INABILITY TO DETECT CYCLIC AMP IN VEGETATIVE OR SPORULATING CELLS OR DORMANT SPORES OF *BACILLUS MEGATERIUM*

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Received March 29, 1973

SUMMARY

Cyclic AMP was not found in vegetative cells or sporulating cells or dormant spores of *Bacillus megaterium* using an assay which would have detected an *in vivo* concentration of $1 - 2 \times 10^{-9}$ M. Adenyl cyclase and cyclic AMP phosphodiesterase were also not detected in sonicates of vegetative or sporulating *B. megaterium* cells.

INTRODUCTION

Several workers have suggested that in *Bacillus* synthesis of sporulation-specific proteins, and therefore, sporulation might be controlled by catabolite repression (1,2). By analogy with the mechanism of catabolite repression in *E. coli* (3) it seemed possible that cyclic AMP (cAMP) might also be involved in the control of sporulation in *Bacillus*. I therefore examined the levels of cAMP in vegetative cells, sporulating cells and dormant spores of *Bacillus megaterium*, and the levels of adenyl cyclase and cAMP phosphodiesterase in cells. Surprisingly, neither cAMP nor the enzymes of its metabolism were detected.

MATERIALS AND METHODS

^{3}H-cAMP (24 Ci/mmole) and ^{3}H-ATP (13.4 Ci/mmole) were purchased from New England Nuclear. The ^{3}H-cAMP was purified by descending paper chromatography on Whatman #1 with n-propanol:NH_4OH:H_2O (7:1:2) as the solvent. Unlabeled cAMP and ATP were products of P-L Biochemicals; adenosine and AMP were obtained from Sigma. cAMP binding protein and an inhibitor of cAMP-dependent protein kinase were purified according to the method of Gilman (4). Dormant spores of *Bacillus megaterium* QM B1551 were produced in supplemented nutrient

broth, harvested, lyophilized, and stored as previously described (5). Cells were grown in the minimal medium of Spizizen (6) supplemented with 0.1% casamino acids. The dry weight of cells was determined on small aliquots following centrifugation and one water wash. Cell number was determined by counting in a Petroff-Hauser chamber. Adenyl cyclase and cAMP phosphodiesterase were assayed as described by Clark and Bernlohr (7), except that separation of the reaction products was by descending paper chromatography as described above Cell extracts for enzyme assays were prepared by sonication in the buffer of Clark and Bernlohr (7), and dormant spores were broken by dry rupture in a Wig-L-Bug with glass beads as the abrasive (8).

cAMP was assayed utilizing a cAMP binding protein as described by Gilman (4). This assay quantitates cAMP by its displacement of known amounts of ^{3}H-cAMP from a specific cAMP binding protein. Assays were conducted at 4° in 200 µl of 50 mM sodium acetate (pH 4.0) containing 0.8 nM ^{3}H-cAMP (24 Ci/mmole) and a maximally effective amount of protein kinase inhibitor. The assay was initiated by addition of sufficient cAMP binding protein to bind 75% of the ^{3}H-cAMP and after the binding reaction had reached equilibrium (60 minutes), the mixtures were diluted to 1 ml with cold 20 mM KPO_4 (pH 6.0), filtered and counted as described by Gilman (4). Using these assay conditions 0.05 pmole of unlabeled cAMP was easily detected; cAMP concentrations of unknown samples were determined from a calibration curve constructed with known amounts of unlabeled cAMP. Glucose was determined by reaction with o-toluidine as described in Sigma Technical bulletin number 635 (Sigma Chemical Company).

Preliminary experiments indicated that cAMP levels were quite low in B. megaterium, and that a significant amount of material was present which interfered with the cAMP assay. Therefore, extracts of cells and spores were purified using a method adapted from that of Hardman et al. (9). Since E. coli B is known to excrete large amounts of cAMP into the growth medium (10), I extracted cells without prior centrifugation to obtain maximum amounts of cAMP. 100 ml of growing cells was added directly to 11 ml of 50% trichloracetic acid,

chilled in ice for 60 min, centrifuged (15 min, 10,000 x g) and the precipitate discarded. Similar treatment was given to the powder from dry rupture of 500 mg of dormant spores which was suspended in 100 ml of 5% trichloracetic acid. 500 mg activated charcoal was added to the supernatant from the previous step, stirred for 60 min at 25°, and the charcoal allowed to settle. The supernatant was discarded, and the charcoal washed four times with 15 ml of 1 mM HCl. The charcoal was eluted twice with 15 ml of ethanol:NH_4OH:H_2O (60:3:40) for 60 min at 25°. The pooled eluates were passed through a filter (Millipore, 0.45 μ) to remove traces of charcoal, and then flash evaporated. The residue was dissolved in 5 ml of 50 mM sodium acetate (pH 4.0) and applied to a Dowex-1-formate column (2.5 ml) which was then washed with 10 ml of water and then with 10 ml of 2 M formic acid. The formic acid eluate was flash evaporated, the residue dissolved in 50 μl of H_2O and then chromatographed (18 hours, descending) on Whatman #1 with n-propanol:NH_4OH:H_2O (7:1:2) as the solvent. cAMP markers were run in tracks adjacent to the sample, and after drying, the region of the paper which should contain cAMP was located from the position of the cAMP markers, cut out and eluted. The eluate was flash evaporated, dissolved in 100 μl H_2O, and 20 μl utilized for cAMP analysis in duplicate.

RESULTS

The procedure used for isolation of cAMP gave recoveries of 40-50% of the cAMP added to initial mixtures of trichloracetic acid and cells (Table I). The recovery was not lowered if cAMP was added to stationary or log phase cells prior to the addition of trichloracetic acid (Table I). It should be noted that extremely small amounts of exogenous cAMP (10-40 pmoles) were added in these experiments.

Despite the good recovery of these small amounts of cAMP in the purification procedure, I could not detect cAMP at any stage of growth of B. megaterium, even at the time at which all glucose in the culture was exhausted (Figure 1, Table II). Material was detected in purified cell and spore extracts which reacted in the cAMP binding assay. However, identical levels of this material also were

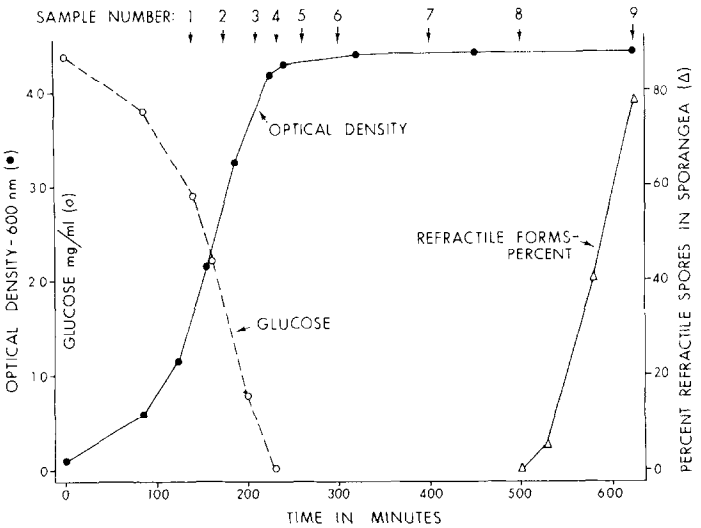

Figure 1. Time course of growth, sporulation and glucose disappearance in a culture of B. megaterium. Cells were grown in Spizizen's medium (6) as described under methods, and the presence of refractile spores in sporangia was determined in the phase contrast microscope. Samples for cAMP analyses were taken at the times indicated by the arrows. The kinetics of growth of E. coli B were similar to those of B. megaterium, but the final optical density attained by E. coli B was only 2.6.

TABLE I

Recovery of cAMP in Purification of Cell Extracts

cAMP added (pmoles)	Stages of growth when cAMP added	Recovery (%)
25 [a,b]	mid log	51
25 [a,b]	1 1/2 hours after log phase	43
40 [c]	2 1/2 hours after log phase	51
10 [a]	2 1/2 hours after log phase	42
20 [a]	10% refractile spores present	46

[a] ^{3}H-cAMP added, recovery determined by counting

[b] ^{3}H-cAMP added 30 min before cells were harvested

[c] Unlabeled cAMP added, recovery determined by cAMP assay

Except where noted the cAMP was added to 100 ml of culture 1 min after addition of trichloracetic acid. Extracts were then purified as described in the text.

TABLE II

Concentrations of cAMP in Different Stages of Growth of *B. Megaterium* and *E. coli*

Stage of Growth		Maximum Intracellular cAMP Concentration [M][a]	
		B. megaterium[c]	*E. coli*
mid log	(1)[b]	$< 2 \times 10^{-9}$ ($< 10^{-11}$)	5×10^{-5} (2×10^{-7})
late log	(2)	$< 10^{-9}$	
early stationary	(3)	$< 10^{-9}$	
early stationary	(4)	$< 10^{-9}$	5×10^{-4}
early stationary	(5)	$< 10^{-9}$	
stationary	(6)	$< 10^{-9}$	10^{-3} (8×10^{-6})
stationary	(7)	$< 10^{-9}$	
late stationary	(8)	$< 10^{-9}$ ($< 2 \times 10^{-11}$)	
late stationary	(9)	$< 10^{-9}$	
purified dormant spores		$< 5 \times 10^{-9}$	

[a] cAMP concentrations were calculated from dry weight using the value for cAMP in an extract, and a value for the amount of cell water calculated by assuming that the water content of cells and spores was 80% and 70% respectively (15). All values were calculated assuming that all cAMP in a culture was intracellular. This is known not to be the case in *E. coli B* (10). Concentrations in parentheses are those calculated for the whole culture.

[b] These numbers correspond to the numbered arrows in Fig. 1 which denote the exact time of sampling of the *B. megaterium* cultures. The *E. coli* culture was analyzed at approximately the same points.

[c] All values are averages of data from at least two separate cultures, and have been rounded off to the nearest integer. They have been corrected for a loss of cAMP during purification of 55% (Table I).

found in purified extracts of log phase and sporulating cells and in samples of sterile growth medium purified identically to cell extracts. The amount of this material varied somewhat, but was approximately equivalent to cAMP concentrations of 5 nM (3 → 6 nM) in the final purified extracts (volume of 100 µl). The material was not destroyed by cAMP phosphodiesterase treatment

sufficient to degrade a 5 nM solution of authentic cAMP. Since the cAMP assay was being used at the limits of its sensitivity, it is not surprising that some non-specific reacting material, possibly introduced in one of the purification steps, was detected.

The non-specific inhibitory material in all purified extracts reduced the sensitivity of cAMP determination since a correction was required for its effect. Taking this correction into account I could have reproducibly detected a cAMP concentration in the final purified cell or spore extracts of about 8 nM. However, I was unable to detect any cAMP in B. megaterium cell or spore extracts, and the limit of detection corresponds to < 1 - 2 nM cAMP in cells, and < 0.5 nM cAMP in spores (Table II). These limits appear even lower when calculated as molecules per organism: less than 22-45 molecules of cAMP/cell and less than 3 molecules/spore. All of these values may be even lower since they were calculated assuming all cAMP in a culture was intracellular.

Although cAMP was not detected in B. megaterium, I did find cAMP in cultures of E. coli B at levels similar to those reported by other workers (10,11). The concentration of cAMP in E. coli cultures was >2 x 10^4 fold higher than those found in cultures of B. megaterium, and values reported for intracellular cAMP levels in E. coli are more than 10^3-10^5 fold higher than the maximum endogenous cAMP concentration in B. megaterium (11,12). Analysis of a 1/1 mixture of stationary phase E. coli B and B. megaterium 30 min after mixing gave a value for cAMP predicted by the amount of E. coli present, indicating that no inhibitor was present.

Consistent with the absence of cAMP from B. megaterium was the lack of detectable adenyl cyclase and cAMP phosphodiesterase in these cells (Table III), although the sensitivity of these assays was not high. Adenyl cyclase and cAMP phosphodiesterase levels were previously found to be low or absent from strains of Bacillus cereus and Bacillus subtilis (< 0.05 nmoles / 30 min / mg protein for adenyl cyclase (13)); however, high levels of both of these enzymes have

TABLE III

Absence of Adenyl Cyclase and cAMP Phosphodiesterase from B. Megaterium

Stage of Growth	Protein Assayed	Enzyme activity nmoles product/30 min/mg proteir	
		Cyclase	Phosphodiesterase
mid log	2 - 100 µg	< 5	< 3
stationary [a]	2 - 100 µg	< 5	< 2

[a] Cells harvested 2 hours after the end of log phase

been reported in B. licheniformis (94 nmoles / 30 min / mg protein for adenyl cyclase (7)). Adenyl cyclase in E. coli B has a specific activity of about 0.8 nmole / 30 min / mg protein (10).

DISCUSSION

The inability to detect cAMP in Bacillus megaterium is certainly surprising in view of the ubiquitous distribution of this compound. Although cAMP was reported to be present in B. licheniformis (7), I have been unable to detect cAMP (<2 nM) in this organism, in B. subtilis SB 133, or in B. cereus T when they are grown in Spizizens medium (6) with 0.1% casamino acids (unpublished results). Furthermore, it has recently been reported that cAMP cannot be detected in a Lactobacillus (14). Therefore, cAMP may not be a universal regulatory molecule, at least under all growth conditions.

These results suggest that cAMP is not involved in the regulation of sporulation in B. megaterium. If cAMP is present in B. megaterium, its levels must be very low (< 22-45 molecules/cell), and it is difficult, though not impossible, to imagine cAMP being an efficient regulatory molecule at this extremely low level. It is more likely, however, that in Bacilli some other small molecule takes the place of cAMP.

ACKNOWLEDGEMENTS

This work was supported by a grant from the University of Connecticut Research Foundation.

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