STUDIES ON CYCLIC NUCLEOTIDE METABOLISM IN RELATION TO CELL CYCLE CONTROL IN TETRAHYMENA PYRIFORMIS

A Thesis submitted to the University of Warwick in fulfilment of the requirements for the degree of Doctor of Philosophy.

The research described in this thesis was carried out in the Department of Molecular Sciences, University of Warwick.

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ABBREVIATIONS AND NOMENCLATURE

This thesis follows the internationally agreed rules of the IUPAC-IUB Commission on Biochemical Nomenclature. References are abbreviated according to Chemical Abstracts. Non-standard abbreviations and nomenclature used in this thesis are defined below:-

Chemicals

PGE_1	prostaglandin E_1
Con A	Concanavalin A
PHA	phytohemagglutinin A
cycloheximide	3-[2,-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] glutarimide
cyclic AMP	adenosine 3':5'-monophosphate
dibutyryl cyclic AMP	N^6,2'-O-dibutyryl-adenosine 3':5'-monophosphate
cyclic GMP	guanosine 3':5'-monophosphate
8-bromo cyclic GMP	8-bromoguanosine 3':5'-monophosphate
theophylline	1,3-dimethylxanthine
PPO	2,5-diphenyloxazole
POPOP	1,4-di 2-(5-phenyloxazolyl) benzene
butyl BPD	2-(4'-t-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole
EDTA	ethylenediamine tetraacetic acid
Tris-HCl	tris(hydroxymethyl) aminomethane hydrochloride
DEAE-cellulose	diethylaminoethyl-cellulose

Enzymes

adenyl cyclase, ATP pyrophosphate-lyase (cyclizing)(EC 4.6.1.1)

guanyl cyclase, GTP pyrophosphate-lyase (cyclizing) (EC 4.6.1.2)

cyclic nucleotide phosphidesterase, 3':5'-cyclic nucleotide 5'-nucleotido-hydrolase (EC 3.1.4.17)

cyclic nucleotide-dependent protein kinase, ATP:-protein phosphotransferase, (3':5'-cyclic nulceotide dependent) (EC 2.7.1.37)

thymidylate synthetase, 5,10-methylene-tetrahydrofolate: dUMP C-methyltransferase (EC 2.1.1.45)

RNA polymerase RNA nucleotidyltransferase (EC 2.7.7.6)

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SUMMARY

A technique for the selection of large scale synchronous populations of Tetrahymena pyriformis was developed in co-operation with M.G. Graves. The technique is a modification of the method published by Hildebrandt and Dusplva (180), but achieves a scaling-up of the quantity of synchronous cells compared with the earlier method. A number of important control experiments were performed which validate this selection synchrony method. The method takes advantage of the phenomenon of division-related cessation of phagocytosis. Cells were incubated with fine particles of iron for 12 minutes; after this time magnets were used to separate excess iron particles and cells which have ingested iron, from cells which have not. The cells not retained by the magnets constitute a synchronous population and were used in studies of the natural cell cycle. A cell cycle time of about 180 minutes with a synchrony index of 0.77 was achieved.

A novel method of induction of division synchrony in Tetrahymena pyriformis was developed and validated. Synchronization was achieved by simply growing-up the organisms with rapid shaking in an orbital shaker to a cell density of about 2.0×10^5 cells/ml, not shaking the cells for a period of 240 minutes, and then reshaking. The 240-minute hypoxic shock results in accumulation of the cells in the G2 phase of the cell cycle. There appears to be a number of similarities between this perturbed cell cycle system and the classical multiple heat-shock method (165) of synchronizing Tetrahymena pyriformis.

Assays were developed for cyclic AMP phosphodiesterase, cyclic GMP phosphodiesterase, neutral protease, and intracellular cyclic AMP and cyclic GMP. Cyclic GMP and cyclic GMP phosphodiesterase were demonstrated for the first time in Tetrahymena pyriformis. Km's of 0.11 mM and 0.59 μM were found for cyclic AMP phosphodiesterase, and a Km of 0.91 mM was found for cyclic GMP phosphodiesterase. It was not possible to demonstrate a low Km form of cyclic GMP phosphodiesterase from this cell. A mechanism of

excretion of cyclic GMP from the cell was postulated to account for the apparent absence of an enzyme capable of hydrolyzing physiological concentrations of cyclic GMP.

Various aspects of cyclic nucleotide metabolism were studied in both the natural cell cycle and in cells synchronized by the single hypoxic shock technique. An attempt was made to implicate a neutral protease in the regulation of cyclic AMP phosphodiesterase activity in the natural cell cycle. The experiments performed suggested the existence of 3 cyclic nucleotide signals important in control of the cell cycle of Tetrahymena pyriformis. These were

1. A certain minimum level of cyclic GMP (possibly about 4 p mol/10^6 cells) in cells that are in the G1 phase of the natural cell cycle.
2. Elevated cyclic AMP and cyclic GMP during cell cycle blockade by hypoxia.
3. A "spike" of cyclic AMP that occurred at the division/G1 interface of the natural cell cycle. Roles are suggested for the cyclic nucleotide signals identified in terms of the "division protein" model (31) of cell cycle control in Tetrahymena pyriformis.

CHAPTER 1

INTRODUCTION

1.1 Models of cell cycle control

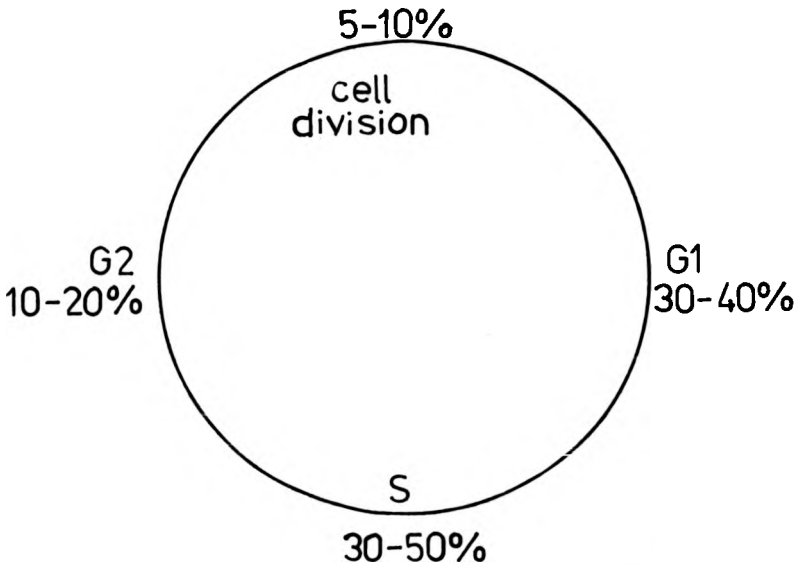

Fig. 1. The cell cycle showing the average times occupied by each phase

Classically the eukaryotic cell cycle has been described as in Fig. 1 (above). The subdivision of the cell cycle into four phases is based on the timing of DNA synthesis and cell division (1). Following cell division there is a gap (G1) during which no nuclear DNA synthesis takes place. This is followed by S phase during which time the DNA content of the nucleus is doubled. G2 is defined as the period between S phase and the ensuing mitosis. The existence of the cell cycle was originally established in different eukaryotes by autoradiographic techniques (1-4). Scoring of labelled mitoses first enabled determination of the phases of the DNA cycle. For such studies cellular DNA is pulse-labelled by incorporation of a radioactive precursor to DNA, (usually tritiated thymidine), and then the proportions of mitoses which are labelled in autoradiographs is determined in cells sampled at various times thereafter (Fig. 2).

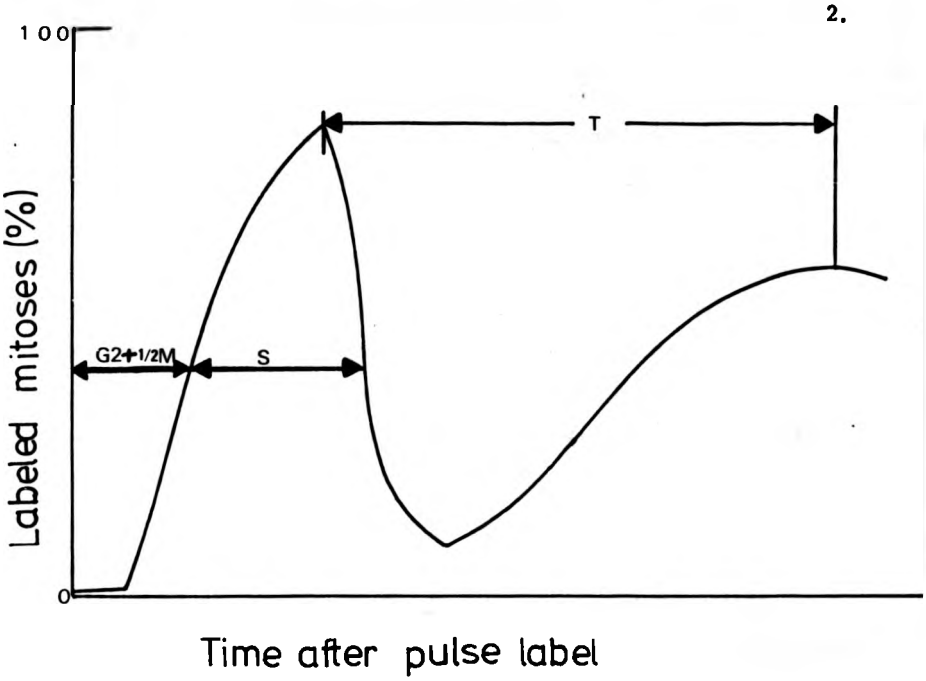

Fig. 2. Diagram of labelled mitoses (metaphases) in successive cell samples after a pulse of tritiated thymidine

The average S period is taken as the time between the two points in the first wave where 50% of the mitoses are labelled. The time between the start of the experiment is equal to G2 + $\frac{1}{2}$M. (Half the mitotic time is added because mitoses are scored in metaphase, thus the cells progress through G2 and prophase before they are scored; prophase represents about one half of the total time occupied by mitosis). The total cycle time (T) is the time between any two identical points in adjacent cycles. The duration of G1 is calculated from G1 + $\frac{1}{2}$M = (T - S - G2 - $\frac{1}{2}$M). The cell division cycle was later confirmed in synchronously growing populations of cells, both in those that naturally undergo synchronous division and in those in which a synchronization procedure has been employed (5, 6).

In a given environment a particular cell type has a defined cell cycle time. Changes in the environment produce changes in the cell cycle time; however, it is found that the duration of S + G2 + M does not alter appreciably; the change in cell cycle time is due almost entirely to an altered G1 (7). Different mammalian cell types have similar S, G2 and M durations but widely varying G1's (8-9). Slowly proliferating cells such as normal hepatocytes, uterine epithelium of

ovarectomised mice, serum-starred or "contact inhibited" cells in culture are mostly in G1. Non-proliferating ("quiescent") cells appear to be arrested in G1 (10-15). From studies based largely on in vivo systems, it has been suggested that if cells cannot proceed through G1 they enter a distinct resting state designated as G0 (16). A detailed consideration of the conceptual problems raised by the many different cell cycle systems outlined above will be given later.

It is clear that the weight of evidence has for a long time favoured the conclusion that the most important cell cycle control mechanism(s) must reside in the G1 phase. This notion has dominated virtually all models of cell cycle control, though it is interesting to note that Schizosaccharomyces pombe (17), Physarum polycephalum (18), Amoeba proteus (19) and a number of mammalian cells (20) apparently lack a G1 phase, and that of course multi-nucleated cells (e.g. the syncytial slime moulds) do not undergo cell division.

The various general models of cell cycle control have been reviewed extensively including mathematical analysis by Fantes et al. (21). The basic concepts are outlined below.

(I) Concentration Models

Essentially a concentration change of an effector produced in the cytoplasm is sensed in the nucleus, and attainment of a critical concentration triggers mitosis.

a) Simple Concentration Model

A single effector substance changes in concentration at a rate in proportion to the mass of the cytoplasm until a triggering concentration is reached. A discontinuous change in concentration accompanies mitosis, the magnitude of which is proportional to the number of nuclei in the cell or to the number of genome equivalents present. Two variations on this model exist: the initiator-accumulation model (22) in which the concentration of effector increases throughout the inter-mitotic period and is "titrated away" by the nuclei at mitosis, and the inhibitor-dilution model (23) in which each nucleus causes the

production of a fixed amount of effector at mitosis, the concentration of which subsequently falls during the intermitotic period due to cell growth or active degradation (24).

b) Linear Exponential Model

In this model the cell is postulated to produce two components. One is an effector which increases in amount at a constant rate proportional to the number of nuclei or genome equivalents present. The other component is produced exponentially in proportion to the cell mass, with the property of titrating the effector or effectively removing the effector in some way. When the two components are present in equal amounts, mitosis is triggered. Following mitosis the rate of synthesis of effector is doubled so that its concentration will initially rise and then fall during each intermitotic period. There is considerable evidence for this type of model, particularly in Schizosaccharomyces pombe where it is well documented that growth in total dry mass is linear over the cell cycle (25) whilst the pattern of synthesis of protein, carbohydrate and RNA approaches exponential rates (26, 27).

c) The Unstable Inhibitor Model

In this model (first postulated by Ycas et al.(28)) nuclei are said to monitor their own concentration throughout the intermitotic period and to vary the time of the next mitosis in response to their concentration. It has been suggested (21) that the absolute amount of an effector must be proportional at all times to the number of nuclei or genome equivalents. The concentration of the effector must therefore decrease by a factor of two during a single intermitotic period. This can only be accomplished by proposing that the effector molecules are unstable, each molecule having a constant half-life independent of concentration. Although such a proposition is attractive, there is not a wealth of experimental data in its support; the decay of m-RNA (29) could represent an analogous situation.

(ii) Structural Models

In structural models subunits of a mitotic structure are said to be made at a rate which is proportional to the cytoplasmic mass. When a certain critical number of subunits per structure have been assembled then mitosis can occur. An essential part of the concept is that after completion of mitosis neither the structure nor its subunits are re-usable; they are thus effectively removed from the division-control system. The structure can either be in the nucleus (30) or the cytoplasm (31). In cell fusion studies it is found that mitosis is synchronous because the nucleus from the more "advanced" cell (i.e. the cell that has progressed further through the cell cycle) is postulated to have structures which are nearer completion. To account for this observation it has been postulated (21) that the subunits are not irreversibly bound to structures, but that an equilibrium exists between the concentration of a pool of free subunits in the cytoplasm and those bound to each mitotic structure.

The various general models of cell cycle control outlined above are almost completely devoted to explaining one question, namely "How do cells cycle ?" ; i.e. they attempt to explain the integration and timing of the various biochemical and morphological events that comprise the cell cycle. However, such an approach does not address itself to the whole of the phenomenon: there is another question that also needs to be asked:- "Why do cells cycle ?" This question attempts to discover why proliferating cells repeatedly grow and divide. It also suggests a consideration of why it is that cells in culture will undergo vegetative proliferation only for so long (dependent upon environmental factors) and then become quiescent (i.e. they show the phenomenon of density dependent inhibition of growth). Smith and Martin have addressed themselves to this question (7, 32). They suggest that the intermitotic period is composed of two fundamentally different parts. One is the orderly sequence of events necessary for division and includes S phase, G2, M and part of G1. These phases are called the B-phase. Some

time after mitosis cells enter the A-state in which they do not progress towards division. A cell may remain in the A-state for any length of time, but always has a certain chance of re-entering the B-phase. This "transition probability" does not change, provided environmental conditions remain constant. It is envisaged that the transition probability is a characteristic of the cell type, and that it is capable of modification by environmental or developmental factors. According to Smith and Martin, modification of transition probability provides a major means of controlling cell proliferation. The frequency distribution of times spent in the A-state has exponential characteristics.

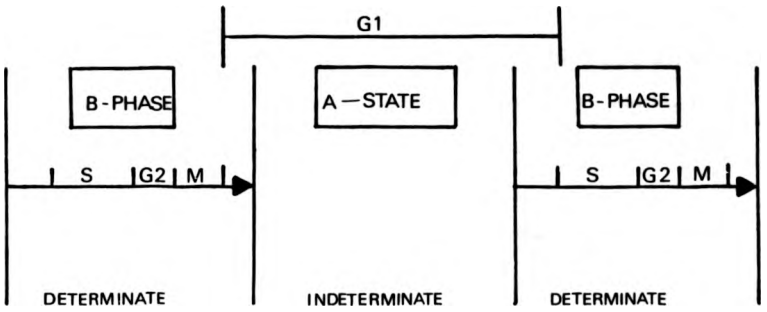

Fig. 3 The cell cycle according to Smith and Martin (7, 32)

The concepts postulated by Smith and Martin have caused considerable controversy among cell biologists. It is argued that their model rests most heavily on the observation that the fraction of undivided cells with time follows a simple negative exponential curve and that it suffers a lack of connection with the biochemistry of the cell cycle (33). After detailed studies using time-lapse video tape analysis and scoring of anaphase figures, Klevecz has proposed that generation times are distributed polymodally and that cell division,

the initiation of DNA synthesis, and the expression of certain enzymes are timed by a system which has some characteristics in common with the circadian clock (33). The mammalian cell cycle is envisaged as being built up of multiples of a fundamental four hour period (Gq). The timekeeping mechanism is reported to be temperature compensated, since the time required to traverse Gq is constant between 34° C and 39° C. It is suggested that cell cycle time increases at lower temperatures, lower serum concentration and high cell densities because the number of rounds of traverse through the subcycle Gq increases. Differences in the length of G1 in mammalian cells of the same culture (which are all in 4-hour units) are viewed as being a consequence of the "gated entry" of cells into S. The exit from Gq into S is probabilistic in the sense that it depends on the environment. G1 arrest or G0 is explained as an indefinite number of Gq cycles in which certain cell functions continue, while those which are dependent upon the initiation of DNA synthesis cease. Whether an analogous situation can be postulated for lower eukaryotes is not clear at this time.

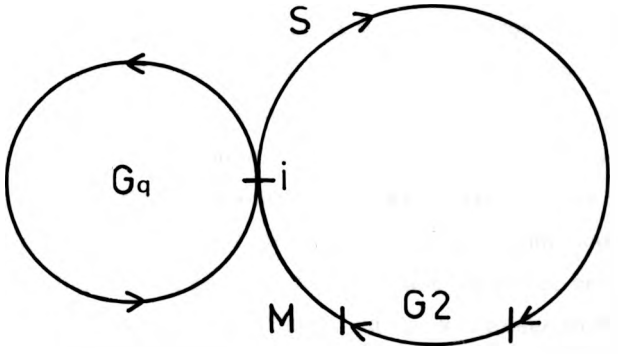

Fig. 4 The mammalian cell cycle according to Klevecz (33)

i is a decision point; should the environment be nonpermissive when the cell reaches i, it enters or re-enters the subcycle Gq. Gq has a traverse time equal to the period of the clock.

1.2. Methods for Studying the Cell Cycle

The individual methods now available for cell cycle analysis are almost innumerable. James has classified the major general methods of obtaining synchronous cultures into eight methods for induction synchrony and four for selection synchrony (5). Besides the use of synchronous cultures it is also possible to study single cells, and cell systems which possess natural synchrony such as the multinucleate slime mold Physarum polycephalum. In the following section I intend to discuss these methods and to outline the advantages and drawbacks of the particular general methods.

(1) Isolated single cells.

In general there is a limited amount of biochemistry that can be performed with isolated single cells because there is insufficient material available for analysis. Of course when studying a single cell each measurement must not result in any damage to the cell or else it will not be available for subsequent measurements. If the cell has to be killed, it is possible to determine its position in the cell cycle by having followed its previous history since the preceding division (usually by observation or time-lapse photography). The chief source of error in such studies is undoubtedly the natural spread of cycle times between individual cells. One way of reducing such error has been by comparing the time from division with the cell cycle time of the "sister" cell rather than using the mean cell cycle time, because in general sister cells are reported to have a higher correlation of their cell cycle times than "unrelated" cells (34). This method can only be used when the sister cell separates at division from the experimental cell. It is plain to see that methods involving the study of isolated single cells are of limited use to the biochemist, and that they can be very time consuming.

The method of cloning of cells falls in between single cell techniques and selection synchrony techniques. Clones are established by transferring single cells from log-phase cultures at random points in the cell cycle. The cells are then grown for a few cell cycles during

which time synchrony is maintained because all the cells derive from one "mother" and thus the tendency for dispersion of cell cycle times is reduced. Micro-determination methods are needed but have been successfully employed in a number of cases, for example in a recent report Zeuthen's group measured glycolytic activity throughout the cell cycle of Schizosaccharomyces pombe by cloning cells into ampulla divers which served both as growth chambers and at the same time as microgasometers (35).

(ii) Induction synchrony methods.

In general it can be stated that induction synchrony methods produce far better synchronization of the cells and greater quantities of synchronous cells than selection methods. It is always possible that the process altered by the induction procedure is the very process that one is trying to examine. For this reason, study of induction-synchronized cells is probably most valuable when compared with the situation that occurs in selection-synchronized cultures.

The general methods of induction synchrony are outlined in Table 1.

TABLE 1

CATEGORY	PRINCIPLE	REPRESENTATIVE REFERENCES
TEMPERATURE METHODS	Temperature changes are used as the treatment.	
Cycles	Culture is subjected to a cold-warm cycle equal to one generation. Cycle is repeated with renewal of medium with each cycle.	(36-39)
Shifts	Temperature is changed from a low to a high level. Divisions occur in warm period.	(40)
Shocks	Temperature is raised and lowered in rapid succession in a series of pulses. Pulses range from optional or suboptional to supraoptional. Divisions occur following a period at the lower temperature.	(41, 42)

/Table continued

Table 1 continued

CATEGORY	PRINCIPLE	REPRESENTATIVE REFERENCES
LIGHT METHODS	Light changes are cue for the entrainment of cell cycle.	
Cycles	Light and dark cycles are used to entrain cultures of photosynthetic cells such that divisions occur one each cycle. Strength and duration of light period must be such as to give balanced growth.	(43-45)
NUTRITIONAL METHODS	Growth on basal medium is followed by addition of enriched medium (a "shift-up") or could use a "shift-down". Shift-up from starvation level to enriched medium are also used.	
Auxotrophic control	Uses a change in a single requirement holding other requirements in excess. Genetic strains with specific requirements are most effective.	(46-49)
Heterotrophic control	Makes use of changes on broad spectrum requirements, i.e. carbon source, nitrogen source. Divisions occur after a lag following enrichment.	(50)
GASOMETRIC METHODS		
Aerobic-anaerobic	Uses single or multiple cycles of bubbling nitrogen, then air, through the culture in a fixed pattern in time to induce synchrony. Controlled gas tensions may also be a mode of control.	(51)
INHIBITORY METHODS	Employs an inhibitor of cell division or a stage in the cell cycle and a washout procedure to accumulate cells at a specific stage of cycle. Excess of natural metabolites are most effective, i.e. thymidine.	(52, 53)

General Methods of Induction Synchrony. (Reproduced from reference (5) with slight modifications).

Two of the most frequently-studied induction synchronized cell systems are quiescent fibroblasts stimulated to divide by addition of fresh serum, and lymphocytes stimulated to divide by addition of mitogens. As stated earlier, resting cells are designated as being arrested in G1 (10-15) or in a distinct G0 state (16). It has been argued that since a cell, once it has entered G0, requires a stimulus to re-enter the proliferative pathway, the processes involved in retrieving cells from G0 need bear no relation to the normal process of unimpeded progress through G1 (16). If this is true, studies of induced proliferation may be irrelevant to the problem of regulating continuously proliferating populations. Smith and Martin have commented that "it is therefore surprising that little attention has been given to the experimental regulation of proliferation rates, other than the 'on/off' systems". (7)

(iii) Selection synchrony methods

The general methods of selection synchrony are outlined in Table 2.

TABLE 2

General methods of selection synchrony. (Reproduced from reference (5) with slight modifications).

CATEGORY	PRINCIPLE	REPRESENTATIVE REFERENCES
SIZING METHODS	Depend on correlation between cell size and cell age. Selects small fraction from a logarithmic culture to start synchronous subculture.	
Filtration	Small cells are obtained by expressing slurry of cells through a filter pad. Care is taken to maintain other conditions constant. Small cells are subcultured.	(54-56)
Sedimentation velocity	Depends on difference of sedimentation rate with cell size. Small cells are removed from upper portion of a density gradient to form synchronous subculture.	(57)
Grow-off methods	Adsorb cells on to filter pack. Parent cell attaches, daughter cell falls off at fission.	(58)

/Table continued

Table 2 continued

CATEGORY	PRINCIPLE	REPRESENTATIVE REFERENCES
Substrate attachment	Tissue culture cells and amoeboid forms attach to the substratum during interphase of the cell cycle. At time of division they tend to round up and detach. Collection of detached forms provides subculture of synchronous cells.	(59)

A major drawback of all selection synchrony techniques compared with systems where cell synchrony is enforced is low yield. By their very nature selection synchrony techniques must only select a small fraction (usually 10% or less) of the total number of cells present in a logarithmically growing population. Scale-up of the sedimentation velocity technique of Mitchison and Vincent (57, 60) has been achieved by using the greater capacity offered by zonal rotors (61, 62). However, the time required for separation can be as much as one hour, during which the organisms are exposed to sub-optimal temperatures and anaerobic conditions; in other words the fundamental requirement of selection synchrony techniques, namely that the cell population during the selection technique shall be free from the distortions that result with changes in temperature, light, nutrition or other factors (5) does not obtain. Furthermore, the method cannot be used with osmotically fragile organisms (e.g. many protozoa) anyway (63). It is reported that the use of high molecular weight gradient media to eliminate high osmotic pressures does not prevent the distortion of the first subsequent cell cycle that occurs with gradients of sucrose (64). It is stated that organisms with rapid motility (such as Tetrahymena pyriformis) cannot be selection synchronized by velocity sedimentation size-selection (63), and anyway it is very difficult to maintain aseptic handling of the organisms for this procedure. The alternative approach of "cell-cycle fractionation" does improve the yield of cells as the whole of an asynchronous starting culture is used and divided into fractions, (each fraction from the gradient representing a

portion of the cell cycle). The quantity of material can be further increased by the use of zonal rotors (65-67) but,as Lloyd et al. have noted, the same criticisms apply as to differential sedimentation methods (63).

To circumvent these difficulties, the elegant technique of continuous flow size selection has been developed to allow large-scale preparations of synchronous cultures of micro-organisms (63). The smallest-sized (recently divided) cells from a logarithmically growing culture are separated by passage through a continuous flow centrifuge rotor under conditions of controlled rotor speed and flow-rate. The great advantage of this method is its rapidity, thus allowing undisturbed cell growth. A very recent report has shown that continuous-flow cell cycle fractionation is possible (205).The authors state that the method has all the advantages of the continuous-flow size selection technique (simplicity, rapidity, no requirement for prior harvesting of cells and no exposure of the cells to density gradients).

Until recently, selection synchrony techniques have not found a great deal of use with higher eukaryotic cells. The main problem has been that tissue culture is usually performed in small petri-dishes or in roller bottles. The amount of cells is always small and selection of a small fraction for subsequent experimentation would further reduce this. However, selection synchrony is now beginning to be used in cell cycle studies of higher eukaryotes with the introduction of "mitotic shake-off" (68,69), though the technique is still often used in combination with inhibitory methods (70).

1.3 The Role of Cyclic Nucleotides in Cell Cycle Control

(i) Fibroblasts

The original observation that implicated cyclic AMP in cellular growth control was made by Burk who showed that caffeine and theophylline (inhibitors of cyclic AMP phosphodiesterase) slowed the growth of normal and transformed BHK cells (71). Subsequently it has been documented that drugs which raise intracellular cyclic AMP (either by inhibition of cyclic AMP phosphodiesterase or by stimulation of adenyl cyclase), analogues of cyclic AMP or cyclic AMP itself, decrease growth

rate in a great many different fibroblast lines. Included in this list are Hela cells (72), L cells (72-80), transformed 3T3 cells (75, 81-85), BHK cells and transformed BHK cells (71, 75, 86, 87), Nil cells (88), embryonic rat cells (89-90), RSV-transformed mouse, rat and hamster cells (76, 77, 91), normal and transformed rat kidney cells (92), human diploid fibroblasts (93), human rhabdomyo sarcoma (94) and CHO cells (95, 96).

The experimental results outlined above strongly suggest that cyclic AMP has a physiological role in regulating the rate of cell division. This has been verified by direct measurement of the cyclic AMP levels in different cell lines dividing at different rates under constant conditions (74, 97-98) in chick embryo cells dividing at different rates in varying concentrations of serum (99), and in BHK cells stimulated to grow more rapidly by the addition of insulin to their growth medium (86).

There are many reports that intracellular cyclic AMP levels rise as normal cells cease growth at confluency (92, 97, 100-101). Transformed cells that do not cease growth do not elevate their cyclic AMP levels (97). Rat kidney cells transformed with a temperature-sensitive Kirsten sarcoma virus show density-dependent inhibition of growth and also increase their cyclic AMP levels at 39° C (the non-permissive temperature), but not at 32° C where they behave like transformed cells in both properties (92).

Quiescent fibroblasts stimulated to divide by addition of serum or insulin show a rapid fall in cyclic AMP levels (93, 98, 100, 102-105). A fall in cyclic AMP levels is apparently important for the initiation of growth because growth stimulation is prevented if cyclic AMP is maintained at high levels following addition of growth-promoting agents. Dibutyryl cyclic AMP will prevent serum-induced DNA synthesis in quiescent 3T3 cells (103, 106), rat embryo fibroblasts (89), chick embryo fibroblasts (99), human diploid fibroblasts (93, 107) and BHK cells (108). Dibutyryl cyclic AMP will also prevent DNA synthesis following protease stimulation in BHK cells (109-111).

(ii) Lymphoblasts

MacManus and Whitfield and their co-workers have performed numerous studies on the effects of cyclic AMP on the proliferation of thymic cells (a mixture of lymphocytes and lymphoblasts). Initial results suggested that physiological concentrations of cyclic AMP (10^{-8} - 10^{-6} M) or dibutyryl cyclic AMP were able to stimulate proliferation of thymic lymphocytes in culture (112). Also it was found that agents which produce an elevation of intracellular cyclic AMP (such as epinephrine or PGE1) cause initiation of DNA synthesis and cell division (113-115). However, concentrations of cyclic AMP above 10^{-6} M were found to inhibit cell proliferation (112). These results were initially interpreted to mean that cyclic AMP was acting as a mitogenic signal to convert the resting lymphocyte to a proliferative lymphoblast. However, since DNA synthesis (as measured by [^{3}H]-thymidine incorporation) occurs within one hour after cyclic AMP treatment and cell division begins within three to four hours, it seems more likely that cyclic AMP rather promotes DNA synthesis and mitosis of a subpopulation of already activated lymphoblasts (116).

Studies with synchronised HeLa cells (117), CHO cells (118), 3T3 cells (109) and human lymphoid cells (119-120) show that intracellular cyclic AMP levels vary throughout the cell cycle. These changes in cyclic AMP levels are thought to regulate passage of the cells through the cell cycle. These cell cycle studies also suggested that cyclic AMP could have either a stimulatory or inhibitory effect on cell cycle progress dependent on the point in the cycle when the cyclic AMP was added. Thus an increase in intracellular cyclic AMP in late G1 might be required to initiate DNA synthesis; this would then explain the stimulatory effects of cyclic AMP on lymphoblast proliferation reported by MacManus and Whitfield (112). In fibroblasts a similar result has been reported in that dibutyryl cyclic AMP added in G1 brought forward the time of commencement of DNA synthesis (106).

Whitfield et al. (116) have presented a model to explain the complicated relationship that appears to exist between calcium and cyclic AMP in the regulation of lymphoblast proliferation (see Fig. 5). It is their opinion

that calcium ion is the prinicpie regulator of cell proliferation both *in vivo* and *invitro* and in some cells calcium may act via cyclic AMP. This notion will be discussed in more detail later when considering Berridge's hypothesis of the control of cell division (section 1.3 (vii)) because the essence of the Berridge hypothesis is that calcium is the primary regulator of cell division (121). The work of Whitfield *et al.* indicates that calcium promotes the initiation of DNA synthesis (which is not necessarily the same as entry into proliferation) by increasing intracellular cyclic AMP levels (122). The calcium concentration in the growth medium can change cyclic AMP from a stimulator to an inhibitor of thymic lymphoblast proliferation (116, 123). Low concentrations (10^{-7} - 10^{-5} M) of exogenous cyclic AMP increase lymphoblast proliferation in low calcium medium. Conversely, the growth of lymphoblasts is inhibited at all cyclic AMP concentrations between 10^{-7} and 10^{-3} M in high calcium (1.5 mM) medium.

The way in which low concentrations of cyclic AMP (10^{-7} - 10^{-6} M) promote cell proliferation is not at all clear because the cyclic AMP does not enter the cell at this extracellular concentration, nor does it cause elevation of intracellular cyclic AMP levels (116, 124). MacManus and Whitfield envisage a binding site for cyclic AMP on the cell surface (116) which is an "activation site" for initiation of DNA synthesis. Pastan *et al.* have commented that if calcium does play a regulatory role in the lymphoblast proliferative process, it is equally likely that low levels of cyclic AMP are able to either mobilise intracellular calcium or enhance calcium uptake from the low calcium medium to elevate calcium ion sufficiently to induce the proliferative response (125). These studies seem to indicate that cyclic AMP is not acting as a mitogen to activate lymphocytes, but is (depending on the calcium concentration) serving as either a positive or negative regulator of the initiation of DNA synthesis in cycling lymphoblasts.

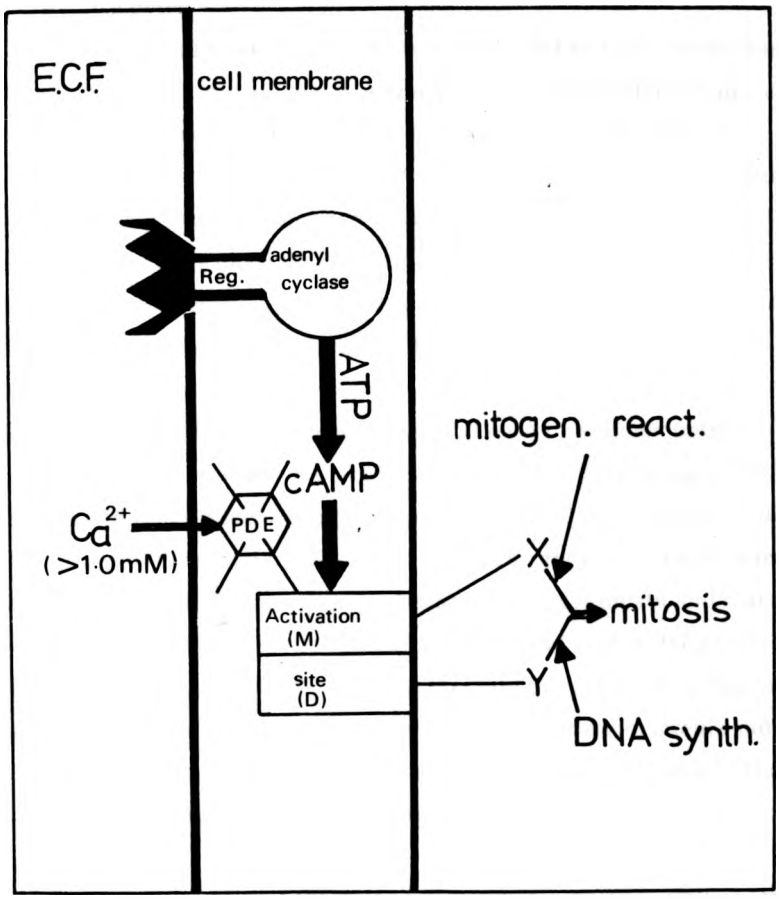

Fig. 5. Summary of the mechanism proposed by Whitfield et al. by which calcium in the extracellular fluid (E.C.F.) stimulates DNA synthesis and proliferation of thymic lymphoblasts. A sudden exposure of the cell to calcium concentrations greater than 1.0 mM inhibits the degradation by an accessible, membrane-bound phosphodiesterase (PDE) of cyclic AMP (cAMP) which is formed in the cell membrane by adenyl cyclase. The adenyl cyclase complex is represented as consisting of an external receptor, which is attached to the catalytic portion by a regulatory portion (Reg.). Cyclic AMP stimulates the mitogenic (M) and DNA synthetic (D) parts of a membrane activation site to produce initiators X and Y which enter the cell and start the mitogenic and DNA processes respectively.
(Reproduced from reference 116 with slight alterations).

(iii) Lymphocytes

When peripheral blood lymphocytes are exposed to high concentrations of phytohemagglutinin A (PHA), a slight rise in intracellular cyclic AMP levels has been observed within a few minutes of mitogen treatment (126-128). Cyclic AMP levels in PHA-activated lymphocytes fall within 6 hours to below the level found in control cultures (127). Novogradsky and Katchalski (129) found that PHA did not elevate cyclic AMP levels in rat lymph node lymphocytes. Hadden et al. noticed a slight increase in intracellular cyclic AMP levels in lymphocytes stimulated with an impure PHA preparation, but no change was observed after stimulation with purified PHA (130). Concanavalin A (Con A), another potent mitogen, does not increase intracellular cyclic AMP even though DNA synthesis and cell division are induced (126, 128, 130). The fact that cyclic AMP and dibutyryl cyclic AMP as well as agents that raise intracellular cyclic AMP do not cause initiation of DNA synthesis and that PHA and Con A do not produce marked elevations of intracellular cyclic AMP suggests that cyclic AMP does not mediate the effect of mitogens in lymphocyte transformation. However, a number of experiments indicate that elevated cyclic AMP levels can inhibit lymphocyte DNA synthesis and prevent mitogen-induced proliferation (131-138).

(iv) Cyclic GMP

Thus far, discussion of the role of cyclic nucleotides in cell cycle control has focussed entirely on cyclic AMP. The great majority of research in this area has until more recently been devoted solely to the study of the role of cyclic AMP. This is almost entirely due to the nature of the discovery of cyclic AMP and cyclic GMP. Cyclic AMP was originally discovered to be an important intracellular "messenger" by Sutherland's group in 1957 in their investigations on the mode of hormone action (139-140). In contrast, cyclic GMP was first discovered in 1963 by Price's group; they first detected it in rat urine but had no idea of its origin or of its importance (141). Thus, as Goldberg has said, "whereas Sutherland's original work might be thought of as starting off with a particular function

(stimulated glycogenolysis) in search of a compound, the cyclic GMP story began with a compound in search of a function". (142). The later discovery and the initial lack of knowledge of a biochemical role of cyclic GMP has always been reflected in the literature which contains far more studies related to cyclic AMP than cyclic GMP. Another stumbling block held back work on cyclic GMP; this was the fact that cyclic GMP levels are often quoted as being up to one hundred times less than cyclic AMP levels in the same tissue (142). Thus although a number of assays for cyclic GMP had been published by 1971 (143-146), the routine assay of cyclic GMP in biological materials was not possible until the major step-forward in assay technology came in 1972 with the radioimmunoassay technique of Steiner et al. (147).

Goldberg's group were the first to implicate cyclic GMP in cellular growth control (130). They found that PHA and Con A stimulation of lymphocytes produced up to a 50-fold increase in intracellular cyclic GMP within 20 minutes of exposure to the mitogens. They proposed the view that cyclic GMP is the active signal to induce proliferation and that elevation of cyclic AMP levels limits or inhibits mitogenic action (130). The idea that cyclic GMP is the "switch-on" signal and cyclic AMP is the "switch-off" signal (148) has gained much support in subsequent studies. Seifert and Rudland showed that cyclic GMP, or its butylated analogues at concentrations greater than physiological, stimulated DNA formation in quiescent BALB 3T3 fibroblasts, and that a few minutes after mitogenic stimulation by addition of serum the intracellular cyclic GMP concentrations rose approximately 10-fold and cyclic AMP levels fell about 2.5-fold (105). In a similar study, the same team of workers measured cyclic AMP and cyclic GMP levels in growing and quiescent fibroblasts. They found that non-transformed cells have a large difference in cyclic AMP : cyclic GMP ratio when comparing growing with quiescent cells, but no such differences were found in transformed cells (149). This idea has been confirmed in prostatic sarcoma tissue (150) and in several strains of Morris hepatoma (151) where a decrease in levels of cyclic AMP and adenyl cyclase

activity and increased cyclic GMP level and guanyl cyclase activity (relative to the normal tissue) have been reported. In the case of the prostatic sarcoma study, it was noted that the protein kinases from the tumour responded to cyclic AMP to the same extent as was observed in the enzyme preparation from the normal tissue, but the protein kinase from the tumour was shown to be more sensitive to cyclic GMP than that from the normal tissue (150). This information indicates how elevated cyclic GMP levels may stimulate cell proliferation. 8-bromo cyclic GMP has been reported to cause time-dependent increases in the synthesis of DNA and RNA in lymphocytes. Stimulation by 8-bromo cyclic GMP paralleled temporally that produced by Con A (152).

Reports from Whitfield's group on the role of cyclic GMP in lymphocyte proliferation are confusing. Initially it was reported that exposure of cycling lymphoblasts to low (10^{-11} - 10^{-10} M) or high (10^{-5} - 5×10^{-6} M) concentrations of cyclic GMP raises intracellular cyclic AMP, and that proliferation is thus stimulated. Concentrations of cyclic GMP in the range 10^{-9} - 10^{-7} M were reported as having no effect (153). Later it was reported that the intermediate concentrations of cyclic GMP (i.e. 10^{-9} - 10^{-7} M) could either stimulate of inhibit the process, depending on the extracellular calcium level and the intracellular cyclic AMP level (154). The same group have reported that cyclic GMP at 10^{-7} M can prevent calcium initiation of DNA synthesis and cell division (116) and that low concentrations of cyclic GMP (5×10^{-11} M) initiates DNA synthesis in lymphoblasts by a calcium-independent process (155). In 1974 it was observed that exposure of thymic lymphocytes in serum-free synthetic medium to Con A caused brief (within the first 8 - 12 minutes) rises in cellular cyclic AMP and cyclic GMP (156). The rise in cyclic GMP level was dependent on extracellular calcium but the cyclic AMP rise was not. These changes were followed in the next hour by calcium-dependent initiation of DNA synthesis by a large fraction of the lymphoblast subpopulation. Other observations suggested that Con A operates by sensitizing lymphoblasts to calcium ions which in turn cause initiation of DNA synthesis by a process mediated by cyclic GMP but not by cyclic AMP (156).

When carefully controlled experiments reveal such a confusing picture as in the case of the involvement of cyclic GMP in lymphocyte proliferation, it is easy to understand why sceptics are quick to try and brush aside all of the work. A number of important publications must be mentioned here. Carchman et al. found that cyclic GMP analogues had no effect on the growth of NRK cells (92) whereas one would have expected promotion of growth. A mutant line of S49 lymphoma cells lacking a cyclic AMP dependent protein kinase were shown to maintain a normal cell cycle even in the presence of high levels of cyclic AMP (157). The authors concluded that periodic fluctuations in the levels of cyclic AMP cannot be required for or determine progression through the cell cycle. Pastan has expressed considerable scepticism about the role of cyclic GMP as a growth promoter. In a study on BALB 3T3 fibroblasts his group of workers reported that addition of serum to nongrowing cells decreased cyclic GMP and cyclic AMP, and promoted cell growth (158). In the same report they observed that cyclic GMP rose as cell growth slowed. Naturally they commented that these results fail to support the idea of cyclic GMP as a promoter of cell growth and suggested that cyclic GMP as well as cyclic AMP may in fact be growth inhibitors. In another study Pastan's group found that the intracellular cyclic GMP level and guanyl cyclase activity in transformed NRK cells were both low when compared with levels measured in confluent (normal) NRK cells (159). Despite these reports (some by distinguished workers) the weight of evidence is still in support of cyclic GMP as a promoter and cyclic AMP as an inhibitor of cell growth.

(v) The Yin Yang Hypothesis of Biological Control

The Yin Yang hypothesis of biological control was proposed by Goldberg et al. (160) as a general theory to account for the control of the many biological systems in which cyclic AMP and cyclic GMP are known to be involved. According to the hypothesis (Fig. 6) the opposing actions of cyclic AMP and cyclic GMP are expressed in systems which are susceptible to both stimulatory and inhibitory controlling influences or those comprised of antagonistic events. The hypothesis envisages

"bidirectionally" controlled systems, the dual opposing cyclic nucleotide signals can enter into a mutual interaction resulting in synthesis. Goldberg et al. have described two types of bidirectionally controlled systems (Fig. 6): those stimulated by an elevation in the level of cellular cyclic AMP (A-type) and those suppressed by an increase in the concentration of cyclic AMP (B-type). The hypothesis suggests that an elevation of cellular cyclic GMP concentration promotes the opposing cellular event or provides the opposing regulatory influence in each type of bidirectionally controlled system. It is clear to see that a reciprocal change in the levels of cyclic AMP and cyclic GMP will allow maximum

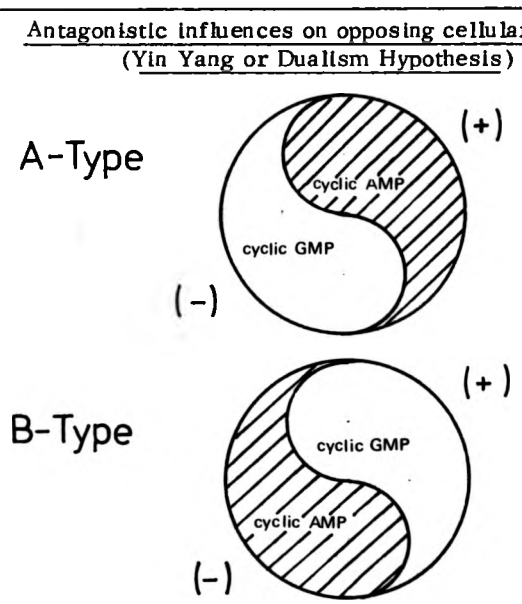

Fig. 6. Biological regulation through cyclic AMP and cyclic GMP. The proposed influences of cyclic GMP and cyclic AMP in the regulation of cell functions. Bidirectionally controlled systems are those comprised of opposing cellular processes. Depending upon the type of bidirectional system, an increase in the concentration of cyclic AMP (A-type) or cyclic GMP (B-type) represents the facilitatory influence, while the suppressive influence and/or opposing cellular event is promoted by an increase in the cellular level of the other nucleotide.
(Reproduced from reference 160).

expression of the incoming signal. The originators of the hypothesis point out that the extent to which there may be a lowering of the cellular concentration of the cyclic nucleotide not linked to the predominant incoming signal will depend upon what the prevailing level may be relative to the basal concentration and probably other factors such as the degree of stimulation from opposing signals. Although the Yin Yang hypothesis was proposed as a general model to embrace many diverse biological systems, the idea has received much attention from workers studying cell proliferation and growth control.

(vi) The Berridge Hypothesis

Berridge has postulated that calcium is the primary division signal in all cells (121). An increase in the intracellular calcium level is viewed as being the primary stimulus to divide. It is envisaged that cyclic AMP can play an important role in many cells by modulating the level of the primary mitogenic signal calcium. Berridge's model can account for cyclic AMP exerting either positive or negative feedback control on the intracellular calcium level (Fig. 7) stimulation of guanyl cyclase by calcium, thereby raising intracellular cyclic GMP is the explanation put forward for the complex relationship that can exist between cyclic AMP and cyclic GMP levels within the cell.

The most important cell cycle decision point is thought to reside in G1 (as explained on page 3). Berridge accounts for the G1 decision point (which he refers to as a short transition phase), and for cellular differentiation in the one model (see Fig. 8). During this short transition phase the cell is said to be faced by two alternatives. It can either remain within the cell cycle by immediately proceeding to another division (path I in Fig. 8) or it can cease proliferation and begin to differentiate (path II in Fig. 8). Quiescent cultured cells are considered to be differentiated. The critical switch during the transition phase is dependent on the level of calcium. Passage through the transition phase in the presence of high calcium commits the cells to another round of cell division, whilst a low level of calcium will switch the cells out of division and towards differentiation. It is proposed that a high level of

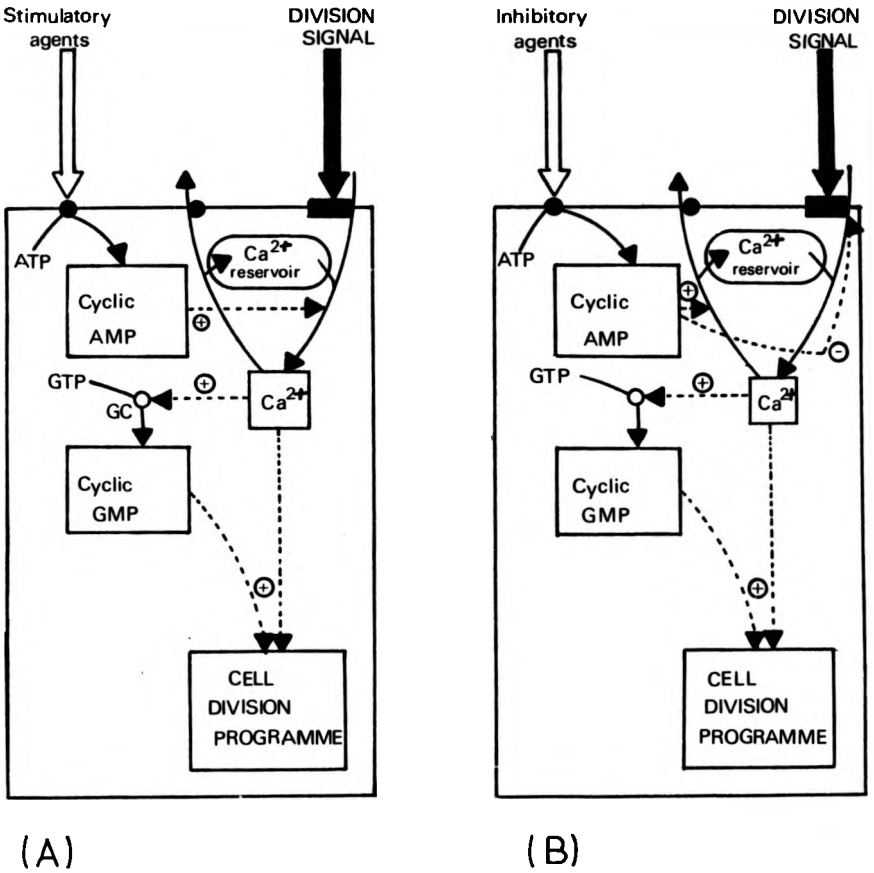

Fig. 7. The Proposed Role of Calcium and Cyclic Nucleotides in the Control of Cell Division.

The major intracellular signal regulating division is calcium which usually enters the cytoplasm from the outside, but it can also be released from intracellular reservoirs. Cyclic AMP may modulate this signal. In (A) cyclic AMP augments the calcium signal by stimulating the release of internal calcium. In (B) cyclic AMP opposes the calcium signal by stimulating its removal from the cytoplasm or by inhibiting the uptake of external calcium. In both cases, a high level of calcium may stimulate guanyl cyclase (GC) to increase the level of cyclic GMP which may function together with calcium to initiate the cell division programme.

(Reproduced from reference 121).

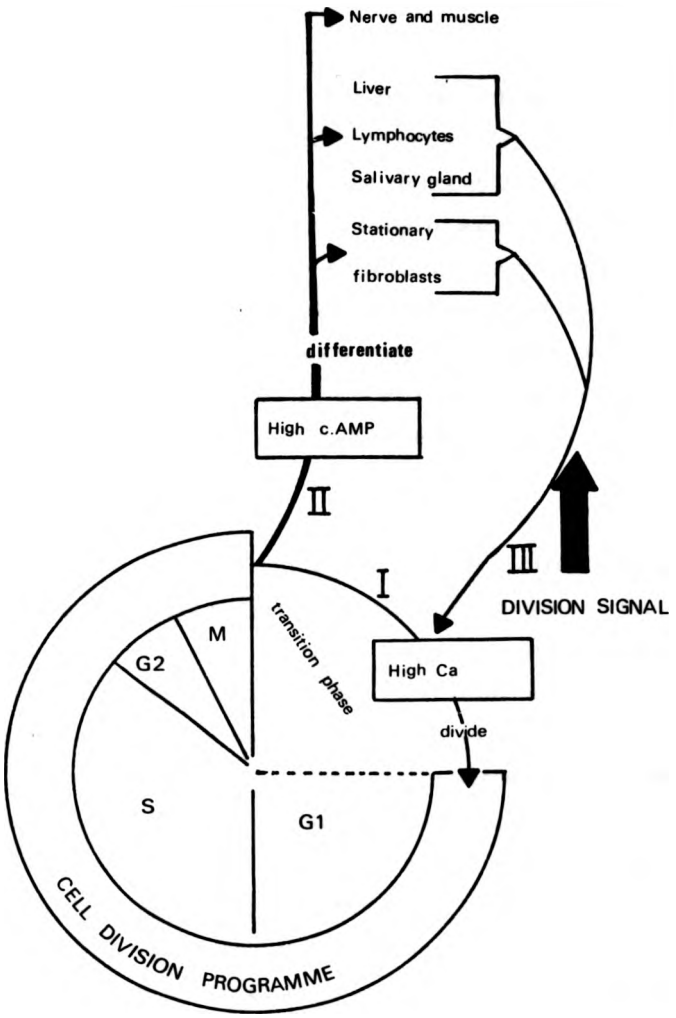

Fig. 8 The Proposed Role of Calcium and Cyclic AMP during the Transition Phase of the Cell Cycle early in G1

A high level of calcium stimulates cells to divide (I), whereas cyclic AMP switches them out of the cell cycle and induces them to differentiate (II). Many differentiated cells can divide if provided with the appropriate division stimulus (III). If a cell commits itself to divide, it embarks upon a set cell division program which terminates with mitosis (M). (Reproduced from reference 121).

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cyclic AMP during the transition phase will assist in lowering the calcium level (necessary to switch the cells out of division), and may also initiate differentiation. The model implies that differentiated cells can be switched back into the cell division cycle. This criticism is answered by

stating that although differentiation is often irreversible (as in the case of nerve and muscle cells), certain specialized cells (e.g. lymphocytes, liver cells and salivary gland cells) do retain the ability to divide and be stimulated to re-enter the cell cycle by a range of external division signals (path III, Fig. 8). Berridge's model of cell cycle control is thus comprised of a short decision-making period followed by a set division program. The model thus has a striking similarity to that proposed by Smith and Martin (7, 32). Cyclic nucleotides are thus proposed as playing a secondary role in that they are related to calcium through the feedback relationships outlined in Fig. 7.

(vii) Summary

Plainly the role of cyclic nucleotides in cell cycle control is not a cut-and-dried story. Some workers view cyclic nucleotides as the universal mediators of biological control mechanisms, whilst others regard calcium ion as the primary effector with cyclic nucleotides playing a secondary role. At the risk of seeming to be dealing solely in semantics, it seems appropriate to comment that perhaps the two opposing attitudes are not really opposing but merely describing different aspects of the same phenomena. Surely cyclic nucleotides can be the universal mediators and calcium ion the primary effector ?

At this point it seems relevant to ask whether the most frequently studied cell systems are in fact the most appropriate for such studies. For instance, Pastan et al. have commented (125) that since it appears likely that mitogen stimulation of lymphocytes first produces an immune response (161), and that the event required to convert resting cells to proceed into G1 may follow some hours later, that one might ask whether the initial changes noted in PHA-stimulated lymphocytes such as the rapid elevation in cyclic GMP levels reported by Hadden et al. (130) actually have any relationship to the initiation of proliferative events which follow some hours later. Furthermore the relevance of studying the G0 to G1 transition has already been questioned (see page 11). A more general point is that the great majority of studies on the role of cyclic nucleotides in cell cycle control have used cells derived from multicellular

organisms. Wolfe has commented (162) that a characteristic of such cells is that they involve some form of cellular interactions. The great majority and variety of effects attributable to cycle nucleotides may be a function of the increased need for regulation of internal metabolism in response to the external stimuli imposed by the condition of multicellularity. In other words, such cells possess intercellular communication phenomena which can involve cyclic nucleotides. In order to study the regulatory role(s) of cyclic nucleotides in fundamental intracellular processes, one needs an experimental system that is independent of cellular interactions. A free-living unicellular eukaryote should be largely devoid of such behaviour, and thus represents a more suitable model system.

1.4 Tetrahymena pyriformis as a model system for studying cell cycle control by cyclic nucleotides

Tetrahymena pyriformis is a free-living unicellular eukaryote and thus fulfils the criteria outlined immediately above (in section 1.3 (vii)) in answer to the objections raised against the cell systems derived from multicellular sources. In addition there are many well-known features of Tetrahymena pyriformis outlined in detail elsewhere (163-164) that render it a most suitable organism for study. These include its ease of culturing and fairly rapid growth (with a cell cycle time of about 3 hours) which means that it is easy to obtain sufficient material for experimentation. The many factors in favour of Tetrahymena pyriformis as a research organism have meant that there is a huge number of reports in the literature on the biochemistry, physiology, morphology and behaviour of this cell. That Tetrahymena pyriformis is so well studied is another good reason for choosing it for cell cycle studies. Furthermore, mass cultures of Tetrahymena pyriformis are amenable to synchronization by both induction techniques and selection techniques.

(i) Synchronization of Cultures of Tetrahymena Pyriformis

(a) Induction synchrony methods

The classical method of induction of cell division synchrony in Tetrahymena pyriformis is the multi heat-shock technique (165). More

recently Zeuthen and co-workers have developed a new repetitive heat shock method (166). Other induction synchrony methods include a multi cold-shock technique (167) and a cold cycling process (168), the use of colchicine or colcemid (169), vinblastine or vincristine (170), starvation-refeeding (171), hypoxia (172-174), high pressure (175), carbon dioxide pulses (186), methotrexate plus uridine (187-188), cycles of light and dark when the cells are grown at 10^{o} C (189) and hydroxyurea (176). In addition, amino-acid starvation followed by multiple heat shocks produces the phenomenon of "synchronous rounding" in which the cells do not divide but periodically "round-up" as though in preparation for division (177).

(b) Selection synchrony methods.

Corbett has described a centrifugal separation of post-division cells from an exponentially-growing culture (178). These cells remained in a 550 x g supernatant, whereas the larger cells sedimented. Bollinger could not repeat this experiment (179). Lloyd et al. have achieved selection of large-scale cultures of synchronous Tetrahymena pyriformis by continuous-flow size selection (63) and by continuous-flow cell cycle fractionation (205). Two other selection synchrony techniques for Tetrahymena pyriformis have been described. They both rely on the fact that Tetrahymena pyriformis is an indiscriminate feeder that will concentrate particles (with or without nutritional value) into food vacuoles and that dividing cells do not form food vacuoles. Hildebrandt and Dusplva added iron particles to an exponentially growing culture and after a few minutes passed the cells through a narrow tube past a magnetic field which separated cells containing iron particles from those without any particles (180). The latter cells were used to start new synchronous cultures. Wolfe has used iron and tantalum particles and separated the feeders from the non-feeders (dividing cells) by means of a discontinuous Ficoll gradient, the cells which have ingested particulate material sedimenting further than those which have not (181). Clearly there are many excellent induction and selection synchrony techniques available for use with this organism.

The most frequently used technique has been multiple heat shocks. Cell cycle experiments with this system have led to the concept of "division proteins".

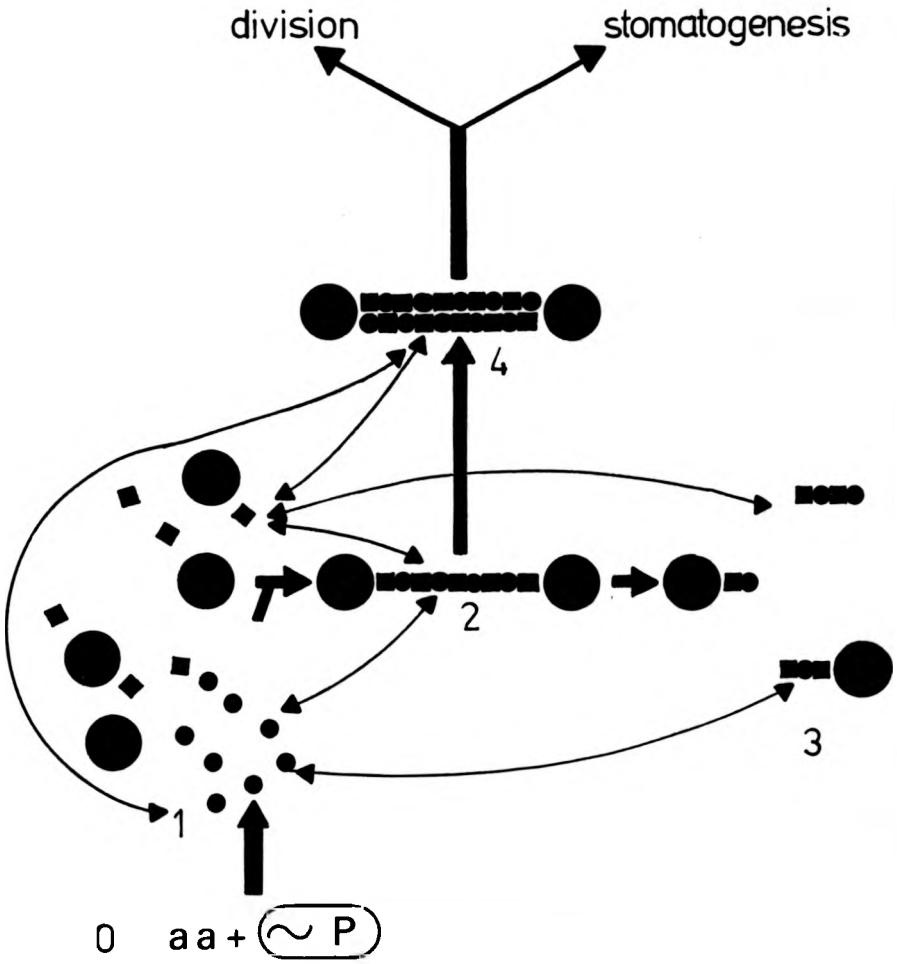

Fig. 9 Diagrammatic representation of the build-up of structures postulated to be essential for cell division and oral morphogenesis in Tetrahymena pyriformis. The heavy arrows between 1 and 2, and 2 and 4 indicate the path of assembly, while the thin arrows denote that proteins already built into more complex units can be in continuous exchange with proteins in the intracellular environment. A heat shock is postulated to open or greatly stimulate the reaction from 2 to 3. (Reproduced from reference 31).

Fig. 9 shows Zeuthen's model for synchronization of cell division in Tetrahymena pyriformis by multiple heat shocks. The model postulates that as a cell proceeds from one division to the next, different kinds of proteins(or organized proteinaceous aggregates)"1" become fitted into a first labile "2" then stable structure"4 ", after which the cell can proceed toward division. It is suggested that temperature changes (multiple heat shocks or multiple cold shocks) cause the structural disturbance that is illustrated by the transition from "2" to "3 " resulting in the trapping of useful proteins in useless fragments "3" (166, 182). The system recovers on return to physiological temperature by a new progression from "1" to "4", or perhaps even from "0" to "4", because recovery requires synthesis of new proteins (183). The model includes the developing oral apparatus that is completed ('stomatognesis") before the cell divides. Structures "2" and "4" are meant to illustrate fibrous connections between centrioles (non-ciliated kinetosomes) of the oral morphogenetic field in the cell. Disruption of these fibres with heat has been observed (184).

The model in Fig. 9 was constructed with the intention of stressing the similarities between the local morphogenetic events in a subpellicular organelle (i.e. the oral apparatus) and the behaviour of centrioles and mitotic fibres in cell division. The thin two-directional arrows in Fig. 9 represent exchanges of protein molecules between the finished oral apparatus and a pool of structural proteins in the cytoplasm, and, through the pool, the exchange of proteins between the old and the developing oral structures that co-exist for some time in the growing cell (185). Both old and new apparatuses are thus highly dynamic structures, and this may aid in explaining why the oral primordium collapses so easily in response to environmental changes. Many of the methods of inducing division synchrony in Tetrahymena pyriformis appear to act through the same mechanism as heat shock (182). Studies involving hydroxyurea-synchronized Tetrahymena pyriformis have led one group of workers to propose that control of nuclear/cytoplasmic ratio is the means of cell cycle control (190).

(ii) Genetic Studies on Cell Cycle Control in Tetrahymena Pyriformis

Although genetic manipulations of Tetrahymena pyriformis have been performed for many years now, and the techniques have reached a high degree of sophistication, genetic dissection of cell cycle control processes had not been described until 1976 when Frankel, Jenkins and de Bault employed some temperature-sensitive mutants of Tetrahymena pyriformis affected in cell division and developmental pathways (191). These studies permitted elucidation of causal dependencies inter-relating micronuclear and macronuclear replication and division, oral development and cytokinesis. The findings suggested that (a) macronuclear division is stringently affected by restriction of cell division, (b) micronuclear division and replication can continue in cells that are undergoing oral development, and (c) macronuclear DNA synthesis can continue in cells regardless of their developmental status. The authors proposed a minimal model of the Tetrahymena pyriformis cell cycle that takes account of all the observed phenomena (Fig. 10).

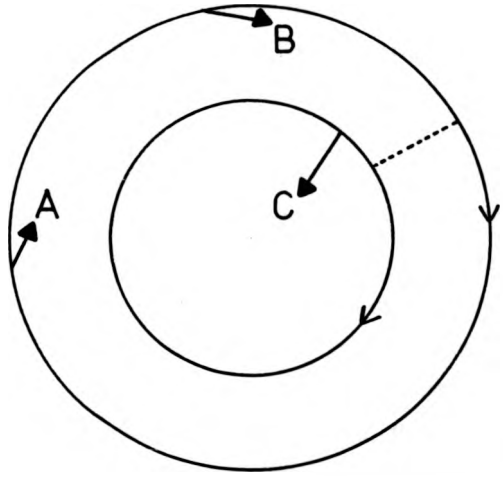

Fig. 10 The Simplest Possible Model to account for Relations of Sequential Events in the Tetrahymena pyriformis Cell Cycle

A, micronuclear division and oral development; B, macronuclear division and cytokinesis; C, macronuclear DNA synthesis. The model involves two parallel independent timer sequences with "check points" (dashed line) affecting phase relations.

(Reproduced from reference 191).

(iii) Cyclic Nucleotides in Control of the Tetrahymena Pyriformis Cell Cycle

Cyclic AMP (162, 192-195), adenyl cyclase (193, 196-199), cyclic AMP phosphodiesterase (192-193, 200-201), and cyclic AMP-dependent protein kinase (202-203) have all been demonstrated in Tetrahymena pyriformis. Prior to this research project cyclic GMP had not been reported in Tetrahymena pyriformis, though there were two publications which suggested the nucleotide was present; cyclic GMP-dependent protein kinase was demonstrated by Murofushi (202) and in 1975 Dickens et al. reported that cyclic GMP induces thymidylate synthetase activity (204).

Stationary phase Tetrahymena pyriformis have been shown to contain higher levels of cyclic AMP than logarithmic phase cells (162, 192-193). Kariya et al. observed that addition of dibutyryl cyclic AMP to Tetrahymena pyriformis synchronized by multiple heat shocks inhibited protein and RNA syntheses in G1 and DNA synthesis in S phase. However, in the same report, they stated that the content of cyclic AMP in the cell increased abruptly in the early exponential phase, and then diminished with further culture. This finding is completely the opposite of that reported by Volchick et al. (192-193). The method of extraction and assay of cyclic AMP by Kariya and co-workers seems most unsatisfactory, and the result must be regarded as uncertain. If selection-synchronized Tetrahymena pyriformis are exposed to 30-minute "pulses" of caffeine at different times in the cell cycle, then the later the pulse is added the greater is the set-back of the subsequent division (162). By comparing the divergence between the theoretical* and the experimental set-back of cell division, it was shown that early in the cell cycle it is possible to set back the cells completely to post-division status, but as the cells progress through the cell cycle, the setback, though quantitatively greater, gets further from a post-division status. In other words, with

* "Theoretical setback" is defined as the setback that would be expected if the excess division delay were equal to the age of the cells when the block was applied.

increased progress towards division, the 30-minute exposure to caffeine is increasingly insufficient to achieve a complete setback in spite of the fact that the setback is quantitatively increased. This reasoning implied that high cyclic AMP levels would maintain the cells in G1.

In this introductory chapter I have tried to outline the ideas currently held on control of the eukaryotic cell cycle. There are technical problems which have prevented the use of selection synchrony techniques with many of the cell systems from higher organisms, and objections by many authors to induction synchrony techniques per se for the investigation of regulatory processes. Interpretation of results can be difficult in some of the most popular cell systems used. Tetrahymena pyriformis as a unicellular eukaryote which can be synchronized by many different methods represents a suitable alternative, but the few preliminary studies to date on the role of cyclic nucleotides in the regulation of the cell cycle in this organism are conflicting. Direct measurements of cyclic nucleotide metabolism in the natural cell cycle of Tetrahymena pyriformis had not been performed before the start of of this project. The report describes the development and validation of suitable assays and synchronizing techniques for use with this cell in an attempt to further our understanding of the role of cyclic nucleotides in the control of the eukaryotic cell cycle.

CHAPTER 2

SYNCHRONIZATION OF CELL DIVISION IN CULTURES OF TETRAHYMENA PYRIFORMIS

2.1 Introduction

Induction and selection synchrony methods with Tetrahymena pyriformis have been outlined previously in section 1.4 (i). As I have pointed out earlier (section 1.2 (ii)), it is always possible that the process altered by an induction procedure is the very process that one is trying to examine. This consideration becomes even more important in the case of control processes. For this reason it seemed preferable to perform the initial measurements on various aspects of cyclic nucleotide metabolism in selection synchronized cultures of Tetrahymena pyriformis to try and identify possible cyclic nucleotide control points in the natural cell cycle. Later experiments would then go on to study induction-sunchronized cells to try and find how perturbation of the normal cycling processes is affected in relation to the patterns of modulation of cyclic AMP and cyclic GMP.

Corbett's centrifugation procedure (178) seemed to be an unreliable technique as Bollinger could not repeat it (179). When this project was commenced in September, 1974, the method of Lloyd et al. to use continuous-flow size selection was known but not in full detail; the report on the method (63) did not appear until 1975. Preliminary investigations on the use of continuous-flow size selection with Tetrahymena pyriformis in our laboratory met with little success (206); it seemed that considerable work on a purely trial-and-error basis was needed, and that the precise conditions were not attainable with the equipment available in this laboratory. This left two selection synchrony techniques as possibly useful methods for the studies envisaged:- the method of Hildebrandt and Duspiva (180) and the method of Wolfe (181).

Clearly, a number of induction synchrony techniques could have been used in the later studies. The technique chosen was a novel method of induction of division synchrony by means of a single (240-minute) hypoxic shock. Other work in progress in our laboratory early in 1975 involved a study of several enzyme changes in Tetrahymena pyriformis when growth conditions were switched from aerobic to anaerobic or from anaerobic to aerobic. In particular it was noticed that a 240-minute period of non-shacking conditions after originally growing the cells with rapid shaking caused a cessation of cell division. When the cells were reshaken, there followed a discrete burst of cell division after a lag period. Cell division appeared to become synchronized by the 240 minutes of non-shaking (207). Part of this research project has involved an evaluation of this synchronization technique.

2.2 Materials and Methods

(i) Growth of Cells

Stock cultures of Tetrahymena pyriformis strain W (from the Cambridge Culture Collection, Cambridge, U.K.) were grown in conical flasks filled to 20% total volume with a growth medium (PPYF) containing 2% proteose peptone, 0.1% yeast extract, and 5 μg/ml $FeCl_3 \cdot 6H_2O$. In later experiments a glucose-enriched (PPYFG) growth medium was employed in which 0.5% w/v glucose was added. Stocks were maintained at room temperature by subculturing at one week intervals.

The cell density of cultures of Tetrahymena pyriformis was determined by fixing aliquots of cells in equal volumes of 20% formaldehyde neutralized with 0.01 M phosphate buffer and counting in a Neubauer haemocytometer. At least 200 cells were counted at each time of sampling. Late division cells were scored as "furrowing cells" because the prominent furrow girdling the cell made identification of this stage unequivocal.

(ii) Chemicals

Proteose peptone ("Difco") was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. Powdered yeast extract was purchased from London Analytical and Bacteriological Media Ltd., London, U.K. Calf

thymus DNA (Type 1: sodium salt "highly polymerized"), bovine serum albumin and ficoll were obtained from Sigma Chemical Company Ltd., Kingston upon Thames, U.K. Oxygen-free grade nitrogen was obtained from the British Oxygen Company Ltd., Coventry, U.K. Finely-powdered iron was from Merck (catalogue number 3819), Darmstadt, West Germany. Tantalum was a gift from Norton Company (Metals Division), Newton, Massachusetts, U.S.A. Acid-washed sand (30-90 mesh and 40-100 mesh) was obtained from Fisons, Loughborough, U.K. All other chemicals used were of the highest purity commercially available.

(iii) Protein Determinations

Protein was estimated by the method of Lowry et al. (208) using bovine serum albumin to construct a standard curve.

(iv) DNA Assays

Cells were harvested at 2000 g for 5 min at growth temperature. Growth medium was carefully removed and the pellet of cells resuspended in a small volume of ice-cold distilled water. The suspension of cells was transferred to a sonication vial and an equal volume of ice-cold 20% trichloroacetic acid added. Sonication was performed at 0° C, using a MSE 150W sonicator at maximum power and amplitude for 1 min, by which time cell disruption was judged to be complete. The trichloroacetic acid-precipitated material was centrifuged and the supernatant discarded. The pellet was washed by resuspending in 5 ml ice-cold 5% trichloroacetic acid and centrifuged again. After removing the supernatant, DNA was extracted from the pellet by heating to 70° C for 15 minutes in the presence 0.5 M perchloric acid. After centrifugation, the pellet was heated to 70° C for a further 15 minutes in the presence of 0.5 M perchloric acid. The supernatants from both perchloric acid steps were combined and DNA was assayed according to the method of Burton (209, 210). Calf thymus DNA was used to construct a standard curve.

2.3 Selection of a Synchronous Population of Tetrahymena Pyriformis by Wolfe's Method

(i) Methods

Tetrahymena pyriformis strain W were grown in PPYF medium in flasks filled to 20% total volume at 28 ± ½°C. Cultures were shaken in a water bath at a rate of 90 strokes/min, the amplitude of each stroke being 4.0 cms. 400 mg of heat-sterilized tantalum was added to a 400 ml culture of cells which was shaken in the presence of the tantalum for 5½ minutes. The cells were then concentrated-up by two centrifugation steps, the first at 1,000 g for 5 min, the second at 1,000 g for 3 min. The cells were then layered on to a single gradient composed of 4 ml of 10%Ficoll in PPYF on top of 4 ml of 20%Ficoll in PPYF in a 15 ml conical centrifuge tube. This was then centrifuged for 4 min at 318 g in a swing-out rotor in a BTL bench centrifuge. After centrifugation tantalum particles and cells which had ingested tantalum were seen as a pellet at the bottom of the tube; cells which had not ingested tantalum formed a layer at the interface between the 10%Ficoll and the 20%Ficoll. The cells at the interface were removed with a Pasteur pipette and innoculated into 40 ml of fresh PPYF medium and shaken as before. Aliquots were removed at intervals for cell counting.

(ii) Results

Fig. 11 shows that a reasonable degree of synchronization was achieved, though a rather long time elapsed between re-innoculation into fresh medium and cell division. The reason for the rather unexpectedly long time between re-innoculation and cell division could be for two reasons. The use of two centrifugation steps prior to the gradient centrifugation itself could mean that the cells become anaerobic; they would then need time to recover when returned to normal growth conditions. Wolfe only used one centrifugation step to concentrate the cells prior to the gradient centrifugation (181) but earlier experiments by myself indicated that to process large volumes of cells (greater than 100 ml) it was preferable to use two prior centrifugations so that all the

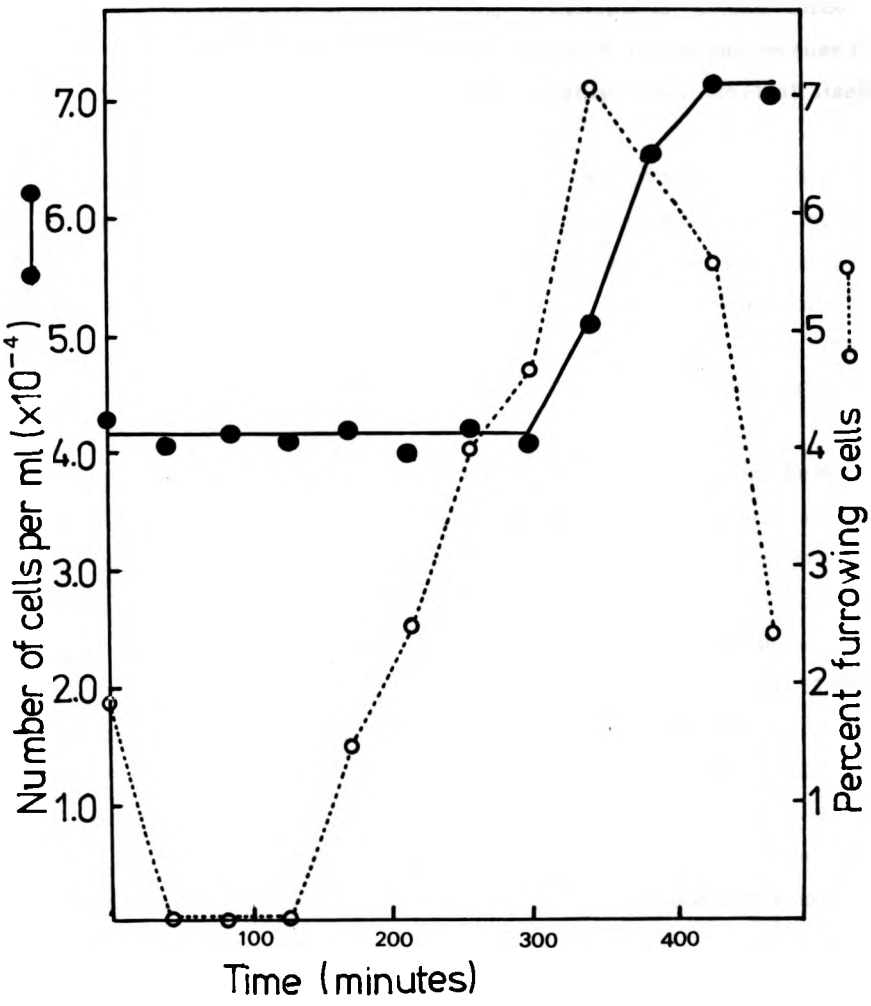

Fig. 11. Preliminary attempt to select a synchronous population of Tetrahymena pyriformis by Wolfe's method

Cells were synchronised as described in section 2.3.(1). Cell counting was performed as described in section 2.2.(1).

cells could be layered on to one gradient. (Attempts to "unload" three or more gradients centrifuged simultaneously proved impossible because of the motility of the cells). The second possibility is that the Ficoll itself is disturbing the cells in some way.

In an attempt to test-out whether the Ficoll is disturbing the cells, the experiment described above was repeated with a washing step included so that the cells recovered from the gradient were washed once for 5 min in growth medium prior to reinnoculation. The results are presented in Fig. 12 and show that the delay in division was reduced by about 30 min compared with the earlier experiment, the division commencing about 180 min after reinnoculation.

Wolfe reported that cell division commenced about 120 minutes after reinnoculation; this (with the timing of the technique involved) is what one might expect. In order to try and reduce further the time between reinnoculation and cell division and to increase the quantity of synchronous cells, the procedure was modified as follows. 2 litres of cells were raised (as 5 x 400 ml batches), pooled, and shaken with tantalum (1 mg/ml of culture) for $5\frac{1}{2}$ minutes. The cells were then concentrated-up by one centrifugation step, and loaded on to 2 gradients in 50 ml tubes and centrifuged in an M.S.E. Mistral 6L centrifuge using an 8 x 50 swing-out rotor. Unfortunately the increased amount of tantalum layered on to each gradient caused disturbance of the gradients which resulted in a poor separation.

(iii) Discussion

Wolfe's technique seems to be well-suited to preparation of small-scale synchronous cultures of Tetrahymena pyriformis. The technique seems to be of no use for the larger quantities of cells required for biochemical studies on the cell cycle. The largest synchronous culture I was able to obtain with this method was 40 ml. Even if the scaling-up to process 2 l of cells had worked, this would still have yielded only 200 ml of cells (since about 10% of cells are selected). The growth conditions for the experiments that were to have been performed if the procedure had proved successful envisaged removal of no more than (say)

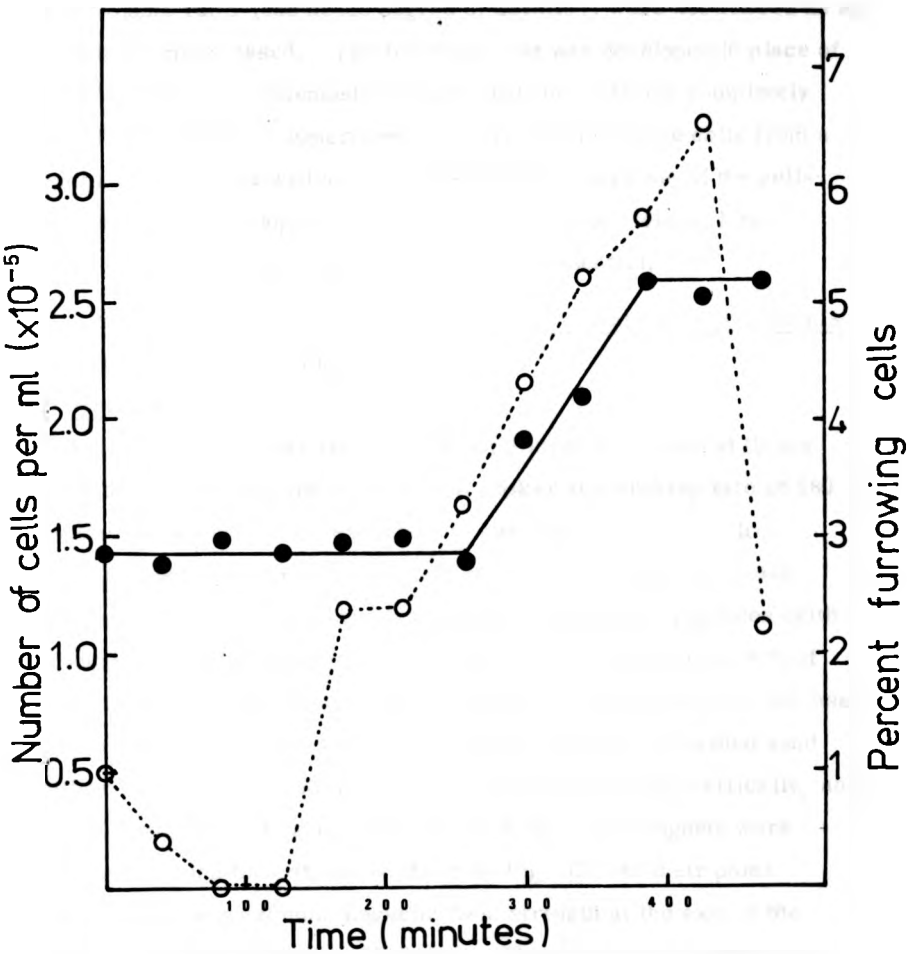

Fig. 12. Further attempt to use the method of Wolfe for the selection of a synchronous population of Tetrahymena pyriformis

Cells were synchronized as described in section 2.3.(i) with an additional washing step (described in 2.3.(ii)) for the removal of ficoll before reinnoculation into fresh growth medium. Cell counting techniques were performed as described in section 2.2.(i).

10% of the synchronous cells throughout an experiment, so that culture/flask volume ratio (and hence degree of aeration) were not altered as an experiment progressed. The technique that was developed in place of Wolfe's method was intended to ensure that the cells are completely aerobic throughout an experiment, thus removal of some cells from a synchronous culture will not alter the degree of aeration of the cells that remain. Development of a method capable of yielding large volumes of synchronous cells was obviously essential.

2.4 Selection of a Synchronous Population of Tetrahymena pyriformis by a Magnetic Method

(i) Methods

Cells were grown at $28 \pm \frac{1}{2}^{o}$ C in PPYF medium in conical flasks filled to 20% total volume in an orbital shaker at a shaking rate of 180 rev/min with a maximum excursion of agitation of 3.1 cm. Iron particles were added (10 mg iron/ml of culture) to logarithmically growing cells. The cells were incubated with the iron particles (with shaking) at growth temperature for 12 minutes, by which time 90% of the cells had ingested iron. Sterile (filtered) compressed air was used to drive the culture onto a 35 x 4.5 cm column of dry acid-washed sand (either 30-90 mesh or 40-100 mesh). The column stood vertically, and was surrounded by 4 barium ferrite magnets. The magnets were arranged around the column as shown in Fig. 13 with their poles horizontal; the maximum magnetic field strength at the axis of the column was approximately 100 Gauss. The two upper magnets were placed with their poles 5.5 cm from the axis of the column, the lower two slightly nearer at 4 cm. Preliminary experiments had revealed a small proportion of the very smallest iron particles was not retained by the 4 magnets arranged round the column, so a sector-shaped magnet was placed against the column outflow pipe to catch these very fine particles. The synchronous cells were collected at growth temperature (with shaking). When sufficient volume of cells had been collected for an experiment, the synchronous culture was divided into separate portions, each culture aliquot being incubated as before. A single small culture was taken at

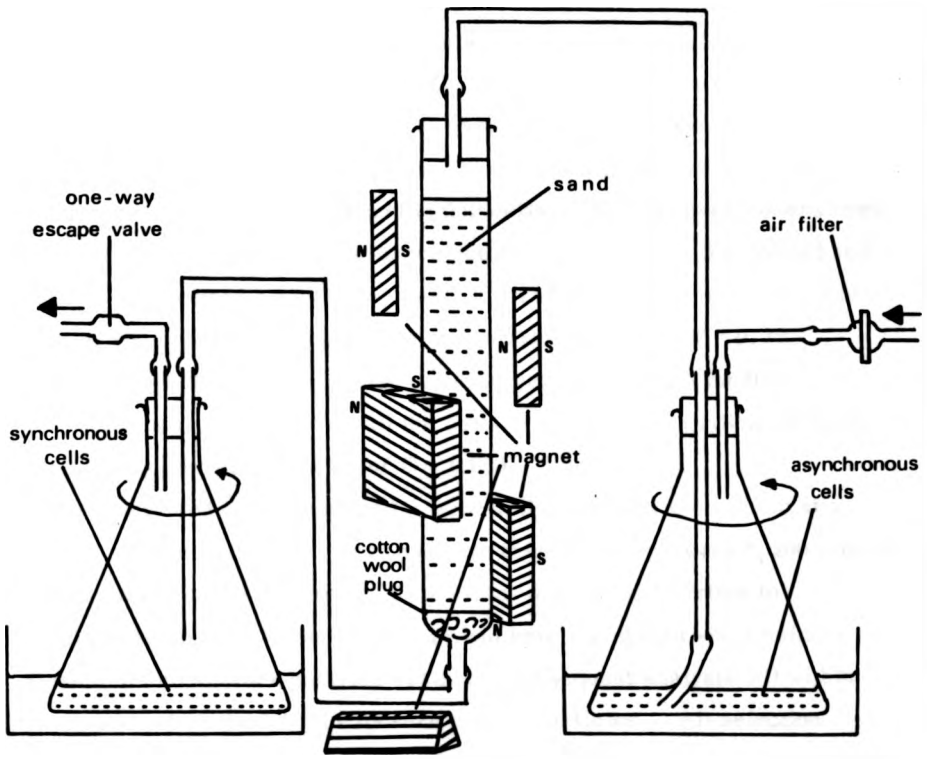

Fig. 13. The apparatus for the magnetic method of selection of a synchronous population of Tetrahymena pyriformis

The use of the apparatus is described in section 2.4.(i).

different times for the various biochemical determinations to be performed. This procedure obviates disturbing unharvested cells, ensures that the culture/flask volume ratio (and hence degree of aeration) is constant for each of the (identical) small cultures, and reduces the risk of contamination which might result from frequent sampling from one culture.

(ii) Results

Fig. 14 shows the synchronization achieved. Use of glucose-supplemented growth medium (PPYFG) appeared to produce better synchrony (Fig. 15), probably because the cells are phogocytosing more actively enabling uptake of more iron by each cell (resulting in better separation of dividing from non-dividing cells). PPYFG medium was used in all cell cycle studies of cyclic nucleotide metabolism. Blumenthal and Zahler have defined a synchrony index F*, (211). Assuming a cell cycle time of 180 minutes, it is possible to calculate a value of F = 0.77 calculated for the division period of cells grown in PPYFG medium; (perfect synchrony i.e. when all the cells divide within an infinitely short time, is equivalent to a value of F = 1.00).

Synchronized cultures of cells were collected from the column at a rate of 130-140 mls/min. It is possible therefore to process 1,200 mls of cells in less than $8\frac{1}{2}$ minutes. This meant that it was possible to synchronize up to 1,200 ml of culture and have the synchronous culture aliquotted into smaller portions ready for subsequent analysis all within 30 minutes after the initial exposure to iron particles. All selection synchrony experiments were started from a "zero time" that corresponded to 30 minutes after the original addition of iron ; this allows for easy comparisons between individual experiments. The rapidity of the technique also means that the cells are out of their normal growth environment for a very short while indeed.

A number of control experiments were performed. Passage of asynchronous cells through the column and magnetic field without prior exposure to iron particles resulted in no synchronization of growth (Fig. 16). Exposure of a logarithmically growing culture to iron particles (10 mg iron/ml

* Explained fully in Appendix.

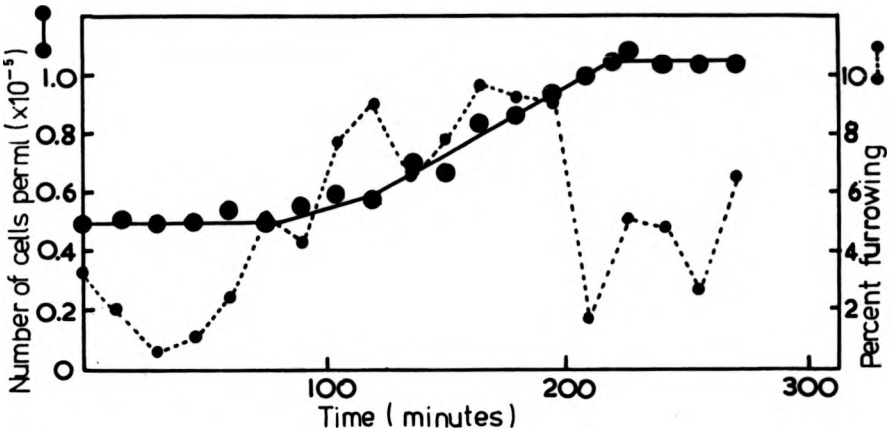

Fig. 14. Synchronization of Tetrahymena pyriformis by the magnetic method: I. Cells grown in basal PPYF growth medium.

The selection synchrony method is described in section 2.4.(i).

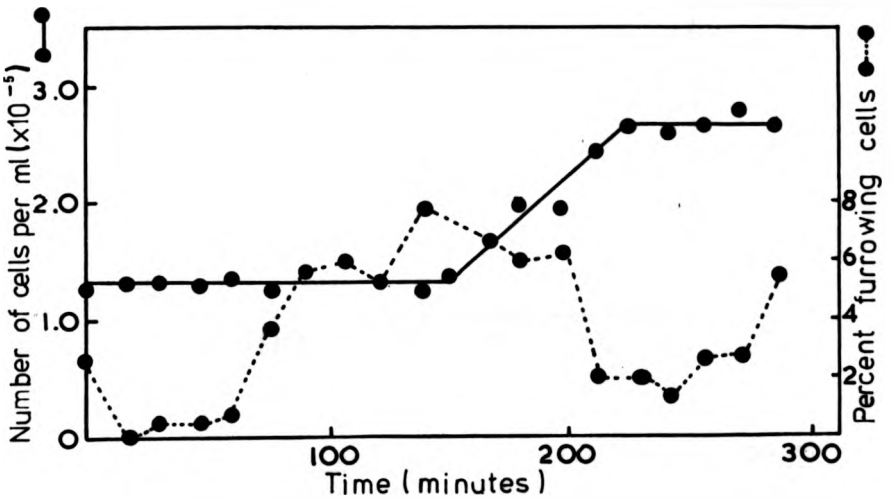

Fig. 15. Synchronization of Tetrahymena pyriformis by the magnetic method: II. Cells grown in glucose - supplemented PPYFG growth medium.

Cells were synchronized as described in section 2.4.(i).

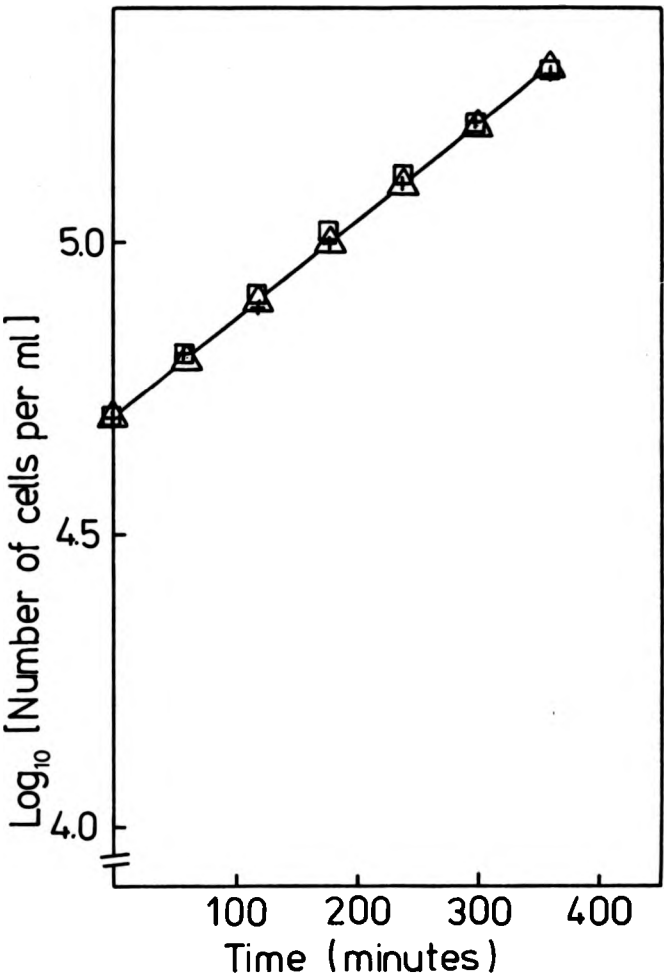

Fig. 16 Demonstration that a magnetic field and iron particles do not cause synchronization of cell division in Tetrahymena pyriformis

A culture was grown-up to a cell density of 5 x 10^4 cells/ml in glucose-supplemented growth medium (as described in section 2.4.(i)). The culture was divided into 3 portions: (i) the control culture (Δ—Δ), (ii) iron particles (10 mg iron/ml of culture) added (+—+), (iii) cells were passed through the sand column and magnetic field without exposure to iron particles (□—□).

culture) for up to 6 hours resulted in normal continuation of logarithmic growth (Fig. 16). If the sand in the column was wetted with growth medium before driving cells through, then synchronization was achieved as before, except the cell density of the synchronous culture obtained was lower than when the column was used dry (Fig. 17). The use of dry sand in the column meant that it was possible to obtain a given number of synchronous cells in a smaller volume (i.e. at greater cell density) than if the sand was pre-wetted. Sand was used dry in all subsequent experiments.

DNA and protein were measured as described in section 2.2 (iii) and 2.2 (iv) throughout the cell cycle of Tetrahymena pyriformis synchronized by the magnetic method. The results shown in Fig. 18 indicate that DNA synthesis is synchronous. This, along with the known timing of cell division, allows identification of cell cycle phase at a particular point in the experiment (1). The major portion of protein synthesis would seem to occur in G2.

(iii) Discussion

The magnetic method of selecting synchronous populations of Tetrahymena pyriformis developed appears to fulfil all the criteria outlined in section 2.3 (iii). The technique would seem to represent an advance on the original method of Hildebrandt and Duspiva (180), which, despite the relatively few experimental details given in that report would seem to be unsuitable for preparation of large quantities of synchronous cells.

2.5 Induction of Division Synchrony in Tetrahymena Pyriformis by a Single Hypoxic Shock

(i) Methods

All experiments involving this method used glucose-supplemented PPYFG medium. Cells were grown up (as described in section 2.4 (i)) as one large batch culture to a cell density of approximately 2.0×10^5 cells/ml.

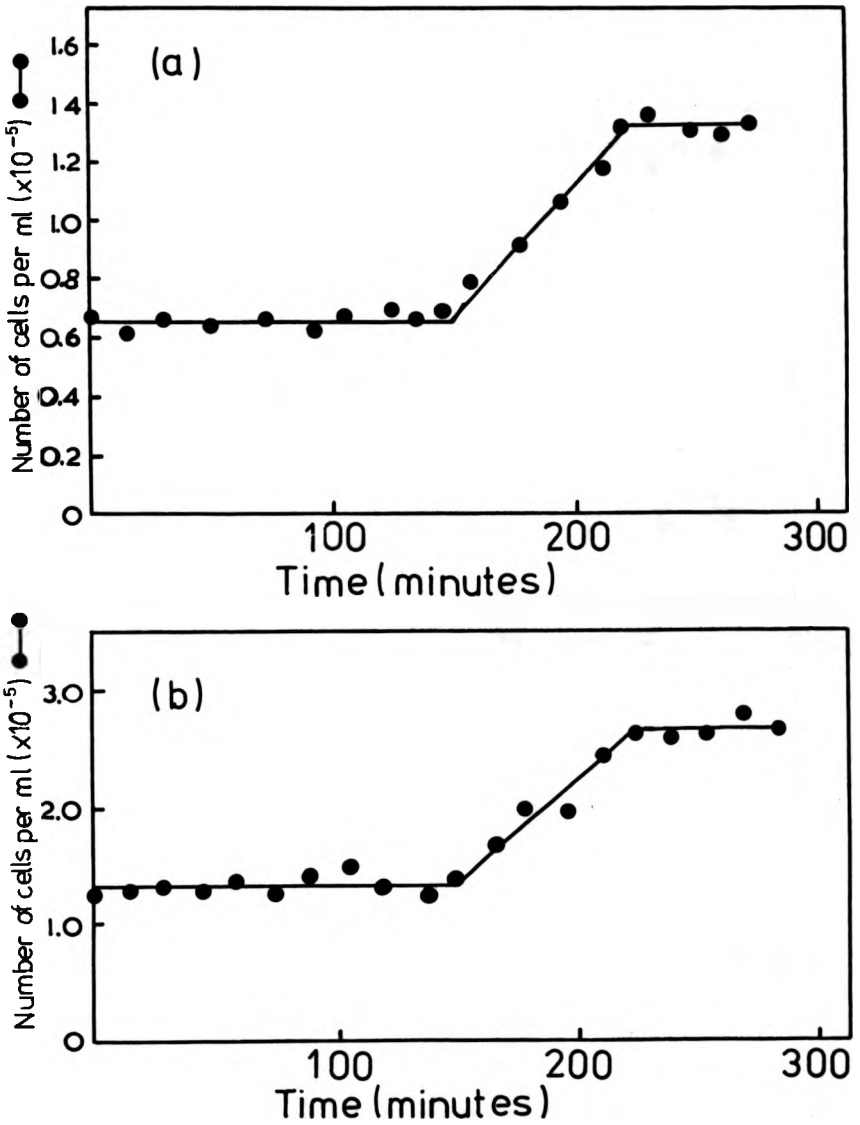

Fig. 17 Synchronization of Tetrahymena pyriformis by the magnetic selection method using dry and wet sand columns.

1600 ml of cells were grown-up to a cell density of 3.3×10^5 cells/ml in glucose-supplemented growth medium. The culture was divided into 2 equal portions and each portion was subjected to the magnetic method of selecting synchronous cells (described in section 2.4.(1)).

(a)The sand in the columns was pre-wetted with growth medium.
(b)Sand in the column used dry.

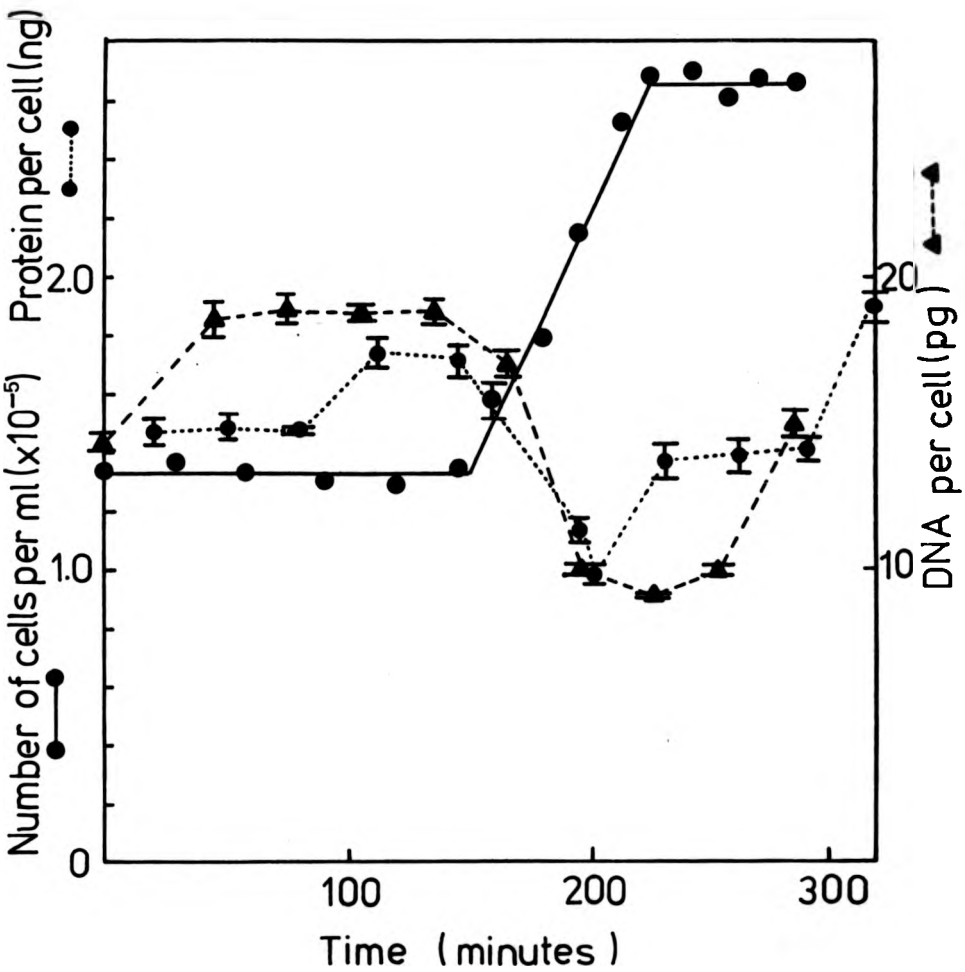

Fig. 18 Analysis of cellular protein and DNA in selection-synchronized Tetrahymena pyriformis

Cells were grown in glucose-supplemented growth medium and synchronized by the magnetic method as described in section 2.4(i). Protein and DNA were determined as described in section 2.2(iii) and 2.2(iv) respectively.

Then smaller quantities of cells were aliquotted into smaller sterile flasks (usually either 50 ml of cells into a 250 ml conical flask or 100 ml of cells into a 500 ml flask). Shaking of the small cultures was then stropped for a period of 240 minutes (cessation of shaking causing hypoxia); the growth temperature was maintained at 28 ± $\frac{1}{2}^{o}$ C throughout. After 240 minutes of hypoxia, the cultures were reshaken. A single small culture was taken at different times for the various assays to be performed. This obviates disturbing unharvested cells and ensures that the culture/flask volume ratio (and hence degree of aeration) remains constant as mentioned in section 2.4 (i). Attention to the degree of aeration is obviously of paramount importance when employing hypoxia.

(ii) Results

Fig. 19(a) shows the effect of a 240 minute hypoxic shock on cell growth. It can be seen that after 120 minutes of hypoxia the cell density has reached a plateau of 2.62×10^5 cells/ml. After commencement of reshaking there is no increase in cell density for about 105 minutes when a burst of synchronous division occurs. All the cells divide within 45 minutes. After completion of the first synchronous division, the cell density remains constant for 55-60 minutes when a second division commences.

Fig. 19(b) shows a control culture of cells (subjected to continuous shaking) continues to grow logarithmically up to a cell density of almost 1×10^6 cells/ml. This shows that the growth medium is not exhausted, and is capable of supporting growth to far greater cell densities.

The atmosphere of a 400 ml culture of cells (in a 2 l flask) at a cell density of 2.0×10^5 cells/ml was gassed with sterile (filtered) oxygen-free grade nitrogen (minimum composition 99.9% N_2) at a flow rate of 30 l/min for 240 minutes under shaking conditions. The atmosphere of the culture vessel was then flushed for 60 minutes with sterile (filtered) compressed air at a flow rate of 12 l/min (with shaking). For the remainder of the time the culture was shaken normally. Fig. 19(c)

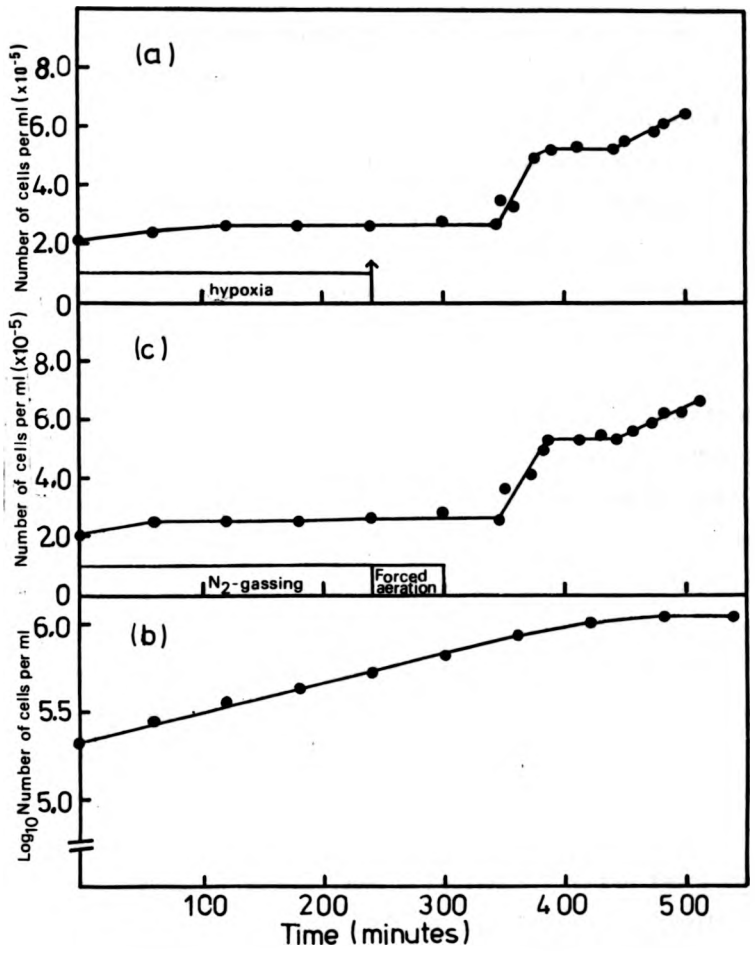

Fig. 19 The effect of a 240 minute hypoxic shock on cell growth

A 100 ml culture of cells was grown-up to a cell density of 2.13 x 10^5 cells/ml and divided into 2 equal portions. (a) Effect of a 240 min hypoxic shock on cell growth. The arrow indicates the point at which reshaking started. (b) Control culture shaken continuously. (c) Separate experiment in which atmosphere of culture vessel was flushed with nitrogen for 240 minutes, followed by 60 minutes of compressed air; culture was shaken throughout.

shows that synchronization of cell division occurred in a similar fashion to that achieved by the non-shaking/reshaking routine.

Using a sterile oxygen electrode, the level of oxygen fell below 0.025 μmol oxygen/ml in the bulk of a culture of cell density 2×10^5 cells/ml 15 minutes after shaking had stopped (212). Warburg manometry indicated that oxygen utilization by cells grown with shaking was 5.33 μl oxygen utilized/10^6 cells/min (212). An experimentally determined rate of diffusion of oxygen from air into still or slowly moving water is of the order of 0.04 - 0.09 μl/cm^2/min at 25° C and zero oxygen tension (213). A 50 ml culture in a 250 ml conical flask has a surface area of 57.15 cm^2. Using the value of oxygen diffusion from air into water of 0.09 μl of oxygen/cm^2/min, this could allow 5.14 μl oxygen to diffuse into the growth medium per minute. Oxygen utilization by the 50 ml culture (at a cell density of 2×10^5 cells/ml) would be 53.3 μl O_2/min. From consideration of these figures it can be seen that when shaking is stopped the cultures rapidly become hypoxic. Furthermore, the oxygen electrode measurements and the control experiment involving gassing of the shaken culture with nitrogen suggest most strongly that hypoxia is the cause of cell cycle blockade.

DNA was measured (as described in section 2.2 (iv) in Tetrahymena pyriformis subjected to this synchronizing technique. The results presented in Fig. 20 show that cellular DNA reaches a plateau 194 minutes after the start of hypoxia. Between this point and the onset of the first synchronous division, there is no increase in cellular DNA. After completion of the first division the DNA content of the cells is halved. In other words, there is no synthesis of DNA between release from hypoxia and cell division. This indicates the cells are trapped somewhere in G2. After the first synchronous division and halving of cellular DNA, the DNA content quickly doubles before the second division. This suggests that G1 is either absent or extremely short. The curve of DNA per ml suggests that DNA synthesis is synchronized by the hypoxic shock. It is possible that this is the primary step in induction of synchrony.

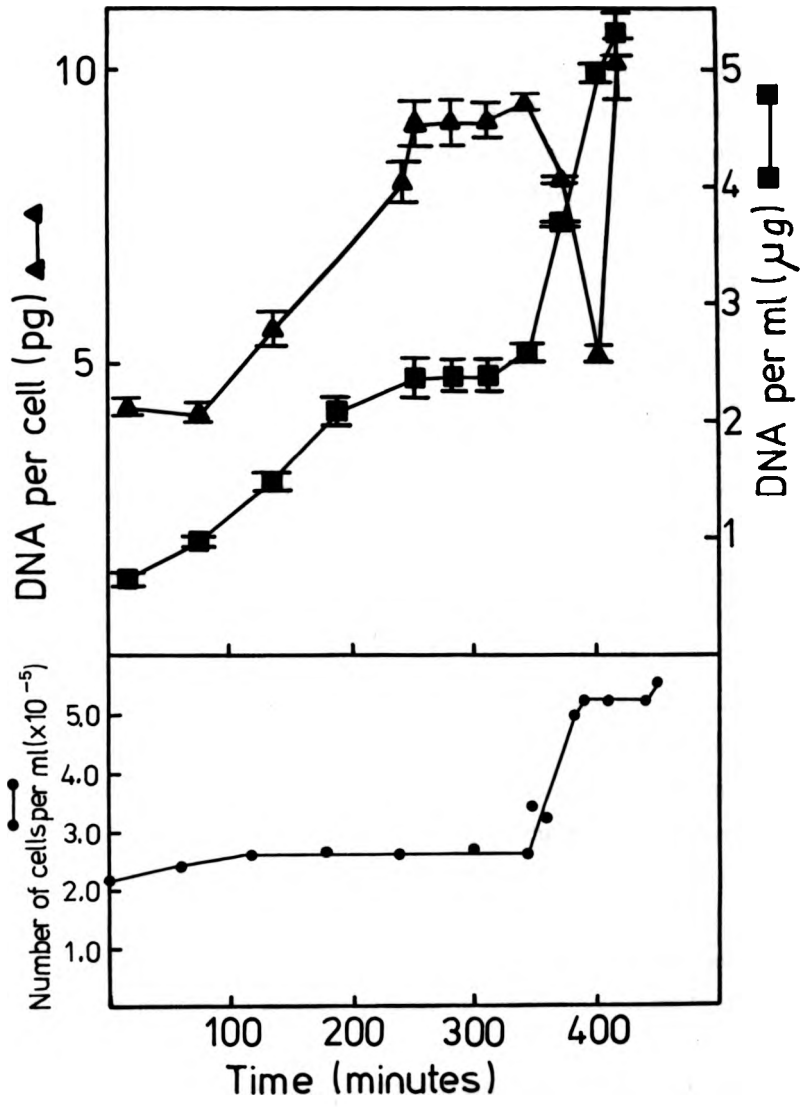

Fig. 20 Characterisation of the point in the cell cycle blocked by a 240 minute hypoxic shock

Cells were synchronized by a single hypoxic shock as described in section 2.5(i). DNA determinations were performed as described in section 2.2(iv).

(iii) Discussion

It is possible to induce division synchrony in Tetrahymena pyriformis by means of a single hypoxic shock. This is achieved by simply growing-up the organisms with rapid shaking in an orbital shaker to a cell density of about 2.0×10^5 cells/ml, not shaking the cells for a period of 240 minutes, and then reshaking. The idea of using anoxia or hypoxia to induce cell synchrony is not new, but this particular method using only a single shock is. Previously Rasmussen has described the use of two 50 minute anoxic shocks separated by a 40 minute growth period (172). Rooney and Eiler have used an even more complicated system of six 45 minute hypoxic shocks with 45 minutes between shocks (173). Later the same workers developed this into an automated technique using seven 45 minute shocks with 45 minutes between shocks (174). This method has as its advantage the fact that gassing of the cells and special apparatus are not required; it is thus far simpler and easier to perform.

The use of synchrony indices needs careful consideration when applied to induction-synchronized cells; such quantities are most useful when studying the natural cell cycle in selection-synchronized cells. With induction synchrony the timing of the first synchronous division that occurs after removal of the blocking process is frequently different from that in the natural cell cycle. However, bearing this in mind, it is possible to calculate a value of $F = 0.81$ for the period of the first synchronous division (assuming a normal cell cycle time of 180 minutes).*

During hypoxia the cells appear to accumulate at a point in the G2 phase of the cell cycle. After the first synchronous division, the subsequent G1 seems to be either extremely short or absent altogether. This observation suggests a strong similarity between the single hypoxic

* The quantity F is the synchrony index of Blumenthal and Zahler (211) which is discussed more fully in the Appendix.

shock technique described here and the classical multiple heat shock technique. If Tetrahymena pyriformis is synchronized by seven heat shocks the G1 phase that follows the first synchronous division is reported to be extremely short or totally absent (214, 215). Another similarity would also seem to exist between the two systems in that it is reported that heat-synchronized Tetrahymena pyriformis can divide synchronously in an inorganic medium (216). If cells are transferred after 240 minutes hypoxia to the non-nutrient medium used by Cameron and Jeter (171), then upon reshaking the cells undergo the first synchronous division (212).

It is to be hoped that the single hypoxic shock technique gains widespread use in other laboratories by virtue of its simplicity. The following chapters of this report describe use of the method in conjunction with studies on the natural cell cycle as a means of dissecting mechanisms of control by cyclic nucleotides.

CHAPTER 3

CYCLIC AMP METABOLISM

3.1. Introduction

The direct measurement of cyclic AMP in synchronous cultures of Tetrahymena pyriformis was clearly an early priority in investigation of the role of cyclic nucleotides in the regulation of the cell cycle of this organism. When faced with performing cyclic AMP assays, the experimenter is faced with a number of choices. A radioimmunoassay for cyclic AMP represents the most convenient method because use of a specific antibody enables measurement of small concentrations of the nucleotide in unpurified samples. Unfortunately preparation of antibody can take quite a long time, and even if commercially prepared antiserum is used, the "shelf life" of the commodity is quite short and the cost is great. Binding protein assays have the advantage that preparation of a cyclic AMP binding protein (the regulatory subunit of a cyclic AMP-dependent protein kinase being the functional protein) is simple and cheap. The "shelf life" of such proteins is in the order of several years (stored at -20^{o} C), so preparation of one batch of binding protein will suffice for many experiments. The major drawback with binding protein assays is that the sample to be assayed for cyclic AMP must be free from many other cellular constituents which can "interfere" with the assay to give spurious results. In other words, purification of the sample is required prior to the assaying procedure.

The saturation binding assay of Brown et al. (217) was chosen for the reasons of cost and "shelf life" of the prepared binding protein. Earlier work by myself (218) using this assay had revealed that although vast excesses of ATP or Mg^{2+} present separately in the assay did not give spurious values for cyclic AMP content, ATP in the presence of Mg^{2+} did. The batch procedure described by Dinnendahl (219) for removal of interfering nucleotides was to have been used. This simply involved whirlimixing of deproteinized cell extract in 200 mM Tris-HCl, pH 7.6, in the presence of one-third volume of neutral alumina. Low-speed

centrifugation enables recovery of the aqueous phase from the alumina. However, I was not able to reproduce this method, and so resorted to the use of columns of neutral alumina according to Ramachandran (220).

Binding protein assays are less sensitive than radioimmune assays so that it is often necessary to concentrate-up the sample before assaying for cyclic AMP. The presence of buffer ions concentrated-up with the cyclic AMP can give rise to spurious results without the use of adequate control procedures. After alumina column chromatography, freeze-drying of material and subsequent resuspension in a smaller volume proved to be necessary to concentrate-up the cyclic AMP prior to assaying. In order to circumvent non-specific effects on the cyclic AMP binding protein due to the concentration of buffer ions, various precautions were taken as outlined by Brown et al. (221) for use with their assay. The development and use of these methods is now described in detail.

3.2 Materials and Methods

(i) Chemicals

All nucleosides, nucleotides, bovine serum albumin and azo albumin were purchased from Sigma Chemical Compnay, Ltd., Kingston upon Thames, U.K. Cycloheximide was from B.D.H. Chemicals, Ltd., Poole, U.K. Neutral alumina, activity grade 1, was obtained from I.C.N. Pharmaceuticals, Eschwege, West Germany. Charcoal ('Norit GSX') was from Hopkin and Williams, Ltd., Chadwell Heath, U.K. [8-^{3}H] cyclic AMP (27.5 Ci/mmole) and [8-^{3}H] cyclic GMP (21 Ci/mmole) were purchased from the Radiochemical Centre, Amersham, U.K. All other chemicals used were of the highest purity commercially available.

Radiochemicals were stored lyophilized and dessicated after being aliquotted into small glass vials. Each vial contained sufficient of the radiochemical for one day's work. This procedure was adopted to reduce the amount of ^{3}H that can exchange with water when [8-^{3}H] cyclic AMP and [8-^{3}H] cyclic GMP are stored in aqueous conditions. The amount of radioactivity present in "blanks" was thereby reduced.

(ii) Enzymes

Beef heart cyclic AMP phosphodiesterase was obtained from Boehringer Corporation (London) Ltd., Lewes, U.K. Bovine adrenal glands (for preparation of cyclic AMP binding protein) were obtained from Coventry Abbatoir, Coventry, U.K.

(iii) Use of columns of neutral alumina for purification of cyclic AMP

14 cm Pasteur pipettes were plugged with glass wool and used as columns. Neutral alumina was packed whilst dry into each column to a height of 6 cm. Columns of alumina were washed before use with a large excess of buffer A (10 mM Tris-HCl, pH 7.4), and then allowed to drain so that effluent had just ceased to flow from the bottom of the column. Then 250 µl of material (either pure bona fide cyclic AMP, cellular extracts or mixtures of nucleotides) was applied to the column and allowed to soak in. Elution was then effected with buffer A. Characterization of elution was achieved either by taking 1 ml fractions and recording the optical density at 260 nm of each fraction in a spectrophotometer, or by applying radioactively-labelled nucleotide to the column, collecting 0.5 ml fractions and then estimating the radioactivity present in each sample by liquid scintillation counting using a Packard 2425 tri-carb liquid scintillation counter. Sample presentation for scintillation counting involved taking 0.1 ml portions of each fraction and mixing with 4 ml of scintillation "cocktail". (1 l of "cocktail" comprised 4 gm PPO, 0.2 gm POPOP, 400 ml 2-ethoxyethanol and 600 ml toluene). All radioactivity determinations were performed in duplicate.

Fig. 21 shows the elution profiles of cyclic AMP, adenosine, cyclic GMP and guanosine. ATP, ADP, AMP, GTP, GDP and GMP were retained by the column under the conditions used. Cyclic AMP eluted from the column earlier than cyclic GMP. Adenosine seemed to be slightly retarded compared to cyclic AMP, and guanosine seemed to elute from the column fractionally later than cyclic GMP. Separation is effected because polyvalent anions are adsorbed by hydrous alumina.

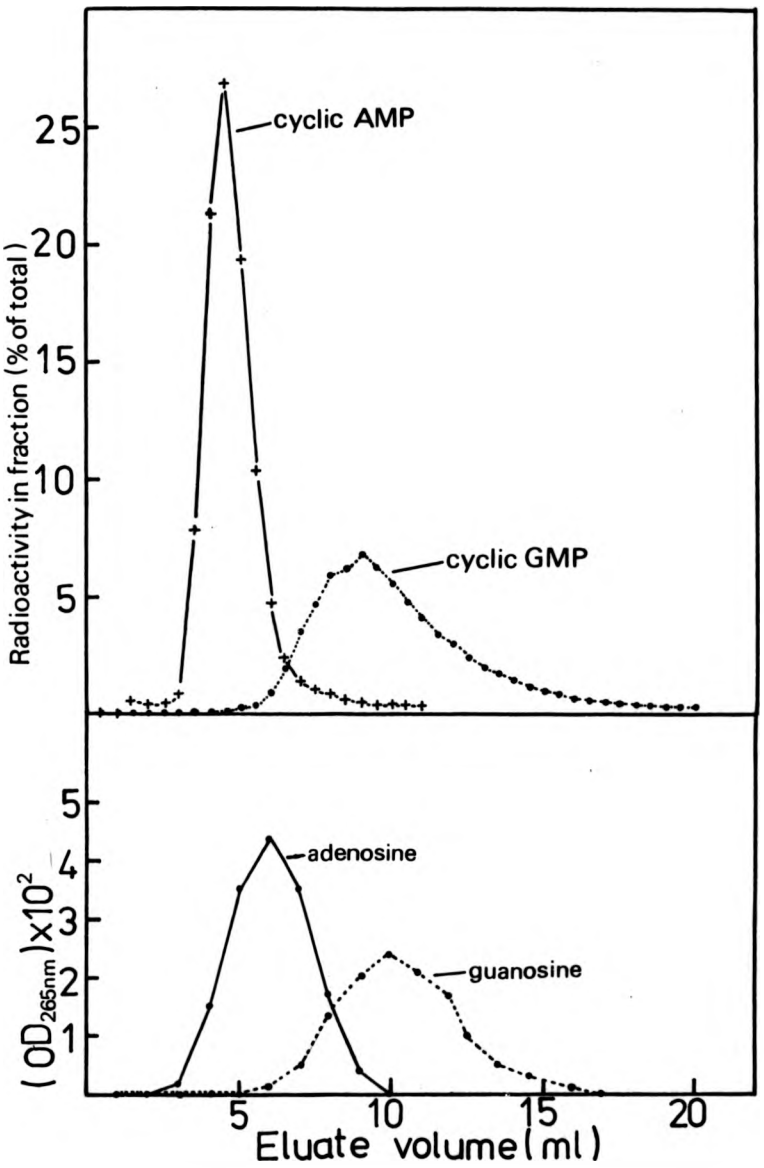

Fig. 21 The elution of nucleosides and cyclic nucleotides from columns of neutral alumina

Columns were eluted with 10 mM Tris-HCl buffer, pH 7.4 (as described in section 3.2(iii)). ATP, ADP, AMP, GTP GDP and GMP were all retained by the columns.

At physiological pH, ATP, ADP, AMP, GTP, GDP, GMP and Pi are all multivalent anions whereas cyclic AMP, cyclic GMP, adenosine and guanosine are monovalent anions. "Characterization" of the columns (by performing many elution profile studies) enables subsequent use of the columns on a batch basis. By collecting the requisite portion of column eluate, cyclic AMP or cyclic GMP can be obtained sufficiently purified for subsequent assay procedures. The presence of some adenosine in samples to be assayed for cyclic AMP is not a problem because the cyclic AMP assay system (detection range 0.05-15 p.mol cyclic AMP) can be used with up to 30 n mol adenosine present per assay tube without giving antefactual results (217). The presence of guanosine in samples to be assayed for cyclic GMP was also permissible as will be discussed in section 4.2(iv).

To purify samples for cyclic AMP determinations, the first 2 ml of column eluate were discarded and the next 7 ml were collected. For cyclic GMP determinations, the first 5.5 ml of eluate were discarded and the next 12 ml were collected. This procedure allows recovery of 99.0 ± 0.2% of all of the cyclic AMP and 72.7 ± 1.3% of all the cyclic GMP applied to the column. The cyclic AMP fraction from neutral alumina chromatography contains 30.3 ± 0.8% of the cyclic GMP fraction. The cyclic GMP fraction contains 22.0 ± 0.1% of the cyclic AMP fraction. This does not cause problems in the assay of cyclic AMP (justified in section 3.2(v) below) nor in the assay of cyclic GMP (see section 4.2(iv)). The rationale for using the alumina columns was <u>not</u> to try and separate cyclic AMP and cyclic GMP from each other prior to assay, but to remove the many other cellular constituents which could give rise to artefacts when assaying the cyclic nucleotides. It was merely fortuitous that neutral alumina column chromatography achieved considerable separation of the two cyclic nucleotides.

(iv) <u>Extraction of cyclic AMP from cells</u>

Cells were harvested at 2000 g for 5 min at growth temperature. Growth medium was carefully removed and the pellet of cells resuspended in 2 ml of ice-cold distilled water. The suspension of cells was transferred

to a sonication vial, and 2 ml of ice-cold 20% trichloroacetic acid was added. Sonication was performed at 0^{o} C using a MSE 150 W sonicator at maximum power and amplitude for 1 min, by which time cell disruption was judged to be complete. After standing for at least 1 hour, denatured cellular material was removed by centrifugation. The supernatant was then washed five times with two volumes of water-saturated diethyl ether to remove the trichloroacetic acid. After freeze-drying, the material was resuspended in 10 mM Tris-HCl, pH 7.4, and cyclic AMP separated by chromatography on columns of neutral alumina as described in 3.2(iii) above.

(v) The assay of cyclic AMP

Cyclic AMP binding protein for use in the assay was prepared as described by Brown et al. (217) and stored at -20^{o} C in 1 ml aliquots. A fresh 1 ml portion of binding protein was taken for each experiment, thawed-out and diluted appropriately. (A preliminary experiment is performed for each batch of binding protein that has been prepared to find the dilution that is required in accordance with the theoretical principles of saturation analysis (222). The experiments described here all used one batch of binding protein requiring a 1:12 dilution in buffer B (50 mM Tris-HCl, pH 7.4, 8 mM theophylline and 6 mM 2-mercaptoethanol) before use of the binding protein in the assay.

The assay comprised 100 µl of diluted binding protein, 50 µl [8-^{3}H] cyclic AMP (60 nM, 27.5 Ci/mmole) in buffer B, 100 µl of buffer B and 50 µl of "unknown" or a standard amount of cyclic AMP in the same buffer. All assay components were precooled to 0^{o} C before being added to precooled tubes. Incubation was performed for 90 minutes at 0^{o} C, after which 100 µl of a suspension of charcoal (Norit GSX) containing 2% (w/v) bovine serum albumin in buffer B was added, whirlimixed, and immediately centrifuged at 2,000 g for 3 min. After centrifugation a 100 µl portion of supernatant was removed and added to a scintillation vial containing 4 ml of scintillation "cocktail". (1 l of "cocktail" comprised 4 gm PPO, 0.2 gm POPOP, 400 ml 2-ethoxyethanol and 600 ml toluene). Radiocativity was then estimated using a Packard 2425 tri-carb liquid scintillation spectrometer. All incubations were performed in duplicate.

Each assay tube contains cyclic AMP-binding protein at a final concentration such that only about 30% of the [^{3}H] cyclic AMP could be bound. Addition of unlabelled cyclic AMP (in standards or unknown samples) competes-out the [^{3}H] cyclic AMP. Equilibrium is achieved in the 90 minute period of the incubation. Addition of charcoal effects removal of unbound nucleotide. The cyclic AMP binding protein is soluble, so centrifugation enables separation of bound and unbound [^{3}H] cyclic AMP. A standard curve is constructed as a plot of radioactivity bound vs known amounts of unlabelled cyclic AMP. Each experiment is performed with two essential controls. A "no binding protein" control (in which 100 µl of buffer B is added instead of binding protein) checks the efficiency of the charcoal and allows a correction to be made for the amount of radioactivity that is adventitiously carried through to the scintillation vial. A "no charcoal" control (100 µl of buffer B is added at the end of the incubation period instead of charcoal suspension) gives a figure for the 100% tracer count. Standard curves were constructed expressing radioactivity bound (corrected for the "no binding protein" control) as a percentage of the "no charcoal" control value. Radioactivity present in "no binding protein" controls was 2.72 ± 0.09% of total ("no charcoal" control) values (in ten separate experiments). The mean deviation of each duplicate assay from its mean was 3.0% calculated from 50 duplicate assays selected at random.

A typical experiment might involve collection of the 7 ml of cyclic AMP fraction from a column of neutral alumina, freeze-drying and resuspension of the sample in 350 µl of buffer B prior to assaying. Thus, the buffer A used for elution on the alumina column has been concentrated-up along with the material for assay by a factor of 20. Fig. 22 shows a cyclic AMP standard curve constructed with and without 50 µl of buffer A concentrated 20 times by this procedure. The presence of 50 µl of 20-times concentrated buffer A causes more [^{3}H] cyclic AMP to be bound than in a normal standard curve. The presence of such concentrated buffer ions (in samples extracted from cells for cyclic AMP determinations) could give rise to artefacts in that it would appear that less cyclic AMP was present in a particular sample than there actually was.

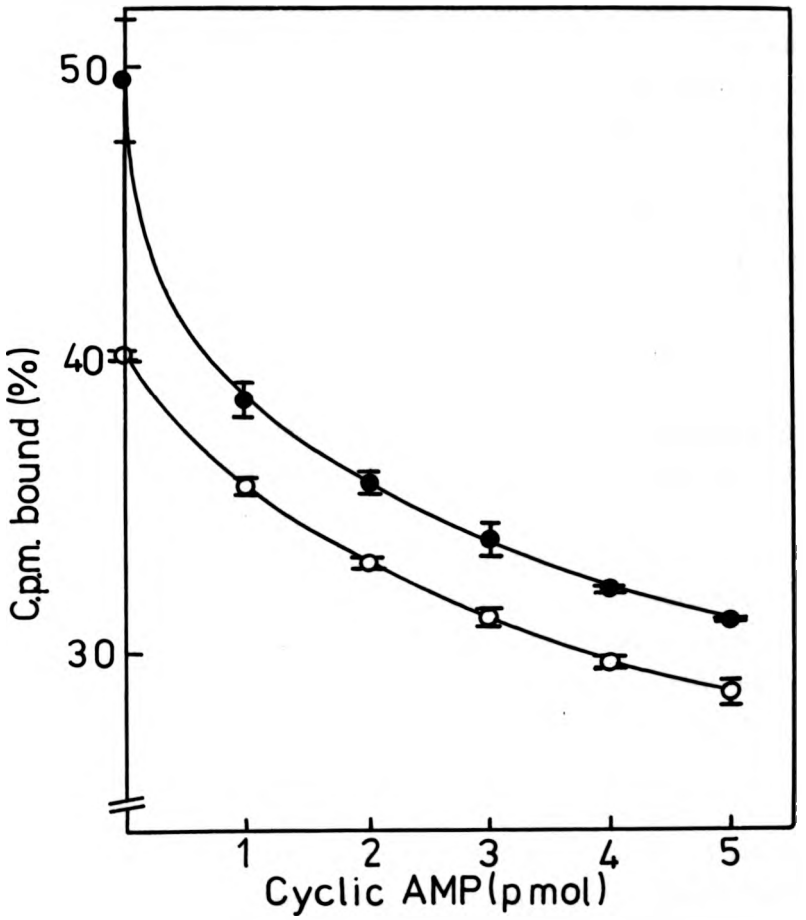

Fig. 22 The effect of concentrated buffer ions on cyclic AMP assays

A cyclic AMP standard curve was constructed with (●—●) and without (O—O) 50 µl of concentrated buffer. Cyclic AMP assays were performed as described in section 3.2(v). Buffer A (10 mM Tris-HCl, pH 7.4) used for elution of neutral alumina columns was concentrated-up 20-fold by freeze-drying and resuspension in a smaller volume, in a similar fashion to the treatment of samples that are to be assayed for cyclic AMP.

In order to avoid such artefacts all cyclic AMP standard curves were constructed so that there was an equivalent amount of concentrated buffer A present in both "unknowns" and cyclic AMP standards. In all other cyclic AMP determinations which involved carry-through of a buffer which was different from the cyclic AMP assay buffer B, standard curves were constructed so that there was an equivalent amount of the different buffer present in both "unknowns" and cyclic AMP standards.

The presence of relatively small amounts of cyclic GMP in samples to be assayed for cyclic AMP content did not seem to be a serious problem. Fig. 23 shows a cyclic AMP standard curve constructed with and without 200 p mol cyclic GMP present. It can be seen that the presence of a minimum 40-fold excess of cyclic GMP produced no cross reaction, the two standard curves were identical within the limits of error of the assay technique.

Recovery of cyclic AMP extracted from cells was monitored by performing cyclic AMP extraction and purification (as described in sections 3.2(iv) and 3.2(iii) respectively) in the presence of known amounts (1.0×10^5 - 1.6×10^5 c.p.m.) of [8-^{3}H] cyclic AMP. Recovery of [8-^{3}H] cyclic AMP in six separate experiments was 71.6 ± 1.5%.

Putative cyclic AMP was extracted from cells, purified on columns of neutral alumina, divided into 2 portions and each aliquot lyophilized. A cyclic AMP determination was performed on one aliquot. A solution of bona fide cyclic AMP was made up in buffer C, (0.1 M glycyl glycine buffer, pH 7.5 containing 0.15 mM $MgSO_4$) and an equal amount of concentrated column elution buffer to a concentration equal to that of the second aliquot of putative cyclic AMP which was also dissolved in buffer C. The two solutions were incubated at 25° C in the presence of beef heart cyclic AMP phosphodiesterase. Samples of the assay mix were removed at intervals, boiled for 3 minutes to terminate the phosphodiesterase reaction, then plunged into ice. Cyclic AMP determinations were performed on all samples. Control incubations comprised putative (or bona fide) cyclic AMP in the presence of buffer C only. Fig. 24

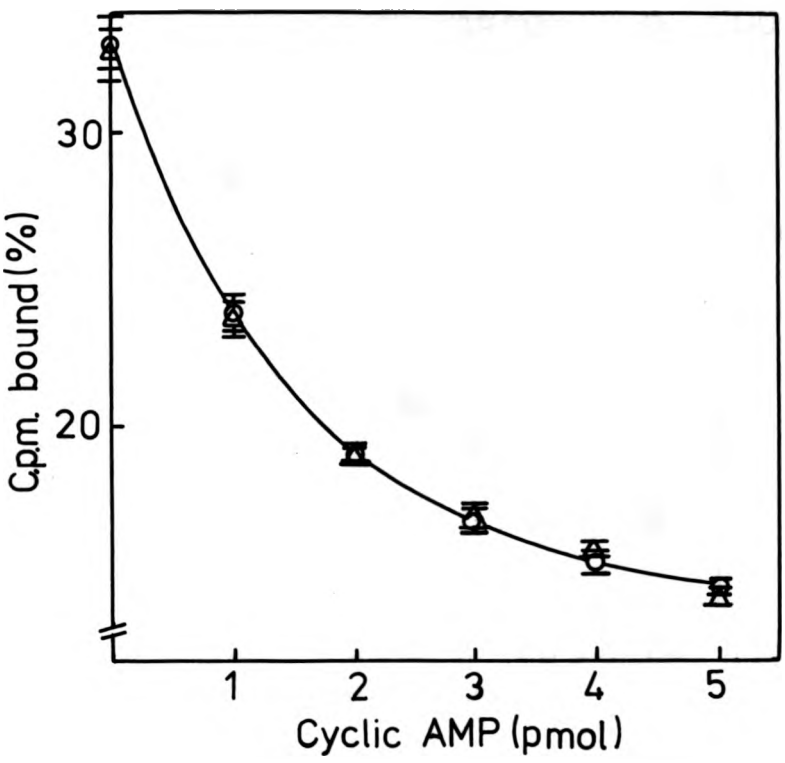

Fig. 23 The effect of 200 p mol of cyclic GMP per assay tube on cyclic AMP assays

A cyclic AMP standard curve was constructed with (Δ—Δ) and without (O—O), 200 p mol cyclic GMP per assay tube. Cyclic AMP was assayed as described in section 3.2(v).

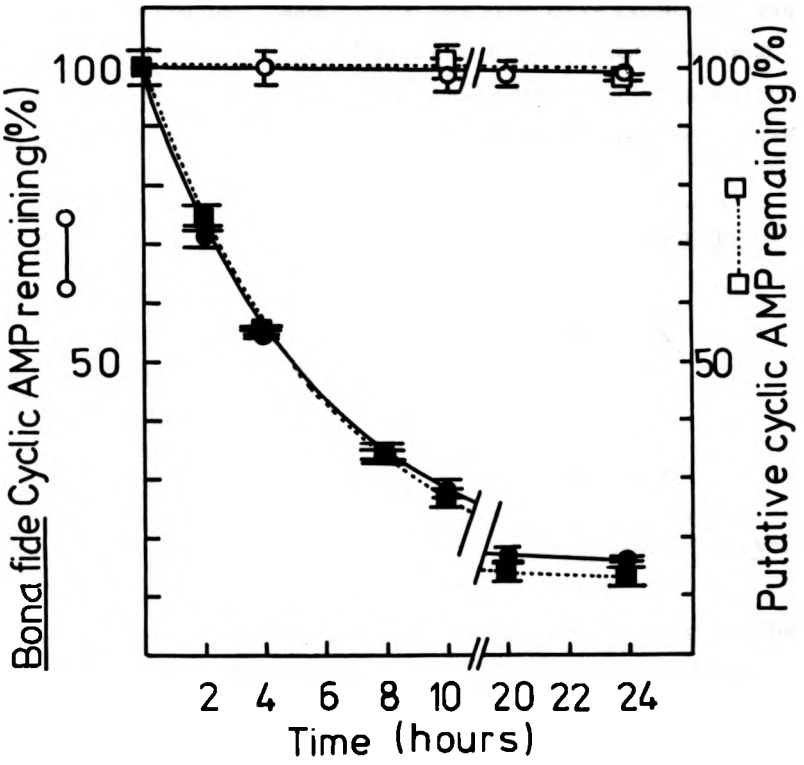

Fig. 24 The destruction of putative cyclic AMP and bona fide cyclic AMP by beef heart cyclic AMP phosphodiesterase

Putative cyclic AMP was extracted from cells and purified on columns of neutral alumina as described in section 3.2. This material was incubated with beef heart cyclic AMP phosphodiesterase (■·····■).

A solution of bona fide cyclic AMP was made up (to the same concentration as the putative cyclic AMP), and was incubated in the presence of cyclic AMP phosphodiesterase (●——●).

Open symbols denote no enzyme controls.

shows that putative cyclic AMP and bona fide cyclic AMP were destroyed in a similar fashion. Material that was assayed as cyclic AMP was further verified to be cyclic AMP by addition of defined amounts of bona fide cyclic AMP to "unknowns" and by dilution of "unknowns"; both tests resulted in the values expected from the calibration curve (see Table 3 below).

Sample	Cyclic AMP content (p mol)
50 µl of "unknown"	2.08 ± 0.06
50 µl of 1/10 diluted "unknown"	0.21 ± 0.00
50 µl of "unknown" plus 2 p mol bona fide cyclic AMP	4.09 ± 0.12
50 µl of 1/10-diluted "unknown" plus 2 p mol bona fide cyclic AMP	2.21 ± 0.06

Table 3 Further verification that material that is assayed as cyclic AMP is cyclic AMP.

(See also Fig. 24). Cyclic AMP was extracted from cells, purified and assayed as described in section 3.2.

(vi) Enzyme assays

All enzyme assays were performed using a similar method for preparation of enzymes. Cells were harvested at 2,000 g for 5 min. at growth temperature, washed in the appropriate assay buffer, then resuspended to 1 ml in ice-cold assay buffer, cooled to 0° C and sonicated in a MSE 150 W sonicator at maximum power and amplitude for 15 sec. These cell homogenates were then used immediately (either neat or diluted as appropriate) for enzyme assays.

(a) Assay of cyclic AMP phosphodiesterase.

Two assays systems were used in the measurement of cyclic AMP phosphodiesterase; both are based on the methods of Butcher and Sutherland (223). The first consisted of measuring the release of inorganic phosphate with the use of an excess of 5'-nucleotidase that is present in the venom of Ophiophagus hannah. The reaction mixture contained 0.36 µmol cyclic AMP in 0.8 ml of buffer D (40 mM Tris-HCl

pH 7.5 containing 2 mM $MgSO_4$). The assay was initiated by addition of 0.1 ml of homogenate diluted as appropriate with buffer D. Incubation was at 30° C for 30 min. After the first 20 min of the incubation, 0.1 ml of a solution of Ophiophagus hannah venom in buffer D was added, containing 0.1 mg of venom. The reaction was terminated by the addition of 0.1 ml of ice-cold 55% trichloroacetic acid. After addition of trichloroacetic acid, the precipitate was removed by centrifugation, and aliquots of the supernatant analyzed for inorganic phosphate in a 1 ml assay, according to the method of Fiske and Subba Row (224). Conditions of the cyclic AMP phosphodiesterase assay was organized such than no more than 10% of the substrate was utilized during the course of the incubation. The quantity of snake venom used was capable of hydrolysing all of the 5' AMP produced in the reaction. Fig. 25 shows that the cyclic AMP phosphodiesterase reaction was linear for at least 30 minutes. (Investigation of the time course of reaction required a modified method with respect to addition of venom and termination of the reaction . The cyclic AMP phosphodiesterase reaction was terminated after a specified time by boiling for 3 minutes. Tubes were then cooled in ice and subsequently allowed to equilibrate to 30° C before the addition of venom. The venom was allowed to hydrolyze the 5' AMP for 10 minutes after which time 0.1 ml of ice-cold 55% trichloroacetic acid was added. Inorganic phosphate was then determined as before). Assays were performed in duplicate; the mean deviation of each duplicate assay from its mean was 4.4% calculated from 60 assays.

The second assay was based on the measurement of cyclic AMP before and after incubation with cyclic AMP phosphodiesterase preparations. This assay was used when low (10^{-8} - 10^{-5} M) concentrations of substrate were employed. The reaction was initiated by addition of 0.1 ml of cell sonicate diluted as appropriate with buffer D to 0.9 ml of substrate in buffer D. Incubation was at 30° C for 30 minutes. The reaction was terminated by boiling for 3 minutes, followed by plunging into ice. Cyclic AMP disappearance was estimated by performing cyclic AMP

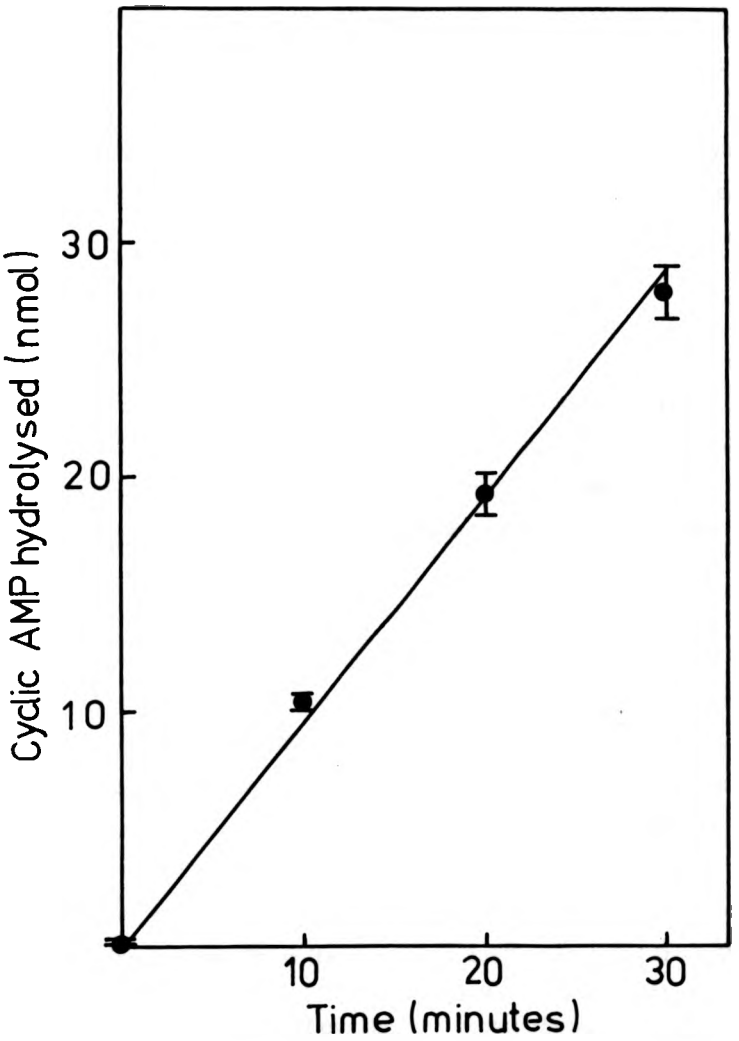

Fig. 25 The time course of cyclic AMP phosphodiesterase, assay 1. Cell homogenate was prepared as described in section 3.2(vi). Cyclic AMP phosphodiesterase activity was measured by monitoring the production of inorganic phosphate in the coupled assay using an excess of 5'-nucleotidase present in the venom of Ophiophagus hannah. Precise assay details are described in section 3.2(vi)(a).

determinations (as described in section 3.2(v)) on samples which had been incubated without and with enzyme, and by subtracting the difference. Again, assay conditions were set up such that no more than 10% of the substrate was utilized during the course of the cyclic AMP phosphodiesterase incubation. Fig. 26 shows that the reaction was linear for at least 30 minutes. Assays were performed in duplicate; the mean deviation of each duplicate assay from its mean was 5.3% calculated from 20 assays.

(b) Neutral protease

The assay used is a modification of that described by Levy et al. (225). The reaction mixture contained 4 mg of azoalbumin in 0.65 ml of buffer E (100 mM Tris-HCl, pH 8.0, containing 1.33 mM 2-mercaptoethanol, 1.33 mM EDTA and 0.08% (v/v) Triton X-100). The assay was initiated by addition of 0.1 ml of homogenate (diluted appropriately in buffer E). Incubation was at 30° C for 60 min. The reaction was terminated by addition of 0.1 ml of ice-cold 55% trichloroacetic acid. After addition of 55% trichloroacetic acid, the precipitate was removed by centrifugation. The supernatant was carefully removed and adjusted to pH 9.0 by the addition of 150 µl of 2M NaOH. The optical density of the fluid was then determined at 520 nm. Activity of the enzyme towards this chromogenic substrate is expressed in terms of O.D. units/hr/mg protein (or O.D. units/hr/10^6 cells) relative to a "no enzyme" control. Assays were performed in duplicate; the mean deviation of each duplicate assay from its mean was 6.9% calculated from 20 assays.

3.3 Some aspects of cyclic AMP metabolism in logarithmically growing Tetrahymena Pyriformis

(i) Cyclic AMP assays on cells grown in basal and glucose-supplemented growth medium

Cyclic AMP was extracted, purified and assayed (as described in section 3.2) in logarithmically growing cells grown in PPYF and 0.5% glucose-supplemented PPYFG growth medium. Table 4 shows that cells grown in the glucose-enriched medium contained only 26.4% of the cyclic

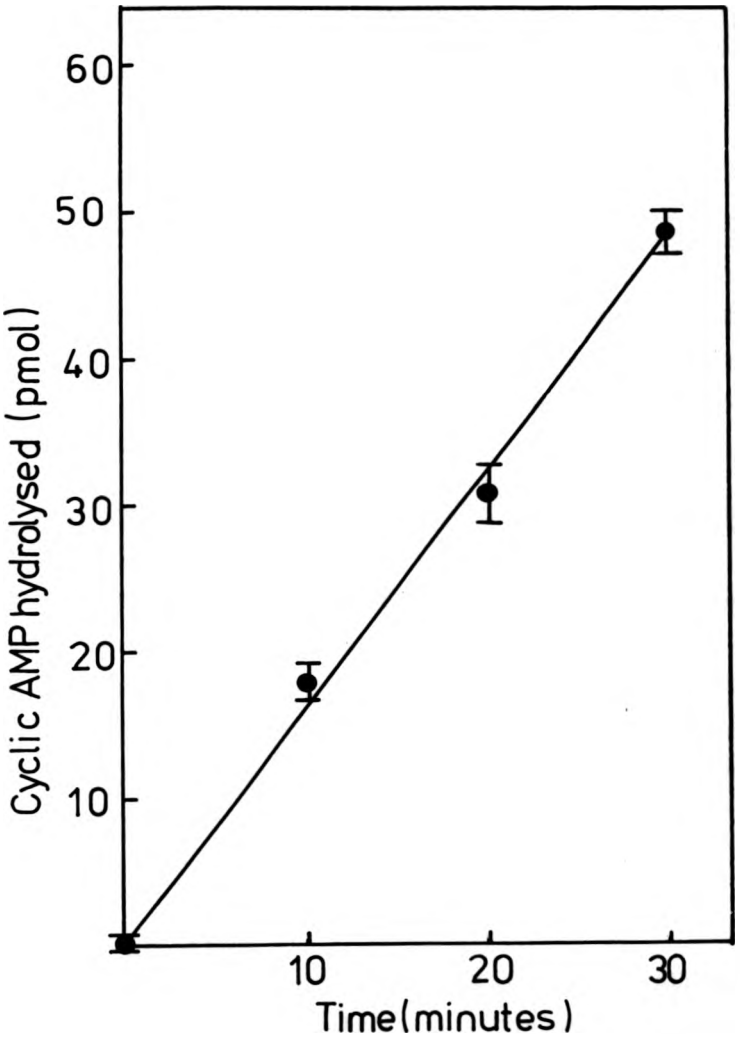

Fig. 26 The time course of cyclic AMP phosphodiesterase, assay 2
Cell sonicate was prepared as described in section 3.2(vi) and cyclic AMP phosphodiesterase was assayed at a substrate concentration of 5×10^{-7} M by measuring the disappearance of cyclic AMP as described in section 3.2 (vi)(a).

AMP to be found in cells grown in basal growth medium.

Growth medium	Number of cells per ml (x 10^{-5})	Cyclic AMP content (p mol/10^6 cells)
Basal PPYF	1.80	10.38 ± 0.31
0.5% glucose-supplemented PPYFG	2.13	2.75 ± 0.08

Table 4. Cyclic AMP content of cells grown in basal and glucose-supplemented growth medium

Cyclic AMP was extracted, purified and assayed (as described in section 3.2) from logarithmically growing Tetrahymena pyriformis strain W, grown in PPYF and 0.5% (w/v) glucose-supplemented PPYFG growth medium.

(ii) The effect of substrate concentration on cyclic AMP phosphodiesterase activity

Cyclic AMP phosphodiesterase was assayed (as described in section 3.2(vi)(a)) over a range of substrate concentrations. The assay based on the production of inorganic phosphate was used for the cyclic AMP concentration range of 5 μM-10mM. The assay based on measuring cyclic AMP before and after incubation with cyclic AMP phosphodiesterase preparations was used for cyclic AMP concentrations from 10 μM to 10 nM. Fig. 27 shows that use of the first assay gives a Km for the enzyme 0.11 mM. The second assay gives a Km of 0.59 μM.

(iii) Discussion

Cyclic AMP content of logarithmically-growing cells grown in glucose-supplemented growth medium is only 26.4% of that in cells grown in the absence of glucose. Volchick et al. reported a similar result (192, 193); they found that Tetrahymena pyriformis strain E, grown in the presence of 1% glucose contained only 19% of the cyclic AMP present in cells grown without glucose supplement. This finding has been correlated with decreased adenyl cyclase and increased cyclic AMP phosphodiesterase in cells grown in the presence of glucose compared to cells grown without glucose supplement (193).

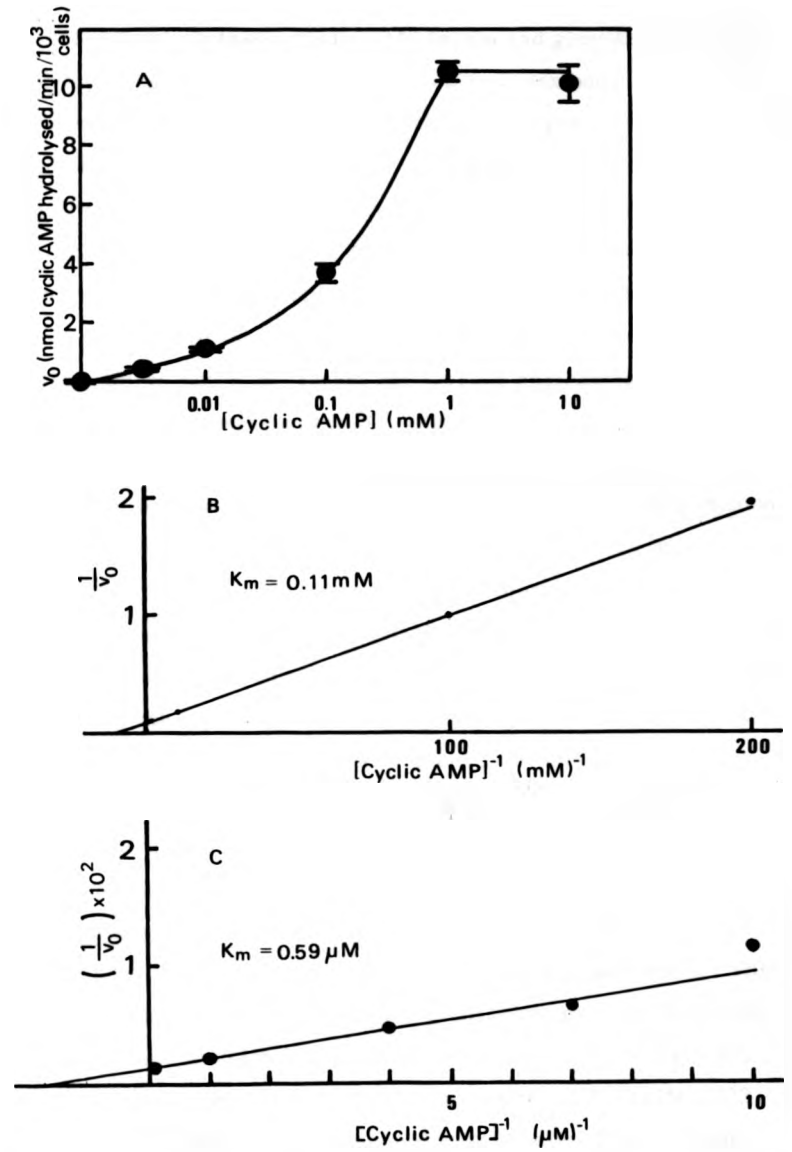

Fig. 27 The effect of substrate concentration on cyclic AMP phosphodiesterase activity.

Cyclic AMP phosphodiesterase was assayed as described in section 3.2(vi)(a). In A) and B) enzyme activity was assayed using the coupled assay and measuring the production of Pi. In C) cyclic AMP disappearance was monitored.

The relationship between cyclic AMP levels and glucose metabolism in Tetrahymena pyriformis appears to be a complex one. It is reported that epinephrine enhances glucose utilisation in Tetrahymena pyriformis (226) and that it produces elevation of intracellular cyclic AMP within the organism (227). Epinephrine had no effect on phagocytotic activity (228) but dibutyryl cyclic AMP enhanced phagocytosis (229). The purported role of epinephrine in this organism is confusing. Janakidevi et al. reported that Tetrahymena pyriformis contains epinephrine (230); however, Lowry and Gordee performed incorporation studies with [^{14}C] tyrosine and [^{14}C] dihydroxyphenylalanine, and found no evidence for the presence of a functional catecholamine system (231). Kindler and co-workers reported that adenyl cyclase from Tetrahymena pyriformis is stimulated in vitro by epinephrine (196, 197) but two other groups of workers using what appear to be far more reliable assay conditions for adenyl cyclase from Tetrahymena pyriformis report that the enzyme is insensitive to epinephrine (198, 199). It seems reasonable to suppose that the experiments involving this mammalian hormone are of pharmacological rather than physiological relevance.

The cyclic AMP phosphodiesterase of Tetrahymena pyriformis strain W was shown to have two Km's, one of 0.11 mM, the other of 0.59 μM. Voichick found that the 22,500 g supernatant of a sonic lysate of Tetrahymena pyriformis contained two cyclic AMP phosphodiesterases, one with a Km of 1.07 mM and the other with a Km of 1.03 μM (192). The phenomenon seems to be a general one; particulate preparations have been shown to contain "high Km" and "low Km" forms of cyclic AMP phosphodiesterase from 3T3 cells, SV40-transformed 3T3 cells (232), rat thymic lymphocytes (233) and pig epidermis cells (234). Most studies have found the "high Km" cyclic AMP phosphodiesterase to be a cytosol enzyme and the "low Km" enzyme to be associated with membranous structures. A study of the subcellular distribution of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities in Tetrahymena pyriformis was made and is presented and discussed in section 4.3(ii)).

3.4 Cyclic AMP metabolism in the natural cell cycle of Tetrahymena pyriformis

Cyclic AMP and cyclic AMP phosphodiesterase activity (at a substrate concentration of 5×10^{-7} M cyclic AMP) were determined (as described in section 3.2) in the natural cell cycle of Tetrahymena pyriformis . Cells were synchronized as outlined in section 2.4. In addition adenyl cyclase activity was measured by M.G. Graves as described elsewhere (198, 235).

The major observation (Fig. 28) was the large peak in intracellular cyclic AMP coincident with cell division. Shortly after division the cells contained little cyclic AMP. The fluctuations in adenyl cyclase and cyclic AMP phosphodiesterase activities can readily account for the observed levels of cyclic AMP. High cyclic AMP levels were associated with high adenyl cyclase activity and low phosphodiesterase activity. Conversely, low cyclic AMP occurred where adenyl cyclase was at a minimum and phosphodiesterase activity had increased.

In Chapter 1 I outlined the evidence derived from studies on a great many different cell types that cyclic AMP levels are high in G1, and that cellular quiescence (when the cell is said to be in G0) correlates with elevated intracellular cyclic AMP. In Tetrahymena pyriformis in particular, stationary phase cells have been shown to contain higher levels of cyclic AMP than logarithmic phase cells (162, 192-193). The observation from this experiment is of a transitory high level of cyclic AMP before the cells progress into G1. This suggests the location of an important control point in the cell cycle whereby the cell may either initiate a further cell division or become quiescent. The observed decline in cyclic AMP may be required to allow the cells to progress into G1. Hence, cells which maintain a high intracellular cyclic AMP level will enter stationary phase.

Cyclic AMP phosphodiesterase activity was also measured in the natural cell cycle using the assay which measures production of inorganic phosphate. This assay is far more simple to perform than the assay

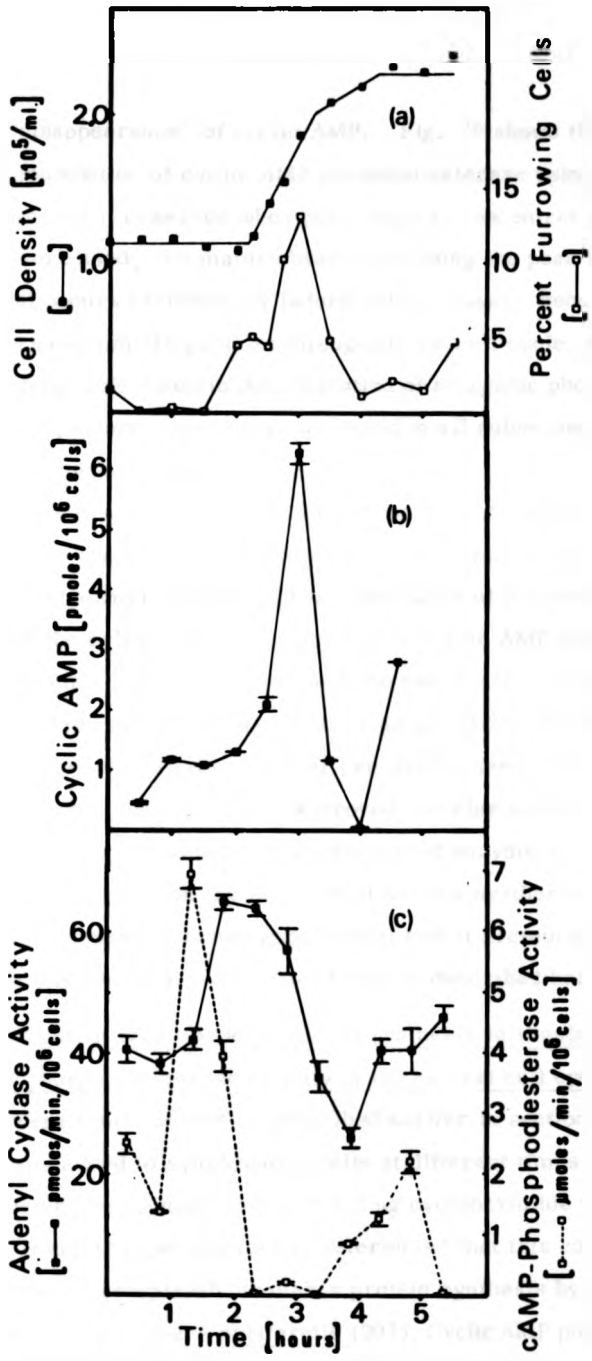

Fig. 28 Cyclic AMP metabolism in the natural cell cycle of Tetrahymena pyriformis.
Synchronous cells were selected as in section 2.4. Cyclic AMP and cyclic AMP phosphodiesterase activity were determined as in section 3.2. Adenyl cyclase activity measured by M.G. Graves as described elsewhere (198, 235). (a) Cell numbers and % dividing cells, (b) Cyclic AMP measured per 10^6 cells, (c) Adenyl cyclase and cyclic AMP phosphodiesterase activities measured at approx. physiological concentrations: (1.2 mM ATP and 5×10^{-7} M cyclic AMP respectively).

based on "disappearance" of cyclic AMP. Fig. 29 shows that the pattern of modulation of cyclic AMP phosphodiesterase using this assay was similar to that observed when physiological concentrations of substrate were used, the major observation being the peak in enzyme activity that occurs immediately before cell division. Because the two assays gave similar profiles throughout the cell cycle, and the assay involving colorimetric determination of inorganic phosphate is simpler to perform, this assay was used in all subsequent studies of cyclic AMP phosphodiesterase.

The modulation of cyclic AMP throughout the natural cell cycle can be explained in terms of the relative activities of cyclic AMP phsopho-diesterase and adenyl cyclase. It was therefore of interest to try and find out how the cell regulates the activity of cyclic AMP phosphodiesterase in the cell cycle. The first question to be asked was:- "How is the peak in cyclic AMP phosphodiesterase activity achieved ?" There seemed to be two possible alternatives. Either pre-synthesized enzyme is first activated and then deactivated or destroyed, or else synthesis of new enzyme molecules is followed by destruction of enzyme or a deactivation process. Cycloheximide (an inhibitor of protein synthesis by cytoplasmic ribosomes) was used to investigate to what extent protein synthesis is involved in this process. The experiment is described below.

3.5 Investigation on the role of protein synthesis in regulation of cyclic AMP phosphodiesterase activity in the natural cell cycle

Cells were synchronized as described earlier in section 2.4. Cycloheximide was added to synchronous cells at different times in the cell cycle to give a final concentration of 2.5 μg cycloheximide per ml of culture. (Earlier experiments had determined that this concentration of cycloheximide completely abolishes protein synthesis by cytoplasmic ribosomes in Tetrahymena pyriformis (207). Cyclic AMP phosphodiesterase was assayed as described in section 3.2(vi)(a)) using the assay based on measurement of inorganic phosphate.

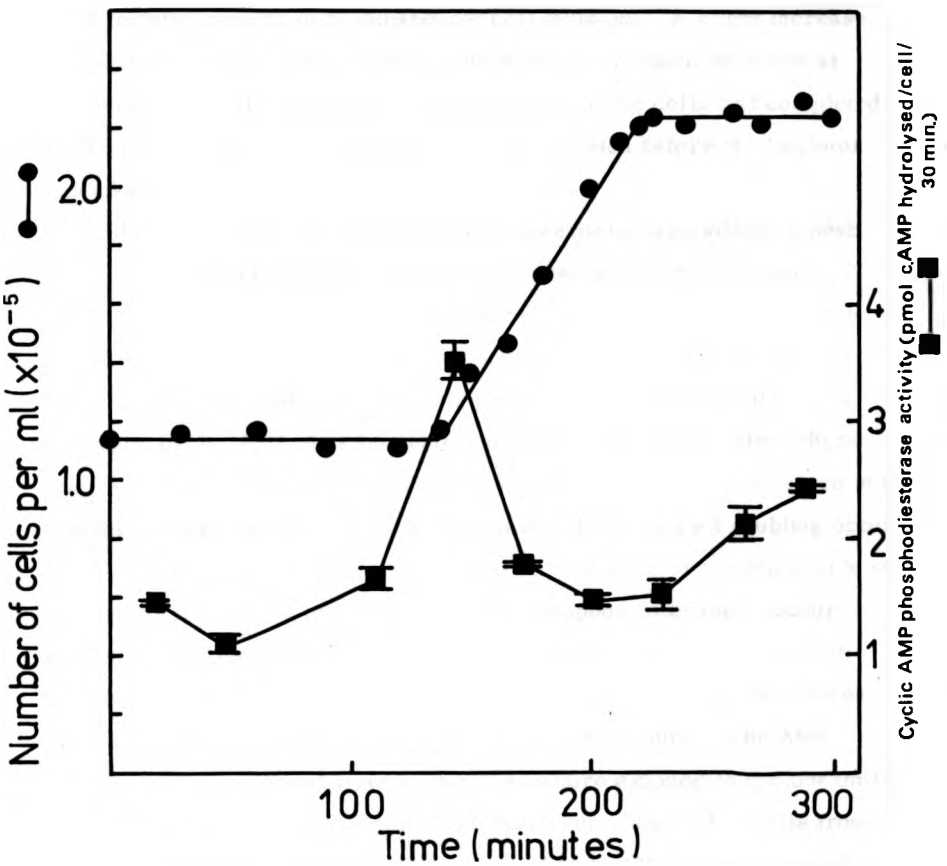

Fig. 29 Cyclic AMP phosphodiesterase activity (assayed at 0.36 mM cyclic AMP) measured throughout the natural cell cycle of Tetrahymena pyriformis

Cells were synchronized as described in section 2.4. Cyclic AMP phosphodiesterase was assayed using the coupled assay measuring production of Pi described in section 3.2(vi)(a).

From Fig. 30 it can be seen that addition of cycloheximide at time 0 or 62 minutes results in no subsequent cell division. A slight increase in cell density occurred in cultures which had cycloheximide added at 105 minutes; this is presumably because some of the cells had completed all the protein synthesis necessary for cell division before the inhibitor was added.

In the control culture (to which no cycloheximide was added) a peak of cyclic AMP phosphodiesterase was seen just before the onset of division. This result concurs with the results presented previously in Fig. 28 and Fig. 29. Addition of cycloheximide at time 0 delayed the appearance of the peak of cyclic AMP phosphodiesterase activity. The maximum cyclic AMP phosphodiesterase activity per cell in the culture drugged at time 0 is more than double the maximum activity observed in the control culture, though it must be remembered that no cell doubling occurred in the culture exposed to drug from time 0. Addition of cycloheximide at 62 minutes caused the rise in cyclic AMP phosphodiesterase to occur earlier than in the control culture, though the actual maximum activity occurred later (the maximum occurring at the same time as in cultures exposed to cycloheximide from time 0). The maximum cyclic AMP phosphodiesterase activity per cell in the culture exposed to cycloheximide from 62 minutes is almost 4 times the maximum observed in cells from the control culture. In the culture drugged from 105 minutes onwards, the rise in cyclic AMP phosphodiesterase activity followed the same pattern up to the maximum as in the control culture, but no subsequent decline in activity occurred.

These results suggested the following conclusions:-

(i) Cyclic AMP phosphodiesterase is synthesized from time 0, but is not fully active until the onset of division.

(ii) It seemed likely that a protease is synthesized from about 62 minutes, this enzyme being responsible for destruction of cyclic AMP phosphodiesterase in cells from control cultures. Addition of cycloheximide from 62 minutes onwards would therefore inhibit synthesis of the protease, and this would explain why cells exposed to cycloheximide have higher maximum cyclic AMP phosphodiesterase activities than in controls.

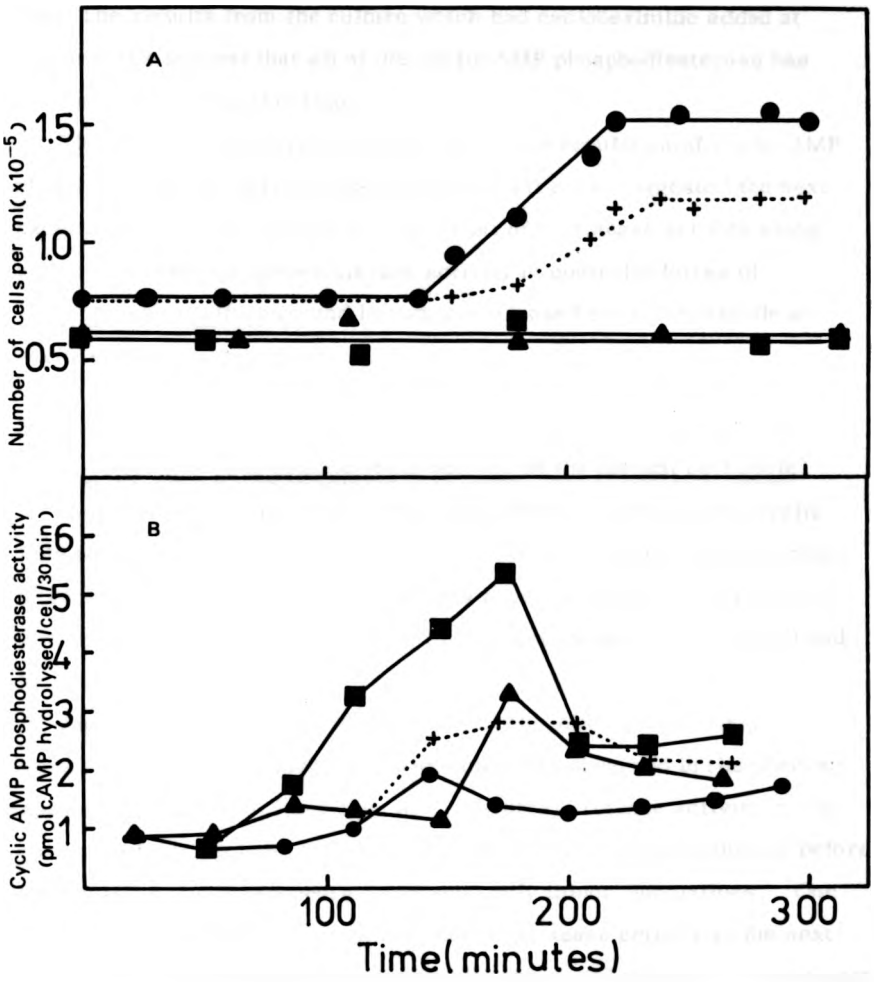

Fig. 30 Investigation on the role of protein synthesis in regulation of cyclic AMP phosphodiesterase activity in the natural cell cycle

Cells were synchronized as described in section 2.4. Cyclic AMP phosphodiesterase activity was assayed using the coupled assay measuring production of Pi described in section 3.3.(vi)(a). Cycloheximide (final concentration 2.5 µg/ml) was added to synchronous cells at zero time (▲——▲), 62 min (■——■) and 105 min (+ ---+). A control culture (●——●) was also studied. (Error bars are omitted from data in this figure for the sake of clarity).

(iii) The results from the culture which had cycloheximide added at 105 minutes suggest that all of the cyclic AMP phosphodiesterase has been synthesized by this time.

The likely involvement of a protease in the regulation of cyclic AMP phosphodiesterase activity throughout the cell cycle suggested the next experiment. This involved measurement of a protease activity along with cyclic AMP phosphodiesterase activity in control cultures of Tetrahymena pyriformis and in cultures exposed to cycloheximide at various times in the cell cycle.

3.6 An attempt to implicate a neutral protease in the regulation of cyclic AMP phosphodiesterase activity in the natural cell cycle

Cells were synchronized as described before (section 2.4), cyclic AMP phosphodiesterase and neutral protease were assayed as described in section 3.2(vi). Cycloheximide was added to synchronous cells (to give a final concentration of 2.5 μg cycloheximide per ml) at time 0 and 62 minutes.

Fig. 31 shows that cell division and cyclic AMP phosphodiesterase occurred in a similar fashion to that which was observed in the previous experiment (section 3.5 and Fig. 30). Neutral protease activity in the control culture declined to a minimum which occurred immediately before cell division and subsequently underwent only minor fluctuations. One would have expected to see a large peak of protease activity in the next cell cycle. Although there was a small peak at 233 minutes, this was not as large as would be expected (even allowing for a decrease in the second cell cycle due to progressive loss of synchrony with time). Cells exposed to cycloheximide from time 0 had a broadly similar pattern of modulation of neutral protease activity except that the initial decline in protease activity occurred slightly later and the minimum of enzyme activity was about 45 minutes later than in the control culture. Addition of cycloheximide at 62 minutes delayed the decrease in protease activity by an even greater time, the minimum protease activity in cells from this culture occurred 90 minutes later than in cells from the control culture.

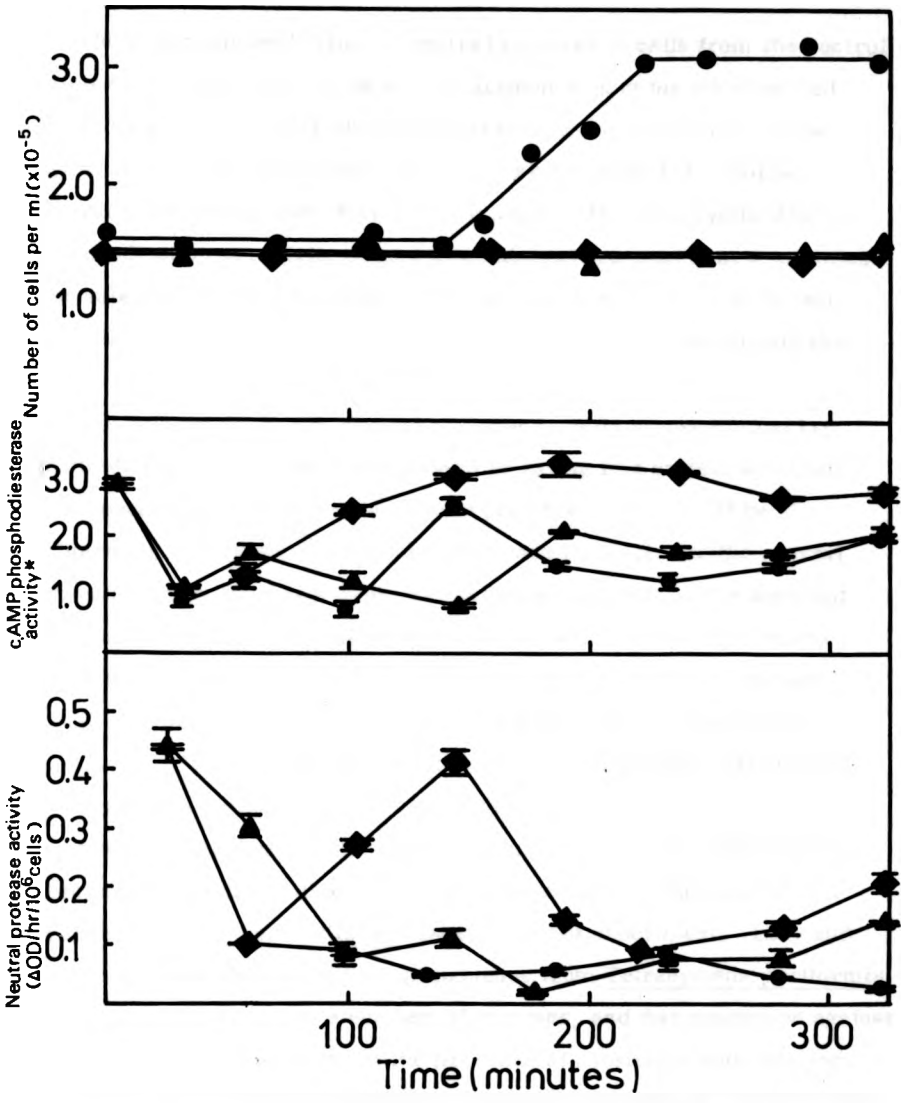

Fig. 31 An attempt to implicate a neutral protease in the regulation of cyclic AMP phosphodiesterase activity in the natural cell cycle

Cells were synchronized as described in section 2.4. Cyclic AMP phosphodiesterase activity was assayed using the coupled assay measuring production of Pi (described in section 3.2.(vi)(a)), and neutral protease activity was assayed as described in section 3.3.(vi)(b). Cycloheximide (final concentration 2.5 μg/ml) was added to synchronous cells at zero time (▲—▲) and at 62 minutes (◆—◆). A control culture (● —●) was also studied.

* units of cyclic AMP phosphodiesterase activity:- p mol cyclic AMP hydrolysed/cell/30 min.

The pattern of modulation of neutral protease in cells from the control culture would only seem to be able to account in part for the observed fluctuations of cyclic AMP phosphodiesterase in the natural cell cycle. Cyclic AMP phosphodiesterase increases slowly up to 111 minutes; neutral protease declines during this interval. Maximal cyclic AMP phosphodiesterase activity occurs when neutral protease is at a minimum. The subsequent small increase in neutral protease might be sufficient to account for the decline of cyclic AMP phosphodiesterase activity that occurs after cell division has started.

The delayed decline in neutral protease in cells exposed to cycloheximide from time 0 can be explained by saying that protein synthesis is necessary for the decline in neutral protease. This could be achieved by the synthesis of another protease (to inactivate the neutral protease) or by synthesis of neutral protease inhibitor. The apparent induction of neutral protease which occurred when cycloheximide was added at 62 minutes cannot be explained at this time. This complex situation meant that no further attempts were made to explore the modulation of cyclic AMP phosphodiesterase in the natural cell cycle by cycloheximide inhibition of protein synthesis.

It was disappointing to be unable to implicate neutral protease in the regulation of cyclic AMP phosphodiesterase activity in the natural cell cycle of Tetrahymena pyriformis by this type of experiment. Levy and McConkey have shown that neutral protease from Tetrahymena pyriformis is capable of inactivating a number of enzymes, and that protection against inactivation can be achieved in the presence of substrates and cofactors of those enzymes (236). Demonstration of in vivo control of cyclic AMP phosphodiesterase by neutral protease along with in vitro protection against inactivation of cyclic AMP phosphodiesterase by cyclic AMP would have represented an elegant model. It is possible that cyclic AMP phosphodiesterase activity is regulated by one of the many other proteases known to be present in Tetrahymena pyriformis (237-240). A similar substrate-protection mechanism could exist, or the specific protease may have its activity regulated by a cyclic nucleotide.

3.7 An investigation of cyclic AMP metabolism in Tetrahymena pyriformis synchronized by a single hypoxic shock

Cells were synchronized as described in section 2.5. Cyclic AMP and cyclic AMP phosphodiesterase activity were determined as described in section 3.2. Adenyl cyclase activity was measured by M.G. Graves by methods described elsewhere (198, 235).

Fig. 32 shows that cellular cyclic AMP is at a low level for the first 136 minutes of hypoxia. Maximum intracellular cyclic AMP is found 194 minutes after onset of hypoxia. Cyclic AMP falls to reach a minimum 73 minutes after the start of reshaking. The first synchronous division follows about 30 minutes after this minimum level of intracellular cyclic AMP. A "spike" of intracellular cyclic AMP is found to coincide with the first division. Cellular cyclic AMP subsequently rises to another maximum at 403 minutes; after 434 minutes it declines progressively.

During hypoxia adenyl cyclase is highest at about 140 minutes. There is then a decline in adenyl cyclase activity to a minimum at 320 minutes or about 25 minutes before onset of the first synchronous division. Subsequently adenyl cyclase activity rises to a maximum at 494 minutes.

During hypoxia cyclic AMP phosphodiesterase activity falls very slowly; the minimum activity occurs 10 minutes after the start of reshaking. Cyclic AMP phosphodiesterase activity then rises dramatically to a maximum at 313 minutes and then falls sharply to a new minimum of 343 minutes. A peak of cyclic AMP phosphodiesterase is subsequently seen with a maximum at 372 minutes.

These results show that when cell cycle blockade by hypoxia occurs in Tetrahymena pyriformis, this is associated with extraordinarily high intracellular cyclic AMP levels. It was previously observed in the natural cell cycle (section 3.4 and Fig. 28) that cyclic AMP is high at a point at the division/G1 interface or in very early G1. It was postulated that this "spike" in intracellular cyclic AMP is a very important signal in the cell cycle; if cyclic AMP remained high, the cells would enter G0 (i.e.

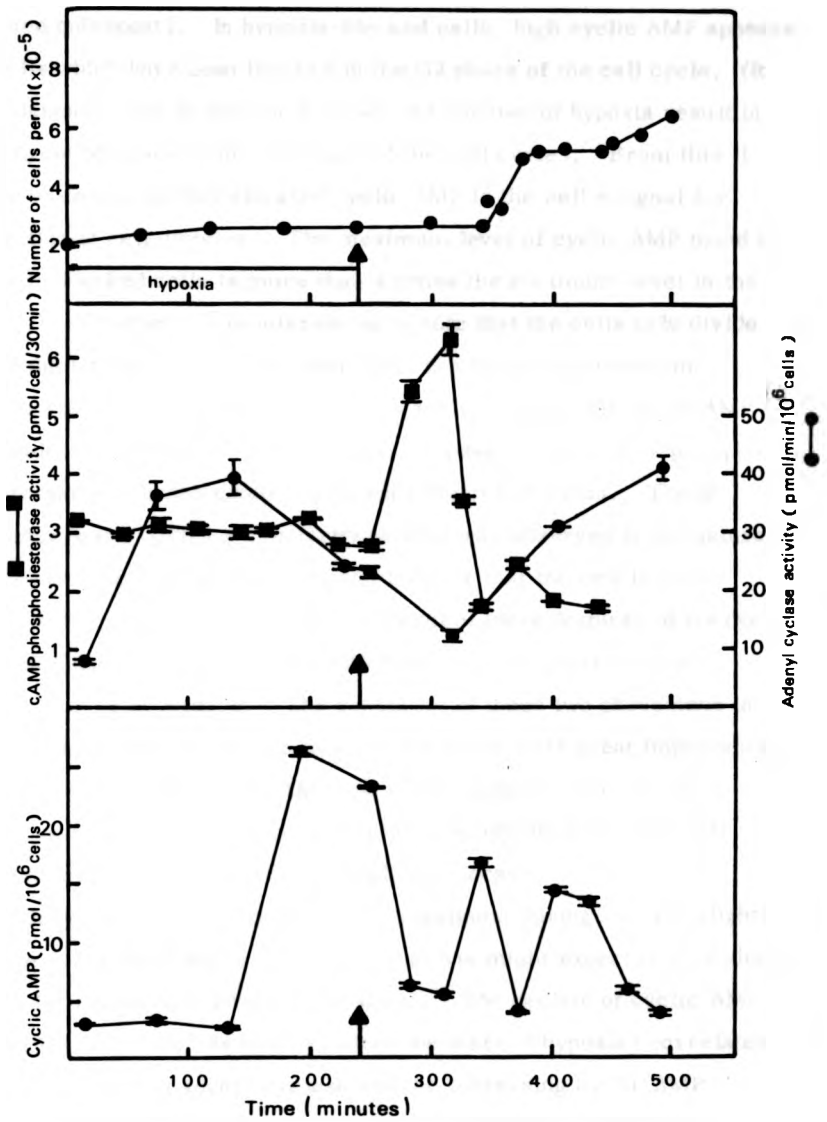

Fig. 32 Cyclic AMP metabolism in Tertahymena pyriformis synchronized by a single hypoxic shock

Cells were synchronized as described in section 2.5. Cyclic AMP and cyclic AMP phosphodiesterase activity were determined as described in section 3.2. Adenyl cyclase activity was measured by M.G. Graves as desribed elsewhere (198, 235). The arrow indicates the point at which reshaking started.

become quiescent). In hypoxia-blocked cells, high cyclic AMP appears in cells which have been blocked in the G2 phase of the cell cycle. (It was demonstrated in section 2.5 that 240 minutes of hypoxia result in cell cycle blockade in the G2 phase of the cell cycle). From this it must be concluded that elevated cyclic AMP is the cell's signal for inhibition of cell division. The maximum level of cyclic AMP found in hypoxia-blocked cells is more than 4 times the maximum level in the natural cell cycle. It is interesting to note that the cells only divide after the intracellular cyclic AMP level has fallen to a minimum.

Two most interesting observations are the "spike" of cyclic AMP coincident with the first division, and the peak of cyclic AMP phosphodiesterase which immediately precedes the cell division. These features bear a striking similarity to what was observed in the natural cell cycle (Fig. 28 and Fig. 29) and indicate that the cell is still capable of a remarkable degree of control of these features of its cyclic AMP metabolism, despite the disturbance it has suffered in the synchronizing procedure. The constancy of these two phenomena in relation to the timing of cell division attests to their great importance. The peak in cellular cyclic AMP that is seen subsequent to the first syncrhonous division presumably reflects readjustment of the cell back towards normal growth as synchrony decays.

Adenyl cyclase activity reaches a maximum during hypoxia slightly before cyclic AMP levels. This is what one might expect (i.e. a short while for cyclic AMP levels to build up). The decline of cyclic AMP (from its maximum 194 minutes after the start of hypoxia) correlates with the decline in adenyl cyclase and the increasing cyclic AMP phosphodiesterase such that cyclic AMP and adenyl cyclase both reach a minimum and cyclic AMP phosphodiesterase reaches a maximum before the onset of the first division. The characteristic "spike" of intracellular cyclic AMP has its maximum coincident with the minimum of cyclic AMP phosphodiesterase seen at that time. The minimum level of intracellular cyclic AMP seen at 374 minutes correlates with the maximum of cyclic AMP phosphodiesterase that

occurs at that point. The peak in cyclic AMP before the second division can also be explained by the respective adenyl cyclase and cyclic AMP phosphodiesterase activities during that part of the experiment.

This experiment also indicates that adenyl cyclase is responsible for regulation of intracellular cyclic AMP levels during hypoxia, (cyclic AMP phosphodiesterase varies little during that period), but that cyclic AMP phosphodiesterase appears to be mainly responsible for controlling the fluctuations in cellular cyclic AMP content during the cell cycle after removal of the hypoxia blockade.

Studies on cyclic AMP metabolism in selection synchronized and induction synchronized Tetrahymena pyriformis suggest most strongly that elevated intracellular cyclic AMP is the signal for inhibition of cell division, and that cell division itself is accompanied by a unique pattern of modulation of cyclic AMP and cyclic AMP phosphodiesterase. It will be of great interest to see whether the use of other methods of induction synchrony yield similar results. Identification of cyclic nucleotide signals in the cell cycle is an important step towards finding the function of these signals.

CHAPTER 4

CYCLIC GMP METABOLISM

4.1. Introduction

The use of a binding protein assay for cyclic GMP determinations was decided upon for the same reasons (see section 3.1) that applied to the assay of cyclic AMP, namely the considerably greater "shelf life" of binding proteins relative to antibodies, and the factor of cost. Originally it was intended to use either the assay of Murad et al. (146) or the assay of Dinnendahl (219). Both assays use cyclic GMP binding protein derived from lobster tail muscle. The assay of Murad et al. uses a relatively crude preparation of binding protein; protein-bound cyclic GMP is separated from unbound nucleotide by means of cellulose ester filters (146). Dinnendahl's assay employs cyclic GMP binding protein which has first been purified by chromatography on DEAE-cellulose; separation of bound and free cyclic GMP is achieved by the use of charcoal (219). I was unable to use either method for the assay of cyclic GMP; both enzyme preparations were completely devoid of cyclic GMP-binding activity. For this reason a commercially available cyclic GMP assay kit was used in all the experiments.

4.2. Materials and Methods

(i) Chemicals

All nucleosides and nucleotides were obtained from Sigma Chemical Company Ltd., Kingston upon Thames, U.K. Neutral alumina, activity grade 1, was purchased from I.C.N. Pharmaceuticals, Eschwege, West Germany. [8-^{3}H] cyclic GMP (21 Ci/mmole) was from the Radiochemical Centre, Amersham, U.K. Cyclic GMP assay kits were supplied by Boehringer Corportaion (London) Ltd., Lewes, U.K. Nonidet P 40 was a gift from Shell Research, Sittingbourne, U.K. All other chemicals used were of the highest purity commercially available.

(ii) Enzymes

Beef heart cyclic nucleotide phosphodiesterase was obtained from Boehringer Corporation (London) Ltd., Lewes, U.K.

(iii) Extraction and purification of cyclic GMP

The extraction procedure was essentially the same as that described for cyclic AMP in section 3.2(iv). Cyclic GMP was purified by chromatography on columns of neutral alumina as described in section 3.2(iii). Cyclic GMP eluted from the column later than cyclic AMP (Fig. 21). It was noted in section 3.2(iii) that the fraction collected from the column containing cyclic GMP also contains some cyclic AMP, adenosine and guanosine. It is shown in section 4.2(iv) (below) that the presence of these compounds in samples to be assayed for cyclic GMP does not give rise to any "cross-reaction". Recovery of cyclic GMP extracted from cells was monitored by performing extraction and purification of cyclic GMP in the presence of known amounts (5.5×10^5 cpm) of [8-^{3}H] cyclic GMP. Recovery of [8-^{3}H] cyclic GMP in six separate experiments was 65.0 ± 1.3%.

(iv) The assay of cyclic GMP

The assay comprised 100 µl "unknown" or a standard amount (0-2 p mol) of cyclic GMP, 20 µl (~9 n Ci) [8-^{3}H] cyclic GMP, 50 µl buffer F (0.2 M sodium acetate buffer, pH 4.0) and 10 µl water. The assay ingredients were mixed, and then 20 µl of cyclic GMP binding protein was added and mixed by carefully tilting the tube. (All assay components were precooled to 0° C before being added to precooled tubes). Incubation was for 20 minutes at 0° C, after which time 0.2 ml of ice-cold 3.8 M ammonium sulphate solution was added followed by careful mixing. After 10 minutes at 0° C, the tubes were centrifuged at 10,000 g for 2 minutes, and the supernatant removed. 0.2 ml of ice-cold 2.7 M ammonium sulphate was then added to the precipitate, and mixed by knocking the test tube. After 5 minutes at 0° C the tubes were centrifuged again at 10,000 g, this time for 1 minute, and the supernatant removed. 1.2 ml of ice-cold water was then added immediately and the precipitate dissolved by vortexing. 1 ml of this solution was then removed and added to 12 ml of scintillation fluid (7 gm butyl-BPD in 500 ml Triton X-100, 1 l toluene). Radioactivity

was estimated using a Packard 2425 tri-carb liquid scintillation counter. All cyclic GMP standard curves were constructed containing similarly concentrated-up alumina column elution buffer in equivalent amounts to those present in "unknowns". This procedure was adopted because preliminary experiments had indicated that the presence of such material could (without these controls) give the appearance of more cyclic GMP being present per assay than was known to be actually present. All determinations were performed in duplicate, the mean deviation of each duplicate assay from its mean was 4.2% calculated from 50 duplicate assays selected at random. The assay is essentially a saturation binding assay; addition of unlabelled cyclic GMP (in standards or unknown samples) competes out the [^{3}H] cyclic GMP. Separation of bound cyclic GMP from unbound nucleotide is achieved by the two ammonium sulphate washes.

Fig. 33 shows that the presence of up to 40 n mol of cyclic AMP, adenosine or guanosine in a cyclic GMP assay produced no cross reaction; the standard curves were identical within the limits of error of the assay technique. The presence of relatively small amounts of these materials co-purified along with cyclic GMP after chromatography on neutral alumina would not give rise to artefacts.

Putative cyclic GMP was extracted from cells, purified on columns of neutral alumina, divided into 2 portions, and each portion lyophilized. A cyclic GMP determination was performed on one aliquot. A solution of bona fide cyclic GMP was made up in buffer C (0.1 M glycyl glycine buffer, pH 7.5 containing 0.15 mM $MgSO_4$) and an equal amount of concentrated column elution buffer to that of the second aliquot of putative cyclic GMP which was also dissolved in buffer C. The 2 solutions were incubated at 25^{o}C in the presence of beef heart cyclic nucleotide phosphodiesterase. Samples of the assay mix were removed at intervals, boiled for 3 minutes to terminate the phosphodiesterase reaction, then plunged into ice. Cyclic GMP determinations were performed on all samples. Control incubations comprised putative

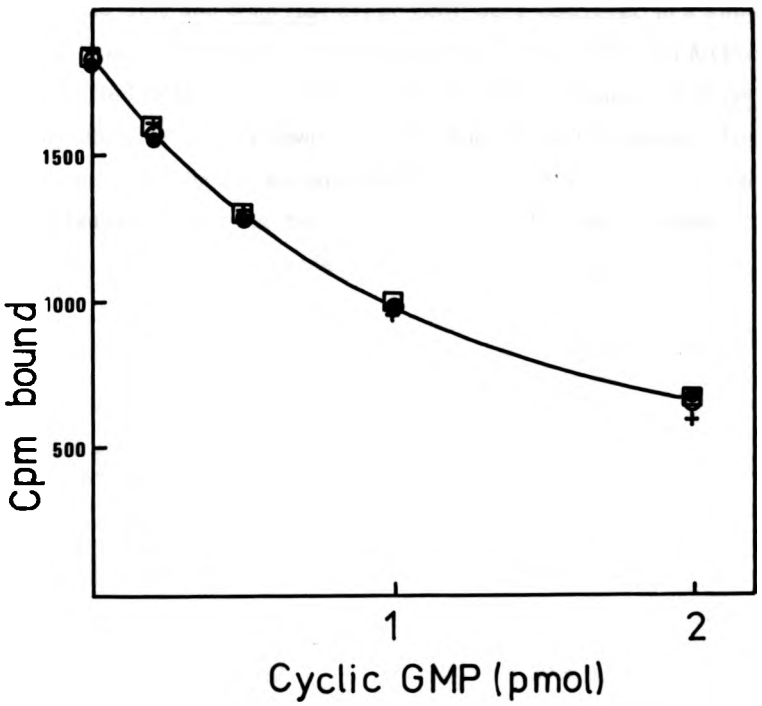

Fig. 33 The effect of cyclic AMP, adenosine and guanosine on cyclic GMP assays

A cyclic GMP standard curve was constructed containing 40 p mol of cyclic AMP (+—+), adenosine (●—●) and guanosine (□—□) per assay. A control standard curve (○—○) was also constructed. Cyclic GMP was assayed as described in section 4.2.(iv).

(or bona fide) cyclic GMP in buffer C. Fig. 34 shows that putative cyclic GMP and bona fide cyclic GMP were destroyed in a similar fashion. Material that was assayed as cyclic GMP was further verified to be cyclic GMP by addition of defined amounts of bona fide cyclic GMP to "unknowns", and by dilution of "unknowns"; both tests resulted in the values expected from the calibration curve (see Table 5 below). This is the first time that cyclic GMP has been demonstrated to be present in Tetrahymena pyriformis.

Sample	Cyclic GMP content (p mol)
100 µl of "unknown"	1.76 ± 0.07
100 µl of 1/10-diluted "unknown"	0.18 ± 0.00
100 µl of "unknown" plus 0.2 p mol bona fide cyclic GMP	1.96 ± 0.08
100 µl of 1/10-diluted "unknown" plus 0.2 p mol bona fide cyclic GMP	0.39 ± 0.02

Table 5 Further verification that material that is assayed as cyclic GMP is cyclic GMP (see also Fig. 34). Cyclic GMP was extracted from cells, purified and assayed as described in section 4.2

(v) The assay of cyclic GMP phosphodiesterase

Cells were harvested and disrupted as described in section 3.2(vi). Cell homogenates were used immediately after sonication for initiation of the cyclic GMP phosphodiesterase assays. Two assay systems were tried out along analogous lines to the assays described for cyclic AMP phosphodiesterase (section 3.2(vi)(a)). The first consisted of measuring the release of inorganic phosphate with the use of an excess of 5' nucleotidase (present in the venom of Ophiophagus hannah). This assay was performed in an identical fashion to the assay of cyclic AMP phosphodiesterase (see section 3.2(vi)(a)), except that 1 mM cyclic

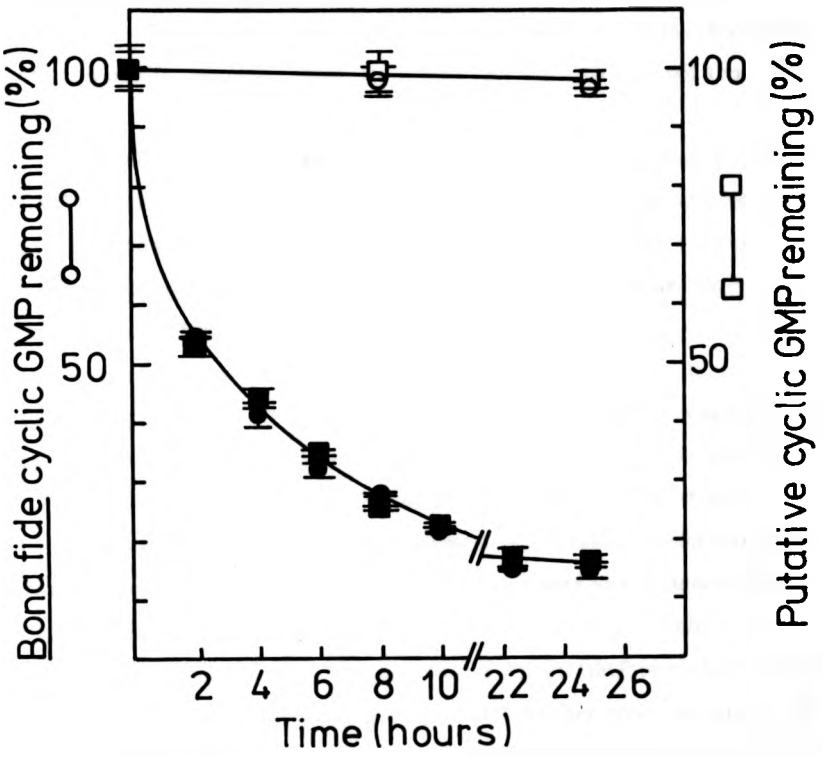

Fig. 34 The destruction of putative cyclic GMP and bona fide cyclic GMP by beef heart cyclic nucleotide phosphodiesterase

Putative cyclic GMP was extracted from cells and purified on columns of neutral alumina as described in section 4.2.(iii). This material was incubated with beef heart cyclic nucleotide phosphodiesterase (■—■). A solution of bona fide cyclic GMP was made up (to the same concentration as the putative cyclic GMP) and was incubated in the presence of cyclic nucleotide phosphodiesterase (●—●). Open symbols denote no enzyme controls.

GMP was used as substrate instead of cyclic AMP. Fig. 35 shows that the cyclic GMP phosphodiesterase reaction was linear for at least 30 minutes. Assays were performed in duplicate; the mean deviation of each duplicate assay from its mean was 2.6% calculated from 40 assays.

The second assay tried was based on the measurement of cyclic GMP before and after incubation with cyclic GMP phosphodiesterase preparations. The assay was initiated by addition of 0.1 ml of appropriately diluted cell homogenate to 0.9 ml of cyclic GMP (2.15×10^{-7} M) in buffer D. Incubation was for 10 mins at 30^{o} C. Boiling for 5 minutes was used to terminate the reaction, after which incubation tubes were plunged into ice. Cyclic GMP disappearance was estimated by performing cyclic GMP determinations (as described in section 4.2 (iv)) on samples which had been incubated without and with cell homogenate, and subtracting the difference. Assay conditions were constructed along similar lines to the equivalent assay described for cyclic AMP phosphodiesterase (section 3.2(vi)(a)). Table 6 shows that it was not possible to demonstrate a cyclic GMP phosphodiesterase activity capable of hydrolysing cyclic GMP at this concentration. Of course, this particular experiment involved dilution of cell homogenate

Concentration of cell homogenate in cyclic GMP phosphodiesterase incubation tube	Cyclic GMP content in 100 µl of 1/12 dilution of aliquot from incubation tube (p mol)
Neat	1.77 ± 0.07
1/10-diluted	1.79 ± 0.07
1/100-diluted	1.78 ± 0.07
No enzyme	1.79 ± 0.07

Table 6 Demonstration of the lack of a low Km cyclic GMP phosphodiesterase in Tetrahymena pyriformis. Cyclic GMP phosphodiesterase activity was measured at a substrate concentration of 2.15×10^{-7} M as described in section 4.2(v).

by a large factor prior to assaying; "neat" cell homogenate in Table 6 actually represents a final dilution of 1/256,000 of 1 ml of sonicate derived from 30 ml of cells of cell density 2.2×10^{5}/ml. If quantities

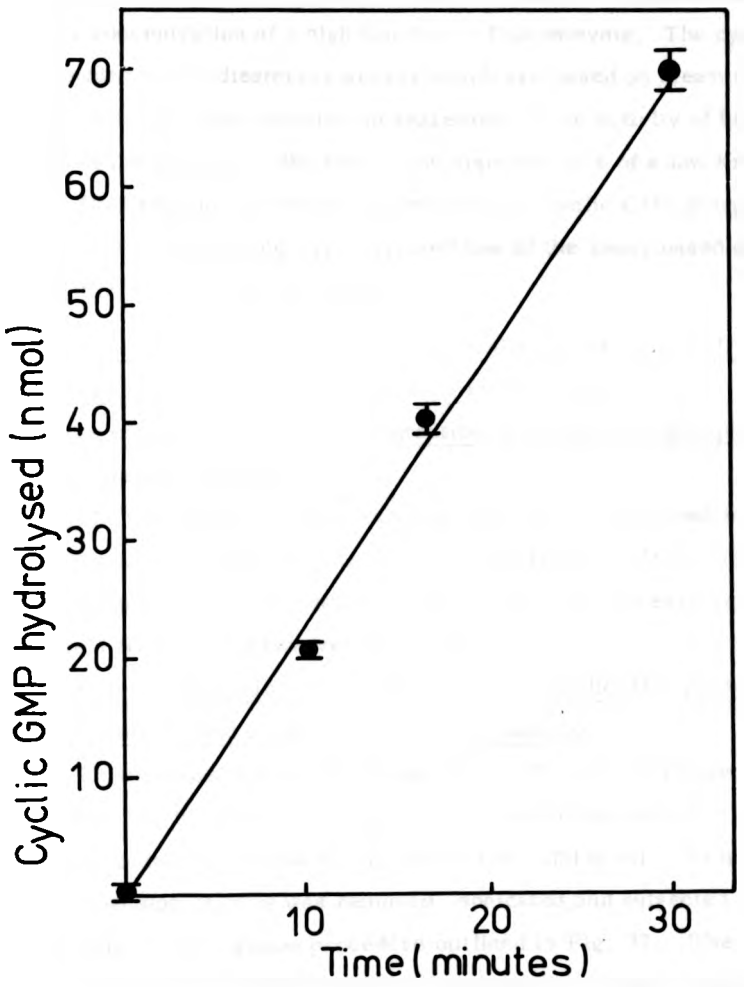

Fig. 35 The time course of the cyclic GMP phosphodiesterase assay
Enzymic activity was measured as described in section 4.2.(v) by monitoring the production of Pi in a coupled assay using an excess of 5'-nucleotidase present in the venom of Ophiophagus hannah.

of cell homogenate greater than this were used, there would be destruction of cyclic GMP, but this would be due to the presence of a large concentration of a high Km form of the enzyme. The cyclic nucleotide phosphodiesterase assays which are based on measurement of inorganic phosphate involve measurement of the activity of high Km forms of the enzymes. Because of the apparent lack of a low Km cyclic GMP phosphodiesterase, all subsequent cyclic GMP phosphodiesterase studies necessarily involved use of the assay based on production of inorganic phosphate.

4.3 Some Studies on Cyclic GMP Phosphodiesterase Derived from Logarithmically Growing Tetrahymena Pyriformis

(i) The effect of substrate concentration on cyclic GMP phosphodiesterase activity.

Cyclic GMP phosphodiesterase was assayed (as described in section 4.2(v)) at cyclic GMP concentrations ranging from 30 μM to 10 mM. Fig. 36 shows that it is possible to obtain a Km for the enzyme of 0.91 mM from the Lineweaver-Burk plot.

(ii) A study of the subcellular distribution of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase

2,750 ml of cells at a cell density of 9×10^5 cells/ml were harvested for 10 mins at 2,000 g at 0-4° C and resuspended in 0.25 M sucrose containing 10 mM $MgCl_2$ to a volume of 180 ml. 10 ml of this suspension of cells was removed, sonicated, and subjected to the differential centrifugation procedure outlined in Fig. 37. The remaining 170 ml of cell suspension was used to prepare intact whole nuclei according to the procedure of Higashinakagawa et al. (241). This involved the addition of 34 ml of 1% Nonidet P 40 in 0.25 M sucrose containing 10 mM $MgCl_2$ to the cell suspension, followed by gentle agitation. The cell membrane is disrupted leaving the nuclear membrane intact. The degree of cell lysis was checked at intervals by microscopical observation, agitation was continued until cell membrane lysis was judged to be complete. The cell lysate was made 2.1 M with respect to sucrose by dissolving solid sucrose, and

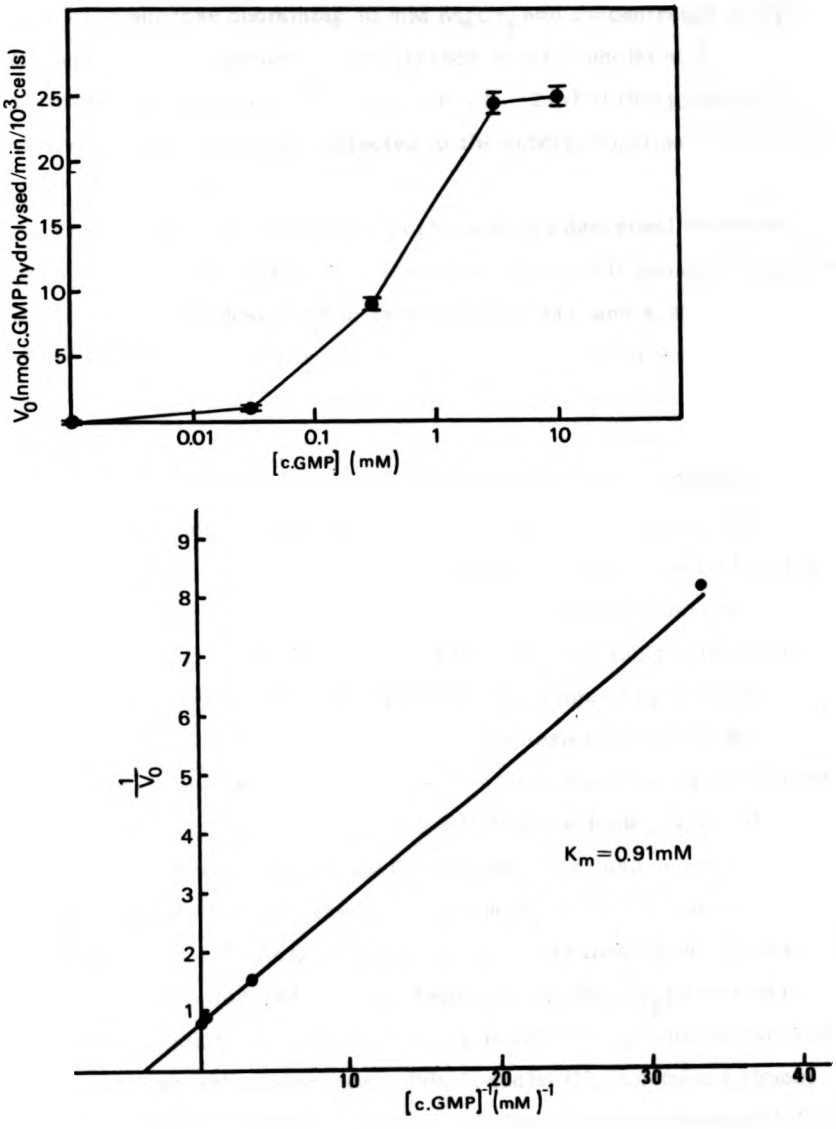

Fig. 36 The effect of substrate concentration on cyclic GMP phosphodiesterase activity

Cyclic GMP phosphodiesterase was assayed as described in section 4.2.(v) using the coupled assay and measuring the production of Pi.

centrifuged at 59,000 g for 1 hr at 0° C. The pellet was resuspended in 0.25 M sucrose containing 10 mM $MgCl_2$ and concentrated-up by low-speed centrifugation. This yielded 10 ml of nuclei at a concentration of 2.46×10^8 nuclei per ml. 2 ml of this preparation of whole nuclei were then subjected to the subfractionation procedures outlined in Fig. 38.

Protein determinations were performed (as described in section 2.2 (iii)), cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase were assayed (as described in section 3.2(vi)(a) and 4.2(v) respectively) on all of the fractions prepared by the procedures outlined above and in Fig. 37 and Fig. 38. The results are presented in Table 7.

Table 7 shows that differential centrifugation of the whole cell sonicate revealed that the greatest percentage of total cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase is to be found in the 50,000 g supernatant; the 1,000 g pellet contained the next greatest amount of both enzymes. Preparation of subfractions from whole nuclei revealed that the greatest percentage of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase was in the fraction N_4; the next greatest amount of both enzymes was contained in fraction N_3. Fraction N_3 is the 2 M MaCl-soluble portion of chromatin and contains principally histones, non-histone chromosomal proteins and RNA polymerase. Fraction N_4 is the "chromatin residue" which is mainly DNA and contains less than 1% of chromosomal proteins (242-243). The chromatin residue (N_4) contained the highest specific activity of both cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase. Whole nuclei (N_1) contained almost the same specific acticity of cyclic GMP phosphodiesterase as did the chromatin residue, but the specific activity of cyclic AMP phosphodiesterase in whole nuclei was only about one half that present in chromatin residue.

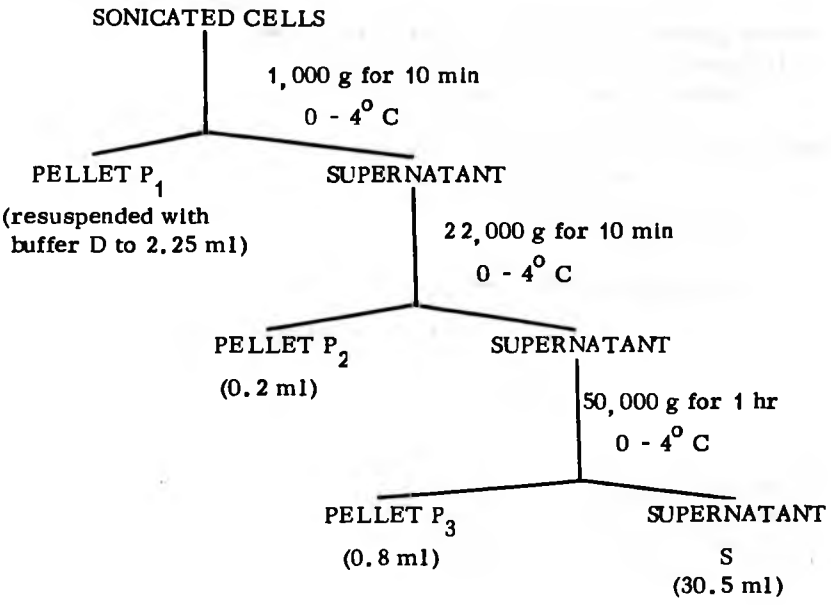

Figure 37.

The differential centrifugation procedure performed on a sonicate of Tetrahymena pyriformis

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Table 8 shows the cyclic AMP phosphodiesterase/cyclic GMP phosphodiesterase activity ratio in each of the cellular subfractions prepared. The fact that this activity ratio varies between fractions suggests most strongly that there are two distinct enzymes and not a general relatively non-specific cyclic nucleotide phosphodiesterase which is capable of hydrolyzing both cyclic AMP and cyclic GMP.

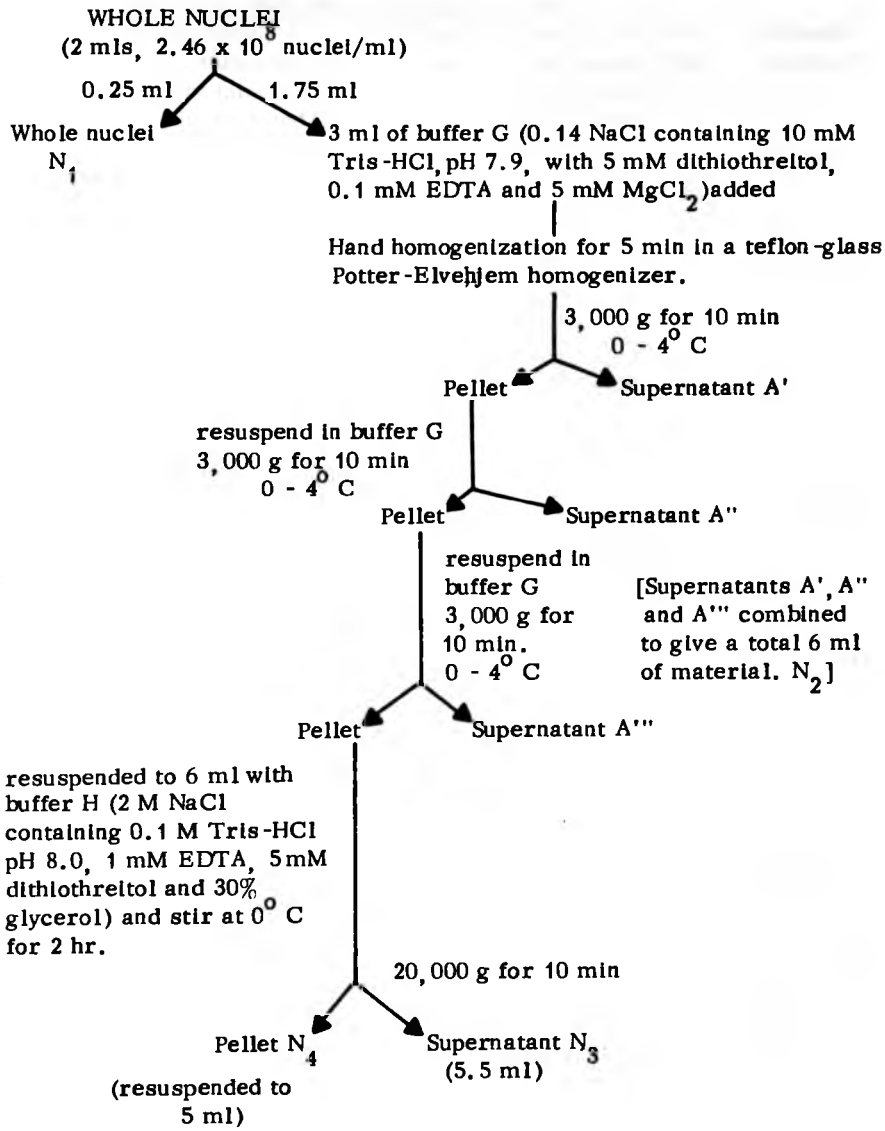

Figure 38

The preparation of nuclear subfractions from intact whole nuclei of Tetrahymena pyriformis

Whole nuclei were prepared as described in section 4.3(ii) according to Higashinakagawa et al. (241).

CYCLIC AMP PHOSPHODIESTERASE

Fraction	Cyclic AMP phosphodiesterase activity (μmol cyclic AMP hydrolyzed/30 min	Protein (mg)	Specific activity (μmol cyclic AMP hydrolyzed/30 min/ mg protein.)	% of total activity in fraction
P_1	25.68	55.12	0.47	25.90
P^1_2	1.30	3.13	0.42	1.31
P^2_3	2.55	15.71	0.16	2.57
S^3	69.62	130.23	0.53	70.21
N_1	1.62	1.02	1.58	100.00
N^1_2	0.85	21.18	0.04	7.48
N^2_3	1.75	4.01	0.44	15.49
N^3_4	4.70	1.50	3.13	41.41

CYCLIC GMP PHOSPHODIESTERASE

Fraction	Cyclic GMP phosphodiesterase activity (μmol cyclic GMP hydrolyzed/30 min	Protein (mg)	Specific activity (μmol cyclic GMP hydrolyzed/30 min/ mg protein.)	% of total activity in fraction
P_1	18.62	55.12	0.34	33.41
P^1_2	1.98	3.19	0.63	3.55
P^2_3	2.67	15.71	0.17	4.78
S^3	32.46	130.23	0.25	58.24
N_1	2.17	1.02	2.12	100.00
N^1_2	0.21	21.18	0.01	1.41
N^2_3	1.45	4.01	0.36	9.55
N^3_4	3.77	1.50	2.51	24.78

Table 7 The subcellular distribution of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase.

Subcellular fractionation was performed as described in section 4.3(ii). Protein was determined as described in section 2.2(iii), cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase were assayed as described in section 3.2(vi)(a) and 4.2(v) respectively. The preparation of fractions P_1-S and N_1-N_4 were separate procedures. The percentages of total activity in the fractions are expressed for each procedure; the percentages of total activity in fractions P_1-S have been normalized.

Fraction	Cyclic AMP phosphodiesterase/ cyclic GMP phosphodiesterase activity ratio
P_1	1.38
P_2	0.66
P_3	0.96
S	2.14
N_1	0.75
N_2	0.40
N_3	1.21
N_4	1.25

Table 8 The relative activities of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase in different subcellular fractions of Tetrahymena pyriformis. Subcellular fraction was performed as described in section 4.3(ii). Cyclic AMP phospho - diesterase and cyclic GMP phosphodiesterase were assayed as described in section 3.2(vi)(a) and 4.2(v) respectively.

(iii) Discussion

Cyclic GMP phosphodiesterase was demonstrated for the first time in Tetrahymena pyriformis. The enzyme (measured in crude homogenates) was shown to have a Km of 0.91 mM. The apparent absence of a low Km cyclic GMP phosphodiesterase would seem to present a problem in explaining how the cell manages to eliminate physiological concentrations of cyclic GMP. One way the cell could circumvent this problem would be to have much larger amounts of the enzyme present in the cell than it would have if a low Km form of the enzyme were present. Another possibility is that the cell does not hydrolyse much of its cyclic GMP, but that cyclic GMP is excreted from the cell. The notion of excretion of cyclic GMP from cells is not without precedent; indeed, this cyclic nucleotide was first found in rat urine (141) as mentioned earlier (page 18).

Most of the cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activity is present in a 50,000 g supernatant, but the highest specific activity of both enzymes is in the chromatin residue. These findings suggest that both cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase are tightly bound to the DNA. The fact that the specific activity of cyclic GMP phosphodiesterase in whole nuclei was almost equal to that in chromatin residue, and that the specific activity of cyclic AMP phosphodiesterase in chromatin residue was almost double that present in whole nuclei, suggests that cyclic AMP phosphodiesterase is more tightly bound to the DNA than cyclic GMP phosphodiesterase. The fact that the 50,000 g supernatant contained so much of the total activity can be explained by the fact that sonication would disrupt chromatin structure (thereby solubilizing the enzymes). The chromatin residue fraction was prepared from cells which were not sonicated, but had the cell membrane lysed to enable preparation of whole nuclei.

The notion that cyclic GMP phosphodiesterase and cyclic AMP phosphodiesterase are integral components of chromatin, and that cyclic AMP phosphodiesterase in particular is tightly bound to the DNA is an attractive one. The most efficient level of control of cellular processes is at transcription. Histones are thought to be the agents that mask DNA, thereby determining whether a particular gene shall be transcribed or not, but the specificity of gene expression is thought to reside with the non-histone chromosomal proteins (244). Phosphorylation of chromatin proteins may play a role in the regulation of transcription (245). Phosphorylation of specific nuclear acidic (non-histone) proteins occurs at times of elevated RNA synthesis during the cell cycle of synchronized HeLa cells (246) and in lymphocytes stimulated to divide (247-248). Cyclic nucleotides may regulate phosphorylation of histones and acidic nuclear proteins by controlling the activity of specific cyclic nucleotide dependent protein

kinases (249-253). It has been observed that cyclic AMP stimulates protein kinase catalyzed phosphorylation of RNA polymerase II preparations from rat liver (254) and calf ovary (255) and thereby enhances RNA polymerase II activity. Cyclic AMP-dependent protein kinase from calf thymus nuclei has been shown to be a nuclear acidic protein capable of binding DNA; in the presence of cyclic AMP, association of the catalytic subunit of the protein kinase with DNA was enhanced (256). Thus, the binding of cyclic nucleotide dependent protein kinases to DNA may be part of a mechanism by which cyclic nucleotides can selectively regulate protein phosphorylation at specific sites in the chromatin. If such a mode of control of transcription exists, (as seems likely), the fine control of cyclic nucleotide concentrations within the chromatin is of key importance. If cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase are integral components of the chromatin in Tetrahymena pyriformis nuclei, modulation of cyclic nucleotide concentrations within the chromatin could be achieved. Adenyl cyclase in Tetrahymena pyriformis is reported to be wholly located in the cortical compartment of the cell (198); this would not seem to represent a convenient location of the enzyme for discrete control of gene expression via cyclic AMP. Furthermore it was observed in section 3.7 that cyclic AMP phosphodiesterase, rather than adenyl cyclase, appears to be mainly responsible for controlling the fluctuations in cellular cyclic AMP during the cell cycle.

4.4 Some aspects of cyclic GMP metabolism in the natural cell cycle of Tetrahymena pyriformis

Cells were synchronized by the magnetic selection method described in section 2.4. Cyclic GMP and cyclic GMP phosphodiesterase were assayed (as described in section 4.2) during the natural cell cycle.

Fig. 39 shows that there was almost a ten-fold variation in cyclic GMP throughout the cell cycle. The range of cyclic GMP concentrations was similar to that described for cyclic AMP in section 3.4. Cyclic GMP was highest during cell division and declined progressively to its

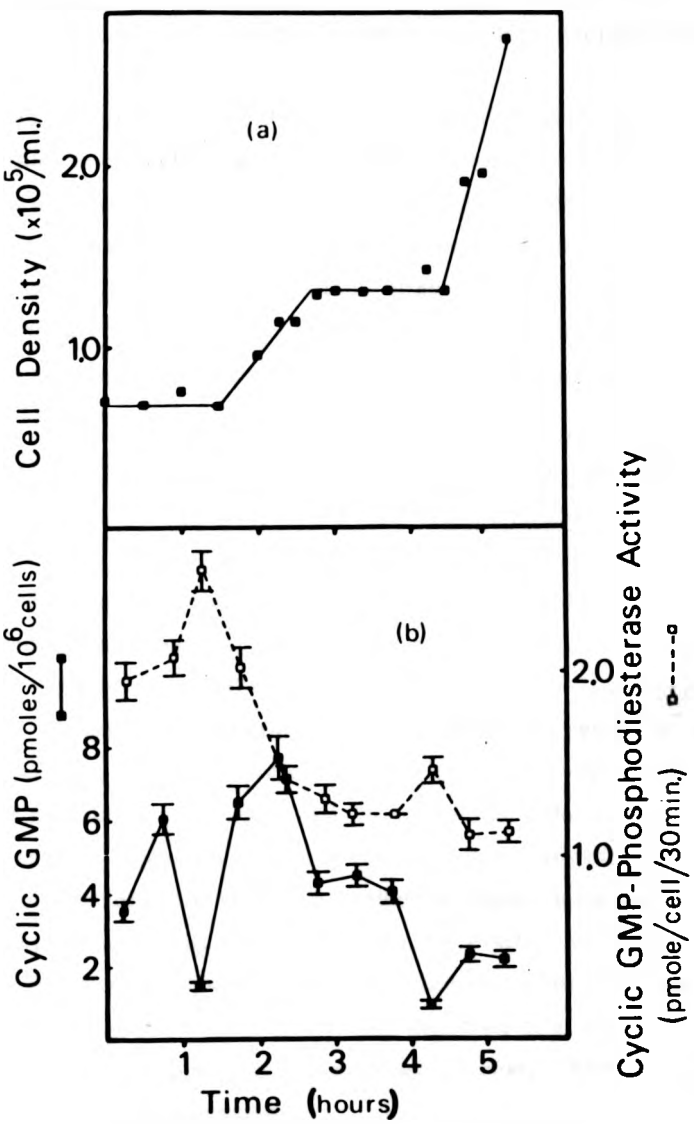

Fig. 39 Cyclic GMP and cyclic GMP phosphodiesterase in the natural cell cycle of Tetrahymena pyriformis

Cells were synchronized by the magnetic selection method described in section 2.4. Cyclic GMP and cyclic GMP phosphodiesterase were assayed as described in section 4.2.

lowest level at a point immediately before the next cell division. The peaks in cyclic GMP phosphodiesterase activity coincided with the cyclic GMP minima.

From this experiment and the experiment on cyclic AMP metabolism in the natural cell cycle (described in section 3.4) it appears that the peaks in cyclic AMP and cyclic GMP occur at very nearly the same point in the cell cycle, although the pattern of modulation of the two nucleotides is different. Regulation of important cell cycle events could be accomplished by the independent activation of cyclic GMP-dependent and cyclic AMP-dependent protein kinases.

4.5 An investigation of cyclic GMP metabolism in Tetrahymena pyriformis synchronized by a single hypoxic shock

Cells were synchronized as described in section 2.5. Intracellular cyclic GMP was determined as described in section 4.2. Cyclic GMP phosphodiesterase and guanyl cyclase were assayed by N.C.C. Gray as described elsewhere (257).

Fig. 40 shows that induction of division synchrony results in the production of a plateau of intracellular cyclic GMP. After commencement of reshaking, cyclic GMP falls to a minimum before cell division. The first synchronous division follows about 30 minutes after this minimum level of cyclic GMP. A "spike" of intracellular cyclic GMP is found to coincide with the first division. Cellular cyclic GMP subsquently rises to another maximum 58 minutes after the onset of cell division. Intracellular cyclic GMP then falls to a new minimum immediately before the start of the second division. After this point, cyclic GMP rises progressively.

After the start of hypoxia, guanyl cyclase rises rapidly and cyclic GMP phosphodiesterase quickly falls. The increased guanyl cyclase and decreased cyclic GMP phosphodiesterase activities are maintained at approximately the same levels throughout the 240 minute hypoxic period. Upon reshaking, guanyl cyclase activity falls to a minimum at 282 minutes, and cylic GMP phosphodiesterase rises rapidly to a

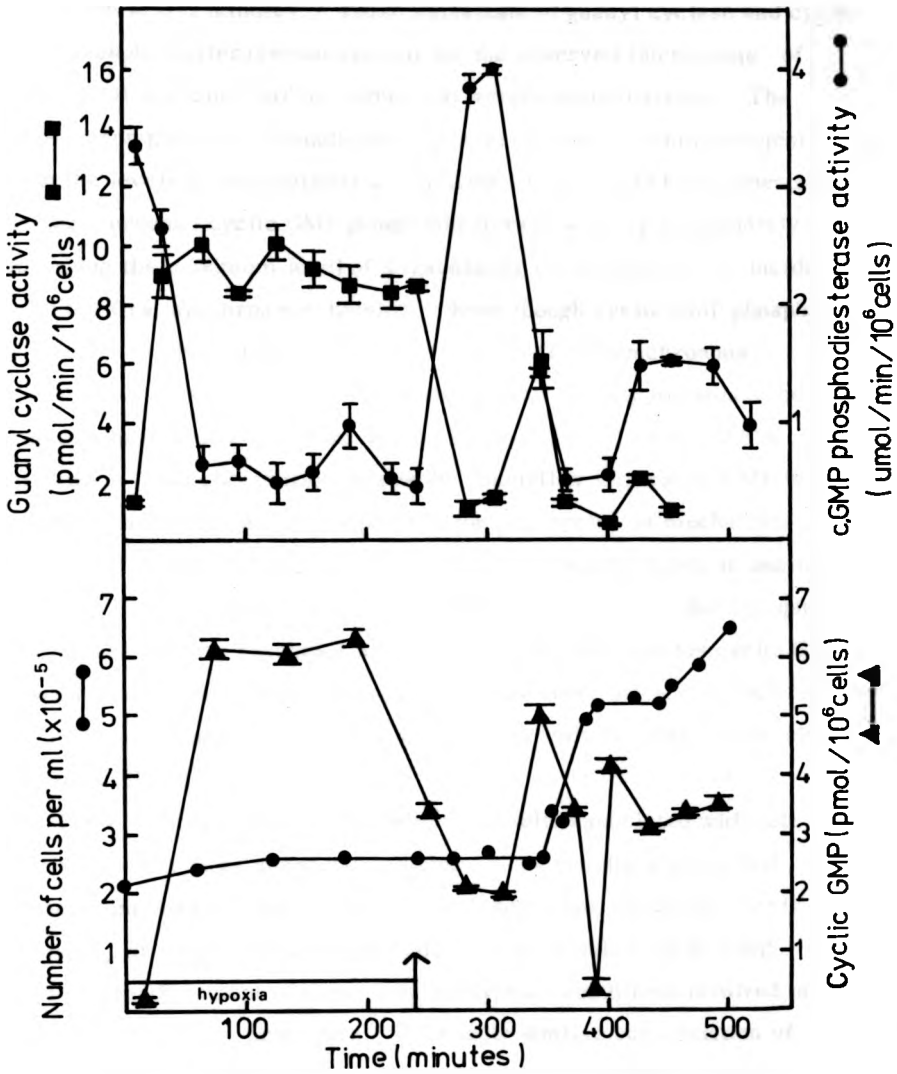

Fig. 40 Cyclic GMP metabolism in Tetrahymena pyriformis synchronized by a single hypoxic shock

Cells were synchronized as described in section 2.5. Intracellular cyclic GMP was determined as described in section 4.2. Cyclic GMP phosphodiesterase and guanyl cyclase were assayed by N.C.C. Gray as described elsewhere (257). The arrow indicates the point at which reshaking started.

maximum at 312 minutes. These variations of guanyl cyclase and cyclic GMP phosphodiesterase can account for the observed fluctuations of intracellular cyclic GMP up to the first synchronous division. The subsequent pattern of modulation of guanyl cyclase is also consistent with the levels of intracellular cyclic GMP. One would have expected to see increased cyclic GMP phosphodiesterase activity immediately following the maximum level of intracellular cyclic GMP that coincides with the first synchronous division. Even though cyclic GMP phosphodiesterase activity does rise shortly after the first synchronous division, it does not appear to rise sufficiently quickly to explain the rapid drop in cyclic GMP that is seen between 343 minutes and 384 minutes. This observation raises the possibility that cyclic GMP is excreted from the cell at this point in the cell cycle; (a mechanism which was postulated might exist in Tetrahymena pyriformis in section 4.3(iii)). Between 422 minutes and 492 minutes, cyclic GMP phosphodiesterase activity reaches a plateau, and after 492 minutes cyclic GMP phosphodiesterase activity falls. This subsequent pattern of cyclic GMP phosphodiesterase activity is consistent with the observed levels of intracellular cyclic GMP.

The elevation of cyclic GMP within the cells associated with cell cycle blockade is an unexpected finding. The results suggest that either high intracellular cyclic GMP is a signal for inhibition of cell division, or that this pattern of modulation of cellular cyclic GMP reflects readjustment of the cell to the hypoxic conditions involved in the synchronization procedure. A broadly similar accumulation of cyclic AMP was observed in hypoxic-shocked cells (see section 3.7). In section 1.3 it was pointed out that one of the most popular ideas has been the notion that cyclic AMP acts as a growth inhibitor and that cyclic GMP antagonizes the actions of cyclic AMP and promotes cellular growth (148, 105). Clearly, the results from this experiment are not in accordance with this idea, for it would appear that both cyclic AMP and cyclic GMP are elevated in the non-dividing hypoxic-shocked cells. Mention was made in section 1.3 that Pastan and co-workers

have made several observations that do not support the idea of cyclic GMP as an antagonist of cyclic AMP action in cell cycle control (158, 159). The study on BALB 3T3 fibroblasts in particular, in which they reported that addition of serum to non-growing cells decreased cyclic AMP and cyclic GMP with promotion of cell growth, along with their observation that cyclic GMP content of the cells rose as cell growth slowed (158), are in agreement with the findings described here.

The "spike" of intracellular cyclic GMP that coincides with the first synchronous division is similar to that which was observed for cyclic AMP in both hypoxia synchronized (section 3.7) and selection synchronized (section 3.4) cultures of Tetrahymena pyriformis. Furthermore, in selection synchronized Tetrahymena pyriformis cyclic GMP was found to be highest during cell division, and then to decline progressively to its lowest level immediately before cell division (see section 4.4). In this experiment cyclic GMP falls to minima which immediately precede the cell divisions. It seems probable that cyclic GMP falls rapidly in hypoxia-synchronized Tetrahymena pyriformis after division, giving the characteristic "spike" pattern observed because the cells have no G1 phase in this cell cycle system. (In section 2.5(ii) it was shown that G1 is either absent or extremely short in the cell cycle that follows the first synchronous division). If G1 were present, the decline of cyclic GMP would presumably be more gradual as it is in the natural cell cycle (see section 4.4).

It is interesting to note that when the cells are made hypoxic, cyclic GMP phosphodiesterase activity declines rapidly, whereas cyclic AMP phosphodiesterase activity declines slowly and by only a relatively small amount (cf. section 3.7). Both adenyl cyclase activity and guanyl cyclase activity rise rapidly after the start of hypoxia. It was suggested in section 3.7 that modulation of cyclic AMP levels during hypoxia is achieved mainly by adenyl cyclase, and that cyclic AMP phosphodiesterase appears to be mainly

responsible for controlling the fluctuations in cellular cyclic AMP content during the cell cycle after release from the hypoxia blockade. It seems that the cellular cyclic GMP content is modulated in hypoxia by both guanyl cyclase and cyclic GMP phosphodiesterase, and that control of guanyl cyclase activity, cyclic GMP phosphodiesterase activity and possibly a mechanism of excretion of cyclic GMP, are all required to achieve the observed modulation of cyclic GMP in the cell cycle after release from hypoxia.

From these findings, it would appear that elevated cyclic AMP and cyclic GMP is the cell's signal for inhibition of cell division. These results do not of course preclude different roles for cyclic AMP and cyclic GMP since differential activation of specific cyclic nucleotide-dependent protein kinases is always possible. Furthermore, it would seem that certain minimum levels of these intracellular nucleotides must be produced in order to allow the subsequent cell division. Although these idease are not in agreement with the notion of simple antagonism between cyclic AMP and cyclic GMP they do not exclude the more fluid concepts involved in the "Yin Yang" hypothesis of biological control (160). The precise reason for the more gradual decline of cellular cyclic GMP observed in the natural cell cycle (section 4.4) is not clear at this time. It may be that a certain level of intracellular cyclic GMP must be maintained in the G1 phase of the natural cell cycle. This possible cyclic GMP signal may be required to ensure that the enzyme thymidylate synthetase is "switched-on" sufficiently early in the cell cycle so that TMP (an essential precursor to DNA synthesis which will occur in the ensuing S-phase) can be made by the cell. There is already evidence for the involvement of cyclic GMP in control of the activity of thymidylate synthetase in Tetrahymena pyriformis for it has been reported that cyclic GMP can induce the synthesis of this enzyme (204). The measurement of thymidylate synthetase activity in the natural cell cycle of Tetrahymena pyriformis should give information as to whether this postulated mechanism exists or not.

Another important experiment would be to investigate whether cyclic GMP is excreted from Tetrahymena pyriformis at certain times in the cell cycle after release from hypoxia. This may be technically quite difficult; the measurement of cyclic GMP in the growth medium could be complicated due to the presence of so many components in the growth medium itself. Elaborate sample purification would be required, and the use of a radioimmunoassay would probably enable more certain measurement of relatively discrete alterations of the cyclic GMP content of the growth medium. It might prove necessary to grow the cells in a medium containing a radioactively-labelled purine, and then to shift the cells to fresh (non-labelled) growth medium in order to detect excretion of radioactively-labelled cyclic GMP.

CHAPTER 5

DISCUSSION

The studies described in this report suggested the existence of 3 cyclic nucleotide signals important in control of the cell cycle of Tetrahymena pyriformis . These were

(1) a certain minimum level of cyclic GMP (possibly about 4 pmol/10^6 cells) in cells that are in the G1 phase of the natural cell cycle. This cyclic GMP signal may be responsible for dictating the timing of DNA synthesis by ensuring that thymidylate synthetase is activated (by a process probably involving both transcription and translation), so that the DNA precursor TMP is available for the subsequent S phase.

(2) Elevated cyclic AMP and cyclic GMP associated with cell cycle blockade in hypoxic-shocked cells. The similarities between the 240-minute hypoxic shock technique and the multiple heat shock technique (outlined in section 2.5(iii)) and the fact that many of the methods of inducing division synchrony in Tetrahymena pyriformis appear to act through the same mechanism (182), suggest that explanation of the role of these cyclic nucleotide signals ought to be possible in terms of Zeuthen's 'division protein' model described in section 1.4(i) and Fig. 9. One should remember too that the eukaryotic cell cycle is not merely a series of inter-related biochemical events, but also comprises a number of dramatic morphological events. It is well documented that cyclic AMP can elicit morphological changes in cells (259-265) as well as biochemical changes, (though of course the morphological events are brought about by biochemical processes). The cyclic AMP-induced morphological changes all involve microtubules, and have been classified into 3 possible mechanisms (266): (a) cyclic AMP permits or promotes the reorientation of assembled tubules, (b) cyclic AMP stimulates the de novo synthesis of tubulin, shifting the equilibrium towards the assembly of microtubules, (c) cyclic AMP stimulates the assembly of

microtubules without affecting the synthesis of tubulin. The elevated cyclic AMP observed in hypoxia could be to direct construction of microtubules that are destroyed during hypoxia. In other words, if hypoxia causes a destruction of division proteins as is postulated to occur with multiple heat shocks (31), then the cell is trying to reassemble these division proteins (microtubules) so that as soon as release from blockade occurs, it can divide. The purpose of elevated cyclic AMP could be to direct synthesis of new tubulin, since recovery from heat shock requires protein synthesis (183).

Yuyama has shown (258) that synthesis of messenger RNA is required after the last heat shock for the first division in multiple heat-shock synchronized Tetrahymena pyriformis. This RNA synthesis appears to be related to the furrowing process, and may be the messenger that codes for synthesis of division protein. If multiple heat shocks and 240 minutes of hypoxia do have a similar effect on cell division in Tetrahymena pyriformis, then it is possible that the elevated cyclic AMP observed in hypoxia is to signal production of this messenger RNA as soon as environmental conditions permit. (The signal could be transmitted via a cyclic AMP-dependent protein kinase which phosphorylates a specific acidic chromosomal protein, thereby directing transcription of a particular gene as described earlier in section 4.3(iii)). The reason that intracellular cyclic AMP levels are so high in hypoxia (more than 4 times the maximum level observed in the natural cell cycle), could be that the cyclic AMP signal is expressed more than once, because a feed-back mechanism which would switch off adenyl cyclase and switch on cyclic AMP phosphodiesterase does not function, due to non-production of the essential cell product.

Elevated cyclic GMP during hypoxia could represent what is normally a G1 signal occurring in G2. There is little or no G1 phase in cells which have been synchronized by a 240-minute hypoxia shock. DNA synthesis starts almost as soon as cell

division is complete. This implies that the cells are already prepared for the next S phase before they have even divided. The occurrence of the postulated (G1) cyclic GMP signal during hypoxia (G2-blocked cells) could account for this.

Brinkley et al. used monospecific antibody directed against tubulin as an immunofluorescent probe to evaluate the distribution of microtubules in various cell lines (267). In all the normal cells observed, it was found that the extensive cytoplasmix microtubule complex disappeared during mitosis. As the cells entered early prophase, the cytoplasmic microtubules began to disappear, by metaphase, microtubules in the cytoplasm had completely disappeared, and most of the fluorescence had become localized within the mitotic spindle. During late telophase-early G1 period, the cytoplasmic tubules reappeared in each daughter cell. Of course, Tetrahymena pyriformis divides amitotically (268), but it has been shown that macronuclear microtubules are required for the terminal stages of nuclear elongation and separation (269).
(3) The spike of cyclic AMP observed in the natural cell cycle that occurred at the division/G1 interface could be responsible for directing reassembly of microtubules for use in the cytoplasm from used tubulin derived from the microtubule network involved in cell division. This could explain the rapid change in cell shape that occurs after completion of division; the new (rounded) daughters quickly assume a pyriform morphology after division. If de novo synthesis of tubulin were required, followed by assembly of new cytoplasmic microtubules, the daughter cells would be expected to remain rounded for a longer time than is observed. Maintenance of high intracellular cyclic AMP levels when the cell becomes quiescent would seem to represent a different type of signal in that the cell is directing the expression of new genes to enable itself to survive in what has become a less favourable environment. The location of a point in G1 where the cell may

either initiate a further round of cell division or become quiescent would seem to represent the most efficient point in terms of its requirement for energy and raw materials. If some preparation towards DNA synthesis or cell division were made before the decision point, this would represent a wasteful use of resources for a cell that is not going to divide but needs to adapt itself to an altered environment. A recently divided daughter cell is complete in all cellular constituents and is thus in a most convenient place to "decide" whether it shall re-enter the proliferative pathway or become quiescent.

APPENDIX

1. Derivation of the synchrony index.

The synchrony index used throughout has been the one developed by Blumenthal and Zahler (211). Their reasoning is as follows:

If the number of cells in a culture increases from N_o to N in an interval, t, less than one generation time, g, then

$$N/N_o - 1$$

is the fraction of cells in the culture which divide during t, and

$$2^t/g - 1$$

is the fraction of cells in the culture which would have divided during t if the culture had been growing logarithmically.

The quantity

$$F = (N/N_o - 1) - (2^t/g - 1)$$

$$= N/N_o - 2^t/g$$

measures the fraction of the population which divides during t in excess of that expected to divide during logarithmic growth in the same interval.

The synchrony index, F, has a maximum value of +1 if the entire population divides within an infinitely short time. F has positive values less than 1 if doubling takes a finite time (which, of course, it always does in reality), or if less than the entire population divides during the measured interval. After a synchronized burst of divisions, the population must increase at a rate less than that of normal logarithmic growth, and F falls to negative values. Thus the criterion for synchronized cell division is a positive value followed by a negative value. (The index should never fall below -1).

2. Synchrony indices and cell cycle times in the synchronous cultures studied.

Synchrony indices were calculated using Blumenthal and Zahler's synchrony index described above. Cell cycle times of selection-synchronized cultures were measured between onset of the first and second synchronous divisions. Cell cycle time has no precise meaning in the hypoxia-synchronized system; the timing of the various events was mentioned in Section 2.5(ii).

Synchronization technique	Growth medium	Cell cycle time (minutes)	Synchrony index (F)
Wolfe's method (181)	PPYF	185 ± 6	0.58
Magnetic method	PPYF	180 ± 5	0.26
Magnetic method	PPYFG	180 ± 3	0.77
Hypoxic shock	PPYFG	–	0.81

APPENDIX Table 1

Synchrony indices and cell cycle times in the synchronous cultures studied

The synchrony index (F) is the quantity devised by Blumenthal and Zahler (211), and which is explained fully in Section 1 of the Appendix. Cell cycle times were measured as described in Section 2 of the Appendix.

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