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THE CONTROL OF MITOSIS IN MAMMALIAN TISSUES

A thesis presented for the degree of

Doctor of Philosophy

by

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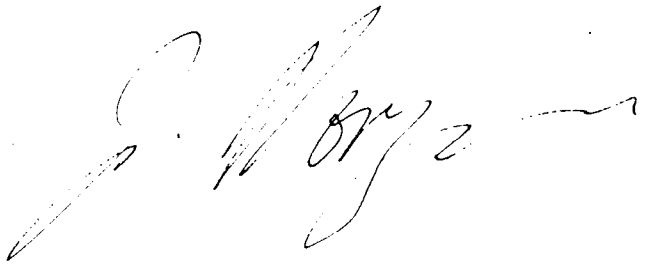
SUMMARY

The question of which factors are central in determining whether a cell will undertake a new round of mitosis or will decycle has been examined in the isolated thymic lymphocyte model. Such cell populations possess both *in vivo* and *in vitro* a subpopulation of quiescent lymphoblasts which may be induced to reinitiate their mitotic programme. In the intact animal the major determinant of proliferative activity is the plasma ionised calcium concentration. However it has been established in culture that a variety of hormones, ions, cyclic nucleotides, plant lectins and ionophores may like calcium elicit a mitogenic response. These agents do not appear however to initiate DNA synthesis in an identical fashion. Rather there are two distinct intracellular mitogenic axes. The first axis includes a number of adenylate cyclase stimulants, cyclic AMP, phosphodiesterase inhibitors and magnesium ions. It was found that all these mitogens required extracellular magnesium ions to exhibit their stimulatory capacity. This dichotomy in mitogenic activity was further emphasised by the observation that these mitogens are all inhibited by testosterone, whilst the magnesium-independent mitogens were insensitive to this androgen. Indeed this second group of stimulatory factors required the presence of calcium ions in the extracellular milieu for activity, and were, in contrast to the magnesium-dependent mitogens inhibited by the presence of oestradiol in the culture. By examining the interrelationships between these various mitogens and inhibitors it has been possible to propose a mechanism to describe the activation process in the thymocyte.

Studies of the metabolism of cyclic nucleotides, membrane potential and transmembrane ion fluxes indicate that there may be a complex relationship between membrane fluidity, ion balance and cyclic

nucleotide levels which may individually or in concert promote the initiation of DNA synthesis. A number of possible mechanisms are discussed to account for these observations.

I declare that the data presented in this thesis has not been submitted for any other degree. The experiments and results cited in Units 6 and 7 were performed in collaboration with Dr. J. Bramhall of the Pharmacy Department, University of Aston in Birmingham. In these studies I accept responsibility for the cell culture techniques and ion transport methodology, but rely on the expertise of my colleague for the assembly and proper functioning of the fluorimetry apparatus. The adenylate cyclase assays reported in Unit 5 were performed in the Department of Chemical Pathology, the University of Sheffield Medical School, using a method already established there. This work was part of a collaborative study with Dr. N.H.Hunt of that Department. Dr. Hunt also performed the cyclic GMP assays cited in Unit 5 on samples prepared by myself at Aston. All other sections of this thesis have been performed independently.



James Ian Morgan. 26·10·76

Acknowledgements

In coming to acknowledge the help and advice one has received during a three year period one is immediately aware of the vast number of people to whom one is indebted. For this very reason it is all too easy to omit acknowledging the services of many acquaintances, to these people I must apologise in advance for my lapse in memory.

Acknowledgements seem to fall into three categories:- (1) Practical (2) Social and (3) Academic and I will express my thanks under these loose headings. Thus I would like to acknowledge the kind gifts of reagents from Drs. Judith Poat, W.A.M. Duncan, H. Sheppard and A.S. Waggoner. I cannot also help but thank Dr. T. Atkins for those occasional few milligrams of obscure compound and his advice on how to dissolve them. It would not be possible to overestimate the help received during my stay at Aston from Mrs Y.M. Pugh in preparing many experiments and lending the extra pair of hands when needed. Under the same heading must come the secretarial and technical staff of the Department of Biological Sciences who have at one time or another proved helpful in a crisis. Of course a University education is also a function of discourse with ones fellow academics, which often seems to occur over a pint of beer after hours. In this context I would like to ackowledge the contribution of many beings to my education these include David Edwards, Tom Brown, Jeremy Ward, Graham Hansen and my good friend Chris Wigham.

For their interest and help in my career I would like to thank Professors A.J. Matty and T.J. Martin and Dr. A.Z. Britten to whom I am indebted for my current position.

This list of acknowledgements contains three notable omissions

to whom I have a particular debt to acknowledge. The first is my friend and colleague John Bramhall, with whom I performed many of the experiments described in the latter sections of this thesis. I thank him for his wit, insight and tolerance as well as his giving me another perspective of science. I should also like to single out for special thanks Dr N.H. 'Pete' Hunt for his help whilst still an inmate of room 321B and even more so on his translation to Sheffield University. This help has been most manifest in his contributions in Unit 5 on adenylate cyclase metabolism and cyclic GMP assays. To him 'cheers skip'. It is customary to acknowledge the contribution of ones supervisor last of all, and I shall not deviate from this convention. The influence of Dr. Alan Perris on my life could well be classed under all the headings listed at the beginning of this text, not the least of these being social. However, I should especially like to thank him for his guidance, scientific training, interest and tolerance. And of course counting those few extra cells at half past five. I cannot express my gratitude in any more positive a way than dedicating this thesis to him. Thanks boss.

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FOREWARD

The biological sciences have surely become the most productive scientific discipline if one gauges this by the large and increasing volume of literature generated during the nineteen sixties and seventies. Given this plethora of information and the somewhat descriptive and qualitative nature of biology, it is not surprising to note a trend towards larger literature surveys in an attempt to reflect this published material. Indeed one can now feel certain that even a small and obscure topic will have attracted sufficient interest to produce a wealth of information. Thus in attempting to introduce a research project the worker often feels like the mythical Sisyphus; thinking he has at last adequately reviewed a particular subject then finding a new publication post-dating previous work or developing another facet of the same problem.

Unfortunately these large introductory dissertations have associated with them a number of disadvantages which are enumerated below:-

(1) For practical reasons the introduction to a thesis is generally the first section to be compiled, and may even be completed many months prior to the termination of experimentation. Hence often quite relevant portions of the literature survey may be outdated, or represent an inadequate appraisal of work carried out subsequent to its completion.

(2) It is very difficult to maintain a theme throughout a fully comprehensive survey. This can unfortunately make for poor reading and comprehension.

(3) Perhaps the most important point is that information relevant to an experiment may have appeared many pages previously, and have been lost amidst a mass of other facts and data.

(4) A reader must find it very tedious to absorb section after section whilst having little foreknowledge of the relative importance of each of these sections to the reported findings.

Since one is therefore torn between producing an adequate literature survey, yet maintaining continuity, readability and comprehensibility, the classical format of introduction, methods, results and discussion has been dispensed with. Rather the findings have been organised into a number of units which represent basically different areas of research. Furthermore these self-contained units have been written to stand by themselves as separate, comprehensive pieces of work, and as such more closely follow the classical layout mentioned above.

The thesis itself is preceded by a general introduction which has developed the theme of cell population dynamics and its regulation from the prokaryotes through lower eukaryotes to the mammals. Diverse aspects of this question of the regulation of cell growth and development have been considered, and an attempt has been made to integrate these facts into a coherent pattern which relate to the particular proliferative problems encountered in different organisms. Much of this evidence is of a conceptual nature and merely forms the theoretical background into which the results reported here fill but a small niche. Indeed as the majority of this data has no direct applicability to this study, no paper published subsequent to March 1st, 1976, has been included.

In contrast each unit contains its own relevant review material appearing alongside pertinent experiments. Although some of this data has been alluded to in the general introduction, where appropriate this has been greatly extended and updated to make each unit as comprehensive as possible. The discussion section of each unit whilst considering predominantly the relationship between the reported results and directly applicable theory, also draws on 'peripheral' evidence where available to indicate possible homologies with other areas of research.

At the end of the thesis a section attempts to integrate the conclusions from each of the units into a cogent hypothesis.

INTRODUCTION

(1) The forces which shape cell proliferation

Implicit in the title of this thesis is the notion that not all organisms or classes of organisms regulate their bodily cell balance in the same fashion. I would suggest that the mode of cell proliferation control adopted by an organism reflects certain constraints or pressures imposed on it by both its external and internal environments and its physical structure. This concept will be demonstrated employing an evolutionary approach to the consideration of cell dynamics and cell cycle regulation. Such a treatment may also serve as a vehicle for the introduction of phenomena thought to be relevant to cell balance systems.

Although no less evolved we generally consider the unicellular organisms to be the most primitive life forms, and that they most closely resemble original primordial life. If one accepts this supposition then one might expect that these organisms would exhibit those features of proliferative regulation which would be considered fundamental and basic to all life forms.

The precise reason why cells divide at all is still a matter of conjecture. However a number of propositions have been put forward relating cell volume to metabolic efficiency. Thus it it has been suggested that as the size of a cell increases so does the physicochemical gradient for gases and nutrients. There will then be some critical cell surface area/volume ratio which when exceeded results in a metabolic decline in a given cell. It is also generally accepted that liposomes and coacervates, artificial lipid systems which are thought to represent early cell membranes,

tend to become unstable and fragment when their diameters reach some critical value. Thus it is feasible that primitive cells exploited this property of coacervates, by modulating its content of lipids to yield a system which would divide when the critical cell volume ratio was exceeded. Or conversely life forms evolved from a coacervate which happened to be unstable at a diameter which was just greater than for instance the effective diffusion gradient for oxygen in a lipid/water environment. It might then be expected that more sophisticated mechanisms would have evolved to facilitate this cell cleavage process.

Amongst the primitive unicellular organisms (prokaryotes) it is apparent that given an adequate supply of nutrients, suitable conditions of temperature and pH, and the correct atmospheric conditions, that anabolism will exceed catabolism. Thus the cell will increase in size. As suggested for the primordial life forms, at some point the cell will experience a difficulty in obtaining nutrients and gases from its surrounding environment because of the slow diffusion rates. Hence it is probable that the cell possesses some mechanism for monitoring its volume, and this might be the same system which triggers cell division. However, since the genetic heritage of the cell is enshrined within a stable DNA (deoxyribonucleic acid) molecule, a further major problem in the division process must be its replication and precise and equal sequestration into two daughter cells. Clearly then the cell not only requires to determine when to initiate division but must also monitor and integrate a number of complex biochemical processes in order to ensure a viable progeny. It might also be pointed out that during this process of DNA replication and segregation there occurs the opportunity for genetic recombination an important feature when

considering the ability of an organism to adapt to its environment. Indeed the bacteria have also developed primitive procedures for enhancing their rate of recombination, thus ensuring greater genetic diversity. However these processes are somewhat fortuitous (e.g. transformation, conjugation and transduction) involving little alteration in their life cycle.

In contrast higher organisms have developed more precise mechanisms for recombination, which involve the expression of alternative life cycle programmes, and which utilize a meiotic as opposed to a mitotic segregation. These unicellular organisms then must not only integrate those cell processes which regulate cell proliferation as in the bacteria, but must also have systems for controlling the transition to different cell cycle patterns. Inasmuch as one cell has the potential to give rise to another cell with a different phenotypic expression without mutation this must be classed as an example of differentiation; and these lower eukaryotes must possess mechanisms for regulating this phenomenon.

However the problem of the control of differentiation is generally more acute in multicellular organisms. The move towards multicellularity has ultimately meant the evolution of organisms in which the fusion of two gametes gives rise to a life form containing many cell types via the manipulation of their genomic expression. These functional cell types have tended to become organised into tissues and organs for the more efficient performance of their designated roles. However these structures must act in a concerted and coordinated fashion for the existence of the organism to be maintained. Thus it is apparent that multicellular organisms must possess mechanisms which not only commit and maintain a cell in a

given avenue of differentiation, but which also ensure that the size and functional output of the tissue is related to demand. Thus one might envisage that a cell population possesses the ability to replace lost differentiated cells, or to enlarge a tissue by tipping the balance of cell birth to cell loss in favour of cell production in response to an increased demand on that tissue. Furthermore if cells divided ad libitum within an organism it is difficult to envisage a system whereby anything but a small number of cell types could be integrated to perform a balanced function. This would be in addition to the difficulty such an organism would find in establishing a stable ecological niche. Certainly a tissue undergoing rapid expansion through uncontrolled cell proliferation would face the problem of nutrient supply to cells at its centre. Thus in a solid cancer necrosis often occurs in the central regions where cells do not recieve adequate substrates, and where toxic wastes accumulate. Thus in higher animals and plants one does not have the problem of initiating cell division but of actually inhibiting its occurence. This problem has been overcome in a number of ways as will be made apparent in later sections of this introduction.

As has been suggested the modes of proliferation regulation found amongst the prokaryotes probably represents the most basic paradigm for growth/division control, and for this reason will be considered first.

(2) The control of division in prokaryotes

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Martin Slater, 1974.

In considering prokaryotic cell division and its regulation, it must be said that the literature deals predominantly with the bacteria to the exclusion of the blue-green algae, and with relatively few bacteria at that. This state of affairs has arisen partly through convenience, but mainly through an adopted convention to work on Escherichia coli; hence most of the data presented here will have been performed on this organism unless otherwise indicated.

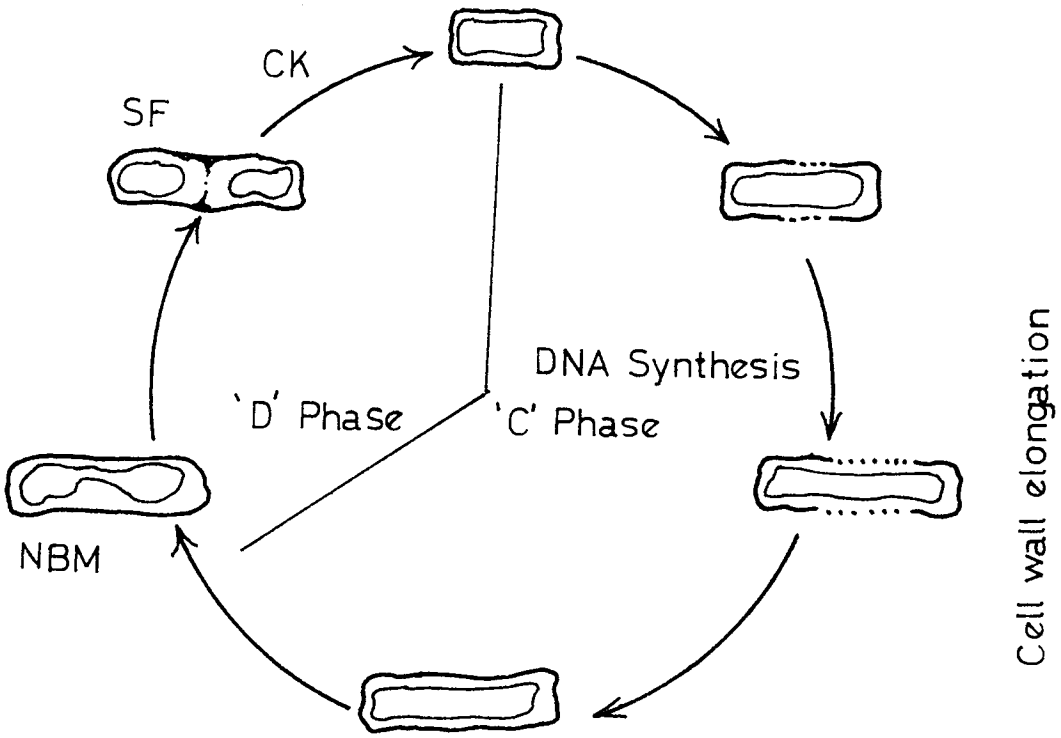
Clearly it is first necessary to define which criteria can be used to assess the action of different agents on bacterial proliferation. One simple indication of division rate is given by monitoring cell numbers. Using this parameter as an index of proliferative activity it has proved possible to demonstrate the action of a number of treatments on cell multiplication. Thus nutrient deprivation (Pritchard and Lark, 1964), inhibition of protein synthesis (Yoshikawa, 1965), temperature alterations (Scott and Chu, 1958) and DNA synthetic inhibitors (Pierucci, 1968) have all been shown to affect the cellularity of bacterial cultures. Thus in general the overall rate of bacterial cell proliferation is determined by the environment in which the organism finds itself. When there are large amounts of nutrients available and conditions

of pH and temperature are suitable, a high rate of division is observed, in less favourable circumstances lower rates are seen. However this might be deemed a somewhat one dimensional approach to the problem of growth control, as we have merely delineated those features which are important, but have not ascertained how or why they should be important. To answer these types of questions requires the knowledge of what changes occur in the bacteria during their cell-division cycle so that specific effects may be related to some particular portion of the bacterial life cycle. It is at this point that the quotation of Slater cited above finds its relevance, since it has proven extremely difficult to discern many features of the bacterial cell cycle, which are readily determined, and which are capable of relating a given agent to bacterial life cycle compartments. Whilst reviews on the topic of bacterial division have been produced recently (Higgins and Shockman, 1971; Bonhoeffer and Messer, 1969) a brief outline will be given here before considering what is known of the control of this process.

The cell cycle of most organisms is generally described from the point at which a daughter cell is formed from its parent undergoing cell fission (cytokinesis). This convention will be adopted in this thesis also. Thus as shown in figure (1) the newly formed cell grows in size, which also requires the rigid bacterial cell wall to be elongated at 'membrane growth sites' to accommodate this increasing mass (Higgins and Shockman, 1971; Ryder, Hirota and Schwartz, 1973). Depending on the growth rate the DNA content of the bacterium is normally replicated in the early part of the cell cycle (Cooper and Helmstetter, 1968), with a cessation of synthesis prior to division itself. The period of DNA synthesis is frequently termed the C phase, whilst the post synthetic gap is often referred

to as the D phase. However if the rate of division is elevated sufficiently a fresh round of DNA synthesis may occur before the cell has undergone cytokinesis, indeed a bacterium may contain many replicating forks (Cooper and Helmstetter, 1968). Thus it is apparent that the rate of cell growth is closely linked to the initiation of DNA synthesis, ensuring that each daughter cell receives its 'genetic dues'.

Figure (1) A Diagramatic Representation Of A Typical Bacterial Cell Cycle.

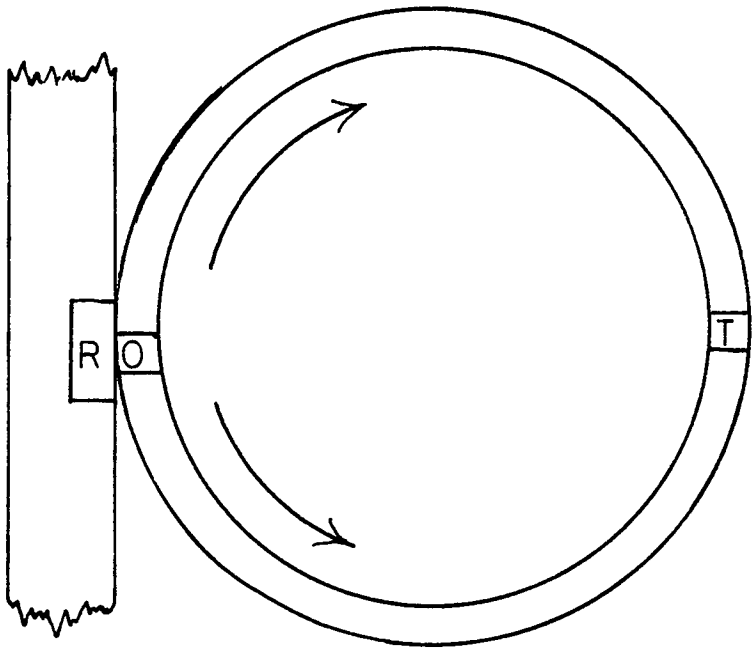


NBM= nuclear body migration and separation; SF= septum formation; CK= cytokinesis;'C' Phase is equated with the DNA synthetic phase and the'D' Phase is the post-synthetic gap.

It might be added that replication in the bacteria is somewhat anomalous, a feature which has contributed largely to their classification as prokaryotes (Stanier, 1961). In E. coli the genome consists of a naked circular strand of DNA (Cairns, 1963) without

the associated histones present in eukaryotes (Bloch and Goodman, 1955). The single chromosome is often observed attached to the bacterial cell membrane, and further evidence (Smith and Hanawalt, 1967; Sueoka and Quinn, 1968) has led to the suggestion that the genome is anchored at its replicating fork to the cell wall (See Figure (2). Furthermore the enzymes responsible for DNA polymerisation

Figure (2) A Scheme To Illustrate The Replicative Apparatus Of E.coli



DNA synthesis is initiated at a common site or origin (O) through the activity of replicative enzymes (R) which lie in the bacterial cell membrane. Replication proceeds in both directions, indicated by arrows, and halts at a distinct terminator site (T).

may themselves be an integral part of the cell wall (Sueoka and Quinn, 1968). Mitchison (1971) discusses a number of models which could operate via such an apparatus to facilitate chromosome separation. It is also apparent that replication always commences at the same point on the chromosome in successive rounds of division,

this region being termed the origin (Pritchard and Lark, 1964). Synthesis then proceeds from the origin in both directions (Masters and Broda, 1971) and is terminated at another distinct point on the chromosome, the terminator (Sueoka and Quinn, 1968; Kuepel, 1973).

During replication there occurs the separation of the nuclear bodies, which are analagous to the nuclei of eukaryotes, but which lack both nuclear membrane and nucleolus. In E. coli this process begins at around the mid-point of the cell cycle (Adler and Hardigree, 1971) that is towards the end of the 'C' phase. Subsequently there is an involution of the bacterial membrane, which precedes the laying down of a cross wall or septum (Figure 1) which partitions the bacterium into two daughter bodies, each containing a copy of the parental genome (Chapman and Hillier, 1953). Finally cell separation is facilitated by the production of hydrolytic enzymes (Fan, 1970).

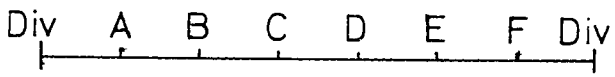
A number of experimental procedures have been employed to ascertain the action of various agents on these discernible cell cycle landmarks. One technique is to inhibit selectively, at various stages of the cycle, metabolic processes such as DNA, RNA or protein synthesis and to monitor the fate of the cells.

In general cell division in the bacteria is closely linked to DNA synthesis, thus treatment of bacterial cultures with nalidixic acid results in the cessation of both DNA synthesis and proliferation (Clark, 1968). In addition a series of bacterial clones have been isolated which bear single gene mutations resulting in the generation of defective gene products which are required for the initiation or maintainance of normal DNA synthesis. However the defective nature

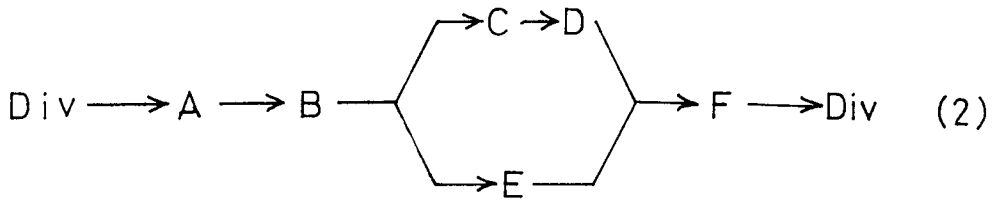
of the product is only apparent under certain well defined 'restrictive conditions', whilst it functions normally under a wide range of other situations. Such mutations are said therefore to be conditional, and the restrictive condition usually exploited is temperature sensitivity. Using such an approach it has been possible to isolate seven distinct mutant bacterial strains for DNA synthesis (designated dna$^-$ A to G). Furthermore transfer of these mutants to restrictive conditions results in the termination of both replication and cytokinesis (Clark, 1968).

However despite this apparent dependence of the bacterial

Figure (3) Possible Causal Relationships In The Cell Cycle



(1)



(2)

Figure (3)-1 indicates that between successive rounds of cell division (div) there are six discernible cell cycle features (A through F) which may be placed in a temporal sequence. However, whilst one can envisage that each landmark is dependent upon the occurence of the one immediately prior to it (i.e. A causes B, B causes C and so on) there is no a priori reason why this should be so. Figure (3)-2 indicates other possible relationships between cell cycle features. Thus it indicates that A and B may be linked causatively, and are therefore part of a dependent pathway, as are C and D. However, the event E occurs independently of C, and is not caused by C or D. Thus the pathways C, D and E are independent. The landmark F, if it requires the completion of both the pathways C, D and E to be executed, is clearly an interdependent stage. In contrast completion of either pathways may be sufficient, in which case it is merely dependent.

fission event upon prior DNA replication, a number of results indicate that there may not be a simple causal chain linking synthesis to cytokinesis (Figure (3)). Firstly, the dna$^-$ mutants, which are unable to synthesize DNA or divide under restrictive conditions, may acquire the ability to divide without prior replication if they also bear a second (div) mutation (Hirota et al., 1968). Secondly, bacteria maintained at high growth rates may replicate their DNA but not undergo cytokinesis, thus forming multinucleate, aseptate filaments (Slater and Schaecter, 1974). Finally again at high growth rates the cell may contain many DNA replicating forks which initiate at different times during the cell cycle (Cooper and Helmstetter, 1968). Therefore it must be concluded that (a) DNA synthesis may occur in the absence of cytokinesis and (b) division may occur in exceptional circumstances without a preceding round of replication.

Whilst inhibition of DNA synthesis normally results in the blockade of septum formation, this treatment does not affect cell wall elongation (Slater and Schaecter, 1974). Thus there may be interactions between the DNA synthetic event and specific aspects of bacterial membrane synthesis. Indeed bacteria which have had their replicative apparatus blocked exhibit distinct physical and chemical differences in their membrane components (Inouye and Pardee, 1970). Recently a protein has been isolated from the outer cell membrane of E. coli which plays a central role in membrane growth and development, and whose synthesis is regulated by the state of replication (James, 1975). This protein appears transiently at the beginning of the cell cycle, where it promotes cell wall elongation. However this agent may be induced at later stages of the growth/division cycle if DNA synthesis is blocked. At these later times it acts to inhibit septum formation. Thus although cell elongation may occur in the absence of

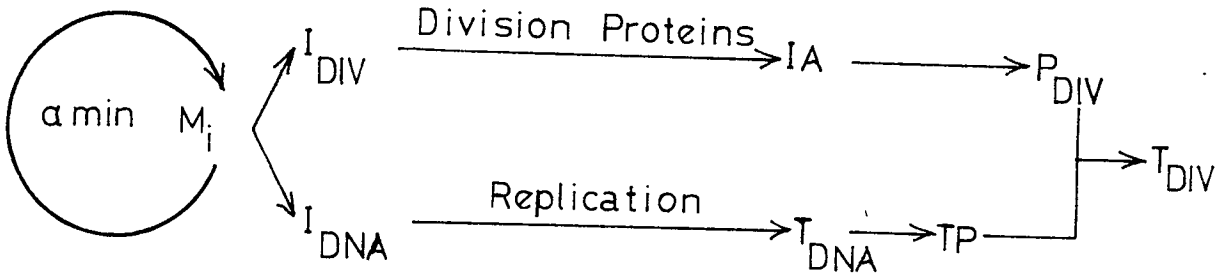
replication, cell death is avoided by preventing cell division in the form of an inhibition of septation.

The synthesis of specific proteins is likely therefore to be a key factor in the timing and integration of the bacterial cell cycle. It has been demonstrated that there is a requirement for ongoing protein synthesis during the 'C'phase, whilst the 'D' phase has no such requirement.(Pierucci and Helmstetter, 1969). Furthermore the initiation of DNA synthesis itself is effected through a specific protein generated at the beginning of the cell cycle (Lark and Renger, 1969). It has been suggested that some of the proteins synthesized during the 'C' phase are intimately involved in the process of cytokinesis (Adler, Fisher and Hardigree, 1969). This notion derives from the observation that filament form bacteria when returned to slower growth conditions undergo rapid septation in the correct positions (Adler et al., 1969). Thus it is as though these bacteria have acquired or accumulated 'division potential' during their phase of rapid growth. This potential is believed to be a protein(s) which both initiates and defines the site for septum formation, but whose activity is nullified under conditions of both very high and zero DNA synthesis (Adler et al., 1969). There are therefore important links between DNA synthesis, membrane growth and development and cell division itself which are mediated by specific proteins.

As mentioned previously the initiation of DNA synthesis is triggered by the production of a specific protein (Lark and Renger, 1969). The stimulus for the production of this gene product is the attainment of a critical ratio between cell mass and number of chromosome origins (Donachie, 1968). It is uncertain how this is effected in practice although the dilution of a gene repressor

substance would be one explanation amongst several (Mitchison, 1971). This gives the bacterium a link between cell volume and the initiation of DNA synthesis and division, a feature which should be of some importance in terms of cell efficiency. Another link between replication and cytokinesis has recently come to light in that there is a short period of RNA and protein synthesis required at the time of chromosome termination (Jones and Donachie, 1973). This protein is essential for some stage in cytokinesis. Figure (4) illustrates the model proposed by Jones and Donachie (1973) to explain these results. They suggest that at every mass doubling time, the ratio of cell mass to chromosome origins reaches a critical value (M_i)

Figure (4) A Model For The Control Of DNA Synthesis And Cytokinesis.



The diagram, modified from Jones and Donachie, 1973 represents a theoretical control model for the cell cycle of E. coli. Alpha minutes is the mass doubling time, and M_i the critical ratio of cell mass to chromosome origins. The latter causes the initiation of both DNA synthesis (I_{DNA}) and the cell division sequence (I_{DIV}) which involves the synthesis of specific division proteins. At the time of the cessation of DNA synthesis (T_{DNA}) a specific termination protein is synthesized (TP). When the concentration of the division proteins reach some critical level they initiate an event (IA) which in turn generates a protein P_{DIV} which interacts with the terminator protein in some way to complete some late stage of division (T_{DIV}).

which causes both the initiation of DNA synthesis and the production of the division proteins mentioned previously. The two pathways being largely independent of one another once initiated. Termination of chromosome replication induces the synthesis of a terminator

protein. Sufficient of this agent is generated within five minutes of chromosome termination for cell division to occur, however the cell fission event also requires that the second 'division protein' pathway should have reached some critical point. That is this second mechanism acts as a timing device for cytokinesis. Termination protein interacts with either a division protein or some other product which is induced by these agents to promote cell division. It is argued that cytokinesis occurs almost immediately after this interaction (Jones and Donachie, 1973). Thus termination protein whilst not strictly timing cell division does ensure that it cannot occur before DNA synthesis has been accomplished.

Clearly this model does not incorporate all the known factors involved in the regulation of bacterial cell division, most notably the initiator protein for cell elongation. However, it does give a basic outline of the possible type of control mechanisms which will account for the initiation of cell division and its integration with the DNA synthetic phase, although it does not account for the numerous alternate sequences which are seen (Slater and Schaecter, 1974).

If I might be permitted to quote once more from the paper of Slater and Schaecter, this might serve as perhaps the best conclusion concerning the current state of the knowledge of the control of bacterial cell proliferation.

> 'The complexity in regulation of cell division in the bacteria may be attributed in part to their relative structural simplicity. Bacteria have not evolved the complex machinery

> that ensures the equipartition of the genome in higher cells. It seems likely that they utilize structures and regulatory mechanisms that may be used also for other cell functions. Therefore it is not surprising that there is not a unique cell cycle but rather a normal sequence of events with alternate sequences should the normal one be interrupted.'

Thus to summarise the most important concepts in the regulation of bacterial proliferation:-

(1) The rate of cell division under normal circumstances is governed by the nutrient status of the extracellular milieu.

(2) The potential of the environment for supporting growth is linked to the initiation of the cell division cycle by some mechanism which titrates cell mass against DNA content.

(3) The attainment of a critical mass/DNA ratio appears to induce the synthesis of specific proteins which initiate two regulatory pathways, one being replication and the other cell elongation. The proteins may be induced through a gene repressor dilution mechanism.

(4) Generally during undisturbed growth DNA synthesis is a prerequisite for cytokinesis. However, membrane growth occurs independently of DNA synthesis and vice versa. Whilst it is apparent that the timing of septation is controlled by the accumulation of proteins generated independently of replication, premature division

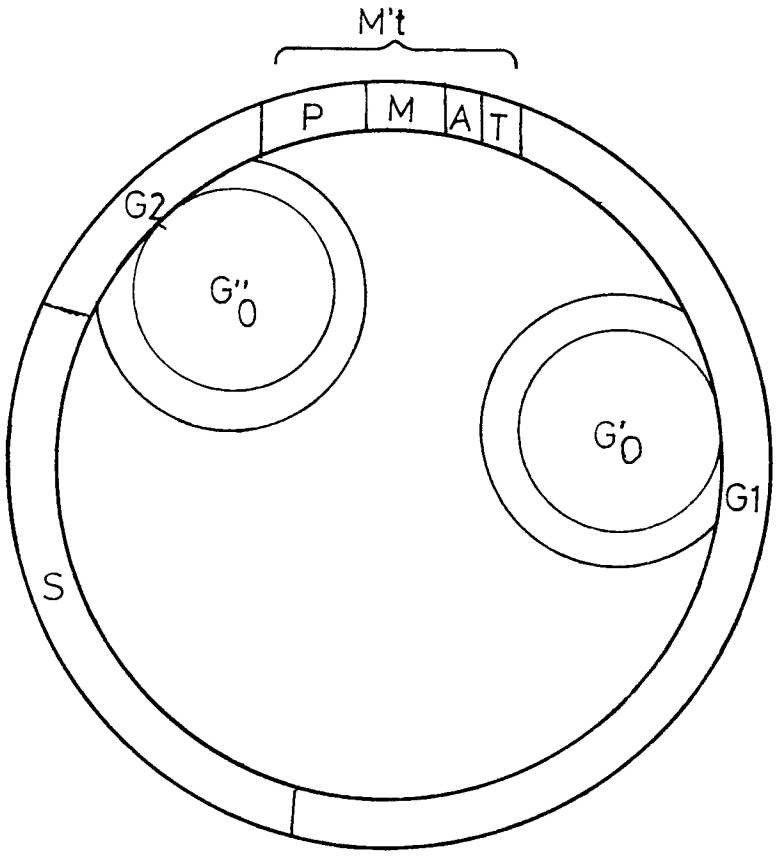
is avoided by the interaction of protein produced at the time of chromosome termination with some aspect of septum formation. Thus in the absence of this protein septum formation is inhibited.

(5) As a general conclusion it should be noted that two types of control mechanisms exist in the bacteria. The first is a mechanism which relates or detects the nutrient status of the environment and relates this to growth rate. The second is probably a series of mechanisms which integrate and time the cell growth/division cycle and its various components.

(3) Cell division in the eukaryotes

Mitotic cell division in the eukaryotes is accomplished through a highly reproducible sequence of events that is common to almost all higher organisms. An interval of time, G1, separates the previous cell division from the initiation of DNA synthesis (Figure 5).

Figure (5) A Schematic Representation Of The Eukaryotic Cell Cycle



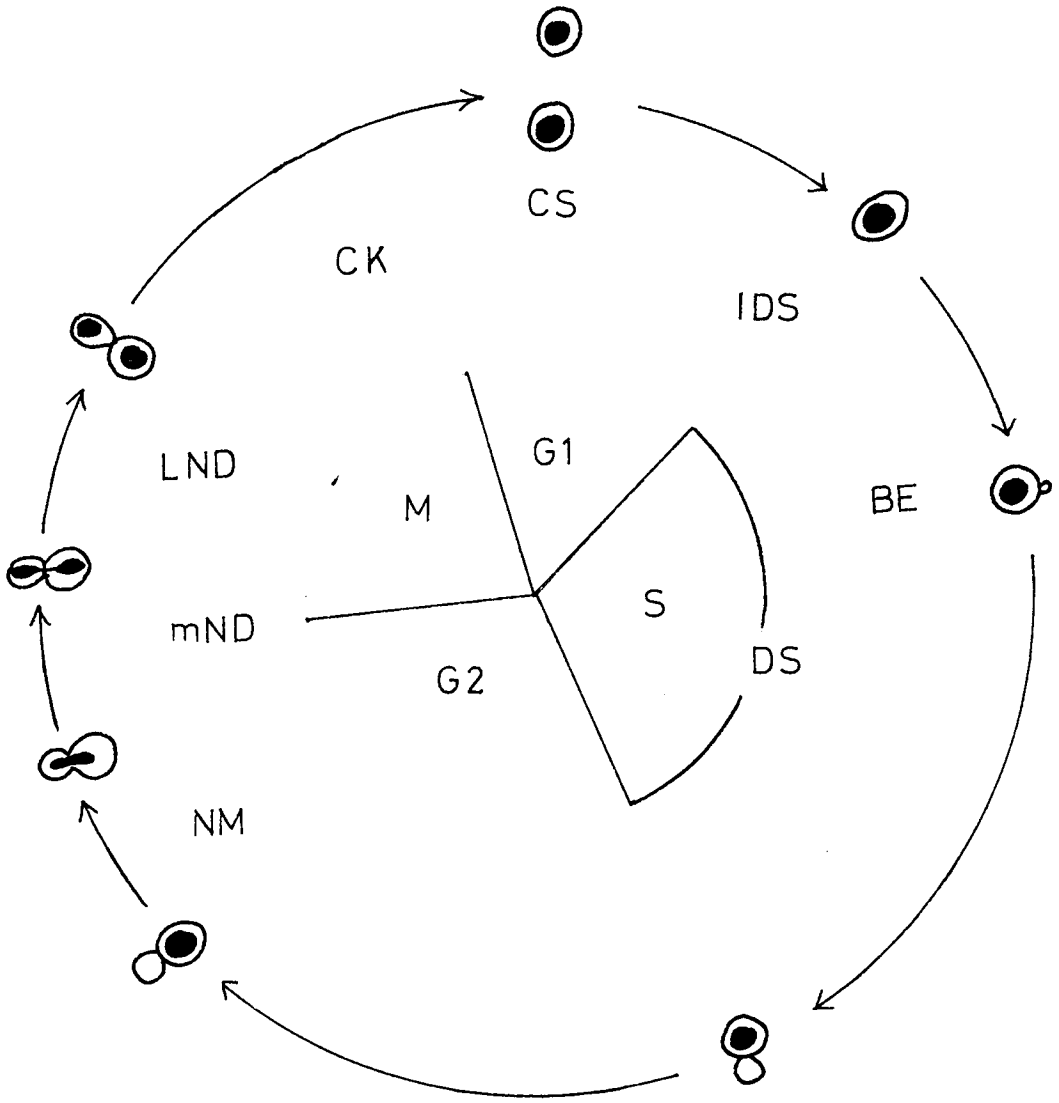
G1= presynthetic gap; S= DNA synthetic phase; G2= post-synthetic gap; M't= mitotic sequence; P= prophase; M= metaphase; A= anaphase; T= telophase; G'_0= G1 resting phase ; G''_0= G2 resting phase.

Chromosome replication is accomplished during the DNA synthetic period (S phase) which typically occupies about one third of the cell cycle (Mitchison, 1971). Another interval of time, G2, separates the completion of DNA synthesis from prophase, the beginning of

mitosis (M). A dramatic sequence of changes in chromosome structure and of chromosome movement characterizes the brief mitotic period that results in the precise separation of sister chromatids to daughter nuclei. Mitosis is followed by cytokinesis, the partitioning of the cytoplasm into two daughter cells with separate plasma membranes. In some organisms the cycle is completed by cell wall separation.

In general, as in the bacterial situation it is no simple matter to detect the position of a given cell precisely in its growth/division cycle. However one notable exception does exist in the budding yeast, Saccharomyces cerevisiae, which may be accurately placed in its cycle by an examination of bud size to parent size (Hartwell, 1974). There are also further cycle landmarks which may be used as indices of a yeasts cell cycle position (Figure 6). For this reason a section will be dedicated to the division control mechanisms of S. cerevisiae.

Figure (6) The Mitotic Cycle Of The Budding Yeast, *Saccharomyces cerevisiae*



At the time of cell separation (CS) the yeast enters its G1 phase, where it remains until it initiates its DNA synthetic period (S). At approximately the same time as the initiation of replication (IDS) bud emergence (BE) occurs. During the S and G2 phases the bud grows and the ratio of bud to parent mass increases. In G2 the nucleus migrates to the neck of the bud (NM, nuclear migration). It subsequently begins to constrict during medial nuclear division (mND) and separates at late nuclear division (LND). These latter karyokinetic events constitute the mitotic phase (M) of the yeast. Subsequently cytokinesis ensues (CK) followed by cell separation (CS) which is the first stage of the new cell cycle.

Redrawn and modified from Hartwell (1974).

(3a) The control of the cell division cycle in yeast

The newly budded cell of S. cerevisiae may undertake a number of possible life cycle programmes (Figure 7). These afford this yeast not only the ability to enhance its rates of recombination through meiotic and conjugation stages, but also to arrest proliferation during periods of low nutrient availability. The selection of a given pathway of differentiation is dependent upon a number of factors, which may be both environmental and intracellular.

Figure (7) A Diagram To Illustrate The Life Cycle Of S. cerevisiae

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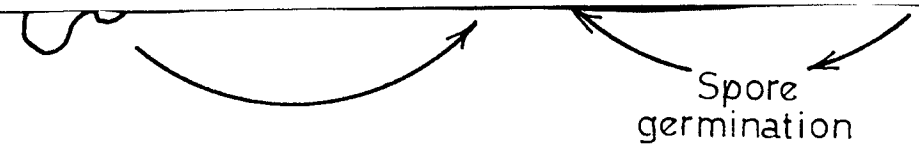
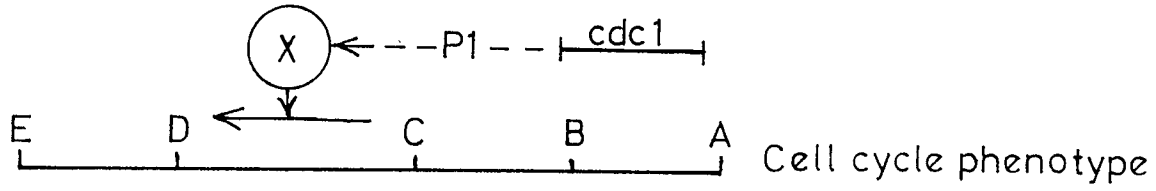


The diagram redrawn from Hartwell (1974) depicts the alternative life cycle programmes of S. cerevisiae. For description see text. The two mating types discussed in this section are designated a and α.

As in the bacteria certain basic and essential nutritional requirements must be met before any progress through the cell cycle may be made. In general the yeast requires carbon, an energy source, nitrogen, sulphur, phosphorus, potassium, and biotin for the growth division cycle to be initiated (Williamson and Scopes, 1962; Hartwell, 1974). That is the yeast makes a decision in the early G1 phase if there are sufficient nutrients available to undertake a fresh round of division. If the medium is depleted of nutrients then the cell enters a stationary phase, or protracted G1, where it continues to enlarge and increase its dry mass, but does not initiate either DNA synthesis or budding (Beam, Mortimer, Wolfe and Tobias, 1954). Besides having a conjugative process like the bacteria, the budding yeast also possess a specific meiotic life cycle programme. The decision to enter both of these alternative and mutually exclusive stages is again taken in early G1 (Hartwell, 1974). For a meiotic programme to be initiated it appears that an alteration of growth conditions is required and this is usually a shift from a depleted to an enriched medium, as evidenced by the higher rate of meiosis seen amongst large stationary cells transfered to more favourable environments (Hartwell, 1974). In S. cerevisiae conjugation may only occur in the G1 phase (Bucking-Throm, Duntze, Hartwell and Manney, 1973). It has been further demonstrated by these workers that cells of opposite mating types produce a 'type specific' protein which synchronizes a yeast cell of the opposite mating type in this part of the cycle by restricting any further progress through the developmental programme. Thus a cell of mating type a when exposed to alpha mating factor will finish its current round of division and become arrested in the G1 period when conjugation may occur.

It is evident therefore that the yeast has several problems to overcome in integrating its life cycle, some of which are not apparent in the bacteria. The yeast has to assure that macromolecular synthesis during the cycle undergoing completion is ordered in such a fashion that each daughter cell contains the correct amount of DNA, membrane components, microtubules etc. This problem is common to the bacteria. However, it also has to regulate the transitions between cell cycle programmes, a feature not evident in bacteria. An approach to the analysis of these functions has been the isolation and assay of cell division cycle (cdc) mutants. Like the dna$^-$ mutants of *E. coli* mentioned previously, these yeast possess a single conditional mutation for a gene product necessary for one of the discontinuous events of the cell cycle. Thus when cells bearing one of these recessive genes are exposed to restrictive conditions (raised temperature) they become arrested at one particular point in the cell cycle, this configuration being termed the terminal phenotype. Clearly each cdc mutant represents a diagnostic landmark in the cell cycle. Once a cell has proceeded beyond this point in the cell cycle even exposure to restrictive conditions will not halt the division process until this gene product is required again in the next cell cycle. (Figure 8).

Figure (8) A Diagram To Illustrate The Features Of A cdc Mutant



The diagram proposes that the product of cdc 1 (P1) controls some function X, which causes the acquisition of a phenotype D. That is cdc 1 regulates the movement from landmark C to landmark D in the cell cycle. In restrictive conditions cells may not move from C to D and thus arrest with a terminal phenotype C. Note however that cdc 1 may have been expressed some time before (the execution point) the events C or D.

Using this technique it has been possible to obtain a collection of cdc mutants which are intimately involved in both cell cycle coordination and programme transition control (Hartwell, 1974). Each of these mutants, as mentioned previously, is associated under restrictive conditions with both a particular bud/parent size ratio as well as other discernible developmental landmarks. Hence it has been possible to compile a map of the apparent, relative positions of the cdc genes in the mitotic cycle (Hartwell, 1974; Hartwell, Culotti, Pringle and Reid, 1974). Whilst many of these genes express functions required for both meiosis and conjugation (Simchen, 1974) only their involvement with the mitotic cycle will be considered here.

The newly formed yeast cell, in early G1, possesses a single gene (cdc 28) whose induction at this point of the cycle results in the commencement of a fresh round of division (Hartwell, Culotti and Reid, 1970; Hartwell, Mortimer, Culotti and Culotti, 1973). This gene only appears to initiate division if there are the correct and sufficient nutrients available in the growth medium (Hartwell, et al., 1974). Furthermore the two mating factors (a and α) act at a point just prior to the expression of the cdc 28 function (Hartwell, 1974). Hence it may be that these agents inhibit the induction of, or suppress the action of, the cdc 28 gene product. In either case the net effect will be to synchronize yeast cells of opposite mating types in early G1, prior to the expression of cdc 28, and where conjugation may occur. However, once the gene product has been generated then the cell is irreversibly committed to a round of mitosis, hence this loci has been termed the 'start' gene (Hartwell et al., 1970). There is also evidence to suggest that before bud emergence or DNA synthesis (events which mark the end of G1) may

occur, some critical cell size must be attained (von Meyenberg, 1968; Beck and von Meyenberg, 1968). This situation is analagous to that occurring in bacteria, where there again appears to be a critical cell mass ratio for the initiation of DNA synthesis and cell division (Donachie, 1968). In this case one might regard cdc 28 as a differentiation gene which commits the cell to mitosis when the prevailing conditions are suitable. Subsequent development may then be geared to a cell volume titration process which initiates further sequences of events. Such a system may be necessary on account of the divergent sizes of daughter cells at mitosis (Fantes, Grant, Pritchard, Sudbery and Wheals, 1975), since control and integration of the cell cycle would presumably be much simpler if all cells started at the same volume. However cell growth alone is not a sufficient stimulus to elicit division since cells certainly increase their dry mass in stationary phase conditions.

If cdc 28 is expressed then the next visible change in the yeast cell is the division of the spindle plaque. This structure has attached to it both the intra- and extranuclear microtubules and lies within the nuclear envelope. At about the time of bud emergence the spindle plaque duplicates and migrates to opposite sides of the nucleus whilst still connected by a continuous fibre of microtubule (Byers and Goetsch, 1973). This structure is termed the complete spindle. In restrictive conditions cdc 28 has been shown to be the gene controlling spindle plaque duplication (Byers and Goetsch, 1973). These workers have also shown that the spindle plaque plays a vital role in bud emergence, and that conditions which disfavour plaque division also inhibit budding. However another conditional mutant, cdc 4, whilst inhibiting spindle migration does not affect bud

formation, indicating that these two events may be located on two independent pathways (Byers and Goetsch, 1973). Furthermore the phenotypes of mutants defective in cdc 4 suggests that some type of clock controls the bud emergence event, because successive rounds of budding at the restrictive temperature maintain a periodicity of about one cell cycle time (Hartwell, 1971). The clock must also run in the absence of DNA synthesis, nuclear division, cytokinesis and cell separation which do not occur in this mutant. In contrast bud emergence does depend on the expression of another gene, cdc 24, which is required neither for DNA synthesis nor nuclear division (Hartwell et al., 1974). Thus there appears to be two genetically controlled cycles running concomitant with one another (Figure 9). The first is bud emergence, nuclear migration, bud growth, cytokinesis, and cell separation. Whilst the second runs spindle plaque separation, DNA synthesis, spindle elongation, medial nuclear division, late nuclear division, cytokinesis and cell separation (Hartwell, 1974; Hartwell et al., 1974). Unfortunately no clear cut mutants are available which control stages in the first of these two pathways, although several (cdc 19, 22, 25, 29 and 32) have been implicated (Hartwell et al., 1973). However, a series have been described which play regulatory roles in the second sequence, and so some time will be spent considering those events which constitute the DNA synthetic mechanism.

Following spindle plaque migration, and at the time of bud emergence the initiation of DNA synthesis occurs, the S phase occupying approximately one quarter of the cell cycle (Williamson, 1965). The initiation of replication is controlled by at least one gene, cdc 7, but whose continued expression is not required for ongoing synthesis (Hereford and Hartwell, 1973). Indeed unlike the bacteria and most

other eukaryotes there is no absolute requirement for protein synthesis in the yeast once replication has been initiated (Hereford and Hartwell,

Figure (9) A Genetic Map Of The Cell Division Cycle Of S. cerevisiae

Figure (9) redrawn from Hartwell (1974) illustrates the cell cycle of S. cerevisiae with the relative positions of each cdc mutant indicated by a number. The block capitals represent visible cell cycle features:- PD= plaque division; PS= plaque separation; iDS= initiation of DNA synthesis; DS= DNA synthesis; mND= medial nuclear division; SE= spindle elongation; LND= late nuclear division; CK= cytokinesis; CS= cell separation; BE= bud emergence; NM= nuclear migration. MF (mating factors), HU (hydroxyurea) and TR (Trenimon) act at the stages indicated. Events in each of the two cycles are dependent upon the event preceding them, but are not dependent prior to late nuclear division on events in the other pathway (i.e. the two pathways are independent). However following the late nuclear division stage the two sequences coalesce to form an interdependent pathway.

1973). Hence all proteins needed both for the initiation and the replication of the 200 replicons of yeast (Petes, Newlon, Byers and Fangman, 1973) must be present at the onset of the S period. Two further mutants of the DNA synthetic phase are cdc 8 and cdc 21, which are thought to express a function required throughout the S phase, as lesions in these genes leads to an immediate cessation of synthesis (Hartwell, 1971). Cells bearing cdc 8 and 21 lesions, as well as those whose DNA synthesis has been blocked with hydroxyurea (Slater, 1973), Trenimon (Hereford and Hartwell, 1973) or fluoro-deoxyuridine (Esposito, 1968) arrest with the same terminal phenotype as cells which have conditional mutations for medial nuclear division. Furthermore many of these mutants have execution points around the time of the initiation of DNA synthesis (Hartwell, 1974). These results have been interpreted as meaning that the proteins encoded by these genes are responsible for the completion of chromosome duplication, and not for DNA polymerisation. It has also been demonstrated that mitochondrial DNA synthesis occurs throughout the cell cycle in S. cerevisiae (Williamson and Moustacchi, 1971) and does not require the presence of functional DNA initiation genes (cdc 4 and 7) whilst cdc 8 and 21 are required (Hartwell, 1974). It should also be noted that the genome of yeast is grossly dissimilar to that of the bacteria, which may account for some of the additional mechanisms apparently present in eukaryotes. Firstly the nucleus is bounded by a double unit membrane, which constitutes the nuclear envelope in which the spindle plaque is embedded. More importantly the DNA is present as a diffuse chromatin, that is the DNA molecule is found in association with a number of proteins (e.g. histones) which influence both the accessability of the DNA for transcription and its structure (Tonino and Rozijn, 1966; Van der Vliet, Tonino and Rozijn, 1969). However it is still uncertain if the chromatin

condenses to form chromosomes during the segregation process (Hartwell, 1974).

When DNA synthesis is complete the nucleus migrates to the neck of the bud. This process is not dependent upon spindle plaque migration or its elongation, and occurs in the absence of DNA synthesis (Hartwell, 1974). Therefore nuclear migration and replication are not dependently linked. Following nuclear migration, and prior to the completion of nuclear division, the complete spindle elongates in the long axis of the cell, as does the nucleus. Indeed spindle elongation may provide the force to effect nuclear division via its microtubule component. Hartwell (1974) has also suggested that this process may facilitate chromosome segregation. Whereas nuclear migration is independent of DNA synthesis, spindle elongation does not occur in the absence of replication or medial nuclear division (Byers and Goetsch, 1973) showing it to be a part of the same dependent cascade pathway as DNA synthesis.

As the nuclear and cytoplasmic membranes pinch in two, vesicles of endoplasmic reticulum accumulate at the neck of the bud (Robinow and Marak, 1966). This stage does not occur in the conditional mutants cdc 5,14 and 15 which arrest at the elongated spindle configuration (Hartwell et al., 1974). It is suggested that these mutants are defective in one of the terminal steps of late nuclear division. The next visible cell cycle feature is cytokinesis, which does not occur if either bud emergence (Hartwell et al., 1973) or the DNA synthetic initiation pathway is inhibited (Hartwell, 1971). Therefore the two pathways converge at or before cytokinesis and form an interdependent pathway, and if either mechanism is interupted then no cell division occurs. Furthermore four mutants (cdc 3,10,11

and 12) have been isolated which act after the convergence of these two pathways (Hartwell, 1971). The execution points for these genes may be early in the cycle near to the time of bud emergence (Hartwell, 1974). The actual process of cell separation has been analysed by Cabib and others (Cabib, Ulane and Bowers, 1974) and they have demonstrated that a primary septum of chitin is laid down first upon which is secreted a secondary septum of glucan. It is also apparent that the formation of the chitinous septum is effected through the action of chitin synthetase, which is normally present as an inactive zymosan (Cabib and Farkas, 1971). The inhibitory subunit may be removed by a proteolytic enzyme found in the bud neck vesicles, thus activating the chitin synthetase (Hasilik and Holser, 1973). In its turn this activator may be inhibited by a third heat stable protein (Cabib and Farkas, 1971). Thus the cdc mutants preventing cell separation may represent mutations in the genes responsible for the production of these protein enzymes.

Before passing to the next section it would seem germane to first summarise the most important features relating to the regulation of yeast cell proliferation.

(1) As in the bacteria there are certain nutritional requirements which have to be met before cell cycle progress may be made.

(2) Yeast have also evolved several life cycle programmes which enable them to exploit their environment more successfully. These alternative developmental sequences include a meiotic and conjugative cycle as well as a resting or stationary phase; the latter may be analagous to the G_o phase of higher cells.

(3) There is a critical decision period in early G1 when the choice of cell cycle programme is made. At this key point a single gene appears to be responsible for integrating the nutrient status of the medium, the induction of its product being an irreversible signal for division.

(4) The yeast also possesses mechanisms which superimpose themselves on the start gene to cause the arrest of the cell in G1. This has the effect of facilitating programme transition to a conjugative cycle.

(5) Once the cell cycle has been initiated, further development requires the acquisition of some critical cell mass as it does in the bacteria.

(6) The division process itself is timed by the production of specific proteins by a rigidly determined set of genes. Disruption of these gene products results in cell cycle arrest.

(7) There are two independent genetically determined pathways running concomitantly during the yeast mitotic cycle. However these two mechanisms have common initiators and both coalesce prior to cytokinesis to constitute an interdependent pathway. Such a system has been proposed to be capable of regulating the correct occurence of cell cycle events, and ensuring equipartitioning of macromolecules into daughter cells.

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Margalef, 1968.

Before examining in detail control mechanisms for modulating cell division rates in mammals, it would first seem pertinent to reiterate the types of processes one might expect to be present in the light of previous sections. Also some benefit might be gained by taking an appraisal of any new factors which might be present in mammalian cell systems which might contribute to the proliferation control mechanisms found.

As in the yeast and bacteria, the cell cycle of mammalian cells is a complex and coordinated series of events which must be integrated and timed in a very precise fashion. For this reason one might expect to find intracellular processes geared to regulating this aspect of cell function. This type of mechanism might be termed an intrinsic control process, and might have a great deal in common with lower organisms. One would also predict that the cell would be both aware of, and responsive to, its nutrient environment. However in itself this feature might be of less importance in mammalian cells as the extracellular environment is far more static than say the external milieu of yeast or bacteria. Nevertheless it would not be inconsistent to expect that agents which reflect exogenous nutrient levels in mammals also influence cell proliferation rates. Like the

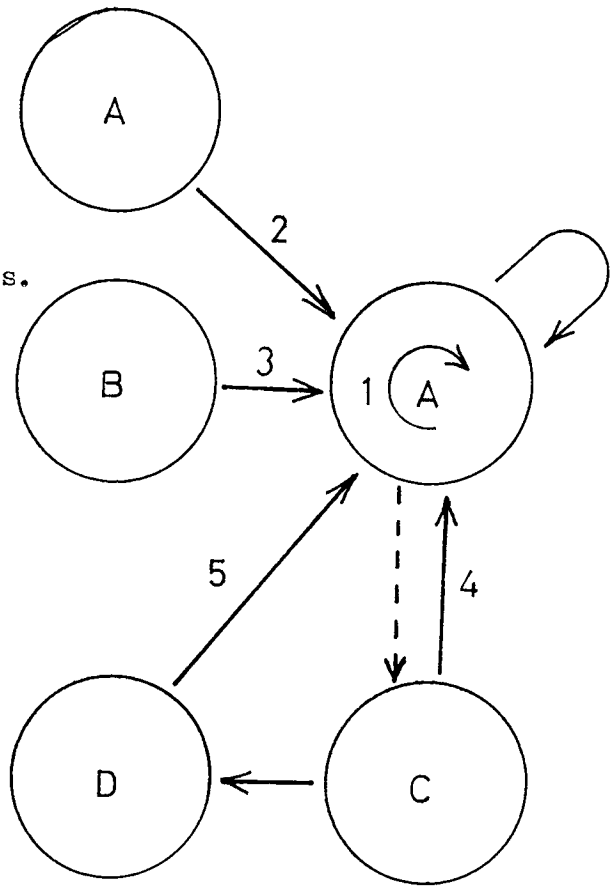
yeast, a mammalian cell may express a number of alternative phenotypes, and the control of cell differentiation is clearly of fundamental importance here as in lower eukaryotes. This process might be all the more important as proliferation and differentiation seem to be intimately linked.

Whereas the yeast is basically a unicellular organism, the mammal is invariably a complex multicellular entity. It consists of a large number of diverse tissues composed of cells with unique functions which are integrated into organs which act in unison with other organs. Clearly there must be mechanisms present controlling both the size of, and division rates within these organs and tissues thus ensuring a balanced function for that organ with respect to the organism as a whole. One can therefore envisage a second basic type of cell recognition system in which there is an interaction between cell types to maintain physiological stasis. Such cell cooperation might be effected through direct cell contact or via soluble products from both the same and dissimilar cells. Furthermore if these emanations were related to the function of a tissue they might provide an apparatus with which to regulate both cell proliferation and tissue activity. Thus the organisation and functional control of tissues and organs is not unlike an ecological matrix where there is a complex series of interdependencies between not in this case organisms but cells possessing different functions.

Figure (10) represents a heuristic diagram to illustrate the possible forms of cellular interactions which might act to regulate cell division and function. Thus there are mechanisms of type (1), which I have termed intrinsic control, which will modulate and integrate the mammalian cell cycle even in the absence of other

cell types. These mechanisms may also titrate extracellular nutrient concentrations and modulate cell division rates in harmony with these various levels. Secondly there are extracellular interrelationships, which represent extrinsic control of proliferation. At the cellular level these exogenous axes may interact with intrinsic control

Figure (10) A Theoretical Scheme Illustrating Possible Cell Interactive Axes.



The diagram proposes the possible modes of interaction of cell types B to D with cell type A. Thus the proliferation of cell type A may be modified by intrinsic mechanisms (1), or even by its own emanations. Alternatively extrinsic axes may apply. Thus either by cell contact or via cell products similar (2) or dissimilar (3) cells may influence cell type A whilst being unaffected by A themselves. In contrast some function of A might cause some other cell type (C) to react by modifying the activity of A (4). In a more indirect scheme (5) the function of A influences cell type C, which in its turn causes cell type D to alter the proliferation of A.

mechanisms, or may have their own distinct points of action. However, these extrinsic interactions may take several forms. Firstly cells of the same type (2) may influence one another through direct contact or by soluble products. Alternatively dissimilar cells may have the same role (3) of modifying the proliferation of a cell pool without being directly affected themselves. A more elegant scheme would be the short feedback loop, where a cell type directly alters a second

cell in such a way that this cell in its turn changes the proliferative activity of the first cell (4). Clearly longer feedback loops may apply (5) where a second cell types influences a third, which in turn modifies the activity of the first. It is also not inconceivable that all the cells illustrated in figure (10) have similar effects upon one another either directly or indirectly and therefore the model becomes far more complex. It is not surprising to find therefore that it is extremely difficult to investigate all of these mechanisms in one cell model due to these diverse interactions. One has to compromise and use a number of techniques and situations to elucidate the nature of these control systems.

(3b-i) The in vitro regulation of cell cycle proliferation

Although with a few exceptions (Pollack, Green and Todaro, 1968; Naha, Meyer and Hewitt, 1975) cell cycle mutants have not yet become available, nevertheless the use of cells in tissue culture has given perhaps the greatest insights into the cellular regulation of proliferation. The areas which have been investigated are certainly somewhat diverse and so an ordered approach to these will be adopted.

(ia) The mammalian cell cycle

Having completed its mitotic sequence, the mammalian cell embarks upon the first stage of its cell/division cycle, the so-called presynthetic gap or G1 (Howard and Pelc, 1953). This is the most variable phase in terms of temporal duration with times ranging from almost zero to weeks or even years (Mitchison, 1971). Other phases show much less heterogeneity of timing (Mitchison, 1971). Not all parts of the G1 phase are necessarily equivalent, and it is likely that this compartment contains a number of transition points in mammalian cells. Recently it has proved possible to partially map the G1 phase using temperature sensitive clones of BALB/3T3 cells (Naha et al., 1975). This work indicates that there are at least four distinct regions of the presynthetic gap each with a characteristic restriction point. These workers also suggest that the acquisition of certain non histone proteins may be the prerequisite for passing the transition point. Thus the divergent timing of this compartment may represent temporary blocks at one of these (and possibly other) sites. There is also a period during late G1 where the irreversible commitment to DNA synthesis is taken (Frankfurt, 1968). It is not clear if this transition point is represented by any of the points

detected in the temperature sensitive clones mentioned previously.

The cell's DNA content is replicated during a discrete phase of the cell cycle, the S period, which varies in duration between four and fourteen hours (Cleaver, 1967). Indeed the lengths of this phase and the subsequent postsynthetic gap (G2) and mitotic sequence are remarkably constant (Mitchison, 1971). This feature has led to the suggestion by some authors that there are in fact only two cell cycle phases (Smith and Martin, 1973). The first or A phase is said to be both indeterminate and equivalent to the G1 period. The rest of the cell cycle comprises the B phase, which is relatively constant in duration. It is proposed that cells exit the A phase randomly, thus making its duration indeterminate, and enter the B phase. The latter is strictly defined and thus is of a fairly consistent length. Hence any agent which alters the probability of a cell leaving the A phase will of necessity change the proliferative activity of the cell population concerned. However a number of criticisms have been levelled at this concept (Wheals, 1976; personal communication) and the standard nomenclature of Howard and Pelc (1953) will be adopted here.

It appears that the signal to initiate DNA synthesis comes from the cytoplasm. This evidence accrues from the observation that in a number of multinucleate cells the S phases occur concurrently (Oehlert, Seemayer and Lauf, 1962). In addition fusion of a G1 cell to an S phase cell results in the premature initiation of DNA replication in the G1 cell (Rao and Johnson, 1970). It has been proposed therefore that the mammalian cell, like the bacteria and yeast, utilize protein inducer compounds to initiate the mitotic cycle (Mitchison, 1971). In support of this notion is the observation

that inhibition of protein synthesis in G1 stops, or substantially reduces, the entry of cells into the DNA synthetic phase (Bloch, MacQuigg, Brack and Wu, 1967). Furthermore cells which have initiated replication are much less susceptible to inhibition of protein synthesis than are cells which have not or are in the early stages of DNA synthesis (Kim, Gelbard and Perez, 1968). There is also a body of evidence to suggest that cells initiate DNA synthesis at the same cell volume (Killander and Zetterberg, 1965). Thus the variability of the G1 phase may represent a function of the time taken for cells to attain a critical cell mass. As there certainly is an uneven partitioning of cytoplasm at cytokinesis (Fantes et al., 1975) this may indeed be an important factor in determining the duration of G1.

When considering the DNA synthetic phase there are two important processes occuring simultaneously. As in the bacteria and most other organisms there is the actual replication of the DNA molecule to be facilitated, and from which this period gains its designation of S phase. However DNA is present in a complex association with proteins of various classes to form chromatin (Elgin and Weintraub 1975). Whereas one group of these proteins (non histones) are synthesized throughout the cell cycle (Cave, 1968), histones are only generated during the S phase (Bloch and Goodman, 1955; Prescott, 1966). Recent evidence also shows that the histones are produced actually at the DNA replicating fork and added back directly to the freshly synthesized DNA (Weintraub, 1973). The molecular basis of this phenomenon is unknown. The importance of this process may be that histones are thought to be intimately related to the expression of the genetic code, and may indeed suppress transcription (Smart and Bonner, 1971; Elgin and Weintraub, 1975). Thus as it is of paramount importance to maintain the differentiated expression characteristic

of the particular cell concerned, which may be dependent upon the histone pattern in the chromatin, this pattern should be copied faithfully at the time of DNA synthesis onto the new daughter strand. Whilst histones are synthesized almost entirely during the S phase, non histone chromosomal proteins (NHC proteins) are produced throughout the cell cycle, as indicated previously (Cave, 1968). However, these latter NHC proteins do have higher rates of synthesis during the G1 period (Stein and Borun, 1972). Furthermore specific NHC proteins have been shown to be produced at distinct times during the mitotic cycle (Borun and Stein, 1972; Bhorjee and Pederson, 1973). Such an example of this specific synthesis may be the non histones associated with the G1 temperature sensitive mutants of BALB/3T3 (Naha et al., 1975). These NHC proteins may represent enzymes such as the nucleic acid polymerases, nucleases as well as enzymes of histone metabolism, they may also have further structural functions within the chromatin (Elgin and Weintraub, 1975). Thus one can envisage that some of the transition points noted may be associated with changes in chromatin structure or the induction of specific nuclear enzymes.

The DNA molecule itself is not replicated in a simple end to end fashion. Rather there appears to be a large number of initiation sites which bind polymerase and replicate bidirectionally (Huberman and Riggs, 1968). Furthermore not all these sites initiate synthesis simultaneously, but show a temporal sequence of cistron replication (Hsu, Schmidt and Stubblefield, 1964; Mueller and Kajiwara, 1966; Braun and Willi, 1969). This conclusion is also supported by the observation of both early and late replicating segments of the human chromosomes (Gavosto, Pegoraro, Masera and Rovera, 1968). Clearly such processes will require a large degree of organisation and control.

One indication of a mechanism which might impart this temporal organisation upon DNA synthesis is given by the properties of membrane-DNA complexes. Chromatin is known to be associated with regions of nuclear membrane or membrane-like components (Comings, 1968; Comings and Okada, 1970; Hildebrand and Tobey, 1973). This interaction is known to increase during the latter portion of the S phase (Hildebrand and Tobey, 1973). Furthermore in regenerating liver, membrane-bound DNA is the last to be replicated (Kay, Haines and Johnstone, 1971). Thus, since heterochromatin is normally the last of the nuclear material to be replicated (Lima-de-Faria and Janorska, 1968) and this is usually associated with the nuclear membrane (Williams and Ockey, 1970) it has been suggested that DNA-membrane complexes represent a mechanism for the suppression or delay of DNA synthesis (Comings and Okada, 1973; Hildebrand and Tobey, 1973; Yamada and Hanoaka, 1973). Thus regulation of enzymes which control DNA-membrane association offers a possible method of controlling a sequential DNA synthetic pattern. Additionally both the state of DNA-membrane complexing (Rose and Frenster, 1965; Harrison, 1971; Tata, Hamilton and Cole, 1972) and chromatin structure (Frenster, 1972) show changes throughout the mitotic cycle. Hence the conformation and association of chromatin with other cell structures may play a vital role in regulating other cell cycle phases.

On exiting the S phase a cell enters the postsynthetic gap, or G2. As mentioned previously this period is of a fairly consistent duration, ranging between zero and eight hours (Mitchison, 1971), but is short in most cells being between one and two hours. This phase does appear to contain at least one transition point which requires ongoing RNA and protein synthesis for its completion (Doida and Okada, 1969). The newly synthesized protein(s) is

presumably required in the G2 phase for the initiation of chromosome condensation or some other process related to transition into the mitotic sequence itself. This chromosome condensation heralds the entry of the cell into the first stage (prophase) of mitosis proper. It is at this time that the chromosomes first become visible under normal conditions. During this phase, which last approximately twenty minutes, the centrosome splits and migrates to opposite poles of the nucleus. Associated with this is the breakdown of the nuclear membrane and the disappearance of the nucleolus. The condensed chromosomes then undergo a complex series of movements facilitated by microtubular structures (spindles) which apparently emanate from the centrioles, and which attach to the chromosomes at the centromeres. These movements of the chromosomes constitute the metaphase, which culminates some ten to fifteen minutes after its initiation with the arrangement of the strands in a flat plane at the cell's equator. There then ensues chromosome separation (anaphase) which takes only a few minutes to occur via microtubule contraction or shortening. Subsequently the chromosomes disappear to take up their interphase state, the nuclear membrane reforms and nucleolus reappears. The cell then undergoes cytokinesis. This latter phase (telophase) takes surprisingly only some four to five minutes to be completed.

The processes which initiate and control these movements are a total mystery at present and represent a fundamental challenge to the cell biologist.

(ib) Enzyme synthesis during the cell cycle

The alteration in chromatin structure and replicative activity outlined in the previous section could clearly be mediated by the action of specific enzymes. Furthermore if these enzymes were only synthesized at specific times during the cell cycle then the pattern of their induction could represent a fundamental control system for the mitotic programme. Thus much attention has been paid to measuring the activity of a large number of enzymes throughout the growth/division cycle.

Table (1) represents a by no means exhaustive list of enzyme activities studied. An examination of the occurence of these enzymes indicates that whilst some are synthesized throughout the cell cycle, others are only evident (or show enhanced activity) during discrete periods. Thus many of the oxidative phosphorylation enzymes are generated continuously, since these are required for general metabolic activity and cell survival. In contrast enzymes associated with the replication of DNA and histone synthesis appear just prior to or during the S phase. Whereas the control of such a periodic function is thought to lie at the transcriptional level in bacteria via oscillatory repression (Mitchison, 1969), it is most likely that this process is regulated at both the transcriptional and translational stages in eukaryotes (Martin and Tomkins, 1970). One theory suggests that enzymes are transcribed in the same order as their linear sequence on the chromosome (Tauro, Halvorson and Epstein, 1968). This would have the effect of producing a sequence of enzymes in a genetically defined order. However, since some enzymes exhibit continuous synthesis there would presumably have to be gene multiplication for some of these enzymes.

Table (1) The Patterns Of Enzyme Synthesis During The Mammalian Cell Cycle

Enzyme	Pattern	Cell Type	Phase	Ref.
NADPH-cytochrome C reductase	C	Mouse P815Y	All	(1)
Uridine diphosphatase	C	" "	"	"
Cytochrome C oxidase	C	" "	"	"
Succinate-cytochrome C reductase	C	" "	"	"
Glucose-6-phosphate dehydrogenase	C	" "	"	"
Lactate dehydrogenase	C	Human KB cells	"	(2)
Fumarase	C	" " "	"	"
Alkaline phosphatase	C	HeLa cells	"	(3)
Histone kinase	P	Lymphocytes	Late G1	(4)
Histone phosphatase	P	"	Late G1	"
RNA-directed DNA polymerase	P	"	Late G1	(5)
" " " "	P	Mouse L cells	G1/S	(6)
" " " "	P	HeLa cells	G1/S	(7)
Ornithine decarboxylase	P	Lymphocytes	Late G1	(8)
Thymidine kinase	P	"	G1/S	(9)
" "	P	Mouse L cells	G1/S	(10)
" "	P	Don C cells	G1/S	(11)
" "	P	HeLa cells	G1/S	(12)
Thymidylate synthetase	P	Lymphocytes	G1/S	(13)
PolyADP-ribose polymerase	P	"	G1/S	(14)
RNA polymerase II	P	"	S	(15)

Table (1) represents the activities of various enzymes measured during the mitotic cycle. Thus enzymes generated throughout the cycle are said to be continuous (C), whilst those produced only at discrete times are deemed to be periodic (P). The phases in which synthesis (or enhanced synthesis) is observed is designated by the standard notation.

Table (1) continued;

Reference code:-

(1) Warmsley, Phillips and Pasternak, 1970.
(2) Bello, 1969.
(3) Griffin and Ber, 1969.
(4) Cross and Ord, 1971.
(5) Penner, Cohen and Loeb, 1971.
(6) Gold and Helleiner, 1964.
(7) Friedman and Mueller, 1968.
(8) Kay and Cooke, 1971.
(9) Wilms and Wilmanns, 1969.
(10) Adams, 1969.
(11) Klevecz, 1969.
(12) Brent, Butler and Crathorn, 1965.
(13) Youdale and MacManus, 1975.
(14) Lehmann and Shall, 1972.
(15) Handmaker and Graf, 1970.

The evidence, although far from conclusive, suggests that in mammalian cell types the synthesis of specific enzymes may be restricted to that period of the mitotic cycle where they would be expected to normally function. However, whilst it is tempting to speculate that it is in fact the induction of these enzymes which initiates cell cycle development this need not be the case. Indeed other signals which integrate the mitotic programme may cause these periodic enzymes to be synthesized, their lack of production during other phases merely being a conservation mechanism. Thus inhibition of DNA synthesis prevents the induction of some enzymes, whereas others appear on schedule (Mitchison, 1971). Hence it must be concluded that there must be a feedback mechanism to the genetic apparatus indicating that some transition point had not been passed, and that further enzyme induction could not take place. That is if one proposes the linear coding model cited previously (Tauro et al., 1968) to control cycle coordination this must be capable of modulation through some repression/derepression feedback which is clearly the fundamental regulation. Furthermore, the very fact that some enzymes are unaffected indicates that there may be more than one control

pathway operating during the mitotic programme. This notion is further emphasized by the observation that the membrane transport of substrates may be restricted to, or enhanced during phases in which they are utilized (Miller, Stone and Prescott, 1964; Adams, 1969; Hwang, Murphree, Shansky and Sartorelli, 1974; Roller, Hirai and Defendi, 1974). Furthermore in the case of thymidine transport, which is elevated during the S phase (Adams, 1969), inhibition of DNA synthesis does not alter these enhanced rates of translocation (Roller et al., 1974). Thus there may be one gene sequence which codes for enzymes which regulate and time DNA replication, and is subject to its own series of feedback effector molecules. There may also be another pathway which operates concomitantly with the DNA synthetic sequence but which is intimately involved with the metabolism and function of the cell membrane. The two may then act in concert to time the normal cell cycle. Certainly such a scenario would not be without precedents in both the yeast and bacteria.

(ic) The cell membrane during the mitotic cycle

In the light of the potential importance of the plasma membrane in the regulation of proliferation much attention has been focused recently on its metabolism and function in normal and malignant cells. These studies have compared normal cell lines with their transformed derivatives, or have monitored the changes which occur during the cell cycle in synchronous cultures.

It was initially noted that in normal fibroblasts the binding of two plant lectins, wheat germ agglutinin (WGA) and concanavalin A (con A), to the plasma membrane was greatly enhanced during the late G2 and mitotic phases of the cell cycle (Fox, Sheppard

Burger, 1971; Shoman and Sachs, 1972). It was therefore postulated that membrane modifications might indeed play a role in integrating the normal cell cycle. Subsequently other phase-specific membrane modulations have been shown to occur. These include increased blood group H activity in HeLa cells during mitosis (Kuhns and Bramson, 1968); decreased expression of the H2 transplantation antigen in S phase mouse cells (Cikes and Friberg, 1971); increased surface charge during G2 and mitosis in HeLa and sarcoma cells (Brent and Forrester, 1967); alterations in the occurence of specific membrane proteins during G1, G2 and M in HeLa cells (Johnsen, Stokke and Prydz, 1975); and alterations in membrane fluidity during the G2 phase of mouse 3T6 cells (Shodell, 1975). An important extension of this work was the discovery that virally transformed fibroblasts bound, and were agglutinated by WGA and con A not only during mitosis as is the case with normal cells but also during G1 (Burger, 1973). This unscheduled binding in transformed cell lines declined during the S phase to reach a minimum during G2 (Smets and De Ley, 1974). These findings indicated that the membrane gave and received information which was central in controlling cell division, and that malignancy might be associated with some defect in this informational transfer to the cell membrane.

At this point a digression will have to be made to describe the notion of contact inhibition. When mouse embryonic 3T3 fibroblasts are cultured <u>in vitro</u>, they proliferate to form a monolayer. At this point division ceases although the cells remain perfectly viable for a number of days (Todaro, Lazar and Green, 1965). Thus the term contact inhibition has been coined to describe the phenomenon whereby cells proliferate until they become confluent whereupon they cease dividing. Although this simple definition will suffice for the experiments reported here, it has a number of criticisms (Dulbecco,

1971).

The cell density at which this arrest of division occurs is not absolute, but is dependent upon a number of factors such as serum concentration in the culture medium (Holley and Kiernan, 1968). Indeed serum addition to a contact inhibited culture results in the arrested cells undertaking a fresh round of division (Todaro et al., 1965). Thus the cells, which are arrested in G1, have a requirement for certain protein constituents of serum which are either depleted at high cell densities, or more likely become difficult to obtain through the close cell packing (Nilausen and Green, 1965; Stoker, O'Neill, Berryman and Waxman, 1968; Holley and Kiernan, 1971). If, however, these 3T3 cells are transformed with a tumour virus, SV 40, they show a much reduced tendency to exhibit density dependent inhibition, which is associated with a reduction in their requirement for serum factors (Temin and Rubin, 1958; Todaro, Green and Goldberg, 1964). This may be explained by postulating that the membrane in the transformed cell line is more permeable to the growth promoting serum factors than it is in the untransformed parent line. The result being that the tumour cells pass some critical transition point in the G1 which requires that the cells be exposed to some minimum level of growth factors. Normal cells at high densities fail to pass this cellular event through inadequate serum factors and become arrested in G1.

Trypsinization of normal fibroblasts leads to the expression of membrane components similar to those apparent in transformed cells (Burger, 1970), and normal cells during mitosis (Smets and De Ley, 1974). It has been suggested therefore that tumour cells fail to return to a normal membrane configuration following mitosis on account

of a high native proteolytic activity in their membranes (Poste, 1972; Schnebli and Burger, 1972). Indeed proteolytic inhibitors return the growth characteristics of transformed cells back to that of the parent cell type (Schnebli and Burger, 1972; Collard and Smets, 1974). The reverse of this situation also obtains where addition of proteases to contact inhibited cultures induces a fresh wave of division to occur (Burger, 1969). This evidence implies that there may be a membrane cycle which influences cell proliferation. Disruption of this cycle may lead to the acquisition of malignant growth characteristics by altering membrane sensitivity to humoral growth factors.

Other evidence implicating the cell membrane in the control of proliferation is the antimitotic activity of the polyanionic surfactants heparin and dextran sulphate (Temin, 1966; Clarke and Stoker, 1971). These agents will prevent the reinitiation of division in quiescent 3T3 cells provoked by serum, so long as they are added within the first three hours of activation (Clarke and Stoker, 1971). Since the cultures become insensitive to the surfactants after three hours it must be inferred that these compounds either only act at one point in the cycle,or disruption of some membrane function is only of any consequence in this part of the mitotic programme.

Using Ruthenium Red staining it has been possible to demonstrate alterations in the membrane following transformation (Torpier and Montagnier, 1969). Thus transformation may lead to an increase in acidic lipopolysaccharides in the outer membrane of the cell. These groups may then protect the polar head groups of phospholipids from degradation (Montagnier, 1970). This notion that membrane phospholipid metabolism might be important in the control of cell proliferation in normal and transformed cells has led a

number of investigators to estimate chemically membrane phosphorylation. Thus 3T3 cells grown to confluency show a marked alteration in their phosphate incorporation into phospholipids when compared to non-confluent cells (Cunningham and Pardee, 1971). This is mainly reflected in the ratios of incorporation into phosphatidyl choline (increased at confluency) and phosphatidyl serine and ethanolamine (both decreased at confluency). In transformed 3T3 cells however there is no change in phosphorylation pattern at cell densities which would lead to density inhibition in untransformed cells (Cunningham and Pardee, 1971). However, whilst sphingomyelin does appear to be more abundant in confluent cells, assay of total phospholipids indicates little difference under the various treatments (Cunningham and Pardee, 1971). Thus suggesting that it is the rate of turnover of lipid which is altered and not absolute amounts of any particular species. Further evidence for the involvement of membrane phospholipids in the growth/division cycle comes from the stimulation of quiescent lymphocytes by lectins. Peripheral lymphocytes appear to be arrested in an extended G1 phase, or G_o, until they are activated by an antigen or other immune agents (Ling and Kay, 1975). On stimulation there is a rapid increase in the activity of a series of enzymes involved with membrane metabolism these include acyl CoA: lysolecithin acyl transferase (Ferber, Reilly, De Pasquale and Resch, 1974); phosphatidyl-choline cytidyl transferase (Nelson and Sribney, 1972); and phosphatidyl-choline glyceride transferase (Nelson and Sribney, 1972). There also occurs an increase in the rate of incorporation of a number of substrates into phospholipids. These have been reviewed by Ling and Kay (1975) and include glucose, glycerol, choline, phosphate, acetate, inositol, oleate and glucosamine.

The next question arises as to how (if at all) these

membrane alterations effect changes in the ability of the cell to progress through the cell cycle.

(id) Cell division and the pleiotypic programme

It has now been established that a diverse range of agents are capable of initiating division in both contact inhibited and quiescent cells (Table 2). The interaction of these mitogenic factors with the cell membrane also results in a change in the rate of transport of various substrates (Table 3). Thus it has been suggested that these transported species are required for certain biochemical processes which are essential for the progress of the cell through some transition point(s) in G1 (Kram and Tomkins, 1973). This schedule of biochemical reactions has been termed the pleiotypic programme (Hershko, Mamont, Shields and Tomkins, 1971; Kram, Mamont and Tomkins, 1973; Kram and Tomkins, 1973).

Whilst it is clear that the transport of some of these species results from alterations in membrane function, it is now becoming evident that some examples of enhanced translocation may be attributable to an elevation of endogenous metabolism of the particular substrate. Thus although there is an increased rate of hexose transport into serum activated cells (Sefton and Rubin, 1971; Kram and Tomkins, 1973) this is associated with an elevation of intracellular phosphorylation of the sugar (Colby and Romano, 1974). This has the effect of decreasing the endogenous glucose concentration, and facilitating the further influx of the compound. Non-metabolisable hexoses such as 3-O-methyl glucose show no such change in translocation rate following cell stimulation (Colby and Romano, 1974). Similarly the apparent increase in phosphate (and perhaps uridine) transport

Table (2) Agents Which Reinitiate The Mitotic Cycle

Mitogenic agent	Cell type	Reference
Serum macromolecular factors	3T3	(1)-(3)
Low molecular weight serum factors	3T3	(4)
Fibroblast growth factor	3T3	(5),(6)
Epidermal growth factor	3T3	(7)
Insulin	3T3	(6),(8)
Hydrocortisone	3T3	(9)
Prostaglandin F2 alpha	3T3	(10)
Cyclic adenosine 3'5'-monophosphate	3T3	(11)
Cyclic guanosine 3'5'-monophosphate	3T3	(12)
Calcium ions	3T3	(13)
Magnesium ions	CEF	(14)
Amino acids	3T3	(6)
Phosphate	3T3	(6)
Glucose	3T3	(6)
Proteolytic enzymes	3T3	(6)

Table (2) indicates mitogenic agents, which will release contact inhibited 3T3 or chick embryo fibroblasts (CEF).

Reference code:-

(1) Todaro et al., 1965.
(2) Holley and Kiernan, 1968.
(3) Temin, 1966.
(4) Holley, Baldwin and Kiernan, 1974.
(5) Gospodarowicz, 1974.
(6) Holley and Kiernan, 1974.
(7) Armelin, 1973.
(8) Yarnell and Schnebli, 1974.
(9) Thrash and Cunningham, 1973.
(10) Jimenez de Asua, Clingan and Rudland, 1975.
(11) Kram and Tomkins, 1973.
(12) Seifert and Rudland, 1974.
(13) Dulbecco and Elkington, 1975.
(14) Rubin and Koide, 1976.

Table (3) Substances Which Exhibit Enhanced Transport Rates Following Stimulation Of Resting Cell Cultures

Substrate	Stimulant	Cell type	Ref.
Uridine	Serum	3T3	(1)
"	Colcemid	3T3	(1)
"	Vinblastine	3T3	(1)
"	PHA	Lymphocytes	(2)
Cytosine	Serum	3T3	(3)
Guanosine	Serum	3T3	(3)
Phosphate	Serum	3T3	(3)
"	PHA	Lymphocytes	(4)
Glucose	Serum	CEF	(5)
"	Trypsin	CEF	(5)
2-deoxy-D-glucose	Serum	3T3	(1)
" "	Colcemid	3T3	(1)
" "	Vinblastine	3T3	(1)
" "	Insulin	3T3	(6)
" "	PHA	Lymphocytes	(2)
Leucine	Serum	3T3	(1)
"	PHA	Lymphocytes	(2)
Calcium	PHA	Lymphocytes	(7),(8)
"	Con A	Lymphocytes	(7)
"	Ionophore A23187	Lymphocytes	(7)
Potassium	PHA	Lymphocytes	(9)
Sodium	PHA	Lymphocytes	(10)
Choline	PHA	Lymphocytes	(2)
Alpha-amino isobutyric acid	PHA	Lymphocytes	(11)

Table (3) represents a list of substrates whose transmembrane transport rate increases when the resting cell population (3T3 or CEF fibroblasts and peripheral lymphocytes) is stimulated to undertake a fresh round of division by a diverse array of agents. These stimulants include serum, PHA (phytohaemagglutinin), Con A (concanavalin A), the calcium ionophore A23187, insulin and the microtubular disaggregators colcemid and vinblastine.

Reference code:- (1) Kram and Tomkins, 1973.
(2) Peters and Hausen, 1971.
(3) Cunningham and Pardee, 1969.
(4) Cross and Ord, 1971.
(5) Sefton and Rubin, 1971.
(6) Bradley and Culp, 1974)

Table (3) continued Reference code (7) Freedman, Raff and Gomperts, 1975.
(8) Allwood, Asherson, Davey and Goodford, 1971.
(9) Quastel and Kaplan, 1971.
(10) Dent, 1971.
(11) Mendelsohn, Skinner and Kornfeld, 1971.

may be attributable to the rapid incorporation of these species into RNA, the metabolism of which is also raised following stimulation (Cunningham and Pardee, 1971). It should also be noted that not only do transformed cells not exhibit contact inhibition but they also have higher intrinsic transport rates for substrates which are involved in G_0 arrest. These nutrients include glucose (Hatanaka, Augl and Gilden, 1970) and 2-deoxy-D-glucose (Isselbacher, 1972). However it is unclear which of these species are essential for, or cause G_0 escape. Certainly increased hexose transport may be dissociated from division by the use of cortisol in 3T3 cells (Thrash and Cunningham, 1974). Thus the observation of higher transport rates for glucose in transformed fibroblasts may not be of any fundamental importance in the abnormal proliferation of these cells. Similarly uridine is not an essential component of the culture medium for lymphocyte stimulation with PHA to occur, although its translocation is enhanced (Peters and Hausen, 1971).

(ie) <u>The role of cyclic nucleotides in the control of cell division and pleiotypic programming</u>

Perhaps the largest single link yet detected between cell division, membrane dynamics and pleiotypic regulation is the action of the cyclic nucleotides, adenosine 3'5'-monophosphate (cyclic AMP) and guanosine 3'5'-monophosphate (cyclic GMP). Although the precise

action of these cyclic purines will be discussed in detail in Units 4 and 5 of this thesis, it would seem pertinent to outline the historical background behind their role in the regulation of cell division.

One distinguishing feature of transformed cells when compared with their parent cell type is that they contain appreciably lower concentrations of cyclic AMP (Sheppard, 1972). Furthermore treatment of transformed cells with cyclic AMP or agents which raise endogenous levels of the nucleotide results in the cells reverting to normal patterns of proliferation (Sheppard, 1971). In addition these cells also exhibit changes in membrane structure, reverting towards that typical of the cell from which they were derived (Burk, 1968; Hsie and Puck, 1971; Puck, Waldren and Hsie, 1972). This has led to the suggestion that this cyclic nucleotide is a differentiating agent, since it appears to arrest growth in favour of morphological changes.

When untransformed cells reach confluency there is an increase in their intracellular cyclic AMP concentration (Bannai and Sheppard, 1974). This subsequently falls on serum readdition (Burger, Bombik, Breckenridge and Sheppard, 1972), as it does with proteases (Burger et al., 1972), insulin (Seifert and Rudland, 1974) and transforming virus (Raska, 1973), agents which will also reinitiate division. Thus a fall in cellular cyclic AMP concentrations or the prevention of a rise in its level may represent a key control stage in cell proliferation. Further evidence (Burger et al., 1972) suggests that cyclic AMP concentrations fluctuate through the cell cycle, there being drops in nucleotide levels prior to DNA synthesis and again during late G2 and mitosis. Thus a fall in endogenous cyclic

AMP may be the effector signal in late G1 for the commitment of the cell to DNA synthesis and a fresh round of mitosis. If the cell does not experience this fall in endogenous cyclic AMP then it will fail to exit the G1 phase and remains in a G_0.

Recently the other naturally occuring cyclic nucleotide, cyclic GMP, which often antagonises the action of cyclic AMP (Goldberg, Haddox, Nicol, Glass, Sanford, Kuehl and Estensen, 1975), has been implicated in the regulation of proliferation. The intracellular concentration of this cyclic purine is thought to rise shortly after stimulation of quiescent cells (Hadden, Hadden, Haddox and Goldberg, 1972; Seifert and Rudland, 1974). However this point is in contention as the opposite effects have been observed (Miller, Lovelace and Pastan, 1975).

Cyclic AMP may have profound effects upon the cell membrane and transport kinetics. It has been observed that when fibroblasts are arrested in G1 by addition of cyclic AMP there is an abrupt decrease in transport activity (Kram et al., 1973). Furthermore exogenous cyclic AMP antagonises both the mitogenic and transport enhancing capacity of serum (Kram and Tomkins, 1973). This action is mirrored by prostaglandin E_1 and theophylline, agents which elevate intracellular cyclic AMP concentrations (Robison, Butcher and Sutherland, 1968). In contrast cyclic GMP, colcemid and vinblastine all synergize the action of serum in enhancing transport rates and inducing a fresh wave of division in contact inhibited cells (Kram and Tomkins, 1973). Thus these agents antagonise the effect of cyclic AMP. These results suggest that since cyclic AMP can induce changes in membrane morphology (Furmanski, Silverman and Lubin, 1971; Hsie and Puck, 1971) and this action appears to be opposed by agents

which provoke microtubule disaggregation, then cyclic AMP (and perhaps cyclic GMP) effects at least part of its action by regulating the state of microtubule assembly. Such a notion would be supported also by the observation that cyclic AMP 'quietens' cell membranes (Johnson, Morgan and Pastan, 1972).

Thus to summarise this somewhat diverse section, it appears that the growth and division of normal cells is regulated by the interaction of the cells with a number of polypeptide hormones or hormone-like factors, which are present in the surrounding fluids. As cells become crowded they require larger amounts of these agents on account of poor accessibility. When supply no longer meets some critical demand level cells opt out of the mitotic cycle. This process may be facilitated through alterations in cyclic nucleotide concentrations which in turn modify membrane structure and transport rates via its ability to regulate microtubule assembly. Tumour cells appear to have lost the requirement for growth promoting factors, and may divide in the absence of these agents until nutrient supplies become exhausted. This property may be the result of changes in cyclic nucleotide metabolism or membrane function.

(3c) The cellular organisation of mammalian tissues..... a possible guide to their proliferative control mechanisms?

On the basis of tritiated thymidine labelling of DNA, Leblond (Leblond, Messier and Kopriwa, 1959) was able to discern three types of tissues. The first category was those tissues which did not incorporate label in the adult animal, that is undetectable levels of cells passed through their DNA synthetic phase during the period of the experiment. Furthermore if these tissues were labelled in the neonatal animal, when there were proliferating cells present, then it was noted that this label was not lost over a long period. It was concluded therefore that tissues such as striated, cardiac and smooth muscle, as well as neurons did not contain proliferating cells in the adult animal. Neither was there any significant cell loss in these tissues since there was no turnover of labelled cells. Such cell populations were termed stable or non growing. However, two other classes of tissues did show incorporation of the radionuclide. In one of these there was always a low level of labelling, with a concomitantly low level of cell loss. Such tissues included the proximal convoluted tubule of the kidney, parenchymal cells of the liver, acinar cells of the islets of Langerhans, epithelial cells of the thyroid and cortical and medullary cells of the adrenals. This data suggested that cells were lost for various reasons at a slow rate and were replaced by proliferation. The final population type described exhibited both rapid incorporation and label loss. Such tissues, termed renewing cell pools, included the Malpighian cells of the tongue, cortical cells of the thymus, bone marrow cells, skin and gastrointestinal epithelium, lymphatic organs and the connective tissue of the derma. These tissues appeared to have a high intrinsic cell turnover as indicated by rapid labelling and low

levels of label retention. However, the distinction between these latter two groups may only be a matter of degree, inasmuch that intestinal mucosa and erythrocytes have a greater rate of attrition than do thyroid cells for instance, although both tissues may contain very similar control systems.

Further work (Gilbert and Lajtha, 1965) has shown that this simple classification belies another order of complexity in cell organisation. There may in fact be at least six different types of cell populations (Figure 11) and any one tissue may contain a number of these basic kinetic units within it. Thus in the embryo and young animal most tissues consist of expanding cell populations

Figure (11) A Diagrammatic Representation Of Possible Cell Populations

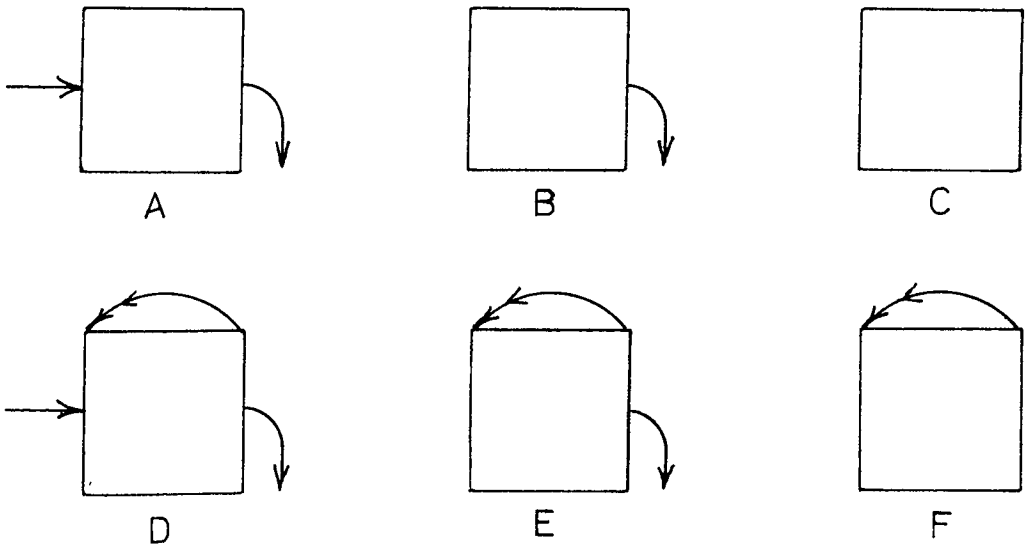


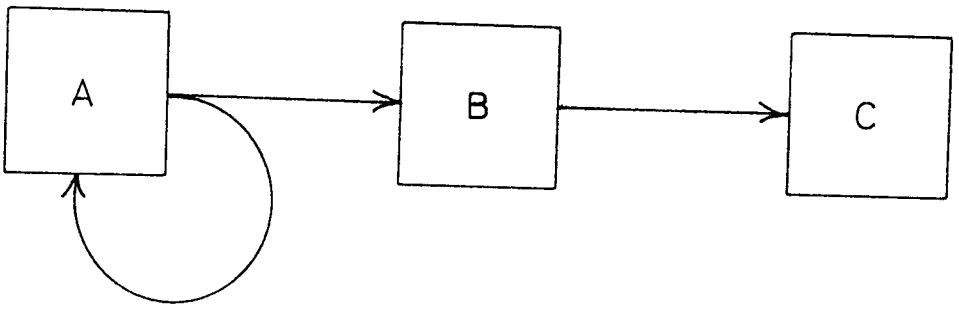
Figure (11) indicates the possible cell kinetic units which may exist within a tissue (modified from Gilbert and Lajtha, 1965). The arrows entering indicates cell input from distal sources, arrows leaving represents cell death and efflux. Proliferation within a population is shown by a double headed arrow. A is a simple transit unit e.g. reticulocytes; B is a decaying population e.g. ovary; C is a closed static pool e.g. neurons; D is a dividing transit population e.g. bone marrow; E is a stem cell unit e.g. bone marrow; F is a closed dividing population e.g. regenerating liver, tumours.

in which the rate of division exceeds cell loss. In the adult however most tissues maintain a steady size either because there is a negligible rate of cell loss and gain or because the rate of cell production always equals the rate of cell attrition and migration. This latter type of cell population in which there is a precise balance between production and loss has been termed a cell renewal system (Patt and Quastler, 1963). The common feature of most renewal systems is that the mature functional cells have only a limited lifetime, and they must be continuously replaced by the proliferation and maturation of other cells in the renewal system (e.g. Cleaver, 1967). Thus it is possible to discern several cell compartments within a tissue based on both proliferative and morphological criteria. Firstly there is a mature functional compartment composed of post-mitotic cells. These terminal cell types are generated by division and differentiation of cells in a dividing maturing compartment. However calculations indicate that the absolute cell numbers present in this latter compartment are inadequate to maintain a steady tissue size throughout the life of the animal (Lajtha, 1970). Thus it has been proposed that these renewal systems must contain a self-maintaining stem cell compartment (Lajtha, Oliver and Gurney, 1962; Patt and Quastler, 1963). These relatively undifferentiated cells are often difficult to identify (Cudkowitz, Upton, Smith, Gosslee and Hughes, 1964) and may be distributed throughout the tissue as is the case in bone marrow (Quastler and Sherman, 1959), although they may have a fixed loci such as the base of the villi in gastrointestinal epithelium (Bond, Fliedner and Archambeau, 1965).

Thus a simple renewal system may be envisaged as comprising three compartments (Figure 12). First it contains a self-maintaining stem cell pool. On average for each division in this compartment one

cell will re-enter the stem cell compartment, thus maintaining it at a constant size, whilst the other daughter cell will be irreversibly committed to a given programme of differentiation in the dividing maturing compartment. In this maturation phase a number of rounds of mitosis may occur before the cell becomes post-mitotic and thus

Figure (12) A Simple Three-Compartment Cell Renewal System



The figure illustrates a hypothetical three-compartment cell renewal system. Compartment A is a self-maintaining stem cell population, as indicated by the circular arrow. Cells exit this cell pool and undergo differentiation and further rounds of division in the maturing dividing compartment (B). These cells ultimately become post mitotic mature functional cells (C). After Cleaver, 1967. For further discussion see text.

it acts to amplify the effect of one cell leaving the stem cell compartment (Cleaver, 1967). Indeed in the erythroid series this amplification factor may be as high as $1:10^6$ (Gordon and Zanjani, 1970), hence a slight alteration in the rate of cell output from the stem cell compartment may be reflected in a large increase in absolute numbers in the mature cell population.

In reality, however, renewal systems are more complex than this simple model. For instance the maturing dividing compartment may contain a number of discernible cell types as is the case in the bone marrow (Bloom and Fawcett, 1962). Furthermore cells in both

the stem and dividing maturation compartments may leave their normal

Figure (13) A Four-Compartment Cell Renewal System

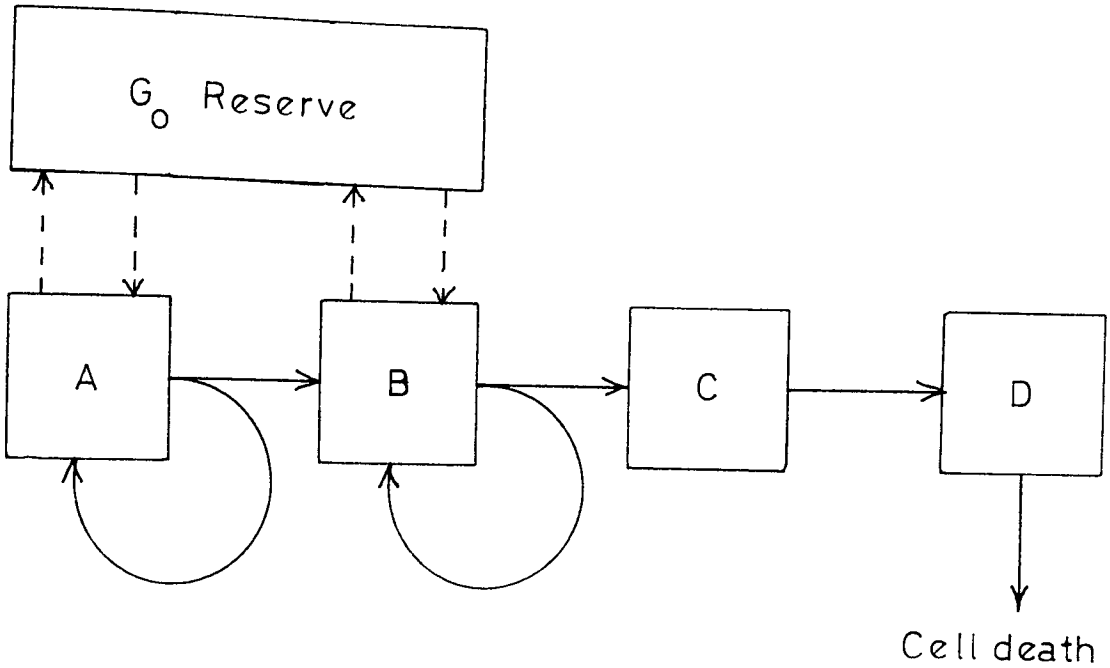


Figure (13) represents a four-compartment cell renewal system. A is a self-maintaining stem cell compartment; B is a maturation pool which does contain dividing cells; C is a maturation population which does not contain any dividing cells; D is a mature functional compartment. The G_o reserve consists of cells which are indistinguishable morphologically from the cells in either A or B so are drawn together. These cells may enter their respective cycling pools when the need arises. In this diagram population B is also designated as being a self renewing cell pool. Modified from Cleaver, 1967.

mitotic cycle and enter either an extended G1 or G2, these phases being designated G'_o and G''_o. The former have been detected in bone marrow (Lahiri and van Putten, 1972), tumour cells (Wilson and Gehan, 1970), chick intestinal epithelium (Cameron and Cleftman, 1964) and blood borne lymphocytes (Knight and Ling, 1968). G''_o populations have been discerned in ear epithelium (Gelfant, 1962). These cells appear to be capable of reinitiating their mitotic programme very rapidly following cell loss or the demand for further cell production.

Such quiescent phases may provide both an emergency store of cells and a mechanism for the maintenance of stem cell compartment numbers.

A somewhat more complex cell renewal system is shown in Figure (13) which encompasses these additional features of cellular kinetics. The population contains a self perpetuating clone of stem cells which may ultimately divide and begin the process of irreversible differentiation. Many of these cells may not be actively cycling at any one time but may be arrested in a G_o, and to re-enter division on demand. The exit of a cell from the stem cell into the maturing dividing compartment provides an amplification stage which enables one precursor cell to generate a large number of functional mature cells. This phase too, may contain resting cell populations which have been committed to a given avenue of differentiation. The final stages of differentiation occur in the absence of proliferation in non-dividing maturation compartments which eventually yields cells with the terminal phenotype characteristic of that tissue.

Several features exist which can therefore be taken as indicators of possible sites for mitotic regulation.

(a) A mechanism must exist for perpetuating the stem cell population.

(b) Once a cell has been committed to some avenue of differentiation there has to be an apparatus for preventing the cell from reverting to more primitive phenotypes.

(c) At some point in the maturation programme there is a process which stops further cell/division cycles occurring.

(d) In both stem cell and committed compartments there has to be the facility for cells which still have the potential to undergo mitosis to enter protracted resting phases. They must also be capable of re-initiating division under suitable conditions.

(e) In terms of the physiological status of the whole organism a renewal system will represent a bodily function, and as such the total output must be geared to the day to day requirements of the animal. Thus although in general a cell pool will remain relatively constant during adult life, fluctuations will occur either through abnormal cell loss or through a change in the demand for the functional output of the system. Therefore there must be mechanisms which relate terminal function to cell proliferation. These processes may act at a number of sites in the renewal system to enhance or reduce cell output.

There are now several types of mechanisms which have been proposed as being capable of regulating proliferation and differentiation within renewal systems in vivo. These however might be grouped into two categories (a) physiological regulation and (b) chalone regulation. The credibility of each of these proposed systems depends upon the model used since certain tissues appear to be better examples for each process. Thus it may be that both these control systems may exist simultaneously.

'La fixite du nombre des cellules est une condition pour la vie inflexible'.

(3d) The physiological regulation of cell proliferation

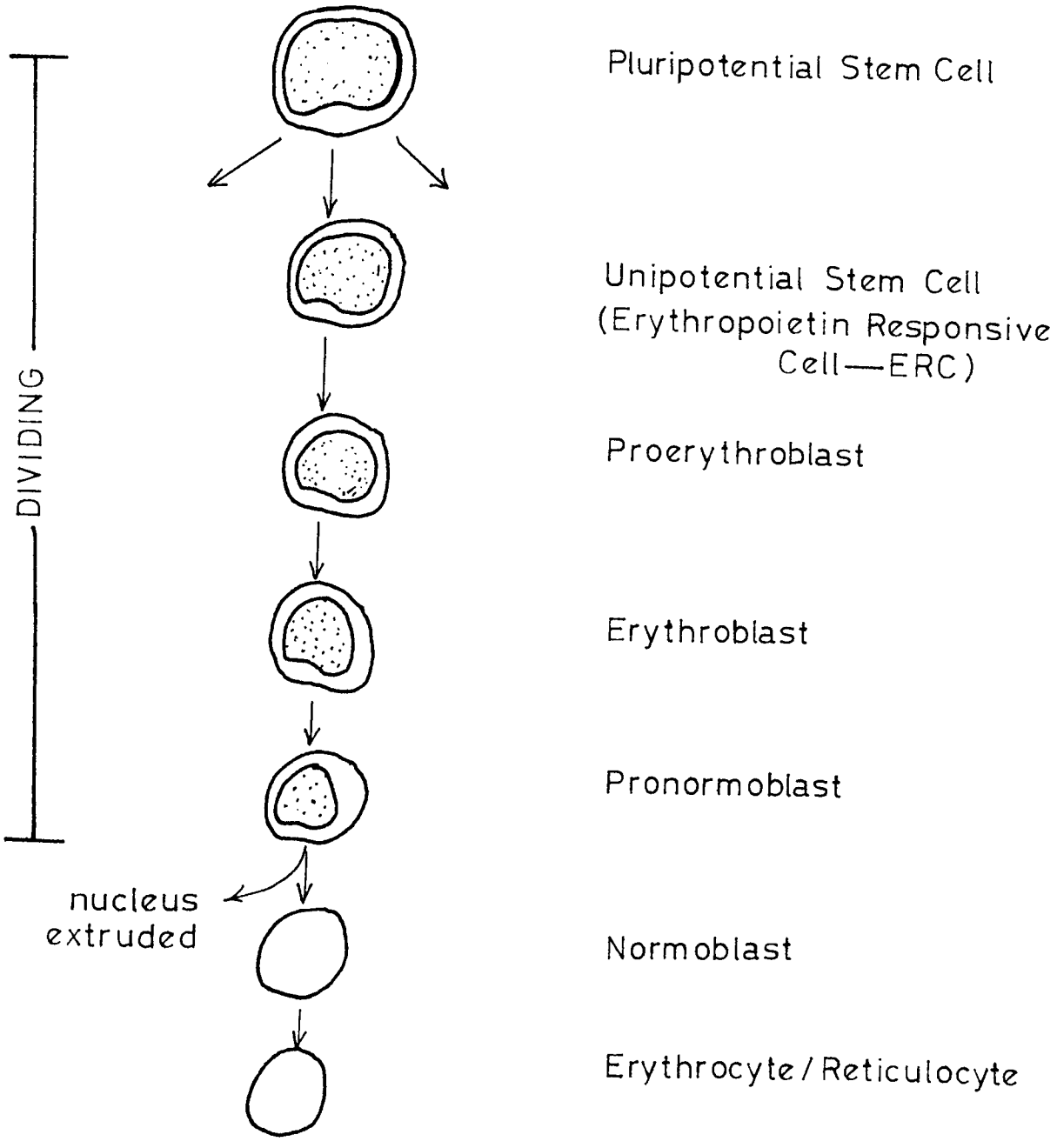
As stated in the previous section the control of proliferation in some tissues can be said with some certainty to be dependent upon one type of mechanism. In other tissues a different control system may be operative. Thus in erythroid, lymphoid, uterine and mammary tissues proliferation is at least partially controlled via the endocrine system. In epithelial cell systems only local effects have been demonstrated for tissue hormones and neurotransmitter substances and such tissues may not therefore be subject to a homeostatic control through the endocrines. Space restricts the analysis of all these renewal systems and so specific examples will be taken to illustrate both types of proliferative control models. Thus the control of erythroid and epithelial cell proliferation will be considered here as examples of these mechanisms. Lymphoid division will be considered in detail in a later section.

The erythroid renewal system as a model for physiological regulation

The terminal phenotype for the erythroid series is the circulating erythrocyte, whose function is the transport of oxygen to the tissues via the haemoglobin contained therein (Harris and Kellermeyer, 1970). These anucleate cells are generated principally in the bone marrow of adult rats (Grant and Roots, 1952) through the division and maturation of precursor cells (Marks and Kovach, 1966). These intermediary cell types of the erythropoietic series are derived in the following sequence: pluripotential stem cell-

unipotential stem cell-proerythroblast-erythroblast-pronormoblast-normoblast-reticulocyte-erythrocyte, as illustrated diagrammatically in Figure (14). Division occurs in all cell types up to and including the pronormoblast (Grasso, Woodward and Smith, 1963); subsequent to this stage nuclear extrusion occurs (Pease, 1956) hence no DNA synthesis or cell division is possible.

Figure (14) A Diagram To Illustrate The Erythroid Series



It has been inferred that the bone marrow must contain at least two cell types whose morphological identities have not been ascertained, and which give rise to the erythroid sequence. These experiments first showed that on transfer of bone marrow to irradiated hosts morphologically identifiable clones of erythroid, granuloid and thromboid cells arose in the spleen of these animals (Till and McCulloch, 1961; Lewis and Trobaugh, 1964). It was suggested therefore that the marrow contained a cell type which had the potential for differentiating into a primitive precursor cell of any of these three mature cell types i.e. it was pluripotential. This cell was termed a colony forming unit or CFU (Till and McCulloch, 1961). Further evidence (Jacobson, Goldwasser, Plzak and Fried, 1957) also indicated the presence of a stem cell population based on the disappearance and reappearance of proerythroblasts following transfusion-induced polycythaemia. Chromosomal marker studies have shown that not only is this CFU pluripotential but that it has the capacity to reproduce itself (Wu, Till, Siminovich and McCulloch, 1967). This evidence has led to the propounding of a composite model for erythropoiesis (Lajtha, 1970). Briefly the pluripotential stem cell or CFU, of which only 10% are actively cycling at one time (Becker, McCulloch, Siminovich and Till, 1965) divides to give a committed erythroid cell whose morphology has likewise not been ascertained. This cell has been characterized by its ability to respond to erythropoietin, thus this committed cell has been named an erythropoietin responsive cell (ERC). This latter cell is unipotential and generates the morphologically distinct proerythroblast. The ERC may divide as many as twenty times before it differentiates into the proerythroblast however, thus providing a large amplification factor in the erythropoietic process. Such amplification may be necessary to counterbalance the large losses of mature erythrocytes

which occur. Hunt (1974) has estimated that the adult 200g rat needs to replace some 10^9 erythrocytes every day of its life. This number may serve to indicate the magnitude of the cellular proliferation required.

It is clear that a major determinant of erythropoietic activity is the oxygen status of the tissues and in particular the kidney (Jacobson, Goldwasser, Fried and Plzak, 1957; Reissmann, Nomura, Gunn and Brosius, 1960). It was found that the kidney elaborates an enzyme, erythrogenin, which acts upon a plasma globulin substrate, erythropoietinogen, to form a hormone erythropoietin (EPO), which in turn regulates erythropoiesis (Gordon and Zanjani, 1970). EPO production is stimulated when the oxygen supply fails to meet the metabolic demand of the tissues (Jacobson and Goldwasser, 1958; Eskuche and Hodgson, 1962). Conversely when the oxygen carrying capacity of the blood is more than adequate (or tissue demand decreases) EPO production is suppressed (Jacobson et al., 1957a).

EPO acts at a number of points in the erythropoietic programme, and these are summarised below.

(a) EPO induces the synthesis of haemoglobin in the committed ERC (Lajtha, Pozzi, Schofield and Fox, 1969)

(b) In addition to this differentiating property EPO increases the number of proerythroblasts in the marrow (Orlic, Gordon and Rhodin, 1968). It appears to facilitate this by stimulating a G_o recruitment (Gross and Goldwasser, 1970) via a cyclic AMP dependent mechanism (Krantz, 1973).

(c) The hormone may decrease the cell cycle time in proliferating erythroblasts (Tarbutt, 1969), thus speeding up the transition to the maturing compartment.

(d) The normoblast maturation phase may be shortened or bypassed by EPO resulting in the release of immature reticulocytes (Krantz, 1973).

(e) EPO is known to cause the release of reticulocytes from the isolated hind limbs of rats (Gordon, LoBue, Dornfest and Cooper, 1962).

Thus to summarise this data, there is a stem cell compartment containing precursor cells which are the progenitors of bone marrow derived cell types. This cell compartment is capable of self renewal, and is not of sufficient size to provide committed precursor cells throughout the life of the animal without this endogenous replacement (Lajtha, 1970). The mechanism which maintains the stability of this cell pool are unknown, as are the causes for the decision to enter the committed cell population as an ERC. Both the ERC and CFU divide even in the polycythaemic mouse, however the former fail to manifest themselves as proerythroblasts (Lajtha et al., 1969) because of the low levels of circulating EPO which exist in these animals. Erythropoietin which is elaborated in the kidney reflects the oxygen supply/demand ratio of the blood and positively effects the differentiation of the ERC to the proerythroblast by inducing haemoglobin synthesis. It may also stimulate a G_o recruitment in the erythroblast compartment. The hormone also has a number of actions on phase compartment lengths, which serve to speed up the maturation of erythrocytes and their subsequent extrusion in the

peripheral circulation. Clearly EPO is therefore a positive effector of both cell proliferation and differentiation in the dividing maturation compartment. This point is worth emphasizing as in other proposed control systems a negative feedback is the dominant feature.

There is also evidence to suggest a positive hormonal control of the development sequences leading to thrombocytes and B lymphocytes (Ebbe and Stohlman, 1965; Miller and Mitchell, 1967; Ebbe, 1968; Gregory and Lajtha, 1968). Evidence for a granulopoietic hormone and ERC equivalent cell is uncertain at this time, however, all these cell types are thought to arise from the same CFU as the erythrocyte series.

More recently another physiological impingement upon erythroid proliferation has come to light in that the calcium homeostatic system appears to be intimately involved in the control of both basal and elevated levels of erythropoiesis. It was first noted that injections of calcium chloride into male rats enhanced mitotic activity in the bone marrow (Perris, Whitfield and Rixon, 1967). This treatment also produced an increase in reticulocytes in both the bone marrow and peripheral blood, as well as stimulating incorporation of ^{59}Fe into peripheral red blood cells (Perris and Whitfield, 1971). Injection of the hypercalcaemic parathyroid hormone (PTH) similarly led to an increase in mitotic activity (Perris and Whitfield, 1967) and ^{59}Fe incorporation (Perris and Whitfield, 1971). In contrast if the parathyroid glands were removed and the rats maintained on a low calcium diet then hypoplasia of the marrow ensued with or as a result of reduced mitotic activity (Rixon, 1968; Perris and Whitfield, 1971). There was a concomitant decrease in ^{59}Fe uptake into peripheral blood erythrocytes (Perris and Whitfield,

1971). If these aparathyroid animals were either fed calcium in their diet or were put on replacement doses of PTH then normal rates of proliferation and ^{59}Fe incorporation were again apparent (Perris and Whitfield, 1971). It is probable from these observations that it is the extracellular calcium ion concentration rather than the PTH itself which has a fundamental role in maintaining proliferation in this tissue. In addition the reduction in bone marrow cellularity was limited to the erythroid and lymphoid series, with the myeloid and thromboid sequences being unaffected (Rixon and Whitfield, 1972a).

Although a primary action of calcium appeared to be the maintainance of normal proliferation in the marrow a further important observation indicated that there might be a more far reaching involvment. It was noted that there developed an hypercalcaemia in rats which had been artificially haemorrhaged by cardiac puncture, these elevated calcium levels persisting until normal circulating erythrocyte numbers had been re-established (Perris, MacManus, Whitfield and Weiss, 1971). If the animal was aparathyroid then this hypercalcaemia did not occur, and the return to normal haematocrit values took much longer (Perris et al., 1971b; Perris and Whitfield, 1971). This response was a direct consequence of the loss of red blood cells rather than hypovolaemia or hypotension. There is thus a link between hypoxia, calcium homeostasis and erythropoiesis. This link was further characterized by the demonstration that injections of EPO into normal rats resulted in an elevation of both bone marrow mitotic activity and plasma ionised calcium concentrations (Hunt and Perris, 1973). Again in aparathyroid animals EPO treatment elicited neither a calcium increase nor an elevation of mitotic activity (Hunt and Perris, 1973). An interpretation of this data could be therefore that hypoxia causes the elaboration of EPO following haemorrhage. The hormone then acts

either directly or indirectly to elevate plasma calcium levels which in turn enhance division rates in the bone marrow. If this is the case then EPO itself might be considered as a differentiating agent and calcium as a proliferogenic one. In addition the hypovolaemia which occurs when blood is removed from the rat releases large amounts of antidiuretic hormone (ADH), and this hormone appears to stimulate bone marrow mitotic activity (Hunt, 1974).

It has now been established that a number of physiological situations exist in which there is a calcium-dependent stimulation of bone marrow mitotic activity. In all cases examined prior parathyroidectomy ablates both the calcium surge generated during these proliferative crises and the enhanced division rates which are normally concurrent with this hypercalcaemia. Table (4) outlines some of these situations which impinge upon the normal erythropoietic process.

Table (4) Parathyroid Dependent Processes Which Are Associated With A Stimulation Of Bone Marrow Proliferation.

Situation	Reference
(1) Erythropoietic insufficiency;	
(a) Haemorrhage	Perris et al., 1971.
(b) Pregnancy	Perris, 1971.
(c) Cobalt chloride injections	Perris, 1971.
(d) Erythropoietin injections	Hunt and Perris, 1973, 1974a.
(2) Immunological challenge	
(a) Humoral antigens (SRBCs, LPS, BSA.	Edwards, Mekori, Atkinson and Perris, 1976.
(b) Xenograft (cell-mediated)	ditto

Table (4) continued.

Situation	Reference
(3) Circadian variations	Hunt and Perris, 1974b
(4) Oestrous cycle changes	Smith, 1976.
(5) Rapid body growth	Perris, Whitfield and Tolg, 1968.
(6) Regenerating liver	Rixon and Whitfield, 1972b

Table (4) enumerates a number of physiological situations which either cause or are associated with changes in plasma calcium levels. In all instances peak calcium titres accompany peak mitotic activity in the bone marrow. Furthermore parathyroidectomy ablates both the calcium changes and mitotic surges in this tissue. The humoral antigens used were SRBCs-sheep red blood cells, LPS-bacterial lipo-polysaccharide, BSA-bovine serum albumin. The xenograft involved the transplant of mouse tail skin to rat flank.

The chalone theory as a mechanism for the control of cell division rates in mammalian epithelial layers.

Some twenty years ago the suggestion that tissue mass might be regulated through the production of a tissue specific antimitotic agent was first propounded by Weiss and Kavanau (1957). This agent, later termed a chalone (Bullough and Laurence, 1960), was proposed to be generated by the cells of a given tissue where it accumulated locally and inhibited division when some critical concentration was exceeded. In the intervening years a range of tissues have been shown to yield extracts capable, when injected back into syngeneic animals, of reducing the levels of mitosis in the parent tissue (Bullough, 1973; Tutton, 1973; Clermont and Mauger, 1974). However, the chemical nature of these factor(s) has proved difficult to ascertain although it has been suggested that they may be proteins or glycoproteins (Hondius, Boldingh and Laurence, 1968; Houck and Daugherty, 1974). Thus it was envisaged that each cell of a tissue generated this specific agent at a constant rate, and therefore as cell numbers increased so did the absolute levels of chalone synthesized. Since it was argued that the chalone accumulated within the cells of the tissue and did not diffuse away, then when the tissue reached some critical size this agent would begin to inhibit division in the tissue since some threshold level had been exceeded. On wounding it was inferred that a drop in chalone concentration occured and that further cells might then initiate their mitotic programmes. A similar arguement was implied for an abnormal cell loss (Bullough, 1973).

Although this notion of a one stage feedback loop regulating division has remained the central common theme of the chalone theory over the years it has proved necessary to invoke further elements

in this model. One new feature was the realisation that in the epithelium of the skin there was an agent exerting a positive proliferative action (Karasek, 1969; Laurence, Randers-Hansen, Christophers and Rytömaa, 1972; Ronzio and Rutter, 1973). One such 'antichalone' is the so-called mesenchymal factor, produced by non-epithelial mesenchymal cells which underly the proliferative layer of the dermis (Ronzio and Rutter, 1973; Bullough, 1975). The suggestion has been made that this proliferogenic factor overides the inhibitory action of the chalone in the cells in the immediate proximity of its production (Bullough, 1975), irrespective of the chalone concentration. There is no substantial evidence available however to indicate why cells further from the site of production are not affected. Clearly explanations could include poor diffusibility of the factor, its rapid metabolism or uptake by cells. It might be recalled that a number of hormones (as well as calcium ions) exert positive proliferative effects and thus a parallel might be drawn between these hormones and the tissue proliferogenic factors.

A second feature which has recently developed is the discovery that a tissue may contain more than one chalone (Marks, 1971, 1973; Elgjo, 1973). The implications of these findings have not yet been adequately incorporated into a unifying hypothesis for chalone action. Furthermore, whilst it has been proposed that it is the intracellular chalone concentration which is the parameter determining mitotic activity (Bullough, 1972), recent evidence suggests an extracellular site of action (Houck, 1973). However, this result would not significantly alter the basis of the theory.

Chalone application appears to arrest the cell at two distinct points in its mitotic cycle. The first site is in late G1

(Frankfurt, 1968) and the second is in the G2 phase (Bullough and Laurence, 1961). Cells which have failed to pass these arrest points have not been designated as having entered a G_o (Bullough, 1972), despite the fact that G''_o populations have been detected in ear epithelium (Gelfant, 1962) and that the cells may under appropriate conditions rapidly reinitiate their mitotic programme (Christophers, 1972). It has been suggested that chalones rather than having an absolute effect on the passage of cells through their cell division cycle merely make it less likely for a given cell to pass one of these two transition points (Bullough, 1972). That is chalones have a stochastic (or probabilistic) rather than a deterministic action on the cell cycle. It has also been observed that other agents may synergize with the action of chalones. Thus corticosteroids are believed to enhance the effect of chalones in arresting mitotic progression (Bullough and Laurence, 1961). Adrenaline was formerly thought to also enhance the effectiveness of epidermal chalone (Bullough and Laurence, 1961), however, this contention has now been discounted (Bullough, 1975).

According to the theory (Figure (15)) the chalones act primarily on cells which are in that region of the G1 phase where a decision is taken to either undertake a fresh round of division, or to 'decycle' and effectively differentiate (Bullough, 1972). Temin (1971) has discerned at least three regions of the G1 period. The first termed G1a is essentially the terminal portion of the previous cell cycle, and is not directed towards division (Smith and Martin, 1973). The second (G1b) is a critical decision period and terminates at the G1 chalone blockage point according to Bullough (1972). The final portion of G1 (G1c) is relatively short and represents the irreversible commitment to DNA synthesis and mitosis (Frankfurt, 1968).

The basic tenet of the theory still holds that if a cell is subjected to a supracritical concentration of chalone, then it will cease to cycle and become post-mitotic. In the epidermis it is

Figure (15) A Diagram To Illustrate The Control Of Epidermal Proliferation By Chalones.

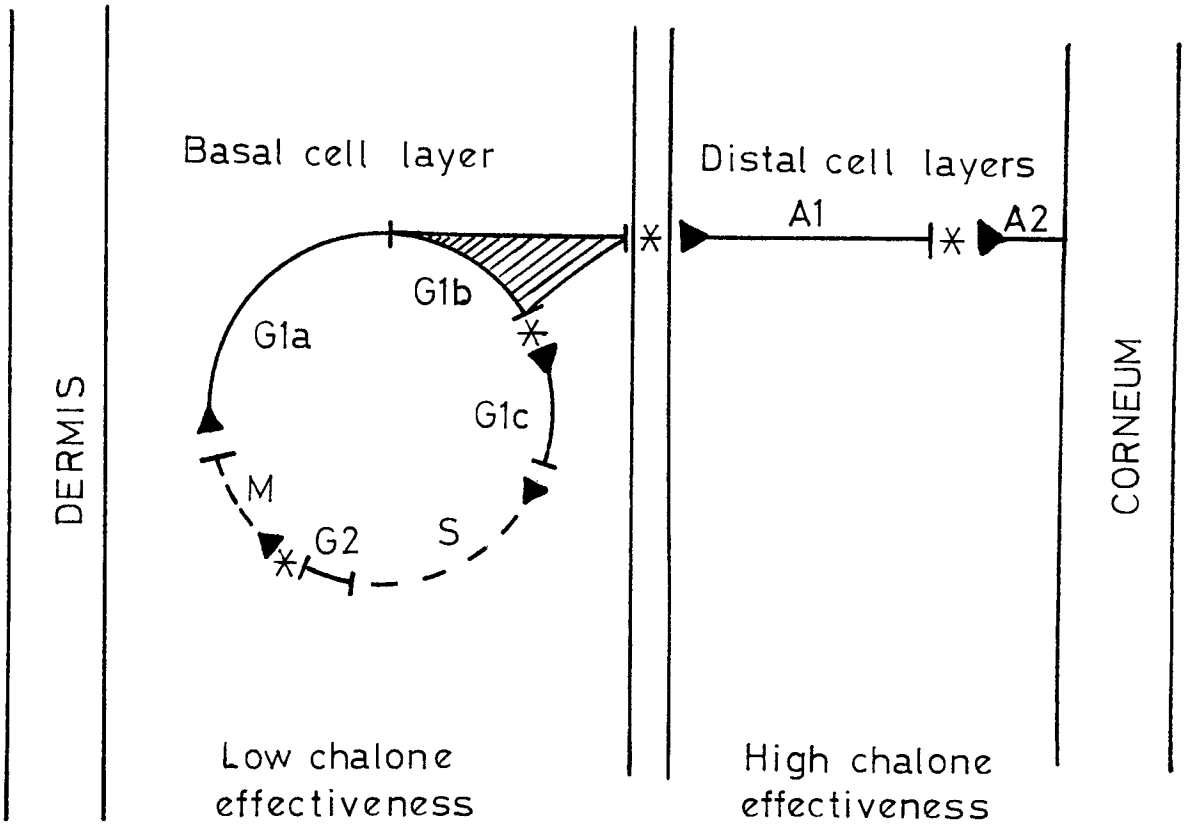


Figure (15) is redrawn from Bullough, 1975, and represents the control of epidermal thickness through a chalone system. The chalones act at the sites in the cell cycle indicated by asterisks. The cell cycle compartments G1a, G1b, G1c, S, G2, M, A1 and A2 are as defined in the text. The basal connective layer (dermis) produces the mesenchymal factor which promotes division in the basal cell layer. Cells which are forced out of the basal layer tend to become post-mitotic due to the action of epidermal chalone, which is more effective in these distal regions. Cells then move outwards to the stratum corneum (corneum) where they are eventually lost. Chalone may also affect the rate of progression to these outer layers and thus alter the ageing process of these cells.

clear that proliferation occurs predominantly in the basal epithelial

cell layer through the proliferogenic action of the mesenchymal factor (Ronzio and Rutter, 1973; Bullough, 1975). Thus in this basal layer of cells chalone has a low effectiveness. However, due to lateral pressure imposed on the basal layer cells by their increase in cell numbers at mitosis post-mitotic cells are forced up into a higher cell stratum (Bullough and Laurence, 1974; Frankfurt, 1967; Bullough, 1975). As these cells move away from the area of mesenchymal factor secretion they enter regions where of necessity the chalone must be more effective. Furthermore it is also claimed that only post-mitotic cells produce chalone (Bullough, 1972) thus it is likely that not only will chalone be more effective in higher layers due to a lower level of mesenchymal factor, but that there will indeed be higher ambient chalone concentrations. Following tissue damage cell division is known to occur in higher cell layers of the stratum spinosum (Epstein and Sullivan, 1964; Bullough, 1969). It is reasoned that cell loss causes the chalone concentration gradient to be broken and for some cells to escape the blockade. It is further proposed that two post-mitotic states exist. The first is designated A1 and represents cells of the stratum spinosum which may revert to their mitotic programme, and which are only maintained quiescent by the chalone concentration (Bullough, 1975). However, cells of the upper stratum spinosum and stratum corneum never divide irrespective of the chalone status, and these cells have been designated as being in an A2 phase (Bullough, 1975). These latter cells may have supressed mitotic genes, and may eventually lose their nuclei (Vaughan and Bernstein, 1971). Chalone has also been proposed to act upon cells in the A1 phase to slow their transition into the A2 compartment (Bullough, 1972). It might therefore be that chalone actually slows down the ageing process.

The chalone theory has been extended to include the genesis and growth of animal tumour cells (Bullough and Laurence, 1968). They suggest that neoplastic cells contain lower concentrations of the particular chalone through an inability to retain this factor. The cells however were still thought to be capable of responding to the chalone albeit at much higher concentrations. Evidence is now available where chalone addition has caused tumour regression (Bichel, 1970, 1971, 1972, 1973) thus substantiating this claim. This data should be treated with care as the injection of a number of non-specific agents such as bacterial toxins also facilitates tumour regression through a stimulation of the immune response.

A number of question still require resolving concerning this particular theory, especially how such a system can possibly operate in a diffuse tissue such as the bone marrow or lymphoid organs, where there is not the clear cellular organisation apparent in epithelia. However it would not be inconsistent to imagine an agent which acted in the G1 phase of the cell cycle and which caused both mitotic arrest and transition of cell cycle programmes in a fashion analagous to the mating factors of yeast. Indeed the chalone theory would represent a simple and effective system for maintaining a tissue at a constant size. It should be noted, however, that such a situation is not perhaps the boon it would be in terms of other physiological parameters since it is advantageous in many circumstances to have a flexible cell population capable of specific enhancement or decrease. This perhaps raises the major criticism against the theory, that it does not apparently contain the facility for external modulation as does a physiological control model which directly relates to cell demand. There is certainly no evidence to indicate that chalone synthesis alters during periods requiring

changes in cellularity, a basic requirement for a true proliferative control system. When this fact is added to the lack of identification and characterisation of the chalones, it is not surprising to note that the theory as a whole has met with not a little scepticism. However, as this theory could represent a fundamental control system for tissue mass and biological ageing regulation one can only hope that some of these difficulties may be resolved in the near future.

A summary of proliferative control

Before embarking upon the experimental section of this thesis it would seem germane to review the important concepts introduced in this first section of the dissertation, and to indicate where the reported results might be relevant.

(1) Cells need not necessarily be actively cycling at all times. They may indeed either become temporarily quiescent (a G_o population) or may permanently decycle, a feature which is frequently associated with the acquisition of the terminal differentiated phenotype of the cell type concerned.

(2) In both the yeast and mammalian cells the G1 period of the cell cycle contains a decisive region where the commitment to undergo a fresh round of mitosis is taken. In the yeast this process is under the control of a single gene, which titrates the extracellular nutrient status. In addition a number of proteinaceous factors also alter the expression of this gene so as to facilitate a transition of cell cycle programmes i.e. they effect differentiation. Similarly (or in an analagous fashion) nutrient deprivation causes many normal cell lines in culture to become arrested in G1, and

subsequent serum addition permits further rounds of division to occur.

(3) In mammals it seems that the control of proliferation may reside at least in part with a number of peptide or polypeptide hormones which act on cells in G1, and which are capable of overcoming a nutrient deprivation inhibition of division.

(4) The cell may undergo a typical biochemical cycle involving changes in nucleotide metabolism and chromatin structure, with defined patterns of enzyme inductions. Extrinsic factors may then superimpose themselves upon this intrinsic order to regulate and integrate tissue proliferation rates. These hormonal effectors may notably affect transition points in the G1 period, which constitute some aspect of the chromatin or membrane cycle.

(5) In many ways differentiation and proliferation are antagonistic processes, or are at least inversely related to one another. Hence at the organism and cellular levels there must be physiological axes which mediate these distinct events.

The experimental section of this thesis will deal almost exclusively with the lymphoid tissue <u>in vitro</u> as a model for the control of proliferation. It will examine the features of a G_0 population in the lymphocytes which relate to the wider questions of intrinsic and extrinsic regulation of division as outlined in the introduction. The areas of study include cyclic nucleotide metabolism, membrane function and hormone/cell interactions. There is also an attempt to invoke a dualistic model for the cellular regulation of proliferation and differentiation.

UNIT 1 The thymus: its kinetics and culture

1•1 The role of the thymus in immunity

No appraisal of thymus kinetics would be complete without first establishing the function of this organ with respect to the whole animal.

It was clear that animals which had been deprived of their thymus glands in neonatal life had low levels of circulating lymphocytes, and were less capable of responding to immunological challenge than normal animals (Gowans and McGregor, 1965). Furthermore, whilst some areas of lymphoid tissues seemed unaffected by this treatment, other regions were notably denuded of cells. These affected regions included cortical areas of lymph nodes, and the periarteriolar sheaths of the spleen (Gowans and McGregor, 1965). For this reason these denuded areas were designated as being thymus-dependent. Further experimentation indicated that the thymus seeded cells into these loci and probably maintained their normal cellularity (Miller and Osoba, 1967).

Not all immunological reactions were found to be equally impaired by neonatal thymectomy, indeed humoral antibody synthesis frequently showed no quantitative change (Humphrey, Parrott and East, 1964). This effect did however depend on which antigen was used to elicit the immune response (Papermaster, Dalmasso, Martinez and Good, 1962). It appeared rather that thymectomy affected the ability of the animal to mount delayed hypersensitivity reactions and to reject grafts. These latter types of immune reaction are said to be cell-mediated immune responses (Roitt, 1972). That is the presence

of cells is required at the site of the antigenic invasion. On the other hand humoral antibody responses can take place some distance away from the cells which synthesize the antibody. Thus in terms of immune reactivity there appears to be a clear cut distinction between two arms of the immune system (although this distinction is rather arbitrary when considering the immune response as a whole). The first limb comprises cells which produce specific globulin proteins which are directed against foreign substances and organisms, which destroys these through a number of mechanisms (e.g. lysis, agglutination and opsonization). The second response depends upon a cellular attack on these foreign bodies both by a direct lymphocyte cytotoxic reaction and polymorph or macrophage assault induced by a lymphocyte or its emanations (lymphokines).

The cells which came from the thymus were shown to be involved directly in cell-mediated responses (Davies, Leuchars, Wallace and Doenhoff, 1971) and did not develop the ability to synthesize antibody (Elves, 1972). Thus it was clear that there were at least two populations of lymphocytes; those derived from the thymus and responsible for cell-mediated immunity; and those which produced antibody. These latter antibody-forming cells were identified as filling those areas of lymphoid tissue not occupied by thymus-derived cells (Elves, 1972). Since in the chicken these lymphocytes also have a distinct site of production in the bursa of Fabricius (Cooper, 1965) they have been designated as B lymphocytes, or bursal-equivalent lymphocytes as no such organ exists in man (Elves, 1972). In contrast the lymphocytes from thymus-dependent areas have been termed T lymphocytes because of their developmental origins (Roitt, 1972). It has been possible subsequently to distinguish these two classes of immunocytes by a variety of surface determinants (Raff and

Cantor, 1971; Waksman, Raff and East, 1972; Greaves, Brown and Hayward, 1976).

The B lymphocytes appear to have a short life span and divide following the appropriate antigenic challenge to yield plasma cells which secrete antibody (Nossal and Makela, 1962). The B lymphocyte population is then constantly replenished by the division of precursor stem cells in the bone marrow (Miller and Mitchell, 1967). The T lymphocytes, on the other hand, may be much longer lived than both B lymphocytes and plasma cells (Buckton, Court-Brown and Smith, 1967). These thymic dependent lymphocytes exhibit diverse functions some being immunosurveillant cells, others may be cytotoxic to a given antigen, whilst others may have the ability to secrete specific factors (lymphokines) which have chemotactic actions on both polymorphonuclear leucocytes and macrophage (Ling and Kay, 1975). Although B lymphocytes are primarily involved with antibody synthesis and T lymphocytes with cell-mediated immune responses this dichotomy of action is not as well defined as might be expected. Indeed the T lymphocyte memory cell, or antigen sensitive cell, is thought to play a role in initiating the more rapid synthesis of antibody during the secondary immune response (Metcalf, 1970). Furthermore injections of T and B lymphocytes together into irradiated animals is more effective in generating antibody synthesis than one or other of the cell types injected singly (Claman, Chaperon and Triplett, 1966). Thus a more realistic (but nevertheless simplistic) scheme for an immune response against a foreign organism might follow the pattern depicted in Figure 1·1(a). First a circulating T lymphocyte or 'memory cell' recognises the foreign material, whereupon it frequently divides to initiate the next stage of the reaction (Davies et al., 1971). This will involve the release of lymphokines which cause both

Figure (1•1(a)) A Scheme To Illustrate A Typical Immune Response

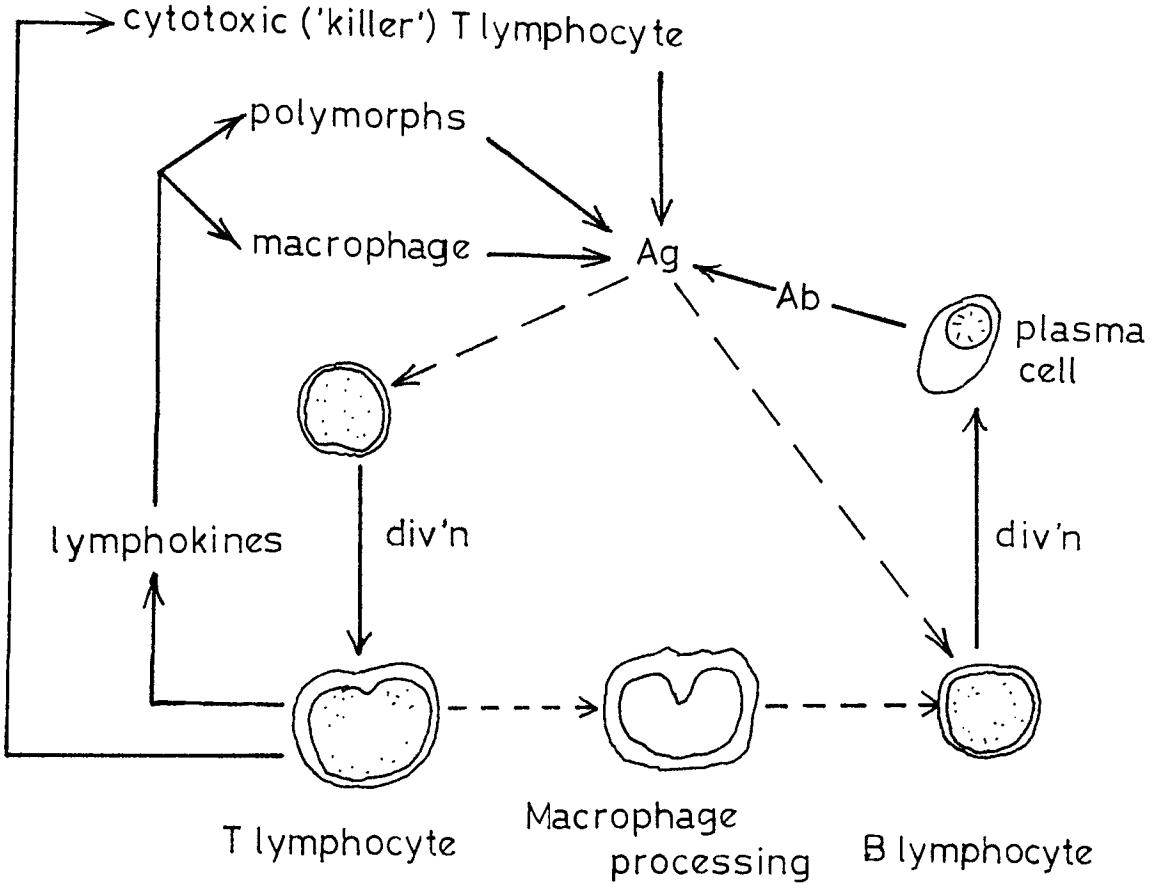


Figure 1•1(a) represents a typical immune response to an antigen (Ag). The foreign material may act upon a circulating T lymphocyte memory cell to induce it to undergo mitosis (div'n) which is a prelude to further responses. The daughter cells formed may directly kill the foreign organism as cytotoxic T lymphocytes. Alternatively or in addition daughter cells may secrete lymphokines which cause macrophage and polymorphonuclear neutrophils to congregate at the site of the antigenic infringement and to secondarily kill the antigen. These reactions constitute cell-mediated immune responses. In contrast the antigen may directly affect B lymphocytes, causing them to divide and differentiate into plasma cells which generate specific antibodies (Ab) directed against the antigen. This process constitutes humoral antibody-mediated immunity. Activated T lymphocytes may either directly or via macrophage processing induce B lymphocytes to undergo a similar transformation as occurs with antigen exposure. This process therefore interlinks cell-mediated and antibody-mediated immunity.

macrophage and polymorphs to localise at the site of the infiltration. These cells may attempt to kill and phagocytose the organism using a variety of proteolytic enzymes. Furthermore primed T lymphocytes which possess specific surface determinants for features of the antigen may also directly attack the foreign organism. Thus the nett effect is the slowing down, walling off and killing of the invading antigen. On the other hand through direct contact of the organism or its emanations with the antibody producing system a clone of plasma cells are produced through the proliferation of B lymphocytes. Some of these cells may also possess some memory capacity as do certain T lymphocytes (Roitt, 1972). On antigenic challenge, therefore, a population of cells are generated which synthesize a specific globulin antibody against the foreign organism. In addition 'memory' T lymphocytes which have previously been exposed to the antigen in question may also induce plasma cell production (Roitt, 1972). This phenomenon may require the processing of the antigen or the cooperation of macrophage with T and B lymphocytes in some way for it to be manifested (Seeger and Oppenheim, 1970, 1972). Thus in most instances an antigen elicits not one but a whole spectrum of immune responses directed against itself.

1•2 The thymus gland; its kinetics

The thymus in mammals is principally a central lymphoid tissue not taking part directly in the immune response (Miller and Osoba, 1967). During embryonic and neonatal life cells from the thymus may be seeded into the peripheral lymphoid tissues to constitute the so-called thymic dependent regions (Nossal, 1964; Gowans and McGregor, 1965). On the other hand precursor cells enter the thymus from the bone marrow to maintain the organ's lymphocyte population steady (Ford and Micklem, 1963). However, during adult life the rate of cell migration from the thymus is low (Davies, 1969) whilst mitotic activity within the gland is very high (Metcalf and Wiadrowski, 1966) far outstripping cell export. This has led to the notion that most cells formed within the thymus must in fact die there (Metcalf, 1966). The reason for this apparently wasteful process is uncertain although it may be related to the removal of 'self-reactive' lymphocytes (Burnet, 1968).

Histological examination of the thymus indicates it to contain predominantly small lymphocytes (Craddock, Nakai, Fukuta and Vanslanger, 1964; Perris and Whitfield, 1967) with the remainder comprising medium and large lymphoblasts retained by a cytoreticular structure. Furthermore the high levels of mitotic activity observed in the gland is seen predominantly amongst the comparatively small lymphoblast population, whilst the small lymphocytes are mitotically inert (Metcalf and Wiadrowski, 1966; Perris and Whitfield, 1967; Hunt, 1974). Indeed it is the repeated division of the larger lymphocytes which ultimately yields the small lymphocyte population (Sainte-Marie and LeBlond, 1958, 1965; Craddock et al., 1964). Labelling studies have further indicated that the turnover time for

the small lymphocyte population is very rapid, being 3 to 4 days in both mice (Metcalf and Wiadrowski, 1966) and rats (Craddock et al., 1964). This again reflecting the high levels of cell attrition which must also occur to balance the overall lymphocyte population.

The sequence large-medium-small thymocyte is biassed towards the production of the latter, with a consequent reduction in large thymocyte numbers (Metcalf, 1966). The thymus does not contain a self-maintaining stem cell compartment, and as mentioned previously a body of evidence points to the fact that the thymus is seeded by precursor cells from the bone marrow (Ford and Micklem, 1963). However several studies have detected resting G_o populations in both mouse (Potmesil and Goldfeder, 1973) and rat (Whitfield, MacManus, Braceland and Gillan, 1972) thymic lymphocyte pools. These quiescent cells may be induced to recycle under appropriate conditions and thus the thymus may possess some endogenous capacity for cell proliferation control, independent of bone marrow replenishment.

Recently another criterion for the classification of thymocytes has been proposed (Shortman and Jackson, 1974) which does not necessarily relate to their size or proliferative ability but rather their immunological capabilities. This division is based on the antigenic properties of the cell membrane of mouse thymocytes. A major population, about 85%, has an antigenic constitution unique to the thymus in possessing the TL antigen, high levels of the theta antigen and low levels of the H2 determinant (Shortman and Jackson, 1974). The minor population has the antigenic properties of peripheral T lymphocytes, namely absence of TL, low levels of theta and high levels of H2 (Cerottini and Brunner, 1967; Raff, 1971). Furthermore the major population is thought to represent the cortisone-sensitive

cortical thymocytes, and these may indeed be separated from the minor population by their greater density (Anderson and Blomgren, 1970; Shortman, Cerottini and Brunner, 1972). The low theta population, unlike its high theta counterpart is active in certain immunological tests (Levey and Burleson, 1972; Shortman et al., 1972).

On the basis of tritiated thymidine labelling of these cells and subsequent purification and characterization of subtypes by their surface markers a model for thymocyte development has been proposed (Shortman and Jackson, 1974). They suggest that a stem cell seeds the thymus where it becomes a pre-T lymphocyte; the antigenic characteristics of this cell type have not been ascertained. Under the influence of the thymic environment this cell differentiates to become an immature reactive thymocyte possessing the low theta, TL^- low density characteristics. If this cell is self-reactive and encounters its appropriate stimulus within the thymus then it divides and differentiates. A similar result is observed with immunocompetent peripheral T lymphocytes as indicated previously (Davies et al., 1971). However this developmental pathway in the thymus leads not to an immune response but to a blocked and inactive cell that is secluded within the thymus and which dies there. Evidence is available to indicate that the thymus does indeed contain self-reactive cells (von Boehmer and Byrd, 1972). The mechanism of censorship is via a shunting of forbidden cells into a 'sterile' differentiation process which involves the acquisition of the TL and high theta antigens. Cells not shunted into this pathway leave the thymus as 'virgin T cells', a relatively immature but reactive lymphocyte, which may proliferate and develop into a small dense, low theta TL^- T lymphocyte.

Kinetically therefore this model predicts that there will

be a minor population of small lymphocytes which pass into the thymic environment and which after a short time leave without undergoing any cell division after some critical point in its life. In contrast there will be a large population of small terminal lymphocytes which are generated from the division of large lymphoblasts, which have encountered their appropriate antigenic stimulus. Thus the model can readily account for the high death rate and high mitotic activity observed in the thymus. Less clear however is the reason for a G_o population in what would ostensibly represent a moribund, self-reactive cell type. One clue may lie in the observation that antigenic stimulus results in an increased cell output from the thymus (Miller, DeBurgh and Grant, 1965). Biological efficiency would dictate that there would not be an increase in the output of self-reactive cells under such conditions but rather an increase in functional T lymphocyte production. This could occur through a number of processes. First there could be an increased proliferation and release of bone marrow precursors following antigenic challenge which enhance the thymic lymphocyte pool. Secondly there may simply be an increase in the rate of functional T lymphocyte release, however, such a mechanism might engender a higher chance of releasing self-reactive cells. Finally there may be a pool of precursor cells within the thymus itself which have not acquired the reactive state, i.e. the so-called pre-T lymphocytes (Shortman and Jackson, 1974) and which have a proportion of their population in a resting phase. These cells may then be rapidly recruited into the cell cycle on challenge where they can undergo differentiation into either terminal thymocytes or immunocompetent peripheral T lymphocytes. Certainly during the immune response the plasma calcium concentration rises (Edwards, et al., 1976) a situation which leads to increased mitotic activity in the thymus (Perris, 1971) under other circumstances. It might

also be noted that during immunological challenge bone marrow mitotic activity is enhanced (Edwards et al., 1976) which might also indicate the potential for enhanced thymocyte precursor production.

1·3 The thymus; its proliferative control

The preceding section begs the question as to what agents provide the stimulus for precursor cells to assume a line of differentiation leading to the acquisition of thymocyte characteristics when they passage through the intrathymic environment. Much evidence has been focused on intrinsic factors which appear to modify both the proliferation of thymic lymphocyte grafts within diffusion chambers (Metcalf, Sparrow, Nakamura and Ishidate, 1961; Metcalf, 1962) and their age dependent involution (Metcalf et al., 1961). This intrinsic control is thought to lie in the epithelial and reticuloendothelial framework of the organ or in emanations from it (Metcalf et al., 1961; Clarke, 1968). This thymic hormone latterly termed thymosin is also believed to act upon peripheral lymphoid cells to induce the capacity of T lymphocyte function (Comsa, 1955; Metcalf, 1958; Levey, Trainin and Law, 1963). This evidence basically derives from experiments where thymus implants in millipore chambers are shown to restore to some degree immunological competence in athymic or irradiated animals. Not only is cellular exchange excluded in this system, but also the lymphocyte element of the implant soon disappears leaving only the reticular epithelial material. Furthermore in many of these experiments peripheral T dependent regions did not become repopulated, although there was an improvement in immunological competence. Thus the suggestion was put forward that the thymus generates a humoral factor which controls lymphopoiesis and the

acquisition of immunocompetence (Miller and Osoba, 1963). Thymosin is thought to be a sulphated PAS positive material produced as suggested previously in the cortisone-resistant epithelial cells of the thymic medulla (Clarke, 1968).

Although endogenous mechanisms regulating thymocyte proliferation and differentiation undoubtedly exist there are also a large number of well established exogenous regulators. As early as 1952 adrenal corticosteroids and sex steroids were known to be inhibitors of thymic lymphopoiesis or to be lymphocytolytic (Dougherty, 1952; Santisteban, 1960; Dougherty, Berliner, Scheebeli and Berliner, 1964). In contrast thyroxine and growth hormone enhanced lymphoid mitotic activity (Dougherty, 1952; Gyllenstein, 1962).

A notable contribution to the understanding of extrinsic control of thymocyte proliferation has evolved from the work of Dr. J.F. Whitfield and his colleagues. It was initially noted that injections of calcium or magnesium chloride into male rats resulted in an elevation of mitotic activity in the thymocyte population as assessed using the colcemid arrest technique (Perris, Whitfield and Rixon, 1967). Furthermore parathyroidectomy, which results in a depression of the plasma calcium concentration, also provoked acute thymic involution through a lowering of mitotic rates (Perris, Weiss and Whitfield, 1970). Moreover a number of physiological situations now exist where elevated plasma calcium concentrations are associated with enhanced proliferation in the thymus as well as other tissues (See Table (4) also Perris, 1971; Hunt, 1974). Thus in vivo the calcium homeostatic system occupies an important position in the maintainance of normal and heightened thymopoiesis. It is not clear however if these effects are mediated through the modulation

of any intrinsic mechanism such as thymosin secretion in the whole animal. Since many of these responses may be mimicked in vitro where there are unlikely to be any reticular cells and where the contamination with any putative thymosin will be negligible might suggest a direct action of calcium on the thymocyte (See Units 2,3 and 4).

The point at which calcium acts in the complex sequence of proliferative and differentiational processes in vivo has not been conclusively resolved. However there are some answers available from in vitro experiments. Evidence suggests that calcium acts on cells in the G1 or G_o phase (Whitfield, Rixon, Perris and Youdale, 1969; Whitfield, Rixon, MacManus and Balk, 1972) and has no action on G2 cells (Whitfield, Perris and Rixon, 1969; Whitfield, Perris and Youdale, 1969). This evidence would indicate that either there is a calcium-dependent transition point in the early G1 of thymic lymphoblasts, or that there is a calcium-dependent trigger which reinitiates the division cycle of quiescent thymocytes. This question is also examined in this Unit. As postulated previously these non-cycling cells may represent primitive T lymphocyte precursors. Certainly when activated to enter their DNA synthetic phase these cells appear as lymphoblasts in autoradiograms (personal observation) indicating them to represent primitive rather than terminal thymocytes. However there is no definitive data available to indicate the precise level in the thymopoietic process at which these cells are positioned.

1•4 Thymic lymphocytes; their culture

To investigate both the kinetics and action of various agents on thymocyte proliferation a primary tissue culture system was evolved. This *in vitro* approach was adopted since to investigate those aspects of biochemistry and biophysics which might be pertinent to lymphocyte activation as discussed in this thesis would prove impossible in view of the great degree of complexity in both the thymus itself and the animal as a whole. However in considering any result obtained *in vitro* it must be remembered that some doubt must inevitably be attached to this data until corroborative *in vivo* evidence becomes available.

1•4•1 The primary cell culture was made initially in a balanced glucose-salts solution (BGS) the composition of which is given in Table 1•4•1(a).

Table 1•4•1(a) The Composition Of BGS Medium For Thymocyte Cultures

KCl	5•0mM
NaCl	120•0mM
$Na_2HPO_4 \cdot 12H_2O$	5•0mM
"Tris" buffer	5•0mM
$CaCl_2 \cdot 2H_2O$	0•6mM
$MgSO_4 \cdot 7H_2O$	1•0mM
Glucose	5•5mM

Buffered to pH 7•2 with 0•1N HCl.

Table 1•4•1(a) The "Tris" buffer used here was tris (hydroxymethyl) aminomethane buffer. All reagents were AnalaR grade from BDH Ltd.

Thymus glands were removed from male albino rats of the Wistar strain (obtained from Bantin and Kingman) whilst under light ether anaesthesia. Ether was chosen as it has no effect on a number of cell parameters including total red and white cell counts, differential white cell count, nor does it affect corticosteroid release which might alter cell proliferation (Grice, 1964; Besch and Chou, 1971). Furthermore barbiturates have been demonstrated to inhibit DNA synthesis in a series of tissues (Baserga and Weiss, 1967). The thymuses were routinely washed three times in isotonic saline to remove superficial erythrocyte and protein contamination before transfer to the BGS where they were finely minced with scissors. The mince was subsequently filtered through moistened cheese-cloth to remove any cellular aggregates and epithelial material. The resultant cell suspension was then diluted further in medium to give a cell density of approximately 4×10^7 cells.ml^{-1}, as assessed using a Coulter ZB1 electronic cell counter (Coulter Electronics, Hialeah, Fla.). Although erythrocyte contamination was inevitable without the purification procedures discussed in Unit 6, this did not exceed 10% of the total count. In view of the deleterious effects hypotonic shock or gradient sedimentation might have on thymocytes it was considered preferable to tolerate this low level of contamination. The cells in five millilitre aliquots were then pipetted into glass boiling tubes and sealed with non-toxic rubber stoppers. The cultures were subsequently rotated about their long axes at 30 r.p.m. and 37^{o}C for the required period in a 'Leec' roller drum assembly.

This procedure is essentially that described by Whitfield and coworkers (Whitfield, Brohée and Youdale, 1964a,b.). However it was considered that the method could be made more economical by the use of smaller cultures. Furthermore although the BGS appeared

to support thymocyte viability and proliferation adequately one criticism might be that one was merely depleting the cells of some essential nutrient which calcium might replace. For this reason a synthetic culture medium was employed in the place of BGS. The medium chosen was Medium 199 (Morgan, Morton and Parker, 1950) as supplied by Burroughs Wellcome Ltd. without antibiotics or serum augmentation to avoid any complications from these additives. The medium was buffered to pH 7·2 with sodium bicarbonate and maintained under an enhanced CO_2 (5%) air (95%) atmosphere. Experience however showed that at these high cell densities this enriched atmosphere was not necessary to maintain the buffering capacity over six hours. Cells were prepared as before except that they were cultured in one millilitre aliquots in plastic sterile tubes (Sterilin Ltd.) sealed with polythene caps.

1·4·2 To assess proliferative activity a number of indices can be used. It is possible to simply measure the increase in cell counts during a unit time. This approach is perfectly valid in a system exhibiting high levels of proliferation such as tumour cell cultures. However, the thymic lymphocyte culture shows low levels of mitotic activity in comparison with tumour cells with only 4 percent of the cell population reaching mitosis during a six hour period. Thus even over a day the cellularity of the culture would only increase some 20%. In addition because of the intrinsically high mortality rate in the thymus mentioned previously and the inherent fragility of these cells in culture such long term experiments are impracticable. Thus other indices must be sought.

The alkaloid colchicine (Ciba Ltd.) causes microtubule disaggregation (Eigsti and Dustin, 1947, 1949). Thus addition of

this agent to thymocyte cultures arrests the progress of the cells through mitosis just prior to metaphase because of spindle disruption. Thus by adding colchicine at a final concentration of $6{\cdot}2 \times 10^{-5}$M to cultures it was possible to accurately monitor the number of cells arriving in the mitotic sequence over various time periods. This was done in practice by removing two drops of cells from the cell suspension and mixing it with one drop of calf serum on a glass microscope slide to form a smear. This was subsequently air dried and fixed in neutral formalin and then stained with Delafield's Haematoxylin (Whitfield et al., 1964a,b). In some cases the synthetic analogue of colchicine, desacetyl-N-methyl colchicine (colcemid, Ciba Ltd.) was used, this has been previously demonstrated to have the same action as colchicine itself with fewer side-effects (Rixon, 1968). Microscopic viewing of the thymocyte smears reveals that mitotic cells become arrested in a typical quasi metaphase configuration which is easily recognised. Thus by scoring these "colchicine metaphases" as a percentage of the total nucleated cell population gives a precise estimate of cell proliferative activity. The statistical validity of this method has been previously established in this laboratory (Hunt, 1974).

Alternatively the passage of cells through their DNA synthetic phase may be assessed by monitoring the incorporation of tritiated thymidine into DNA. This may be done by either extracting the DNA and measuring the levels of radioactive incorporation by liquid scintillation spectrometry or via autoradiographic techniques. The latter procedure has been employed in this study as it relates to cells labelled as opposed to DNA synthesised which is not readily amenable to kinetic analysis in heterogeneous cell populations.

The standard cell culture was treated with 5uCi of tritiated thymidine (Radiochemical Centre, Amersham) having a high specific activity ($26Ci.mmol^{-1}$). The cells after exposure to the radionuclide for the appropriate period of time were fixed whilst in suspension with formalin (buffered to pH 7·0) to give a final formaldehyde concentration of 6·6%. After 30 minutes fixation the cells were centrifuged from suspension at 500 x g for 5 minutes and the supernatant discarded. To remove any labelled nucleoside not incorporated into DNA, or which might be trapped in cellular interstices, the cell pellet was resuspended in aqueous unlabelled thymidine (10mM) at $4^{o}C$ for 5 minutes. This procedure was repeated twice. The cells were finally suspended in distilled water and a few drops of this suspension were spread on a glass slide and permitted to dry. This procedure has been described elsewhere (Whitfield, Perris and Rixon, 1969). Furthermore DNAase digestion of the cell smear at this juncture results in the absence of labelled cells in the final autoradiogram (Whitfield, MacManus, Youdale and Franks, 1971) thus validating the method as reflecting DNA synthesis. The slides were subsequently covered with AR10 stripping emulsion (Kodak Ltd.). After exposure of the film to the radioactive label for two days in a desiccated dust-free container the autoradiograms were developed in Kodak D-19 developer, fixed in metafix (Kodak Ltd.) and washed in tap water for thirty minutes. All procedures were carried out in a dark room under a Wratten number two red filter to avoid excessive background contamination. It was found that if the developer and fixer were chilled to about $14^{o}C$ prior to use better adhesion of emulsion was obtained. Development times of five minutes were found adequate and fixation for 1·75 minutes cleared the film without causing depositions.

When the films had dried the autoradiograms were stained with Nuclear Fast Red and Tartrazine (G.T. Gurr Ltd.) according to the method of Sams and Davies (1967). Alcian Blue was excluded from this method since the tissue carries no collagenous material. The staining protocol was as follows:-

Nuclear Fast Red.....4 minutes
Wash in distilled water.....10 seconds
Tartrazine.....1·5 minutes
50:50 Tartrazine/absolute alc. 30 seconds
Absolute ethanol.....2 minutes.
Dry.

Microscopic examination under oil immersion revealed that many large and medium lymphoblasts had become heavily labelled (it was noted that normally in excess of 40 silver grains were visible over each nucleus) whilst small lymphocytes were very rarely labelled. In addition as reported elsewhere (MacManus, Whitfield and Youdale, 1971) there is an almost total lack of 'background' except for regions where some emulsion bubbling had occured. Thus examination of the number of labelled lymphocytes in 2000 cells by two independent observers provided an adequate indication of the number of cells which were actively engaged in synthesizing their DNA content at the time of exposure to the label.

A note on this method is required since some authors have demonstrated that colchicine and autoradiographic studies are not always in agreement with results obtained with DNA extraction and scintillation counting of thymidine incorporation, the latter being lower than expected (Simnett and Fisher, 1973; Youdale and MacManus,

1975). This discrepancy in thymocytes is attributed to the ability of calcium (and therefore agents which increase intracellular calcium concentrations) to elevate thymidylate synthetase activity (Youdale and MacManus, 1975). This leads to an increase in the deoxythymidine-5'-monophosphate pool by enhancing the rate of metabolism of 2' deoxy uridine-5'-monophosphate. However, thymidine itself enters the DNA synthetic pathway by its conversion to deoxythymidine-5'-monophosphate (dTMP) under the action of thymidine kinase. Thus there is a tendency for the tritiated dTMP formed from tritiated thymidine to be diluted by cold dTMP formed from the metabolism of deoxyuridine-5'-monophosphate by thymidylate synthetase. In addition a product of dTMP, 2'deoxy-thymidine-5'-triphosphate, also inhibits the action of thymidine kinase when it is formed in high concentrations, thus exacerbating this situation (Bresnick and Thomson, 1965). Thus autoradiographic techniques precisely detect cells engaged in DNA synthesis but give no indication of the absolute amounts being produced. In thymocytes at least chemical analysis is clearly inadequate for it reveals only minimal absolute synthesis quite uncommensurate with the extra number of cells known to be entering their S phase (Youdale and MacManus, 1975). Calcium does however increase new DNA synthesis, for ^{14}C-formate was incorporated into dTMP via the tetrahydrofolate dependent step (Friedkin, 1973) in increased amounts following the application of this cation to the thymocytes (Youdale and MacManus, 1975). Thus simply assaying the levels of tritiated thymidine incorporation into DNA without reference to the effects of any treatment upon dTMP pool dynamics is no longer to be considered a valid technique without concurrent checks on cell labelling kinetics.

1·4·3 In order to ascertain further features of cell cycle kinetics, and to elucidate at which point in the cell cycle a given

agent is acting requires the use of compounds which specifically arrest the movement of the cell through some phase of its cycle. Such a stathmokinetic agent, colchicine, has already been mentioned in the preceding section. However further synchronizing compounds are available, these usually halting the cell in late G1 or S phase (Cameron and Padilla, 1966).

It was established that cells treated with excess thymidine became arrested in their DNA synthetic phase (Xeros, 1962). Further data suggested that the increased dTMP pool resulting from this treatment inhibited the synthesis of 2'deoxycytidine-5'-monophosphate from cytidine-5'-monophosphate by the action of the thioredoxin system (Cleaver, 1967). Thus addition of deoxycytidine to such inhibited cells leads to a release from the thymidine block (Xeros, 1962). A second agent which also halts cells in their S phase is 5'fluoro-2'deoxyuridine (Rueckert and Mueller, 1960; Littlefield, 1962) which inhibits the thymidylate synthetase system thus preventing the conversion of 2'deoxyuridine-5'-monophosphate to dTMP and in so doing blocks DNA synthesis (Davidson, 1972). As this agent also affects RNA synthesis (Reiter and Littlefield, 1964) it is usual to feed these cells uracil to alleviate this undesirable side-effect.

Whilst these techniques provide a useful tool for dissecting cell cycle kinetics it should be borne in mind that these agents may always have untoward effects which might vitiate the results obtained. The only practical solution to this problem where no natural synchrony technique is available is to repeat the experiment with as many stathmokinetic compounds as possible.

1·4·4 In thymocyte cultures it was necessary to establish cell viability. This was performed routinely by trypan blue dye exclusion. Cells (1 drop) were mixed with 0·1% trypan blue (BDH Ltd.) in 0·9% saline (1 drop) and allowed to stand for five minutes. The percentage cells excluding the dye was then assessed with optical microscopy. In culture thymocytes exhibited a viability level of 92-98% which did not alter over a range of incubation times and treatments. Similar results have been reported for cultured thymic lymphocytes (MacManus, Whitfield and Youdale, 1971). The small loss in viability may be attributable to the inherent cell death in the thymocyte population mentioned previously or it may be the result of the isolation procedure.

Whilst cell viability did not change under a number of treatments it was still necessary to establish that there was no cell loss in the culture. Furthermore it was desirable to check that any loss was not ascribable to any particular thymic lymphocyte size compartment. This possibility was tested by the use of the size distribution analysing facility of the Coulter ZB1 cell counter. The device was calibrated for thymocytes following the procedure described in the Coulter manual, and total and total cell count and differential size count taken both at the beginning and termination of an experiment. This showed no statistical difference between treated and control cultures in either cellularity or size distribution.

Although cultures showed no signs of cell attrition or loss of viability it is still possible that they may be affected metabolically. This point was emphasized when it was noted that cells isolated at 37°C showed low levels of mitotic activity and

appeared to be unstimulable with standard mitogens when compared to cells isolated at room temperature. This was despite the fact that these cells exhibited almost a perfect cytology and no loss of viability. It was reasoned that these lymphocytes must have either become anoxic or were respiring anaerobically thus restricting the progress of cells into the S phase for purely metabolic reasons. Thus a series of experiments were performed to check that thymocyte metabolism was not impaired under a number of conditions whilst in culture.

These studies took two forms. The first was the metabolism of exogenous glucose in the medium, which was assumed to occur via its transport into the cell and phosphorylation to form the basic substrate for glycolysis. The second was the transport of labelled nutrients. These included ^{14}C-glucose and its non metabolizable analogue ^{14}C-3-O-methyl glucose as well as ^{14}C-leucine. The removal of glucose from the medium was monitored by measuring the glucose content of the medium at the beginning and end of a culture period. The cells were spun out of suspension and the medium analysed for glucose using the Boehringer glucose oxidase method (Boehringer Gmbh) with a Beckman glucose analyser. Isotopic uptake was measured by feeding the cell suspension 0·1uCi of the radionuclide and trapping the cells at various time intervals on a Millipore RAWP 02500 filter under vacuum and washing through with unlabelled medium (2 x 10ml). The filter was dried and dissolved in 1 ml of 2-methoxyethanol (BDH Ltd.) in a scintillation vial to which was subsequently added 10 ml of a standard scintillant. This comprised:-

Toluene.....750 ml

2-methoxyethanol.....250 ml

1,4-Di-2(phenyloxazoyl)benzene.....0·1g
(POPOP)

2,5-Diphenyloxazole (PPO).....3·0g

All scintillant reagents were supplied by BDH Ltd. After mixing radioactivity was determined on a Beckman LS 230 liquid scintillation counter.

1·5 Results and conclusions concerning the culture, kinetics and viability of thymocytes in vitro.

When thymocytes were cultured in BGS there was an approximately linear progression of cells into mitosis (Figure 1·5a). It is clearly important to ascertain that progress is indeed linear since it indicates that (a) the cultured cells behave essentially as they do in vivo under analagous circumstances (Hunt, 1974) that (b) movement through the cell cycle is not arrested abruptly during the culture period (c) that cells must be moving through G2 and the S phase in a linear fashion at these time intervals. As a consequence any treatment which subsequently changes the accumulation rate does not do so by merely providing a general 'corrective' action on the cell. When a similar experiment was performed in Medium 199 essentially the same results were obtained (Figure 1·5b) suggesting that this was also capable of supporting thymocyte division. As a further check cells from one source were cultured either in 5 ml aliquots in BGS or 1 ml aliquots in Medium 199, and subsequently exposed to a mitogenic calcium load (Figure 1·5c). It was observed that there was no significant difference between these two treatments in either basal or stimulated metaphase accumulations at six hours. This again suggests that Medium 199 is just as effective as BGS, and can be used in 1 ml cultures without any apparent loss of culturing efficiency.

Figure 1•5a

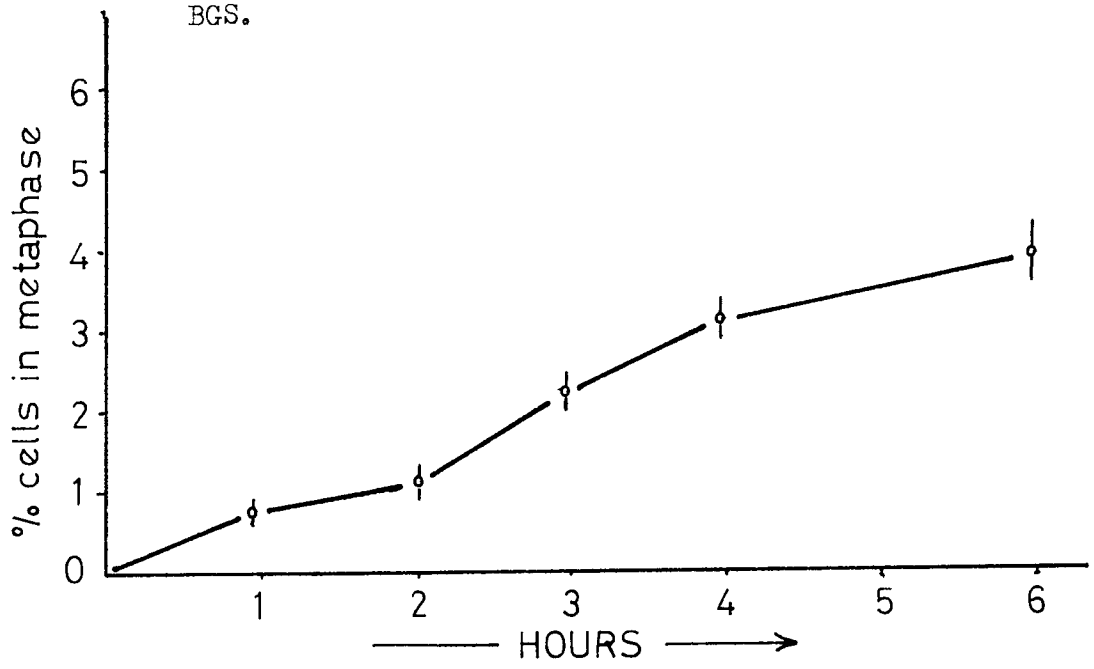


Figure 1•5a. The mitotic accumulation profile of thymocytes cultured in BGS. The medium contained 0•6mM $CaCl_2$ and 1•0mM $MgSO_4$ as well as colchicine. The circles represent the mean of four separate determinations and the vertical bar the standard error of the mean (SEM).

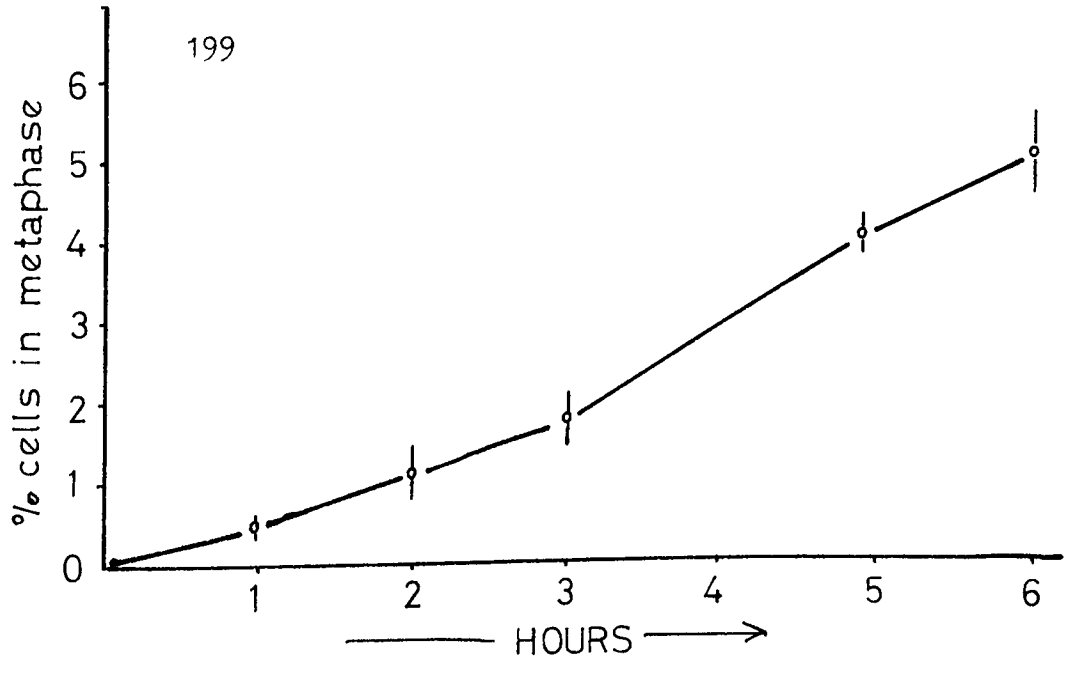


Figure 1•5b. The mitotic accumulation profile of thymocytes cultured in Medium 199. Other details as in Figure 1•5a and text.

Figure 1•5c

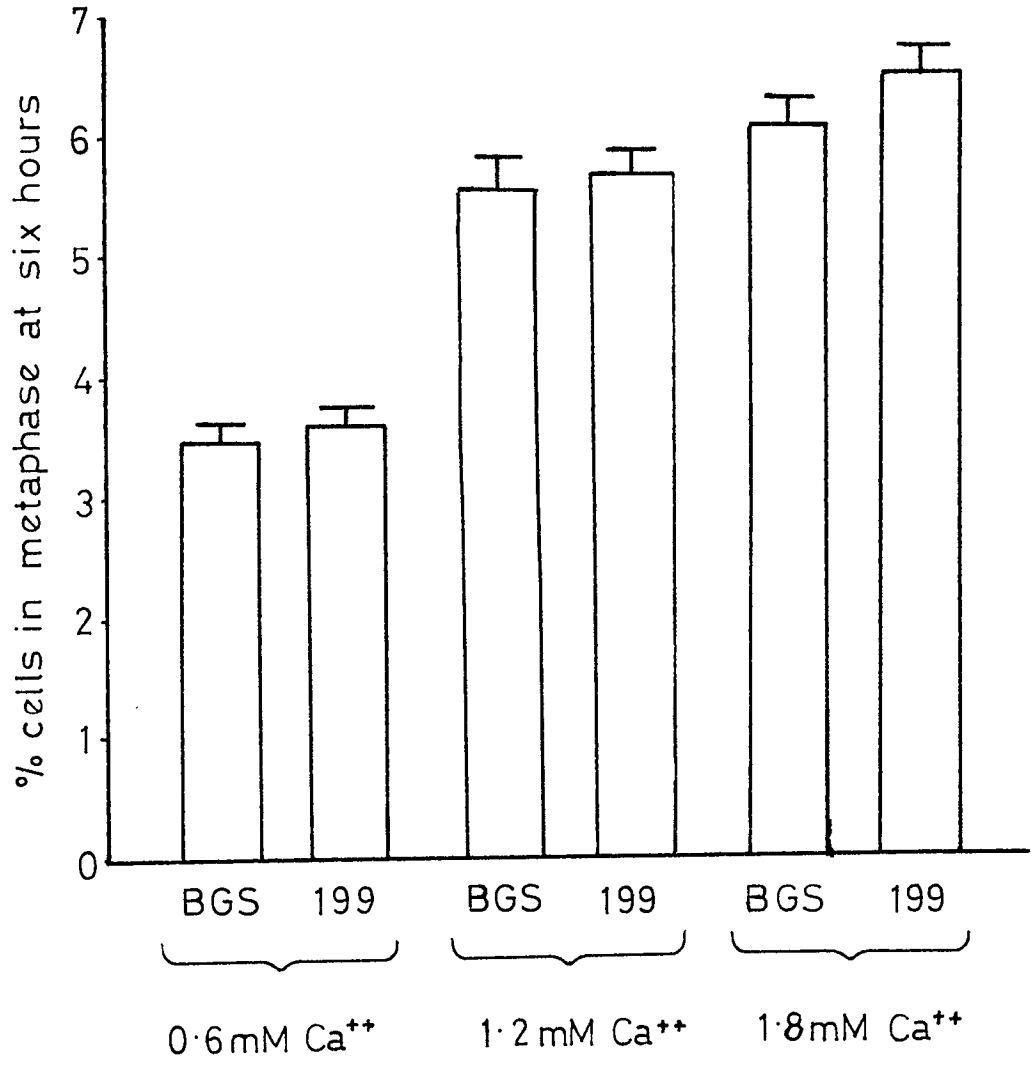


Figure 1•5c. A comparison of the culturing efficiencies of BGS and Medium 199. Cells were either incubated in a balanced glucose salts (BGS) medium or Medium 199 (199). The former were in 5 ml aliquots in glass boiling tubes and the latter in 1 ml aliquots in plastic tissue culture tubes. All cultures contained colchicine, but the calcium concentration in the suspension was adjusted to either 0•6, 1•2 or 1•8mM. The percentage nucleated cells in metaphase was then assessed 6 hours after the start of the experiment. The columns represent the mean and the vertical line with horizontal bar the SEM of four separate estimations of mitotic activity.

Although trypan blue dye exclusion indicated no significant loss of viability over a six hour incubation period, there was a small loss of cellularity as assessed using a Coulter Counter

Table 1·5d An estimation of initial and final thymocyte numbers during a six hour incubation in BGS.

Treatment	Mean initial cell count	Mean final cell count	% cells remaining
1·2mM calcium	$9·33 \times 10^6$	$8·77 \times 10^6$	94% ± 2%
1·2mM calcium + $0·1\mu g.ml^{-1}$ oestradiol	$9·51 \times 10^6$	$8·88 \times 10^6$	94% ± 1·7%

Table 1·5d. The influence of $0·1\mu g.ml^{-1}$ oestradiol on the percentage of the initial cell population remaining in thymocyte cultures maintained in BGS medium containing 0·062mM colchicine and 1·2mM calcium chloride after a six hour incubation. Values are means ± S.E.M. of four separate experiments.

(Table 1·5d). However, this cell loss occured in both control and test cultures which exhibited both enhanced and basal levels of mitotic activity. Therefore the expression of higher levels of proliferation was not associated with any change in cellularity. In addition this cell loss was not attributable to cell attrition in any particular size range (Figure 1·5e). Thus this may represent cells which were moribund at the time of initiation of the culture and which fragmented rapidly during the incubation. Alternatively it may represent erythrocyte contamination, since it was noted that these cells become crenated during an incubation and may indeed become too small to register on the Coulter. Whatever the reason it is unlikely that this small cell loss could account for any of the observed alterations of mitotic activity since it is not ascribable to any particular size compartment. Therefore to artifactually double the mitotic accumulation would require the loss of some 50% of the

Figure 1•5e

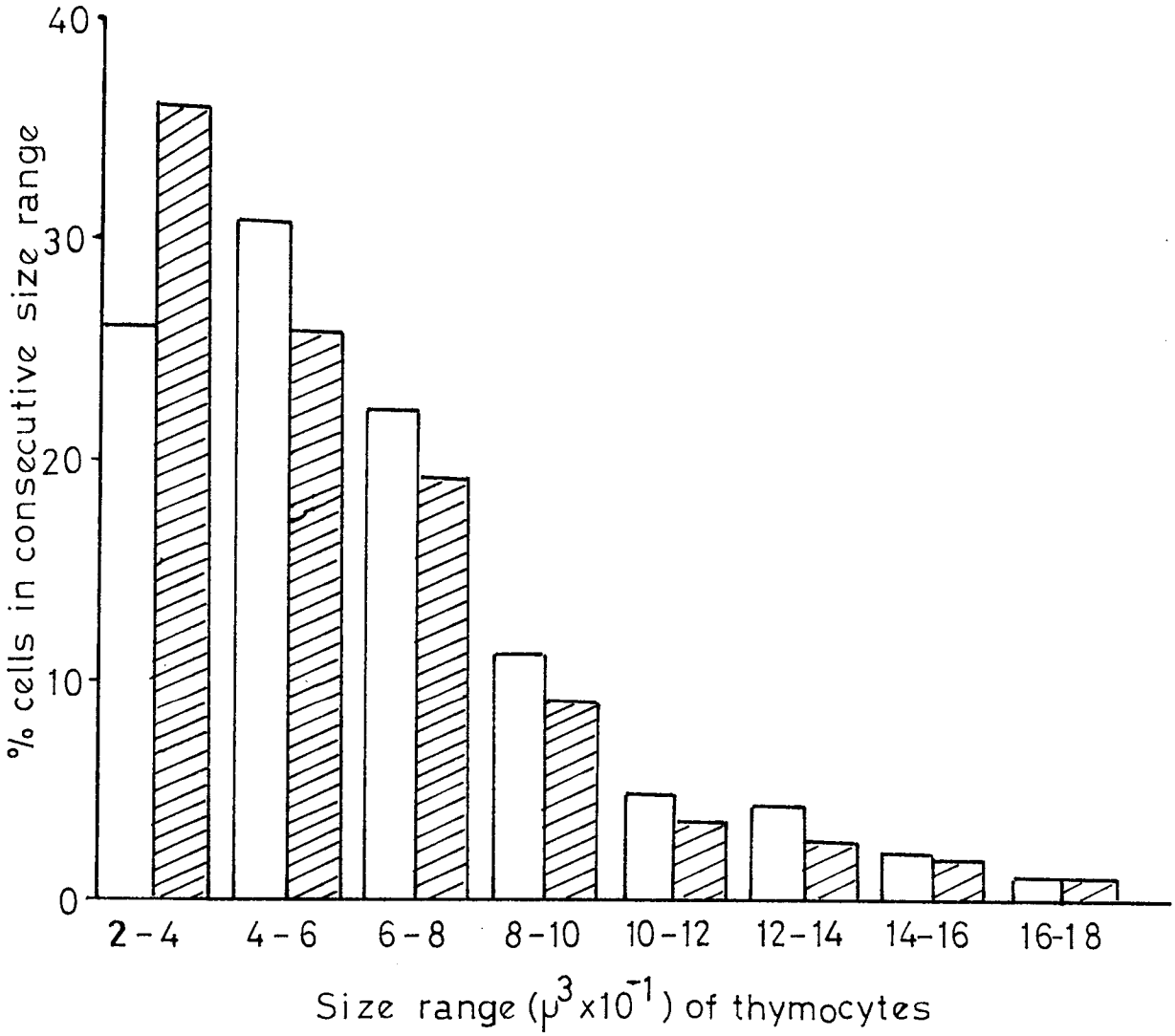


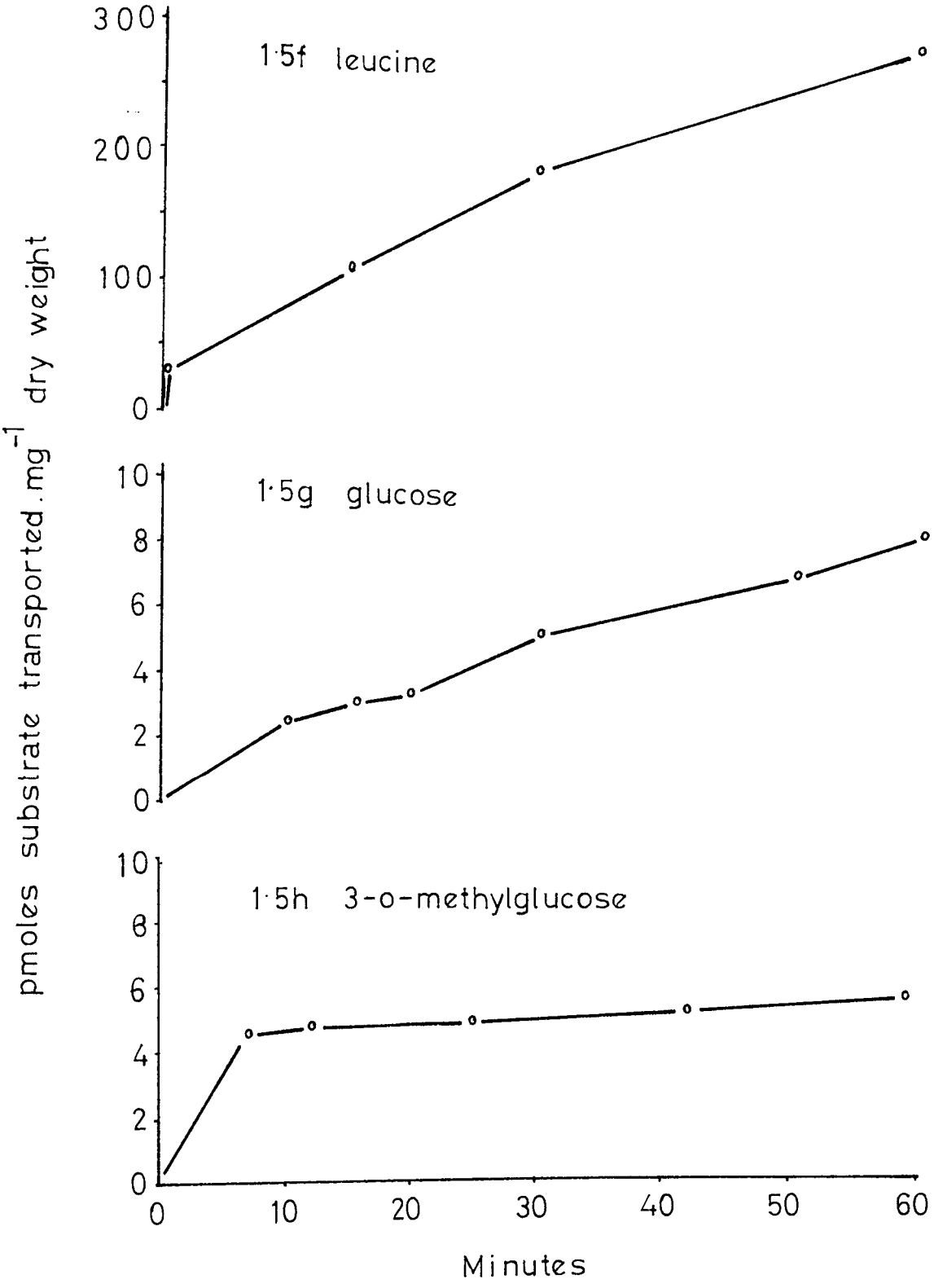
Figure 1•5e. A comparison of the size distribution of thymocytes both before and after a six hour incubation. Cells were cultured in BGS containing colchicine and 0•6mM calcium chloride and sized on a Coulter Counter which had been previously calibrated for thymic lymphocytes. Initial size distribution is given by the open columns and represents the mean of two duplicated experiments, the final size distribution is represented by the hatched columns. Similar results were obtained when cultures also contained 1•2mM calcium chloride and oestradiol-17β (0•1μg.ml^{-1}), data not shown.

mitotically inert small lymphocyte population, which clearly does not occur (Table 1•5d).

The third criteria for methodological validity proposed in section 1•4•4 was that the general metabolic activity of the cells should not be affected by the culture conditions. Thus a series of experiments were performed to ascertain if the transport and utilization of carbohydrates and amino acids were altered by being maintained in suspension culture.

The two nutrients ^{14}C-leucine and ^{14}C-glucose as well as the non-metabolizable form of glucose, ^{14}C-3-O-methylglucose were taken up into thymocytes which had been preincubated for one hour in the absence of the radionuclides (Figures 1•5f-h). The utilization of both glucose and leucine was linear over the one hour period studied indicating no deterioration in the metabolic activity of the thymocytes during this time. In contrast the uptake of 3-o-methyl glucose after an initial rise showed only a relatively small transport rate (Figure 1•5h). This may be an indication that the glucose transfer observed is the consequence of metabolism of the hexose within the cell since its non-metabolizable analogue does not exhibit such a pronounced and protracted uptake. However some of these apparent differences between the two sugars may be a function of relative affinities for their plasma membrane receptor.

A number of agents were tested for their ability to alter transport rates of these three labelled substrates. Thus the action of mitogenic concentrations of calcium chloride (1•8mM), histamine and insulin was examined with respect to leucine and glucose uptake. In all cases no significant effect was observed on the transport of these two nutrients (Figure 1•5i and j) thus indicating that mitogenic compounds did not stimulate division by promoting the uptake of nutrients whose supply may have become limiting. Further-



Figures 1·5f-g. The transport profiles of ^{14}C-leucine, ^{14}C-glucose and ^{14}C-3-o-methylglucose into thymic lymphocytes maintained in BGS and 0·6mM calcium chloride. Cells were treated and isolated as outlined in the text. Each circle is the mean of two individual experiments and has been expressed as pmoles of substrate by conversion of CPM observed from the cited specific activity of the radionuclide.

Figure 1•5i

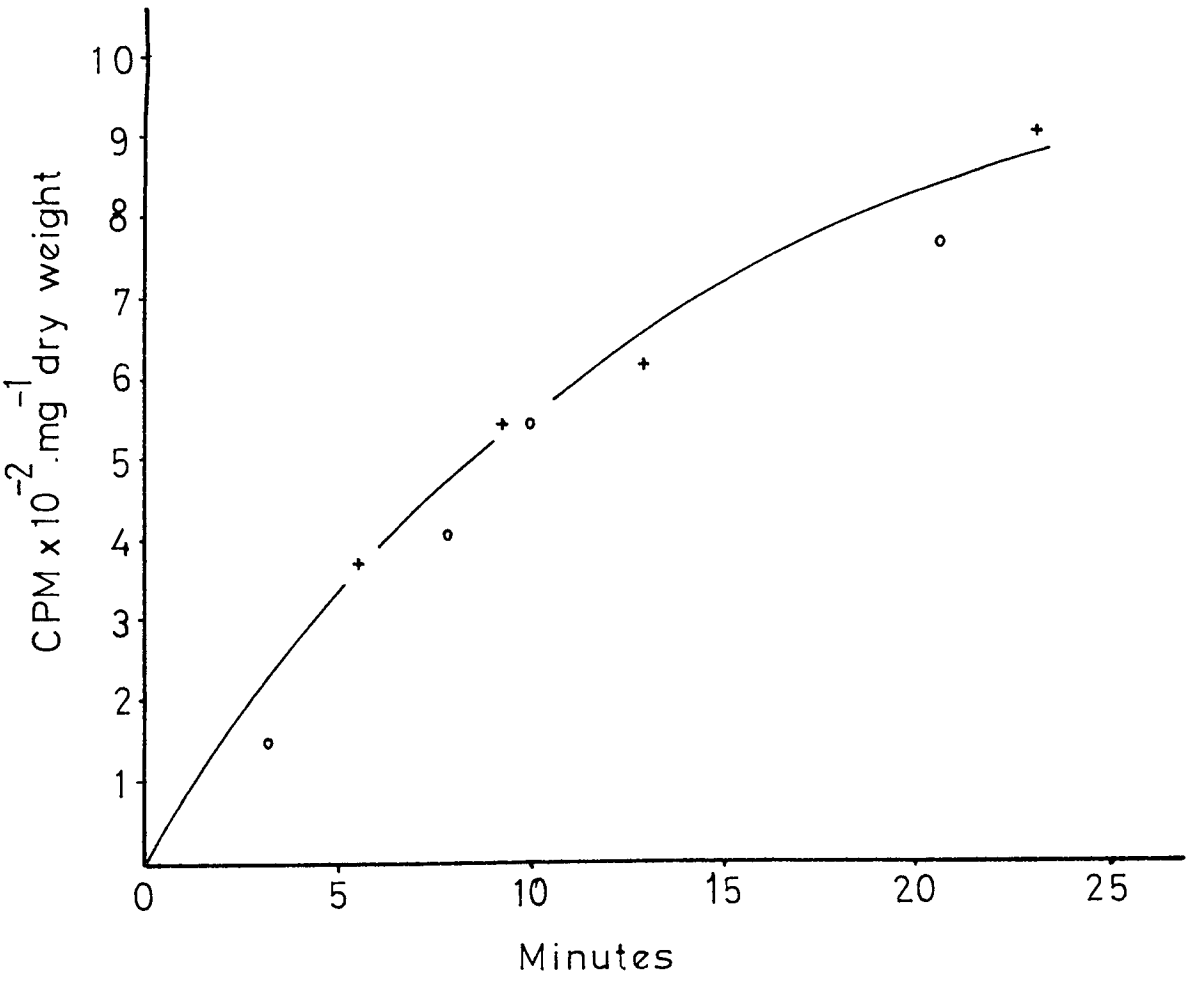


Figure 1•5i. Leucine uptake in the presence of 0•6mM (+) and 1•8mM (o) calcium chloride. Each point is the mean of two separate determinations. Cells were incubated in BGS. Similar results were obtained when histamine (10^{-10}M) was used in place of 1•8mM calcium chloride.

more it would also indicate that a restriction in membrane transport or substrate utilization is unlikely to be the explanation for cells becoming arrested in G_o. It should be pointed out however that alterations in ion distributions do occur on mitogenic challenge as indicated in Unit 7 using slightly more sophisticated methods than those reported here. These latter results are most likely to reflect the behaviour of a small subpopulation of quiescent G_o cells whereas the present ones indicate overall utilization patterns. Nevertheless

Aston University

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Figure 1·5j. The effect of insulin (160μU. ml^{-1}) on the uptake of glucose into thymocytes maintained in Medium 199. Cells were cultured for one hour with 0·01μCi labelled glucose in one millilitre aliquots. The cells were subsequently centrifuged from suspension and washed twice in unlabelled medium. They were finally suspended in 5% trichloroacetic acid and disrupted by ultrasonication at 20KHz on a 3mm microprobe (Ultrasonics, Shipley Yorks.). The protein precipitate was spun down at 3000 x g and the acid soluble supernate counted in 10ml NE 260 liquid scintillant (Nuclear Enterprises, Edinburgh).

it is clear that the cultured cells as a whole transport glucose and leucine during the first three hours of incubation.

In an alternative series of experiments after preincubation in Medium 199 for one hour cells were subsequently treated with insulin (160μUnits.ml^{-1}) , oestradiol-17β, or left without additions (Figure 1·5k). The initial medium glucose concentration was determined by centrifuging untreated cells from suspension and analysing for glucose on a Beckman glucose analyser. After 90 minutes cells were again spun off and the final medium glucose concentration assayed. The results indicate that again cells in culture remove glucose

Figure 1·5k.

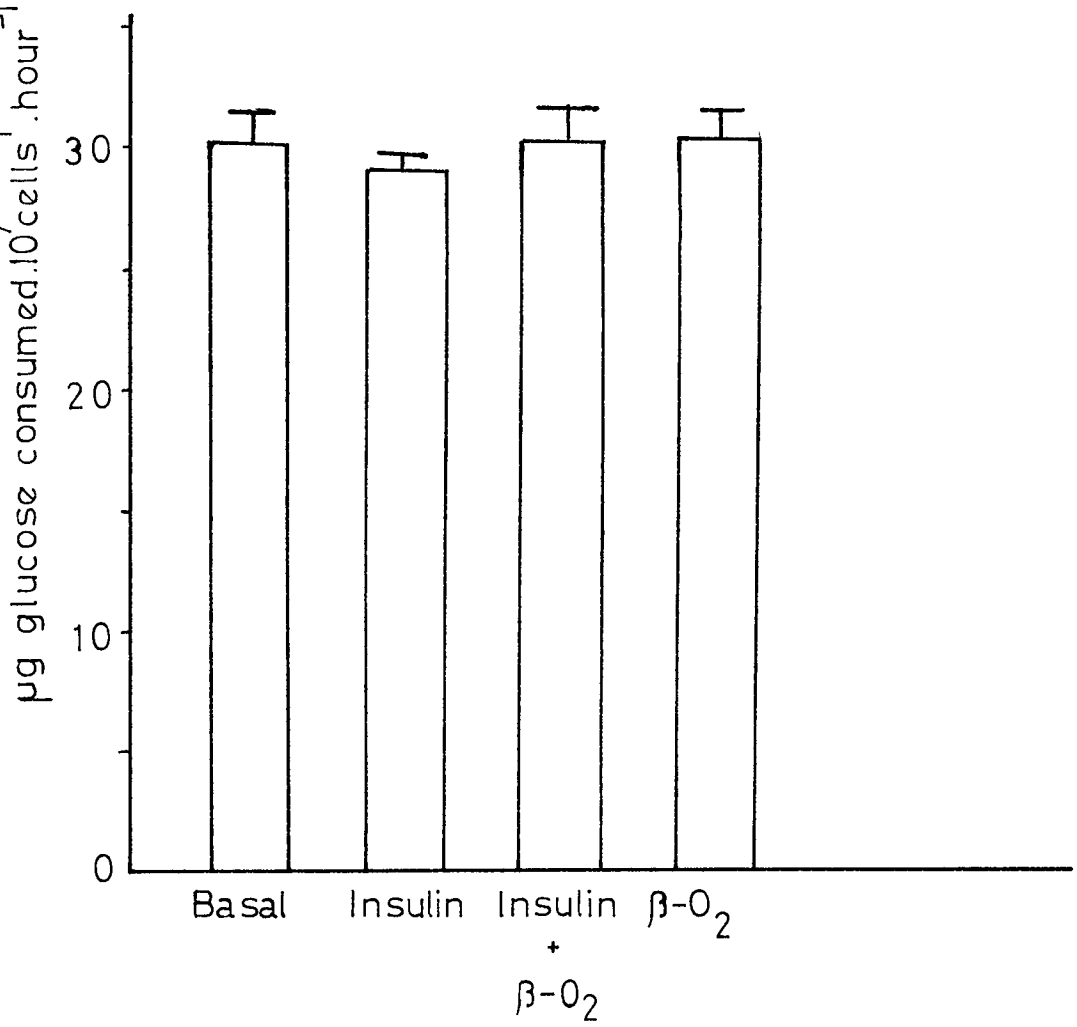


Figure 1·5k. The action of insulin (160μUnits.ml^{-1}, Sigma Chemical Co.), oestradiol-17β (βO_2) on glucose metabolism of thymocytes. Procedural details are as in text. Each column is the mean and the vertical line with bar the S.E.M. of four separate determinations.

from the extracellular milieu. Furthermore a mitogenic concentration of insulin (see Unit 3) did not significantly alter the uptake of glucose over a ninety minute period. Also oestradiol-17β (0·1μg.ml^{-1}) which inhibits the mitogenic action of insulin did not affect glucose utilization (Figure 1·5k). This would indicate that hexose transport is (a) not an essential feature of cell activation and that (b) glucose metabolism is unimpaired during this time period.

Having established that the mitotic increment induced by elevated extracellular calcium concentrations (Figure 1·5c) is not an artifact of technique, it must next be established from which phase of the cell cycle it is derived.

When thymocytes were exposed to 1·2mM calcium chloride there is a lag of some 3-4 hours before the stimulated cells appear Figure 1·51.

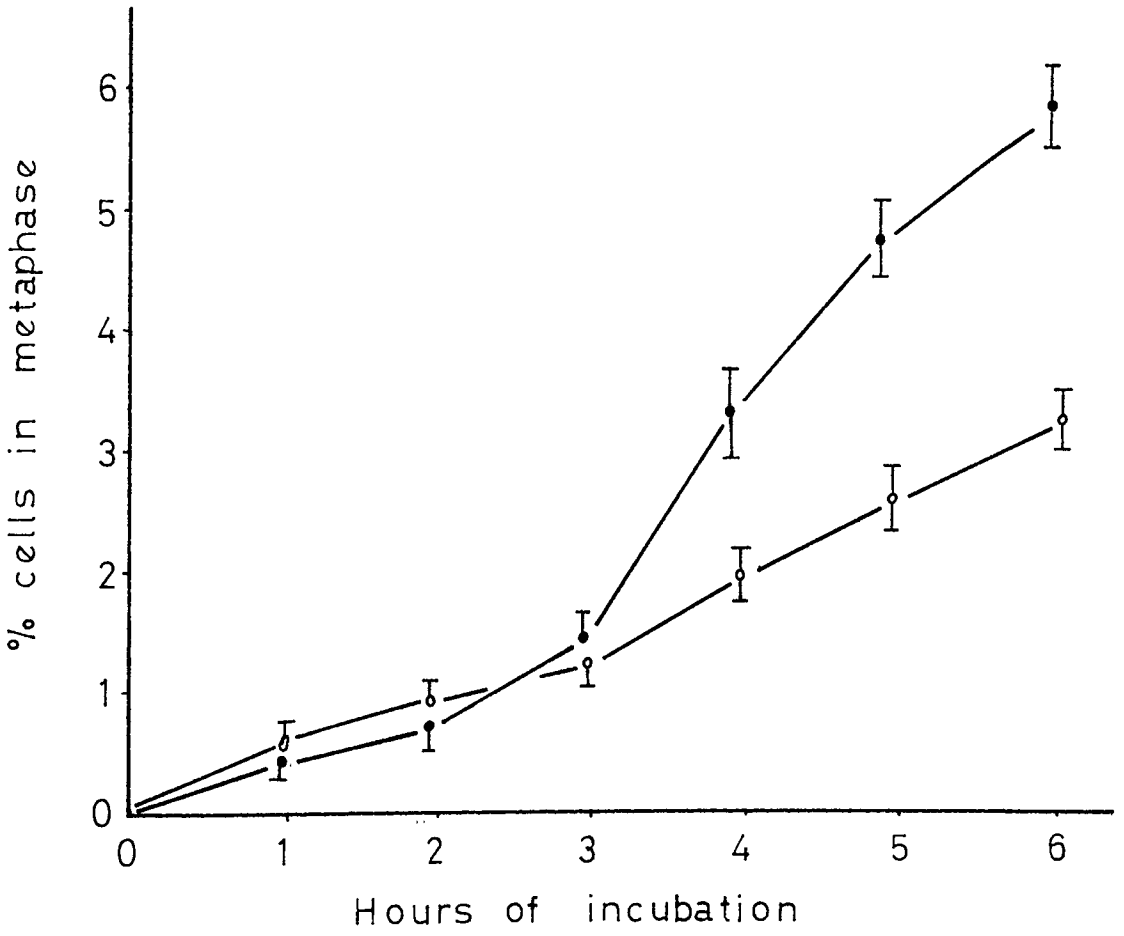


Figure 1·51. The accumulation profiles of thymocytes cultured in Medium 199 with either 0·6mM (o) or 1·2mM (•) calcium chloride, and in the presence of 0·062mM colchicine. Each point is the mean, and the vertical line with horizontal bar the S.E.M. of between 4 and 8 individual determinations.

in metaphase (Figure 1·51). This may indicate that the extra cells emanated from some compartment normally located 3 to 4 hours before

mitosis. Using the values for cell cycle parameters derived by Metcalf (1966) viz S phase 3 hours; G2 1 hour; mitosis 50 minutes

Figure 1·5m.

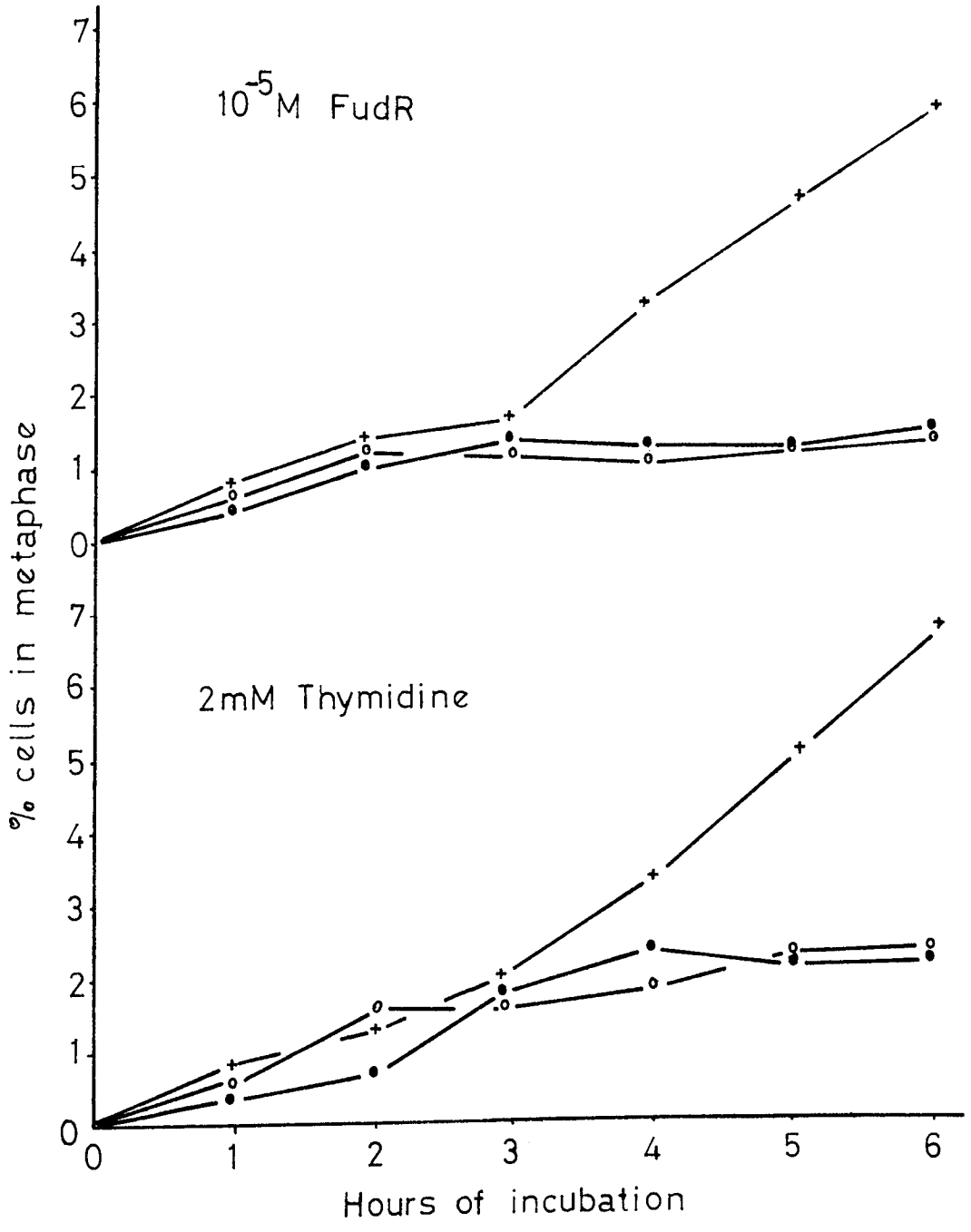


Figure 1·5m. An analysis of the action of histamine (10^{-12}M) on 'colchicine metaphase'accumulations in the presence and absence of the DNA synthetic blocking agents thymidine (2mM) and 5'fluoro-2'-deoxyuridine (FudR, 10^{-5}M) plus uracil 10^{-6}M. Cells were exposed to histamine either in the absence (+) or presence of (•) the two S phase inhibitors. In addition cells in basal Medium 199 were also exposed to these agents as a control (o). Each point is the mean of two experiments.

and G1 3 hours; would indicate that these cells were close to the beginning of the S phase when first exposed to the mitogen. Alternatively one might envisage that the additional cells could have been derived from some quiescent phase much closer to mitosis (e.g. a $G"_o$) and the calcium-induced activation process required 3 to 4 hours to be completed.

To test this latter possibility control and mitogen treated thymocytes were exposed to either excess thymidine or FudR (see section 1•4•3) to prevent the passage of cells through S whilst leaving other cycle phases unaffected. Under these circumstances the same number of cells entered mitosis in both control and mitogen treated cultures (Figure 1•5m). Thus mitogens do not exert their effect in this system on either quiescent or cycling G2 cells, confirming the results of other workers using a different technique (Whitfield, Perris and Youdale, 1969).

To establish whether the mitogen-stimulated dividing cells were derived from a pre-DNA synthetic state autoradiographic techniques were employed. When control cultures were continuously exposed to tritiated thymidine in the presence of colchicine to prevent labelled cells recycling, approximately 7•7% of the cells rapidly (within half an hour) incorporated the label (Figure 1•5n). Subsequently there was a slow rise in the fraction of cells synthesizing DNA which reached a plateau of 9•0% after 3 hours (Figure 1•5n). If the culture contained mitogenic levels of calcium (1•2mM) then there was a greater number of cells rapidly incorporating the label and which reached a new higher plateau value of 11% (Figure 1•5n). This suggested a presynthetic origin for these additional cells. The cessation of incorporation after 3 hours cannot have been due to

Figure 1•5n.

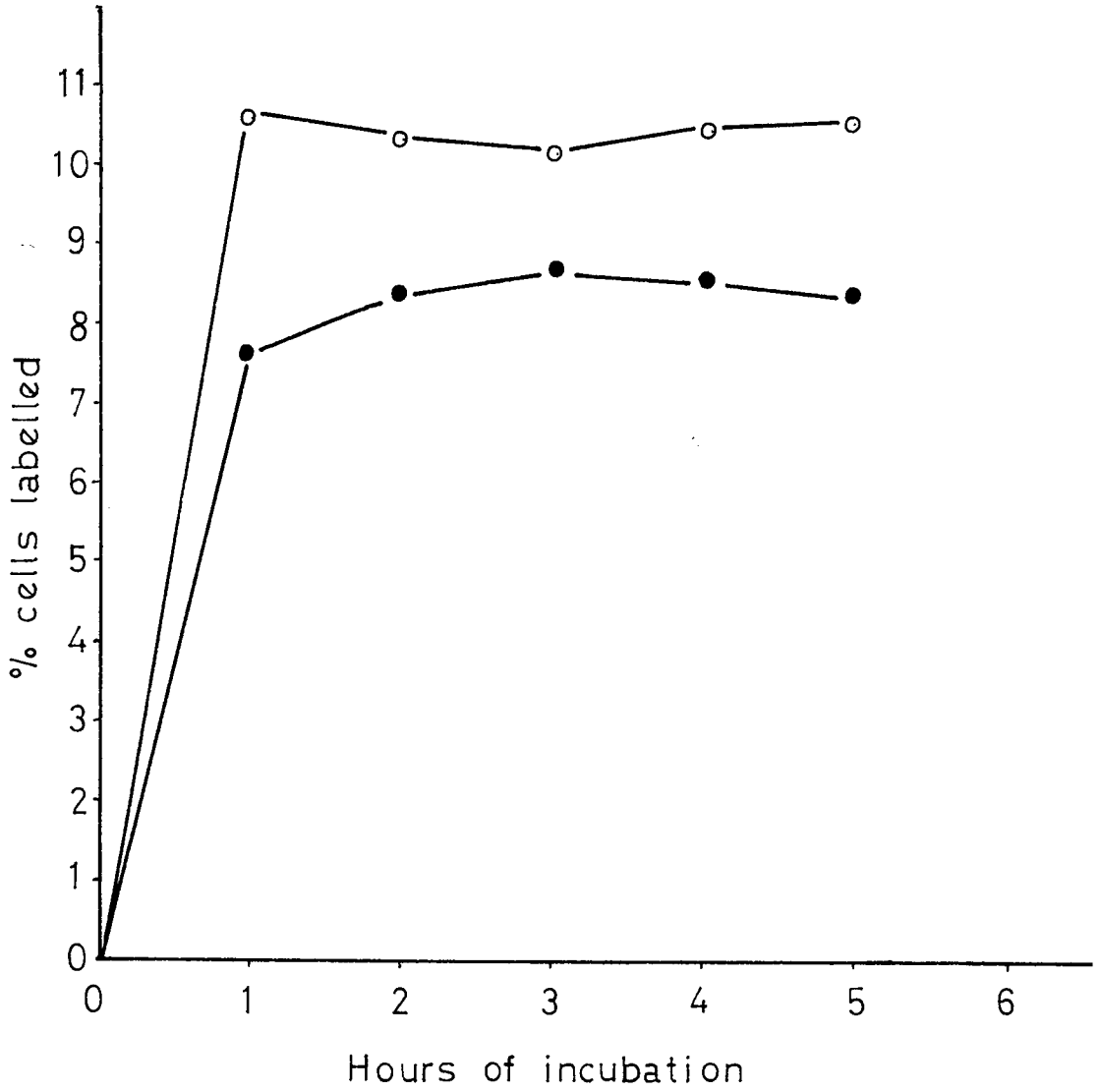


Figure 1•5n. A continuous labelling experiment with thymocytes maintained in Medium 199. Cells were continuously exposed to 5μ.Ci.ml^{-1} tritiated thymidine in the presence of 0•062mM colchicine. Cultures also contained either 0•6mM (●) or 1•2mM (○) calcium chloride. Autoradiograms were subsequently prepared as outlined in section 1•4•2. Each circle represents the mean of between two and three separate determinations.

thymidine exhaustion in the medium as Youdale and MacManus (1975) have shown that with higher cell densities than those used here the activity of thymidine phosphorylase is insufficient to deplete all of the nucleotide. In fact sufficient labelled thymidine was still present after four hours to label a fresh culture. Thus it seems likely that after 3 hours no further cells are able to leave

Figure 1·5o.

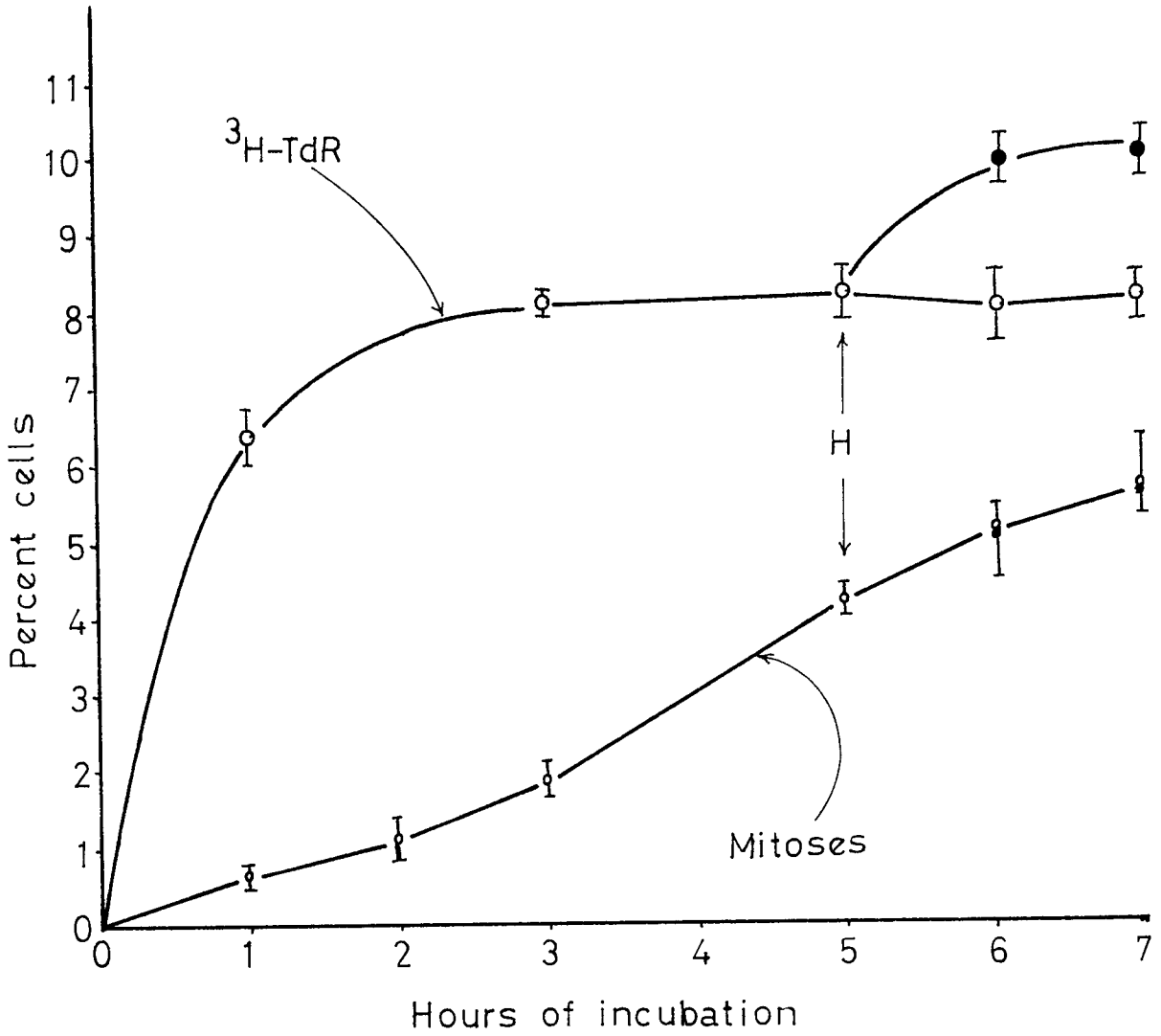


Figure 1·5o. A kinetic analysis of the action of histamine on the entry of cells in metaphase and the DNA synthetic phase *in vitro*. Thymocytes maintained in Medium 199 containing 0·062mM colchicine were exposed continuously to 5μCi.ml^{-1} tritiated thymidine and aliquots were removed at various time intervals and either processed to give autoradiograms (^{3}H-TdR) or metaphase smears (Mitoses). Five hours after the initiation of the culture some tubes were treated with histamine (H) at a mitogenic concentration of 10^{-10}M. Large open circles (o) represent control autoradiographs and closed circles (●) histamine-treated cultures. Small open circles indicate (o) control colchicine metaphase accumulation values and closed small circles (•) histamine stimulated values. All points are the mean, and vertical line with bar the S.E.M. of between 4 and 6 separate experiments.

the G1 and enter S, i.e. G1 may occupy approximately 3 hours in these cells. When cells were maintained for longer than this period (5 hours) in the presence of tritiated thymidine addition of the

mitogen histamine (10^{-12}M) caused an additional cohort of cells to rapidly enter DNA synthesis (Figure 1·5o). Thus since calcium and histamine can induce additional cells to enter S either at the beginning of culture or when there are no further cells initiating synthesis from G1 (Figures 1·5l-o) it is most likely that they are derived from a presynthetic G_o.

Figure 1·5p.

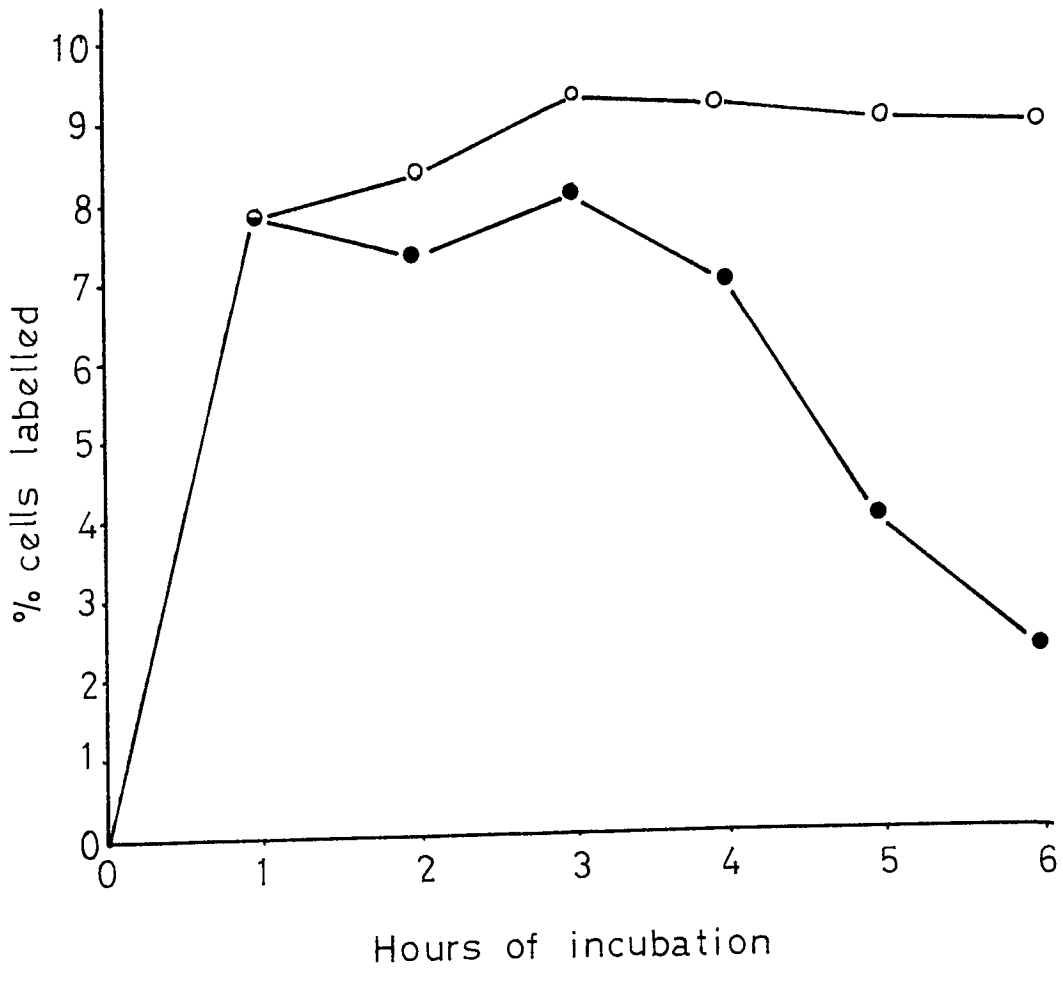


Figure 1·5p. A comparison of pulse labelling versus continuous labelling in unstimulated thymocytes maintained in Medium 199 containing 0·062mM colchicine and 0·6mM calcium chloride. Thymic lymphocytes were either exposed continuously to tritiated thymidine (o) and samples removed at various intervals for autoradiographic processing, or suspension cultures were fed the label for one hour (●) intervals whereupon the cells were fixed and processed as before. Points are the means of two separate determinations.

A further approach to the analysis of cell cycle function and kinetics is the pulse labelling of cells at various times after the initiation of the culture. The percentage of cells incorporating label remains relatively constant at between 7-8% for the first three or four hours of culture, and then progressively declines to reach 2·5% after five to six hours (Figure 1·5p). The G1 period is indicated by the time of constant pulse labelling (i.e. 3-4 hours) and the S phase from the onset of declining labelling to the point where this reaches zero. Extrapolation suggests this phase lasts between 2·5 and 3·5 hours (Figure 1·5p). Clearly these values are only approximations because of the inherent inaccuracies in this technique and the heterogeneous nature of the thymocyte population. However, the values are in good agreement with previous experiments using continuous labelling and metaphase accumulations (Figures 1·5 l-p).

One criticism of these labelling experiments might be that they were conducted in the presence of colchicine which in concert with the mitogens might alter artifactually other cell cycle compartments in addition to the mitotic sequence where it has been assumed to exert its exclusive action. However, even in the absence of this stathmokinetic agent mitogenic levels of calcium and histamine again resulted in an increased recruitment of cells into their S phase (Figure 1·5q). Thus there can be little doubt that the additional cells these mitogens ultimately provoke to division have a pre-DNA synthetic origin.

Figure 1•5q.

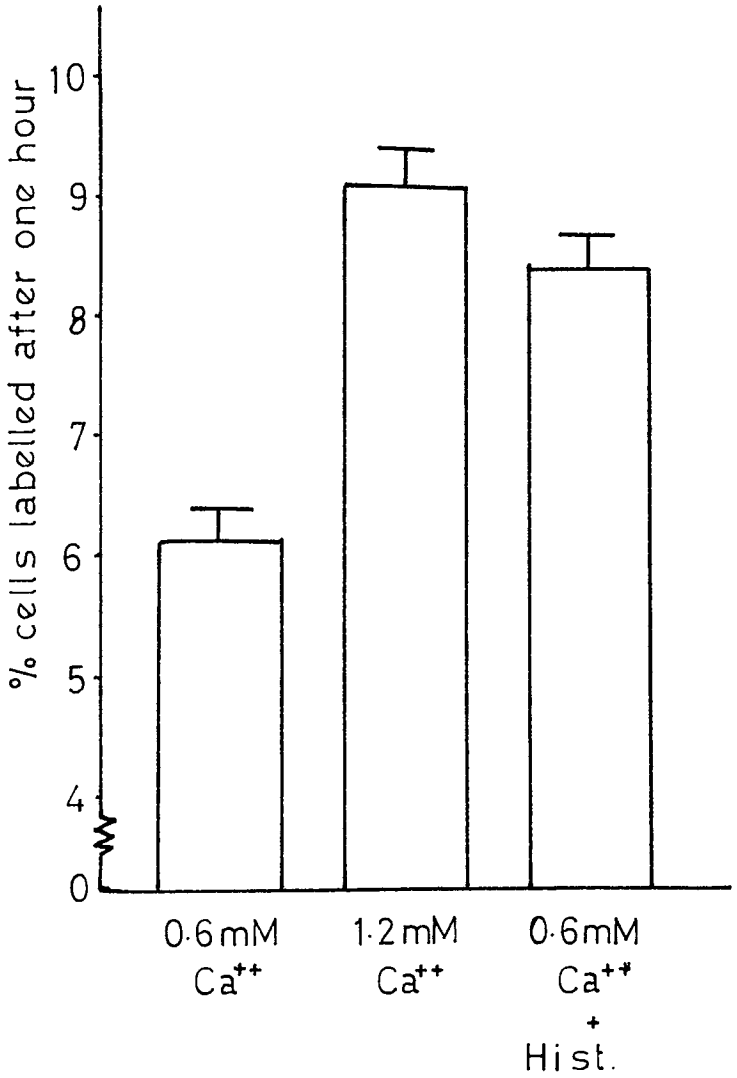


Figure 1•5q. The action of 1•2mM calcium and 10^{-12}M histamine on the percentage of cells becoming labelled after one hours exposure to the mitogens in the absence of colchicine. Cells were pre-incubated in Medium 199 containing no colchicine for 30 minutes. They were then treated with 5μCi.ml^{-1} tritiated thymidine and either 1•2mM calcium or histamine (Hist). One hour later the cultures were processed to yield autoradiograms. Each column represents the mean, and the vertical line with bar the S.E.M. of four separate experiments.

UNIT 2 The Role Of Ions In The Regulation Of Cell Proliferation

2·1 The actions of ions *in vivo*

In the context of the maintainance of normal cell proliferation in the whole animal it has been established that one ion, calcium, plays a central part. Injections of this divalent cation into irradiated rats prolongs survival (Rixon and Whitfield, 1963) whilst in normal animals it provokes an increase in both thymus (Perris and Whitfield, 1967) and bone marrow (Perris, Whitfield and Rixon, 1967) mitotic activity. Furthermore modulation of the calcium status of the adult rat, through manipulation of the calcium homeostatic system, leads to marked changes in cellularity and proliferative activity in several tissues. Thus by injecting the hypercalcaemic agent parathyroid hormone into the rat it is possible to demonstrate an increase in division rates in both thymus and bone marrow (Perris, Whitfield and Rixon, 1967), in a fashion analagous to that observed by injection of calcium chloride. In contrast surgical removal of the parathyroid glands results in the onset of hypocalcaemia in the rat when fed a calcium deficient diet (Perris, 1971; Hunt, 1974). Under these low calcium conditions both the thymus and bone marrow become hypoplastic (Rixon, 1968; Perris, Weiss and Whitfield, 1970) through a reduction in the proliferative rates within these tissues (Rixon and Whitfield, 1972; Hunt, 1974). These low levels of cellularity may be returned at least transiently to near normal values by restoration of plasma calcium concentrations by transfer of the parathyroprivic animal to calcium containing diets or through injections of calcium chloride or PTH (Perris, Weiss and Whitfield, 1970; Perris and Whitfield, 1971). Whilst the liver and gastro-intestinal epithelium do not show marked hypoplasia or involution

following parathyroidectomy, evidence does suggest that in the case of the liver its regeneration following partial hepatectomy may be dependent upon the presence of a functional parathyroid gland within the test animal (Rixon and Whitfield, 1972b). In terms of the small intestinal epithelium it has been established that the calcium homeostatic hormone, vitamin D (or its active metabolite 1, 25 dihydroxycholecalciferol, 1,25 DHCC) does have a major role in proliferation regulation in this tissue (Urban and Schedl, 1969).

2•1•2 Since the calcium homeostatic system appears to play such a fundamental role in the maintainance of normal proliferation in the thymus, bone marrow, liver and perhaps gastrointestinal epithelium some advantage might be gained by considering this process in a little more detail.

As the overall biological involvement of the plasma ionised calcium concentrations in a number of physiological processes outweigh even its modest implication in cell division control, it is perhaps not surprising to note that the regulation of calcium balance is a complex and precise interaction between at least three hormones, viz parathyroid hormone (PTH), calcitonin (CT) and vitamin D. These other calcium dependent situations include membrane integrity, microtubule assembly, enzyme activity, muscle contraction, neurotransmission, blood coagulation, skeleton formation, lactation, hormone-cell interactions and stimulus-secretion coupling to name but a few. The precise manipulation of the calcium burden is beyond the scope of this thesis, the work representing perhaps some of the most elegant endocrine studies yet performed. This remarkable system is all the more intriguing since only some 1% of calcium present in

the body is outside of the skeletal compartment (Catt, 1970). Of this a proportion will be bound to extracellular proteins, cell membranes, other cell components and stored within mitochondria whilst the final circulating ionised plasma calcium concentration is very low (Hunt (1974) indicates this to be 1·2mM in the adult Wistar rat). Despite this apparent concentration gradient between bone and plasma it is in fact believed that plasma is 'supersaturated with respect to bone' (Neuman and Neuman, 1958). Thus calcium is taken up into bone incubated with normal plasma (Talmage, 1967). This tendency of calcium to move into bone is opposed by one of the calcium homeostatic hormones, PTH, mentioned previously which tends to resorb bone (Talmage, 1962). This hormone is a polypeptide secreted from the parathyroid glands (Aurbach, 1959) and which on injection into a number of species produces an hypercalcaemia (Parsons and Robinson, 1971). However this hypercalcaemia is preceded by a hypocalcaemic phase in rats which is thought to represent an important aspect of PTH action (Parsons and Robinson, 1971). This calcium elevating action of the hormone is attributable to three sites of action. PTH stimulates reabsorption from renal tubules (Talmage and Kraintz, 1954) promotes absorption from the gut (Rasmussen, 1959) and stimulates bone resorption (McLean, 1957). All three mechanisms thus tend to conserve plasma calcium. Thus there exists an inverse correlation between plasma calcium levels and parathyroid hormone titres in both man (Berson and Yallow, 1966) and pig (Arnaud, Littledike and Tsao, 1970), which reflects the negative feedback loop controlling PTH release (McLean, 1957). Furthermore perfusion of the parathyroid glands in sheep and goats with elevated calcium perfusates results in the inhibition of PTH secretion (Care, Sherwood, Potts and Aurbach, 1966). In contrast perfusion of the glands with low calcium medium leads to a stimulation of hormone release (Raisz, 1963).

The second calcium homeostatic hormone, calcitonin, is generated in the thyroid and is known to produce an hypocalcaemia in rats (Hirsch, Voelkel and Munson, 1964) and pigs (Care, 1965). CT release shows a linear relationship with plasma calcium in pigs (Care, Cooper, Duncan and Orimo, 1968) rabbits (Lee, Deftos and Potts, 1969) and calves (Care, Bates, Philippo et al., 1970), suggesting that it is the level of this ion in the circulation which regulates the secretion of this hormone. The target for CT is bone (Munson, 1971) whilst it has only a slight action on kidney (Robinson, Martin and MacIntyre, 1966) and no action on intestinal absorption (Cramer, Parkes and Copp, 1969). The action of CT on the skeleton is to promote bone accretion (Foster, Doyle, Bordier and Matrajt, 1966), whilst it may also directly antagonise the peripheral action of PTH (Munson, 1971). The physiological role of calcitonin in the adult animal is unclear, however, since the mature rat can adequately balance its calcium levels without a thyroid gland (Care, 1969) and therefore presumably without CT. It is perhaps that calcitonin only comes into play to augment the natural flux of calcium into the bone in times of high calcium episodes such as post-prandial hypercalcaemia (Gray and Munson, 1969). In this instance it is unclear if the stimulus for CT secretion is an hypercalcaemia or a gastrointestinal hormone such as pancreatic glucagon or gastrin (Gray and Cooper, 1970).

Although PTH produces an hypercalcaemia it requires the presence of vitamin D for this action to be manifest(Harrison and Harrison, 1961). Furthermore rats fed on a vitamin D deficient diet became hypocalcaemic, which was reversible by subsequent vitamin D replacement. Besides the dietary source of the vitamin there may also be a biogenic source in man from the ultraviolet irradiation

of the provitamin, 7-dehydrocholesterol, in the skin (Avioli and Haddad, 1973). It has also been established that vitamin D3 is not the active form of the vitamin, but requires further metabolism to hormonally active products (DeLuca, 1967). It appears that the native vitamin, cholecalciferol, undergoes at least two hydroxylation stages in the body to yield active metabolites. First cholecalciferol is hydroxylated in the liver to 25,hydroxycholecalciferol by a mitochondrial enzyme system (Horsting and DeLuca, 1969). This hydroxylated derivative is the main circulating form of vitamin D3, and is capable of promoting some bone mineral resorption and intestinal calcium absorption (Olson and DeLuca, 1969). However, further hydroxylation within the kidney is now known to occur (Gray, Boyle and DeLuca, 1971) to yield either 1,25DHCC or 24,25DHCC. The 1,25DHCC form of the vitamin has a potent bone resorbing capacity and will also stimulate calcium absorption from the gut (Myrtle and Norman, 1971; Raisz, Trummel, Holick and DeLuca, 1972). Thus it may represent the final effector metabolite of vitamin D3. The action of this hormone on gut and bone however is not well established, although the picture may now be coming clearer. In terms of gut calcium absorption, it has been demonstrated that 1,25DHCC may induce the formation of a calcium transport protein in gastrointestinal epithelial cells (DeLuca, 1971). This protein is known to also interact in some fashion with a calcium binding protein whose synthesis may also be under the regulation of vitamin D metabolites (Wasserman and Corradino, 1974). The nett effect of an increase in circulating 1,25DHCC being an elevation in calcium uptake from the gastrointestinal lumen. In addition the vitamin may also facilitate the transfer of calcium into the gut cell through generalised permeability changes (Patrick, 1973).

In terms of bone resorption it is now becoming apparent that 1,25DHCC is the prime hypercalcaemic agent, much more so than PTH whose activity may be largely due in vivo to enhancing the conversion of 25HCC to 1,25DHCC (Rasmussen, Wong, Bible and Goodman, 1972). Thus under normal calcium status, PTH and CT secretion continue at a low rate, and cholecalciferol is converted to 25HCC independently of these two hormones. Furthermore this reaction shows a marked product inhibition by 25HCC, thus restricting the further and excessive formation of itself and the active 1,25DHCC in the kidney (Horsting and DeLuca, 1969). Hypocalcaemia, however, leads to PTH secretion which promotes the formation of 1,25DHCC from the mono-hydroxylated substrate, thus enhancing bone resorption and calcium uptake from the gut, causing plasma calcium concentrations to rise. Also under these circumstances there will be a rapid utilisation of 25HCC promoting the occurence of more hydroxylation of cholecalciferol since the product inhibition will no longer obtain at low levels of 25HCC. When near normal calcium values are obtained PTH secretion is reduced and CT may be generated in greater amounts. Thus there will ensue a reduction in the rate of 1-hydroxylation , and a flux of calcium into the skeleton. It is as yet not established if PTH or 1,25DHCC is the agent which actually promotes the significant bone resorptive event, however evidence begins to favour the latter of these two.

2·1·3 As indicated in section 2·1·1 modulation of the plasma calcium concentrations via gross disruption of the calcium homeostatic results in marked alterations in mitotic activity in a number of tissues in vivo. However an extension of this work has indicated that at times when there is a requirement for extra cell production in these tissues there may be natural endogenous mechanisms for

producing concomitant alterations in plasma calcium levels. These situations have been outlined in the introduction in table (4) (pages 70-71). Indeed in general the proliferation rate in the thymus and bone marrow correlates closely with the plasma calcium concentration (Rixon, 1968; Perris, 1971). Thus at times when the body growth rate is maximal, there is associated with this an elevated plasma calcium concentration (Perris, Whitfield and Tolg, 1968) and heightened mitotic activity in the thymus and bone marrow. In another instance haemorrhage leads to a protracted hypercalcaemia which persists until normal circulating erythrocyte values are reestablished (Perris, MacManus, Whitfield and Weiss, 1971). This hypercalcaemia causes heightened erythropoiesis and thymopoiesis (Perris, MacManus, Whitfield and Weiss, 1971) and is the consequence of erythropoietin release attributable to hypoxia (Hunt and Perris, 1973). Hypoxia induced by cobaltous chloride injections or pregnancy lead to a similar situation (Perris, 1971) as does injection of erythropoietin itself (Hunt and Perris, 1973). However in the aparathyroid rat these alterations in plasma calcium do not occur and the enhanced proliferation rates do not manifest themselves (Perris, 1971; Perris, MacManus, Whitfield and Weiss, 1971; Hunt and Perris, 1973). This suggests that there is a requirement for functional parathyroids for these proliferative events to be apparent, and that furthermore erythropoietin may act to stimulate PTH release thus generating the observed hypercalcaemia.

Another well documented example where fluctuations in plasma calcium levels closely follow changes in thymus and bone marrow proliferative rates is the diurnal rhythm in these tissues (Hunt and Perris, 1974). Here also elevated plasma calcium values are associated with enhanced proliferation in marrow and thymus and

this periodicity of calcium and mitosis is ablated by parathyroidectomy (Hunt, 1974; Hunt and Perris, 1974). Similar cyclical changes occur during the rat oestrous cycle (Smith, 1976).

A potentially exciting extension of this work has indicated that the immune system may itself possess calcium- and parathyroid-dependent proliferative phases (Edwards et al., 1976; Edwards and Perris, 1976). Thus injection of sheep red blood cells, bacterial lipopolysaccharide or bovine serum albumin all lead to hypercalcaemic states which are associated with enhanced bone marrow mitotic activity. Parathyroidectomy ablates both the calcium increment and enhanced division. However both the genesis of plaque forming cells and haemagglutinin synthesis appears unaffected by this operation. In contrast the cell-mediated response of xenograft rejection is markedly impaired by parathyroidectomy in the rat (Atkinson, 1976; Edwards et al., 1976) as is the hypercalcaemia and bone marrow proliferation which this transplantation elicits. These results would indicate that the T lymphocyte proliferative response upon immunological challenge is impaired by low ambient calcium concentrations in both graft rejection and primary humoral antibody responses. However the transformation and subsequent antibody synthesis of the B lymphocyte system does not appear to be dependent upon calcium, suggesting that at least two proliferative mechanisms exist.

2·1·4 Besides calcium, injections of magnesium chloride into male rats will also elicit a mitogenic response in both thymus and bone marrow (Perris, Whitfield and Rixon, 1967; Smith, Gurson, Riddell and Perris, 1975). However there has as yet been no indication that there are natural modulations of the total plasma magnesium concentration in concert with proliferative changes. This negative result

might emanate however from the present inability to accurately assess the ionised rather than the total magnesium fraction in the plasma. This is possible for calcium by use of a specific ion electrode, which does indicate changes in ionised as well as total calcium in hyperproliferative situations (e.g. Hunt, 1974). When such a technique becomes available for magnesium ions it is hoped that this point will be resolved.

2·2 The role of ions *in vitro*

Although a number of ions will affect cell proliferation *in vitro* when their concentrations are grossly altered it is clear that only calcium and magnesium ions do so within the range which would be considered as representing physiological concentrations in the intact animal. However as will be discussed in Unit 7 the alterations in the transport of various ionic species may accompany cell stimulation, and in this secondary role might represent an important feature of the activation process. In this latter context some time will be spent considering these ion fluxes in the relevant Units, and would be out of place at the present since this Unit is designed to consider the primary level of cell proliferation control *in vitro*.

2·2·1 Both calcium and magnesium have been shown to have profound effects upon cell proliferation in non lymphoid cells including 3T3 fibroblasts (Dulbecco and Elkington, 1975), chick embryo (CEF) fibroblasts (Rubin and Koide, 1976) and even yeast (Duffus and Patterson, 1974). In general addition of calcium to density inhibited 3T3 fibroblasts results in the restoration of division (Dulbecco and

Elkington, 1975). In this respect calcium mirrors the action of serum addition to such arrested cultures as mentioned in the general introduction. However the action of calcium is apparent even in the absence of serum in these cultures and hence is not dependent upon any putative serum factors. Just as the concentration of serum in the medium defines the cell density at which mitotic arrest occurs so the calcium concentration also defines the final cell density attainable in 3T3 cultures (Dulbecco and Elkington, 1975). However these workers were unable to demonstrate any action of the calcium transporting ionophore, A23187, (Reed and Lardy, 1972) on cell proliferation in 3T3 cultures. Evidence presented in this Unit may indicate that this was the result of the excessively high ionophore concentrations used in these studies rather than a true dichotomy of action between intra- and extracellular calcium. An action of calcium on 3T3 proliferation had been indicated previously by the work of Gail et al.(Gail, Boone and Thompson, 1973). These workers demonstrated that chelation of extracellular calcium with EGTA resulted in the diminution of both proliferation and motility of these cells. Furthermore broad changes in both extracellular sodium and potassium concentrations had no effect on division rates in this study unless gross alterations were induced with metabolic poisons. Similar results with respect to monovalent cations have been reported for chinese hamster ovary cells (Cone and Tongier, 1971). Furthermore in the experiments of Dulbecco and Elkington (1975) it was shown that strontium, barium, magnesium and manganese were unable to substitute for calcium in releasing arrested 3T3 cells. In addition baby hamster kidney cells (BHK) and SV40 transformed 3T3 fibroblasts did not have a requirement for calcium in density dependent growth effects, whilst there was a small effect in hamster embryo cells (Dulbecco and Elkington, 1975). Chick embryo fibroblasts

similarly show a reduction in the progression of cells into the S phase if calcium is chelated from the medium (Balk, Whitfield, Youdale and Braun, 1973). The transformation of these cells with a Rous sarcoma virus removes this requirement for calcium (Balk et al., 1973). It has recently been shown in CEF cells that this requirement for low levels of calcium may be related to a more important requirement for magnesium(Rubin and Koide, 1976). They propose that magnesium (or rather its intracellular ionised concentration) is the fundamental regulator of cell proliferation and metabolism, because of its involvement in many metabolic pathways. Calcium availability then merely modulates secondarily intracellular magnesium concentrations. This notion for the coordinate control of cell metabolism comes from a number of observations. First deprivation of magnesium reduces the entry of CEF cells into the S phase as does the chelation of magnesium with pyrophosphate (Rubin, 1975). Secondly a range of metabolic processes are known to be stimulated by serum addition and are regulated coordinatedly by the serum concentration as well as growth factors, pH and cell density (Rubin and Fodge, 1974). The pathways regulated include the metabolism of hexoses (Fodge and Rubin, 1973; Sefton and Rubin, 1973), RNA and protein synthesis (Rubin and Koide, 1975) and the execution of differentiated fibroblast function (Moscatelli and Rubin, 1975). Calcium is not known to play any role in the regulation of these pathways whereas magnesium plays a central part since it is required for all transphosphorylation reactions (Lehninger, 1970). Thus serum (Fodge and Rubin, 1973) and viral transformation (Singh, Singh, August and Horecker, 1974) activate the conversion of glucose to pyruvate. Furthermore limiting the external magnesium supply causes reductions in the rates of DNA, RNA and protein synthesis, glycolysis and the transport of 2'-deoxy-D-glucose in a fashion similar to serum

deprivation (Rubin, 1975). Their arguement then goes that it is the intracellular ionised magnesium concentration which ultimately regulates cell growth, and this may be modulated through cellular calcium levels which compete for divalent cation binding sites. In times of high intracellular calcium, magnesium will be freed from from binding to some degree thus elevating its endogenous ionised concentration and thus stimulating phosphorylation and division. When calcium levels are low the converse would happen. Furthermore through unspecified alterations in the membrane of transformed fibroblasts cation binding is altered such that this calcium releasing action is removed. Although this explanation does have some experimental backing there are perhaps a number of glaring omissions which might contraindicate this simplistic interpretation. First calcium is in fact very important in mitochondrial oxidative phophorylation (Bramhall, personal communication) . Secondly the rather crude interpretation of membrane structure and function adopted in these studies is not a realistic appraisal of transmembrane ion distribution and regulation (Borle, 1970; Weissmann and Claiborne, 1975). Furthermore calcium can itself directly activate enzymes responsible for DNA synthesis (Youdale and MacManus, 1975). This being in addition to its ability to elevate endogenous cyclic AMP via inhibition of cyclic nucleotide phosphodiesterase (MacManus and Whitfield, 1971). The subsequent rises in cyclic nucleotides are then known to regulate a large number of enzyme activities (Langan, 1970). Certainly this mechanism would offer a greater degree of metabolic control than would the intracellular magnesium concentration. Whilst one can identify precedents for a restriction in metabolic activity being the controlling factor in cell proliferation (in the yeast for instance) it is unlikely that the primary event will be a running down of metabolism since this will offer no degree of control in a

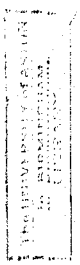
complex tissue containing both terminal differentiated and proliferating cells. And in addition some of these dividing cells may well be entering and exiting resting phases. It would seem likely that cells may not undertake the metabolically costly process of division if their energy supply is low but it would seem equally apparent that extracellular (extrinsic) factors must be capable of inducing division in cells without altering their energetic status. Many of these mitogenic factors furthermore are known to be dependent upon calcium (and one can cite calcium itself as a mitogenic agent) and bring about changes in endogenous calcium concentrations (Units 3,4 and 7). It is not at all clear from Rubin's mechanism why the level of endogenous magnesium should drop in arrested cells, and if this is solely governed by the calcium concentration within the cell then why the calcium level should change. Thus whichever way one analyses this problem calcium must be the prime modulator of cell division and magnesium may play a secondary role in governing basal levels of proliferation.

2•2•2 In a lymphoid tumour line, L5178Y, calcium and magnesium have also been demonstrated to be involved directly in cell proliferation regulation (Brennan and Lichtman, 1973). Both calcium and magnesium were reported as being capable of supporting growth of these cells independently of one another, although magnesium was claimed to be the more effective ion of the two. Further it was indicated that both intra- and extracellular divalent cation concentrations were directly related to cell growth rates. Calcium and magnesium may also be involved in the proliferation of the human Raji lymphoid cell line although the pattern of this involvement is somewhat complex (Grimwade, 1976). However in terms of normal lymphoid proliferation <u>in vitro</u> calcium represents a major determinant

of mitotic activity. Thus calcium is required for the initiation of DNA synthesis in PHA stimulated lymphocytes (Whitney and Sutherland, 1972a) and allogeneic lymphocyte stimulated cells (Whitney and Sutherland, 1972b). Also calcium enhances antibody production both *in vivo* and *in vitro*, a process which entails lymphocyte proliferation (Braun, Ishizuka and Seeman, 1970). It has also been demonstrated that during lymphocyte activation calcium is taken up into these cells (Allwood *et al.*, 1970; Whitney and Sutherland, 1973; Freedman *et al.*, 1975).

In thymocyte cultures both magnesium (Whitfield, Perris and Rixon, 1969) and calcium (Whitfield, Rixon, Perris and Youdale, 1969) can stimulate the entry of quiescent cells into the DNA synthetic phase of their cell cycle. Furthermore a large number of mitogenic agents in this system require the presence of extracellular calcium for their division promoting capacity to be manifest (Whitfield, Perris and Youdale, 1969; Whitfield, MacManus, Youdale and Franks, 1971; Whitfield, Rixon, MacManus and Balk, 1972). In contrast other mitogens do not possess this dependence upon calcium ions (Whitfield, MacManus, Braceland and Gillan, 1972). Whilst magnesium has attracted little attention as a mitogen in these studies, calcium has been extensively investigated because of its established importance *in vivo*. A mechanism has been proposed for calcium's mitogenic activity which involves the metabolism of cyclic AMP (MacManus and Whitfield, 1971). They suggest that this nucleotide mediates the stimulatory action of the ion, and that calcium elevates endogenous cyclic AMP through an inhibitory action on nucleotide phosphodiesterase (Whitfield, Rixon, MacManus and Balk, 1972). This mechanism will be more fully discussed in Units 3 and 4.

The role of magnesium in this proliferative scheme however has remained something of a mystery, but one piece of information indicated that the two divalent cations might not act in an identical fashion. For whilst injections of calcium or magnesium chloride into male rats elicited an enhanced mitotic rate in the thymus and bone marrow, calcium injections were without effect in the female rat (Magnesium-induced mitogenesis being unaffected, Dawson and Perris, 1973). Hence a series of experiments were initiated to investigate this dichotomy of reaction between calcium and magnesium in thymic lymphocyte cultures.



2·3 An investigation of the mitogenic properties of calcium and magnesium in vitro.

Figure 2·3a indicates that when thymocytes were suspended in medium containing 0·6mM calcium chloride and 1·0mM magnesium sulphate some 3·6% of the nucleated cell population were present in

Figure 2·3a. The Influence Of Calcium, Magnesium And Oestradiol On Thymic Lymphocyte Proliferation.

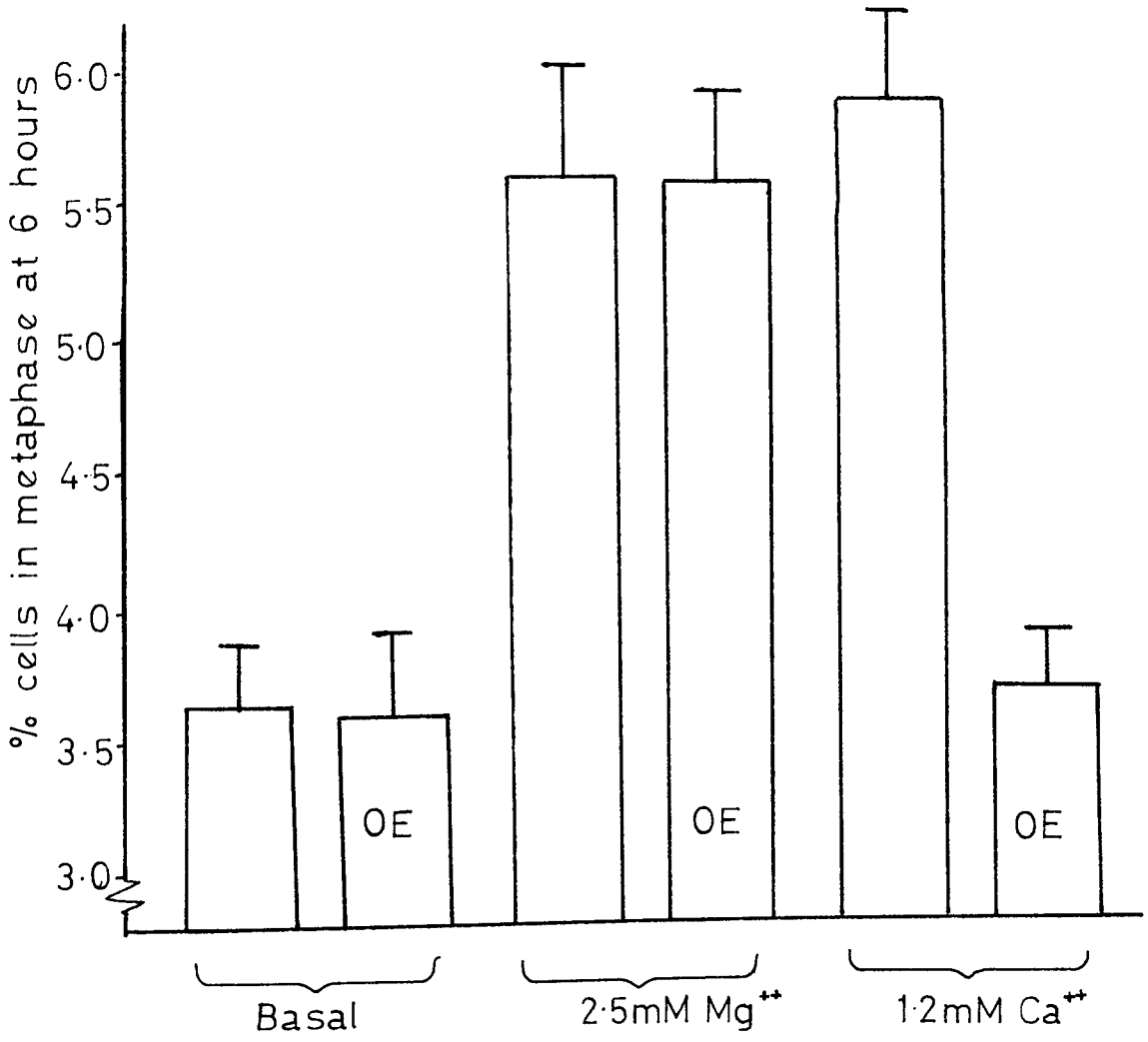


Figure 2·3a. The interaction of calcium, magnesium and oestradiol-17β in thymocyte proliferation. Cells were suspended in BGS containing 0·6mM calcium and 2·5mM 0·6mM calcium and 1·0mM magnesium (Basal); 0·6mM calcium and 2·5mM magnesium (2·5mM Mg^{++}); 1·2mM calcium and 1·0mM magnesium (1·2mM Ca^{++}). In addition cultures marked OE contained oestradiol-17β (0·1μg.ml^{-1}). All cultures contained colchicine at a final concentration of 0·062mM and metaphase accumulations were scored as described. Results are the mean (column) + S.E.M. (vertical line and bar) of 4 experiments.

the metaphase compartment at six hours. However if the level of calcium was raised to 1·2mM, or if the magnesium concentration was elevated to 2·5mM then there was a significant ($p < 0·001$) increase in the 'colchicine metaphase' accumulation. Thus the addition of calcium or magnesium promotes the entry of cells into their DNA synthetic phase and ultimately mitosis as indicated in Unit 1 and also Figure 2·3d. Cells which are already in the S phase at the start of the experiment are unaffected by these ionic alterations and give rise to the basal level of mitotic activity seen in these experiments. Since calcium-induced mitogenesis was not apparent in the female rat it was postulated that female sex steroids could be the causative blocking agent (Dawson and Perris, 1973). Subsequent studies have shown this to be the case *in vivo* by orchidectomy and steroid replacement regimes (Smith *et al.*, 1975). Furthermore addition of oestradiol 17β ($0·1 \mu g.ml^{-1}$) to thymocyte cultures whilst not influencing basal activity significantly reduced calcium stimulated mitosis (Figure 2·3a). Oestradiol had no effect on magnesium-induced mitogenesis. Thus this *in vitro* technique mirrors almost exactly the results obtaining in female rats. It should be pointed out that whilst female thymocytes do not respond to calcium *in vivo* they regain this ability when in culture (Dawson and Perris, 1973; Smith *et al.*, 1975). In the results reported here male thymocytes were used to exclude problems with oestrogen receptors and oestradiol carryover. However, there does appear to be a clear dichotomy in the reaction of calcium and magnesium to oestradiol, enforcing the notion that there may be two mechanisms underlying the activity of these two divalent cations.

The mode of action of oestradiol was further elaborated in another series of experiments. In the first of these cells were preincubated for 30 minutes in basal concentrations of calcium and

and magnesium (0·6 and 1·0mM respectively) then at time 0 (T_o) some of the cultures were treated with further calcium to elevate the ions

Figure 2·3b. A Time Study Of The Inhibition Of Calcium-Induced Mitogenesis By Oestradiol.

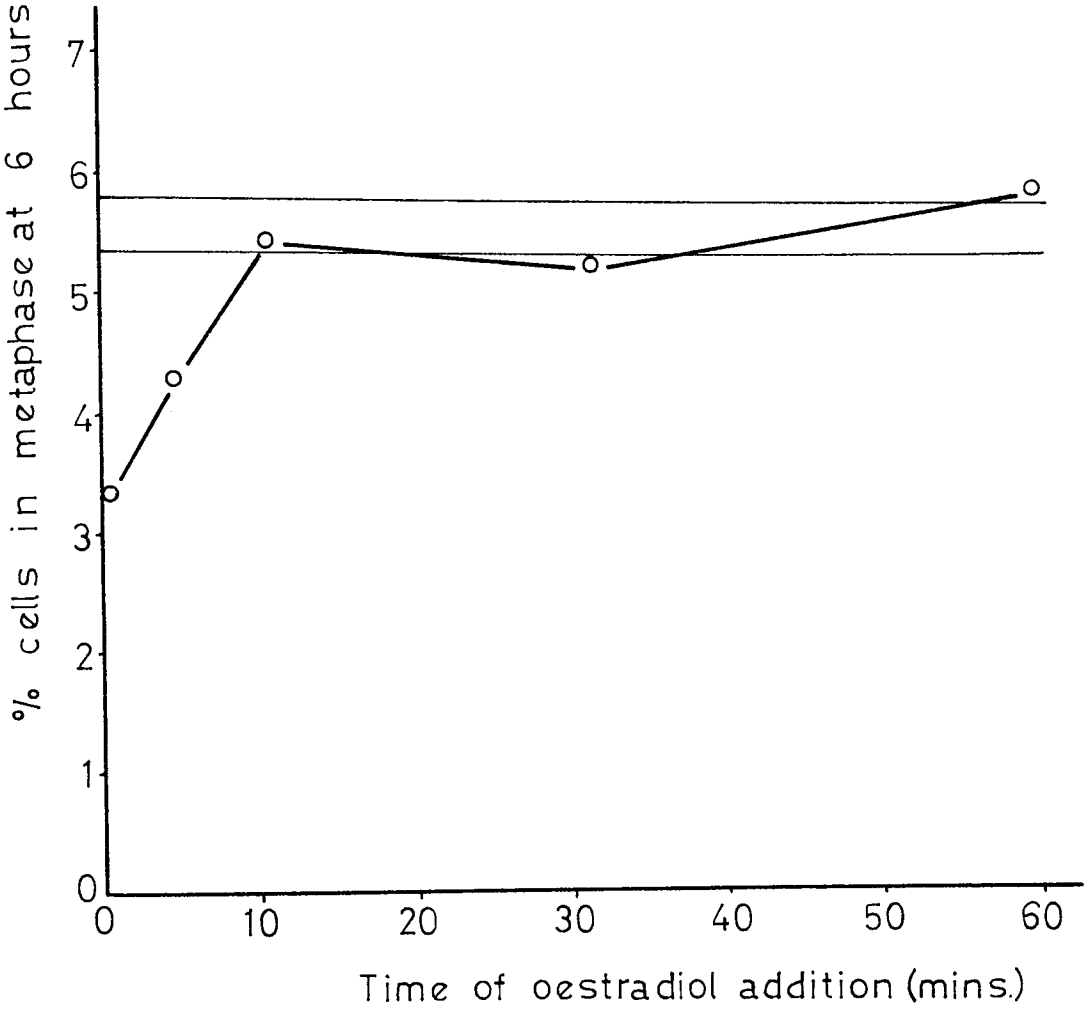


Figure 2·3b. An experiment to determine the reversibility of a calcium-induced mitotic increment by oestradiol. Cells were treated as described in the text in the presence of 0·062mM colchicine. At the indicated times oestradiol was added to calcium stimulated cell suspensions and mitotic activity assessed six hours later. The two horizontal lines represent two S.E.Ms from ten cultures stimulated with 1·2mM calcium, the mean value being the midpoint of these lines. Each experimental point is the mean of two experiments.

concentration to 1·2mM. Either simultaneously or at various times afterwards (T_n) oestradiol-17β ($0·1 \mu g.ml^{-1}$) was added to these cultures and metaphases accumulated for a further six hours. The results indicate (Figure 2·3b) that only if oestradiol was added

along with the mitogenic load was the response completely ablated. If addition was delayed for ten minutes then the response was insensitive to oestradiol treatment, thus indicating that the reaction generated by calcium is rapid, and once stimulated the further passage of thymocytes through the cell cycle is not steroid-sensitive.

Figure 2·3c. A Dose-Response Curve For The Oestradiol Inhibition Of Calcium-Induced Mitogenesis.

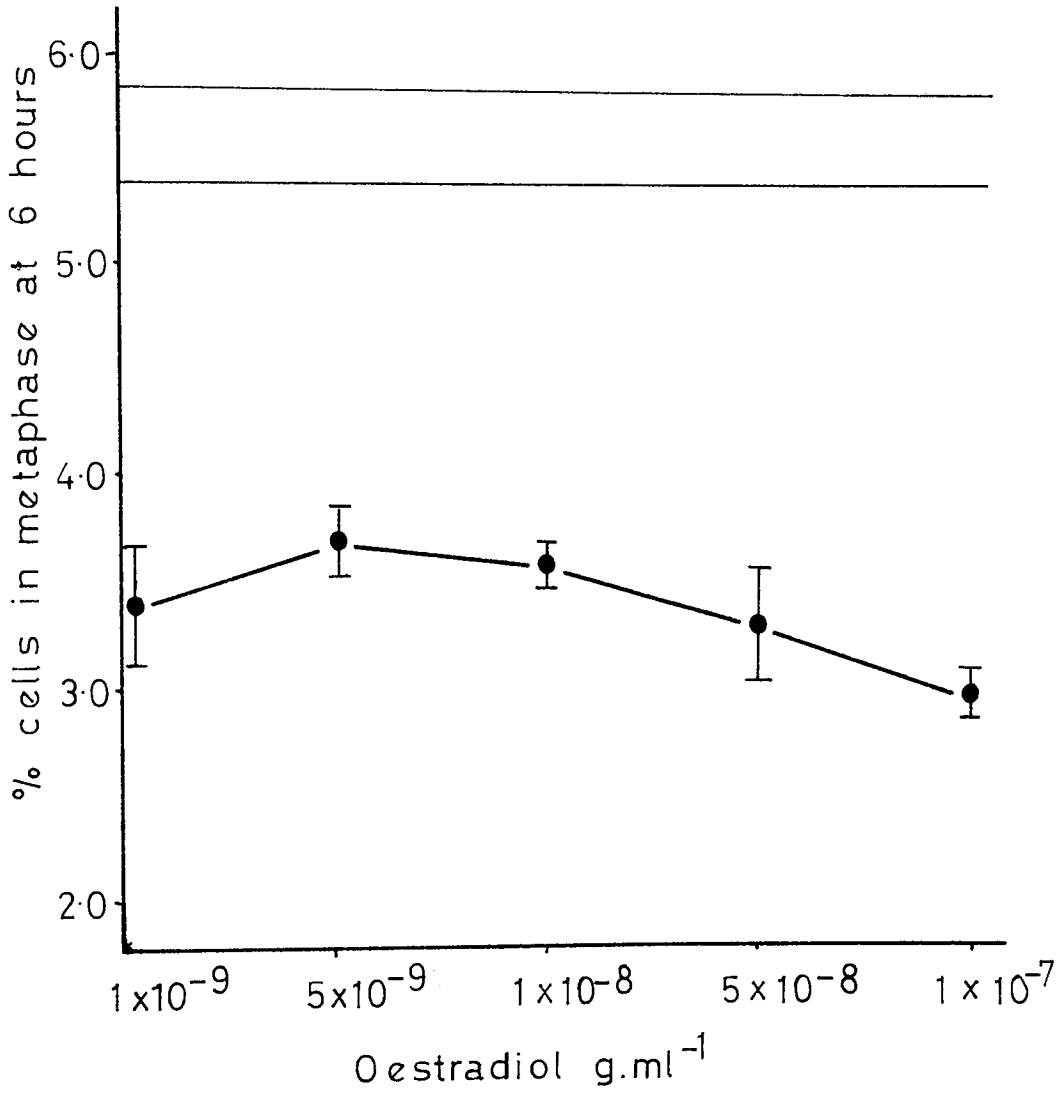


Figure 2·3c. A dose response study of oestradiol inhibition of a calcium-induced mitotic increment. Thymocytes were cultured in BGS containing colchicine and 1·2mM calcium chloride. To these cultures was added oestradiol-17b at various concentrations, and six hour metaphase accumulations performed. Each point is the mean ± 1 S.E.M. of four separate determinations. The two horizontal lines represent the mean ± 1 S.E.M. from 6 cultures containing 1·2mM calcium and no oestradiol.

This oestradiol blockade was effective at steroid concentrations down to $1ng.ml^{-1}$, which is the lowest level tested (Figure 2·3c). It is likely however that lower levels will be also be effective since plasma oestradiol concentrations are in the range 30 to $50pg.ml^{-1}$ of plasma (Brown-Grant, Corker and Naftolin, 1972) and these concentrations appear to be active *in vivo* (Smith et al., 1975). The situation may of course be obscured by the lack of steroid binding globulin in the culture system, and any attempt to correlate *in vitro*

Figure 2·3d. The Action Of Calcium And Oestradiol On DNA Synthesis In Thymocyte Cultures.

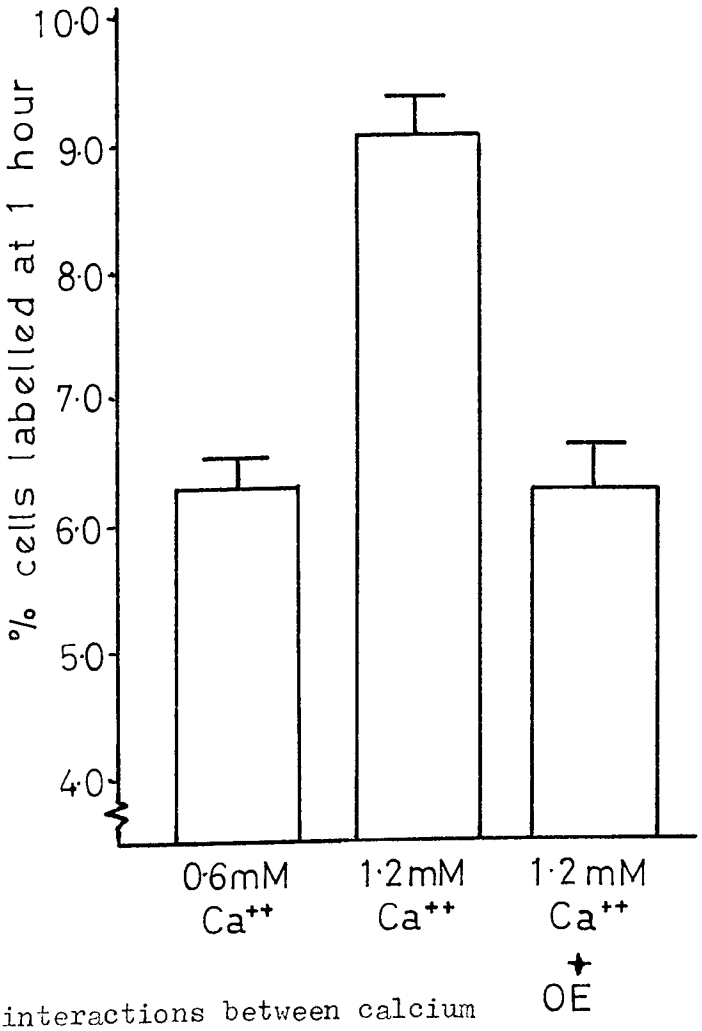


Figure 2·3d. The interactions between calcium and oestradiol in the initiation of DNA synthesis in thymic lymphocyte cultures. Cells were maintained in Medium 199 containing no colchicine and preincubated for thirty minutes. They were subsequently exposed to tritiated thymidine ($5\mu Ci.ml^{-1}$) and either 1·2mM calcium or 1·2mM calcium plus oestradiol ($0·1\mu g.ml^{-1}$). One hour later cells were prepared for autoradiograms. Each result is the mean ± 1 SEM of 4 to 8 experiments.

oestrogen concentrations with those obtaining in the intact animal may be open to criticism at the fundamental level.

It was important to establish whether the oestradiol blockade of calcium-induced mitogenesis was related to an ability to restrict the exit of stimulated cells from a presynthetic G_o. Figure 2·3d indicates that elevation of the extracellular calcium concentration from 0·6 to 1·2mM results in an increase in the number of cells actively incorporating tritiated thymidine into their DNA. Moreover in the presence of oestradiol ($0·1\mu g.ml^{-1}$) elevation of the calcium level fails to enhance thymidine uptake (Figure 2·3d). Thus it would seem likely that the steroid acts upon the calcium initiation process preventing the entry of quiescent cells into DNA synthesis. Since cells cultured in the presence of oestradiol for all but ten minutes of their incubation period show no change in mitotic activity it is unlikely that the oestrogen is having any lymphocytolytic effect. Furthermore evidence presented in Unit 1 shows that viability is the same in both control and oestradiol-treated cultures in terms of cellularity, size distribution and metabolic activity. Indeed another steroid, cortisol, actually stimulates thymocyte proliferation in a calcium dependent fashion (Whitfield, MacManus and Rixon, 1970) indicating a heterogeneity of reaction amongst the steroids with respect to thymic lymphocyte mitotic activity.

If this action of oestradiol on calcium-mediated proliferative events has any relevance it might be expected that situations would exist in the intact animal where oestrogens oppose a calcium stimulation. It is known that the female of a number of species show poorer growth rates and pubertal growth arrest (Harvey, Hervey

and Hutchinson, 1973). This may be the result of oestrogen interactions with calcium-induced proliferative events, remembering that in the rat rapid growth rates, enhanced mitotic activity and hypercalcaemia are closely correlated (Perris, 1971). Furthermore the calcium dependent erythropoietic recovery from haemorrhage is slower in the female compared to the male (Steinglass, Gordon and Charriper, 1941). In terms of lymphocyte proliferation in vivo, evidence is inconclusive concerning the role of oestrogens. However, women are less prone to suffer from many atopic skin allergies and hay fever (Borrie, 1964; Urbach, 1966) which may represent a dampening of T-cell transformation by antigen. Indeed stilboestrol has until recently been used as a treatment for Acne Rosaceae (Borrie, 1964). Furthermore there is an increased incidence of inflammatory skin diseases in women over the age of 40, which is perhaps related to the reduction in oestrogen levels at the menopause (Urbach, 1966). Two other physiological situations exist where there are alterations in circulating oestrogen levels and concomitant changes in a calcium dependent process. First oestrogens antagonise PTH-induced bone resorption (Lafferty, Spencer and Pearson, 1964) a calcium dependent hormone whose action is attributable to the transport of calcium into bone cells (Talmage, Cooper and Park, 1970). Evidence presented in the following Unit will also demonstrate that PTH-induced mitogenesis is similarly blocked by oestradiol. Thus the post-menopausal osteoporotic condition which occurs through reduced oestrogen levels (Gallagher and Nordin, 1972) may be explained also at the cellular level by oestradiol impairment (or in this case lack of impairment) of a calcium dependent hormone. The converse of this situation is postparturient paresis in the cow where an hypocalcaemia ensues despite high PTH levels but which is associated with a large oestrogen surge (Manunta, Saroff and Turner, 1957; Ranney, 1959). Thus the

Table 2·3e. A Comparison Of The Ability Of Various Steroids To Inhibit Calcium- and Magnesium-Induced Mitogenesis.

Steroid	High Calcium (1·2mM) Medium	High Magnesium (2·5mM) Medium
No Steroid Added	6·2 ± 0·48%	5·6 ± 0·10%
Oestradiol-17β	[a]2·9 ± 0·46*	[b]6·1 ± 0·52
Oestradiol-17α	[a]3·8 ± 0·41*	N.T.
Oestradiol-3-benzoate	[a]3·6 ± 0·23*	b5·5 ± 0·30
Oestriol	[a]3·0 ± 0·51*	N.T.
Oestrone	[a]4·4 ± 0·42*	N.T.
Oestrone-3-sulphate	[a]3·9 ± 0·70*	N.T.
Oestradiol-3-glucuronide	[a]5·8 ± 0·41	N.T.
17α-ethyl-β-oestradiol 3-methyl ether	[a]5·7 ± 0·20	N.T.
β-oestradiol 3-methyl ether	[a]6·0 ± 0·42	N.T.
Testosterone	[b]6·0 ± 0·52	[b]3·0 ± 0·10*
5α-dihydrotestosterone	N.T.	[b]4·5 ± 0·52*
Dehydroepiandrosterone	N.T.	[b]5·5 ± 0·74
Androstenediol	N.T.	[b]6·3 ± 0·11
Androstenedione	N.T.	[b]6·2 ± 0·62
Cholesterol	[b]5·4 ± 0·40	N.T.
Progesterone	[b]6·1 ± 0·23	[b]5·6 ± 0·24

Figure 2·3e. The action of various steroids on calcium- and magnesium -induced mitogenesis. Thymocytes were cultured in Medium 199 which contained either 1·2mM calcium or 2·5mM magnesium and colchicine. Six hour metaphase accumulations were then performed on these cell suspensions in the presence or absence of a range of steroids. These are expressed as the mean percentage cells in metaphase at six hours ± 1 S.E.M. derived from between 4 and 19 separate experiments. Where the mitotic accumulation was significantly lower than the ion-stimulated control ($p < 0·01$) this is indicated by an asterisk (*). Where an N.T. appears in the table this indicates that this combination was not tested. The suffix (a) denotes the steroid to be used at $1ng.ml^{-1}$ and (b) at $100ng.ml^{-1}$.

expectation that oestrogens should interact with calcium dependent processes *in vivo* is borne out to varying degrees in a number of situations.

It was next decided to investigate the possibility that other steroids might impair calcium- or indeed magnesium-induced mitogenesis. To this end a wide range of steroid configurations were tested for antimitotic activity in thymocyte cultures (Table 2·3e) against calcium and magnesium stimulated mitosis. It was apparent that a number of other oestrogens could inhibit calcium- but not magnesium-induced mitotic changes in the cell suspensions, whilst other classes of steroids were without effect. The active oestrogens included both α and β oestradiol, oestradiol-3-benzoate, oestriol, oestrone and oestrone-3-sulphate. However oestradiol-3-glucuronide and the 3-methyl ether derivatives of oestradiol and 17-ethyl-oestradiol were ineffective in blocking either calcium or magnesium promoted mitosis (Figure 2·3e). Thus structurally for a steroid to be capable of blocking the calcium response it must be an 18 carbon oestrogen with an aromatic 'A' ring. Since the methyl ether derivatives of oestradiol were non-active it must be interpreted as meaning that there are other additional features of structure which are important since these agents have aromatic 'A' rings. Simple lipid solubility might be one feature since the very water soluble glucuronide conjugate of oestradiol was inactive . Clearly the carbon 3 and 17 positions could be of some importance since alterations of the 3 position substituents may result in loss of activity with no change in lipid solubility (e.g. β-oestradiol 3-methyl ether). Thus it may be that ability to form some type of loose bonding with membrane constituents by groups at carbon 3 and 17 may endow upon the molecule some specificity for plasma membrane sites. Such links might be

hydrogen bonding through hydroxyl groups or negatively charged tautomerising ketone functions at carbon 17, or sulphates and hydroxyl groupings at carbon 3 with positively charged elements of the polar head groups in the outer palisade of the membrane. In this respect it is noticeable that oestrone is less effective than oestradiol in blocking calcium-induced mitosis (Table 2·3e). This may be the result of low levels of the negatively charged O^- radical at carbon 17 in comparison to the uncharged ketone. However it should also be noted that if there is some form of chemical interaction between this group and membrane sites then there will be a tendency to remove O^- forms through bonding thus shifting the equilibrium to generate further O^- groups. Hence in view of the rapidity of the calcium activation process, and its insensitivity to oestradiol within ten minutes of stimulation one might envisage that one feature determining the ability of a steroid to block this process is the rate at which it can bind to such membrane sites. This will of course be restricted by the dissociation of substituent groups at carbon 3 and 17. Another important feature of structure relating to any receptor interaction is the observation that both α and β oestradiol are equally active in blocking calcium-evoked mitotic alterations (Table 2·3e). Since the former relatively rare and inert epimer of oestradiol does not bind to oestrogen cytosol receptors in target tissues (Noteboom and Gorski, 1965; King and Gordon, 1966; Puca and Bresciani, 1969) it must be inferred that these responses are not being mediated through one of these classical receptors (Jensen and Jacobson, 1962; Shymala and Gorski, 1969). It is indeed more likely that the response as a whole represents a superficial membrane action since as stated previously cells obtained from female rats rapidly regain the ability to respond to calcium when placed in culture (Dawson and Perris, 1973).

It must also be added that although many of these experiments were performed using only 1ng.ml^{-1} of steroid the possibility exists that there may still have been contamination with oestradiol in sufficient quantities to inhibit mitosis. Without sophisticated radioimmunoassays or ultrapure steroids this point cannot readily be resolved. Furthermore structure/function relationships cannot be carried too far as one is unsure of the cellular metabolism of the steroids in culture, such as sulphation or hydroxylation.

A further most significant observation in Table 2·3e is that testosterone and to a lesser extent 5α dihydrotestosterone block magnesium-induced but not calcium-induced mitotic changes. This then points to another dichotomy between the mitogenic actions of calcium

Table 2·3f. The Dose-Dependent Inhibition Of Magnesium-Induced Mitogenesis By Testosterone.

Ionic Status Of Medium	No Steroid Added	Concentration Of Testosterone 10ng·ml^{-1}	50ng.ml^{-1}	100ng.ml^{-1}
1·0mM Mg^{++} 0·6mM Ca^{++}	3·2 ± 0·1% (5)	3·1 ± 0·2% (4)	3·0 ± 0·1% (4)	3·3 ± 0·2% (4)
2·5mM Mg^{++} 0·6mM Ca^{++}	5·5 ± 0·1% (5)*	4·8 ± 0·2% (5)*	3·1 ± 0·2% (5)	3·0 ± 0·1% (5)

Figure 2·3f. The ability of various doses of testosterone to inhibit magnesium-induced mitogenesis in thymocyte cultures. Cells were incubated in Medium 199 containing colchicine and either low (1·0mM) or high (2·5mM) magnesium sulphate. To these cell suspensions was added testosterone (Sigma Chemical Co.) at different final doses in ethanolic solution. Control cultures contained ethanol carrier only. Results are the mean ± 1 S.E.M. of the percentage cells in colchicine metaphase at six hours. The number in parentheses represents the number of experiments from which each point was derived. An asterisk indicates that the result was significantly ($p<0·01$) higher than basal.

and magnesium, and provides a further probe with which to dissect the biochemical events behind thymocyte stimulation. The male sex steroid, testosterone, was active down to a concentration of 50ng.ml^{-1} (Table 2·3f) which exceeds normal circulating rat testosterone levels which are in the range 2 to 4ng.ml^{-1} plasma (Cooper and Waites, 1974). This may account for the fact that magnesium injections into male rats does elicit a mitotic response despite the presence of the androgen.

What the developmental significance might be of oestrogens inhibiting calcium-induced phenomena and androgens perhaps preventing magnesium dependent ones is a matter of some speculation but potential importance.

Since evidence was available suggesting that calcium may merely influence endogenous magnesium levels, which was the important parameter with respect to cell proliferation (Rubin and Koide, 1976) some early experiments were performed to establish this point. Thus when cells maintained in either calcium- or magnesium-free medium were treated with mitogenic levels of the other cation it was apparent that both calcium and magnesium could exert their mitogenic effects independently of each other (Figure 2·3g; Riddell, 1971). However, it must be stated that the normally mitogenic calcium concentration of 1·2mM was not effective in magnesium-free medium. Such results could be accounted for in the mechanism proposed by Rubin (Rubin, 1975).

Thyrocalcitonin (CT) is known to inhibit the mitogenic action of a number of agents in thymic lymphocyte cultures. These mitogens include adrenaline (Whitfield, MacManus and Gillan, 1971)

parathyroid hormone and cyclic AMP (MacManus and Whitfield, 1970).

Figure 2·3g. An Analysis Of The Interdependence Of Calcium And Magnesium In Promoting Thymocyte Mitotic Activity.

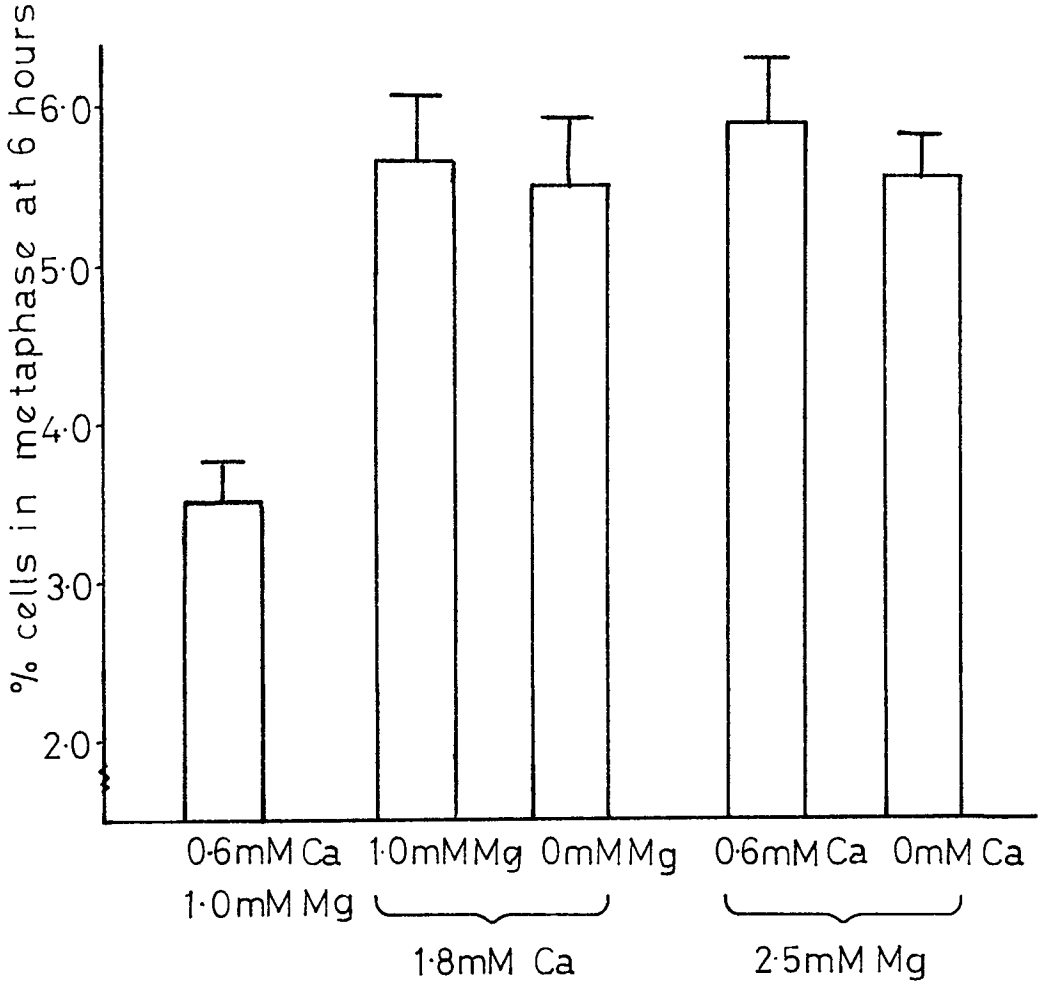


Figure 2·3g. An analysis of the interdependence of calcium and magnesium in the initiation of division in thymic lymphocyte cultures. Thymocytes were suspended in Medium 199 containing colchicine and (a) 0·6mM calcium, 1·0mM magnesium (b) 1·8mM calcium, 1·0mM magnesium (c) 1·8mM calcium, 0·0mM magnesium (d) 2·5mM magnesium, 0·6mM calcium (e) 2·5mM magnesium, 0·0mM calcium. 'Colchicine metaphases' were estimated as a percentage of the total cell population six hours after the start of the experiment. Values represent the mean (column) and 1 S.E.M. (vertical line with horizontal bar) of between 4 and 7 separate experiments.

This inhibitory action of the hypocalcaemic hormone has been attributed to its ability to inhibit the active extrusion of calcium from the lymphoblast (Borle, 1969; Whitfield, MacManus, Franks, Braceland and Gillan, 1972), this resulting in the development of excessively

high, antimitotic, levels of calcium within the cell. Although it should be remembered that modest elevation of endogenous calcium concentrations results in a mitotic stimulation. In order to test

Figure 2·3h. The Action Of Calcitonin Upon Calcium- And Magnesium-Induced Mitogenesis.

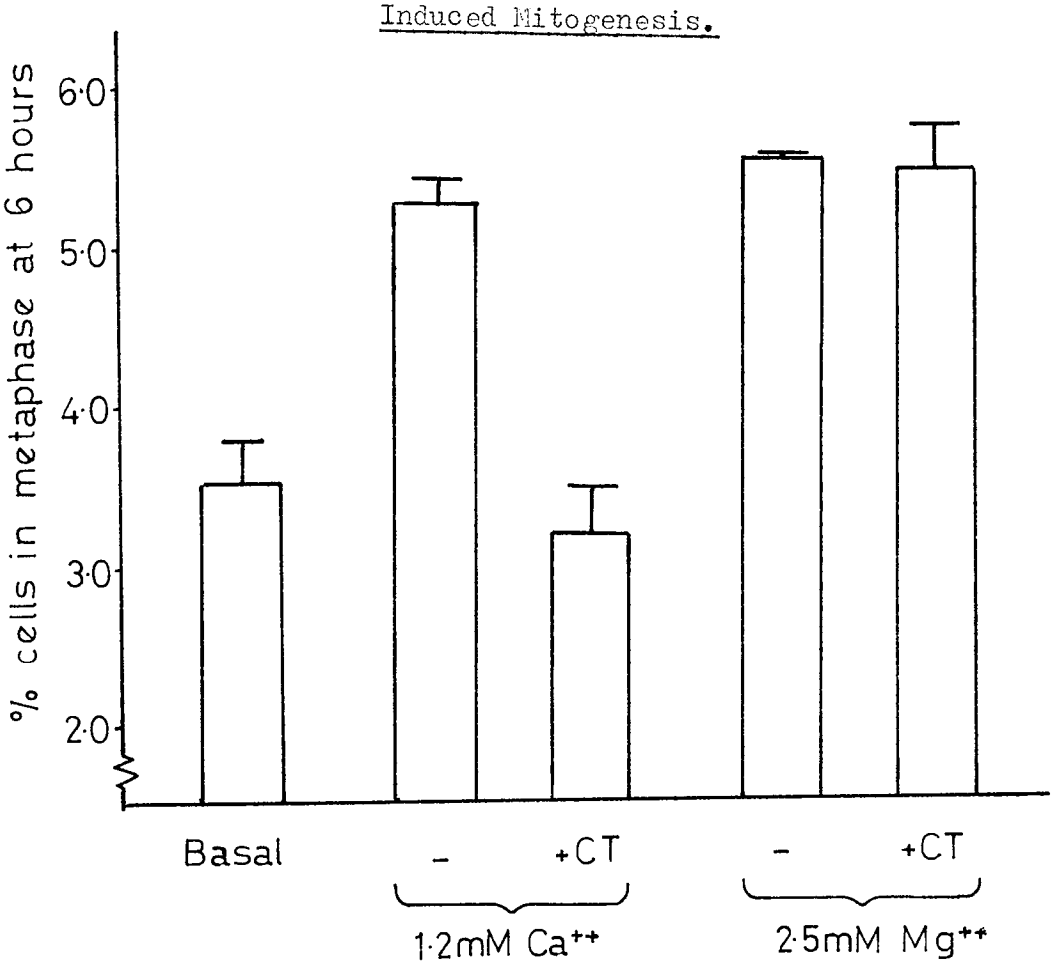


Figure 2·3h. The action of calcitonin on calcium- and magnesium-induced mitogenesis in thymocyte cultures. Cells were maintained in BGS containing colchicine and (a) 0·6mM calcium, 1·0mM magnesium the Basal medium (b) 1·2mM calcium, 1·0mM magnesium (c) 0·6mM calcium, 2·5mM magnesium. Both the high calcium and magnesium cultures were also treated with calcitonin (CT) at a final concentration of 8MRC $mU.ml^{-1}$, whilst others were merely treated with carrier (-). The calcitonin (Wilson Laboratories) was dissolved in a minimum quantity of 0·1M formic acid, and then made up to a stock solution with 0·1M sodium acetate. The initial activity of the preparation was reported as being 0·2MRC units.mg^{-1}. Results are the mean percentage cells in colchicine metaphase at six hours (columns) + 1 S.E.M. of 4 separate experiments.

the possibility that calcium and magnesium acted to stimulate thymocytes through distinct proliferative axes, CT was added to both calc-

ium- and magnesium-stimulated cultures at a final concentration of 8MRC mU.ml^{-1} which inhibits mitosis in the above experiments. The results indicate that whilst calcium-induced mitosis was inhibited by CT, magnesium-induced mitogenesis was completely unaffected (Figure 2·3h). This again emphasised the dichotomy of action between the two ions. Furthermore since calcitonin will inhibit the action of cyclic AMP itself (MacManus and Whitfield, 1970) it is unlikely that magnesium, unlike calcium, is acting via this nucleotide. This suggests a completely new and independent pathway for magnesium on thymic lymphocyte proliferation.

This independence of magnesium from cyclic AMP-mediated mitogenic processes was further enforced by the observation that imidazole could not influence a magnesium-induced mitotic stimulation (Figure 2·3i). In contrast calcium-induced mitogenesis was inhibited (Figure 2·3i). This action of imidazole has been attributed to its ability to reduce intracellular cyclic AMP concentrations by stimulating the enzyme cyclic nucleotide phosphodiesterase (Robison, Butcher and Sutherland, 1968). This enzyme degrades cyclic AMP to 5'AMP (Robison et al., 1968). It would have been expected that if magnesium acted through an elevation of intracellular cyclic AMP then this would also have been inhibited by imidazole. It might be added parenthetically that imidazole did not inhibit magnesium-induced mitogenesis at any concentration up to and including 10mM, thus it is unlikely that a differential sensitivity to this compound could account for the negative result observed following magnesium stimulation.

In recent years calcium has been implicated in the action and activation of guanylate cyclase (Goldberg, O'Dea and Haddox, 1973)

Figure 2·3i. The Influence Of Imidazole On Calcium- And Magnesium-Induced Mitogenesis In Thymocyte Cultures.

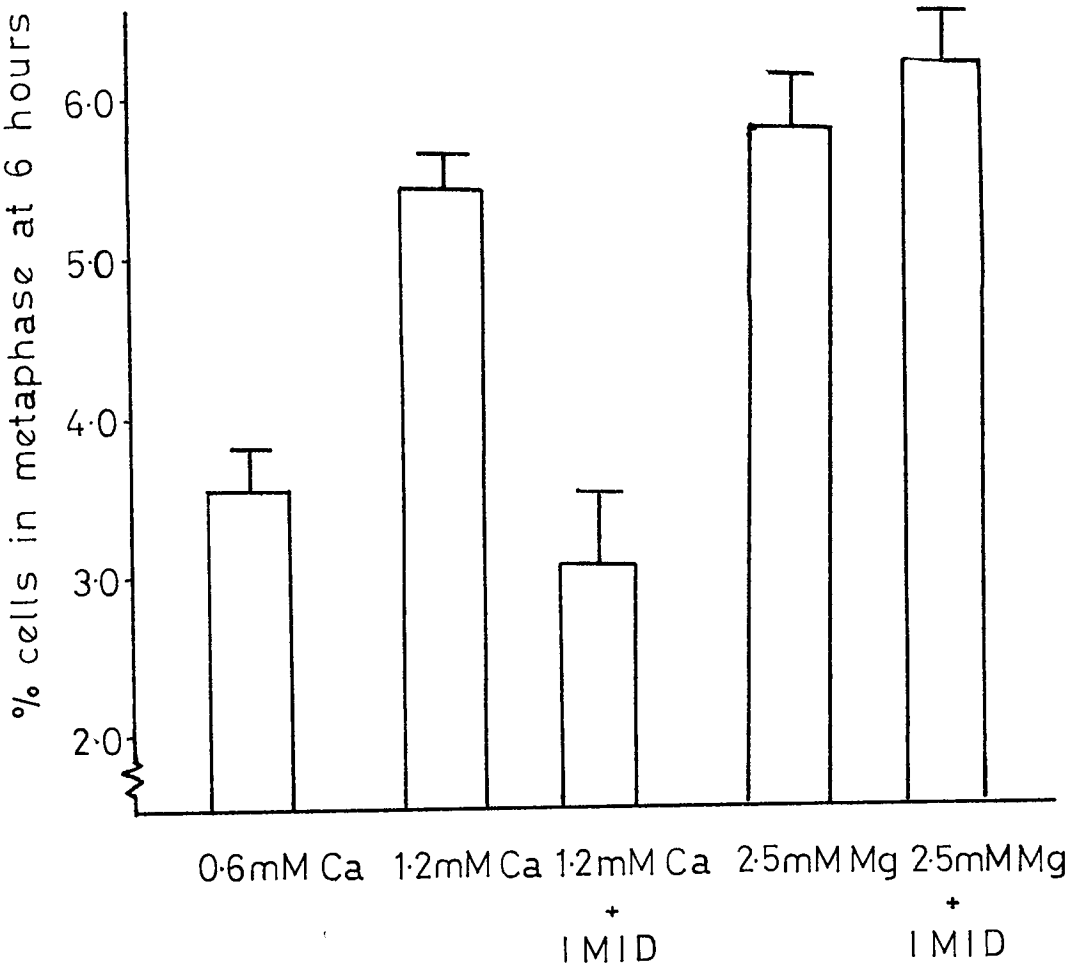


Figure 2·3i. The action of imidazole on calcium- and magnesium-induced mitogenesis in thymocyte cultures. Thymic lymphocytes were maintained in BGS containing colchicine and either high (1·2mM) calcium or high (2·5mM) magnesium. To cultures containing these mitogenic levels of calcium and magnesium was also added imidazole (Sigma Chemical Co.) to give a final concentration of 4mM. The columns represent the mean percentage cells in metaphase at six hours plus 1 S.E.M. (vertical line with bar) of four separate experiments.

the enzyme which converts guanosine-5'-triphosphate (GTP) to cyclic GMP (Ishikawa, Ishikawa, Davis and Sutherland, 1969). This latter cyclic nucleotide may play a central role in the modulation of cell stimulation (Hadden, Hadden, Haddox and Goldberg, 1972; Seifert and Rudland, 1974), the exact action of this cyclic purine will be

discussed in greater detail in Units 3-5. Thus the possibility might exist that calcium elevates endogenous cyclic GMP levels as well as intracellular cyclic AMP concentrations, these nucleotides might then act in concert to produce the activation of quiescent lymphocytes. In order to gain some insight into the possible interaction of the two divalent cations with the guanylate cyclase system, both calcium- and magnesium-stimulated cultures were treated with zinc chloride at concentrations between 10^{-6} and 10^{-4}M. The rationale behind this treatment was that the literature indicated that guanylate cyclase, unlike adenylate cyclase, is particularly sensitive to and inhibited by zinc salts (Hardman and Sutherland, 1969). Thus any agent acting through this enzyme should be inhibited or impaired in the presence of zinc. It is shown in Figure 2•3j that calcium-induced mitogenesis was more sensitive to the presence of zinc than was magnesium-induced mitogenesis, there being some 10 fold difference in sensitivities. It is interesting to note that the concentration of zinc inhibiting the action of calcium is exactly that quoted by Hardman and Sutherland (1969) as causing complete inhibition of a broken cell preparation of guanylate cyclase. However, this data should not be taken as any more than indicating the possibility of the implication of the cyclic GMP system in calcium-induced proliferation, as the zinc could clearly have a number of unknown properties such as competing for calcium binding sites, or at higher concentrations inhibiting oxidative phosphorylation. It might also be noted that cells have a requirement for zinc at low concentrations for proliferation to take place (Chesters, 1972). However these results do serve to further underline the difference between calcium and magnesium in this system, and offer another probe molecule with which to dissect the biochemical pathways stimulated by these ions.

Figure 2•3j. The Action Of Zinc On Calcium- And Magnesium-Induced Mitogenesis.

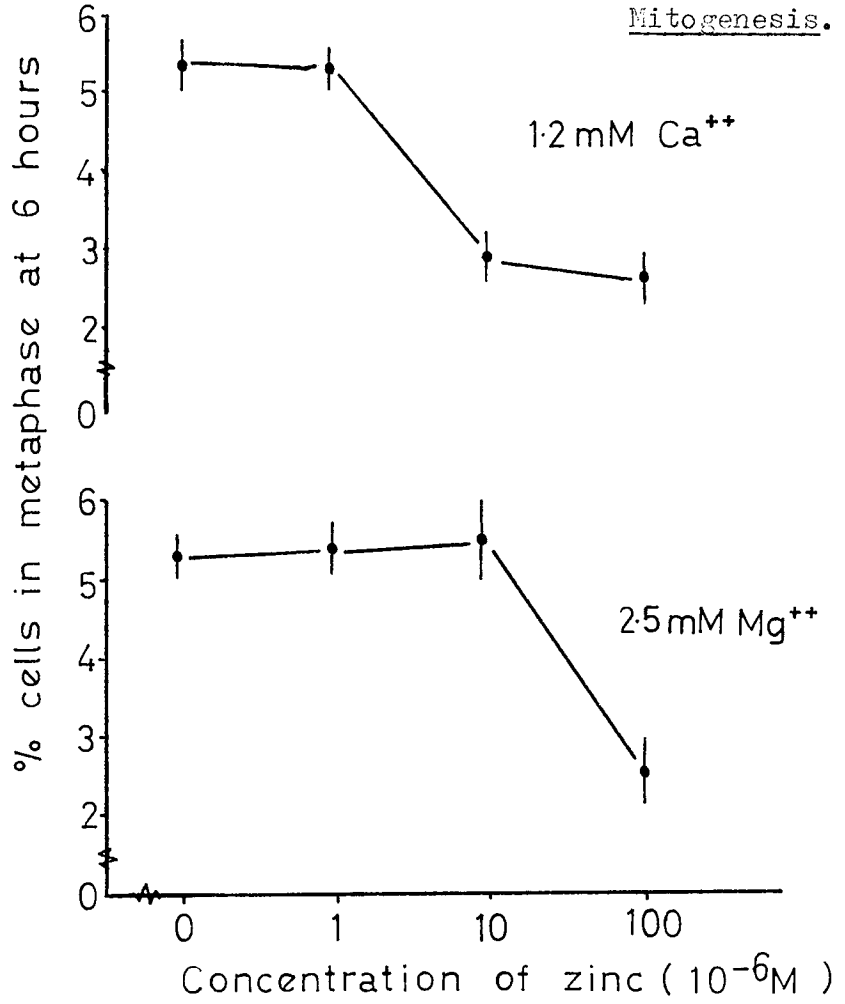


Figure 2•3j. The inhibitory action of zinc upon calcium- and magnesium-induced mitogenesis in thymocyte cultures. Cells were suspended in Medium 199 containing colchicine and high (1•2mM) calcium or high (2•5mM) magnesium. To some of the stimulated cultures was also added zinc chloride at the concentrations indicated and six hour metaphase accumulations performed. Results are the mean (circle) ± 1 S.E.M. (vertical line) of four separate determinations. Zinc at the concentrations used here did not significantly affect basal mitotic activity assessed using the same technique.

In order to decide if the divalent cations needed to enter the thymocyte to elicit their mitogenic propensity intracellular calcium concentrations were elevated by use of the calcium ionophore A23187 (Reed and Lardy, 1972). This agent facilitates the movement of calcium with its concentration gradient which under physiological conditions is into the cell (Borle, 1971). The ion transporting capacity of the ionophore is considered in more detail in Unit 7.

Figure 2·3k. The Mitogenic Characteristics Of A23187.

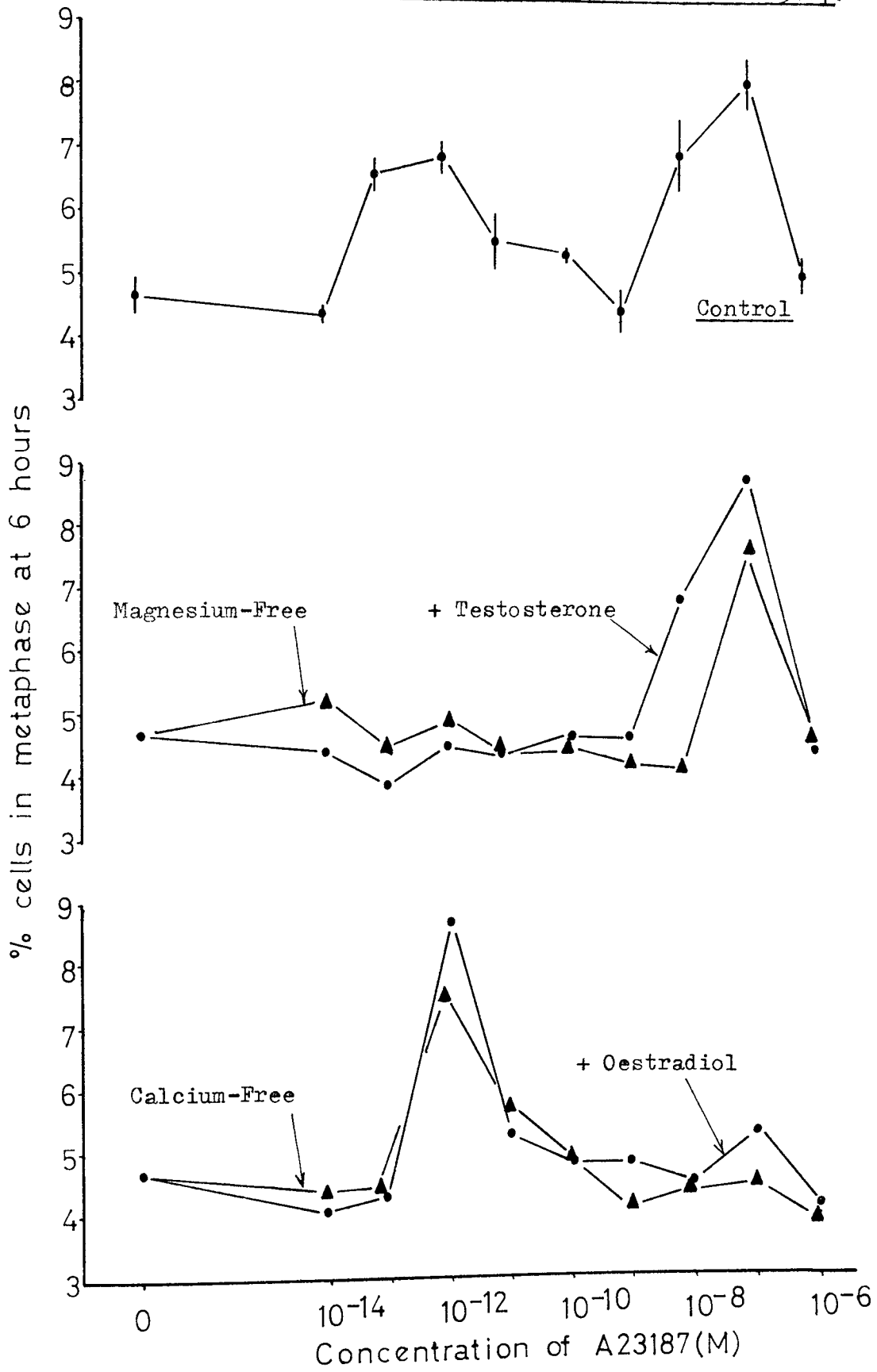


Figure 2·3k. Thymocytes were incubated in Medium 199 containing both colchicine and various combinations of calcium, magnesium. To medium containing the control levels of divalent cations was also added either testosterone or oestradiol (both 0·1μg.ml^{-1}). A23187 was subsequently added to the cultures at the indicated concentrations. Results are the mean percentage cells in metaphase at six hours from either 2 or 4 tests.

When A23187 was applied to thymocytes over a wide range of concentrations it was noted, somewhat surprisingly, that it exhibited two peaks of mitotic activity (Figure 2·3k). Since the ionophore was assumed to be acting primarily as a calcium transporting moiety then its mitogenic capacity should reside in this calcium influx into the thymocyte. Thus cells were cultured in the presence of A23187 but in calcium-free medium (Figure 2·3k). It was apparent that at the higher mitogenic concentration of the agent calcium omission from the medium resulted in the ablation of this response, whereas the lower mitogenic level was unaffected by a deficiency of this cation. This unsuspected observation suggested that perhaps the second mitogenic peak might be magnesium dependent since (a) this ion represents a different proliferative axis and (b) A23187 has been reported as having some affinity for magnesium (Caswell and Pressman, 1972). When thymocytes were exposed to the ionophore in magnesium-free medium this expectation was confirmed (Figure 2·3k). The high mitogenic concentration was unaffected by magnesium omission in this instance whilst the low peak was abolished. Since oestradiol and testosterone have already been shown to block calcium- and magnesium-induced mitogenesis, the next step was to investigate the action of these steroids on the calcium and magnesium dependent mitotic recruitments provoked by the ionophore. When thymic lymphocytes were incubated with the ionophore, A23187, both in the presence and absence of testosterone ($0{\cdot}1\mu g.ml^{-1}$) or oestradiol ($0{\cdot}1\mu g.ml^{-1}$) it was noted that the calcium-dependent peak at high ionophore concentrations was abolished by oestradiol but not testosterone, whereas the magnesium-dependent peak at low ionophore concentrations was inhibited by the presence of testosterone but not oestradiol (Figure 2·3k). Thus oestradiol inhibits calcium induced mitogenesis and that of the calcium-dependent concentration of A23187. On the other

hand testosterone inhibits magnesium-stimulated division and that of the magnesium-dependent concentration of A23187.

The inference in this Unit is that both calcium and magnesium elicit their mitogenic potential via different biochemical pathways, as evidenced by their different susceptibilities to inhibitor compounds which are summarised in Table 2·31. It is also post-

Table 2·31. The Dichotomy Of Action Between Calcium And Magnesium In Eliciting Enhanced Proliferation In Thymocytes

Inhibitor Comound	Calcium-Induced Mitogenesis	Magnesium-Induced Mitogenesis
Oestradiol (other oestrogens)	Inhibited	Not Inhibited
Zinc chloride (10^{-5}M)	Inhibited	Not Inhibited
Calcitonin	Inhibited	Not Inhibited
Imidazole	Inhibited	Not Inhibited
Testosterone (other androgens)	Not Inhibited	Inhibited

ulated that whilst calcium might act through cyclic AMP and cyclic GMP it is unlikely that magnesium does so since it is insensitive to both imidazole and calcitonin, as well as, zinc chloride, all agents which should alter endogenous metabolism and function of these nucleotides. Further aspects of these results will be discussed in the general conclusions at the end of the thesis.

In order to further extend this notion of duality at the cellular level the role of hormones in proliferation was examined in the thymocyte system, this is discussed in the next Unit.

Unit 3. Hormones As Functional Modulators Of Cell Proliferation

3·1 The actions of hormones *in vivo*

For the initiation and maintainance of normal proliferation and differentiation in a number of tissues *in vivo* there is the requirement for the presence of one or more hormones. In the absence of these hormones the tissues and organs either regress or fail to develop normally, because of inadequate levels of proliferation in precursor cell populations. For instance the erythroid series in the bone marrow is depleted in the absence of erythropoietin (Gordon and Zanjani, 1970) the result of inadequate differentiation of the erythropoietin responsive cells to proerythroblasts. Similarly the male accessory sex organs require the continued presence of testosterone for normal levels of proliferationto be maintained (Allison, Appleton, Hanwell and Wright, 1976). Thus in the castrate mouse the prostate, coagulating and preputial glands all become hypoplastic because of reduced proliferation rates of stem cells. In an analagous fashion, the T lymphocyte series and thymocyte population are thought to require the thymic factor, thymosin, for normal function and proliferation (Metcalf, *et al.*, 1961; Levey, *et al.*, 1963). This dependence of some tissues on the presence of hormone activators has been exploited by limiting the output of these agents to specific times in the animals life when there is some requirement for the given tissue to express its differentiated function. Thus mammary development requires both prolactin and progesterone (Chatterton, 1971; Oka and Topper, 1972); these hormones however are only generated in adequate amounts during pregnancy and the immediate post-partum period. Thus mammary gland growth is also restricted to this period, and recession occurs on withdrawal of the hormones after suckling. Similarly uterine development shows a cyclic variation in most

mammalian species (during the oestrous or menstrual cycles) which involves proliferation and differentiation of the endometrium. This complex series of events is coordinated by the proliferative response of these cells to oestradiol (Clegg and Clegg, 1969) which is only produced in critical amounts during key periods in the cycle. There are in addition interactions between other hormones which cause differentiation and subsequently either endometrial regression or stasis if implantation has occured. These examples illustrate the notion of the physiological regulation of cell proliferation as defined in the introduction. Here a proliferogenic hormone concentration is varied either via other integrated hormonal axes, or as in the case of erythropoietin through a product feedback system (the differentiated product in this instance being oxygenation). Another example of a product effect is the role of vitamin D in gastrointestinal epithelial proliferation. In times of hypocalcaemia there are high circulating levels of the vitamin D metabolite 1,25-DHCC, which attempt to increase plasma calcium concentrations by increasing absorption from the gut. This it does by both synthesising calcium transport protein and having a trophic action on epithelial cells, increasing the area for calcium absorption (Urban and Schedl, 1969). Disruption of the insulin/carbohydrate homeostatic system also leads to alterations in the proliferative activity in the epithelium of the small intestine, since hyperplasia of the gut accompanies alloxan-induced diabetes (Jervis and Levin, 1966). Proliferation in the gastrointestinal mucosa is also known to be influenced by cholinergic agents thus representing some neural control of division in this tissue (Tutton, 1973).

In comparison with these specific mitogenic hormones one can contrast the action of apparently non-specific proliferative

hormones. It might be recalled from previous Units that PTH production is required for normal proliferation in several tissues including bone marrow, thymus, lymphoid system, liver and perhaps gut. This action is almost certainly not attributable to a direct action of the hormone but to its ability to modulate plasma calcium concentrations which in turn alters or maintains cell proliferation rates. Along with PTH one might cite the general growth hormone, somatotrophin, which as well as having some well defined sites of action also has a generalised growth promoting activity (Clegg and Clegg, 1969). Thus it is clear that the normal development of a tissue or organ will depend not only upon the presence of specific trophic hormones, but also upon more general, permissive, hormones such as insulin, growth hormone, thyroxine and PTH. This may also be in addition to any endogenous growth modifying agents generated by the tissue in question. It is not surprising to note therefore that few tissues maintained in culture ever exhibit their normal, in vivo, pattern of differentiation (Nevo, Weisman and Sade, 1975) when they are isolated from these potential hormonal influences.

3·2 The mitogenic action of hormones in vitro.

As in the whole animal a large array of hormonal agents are capable of modulating cell proliferation in vitro. However, there is only just beginning to emerge some pattern of action from these studies. The evidence indicates that as in vivo a cell may be exposed to two basically antagonistic classes of effector molecules, namely those hormones which promote differentiation, and those which promote proliferation. In addition it would appear that those agents which increase the differentiated state of a cell tend to elevate intra-

cellular cyclic AMP by stimulating the enzyme adenylate cyclase. Thus prostaglandin E_1, which is a potent cyclase stimulant (Robison et al., 1968), inhibits proliferation in WI 38 cells (Kurtz, Polgar, Taylor and Rutenburg, 1974). Similarly adrenaline causes a reduction in proliferation in skin epidermal cells (Bronstad, Elgjo and Oye, 1971). A number of glial and astrocytoma lines respond to neurotransmitters, and those which increase endogenous cyclic AMP levels tend to reduce division rates whilst inducing characteristics of the differentiated parent cell type (Furmanski, Silverman and Lubin, 1971; Hsie and Puck, 1971; Hsie, Jones and Puck, 1971; Lim and Mitsunobu, 1972). In Cloudman S91 mouse melanoma cells ACTH and MSH not only stimulate adenylate cyclase but also increase melanin output whilst reducing proliferation (Cobb and McGrath, 1974).

These antiproliferative hormones may be contrasted with both specific and non-specific proliferogenic hormones. Thus an ovarian cell line may be stimulated to enhanced division by gonadotrophins (Clark, Jones, Gospodarowicz and Sato, 1972) as they would be *in vivo*. In a non-specific fashion the glucocorticoid hormones cortisol, corticosterone, dexamethasone (Thrash, Ho and Cunningham, 1974) and hydrocortisone (Armelin, 1973) will all release density-inhibited 3T3 cultures. They have similar properties in ovarian cells (Clark et al., 1972) and thymic lymphocyte cultures (Whitfield, MacManus and Rixon, 1970). Insulin is an equally non-specific mitogen stimulating division in 3T3 cells (Temin, 1966; Holley and Kiernan, 1974) an ovarian cell line (Gospodarowicz and Moran, 1974) a plasma cell tumour line (Naseem et al., 1973) smooth muscle cells (Stout et al., 1975) and thymocytes (Morgan and Perris, 1975). In these respects these hormones appear to mimic the action of serum in defining the cell density at which confluency occurs, and in releasing

contact-inhibited cells. Indeed it is now becoming established that a number of peptide and polypeptide factors in the serum of normal animals will stimulate cell proliferation in vitro (Holley, 1974). Whilst undoubtedly some of these reports will be attributable to insulin contamination it is also clear that some of these serum growth factors are devoid of insulin-like activity (Temin, 1971; Uthne, 1973; Antoniades, Stathakos and Scher, 1975). Another series of serum components which stimulate cartilage cell proliferation, the somatomedins, have also been characterised as lacking insulin activity (Van Wyk, Underwood, Hintz, Voina and Weaver, 1974). Further evidence also suggested that serum from hypophysectomised animals possessed less fibroblast stimulating activity than did normal serum (Holley and Kiernan, 1968). Thus the search began for pituitary factors which regulated fibroblast division rates. A number of groups have now identified pituitary fibroblast growth factors which stimulate division in both 3T3 and ovarian cell lines (Armelin, 1973; Armelin and Sato, 1973; Gospodarowicz, 1974, 1975a; Jones et al., 1975). Indeed the serum factor of Antoniades et al. (1975) is claimed to be identical to the pituitary growth factor of Gospodarowicz (1974). These hormone-like agents are also claimed to be distinct from any known hypothalamic hormone (Armelin, 1973). Thus the possibility exists that that the pituitary and hypothalamus play a central role in the maintainance of cell proliferation and differentiation by the production of mitogenic hormones specific for given cell types. Recently two new growth factors have been identified which promote division in brain (Gospodarowicz, 1975b) and liver (Nadal and Boffa, 1975) which adds further weight to this notion of proliferogenic hormones.

Of some interest also would be the way (if any) in which the state of proliferation of say a fibroblast is related to hypothalamic hormone secretion. There may indeed be discrete feedback loops utilizing fibroblast-specific products as effector molecules. On the other hand output may be constant and it is the interaction of secondary effector substances with the target cell which define the level of cell division. In either case it is likely that many more hormones have yet to be identified or implicated in the control of cell proliferation and differentiation.

Thus we have the picture of growth promoting hormones from the hypothalamus interacting with permissive hormones such as insulin, the glucocorticoids, PTH, somatotrophin, and thyroxine to set proliferation rates. There will then be a second axis in which the differentiating hormones specific for that cell type will be titrated against the proliferogenic hormones to balance the functional output of that cell clone. Indeed it might be that it is the differentiational hormones which regulate growth factor secretion. However this notion does augur the presence of at least two interacting axes within the cell; one for proliferation and one for differentiation. In view of the implication of cyclic AMP with differentiation in many cells it might be envisaged that it is the metabolism of cyclic AMP and GMP which represent these two functions at the level of the cell.

3·3 The action of hormones on thymocyte proliferation *in vitro*

The thymocyte model as described in this thesis is remarkably sensitive to a variety of hormonal mitogens, which are listed in Table 3·3a. To these must be added the inhibitory steroids,

Table 3·3a. The Stimulatory Action Of Hormones Upon In Vitro Thymocyte Proliferation.

Hormone	Reference
Cortisol	Whitfield, MacManus and Rixon, 1973.
Bradykinin	Perris and Whitfield, 1969.
Adrenalin	MacManus, Whitfield and Youdale, 1971.
Growth hormone	Whitfield, Perris and Youdale, 1969.
Oxytocin	" " " " "
Prolactin	" " " " "
Parathyroid hormone	" " " " "
Prostaglandin E_1	Whitfield, MacManus, Braceland and Gillan, 1972.
Antidiuretic hormone	Whitfield, Perris and Youdale, 1969.
Calcitonin	Whitfield, MacManus, Franks, Braceland and Gillan, 1972.

oestradiol and testosterone described in Unit 2 with their related derivatives. Although there appears to be no clear pattern in these hormonal mitogens it might be noted that all except adrenalin and prostaglandin E_1 require extracellular calcium ions for their mitogenic capacity to be manifest. Thus considering the clear dichotomy between the action of calcium and magnesium and the requirement shown by these mitogens for calcium the possibility existed that calcium played some key role in the activation process. If this part was similar to that it played when added itself at mitogenic concentrations then these calcium-dependent hormones might also be susceptible to blockade with agents such as oestradiol and imidazole. Thus one extension of this work was to establish the ionic requirements and steroid sensitivity of a range of hormones when added to thymocyte

cultures.

Secondly since most of the hormones listed in Table 3·3a are polypeptides there are no clear receptor antagonists to decide if the action is attributable to some surfactant effect. Therefore it was decided to identify other mitogenic hormones which possess known pharmacological blocking agents, to establish the specificity of the cell/hormone interaction.

3·4·1 Histamine as a mitogen for thymic lymphocytes in culture.

An important component of the immune response is the release of various local tissue hormones and lymphokines, particularly histamine at the site of the reaction (Rocha e Silva and Aronson, 1952; Lichtenstein and Osler, 1964). The increased vascular permeability which ensues may facilitate the access of a variety of leucocytes to the site of an antigenic infringement (Boughton and Spector, 1963; Leonard and McHutchinson, 1964; Elves, 1972).

The demonstration that another vasoactive agent, bradykinin, can stimulate DNA synthesis and division in thymocyte suspensions (Perris and Whitfield, 1969) suggested that histamine might also possess this property. This possibility was tested in the standard thymic lymphocyte culture system described in Unit 1, using both colchicine metaphase accumulations and autoradiography as indices of proliferative activity.

When thymocytes were incubated in Medium 199 containing low (0·6mM) calcium chloride concentrations, some 3·5% of the cells

were in metaphase after a six hour incubation period (Figure 3·4·1a). However if the medium contained histamine at concentrations ranging between 10^{-9} to 10^{-15}M then there was a significant increase in the number of cells present in mitosis (Figure 3·4·1a).

Figure 3·4·1a. The Calcium-Dependent Stimulation Of Thymocyte Mitosis By Histamine.

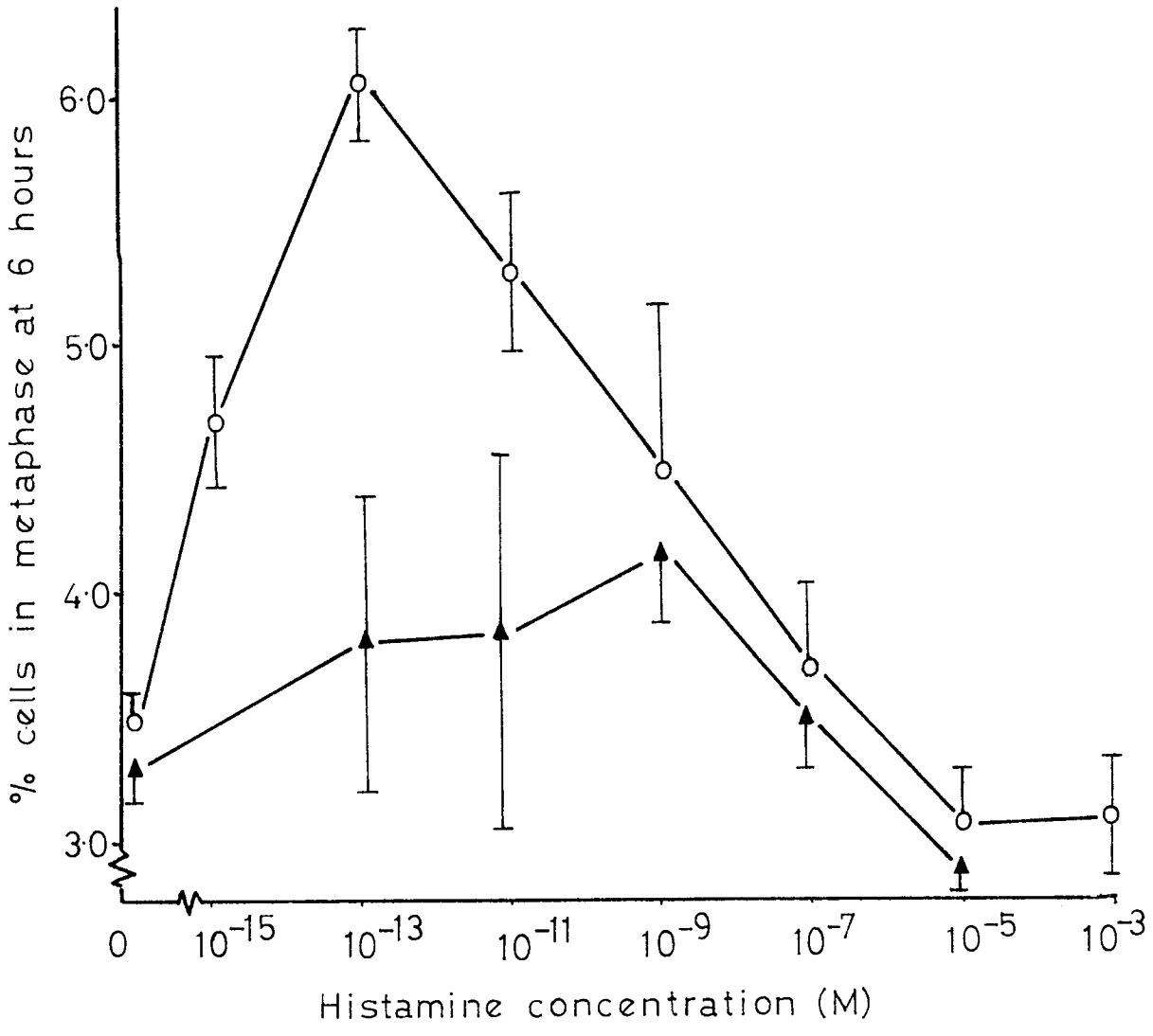


Figure 3·4·1a. The calcium-dependent stimulation of thymocyte mitosis by histamine. Cells were incubated in Medium 199 containing colchicine and either 0·0mM (▲) or 0·6mM (O) calcium chloride. The thymocytes were also treated with histamine (Sigma Chemical Co.) at concentrations between 10^{-15} to 10^{-3}M and six hour accumulations performed. The results are the mean ± 1 S.E.M. of between 4 and 13 separate determinations. Values obtained in 0·6mM calcium with histamine are significantly higher than the histamine-free culture at 10^{-15}- 10^{-11} ($p < 0·001$) and 10^{-9}M ($p < 0·02$). No other results show statistical significance with a t test or F test.

Figure 3·4·1b. The Influence Of Histamine And Oestradiol On Thymic Lymphocyte DNA Synthesis.

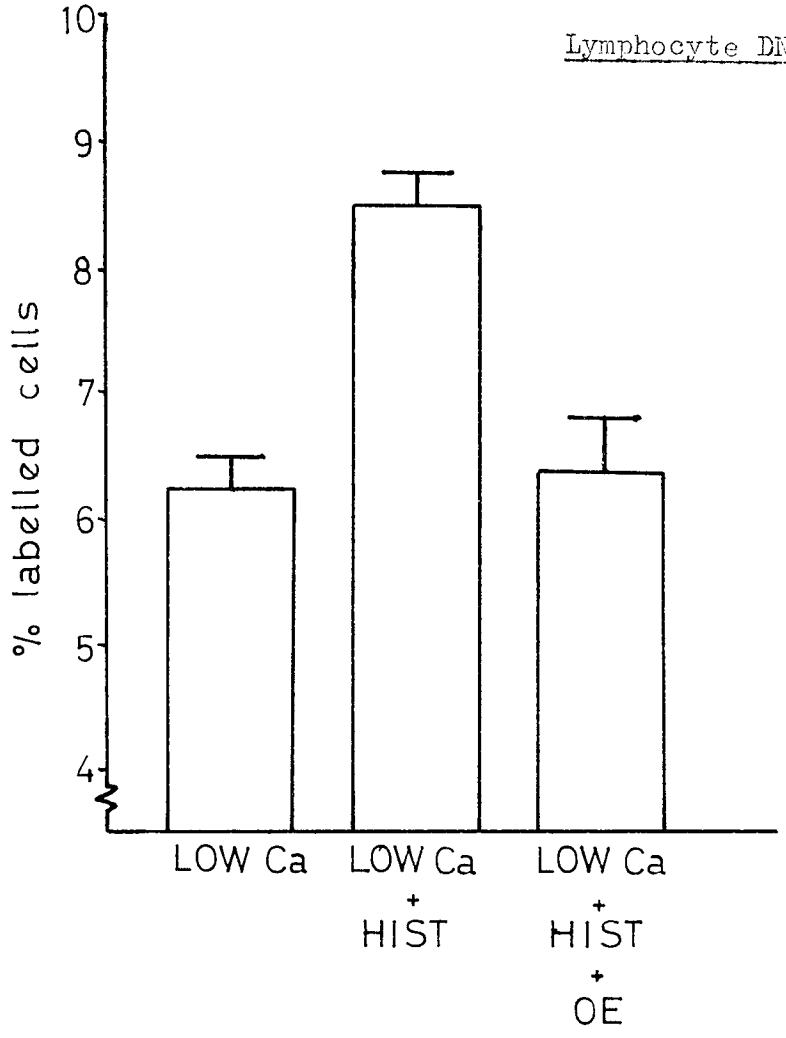


Figure 3·4·1b. The interaction between histamine and oestradiol upon thymocyte DNA synthesis *in vitro*. Thymic lymphocytes were cultured in Medium 199 containing 0·6mM calcium (LOW Ca) and without colchicine. After 30 minutes incubation the cultures were pulsed with tritiated thymidine ($5\mu Ci.ml^{-1}$) and histamine (HIST) at $10^{-13}M$ both in the presence and absence of oestradiol-17β (OE) at a final concentration of $0·1\mu g.ml^{-1}$. One hour later cultures were fixed and prepared as autoradiograms. Results are means (columns) and S.E.M. of between 4 and 9 separate determinations.

This increment in mitotic activity was probably derived from a cohort of cells undergoing unscheduled entry into their DNA synthetic phase. For within one hour of exposure to these mitogenic levels of histamine there was a significant increase in ($p < 0·001$) the number of lymphocytes actively encaged in DNA synthesis (Figure 3·4·1b). A more extensive and definitive demonstration of histamine recruitment of G_o thymocytes was shown in Unit 1.

Table 3·4·1c. The Role Of Ions And Steroids In the Mitogenic Action Of Histamine.

Ionic Or Steroid Status Of Medium.	No Additions	Histamine 10^{-12}M.
0·6mM Ca^{++} 1·0mM Mg^{++}	3·4 ± 0·12%	6·1 ± 0·18% *
0·0mM Ca^{++} 1·0mM Mg^{++}	3·6 ± 0·30%	3·8 ± 0·60%
0·6mM Ca^{++} 1·0mM Mg^{++} + Oestradiol	3·7 ± 0·30%	3·2 ± 0·34%
0·6mM Ca^{++} 0·0mM Mg^{++}	3·7 ± 0·20%	5·9 ± 0·34% *
0·6mM Ca^{++} 1·0mM Mg^{++} + Testosterone	3·5 ± 0·20%	6·9 ± 0·38% *

Table 3·4·1c. The ionic dependence and steroid sensitivity of histamine-induced mitogenesis. Cells were incubated in Medium 199 which contained colchicine and either lacked calcium or magnesium or was supplemented with oestradiol-17β (0·1μg.ml^{-1}) or testosterone (0·1μg.ml^{-1}). Results represent the mean percentage number of cells in colchicine metaphase at six hours ± 1 S.E.M. of between 4 and 22 separate determinations. Where values are significantly higher ($p<0·001$) than the normal basal medium without steroid or histamine addition (0·6mM Ca, 1·0mM Mg) this is indicated by an asterisk (*).

When cells were cultured in the absence of calcium the mitogenic action of histamine was severely muted or ablated (Figures 3·4·1a, c). Thus it is probable that histamine is yet another example of a calcium-dependent hormonal mitogen, comparable with those listed in Table 3·3a. In the few cases where inhibition was incomplete this could be attributable to residual calcium carried over in the cell's glycocalyx and membrane. As with raised calcium itself, the simultaneous presence of oestradiol (0·1μg.ml^{-1}) abolished the mitogenic response to histamine (Figure 3·4·1c) . However neither magnesium omission nor testosterone addition had any effect on histamine-induced proliferative changes (Table 3·4·1c). Furthermore oestradiol

also blocked the histamine-induced increment in labelled cells (Figure 3·4·1b) indicating that it was acting on the recruitment phenomenon and not on subsequent cell cycle compartments.

Thus the important feature has been established that oestradiol has a similar blocking action on both calcium and calcium-

Figure 3·4·1d. The Interaction Of Antihistamines And Imidazole With Histamine-Induced Proliferation.

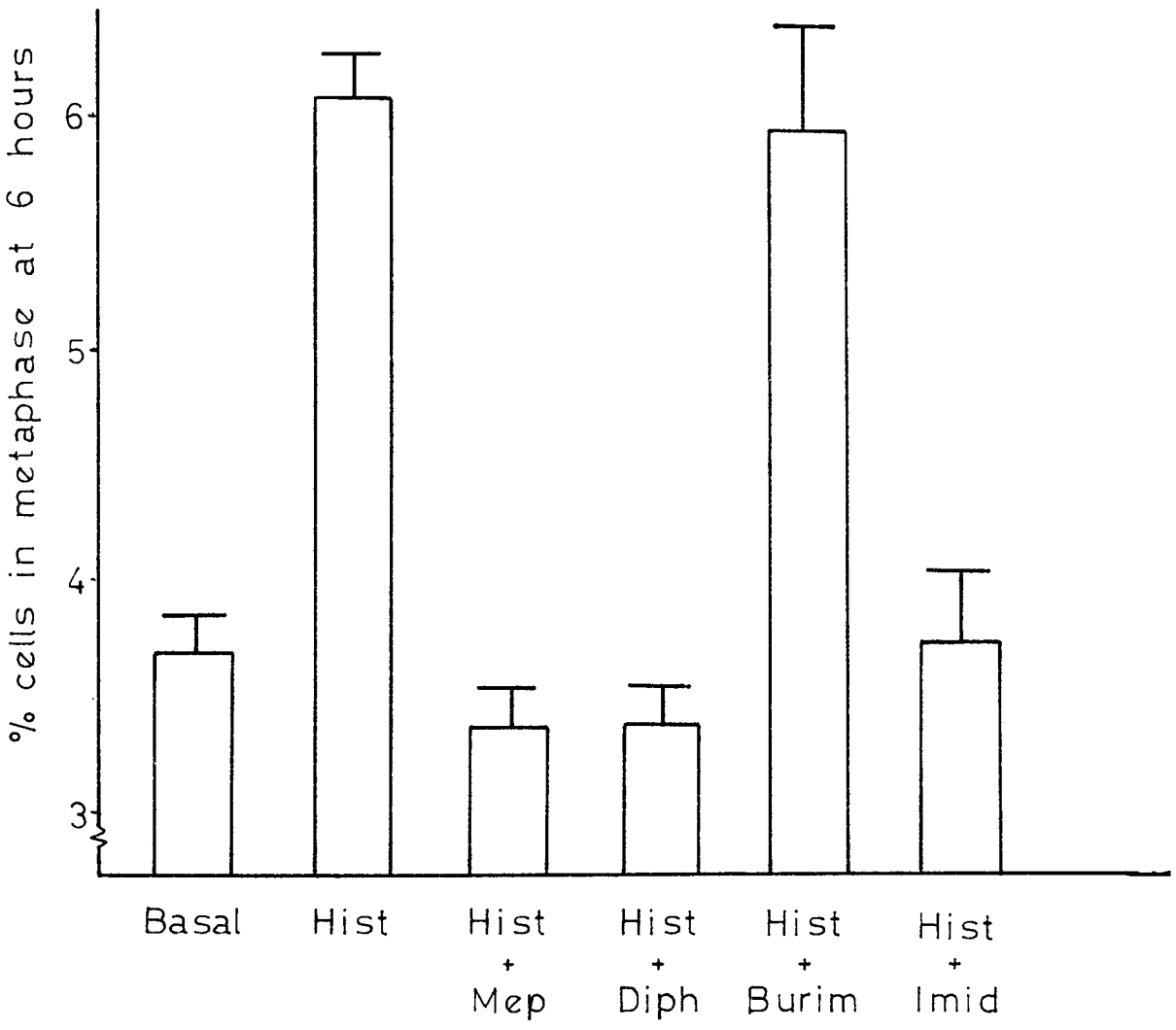


Figure 3·4·1d. The interaction of antihistamines and imidazole with histamine-induced mitogenesis in isolated rat thymic lymphocyte cultures. Cells were maintained in Medium 199 containing colchicine and 0·6mM calcium (Basal). Other cultures were exposed to histamine (Sigma Chemical Co.) at a concentration of 10^{-12}M. These stimulated suspensions also contained mepyramine (Mep) maleate (May and Baker Ltd.) at 10^{-12}M; (Diph) diphenhydramine (Sigma Chemical Co.) at 10^{-12}M; (Burim) burimamide (Dr. W.A.H. Duncan, Smith Klein and French Ltd.) at 10^{-11}M; and imidazole (Imid) at 4mM final concentration. Cont.

Figure 3•4•1d. continued.
Six hour metaphase accumulations were scored and the results represent the mean (column) and 1 S.E.M. of between 4 and 22 separate experiments. It might also be noted that burimamide was tested over a concentration range of 10^{-13} to 10^{-5}M however no effect upon histamine-stimulated proliferation was observed at any concentration. The agent also became somewhat lymphocytolytic at 10^{-5}M, a recommended dose for the blockade of H_2 receptors (W.A.H. Duncan, personal communication).

dependent mitogens. In contrast neither magnesium nor testosterone had any effect on these agents. This correlation was extended by the observation that imidazole also blocked histamine-induced mitogenesis (Figure 3•4•1d), it should be remembered that imidazole will block calcium- but not magnesium-stimulated division (Unit 2).

An important feature of testing the action of histamine as a mitogen is that it possesses a well characterised receptor system at the cell membrane (Melville, 1973). There are indeed two receptor types known for histamine the first being the so-called H_1 receptor, and is antagonised by a variety of antihistamines including mepyramine maleate and diphenhydramine (Melville, 1973). The second is the H_2 receptor (Ash and Schild, 1966; Black, Duncan, Durant, Ganelin and Parsons, 1972) which is blocked by burimamide and metiamide. In the thymocyte mepyramine and diphenhydramine blocked the histamine response whilst burimamide was without effect at any concentration tested down to 10^{-5}M. Thus it must be concluded that histamine is acting via an H_1 receptor (Figure 3•4•1d). It might also be noted that the histamine-induced contraction of the guinea pig ileum is also mediated via an H_1 receptor, and is blocked by both mepyramine and diphenhydramine. Rather interestingly this response may be blocked (or greatly reduced in magnitude) by the addition of oestradiol to the gut bath (unpublished observations). Thus there may be a much more general interaction of oestradiol with calcium-

dependent hormone/cell interactions than are evident from the results obtained from these proliferation studies.

Since histamine requires calcium for activity it could be that part of its mechanism involves the transport of this cation across the cell membrane, and the antihistamine might simply impair this process. However, when cells were stimulated with calcium or PTH, another calcium-dependent mitogen (Whitfield, Perris and Youdale, 1969), addition of either of the antihistamines had no muting effect (Table 3·4·1e). This would indicate that these agents do not antagonise calcium transport per se.

Table 3·4·1e. The Lack Of Effect Of Antihistamines On Calcium- And Parathyroid Hormone-Induced Mitogenesis.

Treatment	Mean percentage cells in colchcine metaphase at six hours ± 1 S.E.M.	n	p*
Low (0·6mM) Ca^{++}	3·4 ± 0·16%	8	
High (1·2mM) Ca^{++}	6·0 ± 0·29%	5	<0·001
High Ca^{++} + 10^{-13}M Diphenhydramine.	6·3 ± 0·50%	4	<0·001
High Ca^{++} + 10^{-12}M Mepyramine maleate	5·8 ± 0·09%	4	<0·001
Low Ca^{++} + PTH (5 MRC Units.ml^{-1})	6·4 ± 0·28%	6	<0·001
Low Ca^{++} + PTH (5 MRC Units.ml^{-1}) + 10^{-13}M Diphenhydramine	5·8 ± 0·25%	4	<0·001

Table 3·4·1e. The lack of effect of antihistamines on calcium- and PTH-induced mitogenesis in isolated thymic lymphocyte cultures. The cells were suspended in BGS containing colchicine and either low (0·6mM) or high (1·2mM) calcium. Low calcium-containing cultures were also exposed to PTH (Eli Lilly) at a final concentration of 5 MRC Units.ml^{-1}. Both calcium- and PTH-stimulated cultures were also treated with the antihistamines mepyramine maleate and diphenhydramine at the concentrations indicated. 'p' values were obtained by the application of a student's t test comparing the relevant result with that for low calcium in all instances. 'n' is the number of individual determinations made.

In order to preclude the unlikely possibility that the antihistamines were associating with histamine or that they were contaminated with antimitotic compounds such as imidazole ascending paper chromatography was employed. Thus histamine both in the presence of diphenhydramine and absence was run on Whatman number 4 paper in either an n-butanol: acetic acid: water solvent system (12:3:5) or an ethanol: water: ammonia system (18:1:1) as described by Smith (1969). The chromatograms were air dried for ten minutes and stained with Pauly's reagent (Smith, 1969) which comprised:-

Sulphanilic acid (9g) dissolved in 90ml concentrated HCl and 900ml water. 1 Volume.

5% aqueous sodium nitrite. 1 Volume.

10% aqueous sodium carbonate. 2 Volumes.

Using the first solvent system there was no difference in Rf values between histamine (0·18) and histamine in the presence of diphenhydramine. In the second system also these was no significant difference in Rf with values of 0·64 (histamine) and 0·65 (histamine in the presence of diphenhydramine) being obtained. In addition it was not possible to detect the likely impurities of imidazole and histidine in either the antihistamines or histamine preparation itself.

It has also been reported that the H_2 receptor is linked to a membrane-bound adenylate cyclase in the gastric mucosa (Douse and Code, 1974). In contrast the H_1 receptor has been implicated with the metabolism of the guanylate nucleotides, notably cyclic GMP (Schultz, Hardman, Schultz et al., 1972; Goldberg et al., 1973).

Such observations would tend to support the notion that calcium, and calcium-dependent mitogens are involved with cyclic GMP metabolism, and might not act via adenylate cyclase.

3·4·2. The role of parathyroid hormone as a calcium-dependent mitogen *in vitro*.

Although PTH has been established as an *in vitro* mitogen for thymocytes it was necessary to reestablish this point for several reasons. Firstly it was important to ascertain if PTH has the same steroid sensitivity as histamine and calcium. Secondly all the early mitogenic reports for this hormone employed an impure commercial preparation and a highly purified MRC research standard PTH was now available, thus it seemed germane to assess the proliferative activity of this preparation.

PTH has already been demonstrated to stimulate mitosis in thymocyte cultures in a calcium-dependent fashion (Whitfield, Perris and Youdale, 1969) and does so by elevating intracellular cyclic AMP concentrations (Whitfield, MacManus and Rixon, 1970; Whitfield, MacManus, Youdale and Franks, 1971). However it has not proved possible to stimulate thymocyte adenylate cyclase with PTH (Whitfield, MacManus, Youdale and Franks, 1971; MacManus, Youdale, Whitfield and Franks, 1972). Rather it has been suggested that PTH provokes an influx of calcium into the thymocyte (Borle, 1967; Rasmussen, 1971) which subsequently inhibits cyclic nucleotide phosphodiesterase (MacManus and Whitfield, 1971; Whitfield, MacManus, Youdale and Franks, 1971) thus resulting in the elevation of endogenous cyclic AMP concentrations.

Figure 3·4·2a. The Influence Of PTH And Oestradiol Upon Thymocyte Proliferation.

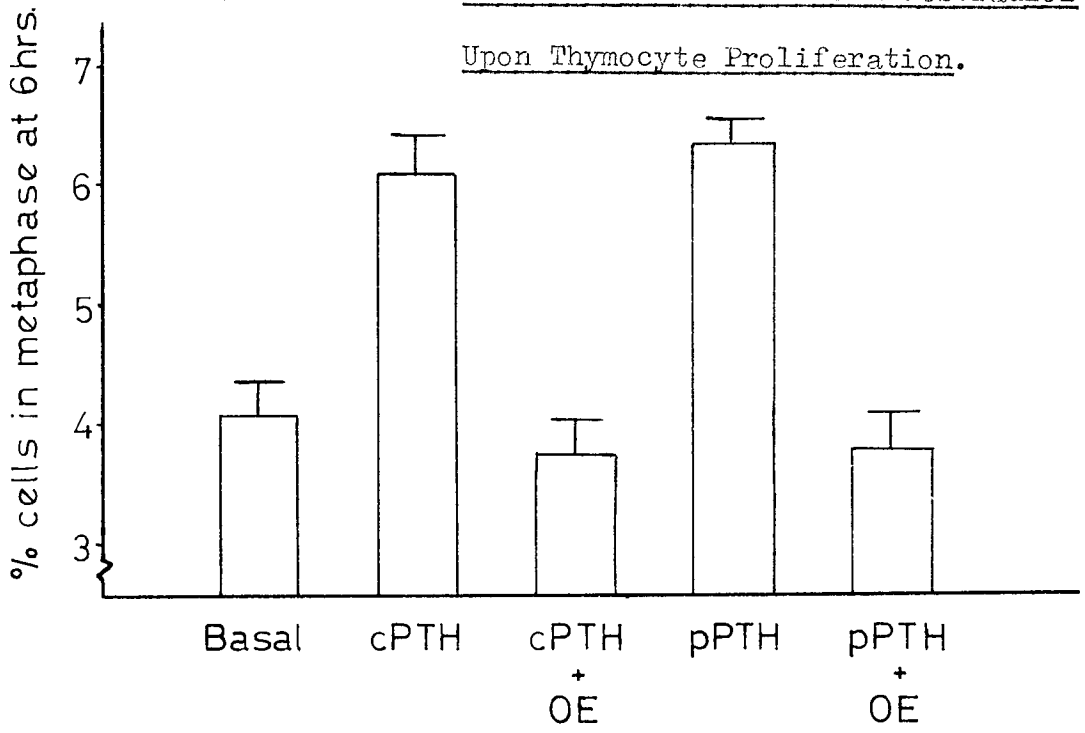


Figure 3·4·2a. The interaction between PTH and oestradiol on mitotic activity in thymic lymphocyte cultures. Thymocytes were cultured in Medium 199 containing colchicine and 0·6mM calcium chloride (Basal). Suspensions were also treated with PTH and oestradiol (OE) at a final concentration of 0·1μg.ml^{-1}. The PTH used came from two sources, the first, a relatively crude industrial preparation (cPTH) was obtained from Eli Lilly and Co. and was diluted with isotonic saline to give a final concentration of 5MRC units.ml^{-1}in the culture. The second was a highly purified preparation from the MRC Standards Institute (pPTH), the generous gift of Dr. J.A. Parsons. Batch number 72/286. This was dissolved as a stock concentrate in a solution comprising 1% sodium acetate, 0·5% L-cysteine hydrochloride and 0·1% crystalline bovine serum albumin, which had been previously heated to 56°C to inactivate peptidases (Parsons and Potts, 1972). This again yielded 5MRC units.ml^{-1}in the cultures. The vehicle had no effect on basal or stimulated mitotic activity. The results represent the mean percentage cells in metaphase at six hours (columns) + 1 S.E.M. of between 4 and 12 separate experiments.

Figure 3·4·2a demonstrates that both a crude PTH preparation obtained from Eli Lilly and Co. and a highly purified PTH from the Medical Research Council Standards Institute (batch number 72/286) stimulated thymocyte proliferation at a concentration of 5MRC units per millilitre of culture. Furthermore this purified preparation also enhanced the number of cells actively engaged in DNA synthesis within one hour of its addition to the suspension (Figure 3·4·2b).

Figure 3·4·2b. The Influence Of PTH And Oestradiol On Thymocyte DNA Synthesis.

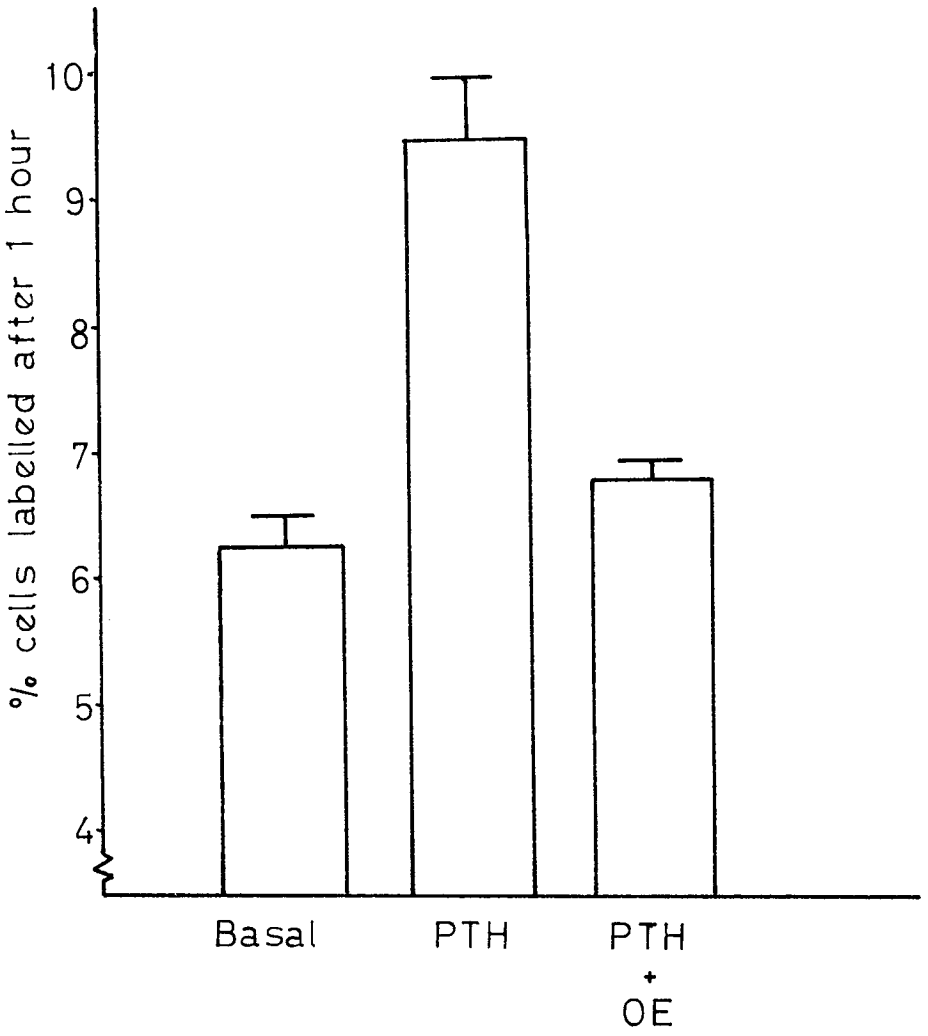


Figure 3·4·2b. The action of PTH and oestradiol on thymic lymphocyte DNA synthesis. Cells were precultured for 30 minutes in Medium 199 containing 0·6mM calcium chloride (Basal). After this time all tubes received 5μCi.ml^{-1} tritiated thymidine and combinations of oestradiol (0·1μg.ml^{-1}) and highly purified PTH (5MRC units.ml^{-1}) dissolved as described in Figure legend 3·4·2a. One hour later cells were fixed and prepared as autoradiograms. Each result is the mean percentage labelled cells (columns) + 1 S.E.M. of between 4 and 8 separate determinations.

Thus it would appear that PTH also elicits a G_o recruitment phenomenon in a fashion similar to both calcium and histamine.

As noted by other workers (Whitfield, Perris and Youdale, 1969) PTH does not stimulate mitosis if calcium is absent from the

Table 3•4•2c. The Role Of Ions And Steroids In The Mitogenic Action Of PTH.

Ionic Or Steroid Status Of Medium	No Additions	PTH 5MRC units.ml^{-1}
0•6mM Ca^{++} 1•0mM Mg^{++}	3•4 ± 0•12%	6•0 ± 0•25% *
0•0mM Ca^{++} 1•0mM Mg^{++}	3•6 ± 0•30%	3•4 ± 0•44%
0•6mM Ca^{++} 1•0mM Mg^{++} + Oestradiol	3•7 ± 0•30%	3•7 ± 0•19%
0•6mM Ca^{++} 0•0mM Mg^{++}	3•7 ± 0•20%	5•5 ± 0•36% *
0•6mM Ca^{++} 1•0mM Mg^{++} + Testosterone	3•5 ± 0•20%	6•0 ± 0•33% *

Table 3•4•2c. The ionic dependence and steroid sensitivity of PTH-induced mitogenesis. Cells were incubated in Medium 199 which contained colchicine and either lacked calcium or magnesium or was supplemented with oestradiol (0•1μg.ml^{-1}) or testosterone (0•1μg.ml^{-1}). Cultures were also exposed to a highly purified PTH preparation at a final concentration of 5MRC units.ml^{-1}. Results are the mean percentage cells in colchicine metaphase at six hours ± 1 S.E.M. of between 4 and 22 separate experiments. Where values differ significantly ($p<0•001$) from the basal medium containing no steroid or PTH this is indicated by an asterisk (*).

medium (Table 3•4•2c). In addition as with both histamine and calcium oestradiol blocks both the mitotic (Figure 3•4•2a, Table 3•4•2c) and DNA synthetic (Figure 3•4•2b) responses to mitogenic levels of PTH. Neither the omission of magnesium from the medium nor the addition of testosterone had any effect on PTH-induced mitogenesis (Table 3•4.2c). The highly significant increase ($p<0•001$) provoked by PTH in mitotic activity (3•37 ± 0•15% to 6•1 ± 0•13%) was not affected by the beta blocker propranolol (10^{-5}M) or the H_1 antagonist diphenhydramine (10^{-13}M). The six hour accumulations in the presence of PTH and these receptor antagonists being 6•4 ± 0•33% and 6•1 ± 0•43% respectively. This would indicate that there is some degree of

specificity in the interaction of PTH with the thymocyte. There is of course no known receptor antagonist of PTH.

3·4·3 Insulin stimulation of thymocyte proliferation.

It was noted in section 3·2 that insulin is a potent mitogen *in vitro*, thus it was tested for activity in this system also. Insulin does indeed stimulate thymocyte mitotic activity at concentrations between 25 and 500μUnits.ml^{-1} (Figure 3·4·3a). These values encompass the normal range for adult rat plasma immunoreactive insulin levels (Dr. C.J. Bailey, personal communication; Smith and Morgan unpublished data). Like histamine and PTH this mitogen has a requirement for

Figure 3·4·3a. The Calcium-Dependent Mitogenic Action Of Insulin.

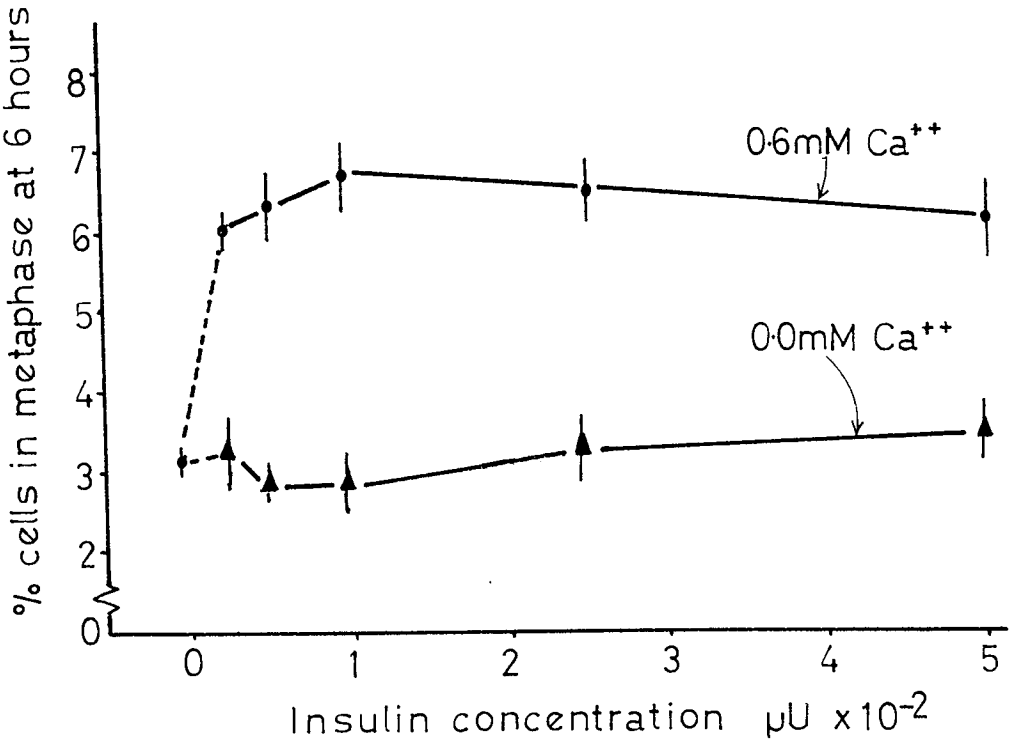


Figure 3·4·3a. The calcium-dependent mitogenic action of insulin. The thymocytes were suspended in Medium 199 containing colchicine and 0·6mM calcium chloride. Cells were also exposed to insulin (Sigma Chemical Co.) at various concentrations. This was dissolved in unbuffered medium. Results are mean ± 1 S.E.M. of 4 to 12 experiments.

Table 3·4·3b. The Role Of Ions And Steroids In The Mitogenic Action Of Insulin.

Ionic Or Steroid Status Of Medium	No Additions	Insulin 100μU.ml^{-1}
0·6mM Ca^{++} 1·0mM Mg^{++}	3·4 ± 0·12%	6·3 ± 0·30% *
0·0mM Ca^{++} 1·0mM Mg^{++}	3·6 ± 0·30%	2·8 ± 0·26%
0·6mM Ca^{++} 1·0mM Mg^{++} + Oestradiol	3·7 ± 0·30%	3·5 ± 0·18%
0·6mM Ca^{++} 0·0mM Mg^{++}	3·7 ± 0·20%	5·9 ± 0·41% *
0·6mM Ca^{++} 1·0mM Mg^{++} + Testosterone	3·5 ± 0·20%	6·1 ± 0·29% *

Table 3·4·3b. The ionic dependence and steroid sensitivity of insulin-induced mitogenesis. Cells were cultured as described in Table 3·4·2c. Insulin (Sigma Chemical Co.) was added to give a final concentration of 100μU.ml^{-1}. The agent was dissolved in unbuffered medium at acid pH. Values are the mean ± 1 S.E.M. of between 4 and 22 separate experiments. An asterisk (*) indicates that a value is significantly higher than ($p<0·001$) the basal six hour accumulation in medium containing no steroid or insulin.

extracellular calcium ions, and is similarly inhibited by the simultaneous presence of oestradiol (Figure 3·4·3a, Table 3·4·3b). Magnesium depletion and testosterone addition have no discernible effect on the insulin-induced mitotic increment (Table 3·4·3b).

Imidazole (6mM) did block insulin-induced mitogenesis perhaps implicating the cyclic nucleotides in the action of this hormonal mitogen (Table 3·4·3c). Additionally insulin was not impaired by the inappropriate receptor antagonist propranolol (10^{-5}M) indicating specificity of reaction (Table 3·4·3c). Thus since insulin

Table 3·4·3c. The Influence Of Imidazole And Propranolol On Insulin-Induced Mitogenesis.

Treatment	Percentage Cells In Metaphase At 6 Hours + 1 S.E.M.
No Additions	3·4 ± 0·11%
Propranolol (10^{-5}M)	2·8 ± 0·25%
Insulin (100μU.ml^{-1})	6·0 ± 0·13% *
Insulin (100μU.ml^{-1}) + Propranolol (10^{-5}M)	6·1 ± 0·26% *
Insulin (100μU.ml^{-1}) + Imidazole (6mM)	3·7 ± 0·34%

Table 3·4·3c. The influence of imidazole and propranolol on insulin-induced mitogenesis. Thymocytes were maintained in Medium 199 which contained colchicine and 0·6mM calcium chloride. The cultures were subsequently treated with combinations of Insulin (Sigma Chemical Co.), propranolol ("Inderal", I.C.I. Ltd.) or imidazole (Sigma Chemical Co.) at the concentrations indicated. The results represent the mean percentage cells in metaphase at six hours ± 1 S.E.M. of between 4 and 8 separate experiments. Where a result is significantly higher ($p < 0·001$) than the basal control (No Additions) this is indicated by an asterisk (*).

acts at a membrane receptor (Gavin, Gordon, Roth, Buell and Archer, 1973) and elevates lymphocyte cyclic GMP levels (Illiano et al., 1973) it is tempting to speculate that calcium provides some link between the surface receptor and guanylate cyclase. Certainly the observation that imidazole blocks the mitogenic action of insulin would implicate cyclic nucleotides in the chain of events culminating in mitosis. However it should also be noted that there is evidence which suggests that imidazole may influence calcium transport directly in isolated sarcoplasmic reticulum vesicles. The compound does in fact inhibit the (Ca-K)-ATPase of these organelles (Dr. P. Barford, personal communication). Thus although imidazole certainly does potentiate cyclic nucleotide phosphodiesterase (Robison et al.,

1968) thus reducing endogenous cyclic purine concentrations, it must be borne in mind that active calcium extrusion from both mitochondria and the cell as a whole may also be affected.

3·4·4 The biphasic stimulation of thymocyte mitotic activity by acetylcholine.

When thymic lymphocytes were incubated with acetylcholine at concentrations ranging between 10^{-13} to 10^{-4}M it was noted that the agent stimulated division at two distinct concentrations (Figure 3·4·4a). It was also noted when performing these experiments that the position of these peaks, especially at the low concentration, varied over a concentration range of up to one hundred fold. It was thought at this time that this could be a difference in the rate of degradation of this neurotransmitter by acetylcholinesterase. Indeed analysis of a crude soluble extract of thymocytes did reveal an appreciable cholinesterase activity as detected using the Boehringer standard assay for this enzyme (data not presented). This notion was reinforced by the observation that carbamylcholine, an acetylcholinesterase-resistant analogue of acetylcholine (Bowman et al., 1970) showed optimal stimulatory activity at a much lower concentration than did acetylcholine itself (Figure 3·4·4a). Furthermore if cells were exposed to acetylcholine plus the cholinesterase inhibitor eserine (Bowman et al., 1970) the mitotic peak was again shifted down to levels comparable with carbamylcholine (Figure 3·4·4a). There would seem little doubt therefore that the preparation does contain cholinesterase activity and this does affect the reproducibility of the results.

Figure 3·4·4a. The Mitogenic Potential Of Acetylcholine And The Influence Of Acetylcholinesterase.

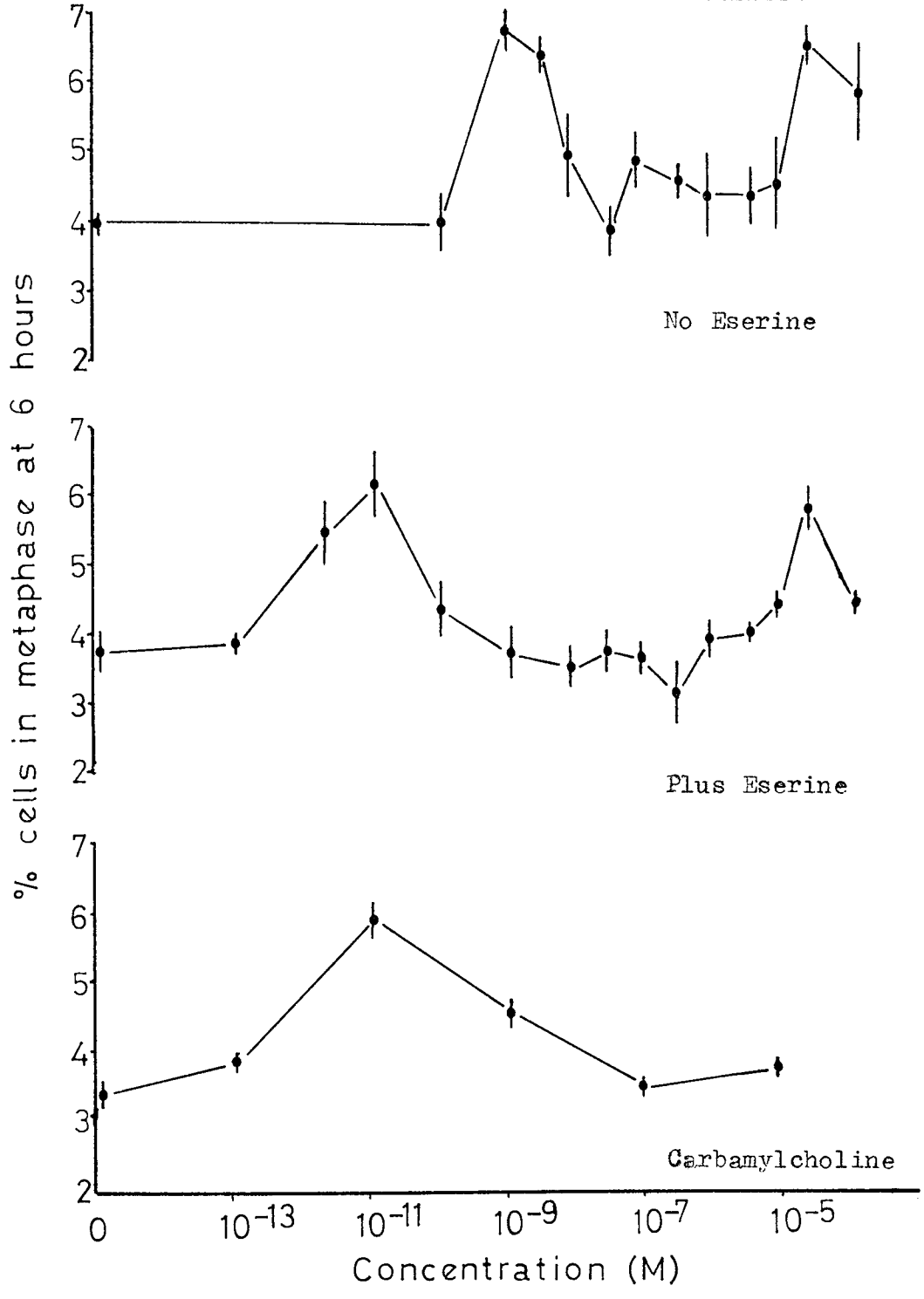


Figure 3·4·4a. The influence of acetylcholine, carbamylcholine and eserine upon thymocyte proliferation. Cells were incubated in Medium 199 containing colchicine and 0·6mM calcium chloride. They were then exposed to various concentrations of carbamylcholine (BDH Ltd.) and acetylcholine either in the presence or absence of eserine (both BDH Ltd.). Results represent the mean percentage cells in metaphase at six hours ± 1 S.E.M. of between 4 and 17 separate experiments.

The biphasic mitogenic action of acetylcholine on thymic lymphocytes is somewhat reminiscent of the pattern of stimulation observed with the calcium ionophore, A23187 (Unit 2). It was established that the two mitogenic peaks attributable to A23187 were dependent upon the presence of either calcium or magnesium, and were selectively inhibited by either oestradiol or testosterone addition to the cultures. Thus the mitogenic requirements of these two stimulatory concentrations of acetylcholine were similarly investigated. In contrast to the

Table 3·4·4b. The Ionic Dependence And Steroid Sensitivity Of Acetylcholine-Induced Mitogenesis.

Ionic Or Steroid Status Of Medium.	No Additions	5×10^{-5}M Ach.	10^{-12}M Ach.
0·6mM Ca^{++} 1·0mM Mg^{++}	3·4 ± 0·12%	6·2 ± 0·45% *	6·0 ± 0·52% *
0·0mM Ca^{++} 1·0mM Mg^{++}	3·6 ± 0·30%	6·8 ± 0·75% *	2·9 ± 0·24%
0·6mM Ca^{++} 1·0mM Mg^{++} + Oestradiol	3·7 ± 0·30%	5·4 ± 0·12% *	2·9 ± 0·21%
0·6mM Ca^{++} 0·0mM Mg^{++}	3·7 ± 0·20%	3·0 ± 0·17%	5·7 ± 0·34% *
0·6mM Ca^{++} 1·0mM Mg^{++} + Testosterone	3·5 ± 0·20%	3·1 ± 0·31%	6·5 ± 0·39% *

Table 3·4·4b. The ionic dependence and steroid sensitivity of acetylcholine-induced mitogenesis. Thymocytes were maintained in Medium 199 containing colchicine and the various combinations of divalent cations and steroids shown in the table. Cells were also exposed to acetylcholine at the concentrations indicated, all cultures containing the anticholinesterase agent eserine (10^{-9}M). Results are the mean percentage cells in metaphase at six hours ± 1 S.E.M. of between 4 and 22 separate experiments. Where a value is significantly higher than the basal control containing no acetylcholine or steroid ($p < 0·001$) this is indicated by an asterisk. Other details are as in Figures 3·4·1c and 3·4·4a.

ionophore, the high mitogenic concentration (5×10^{-5}M) of acetylcholine was magnesium-dependent and testosterone blocked whilst the low (10^{-12}M) concentration was calcium-dependent and oestradiol blocked (Table 3·4·4b). It was therefore possible that acetylcholine could express its mitogenic potential through two distinct, concentration-dependent, mechanisms. To further investigate this dichotomy of action mitogenic concentrations of acetylcholine were tested for their susceptibility to the two antagonists atropine and hexamethonium. The former antagonises the so-called muscarinic receptor and the latter the nicotinic receptor (Bowman et al., 1970).

Table 3·4·4c. The Influence Of Receptor Antagonists On The Mitogenic Action Of Acetylcholine.

Addition	None	5×10^{-5}M Ach.	10^{-11}M Ach.
None	3·4 ± 0·12%	6·2 ± 0·45% *	7·1 ± 0·17% *
10^{-5}M Hex.	4·5 ± 0·29%	3·0 ± 0·30%	6·1 ± 0·39% *
10^{-11}M Hex.	4·3 ± 0·31%	6·8 ± 0·53% *	6·9 ± 0·29% *
10^{-5}M Atr.	4·3 ± 0·60%	6·1 ± 0·35% *	3·5 ± 0·33%
10^{-11}M Atr.	5·0 ± 0·35% *	N.T.	4·0 ± 0·12%

Table 3·4·4c. The influence of the receptor antagonists atropine and hexamethonium on acetylcholine-induced mitogenesis in thymic lymphocyte cultures. Cells were incubated in Medium 199 containing colchicine and 0·6mM calcium chloride. Thymocytes were then exposed in the presence of eserine (10^{-9}M) to various combinations of acetylcholine (Ach.), Hexamethonium (Hex.) obtained through BDH Ltd. or Atropine (Atr.) supplied by Hopkin and Williams Ltd. Results are the mean percentage cells in metaphase at six hours ± 1 S.E.M. of between 4 and 18 separate experiments. Where values obtained differ significantly from the result derived from the culture containing no additions this is indicated by an asterisk. It should also be noted that atropine at 10^{-11}M did have a statistically significant effect ($p < 0·02$) on mitotic activity when present by itself.

Table 3•4•4c indicates that whilst the nicotinic blocker, hexamethonium, inhibited the mitogenic response to the high magnesium-dependent concentration of acetylcholine, the low calcium-dependent concentration was unaffected. It might be proposed, therefore, that the high concentration of this agent (10^{-5}M) is acting through a nicotinic receptor. In addition this receptor is associated with a mechanism which requires magnesium to be present in the extracellular environment and which is inhibited by testosterone. The following three sections of this Unit will demonstrate that known adenylate cyclase stimulants also require magnesium ions and are blocked by the presence of testosterone in the medium. Hence it might be that the nicotinic receptor of the thymocyte is linked with, and possibly activates adenylate cyclase. In contrast the low (10^{-11}M) calcium-dependent peak was blocked by the muscarinic receptor blocker atropine (Table 3•4•4c). This antagonist did not affect the high mitogenic concentration of acetylcholine, further underlining the specific nature of the nicotinic-type interaction. Hence it may be inferred that the low peak of mitotic stimulation by acetylcholine is being mediated via a muscarinic receptor. This mechanism also involves a calcium-dependent event which is blocked by oestradiol in a fashion analagous to that observed with histamine, PTH, insulin and calcium itself. It is also of some considerable interest that what are ostensibly two unique membrane receptors may elicit a response at one concentration whilst not at another. Whilst it is easy to envisage that the nicotinic receptor is insensitive to acetylcholine at 10^{-11}M it is more difficult to countenance the fact that the muscarinic receptor cannot respond to a high concentration of the neurotransmitter. This might indicate that supraoptimal levels of hormone actually switch off a receptor, or that these apparently distinct receptors undergo interconversion in a concentration-dependent fashion.

As indicated the low mitogenic concentration of acetylcholine has the same requirement for calcium as do histamine, PTH and insulin and is like these other mitogens blocked by oestradiol. It was proposed that these other calcium-dependent hormones might be acting through the guanyl nucleotide system, hence the actions of imidazole and zinc chloride were examined upon acetylcholine-induced mitogenesis to further enforce this notion. The presence of imidazole

Table 3·4·4d. The Influence Of Imidazole And Zinc Ions On Acetylcholine-Induced Mitogenesis.

Treatment	Percentage Cells In Metaphase At 6 Hours + 1 S.E.M.
Control	3·4 ± 0·11%
Control + Acetylcholine (10^{-7}M)	5·8 ± 0·37% *
Control + Acetylcholine + Imidazole (6mM)	3·9 ± 0·25%
Control + Acetylcholine + Zinc Chloride (0·1mM)	3·7 ± 0·46%
Control + Acetylcholine + Zinc Chloride (0·01mM)	3·5 ± 0·23%

Table 3·4·4d. The influence of imidazole and zinc chloride on acetylcholine-induced mitogenesis. Cells were incubated in Medium 199 containing colchicine and 0·6mM calcium chloride. Cultures were subsequently stimulated with a calcium-dependent concentration of acetylcholine in the absence of eserine, and treated with imidazole (Sigma Chemical Co.) at 6mM or zinc chloride at final concentrations of 0·01 or 0·1mM. Results are the mean percentage cells in metaphase at six hours ± 1 S.E.M. of between 4 and 11 determinations. Values significantly higher than control are indicated by an asterisk.

at a final concentration of 6mM inhibited the mitogenic potential of a calcium-dependent concentration of acetylcholine (Table 3·4·4d) thus indicating again the possible involvement of the cyclic nucleotides in this response. Furthermore acetylcholine was like calcium

inhibited by zinc chloride at a concentration of 10^{-5}M. This was invoked as being a possible indication of the mediation of this stimulatory action by the cyclic GMP system as this ion is a known inhibitor of guanylate cyclase (Hardman and Sutherland, 1969). In addition magnesium, and isoprenaline are not inhibited by this level of zinc thus pointing to at least a dichotomy of action between these two classes of mitogens.

In Table 3·4·4c it was indicated that atropine at a concentration of10^{-11}M actually promoted a small but significant increase in mitotic activity. Indeed this response was common to a number of antagonists used in this study when present at concentrations about one hundred fold greater than the optimal mitogenic level of the respective agonist. Such receptor blockers exhibiting this property were atropine, hexamethonium and diphenhydramine. However it has proven difficult to obtain clear profiles of these mitogenic responses since the peak varies much more than does the curve for the agonist itself. However where tested the mitogenic stimulus of the receptor blocker invariably operates through the same ion-dependent pathway as the hormone. Thus it must be inferred that these antagonists bind to the receptor and induce at least some of the events generated by the agonist only perhaps with less efficiency. Such observations may be in agreement with the results of Illiano et al., (1973) who noted that acetylcholine, and to a lesser extent atropine stimulated the accumulation of cyclic GMP within lymphocytes. This work would also support the notion that acetylcholine acting via a muscarinic receptor effects its action by influencing cyclic GMP metabolism. Other reports also show that the elevation of cyclic GMP via a muscarinic interaction is a calcium-dependent process (MacManus, Boynton, Whitfield et. al., 1975; Schultz, Hardman, Schultz et al., 1973), supporting these results.

3·4·5 The mitogenic properties of adrenalin and isoprenaline

To contrast the action of acetylcholine both adrenalin and its more stable and specific analogue, isoprenaline, were added

Figure 3·4·5a. The Mitogenic Action Of Adrenalin And Isoprenaline.

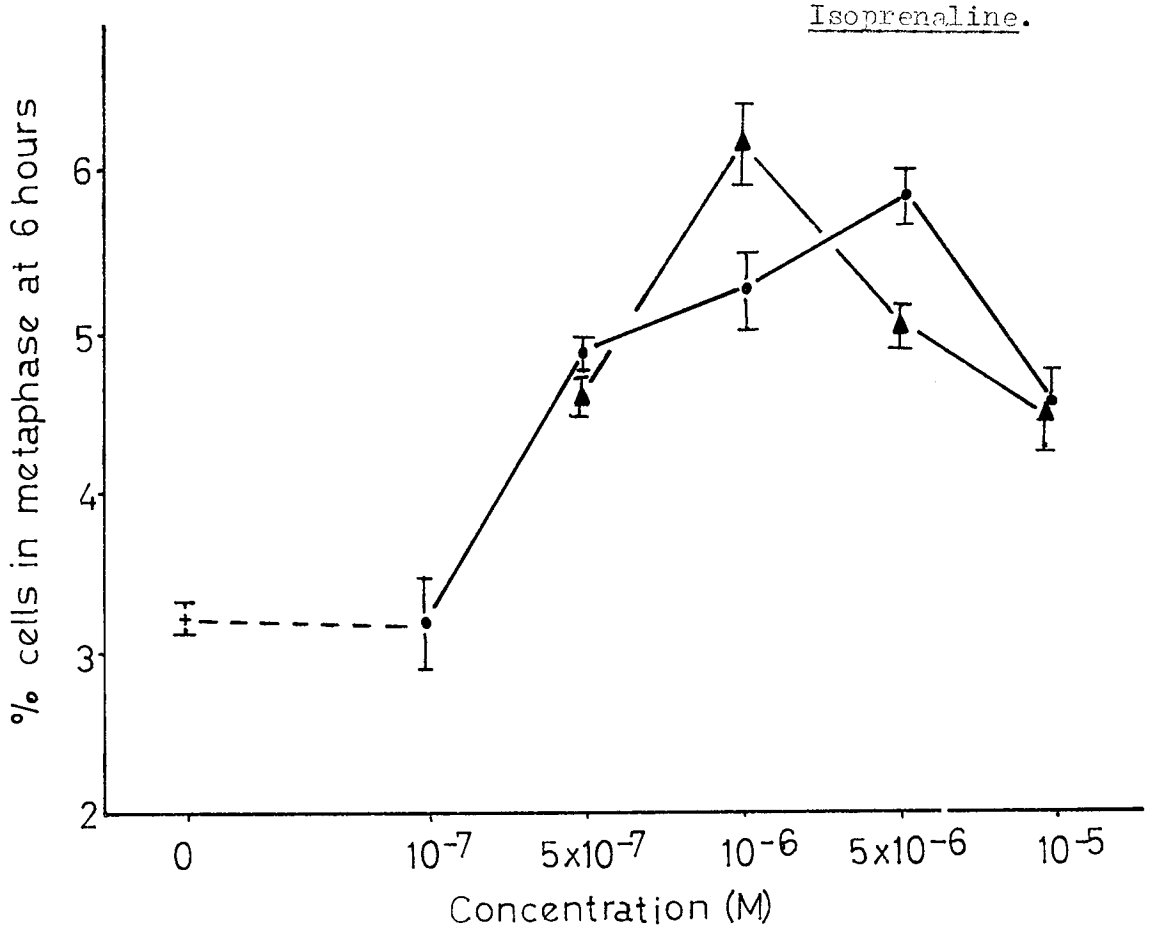


Figure 3·4·5a. The mitogenic actions of adrenalin and isoprenaline. Thymocytes were incubated in Medium 199 containing colchicine and 0·6mM calcium chloride. The suspensions were also treated with either adrenalin (●)suplied by Hopkin and Williams Ltd. or isoprenaline sulphate (▲) obtained from BDH Ltd. at the concentrations indicated. Six hour metaphase accumulations were then performed. The results are the mean ± 1 S.E.M. of between 4 and 55 separate determinations.

to thymocyte cultures (Figure 3·4·5a). It was observed that both agents stimulated mitotic activity, with isoprenaline being the more efficient. Furthermore neither hormone was impaired by calcium omission from the medium nor by oestradiol addition (Table 3·4·5b). In contrast isoprenaline was inhibited by both the presence of testosterone in

Table 3·4·5b. The Role Of Ions And Steroids In The Mitogenic Action Of Adrenalin And Isoprenaline.

Ionic Or Steroid Status Of Medium	No Additions	Isoprenaline 10^{-6}M	Adrenalin 5×10^{-6}M
0·6mM Ca^{++} 1·0mM Mg^{++}	3·4 ± 0·12%	6·1 ± 0·17% *	5·13 ± 0·11% *
0·0mM Ca^{++} 1·0mM Mg^{++}	3·6 ± 0·30%	6·3 ± 0·21% *	5·2 ± 0·18% *
0·6mM Ca^{++} 1.0mM Mg^{++} + Oestradiol.	3·7 ± 0·30%	7·3 ± 0·60% *	5·2 ± 0·22% *
0·6mM Ca^{++} 0·0mM Mg^{++}	3·7 ± 0·20%	3·8 ± 0·22%	N.T.
0·6mM Ca^{++} 1.0mM Mg^{++} + Testosterone.	3·5 ± 0·20%	3·9 ± 0·41%	N.T.

Table 3·4·5b. The ionic dependence and steroid sensitivity of the mitogenic actions of adrenalin and isoprenaline. Thymocytes were cultured in Medium 199 containing colchicine and the various ion and steroid regimes outlined in the table itself. The actions of the two catecholamines was assessed by six hour 'colchcine metaphase' accumulations. The results are the mean percentage cells in metaphase at six hours ± 1 S.E.M. from between 4 and 22 separate experiments. Where a result is significantly higher ($p < 0·001$) than the basal control containing 0·6mM Ca and 1·0mM Mg and no other additions this is indicated by an asterisk. N.T. denotes that this combination of hormone and medium was not tested.

the culture and magnesium omission from the medium (Table 3·4·5b). These latter observations suggested that there might be a magnesium-dependent axis which was blockable with testosterone, and through which extracellular magnesium might act. As far as can be ascertained there are few precedents for the implication of magnesium in hormone action. It is known that the ion is required for hormone receptor binding (Birnbaumer, Pohl, Michiel, Krans and Rodbell, 1970) notably to adenylate cyclase. This is unlikely to be a problem in this case however as even in a magnesium-free medium there will undoubtedly be some magnesium contribution from the cells themselves. In fat

cells it has been shown that adenylate cyclase stimulants enhance the uptake of magnesium into the membrane and cytosol of the lipocyte (Elliott and Rizack, 1974). It is interesting to note in this context that adrenalin stimulates thymocyte adenylate cyclase (MacManus, Whitfield and Youdale, 1971) as indeed does isoprenaline (Unit 5). Thus the magnesium-dependence of these agents may represent some aspect of the cell recognition system which involves the uptake of this divalent cation.

Table 3·4·5c. The Specificity Of Adrenergic Stimulation Of Thymocyte Proliferation.

Treatment	Percentage Cells In Metaphase At 6 Hours + 1 S.E.M.
Control	3·25 ± 0·04% (n=55)
Control + Adrenalin (5×10^{-6}M)	6·0 ± 0·21% (n=26) *
Control + Adrenalin + Propranolol (5×10^{-6}M)	3·1 ± 0·25% (n=4)
Control + Adrenalin + Phentolamine mesylate (5×10^{-6}M)	5·9 ± 0·32% (n=4) *
Control + Isoprenaline (10^{-6}M)	6·2 ± 0·35% (n=12) *
Control + Isoprenaline + Propranolol (10^{-6}M)	3·0 ± 0·10% (n=6)
Control + Isoprenaline + Phentolamine Mesylate (10^{-6}M)	5·4 ± 0·25% (n=4) *

Table 3·4·5c. The characterisation of the stimulation of thymocyte proliferation through a beta adrenergic receptor. Cells were cultured in Medium 199 containing colchicine and 0·6mM calcium chloride, and were treated with adrenalin, isoprenaline, propranolol or phentolamine mesylate (Ciba Ltd.) in the combinations and concentrations shown. Results are the percentage cells in metaphase at six hours ± 1 S.E.M. of the number of experiments in parentheses. Where a value is significantly higher than the control value ($p<0·001$) this is indicated by an asterisk.

The mitogenic actions of both adrenalin and isoprenaline was inhibited if cultures contained the beta blocker, propranolol (Table 3·4·5c), however the alpha blocker, phentolamine mesylate, had no effect on either hormone (Table 3·4·5c). Thus it must be inferred that adrenalin and isoprenaline effect their mitogenic potential through a beta adrenergic receptor. In contrast noradrenalin stimulates mitotic activity in this cell type via an alpha receptor in that its mitogenic action is blocked by phentolamine but not propranolol (data not presented). Rather interestingly noradrenalin is a calcium-dependent mitogen and is blocked by oestradiol addition to the culture system. Thus the beta receptor is linked in some way with a magnesium-dependent process, whereas the alpha adrenergic receptor is associated with a calcium-dependent mechanism.

Since acetylcholine, like calcium itself was sensitive to the presence of zinc in the extracellular medium and this was invoked as a possible indication of the mechanism of these two mitogens it was next decided to examine the influence of zinc upon isoprenaline-induced mitogenesis. Whereas acetylcholine and calcium promoted mito-

Table 3·4·5d. The Inhibitory Action Of Zinc Ions On Isoprenaline-Induced Mitogenesis.

Treatment	Percentage Cells In Metaphase At Six Hours.
Control	3·5 ± 0·10% (n=14)
Control + Isoprenaline (10^{-6}M)	6·0 ± 0·13% (n=19) *
Control + Isoprenaline + $ZnCl_2$ (0·01M)	5·9 ± 0·27% (n=6) *
Control + Isoprenaline + $ZnCl_2$ (0·1M)	4·5 ± 0·65% (n=5)

Figure 3·4·5d. The influence of zinc chloride upon isoprenaline-induced mitogenesis. Cells were cultured in Medium 199 containing 0·6mM calcium, to which was added zinc chloride and isoprenaline. Other details are as in Table 3·4·5c

tic increments were inhibited by the presence of zinc chloride at concentrations of 10^{-5} and 10^{-4}M, isoprenaline-induced mitogenesis was only blocked at the higher of these concentrations (Table 3·4·5d). Thus there is again a parallel between the sensitivity of both magnesium and magnesium-dependent mitogens to zinc and testosterone, which may be contrasted with calcium and calcium-dependent mitogens and their higher sensitivity to zinc and specific blockade with oestradiol.

3·4·6 The magnesium-dependent stimulation of thymocyte proliferation by dopamine.

Structurally dopamine is closely related to both adrenalin and noradrenalin, all being derived from the same precursor 3,4-Dehydroxyphenylalanine (DOPA). Like these other two agents it is also a neurotransmitter and local tissue hormone. Evidence is also available to indicate that dopamine acts at a receptor not altogether different from a beta adrenergic site (Bowman, Rand and West, 1970), which it has been established represents a magnesium-dependent mitogenic axis in thymocytes. Thus dopamine might represent another calcium-independent mitogen with which to contrast the properties of the calcium-dependent group. Indeed addition of dopamine to thymocyte cultures at concentrations between 10^{-6} and 10^{-7}M caused a significant increase in mitotic accumulations over a six hour period (Figure 3·4·6a). This mitogenic action like that of adrenalin was not calcium-dependent and was not influenced by the presence of oestradiol in the culture medium Table 3·4·6b). In contrast dopamine-induced mitogenesis was blocked by both magnesium omission from the medium and testosterone addition to the cultures (Table 3·4·6b). Hence dopamine is a magnesium-dependent mitogen like adrenalin and is similarly inhibited by testosterone.

Figure 3·4·6a. Dopamine-Induced Mitogenesis.

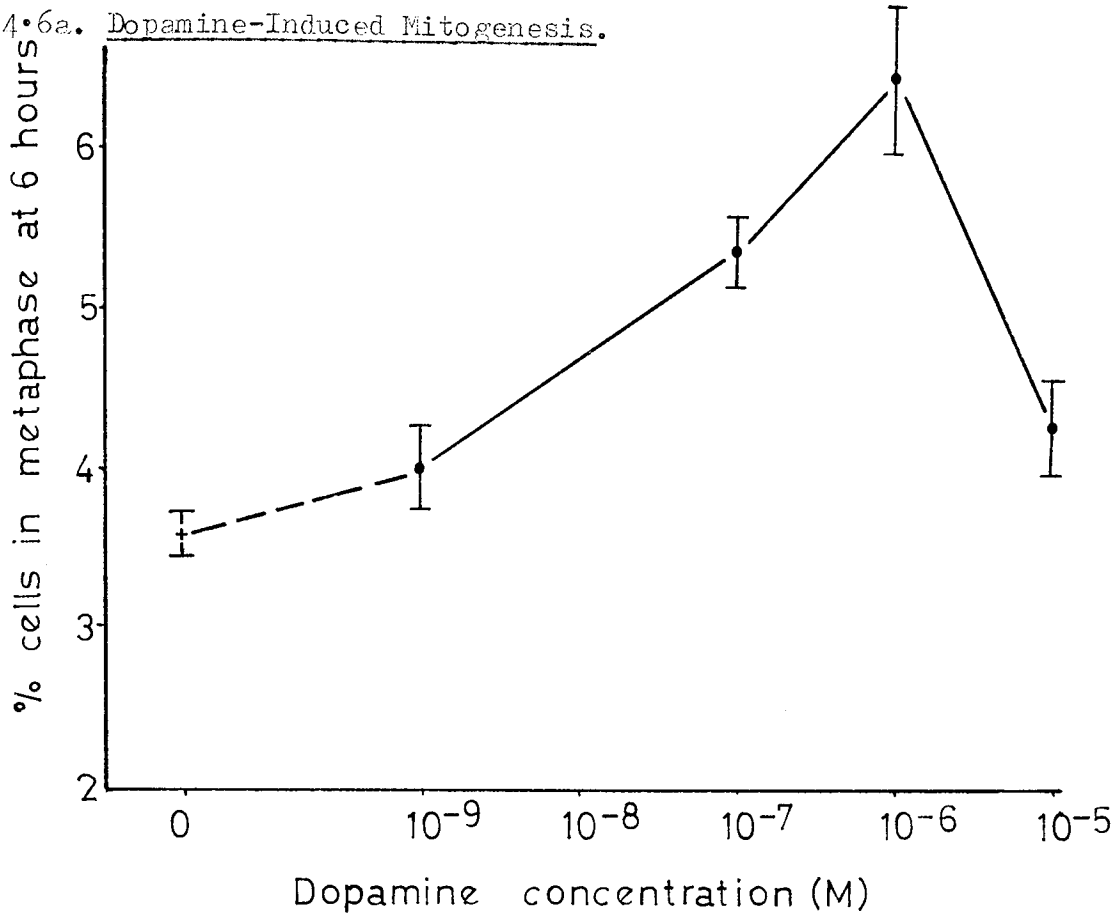


Figure 3·4·6a. The influence of dopamine on thymocyte proliferation. Thymic lymphocytes were cultured in Medium 199 containing 0·6mM calcium chloride and colchicine. The cells were also exposed to dopamine (BDH Ltd.) at the concentrations indicated, and six hour metaphase accumulations performed. Results are the mean ± 1 S.E.M. of 4 separate experiments.

To assess the specificity of the dopamine-cell interaction, the dopamine receptor antagonist, pimozide (Bowman et al., 1970)was added to cultures containing both dopamine and isoprenaline (Table 3·4·6c). It was established that pimozide blocked the mitogenic action of dopamine but not isoprenaline or adrenalin (Table 3·4·6c). Thus in these cells dopamine is acting via a distinct, dopaminergic receptor, which is not the same as the beta adrenergic receptor. This observation taken with the magnesium-dependence of this catecholamine is of some significance since dopamine, like adrenalin, acts through an adenylate cyclase receptor in certain regions of the brain (Phillipson and Horn, 1976) indicating another parallel between the magnesium-dependent agents.

Table 3·4·6b. The Role Of Ions And Steroids In The Mitogenic Action Of Dopamine.

Ionic Or Steroid Status Of Medium.	No Additions	Dopamine (10^{-6}M)
0·6mM Ca^{++} 1·0mM Mg^{++}	3·4 ± 0·12%	5·4 ± 0·14% *
0·0mM Ca^{++} 1·0mM Mg^{++}	3·6 ± 0·30%	5·9 ± 0·42% *
0·6mM Ca^{++} 1·0mM Mg^{++} + oestradiol	3·7 ± 0·30%	5·7 ± 0·39% *
0·6mM Ca^{++} 0·0mM Mg^{++}	3·7 ± 0·20%	3·9 ± 0·50%
0·6mM Ca^{++} 1·0mM Mg^{++} + testosterone	3·5 ± 0·20%	3·6 ± 0·23%

Figure 3·4·6b. The ionic dependence and steroid sensitivity of dopamine-induced mitogenesis. Thymic lymphocytes were cultured in Medium 199 containing colchicine and the combination of ionic and steroid modifications shown in the table. The percentage cells in metaphase at six hours was then assessed in these various media both in the presence and absence of dopamine (10^{-6}M). Results are the mean ± 1 S.E.M. of between 4 and 22 separate determinations. Where values are significantly higher ($p<0·001$) than basal control this is shown by an asterisk.

Table 3·4·6c. Characterisation Of The Thymocyte Dopamine Receptor.

Treatment	Percentage Cells In Metaphase At 6 Hours ± 1 S.E.M.
Control	3·6 ± 0·15% (n=4)
Control + Dopamine (10^{-6}M)	5·4 ± 0·16% (n=6) *
Control + Dopamine + Pimozide (10^{-6}M)	3·8 ± 0·26% (n=4)
Control + Isoprenaline (10^{-6}M)	6·0 ± 0·13% (n=19) *
Control + Isoprenaline + Pimozide (10^{-7}M)	6·4 ± 0·18% (n=4) *
Control + Adrenalin (5×10^{-6}M)	6·0 ± 0·21% (n=26) *
Control + Adrenalin + Pimozide (10^{-7}M)	5·8 ± 0·48% (n=4) *

Figure 3·4·6c. Figure legend details as for 3·4·5c .

3·4·7 The mitogenic potential of glucagon in thymocyte cultures.

Glucagon is the natural antagonist to insulin in both the liver and fat cells (Robison, Butcher and Sutherland, 1971). Thus it seemed germane to investigate the action of this hormone upon thymic lymphocyte proliferation. It was found that glucagon did in fact enhance mitotic activity at a concentration of 10^{-4}M (Table 3·4·7a).

Table 3·4·7a. The Role Of Ions And Steroids In The Mitogenic Action Of Glucagon.

Ionic Or Steroid Status Of Medium	No Additions	Glucagon (10^{-4}M)
0·6mM Ca^{++} 1·0mM Mg^{++}	3·4 ± 0·12%	7·2 ± 0·64% *
0·0mM Ca^{++} 1·0mM Mg^{++}	3·6 ± 0·30%	6·1 ± 0·46% *
0·6mM Ca^{++} 1·0mM Mg^{++} + 9estradiol	3·7 ± 0·30%	6·6 ± 0·54% *
0·6mM Ca^{++} 0·0mM Mg^{++}	3·7 ± 0·20%	3·2 ± 0·17%
0·6mM Ca^{++} 1·0mM Mg^{++} + Testosterone	3·5 ± 0·20%	3·8 ± 0·23%

Table 3·4·7a. The ionic dependence and steroid sensitivity of glucagon-induced mitogenesis. Thymocytes were cultured in Medium 199 containing colchicine and the ionic and steroid combinations indicated in the body of the table itself. The cell suspensions were also treated in some cases with glucagon (Eli Lilly and Co.) at a final concentration of 10^{-4}M. Six hour metaphase accumulations were then performed and these are expressed as the mean percentage cells in metaphase ± 1 S.E.M. of between 4 and 22 separate determinations. Where a result differs significantly from the basal count (3·4 ± 0·12%) this is indicated by an asterisk.

Not only is glucagon the natural antagonist to insulin in many ways *in vivo*, but also in the thymocyte model whereas insulin is a calcium-

dependent mitogen, glucagon is a magnesium-dependent one (Table 3·4·7a). Thus these two mitogens appear to be acting at the cellular level through different mitogenic axes. Furthermore glucagon like adrenalin, dopamine and magnesium is inhibited if the culture contains testosterone (Table 3·4·7a). And glucagon in a number of tissues also stimulates adenylate cyclase (Robison et al., 1971). Thus glucagon fulfills the same mitogenic requirements as these other cyclase stimulating hormonal mitogens in being magnesium-dependent and testosterone blocked, and is independent of calcium and oestradiol.

3·5 Discussion of results and summary

The mitogenic hormones tested fall invariably into two distinct groups. The first class of hormones require calcium ions to be present in the extracellular milieu for their mitogenic potential to be manifest (Table 3·5a). This group could be extended by the inclusion of previously described calcium-dependent mitogens in Table 3·3a. Furthermore these calcium-requiring mitogens are inhibited if the culture also contains the female sex steroid, oestradiol, in a fashion analagous to calcium-induced mitogenesis itself (Table 3·5a). Thus it might be speculated that calcium plays some role in the mitogenic action of these hormones, and that oestradiol impairs some facet of this process which is common to both hormone and calcium itself. This group of hormones, and indeed calcium, is further characterised by their greater sensitivity to the presence of zinc ions in the cell suspension when compared to the calcium-independent hormones. This latter group of mitogens, comprising dopamine, adrenalin, isoprenaline and glucagon are independent of extracellular calcium and are unaffected by oestradiol addition to the culture system. In

contrast these hormones have an obligatory requirement for the presence of magnesium ions in the extracellular medium and are blocked by the androgen, testosterone (Table 3·5a). To this group must also be added acetylcholine when it is acting via a nicotinic receptor. A similar argument might therefore implicate magnesium in the skein of biochemical events initiated by hormone addition to these cells. Testosterone would thus inhibit some common part of the magnesium-dependent mitogenic axis.

Table 3·5a. A Comparison Of The Calcium- And Magnesium-Dependent Mitogenic Hormones.

Calcium-Dependent, Oestradiol-Blocked, Zinc-Sensitive Axis.		Magnesium-Dependent, Testosterone-Blocked, Zinc-Insensitive Axis.	
Hormone	Concentration	Hormone	Concentration
Insulin	10^{-10}M	Glucagon	10^{-4}M
Acetylcholine (muscarinic)	10^{-12}M	Acetylcholine (nicotinic)	5×10^{-4}M
Noradrenalin (alpha receptor)	10^{-12}M	Adrenalin (beta)	5×10^{-6}M
		Isoprenaline (beta)	10^{-6}M.
Histamine (H_1)	10^{-13}M	Dopamine (dopaminergic)	10^{-6}M.
Parathhroid Hormone	10^{-9}M		

Table 3·5a. A comparison of the properties of the mitogenic hormones used in this Unit. Concentrations have all been adjusted to the optimal mitogenic concentration of the hormone in molarity from cited data for the given agent.

Examination of Table 3·5a. also indicates other more subtle differences between calcium- and magnesium-dependent mitogens. It is noticeable that calcium-dependent hormones express their mitogenic action at lower concentrations than magnesium-dependent mitogens.

This is unlikely to be a consequence of degradation alone, although it will play some part, since there is a vast dichotomy in the mitogenic concentrations of adrenalin and dopamine on the one hand and noradrenalin on the other yet all are closely related in structure. In addition acetylcholine may be either magnesium- or calcium-dependent on the basis of which receptor it is activating and this agent is presumably degraded at the same rate at both mitogenic concentrations. Whilst it is clear that acetylcholine does undergo rapid hydrolysis as probably does PTH and glucagon in culture on balance it would seem that magnesium-dependent hormones are less efficient mitogens than calcium-dependent hormones. This may be the result of fewer receptors, or of some reduced intracellular amplification stage in comparison with calcium-requiring mitogens. It might also suggest that the magnesium axis is not normally a mitogenic route.

A second clear feature is that classically antagonistic hormones act through different proliferative axes. Thus on the one hand adrenalin and dopamine act through beta adrenergic and dopaminergic receptors respectively and are magnesium-dependent whilst in contrast the classical antagonists acetylcholine (muscarinic) and noradrenalin (alpha adrenergic receptor) are calcium-dependent. A similar comparison exists between insulin (calcium-dependent) and its natural antagonist glucagon (magnesium-dependent). This notion of antagonism in physiological systems being mediated through two distinct pathways has recently become a central feature of cell biology. The concept has been termed the Yin Yang Hypothesis by N.D.Goldberg (Goldberg, Haddox, Nicol et al., 1975), and it proposes that the response of a given physiological system is the resultant of the antagonistic hormones impinging upon it. Furthermore it is postulated that these hormones act at the cellular level through

modulating the endogenous levels of cyclic AMP or cyclic GMP. Thus although a cell may bear a number of hormone receptors on its membrane these receptors have the capacity to influence either the intracellular cyclic AMP or GMP concentrations. Here one may draw a parallel with the situation in the thymocyte where a large number of hormones all appear to act through one of two possible pathways. This study may have determined therefore a number of probe molecules which interact with either the cyclic AMP or cyclic GMP metabolic processes to disrupt hormone-induced mitogenesis. Clearly the question to ask is if the calcium- and magnesium-dependent pathways are to be equated with either the cyclic AMP or cyclic GMP axes, then which is which?. One indication is that adrenalin, glucagon (Robison et al., 1971) and dopamine are frequently adenyl cyclase stimulants (Phillipson and Horn, 1976). In contrast whilst histamine H_2 receptors are associated with adenylate cyclase in the gastric mucosa (Dousa and Code, 1974) the H_1 receptor found in this study is not (Goldberg, O'Dea and Haddox, 1973). However H_1 interactions are known to lead to an elevation of tissue cyclic GMP concentrations (Kuo et al., 1972; Schultz et al., 1972). This property is shared by insulin (Illiano et al., 1973), acetylcholine when acting through a muscarinic receptor (Illiano et al., 1973; MacManus, Boynton, Whitfield et al., 1975) and PTH when not stimulating adenylate cyclase (Goldberg et al., 1973). Thus it would seem likely that the calcium-dependent axis may be involved with the metabolism of the guanyl nucleotides, whereas the magnesium-dependent axis is synonymous with the adenylate cyclase system. This being the case it is interesting to note that calcium has previously and quite independently been invoked as playing a vital role in the cyclic GMP limb of the Yin Yang Hypothesis (Goldberg et al., 1973). It should also be remembered however that cyclic AMP and calcium have also been linked in some cellular response systems (Rasmussen

et al., 1975).

Whereas cyclic AMP synthesis is via the membrane-bound enzyme, adenylate cyclase (Robison et al., 1971) this does not appear to be the case for the guanylate cyclase moiety; which seems to be present as both soluble cytoplasmic and membrane-associated enzyme (Hardman and Sutherland, 1969; White, Aurbach and Carlson, 1969). As will be discussed in the following two Units there is some controversy over the precise role and activity of these two forms of guanylate cyclase. However it has been established that it is not possible to stimulate broken cell cyclase preparations with hormones known to elevate endogenous cyclic GMP concentrations in the intact cell (Goldberg et al., 1973). Thus it may be that calcium ions provide the key link between the plasma membrane receptor and the guanylate cyclase unit. In favour of such a contention is the observation that calcium ions activate broken cell guanyl cyclase preparations (Hardman et al., 1972), this being in contrast to their potent inhibitory action on adenylate cyclase (Perkins, 1973). In addition the elevation of lymphoblast cyclic GMP concentrations by concanavalin A is also dependent upon extracellular calcium (Whitfield, MacManus, Boynton et al., 1974).

The sites of action of oestradiol and testosterone cannot be decided from these experiments, neither can the calcium and magnesium-dependent stages of the two mitogenic pathways. However it is possible to indicate reactions which might be affected. Thus hormone binding to its receptor may require calcium or magnesium. As indicated previously the so-called calcium-free medium does contain trace amounts of calcium (the same probably applies for magnesium-free media) which is of the order or 5×10^{-5}M determined using the Corning-Eel fluor-

imetric calcium titrator. These levels are adequate for binding to occur. Additionally the insulin receptor does not require either of these divalent cations for binding of insulin (Exton, Jefferson, Butcher and Park, 1966; Gavin et al., 1973). In an analagous fashion the steroids may affect hormone binding to their receptors, although no reports are available concerning this possibility. It would seem most likely that steps subsequent to the initial hormone/cell interaction are being affected by these various inhibitory situations. Clearly one area which might be influenced is the metabolism of the cyclic nucleotides themselves which is known to be modulated by hormones. Thus the next approach will be to examine the role of cyclic nucleotides as regulators of proliferation, and to investigate and ionic dependence or steroid sensitivity these purines might have. These aspects of cell proliferation control will be explored in the following Unit.

Unit 4. The influence of cyclic nucleotides on proliferation.

> '..... and I assert that whenever a dispute has raged for any length of time, there was at the bottom of it, never a problem about mere words, but always a genuine problem about things'.
>
> Kant (1786)

4·1 The role of cyclic nucleotides as regulators of cell proliferation.

The role of cyclic nucleotides in the control of mammalian cell proliferation is certainly contentious. It is all to easy to select from the vast amount of published data that which fits one's own particular model and offer this as a unifying hypothesis for the regulation of division by cyclic nucleotides. It is hoped to avoid this pitfall by adopting an historical and therefore unbiased approach to the involvement of cyclic purines in mitotic control.

The initial observations which implicated these agents in cell proliferation were that caffeine and theophylline slowed the growth of normal and transformed BHK cells (Burk, 1968). These compounds were assumed to raise endogenous cyclic AMP by inhibiting cyclic nucleotide phosphodiesterase (Robison et al., 1971). Thus an anti-proliferative role was assigned to cyclic AMP. The demonstration shortly afterwards that high concentrations of exogenous cyclic AMP inhibited proliferation of HeLa cells confirmed this belief (Ryan and Heidrick, 1968). It should be noted, however, that these workers (Heidrick and Ryan, 1970) obtained inhibition with a range of cyclic nucleotides at rather high concentrations (6×10^{-5} to 3×10^{-4}M) and one must question the physiological specificity of this action. Subsequently a number of cyclic AMP analogues were also shown to inhibit

proliferation in fibroblast cell lines (Sheppard, 1971; Burger et al., 1972; Carchman, Johnson, Pastan and Scolnick, 1974; Froehlich and Rachmeler, 1974). These cyclic AMP derivatives (e.g. dibutyryl cyclic AMP) appeared to be much more effective in arresting division than the free acid itself. This may be related to the more rapid metabolism of the native cyclic AMP, or its poorer penetration into the cell. Two degradation products of cyclic AMP, adenosine and 5'AMP, also inhibit proliferation possibly by depleting the pool of pyrimidine precursors for DNA synthesis (Ishii and Green, 1973). Therefore the specific antimitotic function of exogenous cyclic AMP must again be questioned. However elevation of endogenous cyclic AMP concentrations with PGE_1 (Johnson and Pastan, 1971), cholera toxin (Pastan, Johnson and Anderson, 1975), bacterial glycolipid (Brailovsky, Trudel, Lallier and Nigam, 1973) and phosphodiesterase inhibitors (Burk, 1968) all resulted in a reduction in proliferation rates.

In contrast to these various observations implying an anti-proliferative role to cyclic AMP, addition of low concentrations of cyclic AMP (MacManus and Whitfield, 1969; Rebhun and Monroy, 1970; McCrery and Rigby, 1972) or the cyclic AMP-elevating agents PGE_1 (Whitfield, MacManus, Franks, Braceland and Gillan, 1972) adrenalin (MacManus, Whitfield and Youdale, 1971) and caffeine (Morgan and Perris, 1974) to lymphocyte cultures all stimulated division. Since exogenous cyclic AMP neither raises its own endogenous levels nor enters the cell some cyclic AMP receptor site at the plasma membrane has been postulated for the critical event which initiates the division process (MacManus, Whitfield and Braceland, 1971; MacManus and Whitfield, 1972). A cell cycle arrest in G2 can also be induced in thymocytes by using very high concentrations of cyclic AMP (10^{-5}M) as noted by Whitfield and coworkers (MacManus and Whitfield, 1972; Whitfield,

MacManus, Braceland and Gillan, 1972). Both G1 and G2 arrest by high concentrations of cyclic AMP have also been observed in other cell types (Willingham, Johnson and Pastan, 1972; Froehlich and Rachmeler, 1974). Whether this too is due to some receptor interaction or a non-specific arrest precipitated through a pyrimidine depletion effect is not established.

It is worth noting here that positive proliferative effects of cyclic AMP on thymocytes is the result of a specific activation of quiescent G_o cells. Many cultured cell lines of course do not possess such dormant, recruitable cells. Thus it is perhaps not surprising that nucleotides exert different effects in different cell types.

Experiments in which endogenous cyclic AMP levels have been measured in parallel with altered division rates, and throughout the cell cycle have also assigned a central role to cyclic AMP in mitotic regulation. Thus there is an inverse correlation between the cyclic AMP content of a cell and its proliferation rate (Otten, Johnson and Pastan, 1971). Furthermore as cells approach confluency and division arrest there is an increase in their average cyclic AMP content (Otten, Johnson and Pastan, 1971; Bannai and Sheppard, 1974), with a concomitant fall in their ATP content (Bannai and Sheppard, 1974). It must be pointed out however that some workers have been unable to demonstrate this rise in cyclic AMP at confluency (Burstein, Renger and Basilico, 1974; Oey, Vogel and Pollack, 1974). This increase in cyclic AMP at confluency reported by Otten et al., (1971) and Bannai and Sheppard (1974) may thus be due to not density-dependent inhibition per se but to inadvertant serum starvation. However there is agreement that addition of agents such as insulin and serum, which cause the recycling of inhibited fibroblasts, do cause a drop in cyclic AMP concentration (Otten et al.,

1972; Sheppard, 1972; Oey et al., 1974). In peripheral lymphocyte cultures also the reinitiation of division promoted by phytomitogens is associated with a slight fall in cyclic AMP levels over a six hour period (Smith, Steiner, Newberry and Parker, 1971), although other reports indicate no change in the concentration of this nucleotide (Hadden, Hadden, Haddox and Goldberg, 1972). These results are supported by the observations of Burger and coworkers (Burger et al., 1972) who demonstrated that during the normal cell cycle of 3T3 cells there is an abrupt fall followed by an increase in cell cyclic AMP content just prior to the DNA synthetic phase. This was interpreted as a critical transition point in late G1 which required a drop in endogenous cyclic AMP levels for the commitment to DNA synthesis and mitosis to be taken.

Since transformed cells have lower cyclic AMP contents than normal cells maintained in vitro (Otten et al., 1971) it might be expected that they would freely pass the G1 transition point at least until the nutrient supply in the medium is exhausted. Such a notion is clearly supported by the observation that transformed cells do not exhibit density-dependent inhibition (Todaro, Lazar and Green, 1965).

In addition to these various changes in cyclic AMP levels, cyclic GMP alterations may also be implicated in division control. Thus after Con A addition to both peripheral (Hadden et al., 1972) and thymic lymphocyte cultures (Whitfield, MacManus, Boynton et al., 1974) there was a dramatic rise in cyclic GMP concentrations. Exogenous cyclic GMP also stimulated thymocyte (Whitfield, MacManus, Franks, Gillan and Youdale, 1971) and peripheral lymphocyte proliferation (Weinstein, Chambers, Bourne and Melmon, 1974) and at 10^{-5}M also enhanced the action of serum in initiating DNA synthesis in contact-inhibited 3T3 cells (Schor and Rozengurt, 1974). Thus cyclic GMP in

contrast to cyclic AMP has been invoked as a positive effector of cell proliferation. However a number of other cyclic nucleotides at these concentrations also stimulated division, thus the reaction is not particularly specific. Furthermore Carchman et al., (1974) found no action of cyclic GMP on 3T3 division.

Whereas density-inhibited fibroblasts contain elevated levels of cyclic AMP, the converse has been reported for cyclic GMP (Moens, Vokaer and Kram, 1975). Thus proliferating 3T3 cells contain higher concentrations of cyclic GMP compared with quiescent cells (Goldberg, Haddox, Dunham, Lopez and Hadden, 1974). In addition transformed 3T3 fibroblasts have been claimed to have twice the level of endogenous cyclic GMP found in the untransformed parent cell line (Moens et al., 1975). These reports would indicate that raised cyclic GMP concentrations are associated with proliferating cells. Two studies also claim that serum addition to serum starved (Seifert and Rudland, 1974) or density-inhibited fibroblasts (Moens et al., 1975) leads to a rapid increase in cyclic GMP levels which precede DNA synthesis. Like serum, fibroblast growth factor also reinitiates proliferation in arrested 3T3 cultures and elevates endogenous cyclic GMP (Rudland, Gospodarowicz and Seifert, 1974) by stimulating guanylate cyclase (Rudland, Hamilton, Hamilton et al., 1975). However these observations must be balanced against a number of reports which do not demonstrate a positive role for cyclic GMP in proliferation control. Thus serum addition to arrested fibroblasts leads in these studies to a fall in both cyclic AMP and cyclic GMP concentrations (Miller, Lovelace, Gallo and Pastan, 1975). In addition they claim that proliferating cells contain lower levels of cyclic GMP than do arrested cells, and that the contact-inhibition event is associated with an increase in intracellular cyclic GMP concentrations (Miller et al., 1975). This increase in cyclic GMP in

normal cells has been attributed to an elevation of guanylate cyclase activity at confluency (Nesbitt, Miller, Russell and Pastan, 1975). Indeed not only do this group also find transformed fibroblasts to contain lower levels of cyclic GMP compared to normal cells (Miller et al., 1975) but these tumour cells also have a lower guanylate cyclase activity (Nesbitt et al., 1975).

Thus some picture begins to emerge, supported by many workers of a decrease in cyclic AMP levels and an increase in cyclic GMP concentrations being associated with critical events for division initiation and the reverse for density-dependent inhibition. These changes may be variously ascribed to the respective phosphodiesterase and cyclase enzymes. Thus adenylate cyclase activity (Makman, 1971; Anderson, Russell, Carchman and Pastan, 1973) and phosphodiesterase activity (Anderson et al., 1973) increase with increasing cell density. However, at confluency there is a fall in phosphodiesterase activity whilst cyclase function continues to rise, resulting in an elevation of cyclic AMP levels (Anderson et al., 1973). Furthermore in general transformed cells possess lower adenylate cyclase activity than the parent cell line, which might contribute to their lower cyclic AMP content (Pastan, Johnson and Anderson, 1975). Adenylate cyclase activity also alters through the cell cycle, as do cyclic AMP concentrations. Thus cyclase activity is high during G1 (Raska, 1973; Millis, Forrest and Pious, 1974) falls during the S phase (Makman and Klein, 1972; Raska, 1973; Millis et al., 1974) and rises to a maximum during G2 and mitosis (Makman and Klein, 1972; Millis et al., 1974). Such changes in the activity of this enzyme could be the causative factor in the cell cycle fluctuations of cyclic AMP levels observed by Burger et al., (1972). It must be emphasised however that equally reputable workers find no evidence for cyclic nucleotide involvement in proliferative

events. Perhaps the most persuasive of these contrary views is the recent paper by Coffino, Gray and the late Gordon Tomkins (1975). The notion that cyclic AMP might not play any functional role in cell cycle regulation derives from experiments with revertant cell lines which lack a protein kinase sensitive to cyclic AMP. These cells proliferate quite normally even in the presence of high cyclic nucleotide concentrations, whilst the parent cell is inhibited in G1. Thus they argue that any cell cycle dependent fluctuation in cyclic AMP cannot be essential for the control of the growth/division cycle, and

> 'cannot be required for or determine progression through the cell cycle.'

Such an apparently clear cut exclusion of a cyclic nucleotide in initiatory events cannot be made so emphatically for cyclic GMP. Although cyclic GMP has been variously reported to stimulate, inhibit and have no effect on fibroblast proliferation, evidence for it being a positive factor in lymphocyte activation is persuasive. The ability of cyclic GMP and calcium ions to stimulate RNA polymerase I activity and decrease RNA polymerase II, events associated with the initiation of DNA synthesis (Johnson and Hadden, 1975) would certainly render it a good candidate. In addition calcium ions, which have been linked previously with cyclic GMP metabolism, will stimulate DNA synthesis in isolated thymocyte nuclei (Burgoyne, Waqar and Atkinson, 1970). It might also be added that cyclic AMP in this latter study had no action on DNA synthesis.

Perhaps the resolution of these various antagonisms(highlighted in thymocytes where both nucleotides may stimulate or even inhibit division depending on their concentration) may lay in a consideration of the balance between these two nucleotides or consequences of this balance.

Such an approach might also be regarded as a more valid interpretation of the Yin Yang hypothesis since one is not merely reflecting upon a perhaps transient alteration in one nucleotide with no regards to the status of the other. It might also be remembered that in the thymocyte model one is not dealing with a terminal cell type such as a fibroblast, but a primitive precursor cell part of whose differentiated function will be to divide. Thus reverting to the notion of cyclic AMP mediating the differentiational axis and cyclic GMP the proliferative axis, one might well imagine that stimulation of differentiation might also lead to proliferation in this particular cell type.

4·2 The interaction of ions and steroids with cyclic nucleotide-induced mitogenesis.

Addition of either cyclic AMP (Figure 4·2a) or cyclic GMP (Figure 4·2b) to cultures in a basal medium (0·6mM Ca; 1·0mM Mg) caused a biphasic mitotic stimulation. Thus high (between 10^{-6} and 10^{-8}M) and low ($10^{-10}$to 10^{-13}M) concentrations of cyclic AMP and cyclic GMP are mitogenic. Since it had been previously established that the distinct mitogenic concentrations of both acetylcholine and A23187 were dependent upon the presence of either calcium or magnesium ions and were blocked by the presence of oestradiol or testosterone a similar study was performed with these two cyclic nucleotides. Thus omission of magnesium ions from the medium resulted in the mitotic response to high levels of both cyclic AMP (10^{-7} to 10^{-8}) and cyclic GMP (10^{-6}M) being muted (Figure 4·2a and b). The stimulation induced by the low concentrations of cyclic nucleotides however was uninfluenced by lack of this ion. In a superficially analagous fashion addition of testosterone to suspensions of thymocytes maintained in basal medium also abolished

Figure 4·2a. The interactions amongst cyclic AMP, steroids and divalent cations.

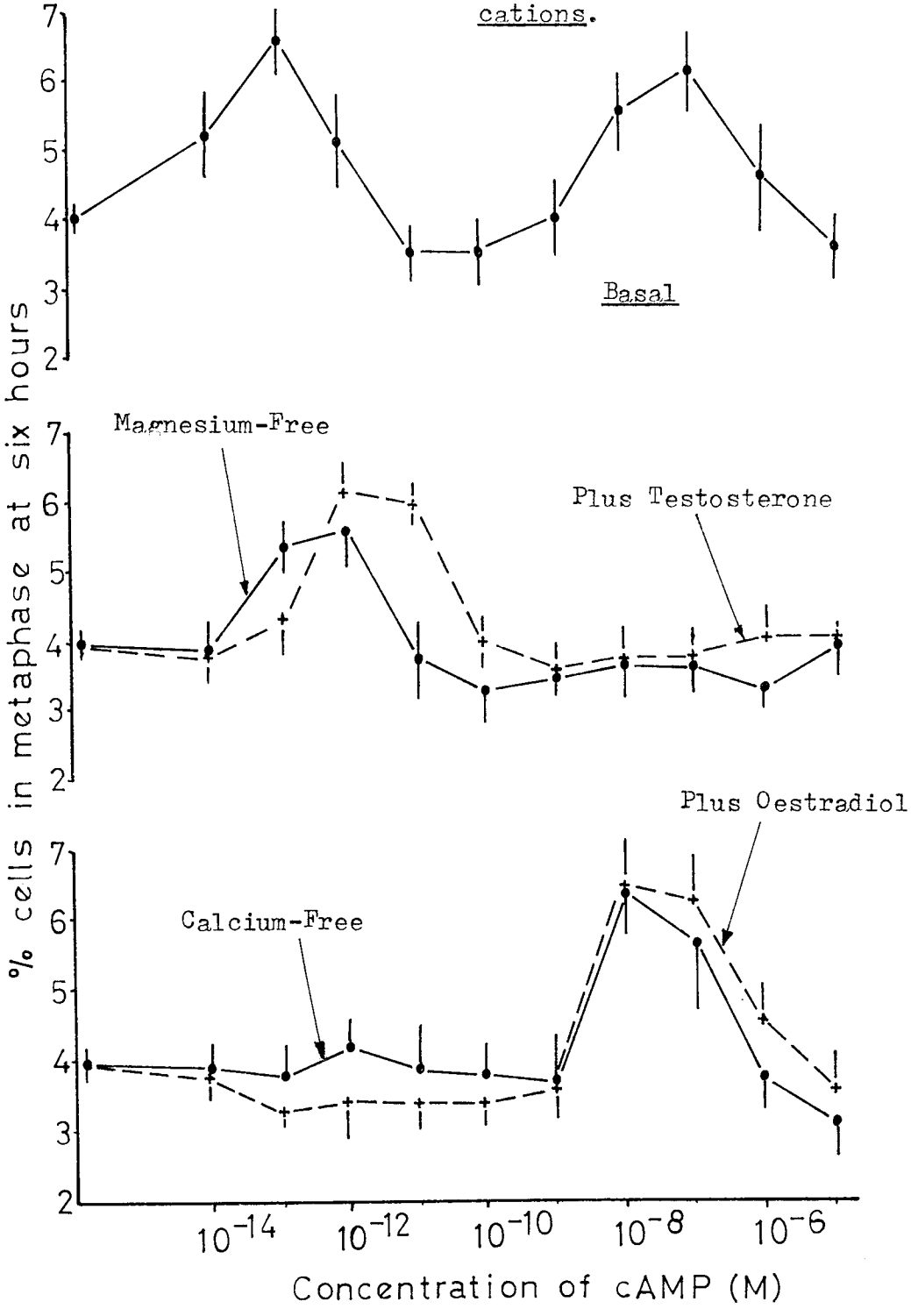


Figure 4·2. The influence of steroids and ions upon cyclic AMP-induced mitogenesis. Thymocytes were cultured in Medium 199 containing colchicine and either basal (0·6mM calcium, 1·0mM magnesium) or calcium- and magnesium-free conditions. In addition some basal cultures also contained oestradiol and testosterone ($0·1\mu g.ml^{-1}$). All cultures then recieved cyclic AMP at varying concentrations and six hour metaphase accumulations were performed. Each point is the mean and ± 1 S.E.M. of between 4 and 20 separate determinations.

Figure 4·2b. The influence of ions and steroids upon cyclic GMP-induced mitogenesis.

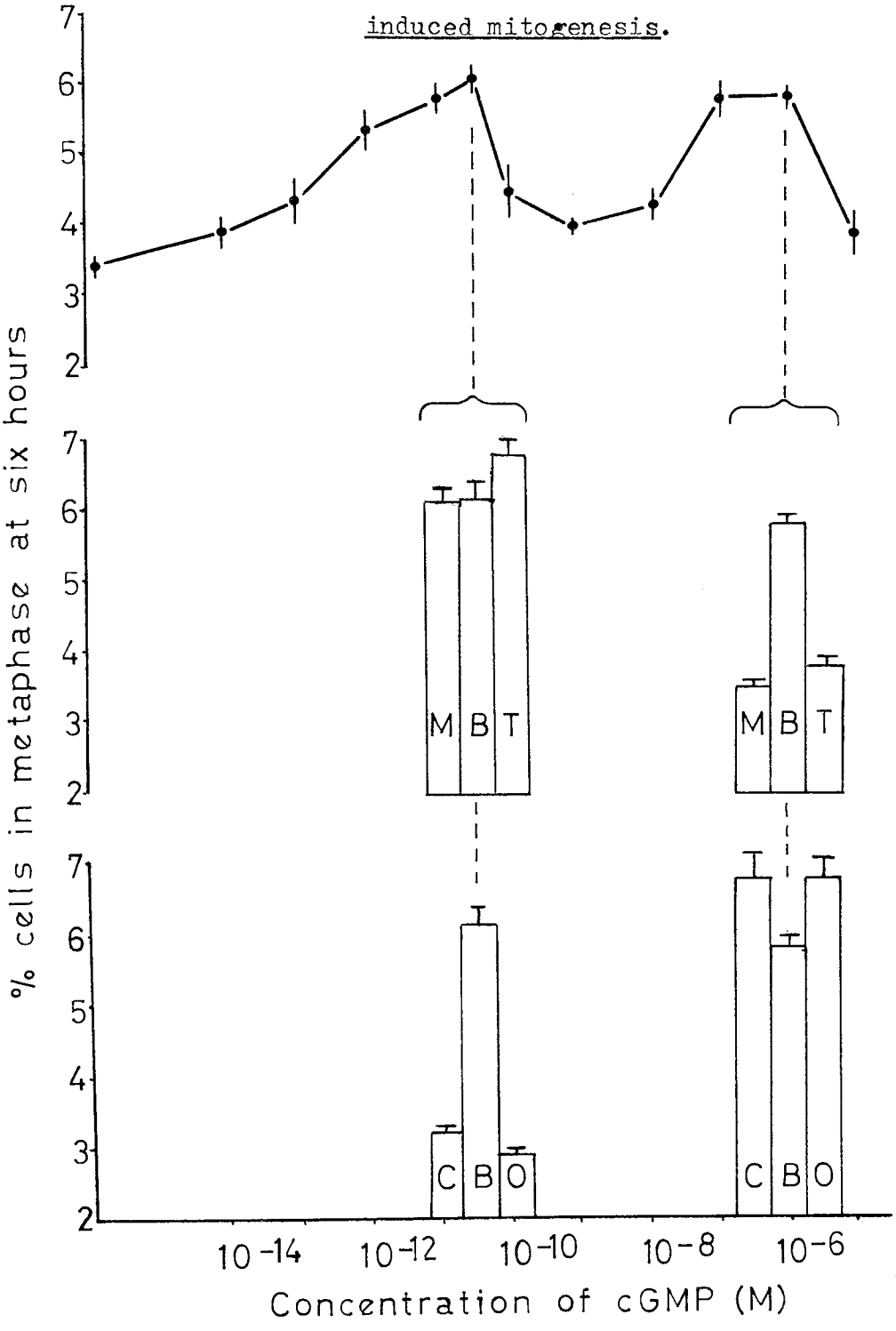


Figure 4·2b. The ionic dependence and steroid blockade of cyclic GMP-induced mitogenesis. Cells were incubated in Medium 199 containing basal calcium and magnesium (B) or which lacked calcium (C) or magnesium (M). Some basal cultures also contained oestradiol (O) or testosterone (T) at a final concentration of 0·1μg.ml^{-1}. The action of cyclic GMP upon thymocyte proliferation was studied by adding various doses of the nucleotide to cells in basal medium and scoring six hour metaphase accumulations. When studying the action of ions and steroids upon cyclic GMP-induced mitogenesis, the purine was added at either 10^{-6} or 5 x 10^{-11}M, the peak stimulatory concentrations. Results are the means $\pm$ 1 S.E.M. of between 4 and 18 separate determinations.

the response to the high but not the low concentrations of cyclic AMP and cyclic GMP (Figures 4·2a,b).

Conversely in a calcium-free medium (Figures 4·2a,b) the low and normally mitogenic levels of cyclic AMP (10^{-13} to 10^{-14}M) and cyclic GMP (5×10^{-11}M) failed to stimulate mitosis. Calcium lack did not impede the action of the high stimulatory concentrations of nucleotide (Figures 4·2a,b). As an additional check on calcium requirements, stimulatory concentrations of cyclic GMP were tested in calcium-free medium containing 0·2mM EGTA (ethylene bis(oxyethylenenitrilo) tetra acetic acid) to chelate any residual calcium. Again the low concentration of cyclic GMP was clearly found to be calcium-dependent, and the high mitogenic concentration calcium-independent. In contrast to the testosterone action which only blocked the mitogenicity of the magnesium-dependent high concentrations of cyclic nucleotides, oestradiol only inhibited the calcium-dependent low concentrations (Figures 4·2a,b).

In an attempt to elevate endogenous or intracellular cyclic nucleotide levels without implicating adenylate or guanylate cyclase (as might be the case with hormonal treatments) the phosphodiesterase inhibitor (Sheppard, Wiggan and Tsien, 1972) Ro 20-1724 was added to the cultures. At concentrations between 10^{-5} and 10^{-8}M a significant increase in mitotic activity was evident (Figure 4·2c).

As with the high concentrations of the cyclic nucleotides themselves, the optimal (10^{-7}M) stimulatory level of Ro 20-1724 was inhibited by either testosterone addition or magnesium omission (Figure 4·2d). No blockade was induced by either calcium-free medium or oestradiol addition to the cell suspensions.

Figure 4·2c.

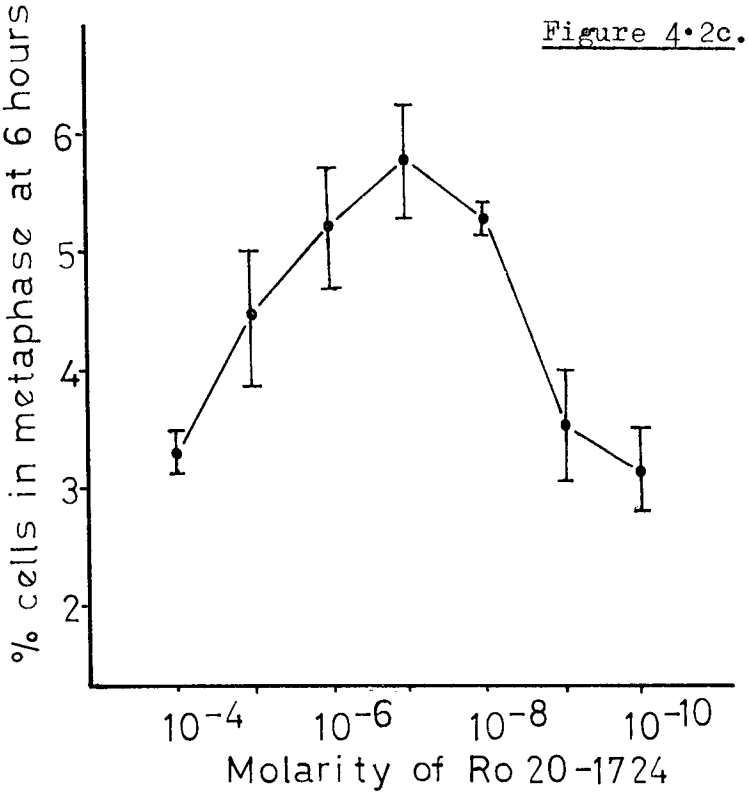


Figure 4·2d.

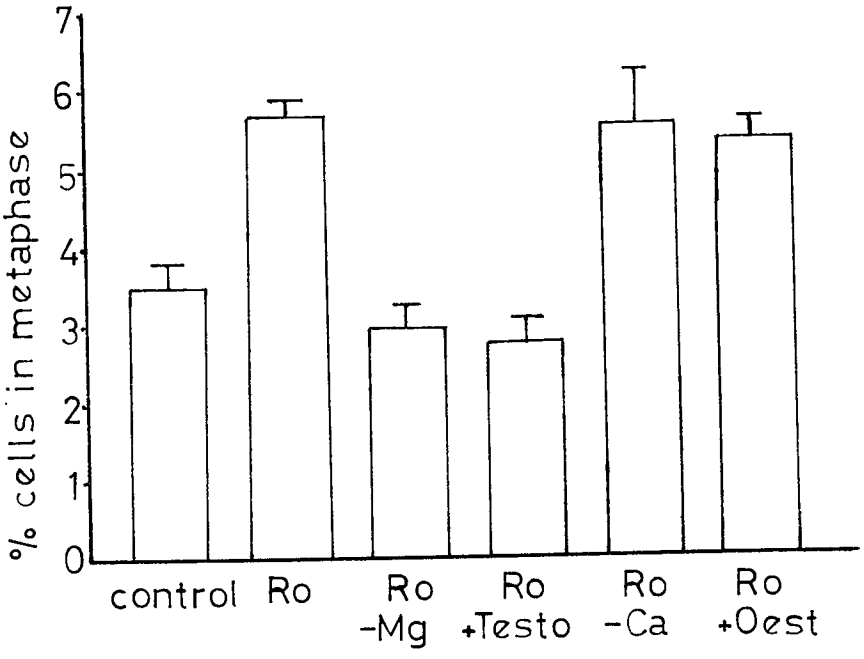


Figure 4·2c. The mitogenic action of Ro 20-1724. Cells were incubated in Medium 199 containing 0·6mM calcium chloride and colchicine and exposed to various concentrations of Ro 20-1724. This was the gift of Dr. H. Sheppard of Hoffman-La Roche Inc. and was dissolved in ethanol. Results are the mean ± 1 S.E.M. of the percentage cells in 'colchicine metaphase' at six hours of 4 experiments.

Figure 4·2d. The ionic requirements and steroid sensitivity of Ro 20-1724-induced mitogenesis. Cells were cultured in Medium 199 containing colchicine and either 0·6mM (control) or calcium-free (-Ca) conditions and in which the magnesium levels were adjusted to 1·0mM (control) or zero (-Mg). In addition cultures also contained testosterone (Testo) or oestradiol (Oest) at 0·1$\mu g.ml^{-1}$. These suspensions were then exposed to Ro 20-1724 (10^{-7}M). Results are means +1 S.E.M. of 6 determinations.

Table 4·2e. The Mitogenic Potential Of Various Nucleotides.

Nucleotide	Magnesium-dependent mitogenic concentration	Calcium-dependent mitogenic concentration
Cyclic adenosine 3',5'-monophosphate	10^{-7}M	10^{-13}M
Dibutyryl cyclic adenosine 3',5'-monophosphate	10^{-7}M	10^{-13}M
Cyclic adenosine 2',3'-monophosphate	10^{-5}M	10^{-8}M
2'-deoxy cyclic adenosine 3',5'-monophosphate	10^{-6}M	10^{-11}M
Adenosine 5'-monophosphate	N.D.	N.D.
Adenosine 5'-diphosphate	N.D.	N.D.
Inosine 5'-triphosphate	N.D.	N.D.
Cyclic guanosine 3',5'-monophosphate	10^{-6}M	10^{-11}M
Dibutyryl cyclic adenosine 3',5'-monophosphate	10^{-6}M	10^{-11}M
Guanosine 5'-diphosphate	N.D.	N.D.
Guanosine 5'-monophosphate	N.D.	N.D.
Cyclic guanosine 2',3'-monophosphate	N.D.	N.D.
Cyclic cytidine 3',5'-monophosphate	N.D.	N.D.

Table 4·2e. The mitogenic potential of various nucleotides. Cells were incubated in Medium 199 containing colchicine and various regimes of calcium and magnesium in order to ascertain the concentrations at which any of the tested nucleotides stimulated mitosis, and if this action depended on the presence of either magnesium or calcium. The nucleotides listed were added at 10-fold concentration steps over the range 10^{-14} to 10^{-5}M. The optimum mitogenic concentration at which an agent stimulated mitotic recruitment in an ion-dependent fashion is listed and is derived from between 4 and 22 separate determinations. Where mitogenic concentrations proved to be magnesium- or calcium-dependent as shown they were invariably blocked by testosterone and oestradiol respectively ($0·1\mu g.ml^{-1}$). N.D. indicates no detectable mitogenic potential over the concentration range cited above. All nucleotides were supplied by the Boehringer Corporation.

In an attempt to further characterise the interactions of the cyclic nucleotides with the thymic lymphocyte a range of other nucleotides were tested for their mitogenic potencies as indicated in Table 4·2e. The dibutyryl derivatives of the cyclic purines also exhibited a biphasic mitogenic action. The effective concentrations were similar to those of the parent nucleotides. The high mitogenic levels again required the presence of magnesium and could be blocked by testosterone. The low concentrations required calcium ions and were inhibited by the presence of oestradiol. This pattern of ionic dependency and steroid blockade was also shown by 2'-deoxy cyclic adenosine 3',5'-monophosphate and cyclic adenosine 2',3'-monophosphate (Table 4·2e). However markedly higher concentrations of these nucleotides had to be used to elicit comparable stimulation of mitosis. A series of other cyclic and non-cyclic nucleotides over a concentration range between 10^{-5} to 10^{-14}M had no discernable effect on mitotic activity (Table 4·2e).

4·3 Some implications of cyclic nucleotide-induced mitogenesis.

Both cyclic AMP and cyclic GMP exhibit mitogenic activity over two distinct concentration ranges; thus high (between 10^{-6} and 10^{-8}M) and low (between 10^{-10} and 10^{-13}M) concentrations of these cyclic purines enhance thymocyte proliferation. The high exogenous concentration of cyclic AMP and the low exogenous concentration of cyclic GMP are thought to approximate to the normal intracellular levels of these nucleotides (Goldberg, O'Dea and Haddox, 1973). Indeed an endogenous increase in their concentration presumably either by hormonal activation of the appropriate cyclase enzymes (MacManus, Whitfield and Youdale, 1971; MacManus, Boynton, Whitfield et al., 1975)

or by phosphodiesterase inhibition by Ro 20-1724 or methyl xanthines (Units 2 and 5; Figure 4·2c) is likewise accompanied by a stimulation of thymocyte mitosis. Thus cyclic AMP and cyclic GMP may indeed be the common factors in the initiation of events which lead ultimately to mitosis in these different situations.

The agents glucagon, dopamine, adrenalin and acetylcholine which have been proposed to stimulate thymocyte adenylate cyclase at the appropriate concentration also have in common with the high mitogenic concentration of cyclic AMP a requirement for magnesium ions. High concentrations of cyclic GMP fall into this same magnesium-requiring category (Figure 4·2b). It has been previously demonstrated that this unphysiological concentration of the nucleotide elevates endogenous cyclic AMP levels by inhibiting cyclic AMP-phosphodiesterase (Whitfield, MacManus, Franks, Gillan and Youdale, 1971). Thus cyclic AMP would act as the ultimate magnesium-dependent mitogen in this instance. If cyclic AMP is the ultimate magnesium-dependent mediator of these presumptive cyclase stimulators it might be expected that the compound Ro 20-1724, which inhibits predominantly the cyclic AMP- rather than the cyclic GMP-phosphodiesterase (Sheppard et al., 1972) would be a magnesium-requiring mitogen also. This predicted property was in fact clearly demonstrated (Figures 4·2c,d).

Although the activation of adenylate cyclase by compounds such as glucagon, isoprenaline and adrenalin is itself a magnesium-dependent event (Unit 3) there must be a second reaction which is triggered by the rise in endogenous cyclic AMP, or by high exogenous concentrations of cyclic AMP as in the present study, which also needs magnesium and is clearly blocked by testosterone. Evidence from adipocytes in which there is increased magnesium uptake following exposure

to the adenylate cyclase stimulants adrenalin and adrenocorticotrophic hormone (Elliott and Rizack, 1974) supports the notion that cyclic AMP may regulate magnesium ion transfer in these cells. Thus in the thymic lymphoblast when exposed to mitogens which stimulate adenylate cyclase one might visualise an increment in cyclic AMP and an enhanced magnesium influx to the cell which initiates the events culminating in mitosis. Testosterone since it blocks the mitogenic action of high exogenous cyclic AMP and cyclic GMP, adenylate cyclase-stimulating hormonal mitogens, Ro 20-1724 and raised extracellular magnesium concentrations must then either block the magnesium influx or a step subsequent to it.

In an analagous fashion low exogenous cyclic GMP and cyclic AMP concentrations and calcium-dependent hormonal mitogens such as PTH, insulin, acetylcholine and histamine (possibly by stimulating guanylate cyclase and increasing intracellular cyclic GMP) could provoke a calcium influx in these cells. Certainly PTH, insulin and dibutyryl cyclic GMP are known to do this in certain cell types including splenic T lymphocytes (Borle, 1970; Kafka and Pak, 1969; Freedman et al., 1975). Oestradiol would then perhaps impede this calcium movement and in so doing block the mitogenic action of all these agents.

It should be pointed out that although these results agree with other authors (Whitfield, MacManus, Rixon and Gillan, 1973) that exogenous cyclic GMP is mitogenic over two distinct concentration ranges, they believe that the low mitogenic concentration of cyclic GMP is calcium-independent and the high concentration calcium-dependent, i.e. the reverse of the findings reported here. Even when experiments were repeated with cultures containing EGTA to remove any trace amounts of calcium, our results were substantiated. Furthermore, oestradiol

which only inhibits calcium-dependent mitogens clearly blocked the action of the low, but not the high concentrations of both cyclic GMP and cyclic AMP. Differences in buffering systems, cell densities and strain of rat used may all account for the opposite results of the two laboratories.

These findings would be consistent with an action of cyclic AMP and cyclic GMP on two separate kinase enzymes associated with the plasma membrane (Figure 4·3a). These enzymes might be involved in the transmembrane movement of calcium and magnesium and possibly other ions. Alternatively calcium and magnesium may simply be obligatory cofactors for the kinase activations. The relatively poorer mitogenic potential of cyclic adenosine 2',3'-monophosphate and 2'-deoxy cyclic adenosine 3',5'-monophosphate may be a reflection of the relative affinities of the kinases for the cyclic nucleotide concerned. In addition since the breakdown products of the two nucleotides are without effect on mitotic activity it must be inferred that the response to the cyclic nucleotides is a specific interaction and not the result of some secondary metabolic action as may be the case for cyclic AMP used in high concentrations in other cell types (Ishii and Green, 1973). Indeed this kinase system could represent the membrane activator site for cyclic AMP proposed by other workers (MacManus, Whitfield and Braceland, 1971; MacManus and Whitfield, 1972). It is possible therefore that physiological variations in cyclic nucleotide concentrations within the cell, or in compartments thereof, might determine the divalent cationic climate within the cell. In turn intracellular calcium and magnesium levels might dictate the movements of other ions and whether or not certain quiescent cells will initiate DNA synthesis and go on to divide. Such mechanisms could be of fundamental importance in the mitotic economies of normal and malignant cells, and their response to hormonal stimuli.

Figure 4·3a. A Model To Illustrate The Possible Relationships Between Hormones, Ions And Cyclic Nucleotides In Thymocyte Division.

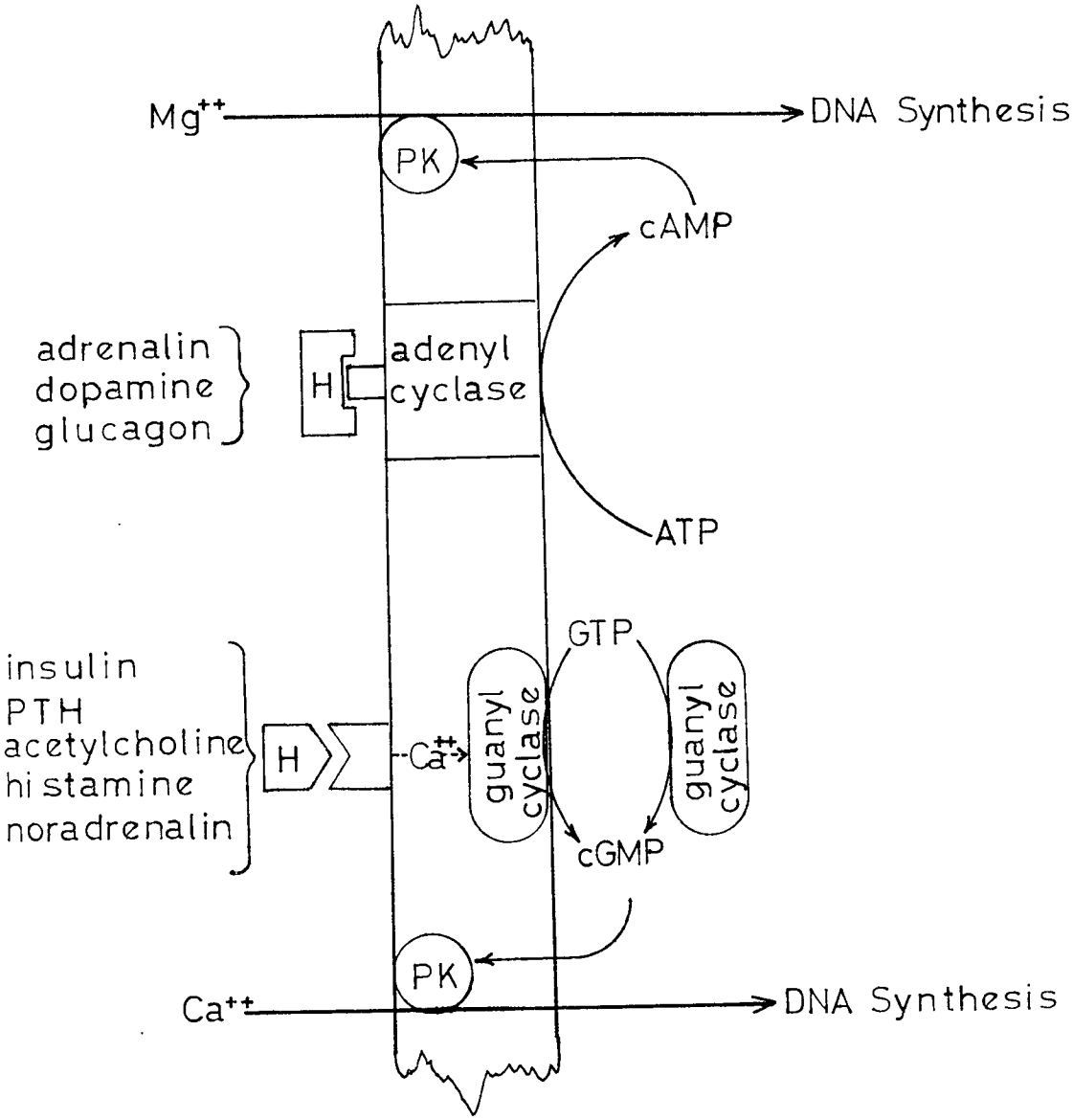


Figure 4·3a. A possible mechanism which would account for the observed ionic dependencies and steroid blockade of ionic, hormonal and cyclic nucleotide stimulated mitotic activity in thymocytes. The hormones adrenalin, dopamine and glucagon stimulate adenyl cyclase via specific membrane receptors thus raising endogenous cyclic AMP levels. The cyclic AMP thus generated alters the activity of a membrane-bound protein kinase (PK) which initiates some process which either transports or has a requirement for magnesium ions. The consequence of this interaction is the entry of quiescent cells into their DNA synthetic phase. Testosterone blocks this process at or after the magnesium-dependent step. In contrast insulin, PTH, acetylcholine, histamine and noradrenalin stimulate guanylate cyclase via their receptor system. Since this enzyme may be soluble or membrane bound, the receptor interaction may promote a calcium movement into the cell which stimulates both types of guanyl cyclases. In an analagous fashion the cyclic GMP formed stimulates a different kinase which involves calcium transport and which is blocked

Figure 4·3a continued. at the calcium-dependent stage or subsequent to it by oestradiol. This process also leads via unknown mechanisms to the initiation of DNA synthesis.

Unit 5. The metabolism of cyclic nucleotides.

The level of a cyclic nucleotide at any one point in time is the resultant of its synthesis via the respective cyclase enzyme and loss whether this be through degradation by cyclic nucleotide phosphodiesterases, removal by binding to enzymes or other protein moieties or translocation across the plasma membrane. These various possibilities will be dealt with in turn, with special reference to potential involvement in proliferative control.

5·1 The enzyme adenylate cyclase; its function and regulation.

Adenylate cyclase is a particulate enzyme which catalyses the conversion of Mg-ATP to cyclic AMP and pyrophosphate (Rall and Sutherland, 1962). The enzyme is normally activated upon receipt of a hormonal stimulus by the target cell. Although the catalytic action is identical in many cell types the increased activity may only be expressed following the highly stereospecific interaction of a triggering hormone at the cell surface. Thus it was reasoned that the enzyme comprised at least two subunits. The first, which was orientated towards the outer surface of the cell was a receptor unit which was characteristic for a given hormone and therefore cell specific. The second was the catalytic unit which was located towards the inner membrane palisade and was common to most cell types (Robison, Butcher and Sutherland, 1967). It was proposed that the receptor unit was linked in some way to the catalytic unit and regulated its activity (Robison et al., 1967).

It now seems likely that a third so-called regulatory or

transducer subunit may provide a link between receptor and catalytic subunits of the complex (Birnbaumer, Pohl, Krans and Rodbell, 1970). The interaction of GTP with the nucleotide regulatory site (a functional locus of the transducer portion of the enzyme) enhances the response of the enzyme to hormonal activation (Rodbell, Lin and Salomon, 1974; Aurbach et al., 1975; Schramm, 1975). In addition an analogue of GTP, 5'-guanylylimidodiphosphate (Gpp(NH)p) which is not degraded by nucleotide phosphohydrolases present in great amounts in membrane preparations, causes a marked elevation of adenylate cyclase activity even in the absence of a hormone (Rodbell, Lin, Salomon et al., 1975). This action of Gpp(NH)p is competitively inhibited by GTP and ITP, hence it is inferred that GTP and ITP also act at the nucleotide regulatory site (Bilezikian and Aurbach, 1974; Rodbell et al., 1975). It might also be noted that GDP antagonises both the stimulatory actions of hormones and guanyl triphosphates (Rodbell et al., 1975). It has been proposed that the nucleotide regulatory site affects the sensitivity of the catalytic subunit to hormonal activation of the receptor unit by bringing about conformational changes in the enzyme, these different configurations of the enzyme being termed multisite transition states (Rodbell et al., 1975).

A further source of adenylate cyclase control is thought to be via the so-called feedback regulator (FR). This low molecular weight agent is synthesised in response to cyclic AMP (Ho and Sutherland, 1971) and acts on the plasma membrane to inhibit cyclase activity in a non-competitive fashion (Ho and Sutherland, 1975). It also acts at other intracellular loci to enhance the action of cyclic AMP on target enzymes (Ho and Sutherland, 1975). Such an action distinguishes FR from prostaglandins and adenosine, which also exhibit negative feedback control of adenylate cyclase (Robison et al., 1971).

5·2 Guanylate cyclase; an enzyme catalysing the formation of cyclic GMP.

The formation of cyclic GMP from GTP is via the enzyme guanylate cyclase (Hardman and Sutherland, 1969). There is some controversy at present as to the location of the enzyme itself, although it does appear as if it may be both soluble and particulate (Hardman and Sutherland, 1969; White, Aurbach and Carlson, 1969). More recent reports indicate that it may have a greater association with membrane components than formerly thought (White, 1975) however the actual site is still believed to be different from that of adenylate cyclase which is found predominantly in the plasma membrane. White (1975) suggests that it is the soluble form of the guanylate cyclase enzyme which is active, and that agents which promote the solubilisation of the enzyme from its interaction with membrane-like units will elevate endogenous cyclic GMP concentrations. Such a mechanism is proposed to account for the slow elevation of uterine cyclic GMP levels by oestrogens (Kuehl, Ham, Zanetti et al., 1974).

Calcium is known to be able to stimulate the activity of both soluble (Hardman, Beavo, Gray et al., 1971; Hardman et al., 1972) and particulate guanylate cyclase (Schultz, Hardman, Schultz et al., 1973). Thus the rapid increase in cyclic GMP levels following application of either cholinergic agents or high levels of potassium which is mediated through a calcium redistribution (Schultz et al., 1973) may represent a direct stimulation of cyclase by calcium and not a solubilisation of microsomal-bound enzyme. Alternatively it may indicate an action on cyclic GMP-phosphodiesterase by calcium ions, which are certainly known to modulate the activity of this enzyme (Kakiuchi, Yamazaki and Teshima, 1972). Such possibilities will now be considered in the section on phosphodiesterases and their regulation.

5·3 Cyclic nucleotide phosphodiesterases; their action and regulation.

The main problem in dealing with the regulation of phosphodiesterase activity is one of classification since there are a spectrum of enzymes which variously convert 3',5'-cyclic nucleotides to their 5'-derivatives. These enzymes may be divided into three basic categories (1) those which hydrolyse cyclic GMP with some specificity (cyclic GMP-phosphodiesterases); (2) those which convert both cyclic AMP and cyclic GMP to their 5'-derivatives with roughly equal efficiency and (3) those which degrade cyclic AMP preferentially (Russell, Terasaki and Appleman, 1973). In addition these various classes themselves contain multiple enzyme forms with specific activities and substrate affinities for given nucleotides (Weiss, 1975). It is likely that it is the unique pattern of phosphodiesterases within a particular cell which gives it many of its differentiated properties.

Cyclic AMP-PDE (DIII) is thought to be under negative cooperative control by its own substrate, cyclic AMP (Russell, Thompson, Schneider and Appleman, 1972). The enzyme is particularly associated with the plasma membrane (Appleman and Terasaki, 1975) and it is believed that treatment of cells with various hormonal stimuli will induce further enzyme formation although the mechanism is not well understood (House, Poulis and Wiedemann, 1972). The mainly soluble PDE DII (Weiss, 1975) hydrolyses both cyclic AMP and cyclic GMP at roughly equal rates and indeed each nucleotide inhibits the degradation of the other in a competitive fashion (Appleman and Terasaki, 1975). In addition cyclic GMP at low and possibly physiological concentrations also activates PDE DII via an allosteric activator site, cyclic AMP does not share this property (Russell *et al.*, 1973; Appleman and Terasaki, 1975). Thus changes in cyclic GMP concentrations may influence

cyclic AMP metabolism through a positive cooperative mechanism. Perhaps the most interesting of the phosphodiesterases is DI, the 'cyclic GMP-phosphodiesterase'. This enzyme has a protein activator which was formerly thought to be regulating the activity of a cyclic AMP phosphodiesterase (Cheung, 1971). The activation of the PDE by this factor, P.A.F. (phosphodiesterase activating factor) requires calcium, and indeed calcium appears to stimulate the activity of PDE in heart (Kakiuchi, Yamazaki, Teshima and Ueneshi, 1973). Thus the mechanism (after Teshima and Kakiuchi, 1974) shown below has been proposed to be operative:-

$$(\text{Enzyme})_{\text{inactive}} + \text{PAF} + Ca^{++} \rightleftharpoons (\text{Enzyme-PAF-}Ca^{++})_{\text{active}}$$

Thus it is thought that calcium levels will determine the equilibrium point at which this reaction is set.

Clearly the overall balance of cyclic nucleotide hydrolysis will be a complex interaction between these various classes of phosphodiesterases.

5·4 Cyclic nucleotide-dependent protein kinases.

A large number of physiological processes influenced by cyclic AMP and cyclic GMP are known to be modulated by the enzymatic phosphorylation of proteins (Krebs, 1972). The enzymes involved in these processes are termed cyclic nucleotide-dependent protein kinases, and the mode of action of cyclic purines upon these enzymes has been partially resolved. Thus in a number of situations the protein kinase consists of two subunits, one the catalytic unit is unaffected by cyclic AMP, but it is found in association with a second regulatory

subunit which will bind cyclic AMP and upon doing so dissociates from the catalytic unit thus activating it (Krebs, 1972). This may be depicted:-

$$R \cdot C + cAMP \rightleftharpoons R \cdot cAMP + C$$

where R is the regulatory subunit; C is the catalytic unit of the protein kinase; and R•C is the inactive form of the complex. This is an equilibrium and factors other than the concentrations of cyclic AMP and regulatory and catalytic subunits may influence the position of the equilibrium point. Such effectors include Mg-ATP which decreases the affinity of the kinase for cyclic AMP, and thus promotes the recombination of subunits (Beavo, Bechtel and Krebs, 1975). Such mechanisms also serve to sequester cyclic AMP, hence lowering its effective concentration. It might also be noted in passing that these cyclic nucleotide-dependent kinases have been used as the basis of a number of assays for cyclic AMP and indeed cyclic GMP.

It has also been suggested that not only do protein kinases bind cyclic AMP, but there may be translocation of these complexes to other intracellular sites in a manner analagous to the steroid-receptor protein mechanism (Jungman, Lee and DeAngelo, 1975). Such phenomena are thought to be able to account for the apparent action of cyclic nucleotides upon genetic transcription.

5·5 The role of guanyl nucleotides in the regulation of thymocyte proliferation.

Somewhat paradoxically the first results to be reported here do not involve the assay of cyclic nucleotide metabolism but are rather an extension of the previous Unit relating to the role of guanine nucleotides in thymic lymphocyte proliferation. Whilst cyclic AMP and cyclic GMP as well as a number of their analogues stimulated mitotic activity in thymocyte cultures, it was noted that other non-cyclic guanyl nucleotides had stimulatory actions on the proliferation of these cells. Figure 5·5a indicates that both GTP and the GTP-phosphohydrolase-resistant analogue, Gpp(NH)p, mentioned in section 5·1 stimulate division at a concentration of 10^{-7}M. In this prior section it was mentioned that Gpp(NH)p could indeed enhance adenylate cyclase activity in a number of broken cell preparations (Rodbell *et al.*, 1975) and that GTP could synergise with cyclase-stimulating hormones to raise the activity of the enzyme (Rodbell *et al.*, 1974). Thus it could be that these two nucleotides are acting as adenylate cyclase stimulants, provoking an increase in endogenous cyclic AMP and initiating a G_o recruitment. This possibility is supported by the observations that both nucleotides are magnesium-dependent and their mitogenic activity is muted by the presence of testosterone in the culture medium (Table 5·5b) as were the adenylate cyclase activating hormones, the phosphodiesterase inhibitors and cyclic AMP itself. In addition it was noted that whilst ITP did not influence mitotic activity *per se* (Figure 5·5a) it did ablate the mitogenic ability of both GTP and Gpp(NH)p, GDP however had no effect (Figure 5·5c). It might be recalled that ITP could compete with both Gpp(NH)p and GTP for the nucleotide receptor site of the erythrocyte adenylate cyclase (Bilezikian and Aurbach, 1974). It was somewhat surprising that GDP did

Figure 5·5a. The influence of GTP, Gpp(NH)p, ITP and GDP on thymocyte proliferation.

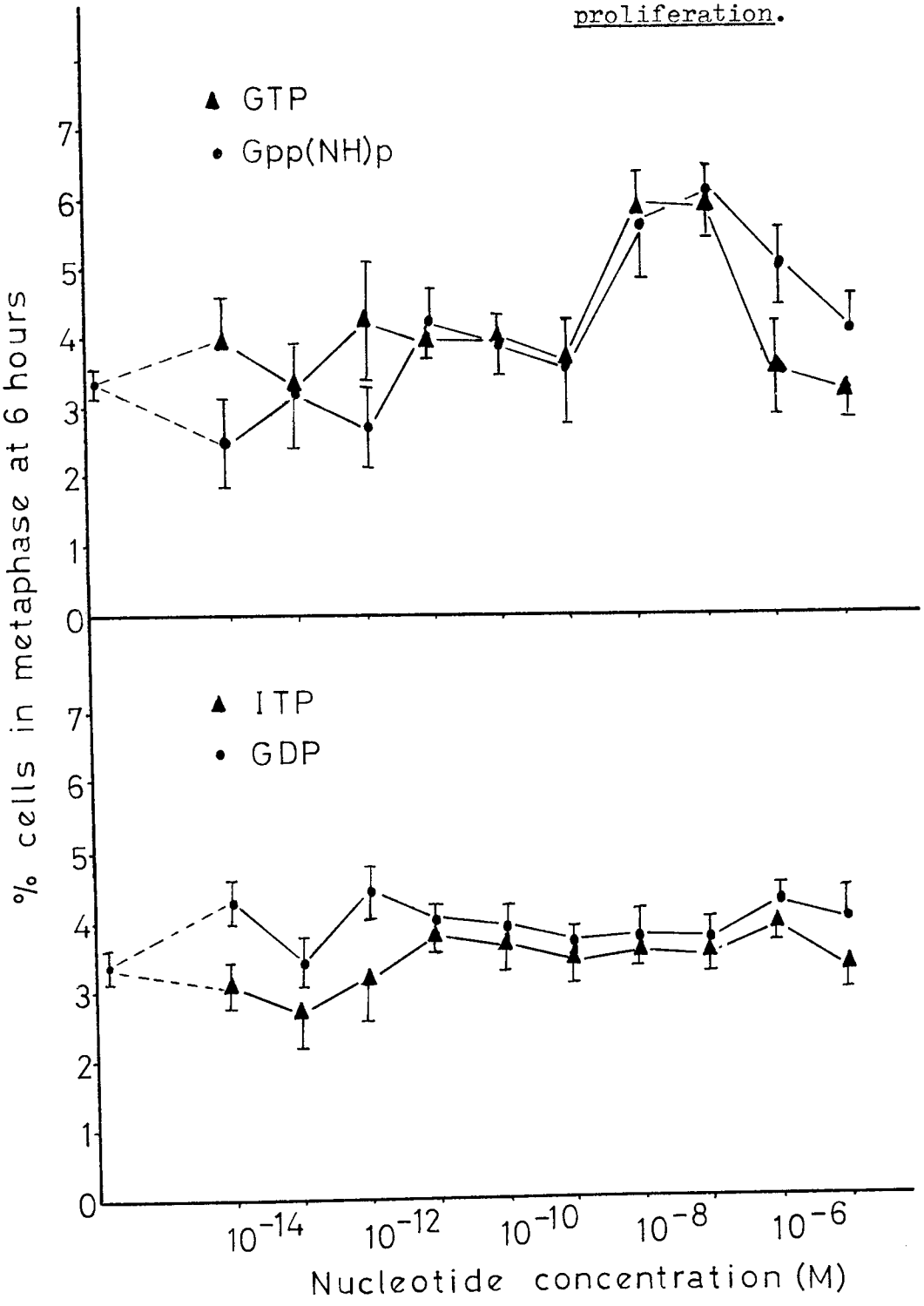


Figure 5·5a. The influence of guanyl nucleotides and ITP on thymocyte proliferation. Cells were incubated in Medium 199 containing colchicine and basal levels of calcium and magnesium. Cultures were then exposed to GTP, Gpp(NH)p, GDP or ITP at various concentrations (all nucleotides were obtained from the Boehringer Corporation). The results represent the mean percentage cells in metaphase at six hours, and are derived from between 4 and 6 separate experiments. Vertical lines with bar denote the standard error of the mean.

Table 5·5b. The Ionic Dependence And Steroid Blockade Of Guanyl Nucleotide-Induced Mitogenesis.

Ionic Or Steroid Status Of Medium	Gpp(NH)p 10^{-7}M	GTP 10^{-7}M
0·6mM Ca^{++} 1·0mM Mg^{++}	5·7 ± 0·23% *	6·1 ± 0·27% *
0·0mM Ca^{++} 1·0mM Mg^{++}	5·8 ± 0·46% *	5·3 ± 0·24% *
0·6mM Ca^{++} 1·0mM Mg^{++} + Oestradiol.	5·4 ± 0·33% *	6·1 ± 0·43% *
0·6mM Ca^{++} 0·0mM Mg^{++}	3·3 ± 0·24%	3·1 ± 0·30%
0·6mM Ca^{++} 1·0mM Mg^{++} + Testosterone	3·5 ± 0·25%	4·1 ± 0·40%

Table 5·5b. The influence of divalent cations and steroids on guanyl nucleotide-induced mitogenesis. Thymocytes were cultured in Medium 199 containing colchicine and the combinations of steroids ($0·1 ug.ml^{-1}$) and ions indicated in the table. Cultures were then exposed to GTP or Gpp(NH)p at a concentration of 10^{-7}M and six hour metaphase accumulations performed. The results are the mean percentage cells in metaphase at six hours ± 1 S.E.M. derived from between 4 and 6 experiments. Where a value is significantly higher than the basal control (3·4 ± 0·16%) this is indicated by an asterisk.

not inhibit this action of guanyl nucleotides since it is, in broken cell preparations, a potent inhibitor of both hormonal and nucleotide-stimulated adenylate cyclase activity (Rodbell et al., 1975). This lack of effect could be attributable to various factors (1) poor penetration of this nucleotide into the cell, or its rapid metabolism (2) GDP may not in this cell type possess the blocking activity which was observed in fat and liver cells (3) the action might be an artifact of the broken cell assay (4) the guanyl nucleotides might not be acting upon the cyclase at all but at some other site such as the membrane

Figure 5·5c. The influence of ITP and GDP on guanyl nucleotide-induced mitogenesis.

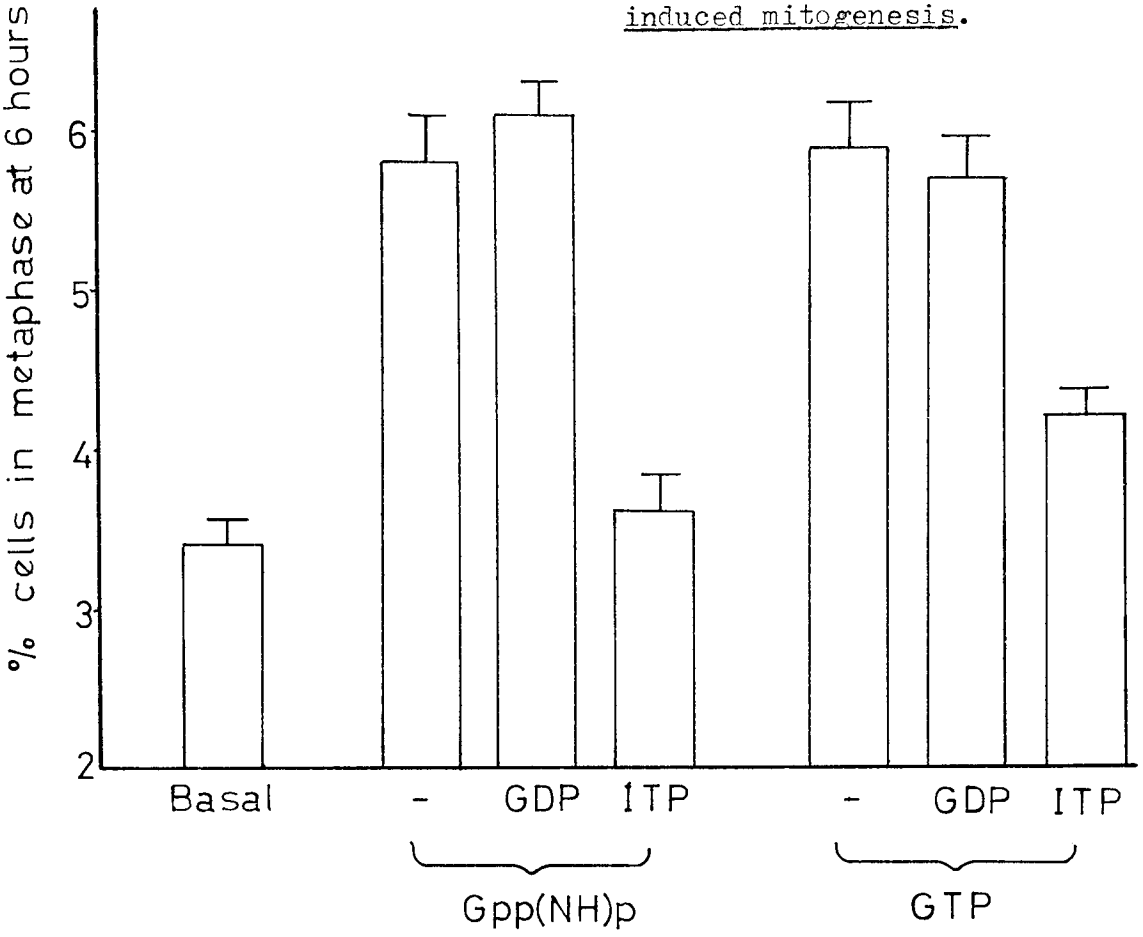


Figure 5·5c. The inhibitory action of ITP upon GTP and Gpp(NH)p stimulated mitotic activity. Thymocytes were maintained in Medium 199 containing colchicine and calcium (0·6mM). Cultures were then exposed to combinations of GTP, Gpp(NH)p, ITP and GDP (all 10^{-7}M) and six hour metaphase accumulations performed. Where a horizontal dash (-) appears below a column this indicates that the guanyl nucleotide was tested in the basal medium (0·6mM calcium, 1·0mM magnesium) with no further additions. Results are the mean (column) plus one S.E.M. (vertical line with horizontal bar) of between 5 and 8 separate experiments.

receptor postulated to exist in Unit 4. In an attempt to preclude the latter possibility ITP was next tested for its ability to inhibit other mitogens (Table 5·5d). It was clear that it only inhibited the adenylate cyclase stimulator, isoprenaline, and the high, magnesium-dependent concentration of cyclic GMP. The calcium-dependent mitogen, histamine, was unaffected by ITP as was calcium itself and magnesium. Cyclic AMP at its high mitogenic concentration and both cyclic AMP and cyclic GMP at their low, calcium-dependent concentrations were also insensitive to

Table 5·5d. The Influence Of ITP Upon The Ability Of Various Agents To Initiate Thymocyte Proliferation.

Mitogen	No ITP	ITP 10^{-7}M
None	3·9 ± 0·28%	3·7 ± 0·22% *
Isoprenaline 10^{-6}M	5·6 ± 0·24%	3·4 ± 0·22% *
Histamine 10^{-12}M	5·5 ± 0·21%	5·8 ± 0·33%
Calcium chloride 1·2mM	5·3 ± 0·24%	5·6 ± 0·19%
Magnesium sulphate 2·5mM	5·4 ± 0·27%	6·2 ± 0·32%
Cyclic AMP 10^{-7}M	7·1 ± 0·62%	6·9 ± 0·63%
Cyclic AMP 10^{-13}M	7·2 ± 0·31%	6·6 ± 0·59%
Cyclic GMP 10^{-6}M	6·7 ± 0·66%	4·3 ± 0·43% *
Cyclic GMP 10^{-7}M	7·5 ± 1·10%	4·8 ± 0·75% *
Cyclic GMP 10^{-11}M	6·5 ± 0·26%	6·0 ± 0·26%
Cyclic GMP 10^{-12}M	7·2 ± 0·67%	7·6 ± 0·93%

Table 5·5d. The influence of ITP on mitogen-induced proliferation in thymic lymphocyte cultures. Thymocytes were maintained in Medium 199 containing colchicine and basal calcium and magnesium. The cells were then exposed to a range of mitogens both in the presence and absence ITP (10^{-7}M) and six hour metaphase accumulations scored. The results represent the means ± 1 S.E.M. of the percentage cells in metaphase at six hours from between 4 and 10 separate experiments. Where a value is not significantly higher than basal control containing no mitogen or ITP ($p > 0·1$) this is indicated by an asterisk.

the presence of ITP in the cultures. It would have been anticipated that if ITP were an antagonist for the membrane receptor, then it would have inhibited the action of cyclic AMP at its magnesium-dependent concentration. Thus it would seem likely that Gpp(NH)p and GTP stimulate thymocyte adenyl cyclase, and that this should be inhibited in some way by ITP. In addition it would then follow that the high

concentration of cyclic GMP might directly, or after further metabolism stimulate adenylate cyclase, thus proffering a mechanism for this apparently inappropriate response. Some of these speculations could be resolved by directly assaying membrane adenylate cyclase activity. Thus a number of experiments were performed at the University of Sheffield Department of Chemical Pathology to elucidate some of these propositions.

5·6 The assay of adenylate cyclase.

An exhaustive description of the adenylate cyclase assay would be out of place in this thesis as it has been described in detail elsewhere (Rodbell, 1972). Only a brief outline will be given here pointing out the most important features of the analysis of the activity of this enzyme. Most cyclase assays depend on the conversion of radiolabelled ATP to labelled cyclic AMP by broken cell preparations (Rodbell, 1972). The most frequently used isotope is ^{32}P-ATP with the label in the alpha position. The ^{32}P-cyclic AMP thus generated has to be separated from both inorganic ^{32}P as well as remaining labelled ATP and many of its degradation products. This is carried out by column chromatography first on neutral alumina and then Dowex 50 as described by Salomon et al (Salomon, Londos and Rodbell, 1974). The ^{32}P-cyclic AMP fraction is collected and counted in Brays scintillant (Bray, 1960).

The assay mixture needs to contain not only ^{32}P-ATP and buffer but also a phosphodiesterase inhibitor. In the assays reported here theophylline was employed at a final concentration of 3mM, and in all cases except those in which the effects of cyclic AMP itself on adenyl cyclase were to be examined the mixture also contained 1mM cyclic AMP.

At this concentration the nucleotide inhibits phosphodiesterase and clearly also acts as a carrier to protect labelled cyclic AMP generated by the system. A further important consideration is the action of membrane ATPases which show great activity in such preparations and rapidly degrade this nucleotide. Thus agents which alter ATPase activity might ostensibly alter adenylate cyclase function by changing ATP substrate availability. Thus the assay also employs an ATP regenerating system. This was either pyruvic kinase/phosphoenolpyruvate or creatine kinase/phosphocreatine, in addition the mix also contained excess ATP in order to further protect labelled nucleotide.

Cell membranes were prepared by mincing thymuses, and then disrupting them by four strokes of a Dounce homogeniser. The cell homogenate in cold ($4^{o}C$) 25mM Tris-HCl, pH 7·4, containing 0·25M sucrose and 1mM tetrasodium EDTA was centrifuged in the SS34 rotor of a Sorvall RC2-B centrifuge, also at $4^{o}C$. The centrifuge was switched on, allowed to reach 2,000 rpm (480g) and immediately slowed with the brake applied. The supernatant was further centrifuged (same rotor) at 2,200 x g for 10 minutes at $4^{o}C$ and the resultant pellet then resuspended in 25mM Tris-HCl, pH 7·4, buffer containing 0·013% (w/v) bovine serum albumin (Armour Ltd.); 30mM KCl and 4·5mM $MgCl_2$ to give a partially purified preparation of plasma membranes. The protein concentration of this preparation was estimated by the method of Hartree (1972) with bovine serum albumin as standard.

Typically assay mixtures consisted of the following components in a volume of 0·1ml: 25mM Tris-HCl, pH 7·4; 1mM ($-^{32}P$)-ATP; 1mM cyclic AMP; 2·5mM phosphoenolpyruvate; 1·5 i.u. pyruvate kinase; 0·013% bovine serum albumin; 30mM KCl; 4·5mM $MgCl_2$; 150-400ug membrane protein preparation; and agent under test. The reaction was started by adding

the plasma membrane suspension, continued at 37°C for 12 minutes and terminated by addition of 0·1ml 40mM ATP and boiling for 2 minutes. After removal of the membranes by centrifugation, the ^{32}P-ATP was separated by chromatography as described.

All assays were performed in triplicate and results in pmoles of cyclic AMP generated were calculated from the equation:-

$$\text{Cyclic AMP formed (pmoles)} = \frac{(\text{mean of treatment counts}) - \text{background}}{\text{total cpm} \times 10^{-5}}$$

The amount of nucleotide generated during the 12 minutes of assay was finally corrected for protein content and expressed as pmoles of cyclic AMP.minute^{-1}.mg protein^{-1}.

5·6·2 The action of guanyl triphosphates on thymocyte adenylate cyclase.

Gpp(NH)p provoked a large increase in adenylate cyclase activity at concentrations of 10^{-5} and 10^{-4}M, the response being marginal at 10^{-6}M (Figure 5·6·2a). GTP, however, only showed a slight increase over basal activity at 10^{-4}M, which was not significant ($p>0·2$). ITP similaraly showed a small effect at 10^{-4}M, this being at the limits of significance (Figure 5·6·2a). The mitogenic action of Gpp(NH)p could thus be explained by a stimulation of adenylate cyclase. It is unlikely that this could be the explanation for GTP unless of course the mitotic system contained some intrinsic thymocyte adenylate cyclase stimulant which could be made more effective by the presence of GTP. Such an agent might be thymosin. As in other broken cell systems (Bilezikian and Aurbach, 1974; Rodbell et al., 1975) GTP inhibits the stimulatory action of Gpp(NH)p in a dose-dependent fashion (Figure

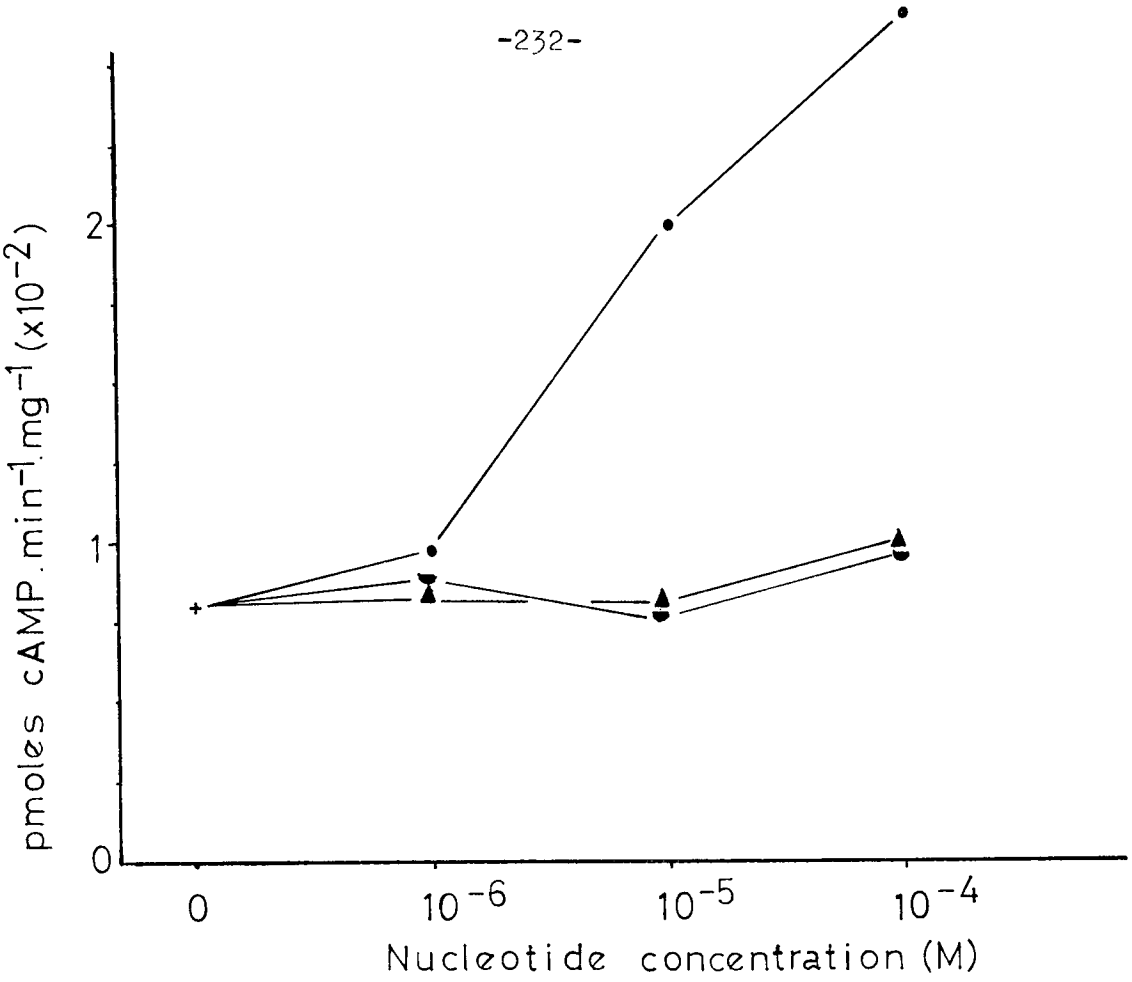


Figure 5·6·2a. The action of GTP, Gpp(NH)p and ITP on thymocyte adenylate cyclase activity. Assays were performed as described in the text with incubation mixtures containing Gpp(NH)p (•), GTP (◒) ITP (▲) at the concentrations indicated, or with no further additions (+). Each point is the mean of two values performed in triplicate.

5·6·2b). Hence it might be inferred that 5'-guanylylimidodiphosphate is acting through the nucleotide receptor site of adenylate cyclase. However whilst ITP blocked the mitogenic action of Gpp(NH)p and GTP it did not inhibit the stimulatory action of the former upon adenyl cyclase (Figure 5·6·2b). Hence it is unlikely that the antimitotic activity of this nucleotide derives from any interaction with the transducer subunit of the cyclase enzyme.

The presence of isoprenaline, glucagon or dopamine (all at 10^{-4}M) in the assay system all resulted in a significant increase in cyclic AMP formation (Table 5·6·2c). This finding corroborates the contention made in the preceeding Units that these magnesium-dependent

Figure 5•6•2b. The interaction between GTP, Gpp(NH)p and ITP on thymocyte adenylate cyclase activity.

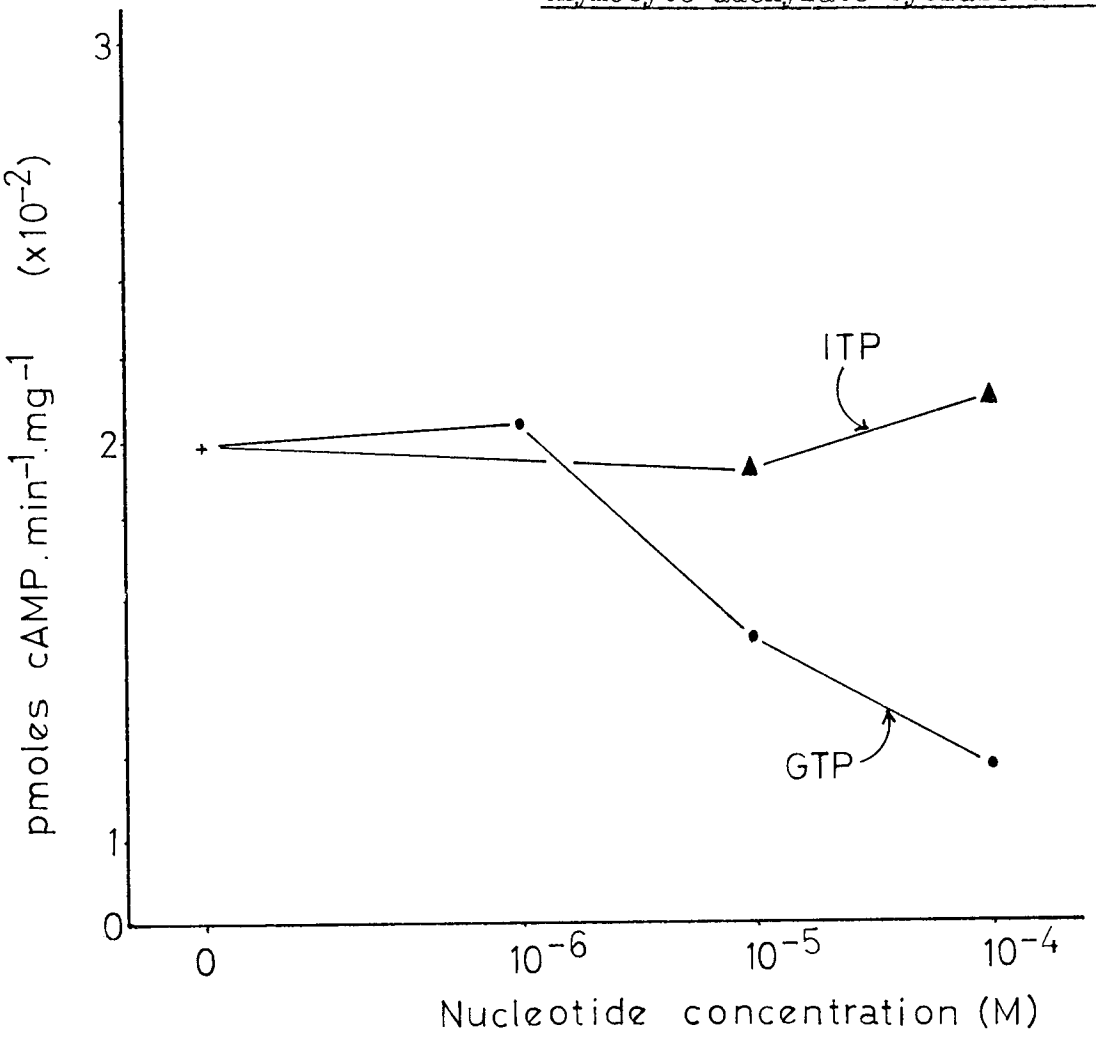


Figure 5•6•2b. The influence of ITP and GTP upon Gpp(NH)p-stimulated adenylate cyclase activity. Assays were performed as described in the text. All incubation mixtures contained Gpp(NH)p at a concentration of 10^{-5}M, and were also exposed to ITP or GTP at various concentrations. Basal cyclase activity was 81pmoles cyclic AMP.min^{-1}.mg^{-1} and the Gpp(NH)p-stimulated value (+) 199 pmoles cyclic AMP.min^{-1}.mg^{-1}. Results are the mean of three determinations.

hormones are indeed adenylate cyclase stimulants. It was further noted that whilst ITP blocked the mitogenic action of isoprenaline, it did not influence the ability of this, or other hormones, to stimulate adenylate cyclase activity (Table 5•6•2c). This would further imply that the site of action of ITP is not the nucleotide receptor site. Hence the evidence might begin to swing in favour of the guanyl nucleotides acting at another point in the proliferative axis. However,

Table 5•6•2c. The action of hormones and ITP upon thymocyte adenylate cyclase activity.

Hormone added	pmoles cyclic AMP generated.min^{-1}.mg^{-1}	
	No ITP	ITP 10^{-5}M
None	81 (n=3)	79 (n=3)
Isoprenaline 10^{-5}M	119 (n=3)	161 (n=6)
Glucagon 10^{-5}M	130 (n=6)	128 (n=3)
Dopamine 10^{-5}M	102 (n=3)	98 (n=3)

Table 5•6•2c. The interaction between ITP and magnesium-dependent hormones on thymocyte adenylate cyclase activity. Assays were performed as outlined in the text. Incubation mixtures were exposed to the various combinations of hormones and ITP indicated in the table and cyclase activity assessed. Results are the mean amounts of cyclic AMP generated from the number of assays indicated in parentheses.

it should be borne in mind that evidence obtained from the broken cell system might not bear any direct relationship to the situation obtaining in the intact cell.

5•6•3 The role of cyclic nucleotides and their degradation products in the regulation of adenylate cyclase activity.

In order to ascertain whether cyclic GMP stimulated cyclase activity a number of experiments were carried out employing this nucleotide as well as a range of other cyclic and non-cyclic purines. However it did not prove possible to stimulate the activity of the preparation with cyclic AMP, cyclic GMP or the degradation products of cyclic AMP namely adenine, adenosine and 5'AMP (Figure 5•6•3a). Thus the mitogenic activity of high concentrations of cyclic GMP are

Figure 5·6·3a. The influence of nucleotides upon thymocyte adenylate cyclase activity.

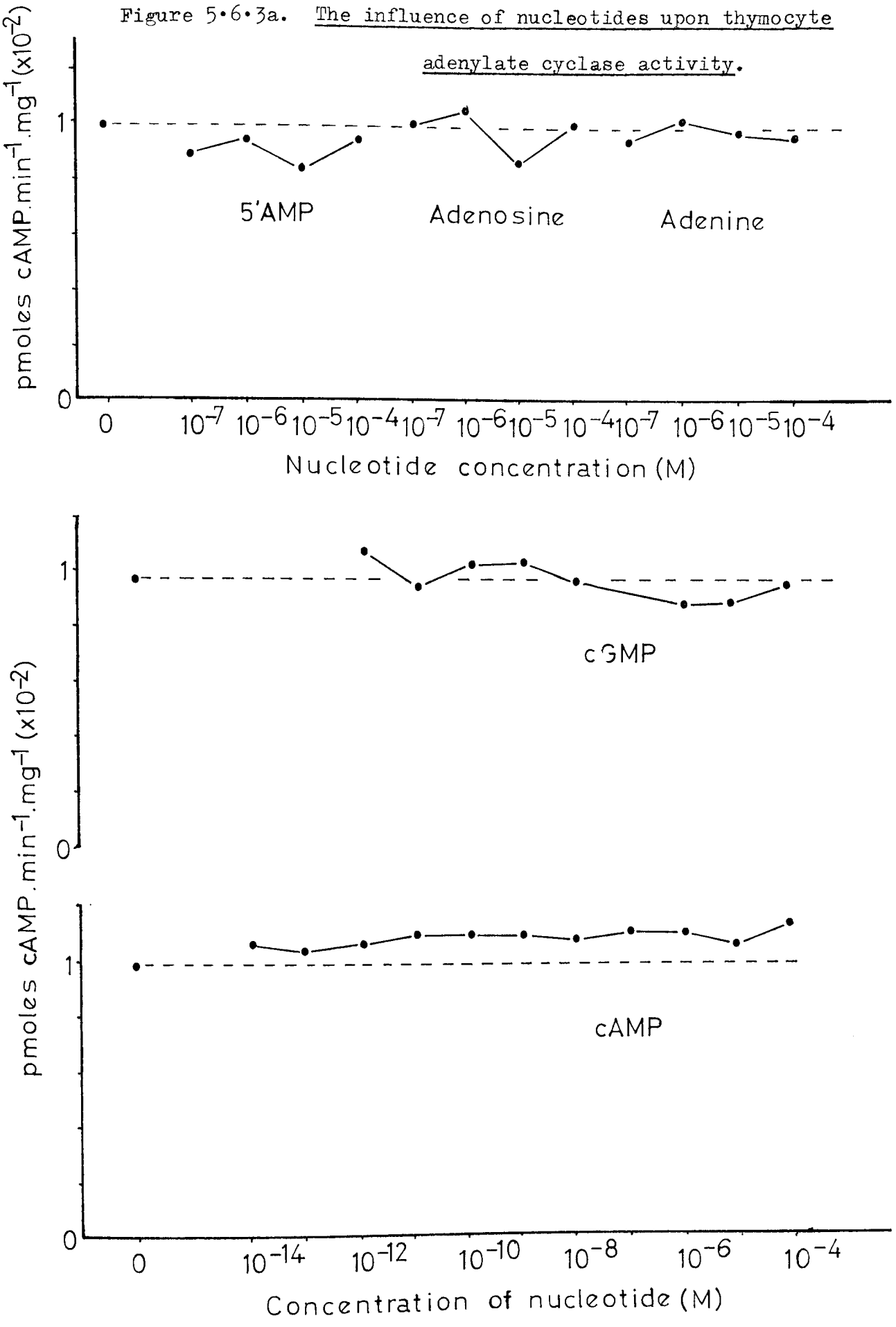


Figure 5·6·3a. Legend over page:-

Figure 5•6•3a. The influence of nucleotides upon thymocyte adenylate cyclase activity. Assays were performed as described in the text. Incubation mixtures were exposed to the nucleotide under test (all obtained through Boehringer) at the concentrations indicated and the cyclase activity determined. Each point is the mean cyclic AMP generated from three experiments. The dotted line represents the basal adenylate cyclase activity.

unlikely to be attributable to a stimulation of adenylate cyclase. Furthermore, breakdown products of cyclic AMP have no stimulatory capacity hence cyclic AMP almost certainly has a specific effect on the thymocyte to induce proliferation.

5•6•4 The interaction between steroids and hormones in the activation of thymocyte adenylate cyclase.

As mentioned in section 5•6•2, the hormones glucagon, dopamine and isoprenaline all stimulate thymocyte adenylate cyclase activity (Figure 5•6•4a). In addition it has already been shown (in the mitogenic context) that these hormones are magnesium-dependent and testosterone blocked, and it was speculated that this indicated some property of their ability to alter the metabolism of cyclic AMP (Unit 3). It was also reasoned that testosterone blocked hormone-induced mitogenesis subsequent to the elevation of cyclic AMP concentrations (Unit 4). Thus it might be expected that this steroid would not interfere with either basal or stimulated cyclase activity. This result was infact obtained when all these mitogens were added to the cyclase assay system both in the presence and absence of testosterone and oestradiol ($0•1\mu g.ml^{-1}$) as well as in basal assays (Figure 5•6•4a). It was also predicted that histamine was not an adenylate cyclase activator. Again it was impossible to stimulate thymocyte cyclase with histamine at concentrations between 10^{-12} and 10^{-4}M (Figure 5•6•4b). Thus it would appear that magnesium-

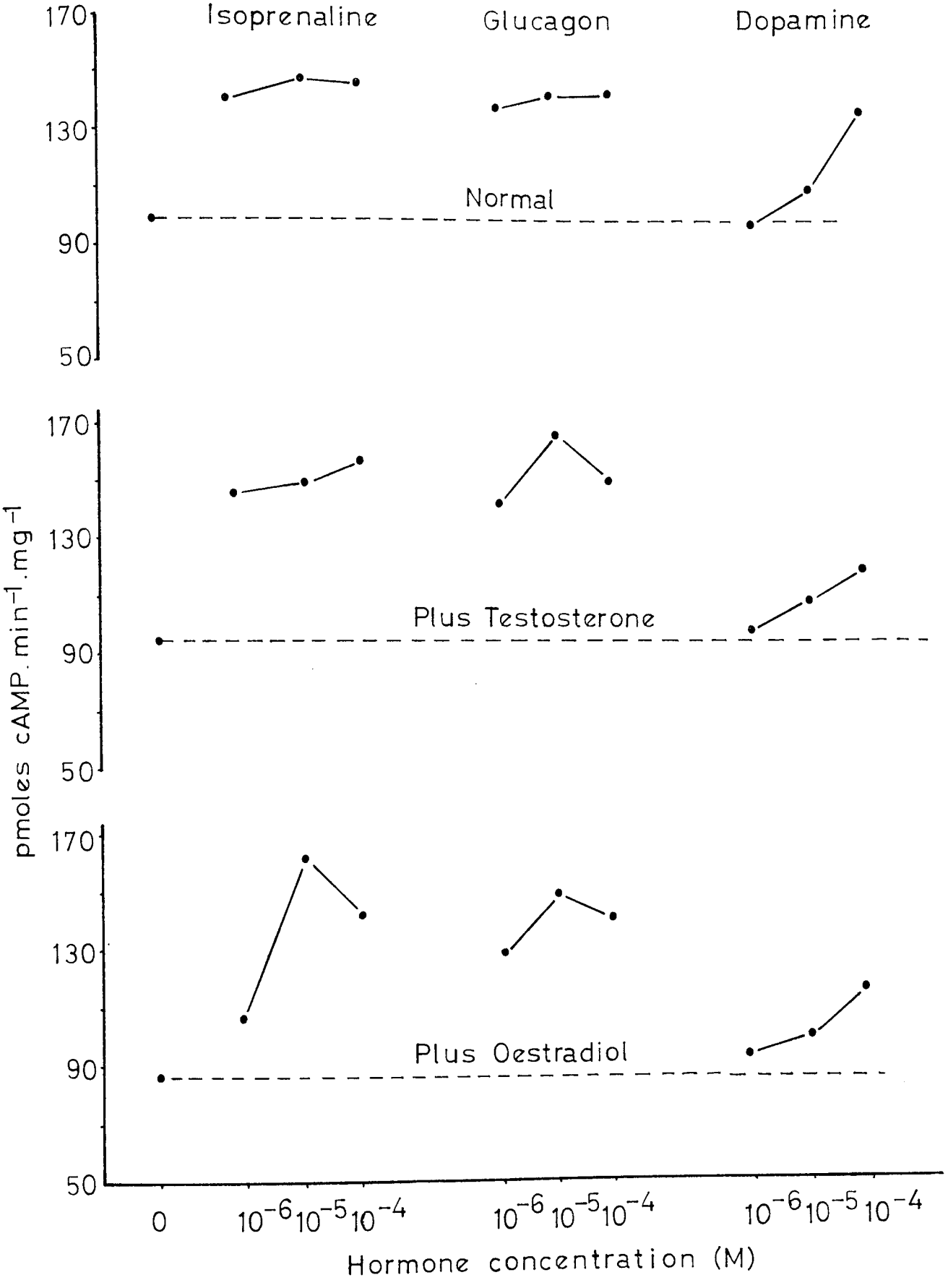


Figure 5·6·4a. The influence of hormones and steroids on thymocyte adenylate cyclase activity. Membranes were incubated in normal medium or buffer containing testosterone or oestradiol ($0{\cdot}1\mu g.ml^{-1}$). These were then exposed to isoprenaline, dopamine or glucagon at the levels

Continued over page:-

Figure 5•6•4a continued:
indicated in the figure and cyclase activity assessed by the procedure outlined in the text. Points are the mean of three determinations.

dependent mitogens are indeed adenylate cyclase stimulants, whereas calcium-dependent mitogens are not. Further the testosterone blockable stage of the magnesium-dependent mitogenic axis is not represented by

Figure 5•6•4b. The influence of histamine on thymocyte adenylate cyclase activity.

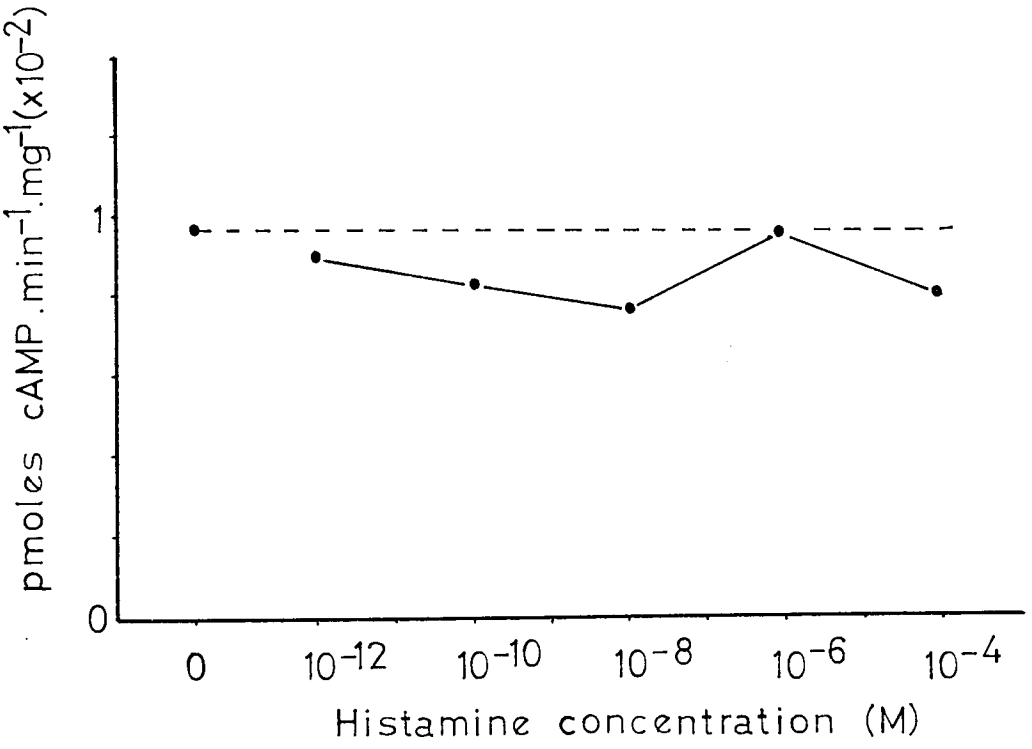


Figure 5•6•4b. The lack of stimulation of adenylate cyclase by histamine. Standard membrane preparations were exposed to histamine over a range of concentrations and cyclase activity assessed as described previously. Each point is the mean of three determinations. The dotted line indicates the basal adenylate cyclase conversion rate.

the hormonal elevation of membrane adenylate cyclase.

5·7 The assay of endogenous cyclic AMP concentrations.

Whilst the measurement of broken cell adenylate cyclase activity can provide a number of answers to the questions posed in the foregoing Units, by its very nature the assay can make few predictions about the response of the intracellular levels of cyclic nucleotides to hormonal mitogens. Thus the endogenous concentrations of cyclic AMP were measured following cell activation.

The analytical method employed was that of a specific protein binding assay. The protein was a highly purified cyclic AMP-dependent protein kinase extracted from bovine cheek muscle, and was the generous gift of Dr. Judith Poat of Southampton University. The assay method is essentially that described by Gilman (1970) except that the binding protein-cyclic AMP complex was retained on a Millipore, cellulose ester filter (HAWP 02500, Millipore Ltd.) as described by Weinryb, Michel and Hess (1972). Briefly samples were diluted in 50mM sodium acetate buffer, pH 4·0, such that 0·02ml aliquots contained between 0·5 and 4·0pmoles of cyclic AMP. Each 0·02ml aliquot was incubated with 0·01ml ^{3}H-cyclic AMP diluted in acetate buffer such that the 0·01ml contained 0·5pmoles cyclic AMP as estimated from the cited specific activity. To this mixture at 0°C was added 0·02ml binding protein diluted in 5mM potassium phosphate buffer, pH 7·0, such that this amount of kinase would complex between 20 and 30% of the total counts added. After one hours incubation the reaction was stopped by addition of 1ml 20mM potassium phosphate buffer, pH 6·0, at 0°C for four minutes. The protein kinase and its associated labelled cyclic AMP was then trapped on a Millipore filter, and dissolved in 1ml of 2-methoxyethanol. This was subsequently diluted with 10ml of scintillant comprising:-

0•1g POPOP

3•0g PPO

250mls 2-methoxyethanol

750mls toluene

The samples were then counted for their radioactive content. Unknown values were quantified against a standard curve obtained by adding 0•02ml of cyclic AMP solutions containing 4, 2, 1 or 0•5pmoles per 0•02ml. Counts derived from these assays were plotted as a log.log graph and unknowns read off this standard curve. For increased accuracy the 0•5pmoles cyclic AMP present in the labelled nucleotide component was added onto standard values before graphing.

Sample preparation is of some importance when assaying cyclic nucleotide concentrations for a number of reasons. First, if the method is not rapid then any transient change in cyclic AMP levels may have disappeared. Second, and allied to the first, since the cell contains high levels of cyclic nucleotide phosphodiesterase activity this must be rapidly and effectively inactivated to protect any increased cyclic AMP from being degraded. Finally it must be ensured that the fixation process does not affect the assay. With these criteria in mind the following protocol was evolved:-

(1) Rapidly spin down cells and pour off medium.

(2) Fix with 1ml 80% ethanol containing 1mM theophylline at 0°C.

(3) Sonicate for 30 seconds on a 3mm microprobe at 40% maximum efficiency (approx. 10KHz)

(4) Centrifuge at 12,000 x g for 10 minutes.

(5) Decant supernatant in LP3 plastic tube.

(6) Evaporate to dryness at 75°C under a stream of nitrogen.

(7) Cap tube and store dry at -20°C until required for assay.

This method showed almost negligible loss of tracer cyclic AMP added at the ethanol fixation stage and recovered by Dowex AG 50 chromatography after drying, thus showing good recovery of cyclic AMP. In addition when assays were performed using increasing dilutions of samples a linear decrease in cyclic AMP concentration was observed indicating no interference with the assay from either fixative or other contaminants. It might be added that in preliminary studies when trichloroacetic acid (5%) was used as fixative, even after five extractions in water saturated ether and subsequent freeze drying there was a considerable displacement of the standard curve by this procedure.

5·7·2 The influence of Gpp(NH)p on thymocyte cyclic AMP levels.

Since Gpp(NH)p does stimulate thymocyte broken membrane adenylate cyclase it might be expected that this would reflect itself in an increase in intracellular cyclic AMP levels in the intact cell. However, when thymic lymphocytes were exposed to the guanyl nucleotide at the relatively high concentration of 10^{-5}M there was no detectable alteration in cell cyclic AMP content over a thirty minute period (Figure 5·7·2a). In addition the presence of testosterone, an agent which blocks the mitogenic action of Gpp(NH)p does not influence cyclic AMP metabolism. These results would suggest that (a) Gpp(NH)p is not an activator of whole cell cyclase activity and (b) the antimitotic activity of testosterone does not derive from an ability of the steroid to interfere with cyclic AMP metabolism. Hence, Gpp(NH)p and probably GTP also act at the membrane receptor sites proposed to exist in the

Figure 5•7•2a.

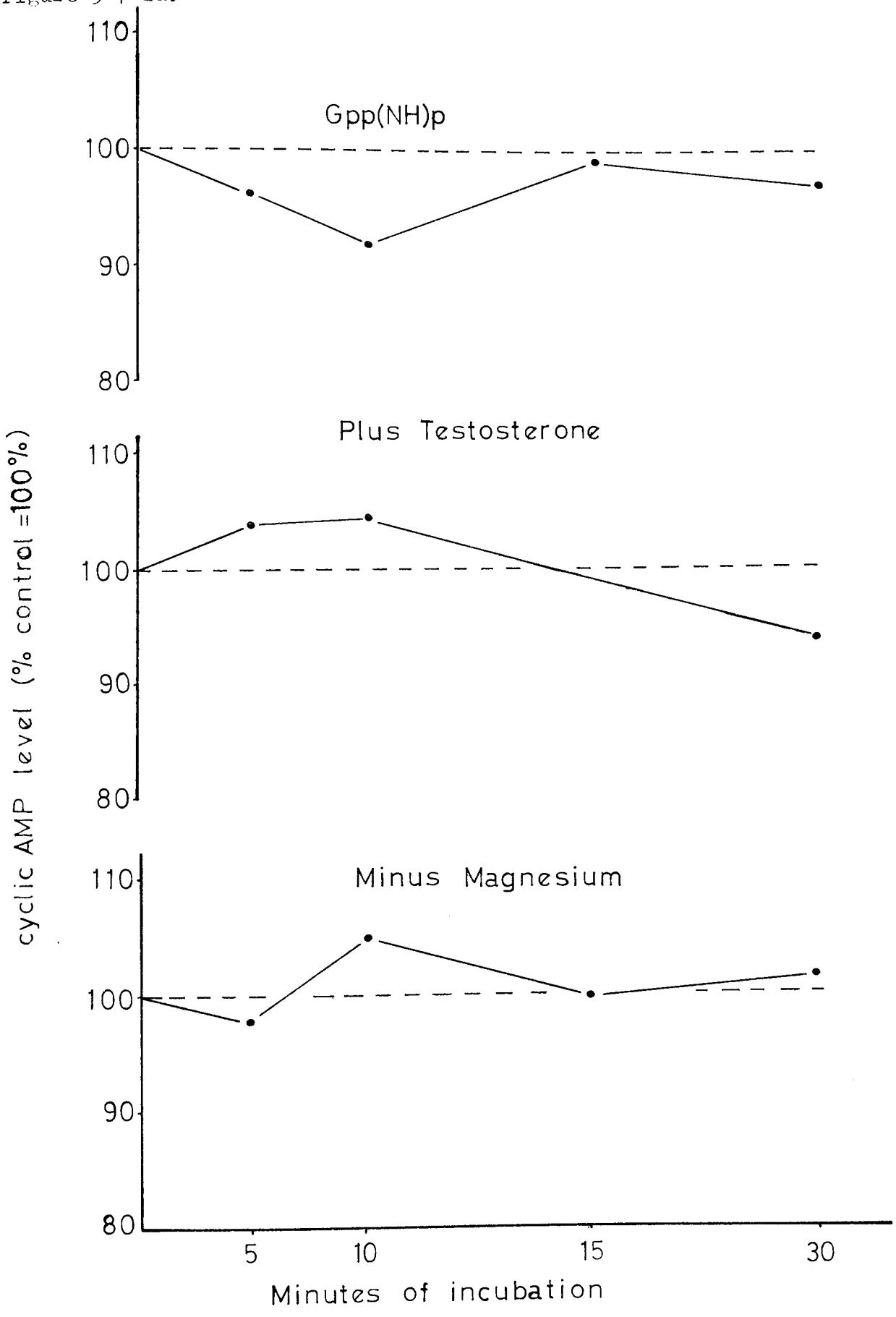


Figure 5•7•2a. The interaction between Gpp(NH)p, ions and steroids in modulating intracellular cyclic AMP concentrations. Figure legend over page.

Figure 5·7·2a continued:-
Thymocytes were preincubated for thirty minutes in Medium 199 which contained 0·6mM calcium chloride and either 1·0 or 0·0mM magnesium sulphate (minus magnesium). At time zero cultures were treated with Gpp(NH)p at a concentration of 10^{-5}M (Gpp(NH)p) or Gpp(NH)p plus testosterone (0·1μg.ml^{-1}). At the times indicated the cyclic AMP content of the cells were assessed as outlined in the text. Results are expressed as the percentage cyclic AMP level found in untreated control cultures, which are deemed as containing 100% cyclic AMP. The values are the mean percentage cyclic AMP present in between 2 and 4 cultures assayed in duplicate. The dotted line denotes the 100% control value.

previous Unit, this interaction being inhibited by ITP. Certainly it is difficult to envisage how a large charged molecule like Gpp(NH)p or indeed GTP could enter the cell to stimulate the adenylate cyclase moiety.

5·7·3 The role of magnesium-dependent mitogens as modulators of cyclic AMP concentrations.

Application of prostaglandin E_1 caused a rapid and marked elevation of intracellular cyclic AMP concentrations, which rose to to some fifty times that of control values within five minutes and which persisted at these increased levels for thirty minutes (Figure 5·7·3a). This increase is attributable to the ability of this agent to powerfully stimulate adenylate cyclase (Robison et al., 1971). As in the previous section the ability of the prostaglandin to raise intracellular cyclic AMP concentrations was unimpaired by the presence of testosterone in the culture (Figure 5·7·3a). This again points to some action of the steroid at a stage subsequent to elevation of cyclic nucleotide levels. Like the prostaglandin, adrenalin has been established as an adenylate cyclase stimulant. In addition this mitogenic hormone is known to be magnesium-dependent and testosterone blocked. Indeed

isoprenaline (10^{-5}M) caused a large increase in cyclic AMP levels during the first ten minutes of exposure (Figure 5·7·3b). This action was not influenced by testosterone (Figure 5·7·3b). Thus testosterone does not affect either the ability of the hormone to cause an increase in adenylate cyclase activity or the ability to elevate endogenous cyclic AMP levels. In addition the magnesium-dependent, testosterone-

Figure 5·7·3a. The influence of prostaglandin E_1 and testosterone on thymocyte cyclic AMP concentrations

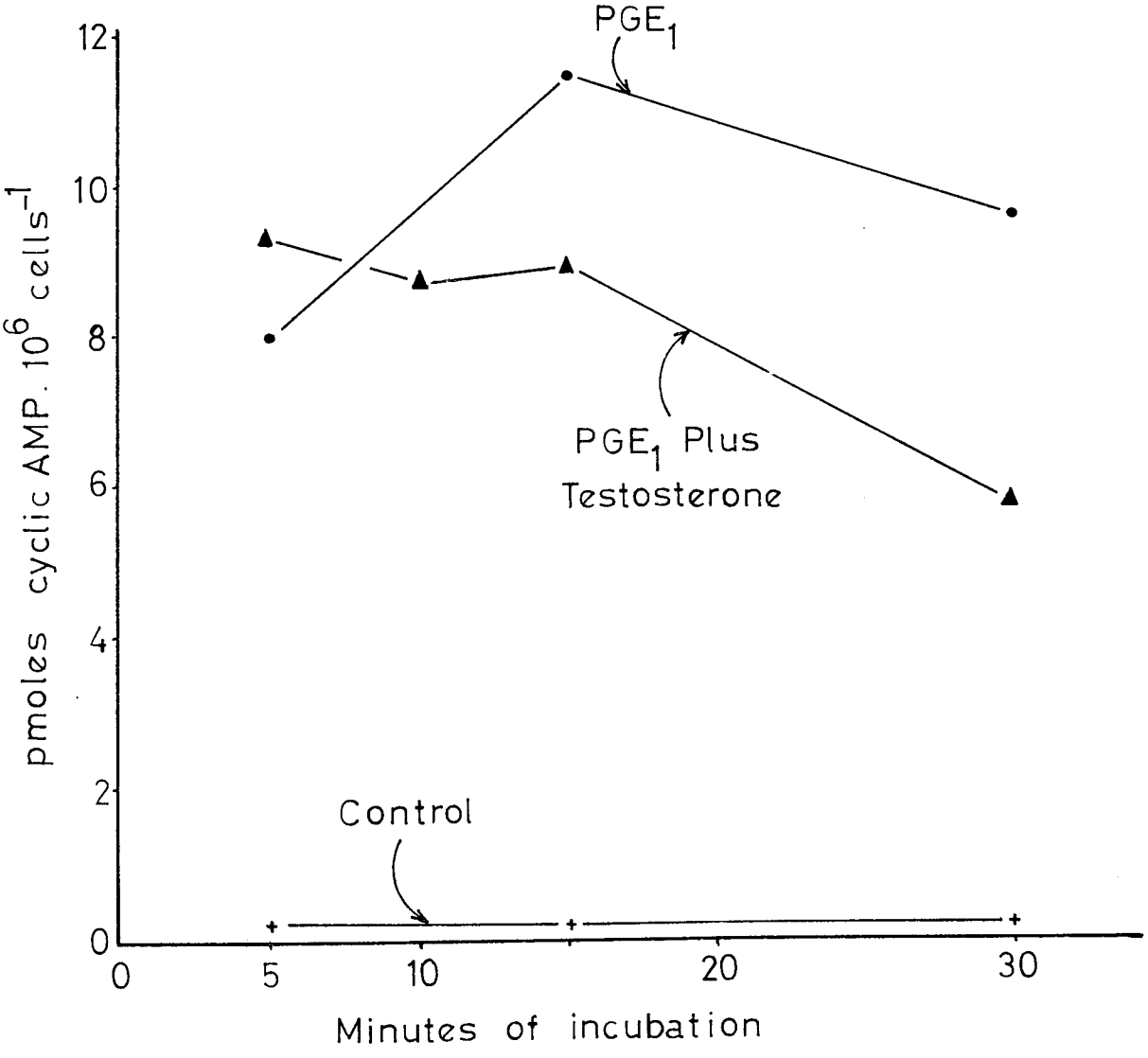


Figure 5·7·3a. The influence of prostaglandin E_1 and testosterone upon thymocyte cyclic AMP concentrations. Thymic lymphocytes were preincubated for thirty minutes in Medium 199 containing no colchicine and 0·6mM calcium and 1·0mM magnesium. At time zero cultures were exposed to combinations of PGE_1 ($5\mu g.ml^{-1}$) and testosterone ($0·1\mu g.ml^{-1}$) and intracellular cyclic AMP concentration assessed. Results are the mean levels of cyclic AMP (pmoles) present in two cultures assayed in duplicate.

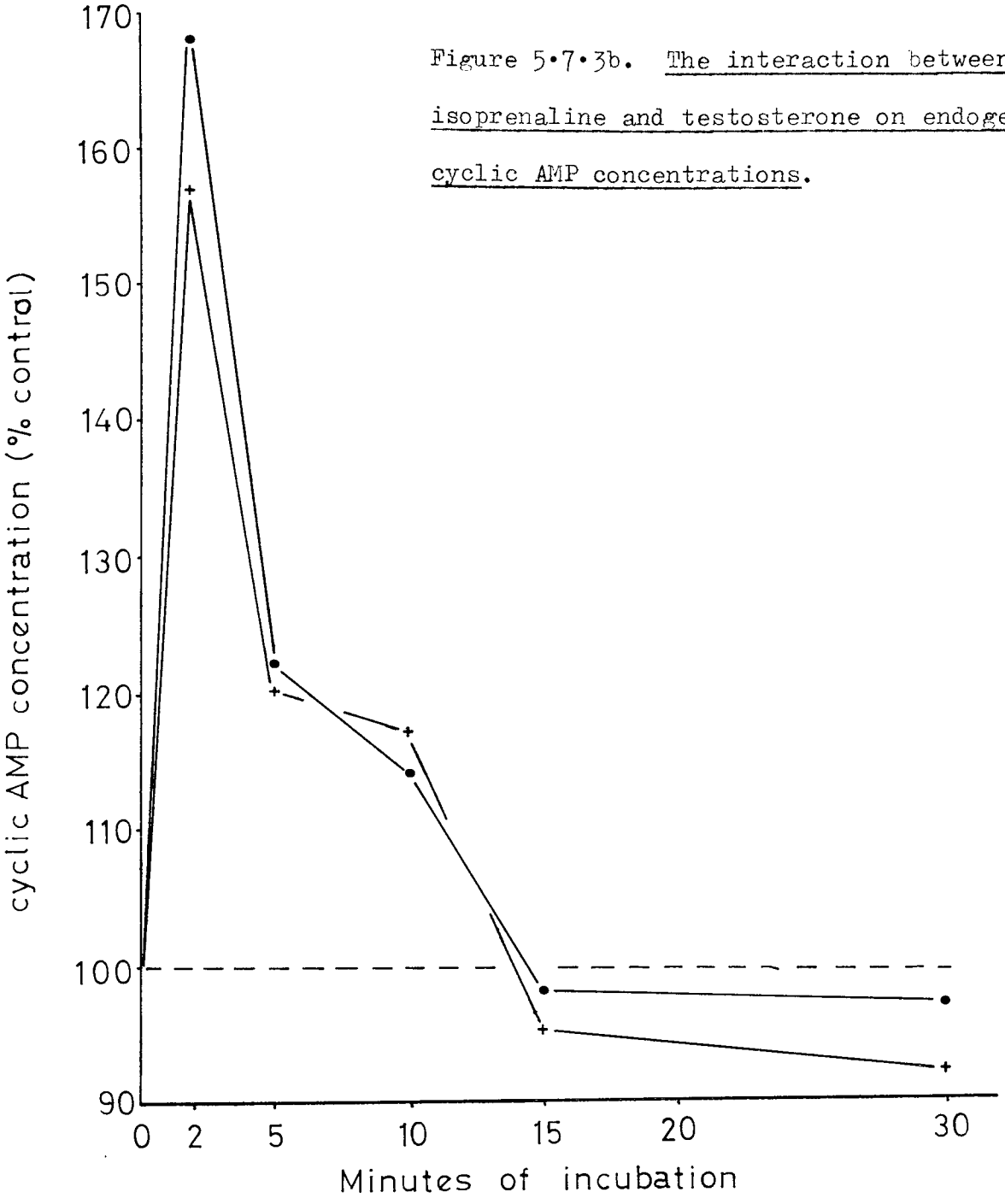


Figure 5•7•3b. The interaction between isoprenaline and testosterone on endogenous cyclic AMP concentrations.

Figure 5•7•3b. The lack of affect of testosterone upon isoprenaline-stimulated cyclic AMP concentrations. Cells were preincubated for thirty minutes in Medium 199 containing no colchicine and 0•6mM calcium, 1•0mM magnesium. At time zero cells were either left untreated (hatched control line) or exposed to isoprenaline (•) at a concentration of 10^{-5}M or isoprenaline plus testosterone (+) at a concentration of 0•1μg.ml^{-1}. Results are the means derived from between 2 and 5 experiments performed in duplicate of the cyclic AMP content of thymocytes. These are expressed as the percentage of the control value at each time.

inhibited mitogen Ro 20-1724, which was thought to act via an increase in endogenous cyclic AMP levels, did indeed promote the elevation of this nucleotide (Figure 5•7•3c). This is thought to occur through an

inhibition of cyclic AMP-phosphodiesterase (Sheppard et al., 1972). As with the other cyclic AMP elevating agents, testosterone did not

Figure 5·7·3c. The influence of Ro 20-1724 and testosterone upon thymocyte cyclic AMP concentrations.

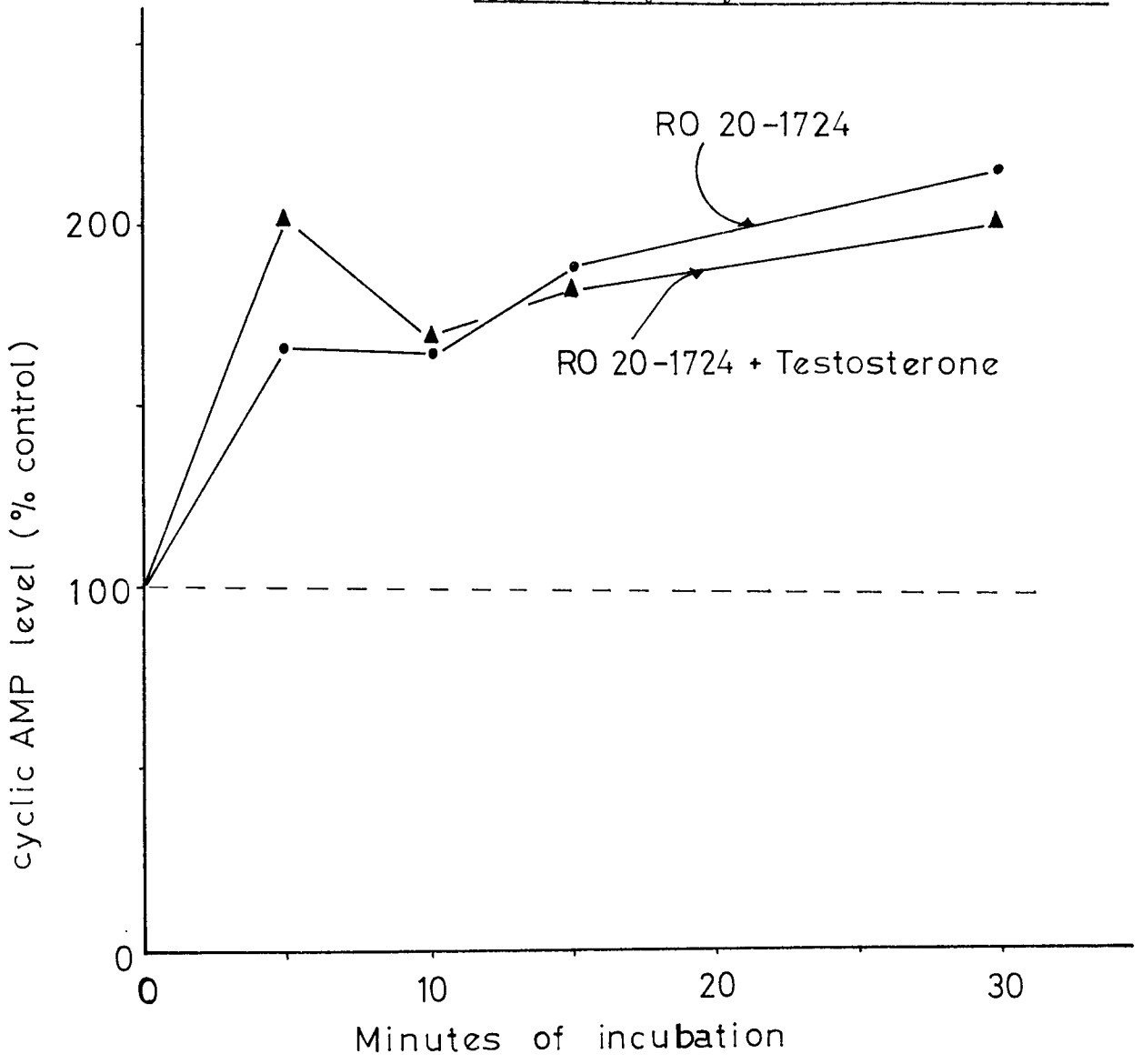


Figure 5·7·3c. The influence of Ro 20-1724 and testosterone upon thymocyte cyclic AMP levels. Cells were preincubated in Medium 199 containing 0·6mM calcium and 1·0mM magnesium for thirty minutes. The cultures were subsequently exposed to Ro 20-1724 (10^{-5}M) or Ro 20-1724 plus testosterone (0·1μg.ml^{-1}) and the cyclic AMP content of the cells assessed at the times indicated. The results derived from two cultures per point, assayed in duplicate, are the percentage cyclic AMP present compared with the untreated control at the respective time.

affect the ability of Ro 20-1724 to raise cyclic AMP levels (Figure 5·7·3c). Clearly, the elevation of endogenous cyclic AMP concentrations

whether this be via a stimulation of adenylate cyclase through either the receptor or catalytic portions or via an inhibition of cyclic nucleotide phosphodiesterase is not sensitive to testosterone which does block the mitogenic capacities of these agents.

5·7·4 The influence of calcium-dependent mitogenic hormones on endogenous cyclic AMP concentrations in thymocytes.

Whereas the alterations in cell cyclic AMP levels following the application of a magnesium-dependent mitogen were clear and characteristic for a given hormone, this was not the case with calcium-dependent mitogenic hormones. And indeed the observed changes in cyclic AMP concentrations were small in comparison to those induced by magnesium-dependent hormones. However exposure of cells to mitogenic levels of histamine (10^{-10}M) or insulin (500μU.ml^{-1}) resulted in fluctuations of cyclic AMP levels about the basal values (Figures 5·7·4a-d). In all cases except one, application of histamine or insulin caused an initial depression of intracellular cyclic AMP concentrations by some 7 to 10% (Figures 5·7·4a,b and c). This initial fall in cyclic AMP was followed, some ten to fifteen minutes after mitogen addition, by a modest elevation of this nucleotide's concentration to between 109 and 130% of that observed in control cultures (Figures 5·7·4 a-d). In one experiment however, insulin (500μU.ml^{-1}) provoked a rapid rise in cyclic AMP concentration, which was followed by a fall to 85% of the control value at ten minutes after mitogen addition (Figure 5·7·4d). This fall in cyclic AMP was, as in the other three experiments, followed by an elevation of this nucleotide's concentration (Figure 5·7·4d). Thus the possibility exists that the calcium-dependent mitogens provoke a rapid rise in cyclic AMP levels which went undetected in most of the

experiments performed here, and which is followed by a phase of

Figure 5•7•4a. The influence of histamine and calcium upon thymocyte cyclic AMP concentrations.

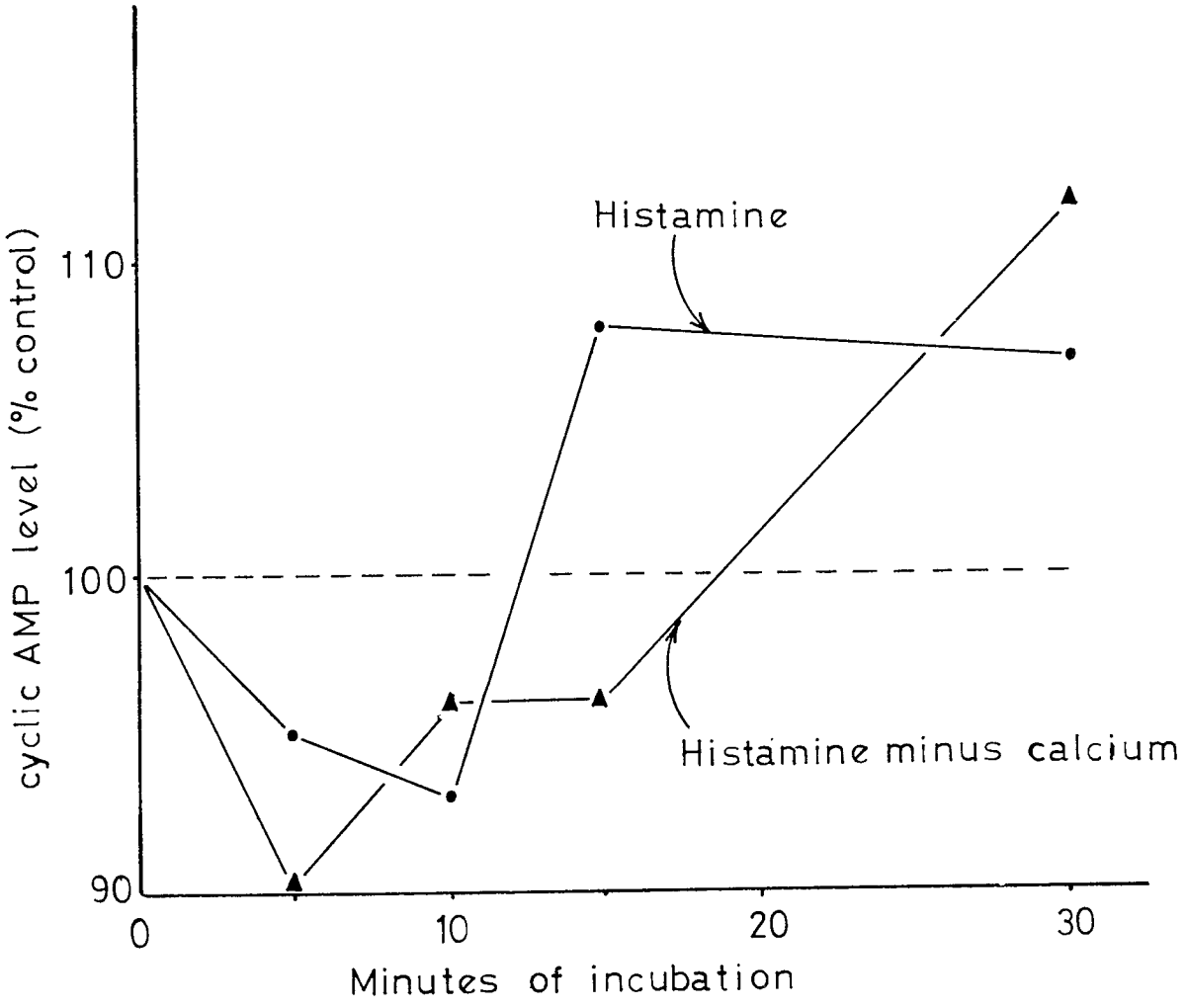


Figure 5•7•4a. The interaction between calcium and histamine upon thymocyte cyclic AMP concentrations. Cells were incubated in Medium 199 containing no colchicine and 1•0mM magnesium for thirty minutes before treatment. At time zero cells suspended in medium containing either 0•6 or 0•0mM calcium chloride were exposed to histamine (10^{-10}M) and cell cyclic AMP levels assessed over the next thirty minutes. The results are the mean cyclic AMP content of two cultures assayed in duplicate and expressed as a percentage of the control value at the respective time.

depressed nucleotide concentrations. This fall in cyclic AMP is in its turn followed by a period of raised cyclic AMP values. That is there is possibly a biphasic elevation of cyclic AMP levels by calcium-dependent mitogens. It might be noted that in a number of experiments with histamine using a methodology which was eventually discarded just such a bi-

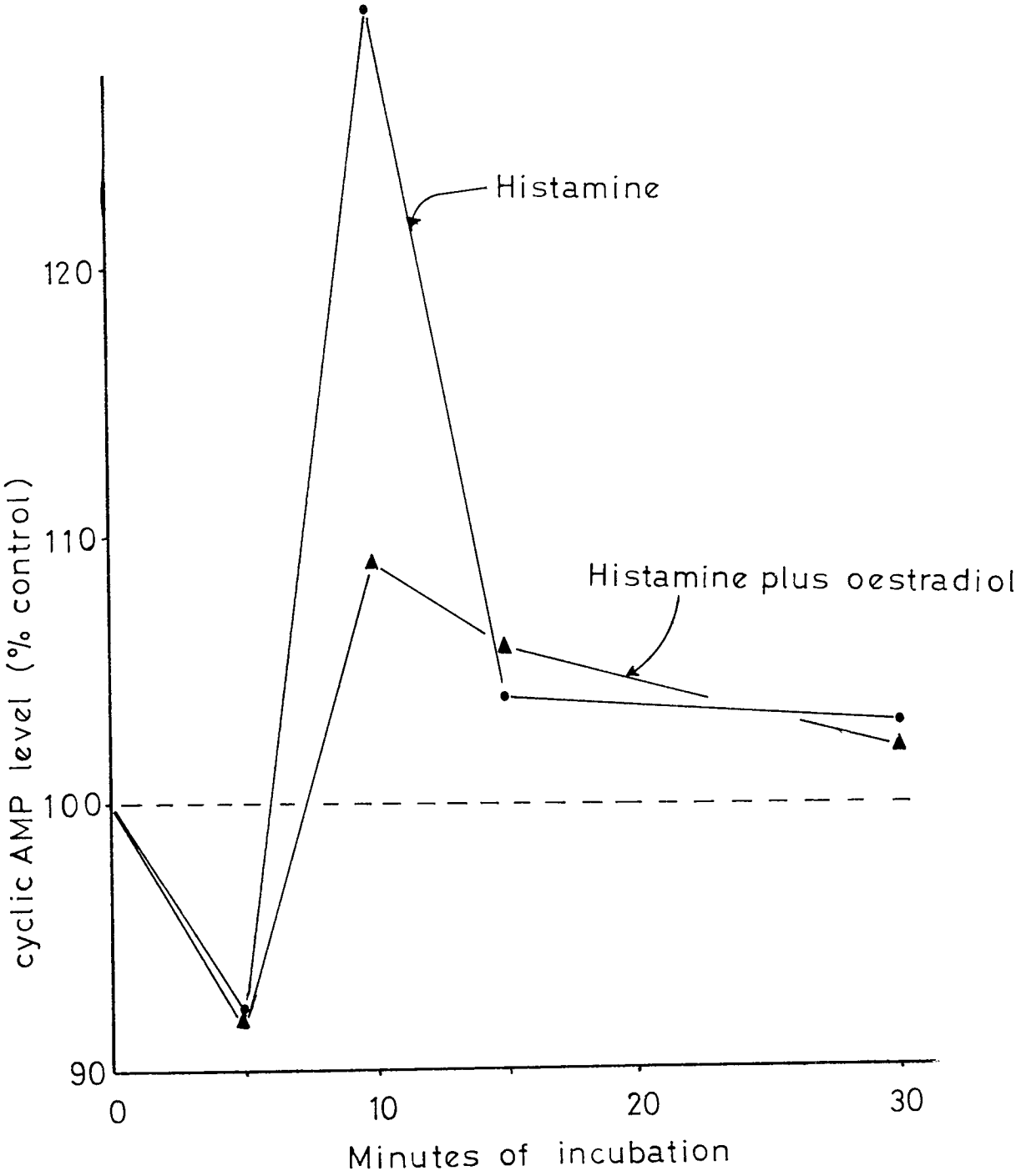


Figure 5·7·4b. The interaction between oestradiol and histamine on thymic lymphocyte cyclic AMP concentrations. Thymocytes were incubated in Medium 199 containing 0·6mM calcium chloride and 1·0mM magnesium sulphate and no colchicine. Thirty minutes after the initiation of the culture cells were exposed to histamine (10^{-10}M) or histamine plus oestradiol ($0·1\mu g.ml^{-1}$). Cyclic AMP concentrations were then monitored over the subsequent thirty minutes. Results represent the mean cyclic AMP content of two cultures assayed in duplicate and expressed as a percentage of the respective control culture cyclic AMP content.

phasic pattern was observed.

The omission of calcium from the incubation medium, did not result in any clear modification of the changes in cyclic AMP metabolism

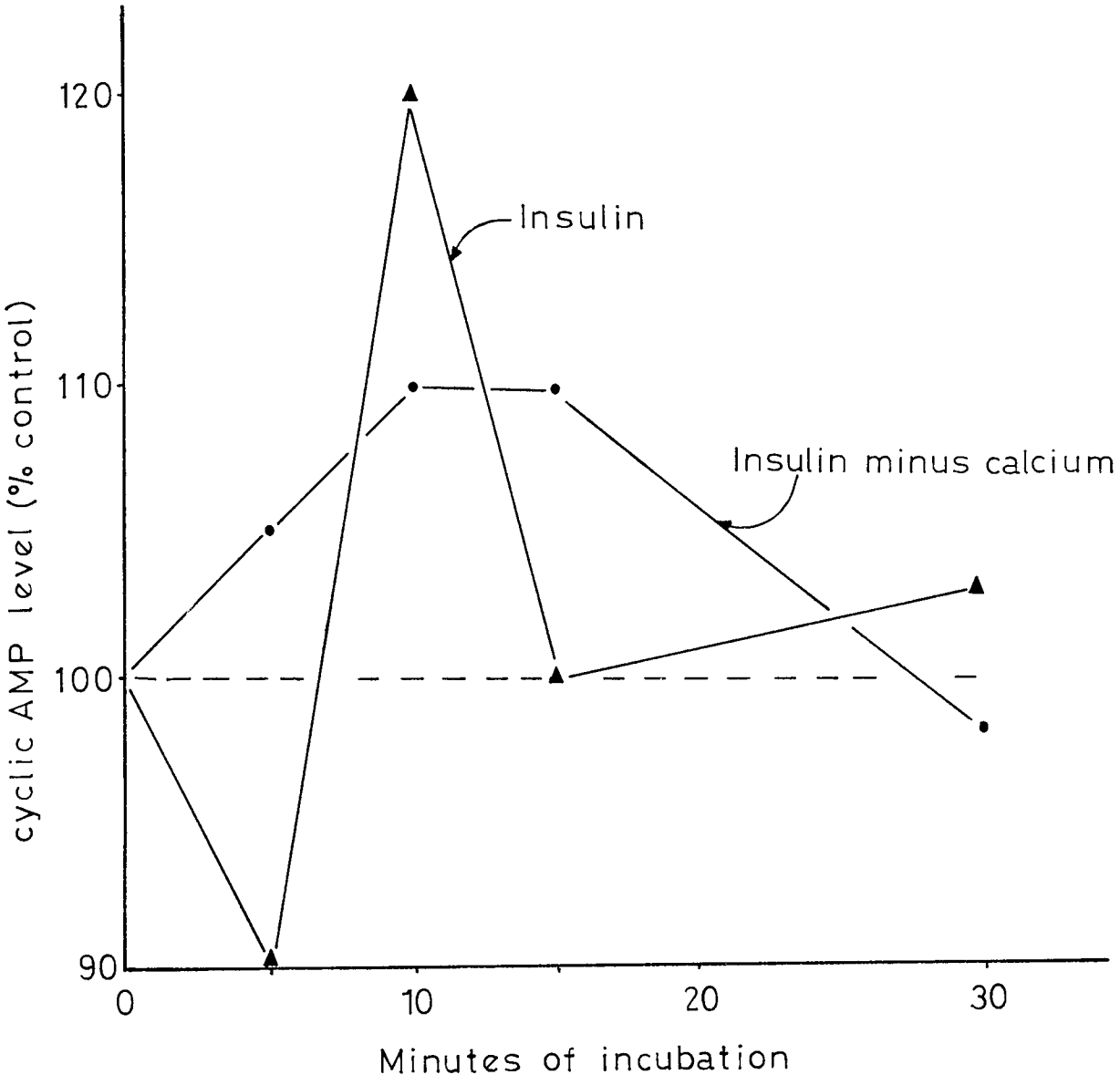


Figure 5•7•4c. The interaction between insulin and calcium upon thymic lymphocyte cyclic AMP concentrations. Cells were preincubated for thirty minutes in Medium 199 containing 1•0mM magnesium sulphate and either 0•6 or 0•0mM calcium chloride. The cultures were then exposed to insulin at a concentration of 500μU.ml^{-1}, and cyclic AMP values monitored over the succeeding thirty minutes. Results are the mean cyclic AMP content of two cultures assayed in duplicate expressed as the percentage of the observed value in control cultures.

induced by histamine or insulin (Figures 5•7•4a,c). However there may be some indication that in the calcium-free medium the elevation phase may be somewhat shifted to the right (Figures 5•7•4a,c). It is possible that such an action could account for the failure of histamine and insulin to stimulate division in calcium-deficient conditions. In contrast the other inhibitor of calcium-dependent mitogens, oestradiol,

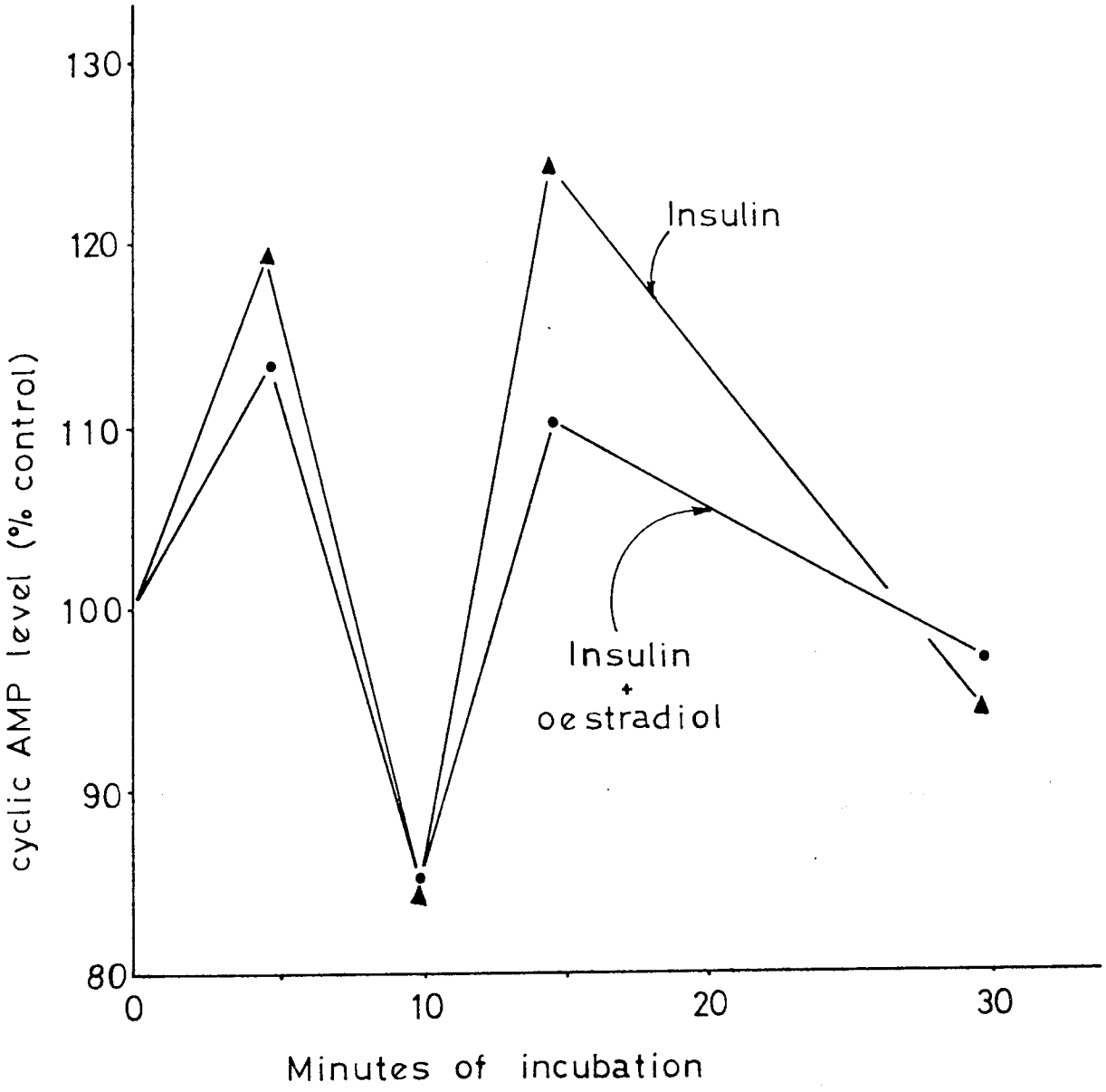


Figure 5·7·4d. The interaction between oestradiol and insulin upon thymocyte cyclic AMP content. Cells were incubated in Medium 199 which contained 0·6mM calcium chloride and 1·0mM magnesium sulphate for thirty minutes before treatment. The cultures were then exposed to either insulin ($500uU.ml^{-1}$) or insulin plus oestradiol ($0·1ug.ml^{-1}$) and cyclic AMP concentrations assessed over the next thirty minutes. Results are the mean cyclic AMP content of two experimental cultures assayed in duplicate and expressed as a percentage of their respective control value.

did appear to reduce the cyclic AMP elevation provoked by both insulin and histamine (Figures 5·7·4b,d). In the case of insulin it appeared that it was the second cyclic AMP increment which was most affected (Figure 5·7·4d). Again it might be added that in the preliminary studies mentioned previously oestradiol greatly reduced the second

cyclic AMP peak generated by histamine, the first peak as with insulin being largely unaffected. Thus the antimitotic ability of oestradiol may reside in a capacity to ablate some aspect of cyclic AMP metabolism which produces a secondary rise in cyclic AMP levels. It is curious however that the first rise in cyclic AMP levels should not be sufficient to induce a mitotic recruitment under these conditions since it has been established that within five minutes of calcium addition, oestradiol cannot reverse the entry of cells into their S phase. Thus this action of the steroid on cyclic AMP levels may not have any causal link with its antimitotic activity but may be a passive consequence of some other aspect of its interaction with the thymocyte. Furthermore it has not been possible to demonstrate any significant alteration in cyclic AMP concentrations with either of the mitogenic cations, calcium or magnesium. Therefore it is unlikely that oestradiol could impair calcium-induced mitogenesis by changing cyclic AMP metabolism. This data would also indicate that calcium, and indeed magnesium, act either independently of cyclic AMP or as a consequence of its metabolism. The latter case would of course fit the notion of a membrane kinase system involving divalent cation transfer or activation as suggested in the previous Unit.

5·7·5 The influence of calcium-dependent mitogens on cyclic GMP metabolism in the thymic lymphocyte.

Since in Unit 4 cyclic GMP was invoked as being a possible intracellular mediator of calcium-dependent mitogens, the levels of this nucleotide were assessed following the application of histamine and acetylcholine to thymic lymphocyte cultures. The assay procedure

was that of radioimmunoassay using a standard commercial kit (Radiochemical Centre, Amersham). The preparation was also acetylated to yield a greater sensitivity, as recommended by the suppliers. These procedures will not be described here.

Since there appeared to be pronounced changes in cyclic AMP levels during the first five minutes of exposure to histamine and insulin it was decided to concentrate on the metabolism of cyclic GMP during this period. However, the presence of histamine in the culture did not cause any change in cyclic GMP levels (Figure 5·7·5a) and neither did acetylcholine (Figure 5·7·5b). Therefore it is possible that this nucleotide does not play a role in the action of either of these mitogens. It should also be remembered that the measurement of endogenous cyclic GMP concentrations is notoriously difficult, and open to criticism. Therefore these negative results may emanate from some technical difficulty in protecting extra cyclic GMP generated from enzymatic or chemical degradation. Alternatively the time scale studied may not include the periods of altered cyclic GMP metabolism. However other workers (Whitfield, MacManus, Boynton et al., 1974) have demonstrated changes in cyclic GMP concentrations during the first five minutes of exposure of thymocyte cultures to a calcium-dependent mitogen, concanavalin A. Thus the implication of cyclic GMP in the mitogenic actions of calcium-dependent agents must still remain a matter of speculation.

5·8 Summary of cyclic nucleotide metabolism following mitogen addition to thymic lymphocyte cultures.

Magnesium-dependent mitogens such as isoprenaline, glucagon

Figure 5·7·5a. The interaction between histamine and oestradiol on thymocyte cyclic GMP content

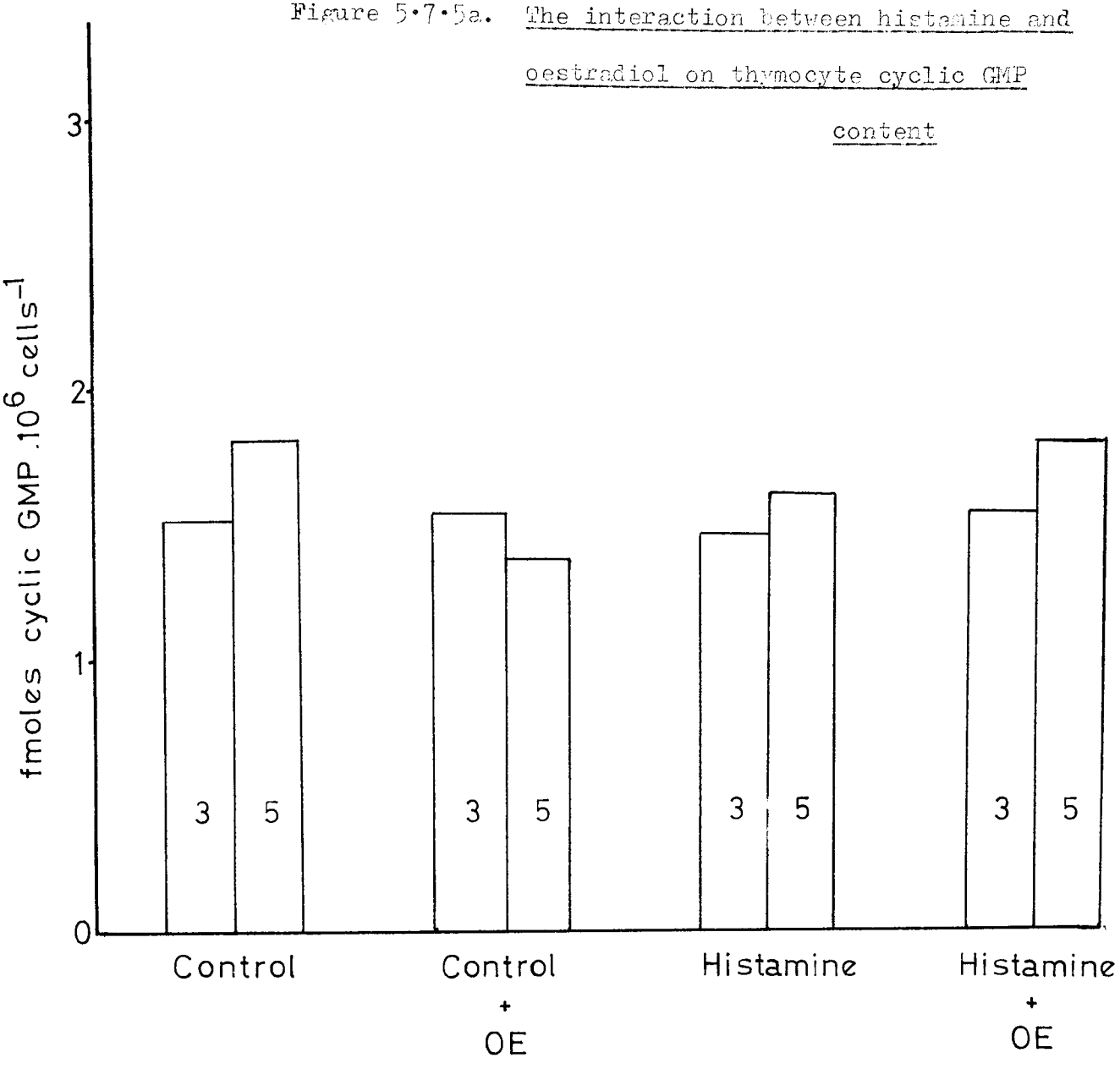


Figure 5·7·5a. The lack of affect of histamine and oestradiol on thymic lymphocyte cyclic GMP concentrations. Cells were incubated in Medium 199 containing 0·6mM calcium chloride and 1·0mM magnesium sulphate and no colchicine. After a thirty minute preincubation cultures were treated with combinations of histamine (10^{-10}M) and oestradiol (0·1μg.ml^{-1}) and intracellular cyclic GMP levels assessed 3 or 5 minutes after mitogen addition. These times are indicated by the numbers in the columns. Cell fixation was by ice cold 80% ethanol containing 1mM theophylline. Cyclic GMP was assayed by R.I.A. (Radiochemical Centre, Amersham) and the sample cyclic GMP content was acetylated prior to estimation to increase sensitivity. The results are the mean of two experiments determined in triplicate on separate occasions, and are expressed as fentomoles of cyclic GMP present per million cells in culture.

Figure 5·7·5b. The interaction between acetylcholine and oestradiol on thymocyte cyclic GMP content.

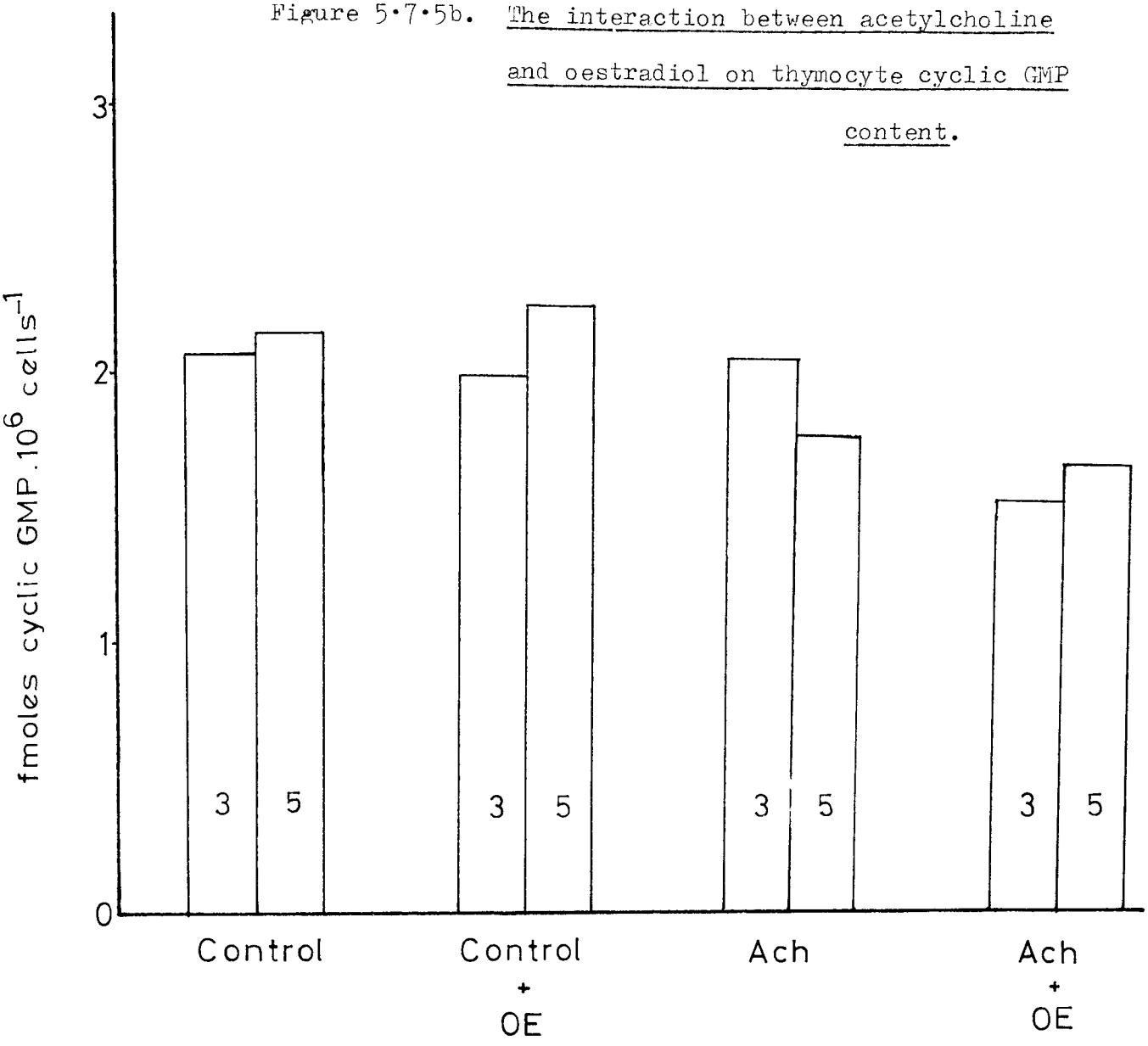


Figure 5·7·5b. The lack of affect of acetylcholine and oestradiol on thymic lymphocyte cyclic GMP content. Cells were preincubated for thirty minutes in Medium 199 containing 0·6mM calcium chloride and 1·0mM magnesium sulphate and no colchicine. After this period cultures were exposed to various combinations of acetylcholine (10^{-10}M) and oestradiol ($0·1\mu g.ml^{-1}$) and cyclic GMP levels estimated at 3 and 5 minutes after mitogen addition. These cultures also contained eserine (10^{-9}M) to inhibit acetylcholinesterase activity in these cell suspensions. The results are the mean cyclic GMP content of 2 cultures assayed in triplicate, and expressed in fentomoles of cyclic GMP per million cells.

dopamine and Gpp(NH)p all increase the activity of adenylate cyclase in a broken cell preparation of thymocytes. However, whilst isoprenaline and presumably glucagon and dopamine increase intracellular cyclic AMP concentrations this is not the case for Gpp(NH)p. Furthermore ITP, which

inhibits the mitogenic action of Gpp(NH)p, does not affect the ability of this guanyl nucleotide to stimulate broken cell cyclase activity. It is argued therefore that Gpp(NH)p does not act on adenylate cyclase in the intact cell but on a membrane receptor which involves the transfer of magnesium ions into the cell, or which requires the presence of extracellular magnesium for its activation by nucleotides. ITP might then antagonise the interaction of Gpp(NH)p with this receptor.

Testosterone, which inhibits the mitogenic action of the magnesium-dependent mitogens does not influence the ability of these agents either to stimulate adenylate cyclase, or to bring about changes in endogenous cyclic AMP concentrations. Therefore it is reasoned that either the steroid acts at a point subsequent to the elevation of cyclic AMP levels, or it influences some other process which is unrelated to cyclic nucleotide metabolism.

The calcium-dependent mitogens bring about no changes in adenylate cyclase activity, but do seem capable of inducing small changes in endogenous cyclic AMP levels. This is characterised by phases of depressed and then elevated nucleotide concentrations. Since these hormones do not appear to be capable of stimulating adenylate cyclase activity per se it must be inferred that they influence the degradation of cyclic AMP by modifying phosphodiesterase activity in some way. Calcium may play a central role in such a secondary action. Certainly the omission of calcium from the medium does result in the shifting of the response curve of cyclic AMP to the calcium-dependent mitogens. Oestradiol, which also inhibits calcium-dependent stimulation of mitotic activity is capable of reducing the secondary rise in cyclic AMP concentrations observed with histamine and insulin. However it is known that the antimitotic action of oestradiol resides in some property

of the steroid which is expressed within the first five minutes of mitogen addition to the cell culture. Thus it would seem unlikely that this action of oestradiol in modifying the late cyclic AMP response could account for its inhibitory action. In addition calcium itself at mitogenic concentrations does not influence cyclic AMP levels, yet it too is inhibited by oestradiol. These results would substantiate the mechanism proposed in Unit 4 in which hormones elevate endogenous cyclic nucleotide concentrations, and these raised cyclic purine levels influence membrane kinases which either stimulate the transport of divalent cations or which require calcium or magnesium for their activation by the appropriate cyclic nucleotide.

It was not possible to demonstrate any affect of calcium-dependent mitogens upon thymic lymphocyte cyclic GMP content over a short time course. However these results may have derived from a technical difficulty in protecting cyclic GMP generated by the cells. Alternatively, contrary to the findings of other workers, cyclic GMP concentrations may become elevated at a later time.

An intriguiging question raised by the observations on the effects of calcium-dependent hormones on cyclic AMP metabolism is how these agents might effect such a change in the absence of any adenyl cyclase receptor. Since such a property might reside in an ability to alter ion movements, especially calcium ions, it was decided to examine the influence of calcium ions and steroids upon membrane potential and transmembrane ion fluxes. These aspects will be dealt with in Units 6 and 7.

Unit 6 The role of the plasma membrane in lymphocyte activation: I. an analysis using a membrane fluorescent probe.

It is now becoming established that the cell plasma membrane does not simply represent a passive physical barrier to various molecules, but is rather a dynamic structure which both gives and receives information from the rest of the cell body. Indeed as outlined in the introduction (pp 44-55) the cell membrane plays a vital role both in the normal mitotic cycle and during G_o recruitment phenomena. An attempt will now be made to discuss some aspects of membrane structure and function which appertain to the problem of thymocyte G_o activation.

6·1 The cell membrane; its structure.

The accepted structure of the cell membrane is that of the 'fluid mosaic' first proposed by Singer and Nicolson (1972) which is illustrated in Figure 6·1(a). The model indicates that the membrane consists of a lipid bilayer in which is embedded globular protein molecules. The lipids associated with the cell membrane are predominantly polar phospholipids and to a lesser degree steroids such as cholesterol. These compounds then orientate themselves with their hydrophobic carbon tails facing towards the centre of the bilayer and their charged polar head groups facing the aqueous intra- or extracellular environments. Thus there is a hydrophobic interface between the two sets of head groups which represents a significant barrier to the diffusion of many small molecules and ions. This being attributable to the large amount of electrostatic free energy required to bring an ion from aqueous solution ($\epsilon = 80$) into a lipid

phase of low dielectric constant (ϵ = 2-3) which can be calculated to be of the order of 46Kcal.mole^{-1}. The lipid matrix is not a rigid structure; indeed there is continual motion within it (Lee, 1975). First there is rapid motion within each lipid molecule represented by the bond rotation about C-C bonds in the lipid tails. Superimposed upon these oscillations is the tendency of C-C bonds to 'jump' from trans to gauche conformations. Thus if two adjacent carbon-carbon bonds

Figure 6·1 (a) A Diagrammatic Representation Of The 'Fluid Mosaic'.

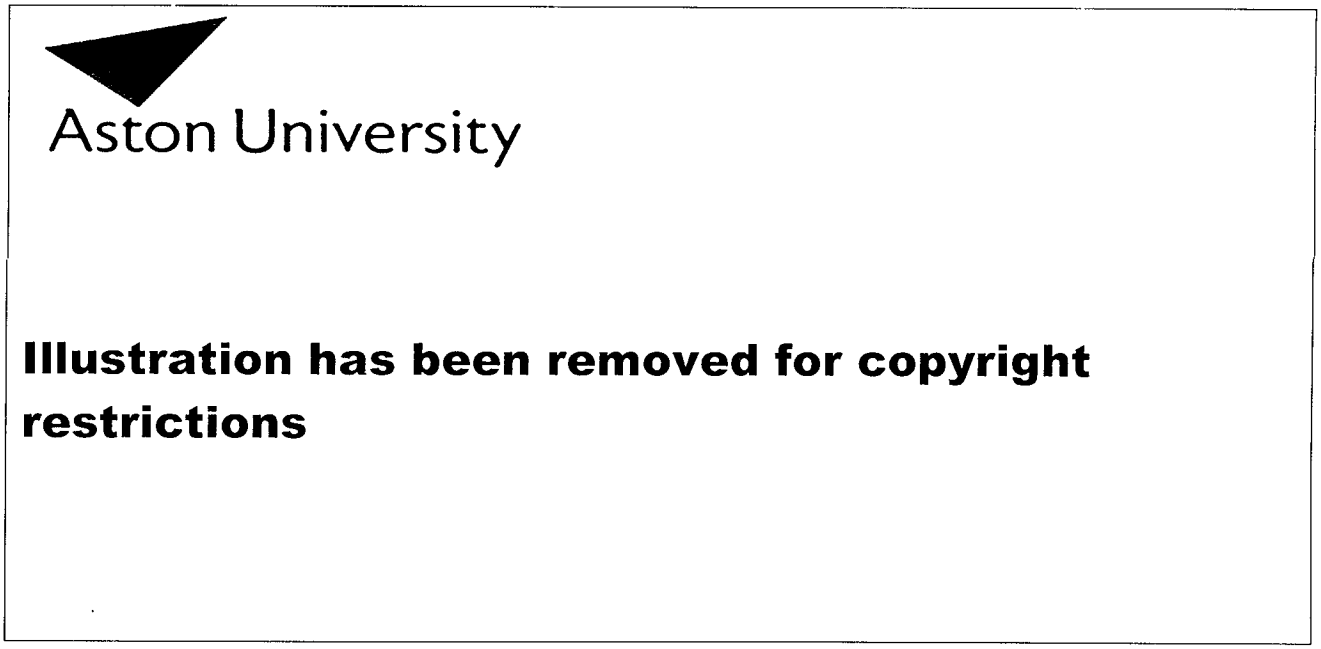


Figure 6·1(a) The fluid mosaic of Singer and Nicolson, 1972. The model shows a lipid bilayer composed of polar phospholipids and steroids (shaded molecules) in which are embedded a number of proteins. Some of these proteins traverse both lipid palisades and effectively link the intra- and extracellular environments, whilst others are only associated with one face of the membrane.

are rotating in a single chain to the gauche conformation this will give rise to a 'kink' in the chain (the so-called 2g-1 kink, Trauble and Haynes, 1971) and will decrease chain length. The formation of such kinks is a dynamic process with kinks forming and disappearing at any one point in the chain. However there is more freedom for

for them to form near the terminal methyl ends of the chain due to reduced steric hindrance (Rotham, 1973). Such motions are thought to be involved in the formation of 'statistical pores' which have been invoked to explain some transmembrane diffusion phenomena (Harrison and Lunt, 1975). This process will be discussed later with reference to the action of calcium and oestrogens upon thymic lymphocytes. The movement within the lipid matrix is determined in part by the chemical composition of the phospholipid and in part by its physical state (Hinz and Sturdevant, 1972). Below their transition (melting) temperature the fatty acids are packed in highly ordered hexagonal arrays (Dodds, 1975). Motion of the chains in this gel phase is very restricted. In the liquid crystaline phase (above the transition temperature) whilst the bilayer configuration is retained the arrays become disordered because of C-C bond rotation (Salsbury and Chapman, 1968). This disorder represents the lateral diffusion of lipid molecules in the plane of the bilayer. This diffusion may also be facilitated by the movement of lipid molecules into adjacent vacant sites in the lattice (Chadwick and Sherwood, 1971). The addition of various agents may also directly influence the fluidity of the membrane. Thus cholesterol reduces membrane fluidity in a liquid crystalline phase (Phillips, 1972).

We can envisage the cell membrane therefore to be composed of lipid and protein. These proteins may represent enzymes, receptors or transport complexes. The bulk of the lipid is present in the bilayer form, with perhaps a fraction associated with (and immobilized) and around intrinsic proteins. Regions of the lipid may be in the gel phase, whilst others may be more fluid, and therefore agents such as cholesterol may preferentially associate with lipids which are in one or the other state. Both the lipids and proteins undergo

constant movement within the matrix resulting in the dynamic equilibrium structure for the membrane as a whole.

6·2 The cell membrane; its role in ion distributions.

Within the cell and its various compartments there exists a nett surplus of negatively charged macromolecules which are not free to move across the cell membrane. The surfeit of negative charge is in its turn balanced of necessity by an equivalent number of mobile positive species. This then leads at equilibrium to an unequal distribution of ions across the membrane which is described as a Donnan equilibrium (Goldman, 1943). When the activities of the mobile ions on either side of the membrane are not equal there is set up an electrical transmembrane potential.

In most cells the observed membrane potential is the resultant of the sum of the pressures of each mobile species. However, this can be reduced to a simple Donnan potential, as has been done by Goldman (1943), to produce a constant field equation:-

$$E = \frac{RT}{F} \ln \left\{ \frac{P_c(C)_o}{P_c(C)_c} + \frac{P_a(A)_c}{P_a(A)_o} \right\}$$

Where, R = gas constant; T = absolute temperature; F = Faraday's constant; and P_c and P_a the permeability constants for cations and anions respectively. $(C)_o$ and $(A)_o$ are the concentrations of cations and anions respectively (activities) in the extracellular medium; and $(C)_c$ and $(A)_c$ are those of the cell water.

In many cell types potassium and chloride ions are the only species which display any appreciable movement across the cell membrane. Therefore it is justified to reduce the Goldman equation to:-

$$E = \frac{RT}{F} \ln \frac{a(K)_o + (Cl)_c}{a(K)_c + (Cl)_o}$$

$$\text{Where } a = \frac{P_K}{P_{Cl}} \quad .$$

It has been shown (Hunter, 1971) that in somatic cells P_{Cl} is about 100 times greater than P_K, thus, provided that the chloride is distributed passively the membrane potential should be equivalent to the chloride equilibrium potential.

However it should be noted again that the membrane is not passive in this action of ionic balance. It does not merely represent a barrier to large and or hydrophilic molecules. For whilst changes in the gel-liquid crystal structure can bring about changes in membrane permeability (Harrison and Lunt, 1975) it is also clear that the biological membrane can discriminate between different molecules and molecular species. Some of this selectivity undoubtedly lies in the lipid composition of the membrane (Papahadjopoulos, 1971) but the majority must surely reside in the range of 'carrier' molecules and transport enzymes which are to be found associated with the plasma membrane. Thus although the permeability of the membrane to sodium and potassium is low, there still occurs a nett passive leak of potassium out of the cell and an opposite but smaller flow of sodium in. This leakage has to be compensated for by the expenditure of energy in a sodium-potassium {(Na-K)-ATPase} adenosine triphosphatase

ATP phosphohydrolase EC 3·6·1·3, which selectively recaptures lost potassium and expels sodium stoichiometrically (Hoffman, 1975). In addition the activity of this pump may be regulated not only by the endogenous potassium concentration but also by such intracellular messengers as calcium and cyclic AMP (Hoffman, 1975). To take another relevant example, the membrane is very impermeable to the passive diffusion of calcium ions, but it possesses a facilitated diffusion system involving membrane 'carrier' molecules (Borle, 1970). Similar carrier systems exist for a number of somewhat more exotic molecules such as glucose and leucine, these also seem to be linked to sodium transport (Schultz and Curran, 1970). The cell also possesses an active pump for excluding calcium which exhibits great ionic specificity and is regulated by the intracellular calcium concentration (Vincenzi, 1971).

On activation of both quiescent 3T3 cells and lymphocytes by mitogens there are known to be alterations in transmembrane ion distributions (Table 3 of the introduction) as well as other non-charged solutes. And indeed the altered transport rate of these various species may represent important aspects of cell stimulation. The preceding Units have also demonstrated that magnesium and calcium ions play some essential role in thymocyte G_o recruitment, and this role may involve the transfer of these divalent cations across the cell plasma membrane. When 3T3 cells become arrested at confluency the activity of their (Na-K)-ATPase decreases, this activity could be returned to its value in exponentially growing cells by serum addition (Elligsen, Thompson, Frey and Kruuv, 1974). These workers concluded that the reduction in ATPase activity was associated with the cessation of division rather than contact inhibition itself since the phenomenon was also apparent in SV40 transformed fibroblasts which

had been arrested by serum starvation. Similar results have been obtained in a plasmacytoma line (Lelievre and Paraf, 1973). Such a change in ATPase activity might be expected to cause changes in ion distributions such as those observed following mitogenic stimulation; indeed fast growing, in comparison with contact arrested ME_2 cells show a greater efflux rate of potassium (Ducouret-Prigent, Lelievre, Paraf and Kepes, 1975). However in this study absolute cell potassium levels were unaltered, thus suggesting that membrane permeability as well as (Na-K)-ATPase activity had changed. This being the case it might be speculated that the initial event was an alteration in membrane permeability to monovalent cations, which provoked a subsequent enhancement of ATPase activity to return the ionic equilibrium to normal.

Whether an alteration of ATPase activity via one or more mechanisms actually dictates G1 arrest or G_o activation respectively is unclear. However there is no doubt that changes in membrane permeability are intimately associated with altered growth states. Changes in intrinsic membrane structure are known to occur at confluency (Scott, Furcht and Kersey, 1973). These changes may result not only in architectural alterations but also in changes in fluidity (Barnett, Furcht and Scott, 1974). Thus in both exponentially growing and transformed 3T3 cells membrane fluidity is high (i.e. tending towards the liquid crystalline state). However at contact inhibition cell membranes undergo a transition to the less fluid and more compact gel state. Such changes could well be associated with permeability alterations. A second source of evidence indicates that Chinese hamster ovary cells exhibit a six fold increase in transmembrane potential at confluency (Cone and Tongier, 1973). This rise in potential is associated with a loss of sodium from these cells (Cone

and Tongier, 1973). Furthermore imposition of conditions upon log phase cells which correspond to an increased membrane potential difference result in division arrest in G1, thus imitating a contact inhibition situation (Cone and Tongier, 1971). In agreement with these results are the observations of Adam and Adam (1975) who demonstrated that stimulation of contact inhibited 3T3 cells by a range of agents provoked a change in surface charge. Transformed fibroblasts, however, did not exhibit this charge alteration when treated with these mitogens.

Altered sodium (Dent, 1971) and potassium (Quastel and Kaplan, 1971) movements have also been noted in lymphocytes stimulated to leave G_o by PHA. Indeed if this potassium movement is blocked by ouabain, protein and DNA synthesis does not occur (Kay, 1972). Thus the ion movements or rearrangements seem to be critical factors in the activation process, and may either directly or indirectly via an alteration in cell metabolism bring about G_o escape. It is easy to visualise a similar system operating in normal cycling cells. Intrinsic to the cell cycle may be sequential alterations in the membrane conformation (i.e. a membrane cycle as suggested previously) which normally bring about changes in ion balance. Such changes in G1 might either commit or inhibit the cell from undertaking further rounds of division. With reference to this notion of changes in membrane permeability throughout the cell cycle recent work indicates that both passive diffusion and active transport characteristics of a number of solutes show marked cell cycle dependence (Tupper, Mills and Zorgniotti, 1976).

It was decided to investigate the possibility that thymocytes might similarly exhibit changes in transmembrane potential

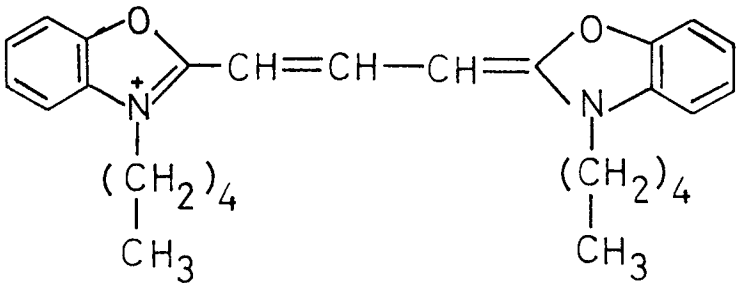
difference with special reference to the actions of divalent cations and steroids as mitotic and antimitotic agents. Such a study might also help to answer the perplexing problem of which point in the mitotic activation process oestradiol and testosterone act, and might also ascribe a role to the calcium and magnesium dependent processes apparent in these cells.

6·3 The membrane fluorescent potential probe 3,3'-dipentyloxacarbocyanine.

'Sophisticated does not necessarily mean elegant!

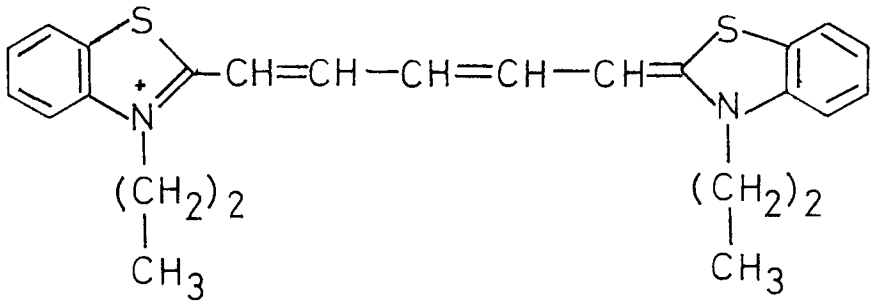
Because of the heterogeneity of the thymocyte population it is clearly impracticable to monitor transmembrane potential with a microelectrode. Thus another approach was adopted which involved the use of a fluorescent dye (CC_5) 3,3'-dipentyloxacarbocyanine iodide (Figure 6·3a). Dyes of the carbocyanine family are known to reflect transmembrane potential difference (Sims, Waggoner, Wang and Hoffman, 1974) and CC_5 in particular has been used most effectively in erythrocytes (Hoffman and Laris, 1974). Their mechanism of action however remains unclear. It has been suggested that the formation of

Figure 6 3a The structure of CC_5



non-fluorescent aggregates in the membrane occurs when the association of the dye with the membrane is promoted by the existence of a potential across the plasma membrane. Because of the delocalised positive charge on the dye molecule, hyperpolarisation of the cell will encourage movement of the dye into the membrane where aggregation reactions will tend to decrease the observed emission intensity. Whilst the closely related dye, 3,3'-dipropylthiocarbocyanine (Figure

Figure 6·3b. The Structure Of 3,3'-dipropylthiocarbocyanine.



6·3b) does indeed form higher aggregates (Figure 6·3c) confirming the results of Sims et al., (1974), CC_5 itself does not form such polymers under a wide range of conditions (Figure 6·3d). Clearly CC_5 has an affinity for the hydrophobic environment of the plasma membrane in which it fluoresces more intensely than in the aqueous milieu (Sims et al., 1974). Thus the nett fluorescence observed will be the resultant of a partitioning of dye between the extra- and intracellular compartments and the plasma membrane. We suggest that this partitioning will be altered changing the potential difference across the membrane (Figure 6·3e) and which will therefore be reflected as a change in fluorescence emission.

The fluorescence experiments were performed on a specially modified spectrofluorimeter (Aminco-Bowman) equipped with an EMI 9781B

Figure 6•3c (i) An absorption spectra of the dye 3,3'-dipropylthiocarbocyanine.

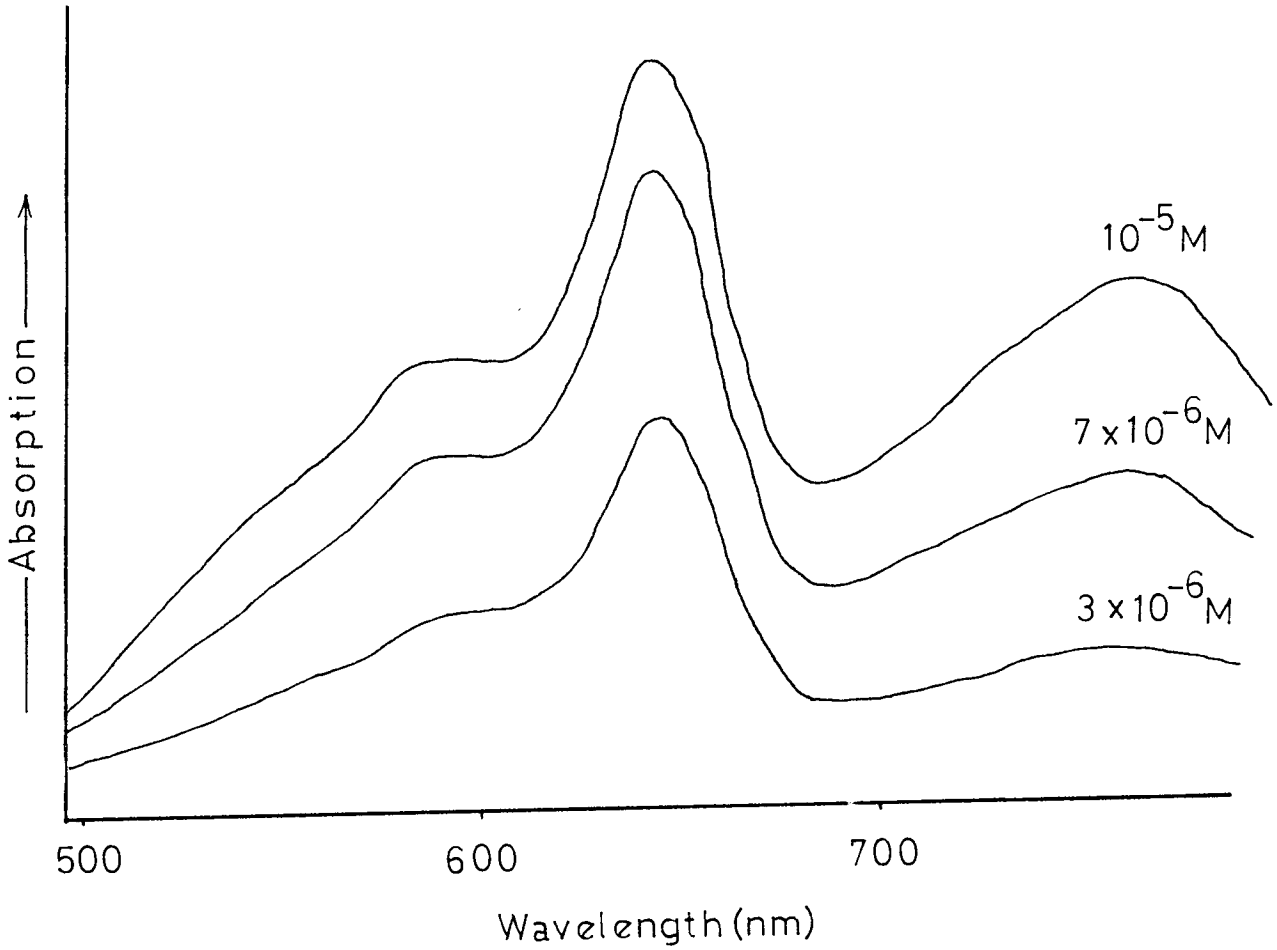


Figure 6•3c (i) An absorption spectra of 3,3'-dipropylthiocarbocyanine at different concentrations in a sodium chloride/Tris buffer at pH 7•4. The experiment was also performed in a range of other buffer systems. The shoulder peak at 583nm represents a non-fluorescent dimer. The peak at 750nm is a higher order aggregate.
Figure 6•3c (ii) Indicates the favouring of dimer formation against the principal peak at 643nm at higher dye concentrations.

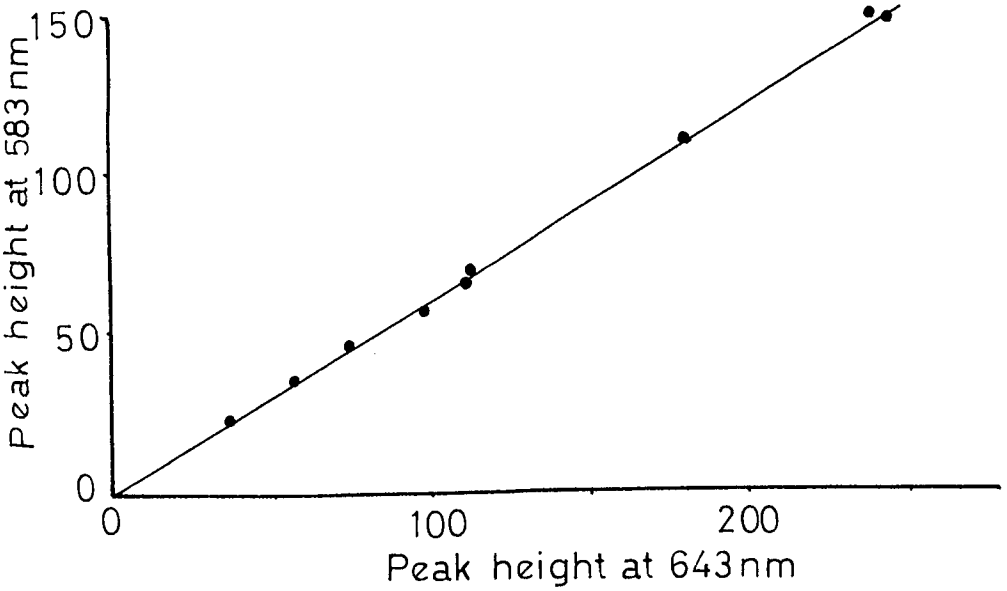


Figure 6·3d. The absorption spectra of 3,3'-dipentyloxacarbocyanine at increasing concentrations.

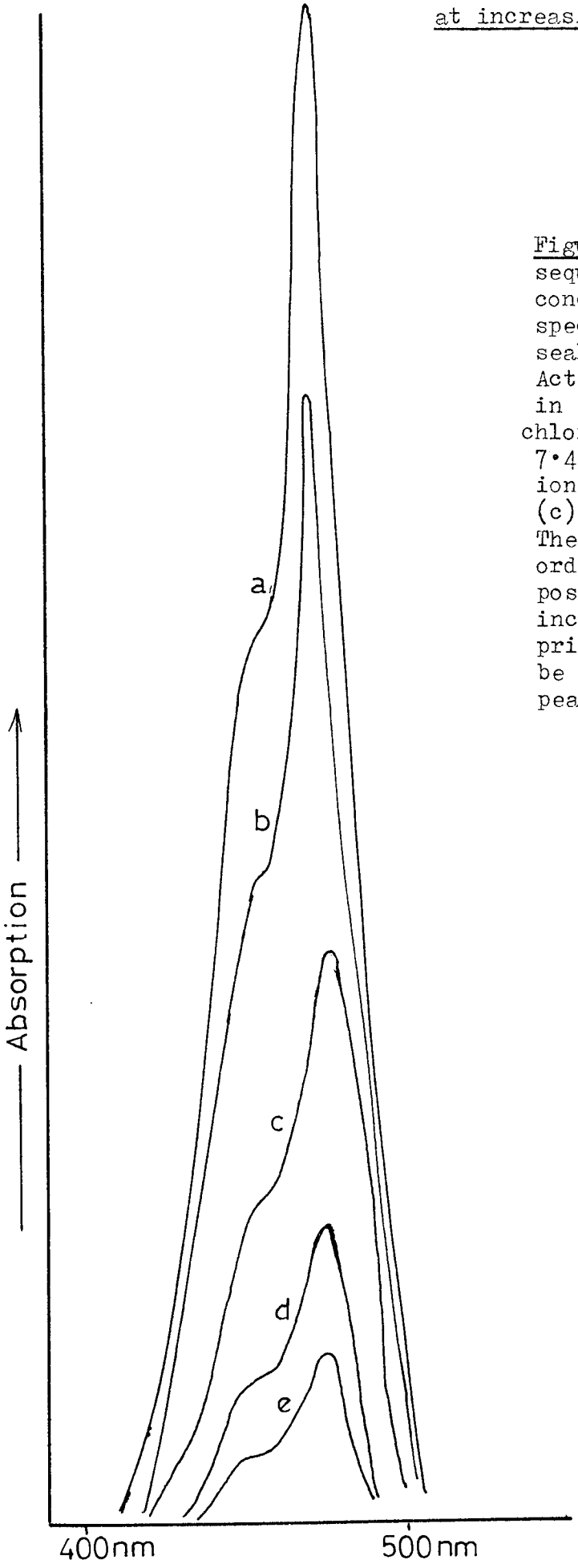


Figure 6·3d. The action of a sequential increase in CC_5 concentration upon its absorption spectrum. Dye was added to a sealed quartz cuvette in a Beckman Acta IV absorption spectrometer in an aqueous solution of sodium chloride and tris, buffered to pH 7·4 with HCl. The dye concentrations were (a) 10^{-4}M; (b) 5×10^{-5}M; (c) 10^{-5}M; (d) 5×10^{-6}M; (e) 10^{-6}M. There was no evidence of higher order aggregate formation, and the possible shoulder peak does not increase as a proportion of the principal peak, and hence must be regarded as part of the main peak.

Figure 6·3e. A Diagram To Illustrate The Possible Mode Of Action Of CC_5 In Detecting Membrane P.D.

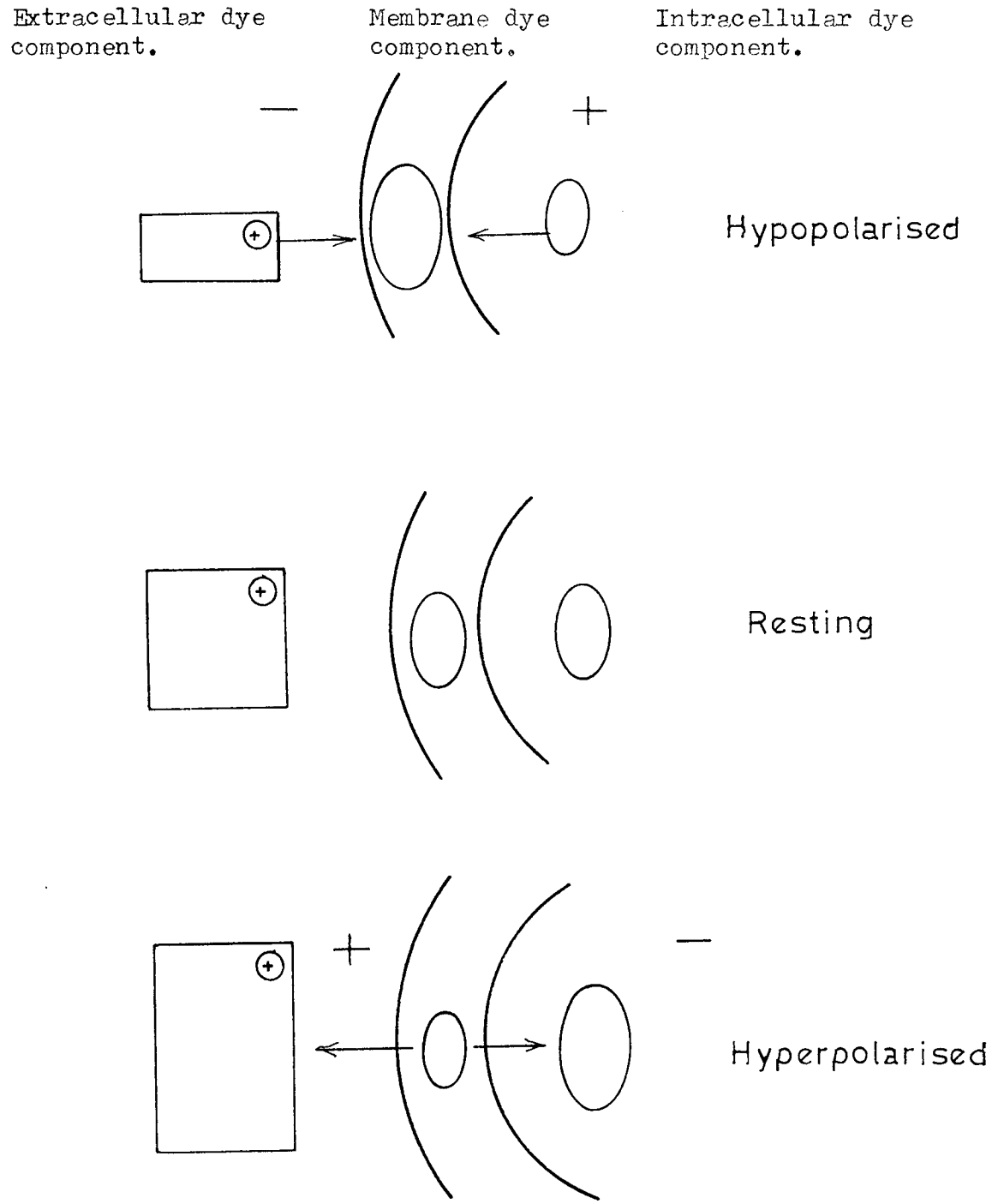


Figure 6·3e. A possible scheme whereby CC_5 could detect changes in transmembrane potential, and reflect this as an alteration in its fluorescence intensity emission. It is suggested that the dye in the resting condition attains some equilibrium distribution with a typical fluorescence emission for that distribution. However if the cell becomes hypopolarised, then the inside of the membrane becomes more positive (+) and the outside more negative (-), because of the small nett positive charge on the dye, this attracts CC_5 into the membrane from both the extracellular pool and cytosol. The converse occurs in hyper

Figure 6·3e continued.
polarisation, where the increase in intracellular negativity (-) tends to favour movement of dye into the cytosol where its fluorescence is quenched for purely optical reasons. Furthermore because the outer face of the membrane now carries a more positive (+) charge this disfavours movement of dye into the membrane from the extracellular compartment through mutual repulsion. Since the dye does fluoresce much more intensely in the membrane than either the extra- or intracellular environments, any treatment which reduces or increases the amount of dye associated with the membrane will therefore directly alter the observed fluorescence emission. The areas representing the dye components are in no way meant to indicate absolute dye levels, but are merely indicative of the direction of change of a given dye pool. Furthermore even in resting cells, the outside of the membrane will be more positive than the inside, thus the changes in polarity (+) or (-) are only relative alterations with respect to the resting potential.

photomultiplier tube. The excitation source was a 250W Xenon arc (Englehard Hanovia Inc.) focussed at the cathode spot. Figure 6·3f indicates that the peak excitation and emission wavelengths of the dye are 470 and 510nm respectively. Thus the system was monochromated

Figure 6·3f. Emission Spectrum Of 3,3'-dipentyloxacarbocyanine.

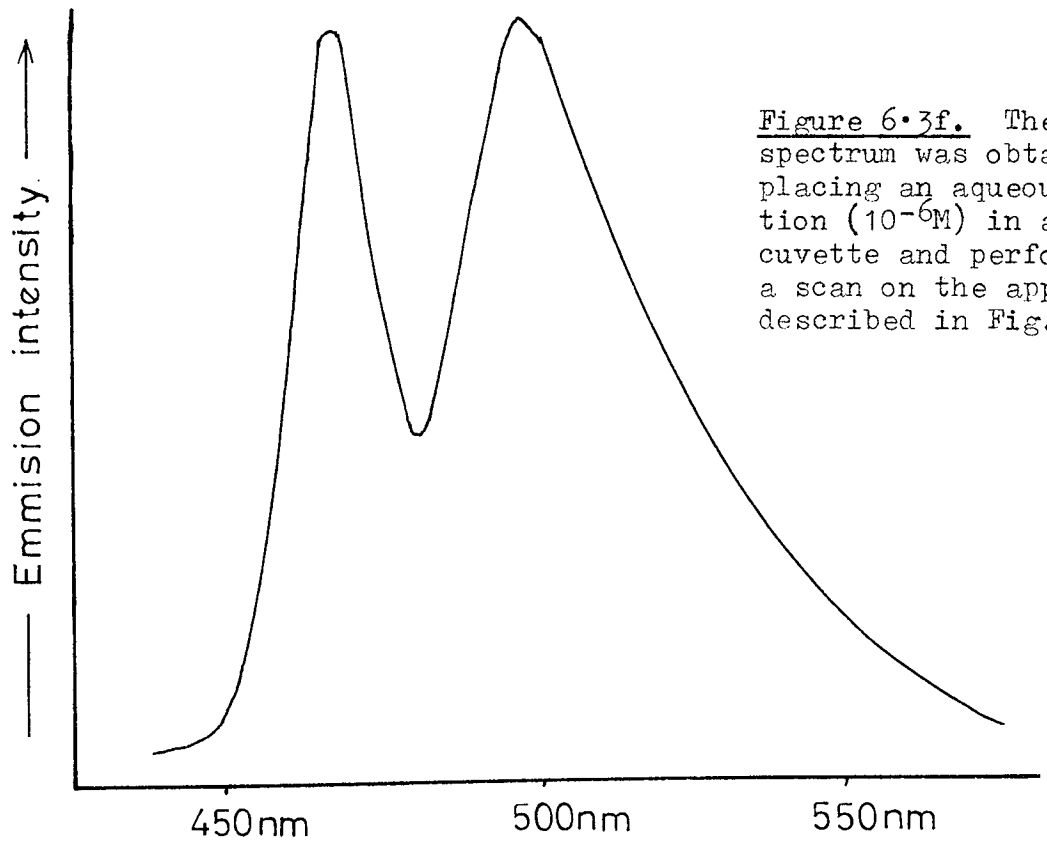


Figure 6·3f. The emission spectrum was obtained by placing an aqueous solution (10^{-6}M) in a 1cm cuvette and performing a scan on the apparatus described in Fig. 6·3g.

Figure 6·3g. A Flow Diagram Of The Fluorescence Apparatus.

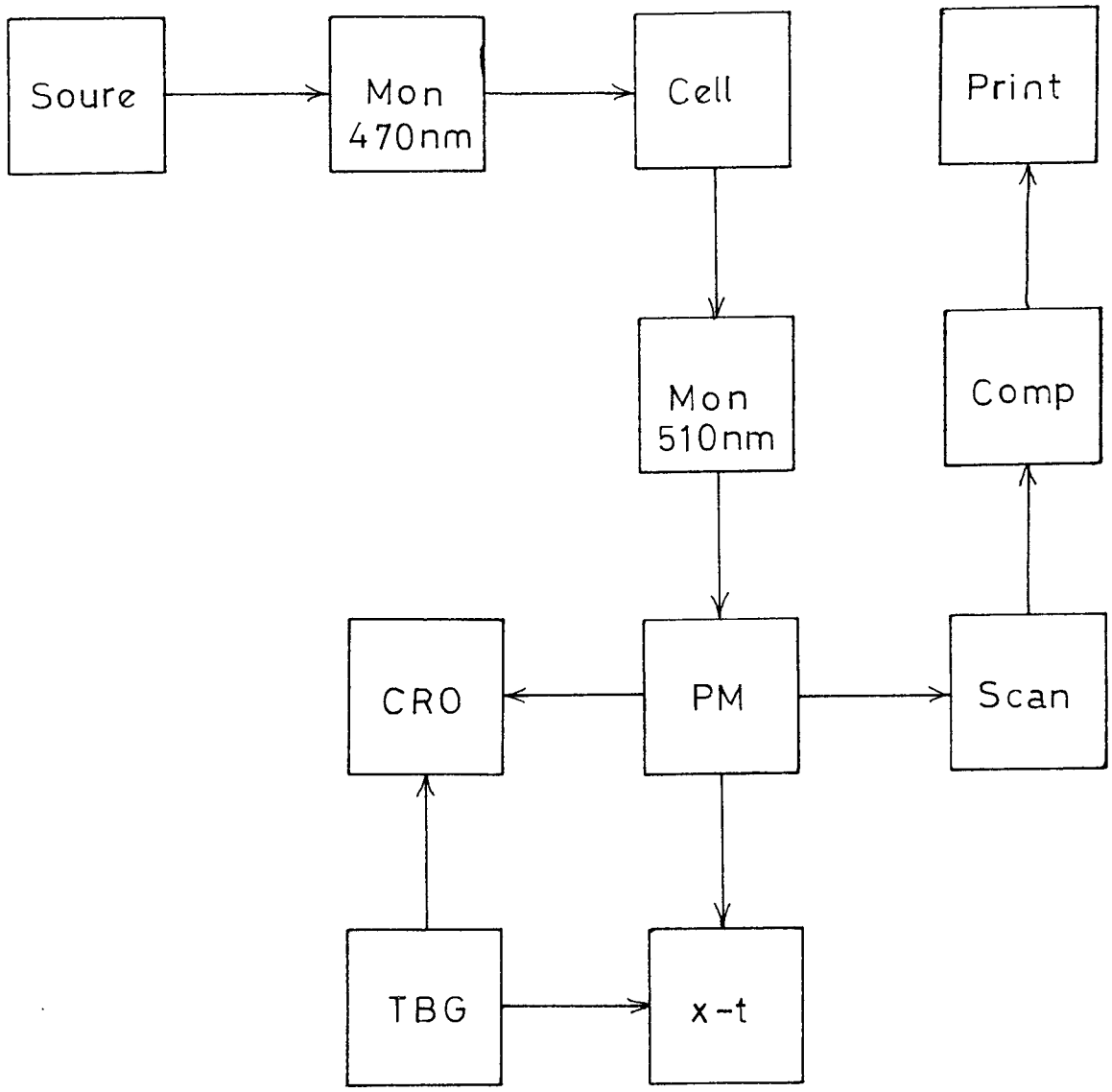


Figure 6·3g. A Flow diagram to show the organisation of the apparatus employed for detecting alterations in CC_5 fluorescence intensity. Light from the Xenon arc (Source) passes through the emission monochromator set for 470nm and into a 1cm light path quartz cuvette containing the thymocytes (Cell). Fluorescent and scattered light is received at right angles to the source and is monochromated at its emission peak (Mon 510nm). The light then passes to the photomultiplier (PM). The signal may then be recorded directly on a Beckman x-t recorder. In addition rapid changes may be monitored with an oscilloscope (CRO) with an associated time-base generator (TBG). Spectral emission scans mat be performed by use of a second recording (Hewlett Packard) device designed for this procedure. These emission spectra may then be processed by a computer programme (Comp) and obtained back as a corrected scan (Print).

to yield these required optimum wavelengths. Fluorescent and scattered light after passage through the photomultiplier was recorded on an x-t recorder (Beckman). Figure 6•3g shows the full apparatus with ancillary equipment for monitoring rapid changes in fluorescence, or for obtaining emission spectra. It might also be added that these spectra were corrected using a standard computer programme (FLUCORR) which calculated the energy transfer efficiencies of the apparatus at differing wavelengths. The apparatus as a whole thus permitted the continuous recording of fluorescence intensity (trace height) against time.

Using a cell-free system a fluorescence yield against dye concentration plot was performed (Figure 6•3h). On the basis of this

Figure 6•3h. A Comparison Between Fluorescence Yield And Dye (CC_5) Concentration.

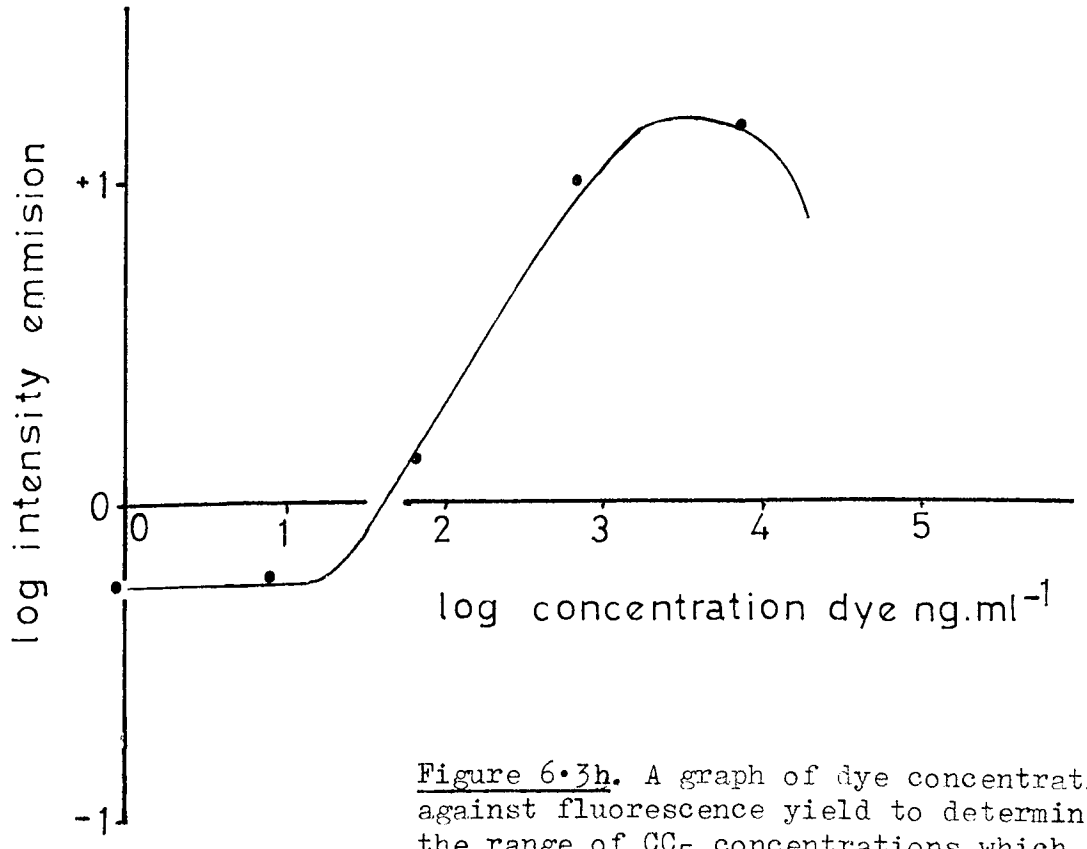


Figure 6•3h. A graph of dye concentration against fluorescence yield to determine the range of CC_5 concentrations which exhibit a constant quantum yield. CC_5 was prepared as an aqueous solution and placed in a 1cm cuvette in the fluorimeter and yield estimated at 510nm after excitation at 470nm.

water jacket system. They were also gently stirred with a motorised glass rod to prevent sedimentation during the experiment.

Addition of thymocytes to the fluorimeter resulted in an abrupt increase in fluorescence emission (Figure 6·3i). It is known that a number of agents may selectively alter the membrane permeability

Figure 6·3i. The Effect Of Adding Thymocytes To A CC_5/Medium Mixture In The Spectrofluorimeter.

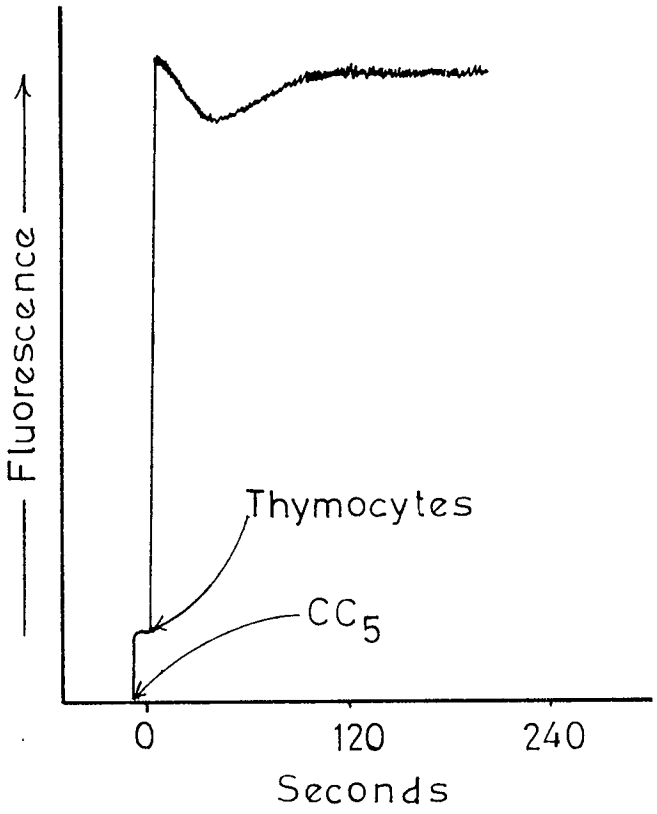


Figure 6·3i. The effect of adding thymocytes on the observed fluorescence from a dye/buffer system. CC_5 was added to a cuvette containing the modified Earle's salts solution (pH 7·3) in the standard spectrofluorimetry system to give a final dye concentration of 2×10^{-6} M. This caused an increase in fluorescence. Addition of thymocytes in the medium described previously however promoted a large rise in fluorescence intensity followed by a characteristic emission profile before a stable plateau was attained after about 2 minutes.

to a given ionic species. One such compound is valinomycin, which increases the membrane permeability to potassium (Andreoli, Tieffenberg and Tosteson, 1967). In thymocytes also this agent promotes the marked

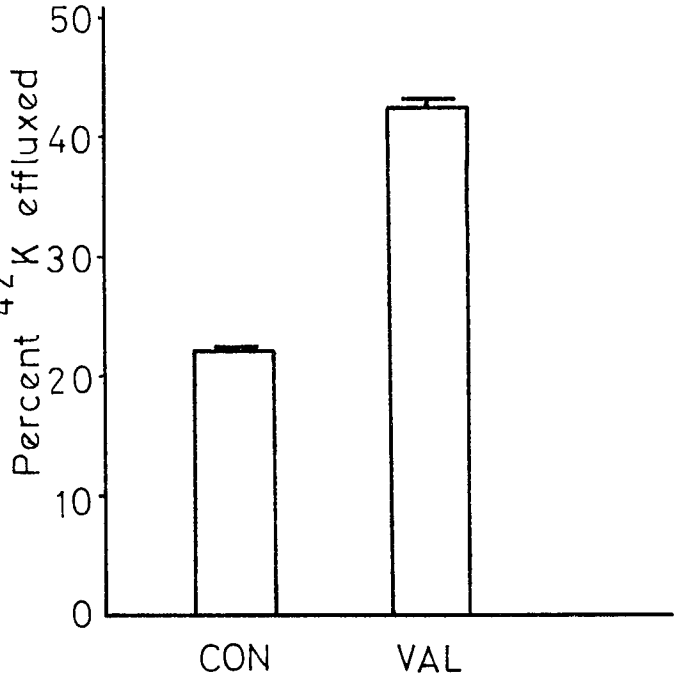


Figure 6·3j. The influence of valinomycin on the efflux of ^{42}K from thymocytes. Thymocytes maintained in modified Earle's salts solution were prelabelled for 50 minutes with 1μCi.ml^{-1} of the isotope. Cells were subsequently washed twice in unlabelled medium and then incubated for a further twenty minutes in fresh salts solution to permit recovery from centrifugation. After this period some cultures were treated with valinomycin (Sigma Chemical Co.) at a final concentration of 10^{-5}M whilst others acted as controls (CON). 30 minutes later the cells and supernatant were isolated by microfugation as outlined in Unit 7, and counted for radioactivity on a Tracerlab Gamma Set 500, gamma emission spectrometer. The results have been expressed as the percentage label having appeared in the supernate, thus correcting for dispensing errors in cell numbers. Each column is the mean and vertical bar with horizontal line the SEM of 4 separate experiments. Counts were corrected for decay during the experiment and whilst counting.

efflux of ^{42}K from prelabelled cells (Figure 6·3j). This action will clearly alter the transmembrane potential as now the permeability coefficient for potassium becomes large and a significant function in the Goldman equation (Goldman, 1943). In fact this ionophore induces induces an hyperpolarisation when it is added to a number of systems (Andreoli et al., 1967) since it leaks positively charged potassium ions from the cell into the surrounding medium. This aspect of valinomycin's action enables characterisation of the fluorescence changes which occur in this system.

Figure 6·3k. The Effect of Valinomycin On Fluorescence From A Variety Of Cell Types.

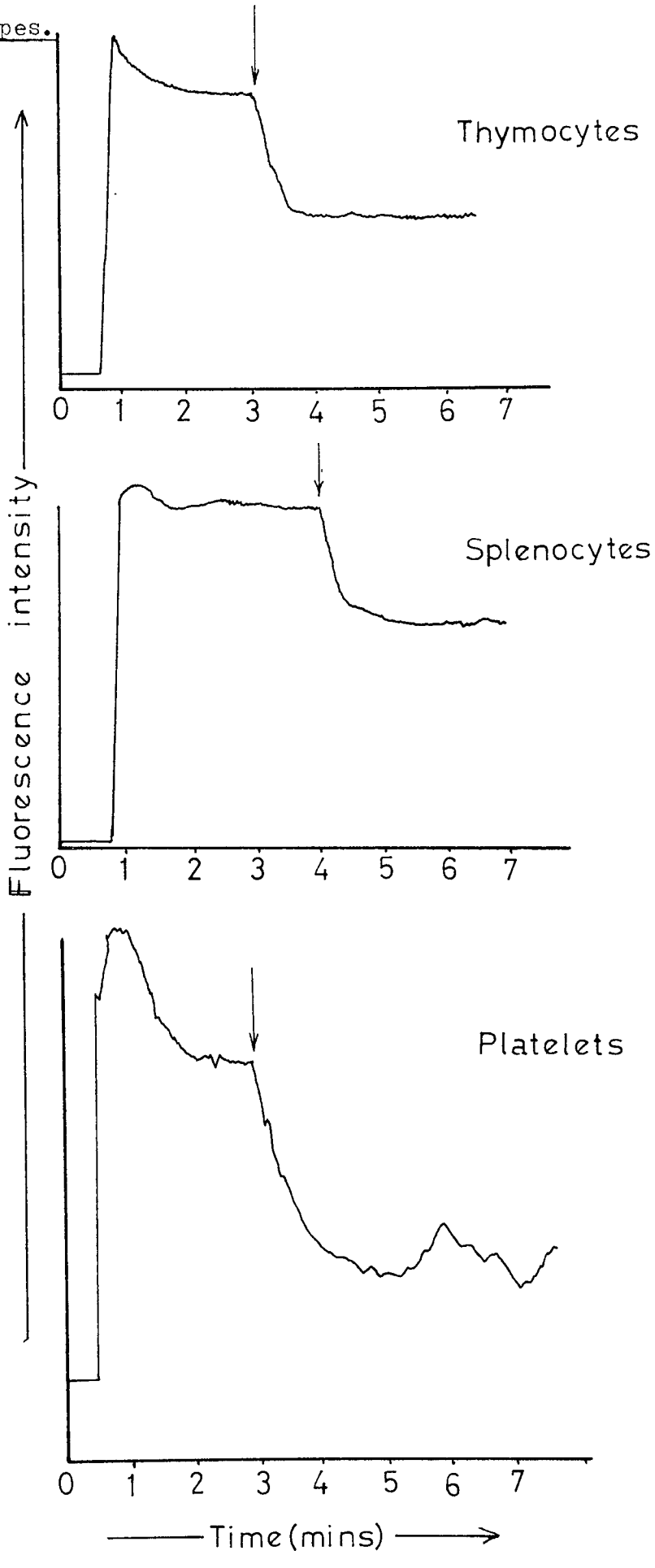


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Figure 6·3k. The action of valinomycin on the observed CC_5 fluorescence emission from thymocytes, splenic lymphocytes and platelets. Cells were preincubated and then equilibrated with dye (2×10^{-6}M) in the spectrofluorimeter. They were then treated with valinomycin (10^{-5}M) indicated by an arrow on the diagrams, and further fluorescence changes monitored.

Thymocytes were prepared as described previously. Splenic lymphocytes were obtained by extruding rat spleens through a fine mesh steel filter. Erythrocytes were removed by hypotonic shock in 0·76% ammonium chloride buffered to pH 7·3 with Tris-HCl. They were then diluted to the desired concentration in the standard salts solution. For platelets, human venous blood was diluted 1:1 with phosphate buffer (pH 7·4) and aliquots layered over a column which had been prepared previously by mixing 9% aqueous Ficoll 400 (24 parts) with 34% aqueous sodium metrizoate (3 parts). The column was spun at 400 x g for 20 minutes, and the platelet layer was aspirated away and resuspended in salts solution. This solution was spun at 400 x g for 5 minutes, and the supernatant, containing purified platelets, was diluted with salts solution and used in subsequent experiments.

When thymocytes, splenic lymphocytes or platelets which had been equilibrated with the dye were exposed to valinomycin (10^{-5}M) there was a marked and rapid decrease in fluorescence intensity (Figure 6·3k). This can be designated as being an hyperpolarisation. As a check on this observation the potassium concentration in the Earle's medium was varied at the expense of the sodium concentration. Thymocytes could then be exposed to valinomycin under various potassium regimes. The results indicate (Figure 6·31) that as the potassium concentration was increased there was a concomitant overall reduction in the nett fluorescence decrease observed on addition of valinomycin. Indeed at 160mM potassium the observed fluorescence change was a positive one suggesting that this level of potassium was greater than the intracellular burden of this cation and that ions were now moving into the cell thus generating a hypopolarisation. Hence in this fluorescence system a decrease in fluorescence intensity is interpreted as an hyperpolarisation, whilst an increase in emission is indicative of an hypopolarisation.

Another ion-specific ionophore is A23187 (Reed and Lardy,

1972) which was utilised in Unit 2, and has been found to be mitogenic. This agent preferentially transports calcium and to a much lesser extent magnesium (Caswell and Pressman, 1972) with its concentration gradient (i.e. into the cell under normal circumstances). When thymocytes were treated with this ionophore, the observed

Figure 6·31. The Influence Of The Extracellular Potassium Concentration On The Hyperpolarising Activity Of Valinomycin.

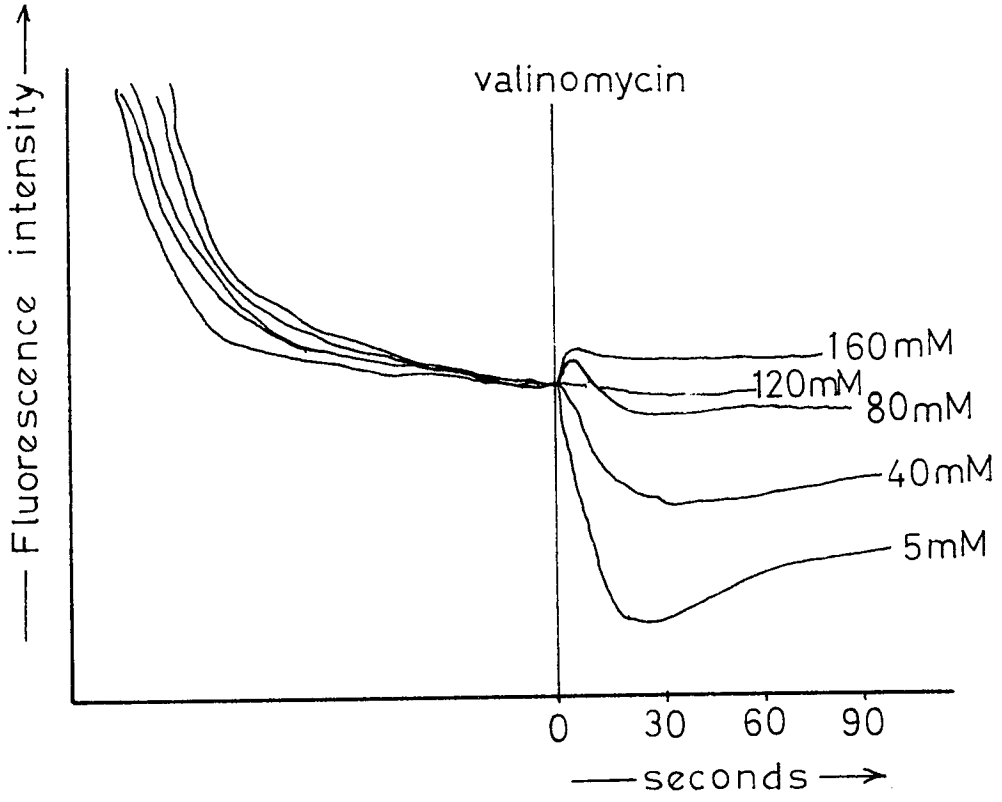


Figure 6·31. The role of extracellular potassium in the action of valinomycin. Thymocytes were incubated in salts medium in which the potassium concentration had been adjusted to the values marked on the figure. These various cultures were subsequently treated with valinomycin (10^{-5}M) and fluorescence monitored. To obtain the figure illustrated traces were overlayed, such that the time of addition of valinomycin and their equilibrated fluorescence intensity coincided, a copy was then made of these plots manually.

fluorescence change was dependent upon the extracellular calcium concentration (Figure 6·3m). This response is directly analagous to that observed with valinomycin and potassium except that now

calcium is the principal permeant ion. The results would again indicate

Figure 6·3m. The Interaction Of Calcium And A23187 On Fluorescence

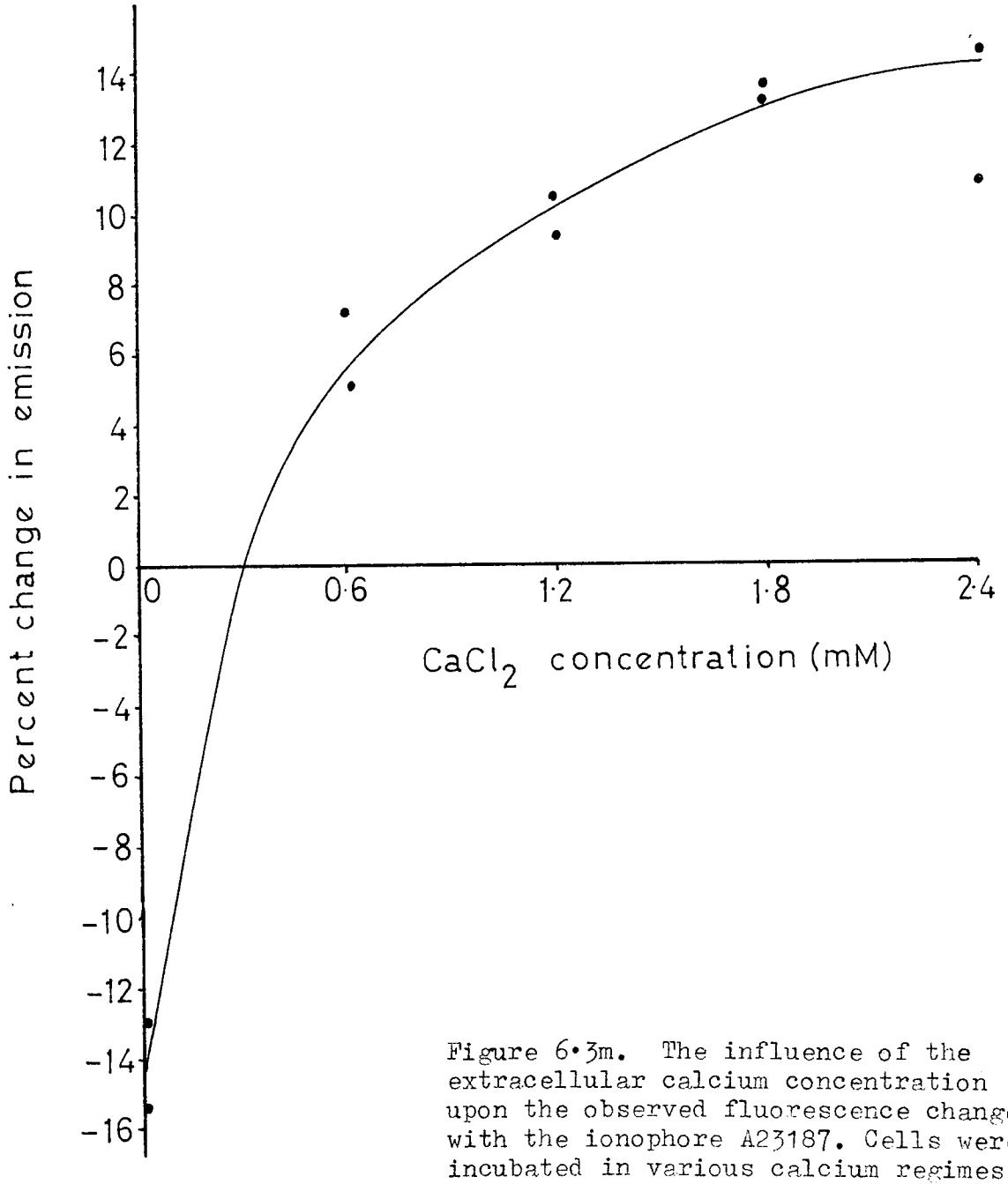


Figure 6·3m. The influence of the extracellular calcium concentration upon the observed fluorescence change with the ionophore A23187. Cells were incubated in various calcium regimes and subsequently treated with A23187 (10^{-5}M) and the maximum fluorescence change recorded. This was converted to a percentage change over the fluorescence in a cell-free system. Each point is one determination.

that a positive fluorescence change is associated with a situation where positively charged ions are entering the cell i.e. an hypo-

polarisation. In addition when calcium ions were induced to leave the cell by the ionophore because the extracellular medium contained no calcium an hyperpolarisation and decrease in fluorescence was observed.

When cells which had been preincubated in the absence of calcium were equilibrated with CC_5 and then exposed to 1·8mM calcium

Figure 6·3n. The Influence Of Calcium On Transmembrane Potential

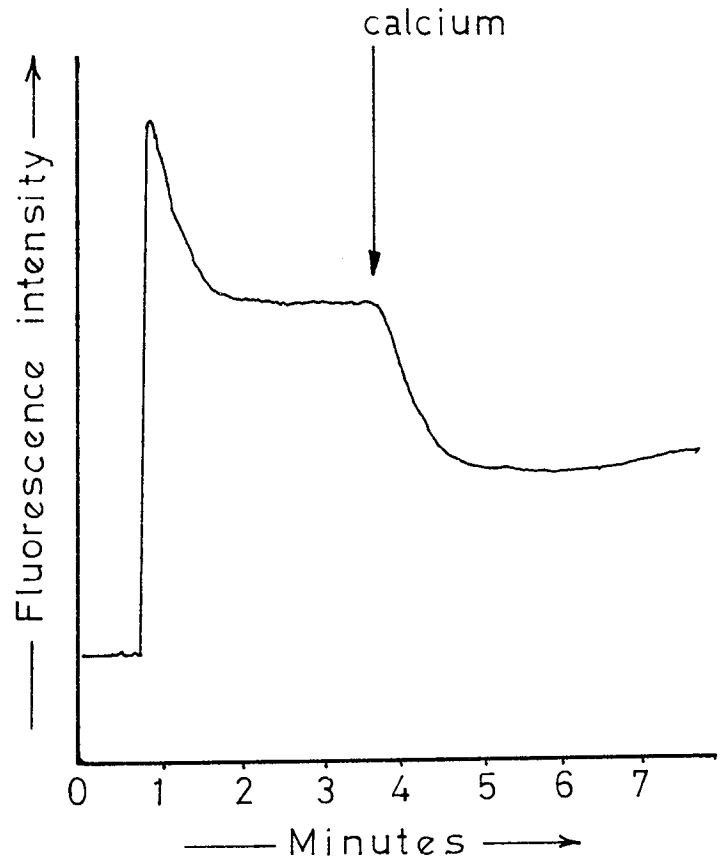


Figure 6·3n. The hyperpolarising action of calcium on the thymocyte membrane. Cells were preincubated in calcium-free Earle's salts and subsequently equilibrated with CC_5 and then exposed to 1·8mM calcium. The figure represents a tracing of a typical fluorescence profile.

chloride there was a marked decrease in fluorescence which reached a maximum value within 120 seconds (Figure 6·3n). Thus calcium addition had established a membrane hyperpolarisation.

Figure 6·3o. A Dose-Response Study Of Calcium Upon Fluorescence

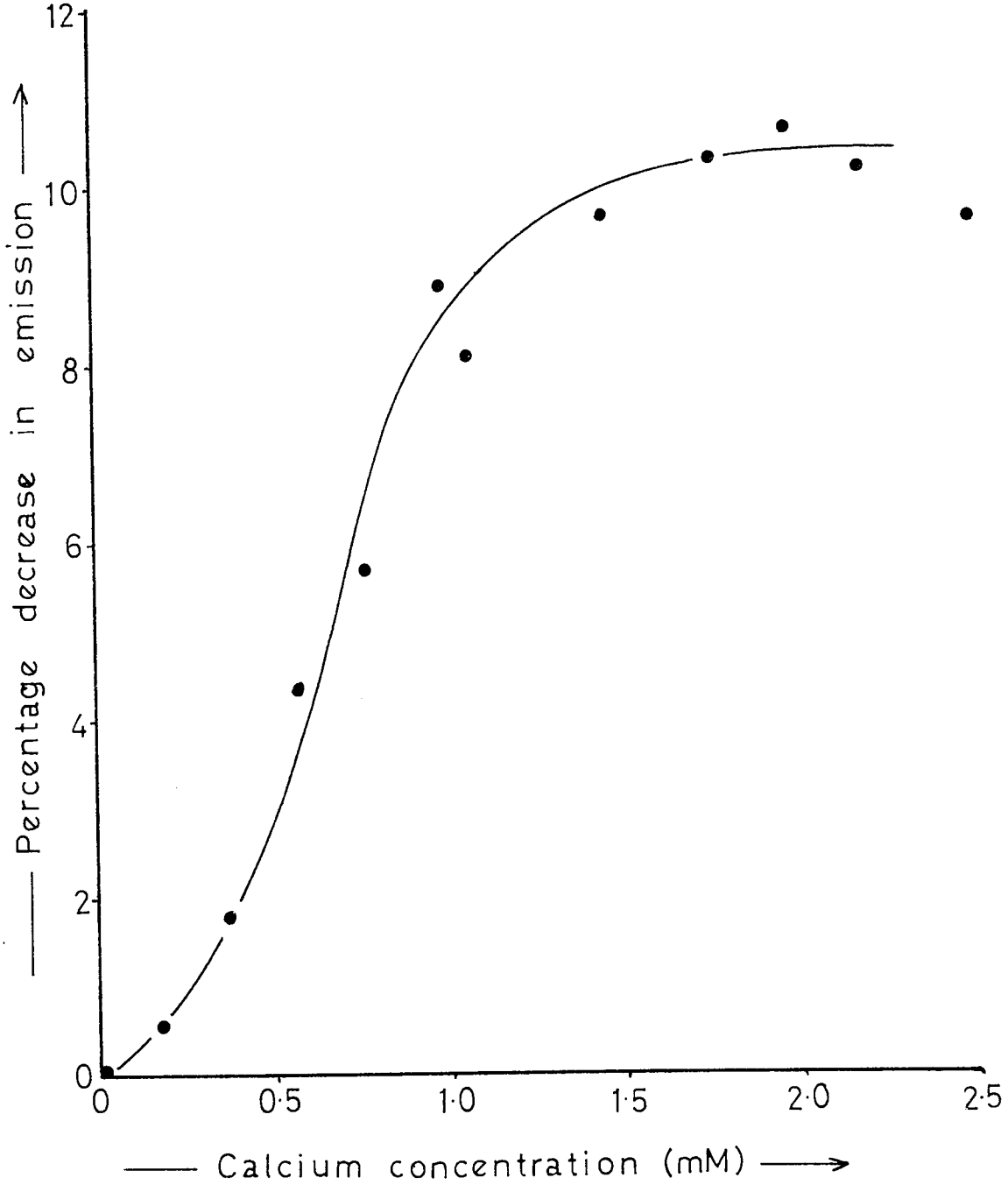


Figure 6·3o. The effect of adding increasing calcium concentrations on the fluorescence emission of CC_5 in thymocyte cultures. Cells were preincubated in calcium-free Earle's salts and then equilibrated with dye. The results represent the mean of three determinations corrected to a percentage value to reduce error in pipetting.

To further characterise this response to calcium, thymocytes were treated in the fluorimeter with smaller incremental additions

of calcium. This revealed a progressive increase in hyperpolarisation with the maximum rate of change of fluorescence occuring between 0·2 and 1·1mM. Maximum values were obtained at 1·8 to 2·0mM (Figure 6·3o). Thus the response appeared to be saturable at around 1·8mM calcium. Of some interest is the subsequent report of the induction of an hyperpolarisation in Amphiuma erythrocytes by addition of extracellular calcium (Lassen, Pape and Vestergaard-Bogind, 1976) which shows very similar kinetics to those detected here.

A number of divalent cations were tested for their ability to induce changes in the thymocyte membrane potential. Certainly the closely related species magnesium and strontium had no effect whatsoever and neither did zinc, manganese and tin. The case with barium and lanthanum was confused as both ions caused rapid cell aggregation which resulted in a large increase in 'noise' and which made any objective interpretations of the data impossible. In addition a number of these other ions showed poor solubility properties at pH 7·3 and there was a tendency for precipitation to occur, which also increased the scattered light. However with the exception of barium, it could be stated unequivocally that the most closely related cations to calcium (i.e. magnesium and strontium, the latter of which competes for calcium carriers, Vincenzi, 1971) did not themselves influence transmembrane potential as measured by the CC_5 system. However, when cells were incubated in 0·6mM calcium chloride and varying amounts of magnesium sulphate it was noticed that at high extracellular magnesium concentrations (3mM) there was a reduction in the maximal hyperpolarisation achieved with subsequent calcium additions (Figure 6·3p). This indicated that magnesium might be antagonising this potentiogenic action of calcium. Indeed this might be the first clear indication of a direct antagonistic link

between the two mitogenic divalent cations at the cellular level, and it might be re-called that such a relationship was proposed to exist on theoretical grounds. Furthermore since cells were cultured in the presence of calcium in these experiments this also precludes the possibility that calcium deprivation itself leads artifactually to these observed calcium-induced hyperpolarisations. Thus since the normal ionised calcium values in the plasma range between 1·0 and 1·2mM (Hunt, 1974) this highly specific calcium-induced hyperpolarisation of the thymocyte plasma membrane occuring over this

Figure 6·3p. The Influence Of Magnesium On Calcium-Induced Fluorescence Alterations In Thymocytes

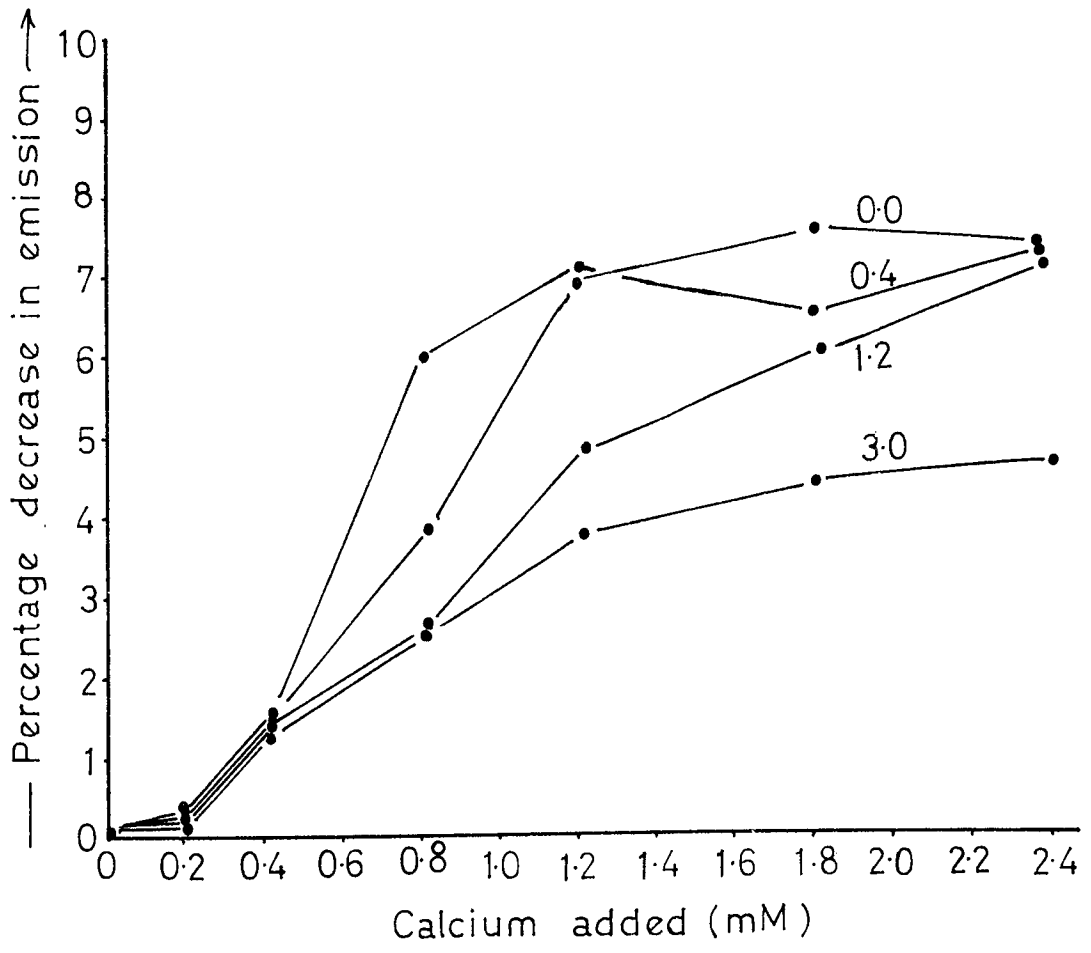


Figure 6·3p. The interaction between calcium and magnesium in the observed potential difference of the thymocyte plasma membrane. Cells were incubated in 0·6mM calcium and various (0, 0·4, 1·2 or 3·0mM) concentrations of magnesium. The cells were subsequently exposed to different loads of calcium (Calcium added) and fluorescence plotted. Each point is the mean of three determinations.

range (Figure 6·3o) may well represent a true physiological role for this ion in membrane function.

Once established, a calcium triggered potential change could not be reversed by addition of the calcium chelating agent EGTA (Figure 6·3q). Thus calcium seems to initiate some event which subsequently generates and maintains the observed potential alteration. Certainly it is not related to a simple diffusion of more calcium ions into the cell, for when such movement was provoked with some specificity by addition of the calcium ionophore A23187 (Reed and Lardy, 1972), this results in an increase in fluorescence

Figure 6·3q. The Influence Of EGTA On Calcium- And Ionophore-Induced Fluorescence Changes.

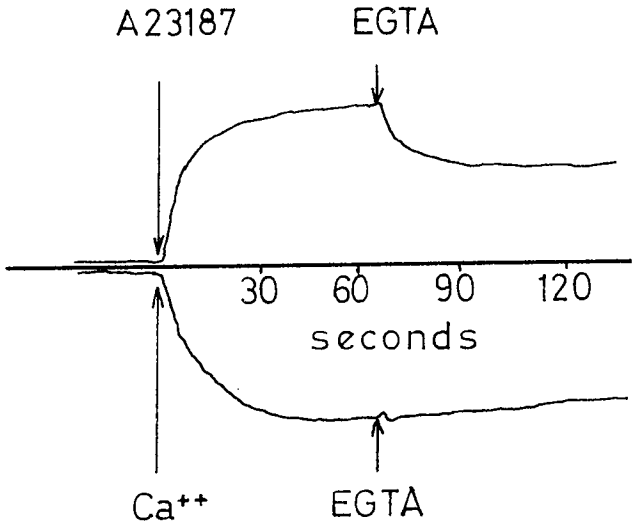
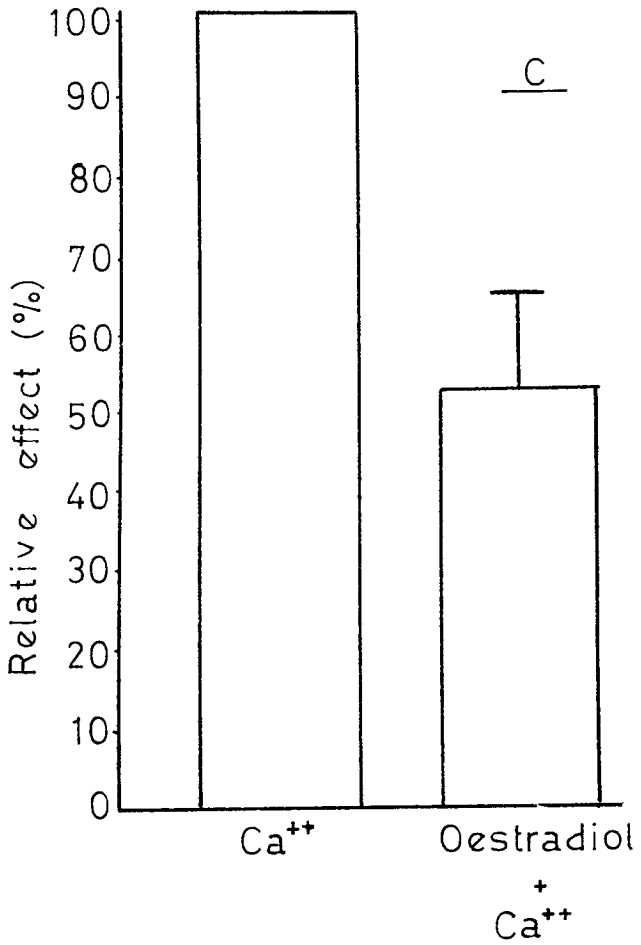


Figure 6·3q. The action of EGTA upon calcium- and ionophore-induced fluorescence changes in thymocytes. Cells were incubated in 0·6mM calcium and then equilibrated with CC5. They were subsequently treated with either 1·2mM calcium chloride (Ca^{++}) or A23187 (10^{-5}M) obtained from the Lilly Research Centre. One minute later the suspensions were exposed to 30µl of a saturated aqueous solution of EGTA and any fluorescence changes monitored. None of these agents interfere with or are fluorescent at these wavelengths.

Figure 6.3r. The influence of oestradiol on calcium-induced hyperpolarisation



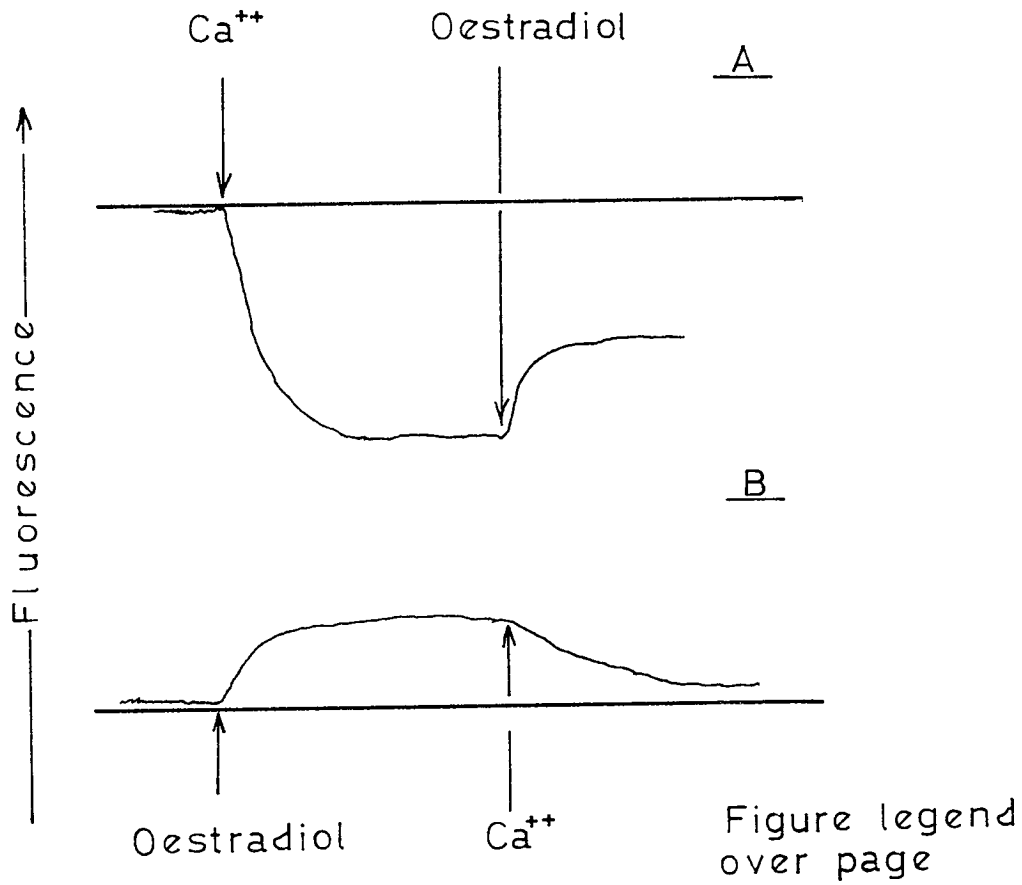


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Figure 6·3r. The influence of oestradiol-17β on transmembrane potential and calcium-induced hyperpolarisation. Thymocytes were incubated in Earle's salts solution containing 0·2mM calcium chloride and then equilibrated with CO_2. Cultures were either treated with oestradiol (10^{-5}M) followed by calcium chloride (1·8mM) as depicted in Figure B; or the reverse procedure was employed as shown in Figure A. In addition some cultures were divided into two, one half received 1·8mM calcium chloride and the maximum fluorescence change noted, whilst the second aliquot received oestradiol-17β (10^{-5}M) followed 90 seconds later by 1·8mM calcium and again the maximum fluorescence change with this calcium load noted. In all cases the observed change with calcium alone was taken as the 100% value and the fluorescence change with oestradiol calculated as a percentage of this index. Thus Figure C represents the mean of five estimations ± SEM of the effect of oestradiol on calcium-induced fluorescence alterations as a percentage of non-steroid treated controls (100%).

or hypopolarisation (Figure 6·3q), i.e. exactly the opposite observed with calcium. Furthermore addition of EGTA to ionophoretically hypopolarised thymocytes resulted in at least a partial reversion of this potential change.

Since oestradiol can inhibit calcium-induced mitogenesis in isolated thymic lymphocyte cultures (Unit 2) this steroid was added to the cell suspension in the fluorescence system both before and after calcium addition. In each case when oestradiol-17β (10^{-5}M) was added to cultures a small but significant hypopolarisation was observed (Figure 6·3r). Furthermore when calcium (1·8mM) was added to the cultures after oestradiol the hyperpolarisation induced by the ion was reduced to some 50% of that in paired suspensions containing no steroid (Figure 6·3r). In addition the characteristics of the calcium-provoked hyperpolarisation were certainly modified by the presence of oestradiol. It might also be noted that the polarity of the oestrogen-induced potential change is in the opposite direction to that generated by calcium. Additionally oestradiol-17β did not block an ionophore-induced hypopolarisation. It is not established however whether the hyperpolarisation and its presumptive ion fluxes

which calcium causes are related to, or essential for this ion's ability to recruit cells into their DNA synthetic phase. In an attempt to provide a partial answer to this question a range of steroids was examined to see whether an ability to inhibit calcium-induced mitogenesis was always associated with a capacity to block calcium-induced hyperpolarisation.

Several other oestrogenic molecules had the ability to inhibit calcium-induced proliferation as was reported in Unit 2, and are also depicted in Table 6•3s. When carrying out a similar analysis for the capacity to mute calcium-induced fluorescence changes it was apparent again that only 18 carbon oestrogens were active. Thus an initial close resemblance was noted between the requirements for activity in blocking calcium-induced mitogenesis and hyperpolarisation. Remarkably the biologically inactive alpha epimer of oestradiol was was again equally potent which also suggests a superficial site of action for the steroid, not involving a classical cytosol receptor (Noteboom and Gorski, 1965; King and Gordon, 1966). Furthermore, oestrone and oestriol possessed some activity in muting the calcium-induced hyperpolarisation, as did the 3-sulphate derivative of oestrone. It would appear that the common feature of these steroids is their aromatic 'A' ring, which might be an important component in structure activity. However, two steroids namely the 3-methyl ether of oestradiol and the 3-methyl ether of 17-ethyl-oestradiol whilst blocking calcium-induced potential changes, were not anti-proliferative (Table 6•3s), yet these steroids do possess an aromatic 'A' ring. Indeed anisole which essentially represents the 'A' ring configuration of oestradiol also had no effect on mitosis nor upon fluorescence. Thus it would seem that other aspects of structure are important. One such parameter might be the groups present on

Table 6•3s. A Comparison Of The Ability Of Various Steroids To Inhibit Calcium-Induced Mitogenesis And Hyperpolarisation.

Steroid	Blocks Mitosis	Blocks Hyperpol'n	Increases/Decreases fluoresc'n
Oestradiol-17β	++	++	Increase
Oestradiol-17α	++	++	Increase
Oestradiol-17β-3-benzoate	++	++	Increase
Oestriol	++	+	Increase
Oestrone	+	+	Increase
Oestrone-3-sulphate	++	+	Increase
17α-ethyl-β-oestradiol 3-methyl ether	-	++	Increase
β-oestradiol 3-methyl ether	-	++	Increase
β-oestradiol-3-glucuronide	-	-	No change
β-oestradiol-17-glucuronide	-	-	No change
Testosterone	-	-	Decrease
Androstenedione	-	-	No change
Androstenediol	-	-	No change
Dehydroepiandrosterone	-	-	No change
Progesterone	-	-	No change
Cholesterol	-	-	No change
Anisole	-	-	No change

Table 6•3s. A comparison of the capacity of different steroids to block calcium-induced mitogenesis and hyperpolarisation, and to cause changes in membrane potential. In the mitotic system all steroids (Sigma Chemical Co.) were dissolved in absolute ethanol and added to the cultures to give a final concentration of 3×10^{-9}M. This small amount of ethanol is known not to affect proliferation. Agents that are as effective as oestradiol in blocking calcium-induced mitogenesis are indicated by ++, those which are less effective are denoted by + and those which are inactive are indicated by -. A similar classification has been adopted to denote the ability to mute calcium-induced fluorescence alterations. Here steroids were used at a final concentration of 10^{-5}M.

carbon atoms 3 and 17. Since the alpha and beta epimers are equipotent clearly the spacial configurations of the 17-hydroxyl function is unimportant. This hydroxyl group may also be replaced with a ketone with only a slight loss of activity in both systems, and as seen the additional hydroxylation of carbon 16 does not seem to appreciably affect activity. In terms of substitution at carbon 3, a sulphate group does not seem to impair function whereas a glucuronide does. The latter observation may relate to the poor lipid solubility of glucuronides, an important feature in their excretion (Cohen, 1950). The 3-benzoate derivative of oestradiol blocked both calcium-induced mitogenesis and hyperpolarisation, whereas the 3-methyl ether derivative only blocked the latter. Thus since both compounds are particularly lipid soluble (as indeed are most of the other non-active steroids) it is unlikely that this property is a primary factor governing mitotic blockade or indeed fluorescence changes. It is possible that the important features include an aromatic 'A' ring, reasonable lipid solubility and groups at positions 3 and 17 which are capable of some interaction with elements of the membrane structure and which do not significantly increase the water solubility of the steroid.

It was also noted that testosterone whilst not influencing either calcium-induced mitogenesis or hyperpolarisation did on its own generate a small hyperpolarisation. This is of the opposite polarity to that evoked by oestradiol. Such activity might indicate some importance for this steroid in membrane function.

It should be borne in mind that structure activity studies are prone to a number of criticisms which were outlined in Unit 2. Hence an appeal to these correlations as an indication of causation

Figure 6•3t. The Action Of Cyclic Nucleotides On Membrane Potential.

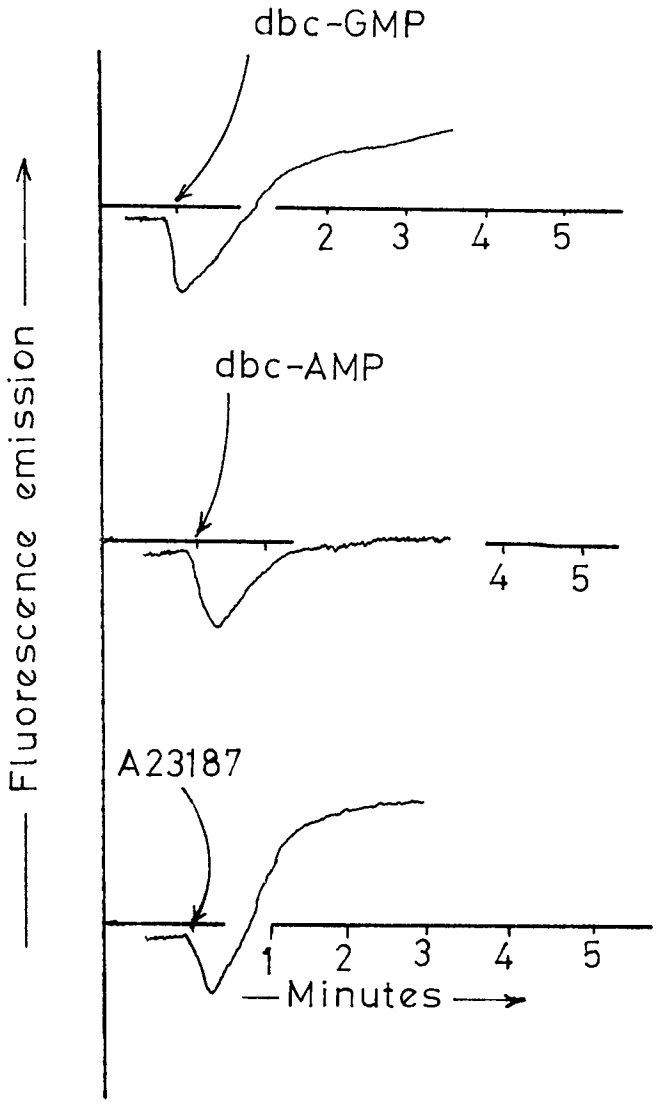


Figure 6•3t. The action of cyclic nucleotides on the observed CC_5 fluorescence in thymic lymphocyte cultures. The cells were incubated in medium containing 0•6mM calcium chloride and then exposed to (a) dibutyryl cyclic GMP (10^{-3} M) (b) dibutyryl cyclic AMP (10^{-2}M) or (c) A23187 (10^{-5}M). All nucleotides were supplied by the Boehringer Corp. and dissolved in ethanol. Emission intensity was then monitored after these additions typical profiles being shown here.

are at best tenuous.

Finally the action of cyclic nucleotides on membrane polarity was studied using the calcium-induced hyperpolarisation as an index. Addition of either dibutyryl cyclic AMP or GMP at high concentrations (10^{-2} and 10^{-3}M respectively) resulted in a characteristic pattern of fluorescence changes (Figure 6•3t), there being an initial hyperpolarisation followed by an hypopolarisation. In many respects this mirrors the response of thymocytes to A23187 in intermediary

(0•6mM) calcium concentrations, where there is an initial drop in fluorescence followed by a protracted hypopolarisation. This action of the cyclic nucleotides was only evident as a small inflection in

Figure 6•3u. The Influence Of Cyclic Nucleotides On A Calcium-Induced Hyperpolarisation.

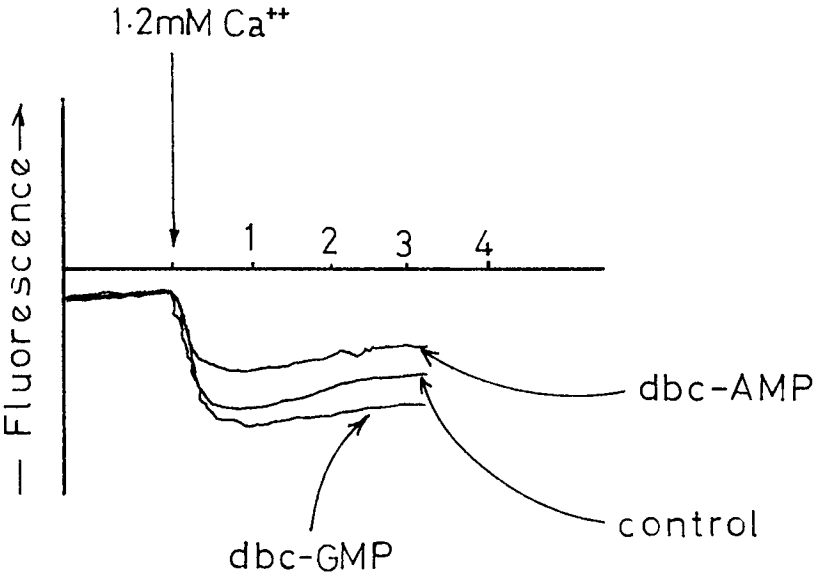


Figure 6•3u. The effect of prior addition of cyclic nucleotides on the observed fluorescence change induced with calcium. Cells were incubated in the presence of 0•2mM calcium chloride in the salts solution and then equilibrated with CC_5. The standard response of the system was established by adding 1•2mM calcium chloride (control) to the cells and recording the fluorescence change. Further aliquots of thymocytes were treated with either dibutyryl cyclic AMP or GMP (both 10^{-5}M) and then subsequently exposed to 1•2mM calcium chloride and fluorescence alterations plotted. The responses from three typical traces are shown in the diagram, basal fluorescence lines have been superimposed to indicate differences.

the line at 10^{-5}M and hence studies on the influence of these agents on calcium-induced hyperpolarisations were carried out at this concentration. It was established over a number of experiments that dibutyryl cyclic GMP, and its free acid, could augment the fluorescence response to 1•2mM calcium, although the maximum response obtainable with 1•8mM calcium did not appear to be increased (Figure 6•3u). In contrast dibutyryl cyclic AMP appeared to reduce the magnitude of

the 1·2mM calcium response (Figure 6·3u). These results , although poorly characterised, are potentially interesting for a number of reasons. First in the mitotic system high concentrations of cyclic AMP are dependent upon the presence of magnesium ions in the extracellular milieu, and magnesium like cyclic AMP appears to be able to mute the calcium-induced hyperpolarisation. This would suggest again that the magnesium-cyclic AMP axis possibly antagonises the calcium axis. And since cyclic GMP enhances the membrane action of calcium this might also implicate these two agents in the same mitogenic mechanism. Indeed it has been reported that cyclic GMP enhances the gating of calcium in lymphocytes (Freedman et al., 1975) which may be its action here. Since calcium apparently triggers the membrane hyperpolarisation this may represent the activation (or indeed inhibition) of some membrane enzyme which is involved directly or indirectly in ion transport or permeability, and which is also regulated by cyclic nucleotides since these purines affect the response. Certainly an ATPase activity has been detected at the surface of astroglial cells (Mandel, Stefanovic, Ciesielski-Treska and Ebel, 1974) and cyclic AMP has been reported to control a protein kinase which is thought to regulate the (Na-K)-ATPase (Dowd and Schwartz, 1975). One attractive feature for calcium possibly modifying the activity of the (Na-K)-ATPase is that calcium can in fact lower the potassium requirement for the pump. That is the pump is more (twice) active in the presence of high calcium concentrations for a given potassium concentration (Lindenmeyer and Schwartz, 1975). In addition the calcium-induced hyperpolarisation observed in amphibian erythrocytes is mediated by potassium (Lassen et al., 1976). Calcium may also be capable of indirectly affecting ATPase activity as well as membrane permeability. This derives from the ability of calcium to become associated with negatively charged polar head

groups, thus reducing the nett electrostatic charge of the membrane. This in its turn removes a repulsive force between like-charged head groups, and they tend to move closer together thus resulting in a restriction of C-C bond oscillation in the lipid chains because of enhanced steric hindrance. That is in the final analysis calcium stabilises membrane structure (Ohnishi and Ito, 1974). Under such stabilised conditions the (Na-K)-ATPase is known to function more efficiently because of cooperative effects between enzyme molecules (Massa, Morero, Bloj and Farias, 1975). And certainly alterations in the gel-liquid crystal state may lead directly to changes in membrane permeability (Harrison and Lunt, 1975). Oestrogens may be able to oppose this action of calcium by interacting with the outer palisade of the membrane (Khaiat, Ketevi, Ter-Minassian-Saraga, et al., 1975) in such a way as to antagonise these fluidity changes. Certainly steroids exert anaesthetic effects (Seeman, 1972) and for example protect erythrocytes from lysis (Seeman, 1966). Furthermore the anaesthetic capacity of many molecules derives from their ability to displace calcium ions from their association with anionic groups of acidic phospholipids (Papahadjopoulos, 1972). Thus it might not be inconsistent to imagine that oestrogens float on the membrane (rather than in it as many other steroids do, Huang, 1976) and also displace calcium ions from their interaction with polar head groups, therefore, counteracting this cations stabilising action.

Since the evidence presented in this Unit appeared to implicate ion movements and changes in membrane permeability with thymocyte activation by mitogens it was next decided to directly monitor these using radiotracer techniques. This is the substance of Unit 7.

Unit 7. The role of the plasma membrane in lymphocyte activation. II; an analysis of ion movements.

"All is flux, nothing is stationary."
Heracleitus.

The previous Unit clearly demonstrated the involvement of ion fluxes in the stimulus-division coupling phenomenon. The opportunity will therefore be taken here to elaborate on certain relevant aspects of ion transport and to indicate other biological phenomena in which there are parallel changes in transmembrane ion distributions.

7·1 Clearly the best established example of the involvement of transient ion movements in a stimulus-response situation is during neurotransmission (Hodgkin and Huxley, 1952; Hodgkin, 1957). Many other biological processes however also have components utilizing ion transport alterations. For instance the secretion of histamine from the mast cell provoked by an ionophore or a reagin is dependent upon an influx of calcium into the cell (Foreman, Mongar and Gomperts, 1973). Inhibition of this calcium uptake by lanthanum prevents the histamine release (Lichtenstein, 1976). It is of interest that cyclic AMP can also block this release perhaps suggesting some causal link between cyclic nucleotide metabolism and ion movement. Support for such a suggestion is derived from studies on blowfly salivary glands where 5-hydroxytryptamine causes both an elevation of cyclic AMP and calcium efflux (Prince and Berridge, 1973). Similar changes are seen in rat liver when gluconeogenesis is stimulated with glucagon (Friedmann and Rasmussen, 1970). Calcium influx and efflux in cultured kidney cells is enhanced by the adenylate cyclase stimulant PTH (Borle, 1973a).

Insulin release from the beta cells of the Islets of Langerhans is another secretory process with an obligatory requirement for calcium uptake (Matthews, 1975), and when such secretion is occasioned by adrenalin application there is additionally an associated potassium efflux. Potassium efflux from turkey erythrocytes can also be provoked by adrenalin and cyclic AMP (Aurbach, Spiegel and Gardner, 1975) as can bidirectional sodium movements (Gardner, Klaeveman, Bilezikian and Aurbach, 1974). Thus an important aspect of hormone function via cyclic nucleotide metabolism may well be the control of calcium and monovalent cation transport.

7·2 Some mechanistic aspects of ion transport and its regulation.

Like the hormonal stimuli discussed in section 7·1 it is evident that the mitogenic stimulus is likewise associated with altered ion movements. Thus mitogens also provoke calcium uptake (Asherson et al., 1970; Whitney and Sutherland, 1973; Freedman, Raff and Gomperts, 1975) and sodium and potassium rearrangements (Dent, 1971; Quastel and Kaplan, 1971) as well as changes in cyclic nucleotide levels in lymphocytes (Hadden et al., 1972). In the previous Unit a number of mechanisms were discussed which might account for such phenomena. In the context of this Unit some of these will be expanded, perhaps to the point of speculation, and an attempt to link the divalent with the monovalent cationic fluxes will be made.

Perhaps the least understood question is how a mitogen might actually promote calcium uptake. Calcium enters the cell both by passive diffusion as well as a facilitated transport process (Borle, 1970). To say that a mitogen 'stimulates gated calcium ion flux' serves only to veil our ignorance of the underlying mechanism.

A similarly obscure 'transient opening of specific calcium pores' has been suggested as the initial action of mitogenic plant lectins (Freedman et al., 1975).Other authors have felt that the physical properties of a protein calcium carrier might be temporarily altered by a lectin since phytohaemagglutinin decreases the Km for calcium transport while leaving Vmax and active calcium efflux unaffected (Whitney and Sutherland, 1973). Clearly the activity of a mitogen at the cell surface could modify endogenous calcium levels by changing the membrane permeability to calcium, altering the characteristics of a carrier system or by affecting the activity of the calcium extrusion pump. A number of workers appear to have lost sight of the latter possibility and have only measured calcium uptake rates (Freedman et al., 1975). Normally the majority of calcium entering the cell is reexported via this active extrusion mechanism involving the Ca-ATPase system which is activated by an increase in endogenous ionised calcium (Vincenzi, 1971). Intracellular calcium homeostasis is also maintained by the sequestration of the ion by the mitochondria as a non-ionic rapidly exchangeable phosphate salt, so that there is an asymmetrical distribution of calcium within the various subcellular compartments. This distribution is maintained by a complex set of factors including the energy charge of the cell, the sodium distribution across the membrane, extracellular pH, phosphate concentration and cyclic nucleotide levels (Borle, 1967, 1973a; Rasmussen, 1971; Rasmussen, Jensen, Lake, Friedmann and Goodman, 1975). The latter compounds appear to regulate an active pump-leak exchange of calcium between the mitochondria and the cytoplasm (Borle, 1973b; Rasmussen et al., 1975). Thus cyclic AMP can stimulate the release of calcium from mitochondria by inhibiting the active uptake mechanism and/or it may alter the permeability of the mitochondrial membranes to calcium (Borle, 1973b). It has also been suggested that cyclic GMP

has the opposite effect, stimulating the active sequestration of calcium into mitochondria (Rasmussen et al., 1975). With the discovery of plasma membrane protein kinases which are under the control of cyclic nucleotides (Dowd and Schwartz, 1975) it is not unreasonable to contemplate a link between these enzymes and the Ca-ATPase of the extrusion pump. A combination of any or all of these factors influencing cellular calcium metabolism at the mitochondrial or plasma membrane locations can result in the transient increase in ionised calcium in the cytosol, and it is this feature which is the critical factor in stimulus-division or stimulus-secretion coupling. Calcium may directly affect enzymes involved with the stimulus-response event or may indirectly effect a response via its links with monovalent cation homeostasis which will now be discussed.

The control of the (Na-K)-ATPase may be central to our understanding because of its implication in ion balance and the growth division cycle (Elligsen et al., 1974; Hoffman, 1975). The observation that insulin, which is a thymic lymphocyte mitogen (Unit 3), can increase cyclic GMP concentrations (Illiano et al., 1973) and elevate membrane ATPase activity in lymphocytes (Hadden, Hadden, Wilson and Good, 1972) lends support to this contention. One view of the control of the (Na-K)-ATPase cycle is shown in Figure 7·2a (modified from Hoffman, 1975). It suggests that the initial events are the binding of a sodium ion to the ionophore portion, and the binding of the Mg-ATP substrate to the catalytic portion of the complex. These events result in the ATPase-ionophore becoming phosphorylated with the consequentially formed ADP being released on the inside of the membrane. The ionophore is then supposed to undergo a configuration change moving it to the outer face of the membrane, a step which can be impeded by oligomycin. The phosphorylation also

Figure 7·2a. A Diagrammatic Representation Of The (Na-K)-ATPase Cycle.

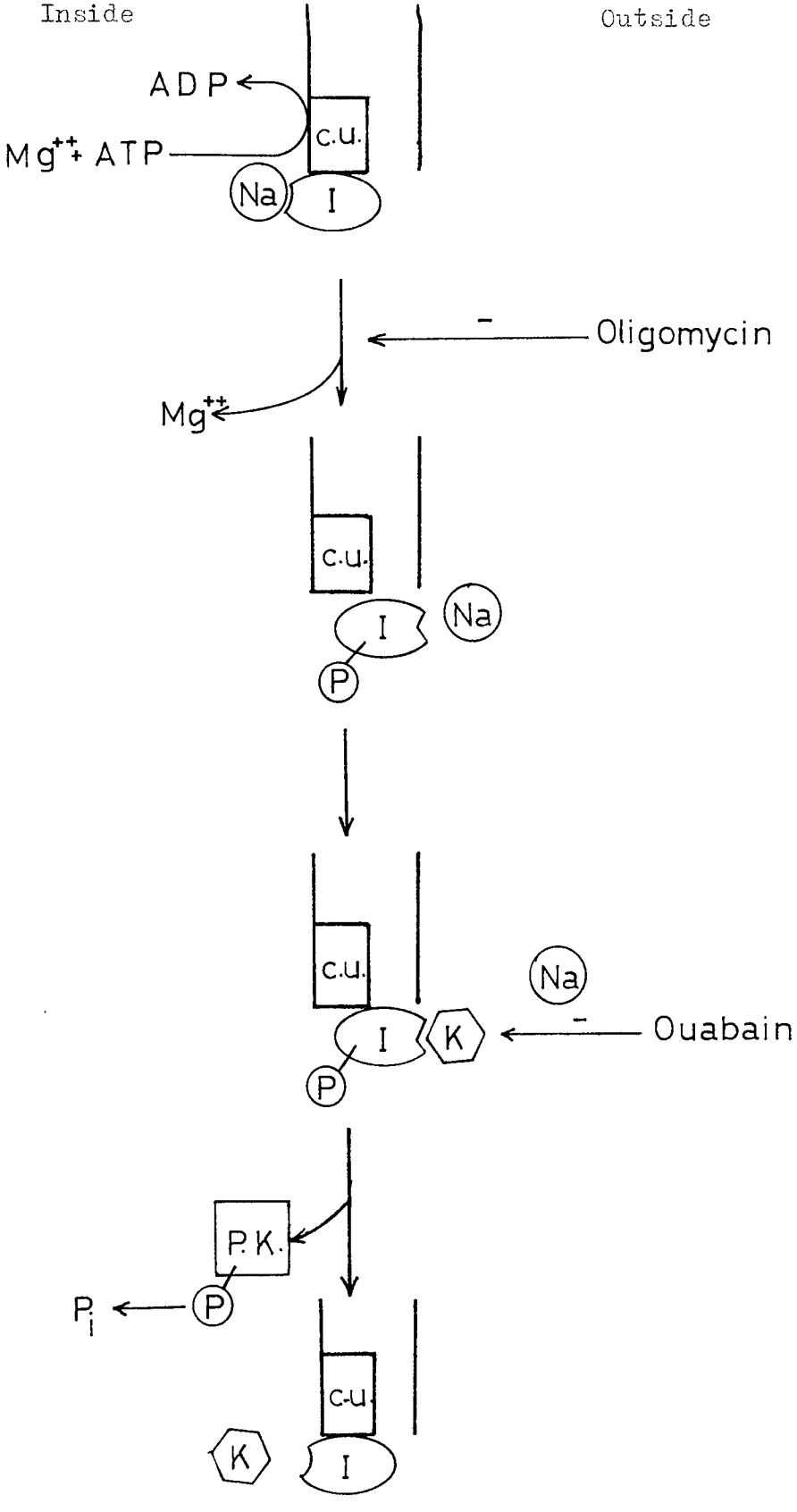


Figure 7·2a. The (Na-K)-ATPase cycle modified from Hoffman, 1975. c.u. is the catalytic unit; I is the ionophore portion. P.K. is a protein kinase. Other details are as in the text.

alters the affinity of the carrier receptor for sodium such that this monovalent cation exchanges for potassium at the outside face of the cell membrane. Ouabain may inhibit the action of the ATPase by binding to the outer potassium receptor site. The return of the (Na-K)-ATPase to its ground state requires that it be dephosphorylated; such a process facilitating potassium movement into the cell. It has been suggested that the ATPase is in fact the substrate for a protein kinase which mediates this dephosphorylation stage. Clearly such a process could be regulated by activation or inhibition of the protein kinase moiety by cyclic nucleotides, which would also offer a direct link between mitogen activation and monovalent cation homeostasis. Details of the effect of cyclic nucleotides upon the (Na-K)-ATPase and Ca-ATPase are currently scarce, but there do appear to be certain mechanistic links between the two transport complexes. Thus when endogenous calcium levels are high (as would be the case following hormonal and mitogenic activation) the calcium extrusion pump becomes more active (Vincenzi, 1971) whilst the (Na-K)-ATPase becomes less active (Hoffman, 1975). Indeed calcium inhibits the dephosphorylation stage in the ATPase cycle (Hoffman, 1975). This is associated with a switch in phosphorylation pattern of the membrane from a protein thought to be associated with the (Na-K)-ATPase to one which is believed to be the calcium extrusion pump. In contrast it has been established in the erythrocyte membrane that cyclic AMP enhances the action of the (Na-K)-ATPase (Hoffman, 1975). It is therefore beginning to become established that calcium, cyclic nucleotides, hormones and mitogens may influence the cellular divalent and monovalent cationic climate by their interaction with a variety of control systems. The ensuing ion redistributions may be of considerable significance in both the stimulus-division and stimulus-secretion events.

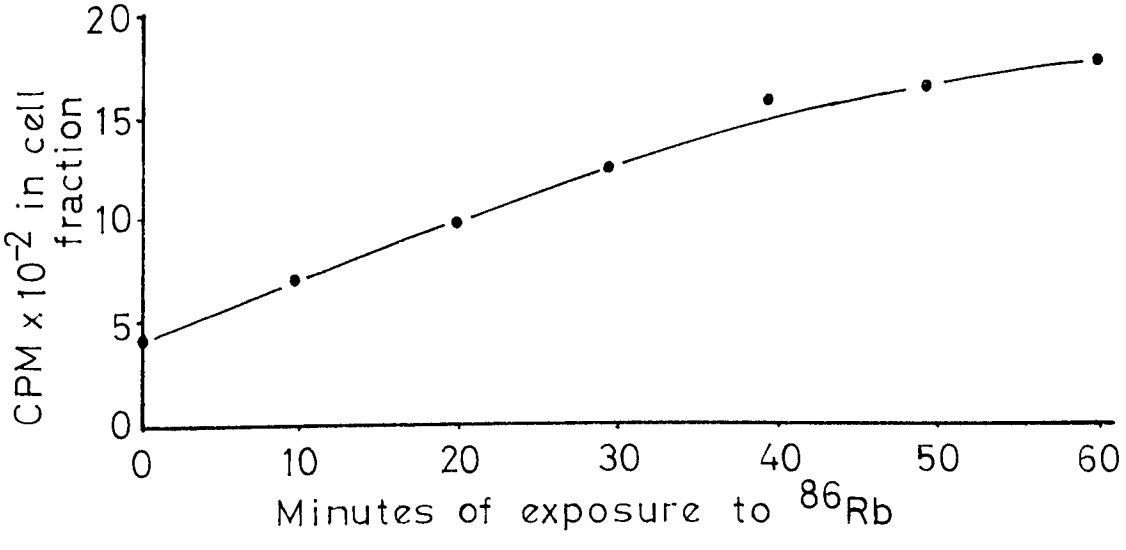
7·3 Some observations concerning the transport of ions in thymocytes maintained in suspension culture following mitogenic stimulation.

When attempting to detect changes in ion flux rate any delays in technique, and the washing of cells in unlabelled medium (often of a different temperature) to remove non-incorporated isotopes are major potential sources of error. The method adopted in these studies at least counters the difficulty of having to wash the cells free of excess label. It is also relatively fast when compared to a number of other radiotracer methods. The technique is basically that described in several publications (Harris and Berenti, 1969; Foreman et al., 1973; Freedman et al., 1975). Cells were cultured in the conventional way but at the required time aliquots of cells (0·2ml) were accurately transferred to a Beckman Microfuge tube (PRO 22) which contained at its base 0·05ml 98% formic acid overlayed with 0·1ml DC 550 (Hopkin and Williams Ltd.) silicone fluid. Centrifugation for two minutes at 12,000 x g in a Beckman Microfuge caused the cells to pass through the oil layer into the formic acid leaving the culture medium behind. It was thus possible to separate the cells from their isotopically labelled medium. As the cells passed into the formic acid they were lysed, and released their radioactive contents into this aqueous phase. After freezing in liquid nitrogen the tube was cut in two so that the lower portion contained only isotope retained by the cells, and the upper portion that in the medium. Thus both supernatant and cells may be counted for radioactivity. For beta-emitting isotopes the respective part of the tube was immersed in NE 260 scintillant (Nuclear Enterprises, Edinburgh) and then counted in a Beckman LS 230 liquid scintillation spectrometer. NE 260 was found to be the only suitable scintillant for counting the cell pellet. Where a gamma-emitter was used, Microfuge tubes were simply cut off

and placed inside sealed plastic containers and counted directly on a Tracerlab Gamma Set 500 gamma-emission spectrometer. When counting ^{22}Na and ^{42}K preset emission window keys were employed, for ^{86}Rb an adjustable window was set for the high emission peak of this isotope as described in the Tracerlab manual. When calculating results only ^{42}K was corrected for decay because of its very short half-life. Results for beta-emitters were uncorrected for quenching as preliminary 'spiking' procedures had indicated no significant variation between vials. All isotopes used in this thesis were obtained from the Radiochemical Centre, Amersham.

When studying the uptake of an isotope, cells were cultured in the modified Earle's salts solution described in the previous Unit at a density of 10^8 cells.ml^{-1}, and exposed in all cases to 1μCi.ml^{-1} of the radionuclide of interest. Similarly when monitoring efflux rates, cells were prelabelled for fifty minutes with 1μCi.ml^{-1} of the isotope and then washed twice, and finally resuspended in unlabelled medium at 37°C. Following this procedure the cells were left for twenty minutes to recover from this trauma and then subsequently treated as desired. Figure 7·3a shows a typical ^{86}Rb loading profile after two washes.

Figure 7·3a. A Loading Profile For ^{86}Rb In Thymocyte Suspensions



The initial experiment was designed to establish whether calcium uptake and efflux was affected by the treatment of the cells with a mitogenic agent. Two calcium-dependent mitogens (Unit 2 and unpublished data) ionophore A23187 (10^{-5}M) and concanavalin A ($5\mu g.ml^{-1}$) both provoked an increased efflux (Figure 7·3b) and uptake of ^{45}Ca (Figure 7·3c) within the first ten minutes of exposure. The ingress of calcium promoted by concanavalin A however appeared to be only transient, which is in contrast to other work (Freedman et al., 1975) which indicated it to persist for a number of hours. The reason for this discrepancy is unknown, but may be related to the higher levels of extracellular calcium (1·8mM) used in these other studies. Indeed the only clear explanation for the observed fall in uptake after ten minutes is the increasing efflux rate at this time (Figure 7·3b). Thus although ConA may still be causing an enhanced entry of calcium into the thymocyte, because of a higher rate of active extrusion this is obscured at later times. However since these mitogens are calcium-dependent one might suppose that this calcium transient was a causal factor linking the mitogen to the initiation of DNA synthesis and mitosis. In view of the enhanced efflux rate there is of course no direct evidence to prove that endogenous calcium levels have in fact been elevated. Yet this heightened extrusion could itself indicate that intracellular ionised calcium levels have risen and have subsequently stimulated the Ca-ATPase. Alternatively the data might be interpreted as representing a general increase in membrane permeability to calcium.

When the action of these two mitogens on ^{32}P transport was examined, no clear pattern emerged. For whilst A23187 stimulated ^{32}P release (Figure 7·3d) it inhibited uptake (Figure 7·3e). Con A on the other hand stimulated uptake (Figure 7·3e) and had little or

Figure 7·3b. The Influence Of ConA And A23187 On The Efflux Of ^{45}Ca From Preloaded Thymocytes.

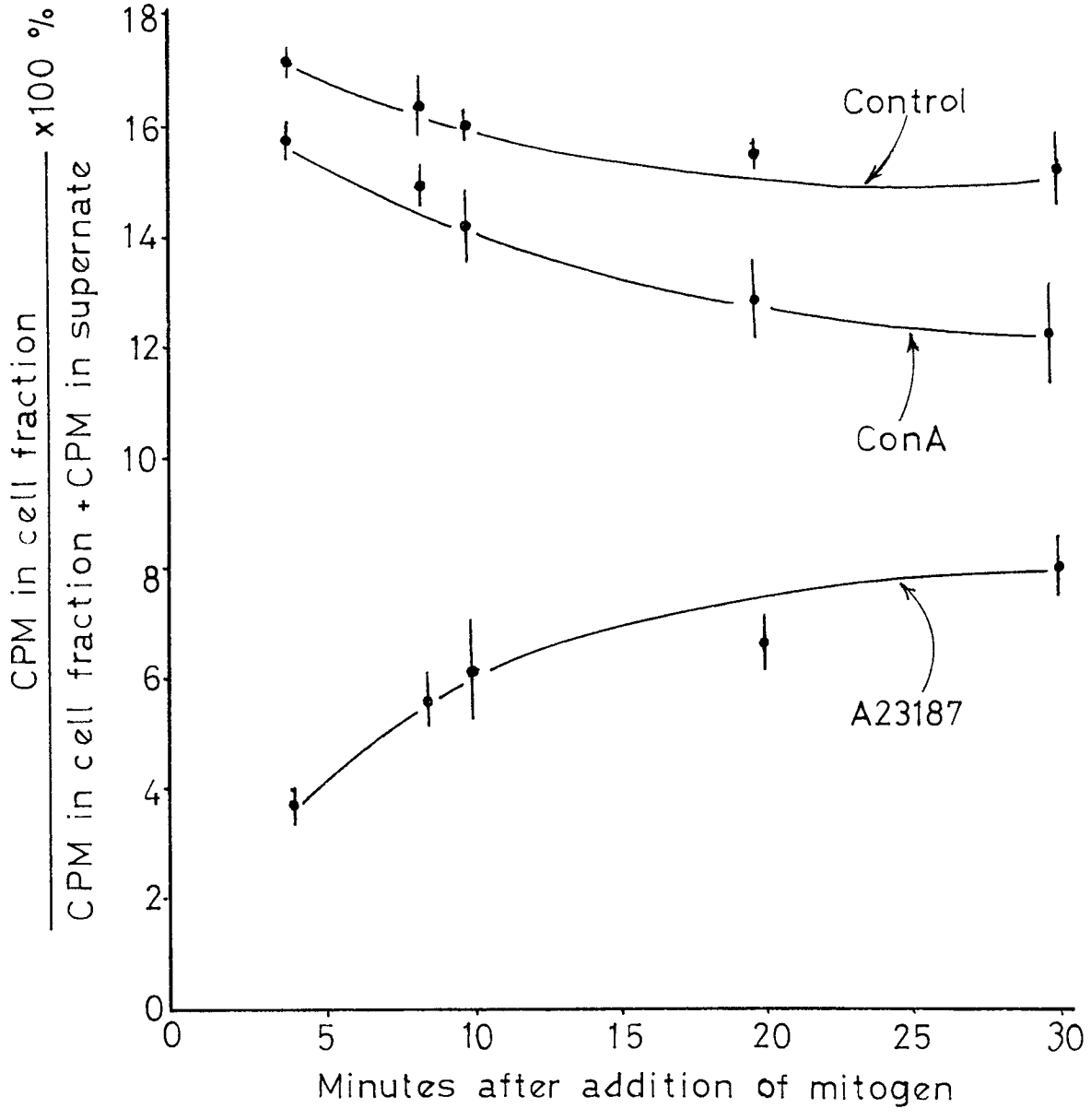


Figure 7·3b. The action of concanavalin A and A23187 on the egress of ^{45}Ca from prelabelled thymocytes maintained *in vitro*. Cells were cultured in Earle's salts solution containg 0·6mM calcium and radiocalcium as described in the text. At time zero cells were exposed to concanavalin A (Pharmacia Ltd.) at a concentration of $5\mu g.ml^{-1}$, A23187 (Lilly Research Centre) at a concentration of $10^{-5}M$, or saline carrier control. Cells and supernatant were subsequently harvested by microfugation and counted for ^{45}Ca as described previously. Results are expressed as the percentage of the total radioactivity present in the cell fraction at the respective time, and represent the mean ± 1 S.E.M. of 5 separate experiments.

Figure 7·3c. The Influence Of ConA And A23187 On The Uptake Of ^{45}Ca Into Thymocytes.

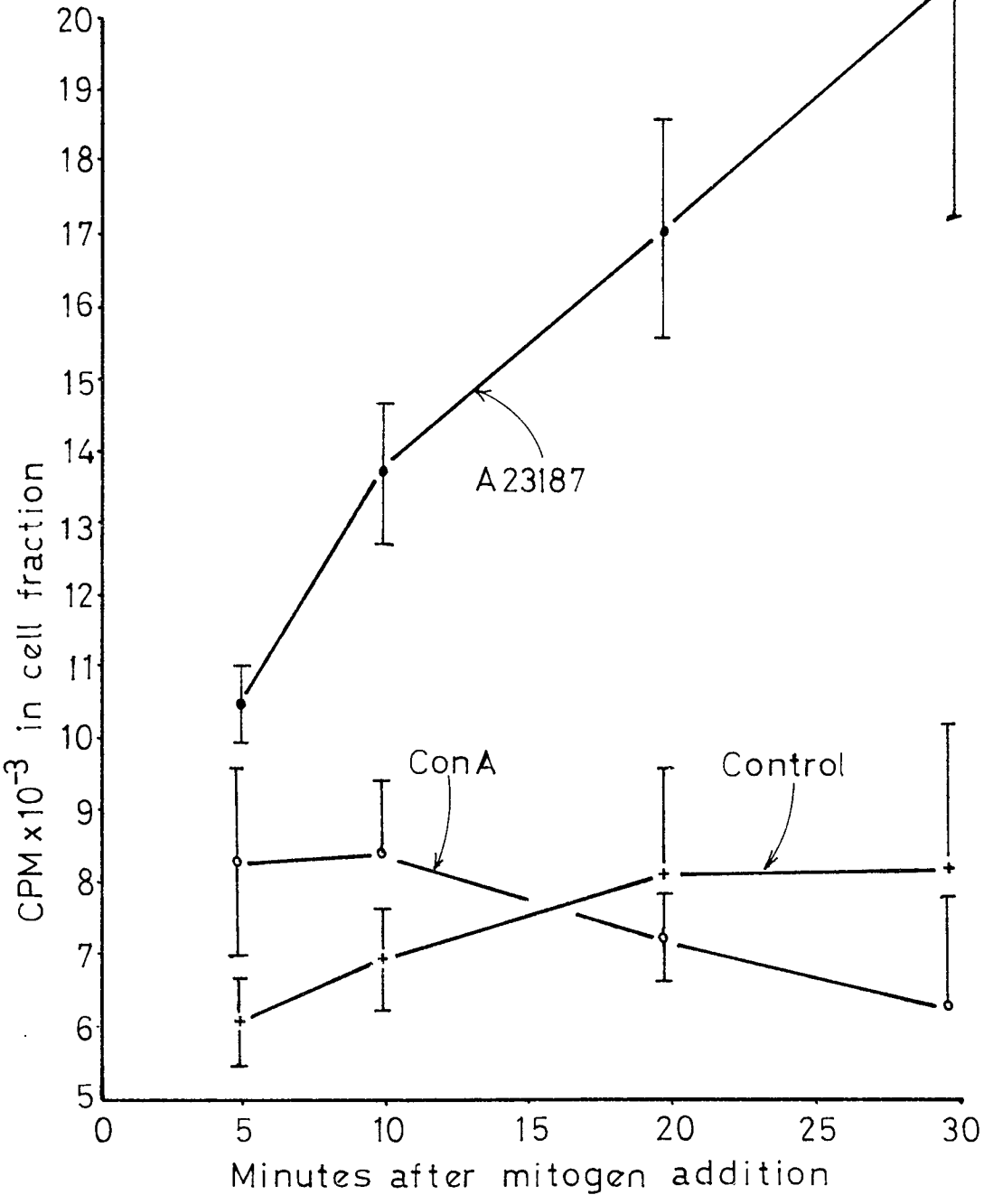


Figure 7·3c. The action of A23187 and concanavalin A on the uptake of ^{45}Ca into thymocytes. Cells were cultured in Earle's salts solution containing 0·6mM calcium chloride as described. At time zero they were exposed to A23187 (•). concanavalin A (o) or carrier control (+). Details as in figure 7·3b. Cells were harvested at the required time and counted for their radiocalcium content. Results are the mean of 5 duplicated determinations ± 1 S.E.M.. The experiment has been repeated on a number of occasions with qualitatively similar results being obtained.

Figure 7·3e.

The Uptake Of ^{32}P Following Mitogen Addition

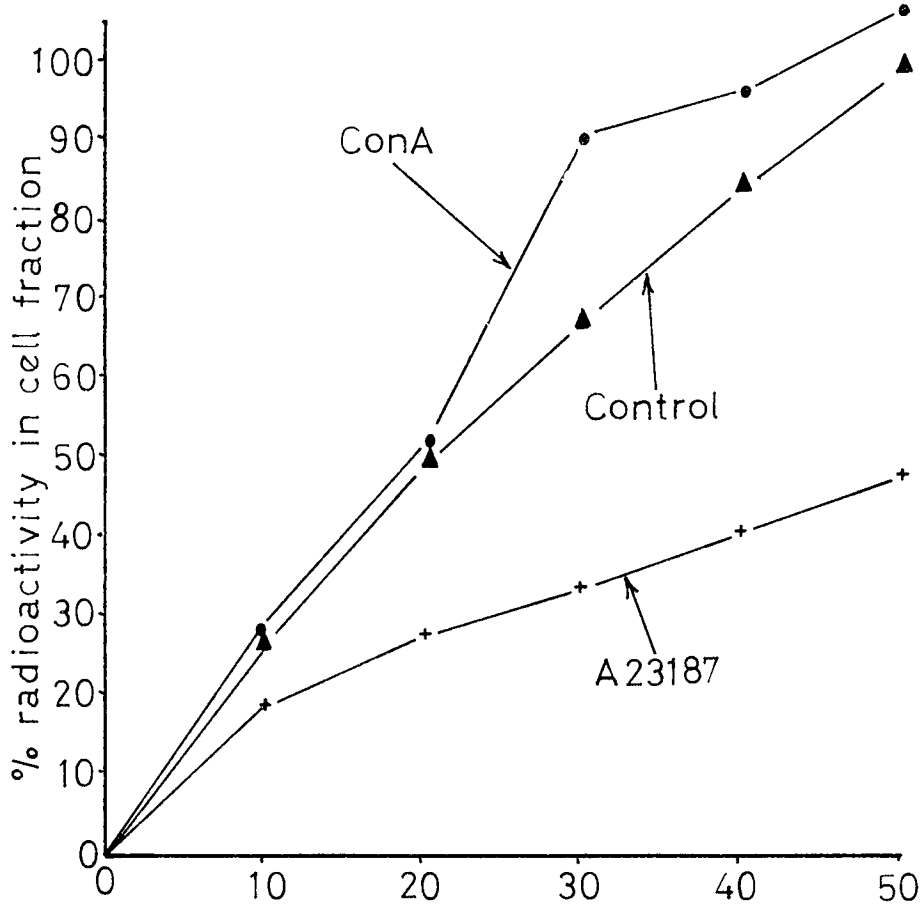


Figure 7·3d.

The Efflux Of ^{32}P Following Mitogen Addition

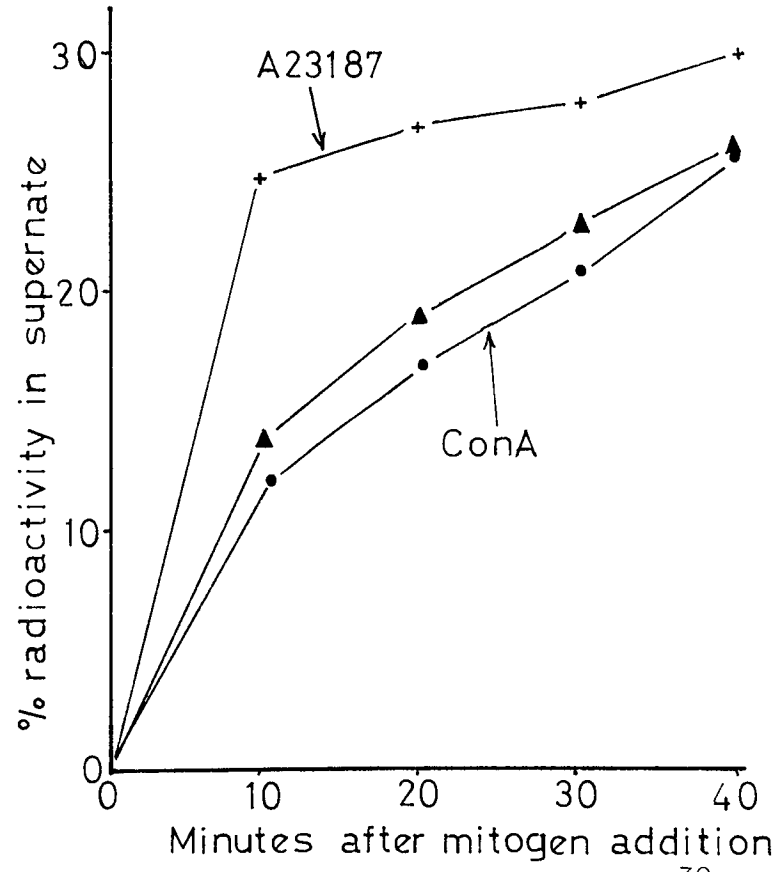


Figure 7·3e. The uptake and Figure 7·3d. the efflux of ^{32}P in thymic lymphocytes stimulated with Con A and A23187. Details as in 7·3b. Results are the mean of two duplicated experiments. When calculating uptake the value obtained for controls at 50 minutes was taken as 100%.

no effect on efflux of the label from thymocytes (Figure 7·3d). Thus it is unlikely that the altered transport of phosphate is a key event in the activation of these cells.

Since a variety of agents have been reported to alter sodium and potassium transport, the movements of these ions were monitored using ^{22}Na for sodium and ^{42}K or ^{86}Rb for potassium. The validity of using ^{86}Rb as an indicator of potassium movements in these cells has been established elsewhere (Segel, Gordon, Lichtman, Hollander and Klemperer, 1976). Thus exposure of thymocytes to both

Figure 7·3f. The Influence Of Mitogens On Sodium Uptake Into Thymocytes

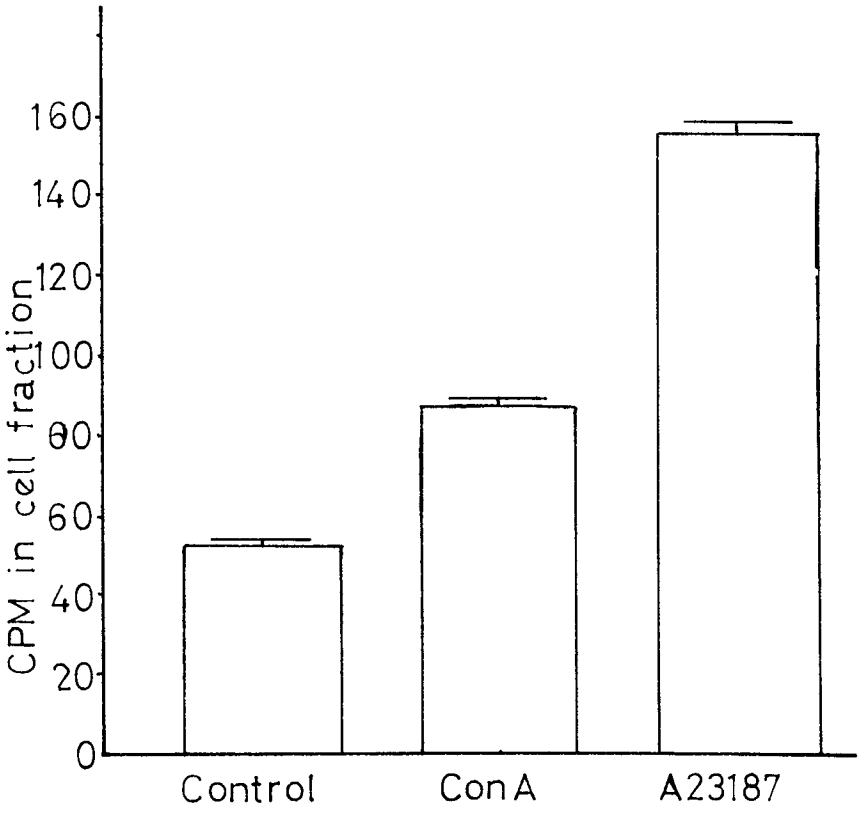


Figure 7·3f. The influence of Con A and A23187 on ^{22}Na uptake into thymocytes. Cells were cultured and exposed to mitogens and isotope as described in Figure 7·3b. and in the text. The results indicate the counts per minute per culture (cell fraction) detected after 50 minutes exposure to mitogens or saline control. Values are mean (column) + 1 S.E.M. of four separate experiments.

Figure 7·3g

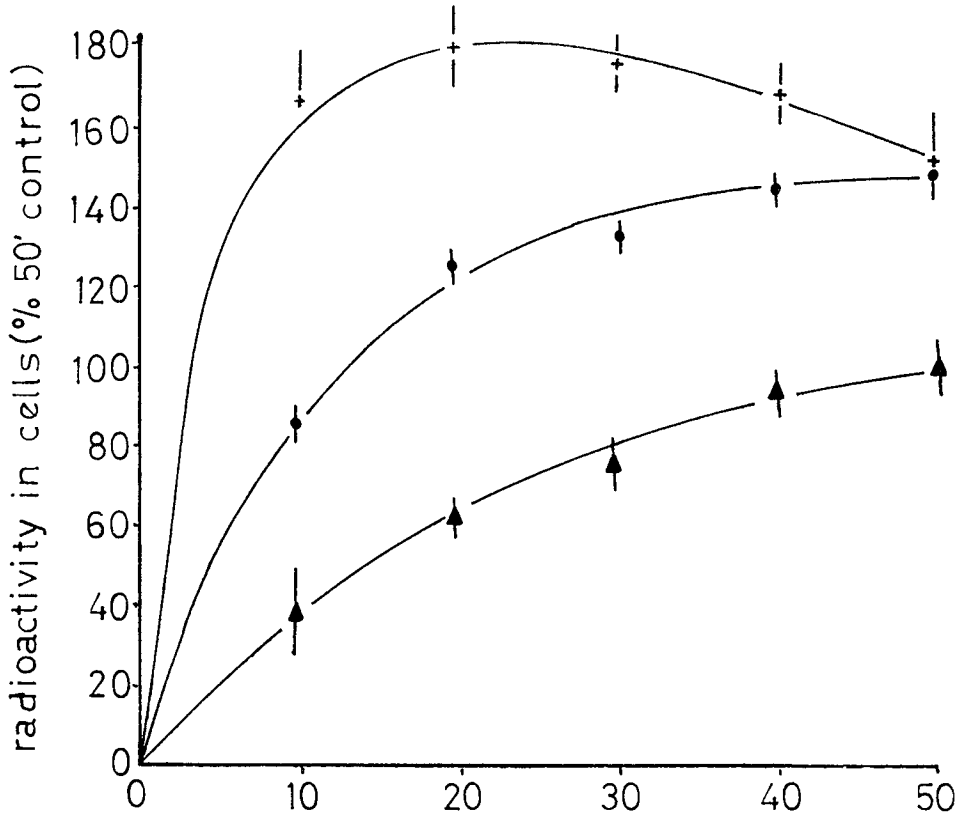


Figure 7·3h.

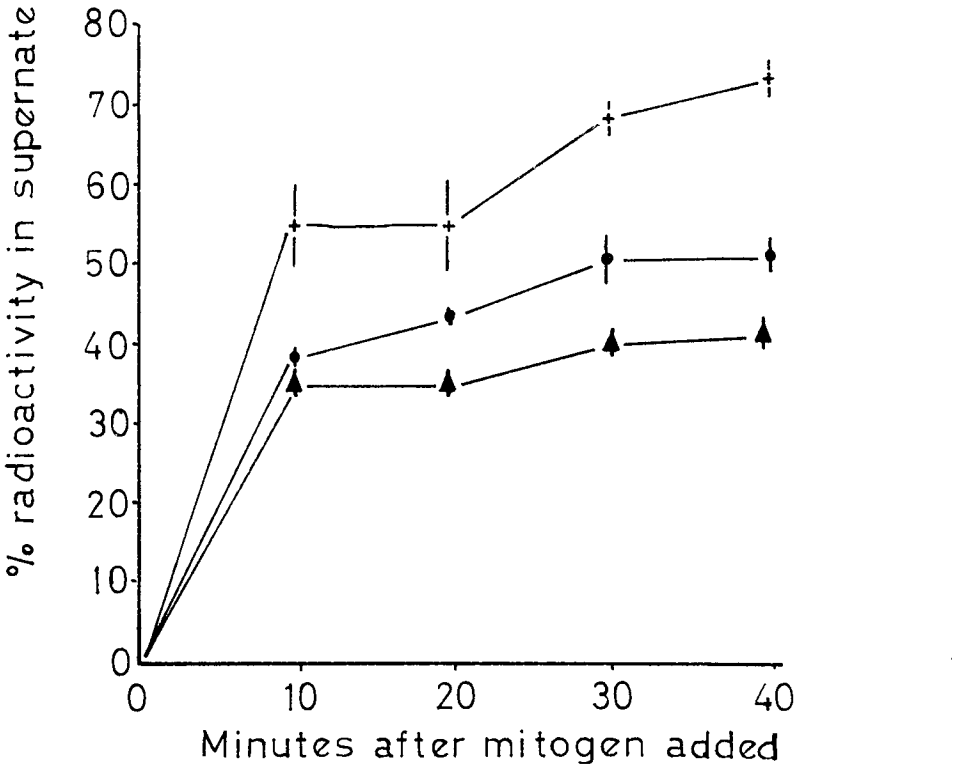


Figure 7·3g. The influence of concanavalin A and A23187 on ^{42}K uptake into thymocytes. Details of culture and mitogen and isotope levels are as in Figure 7·3b. (+) is A23187; (•) is concanavalin A; (▲) is the control value. Results are expressed as the percentage of the 50 minute control value (100%) and represent the mean ± 1 S.E.M. of 5 individual experiments.

Figure 7·3h. The influence of mitogens on ^{42}K efflux from thymocytes. Details as in 7·3b. and 7·3g. for mitogens and code to graph. Results are the mean ± 1 S.E.M. of the percentage total label appearing in the supernatant. Derived from 5 individual experiments.

A23187 (10^{-5}M) and concanavalin A (5ug.ml^{-1}) stimulated the uptake of sodium (Figure 7·3f) and potassium (Figure 7·3g) as well as provoking a marked efflux of potassium from these cells (Figure 7·3h). It was not found practicable to measure sodium efflux on account of the low transport coefficient for this ion which made it difficult to load the intracellular pool without resorting to manipulations of the medium. Such procedures might well destroy those aspects of membrane function in which we were interested. As the data stands it is difficult to decide if there has ensued an increase in the membrane permeability to the two monovalent ions, or if the activity of the (Na-K)-ATPase has been altered, or both. However the relatively larger effect of the two mitogens on potassium transport would indicate (in view of the stoichiometry of the pump:- $3Na^+$: $2K^+$: 1ATP) that there must have been at least an alteration in membrane permeability, and that this might have been relatively greater for potassium than sodium.

Whilst 5μg.ml^{-1} Con A is a mitogenic concentration, 10^{-5}M A23187 is somewhat high (Unit 2), hence a range of concentrations of these agents were tested for their ability to efflux ^{86}Rb from thymocytes. Con A at 5 and 50μg.ml^{-1} caused a release of rubidium from preloaded thymic lymphocytes, with the higher dose causing the greater efflux (Figure 7·3i). The ionophore provoked an efflux of isotope at the standard 10^{-5}M and also at the mitogenic concentration of 10^{-7}M, however, the response was not apparent at 10^{-9}M (Figure 7·3i). Thus both agents do in fact initiate these unscheduled ion fluxes at mitogenic concentrations.

In an effort to ascertain whether or not the (Na-K)-ATPase was involved in these monovalent cation fluxes, the ATPase inhibitor

Figure 7·3i. The Ability Of Various Concentrations Of Concanavalin A And A23187 To Release ^{86}Rb.

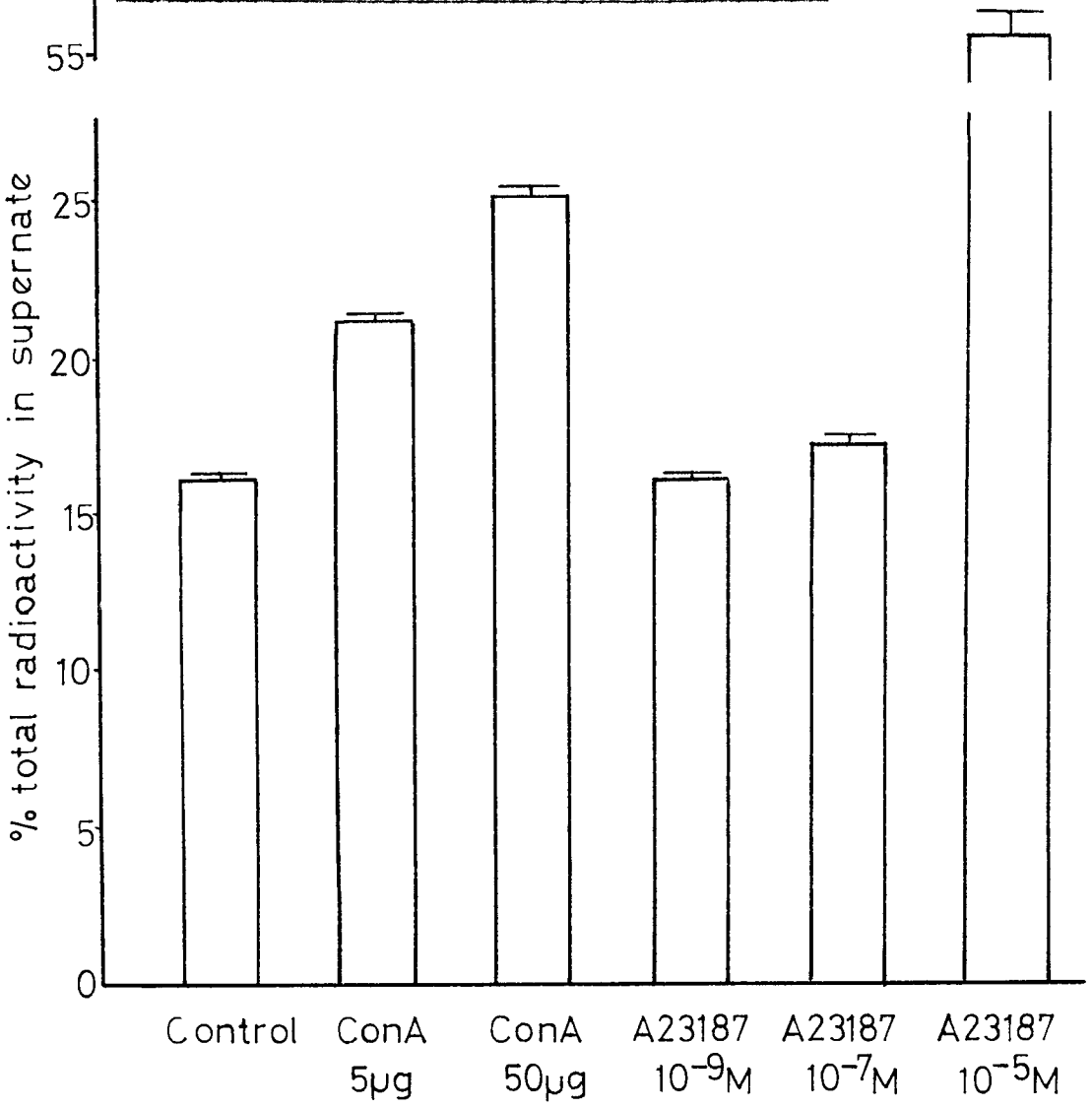


Figure 7·3i. A dose-response experiment between concanavalin A and A23187 in their ability to release ^{86}Rb from prelabelled thymocytes. Cells were incubated in Earle's salts containing 0·6mM calcium chloride and prelabelled with rubidium as described in the text. The cultures were subsequently exposed to concanavalin A at concentrations of 5 or 50µg.ml^{-1} or A23187 at 10^{-5}, 10^{-7} or 10^{-9}M for a period of 30 minutes and the percentage label effluxed determined. Results are the mean (column) + 1 S.E.M. of four separate determinations.

ouabain was employed at a final concentration of 10^{-4}M. Although A23187 clearly stimulated ^{22}Na uptake ouabain only reduced this increment by some 19% (Table 7·3j). Thus it is unlikely that the ATPase is involved in the enhanced sodium uptake occasioned by the mitogen. It is more probable that A23187 in some way alters the permeability coefficient of the cells to sodium and potassium and

of course calcium. It is hoped to repeat this experiment using potassium isotopes which should give a much clearer picture of any (Na-K)-ATPase interaction in this phenomenon.

Table 7·3j. The Interaction Between A23187 And Ouabain In Effecting The Enhanced Uptake Of ^{22}Na Into Thymocytes.

Treatment	% ^{22}Na radioactivity present in cells after 5 minutes ± 1 S.E.M.
Control	0·440 ± 0·001% (n=10)
Control + Ouabain ($10^{-4}M$)	0·455 ± 0·006% (n=5)
Control + A23187 ($10^{-5}M$)	0·600 ± 0·008% (n=10)
Control + A23187 + Ouabain	0·569 ± 0·009% (n=9).

Table 7·3j. The action of ouabain on ionophore-induced ^{22}Na uptake into thhmocytes. Cells were cultured in Earle's salts solution containing 0·6mM calcium chloride as outlined previously. At time zero they were exposed to radio-sodium and combinations of ouabain (Calbiochem Ltd.) at $10^{-4}M$, A23187 ($10^{-5}M$) or carrier control. Five minutes later cells were harvested and counted for their content of ^{22}Na. This was expressed as the percentage of the total label in a sample present in the cell fraction. Results are the mean ± 1 S.E.M. of the number of determinations shown in parentheses.

Since the mitogens provoked not only a sodium and potassium rearrangement but also an alteration in calcium transport, it seemed germane to establish whether in fact the calcium phenomenon was causally linked to the monovalent ion redistribution. Thus thymocytes were incubated in the presence of the two calcium-dependent mitogens A23187 and Con A, and the extracellular calcium concentration adjusted to either 0·6mM or zero. ^{86}Rb efflux was then ascertained under these various calcium regimes. In the presence of calcium, both mitogens provoked an enhanced release of isotope as indicated previously (Figure 7·3k). However, calcium omission from the medium resulted

Figure 7·3k. The Calcium-Dependent Stimulation Of ^{86}Rb Release From Thymocytes By Concanavalin A And A23187.

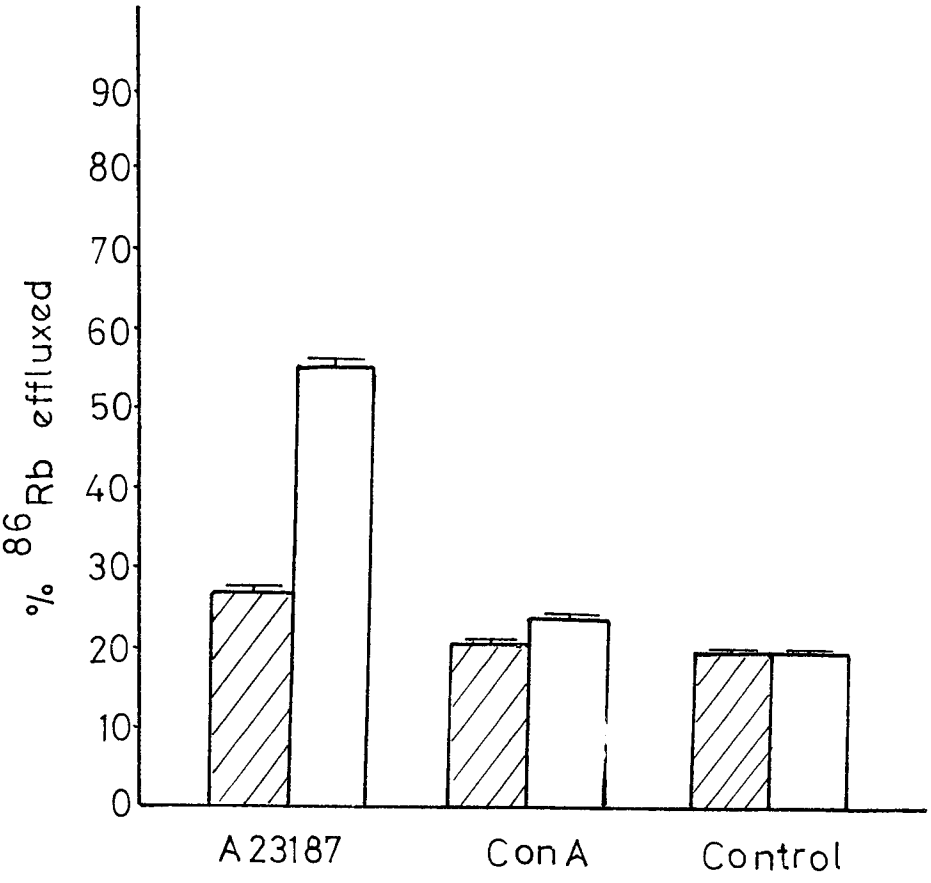


Figure 7·3k. The influence of the extracellular calcium concentration on the ability of Con A and A23187 to stimulate the efflux of ^{86}Rb from preloaded thymocytes. Cells were incubated in Earle's salts solution containing either 0·6mM (open columns) or zero (hatched columns) calcium chloride. To the cultures was then added either A23187 ($10^{-5}M$) or Con A ($5\mu g.ml^{-1}$) and the cells incubated for a further 30 minutes. After this period the radioactivity in the cell pellet and supernatant was assessed and the results expressed as the % of the total isotope present in the supernate. Each column is the mean, and line with bar 1 S.E.M. of four separate determinations. Stock cell cultures were preloaded in the presence of 0·6mM calcium, zero calcium cultures being obtained by subsequently resuspending these cells in calcium-free medium.

in an almost complete ablation of this stimulated egress of rubidium (Figure 7·3k). These results suggest that it is the ingress of calcium into the thymocyte which provokes this potassium permeability change. In addition Figure 7·31 indicates that in a calcium-free medium the ionophore, A23187, shows a much reduced ability to enhance ^{22}Na uptake,

Figure 7·31. The Calcium-Dependent Stimulation Of ^{22}Na Uptake Into Thymocytes By A23187.

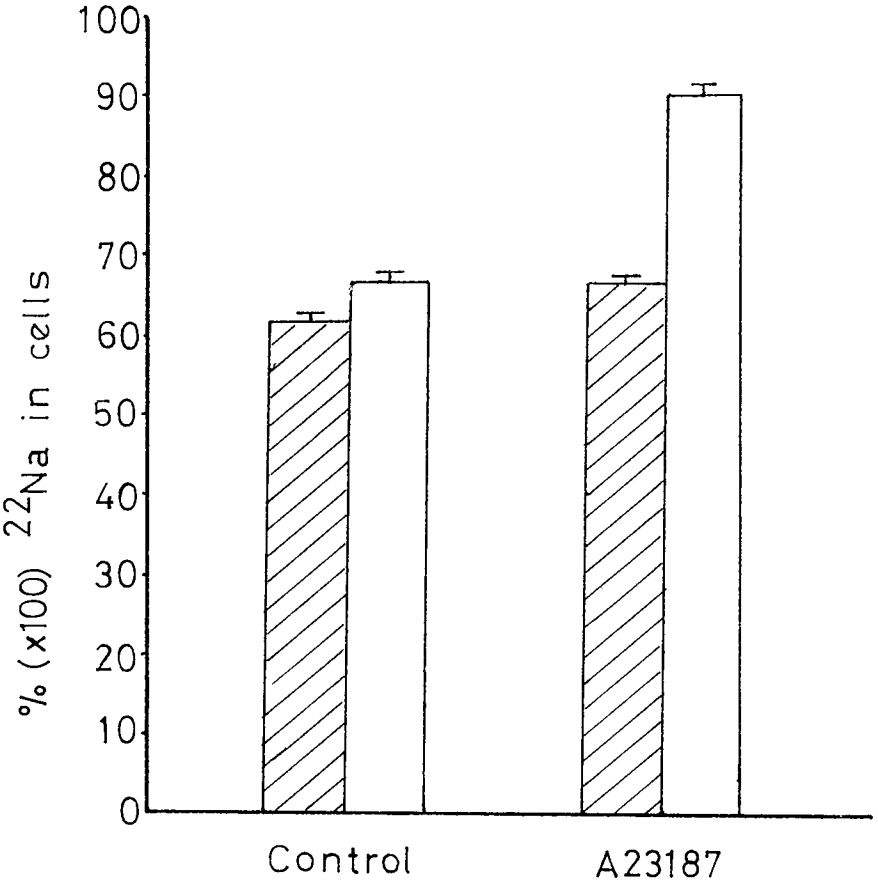


Figure 7·31. The influence of the extracellular calcium concentration on the ability of A23187 to promote the uptake of ^{22}Na into thymocytes. Cells were incubated in Earle's salts solution containing either zero (hatched columns) or 0·6mM (open columns) calcium chloride. At time zero cultures were treated with isotope and either A23187 ($10^{-5}M$) or carrier. 5 minutes later cells were harvested and their radioactive content assessed. Results are expressed as % of the total activity in each culture present in the cells (x 100). These are derived from 5 experiments, and represent the mean + 1 S.E.M. as in the previous figure.

thus corroborating the findings with ^{86}Rb.

In the mitotic system oestradiol-17β is capable of inhibiting the mitogenic action of the two calcium-dependent mitogens , A23187 and Con A (Unit 2 and unpublished data). Furthermore it has been established that these mitogens promote the uptake of calcium into thymic lymphocytes, and this calcium increment appears to provoke a

Figure 7·3m.

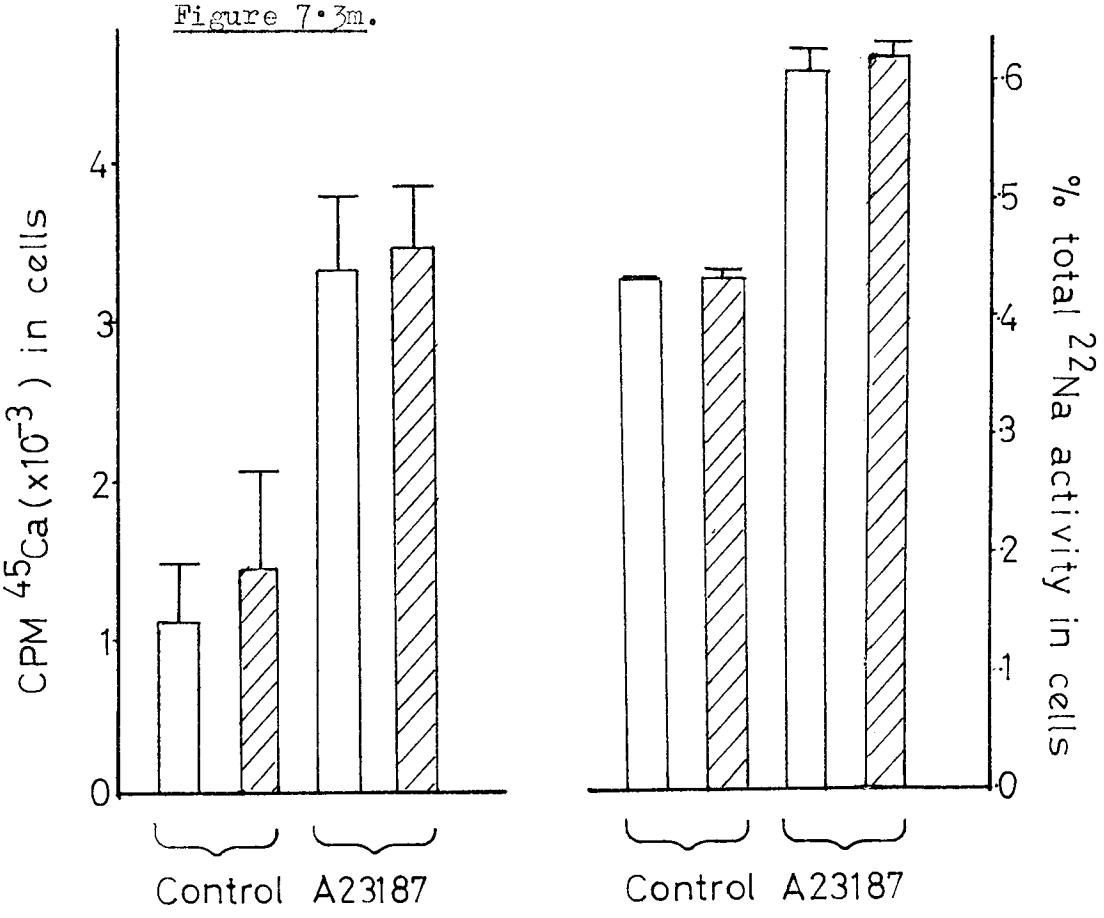


Figure 7·3m. The lack of influence of oestradiol on A23187-induced sodium and calcium uptake into thymocytes. Cells were cultured in Earle's salts solution containing 0·6mM calcium chloride and at time zero were treated with either ^{45}Ca or ^{22}Na. Simultaneously the ionophore, A23187 (10^{-5}M) was added to half of the cultures. In addition some cultures contained oestradiol-17β (hatched columns) at a final concentration of 0·1μg.ml^{-1}, other cultures contained the ethanol carrier (open columns) as controls. Results for calcium uptake are expressed as CPM in the cell fraction, whilst sodium uptake is expressed as the percentage of the total label present in the cell pellet both at five minutes. Each column is the mean of five determinations, the vertical line with bar being 1 S.E.M.

monovalent cation redistribution. Thus it would not seem inconsistent to imagine that oestradiol blocked this calcium uptake and therefore the subsequent ionic rearrangements. Cells were therefore incubated in the presence of A23187 both with and without oestradiol-17β (0·1 μg.ml^{-1}) and the uptake of ^{45}Ca and ^{22}Na monitored (Figure 7·3m). It

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is clear from these experiments that oestradiol blocked neither the calcium uptake promoted by the ionophore nor the subsequent sodium flux, thus contraindicating the notion that the steroid blocks these initial events.

The problem of the action of oestradiol became even more perplexing when the action of high extracellular calcium levels upon ion movements was examined. It was found that cells incubated in the presence of high (1·8mM) calcium showed lower rates of ^{42}K uptake and marginally lower rates of efflux of the same isotope (Figure 7·3n) when compared with cells incubated in 0·6mM calcium. Similarly the uptake of sodium appeared to be lower in high calcium containing medium (Figure 7·3o). Thus a picture is beginning to unveil itself where the presence of extracellular calcium ostensibly reduces the transport rates of sodium and potassium. This is in direct contrast to the action of the calcium dependent mitogens, which on elevating endogenous calcium levels provoke an increase in the permeability of the membrane to monovalent cations. Such results might indicate that these monovalent ion redistributions are therefore inconsequential to the mitogenic activation process. It might also be added that high calcium certainly did not increase phosphate uptake or efflux, and indeed in both instances values obtained were lower in medium containing 1·8mM calcium than in that containing 0·6mM calcium, this being most marked for efflux (data not presented).

These results in some senses parallel the fluorescence studies in which calcium caused a cell hyperpolarisation whereas A23187 provoked cell hypopolarisation. These differences could derive from the fact that A23187 clearly provoked a nett potassium efflux from the cell whilst calcium itself caused only minor and vectorially

Figure 7·3n. The Influence Of High Extracellular Calcium Concentrations On The Uptake And Efflux Of ^{42}K.

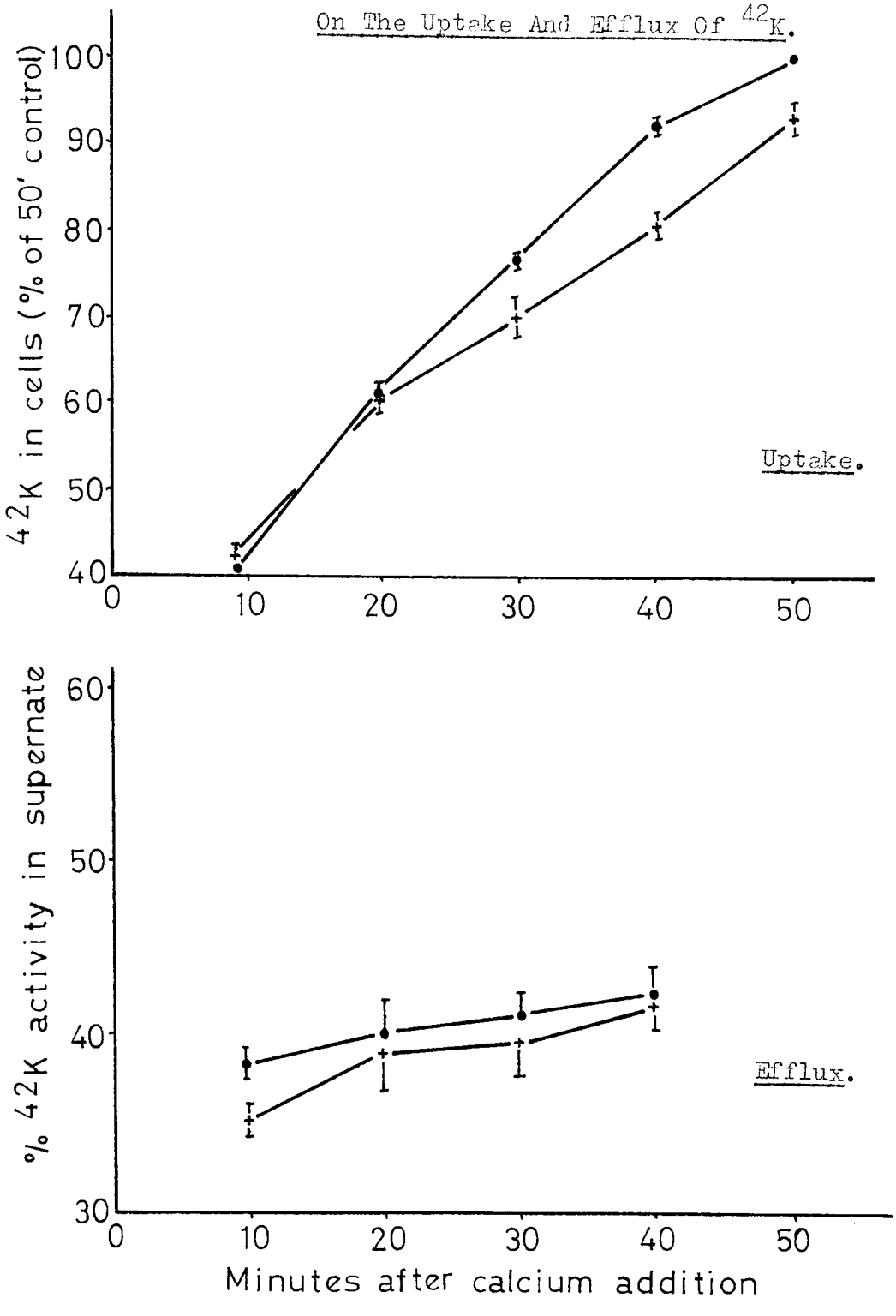

Figure 7·3n. The action of high extracellular calcium levels upon the uptake and efflux of ^{42}K in thymocytes. All cells were initially cultured in Earle's salts solution containing 0·6mM calcium. For the uptake experiment at time zero half the cultures were treated with 1·8mM calcium chloride (+) whilst the other half only received isotope (•). Cells were then harvested at the desired time. For efflux determinations cells preloaded and resuspended in 0·6mM calcium were exposed at time zero to either 1·8mM calcium chloride (+) or saline carrier (•). All results are the mean ± 1 S.E.M. of four separate determinations.

Figure 7·3o.

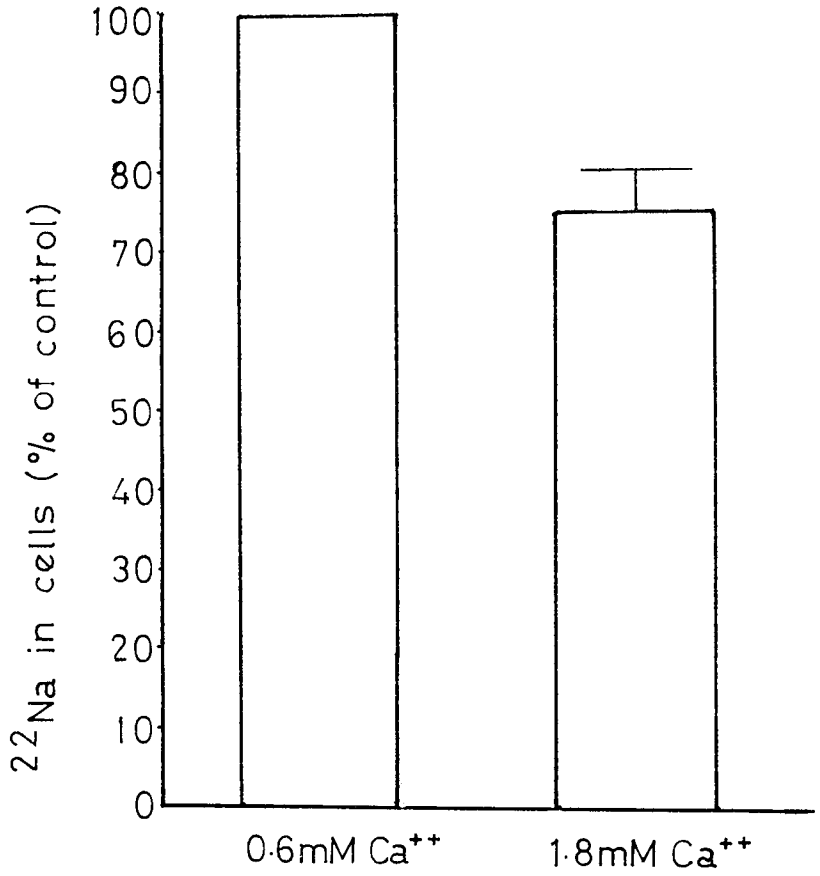

Figure 7·3o. The effect of high extracellular calcium concentrations on the uptake of ^{22}Na into thymocytes. Cells were incubated in either 0·6mM or 1·8mM calcium chloride and exposed to the isotope at time zero. 40 minutes later the cells were spun down and counted for their radioactive content, results being expressed as the % label in the sample compared to the 0·6mM calcium control culture (100%). Each column is the mean and vertical line with bar 1 S.E.M. of 4 determinations.

opposite modifications of ion distributions. Such differences may relate to the action of exogenous calcium upon the (Na-K)-ATPase which might tend to obscure the results obtained, and upon the movement of other ions which have not as yet been monitored (e.g. Cl^- or H^+). It does appear however as if intracellular calcium has a different effect upon the membrane than does extracellular calcium, a point which has not been made by other workers. In addition the small inhibition of potassium uptake apparent with 1·8mM calcium was reversed by the simultaneous presence of oestradiol in the cell suspension (Figure 7·3p). This may therefore be the first indication that oestradiol may either influence the action of calcium upon the outer face of the membrane, or may interfere in some specific way with potassium

Figure 7·3p.

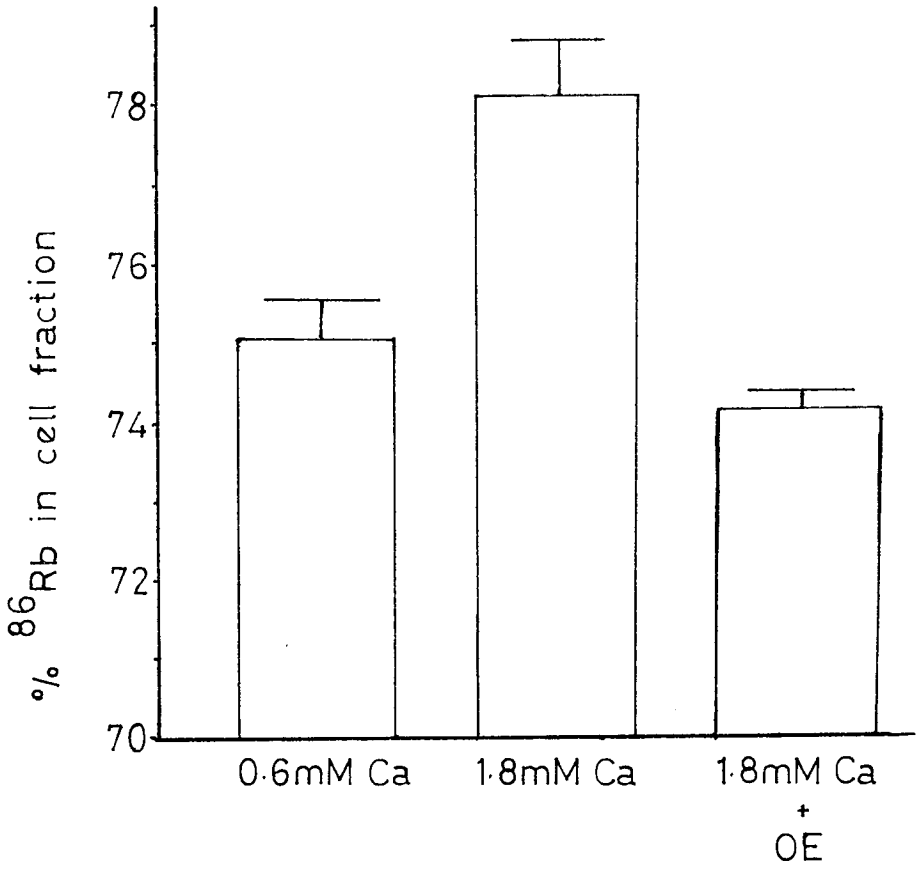

Figure 7·3p. The interaction of oestradiol and high calcium on the efflux of ^{86}Rb from thymocytes. Cells were preincubated and loaded with isotope in Earle's salts solution containing 0·6mM calcium chloride. After washing and resuspension in similar medium at time zero the experiment was commenced by adding either high calcium (1·2mM) or high calcium plus oestradiol-17β (0·1μg.ml^{-1}), other cultures being untreated. 30 minutes later the cells and supernatant were separated and the percentage label remaining in the cells determined. Results are the mean (column) and 1 S.E.M. of between 4 and 8 separate experiments.

transport. In this latter context it might be noted that the cardiotonic steroids (e.g. ouabain) also interfere with monovalent ion balance by binding at the external face of the cell membrane to the potassium acceptor site of the (Na-K)-ATPase (Hoffman, 1975). Thus experiments must be performed to determine whether or not oestrogens do interfere with potassium transport specifically (it is hoped to perform these in the near future). It has already been established that oestradiol does not influence calcium transport per se (Figure 7·3m).

One other series of experiments are worth note in that they may demonstrate some antagonistic action of magnesium upon calcium transport. When cells were incubated with the ionophore, A23187, in medium containing either zero or 1·0mM magnesium sulphate, there was a markedly lower stimulation of ^{45}Ca uptake in the magnesium-containing cultures (Figure 7·3q). We are as yet uncertain if these differ-

Figure 7·3q. The Interaction Between Extracellular Magnesium Levels And A23187-Induced ^{45}Ca Transport Into Thymocytes.

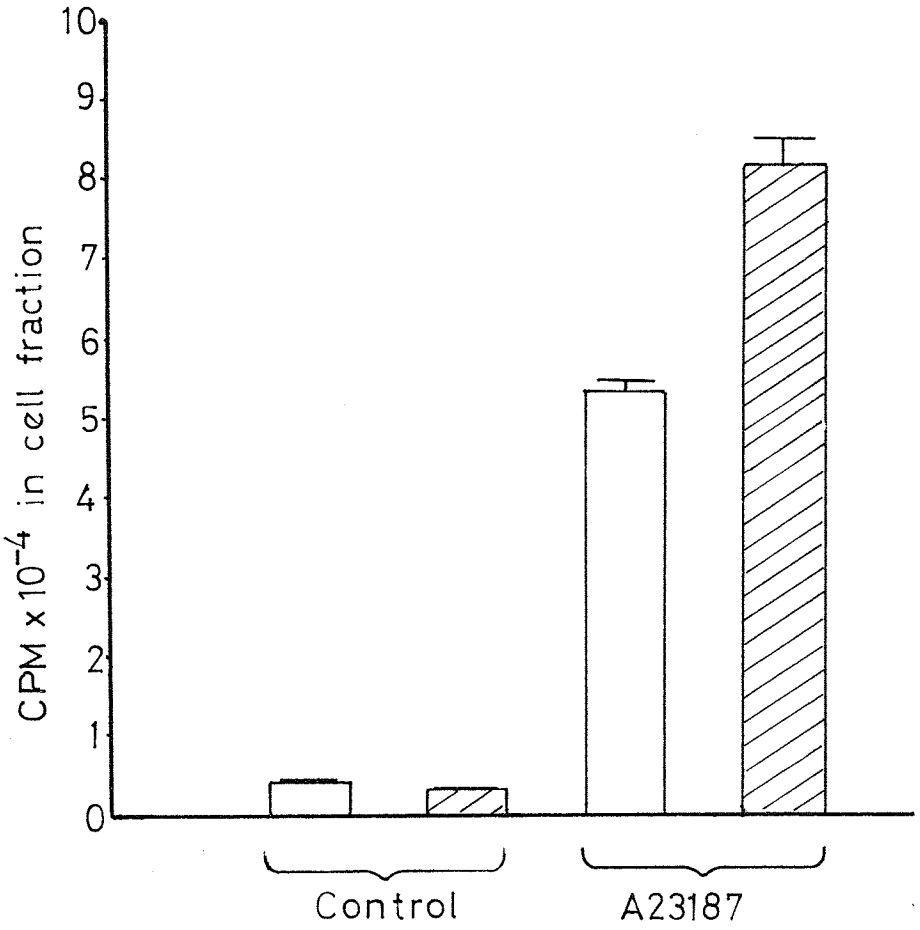

Figure 7·3q. The influence of extracellular magnesium to impair the ability of A23187 to transport calcium into the thymocyte. Cells were incubated in Earle's medium containing 0·6mM calcium and either 1·0mM (open columns) or zero magnesium (hatched columns). At time zero ^{45}Ca was added to test cultures along with A23187 (10^{-5}M) and 5 minutes later the calcium content of the cells determined. Results are the mean CPM in the cells (columns) + 1 S.E.M. of 10 experiments.

ences represent a true antagonistic action, or if they are produced by some artifactual mode of action of the ionophore.

Thus to summarise this Unit, it appears that calcium-dependent mitogens provoke an increase in both the uptake and efflux of calcium in thymocytes. They also cause a rearrangement of the monovalent cationic climate within the cell. This action on sodium and potassium balance is a consequence of calcium entering the cell, since these agents cannot elicit this response in a calcium-free medium. In addition whilst oestradiol blocks the mitogenic actions of these two proliferogenic species it does not appear to impair their ability to stimulate the ingress of calcium or sodium into the thymic lymphocyte. Therefore a simple rise in the endogenous calcium concentration cannot be the final initiator of DNA synthesis, since under these experimental conditions thymocytes do have elevated intracellular calcium levels but do not proceed on to mitosis. In contrast calcium when added exogenously elicits only small changes in monovalent cation distributions, which are also in the opposite direction to those caused by the calcium-dependent mitogens A23187 and Con A. This may also be reflected in the fluorescence system where opposite polarity responses were observed with calcium and the ionophore. In addition oestradiol appears to be capable of reversing this calcium action on potassium movement. This may be a reflection of the proposed ability of oestradiol to interact at the cell surface with the polar head groups to counteract the membrane stabilising activity of calcium. Alternatively it may represent a fundamental and specific effect of the steroid on potassium transport which could be a crucial event in mitotic induction.

Unit 8 General Discussion

The critical decision of whether a cell undertakes a fresh round of mitosis or remains in G1 as a functional differentiated cell is the resultant of a complex series of factors. Such factors might include the general metabolic state of the cell, its nutrient supply, cell to cell contact and genetic determinants. However in this thesis the problem of mitotic control has been studied in the thymocyte model where the critical decision to reinitiate division is taken by a small subpopulation of thymic lymphoblasts which have been shown to be in a quiescent (G_o) phase. Such cells probably maintain the normal levels of cellularity in the thymus gland itself by entering their DNA synthetic phase upon demand. It has been shown by other workers that the normal physiological demand mechanism on these cells in a number of situations is the calcium homeostatic system (Perris, 1971; Hunt and Perris, 1974; Edwards et al., 1976). Indeed when maintained in vitro thymocytes which are in G_o may be induced to reinitiate mitotic cycling by simply raising the extracellular calcium ion concentration, thus mimicking the situation obtaining in vivo. In the in vitro context a number of other agents have been shown to stimulate mitotic activity. These include magnesium ions, the calcium ionophore A23187, a range of hormones and cyclic nucleotides and the plant lectin concanavalin A. By the use of this panel of mitogens it has been possible to evolve a model to describe the activation process following cell stimulation. This task has been aided by the use of the steroids oestradiol and testosterone which selectively inhibit certain of these mitogens. Thus it has been found that all mitogens may be classed into two categories:- the first require extracellular calcium ions for their mitogenic activity to be expressed and are inhibited by oestradiol. The second have no such requirement for calcium ions but do require magnesium ions. These mitogens are inhibited by the

androgen testosterone.

Biochemical studies have indicated that the magnesium-dependent mitogenic hormones elevate intracellular cyclic AMP levels by stimulating the enzyme adenylate cyclase. Furthermore this cyclic nucleotide may play a key role in initiating DNA synthesis in the quiescent lymphoblast since exogenous addition of cyclic AMP will stimulate thymocyte mitotic activity. Certainly elevation of endogenous cyclic AMP concentrations via an inhibition of cyclic nucleotide phosphodiesterase with Ro 20-1724 also leads to a stimulation of mitotic activity. However, not only do the hormones require extracellular magnesium ions for their mitogenic activity to be manifest but also exogenously applied cyclic AMP and the phosphodiesterase inhibitor Ro 20-1724 need this ion to be present in the culture medium to elicit their responses. Testosterone is also capable of blocking the mitogenic activities of hormones, cyclic AMP and the phosphodiesterase inhibitor. This being in addition to its ability to inhibit magnesium-induced mitogenesis itself.

One interpretation of this data would indicate the following course of events on cell stimulation by a magnesium-dependent hormonal mitogen:-

(1) Hormone stimulates adenylate cyclase by binding at a membrane receptor. This binding is not impaired by testosterone.

(2) Cyclase stimulation leads to an elevation of endogenous cyclic AMP levels. Testosterone does not appreciably alter the surge of cyclic AMP generated by the hormone, or the phosphodiesterase inhibitor Ro 20-1724. Thus indicating that testosterone must act subsequent to this event, or on an entirely different process.

(3) The elevated cyclic AMP levels then initiate reactions which culminate in the entry of quiescent lymphoblasts into their DNA synthetic phase. One or more of these processes elicited by the cyclic nucleotide must be magnesium-dependent and testosterone-blocked. Therefore if the mitogenic action of magnesium is attributable to its acting on this same magnesium-dependent reaction, it would not be expected that elevation of exogenous magnesium concentrations would alter cyclic AMP levels. Indeed it did not prove possible to detect any changes in cyclic AMP metabolism with magnesium at mitogenic concentrations, thus supporting the proposed scheme. Furthermore, since magnesium-induced mitogenesis is blocked by testosterone it might be inferred that the testosterone-blockable stage lies subsequent to (or is the same as) the magnesium-dependent reaction.

(4)As a number of cyclic and non-cyclic nucleotides also possess the ability to initiate division in a magnesium-dependent, testosterone-blockable fashion (albeit at much higher concentrations than cyclic AMP itself) it has been proposed that these other agents have some affinity for a membrane receptor which is specific under normal circumstances for cyclic AMP. Such a moiety may be a protein kinase which either requires magnesium ions for its activation by cyclic AMP, or which regulates a mechanism for transporting this divalent cation into the cell. The latter action may of course require a number of intermediate steps. Certainly evidence from both ion transport experiments and membrane potential assessments suggest the possibility that cyclic AMP and magnesium ions have a similar action upon the membrane in muting calcium-dependent responses. This interaction may result in the case of magnesium in a suppression of calcium transport into the thymic lymphocyte promoted by the calcium-dependent mitogen, A23187. Furthermore both magnesium and cyclic AMP have been

demonstrated to mute the hyperpolarisation induced by calcium addition to thymocyte suspensions. Thus alterations in membrane permeability or function may represent key events in the mitotic activation process. Indeed whilst testosterone did not significantly alter cyclic AMP metabolism, it was detected as having a small effect upon membrane potential. Therefore it would seem likely that this androgen derives its antimitotic capacity from an ability to alter some aspect of membrane function. And these changes in membrane function are also likely to be of opposite polarity to those induced by oestradiol, further underlining the dichotomy of action between these two agents.

In view of the functional differences between the magnesium- and calcium-dependent mitogenic axes and the actions of cyclic nucleotides and ions upon membrane phenomena it would seem likely that at the cellular levels both mechanisms interact in a reciprocal harmonic fashion. This is in spite of the fact that both axes result in a common event, DNA synthesis. The mechanisms underlying this antagonism may be more clearly understood by next considering the calcium-dependent mitogens.

In contrast to the magnesium-dependent mitogens there are a number of agents which constitute the so-called calcium-dependent axis. Factors in this group comprise polypeptide hormones, neurotransmitters, calcium ionophores, cyclic GMP and concanavalin A. As mentioned previously this group of mitogens require extracellular calcium ions to elicit their mitogenic response and are inhibited by the presence of oestradiol in the culture system. A skein of events analagous to those described for magnesium-dependent mitogens has been invoked to account for the actions of these mitogens.

(1) Since there is no evidence for the stimulation of adenyl

cyclase by the calcium-dependent mitogens it has been suggested that these agents act via the metabolism of the guanyl nucleotides. In addition, cyclic GMP at concentrations approximating to physiological levels, will stimulate thymocyte proliferation in a calcium-dependent, oestradiol-blockable fashion. It is envisaged therefore that calcium-dependent hormones stimulate cyclic GMP production by activating either a membrane bound guanylate cyclase, or via an influx of calcium to the cell, a soluble cyclase moiety. It must be added however that it has not proved possible to detect any alteration in absolute cyclic GMP levels following the application of histamine or acetylcholine to the thymocyte suspensions. Although such a result may have accrued through a technical difficulty in measuring endogenous cyclic GMP concentrations.

(2) Since exogenous cyclic GMP does stimulate division in a calcium-dependent manner, and is inhibited by oestradiol it has been argued that it too (like cyclic AMP) activates some membrane kinase which initiates DNA synthesis. This enzyme either transfers calcium ions into the thymocyte, or may require these ions for its activation. Membrane physicochemical studies have revealed that cyclic GMP and calcium cooperate to induce changes in membrane potential. Such an action may represent a function of the proposed kinase enzyme.

(3) Oestradiol, in a manner analagous to testosterone, must derive its antimitotic ability by interfering with some process subsequent to the calcium-dependent reaction initiated by cyclic GMP. For although this steroid does alter the long-term cyclic AMP changes induced by two calcium-dependent hormones (histamine and insulin) time course studies of the oestradiol reversibility of calcium-induced mitogenesis would indicate such alterations to be too slow. In addition calcium itself does not produce any marked changes in cyclic AMP

metabolism, indicating that oestradiol does not inhibit the mitogenic action of this divalent cation by changing nucleotide metabolism. It has been established however that oestradiol does markedly reduce the membrane hyperpolarisation induced by calcium addition to thymocytes. Such an action of this steroid may derive from an ability to prevent the association of calcium with anionic polar head groups of the outer palisade of the cell membrane. Thus oestradiol would reduce the membrane stabilising action of calcium which would otherwise have brought about changes in membrane ion permeabilities, which could themselves play a central role in the initiation of division. Alternatively or in addition this reduction in calcium associated with the plasma membrane may result in some change in the activation characteristics of the cyclic GMP-dependent protein kinase.

(4) Two calcium-dependent mitogens, A23187 and concanavalin A, have been shown to provoke marked changes in membrane permeability to a number of ionic species including calcium, sodium, potassium and phosphate. In addition this ion transporting activity, like their mitogenic action, is dependent upon the presence of extracellular calcium ions. Therefore as these agents have been shown to promote calcium uptake it must be inferred that it is an increase in cytosolic calcium levels which triggers these unscheduled ion movements. Exogenous calcium does not provoke such a clear alteration in membrane permeability to these ions, and may indeed initiate changes of opposite polarity. However, oestradiol did appear to abolish the small decrease in potassium transport caused by calcium addition to the culture. Thus it may be that calcium, by altering membrane fluidity, brings about changes in cellular potassium balance which determines the entry of quiescent lymphoblasts into their DNA synthetic phase. Such permeability changes are antagonised by oestradiol, thus inhibiting the initiation of proliferation.

(5) Such a scheme where hormones trigger monovalent ion fluxes via calcium transients may account for the rather curious changes in cyclic AMP levels following the addition of calcium-dependent hormones to thymocyte cultures. For these oscillations in cyclic AMP values may reflect not a direct consequence of a hormone-cell interaction, but rather a mechanism for balancing intracellular ion concentrations disrupted by this entry of calcium into the cell or cell membrane. Thus calcium entering the cell would be expected to:-

(a) Increase membrane permeability to sodium and potassium, causing a nett influx of the former and a nett efflux of the latter.

(b) Increasing the ionised calcium concentration in the cytosol would also be expected to reduce the activity of the (Na-K)-ATPase thus exacerbating the monovalent ion imbalance.

(c) The switch of phosphorylation from the (Na-K)-ATPase to the Ca-ATPase, extrusion pump, would be one mechanism for lowering the calcium load in the cytoplasm.

(d) Increasing the intracellular calcium concentration would also tend to increase cyclic GMP levels by activating guanylate cyclase. This nucleotide is known to promote the sequestration of calcium into mitochondria (Rasmussen et al., 1975).

(e) Cyclic GMP will also lower cyclic AMP concentrations in the thymocyte by stimulating phosphodiesterase activity. In terms of calcium homeostasis this makes good sense as cyclic AMP promotes the efflux of calcium from mitochondria which would tend to compound the problem with which the cell is faced. Since cyclic AMP is also a positive regulator of (Na-K)-ATPase activity it would be expected that there would be a fall in the functioning of this enzyme during the phase of low cyclic AMP.

(f) As cytosolic calcium levels fall, there should be a concomitant drop in cyclic GMP concentrations. This should be associated with a rise in cyclic AMP levels. Furthermore cyclic AMP levels may actually overshoot basal values for a number of reasons. First calcium will inhibit phosphodiesterase thus elevating endogenous cyclic AMP levels (this action was masked by cyclic GMP also activating this enzyme in the initial phase). At reduced substrate levels of cyclic AMP, cyclic GMP will begin to inhibit cyclic AMP-phosphodiesterase thus raising cyclic AMP values. Such an overshoot also makes sense since it will promote the activity of the (Na-K)-ATPase thus reestablishing the monovalent ion balance more rapidly.

(g) When normal ionised calcium levels are obtained, cyclic GMP values will be back to normal and the calcium-ATPase will be reduced in activity. Eventually cyclic AMP levels return to basal concentrations when the monovalent ion distribution is reestablished via both an increase in (Na-K)-ATPase activity by cyclic AMP and a reduction in membrane permeability to sodium and potassium through the sequestration of free calcium.

If this rationale is correct, and if oestradiol selectively prevents a potassium rearrangement then the following would be expected to occur. First a calcium-dependent mitogen would provoke a calcium influx to the thymic lymphocyte. This is known not to be influenced by oestradiol. Thus one would expect to observe the initial rise in cyclic GMP with the associated fall in cyclic AMP. However, oestradiol is likely to oppose the secondary monovalent rearrangement preventing the imbalance observed in its absence. Thus whilst one would expect to observe a small overshoot in cyclic AMP levels following the depression through calcium ingress it would not make sense for there to be a gross rise in the absence of a monovalent ion imbalance. Mechanistically the

observed decrease in the late cyclic AMP peak may have a number of explanations. First the calcium transport system of the mitochondria is known to be sensitive to the sodium/potassium balance of the cytosol. Thus the altered pattern of calcium movement into and out of the mitochondrial pool may serve to dampen the cyclic AMP surges. Secondly potassium is also known to elevate endogenous cyclic GMP levels and may thus contribute with calcium to the proposed elevation of cyclic GMP. In the absence of a potassium movement it is possible that the kinetics of the cyclic GMP phase are modified in such a way as to reduce the subsequent rise in cyclic AMP. The former of these two possibilities would seem the more likely since a slower removal of calcium from the cytosol into mitochondria would be expected to reduce the second cyclic AMP peak.

Alternatively the membrane stabilising action of calcium may initiate division in an as yet unknown fashion. Thus by simply opposing this change oestradiol would prove antimitotic. Calcium-dependent mitogens might also promote the entry of quiescent lymphoblasts into their DNA synthetic phase by provoking an association of calcium with some part of the cell membrane which leads to the same changes elicited by calcium itself. It is not clear if these changes require the complex changes in cyclic nucleotide metabolism and transmembrane ion distribution observed to occur on mitogen addition. Or if any or all of these changes act singly or in concert to bring about the initiation of mitosis. Such questions must be left to be answered in the future.

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CALCIUM AND OESTROGEN INTERACTIONS UPON THE RAT THYMIC LYMPHOCYTE PLASMA MEMBRANE

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THE USE OF A FLUORESCENT PROBE TO MONITOR ALTERATIONS IN TRANS-MEMBRANE POTENTIAL IN SINGLE CELL SUSPENSIONS

J.S. BRAMHALL[1], J.I. MORGAN[2], A.D. PERRIS[2], and A.Z. BRITTEN[1]

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The Interaction of Divalent Cations, Hormones and Cyclic Nucleotides in the Control of Mitosis

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Structural requirements for oestrogenic activity at the plasma membrane surface determined using the carbocyanine fluorescent probe. By J. S. Bramhall, J. I. Morgan, A. Z. Britten and A. D. Perris. *Departments of Pharmacy and Biological Sciences, The University of Aston, Gosta Green, Birmingham, B4 7ET*

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The use of a fluorescent probe to demonstrate the interaction of calcium and oestradiol-17β in the thymic lymphocyte plasma membrane. By J. I. Morgan, J. S. Bramhall, A. Z. Britten and A. D. Perris. *Department of Biological Sciences and Pharmacy, The University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET*

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Vol. 66, No. 1, 1975 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

REQUIREMENTS FOR DIVALENT CATIONS BY HORMONAL MITOGENS AND THEIR INTERACTIONS WITH SEX STEROIDS

J.I. MORGAN, A.K. HALL and A.D. PERRIS

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The Influence of Sex Steroids on Calcium- and Magnesium-induced Mitogenesis in Isolated Rat Thymic Lymphocytes

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