Cyclic AMP and Cyclic GMP Control of Synthesis of Constitutive Enzymes in *Escherichia coli*

By PETER H. CALCOTT†

Department of Biological Sciences, Wright State University, Dayton, Ohio 45435, U.S.A.

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*Escherichia coli* was grown in chemostat culture under glycerol-limited and ammonium-limited conditions at growth rates between 0.1 and 0.5 h⁻¹. At steady state, the concentrations of cyclic AMP and cyclic GMP and the activities of four constitutive enzymes (glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, NADH oxidase and cyclic phosphodiesterase) were determined in the organism. Addition of exogenous cyclic AMP, cyclic GMP or phencyclidine perturbed the steady state and caused inhibition or stimulation of synthesis of phosphodiesterase and isocitrate dehydrogenase. A novel hypothesis is proposed to account for the ability of bacteria to regulate the synthesis of constitutive enzymes with cyclic nucleotides and possibly other small molecules.

INTRODUCTION

When bacteria grow, they can modulate their enzyme complement to adjust to changing environments. With a few exceptions, the precise factors which stimulate or repress synthesis of enzymes are unknown (Chock *et al.*, 1980; Drew & Demain, 1977; Matin, 1981; Nierlich, 1978; Pastan & Adhya, 1976; Rickenberg, 1974). Matin (1981), while reviewing control of enzyme synthesis in microbes grown in continuous culture, concluded that small molecules acted as effectors in controlling the synthesis of a variety of constitutive enzymes. He postulated that cyclic AMP might be one of these small molecules. Cyclic AMP is a potential effector since it has been implicated in the control of numerous inducible enzymes and to a lesser degree in constitutive enzymes (Aono *et al.*, 1978; Calcott & Calvert, 1981; Dietzler *et al.*, 1977; Pastan & Adhya, 1976; Peterkofsky, 1976; Rickenberg, 1974). Cyclic AMP concentrations and adenylate cyclase activities have been shown to be modulated by growth rate in chemostat populations of *Escherichia coli* (Botsford & Danley, 1975; Harman & Botsford, 1979). More recently, cyclic GMP has been implicated in cell metabolism, though the evidence is less strong (Black *et al.*, 1980; Cook *et al.*, 1980; Goldberg *et al.*, 1975; Gonzalez & Peterkofsky, 1975; Pastan & Adhya, 1976; Peterkofsky, 1976).

In the work described here, the relationship was investigated between the activities of four constitutive enzymes and the concentration of cyclic AMP and cyclic GMP in *E. coli* grown under a variety of growth conditions in continuous culture. In addition, the effect of exogenous cyclic nucleotides and phencyclidine on the synthesis of two of the enzymes was investigated.

METHODS

Organism and cultural conditions. *Escherichia coli* (451-B variant) was maintained on nutrient agar at room temperature after growth at 37 °C for 24 h. Organisms were grown aerobically in chemostats of effective volume 500 ml (except in certain experiments where it was lowered to 50 ml) at 37 °C, at growth rates from 0-10 to

† Present address: CR-Bioproducts Laboratory, The Dow Chemical Company, Midland, MI 48640, U.S.A.
0.5 h⁻¹ (Calcott & MacLeod, 1974) in a simple salts/ammonium/glycerol-based medium (Postgate & Hunter, 1962). Glycerol-limiting medium contained 2 g glycerol l⁻¹ and 50 mM-ammonium, while ammonium-limiting contained 10 g glycerol l⁻¹ and 6 mM-ammonium ions. Cultures were grown in the chemostat to steady state (as judged by a constant turbidity over a 24 h period) before samples were removed for analysis. In all experiments, steady states were obtained at the lowest growth rate first (0-10 h⁻¹). Once analysis had been performed, a new steady state was established at a specific growth rate (μ) of 0.20 h⁻¹, then at 0-30 h⁻¹ and finally at 0.50 h⁻¹. After analysis, the growth rate was then decreased to 0-10 h⁻¹ and the steady state reestablished. The data for this steady state never deviated by more than 10% from those obtained from the population initially at μ = 0.1 h⁻¹. In general, steady states required more than seven replacements of the culture to establish. Chemostats were never operated for more than one month. In certain experiments, steady states were perturbed by addition of cyclic AMP (10 mM), cyclic GMP (10 mM) or phencyclidine (0.1 mM) to the culture vessel and by changing the inflow medium to one with the additive at the appropriate concentration. At various times, samples were removed for analysis. Under no growth condition did the bacterial carbohydrate content determined by the anthrone method (Calcott, 1974) exceed 10% dry weight. This prevents complications in interpreting the concentrations of nucleotides and enzymes in the organism.

Enzyme and protein assays. Isocitrate dehydrogenase (EC 1.1.1.42), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 2’3’-cyclic nucleotide 2’-phosphodiesterase (EC 3.1.4.16) and NADH oxidase (EC 1.6.99.3) were assayed as described before (Calcott, 1981a; Calcott & Calvert, 1981) on extracts prepared by ultrasonic disruption. Protein was determined by the Lowry method with bovine serum albumin as standard.

Cyclic nucleotide extraction and determination. Samples of the culture were centrifuged and washed in growth medium without carbon source before the organisms were resuspended in 5% (w/v) trichloroacetic acid (Gersch et al., 1978). The preparations were then frozen at −80 °C, thawed and sonicated for 6 min (Gersch et al., 1978). The extract was centrifuged to remove debris and washed thrice in diethyl ether to remove lipids; the fluid was then applied to a Dowex (1 x 2-200) column. The column was eluted sequentially with distilled water, 50 mM-HCl (to remove cyclic AMP) and then 500 mM-HCl (to remove cyclic GMP). The acid fractions were collected and freeze-dried before reconstitution with boiled, glass-distilled water. Recovery of cyclic nucleotides, which was between 80 and 90%, was determined by monitoring recovery of [³H]cyclic AMP or [³H]cyclic GMP from parallel samples taken through the freezing, sonication, ether washing, chromatography and freeze-drying steps. No degradation of the cyclic nucleotide could be detected in the extraction and purification procedures as determined by paper chromatography (Calcott & Calvert, 1981). Cyclic AMP and cyclic GMP were determined by protein-binding assay systems (Boehringer-Mannheim); 0.5 pmol cyclic AMP and 0.1 pmol cyclic GMP could be detected. The authenticity of the extracted nucleotides was confirmed since they co-chromatographed with authentic nucleotide and were at least 90% degraded by commercial cyclic phosphodiesterase (Sigma: no. P0134). Cyclic nucleotide contents of bacteria were expressed as pmol or fmol (mg dry wt)⁻¹. Bacterial dry weights were determined turbidimetrically from a calibration curve.

Chemicals. All chemicals were purchased from Sigma or from Fisher Chemical Company, Cincinnati, Ohio, except phencyclidine (1-phenylcyclohexylpiperidine) which was purchased from Applied Science Division of Milton Roy Company, State College, Pennsylvania.

RESULTS

When Escherichia coli was grown in chemostat culture, the concentrations of cyclic AMP and cyclic GMP were dependent on both growth rate and nutrient limitation (Figs 1 and 2). For glycerol-limited cultures the concentrations of cyclic AMP and cyclic GMP decreased as growth rate increased (Fig. 1), while for ammonium-limited cultures, the cyclic GMP increased and the cyclic AMP was unchanged (Fig. 2). The concentrations of cyclic nucleotides were higher in glycerol-limited cultures than in ammonium-limited ones. The concentration of cyclic AMP also decreased with increasing growth rate in glucose-limited and lactose-limited cultures in a similar fashion, with glucose-limited cultures containing low (16 pmol mg⁻¹) and lactose-limited cultures intermediate (52 pmol mg⁻¹) concentrations of cyclic AMP at a specific growth rate of μ = 0.1 h⁻¹.

The activities of four constitutive enzymes, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, NADH oxidase and phosphodiesterase, were investigated in organisms grown in continuous culture (Figs 1 and 2). In general, highest activities were detected in glycerol-limited organisms grown at the highest rates. In ammonium-limited cultures, a similar trend was noted. Under both nutrient limitations, each enzyme appeared to be regulated independently.
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Fig. 1. Effect of growth rate on the activities [units (mg dry wt)$^{-1}$] of four constitutive enzymes and the concentrations of cyclic AMP and cyclic GMP in glycerol-limited chemostat cultures of Escherichia coli at steady state. Samples were removed and assayed for isocitrate dehydrogenase (●), glucose-6-phosphate dehydrogenase (○), phosphodiesterase (△), NADH oxidase (▲), cyclic AMP (■) and cyclic GMP (□) as described in Methods. The bars represent the range of results for each point.

Fig. 2. Effect of growth rate on the activities [units (mg dry wt)$^{-1}$] of four constitutive enzymes and the concentrations of cyclic AMP and cyclic GMP in ammonium-limited chemostat cultures of Escherichia coli at steady state. Samples were removed and assayed for isocitrate dehydrogenase (●), glucose-6-phosphate dehydrogenase (○), phosphodiesterase (△), NADH oxidase (▲), cyclic AMP (■) and cyclic GMP (□) as described in Methods. The bars represent the range of results for each point.
Table 1. Statistical relationships between the activities of four constitutive enzymes and cyclic nucleotide concentrations in E. coli

Enzyme activities and cyclic AMP and cyclic GMP concentrations were determined in steady-state populations of bacteria grown in chemostat. The data were fitted to the equation \[ z = c + a_1 x + a_2 y, \]
where \( z \) represents enzyme activity, \( x \) and \( y \) represent the concentrations of cyclic AMP and cyclic GMP, respectively, and \( c, a_1, \) and \( a_2 \) are constants (see text). The statistical analysis was made by multiple linear regression analysis by the methods of least squares; \( r^2, \) degrees of freedom (DF) and \( t \) values were determined and the \( t \) value was converted to probability with Student \( t \)-tables.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( c )</th>
<th>( a_1 )</th>
<th>( a_2 )</th>
<th>( P )</th>
<th>DF</th>
<th>( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphodiesterase</td>
<td>4.0</td>
<td>-0.032</td>
<td>+0.01</td>
<td>&lt;0.088</td>
<td>8</td>
<td>0.60</td>
</tr>
<tr>
<td>Isocitrate dehydrogenase</td>
<td>4.4</td>
<td>-0.007</td>
<td>-0.023</td>
<td>&lt;0.0076</td>
<td>8</td>
<td>0.86</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>2.43</td>
<td>+0.019</td>
<td>-0.065</td>
<td>&lt;0.058</td>
<td>8</td>
<td>0.78</td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>-2.22</td>
<td>-0.02</td>
<td>+0.03</td>
<td>&lt;0.0001</td>
<td>8</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of exogenous cyclic AMP and cyclic GMP on the activities of isocitrate dehydrogenase and phosphodiesterase in chemostat cultures of *Escherichia coli*. The bacteria were grown to steady state in glycerol-limiting chemostat culture of 50 ml effective volume at a growth rate of 0.50 h\(^{-1}\). At time 0, cyclic AMP (a) or cyclic GMP (b) was added to the growth vessel to a final concentration of 10 mM and the inflow medium changed to one containing the cyclic nucleotide. At various times samples were removed and assayed for phosphodiesterase (●) and isocitrate dehydrogenase (○). The dotted lines represent variation (range of values) experienced in a parallel unperturbed culture. The mean control activities were: phosphodiesterase, 3-2 units (mg dry wt\(^{-1}\)); isocitrate dehydrogenase, 9 units (mg dry wt\(^{-1}\)). The bars represent the range of results for each point.

Since there appeared to be a linear relationship between cyclic nucleotide concentrations and certain of the enzyme activities, a linear model, as a first-order approximation, was tested. Multiple regression analysis of the relationships between the concentrations of the two cyclic nucleotides and the activities of the four enzymes yielded regression equations of the general form \( z = c + a_1 x + a_2 y, \) where \( z \) was the enzyme activity (\( \mu \)mol substrate consumed or product formed min\(^{-1}\) mg\(^{-1}\)), \( c \) (\( \mu \)mol substrate consumed or product formed min\(^{-1}\) mg\(^{-1}\)), \( a_1 \) [\( \mu \)mol substrate consumed or product formed (pmol cyclic AMP\(^{-1}\))], and \( a_2 \) [\( \mu \)mol substrate consumed or product formed (pmol cyclic GMP\(^{-1}\))] were constants and \( x \) and \( y \) were the concentrations of the cyclic AMP (pmol mg\(^{-1}\)) and cyclic GMP (fmol mg\(^{-1}\)), respectively (Table 1). The relationships were statistically significant for each set of eight data points at least at the 0.1 level. From this analysis, between 60 and 99% of the variation in enzyme activity could be explained by altered cyclic nucleotide concentrations. Although the activities of the four enzymes could be predicted by the concentrations of the two cyclic nucleotides, each enzyme exhibited a different relationship with different values of \( a_1 \) and \( a_2 \), both positive and negative.
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Fig. 4. Effect of phencyclidine on the activities of isocitrate dehydrogenase and phosphodiesterase, and the bacterial concentration of cyclic AMP and cyclic GMP, in chemostat cultures of Escherichia coli grown as described for Fig. 3. At time 0, phencyclidine was added to the growth vessel to a final concentration of 0.1 mM and the inflow medium changed to one containing the drug. At various times samples were removed and assayed (a) for isocitrate dehydrogenase (○) and phosphodiesterase (●) activities, and (b) for the concentrations of cyclic AMP (△) and cyclic GMP (▲). The dotted lines represent variation (range of values) experienced in a parallel unperturbed culture. The mean control activities were: isocitrate dehydrogenase, 8.6 units (mg dry wt)^-1; phosphodiesterase, 3.5 units (mg dry wt)^-1. The bars represent the range of results for each point.

While relationships were found between enzyme activities and cyclic nucleotide concentrations, causality could not be determined with the data presented. To determine whether the observed enzyme activities were direct results of the cyclic nucleotide concentrations, cyclic AMP or cyclic GMP was added to steady-state cultures of E. coli and cyclic phosphodiesterase and isocitrate dehydrogenase were monitored (Fig. 3). When the culture was perturbed by cyclic AMP, there was an inhibition in the synthesis of the phosphodiesterase while the synthesis of isocitrate dehydrogenase was not affected. In a similar fashion, perturbation of the culture with cyclic GMP caused a stimulation in synthesis of the phosphodiesterase and an inhibition in synthesis of the isocitrate dehydrogenase. This is consistent with the values in Table 1, where phosphodiesterase activity was negatively related to cyclic AMP and positively related to cyclic GMP concentrations. Similarly, the effects of perturbation by cyclic nucleotides on isocitrate dehydrogenase activities were as predicted from Table 1, where enzyme activities were negatively related to both cyclic AMP and cyclic GMP concentrations, with the effect of cyclic AMP, though statistically significant, being smaller than that for cyclic GMP (Table 1). Additions of cyclic nucleotides did not in themselves stimulate or inhibit the observed enzyme activities in cell extracts (results not shown). This indicated that the cyclic nucleotide concentrations could be directly controlling the synthesis or degradation of the enzymes.

To test further the relationships between enzyme activities and cyclic nucleotide concentrations, the effect of inhibitors of cyclic nucleotide metabolism on enzyme synthesis was sought. While methyl xanthines are effective inhibitors of cyclic phosphodiesterases in animal systems, Klebsiella and Serratia, they are not in E. coli (Calcott & Calvert, 1981; Peterkofsky, 1976). However, phencyclidine has been shown to inhibit guanylate cyclase in animal cells (Vesely, 1979). Phencyclidine was added to a steady-state culture of E. coli and the concentrations of the two cyclic nucleotides and the activities of the two enzymes, phosphodiesterase and isocitrate dehydrogenase were followed (Fig. 4). Phencyclidine caused a rapid and similar decrease in the concentrations of both cyclic nucleotides, which appeared in the growth medium indicating that the mechanism of action of the drug might be by stimulating excretion. During these decreases in cyclic nucleotide concentration, isocitrate dehydrogenase synthesis was stimulated and that of phosphodiesterase inhibited. This result is consistent with the values in Table 1.
Matin (1981) has reviewed the control of enzyme synthesis in microbes grown in continuous culture. Of 51 bacterial enzyme systems studied, 95% showed enzyme synthesis influenced by growth rate and/or nutrient limitation, whereas of 47 eukaryotic systems studied, 77% were influenced by these two parameters. Matin (1981) predicted that small molecules, such as cyclic AMP, could play crucial roles in regulating enzyme synthesis. In fact, cyclic AMP concentrations and the activity of adenylate cyclase were growth-rate dependent in chemostat cultures of *E. coli* (Botsford & Danley, 1975; Harman & Botsford, 1979). The observation of modulation of cyclic AMP and cyclic GMP concentrations by growth rate and nutrient limitation in chemostat cultures reported here not only confirms Botsford’s studies and Matin’s predictions, but extends them to include cyclic GMP.

Pastan & Perlman (1967) proposed four criteria to implicate cyclic nucleotides in mediation of cell phenomena, namely: (1) nucleotide concentrations must correlate with observed cell function; (2) addition of exogenous nucleotides must affect in a predictable manner the cell response; (3) inhibitors of cyclic nucleotide metabolism must similarly affect the phenomenon; and (4) the cyclic nucleotide synthesis and degradation enzymes should also be modulated during the phenomenon. This study has satisfied the first three but has not tested the fourth criterion. Thus, the four enzyme activities or their rates of synthesis in chemostat populations were the direct result of the concentrations of the cyclic nucleotides in the organisms. While these two cyclic nucleotides have been implicated in the synthesis of the enzymes, each enzyme shows a different relationship between enzyme activity and cyclic nucleotide concentration (Table 1). Although 60–99% of the variability in enzyme activity could be explained by fluctuations in cyclic nucleotide concentrations, 1–40% of the variability must be due to other factors, such as other small molecules. Nevertheless, the two cyclic nucleotides play important roles in governing the levels of four distinct constitutive enzymes in *E. coli* and logically might also play roles in general metabolism of this organism. The precise steps in enzyme synthesis that are controlled by cyclic AMP and cyclic GMP cannot be determined from these data.

With these data, it is possible to construct a working hypothesis, which I term the ‘permutation hypothesis’, to relate cyclic nucleotide concentration to cell metabolism. In bacteria, there are blocks of constitutive enzymes, which may or may not represent metabolic pathways. The synthesis of these blocks of enzymes is controlled at the transcriptional level by the concentration of at least two small molecules, cyclic AMP and cyclic GMP. For instance, synthesis of one block might be stimulated by low cyclic AMP and high cyclic GMP concentrations and repressed by the opposite. An example of an enzyme such as this would be phosphodiesterase. On the other hand, the synthesis of another block might be stimulated by both high cyclic AMP and cyclic GMP concentrations. The example in this system would be glucose-6-phosphate dehydrogenase. The synthesis of another enzyme might be relatively unaffected by cyclic AMP yet be stimulated by cyclic GMP (e.g. isocitrate dehydrogenase). With two nucleotides, e.g. cyclic AMP and cyclic GMP, and three possible effects, a positive, a negative and a neutral, nine permutations or blocks of enzymes under separate control are possible. Most probably, the cell contains more than nine blocks of enzymes. By introducing a third regulator, the number would increase to 27; a fourth would increase it to 81. The other regulators could be other cyclic nucleotides such as cyclic TMP, cyclic IMP, cyclic UMP, cyclic CMP, or highly phosphorylated compounds such as guanosine tetraphosphate or pentaphosphate (Gallant, 1979; Silverman & Atherley, 1979) or catabolite modulator factor (Dessein et al., 1978). Thus these small molecules could play roles in control of enzyme synthesis with other, cruder, control being accomplished by intermediates of metabolism or inducers, e.g. lactose for the lac operon (Pastan & Adhya, 1976; Pastan & Perlman, 1967; Peterkofsky, 1976; Rickenberg, 1974). Once the absolute concentration of enzyme has been defined, the organisms’ fine control mechanisms, e.g. allostery, could be used to produce *in vivo* activity.
The data and the hypothesis presented in this paper pertain to this population of *E. coli* when growing at steady state in either glycerol- or ammonium-limited chemostat culture. Several questions arise, namely: is the hypothesis relevant (1) for other nutrient limitations such as sulphate, potassium or magnesium, (2) for growth conditions where the carbon source is changed, for example to glucose or lactose, and (3) for other strains of bacteria? At present no data are available to answer any of these questions. However, enzyme activities are modulated in bacteria grown under nutrient limitations other than carbon or ammonium (see Matin, 1981) but no data are available on the effects on cyclic nucleotide concentrations. When bacteria are transferred from one carbon source to another, large changes in cyclic nucleotide, particularly cyclic AMP, concentration are observed for batch-grown (Gonzalez & Peterkofsky, 1975) and chemostat-grown (Wright *et al.*, 1979; this paper) cultures. Under conditions when one cyclic nucleotide concentration is abruptly changed, the hypothesis would require the concentration of the other effectors to be modulated to compensate; Gonzalez & Peterkofsky (1975) have observed this modulation of cyclic AMP and cyclic GMP in *E. coli* during carbon source change. While this hypothesis was developed to describe the response of one particular strain of *E. coli* grown in chemostat culture, the study of the response of other strains, for instance those defective in cyclic nucleotide metabolism, should be fruitful. While bacterial physiology is rigorously controlled by the constant environment attained at steady state in a chemostat culture, batch-culture grown organisms are in an ever-changing environment which directly alters their physiology (Calcott, 1981b). Thus interpretation of the relationship between enzyme activities and cyclic nucleotide concentrations might be extremely difficult in batch-grown organisms.

When the concentration of carbon source was very high (ammonium-limiting conditions) the cyclic AMP concentration was very low. Under these growth conditions, growth rate does not influence the concentration of carbon source in the growth vessel (Calcott, 1981a) and did not affect the cyclic AMP concentrations in the organism. However, under glycerol limitation, the concentration of carbon source in the growth vessel would be much lower than that in ammonium-limited cultures and was also growth rate dependent. When organisms grew in chemostat at slow rates, the concentration of carbon source would be low while the cyclic AMP concentration was high. As growth rate increased, the concentration of carbon source would increase and cyclic AMP concentration decreased. Not only was cyclic AMP concentration apparently governed by sugar concentration, but also by the nature of the sugar. Glucose-limited cultures contained lower concentrations of the nucleotide than glycerol-limited cultures, with lactose-limited cultures containing intermediate concentrations. This correlated with the relative efficiency of the sugars as catabolite repressors, with glucose being the most effective, glycerol the least and lactose intermediate (Pastan & Adhya, 1976; Pastan & Perlman, 1967; Peterkofsky, 1976; Rickenberg, 1974). With the data presented, it is impossible to predict whether cyclic AMP concentrations were controlled by adenylate cyclase activity as proposed by Peterkofsky and colleagues (Peterkofsky, 1976, 1977; Peterkofsky & Gazdar, 1979), by the phosphodiesterases (Buettner *et al.*, 1973; Calcott & Calvert, 1981) and/or by excretion of the nucleotide (Goldenbaum & Hall, 1979). From the data presented, it is apparent that the control of cyclic GMP concentrations in *E. coli* was more complex than that of cyclic AMP, with both nutrient concentrations and limitations, as well as growth rates, playing roles.

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**REFERENCES**


