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NUCLEOTIDE CYCLASES AND CYCLIC NUCLEOTIDE BINDING PROTEINS IN MYXOCOCCUS XANTHUS.

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NUCLEOTIDE CYCLASES AND CYCLIC NUCLEOTIDE BINDING PROTEINS IN *MYXOCOCCUS XANTHUS*

by

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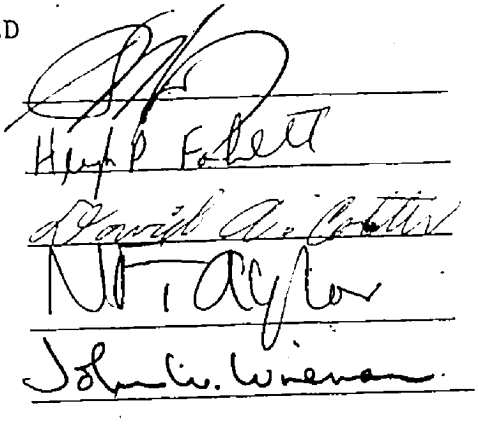

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ABSTRACT

Myxococcus xanthus vegetative cells contained significant amounts of nucleotide cyclase activities and cyclic nucleotide binding proteins. The guanylate cyclase activity was distributed between the 100,000g supernatant and pellet fractions, required divalent cation and exhibited an apparent K_m of 1.0mM.

Adenylate cyclase activity was detected both in the 100,000g supernatant and pellet. The supernatant enzyme had an apparent K_m of 220uM with a Hill coefficient of 1.9, whereas that of pellet fraction had an apparent K_m of 72uM and a Hill coefficient of 1.0. The enzymes differed in their pH optima and divalent cation requirements.

Three binding activities, one specific for guanosine 3',5'-monophosphate (cGMP) and two specific for adenosine 3',5'-monophosphate (cAMP) have been partially purified. The cyclic GMP binding activity exhibited high specificity and affinity towards cGMP with a K_D of 42nM. Scatchard analysis of the data indicated a single class of binding sites.

The two cAMP binding activities were physically distinct as indicated by their cellular locations and dissociation constants. The cytoplasmic binding protein exhibited a

K_D of 57nM whereas that of the periplasm had a lower affinity with a K_D of 1uM.

During development, the nucleotide cyclases and cyclic nucleotide binding proteins exhibited changes in activities that are consistent with the previous proposal for the involvement of cyclic nucleotides in development (McCurdy, Ho and Dobson, 1978).

ACKNOWLEDGEMENTS

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Chapter I

INTRODUCTION

Myxococcus xanthus is a Gram-negative bacterium that possesses a complex life cycle (McCurdy, 1974). When nutrients are depleted in a solid medium, cells glide towards aggregation centers forming hemispherical mounds called 'Fruiting bodies' within which the rod shaped cells convert to ovoid, environmentally resistant microcysts. M. xanthus is therefore regarded as an attractive prokaryotic system for the study of morphogenesis and cellular differentiation.

The developmental cycle of M. xanthus shows several remarkable similarities to that of cellular slime mold, Dictyostelium discoideum (Newell, 1977). When deprived of a food source the initially free-living D. discoideum amoebae differentiate into aggregation-competent cells that chemotactically respond to pulses of cAMP to form a multicellular fruiting body (Konijn, et al. 1967). cAMP phosphodiesterases (Toorchen, et al. 1976) play a significant role in the cellular response presumably by modulating the concentration of cAMP to maintain it within the sensitive range.

During the onset of aggregation competence, D. discoideum synthesises a carbohydrate binding protein (discoidin) at the cell surface which is presumed to play a role in cell to cell adhesion during aggregation (Barondes, 1978).

The finding of a similar development-specific lectin [Myxobacterial hemagglutinin (MBHA)] in M. xanthus (Cumsky and Zusman, 1979) suggested that the similarities between D. discoideum and M. xanthus were not merely superficial but reflected similar morphogenetic mechanisms. Hence the search for other mechanistic similarities was encouraged.

A number of workers examined the possibility that cAMP played a role in M. xanthus. First, it was observed that cAMP and ADP stimulated fruiting in M. xanthus (Campos and Zusman, 1975). Subsequently, Parish, et al. (1976) and Yajko and Zusman (1978) demonstrated the presence of cAMP in M. xanthus and Zusman (1978) reported cAMP phosphodiesterase activity in cell free extracts.

In D. discoideum exogenous phosphodiesterase hastened aggregation (Alacantra and Brazil, 1976) and cAMP suppressed the PD effect (Wier, 1977). In M. xanthus, however, it was found that cGMP rather than cAMP delayed the appearance and depressed the number of fruiting bodies, hence suggesting that cGMP not cAMP functioned as the fruiting chemoattractant in M. xanthus (McCurdy, et al. 1978).

These observations contributed to the proposal of a model for the roles of cyclic nucleotides during development in M. xanthus. This model incorporated the suggestion of Yajko and Zusman (1978) that cAMP functions as derepressor of development specific operons and proposed further that cGMP functions as a aggregation chemoattractant (McCurdy, et al. 1978).

Consistent with this proposal it was subsequently reported that cAMP concentration peaked immediately after induction, that cGMP peaked intracellularly between 18-28h and that the latter also exhibited an extracellular peak corresponding to aggregation. Further it was found that cyclic nucleotide PD activities were maximal at the time of maximal aggregation (Ho and McCurdy, 1980). It was also confirmed that cGMP is indeed a chemoattractant for M. xanthus under nutrient deprived (i.e. fruiting) conditions. (Ho and McCurdy, 1979).

During development in D. discoideum adenylate cyclase (Klein, 1976), guanylate cyclase (Mato and Konijn, 1977) and cAMP surface receptors (Malchow and Gerisch, 1974) exhibit changes in activity that reflect critical roles in development. In the following we report the results of an examination of nucleotide cyclases and cyclic nucleotide binding proteins in M. xanthus which suggest that they may play a similar critical role in the development of this prokaryote.

Chapter II

MATERIALS AND METHODS

Organism and growth conditions

Myxococcus xanthus strain M300 (McCurdy, et al. 1978) was used for all experiments.

Working cultures were cultivated on SP agar plates (McCurdy, 1963) or in 30 mls of liquid SP in 125 ml screw cap flasks with shaking at 30°C in the dark. Stock cultures were maintained either on SP slants at 18° for weekly transfers or frozen at -70°C.

Studies on fruiting body formation

Fruiting medium, FM (McCurdy, et al. 1978) contained the following per litre of distilled water: K_2HPO_4, 1.4g; KH_2PO_4, 0.35g; Casitone (Difco, Detroit, Mich), 0.4g; $MgSO_4 .7H_2O$, 1.0g; agar, 15g; pH 7.3. Three milliliter quantities of autoclaved FM were aseptically dispensed onto 50 x 9mm petri dishes (Falcon, Oxnard, CA., Type 1006). After the agar plates had solidified, the condensate was allowed to dry for 3 to 6h with covers partially open.

FM plates were each spot-inoculated with 7×10^6 cells and, after brief drying, incubated at 28°C. After various time intervals a number of replicate plates were harvested by scraping the cells from the agar surfaces and resuspend-

ing them in a buffer containing 0.05M Tris-hydrochloride, pH 7.5; 0.01M Mg^{++} and 3mM mercaptoethanol.

Protein Determinations

The method of Lowry, et al. (1951) was used for protein estimations. Bovine serum albumin was used as standard.

Biochemicals

The following compounds were obtained from Sigma chemical Co., [St. Louis, MO]: Adenosine 5'-triphosphate, sodium salt (ATP); Guanosine 5'-triphosphate, lithium salt (GTP); Adenosine 3',5'-monophosphate (cAMP); Guanosine 3',5'- monophosphate (cGMP); Adenosine monophosphate (AMP); Guanosine monophosphate (GMP); 1-Methyl-3-isobutyl xanthine (IBMX); 2-Mercaptoethanol; Phenylmethanesulfonyl fluoride (PMSF); Sodium azide; Creatine kinase; Phosphocreatine; Tris (hydroxy methyl) amino methane; HEPES; Maleic acid and Glycine. The cAMP and cGMP radioimmune assay (RIA) kits were obtained from New England Nuclear Corp., [Lachine, Quebec].

(8-^{3}H) Guanosine 3',5'-monophosphate (18ci/mmol) and (8-^{3}H) Adenosine 3',5'-monophosphate (20ci/mmol) were obtained from Amersham Corp., Oakville, Ontario]. The DEAE cellulose, CM cellulose and Sephadex G-200 were products of Whatman Corp., [Clifton, New Jersey]. Millipore filters were from Millipore Co., [Bedford, Mass.].

For liquid scintillation counting, the filmware plastic bags (20 ml) were obtained from Nalge Co., [Rochester, New York]. Triton X-100 was obtained from Rohm and Haas Co., [West Hill, Ontario]. 2,5-oxazole (PPO) and 1,4-2(5-phenyl oxozolyl) benzene toluene was obtained from Fisher Chemical Co.,[Don Mills, Ontario].

Enzyme preparation

Toluene treatment of cells was done according to Harwood and Peterkofsky (1975). Ten µls of toluene were added to 1 ml of cell suspension containing approximately 0.5 mg/ml protein, and incubated at 30°C for 10 minutes.

For preparation of crude homogenate and preliminary fractionation, cells were suspended in homogenizing buffer consisting of 50mM Tris-hydrochloride, pH 7.5; 10mM $MnSO_4$; 3mM mercaptoethanol and 20% v/v glycerol. The cells were broken by two, twenty second bursts of sonication with a Bronwill Biosonik (Bronwill Scientific, Rochester, New York), set at full power. The cell free extract obtained after low speed centrifugation (7000 g) was centrifuged at 100,000 g for 90 min at 4°C. The clear supernatant was removed and the following procedure was undertaken to wash the thin pellet adhering to the inner wall of the centrifuge tube. The tube was inverted to drain off all the remaining supernatant and the sides of the inverted tube including the surface of the pellet were washed quickly with the homogenizing buffer. After removing the buffer completely the pellet was suspended in homogenizing buffer to a final volume equivalent to original volume of 5 mls. This wash procedure was employed mainly to minimize the loss of enzyme activity that occurs even at 4°C.

Enzyme Assays

Adenylate cyclase: The enzyme assay was essentially by the method of Terasaki, et al. (1979). The 100 µl reaction

mixture contained 50 mM Tris-hydrochloride, pH 7.5; 2mM $Mnso_4$; 2mM Creatine kinase; 2mM Phosphocreatine, 3mM Sodium azide; 3mM 1-Methyl-3-isobutyl xanthine (IBMX is a cyclic nucleotide phosphodiesterase inhibitor in Myxococcus xanthus, Ho and McCurdy, 1980); 2mM Mercaptoethanoel and 0.1mM ATP. The reaction was carried out at 30°C for 10 min and then stopped by adding 85 µl of 0.2M acetic acid at 4 C followed by boiling for 3 minutes. After cooling 15 µl of 0.8 N NaOH were added to neutralize the reaction mixture and cAMP determinations were made by using the radioimmune assay of Steiner, et al. (1972). The data analysis was made using the computer program of Brooker, et al. 1979.

Guanylate Cyclase Assay

The enzyme assay was essentially by the method of Garbers and Murad (1979). The reaction mixture was same as above except that 1mM GTP was used as substrate and again the determinations were made using the radioimmune assay of Steiner, et al. (1978) and the data analysis using a computer program of Brooker, et al. 1979.

In the assays of both adenylate and guanylate cyclases Tris-HCl, Tris-maleate, Glycine-NaOH and HEPES buffers were used for pH optimum determinations. The enzyme activity was linear with respect to protein concentration (up to 3.33µg/µl) and time (up to 30 min) in the assay.

Preparation of Periplasmic Shock Fluid

Osmotic shock was carried out by the method of of Nossal and Heppel (1966), as modified by Burchard (1974) for use in Myxococcus xanthus. The technique is operationally considered to release the fluids located in the periplasmic space although it cannot be precluded that small amounts of other proteins (i.e. some located in the outer membrane) are released. We shall use the term 'Periplasmic protein' based upon the operational definition in common use. In any case it is the peripheral location that was of importance here.

Vegetative cells were collected by centrifugation at 7,000 g for 10 min at 4°C and washed once with T buffer (10mM Tris HCl, pH 7.5). The washed cells were collected by centrifugation and resuspended in 40 volumes of T buffer. After gentle mixing of the cell suspension for 20 min, an equal volume of 40% sucrose in T buffer was added with stirring and cells were incubated for 10 min with gentle shaking. The bacteria were collected by centrifugation and then shocked with 80 volumes of cold deionized water containing 0.5mM $MgCl_2$, at 0°C. The cells were pelleted by centrifugation and the pellet was saved. The shock fluids were routinely concentrated 20 fold by lyophilization. EDTA was omitted from the shock procedure as it induced lysis in M. xanthus. More than 90% of the shocked cells were viable as determined by viable counts on SP agar.

The post-shocked pellet was suspended in T1 buffer (T buffer containing 5mM $MgCl_2$ and 3mM mercaptoethanol) and

broken by 2, 15 second bursts of sonication with a Brownhill Biosonik (Bronwill Scientific, Rochester, New York) set at full power. The cell free extract was obtained after low speed centrifugation (7000 g for 10 min). The supernatant fraction was dialyzed overnight against 100 volumes of T1 buffer.

Binding Assay

The assay of cyclic nucleotide binding proteins was a modification of the procedure described by Gilman (1970).

The 250 ul reaction mixture contained 10mM Tris-HCl, pH 7.5; 5mM $MgCl_2$; 3mM Mercaptoethanol; 3mM 1-Methyl-3-isobutyl xanthine (IBMX); 1mM Phenyl methane sulfonyl fluoride (PMSF, a protease inhibitor); 20 nM tritiated cyclic nucleotide (10,000 cpm / pmole) and 200 to 800 ug of cell protein. The cytoplasmic cAMP binding assay contained the same reaction mixture except that 200 nM tritiated cyclic nucleotide (10,000 cpm/pmole) was used.

The reaction mixture was incubated for 10 min at 0°C and the assay was terminated by passing the mixture over a membrane filter (Millipore corp. Type HAWMP, 0.45µM pore size) and washing immediately with 3 ml of ice cold T1 buffer. The filter was dried under a heat lamp and counted in a scintillation counter (Beckman, LSC Model 3150P). Controls containing high concentrations of non - ra-

dioactive cyclic nucleotides were run in parallel with binding assays. Determinations containing boiled proteins were equivalent to controls containing high concentrations of non-radioactive cyclic nucleotides.

Purification procedure

The purification procedure was essentially that of Willis, et al. (1974). The shock fluid concentrated by lyophilization was passed through a Sephadex G-200 column equilibrated with T buffer. A single broad protein peak was obtained. The fractions with binding activity were pooled and the pH adjusted to 4.5 with 7.5% acetic acid. The precipitate was separated by centrifugation and the supernatant was loaded onto a high flow-rate CM cellulose column (1 ml resin bed volume, 6-8 mg of protein to be fractionated; column height:diameter 3:1) equilibrated with CM buffer (10mM sodium acetate, pH 4.5). The column was washed with 3-5 volumes of CM buffer followed by elution with 3.5 volumes of 0.3M NaCl in CM buffer. About 20% of the binding activity was absorbed and subsequently eluted from the the column. When the effluent was again passed through a column the same recovery was obtained. Hence it appeared unlikely that there was selective adsorpton between more than one binding protein. The eluted fractions containing all binding activity were pooled and the pH was adjusted to 7.5 with concentrated ammonium hydroxide and then dialysed overnight against T buffer at 4°C. The dialysate was loaded onto a

DEAE cellulose column (1 ml resin bed volume, 4-6 mg protein to be fractionated; column height:diameter 12:1) previously equilibrated with T buffer. The column was washed with 5 bed volumes of cold buffer and activity was eluted with a linear gradient of 0-0.4M KCl in T buffer. The flow-rate was kept at 8-10 mls/h. The cytoplasmic cAMP binding protein eluted between 0.1 to 0.15M KCl, whereas the cAMP binding protein from shock fluid eluted between 0.17 to .23M KCl. The cGMP binding protein eluted between 0.15 to 0.17M KCl. All active fractions were pooled for activity and dialysed overnight against T buffer.

Chapter III

RESULTS

PART I: STUDIES ON ADENYLATE AND GUANYLATE CYCLASE ACTIVITIES

Enzyme Activities During Development

When *Myxococcus xanthus* M300 was induced to fruit by inoculation onto FM, maximum aggregation occured at approximately 40 hours. Fruiting bodies were clearly delimited at about 60h and myxospore differentiation was completed between 75-85 hours.

Both adenylate and guanylate cyclase were detected at levels of activity which varied with the time of development.

The level of adenylate cyclase activity in toluenized vegetative cells was 2-4 pmole/mg protein/min, but during development it exhibited much higher peaks between 10-20h and between 40-50h (Fig. 1). There was also a third smaller but reproducible peak at about 75 hours.

Initial guanylate cyclase activity in toluenized cells was about 3-4 pmole/mg protein/min but then rose sharply to a peak between 8-20h, decreased until 40h and then rose again to a less dramatic maximum at about 75h (Fig. 2).

Guanylate Cyclase

Guanylate cyclase activity was detectable in both the 100,000g supernatant and pellet (Table 1). Both activities exhibited concave substrate-velocity curves, however, the insolubility of the substrate prevented the obtaining of data from higher concentration which with the usual saturation kinetics would have yielded sigmoidal curve. Both the supernatant and pellet enzymes exhibited a K_m of 1mM and a Hill coefficient of 2.6 (Fig. 3). Accordingly, it appears that the same enzyme is detected in both fractions and it probably exhibits positive cooperativity. Guanylate cyclase was dependent on divalent cations: Mn^{++} was more effective than Mg^{++} or Ca^{++}, producing optimal activity at 0.15mM whereas the concentration of Mg^{++} for optimal activity was 1.5mM (Fig. 4). The enzyme exhibited a sharp pH optimum at pH 7.0. It was stimulated by cAMP and pyruvate, but neither ATP (Macchia, et al. 1975) nor fluoride ion (Macchia, et al. 1981; Sun, et al. 1974) had any effect (Table 2).

Adenylate Cyclase

Adenylate cyclase activity was located in both the 100,000g supernatant (soluble enzyme) and pellet (particulate enzyme) (Table 1). The apparent K_m for the latter was 72uM and the Hill coefficient, N=1. The supernatant enzyme had an apparent K_m of 220uM and a Hill coefficient of N=1.9 which may indicate positive cooperativity towards the substrate (Fig. 5).

The supernatant enzyme exhibited a sharp pH optimum at pH 8.5 whereas the pellet enzyme had maximal activity between pH 7.5 to 9.0. Both enzymes required Mn^{++} or Mg^{++} with Mn^{++} being more effective than Mg^{++} (Table 3). Ca^{++} had no effect on either the soluble or particulate enzyme.

Soluble enzyme was stimulated by pyruvate, NaF and GTP while that of the particulate enzyme was not (Table 3).

To determine if the activity peaks seen during development reflected one or both enzymes, changes in soluble and particulate enzyme activities were separately examined during development. It was observed that soluble activity exhibited two peaks, one around 8h and another at approximately 75h (Fig. 6). Pellet enzyme activity was initially high in the vegetative cells, declined and then rose to a peak around 40h (Fig. 7). Hence, while the specific activities determined using toluenized developing cells are not the result of the combined specific activities of pellet and supernatant enzymes (the two enzymes perhaps, being differentially extracted), the results do seem consistent with the assumption that the 8h and 75h peaks observed are attributable to supernatant enzyme while the 40h peak is attributable to the pellet enzyme.

Figure 1: Cellular adenylate cyclase activity in toluenized Myxococcus xanthus, during development. The enzyme was assayed as described under 'Materials and Methods'. Specific activity is expressed as pmole cAMP/ mg protein/min and plotted as the mean with bars showing highest and lowest values detected. Each point represents an average of 5-6 separate determinations.

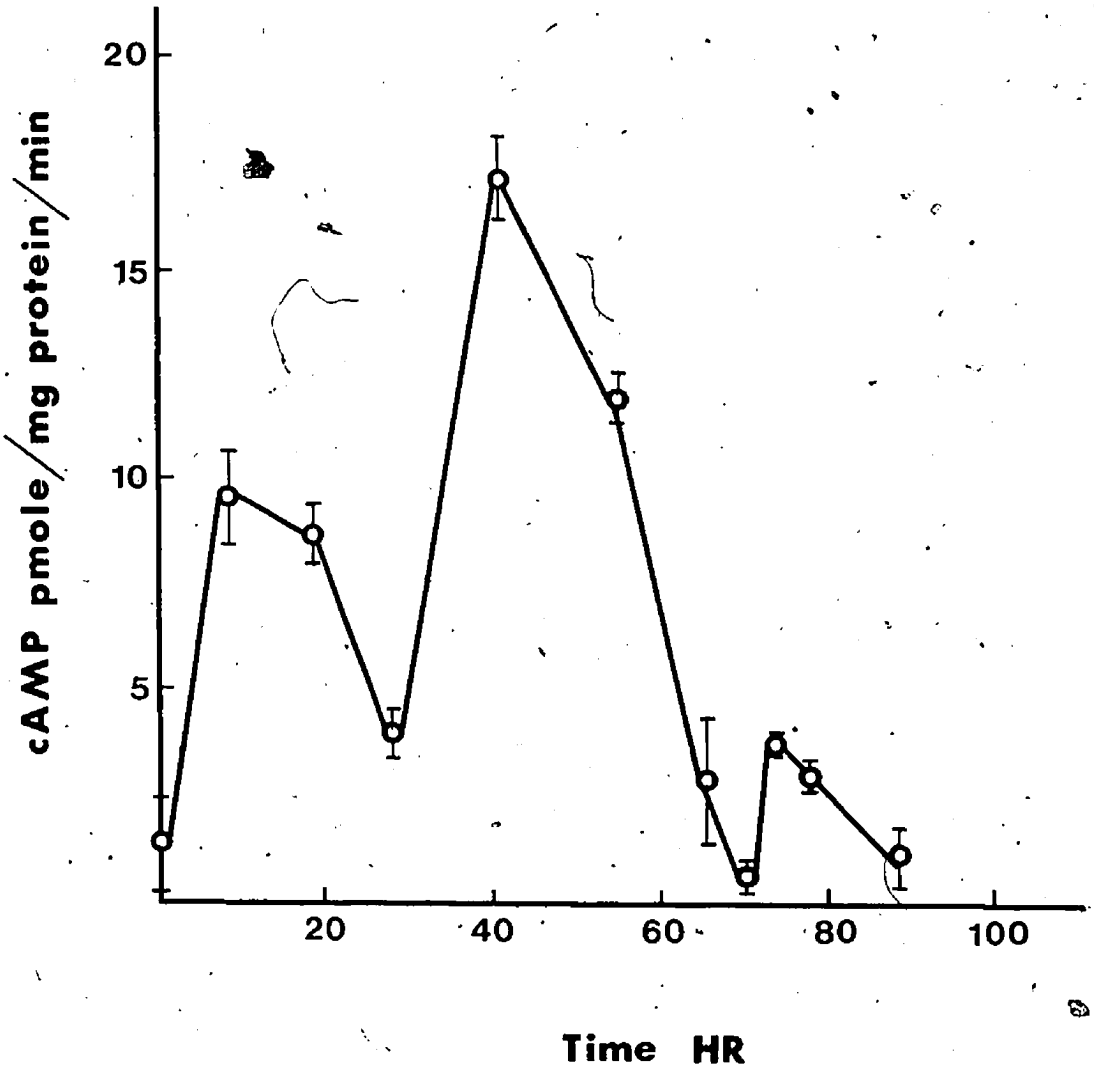

Figure 2: Guanylate cyclase activity in toluenized *Myxococcus xanthus*. The enzyme was assayed as described under 'Materials and Methods'. The specific activity was expressed as pmole cGMP/mg protein/min and plotted as a mean with bars showing highest and lowest detected values. Each point represents an average of 5-6 separate determinations.

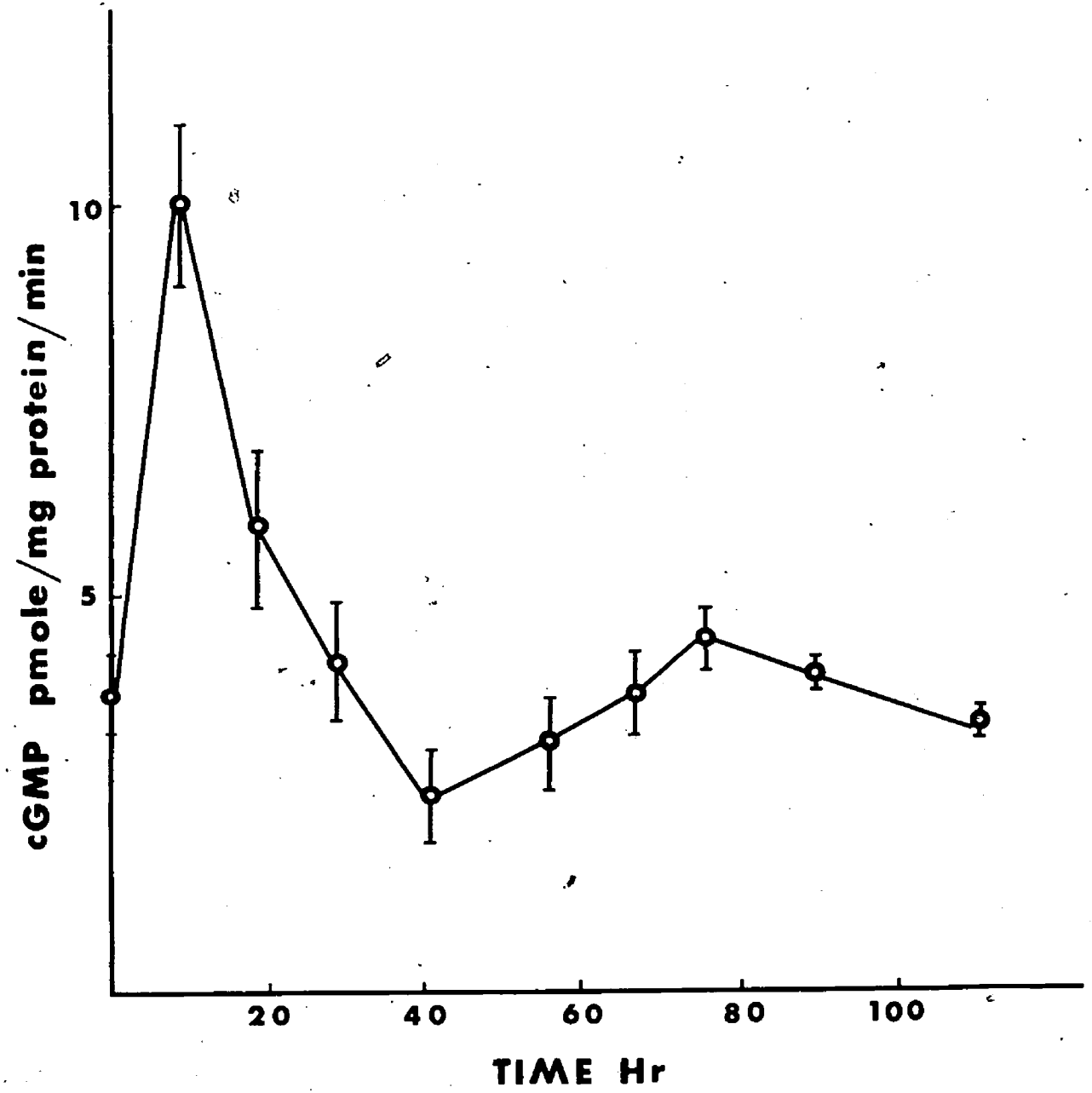

Figure 3: Substrate-velocity plot of guanylate cyclase activity The enzyme was assayed in the 100,000g soluble (o) and particulate (•) fractions of M. xanthus. Substrate concentration was varied from 10^{-2} M to 10^{-6} M. Inset, Hill plot of guanylate cyclase activity obtained using 100,000g soluble (o) and particulate (•) fractions at various concentrations of GTP from 10^{-2} M to 10^{-6} M.

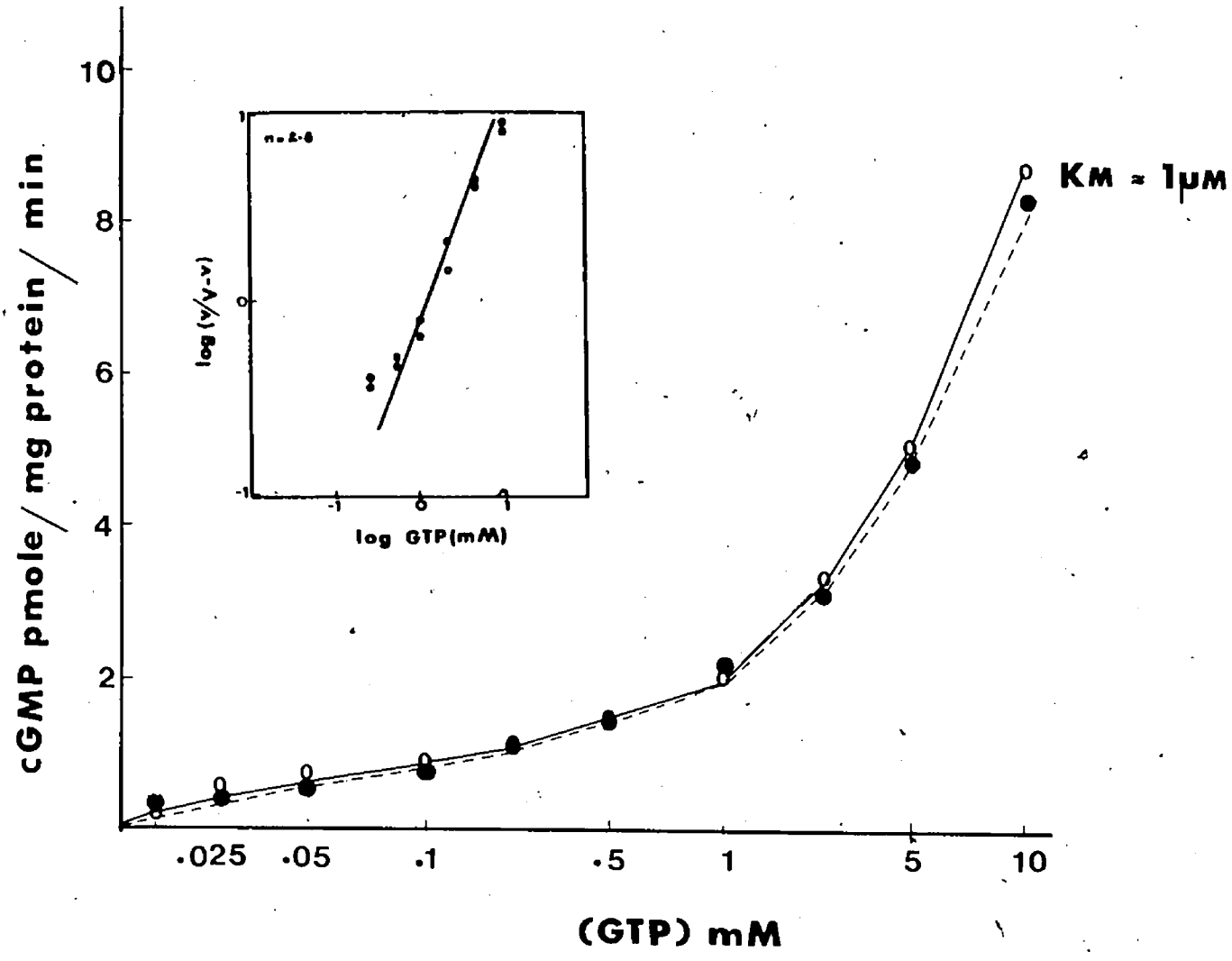

Figure 4: Effect of Mg^{++} and Mn^{++} on guanylate cyclase activity. The reaction was carried out at 30°C under standard conditions, using toluene-treated cells, except that indicated concentrations (0-15mM) of $MnSO_4$ (●) or $Mgcl_2$ (o) were used.

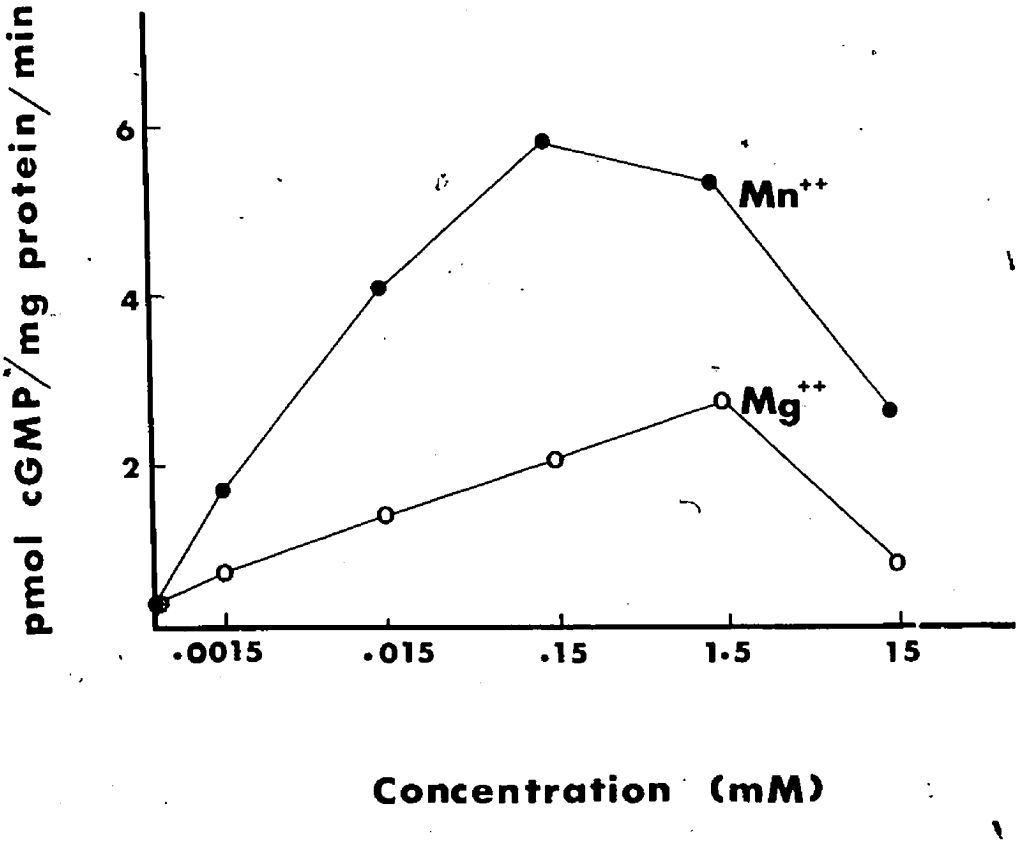

Figure 5: Substrate-velocity plot of adenylate cyclase activity. The enzyme was assayed in the 100,000g soluble (o) and particulate (▲) fractions from the vegetative cells of M. xanthus. Substrate concentrations were varied from 0.5×10^{-2}M to 0.6×10^{-6}M. Inset; Hill plot of adenylate cyclase activity obtained using 100,000 g soluble (o) and particulate (▲) fractions at various concentrations of ATP from 0.5×10^{-2}M to 0.6×10^{-6} M.

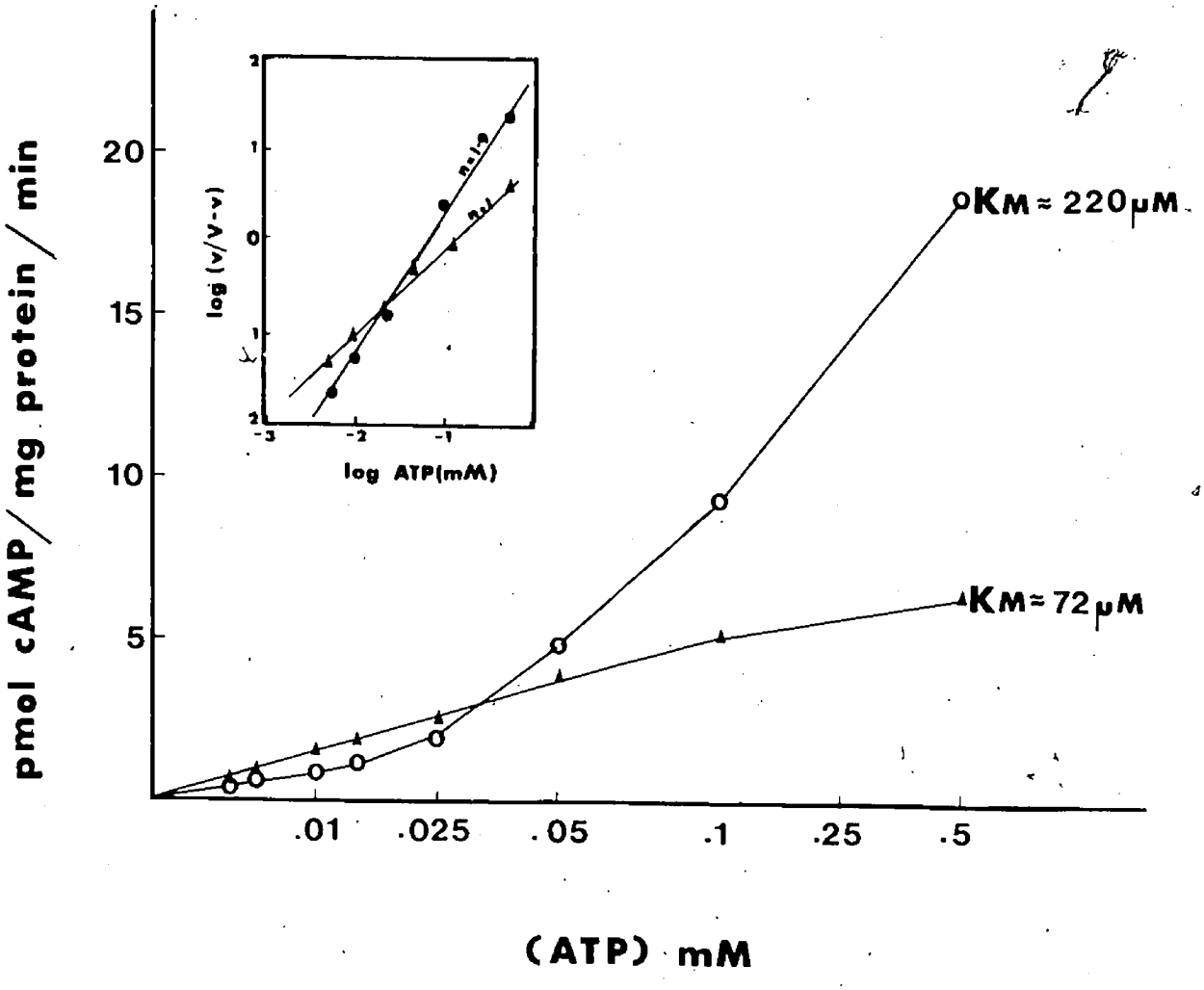

Figure 6: Adenylate cyclase activity in 100,000g soluble fraction of Myxococcus xanthus during development. The enzyme assays were carried out as described under 'Materials and Methods'. The enzyme activity is expressed as pmol cAMP/ mg protein/min, and of two separate determinations.

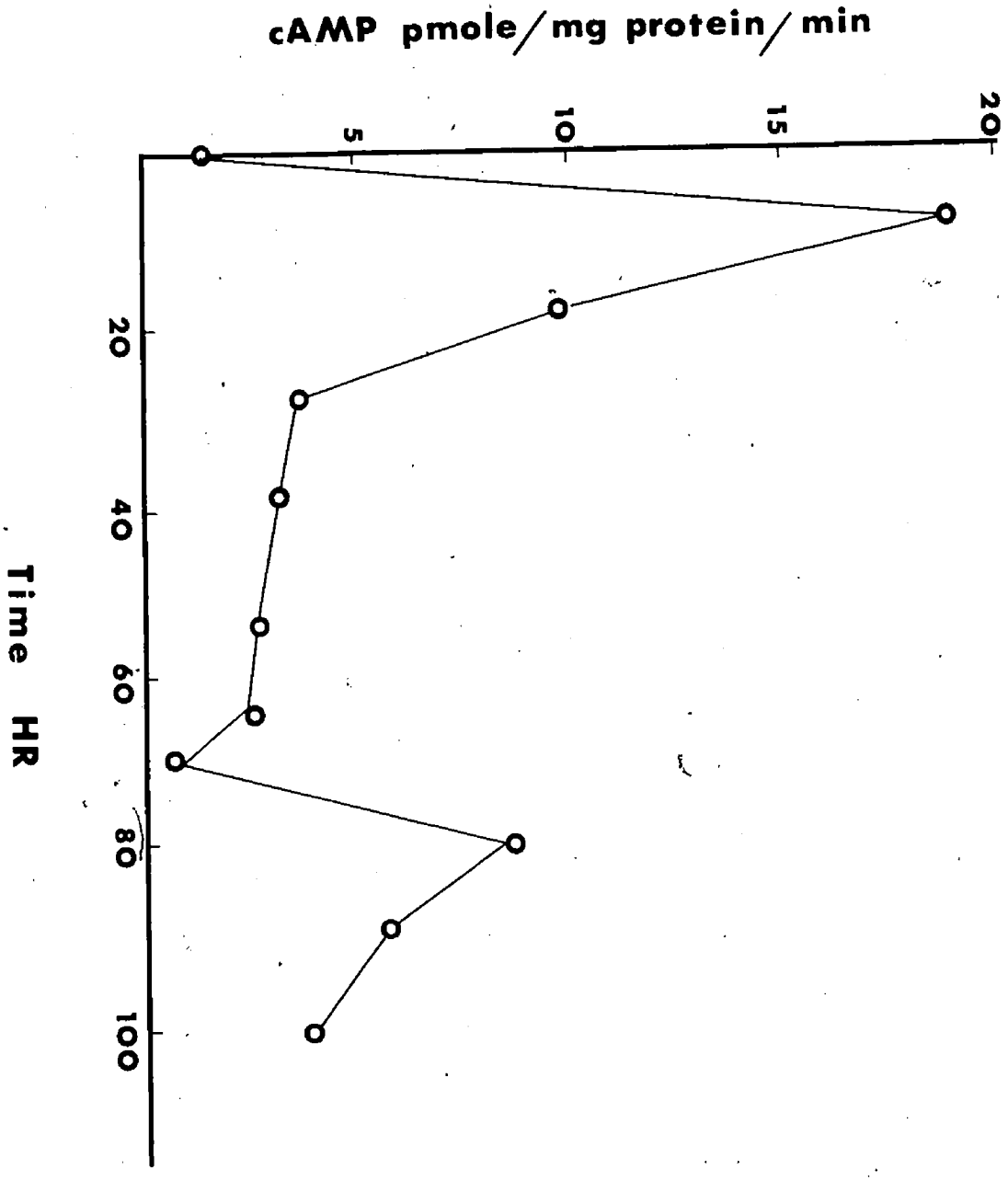

Figure 7: Adenylate cyclase activity in 100,000g particulate fraction of *Myxococcus xanthus* during development. The assay was carried out as described under 'Materials and Methods.' The enzyme activity is expressed as pmol cAMP/mg protein/min and of two separate determinations.

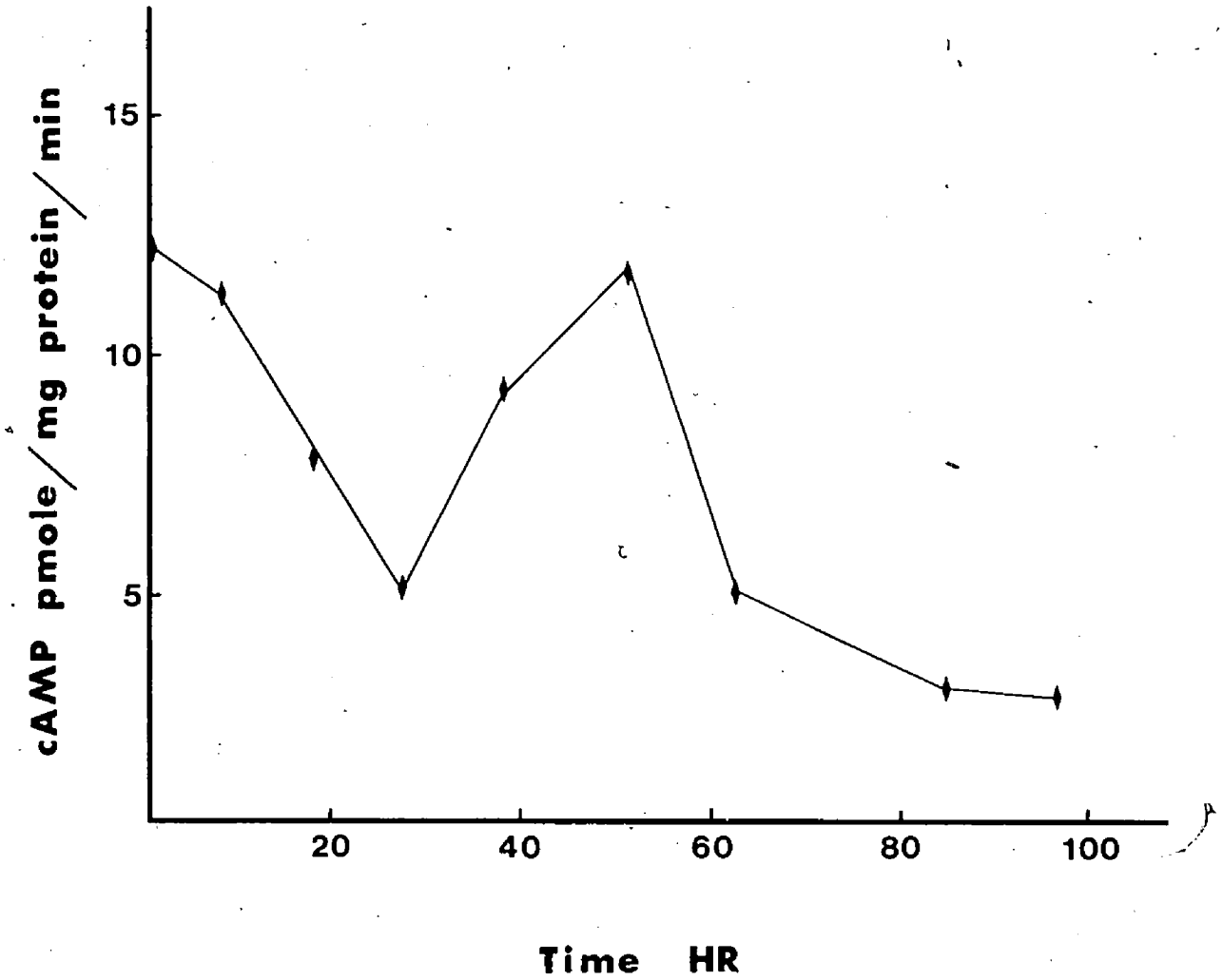

TABLE 1

Cyclase activities in the 100,000g supernatant and pellet fractions

	Specific activity pmol/mg protein/min	Total activity pmol
Adenylate cyclase		
supernatant fraction	1.81	4.52
pellet fraction	12.1	18.15
Guanylate cyclase		
supernatant fraction	3.3	8.25
pellet fraction	4.3	6.73

TABLE 2

The effect of pyruvate, GTP, NaF and cAMP on guanylate cyclase activity

Addition	Concentration mM	Guanylate cyclase activity %
Control		100
Pyruvate	4.5	223
cAMP	5	200
NaF	5	99
ATP	1	100

TABLE 3

The effect of pyruvate, ATP and NaF on soluble and particulate adenylate cyclase activity

Addition	Concentration mM	Adenylate cyclase activity Supernatant %	Pellet %
Control		100	100
Mn^{++}	1.5	736	163
Mg^{++}	1.5	271	105
Pyruvate	4.5	270	103
NaF	5	562	98
GTP	0.05	1200	101

PART II: STUDIES ON CGMP AND CAMP BINDING PROTEINS

Purification of cAMP and cGMP binding proteins

cAMP and cGMP binding activity present in the crude shock fluid was partially purified as described in 'Materials and Methods'. The concentrated shock fluid was passed through a Sephadex G-200 column equilibrated with T buffer. The activity peak was loaded on to a CM cellulose column. The small amount (20%) of activity bound was eluted with 0.3M NaCl in CM buffer. Binding activity was further purified by passing it over a DEAE cellulose column and elution of the bound activity with a linear salt gradient. A peak of cAMP binding activity was found to elute between .17 to .23M KCl and a cGMP binding activity peak eluted between .15 to .17M KCl from DEAE cellulose column.

When the cell free extract from the post-shocked pellet was passed through the purification steps as above, a single peak of cAMP binding activity was eluted between .1 to .15M KCl from DEAE cellulose column.

The purification data are summarized in table 4. The recovery of periplasmic cAMP binding protein was 26% whereas that of the cGMP binding protein was 7%. The purification of cytoplasmic cAMP binding protein was 5 fold whereas that of the periplasm was 30 fold.

Characterization of cGMP binding protein

90% of cGMP binding activity found in the vegetative cells was recovered in periplasmic shock fluid (Table 5).

The binding was complete within 10 seconds and stable for up to 30 mins (Fig. 8). No appreciable cGMP hydrolysis was detected under the assay conditions. cGMP binding was linear with increasing amounts of protein (Fig. 9). It was found in competitive binding assays that the binding was not inhibited cAMP, GTP or GMP (Fig. 10). All the binding activities were corrected for the non-specific binding of labelled cGMP. The Scatchard analysis of the binding data for cGMP yielded a straight line suggesting the presence of a single class of binding sites (Fig. 11). The apparent dissociation constant (K_D) was calculated to be 42nM.

During development M. xanthus exhibited two peaks of cGMP binding activity; a minor but reproducible peak was observed at about 18h and a major peak occurred around 40h, the time of maximum aggregation (Fig. 12). Thereafter the binding decreased to initial levels.

Properties of cAMP binding proteins

cAMP binding was also detected in the periplasmic shock fluid (Table 5). The binding was linear with increasing protein concentrations (Fig. 13) and binding was complete within 10 seconds at 4°C (Fig. 14). Binding of labelled cAMP was not inhibited by cGMP, ATP or AMP over the concentration ranges tested (Fig. 15). Scatchard analysis of the partially purified periplasmic cAMP binding protein indicated the presence of only one class of binding sites with an apparent dissociation constant (K_D) of 1μM (Fig. 16).

cAMP binding was also detected in the post-shocked pellet (Table 5). This activity had properties which were virtually identical to the cytoplasmic cAMP binding activity reported by Orlowski (1980), i.e. cGMP, ATP nor AMP had any effect on cAMP binding at 50μM (Table 6) and a Scatchard plot of cAMP binding activity was linear with a dissociation constant (K_D) of 57nM (Fig. 16).

During development, cytoplasmic cAMP binding activity exhibited a peak early in development around 8-18 h and a subsequent decline to relatively constant levels (Fig. 17). The periplasmic cAMP binding activity however, exhibited a peak around 45-55h.

Figure 8: Cyclic GMP binding as a function of time. The binding assay was performed as described in the text. The binding reaction was started by the addition of concentrated shock fluid to [^{3}H]cAMP (20nM final concentration) at 4°C. Each point represents an average of three separate determinations.

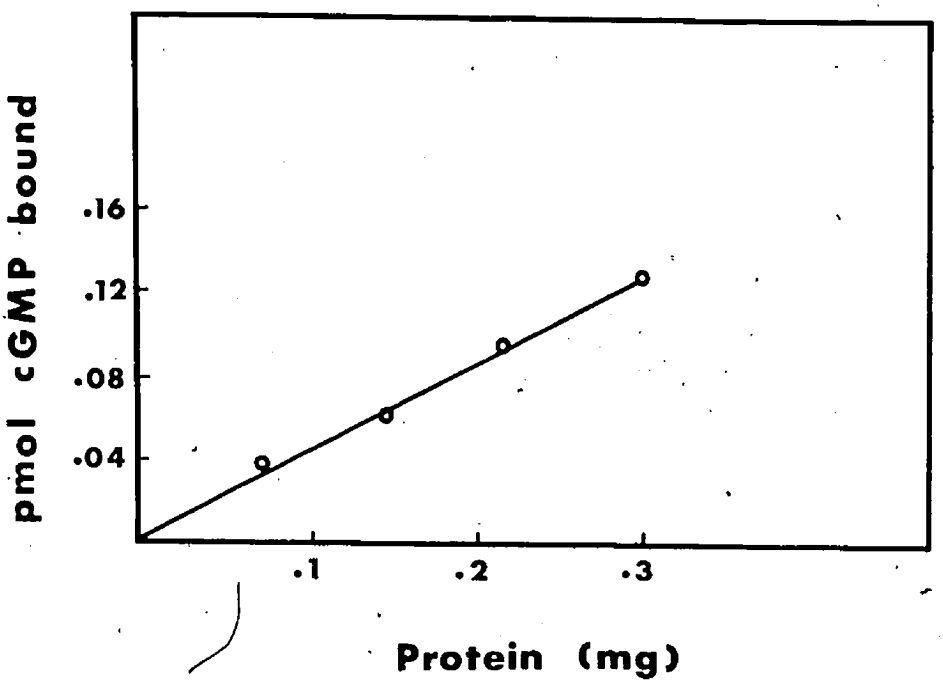

Figure 9: cGMP binding as a function of protein concentration. The assay was started by adding concentrated shock fluid to reaction mixture. The value on the abscissa represents the amount of protein in 250µls reaction mixture.

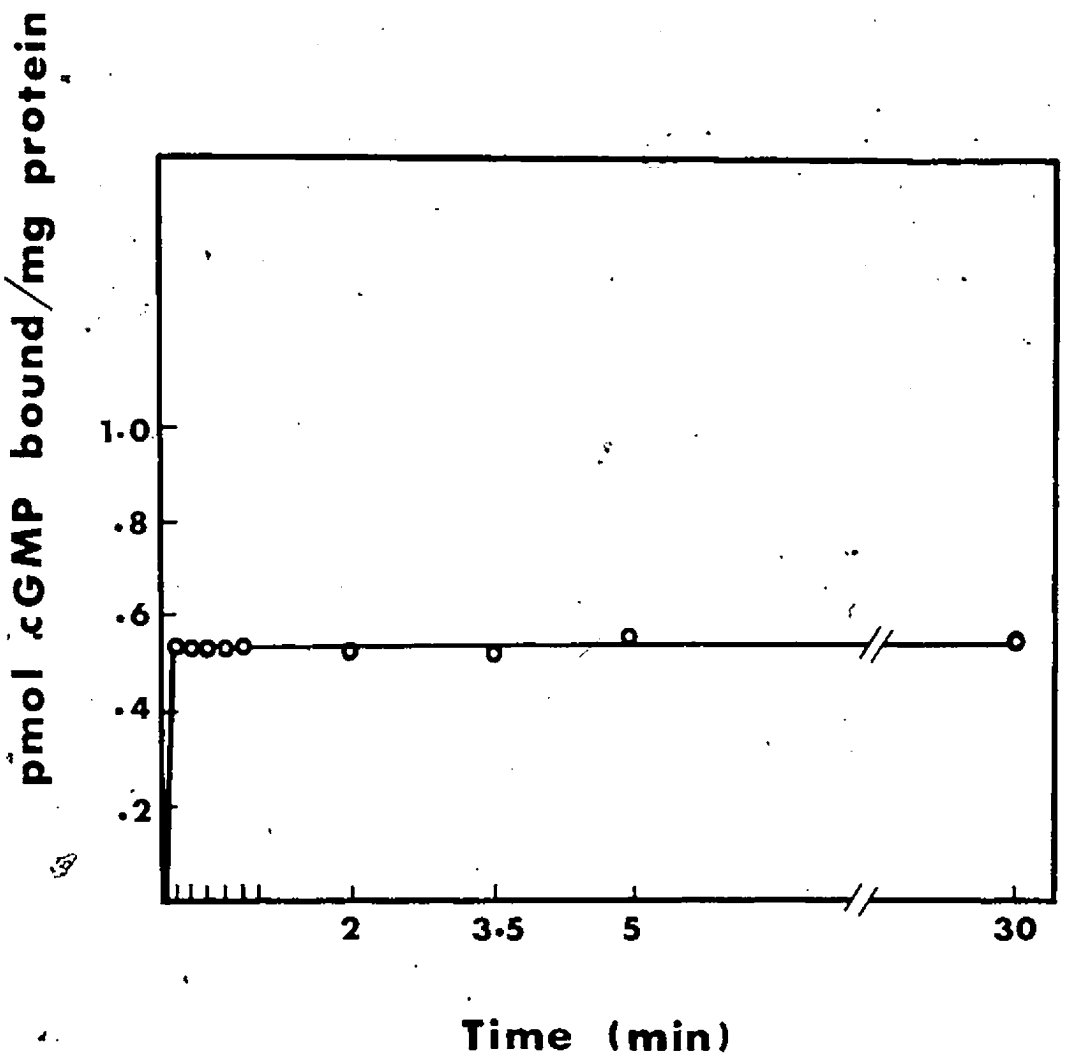

Figure 10: The effects of non-radioactive cGMP (▲), cAMP (▲), GTP (●) on binding of labelled cGMP. The nucleotides were present in increasing concentrations in the presence of 20 nM (^{3}H) cGMP. The binding assay was performed as described under 'Materials and Methods'.

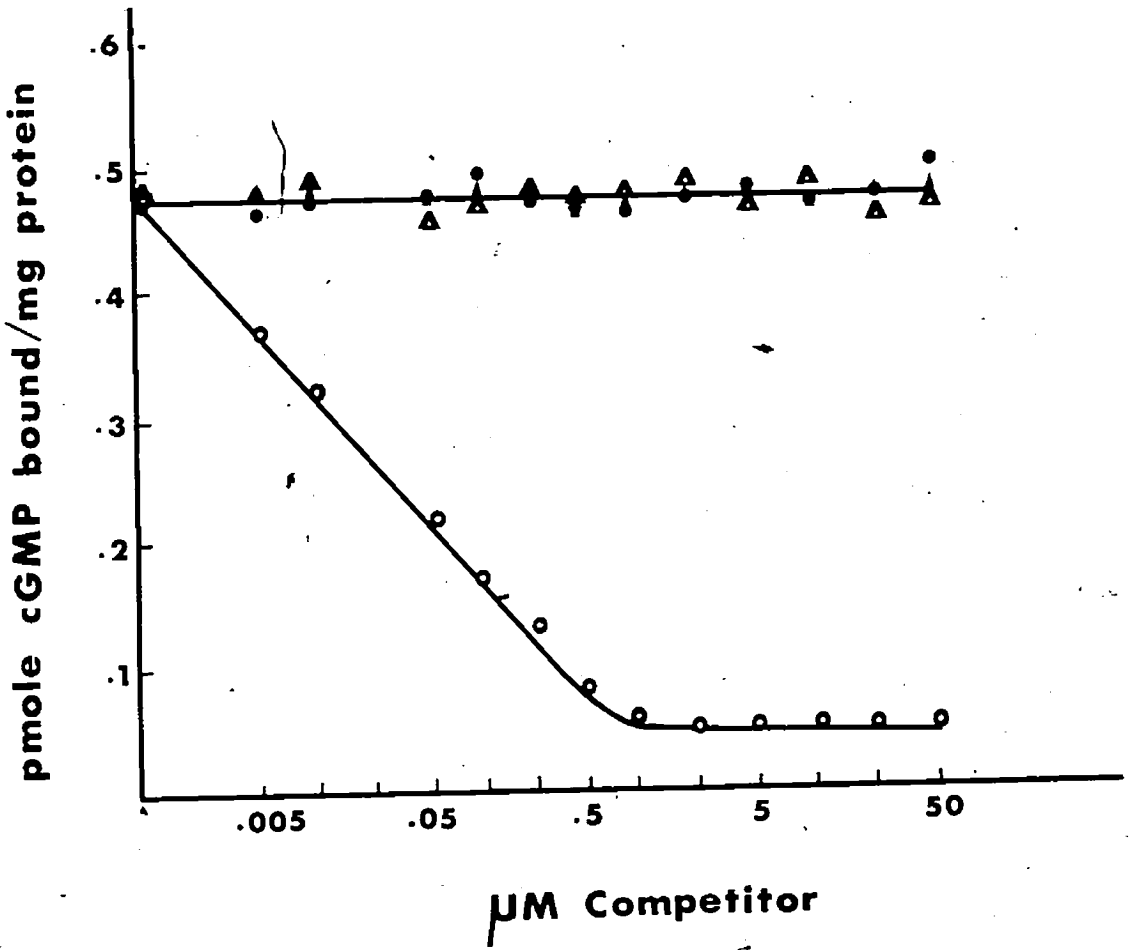

Figure 11: Scatchard plot of [^{3}H]cGMP binding by shock fluid. The binding assay was performed as described in the text. The 250 ul incubation mixture contained 200µg protein and the amount of cGMP bound was calculated from the amount of radioactivity retained by the millipore filter.

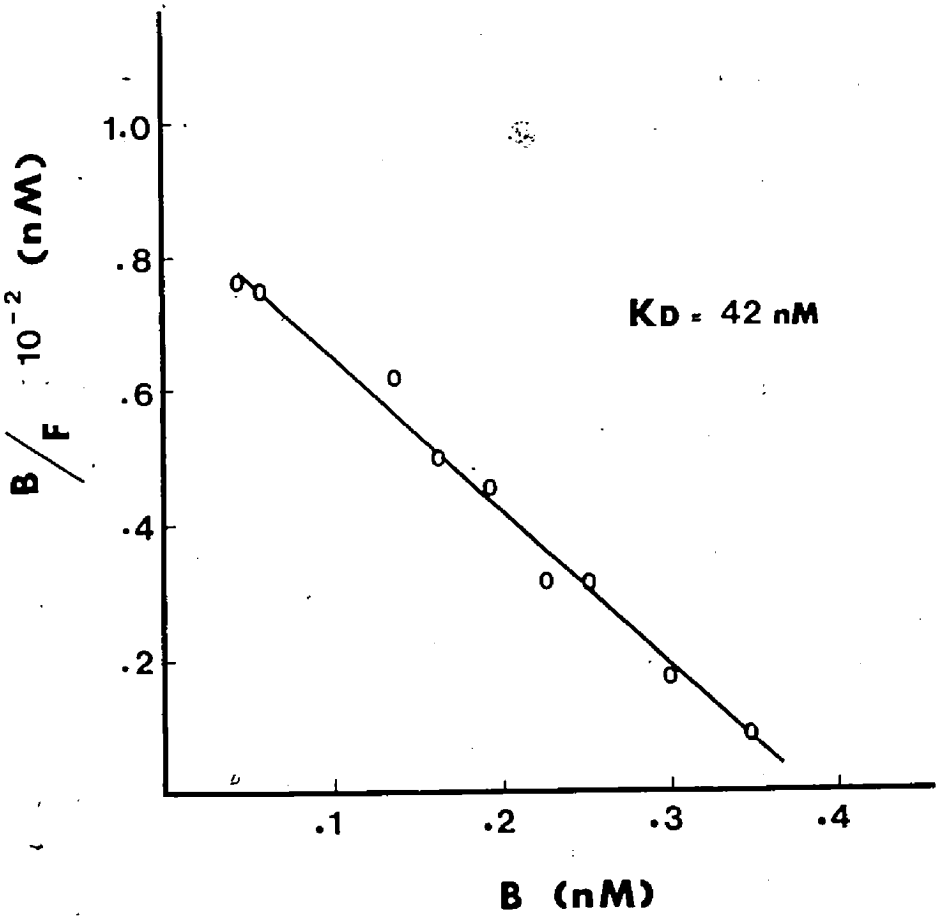

Figure 12: cGMP binding activity during development of M. xanthus. The binding assay was performed as described in 'Materials and Methods'. Concentrated periplasmic shock fluids were prepared at the times indicated after inoculation on FM. Bound cGMP is plotted with the bars showing highest and lowest detected values in 4-5 separate determinations.

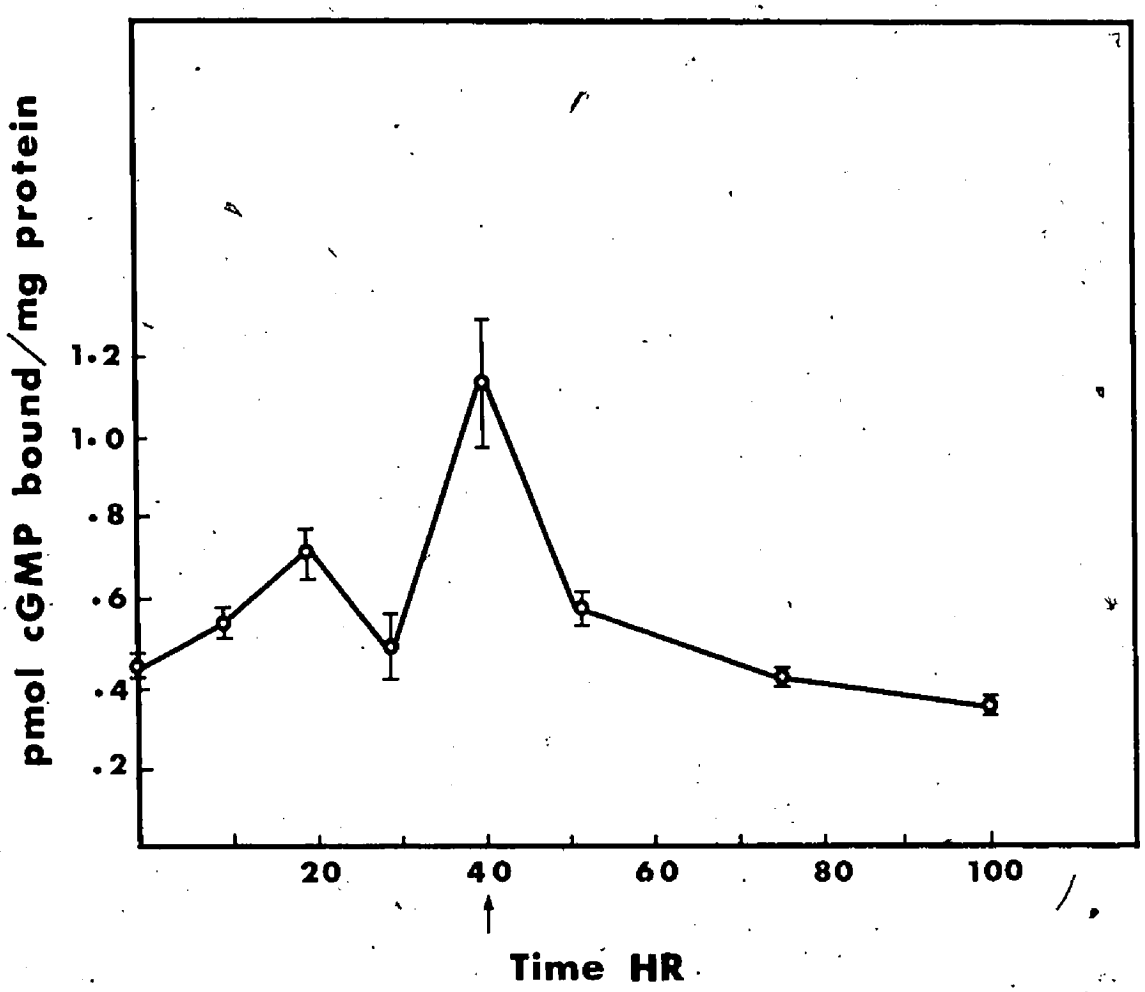

Figure 13: cAMP binding as a function of protein concentration. The assay was performed as described in 'Materials and Methods', boiled protein is used as control. The value on the abscissa represents the amount of protein in 250µls reaction mixture.

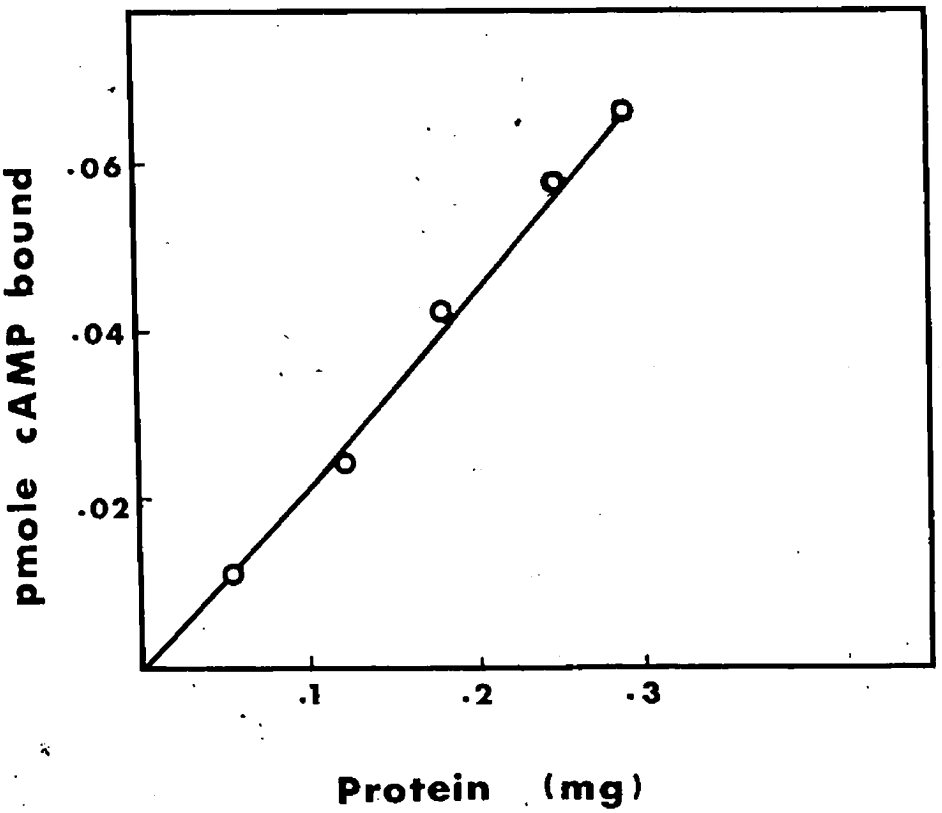

Figure 14: cAMP binding as a function of time. Details of the assay are presented in the text. The binding reaction was started by addition of cell free extract to [^{3}H]cGMP (200nM final concentration) at 4° C.

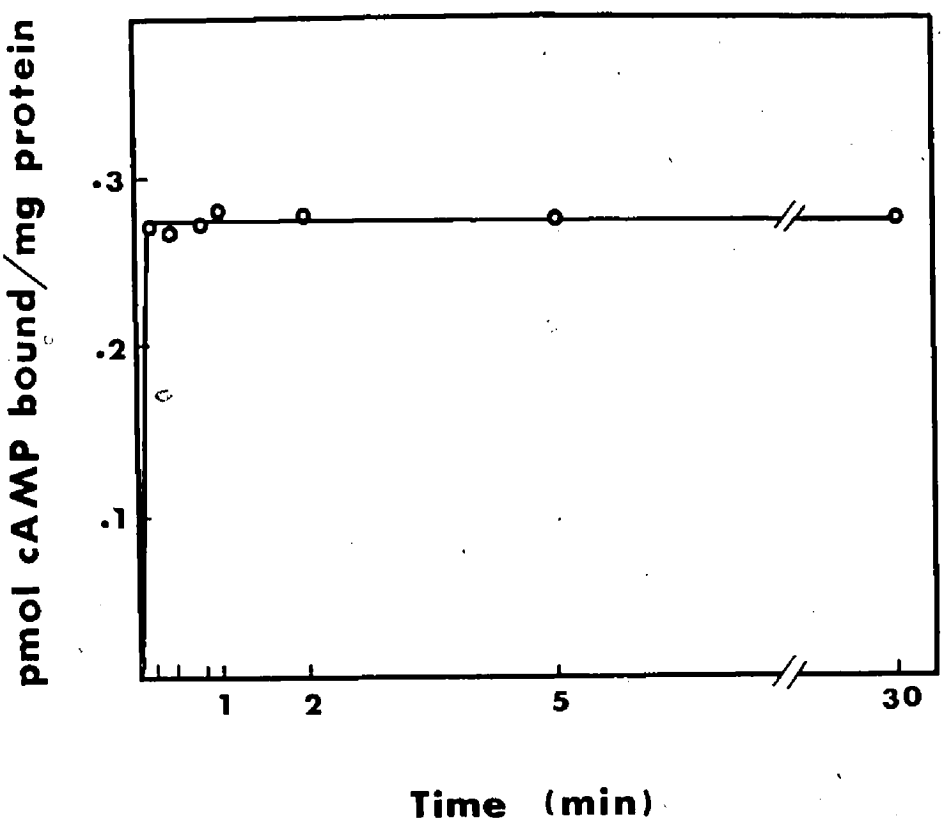

Figure 15: Specificity of cAMP binding activity. The unlabelled cAMP (O), cGMP (●), ATP (Δ) and AMP (▲) were present at increasing concentrations. The standard binding assay described in the text was used.

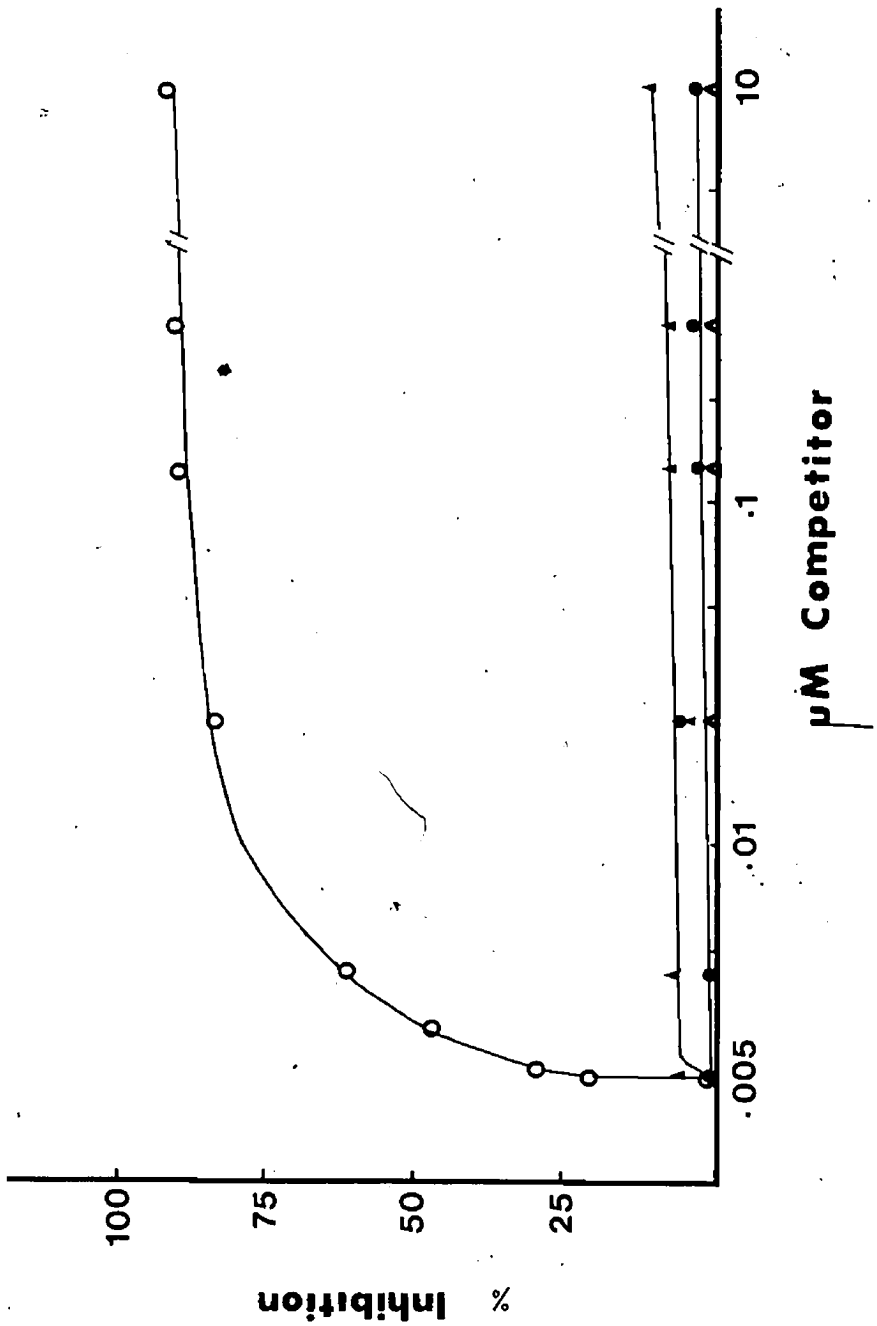

Figure 16: Scatchard plot of cAMP binding activities. The assay was performed using periplasmic shock fluid (▲) and shock pellet cell-free extract (O) as described in 'Materials and Methods'. The amount of cAMP bound was calculated from the amount of radioactivity retained by the filter. The amount of free cAMP was calculated from the total amount of cAMP added to the mixture less the amount bound.

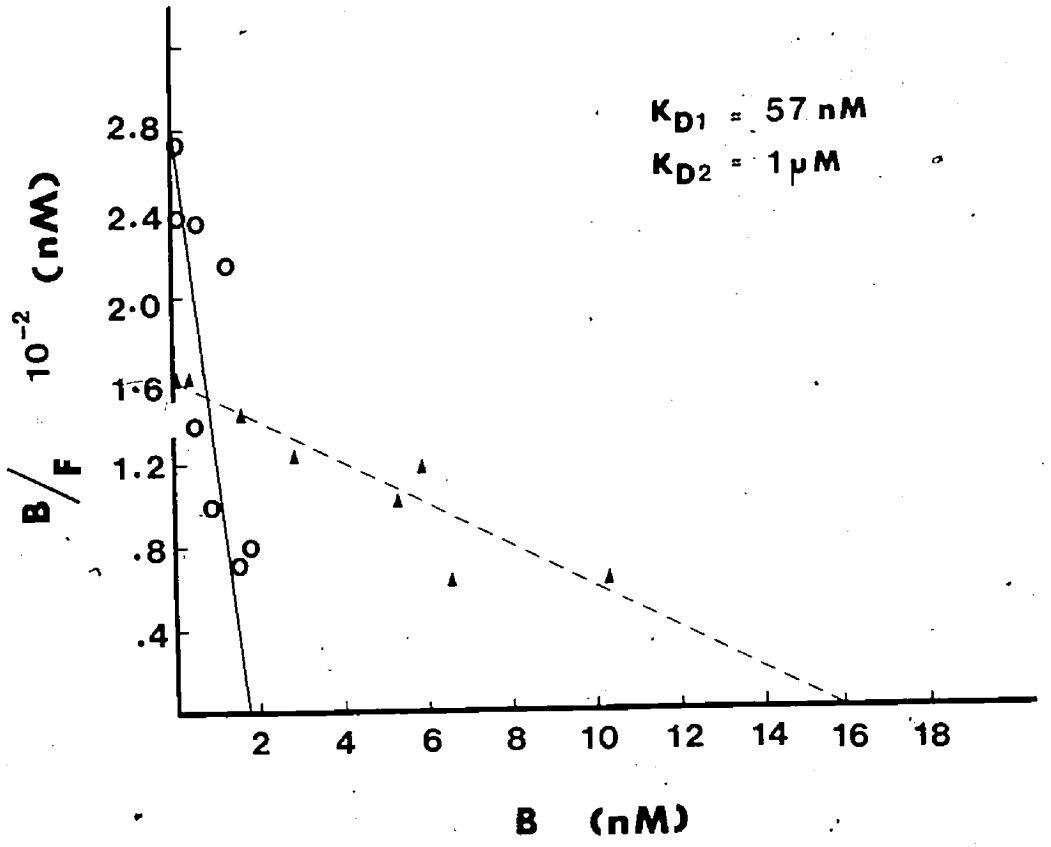

Figure 17: cAMP binding activities during development of M. xanthus. Periplasmic shock fluid (▲) and post-shock pellet cell free extract (O) were obtained from the same batch of cells immediately after harvesting. Binding data represent average of four separate determinations, bars indicate the highest and the lowest values detected.

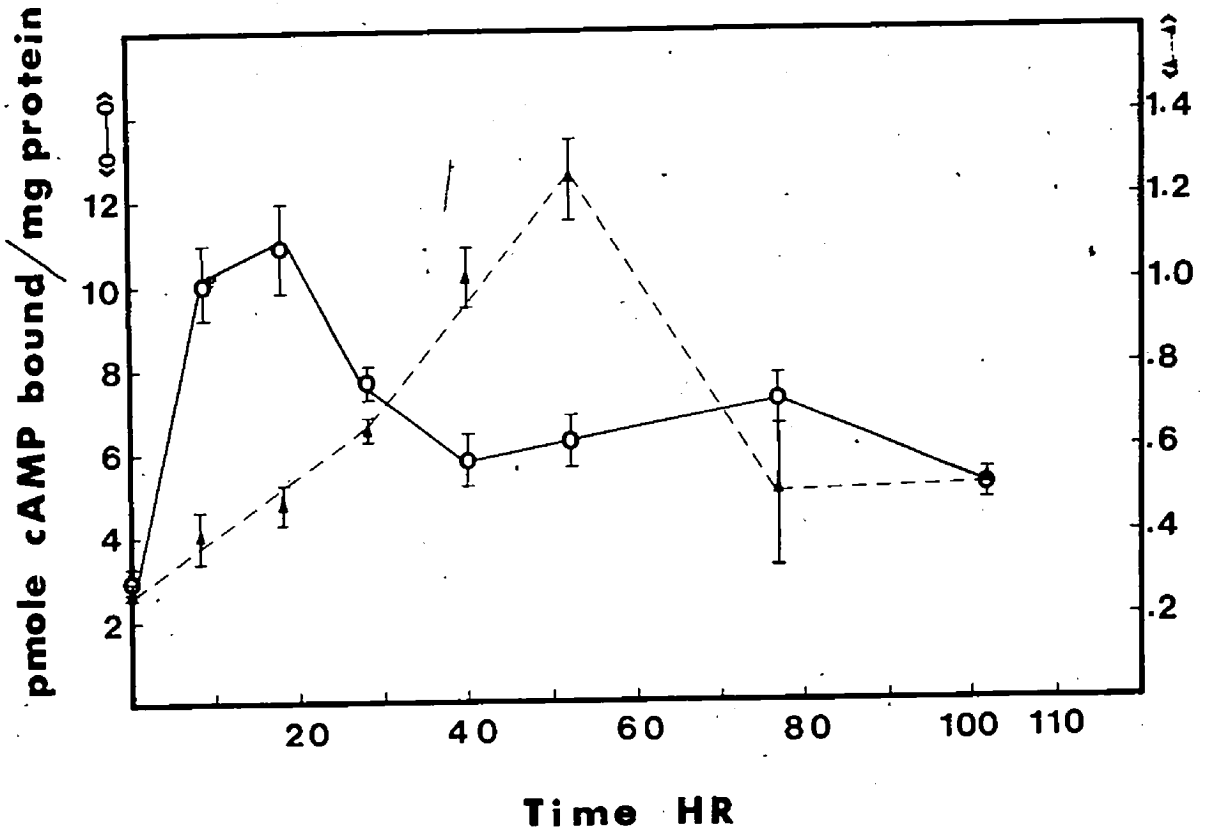

TABLE 4

Partial purification of binding proteins.

	Vol mls	Total Units	Protein mg/ml	Specific Activity pmoles bound mg protein^{-1}	Purification	Recovery %
Periplasmic cGMP Binding protein Shock fluid (concentrated)	37	16.00	0.93	0.46	1	100
G-200	20	11.40	0.60	0.95	2.10	71.2
CM-cellulose	10	2.43	0.09	2.70	5.86	15.18
DEAE-cellulose	5	1.13	0.07	3.80	8.26	7.06
Cytoplasmic cAMP Binding protein Cell free extract	45	404.1	5.98	1.5	1	100
G-200	25	209.0	3.80	2.2	1.46	51.7
CM-cellulose	10	43.44	1.81	3.0	2	10.7
DEAE-cellulose	5	8.4	0.3	8.0	5.3	2.1
Periplasmic cAMP Binding protein Shock fluid (concentrated)	37	9.6	0.93	0.28	1	100
G-200	20	6.72	0.30	1.12	4.0	70.0
CM-cellulose	10	4.96	0.09	5.52	19.7	51.6
DEAE-cellulose	5	2.49	0.06	8.3	29.6	25.9

TABLE 5

The cellular distribution of binding proteins.

	Periplasmic shock fluid	Post-shock pellet	Total*
cGMP binding activity (pmole bound)	0.388	0.042	0.43
cAMP binding activity (pmole bound)	1.13	2.03	1.4

* The total cyclic nucleotides bound was obtained from sonicating and subsequently assaying the sonicated extracts of vegetative cells.

TABLE 6

Specificity of cAMP binding.

Nucleotide added	% cAMP binding
None	100
cGMP (5×10^{-5} M)	100.2
ATP (5×10^{-5} M)	95
5'AMP (5×10^{-5} M)	101
GTP (5×10^{-5} M)	98
GMP (5×10^{-5} M)	100.7

Chapter IV

DISCUSSION

The guanylate cyclase from vegetative cells of Myxococcus xanthus has many properties in common with the enzymes previously reported in prokaryotes: it is not stimulated by fluoride- an agent known to activate guanylate cyclase of eukaryotes; it requires Mn^{++} for activity and does not use ATP as substrate. The K_m for GTP of 1mM is in the range of values so far reported for guanylate cyclases of prokaryotes [0.1 to 5mM] (Clark and Bernlohr, 1972, Macchia, et al. 1975 and Sun, et al. 1974).

On the other hand it resembles the eukaryotic enzyme in that it seems to exhibit cooperativity towards its substrate (Garbers, et al. 1975; Chrisman, et al. 1975; Craven, et al. 1975).

At least two forms of adenylate cyclase activity can be separated by high speed centrifugation of cell free extracts.

While the terms "soluble and particulate", may not precisely reflect the in vivo nature of the enzyme, it is likely that there is compartmentalization. In this connection the observations of [illegible] are relevant.

Ide examined the lysates of 21 bacterial species for adenylate cyclase activity and found that the bacterial enzymes generally fell into two groups: (i) predominantly soluble in cell lysates and stimulated by pyruvate and (ii) primarily membrane-associated, not pyruvate stimulated. Similarly we found that the soluble form of adenylate cyclase was stimulated by pyruvate whereas pyruvate had no effect on pellet activity. The soluble form of adenylate cyclase resembles the eukaryotic enzyme in that it is stimulated by GTP and NaF (Bhat, et al. 1980).

The soluble and particulate adenylate cyclase activities are also distinguishable by their kinetic behaviour. The presence of two different adenylate cyclase activities exhibiting different K_ms has also been reported for Streptococcus salivarius (Khandelwal and Hamilton, 1979), for which it was suggested that the presence of two activities reflected their association with different subcellular fractions.

Adenylate cyclase activity exhibited peaks at critical periods in development. The first peak occuring around 8h could be responsible for the peak of cAMP concentrations previously reported to occur between 0 and 20h (Ho and McCurdy, 1980). The 40h peak while coincident with aggregation does not correlate with any observed increase of cAMP concentrations (Yajko and Zusman, 1978; Ho and McCurdy, 1980). This may be due to high phosphodiesterase activity

observed at that stage (Ho and McCurdy, 1980). The third peak occured around 75h when microcyst formation within fruiting bodies is initiated. Previous studies (McCurdy, et al. 1978; Yajko and Zusman, 1978) have demonstrated a sharp peak in cAMP content during early development of glycerol induced myxospores.

It was hypothesized (McCurdy, et al. 1978) that cAMP is involved in derepressing differentiation specific operons at two critical times. First, shortly after induction of fruiting body formation; second, on initiation of myxospore induction. It is consistent with these expectations that it is the soluble, apparently allosteric form of adenylate cyclase which exhibits maxima at these critical times.

Guanylate cyclase activity exhibited a peak of activity ty between 0 and 20h, a time which slightly preceeds the intracellular and subsequent extracellular accumulation of cGMP associated with aggregation (Ho and McCurdy, 1980). Similarly the second peak in the activity corresponds to the second rise in cGMP concentration occuring late in development as reported earlier (Ho and McCurdy, 1980).

If one takes into account the phosphodiesterase activities, it appears that cyclic nucleotide accumulation is a reflection of cyclase activities in M. xanthus.

The studies on binding proteins indicated the presence of a cGMP specific periplasmic binding protein and two cAMP binding proteins distinguishable by their elution patterns

on DEAE cellulose, their dissociation constants and their cellular locations. The cytoplasmic cAMP binding protein has been identified with that previously reported by Orlowski (1980).

The properties of the binding proteins are consistent with the premise that they function as receptors which mediate the physiological effects of their ligands. Among these are their high specificities and affinities, although the periplasmic cAMP binding activity is marginal in this respect with its K_D of 1μM. It is also unlikely that the binding activities are identifiable with enzymes attacking the ligands. Ligand binding was stable in all cases for at least 30 mins with no evidence of hydrolysis and was not affected by the corresponding nucleotide monophosphates. PDs were inhibited in the reaction mixtures but in any case exhibited different patterns during development from those of the binding proteins (Ho and McCurdy, 1980). The K_D of cAMP binding protein (57nM) differed significantly from the K_m for cAMP-PD (24μM, Yajko and Zusman, 1978). It is unlikely therefore that PD interfered with the assay.

It would be consistent with a physiological receptor function if the location and levels of activity of the binding proteins corresponded to the site of action of their ligands as well as changes in their concentration levels.

Accordingly, the periplasmic location of cGMP binding protein makes it accessible to its extracellular and chemo-

tactic ligand (Ho and McCurdy, 1979). Furthermore, as would be expected, it increases in activity concomittant with the increase in extracellular cGMP concentration which is associated with fruiting body aggregation (Ho and McCurdy, 1980).

Similarly the cytoplasmic cAMP binding protein increased concomittantly with the increase in intracellular cAMP levels observed upon fruiting body induction, an observation that supports the premise that it functions with cAMP in derepression of morphogenesis in a manner analogous to CAP protein in E.coli (Zubay, et al. 1970).

The periplasmic cAMP binding protein exhibits a peak of activity at the later stages of development which corresponded to a significant increase in extracellular concentrations of cAMP (Ho and McCurdy, 1980). Whether such a correlation is of significance in fruiting body formation is unknown.

In conclusion, this study was based upon the assumption that nucleotide cyclases and cyclic nucleotide binding proteins play a role in development of M. xanthus. In general, our results are consistent with that proposition although only correlative in nature. What is now required is an examination of aggregation-defeicient mutants of M. xanthus M300 to determine whether their developmental defects correspond to deficiencies in the elements of cyclic nucleotide metabolism which were examined in this dissertation study.

Should this be found to be the case more detailed examinations of the distribution, physical characteristics and mechanisms of action of these activities would be justified.

SUMMARY

Activities of nucleotide cyclases and cyclic nucleotide binding proteins and their behaviour during development were examined. The cyclases were distributed between 100,000g supernatant and pellet fractions. The adenylate cyclase activity of the latter differed from that of the soluble fraction in its kinetic behaviour. Both the supernatant and the pellet fractions contained the same guanylate cyclase activity.

The cyclic nucleotide binding proteins exhibited affinities, specificities and cellular distribution expected of physiological receptors. The two cAMP binding proteins were distinct as indicated by their elution patterns from DEAE cellulose column, cellular location and affinities towards cAMP. Only one cGMP binding protein was detected and it was located in the periplasm.

The activities of nucleotide cyclases and cyclic nucleotide binding proteins exhibited changes during development that suggest their involvement in morphogenesis in M. xanthus.

ABBREVIATIONS

AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
cAMP	Adenosine 3',5'-monophosphate
cGMP	Guanosine 3',5'-monophosphate
CM-cellulose	Carboxy-methyl cellulose
CM-buffer	10mM Sodium acetate, pH 4.5
DEAE-cellulose	Diethylaminoethyl cellulose
EDTA	Ethylenediaminetetraacetic acid
FM	Fruiting medium
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
HEPES	N'-2-Hydroxyethylpiperazine N'-2-ethanesulfonic acid
IBMX	1-Methyl-3-isobutyl xanthine
PD	Phosphodiesterase
PMSF	Phenyl mehane sulfonyl fluoride
Tris HCl	Tris(hydroxymethyl)aminomehtane hydrochloride
T buffer	10mM Tris HCl, pH 7.5
T1 buffer	10mM Tris HCl, pH 7.5; 5mM $MgCl_2$; 3mM mercaptoethanol

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