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A STUDY OF CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE AND MORPHOGENESIS IN ARTHROBACTER CRYSTALLOPOIETES (ARTHROBACTER GLOBIFORMIS)

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A STUDY OF CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE AND MORPHOGENESIS IN ARTHROBACTER CRYSTALLOPOIETES (ARTHROBACTER GLOBIFORMIS)

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CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE IN *ARTHROBACTER CRYSTALLOPOIETES* (*ARTHROBACTER GLOBIFORMIS*)

INTRODUCTION

The genus *Arthrobacter* was described by Conn and Dimmick (8) in 1947 as pleomorphic, Gram variable, soil bacteria and placed in the *Corynebacteriaceae* (19). One of the distinguishing characteristics of *Arthrobacter* has been the morphogenic cycle exhibited during growth (45). Distinct cell types occur sequentially during the life cycle: cocci elongate to rods which grow and divide until late in log stage when they fragment, become shorter and revert to cocci. This simple morphological variation and facile growth and synchronization have made *Arthrobacters* an advantageous system in which to study morphogenesis, its regulation and control (6).

The species *Arthrobacter crystallopoietes* (*Arthrobacter globiformis*) (19) was first isolated from soil and described by Ensign and Rittenberg, (11) in 1963. It was characterized by the production of a blue pigment when utilizing 2-hydroxypyridine as a carbon source. Later, this pigment production was shown to be a plasmid associated phenomenon (46). More recently, Keddie (19) has classified *Arthrobacter crystallopoietes* as a strain of the type species *Arthrobacter*

globiformis, and placed the genus in the coryneform group of bacteria. For the purpose of continuity, both names will be cited throughout the following manuscript. Ensign and Wolfe (12) reported that the morphogenic cycle in Arthrobacter crystallopoietes could be nutritionally controlled. In a defined medium containing certain substrates, for example, glucose, cells could be grown as cocci only. The addition of one of a number of apparently unrelated compounds induced the formation of rod shaped cells. Nutritional control was a helpful finding in that a system was provided in which cellular changes associated with morphogenesis could be compared to changes due to growth and aging.

The mechanism of nutritional control has not been ascertained. Once coccoidal cells have been induced to form rods, the presence or absence of the inducing compound did not appear to directly initiate the other major morphologic event, that is, the fragmentation of the rods and eventual reversion to cocci (16). Fragmentation did not result from the exhaustion of the inducing compound. Therefore, although initiation of the morphogenic cycle was under nutritional control, subsequent stages were not controlled directly by the inducing compound. This did not preclude the role of the compounds as precursors of cell metabolites which may play a regulatory role.

There has been some controversy as to whether these compounds actually induce the morphogenic cycle. Luscombe and Gray (26) and Crombach (9) proposed that the morphology of certain Arthrobacters was a function of the growth rate and that specific inducers are not required. However, in substrate levels which barely supported growth as measured by increase in absorbance, coccoidal A. crystallopoietes

cells were induced to the rod stage (C. Kimberlin-Hariri, M.S. thesis, University of Oklahoma, Norman, 1974). Also, growth rates were achieved in non-inducing substrates which surpassed those obtained in inducing media but still the cells remained coccoidal. Ensign and St. John (44) showed that coccoidal cells in an appropriate, inducing medium become rod shaped in the presence of a DNA inhibitor although they neither divide nor fragment. Therefore the morphogenic change from coccus to rod preceded cell division and was independent of the rate of cell division or growth rate. However, there could have been a direct relationship between the rate of increase in cell mass and morphogenesis, but no evidence has suggested that a causal relationship occurs. As Kvasnikov et al. (24) pointed out in their discussion of the nutritional control of morphogenesis in Arthrobacter simplex (Jensen) Lochhead, there is at present no unanimous opinion concerning morphogenesis and its occurrence or induction in the different Arthrobacter species.

Once the cycle has been induced, the morphological events of elongation, fragmentation and reversion are accompanied by biochemical changes in structural and functional cell components. A number of biochemical differences have been observed between coccoidal and rod cells. Krulwich et al. (20,21) reported that the polysaccharide backbones of rod cell walls were longer and more homogeneous than those in coccoidal cells, and the amino acid cross-linking in the peptidoglycan was slightly greater in the rods. N-acetyl muramidase activity was four to five times higher in the spheres (22). Although no significant variation was found in phospholipid or glycolipid composition when lipids were extracted during rod and sphere stages (43),

Meyers (30) reported that one neutral lipid, a diglyceride, appeared only in cells just beginning to fragment.

Changes in morphology were accompanied by changes in metabolism as well. Ferdinandus (J. Ferdinandus, Ph.D. thesis, University of Oklahoma, Norman, 1969) reported that fats were accumulated during rod formation and utilized as an endogenous energy supply during fragmentation. Lipogenic enzymes reached their highest specific activity during rod stage, while lipase and the gluconeogenic enzymes had their highest specific activity during fragmentation. One enzyme, the malic enzyme (L-malate: NADP oxidoreductase (decarboxylating); E.C. 1.1.1.40.), was found to be active only during the rod stage. At present, this is the only rod specific marker available during the morphogenic cycle of Arthrobacter crystallopoietes. Ferdinandus suggested a model to explain morphogenic control at the substrate level via feedback inhibition of key enzymes. The enzymes of glycolysis and lipogenesis would be selectively inhibited by free fatty acids while gluconeogenic enzymes would be unaffected.

Ferdinandus and Clark (14) reported that the phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase; E.C. 2.7.1.11) found in Arthrobacter crystallopoietes cells which have undergone morphological changes was not inhibited by high concentrations of ATP. Later, Lawrence (1974, M.S. thesis, University of Oklahoma, Norman) investigated this enzyme and adenylate control in cells which were grown only as cocci and found the phosphofructokinase to be a regulatory enzyme. Apparently, there were two different phosphofructokinases in this organism, and they may play a role in the morphogenic cycle.

Other work has been done on the metabolism of Arthrobacter crystallopoietes. This species has the enzymes of the Krebs cycle, the Embden-Meyerhoff-Parnas pathway and the hexose monophosphate shunt pathway (2,23). Schecter, et al. (40) reported that the major catabolite effects on inducible enzyme synthesis involved the uptake of the inducer and a severe catabolite repression (as seen with L-serine hydrolyase deaminating dehydratase EC 4.2.1.13). The extent of catabolite repression by a compound had nothing to do with its utilization as a substrate but was apparently related to the pathway by which it was metabolized. Cyclic adenosine 3':5'-monophosphate (cAMP) did not reverse the catabolic effects. They suggested that cAMP may possibly effect metabolism at the enzyme level via protein kinases.

Little is actually known of the control mechanisms of morphogenesis in Arthrobacter crystallopoietes. Induction of morphogenesis by specific compounds is not understood. Changes in the specific activities of enzymes during morphogenesis may be due to either metabolic or genetic control, or both. There is evidence to support the concept that control occurs at both levels. Competitive hybridization studies by Massey, Clark and Jacobson (29) indicated that significant qualitative changes in the mRNA population occur during morphogenesis, suggesting regulation by differential transcription. Differential transcription can result from changes in the existing RNA polymerase and its specificity as well as synthesis of a new RNA polymerase (10). Two DNA-dependent RNA polymerases have been isolated from Arthrobacter crystallopoietes by Jacobson and Mazukelli (17). These enzymes differed in their template specificity and other characteristics.

The majority of the investigations of morphogenesis in Arthrobacter crystallopoietes produce similar queries: how is the morphogenic cycle regulated; when does the regulation occur and, at what level?

A search was begun to find a metabolite capable of cell regulation at various levels, one that was known to be involved in morphogenesis and one that not only regulated cell metabolism but can itself be regulated by the cell's environment. Especially of interest was a metabolite which influenced morphogenesis while not affecting growth of the organism as a non-morphogenizing entity. Cyclic adenosine 3':5'-monophosphate (cAMP) was selected as the metabolite to be investigated, since it had all of the above capacities.

Cyclic adenosine 3':5'-monophosphate (cAMP) is an important regulatory molecule in animal cells (37). Discovered by Sutherland et al. (36) as the intermediate in hormone activation of liver phosphorylase, cAMP has been found to play an important role in many hormonal actions and regulatory mechanisms of the animal body (37). This cyclic nucleotide has been found in all animal cells (38), in many plants (3,15), in the green algae (1) and in the procaryotic blue-green alga, Anacystis nidulans (38). In 1963, cAMP was identified in Escherichia coli (28) and in the culture fluid of Brevibacterium liquiefaciens (31). Since then, cAMP has been demonstrated in numerous procaryotes (34, 38).

In the procaryotic cells, cAMP has exerted its effects primarily by stimulating the synthesis of a number of proteins which were not essential for all growth conditions. cAMP overcame severe transient

repression (18) and the less severe catabolite repression (27,32) of many inducible enzymes. Makman and Sutherland have (28) found that cellular cAMP concentrations depended greatly upon growth conditions. In poor or limiting substrates, cells accumulated large amounts of cAMP and the addition of a readily utilizable substrate usually resulted in a decrease in the intracellular cAMP concentration.

cAMP has been shown to affect several procaryotic systems which undergo morphogenesis or structural changes. It was required for the synthesis and formation of flagella in Escherichia coli and Salmonella typhimurium (48). Vegetative cells of Myxococcus xanthus were enhanced to swarm and form fruiting bodies by this cyclic nucleotide (5). Shapiro et al. (42) demonstrated that cAMP hastened the morphological differentiation of Caulobacter crescentus. In Bacillus licheniformis, cAMP was found only at the end of active cell division and growth (6). The morphology of simple eucaryotes has also been shown to be affected by cAMP. Hypha formation in Mucor racemosus was inhibited by cAMP (25). In the life cycle of Dictyostelium discoideum, cAMP was found to activate aggregation and culmination (2,39). Compounds which either reduced or increased endogenous cAMP produced morphological abnormalities in Neurospora crassa (41). The mechanisms of many of these actions have not been elucidated. It may be possible, that some mechanisms of cAMP mediated effects in procaryotes are similar to those in eucaryotes.

cAMP and its relationship to morphogenesis in Arthrobacter crystallopoietes has not been previously studied. Investigations were undertaken in an effort to establish not only if cAMP is involved in

this system, but how it is involved and by what mode(s) of action. The results presented in the following manuscripts answer to some degree these questions and serve, as well, to indicate Arthrobacter as a promising system in which to study morphogenic changes, their regulation and control.

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PAPER I

INHIBITION OF MORPHOGENESIS IN A PROCARYOTE, *ARTHROBACTER CRYSTALLOPOIETES* (*A. GLOBIFORMIS*), BY EXOGENOUS CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE

INHIBITION OF MORPHOGENESIS IN A PROCARYOTE, *ARTHROBACTER CRYSTALLOPOIETES* (*A. GLOBIFORMIS*), BY EXOGENOUS CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE

ABSTRACT

Exogenous cAMP inhibited the elongation of coccoidal cells into rod forms. Theophylline delayed elongation and fragmentation of the rod cells. Cyclic GMP produced no apparent effects on the morphogenesis when tested under similar conditions.

INTRODUCTION

The functions of cAMP in procaryotic cells are diverse and numerous (10). However, morphogenic effects have been demonstrated in relatively few cases. These effects were for the most part "positive" in relation to morphogenic events and included such observations as the requirement of cAMP in flagellation in coliforms (12), the induction of fruiting body formation in Myxococous xanthus (1), and the enhancement of differentiation in Caulobacter crescentus (11). In contrast, this paper reports the inhibition of morphogenesis in Arthrobacter crystallopoietes (4, 6) by cAMP, a "negative" effect on a morphogenic event, demonstrating diversity in the regulatory roles of cAMP in procaryotic morphogenic systems. In addition, the effect of theophylline, a cAMP specific phosphodiesterase inhibitor, was studied, and the observed effects on morphogenesis were thought to result from in vivo prevention of cAMP destruction and thus an increase in intracellular levels of cAMP.

Arthrobacter crystallopoietes has a life cycle with distinct morphological cell types: early cocci elongate to rods which grow and divide until late in log stage when they fragment, become shorter rods and eventually revert to cocci (2). This species offered the advantage of a nutritionally controllable morphogenic cycle since the cells could be grown only as cocci or induced to undergo a morphogenic cycle by

the addition of certain substrates.(5). In conjunction with the morphological stages, biochemical and physiological changes occured which were absent or less pronounced in the coccoidal control cells (2).

METHODS AND MATERIALS

Arthrobacter crystallopoietes (ATCC 15481) (A. globiformis) (6) coccoidal cells from a synchronous 24 hour culture were treated by a modified TRIS-EDTA method (7) which made them more permeable. Treated cells were inoculated into flasks containing 0.5% (w/v) glucose minimal salts medium (GMS) (5), in which cells were coccoidal only, and into three media in which cells underwent morphogenesis: 0.5% L-asparagine minimal salts (AMS), 0.5% succinate minimal salts (SMS) and Tryptone Glucose Yeast broth (Baltimore Biological Laboratories). Initial absorbance was standardized to 0.025 at 485 nm (Bausch and Lomb Spectrophotometer 20).

At the time of inoculation and at 2 hour intervals thereafter, cAMP or cGMP (Sigma Chemical Company) was added to flasks containing the previously described cells and media. Final cyclic nucleotide concentrations of 0.1, 0.5, 1.0, 2.0 and 5.0 mM were maintained. Cell growth was measured by absorbance at 485 nm and morphology was determined by 0.1% methylene blue stained slide preparations.

Controls utilized media to which no cyclic nucleotide had been added and media to which nucleotides previously treated with phosphodiesterase had been added. Similar experiments were performed adding 10 mM theophylline at the various time intervals to the four media containing the TRIS-EDTA treated cells.

Experiments were repeated using cells allowed to grow to a certain stage of the life cycle, i.e. early rods, mid-log phase rods and fragmenting rods reverting to cocci. The cells at the various stages were treated with TRIS-EDTA and were reinoculated into their

original media which had been titrated from an alkaline pH to the original pH of the medium (7.2). To these cultures, 5.0 mM cAMP, cGMP or 10 mM theophylline was added, and observations of the life cycle were continued.

RESULTS

The TRIS-EDTA treatment itself had no effect on the morphogenic cycle, but a slightly higher maximum growth was achieved as compared to untreated control cells. Without this treatment, the cells were apparently impermeable to cAMP and theophylline since no effect was observed in cultures not pretreated with TRIS-EDTA. Cyclic cGMP did not effect the growth or morphogenesis of treated or untreated cells under any of the conditions used. The morphogenic cycle in the three media is shown in Fig. 1. Cells remained coccoidal in the GMS medium only.

No effect of cAMP was seen in the 0.5% GMS medium in which cells were coccoidal only, or in any medium in which the cAMP concentration was below 1.0 mM, or when phosphodiesterase was used to break down the cAMP before its addition to the medium.

Above a 1.0 mM concentration of cAMP, elongation of the cocci was delayed in the morphogenic cultures and rods did not appear until approximately 16 hours (Fig. 2). This delay continued through fragmentation which occurred at 20-22 hours as compared to 18-22 hours in the controls. However, if cAMP was not added utnil after 8 hours of growth, it had no observable effects on the morphogenic cycle. There were several possible explanations for this loss of effect. The compound could have been unstable in the increasingly alkaline media, a critical threshold level might have been reached intracellularly, there could have been only a short time span during which cAMP was effective, or after 8 hours the cells might no longer have been permeable to exogenous cAMP.

To test this last possibility, cells were allowed to reach a particular stage in the morphogenic cycle, then were treated with TRIS-EDTA, were reinoculated into the various media, and cAMP was added as described in METHODS. These experiments demonstrated that once rod formation had begun exogenous cAMP did not delay the remaining morphogenic stages of fragmentation and reversion to cocci.

Theophylline inhibits cAMP specific phosphodiesterase, an enzyme which catalyzes the breakdown of intracellular cAMP. This methylxanthine has been shown to inhibit in vitro the phosphodiesterase isolated from Serratia marcensens (8), and was used in these experiments to maintain intracellular cAMP levels during the morphogenic cycle. In the presence of 10 mM theophylline, cells which had been grown in 0.5% GMS medium, remained coccoidal and grew at the same rate as the control, apparently unaffected.

In the morphogenic cultures, theophylline delayed rod formation approximately 2-8 hours. Once rods were formed they failed to fragment and revert to cocci until 32-36 hours while controls without the theophylline fragmented at 18-22 hours (Fig. 2). When added to TRIS-EDTA treated cells which had already reached rod stage, theophylline delayed fragmentation for a 2-8 hours period. The rod cells then fragmented and reverted to cocci.

DISCUSSION

These observations indicate that exogenous cAMP inhibits the normal initiation of the morphogenic event of elongation of cocci to rods in the life cycle of *Arthrobacter crystallopoietes* and that theophylline, a cAMP phosphodiesterase inhibitor, has a similar effect, as well as causing a long delay in the process of fragmentation of rods to cocci. No effect was observed as a result of exogenous cGMP in these experiments. This does not imply, however, that this nucleotide does not have a possible regulatory function, as yet undetermined, during the morphogenic cycle. We assume, from these results, that increased levels of cAMP *in vivo* may inhibit the elongation of cocci and sequential morphological transitions. A measurement of cAMP levels during morphogenesis may help to substantiate this assumption.

FIGURE LEGENDS

Fig. 1. Arthrobacter crystallopoietes was grown in various media, with growth assessed through the increase in absorption at 485 nm: (□— □) tryptone, glucose and yeast (TGY) broth; (● — ●) 0.5% L-asparagine with minimal salts (AMS); (○—○) 0.5% succinate with minimal salts (SMS); and (■ — ■) 0.5% glucose with minimal salts (GMS). Morphological changes are described and drawn as viewed on preparations stained with 0.1% methylene blue using light microscopy.

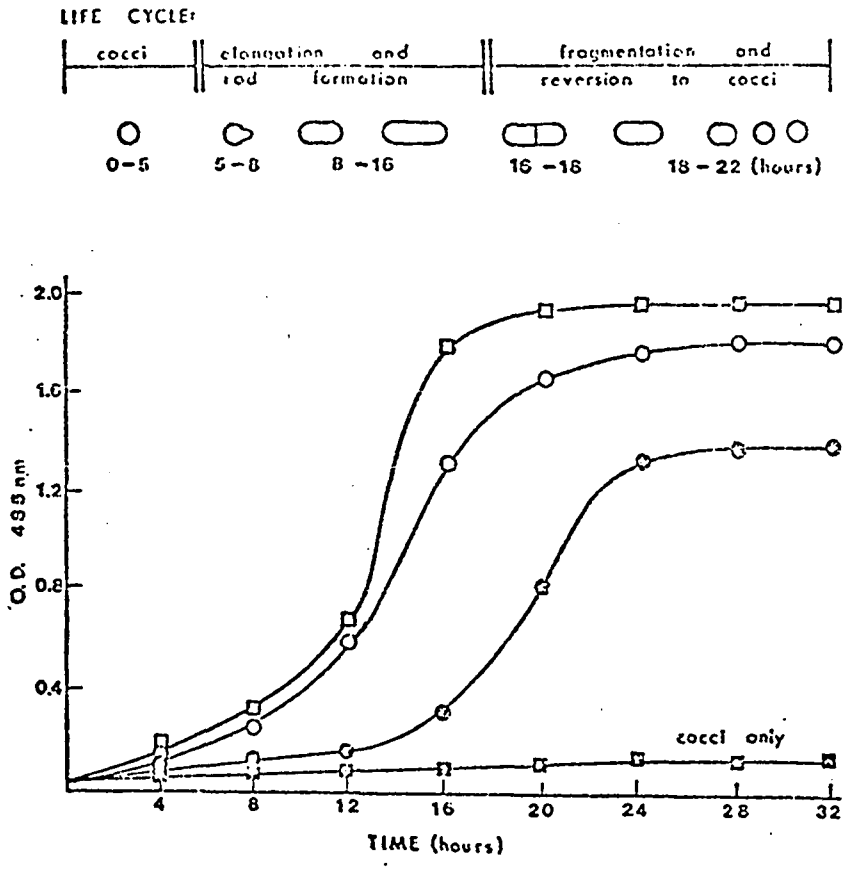
LIFE CYCLE:
cocci
elongation and rod formation
fragmentation and reversion to cocci
0-5
5-8
8-16
16-18
18-22 (hours)
O.D. 495 nm
2.0
1.6
1.2
0.8
0.4
cocci only
4
8
12
16
20
24
28
32
TIME (hours)

Figure 1

Fig. 2. This diagram represents the inhibitory effects of either 3',5'-cyclic AMP or theophylline added at the beginning of the life cycle (time = 0 h) to cells which were previously treated with TRIS-EDTA. A. Morphogenetic cycle of control cells grown in SMS, AMS, TGY, with or without the TRIS-EDTA treatment. B. Inhibition due to 1mM-5mM 3'-5' cAMP in the same media. C. Inhibition due to addition of 10mM theophylline in the same media. The effects seen in B and C occur when cAMP or theophylline were added between 0-8 hours but not afterwards. Rod stage cells were delayed in their fragmentation when TRIS-EDTA treated and theophylline was added.

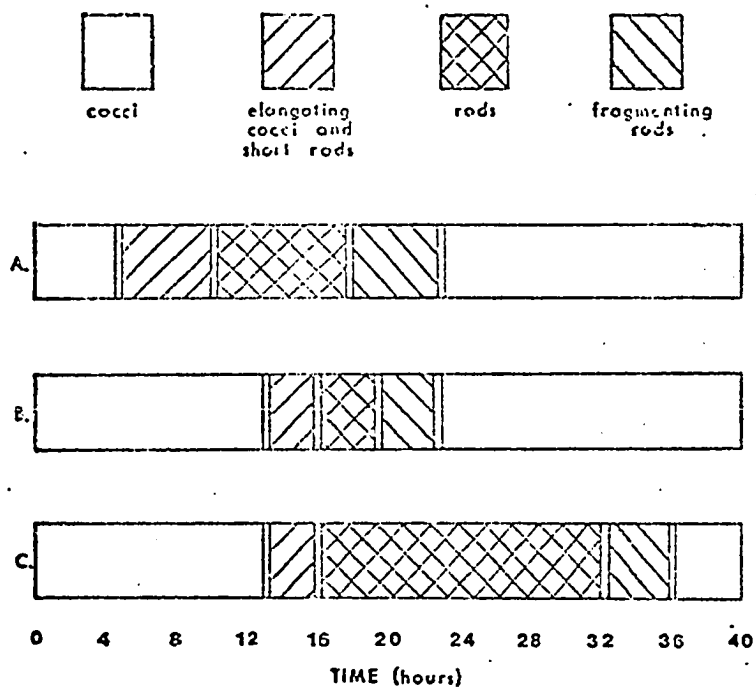
cocci
elongating cocci and short rods
rods
fragmenting rods
A.
B.
C.
0
4
8
12
16
20
24
28
32
36
40
TIME (hours)

Figure 2

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PAPER II

INTRACELLULAR AND EXTRACELLULAR LEVELS OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE DURING MORPHOGENESIS IN *ARTHROBACTER CRYSTALLOPOIETES* (*A. GLOBIFORMIS*) AS COMPARED TO LEVELS DURING NON-MORPHOGENIC GROWTH

INTRACELLULAR AND EXTRACELLULAR LEVELS OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE DURING MORPHOGENESIS IN ARTHROBACTER CRYSTALLOPOIETES (A. GLOBIFORMIS) AS COMPARED TO LEVELS DURING NON-MORPHOGENIC GROWTH

ABSTRACT

Intracellular cAMP levels rapidly and markedly increased in the coccoidal cells which were elongating to form rods. Endogenous cAMP peaked and returned to levels in the rod cells comparable to that found in pre-transition cocci. This level was maintained throughout the remaining morphogenic cycle. Increases in the extracellular cAMP levels were found at early rod and fragmentation stages. During the non-morphogenic growth cycle, cells were coccoidal only. Intracellular cAMP levels remained relatively constant while extracellular cAMP levels gradually increased after log stage.

INTRODUCTION

Arthrobacter crystallopoietes (A. globiformis) (7) has been in part characterized by its nutritionally controlled morphogenic cycle (5). In glucose minimal salts medium, the cells grow as cocci. Upon the addition of a number of seemingly unrelated compounds, the cells elongate to rods which grow and divide until late in log stage when they fragment, become shorter rods and eventually revert to cocci. A. crystallopoietes provides a useful system in which morphogenic changes can be compared to changes occuring during growth and aging. (2).

Evidence has accumulated indicating that adenosine 3':5'-cyclic monophosphate (cAMP) has specialized and regulatory functions related to growth and differentiation in lower eucaryotic and procaryotic organisms. Examples included, hypha formation in Mucor (8); aggregation and culmination in Dictyostelium (1, 10) flagellation in coliforms(14); differentiation in Caulobacter crescentus (13); and fruiting body formation in Myxococcus xanthus (9). In Neurospora crassa, drugs which altered intracellular cAMP levels led to abnormalities in morphology (12). In Bacillus licheniformis, a representative of procaryotic morphogenesis via sporulation, endogenous cAMP has been found at the end of growth and cell division (4). Although cAMP has often been correlated with morphogenesis in procaryotes, it has also

been shown to regulate non-morphogenic systems as well. It has been stated that cAMP plays a regulatory role in bacteria but is not essential for growth and viability (9).

By employing the Arthrobacter system, changes in cAMP levels could be measured and compared in the morphogenic and non-morphogenic cells. The purpose of this investigation was to determine if changes in cAMP levels occur during morphogenesis which vary from levels found in the cells grown as cocci only.

MATERIALS AND METHODS

Organism and growth conditions. Arthrobacter crystallopoietes ATCC 15481 (A. globiformis) (7) cells were synchronized in their morphogenic cycle by transfer of 24 h coccoidal cells into fresh medium in which the cycle occurs. Cells were transferred at 48 h intervals into 0.5% (w/v) glucose minimal salts (GMS) medium in which cells grow only as cocci. The minimal salts medium (5) contained either 0.5% glucose or one of two morphogenic inducing compounds; 0.1% (w/v) L-asparagine of 0.05% (w/v) succinate. Incubation was aerobic at 30 C [illegible]. New Brunswick Controlled Environment Incubator Shaker at 225 RPM. [illegible]ocula consisted of coccoidal cells washed x 3 with 0.03 M phosphate buffer (pH 7.0) and were added to 200 ml medium to attain a 0.025 absorbance at 485 nm (Bausch and Lomb, Spectrophotometer 20). Bacterial numbers were determined by colony forming units on Plant Count agar (Difco). Growth was measured by absorbance at 485 nm. Protein concentrations were quantitated by the method of Schacterle and Pollack (11). DNA was quantitated by the method of Burton (2).

Morphological stages, as determined by 0.1% methylene blue stained slide preparations, selected for this study were as follows: young cocci (lag stage); elongating cocci (early log stage); rods (mid-log stage); fragmenting rods (late-log stage); and newly formed cocci (stationary stage cells) (Fig. 1). Growth stages of the cells grown in 0.5% GMS included; lag stage cocci; early, middle and late log stage cocci; and stationary stage cocci.

Measurement of cAMP. Samples were taken at 2 h intervals and/or

at the appropriate morphological stage. For the determination of cAMP levels, 10 ml aliquots of culture were rapidly filtered (Millipore HAWP 02500; AA 0.45 μm membrane filter). A portion of the filtrate was removed for determination of the extracellular cAMP level. Filters were washed x 2 with 10 ml of 20 mM phosphate buffer (pH 7.0) and were placed into 1.0 ml of 0.1 N HCl in a boiling water bath for 10 minutes. Extracts were dried in a stream of air and diluted with 0.5 ml of 50 mM acetate buffer (pH 4.5).

Triplicate samples (10 μl) were assayed for cAMP concentration. In addition, controls consisting of 10 μl of each sample were reacted with cAMP dependent phosphodiesterase (30 min, 30 C) to destroy all of the cAMP present and assure that what was measured in the test sample was actually cAMP. Known quantities of cAMP were prepared as standards in a range of 1-20 picomoles and were repeatedly assayed at random throughout the testing. Known amounts of cAMP were added to samples of test filtrates randomly selected to determine recovery efficiency and compared to a control test containing no added cAMP.

A modification of the Gilman (6) cAMP protein binding assay was employed. The reaction mixture included: 50 mM acetate buffer (pH 4.5); 10 μl [^{3}H] cAMP (38.4 Ci/mM); 10 μl protein kinase inhibitor (4 μg/ml); 10 μl protein kinase (binding protein) (2 μg/ml); 10 μl sample, standard or control. Binding protein was added to initiate the reaction. Incubation was for 2 h at 0 C. The reaction was terminated with 1.0 ml cold 20 mM phosphate buffer (pH 6.0). Each test was filtered (Millipore HAWP 02500). Filters were washed x 4 with 10 ml of 20 mM phosphate buffer (pH 6.0), dried in an air stream, placed in vials

to which 12 ml Aquasol (New England Nuclear) scintillant was added. Radioactivity of the [^{3}H]-cAMP bound to the protein collected on the filter was determined by scintillation counting using a Beckman DPM-100 liquid scintillation system. Concentrations of cAMP were calculated by reference to a standard curve based on a decrease in the amount of bound [^{3}H]-cAMP due to the presence of unlabeled cAMP. A standard curve was established for each assay.

Chemicals. cAMP, protein kinase, protein kinase inhibitor, and cAMP dependent phosphodiesterase were purchased from the Sigma Chemical Co., St. Louis, Mo. Aquasol scintillant and [^{3}H]-cAMP (38.4 Ci/mM) were purchased from the New England Nuclear, Corp., Boston, Mass.

RESULTS

During the morphogenic cycle, the intracellular level of cAMP increased to 800 nM/mg protein when coccoidal cells elongated (Fig.1a). As rod forms predominated, the cAMP level decreased to less than 50 nM /mg protein and remained relatively constant throughout fragmentation of the rods and reversion to cocci. Intracellular cAMP levels were calculated per weight of wet cells, mg protein, μg DNA and per number of viable cells: in each instance the resulting changes in cAMP levels remained similar quantitatively; therefore we arbitrarily reported the cAMP levels per mg protein. Samples were measured initially at 2 h intervals. Once it had been determined that a peak occurred in the endogenous cAMP level, assays were repeated on samples at 30 min intervals. Cyclic AMP was released into the medium when rod cells had formed and again when rod cells were fragmenting (Fig.1a).

In the GMS medium, in which cells grow as cocci only, there was a lag period for approximately 18 h during which the cAMP concentration remained at approximately 50 nM/mg protein. No exogenous cAMP was detected at this stage (Fig.1b). Endogenous cAMP decreased slightly from early to midlog stage while a gradual increase was detected during late log to stationary stage. Small amounts of cAMP were detected extracellularly after mid log stage of the cocci.

DISCUSSION

The ubiquity of cAMP and its correlation with cellular changes in eucaryotic and procaryotic organisms prompted this investigation of cAMP levels during morphogenesis in Arthrobacter crystallopoietes as compared to levels found during the non-morphogenic growth cycle. Inherent in the morphogenic cycle of A. crystallopoietes are specific changes in cell structure: coccoidal cells elongate to form rods; after a period of rod growth and division, rod cells fragment and eventually revert to cocci. The hypothesis, that cAMP was involved in the regulation of these morphological transitions, led to these questions: (1), could exogenous cAMP affect the morphogenic cycle; (2), could changes in the cAMP levels occur during morphogenesis which were different from changes in cAMP levels measured during non-morphogenic growth; (3), how are cAMP levels controlled in the cell; and (4), by what mode of action does cAMP affect morphogenesis in this system?

It has been shown (C. Kimberlin-Hariri, Ph.D. dissertation, 1975, University of Oklahoma, Norman, Okla.) that 10^{-3} M exogenous cAMP delayed the elongation of TRIS-EDTA treated cocci. As a result of the initial delay, the fragmentation process was slightly delayed also by exogenous cAMP. Under the same conditions, exogenous cGMP did not produce any observable effects on morphogenesis. Theophylline, a cAMP dependent phosphodiesterase inhibitor, delayed elongation, and fragmentation as well, presumably by increasing the intracellular cAMP concentration (by preventing cAMP destruction resulting from phosphodiesterase activity). Therefore, it was apparent that cAMP was involved in the morphogenesis of A. crystallopoietes. This paper

reports that intracellular and extracellular cAMP levels fluctuated significantly during the transition of cocci to rods. Although this seems to be in contrast to the delay of coccoidal elongation by exogenous cAMP, there are possible explanations for these results. High concentrations of cAMP *in vivo* may inhibit elongation, while an increase followed by a decline in cAMP concentration may trigger elongation. Perhaps release of cAMP from inside the cell is necessary before the elongation process can occur. These are speculations and more evidence is needed to clarify the results. During fragmentation of the rods, increased levels of cAMP were found only extracellularly. The pattern of cAMP levels detected during morphogenesis were different from that found in cells grown as cocci only.

Morphogenic-related changes in cAMP levels were not detected in the *Caulobacter crescentus* morphogenic cycle (13). However, in that system, 3×10^{-3}M dibutyryladenosine 3':5'-cyclic monophosphate was found to stimulate the growth and differentiation of the cells. The contrasts of these two procaryotic systems indicate the diversity of cAMP actions. In the *Arthrobacter crystallopoietes* system, 10^{-3} M (or greater) exogenous cAMP delayed the elongation of cocci, while intracellularly, a rapid increase, followed by an equally rapid decline, in the cAMP levels occurred during coccoidal elongation. It is necessary then to elucidate the mode(s) of action of these diverse effects in relation to procaryotic morphogenesis.

Arthrobacter crystallopoietes will provide a useful system in which to study these actions for the following reasons: one, it has a nutritionally controllable morphogenic cycle which allows non-morphogenic growth and could related morphogenesis-inducing compounds to cAMP

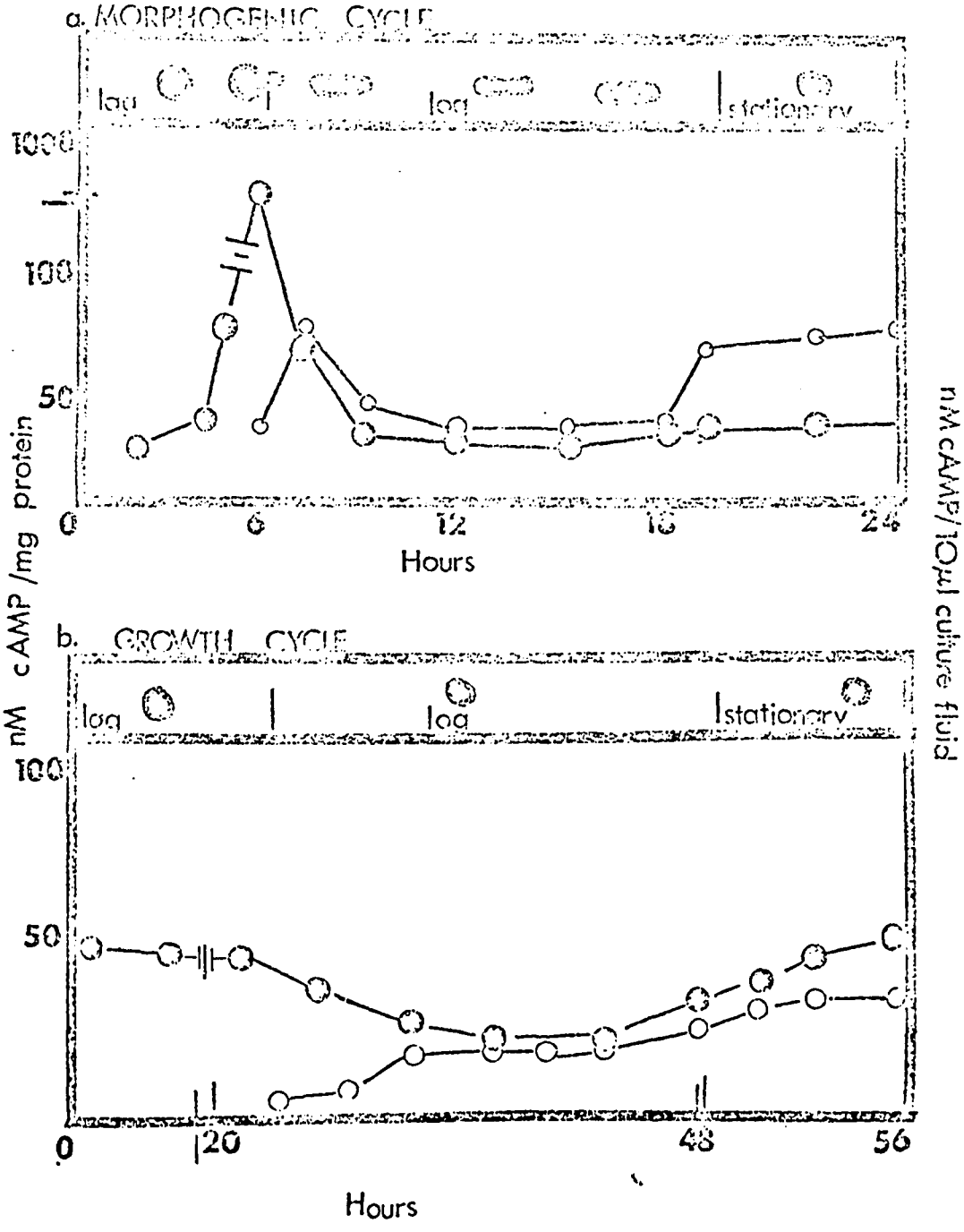
regulation; two, since abrupt changes in cAMP levels occur which are apparently morphogenesis-related, there must exist certain controls or regulation of endogenous and exogenous cAMP levels; and three, there exists a key enzyme active only during rod stage (unpublished data, J. Ferdinandus, Ph.D. thesis, University of Oklahoma, Norman, Okla. 1969), which may or may not be regulated by cAMP.

FIGURE LEGENDS

Fig. 1a. Morphogenic cycle depicted at top. (●———●) Intracellular levels of cAMP during the cycle (nM/mg protein). (o———o) Extracellular levels of cAMP (nM/10 μl culture fluid).

Fig. 1b. Growth stages depicted at top. (●———●) Intracellular cAMP levels (nM/mg protein). (o——— o) Extracellular levels of cAMP (nM/10 μl culture fluid).

Figure 1


a. MORPHOGENIC CYCLE
lag
log
stationary
1000
100
50
0
6
12
18
24
Hours
nM cAMP /mg protein
nM cAMP/10µl culture fluid
b. GROWTH CYCLE
lag
log
stationary
100
50
0
20
48
56
Hours

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PAPER III

REGULATION BY ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE AND GUANOSINE 3',5'-CYCLIC MONOPHOSPHATE OF THE MALIC ENZYME IN *ARTHROBACTER CRYSTALLOPOIETES*, A MORPHOGENIC BACTERIUM

REGULATION BY ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE AND GUANOSINE 3',5'-CYCLIC MONOPHOSPHATE OF THE MALIC ENZYME IN ARTHROBACTER CRYSTALLOPOIETES, A MORPHOGENIC BACTERIUM

ABSTRACT

When L-malate was the varied substrate and NADP was in excess, c-AMP and cGMP competitively inhibited the malic enzyme of a crude extract from rod-shaped Arthrobacter crystallopoietes. As the cyclic nucleotide concentration was increased from 10^{-4}M to 10^{-2}M, the inhibition disappeared. However, when L-malate was at saturation levels, and $NADP^{+}$ was the varied substrate, the competitive inhibition increased as the cyclic nucleotides concentrations increased. Regulation by these compounds of the malic enzyme may play a role in the morphogenic cycle of Arthrobacter crystallopoietes.

INTRODUCTION

Arthrobacter crystallopoietes (A. globiformis) (7) is a bacterium which undergoes morphogenesis under certain nutritional conditions (2). In the presence of a number of seemingly unrelated compounds, coccoidal cells elongate to rods which grow and divide until late in log stage when they fragment, become shorter and eventually revert to cocci. However, the cells can be grown as cocci only when certain other substrates are employed.

The morphogenic cycle of A. crystallopoietes has been characterized by changes in enzyme activities. One enzyme, the malic enzyme, [L-malate: NADP oxidoreductase (decarboxylating): E.C. 1.1.1.40.], was detected only in rod shaped cells, having had the highest specific activity during rod stage and decreased specific activity during early stages of elongation or the later stages of fragmentation. Coccoidal cells which had been in the presence of an inducer (post-reversion cocci) and those grown in a medium which did not induce morphogenesis, failed to exhibit any malic enzyme activity.

Having been found only during one morphologic stage, the malic enzyme could be considered an important enzyme of A. crystallopoietes morphogenesis. This enzyme has been shown to be mainly concerned with the generation of pyruvate and NADPH for lipogenesis occurring when there is an abundance of C_4 compounds (5). During rod formation

lipolysis and gluconeogenesis occur (1, 3). Ferdinandus and Clark (4) reported that free fatty acids inhibited the enzymes involved in lipogenesis (glucose-6-phosphate dehydrogenase, phosphofructokinase, pyruvate kinase, fumarase, lactate dehydrogenase and the malic enzyme) but did not inhibit non-lipogenic enzymes. Apparently, malic enzyme activity and other enzymes of lipogenesis were regulated to some degree at the enzyme-substrate level. The purpose of this investigation was to determine if the malic enzyme could be affected by cAMP at the substrate level, for it has been shown that cAMP was apparently involved in the morphogenic cycle of A. crystallopoietes, albeit the mode of action was unknown (C. Kimberlin-Hariri, Ph.D. thesis, University of Oklahoma, Norman, 1975).

In only one procaryote has cAMP been shown to affect an enzyme at the substrate level (12) rather than at the transcriptional level (6). Sanwal and Smando (12) reported that in Escherichia coli, cAMP competitively inhibits the malic enzyme when malate was the varied substrate and noncompetitively inhibits the enzyme when $NADP^+$ was the varied substrate. They described the malic enzyme as a dispensable one in that it was not essential for cell growth. Control of the E. coli malic enzyme by cAMP was suggested as a protective mechanism of the cell economy during unfavorable circumstances.

This concept of cell economy was applicable to Arthrobacter crystallopoietes as well. The cells may be grown as only cocci and not exhibit malic enzyme activity. When there was ample energy available or by means of some signal, the cells became rod shaped and malic enzyme activity appeared. This paper reports the results of our

investigation of the affects of cAMP and cGMP on the malic enzyme in a crude extract from Arthrobacter crystallopoietes rod shaped cells and the possible function of these effects during morphogenesis.

MATERIALS AND METHODS

Preparation of cell free crude extracts. Arthrobacter crystallopoietes ATCC 15481 (A. globiformis) (7) was grown at 30 C in tryptose, glucose, yeast extract broth (BBL DIFCO), pH 7.0. The cells were synchronized in the morphogenic cycle by transfer of 24 h coccoidal cells (washed x 2 with 0.03M phosphate buffer, pH 7.0) into fresh TGY medium at 24 h intervals. Cells were harvested at rod stage (mid-log stage) by centrifugation at 10,000 x g for 20 min in a Sorvall RC2-B centrifuge at 0 C. Cells were washed twice in 0.05M tris (hydroxymethyl)-amino-methane(TRIS)-chloride (pH 7.6), resuspended in a small volume of the same buffer and disrupted by sonication (Blackstone, Model BP-2) with 10 sec pulses at 4 C for 10-15 minutes. The extract was centrifuged at 30,000 x g for 30 min at 0 C (Sorvall RC2-B) and the supernatant was used for the malic enzyme assay. Extracts were measured promptly for enzyme activity, as malic enzyme was unstable after approximately 12 h at 25 C or even upon storage for the same period of time at 0 C. Protein was determined by the method of Schacterle and Pollack (13).

Assay of malic enzyme. [L-malate: NADP oxidoreductase (decarboxylating): EC 1.1.1.40.] enzyme activity was measured using a modified Ochoa (8) method. The reaction mixture in Beckman 1.0 cm silica cuvettes included 0.3 ml glycylglycine buffer, pH 7.4 (75 μM), 0.06 ml $MnCl_2$ (3.0 μM), varying concentrations of NADP (when saturating, 0.54 μM), varying concentrations of L-malate, (when saturating, 18 μM), enzyme and water to a final volume of 3.0 ml. The assay was carried

out at 25 C. The reaction was initiated by the addition of L-malate, and absorbance readings were measured spectrophotometrically at 340 nm using a Gilford model 2000 recording spectrophotometer.

One enzyme unit was defined as that amount of enzyme causing an increase in absorbance of 0.01/min. Blanks consisted of the reaction mixture minus NADP . Controls included the inactivated enzyme (heated at 100 C for 10 min) and an endogenous control which contained all constituents except L-malate. Neither of these controls displayed absorbance changes when monitored spectrophotometrically at 340 nm during a three min reaction period.

Inhibition studies. cAMP and cGMP was added to the reaction mixture in 0.1 ml volumes to give the final concentrations indicated in the figures. Portions of a crude cell extract, prepared in the previously described manner, were incubated for 12 h at 0 and 25 C in order to inactivate the malic enzyme present. These preparations were added (1:1 v/v) to a fresh crude extract and their activities were compared in the presence and absence of cAMP and cGMP. This was done in order to determine if an intermediate protein may serve as a target of cyclic nucleotide action and consequently affect enzyme activity.

Chemicals. The chemicals used in this study were purchased from the Sigma Chemical Company, St. Louis, Mo.

RESULTS

The data indicate that cyclic AMP competitively inhibited the malic enzyme from crude extracts of rod shaped Arthrobacter crystallopoietes when either L-malate or NADP was used as the varying substrate. As seen in Figures 1a and b, competitive inhibition increased as the cyclic nucleotide concentration decreased when L-malate was the varying substrate and NADP was in excess. Usually, when competitive inhibition has been described, the inhibition has increased in proportion to an increase in inhibitor concentration. This was observed in our system when NADP was the varied substrate and L-malate was in excess; competitive inhibition increased as the cyclic nucleotide concentration increased (Fig. 2a and b). As can be seen from the Lineweaver-Burke plots, there apparently was no cooperativity between substrate or effector and enzyme. The enzyme under all the described test conditions exhibited normal Michaelis-Menten kinetics.

A ratio of cAMP and cGMP were added to the enzyme mixture. The competitive inhibition by the combined nucleotides was not equal to the inhibition which resulted from the same concentration of either cyclic nucleotide alone or that which resulted from the sum of their concentrations (Fig. 1a and b).

The term K_i, a constant value, has been defined as the affinity constant for the binding of the ligand to the enzyme. K_i values were determined for cAMP and cGMP and were found to vary in relation to the concentration of the particular inhibitor instead of remaining constant regardless of inhibitor concentration. When L-malate had served as the varied substrate, the K_i values decreased as the

inhibitor concentration increased. When NADP had been employed as the varied substrate, the K_i values increased in proportion to an increased inhibitor concentration.

Protein extracts from rod cells were prepared which lacked malic enzyme activity as a result of incubation for 18 h at temperatures which destroy malic enzyme activity (-0 and 25 C). When this extract was added to fresh preparation and assayed for malic enzyme activity, no effect on activity resulted when L-malate or NADP was the varying substrate. The protein extract did not alter the effects of the cyclic nucleotides on enzyme activity.

Sanwal and Smando (10, 11) reported that the malic enzyme in Escherichia coli was activated by 0.66 M glycine and that desensitization to many effector molecules occurred in its presence. They suggested glycine may have caused dissociation of the enzyme subunits. The malic enzyme from A. crystallopoietes was not activated by glycine (0.66 M) nor desensitized to regulation by the cyclic nucleotides tested.

DISCUSSION

It had previously been shown by this laboratory that exogenous cAMP delayed coccoidal elongation when added to D-8 h TRIS-EDTA treated Arthrobacter crystallopoietes cells. In addition, we had detected a rapid increase and decline in the intracellular cAMP levels during the coccoidal elongation (pre-rod) stage (C. Kimberlin-Hariri, Ph.D. thesis, University of Oklahoma, Norman, 1975). Ferdinandus (Ph.D. thesis, University of Oklahoma, Norman, 1969) had found that malic enzyme [L-malate: NADP oxidoreductase (decarboxylating); EC 1.1.1.40] was active only in rod shaped cells. We were therefore interested in investigating the affects, if any, by cAMP on malic enzyme activity in vitro. Sanwal and Smando (12) had reported that the malic enzyme in Escherichia coli was the first enzyme reported in the literature to be inhibited by cAMP at the substrate level. Thus, we wondered if control by cAMP at the substrate level occured in A. crystallopoietes as well.

The malic enzyme from crude extracts of rod shaped Arthrobacter crystallopoietes cells exhibited normal Michaelis-Menten kinetics when either L-malate or NADP was used as the varying substrate while the other was in excess. This suggests that cooperativity does not occur between these substrates. When NADP was at a saturating level, cAMP and cGMP competitively inhibited malic enzyme with L-malate as the substrate. However, this inhibition was lost at higher concentrations of these cyclic nucleotides. Competitive inhibition by cAMP of malic enzyme in Escherichia coli was reported by Sanwal and Smando (12). The concentration of inhibitor was in the range of 10^{-4} M cAMP

and as it increased, inhibition of enzyme activity increased, as has usually been reported with competitive inhibition.

There are many dissimilarities between the two enzymes. When L-malate was kept at saturating levels and NADP was varied, the cyclic nucleotides competitively inhibit malic enzyme from Arthrobacter, the inhibition increased as the inhibitor concentration was increased. However, in E. coli, cAMP non-competitively inhibited malic enzyme when NADP was the varying substrate (12). Glycine failed to activate or desensitize the malic enzyme of A. crystallopoietes. Glycine was thought to have had an affect on malic enzyme stability in E. coli (11). There may be other factors, however, which would serve to stabilize or dissociate this enzyme in A. crystallopoietes which could shed light on its obvious physical instability and its unusual activity in the presence of cAMP and cGMP.

Addition of protein extract lacking malic enzyme activity to fresh preparations which were active did not produce any apparent effects on malic enzyme activity, alone or in the presence of cAMP or cGMP. This suggested that an intermediate protein was not serving as the target of cyclic nucleotide regulation and in turn acting on malic enzyme, but did not preclude the possibility that the intermediate itself may have been labile at those temperatures of incubation. Thus denatured, it could have failed to act in competition with either cyclic nucleotides for an effector site or react with the enzyme in the absence of the cyclic nucleotides.

The variations in K_i values could, result from several factors, i.e. (1) the enzyme was contained in a crude extract which may have had

constituents affecting the affinities of inhibitors to the enzyme or (2) simultaneous reactions occuring in the extract could have limited one or a number of compounds which were essential for the malic enzyme activity.

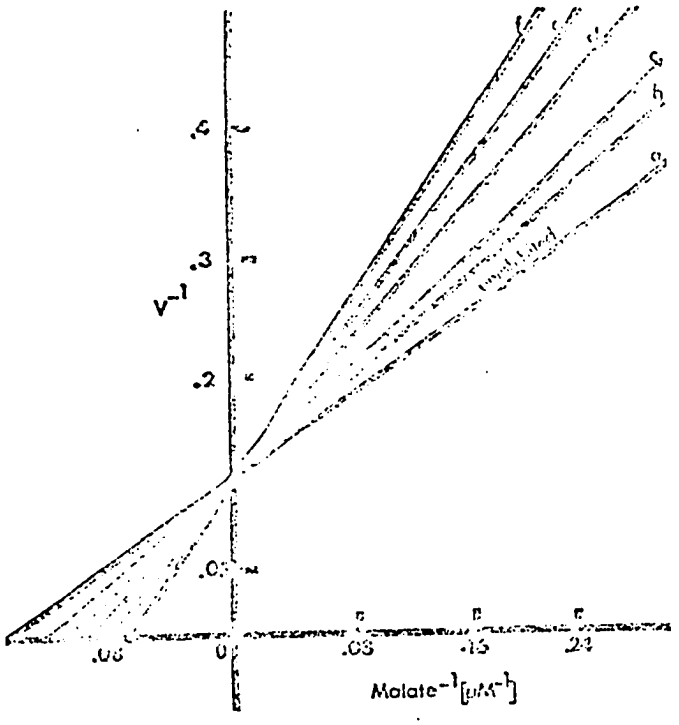
This paper reports an effect on the malic enzyme activity by cAMP and cGMP using crude extracts of rod shaped cells of Arthrobacter crystallopoietes. We were interested in determining if this rod-specific enzyme was affected by cAMP since this cyclic nucleotide was obviously involved in the morphogenic cycle. However, we report that similar effects on malic enzyme activity were seen when cGMP was used as the inhibitor, although this cyclic nucleotide has not been shown, as yet, to be a regulator of morphogenesis in A. crystallopoietes. Further work is necessary to elucidate the mode of action of cAMP and cGMP inhibition of the malic enzyme in this system. Knowledge of the enzyme structure, binding sites, etc., acquired from investigations using a more purified enzyme will certainly help to clarify these matters. Therefore, we have observed the reported effects although their real significance in relation to morphogenesis and its control in Arthrobacter crystallopoietes cannot, at this time, be fully ascertained.

FIGURE LEGENDS

Fig. 1a. Reciprocal plot of velocity versus L-malate concentration, alone and in the presence of various concentrations of cAMP or cAMP + cGMP. (a = 10^{-1} M; b = 10^{-4} M cAMP + 10^{-2} M cGMP; c = 10^{-2} M cAMP; d = 10^{-3} M cAMP; e = 10^{-4} cAMP + 10^{-4} cGMP; f = 10^{-4} cAMP.)

Fig. 1b. Reciprocal plot of velocity versus L-malate concentration alone and in the presence of various concentrations of cGMP, or cGMP + cAMP. (a. 10^{-1} M; b. 10^{-2} M; c. 10^{-2} M cGMP + 10^{-4} M cAMP; d. 10^{-3} M cGMP; e. 10^{-4} M cGMP + 10^{-4} M cAMP; f. 10^{-4} M cGMP.)

a.


.6
.3
V^{-1}
.2
.08
0
.08
.16
.24
Malate^{-1} [μM^{-1}]
f
e
d
c
b
a
unlabeled

b.

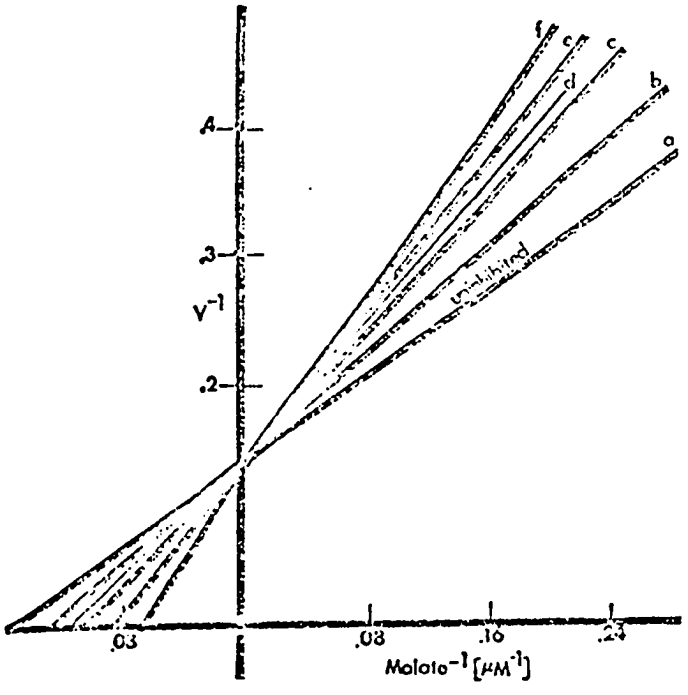
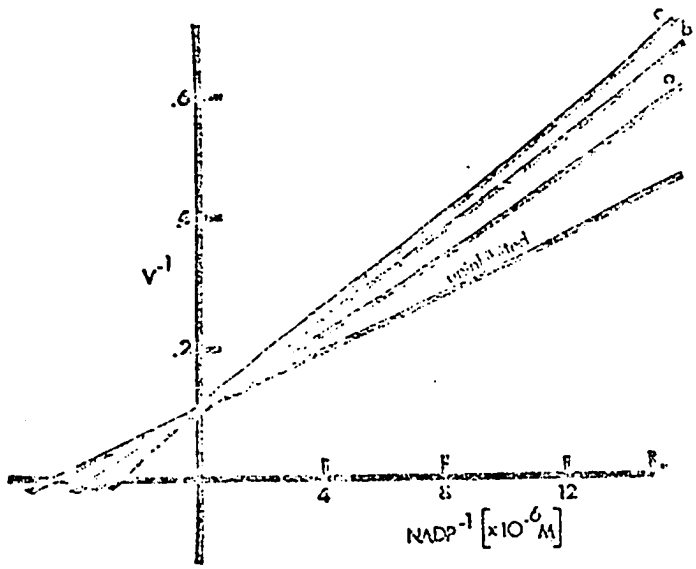

.4
.3
V^{-1}
.2
.03
.08
.16
.24
Malate^{-1} [μM^{-1}]
f
e
d
c
b
a
unlabeled

Figure 1

Fig. 2a. Reciprocal plot of velocity versus NADP concentration alone and in the presence of various concentrations of cAMP. (a = 10^{-4} M cAMP; b = 10^{-3} M cAMP; c = 10^{-2} M cAMP).

Fig. 2b. Reciprocal plot of velocity versus NADP concentration alone and in the presence of various concentrations of cGMP. (a = 10^{-4} M cGMP; b = 10^{-3} M cGMP; c = 10^{-2} M cGMP).

a.


c
b
a
.6
.4
V⁻¹
.2
uninhibited
4
8
12
NADP⁻¹ [x10⁻⁶M]

b.

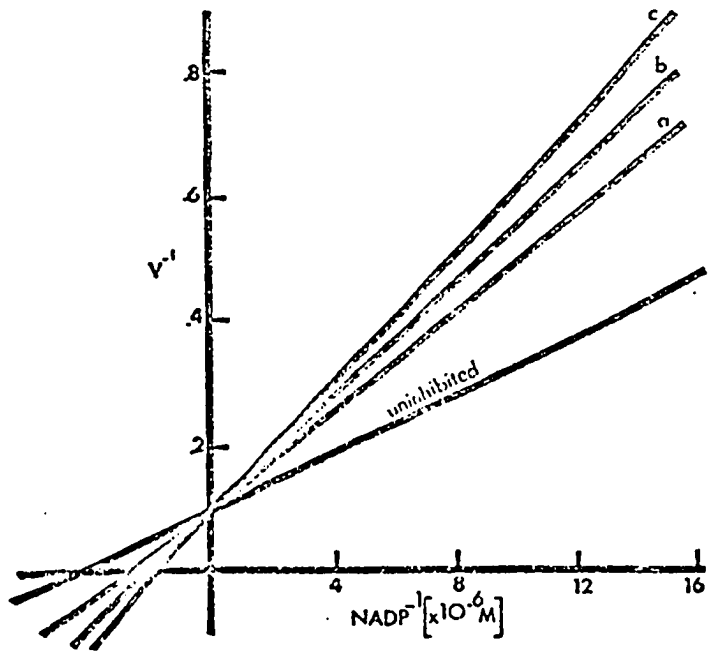

c
b
a
.8
.6
V⁻¹
.4
.2
uninhibited
4
8
12
16
NADP⁻¹ [x10⁻⁶M]

Figure 2

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PAPER IV

ADENYL CYCLASE ACTIVITY AND cAMP-PHOSPHODIESTERASE ACTIVITY, AND ACCUMULATION AND RELEASE OF cAMP FROM MEMBRANES: CONTROL OF ENDOGENOUS cAMP LEVELS DURING MORPHOGENESIS IN *ARTHROBACTER CRYSTALLOPOIETES* (*A. GLOBIFORMIS*)

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ABSTRACT

Coccoidal cells exhibited greater adenyl cyclase activity than rod stage cells. In all cases, adenyl cyclase activity was stimulated by pyruvate but not by glucose, succinate, malate or lactate. Cyclic AMP-phosphodiesterase activity did not vary during the morphogenic or growth cycle. This enzyme activity was found in the soluble fraction only and was inhibited by theophylline but not affected by pyruvate, glucose, malate, succinate or lactate. Coccoidal membranes were able to accumulate more [^{3}H]-cAMP than rod stage membranes. Various effects resulted when glucose, succinate, pyruvate, malate and lactate were added to membrane fractions, to determine their affect on accumulation and release of [^{3}H]-cAMP.

INTRODUCTION

Arthrobacter crystallopoietes (A. globiformis) (9) is a morphogenic bacterium which exhibits distinct structural changes during its life cycle: coccoidal cells elongate to rods which grow and divide until late in log stage when they fragment, shorten and revert to cocci (3). This simple morphogenic cycle can be nutritionally controlled (5). Changes in the intracellular and extracellular cAMP levels have been found to occur during morphogenesis which were different from those measured during growth as cocci (C. Kimberlin-Hariri, Ph.D. thesis, University of Oklahoma, Norman, Oklahoma, 1975). A significant increase in endogenous cAMP has been detected in coccoidal cells elongating to rod forms. Once rods were predominant, the cAMP level returned to the pre-transition coccoidal concentration while the exogenous cAMP level increased. Intracellular cAMP levels remain relatively constant during rod growth, fragmentation and reversion to cocci. Extracellular cAMP appears in increased amounts during and after fragmentation. If cAMP levels changed during morphogenesis, the cyclic nucleotide must have been subject to control or regulation of some kind. Regulation of intracellular cAMP could be accomplished by the action of adenylate cyclase (E.C. 4.6.1.1.), phosphodiesterase (E.C. 3.1.4. D), or by a change in the release of cAMP from the bacteria into the surrounding medium (10,13). Extrusion from the cell may

involve the membrane, of course, and in relation to this, it is possible that cAMP levels could be regulated there. Seto et al. (18) have reported that glucose affects the accumulation and release of [^{3}H]-cAMP in membrane fractions of Escherichia coli. Repression of B-galactosidase in that system was associated with an abrupt decrease in intramembranal cAMP levels as a result of the presence of glucose-6-phosphate. These fractions retained less than 10% of the cellular phosphodiesterase activity.

As previously stated, the morphogenic cycle of Arthrobacter crystallopoietes is at least in part under nutritional control (5). In a glucose minimal salts medium for example, cells grow and divide as cocci only. However, when one of a number of seemingly unrelated compounds was added the cells underwent a simple, but distinct, morphogenic cycle: cocci elongate to form rods, which grow and divide until late in log stage, when they fragment, become shorter and eventually revert to cocci. Several of these compounds were selected for this study in order to determine their effect on adenyl cyclase and phosphodiesterase activities in morphogenic and non-morphogenic A. crystallopoietes cells. Using the membrane fractions, we decided to determine if any of these compounds could cause increased accumulation of cAMP at the membrane or would stimulate release of the cAMP from the membrane. This would offer the cell another means of regulating the intracellular levels of cAMP as well as possibly elucidating nutritional induction of morphogenesis as a membrane associated phenomenon.

The first procaryotic adenyl cyclase was detected in

Brevibacterium liquefaciens by Okabayaski (12) and partially purified by Hirata and Hyashi (7). The enzyme has been shown to be activated by pyruvate but not by glucose (20). Activation of adenyl cyclase by pyruvate has been demonstrated in many bacteria including Arthrobacter globiformis (ATCC 8010) and A. citreus (ATCC 11624) (8). A particulate but readily solubilizable adenyl cyclase was detected in Escherichia coli (1), which showed little regulation except in the presence of high concentrations of effectors (10). At present, therefore, the physiological significance of these effects is unknown. Makman and Sutherland (10) concluded from their studies using chloramphenicol, that increases in cAMP levels in E. coli were not dependent on de novo synthesis of adenyl cyclase. Peterkofsky and Gazdar (14, 15) concurred that variations in cAMP levels in E. coli were not dependent on the de novo synthesis of either adenyl cyclase or phosphodiesterase. They also reported that glucose did not inhibit adenyl cyclase activity.

A soluble cAMP-phosphodiesterase in E. coli was first reported by Brana and Chytil (2). Later, the enzyme was purified from Serratia marcescens and found to be 3'5'-cyclic nucleotide specific and theophylline sensitive (12). Ide (8) reported that soluble c-AMP-phosphodiesterase was found in several bacteria including A. globiformis (ATCC 8010) and A. citreus (ATCC 11624) but none was found in several species of Bacillus and in several strains of E. coli. However, Clark and Bernlohr (4) reported that in Bacillus licheniformis, membranal cAMP-phosphodiesterase and adenyl cyclase are present, although their specific activities did not change in relation to intracellular levels of cAMP changing.

Conflicting reports as to whether these enzymes really regulate intracellular cAMP levels exist, and very little has been done to study their activities in procaryotic morphogenic systems. For example, E. coli AB257^{PC-1}, a mutant lacking phosphodiesterase activity, contained twice as much intracellular cAMP as the wild type, AB257 (6). However, when starved on a poor carbon source, both strains showed equal increases in their cAMP concentrations. Rickenberg (16) concluded from this that the cAMP levels were not regulated by the phosphodiesterase. It has been observed that adenyl cyclase activity varies during the life cycle of Caulobacter crescentus, a morphogenic bacterium (19), although intracellular changes in the cAMP levels die not occur in correlation with morphogenesis.

This paper reports the results of adenyl cyclase and phosphodiesterase activities assayed at various stages of the morphogenic cycle of A. crystallopoietes and in the presence of certain effector molecules. In addition, we have studied the ability of membrane fractions to accumulate and release [^{3}H]cAMP alone and in the presence of these effectors.

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MATERIALS AND METHODS

Organism and growth conditions. Arthrobacter crystallopoietes ATCC 15481 (A. globiformis) (9) cells were synchronized in their morphogenic cycle by transfer of 24 h coccoidal cells into fresh Plate Count broth (DIFCO) in which the morphogenic cycle occurs. Cells were transferred at 48 h intervals into 0.5% glucose minimal salts (GMS) medium (5) in which the cells grow as cocci only. Incubation was aerobic at 30 C in a New Brunswick Controlled Environment Incubator Shaker at 225 RPM. From the plate count broth, cells were harvested during the following morphological stages (as determined by 0.1% methylene blue slide preparations): early cocci, elongating cocci, mid-log rods, fragmenting rods and reversion-cocci. From GMS medium, mid-log cocci and stationary phase cocci were harvested. Coccoidal cells from GMS medium were considered to be non-induced to undergo morphogenesis, although capable of doing so. To insure this, an inoculum was removed and placed in plate count broth which induced the cells to elongate and undergo the morphogenic cycle. Coccoidal cells harvested from plate count broth were considered to be morphogenically induced even though morphologically they were still coccoidal.

Preparation of extracts and membrane samples for enzymatic analysis. Cells, washed x 2 in 50 mM Tris-HCl buffer pH 9.0, were resuspended in 2 vol of the same buffer containing 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 10 μg/ml deoxyribonuclease. This cell suspension was disrupted by sonication (Blackstone, Model BP-2) using 10 second pulses with 30 second rests at 0-4 C. Extracts were centrifuged at

30,000 x g for 30 min at 0°C in a Sorvall RC2-B centrifuge. The resultant supernatant and the precipitate (resuspended in 1 vol same buffer) were assayed for adenyl cyclase and phosphodiesterase activity. Samples could be stored at -0 C without loss of activity.

Adenyl cyclase activity. The assay method of Ide (8) was used with a few modifications. Enzyme activity was measured by determining the radioactivity of the cAMP-[^{14}C] formed from ATP-[^{14}C]. The product was separated by chromatography and the spot was identified by the following means: co-chromatography of cAMP-[^{3}H]; and selecting one of the triplicate samples for treatment and destruction by cAMP-phosphodiesterase after the adenyl cyclase reaction occurred. Heated enzyme controls were also assayed and found to produce no labeled product at this location on the chromatograph.

The standard reaction mixture contained the following in a total volume of 0.05 ml: 50mM TRIS-HCl, pH 9.0; 2.5 mM ATP-[^{14}C] (4 μCi/μM); 30 mM $MgCl_2$; 5mM 2-mercaptoethanol; and 0.02 ml of enzyme preparation (200 μg protein). Concentration of effector compounds when added were 30 mM. Controls consisted of heated enzyme preparations (100 C, 10 min). The reaction was carried out at 30 C for 30 min and was terminated by adding 40 μl of 40% cold trichloroacetic acid, containing 10 mM [^{3}H]cAMP as a marker compound. This mixture was centrifuged (5,000 x g, Sorvall, Model GLC-1) and 20 μl of the supernatant was spotted on Whatman No. 3 MM filter paper (40 x 40 cm) and chromatographed using a solvent consisting of isopropyl alcohol, ammonia and water (7:1:2 v/v) (1, 8). The spot corresponding to the [^{3}H]-cAMP control was cut out (5 cm^2), placed in a vial, dried in an air stream,

resuspended in toluene based fluor, and its radioactivity was determined by using a Beckman DPM-100 liquid-scintillation counter.

Assay of cAMP-phosphodiesterase. Degradation of [^{3}H]-cAMP was measured to determine phosphodiesterase activity. The assay mixture contained in a total volume of 0.05 ml included: 50 mM Tris-HCl, pH 9.0; 0.6 mM ^{3}HcAMP (37.7 Ci/mM); 30 mM $MgCl_2$; 5 mM 2-mercaptoethanol; and 0.02 ml enzyme preparation (200 μg protein). The reaction was carried out at 30 C for 30 min and terminated with the addition of 20 μl cold 40% (w/v) trichloroacetic acid. The mixture was centrifuged (5,000 x g, 5 min, Sorvall, Model GLC-1) and 20 μl of the supernatant was applied to a Whatman No. 3 MM filter paper (40 x 40 cm), and chromatographed using the same solvent used in the adenyl cyclase assay. The spot corresponding to [^{3}H] cAMP was cut out and measured for its decrease in radioactivity. Controls consisted of heated enzyme preparations (100 C, 10 min) which showed relatively no decrease in cpm as compared to the [^{3}H] cAMP standard used. This reading was used as the 100% value to determine the amount of decrease of ^{3}HcAMP. In addition, a standard was assayed containing 200 μg/.05 ml cAMP-phosphodiesterase (Sigma Chem. Co.) and 0.6 mM [^{3}H]-cAMP and chromatographed under test conditions. Effectors employed in this study included: 10^{-2} M theophylline, 30 mM of either glucose, pyruvate, malate, lactate or succinate.

All chromatograms were developed using an ascending method for 16 h at 25 C. Chromatograms were then dried overnight before being cut into assay samples (approximately 5 x 5 cm) and placed in vials. Protein was measured using the method of Schacterle and Pollack (15).

Preparation of the membrane systems. Membrane fractions of the various stages of A. crystallopoietes cells were prepared with a modified method of Maruo et al. (11), the description of which follows. Cells were harvested by centrifugation (8.000 x g, 15 min, 4 C, using a Sorval RC2-B centrifuge). Cells were resuspended in 0.025 M Tris-HCl buffer, pH 8.0, containing 400 μg/ml ethylenediaminetetracetate (EDTA), 25 μg/ml lysozyme, 0.05 M $MgCl_2$ and incubated at 30 C for 30 min with gentle shaking. The suspension was centrifuged at 20,000 x g, 15 min, 0 C, and the pellet was resuspended in ice cold 0.05 M Tris-HCl, pH 7.6, containing 0.05 M $MgCl_2$. The suspension was sonicated very gently at 0.4 C with 5 sec pulses and 30 sec rests using a Blackstone Model BP-2 sonicator, and centrifuged (15,000 x g, 20 min, 0-4 C). The pellet was resuspended in 0.05 M Tris-HCl, pH 7.6, containing 0.005 M $MgCl_2$, diluted to a standard protein concentration of 100 μg/0.1 ml and considered as the membrane system. Protein was measured by the method of Schacterle and Pollack (17).

Accumulation of [^{3}H] cAMP. Accumulation of [^{3}H]-cAMP in the membrane fraction was measured by the Millipore filtration method (18). One milititer of the reaction mixture included: 0.5 ml membrane fraction (500 μg protein); 0.2 mM potassium phosphate buffer, pH 7.0; 10 μM $MgCl_2$; [^{3}H]-cAMP (2 x 10^{-6}M, 37.7 Ci/mM); and in some cases, 30 mM of either glucose, succinate, malate, lactate or pyruvate. The reaction was carried out at 30 C, 30 min and terminated with ice cold 0.1 M potassium phosphate buffer, pH 7.0. The diluted samples were placed on ice, filtered as soon as possible with membrane filters (HAWP 0.45 μm, Millipore Filter Co., Bedford, Mass.) and washed with

an equal volume of cold buffer. The filters were placed in vials, dried in an air stream, and radioactivity was determined by scintillation counting in a toluene-based scintillant using a Beckman DPM-100 liquid scintillation counting system.

Release of [^{3}H]-cAMP retained in the membranes. The membrane fractions were incubated at 30 C for 30 min in the presence of 2 x 10^{-6}M [^{3}H]-cAMP (37.7 Ci/mM). After this period, they received either 0.2 mM phosphate buffer pH 7.0, or 30 mM of either glucose, succinate, malate, lactate or pyruvate. The reaction mixture was contained in a total volume of 1.0 ml, and included: 0.5 ml membrane fraction (500 µg protein) preincubated with [^{3}H]-cAMP as mentioned; 0.2 mM phosphate buffer, pH 7.0; 10 µmol $MgCl_2$ and in some cases 30 mM of the previously listed carbon compounds. This mixture was incubated for 30 min at 30 C, the reaction was terminated and membranes were collected as previously described.

Chemicals. All chemicals were purchased from the Sigma Chemical Company, St. Louis, Mo. Radioactive materials ATP-^{14}C (52.8m Ci/mM) and cAMP-[^{3}H] (37.7 Ci/m mole) were purchased from New England Nuclear, Boston, Mass.

RESULTS

Formation of cAMP from ATP. The separation of cAMP from ATP occurred with good resolution in the solvent system used in this study: the R_F value of cAMP was 0.45 while the R_F value of ATP was 0.10. Samples treated with phosphodiesterase after the adenyl cyclase reaction had occurred and before termination with cold TCA showed no ^{14}C-labeled product at the spot corresponding to the chromatographed [^3H]-cAMP marker. Since the same amount of protein/reaction mixture was used in all samples and the same amount of ATP-^{14}C was added to all, the difference in cpm/30 min reaction time was used to estimate enzyme activity. A comparison of adenyl cyclase activity of various stages of the morphogenic and growth cycle is shown in Table 1. All stages involving coccoidal cells were more active than rod stages. Pyruvate greatly stimulated adenyl cyclase activity of all the fractions showing activity. Little or no affect on enzyme activity was exhibited in the presence of 30mM of glucose, succinate, malate, or lactate.

Degradation of cAMP by phosphodiesterase. Phosphodiesterase activity was determined by calculating the % decrease in cpm as compared to cpm of a standard amount of [^3H]-cAMP which had been reacted with heat inactivated enzyme. The same amount of protein and [^3H] cAMP was added to all reaction mixtures, and the % decrease in radioactivity of the [^3H]-cAMP was determined for each morphological and growth stage. Results are shown in Table II. Activity was found in the supernatant fractions only. There was no remarkable change in activity throughout the morphogenic or growth cycle. The enzyme was inhibited by 10^{-2} M

theophylline but no effect was seen on enzyme activity when 30 mM of pyruvate, glucose, malate, succinate or lactate was added.

Accumulation of ^{3}HcAMP in membranal systems. Coccoidal membranes from A. crystallopoietes cells accumulated approximately the same amount of [^{3}H]-cAMP per μg protein regardless of their growth or morphological stage. Cocci which had been in presence of inducing agents or had undergone morphogenesis accumulated slightly less than non-morphogenic cocci. (Table 3). This was in marked contrast to the small amount of [^{3}H]-cAMP accumulated by the rod membranes. Glucose stimulated accumulation of the cyclic nucleotide slightly in coccoidal cells but by a factor of four in rod cells. Malate had little or no effect. Succinate stimulated the accumulation of cAMP in the rods (x7), had little effect on induced-reversion cocci, and slightly lowered that in the non-morphogenic cocci. Lactate lowered [^{3}H]cAMP accumulation in the non-morphogenic cocci. Pyruvate lowered accumulated cAMP in coccoidal cells but slightly raised the level achieved in the rods. These results were consistent after 10 min and 30 min incubation periods.

Release of [^{3}H]cAMP from membranal systems. When membranes were incubated for two 30 min periods at 30 C in the presence of [^{3}H] cAMP diverse effects resulted. Cocci which had been induced to undergo morphogenesis or those which had just reverted from fragmenting rods spontaneously released more than 50% of their accumulated [^{3}H]cAMP. Glucose and succinate prevented this loss and actually stimulated greater accumulation, while the other compounds showed no effect. Rod membranes however lost less [^{3}H]cAMP upon continued incubation. Again

glucose and succinate and in addition malate, tended to stimulate accumulation while lactate and pyruvate caused release of the cyclic nucleotide. In the non-induced coccoidal membranes large amounts of [^{3}H]-cAMP accumulated during the second incubation period. Incubation with glucose, succinate and malate resulted in some release from the membrane, while lactose and pyruvate resulted in almost complete release from the membrane of the total amount of accumulated [^{3}H] cAMP. No release from the membrane could be detected until at least 10 min incubation had occurred with maximum release measured after 20 min.

DISCUSSION

During the morphogenic cycle of Arthrobacter crystallopoietes, changes were found to occur in the intracellular and extracellular cAMP levels which did not occur during growth as only cocci (C. Kimberlin-Hariri, Ph.D. thesis, University of Oklahoma, Norman, 1975). As coccoidal cells elongated, endogenous cAMP levels increased from 50 nM to 800 nM/mg protein. When cells were rod forms, the cAMP level had returned slightly less than 50 nM and remained constant throughout fragmentation and reversion to cocci. We had previously shown that exogenous cAMP can delay elongation of cocci. Apparently, increased intracellular cAMP levels inhibit elongation while the rapid decline in the level may trigger elongation to occur. Further investigations are needed to substantiate this possibility. Extracellular cAMP was increased in the medium just after elongation and again during and after fragmentation. Intracellular cAMP levels could be regulated by adenyl cyclase, phosphodiesterase or by release of the cAMP into the medium. This paper reports the results of a comparative study of adenyl cyclase and phosphodiesterase activity in membrane and soluble fractions of cells from various morphogenic and growth stages.

Adenyl cyclase activity had previously been detected in stationary cocci of two Arthrobacter species by Ide (8). A. globiformis ATCC 8010 exhibited adenyl cyclase activity in the supernatant and precipitate fractions while A. citreus ATCC 11624 showed activity in the precipitate only in the presence of pyruvate. Pyruvate activated the adenyl cyclase activity of both fractions from A. globiformis ATCC 8010. As seen in Table 1, Arthrobacter crystallopoietes ATCC 15481

exhibited adenyl cyclase activity in both particulate and soluble fractions when the cells were coccoidal. However, during rod stages adenyl cyclase activity was greatly diminished and found only in the membrane samples. The enzyme in all fractions was greatly activated by the presence of 30 mM pyruvate. The same concentration of glucose, malate, succinate, or lactate did not affect enzyme activity as assayed. Since the activity of adenyl cyclase was measured by the amount of cAMP formed, the decrease in activity seen at rod stage could have been produced by phosphodiesterase in the preparation. However this appears unlikely as rod membranes showed little or no phosphodiesterase activity.

Cyclic AMP-phosphodiesterase activity was detected in all stages of cells but only in the soluble fractions. No significant change in activity occurred as a function of growth or morphogenic stage. This enzyme was sensitive to theophylline in vitro. Earlier studies had shown that exogenous theophylline caused a delay of the normal morphogenic cycle at the elongation and fragmentation stages. (C. Kimberlin-Hariri, Ph.D. thesis, University of Oklahoma, Norman, 1975). This suggests that, although the activity of the cAMP phosphodiesterase does not change, it may play a role in regulating intracellular cAMP levels which in turn can affect the morphogenic cycle.

No definite pattern of accumulation of cAMP in membranes and release of cAMP from membranes at various stages during the life cycle of Arthrobacter crystallopoietes was observed. Rod membranes did not accumulate as great an amount of [^{3}H]-cAMP/μg protein as coccoidal membranes accumulated. In membranes collected during all stages of morphogenesis, glucose, succinate and malate either stimulated or had

no effect on accumulation of intramembranal cAMP concentration. In the non-induced coccoidal membranes, these compounds maintained the concentration within a certain range.

Release of cAMP from the membrane was especially observable in membranes of non-induced coccoidal cells in the presence of all substrates tested. Release of cAMP from the membranes of induced cocci and rods was not affected greatly by the presence of the substrates.

Although no definite pattern had been established in relation to the non-inducing compounds, glucose and pyruvate, and the inducing compound malate, succinate and lactate, it was obvious that the control of intracellular and intramembranal levels of cAMP may depend on more than the adenyl cyclase and phosphodiesterase activities. These membranal fractions were found not to contain phosphodiesterase activity; therefore, we assume that release from the membrane was a result of some other mechanism. Heated membrane controls accumulated little or none of the [^{3}H] c AMP (20-50cpm/500 μg) which suggests this action with cAMP was not mere adsorption although it did not completely rule out non-specific adsorption on the intact membrane. During incubation, the membranes are capable of enzymatic activity (18) and thus many reactions could be occurring in the presence of these various substrates which could involve or effect the intramembranal cAMP level. Much more information is needed on the metabolic role of these compounds, their appearance in the cycle, and resulting morphogenic changes which may occur. However, it is obvious that the membrane is a candidate for a regulator of intracellular cAMP levels in addition to the other known mechanisms.

It is difficult to ascribe the exact regulatory roles of the adenyl cyclase and cAMP-phosphodiesterase in the morphogenic cycle of Arthrobacter crystallopoietes. We can only report what is known to date and suggest possible actions. A summary of this information on cAMP levels and the enzymes or actions which could regulate them is shown in Table III. It was hoped that a correlation could be found between adenyl cyclase activity and the presence of compounds which can induce morphogenesis (succinate, malate, lactate) or the presence of those compounds which support growth of the cells as cocci only (glucose, pyruvate). However the only effect seen was a great stimulation of adenyl cyclase activity by pyruvate. The significance of this at present is undetermined. More knowledge of the metabolic role of pyruvate as a key intermediate metabolite during morphogenesis and its effect on other enzymes would perhaps elucidate its physiological role as an activator of adenyl cyclase.

TABLE I

Adenyl cyclase activity in membrane and supernatant fractions of cells from various stages during growth and morphogenesis in A. crystallopoietes

Arthrobacter crystallopoietes Morphogenic - or- Growth Stage		Adenyl cyclase activity cpm		cAMP/30 min/mg protein	
		Membrane Fraction		cpm x 10^3 Supernatant fraction	
			+ Pyruvate		+ Pyruvate
Induced Cocci		50	250	50	250
Elongating Cocci		50	250	50	250
Rods (Mid-log)		8	30	0	0
Fragmenting Rods		2	10	0.8	0.8
Reversion Cocci		40	250	60	300
Non-induced, mid-log cocci		50	250	50	200
Non-induced, stationary cocci		50	200	60	250

TABLE II

cAMP-phosphodiesterase activity during various stages of growth and morphogenesis in A. crystallopoietes

A. crystallopoietes Morphogenic or Growth stages		cAMP-phosphodiesterase activity % decrease cpm/30 min reaction/mg protein		
		Membranes	Supernatant	10^{-2} M Theophylline
Induced cocci		0-1%	90%	5%
Elongating cocci		0-2%	87%	4%
Rods		0-1%	92%	5%
Fragmenting rods		0-1%	89%	1%
Reversion cocci		0-2%	85%	3%
Non Induced cocci (mid-log)		0-3%	90%	5%
Non Induced cocci (Stationary)		0-2%	90%	5%
Control		97% reduction in cpm[^{3}H]cAMP		7%

TABLE III

Amounts of [^{3}H]-cAMP accumulated per 500 μg membrane protein/30 min at various morphogenic and growth stages, alone, and in presence of 30 mM of substrates listed

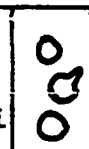
		Accumulated [^{3}H]-cAMP in protein-membrane fraction (cpm x 10^3/500 μg protein) reacted 30 min, 30 C					
		Membrane Alone	30mM Glucose	30mM Malate	30mM Succinate	30mM Lactate	30mM Pyruvate
Induced cocci Reversion cocci		11-12	15	14	14.5	12	8.5
Rods		1.4-1.5	4	1.5	7	0.4	2
Non-induced cocci All stages		15-20	17	15	12	7	2

TABLE IV

Amount of [^{3}H]-cAMP present in the membranes (which had been preincubated 30 min with [^{3}H]cAMP), after 30 min added incubation alone and in the presence of the effectors listed

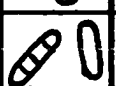
		Release of [^{3}H]-cAMP from membranes preincubated 30 min — reacted 30 min in presence of [^{3}H]-cAMP: (cpm x 10^3/500 μg protein)					
		Membrane alone	30mM Glucose	30mM Malate	30mM Succinate	30mM Lactate	30mM Pyruvate
Induced & Reversion cocci		4.0	40	4.7	3.0	4.0	4.0
Rods		1.0	13	10	8.0	0.3	0.3
Noninduced cocci all stages		30	12	12	7.0	3.0	3.0

TABLE V

Summary of data showing cAMP-related

effects during morphogenesis in A. crystallopoietes

Arthrobacter crystallopoietes morphogenic cycle					
Inhibition of morphogenesis by Exogenous cAMP In Vivo	X	X	—	—	—
Increased endogenous cAMP In Vitro	—	X	—	—	—
Adenyl cyclase activity, In Vitro	X	X	—	—	X
Extrusion of cAMP from cell, In Vivo	—	—	X— early	—X late	X
cAMP-phosphodiesterase activity In Vitro	X	X	X	X	X
Delayed morphogenesis by theophylline In Vivo	X	X	—	X	X
Membranes accumulate large amounts of cAMP In Vitro	X	X	—	—	X

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SUMMARY

The study of morphogenesis is an attempt to discover, describe and explain a sequence of morphological transitions which are accompanied by biochemical and metabolic changes. Arthrobacter crystallopoietes is a good system with which this type of investigation can be performed for these reasons: (1) it has a simple morphogenic cycle; (2) it can be nutritionally induced to undergo morphogenesis or be grown as a non-morphogenic system which provides a controlled situation for the comparison of aging phenomena versus morphogenic phenomena and (3) changes in metabolism occur which include the establishment of varying enzymatic patterns obviously involving regulation at some level, either gene or substrate or both.

The research reported in this dissertation is the result of questions which were asked in relation to the regulation of morphogenesis in Arthrobacter crystallopoietes: (1) is cAMP, which is known to be an important regulatory molecule in animal, plant, and bacterial cells, involved in the morphogenic cycle of A. crystallopoietes? (2) if it is involved, how does cAMP exert its regulatory effects? (3) do cAMP levels during morphogenesis fluctuate or remain constant? (4) how are cAMP levels regulated by the cells and finally, (5) how does this system of morphogenesis compare to others in relation to regulation by cAMP?

We have shown that exogenous cAMP, when added to cells treated to make them more permeable to the cyclic nucleotide, can delay a morphogenic event, i.e., the elongation of cocci. This delay is time dependent (0-8 hr) dosage dependent (greater than 1mM cAMP), and apparently cAMP specific since cGMP produced no similar delay. How does this delay relate to the peak of cAMP measured intracellularly? Although it is speculative, we propose that if, after the cAMP level peaked, it did not decline, then perhaps rod formation would not occur. What controls the decrease in the cAMP level at this time? Although no change in the cAMP-dependent-phosphodiesterase specific activity occurred as a function of morphologic changes, when this enzyme was inhibited in vivo by theophylline, rod formation did not occur. We presume this resulted from high cAMP concentrations due to phosphodiesterase inhibition. The cell also decreases its intracellular cAMP level by regulating its flow out of the cell into the medium at this time. In addition, once cells become rod stage, they: (a) have less adenyl cyclase activity, (b) fail to accumulate cAMP at the membrane and (c) are still capable of cAMP destruction by phosphodiesterase or are capable of extruding cAMP into the medium. These events all produce the condition of low intracellular cAMP.

One may ask, why then are intracellular levels at this time comparable to that found in non-morphogenic cocci? One possible explanation is that cAMP levels are high in these non-morphogenic cocci but were undetected by our methods due to the enhanced binding of cAMP to these coccoidal membranes. Another explanation is that actually a

significant change in cAMP levels triggers morphogenic events and not simply particular levels of the cyclic nucleotide.

There is a slight increase in extracellular cAMP at the fragmentation stage which is comparable but not equal to the increase seen at late log and stationary stages during non-morphogenic growth. However, since theophylline delayed fragmentation, we suspect that cAMP is involved in regulating this stage albeit we can not fully explain it at this time. We feel that clarification of the malic enzyme work using a more purified preparation may produce results which will better fit the pattern of data thus far accumulated. However, it is significant that this rod-stage specific enzyme was shown to be affected by cAMP at the substrate level.

Out findings are significant, as compared to those resulting from studies of cAMP in procaryotic morphogenic systems, for the following reasons:

1. we have shown, a somewhat infrequently observed, negative effect on initiation of morphogenesis;
2. we have shown changes in intracellular cAMP levels and adenyl cyclase activity which appears to be a function of morphogenesis and not aging and,
3. our data suggests that regulation by cAMP occurs at both gene and substrate levels.

It is unfortunate that no pattern of association evolved between morphogenesis-inducing agents and cAMP regulation. This nutritional control, along with numerous other areas, are still to be explained. The answers, which accumulate from investigations of the

regulation of morphogenesis in Arthrobacters, will perhaps apply to other basic biological systems as well.