CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE AND CYCLIC GUANOSINE 3':5'-MONOPHOSPHATE METABOLISM DURING THE MITOTIC CYCLE OF THE ACELLULAR SLIME MOULD PHYSARUM POLYCEPHALUM (SCHWEINITZ)

by

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ABSTRACT.

Several methods have been assessed and the most effective used for the extraction, purification, separation and assay of cyclic AMP and cyclic GMP from the acellular slime mould Physarum polycephalum.

Both cyclic nucleotides have been measured at half hourly intervals in synchronous macroplasmodia. Cyclic AMP was maximal in the last quarter of G2 while cyclic GMP showed two maxima, one occurring during S phase and the other late in G2.

Enzymes synthesising and degrading these cyclic nucleotides and their sensitivity to certain inhibitors and cations have been assayed by new radiometric methods in which the tritium labelled substrate and product are separated by thin layer chromatography on cellulose.

Synchronous surface plasmodia have been homogenised and separated into three fractions by differential centrifugation.

Phosphodiesterase activity of each fraction has been measured using tritium labelled cyclic AMP and cyclic GMP as substrate. During the 8 hour mitotic cycle, no significant change of either enzyme in any fraction was detected.

Adenylate and guanylate cyclase activity has been measured using the tritium labelled imidophosphate analogues as substrate.

Adenylate cyclase activity in a low speed particulate fraction increased by about 50% in late G2. No significant changes were detected at any time in the high speed particulate or soluble fractions.

Guanylate cyclase activity in all fractions rose and fell in the S phase and in the last quarter of G2, at times coincident with the time of maximal cyclic GMP content.

The role of cyclic nucleotides in the cell cycle and their function in controlling the mitotic cycle in Physarum is discussed in the light of these findings.

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ABBREVIATIONS.

Aden.	Adenosine.
AMP	Adenosine monophosphate
AMP PNP	β-γ imido adenosine 5' triphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Av..	Average
BHK cells	Baby hamster kidney cells.
B.P.	Binding protein
BSA	Bovine serum albumin
cAMP, cyclic AMP	Cyclic adenosine 3':5'-monophosphate
cAMP PDE	Cyclic adenosine 3':5'-monophosphate phospho-diesterase.
cGMP, cyclic GMP	Cyclic guanosine 3':5'-monophosphate
cGMP PDE	Cyclic guanosine 3':5'-monophosphate phospho-diesterase.
CHO cells	Chinese hamster ovary cells.
Ci	Curie
cm	Centimetre
c.p.m.	Counts per minute
dbcAMP	Dibutyryl cyclic AMP
DEAE cellulose	Diethylaminoethyl cellulose
d.p.m.	Disintegrations per minute
E.S.R.	External standard ratio
g	Acceleration due to gravity, gram
GMP PNP	β-γ imido guanosine 5' triphosphate
Guan.	Guanosine
IBMX	3-isobutyl-1-methyl xanthine
IMP	Inosine monophosphate.

IR	Infra red
K_m	Michaelis constant
L.S.C.	Liquid scintillation counting
M	Molar, mitosis
M2,M3	Second and third post fusion mitosis
mg, ml, mm, mM	Milli-, gram, litre, metre, molar
N	Normal
n	Number, nano
NADP	Nicotinamide-adenine dinucleotide phosphate
NADPH	Nicotinamide-adenine dinucleotide phosphate, reduced
NRK cells	Normal rat kidney cells
p	Pico
QAE	Quaternary aminoethyl
r^2	Regression coefficient
RIA	Radioimmunoassay
rpm	Revolutions per minute
S.D.	Standard deviation
S.D.M.	Semi defined medium
sec	Second
SQ 20009	1-Ethyl-4-(isopropylidenehydrzino)-1H-pyra-zolo-(3, 4-b)- pyridine-5-carboxylic acid, ethyl ester hydrochloride
S.W.G.	Standard wire gauge
T	Toluene scintillant
TCA	Trichloroacetic acid
TE	Toluene ethanol scintillant

TES	N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid
t.l.c.	Thin layer chromatography
TTX-100	Toluene Triton X-100 scintillant
UV	Ultra violet
vol	Volume
wt.	Weight
w/v	weight/volume
μ	micro

CHAPTER 1

INTRODUCTION.

1•10 Introduction.

Adenosine 3':5'-monophosphate has been accepted as a ubiquitous central regulator of many metabolic processes (Robison *et al.* 1968). Moreover, cyclic AMP and cyclic GMP appear to be important in the regulation of cell growth (Goldberg *et al.* 1974).

The use of synchronised cell populations greatly facilitates the anaylsis of cyclic nucleotide function. The first indication that cyclic AMP may play a regulatory role was the finding that its concentration varies in different stages of the cell cycle, an extensive study has been made with HeLa cells by Zeilig *et al.* (1976). However, treatments that are used to induce synchrony may result in a perturbation of the normal biochemistry of the cell (Mitchison 1970).

To avoid these problems use has been made of the acellular slime mould *Physarum polycephalum*, in which the nuclei show natural synchronous mitosis. *Physarum* may be grown to provide ample material for biochemical analysis of the mitotic cycle. Almost no work has been done on cyclic nucleotides or their role in the mitotic cycle of this eukaryote.

1•20 Cyclic nucleotides.

Studies on the stimulation of glycogenolysis by adrenaline (epinephrine) and glucagon by Sutherland (1951) led to the discovery that these hormones were responsible for the activation

of the enzyme, phosphorylase. Later, Rall et al. (1957) were able to show that the activation of this enzyme was mediated by a low molecular weight heat stable factor. Independently, Cook et al. (1957) described the formation of an hitherto unknown adenine nucleotide when adenosine triphosphate (ATP) was treated with barium hydroxide. Sutherland and Rall (1957) showed that this preparation had the same biological properties as their heat stable factor. Subsequently Lipkin et al. (1959) confirmed the structure of this factor as adenosine 3':5'-monophosphoric acid (cyclic AMP).

Later, in 1963, a different cyclic nucleotide was discovered among (^{32}P) containing compounds in the urine of rats following the administration of labelled inorganic phosphate (Ashman et al. 1963). This was guanosine 3':5'-monophosphate (cyclic GMP) and like cyclic AMP it has been found in all organisms examined to date. By comparison with cyclic AMP, little is known about the action of cyclic GMP in cellular metabolism. This is partially because of the difficulty of measuring the very low concentrations of cyclic GMP present. Only recently have sensitive radioimmunoassay (RIA) methods become available. A comparison of the concentrations of cyclic AMP and cyclic GMP in various rat tissues and human body fluids is shown in Table 1·1.

Several studies have suggested that cyclic nucleotides play an important role in the control of cell growth. In 1968, Burk noted that the cyclic AMP phosphodiesterase inhibitors caffeine and theophylline slowed the growth of normal and transformed baby hamster kidney cells (BHK). At about the same time Ryan and Heidrick (1968) reported that cyclic AMP inhibited the growth of HeLa cells. Since these early studies, numerous reports have appeared to support these

observations, namely that the addition of analogues of cyclic AMP, cyclic AMP or agents that raise the intracellular concentration of cyclic AMP decrease the growth rate of numerous cell lines grown *in vitro*.

Table 1·1 Concentrations of cyclic AMP and cyclic GMP in rat tissues and human body fluids.

Tissue	Cyclic AMP	Cyclic GMP
Rat	(pmole / g wet wt.)	
Liver	925	15
Kidney	910	40
Fat cells	55	3
Human	(pmole / ml)	
Urine	1000	2
Plasma	14	9

(Steiner *et al.* 1972).

The role of cyclic GMP in the control of growth is still obscure. Goldberg and his collaborators have suggested that cyclic AMP and cyclic GMP function together in opposing roles and have emphasized that it is the ratio of these two cyclic nucleotides that is important (Goldberg *et al.* 1974).

If cyclic GMP acts in the opposite way to cyclic AMP then it might be expected that the addition of cyclic GMP or analogues of cyclic GMP to cell cultures might promote growth. However Carchman *et al.* (1974) found no effect of cyclic GMP analogues on the growth of normal rat kidney (NRK) cells.

In addition to cyclic AMP and cyclic GMP other cyclic nucleotides occur in nature.

Cyclic cytidine 3':5'-monophosphate (cyclic CMP) has been isolated from leukemia L 1210 cells, where it is present in substantial quantities, although precise concentrations have not been reported (Bloch 1974).

Cyclic uridine 3':5'-monophosphate (cyclic UMP) has been isolated from rat liver, it is present in concentrations between 3 - 6 nmole / g wet wt. Cyclic UMP has also been detected in the urine of leukemia patients (Bloch 1975).

Both these cyclic nucleotides have also been isolated from the culture fluid of Corynebacterium murisepticum and Microbacterium sp. (Ishiyama 1975).

Enzymes from sea urchin sperm have also been shown to catalyse the formation of cyclic inosine 3':5'-monophosphate (cyclic IMP) and cyclic 2'deoxyguanosine 3':5'-monophosphate (cyclic dGMP) from their respective triphosphates (Garbers et al. 1975).

These and other cyclic nucleotides may be quite widespread but in the absence of sensitive assay methods our knowledge of them or their metabolic role remains very fragmentary.

1•30 Metabolism of cyclic nucleotides.

Cyclic AMP and cyclic GMP are formed from their respective triphosphates enzymically by adenylate cyclase or guanylate cyclase. In animal cells, adenylate cyclase is an integral part of the plasma membrane (Perkins 1973) and associated with hormone receptor molecules which modulate its activity. Guanylate cyclase, in mammalian cells is found to be a mainly soluble enzyme, little activity being located in the particulate fraction of homogenates (Hardman and Sutherland 1969)

Cyclic AMP is degraded to 5'AMP by cyclic AMP phosphodiesterase (cyclic AMP PDE), a predominently soluble enzyme. While a single enzyme may degrade both cyclic AMP and cyclic GMP, most tissues contain several PDE's, differing in their substrate specificities. Thus in rat liver, three fractions - two soluble and one particulate can be separated by DEAE cellulose chromatography (Russell et al. 1973). One of the soluble fractions hydrolyses cyclic AMP but not cyclic GMP and its activity is unaffected by cyclic GMP. The other soluble fraction hydrolyses both cyclic AMP and cyclic GMP, each substrate acts as a competitive inhibitor for the hydrolysis of the other. The particulate fraction contains a cyclic AMP PDE that is inhibited by cyclic GMP.

Moreover many cyclic nucleotide PDE's exhibit non linear Michaelis - Menten kinetics due to the presence of low and high K_m forms of the enzyme(s). This and other evidence suggests that cyclic nucleotide PDE's are probably a mixture of isoenzymes. (Russell and Pastan 1974).

Cyclic nucleotides act by influencing the activity of protein kinases (Krebs 1972), These consist of a catalytic (C) and a regulatory (R) subunit, which when associated are inactive. Cyclic AMP or cyclic GMP, on binding to the R subunit, cause it to dissociate from C. The catalytic protein kinase subunit can now catalyse the transfer of phosphate from ATP to a protein. This may have far reaching effects on the activity of the protein.

These aspects of the metabolism of cyclic AMP are shown in Figure 1·1.

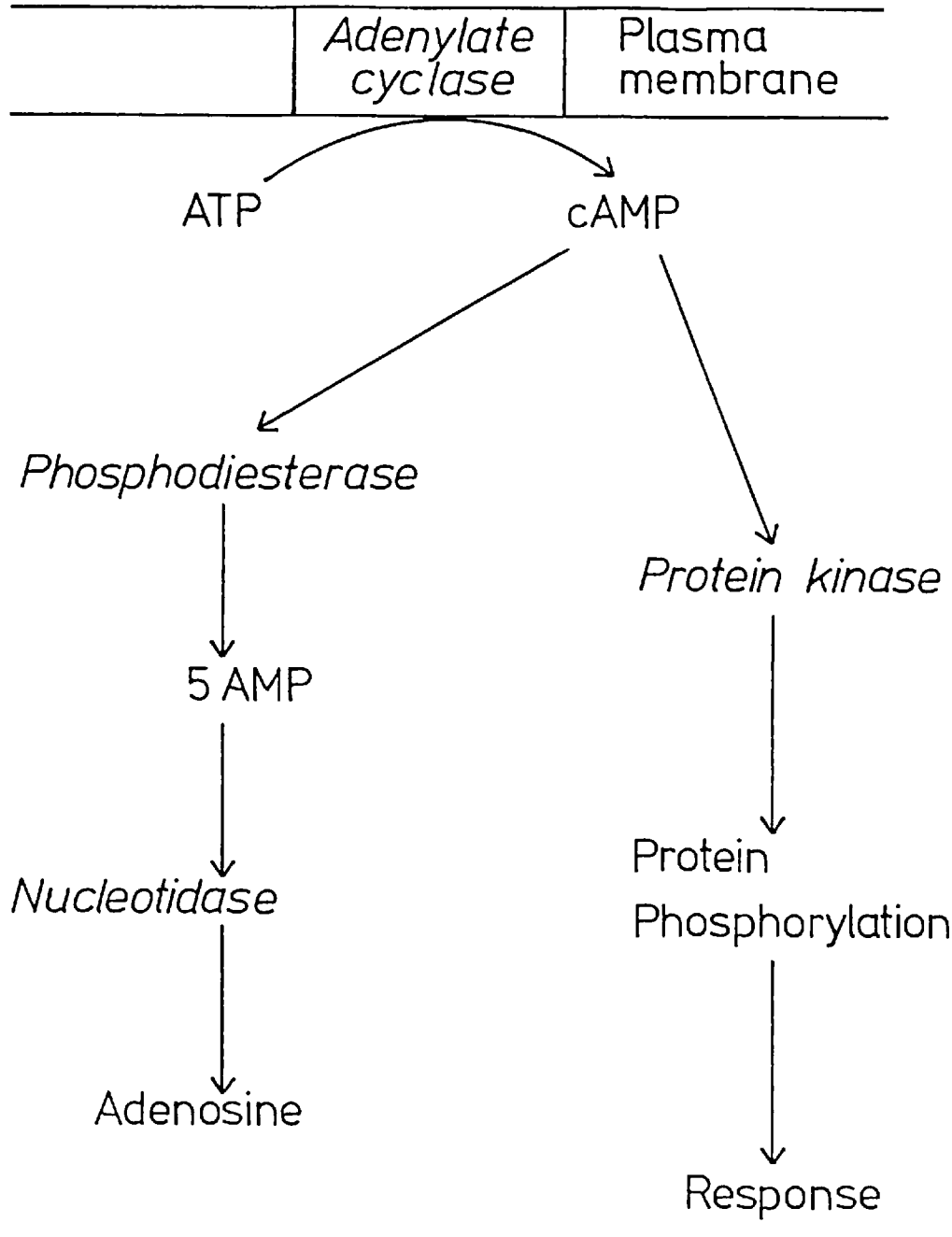


Figure 1•1

Metabolism of cyclic AMP.

1•40 The cell cycle.

The cell cycle may be defined as series of sucessive phases during the reproduction of the cell. During each phase the cell accomplishes a necessary part of the overall process at a particular time.

One way to study the cell cycle is to use a synchronous population of cells. As native cells are rarely synchronous, various procedures such as thymidine block or mitotic selection have been used to obtain synchronous populations (Mitchison 1970).

The cell cycle may be divided into a number of distinct phases between one mitosis and the next, although not all these phases are found in all cells.

1) Mitosis.

Mitosis is the stage of the cell cycle that can be determined visually by the appearance, separation and disappearance of chromosomes. Mitosis is conveniently divided into four distinct phases.

In prophase, the chromosomes condense and become visible. In metaphase, the nuclear membrane breaks down, the spindle apparatus appears and the chromosomes, now seen to be composed of two chromatids, gather on the equatorial plate where each centromere divides. In anaphase, one chromatid passes to each pole of the cell. This process continues through to telophase when separation of the chromosomes is completed and new nuclear membranes are formed. Following telophase the chromosomes elongate and become diffuse. The time between two mitoses is usually called interphase, which may be divided into the following phases.

2) G1 Phase.

In most mammalian cells the chromosomes are no longer condensed in the G1 phase and are not recognisable as distinct entities. A number of events occurring in the G1 phase are related to the subsequent onset of DNA synthesis, specific RNA, specific proteins and histone mRNA are all synthesised in the G1 phase. Conversely not all events may be in preparation for DNA synthesis, the phosphorylation of histones, more specifically H1 (F1) histone, although beginning at the end of G1 is probably more related to mitosis than the onset of DNA synthesis.

The G1 phase is absent in Physarum, after mitosis DNA synthesis begins immediately, there is no discernible interval between the end of mitosis and the onset of DNA synthesis. This phase is also absent in other organisms including Schizosaccharomyces pombe (Bostok 1970) and Amoeba proteus (Ron and Prescott 1969). The absence of a G1 phase suggests that events necessary for DNA synthesis need not occur after mitosis but before it, in the G2 of the preceding cycle. The duration of G1 and other phases of the cell cycle in various cell types are shown in Table 1•2

Table 1•2 Duration of phases of the cell cycle in various cell types.

Cell type	Phase time (in hours)				
	G1	S	G2	M	TOTAL
Mouse corneal epithelium	87	11	35	0•5	102
Human kidney	10	9	4	1•5	24-25
HeLa cells	10•5	10	4	0•5	25
WI-38	6	6	4	0•8	17
Physarum	-	3	5	0•3	8-8•5

(F.A.S.E.B. Cell Biology Vol 1, Biological Handbooks 1976).

3) S Phase.

The S phase is defined as that part of the cell cycle during which DNA is synthesised. Several other events accompany DNA synthesis in the nucleus. For instance, in diploid cells the synthesis of microtubular proteins begins at mid S eventually to continue throughout G2 (Forrest and Klevecz 1973) and centrioles present in G1 commence replication at about the same time as DNA synthesis begins (Robins et al. 1968). In mouse L cells, poly (ADP) ribose is synthesised in short bursts during the S phase. (Colyer et al. 1973).

4) G2 Phase.

When DNA synthesis is completed the cell progresses into the G2 phase.

There are indications that some of the proteins synthesised during G2 are necessary for mitosis, Jockush et al. (1970) reported the appearance in G2 plasmodia of Physarum polycephalum of a nuclear protein (or class of proteins) not detectable in other phases of the mitotic cycle. Modification of pre-existing proteins may be just as important as the synthesis of new proteins. The phosphorylation of non-histone nuclear proteins reaches a maximum in late G2 (Karn et al. 1974), while the phosphorylation of H1 (F1) histone continues as in the S phase; so that prior to mitosis H1 histone is maximally phosphorylated (Marks et al. 1973). In fact according to Bradbury et al. (1974a and 1974b) the phosphorylation of H1 histone represents the initiation step for mitosis.

1•50 Cyclic nucleotides and the cell cycle.

Studies on the role of cyclic nucleotides in the control of the cell cycle have employed animal cells grown in vitro, including fibroblasts, lymphocytes, melanocytes and HeLa cells. These cells are not naturally synchronous and are usually synchronised by the use of external agents e.g. thymidine block - such treatments, while aligning the cell cycle, may interfere with other cellular processes. Unless the synchrony is good any possible changes in cyclic nucleotide levels during the cell cycle will be blurred. Both problems may be avoided by the use of Physarum, in which there is a very high degree of natural synchrony.

All studies seem to agree that the levels of cyclic AMP are elevated during G1 and decreased in mitosis. The reasons for the low level of cyclic AMP during mitosis are unknown but may be necessary for the expression of other systems cyclic AMP inhibits. There is some disagreement with regard to the fluctuations of cyclic nucleotides in S and G2 phases. Addition of cyclic AMP (or dibutyryl cyclic AMP, dbcAMP) to cells at different stages of the cell cycle may block progress through the cell cycle. Pardee (1974) observed that there was a lag period of 8 hours before DNA synthesis starts in BHK cells after the removal of a dbcAMP block. It is suggested that there is a common restriction point in the G1 phase. Seifert and Rudland (1974) have observed a similar effect in BALB 3T3 cells.

Many studies indicate that cyclic AMP arrests cell growth in G1 or G2. However, in Reuber hepatoma cells, growth inhibition in S phase was observed (Van Wijk et al. 1973). Other data suggests that cyclic AMP may actually have a stimulatory role if added late in G2 phase (Willingham et al. 1972). Clearly cyclic nucleotides play an important role in the regulation of growth.

1•60 Physarum polycephalum.

The slime moulds or Mycetozoa are eukaryotes which exhibit the basic life cycle of eukaryotes in what is, perhaps, its simplest form. The Mycetozoa may be subdivided in to two major groups, the Acrasiales or cellular slime moulds e.g. Dictyostelium discoidium and the Myxomycetes or acellular slime moulds e.g. Physarum polycephalum.

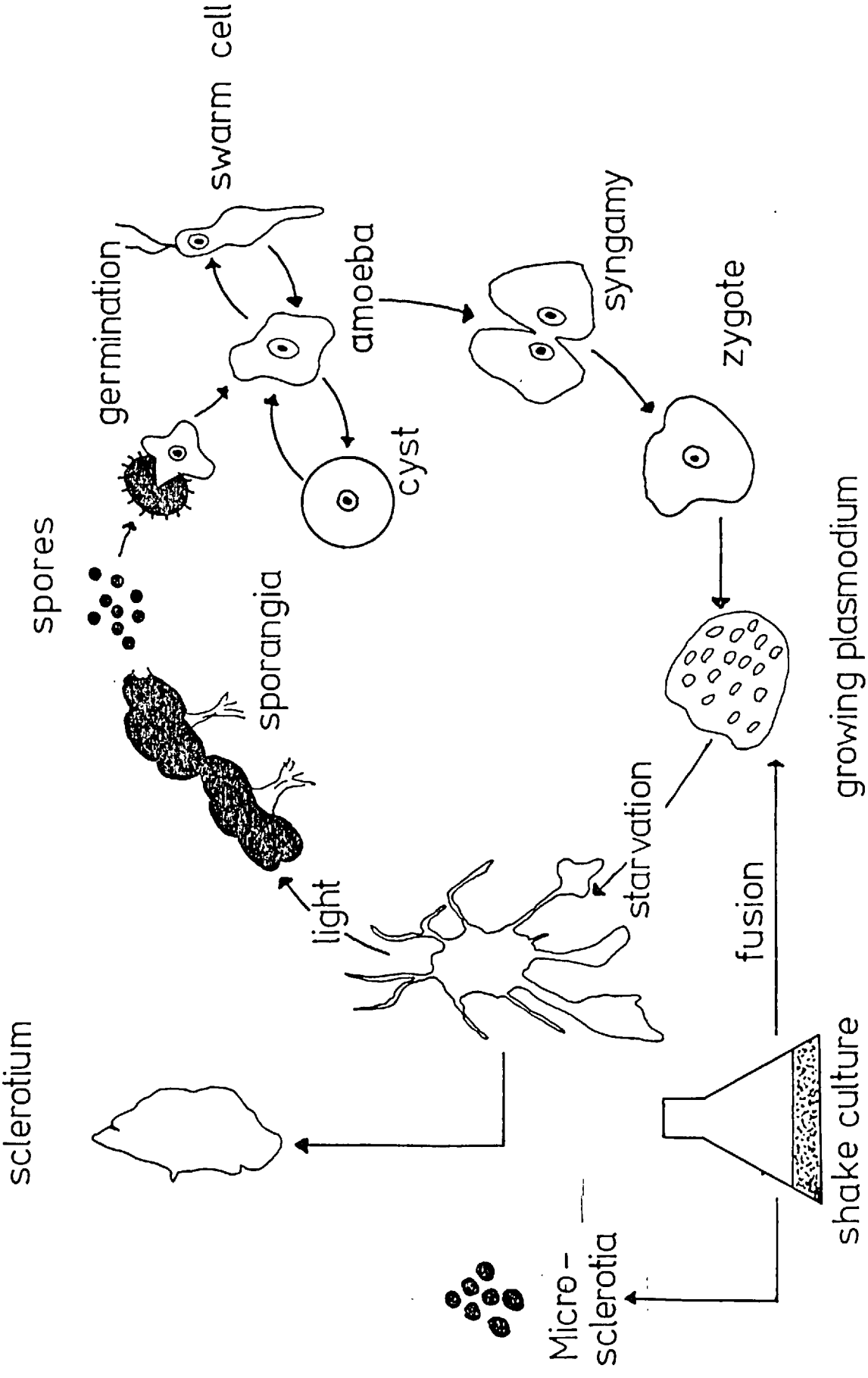
The life cycle of Physarum is shown in Figure 1•2, comprises a unicellular amoeboid stage, a multinucleate plasmodium stage and a sporulation stage. The process by which amoebae form plasmodia differ in different species and are in many cases unknown. In all however the plasmodium is a syncytium in which all the nuclei divide synchronously.

The stimuli which lead the plasmodium to form fruiting bodies also differ in different species, but lack of food and changes in light intensity are often important.

During the vegetative period the plasmodium continues to grow as long as nutrients are adequate. When food becomes depleted the plasmodium may form sclerotia, which consist of many small spherules each containing several diploid nuclei. Or it may sporulate providing the plasmodium has been starved for about four days and exposed to light for a few hours. Spores containing a single haploid nucleus germinate to form amoebae (myxamoebae) when they comes into contact with a suitable moist environment. Amoebae may also survive adverse conditions by encysting. After a certain period, two amoeba may fuse to form a zygote, which forms a plasmodium by nuclear division and subsequent growth without cell division.

Figure 1•2

Life cycle of the acellular slime mould Physarum polycephalum.



As mitosis is unaccompanied by cell division it is more correct to speak of a mitotic cycle rather than a cell cycle in Physarum. The mitotic cycle of Physarum lasts about 8 - 12 hours depending on the strain, $8\frac{1}{4}$ for the strain used by the author. After mitosis, DNA synthesis commences immediately and lasts for about 3 hours. The G2 phase lasts for about 5 hours and is followed by mitosis itself, which takes about 20 min.

CHAPTER 2

LITERATURE SURVEY.

2·10 Introduction.

The literature on cyclic nucleotide methodology and function is extensive and no attempt has been made to review it. What has been attempted is a review of those aspects which clearly come within the ambit of this thesis.

With respect to Physarum the literature review is restricted to those events occurring during the mitotic cycle which may be implicated in the control of, or subjected to control by cyclic nucleotides.

2·20 Fixation and purification of cyclic nucleotides from biological materials.

Cyclic AMP, although a very stable nucleotide in the chemical sense (Sutherland and Rall 1958), is quite labile in the presence of cyclic nucleotide phosphodiesterase, even at $0^{o}C$ (O'Dea et al. 1970). In addition, since cyclic nucleotides levels can change markedly within seconds after hormonal stimulation, proper fixation is essential when tissue or enzymically produced cyclic nucleotides are being measured.

2·21 Fixation.

Liquid nitrogen is not satisfactory for rapid freezing of biological materials because nitrogen gas is formed as an insulating layer when a warm piece of tissue is immersed in the liquid.

This delays freezing of the tissue which may be long enough to allow biochemical changes to occur.

Immersion of the tissue sample in liquids or gases cooled to below their boiling point in liquid nitrogen is satisfactory. The gas dichlorodifluoromethane (Freon 12) has been used after liquification by passing it through a metal coil immersed in liquid nitrogen. But it is difficult to use because it is a gas. Isopentane (2 methylbutane) to which has been added methylcyclohexane (to depress the freezing point to -175°C) may be cooled to near to this temperature by holding it over liquid nitrogen has been used in the present study. This mixture is slightly viscous at low temperatures but is easy to handle.

Another method worthy of mention is microwave irradiation (Schmidt et al. 1971). Although not as fast as freezing to liquid nitrogen temperatures it offers the hope of more rapid fixation when the tissue is not readily accessible. It will of course denaturate the enzymes responsible for the synthesis and degradation of cyclic nucleotides.

2·22 Extraction.

Extreme care must be taken when frozen tissue is extracted since upon thawing enzymic hydrolysis of the endogenous cyclic AMP can occur (Kakiuchi and Rall 1968).

The use of Polytron type homogenisers has simplified tissue homogenisation procedures. Frozen tissue (upto 100mg) can be readily homogenised at 0°C in 3ml of 5% trichloroacetic acid within 3 to 5 seconds. (Brooker 1971).

Gilman and Nirenberg (1971) introduced a technique of extracting cyclic AMP from monolayer cultures, fixed by adding 5% TCA to the culture. The cells appear to be fixed almost instantaneously by the method and recovery is quantitative as verified by the use of radioactive cyclic AMP and also by exhaustive extraction. Once the cyclic nucleotide is in TCA solution it is relatively stable when stored frozen. Perchloric acid or ethanol may be used in the same manner (Albano et al. 1974). These techniques serve both for fixation and extraction.

2·23 Purification.

Some assay methods require extensive previous purification to work reliably whereas other newer methods work quite well with little or no previous purification of the sample. The degree of purification is inversely related to the selectivity of the assay method.

Krishna et al. (1968) discovered that the Somogyi precipitation method, long used to prepare protein free filtrates of blood, also precipitated nucleotides and many other substances, yet left cyclic AMP in the supernatant. This was applied to the assay of adenylate cyclase, an important feature was the preliminary separation of the substrate, ATP, from the product formed (cyclic AMP) by chromatography over a Dowex 50 cation exchange column to prevent the non enzymic production of cyclic AMP. The initial observation of Cook et al. (1957) that hot alkaline hydrolysis of ATP by barium hydroxide solution produced cyclic AMP underlies the danger of treating tissue extracts or solutions containing ATP with barium hydroxide. Treatment of ATP solutions at $0^{\circ}C$ in this way only reduces and does not eliminate the non enzymic formation of cyclic AMP from ATP (Brooker 1971).

Additionally, since barium hydroxide is used in conjuction with zinc sulphate to generate barium sulphate, these metal ions markedly affect some saturation assay systems (Albano et al. 1974).

Rall and Sutherland (1958) described the assay and isolation of cyclic AMP from liver homogenates utilising Dowex 2 anion exchange and Dowex 50 cation exchange resins to isolate cyclic AMP before assay. Numerous workers have since used ion exchange columns to purify extracted cyclic nucleotides. Cyclic AMP and cyclic GMP may be separated by varying the ionic strength of the eluting buffer or by using QAE Sephadex: cyclic AMP and cyclic GMP are easily separated by elution with ammonium formate at different pH's. (Schultz et al. 1973).

2·30 The assay of cyclic nucleotides.

A number of methods have been described for the analysis of cyclic AMP and cyclic GMP in cell extracts. Newer methods have been developed to gain higher sensitivity and, it is hoped, to eliminate the need for purification of samples before assay.

Cyclic nucleotide assay methods can be categorised as follows: phosphorylase activation of protein kinase activation (Rall and Sutherland 1958, Brown et al. 1963), enzyme cycling (Hardman et al. 1966), competition protein binding (Gilman 1970), slime mould aggregation (Konijn 1970), gas chromatography (Krishna 1968) and liquid chromatography (Brooker 1971).

At the present time, the competitive protein binding assay of Gilman (1970) and the RIA of Steiner et al. (1969) are used extensively.

The merits of some of these newer methods will now be discussed.

2·31 Conversion of cyclic nucleotides to other nucleotides.

The measurement of cyclic AMP by this method is based on the amount of cyclic AMP converted to ATP and, hence, glucose-6-phosphate. Glucose-6-phosphate in the presence of NADP is quantitatively converted to 6-phosphogluconate and NADPH, the latter being fluorescent. After conversion of cyclic AMP to ATP a firefly assay has been used to measure ATP (Johnson et al. 1970).

In general, methods wherein cyclic nucleotides are to be assayed, which are found in an abundance of biological materials, require initial purification of reagents and unknowns for best results. In fact the reagent blank in these methods is the limiting factor, since the potential sensitivity is theoretically many orders of magnitude higher than

the optimal 10^{-14} mole blank (Goldberg et al. 1969). The blank can be much higher because of contamination of the reagent enzymes with other adenine nucleotides. Another major disadvantage of this general type of methodology is that many steps are required for each analysis, a feature which inherently makes the variance large for these methods.

2·32 Protein binding isotope displacement.

The method of Gilman (1970), wherein cyclic AMP displaces labelled cyclic AMP of high specific activity from the regulatory subunit of bovine skeletal muscle protein kinase under saturation conditions is at present used extensively for the assay of cyclic AMP.

Little, if any, sample purification is necessary, the reagents are easily prepared, the assay readily performed. Labelled cyclic AMP and cyclic AMP compete for sites on a specific binding protein. Bound cyclic AMP is separated from the unbound nucleotide by filtration on Millipore cellulose ester filters. The bound nucleotide is quantitively retained on the filter.

Brown et al. (1970) have also described a cyclic AMP protein binding assay. The protein was obtained from bovine adrenal cortex. The free (^3H) cyclic AMP was absorbed on charcoal and the bound isotope, in the supernatant, counted. Since tritiated water is always a contaminant of (^3H) cyclic AMP, higher backgrounds are seen with this method because tritiated water passes through the Millipore filter but remains in the charcoal supernatant.

Murad et al.(1971) have described a protein binding assay for cyclic GMP using the cyclic GMP protein kinase from lobster muscle. The method is similar to the protein binding assay for

cyclic AMP described by Gilman. Murad and Gilman (1971) have described a simultaneous protein binding assay for cyclic AMP and cyclic GMP, using (^{32}P) cyclic AMP and (^{3}H) cyclic GMP. This method eliminates the necessity of two separate assays when both cyclic nucleotides are measured in one sample.

2·33 Radioimmunoassay (RIA).

Steiner et al. (1969) developed an immunoassay for cyclic nucleotides. They succeeded in making antisera quite specific for cyclic AMP, cyclic GMP, cyclic IMP and cyclic UMP. Antibodies to the cyclic nucleotides were made by immunising rabbits with the 2'-O-succinyl derivative of the cyclic nucleotide, conjugated to human serum albumin or keyhole limpet haemocyanin. High specific activity derivatives of the cyclic nucleotides were prepared by radio-iodinating the tyrosine methyl ester of the succinyl derivatives. Bound cyclic nucleotide was separated by precipitation with ammonium sulphate. Steiner and his associates subsequently described the use of his methodology for analysis of cyclic GMP and cyclic AMP in mammalian tissues and fluids (1972). The immunoassay is sensitive and easy to perform. However, reagent preparation is more difficult than in the Gilman assay, since the cyclic nucleotide antigenic derivatives must be synthesised, the antibody raised in rabbits and the ^{125}I derivatives prepared.

Since the antibody is prepared against the 2'-O- cyclic nucleotide derivative, the cyclic nucleotide compounds with 2'-O-substitutions have the greatest affinity for the antibody. Succinyl-ation of cyclic nucleotides in unknown samples in a similar manner yields greater sensitivity.

A simultaneous RIA for cyclic AMP and cyclic GMP, using the respective antibodies with ^{131}I and ^{125}I -2'-O- succinyl labelled tyrosine methyl ester cyclic nucleotide derivatives, has recently been described by Wehmann et al. (1972). This combined method gave results essentially similar to those obtained when the assays were done individually.

2·34 High pressure liquid chromatography.

High pressure anion exchange chromatography assay of cyclic AMP was developed by Brooker (1971). The disadvantages are the need for extensive purification of samples of biological materials before injection into the chromatograph for quantitation. Both the protein binding assay and RIA are more sensitive at present than this method since a sample should contain 10 - 15 pmoles of cyclic AMP or cyclic GMP. However, the high pressure method method is more reproducible than either of the other two methods.

As little as 0·2 pmole cyclic AMP and 0·5 pmole cyclic GMP can easily be detected using protein binding assays (Gilman and Murad 1974). Approximately 0·025 pmole cyclic AMP and 0·03 pmole cyclic GMP can be detected using a RIA.(Steiner et al. 1969).

2·40 Determination of adenylate cyclase and guanylate cyclase activities.

The cyclase reaction consists of the conversion of the nucleotide triphosphate to pyrophosphate and the respective 3':5' cyclic phosphate nucleotides.

Originally Rall and Sutherland (1958) and Sutherland and Rall (1958) incubated liver homogenates with ATP, magnesium and hormones or sodium fluoride and terminated the reaction by heating for 3min in a boiling water bath. The heated extracts were assayed for cyclic AMP by the liver phosphorylase activation method. Many methods have since been described to measure this reaction.

2·41 Using non radioactive nucleotide triphosphate substrates.

The first type of method, as Rall and Sutherland initially used it, can serve to generate cyclic AMP from ATP; the cyclic AMP produced is measured by one of the many assay methods available. The sample must be purified since the ATP will interfere with most assay methods. Simple zinc - barium precipitation, which will remove ATP, will also non enzymically generate cyclic AMP (Brooker 1971). Under certain conditions, samples treated in this way show lower cyclic AMP levels in the specimens containing enzyme than the controls. The reason for this is that the ATP concentration would be lower in the enzyme containing samples than in the controls because of ATPase contamination, and since barium catalysed cyclic AMP production is concentration dependent, less would be produced in the experimental sample if less cyclase activity existed.

Steiner et al. (1972) have reported that the product of adenylate and guanylate cyclase activity could be detected directly in boiled supernatants. The resolution of the RIA method between

cyclic nucleotides and their respective mono-, di- and triphosphates means that no previous purification of the sample is necessary.

Maguire and Gilman (1974) have reported that adenosine $\beta-\gamma$ 5' triphosphate (AMP PNP) is good substrate for adenylate cyclase: additionally it is undegraded by ATPase.

2·42 Using radioactive nucleotide triphosphates as substrates.

Until the time that Krishna et al. (1968) introduced a simple radioisotopic assay for adenylate cyclase, measurement of this reaction with radioactive substrates usually required several thin layer or paper chromatography steps to obtain reliable results.

Many types of labelled ATP have been used for the assay: however it appears that $\alpha\,^{32}P$ ATP is the isotope of choice since fewer by-products are possible and higher specific activities are available.

If an ATP regenerating system is included to maintain the ATP concentration constant during the incubation period, the specific activity of ATP is not altered because removal and addition of the terminal phosphate from ATP does not involve the isotope. Since most adenylate cyclase preparations contain a large amount of ATPase, it is sometimes difficult to maintain ATP concentrations even with a regenerating system. The phosphoenolpyruvate - pyruvate kinase ATP regenerating system produces pyruvate as a side product: this has been reported to inhibit some adenylate cyclases.
Therefore, creatine phosphate and creatine kinase is probably preferable. (Schultz 1974).

Rodbell et al. (1971) have used $\alpha^{32}P$ AMP PNP as a substrate for adenylate cyclase.

2•43 Precautions for adenylate cyclase assay methods.

If non radioactive substrates are chosen for cyclase assays phosphodiesterase inhibitors should be used to inhibit phosphodiesterase activity, which is almost always present in adenylate cyclase preparations. The classical inhibitor, theophylline (Butcher and Sutherland 1962), has been shown to inhibit adenylate cyclase (Sheppard 1970). A more potent phosphodiesterase inhibitor such as SQ 20009 (Chasin et al. 1972), might be used since lower concentrations effectively inhibit phosphodiesterase.

When radioactive substrates are used, some workers (Bär and Hechter 1969) have added a large excess of the cyclic nucleotide to prevent hydrolysis of the newly formed radioactive cyclic nucleotide. and to act as a trap for the radioactive cyclic nucleotide product, however added cyclic nucleotide may inhibit the cyclase.

2·50 Measurement of phosphodiesterase.

The cyclic nucleotide phosphodiesterase hydrolyses the 3' bond of 3':5' cyclic nucleotides.converting them to their respective 5' mononucleotides. Several basic methods have been used to measure this reaction. The earliest method (Butcher and Sutherland 1962) relied on converting the 5'AMP formed by the reaction to inorganic phosphate and adenosine with snake venom nucleotidase. The inorganic phosphate was then measured colourimetrically (Butcher and Sutherland 1962). Another method (Drummond and Perrot - Yee 1961) measures the 5'AMP formed by the PDE by deaminating it to 5'IMP and following the decrease in absorbance at 265nm. Still another method (Goldberg and O'Toole 1971)measures the 5'AMP product by converting it to ADP with adenylate kinase. The ADP formed is measured fluorometrically by the coupled pyruvate kinase - lactic dehydrogenase system. Cheung (1969) has developed a continuous titrimetric assay for PDE wherein the proton generated by the hydrolysis of cyclic AMP is continuously titrated with sodium hydroxide to maintain a constant pH in the unbuffered reaction solution. The amount of base added per unit time is monitored on a strip chart recorder. These methods require substrate concentrations 1 to 3 orders of magnitude above the K_m for the cyclic AMP PDE (Brooker *et al.* 1968) and are not recommended in the light of the fact that PDE's have multiple forms and K_m values (Thompson and Appleman 1971a and 1971b). However, Weiss *et al.*(1971) have reported a more sensitive coupled enzyme PDE assay method wherein the 5'AMP formed is measured by conversion to ATP with adenylate kinase and pyruvate kinase. The ATP is then measured by firefly luminescence.

Even though this assay can be run at low substrate concentrations, which are needed to assay cyclic AMP specific PDE, substances that alter these enzyme activities can interfere with this method.

A more feasible and very sensitive assay method relies on the conversion of (^{3}H) cyclic AMP to (^{3}H) 5'AMP. The (^{3}H) 5'AMP is either isolated or converted to (^{3}H) adenosine by nucleotidase, and the (^{3}H) adenosine separated from 5'AMP and cyclic AMP. Appleman and Kemp (1966) first used the latter approach to measure rat brain PDE and isolated the (^{3}H) adenosine product on small Dowex anion exchange columns. Huang and Kemp (1971) later found that DEAE Sephadex A 25 reduces the volume of buffer necessary to elute (^{3}H) adenosine. Brooker et al. (1968) found that the complete phosphodiesterase reaction could be run in one step in a liquid scintillation vial. The reaction was stopped by the addition of a slurry of Dowex resin. Dioxane based liquid scintillation cocktail was added and the samples counted. The (^{3}H) cyclic AMP radioactivity is quenched by about 90%, whereas the product, (^{3}H) adenosine, is not. Monard et al. (1969) have reported a similar procedure. Thompson and Appleman (1971a) have modified this method further and heat inactivated the PDE before the nucleotidase is added. In addition, they add the Dowex resin slurry to terminate the nucleotidase reaction and count an aliquot of the supernatant of the resin - reaction mixture. This would seem to be a most reliable and sensitive assay method for cyclic nucleotidase, since it works also for cyclic GMP PDE, using (^{3}H) cyclic GMP as substrate (Brooker et al. 1968 and Thompson and Appleman 1971b). However, the resin absorbs some (^{3}H) adenosine (Boudreau and Drummond 1975) thus underestimating enzyme activity. Inclusion

of a known amount of (^{14}C) guanosine in the second step (with nucleotidase) has been used to monitor absorption of (^{3}H) guanosine (Craven and DeRubertis 1976).

Multiple molecular forms of PDE as first kinetically demonstated (Brooker et al. 1968) have now begun to be resolved by various chromatographic means. Thompson and Appleman (1971a and 1971b) have applied agarose and DEAE cellulose columns to separate multiple forms of PDE. Kakuchi et al. (1972) have employed Sepharose columns to separate multiple molecular forms of rat brain PDE.

2·60 Cyclic nucleotides and the cell cycle.

Implication of cyclic nucleotides in the control of cell division has been suggested by a number of studies on synchronously growing cells.

A number of cell types have been used to assess this role. First indications that cyclic nucleotides might play a role in the control of the cell cycle appeared in 1972 in reports by Burger et al., Makman and Klein, Millis et al., Sheppard and Prescott, and Zeilig et al. These studies, using fibroblasts from various sources, dealt primarily with changes in cyclic nucleotide levels after release from "quiescence". Burger et al.(1972) used synchronised 3T3 Swiss mouse fibroblasts released from quiescence by the addition of serum or by trypsinisation. After trypsinisation there was a rapid decrease in cyclic AMP content of about 70%, which began to rise again to the initial level. A second decrease in cyclic AMP occurred after 28 hours, at mitosis. Subsequently, cyclic AMP content returned to almost its initial value by 34 hours. Similar results have been reported by Seifert and Rudland (1974) concerning cyclic AMP and cyclic GMP levels after release from quiescence of BALB/C 3T3 cells. Cyclic AMP and cyclic GMP levels were determined in the first cell cycle following the release from quiescence. Cyclic AMP rapidly decreased to 60% of its initial value, concurrently cyclic GMP content increased 10 fold. Within 1 hour cyclic GMP had decreased to the initial level, further changes in cyclic GMP were not detected. Cyclic AMP returned to its initial concentration and there remained until the next mitosis when a second decrease was noted.

A similar decrease in cyclic AMP occurs in Chinese Hamster Ovary (CHO) cells during mitosis (Sheppard and Prescott 1972),

a peak of cyclic AMP was noted in early G1 phase. Work on the V79 line of CHO cells failed to show a rise in cyclic AMP during early G1 (Russell and Stambrook 1975). Instead the concentration of cyclic AMP rose steadily through G1 to reach a peak in early S phase. A similar pattern was observed in HeLa cells (Zeilig et al. 1976)

In summary, a number of studies have suggested that the intracellular concentrations of cyclic AMP and cyclic GMP vary during the cell cycle. All agree the levels of cyclic AMP are low in mitosis and elevated in G1. There is no agreement on fluctuations of cyclic AMP or cyclic GMP in S phase or G2 phase of the cell cycle.

2·61 Adenylate cyclase and cyclic AMP phosphodiesterase during the cell cycle.

The fluctuations of adenylate cyclase and cyclic AMP PDE during the cell cycle have only been studied in a limited number of cell types.

Millis et al. (1972) measured both adenylate cyclase and cyclic AMP PDE during the cell cycle of synchronous human lymphoid cells. Cells synchronised at the beginning of S phase with thymidine showed fluctuations in fluoride - stimulated adenylate cyclase that paralleled the changes in cyclic AMP, namely, initially low and increasing to a maximum coincident with the peak of cyclic AMP levels in late S or G2 phase and then decreasing to a low point coinciding with mitosis. In contrast, cyclic AMP PDE activity decreased in early S and increased through the remainder of S and G2 reaching a maximum just after mitosis. Unfortunately these studies are incomplete since adenylate cyclase measurements were only of

fluoride stimulated activity. Measurements were not made in the second half of G1 phase. Also no attempt was made to differentiate the different forms of cyclic AMP phosphodiesterase.

Dickinson et al. (1976) have attempted to analyse the cyclic AMP - adenylate cyclase - phosphodiesterase system in Tetrahymena pyriformis, a unicellular eukaryote. In contrast to mammalian cells a large increase in intracellular cyclic AMP was associated with mitosis and the observed levels of cyclic AMP PDE and adenylate cyclase account for the fluctuations in cyclic AMP. High levels of cyclic AMP were associated with high adenylate cyclase activity and low cyclic AMP PDE activity. Conversely low cyclic AMP occurred where adenylate cyclase activity was minimal and cyclic AMP PDE activity had increased.

2·62 Guanylate cyclase and cyclic GMP phosphodiesterase during the cell cycle.

Little information is available as regards the fluctuations of guanylate cyclase and cyclic GMP phosphodiesterase during the cell cycle.

Graves et al. (1976) have determined the concentrations of cyclic GMP and the activity of cyclic GMP PDE during the cell cycle of Tetrahymena pyriformis. Cyclic GMP was highest during mitosis and declined to a minimum immediately before the next mitosis. The maxima of cyclic GMP PDE activity coincided with the cyclic GMP minima. They did not report the activity of guanylate cyclase during the cell cycle of this organism.

2·63 Summary.

Reliable data on the role of cyclic nucleotides in the cell cycle are still fragmentary. Many conclusions have been drawn from poorly controlled studies that relied on the use of dibutyryl cyclic AMP or phosphodiesterase inhibitors and very often reports failed to give details of synchronisation techniques or the degree of synchrony attained. It is clear that the cell cycle of different cell types may be regulated by different mechanisms involving cyclic nucleotides.

None of the possible underlying mechanisms have been established by which cyclic nucleotides might influence a given cell cycle event.

2·70 The mitotic cycle of Physarum.

Plasmodia of Physarum are syncytcia and exhibit exceptionally good, natural mitotic synchrony. All the nuclei traverse the mitotic cycle in synchrony. Microplasmodia may be allowed to establish a macroplasmodium, the second and third mitotic divisions of which are highly synchronous. Such plasmodia yield milligram quantities of protein and RNA and several hundred micrograms of DNA (Rusch 1969).

The synthesis of DNA begins immediately after telophase of mitosis and continues for about three hours, occupying about 30 - 35% of the total cycle time. The G2 phase lasts for about five hours and mitosis, about twenty minutes.

2·71 Mitosis.

After nuclei have divided, many densely staining irregular bodies appear in the nucleus which gradually fuse over a period of about two hours to form the nucleolus. The period of nuclear reconstruction coincides with the start of the S phase. One of the unusual features of mitosis in Physarum is that the nuclear membrane does not disintegrate (Guttes et al. 1961). Examination of the nuclear membrane with the electron microscope seems to show that it may break down during mitosis (Guttes et al. 1968).

2·72 S Phase.

Once initiated in the late telophase, DNA synthesis is unaffected by the cytoplasmic environment. This conclusion has been drawn from fusion experiments (Guttes and Guttes 1968) and from experimentally desynchronised plasmodia (Guttes and Telatnyk 1971).

In the first series of experiments, G2 phase nuclei were not turned on to make DNA in an S phase cytoplasm and S phase nuclei were not turned off in G2 phase cytoplasm. In the second experiment when a plasmodium, in prophase was covered with a membrane, only nuclei in the periphery divided on time while mitosis was delayed in the central part of the plasmodium. Prophase nuclei from the retarded area were transported into the periphery by cytoplasmic streaming, where postmitotic nuclei had already entered S phase. In these translocated prophase nuclei no chromosomal DNA synthesis was detected. This experiment suggests that mitosis must precede nuclear DNA synthesis and eliminated the possibility that a sudden burst of DNA polymerase activity alone defined S phase.

There are two classes of nuclear proteins that are synthesised during S phase: histone and acidic proteins. No changes in the composition of a phenol soluble fraction of acidic protein were observed during S phase (LeStourgeon and Rusch 1971). Histones will be dealt with in more detail in a separate section (2·73)

A number of enzyme activities have been measured during the mitotic cycle. There is a well established correlation of thymidine kinase with mitosis and the S phase. (Sachsenmaier and Ives 1965), similarly NAD pyrophosphorylase, which is located in the nucleus, is also correlated with the S phase (Solao and Shall 1971). A number of enzymes show no variation during the mitotic cycle, these include:- glucose-6-phosphate dehydrogenase (Sachsenmaier and Ives 1965), isocitrate dehydrogenase, acid phosphatase, phosphodiesterase, β glucosidase and histidase (Hütterman et al. 1970).

Total assayable ornithine decarboxylase increases in a step

pattern during the mitotic cycle, the bulk of which is synthesised within a short period during the S phase (Sedory and Mitchell 1977).

2·73 Histones and the mitotic cycle.

Physarum contains five major histone fractions and equal amounts of histone and DNA. During the mitotic cycle no qualitative changes in any of the major fractions were observed, but the very lysine rich fraction changed just before mitosis (Mohberg and Rusch 1970).

Histone H1 in Physarum is considerably phosphorylated, and the number of phosphate groups per molecule increases just before mitosis when the chromosomes condense. Consequently, Bradbury et al. (1973) proposed that the very lysine rich histone is phosphorylated as part of chromosomal condensation leading to mitosis. The phosphorylating activity of nuclei of Physarum has been measured at defined times in the mitotic cycle, by their effect on added calf H1 histone (Bradbury et al. 1974a). The phosphorylation of H1 histone from calf thymus increased steadily during the mitotic cycle from a minimum near metaphase to a maximum in late G2. After that time it fell rapidly to a minimum near metaphase. The peak in activity preceded the maximum in the phosphate content of Physarum H1 histone. Furthermore, histone phosphokinase, the enzyme responsible for the phosphorylation of histones is cyclic AMP dependent (Matthews 1976).

2•80 Physarum and cyclic nucleotides.

Almost no work has been carried out on cyclic nucleotides or their role in the mitotic cycle of Physarum.

Murray et al. (1971) reported the presence of a cyclic AMP phosphodiesterase released by Physarum into the growth medium similar in kinetic properties to the particulate enzyme.

Adenylate cyclase in Physarum (Atmar et al. 1976) has been found to consist of two distinct enzymes. One, nuclear, is potently inhibited by an equimolar combination of the three polyamines, putrescine, spermidine and spermine. The other enzyme is particulate and insensitive to these polyamines.

A protein kinase in Physarum has been found to exhibit cell cycle dependence with respect to inhibition by cyclic AMP (Kuehn 1972). The inhibitory response of the kinase to cyclic AMP was decreased from mitosis to mid S phase. For one hour during mid S, the kinase was independent of cyclic AMP, and thereafter the capacity of cyclic AMP to inhibit the protein kinase was restored, the completion of restoration coinciding with the end of S phase.

When cultures of Physarum are incubated with the cyclic nucleotide PDE inhibitors caffeine or theophylline for over 100min before mitosis, mitosis was delayed. A similar effect was observed if cultures were starved for longer than 3 hours before mitosis. The addition of dbcyclic AMP appeared to stimulate nucleolar reconstruction (Trevithick and Braun 1977).

CHAPTER 3

MATERIALS AND METHODS.

3·10 Physarum polycephalum.

Physarum polycephalum, strain M_3c, supplied by Dr. Joyce Mohberg was used in these studies. For early work, on the extraction of cyclic AMP from Physarum strain i+29, supplied by Dr. M. J. Carlile, was used. In all culture work with Physarum, sterile manipulative techniques were used.

Physarum was grown in the following semi - defined medium (SDM)

Table 3·1 Semi - defined medium for the culture of Physarum.

Difco Bacto tryptone	10·000g
Difco yeast extract	1·500g
Glucose	10·000g
KH_2PO_4	2·000g
$CaCl_2.\ 2H_2O.$	0·600g
$MgSO_4.\ 7H_2O$	0·600g
$FeCl_2.\ 4H_2O$	0·600g
$MnCl_2.\ 4H_2O$	0·085g
$ZnSO_4.\ 7H_2O$	0·035g
Citric acid	3·500g
Distilled water	1000ml

The constituents were dissolved in distilled water and the pH adjusted to 4·6 with 1N NaOH, autoclaved for 15min at 15 p.s.i. and removed from the autoclave as soon as possible.

Physarum microplasmodia were grown in 500ml conical flasks containing 50ml SDM to which 1ml of hemin (B.D.H., 50mg per ml in 1% w/v NaOH) was added before use. Between 1 and 2ml of culture was used to inoculate a flask of fresh medium every 3 to 4 days. Culture flasks were incubated in the dark on an orbital shaker at 26°C (at approximately 200 revolutions per min).

3·11 Synchronous surface plasmodia.

For the establishment of synchronous surface cultures (macroplasmodia), 1ml of a young dense microplasmodial culture was transferred into 20ml SDM containing 0·5ml hemin in a 500ml conical flask with four indentations in the base (each 1cm high), and incubated as above for 2 days.

The culture was centrifuged in conical centrifuge tubes for 1min at about 50g in a BTL bench top centrifuge at room temperature. For the best results approximately 0·7 - 0·9ml of compacted microplasmodia were obtained from 10ml of culture. The pellet was gently suspended in 20 volumes of sterile distilled water and centrifuged as before. Nearly all the supernatant was discarded and the pellet mechanically agitated for a few seconds, 0·3ml was removed with a wide oriface pipette and spread evenly in the centre of an 8cm filter paper (Schleicher and Schuell No. 1575) supported on a disk of ¼in. stainless steel (S.W.G. 18) mesh in a 9cm petri dish.

As an alternative, to provide more material, the inoculum could be increased to 0·9ml and spread on the filter paper in the form of a ring 5mm wide and 40mm internal diameter.

The microplasmodia were allowed to coalesce, on the filter paper, by starvation in the dark at 26°C for approximately 60min,

when the inoculum had assumed an even velvet - like appearance SDM containing hemin was added to the dish so as to wet the filter paper from below. Any air bubbles were removed by lifting the filter paper by the edge, with sterile forceps, and replacing slowly, thus forcing any air bubbles from under the paper.

The time of feeding was noted. If the M2 - M3 mitotic cycle was being studied, the macroplasmodia were refed 60 - 90min before M2.

3·12 Growth of spherules of Physarum.

A shake culture was centrifuged for 1min at 50g. The pellet was washed in 2vol of the following salts medium.

Table 3·2 Physarum salts medium.

Citric acid	4·000g
$FeCl_2 . 4H_2O$	0·060g
$MgSO_4 . 7H_2O$	0·600g
$CaCl_2 . 2H_2O$	1·200g
$MnCl_2 . 4H_2O$	0·084g
$ZnSO_4 . 7H_2O$	0·034g
KH_2PO_4	0·040g
Distilled water	1000ml

The components were dissolved in the above order to avoid precipitation of metal salts and the pH was adjusted to 3·8 with 1N KOH.

Pellets of compacted microplasmodia were resuspended in 1vol of salts medium and inoculated into 20ml of salts medium in a 500ml conical flask and incubated on a rotary shaker at 26°C for 3 days.

The culture was centrifuged as before and the pellets washed with 2vol of salts medium and recentrifuged. After resuspension in 0•5vol salts medium the spherules were streaked onto 1 x 2cm strips of Oxoid cellulose acetate electrophoresis strips. The strips were dried in a petri dish in the dark for 2 days and stored in sealed sterile 10ml MaCartney bottles at 4°C in the presence of $CaCl_2$.

Cultures of microplasmodia were re - established by placing a cellulose acetate strip bearing spherules in a sterile tube and carefully adding SDM + hemin until the lower edge of the strip was moistened. After incubation in the dark at room temperature for 2 days, the spherules had germinated and then transferred to a conical flask containing 50ml SDM + hemin and incubated as described above.

3•13 Observation of mitosis.

A small piece of a macroplasmodium was removed and squashed onto a clean dry microscope slide, fixed by brief immersion in 95% ethanol and mounted in a drop of glycerol : ethanol (1 : 1 v/v). Nuclei were observed under oil immersion (x 1000) with phase contrast optics using a Gillett and Siebert binocular microscope. Observations were made approximately every 10min.

The first post fusion mitosis occurred about 5 hours after feeding. Synchrony was very poor at this stage (less than 40% of nuclei entered metaphase at the same time). The next mitosis (M2) occurred about 8 - 8¼ hours later showing good synchrony (greater than 95% of the nuclei entered metaphase at the same time). If refed 60 - 90min before M2 the third post fusion mitosis (M3) occurred 8 - 8¼ hours after M2. The events leading upto mitosis are shown in Table 3•3.

Table 3·3 Description of events leading upto mitosis.

M2 -70min	Nucleolus assumes an eccentric position in nucleus.
-50min	Nucleolus enlarges.
-40min	Clear area around the nucleolus diminishes.
-30min	Clear area no longer visible
-10min	PROPHASE. Chromosomes begin to condense, nucleus begins to lose granular appearance.
0min	METAPHASE. Metaphase plate clearly visible.
+ 7min	ANAPHASE. Separating chromatids clearly visible.
+10min	TELOPHASE.
+20min	Nucleolar reconstruction very clearly visible. Nucleolus fragmented
+60min	Nucleolar reconstruction now complete.

3·14 Sampling of surface cultures during the mitotic cycle.

One and a half hours before the second post fusion mitosis (M2) the medium was discarded and the cultures refed with fresh medium.

Samples were taken at 30min intervals from 1 hour before M2 until about 1 hour after M3.

1). For cyclic nucleotide analysis.

Plasmodia on filter paper were frozen for 5min in isopentane - methylcyclohexane (92 : 8 v/v) at the temperature of liquid nitrogen. The filter papers were withdrawn and the inoculum discarded, the remaining material was scraped off and used for the extraction of cyclic nucleotides.

2). For the analysis of enzyme activities during the mitotic cycle.

One macroplasmodium, on filter paper, was frozen for 5min in

liquid nitrogen. Submersion in isopentane - methylcyclohexane was not used to avoid possible permanent damage to enzymes. The filter paper was withdrawn and the inoculum discarded. The remaining material was scraped off and plunged into 4vol of extraction buffer (0•25M sucrose, 5mM $CaCl_2$, 1mM dithiothreitol, 50mM tris - HCl pH 7•5) and gently dispersed by 12 - 15 strokes in a Potter - Elvejham homogeniser at 0°C. After centrifugation for 15min at 10000g at 4°C the supernatant was recentrifuged in a Spinco 65 rotor for 2 hours at 100000g at 4°C in a Model L ultracentrifuge. The pellet from both the low speed and high speed centrifugation steps were resuspended in two and one vol of extraction buffer respectively. Portions of each fraction were stored in liqiud nitrogen. Fractions obtained from the same cultures were used for the assay of adenylate cyclase, guanylate cyclase, cyclic AMP PDE and cyclic GMP PDE.

3•15 Determination of the duration of S phase of the mitotic cycle.

The method used to determine the duration of the S phase of the mitotic cycle is essentially that reported by Braun and Wili (1969).

At the times indicated, one macroplasmodium was transferred to a petri dish containing SDM + hemin supplemented with (^3H) thymidine (20Ci/mmol, used at a concentration of 5μCi/ml) for 15min. The macroplasmodium was scraped off the filter paper and plunged into 30ml TCA - acetone (4g TCA in 100ml acetone : water 1 : 1 v/v) at 0°C and homogenised for 30sec with an ILA X-1020 mixer (Internationale Laborapparat, GmbH, Dottingen, West Germany). A 5ml aliquot was then removed and filtered through a GF/C Whatman glass fibre filter disk (2•5cm dia.) supported in a Millipore filter holder. The filter was washed with 20ml TCA acetone - water mixture followed by 10ml absolute ethanol. After drying under an infra - red (IR)

lamp for at least one hour, each filter was placed in a vial containing 10ml T scintillant and the radioactivity determined. The radioactive medium was discarded after it had been used 5 times. The results are shown in Figure 7·2.

3·20 Measurement of protein.

The method of Lowry et al. (1951) was used to measure protein using bovine serum albumin (Fraction V) as standard.

The following solutions were prepared and stored indefinitely at 4°C.

1). Solution A	Na_2CO_3	20·0g
	Sodium potassium tartrate	0·2g
	Distilled water	1000ml
2). Solution B	$CuSO_4 . 4H_2O$	5·0g
	Distilled water	1000ml
3). Solution C	was prepared by mixing 50vol of Solution A with 1vol of Solution B just before use.	

4). Folin Ciocalteu reagent (B.D.H.) whose normality had been determined by titration against 1N NaOH, was made 1N by dilution with distilled water. This reagent, on storage, developed a green tinge; by refluxing with a drop of bromine the original yellow colour could be restored.

The assay was conducted as follows:

Method

To 1vol of sample (200µl), 5vol of solution C was added. After at least 10min at room temperature, 0·5vol of Folin Ciocalteu reagent was added and the tube contents vigourously mixed on a vortex mixer. After at least 30min at room temperature the absorbance at 650nm was measured. The protein content of unknowns and dilutions

($\frac{1}{2}$, $\frac{1}{4}$ etc.) were always determined in order to minimise the effects of interfering substances and to bring the protein concentration within the assay range, 20 - 200μg per 200μl. On each occasion a standard graph was constructed by dilution of a stock solution with 0·4N NaOH. A typical standard graph is shown in Table 3·4.

Some samples contained tris buffer, this was found to give erroneous results, also shown in Table 3·4. The effect of tris buffer could be removed by precipitation of the protein with 1vol of 10% TCA. The precipitate was collected by centrifugation for 5min at 5000rpm in a BTL bench top centrifuge at room temperature. The precip -itate was dissolved in the original volume of 0·4N NaOH. Protein fractions contained in tris buffer were always treated in this way.

Table 3·4 Lowry protein assay - standard graph and the effect of tris buffer.

(50mM tris - HCl pH 7·5)

Standard graph.

μg BSA	50	100	150	200
E_{650}	0·271	0·550	0·843	1·211

With TCA precipitation. (figures in brackets = without TCA treatment)

Sample	E_{650} Dilution $\frac{1}{2}$	E_{650} Dilution $\frac{1}{4}$	μg protein Dilution $\frac{1}{2}$	μg protein Dilution $\frac{1}{4}$
10000g	0·616	0·415	108	62
pellet	(0·601)	(0·328)	(106)	(57)
100000g	0·197	0·069	35	11
pellet	(0·070)	(0·061)	(12)	(10)
100000g	0·472	0·223	80	38
supernatant	(0·231)	(0·163)	(40)	(28)

3·30 Freeze - drying.

The eluate from a column was collected directly in a 10ml ampoule prepared for sealing by drawing out the neck. Each ampoule was frozen in liquid nitrogen while rotating it at an angle of about 30° so that the liquid was distributed as an even thin layer over the wall of the ampoule.

Freeze - drying was carried out on a commercial freeze - drying apparatus (Biochemistry Dept.). When completely dry the neck of the ampoule was sealed in a gas jet and the ampoule stored at $-20^{\circ}C$.

The neck of the ampoule was scored with a diamond and a hot glass rod touched on the score line. The required amount of buffer was then added and the ampoule rotated repeatedly to ensure that the small amount of buffer washed the whole inner surface of the ampoule. The buffer was collected by brief centrifugation.

3·40 Rotary evaporation.

Liquids were reduced in volume or taken to dryness by rotary evaporation. The condenser of a Buchi Rotavapour (RE) was cooled to about $-35^{\circ}C$ by circulating methanol through it from a Dewar flask containing the cooling probe of a Neslab Cryocool.
A partial vacuum was maintained with a mechanical pump. Air entering the system was dried by passing it over finely granulated $CaCl_2$.
The sample was maintained at about 30 - $35^{\circ}C$ in a water bath.

3·50 Spectrophotometry.

All spectral measurements were carried out on a Gilford 240 spectrophotometer the wavelength of which was checked at 6 monthly intervals with a holmium oxide filter. Absorbance was checked with

standards supplied with the instrument. The purity of cyclic nucleotides and enzyme substrates was checked by comparing their ultra - violet (UV) spectra with those on the following pages (Figures 3·1, 3·2, 3·3 and 3·4)

Concentrations were checked by measuring the absorbance at a particular wavelength and using published extinction coefficients (Specifications and Criteria of Biochemicals and Biological Compounds: National Science Council, U.S.A., 3rd edition, 1976).

Table 3·5 Extinction coefficients of cyclic nucleotides and enzyme substrates.

Compound	Extinction 260nm	Compound	Extinction 260nm
Cyclic AMP	15000	AMP PNP	15000
Cyclic GMP	15000	GMP PNP	11800

3·60 Liquid scintillation counting.

Radioactivity was estimated by liquid scintillation counting at first using an ICN scintillation spectrometer using settings which gave 21% efficiency for (^{3}H) with quenching monitored by the use of an external standard.

Most of the samples were counted in a Packard Tricarb liquid scintillation spectrometer Model 2425, using the preset tritium mode, at an efficiency of approximately 31%. All samples were counted in Packard plastic scintillation vials which were washed for 2 days in industrial methylated spirits followed by immersion in Decon (2%) for a further 2 days and washing in distilled water.

Figure 3•1, 3•2, 3•3.and 3•4.

Ultra violet (UV) absoption spectrum of β-γ imido adenosine 5' triphosphate (AMP PNP), β-γ imdio guanosine 5' triphosphate (GMP PNP), cyclic AMP (cAMP) and cyclic GMP (cGMP).

Figure 3•1

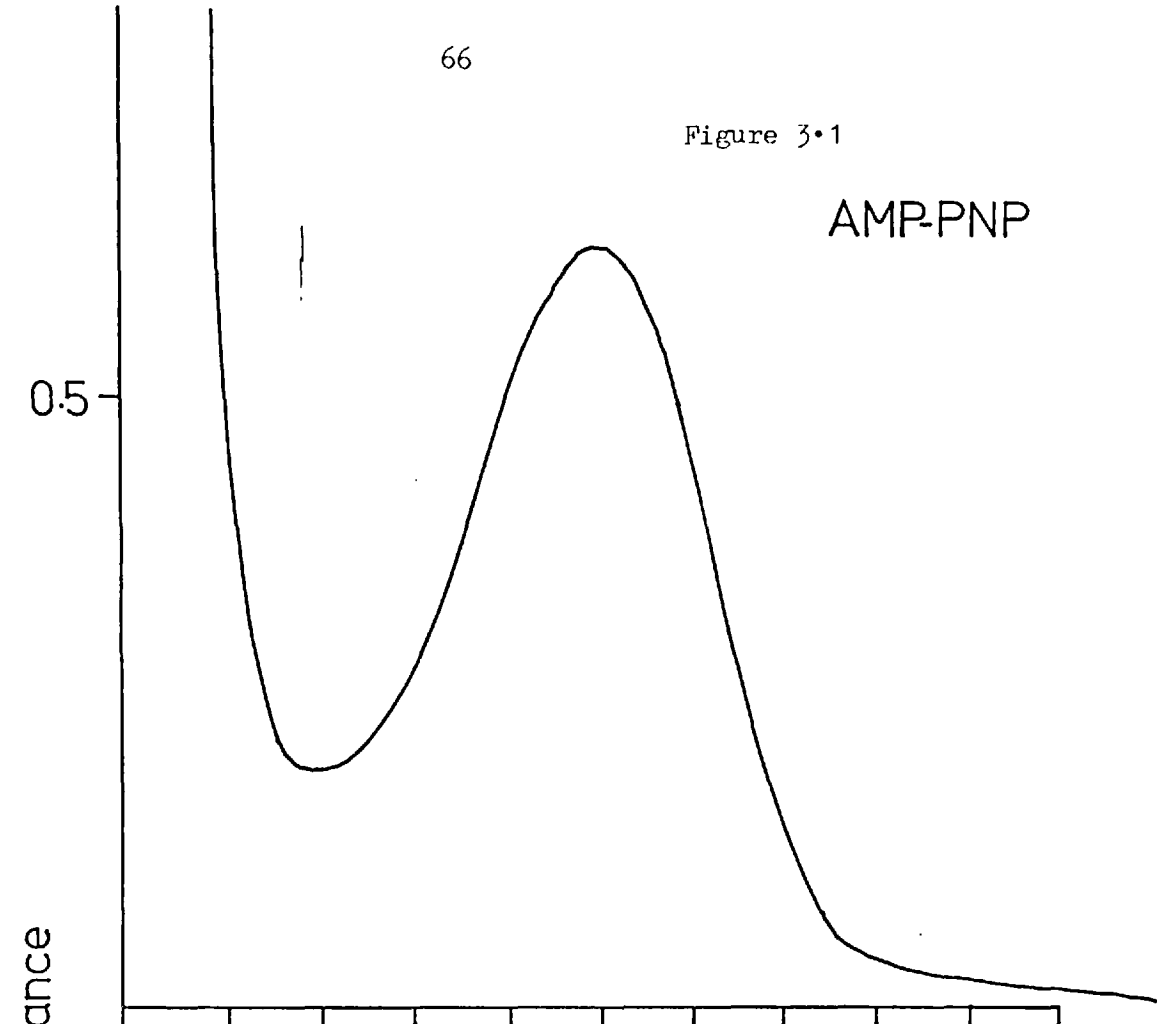


Figure 3•2

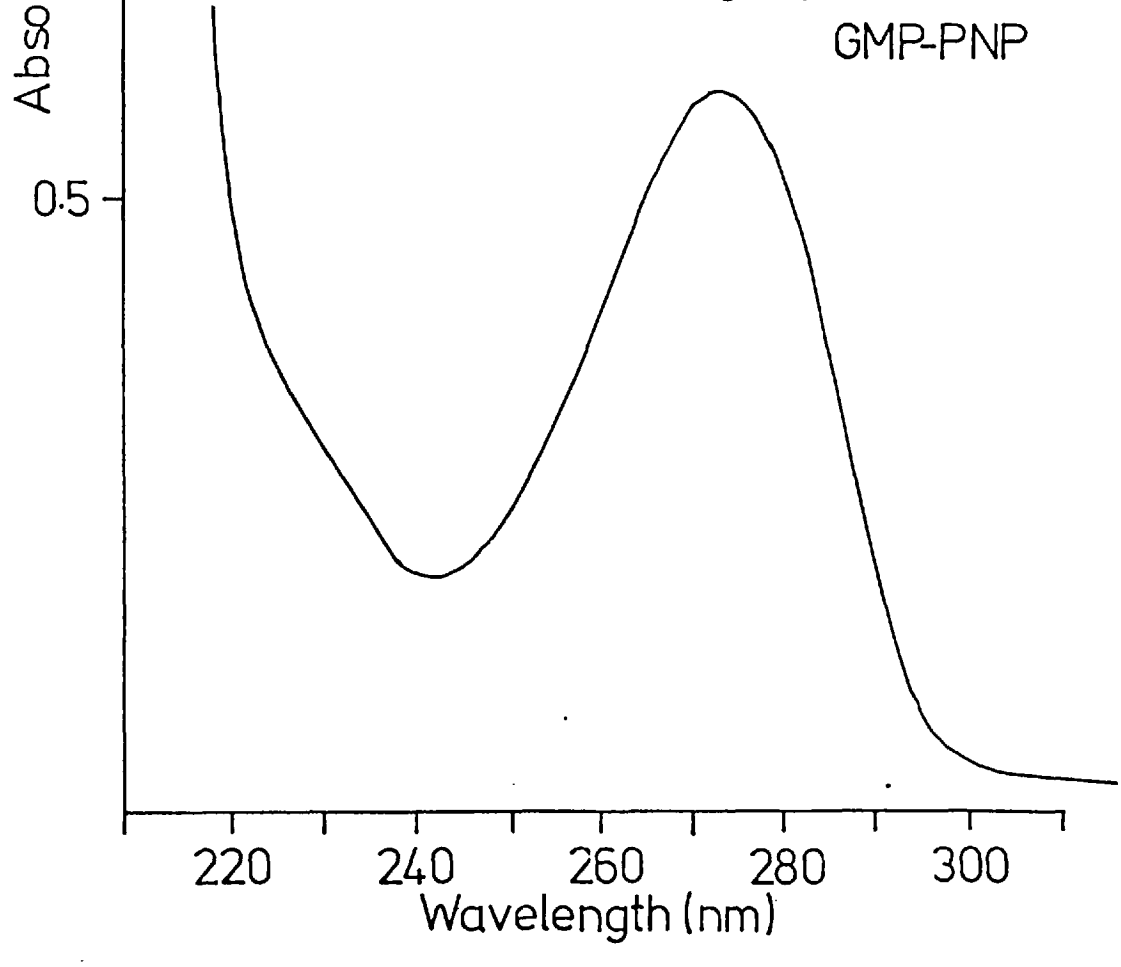


Figure 3·3

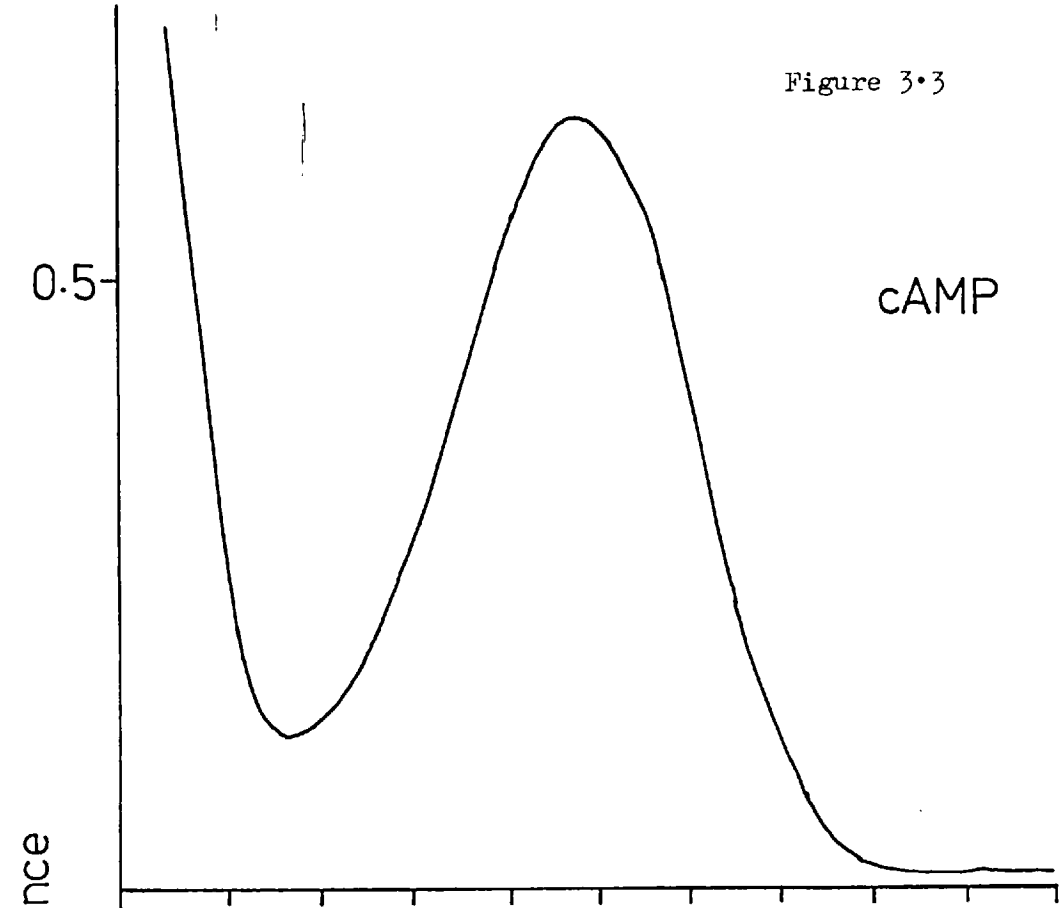
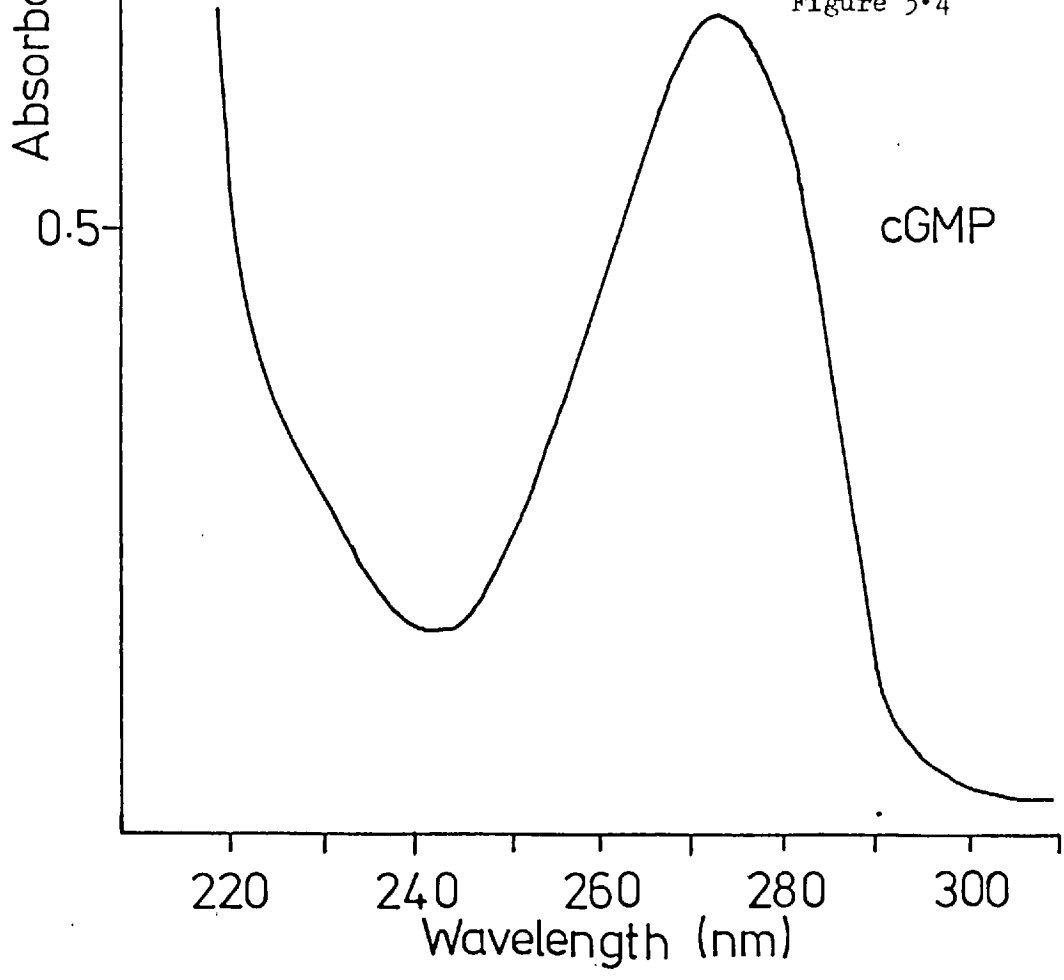


Figure 3·4



Non aqueous samples e.g. filter paper disks, were counted in scintillant T,

Toluene (Sulphur - free)	1000ml
2, 5-Diphenyloxazole (PPO)	4·00g
1, 4-Bis-(5-phenyloxazol-2-yl) benzene (POPOP)	0·05g

Aqueous samples were initially counted in scintillant TE,

Toluene (Sulphur - free)	800ml
Ethanol	200ml
PPO	4·40g
POPOP	0·05g

Because 10ml of this scintillant could not accept 250µl of aqueous sample its use was discontinued in favour of the following scintillant (TTX-100)

Toluene (Sulphur - free)	1000ml
Triton X-100 (Scintillation grade)	500ml
PPO	4·40g
POPOP	0·10g

Eight millilitres of this mixture could accept 1ml of sample, upon shaking a clear solution was obtained. When mixed with aqueous sample, solutions or emulsions result depending on the amount of water in the system. Between 0 - 4% and 14 - 20% water causes the formation of unstable emulsions which soon collapse into two phases and are unsuitable for counting owing to the large and varying amounts of self absorption which occur in the aqueous phase. Samples containing between 4 and 14% water behave as a solution. For these reasons, in protein binding assays the sample (250µl) was added to 8ml TTX-100 scintillant containing 0·8ml water (10%), thus giving a solution containing about 12·8% aqueous sample: after shaking a clear solution resulted.

This property was exploited in thin layer chromatography on cellulose where the area containing the labelled compound of interest could be cut out and eluted directly in a scintillation vial, the scintillant being added later and the sample then counted.

To monitor, and if necessary correct for, any quenching of samples the external standard ratio (E.S.R.) was measured. To do this 5µl (^3H) toluene (33811 d.p.m.) was added, by weight, to a plastic scintillation vial containing 8ml TTX-100 scintillant and 0·8ml water and the sample counted for 10min with E.S.R. Then 25µl of diluted binding protein (beef adrenal binding protein) was added and recounted. This was repeated until the vial contained a total of 300µl of binding protein. The results are shown in Figure 3·5. A correction was applied if the E.S.R. was lower than 0·63.

3·70 Preparation and use of ion - exchange resins.

Cation exchange resins.

1). Dowex 50 (AG50-X8) 100 - 200 mesh H^+ and 200 - 400 mesh H^+ form

The resin was continually stirred with 5vol of fresh 1N NaOH using an overhead stirrer, for 30min. After settling the supernatant was discarded. The resin was washed repeatedly with 5vol of glass distilled water until the pH of the supernatant was constant (pH 5). The resin was again stirred with 5vol of 1N HCl for 30min and the washing treatment repeated. The resin was stored in sterile distilled water containing a trace of sodium azide.

2). Dowex 50 (AG50W-X8) 100 - 200 mesh ammonium form.

This was prepared by stirring as much as required of preparation 1 for 30min with 5vol of 1N NH_4OH followed by the washing procedure described above.

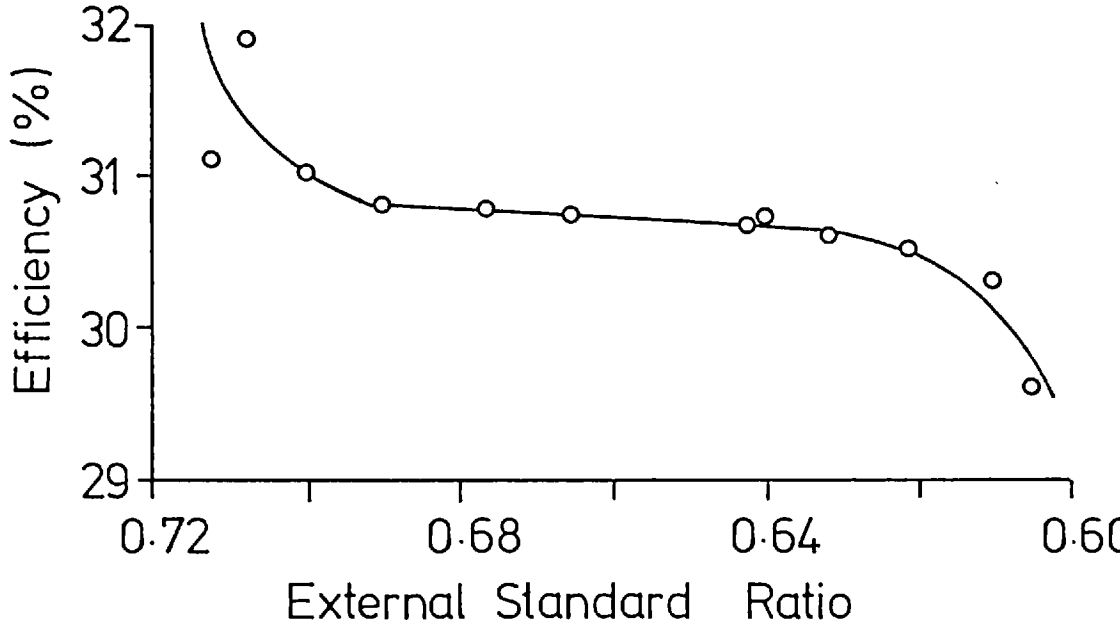


Figure 3·5

Quench correction curve for TTX-100 scintillant using diluted binding protein as quenching agent.

Anion exchange resin.

1). Dowex 1-X8 100 - 200 mesh Cl^- form.

This was prepared by washing with 4vol of 0•5N NaOH, 8vol deionised water, 4vol 0•5N HCl and allowed to stand overnight at room temperature. The resin was then washed repeatedly with deionised water on a large sintered glass funnel until the pH of the eluate was constant. The resin was suspended in 3vol of deionised water and allowed to stand for 2 days before use. The resulting pH of the supernatant was 4•62.

QAE Sephadex A 25.

About 10g of QAE Sephadex (Pharmacia) was allowed to swell overnight in 50vol sterile distilled water then washed with 100vol of 0•1N NaOH. The resin was packed into a 2•5 x 60cm glass column and washed with deionised water to equilibrium. The removal of Cl^- ions was monitored by adding a drop of saturated silver nitrate to a few millilitres of the eluate. The resin was then washed with 100vol of 0•1N formic acid then with distilled water to constant pH. The resin was stored in sterile bottles at 4^oC in the presence of a small amount of sodium azide.

DEAE Cellulose.

DE 52 is a microgranular, pre - swollen, anion exchange resin, as such, pre - cycling is unnecessary.

Fines were removed by allowing the resin to settle after dispersion in 50vol of buffer (50mM tris - HCl pH 7•5). The supernatant was removed by aspiration and the process repeated twice. After removal of fines, the resin, in 1•5vol buffer, was degassed by applying a vacuum until bubbles ceased to appear on shaking and the treatment was terminated before boiling occurred. The resin was packed into a silicondised glass column and washed with 50vol of the above buffer before the application of the sample (See effect of Lubrol on adenylate cyclase).

3·80 Buffers.

1) Cyclic AMP protein binding assay buffer.

4mM EDTA

50mM tris - HCl pH 7·5

2) Cyclic AMP protein binding assay buffer (B.D.H.)

4mM β mercaptoethanol

8mM theophylline

50mM tris - HCl pH 7·4

3) Beef adrenal binding protein extraction buffer.

0·25M sucrose

25mM KCl

5mM $MgCl_2$

50mM tris - HCl pH 7·4

4) Cyclic AMP PDE extraction buffer (Resin assay method).

50mM tris - HCl pH 8·0

5) Cyclic AMP PDE asaay buffer (Resin assay method).

120mM $MgCl_2$

2·5mM EDTA

120mM tris - HCl pH 8·0

6) Cyclic AMP PDE and cyclic GMP assay buffer (t.l.c. method).

5mM $MgCl_2$

50mM tris - HCl pH 7·5

7) Adenylate cyclase, guanylate cyclase, cyclic AMP PDE and cyclic GMP PDE extraction buffer (t.l.c. assay method).

0·25M sucrose

5mM $CaCl_2$

1mM dithiothreitol

50mM tris - HCl pH 7·5

8) Adenylate cyclase assay buffer.

5mM $MgCl_2$

1mM 3-isobutyl-1-methylxanthine (IBMX)

50mM tris - HCl pH 8·0

9) Guanylate cyclase assay buffer.

1mM SQ20009

50mM TES - HCl pH 8·5

3·90 Chemicals.

From Aldrich Chemical Co. : IBMX.

From The Boehringer Corporation London Ltd. : β-γ imido adenosine- and β-γ imido guanosine 5' triphosphate.

From B.D.H., Poole, Dorset or Koch - Light Laboratories Ltd., Colnbrook, Bucks. : Triton X100 (scintillation grade)

From Sigma London Chemical Co. Ltd., Kingston-upon-Thames, Surrey. : Cyclic AMP, cyclic GMP, nucleotides and nucleosides.

SQ20009 was a kind gift of the Squibb Medical Research Insitute, New Jersey, U.S.A.

All other chemicals were of reagent grade.

3·91 Materials.

From Anderman and Co. Ltd., East Molesey, Surrey. : Schleicher and Schuell cellulose (F1440/LS 254) and PEI cellulose (F1440/LS 254) thin layer chromatography plates.

3·92 Radioactive chemicals.

Tritiated toluene ($2{\cdot}18 \times 10^6$ d.p.m./g $\pm$ 3%, 25/10/74) was obtained from The Packard Instrument Co.

All other radiochemicals were purchased from the Radiochemical Centre, Amersham, Bucks.,

(8-^{3}H) Adenosine 3':5'-monophosphate, ammonium salt. TRK 304 27·5Ci/mmol. Diluted to 0·5μCi/ml with ethanol - water (1:1 v/v) and stored in capped polystyrene tubes at -20^{o}C. The tubes were sterilised by irradiation from a ^{60}Co source (about 2·5 megarads).

(8-^{3}H) Guanosine 3':5'-monophosphate, ammonium salt. TRK 392, 19Ci/mmol. Diluted and stored as described above.

$\beta-\gamma$ Imido (8-^{3}H) adenosine 5' triphosphate (AMP PNP), ammonium salt. TRK 466, 15Ci/mmol and 20·3Ci/mmol. Diluted to 5μCi/ml with ethanol - water (1:1 v/v) and stored at -20^{o}C.

$\beta-\gamma$ Imido (8-^{3}H) Guanosine 5' triphosphate, ammonium salt. TRK 467, 10·8Ci/mmol. GMP PNP was diluted and stored in the same manner as AMP PNP.

(6-^{3}H) Thymidine, TRK 296, was stored at 4 - 6^{o}C without dilution.

Cyclic AMP assay kit (TRK 432) and cyclic GMP RIA kit (TRK 500) were also obtained from the Radiochemical Centre and used as directed.

3·93 Pipettes.

Throughout this work, to accurately dispense microlitre volumes, micropipettes supplied by "Oxford","Eppendorf or"Gilson" were used. Each pipette was checked either by the weight of water dispensed or the number of c.p.m. per particular volume. The variance was usually found to be less than 4% from the stated volume dispensed.

CHAPTER 4

THIN LAYER CHROMATOGRAPHY.

4·10 Introduction.

In this work thin layer chromatography (t.l.c.) was used extensively for the separation of enzyme reaction products. As this forms a major part of the thesis it was thought useful to collect all the t.l.c. data into a separate chapter.

4·20 Separation of reaction products by thin layer chromatography.

Thin layer chromatography was used to separate the reaction products of cyclic AMP PDE, cyclic GMP PDE, adenylate cyclase and guanylate cyclase. Five microlitres of the enzyme reaction mixture was spotted onto a 20 x 20cm cellulose t.l.c. plate 2cm from the bottom, scored into 15mm wide vertical channels. After air drying, two more 5μl aliquots were added in the same manner. Fifteen microlitres of the reaction mixture was spotted, in the same way, onto a 15mm square of a cellulose t.l.c. plate for the determination of total counts. Cellulose t.l.c. plates were developed overnight in pre - equilibrated glass t.l.c. tanks in the dark at 4 - 6^{o}C. Polyethyleneimine (PEI) plates were developed for 1 - 2 hours in the light at room temperature. After development the plates were air dried and the markers detected under UV light (254nm). Regions containing the markers were cut out and individually eluted for at least 20min with 0·8ml distilled water in plastic scintillation vials. Total count squares were treated in the same way. The radioactivity was then determined after the addition of 8ml TTX-100 scintillant.

By determining the total counts present and knowing that elution was complete it was possible to account for all the substrate undegraded and the one or more degradation products. An example is shown below.

Adenylate cyclase.

(^{3}H) AMP PNP	(^{3}H) cAMP	TOTAL COUNTS	(^{3}H) AMP PNP + (^{3}H) cAMP
10539	3711	14397	14250

(all values in c.p.m.)

There is about 1% difference between the total counts and the sum of the counts in the cyclic AMP and AMP PNP regions.

A number of solvent systems using cellulose or PEI cellulose were tested to determine the best way of separating substrate from product(s) in enzyme assays. The results of this study are shown in Tables 4·1 and 4·2.

It may be seen from Table 4·2 that the best system of the 22 tested for;

Adenylate cyclase assays is system 16.

Cyclic AMP PDE assays is system 16.

Guanylate cyclase assays is system 17.

Cyclic GMP PDE assays is system 20.

System 20 also gives good separation of guanylate cyclase reaction products, however the GMP PNP region was approximately twice the diameter as in system 17.

A more detailed examination of the efficiency of these separation systems was undertaken.

Table 4·1 Solvent systems used in thin layer chromatography.

System number	Solvent
1	0·2M Ammonium carbonate
2	0·5N Lithium chloride in 2M formic acid
3	1M Tris - HCl pH 7·6
4	1M Tris - HCl pH 8·0
5	25mM Ammonium carbonate pH 8·0
6	1·5M Ammonium sulphate
7	2·5M Ammonium sulphate
8	50mM Acetic acid
9	100mM Acetic acid
10	200mM Acetic acid
11	60mM Sodium acetate pH 5·4
12	25mM Sodium formate pH 4·2
13	Ammonium acetate : 95% ethanol (30 : 75 v/v)
14	2M Acetic acid : 95% ethanol : 2M ammonia (19 : 100 : 20 by vol)
15	As 14 (20 : 100 : 20 by vol)
16	Butanol : acetic acid : water (2 ; 1 : 1 by vol)
17	2-Propanol : dimethylformamide : ethyl-methyl ketone water : ammonia (20 : 20 : 20 : 39 : 1 by vol)
18	1-Propanol : ammonia : water (6 : 3 : 1 by vol)
19	2-Propanol : ammonia : water (55 : 10 : 35 by vol)
20	95% Ethanol : 2M ammonia : 2M acetic acid (100 : 20 : 20 by vol)
21	As 20 (100 : 40 : 20 by vol)
22	As 20 (100 : 20 : 40 by vol)

Table 4·2 Rf values of adenine and guanine nucleotides in various solvents.

System	Compound.							
PEI cellulose	cAMP	AMP PNP	AMP	Aden.	GMP PNP	cGMP	GMP	Guan.
1	0·57	0·43						
2	0·75	0·42						
3	0·75	0·11	0·58	0·54				
4	0·53	0·43						
5	0·56	0·43						
6	0·20		0·42	0·24	0·31	0·25	0·47	0·55
7	0·16		0·39	0·18	0·44	0·19	0·50	
8	0·07		0·00	0·43				
9	0·10		0·00	0·43				
10	0·14	0·50	0·08	0·58				
11	0·10		0·00	0·40	0·00	0·10	0·00	
12	0·11		0·00	0·45	0·00	0·07	0·00	
Cellulose.								
13	0·29	0·65	0·00	0·44	0·00	0·22	0·00	
14	0·55	0·00						
15	0·25	0·00	0·00	0·40	0·00	0·22	0·07	
16	0·37	0·00	0·26	0·64	0·10	0·21	0·16	
17					0·00	0·51	0·00	0·51
18					0·00	0·16	0·00	0·17
19					0·20*	0·39	0·11	0·39
20					0·07	0·35	0·12	0·48
21					0·15	0·40	0·10	0·43
22					0·03	0·24	0·39	0·24

* GMP PNP - two regions that absorb UV light (Rf = 0·20, 0·08)

4·21 Adenylate cyclase and cyclic AMP phosphodiesterase.

To test the ability of system 16 to separate cyclic AMP, adenosine, 5'AMP and AMP PNP, a mixture of all four compounds each tritium labelled and unlabelled were chromatographed. Tritiated AMP was prepared in the laboratory from a digest of (^{3}H) cyclic AMP by beef heart cyclic nucleotide PDE. Tritiated adenosine was prepared with (^{3}H) cyclic AMP and an homogenate of Physarum containing 5' nucleotidase. The cellulose t.l.c. plate was loaded with the four compounds and after development the 15mm wide strip was cut into 5mm wide pieces parallel to the solvent front. Each piece was eluted in a scintillation vial with 1ml distilled water for 30min, 500µl was removed and the absorbance determined, to the remaining 500µl, 8ml TTX-100 scintillant was added and the radioactivity determined. The results are shown in Figure 4·1.

The efficiency of eluting these compounds was measured by comparing similar amounts counted directly. The results are shown in Table 4·3.

Table 4·3 Elution of adenine nucleotides from cellulose.

Compound	Direct c.p.m.	Av.	t.l.c. c.p.m.	Av	$\frac{\text{t.l.c.}}{\text{Direct}} \times 100$
(^{3}H) cAMP	12039 12111	12075	12013 11899	11956	99·0%
(^{3}H) Adenosine	737 752	747	727 719	723	97·2%
(^{3}H) 5'AMP	1032 1079	1055	993 1009	1001	94·9%
(^{3}H) AMP PNP	24730 25002	24866	24621 24790	24705	99·4%

Elution of these compounds was regarded as being complete.

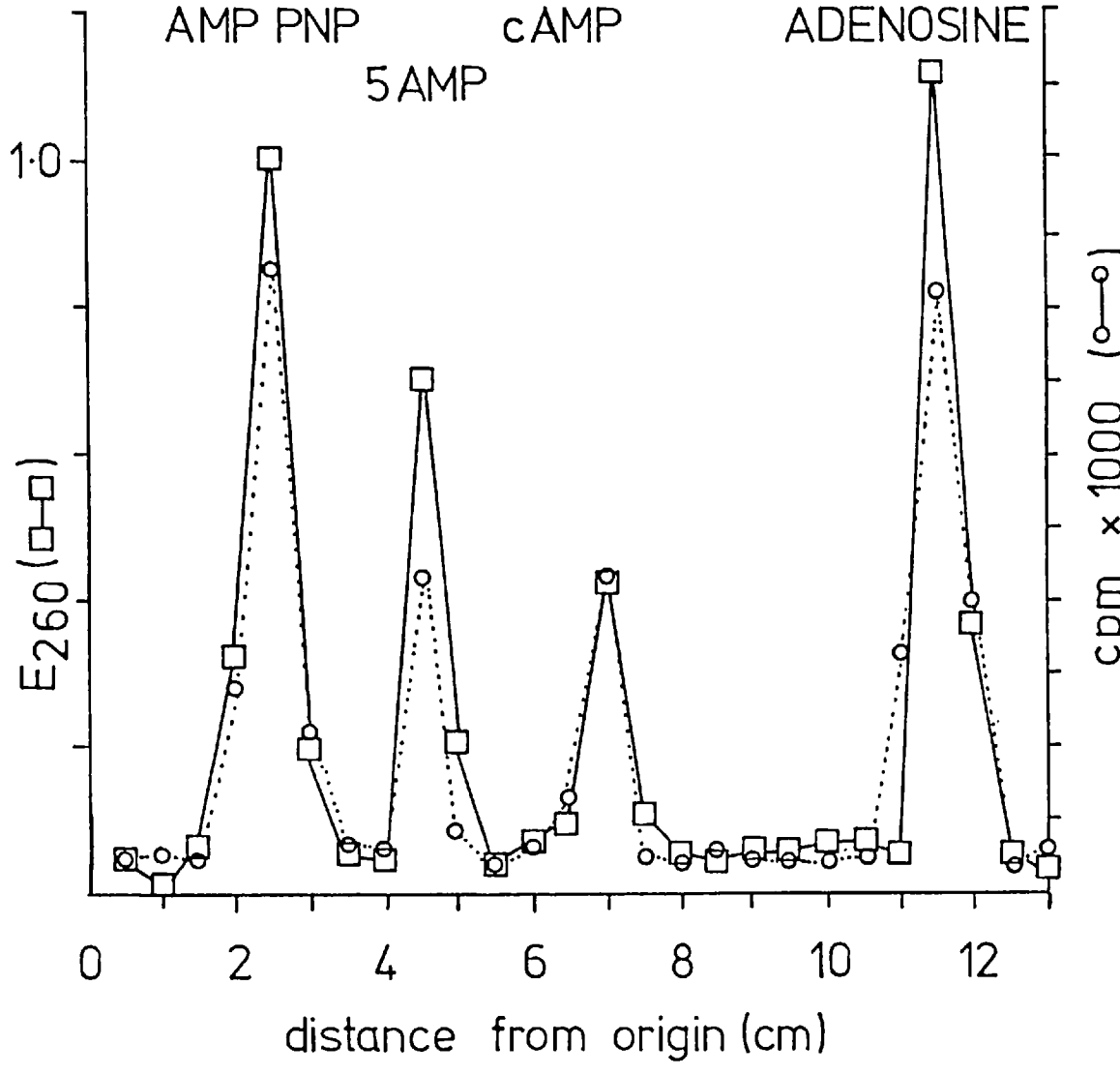


Figure 4·1

Chromatography of labelled and unlabelled AMP PNP, cyclic AMP, 5'AMP and adenosine in system 16. No significant radioactivity or absorbance was detected in the final 5cm of the cellulose t.l.c. plate.

4·22 Guanylate cyclase.

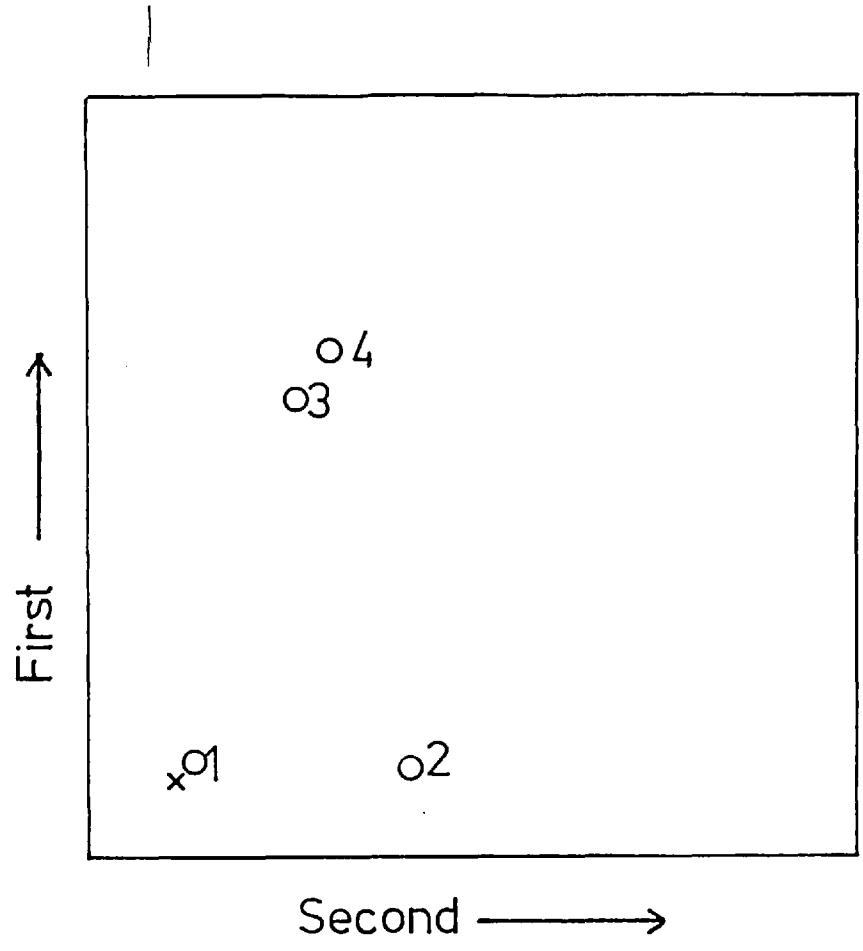
An early attempt to separate (^{3}H) GMP PNP and (^{3}H) cyclic GMP on Whatman No. 1 paper developed in isobutyric acid : ammonia : water 0·1M EDTA (100:56:4·2:1·6 by vol) by descending chromatography at room temperature was unsatisfactory because a considerable proportion (about 50%) of the reaction mixture remained at the origin.

This was found not to be so using t.l.c. on cellulose developed in system 17, GMP PNP and 5'GMP remained at the origin while cyclic GMP and guanosine had an Rf of 0·51. However all four compounds could be resolved by two dimensional t.l.c. Tritiated GMP PNP (8750 - 8800 c.p.m.) was incubated with a sample of homogenised macroplasmodium of Physarum for 30min at 30°C. After termination of the reaction with an equal volume of 30% TCA (w/v) containing GMP PNP, 5'GMP, cyclic GMP and guanosine as chromatographic markers (all approximately 1mM), it was chromatographed on cellulose in system 17 followed by drying and development at right angles to the first solvent front, in system 20. No significant amounts of (^{3}H) 5'GMP or (^{3}H) guanosine were formed. This can be seen in Figure 4·2. In view of this the one dimensional separation system 17 was used in the assay of guanylate cyclase. It may be noted that enzyme assays were carried out with 10min incubations.

The elution of (^{3}H) GMP PNP and (^{3}H) cyclic GMP was measured by comparing the radioactivity eluted from a 15mm cellulose square with 1ml water with the radioactivity in an aliquot counted directly in the same scintillant (TTX-100). The results are shown in Table 4·4. Elution was regarded as being complete.

Figure 4·2

Two dimensional thin layer chromatography of guanylate cyclase reaction products. The t.l.c. was developed in the first direction in system 17 and after drying it was developed at right angles in system 20. Spots 1 to 4 indicate the UV absorbing areas, the radioactivity present in each region is shown in the table below.



Compound	cpm	% Total
1 GMPPNP	6814	78·39
2 5 GMP	26	0·29
3 cGMP	1619	18·62
4 GUA.	42	0·48
× Origin	20	0·23
Total	8693	

Table 4·4 Elution of (^{3}H) cyclic GMP and (^{3}H) GMP PNP from cellulose.

Compound	Direct c.p.m.	Av.	t.l.c. c.p.m.	Av	$\frac{\text{t.l.c.}}{\text{Direct}} \times 100$
(^{3}H) cGMP	12623 10525	11574	11371 11574	11472	99·1%
(^{3}H) GMP PNP	30185 27153	28669	31199 26613	28906	100·8%

4·23 Cyclic GMP phosphodiesterase.

An experiment similar to that described for cyclic AMP PDE was carried out to determine the precise limits of the cyclic GMP, 5'GMP and guanosine regions using cellulose developed in solvent system 20. The labelled compounds coincided with the unlabelled UV absorbing compounds. Elution of cyclic GMP, 5'GMP and guanosine was determined to be complete, greater than 98% in each case.

It may be noted here that in experiments to determine the activity of these enzymes during the mitotic cycle the elution of these compounds was checked again and similar results obtained.

CHAPTER 5

CYCLIC AMP AND CYCLIC GMP.

5·10 Introduction.

Accurate measurement of cyclic nucleotide concentrations in cells and biological materials necessitates the following;

1). A rapid fixation method so that cyclic nucleotides are not degraded by phosphodiesterases.

2). Extraction of cyclic nucleotides, which should be complete.

3). Efficient purification methods so that substances interfering with the assay, are removed.

4). Sensitive and accurate assay methods suited to the processing of many samples.

Various techniques of fixation, extraction, purification and assay were investigated to determine the best ways of achieving these aims. However, before the efficiency of extraction and purification methods can be measured, the cyclic AMP must be quantified.

The extraction and purification methods were worked out for cyclic AMP because at the time the radioimmunoassay (RIA) for cyclic GMP was not availible.

In the first part of this section, concerning cyclic AMP and cyclic GMP assays, experimental data are provided as well as graphs - this is to demonstrate the agreement between duplicates.

5·20 Extraction of cyclic AMP binding protein from beef adrenal glands.

Fresh bovine suprarenal glands (adrenal glands) were collected from the Guildford abbatoir and transported in ice in a Dewar flask to a cold room at the laboratory.

The glands were stripped of fat and the outer cortex removed from the medulla and finely chopped with scissors. The cortices were resuspended in 1·5vol of ice cold buffer (0·25M sucrose, 25mM KCl, 5mM $MgCl_2$, 50mM tris - HCl pH 7·4) and homogenised in an M.S.E. Atomix at high speed for 2min. After centrifugation for 15min at 1250g at 4°C the supernatant was recentrifuged for 15min at 5000g at 4°C. The final supernatant was divided into 0·5ml portions and stored in sterile capped plastic tubes at -20°C.

5·21 Binding of (^{3}H) cyclic AMP to beef adrenal binding protein.

To plastic tubes (7 x 62mm, Luckham Ltd.) containing 50µl (^{3}H) cyclic AMP (= 0·3 pmole = 8nCi = 6000 c.p.m.) and 150µl cyclic AMP assay buffer (4mM EDTA, 50mM tris - HCl pH 7·4) was added 100µl binding protein diluted as necessary with assay buffer. After incubation for 2 hours at 0°C, 100µl of charcoal - BSA mixture (520mg Norit GSX charcoal, 0·4g BSA, Fraction V, in 20ml cyclic AMP assay buffer) was added and the tubes vortex mixed for 10sec then centrifuged for 5min at 5000 r.p.m. in a BTL bench centrifuge and 250µl of the supernantant removed for counting in 8ml TTX-100 scintillant plus 0·8ml water. The results are shown in Table 5·1 and Figure 5·1.

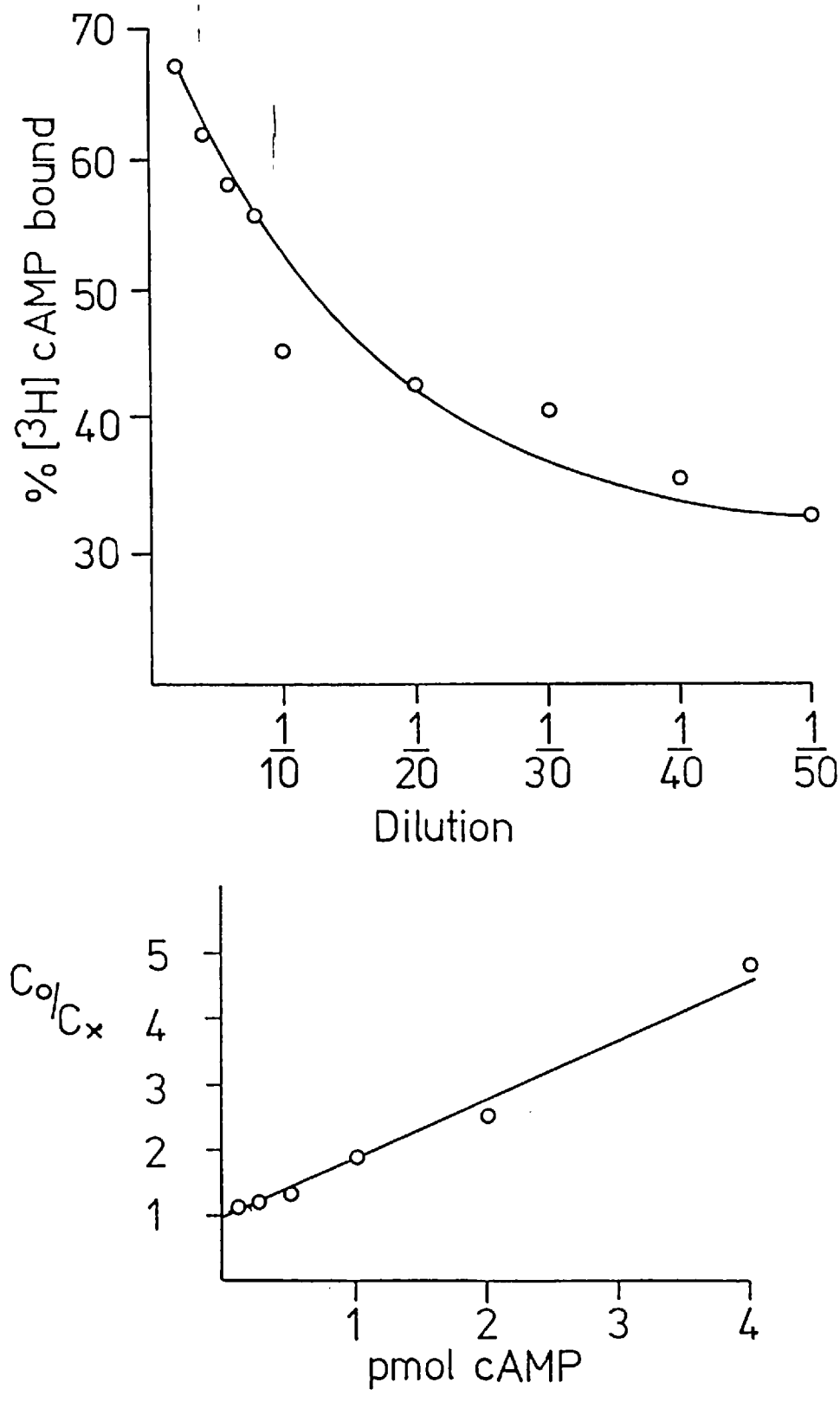


Figure 5•1. Binding of (^{3}H) cyclic AMP to beef adrenal binding protein dilutions (upper panel) and standard graph using this binding protein. (lower panel)

Co = cpm [^{3}H] cAMP bound by protein in absence of unlabelled cAMP
Cx = cpm [^{3}H] cAMP bound by protein in presence of unlabelled cAMP

Table 5·1 Binding of (^{3}H) cyclic AMP to binding protein dilutions.

Dilution	c.p.m. bound	Av c.p.m. bound	% bound
1/2	4163 4097	4130	67·1
1/4	3855 3814	3834	62·3
1/6	3616 3536	3576	58·1
1/8	3360 3485	3422	55·6
1/10	2755 2821	2788	45·3
1/20	2660 2647	2653	43·1
1/30	2596 2487	2542	41·3
1/40	2155 2227	2191	35·6
1/50	2006 1969	1988	32·3*
1/100	1259 1209	1237	20·1
Total counts	6200 6109	6155	

* This dilution was used in subsequent assays.

A standard graph using beef adrenal binding protein was constructed as follows :

The incubation conditions were as outlined above but the amount of cyclic AMP in the assay was varied. The reaction mixture contained the following :

50µl (^{3}H) cyclic AMP (= 8nCi = 6000 c.p.m.)

50µl cyclic AMP (0 - 16pmoles)

100µl assay buffer.

100µl binding protein (stock diluted 1/50 with assay buffer)

The cyclic AMP standards were made up as follows :

A few crystals of cyclic AMP were dissloved 10ml distilled water, scanned in the UV to check the purity of the cyclic AMP, and its absorbance at 260nm measured. Using an extinction coefficient of 15000 it was possible to calculate the concentration of cyclic AMP since :

$a = c \times l \times E_{260}$

c = concentration (moles / litre)

a = absorbance

l = path length (1cm)

E_{260} = extinction coefficient at 260nm.

The results are shown in Table 5·2.

Table 5·2 Standard graph using beef adrenal binding protein.

pmoles cAMP	(^{3}H) cAMP bound c.p.m.*	Av c.p.m.	Co/Cx
0	2137 2003	2070	1·0
4	457 482	432	4·8
2	791 839	815	2·5
1	1001 1167	1084	1·9
0·5	1510 1626	1568	1.3
0·25	1751 1669	1710	1·2
0·125	1721 1757	1739	1·2

* A blank (= 101 c.p.m.) has been substracted from the values shown above

These results show the effect of cyclic AMP on the amount of

(^3H) cyclic AMP bound to the protein ; they are plotted in Figure 5·1.

5·22 Dissociation of the cyclic AMP - binding protein complex.

In the assay of cyclic AMP, charcoal was added only to enough tubes as could be centrifuged. As this was usually less than the total, some samples were incubated for longer than others. If binding changes with time, erroneous results may be produced. To test this, tubes were incubated in duplicate for various times between 0 and 8 hours before the addition of charcoal. Each tube contained approximately 4 pmoles cyclic AMP. The results are shown in Table 5·3.

Table 5·3 Dissociation of cyclic AMP - binding protein complex.

Incubation time (hours)	(^3H) cAMP bound c.p.m.*	Av. c.p.m.
0·0	886 888	887
1·5	1552 1592	1573
2·0	1660 1644	1652
3·0	1636 1644	1640
4·0	1649 1625	1637
5·0	1690 1628	1659
6·0	1631 1686	1658
7·0	1650 1655	1653
8·0	1550 1685	1618

* A blank value (93 c.p.m.) has been subtracted from these values.

S.D. (excluding 0 and 1·5 hour values) = 29·8 c.p.m.

5·23 Reliability of cyclic AMP assay.

In order to determine the statistical variation in the assay of cyclic AMP, 22 identical samples containing approximately 1·37 - 1·38 pmoles cyclic AMP were assayed (Cx) and 10 samples without added cyclic AMP (Co). The results are shown in Table 5·4.

Table 5·4 Statistical variation in the cyclic AMP assay.

	Mean c.p.m.	S.D.	n
Co	6097	93·50	10
Cx	3416	132·50	22
Co (Av)/Cx	1·79	0·07	22
pmoles cAMP	1·37	0·15	10

The first attempt at the extraction of this binding protein was successful: the second was not, other workers have also encountered this problem' (Dr. B. Brown personal communication). To avoid this problem, whose cause is difficult to ascertain - a commercial preparation of greater purity was used in subsequent work.

5·30 Beef adrenal binding protein supplied by B.D.H.

The cyclic AMP binding protein used in the rest of this work (except where stated) was obtained from B.D.H., as an ammonium sulphate solution and stored at 4°C. Tritiated cyclic AMP was used at a concentration of 1ng/50µl (= 1·8 pmole = 35000 c.p.m.) in the following buffer: 8mM theophylline, 6mM mercaptoethanol, 50mM tris - HCl pH 7·4. The incubation mixture contained the following:

50µl (^{3}H) cyclic AMP (= 35000 c.p.m.)

50µl cyclic AMP (standards or samples)

100µl assay buffer (as above)

100µl binding protein (various dilutions)

After 120min (± 10min) incubation on ice, bound cyclic AMP was separated from free cyclic AMP by the addition of 100µl of charcoal suspension (10% w/v Norit GSX charcoal in assay buffer containing 2% BSA (Fraction V). After centrifugation for 5min at 5000 r.p.m., 250µl of the supernatant was removed for counting.

Total counts were determined by removing 250µl from a mixture of 50µl (^{3}H) cyclic AMP and 350µl assay buffer.

The results are shown in Table 5·5 and Figure 5·2. The stock binding protein was diluted 1/12½ with assay buffer for routine use.

Table 5·5 Binding of (^{3}H) cyclic AMP to binding protein dilutions.

Protein dilution	(^{3}H) cAMP bound c.p.m.	Av c.p.m.*	% bound
1/5	10083 9993	9970	45·7
1/10	7675 7719	7629	35·0
1/15	5515 5593	5486	25·2
1/20	2118 2034	2008	9·2
Total counts	22312 21438	21808	

* A blank value (= 68 c.p.m.) has been subtracted from these values.

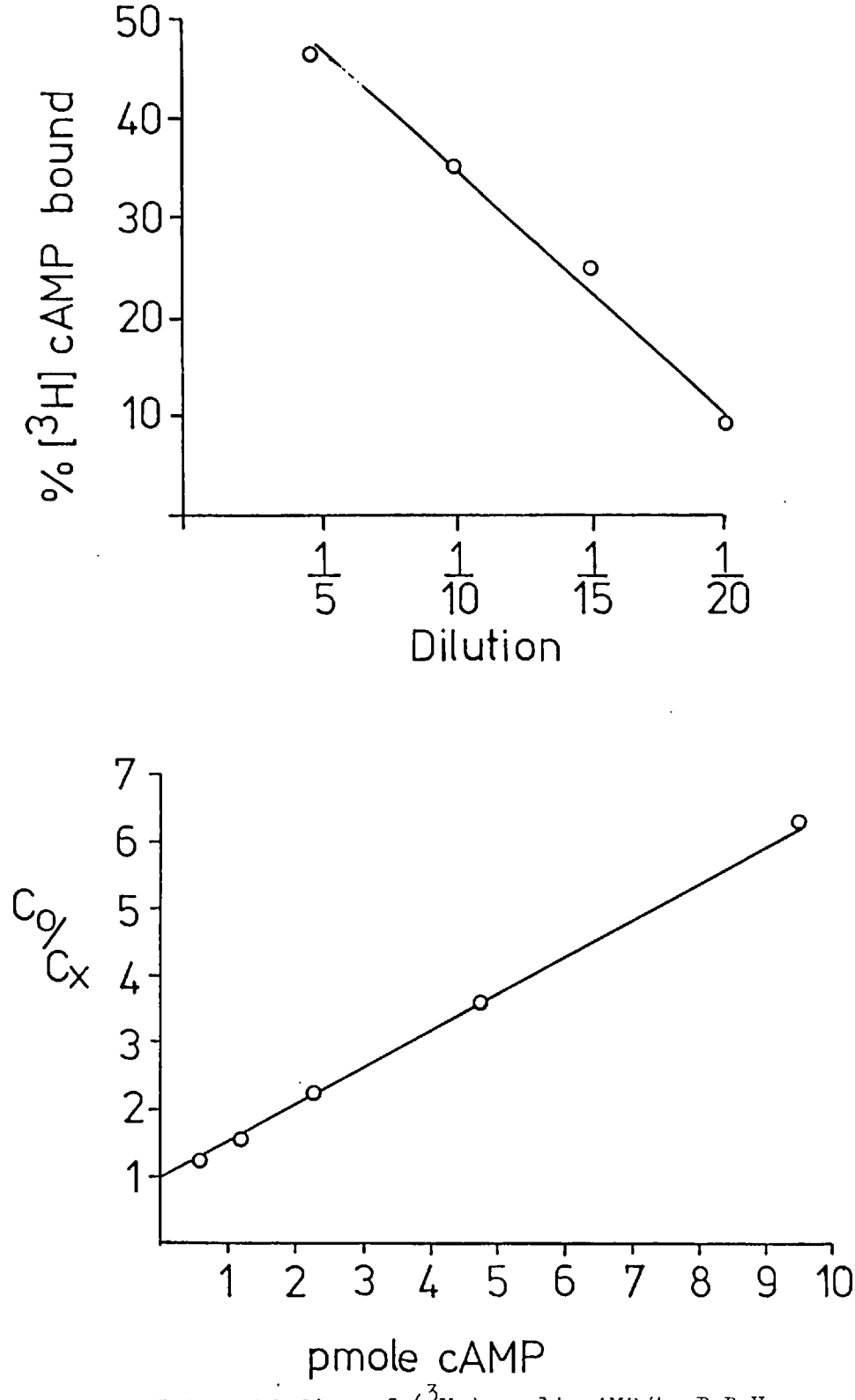


Figure 5·2 Binding of (^{3}H) cyclic AMP to B.D.H. binding protein dilutions (Upper panel) and Standard graph (Lower panel).

5·31 Standard graph using B.D.H. binding protein.

The incubation mixture contained the following :

50µl cyclic AMP (0 - 19 pmoles)

50µl (^{3}H) cyclic AMP (35000 c.p.m.)

100µl assay buffer

100µl binding protein (diluted 1/12½ with assay buffer).

The incubation and separation procedure were as described in section 5·30.. The results are shown in Table 5·6 and Figure 5·2.

Table 5·6 Standard graph using B.D.H. binding protein.

pmoles cAMP	(^{3}H) cAMP bound c.p.m.	Av c.p.m.*	Co/Cx observed	Co/Cx** calculated
0	5291 5262 5337 5450	5193 (C_o)	1	1 (Co)
19	690 635	532 (C_x)	9·76	-
9·5	916 992	824 (C_x)	6·30	6·27
4·75	1657 1661	1529	3·39	3·59
2·37	2298 2241	2140	2·43	2·25
1·18	3074 3221	3018	1·72	1·58
0·59	4660 4924	4662	1·11	1·25

* A blank value (= 130 c.p.m.) has been subtracted fron these values.

** r^2 (regression coefficient) = 0·994. The value for 19 pmoles was not used in calculating the regression, the assay was only linear over the range 0.5 - 10 pmoles.

The line, shown in Figure 5·2, was calculated by linear regression using a Hewlett Packard HP 25 programmable calculator.

The data are taken from a standard assay carried out during the analysis of samples harvested throughout the mitotic cycle.

It may be noted here that a third method of determining cyclic AMP - with a kit from the Radiochemical Centre - was also used as an additional check on the above method. It was used exactly as directed in the accompanying instructions.

5·4 Radioimmunoassay of cyclic GMP.

Cyclic GMP was measured using a radioimmunoassay (RIA) kit obtained from the Radiochemical Centre.

5·41 Assay protocol.

The freeze dried reagents provided were reconstituted in sterile distilled water as described in the manual provided with the kit.

Into each conical centrifuge tube (Sarstedt 39/10A) cooled on ice was pipetted 50µl (^{3}H) cyclic GMP (= 6000 c.p.m.), 100µl assay buffer (4mM EDTA, 50mM tris - HCl pH 7·5), 50µl cyclic GMP (0 - 8 pmoles) or 50µl sample and 50µl antiserum. After incubation on ice for 120min, 1·0ml of cold 60% saturated ammonium sulphate was added and the tube centrifuged in an Eppendorf 1200 Zentrifuge for 2min at room temperature. The supernatant was carefully decanted, the tube inverted and allowed to drain. The inside of the tube was wiped dry with a Kleenex paper tissue.

The precipitate was dissolved in 200µl distilled water and 1·2ml TTX-100 scintillant added and the tube capped. After shaking, the tube was placed in an empty plastic scintillation vial and the radioactivity determined.

After counting, each tube was emptied into a vial and rinsed twice with 1ml scintillant, the washings were added to the vial.

A further 5ml of scintillant was added to the vial together with 0•8ml water and the sample counted. Table 5•7 and Figure 5•3 shows a comparison of these results.

The ammonium sulphate precipitates of samples from the mitotic cycle experiment were dissolved in 800µl distilled water and emptied into a scintillation vial, the assay tube washed 5 times with 1•5ml TTX-100 scintillant; each wash being added to the vial.

Table 5•7 Standard graph of cyclic GMP radioimmunoassay; comparison of counting methods.

pmole cGMP	c.p.m. bound*	c.p.m. bound**	Av c.p.m.*	Av c.p.m.**	Co/Cx*	Co/Cx**
0	2321 2025	2609 2186	2070	2277	1	1
8	266 308	304 307	184	184	11•25	12•38
4	347 403	416 587	272	380	7•61	5•90
2	535 557	625 815	443	599	4•67	3•80
1	862 975	970 1091	815	909	2•54	2•50
0•5	1215 1212	1372 1299	1130	1215	1•80	1•87
Blank	100 107	127 114	103	121	-	-
Total counts	6080 5271	6566 5953				

* = samples counted in assay tube

** = samples counted after emptying into scintillation vial.

The respective blank value has been subtracted from each series of Av. c.p.m. values.

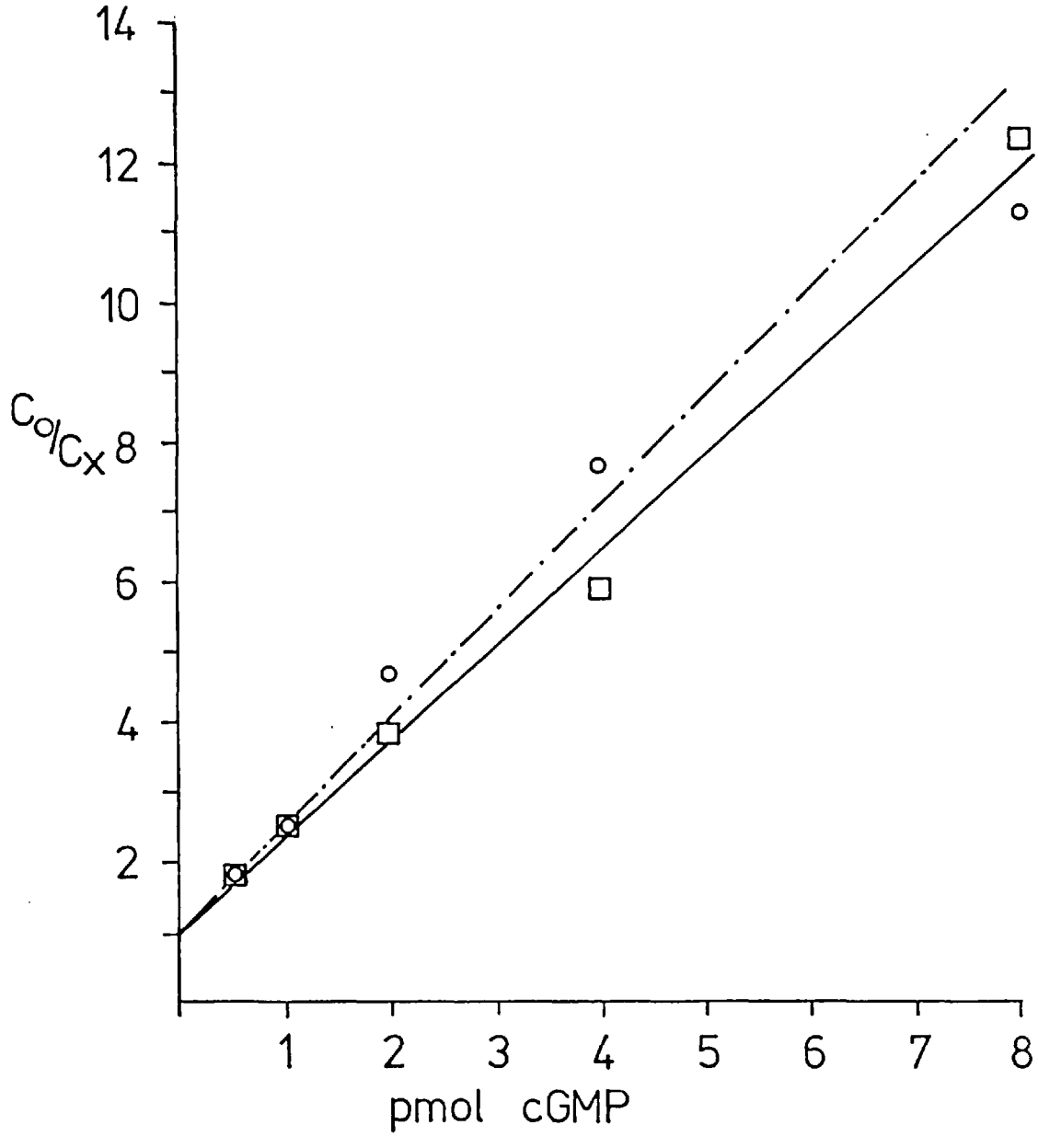


Figure 5•3

Standard graph of radioimmunoassay of cyclic GMP.

Open symbols = samples counted in 8ml TTX-100 scintillant.

Square symbols = samples counted in assay tube.

5•50 Extraction of cyclic AMP from Physarum : comparison of solvents.

Once a reliable and sensitive assay for cyclic AMP had been developed attention was turned to the evaluation of various methods of extracting cyclic AMP from Physarum.

Trichloroacetic acid (TCA), perchloric acid, ethanol and methanol were used to extract cyclic AMP from Physarum.

Twelve reasonably uniform macroplasmodia were harvested as de described in Chapter 3•14. Three macroplasmodia were plunged into 10ml 5% TCA, three into 10ml 5% perchloric acid, three into absolute ethanol and three into absolute methanol. To monitor recovery, 10nCi (^{3}H) cyclic AMP (= 7500 c.p.m.) was added to each extract. Further treatment was as follows:

1) Trichloroacetic acid.

Following mechanical homogenisation with an ILA mixer for 30sec the extract was centrifuged for 15min at 10000g at 4°C. The supernatant was decanted and the pellet dissolved in 4ml 0•4N NaOH for protein analysis. To remove TCA, an equal volume of diethyl ether, saturated with water, was added to the supernatant and after vigourous shaking for 30sec the extract was centrifuged for 5min at 5000 r.p.m. at room temperature. The upper layer was removed and discarded. Extraction with ether was repeated four times.

i) Ether extraction of trichloroacetic acid.

To examine whether cyclic AMP dissolves in the ether used to extract TCA the following experiment was conducted.

A Physarum extract, to which (^{3}H) cyclic AMP (253271 c.p.m.) had been added, was treated with 1vol diethyl ether saturated with water and shaken. After centrifugation as before the upper layer was removed and placed in a scintillation vial. This was repeated ten times. The ether extracts were evaporated to dryness in a water

bath at 60°C for 60min. To the dry vials, 0·8ml water and 8ml TTX-100 scintillant was added and the radioactivity determined. The results are shown in Table 5·8

Table 5·8 Loss of cyclic AMP during ether extraction.

Extract no.	c.p.m. (- blank, 19 c.p.m.)
1	30
2	20
3	60
4	134
5	253
6	152
7	201
8	288
9	799
10	884
Total	2822
Total c.p.m. added	253251
% removed	2822/253251 x 100 = 1·11%

This loss to the ether is negligible.

2) Ethanol and methanol.

After homogenisation and centrifugation as above the supernatant was removed and evaporated to dryness. The pellet was dissolved in 4ml 0·4N NaOH for protein analysis.

3) Perchloric acid.

After homogenisation etc. as before the supernatant was removed, to it was added 1vol 1M K_2CO_3, a precipitate, potassium perchlorate, was immediately formed. After centrifugation as before the supernatant

was carefully removed and evaporated to dryness.

Each extract was taken up in 1ml cyclic AMP assay buffer, 500µl was removed diluted and assayed, the remaining 500µl was loaded onto a 10 x 1cm column of Dowex AG50 X4 200 - 400 mesh H^+ form and eluted with 0·1N HCl. The first 6ml were discarded, the next 12ml, containing cyclic AMP were pooled and evaporated to dryness. Each extract was taken up in 500µl cyclic AMP assay buffer. Twenty microlitres was removed from each purified extract to monitor recovery. The results are shown in Table 5·9.

Table 5·9 Extraction of cyclic AMP; comparison of solvents.

Solvent	pmole cyclic AMP / mg protein						Recovery
	without Dowex treatment			treated			
	1	½	¼	1	½	¼	
TCA	22	18	20	26	17	12	71%
PCA	19	21	12	17	16	10	45%
Ethanol	13	12	9	14	10	7	36%
Methanol	9	8	8·5	7	5	2	25%

(1, ½, ¼ indicates dilution of original sample.)

Extraction of cyclic AMP from Physarum macroplasmodia with 5% TCA followed by ether extraction appears to be the best method as judged by the recovery. This method was used for studies on the mitotic cycle but with a more extensive purification of the sample and separation of cyclic AMP and cyclio GMP.

The other solvents tested appeared to result in low recovery. Upon dilution of the sample, the amount of cyclic AMP present did not change by the same degree, this is probably due to interfering substances present in the sample. Additionally, extraction with

ethanol precipitated polysaccharides and proteins, polysaccharides may interfere with protein analysis.

5·60 Extraction and separation of cyclic AMP and cyclic GMP.

Four macroplasmodia were harvested and extracted with 10ml ice cold 5% TCA as described above. To monitor recovery, 50nCi (^{3}H) cyclic AMP (= 37000 c.p.m.) was added and allowed for in the subsequent assay steps. Instead of (^{3}H) cyclic AMP, 8nCi (^{3}H) cyclic GMP (= 6000 c.p.m.) was added to monitor the recovery of cyclic GMP. After shaking with ether five times the aqueous extract was taken to dryness in a rotary evaporator. When dry the residue was dissolved in 1ml 50% ethanol (v/v) and loaded onto a 1 x 2·5cm column of Dowex AG50 X4 100 - 200 mesh resin ammonium form. The tube containing the extract was carefully washed out with another 1ml 50% ethanol and this added to the column. After the sample had drained into the resin it was washed with 10ml sterile distilled water and the eluate allowed to drip into a 1.5 x 2.0cm column of QAE Sephadex A 25 formate form. This column was washed with 20ml sterile distilled water and the eluate discarded. Cyclic AMP was eluted with 18ml 0·1N ammonium formate pH 9·0. A further 10ml 0·1N ammonium formate pH 9·0 was applied to the column. Cyclic GMP was then eluted with 18ml 0·1N ammonium formate pH 6·0.

In a separate experiment a sample treated in the same way, containing (^{3}H) cyclic AMP and (^{3}H) cyclic GMP, was eluted from QAE Sephadex A 25 as described above, fractions collected, a sample of each removed and the radioactivity determined. The elution of cyclic AMP and cyclic GMP from QAE Sephadex A 25 is shown in Figure 5·4.

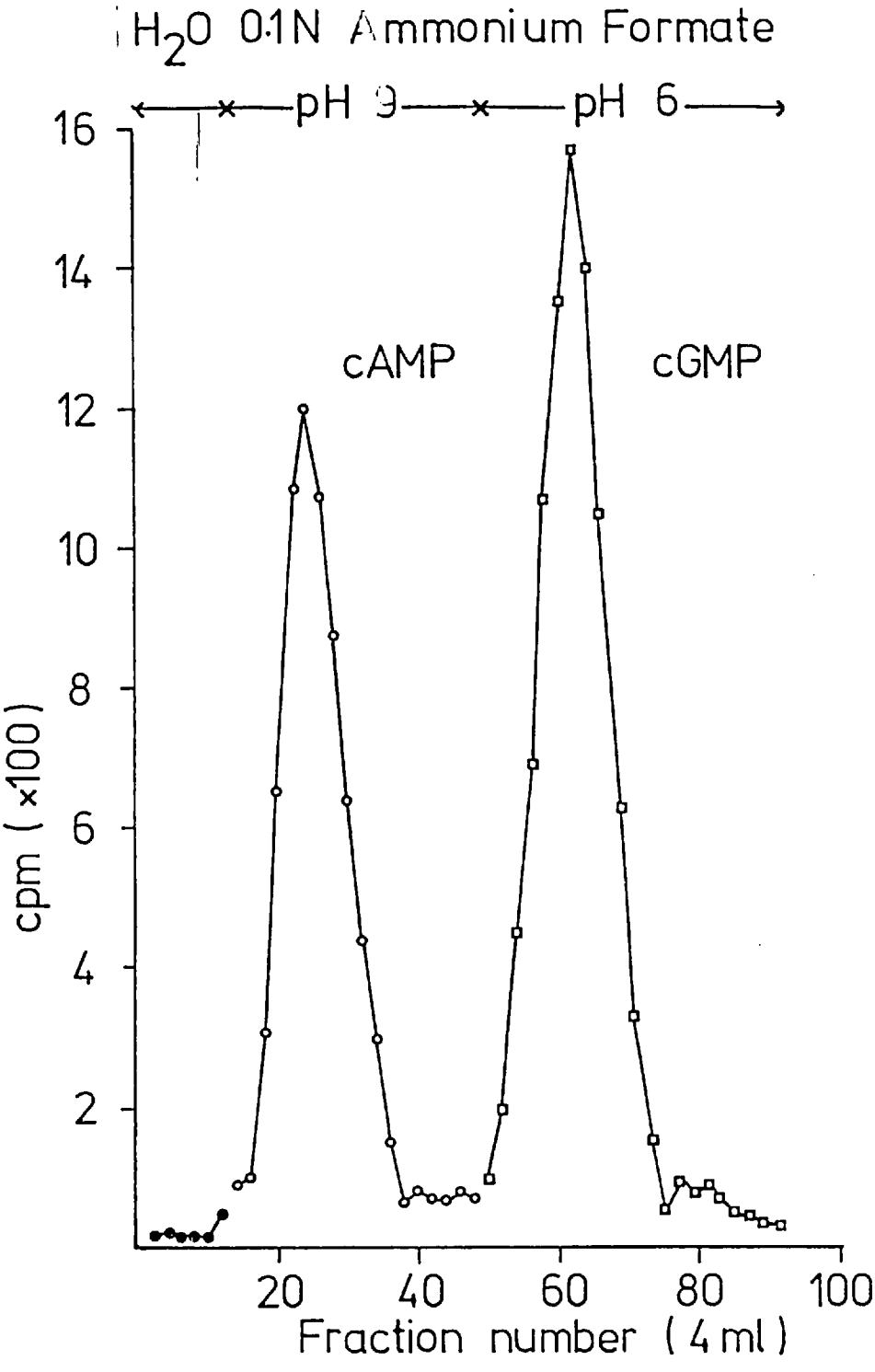


Figure 5·4

Elution of cyclic AMP and cyclic GMP from QAE Sephadex A 25 formate form.

Fractions containing cyclic AMP (or cyclic GMP) from QAE Sephadex were collected on a 1•5 x 5•0cm column of Dowex AG50 X4 200 - 400 mesh H^+ form. Cyclic AMP (or cyclic GMP) was eluted with 12ml 0•1N HCl.

Again in a separate experiment, an homogenate of Physarum containing (^3H) cyclic AMP and (^3H) cyclic GMP was chromatograhed as described above, the eluate, in 0•1N HCl, from the second Dowex column was fractionated and a sample from each fraction removed and the radioactivity determined. The results are shown in Figures 5•5 and 5•6.

The eluate from the second Dowex column was lyophilised and stored at -20°C until assayed.

5•70 Assay of cyclic AMP and cyclic GMP.

1) Cyclic AMP.

The freeze dried cyclic AMP extracts were taken up in 200µl cyclic AMP assay buffer (8mM theophylline, 6mM mercaptoethanol, 50mM tris - HCl pH 7•4) and two 50µl aliquots removed, from each 5µl was removed to monitor recovery of cyclic AMP. To the remaining 100µl of extract, 100µl assay buffer was added, after mixing, two more 50µl aliquots were removed. This was repeated until four dilutions (in duplicate) of each sample had been obtained.

As 50nCi (^3H) cyclic AMP (37034 c.p.m.) was added to each extract, 200µl should contain 37034 c.p.m., assuming 100% recovery, similarly, 5µl would be expected to contain 926 c.p.m.. A few representative results are shown in Table 5•10 for samples taken during the analysis of cyclic AMP and cyclic GMP in the mitotic cycle.

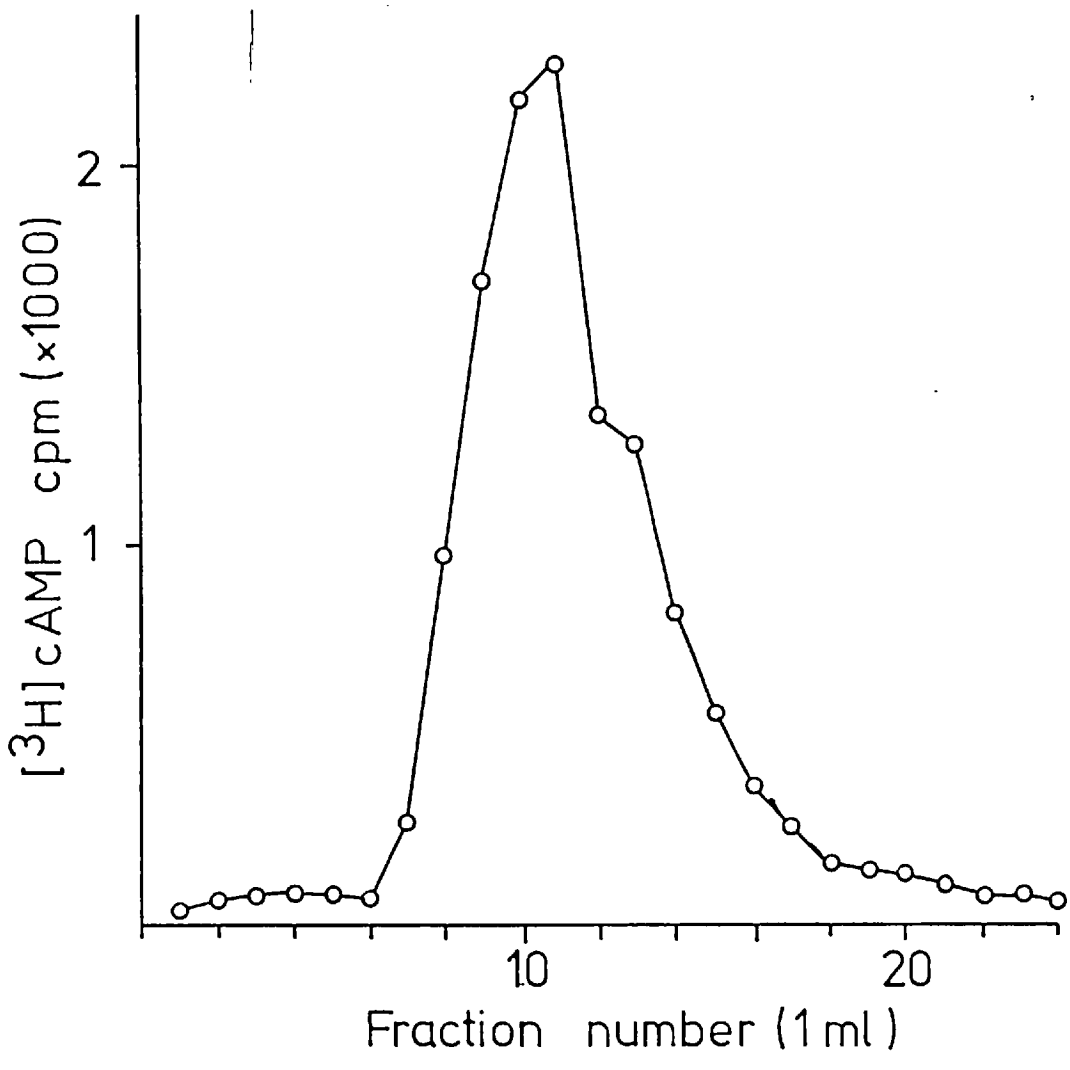


Figure 5·5

Elution of (^{3}H) cyclic AMP from Dowex AG50-X4 200 - 400 mesh H^{+}form with 0.1N HCl.

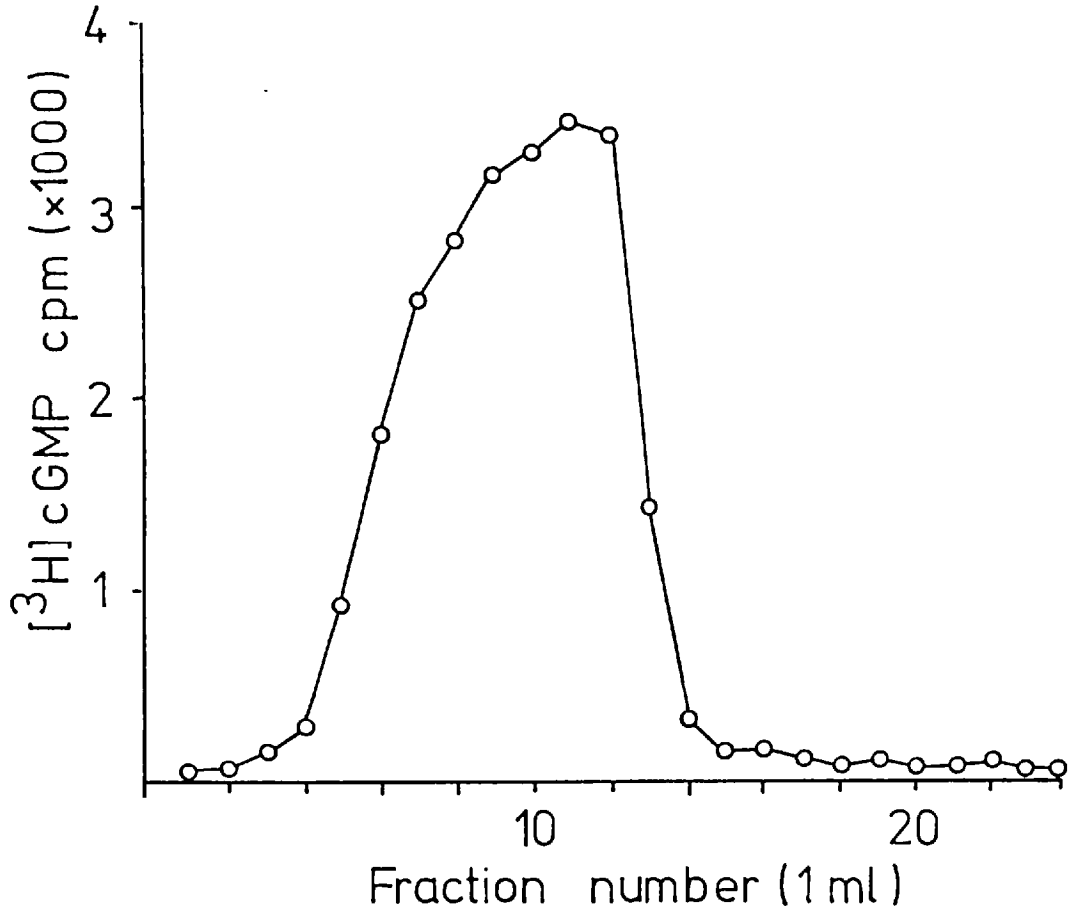


Figure 5·6

Elution of (^{3}H) cyclic GMP from Dowex AG50-X4 200 - 400 mesh H^{+}form with 0·1N HCl

Table 5·10 Recovery of cyclic AMP.

(^{3}H) cAMP/5µl c.p.m.	Sample time	% recovery
723	M2-60min	78
694	M2-30min	75
705	M2	76

The recovery of cyclic AMP varied between 75% and 79% (mean = 78%, S.D. = 0·87, n = 24). Consequently each dilution contained some (^{3}H) cyclic AMP, this was allowed for in the assay. The results are shown in Table 5·11.

Table 5·11 (^{3}H) cyclic AMP content of sample dilutions.

Dilution	(^{3}H) cAMP/50µl
x1	7230
x2	3615*
x4	1808*
x8	904*

* not measured, these results were obtained from a sample harvested 60min before M2.

In the assay procedure previously described for the beef adrenal binding protein supplied by B.D.H., about 4200 c.p.m. were determined to be the total counts per assay tube. From 400µl, 250µl was removed for counting, thus each tube contained approximately 7000 c.p.m. In the assay of cyclic AMP the first dilution of sample M2-60min contained 7230 c.p.m. (^{3}H) cyclic AMP, therefore no more (^{3}H) cyclic AMP was added, the incubation volume was made up with 50µl assay buffer. The second (x2) dilution however contained less (^{3}H) cyclic AMP than was needed, therefore this was supplemented with approximately 3400 c.p.m. (^{3}H) cyclic AMP. In this way each

dilution was assayed in the presence of about the same amount of (^{3}H) cyclic AMP - enough to give 25% - 30% zero dose binding.

The assay results for the first dilution were usually ignored for the following reasons;

1) 10% of the sample volume had been removed to monitor recovery

2) Interfering substances were probably still present in sufficient concentrations to affect the results of the assay.

Subsequent dilution readings were used to calculate the final concentration of cyclic AMP in the extract. The effect of sample dilution on cyclic AMP content can be seen in Table 5·12.

Table 5·12 Effect of sample dilution on cyclic AMP content.

Dilution	pmole cAMP/ extract.
x1	61·3
x2	34·1
x4	16·5
x8	8·0

Each value is an average of two determinations on a sample taken 7½ hours after M2, not corrected for recovery.

A further dilution of the cyclic AMP extracts was made and assayed using a kit supplied by the Radiochemical Centre, which uses a beef skeletal binding protein (zero dose binding = 57%).

A comparison of results using these two binding proteins is shown in Table 5·13 (duplicate samples, corrected for dilution but not recovery).

Table 5·13 Comparison of cyclic AMP content using B.D.H. and the Radiochemical Centre binding proteins.

Sample time	Beef adrenal B.P.		Beef skeletal muscle B.P.	
(hours, M2 = 0)	pmole cAMP		pmole cAMP	
-1	20·4	20·3	21·1	19·8
-½	9·5	9·1	10·0	8·8
+7	73·6	77·1	77·8	72·1
+7½	61·0	61·5	61·9	60·4

Cyclic AMP concentrations were read off from a standard graph plotted using the least squares method.

2) Cyclic GMP.

Tritiated cyclic GMP (8nCi = 6000 c.p.m.) was added to only one sample in order to monitor recovery. At the time the mitotic cycle samples were taken the exact particulars of the assay method were unavailable.

The cyclic GMP extracts were taken up in 400µl cyclic GMP assay buffer (4mM EDTA, 50mM tris - HCl pH 7·5). Two 100µl aliquots were removed for assay and 200µl assay buffer added to the remainder, a further two 100µl aliquots were removed for assay. Only two dilutions of cyclic GMP extracts were made because the sample volume in each assay tube was 100µl and it was thought that there may have been less cyclic GMP than cyclic AMP.

To a sample taken at M3+45min, 8nCi (^{3}H) cyclic GMP (6000 c.p.m.) was added to monitor recovery. This sample was reconstituted in 400µl cyclic GMP assay buffer and two 100µl aliquots removed and the radioactivity determined. The results are shown in Table 5·14

Table 5·14 Recovery of cyclic GMP.

(^{3}H) cGMP added c.p.m.	(^{3}H) cGMP recovered c.p.m.	(^{3}H) cGMP in extract	% recovery
5925	842 817	3318	56

Because the recovery of cyclic AMP was constant it was assumed that the recovery of cyclic GMP was also constant (56%). The effect of sample dilution on cyclic GMP content is shown in Table 5·15.

Table 5·15 Effect of dilution on cyclic GMP content.

Dilution	pmole cGMP/extract
x1	1·9
x2	0·9

A summary of the extraction and purification of cyclic AMP and cyclic GMP from Physarum is shown in Table 5·16.

5·80 Proof of identity of cyclic AMP extracted from Physarum.

Cyclic AMP was extracted from Physarum and purified as described previously. The lyophilised extract was taken up in 200µl 50mM tris - HCl pH 7·4, 100µl was removed and added to 100µl of the same buffer containing approximately 2µg beef heart cyclic nucleotide phosphodiesterase and incubated for 60min at 30°C followed by boiling for 5min. To the remaining extract 100µl buffer was added and boiled as before.

Both the treated and untreated extracts were diluted with cyclic AMP assay buffer and 50µl portions of each dilution assayed. The results are shown in Table 5·17

Table 5·16 Summary of extraction and purification of cyclic AMP and cyclic GMP from Physarum.

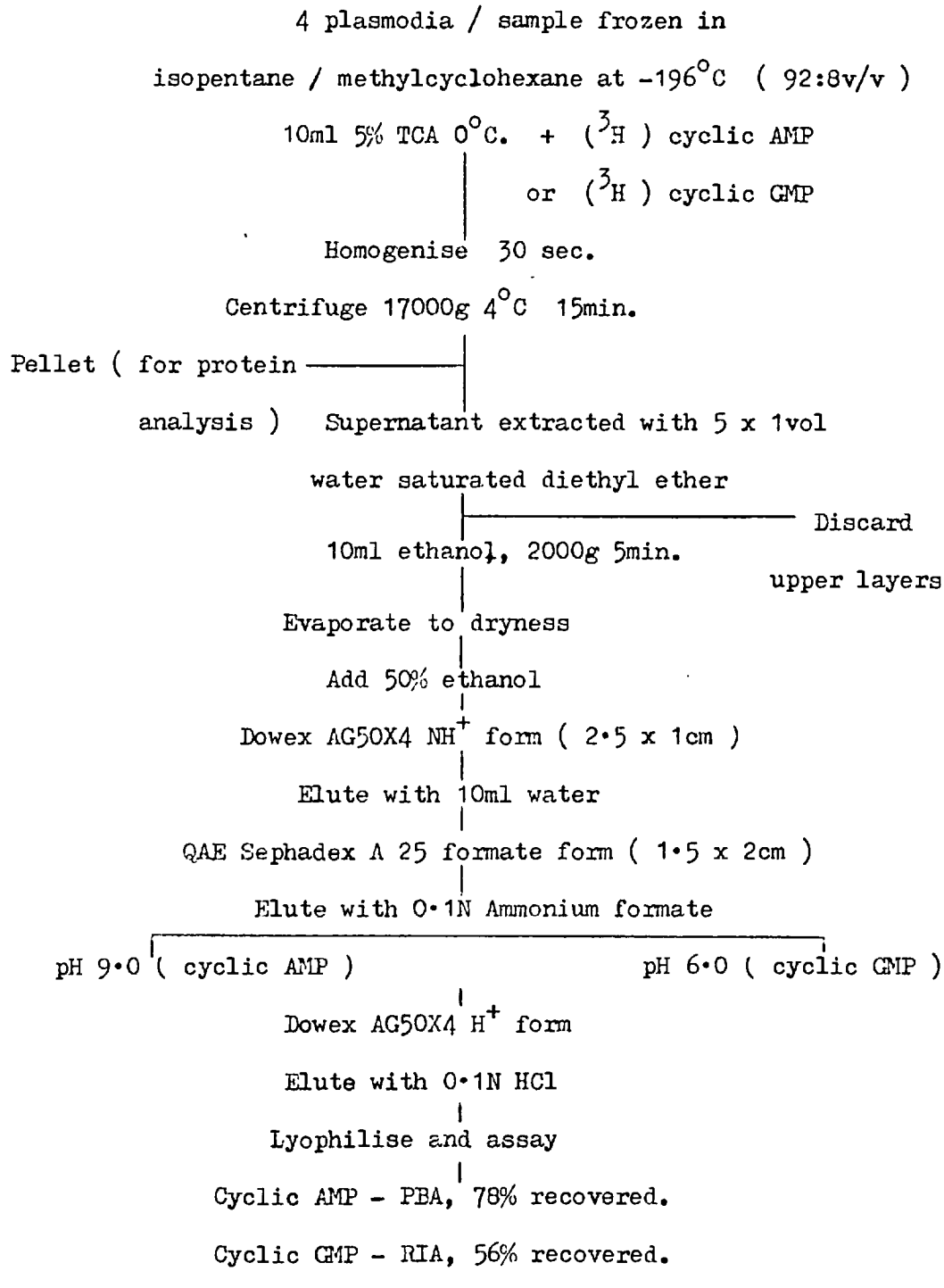


Table 5·17 Degradation of Physarum cyclic AMP by cyclic nucleotide phosphodiesterase.

Dilution	pmole cAMP		$\frac{-PDE}{+PDE} \times 100$
	+PDE	-PDE	
x1	0·07	72·30	0·097
x2	0·06	31·71	0·198
x4	0·00	15·80	0·000
x8	0·00	8·05	0·000

Thus the trace of cyclic AMP detected after phosphodiesterase treatment represents a very small proportion of the total cyclic AMP in the control extracts not treated with the enzyme. The variation in cyclic AMP remaining is not thought to be significant and is so low as may be disregarded.

5·90 Summary.

A number of assay methods for cyclic AMP have been demonstrated and used to determine the optimum extraction and purification methods for cyclic AMP from Physarum.

CHAPTER 6

THE METABOLISM OF CYCLIC AMP AND CYCLIC GMP.

6·10 Introduction.

This chapter describes work on four enzymes likely to have a profound effect on cyclic nucleotide levels in *Physarum*. They are : cyclic AMP phosphodiesterase (cyclic AMP PDE), cyclic GMP phosphodiesterase (cyclic GMP PDE), adenylate cyclase and guanylate cyclase.

In some experiments done at the beginning of this work an established method was used to measure the activity of cyclic AMP PDE. However, at the time it was realised and indeed later demonstrated that it underestimated enzyme activity. To circumvent this a new method was developed in which the reaction products were separated by thin layer chromatography (t.l.c.) on cellulose. A similar method was applied to the determination of cyclic GMP PDE activity.

Adenylate cyclase and guanylate cyclase activity was measured using the tritiated imidophosphate analogue of their respective triphosphates as substrate. The natural substrate, ATP or GTP, may be degraded by triphosphatases so continually reducing the substrate concentrations during the assay. Imidophosphates are unaffected by such enzymes. The reaction products were subsequently separated by t.l.c. on cellulose.

The next four sections deal with these enzymes.

Cyclic AMP phosphodiesterase.

6·20 The resin assay method.

The radiometric assay method for cyclic AMP PDE is derived from the methods described by Butcher and Sutherland (1962) and Thompson and Appleman (1971a) later modified by Chasin and Harris (1972).

Tritiated cyclic AMP is degraded by cyclic AMP PDE to (^{3}H) 5'AMP. This is converted stoichiometrically to (^{3}H) adenosine by the addition of excess 5' nucleotidase present in the venom of the King cobra (Ophiophagus hanna). On addition of Dowex resin, (^{3}H) cyclic AMP and (^{3}H) 5'AMP are bound but (^{3}H) adenosine is left in the supernatant. After centrifugation this adenosine may be estimated by taking a sample of the supernatant for determination of radioactivity by liquid scintillation counting (L.S.C.).

Work by Boudreau and Drummond (1975) showed that the resin binds some of the (^{3}H) adenosine, so enzyme activity will be underestimated. Nevertheless the method is rapid and useful for comparative purposes. It has been applied to study the activity of ammonium sulphate precipitated fractions of the enzyme present in the growth medium, the effect of various agents on the external and internal and external enzymes and the effect on enzyme activity of certain metal ions.

6·21 Thin layer chromatography of reaction products.

A more accurate method for the assay of cyclic AMP PDE was developed by the author in which cyclic AMP, 5'AMP and adenosine were separated by t.l.c. on cellulose so that each may be eluted and the radioactivity determined by L.S.C.

6·22 Resin assay conditions.

To 50µl (^{3}H) cyclic AMP (10nCi = 7500 c.p.m.) in assay buffer (120mM $MgCl_2$, 2·5mM EDTA, 120mM tris - HCl pH 8·0) was added 100µl assay buffer and 100µl Physarum homogenate (or growth medium). Any additions, such as inhibitors, were present in the assay buffer. After incubation at 30°C for 10min the reaction was terminated by the addition of 500µl slurry of Dowex resin (Dowex AG1 X8 100 - 200 mesh Cl^- form), kept constantly stirred. After agitation on a rotary mixer the assay tubes were centrifuged for 15min at 5000 r.p.m. at room temperature and 250µl of the supernatant removed for counting in 8ml TTX-100 scintillant plus 0·8ml distilled water.

The volume of resin slurry used was decided upon after a pilot experiment, in which the amount of Dowex resin was varied. To each of eight tubes was added approximately 10nCi (^{3}H) cyclic AMP followed by Dowex resin slurry as shown in Figure 6·1. After incubation at 0°C for 2min the tubes were centrifuged as above and 250µl of the supernatant removed for counting.

Because the same volume and not the same proportion of the total volume was removed for counting a correction must be applied to calculate the total activity in the supernatant. This was done in the following way:

100µl Dowex suspension (67µl water, 33µl resin)

Total volume = 67 + 250µl reaction volume

= 317µl total supernatant volume.

For L.S.C. 250µl was removed, this is 250/317 x 100 = 78·86%, only this proportion of the supernatant was removed, the correction factor = 1·268. Corrected and uncorrected results are shown in Figure 6·1.

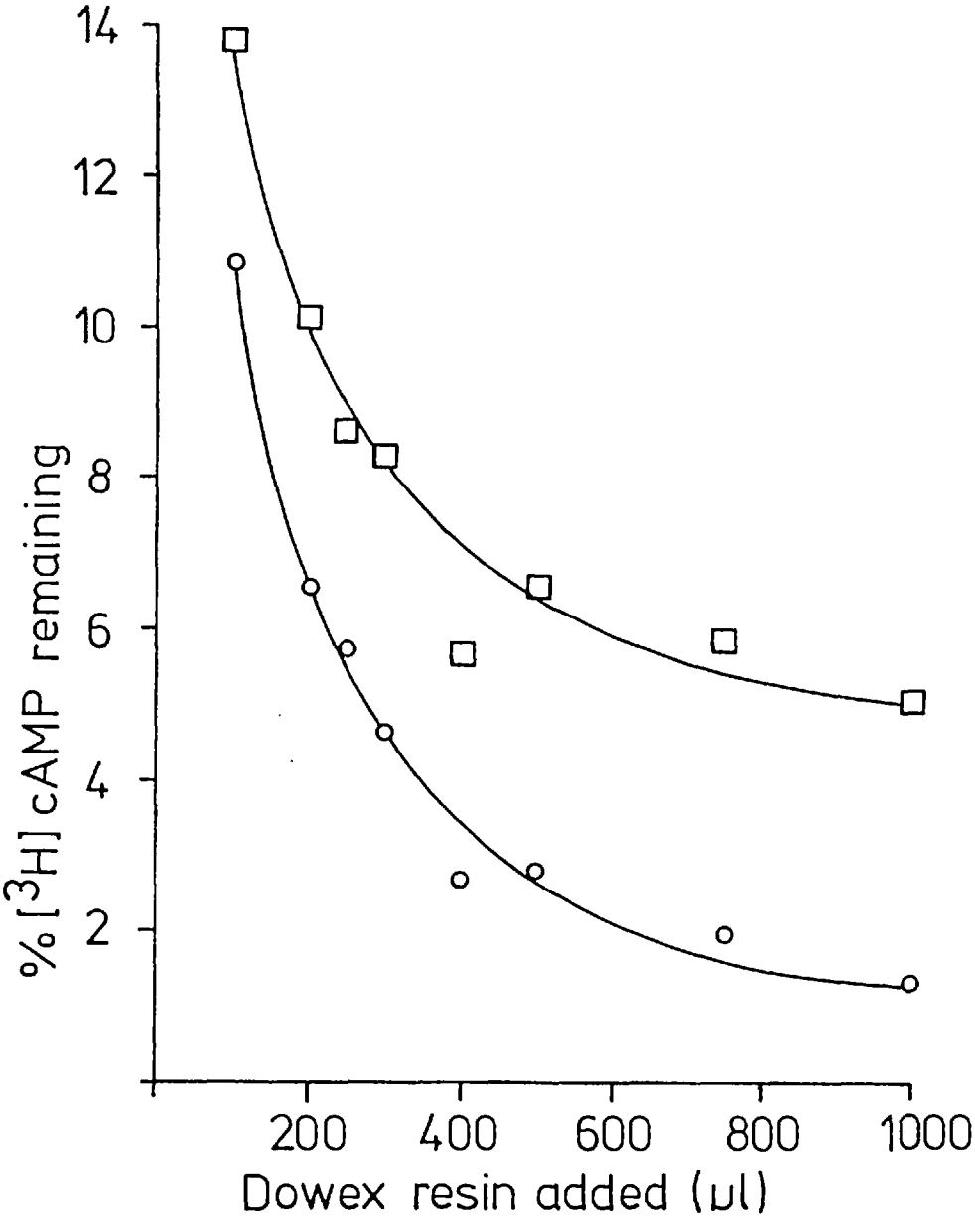


Figure 6•1 Amount of (^{3}H) cyclic AMP remaining in the supernatant. o Without correction factor applied

□ correction factor applied

This demonstrates that over 6% of the substrate remains in the supernatant after centrifugation. This will contribute to the radioactivity attributed to the unquenched (^{3}H) adenosine. Removal of (^{3}H) adenosine by the resin is known to occur (Boudreau and Drummond 1975) but has not been studied or allowed for here.

6·23 Isolation of the external cyclic AMP phosphodiesterase.

It was found that the cyclic AMP PDE secreted into the growth medium could be partially removed by precipitation with ammonium sulphate.

A three day old culture of microplasmodia was centrifuged for 5min at 50g at room temperature and the supernatant retained. To the stirred supernatant , at 4 - 6°C, was added enough solid ammonium sulphate to make the solution 10% saturated. After 30min the solution was centrifuged for 15min at 10000g at 4°C, the supernatant was removed and the pellet resuspended in 5vol of cyclic AMP PDE assay buffer. Ammonium sulphate was added to the remaining supernatant to make it 20% saturated. The above procedure was repeated until 10 ammonium sulphate fractions had been obtained. Each fraction was dialysed against 500vol of assay buffer overnight at 4°C before assay for cyclic AMP PDE using the resin method. This procedure is shown in Table 6·1.

The amount of ammonium sulphate added was calculated from tables. The results are shown in Figure 6·2

Figure 6·2. Ammonium sulphate precipitation of cyclic AMP phosphodiesterase from the growth medium of Physarum.

Abscissa : % ammonium sulphate added, RS = remaining supernatant after 100% ammonium sulphate precipitation step, GM = unpurified growth medium.

Ordinate : Enzyme activity, units x 100, 1 unit = 1 pmole formed per 10min per mg. protein.

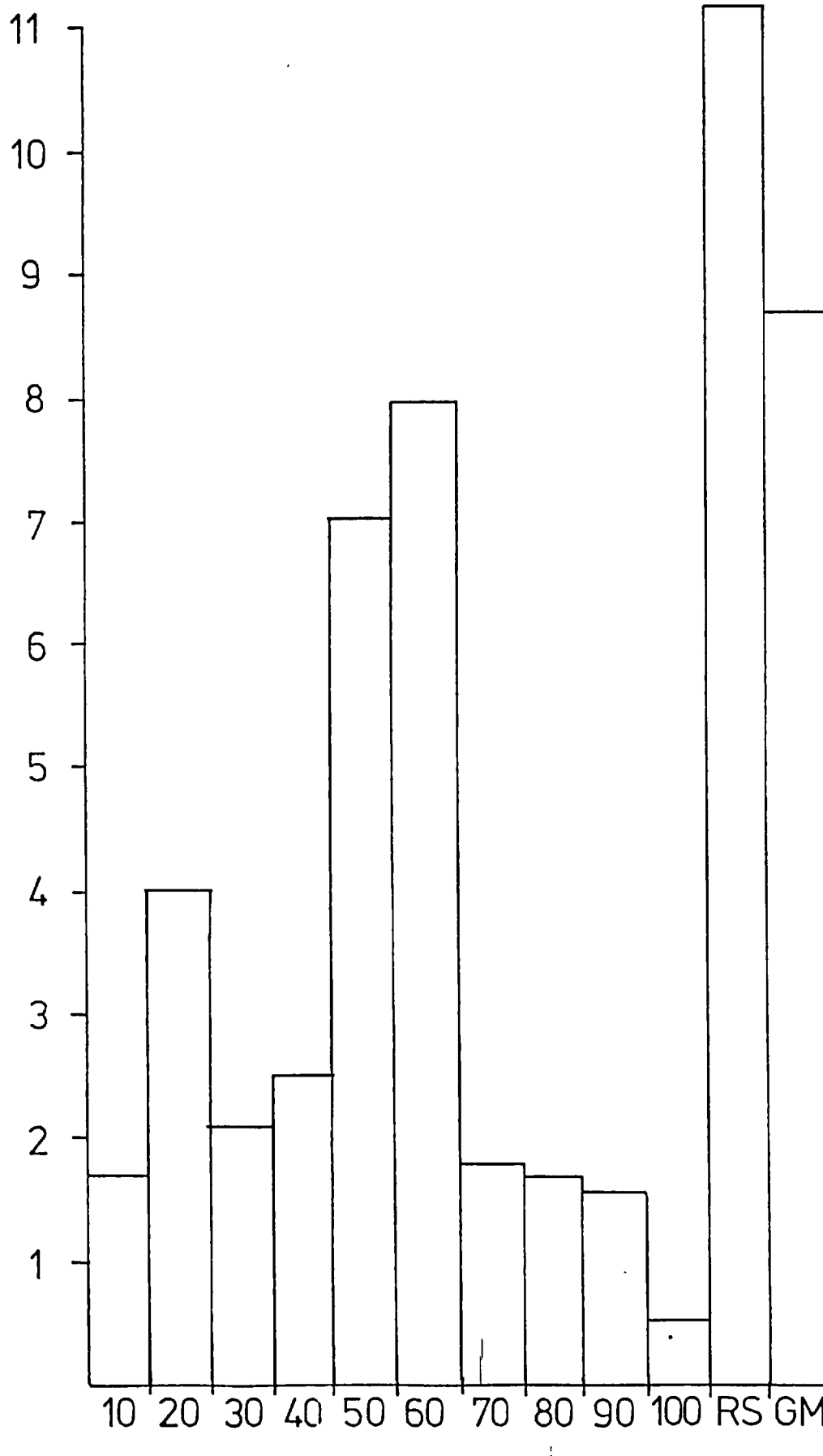
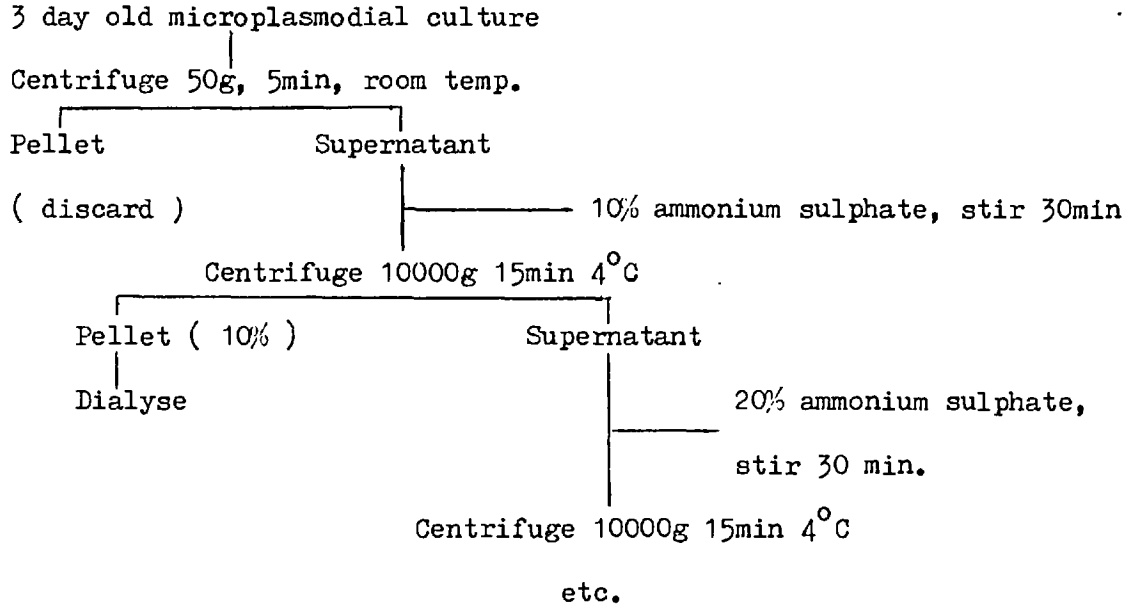

11
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100
RS
GM

Table 6·1 Ammonium sulphate precipitation of growth medium cyclic AMP phosphodiesterase.



Cyclic AMP PDE activity is located in the 50 - 60% ammonium sulphate fraction, a large amount (37%) remains in the supernatant.

The activity in the unpurified growth medium is less than the total activity in the ammonium sulphate fractions. This may be because ammonium sulphate precipitates the enzyme but not the enzyme inhibitors which are left in the supernatant. Another possibility is that interfering substances may be present in the growth medium that affect the protein assay.

6·24 Physarum cyclic AMP phosphodiesterase.

1) A number of likely inhibitors were tested at two concentrations for their effect on both the external (50 - 60% ammonium sulphate precipitate) and the ~~e~~internal enzyme. The internal enzyme was located in the particulate fraction of an homogenate of Physarum and isolated as follows.

A 3 day old culture of microplasmodia was centrifuged for 5min

at 50g at room temperature. The pellet was suspended in 5vol of cyclic AMP PDE extraction buffer (50mM tris - HCl pH 8·0) and homogenised for 30sec with an ILA X1020 mixer. After centrifugation for 10min at 4°C, the pellet was resuspended in 1vol assay buffer. The results are shown in Table 6·2.

Table 6·2 Effect of certain inhibitors on cyclic AMP phosphodiesterase activity.

Compound	Concentration (mM)	% control Internal	% control External
Theophylline	1	54·6	N.D.*
	2·5	56·5	N.D.
EDTA	1	66·5	94·6
	2·5	68·9	71·4
Papaverine	1	76·5	95·2
	2·5	57·6	93·3
Imidazole	1	82·7	86·0
	2·5	70·0	102·0
Caffeine	1	47·4	92·1
	2·5	54·5	79·5
ATP	1	42·2	75·9
	2·5	66·8	62·6
SQ20009	1	76·8	66·6
	2·5	52·7	50·1
IBMX	1	34·5	44·3
	2·5	8·0	30·1

* N.D. = not determined. Internal enzyme control = 274 units, External enzyme control = 3502 units (1 unit = 1 pmole adenosine formed/10min/mg protein.

2) Effect of Ca^{++} and Mn^{++} on cyclic AMP phosphodiesterase activity.

The effects of Ca^{++} and Mn^{++}, added to the assay buffer, on the internal cyclic AMP PDE activity are shown in Figure 6·3. Control activity was determined in the absence of added metal ions in the assay buffer.

Calcium alone inhibited activity at low concentrations (10mM). Increased concentrations of Ca^{++} stimulated activity. Manganese at 10mM enhanced activity nearly five fold but this decreased with increasing Mn^{++} concentration. When both ions were present at 40mM a drastic reduction in activity was noted.

6·25 Assay conditions for thin layer chromatographic analysis of cyclic AMP phosphodiesterase.

1) Termination of the reaction.

The following reaction mixture was set up in duplicate:

20µl (^3H) cyclic AMP (= 8000 c.p.m.) in assay buffer

20µl assay buffer (1mM $MgCl_2$, 10mM tris - HCl pH 7·5)

20µl Physarum homogenate (100000g supernatant)

The reaction mixture was incubated at 30°C for 0, 2·5, 5 and 10min and terminated by the addition of 20µl of 30% TCA containing cyclic AMP, 5'AMP and adenosine (approximately 5mM) as chromatographic markers. To investigate whether changes took place after the addition of TCA some tubes were incubated for 10min, the reaction stopped and incubation continued for a further 5, 10, 15 or 20min. Fifteen microlitres was removed from each tube and analysed by t.l.c. The results are shown in Figure 6·4. The addition of TCA did not induce any changes in the composition of the reaction mixture.

Figure 6·3 Effect of metal ions on cyclic AMP PDE activity.

Ordinate = % Control activity. 100% = Control activity determined in the absence of added ions.

Abscissa = Concentration of ion in assay buffer.

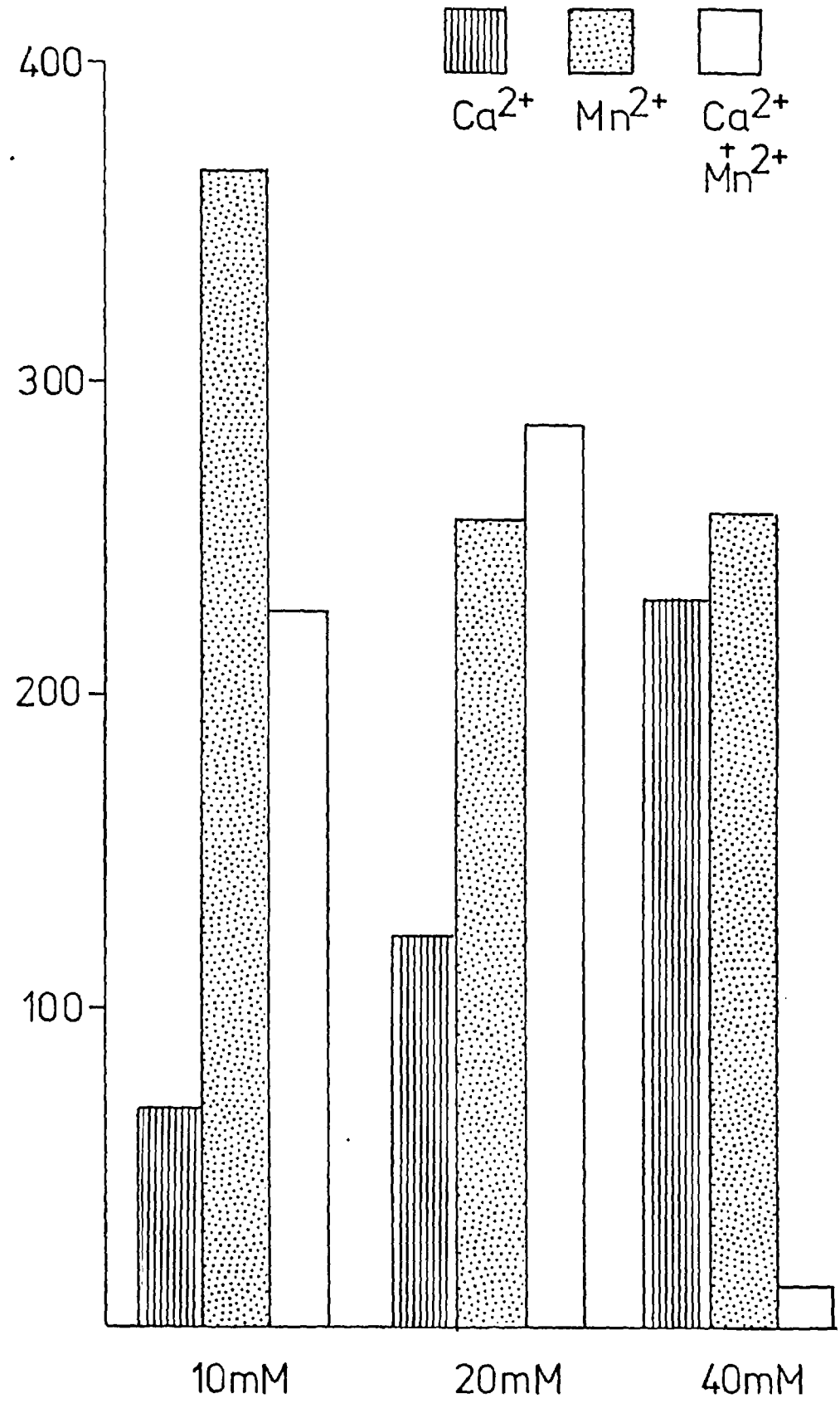


Figure 6•3

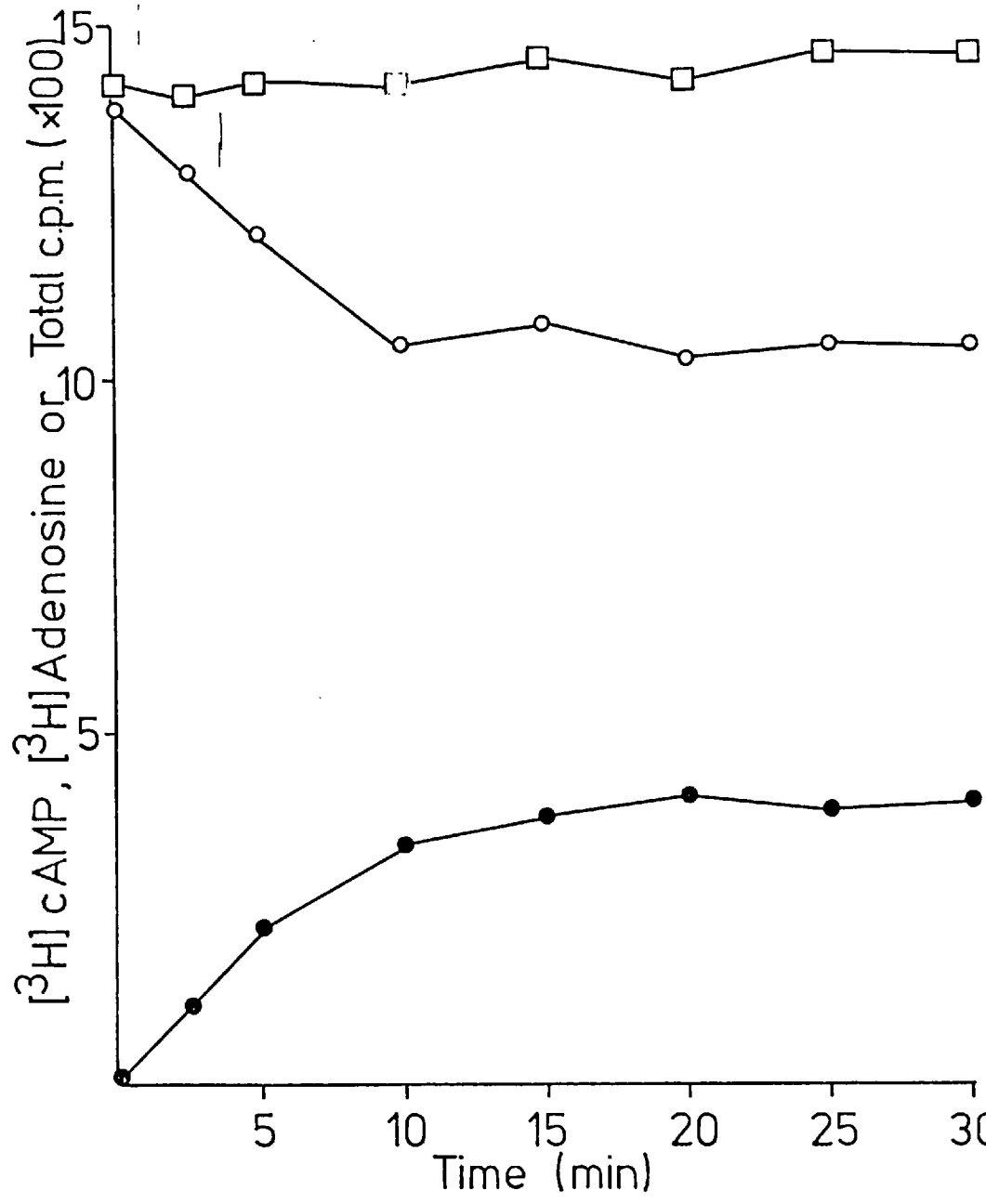


Figure 6·4 Termination of cyclic AMP PDE reaction.
by the addition of 15µl 30% TCA. Added at 10min, continued incubation for the times indicated, also for 2·5 and 5 min. Blank (98 c.p.m.) subtracted. ● (^{3}H) adenosine, □ Total counts, o (^{3}H) cyclic AMP. No (^{3}H) 5'AMP was detected

In subsequent work the reaction mixture composition was changed to:

15µl 0·8mM cyclic AMP containing 60nCi (^3H) cyclic AMP (= 44400 c.p.m.)

15µl assay buffer (5mM $MgCl_2$, 50mM tris - HCl pH 7·5)

15µl Physarum homogenate

The reaction was terminated by the addition of 15µl 30% (w/v) TCA and 15µl removed for analysis by t.l.c.

2) Linearity of the reaction with time.

Linearity of adenosine formation with incubation time up to 12min is demonstrated in Figure 6·5. The reaction mixture contained 11µg protein from the 10000g pellet.

3) Protein content.

Figure 6·5 shows the relationship between protein content and cyclic AMP PDE activity (using 10000g pellet), this is linear up to 45µg protein per assay tube. Typically between 10 - 20µg protein was included in each assay tube.

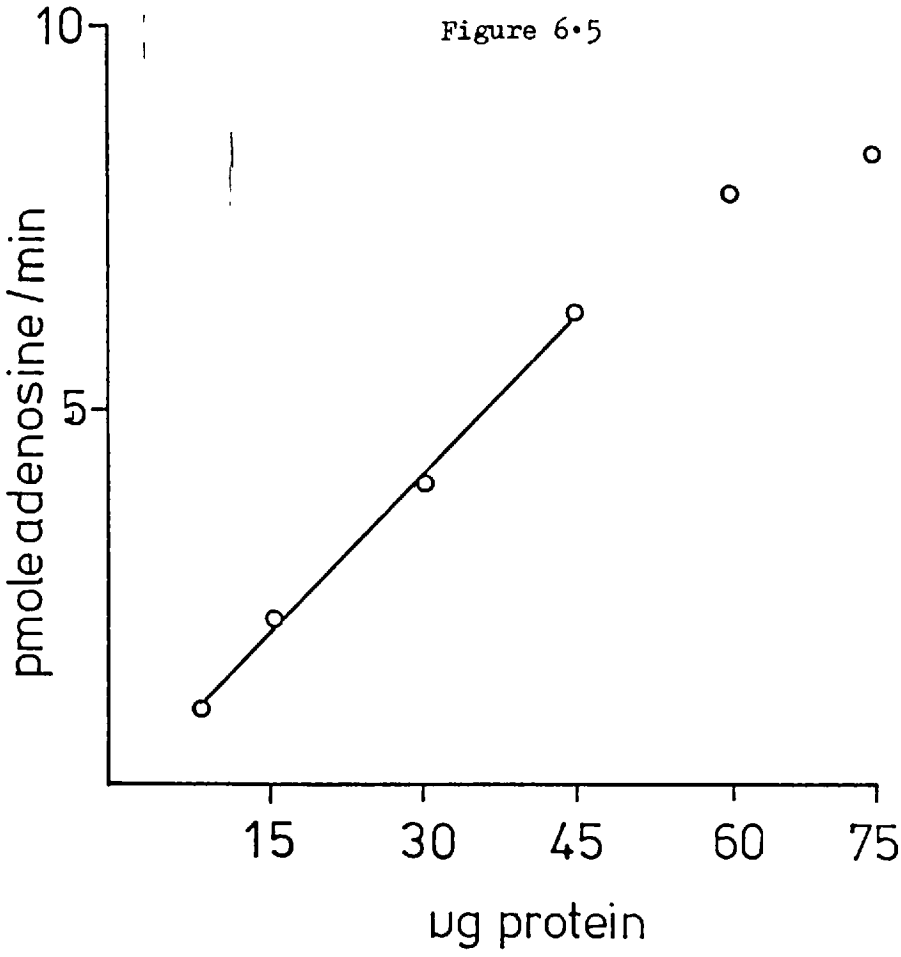
6·26 Extraction of internal and growth medium cyclic AMP phosphodiesterase.

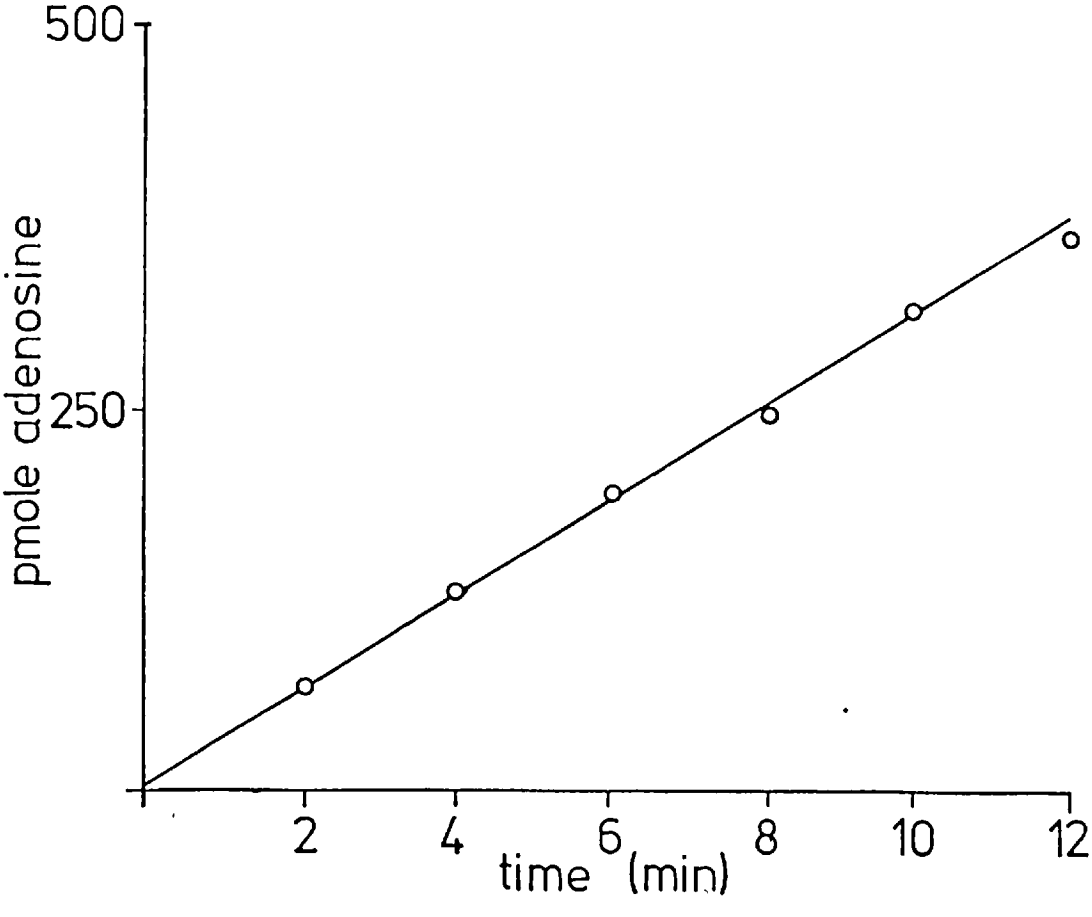
A 3 day old culture of microplasmodia was collected by centrifugation for 5min at 50g at room temperature and the supernatant removed. A portion of the supernatant was stored in liquid nitrogen for assay later. The remaining supernatant was filtered through a 0·45µm Millipore filter. The pelleted material was suspended in 4vol adenylate cyclase extraction buffer.(0·25M sucrose, 5mM $CaCl_2$, 1mM dithiothreitol, 50mM tris - HCL pH 7·5) and homogenised at 0°C. After centrifugation for 5min at 2500g the supernatant was removed and used as the source of the external enzyme. This method was only

Figure 6·5 Upper panel : Effect of protein concentration on cyclic AMP PDE reaction. Usually 10 - 20μg protein was included in the reaction tube.

Lower panel : Production of adenosine with respect to time. Reaction tubes were incubated for 10min.

Figure 6·5


10
5
pmole adenosine / min
15
30
45
60
75
µg protein


500
250
pmole adenosine
2
4
6
8
10
12
time (min)

used once and so is described here.

Cyclic AMP PDE activity in filtered growth medium, unfiltered growth medium and the supernatant of homogenised plasmodia was assayed as previously described. The results are shown in Table 6·3

Table 6·3 Growth medium and plasmodial cyclic AMP phosphodiesterase.

Enzyme source	(^{3}H) adenosine	(^{3}H) cAMP	(^{3}H) 5'AMP	Total
	c.p.m.	c.p.m.	c.p.m.	c.p.m.
Filtered G.M.	41	1588	564	2170
	32	1420	618	2105
Unfiltered G.M.	67	1467	517	2075
	45	1334	593	1928
Internal enzyme	455	1459	47	1926
	420	1430	62	1947
Zero time	37	1991	72	1937
	49	2002	63	2107

G.M. = growth medium. Reaction was initiated by the addition of 12μg protein and incubated for 10min at 30°C.

These results demonstrate that Physarum secretes a cyclic AMP PDE into the growth medium but not a 5' nucleotidase although the latter is present in the homogenate and apparently capable of converting all 5'AMP to adenosine. This was particularly useful as it obviated the need to add 5' nucleotidase to the assay buffer.

6·27 Summary.

Two methods were used to measure cyclic AMP PDE activity. The method intially used employed the batch use of an ion exchange resin to quench (^{3}H) cyclic AMP and (^{3}H) 5'AMP and has been found to be unsatisfactory by a number of workers. This method was used to detect gross changes in enzyme activity for comparative purposes only.

A second method, developed by the author, using t.l.c. on cellulose to separate the reaction products worked well. Cyclic AMP could be separated from 5'AMP and adenosine (unlike the resin method). It was found that no 5'AMP could be detected with cyclic AMP PDE from homogenised plasmodia because it was wholly converted to adenosine by a potent 5' nucleotidase. The growth medium is a potent source of the cyclic AMP PDE and here the reaction product was 5'AMP because Physarum does not secrete a 5' nucleotidase into the growth medium.

Using this t.l.c. method, the activity of cyclic AMP PDE in three fractions of Physarum homogenates separated by differential centrifugation was measured throughout the mitotic cycle.

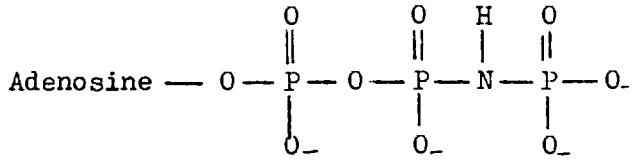
ADENYLATE CYCLASE

6·30 Problems relating to the assay of adenylate cyclase.

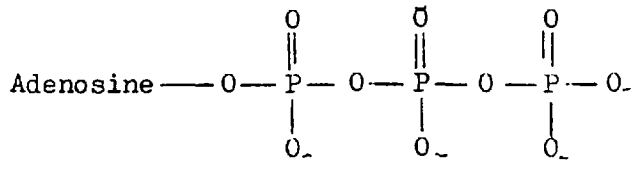
Cyclic AMP is formed from adenosine triphosphate (ATP) by the enzyme adenylate cyclase (Sutherland et al. 1962).

A number of problems, outlined below, are associated with the assay of this enzyme.

1) The enzyme is usually difficult to isolate as it is membrane bound. This means crude homogenates are used as a source of activity. This results in the substrate being degraded by contaminating enzymes e.g. ATPase, the activities of which may be greater than that of adenylate cyclase. The imidophosphate analogue of ATP - β-γ imido adenosine 5' triphosphate (AMP PNP) - can act as substrate for adenylate cyclase (Yount et al. 1971). AMP PNP is not degraded by ATPase; indeed it can act as a competitive inhibitor of mitochondrial ATPase (Penefsky 1974). The K_m of adenylate cyclase using ATP and AMP PNP as substrates have been reported to be similar (Rodbell et al. 1971) although the V_{max} values may differ, being lower when AMP PNP is used (Maguire and Gilman 1974). The structure of AMP PNP and ATP is shown below.



AMP PNP



ATP

2) The product, cyclic AMP, may be degraded by cyclic AMP phosphodiesterase (cyclic AMP PDE). This may be prevented by the use of a suitable PDE inhibitor which, clearly, must not affect adenylate cyclase.

The following sections describe the development of a new assay method for adenylate cyclase using (^{3}H) AMP PNP as substrate.

6·31 Separation of (^{3}H) AMP PNP and cyclic AMP.

Cyclic AMP cannot be determined in the presence of AMP PNP by a protein binding assay method because AMP PNP also binds to the protein, so some way of separating the two compounds was sought. Three methods were examined for their suitability in separating (^{3}H) AMP PNP from cyclic AMP.

1) Cellulose acetate electrophoresis.

Cellulose acetate electrophoresis was carried out exactly as described by DeLaage et al. (1974).

Five microlitres of a mixture of (^{3}H) AMP PNP (= 12000 c.p.m.) and cyclic AMP (5mM) was applied as a spot onto a blotted cellulose acetate (Oxoid) strip (12 x 2cm) near the cathode (-), that had been soaked overnight in 10mM sodium pyrophosphate, 7% glycerol containing 20mg/l proflavine (adjusted to pH 8·6 with 4N HCl). Electrophoresis was carried out in a water cooled, flat bed, Camag apparatus using the above buffer for 35min at 25 V/cm. The position of cyclic AMP was detected under UV (254nm) light. The cellulose acetate was dried and cut, parallel to the origin, into 5mm wide strips. Each piece was solubilised in 1ml dimethylsulphoxide (DMSO) and after 60min 10ml TE scintillant was added.and the radioactivity determined. The results are shown in Figure 6·6.

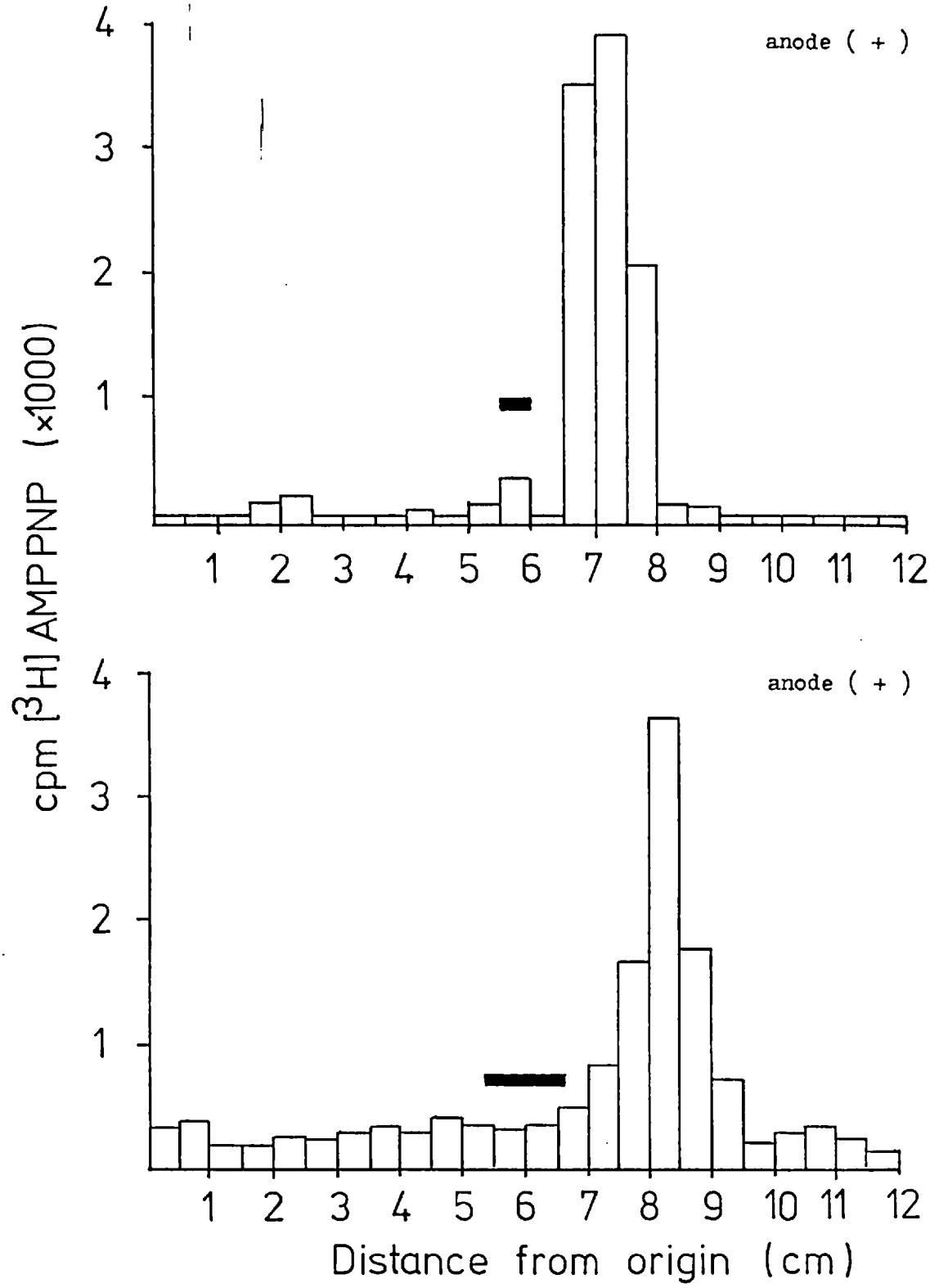


Figure 6·6 Cellulose acetate electrophoresis (35min at 25 V/cm) of (^{3}H) AMP PNP and cyclic AMP (black bar) - upper panel. Lower panel - paper electrophoresis of the same mixture (6min at 40 V/cm)

2) Paper electrophoresis.

Strips of Whatman 3MM paper (20 x 4cm) were soaked overnight in 25mM ammonium carbonate pH 8·0 and then blotted to remove excess liquid. The sample (see cellulose acetate section) was applied as before. Electrophoresis was carried out in a Shandon electrophoresis tank using the above buffer, for 6min at 40V/cm. After air drying the strip was again cut up as before and each piece eluted with 1ml distilled water in a scintillation vial for 60min, after the addition of 10ml TTX-100 scintillant the radioactivity was determined. The results are shown in Figure 6·6 (lower panel).

Neither method 1 nor 2 gave the degree of separation required and the isolated substances were diffuse.

3) Thin layer chromatography.

Thin layer chromatography (t.l.c.) of (^{3}H) AMP PNP and cyclic AMP on polyethyleneimine (PEI) cellulose in either 0·5N LiCl in 2M formic acid (system 2) or 1M tris - HCl pH 7·6 (system 3) revealed the presence of a radioactive impurity, probably 5' adenylyl phosphoramidate, that had the same mobility as cyclic AMP. If the (^{3}H) AMP PNP was eluted with water and re-run under identical conditions in either system the impurity was still detected. The impurity represented about 8% of the total counts applied and was probably due to breakdown of (^{3}H) AMP PNP on the plate. The profile of (^{3}H) AMP PNP chromatographed on PEI cellulose in system 3 can be seen in Figure 6·7. Chromatography in system 2 gave a similar profile (Rf (^{3}H) AMP PNP = 0·42, cyclic AMP = o·75).

Additionally, chromatography on PEI cellulose yielded very variable results. With one batch of plates chromatographed in system 3 the Rf for cyclic AMP was between 0·23 and 0·60

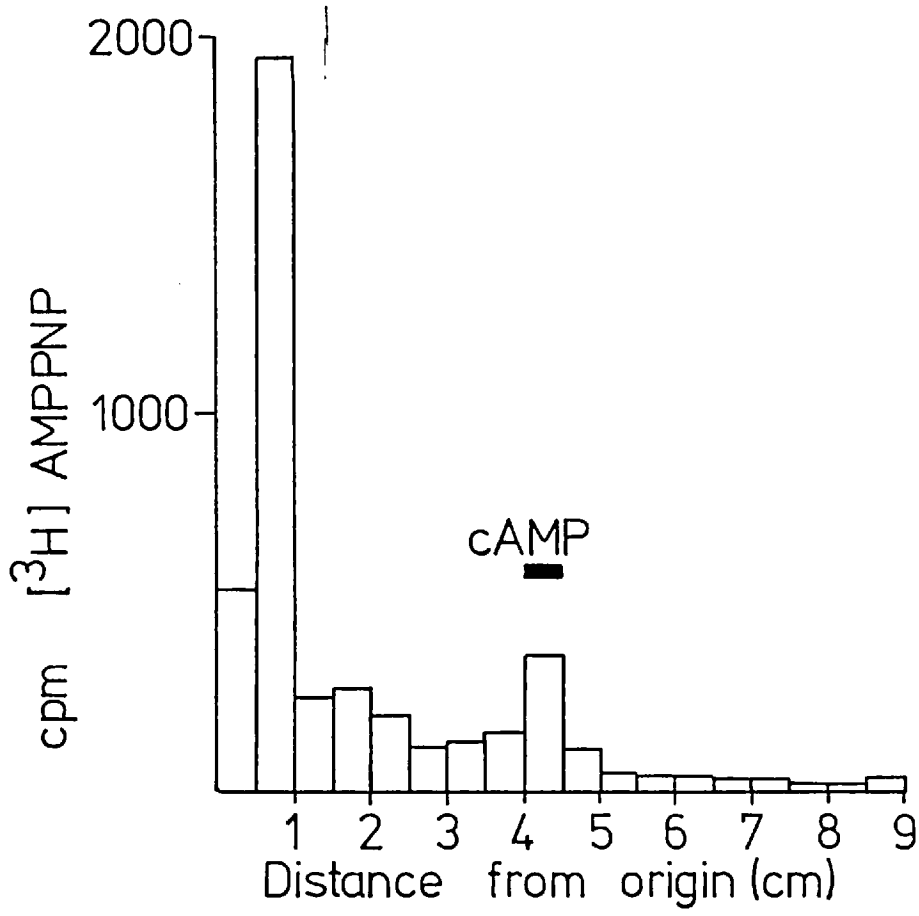


Figure 6•7 Thin layer chromatography of (^{3}H) AMP PNP and cyclic AMP on PEI cellulose in 1M tris - HCl pH 7•6 (system 3).

(mean = 0·49) and for (^{3}H) AMP PNP, 0·04 to 0·15 (mean = 0·13), from five determinations,

For these reasons chromatography of cyclic AMP and AMP PNP on PEI cellulose was considered unsatisfactory as a means of separating the two compounds.

6·32 Purity of (^{3}H) AMP PNP.

Although (^{3}H) AMP PNP has been shown to be subject to breakdown on the t.l.c. plate under certain conditions the possibility remains that there may be other impurities not being revealed by t.l.c. Tritiated AMP PNP and AMP PNP were chromatographed on a column of DEAE cellulose and (^{3}H) AMP PNP on Dowex AG50 100 - 200 mesh H^+ form.

1) DEAE cellulose.

A 0·5 x 5·0cm column of DEAE cellulose was eluted with a 0 - 0·5M linear gradient of ammonium formate, 1ml fractions were collected and the radioactivity and absorbance at 260nm of each fraction determined. Both AMP PNP and (^{3}H) AMP PNP showed a similar elution pattern, both were eluted at approximately 0·3M ammonium formate. This result agrees with that reported by Rodbell et al. (1971). Recovery of (^{3}H) AMP PNP from DEAE cellulose was quantitative (= 104%). The elution is shown in Figure 6·8.

2) Dowex.

The elution of (^{3}H) AMP PNP from this resin by water is shown in Figure 6·9. One major but very broad peak was revealed, recovery was poor at 35%.

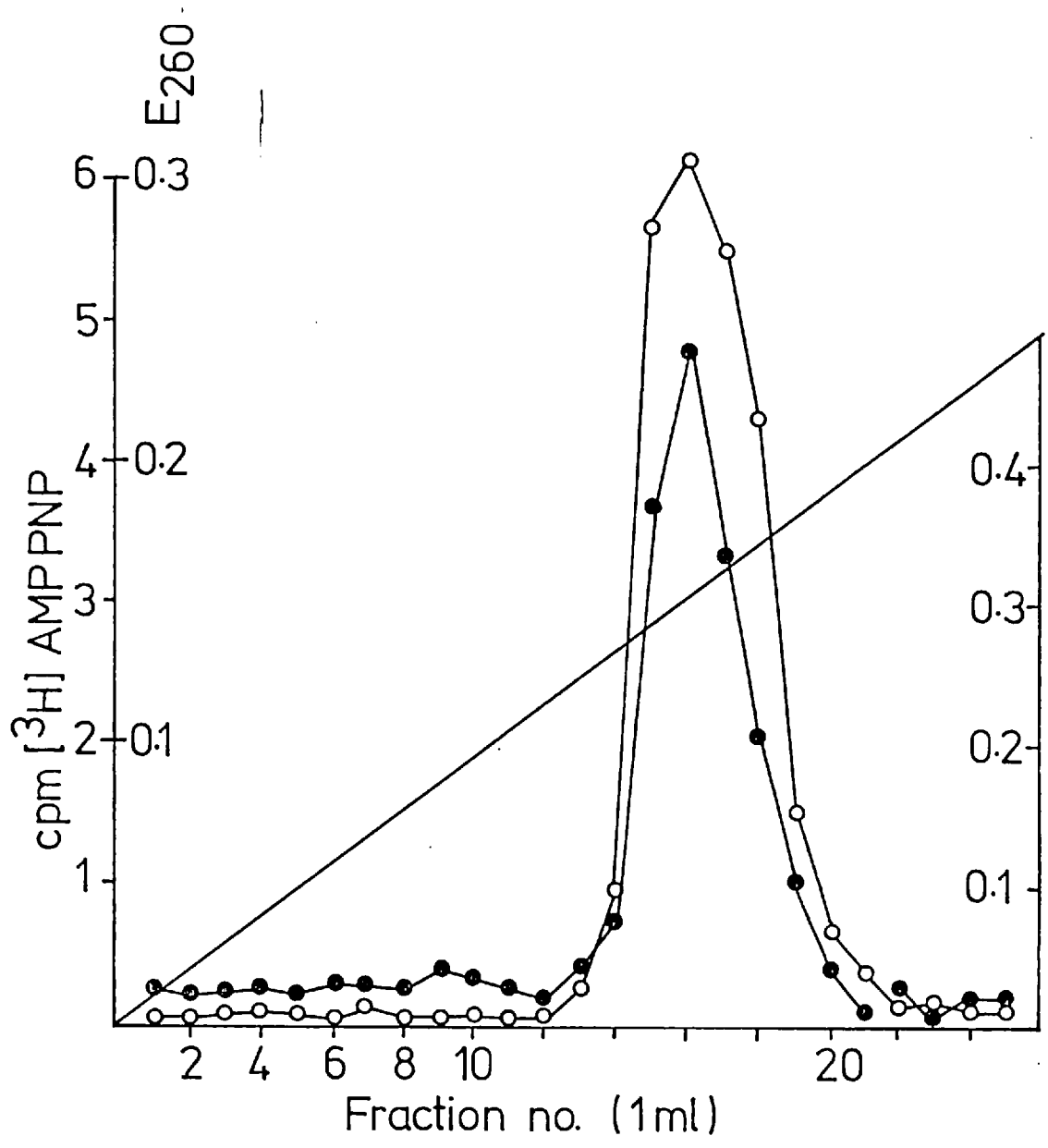


Figure 6·8 Elution of (^{3}H) AMP PNP and AMP PNP from DEAE cellulose with a linear gradient of 0 - 0·5 M ammonium formate

Open circles = (^{3}H) AMP PNP (c.p.m.)

Closed circles = Absorbance at 260nm (E_{260}) of AMP PNP.

About 104% of the applied counts were recovered.

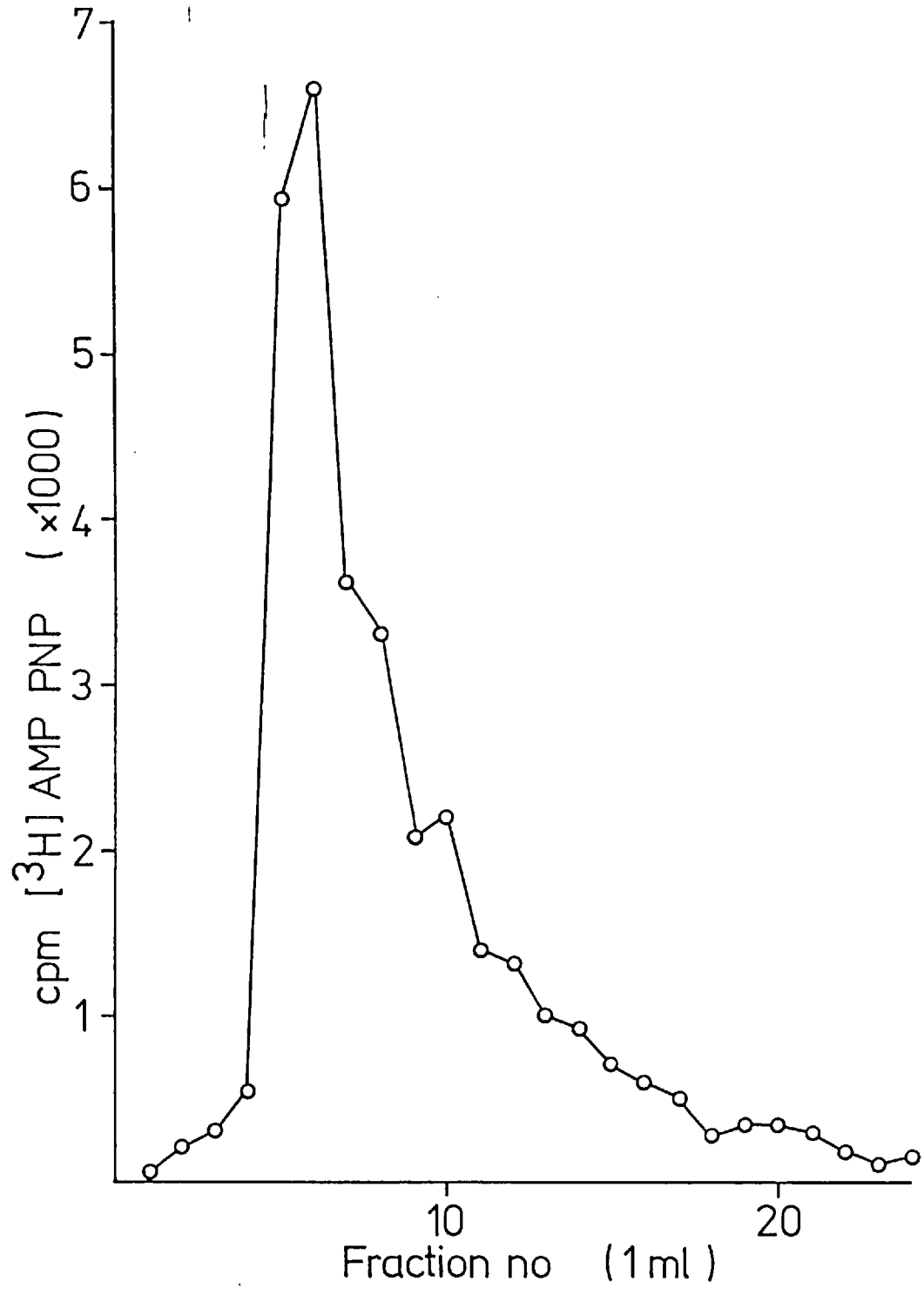


Figure 6·9 Elution of (^{3}H) AMP PNP from Dowex AG 50 100 - 200 mesh H^{+} form by water. Recovery was poor at 35%.

6•33 Summary.

These tests indicate that (^{3}H) AMP PNP and AMP PNP are pure as judged by elution from DEAE cellulose. However the peak is broad but further work in a number of t.l.c. systems never revealed any impurities (except in those systems already described, where breakdown is taking place in the solvent system used).

Tritiated AMP PNP is subject to degradation when chromatographed on PEI cellulose in either system 2 or 3. Moreover, because the impurity had the same mobility as cyclic AMP in both systems these could not be used to separate cyclic AMP in adenylate cyclase assays.

Low voltage electrophoresis on either cellulose acetate or paper was also impractical for the following reasons:

1) Separation of cyclic AMP from AMP PNP was poor.

2) The background values with paper electrophoresis were very high (about 300 c.p.m. = 10% total counts applied.).

3) Only a small amount (5μl) of the reaction mixture could be applied and used for the separation of AMP PNP and cyclic AMP by these methods.

The system finally selected for the separation of cyclic AMP, AMP PNP, 5'AMP and adenosine was the one described in Chapter 4, namely t.l.c. on cellulose developed in butanol : acetic acid : water (2 : 1 : 1 by vol.).

6·34 The extraction and assay of adenylate cyclase from Physarum.

1) Extraction of adenylate cyclase.

The same extraction procedure described for cyclic AMP PDE was used for adenylate cyclase (Chapter 3·14).

2) Assay of adenylate cyclase.

To 15µl of 0·2mM AMP PNP (containing approximately 30nCi (^{3}H) AMP PNP = 22220 c.p.m.) and 15µl assay buffer (5mM $MgCl_2$, 1mM isobutylmethylxanthine, 50mM tris - HCl pH 8·0) was added 15µl Physarum homogenate. All assays were run in duplicate. After incubation at 30°C for 10min the reaction was terminated by the addition of 15µl 4N HCl containing, as chromatographic markers, cyclic AMP, 5'AMP, AMP PNP and adenosine (approximately 5mM). Fifteen microlitres of the reaction mixture was chromatographed on cellulose as described in Chapter 4, 15µl was also spotted onto a 15mm square of cellulose for the determination of total counts. Often one channel was loaded with (^{3}H) AMP PNP and another with (^{3}H) cyclic AMP, after development these were cut into 5mm strips and individually eluted and counted. This was to determine the precise boundary of the radioactive cyclic AMP and AMP PNP spots and to check that spots of labelled and unlabelled compounds coincided; this was always found to be so.

After detection under UV light regions containing cyclic AMP and AMP PNP were eluted for at least 20min with 0·8ml distilled water in plastic scintillation vials. Total count squares were treated in the same way. Eight millilitres of TTX-100 scintillant were added to each vial which was thoroughly shaken and the radioactivity determined as described in Chapter 3·60.

6·35 Adenylate cyclase assay conditions.

1) Identity of reaction product.

Incubation of 15µl (^{3}H) AMP PNP (30nCi = 22220 c.p.m.) and 15µl assay buffer with 15µl Physarum homogenate (100000g supernatant) at 30°C for 0, 2, 4, 6, 8 and 10min in duplicate followed by termination with 15µl 4N HCl (containing no markers) resulted in the formation of a labelled compound thought to be (^{3}H) cyclic AMP.

Each t.l.c. plate had two channels run with unlabelled cyclic AMP (approximately 5mM) in order to locate the position of the (^{3}H) cyclic AMP, the areas in the same position as those containing cyclic AMP were cut out and eluted with 1ml distilled water. Two 250µl aliquots were removed and treated as follows. To one series, an equal volume of beef heart PDE (20µg/ml) in adenylate cyclase assay buffer without IBMX was added and incubated for 60min at 30°C, followed by boiling for 2min to inactivate the PDE. The other 250µl sample acted as a control and was boiled for 2min. Fifty microlitres from each series of samples were added to tubes containing 50µl cyclic AMP binding protein (Radiochemical Centre) and 50µl assay buffer. After incubation for 2 hours at 0°C, 100µl of charcoal - BSA mixture was added and the tubes shaken and then centrifuged for 5min at 2500g at room temperature. A 100µl aliquot of the supernatant was removed from each tube and added to 8ml TTX-100 scintillant containing 0·8ml distilled water and the radioactivity determined. The results are shown in Figure 6·10.

The radioactive substance is in fact (^{3}H) cyclic AMP. If so then it will be specifically degraded by PDE and so the protein binding assay should reveal little, if any, cyclic AMP present.

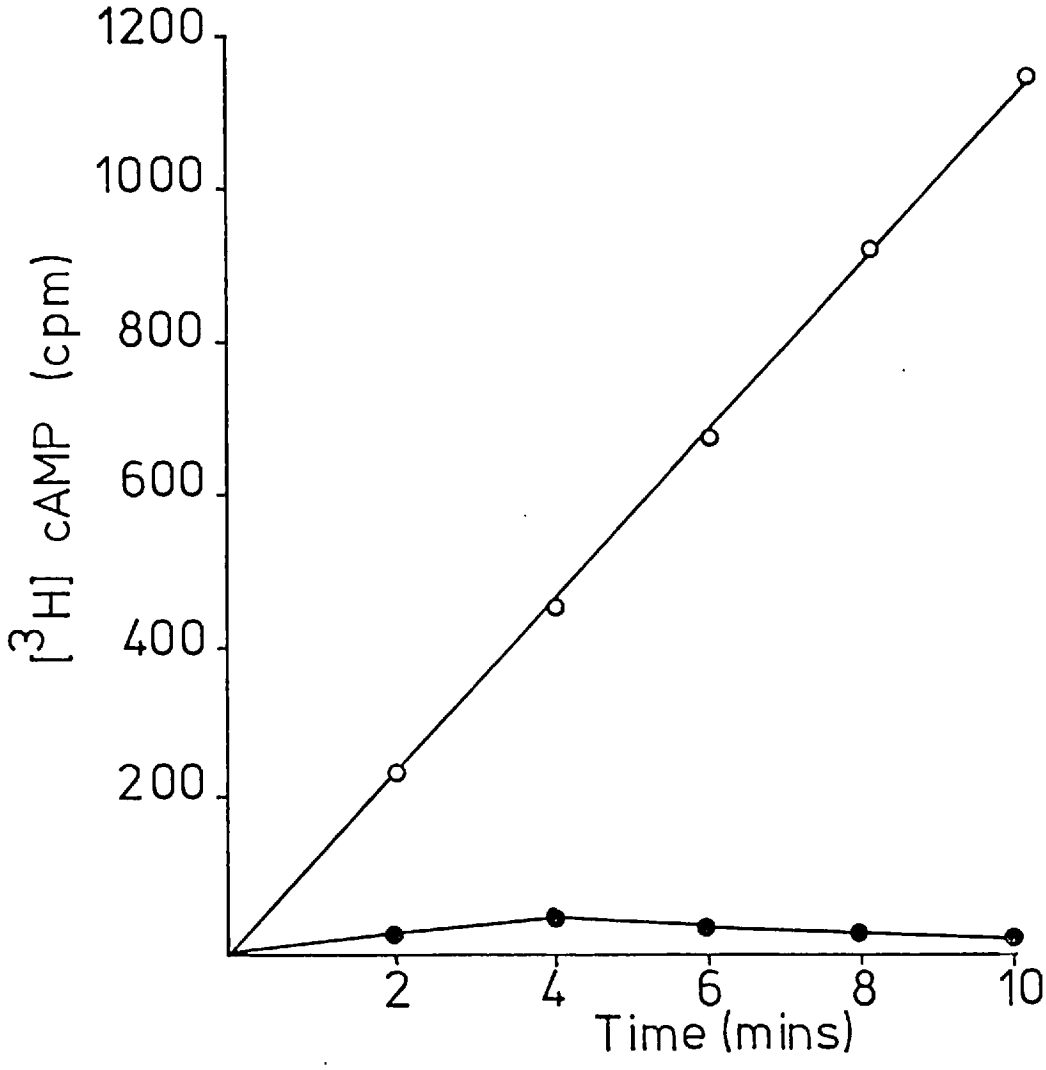


Figure 6·10 Identity of adenylate cyclase reaction product.

Open circles = (^{3}H) cyclic AMP bound to cyclic AMP binding protein.

Closed circles = Binding of (^{3}H) cyclic AMP after treatment with beef heart cyclic nucleotide phosphodiesterase.

Unlabelled cyclic AMP was excluded because it would have competed with (^{3}H) cyclic AMP and reduced sensitivity.

If AMP PNP has no affinity for the cyclic AMP binding protein then it would be possible to measure cyclic AMP in a mixture of substrate and product and there would be no need to separate them. To investigate this, binding protein was added to tubes containing 50µl (^{3}H) AMP PNP (= 30nCi) and treated as described before. It was found that (^{3}H) AMP PNP was bound to the binding protein. These results are shown in Table 6·4.

Table 6·4 Binding of (^{3}H) AMP PNP to cyclic AMP binding protein.

Counts added (c.p.m.) (100µl from 250µl)		Counts in supernatant (c.p.m.) (100µl)
27052		2367
26130		2531
26591	Average value	2449

These results show that 10·86% of the total counts were bound to the protein so it is clearly necessary to separate cyclic AMP from the substrate if a protein binding assay for cyclic AMP is employed.

2) Termination of the reaction.

Termination of the reaction by adding 15µl 30% (w/v) TCA or by placing the assay tubes in boiling water for two minutes produced unacceptably high blanks for the cyclic AMP region, amounting to 4 - 5% of the total counts applied. It was demonstrated that boiling resulted in the non enzymic formation of cyclic AMP. Termination of the reaction by adding 15µl 4N HCl or 20% (w/v) sodium dodecyl sulphate (SDS) or 100mM EDTA gave low blanks, representing about 0·5% of the counts applied. The results are

shown in Table 6•5.

Table 6•5 Termination of the adenylate cyclase reaction.

Stopping agent	Blank value (c.p.m.)	% Total counts.
30% TCA	732	4•95
100mM EDTA	92	0•62
20% SDS	67	0•45
4N HCl	69	0•46
Boiling	721	4•87
Without enzyme	29	0•19
Without (^{3}H) AMP PNP	18	0•12
Total counts applied	14783	-

A background value (18 c.p.m.) has been subtracted from these values.

3) Effect of protein concentration.

The effect of protein concentration on the reaction is shown in Figure 6•11. Typically reactions were initiated by the addition of between 10 and 30μg of protein.

4) pH optima

Figure 6•12 shows the effect of assay buffer pH on adenylate cyclase activity. Each fraction, 10000g pellet, 100000g pellet, and 100000g supernatant, has a pH optimum at pH 8•00. This differs from that of pH 7•2 used by Atmar et al. (1976) who used ATP as substrate. This may result from the higher pK_a value of the β-γ phosphate in AMP PNP (7•7) compared with ATP (7•1) (Yount et al. 1971). The plasmodium used in this particular study was 31 hours old when harvested, this may account for the slightly low activity in the 10000g pellet.

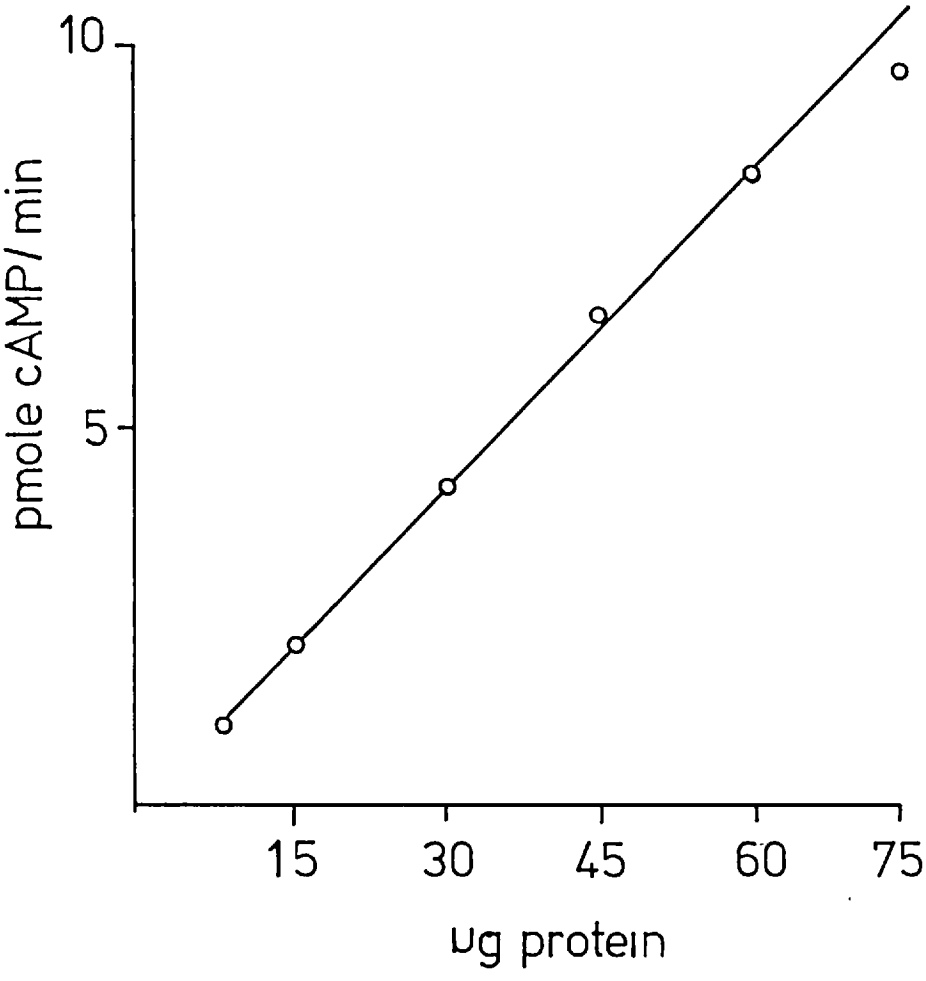


Figure 6·11 Effect of protein concentration on adenylate cyclase reaction. Typically reactions were initiated by the addition of between 10 and 30µg of protein.

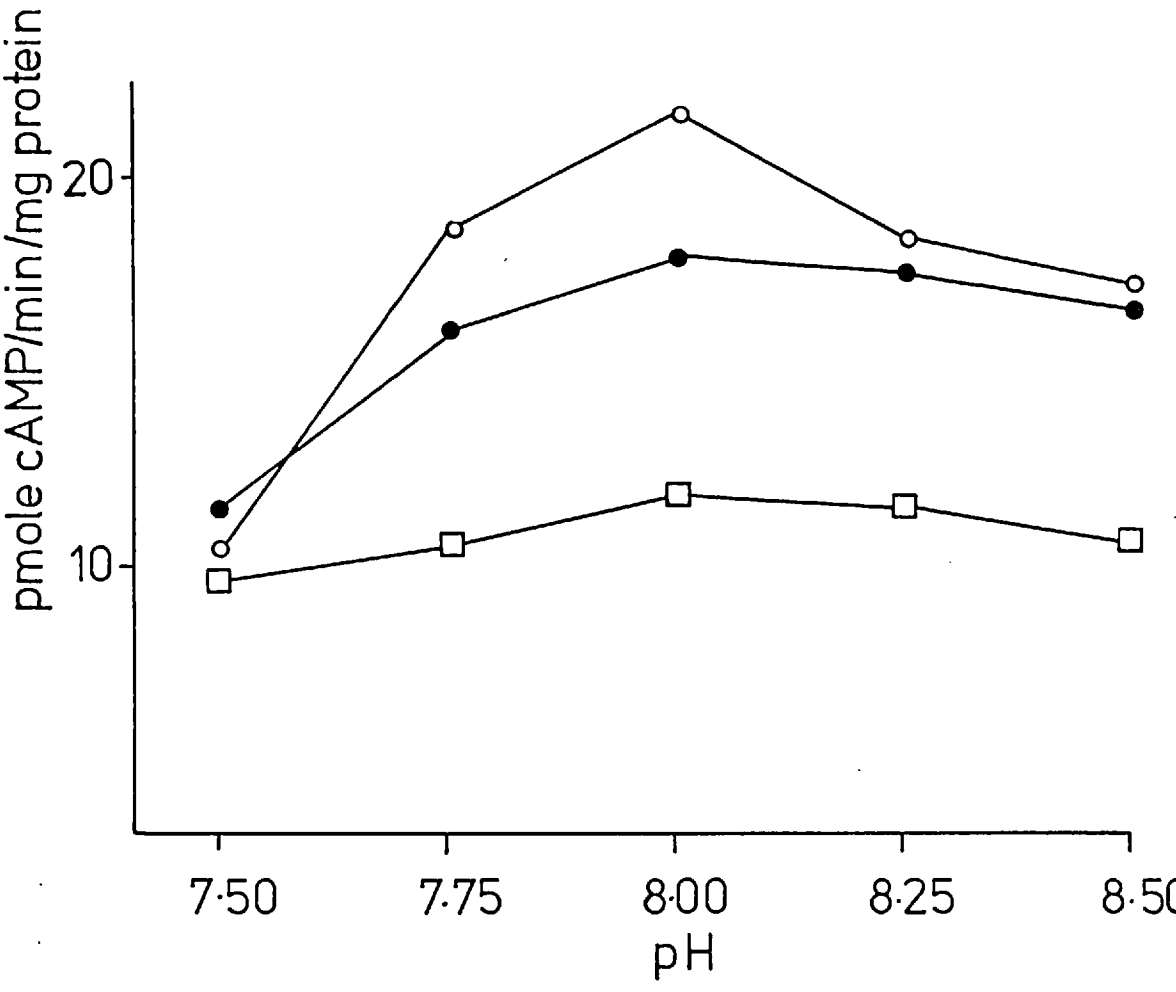


Figure 6•12 pH optima of adenylate cyclase. The pH of the assay buffer is indicated on the abscissa.

- o 10000g pellet.
- ● 100000g pellet.
- □ 100000g supernatant.

The plasmodium used in this experiment was 31 hours old, this may account for the low activity in the 10000g pellet fraction.

5) Standard deviation of assay results.

The mean and standard deviation of twelve identical assays run in parallel is shown in Table 6·6.

Table 6·6 Standard deviation of assay results.

	mean	S.D.
Total counts	4660·0	33·0
AMP PNP	4382·0	26·5
Cyclic AMP	284·0	26·2

The low speed particulate fraction was used in this experiment: all values are in c.p.m.

6) Controls.

The controls for this experiment were:

i) Zero time.

Addition of 15µl 4N HCl to buffer containing (^{3}H) AMP PNP followed by the addition of Physarum homogenate and incubation for 10min at 30°C. The results are shown in Table 6·7

Table 6·7 Zero time controls.

Fraction	c.p.m. in cyclic AMP region.	
10000g pellet	69	53
100000g pellet	63	66
100000g supernatant	61	65

An average value of 63 c.p.m. was subtracted from reaction values.

ii) Cross talk.

Chromatography of the following two mixtures was carried out to measure the cross talk between substrate and product.

i) (^{3}H) AMP PNP and cyclic AMP

ii) (^{3}H) cyclic AMP and AMP PNP

The results are shown in Table 6·8.

Table 6·8 Cross ~~talk~~ contamination between substrate and product.

Sample applied	AMP PNP	Cyclic AMP
(^{3}H) AMP PNP + Cyclic AMP	161	9376
(^{3}H) cyclic AMP	10107	39
AMP PNP		

7) Inhibition of cyclic AMP phosphodiesterase activity.

1) Effect of phosphodiesterase on AMP PNP.

Beef heart cyclic nucleotide phosphodiesterase (20µg/ml) was incubated under the usual conditions with 160nCi (^{3}H) AMP PNP (= 118506 c.p.m.) in adenylate cyclase assay buffer with or without 1mM IBMX. This was chosen because of its ability to inhibit Physarum cyclic AMP PDE. (Chapter 6·25, Table 6·2). The results are shown in Table 6·9.

Table 6·9 Effect of phosphodiesterase on AMP PNP.

(^{3}H) AMP PNP	(^{3}H) cAMP	(^{3}H) Adenosine	(^{3}H) 5'AMP	
29630	42	61	29	+ IBMX
28011	61	39	543	- IBMX

All values are averages of triplicate determinations and are expressed in c.p.m. (background not subtracted).

Thus 1mM IBMX effectively inhibits degradation of (^{3}H) AMP PNP by the beef heart enzyme. An experiment described in the next section indicates that IBMX is equally effective with Physarum PDE.

ii) Effect of IBMX on adenylate cyclase activity.

While IBMX effectively inhibits the degradation of AMP PNP by cyclic nucleotide PDE it may also affect the activity of

Figure 6•13 The effect of 1mM isobutyl methyl xanthine on adenylate cyclase activity. The assay buffer contained 5mM $MgCl_2$, 50mM tris - HCl pH 8•0 + 1mM IBMX (upper panel) and no IBMX (lower panel) In the presence of 1mM IBMX the reaction product is cyclic AMP whereas in its absence the reaction product is adenosine. Cyclic AMP is degraded to 5'AMP by cyclic AMP phosphodiesterase this in turn is degraded to adenosine. The utilisation of substrate is unaffected.

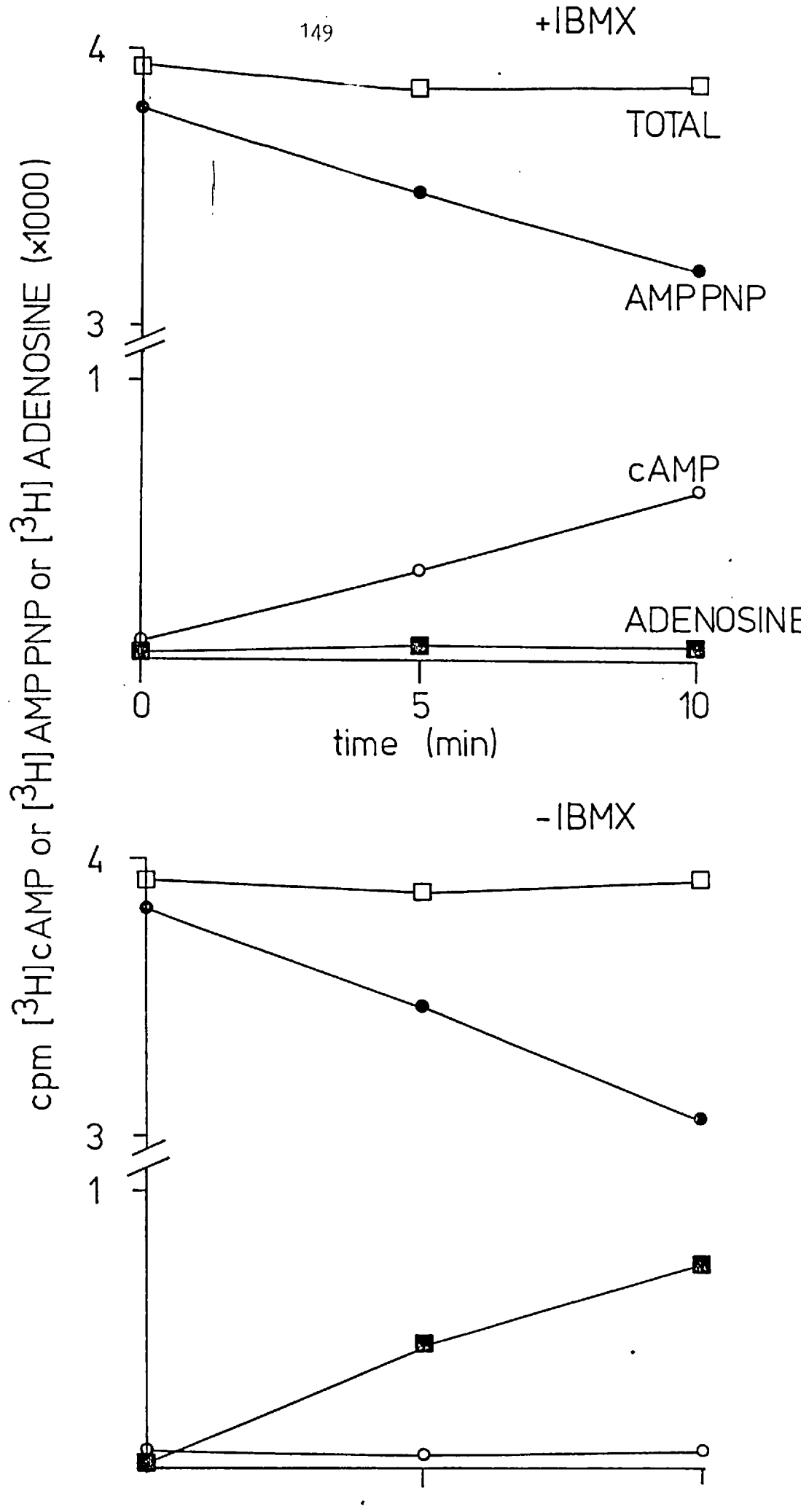
□ Total counts.

● AMP PNP.

o Cyclic AMP.

■ Adenosine.

Symbols are the same in each diagram as is the abscissa (incubation time in min.).


+IBMX
TOTAL
AMP PNP
cAMP
ADENOSINE
4
3
1
0
5
10
time (min)
-IBMX
cpm [3H]cAMP or [3H] AMP PNP or [3H] ADENOSINE (×1000)

adenylate cyclase. Figure 6·13 shows the effect of IBMX on adenylate cyclase activity. In the absence of 1mM IBMX adenosine is the final product, because of the degradation of cyclic AMP by the action of PDE and 5' nucleotidase. In the presence of IBMX cyclic AMP is not degraded to adenosine, but it would seem that there is no inhibitory effect on adenylate cyclase.

Thus 1mM IBMX was routinely included in adenylate cyclase assay buffer for the following reasons:

i) Cyclic AMP PDE activity was inhibited

ii) Adenylate cyclase activity, as determined by substrate utilisation was unaffected.

iii) Protection of the substrate from degradation by PDE.

8) Substrate utilisation.

The utilisation of substrate with respect to time is shown in Figure 6·14. For this experiment the enzyme used was obtained from the 100000g supernatant of homogenised macroplasmodia harvested approximately 1 hour before M3.

The utilisation of (^{3}H) AMP PNP without addition of unlabelled AMP PNP is linear for approximately 10min (open circles). Addition of 0·2mM AMP PNP (closed circles) resulted in a decrease in the rate of change of both substrate and product (square symbols) as expected.

Although not determined in this experiment the total counts present during the assay was the same. Thus the sum of (^{3}H) AMP PNP and (^{3}H) cyclic AMP was constant. In Table 6·10 full details of the assay results are given, so that the computations given in later experiments may be followed.

Table 6·10 Utilisation of AMP PNP by adenylate cyclase.

Incubation time (min)	Cyclic AMP c.p.m.	Cyclic AMP Av.	AMP PNP c.p.m.	AMP PNP Av.	Sum
With 0·2mM AMP PNP (containing 30nCi (^{3}H) AMP PNP, 0·033μM)					
0	17 9	13	15932 15838	15885	15902
2	312 286	299	15607 15531	15569	15868
5	423 391	407	15593 15517	15555	15962
7	547 517	532	15473 15530	15501	16033
10	691 654	673	15201 15039	15120	15793
15	960 1001	981	15091 14927	15009	15990
20	1129 1091	1110	14603 14393	14498	15608
30	1761 1703	1732	14209 13999	14102	15863
With 30nCi (^{3}H) AMP PNP (0·033μM)					
0	57 93	75	15555 15567	15561	15636
2	1761 1721	1741	14132 14168	14150	15893
5	3899 3873	3886	12091 11923	12007	15893
7	5091 5175	5133	10731 10789	10760	15864
10	6999 6972	6986	8971 8943	8957	15943
15	9251 9189	9220	6683 6694	6688	15908
20	10692 10632	10662	5421 5240	5330	15992
30	12793 12749	12771	3179 3215	3197	15968

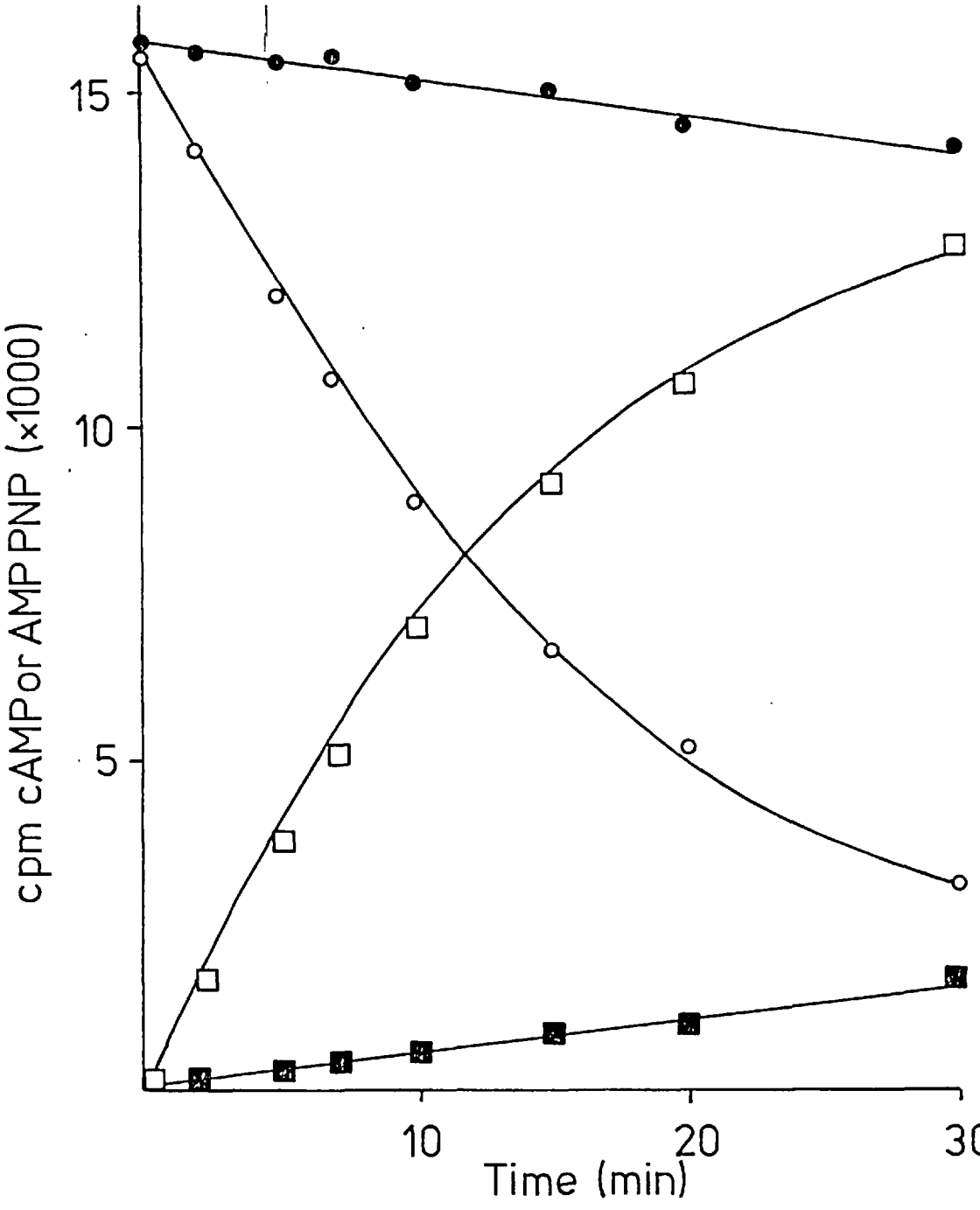


Figure 6·14 Utilisation of AMP PNP by adenylate cyclase.

The results given in Table 6·10 are plotted here

Closed symbols ● (^{3}H) AMP PNP + 0.2mM AMP PNP

■ Cyclic AMP formed.

Open symbols ○ (^{3}H) AMP PNP only (0·033 M)

□ Cyclic AMP formed

6·36 Physarum adenylate cyclase.

1) Effect of sodium fluoride on adenylate cyclase activity.

The activity of the majority of mammalian adenylate cyclases are stimulated by the presence of 1 - 10mM sodium fluoride (Perkins 1973).

If 10mM NaF was included in the assay buffer or if Physarum macroplasmodia were homogenised in extraction buffer containing 10mM NaF, no stimulation of adenylate cyclase activity was apparent. Indeed, when homogenised in extraction buffer containing 10mM NaF, Physarum adenylate cyclase appeared to be inhibited. These results are shown in Figure 6·15.

Other workers (Atmar et al. 1976) also noted that NaF did not stimulate Physarum adenylate cyclase.

2) Effect of Ca^{++}, Mg^{++} and Mn^{++} on adenylate cyclase activity.

If plasmodia were homogenised either in buffer without $CaCl_2$ or in buffer without $CaCl_2$ but containing 2mM EGTA, no adenylate cyclase activity could be detected in any fraction. In the presence of 1mM $CaCl_2$ in the extraction buffer, adenylate cyclase activity could be detected in all fractions. The optimum concentration of $CaCl_2$ in this buffer for all fractions was found to be 5mM. In view of this requirement for Ca^{++} the extraction buffer contained 5mM $CaCl_2$.

If plasmodia were homogenised in extraction buffer containing 5mM $CaCl_2$ but assayed in the absence of Mg^{++} no adenylate cyclase activity could be detected. Thus it was necessary to homogenise plasmodia in the presence of Ca^{++} and assay in the presence of Mg^{++}. Reversal of this combination of Ca^{++} and Mg^{++} was not tested. The effect of certain concentrations of these divalent cations on adenylate cyclase activity is shown in Table 6·11.

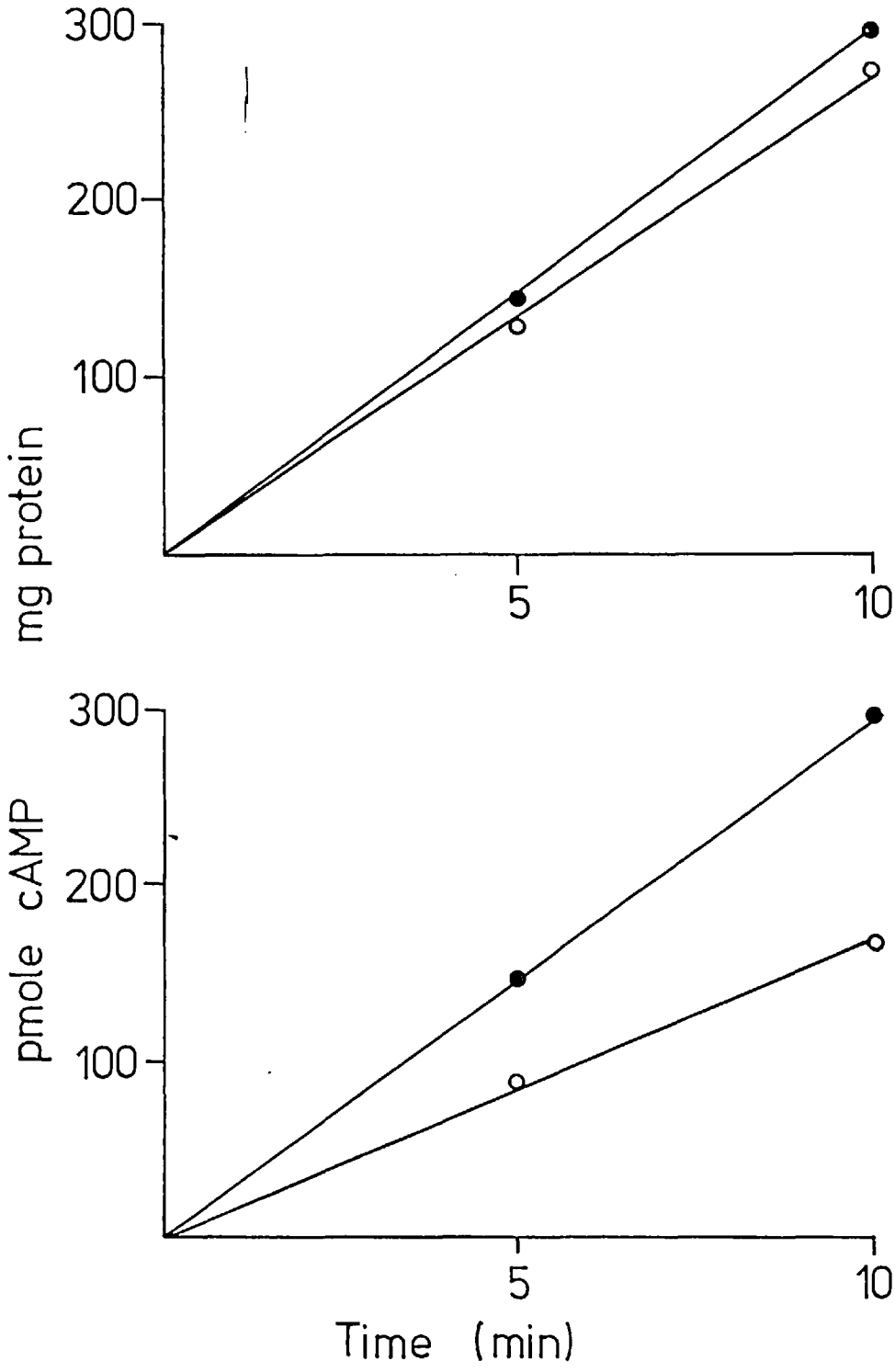


Figure 6·15 The effect of 10mM sodium fluoride on adenylate cyclase activity. Upper panel, when assayed in assay buffer containing 10mM NaF. Lower panel, when adenylate cyclase was extracted in extraction buffer containing 10mM NaF.

● Control (no NaF)

o + 10mM NaF

Table 6·11 Effect of certain metal ions on adenylate cyclase activity.

Extraction buffer		Assay buffer			Activity*
Ca^{++}	Mg^{++}	Mn^{++}	Ca^{++}	Mg^{++}	
0	0	0	0	5	0
5	0	0	0	0	0
1	0	0	0	1	7
5	0	0	0	5	331
10	0	0	0	0	178
5	0	0	0	0	36
5	0	5	0	5	205

* Activity = pmole cyclic AMP / 10min / mg protein.

Extraction buffer = 0·25M sucrose, 1mM dithiothreitol, 50mM tris - HCl pH 7·5, Assay buffer = 1mM IBMX, 50mM tris - HCl pH 8·0.

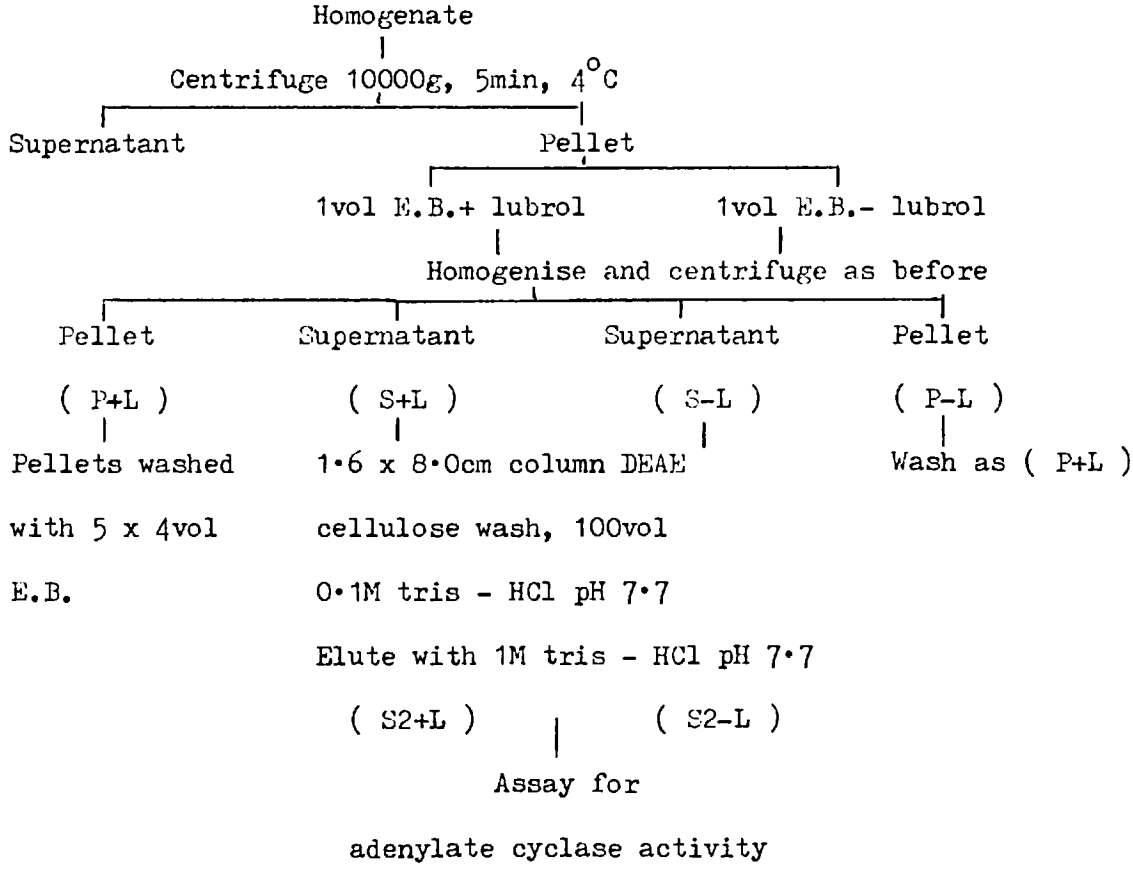
All ion concentrations = mM, 100000g supernatant enzyme, extracted 180min before M3 was used.

3) Attempt at the solubilisation of adenylate cyclase by the use of a nonionic detergent.

Four macroplasmodia were harvested in the usual way and homogenised in 4vol of adenylate cyclase extraction buffer. After centrifugation for 5min at 10000g at 4°C the pellet was resuspended in 1vol of extraction buffer. The suspension was divided into two equal portions, to one portion was added 1vol of extraction buffer containing 0·2% lubrol, to the other portion was added 1vol of extraction buffer. Both portions were again homogenised as before. After a second centrifugation, like the first, the pellets were washed five times with 4vol of extraction buffer and finally resuspended in 1vol of the same buffer. Portions of the pellets and supernatants were removed and stored at -196°C for assay later.

Each supernatant was applied to a 1•6 x 8•0cm column of DEAE cellulose at 4 - 6°C. After allowing the supernatants to drain into the resin, each column was washed with approximately 100 column volumes of 0•1M tris - HCl pH 7•7. Protein was eluted with 1M tris - HCl pH 7•7, the elution of protein is shown in Figure 6•16.. Activity was detected in the main peak (fraction 9 - 14). Portions of this peak were taken and stored at -196°C for assay later. This procedure is shown more clearly in the flow diagram below (Table 6•12).

Table 6•12 Attempt at the solubilisation of adenylate cyclase.



E.B. = adenylate cyclase extraction buffer.

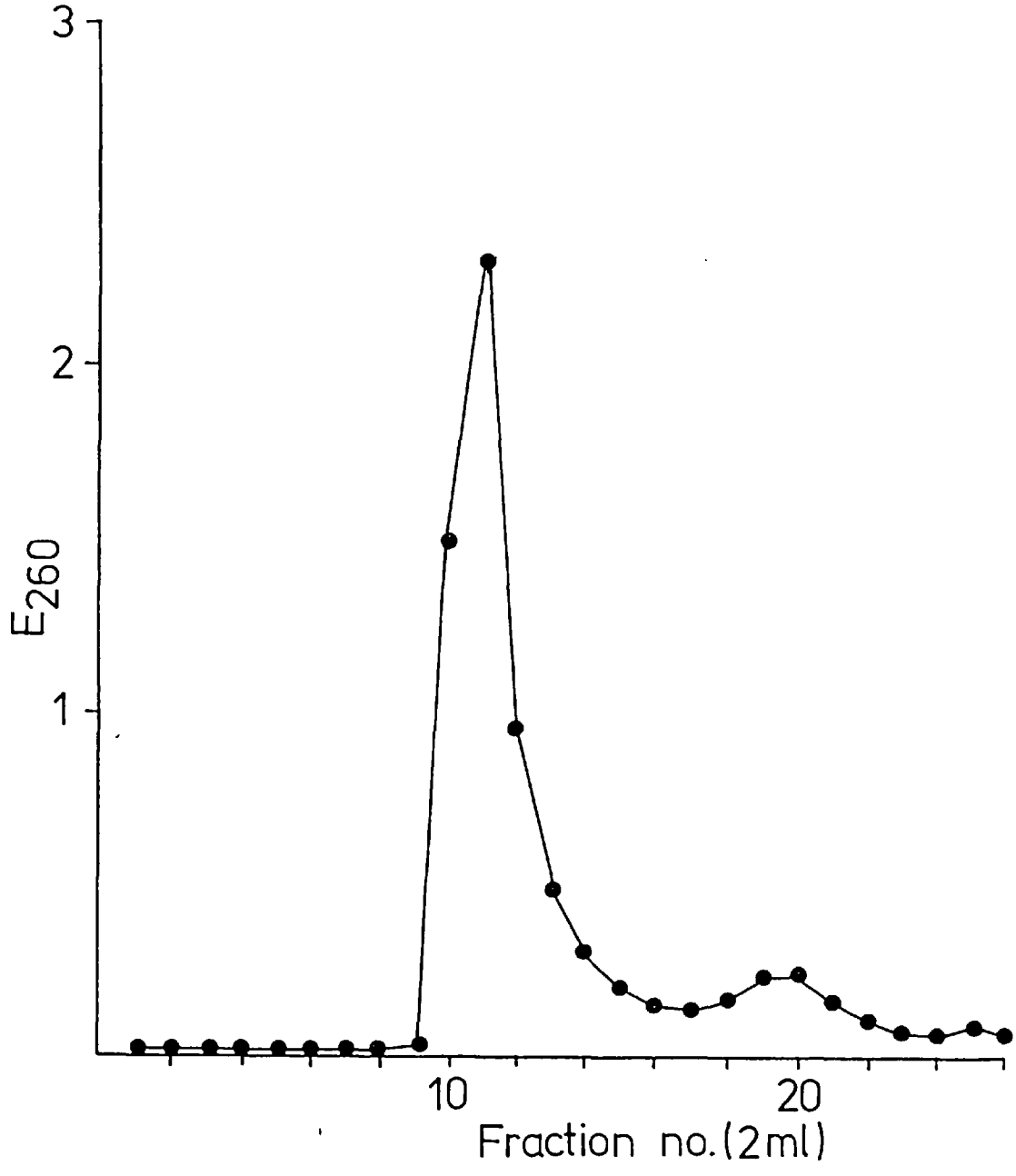
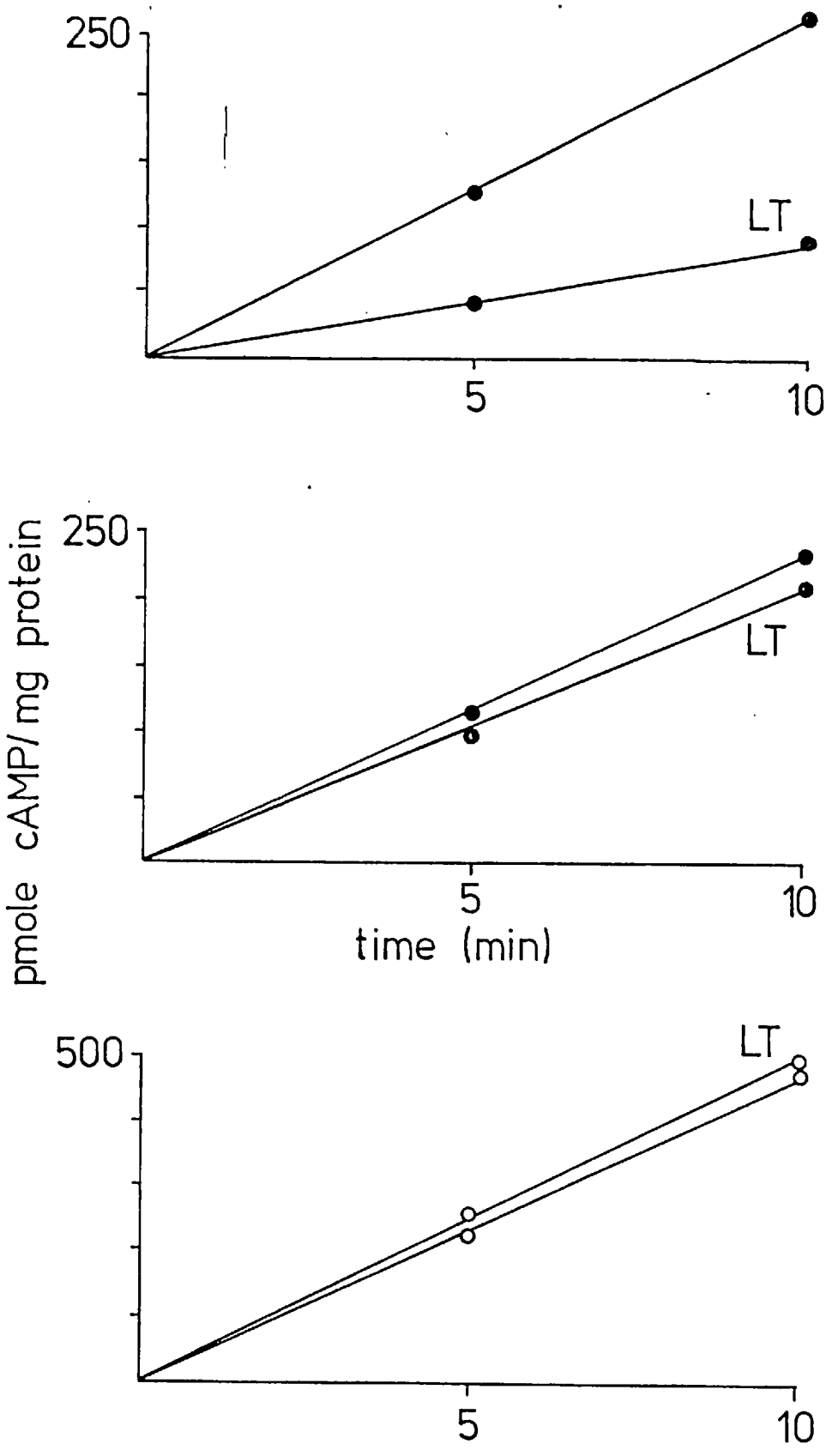


Figure 6•16 Elution of protein from a 1•6 x 8•0 cm column of DEAE cellulose with 1M tris - HCl pH 7•7. Adenylate Cyclase activity was detected in the main peak (Fractions 9 - 14).

Figure 6•17 Effect of 0•1% lubrol on adenylate cyclase activity. Upper panel = Effect of lubrol on 10000g supernatant fraction before chromatography on DEAE cellulose. LT = lubrol treated.

Middle panel = Activity of the 10000g supernatant after chromatography on DEAE cellulose. Lubrol is removed by this treatment.

Lower panel = Activity of the washed 10000g pellet following homogenisation in 0•1% lubrol.


250
LT
5
10
250
LT
5
10
pmole cAMP/ mg protein
time (min)
500
LT
5
10

Portions of the supernatants S+L, S-L, S2+L and S2-L were assayed for adenylate cyclase activity. The results are shown in Figure 6·17. The presence of 0·1% lubrol inhibits adenylate cyclase activity (upper panel). Chromatography on DEAE cellulose as described by Levey (1970) removes the detergent in the washing buffer (0·1M tris - HCl pH 7·7).

These results show that adenylate cyclase activity in the particulate fraction is not solubilised by homogenisation in the presence of 0·1% lubrol.

6·37 Summary.

A new method has been described for the assay of adenylate cyclase using β-γ imido (8-^{3}H) adenosine 5' triphosphate as substrate. A number of methods were investigated as a means of separating cyclic AMP from (^{3}H) AMP PNP, thin layer chromatography on cellulose was found to resolve not only AMP PNP and cyclic AMP but also 5'AMP and adenosine.

Tritiated AMP PNP, when incubated with a Physarum homogenate, is partially converted to (^{3}H) cyclic AMP. Cyclic AMP was identified by its affinity for a specific binding protein and its sensitivity to cyclic nucleotide phosphodiesterase.

The assay conditions have been carefully worked out with respect to protein concentration, pH optima and utilisation of substrate.

Adenylate cyclase, not stimulated by 10mM NaF, was found to be present in homogenised Physarum plasmodia separated into three fractions by centrifugation. The low speed particulate fraction contained adenylate cyclase insensitive to solubilisation by the

detergent, lubrol.

Fractions prepared as above, from Physarum macroplasmodia harvested throughout the mitotic cycle from before M2 until after M3 were used to measure adenylate cyclase activity. An increase in adenylate cyclase activity in the particulate fraction was noted approximately 7½ hours after M2. A decrease in activity before M2 suggests a similar peak of activity at the same point in the previous mitotic cycle.

Further details of the mitotic cycle activity of this enzyme are given in Chapter 7.

Cyclic GMP phosphodiesterase.

6·40 Introduction.

Cyclic GMP phosphodiesterase activity was measured using a radiometric assay similar to that used for cyclic AMP phosphodiesterase. Separation of the reaction products was by t.l.c. on cellulose. The use of a resin method was not attempted.

6·41 Assay conditions.

1) The same protocol was used as described for cyclic AMP PDE, with the following exceptions.

The reaction mixture consisted of:

15µl Assay buffer (5mM $MgCl_2$, 50mM tris - HCl pH 7·5)

15µl Cyclic GMP in assay buffer (6·75 - 8·00mM cyclic GMP containing about 100nCi (^3H) cyclic GMP.

15µl Physarum homogenate.

The reaction was terminated by the addition of 15µl of 30% TCA, after incubation at 30°C for 10min. Approximately 5mM cyclic GMP, 5'GMP and guanosine were included in the stopping solution as chromatographic markers. The reaction was analysed by t.l.c. on cellulose developed in system 20.

2) Under the conditions described the reaction was found to be linear with upto 45µg protein per assay tube. With respect to time, the reaction was linear for upto 15min. (data not shown).

6·42 Inhibition of cyclic GMP phosphodiesterase activity.

A number of likely compounds were tested (at 1mM) for their effect on cyclic GMP PDE activity. The aim of this experiment was to

find an inhibitor that was without inhibitory effect on guanylate cyclase but had a profound effect on cyclic GMP PDE. The results are shown in Table 6·13.

Table 6·13 Inhibition of cyclic GMP phosphodiesterase activity.

Compound	% control activity.
IBMX	23·8
Caffeine	127·0
Theophylline	16·6
SQ20009	18·9
Papaverine	90·1
Imidazole	107·4
EDTA	70·8

Because SQ20009 inhibited cyclic GMP PDE (18·9% of control) but had little effect on guanylate cyclase (103% of control) it was used in guanylate cyclase assays to prevent product degradation.

6·43 Growth medium cyclic GMP phosphodiesterase.

The experiment described in Chapter 6·26 with cyclic AMP as substrate for the growth medium phosphodiesterase was repeated using cyclic GMP as substrate. The reaction products were chromatographed on PEI cellulose in system 6. The results are shown in Figure 6·18. The growth medium phosphodiesterase failed to hydrolyse (^{3}H) cyclic GMP to (^{3}H) 5'GMP.

Thus Physarum secretes a potent cyclic AMP phosphodiesterase into the growth medium, cyclic GMP is not hydrolysed by this enzyme.

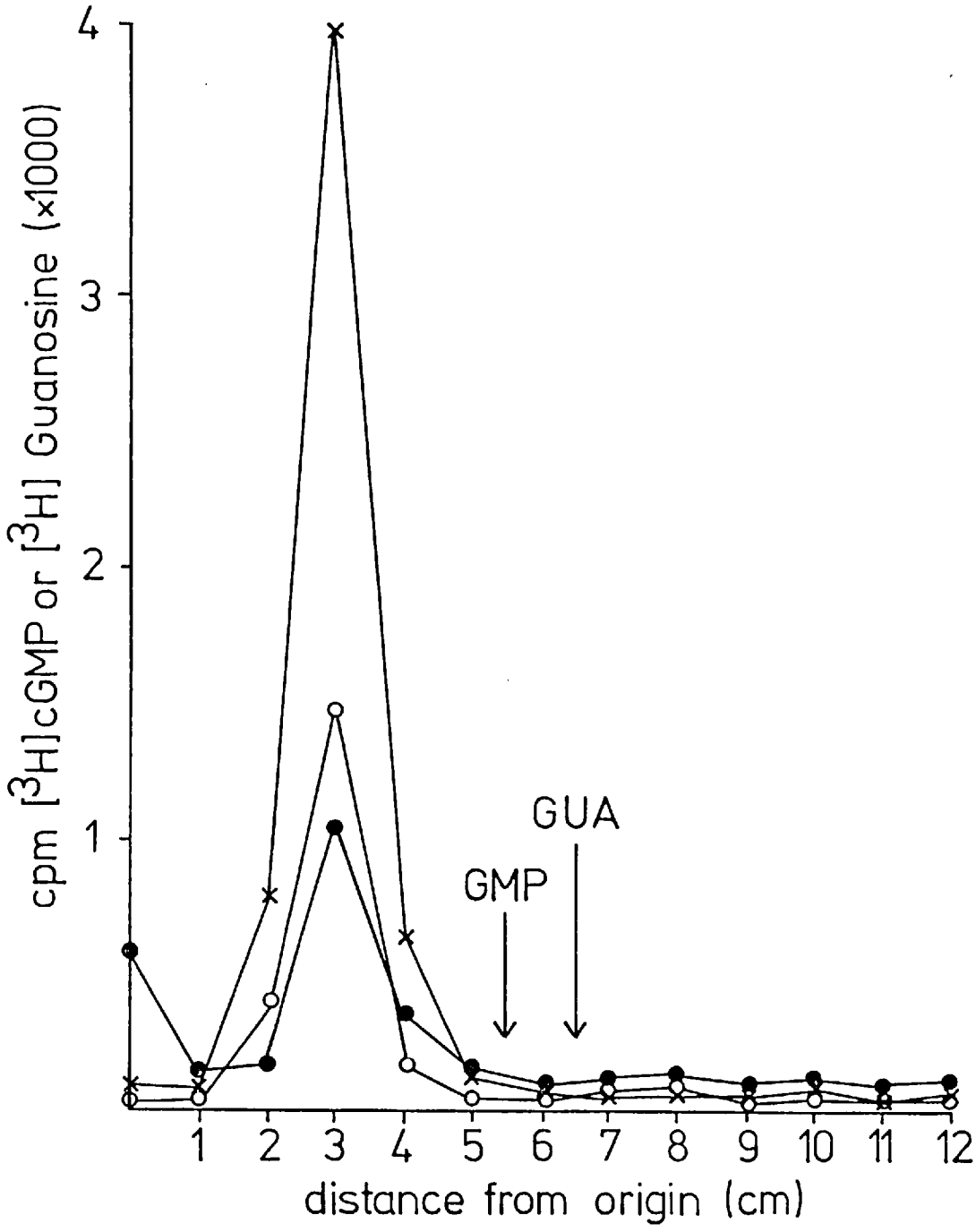


Figure 6·18 Cyclic GMP PDE in Physarum growth medium.

× (^{3}H) cyclic GMP marker, o filtered growth medium

● 50 - 60% ammonium sulphate fraction. GUA and GMP indicate the position of unlabelled guanosine (GUA) and guanosine monophosphate (GMP).

6•44 Summary.

Cyclic GMP phosphodiesterase activity has been measured using a radiometric method, with separation of substrate and product(s) by t.l.c. on cellulose developed in solvent system 20 (95% ethanol : 2M ammonia : 2M acetic acid 100:20:20 by vol).

Activity was detected in the particulate fraction and soluble fractions of homogenates. Activity was not demonstrable in the growth medium - a source of a potent cyclic AMP phosphodiesterase.

The activity of cyclic GMP phosphodiesterase has been measured during the mitotic cycle in three fractions of homogenates. The results of this work are presented in Chapter 7.

Guanylate cyclase.

6·50 Introduction.

In 1969 three groups of workers published reports of an enzyme, guanylate cyclase, that catalysed the formation of cyclic GMP from GTP in cell free systems (Hardman and Sutherland, White and Aurbach and Ishikawa et al.).

Many of the problems associated with the assay of adenylate cyclase are also encountered in the assay of guanylate cyclase. Additionally, unlike adenylate cyclase, there is no effective substrate recycling system as is available for adenylate cyclase.

As with adenylate cyclase so with guanylate cyclase the imidophosphate analogue has been used as substrate with a suitable inhibitor to prevent the degradation of cyclic GMP by cyclic GMP phosphodiesterase.

6·51 Assay conditions.

The assay procedure is similar to that used for adenylate cyclase, with the following exceptions.

The reaction mixture contained:

15µl Assay buffer (1mM SQ20009, 25mM N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid. pH 8·25)

15µl (^{3}H) GMP PNP (100 - 102nCi) containing approximately 6·7mM GMP PNP.

15µl Physarum homogenate.

The reaction mixture was incubated at 30°C for 10min and the reaction terminated by the addition of 15µl 1N HCl containing GMP PNP and cyclic GMP as chromatographic markers (approximately 5mM).

The buffer, N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid, TES, was used because it is a very weak metal binding agent (Garbers et al. 1976). Additionally, guanylate cyclase has been reported to be very sensitive to metal ions (Chrisman et al. 1975). It was thought that Physarum guanylate cyclase might be sensitive to metal ions and thus a weak metal binding buffer that would not remove endogenous cations from solution, was used. It was hoped that this would lead to the expression of native guanylate cyclase activity.

1) Identity of reaction product.

The procedure was exactly the same as used with adenylate cyclase (Chapter 6·35) with the following exceptions. Assays were run in triplicate, each of the replicates were treated in one of three ways: putative (^{3}H) cyclic GMP was eluted and counted, eluted and bound to a cyclic GMP antibody or eluted, treated with beef heart cyclic nucleotide phosphodiesterase (20μg/ml) and bound to a cyclic GMP antibody (Binding of cyclic GMP is described more fully in Chapter 5·41). The results are shown in Figure 6·19.

That the product of the reaction is indeed (^{3}H) cyclic GMP is judged from its binding to a specific antibody and its sensitivity to cyclic nucleotide phosphodiesterase.

2) pH optima.

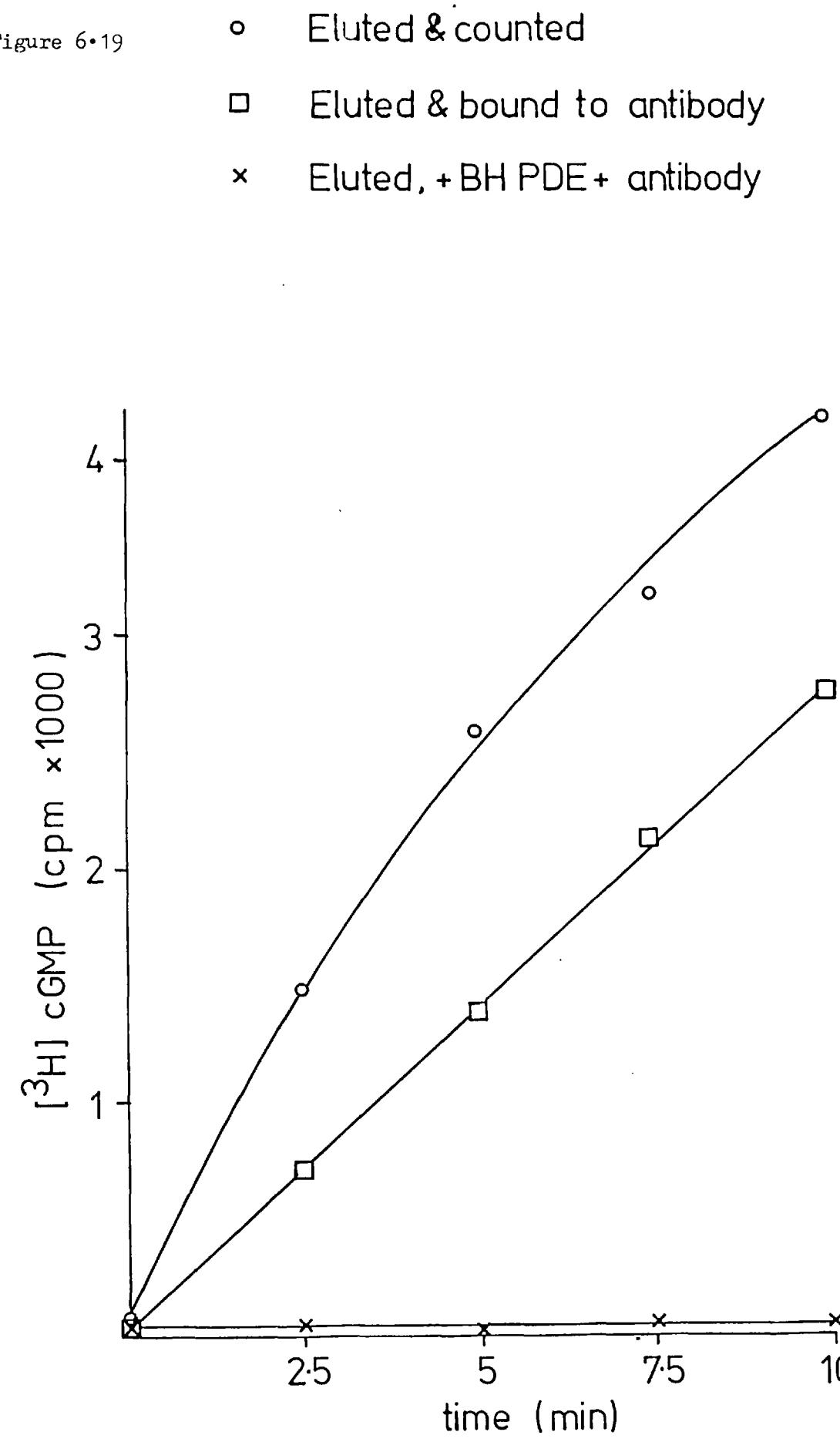
The pH optima of guanylate cyclase in the three fractions of an homogenate of Physarum is shown in Figure 6·20. Guanylate cyclase from all three fractions have a pH optimum of about 8·25.

3) Protein concentration.

The reaction was found to be linear with upto 45μg protein per assay tube (data not shown).

Figure 6•19 Identity of the guanylate cyclase reaction product. Putative (^{3}H) cyclic GMP was bound to a cyclic GMP specifc antibody (□) or degraded by cyclic nucleotide phosphodiesterase and then exposed to the antibody (X) or eluted and counted. (O).

Figure 6·19


o Eluted & counted
□ Eluted & bound to antibody
× Eluted, + BH PDE + antibody
[3H] cGMP (cpm ×1000)
4
3
2
1
2·5
5
7·5
10
time (min)

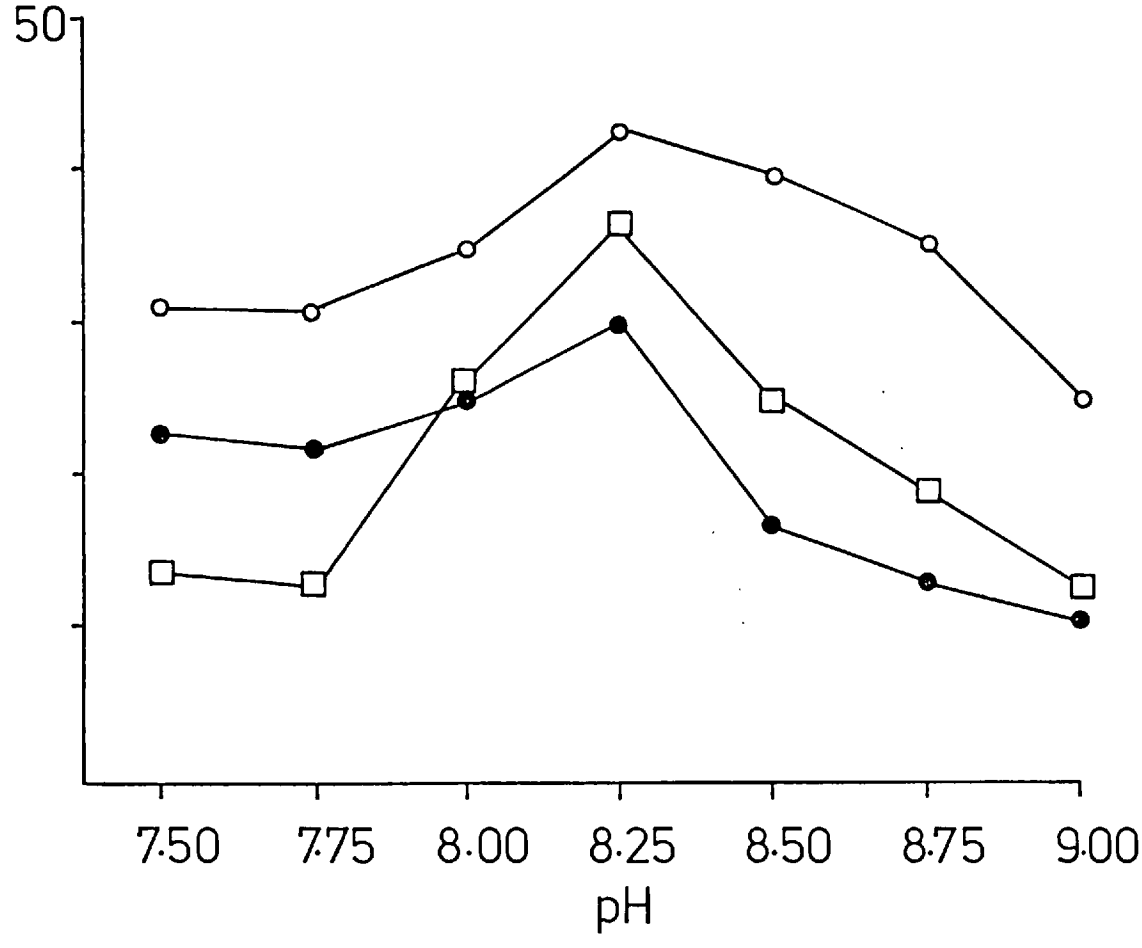


Figure 6-20 pH optima of guanylate cyclase from fractions of a Physarum homogenate. The plasmodium was homogenised about 10min before M3.

□ 10000g pellet

○ 100000g pellet

● 100000g supernatant.

Ordinate : pmole cyclic GMP per 10min per mg protein.

4) Utilisation of substrate.

Figure 6·21 shows the utilisation of substrate and the appearance of product. The reaction, under these conditions, is linear for upto 20min.

In later experiments the amount of (^{3}H) GMP PNP was increased to about 150nCi per assay tube to improve sensitivity. Although only making a small difference, the increase in total GMP PNP present was taken into account.

5) Blank reaction in guanylate cyclase assays.

Using enzyme from the 100000g supernatant fraction, twelve identical assays were run for zero time i.e. 15µl 1N HCl was added before the enzyme. Separation of product and substrate was as described previously. The results are shown in Table 6·14.

Table 6·14 Zero time blank reaction.

	(^{3}H) cGMP c.p.m.	(^{3}H) GMP PNP c.p.m.
Mean	39·38	15606
S.D.	6·72	1080

(n = 12)

Three controls were routinely carried out:

i) Zero time, addition of 15µl 1N HCl before the addition of Physarum homogenate and incubation for 10min at 30°C. The results are shown in Table 6·15

Table 6·15 Zero time control.

Fraction	gross c.p.m.		Av. c.p.m.
10000g pellet	69	102	85·5
100000g pellet	37	49	43·0
100000g supernatant	109	82	45·5

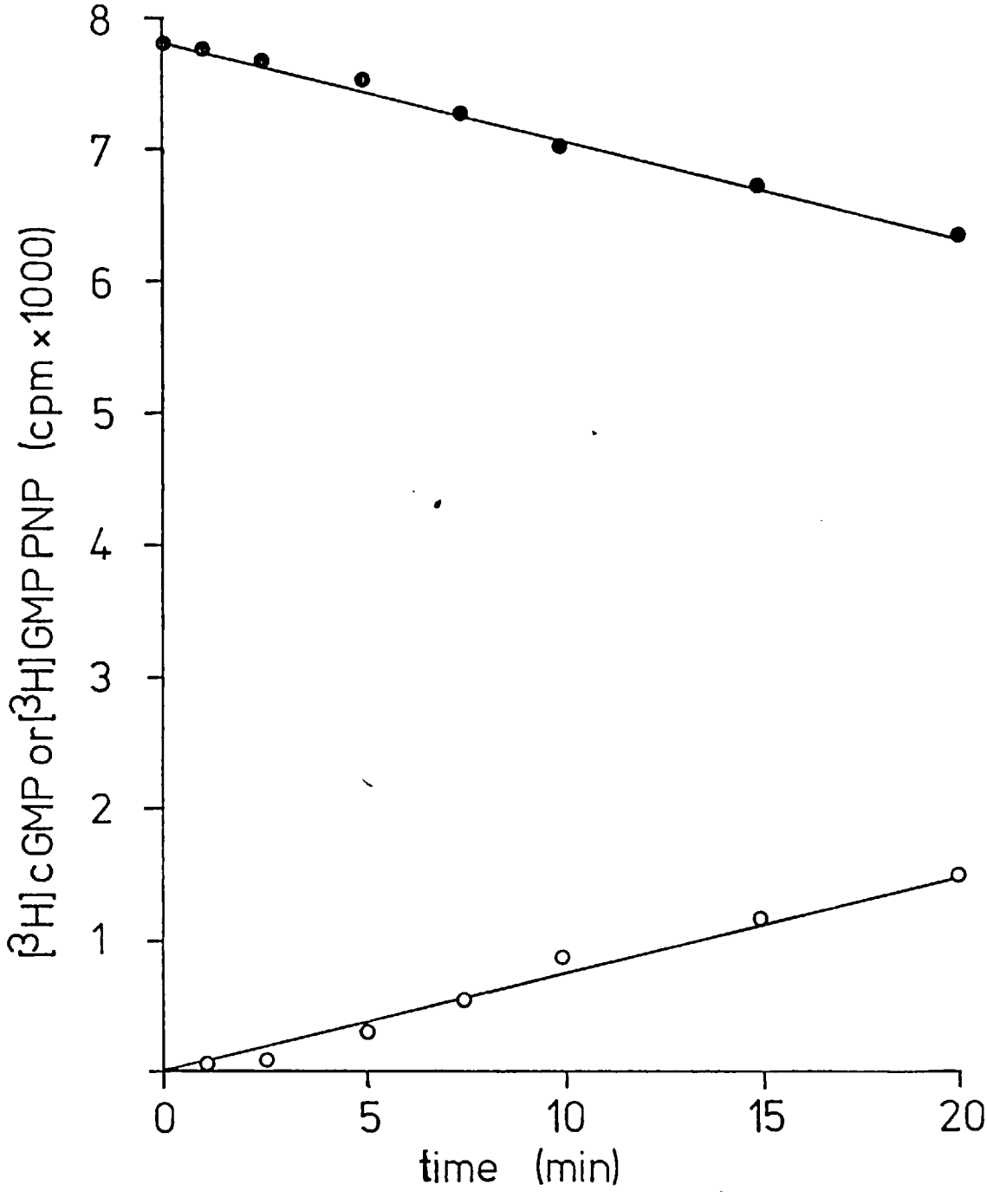


Figure 6·21 Utilisation of GMP PNP by guanylate cyclase. 6·7mM GMP PNP containing 100 - 102nCi (^{3}H) GMP PNP was incubated with 11μg protein from 100000g pellet for the times indicated.

These average values were subtracted fron the values obtained with the active enzyme.

ii) The radioactivity of a sample of (^{3}H) cyclic GMP and of (^{3}H) GMP PNP eluted from cellulose was compared with aliquots counted by adding directly to scintillant. The results are seen in Table 6·16

Table 6·16 Elution of (^{3}H) cyclic GMP and (^{3}H) GMP PNP from cellulose.

(^{3}H) cyclic GMP

Direct c.p.m.	Av c.p.m.	Eluted c.p.m.	Av c.p.m.
338971	338451	327631	332524
337931		333173	
		329983	
		339311	

(^{3}H) GMP PNP

Direct c.p.m.	Av c.p.m.	Eluted c.p.m.	Av c.p.m.
537361	545541	553712	541496
553722		521736	
		543217	
		547319	

For (^{3}H) cyclic GMP, 98·25% of the counts applied were eluted while for (^{3}H) GMP PNP the recovery was 99·26%.

iii) Cross talk, tritiated cyclic GMP and GMP PNP were chromatographed together as were (^{3}H) GMP PNP and cyclic GMP. The regions containing GMP PNP and cyclic GMP were cut out and the radioactivity determined after elution. The results are shown in Table 6·17.

Table 6·17 Cross ~~talk~~ contamination between labelled and unlabelled cyclic GMP and GMP PNP.

Compound	c.p.m.		Av c.p.m.
GMP PNP	71	51	61·5
(^{3}H) cGMP	339721	393127	366424·0
(^{3}H) GMP PNP	556317	559981	558149·0
cGMP	90	79	84·5

The results in this table show that approximately 0·0168% (^{3}H) cyclic GMP was located in the GMP PNP region while 0·0151% (^{3}H) GMP PNP was located in the cyclic GMP region.

6·52 Physarum guanylate cyclase.

1) Effect of Ca^{++}, Mg^{++} and Mn^{++} ions on guanylate cyclase activity.

Guanylate cyclase was extracted in the way described for adenylate cyclase but without $CaCl_2$ in the extraction buffer. No Ca^{++} ions were present in the assay buffer yet activity was easily detectable. The effect of adding Ca^{++}, Mg^{++} or Mn^{++} to the assay buffer can be seen in Figure 6·22. Using the 100000g pellet, the most active fraction, as enzyme source. Control activity was 163 pmole cyclic GMP formed per 10min per mg. protein. The effect of Ca^{++} or Mg^{++} were not very marked (c.f. adenylate cyclase), Mn^{++} at high concentrations (100mM) inhibits guanylate cyclase activity.

2) Effect of cyclic GMP phosphodiesterase inhibitors on guanylate cyclase activity.

To protect the reaction product from degradation by PDE, this enzyme must be inhibited. The inhibitor must not affect guanylate cyclase. A number of likely inhibitors were tested for their effect

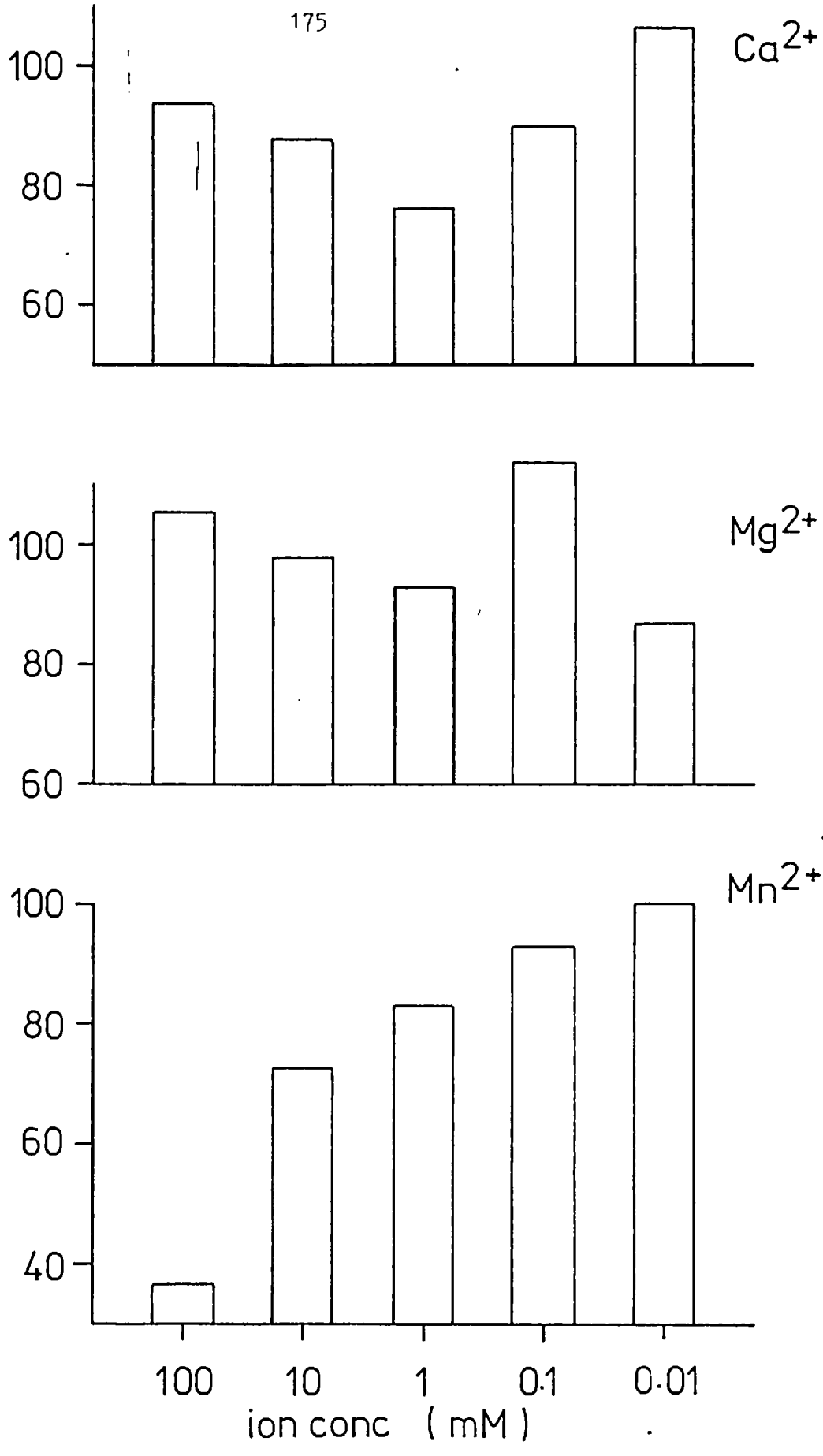


Figure 6·22 Effect of metal ions on guanylate cyclase activity

Ordinate = % control activity, abscissa = ion concentration.

on guanylate cyclase activity. The results are shown in Table 6·18. All the inhibitors were tested at the same concentration, 1mM.

Table 6·18 Effect of cyclic GMP phosphodiesterase inhibitors on guanylate cyclase activity.

Inhibitor (1mM)	% control activity
IBMX	90
Caffeine	23
Theophylline	35
SQ20009	103
Papaverine	79
Imidazole	103
EDTA	61

Consequently 1mM SQ20009 was included in the assay buffer to protect cyclic GMP from cyclic GMP phosphodiesterase while having no obvious effect on guanylate cyclase activity.

6·53 Summary.

A new assay method for guanylate cyclase has been developed using $\beta-\gamma$ imido guanosine 5' triphosphate as substrate and separation of substrate and product by t.l.c. on cellulose in solvent system 17. (2-propanol : dimethylformamide : ethyl-methyl ketone : water : ammonia (20:20:20:39:1 by vol).

This method has been used to measure enzyme activity in three fractions of a Physarum homogenate throughout the mitotic cycle. Unlike adenylate cyclase it was noted that this enzyme is not very sensitive to Ca^{++} or Mg^{++}.

CHAPTER 7

THE MITOTIC CYCLE.

7·10 Introduction.

This chapter contains the results of experiments on the mitotic cycle as revealed by the analytical methods described in Chapters 5 and 6.

This is preceded by two short sections. The first gives the results of protein content of synchronous plasmodia measured through the mitotic cycle. The other section gives an estimate of the duration of the S and G2 phases in such plasmodia.

7·20 Protein content during the mitotic cycle.

The protein content on individual plasmodia homogenised and centrifuged as described in Chapter 3·14 is shown in Figure 7·1. The same plasmodia were used to measure adenylate cyclase, guanylate cyclase, cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activity during the mitotic cycle.

7·30 DNA synthesis.

The synthesis of DNA during the mitotic cycle was measured by following the incorporation of (^{3}H) thymidine into TCA - acetone precipitable material (Braun and Wili 1969, Chapter 3·15).

The results are shown in Figure 7·2. The levels of cyclic AMP and cyclic GMP were determined on a different batch of cultures set up in the same manner.

These results show that S phase lasts for about 3 - 3·5 hours and G2 for about 5 hours. Mitosis was completed, typically, within

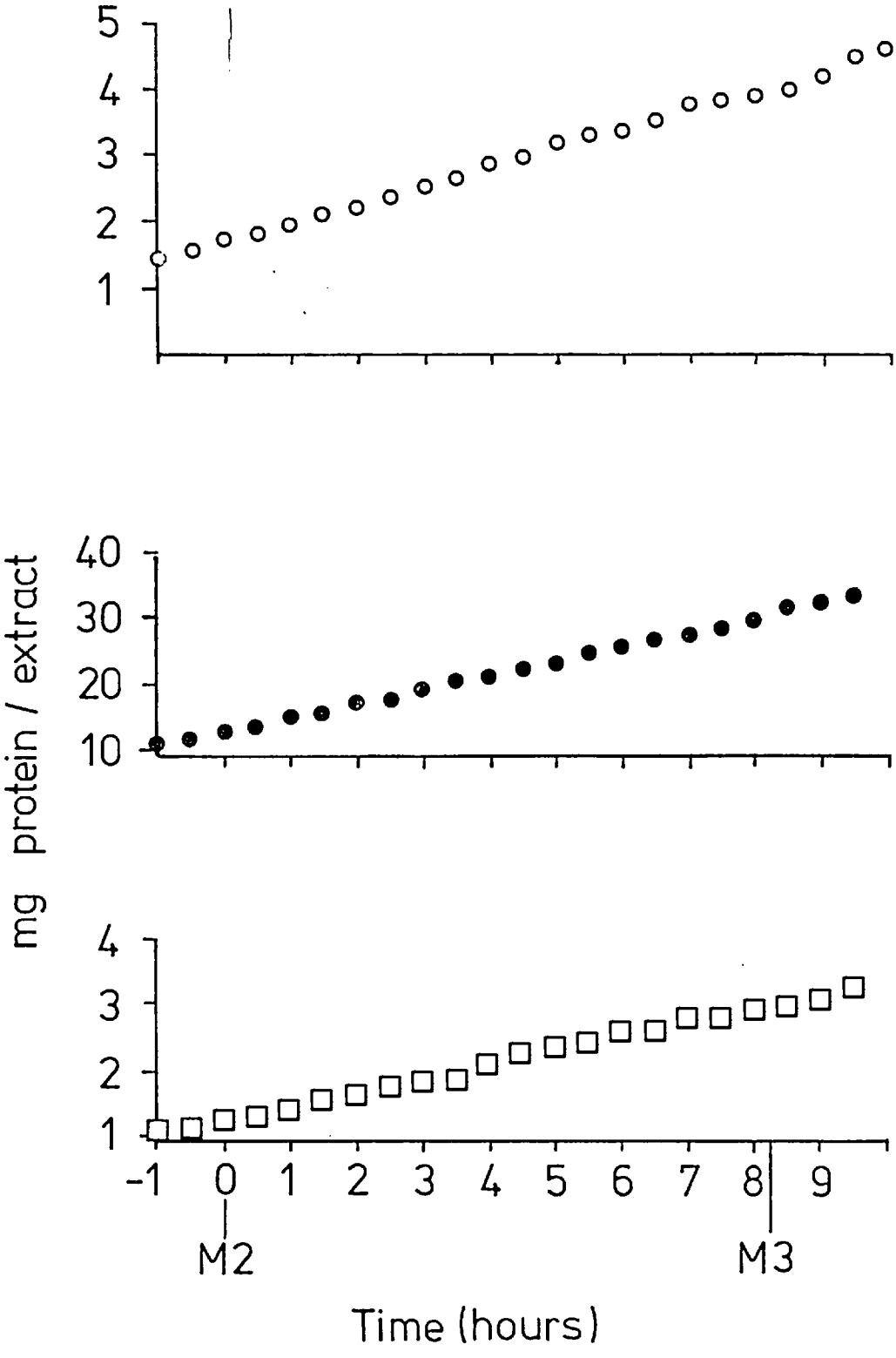


Figure 7·1 Protein content of plasmodia during the mitotic cycle. ○ 10000g pellet, ● 100000g supernatant □ 100000g pellet.

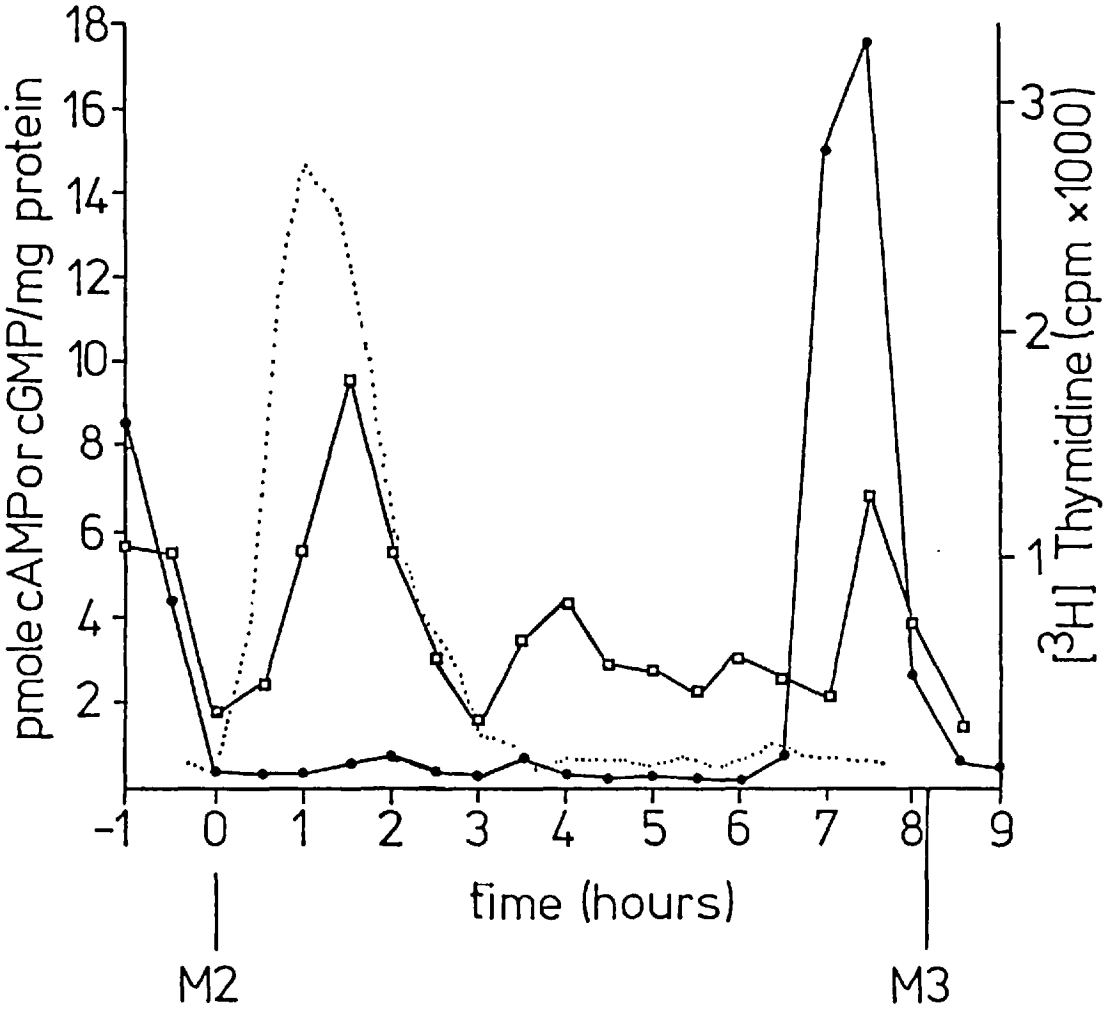


Figure 7·2

Cyclic AMP (•) and cyclic GMP (▫) content of synchronous plasmodia of Physarum. Uptake of (^{3}H) thymidine (·····) by synchronous plasmodia (determined in a different batch of cultures).

20 - 25min. Nucleolar reconstruction lasted for about 60min. These results are in agreement with the observations of other workers with this strain of Physarum.

7•40 Cyclic AMP and cyclic GMP content during the mitotic cycle.

Figure 7•2 shows that during mitosis, the S phase and the first three quarters of G2 cyclic AMP remained constant at less than 1 pmole cyclic AMP per mg. protein. Within the last quarter of G2 cyclic AMP rose rapidly to 17 pmole per mg. protein to return to a basal level by M3. Two samples taken before M2 showed a similar decrease earlier, so a similar peak may have occurred before M2. Two clear peaks of cyclic GMP were detected. The duration of the larger coincided with the S phase rising to 9 pmole per mg. protein at about the time of maximum incorporation of labelled thymidine. The smaller peak (7 pmole cyclic GMP per mg. protein) coincided with that of cyclic AMP late in G2 but showed a 30min lag in its onset. Again results for the hour before M3 parallel those for the same period before M2.

7•50 Adenylate cyclase activity during the mitotic cycle.

Figure 7•3 shows the activity of adenylate cyclase for each fraction during the mitotic cycle. Changes in adenylate cyclase activity during the mitotic cycle were confined to the particulate fraction (10000g). Enzyme activity of this fraction remained between 330 and 380 units (1 unit = 1 pmole cyclic AMP formed per 10min per mg. protein) during mitosis, the S phase and the first three quarters of G2. During the last quarter activity reached a maximum of 476 units 90min before M3. Samples taken before M2 show a decrease in activity suggesting a similar peak before M2.

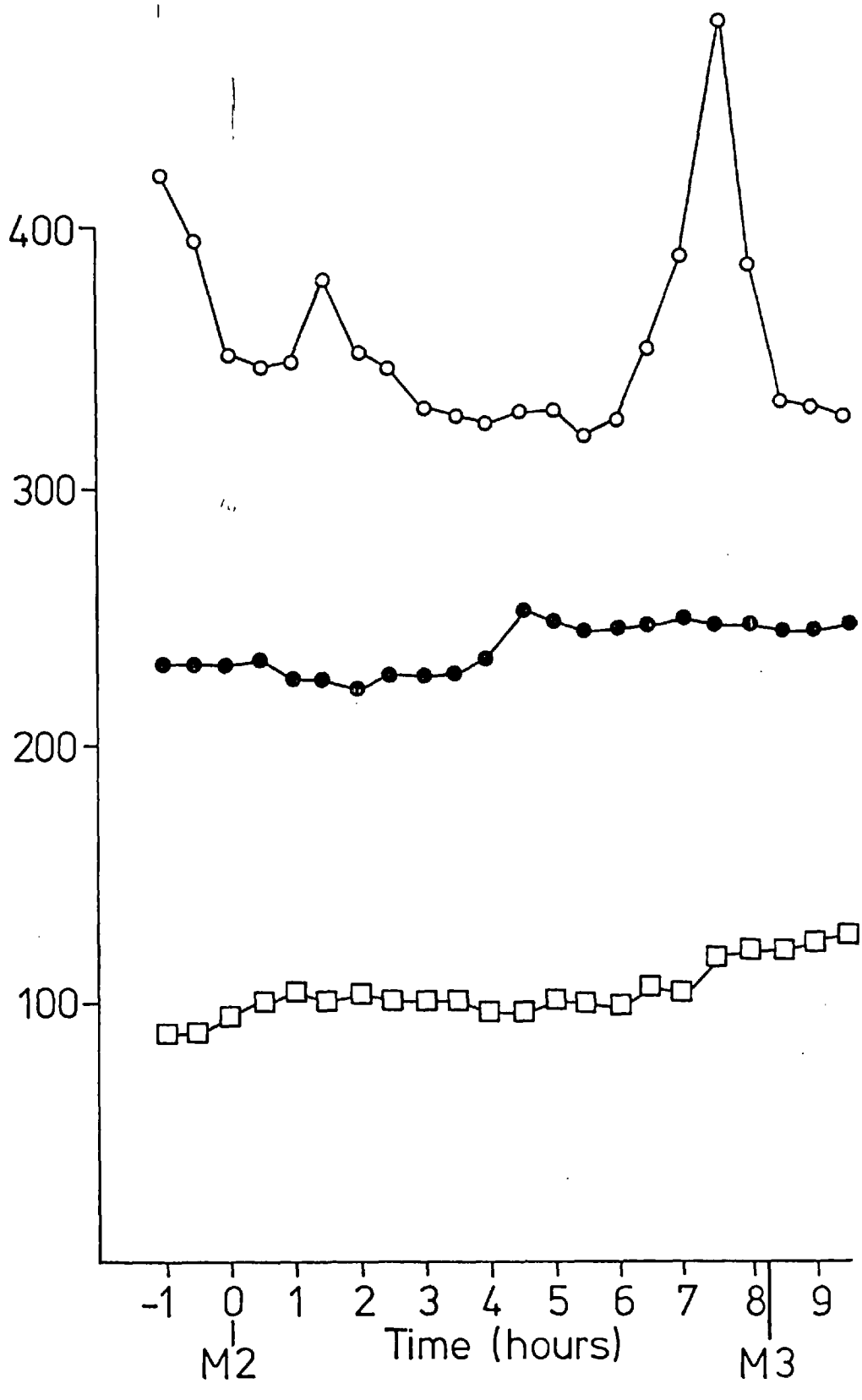


Figure 7•3

Activity of adenylate cyclase during the mitotic cycle.

o 10000g pellet, □ 100000g pellet, ● 100000g supernatant.

Ordinate = pmole cyclic AMP per 10min per mg. protein

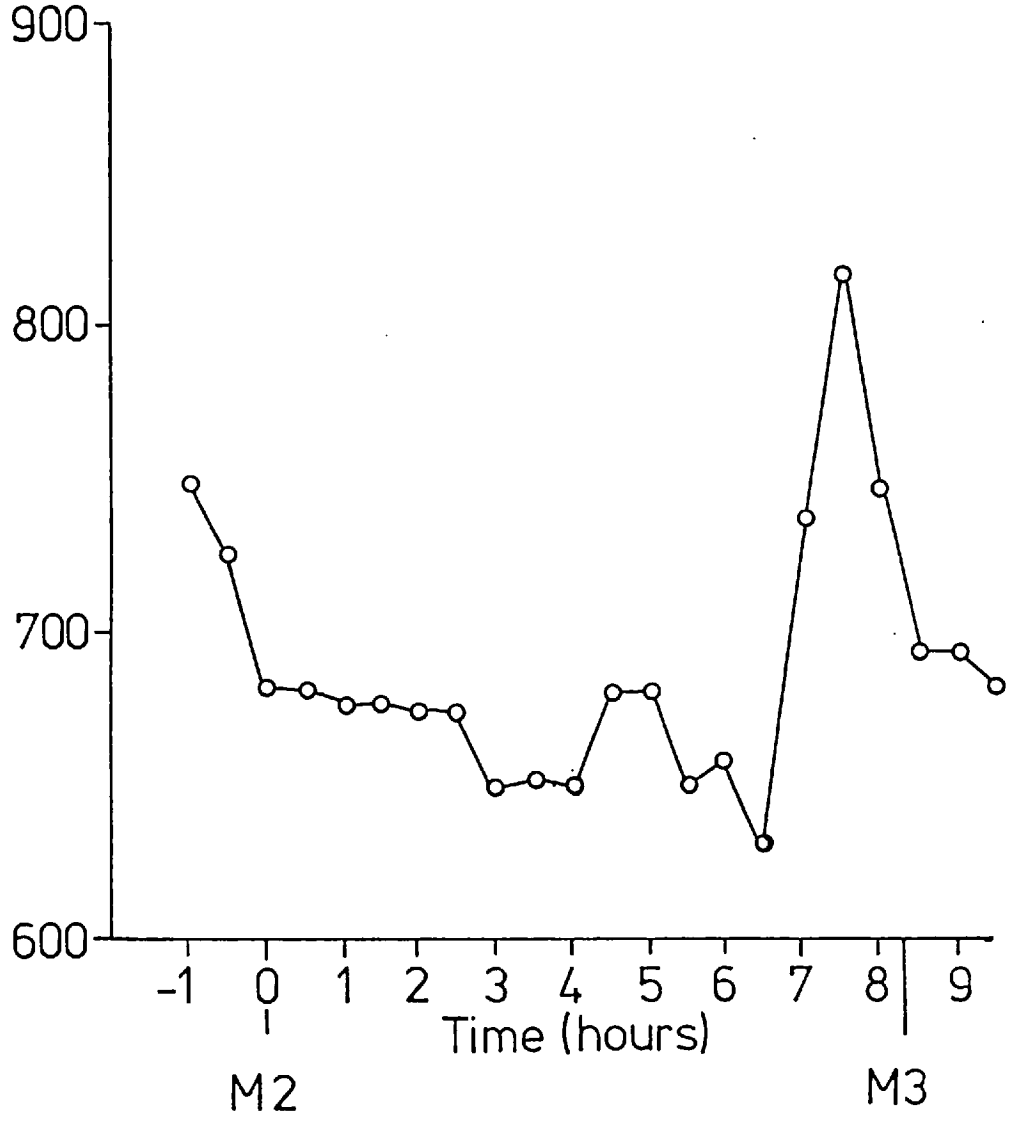


Figure 7•4

Total adenylate cyclase activity during the mitotic cycle.

(Sum of the activity of three individual fractions).

Ordinate = pmole cyclic AMP per 10min per mg. protein.

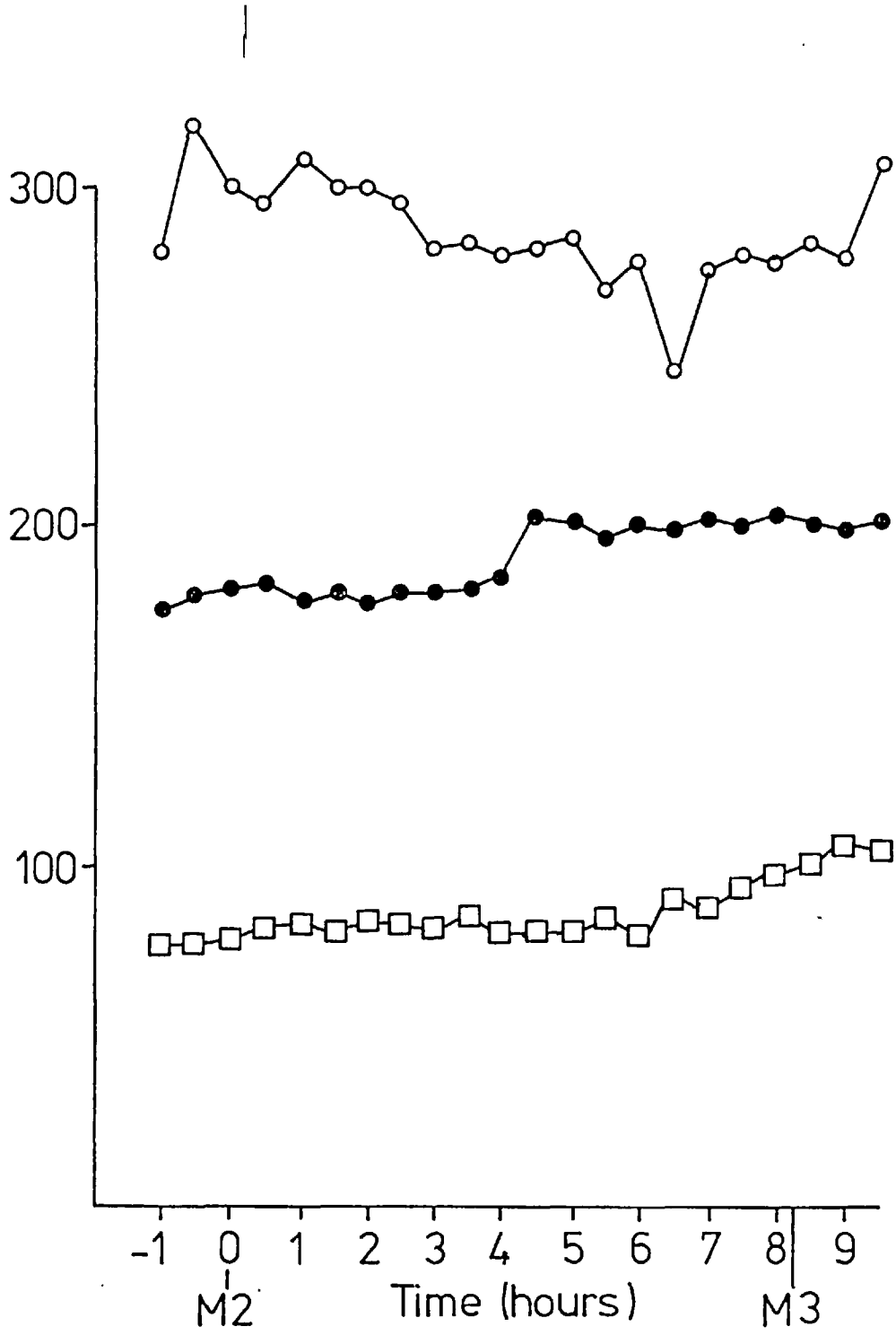


Figure 7·5

Cyclic AMP phosphodiesterase activity during the mitotic cycle. Ordinate = pmole adenosine per 10min per mg protein.

○ 10000g pellet, ● 100000g supernatant, □ 100000g pellet.

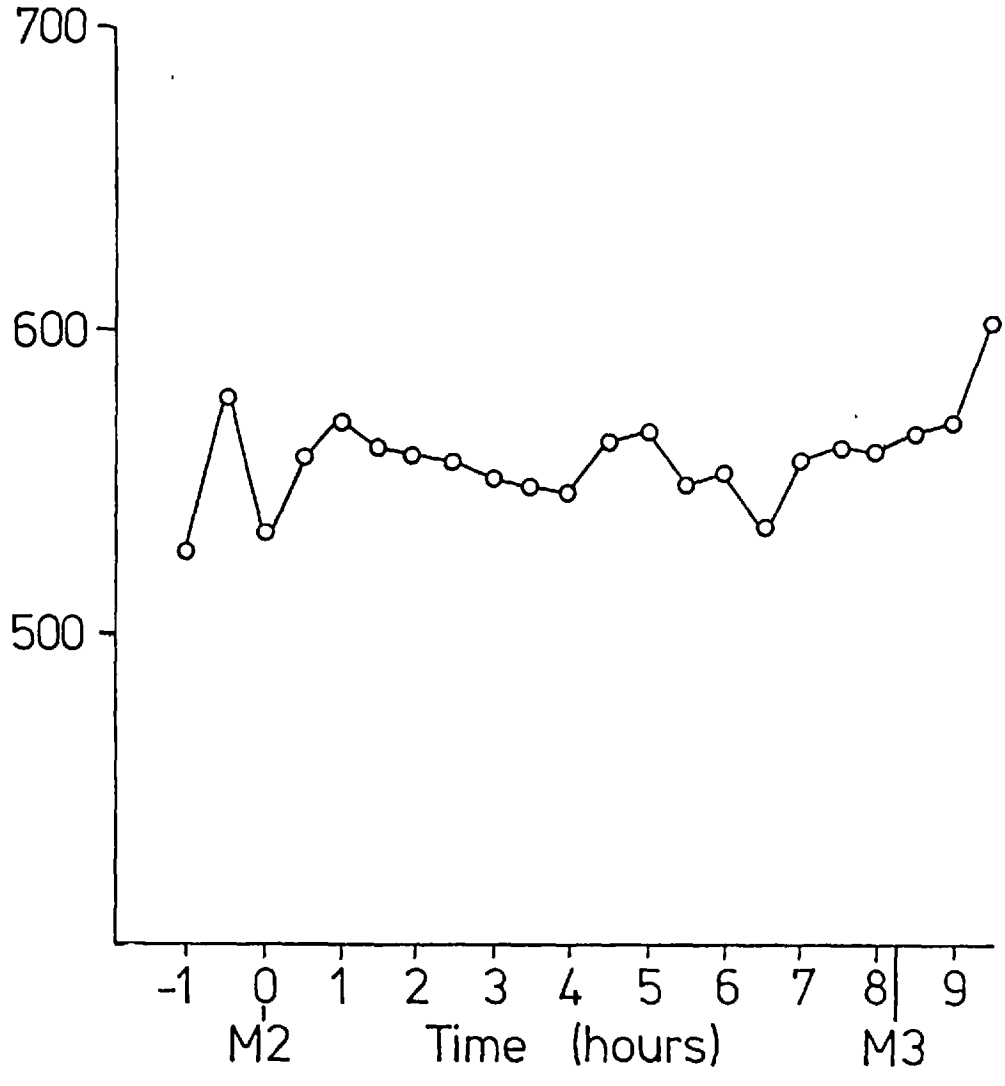


Figure 7·6

Total cyclic AMP phosphodiesterase activity during the mitotic cycle. Obtained by summing the activities of the three individual fractions. Ordinate = pmole adenosine per 10min per mg. protein.

Total adenylate cyclase activity can be seen in Figure 7·4.

7·60 Cyclic AMP phosphodiesterase activity during the mitotic cycle.

The activity of cyclic AMP phosphodiesterase (cyclic AMP PDE) during the mitotic cycle is shown in Figures 7·5 and 7·6.

Figure 7·5 shows the activity in individual fractions, the high speed pellet and supernatant show little variation during the mitotic cycle, remaining constant at 80 - 100 and 180 - 200 units (1 unit = 1 pmole adenosine formed per 10min per mg protein) respectively. Activity in the low speed pellet show more irregular behaviour but no clear trends are discernable.

Figure 7·6 shows the sum of the activities of the individual fractions. Activity remains constant throughout the mitotic cycle at about 550 units.

It must be stressed that at no time was any (^{3}H) 5'AMP detected in significant amounts (i.e. above background) thus demonstrating that a 5' nucleotidase converted all the 5'AMP to adenosine.

7·70 Guanylate cyclase activity during the mitotic cycle.

Guanylate cyclase activity during the mitotic cycle is shown in Figure 7·7.

Of the three fractions assayed, the high speed pellet is the most active showing maximal activity during the S phase and late G2. The enzyme in the 10000g pellet and 100000g supernatant have approximately the same activity, at about 10 units (1 unit = 1 pmole cyclic GMP formed per 10min per mg. protein). The peaks of guanylate cyclase in these low activity fractions during the S phase are different by 30min: the peak in the 10000g pellet

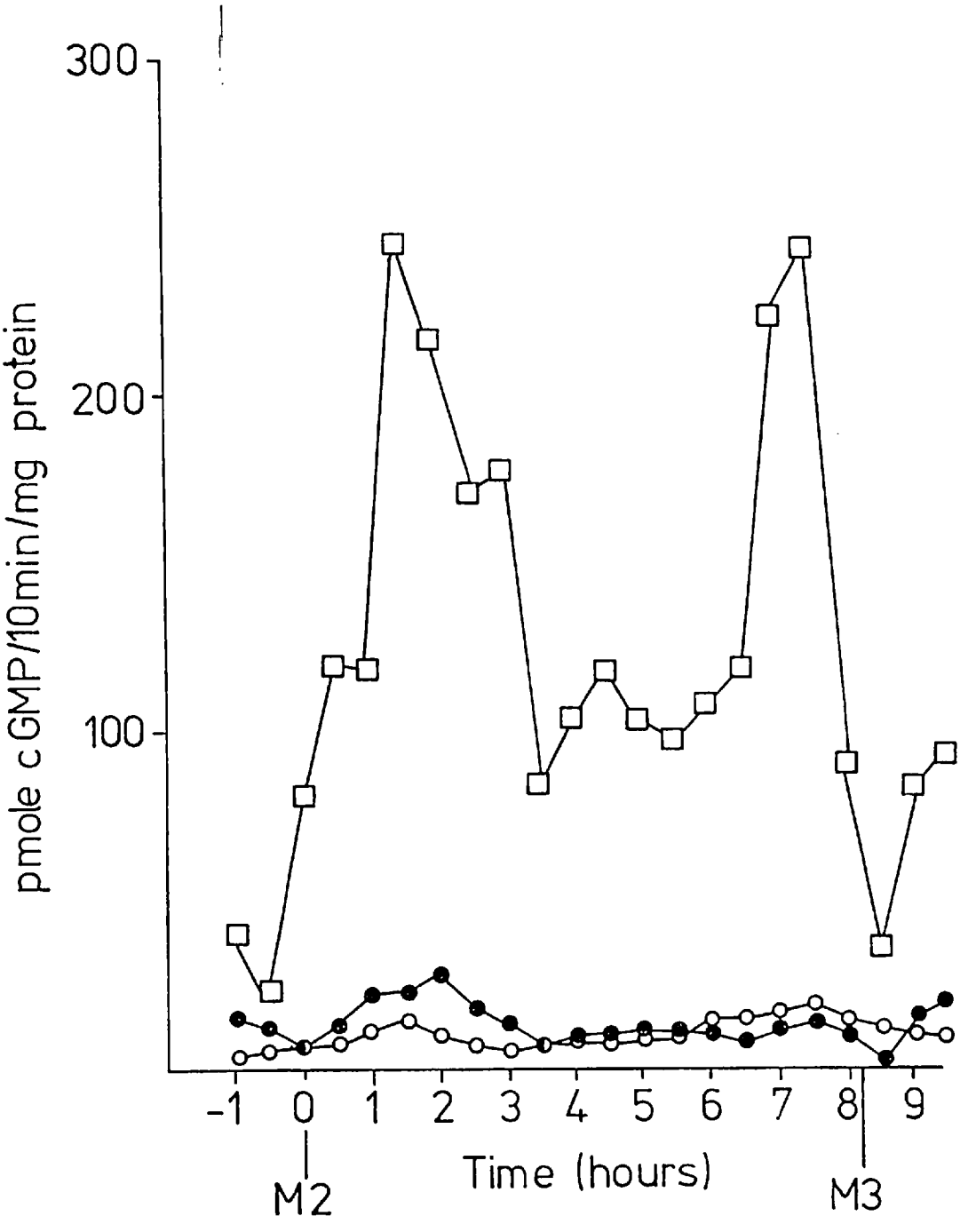


Figure 7·7 Guanylate cyclase activity in fractions of an homogenate of Physarum during the mitotic cycle.

○ 10000g pellet, □ 100000g pellet, ● 100000g supernatant.

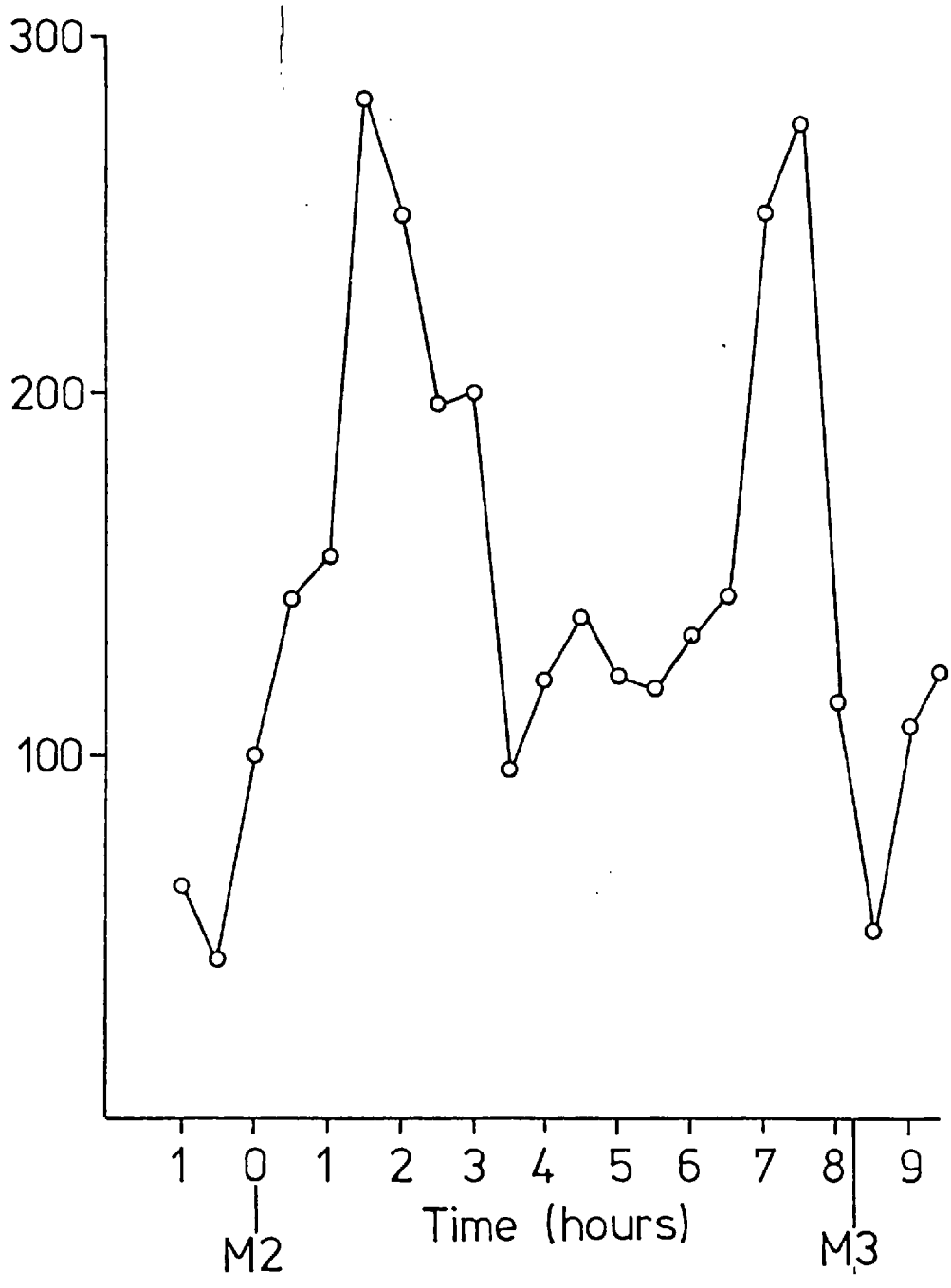


Figure 7·8 Guanylate cyclase activity during the mitotic.

Each point is a sum of the activity found in each fraction.

Ordinate = pmole cyclic GMP formed per 10min per mg. protein.

occurring first, although this may not be significant. During late G2 two peaks coincide but again this may not be significant.

Total guanylate cyclase activity, a summation of the activity in the three fractions, is shown in Figure 7·8

7·80 Cyclic GMP phosphodiesterase activity during the mitotic cycle.

The activity of cyclic GMP phosphodiesterase during the mitotic cycle is shown in Figures 7·9 and 7·10

Figure 7·9 shows the activity of the enzyme in the individual fractions. At no time, in any fraction did the activity change significantly. As with cyclic AMP PDE, because of the presence of an active 5' nucleotidase, the reaction product was guanosine, no 5'GMP was detected in significant amounts in any fraction at any time.

Total cyclic GMP PDE activity is shown in Figure 7·10.

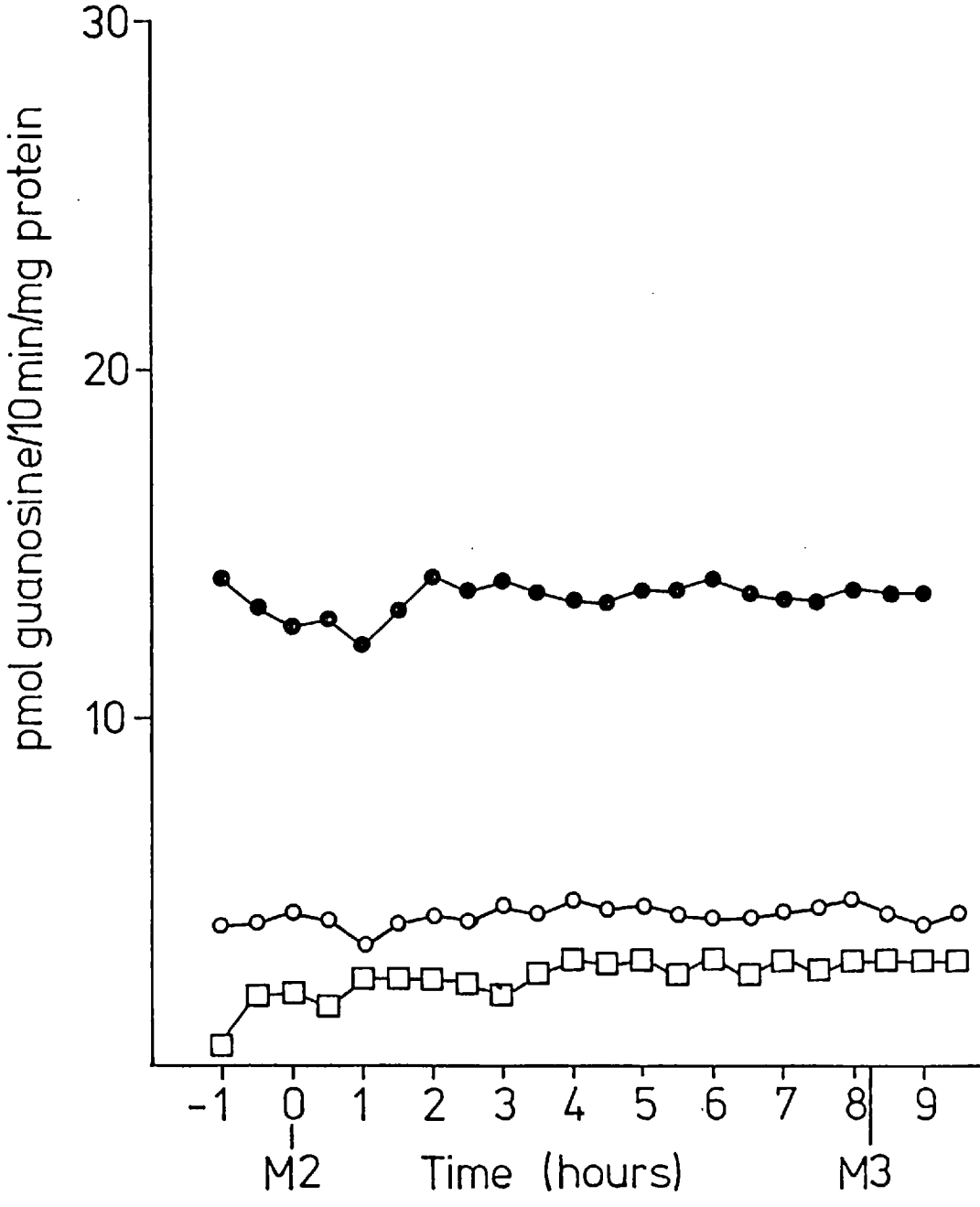


Figure 7·9 Activity of cyclic GMP phosphodiesterase during the mitotic cycle.

○ 10000g pellet, □ 100000g pellet, ● 100000g supernatant

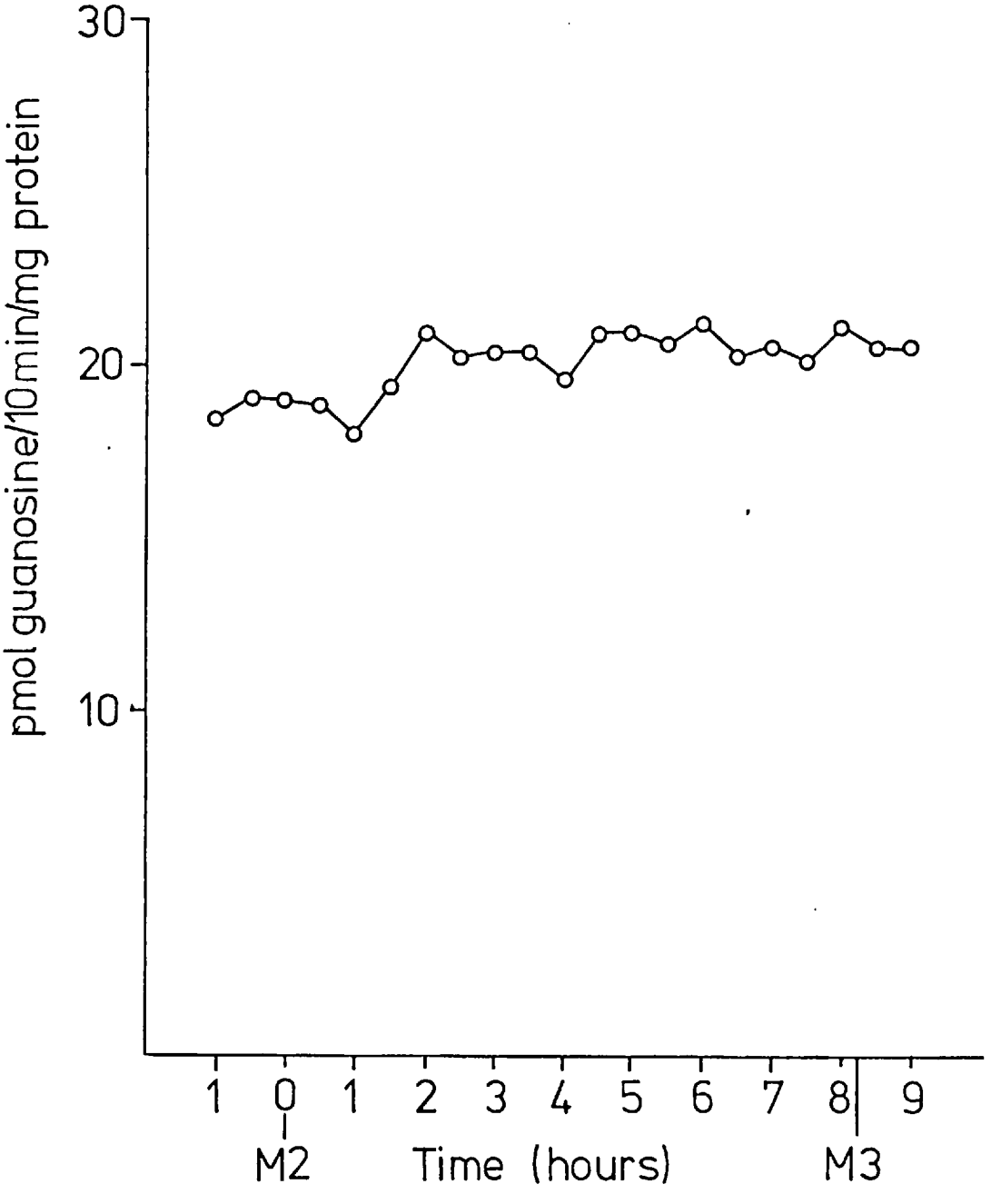


Figure 7•10 Total cyclic GMP phosphodiesterase activity during the mitotic cycle.

CHAPTER 8

DISCUSSION.

In the first part of this chapter the significance of the changes in cyclic AMP and cyclic GMP levels will be discussed with respect to other mitotic cycle events in Physarum polycephalum. Where relevent the results of similar studies on other cell systems will be mentioned.

In the second part, attention will be directed to those enzymes of cyclic nucleotide metabolism which either alone or in conjunction with other factors, regulate cyclic nucleotide levels in living cells.

The levels of cyclic AMP remained relatively constant during mitosos, S phase and the first three quarters of G2. Other workers (Friedman et al. 1976).have noted, that in animal cells, cyclic AMP is low during mitosis. The reasons for this are still obscure, it has been suggested that is is necessary for the expression of systems inhibited by cyclic AMP.

Peaks of cyclic AMP and cyclic GMP during the mitotic cycle in Physarum may be associated with the activation of cyclic nucleotide dependent protein kinases involved in the phosphorylation of histones (Langan 1971), membrane (Weller and Rodnight 1970), microtubule (Gillespie 1975), ribosomal (Jungman et al. 1975) and non histone proteins (Johnson and Hadden 1975). Some specific examples of cyclic nucleotide mediated protein kinase activity will now be discussed. Before doing so it seems only fair to point out that a functionally active form of cytoplasmic protein kinase may not be essential for cell division. Mutant mouse lymphome cells (Coffino et al. 1975) with little intracellular cyclic AMP and

no detectable cyclic AMP protein kinase activity have an intermitotic time similar to normal cells. In addition, the existance of several cell lines that exhibit subnormal cyclic AMP (Granner et al. 1968) or cyclic GMP (Nesbitt et al. 1976) levels has been used to argue against an obligatory role of cyclic nucleotides in the control of the cell cycle.

Be that as it may, several groups of workers (Otten et al. 1972, Burger et al. 1972 and Kram et al. 1973) have proposed that the rapid and transient change in cyclic AMP concentration in fibroblasts after release from "quiescence" may act as a signal that triggers the initiation of the reactions leading to DNA synthesis.

Very low concentrations of either cyclic AMP or cyclic GMP cause thymic lymphoblasts in calcium free medium to initiate DNA synthesis. These cyclic nucleotides also stimulate the initiation of DNA synthesis and cell proliferation in human and rat bone marrow cells in vivo and in vitro (Whitfield et al. 1976). Such observations therefore clearly recommend cyclic AMP and cyclic GMP as possible intracycle regulators of cell proliferation.

The peak of cyclic AMP late in G2 phase is roughly coincident with the maximum phosphorylation of H1 (F1) histone (Bradbury et al. 1973). Langan (1971) reported that cyclic AMP stimulates histone phosphorylation in liver.

Histone H1 phosphorylation in late G2 phase has been implicated in the initiation of nuclear division in Physarum (Inglis et al. 1976). The enzyme responsible is the growth associated histone phosphokinase (HKG) which is only slightly stimulated by cyclic AMP. Two other histone phosphokinases are found in non dividing or interphase cells (Langan 1971) both are cyclic AMP dependent.

Growth associated histone phosphokinase (HKG) from Physarum has been resolved into two different components with different substrate specificities and times of appearance during the mitotic cycle (Hardie et al. 1976). These two enzymes are cyclic AMP independent in vitro but the authors note that this does not necessarily mean that cyclic AMP has no effect in vivo as putative regulatory subunits may have been lost during extraction.

While protein kinases may be cyclic nucleotide dependent or independent, Kuehn (1972) has described a protein kinase in Physarum that is inhibited by cyclic AMP. For one hour in mid S phase, a time when cyclic AMP levels are low, the kinase was independent of cyclic AMP. During the rest of the mitotic cycle the kinase is inhibited by cyclic AMP. The significance of this finding remains to be determined.

In eukaryote cells, microtubules are ubiquitous structural organelles playing an essential role in the maintainence of cellular morphology and mitosis. One of the effects of cyclic AMP is the promotion of microtubule assembly (Puck et al. 1972). Cyclic AMP has been reported to stimulate the phosphorylation of tubulin, the major microtubule protein (Goodman et al. 1970). Moreover, microtubule preparations have been found to contain a cyclic AMP dependent protein kinase that can catalyse the phosphorylation of tubulin (Soifer 1973). The phosphorylation could affect the initiation or elongation steps of microtubule formation. In Physarum spindle microtubules are formed about 25 - 30 min before metaphase and are completed about 5 min before metaphase (Sakai and Shigenaga 1972). Thus the peak of cyclic AMP observed during late G2 may be related to the start of spindle formation.

The peaks of cyclic GMP in S and G2 phases may betray an involvement in the phosphorylation of ribosomal proteins because the high speed pellet is largely composed of ribosomes and polyribosomes. Ribosomal proteins from various tissues have been shown to be phosphorylated *in vitro* by a cyclic AMP dependent protein kinase (Walton *et al.* 1971). *In vivo* stimulation of ribosomal protein synthesis has also been found in anterior pituitary, mammary gland and reticulocytes (Zalthen *et al.* 1972). It seems probable that cyclic GMP dependent protein kinases may also be involved in this process, although this has not been reported.

Cyclic GMP has been shown to stimulate (^{3}H) uridine incorporation into RNA (Hadden *et al.* 1972). In *Physarum* the synthesis of RNA shows a rhythmical pattern. The incorporation of (^{3}H) uridine during mitosis is very low but increases immediately after telophase (simultaneously with the incorporation of thymidine into DNA) reaches a peak about 2 to 2·5 hours after mitosis, decreases to a lower level and them begins to rise again about 2 hours before metaphase (Mittermayer and Braun 1964 and Braun 1966).
Fifty percent of the RNA synthesised in S phase nuclei is sensitive to α amanitin, whereas G2 phase nuclei are insensitive to α amanitin, suggesting that G2 nuclei exhibit almost entirely nucleolar type RNA synthesis. (Grant 1973).

Cyclic AMP is reported to inhibit RNA synthesis by the nucleoplasmic RNA polymerase II in prostrate gland nuclei (Hechter personal communication in Anderson 1975) while in lympocyte nuclei cyclic GMP increased RNA synthesis by the same enzyme (Hadden *et al.* 1974). Phosphorylation of RNA polymerase I (nucleolar) and

RNA polymerase II increased transcription ability (Jungman et al. 1974, Martelo and Hirsch 1974).

In prokaryotes much more is known about the regulation of gene activity than in eukaryotes. In E. coli glucose is preferentially metabolised and, for example, the lac operon is supressed. The inhibitory action of glucose on transcription of the lac operon is partially due to increased excretion of cyclic AMP into the medium. Expression of the lac operon requires a cyclic AMP receptor protein (CRP or catabolite activator protein, CAP) which binds to a specific site adjacent to the RNA polymerase binding site necessary for transcription of the operons. Although evidence for the existance of operons in eukaryotes is less compelling cyclic AMP may act in a similar way (Nakanishi et al. 1973).

Polyamines have been implicated in the control of cell growth (Russell 1970). Sedory and Mitchell (1977) note that cyclic GMP concentrations during the mitotic cycle of Physarum parallel the activity of the more active A form of ornithine decarboxylase. Cyclic GMP may be involved in the activation of this enzyme which converts ornithine to putrescine. Heby et al. (1976) suggest that putrescine may play an important role in the initiation of DNA synthesis and mitosis. Two peaks of putrescine biosynthesis were observed in CHO cells; one during late G1 phase and the other just before mitosis. Heby et al. also suggested that the second peak may be necessary for mitosis. Kuehn (personal communication in Sedory and Mitchell 1976) has also observed a peak of putrescine, spermidine and spermine just before mitosis in Physarum.

If polyamines are involved in mitosis this may explain why cyclic GMP may be necessary because it activates ornithine decarboxylase at this time. Atmar and Kuehn (1977) have suggested that

polyamines mediate changes in nuclear protein phosphorylation by affecting nuclear cyclic AMP levels.

Reports on the effects of cyclic AMP on eukaryote DNA synthesis and on mitosis are contradictory. The majority of them suggest that cyclic AMP inhibits both events (Posternak 1974). However, some evidence suggests that even *in vivo* cyclic AMP accelerates DNA synthesis and mitosis in some types of normal mammalian (MacManus *et al.* 1971) and plant cells (Truelsen *et al.* 1974). DNA synthesis may be stimulated in normal proliferating cells while cells in G_0 state may be hastened into DNA synthesis. It thus appears at first sight that the role played by cyclic AMP in DNA replication and cell proliferation in normal eukaryotic cells is obscure. This is not surprising in view of the diversity of cell types studied and the complexity and interdependence of biochemical processes. There would seem no *a priori* reason why cyclic AMP should perform the same function at the same time in the cell cycle of all cells.

Furthermore, Prescott (1976) has suggested that in cells lacking a G1 phase such as *Physarum* those events necessary for the onset of DNA synthesis may be completed in late G2 phase of the previous cell cycle. If the initiation of DNA synthesis in *Physarum* is a cyclic AMP dependent event, the peak of cyclic AMP in late G2 may be responsible for this.

Many workers have investigated the effect of adding cyclic AMP or dibutyryl cyclic AMP to enhance cellular levels of cyclic AMP. As an alternative, phosphodiesterase inhibitors have been used in the expectation of raising intracellular cyclic AMP levels. Thus in *Physarum* Trevithick and Braun (1977) have attempted to raise the

intracellular concentrations of cyclic AMP by incubating macroplasmodia on medium containing caffeine or theophylline and observed that mitosis was retarted. Treatment had to begin 100 min before the expected mitosis for any effect to be observed. The delay in mitosis was found to be proportional to the length of exposure to the PDE inhibitor. However, these and other inhibitors are known to inhibit the nuclear enzyme poly (ADP) ribose polymerase in Physarum (Shall personal communication). This enzyme is tightly bound to chromatin and modifies nuclear proteins by covalently coupling a polymer (ADP - ribose) to them. The enzyme has been implicated in the control of DNA synthesis.(Whish and Shall 1974). It is known that these inhibitors have other effects on cellular metabolism - theophylline inhibits uridine transport (Kram et al. 1973) - so the unequivocal interpretation of such experiments is difficult. For this reason in vivo experiments of this kind have not been undertaken by the author.

The peak of cyclic GMP coincident with the peak of cyclic AMP in late G2 may also be linked to mitosis and DNA synthesis. Cyclic GMP dependent protein kinases have been purified from lobster tail muscle (Myamoto et al. 1973), bovine cerebellum (Takai et al. 1975), guinea pig fetal lung (Kuo et al. 1976) and silkworm pupae (Inoue et al. 1976) and are clearly distinguishable from cyclic AMP dependent protein kinases with respect to substrate specificity, biochemical properties (Takai et al. 1975) and possibly, the mechanism of cyclic nucleotide activation (Takai et al. 1976).

In contrast to the ready dissociation of regulatory (R) and catalytic (C) subunits of cyclic AMP dependent protein kinases by cyclic AMP, the cyclic GMP dependent enzyme isolated from silkworm

could not be dissociated by cyclic GMP, salt or histone either alone or in combination (Takai et al. 1976). Although Kuo et al. (1975) reported the dissociation into subunits of mammalian lung cyclic GMP dependent protein kinase by a combination of histone and cyclic GMP. De Jonge and Rosen (1977) have reported that bovine lung cyclic GMP dependent protein kinase is able to phosphorylate itself and is, in this instance, affected by cyclic AMP, cyclic GMP and histone.

Cyclic GMP has been shown to stimulate DNA synthesis in splenic leucocytes from BALB/C mice (Weinstein et al. 1974). After the adminisration of cyclic GMP or 8 bromo cyclic GMP to resting cells incorporation of (^{3}H) uridine or (^{3}H) thymidine into acid precipitable material was increased ten fold, thus it appears that cyclic GMP stimulates the synthesis of both DNA and RNA.

It is well known that divalent cations, particularly calcium, are involved with cyclic nucleotides in the control of cell division (Berridge 1975). The enzymes responsible for the degradation and synthesis of cyclic nucleotides in Physarum appear to be sensitive to calcium (Chapter 6). Cyclic AMP PDE activity is inhibited by Ca^{++} at low concentrations while increasing concentrations stimulated activity. However, manganese ions at low concentrations stimulated activity, but this was reduced with increasing concentrations. Both these ions drastically inhibited enzyme activity when present together. Calcium and magnesium were shown to be essential for maximal adenylate cyclase activity. Guanylate cyclase appeared not to be as sensitive to Ca^{++}, Mg^{++} and Mn^{++} ions. Clearly divalent cations, particularly

Ca^{++}, could be involved in the regulation of these enzymes. Unfortunately, intracellular concentrations or movements of these ions during the mitotic cycle have not been studied. Changes in Ca^{++} and cyclic AMP concentrations are related to microtubule functions; Ca^{++}, is required for microtubule assembly and both Ca^{++} and cyclic AMP are required for spindle formation (Rasmussen and Goodman 1975).

A peak of adenylate cyclase activity in the low speed particulate fraction was detected in late G2 phase. This was coincident with the rapid increase in cyclic AMP observed in the mitotic cycle. Activity in the high speed soluble and particulate fractions showed no significant changes during the mitotic cycle. Adenylate cyclase in Physarum has also been studied by Atmar et al.(1976) using ATP as substrate, they reported activities of the same order as in this study where AMP PNP was used.

Cyclic AMP phosphodiesterase was detected in the growth medium, its activity being greater than that of the internal enzyme, this potent external enzyme protects against exogenous cyclic AMP. While a 5' nucleotidase was detected in both soluble and particulate fractions of homogenised plasmodia it was absent from the growth medium. In animal cells this enzyme is often used as a marker for plasma membranes (Hardie and Stansfield 1977); in Physarum its location would not seem to be so restricted.

Cyclic AMP PDE activity in all three fractions showed no change during the mitotic cycle, this agrees with a previous report by Murray et al. (1971). It would appear that fluctuations in cyclic AMP are modulated by adenylate cyclase. This contrasts with the situation in Tetrahymena (Dickinson et al. 1976) and human lymphoid

cells (Millis et al. 1972) where an increase in adenylate cyclase and a decrease in cyclic AMP PDE activities are associated with increases in cyclic AMP. Adenylate cyclase activity in all three fractions exceeded cyclic AMP PDE activity.

The metabolism of cyclic GMP in Physarum would appear to be mediated by changes in guanylate cyclase. Activity was detected in all three fractions but a significant change in activity (2 - 3 fold) occurred in the high speed particulate fraction coincident with the changes in cyclic GMP observed in S phase and late G2 phase.

As with cyclic AMP PDE so with cyclic GMP PDE, no change in activity occurred during the mitotic cycle. Graves et al. (1976) studied cyclic GMP and cyclic GMP PDE, but not guanylate cyclase, during the mitotic cycle of Tetrahymena. Cyclic GMP varied during the cell cycle, high levels being associated with low cyclic GMP PDE activity. Again this situation was different from that in Physarum.

Cyclic nucleotides levels may also be regulated by the action of cyclic nucleotide binding proteins (Simantov and Sachs 1975), such proteins have been detected in homogenates of Physarum (Threlfall personal communication) but, as yet, little is known about them.

While it is clear that the activity of several biochemical processes can be influenced by cyclic nucleotides it is less apparent how these levels of cyclic nucleotides are regulated. In Physarum, the synthesising enzymes for both cyclic AMP and cyclic GMP seem to be more important than the degrading enzymes.

Whether cyclase activity is modulated by intracellular redistribution of divalent cations, by organic molecules or arises from

de novo synthesis at specific times remains unknown.

CHAPTER 9

SUMMARY.

9·10 Established methods for the extraction, purification and separation of cyclic AMP and cyclic GMP have been tested, improved and modified for use with Physarum. The recovery of cyclic nucleotides was monitored by the inclusion of (^{3}H) cyclic AMP or (^{3}H) cyclic GMP in the homogenate, typically recovery was 78% for cyclic AMP and 56% for cyclic GMP.

9·11 Cyclic AMP was assayed by a protein binding method using beef skeletal muscle or beef adrenal gland protein. Agreement between the two methods was excellent. Analysis of cyclic GMP was by a radioimmunoassay.

9·12 The concentrations of cyclic AMP and cyclic GMP during the mitotic cycle of Physarum have been measured at half hourly intervals from one hour before M2 until 45 min after M3 in synchronous macroplasmodia. The content of cyclic AMP was less than 1 pmole per mg. protein during mitosis, the S phase and the first three quarters of G2. During the last quarter of G2 cyclic AMP increased rapidly to reach a maximum of 17 pmoles per mg. protein at 45 min before M3 then fell to basal levels at M3.

Cyclic GMP showed two peaks, one of about 9 pmoles per mg. protein halfway through the 3 hour S phase, the other of about 6 pmoles per mg. protein roughly coincident with the cyclic AMP peak.

9·20 Cyclic AMP phosphodiesterase activity has been measured using two methods.

The well known resin method depends on the presence of a nucleotidase to convert 5'AMP to adenosine which alone remains in solution on the addition of Dowex resin. This method is known to be unsatisfactory because some (^{3}H) adenosine is removed by the resin. It has been shown here that some of the substrate remains in solution, leading to an underestimation of enzyme activity.

This method has been used to detect phosphodiesterase activity in the growth medium, ammonium sulphate fractionation succeeded in removing 63%. This enzyme and the one contained in the plasmodium have been tested for their sensitivity to isobutyl methyl xanthine, SQ20009, ATP, EDTA, imidazole, papaverine and caffeine. The internal enzyme was also found to be sensitive to Ca^{++} and Mn^{++}.

9·21 A new method was developed to separate all reaction products of the PDE action by t.l.c. on cellulose. This method has been used to measure activity during the mitotic cycle of three fractions of Physarum isolated by differential centrifugation. These were 10000g particulate (A), 100000g supernatant (B) and 100000g particulate (C) fractions. No significant change could be detected at any time. Activities of about 300 units (1 unit = 1 pmole adenosine formed per 10 min per mg. protein) (Fraction A), 180 - 200 units (B) and 80 - 100 units (C) were estimated. As Physarum contains a potent 5' nucleotidase, the reaction product of the cyclic AMP PDE present in homogenised plasmodia was adenosine, whereas the product from the enzyme in the growth medium was 5'AMP because the nucleotidase is not secreted into the growth medium.

9·30 Cyclic GMP PDE activity has been measured using a new t.l.c. method. No change in activity during the mitotic cycle could be detected in any fraction. The activity of fraction A was about 15 units (1 unit = 1 pmole guanosine formed per 10min per mg. protein), the activity in fractions B and C was about 4 - 5 units. No cyclic GMP PDE activity could be detected in ammonium sulphate fractions of growth medium.

9·40 A new method, using (^{3}H)β-γimido adenosine 5' triphosphate as substrate has been developed to measure adenylate cyclase activity with separation of substrate and product by t.l.c. on cellulose and followed by liquid scintillation counting of the isolated compounds. Degradation of cyclic AMP was prevented by 1mM IBMX.

9·41 Adenylate cyclase activity was not stimulated by fluoride. Attempts to solubilise activity in fraction A with the non ionic detergent, lubrol, were not successful.

9·42 Enzyme activity could be detected in fractions A, B and C of homogenised plasmodia. Approximately 350 - 400 units (1 unit = 1 pmole cyclic AMP formed per 10min per mg. protein) of activity was found in fraction A, 220 units in fraction B and 100 units in fraction C.

9·43 Adenylate cyclase was found to be very sensitive to the presence of divalent cations. Calcium (5mM) in the extraction buffer and magnesium (5mM) in the assay buffer, were essential for the expression of activity. Higher concentrations of calcium were found to inhibit activity.

9·44 Adenylate cyclase activity has been measured at half hourly intervals during the mitotic cycle. No change occurred in fractions B or C at any time. Activity in fraction A increased late in G2 from about 330 units to 475 units.

9·50 Guanylate cyclase activity has been measured using (^{3}H)β-γ imido guanosine 5' triphosphate as substrate. Activity in three fractions of homogenates during the mitotic cycle have been measured. Fraction C shows the greatest activity, capable of synthesising 100 - 200 pmole cyclic GMP per 10min per mg. protein. Activity in fraction A and B was similar at about 15 - 20 units. (1 unit = 1 pmole cyclic GMP formed per 10 min per mg protein.). Maximum activity occurred in the S phase and late G2 phase, coincident with the intracellular increases in cyclic GMP .

9·60 The relevance of enzyme changes to fluctuations in cyclic nucleotide levels in Physarum is discussed in relation to temporal biochemical changes in the mitotic cycle. A comparison is made between the situation in Physarum and that in other cell systems.

APPENDIX.

Vol. 71, No. 3, 1976 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

FLUCTUATIONS IN CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE AND CYCLIC GUANOSINE 3':5'-MONOPHOSPHATE DURING THE MITOTIC CYCLE OF THE ACELLULAR SLIME MOULD *PHYSARUM POLYCEPHALUM*

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SUMMARY Cyclic adenosine 3':5'-monophosphate (cyclic AMP) and cyclic guanosine 3':5'-monophosphate (cyclic GMP) have been determined at half-hourly intervals throughout the mitotic cycle of *Physarum polycephalum*. Cyclic AMP was constant at 1pmole/mg protein throughout except for a transient peak of 17pmoles/mg protein in the last quarter of G2. Cyclic GMP was more variable (2-4pmole/mg protein) rising to 9.5pmole/mg protein during the 3 hour S period and to 7pmole/mg protein during the last hour of G2. The significance of these changes is discussed.

INTRODUCTION Variations in the intracellular levels of cyclic AMP during the cell cycle of animal and human cells *in vitro* have been described (1-3) and reviewed (4-6). These changes reflect the regulatory function of cyclic AMP on cell growth (6) and morphology (7,8) and, more specifically, on several processes with distinct temporal patterns of activity in synchronously dividing cell populations. Recent reports suggest an equally important role for cyclic GMP in the cell cycle (9,10). One of the important functions of cyclic AMP and cyclic GMP is the activation (or inhibition) of protein kinases and thus the control of phosphorylation of F1 histone (11-13), nuclear acidic (14), membrane (15,16), microtubule (17-19) and ribosomal proteins (20). The natural mitotic synchrony of plasmodia of the acellular slime mould *Physarum polycephalum* provide excellent material for studies of the mitotic cycle.

MATERIALS AND METHODS (a) Culture of Physarum. Strain M_3c was a kind gift of Dr. Joyce Mohberg. Microplasmodia were grown in a partially defined medium with hemin (21). Surface plasmodia were established by inoculating a washed suspension of microplasmodia on to the centre of a filter paper disk supported on a stainless steel mesh in a 9cm petri dish. After 70 minutes the microplasmodia had fused and medium was added to wet the paper from below. The first post-fusion mitosis (M1) occurred $4\frac{3}{4}$ hours after feeding, the second (M2) 8 hours later and the third (M3) $8\frac{1}{4}$ hours after the second. Half-an-hour before M2 the medium was discarded and cultures refed with fresh medium. The time of metaphase was determined by phase contrast observation of ethanol fixed smears of small pieces of plasmodia mounted in glycerol-ethanol (1:1 v/v). Measurement of cyclic AMP and cyclic GMP was made on a single batch of cultures; determination of the duration of S period was made on a similar batch on another occasion.

(b) Extraction of cyclic AMP and cyclic GMP. Samples were taken at thirty minute intervals from one hour before M2 to one hour after M3. Four plasmodia on filter paper were frozen for five minutes in isopentane-methylcyclohexane (92:8 v/v) at the temperature of liquid nitrogen. The filter papers were withdrawn and the inoculum discarded. The remaining material was scraped off and plunged in 10ml ice-cold 5% (w/v) trichloroacetic acid (TCA) and mechanically homogenised at 0°C for 30 seconds using an ILA x-1020 mixer. 50nCi (^{3}H)cyclic AMP was added to all samples in order to monitor recovery and allowed for in the subsequent assay. To one sample only 8nCi(^{3}H) cyclic GMP was added for the same reason. The extracts were centrifuged at 17,000g for 10 minutes at 4°C and the supernatant carefully removed. The pellet was allowed to dissolve in 4ml 0.4N NaOH at room temperature for 48 hours. Protein was determined by the method of Lowry *et al.* (22) using bovine serum albumin as standard. TCA was removed from the supernatant by five extractions with an equal volume of water saturated diethyl ether and the ether layer discarded. Polysaccharides were removed by precipitation with 10ml ice-cold 95% (v/v) ethanol followed by centrifugation at 2500g for 5 minutes at room temperature. The supernatant was removed and taken to dryness in a rotary evaporator. The residue was dissolved in 1ml 50% (v/v) ethanol and loaded on to a 1x2.5cm column of Dowex AG50 X4 100-200 mesh resin ammonium form.

The tube containing the extract was carefully washed out with another 1ml 50% ethanol and this added to the column. After the sample had drained into the resin it was washed with 10ml sterile distilled water and the eluate allowed to drip into a 1.5x2cm column of QAE Sephadex A25 formate form. This column was washed with 20ml sterile distilled water and the eluate discarded. Cyclic AMP was eluted with 18ml 0.1N ammonium formate pH 9.0. Cyclic GMP was then eluted from the column with 18ml 0.1N ammonium formate pH 6.0. Each fraction was collected on a separate 1.5x5cm column of Dowex AG50 X4 200-400 mesh H^+ form. Cyclic AMP (or cyclic GMP) was eluted with 12ml 0.1N HCl. The eluate was lyophilized and stored at -20°C until assayed.

(c) Assay of cyclic AMP and cyclic GMP. Cyclic AMP was measured using a binding protein obtained from beef adrenal glands (23). Zero dose binding was 27% and the assay linear

over the range 0.3 to 8pmole cAMP per assay tube. Cyclic AMP in each extract was also measured using a commercially available kit; zero dose binding was 57% and the assay linear over the range 0.25 to 16pmole cAMP per tube. With both methods the standard curve was fitted by the method of least squares, r=0.996 and r=0.994 for the adrenal protein and kit assay respectively.
The extract was taken up in 200µl assay buffer (4mM EDTA 0.05M Tris-HCl pH 7.5) and two 50µl aliquots removed. From each 5µl was removed in order to monitor recovery. 100µl buffer was added to the remaining extract and again two 50µl aliquots removed for assay. This was repeated until four dilutions of each sample were obtained.
For the assay of cyclic GMP the extract was taken up in 400µl assay buffer and two 100µl portions removed for assay. 200µl buffer was added and again two 100µl portions removed. Cyclic GMP was measured using a commercially available kit. Zero dose binding was 37%. The assay was linear over the range 0.5 to 8pmole cGMP per assay tube; the standard curve was fitted as described above (r=0.993).
Radioactive samples were added to 8ml scintillation fluid (Sulphur-free toluene 1000ml, Triton X-100 500ml, PPO 4.4g, POPOP 0.1g) in plastic vials to which 0.8ml distilled water was then added. Radioactivity was measured to better than 2% SD in a Packard Tri-Carb Model C2425 scintillation spectrometer at 30.5% efficiency.
(d) Determination of S period. This was with the following minor modifications based on the method of Braun and Wili (24). A single synchronous plasmodium was transferred to a medium containing 5µCi (^{3}H) thymidine/ml at intervals of 20 minutes. After 15 minutes the plasmodium was plunged in 30ml ice-cold TCA-acetone (TCA 4g, acetone 50ml, distilled water 50ml). After mechanical homogenisation duplicate 5ml samples were filtered through 2.5cm glass-fibre disks GF/C(Whatman) washed twice with 10ml TCA-acetone and finally with 10ml ethanol. After drying each disk was placed in 10ml scintillation fluid (Sulphur-free toluene 100ml, PPO 4g, POPOP 50mg) contained in a plastic vial and radioactivity determined as above.
(e) Chemicals. Cyclic AMP assay kit (TRK 432), cyclic GMP assay kit (TRK 500), (^{3}H) cyclic AMP (27.5Ci/mmol), (^{3}H) cyclic GMP (19Ci/mmol) and (^{3}H) thymidine (5Ci/mmol) were purchased from the Radiochemical Centre, Amersham, U.K. Bovine adrenal cyclic AMP binding protein was purchased from B.D.H., Poole, Dorset, U.K. Other chemicals were of reagent grade and obtained from B.D.H. or Sigma (London) Chemical Co., Kingston-on-Thames, Surrey, U.K.

RESULTS AND DISCUSSION Recovery of cyclic GMP was 56%, that of cyclic AMP 75% to 79% (mean 78% SD 0.87). The results in Table 1 demonstrate the good agreement between results obtained with the two methods of analysis. Purity of the extracts and absence of interfering compounds is shown in Table 2 by the agreement between dilution and cyclic nucleotide content.

Table 1. Determination of cyclic AMP by two Methods

Sample Time (hr) M2=0	Beef Adrenal Binding protein assay (pmole)		Commercial Kit assay (pmole)	
-1	20.4	20.3	21.1	19.8
-½	9.5	9.1	10.0	8.8
+7	73.6	77.1	77.8	72.1
+7½	61.0	61.5	61.9	60.4

Duplicate samples not corrected for recovery.

Table 2. Effect of Dilution on Cyclic Nucleotide Content

Dilution	Cyclic AMP* (pmole)	Cyclic GMP (pmole)
1/1	61.3	1.9
1/2	34.1	0.9
1/4	16.5	-
1/8	8.0	-

Each value an average of two determinations on +7½ hr. sample not corrected for recovery.

*beef adrenal binding protein assay.

Figure 1 shows that during mitosis, the S period and the first three-quarters of G2 cyclic AMP remained constant at less than 1 pmole cyclic AMP per mg protein. Within the last quarter of G2 cyclic AMP rose rapidly to 17pmole per mg protein to return to a basal level by mitosis 3. Two samples taken before M2 showed a decrease from a high level; this suggests that a similar peak occurred before M2. Two clear peaks of cyclic GMP were detected. The duration of the larger coincided with

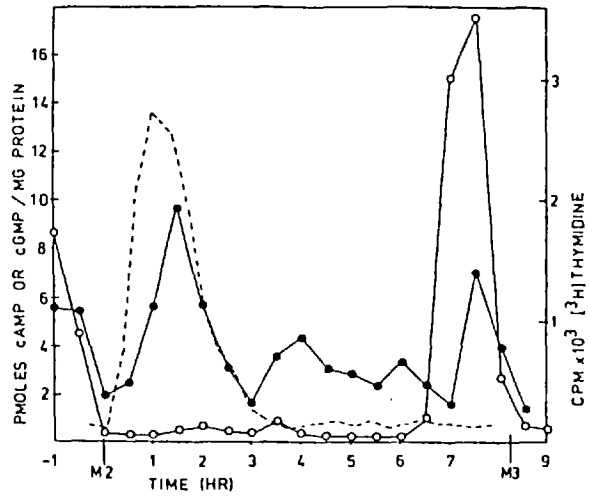

FIGURE 1. Cyclic AMP (open circles) and cyclic GMP (solid circles) content of synchronous plasmodia of the slime mould *Physarum polycephalum* from 1 hour before M2 to 1 hour after M3 (M2 = 0 hr.). Uptake of tritiated thymidine (dotted line) by synchronous plasmodia (See methods). Duration of S period 3 hours, G2 period 5 1/4 hours.

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the S period rising to 9pmole cyclic GMP per mg protein at the time of maximum incorporation of labelled thymidine. The smaller peak (7pmole cyclic GMP per mg protein) coincided with that of cyclic AMP late in G2 but showed a thirty minute lag in its onset. Again results for the hour before M3 parallel those for the same period before M2.

Bradbury *et al.* (11) have shown nuclear histone phosphorylating activity in *Physarum polycephalum* increases from a minimum at metaphase to a maximum late in G2. The peak of cyclic AMP and cyclic GMP is at a time when F1 histone phosphorylation is maximal and probably betrays the involvement of these cyclic nucleotides in the regulation of distinct protein kinases.

The time of mitosis in a plasmodium early in G2 can be accelerated by the application of homogenised material; that from a plasmodium late in G2 is more effective than that from a plasmodium early in G2 (25). This may result from the

addition in the homogenate of cyclic AMP and cyclic GMP and (or) enzymes capable of their net synthesis. This hypothesis may also explain why, when halves of two plasmodia each in a different phase of the mitotic cycle are fused, all the nuclei divide synchronously at a time intermediate between the time of mitosis in the unfused halves (26). Clearly the regulation of cyclic AMP and cyclic GMP will depend not only on the kinetics of the regulatory systems and their distribution within the cell but also on how they interact when two plasmodia are fused.

Recently cyclic GMP has been implicated in the initiation of cell proliferation (9,10). In Physarum the peak of cyclic GMP during the S period suggests that it is tied in some way to DNA synthesis. One way in which it might act is to stimulate DNA dependent RNA polymerase and RNA synthesis as has been shown to occur in lymphocytes (10,27). The RNA so formed could act as a primer of DNA synthesis. Evidence for this in Physarum is provided by the observation that pulse labelled DNA is attached to RNA (28). The role of cyclic GMP during the S period is likely to be quite distinct from the part it plays late in G2.

Some of these problems mentioned above are under further investigation in our laboratory.

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