

Cellular Levels, Excretion, and Synthesis Rates of Cyclic AMP in *Escherichia coli* Grown in Continuous Culture

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Changes in dilution rate did not elicit large and systematic changes in cellular cyclic AMP levels in *Escherichia coli* grown in a chemostat under carbon or phosphate limitation. However, the technical difficulties of measuring low levels of cellular cyclic AMP in the presence of a large background of extracellular cyclic AMP precluded firm conclusions on this point. The net rate of cyclic AMP synthesis increased exponentially with increasing dilution rate through either the entire range of dilution rates examined (phosphate limitation) or a substantial part of the range (lactose and glucose limitations). Thus, it is probable that growth rate regulates the synthesis of adenylate cyclase. The maximum rate of net cyclic AMP synthesis was greater under lactose than under glucose limitation, which is consistent with the notion that the uptake of phosphotransferase sugars is more inhibitory to adenylate cyclase than the uptake of other carbon substrates. Phosphate-limited cultures exhibited the lowest rate of net cyclic AMP synthesis, which could be due to the role of phosphorylated metabolites in the regulation of adenylate cyclase activity. Under all growth conditions examined, >99.9% of the cyclic AMP synthesized was found in the culture medium. The function of this excretion, which consumed up to 9% of the total energy available to the cell and which evidently resulted from elaborate regulatory mechanisms, remains entirely unknown.

In a previous study (12), we showed that two bacteria exhibited markedly higher rates of synthesis of several enzymes of intermediary metabolism when grown at low dilution rates (*D*) in a chemostat under carbon, nitrogen, or phosphorus limitation. Such an increase in rates of enzyme synthesis in slowly growing bacteria appears to be a general phenomenon (9, 10, 12), and we speculated (12) that this may be a consequence of increased cellular cyclic AMP levels at low growth rates. This speculation was consistent with the available evidence that an inverse relationship exists between growth rate and cellular cyclic AMP levels of bacterial and mammalian cells (2, 13-15) and that in mammalian cells contact inhibition of growth is accompanied by a rise in cellular cyclic AMP levels (14, 15). In fact, extensive studies in mammalian cells, spurred by these observations, have led to the postulation that cellular cyclic AMP levels play a central role in growth regulation and neoplastic transformation (14, 15).

However, the evidence for an inverse relationship between cellular cyclic AMP levels and growth rates was obtained with batch cultures, which are not suitable for such studies. The environmental conditions in such cultures change continuously and drastically with time, leading to significant changes in cellular cyclic

AMP levels even during the logarithmic phase (1), and a change in growth rate can only be achieved by qualitative changes in the environment, which may have effects independent of growth rate. Thus, continuous culture (chemostat) studies of the effect of growth rate on cyclic AMP levels are of interest. This technique permits microbial growth under steady-state conditions and wide changes in growth rate with only a slight alteration in the concentration of a growth-limiting nutrient.

While our studies were in progress, Harman and Botsford (5) and Wright et al. (26) published their chemostat work dealing with cyclic AMP in bacteria. The studies reported here include two growth conditions—lactose and phosphate limitations—not examined by these workers, and the results differ from their findings in some respects.

MATERIALS AND METHODS

Organism and culture conditions. *Escherichia coli* K-12 was obtained from our departmental culture collection. The basal salts solution employed was very similar to that described previously (12), except for the following modifications. The NaCl concentration used was 0.03 g/liter, and phosphate-limited media contained 0.17 g of $K_2 HPO_4 \cdot 3H_2O$ per liter and 0.011 g of KH_2PO_4 (anhydrous) per liter. The glucose-limited

media contained 0.5 g of glucose per liter; lactose-limited media contained 0.5 g of lactose per liter; and phosphate-limited media contained 1.5 g of lactose per liter. The carbon source and phosphates were autoclaved separately and added aseptically to the basal salts. The bacteria were grown at 37°C ($\pm 0.5^\circ\text{C}$) in a New Brunswick C-30 Bioflo chemostat with a working volume of 350 ml. The pH was automatically maintained (11, 12) at 6.8 ± 0.1 by the addition of 5% Na_2CO_3 solution. The aeration rate was 0.5 liter/min, and agitation was maintained between 350 and 400 rpm. The cells were allowed to grow at a given D value for at least five volume changes (approximately seven generations) before being considered to be in the steady state. Biomass was monitored by optical density measurement, and the optical density units were converted to protein values by use of a standard curve that related these two parameters; according to our previous studies (12), the optical density-to-protein ratio changes very little in response to D in bacteria. The steady-state biomass varied from 0.17 to 0.19 and from 0.21 to 0.35 mg of protein per ml of culture under carbon and phosphate limitations, respectively.

Stock cultures were maintained on lactose and glucose agar plates of the same composition as those used in chemostat cultivation, except that agar (20 g/liter) was added.

Culture sampling and assay of cyclic AMP. The connection of a repeating dispenser pipette (Minipet; Manostat, New York, N.Y.) to the sampling port of the chemostat made it feasible to remove rapidly aliquots of steady-state cultures. For total culture cyclic AMP determination, 1-ml aliquots were collected in tubes containing 1 ml of 1.2 N HClO_4 . To determine intracellular cyclic AMP, 5-ml aliquots were dispensed over prewashed 0.45- μm membrane filters (Millipore Corp., Bedford, Mass. kept over a vacuum. The filters were allowed to drain thoroughly, which required 2 min, and then quickly transferred to tubes containing 4 ml of 0.6 N HClO_4 . For reasons discussed below, washing of the filters was omitted. However, since the extracellular fluid was found to contain a large amount of cyclic AMP, it was necessary to estimate the amount of extracellular fluid that remained on the filters under these conditions. This was done by filtering cell suspensions containing a known amount of ^3H -labeled cyclic AMP and determining the counts that remained on the filter. The filters retained ca. 20 μl of extracellular fluid and, accordingly, the corresponding amount of cyclic AMP was subtracted from the amount of the nucleotide recovered from the filters (see Table 2). In several experiments, culture filtrates were also analyzed; the results agreed closely with the values predicted by total cell culture measurements. A known amount of ^3H -labeled cyclic AMP was added to each sample to check the recovery of the cyclic nucleotide during chromatographic purification (see below). Each sample was analyzed at least in duplicate, and often in quadruplicate; average values with standard deviations are presented.

After mixing in a Vortex mixer for 3 min, the preparations were centrifuged at $3,000 \times g$ for 15 min at 4°C , and 1 ml of the supernatant was layered on a glass column (0.4 by 15 cm) containing HCl-treated Dowex 50W $\times 4 - \text{H}^+$ resin of 200 to 400 mesh (Bio-Rad Laboratories, Richmond, Calif.). The column was eluted in two volumes of distilled water (3 and 6 ml,

respectively), the second eluate containing the cyclic AMP. Nearly all of the radioactive cyclic AMP that had been added at the time of harvest (see above) was present in this fraction, indicating that the recovery of cyclic AMP from the column was very close to 100%. Two milliliters of this fraction were dried in a stream of warm air and redissolved in 0.8 ml of 50 mM sodium acetate buffer (pH 6.2). Control experiments, in which known amounts of cyclic AMP were subjected to drying and resuspension, showed that no loss of the nucleotide occurred during this step.

Cyclic AMP was assayed by the radioimmunoassay of Steiner et al. (22). The assay protocol was followed exactly according to the recommendation of the assay kit supplier (New England Nuclear Corp., Boston, Mass.), except that, to increase the sensitivity of the assay (6), 5 μl of a freshly prepared mixture of acetic anhydride and triethylamine (1:2 vol/vol) was added to each assay tube; this modification increases the sensitivity of the assay to 5 fmol. Five known concentrations of cyclic AMP ranging from 5 to 100 fmol were included in each assay, and the resulting standard curve was used to determine the cyclic AMP concentration in the samples. The results were corrected for the labeled cyclic AMP added at the time of harvest (see above). To calculate the concentration of cyclic AMP in the cells, it was assumed that 1 mg of cell protein corresponded to 5.4 μl of cell water (25).

The net rate of cyclic AMP synthesis (P) was calculated by the equation:

$$P = \frac{Z \times D}{\bar{x}} \quad (1)$$

where Z is the steady-state total cyclic AMP content of the culture, \bar{x} is the steady-state cell biomass in milligrams of protein, and D is the dilution rate. The specific rate of consumption of the limiting substrate (q) was calculated by the following equation:

$$q = \frac{S_R - \bar{s}}{\bar{x}} \times D \quad (2)$$

where S_R is the concentration of the growth-limiting substrate in the inflow medium, \bar{s} is the residual steady-state concentration of this substrate in the culture medium (assumed to be zero), and \bar{x} and D are as defined for equation 1.

RESULTS

Cellular cyclic AMP levels. Initial experiments showed that an excess of cyclic AMP was present in the extracellular fluid under the various growth conditions employed, so that washing of the filters used in estimating cellular cyclic AMP levels appeared to be necessary. However, washing involves further disturbance of the culture steady state and could therefore influence the cellular cyclic AMP levels. A control experiment was done to check this possibility. Organisms were grown under glucose limitation at various D values, and each steady-state culture was sampled repeatedly by filtering 5-ml aliquots through several separate filters. These

TABLE 1. Changes in cellular cyclic AMP levels at various D values produced by washing of filters used in collecting cells^a

$D(\text{h}^{-1})$	Cellular cyclic AMP level after indicated no. of washes (% of that estimated with unwashed filters)		
	1	2	3
0.05	50 ± 15	25 ± 3	40 ± 2
0.10	71 ± 1	57 ± 4	28 ± 0
0.20	230 ± 62	280 ± 11	250 ± 16
0.50	280 ± 79	160 ± 20	172 ± 17

^a Aliquots from a steady-state culture were filtered with separate filters. One milliliter of basal medium (i.e., complete medium without carbon source) was used for each wash. The limiting nutrient was glucose.

filters were analyzed for cyclic AMP after zero, one, two, and three washes. The results (Table 1) showed that washing did indeed profoundly influence cellular cyclic AMP levels; more importantly, the effect produced by washing changed with the D value at which the culture had been grown. At low D values (0.05 and 0.10 h^{-1}), the cyclic AMP levels decreased with an increasing number of washes, and the observed decrease appeared to be greater than that expected merely by the removal of extracellular cyclic AMP. For instance, at $D = 0.05 \text{ h}^{-1}$, two washes reduced the filter cyclic AMP content by

75%, yet, according to the data shown in Table 2, the extracellular cyclic AMP constituted no more than 66% of the total cyclic AMP on the filter under these conditions. At higher D values, the washings actually increased the cellular cyclic AMP, indicating net synthesis. Because of the complex effects of washing on the measured cellular cyclic AMP levels, it was decided to omit this step and to apply a correction for the contaminating extracellular nucleotide, as described above.

The cellular cyclic AMP level was high at the lowest D value tested under lactose limitation (Table 2). At $D = 0.25 \text{ h}^{-1}$, the content decreased by about one third and changed relatively little at the high D value of 0.5 h^{-1} . Under glucose limitation, the levels were similar to those found in higher D value lactose-limited cultures and showed relatively little change in response to D . Under phosphate limitation, except for $D = 0.10 \text{ h}^{-1}$, the cellular cyclic AMP level was lower than that found under other conditions. Under conditions in which the extracellular cyclic AMP was very high ($D = 0.40$ and 0.20 h^{-1} under lactose and glucose limitations, respectively), the corrected values for cellular cyclic AMP were negative; the implications of this are considered below.

Total and extracellular concentration and net synthesis rate of cyclic AMP. The steady-state

TABLE 2. Steady-state cellular and extracellular cyclic AMP levels at various D values under different limitations

$D(\text{h}^{-1})$	Cellular				Extracellular	
	Amt on filter (pmol) ^a	Residual extracellular on filter ^b (pmol)	Corrected amt (pmol)	μM^c	Amt per ml (nmol)	μM
Lactose limitation						
0.05	24.3 ± 1.0	5.4	18.9	3.8	0.27 ± 0.008	0.27
0.25	15.1 ± 0.2	8.6	6.5	1.3	0.43 ± 0.009	0.43
0.30	— ^d	—	—	—	0.60 ± 0.006	0.60
0.40	17.5 ± 0.5	25.0	-7.5	<0	1.25 ± 0.037	1.25
0.50	20.5 ± 0.4	11.2	9.32	1.9	0.56 ± 0.022	0.56
Glucose limitation						
0.05	17.4 ± 0.5	11.6	5.8	1.2	0.58 ± 0.003	0.58
0.10	—	—	—	—	0.84 ± 0.03	0.84
0.20	17.5 ± 0.3	22.0	-4.5	<0	1.10 ± 0.03	1.10
0.50	19.0 ± 0.7	9.2	9.8	2.0	0.46 ± 0.02	0.46
0.60	17.4 ± 0.3	8.6	8.8	1.8	0.43 ± 0.02	0.43
Phosphate limitation						
0.05	4.7 ± 0.0	1.0	3.7	0.7	0.05 ± 0.002	0.05
0.10	6.0 ± 0.2	0.8	5.2	1.0	0.04 ± 0.001	0.04
0.50	2.8 ± 0.1	1.4	1.4	0.29	0.07 ± 0.001	0.07
0.60	—	—	—	—	0.07 ± 0.001	0.07

^a Amount of culture filtered was 5 ml (see text).

^b The amount of contaminating extracellular cyclic AMP remaining on the filters was estimated from the finding that the filters retained 20 μl of water.

^c Calculation based on the assumption that 1 mg of cell protein corresponded to 5.4 μl of cell water (25).

^d —, Not determined.

levels of total cyclic AMP in culture varied markedly with the D value under all three individual limitations (Fig. 1). The maximum level of cyclic AMP was exhibited at an intermediate D value in carbon-limited cultures, at $D = 0.2 \text{ h}^{-1}$ in glucose-limited cultures, and at $D = 0.4 \text{ h}^{-1}$ in lactose-limited cultures. Under phosphate limitation, the total culture cyclic AMP appeared to increase with increasing D , but the magnitude of the change was small. The highest peak level of total culture cyclic AMP was found under lactose limitation, and the lowest was found under phosphate limitation. At all D values and regardless of the nature of limitation, >99.9% of the total cyclic AMP synthesized was found in the extracellular fluid (Fig. 1 and Table 1). Previous batch culture studies have created the impression that excretion of cyclic AMP occurs primarily in the stationary phase of growth (17), but these studies show that extensive excretion occurs during logarithmic growth as well.

The effect of D value on the rate of net cyclic AMP synthesis (equation 1) under the three limitations is shown in Fig. 2. In all three cases, there was an exponential increase in this rate in response to culture D , but the range of D values to which this response extended varied with the type of limitation. Under glucose limitation, the exponential response was confined to D values below 0.2 h^{-1} , with the rate remaining essentially constant at ca. $0.25 \mu\text{mol h}^{-1} (\text{mg of protein})^{-1}$ above this D value. Under lactose limitation, the exponential increase continued up to $D = 0.4 \text{ h}^{-1}$, declining somewhat above this D value, and under phosphate limitation the exponential increase appeared to extend to the entire range of D values tested. The highest rate of synthesis was observed at $D = 0.4 \text{ h}^{-1}$ under

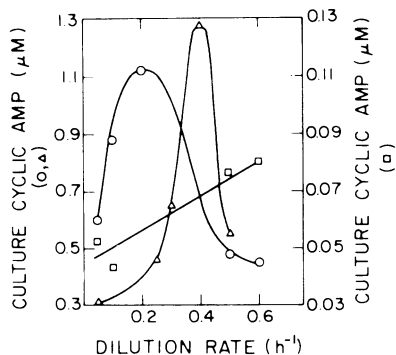


FIG. 1. Steady-state levels of total cyclic AMP in culture at various D values under glucose (\circ), lactose (Δ), or phosphate (\square) limitation. The values given are for a steady-state cell biomass equivalent to 1 mg of cell protein per ml of culture.

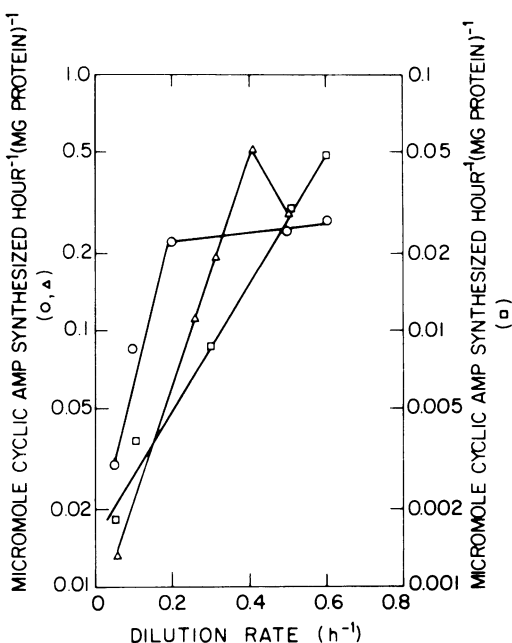


FIG. 2. Steady-state rates of cyclic AMP synthesis at various D values under glucose (\circ), lactose (Δ), or phosphate (\square) limitation.

lactose limitation ($0.5 \mu\text{mol h}^{-1} [\text{mg of protein}]^{-1}$), and this rate was twofold higher than the peak rate of synthesis under glucose limitation. Phosphate-limited cells had the lowest rate of synthesis, approximately 3 to 10% of the rate of carbon limited cultures at corresponding D values.

Energy requirement for cyclic AMP synthesis.

It was intriguing that under all growth conditions examined, almost all of the cyclic AMP synthesized was excreted into the medium. The proportion of the total available energy that this excretion represented was calculated for selected instances. It was assumed for these calculations that oxidation of 1 mol of glucose generated 26 mol of ATP (i.e., the organism possessed two proton translocating loops [7]) and that synthesis of a complete molecule of ATP (or cyclic AMP) from glucose required 14 mol of ATP (3).

The specific rate of glucose consumption was calculated from equation 2. At $D = 0.2 \text{ h}^{-1}$, this value was $3.1 \mu\text{mol of glucose h}^{-1} (\text{mg of protein})^{-1}$. Yield calculations showed that 44% of the carbon source was used in energy generation under these conditions, the rest being assimilated; thus, $1.36 \mu\text{mol of glucose h}^{-1} (\text{mg of protein})^{-1}$ was actually used in energy generation. Consequently, ATP at this D value was produced at a rate of $(1.36 \times 26) = 35.36 \mu\text{mol of}$

ATP h^{-1} (mg of protein) $^{-1}$. The rate of net cyclic AMP synthesis (Fig. 2) under these conditions was $0.224 \mu\text{mol}$ of cyclic AMP h^{-1} (mg of protein) $^{-1}$, which must have required $(0.224 \times 14) = 3.14 \mu\text{mol}$ of ATP h^{-1} (mg of protein) $^{-1}$. Hence, approximately 9% of the total energy produced was used for cyclic AMP synthesis under these conditions. Similar calculations showed that at $D = 0.05 \text{ h}^{-1}$, under glucose limitation, cyclic AMP synthesis consumed 5% of the total energy available to the cell and 8% at $D = 0.4 \text{ h}^{-1}$ under lactose limitation. The values represent only the energy demand for net cyclic AMP synthesis; depending on the extent to which cyclic AMP phosphodiesterase was active under these conditions, the actual rate and consequently the energy demand for cyclic AMP synthesis would be higher.

DISCUSSION

A major objective of this study was to determine the relationship between growth rate and cellular cyclic AMP levels in *E. coli*. However, the presence of an enormous background of extracellular cyclic AMP under all conditions tested complicated these measurements. Washing of the filters used in cellular cyclic AMP assays altered the results profoundly in ways that depended on the antecedent D value of the culture and had to be abandoned. Instead, we corrected the measured values for contaminating extracellular cyclic AMP that remained on the filter. Two findings indicate, however, that this method may not be reliable. First, we found that at $D = 0.40 \text{ h}^{-1}$ (lactose limitation) and 0.20 h^{-1} (glucose limitation), where the extracellular cyclic AMP levels were very high (Table 2), the application of the correction gave zero or even slightly negative values for cellular cyclic AMP. Second, Buettner et al. (2), who also omitted the washing step in cellular cyclic AMP measurement, reported a much lower value for contamination by extracellular cyclic AMP. Their reconstitution experiments, which involved the use of an adenylate cyclase-negative mutant, showed that the contaminating extracellular cyclic AMP constituted no more than 2 to 3% of the cellular cyclic AMP on the filter. Yet, if our correction method is applied to their data (Fig. 2, 200-min point), a contribution by extracellular cyclic AMP equal to some 55% of the cellular nucleotide would be indicated. Both of these considerations suggest that, for unknown reasons, the correction method used here leads to an underestimation of cellular cyclic AMP levels.

These uncertainties preclude firm conclusions about cellular cyclic AMP levels. It seems clear, however, that changes in D do not elicit large and systematic changes in cellular cyclic AMP levels in *E. coli*. A similar conclusion was drawn

by Wright et al. (26) for this organism grown under glucose or ammonium limitation. These workers did wash the filters employed in cellular cyclic AMP measurements, but reported no control studies on the effect of washing on these measurements. For this reason and in the light of our results, their findings should also be considered tentative. We conclude that none of the available evidence permits unambiguous conclusions about the relationship between cellular cyclic AMP levels and growth rate in bacteria: the chemostat studies suffer from technical difficulties arising from the presence of a high background of extracellular cyclic AMP, and the batch culture studies are unsatisfactory because of insufficient control of several variables, as discussed above.

Two conclusions appear certain, however: at all growth rates and under all conditions tested, >99.9% of the cyclic AMP synthesized is excreted into the medium, and the net rate of cyclic AMP synthesis increases with increasing growth rates, regardless of the nature of limitation. In this respect, our results confirm those of Wright et al. (26) and Harman and Botsford (5). The former showed excretion of a large amount of cyclic AMP by *E. coli* grown under glucose, succinate, or ammonium limitation, and the latter showed increased synthesis rates of cyclic AMP in response to increasing D in a phosphodiesterase-deficient mutant of *Salmonella typhimurium*, although they found a linear relationship between this increase and the D value which we did not find.

The advantage to the organism of elevating the rate of cyclic AMP synthesis in response to increasing growth rate, when nearly all of the nucleotide synthesized was excreted into the medium, is not at all clear. This excretion consumed up to 9% of the total energy ration of the cell and thus represented a substantial drain on its resources. When the selective pressures that imperil the survival in nature of even a slightly wasteful organism (4, 27) are considered, it must be assumed that the excretion of cyclic AMP has a salutary effect. What this effect may be remains entirely unknown. This excretion, especially if it is symptomatic of excretion of other compounds as well (8), also casts doubt on the validity of the Pirt method (20) for the calculation of energy of maintenance and theoretical maximal growth yields. This method assumes that all of the energy produced is coupled to growth or maintenance processes and that the amount of energy not linked to growth processes is independent of growth rate. This is clearly not true for the energy consumed in cyclic AMP synthesis: it can have neither a growth nor a maintenance function, nor does it appear to be constant.

Harman and Botsford (5) pointed out that their finding of a direct relationship between the cyclic AMP synthesis rate and culture D could not be explained solely on the basis of the current view of the regulation of cyclic AMP synthesis. This view holds that adenylate cyclase is the main locus of this regulation and that the activity of this enzyme is inhibited by the uptake into the cells of carbon substrates, particularly that of sugars of the phosphotransferase system (16, 18, 19). Such a mechanism would predict enhanced synthesis rates of the nucleotide at low D values by carbon-limited cultures, i.e., the precise opposite of what was observed. They suggested that the specific activity of adenylate cyclase increases with the growth rate (5). If it is assumed that adenylate cyclase remained the primary locus of the regulation of cyclic AMP synthesis during nutrient-limited growth of our wild-type strain, our results appear to be consistent with this postulation. Experiments are now in progress to determine whether adenylate cyclase levels decrease in carbon-limited organisms as they are grown at progressively lower D values. If variation in the specific activity of adenylate cyclase at different D values is indeed the cause of the observed rates of cyclic AMP synthesis, then our data show that at very low growth rates ($<0.3 \text{ h}^{-1}$) glucose is a better inducer of this enzyme than is lactose.

The fact that phosphate-limited cultures exhibited markedly depressed rates of cyclic AMP synthesis compared with carbon-limited cultures could be interpreted as being consistent with the view that the phosphorylated form of enzyme I of the phosphotransferase system stimulates adenylate cyclase activity (16, 21). Phosphate limitation forces the bacteria to dispense with nonessential phosphorylated metabolites from cellular pools (23, 24). Since phosphorylated enzyme I of phosphotransferase system has no evident role during growth on lactose, which was the carbon source employed, it is reasonable to assume that its pool levels would be low under phosphate limitation and to suspect that this accounted for the lower rates of cyclic AMP synthesis under these conditions. However, an alternate interpretation is that saturating levels of the carbon substrate (lactose) present at all D values under these conditions directly inhibited adenylate cyclase activity. Studies are now in progress to distinguish between these possibilities.

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LITERATURE CITED

- Bernlohr, R. W., M. K. Haddox, and N. D. Goldberg. 1974. Cyclic guanosine 3',5'-monophosphate in *Escherichia coli* and *Bacillus licheniformis*. *J. Biol. Chem.* **249**:4329-4331.
- Buettner, M. J., E. Spitz, and H. V. Rickenberg. 1973. Cyclic adenosine 3',5'-monophosphate in *Escherichia coli*. *J. Bacteriol.* **114**:1068-1073.
- Forrest, W. W., and D. J. Walker. 1971. The generation and utilization of energy during growth. *Adv. Microb. Physiol.* **5**:213-274.
- Harder, W., J. G. Kuenen, and A. Matin. 1977. Microbial selection in continuous culture. *J. Appl. Bacteriol.* **43**:1-24.
- Harman, J. G., and J. L. Botsford. 1979. Synthesis of adenosine 3',5'-cyclic monophosphate in *Salmonella typhimurium* growing in continuous culture. *J. Gen. Microbiol.* **110**:243-246.
- Harper, J. F., and G. Brooker. 1975. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2' O acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.* **1**:207-218.
- Jones, C. W. 1977. Aerobic respiratory systems in bacteria. *Symp. Soc. Gen. Microbiol.* **27**:23-59.
- Landwall, P., and T. Holme. 1977. Removal of inhibitors of bacterial growth by dialysis culture. *J. Gen. Microbiol.* **103**:345-352.
- Leefeldt, R. H., and A. Matin. 1980. Growth and physiology of *Thiobacillus novellus* under nutrient-limited mixotrophic conditions. *J. Bacteriol.* **142**:645-650.
- Matin, A. Microbial regulatory mechanisms at low nutrient concentrations as studied in chemostat. *Life Sci. Res. Rep.* **13**:323-339.
- Matin, A., and J. C. Gottschal. 1976. Influence of dilution rate on NAD(P) and NAD(P)H concentrations and ratios in a *Pseudomonas* sp. grown in continuous culture. *J. Gen. Microbiol.* **94**:333-341.
- Matin, A., A. Grootjans, and H. Hogenhuis. 1976. Influence of dilution rate on enzymes of intermediary metabolism in two fresh water bacteria grown in continuous culture. *J. Gen. Microbiol.* **94**:323-332.
- Otten, J., G. S. Johnson, and I. Pastan. 1971. Cyclic AMP levels in fibroblasts: relationship to growth rate and contact inhibition of growth. *Biochem. Biophys. Res. Commun.* **44**:1192-1198.
- Pastan, I., and G. S. Johnson. 1974. Cyclic AMP in the transformation of fibroblasts. *Adv. Cancer Res.* **19**:303-329.
- Pastan, I., G. S. Johnson, and W. B. Anderson. 1975. Role of cyclic nucleotides in growth control. *Annu. Rev. Biochem.* **44**:491-522.
- Peterkofsky, A. 1978. Transmembrane signalling of *Escherichia coli* adenylate cyclase via a cascade reaction with sugar transport systems. *Adv. Cyclic Nucleotide Res.* **9**:611-619.
- Peterkofsky, A., and C. Gazdar. 1971. Glucose and metabolism of adenosine 3',5'-cyclic monophosphate in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **68**:2794-2798.
- Peterkofsky, A., and C. Gazdar. 1973. Measurements of adenosine 3',5'-cyclic monophosphate in intact *Escherichia coli* B. *Proc. Natl. Acad. Sci. U.S.A.* **70**:2149-2152.
- Peterkofsky, A., J. Harwood, and C. Gazdar. 1975. Inducibility of sugar sensitivity of adenylate cyclase of *E. coli* B. *J. Cyclic Nucleotide Res.* **1**:11-20.
- Pirt, S. J. 1975. Principles of microbe and cell cultivation, p. 63-80. John Wiley & Sons, Inc., New York.
- Saier, M. H., Jr. 1977. Bacterial phosphoenolpyruvate:sugar phosphotransferase system: structural, functional, and evolutionary interrelationships. *Bacteriol. Rev.* **41**:856-871.
- Steiner, A. L., C. W. Parker, and D. M. Kipnis. 1972. Radioimmunoassay of cyclic nucleotides. *J. Biol. Chem.* **274**:1106-1113.
- Tempest, D. W. 1970. The place of continuous culture in

- microbial research. *Adv. Microb. Physiol.* **4**:223-250.
24. **Tempest, D. W., J. L. Meers, and C. M. Brown.** 1970. Influence of environment on the content and composition of microbial free amino acid pools. *J. Gen. Microbiol.* **64**:171-185.
25. **Winkler, H. H., and T. H. Wilson.** 1966. The role of energy coupling in the transport of β -galactosides by *Escherichia coli*. *J. Biol. Chem.* **241**:2200-2211.
26. **Wright, L. F., D. P. Milne, and C. J. Knowles.** 1979. The regulatory effects of growth rate and cyclic AMP levels on carbon catabolism and respiration in *Escherichia coli* K-12. *Biochim. Biophys. Acta* **583**:73-80.
27. **Zamenhoff, S., and H. H. Eichhorn.** 1967. Study of microbial evolution through loss of biosynthetic functions: establishment of "defective" mutants. *Nature (London)* **216**:456-458.