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Measurements of Rates of Adenosine 3′:5′-Cyclic Monophosphate Synthesis in Intact *Escherichia coli* B

(adenylate cyclase/glucose utilization)

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ABSTRACT **A method for labeling adenosine 3′:5′-cyclic monophosphate *in vivo* from precursor adenosine followed by quantitative analysis of the labeled nucleotide is described. When the labeling period is short and the specific activity of the ATP pool is determined, the rate of incorporation of radioactivity corresponds to a determination of adenylate cyclase activity *in vivo*. In *E. coli* B the specific activity of adenylate cyclase *in vivo* varies under different growth conditions. Under all conditions tested, the adenylate cyclase activity *in vivo* is great enough to account for the pattern of accumulation of cyclic AMP. This is in contrast to previous adenylate cyclase assays *in vitro*, where the measured enzyme activities were insufficient to account for the amounts of cyclic AMP accumulated.**

We have previously reported that *Escherichia coli* B accumulates substantial amounts of cyclic AMP (cAMP) extracellularly (1). During typical growth conditions in glucose-minimal medium, the extracellular cAMP concentration increases dramatically at the end of exponential growth. The coincidence of cAMP accumulation and glucose depletion of the medium (1, 2) indicates that cAMP metabolism is influenced by glucose. In our previous study, we explored the possibility that glucose exhaustion derepresses the synthesis of adenylate cyclase. However, measurements of adenylate cyclase activity in extracts of logarithmic or stationary phase cells gave no indication of higher adenylate cyclase activity in stationary or glucose-starved cells than was found in extracts of logarithmic phase cells. These data were, however, complicated by the fact that the measured activities of adenylate cyclase in extracts were insufficient to account for the amounts of cAMP which accumulated.

With the intention of further studying the mode of regulation of adenylate cyclase under conditions where the enzyme activity is maximally expressed, we have devoted our recent attention to studies in intact cells. The present report details a method whereby rates of cAMP formation can be measured in intact cells. We have termed this the "*in vivo*" assay for adenylate cyclase. Under these conditions, we show that adenylate cyclase activity is sufficiently high to account for the rate of accumulation of the nucleotide under various growth conditions. Further, we demonstrate that when glucose is depleted from growth medium, there is an increase in the adenylate cyclase activity *in vivo* sufficient to fully account for the increased accumulation of cAMP.

A portion of this work has been published in abstract form (3).

METHODS

All growth experiments reported here were done with *E. coli B* cultured in a minimal salts medium (4) supplemented with the indicated concentrations of glucose. Growth was determined turbidimetrically at 650 nm in a Gilford model 300 spectrophotometer. Concentrations of unlabeled cAMP were determined by the Gilman procedure (5) as previously described (1). ATP concentrations were measured by the luciferase procedure, with firefly lantern extract (Sigma) by the procedure of Klofat *et al.* (6). Radioactivity in ATP was determined after thin-layer chromatography of an aliquot of the HCOOH extract of cells by the method of Cashel *et al.* (7). [^{3}H]Adenosine (17.7 Ci/mmol) and [^{14}C]cAMP (52.5 Ci/mol) were obtained from New England Nuclear Corp. Dowex-50, analytical grade, was obtained from Bio-Rad. Beef-heart 3′:5′-cyclic nucleotide phosphodiesterase was a Sigma product. Polyethyleneimine thin-layer chromatography plates (Polygram Cel-300 PEI) were from Brinkmann.

Assay for cAMP formation in intact cells

A 10-ml aliquot of *E. coli B* in salts medium (4) supplemented with glucose was placed in a 50-ml beaker and subjected to shaking at 37° in a reciprocating water-bath shaker. Under continuous shaking, an aliquot (0.5 ml) of [^{3}H]adenosine (17.7 Ci/mmol, 1 mCi/ml) was added to the sample. For determination of ATP concentration and specific activity, samples (0.1-ml) were withdrawn into tubes containing 0.05 ml of 2 N HCOOH at 20-sec intervals during the reaction. The balance of the incubation was terminated usually at 1 min by addition of 5 ml of 2 N HCOOH. The acidified samples were frozen and thawed, then centrifuged to remove the cell debris.

Determination of [^{3}*H*]*cAMP*. The supernatant solutions (usually about 15 ml), after dilution to 20 ml, were adjusted to pH 1.54–1.6 with HCl. An aliquot (0.01 ml) of [^{14}C]cAMP (52.5 Ci/mol, 10 μCi/ml) was added, and the sample was applied to a column of Dowex-50 (AG50W-X2, 200–400 mesh), 1.5 × 23 cm, that had been equilibrated with HCl solution (pH 1.54–1.6). All column equilibrations, washings, and elutions were with this same HCl solution. 10-ml Fractions were collected. Those fractions containing the [^{14}C]cAMP were pooled and lyophilized (see Fig. 1*A*). The dried sample was dissolved in 6 ml of 0.05 M Tris·HCl buffer (pH 7.5). Aliquots (2.5-ml) of this solution were incubated with 0.05 ml of 0.1 M $MgCl_2$ in the presence or absence of 0.4 mg of 3′:5′-cyclic nucleotide phosphodiesterase from beef heart (0.45 units/mg

of protein) for 30 min at 30°. After this incubation period, about 60 A_{260} units of unlabeled 5′-AMP was added to each sample. 15 ml of HCl was added, and the solutions were adjusted to pH 1.57 with HCl. These samples were applied to columns (0.9 × 21 cm) with an attached reservoir (3 × 20 cm) containing a 9-ml volume of Dowex-50 (AG50W-X8, 100–200 mesh) equlibrated with HCl. The columns were washed with 175 ml of HCl; then a further fraction of 150 ml was collected. On some occasions, as in Fig. 1 *B* and *C*, 3.5-ml fractions were collected.

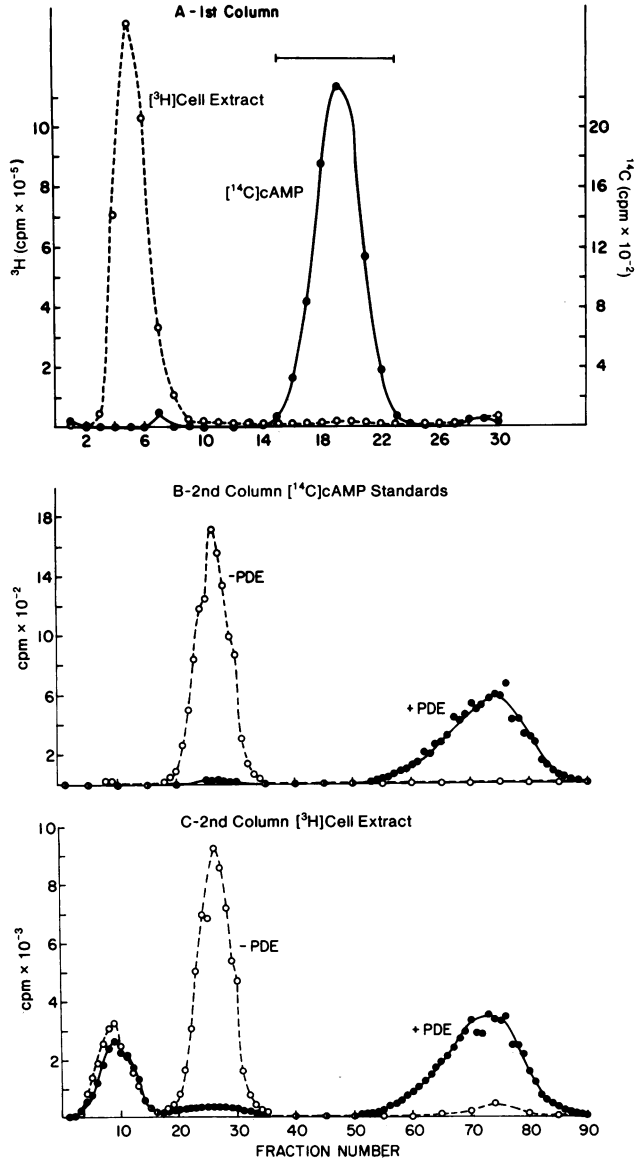

FIG. 1. Fractionation of radioactive cAMP extracted from *E. coli* B cells exposed to [³H]adenosine for 1 min. A 10-ml aliquot of *E. coli* B at stationary phase, which shows high amount of *in vivo* adenylate cyclase, was exposed to [³H]adenosine. Fractionation on Dowex-50 columns was as described in *Methods*. (*A*) O- - -O, ³H-labeled cell extract; ●——●, ¹⁴C-labeled cAMP standard. (*B*) O- - -O, [¹⁴C]cAMP standard, recovered from first column; ●——●, [¹⁴C]cAMP standard, recovered from first column, then treated with cAMP phosphodiesterase (*PDE*). (*C*) O- - -O, Crude cAMP fraction recovered from ³H-labeled cell extract; ●—— ●, crude cAMP fraction recovered from ³H-labeled cell extract, then treated with cAMP phosphodiesterase.

Measurement of the A_{260} of the second fraction usually indicated that 90–100% of the added 5′-AMP was recovered. Aliquots (3-ml) of the fractions containing 5′-AMP were counted in a scintillation counter, under conditions that detected both ¹⁴C and ³H. After correction for recovery of the added 5′-AMP, the difference in the ³H counts between the enzyme-treated and untreated samples was calculated. This difference was assumed to correspond to the amount of labeled cAMP originally in that aliquot. The ¹⁴C radioactivity recovered was used to calculate the amount of radioactive cAMP in the total original sample.

Determination of ATP Specific Activity. The aliquots (0.15-ml) of the HCOOH-treated cells were lyophilized to dryness, then dissolved in 0.05 ml of H_2O. Standard solutions of ATP were similarly treated. ATP concentration was determined by a luciferase method (6) on 0.01-ml aliquots of these samples. Thin-layer chromatography on polyethyleneimine plates was done by spotting 0.005 ml of ATP (5 μmol/ml) and 0.005 ml of the radioactive samples in each channel (7). The plates were first run with H_2O, then with 1 M KH_2PO_4 (pH 3.4). The ATP spots were made visible under ultraviolet light, then cut out and counted with 10 ml of toluene scintillator. The specific activity of ATP was determined from these counts and the ATP concentrations. The specific activity of ATP was used to convert the expression of cAMP radioactivity into concentration (usually pmol).

RESULTS

Measurement of *in vivo* cAMP formation

The assay for detection of radioactive cAMP synthesized in short time intervals in intact cells was based on the extraction of acid-soluble compounds from cells followed by chromatographic isolation of a fraction enriched in cAMP. Isolation of the 5′-AMP produced by treatment of this fraction with 3′:

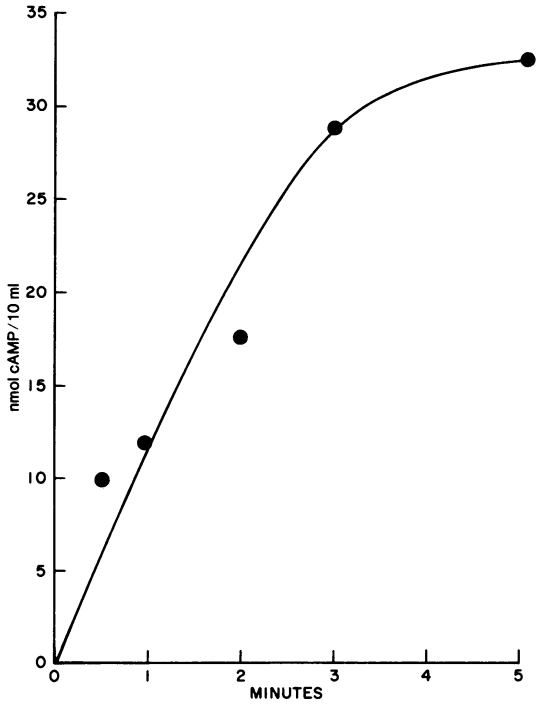

FIG. 2. Kinetics of adenylate cyclase activity *in vivo*. Aliquots (10-ml) of *E. coli* B at stationary phase were incubated in the presence of [³H]adenosine for the indicated times.

5'-cyclic nucleotide phosphodiesterase was used as a final purification step. A determination of the specific activity of the ATP pool that served as the precursor of the measured cAMP provided the basis for calculating the amount of newly synthesized cAMP.

Fig. 1 shows the results of a representative fractionation. The major portion of the tritium-labeled radioactivity was resolved from cAMP in the first column fractionation (panel *A*). The cAMP fraction was treated as described in *Methods* and rechromatographed. Fig. 1*B* shows the behavior of the standard [^{14}C]cAMP. In the absence of phosphodiesterase treatment, the radioactivity was eluted in the first fraction, characteristic of cAMP. After phosphodiesterase treatment, the radioacitity eluted later in the second fraction, characteristic of 5'-AMP. Fig. 1*C* details the behavior of the ^{3}H-labeled sample recovered from the first column. The sample not treated with phosphodiesterase, while not homogeneous, had a main component that chromatographed mainly in the cAMP region. After treatment with phosphodiesterase, essentially all of this main peak shifted to the region characteristic of 5'-AMP. The radioactivity in the 5'-AMP region that specifically resulted from phosphodiesterase treatment was taken as a measure of the amount of cAMP in the original ^{3}H-labeled extract.

Kinetics of cAMP formation in intact cells

Using the radioactive labeling method described in the preceding section, we determined the time course of labeled cAMP synthesis after a single addition of radioactive adenosine (Fig. 2). The kinetics are curvilinear for 2–3 min. The departure from linearity after that time probably reflects an equilibration of the radioactivity in the ATP pool with that in the cAMP pool. The specific activity of the ATP pool remains essentially constant (about 45 cpm/pmol) from the period 1 through 5 min. To avoid complications due to nonlinear incorporation, the studies to be described were done with 1-min labeling periods.

***In vivo* cAMP synthesis as a function of the growth stage**

By use of the assay just described, the initial rates of cAMP synthesis at different stages of growth and under different growth conditions were determined (Table 1). As we have previously shown (1), cells in the logarithmic phase accumulate only small amounts of cAMP (72 pmol/hr per mg of protein). During the transition to stationary phase, in concert with depletion of glucose, there was a marked increase in cAMP accumulation (1425 pmol/hr per mg of protein). This increased accumulation rate was transient, since after overnight incubation, the accumulation rate was decreased to about the logarithmic level (46 pmol/hr per mg of protein). Measurements of the adenylate cyclase activity *in vivo* showed a parallel to the pattern of cAMP accumulation. At the logarithmic stage of growth, the *in vivo* activity was 1740 pmol/hr per mg of protein, a value substantially higher than necessary to account for the accumulated nucleotide. We interpret this to mean that the 1-min measurement *in vivo* describes the initial rate of adenylate cyclase uncomplicated by degradation, while the measurement of the accumulated nucleotide reflects both synthetic and degradative processes. Therefore, the reason that logarithmic cells show no accumulation of cAMP is that their synthetic capacity (1700 pmol/hr per mg of protein) is balanced by an equivalent degradative activity.

TABLE 1. *cAMP synthesis as a function of growth stage*

	Stage of growth		
	Logarithmic	Stationary	Overnight
0.3% Glucose medium			
A_{650}	0.51	2.2	2.2
ATP concentration (M × 10^{-4})	3.8	1.1	0.4
ATP specific activity*	716	135	46
In vivo adenylate cyclase†	1740	3972	18
cAMP accumulation in medium‡	72	1425	46
3% Glucose medium			
A_{650}	0.46	2.4	2.4
ATP concentration (M × 10^{-4})	3.3	0.9	0.4
ATP specific activity	868	606	25
In vivo adenylate cyclase	1200	144	644
cAMP accumulation in medium	100	0	0

* Specific activity is expressed as cpm/pmol.

† Determined as described in *Methods*. The unit of specific activity is pmol/hr per mg of protein.

‡ Determined by the procedure of Gilman (5). For comparison with the *in vivo* adenylate cyclase activity, the data are also expressed as pmol/hr per mg of protein. While an aliquot of the total culture is sampled, most of the cAMP is found in the medium.

At the stationary phase of growth, the adenylate cyclase activity *in vivo* was markedly increased (3972 pmols/hr per mg of protein). This activity was clearly great enough to account for the measured increased rate of accumulation of cAMP characteristic of stationary phase. The data suggest that if the degradative activity were essentially unchanged in the transition from logarithmic to stationary phase, the increase in synthetic activity (3972–1740 = 2232) could reasonably explain the increase in accumulation rate (1425–72 = 1353).

Our previous study (1) showed that the concentration of cAMP rose substantially for a brief period at stationary phase and then remained stable. This marked decrease in the rate of cAMP accumulation in overnight cultures (46 pmol/hr per mg of protein) was paralleled by a similar marked decrease in the synthetic capacity (18 pmol/hr per mg of protein).

We have shown before that *E. coli* grown in salts-medium supplemented with 3% glucose have a growth curve similar to that of cells grown in 0.3% glucose medium. However, in the high glucose medium the characteristic burst of cAMP accumulation in stationary phase was absent (1). Measurements of the adenylate cyclase activity *in vivo* at different stages of growth in the high glucose medium provided an explanation for this inhibition. At logarithmic phase, the adenylate cyclase activities *in vivo* of 0.3 and 3% glucose cultures were comparable and both cultures had correspondingly low accumulation patterns. This finding suggests a similarity in the balance between synthesis and degradation in logarithmic phase independent of the glucose concentration in the medium. However, in stationary phase, the *in vivo* adenylate cyclase activity decreased in the high glucose medium compared to an increase in the low glucose medium. It should be noted that glucose was exhausted from the medium at stationary phase only in the 0.3% glucose culture. Overnight

cultures of cells grown in either medium had low activities of adenylate cyclase *in vivo*. Overnight cultures of *E. coli* grown in 3% glucose were relatively inactive in their capacity to convert [^{3}H]adenosine to [^{3}H]ATP (note the low specific activity of ATP, 25 cpm/pmol). Thus, the value for adenylate cyclase activity in overnight cells grown in 3% glucose is relatively unreliable.

Intracellular ATP concentration as a function of the growth stage

The mechanism by which the *in vivo* adenylate cyclase activity varies in the three stages of growth is not clear. We did examine the possibility that the variations in enzyme activity resulted from changes in ATP levels. This hypothesis predicts that cells with low adenylate cyclase activity *in vivo* (logarithmic cells in either medium or stationary cells in 3% glucose medium) should have lower ATP levels than cells with high adenylate cyclase activity *in vivo* (stationary cells in 0.3% glucose medium). This prediction was not borne out by the data shown in Table 1. Intracellular ATP concentration progressively decreased through the growth stages, whether the cells were grown in 0.3 or 3% glucose. Thus, a limitation in ATP did not explain the lower adenylate cyclase activity in logarithmic cells than in stationary cells.

DISCUSSION

The assay method described here for measuring cAMP synthesis has several advantages. The use of the radioactive label allows us to measure small amounts of synthesis in the presence of a substantial pool of cAMP, a feature not offered by methods based on competion of binding (e.g., ref. 5). Since growing cells can be pulsed with a trace amount of radioactive adenosine with no apparent metabolic perturbations, it is possible to make measurements of adenylate cyclase activity under physiological conditions. Adenylate cyclase activity can thus be measured under various experimental conditions.

A procedure for determining levels of cAMP after prior labeling with [8-^{14}C]adenine was previously described by Kuo and De Renzo (8). The procedure described here, while more time consuming, should be more universally applicable on two grounds. The authors measured only radioactivity in cAMP without correction for changes in specific activity of the ATP pool. We incorporate a determination of the ATP specific activity in our calculations. More importantly, the isolation of radioactivity in cAMP reported here is designed to separate very small amounts of radioactive cAMP from a large pool of radioactive contaminants. When we tried a $BaSO_4$ fractionation procedure (9) similar to that used by Kuo and De Renzo for purification of radioactive cAMP formed in short-term studies in *E. coli*, the cAMP fraction was still considerably contaminated. Therefore, the double-column procedure used here, which takes advantage of the specificity of 3′5′-cyclic nucleotide phosphodiesterase, has a greater resolution potential than the $BaSO_4$ procedure.

Our previous attempts to measure adenylate cyclase *in vitro* (1) were not satisfactory on several grounds. Assays in cell extracts tended to be variable and, at best, were too low to account for the level of cAMP that the cells actually accumulated. The *in vivo* assay does not suffer from these limitations. Not only are the determinations reproducible, but the specific enzyme activities are always high enough to account for the accumulation pattern. These studies have shown that the enzyme activity varies as a function of growth stage as well as the conditions for growth. This is compatible with the idea that the observed regulation of cAMP levels is due to changes in adenylate cyclase activity.

The observed difference in the *in vivo* adenylate cyclase activity and the amount of cAMP accumulated points up the presence of an active turnover or degradation mechanism. This may be due, in part, to cAMP phosphodiesterase activity. Our demonstration that a substantial part of the cAMP that accumulates is extracellular (1) and resistant to degradation (unpublished results) provides another parameter by which cAMP levels can fluctuate.

These studies do not provide an explanation for the changes in activity of adenylate cyclase under various growth conditions. Variations in ATP concentration clearly do not explain the changes in enzyme activity. The intracellular ATP concentrations are similar throughout growth in both high and low glucose medium, even though the enzyme activities vary markedly in the two growth media. The ATP concentration in stationary phase does, however, appear to be limiting for maximum enzyme activity (unpublished experiments) and provides some possibility for metabolic control.

The increase in adenylate cyclase activity in the transition from logrithmic to stationary phase might be due to (*a*) an increase in the amount of enzyme or (*b*) an inhibition of the enzyme activity by glucose or one of its metabolites. The depletion of glucose in stationary phase might then allow the enzyme to express its full activity.

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