.

MATERIALS AND METHODS

Strains. All strains are K-12 derivatives. ATCC 23804 (*lacY*$^-$) and W3100 (K-12 wild type) came from American Type Culture Collection, Rockville, Md. Dr. M. Saier sent us strain 1100 and two mutants derived from it, 5333 (*crp*$^-$) and 5336 (*cya*$^-$) (5, 12).

Growth Conditions. We grew cells in 1% (w/v) glucose or 1% (v/v) glycerol medium containing, per liter, 14.0 g of K_2HPO_4, 6.0 g of KH_2PO_4, 2.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, and 100 μg of thiamine with a final pH of 7.0. Adenine (20 μg/ml) was added for strain 23804.

Individual colonies were grown to log phase at 37° in nutrient broth, diluted into minimal medium and grown overnight. In the morning we diluted the culture 1:10 into fresh medium. We grew the cells in flasks with a capacity twice the volume of medium, agitated at 150 rpm in a gyrotory shaker at 37°. An OD_{550} of 1.0 corresponds to 1.4×10^9 cells per ml or 1.62 mg of wet weight per ml. Cellular concentrations were calculated from levels of cAMP and wet weight, assuming a cell density of 1.

Chemicals. Isopropyl-β-D-thiogalactopyranoside (IPTG), *o*-nitrophenyl-β-D-galactoside (ONPG) and cAMP came from Sigma; [^{3}H]cAMP (12.8 Ci/mmol) from Schwarz/Mann; Omnifluor scintillant from New England Nuclear Corp. Dowex AG 50W-X8 resin, 100-200 mesh, was from Bio-Rad. The resin was washed with 0.1 N NaOH, 0.1 N HCl, and deionized water before use.

β-Gal Assay. We adapted Pardee and Jacob's method (19) by agitating either 0.5 ml or 50 μl of cells with 25 μl of toluene, adding 0.2 ml of 4 mg/ml of ONPG in 0.25 M sodium phosphate buffer, pH 7.0, and incubating at 37° for 5–10 min. The assay was stopped with 0.5 ml of 1 M Na_2CO_3. One unit of enzyme hydrolyzes 1 nmol of ONPG per min.

Measurement of Cellular cAMP. We filtered approximately 3×10^9 cells onto nitrocellulose filters (24 mm; 0.45 μm pore diameter) that had been boiled 5 min in water. Filtering took from 30 sec to 2 min. Cells were immediately washed with 5 ml of prewarmed medium of the same composition as that from which the cells were taken. Filters were then immersed in 5 ml ice cold 5% CCl_3COOH (TCA) containing 1 pmol of [^{3}H]cAMP. We used three filters per sample point

TABLE 1. *Effect of washing on apparent cellular concentration of cAMP*

Wash (ml)	cAMP (μM)
0	19.8
1	10.8
2	8.8
5	7.6
10	8.6
20	7.8

Cells of strain 23804 were grown in glucose medium to an OD_{550} of 0.697. Aliquots (5 ml) were filtered, washed with prewarmed fresh medium, extracted in boiling 0.1 N HCl, and assayed for cAMP.

Abbreviations: *cya*, gene coding for adenylate cyclase-deficient phenotype; *crp*, gene coding for cAMP receptor protein; CRP, cAMP receptor protein; IPTG, isopropyl-β-D-thiogalactopyranoside; ONPG, *o*-nitrophenyl-β-D-galactoside; TCA, CCl_3COOH; β-gal, β-galactosidase.

TABLE 2. *Cellular and extracellular concentration of cAMP in strains of E. coli*

Strain	Carbon source	Cellular cAMP (μM)		Extracellular cAMP (pmol/ml per OD_{550})	
		mean	range	mean	range
1100	Glucose	5.2 (7)	2.8–9.8	182 (6)	143–251
	Glycerol	4.2 (6)	1.4–6.2	548 (4)	371–926
5333	Glucose	61.8 (2)	52.2–70.4	1210 (2)	1111–1309
	Glycerol	18.2 (1)	—	6830 (1)	—
5336	Glucose	2.6 (3)	0–4.8	38 (3)	9.1–69.6
	Glycerol*	—	—	—	—
23804	Glucose	9.4 (3)	4.4–19.2	1594 (7)	1069–2915
	Glycerol	18.8 (5)	11.6–23.6	3483 (11)	1500–6200

Cultures were grown many generations in the indicated media and assayed for cellular and extracellular cAMP. The number of experiments is indicated in parentheses.

* Strain 5336 (*cya*$^-$) fails to grow on glycerol.

for strains 23804 and 5333, six to eight filters for strains 1100, 5336, and W3100. The sample was frozen and thawed 3 times, spun briefly and decanted onto Dowex AG 50W-X8 columns, 200 × 8 mm (18). Results were similar when filters were boiled in 0.1 N HCl or in H_2O instead of being frozen and thawed. cAMP was eluted with water and the fraction was evaporated to dryness. In each experiment we ran a blank column with TCA and H_2O. All samples were resuspended in 250 μl of 0.02 M Tris·HCl, pH 7.3, assayed in duplicate (60-μl aliquots) for cAMP using the Gilman technique (20), and monitored for recovery (average, 45% after all steps). The Dowex blank (21) ran at 6–9 pmol of cAMP equivalents per 60-μl aliquot.

Two hundred micrograms of cyclic nucleotide phosphodiesterase (EC 3.1.4.17) (22) with specific activity = 1 μmol of cAMP hydrolyzed per min/mg of protein at 30°, digested 95% of the Dowex blank, as well as the cellular cAMP in a 30-min incubation at 35°.

We found that washing was required to free cells of medium contamination before measuring cellular cAMP. Since previous authors have reported otherwise (14, 16), we present our control experiments.

Table 1 shows that 5 ml of rinse removes a leachable fraction of cellular cAMP and leaves a stable fraction. To verify that the leachable fraction came from the medium and not from a labile cellular pool, we filtered increasing quantities of cells, omitted washing, and assayed for cAMP. Total cAMP for each filter consisted of cellular cAMP plus medium contamination. A plot of cells filtered versus cAMP extracted (Fig. 1) gives a straight line; extrapolation to zero cells filtered gives the total contamination. The average filter retains 50 μl of fluid*; at an extracellular concentration of 1 μM (the level estimated for this culture; compare Table 2), each filter retains about 50 pmol of cAMP. The line in Fig. 1 extrapolates to 65 pmol of cAMP retained per filter. Cells (2.4 units: see legend to Fig. 1) yielded 62 pmol of cAMP after washing. We anticipate, then, 127 pmol of cAMP from 2.4 units of unwashed cells (*filled circle*, Fig. 1). Note that this is close to the observed line for unwashed cells. When increasing numbers of cells over the range shown in Fig. 1 were filtered and washed, we obtained a straight line extrapolating back to zero cAMP at zero cells (not shown).

* We determined retention by filtering a 1 μM solution of [^{3}H]cAMP and measuring the radioactivity on the filter.

Measurement of Extracellular cAMP. We added 1–2 ml of cell culture to 1 pmol of [^{3}H]cAMP, chilled and spun the sample to precipitate cells, and decanted the supernatant into 4 ml of cold 5% TCA. Samples were otherwise treated as for cellular levels.

RESULTS

The "Makman–Sutherland" Experiment. Makman and Sutherland (14) reported that cells grown in glucose elevate their cellular cAMP when suspended in phosphate buffer; this elevation fails to occur if glucose is present (Fig. 2 and ref. 14). Both cellular and extracellular cAMP show this response in strains 23804 (Fig. 2*A* and *B*) and 1100 (Fig. 2*C* and *D*). Strain 5333, a *crp*$^-$ derivative of strain 1100, contained over 10 times the basal cellular and extracellular cAMP of its parent, and doubled its cellular cAMP transiently in response to carbon source deprivation (Fig. 3*A* and *B*).

As expected, strain 5336 (*cya*$^-$) evinced no appreciable cAMP synthesis in response to carbon source deprivation (Fig. 3*A* and *B*).

When glucose-grown strain 1100 was resuspended in medium lacking glucose but containing glycerol, cellular cAMP levels rose more than 10-fold, but returned to basal levels by 60 min. The rate of extracellular cAMP accumulation also slowed after 30 min (Fig. 4*B*).

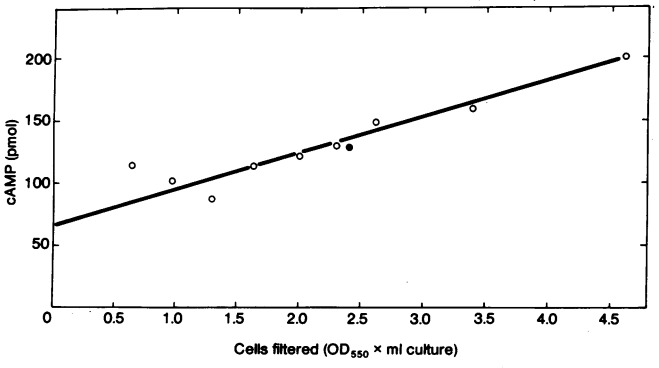

FIG. 1. cAMP associated with unwashed cells. Strain 23804 was grown in glycerol medium. Mid-log cells were filtered and assayed for cAMP. The abscissa gives total cell mass/filter in units of OD_{550} × ml of culture filtered. Each point is the average of two separate samplings, assayed in duplicate. O, cells filtered and assayed for cAMP without an intervening wash step; ●, 2.4 units of washed cells plus estimated filter blank (see *text*).

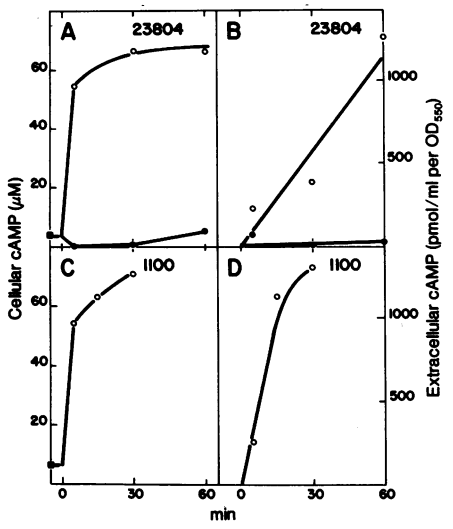

FIG. 2. The "Makman–Sutherland" experiment in strains 23804 and 1100. Figs. 2*A* and *C* show cellular cAMP concentrations; 2*B* and *D* show medium levels. ■, log phase cells in glucose medium; O, cells resuspended in phosphate buffer; ●, cells resuspended in phosphate buffer plus 1% glucose. Mid-log cells were grown in glucose medium. At 10 min, aliquots were sampled and cellular cAMP determined. The remainder was spun at room temperature and resuspended in 0.125 M potassium phosphate buffer, pH 7.0, with or without 1% (w/v) glucose. The cells were immediately spun again and resuspended (0 min) in the same buffer with or without glucose. OD_{550} of final suspension: 2*A* + *B*, 0.279 (phosphate), 0.250 (phosphate + glucose); 2*C* and *D*, 0.422. Samples for cAMP determination were taken at the indicated times. The strain number is in the upper right-hand corner of each graph.

Extracellular cAMP and Carbon Source. Extracellular cAMP accumulates linearly with total cell mass (Fig. 5) and is subject to both a severe transient and a moderate catabolite repression. For glycerol-grown strain 23804 (Fig. 5) the rate of accumulation is more than 3 times that for glucose-grown cells. Glucose added to glycerol-grown cells represses extracellular cAMP accumulation for 30–60 min before accumulation resumes at half the rate of cells grown on glucose alone. Glucose addition to glucose-grown cells is without effect.

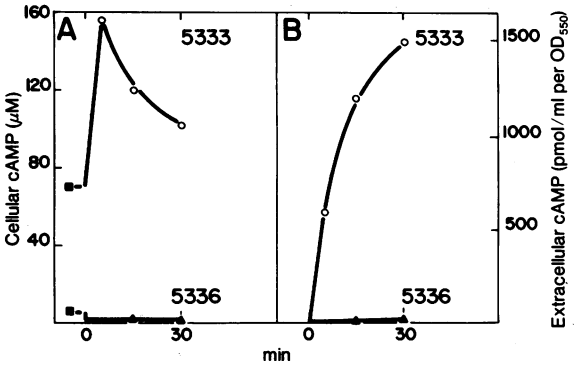

FIG. 3. The "Makman–Sutherland" experiment in strains 5333 and 5336. Fig. 3*A*, cellular cAMP; Fig. 3*B*, extracellular cAMP. ■, log phase *crp*$^-$ cells (strain 5333) in glucose medium; O, *crp*$^-$ cells resuspended in phosphate buffer, OD_{550} = 0.608; ■, log phase *cya*$^-$ cells (strain 5336) in glucose medium; ▲, *cya*$^-$ cells in phosphate buffer, OD_{550} = 0.469.

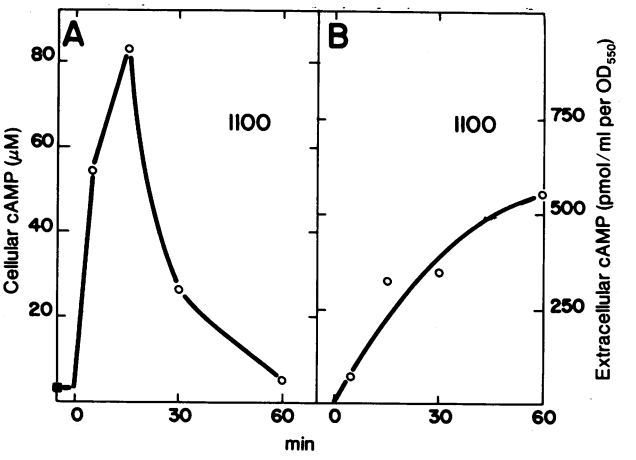

FIG. 4. Shift-down of strain 1100 from glucose to glycerol medium. Cells of strain 1100 were grown in glucose medium, spun, resuspended in glycerol medium, spun again, and resuspended in glycerol medium. Aliquots of cells and of medium were assayed for cAMP. The final suspension in glycerol medium (0 min) was at OD_{550} = 0.447 and no growth occurred during the experiment.

Extracellular cAMP accumulation also varies with the strain (Table 2). In all cases, glycerol-grown cells secrete cAMP at 2–5 times the rate of glucose-grown cells.

Cellular cAMP, Transient and Catabolite Repression. The addition of glucose to induced, glycerol-grown cells represses their ability to synthesize β-gal (Fig. 6*B*). The first 10–30 min of repression is complete (transient repression), after which there is partial recovery (catabolite repression). The duration of transient repression in strain 23804 is independent of time of addition of glucose relative to inducer (Fig. 6*B*).

In strain 23804, transient repression correlates with a drop in cellular cAMP from 14 μM to 2.0 μM (Fig. 7*A*). A less dramatic fall from 5.4 μM to 4.0 μM was seen with strain 1100 (Fig. 7*B*). The cellular cAMP remains low long after the bacteria overcome transient repression and enter into catabolite repression. We have already seen (Fig. 5) that glucose transiently halts all extracellular cAMP accumulation, but that secretion resumes, albeit at a lower rate, concomitant with β-gal synthesis (Fig. 5, Fig. 6*B*).

Tyler *et al.* (23) reported that transient repression occurs only when glycerol-grown strain 3000 is transferred to medium containing glycerol in addition to glucose. That is, the continued presence of glycerol was necessary for transient repression to occur. Immunity to the transient repression ordinarily

TABLE 3. *β-Gal synthesis during growth on glucose or glycerol*

Strain	Carbon source	β-Gal synthesis*
23804	Glucose	62
	Glycerol	448
1100	Glucose	670
	Glycerol	1825

Cultures were grown on media with the indicated carbon source. IPTG (1 mM) was used as inducer; β-gal was assayed as described in *Methods*.

* Rate of enzyme synthesis = units of β-gal/unit increase in OD_{550} of culture.

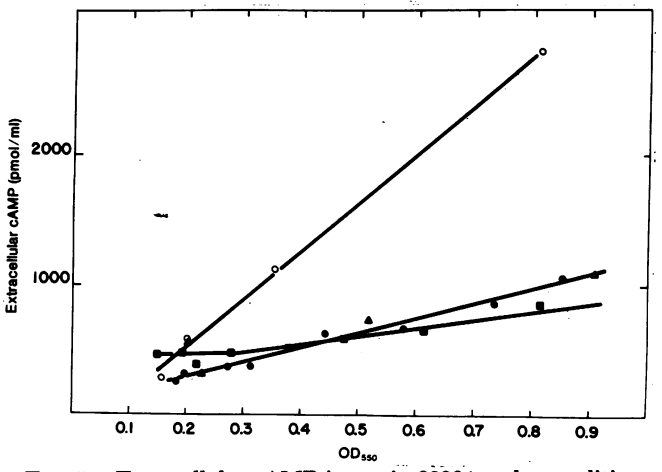

FIG. 5. Extracellular cAMP in strain 23804 under conditions of transient and catabolite repression. Shown is one of several experiments yielding the same pattern. Cells were grown in either glycerol or glucose medium and assayed for extracellular cAMP. After the first sample each of the two cultures was split into two. One portion was left unchanged, and glucose (1 g/100 ml) was added to the other. Final cultures were therefore: O, 1% glycerol; ■, 1% glycerol plus 1% glucose; ▲, 1% glucose; ●, 2% glucose.

seen in cells exposed to glucose might be caused by the maintenance of high cellular levels of cAMP. Cells transferred to glucose showed a sustained elevation in cellular cAMP when compared to those transferred to glucose plus glycerol (Fig. 8). Only these latter cells experienced transient repression; both cultures underwent catabolite repression (not shown).

α-Methylglucoside produces transient repression unaccompanied by permanent repression (23). Addition of this glucose analog to a glycerol-grown culture produces a brief drop (in a single experiment, 6 μM → 4 μM) in cellular cAMP in strain W3100, followed by recovery to the glycerol level.

Cellular cAMP, Carbon Source, and β-Gal Synthesis. Cells of strains 23804 and 1100 synthesize β-gal at a higher rate

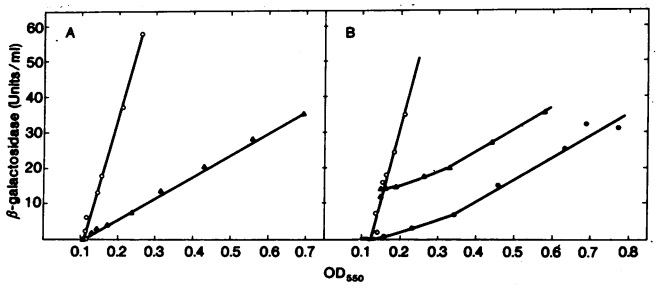

FIG. 6. (*A*) Transient and catabolite repression in strain 23804. Cells were grown in either glycerol or glucose medium. A 500-μl aliquot was sampled for β-gal activity; IPTG was immediately added to a final concentration of 1 mM, and subsequent 500-μl aliquots were assayed for enzyme. O, β-gal activity in glycerol medium; ▲, β-gal in glucose medium. (*B*) Cells were grown in glycerol medium. The culture was induced with IPTG (1 mM) and then split into two. One portion was left unchanged and glucose (1 g/100 ml) was added to the other. After 30 min glucose (1 g/100 ml) was added to a portion of the unmodified culture. O, β-gal in glycerol; ●, β-gal in glycerol to which glucose and IPTG were added simultaneously; ▲, β-gal in glycerol to which glucose was added 30 min after IPTG.

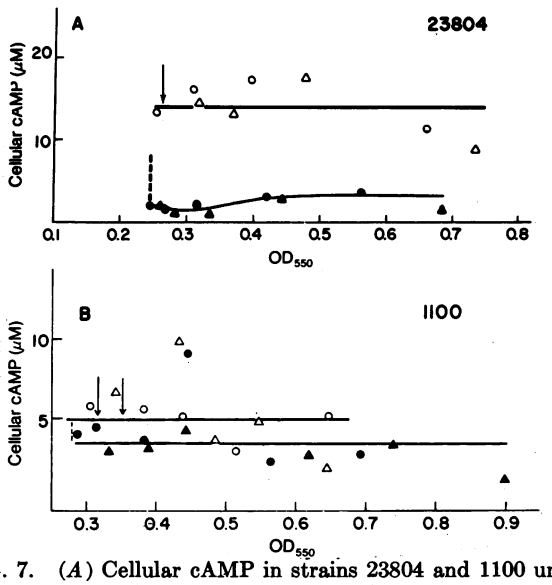

FIG. 7. (*A*) Cellular cAMP in strains 23804 and 1100 under conditions of transient and catabolite repression. Strain 23804 was grown in glycerol medium. An aliquot was sampled for cellular cAMP and the culture was then divided into two (*arrow*). One part was left unchanged, the other made 1% in glucose. Cellular cAMP was assayed at 3, 15, 30, 60, 90, and 120 min after division. Two separate experiments are shown here. O, cells in glycerol, experiment 1; ●, cells in glycerol plus glucose, experiment 1; Δ, cells in glycerol, experiment 2; ▲, cells in glycerol plus glucose, experiment 2. (*B*) Same as Fig. 7*A*, but using strain 1100.

(7- and 2- to 3-fold) when grown many generations in glycerol than when grown in glucose for a comparable period (Fig. 6*A*, Table 3). In strain 23804, cellular cAMP is twice as high in glycerol-grown cells; the same holds for extracellular cAMP (Table 2). In contrast, cellular cAMP is actually *lower* in glycerol-adapted strains 1100 and 5333 (*crp*⁻) (Table 2). The wild-type W3100 shows little or no difference between glucose and glycerol (not shown). Since strains 1100 and W3100 show normal catabolite repression, cellular cAMP does not always follow the same kinetics as ability to synthesize β-gal. Extracellular cAMP accumulation in strains 1100 and 5333 follows the same pattern as in strain 23804,

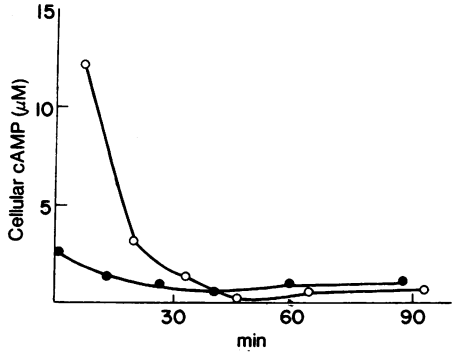

FIG. 8. Cellular cAMP in strain W3100 under conditions where transient repression is prevented. Strain W3100 was grown in 0.4% glycerol medium; the cellular cAMP was measured as 9.3 μM. The cells were spun, resuspended in salts, and incubated at 37° for 5 min. The culture was then divided into two, one half receiving glucose to 0.4% concentration (⊙), the other 0.4% glucose and 0.4% glycerol (●). Aliquots of cells were assayed for cAMP.

that is, it is 3- to 5-fold greater in glycerol-containing medium than in glucose medium (Table 2).

DISCUSSION

We have found that a drop in cellular cAMP correlates well with transient repression. (*a*) Addition of glucose or α-methylglucoside to cultures growing on glycerol produces both transient repression and a fall in cellular cAMP. (*b*) Direct transfer of cells from glycerol to glucose medium, which prevents transient repression, also prevents an abrupt fall in cellular cAMP (Fig. 8). The degree of fall associated with transient repression is strain-dependent, and recovery of β-gal synthesis is not always accompanied by an increase in cellular cAMP levels.

Our results lead us to question the primacy attributed to cAMP in permanent repression. Cellular concentrations of cAMP do not always correlate with ability to synthesize β-gal (Tables 2 and 3). In strains 1100 and its derivative, 5333, the cellular cAMP levels in glucose are, if anything, somewhat higher than those in glycerol-grown cells (Table 2). β-Gal is synthesized about 3-fold more rapidly in strain 1100 grown in glycerol when compared to glucose (Table 3).

Previous authors have reported correlations between cellular cAMP and medium composition (1, 14–17). We believe our results differ from theirs because (*a*) we wash our cells (see *Methods*), whereas they claim either that washing leaches out cellular cAMP (14) or else that it is unnecessary (16); and (*b*) we studied several strains of *E. coli* instead of one (14, 17).

cAMP was first implicated in the two phases of the glucose effect by the observation that 1 mM cAMP overcomes transient repression, while 5 mM cAMP overcomes catabolite repression (3, 24). Since 5 mM cAMP inhibits growth of *E. coli* 3000 by 50% in glucose medium (25), experiments employing such high levels of cAMP are difficult to interpret†. Magasanik (26) and Ullmann (27) pointed out that confirmation of the role of cAMP in repression awaited accurate measurements of cellular cAMP.

The control of cellular cAMP levels is still mysterious. Strain 5333 (*crp*$^-$) demonstrates markedly elevated cAMP both in the cells and in the medium (Table 2, Fig. 3). The relationship between the *crp*$^-$ phenotype and control of cellular cAMP in a number of strains remains to be investigated. Daniel *et al.* (28) report that mouse lymphoma cells deficient in cAMP-binding and protein kinase activities show reduced phosphodiesterase activity and higher cAMP on stimulation by prostaglandin E_1 and isoproterenol. Perhaps cAMP-binding proteins are involved in regulating cAMP levels in animal and bacterial cells.

† α-Methylglucose (1%) inhibited growth of strain 23804 on 1% glucose and substantially relieved catabolite repression in these cultures, concomitant with elevation of intracellular cAMP to about 40 μM. It is not clear whether those conditions which are both growth-limiting and which relieve catabolite repression all produce elevated intracellular cAMP.

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