PRESENCE OF CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE IN *ANABAENA VARIABILIS*

By

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PRESENCE OF CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE

IN ANABAENA VARIABILIS

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Finally, I wish to dedicate my thesis to my husband, Kendall, whose love of nature inspires my own and sparks my constant desire to know more, and to my son, Kyle, who is my link to the future.

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ABBREVIATIONS

5'-AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
cpm	counts per minute
cyclic AMP	cyclic adenosine 3',5'-monophosphate
cyclic GMP	cyclic guanosine 3',5'-monophosphate
ppGpp	guanosine 5'-diphosphate 3'-diphosphate
pppGpp	guanosine 5'-triphosphate 3'-diphosphate
MS I and II	magic spot nucleotides: ppGpp and pppGpp respectively.
MSO	L-methionine-DL-sulfoximine
PDE	cyclic 3',5'-nucleotide phosphodiesterase
pmoles	picomoles
TCA	trichloroacetic acid

CHAPTER I

INTRODUCTION

Regulation of the genetic code is the key to cellular differentiation and consequently the emergence of complex life forms. Two of the simplest systems in which to study cellular differentiation are those prokaryotic eubacteria which alter their cellular morphology (i.e. Arthrobacter crystallopoites, Hamilton et al., 1977) and those cyanobacteria which contain only two different cell types, i.e., vegetative and heterocystous. The latter system is dealt with in these studies.

Tomkins (1975) has suggested that the mechanism of biological regulation is universal and that cyclic adenosine 3',5'-monophosphate (cyclic AMP) and the magic spot nucleotides, guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) (MS I and II, respectively) are the primary regulators. He stated that the universality of cyclic nucleotides (and the magic spots) is due to the survival advantage of those organisms in which they first appeared. However important these regulators may be in animals (especially cyclic AMP, Pastan and Perlman, 1975) and in bacteria (Rickenberg, 1974; Nierlich, 1978), the fact remains that very little is known about their presence or absence in photoautotrophic organisms (Rickenberg, 1974; Amrhein, 1977). Therefore, broad statements such as those made by Tomkins (1975) concerning their

universality should be made with caution.

Cyclic AMP is a small nucleotide which is synthesized from adenosine 5'-triphosphate (ATP) by adenyl cyclase. It is hydrolyzed by cyclic 3',5'-nucleotide phosphodiesterase (PDE) to adenosine 5'-monophosphate (5'-AMP). It was first reported by Rall et al. (1957), in rat tissue, and its chemical structure was established by Sutherland and Rall (1958) and Lipkin et al. (1959). The magic spot nucleotides are also small molecules which have been shown to accumulate in a variety of bacteria in response to amino acid starvation when there is an excess of polyribosomes present in the cell and changes in transcription occur (see Nierlich, 1978, for review). This accumulation was termed stringent control by Stent and Brenner (1961). Magic spot nucleotides have also been found in the cyanobacterial species Anacystis nidulans (Mann et al., 1975), Nostoc sp. and Anabaena cylindrica (Adams et al., 1977).

In contrast to rather limited investigations of the magic spot nucleotides, investigations abound on the presence of and mode of action of cyclic AMP in various organisms. Presence of cyclic AMP in animals and its mode of action as a hormone second messenger is well documented (see Pastan and Perlman, 1975, for review). Cyclic AMP or its metabolic enzymes have also been reported in all types of eubacteria (Ide, 1971; Rickenberg, 1974), eukaryotic algae (Amrhein and Filner, 1973; Keirns et al., 1973; Berchtold and Bachofen, 1977; Bressan et al., 1980), slime molds (Malkinson and Ashworth, 1973; Lovely and Threlfall, 1976), true fungi (Silverman and Epstein, 1975), and higher plants (Rao and Khan, 1975; Ownby et al., 1976; Ashton and Polya, 1977, 1978).

The suggestion that cyclic AMP might also be a second messenger for plant hormones has been brought under attack recently, especially since reports of its presence in plant tissue have been challenged (Bressan et al., 1976; Amrhein, 1977). In species of the cellular slime mold, Dictyostelium, cyclic AMP acts as a chemotactic agent triggering aggregation of cells in response to nutritional deficiencies (Malkinson and Ashworth, 1973). Cyclic AMP is associated with cyto-differentiation in the aquatic fungus, Blastocladiella (Silverman and Epstein, 1975). Several processes in eukaryotic algae have been associated with changes in level of cyclic AMP. As conditions become less favorable for growth, cyclic AMP levels increase in a variety of eukaryotic micro-organisms (Silverman and Epstein, 1975).

Among the prokaryotes, cyclic AMP is best known for its role in catabolite repression in Escherichia coli. When it binds to a cyclic AMP receptor protein, it changes the protein conformation thus enabling it to bind to a promoter region on the bacterial genome, allowing transcription of inducible genes (Eilen and Krakow, 1977). Dills and Dobrogosz (1977) observed in E. coli that mutants lacking adenylate cyclase were unable to generate a proton motive force and consequently were unable to make ATP by oxidative phosphorylation. Flagellar production and consequent motility of E. coli are also subject to control by cyclic AMP (Yokota and Gots, 1970; Dobrogosz and Hamilton, 1971). Morphological changes in both Arthrobacter crystallopoites (Hamilton et al., 1977) and Caulobacter crescentus (Shapiro et al., 1972) have been associated with changes in levels of cyclic AMP.

The system in question in these studies was the nutritional shift-down in Anabaena variabilis effected by removal of its source of

combined nitrogen. This treatment was shown to induce the cellular differentiation process resulting in heterocyst formation (Fogg, 1949). These differentiated cells are the site of nitrogen-fixing activity (reduction of N_2 to NH_3) for this and all heterocystous organisms (Fay et al., 1968).

Some biochemical and ultrastructural events leading to mature heterocysts are described below. When the combined nitrogen source is removed from the growth medium of a heterocystous cyanobacterium, one of the first events to occur in all cells is the breakdown of existing storage proteins (phycobiliproteins). From the amino acids liberated in this process, synthesis of the nitrogenase enzyme complex begins. These are the enzymes of nitrogen fixation. In those cells which are competent to become heterocysts, a complex three-layered envelope develops outside the existing cell wall (Lang and Fay, 1971). This renders the heterocyst impermeable to oxygen and other gases. Uptake of molecular nitrogen must then occur through the microplasmodesmata which develop between heterocysts and adjacent vegetative cells (Lang and Fay, 1971). The heterocyst has a weakly pigmented appearance which is correlated with a loss of photosystem II activity as well as the loss of phycobiliproteins (Stanier and Cohen-Bazire, 1977). These specialized cells do retain photosystem I activity and consequently make ATP from cyclic photophosphorylation (Stewart et al., 1975). A refractile granule which Fogg (1951) has shown to be arginine-rich develops at each junction with a vegetative cell.

Regulatory control of this differentiation process was the question of interest. Cyclic AMP and/or ppGpp and pppGpp were postulated as possible regulatory nucleotides. No evidence for the

presence of magic spot nucleotides in Anabaena variabilis in response to nitrogen starvation was observed (J. D. Ownby and E. E. Hood, unpublished data). Therefore, the presence of cyclic AMP and changes in its concentrations during the differentiation process were the lines of investigation pursued.

CHAPTER II

MATERIALS AND METHODS

Anabaena variabilis strain 377 was originally obtained from the University of Texas culture collection of algae. The algae were routinely cultured at 27 C in the medium of Allen and Arnon (1955) (Appendix A) supplemented with 2 mM NH_4Cl and 1 mM $NaNO_3$, on a reciprocating shaker (90 cycles min^{-1}) with continuous illumination at 40-60 μE m^{-2} s^{-1} from 2 Westinghouse "Agro-Lite" 40-W tubes, or in a New Brunswick Fermentor at 28 C, 200 rpm, and 100-300 μE m^{-2} s^{-1}. Nitrogen starvation was induced by one of two methods, either addition of L-methionine-DL-sufoximine (MSO) to a final concentration of 0.5 μM, or resuspension in nitrogen-free medium following centrifugation at 480 x g, 5 min at 4 C. MSO is a structural analog of glutamine and binds irreversibly to glutamine synthetase preventing the utilization of combined nitrogen (Ronzio and Meister, 1968), thus nitrogen starving the cells in the presence of ammonia (Ownby, 1977). Cultures containing 400 ml of algae (A_{660}=0.2) were harvested at various times over a 24 h period for the cyclic AMP assays.

In measuring cyclic AMP in this alga (or any cyanobacterial species) the possibility that the cyclic AMP might arise from eubacterial contamination was a major concern. A culture of A. variabilis strain 377 which had been freed of bacterial contaminants by UV irradiation (Mehta and Hawxby, 1977) was obtained from R. Mehta. Stock and experi-

mental cultures were routinely assayed for sterility by plating aliquots onto DIFCO nutrient agar, brain heart infusion agar, and agar containing 1% sucrose and 0.5% peptone. A bacterial contaminant occasionally encountered in this study was a nonmotile, gram-negative rod identified as Flavobacterium sp. In preliminary work we observed that cultures of Anabaena heavily contaminated with Flavobacterium contained approximately the same levels of cyclic AMP as did sterile cultures. Production of cyclic AMP by Flavobacterium sp. has not been reported. However, the contamination by this organism was low enough in comparison to algal cell concentration not to contribute significantly to levels of cyclic AMP detected.

Cyclic AMP was partially purified from cyanobacterial cultures by the following procedure. The filaments were allowed to settle out of the growth medium for 10 min and the excess medium was decanted and assayed separately for cyclic AMP. Since the flasks were removed from the reciprocating shaker (or alga removed from the fermentor) for these 10 min, light intensity and agitation were reduced. No controls were employed to determine the effects, if any, of these harvesting procedures on release or breakdown of cyclic AMP. One ml of the concentrated cells was removed for later protein determination. Perchloric acid was added at a final concentration of 0.4 N to the concentrated cells to lyse them and precipitate the polynucleotides and proteins. Approximately 2.5 pmoles of 3',5'-cyclic AMP-2,8-^{3}H (50,000 cpm) (New England Nuclear, 30-50 Ci mmole^{-1}) were added as an internal standard from which to calculate recovery throughout purification.

After cooling to 4 C, the concentrated cells were sonicated with a Branson Model 185 cell disruptor 2 times at #7 for 10 s each to further

disrupt cells and minimize adsorption to large debris particles. Homogenates were centrifuged at 8000 x g, for 10 min at 4 C, and the acid-soluble extract was neutralized with KOH. The $KClO_4$ which precipitated was removed by centrifugation as above. Nucleotides from algal and medium extracts were adsorbed onto washed Norit A activated charcoal as described by Hardman et al. (1969). The charcoal selectively adsorbed nucleotides but not pyrophosphate, sugars, amino acids, and mineral salts. This step allowed the experimentor to reduce greatly the volume of extract since all supernatant after centrifugation (as above) was discarded. The charcoal pellets were rinsed in 10 ml each of succeedingly more dilute HCl (0.01 N, 0.001 N) to remove ions and generally clean up the charcoal.

Nucleotides were subsequently eluted from the charcoal at 27 C with ethanol:NH_4OH:H_2O (63:4:33, v/v). The eluate was air-dried at room temperature and the nucleotides were redissolved in 0.6 ml distilled water. To determine if the compound identified by the binding assay was indeed cyclic AMP, one half of each sample was treated for 30 min to 4 h with 100 μg commercial beef heart cyclic nucleotide phosphodiesterase (PDE), the enzyme which hydrolyzes it to 5'-AMP, (Sigma Chemical Co.). The enzyme was dissolved in 20 mM ammonium acetate, pH 7.5. The enzyme treated samples were placed in boiling water for 3 min and denatured protein was removed by centrifugation at 8000 x g for 10 min at 4 C. These samples and the remaining halves to which an equal quantity of boiled PDE was added were then applied to Bio-RAD AG-50 ion exchange resin columns (100-200 mesh, 0.5 x 4 cm) in the H^+ form and eluted with distilled water. This step gave partial resolution of cyclic from non-cyclic nucleotides. To obtain an elution profile for this column

material, 2.5 pmoles ^{3}H-cyclic AMP were loaded onto a column and eluted with water into fifteen 1 ml fractions. Those fractions containing radioactivity (i.e., 3-7) were combined in subsequent experiments, air-dried at room temperature and redissolved in 0.2 ml distilled water. Aliquots (50 µl) were assayed for cyclic AMP using the protein binding assay of Gilman (1972) as modified by Bressan et al. (1976).

A simplified diagram of the protocol for the cyclic AMP assay is shown in Figure 1. The order of addition of the components was critical in the assay. The ^{3}H-cyclic AMP must be well mixed with any unlabeled cyclic AMP whether in a standard solution or an algal or medium extract, before the binding protein mixture (binding protein, BSA, and NaOAc buffer) can be added. The reaction was initiated with this addition. All reaction tubes were set up while emersed in ice water and incubation occurred at 0 C for 60-90 min. Binding was terminated by diluting each sample with 2 ml of 5 mM potassium phosphate buffer pH 7.2 (Appendix C). Samples were then filtered through 0.45 µ Millipore filters which adsorb the binding protein with or without bound cyclic AMP but did not adsorb unbound ligand. Since both labeled and unlabeled cyclic AMP are present in the reaction tubes, a competition for binding sites on the protein occurs. Concentration of labeled ^{3}H-cyclic AMP is held constant. Therefore, as the amount of unlabeled cyclic AMP increases, fewer molecules, and thus fewer cpm, of ^{3}H-cyclic AMP will be bound to the protein. By using known concentrations of unlabeled cyclic AMP (Figure 1), a standard curve can be generated (Figure 2). Values of pmoles of cyclic AMP present in algal or medium extracts can be extrapolated from this standard curve by comparing cpm values obtained in the binding assay to those in the standard curve.

ORDER OF ADDITION OF COMPONENTS

Standard Assay		Experimental Assay
*	1 pmole ^{3}H-cAMP (20,000 cpm)	*
*	unlabeled cyclic AMP in the following amounts	
	0.5 pmoles	
	1.0 pmoles	
	2.0 pmoles	
	10.0 pmoles	
	20.0 pmoles	
	partially purified algal or medium extracts	*
*	50 µg binding protein	*
*	300 µg bovine serum albumin	*
*	38 mM NaOAc pH 4.5	*

Incubate at 0 C for 60-90 min. To stop reaction, add 2 ml potassium phosphate buffer, pH 7.2. Filter through Millipore (0.45 µ) filters with 9 ml buffer. Dry filters. Assay radioactivity on filters by counting in 10 ml scintillation counting fluor.

Figure 1. Diagram of Simplified Protocol for a Typical Cyclic AMP Assay

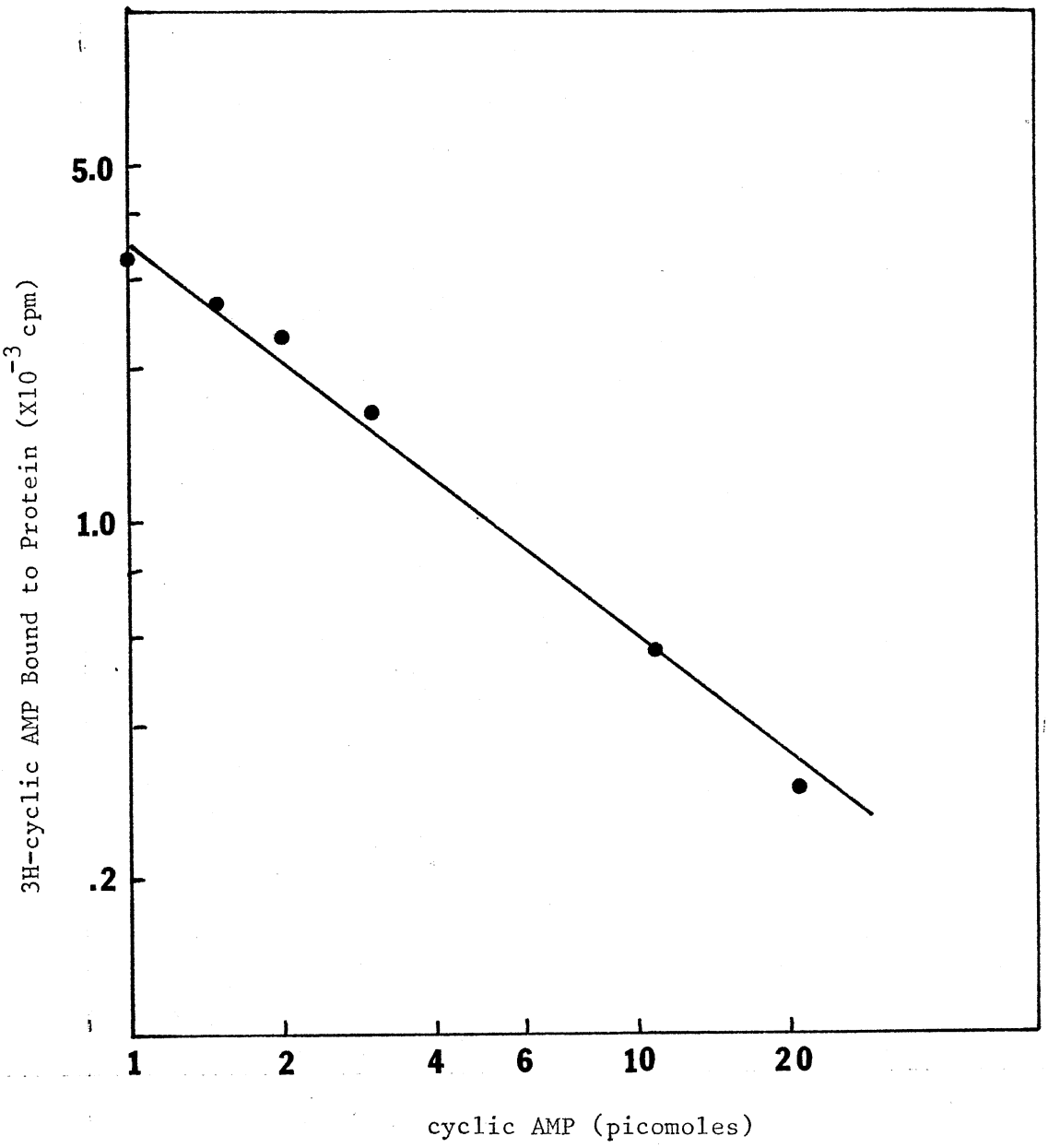

Figure 2. A Typical Standard Curve for the Gilman Binding Assay. Both abscissa and ordinate are logarithmic scales. Each reaction tube contained 1 pmole ^{3}H-cyclic AMP and varying amounts of unlabeled cyclic AMP to give the total amounts shown on the abscissa.

At each step of purification, recovery of marker ^{3}H-cyclic AMP was monitored by spotting 50 µl aliquots onto Millipore filters and measuring radioactivity in a toluene based scintillation cocktail (Appendix B). All estimates of cyclic AMP concentration were corrected for loss as determined by recovery of the ^{3}H-cyclic AMP marker.

In all experiments, protein determinations were by the method of Lowry et al. (1951). The volume of the 1 ml cell concentrate removed before addition of perchloric acid was increased to 5 ml with distilled water, then 1 ml of 30% TCA was added. These samples were chilled and sonicated 2 times at #7 for 10 s each on the Branson sonifier to break the cells and release protein. The proteins were allowed to precipitate at 0 C for 30 min before centrifugation. The supernatant was discarded. The pellet was redissolved in 2 ml of 0.5 N NaOH and the Lowry test was completed with this preparation.

Data on cyclic AMP levels in Escherichia coli B were collected in order to assess the accuracy of the binding assay used in these experiments. The bacteria were grown in minimal salts medium containing 1% glucose. In order to release them from catabolite repression, they were centrifuged at 480 x g for 5 min at 4 C and resuspended in 0.125 M KH_2PO_4 buffer, pH 7.0. Half of the cells were harvested immediately and the remaining half were harvested after 1 h in buffer without agitation at 37 C. A 1 ml protein sample was taken at 0 h and analyzed by the Lowry method. Partial purification of the bacterial cells was as described for the cyanobacterial filaments.

Two experiments were conducted to assess the effect, if any, of exogenously applied cyclic AMP or 5'-AMP on differentiation with or without MSO-induced nitrogen starvation. Cultures of 15 ml of A.

variabilis were treated with 0.5 μM MSO, 1 mM cyclic AMP, MSO plus 1 mM cyclic AMP or MSO plus 1 mM 5'-AMP. The pH of each stock solution of nucleotide was adjusted with NaOH to 7.0. Aliquots of cells were taken at 0, 12, 24, and 36 h to determine the percentage of cells differentiated into proheterocysts and heterocysts. Approximately 1500 to 2000 cells per sample were counted. Heterocysts were identified by their "cleared" appearance and content of two polar nodules. Cells which were rounded and enlarged but could not be identified by the above characteristics were termed proheterocysts.

CHAPTER III

RESULTS

Levels of Cyclic Adenosine 3',5'-Monophosphate in Anabaena variabilis

Much criticism has been expressed recently on the validity of the Gilman binding assay for detection of cyclic AMP in green organisms (Bressan et al., 1976; Amrhein, 1977). However, even though the organism used in these experiments contains chlorophyll a, its characteristics are more similar to those of true bacteria than to those of higher plants. Since the binding assay is considered valid for animal tissue (Gilman, 1972) as well as for eubacterial extracts, it is probably valid for detection of cyclic AMP in this cyanobacterium, also.

The cyclic AMP binding protein used in these assays was isolated from rabbit muscle (Bressan et al., 1976) and was a gift from R. A. Bressan. The amount of protein added to these reaction tubes was 50 μg. It was determined that this amount would bind only 15% of the 1 pmole of ^{3}H-cyclic AMP present when the latter was the only ligand in the reaction tube. With this amount of protein, all reaction tubes contained saturating amounts of cyclic AMP, labeled plus unlabeled. This aspect of the assay was critical if one desired to obtain a straight line logarithmic standard plot (Figure 2) from which quantitative interpretation of unknowns might be achieved.

The protein binding assay for cyclic AMP detection is a negative assay. The experimental numbers obtained decrease in proportion to an increase in unlabeled standard or putative organismal cyclic AMP. Many different things can interfere with the results of the assay and thus would be interpreted as cyclic AMP. Partial purification of biological materials is critical because it removes many nucleotides or other competitively binding molecules from the assay tube. Saturating amounts of cyclic AMP in the reaction mixture also help reduce competitive binding by contaminants in the partially purified extracts (Gilman, 1972). Another interfering compound in this assay is trichloroacetate which can bind to the binding protein and give inflated estimates of cyclic AMP (Wright, 1975). Care should be taken to exclude this from purification procedures.

Several preliminary experiments revealed some problems which required modification of the extraction procedure. The most apparent was a need for very large volumes of algal culture (400 ml, A_{660} = 0.2) to yield detectable levels of cyclic AMP. Secondly, the growth medium contained a large amount of this nucleotide, apparently secreted by the cells, and measurement of extracellular cyclic AMP was required in addition to the assay for intracellular cyclic AMP.

At various times throughout the partial purification steps, recovery of the marker ^{3}H-cyclic AMP was monitored (Table I). Fifty thousand cpm were added to the perchloric acid-treated algal cell concentrates and to the growth medium, this being considered 100% of the internal standard. Losses of algal cyclic AMP occurred during purification steps and this marker was used to determine the extent of such losses. Recoveries after ethanol:NH_4OH:H_2O elution from

TABLE I

RECOVERY OF MARKER 2,8-^{3}H-CYCLIC AMP AFTER VARIOUS PURIFICATION STEPS

Purification Step		+ MSO						
		0 h	1 h	2 h	4 h	8 h	12 h	24 h
Perchloric Acid treated algal cell concentrate	Cpm	50,000	50,000	50,000	50,000	50,000	50,000	50,000
	% Recov	100	100	100	100	100	100	100
EtOH eluate from charcoal	Cpm	41,000	34,020	31,752	20,250	28,269	43,821	30,220
	% Recov	82	68	64	40	56	88	60
5-ml eluate from BIO-RAD AG-50	Cpm	41,000	9,960	15,080	17,240	20,680	31,360	31,400
	% Recov	82	20	30	34	41	63	63

charcoal and after Bio-RAD column chromatography were recorded. Values ranged from 20 to 82% recovery after the final chromatographic step, the average being 48%. Sampling times varied between experiments and not all those shown in Table I were from a single experiment.

When cultures of A. variabilis were starved of nitrogen by transfer to fresh medium lacking in combined nitrogen, intracellular levels of cyclic AMP increased from 0.031 pmol ml^{-1} culture at 0 h to a high of 0.14 pmol ml^{-1} culture at 4 h (Figure 3). The level at 6 h was the lowest but after 24 h nitrogen starvation this amount increased to 5.5 times the 6 h level. Concentration of extracellular cyclic AMP was approximately the same as the intracellular level of nucleotide at 0, 2, 4, and 24 h. The drop in extracellular cyclic AMP at 6 h was not as dramatic as that for the intracellular level. Extracellular cyclic AMP was 37% of the total at time zero, reaching a high of 84% at 6 h, and finally declining to 56% of the total after 24 h.

Flask-grown algae which were nitrogen-starved with 0.5 μM MSO, had a low level (0.066 pmol ml^{-1} culture) of intracellular cyclic AMP at time zero (Figure 4). This increased during the 24 h period of nitrogen starvation to 0.22 pmol ml^{-1} culture. Extracellular cyclic AMP concentration corresponding to these data were 9 to 10 times higher at 0, 1, and 2 h, 5 times higher at 4 h, and approximately 3 times higher at 24 h. Extracellular cyclic AMP as a percentage of total cyclic AMP ranged from 90% in the early hours to 73% after 24 h nitrogen deprivation. These data are in contrast to those in Figure 3 where the lowest percent extracellular cyclic AMP (37%) occurred at zero time just after the cells were resuspended in nitrogen-free medium.

Fermentor-grown algae had a low level (0.050 pmol ml^{-1} culture)

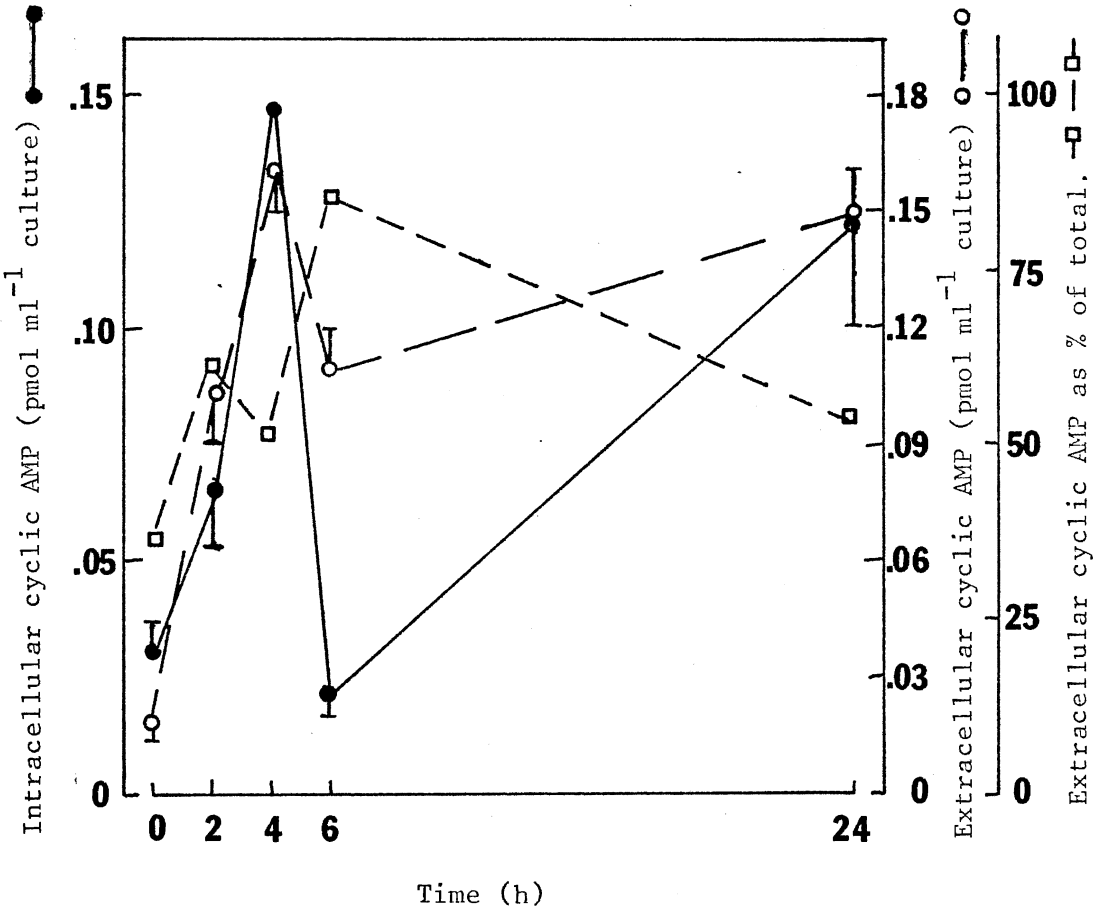

Figure 3. Intracellular and Extracellular Concentrations of Cyclic AMP When Nitrogen Starvation was Initiated by Transfer of Cells to Nitrogen-Free Medium. Also shown is extracellular cyclic AMP as percent of total measured in binding assay. Values are mean and S.D. of 2 replicates.

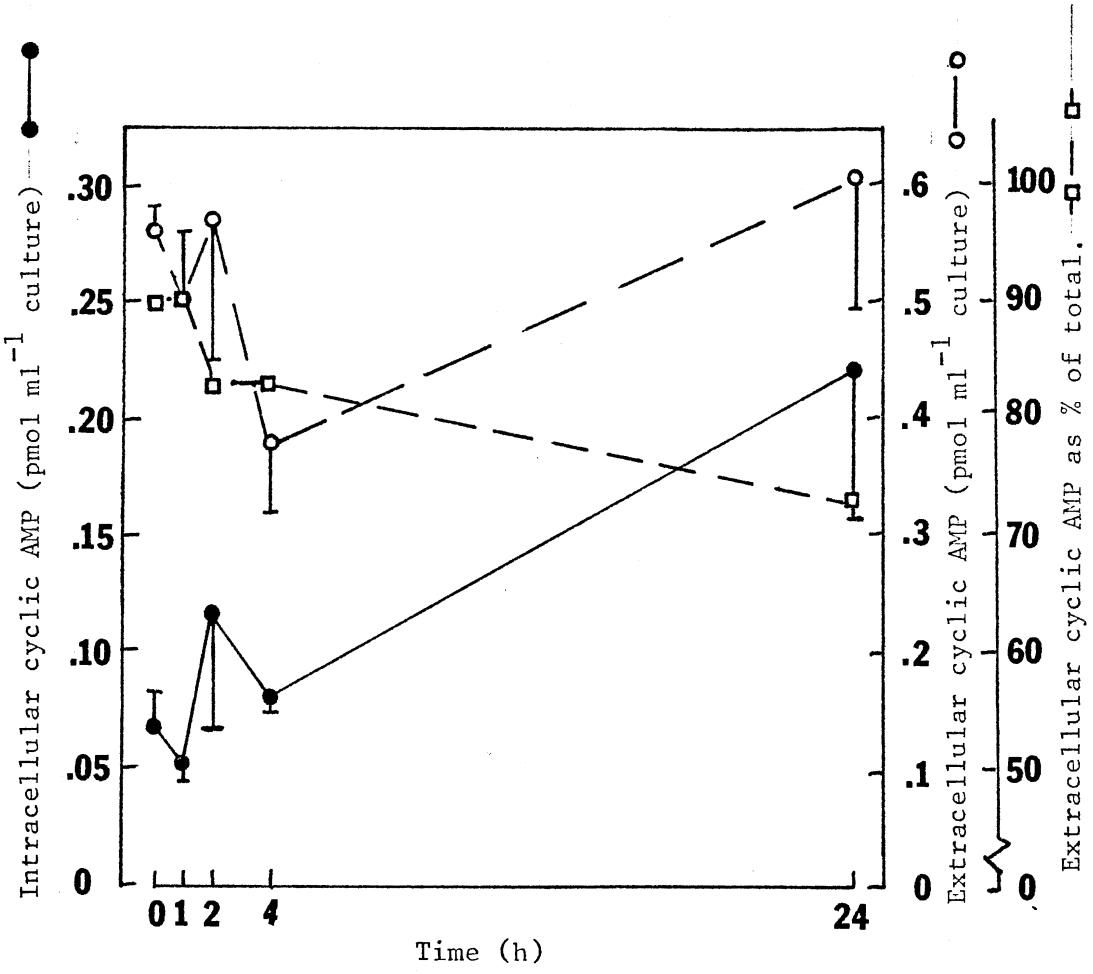

Figure 4. Intracellular and Extracellular Concentrations of Cyclic AMP When Nitrogen Starvation was Initiated by Addition of MSO to a Final Concentration of 0.5 μM to the Growth Medium. Also shown is extracellular cyclic AMP as percent of the total measured in binding assay. Values are mean and S.D. of 4 replicates.

of intracellular cyclic AMP at 2 h (Figure 5) which increased sixfold to a substantial peak after two more hours of nitrogen starvation. Levels at 8, 12, and 24 h were comparable to the level at 2 h. Concentration of extracellular cyclic AMP increased 1.5 times from 0 to 12 h with a slight decline between 12 and 24 h. In this experiment, extracellular cyclic AMP ranged from seven times greater than intracellular nucleotide at 4 h to 90 times greater at 12 h. Therefore, extracellular cyclic AMP represented the majority of this nucleotide in fermentor-grown algae. (Note expanded scale for percent extracellular cyclic AMP.)

Cyclic 3',5'-Nucleotide Phosphodiesterase Hydrolysis of Putative Cyclic Adenosine 3',5'-Monophosphate

Enzymatic hydrolysis of putative cyclic AMP to 5'-AMP with PDE is a necessary step in all experiments involving detection of cyclic AMP to provide further evidence of its identity. In different experiments in which the time of incubation of partially purified extract and PDE was varied, values for percent hydrolysis of marker ^{3}H-cyclic AMP were not correlated with time of incubation. Therefore, the percentage of hydrolysis of marker ^{3}H-cyclic AMP was compared to percent reduction of binding activity (below non-PDE treated controls) to express breakdown of putative cyclic AMP by PDE (Figure 6). In other words, a sample showing 75% hydrolysis of its marker (and presumably 75% hydrolysis of unlabeled algal cyclic AMP) should exhibit binding activity equal to only 25% of the unhydrolyzed control. These two parameters displayed reasonably good correlation ($r = 0.94$).

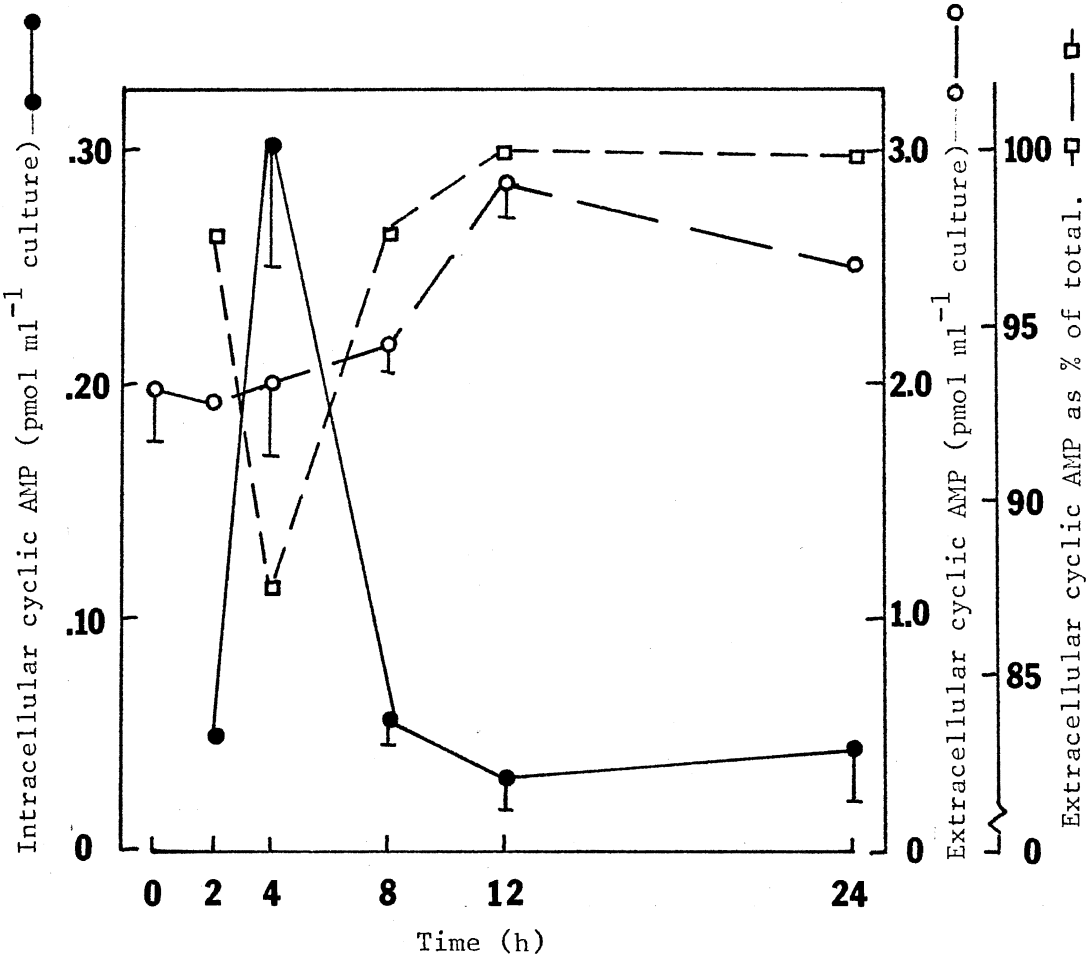

Figure 5. Intracellular and Extracellular Concentrations of Cyclic AMP of A. variabilis Grown in a New Brunswick Fermentor. Nitrogen starvation was initiated by adding MSO. Also shown on an expanded scale is extracellular cyclic AMP as percent of total measured in binding assay. Values are mean and S.D. of 4 replicates.

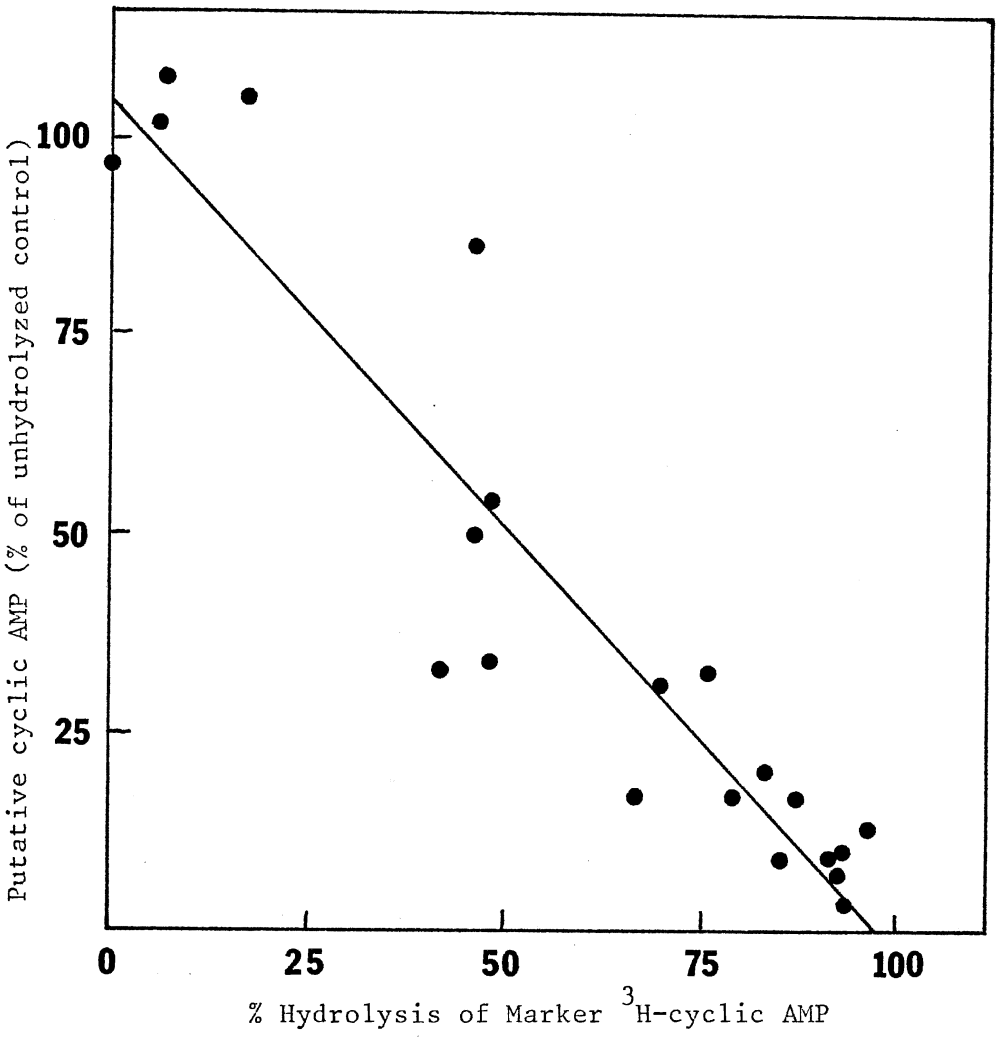

Figure 6. Correlation Between Hydrolysis of Marker ^{3}H-cyclic AMP and Reduction in Putative cyclic AMP (as Determined by the Gilman Assay) in Extracts Treated With Phosphodiesterase. Correlation coefficient = 0.94.

Effects of L-methionine-DL-sulfoximine, Cyclic Adenosine 3',5'-Monophosphate, and/or Adenosine 5'-Monophosphate on Heterocyst Development

Many biochemical and ultrastructural changes occur during the differentiation of heterocysts. A regulator probably activates the genes involved in heterocyst development in those cells competent to become heterocysts. The hypothesis for the experiments described below was that cyclic AMP is that regulator. In order to test this hypothesis, cyclic AMP was added to cultures of Anabaena with or without MSO, to determine its effects on heterocyst development. To determine if the product of breakdown of cyclic AMP might have an effect on heterocyst development, 5'-AMP was also added to cultures in combination with MSO.

Four different treatment regimes were employed in these experiments: 1) 0.5 μM MSO alone; 2) 1 mM cyclic AMP alone; 3) 0.5 μM MSO plus 1 mM cyclic AMP; or 4) 0.5 μM MSO plus 1 mM 5'-AMP. Treatment one represents the usual nitrogen starvation condition employed. The concentration of nucleotides (1 mM) added to the algal cultures in these experiments was 4×10^6 times greater than the endogenous levels measured. This amount was probably necessary to achieve uptake of enough cyclic AMP and 5'-AMP to simulate endogenous levels, although no data on uptake were obtained. Exogenous levels of nucleotide added to Anabaena in these experiments was comparable to those added to Chlorella (Berchtold and Bachofen, 1977) and Caulobacter (Shapiro et al., 1972). A typical percentage of cells (3 to 4%) differentiated

into heterocysts and proheterocysts under usual nitrogen starvation conditions (Figure 7). The increase in percentage of heterocysts in MSO-starved cultures at 36 h is most likely due to maturation of existing proheterocysts at 24 h plus those heterocysts already matured (Figure 7A). Cyclic AMP did not override the presence of ammonia in the growth medium and no cells differentiated (Figure 7B). In those cultures to which cyclic AMP was added with MSO, the percentage of differentiated cells was approximately half (1.5%) of control cultures after 24 h treatment (Figure 7C). At this time, proheterocysts were the major form of the differentiated cells. After 36 h treatment, it appeared that the proheterocysts had matured to heterocysts but the total percentage of differentiated cells was still only half (2.25%) of control cultures. The data suggest that cyclic AMP delayed heterocyst development by 12 hours. Those cultures to which 5'-AMP was added with MSO did not show the typical pattern of differentiation (Figure 7D). The 5'-AMP was strongly inhibitory of proheterocyst and heterocyst development.

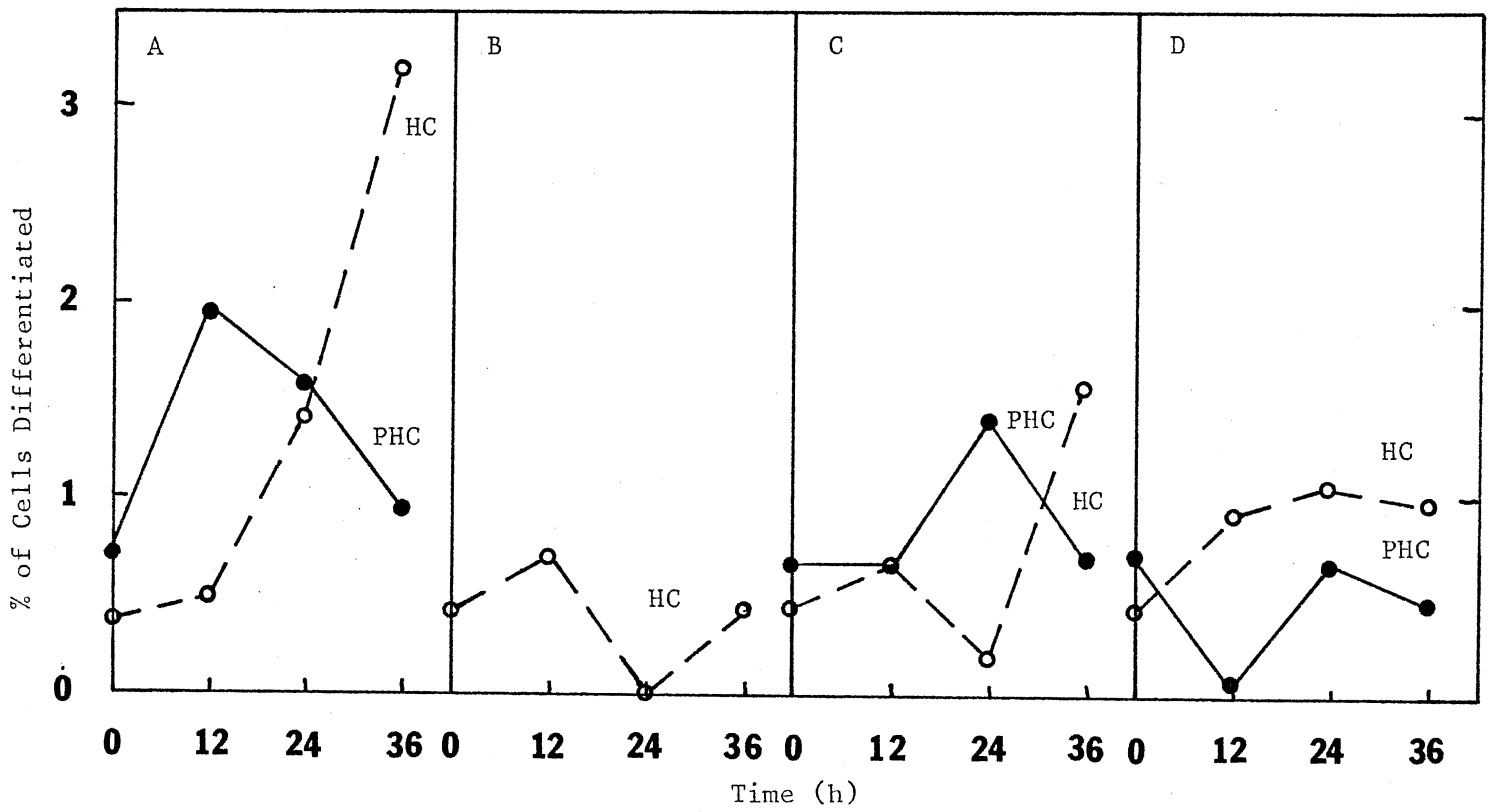

Figure 7. Effects of Several Compounds on the Differentiation of Heterocysts and Proheterocysts. A) 0.5 µM MSO; B) 1 mM cyclic AMP; C) 0.5 µM MSO plus 1 mM cyclic AMP; D) 0.5 µM MSO plus 1 mM 5'-AMP.

CHAPTER IV

DISCUSSION

Results in Figures 3, 4, and 5 demonstrate presence of nanomolar quantities of both intracellular and extracellular cyclic AMP in Anabaena variabilis as measured by the Gilman protein binding assay. Because the experimental conditions utilized were varied, it is difficult to compare directly the results obtained. Flask-grown algae which were treated with 0.5 μM MSO to induce nitrogen starvation showed the least response in terms of cyclic AMP accumulation (Figure 4). The intracellular nucleotide increased over the 24 hour period observed during which the pattern of differentiated cells usually becomes established (Wilcox, 1970; Hood et al., 1979). The extracellular concentration decreased slightly from 0 to 4 hours but appeared to increase again at 24 hours (Figure 4).

In contrast to these data, the intracellular cyclic AMP concentrations in flask-grown algae resuspended in nitrogen-free medium (Figure 3) and in fermentor grown algae to which 0.5 μM MSO was added (Figure 5) both showed a peak after 4 hours nitrogen starvation which was 5 or 6 times, respectively, the cyclic AMP concentrations at time zero. The intracellular cyclic AMP level dropped to basal levels in both treatments after 6 to 8 hours and continued to be low in the fermentor-grown algae for the remainder of the sampling period (Figure 5). However, the 24 h values for algae in nitrogen free medium were

four times the basal level. When nitrogen starvation was initiated by transfer of cells to nitrogen-free medium, the filaments recovered growth competence after 18 to 24 hours whenever heterocysts became functional and began providing nitrogen to the vegetative cells. Those cells starved of nitrogen by addition of MSO did not recover from this condition for several days (data not shown). Consequently, no conclusions can be drawn about cellular events in MSO-treated cultures beyond the 24-hour period required for heterocyst formation. These observations could account for the differences in intracellular cyclic AMP levels at 24 hours under these two nitrogen starvation regimes. Another observation which needs to be mentioned is that when Anabaena was grown in the fermentor, development of heterocysts appeared to be delayed up to 12 hours over those flask-grown algae (data not shown). Therefore, data in Figures 3, 4, and 5 may not be directly comparable, although general patterns should be noted.

Early observations of cyclic AMP in the growth medium led to these experiments in which extracellular cyclic AMP was assayed. These data suggest that cyclic AMP was released by the cells into the growth medium. Extracellular cyclic AMP was present in very low concentrations in those cultures which had been resuspended in fresh (nitrogen-free) medium. This was probably due to washing the filaments and dilution of any remaining extracellular cyclic AMP by the new medium. In fermentor-grown algae, extracellular cyclic AMP comprised a significant fraction of the total nucleotide present. This phenomenon of excretion of cyclic AMP into the growth medium was also reported for Brevibacterium liquefaciens (Okabayashi et al., 1963) although the levels of nucleotide were much higher (Table II) than those in

TABLE II

CONCENTRATION OF CYCLIC AMP OBSERVED IN VARIOUS ORGANISMS

Organism and Source	Condition	Cyclic AMP Concentration
Lolium multiflorum (Ashton and Polya, 1978)	endosperm cell suspension culture	2-12 pmol gFW^{-1}
Kolanchoe	normal growth	2-6 pmol gFW^{-1}
Agave (Ashton and Polya, 1977)		1 pmol gFW^{-1}
Avena sativa (Ownby, Ross, and Key, 1975)	dark grown coleoptiles	7-11 pmol gFW^{-1}
Physarum polycephalum (Lovely and Threlfall, 1976)	cell cycle events	1 pmol mg pro^{-1} (constant)
Dictyostelium discoideum (Malkinson and Ashworth, 1973)	development of myxamoebae	7.04-15.1 pmol mg pro^{-1} (intracellular)
Blastocladiella emersonii (Silverman and Epstein, 1975)	sporulating cells	1.1 nmol ml^{-1}
Chlamydomonas reinhardtii (Amrhein and Filner, 1973)	basal level	25 pmol gDW^{-1}
	+1 h in aminophylline	250 pmol gDW^{-1}
Ochromonas malhamensis (Bressan et al., 1980)	stationary phase cells	3-3000 pmol gFW^{-1}
	growth medium	$\leq$ 20 times cells
Acetabularia mediterranea (Driessche, 1976)	5-10 mm long	54 pmol gFW^{-1}
	20-30 mm long	111 pmol gFW^{-1}

TABLE II (Continued)

Organism and Source	Condition	Cyclic AMP Concentration
Chlorella fusca (Berchtold and Bachofen, 1977)	normal growth	0.2-2.0 nmol gDW^{-1}
Euglena gracilis (Keirns et al., 1973)	normal heterotrophic growth	15 pmol mg pro^{-1}
Escherichia coli B (Shapiro et al., 1972)	in PO_4	75.79 nmol ml^{-1}
	in PO_4 + glucose	.93 nmol ml^{-1}
Pseudomonas aeruginosa (Shapiro et al., 1972)	in PO_4	2.87 nmol ml^{-1}
	in PO_4 + glucose	0.30 nmol ml^{-1}
Caulobacter crescentus (Shapiro et al., 1972)	in PO_4	0.15 nmol ml^{-1}
	in PO_4 + glucose	0.11 nmol ml^{-1}
Bacillus megaterium (Setlow, 1973)	vegetative or sporulative cells	----
Brevibacterium liquefaciens (Okabayashi, 1963)	extracellular	60-200 nmol ml^{-1}
	intracellular	30-55 µmol gDW^{-1}
Arthrobacter crystallopoietes (Hamilton et al., 1977)	in glucose (spheres)	7 pmol $mgDW^{-1}$
	to succinate (rods)	240 pmol $mgDW^{-1}$

Anabaena. (Use Appendix D to compare the nucleotide levels in Anabaena to those reported for other organisms in Table II.)

The intracellular levels of cyclic AMP in Anabaena reported here range from a low of 0.02 pmol ml^{-1} culture to 0.3 pmol ml^{-1} culture. As interpreted from the table in Appendix D, these levels correspond respectively to: 0.74 pmol mg pro^{-1} to 7.0 pmol mg pro^{-1}, 50 pmol g FW^{-1} to 460 pmol g FW^{-1}, and 0.25 pmol mg DW^{-1} to 2.3 pmol mg DW^{-1}. On comparison of these levels in Anabaena with those reported for other organisms listed in Table II, one can observe that they are 50 to 400 times greater than the levels in higher plants, approximately the same as those in Physarum and Dictyostelium (slime molds), and 1000 times less than those in Blastocladiella (an aquatic fungus). Anabaena has concentrations of cyclic AMP similar to all the eukaryotic algae in Table II except Euglena whose level is 2 to 5 times higher. All reports of cyclic AMP in bacteria listed in Table II show much higher levels ($\geq 10^3$ times) than those in Anabaena.

One would expect cyclic AMP concentrations and mechanisms of action in cyanobacteria to be similar to those in eubacteria because of the biochemical and structural similarities among these prokaryotes. Cyclic AMP is associated with regulation of several systems in bacteria including release from catabolite repression in E. coli (Eilen and Krakow, 1977), and shift in carbohydrate source utilization and consequent morphological changes in Arthrobacter (Hamilton et al., 1977). Shifts in cyclic AMP concentration in these two organisms are immediate (20 min to 1 h) and of great magnitude (30 to 60-fold increase). Shifts in cyclic AMP levels in Anabaena may be neither fast enough (peak after four hours nitrogen starvation) nor large enough (5 to

6-fold) to be involved in the cellular differentiation process. However, the doubling time of Anabaena (18-24 hours, Wilcox, 1970) is much greater than that of bacteria (30 min to 1 h). Therefore, regulatory events may not need to occur so rapidly in Anabaena, although the small change in level is still troublesome.

How convincing is the Gilman assay itself and is it actually measuring authentic algal cyclic AMP? Several compounds could potentially interfere with the protein binding assay and would be interpreted as cyclic AMP. This is apparently very critical in isolating cyclic AMP from higher plants (Bressan et al., 1976). These interfering compounds include such things as other nucleotides (Gilman, 1972) and trichloroacetate (Wright, 1975). The acid precipitation, charcoal, and Bio-RAD AG-50 steps in the partial purification procedure removed many contaminating materials from the algal cell suspension and enriched the cyclic AMP fraction. No TCA was used in purification so this compound need not be considered as a contaminant in this case. The specificity of the binding protein is such that it does not bind cyclic GMP to any appreciable amount (Gilman, 1972), but may bind cyclic 2',3'-AMP (Niles and Mount, 1974). The assay itself was checked by the experimentor with material partially purified from E. coli B before and during its release from catabolite repression. The levels measured by the assay, 11.1 ± 1.6 and 687 ± 87 pmol mg pro^{-1}, are very similar to the levels reported by Makman and Sutherland (1965). Therefore, the binding assay as used in these experiments appears to be accurate.

A substantial source of error in interpretation of results from the binding assay was the presence of marker ^{3}H-cyclic AMP in the algal

or medium extracts. The data shown in Figures 3, 4, and 5 were not corrected for these additional cpm added to the assay tubes. A typical recovery of 50% represented 0.62 pmoles of ^{3}H-cyclic AMP. One-fourth of the final algal or medium preparation was added to each assay tube. In this case, the marker would compose 12% (0.16 pmol) of the ^{3}H-cyclic AMP present in the reaction mixture. However, although the average recovery in these experiments was approximately 50%, the majority of recoveries were below this value. Therefore, low recoveries actually improved the accuracy of the assay results for the reasons stated above.

Problems with PDE activity were also a source of error in this work. The PDE used in these experiments was from mammalian tissue and should have been specific for cyclic 3',5'-AMP and not cyclic 2',3'-AMP (Niles and Mount, 1974). Conditions were optimized as described by Bressan et al. (1976) for proper enzymatic activity. However, hydrolysis of cyclic AMP as interpreted by hydrolysis of marker ^{3}H-cyclic AMP was quite erratic (data not shown). Niles and Mount (1974) were able to show breakdown of marker ^{3}H-cyclic AMP added to broad bean extracts but showed no reduction in binding in the Gilman assay. They concluded that the Gilman assay was measuring cyclic 2',3'-AMP and that it was derived from RNA breakdown as suggested by Lin and Varner (1972). Although for A. variabilis PDE activity was erratic, a correlation was established between hydrolysis of marker ^{3}H-cyclic AMP and reduction of activity in the binding assay (Figure 6). These data provide further evidence for presence of cyclic 3',5'-AMP in A. variabilis.

Bressan et al. (1976) criticized the use of the Gilman binding

assay for detection of cyclic AMP in higher plants because a competitor which could eventually be resolved from true cyclic AMP after extensive chromatography was present in partially purified extracts. Data shown in Figure 6 which compare hydrolysis of marker ^{3}H-cyclic AMP with reduction of binding activity in the Gilman assay rule out the presence in these extracts of any competitor except one sensitive to PDE hydrolysis.

The evidence for endogenous cyclic AMP in A. variabilis presented here is supported by evidence from an isotopic labeling method showing 8-^{14}C-adenine incorporation into cyclic AMP (Hood et al., 1979). These data showed low levels of a ^{14}C-labeled compound, in nitrogen-starved and non-nitrogen-starved algae, which co-migrated with marker ^{3}H-cyclic AMP throughout six sequential purification systems. These fractions also displayed activity in the protein binding assay. However, Lin (1974) criticized this method of detection of cyclic AMP because of the limitations of the amount and specific activity of the precursor taken up and its conversion to product.

Exogenous cyclic AMP (1 mM) added to cultures of A. variabilis with 0.5 μM MSO appeared to delay heterocyst maturation by 12 hours and reduce by one half the total percentage of differentiated cells (heterocysts plus proheterocysts; see Figure 7). The observed effects may be due to hydrolysis of cyclic AMP to 5'-AMP since exogenous 5'-AMP (1 mM) inhibited differentiation and heterocyst maturation (Figure 7D). The exogenous concentrations used were much greater than the endogenous levels measured as reported above. However, uptake of this nucleotide was probably quite poor as was shown for Euglena (Keirns et al., 1973). Keirns et al. (1973) showed a stimulation of chloroplast RNA synthesis

by physiological levels of cyclic AMP added to the growth medium. Berchtold and Bachofen (1977) suggested that cyclic AMP may act in a system controlling the chlorophyll content of Chlorella cells since cyclic AMP levels were high with low nitrate concentration or high light intensity. These levels appeared to stimulate chlorophyll synthesis. Since there is an alteration in photosystems I and II in the mature heterocyst (Haselkorn, 1978) perhaps a similar sort of regulatory mechanism is operating in Anabaena when the level of cyclic AMP increases during nitrogen starvation.

Another possible role of cyclic AMP in A. variabilis (and other heterocystous species) could be its involvement in the regular spacing pattern of heterocysts described by Mitchison and Wilcox (1972). Fogg (1949) first suggested the presence of a diffusible inhibitor in the mature heterocyst which is transported to neighboring vegetative cells to prevent their differentiation. He suggested that this was a product of nitrogen fixation. However, it is now known that the pattern of heterocyst spacing is established long before nitrogen fixation competence is established and is not dependent on its products (Haselkorn, 1978). Haselkorn (1978) suggests that the glutamine liberated by protease action at the onset of nitrogen starvation is a reasonable candidate for the diffusible inhibitor, which is destroyed or metabolized by the vegetative cells and put out continually by the heterocyst. Since exogenous cyclic AMP delayed heterocyst maturation, and its hydrolytic product (5'-AMP) reduced by half the number of differentiated cells, the possibility exists that cyclic AMP may be involved in this inhibitory mechanism. This hypothesis suggests some interesting experiments. First, vegetative cells and differentiated cells should be

separated using established techniques (Fay and Lang, 1971) and assayed separately for cyclic AMP as well as for activity of adenyl cyclase (the enzyme of its formation) and PDE. Second, a cytochemical localization technique would be helpful in establishing intrafilamentar location of cyclic AMP and/or the enzymes of its metabolism.

Further research on cyclic AMP in this organism, then, would include establishing the presence of adenyl cyclase and PDE, further purification of crude extracts to reduce variability of PDE hydrolysis, establishment of any differences in concentration of this nucleotide in vegetative and differentiated cells and establishment of a concentration curve for effects of exogenous cyclic AMP and 5'-AMP on heterocyst differentiation.

CHAPTER V

SUMMARY AND CONCLUSIONS

Presence of the regulatory nucleotide, cyclic AMP, was investigated in the cyanobacterium, Anabaena variabilis. Of particular interest was its possible involvement in heterocyst differentiation in response to nitrogen starvation.

Nanomolar quantities of intracellular and extracellular cyclic AMP were observed, using the Gilman protein binding assay on partially purified algal and medium extracts. These levels are more similar to those reported for slime molds and eukaryotic algae than to those in bacteria or higher plants. In two experiments, a peak in cyclic AMP concentration was observed after 4 hours of nitrogen starvation initiated by two different methods. In a third experiment, an increase in intracellular cyclic AMP occurred during the 24-hour period in which samples were taken. Delay of heterocyst maturation was observed in an experiment where exogenous cyclic AMP was supplied to nitrogen-starved cultures of Anabaena variabilis. Inhibition of heterocyst formation was observed when 5'-AMP and MSO were added to this organism.

Criticisms of the procedures used in these experiments are reviewed in Chapter IV. Possible roles of cyclic AMP in this organism are also discussed.

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APPENDIXES

APPENDIX A

COMPONENTS OF THE MINIMAL SALTS MEDIUM OF ALLEN AND ARNON

Ingredient*	Final Concentration
$CaCl_2$	0.5 mM
NaCl	4.0 mM
$MgSO_4$	1.0 mM
$FeCl_3$	75.0 µM
Na_2EDTA	80.0 µM
Na_3 Citrate $2H_2O$	110.0 µM
K_2HPO_4	2.0 mM
$NaNO_3$	1.0 mM
NH_4Cl	2 mM
H_3BO_3	50 µM

Micronutrients:

$MnSO_4$ $4H_2O$	2.50 µM
$ZnSO_4$ $4H_2O$	0.20 µM
MoO_3	0.70 µM
$CuSO_4$ $5H_2O$	0.125 µM
H_3BO_3	0.080 µM

Adjust pH to 7.2 with HCl.

*Added in order listed.

APPENDIX B

COMPONENTS OF LIQUID SCINTILLATION COCKTAIL

Cocktail in which all Millipore filters were counted. The methyl-cellosolve dissolves the filters.

Ingredient	Amount per 1 ℓ cocktail
toluene	750 ml
methyl-cellosolve	250 ml
PPO*	7.0 g
POPOP*	0.2 g

* PPO = 2,5-diphenyloxazole

POPOP = p-bis 2-(5-phenyloxazolyl) -benzene

APPENDIX C

COMPONENTS OF POTASSIUM PHOSPHATE BUFFER FOR THE PROTEIN BINDING ASSAY

1. 340 mg KH_2PO_4 in 500 ml H_2O
2. 435 mg K_2HPO_4 in 500 ml H_2O
3. Add solution #2 to #1 until pH reaches 7.2.

APPENDIX D

INTRACELLULAR CONCENTRATIONS OF CYCLIC AMP

EXPRESSED IN VARIOUS UNITS FOR ANABAENA

Time (h)	Cyclic AMP Concentration			
	pmol ml^{-1} culture	pmol $mgpro^{-1}$	pmol gFW^{-1}	pmol $mgDW^{-1}$
		+ MSO in Fermentor		
2	0.050	1.0	66	0.33
4	0.30	7.0	460	2.3
8	0.053	0.78	52	0.26
12	0.031	0.51	34	0.17
24	0.043	0.63	42	0.21
		Nitrogen-Free Medium		
0	0.031	0.91	600	3.0
2	0.065	1.3	86	0.43
4	0.14	3.2	220	1.1
6	0.021	0.74	50	0.25
24	0.12	3.3	220	1.1

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