MEASUREMENT OF CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE SYNTHESIS IN GROWING ESCHERICHIA COLI

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Summary

The net synthesis of cAMP by an adenine auxotroph of Escherichia coli was measured by assaying the incorporation of tritium from [^{3}H]-adenine into cyclic [^{3}H] AMP during exponential growth. Synthesis of cAMP ceased abruptly when glucose was added to cells growing in glycerol and then recovered to an intermediate rate of synthesis after 0.5-1.0 generation. Cyclic AMP appeared to be synthesized from a precursor pool that turned over more rapidly than total cellular ATP. The rates of cAMP synthesis measured by this technique are compatible with the cellular levels of cAMP previously measured in this strain(3).

Cyclic 3',5'-adenosine monophosphate (cAMP)[1] is thought (1,2) to regulate transient and catabolite repression in Escherichia coli. Previously we measured cAMP levels in several strains of E. coli and found that transient repression was associated with a fall in cellular cAMP but that a consistent correlation could not be made between catabolite repression and the concentration of cAMP (3).

Peterkofsky and Gazdar (4,5) using [^{3}H]-adenosine as a precursor for the synthesis of [^{3}H]-cAMP in vivo showed that the high rate of cAMP formation, characteristic of stationary phase E. coli that have recently exhausted their glucose supply, is strongly inhibited by addition of glucose and several other sugars. We now report studies of cAMP formation in growing E. coli during conditions of transient and catabolite repression. By labeling an adenine auxotroph with [^{3}H]-adenine, we show: (1) that net synthesis of cAMP stops

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[1] The abbreviation used is: cyclic 3',5'-adenosine monophosphate, cAMP.

within a half minute of addition of glucose to cells growing in glycerol; (2) that net cAMP synthesis recovers to an intermediate rate after 0.5-1 generation, and (3) that cAMP appears to be synthesized from a precursor pool that turns over faster than does total cellular ATP. Rates of net cAMP synthesis during transient and catabolite repression obtained by this labeling technique are consistent with our earlier measurements of cellular cAMP using a modification (3) of the cAMP-binding protein assay of Gilman (6).

Materials and Methods

Growth and Labeling of Cells. American Type Culture Collection strain #23804 (K-12, lacY$^-$, ade$^-$, thi$^-$) was grown at 30° or 37° in minimal medium containing, per liter, 14.0 g K_2HPO_4, 6.0 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4$, 100 µg thiamine, 10 mg adenine, and 4 ml glycerol. Growth was followed turbidimetrically at 550 nm. Labeling was initiated by addition of 0.5 mCi/ml [^{3}H]-adenine to mid-log cells. The [^{3}H]-adenine (31.7 Ci/mmole, New England Nuclear Corp.) was purified before use by thin layer chromatography on polyethyleneimine-impregnated plates, 20 x 20 cm (CEL PEI, Brinkman Instruments), developed 3 hours in ethanol : 1 M ammonium acetate (70:30).

Cyclic [^{14}C]-AMP and Omnifluor were purchased from New England Nuclear Corp. Cyclic nucleotide phosphodiesterase (0.2 units/mg) was obtained from Sigma Chemical Co.

ATP Determinations. Aliquots [100 µl) of cells were mixed with 50 µl of 2 N HCOOH containing 10,000 dpm of [^{14}C]-ATP as tracer, and frozen immediately in dry ice - isopropanol. The samples were lyophilized to dryness, resuspended in 25 µl H_2O and chromatographed with 50 nmoles ATP on the PEI thin layer plates mentioned above. The plates were developed 3 hours by ascending chromatography in a first dimension with ethanol : 1 M ammonium acetate (70:30). This step was necessary to separate adenosine from the nucleoside triphosphates, which remained at the origin. The plates were then rotated 90° and developed 2 hours by ascending chromatography in a second dimension with 1 M KH_2PO_4, pH 3.4 to separate the triphosphates (7). The ATP-containing region was located under ultraviolet light; 1-cm long strips were cut out corresponding to this region and to the areas immediately surrounding it. Each strip was placed in a scintillation vial; 1 ml of 1 N HCl was added and left for 30 min to elute the ATP. Triton-toluene scintillant (700 ml toluene, 300 ml Triton X-100, 4 g Omnifluor) was added, and the radioactivity determined with a Packard liquid scintillation counter. Total synthesis of [^{3}H]-ATP was calculated on the basis of [^{14}C]-ATP and [^{3}H]-ATP in the peak strip.

Measurement of Cellular cAMP. 2 ml of culture was filtered on Millipore filters and the filter immersed in 5 ml 5% trichloroacetic acid containing tracer ^{14}C-cAMP and 100 nmoles unlabeled cAMP carrier. Where indicated, the filter was washed with 5 ml pre-warmed medium before acid extraction. Samples were frozen and thawed three times, centrifuged briefly, and the supernatants decanted onto 200 x 8 mm columns of Dowex AG50W-X8 resin, 100-200 mesh (Bio-Rad). The columns were eluted with H_2O and the cAMP-containing fraction evaporated and chromatographed on Whatman #1 paper in ethanol : 1 M ammonium acetate (70:30). Cyclic AMP-containing spots were located under ultraviolet light, cut out and eluted with H_2O, evaporated and spotted on fresh sheets of Whatman #1 paper. The second sheet was developed 24 hours by descending chromatography in isobutyric acid : 2 N ammonium hydroxide (66:34) (8). The

cAMP region was located under ultraviolet light; 1-cm strips were cut and counted in 2 ml H_2O and 17 ml Triton-toluene. Overall recovery was 20%.

Measurement of Total cAMP. 2 ml of culture was added directly to 0.2 ml 50% trichloroacetic acid containing tracer cyclic [^{14}C]-AMP and carrier cAMP. Samples were then treated precisely as cellular samples. Rates of total cAMP accumulation can vary several fold from experiment to experiment in the same strain (3). We have no simple explanation of this but it points out the need for multiple experiments before making quantitative comparisons of total cAMP content.

Results

In the first few minutes of labeling, the incorporation of radioactivity into [^{3}H]-cAMP is affected by (1) the net rate of accumulation of cAMP (a constant under steady-state conditions), (2) the changing specific activity of the immediate precursor to cAMP, and (3) the size and turnover rate of the cellular cAMP pool. If only a minor fraction of the labeled cAMP synthesized at early times is cellular, then the kinetics of equilibration of the cellular cAMP pool will have little effect on the kinetics of accumulation of total cAMP. To obtain an estimate of the effects of these three processes, we labeled cells with [^{3}H]-adenine and isolated total [^{3}H]-ATP, cellular [^{3}H]-cAMP, and total [^{3}H]-cAMP at different times.

At 30^{o}C it takes 10-15 min for the cellular ATP pool of strain 23804 to equilibrate with added [^{3}H]-adenine (Fig. 1). It takes slightly less time, 5-10 min, for equilibration to occur at 37^{o}C. The time for equilibration and the final concentration obtained (3 mM)[2] were the same in glycerol-grown cells, whether or not glucose was added to the medium (Fig. 1). Similar results were obtained using [^{14}C]-adenine as the precursor for [^{14}C]-ATP (not shown).

Surprisingly, incorporation into total cAMP (both cells and medium) begins almost immediately after addition of [^{3}H]-adenine and is maximal by 2 or 3 min (Fig. 2, insert). The steady-state rate of total cAMP synthesis reached by the glycerol control of Fig. 2 is 4114 pmole/ml per O.D.$_{550}$, assuming that the specific activity of newly synthesized cAMP equals that of the exogenous

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2 Calculated on the basis of tritium incorporated in ATP, the specific activity of the exogenous [^{3}H]-adenine and the O.D.$_{550}$ of the culture (3).

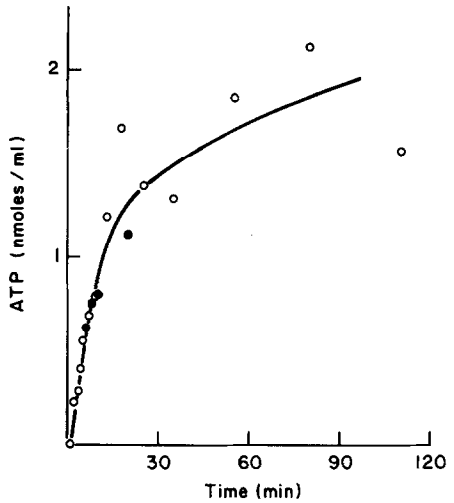

Fig. 1. Equilibration of ATP with [^{3}H]-adenine. Cells of strain 23804 were grown at 30°C in 100 ml minimal medium containing 0.4% glycerol and 5 µg/ml adenine. At zero times, the [^{3}H]-adenine was added to the culture and 20 ml was withdrawn to a separate flask to serve as control. At 3.5 min after addition of label, glucose was added to the experimental flask to a concentration of 0.4%. Aliquots (100 µl) were withdrawn for ATP determinations as described in Materials and Methods. (o), control, cultured in glycerol; (•), culture growing in glycerol to which glucose was added at 3.5 min.

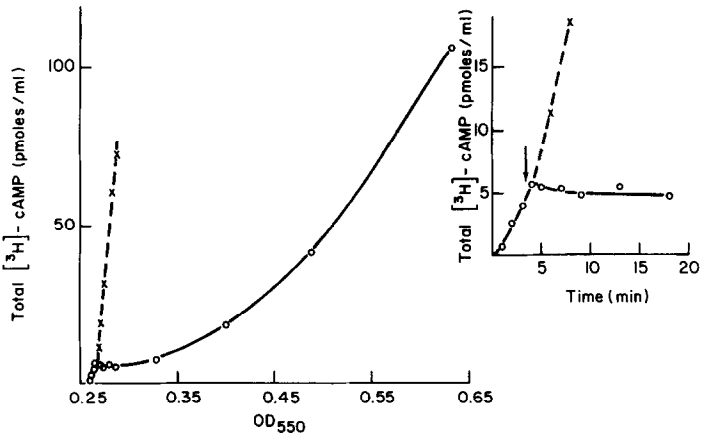

Fig. 2. Total synthesis of [^{3}H]-cAMP. Strain 23804 was grown at 37°C in 100 ml minimal medium containing 0.4% glycerol and 10 µg/ml adenine. [^{3}H]-adenine (5 mCi) was added to initiate the experiment. The culture was divided as in the legend to Fig. 1; aliquots (2 ml) were withdrawn for cAMP determination as described in Materials and Methods. Samples were taken from the control (glycerol only) flask at 6,8,10,15 and 20 min; from the experimental (glycerol followed by glucose) culture at 1,2,3,4,5,7,9,13,18,25, 35,55,75 and 100 min. (x), glycerol growing culture; (O), culture growing in glycerol with glucose added at 3.5 min.

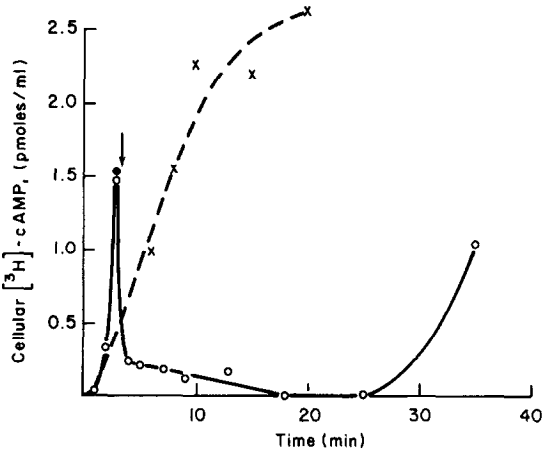

Fig. 3. Accumulation of cellular [^{3}H]-cAMP. The experiment is that described in the legend to Fig. 2. Aliquots (2 ml) were filtered and immediately immersed in 5 ml 5% trichloroacetic acid. A sample at 3 min (filled circle) was washed with 5 ml pre-warmed medium before immersion in trichloroacetic acid; other samples were not washed. Symbols are the same as those used in Fig. 2.

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adenine as would be the case if cAMP is synthesized from an ATP pool which equilibrates faster than the total ATP pool. This figure compares well with the average value of 3483 pmole/ml per $O.D._{550}$ obtained by measuring total cellular cAMP using a protein binding assay (3). The purified cyclic [^{3}H]-AMP was quantitatively converted to [^{3}H] adenosine (2.4%) and [^{3}H]-5'-AMP (93.7%) after 60 min incubation at 37^o with 20 μg of cyclic nucleotide phosphodiesterase, confirming its identification as [^{3}H]-cAMP.

Addition of glucose to a culture actively synthesizing [^{3}H]-cAMP arrests accumulation within a half minute (Fig. 2, insert). [^{3}H]-cAMP synthesis is severely repressed for 35 min and then recovers to a moderate level (Fig. 2).

Cellular [^{3}H]-cAMP was determined by filtering cells and extracting them with acid. Washing of cells (3) at early time points was not necessary, since contamination of the cells by [^{3}H]-cAMP in the medium was negligible (Fig. 2 and the filled circle of Fig. 3). Cellular cAMP was also synthesized rapidly from zero time; addition of glucose led to an immediate and dramatic decline in cellular cAMP (arrow, Fig. 3). Even at 3 min, however, the bulk of the [^{3}H]-cAMP is in the medium rather than in the cells, making it impos-

sible to say with certainty whether the glucose-induced diminution of cellular cAMP was brought about by secretion or degradation. After 25 min the cells began to reaccumulate [^{3}H]-cAMP; this accumulation precedes the rise in medium cAMP (Figs. 2 and 3).

Discussion

The cellular level of cAMP in E. coli is determined by three processes: synthesis, degradation and secretion. There is presently no evidence that secretion is subject to any regulatory influences. The abrupt drop in cellular cAMP which follows glucose addition to glycerol-grown cells (Fig.3) cannot be caused by enhanced secretion alone since extracellular accumulation also stops (Fig. 2). The fall in cellular cAMP and the cessation of de novo accumulation can both be the result of adenylate cyclase inhibition; however, an effect on cyclic nucleotide phosphodiesterase cannot be ruled out. Similarly, it is not possible in these kinds of experiments to assess the relative activities of adenylate cyclase and phosphodiesterase in maintaining cellular cAMP levels in glycerol-grown cells.

The results we report here agree qualitatively and quantitatively with those obtained previously by measuring the cAMP content of cells and medium in growing E. coli (3). They confirm the finding that cellular cAMP levels fall when glycerol-grown cells are exposed to glucose (1,3). Recently, Bernlohr et al. (9), using a technique similar to the one we developed (3), observed an abrupt fall in cellular cAMP after glucose addition to succinate-grown E. coli.

Peterkofsky and Gazdar (5) were unable to detect [^{3}H]-cAMP synthesis in exponentially growing E. coli B labeled with [^{3}H]-adenosine. They found high activity in stationary phase E. coli B that had exhausted their glucose supply. In conditions of acute glucose deprivation, cells are known to produce extremely large amounts of cAMP (4-6). We think that our ability to detect synthesis in growing cells is due to: (1) strain differences - strain 23804 contains about

10 more cAMP than strain B (10), and (2) utilization of a more extensive purification technique for [^{3}H]-cAMP.

It is interesting that cAMP is apparently synthesized from an ATP pool that equilibrates more rapidly with exogenous adenine than does total ATP (Figs. 1 and 2). A similar phenomenon has been reported in slices of guinea pig cerebral cortex labeled with [^{3}H]-adenine (11). Adenylate cyclase may utilize distinct, rapidly equilibrating, precursor pools.

Adenylate cyclase has been partially purified from E. coli (12,13). It has not been possible, however, to demonstrate any effect of glucose or other sugars on its activity assayed in vitro. The components of the adenylate cyclase system responsible for regulation in vivo appear to be either destroyed or not present in the in vitro preparations described to date. The labeling techniques described by Peterkofsky and Gazdar (4,5) and in this paper should make it possible to elucidate the physiological variations of adenylate cyclase, cyclic nucleotide phosphodiesterase, and secretion of cAMP in response to changes in growth conditions. These assays could provide clues for ultimately studying regulation in vitro with adenylate cyclase isolated by other, possibly gentler, techniques.

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