CHARACTERIZATION AND LOCALIZATION OF ADENYLATE CYCLASE DURING DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

by

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ABBREVIATIONS

DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
EGTA	-	Ethyleneglycol-bis (β-amino-ethyl ether) N, N' tetraacetic acid
Gpp(NH)p	-	Guanyl-5'-yl imidodiphosphate
RIA	-	Radioimmunoassay
ScAMP-TME	-	Succinyl cyclic adenosine monophosphate-tyrosine methyl ester
TCA	-	Trichloroacetic acid
Tris	-	Tris (hydroxymethyl) aminomethane

1. INTRODUCTION

Development has been defined as the "regulation of the compartmentalization of specific molecules in the cells" of the organism (Ebert and Okada 1979). One of the fundamental processes of development is pattern formation, or the mechanisms by which cells are correctly ordered in space. The result is cellular expression of this determined status by differentiation into the appropriate cell type (Ashworth 1973). Differentiation involves the appearance of biochemical and structural properties unique to a particular cell type. The central questions of pattern formation and differentiation are embodied in how the cells are provided with information as to their position within an organism, and how the cells interpret this information in order to take the correct developmental pathways (Deak 1981).

The answers to these questions may help to elucidate the regulatory course of differentiating cell processes including embryogenesis, senescence and neoplastic transformation. Since many developmental phenomena are not easily probed in complex multicellular organisms, it is desirable to be able to study them in a simpler, model system.

The cellular slime mold, Dictyostelium discoideum, provides such a model system for the study of developmental processes. Development commences approximately seven hours after the identical vegetative amoebae are deprived of a food source. The cells aggregate in response to the chemotactic agent cyclic AMP (Konijn et al. 1968) by streaming radially towards central collecting points and forming groups of up to 10^5 cells (Loomis 1975). At the completion of aggregation the cell

mass, integrated by a slime sheath and now called a slug, begins a period of migration. Evidence from biochemical (Brenner 1977), histochemical (Bonner 1971, Farnsworth and Loomis 1974), and morphological studies (Gregg and Badman 1970) shows that the anterior third of the slug is composed of presumptive stalk cells whereas the posterior two-thirds is composed of presumptive spore cells. Differentiation of the two cell types occurs at the culmination stage which takes place approximately 21 hours into the developmental cycle. During culmination the pre-stalk cells migrate up toward the apical tip and then down through the pre-spore cells into a central cellulose sheath. The stalk cells develop rigid cell walls and become vacuolated (Raper and Fennell 1952). Differentiation of the spore cells involves formation of a protective spore coat (Hohl and Hamamoto 1969). Migration of the pre-stalk cells into the stalk sheath lifts the pre-spore cell mass off the substrate to its final apical position (Bonner 1971). Only the spore cells are capable of germinating and reinitiating the life cycle; the stalk cells show aging effects and eventually die. Thus, identical precursor cells may enter one of two alternate differentiation routes. Although the initiation of development, the aggregation process, has been extensively studied, less attention has been focused on the regulation of the terminal differentiation steps.

There are several lines of evidence that suggest a role for cyclic AMP in the developmental processes that occur subsequent to aggregation. For example, periodic movements similar to those of amoebae undergoing chemotaxis are observed during culmination in the pre-stalk region

(Durston et al. 1976). Matsukuma and Durston (1979) showed that from mounds containing disaggregated presumptive stalk and spore cells the former type will sort out by moving towards an external source of cyclic AMP. Depending upon the conditions undifferentiated cells can be induced by cyclic AMP to become stalk-like cells (Bonner 1970) or spore cells (Kay 1979). Feit et al. (1978) found that implantation of cyclic AMP-soaked Sephadex particles into slugs which were conditioned to suppress differentiation caused the induction of clusters of stalk cells and spore cells.

There is also direct evidence that cyclic AMP affects gene expression after the aggregation process. A peak of gene activity, detected by in vivo labeling or by translation of stage-specific mRNA, was found to occur concurrently with tip formation of tight aggregates (Alton and Lodish 1977). It was discovered that the continued synthesis of these developmentally regulated proteins was dependent upon continued cell contact: If the aggregates were disrupted synthesis was arrested. However, Landfear and Lodish (1980) reported that maintenance of synthesis of the developmentally controlled proteins was possible in disaggregated cells if exogenous cyclic AMP was supplied to the cells. Their results indicate that cyclic AMP acts at the level of the messenger RNA although the precise control point of the effect has not been determined. Thus cyclic AMP seems to be involved in these later steps of morphogenesis.

Cyclic AMP serves an important first messenger role in Dictyostelium discoideum, analogous to hormones in mammalian systems (Konijn 1972).

It follows that the cyclic AMP synthetic enzyme, adenylate cyclase, in conjunction with the degradative enzyme, cyclic AMP phosphodiesterase, must be regulated so as to maintain levels of cyclic AMP which are appropriate as a morphogen. Dinauer et al. (1980) suggest that adenylate cyclase activity is the main determinant of cyclic AMP secretion in the signalling response of amoebae. Dictyostelium adenylate cyclase has been characterized to some extent, with these studies concentrating on the enzyme at the aggregation stage. The reports are contradictory. Rossomando and Sussman (1973) reported the existence of a soluble enzyme that could be activated by 5'AMP. However, preparation of plasma membranes with amphotericin B resulted in a membrane-bound activity that could not be stimulated by 5'AMP (Rossomando and Cutler 1975). Klein (1976) reported that in the presence of comparable amounts of 5'AMP adenylate cyclase activity was inhibited. She points out that this may represent a means of regulating the enzyme. Rossomando and Sussman (1973) observed that enzyme activity was maximal at 37°C and would tolerate a 20 minute preincubation at this temperature. Klein (1976), on the other hand, noted an activity that was maximal at 27°C and thermolabile. Klein (1976) ascribes these discrepancies to be possibly due to differences in assay conditions.

Although adenylate cyclase has resisted purification due to its instability, studies on its modulation have been carried out using living cells. Cell suspensions that were stimulated by a constant level of cyclic AMP showed a transient increase in adenylate cyclase

activity which peaked sharply within one minute then fell to nearly basal (pre-stimulus) levels within four minutes (Dinauer et al. 1980).

In contrast to the situation in Dictyostelium discoideum, a great abundance of data is available on the adenylate cyclase in other systems, particularly the mammalian, hormone-sensitive enzyme. A recent review by Ross and Gilman (1980) summarizes the most pertinent data. Three protein components comprise the system. One of these is the catalytic unit (C) with a molecular weight of 190,000. By itself it cannot be stimulated by hormones, fluoride or guanine nucleotides. It exhibits maximal activity with Mn·ATP serving as substrate; with Mg·ATP less than 10% of the maximal activity is observed. A regulatory protein, the guanine nucleotide-binding protein (G/F), confers upon C the ability to use Mg·ATP as substrate, binds guanine nucleotides and fluoride, and modulates the catalytic activity by these compounds. The regulatory protein has a GTPase function and serves as the substrate for a cholera toxin-catalyzed covalent modification. This modification, an ADP-ribosylation, leads to a persistent activation of adenylate cyclase by blockage of the GTP hydrolysis function of the regulatory protein. The third component of the enzyme system is the hormone receptor which contains a specific hormone binding site on its extracellular surface. The presence of a guanine nucleotide in addition to the substrate, ATP, is required to elicit hormonal stimulation.

The "floating receptor" model is a currently accepted hypothesis to explain the mode of adenylate cyclase regulation. The model predicts that the component proteins are discrete molecules able to

freely diffuse and interact within the plane of the membrane bilayer. The overall mechanistic scheme describes the catalytic unit as being relatively inactive. When the regulatory unit, G/F, binds GTP it can stimulate the catalytic moeity. Activation is terminated when the GTP is hydrolyzed, and can be reinitiated when G/F binds another GTP. The receptor-hormone complex binding stimulates the dissociation rate of GDP and thus catalyzes the regeneration of G/F·GTP. Membrane integrity is required for this regulatory cycle as hormonal stimulation is not observed upon solubilization of the enzyme.

Adenylate cyclase has been characterized to a lesser extent in some other eukaryotic systems. Adenylate cyclase in Xenopus laevis oocytes is found as a soluble (50-65%) as well as a particulate (20-30%) form (Finidori-Lepicard et al. 1981). Both are equally active in the presence of Mg^{+2} or Mn^{+2} but only the particulate activity could be stimulated by NaF. The steroid hormone progesterone inhibited the enzyme whereas the GTP analogue Gpp(NH)p stimulated it. Fluoride is also able to stimulate the adenylate cyclase activity of the molluscan heart (Higgins et al. 1978). Morishima (1978) examined the properties of adenylate cyclase in the silkworm pupal fat body and found that the activity was predominantly particulate and that the Mn-dependent activity was 2-fold higher than the Mg-dependent activity. Fluoride stimulated both cation-dependent activities, and millimolar levels of calcium strongly inhibited the Mg-dependent activity. The GTP analogue Gpp(NH)p caused 20-fold stimulation of the particulate adenylate cyclase from the planaria Polycelis tenuis (Franquinet et al. 1978). Gomes et

al. (1978) observed that the particulate enzyme from Blastocladiella emersonii could be solubilized by 1% Triton X-100. Particulate, non-solubilized enzyme required Mn^{+2} for maximal activity and Mg^{+2} could not substitute as the divalent cation. Additionally, they found no enhancement of activity by NaF. Pall (1981) reported that Neurospora and Saccharomyces cerevisiae adenylate cyclases are dependent on Mn·ATP and are stimulated by excess levels of Mn^{+2}. The enzyme from these organisms, like that from Blastocladiella could not be stimulated by fluoride or GTP. The enzyme from these fungi as well as from M. rouxii are manganese dependent; they do not exhibit much, if any, activity with Mg·ATP serving as substrate. On the other hand, Phycomyces blakesleeansus adenylate cyclase is dependent upon magnesium and is inhibited by manganese. Additionally, it is stimulated by GTP. Pall mentions unpublished data indicating that under certain conditions Neurospora adenylate cyclase exhibits a Mg-dependent activity which can be stimulated by guanine nucleotides. However, the details of this finding are not provided.

Thus it is clear that adenylate cyclase occurs in many systems; the consensus of the reports is that the enzyme is the most important control point of cyclic AMP levels in these diverse organisms. The overall purpose of the present study was to examine the role of adenylate cyclase as a regulatory enzyme involved in pattern formation and differentiation of the two cell types in the cellular slime mold Dictyostelium discoideum. The specific objectives were to: (1) characterize the regulation of adenylate cyclase by modulatory ligands

at the culmination stage of development, (2) determine the distribution of enzyme activity within the developing organism, (3) measure the levels of cyclic AMP in individuals during development, and (4) examine the distribution of cyclic AMP within the developing organism.

2. MATERIAL AND METHODS

2.1. Growth and Differentiation of Cells

Amoebae of Dictyostelium discoideum (wild-type NC-4) were grown from spores in association with Escherichia coli on nutrient agar at 23°C as described by Rutherford (1976a). The amoebae were harvested after 48 hours of growth at 23°C then washed three times (1800 x g for 1 minute) with ice-cold salt solution (Bonner, 1947) to remove the bacteria.

For mass culture studies the cells were spread directly on non-nutrient agar, and were allowed to differentiate to various stages of development at 19°C. When the cells reached the desired developmental stage they were harvested, washed with double-distilled water (1800 x g, 5 minutes), frozen, lyophilized, and stored under vacuum at -70°C.

For the study of individuals, the amoebae were harvested and washed as above, but were plated on Whatman Number 50 filter paper that was overlaid on non-nutrient agar (Rutherford and Harris, 1976), and allowed to differentiate at 19°C. At various stages of development the organisms were frozen quickly by removing the filters to a metal surface at -70°C. The individuals were then lyophilized while still on the filters, and stored under vacuum at -70°C.

2.2. Preparation of Uninduced and Induced Slugs

The induced slug series was developed in the laboratory of Dr. M. Sussman at the University of Pittsburgh by K. Deml. Dictyostelium

discoideum strain NC-4 was grown in association with Enterobacter aerogenes, harvested after 24 hours growth at 22°C, and washed three times by centrifugation in cold water (Schindler and Sussman, 1977). 20 µl of 9 x 10^8 cells/ml were transferred to black Whatman no. 50 filters which were then placed in Lucite incubation chambers (Schindler and Sussman, 1977) and kept in the dark. After the cells developed to the slug stage induction was initiated by transferal of the filter to an absorbent pad in a petri dish containing 2 ml of a buffered salt solution, "LPS" (1.5 mg/ml KCl, 0.5 mg/ml $MgCl_2 \cdot 6H_2O$, 0.5 mg/ml streptomycin sulfate, and 40 mM sodium/potassium phosphate, pH 6.5). The dish was exposed to overhead light, and excess LPS was removed. After the slug reached the desired stage of induction the filter was removed to a metal surface at -80°C. The individuals were lyophilized while still on the filters and stored under vacuum at -80°C. The stages of induction were described according to their morphological features and are assigned a numerical designation: (1) slug, uninduced, (2) tip up 45°, (3) bulge behind tip, (4) bottle, (5) hat.

2.3. Adenylate Cyclase Assay

2.3.1. Tissue homogenates: Adenylate cyclase activity was measured by radioimmunoassay of the cyclic AMP reaction product. Extracts from mass cultures were prepared by homogenization of the lyophilized cells in 100 mM Tris-HCl buffer, pH 7.5 containing 3 mM DTT ("homogenization buffer"). 10 µl of this extract was added to a 6 x 50 mm test tube. The adenylate cyclase reaction was initiated by the addition of 27.6 µl of a reaction

mixture containing 13 mM DTT, 1 mg/ml BSA, 1.5 mM ATP, 0.5 mM EDTA, and 2.5 mM $MnCl_2$ in 100 mM Tris-HCl, pH 7.5. After incubation at 23°C for various periods of time the reaction was terminated by acetylation of the cyclic AMP by addition of 3.8 µl of a 2:1 (v/v) solution of triethylamine and acetic anhydride. Both the reaction mixture and the acetylating reagent were prepared freshly at the time of the experiment.

The acetylated cyclic AMP was measured by the addition of 12.1 µl of [^{125}I]-Succinyl cyclic AMP-tyrosine methyl ester ([^{125}I]-ScAMP-TME) diluted in 50 mM sodium acetate buffer, pH 6.2, containing 5 mg/ml bovine serum albumin to give 5,000 to 20,000 cpm/reaction tube. This was followed by the addition of 12.1 µl of cyclic AMP antiserum diluted in 50 mM sodium acetate buffer, pH 6.2, containing 30 mg/ml BSA. The dilution was adjusted to bind 50% of the total cpm per blank reaction tube (that is, one lacking enzyme or added cyclic AMP standard).

The samples were allowed to equilibrate at 6°C for at least 15 hours before adding 300 µl of an activated charcoal solution (1% w/v) in 50 mM sodium acetate buffer, pH 6.2, containing 10 mg/ml BSA. The samples were centrifuged at 1800 x g for 20 minutes, the supernatant fractions were removed to gamma vials, and the radioactivity was determined in a Beckman Biogamma II counter.

Standard curves (0-900 fmol) were generated by the addition of 10 µl of cyclic AMP to 27.6 µl reaction mixture followed by acetylation and radioimmunoassay. The controls that were routinely included were enzyme blanks and cyclic AMP antiserum blanks. Cyclic AMP was quantified by subtraction of the antiserum blank (limit of charcoal

binding) from all samples. The cyclic AMP standard blanks were considered to represent the maximum counts that could be bound by the antibody (usually equal to 50% of the total cpm added to each reaction tube). The standards were plotted as the log of fmol cyclic AMP versus the percentage maximum counts bound. All experimental tubes were calculated as percentage of maximum counts bound, and were then quantified using the standard curve. Separate standard curves were generated for each experimental condition.

2.3.2. Tissue sections: Lyophilized sections, cut and weighed on the day of the assay, were placed in the bottom of 6 x 50 mm test tubes. To initiate the adenylate cyclase reaction 37 µl of a 1:2.7 (v/v) mixture of homogenization buffer (100 mM Tris, pH 7.5, containing 3 mM DTT) and reaction mixture (as previously described) was added carefully to each tube. The tube was viewed through a dissecting microscope to ascertain that the section was immersed in the reaction mixture, and then was incubated at 23°C. The reaction was terminated by acetylation and cyclic AMP was determined by radioimmunoassay as described above.

2.4. Iodination and Purification of [^{125}I]-ScAMP-TME

Iodination of cyclic AMP was accomplished as described by Brooker et al. (1979). High concentration, reductant-free Na ^{125}I was purchased from New England Nuclear as a 1 mCi aliquot contained in a Combi-V-vial. Twenty microliters of 0.5 M potassium phosphate buffer, pH 7.0 was added to the Na^{125}I. Twenty microliters of 60 µM ScAMP-TME

(approximately 40 µg/ml of 5 mM sodium acetate, pH 4.75) was then added; the vial was capped and vortexed. Five microliters of chloramine T (1 mg/ml in 0.5 M potassium phosphate buffer, pH 7.0) was added to initiate iodination and the vial was capped and vortexed. After 60 seconds 50 µl sodium metabisulfite (5 mg/ml) was added to stop the reaction and allow volatilization of free $^{125}I_2$. The above steps were carried out in a fume hood.

Purification of ^{125}I-cyclic AMP was carried out by streaking the reaction mixture in a horizontal line onto a 2 x 43.5 cm strip of Whatman 31 ET paper. The paper was allowed to dry then developed by descending paper chromatography for approximately 4.5 hours (until the solvent front is within 1 cm of the bottom of the paper). The solvent used was 1-butanol: glacial acetic acid: water in a ration of 12:3:5. The strip was removed from the tank and allowed to dry. One-centimeter sections of the strip were placed in gamma vials and eluted in 2 ml 50 mM sodium acetate buffer, pH 4.75, at 4°C for 1 hour. One microliter from each fraction was removed to a second group of gamma vials for determiantion of radioactivity. The iodinated nucleotide derivative was found as a peak of radioactivity nearest to the solvent front. The fraction containing the peak was then divided as 20 µl aliquots in 6 x 50 mm disposable tubes and stored at -70°C.

2.5. Description of the Microtechnique

Lyophilized individuals, developed on filter papers and stored at -70°C under vacuum, were brought to room temperature. Individuals at

the desired stage of development were selected and removed from the vials under a dissecting microscope. Sections were cut from the individual by dissection using a microscalpel composed of a small section of razor blade attached to a copper wire embedded in a dowel (Rutherford 1976b). The sections, weighing from 50 nanograms, were transported by a hair tip. The weight was measured on a quartz-fiber microbalance as described by Lowry and Passonneau (1972). Sections were cut to the same size as nearly as possible to avoid artifactual differences in enzyme activity or cyclic AMP levels due to the amount of tissue used. Careful drawings were made at the time of dissection in order to express activity or cyclic AMP levels with relation to the location of the section within the individual.

2.6. Measurement of Cyclic AMP in Microsections

Freeze-dried tissue sections, cut and weighed on the day of assay, were placed in the bottom of 6 x 50 mm glass tubes. Cyclic AMP was extracted from the tissue sections by a modification of the method of Ferrendelli et al. (1977). 14.8 µl of 5% TCA (prepared freshly for each experiment from a 100% stock solution) was added to each tube containing sections as well as to tubes containing 10 microliter volumes of standards (containing 1 to 180 fmol cyclic AMP). After a 15 minute incubation at 4°C, 2.0 µl of 1 M HCl was added to each tube. Tubes with tissue sections also received 10 µl of H_2O to correct for the volume in the tubes which contained the cyclic AMP standards. All samples were then dried under vacuum on a Virtis centrifugal bio-dryer

to accomplish TCA removal. The dried residues were dissolved in 30 µl of 50 mM sodium acetate buffer, pH 6.2, and acetylated with 1.56 µl of a 2:1 (v/v) solution of triethylamine and acetic anhydride. The radioimmunoassay for cyclic AMP was carried out as described above for tissue homogenates. Cyclic AMP levels in the sections were quantified on the basis of section weight and were expressed as femtomoles per microgram dry weight.

3. RESULTS

3.1. Assay of Adenylate Cyclase Activity

A radioimmunoassay for the reaction product cyclic AMP was used to measure adenylate cyclase activity. Initially I wanted to determine the optimal conditions for measuring enzyme activity in lyophilized culmination stage tissue. Tris-HCl or glycyl-glycine buffer systems are usually employed for adenylate cyclase assay (Perkins 1973). I observed only a 10% difference in activity when the tissue was homogenized in 100 mM Tris-HCl buffer, pH 7.5, as compared to homogenization in 100 mM glycylglycine buffer, pH 7.5 (Fig. 1). Specific activities of 1.8 and 1.6 pmol min^{-1} mg^{-1} were obtained respectively. I decided to use Tris-HCl as the buffer for all subsequent assays.

The results of Pahlic and Rutherford (1979) indicated that the recovery of enzyme activity was dependent on the treatment of the crude homogenate. Their results suggested that centrifugation affects the activity. I compared two crude homogenates--one that was incubated on ice while the other was spun at 7500 x g for 15 minutes followed by resuspension of the pellet in the supernatant fluid (Fig. 2). Centrifugation resulted in a 33% decrease in activity. Additional spins resulted in further losses in activity that could not be accounted for merely by enzyme lability over time (data not shown). Because of this, in most experiments I used a crude homogenate that had not been subjected to centrifugation.

Reaction mixtures for the assay of adenylate cyclase activity

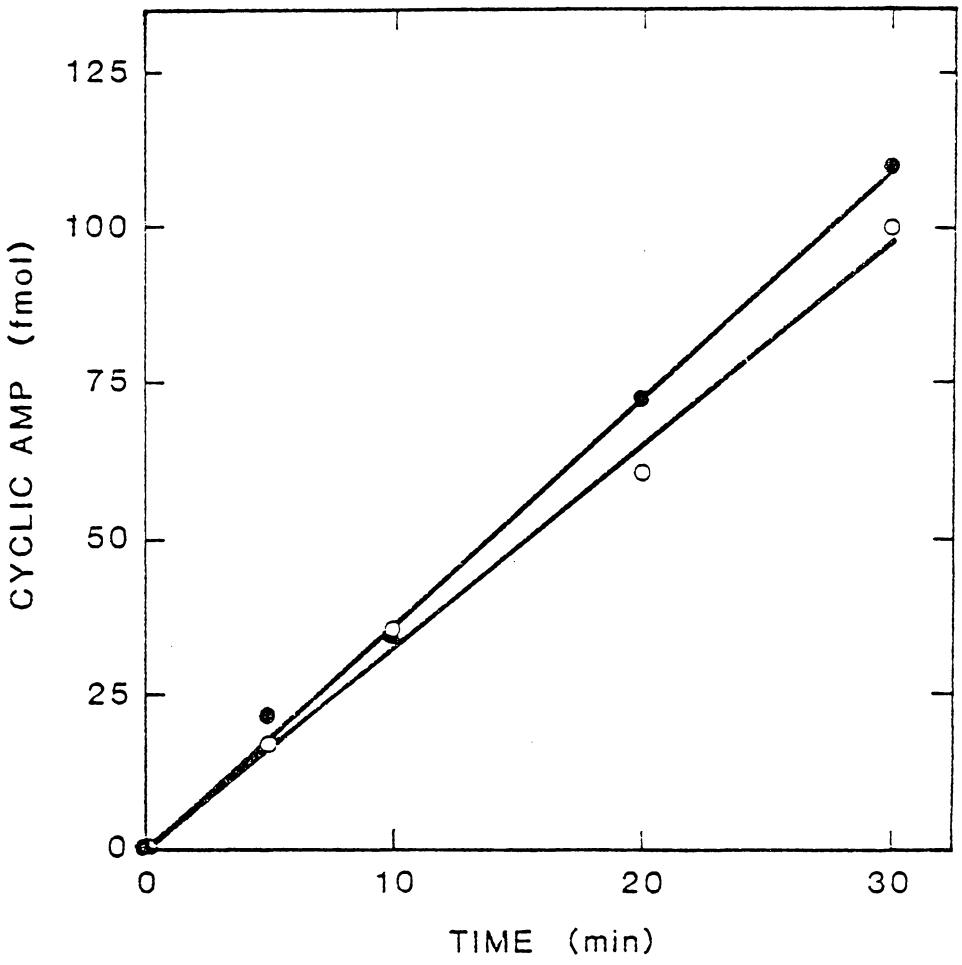

Figure 1. Effect of buffer system on adenylate cyclase activity. Culmination stage enzyme was homogenized in buffer composed of 100 mM Tris (•) or glycyl-glycine (o) at pH 7.5 and 3 mM DTT at a concentration of 0.2 mg dry weight/ml. 10 µl of this preparation was assayed in 27.6 µl of a reaction mixture containing 1.5 mM ATP and 2.5 mM $MnCl_2$ as described in "Methods". Incubation was at 23°C. Points are the average of two determinations. Best fit lines were determined by a least-squares linear regression.

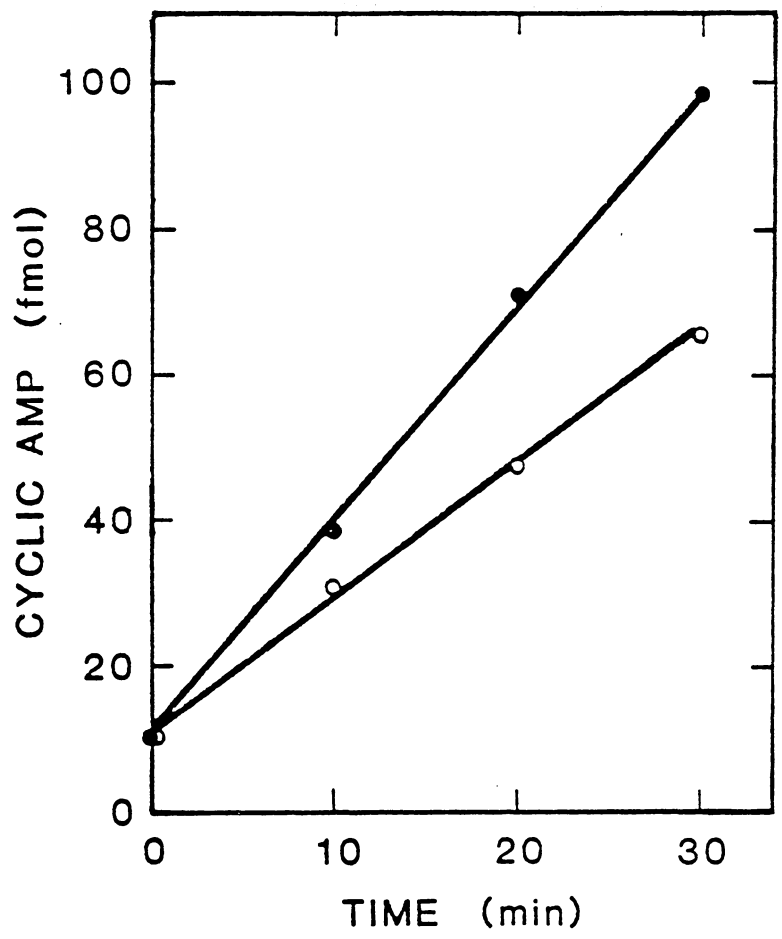

Figure 2. Effect of centrifugation on adenylate cyclase activity. Culmination stage tissue was homogenized in 100 mM Tris-HCl buffer, pH 7.5 containing 3 mM DTT at a concentration of 0.2 mg dry weight/ml. This preparation was divided into 2 aliquots. One aliquot was incubated at 4°C while the other was centrifuged at 7500 x g for 15 minutes. The pellet of the latter was resuspended in the supernatant fluid. 10 µl of each preparation was assayed for adenylate cyclase activity as described in "Methods", (●), incubated homogenate; (o), centrifuged preparation. Each point represents the average of two determinations.

generally include an ATP-regenerating system to counteract the effects of ATPases that may be present in the enzyme extracts (Perkins 1973). Substrate can thus be maintained at saturating levels. Rossomando and Cutler (1975) had reported that a regenerating system was not beneficial to the assay of the Dictyostelium enzyme by a TLC method. I examined the effects on the Dictyostelium enzyme of the inclusion and exclusion of such a regenerating system using my assay method. The regenerating system consisted of 0.25 mg/ml creatine phosphokinase and 30 mM phosphocreatine. The results of this experiment are illustrated in Figure 3. The inclusion of the regenerating system led to a 27% decrease in activity and therefore was not routinely included in subsequent assays.

Figure 4 shows the relationship of tissue concentration to linearity of enzyme activity. An extract dilution curve should be linear in order to demonstrate the absence of non-specific inhibitors in the tissue extract (Brooker et al. 1979). Over the range of 0 to 10.0 micrograms dry weight of tissue the linearity was maintained. Addition of greater than 10.0 micrograms gave zero-time incubation values significantly different from blank values (data not shown). This indicated a probable interference with the antibody binding reaction.

I next examined the linearity of the adenylate cyclase reaction over time. Homogenates at concentrations of 0.1 to 0.5 mg/ml were generally linear over a 60 minute incubation period at 23°C. Figure 5 shows adenylate cyclase activity from a culmination extract of 0.2

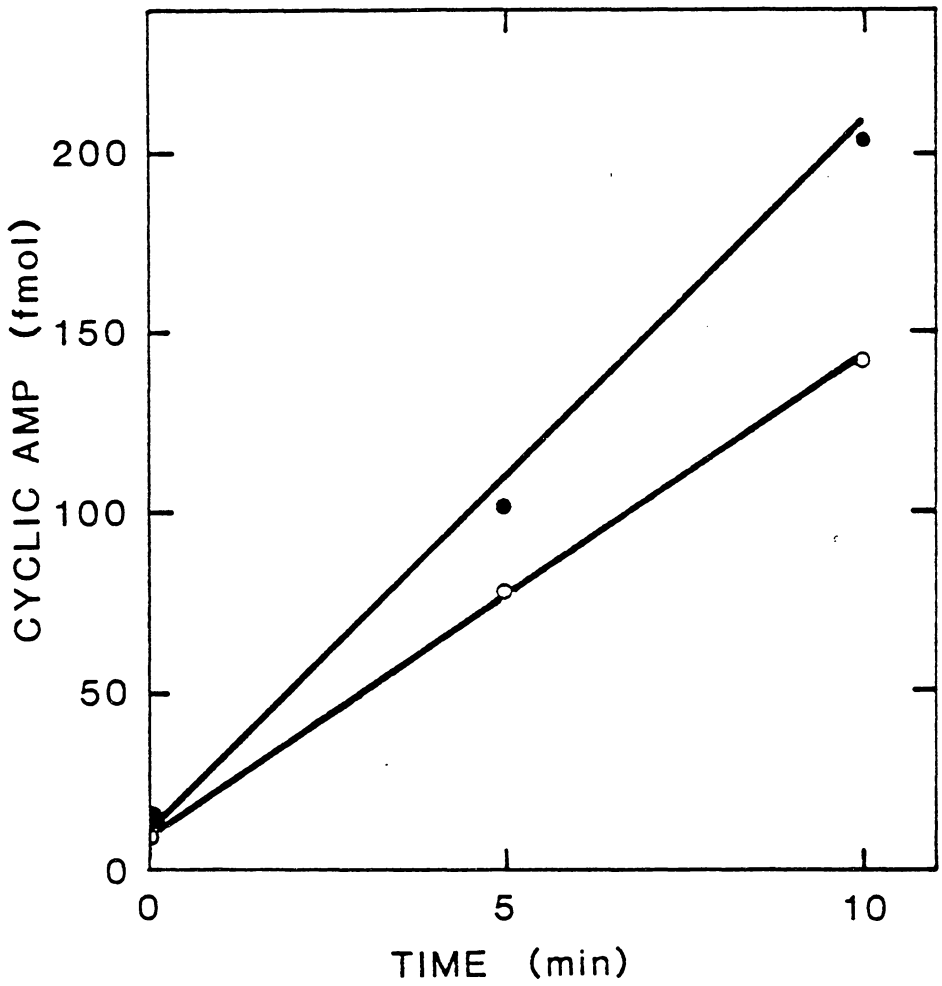

Figure 3. Effect of ATP-regenerating system on adenylate cyclase reaction. Culmination stage tissue was homogenized in 100 mM Tris-HCl, pH 7.5, containing 3 mM DTT at a concentration of 1 mg/ml. 10 µl was added to a test tube. Adenylate cyclase reactions were initiated by adding 27.6 µl reaction mixture as described in "Methods" but containing 2.5 mM $MgCl_2$ (•) or containing 2.5 mM $MgCl_2$ and an ATP-regenerating system consisting of 0.25 mg/ml creatine phosphokinase and 30 mM phosphocreatine (o). Incubation was carried out at 23°C. Points represent the mean of two determinations. Best-fit lines were calculated by a least-squares linear regression.

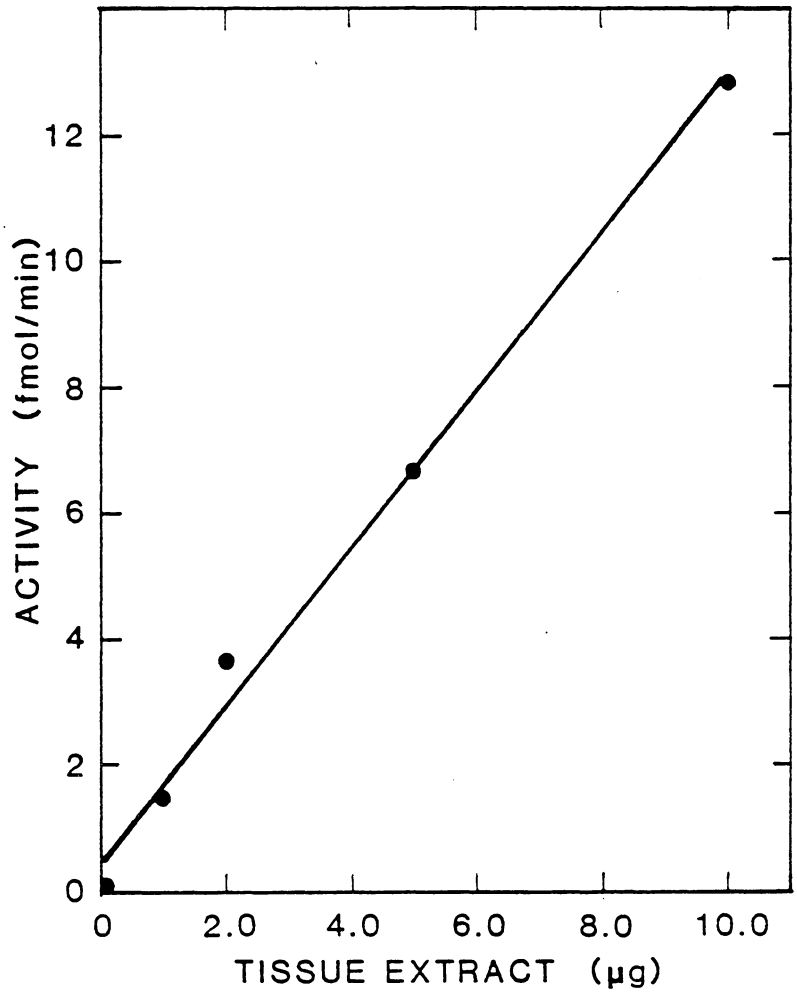

Figure 4. Linearity of adenylate cyclase reaction with concentration of tissue. 10 µl of culmination stage enzyme in crude homogenate at various concentrations were assayed in 27.6 µl of a reaction mixture containing 1.5 mM ATP as described in "Methods". The reaction was buffered at pH 7.5 and contained 2.5 mM $MnCl_2$. Incubations were carried for 10, 20, and 30 minutes at 23°C, and rate determinations were calculated from the slopes of the best-fit lines as determined by a least-squares linear regression. The best-fit line from the activity vs. concentration was determined in the same manner.

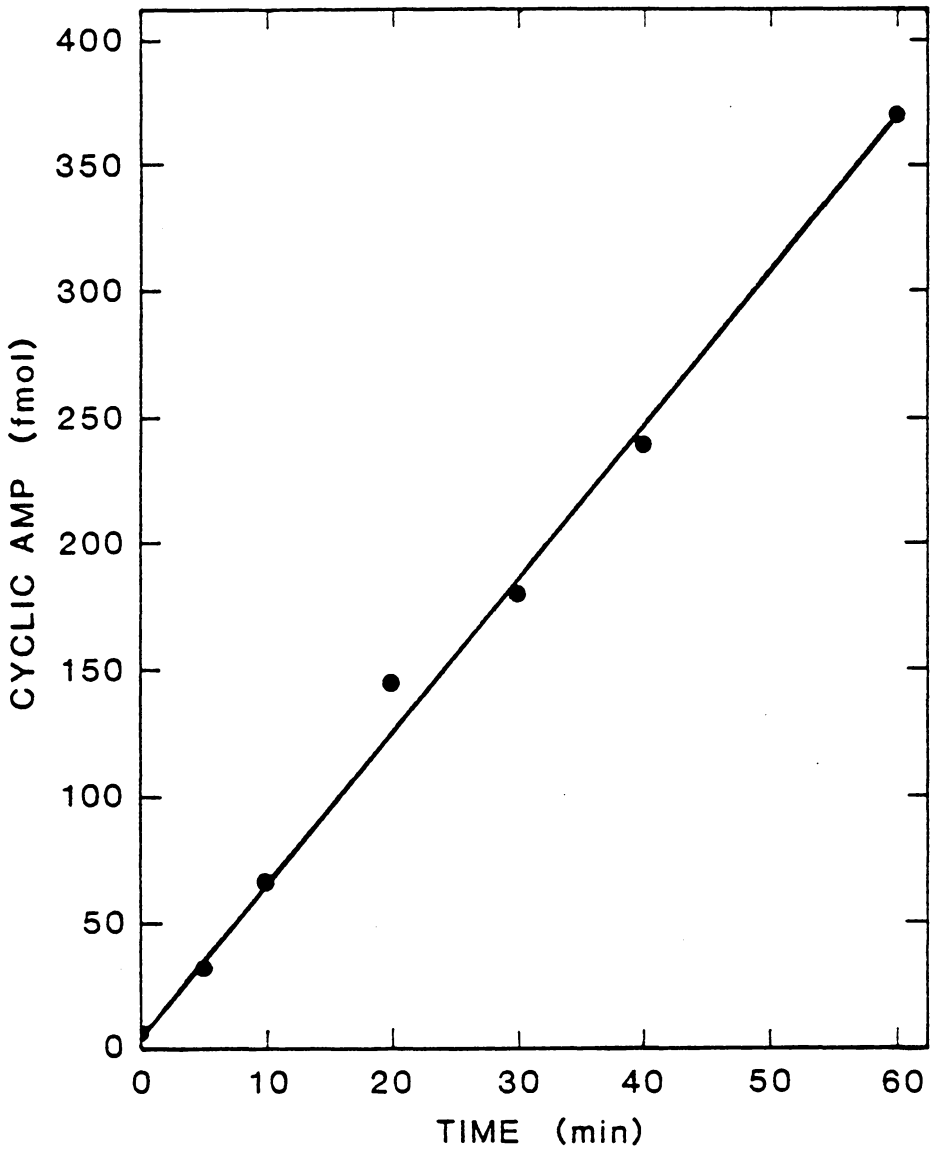

Figure 5. Linearity of adenylate cyclase reaction with time of incubation. 10 µl of culmination stage enzyme in crude homogenate (0.2 mg dry weight/ml homogenization buffer) was assayed in 27.6 µl of a reaction mixture containing 1.5 mM ATP as described in "Methods". Incubation was at 23°C. Points are the average of 2 determinations. The best-fit line was calculated from a least-squares linear regression.

mg/ml. The reaction mixture contained Mn^{+2} as the divalent cation. In general, the enzyme from all developmental stages, and with Mn^{+2} or Mg^{+2} serving as the divalent cation in the reaction mixture, exhibited linearity at least over a 30 minute incubation period (data not shown). For all experimental studies of Dictyostelium adenylate cyclase I took into consideration the constraints of reaction linearity in relation to tissue concentration and incubation time.

Characterization of adenylate cyclase with regard to potential modulators of activity could involve preincubation of the enzyme with various ligands. I thus thought it would be important to examine the stability of the Dictyostelium enzyme in the preincubation buffer (homogenization buffer) at 4°C and 23°C. Figure 6 illustrates the results of this study. Forty-six percent of the control (non-preincubated) activity remained after 50 minutes of preincubation at 4°C. However, no activity was detectable after 33 minutes of preincubation at 23°C. This lability at 23°C contrasts to the linearity of the reaction over a 60 minute incubation period during which the enzyme is exposed to the reaction mixture as well as the homogenization buffer. This instability of adenylate cyclase exposed to homogenization buffer is depicted by another experiment. As shown in Figure 7, sections of lyophilized tissue were either allowed to incubate in homogenization buffer for three minutes before reaction initiation, or they were assayed immediately by the addition of reaction mixture and homogenization buffer in one step. The pieces exposed to homogenization buffer before reaction initiation exhibited only 63% of the

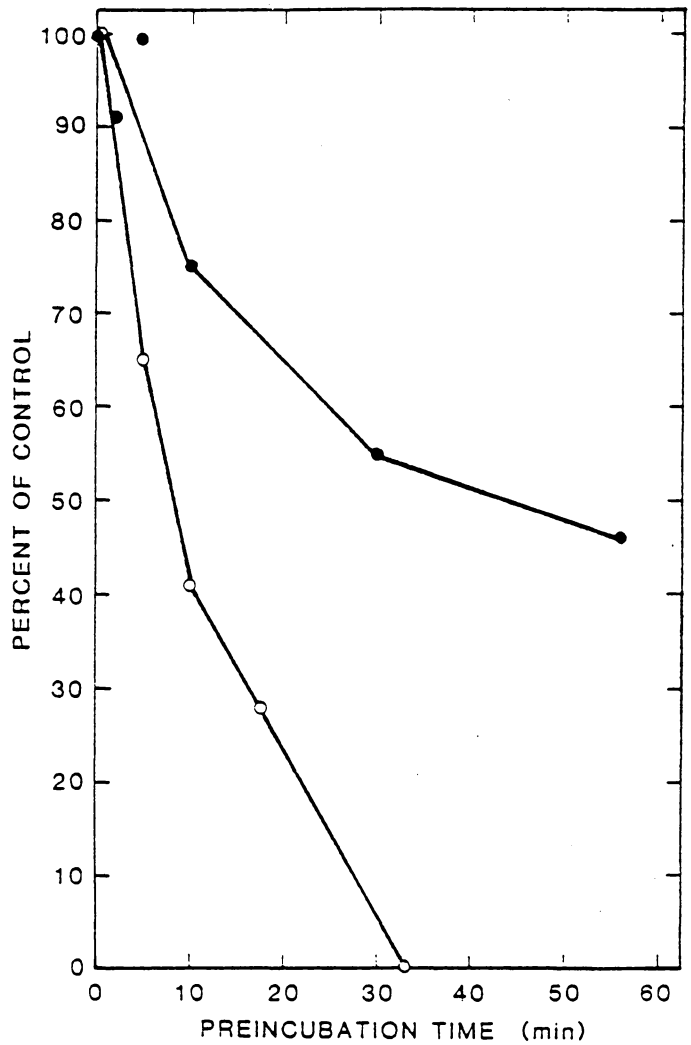

Figure 6. Temperature stability of adenylate cyclase. 10 µl aliquots of culmination stage enzyme in crude homogenate (0.2 mg/ml homogenization buffer) were preincubated at 4°C (●) and 23°C (o) and for the times indicated. Reaction mixture (see "Methods") was added to initiate adenylate cyclase reactions, and preparations were assayed in duplicate for 0, 15, and 30 minutes. Activities in pmol min^{-1} mg^{-1} are expressed as the percentage of non-preincubated controls.

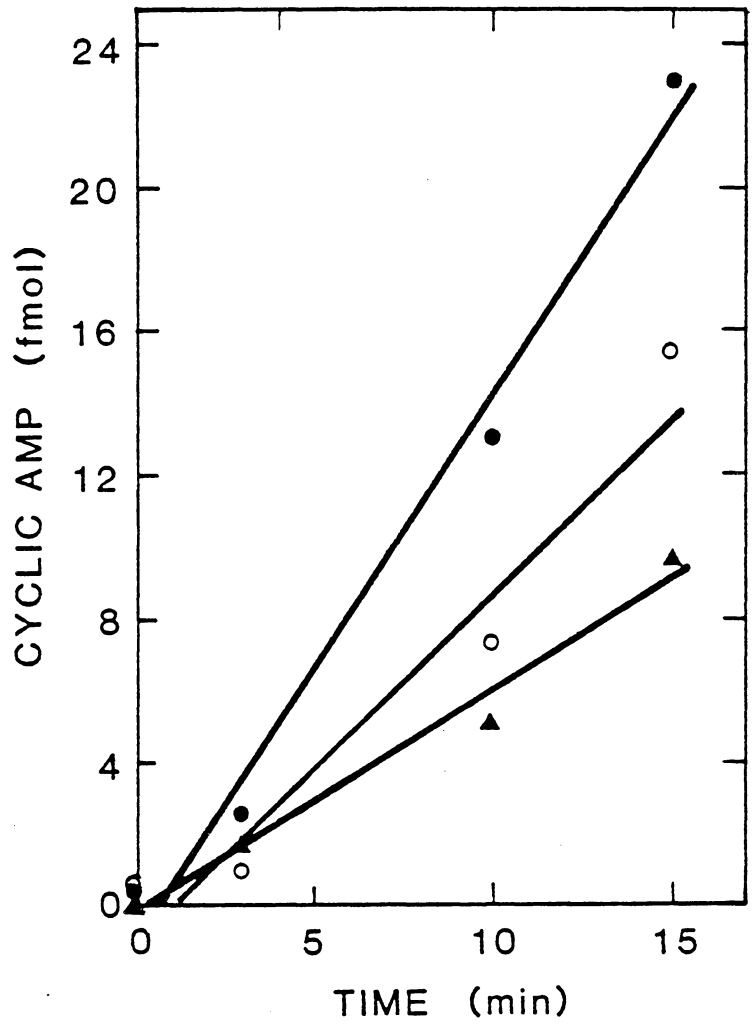

Figure 7. Effect of homogenization on enzyme activity. Sections of lyophilized culmination stage tissue were chosen such that the final concentration in 9.6 µl of homogenization buffer was approximately 0.25 mg/ml (the actual concentration of the samples was 0.25 ± 0.03 mg dry wt/ml). The sections were placed in 6 x 50 mm test tubes. Half of the tubes received 37 µl of 1:2:8 (v/v) mixture of homogenization buffer and reaction mixture (●) and were incubated at 23°C for the times shown. The other half received 9.6 µl homogenization buffer (o), were preincubated at 4°C for 3 minutes, and then received 27.6 µl reaction mixture, and were incubated at 23°C for the times shown. A crude tissue homogenate at 0.25 mg/ml was prepared at the same time. 9.6 µl aliquots of this preparation were distributed to 6 x 50 mm tubes, and the adenylate cyclase reaction was initiated by the addition of 27.6 µl reaction mixture and incubated at 23°C (▲).

activity of those pieces not exposed. For comparison, a crude tissue homogenate at an equivalent concentration was prepared in the usual manner, that is by weighing milligram quantities of lyophilized mass-culture, culmination stage tissue, and homogenizing it in buffer. This preparation exhibited only 41% of the activity. These results led me to conduct all characterization studies with care not to allow the enzyme to be exposed to homogenization buffer more than a minimal length of time. Additionally, controls were always planned such that tissue preparations were exposed to homogenization buffer for the same length of time regardless of other experimental parameters.

The above observations also led me to question whether some component of the reaction mixture might act to stabilize the enzyme. I included components of the reaction mixture, singly and in combination, in preincubation mixtures to test this possibility. However, I did not observe any increase in stability (data not shown). I could not include ATP in these mixtures as some cyclic AMP would be made under the pre-incubation conditions and the appropriate controls would have been difficult to perform. Since substrates are known to stabilize some enzymes I examined the effects of ATP during a preincubation by using the procedure of Richards et al. (1981). They observed a 4- to 7-fold persistent activation of rat-liver adenylate cyclase by ATP. This was accomplished by preincubating purified rat liver plasma membranes with $MgCl_2$, ATP and an ATP-regenerating system for 24 hours at 4°C. The membranes were washed after preincubation and assayed for adenylate cyclase activity. I performed a similar experiment using crude

homogenates from culmination stage Dictyostelium as shown in Table 1. After 4 hours of preincubation in homogenizing buffer only 9% of the original activity was remaining. Preincubation in the presence of $MgCl_2$ and ATP with or without a regenerating system seemed to stabilize the enzyme somewhat; in these cases 19% and 24% of the control activity was recovered respectively. Thus a 2-fold increase over the "no addition" preincubated activity was seen. After a 24 hour incubation very little activity was recovered in any case. Thirteen percent of the control activity was recovered when magnesium was included in the presence of a regenerating system. Certainly no activation was noted in any of the preparations.

3.2. Characterization of Adenylate Cyclase

3.2.1. Preparation of plasma membranes: Adenylate cyclase is a plasma membrane-bound enzyme in most eukaryotic cells (Perkins 1973). By using a cytochemical localization procedure, Cutler and Rossomando (1975) showed that adenylate cyclase of stationary phase cells of Dictyostelium (axenic strain AX-3) is membrane-bound and that the probable active site faces intracellularly. Pahlic and Rutherford (1979) have shown that the enzyme in strain NC-4 at both the aggregation and culmination stage is almost entirely bound to a 25,000 x g pellet fraction.

I prepared culmination stage Dictyostelium plasma membranes according to the procedure of Parish and Müller (1976). Their method employs Concanavalin A to stabilize the plasma membrane and Triton X-100 to release the cell contents. As Table 2 shows, no adenylate cyclase

Table 1

Stabilization of Adenylate Cyclase during Preincubation

	Additions to Homogenate[a]			Relative Adenylate Cyclase Activity[c]	
	PC-CPK[b]	$MgCl_2$ (5 mM)	ATP (1 mM)	Preincubation 4 hr	Preincubation 24 hr
a				9.0 ± 0.9	4.2 ± 1.4
b	+	+	+	19.3 ± 2.5	5.3 ± 1.2
c	+			11.8 ± 0.3	3.3 ± 0.6
d	+	+		9.7 ± 0.6	13.0 ± 1.0
e		+		8.8 ± 1.0	5.5 ± 0.5
f			+	7.0 ± 1.7	6.3 ± 1.5
g		+	+	24.0 ± 1.7	6.3 ± 1.5

[a] Crude homogenates of culmination stage tissue were incubated in homogenizing buffer at a concentration of 0.5 mg/ml after addition of the other components listed for 4 hr or 24 hr at 4°C. The particulate fraction was pelleted at 25,000 x g for 15 minutes, resuspended in fresh homogenizing buffer and assayed for adenylate cyclase as described in "Methods".

[b] PC is phosphocreatine, present in the final mixture at 30 mM. CPK is creatine phosphokinase, present in the final mixture at 0.25 mg/ml. All other concentrations given are those present in the final mixture.

[c] The initial activity of a particulate fraction not subjected to preincubation was 2.34 pmol min^{-1} mg^{-1}. Percent activity relative to that value is presented as the mean ± standard deviation of triplicate values.

Table 2

Adenylate Cyclase Activity of Purified Plasma Membrane Fraction

Preparation	Specific Activity (pmol $min^{-1}mg^{-1}$)
Untreated Control[a]	9.8
Plasma Membrane Plus Concanavalin A and Triton X-100[b]	Not detectable
Minus Concanavalin A and Triton X-100[c]	10.0

[a]Cells at the tight aggregate stage were harvested as described in "Methods". They were kept on ice during the preparation of plasma membranes and then lyophilized.

[b]Cells at the tight aggregate stage were harvested and the protocol of Parish and Muller (1976) was followed: Cells were resuspended at 5 x 10^6 cells/ml in H_2O and shaken for 10 minutes. The cells were sedimented at 1800 x g for 5 minutes then resuspended at 5 x 10^7 cells/ml in PDF buffer (50 mM Na phosphate, pH 6.5, containing 20 mM KCl and 25 mM $MgCl_2$) containing 100 µg/ml Concanavalin A. The suspension was incubated for 5 minutes then diluted with 0.1 M Tris-HCl, pH 8.5. The cells were washed twice at 300 x g for 1 minute then resuspended at 5 x 10^7 cells/ml in Tris-HCl, pH 8.5 containing 0.2% Triton X-100. This was diluted with Tris-HCl, pH 8.5, centrifuged at 300 x g for 10 seconds to remove whole cells. The supernatant was then centrifuged at 1700 x g for 2 minutes. The pellet was resuspended in Tris-HCl and washed 3 times and then lyophilized.

[c]Cells were prepared as in "b" but the Concanavalin A and Triton X-100 were omitted from the steps using them.

activity was recovered after this procedure was followed. Since activity was recovered from tissue that had been subjected to a similar treatment except without the use of the Concanavalin A or Triton X-100, it appeared that one or both of these substances had a deleterious effect on the enzyme. Moreover, the physical preparation itself was not a problem as the activity recovered was equivalent to an untreated control. Since I was unable to recover activity from a membrane preparation I decided to work with the crude homogenate as the enzyme source.

3.2.2. Treatment with detergent: In order to partially purify the enzyme for characterization studies I wanted to separate the enzyme from the membrane. Such solubilization of proteins can be effected by the use of non-ionic detergents (Neer 1977). Two detergents, Triton X-100 and Lubrol PX have been widely used and found to be effective for solubilizing proteins, including adenylate cyclase, from eukaryotic cell membranes (Neer 1977). Another Dictyostelium membrane-bound enzyme, 5'AMP nucleotidase, had been solubilized in our laboratory using Triton X-100 (Armant and Rutherford, 1981), and so I decided to try this detergent. As shown in Figure 8, inclusion of Triton X-100 in a solubilization wash led to almost complete elimination of adenylate cyclase activity. The activity was not recovered in the 3000 x g supernatant fractions. Solubilization with subsequent centrifugation may have compartmentalized factors which, when combined as they normally are, exhibit adenylate cyclase activity. However, resuspension of the Triton-treated pellet in the supernatant fluid did not lead to recovery

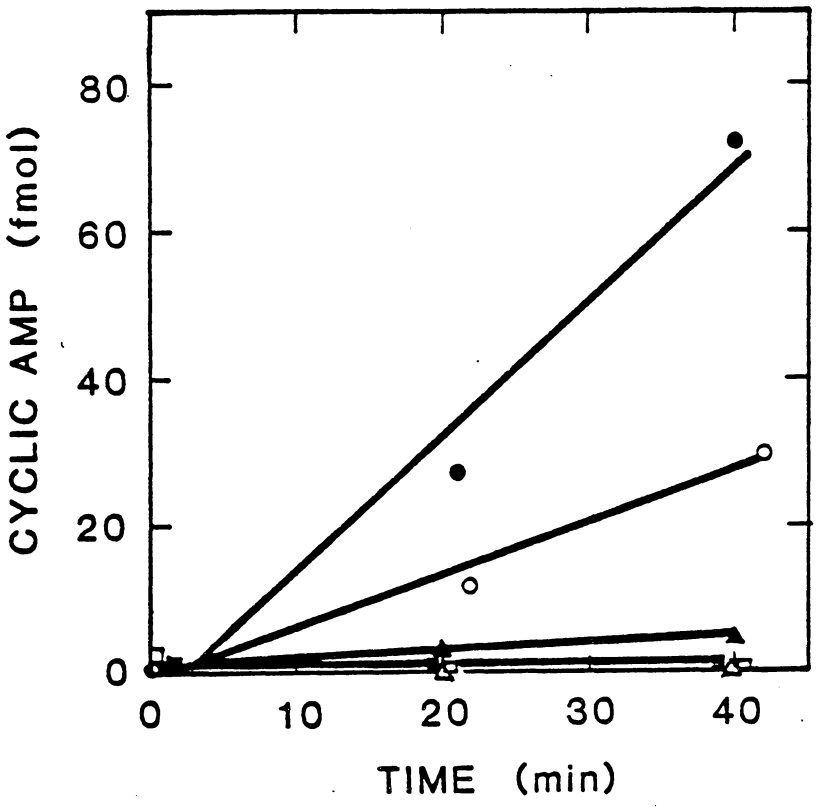

Figure 8. Effect of Triton X-100 on twice-washed crude pellet adenylate cyclase activity. Culmination stage tissue was prepared in homogenization buffer at a concentration of 5 mg/ml, and centrifuged at 8000 x g for 15 minutes. The supernatant was discarded and the pellet was resuspended in 2 ml of the same buffer. This homogenate was divided in half and each aliquot was centrifuged at 8000 x g for 15 minutes. The supernatants were discarded. One pellet was resuspended in 100 mM Tris-HCl, pH 7.5, ("untreated"); the other pellet in the same buffer but with 0.5% Triton X-100 (detergent "treated"). Both preparations were centrifuged at 8000 x g for 15 minutes. The supernatants were saved and both pellets were resuspended in 100 mM Tris-HCl, pH 7.5. Each pellet homogenate and supernatant fraction was assayed as prepared at a concentration of 25 mg/ml (closed symbols) and as a 1:2 dilution (open symbols). Circles represent the untreated pellets; triangles represent the detergent-treated pellets; squares represent the supernatant fractions.

of activity indicating that there probably was not a loss of membrane factors during the solubilization.

Investigators have used Lubrol-PX for the solubilization of adenylate cyclase from several systems such as bovine corpus-luteum (Young and Stansfield 1978) and rat liver (Newby and Chrambach 1979). I attempted to solubilize adenylate cyclase from Dictyostelium using a 0.1% concentration of this non-ionic detergent. After treatment I was unable to detect any activity from either a 100,000 x g pellet or supernatant fraction. Measurable activity was obtained from the 100,000 x g pellet fraction of a preparation treated in the same manner but in the absence of detergent. These results suggest a detergent effect on the enzyme activity.

A possibility that could account for the loss of adenylate cyclase activity after detergent treatment was that solubilization exposed the enzyme to cellular proteases. Before such release the membrane may have served to protect the enzyme from proteolysis. To counteract this problem I again tried a solubilization procedure utilizing Triton X-100 but with the inclusion of the protease inhibitor benzamidine, an inhibitor which has proven effective in the recovery of cyclic AMP-dependent protein kinase in Dictyostelium (Rutherford unpublished). The results of this experiment are shown in Table 3. In order to facilitate the recovery of observable activity in the supernatant fractions I used a high concentration of tissue (10 mg/ml). This enrichment resulted in a yield of cyclic AMP in the crude homogenates that was beyond the limits of the standard curve. It can be seen, nevertheless, that solubilization

Table 3

Solubilization in the Presence of Benzamidine

Treatment	Specific Activity[a]
Crude Homogenate[b]	>0.40
Crude Homogenate + Benzamidine[c]	>0.40
Solubilized[d]	
Supernatant	0.14
Pellet	0.40
Not Solubilized + Benzamidine[e]	
Supernatant	0.14
Pellet	>0.40
Not Solubilized[f]	
Supernatant	0.16
Pellet	>0.40

[a]Enzyme preparations were assayed as described in "Methods". The activity is expressed in pmol min^{-1} mg^{-1}. Each value represents the average of triplicate determinations from 0 to 10 minute incubations.

[b]A crude homogenate was prepared by homogenizing culmination stage tissue in homogenization buffer at a concentration of 20 mg/ml. 0.1 ml was removed to a tube and diluted with the same buffer to a final concentration of 10 mg/ml. This was kept at 4°C during solubilization preparations.

[c]0.1 ml of the 20 mg/ml crude homogenate in "b" was diluted such that the final concentration was 10 mg/ml in homogenization buffer containing 10 mM benzamidine. This was kept at 4°C before assay.

[d]1.0 ml of the 20 mg/ml crude homogenate in "b" was diluted such that the final concentration was 10 mg/ml in homogenization buffer containing 10 mM benzamidine and 0.2% Triton X-100. This preparation was centrifuged at 25,000 x g for 15 minutes. The supernatant was removed and the pellet was resuspended in the same volume in homogenization buffer with 10 mM benzamidine.

[e]Tissue preparation was as described in "d" except that no Triton X-100 was included.

[f]Tissue preparation was as described in "d" except that Triton X-100 and benzamidine were omitted.

in the presence of benzamidine did not lead to an increased recovery of adenylate cyclase activity in the supernatant fraction (as compared to the equivalent non-solubilized supernatant fraction that was prepared with benzamidine present). It is also evident that the detergent treatment almost completely eliminated activity from the pellet fraction. Additionally, the presence of benzamidine did not make a difference in the detectable activity of non-solubilized preparations.

Examination of all possible detergent-related inactivation parameters could have required extensive testing. Since one of my original goals was to compare a partially purified, solubilized enzyme activity with the membrane-bound activity at this point I decided to pursue the characterization of the membrane-bound activity.

3.2.3. Divalent cation requirement of adenylate cyclase: Adenylate cyclase in most systems requires a divalent cation for activity (Perkins 1973). I therefore tested the effects of several divalent metal cations on the Dictyostelium enzyme as shown in Table 4. When no cation was present I was unable to detect any activity. The greatest activity was obtained when Mn^{+2} served as the cofactor. Magnesium-supported adenylate cyclase activity was over 4-fold less than that of the manganese-supported activity. Inclusion of a higher concentration (45 mM) of Mn^{+2} or Mg^{+2} appeared to have inhibited the Dictyostelium enzyme. My results show that calcium will not satisfy the divalent cation requirement of the enzyme. Calcium may actually cause some inhibition of activity as when it is present along with Mn^{+2} there is a

Table 4

Effect of Divalent Cations on Adenylate Cyclase Activity

Me^{+2}	Final Concentration[a] (mM)	Specific Activity[b] (pmol min^{-1} mg^{-1})
None		Not detectable
Mg	4.5	0.70 ± 0.10
Mn	4.5	3.0 ± 0.47
Mn	45.0	0.50 ± 0.10
Mg	45.0	0.44 ± 0.05
Mn, Ca	4.5, 4.5	2.5 ± 0.28
Ca	4.5	Not detectable

[a]10 µl of culmination stage enzyme in crude homogenate (0.2 mg dry weight/ml homogenization buffer) was assayed in 27.6 µl of a reaction mix containing components described in "Methods", and divalent cation as indicated. Incubation was carried out in duplicate at 23°C for 20 minutes.

[b]Activity values are the average of at least two separate experiments. Values represent the mean ± the standard deviation.

depression in activity (as compared with the activity in the presence of Mn^{+2} alone).

3.2.4. Kinetic parameters: I next wished to examine the reaction kinetics of adenylate cyclase. Pahlic and Rutherford (1979) found maximal activity at ATP substrate levels from 1 to 4 mM with 8 mM $MgCl_2$. They showed diminution of activity at substrate levels above 6 mM. I performed a similar experiment using 2.5 mM $MnCl_2$ as the divalent cation. Figure 9 shows similar results to those obtained by Pahlic and Rutherford (1979). Maximum velocity is reached at 1.5 mM ATP and the activity drops off at substrate levels between 2.0 and 10.0 mM. A Lineweaver-Burk double-reciprocal plot (Figure 9 B) indicates substrate inhibition, possibly due to competition of uncomplexed ATP with the true substrate $ATP \cdot Me^{+2}$ (Dixon and Webb 1979).

I performed a similar experiment with the provision of a 3 mM excess of divalent cation over each substrate concentration. Figure 10 shows that the ATP inhibition can be overcome when sufficient divalent cation is present. The K_m value obtained from the double reciprocal plot was 0.53 mM ATP.

3.2.5. Effect of guanine nucleotides on activity: Hormonal stimulation of mammalian adenylate cyclase requires the presence of a guanine nucleotide in addition to the substrate. GTP and various analogues of GTP can stimulate the activity of adenylate cyclase in the absence of hormones as well (Ross and Gilman 1980). I therefore decided to test the effects of the non-hydrolyzable analogue guanyl-5'-yl-imidodiphosphate

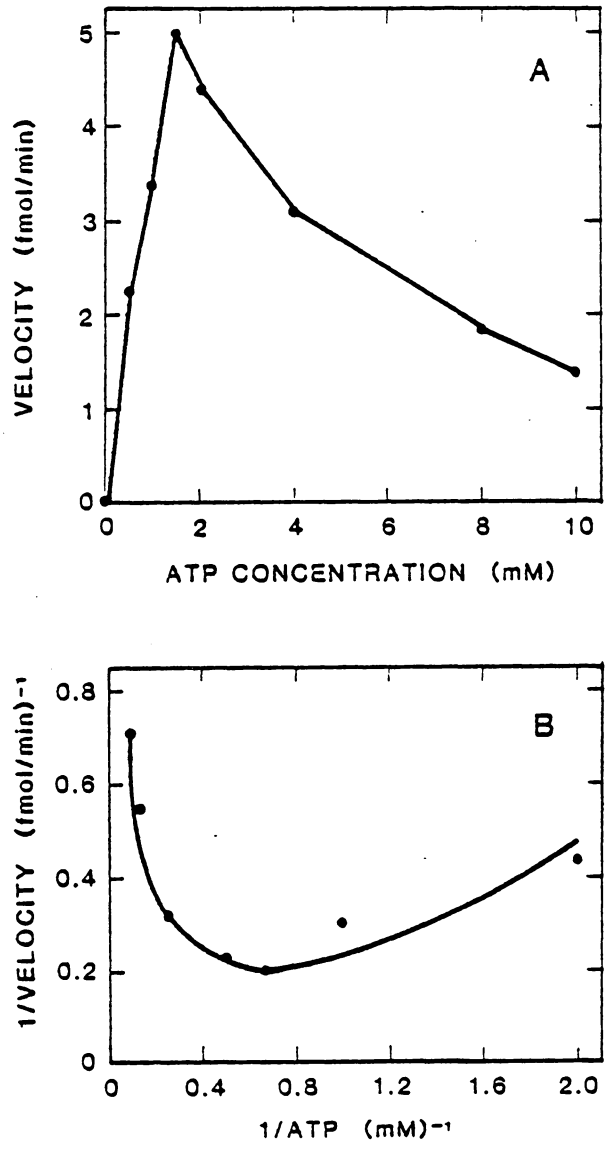

Figure 9. Kinetics of adenylate cyclase reaction with limiting divalent cation. Culmination tissue was prepared in homogenization buffer at a concentration of 0.2 mg dry wt/ml. Reaction mixture was prepared without ATP and containing 2.5 mM $MnCl_2$. ATP was added to reaction mixture aliquots at the concentration indicated. ATP was prepared freshly the day of the experiment, and was standardized spectrophotometrically. Cyclic AMP standards were run with each reaction mixture. "A" represents a plot of substrate concentration versus specific activity. Velocity values are based on the rate determinations from incubations at each substrate concentration at 0, 15, 30, and 45 minutes. "B" is a Lineweaver-Burk double reciprocal plot of the data.

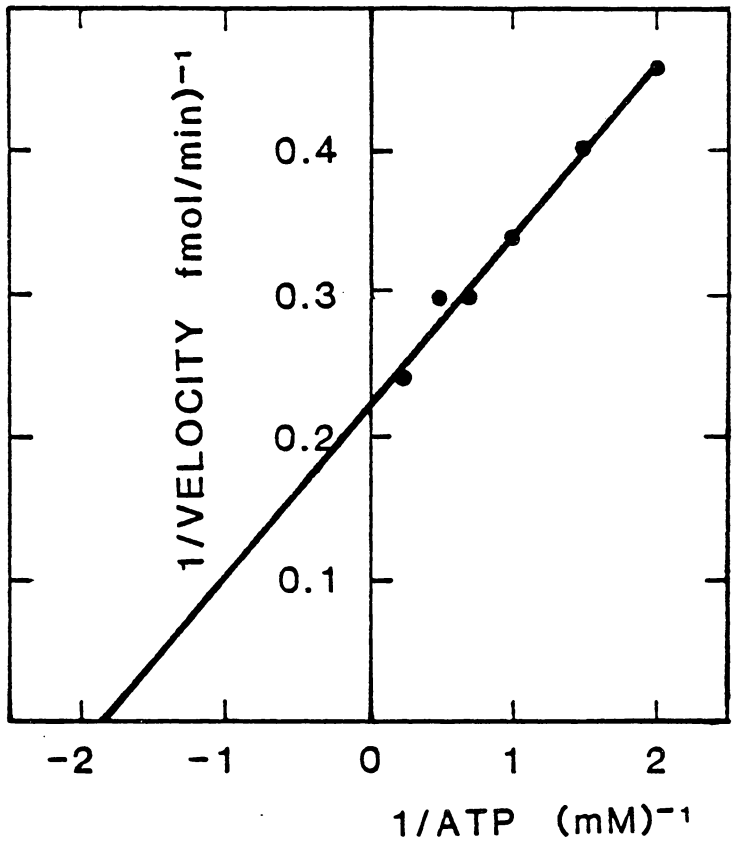

Figure 10. Kinetics of adenylate cyclase with excess divalent cation. Lineweaver-Burk double reciprocal plot of activity of crude adenylate cyclase in culmination stage tissue. The substrate range was 0.50 - 4.0 mM ATP with a 3 mM excess of $MnCl_2$ provided over the ATP concentrations. Activity was assayed as described in "Methods". Points are the average of 2 determinations, and the best-fit line was calculated by a least-squares linear regression.

(Gpp(NH)p) on the adenylate cyclase from three developmental stages of Dictyostelium discoideum. Since Mn^{+2} may have elevated activity to fully stimulated levels I tested the analogue using Mg^{+2} as the divalent cation as well. The results, shown in Table 5, are variable. In no case was the striking activation typical of hormone-sensitive adenylate cyclase by this nucleotide seen with the Dictyostelium enzyme. A slight stimulatory effect was noted at aggregation with a concentration of Gpp(NH)p of 100 μM. At preaggregation and culmination however, increasing amounts of Gpp(NH)p led to a parallel decrease of activity. This was seen in the presence of either divalent cation.

To ensure that the substrate remained at a constant, high level I performed a similar experiment but with the inclusion of an ATP-regenerating system. As shown in Figure 11, the presence of Gpp(NH)p gave a specific activity of 1.8 pmol min^{-1} mg^{-1}, and insignificant increase over the control activity of 1.6 pmol min^{-1} mg^{-1}. Typical stimulation of mammalian adenylate cyclase by the analogue in the absence of hormone is 2.5 to 3-fold over basal activity (Birnbaumer 1977).

I also tested the effects of GTP itself on the culmination stage enzyme. Inclusion of 0.01, 0.1, and 1.0 mM concentrations of the nucleotide did not lead to any change from the basal level of the Mn- or the Mg-dependent activities.

It was possible that preincubation of the enzyme with the guanyl nucleotide was a prerequisite for activation. I therefore designed several experiments to test this possibility. Preincubation with or

Table 5

Effect of Gpp(NH)p on Adenylate Cyclase Activity

Developmental Stage	Divalent Cation[a]	Gpp(NH)p (μM)[b]	Activity[c]	%Basal Activity[d]
Pre-aggregation	Mg	1	0.30	111
		10	0.22	81
		100	0.17	63
Aggregation	Mg	1	0.35	100
		10	0.30	86
		100	0.56	160
		1000	0.25	71
		10000	0.04	11
	Mn	1	2.3	110
		100	3.1	148
Culmination	Mg	1	1.7	85
		10	1.5	75
		100	1.4	70
		1000	0.2	10
	Mn	10	1.8	86
		100	1.6	76

[a]Either divalent cation was included in the reaction mixture as the Cl salt at a concentration of 2.5 mM.

[b]Gpp(NH)p was included in the reaction mixture. The concentrations represent final levels after enzyme addition.

[c]Activity is expressed as pmol min^{-1} mg^{-1}.

[d]Basal activity was measured in the absence of Gpp(NH)p for each developmental stage and with the two divalent metal ions.

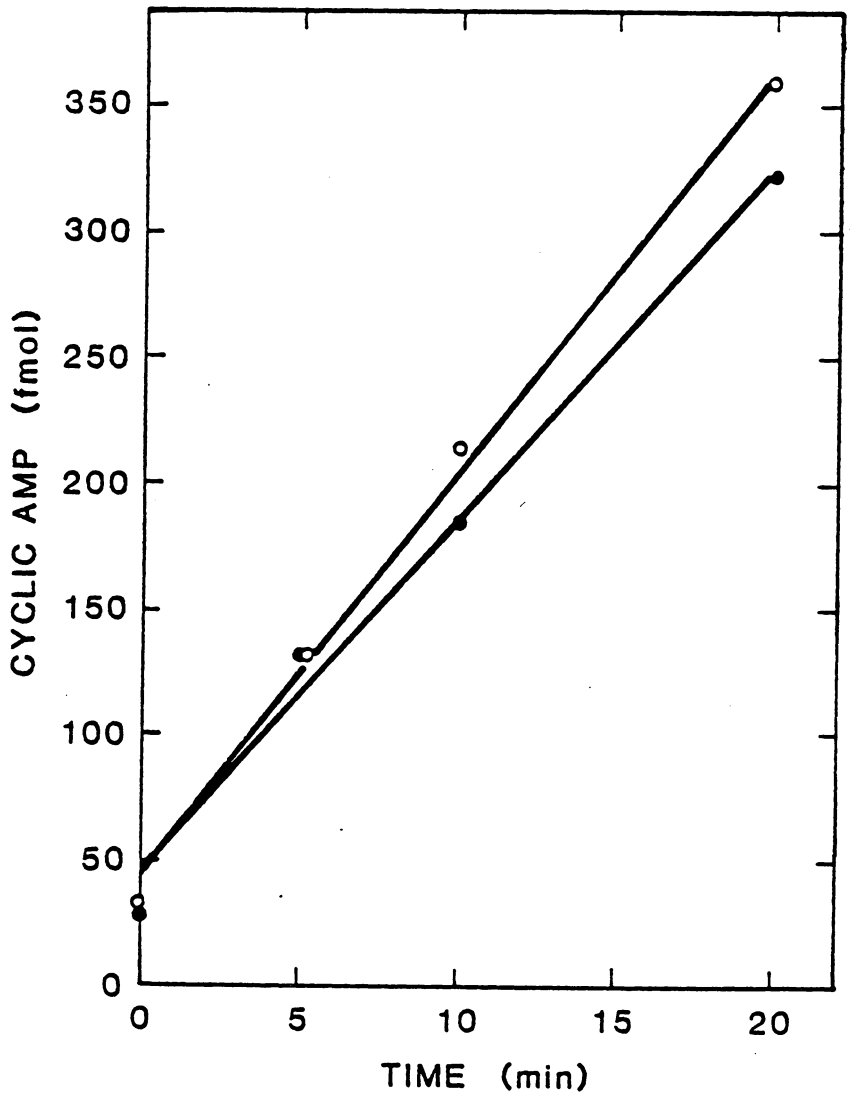

Figure 11. Effect of Gpp(NH)p on adenylate cyclase activity with inclusion of an ATP-regenerating system. Culmination stage tissue was prepared in homogenization buffer at a concentration of 1 mg/ml. 10 µl was added to test tubes. To initiate adenylate cyclase reactions, 27.6 µl of reaction mixture containing 2.5 mM $MgCl_2$ was added. Reaction mixture contained other components as described in "Methods" plus an ATP regenerating system (25 mg/ml creatine phosphokinase and 30 mM phosphocreatine). Open circles represent inclusion of 10.0 µM Gpp(NH)p, closed circles, no addition. Points are the average of duplicates.

without Gpp(NH)p for 20 minutes at 25°C of the Mg-dependent enzyme led to the total loss of activity. Preincubation under the same conditions of the Mn-dependent enzyme led to recovery of 43% of the non-preincubated control activity regardless of the presence or absence of the analogue. Similar experiments were carried out except that the preincubation was at 4°C. Greater overall activity was recovered but here again Gpp(NH)p did not effect any observable stimulation.

3.2.6. Effect of fluoride on activity: Sodium fluoride has been found to stimulate adenylate cyclase from virtually all sources. The maximally effective concentration range is 3 to 15 mM (perkins 1973). Rossomando (1974) had reported the stimulation of Dictyostelium discoideum membrane-bound adenylate cyclase by this compound although Klein (1976) had reported no stimulation. I examined the effect of 10 mM fluoride on the culmination stage enzyme with either Mg or Mn serving as the metal cation (Table 6). Inclusion of NaF in the reaction mixtures led to no significant increase in activity with Mg and Mn over the basal levels. Other systems show 2- to 20-fold increases (Perkins 1973).

As with Gpp(NH)p I also tried preincubating the enzyme in the presence of NaF. This method did not yield any observable stimulation. I also tried including a divalent cation (Mn^{+2}) in the preincubation buffer. I did not detect any significant stimulation by NaF in this instance either.

Table 6

Effect of NaF on Adenylate Cyclase Activity

NaF[a]	Divalent Cation (2.5 mM)	Specific Activity[b]
-	Mg	0.73 ± 0.15
+	Mg	0.85 ± 0.08
-	Mn	2.1 ± 0.3
+	Mn	2.8 ± 0.8

[a]NaF was included as a component in the reaction mixture at a final concentration of 10 mM.

[b]Production of cyclic AMP was measured by the RIA as described in Methods. Specific activity is expressed as pmol min^{-1} mg^{-1}. Values represent the mean ± standard deviation of triplicate determinations of 15 minute incubations.

3.2.7. Validation of the assay method: The inability to demonstrate any significant stimulatory effects by guanyl nucleotides or fluoride on the Dictyostelium enzyme led me to conduct a parallel study using rat cerebrum as a control tissue. I did this to verify that the lack of stimulation was an organism-specific effect rather than a phenomenon due to the assay conditions. A frozen sample of a washed particulate fraction of rat cerebrum (Johnson and Sutherland 1974) was diluted such that activity would fall in the measurable range of the RIA. The tissue was homogenized in a manner analogous to that of Dictyostelium tissue preparation. Assay of adenylate cyclase activity gave results consistent with what would be expected for mammalian enzyme. Inclusion in the reaction mixture of 10 μM Gpp(NH)p and of 10 mM NaF led to 2.5-fold and greater than 4-fold activation respectively of the basal Mg-dependent activity. With both compounds present together over 4-fold activation was apparent. I concluded that there were no problems inherent in the assay method I was using.

3.2.8. Effect of cholera toxin: The exotoxin produced by the bacterium Vibrio cholerae irreversibly activates vertebrate adenylate cyclase (Gill 1977). Fragment A_1 of the toxin catalyzes an ADP-ribosylation of the 42,000 MW GTP-dependent regulatory protein (Gill and Meren 1978) thereby altering the GTPase function of the regulatory moeity. The GTPase activity is hypothesized to be necessary for the deactivation of adenylate cyclase (Cassel and Selinger 1977). The result of the covalent modification is the maintenance of the catalytic unit in a

persistently activated state. Leichtling et al. (1981) have reported the occurrence of a membrane-associated GTP-binding protein in Dictyostelium discoideum. This protein has a molecular weight of 42,000 daltons and served as substrate for ADP-ribosylation catalyzed by cholera toxin. As in the vertebrate systems this ribosylation was NAD^+-dependent. The authors speculated that their GTP-binding protein might be a component of the Dictyostelium adenylate cyclase and may function to mediate the stimulation of this enzyme by cAMP in a manner analogous to stimulation of the vertebrate enzyme by hormones. I therefore thought it would be of interest to see if cholera toxin could actually stimulate the Dictyostelium enzyme, especially in light of the fact that I had not been able to demonstrate activation of the enzyme by guanyl nucleotides. In vertebrate systems the toxin will stimulate the basal adenylate cyclase activity as well as the hormonally stimulated activity (Enomoto and Gill 1980); I thus felt that such an activation was possible in Dictyostelium even in the absence of exogenous cyclic AMP "hormone".

The following protocol for the cholera toxin experiments was derived from several published methods (Cassel and Selinger 1977, Enomoto and Gill 1980, Leichtling et al. 1981a): Preactivation of cholera toxin (0.5 mg/ml) was accomplished by incubating it with 20 mM DTT at 37°C for 30 minutes. After this preactivation step, which liberates the active fragment A_1, the activated toxin was added to reaction mixtures containing 1.5 mM NAD^+ with or without 10 μM GTP. GTP is required for the activation of adenylate cyclase by the toxin

and for the expression of maximum catalytic activity of the toxin-activated enzyme (Nakaya et al. 1981). Enomoto and Gill (1980) have reported that the GTP analogue, GPP(NH)p, at a final concentration of 1 to 3 μM will also support the toxin-catalyzed reaction so I also tried the experiments including this compound. I utilized an extended incubation period in case the activation step required more time than the ten to thirty minute span over which I usually assayed adenylate cyclase activity.

As Figure 12 shows, in the absence of a guanyl nucleotide, culmination stage, Mn-dependent adenylate cyclase is not affected by the toxin, even over a 60 minute incubation period. Figure 13 shows the results of another experiment in which either GTP or the analogue were included along with the toxin. The basal Mn-dependent adenylate cyclase activity was 3.0 pmol min^{-1} mg^{-1}. With the inclusion of toxin and GTP in the reaction mixture I observed an initial rate (stable over at least 20 minutes) of 3.5 pmol min^{-1} mg^{-1}; not significantly greater than the basal level. Inclusion of the analogue, Gpp(NH)p, led to a specific activity of 1.5 pmol min^{-1} mg^{-1}, a decrease equal to half the basal rate. I also tested the effect of cholera toxin on the Mg-dependent activity. As Figure 14 shows, very little difference between the initial rates of the basal activity (1.3 pmol min^{-1} mg^{-1}) and the toxin-treated enzyme (1.1 pmol min^{-1} mg^{-1}) was noted. I concluded that there was no demonstrable toxin activation under any of the tested conditions.

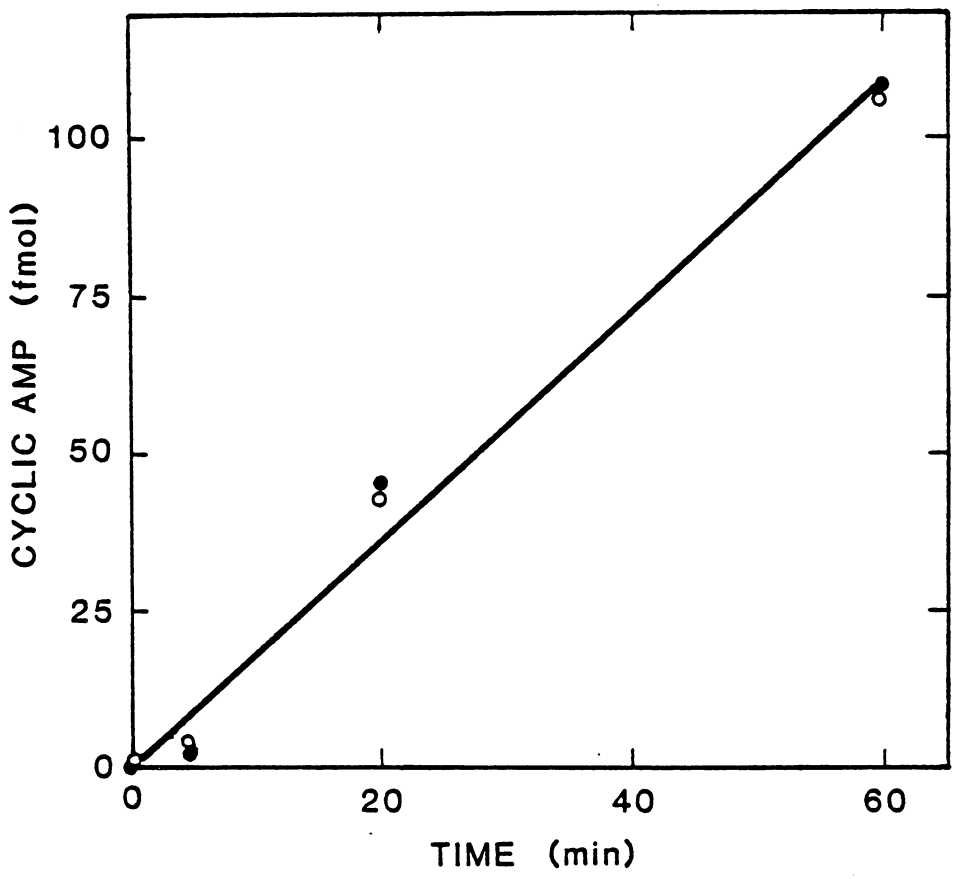

Figure 12. Effect of cholera toxin on culmination Mn-dependent adenylate cyclase activity. I. Absence of guanyl nucleotide. 0.1 mg/ml culmination stage tissue was incubated at 23°C for the times indicated in reaction mixture containing (o) or not containing (•) activated cholera toxin and NAD^+ (see "Results" for protocol). Points represent the mean of 2 determinations. This line, which is identical for the separate conditions, is the best-fit determined by a least-squares linear regression.

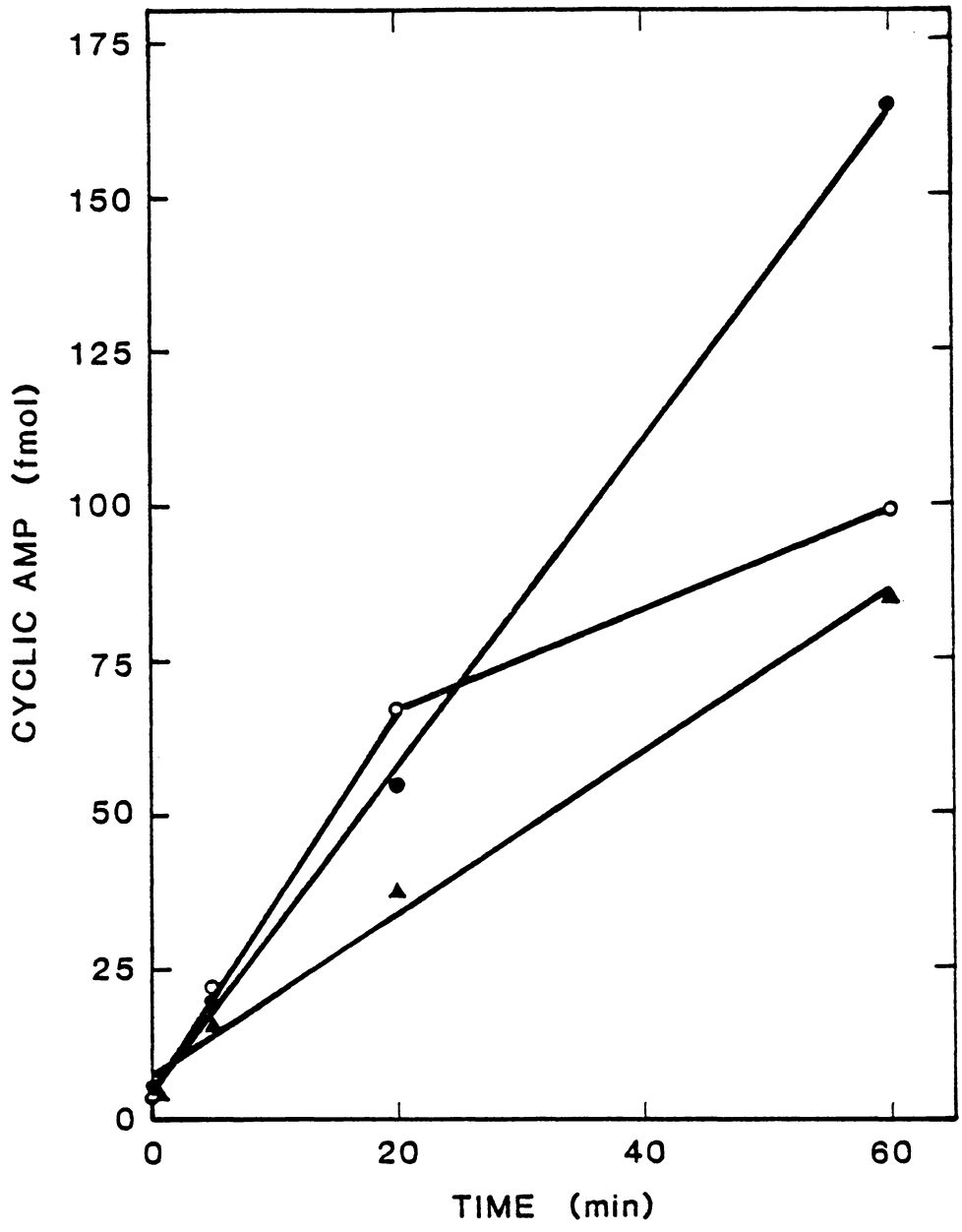

Figure 13. Effect of cholera toxin on culmination Mn-dependent adenylate cyclase activity. II. Presence of guanyl nucleotide. 0.1 mg/ml culmination stage tissue was incubated at 23° for the times indicated in reaction mixture with either no addition (●) or containing activated cholera toxin, NAD^+ and with either 10 μM GTP (o) or with 10 μM Gpp(NH)p (▲). Points represent the mean of two determinations, and the best-fit lines were calculated by a least-squares linear regression.

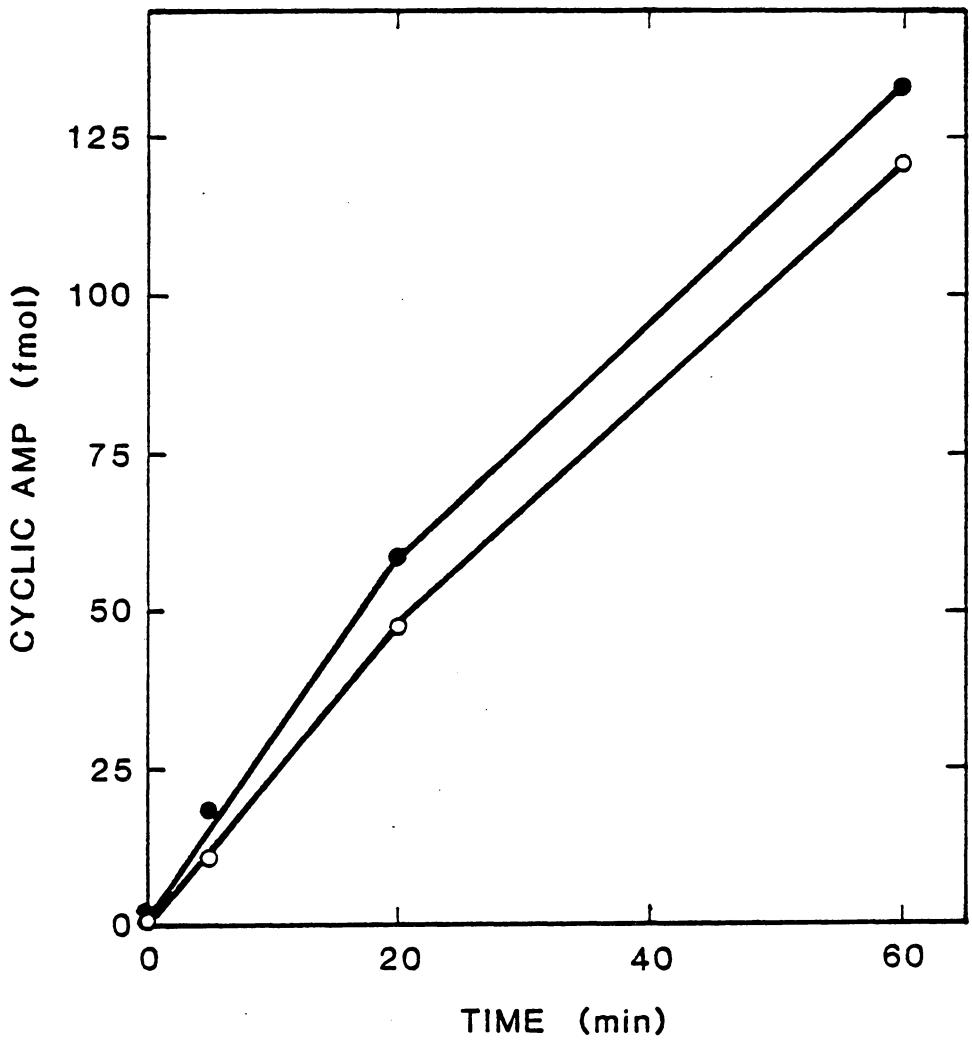

Figure 14. Effect of cholera toxin on culmination Mg-dependent adenylate cyclase activity. 0.5 mg/ml culmination stage tissue was incubated at 23°C for the times indicated in reaction mixture with no addition (●) or containing activated cholera toxin, NAD^+ and 10 μM GTP (o). Points represent the mean of two determinations, and best-fit lines (0 through 20 minutes) were calculated by a least-squares linear regression.

3.2.9. Effects of ammonia and of ionic strength on adenylate cyclase activity: Schindler and Sussman (1979) have suggested that ammonia, by inhibiting the production or the release of cyclic AMP, causes localization of cyclic AMP within the developing multicellular aggregate. This localized accumulation of the morphogen can thereby control the course of pattern formation. I examined the interaction of ammonia and adenylate cyclase to see if this compound was preventing cyclic AMP production by inhibiting its synthesis. Ammonium chloride was included in the enzyme assay at concentration from 0 to 140 mM (Fig. 15). A loss of activity resulted when greater than 14 mM was present. Only 4% of the control activity remained when 140 mM ammonium chloride was included. Such levels of ammonia accumulate in the stalk cells at the culmination stage (Wilson and Rutherford 1978). Since I was using a chloride salt I decided to look at the effect of increased ionic strength on adenylate cyclase using NaCl. I also tested another ammonium compound, ammonium acetate (CH_3COONH_4) for its effect on activity. Figure 16 shows that 100 mM NaCl causes a 58% loss in activity, 140 mM a 71% loss. CH_3COONH_4 at 100 mM and 140 mM caused a 36% and 82% loss in activity respectively. It appears that increasing ionic strength has a deleterious effect on enzyme activity, and that the inhibition by ammonium chloride may actually be a manifestation of ionic strength. If ammonia does have an effect it will be difficult to demonstrate because of this effect of ionic strength on the enzyme activity.

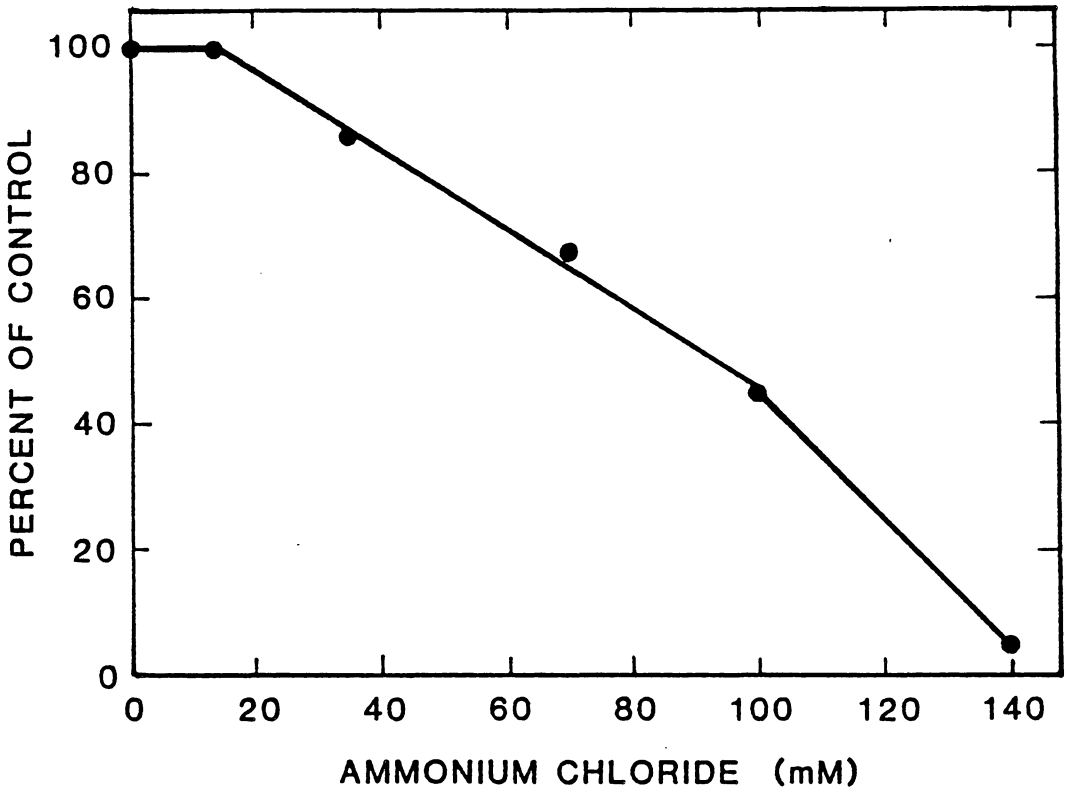

Figure 15. Dose response curve for ammonium chloride effect on adenylate cyclase. Culmination stage tissue (0.3 mg/ml) was assayed for Mg-dependent adenylate cyclase activity in the presence of the indicated concentrations of NH_4Cl. Incubations were carried out for 10 minutes at 23°C. Points represent the average of three determinations.

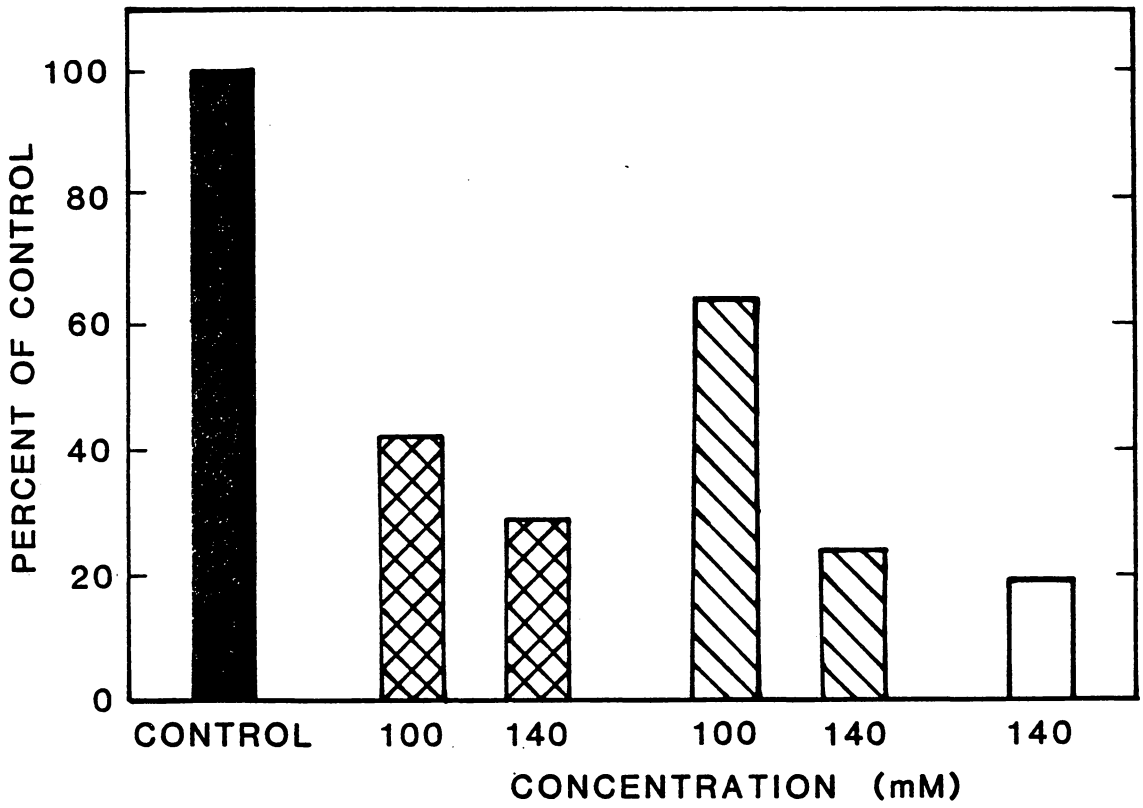

Figure 16. Effect of ammonia and of salt on adenylate cyclase. Culmination stage tissue (0.3 mg/ml) was assayed for Mg-dependent adenylate cyclase activity in reaction mixtures containing no addition (closed column), sodium chloride (cross-hatched column), ammonium acetate (hatched column), or ammonium chloride (open column). The concentrations of the compounds are those after enzyme addition. Incubations were carried out at 23°C for 10 minutes in triplicate.

3.2.10. Effects of EGTA and calcium: Calcium activator proteins (calmodulin) have been implicated in the modulation of phosphodiesterase and adenylate cyclase activities from various sources (Klee et al. 1980, Wang and Waisman 1979). Calmodulin has been identified and characterized in Dictyostelium discoideum (Clarke et al. 1980, Bazari and Clarke 1981). It behaves similarly to bovine brain calmodulin when subjected to ion exchange and gel filtration chromatography and on isoelectrofocusing gels, but it has only one-third the specific activity for the activation of brain phosphodiesterase. However, no data ascribe a regulatory function to the protein with respect to any Dictyostelium enzymes. I wanted to examine the possible role calcium and calmodulin might play in regulating adenylate cyclase.

If Dictyostelium adenylate cyclase was being activated by a calcium-dependent protein (presumably present in the crude homogenates), addition of the calcium chelator EGTA to the reaction mixture would lead to a diminution of activity. Lynch et al. (1977) showed that in rat brain 40 μM is sufficient to reduce adenylate cyclase to a basal level of activity. They accomplished a titration over the range of 0 to 200 μM EGTA. I performed a similar titration of the Dictyostelium Mg-dependent adenylate cyclase by adding EGTA to the reaction mixture at concentrations of 0, 10, 20, 40, 60, 80, 100, 250, and 500 μM. I then incubated triplicate samples for 10 minutes and assayed for activity. The basal level of activity was 1.4 pmol min^{-1} mg^{-1}. There was no obvious trend of an activity decrease as increasing amounts of EGTA were added. The activities fluctuated between 0.9 and 1.3 pmol

min^{-1} mg^{-1}. Also, homogenization of the tissue with EGTA present (to bind the available calcium) followed by assay in the presence of micromolar levels of calcium did not lead to any significant stimulation or inhibition of activity (data not shown). Thus I have been unable to demonstrate any obvious regulatory role for calcium with respect to the adenylate cyclase in Dictyostelium.

3.2.11. Effect of folic acid on adenylate cyclase: Folic acid, acting as a chemoattractant for Dictyostelium amoebae, probably functions to localize the bacterial food source for the organism (Van Driel 1981). Repeated pulses of folic acid (or cyclic AMP) stimulate cell development to aggregation competence (Wurster and Schubiger 1977). Wurster et al. (1979) also showed that such pulses of folic acid will induce developmental processes in agip 71, a morphogenetic mutant of D. discoideum; cells of this mutant normally do not become aggregation competent. Bernstein et al. (1981) showed that the two chemoattractants (cyclic AMP and folic acid) cross-react in the regulation of their inactivating enzymes, folate deaminase and cyclic nucleotide phosphodiesterase. I thus thought it would be worthwhile to examine the effect of folic acid on another component of the biochemical network controlling cyclic AMP, namely, adenylate cyclase. I included folic acid in the reaction mixtures at final concentrations of 5×10^{-10}, 5×10^{-7}, and 5×10^{-4} M. The two lower concentrations had no effect at all on the enzyme activity, but at 5×10^{-4} M a 70% decrease in activity was observed. I do not know if this represents a specific inhibition of the enzyme.

3.2.12. Effects of other compounds on activity: Anderson et al. (1978) have found that low concentrations (1-2.4 μg/ml) of trypsin added to incubation mixtures stimulate adenylate cyclase activity in cultured fibroblasts, whereas high concentrations progressively inactivate the enzyme. This effect is also observed with other endopeptidases (elastase, papain, and α-chymotrypsin). The effect was found to be maximal in the presence of GTP. I tested trypsin alone and in combination with GTP to determine the effect on the Dictyostelium enzyme. No activation was seen either by inclusion of these components in the reaction mixture or in a preincubation step (data not shown). Some inhibition was observed; it was probably due to proteolysis of the catalytic unit itself.

Several other possible modulators of adenylate cyclase were tested for their effects on the Dictyostelium enzyme. Inorganic phosphate (supplied as sodium phosphate) and adenosine at 100 mM and 1 mM, respectively, were included as reaction mixture components or in a preincubation step. Neither of the compounds elicited changes from the control activity (data not shown).

The mammalian hormone, insulin, has been found to occur in simple, unicellular eukaryotes including Neurospora and Aspergillus as well as in the extracts of the prokaryote, Escherichia coli K-12 (LeRoith et al. 1980). Abou-Sabé and Reilly (1978) found that insulin had a biphasic effect on the membrane-bound adenylate cyclase of E. coli, inhibiting at concentrations of 3.75 to 25 μ-units and stimulating activity at concentrations of 225 and 375 μ-units. Insulin inhibits the adenylate

cyclase activity of plasma membranes isolated from adipocytes (Torres et al. 1978), liver cells (Illiano and Cuatrecasas 1972) and fibroblasts (Jimenez de Asua et al. 1973). I was interested to see the effect that insulin had on the Dictyostelium in light of the variety of systems the peptide hormone either occurs in or has effects on adenylate cyclase activity. Addition of 10 or 100 μ units had no effect on activity while addition of 1000 μ-units caused a 40% decrease in activity from the control levels. Whether this inhibition is significant in vivo is open to question, as I am unaware of the existence of insulin in D. discoideum.

3.2.13. Endogenous modulator of adenylate cyclase activity: As I was unable to find any modulation of enzyme activity by ligands known to affect adenylate cyclase of other eukaryotic systems, I decided to look for an endogenous molecule or "hormone" that might function in the control of the Dictyostelium enzyme. Since a peak of adenylate activity occurs during chemotaxis and aggregation (Pahlic and Rutherford 1979, Klein 1977) I reasoned that a stimulatory modulator might be present at this stage. I collected the extracellular fluid from aggregated cells by harvesting them in a minimal amount of water and saving the 1800 x g supernatant fluid. This material, as well as the cells, was lyophilized. The lyophilized extracellular material was reconstituted at 100 mg/ml in Tris-HCl buffer, pH 7.5, and a 1:1 preincubation mixture was prepared consisting of this extracellular material and either an aggregation or culmination stage tissue homogenate. Preincubation was conducted at 4°C for 20 minutes, then the samples were assayed for adenylate cyclase

activity in the presence of Mg^{+2}. The enzyme was completely inhibited by the extracellular material. I found that the inhibitory activity was not dialyzable and that it contained some phosphodiesterase activity. However, after boiling the preparation I could recover 86% of the inhibitory activity and this heat-treated preparation would not degrade authentic cyclic AMP. After boiling, a precipitate was present which would go into solution upon mixing. Separation of the sedimentable material by centrifugation at 20,000 x g showed that the supernatant fraction contained the inhibitory activity. I changed the protocol for inhibitor collection such that the extracellular wash was centrifuged at 20,000 x g for 15 minutes before lyophilization. The inhibitor, made by reconstituting the lyophilized material in Tris-HCl buffer, pH 7.5, boiling for 20 minutes and collecting the 20,000 x g supernatant fraction, was stable for at least 10 days at 7°C.

Characterization of the inhibitor revealed that a preincubation step was not required for demonstration of adenylate cyclase inhibition. The extracellular material was capable of inhibiting adenylate cyclase from any stage in the life cycle. The maximum inhibitory activity was retained by an XM 100A Amicon membrane which has a retentivity of greater than 100,000 daltons. However, about 40% of the maximal inhibitory activity was also found in the eluates from the XM 100A and an XM 50 membrane. This may indicate that the inhibitor is actually a small molecule that either forms self-aggregates or binds to another, larger molecule. Further characterization of this inhibitor was carried out by Cripps and Rutherford (1981) using washed cell extracts.

3.3. Cell Specific Localization of Adenylate Cyclase Activity

3.3.1. Microassay of adenylate cyclase in culminating individuals: I adapted the radioimmunoassay to measure adenylate cyclase in single slime mold individuals or in dissected pieces weighing from 0.04 μg. Initially I looked for a correlation between the weight and enzyme activity in sections dissected from the spore masses of culminating individuals. I expected to see a positive correlation, that is, as the weight increased activity would increase. However, as Figure 17 shows, this was not the case. A correlation coefficient of +0.19 ($p>0.1$) was obtained indicating an insignificant, low positive correlation. Since there was not a strong correlation I reasoned that another factor might be involved; perhaps position within the spore mass. I then dissected culminating individuals keeping track of the location of each section. Assay of ten individuals gave good evidence that a gradient of adenylate cyclase activity existed. The highest activity was at the base of the spore mass and decreased towards the tip (Fig. 18). The correlation coefficient of the relationship between location and specific activity was +0.92, a strong, significant ($p<0.001$) correlation. Another obvious result is that enzyme activity was localized in the spore mass and appeared to be completely absent in the stalk.

To confirm the cell specific localization I assayed spore mass sections and stalks. I pooled stalks to yield weights comparable to those of the spore mass sections. I thus avoided artifactual differences that could be attributable to the disparity between very light stalks and heavier spore pieces. The results are shown in Table 7.

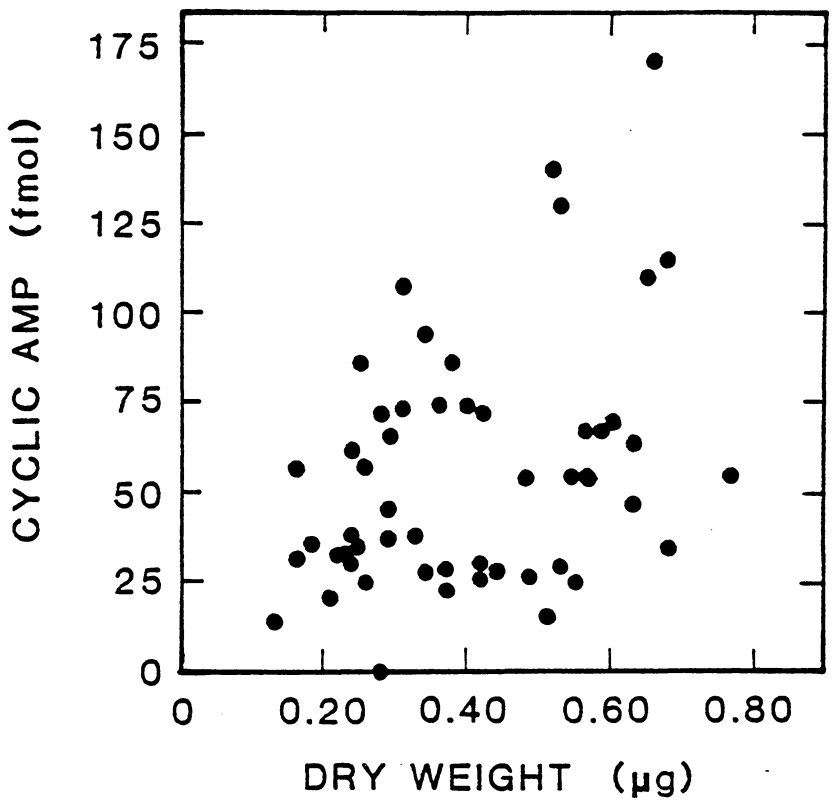

Figure 17. Relationship between weight of section and enzyme activity. Scatter diagram of dry weight of sections in micrograms plotted against adenylate cyclase activity represented by femtomoles of product (as determined in a 60 minute assay). The data are the results of two experiments. The best-fit line was calculated by a least-squares linear regression. The correlation coefficient is +0.19. The mean weight of the sections was 0.40 $\pm$ 0.16 µg.

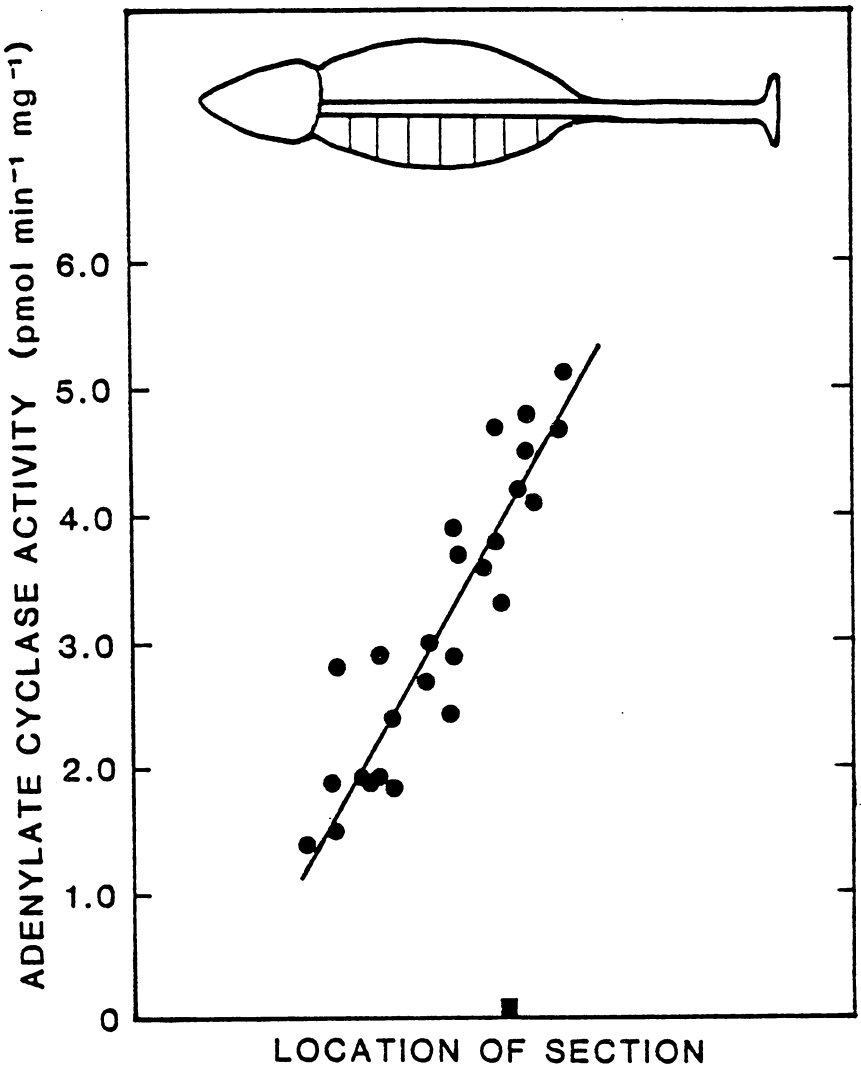

Figure 18. Localization of adenylate cyclase activity in the culmination stage. Data from a single individual plotted to show representative pattern found in ten replicates. Spore masses were halved in the longitudinal axis. The stalks were removed, then the half was bisected along the longitudinal axis (yielding quarter spore mass sections). Adenylate cyclase activity of serial sections of each spore mass quarter (o), and isolated stalk (■), sections are represented according to position in individual. The average weight of the sections was 0.26 ± 0.07 µg. The best-fit line was calculated by a least-squares linear regression, and was found to have a correlation coefficient of + 0.92.

Table 7

Localization of Adenylate Cyclase

I. Spore Mass Sections[a]		
Individuals Assayed	Weight (μg)	Specific Activity[b]
1	0.33	2.9
2	0.15	2.5
3	0.35	2.0
4	0.22	1.4
5	0.21	2.2
6	0.22	1.7
7	0.26	1.4
8	0.24	2.2
		$\bar{x} = 2.0 \pm 0.5$
II. Stalks		
Number of Stalks Assayed[c]	Weight (μg)	Specific Activity
3	0.24	Not detectable[d]
4	0.27	1.1
4	0.24	Not detectable
3	0.25	Not detectable
3	0.22	Not detectable
5	0.27	Not detectable

[a] A section from the middle of the prespore mass of culminating individuals was dissected out, weighed, and assayed as described in "Methods".

[b] Specific activity is expressed as pmol min^{-1} mg^{-1} dry weight of section.

[c] Stalks from several individuals were pooled in order to give weights comparable to those of the spore mass sections.

[d] Levels of cyclic AMP less than 18 femtomoles were not detectable.

In all cases but one there was no detectable activity for the stalks as compared to a mean activity of 2.0 $\pm$ 0.5 pmol min^{-1} mg^{-1} for the mid-spore mass sections.

Since phosphodiesterase activity is known to be present in the stalk (Brown and Rutherford 1980) I tested whether, under my assay conditions, the lack of apparent adenylate cyclase activity was due to the degradation of the cAMP reaction product by the phosphodiesterase. Table 8 shows the results of an experiment in which I incubated stalks or spore sections in reaction mixture lacking substrate (ATP) and with or without added authentic cyclic AMP at the same magnitude as the cyclic AMP level produced during the 60 minute incubation period of tissue sections in the presence of complete reaction mixture. With either cell type cyclic AMP levels equivalent to those of the control (no tissue present) were recovered, thereby showing that no product degradation occurred under these conditions.

I also tested whether endogenous levels of cyclic AMP in the pre-spore tissue contributed to the adenylate cyclase activity measured. The fact that sections not subjected to incubation in reaction mixture (that is, reaction mixture addition was followed immediately by addition of acetylating reagent) did not yield apparent activity contradicted this possibility. Another approach taken to eliminate this possibility was the use of paired tissue sections, that is serial sections of the pre-spore mass that were bisected such that each half of the pair was considered equivalent and could serve as its own control. I incubated a half of the paired sections in reaction mixture in the presence and

Table 8

Control for Phosphodiesterase Degradation of Adenylate Cyclase Reaction Product

Cell Type	Assay Conditions[a]	cAMP Recovered[b]
Control - No tissue	+ cyclic AMP	77 ± 8.1
Stalk[c]	+ cyclic AMP	78 ± 9.9
	- cyclic AMP	0
Spore[d]	+ cyclic AMP	78 ± 8.1
	- cyclic AMP	0

[a]Tissue pieces were incubated at 23°C for 60 minutes in adenylate cyclase reaction mixture as described in "Methods" except that substrate (ATP) was not included. Approximately 80 femtomoles of cyclic AMP was included where indicated by a +.

[b]These data represent the mean of 9 replicates in femtomoles as measured by radioimmunoassay.

[c]Stalks were pooled to give a mean dry weight per tube of 0.23 ± 0.09 µg.

[d]Spore mass sections had a mean dry weight per tube of 0.28 ± 0.09 µg.

the other half in the absence of the ATP substrate. If endogenous levels of cyclic AMP were being measured I should have been able to detect them even if the enzyme substrate was not included in the reaction mixture. The results of this experiment, shown in Table 9, indicated that this was not the case. Except for one instance out of eleven, cyclic AMP was detected only when the substrate for adenylate cyclase was present during the incubation.

3.3.2. Microassay of adenylate cyclase in pre-culmination stages: In order to relate the gradient found in the terminal differentiation stage to the overall scheme of progressive development and pattern formation I wanted to determine if a localization (and gradient) existed in stages prior to culmination. Upon examination of the preculmination "Mexican-hat" stage I found no obvious gradient across the individual. I decided to look for differences between the tip, "sub-tip" (region below the tip) and the edge of the individual (Fig. 19). Table 10 shows that the cells of the tip (pre-stalk cells) have the lowest specific activity, whereas those from the edge of the mass (pre-spore cells) have an activity that is over 2-fold greater. Analysis of the data with a paired t-test showed that there is a significant ($p<0.001$) difference in activity between these regions. Those sections assayed in the region below the tip gave variable activities with the mean intermediately between those of the tip and edge. The variability was probably due to the slight differences in the actual location dissected or to the developmental asynchrony of the different individuals.

Table 9

Control for Endogenous Cyclic AMP using Spore Mass Paired Sections[a]

Paired Sections	Enzyme Activity[b]					
	+	-	+	+	-	-
1	3.0	ND				
2	3.3	ND				
3	1.2	ND				
4	ND	ND				
5	0.9	ND				
6	1.8	ND				
7	1.8	1.3				
8	3.8	ND				
9	3.1	ND				
10	2.7	ND				
11	1.4	ND				
12			1.7	ND		
13			2.7	2.6		
14			2.8	3.8		
15					ND	ND

[a]The base and tip were removed from culmination spore masses. The spore mass was then halved longitudinally, the stalk removed, and the mass was then sectioned serially. Each serial section was then bisected. Each half of the pair was considered equivalent and served as its own control.

[b]Halves of the spore mass pairs were assayed in reactions mixture either containing (+) or not containing (-) 1.5 mM ATP substrate. Adenylate cyclase specific activity is expressed as pmol min^{-1} mg^{-1}. ND means not detectable and indicates cyclic AMP levels less than 20 femtomoles.

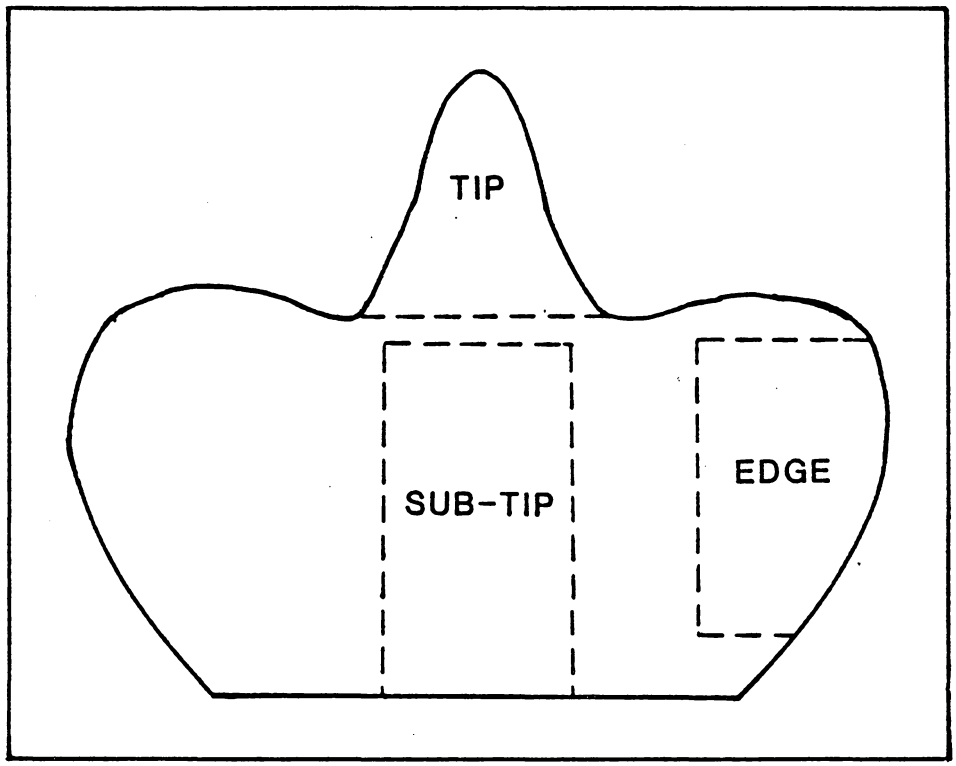

Figure 19. Regions of the pre-culmination Mexican hat. Areas indicated were dissected from individuals as described in "Methods".

Table 10

Localization of Adenylate Cyclase in Preculmination Stage

Individual[a] #	Activity (pmol min^{-1} mg^{-1}) Tip[b]	Sub-Tip	Edge
1	1.4	1.7	2.4
2	1.4	1.4	2.4
3	1.1	2.4	2.8
4	1.0	1.1	2.3
5	1.3	2.9	3.6
6	1.2	3.4	3.6
7	2.4	---	3.5
8	1.2	1.8	2.7
9	2.5	4.7	4.6
10	3.0	3.3	5.2
11	1.9	4.3	3.3
12	1.8	2.4	2.6
13	0.8	1.4	1.9
14	0.9	---	3.2
15	0.6	3.3	2.8
16	1.6	2.8	3.4
17	0.8	---	1.9
18	1.3	1.9	2.9
19	1.6	2.6	2.9
20	1.0	2.7	1.8
21	0.6	1.2	1.2
Σ	29.4	45.3	61.0
$\bar{x}$	1.4 $\pm$ 0.6	2.5 $\pm$ 1.0	2.9 $\pm$ 0.9

[a]Sections from 21 individuals were assayed for adenylate cyclase as described in "Methods". The data from three experiments are tabulated.

[b]The average weights of the tip, sub-tip, and edge sections were 0.38 $\pm$ 0.16, 0.44 $\pm$ 0.18, and 0.40 $\pm$ 0.13, respectively.

The next developmental stage I examined was the slug. This study was carried out in response to a request to collaborate with K. Deml from M. Sussman's laboratory at the University of Pittsburgh. I was provided with a series of induced slugs as described in "Methods". The slugs, developed on unbuffered agar, migrate indefinitely unless provided with certain environmental cues. These include the presence of buffered agar and exposure to overhead light. Figures 20 through 23 show representative results of the assay for adenylate cyclase from the slug series. No obvious localization was apparent in any of the induced stages. The same "pattern" was found with slugs grown in our laboratory (Fig. 24). It may have been the case that the Mn^{+2}-dependent activity is in a fully activated state, and differences in activity levels due to regulatory phenomena would only be expressed when Mg^{+2} was present as the required divalent cation. Figure 25 shows that when Mg^{+2} was used as the divalent cation instead of Mn^{+2} in the reaction mixture, a similar activity distribution was obtained but at an overall lower activity level.

The initial developmental stage was also examined. I did not find any activity localization in the streaming aggregate. A representative individual at this stage is shown in Figure 26.

3.3.3. Mechanism of the adenylate cyclase localization: The absence of adenylate cyclase activity in the stalk cells could be due to the presence in this cell type of an inhibitor such as that previously described (see Results 3.2.13). To test this possibility I bisected pre-spore mass sections and assayed half for adenylate cyclase as a

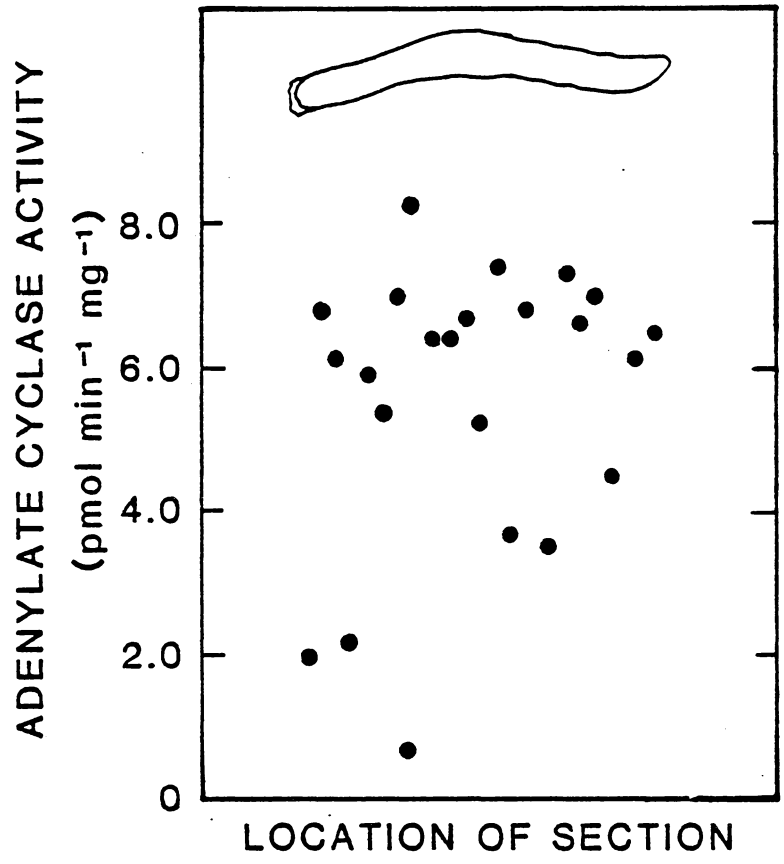

Figure 20. Adenylate cyclase activity distribution in uninduced slug 1-2. Data from a single individual plotted to show a typical pattern found in six replicates. Serial sections of the slug are represented according to position in the individual. The average weight of sections was 0.28 ± 0.08 µg. The slug was developed in the laboratory of Dr. M. Sussman, University of Pittsburgh as described in "Methods".

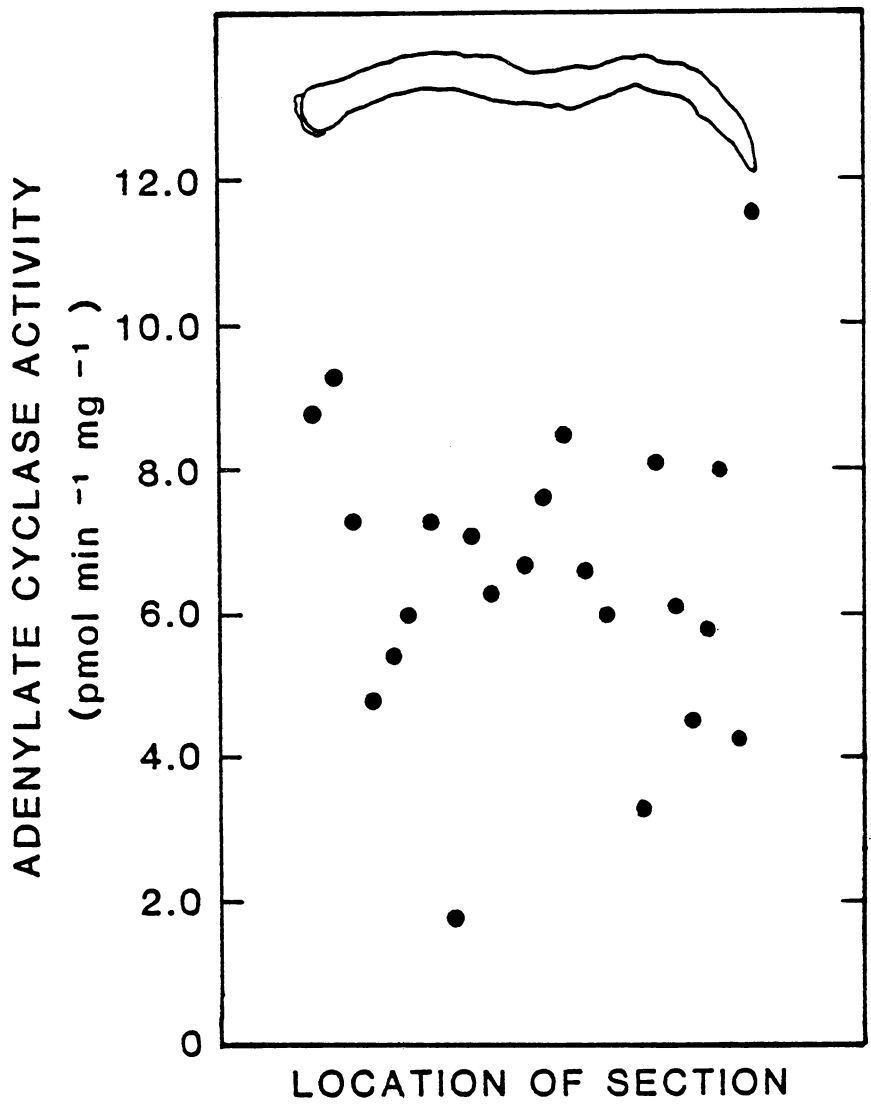

Figure 21. Distribution of adenylate cyclase activity in an induced slug ("tip up") 2-2. Data from a single individual plotted to show a typical pattern found in five replicates. Serial sections of the slug are represented according to position in the individual. The average weight of the sections was 0.34 $\pm$ 0.10 µg.

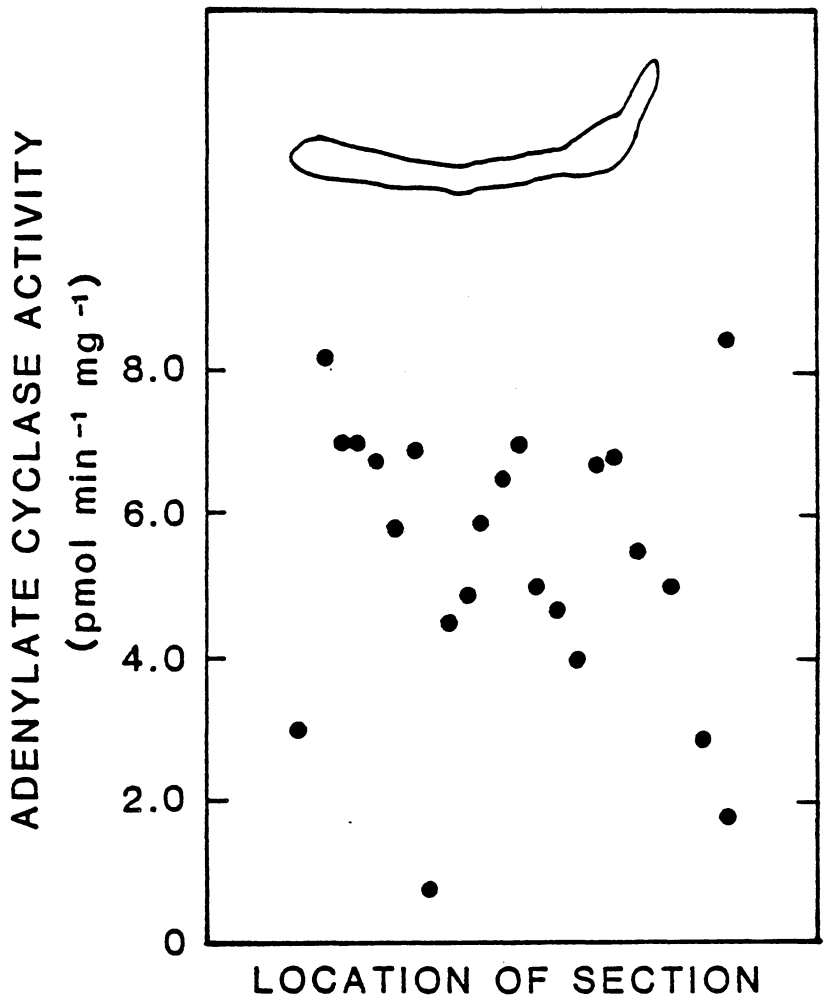

Figure 22. Distribution of adenylate cyclase activity in an induced slug ("bulge behind tip") 3-6. Data from a single individual plotted to show a typical pattern found in four replicates. Serial sections of the slug are represented according to position in the individual. The average weight of the sections was 0.30 $\pm$ 0.10 µg.

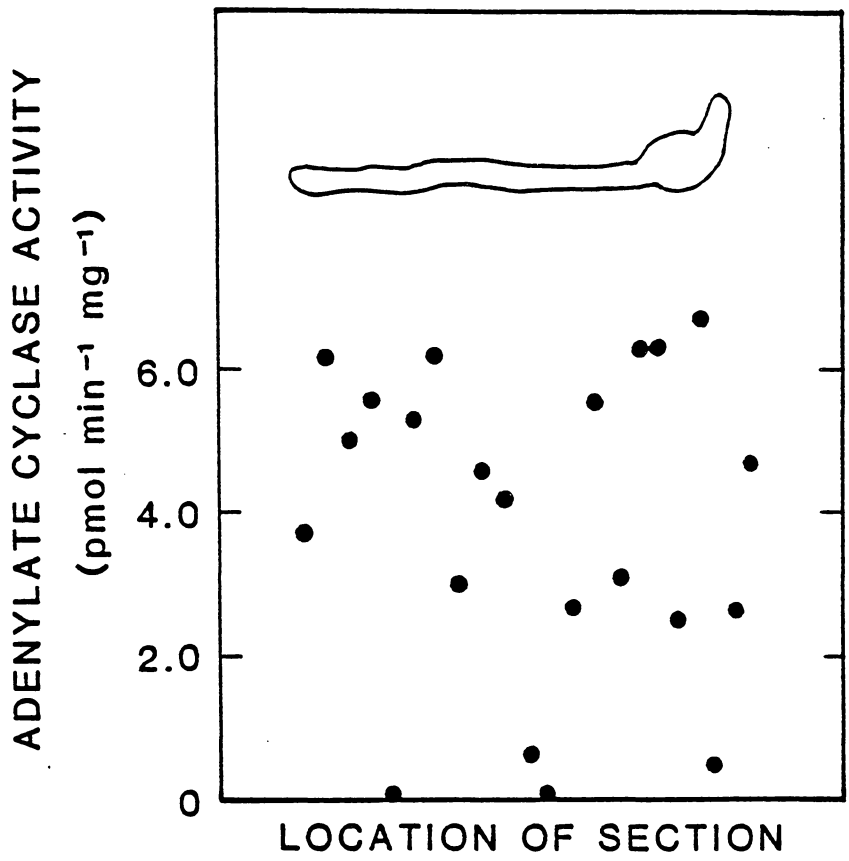

Figure 23. Distribution of adenylate cyclase activity in an induced slug ("bottle") 4-1. Data from a single individual plotted to show a typical pattern found in two replicates. Serial sections of the slug are represented according to position in the individual. The average weight of the sections was 0.15 $\pm$ 0.10 µg.

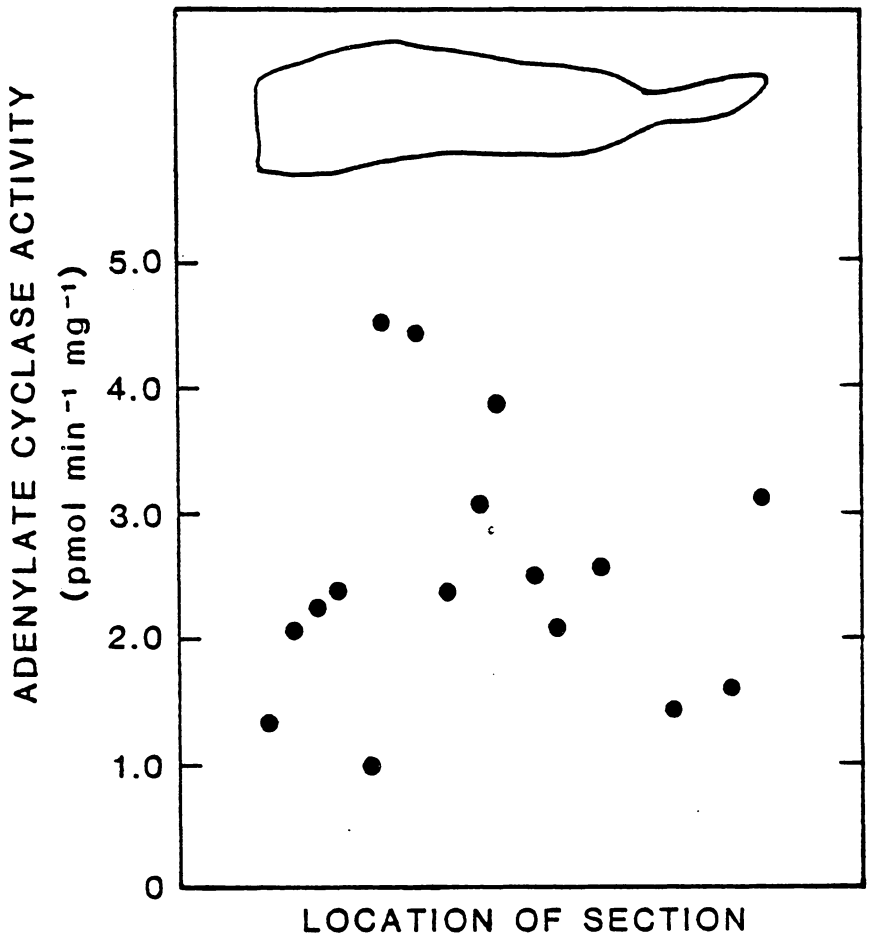

Figure 24. Localization of adenylate cyclase activity in the migrating slug. Data from a single individual plotted to show a typical pattern found in six replicates. The specific activity in serial sections of the slug are represented according to position in the individual. The average weight of sections was 0.45 $\pm$ 0.21 µg.

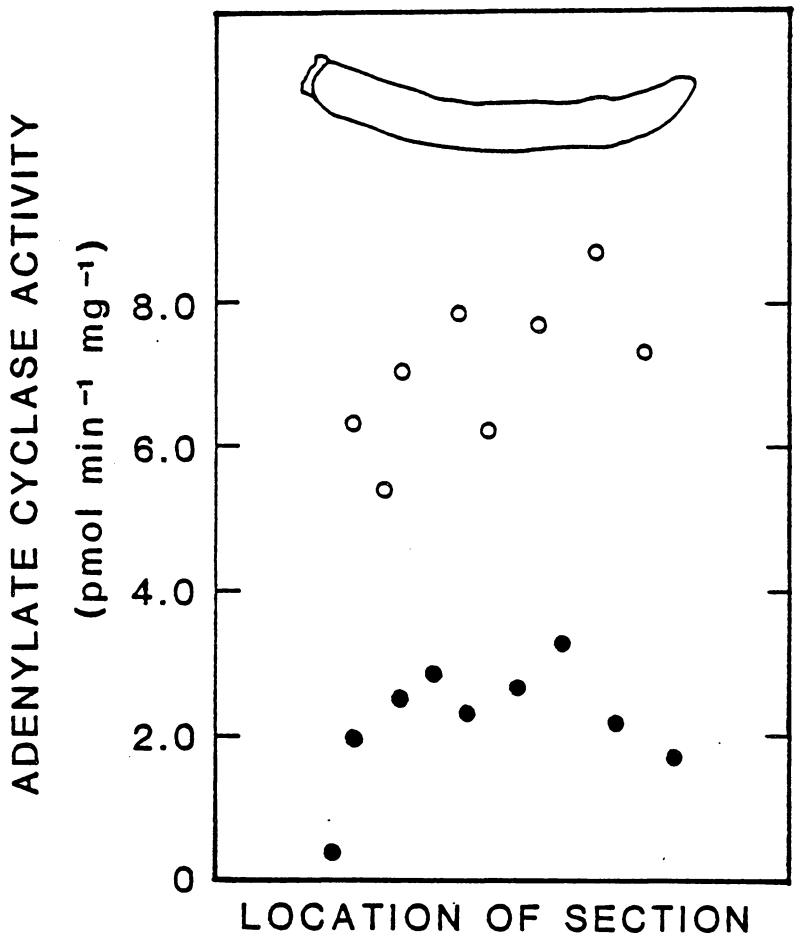

Figure 25. Distribution of adenylate cyclase activity in an uninduced slug with $ATP \cdot Mn^{+2}$ or $ATP \cdot Mg^{+2}$ as substrate. Data from a single individual plotted to show a typical pattern found in four replicates. Serial sections of the slug are represented according to position in the individual. The average weight of sections was 0.31 ± 0.10 µg. Alternate sections were incubated for 20 minutes in a reaction mixture containing 2.5 mM $MgCl_2$ (•), the other sections in a reaction mixture containing 2.5 mM $MnCl_2$ (o). The slug was grown in the laboratory of Dr. M. Sussman, University of Pittsburgh, as described in "Methods".

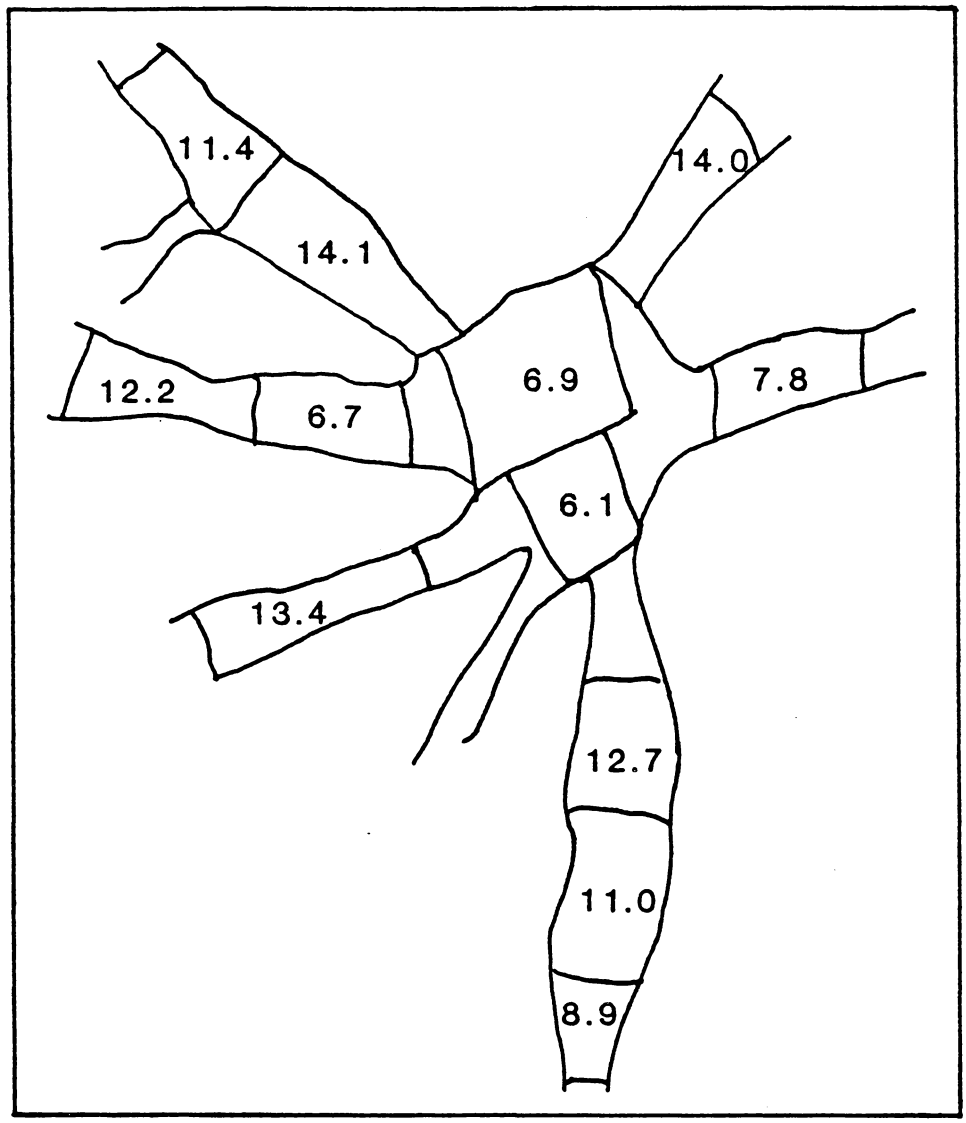

Figure 26. Adenylate cyclase activity in sections of the streaming aggregate. Data from a single individual showing a representative pattern found in two replicates. Activity values are in pmol min^{-1} mg^{-1} dry weight of section. The average weight of sections was 0.13 $\pm$ 0.08 µg.

control and assayed the other half in the presence of a section of stalk cells. No difference in activity was found with the inclusion of the stalks (data not shown).

I also attempted to show that the inhibitor was present in the stalk by collecting large quantities of stalk cells. I harvested individuals at the culmination stage and homogenized them using a power-driven teflon pestle in a Potter-Elvehjem tissue grinder to dislodge the spore masses from the stalks. The stalks remained intact throughout this treatment as ascertained by microscopic examination. The homogenate was filtered as described by Gregg (1967) to remove prespore cells and any bacteria that might be present. This stalk preparation was then lyophilized. The lyophilized stalks were tested for their inhibitory activity by boiling the stalk homogenate and preincubating it with aggregation stage tissue as described by Cripps and Rutherford (1981). As shown in Figure 27, the stalk homogenate elicited a 31% inhibition of the control activity. I thus concluded that there is inhibitor present at the culmination stage, which could be demonstrated under conditions that provided for an enrichment of stalk material.

I also tested for the action of phosphodiesterase in the stalks by incubating individual stalks with authentic cyclic AMP (in amounts comparable to that made during the adenylate cyclase incubation period) in adenylate cyclase reaction mixture lacking ATP and DTT (DTT is an inhibitor of Dictyostelium phosphodiesterase). Under these conditions there was complete recovery of the added cyclic AMP indicating that phosphodiesterase was not responsible for the lcak of measurable adenylate cyclase activity in the stalk.

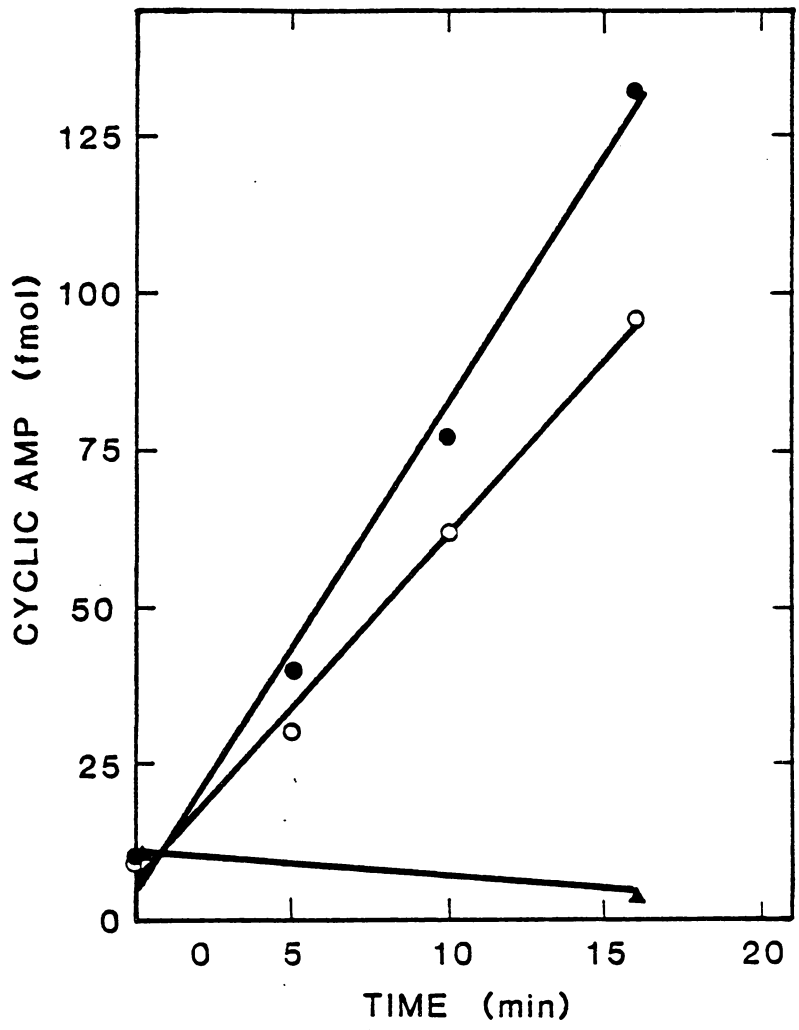

Figure 27. The effect on adenylate cyclase activity of a heat-treated stalk extract. Adenylate cyclase activity (fmol cyclic AMP) was determined in homogenates of the aggregation stage (0.2 mg/ml) after preincubation for 30 minutes at 4°C in the presence of homogenizing buffer (●) or a stalk extract (o). The stalk extract was boiled for 20 minutes prior to preincubation, and it was also assayed for adenylate cyclase activity after a similar preincubation period (▲). Points represent the means of duplicates and the best-fit lines were calculated by means of a least-squares linear regression.

3.4. Microassay of Cyclic AMP

Since both the cyclic AMP synthetic and degradative enzymes, adenylate cyclase and phosphodiesterase are highly localized at the culmination stage of development, it was of great interest to examine the distribution within developing individuals of the suspected morphogen itself. However, measurement of endogenous levels of cyclic AMP in tissue sections was technically difficult due to the limits of detection of the radioimmunoassay. By adjusting the concentrations of ^{125}I-cyclic AMP and the antibody I was able to modify the assay such that a sensitivity of 1 femtomole was attained.

3.4.1. Validation of extraction method: In order to assay cyclic AMP levels in individuals and tissue sections I modified the method of Ferrendelli et al. (1977) for use in the Dictyostelium system. Their method employs a TCA extraction of the cyclic AMP. I found that TCA interfered with the radioimmunoassay thus necessitating its removal. Ether extraction or evaporation are commonly followed procedures for this purpose, however since I was dealing with very small volumes I used evaporation via lyophilization. Initially I tested the effectiveness of TCA removal via lyophilization in a centrifugal biodryer by comparing TCA-treated cyclic AMP standards to those that were not treated. Theresults, illustrated in Figure 28, showed virtually no differences between the cyclic AMP levels in the untreated and TCA-treated samples.

To validate that the TCA extraction product was indeed cyclic AMP

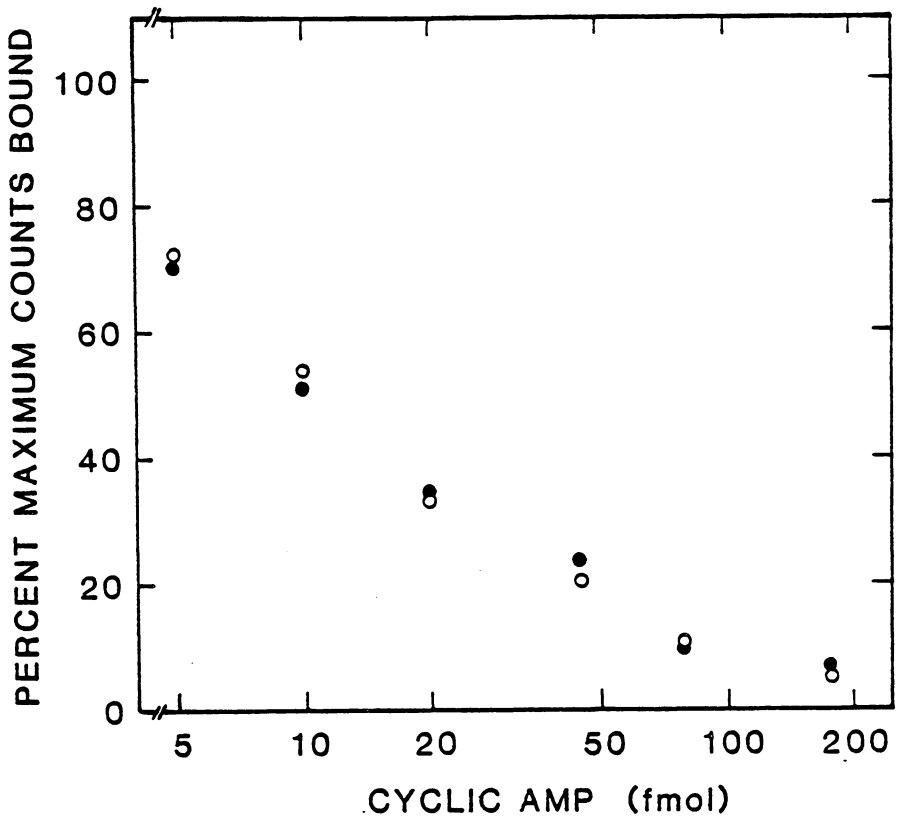

Figure 28. Validation of TCA removal procedure. 10 µl of cyclic AMP standards (0-180 fmol) is added to reaction tubes. 15.0 µl of 5% TCA was added to half the tubes (●). These were incubated for 15 minutes at 4°C, and then 2.0 µl of 1 M HCl was added. To the other tubes (o) 17.0 µl double-distilled H_2O was added. These were then incubated at 4°C for 15 minutes. All of the samples were then lyophilized in a centrifugal biodryer for 30 minutes. The dried residues were dissolved in 30.0 µl 50 mM sodium acetate buffer, pH 6.2. 1.6 µl acetylating reagent was added and cyclic AMP was determined by radioimmunoassay as described in "Methods". The figure represents a typical standard curve with fmol of cyclic AMP plotted on a log scale versus the percentage of maximum counts bound by the antiserum. Each point represents the mean of duplicates.

I tested to see if it was degradable by cyclic AMP phosphodiesterase (Table 11). I accomplished this by the addition of 0.025 to 0.100 U/ml of commercial bovine heart phosphodiesterase to the reaction mixture. Control tubes, either with the usual NaAc buffer or with "PDE buffer" (100 mM Tris-HCl, pH 7.5) yielded the expected 20 fmol after assay. Those tubes subjected to incubation with PDE showed decreased levels of cyclic AMP indicative of degradation. Those samples incubated with inactivated (boiled) enzyme exhibited apparently greater cyclic AMP levels than the controls. This is most likely due to presence of denatured protein interfering with the antibody binding of the RIA. I have observed this phenomenon in other instances where either large amounts of protein or boiled material is added to the reaction tubes. In any case, degradation is not effected by the boiled PDE, and I concluded that the assay method was valid.

I next measured levels of cyclic AMP in tissue sections using spore mass pairs and tips, and checked to see if the extraction product from these were also degradable by phosphodiesterase. The results are presented in Table 12. Cyclic AMP was not detectable in those tip sections that had been incubated with PDE, and reduced levels of cyclic AMP were found in the spore mass sections that were treated as compared to those that had not.

The method of Ferrendelli et al. (1977) calls for the removal of the supernatant fraction to a second reaction tube after TCA extraction. They caution one to be careful not to remove the tissue along with the supernatant but give no reasons for this precaution. I found this step

Table 11

Validation of Cyclic AMP Radioimmunoassay after TCA Extraction: Extraction Product is Degradable by Phosphodiesterase

Treatment[a]		cAMP Recovered (fmol)[b]
NaAc buffer[c]		
4°C, 15 minutes		20
37°C, 15 minutes		20
Tris-HCl buffer + NaAc buffer[d]		
37°C, 15 minutes		20
PDE[e]		
37°C, 15 minutes	0.025 U/ml	4
	0.050 U/ml	4
	0.100 U/ml	4
Boiled PDE[f]		
37°C, 15 minutes	0.025 U/ml	28
	0.050 U/ml	25
	0.100 U/ml	25

[a] 10 µl of cyclic AMP (20 fmol) was added to each tube. 14.8 µl of 5% TCA was added, and each sample was allowed to incubate for 15 minutes at 4°C. 2.0 µl 1 M HCl was added to all samples and the samples were then lyophilized in a centrifugal biodryer for 30 minutes. The samples were treated with PDE or buffer as indicated.

[b] Cyclic AMP was measured by radioimmunoassay as described in "Methods" using 1.6 µl acetylating reagent. Numbers represent the average of two determinations.

[c] After lyophilization 30.8 µl 50 mM NaAc buffer, pH 6.2, was added to each sample. The samples were then incubated as indicated.

[d] After lyophilization, 13.0 µl of 100 mM Tris-HCl, pH 7.5, was added to each sample. After the samples were incubated as indicated, 18.0 µl of 50 mM NaAc buffer, pH 6.2, was added to each.

[e] Commercial bovine heart phosphodiesterase was prepared in 100 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$ at the concentrations indicated. 13.0 µl of the designated concentration was added to each lyophilized sample. The samples were incubated as indicated, then 18.0 µl of 50 mM NaAc buffer, pH 6.2, was added to each.

[f] Phosphodiesterase was prepared as in "e", then boiled for 10 minutes before addition to lyophilized samples.

Table 12

Validation that TCA Extraction Product of Tissue is Degradable by Phosphodiesterase

Culmination Tissue Section	Weight (μg)	PDE Treatment[a]	cAMP (fmol/μg)
I. Spore Mass Pair[b]	1.81	+	4
	1.38	-	17.0
II. Spore Mass Pair	0.95	+	4
	1.29	-	13.2
III. Tip	0.65	-	17.6
IV. Tip	0.50	-	10.0
V. Tip	0.69	-	9.8
VI. Tip	0.30	+	Not detectable
VII. Tip	0.53	+	Not detectable

[a] 14.8 μl of 5% TCA was added to each type to extract the cAMP. Samples were incubated at 4°C for 15 minutes, then 13.0 μl of the supernatant fluid was removed to a second tube. 2.0 μl of 1 M HCl and 9.6 μl double distilled H_2O was added to each tube. The samples were lyophilized in a centrifugal biodryer, and cyclic AMP was measured by radioimmunoassay as described in "Methods".

[b] The base and tip were removed from culmination spore masses. The spore mass was then halved longitudinally, the stalk removed, and the mass was then sectioned serially. Each serial section was then bisected. Each half of the pair was considered equivalent and served as its own control.

to be very tedious when dealing with extremely small sections, and I therefore wished to determine whether it was actually necessary. I first needed a method to obtain paired control sections. I cut sections of the mid-spore mass from individuals and halved them. I then extracted the cyclic AMP and removed the supernatants as described in the published procedure. Table 13 shows the results of assay of 16 paired sections. After correction of cyclic AMP levels by expressing them as femtomoles per microgram dry weight it appears that there is little difference between the halves of a pair. This was confirmed statistically: I employed a paried t-test to validate the null hypothesis that there was no difference between the levels of cyclic AMP in each half of the spore mass section. The value for t was found to be equal to 0.77 ($p>0.20$), therefore I concluded that there was, in fact, no significant differences between the halves. I could thus use half of a pair as a control for the other.

I then tested to see if any differences in cyclic AMP levels were found if one removed the TCA-extract supernatant fluid and lyophilized it as opposed to lyophilization of the supernatant fluid containing the tissue section. The results are shown in Table 14. Again I used a paired t-test, this time to test the null hypothesis that there was no difference between the two treatment methods. A value for t was found to be 0.49 ($p>0.20$). I therefore concluded that there was no significant difference between the two. Since it was much easier to leave the small sections in the test tubes with the TCA extract fluid this method was adopted for all subsequent cyclic AMP determinations.

Table 13

Demonstration that Spore Mass Pairs have Equivalent Levels of Cyclic AMP

Spore Mass Pair[a]	Weight[b]	Cyclic AMP[c]	Spore Mass Pair	Weight	Cyclic AMP
I	3.27	29.4	IX	1.89	13.7
	3.01	25.9		2.12	8.3
II	2.58	39.1	X	0.86	13.0
	1.81	30.9		1.58	15.3
III	4.13	18.9	XI	0.21	20.7
	1.46	11.6		0.28	21.6
IV	1.90	9.0	XII	0.39	17.7
	1.80	12.3		0.46	15.5
V	2.50	18.7	XIII	0.88	27.6
	2.20	16.2		1.07	20.6
VI	2.20	6.9	XIV	0.74	5.9
	2.30	8.3		1.14	14.5
VII	1.30	6.3	XV	1.12	10.4
	1.40	5.8		0.69	9.6
VIII	0.90	37.2	XVI	2.58	12.0
	1.20	30.3		2.75	10.2

[a] Individuals at the culmination stage were picked, and the tips and basal disks were removed. The individuals were cut longitudinally and the stalks removed. Each numeral represents the spore mass halves of a single individual. The data represent the results of four separate experiments.

[b] Weight is expressed in micrograms.

[c] Cyclic AMP was extracted by adding 14.8 µl 5% TCA to each tube containing tissue or standard and incubating the samples at 4°C for 15 minutes. 13.0 µl of the supernatant fluid was removed to a second tube to which 2.0 µl of 1 M HCl and 9.6 µl H_2O were added. The samples were lyophilized in a centrifugal biodryer, and cyclic AMP was measured by radioimmunoassay as described in "Methods". Data is expressed as fmol/µg.

Table 14

Validation of Cyclic AMP Assay Modification

Spore Mass Pair[a]	Cyclic AMP (fmol/μg) Supernatant Removed[b]	Tissue Left in Tube[c]
I	18.9	16.7
II	7.6	6.1
III	6.1	8.8
IV	18.8	19.4
V	8.1	7.3
VI	20.6	25.6
VII	14.5	10.9
VIII	7.3	10.9
IX	10.1	10.5

[a]Individuals at the culmination stage were bisected longitudinally after removal of the tips and basal discs. The stalks were removed. Each numeral represents the spore mass halves of a single individual. The data represent the results of two separate experiments.

[b]Cyclic AMP was extracted by adding 14.8 μl 5% TCA to each tube and then incubating the tubes at 4°C for 15 minutes. 13.0 μl of the supernatant fluid was removed to a second tube to which 2.0 μl 1 M HCl and 9.6 μl H_2O were added. The samples were lyophilized in a centrifugal biodryer, and cyclic AMP was measured by radioimmunoassay as described in "Methods".

[c]Cyclic AMP was extracted as in "b". After incubation at 4° for 15 minutes 2.0 μl of 1 M HCl and 9.6 μl H O were added directly to the extraction mixture containing tissue. The samples were then treated as in "b".

3.4.2. Levels of cyclic AMP during development: Pahlic and Rutherford (1979) measured endogenous levels of cyclic AMP during development of Dictyostelium discoideum using synchronous mass cultures of the organism. They used the protein binding assay of Gilman (1970) to measure yclic AMP which allowed a sensitivity of detection of 0.5 pmol. I did a similar stage study but with single individuals and the use of the radioimmunoassay to measure cyclic AMP. The results from one stage study are illustrated in Figure 29. A separate stage study, with another set of tissue, revealed the same pattern of cyclic AMP levels over the course of development (data not shown). I observed a sharp peak at 10 hours of development similar to that reported by Pahlic and Rutherford. However, at this stage of development they measured 24 pmol cyclic AMP/mg dry weight whereas I found about half that amount. They then showed a drop to near 6 pmol/mg which was maintained throughout the remainder of the developmental cycle. I found an initial peak of 9.6 $\pm$ 3.1 pmol/mg at aggregation; this level was then maintained until a second, higher peak of 29.1 $\pm$ 9.6 pmol/mg is evident at the culmination stage of development. Analysis of this second peak using a Mann-Whitney U test indicated that the level of cyclic AMP is significantly ($p<0.001$) greater at this stage than at the preculmination stages.

3.4.3. Cyclic AMP distribution within developing individuals: After determining that cyclic AMP could be measured in tissue sections that weighed as little as 0.10 μg, I examined the distribution of cyclic AMP in the different regions of the culminating individual. A comparison of the middle pre-spore regions of 33 early and 78 mature culminating

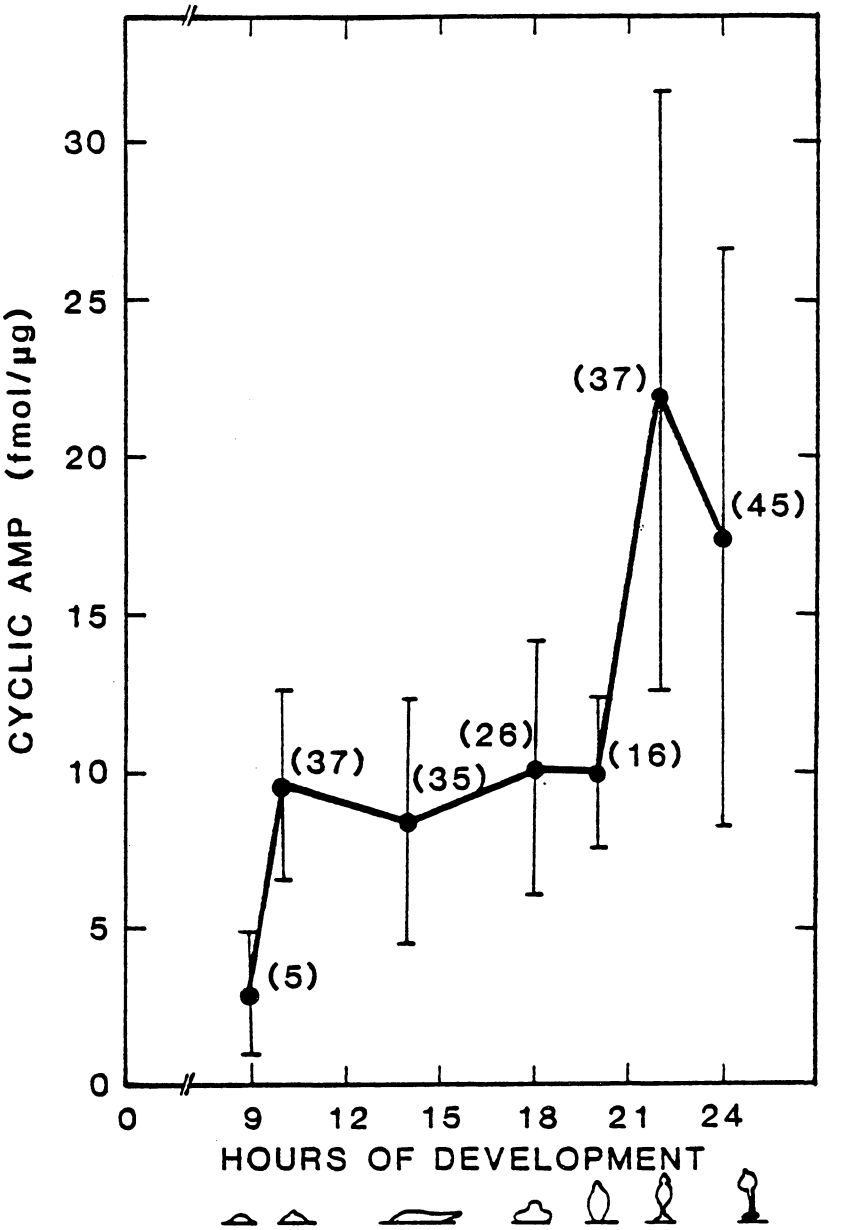

Figure 29. Levels of cyclic AMP in individuals during development. 201 individuals from a stage study were assayed for cyclic AMP as described in "Methods". Numbers in parentheses refer to the number of individuals at that particular developmental stage. The bars represent the standard deviations. Data was from eight experiments.

individuals showed significantly ($p<0.02$) higher levels in the latter than in the former as analyzed using a Mann-Whitney U test. This finding lends additional support for the rise in cyclic AMP levels in the whole individual seen at culmination.

Since adenylate cyclase had been found to be localized in the pre-spore region and absent in the stalk of the culminate I wanted to compare the cyclic AMP levels in these two tissue types. Surprisingly, examination of 49 stalks and 78 pre-spore sections revealed no significant ($p = 0.41$) difference between the values. The average level of cyclic AMP in the pre-spore sections was 16.4 ± 9.7 fmol/μg, for the stalks 14.8 ± 8.2 fmol/μg. Comparison of stalks and mid-pre-spore sections from the same individual using the Wilcoxon matched-pairs signed-ranks test corroborated this result; no significant ($p>0.05$) differences between the two tissue types.

Since cyclic AMP phosphodiesterase is distributed in a gradient along the stalk (highest at the base) I wanted to see if any differences in cyclic AMP were apparent between the upper and lower regions of the stalk. The basal portion of the stalk was cut just below the pre-spore mass and did not include the basal disk. The remaining upper stalk was removed from the surrounding spore mass and did not include the tip. A comparison of 11 upper stalks (mean = 24.4 ± 12.5 fmol cyclic AMP/μg) and 17 lower stalk regions (mean = 9.5 ± 7.9 fmol cyclic AMP/μg) using the Mann-Whitney U test showed that the upper stalks have significantly ($p<0.002$) and appreciably ($r_m>0.48$) more cyclic AMP than do the basal portions.

Cyclic AMP levels in the tip region were compared to a section from the middle of the pre-spore region. Comparison was made within 62 individuals using the Wilcoxon matched-pairs signed-ranks test which tests two related samples for both the direction and size of the differences. It was found that the tips had cyclic AMP levels that were significantly ($p<0.01$) and moderately ($r_m>0.30$) greater than the levels of the mid-spore sections.

Since I had observed a gradient of adenylate cyclase within the pre-spore mass of culminating individuals it was of interest to look at the distribution of cyclic AMP within this region. Of the twelve individuals examined eight out of the twelve exhibited a gradient as illustrated in Figure 30. This gradient ran in the same direction as enzyme activity did, highest at the base of the spore mass, lowest near the tip. An analysis of the relationship between the weight of the pieces and the cyclic AMP levels (fmol/μg) revealed no correlation ($r = 0.17$, $p>0.1$) indicating that the gradient did not result from such an artifact.

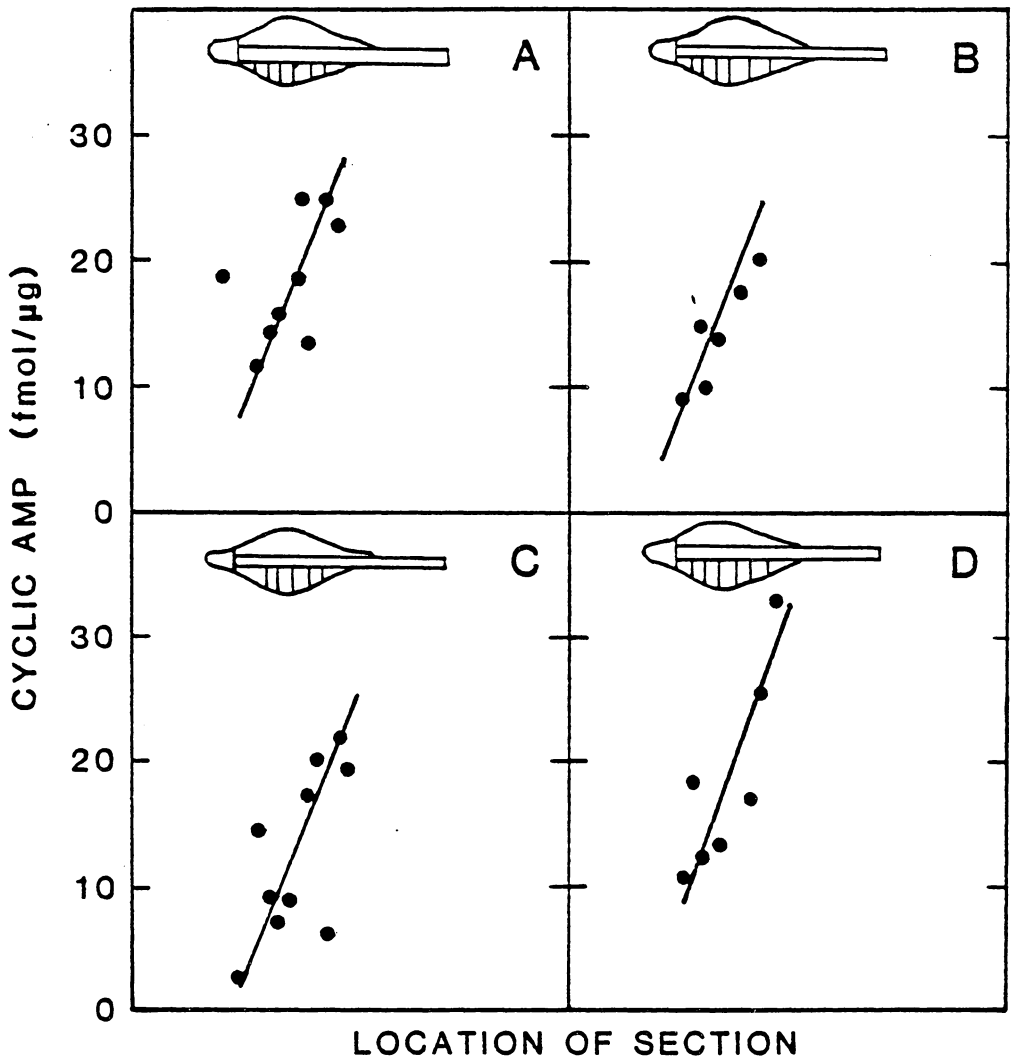

Figure 30. Cyclic AMP distribution within the prespore mass of culminating individuals. Data from four individuals plotted to show a representative pattern found in 8 out of 12 replicates. Spore masses were halved in the longitudinal axis. The stalks were removed, then the halves were sectioned serially. The average weight ± the standard deviation in µg of the sections were as follows: (A) 0.52 ± 0.12, (B) 0.41 ± 0.10, (C) 0.23 ± 0.09, (D) 0.23 ± 0.08.

4. DISCUSSION

4.1. Regulatory Role of Cyclic AMP

Cyclic AMP plays a central role in the initiation of development of Dictyostelium discoideum by serving as the chemotactic agent for aggregating amoebae. Several laboratories have provided evidence that this molecule also plays a regulatory role in the later processes of differentiation as described in the "Introduction". When cyclic AMP was determined by measurement in single individuals, a peak level was found to exist at the culmination stage of development. Such a peak in cyclic AMP at the same developmental stage has been reported by Brenner (1978) as well. He measured cyclic AMP in groups of organisms rather than in individuals. This second peak at the culmination stage further supports a role for cyclic AMP in the differentiation of the two cell types. It is also at this stage of development that a distinct localization of the cyclic AMP synthetic enzyme, adenylate cyclase, was apparent. Additionally, within the pre-spore mass, a gradient of this activity was found. Furthermore, this gradient was paralleled by one of cyclic AMP itself. These observations indicated that the enzyme was being modulated, and that cyclic AMP may play a regulatory role in the later developmental processes of Dictyostelium discoideum. Supporting evidence for these hypotheses include the existence of a specific inhibitor of adenylate cyclase activity, and a strong localization of the degradative enzyme, cyclic AMP phospho-

diesterase, in the stalk of the culminating sorocarp (Brown and Rutherford 1980).

4.2. Enzyme Characterization

One of the original goals of this research was to partially purify adenylate cyclase for characterization studies. Mammalian hormone-sensitive adenylate cyclases are notoriously difficult to work with as documented by many workers. The difficulty stems from the fact that the enzyme is labile in detergent solutions and is composed exclusively of intrinsic membrane proteins that make up only a minor percentage of the total protein content of the membrane (Ross and Gilman 1980). The consequences of these properties were evident in my study of the Dictyostelium enzyme. For example, the enzyme was found to be labile in the absence of substrate. After a 33 minute incubation at 23°C no enzyme activity was detectable. Physical treatment by centrifugation also resulted in decreases in activity, possibly indicating the separation of soluble factors required for activation or for the stabilization of the membrane-bound activity.

Detergent treatment led to the loss of enzyme activity from the pellet and did not result in detectable solubilized activity. An explanation for this finding is that the detergents directly inactivated the enzyme. Some detergents, including Triton X-100, bind to high affinity hydrophobic sites on a protein's surface. Such binding, according to Neer (1977) is probably not cooperative and therefore not necessarily accompanied by a conformation change in the protein that

could result in inactivation. Thus direct inactivation is probably unlikely. However, Hinterman and Parish (1979) indicated that the use of 0.2% Triton X-100 during a membrane isolation procedure may have led to the partial inhibition of D. discoideum adenylate cyclase. Young and Stansfield (1978) have proposed that the successful solubilization of adenylate cyclase from its membrane location may depend on the state of enzyme activation. That is, activation of the enzyme, effected by regulatory ligands, could stabilize the enzyme during detergent treatment. Guillon et al. (1978) point out that a marked decrease in adenylate cyclase activity is often observed upon solubilization. They showed that the inhibition by detergents was not related to solubilization events, but rather to the nature of the hydrophobic environment of the enzyme and to the effect of the micellar concentration of the detergent. It is very likely that once the enzyme is released from its membranous environment it loses its physiological properties. If detergent treatment actually accomplished the desired solubilization of the Dictyostelium enzyme, the lack of detectable activity may have been due either to the loss of membrane factors required for activity or to a disruption of the membrane integrity and normal hydrophobic environment required to maintain the enzyme in an active conformation. Simple recombination of the soluble and insoluble fractions did not lead to the recovery of enzyme activity which tends to refute the former possibility. It is possible that either the pre-treatment of the enzyme with a stabilizing factor or the use of

another detergent besides Triton X-100 or Lubrol would result in the successful recovery of soluble activity.

Like the adenylate cyclase from other systems (Perkins 1973) the Dictyostelium enzyme was shown to require a divalent cation for activity. The manganese-dependent activity was found to be over 4-fold greater than the magnesium-dependent activity. This situation resembles the characteristics of the mammalian catalytic component (Ross and Gilman 1980). These authors showed that in the absence of the regulatory component high Mn-dependent activity results, whereas the Mg-dependent activity is less than 10% of this level. Ross et al. (1978) suggest that the Mn-dependent activity of the catalytic protein is not physiologically significant since the presence of the G/F regulatory component of the mammalian enzyme is required in vitro for the exhibition of substantial magnesium-dependent activity as well as for stimulation by various modulators and hormones. Ross et al. (1978), using a clone of cultured cells from an S49 mouse lymphoma variant, cyc^-, which is phenotypically deficient in adenylate cyclase activity, found that it contains a manganese-dependent activity and therefore proposed that cyc^- contains the catalytic component but lacks the G/F regulatory unit. The Dictyostelium enzyme does not appear to be regulated by guanyl nucleotides or fluoride and therefore resembles the cyc^- phenotype. This indicates that either the regulatory subunit is lost or inactivated during cell extract preparation, or that the regulatory unit as such does not exist in the organism. Since, in mammalian systems, the regulatory component is relatively stable

(Northup et al. 1980), as compared to the catalytic protein which is sensitive to mild heating and low concentrations of sulfhydryl reagents (Ross and Gilman 1980), it seems unlikely that the catalytic activity would be retained after extract preparation and not the regulatory function as well. It is more likely, from the evidence in this study of the Dictyostelium enzyme, that such a regulatory component is not present in the organism. This of course does not rule out the possibility of another means of enzyme regulation. In fact, Dictyostelium adenylate cyclase was shown to be stimulated by its product cyclic AMP (Klein and Darmon 1979). It is certainly possible that this modulation by cyclic AMP is mediated via a cyclic AMP receptor protein that is bound to the plasma membrane.

Preliminary evidence of a GTP-binding regulatory component in Dictyostelium is provided by Leichtling et al. (1981a). They reported the occurrence of a 42,000 dalton, GTP-binding protein that served as a substrate for a cholera toxin-catalyzed ADP-ribosylation. It was speculated that this protein was equivalent to the G/F protein of mammalian adenylate cyclase. Nakaya et al. (1981) indicate two criteria used to establish that a protein is a subunit of G/F. These are that the protein copurifies with G/F activity or that toxin treatment of intact cells prevents subsequent toxin-catalyzed ADP-ribosylation of specific proteins in isolated fractions. It has not yet been demonstrated in Dictyostelium that there is such an adenylate cyclase-associated G/F activity. In addition, no evidence has been provided by Leichtling et al. (1981a) that the 42,000 dalton protein is

part of the adenylate cyclase system in Dictyostelium. Moreover, I could not demonstrate any activation of the enzyme in this organism by cholera toxin. Membrane proteins other than G/F are also subject to covalent modification by cholera toxin (Cassel and Pfeuffer 1978, Leichtling et al. 1981b) so this GTP-binding protein is not necessarily part of the adenylate cyclase system. It just may be fortuitous that its molecular weight corresponds to that of the mammalian G/F unit; 40,000 daltons is a common molecular weight for protein subunits. It is interesting to note that Leichtling et al. (1981b) have also reported on another 42,000 dalton protein in Dictyostelium, a cytoplasmic component that binds cyclic AMP. This protein is capable of inhibiting bovine protein kinase catalytic subunit suggesting that it functions as the regulatory subunit of a cyclic AMP-dependent protein kinase in Dictyostelium. Wallace and Frazier (1979) have identified a membrane counterpart by photoaffinity labeling, a membrane-bound cyclic AMP binding protein of 40,000 daltons. Perhaps it is this receptor which functions as the modifier of adenylate cyclase activity.

Another approach to the determination of the mode of regulation of Dictyostelium adenylate cyclase could be to "reconstitute" a holoenzyme by adding mammalian G/F regulatory protein to the Dictyostelium catalytic unit. This could be done by adding purified G/F from, for example, turkey erythrocyte as described by Hanski et al. (1981) to a Dictyostelium cell extract and assaying the enzyme in the presence of fluoride or guanyl nucleotides. Stengel and Hanoune (1981) reconstituted ram sperm adenylate cyclase, an enzyme that exhibits

maximal activity with Mn^{+2} and that cannot be stimulated by fluoride, guanyl nucleotides or cholera toxin, with G/F component from human erythrocytes. They accomplished this by cell fusion. Since human erythrocytes are enriched in the G/F component as compared to the catalytic unit this could be an ideal source of the regulatory protein, especially as Kuhn and Parish (1981) have published a method for the fusion of cells from Dictyostelium with erythrocyte ghosts. A positive result, that is, activation of the Dictyostelium enzyme through the added regulatory component, would at least indicate whether the enzyme is capable of being regulated in a fashion similar to that of the mammalian enzyme.

The effect of calcium on the Dictyostelium adenylate cyclase was examined since in mammalian systems Ca^{+2} and its protein receptor, calmodulin, have recently been shown to play a role in mediating many cellular functions including synthesis and degradation of cyclic nucleotides (Klee et al. 1980). Calcium appeared to inhibit the Dictyostelium enzyme activity but this may have been due to the formation of Ca·ATP complexes which competed with the substrate Mn·ATP. This was indicated by the results of Table 4 which showed that calcium alone will not serve as the required divalent cation, and supported by Loomis et al. (1978) who demonstrated that inhibition of adenylate cyclase activity by calcium could be overcome by manganese. Calcium has been shown to increase the number of cell-surface cyclic AMP binding sites (Juliani and Klein 1977). It could play a role in adenylate cyclase modulation by this means if this receptor were involved in

mediating cyclic AMP stimulation as discussed previously. A further modifier in the system could be calmodulin which is known to exist in Dictyostelium (Clarke et al. 1980). However, a precise function for calmodulin in this organism has not been defined; in fact, it had no effect on D. discoideum phosphodiesterase even though its existence was discovered by its ability to activate mammalian cyclic nucleotide phosphodiesterase. This ubiquitous mediator of eukaryotic calcium function has been shown to have a role in modulating such enzymes as adenylate and guanylate cyclase, protein kinase, and phosphorylase kinase, as well as cyclic nucleotide phosphodiesterase in mammalian systems (Cheung 1980). I was unable to demonstrate an effect of calmodulin on the activity of Dictyostelium adenylate cyclase. With most calmodulin-regulated enzymes calmodulin is easily dissociated in the presence of the calcium chelator EGTA (Cheung 1980). Calmodulin binds tightly to phosphorylase kinase (Cohen et al. 1978) however, and this may have been the case with Dictyostelium adenylate cyclase. Calmodulin, remaining bound to the enzyme, would maintain it in an activated state and thereby prevent visualization of a specific calcium effect. Thus, if calmodulin-mediated calcium regulation of Dictyostelium adenylate cyclase does exist in vivo it cannot be demonstrated by the methods used to show its effects in mammalian tissues.

Another possible modifier of adenylate cyclase activity examined was ammonia. Sussman and Schindler (1978) proposed a model for the regulation by this compound on Dictyostelium discoideum morphogenesis. Ammonia was proposed to inhibit cyclic AMP production and thereby

restrict accumulation of cyclic AMP to certain areas of the organism. Cyclic AMP could then direct movement of cells within the aggregate and/or trigger spore and stalk cell differentiation. This model is intriguing in light of the finding that adenylate cyclase and cyclic AMP phosphodiesterase are highly localized in the culmination stage, and that there are regions of high cyclic AMP. I was unable to ascertain whether ammonia has a specific effect directly on the synthesis of cyclic AMP, for although ammonium salts inhibited adenylate cyclase activity so did other salts at similar ionic strengths. Schindler and Sussman (1979) showed that exogenous NH_3 does reversibly inhibit the intracellular accumulation of cyclic AMP, however they point out that the mechanism of action is not necessarily via the synthetic enzyme. They also say that the effector molecule may be a metabolite derived from NH_3 rather than NH_3 itself. If this was the case NH_3 would not affect adenylate cyclase *in vitro*.

A means of regulating adenylate cyclase activity in *Dictyostelium* is provided by the endogenous inhibitor substance described herein and by Cripps and Rutherford (1981). Since the extracellular material was capable of inhibiting enzyme activity from all stages of development, a potential exists for regulation of culmination differentiation processes. The preliminary evidence (see Results 3.3.3.) suggests that this inhibitor is present in the stalk and may explain why no adenylate cyclase activity is detected there. Although the identity of the inhibitor as well as its mechanism of action remains to be elucidated, it is not without some basis that I suggest that inhibition of adenylate

cyclase activity in stalk cells can occur. In fact, cyclic AMP phosphodiesterase, the degradative enzyme, has been shown to be masked in pre-spore cells, yet is active in the stalk (Rutherford and Brown 1982). The lack of activity in the pre-spore tissue was due to regulation by a heat-stable inhibitor. Specific binding of this inhibitor to the cyclic AMP phosphodiesterase causes conversion of the enzyme from a low to a high K_m form. Thus the mechanism of action is to alter the enzyme's affinity for its substrate. Should the activities of adenylate cyclase as well as cyclic AMP phosphodiesterase be modulated by endogenous inhibitors, a model would be provided for the regulation of these two opposing activities.

4.3. Localization of Enzyme Activity and Cyclic AMP

Morphogenesis, or expression of form, must be explained by mechanisms which give rise to ordered movements of cell masses (Waddington 1966). Differentiation, or cell specialization, on the other hand, must be explained in terms of chemical mechanisms that give rise to specific structures and specific metabolic processes. One cannot focus on a single event in a series and point to it as the cause of development (Wright 1979). Rather, development involves a complex set of coordinated interactions encompassing changes in enzyme activities, levels of metabolites, substrates and inhibitors, as well as alterations in the control of gene expression (Wright 1966). Models for the development of Dictyostelium discoideum must account for both the processes of morphogenesis and of cellular specialization. As

Bonner (1974) points out, few attempts have been made to integrate biochemical and pattern information. Wolpert (1971) suggests that cells are assigned positional information by which they can interpret their position in a field. A cell differentiates because it has positional information which then leads to an appropriate molecular response. The problem of integrating biochemical and pattern data is hardly surprising when one realizes, as Wolpert (1969) points out, that the fields comprise distances of less than 100 cells.

Gradients have long been theorized to play a role in embryonic development (Child 1941, Summerbell et al. 1973). Positional information is given by a linear gradient set up by "source" cells producing a diffusible substance (or morphogen). At the other end of the gradient the extreme cells act as a "sink", that is, destroying the morphogen and maintaining a fixed low level (Crick 1970). I have found that adenylate cyclase activity is localized in the pre-spore tissue and is completely absent in the stalk. Additionally, a gradient of enzyme activity is apparent within the pre-spore mass with the highest activity at the base and decreasing toward the tip. In comparison, the localization pattern of the cyclic AMP degradative enzyme, phosphodiesterase, is just the opposite (Brown and Rutherford 1980): High levels of phosphodiesterase activity are seen in the stalk, highest at the base, and no detectable activity in the pre-spore tissue. Thus a source of cyclic AMP is provided at the base of the spore mass by adenylate cyclase and a sink at the base of the stalk by phosphodiesterase. These results show that an enzymatic potential

exists for establishing a gradient of this diffusible molecule. In fact, examination of levels of cyclic AMP within the culminating individual revealed a parallel gradient with the pre-spore mass (Fig. 30). Several models of Dictyostelium development propose that the tip of the organism behaves as a classical embryonic organizer region. Rubin (1976) has shown that amoebae are chemotactic toward tips transplanted within their midst, and that addition of bovine cyclic AMP phosphodiesterase can decrease the chemotactic range of the cells. These data suggest that the tip is a source of cyclic AMP. My data also provide evidence for localized levels of cyclic AMP in this region; at least the levels there were significantly higher than those of the middle pre-spore region. The data on the adenylate cyclase activity in the tips, however were not consistent. Variable levels were seen, some high, some low, depending upon the experiment. It may be that the enzyme is regulated in vivo by mechanisms that are not detected in vitro. Additionally, the cyclic AMP signal may be modulated through specific receptors. This could also account for the discrepancy in the detection in the stalks of cyclic AMP but not enzyme activity. Cyclic AMP, a small diffusible molecule, may have been bound to the cellular receptors after having been synthesized elsewhere. Another explanation for the lack of enzyme activity in the stalk is that, as the pre-stalk cells migrate towards the tip into the stalk region, adenylate cyclase activity is inhibited, but the cyclic AMP made at an earlier point in time has not yet been completely degraded. Such degradation could take place as stalk formation ensues.

This is supported by two lines of data: The levels of cyclic AMP phosphodiesterase are higher at the base of the stalk (Brown and Rutherford 1980) and cyclic AMP levels are greater in the upper stalk than in the lower part (see Results 3.4.3.).

Several possible functions for the cyclic AMP gradient can be suggested. One could be to control the chemotactic movements of the pre-stalk cells at culmination. A differential level of cyclic AMP between the tip and the pre-stalk cells would ensure that the latter can migrate into the stalk sheath. Matsukuma and Durston (1979) have shown that after aggregation occurs only the pre-stalk cells retain an attraction for cyclic AMP. Another function for the cyclic AMP gradient would be to provide a signal for the specific changes leading to spore cell differentiation. This information could be transferred to the gene level via a cyclic AMP-dependent protein kinase (Rutherford et al. 1982). Another possibility is that the regulation of intracellular metabolism could be controlled via cyclic AMP. For example, glycogen breakdown may be regulated as in the amplification cascade in the mammalian liver through the action of cyclic AMP on the enzymes protein kinase, phosphorylase kinase and glycogen phosphorylase. At the present however there is no data to back this supposition. In addition, Sussman and Schindler (1978) suggest that cyclic AMP triggers release of structures necessary for terminal spore differentiation, that is, spore-specific vacuoles, granules, and fibrils.

Certainly no definitive answers on specific developmental processes are provided by this data. However, the existence of an adenylate

cyclase gradient and a corresponding cyclic AMP gradient, and the possibility of modulating the enzyme activity by an endogenous inhibitor support the hypothesis that cyclic AMP is involved in the differentiation of the two cell types of Dictyostelium discoideum. More work is needed to precisely define the mechanism of enzyme localization as well as to ascertain the means by which the cyclic AMP signal is transduced.

Although much remains to be learned about the specific roles of cyclic AMP in the terminal stages of development in Dictyostelium discoideum, the results reported in this thesis must be considered within the perspective of developmental biology and embryology as a whole. The fundamental problem in these fields of study is how all the signals are controlled such that differentiation occurs at the right place and time to produce consistent patterns. Although the idea of gradients has been proposed since Child (1941) suggested them, little progress has been made at understanding the biochemical nature of such gradients. The reasons for the lack of information stems from such limitations as the small number of cells involved, the great variety of cell types in most organisms, and the unknown identity of the organizer molecules that attract embryonic cells to move to specific locations within developing multicellular bodies. The relatively simple, multicellular eukaryotic organism, Dictyostelium discoideum, has offered several advantages for the examination of developmental regulatory processes. A morphogen, cyclic AMP, has been identified, only two different cell types are involved, and only one cell type, the pre-stalk cell, is chemotactic and capable of movement. Research in

our laboratory has yielded data indicating the striking localization of enzyme activities as well as cyclic AMP itself. Despite the fact that we have barely begun to delineate the complex processes of cell differentiation and embryonic development, our data may represent a beginning for their eventual elucidation.

5. LITERATURE CITED

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CHARACTERIZATION AND LOCALIZATION OF ADENYLATE CYCLASE DURING DEVELOPMENT OF *DICTYOSTELIUM DISCOIDEUM*

by

Roberta Gayle Kurpit Merkle

(ABSTRACT)

Cyclic AMP functions as the chemotactic signal during aggregation of single-celled amoebae of the cellular slime mold *Dictyostelium discoideum*. Evidence suggests that cyclic AMP also acts as a regulatory molecule during *Dictyostelium* multicellular differentiation. Biochemical characterization of adenylate cyclase, the cyclic AMP synthetic enzyme, was accomplished using a sensitive radioimmunoassay. The enzyme was found to be pellet-bound. The non-ionic detergents, Triton X-100 and Lubrol PX, were not effective for solubilizing this activity. Magnesium or manganese could serve as the required divalent cation, with the Mn-supported activity over 4-fold greater than the Mg-supported activity. Typical mammalian adenylate cyclase modulators such as guanyl nucleotides, fluoride, and cholera toxin did not activate the *Dictyostelium* enzyme. Calcium, in conjunction with its protein receptor calmodulin, did not appear to regulate the enzyme. An endogenous extracellular, heat-stable substance was found to inhibit *Dictyostelium* adenylate cyclase.

By use of ultramicrotechniques adenylate cyclase activity was localized in the pre-spore cells of the culminating individual with no

activity detected in the pre-stalk region. Lack of detectable activity in the pre-stalk cells may be due to a masking by the endogenous inhibitor. An increasing gradient of activity was found in the pre-spore mass with activity increasing from the uppermost area to the base. No striking localization was seen prior to the pre-culmination stage of development. Two peaks in cyclic AMP levels, as measured in individuals were found during development. One coincided with aggregation, the other occurred at the culmination stage. A gradient of cyclic AMP within the culminating individual paralleled the gradient of adenylate cyclase activity. The tip of the individual had greater levels of cyclic AMP than the middle pre-spore region, and the upper stalks had higher levels than the lower stalks.

These data indicate an enzymatic potential for establishing a gradient of cyclic AMP. At the culmination stage of development this molecule could act to direct the chemotactic movements of the pre-stalk cells as well as provide positional information for the terminal differentiation of the pre-spore cells into mature spores.