STUDIES ON THE ADENYLATE CYCLASE SYSTEM OF *TETRAHYMENA PYRIFORMIS*

by

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ABBREVIATIONS

Abbreviations

Abbreviations were used as recommended by the instructions to authors of the Biochemical Journal. However, some non-standard abbreviations and abbreviations in regular use throughout this thesis are included below.

Organisms;

E.coli	;	Escherichia coli
T.pyriformis	;	Tetrahymena pyriformis
S.cerevisiae	;	Saccharomyces cerevisiae

Enzymes;

adenylate cyclase	;	EC 4.6.1.1 ATP pyrophosphate-lyase(cyclizing)
cyclic nucleotide phosphodiesterase	;	EC 3.1.4.c N-ribosyl(deoxyribosyl)-purine-3';5'-cyclic phosphate N-ribosyl(deoxyribosyl)-purine-5'-phosphate phosphohydrolase
cyclic nucleotide-dependent protein kinase	;	EC 2.7.1.37 ATP protein phosphotransferase

in tables only;

A.phos	acid phosphatase
5'Nu	5' nucleotidase
Arg.K	arginine kinase
LDH	lactate dehydrogenase
SDH	succinate dehydrogenase

Chemicals;

ATP & deoxyATP	adenosine 5' triphosphate (&2'deoxy ATP)
5'AMP & 5'dAMP	adenosine 5' monophosphate (&2'deoxy AMP)
BSA	bovine serum albumin
Butyl BPD	2-(4'-t-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole
cyclic AMP	adenosine 3';5' monophosphate
cyclic GMP	guanosine 3';5' monophosphate
dibutyryl cyclic AMP	N^6-2'-O-dibutyryl cyclic AMP
DEAE-cellulose	diethylaminoethyl-cellulose
DCPIP	2,6-dichlorophenolindophenol
DNA	deoxy ribonucleic acid
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid

$G_{pp}(NH)_p$	5'guanylyl imidodiphosphate
GTP	guanosine 5' triphosphate
NADH	nicotinamide adenine dinucleotide (reduced)
NAD	nicotinamide adenine dinucleotide
P_i	inorganic phosphate
PP_i	pyrophosphate
PPO	2,5-diphenyloxazole
POPOP	1,4-di 2-(5-phenyloxazolyl) benzene
PMS	phenazine methosulphate
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
Tris HCl	tris(hyroxy methyl) aminomethane hydrochloride

SUMMARY

An assay suitable for the estimation of adenylate cyclase activity from Tetrahymena pyriformis has been developed and validated.

A proportion of adenylate cyclase activity was found to be isolated in the cilia of T.pyriformis and a reasonable interpretation of the results of cell fractionation would be that adenylate cyclase was wholely located in the cortical compartment of T.pyriformis. Intact isolated cilia proved to be the only stable preparation of adenylate cyclase from this organism. ATP can enter isolated cilia, therefore, adenylate cyclase can be assayed without the disruption of membrane system. Thus intact isolated cilia may prove to be a useful model system for this enzyme in vivo.

Adenylate cyclase activity was supported by a number of divalent metals. At the same concentration in excess of ATP, Mn^{2+}, Mg^{2+}, Cd^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+} supported activity,which was greatest with Mn^{2+}(75 pmole/min/10^6cells) and declined in this order to Zn^{2+}(6 pmole/min/10^6cells). The inhibitory effect of an endogenous cation was removed by 1mM EDTA or EGTA. This was probably calcium. Cations which did not support adenylate cyclase activity were Ca^{2+}, Cu^{2+}, Ni^{2+}.

Of the hormones tested, only serotonin in the presence of GTP or $G_{pp}(NH)_p$ activated adenylate cyclase activity reproducibly. At 10^{-4}M serotonin and 10^{-5}M guanine nucleotide this activation was still only 10%. This is the first reliable demonstration of hormone activation of adenylate cyclase in T.pyriformis and the first example of a synergism between a hormone and guanine nucleotides in a microorganism. 10mM NaN_3 stimulated adenylate cyclase activity by 30%. 5mM NADH inhibited activity by 40% and in contrast to the other reports of adenylate cyclase from T.pyriformis,10mM NaF inhibited activity by 70%. Compounds which were found to be ineffective were l-epinephrine, with and without GTP or $G_{pp}(NH)_p$, these nucleotides alone, glucagon, spermidine HCl, glucose-6-phosphate, pyruvate and adenosine.

Adenylate cyclase activity reached a maximum during the deceleration of cell proliferation in asynchronous batch cultures grown with shaking on glucose supplemented medium. This variation in activity could be induced by making a rapidly proliferating culture hypoxic. This was prevented by 2.5μg/ml cycloheximide.

A technique suitable for the selection of large scale synchronous populations of T.pyriformis was developed in co-operation with J.R.Dickinson. This is essentially the same as that described by Hildebrandt and Duspiva (38) and depends upon the variation of phagocytotic activity during the cell cycle. After incubating the cells with iron particles for 12 min only cells in amitosis, which have not ingested iron, can escape a magnetic field. Analysis of the subsequent cell cycle showed that adenylate cyclase activity was lowest immediately after cell division and reached a maximum just before cell division.

The present state of our knowledge of the cyclic AMP metabolism of T.pyriformis has been discussed with particular reference to the cell cycle. A novel model for the control of the cell cycle by cyclic AMP has been proposed.

PART I
INTRODUCTION AND LITERATURE REVIEW

Introduction

Since its discovery in 1957 (1), cyclic AMP has provided much material to foster controversy among Biochemists. Could cyclic AMP be the single major controlling element in the eukaryotic cell or were the observed fluctuations in the cyclic AMP content of cells merely a secondary effect? Other regulators have been proposed, such as metal ions, energy balance and other cyclic nucleotides, which may be equally controversial. Let us agree that the many processes of the eukaryotic cell must be regulated and controlled by a number of elements and that these elements may act independently, in a conflicting manner or in a co-operative manner to maintain the viability of the cell.

It is now widely recognized that cyclic nucleotides provide an important part of the regulatory capacity of both prokaryotic and eukaryotic cells, although the importance of cyclic nucleotides in the higher plants is still unclear. Cyclic AMP can alter the flux of metabolic pathways of eukaryotes by regulating the activity of enzymes, the amount of enzyme present or substrate availability (2,3). The mechanism of this action has been very fully defined in a number of cases but, where information is still vague, the possibility that cyclic AMP is functioning by the mobilization of some other primary effector, such as calcium, cannot be discarded.

Many investigators have used cells derived from the higher eukaryotes when studying the function of cyclic AMP. A primary feature of these cells is their ability to communicate via the hormone system. Many hormones elicit a response in their target tissue by activating a plasma membrane bound adenylate cyclase. This aspect of a differentiated organism may mask an important role for cyclic AMP in the maintenance of "balanced growth" of individual cells. To investigate the function of cyclic AMP in an organism where intercellular communication should be of little importance, a unicellular eukaryote was chosen. The protozoan, Tetrahymena pyriformis, represents a well-studied and convenient organism for such research.

In my discussion of the protozoan, T.pyriformis, I shall concentrate on the role of cyclic AMP and adenylate cyclase, although other elements of the regulatory system of this organism may interact with cyclic AMP in some way, as yet undefined. N.D.Goldberg has suggested that cyclic AMP and cyclic GMP may operate in a co-ordinate, yet opposing manner to influence the metabolism of a cell (4). Cyclic GMP, cyclic GMP phosphodiesterase and cyclic GMP-dependent protein kinase have been reported in Tetrahymena (5,6).* The induction of thymidylate synthetase activity in Tetrahymena apparently involves cyclic GMP (7). M.J.Berridge has reviewed the proposals that Ca^{2+} may play a central role in the mediation of the effects of cyclic nucleotides, particularly on the control of cell division (8). T.pyriformis will accumulate calcium in storage granules under certain conditions (9) and cyclic nucleotides may be involved in the mobilization of this store. Where relevant, I shall include short summaries of work carried out on the higher eukaryotes and discuss the importance of this work and its relationship to T.pyriformis.

In Chapter 2, I shall discuss the adenylate cyclase system of mammalian cells, of the bacteria and of eukaryotic microorganisms. In this way, one should be able to predict some of the properties of adenylate cyclase from T.pyriformis.

*(Cyclic AMP and cyclic GMP have opposing effects on ciliary regeneration in T.pyriformis (70).)

Chapter 1

Tetrahymena pyriformis

The role and metabolism of cyclic AMP in this organism

Section 1.1 Tetrahymena pyriformis (10,11)

Although most commonly a free-living, microphagous organism in its natural freshwater habitat, T.pyriformis can survive as an accidental parasite in the body cavity of aquatic invertebrates, where it can utilize a purely liquid diet. This normally results in the fatality of the host.

The development of axenic media suitable for T.pyriformis has allowed the convient growth of this organism in the laboratory and the production of large quantities of biological material when required. Successful fully-defined media have contributed to the knowledge of the biochemistry of this organism.

Section 1.1i The life cycle

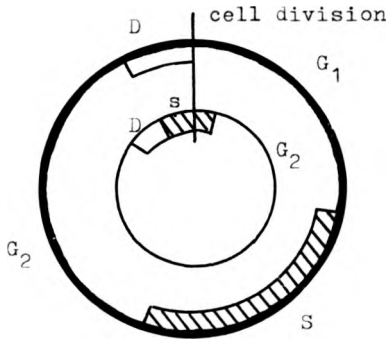


Fig.1.1 The cell cycle of a micronucleate strain of T.pyriformis
outer circle; macronucleus
inner circle; micronucleus

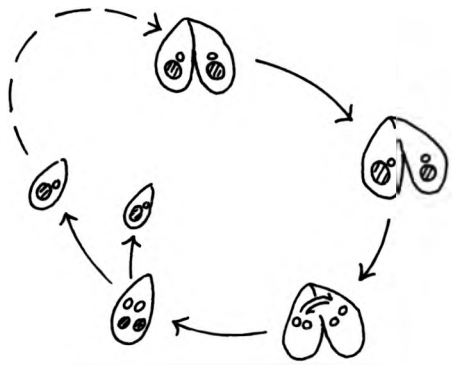


Fig.1.2 The life cycle of a sexually competent strain of T.pyriformis
⊘ ; macronucleus
o ;micronucleus

Most natural strains of T.pyriformis contain two nuclei which replicate their DNA and divide at different times during the cell cycle, in normal vegetative growth (Fig.1.1). Under conditions unfavourable for vegetative growth, opposite mating types of strains within the same syngen will undergo sexual reproduction (Fig.1.2). Following conjugation, the micronuclei undergo meiosis and two

fusion nuclei are formed from the meiotic products. Two mitoses of each fusion nucleus produces four nuclei per cell, two micronuclei and two to become macronuclei to replace the old macronucleus which degenerates. Cell division finishes the procedure which may not be repeated until the cells become sexually mature, after several generations of vegatative growth.

Some amicronucleate strains are found in the wild and long term growth under laboratory conditions has produced several more strains deficient in a micronucleus. Amicronucleate strains are asexual and always reproduce by vegetative growth. The amicronucleate strain T.pyriformis strain W has been used throughout my research and is a far simpler organism to handle and to study, than the micronucleate strains.

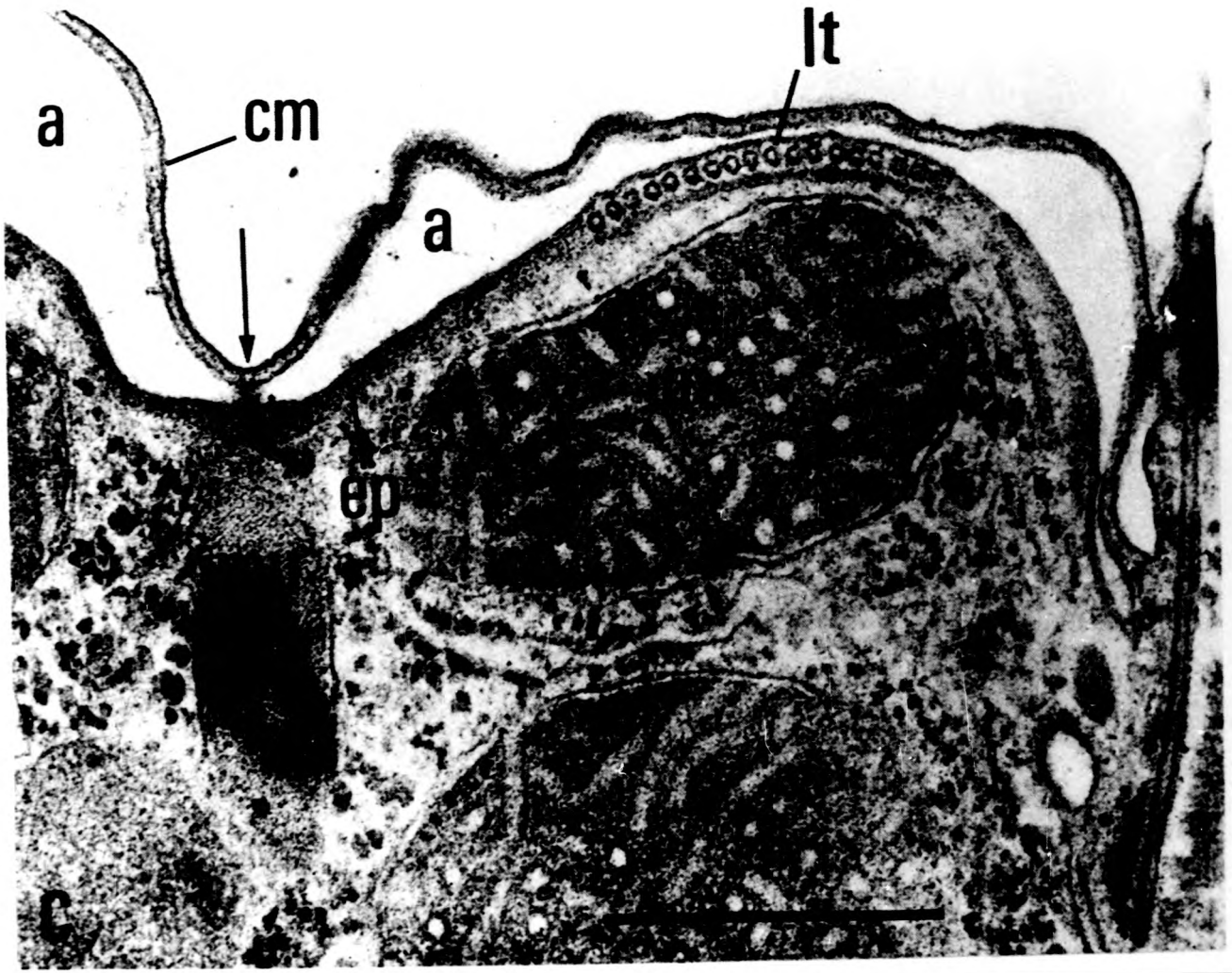
Section 1.1ii The morphology of Tetrahymena

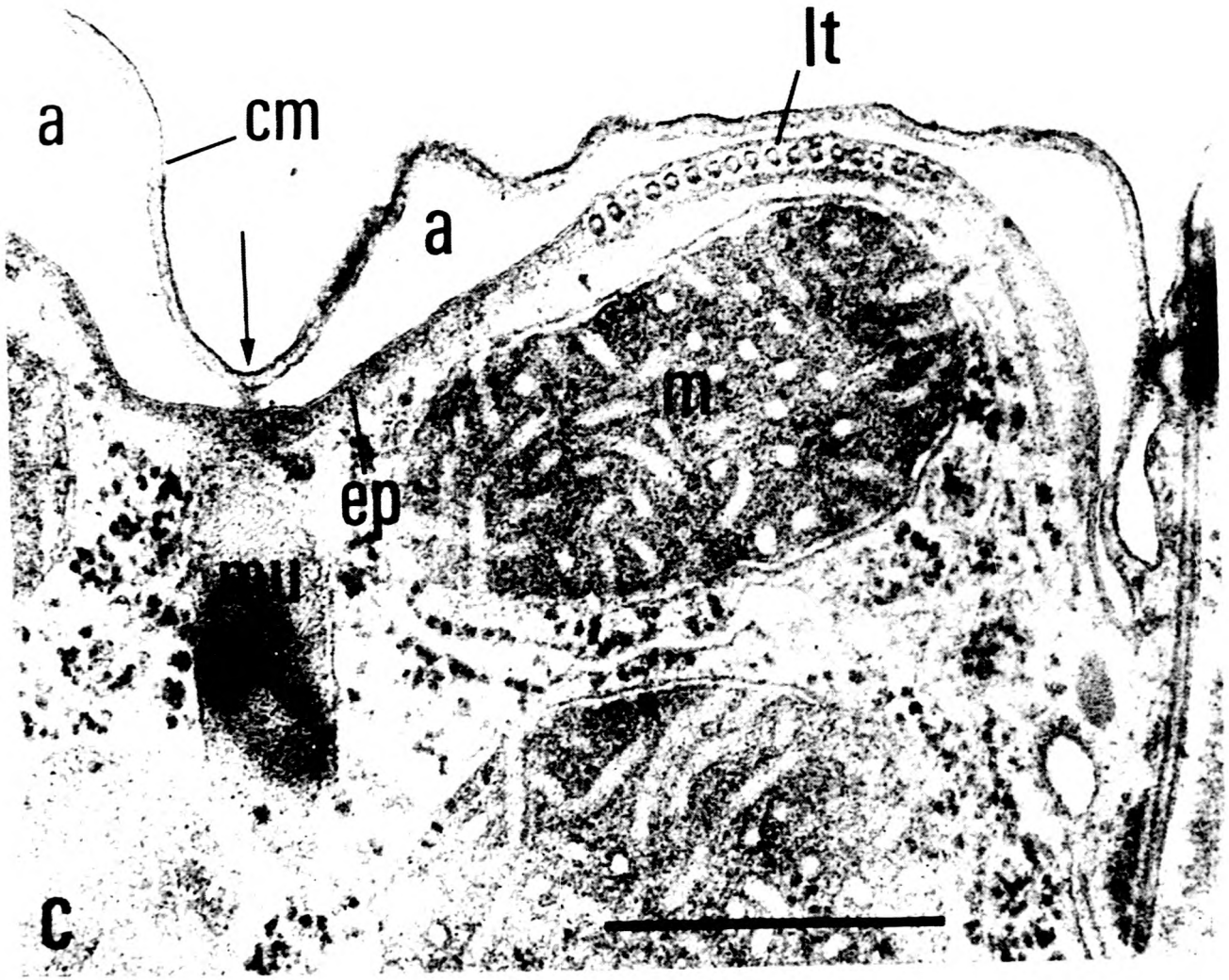
The morphology of T.pyriformis has been reviewed in detail A.M.Elliot and J.R.Kennedy (12), however I shall present some relevant aspects of the morphology.

(a) The nuclei

If present, the micronucleus shows five pairs of chromosomes during mitosis. In vegetative growth, this nucleus is inactive and shows no messenger RNA synthesis. The macronucleus contains a similar histone complement to the higher eukaryotes, however H1 and H3 are absent from the micronucleus (13,14). The ability to transcribe DNA may be a function of chromatin structure in T.pyriformis. Although obviously containing the same genetic material, the macronucleus is hyperpolyploid with approximately 45to 90 granular chromatin aggregates visible with Feulgen staining. Nucleoli, 500-1000, are peripherally located in the macronucleus and represent sites of RNA synthesis. Although division of the macronucleus is amitotic, microtubules are apparently essential for this process as colchicine inhibits cell division by blocking amitosis rather than cytokinesis.

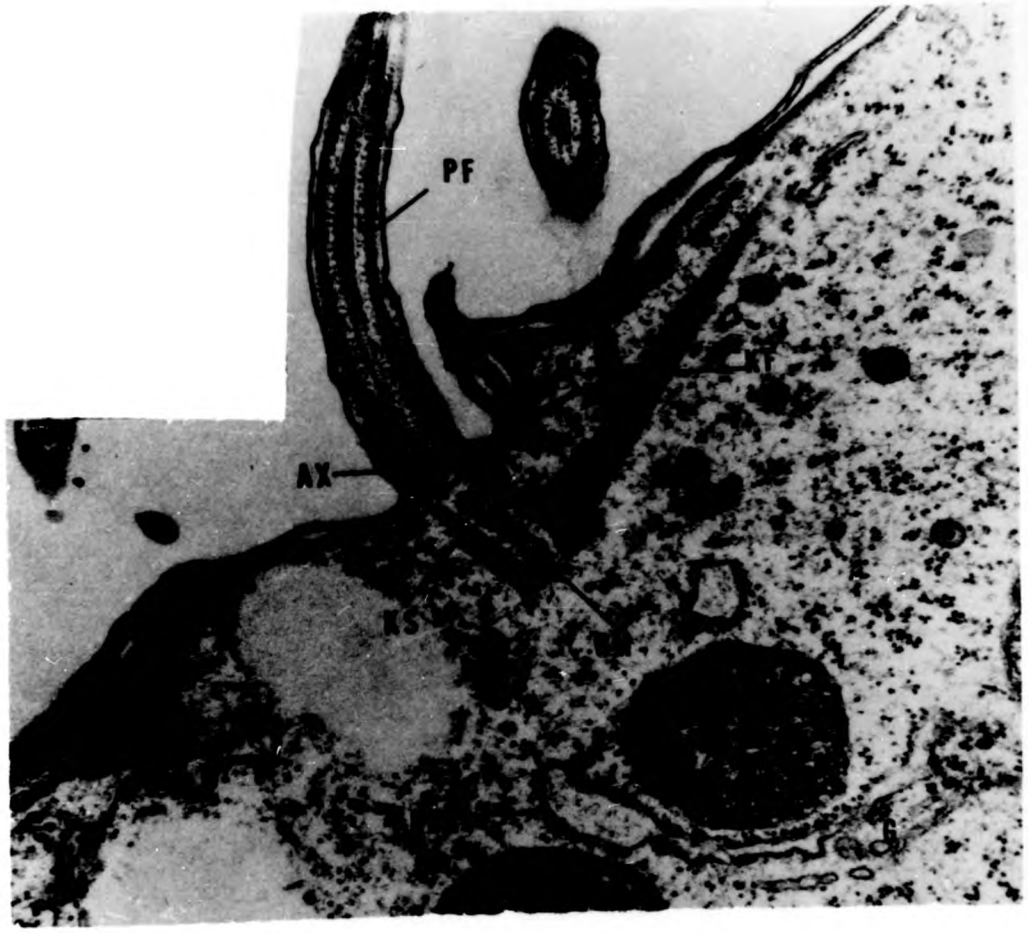
Fig. 1.3 The bounding membrane system of T.pyriformis. Magnification x 62,000. The outer-limiting membrane has been identified by"cm", note that this is continuous with the ciliary membrane. The outer and inner alveolar membranes surround the alveolar sac, "a". Mitochondria, "m", and a mucocyst, "mu", are also present. The microtubular structure underlying the inner alveolar membrane, "lt", is interesting and may be involved in the determination of cell shape, in transmembrane control etc. (Electronmicrograph courtesy of ref. 193)




a
cm
lt
a
m
ep
mu
c

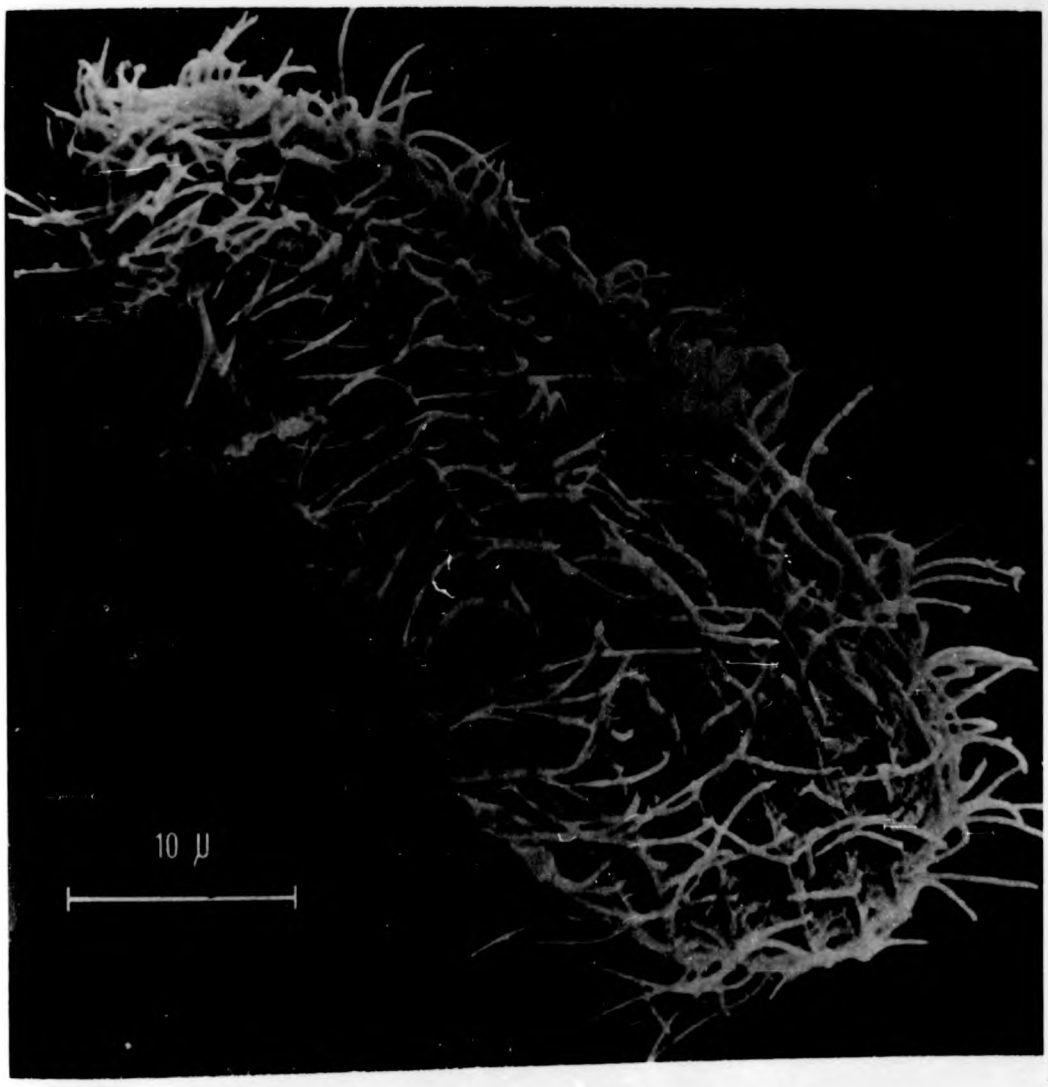
lt
a
cm
a

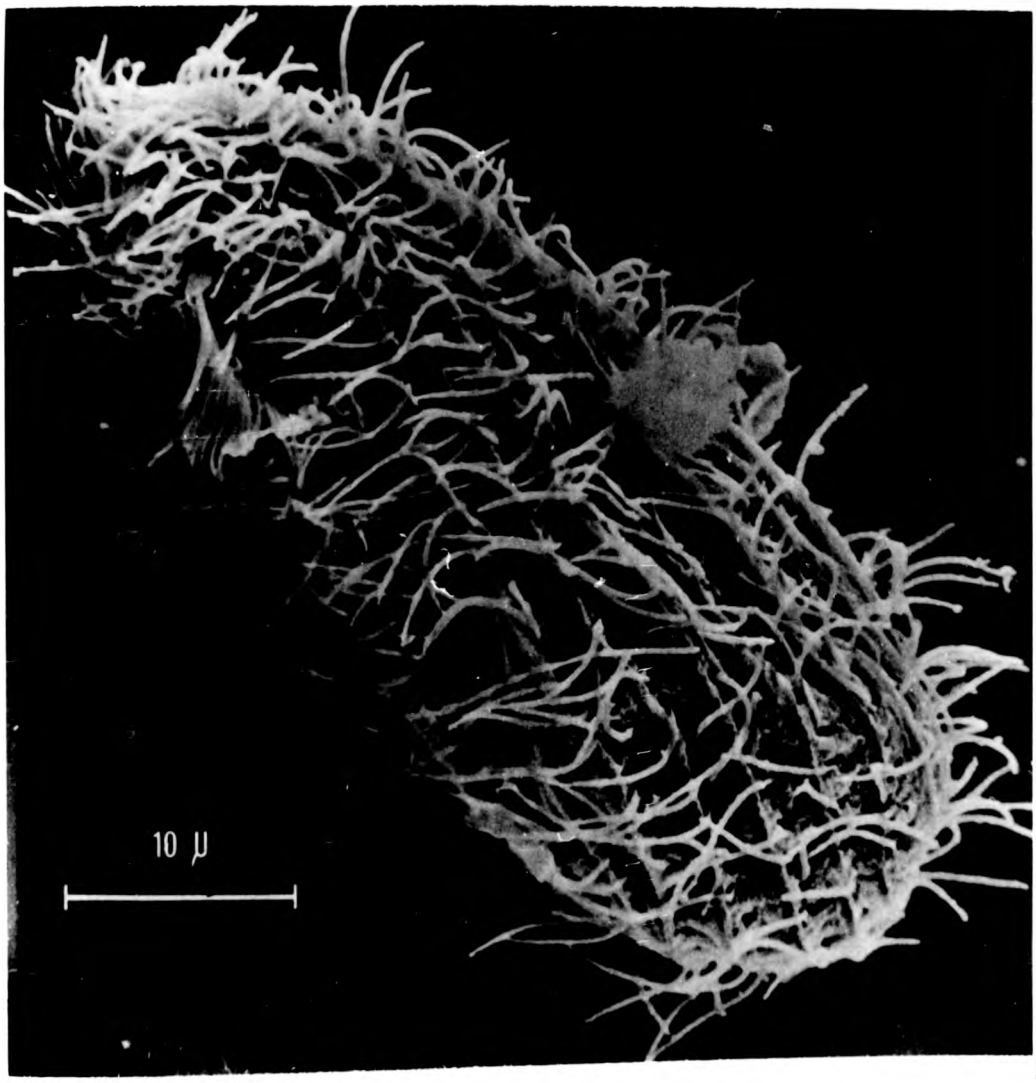
Fig. 1.4 A cilium of T.pyriformis. Magnification x35,000 approx. The longtitudinal section of a cilium shows the structure of the basal body clearly, with kinetosome, "KS", template, "TP", and matrix, "GM". Kinetosomal fibre, "KF", and axenome, "AX" are also clearly depicted. A second cilium is present in cross-section. (Electronmicrograph reproduced from ref.10).

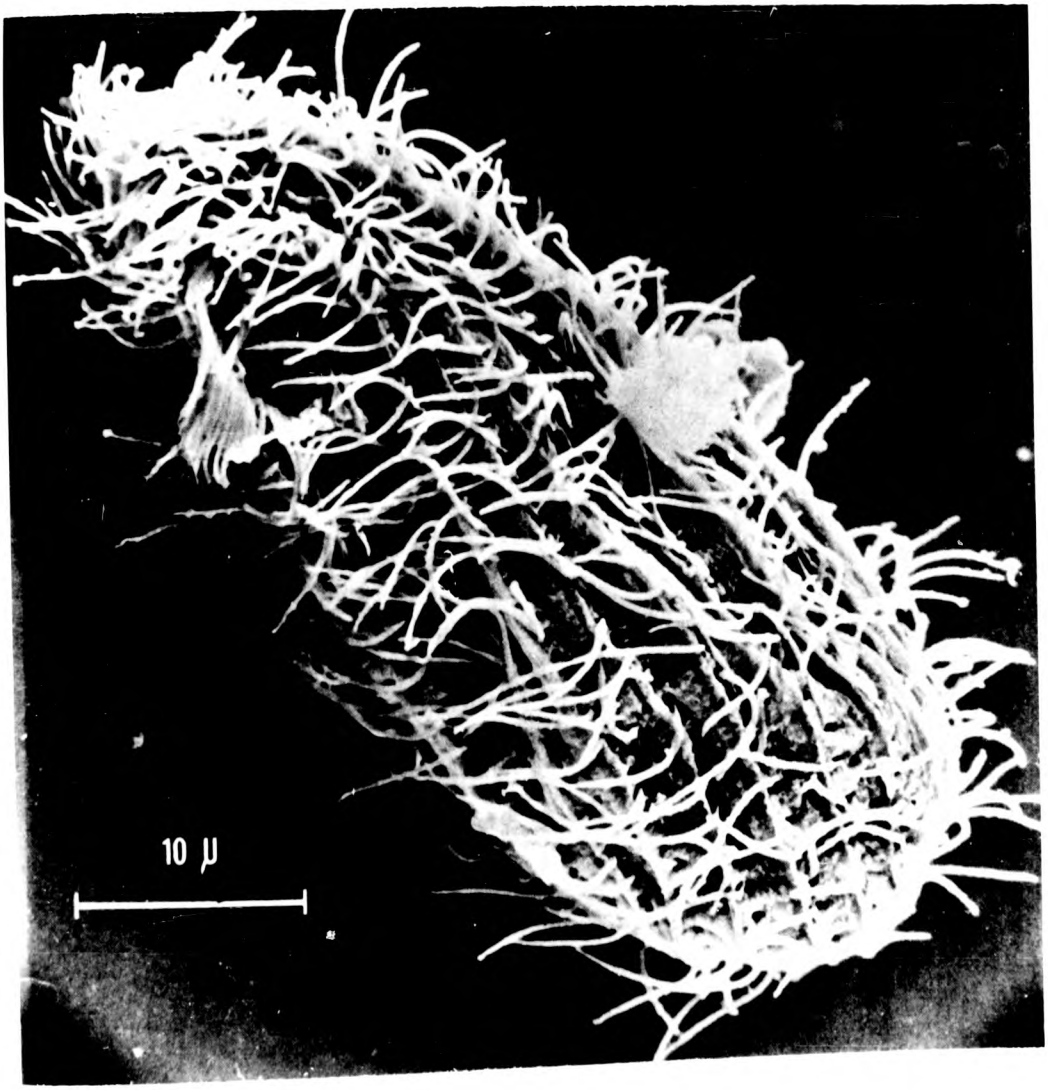



PF
TP
KF
AX
KS
GM
M
PER
G

Fig. 1.5 Tetrahymena pyriformis. (Scanning electronmicrograph reproduced from ref.183).






10 μ

(b) The cortex

The cell is bounded by a system of three membranes and the ciliary components which make up the cortex. The outer-limiting membrane is continuous with the single ciliary membrane. The outer alveolar membrane and the inner alveolar membrane, which are continuous, surround the alveolar sac which lies beneath the outer limiting membrane (Figs.1.3 & 1.4).The outer limiting membrane and outer alveolar membrane are connected by a series of cross-bridges which maintain a constant distance between these membranes. The outer limiting membrane presumably contains the components of the transport systems which control the intracellular environment.

The functional repeating unit of the cortex includes the kinetosome, its cilium and an associated system of fibres and microtubules. This unit is organized with the other cortical components to produce the kineties, the primary meridians visible on the cell surface (Fig.1.5). The kinetosome is rather interesting because of the reports that this organelle carries its own DNA complement and thus may function with some independence of the nuclear genetic material (15).

The oral apparatus of the cell is a complex organization of ciliary membranes and associated kinetosomes, which directs food to the cytopharynx, the site of food vacuole formation. The oral apparatus is at the anterior of the cell. A daughter oral apparatus must be formed before cell division can occur.

(c) The membrane-bound organelles

The membrane system of T.pyriformis has been shown in electronmicrographs to be continuous between several organelles. The outer membrane of the macronucleus and micronucleus are continuous and may be linked to the endoplasmic reticulum of the cell. The smooth endoplasmic reticulum has been observed associated with the kinetosomes during cilia formation and a portion of the rough endoplasmic reticulum links the vesicles of the Golgi apparatus to mitochondria. Continuity between the rough endoplasmic reticulum and primary lysosomes has also been observed. Even so lipid and enzyme distribution does vary amongst the organelles (16). Ciliary membranes are enriched in phosphonolipids, mitochondria in cardiolipin etc..

The cellular organelles are subject to some sort of organization, presumably co-ordinated with the function of the organelle. For instance, during rapid proliferation, mitochondria are distributed just beneath the cell membrane but they move inwards as the rate of proliferation decreases. Portions of rough endoplasmic reticulum underly the cell membrane and are adjacent to kinetosomes and mitochondria. The control of the distribution of the organelles is unknown.

Mucocysts, pinocytotic vacuoles, autophagic vacuoles, contractile vacuoles and peroxisomes have been observed in T.pyriformis. Peroxisomes are more commonly found in plants and endow upon Tetrahymena the ability to convert lipids to glycogen by shunting acetate, via the glyoxylate pathway, to the glyconeogenic pathway of the rest of the cell. Glycogen storage granules are membrane bound and contain enzymes required in the metabolism of glycogen.

Section 1.2iThe cell cycle of T.pyriformis

Cells growing under conditions which minimise intercellular interactions and with sufficient nutrients will double their contents, approximately, between successive cell divisions. This constitutes the cell cycle. Classically, the cell cycle is described by the character of DNA synthesis. This is confined to a period shorter than the cell cycle (Fig.1.6). Many other processes show periodicity with respect to the cell cycle, for instance, morphological changes and the synthesis of other macromolecules.

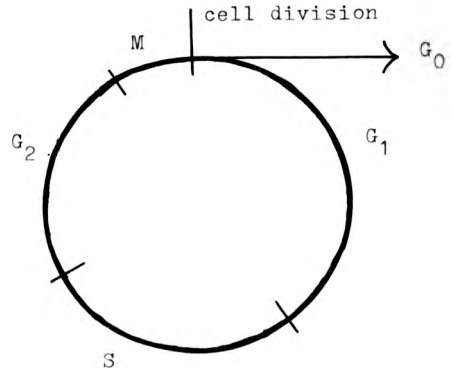


Fig.1.6 The classical cell cycle of a typical eukaryotic cell, using G_1,S,G_2,M to describe the position of DNA synthesis(S) and mitosis(M) G_0 represents a quiescent or stationary phase.

It appears that the cell cycle of mammalian cells includes a highly co-ordinated and well-ordered sequence of events, starting before or coincident with the beginning of S phase and terminating at or just after cell division. The time taken to accomplish this sequence is relatively independent of external conditions and is characteristic of the cell type. In contrast, the duration of G_1 is extremely variable, even in the same population of cells (17). The process of initiation of this sequence of events should determine the length of G_1 and thus the cell cycle (Fig.1.7). Quiescent or stationary phase cells (G_0 phase) normally contain G_1 levels of DNA. These cells may be considered as occupying the indeterminate state of the intermitotic period. When stimulated to proliferate, for instance by serum addition in the case of fibroblasts, these cells become committed to replication and cell division by transition to the determinate state (19).

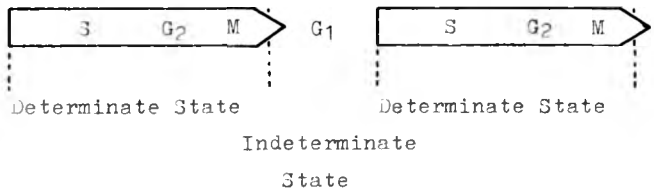


Fig.1.7 A model proposed by J.A.Smith and L.Martin (18) for the intermitotic period, showing the approximate location of the classical phases of the cell cycle. They also proposed that the transition to the determinate state should be governed by a probability function that could be modified by environmental factors.

The determinate period of the mammalian cell division cycle may be blocked by inhibitors of DNA synthesis, mitosis, cell division etc., The induction of cell synchrony by these agents relies upon the cells being blocked at a particular point in the cell cycle. Thus, once the cells have progressed to the block, the following sequence of events in the determinate state cannot be completed. Inhibitors of a particular process do not necessarily halt all the processes of the cell cycle. For instance, cell growth and periodic

enzyme synthesis can continue unaltered for some time after treatment with inhibitors of DNA synthesis. However, DNA replication is required before mammalian cells or yeast cells can progress towards cell division (20,21). This is not the case in Tetrahymena. In this organism, environmental conditions may be adjusted such that DNA synthesis can occur in the absence of cell division and vice versa (21,22). Thus, it would appear that the cell cycle may be broken down further into sequences which are usually coincident, but which may be uncoupled (Fig.1.8). This flexibility is a great advantage in the investigation of cell cycle controls.

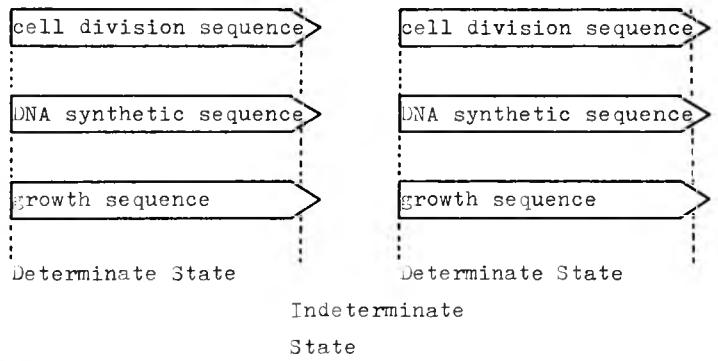


Fig.1.8 A model for the inter-division period of T.pyriformis. The signal for the initiation of each sequence may be identical although each sequence must then respond in a different manner to this signal. Alternatively, a separate signal may initiate each sequence. Further controls are required to establish and maintain the normal relationship between the sequences.

There have been a number of proposed controlling elements which may be involved as signals for the transition to the determinate state. Studies with multinucleate cells and fused cells have indicated that a signal should be a feature of the cytoplasm but should be able to influence the nucleus and initiate DNA synthesis (23,24). Cell volume (25) and nuclear DNA/ cytoplasmic volume ratio (23,26) have been proposed as major factors. Other proposals have involved the build-up or decline of some component of the cell to a critical level , e.g. DNA binding protein (17), Ca^{2+} (8) and cyclic nucleotides (27,28,29)

In Tetrahymena, G_1 normally occupies less than 30% of the cell division cycle. Thus the cells should remain in the indeterminate state for a short period only. In this case, one may be able to identify the physical basis to the necessarily high transition probability to the determinate state with greater ease than with other cell systems.

Section 1.2iiThe growth of T.pyriformis in batch culture

In batch cultures of asynchronous T.pyriformis a typical growth curve is observed (Fig.1.9). The growth curve may be divided into three major phases ; lag phase, logarithmic phase (log phase), and stationary phase. Before the onset of rapid proliferation the inoculated cells require a certain length of time to adapt to their new environment. This lag phase may be reduced by inoculating from a log phase culture. I will define the log phase strictly, as the period when the relationship between cell density and time is exponential. This leaves a transition period or deceleration phase, where the rate of proliferation declines, before the culture enters stationary phase. The stationary phase can last for several hours or days according to the growth conditions, before decline and cell death occur.

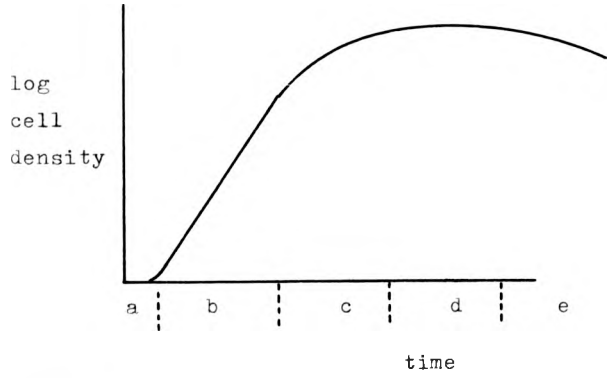


Fig.1.9 A typical growth curve for T.pyriformis; a, lag phase; b, log phase; c, deceleration phase; d, stationary phase; e, decline.

Stationary phase can be caused by nutrient or oxygen deficiency. These factors may block different processes and probably cause different metabolic states in stationary phase. T.pyriformis in nutrient-deficient stationary phase contain G_1 levels of DNA and protein, whereas oxygen-deficient cells contain approximately double these values, similar to G_2(30). It is of great importance, when interpreting data, to know the precise nature of the growth medium,type of aeration and age of inoculum used. Unfortunately, many authors fail to give sufficient details in their published work.

Section 1.2iii The production of synchronous populations of T.pyriformis

Mass cultures of T.pyriformis are amenable to selection methods of producing synchronous populations. Thus, the natural cell division cycle of this organism may be studied. However, the comparison of the natural cell division cycle with the perturbed cycles produced by induction techniques should be beneficial.

(a) Induction techniques.

Synchronous populations of T.pyriformis may be induced by a number of means. The most popular of these methods has been repetitive heat shock. This consists of the transfer of cells from optimal to cytostatic temperature for up to seven periods of 30 min, at 30 min intervals. Heat shock results in a progressive set-back of the cells from division, according to how far the cell has progressed towards cell division when the shock is applied. After a transition point,cell division becomes insensitive to heat shock. A theoretical basis to this technique has been proposed that involves the concept of a division protein, which is synthesized continuously until the transition point, when it becomes stable (20,31). DNA synthesis continues during the heat shocks and the first cell division cycles after reestablishing the optimal growth temperature possess shortened generation times and reduced poorly-synchronized DNA synthesis. Cell growth also continues during the heat shocks, thus, according to Fig.1.8 heat shock specifically blocks the cell division sequence. Synthesis of the division protein must be part of this sequence.

The transfer of cells from a well-aerated to an oxygen-deficient environment has been used to block Tetrahymena at one point of the cell division cycle. This block may be in G_2 and due to energy deprivation of cell division, however, because of the possible uncoupling of DNA synthesis and cell division, this cannot be stated with full confidence (30). Repeated periods of hypoxia have been used to generate synchronous populations of T.pyriformis using complex techniques involving the replacement and control of the gaseous environment (32,33). In our laboratory we have developed a simple approach to hypoxic shock. Under standard conditions, described in Chapter 3, shaken cultures of T.pyriformis will grow exponentially to $8x10^5$cells/ml and will achieve $2x10^6$cells/ml in stationary phase. The maximum stationary phase density of unshaken cultures is $5x10^5$cells/ml. Therefore, cultures may be grown to $2x10^5$cells/ml with shaking, then allowed to stand and thus are effectively being transfered from log phase to decleration phase. A four hour period of hypoxia is sufficient to halt cell division and results in synchronous cell division cycles after reaeration. If the cells are transfered to starvation medium immediately before reaeration, a single sychronous cell division occurs in the absence of DNA synthesis (34).

Other methods for the induction of cell synchrony have been reviewed elsewhere (35).

(b) Selection techniques.

For the study of the natural cell division cycle of T.pyriformis, synchronous populations must be selected with the minimal disturbance of their environment. Suitable techniques are now available which take advantage of the cell cycle dependency of cell size and phagocytotic activity. Two techniques are particularly attractive because they select a synchronous population very rapidly without the long period of anaerobiosis that may accompany techniques involving batch centrifugation. Continuous flow centrifugation has been used to select early G_1 cells, which sediment at a slower rate than the larger cells of the rest of the cell cycle (36). During cell division T.pyriformis loses its ability to ingest particulate matter (37), thus only these cells can escape a magnetic field after incubating with iron particles (38).

Section 1.3 The role of cyclic AMP in T.pyriformis

Tetrahymena pyriformis possesses all the necessary components of a fully functioning cyclic AMP regulatory system. Cyclic AMP (39-46), adenylate cyclase (40,44,45,47,48), cyclic AMP phosphodiesterase (39,40,45,49,50) and cyclic AMP-dependent protein kinase (6 ,52) have all been observed in T.pyriformis. However, the role that cyclic AMP plays in this organism is still unclear.

Section 1.3i Cyclic AMP and cell proliferation

Several authors have studied the relationship between intracellular cyclic AMP concentration and the rate of proliferation of Tetrahymena. Although not all authors have fully defined the conditions of growth of the cells, some consistency can be seen. S.J.Voichick et al, using unshaken cultures of T.pyriformis, strain E found a dramatic rise in cyclic AMP content upon entry into stationary phase and during the lag phase (39,40). J.Wolfe used T.pyriformis GL-C and showed that stationary phase cells contained more cyclic AMP than log phase cells (42), but information on the growth conditions and precise timing of these experiments were limited. Kariya et al have published results conflicting these two reports, showing lower levels of cyclic AMP in stationary phase than at any other time in the growth curve (43). However, the methods of purification and estimation of cyclic AMP used in this publication are uncertain and any attempt to interpret this result would not be productive.

T.pyriformis in log phase can be induced to enter stationary phase by the addition of caffeine or theophylline (42,49,53). Although cyclic AMP phosphodiesterase may not be the only site of action of these compounds, an important effect of methylxanthines is to raise intracellular cyclic AMP levels. In fact, the co-operative inhibition of cell division by caffeine and dibutyryl-cyclic AMP tends to confirm that cyclic AMP is the major factor blocking division (42). Release from caffeine blockade allowed a synchronous division 3 to 4 hours afterwards. If administered to a synchronous culture of T.pyriformis, caffeine produced a progressive set-back of the recovery division throughout the cell cycle (42).

The correlation between high cyclic AMP levels and non-proliferative phases of culture growth agrees with the studies on mammalian tissues and,in particular, tissue culture (27,28,29). In transformed cell lines, cyclic AMP levels are abnormally low (54). These cells have lost density dependent inhibition of growth and will grow to abnormally high cell density. Assuming that high levels of cyclic AMP are involved in halting cell proliferation, one may propose a target for cyclic AMP. As yet, the nature of this target is uncertain and the mechanism by which adenylate cyclase and cyclic AMP phosphodiesterase are controlled is not known. Authors have attempted to correlate G_0 levels of cyclic AMP with levels found in the phases of the cell cycle. Chinese hampster ovary cells may be synchronized by "mitotic shake-off". The level of cyclic AMP in the subsequent cell division cycle has been reported to reach a maximum in early G_1 (55), mid to late G_1 (56) and S phase (57). This situation is of course unsatisfactory.

Section 1.3ii The effect of hormones on cell proliferation

T.pyriformis can synthesize l-epinephrine, l-norepinephrine and serotonin (58,59) and the rate of synthesis of these compounds varies during culture growth (60,61). It appears that the adrenergic metabolism is well developed in T.pyriformis (62),but it is still uncertain whether these compounds elicit a response in T.pyriformis by elevating intracellular cyclic AMP. It has been recently reported that serotonin and histamine increased intracellular cyclic AMP (46) and that serotonin antagonists decreased cyclic AMP levels. This does not necessarily mean that any effects of these compounds should be mediated by cyclic AMP. In mammalian tissues l-epinephrine, l-norepinephrine and serotonin often stimulate adenylate cyclase, although hormone regulation of cyclic nucleotide phosphodiesterase has been observed (63,64,65).

Certain drugs known to effect the uptake, metabolism and storage of catechol amines or serotonin in the higher metozoa have been shown to regulate the glycogen content of Tetrahymena by the co-ordinate regulation of glycogen phosphorylase, glycogen synthetase and peroxisomal isocitrate lyase. Some but not all of these compounds also inhibited the proliferation of T.pyriformis (62,66-68).

Section 1.3iii Cyclic AMP and ciliary regeneration

Ciliary regeneration in deciliated T.pyriformis presumably requires the increased synthesis of tubulin and/or the increased incorporation of tubulin into the microtubules of the cilia. Colchicine, which inhibits microtubule formation (69), inhibits ciliary regeneration. Cyclic AMP and D_2O, which stimulate microtubule formation in other cells, also inhibit ciliary regeneration (42). This may be because excessive microtubule formation inhibits ciliary regeneration or because there is some other site of action for these compounds. Inhibitors of ciliary regeneration, cyclic AMP, dibutyryl cyclic AMP, caffeine and theophylline, have been shown to inhibit the formation of and synthetic capacity of a heavy polysome which becomes predominant in the polysome population after deciliation of T.pyriformis (70). This polysome may be responsible for the synthesis of tubulin and may have a characteristic sensitivity to cyclic AMP. Adenosine and calcium can stimulate the activity of this polysome but, although adenosine shortens the ciliary regeneration period, calcium had no effect on the regeneration period except at high concentrations, it was inhibitory. Cyclic GMP and dibutyryl cyclic GMP stimulated ciliary regeneration but had no effect on the synthesis or activity of this polysome (70).

Section 1.3iv The effect of hormones on other processes in T.pyriformis

The regulation of glyconeogenesis by hormones has been refered to in Section 1.3ii. The effect of theophylline on glyconeogenesis and the relationship between intracellular cyclic AMP and glyconeogenesis during culture growth would infer that if cyclic AMP is involved in the regulation of this process, it acts to inhibit glyconeogenesis. This is a direct contradiction to mammalian metabolism (40,62).

Very low concentrations of several hormones enhance the phagocytotic activity of T.pyriformis. l-Epinephrine, insulin, serotonin, histamine, thyroxine and iodotyrosine compounds produced reproducible if not particularly large activation of phagocytosis at less than 10^{-6}M (71). This effect has been

mimiced by the addition of theophylline and/or dibutyryl cyclic AMP. The co-operative action of theophylline and histamine suggests that phagocytotic activity is regulated by cyclic AMP(72). Phagocytotic activity varies throughout the cell cycle of T.pyriformis (37), therefore any treatment that alters the distribution of asynchronously growing cells about the cell cycle may effect phagocytosis.

The extracellular release of acid hydrolases and the ejestion of food vacuole contents are sensitive to several adrenergic drugs, but these processes are not sensitive to catechol amines, serotonin, dibutyryl cyclic AMP or caffeine (73). Terefore, it appears that the processes of phagocytotic ingestion and vacuole ejestion are controlled by different mechanisms. Microtubule and microfilament disrupting agents reduce ingestion, but not ejestion.

Insulin has been shown to stimulate glucose uptake from the medium by T.pyriformis (74).

Section 1.4 The mechanism of action of cyclic AMP in T.pyriformis

Cyclic nucleotides regulate many of the metabolic processes which are under their control, by modifying the activity of protein kinases, in mammalian cells and other eukaryotes. Cyclic nucleotide-dependent protein kinases probably recognize a specific short amino acid sequence, which may be present in a number of proteins (75). These enzymes are normally assayed by their ability to phosphorylate histone H_1, although this procedure may overlook abnormally specific protein kinases. The use of radioactive phosphate to label whole cell nucleotides, can show cyclic AMP sensitive phosphorylation of endogenous proteins. Membrane proteins histones, nuclear acidic proteins, microtubule associated protein and ribosomal protein have been identified as substrates for cyclic AMP-dependent protein kinases, in vivo. The activity of cyclic nucleotide-dependent protein kinases have been observed to vary during the cell cycle of Chinese hampster ovary cells (76, 77). It would appear that the selective synthesis of protein kinases with different substrate specificity at different times in the cell cycle may represent an important feature of cell

cycle control. A similar situation may be apparent in T.pyriformis.

It has been reported that cyclic AMP-dependent protein kinase activity may be isolated in a number of the organelles of T.pyriformis, but that the substantial proportion is cytoplasmic (52). The isolation techniques used in this publication may allow the lost of activity from organelles, but activity remained in nuclear fragments and mitochondria. Cyclic AMP-dependent protein kinase has also been reported in cilia of T.pyriformis (6). Two purified protein kinases from the cytosol vary in a similar fashion during culture growth (52). Highest activity was attained just before and continuing into stationary phase. Stationary phase cells used as an inoculum showed a decline in protein kinase activity during lag phase (52). Endogenous protein phosphorylation occured most rapidly in lag phase, declined along with cyclic AMP-dependent protein kinase activity but did not increase in stationary phase. This may be due to cyclic AMP-independent protein kinase activity, a variation in phosphatase activity or the presence of other control factors, in vivo. The sites of endogenous protein phosphorylation included histones, nuclear acidic proteins, mitochondria,ribosomal and cytoplasmic proteins, but no cyclic AMP sensitivity was established (52). Protein kinase activity continued to rise during repetitive heat shock treatment of T.pyriformis (52). This would infer that protein kinase activity as measured from the cytosol, is synthesized independently of the cell division sequence, which was blocked.

The functional state of chromatin may be modified by phosphorylation of constituent proteins. This may regulate the activity of the genome and provide a basis for cell cycle control. Ciliary tubulin acted as a substrate for protein kinase located on the ciliary axenome (6,51). The phosphorylation of tubulin may be involved in the regulation of motility and/or ciliary regeneration. Several of the processes of the metabolism of T.pyriformis that require microtubules or microfilaments, that is ciliary regeneration, phagocytosis and cell division, can be blocked by elevating intracellular cyclic AMP. One is tempted to suggest the involvement of cyclic AMP-dependent phosphorylation of microtubule proteins as a common mechanism for the regulation of these processes (78).

Section 1.5 The control of cyclic AMP levels in T.pyriformis

The enzymes adenylate cyclase and cyclic AMP phosphodiesterase together with the transport systems of the cell, are resposible for the regulation of intracellular cyclic AMP levels. Nothing is known of the cyclic AMP transport systems in T.pyriformis, but adenylate cyclase and cyclic AMP phosphodiesterase have been studied.

Section 1.5i Cyclic AMP phosphodiesterase

Cyclic AMP phosphodiesterase activity from T.pyriformis was first reported by J.J.Blum, using strain HSM, (49) and was sensitive to inhibition by methylxanthines. T.pyriformis, strain E contains apparently at least two cyclic AMP phosphodiesterases in the 22,500xg supernatant of sonic lysates (39). These were held responsible for the apparent K_m values of 1.03μM and 1,07mM. Three cyclic AMP phosphodiesterases were separated on Sephadex G-200 from the 105,000xg supernatant of T.pyriformis, strain GL (50). In other tissues multiple forms of cyclic nucleotide phosphodiesterase have different substrate specificity, such that , at physiological cyclic nucleotide concentrations, different enzymes are responsible for the hydrolysis of cyclic AMP and cyclic GMP. This appears to be the case in T.pyriformis, strain W (79).

Section 1.5ii Adenylate cyclase

Three research groups have published their findings on the adenylate cyclase system of T.pyriformis. S.H.Kindler and coworkers at the Tel-Aviv University, Israel have claimed the existence of a hormone-sensitive adenylate cyclase in the 610xg particulate matter of sonic lysates of T.pyriformis, strain W. This could be stimulated several fold by sodium fluoride, l-epinephrine and serotonin, but not by l-norepinephrine. l-Epinephrine stimulated activity was abolished by propranolol and as such appeared to be mediated via a β-adrenergic receptor(47).

Adenylate cyclase activity could be solubilized by repeated washing or by treatment with Triton X100. The detergent solublilized enzyme was still sensitive to sodium fluoride and serotonin and could be purified by DEAE cellulose chromatography (48). However, the assay methods that were used by this research group were unsound and their results cannot be accepted without independent verification. My major critism of this research is the lack of proper substrate and product stability under the conditions of assay. Particulate fractions from T.pyriformis, strain W contain potent ATPase activity, capable of detroying all the substrate during the course of an adenylate cyclase assay (80). This would not only limit the substrate availability, but would give rise to a number of hydrolysis products, which would be difficult to separate from cyclic AMP by the technique chozen by this research group. The survival of cyclic AMP produced in the assay is also doubtful, because the cyclic AMP phosphodiesterase content of particulate fractions of T.pyriformis, strain W, is sufficient to overcome the effect of caffeine, which was used in an attempt to protect the product (80). Thus, if the estimation of cyclic AMP were valid, the measured hormone-sensitivity may have been a function of phosphodiesterase activity or ATPase activity.

Investigations carried out with E.Shrago at the University of Wisconsin, USA are more satisfactory. Adenylate cyclase was estimated in the 12,000xg precipitate from T.pyriformis, strain E grown without shaking. The assay for adenylate cyclase included an ATP regenerating system and an extraordinarily large concentration of theophylline to inhibit cyclic AMP phospho-diesterase.(40). The reaction was incubated at 37°C, which is rather high for an enzyme isolated from an organism with an optimum growth temperature of less than 30°C. The instability of adenylate cyclase at this temperature is probably indicated by the length of the incubation, which was only 3 min. Adenylate cyclase activity was found to be stimulated by sodium fluoride and dependent upon the growth medium and upon the stage of culture growth. The co-ordinate variation of cyclic AMP phosphodiesterase and adenylate cyclase throughout culture growth was consistent with the variation in cyclic AMP content, discussed in Section 1.3i. (40).

A third group, working at the University of Wurtzburg, W.Germany, were already established in the field of mammalian adenylate cyclase before they investigated the system in T.pyriformis (44). Although this research was first published in 1974, this was in a rather obscure journal and it has only recently come to my notice through the abstracting journals. This work has also recently been refered to in a review of adenylate cyclase by this research group (81). They found that the enzyme was insensitive to hormones and sodium fluoride, but was inhibited by low concentrations of Ca^{2+} ($5x10^{-8}$M). They determined that Mn^{2+} would support a much higher activity of adenylate cyclase than Mg^{2+}, which was used in the other investigations of adenylate cyclase in T.pyriformis.

The adenylate cyclase activity measured by each of these research groups was different. Basal activities were 60 pmole/min/mg protein @ 30°C (47,48), 2.5 to 15 pmole/min/mg @37°C (40) and 96 pmole/min/mg @ 30°C (44,81) respectively. The difference between these values may be explained to some extent by uncontrolled calcium inhibition, however, even at 10^{-5}M, calcium inhibition was reported to be only 37% (81). No reliable demonstration of hormone sensitivity of adenylate cyclase from T.pyriformis has been reported, in vitro. However, serotonin and histamine increased the cyclic AMP content of whole cells (46). The effects of these agents on phagocytosis has been discussed in section 1.3iv. Using hormone analogues, it would appear that only serotonin binds to a specific receptor in its regulation of phagocytosis(82) and cyclic AMP (46). The site of this receptor is uncertain, because serotonin may enter T.pyriformis (83). Histamine does not enter the cell and therefore appears to be capable of regulating phagocytosis and cyclic AMP content of the cell by interacting with the surface of T.pyriformis (83). This must be a non specific action (82).

The lack of agreement between the investigations of adenylate cyclase from T.pyriformis calls for a definitive characterization of this enzyme. Of utmost importance is the validation of the assay technique used in such a study. This I have done in detail.

Section 2.1 Mammalian adenylate cyclase

The majority of adenylate cyclase systems studied have been isolated from multicellular organism and especially from mammals, therefore a great deal of our knowledge of this enzyme system depends upon these investigations. These studies have been extensively reviewed (81,84-86). One may suspect that a major factor which determines the properties of the enzyme in these organisms is the role that adenylate cyclase and cyclic AMP play in the intercellular communication of differentiated cells. It is, therfore, difficult to estimate the degree of similarity that may exist between protozoan and mammalian adenylate cyclases.

Section 2.1i Physical characteristics

Hormone-responsive adenylate cyclase is found predominantly in the plasma membrane of eukaryotic cells and is orientated such that the binding of hormone to external receptors can be translated into an increased rate of conversion of cytoplasmic ATP to cyclic AMP (84,87,88). In the other compartments of mammalian cells, adenylate cyclase is always membrane bound and has been reported to have similar or different sensitivity to that of the plasma membrane (89-92).

Several estimates of the size of detergent-dispersed adenylate cyclase have been made. These preparations have normally, but not always, lost their hormone sensitivity, so it is unclear what components of the system are still present. Gel filtration has indicated a molecular weight of between 38,000 D (93) and 800,000 D (94), although a value of 100,000 to 200,000 D is more common (93,95,96). Triton X-100 dispersed adenylate cyclase from rat renal medula has been reported to contain only sufficient detergent to interact with 5% of the enzyme surface. It was suggested that this may indicate that only a small portion of adenylate cyclase interacts with the membrane (93).

Detergent and enzymic destruction of glucagon sensitivity in liver plasma membranes has suggested that this receptor component is a lipoprotein (97). This may be a general phenomenon (84). The only hormone receptor to be purified to homogeneity

Chapter 2

Adenylate cyclase from other organisms

is that for insulin (98). This is an asymetrical glycoprotein of 300,000 D. Although the relationship between this receptor and adenylate cyclase is unclear, certainly insulin appears to reduce cyclic AMP content of mammalian cells, there may be some similarity between different hormone receptors. Cholera toxin, which can irreversibly activate the adenylate cyclase of most tissues, binds to a GM_1 ganglioside (99-101) and then activates adenylate cyclase by a process which involves NAD (101). Sequence homology with glycoprotein hormones suggests that this type of receptor may be of widespread importance (102).

An interesting structural model for adenylate cyclase has come from the purification of this enzyme from brain by Blue Dextran affinity chromatography (103). This type of chromatography is specific for enzymes which contain the "dinucleotide fold", an NAD binding site common to many enzymes. It was suggested that adenylate cyclase was an 8 subunit enzyme, all of which are identical and evolutionarily related to the NAD enzymes. Noradrenalin is structurally similar to NAD and the dinucleotide fold is known to bind nucleotides, therefore the identical site on different subunits of adenylate cyclase could function as hormone receptor and catalytic site.

Section 2.1ii Catalytic properties

Certain properties of adenylate cyclase appear to be independent of hormone sensitivity and observed in many tissues. These include substrate specificity, the action of certain anions and nucleotides and the sensitivity to sulphhydryl reagents. The substrate for the enzyme is $ATP.M^{2-}$, where the nucleotide acts as a ligand of a divalent metal, frequently Mg^{2+} although Mn^{2+} and Co^{2+} can often replace Mg^{2+} (104,84). $DeoxyATP.M^{2-}$ cannot be utilized and free ATP is a competitive inhibitor of the enzyme (105). Adenylate cyclase is stimulated several fold by sodium fluoride, 10mM, and sodium azide, 10mM, but probably by different mechanisms (106). Many adenylate cyclases can be activated by GTP and particularly by the non-hydrolysable synthetic compound $G_{pp}(NH)_p$. A guanyl nucleotide binding protein has been separated from adenylate cyclase, and purified (81). Sensitivity to

sulphhydryl reagents would suggest that one or more suphhydryl groups are crucial for the viability of the enzyme or are important in catalysis.(107).

Section 2.1iii Hormone activation

Although knowledge of the actual nature of hormone activation must await the separation and successful recombination of the components of the adenylate cyclase system, sufficient kinetic and binding data has been collected to allow the proposal of certain models. Unfortunately much of this data is not only tissue specific, but also contradictory (108). Dephosphorylation of a deactivated enzyme in the presence of hormones, fluoride etc. may be a possible mechanism (81), but most models involve the regulation of enzyme activity by conformational changes (110). There is some evidence for conformational changes during enzyme activation (107), although more recent work indicated that these changes probably occured at the receptor rather than at the catalytic site (109).

A stable receptor-enzyme complex could be activated by a typical heterotropic allosteric mechanism , however some cells contain adenylate cyclase systems which are sensitive to several hormones (87). These hormones bind to separate receptors but, apparently interact with the same adenylate cyclase system. The stable receptor-enzyme model would require a most complicated and extensive aggregation of subunits. A more simple model can be achieved if the receptors can diffuse in the plane of the membrane, independently. The reversible association of the receptors with the enzyme component could be responsible for enzyme activation and may be favoured by hormone occupation of the receptors (98,111). Alternatively, a disturbance of the lipid bilayer caused by the binding of the hormone may activate adenylate cyclase (112). Recent evidence that hormone binding creates a linkage between the outer and inner lipid layers of the membrane substantiates the concept of receptor mobility and reversible interaction between receptor and enzyme components (113). This linkage appears to be only a part of hormone activation and does not completely describe the process.

Membrane composition is known to play an important role in the transduction of the hormone binding stimulus. There appears to be some degree of specificity of the lipid head group in the expression of hormone activation (114-116). Cholesterol may closely associate with the adenylate cyclase system and may be involved in hormone activation (81). Microtubules and microfilaments underlying many plasma membranes are required in the aggregation of Cholera toxin on the cell surface, which is part of the process by which the toxin activates adenylate cyclase (101) and resembles the capping of phytohaemaglutinins on lymphocyte membranes (117).

The properties of adenylate cyclase may be complex and depend largely on the tissue. The proceeding discussion was intended to concentrate on properties common to most mammalian adenylate cyclases. Some idea of complex properties that must be included in the models for adenylate cyclase can be gained from Table 2.1.

Table 2.1 The complex behaviour of adenylate cyclase systems isolated from mammalian cells.

apparent heterogeneity of receptors for individual hormones	adrenal	ACTH	(118)
apparent spare receptors for individual hormones		catecholamines	(119)
apparent +ve or -ve co-operativity between individual receptors	erythrocyte	adrenergic	(120)
cholera toxin activation of basal and hormone stimulated activity	liver	glucagon	(101)
co-operativity between guanyl nucleotides and hormone activation	liver	glucagon	(121)
time lag before the onset of hormone activation	liver	glucagon	(121)
incompatible activation by different stimulators of the enzyme	adipocyte	various	(84)
temperature dependent changes in hormone activation	liver	epinephrine glucagon	(113)
regulation by a protein ; feedback regulator	adipocyte	epinephrine	(122)
Ca^{2+} dependent protein activator	brain	-	(123)
Inhibition by small molecules;	lung	adenosine	(124)
	adipocyte	fatty acids	(125)
Activation by small molecules;	kidney	hexose-P	(126)
	liver	adenosine	(127)
	thyroid	spermine	(128)
Ca^{2+} inhibition	erythrocyte	-	(129)
purification on Blue Dextran affinity columns	brain	-	(103)
inhibition by NADH	adipocyte	-	(106)

Section 2.2 Bacterial adenylate cyclase

Adenylate cyclase has been isolated from many bacteria and may be particulate or soluble (130). Brevibacterium liquefaciens releases a soluble adenylate cyclase on cell disruption, which may be activated by pyruvate, serveral other α-keto acids, several monocarboxylic acids and amino acids (131,132). This enzyme has been purified to homogeneity (133) and a number of its properties are pertinent to this discussion. It is an highly asymmetrical molecule with a molecular weight of 92,000 D and two identical subunits. The enzyme activity may be abolished by neuraminidase, phospholipase C and phospholipase A. It is therefore considered to be a lipoglycoprotein (134). This has led to the suggestion that the enzyme is membrane bound in vivo. The substrate for the enzyme may be ATP.M^{2-} or deoxyATP.M^{2-}, where the metal requirement is Mg^{2+}, Mn^{2+}, Co^{2+}. The reverse reaction may also be followed. The enzyme is also sensitive to sulphhydryl reagents.

The particulate adenylate cyclase isolated from Norcadia erythropolis shows properties typical of bacterial membrane-bound adenylate cyclases (130). It is pyruvate independent and will utilize ATP.Mn^{2-} in preference to ATP.Mg^{2-}. Other metal dications, Ni^{2+}, Cu^{2+}, Zn^{2+}, other nucleotides, phosphoglycerates, oxaloacetate and pyridoxal phosphate inhibit the enzyme. Several mammalian hormones and sodium fluoride were without effect.

Escherichia coli B has a membrane-bound adenylate cyclase in vitro (130). Sugars which are transported into E.coli B via the phosphoenolpyruvate : sugar phosphotransferase (P.T.) system can regulate the activity of other carbohydrate permeases and adenylate cyclase in a co-ordinate fashion. The mechanism by which this takes place requires the presence and functional integrity of the sugar specific Enzyme II of the P.T. system (135).(Fig. 2.1)

$$PEP + HPr \xleftrightarrow{\text{Enzyme I}} P\ HPr + pyruvate$$

$$P\ HPr + sugar_{(out)} \xleftrightarrow{\text{Enzyme II}} sugar\text{-}P_{(in)} + HPr$$

Fig 2.1 Phosphoenolpyruvate : sugar phosphotransferase system of E.coli B . HPr and Enzyme I are general proteins, common to all the sugar permeases.

The classical effector of catabolite repression, glucose, will inhibit adenylate cyclase in whole E.coli B. Most of this response is lost in broken cell preparations (136-137). Adenylate cyclase of membrane preparations can be stimulated by several sugars, succinate and glucagon. The adenylate cyclase system from an E.coli mutant which is insensitive to glucose catabolite repression was stimulated by glucose, in vitro (138). E.coli adenylate cyclase was inhibited by sodium fluoride (130) and was insensitive to guanyl nucleotides (139).

Table 2.2 Adenylate cyclase from eukaryotic microrganisms

Organism (ref)	Relevant growth condition	Location (!cytochemical)	Prefered M^{2+}	Optimal pH		Inh (co
Neurospora crassa (140-142)	-	15,000-165,000xg ppt (plasma membrane)	Mn	5.5-6.3		glu ins chl NaF
Saccharomyces cerevisiae (143)	+5%glucose	(intracellular membranes)	Mn,Mg*	6.0*		glu MgC ser
Saccharomyces fragilis (144)	+ O_2;+1.5% or+10% glucose	1,000xg ppt (cell membrane)	Mg*	6.5		NaF PPi gro
Acanthamoeba palestinensis (145,146)		2,000-100,000xg ppt (rough endoplasmic reticulum)	Mg*	7.4*		
Euglena gracilis (147)	+light	650-30,000xg ppt (cellular membranes)	Mn⟩Mg	9.0		pro dop γam all ace
Dictyostelium discoideum (148-152)	axenic	inner surface of plasma membrane ! dislodged by cell breakage	Mn*	7.4*		com gro

* n

isms

* not determined, the metal or pH used is given

ptimal pH	Inhibitors and Activators (concentration, percent basal), if no statement then compound ineffective
6-6.3	glucagon(10nM,140%) insulin(0.33nM,43%)theophylline(10mM,49%) quinidine(2.5mM,54%) chloroquine(2.5mM,55%) quinine(2.5mM,60%) atropine(10mM,63%) NaF, serotonin, histamine, catecholamines
.0*	glucagon(5μg/ml,110%) acetate(0.125M,128%) $MgCl_2$+PP_i(2mM,16%) glucose(0.25M,66%) NaF(1mM,77%) aminophylline(2.6mM,89%) serotonin, concanavalin A.
.5	NaF(5mM,120%) PPi(0.5mM,80%) growth phase dependent rise in stationary phase, increased +lactate or +O_2
.4*	
.0	prostaglandins F_2,E_1,A_2(28μM,125%,122%, 119%) l-epinephrine(25μM,121%) dopamine(25μM,119%) l-norepinephrine(25μM,115%) serotonin(25μM,111%) γaminobutyric acid(25mM,119%) alloxan(10mM,79%) NaF(10mM,83%) glucagon(25μM,95%)α-ketoglutarate(5mM,92%) acetate, pyruvate
.4*	completely dependent on the presence of 5'AMP or 5'dAMP <u>in vitro</u> growth cycle dependent

Section 2.3 Adenylate cyclase from eukaryotic microorganisms

Table 2.2 lists the properties of adenylate cyclase from several eukaryotic microorganisms. Each of these enzymes appears to have adapted to some specific role in the organism concerned and they do not show particularly similar properties. However, none of these organisms contain an adenylate cyclase system typical of the higher eukaryotes.

Section 2.3i The location of adenylate cyclase

Adenylate cyclase from Dictyostelium discoideum can be dislodged from the plasma membrane by sonication and has only recently been isolated in this organelle from cells lysed by a polyene antibiotic (151). The loose binding of adenylate cyclase to the plasma membrane is reminiscent of the situation proposed to exist in Brevibacterium liquefaciens and can cause problems in defining the location of the enzyme in vivo. Non-specific binding of a solubilized adenylate cyclase to intracellular membranes could explain the reported location of this enzyme in the other microorganisms. Only careful cytochemical studies may resolve this dilemma. A plasma membrane-bound adenylate cyclase is more likely to form part of a system of intercellular communication whereas, a soluble enzyme or one associated with intracellular membranes may function independently of neighbouring cells.

Section 2.3ii The hormone sensitivity of adenylate cyclase

The majority of mammalian hormones are not synthesized by these microorganisms, so one would not expect that such alien molecular species would have any effect on adenylate cyclase. It is surprising to find even small changes in activity caused by these hormones and the physiological significance of such effects is unclear. One may propose that, if not directly relevant to the particular organism, hormone sensitivity could represent a dormant response which has gained significance only in the higher evolutionary hierachy.

Section 2.3iii Catalytic properties

The mixed response of these adenylate cyclase systems to sodium fluoride is an indication of the difference that may exist between the nature of this enzyme in the higher and lower eukaryotes. None of the eukaryotic microorganisms possess an adenylate cyclase which may be stimulated several fold by sodium fluoride. This is a prominent feature of mammalian adenylate cyclase. The eukaryotic microorganisms do, in fact, more nearly resemble the bacteria in this respect.

The metal requirement of adenylate cyclase from these organism also resembles that of the bacterial enzyme. Manganese is much more commonly favoured by the adenylate cyclase of the microorganisms than that of the metazoa.

Section 2.3iv The role of cyclic AMP in eukaryotic microorganisms

The properties of a particular adenylate cyclase should be related to the role that cyclic AMP plays in the organism. I shall briefly mention what is known of the cyclic AMP metabolism of these microorganisms.

Of these organisms, only Neurospora crassa and Acanthamoeba have been shown to react to high cyclic AMP content by increasing the rate of glycogen breakdown (153-155). This is the classical response to cyclic AMP in mammalian tissues. The activation of glyconeogenesis by glucagon in intact Neurospora crassa proceeds via the co-ordinate regulation of glycogen synthetase and glycogen phosphorylase by cyclic AMP. This is similar to the situation in mammalian cells, but it is not known whether Neurospora crassa contains cyclic AMP-protein kinase (156).

Cyclic AMP is apparently involved in the regulation of the steady state levels of a number of enzymes in Neurospora crassa. There is direct evidence for the induction of tyrosinase by cyclic AMP (157). The colonial properties of Neurospora crassa is dependent upon intracellular cyclic AMP. Lower than average cyclic AMP levels produces several small colonies, rather than one per plate (142,158). These morphological abnormalities can be induced in the wild type by inhibitors of adenylate cyclase or activators of cyclic nucleotide phosphodiesterases.

Mutants which show these abnormalities have low cyclic AMP levels. Several of these mutants possess defective enzymes of the carbohydrate metabolism. The involvement of cyclic AMP in the circadian periodicity of Neurospora has been proposed (159).

Under nutrient starvation, Acanthamoeba castellani will enter a stationary phase then produce cysts. This process is associated with a transient increase in cyclic AMP and a simultaneous breakdown of glycogen, presumably to provide the energy requirement of the cell. Adenylate cyclase was thought to play an important role in the production of this pulse of cyclic AMP, but how the activity of this enzyme may be modified is unknown (155). In older cultures, Acanthamoeba castellani will aggregate, possibly to allow macromolecular transfer between amoebae. This process depends on the hydrolysis of phospholipids by a cyclic AMP-activated phospholipase A (160). Theophylline induced phagocytosis in Acanthamoeba castellani (161).

Several of the yeasts show catabolite repression reminiscent of that demonstrated in E.coli. Glucose grown Schizosaccharomyces pombe cells have low intracellular cyclic AMP and repressed invertase, NADH-ferricyanide reductase and glucosidase. Glycerol and maltose grown cells showed higher cyclic AMP levels and derepression of these enzymes (162). The mechanism of catabolite repression has been elucidated in E.coli and consists of a cyclic AMP receptor protein, which,in the presence of cyclic AMP, will facilitate the transcription of the lac operon. A cyclic AMP binding protein has been isolated from yeasts, but its function was not determined (163). Unlike E.coli, S.cerevisiae contains a soluble cyclic AMP-dependent histone kinase (164). Phosphofructokinase from S.cerevisiae exists in two interconvertible forms. Cyclic AMP stimulates the production of one form which is hypersensitive to ATP inhibition and is thus under the control of the energy balance of the cell (165). Cyclic AMP, 1mM, has been reported to stimulate active uptake of amino acids and nucleotides (166). Cyclic AMP may be involved in the sporulation of yeasts (167).

It has been suggested that cyclic AMP may play a role as a physiological regulator of chloroplast development in Euglena gracilis (147). Chloroplasts from this organism contain cyclic AMP

binding protein and cyclic AMP phosphodiesterase (168). Cyclic AMP has been shown to stimulate RNA synthesis in chloroplasts. Environmental control of cyclic AMP levels could not be demonstrated in Euglena.

At the onset of exhaustion of their food supply, Dictyostelium discoides aggregate in radiating cell streams around nascent centres and then differentiate to produce multicellular fruiting bodies (169). The wave-like response of the cells suggests that cyclic AMP, the chemotactic agent, is pulsed. Such oscillations could be explained by the co-ordinate regulation of the 5'AMP dependent adenylate cyclase and the cyclic AMP-dependent ATP pyrophosphohydrolase, which have been copurified from the 100,000xg supernatant of sonicated cells (150,170). The cell-surface chemotactic receptor has been proposed to be a membrane-bound cyclic AMP phosphodiesterase (171,172), but the mechanism of chemotaxis has not been elucidated. Cyclic AMP is also thought to be involved in the differentiation of Dictyostelium (173). A number of workers are attemping to characterize this system as a model for the higher eukaryotes.

Section 2.4 Evolutionary aspects

Some discussion of the possible evolution of adenylate cyclase is relevant.

On the molecular level, the adenylate cyclase of Brevibacterium liquefaciens appears to be a relatively simple dimeric allosteric enzyme, although possible interactions with the cell membrane may be important in regulating the activity of this enzyme. In E.coli, adenylate cyclase has formed a more stable association with the cell membrane and can respond to external stimuli by the interaction with the various permeases of the cell membrane. The complex molecular organization of the mammalian adenylate cyclase system is probably related to the various regulatory constraints of the enzyme.

If one proposes that there should be a fair amount of amino acid conservation at the active site of the adenylate cyclases then,one should explain the variety of substrate specificities and sensitivities to agents supposed to act at the active site. This may be a function of the molecular complexity of the enzyme system or may indicate some form of convergent evolution of different adenylate cyclase molecules.

The adenylate cyclases of the eukaryotic microorganisms combine a wide substrate specificity with a well-developed regulatory capacity. As such they lie somewhere between the prokaryotes and higher eukaryotes, in evolutionary terms. This is not surprising. Within this group, Neurospora crassa and Dictyostelium have some form of colonial existence. The increased likelihood of intercellular communication with these organisms may require a more developed adenylate cyclase system. Acanthamoeba can also aggregate in response to environmental conditions, but this does not consitute colonial existence. In Tetrahymena in log phase of culture growth, intercellular communication is of little importance and one may suspect that the regulatory capacity of adenylate cyclase from this organism to be less well developed.

PART II

MATERIALS AND METHODS

Chapter 3

The manipulation of *Tetrahymena pyriformis*

Section 3.1 Materials

Tetrahymena pyriformis, strain W was obtained from the Culture Centre of Algae and Protozoa, Cambridge, England. Ref.No. L1630/1.

Iron particles, for selection synchrony, were obtained from Merck, Darmstadt. Merck No.3819.

Fisons,Loughborough, England supplied; Acid washed sand (30-90 mesh), crystalline ferric chloride, glycerol, diphenylamine.

Difco Laboratories, Detroit, USA supplied; proteose peptone, yeast extract.

Sigma (London) Chemical Co. Ltd. supplied; sodium pyruvate, sodium succinate, p-nitrophenyl phosphate, l-arginine, 5'-AMP.

BDH Chemicals Ltd., UK supplied; glucose, SDS.

Boehringer Corp. (London) Ltd., UK supplied; NADH

Section 3.2 The growth of Tetrahymena pyriformis, strain W

Stock cultures of this organism were maintained by weekly subculture, in 250 ml conical flasks containing 50 ml of growth medium consisting 2% Proteose Peptone (Difco), 0.1% yeast extract and 5μg/ml crystalline ferric chloride. Cultures were kept at room temperature, without shaking. Under these conditions a stationary phase cell density of approximately $5x10^5$ cells/ml was obtained after 2 to 3 days and cells remained viable for a further 7 days, at least.

Experimental cultures were inoculated from stock, such that the cultures were still in log phase after several generations. This was to minimise effects due to lag phase and the adaptation of the cells to their new environment. Aeration of experimental cultures was achieved by orbital shaking at 180 rev./min of conical flasks filled to 20% of their maximum volume with growth medium. The temperature was maintained at 28±½ °C. Glucose supplemented medium (+0.5% glucose) was used only in experiments where this has been stated. Under these conditions, cultures grew exponentially to $8x10^5$ cells/ml and could achieve a stationary phase density of $2x10^6$ cells/ml. The mean doubling time of log phase cultures was 150-160 min.

Section 3.2ii The estimation of cell density and percent furrowing cells

The cell density of cultures of T.pyriformis was determined by fixing aliquots of cells in an equal volume of 20% formaldehyde neutralized with 0.01M phosphate buffer and counting in an improved Neubauer haemocytometer. More than 200 cells were counted for each sample and the mean of duplicate samples was taken as best estimate. Late division cells were scored as "furrowing cells" because the prominent furrow girdling the cell made identification unequivocal. The population of furrowing cells in a log phase culture was approximately 3.7% . Thus furrowing, determined as above, represents 5.3% of the cell cycle or approximately 8 min . The proportion of furrowing cells rarely exceeded 10% of cells scored and thus estimates of this function were never statistically accurate.

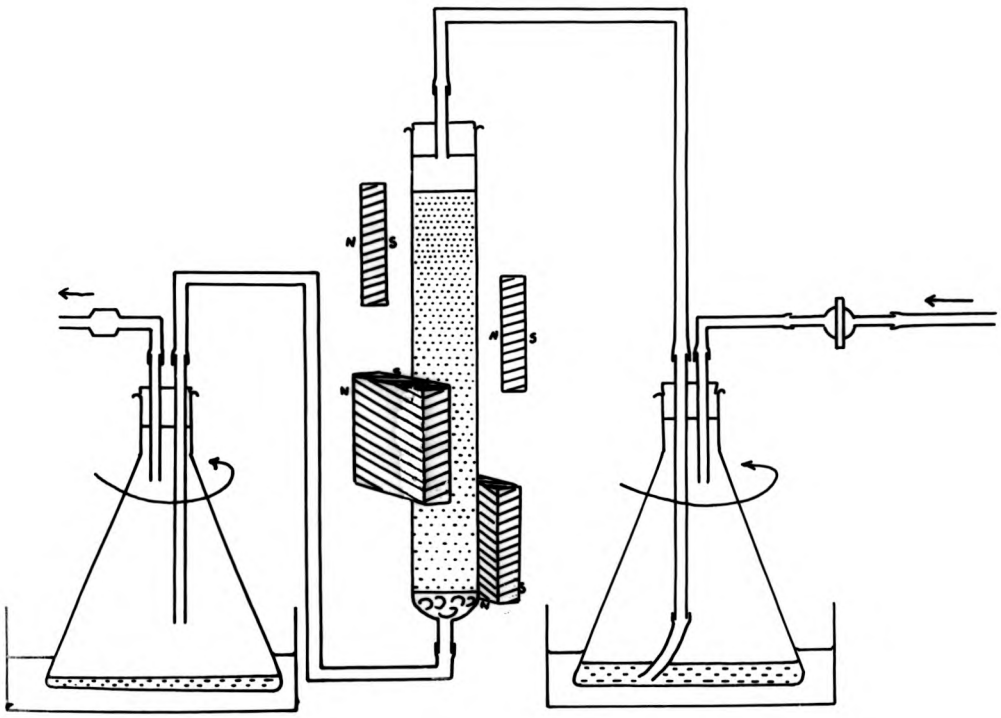


Fig. 3.1 The apparatus for the selection of amitotic cells.

Section 3.3i Selection of a synchronous population of T.pyriformis

Cells undergoing amitosis are unable to ingest particulate material (37). Cells at this stage of the cell division cycle were selected by a procedure essentially the same as that descibed by Hildebrandt and Duspiva (38). The procedure adopted will be described in some detail as it contains some modifications to that reported.

Log phase cells (10^5 to $2x10^5$ cells/ml) were incubated at 28°C with shaking, with 10g/l iron particles for 12 min. At this time more than 90% of the cells had ingested iron. A slight positive pressure was used to drive the culture onto a 35x4.5cm column of dry, acid-washed sand (30-90 mesh), which stood vertically in a magnetic field of approximately 100 Gauss (Fig 3.1). The magnetic field was achieved by positioning 4 magnets around the column, with their poles horizontal, at a distance of 5 cm from the axis of the column. The synchronous population was collected and maintained at 28°C with shaking, before being separated into several aliquots, which were incubated as usual. and taken one at a time for cell cycle analysis.

This method provides a rapid selection of a synchronous population of T.pyriformis with the minimum disturbance of the cells. The flow rate of the column was approximately 100ml/min for up to 900 ml of culture. Therefore, the cells leave their aerobic environment for a very short period. Only cells which have not ingested iron are selected by this procedure. The magnetic field strength was adjusted so that a minimum amount of iron particles escaped into the collection vessel (<1%), but also so that those cells held by the magnetic field were not liable to damage. The selected culture still contained sufficient nutrients to raise several generations of cells, therefore this population of cells was obtained in an environment essentially the same as that of a log phase culture.

Fig. 3.2 shows the synchrony achieved by this method. The use of glucose supplemented medium appeared to improve the degree of synchrony obtained, therefore this was used routinely. The synthesis of ~~macromolecules~~ DNA was also synchronous in these cultures (Fig. 3.3). This would seem to give some validity to the claim that analysis of these cultures provides an insight to the natural

cell cycle of T.pyriformis. The recovery of cells by this procedure appears to be high. This was because the dry sand column absorbed a large volume of medium, thus concentrating the cells. Futhermore, this procedure selects cells just nearing cell division allowing a doubling of the population to occur before the start of cell cycle analysis. Far from being a disadvantage, this provided a synchronous population that could be analysed with ease.

Section 3.3ii Induction of a synchronous population of T.Pyriformis

Log phase cultures in glucose supplemented medium at $2x10^5$ cells/ml were stopped shaking, but maintained at 28^oC. The rate of diffusion of oxygen into the medium is far slower than the rate of utilization of oxygen by T.pyriformis at this density, thus the culture quickly becomes oxygen-deficient or hypoxic. After some block point in the cell cycle, cells can complete division and thus the proportion of furrowing cells drops fairly rapidly in the first hour of hypoxia and eventually drops to zero (Table 3.1). Cells which approach the surface of the culture may uptake sufficient oxygen to escape the block, therefore the increase in cell number during hypoxia may be larger than one may have predicted. After 4 hours of hypoxia, cultures underwent a first synchronous division 2 to 2½ hours after reaeration (34).

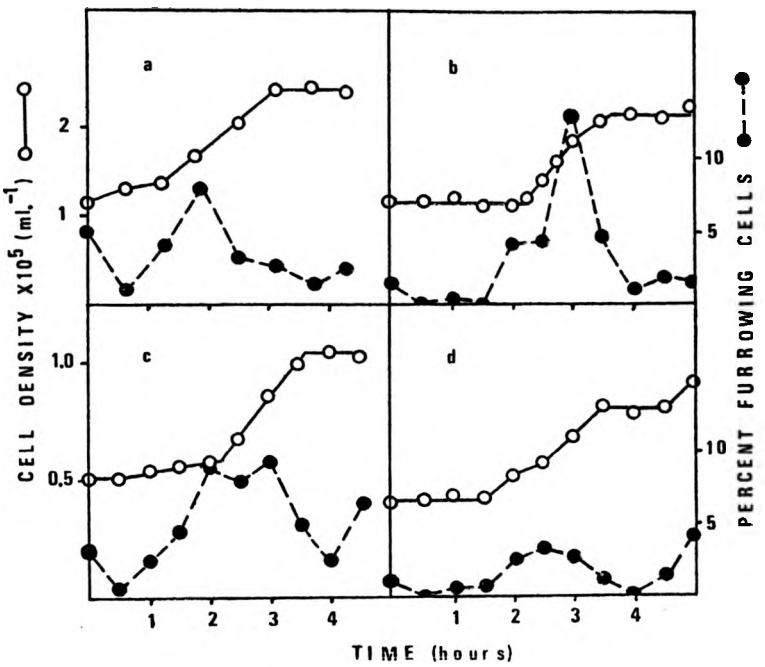


Fig.3.2 Synchrony achieved by the selection of amitotic cells. Synchronous populations of T.pyriformis were selected as described in section 3.3i from log phase cultures (1-2.5x10^{5}cells/ml). In experiments b and d, glucose supplemented medium was used.

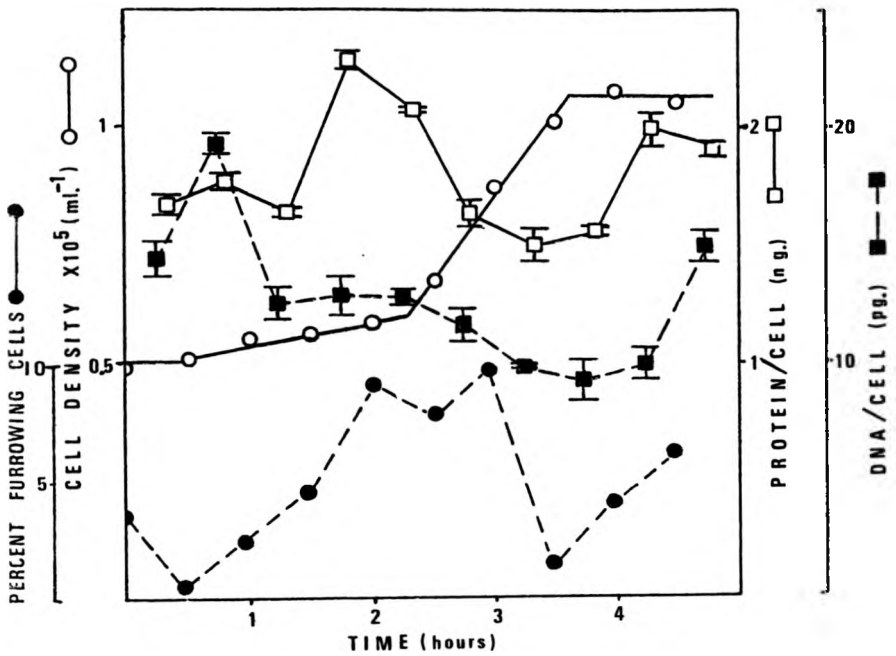


Fig.3.3 The DNA and protein content of a synchronous population of T.pyriformis attained by the selection of amitotic cells. One portion of cells were harvested, washed and disrupted as described in section 4.4. 0.05ml of the sonic lysate was denatured by 0.05ml of 4% SDS, diluted to 0.5ml with water and assayed for protein as described in section 3.5i. A second portion of cells were harvested and assayed for DNA as described in section 3.5ii.

Section 3.4 Isolation and purification of cellular material

In general, the procedure used in the isolation of cellular material will be described in the relevant results section, however two lengthy procedures will be described here. Preparative centrifuges used in the course of this research were; the Sorval RC2-B (ss34 and GSA rotors), the MSE Mistral (6x250ml and 8x30ml, swing-out, rotors) and the Beckmann L-50 and L-250 preparative ultracentrifuges (30,40 and50Ti rotors).

Section 3.4i Isolation of cilia from T.pyriformis (174)

Log phase cultures were harvested at 2,000xg for 10 min and washed with 25mM Tris HCl buffer, pH7.5, containing 0.25 M sucrose, resuspended to approximately $3x10^7$ cells/ml in this buffer then cooled to 0^oC. One volume of the cell suspension was added to a 45ml centrifugation tube (SS34) containing 5 volumes of "glycerol medium", consisting 70% glycerol, 25mM $MgSO_4$, 50mM KCl, 20mM thioglycolic acid, pH8.3 with Tris base, at -20^oC. The mixture was vortexed immediately to avoid freezing the cells, then it was maintained at -20 to -15^oC for up to 30 min. Deciliation was aided by vortexing for 30 sec on two occasions during this time. Cell bodies were removed by centrifugation at 5,000xg for 15 min at -15^oC and cilia were collected at 75,000xg for 30 min at 4^oC. If resuspended in glycerol medium and stored at -20^oC, cilia maitained their shape for several days.

Phase contrast microscopy showed that cilia preparations were usually free from cell debris. If the temperature of the cell bodies was allowed to rise much above -10^oC, cell lysis could occur and contaminate cilia preparations. If this did occur cilia were discarded.

Section 3.4ii Isolation of macronuclei from T.pyriformis (175)

Early stationary phase cultures were harvested at 2,000xg for 10 min, washed and resuspended in 12.5mM sodium phosphate buffer pH7.3, containing 47mM NaCl, 2mM KCl, and 1mM $MgSO_4$, to a density of $4x10^6$ cells/ml. To this an equal volume of cold 0.5% Triton X-100, containing 0.5M sucrose, was added slowly, with shaking. Then, after 5 min two volumes of filtered growth medium

was added to stabilize the nuclei. Nuclei were collected through a discontinuous gradient consisting 1 vol. 1M sucrose in buffer, 3vols. 2% Triton X-100,0.5M sucrose and 4 vols. nuclei preparation, at 800xg for 15 min. Nuclei were resuspended in 1M sucrose and appeared free from contamination under phase contrast microscopy.

Section 3.5 The assay of macromolecules from T.pyriformis

Section 3.5i Protein

Protein was estimated essentially as described by Lowry et al. Standard curves were set up in blank reagents as far as possible. If the protein sample contained interfering components, such as glycerol, the TCA denaturable protein was assayed as follows; 1ml 20% TCA was added to 0.05ml protein sample and this was heated to 100°C for 5 min. The denatured protein was collected by centrifugation, washed with 1ml cold distilled water,then resuspended by heating at 100°C with 0.1ml 2% S.D.S for 5 min. This was diluted to 1ml with distilled water and 0.1ml used in the determination of protein. Estimations were carried out in duplicate. S.E.M. were less than 5%.

Section 3.5ii DNA

Cells were harvested at 2,000xg for 5 min at growth temperature then resuspended in cold 10% TCA and sonicated in a MSE 150W sonicator at maximum power and amplitude for 2 min. Denatured cellular material was collected by centrifugation and washed with 5ml cold 5% TCA to remove free deoxynucleotides. DNA was extracted from the precipitate by heating at 70°C with 0.5M perchloric acid for 15 min. This was repeated and 0.35ml of the combined supernatants was used in the assay of DNA. DNA standards were made up in 0.5M perchloric acid and heated to 70°C for 15 min. 0.7ml of diphenylamine reagent was added to 0.35 ml sample and incubated overnight at 30°C. Optical density was measured at 578 nm. The standard curve was linear between 0 and 75 µg DNA/assay. (Diphenylamine was recrystallized from hexane and dissolved in glacial acetic acid, 1g/100ml. To this conc.H_2SO_4 was added,2.75ml.)

Section 3.6 The estimation of marker enzymes from T.pyriformis

Section 3.6i lactate dehydrogenase

The incubation was initiated by the addition of 0.1ml 60mM sodium pyruvate to the assay mixture which consisted; 70mM sodium phosphate buffer, pH 7.2, 1.3mM sodium cyanide, 0.17mM NADH and enzyme in a total volume of 3ml. The incubation was at 25°C and the oxidation of NADH was monitored continuously at 366nm. Blank rates, in the absence of pyruvate, were less than 10% of lactate dehydrogenase activity.

Section 3.6ii succinate dehydrogenase

The incubation was initiated by the addition of 0.1ml sodium succinate, 60mM, to the assay mixture which consisted; 70mM sodium phosphate buffer, pH 7.2, 1.3mM sodium cyanide, 0.5mM EDTA, 0.045mM DCPIP, 50µg PMS and enzyme in a total volume of 3ml. The incubation was at 25°C and the reduction of DCPIP was monitored at 600nm.

Section 3.6iii acid phosphatase

The reaction was initiated by the addition of enzyme to the assay mixture which consisted 0.1M sodium acetate buffer, pH4.0, 5mM p-nitrophenylphosphate in a total volume of 1ml. The reaction was terminated after 10 min at 30°C by the addition of 0.5ml 2M NaOH. The production of p-nitrophenoxide was determined at 430nm.

Section 3.6iv arginine kinase

The reaction was initiated by the addition of enzyme to the assay mixture. This consisted 20mM l-arginine, pH 8.6, 0.8mM ATP, 0.8mM $MgSo_4$ and enzyme in a total volume of 0.1ml. The reaction was terminated after 10 min at 30°C by the addition of 0.5ml 8% TCA and heated to 100°C for 2 min. Phosphate was determined according to Fiske and Subbarow. An assay omitting the heating stage was used as blank, a more satisfactory blank could have been obtained by the omission of arginine.

Section 3.6 v 5'nucleotidase

The reaction was initiated by the addition of enzyme. The assay consisted 0.1M glycine NaOH buffer, pH9.1, 5mM 5'AMP, 10mM $MgSO_4$ and enzyme in a total volume of 0.1 ml. The reaction was terminated after 15 min at 30°C by the addition of 0.5 ml 8% TCA and heating at 100°C for 2 min. Phosphate was determined according to Fiske and SubbaRow.

Section 3.7 determination of phosphate (177)

0.5ml of the terminated assay mixture were diluted with 0.35ml of distilled water. To this was added 0.1ml of 2.5% ammonium molybdate in 5N sulphuric acid. After mixing, 0.05ml ANSA reagent was added and after 10 min Optical density at 660nm was measured. ANSA reagent was 0.5g 1-amino-2-naphthol-4-sulphonic acid, 30g $NaHSO_4$ and 6g crystalline $Na_2SO_4.7H_2O$ in water, made up to 250 ml.

Chapter 4

The assay of adenylate cyclase from *Tetrahymena pyriformis*

Section 4.1 Materials used in the assay of adenylate cyclase

(a)enzymes

Rabbit muscle creatine phosphokinase was purchased from the Sigma (London) Chemical Co. Ltd., London. Beef heart cyclic AMP phosphodiesterase was purchased from the Boehringer Corp. (London) Ltd., London.

(b)radiochemicals

2-^{3}H ATP (18 Ci/mmole) and 8-^{3}H cyclic AMP (27.5 Ci/mMole) were purchased from the Radiochemical Centre, Amersham, Bucks.,UK. ^{3}H ATP was diluted to 20μCi/ml with double distilled water, 2μCi was added to disposable, heat-resistant polythene tubes suitable for the assay of adenylate cyclase and then freeze-dried. The tubes were stored desiccated at -20^{o}C. ^{3}H cyclic AMP was aliquoted, freeze-dried, stored desiccated at -20^{o}C and resuspended immediately before use.

A Packard 2425 tri-carb liquid scintillation counter was used with automatic standardization. The S.E.M. of the A.S. from 50 samples was less than 0.5% therefore, Quench adjustment was unnecessary.

(c)chromatography resins

Neutral alumina, activity grade 1 was purchased from ICN Pharmaceuticals, West Germany. Dowex 50X4-400 H^{+} form (dry mesh 200-400) was purchased from the Sigma (london) Chemical Co. Ltd. Polyethyleneimine (PEI) plates were Polygram CEL 300 PEI/UV_{254} purchased from Camlab, Cambridge.

(d)chemicals

The following compounds were purchased from Sigma (london) Chemical Co. Ltd.; cyclic AMP, cyclic GMP, all other nucleotides nucleosides, phosphocreatine, dithiothreitol, sodium pyruvate, glucagon, l-epinephrine, serotonin, spermidine HCl, glucose-6-phosphate, EGTA.Na_2., sodium azide, NADH and $G_{pp}(NH)_p$ were purchased from Boehringer Corp. (London) Ltd. SDS, EDTA.Na_2 and imidazole were purchased from BDH Chemicals Ltd., UK.

Section 4.2 The design of the assay

Adenylate cyclase activity was measured using radiolabelled substrate. The formation of cyclic AMP was estimated, after thorough purification on two separate ion exchange columns, by liquid scintillation counting. This is the technique of choice for reasons which I shall discuss. As adenylate cyclase is central to my research, I have evaluated this assay in detail and I shall present my results here.

The specific activity of adenylate cyclase in eukaryotic tissues is very low and, under assay conditions, the conversion of substate to product is rarely greater than 1%. Therefore, a precise and sensitive assay is required for this enzyme. Specific binding-protein assays and radioimmunoassays, that can estimate picomole quantities of cyclic AMP with precision, have been of great benefit in the assay of adenylate cyclase. However, Tetrahymena pyriformis contains a potent low K_m cyclic AMP phosphodiesterase that is capable of hydrolyzing any cyclic AMP, as it is produced by adenylate cyclase in vitro. This enzyme is predominantly soluble, but that present in particulate preparations can interfer drastically with the assay of adenylate cyclase. Methylxanthines and other inhibitors of cyclic AMP phosphodiesterase can be used to reduce product hydrolysis, but these do not eliminate the problem.

If radiolabelled ATP is used as substrate, then a "cold trap" of cyclic AMP may be used to advantage. In the presence of 10mM theophylline and 5mM cyclic AMP, adenylate cyclase may be assayed in the crude homogenates of T.pyriformis with 95 to 99% recovery of cyclic AMP.

Several techniques for the purification of the radioactive product of adenylate cyclase assays are now available. I consider that those using only one ion-exchange column or only thin-layer or paper chromatography lack specificity. A technique developed by Krishna (178), using Dowex 50W cation exchange resin and the precipitation of nucleotides other than cyclic AMP by the nascent production of $BaSO_4$, has been used widely. It has been reported that Ba^{2+} will catalyse the nonenzymic production of cyclic AMP (179), thus anomalous actvity may be measured. I have used two separate

ion-exchange columns in an assay essentially the same as that reported by Salomon et al (180), but which required several minor alterations. This basic assay may be manipulated to allow the estimation of the recovery of both substrate and product. It is also free from any anomolies that the alteration of assay constituents may have.

Section 4.3 Priciples of chromatography and preparation of columns

The functional group of the cation-exchange resin Dowex 50 is a sulphonic acid, which rapidly excludes highly charged anions from the column. The nucleotides are resolved by their charge content and also by their mobility on the resin. Nucleosides and the heterocyclic bases are retained by the column. Cyclic GMP eluted before cyclic AMP and separate from cyclic AMP (Fig.4.1).

Multivalent anions are strongly adsorbed on neutral alumina (181) and this ~~resin~~ will therefore separate cyclic AMP from the nucleotides. Adenosine does not bind to the column and cyclic GMP is eluted after cyclic AMP (Fig.4.2).

Dowex 50W and alumina columns were prepared in 14x0.5cm pasteur pipettes with glass wool plugs. Dowex 50w was defined in distilled water and added as a suspension in distilled water to give a bed hight of 2cm. These columns were washed with 2ml of 1M HCl and 10ml distilled water before use and could be reused without increasing blanks if also washed with 2ml 1M HCl after use and stored at 4°C in a sealed container. Distilled water was used to elute the columns.

Neutral alumina columns were prepared by adding a suspension of 1g ~~of resin~~ in 0.1M imidazole buffer, pH7.5, to the columns through buffer. These were washed with 8ml of imidazole buffer before use and discarded after use. This same buffer was used to elute the columns.

In the purification of cyclic AMP from adenylate cyclase assays, fraction volumes were determined by adding the requisite amount of water or buffer to the column, then allowing the column to "run to dryness". The columns often remained dry for several minutes without detrimental effects.

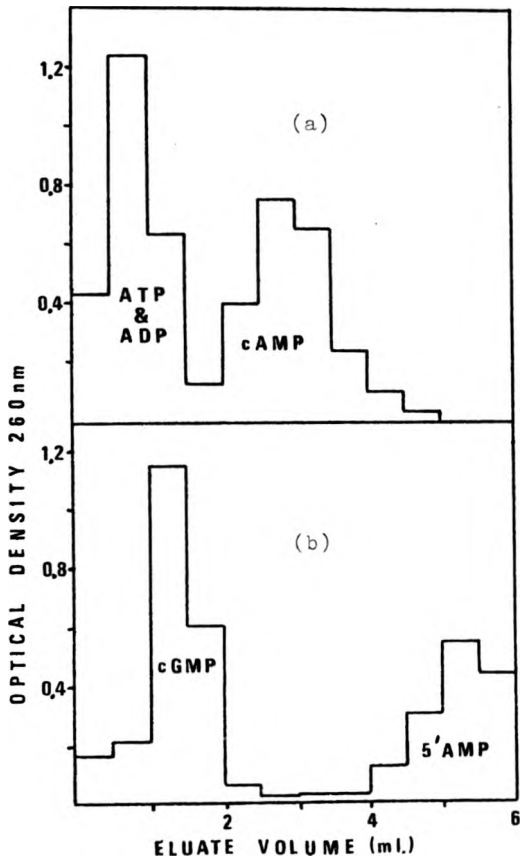


Fig. 4.1 The elution of nucleotides from 2.0x0.5cm Dowex 50X4 columns. Theophylline, adenosine and adenine were retained by the columns. (a)ADP, 0.1mM; ATP, 0.1mM; cyclic AMP, 0.15mM; EDTA, 3mM; SDS,.2%; pH7.5; 1ml. (b) cyclic GMP, 0.2mM; 5'AMP, o.15mM; EDTA, 3mM; SDS, 0.2%; pH7.5; 1ml. Eluant was distilled water in each case.

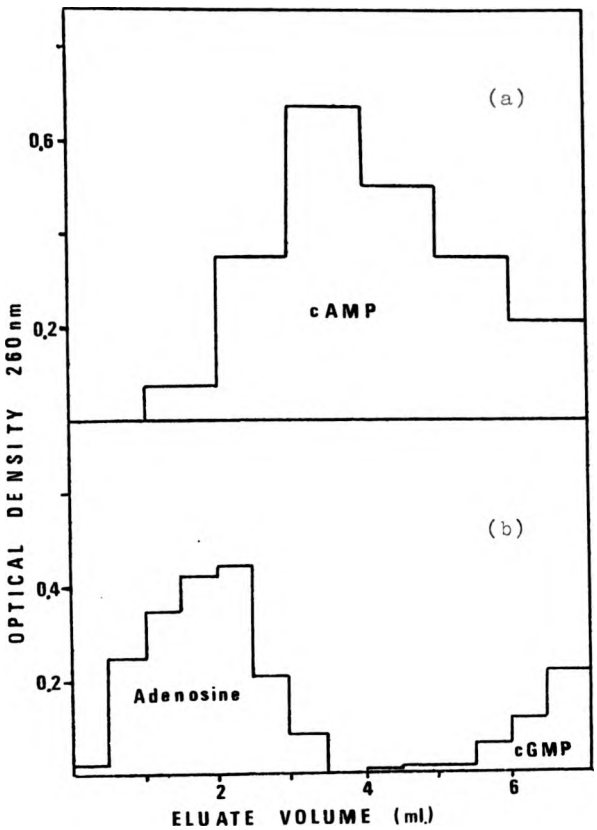


Fig.4.2 The elution of nucleotides and nucleosides from neutral alumina columns. ATP, ADP and AMP were retained by the columns. (a) cyclic AMP, 0.04mM; Imidazole HCl buffer, 0.1M; pH7.5; 2.5ml. (b) adenosine, 0.02mM; cyclic GMP, 0.02mM; Imidazole HCl buffer, 0.1M; pH7.5; 2.5ml. Eluant was 0.1M imidazole HCl buffer, pH7.5.

Section 4.4 the basic adenylate cyclase assay

Cells were harvested at 2,000xg for 5 min at growth temperature and washed with 25mM tris HCl buffer, pH 7.5, containing 0.25M sucrose, 2mM EDTA and 0.2mM dithiothreitol then resuspended in the same buffer, cooled to 0°C and sonicated in a MSE sonicator (150W) at maximum power and amplitude for 10 sec. This cell homogenate was used immediately in the assay of adenylate cyclase which comprised; 40mM Tris HCl buffer, pH7.5 5mM cyclic AMP, 5mM $MgSO_4$, 10mM theophylline, 0.04% BSA (fraction V), 1.2mM 2-^{3}H ATP (17.5 Ci/mole), 25mM phosphocreatine, 50 units/ml creatine phosphokinase and 0.05ml of homogenate in a final volume of 0.1ml. The incubation was terminated after 10 min at 30°C by the addition of 1ml of 3mM EDTA, containing 0.2% SDS and Tris base, pH7.5. 1ml of the reaction mixture was subjected to chromatography.

Initial purification of the reaction mixture was achieved on Dowex 50". The sample eluate and that from a further 1ml of water contained more than 95% of the ATP and ADP in the sample and were discarded. The next 2.5ml was run directly onto alumina column. The eluate from the alumina column was discarded, then 4ml of buffer was added to the alumina column and the eluate of this was collected. 12ml of scintillation fluid (7g butyl-PPD in 500ml Triton X-100 and 1l toluene) was added to this fraction to provide a stable emulsion suitable for liquid scintillation counting.

Serial dilutions of 2-^{3}H ATP in 4ml of 0.1M imidazole buffer pH7.5 were made and 12ml of scintillation fluid was added. It was estimated that each assay contained 1.05×10^6 cpm.

Section 4.5 Blank controls

Incubation blanks, terminated prior to the addition of enzyme, contained a similar amount of radioactivity as controls in which the enzyme or $MgSO_4$ was omitted from the assay. These were 50 to 200 cpm (0.004 to 0.02% total radioactivity). The actual blank value depended upon the batch of ^{3}H ATP used and increased to the top of this range on storage of 2-^{3}H ATP for several months. Even so, blanks were much lower than those attained by other

methods of purifying the reaction product. Much lower blanks could have been achieved by the use of α-^{32}P ATP, but this was thought to be unnecessary.

Section 4.6 Recovery controls

(a) cyclic AMP recovery

The basic assay was repeated in all details except that ^{3}H ATP was replaced by 0.02 μCi 8-^{3}H cyclic AMP. 1.2mM ATP was retained. In replicas of incubation blanks, the recovery of cyclic AMP was 67.5±2.0% in ten separate experiments and was not effected by the presence of Tetrahymena homogenate containing up to 0.6mg protein/assay. This is consistent with an efficiency of chromatography of 74.2%. Adenylate cyclase activity quoted in this report has been corrected for the loss of cyclic AMP during the transfer of samples and during chromatography. In assay replicates, recovery controls were indistinguishable from the recovery controls of incubation blank replicas.

(b) ATP recovery

The basic assay was replicated in all details except that the stopping solution also contained ATP, AMP and ADP at 5mM and omitted SDS. Assays were heated to 100°C for 4 min to destroy creatine phosphokinase activity and protein was removed by centrifugation. 1 μl of the terminated reaction mixture was subjected to polyethylene imine thin-layer chromatography with 1M LiCl as eluant. Nucleotide regions were observed under ultraviolet light. R_f values were 0.17±0.08 (ATP), 0.50±0.04 (ADP) and 0.65±0.03 (AMP). These regions were cut out and imersed in 5ml scintillation fluid (5g/l PPO, 0.25g/l POPOP in toluene) for the estimation of radioactivity. The ATP-regenerating system was sufficient to maintain the ATP concentration throughout the course of the assay. If this regenerating system was omitted, ATP was completely destroyed by the cell homogenate.

Section 4.7 Identification of the product of the adenylate cyclase assay

The identity of the radioactive product of this adenylate cyclase assay was determined by hydrolysis with beef heart cyclic AMP phosphodiesterase. Of course, this product does elute with bone fide cyclic AMP from both Dowex 50w and alumina columns, but this does not prove that the product is cyclic AMP. Evidence that this product and cyclic AMP are hydrolysed in an identical manner by cyclicAMP phosphodiesterase resolves this dilema. Cyclic GMP is also hydrolysed by this enzyme, but this nucleotide elutes in a different manner from Dowex 50W and alumina chromatography.

A cell homogenate was prepared and used in the assay of adenylate cyclase and in cyclic AMP recovery controls. These assays were terminated by 1ml of 3mM EDTA alone, so that the subsequent cyclic AMP phosphodiesterase incubation was not inhibited by SDS. The phosphodiesterase incubation was carried out between the Dowex 50W and alumina chromatography steps. By this means, theophylline was removed.

The "cyclic AMP" fraction from Dowex 50W chromatography (2.3ml in this case) was buffered to 0.1M imidazole, pH7.5 with concentrated buffer and $MgSO_4$ was added to a concentration of 1mM, an excess of Mg^{2+} over the EDTA present in this fraction. Beef heart cyclic AMP phosphodiesterase, 0.05ml of 1/5 diluted enzyme, in 0.1M imidazole buffer, pH7.5 was added to initiate the incubation, which was at 30°C and a total volume of 2.5ml. The hydrolysis of the product from duplicate adenylate cyclase assays and the hydrolysis of cyclic AMP from duplicate recovery controls were terminated after 0, 15 min, 30 min and 18 hours by heating to 100°C for 3 min. Fig4.3 shows that product and cyclic AMP were destroyed in an identical fashion. Controls without cyclic AMP phosphodiesterase showed little hydrolysis.

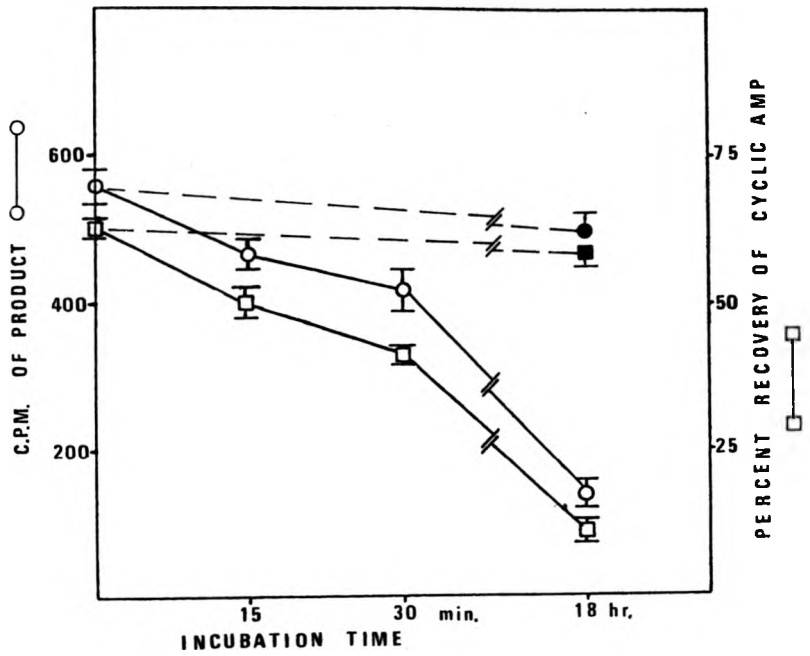


Fig.4.3 The destruction of the product of adenylate cyclase assays by beef heart cyclic AMP phosphodiesterase. Closed symbols represent control experiments in the absence of beef heart cyclic AMP phosphodiesterase.

Section 4.8 The time course of the assay

Fig.4.4 shows the time course of a typical adenylate cyclase assay. The assay was linear for the first ten minutes of the incubation, therefore, a ten minute assay was used routinely.

Section 4.9 The effect of enzyme concentration on the assay

Fig.4.5 shows the production of cyclic AMP as a function of protein concentration. This was linear for up to 0.75mg/assay,

Section 4.10 The accuracy of the assay

For 50 duplicate assays,selected at random from the period of this research, the mean deviation of each duplicate assay from its mean was 9.32%. This value was effected, unduly, by 8 estimates with greater than 10% deviation. If these are omitted the mean deviation of the remainder becomes 4.53%.

It is obvious that with this sort of assay, where control blanks must be subtracted from assay values, that the error involved in estimating low activity will be large. Normally the amount of enzyme added was such that the assay values were greater than five times control blanks. This could not be achieved at all times and consequently some estimates had disproportionately large errors.

In general, each assay was carried out in duplicate and the quoted errors represent the individual estimates. Where greater accuracy was desired, triplicate assays were prefered. In these cases the mean deviation of the estimates has been quoted.

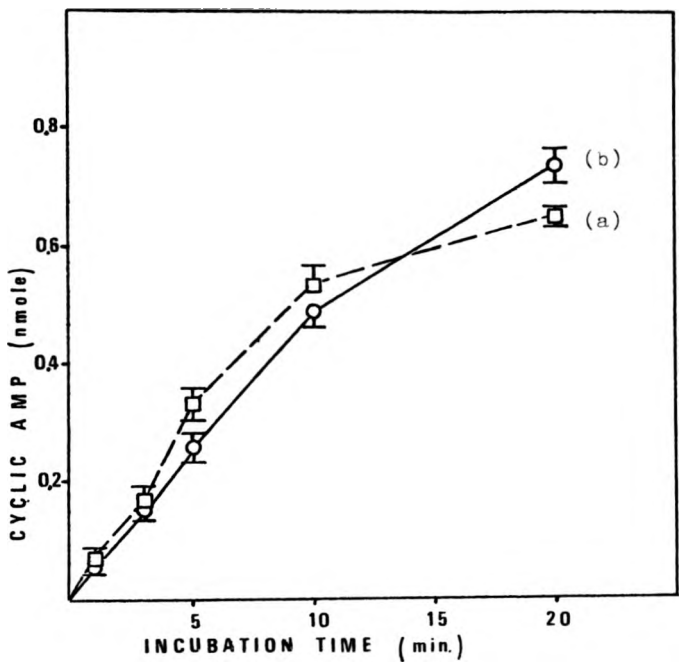


Fig.4.4 The time course of adenylate cyclase assays. Log phase cells were harvested and resuspended in 25mM Tris HCl buffer, pH7.5, containing either (a) 0.25M sucrose or (b) 0.25M sucrose and o.2mM dithiothreitcl. The cells were sonicated and the residue from $3x10^5$ cells was used immediately in several duplicate assays for adenylate cyclase, which were terminated after different length incubations at 30^oC.

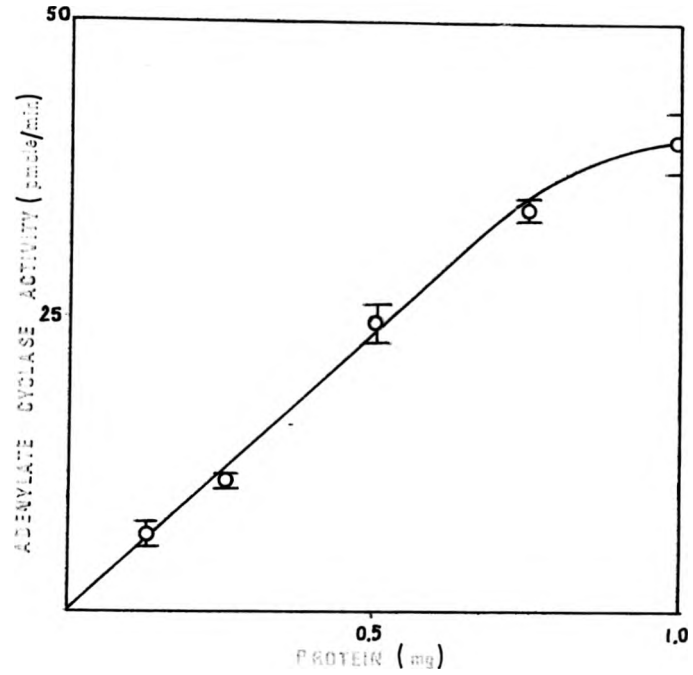


Fig. 4.5 The effect of enzyme concentration on the assay of adenylate cyclase. Enzyme prepared from log phase cells (2.9×10^5/ml) and assayed as described in section 4.4.

Section 4.11 Factors that may be important in the assay of adenylate cyclase from T.pyriformis

(a) harvesting and washing the cells

T.pyriformis may be harvested at lower relative centrifugal forces than 2,000xg, but to do this cells must be chilled to 4°C and up to 15 min centrifugation may be required. Any centrifugation may upset the metabolism of T.pyriformis, therefore, this proceuure should be standardized. In chapter 6, I shall describe the elevation of adenylate cyclase activity caused by hypoxia. T.pyriformis should be harvested and washed as rapidly as possible to limit the length of hypoxia before assay. This was not possible where large batches of cells were harvested.

Several buffers may be used to wash T.pyriformis and, so long as cell lysis is avoided, the choice of buffer is fairly arbitrary. KCl, 0.15M, and Tris HCl buffers containing sucrose were used predominantly. In cases where the wash buffer may have proved to be a substantial contaminant of the enzyme preparation, the same buffer as used in the cell disruption was used to wash the cells.

(b) disruption of T.pyriformis

Four techniques have been tried. Only sonication in a MSE 150W sonicator at maximum power and amplitude for 10 to 15 sec at 0°C, was found to produce efficient and reproducible cell breakage. This was used routinely in the estimation of total adenylate cyclase activity from T.pyriformis. However, sonication causes severe disruption of cell organelles, so two milder techniques were tried for cell fractionation. The passage of cells through sintered glass funnels proved to be inefficient and caused substantial loss of cellular material. Potter-Elvehjem homogenization was prefered, although cell breakage had to be monitored in every experiment. This technique is less rapid than sonication and thus extends the period of hypoxia before assay. Cell lysis with Triton X-100 was used to isolate nuclei.

The effect of prolonged sonication on adenylate cyclase activity measured in whole cell homogenates was determined.

Between 10 and 15 sec, activity reached a broad maximum. After 15 sec, activity fell rapidly and was 50% of the maximum after 60 sec sonication. 10 was used routinely.

(c) disruption buffer

EDTA, 2mM, was used in disruption buffers to avoid aggregation of cellular material (182). It was found that this increased the estimate of adenylate cyclase activity, see chapter 5. This effect was probably due to the relief of calcium inhibition (44,81). EDTA may also effect the apparent location of adenylate cyclase *in vitro*. EDTA, 2.7mM is used in calcium shock deciliation of *T.pyriformis* and prevents the release of cilia from the cell bodies before the addition of calcium (183). EDTA, 0.1mM is used in low ionic strength buffers to lyse isolated cilia and aid solubilization of the arms and central pair of microtubules of the cilia (183), see chapter 5.

Dithiothreitol, 0.2 to 2mM, in disruption buffers, prolonged the linearity of the adenylate cyclase assay to some extent (Fig.4.4). This allowed the adoption of a ten minute incubation. This reagent should help to stabilize adenylate cyclase, but, even at 2mM dithiothreitol, particulate preparations of adenylate cyclase could not be stored overnight at 4°C, at 20°C or in liquid nitrogen, without almost complete loss of activity. $MgSO_4$, 5mM, and NaF, 10mM, have been recommended as stabilizing agents for several mammalian adenylate cyclases (81), however, fluoride was found to be an inhibitor of the enzyme from *T.pyriformis*, chapter 5. Removal of this ion by dialysis before assay would have resulted in a low recovery of enzyme. $MgSO_4$ did not improve the stability of adenylate cyclase from *T.pyriformis* on its own. In general, enzyme preparations were assayed immediately after preparation. Any procedure which took more than an hour to complete, for instance high speed centrifugation or chromatography, was accompanied by a low yield of adenylate cyclase.

(d) assay constituents

$MgSO_4$ was used in preference to $MgCl_2$ because of the reported stimulation of rat liver adenylate cyclase by chloride and other halides (184). This was not observed for *T.pyriformis*.

Sulphate can inhibit creatine phosphokinase, but, at the level used in the assay, creatine phosphokinase activity was sufficient to maintain the concentration of ATP, see section 4.6(b).

Cyclic AMP, 5mM, and theophylline, 10mM, were required to avoid the destruction of cyclic AMP by phosphodiesterases present in whole cell homogenates. For particulate preparations the concentration of the cold cyclic AMP trap could be reduced to 1mM. The activity of adenylate cyclase in a 2,000xg precipitate from sonic lysate of T.pyriformis was 10.9±0.4 pmole/min/10^6 cells in the presence of 1mM cyclic AMP and was 9.3±0.5 pmole/min/10^6 cells in the presence of 5mM cyclic AMP. Therefore, although cyclic AMP may inhibit adenylate cyclase this is unlikely to be substantial. The extent of cyclic AMP inhibition could have been determined only in preparations free from phosphodiesterase contamination. This was not achieved.

In those cases where EDTA was omitted from the disruption buffer, EDTA or EGTA at 1mM were added to the assay. In some cell systems, calcium is important in allowing hormone stimulation of adenylate cyclase in vitro (81), therefore, hormone sensitivity was sought in the presence and absence of chelator, chapter 5.

(e) assay pH

The measured production of cyclic AMP from sonic lysates of T.pyriformis was a function of the pH of the assay buffer (Fig.4.6). This is not claimed to be a pH profile of the enzyme because such factors as the rate of enzyme degradation, the efficiency of the ATP-regenerating system and the recovery of cyclic AMP from chromatography, could be pH dependent. Tris-HCl buffer was prefered because of its superior buffering capacity compared to glycyl-glycine. The most satisfactory standard pH was considered to be pH 7.5 because of the sharp drop in measured activity above pH8.

(f) incubation temperature

Adenylate cyclase from T.pyriformis was found to be particularly unstable and the loss of activity was temperature dependent. Activity was constant at 4°C for 20 min, but at 30°C 42% of activity was lost in this time and at 37°C 66% of activity was lost. Thus 30°C was considered to be the maximum suitable temperature for the assay of adenylate cyclase.

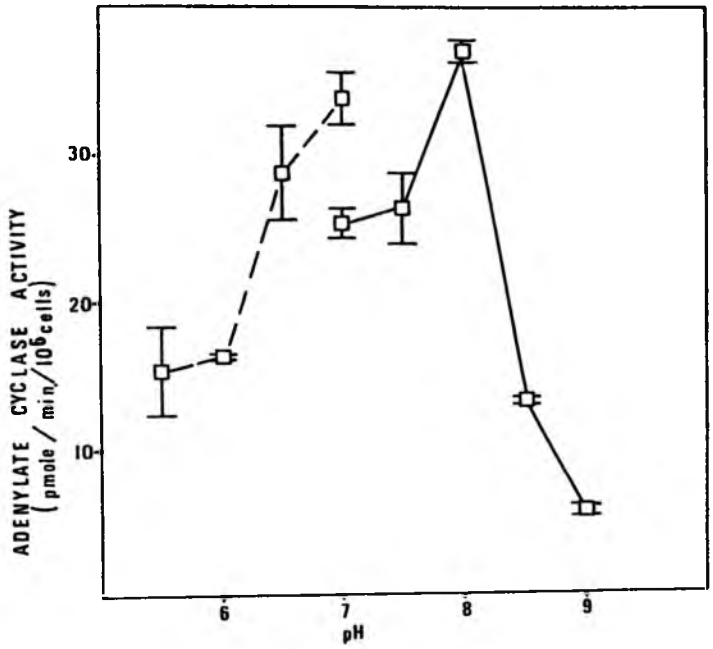


Fig. 4.6 The estimated production of cyclic AMP from the adenylate cyclase assay as a function of pH. Log phase cells ($5x10^5$/ml) were harvested, washed and resuspended in 2.5mM Tris HCl buffer, pH7.5 containing 0.1mM dithiothreitol. Adenylate cyclase activity was measured as described in section 4.4. For pH5.5 to pH7.5, the Tris HCl buffer of the assay cocktail was replaced by 40mM glycyl glycine buffer.

(g) termination of the assay

The incubation may be terminated by several means. The prefered method was the addition of 0.5ml or 1.0ml of 3mM EDTA, pH7.5, which could include 0.2% SDS. Before chromatography, protein had to be denatured by SDS or removed by heating to 100°C for 4 min and centrifugation. EDTA alone stopped the adenylate cyclase assay very effectively, but other enzymic reactions which would not require metal ions would have continued. In experiments where contamination of the cell homogenate by divalent metal ions could occur, the use of EDTA alone could not be recommended. For instance, ferrous ions may displace sufficient Mg^{2+} from EDTA to allow the magnesium-dependent cyclic AMP phosphodiesterase to function. This would be disasterous if terminated adenylate cyclase incubations were stored overnight before processing, even at 4°C.

TCA could not be used to terminate the reaction because it interfered with chromatography.

(h) conclusion

Standardization of all procedures in the assay of adenylate cyclase was very important. Even so, the measured activity of adenylate cyclase from cells at the same stage of culture growth varied as much as five fold, although values of 25 to 50 pmole/min/10^6 cells for log phase cells were most common. The reasons for this could not be determined but the response of adenylate cyclase to hypoxia or calcium could have been involved. However, duplicate experiments carried out on the same culture were reproducible, thus the trends observed during the culture growth and cell cycle are valid.

PART III
RESULTS AND DISCUSSION

PART III
RESULTS AND DISCUSSION

Chapter 5

The characterization of adenylate cyclase from *Tetrahymena pyriformis*

Section 5.1 The location of adenylate cyclase in T.pyriformis

Evidence will be presented which indicates that adenylate cyclase is associated with cilia and probably other parts of the cortical structure of T.pyriformis. 5'nucleotidase has been used as a marker enzyme for the plasma membrane. Cilia, which may be associated with or free from the isolated pellicle, have been identified by arginine kinase activity. This enzyme may be extracted from cilia by lysis with low ionic strength buffers in the presence of EDTA (185), so some care must be taken with the interpretation of these results. Lactate dehydrogenase and succinate dehydrogenase were measured to locate mitochondria. Lysosomes were identified by acid phosphatase activity.

Section 5.1i Adenylate cyclase is an intracellular enzyme

Whole cells, harvested, washed and resuspended as in section 4.4, may be introduced into the basic adenylate cyclase assay and incubated for 10 min at 30°C without substantial cell lysis. Under these conditions, intracellular activity would not be measured because ATP does not enter cells efficiently. No cyclic AMP was detected from such an incubation, whereas a duplicate assay, in which cells were first sonicated, allowed the estimation of 36.1±2.8 pmole of cyclic AMP/min/10^6 cells. Therefore, adenylate cyclase is certainly contained within T.pyriformis.

Section 5.1ii The purification of adenylate cyclase in cilia

Cilia, prepared by glycerination, section 3.4i, were washed and resuspended in glycerol medium and assayed for adenylate cyclase. In a number of experiments this resulted in a 2 to 6 fold purification of adenylate cyclase (Table 5.1). The recovery of adenylate cyclase was never more than 19% and resuspended cell bodies retained a large portion of adenylate cyclase activity. However, phase contrast microscopy showed that the cell bodies were heavily contaminated with cilia. Arginine kinase activity was consistent with this (Table 5.1). Cell bodies could not be prepared free from cilia without lysis of the cells, therefore it is uncertain whether adenylate cyclase is located entirely in the cilia of T.pyriformis.

Section 5.1iii The characterization of adenylate cyclase from isolated cilia

(a) stability of activity

If resuspended in glycerol medium and stored at -20°C, cilia retained their viability for several days. Adenylate cyclase activity was stable under these conditions (Table 5.2).

(b) estimation of activity in undisrupted cilia

The motility of isolated cilia, prepared by glycerination, may be reactivated by the addition of ATP.Mg^{2-}. Therefore, it appears that ATP.Mg^{2-} can enter isolated cilia. Adenylate cyclase activity, estimated in cilia, was identical before and after sonication (Table 5.2). Isolated, intact cilia thus represent a useful model system of the adenylate cyclase, in vivo. It is possible that glycerol may perturb the ciliary membrane.

(c) sensitivity of adenylate cyclase in isolated cilia

Ciliary adenylate cyclase showed a similar sensitivity to fluoride, azide, hormones and $G_{pp}(NH)_p$ as the enzyme measured in the whole cell homogenate, see section 5.4. This would infer that there is only one adenylate cyclase in T.pyriformis, although more detailed experiments would be reqired to confirm this, such as metal dependency, apparent K_m for substrate etc..

(d) the lysis of isolated cilia

If cilia are transfered to a low ionic-strength medium containing EDTA, they round-up and lyse. Glycerinated cilia were dialysed at 4°C for 6 hours against three changes of 1mM Tris HCl buffer, pH8.3, containing 0.1mM EDTA and 0.1mM dithiothreitol. The adenylate cyclase activity was compared in the precipitate and supernatant after centrifugation at 100,000xg for 1 hour. The recovery of adenylate cyclase activity in the supernatant was almost three times the activity in the precipitate (Table 5.3). The supernatant enzyme was unstable and unfortunately not suitable for further purification, however, this experiment does imply that adenylate cyclase in cilia is either soluble or only very loosely attached to the membrane. This has repercussions on the further localization of this enzyme in T.pyriformis, for how can one

differentiate between an enzyme which is particulate *in vivo* but can be easily solubilized and an enzyme which is soluble *in vivo* but can form non-specific attachments to particulate matter *in vitro*.

Section 5.1iv The cell fractionation of *T.pyriformis*

The localization of adenylate cyclase among cell fractions was attempted, but this was fraught with difficulties. Deciliated cell bodies were never isolated free from contamination by cilia, therefore any cell fractions may have been contaminated by cilia. The cortical structures may contain attached cilia and cilia, which precipitate predominantly at greater than 10,000xg, will precipitate at less than 5,000xg and thus contaminate any mitochondrial preparations. Furthermore, the enzyme may be released from cilia and form non-specific attachments to other organelles.

(a) differential centrifugation of disrupted cells

When *T.pyriformis* were disrupted by Potter-Elvehjem homogenization, adenylate cyclase activity was recovered, predominantly in an 800xg particulate fraction, however, sonication disrupted cells such that adenylate cyclase was found in the 800xg supernatant (Table 5.4). The precise distribution of adenylate cyclase activity among fractions from differential centrifugation of sonicated cells was not reproducible. Between 29% and 65% of total activity could be recovered in a 2,000xg precipitation. Adenylate cyclase activity, released into a 2,000xg supernatant from sonicated cells did not precipitate at 20,000xg and was detected predominantly in a 100,000xg supernatant (Tables 5.5 &5.6). Adenylate cyclase released into a 2,000xg supernatant from homogenized cells could be collected at 12,000xg (Table 5.6).

(b) discontinuous sucrose density gradient separation

On the basis of lactate dehydrogenase, succinate dehydrogenase and 5'nucleotidase distribution, mitochondria and plasma membrane were reasonably well separated on a discontinuous sucrose density gradient (Table 5.7). However, adenylate cyclase activity was present in both fractions. Arginine kinase activity was distributed among all the fractions, suggesting that the

cilia were present in the pellicle and mitochondrial fractions, but also that cilia had been lysed to allow the recovery of arginine kinase activity in the supernatant.

Section 5.1v The isolation of the nucleus of T.pyriformis

The previous cell fractionation schemes involve the disruption of the nucleus. The nucleus was isolated separately from cells lysed by Triton X-100. No adenylate cyclase activity was found in the nucleus (Table 5.8).

Section 5.1vi conclusion

A reasonable interpretation of these results is that adenylate cyclase is attached to the outer-limiting membrane and ciliary membrane of T.pyriformis, in vivo. One may present other interpretations, for instance that adenylate cyclase is also associated with mitochondria, but the location of a single type of adenylate cyclase in a single continuous membrane is attractive.

Table 5.1 The purification of adenylate cyclase in cilia

Two experiments in which the activity of adenylate cyclase was monitored throughout the purification of cilia by glycerination, section 3.4i. Total cells, cell bodies and cilia were resuspended in glycerol medium and sonicated immediately before assay. TCA-denaturable protein, arginine kinase and acid phosphatase were assayed as described in chapter 3. Activity has been normalized to 10^6 cells of starting material. Adenylate cyclase assays contained 1mM EDTA supplement.

Experiment I cell density; 1.2×10^5 cells/ml

	protein mg	adenylate cyclase activity				arginine kinase				acid phosphatase	
		pmole/min	%	pmole/min/mg	pur.*	μmole/10min	%	μmole/10min/mg	pur.*	μmole/10min	%
Total cells	1.39	56.9 3.9	100	40.9 2.8	1.0	0.71	100	0.51	1.0	0.60	100
Cell bodies	1.08	35.7 3.4	63	33.1 3.2	0.8	0.33	46	0.31	0.6	0.58	97
Cilia	0.04	10.9 1.0	19	272.5 25.7	6.7	0.05	7	1.25	2.4	0.04	7
Ciliary supernatant	0.03	1.3 0.5	2	43.3 16.4	1.1	0.10	14	3.33	6.5	0.04	7

Experiment II cell density; 1×10^5 cells/ml

	protein mg	adenylate cyclase activity				arginine kinase				acid phosphatase	
		pmole/min	%	pmole/min/mg	pur.*	μmole/10min	%	μmole/10min/mg	pur.*	μmole/10min	%
Total cells	1.31	38.2 2.0	100	29.2 1.5	1.0	0.68	100	0.52	1.0	0.86	100
Cell bodies	1.20	35.8 3.1	94	29.8 2.6	1.0	0.38	56	0.31	0.6	0.55	64
Cilia	0.05	3.7 0.2	10	73.9 4.9	2.5	0.06	9	1.20	2.3	0.04	5
Ciliary supernatant	0.10	1.2 0.1	3	12.2 0.3	0.4	0.12	18	1.20	2.3	0.04	5

* purification.
specific activity; per mg (protein).

Table 5.2 Storage of isolated cilia / adenylate cyclase of intact cilia

Cilia were prepared as described in section 3.4i and assayed for the adenylate cyclase in the presence of 1mM EDTA.

sonicated cilia;	198±23 pmole/min/mg
unsonicated cilia;	187±15 "
intact cilia stored for 4 days;	180±12 "

Table 5.3 Lysis of isolated cilia

Cilia were prepared as described in section 3.4i and resuspended in glycerol medium. This was removed by dialysis against 1mM Tris HCl buffer, pH8.3, containing 0.1mM EDTA and 0.1mM dithiothreitol. The adenylate cyclase activity, measured in the presence of 1mM EDTA, was compared after high speed centrifugation.

activity before dialysis *	8.6 pmole/min/10^6 cells
100,000xg supt. after dialysis *	2.3±0.4 "
100,000xg ppt. " " *	0.8±0.2 "

* in glycerol medium

* in dialysis buffer

Table 5.4 Fractionation of T.pyriformis by differential centrifugation

Log phase cells ($0.8x10^5$/ml) were grown, harvested and washed as described for the basic adenylate cyclase assay, then resuspended in 25mM Tris HCl buffer, pH7.5 containing 2mM EDTA, 0.2mM dithiothreitol with or without 0.25M sucrose and cooled in ice. Disruption was achieved by sonication or Potter-Elvehjem homogenization. A particulate fraction was collected at 800xg for 10 min at 4°C and resuspended in the same buffer.

sucrose & disruption	% total protein		adenylate cyclase pmole/min/10^6cells		
	ppt.	supt.	total	ppt.	supt.
+ /son.	13	95	44.4±5.9	11.4±1.6	61.1±2.9
- /son.	10	92	49.5±2.7	0 ±1.3	65.2±3.1
+ /P-E	33	71	65.6±3.0	32.5±2.6	15.3±0.9
- /P-E	32	74	76.5±1.9	45.9±2.4	12.8±1.4

Table 5.5 Fractionation of T.pyriformis by differential centrifugation ii

Log phase cells were harvested as before, washed with 0.15M KCl, resuspended in 25mM Tris HCl buffer, pH7.5, containing 0.25M sucrose, cooled in ice then sonicated. The 2,000xg for 10 min pellet was washed four times with the same buffer and the combined washings were subjected to centrifugation at 20,000xg for 15 min. EDTA was not present in the assay of adenylate cyclase.

fraction	adenylate cyclase pmole/min/10^6cells
2,000xg ppt.	1.7±0.07
2,000xg supt.	4.1±0.01
washed 2,000xg ppt.	0.62±0.07
washings	0.79±0.09
total cells	5.1±0.1

Table 5.6 Fractionation of T.pyriformis by differential centrifugation iii

In two separate experiments log phase cells were resuspended in 25mM Tris HCl buffer, pH7.5, containing 2mM EDTA and 0.2mM dithiothreitol, cooled in ice and disrupted by Potter-Elvehjem homogenization or sonication. Particulate fractions were resuspended in the same buffer.

cell density	0.2×10^5	5×10^5
disruption	P-E	son.
total adenylate cyclase	37.4±0.8	24.7±1.4
percent total;		
2,000xg ppt.	55±2	65 1
12,000xg ppt.	24±2	5.5±1.1
100,000xg ppt.	0	9.5±0.6
100,000xg supt.	0	18.5±0.7

Table 5.7 Fractionation of T.pyriformis by discontinuous sucrose density gradient centrifugation

log phase cells ($4x10^5$/ml) were harvested and washed with 50mM Tris HCl buffer, pH7.5, containing 3mM EDTA, then resuspended in the same buffer, cooled in ice and disrupted by Potter-Elvehjem homogenization. 4.5ml of this homogenate were added to a discontinuous sucrose gradient consisting 15ml of 1.46M, 8ml of 1.0M and 9ml 0.34M sucrose in buffer. After centrifugation at 4,000xg for 15 min at $4^{\circ}C$, the sample and 0.34M sucrose bands should have contained mitochondria and microsomes, which were separated by diluting this fraction three fold with buffer and centrifuging at 19,600xg for 20 min. The pellicle fraction collected at the interface between 1.0M and 1.46M sucrose.

	LDH*	SDH*	A.Phos.	5'Nu.	Arg.K.	adenylate cyc.	
"pellicle"	0.04	0.15	0.44	0.35	0.11	30.5	1.9
"mitochondria"	0.30	0.51	1.54	0.10	0.11	39.7	2.1
"mitochondrial supernatant"	0.03	0	0.27	0.08	0.26	0	0.9

units; mole/10min/mg except * mole/min/mg and adenylate cyclase which was pmole/min/mg

Table 5.8 Isolation of the nucleus from T.pyriformis

Cells were lysed by Triton X-100 and purified as described in section 3.4ii. Samples were sonicated before adenylate cyclase assay.

	adenylate cyclase
Total lysate/0.25% Triton X-100, 0.25M sucrose	25.6±1.8
nuclei resuspended in 1M sucrose	0.3±0.7
Cells resuspended and sonicated in 1M sucrose	27.9±2.2

adenylate cyclase assay was carried out in the presence of 1mM EDTA and units of activity were pmole/min/10^6cells.

Section 5.2 The metal requirement of adenylate cyclase from T.pyriformis

Throughout the course of this research $MgSO_4$ was used to supply the metal requirement of adenylate cyclase activity, however, several other divalent metals could support activity; Mn^{2+}, Cd^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}. Three cations from those tested were not utilized; Ni^{2+}, Ca^{2+}, Cu^{2+} (Table 5.9). This pattern may be a fundamental property of the enzyme, of the metal-ATP complex or, more probably, a combination of these factors.

In the higher eukaryotes, adenylate cyclase has developed a more rigorous requirement for the metal ion. In general, only Mg^{2+}, and to a smaller extent Mn^{2+} and Co^{2+}, can support activity. An exception to this is beef sperm adenylate cyclase, which will also utilize Zn^{2+}, Cd^{2+} and Ca^{2+} (186). In the lower eukaryotes and the prokaryotes, the metal ion requirement may be more a feature of the suitability of the substrate complex for the production of cyclic AMP, than the selectivity of the enzyme. Boiling alkaline barium hydroxide will catalyse the non-enzymic production of cyclic AMP from ATP (179), but Mg^{2+} will not catalyse this reaction. However, the conformation of the substrate complex will be important in the enzyme catalysed reaction and will be dependent on the metal. Mg^{2+} and Ca^{2+} interact only with the β and γ phosphates of ATP; recently it was reported that Mg^{2+} only interacted with the β phosphate, whereas the transition metals may also interact with N-7 of adenine (187,188). Moreover, Mn^{2+} and Co^{2+} interact with all three phosphates (187) to produce what may be a particularly favourable conformation for the requirements of adenylate cyclase.

The metal ion requirement of mitochondrial ATPase has been correlated with the ionic (crystal) radius of the metal (189) (Fig.5.1). By comparison, there is a poor correlation between adenylate cyclase activity and the size of the metal, however the unpredictably high activities obtained with Mn^{2+} and Cd^{2+} could indicate particularly suitable substrate conformations for the production of cyclic AMP.

The selection of the metal ion requirement of an adenylate cyclase may be achieved by an allosteric site for metal ions. Adenylate cyclase requires an excess of metal ion over ATP for maximum activity. It is still uncertain whether this is because excess metal ions reduce the concentration of free ATP, which may

be a competitive inhibitor of adenylate cyclase, or because there is an allosteric site for metal ions which enhances activity. The possible existence of a binding site for divalent cations which can effect adenylate cyclase activity from T.pyriformis has been indicated by the inhibition of the enzyme by low concentrations of calcium (44,81). I have found that 1mM EDTA and 1mM EGTA stimulated adenylate cyclase activity from T.pyriformis (Table 5.10). In the presence of 1mM EGTA, 1mM $CaCl_2$ produced a 23% inhibition of adenylate cyclase (Table 5.10). Under these conditions the concentration of free Ca^{2+} would be much less than 10^{-6}M. The actual nature of this effect is unknown. Calcium may act via a tightly associated allosteric site of adenylate cyclase or via a more indirect mechanism.

Table 5.9 The metal requirement of adenylate cyclase from T.pyriformis

Log phase cells (2.5×10^5/ml) were harvested, washed and resuspended in 25mM Tris HCl buffer, pH7.5, containing 1mM dithiothreitol, cooled in ice, then disrupted by sonication. This sonic lysate was used to initiate the assay of adenylate cyclase which consisted 40mM Tris HCl buffer, pH7.5, 5mM cyclic AMP, 10mM theophylline, 0.04% BSA, 1mM EDTA, 1.2mM 2-^{3}H ATP (17.5 Ci/ mole), 25mM phosphocreatine, 50units/ml creatine phosphokinase and 0.05ml of homogenate in a final volume of 0.1ml. The reaction was incubated and terminated as previously. A zero-time control, in the presence of 5mM $MgSO_4$, was used as a blank.

additions at 5mM	adenylate cyclase pmole/min/10^6cells
none	<0.5
$NiSO_4$	<0.5
$CaCl_2$	<1.5
$CuSO_4$	<1.5
$ZnSO_4$	6.3±0.1
$CoSO_4$	13.6±2.9
$FeSO_4$	21.6±3.7
$CdSO_4$	32.0±0.7
$MgSO_4$	42.0±0.3
$MnSO_4$	74.8±1.0

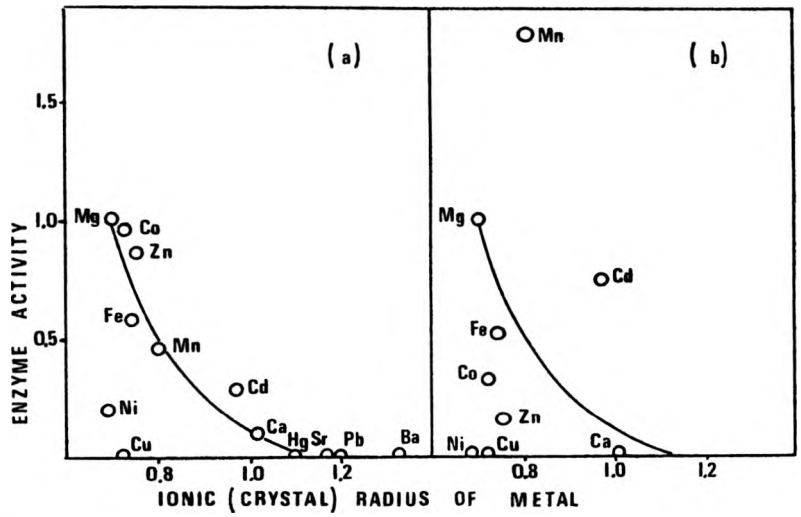


Fig.5.1 The metal requirement of adenylate cyclase from T.pyriformis and of mitochondrial ATPase (189) as a function of the ionic (crystal) radius of the metal. Details of the preparation and assay of adenylate cyclase activity are presented in the legend to table 5.9.
(a)beef heart mitochondrial ATPase from ref.189
(b)adenylate cyclase activity from T.pyriformis
Activity was normalized with respect to the activity in the presence of Mg.

Table 5.10 The effect of Ca^{2+} and metal chelators on adenylate cyclase activity from T.pyriformis

Log phase cells ($1.5x10^5$/ml) were harvested, disrupted and assayed for adenylate cyclase as described in the legend to table 5.9, except that EDTA was omitted from the basic assay cocktail.

additions	adenylate cyclase pmole/min/10^6cells
EDTA (1mM)	<0.5
EGTA (1mM)	6.9±0.4
$MgSO_4$ (5mM)	13.6±0.5
" + EDTA (1mM)	36.3±1.7
" + EGTA (1mM)	31.4±2.1
" + " + $CaCl_2$ (0.5mM)	32.1±1.8
" + " + $CaCl_2$ (1mM)	24.2±1.5

Section 5.3 The effect of substrate concentration on adenylate cyclase activity

The enzyme activity as a function of substrate concentration is shown in Fig. 5.2. The ratio of Mg^{2} to ATP was held constant, therefore the proportion of free ATP to viable substrate would have been the same in each assay. ATP recovery controls were carried out as described in section 4.6. The substrate concentration was not stable at less than 1mM ATP (Table 5.11). This must be taken into account when interpreting Fig. 5.2.

The Eddie-Hofstee plot was preferred for estimations of kinetic constants, because this placed more bias on the higher substrate concentrations, which suffered less from the anomalies caused by substrate instability. The apparent K_m was 6.9mM, rather high for adenylate cyclases, and V_{max} was 110 pmole/min/10^6cells. The latter value was used to estimate the apparent co-operativity between substrate utilization. This was positive at high substrate concentration.

The results presented here, were to have been a preliminary investigation into the kinetics of this enzyme, however, although they indicated some apparent co-operativity, which may have proved of interest, the inherent pratical difficulties of this work would have made further investigations unrewarding. The crude enzyme preparation that was available was unstable and contained high ATPase activity and was thus unsuitable for kinetic analysis of adenylate cyclase.

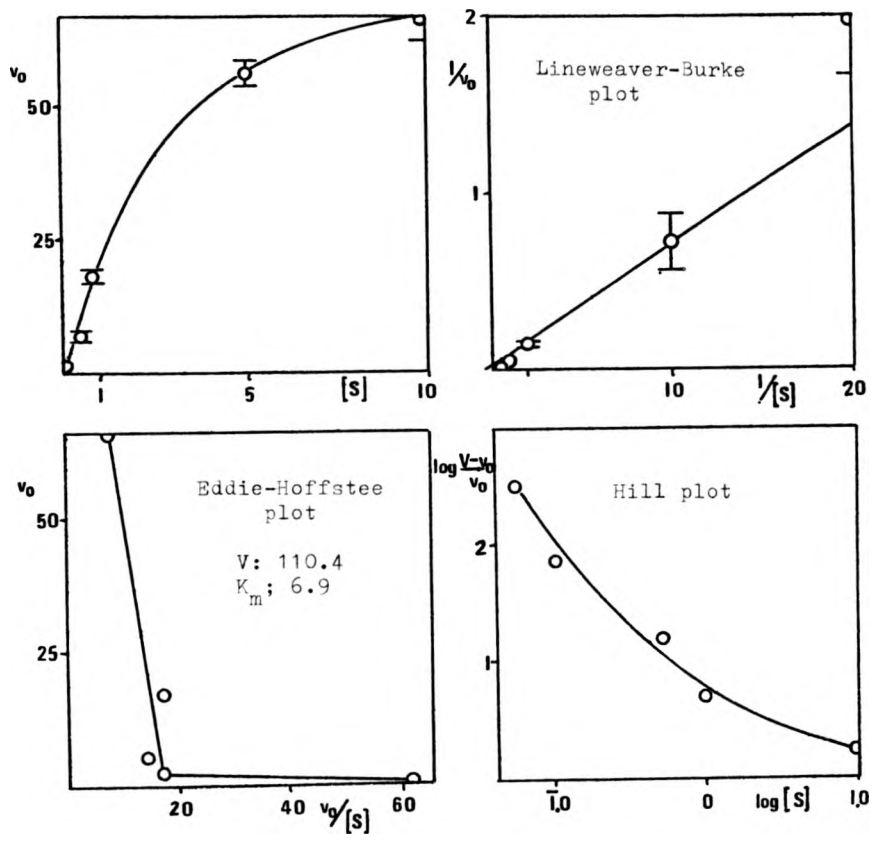


Fig.5.2 The effect of substrate concentration on the assay of adenylate cyclase activity from T.pyriformis. A sonic lysate of T.pyriformis was prepared and assayed as described in section 4.4 from log phase cells (2.3×10^5/ml). Axes are labelled using the abbreviations; v_o, enzyme activity measured over 10min (pmole/min/10^6cells); S, concentration of ATP, for concentration of Mg see table 5.11 (mM); V, maximum enzyme activity ; K_m, Michaelis-Menten constant.

Table 5.11 The recovery of ATP from adenylate cyclase incubations at different substrate concentrations

Sonic lysate of T.pyriformis was prepared as described in the legend to Fig. 5.2 and the residue from 3.2×10^5 cells was added to ATP recovery controls as described in section 4.6. The assay contained 1mM EDTA, therefore a minimum 1mM concentration of $MgSO_4$ was maintained to allow for chelation by EDTA. This may have been an overestimate because of endogenous divalent cations in the sonic lysate

$MgSO_4$ mM	ATP mM	% recovery of ATP
1.2	0.05	18
1.4	0.1	49
3.0	0.5	83
5.0	1.0	100
11.0	10.0	100

Section 5.4 The sensitivity of adenylate cyclase from T.pyriformis

Adenylate cyclase from T.pyriformis was not particularly sensitive to mammalian hormones, although, in the presence of GTP or $G_{pp}(NH)_p$, serotonin activated the enzyme by approximately 10% (Table 5.12). NaF, 10mM, and NADH, 5mM, inhibited adenylate cyclase by approximately 70% and 40% respectively (Tables 5.12-5.14). NaN_3 stimulated the enzyme by approximately 30% at 10mM (Table 5.12). Compounds which were ineffective were; adenosine, 1.2mM; glucagon, 18µg/ml; glucose-6-phosphate, 0.1mM; pyruvate, 10mM; spermidine HCl, 1mM; adrenaline, 0.1mM. (Tables 5.12-5.14).

Several preparations of adenylate cyclase from T.pyriformis were tested and each had similar sensitivity to fluoride, serotonin and adrenaline. Perhaps the most suitable system for testing hormone sensitivity was isolated intact cilia, where the enzyme system should be relatively similar to the in vivo situation.(Table 5.12).

Total adenylate cyclase activity, estimated from T.pyriformis did not appear to be sensitive to the presence of 0.5% glucose in the growth medium. Under these conditions the cyclic AMP content of the cells was lower than that of cells grown on unsupplemented media (79). This could indicate that adenylate cyclase activity was modified by glucose only in vivo, or that glucose was effecting cyclic AMP phosphodiesterase activity or cyclic AMP eflux from cells.

Table 5.12 The sensitivity of adenylate cyclase from sonic lysates and isolated intact cilia of T.pyriformis

(a) Cells grown into stationary phase, without shaking, 4.8×10^5/ml, were harvested, washed with 0.15M KCl and resuspended in 25mM Tris HCl buffer, pH7.5, containing 0.25M sucrose. The sonicate was used in the assay of adenylate cyclase, in the absence of EDTA.

(b) Cilia were prepared as described in section 3.4i, resuspended in glycerol medium and used in the assay of adenylate cyclase, in the presence of 1mM EDTA.

additions	guanine * nucleotide?	adenylate cyclase (a) pmole/min/mg:	(b) basal act.
none	-	7.2±0.1	112 ± 4
10mM NaF	-	-	13.8±5.9%
10mM NaN_3	-	130.3±1.2%	128.7±0.2%
0.1mM l-epinephrine	-	97.5±5.1%	98.9±0.3%
0.1mM l-epinephrine	+	106.9±3.2%	105.0±3.5%
0.1mM serotonin	-	98.6±3.1%	100.8±0.8%
0.1mM serotonin	+	111.8±1.2%	109.2±1.4%
none	+	96.4±4.1%	101.3±1.3%
0.1mM glucose-6-P	-	98.0±2.9%	-

* For preparation (a), 10μM GTP was present where indicated.
For preparation (b), 10μM $G_{pp}(NH)_p$ was present where indicated.
N.B $G_{pp}(NH)_p$ was supplied as the lithium salt. 40μM $Li(SO_4)_2$ had no effect on the assay of adenylate cyclase from intact cilia.

Table 5.13 The sensitivity of adenylate cyclase in the fractions from differential centrifugation of a sonic lysate of T.pyriformis.

Legend as in Table 5.5. The activity of each fraction without additions was given in Table 5.5.

fraction	adenylate cyclase percent basal activity	
	10mM NaF	0.1mM epinephrine
total homogenate	28.5±0.6	103.1±2.2
2,000xg ppt.	32.9±0.2	95.5±2.7
2,000xg supt.	30.2±0.3	106.1±0.1
washed 2,000xg ppt.	25.1±7.1	118.0±11.3
20,000xg supt. of the washings	37.1±3.3	140 ±1.4

Table 5.14 The sensitivity of adenylate cyclase activity from sonic lysates of T.pyriformis.

Cells from the deceleration phase of culture growth, in glucose supplemented medium, 1.1×10^6 cells/ml, were harvested, washed, sonicated and assayed as described in section 4.4.

addition	adenylate cyclase	
none	22.6±1.6	pmole/min/10^6 cells
spermidine HCl, 1mM	21.2±1.4	"
glucagon, 18 µg/ml	19.0±1.7	"
adenosine, 1.2mM	24.2±1.5	"
pyruvate, 10mM	21.5±0.9	"
NADH, 5mM	12.7±0.6	"

Chapter 6

The control of adenylate cyclase activity in *Tetrahymena pyriformis*

Section 6.1 The variation of adenylate cyclase activity during the growth of cultures of T.pyriformis

As measured by the methods described in chapter 4, adenylate cyclase activity in log phase cultures was usually 25 to 50 pmole/min/10^6cells. The activity from stationary phase cultures was not significantly different from this. However, adenylate cyclase activity did vary as a function of the growth of cultures (Fig.6.1). The cells were grown on glucose supplemented medium and adenylate cyclase activity was estimated in the presence of 1mM EGTA, in this experiment. Adenylate cyclase reached a maximum during the deceleration of culture growth.

Section 6.2 The response of adenylate cyclase activity to a period of hypoxia

Logarithmically growing cultures were forced to enter a stationary phase by hypoxic shock, section 3.3ii. A transient high level of adenylate cyclase activity was measured in the first 90 min of hypoxia, then the activity fell to a level not much higher than a control culture (Fig.6.1 & 6.2). The increase in activity could be reduced by 0.5 or 1.0μg/ml of cycloheximide and totally abolished by 2.5μg/ml cycloheximide(Fig.6.3) Thus, de novo protein synthesis was involved in the response of adenylate cyclase activity to hypoxia. This was not necessarily synthesis of adenylate cyclase. The inhibition of protein synthesis,which blocks the completion of division by furrowing cells and blocks the snout formation that normally accompanies hypoxia (Table 6.1), could influence adenylate cyclase activity by some less direct mechanism. For instance, protein synthesis may be involved in the alteration of membrane composition or physical properties or may be involved in the synthesis of some effector of adenylate cyclase.

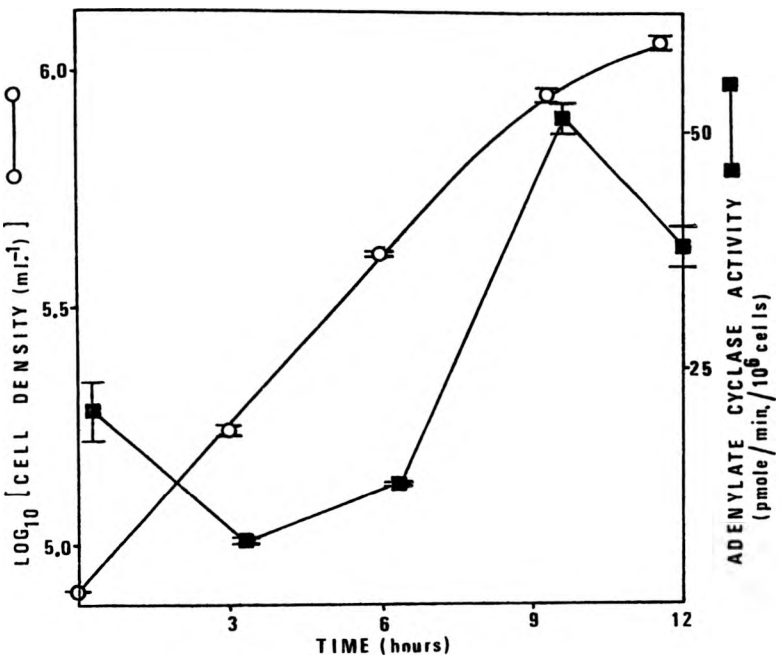


Fig.6.1 The variation of adenylate cyclase activity during the growth curve of T.pyriformis. Cells were grown in glucose supplemented medium as described in section 3.2, then harvested at different times in the growth curve, resuspended in 25mM Tris HCl buffer, pH7.5, containing 1mM dithiothreitol, sonicated and assayed for adenylate cyclase activity immediately, in the presence of 1mM EGTA.

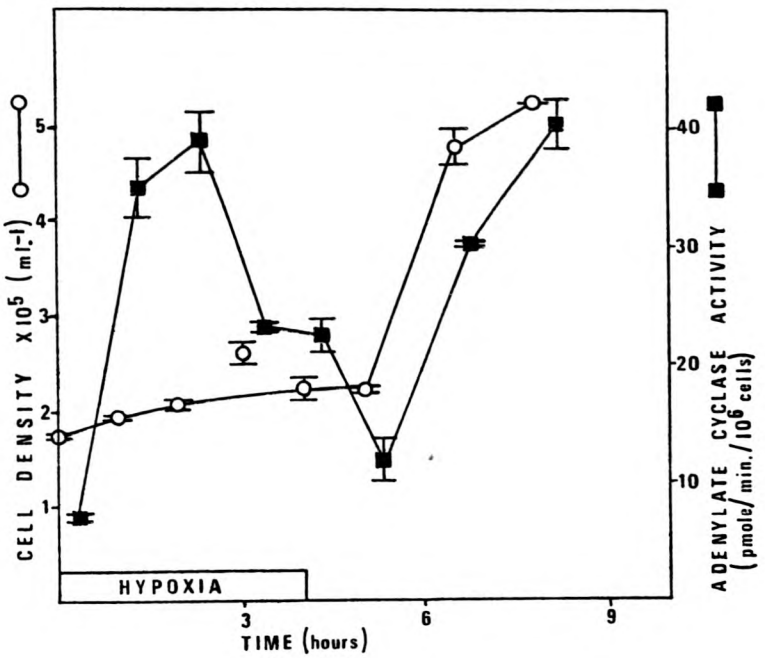


Fig.6.2 The response of adenylate cyclase activity to a 4 hour period of hypoxia and release from this. Fig.6.1 was a control experiment for this figure and the details of the growth of T.pyriformis and assay of adenylate cyclase is given in the legend to fig.6.1. Hypoxia was administered as described in section 3.3ii.

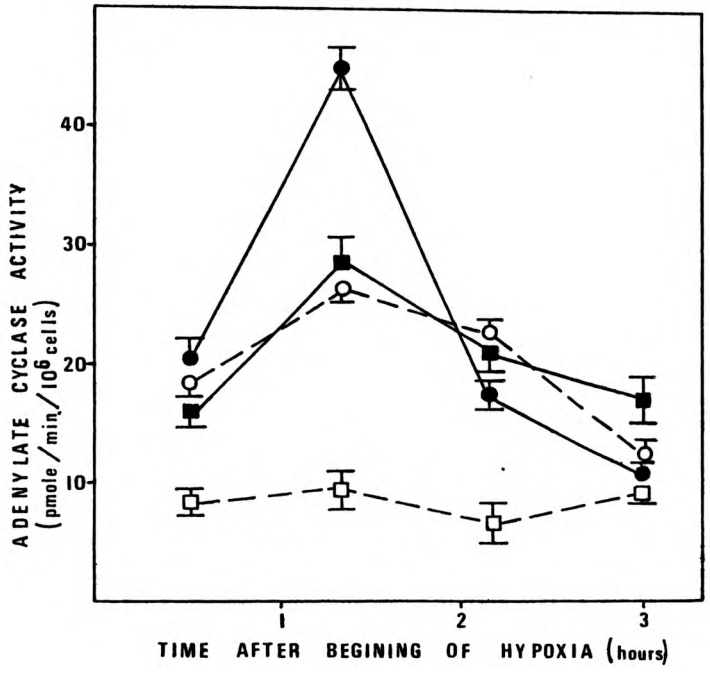


Fig.6.3 The effect of cycloheximide on the response of adenylate cyclase activity to hypoxia in T.pyriformis. Cells were grown in glucose supplemented medium to a density of 2.6×10^5/ml and stopped shaking to initiate hypoxia. Cycloheximide was administered at this time, in the quantities; none ● ; 0.5 μg/ml ○ ; 1.0 μg/ml ■ ; 2.5 μg/ml □ . Cells were harvested, washed and resuspended in 25mM Tris HCl buffer, pH7.5, containing 1mM dithiothreitol, and assayed for adenylate cyclase immediately after sonication, in the presence of 1mM EDTA.

Table 6.1 The effect of cycloheximide on the response of cell density, percent furrowing cells, production of snouted cells and protein content to hypoxia

In the experiment described in fig.6.3 the following functions were monitored.

time after start of hypoxia (min)	cell density x10 (/ml) 0	0.5	1.0	2.5	percent furrowing cells 0	0.5	1.0	2.5
0	2.60	2.68	2.64	2.66	3.5	2.7	1.9	2.2
54	2.64	2.56	2.69	2.52	1.4	2.0	0.8	1.5
104	2.57	2.92	2.60	2.73	1.0	1.5	4.3	0.6
151	2.72	2.72	2.74	2.60	0	0.6	2.0	1.9
	percent snouted cells 0	0.5	1.0	2.5	protein/cell (ng) 0	0.5	1.0	2.5
0	0	0	0	0	1.55	1.45	1.51	1.57
54	1.7	0.2	0	0	2.03	2.03	1.73	1.59
104	4	0.4	0.9	0.2	2.00	1.73	1.72	1.77
151	15	0.7	0.6	0.4	2.13	1.86	1.86	1.86

Cycloheximide concentrations are given as 0, 0.5, 1.0, 2.5 μg/ml. Protein estimation was carried out on the enzyme preparation (fig.6.3) and was manipulated as described in the legend to fig.3.3. Cell scored as "snouted" were those which showed prominant morphological changes, to make identification unequivocal.

Section 6.3 The variation of adenylate cyclase during the natural cell cycle of T.pyriformis

Synchronous populations of T.pyriformis were selected on the basis of phagocytotic activity, section 3.3i, and adenylate cyclase activity was determined,as described in section 4.4, during the subsequent cell division cycle. The degree of synchrony in the two experiments presented in Figs. 6.4 & 6.5 was not identical, therefore the variation of adenylate cyclase activity was not reproduced exactly, however, two major characteristic were seen in both experiments. These were a peak in adenylate cyclase activity immediately prior to or during cell division and a minimum activity immediately following division. This was consistent with the variation in cyclic AMP levels throughout the cell cycle (45), (Fig.7.3).

Section 6.4 The variation of adenylate cyclase during the synchronous recovery of T.pyriformis from hypoxia

Upon release from hypoxia, adenylate cyclase activity declined to a minimum immediately before cell division, then increased dramatically during division and continued increasing after cell division. This perturbed cell division cycle was not studied in detail, therefore, more rapid fluctuations in adenylate cyclase activity could have been overlooked. The rise in adenylate cyclase activity could have been prolonged because the culture had reached a density consistent with deceleration phase (Fig. 6.2).

Section 6.5 Discussion

The measurement of total adenylate cyclase activity per cell during the cell cycle and culture growth may have been influenced by the period of hypoxia during harvest and before disruption of the cells. In fact, one may explain the results presented in this chapter by the variation in the sensitivity of adenylate cyclase activity to hypoxia during the cell cycle and during culture growth. There is no defence to this argument, however I believe that these results may reflect the variation of enzyme activity, in vivo.

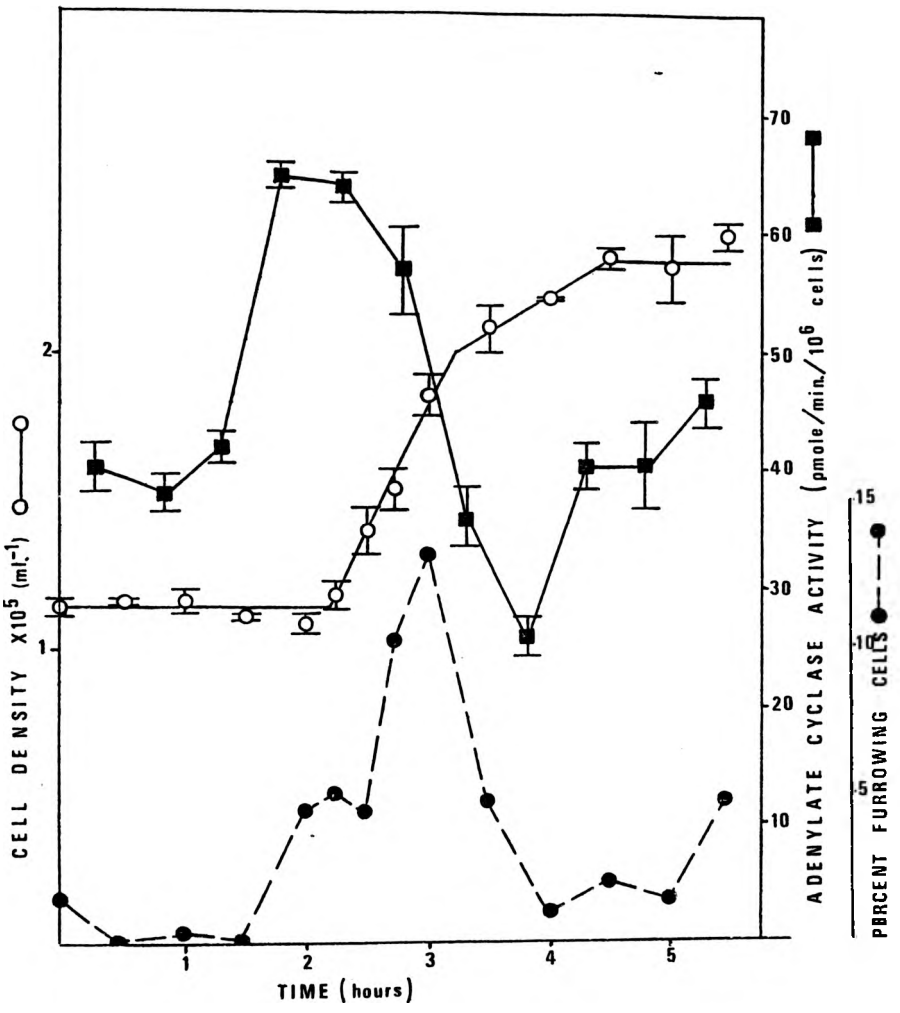


Fig.6.4 The time course of a synchronous culture of T.pyriformis, in glucose supplemented medium, selected on the basis of phagocytotic activity, showing the variation of adenylate cyclase activity. Cells were harvested and assayed for adenylate cyclase activity as described in section 4.4. Each value of enzyme activity was the mean of three estimates.

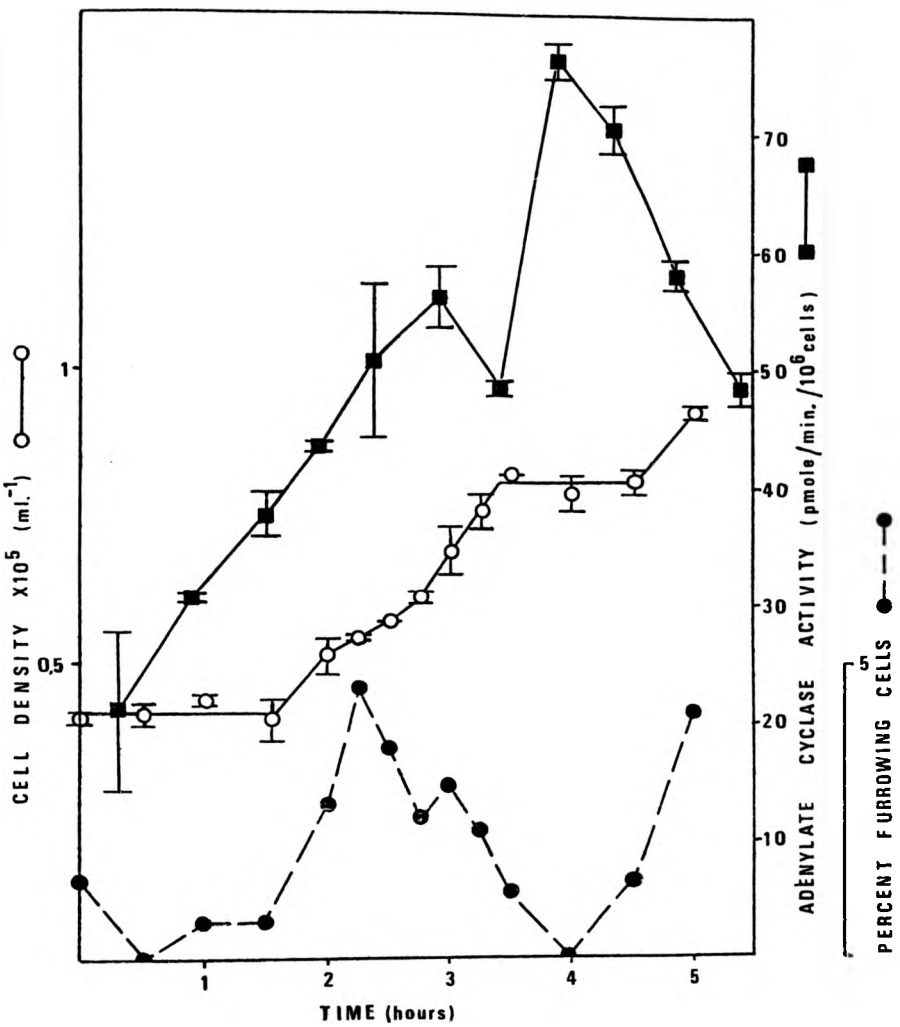


Fig.6.5 The time course of a synchronous culture of T.pyriformis, in glucose supplemented medium, selected on the basis of phagocytotic activity, showing the variation of adenylate cyclase activity. Cells were harvested and assayed for adenylate cyclase as described in section 4.4. Each value of enzyme activity was the mean of three estimates.

Chapter 7
Discussion of results

A specific assay for adenylate cyclase from T.pyriformis was developed and proved to be valid. The results of this investigation can be regarded as the characterization of the true properties of this enzyme in vitro. One can never say how closely in vitro studies reflect the properties of an enzyme in vivo. The use of intact isolated cilia in part of this research as a source of adenylate cyclase activity was probably the best model system available, but, in this case, the membrane system may have been perturbed by glycerol.

One may say that in the discussion of results, one should limit hypothesis and let "the results speak for themselves". However, this would leave a reader, uninitiated to the realm of cyclic nucleotides, without much insight into the possible interpretation of these results. In the following discussion, I may leave myself open to the criticism of hypothesis on too little information. To this I offer no defence, except that one should only accept a hypothesis on the basis of the evidence and that, once proposed, a hypothesis may stimulate its own destruction.

Section 7.1 The localization of adenylate cyclase in T.pyriformis

The complete localization of adenylate cyclase was not achieved, however, a reasonable interpretation of the results of cell fractionation is that the enzyme is associated with the continuous membrane which represents the outer-limiting membrane and ciliary membrane. The association between adenylate cyclase and membrane structures could be broken upon cell lysis. Sonication of the cells was efficient in solubilizing a portion of enzyme activity, whereas lysis of isolated cilia by low ionic strength buffers released the major portion of adenylate cyclase activity into a 100,000x*g* supernatant.

The localization of adenylate cyclase in the ciliary membrane and outer-limiting membrane of T.pyriformis, in vivo, would lead to the compartmentalization of cyclic AMP. Cyclic AMP phosphodiesterase activity can be isolated predominantly from the cytosol of T.pyriformis and as measured in vitro from log phase asynchronous cultures the activity of phosphodiesterase is far larger than the activity of adenylate cyclase, at physiological substrate concentrations. During the cell division cycle the activity of cyclic AMP phosphodiesterase varies dramatically (45). When phosphodiesterase activity is high,

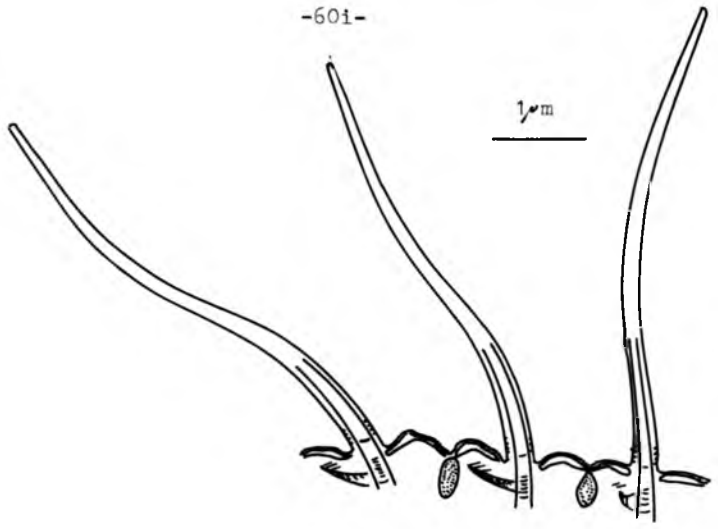


Fig.7.1 A schematic view of the cortical compartment of T.pyriformis. For simplicity the inner pair of microtubules of the cilia are omitted. See the electron-micrographs,figs. 1.3 & 1.4, for a detailed view of the cortical components.

the concentration of cyclic AMP in the cytosol should be determined primarily by the K_m for cyclic AMP of the phosphodiesterase, unless certain control features are absent *in vitro*. One control feature which was certainly absent *in vitro* was compartmentalization. The three membrane system of the cortex of *T.pyriformis* may act as a barrier, which would allow adenylate cyclase activity to outweigh the local activity of phosphodiesterase. Thus, adenylate cyclase may be capable of the control of localised fluctuations of cyclic AMP levels in the cilia and cortex of *T.pyriformis*. When the activity of cyclic AMP phosphodiesterase is low in the cell division cycle of *T.pyriformis*, adenylate cyclase may determine the level of cyclic AMP in the whole cell (Fig.7.1).

This would suggest that the cyclic AMP metabolism of *T.pyriformis* is capable of the independent control of cortical and cellular functions. Further evidence may be forthcoming which may extend the areas of independent control to other compartments of the cell. Also adenylate cyclase activity may be the dominant controlling element of cyclic AMP levels in different compartments of the cell at different times in the cell division cycle.

The ciliary membrane is enriched in phosphonolipids (16). Surely the distribution of the lipids among the membrane structures of the cell may provide the most suitable lipid environment for the membrane bound enzymes of these structures. The adenylate cyclase system of *T.pyriformis* does not form a particularly tight association with the membrane *in vitro*, but the lipid composition of the membrane may still be an important factor in the control of adenylate cyclase activity *in vivo*. This control may or may not be apparent in *in vitro* estimations of enzyme activity.

Section 7.2 The sensitivity of adenylate cyclase of *T.pyriformis*

All preparations of adenylate cyclase activity from *T.pyriformis* showed great sensitivity to sodium fluoride. At 10mM, sodium fluoride inhibited adenylate cyclase activity by approximately 70%. This is inconsistent with the results of other investigations. However, similar fluoride sensitivity has been measured in adenylate cyclase from other microorganisms. Bacterial adenylate cyclases are commonly inhibited by sodium fluoride and adenylate cyclase activity

from Saccharomyces cerevisiae and Euglena gracilis were inhibited by fluoride (130,143,147). The presence of a sodium fluoride inhibited adenylate cyclase in mammalian tissues has been reported. Data obtained from beef brain cortex was rationalized in terms of two independent enzyme activities, one of which should be inhibited by fluoride (190).

Fluoride does not increase cyclic AMP levels in intact mammalian cells, although fluoride may enter cells and can stimulate adenylate cyclase activity in vitro. Although other interpretations may be presented, it has been suggested that in vitro sensitivity to fluoride may be a consequence of a change in the lipid environment that occurs in the preparation of the membranes (81). If this is correct, then it is not surprising to find substantial differences between the fluoride sensitivity of mammalian adenylate cyclase and that of T.pyriformis, because the association between the membrane and enzyme is quite different in these two systems.

Adenylate cyclase activity from T.pyriformis was stimulated 10% by 10^{-4}M serotonin. This was observed only in the presence of GTP or $G_{pp}(NH)_p$ at 10μM. This sensitivity may explain the 30% increase in cyclic AMP levels as a response to 10^{-8}M serotonin during a 10 min incubation, which has been reported in T.pyriformis, strain GL (46). Of particular interest in this recent report was the effect of serotonin antagonists on cyclic AMP levels in T.pyriformis. At 10^{-8}M, three different serotonin analogues reduced cyclic AMP levels to 12-18% of controls in 10 min. This would suggest that endogenous serotonin plays a leading role in the maintenance of cyclic AMP levels in T.pyriformis. The serotonin content of cells varied during culure growth (61), this was low in log phase, incresed 2 to 8 fold upon entry to stationary phase then declined as stationary phase progressed. This was with cultures grown with a glucose supplement and aerated by stirring. The growth conditions of the culture monitored for adenylate cyclase activity through culture growth(Fig.6.1) were similar to this report. The activity of adenylate cyclase appears to be closely related to the serotonin content of T.pyriformis. However, the importance of serotonin in the regulation of cyclic AMP during culture growth may be questioned because the actual level of serotonin per cell was not reproducible in cultures grown identically (61). The existence of hormone sensitivity in adenylate cyclase from

T.pyriformis may suggest that intercellular communication is after all an important feature of the cyclic AMP metabolism of this organism and that the assumption that the role of cyclic AMP in this organism is concerned solely with the metabolism of the individual cell, may be invalid. However, the other major homogeneous populations of cells that have been used as model systems for intact tissues are tissue cultures. In these systems, the hormone sensitivity of the cyclic AMP metabolism is far more startling than that of T.pyriformis. A 10 to 20 fold increase in intracellular cyclic AMP levels of intact "cells" within a 10 min incubation, in response to added hormones is considered a modest increase in these systems (191). Therefore, T.pyriformis, particularly in log phase, probably represents one of the best systems in which to study the metabolism of cells in relative isolation.

Guanine nucleotides appear to be universal effectors of hormonally-stimulated adenylate cyclases from the higher eukaryotes (81). They commonly activate the enzyme in the absence of hormone, but, in the presence of hormone, act in a synergistic fashion. Such an effect has not been observed on an adenylate cyclase system from a microorganism, previous to this report. A GTP binding-protein has been separated from adenylate cyclase by affinity chromatography (81). The dissociation of these two components may represent the activation of adenylate cyclase by GTP and may increase the sensitivity of the enzyme to hormones (81). Rodbell and coworkers have sought to explain the effect of guanine nucleotides and hormones in terms of the facilitated transition of the enzyme between two or more activity states (110). The physiological importance of the regulation of adenylate cyclase activity by GTP is unclear at present, but this may represent a means by which the cell may modify its response to hormone stimuli.

Other hormones and small molecules were tested for their effects on adenylate cyclase from T.pyriformis. At concentrations which have produced pronouced effects in other tissues, l-epinephrine glucagon, spermidine HCl, adenosine, GTP or $G_{pp}(NH)_p$ alone, pyruvate and glucose-6-phosphate were ineffective. NADH, 5mM, inhibited adenylate cyclase activity from T.pyriformis. This is consistent with the effect of NADH on adenylate cyclase activity from rat adipocyte plasma membranes (106) and the proposed evolution of

adenylate cyclase from the primitive dinucleotide binding protein(103).

Sodium azide, 10mM, stimulated adenylate cyclase from T.pyriformis by 30%. Azide stimulation has been reported in a number of tissues. It is thought to interact with adenylate cyclase by a different mechanism to fluoride(106). The observation that azide stimulates the enzyme from T.pyriformis, while fluoride inhibits this enzyme, supports this view.

Section 7.3 The effects of divalent metals on adenylate cyclase activity from T.pyriformis

Adenylate cyclase activity from T.pyriformis was inhibited by calcium. This has been reported previously (44,81) and low concentrations of calcium were found to be effective , $5x10^{-8}$M. The concentration of free Ca^{2+} in T.pyriformis is $4to5x10^{-4}$M. Therefore, adenylate cyclase will be continuously inhibited. An enormous reduction in Ca^{2+} concentration would be required for full expression of adenylate cyclase activity.

The metal requirement of adenylate cyclase from T.pyriformis has been characterized. Mn^{2+}, Mg^{2+}, Cd^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+} at the same concentration allowed the expression of adenylate cyclase activity. The measured activity was greatest with Mn^{2+} and decreased in the order presented. Cu^{2+}, Ni^{2+} and Ca^{2+} did not support activity. The physiological importance of the metal requirement of adenylate cyclase from T.pyriformis is unknown. Even though Tetrahymena possess the ability to accumulate Mn, Zn, and Co against concentration gradients (30), one would expect that the major substrate for adenylate cyclase should be $ATP.Mg^{2-}$.

Section 7.4 The cyclic AMP metabolism in the cell cycle of T.pyriformis

In the initial studies on the cyclic AMP metabolism of the cell division cycle of T.pyriformis certain questions required answers. Foremost of these was, of course, whether cyclic AMP played some essential role in the control of cell cycle events. If this is so, then the levels of cyclic AMP in the whole cell or in the compartments of the cell should vary during the cell cycle. Also, the activity of the enzymes governing the level of cyclic AMP should

vary. Therefore, the first experiment was planned to determine the level of cyclic AMP, adenylate cyclase and phosphodiesterase activity during the cell cycle of a synchronous population of T.pyriformis obtained by selection.

Another member of my laboratory, J.R.Dickinson, had a working knowledge of the determination of cyclic AMP from T.pyriformis and had an interest in cell cycle control. I had, by this time, developed and validated the assay of adenylate cyclase from T.pyriformis. Therefore, our cooperation in this investigation was of great benefit. The magnetic selection of cells undergoing amitosis, chosen for this research, had been reported previously (38), but this omitted some details. Preliminary investigations into the feaibility of this method were required. We are grateful to P.S.McConnell for his efforts and success in obtaining small scale synchronous cultures by this method. However, as in all experiments, scaling-up uncovered further problems which had to be dealt with. The routine presented in section 3.3 represents the fruits of a number of experiments.

We have reported the rapid fluctuation of the cyclic AMP content of T.pyriformis during the natural cell cycle.(45) The maximum level, 6.3 pmole/10^6cells, was coincident with cell division. No cyclic AMP could be detected one hour after this maximum (45,fig.7.2). Therefore, a potent cyclic AMP phosphodiesterase must be present, in vivo, particularly in the early part of the cell cycle. As measured, in vitro at $5x10^{-7}$M substrate concentration, phosphodiesterase activity varied dramatically during the cell cycle. A rapid fall in activity to below the limit of detection coresponded with the beginning of the cyclic AMP peak. Cyclic AMP phosphodiesterase activity rose again and could account for the destruction of the elevated level of cyclic AMP. Cyclic AMP phosphodiesterase activity reached a maximum towards the end of the cell cycle. Adenylate cyclase activity per cell was not subject to such rapid fluctuations and did little more than double during the cell cycle. However, this provides the cell with its greatest capacity to synthesize cyclic AMP at the end of the cell cycle and thus is consistent with the observed fluctuations of this cyclic nucleotide. This experiment cannot, by itself, provide an answer to the question of how essential cyclic AMP is in the control of cell cycle events. However, it is an indication that this may be so.

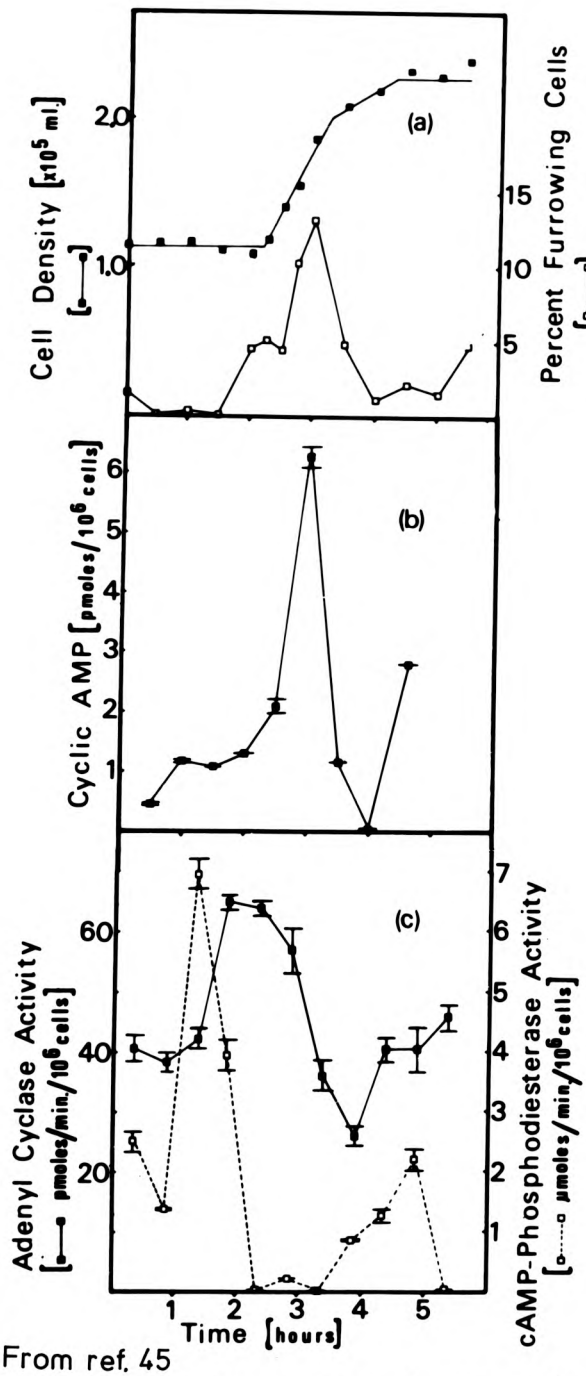


Fig 7.2 From ref. 45

Fig.1. The time course of a typical synchronous culture of *T. pyriformis* W. (a) Cell numbers and percentage dividing cells. (b) Cyclic AMP measured per 10^6 cells. (c) Adenyl cyclase and cyclic AMP phosphodiesterase activities measured at approx. physiological substrate concentrations; (1.2 mM ATP, and 5×10^{-7} M cyclic AMP respectively). These results represent the mean of at least 2 experiments, ± S.E.M.

Some insight into the cyclic AMP metabolism of T.pyriformis can be gained from this analysis of the cell cycle. The level of cyclic AMP is low, during the majority of the cell cycle, and is probably under the control of cyclic AMP phosphodiesterase activity. However, at division the activity of cyclic AMP phosphodiesterase drops so that net synthesis of cyclic AMP can occur. It may be that, whereas the rapid fluctuations of cyclic AMP phosphodiesterase determine the timing of the peak in cyclic AMP, the level of adenylate cyclase activity may determine the size of this peak. But, how are the variations in activity of the enzymes of the cyclic AMP metabolism produced? The preparation of cellular material for the assay of these enzymes involves the dilution of intracellular components by 5 to 10 fold, in the case of adenylate cyclase, and by a factor greater than 10^6 for phosphodiesterase. Therefore, the observed fluctuation of cyclic AMP phosphodiesterase activity can not be explained by the variation in the concentration of some effector, unless the affinity between the enzyme and effector was extraordinarily large. It would appear likely that the observed fluctuation of cyclic AMP phosphodiesterase was due to the net synthesis and net destruction of the enzyme during the cell cycle. This would require the activation or synthesis of some specific proteinase towards the end of the cell cycle. In the case of adenylate cyclase, the dilution of cell components was only small, therefore, the modulation of activity by effectors should have been reduced but not totally removed. The measured activity did little more than double during the cell cycle. However, the assay of adenylate cyclase contained 1mM EDTA, sufficient to chelate endogenous Ca^{2+} therefore, variations in enzyme activity due to Ca^{2+} inhibition may have been overlooked.

The peak in total cyclic AMP, coincident with cell division, may represent an important control feature of the cell cycle of T.pyriformis. The site of action of cyclic AMP has yet to be fully defined, but it has been fairly well established that cyclic AMP levels are involved in the regulation of the proliferation of T.pyriformis (section 1.3i). This may be due to a direct interaction with cell division or some process essential for cell division. Using a selected synchronous population of T.pyriformis, J.Wolfe found that caffeine pulses administered later in the cell cycle caused an increased delay before the subsequent cell division (42).

Assuming that the sole action of caffeine is to inhibit phosphodiesterases and that uptake of this compound by the cell is not altered during the cell cycle, the following alternative proposals may be made; (a) Some process towards the end of the cell division sequence (Fig.1.8) is required for cell division and is inhibited by elevated levels of cyclic AMP or cyclic GMP. Thus elevating the relevant cyclic nucleotide should cause a classical block in the cell cycle. (b) Some ongoing process, started early in the cell division sequence and completed towards the end of this sequence, may be disrupted by elevated levels of one of the cyclic nucleotides. When the level of the relevant cyclic nucleotide falls, this process should restart at the beginning, analogous to the heat sensitive division protein. This second proposal necessarily involves an increased delay in cell division if caffeine is administered later in the cell cycle. The first proposal should also produce increased delay because, if caffeine is administered early in the cell cycle, the cells have time to reduce the level of cyclic nucleotides before the block point is reached. This time is progressively reduced if caffeine is administered later in the cell cycle. The increased delay of cell division of both proposals may be influenced by the sensitivity of the level of cyclic nucleotides to caffeine, which may vary during the cell cycle. This experiment does not allow the differentiation between the effects of cyclic AMP and cyclic GMP, but, whereas there is substantial evidence that elevated levels of cyclic AMP inhibit cell division, there is as yet no similar evidence of a role for cyclic GMP. One would expect that elevated levels of cyclic GMP might, in some way, stimulate cell division. Therefore, it would seem likely that the relevant nucleotide in the proposals above should be cyclic AMP. The sensitivity of cyclic AMP levels, in the cell cycle, to caffeine will vary. Obviously when cyclic AMP phosphodiesterase activity is very low, caffeine should have little effect on the level of cyclic AMP. In fact the destruction of cyclic AMP phosphodiesterase activity at the end of the cell cycle may represent a transition point in the sensitivity of cell division to caffeine.

One may develop a model for cell cycle control, whereby the level of cyclic AMP must be reduced from the elevated level at cell division to allow the successful initiation or completion of the cell division sequence. As the cyclic AMP content of T.pyriformis is reduced very rapidly early in the cell cycle, this would appear

to be an attractive position for a control point or decision-making process. I have proposed that the elevated level of cyclic AMP may represent a transitory state from which cells may progress through a subsequent cell division cycle, only if this level is reduced (45). If the cyclic AMP level remains elevated then the cell no longer divides and the culture enters stationary phase. This transitory phase may be synonymous with the indeterminate state proposed by J.A.Smith and L.Martin from data obtained from mammalian cells (18). In fig.7.3, I have superimposed the measured fluctuation of cyclic AMP on a model for the interdivision period of T.pyriformis.

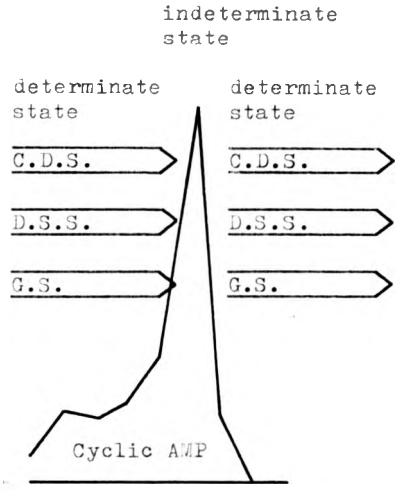


Fig. 7.3 A model for the interdivision period of T.pyriformis and the intracellular level of cyclic AMP. C.D.S., cell division sequence; D.S.S., DNA synthetic sequence; G.S., growth sequence.

As a culture approaches stationary phase, the level of adenylate cyclase activity is increased. Therefore, the peak hight of cyclic AMP may be increased. It should then take longer for the level of cyclic AMP to be reduced, the transition to the determinate state should be delayed and the mean generation time increased. Ultimately, the maintenance of low cyclic AMP phospho-

diesterase activity should trap cells in the indeterminate state and signal stationary phase.

The stationary growth phase of T.pyriformis involves high levels of intracellular cyclic AMP (40,42). These reports do not give sufficient details of growth conditions for one to establish the nature of the stationary phases. It would seem likely that S.J.Voichick et al were observing the increase in cyclic AMP as cells entered an oxygen-deficient stationary phase. If oxygen-limitation blocks the cell division sequence towards the end of this sequence then, why does the level of cyclic AMP rise? Are the cells being blocked at some stage of the cell cycle when the levels of adenylate cyclase and cyclic AMP phosphodiesterase are such that an elevation of cyclic AMP necessarily follows? Is the elevation of cyclic AMP an important part of the control mechanism by which oxygen-limitation halts cell proliferation or just a secondary effect that may be important in the adaptation of the cells to a new environment?

In chapter 6, I have described the response of adenylate cyclase activity as rapidly proliferating cells in glucose supplemented medium are made hypoxic. Within 75 min of hypoxia the measured activity of adenylate cyclase, in the presence of 1mM EGTA, increased almost 5 fold. This was due to de novo protein synthesis and was similar to the rise in activity of glucose grown cells upon entry to stationary phase. One would not expect such a large increase if the cells were merely accumulating late in the natural cell cycle. Furthermore, this increase was only transitory in both cases. This is inconsistent with the variation of enzyme activity measured by S.J.Voichick et al as cells without a glucose supplement entered stationary phase (40).

If grown on a medium unsupplemented by glucose, an important response of T.pyriformis to hypoxia is an increased rate of synthesis of glycogen (30). In this way a glycogen store is accumulated at the expence of cell lipids in early stationary phase, which can be mobilized for the energy requirement of the cell later in stationary phase. Elevated levels of cyclic AMP may increase the glyconeogenic capacity of the cell by inducing the rate limiting enzymes, such as isocitrate lyase, malate synthetase and/or phosphoenolpyruvate carboxylase (62,40). However, when grown with a glucose supplement, T.pyriformis contain high levels of glycogen

in log phase, thus further accumulation of glycogen in response to hypoxia appears to be unnecessary and the induction of glyconeogenic enzymes is not observed (30). Therefore, the response of adenylate cyclase in glucose grown T.pyriformis to hypoxia may be a futile signal for glyconeogenesis which is blocked further along the regulatory chain. The reduction of adenylate cyclase after its initial rise, only observed in glucose grown cells, may be an indication of glucose-dependent feedback regulation of adenylate cyclase activity.

One cannot always equate stationary phase cells of T.pyriformis with cells trapped in the indeterminate state by elevated cyclic AMP. The cyclic AMP metabolism may also be modified to allow the cell to adapt to new environments. However, let us reconsider the natural cell cycle and the model presented in fig. 7.3. In chapter 1, I reviewed the areas of the metabolism which may be controlled by cyclic AMP. These were cell proliferation and cell division, ciliary regeneration, phagocytosis and glyconeogenesis. Cell division is obviously a cell cycle event, amitotic cells do not ingest particles, cilia are probably synthesized towards the end of the cell cycle, but it is not known if glyconeogenic activity varies through the cell cycle. Furthermore, the synthesis of cyclic AMP-dependent protein kinase of the cytosol of T.pyriformis appears to be independent of the cell division sequence and shows more affinity to the DNA synthetic sequence. This is dicussed below. Therefore, a single peak in cyclic AMP is apparently important in the control of several processes.

I base my statement that cyclic AMP-dependent protein kinase of the cytosol may be synthesized in association with the DNA synthetic sequence on the data presented by Majumber et al (52). The activity of these enzymes was measured during the application of repetitive heat shocks and during the first synchronous cell division cycle. Heat shocks allow the continued synthesis of DNA, RNA and protein whilst blocking division. DNA synthesis eventually decelerates and is essentially blocked after 5 or 6 heat shocks (Fig.7.4). Upon release from heat shocks DNA synthesis is much reduced and is poorly synchronized. Cyclic AMP dependent protein kinase activity closely followed the DNA content of the cell. This would suggest that the synthesis of cyclic AMP dependent protein kinase of the cytosol was not part of the cell division sequence and thus remained relatively asynchronous throughout the experiment.

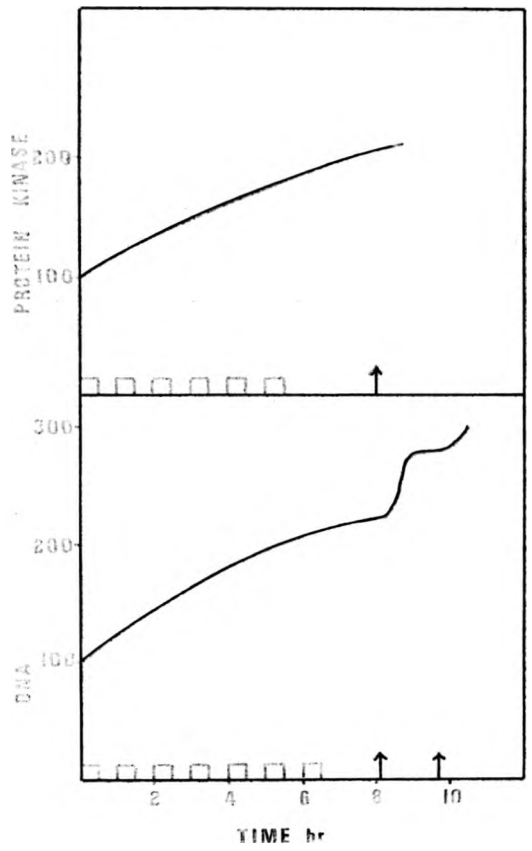


Fig. 7.4 The percentage of cytoplasmic cyclic AMP dependent protein kinase activity during repetitive heat shocks compared to 100% at time 0. (52). DNA content of T.pyriformis during repetitive heat shock (35). Arrows indicate the peaks in division index.

A single peak in cyclic AMP may control several processes if; (a) the processes are part of the same gross sequence of the interdivision period,(b) they are temporally related,(c) they react in a different manner to cyclic AMP or (d) local fluctuations of cyclic AMP are important. The control of cell division and the possible involvement of cyclic AMP in the DNA synthetic sequence should be independent, but these two processes may be temporally related. I have discussed the control of cell division by cyclic AMP in terms of a transition from the indeterminate state to the cell division sequence. This may occur early in the natural cell cycle and coincident with the transition to the DNA synthetic sequence. Furthermore, these two sequences may react in a different manner to cyclic AMP. The effects of cyclic AMP on phagocytosis may or may not be of direct importance in the cell cycle. The measured effects in asynchronous log cultures of T.pyriformis are small and could be related to the alteration of the distribution of the cells about the cell cycle. Cyclic AMP may be important in the synthesis and function of the cilia and cortical components of T.pyriformis. This may remain independent of other cyclic AMP controlled events by the localization of cyclic AMP levels.

The level of adenylate cyclase activity may be important in determining the local concentration of cyclic AMP. As discussed in section 7.1, the cortical structure of T.pyriformis may represent a compartment of the cyclic AMP metabolism. The rate of incorporation of radiolabelled phosphate into the phospholipids of cilia of heat shock synchronized T.pyriformis, strain GL, reaches a maximum at division and incorporation of radiolabelled leucine into ciliary protein immediately preceeded this (192). Therefore, the cortical compartment is increasing most rapidly towards the end of the cell cycle. The relative concentration of adenylate cyclase in this compartment should be important in determining the local cyclic AMP concentration. The actual nature of the variation was not totally reproduced in two separate cell cycle experiments, therefore it is not certain how exactly the cortical components and adenylate cyclase activity vary in relation to each other. However, a rapid rise in adenylate cyclase activity early in the cell cycle should cause an increase in the local concentration of cyclic AMP. A rapid rise in activity towards the end of the cell cycle should maintain the local level of cyclic AMP. Possible roles for cyclic AMP in the

cortex would be concerned with the synthesis or incorporation of cortical components, the regulation of motility, the regulation of membrane transport etc..

T.pyriformis represents a most suitable organism for the cell cycle investigations becuse one may perturb the natural cell cycle and observe the consequences. One may block DNA synthesis or cell division independently and hence hope to observe whether cyclic nucleotides are involved in either or both of these sequences. The control elements will eventually realign these sequences thus, blocking one sequence will eventually inhibit the other sequences but this may take some time. The rapid response of cyclic AMP to a cell cycle block and the synchronization of cyclic AMP levels by the removal of the block should imply a close relationship between cyclic AMP and the blocked process. One may repeat such experiments observing the activity of the enzymes of the cyclic AMP metabolism. Two such experiments have been discussed, the response of adenylate cyclase to induced hypoxia and the response of cyclic AMP-dependent protein kinase to heat shock. Neither of these experiments can be regarded as particularly satisfactory because hypoxia and heat shock are not particularly specific. But hypoxia, which blocks cell division causes a rapid elevation of adenylate cyclase activity and upon release adenylate cyclase activity appeared to be synchronous (section 6.2 &6.4). Heat shocks, which also inhibit division, did not cause an immediate response in cyclic AMP protein kinase nor did heat shocks synchronize protein kinase activity. More experiments of this sort are required to fill in the picture of the cyclic AMP metabolism in the cell cycle of T.pyriformis.

Particularly good systems for such research may be the starvation-refeeding synchrony of Cameron (22) and the application of hydroxyurea. The latter may be particularly rewarding. During a 10 hour treatment with hydroxyurea DNA synthesis is inhibited but RNA and protein synthesis continue. After the release from hydoxy-urea block T.pyriformis accomplished several cell division cycles without a G_1 phase (26). The authors suppose that the nuclear DNA: cytoplasmic volume ratio exherts an influence upon DNA synthesis such that when this ratio is reduced to a certain level DNA synthesis is initiated. In the natural cell cycle growth in G_1 achieves this reduction, however, after hydroxyurea treatment this ratio is already low and DNA synthesis can occur immediately after division. If one

accepts this explanation, surely one must attempt to define the means by which nuclear DNA ; cytoplasmic volume ratio exherts its influence over DNA synthesis. Cyclic nucleotides may be important in this process.

If cyclic AMP is important in the control of nuclear processes, then what should be the situation in micronucleate strains of T.pyriformis? For instance, the cytoplasmic concentration of cyclic AMP may control DNA replication, RNA synthesis or nuclear division. The macronucleus and micronucleus show substantial differences in these processes. The micronucleus is inactive with respect to RNA synthesis and replicates its DNA complement and divides at different times in the cell cycle compared to the macronucleus (Fig.1.1). Therefore, one must propose that these processes are under the control of different effectors for each nucleus or that each nucleus can respond in a different manner to the same effector. The micronucleus is deficient in histones H1 and H3 (13,14). This may be indicative that some process absent from the micronucleus, such as RNA synthesis, is dependent upon the interaction of some cytoplasmic effector and histones H1 and H3. T recent years the concept of the control of genome expression throughout the cell cycle by the cyclic AMP dependent phosphorylation of histones has gone out of vogue. This was because the histones appear to be modified soon after synthesis and not correlated with DNA synthesis. However, cyclic AMP dependent phosphorylation may still have relevance in determining the activity of the nucleus of the eukaryotic cell.

Finally, the nature of the interaction between cyclic AMP and the other control elements of the cell should be considered. Cyclic GMP and cyclic GMP phosphodiesterase activity have been measured during the natural cell cycle of T.pyriformis. The variations observed were by no means as dramatic as the cyclic AMP metabolism (70). However, this cyclic nucleotide may be important in the control of the cell cycle, either in isolation from or in cooperation with cyclic AMP. It is interesting that the mean level of cyclic GMP in this rapid proliferating organism is of the same order of magnitude as cyclic AMP. In slowly proliferating organisms cyclic GMP levels are much lower than cyclic AMP. This is consistent with a role for cyclic GMP in the stimulation of proliferation. No information is available on the mobilization of Ca^{2+} during the cell cycle of T.pyriformis, however this cation may play a central role in the control of the cell cycle.

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