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Adenosine 3′:5′-Cyclic Monophosphate as Mediator of Catabolite Repression in *Escherichia coli*

(transient repression/β-galactosidase/intracellular cAMP/cAMP excretion)

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ABSTRACT **Measurements of intracellular adenosine 3′:5′-cyclic monophosphate (cAMP) concentrations in *E. coli* under a variety of conditions show that levels of this nucleotide are well correlated with the rate of synthesis of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) in both catabolite repression and transient repression. These results, combined with extensive genetic and *in vitro* studies from a number of laboratories on the role of cAMP in *E. coli*, provide strong support for the concept that intracellular cAMP levels mediate the effects of catabolite and transient repression on rates of enzyme synthesis. Under all conditions studied, excretion can be described by a single rate constant, 2.1 min^{-1} at 37°, indicating that intracellular levels cannot be regulated by alterations in the rate of cAMP excretion. Our data are fully consistent with the idea that carbon sources control intracellular cAMP levels by effects on its synthesis.**

Adenosine 3′:5′-cyclic monophosphate (cAMP) interacts with a specific binding protein called cAMP receptor protein (CRP) to stimulate transcription from many operons of *Escherichia coli* (1). Enzymes coded for by cAMP-dependent operons are subject to catabolite repression, a nonspecific control of enzyme synthesis exerted by the carbon source and other variables (2). The addition of cAMP to the growth medium can reverse both the transient and permanent types of catabolite repression (3). These results led to the suggestion that catabolite repression controls enzyme synthesis by regulating the intracellular level of cAMP (3). A prediction of this model is that intracellular levels of cAMP will be correlated with the rates of synthesis of cAMP-dependent enzymes under different degrees of catabolite repression. Failure to detect such a correlation has raised doubts about the role of cAMP in catabolite repression (4–6).

We have measured intracellular concentrations and rates of excretion of cAMP in *E. coli* K-12 under a variety of conditions characterized by different degrees of catabolite repression. We find a good correlation between intracellular concentrations of cAMP and the extent of catabolite repression, leading us to conclude that intracellular cAMP is the chemical signal mediating the phenomenon of catabolite repression. The difference between our results and those of other investigators appears to lie in the manner in which cell samples are prepared for analysis. Contamination with extracellular cAMP and high blank values are two major sources of error. cAMP excretion rates are linearly dependent on the cell concentration of cAMP. Our data are consistent with suggestions that cAMP levels are regulated chiefly by changes in the rate of synthesis (7).

Abbreviations: CRP, the cAMP binding protein of *E. coli*, also referred to in other work as CAP; cAMP, adenosine 3′,5′-cyclic monophosphate; genetic symbols written in italics are those of Taylor and Trotter (8).

MATERIALS AND METHODS

Steady-state measurements in cells growing on different carbon sources were made with *E. coli* strain X9250 (F^- *srl thi* $laci_3$ $strA^r$) which makes β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) constitutively. Steady-state measurements for several selected carbon sources and transient repression experiments were performed with four strains in which β-galactosidase synthesis is inducible: X9251, isogenic with X9250 except that it is $laci^+$; CHE9 (9); 1100, one of the strains used by Wayne and Rosen (5); and AB257, used by Buettner *et al.* (4). β-galactosidase was induced with 10^{-3} M isopropyl-β-D-thiogalactoside (IPTG). This concentration is fully effective under all conditions as demonstrated by a comparison of induced rates of synthesis in X9251 with constitutive levels in X9250. Strain 5336, a *cya* point mutant strain (10), and LU124 and LU135, both constitutive for β-galactosidase synthesis ($laci_3$) and carrying the *cya* deletion mutation from strain CA8306 of Beckwith (11), were used in some experiments.

Except as otherwise noted, cells were grown at 37° with shaking in K115 medium (12) containing, as carbon source, 2 g/liter of sugars or 10 mM of the sodium salts of acids. Measurements were performed on mid-logarithmic phase cultures containing between 120 and 240 μg dry weight per ml, corresponding to 3 and 6 × 10^8 cells per ml, respectively. β-galactosidase was assayed at 37° after toluene treatment of cells as previously described (13). One unit of β-galactosidase produces 1 μmol of product per min at 37°. Culture density was determined by measuring turbidity at 610 nm in 18 mm tubes in a Bausch and Lomb Spectronic 20 colorimeter. The values were converted to dry weight from a calibration curve. The differential rate of β-galactosidase synthesis is expressed as increase in units of enzyme/increase of bacterial dry weight in mg.

Culture supernatants were obtained by rapid filtration through 0.45 μm pore size filters (Millipore, type HA) or by rapid chilling and centrifugation. Up to 75 μl of supernatant was dried for assay; the amount of salts in this volume does not produce any interference in the binding assay. Cell samples were collected by rapid filtration of a volume of culture containing approximately 2 mg (dry weight) of cells on 47 mm diameter, 0.65 μm pore size filters (Millipore, type DA),

and were washed briefly with cold* 0.2 M Na_2SO_4. The 0.65 μm filters allowed rapid collection of samples while still providing quantitative recovery of cells. The cells were immediately extracted in 1.2 ml of cold 0.2 N H_2SO_4 containing a standard amount, approximately 5000 cpm, of [^{3}H]cAMP of the same specific activity as used in the binding assay. The acid extract was centrifuged, the clear supernatant absorbed with charcoal (0.07 ml of a 10% suspension in 0.01 M HCl of activated charcoal that had been exhaustively washed by centrifugation with 1 M NH_3 in 50% ethanol) and the charcoal washed with water. The nucleotides were then extracted with 1 M NH_3 in 50% ethanol (v/v), and the charcoal removed from the eluate by filtration through 0.45 μm filters. We dried a small portion of the eluate in scintillation vials to measure recovery of added cAMP; the rest was dried for cAMP assay. Many batches of membrane filters contain material which interferes in the binding assay for cAMP and produced blank values equivalent to the concentrations found in cells (approximately 1 μM). This problem was eliminated by washing filters with 0.2 M acetic acid, water, and lastly 1 M NH_3 in 50% (v/v) ethanol.

Our method of preparing cell extracts has a small blank. Assay of 13 samples prepared in the usual way from the *cya* deletion mutant strain LU135 yielded a value for intracellular cAMP of 0.17 $\pm$ 0.12 (SD) μM. When large amounts of extract were collected and analyzed by thin-layer chromatography on cellulose (see below), we were unable to demonstrate that any of this activity was due to cAMP. The amount of this activity is not proportional to the amount of LU135 cell extract assayed. We assume this blank is additive with cAMP in the assay and have corrected all our values accordingly by subtracting it.

Reconstruction experiments show that our washing procedure did not entirely eliminate contamination of cell samples by cAMP in the medium. Cell samples of *cya* mutant strain LU135 grown in glucose medium with 2 μM cAMP contained cAMP equivalent to contamination with 0.5 μl of medium per ml of culture filtered. We do not believe this represents cAMP in the cells because, under these conditions, there is no detectable stimulation (less than 2% increase) of β-galactosidase synthesis. Since concentration of cAMP in the medium is proportional to the total amount of this nucleotide in the cell during exponential growth (see *Results*), the resulting error is proportional to the amount found and is estimated at slightly under 10%.

cAMP was assayed by a binding method in which human erythrocyte ghosts are used (14). Membranes, prepared by osmotic lysis (15) of outdated blood obtained from a blood bank, were stored at $-20°$. Immediately before use, the membranes were extracted with NH_4Cl as described by Rubin *et al.* (14). Each assay contained (in a total volume of 100 μl): 50 mM potassium phosphate buffer (pH 7.5), 10 mM $MgCl_2$, 0.87 pmol [8-^{3}H]cAMP (26 Ci/mmol, New England Nuclear), sample for assay, and, added last, extracted membrane suspension containing approximately 40 μg of protein. After a 60 min incubation at 0°, the membranes and bound cAMP were collected on membrane filters, washed with 20 mM potassium phosphate buffer (pH 6), dried, and counted in a liquid scintillation spectrometer. Details of this assay will be published elsewhere (16). Results for cell assays were corrected for recovery of the added radioactive cAMP and for the presence of extra radioactive cAMP in the extracts. We expressed the values as intracellular concentration by using data that relate dry weight to intracellular volume (17).

Selected samples, as well as radioactive and unlabeled cAMP used in the assay, were analyzed by cellulose thin-layer chromatography (Eastman no. 13255) in solvent system A (*n*-butanol 5, glacial acetic acid 2, water 1; v/v/v) and/or system B (isopropanol 7, concentrated ammonia [15 M] 1, water 2; v/v/v) and by electrophoresis on Whatman 3MM paper in 0.05 M sodium borate (pH 9.5).

Both [8-^{3}H]cAMP from Schwarz/Mann (28 Ci/mmol) and in later work [*U*-^{3}H]cAMP from New England Nuclear (38 Ci/mmol) were used in the binding assay. Unlabeled cAMP was obtained from Sigma. None of these contained impurities that interfered in the assay. [*U*-^{3}H]cAMP was purified by chromatography in solvent system A for the studies of stability of cAMP in the medium.

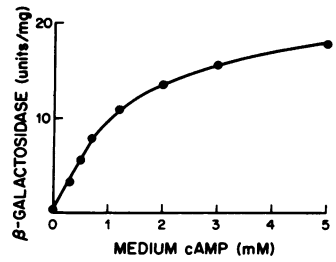

FIG. 1. Dependence of the differential rate of β-galactosidase synthesis on concentration of cyclic AMP in medium in an adenylate cyclase deficient mutant. Mutant strain LU124 (pertinent genetic characteristics are F$^-$ *laci*$_3$ *cya*$_{del}$) was grown in glucose K115 medium to logarithmic phase, then diluted to a density of approximately 8×10^7 cells per ml in the same medium containing the indicated concentrations of cAMP. The average rate of growth and of β-galactosidase synthesis were measured for approximately two generations and expressed as a differential rate.

RESULTS

The rate of synthesis of β-galactosidase was used as a measure of catabolite repression because this enzyme is easy to assay, its regulation is well understood, and its rate of synthesis is very sensitive to catabolite repression. Full induction can be assured by using either a strain which produces the enzyme constitutively, or high concentrations of a potent inducer such as isopropyl-β-D-thiogalactoside where the inducing effect is not subject to interference by any of the carbon sources we used (see *Materials and Methods*). The expected relationship between intracellular cAMP concentration and rate of β-galactosidase synthesis is suggested by an experiment in which the rate of synthesis as affected by *external* cAMP is measured in a mutant strain unable to make cAMP (Fig. 1). The curve shows saturation without evidence of cooperativity at low concentrations of cAMP. Assuming that intracellular cAMP concentrations are linearly dependent on those outside, a curve of similar shape should relate enzyme synthesis to intracellular cAMP concentration. The conclusion that

* We found (as did Wayne and Rosen, ref. 5) that washing was necessary to obtain proportionality between culture volume sampled and cAMP recovered, but that washing the cells with a cold solution which should rapidly arrest synthesis or excretion of cAMP was more satisfactory than washing with solutions at room temperature or 37°. Cold solutions gave consistent results as long as osmotic shock was avoided; 0.4 M mannitol was just as satisfactory as 0.2 M Na_2SO_4 for washing.

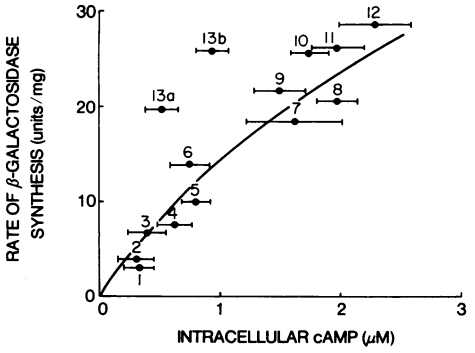

FIG. 2. Relationship of the rate of β-galactosidase synthesis to intracellular concentration of cAMP in strain X9250. Cells were grown at 37° in K115 medium containing different carbon sources. Samples were taken for determination of the rate of enzyme synthesis and cAMP analysis near the middle of the logarithmic phase of growth. Errors in the assay of enzyme synthesis rates were under 5% and are not shown. Error bars for the cAMP determinations represent the standard deviations of the analysis, most of which represent averages of four samples; thus, these represent practical confidence limits equivalent to twice the standard error of the mean. Numbers refer to the carbon source as follows: 1, glucose-6-*P*; 2, glucose + casamino acids; 3, glucose + gluconate; 4, glucose; 5, mannitol; 6, gluconate; 7, fructose; 8, lactate; 9, succinate; 10, glycerol; 11, casamino acids; 12, glucosamine; 13a and 13b, xylose (two separate cultures). The curve is based on a linear fit to a double reciprocal plot of the data excluding that for xylose; it does not differ significantly from a straight line through the origin.

effects of cAMP on expression of the lactose operon are not cooperative is supported by the linear dependence of *in vitro* expression of this operon on cAMP at low concentrations of the nucleotide (18), and the observation that binding of cAMP or CRP is not cooperative *in vitro* (19, 20).

Results of the measurements of intracellular cAMP concentrations in strain X9250 are shown in Fig. 2. There is a good correlation between cAMP and rates of β-galactosidase synthesis for almost all of the carbon sources. The curve shown is based on a linear fit to a double-reciprocal plot of the data. Results for xylose-grown cells appear to be discrepant.

We examined three strains not closely related to X9250 to see if the correlation of Fig. 2 was general. Two of the strains, AB257 and 1100, have been used in investigations of cAMP metabolism by others (4, 5). For each strain, steady-state data were obtained for cells growing on three carbon sources: glycerol, glucose, and glucose-6-*P* which produce weak, strong, and very strong catabolite repression, respectively. The results shown in Table 1 fit, within experimental error, the curve drawn in Fig. 2. Thus, this correlation appears to be true for many different strains of *E. coli* K-12.

The most severe form of catabolite repression is seen in the phenomenon of transient repression, which is produced when a strongly repressing carbon source, such as glucose, is added to a culture growing on glycerol or other compounds which repress only mildly (21). The effect lasts approximately 30 min; the rate of enzyme synthesis rises thereafter to approach the value typical of steady-state growth on glucose. Data for cAMP levels during transient repression in four different strains are also shown in Table 1. A marked decrease in intracellular cAMP concentration occurs in each strain during transient repression. This decrease ranges from 4- to over 10-fold as compared to the control cells growing in glycerol medium.

TABLE 1. *Catabolite and transient repression in four inducible strains of E. coli*

Strain	Growth conditions*	Differential rate of β-galactosidase synthesis (units/mg per min)	Intracellular cAMP (μM)
CHE9	Glucose-6-*P*	3.2	0.38 ± 0.14 (SD)
	Glucose	6.1	0.39 ± 0.14
	Glycerol	18.3	1.3 ± 0.4
	Transient repression	0.9	0.10 ± 0.14
AB257	Glucose-6-*P*	6.2	0.35 ± 0.13
	Glucose	8.5	0.52 ± 0.15
	Glycerol	25.5	2.7 ± 0.7
	Transient repression	1.1	0.24 ± 0.15
1100	Glucose-6-*P*	1.9	0.12 ± 0.13
	Glucose	6.3	0.38 ± 0.16
	Glycerol	19.8	1.53 ± 0.24
	Transient repression	1.2	0.23 ± 0.14
X9251	Glycerol	20.6	1.73 ± 0.34
	Transient repression	2.0	0.45 ± 0.13

* Cells were grown in K115 medium with the indicated carbon source. Transient repression data are from an experiment in which the cells used for the glycerol data shown, received glucose (final concentration 2 g/liter), and are averages for the 20 min period after glucose addition.

Chromatographic analysis (see *Materials and Methods*) revealed that virtually all material assayed as cAMP in samples of cells grown on glycerol and cells grown on glucose was cAMP. However, almost half of the activity in cells grown on glucose-6-*P* was not cAMP. This correction corresponds to the blank correction (see *Materials and Methods*) and thus confirms that the blank is not due to cAMP.

A description of cAMP metabolism in *E. coli* is not complete without consideration of cAMP in the medium. We find that the rate of excretion of cAMP into the medium is constant during the exponential phase of growth, a finding already reported by others (5). We have compared the rate of excretion in a given medium with the intracellular cAMP concentration characteristic of that medium (Fig. 3). As seen in the figure, these two parameters are linearly correlated in steady-state growth and during transient repression.

The concentration of cAMP found in the medium during exponential growth (less than 2 μM under all conditions we examined), is stable and does not have a detectable effect on intracellular events. Over 99% of a small amount of [^{3}H]-cAMP added to a culture of strain X9250 growing in glucose medium was recovered unchanged after three generations of growth. Identity with cAMP was confirmed by thin-layer chromatography in two solvent systems and borate electrophoresis as described in *Materials and Methods*. The conclusion that these low concentrations of external cAMP are without effect in the cell is based on our inability to detect any stimulation of β-galactosidase synthesis by 2 μM cAMP (see *Materials and Methods*). Thus, endogenously produced cyclic AMP in the medium is an end product of metabolism, accumulating but having no detectable effect on intracellular events at these low concentrations.

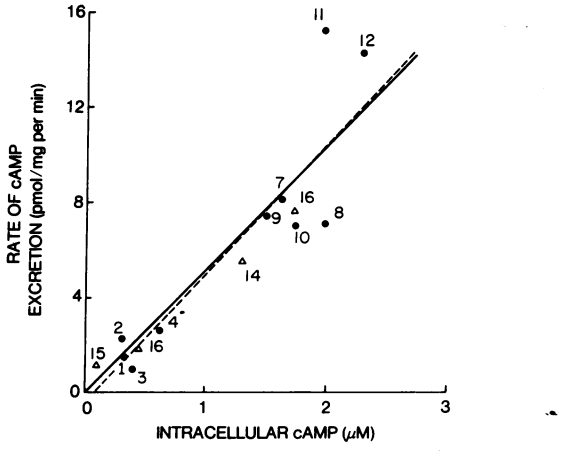

FIG. 3. Relationship of cAMP excretion rate to intracellular concentration of the nucleotide. Steady-state data are for strain X9250: intracellular concentrations are from Fig. 2, while excretion rates were measured in separate experiments under identical conditions. The carbon sources are indicated by numbers corresponding to those of Fig. 2. Transient repression data are: for strain CHE9, glycerol control was 14, transient repression was 15; for strain X9521, control was 16, transient repression was 17. The dashed line, drawn by the method of least squares, does not differ significantly from the line passing through the origin that corresponds to a single excretion rate constant of 2.1/min at 37°.

We examined the rate at which intracellular cAMP falls during transient repression. For this experiment the temperature was 21°, and strain AB257 was chosen since it has rather high cAMP levels when grown on glycerol and thus allows accurate analysis on small samples which can be collected very rapidly. Fig. 4 shows that intracellular levels fall exponentially, approaching a new steady-state level with a half-time of 0.44 min.

DISCUSSION

Our results differ from those reported in other studies of this subject. We believe that three technical reasons account for these differences: (*i*) inducer-specific effects on enzyme synthesis, (*ii*) contamination of cell samples by extracellular cAMP, and (*iii*) artefacts introduced in processing samples for assay.

Inducer-specific effects are known to occur with certain inducers (2), notably methyl-β-thiogalactoside used by Buettner *et al.* (4). This probably accounts for the low rates of β-galactosidase synthesis they observed in strain AB257 grown on glucose; we have confirmed similarly low rates of synthesis when this inducer is used in strain X9251 when grown on glucose. These problems are avoided by using a genetically constitutive strain or saturating levels of a potent inducer such as isopropyl-β-D-thiogalactoside whose effect is not blocked by sugars inhibiting inducer entry.

The importance of washing has already been amply stressed by Wayne and Rosen (5). In steady-state growth, the error produced by not washing will be approximately proportional to the amount of cAMP in the cells, since concentration in the medium is correlated with total cAMP in the cells (see under *Results*). However, the absolute values will be falsely high, and will depend on the volume filtered. A more serious problem arises when sampling during transient repression, where

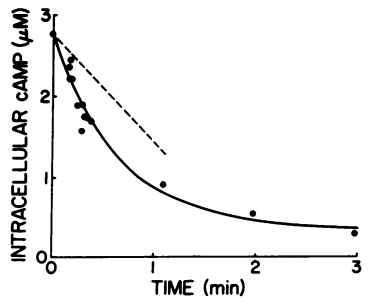

FIG. 4. Changes in intracellular cAMP early in transient repression. Strain AB257 was grown at 21° in K115 glycerol medium. When the culture reached a density near 180 μg/ml (dry weight), repeated small portions received glucose to a final concentration of 0.2% and were rapidly sampled for cell cAMP determination. Data for zero time are average and standard deviation for six control samples. The smooth curve is an exponential with a half-time of 0.44 min; it was drawn by fitting the data to a straight line on a semi-logarithmic plot and assuming a final concentration of 0.35 μM for cAMP; this corresponds to a rate constant of 1.60/min. The dashed line represents the rate of fall expected due to excretion alone, and corresponds to an initial rate constant of 1.09/min.

cAMP in the cell drops precipitously (Fig. 4) while concentration in the medium does not fall. Under these conditions, a much higher fraction of the cAMP measured in unwashed cell samples will represent contamination. This error probably accounts for the higher intracellular cAMP concentrations reported by Buettner *et al.* (4) and their failure to observe a large drop in intracellular cAMP levels during transient repression. Our data for transient repression also over-estimate the true values, possibly by as much as a factor of two, because the relatively small contamination in washed samples becomes quite large during transient repression. We did not attempt to correct for this systematic error because statistical variations in this range of values are so large.

The manner of preparation of cell samples must give reproducible results and low blank values. Rapid collection of cells and prompt washing with a cold solution minimizes changes in intracellular cAMP during sampling, as long as osmotic shock is avoided. Then, rapid inactivation of the enzymes synthesizing or degrading cAMP and destruction of the cell permeability barrier can be effected by a variety of methods. We arbitrarily chose H_2SO_4, although other mineral acids gave identical results. Absorption to well-washed charcoal and elution resulted in reproducible and high recoveries of cAMP. We encountered problems due to the elution of interfering material from membrane filters during separation of charcoal from the ammonical ethanol eluate. Washing the filters eliminates this problem.

The Dowex purification method used by Wayne and Rosen (5) probably accounts for differences between their data and ours. While their blank values ranged from 6 to 9 pmol per assay, our blanks (without cells) were below 0.06 pmol, the amount equivalent to a cell concentration of less than 0.03 μM. Our assay of *cya* mutant strain 5336 gave values (before correction for the 0.17 μM blank value) of 0.08 ± 0.02 (SD) μM; Wayne and Rosen reported an average of 2.6 μM with a range from 0 to 4.8. Presumably, most of the results of these authors include a large and variable blank which obscures the somewhat smaller differences in intracellular cAMP. The

large range of values reported by Wayne and Rosen for a given condition supports this interpretation of their data.

Our data provide good support for a simple model to explain catabolite repression, namely, that this phenomenon is mediated by changes in the intracellular concentration of cAMP. This regulation can be effected by changes in the rate of: (*i*) synthesis, (*ii*) degradation, or (*iii*) excretion of cAMP. Our data exclude the third possibility, for that model predicts that net rates of synthesis would be constant under different conditions of catabolite repression, the only differences being that cell levels fluctuate. Regulation by (*iii*) requires that the rate constant for excretion be high under strong catabolite repression, and low when there is little or no catabolite repression. Instead, we find that excretion rates can be described by a single rate constant under all conditions tested; this suggests that exit is determined simply by the intracellular concentration and is not subject to significant control by the carbon source. If there is a deviation, it is in the opposite direction (Fig. 3), namely, that the rate constant may be higher when intracellular cAMP concentrations are high.

The experiment of Fig. 4 supports control at the level of synthesis. If glucose affects only synthesis, not degradation, the expected initial rate of fall in cAMP (due solely to continued excretion) can be calculated from the excretion rate measured in the control culture growing on glycerol. Excretion accounts for a drop described by a rate constant of 1.09/min, representing almost 70% of the observed rate. A rather low rate of degradation, rate constant 0.51/min, then fully accounts for the observations. If, on the other hand, glucose controls degradation but not synthesis, then the sum of the rate constants for degradation and excretion must rise to 8.72 (eight times the excretion rate constant of 1.09) to explain the 8-fold reduction (from 2.79 to 0.35 μM) in steady-state levels. However, such a high rate constant for degradation requires that the initial rate of fall should be 8.7/min, which is over five times the rate observed. Thus, our data are most consistent with control of synthesis. This model is especially attractive since Peterkofsky and Gazdar have demonstrated an immediate and large inhibition of cAMP synthesis *in vivo* during transient repression (7).

Our data support the model discussed above, but they do not prove it. Other factors may play significant roles under some conditions. The errors in our cAMP analyses are relatively large when the absolute values are low, so that small but significant deviations from those expected could be obscured. At higher intracellular concentrations, cAMP fully explains catabolite repression for most carbon sources. Growth on xylose may well be an exception, since we doubt that the data deviate due to errors in the analysis. Growth on xylose may promote the participation of other factors which either enhance the sensitivity (of *lac* operon expression) to stimulation by cAMP and CRP, or partially substitutes for these. An example of the latter has been reported (22).

Finally, it appears that transient and permanent catabolite repression are similar in mechanism, both being explained (within the limits of error) by changes in intracellular cAMP. Whether both involve the same mechanism connecting carbon source metabolism and changes in cell cAMP is not known, but the correlation with net synthesis rates (Fig. 3) suggests that both involve controls on synthesis and that transient repression is more severe simply because two carbon sources are being metabolized at the same time.

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