STIMULUS-MITOSIS COUPLING IN THE RAT THYMIC LYMPHOCYTE

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SUMMARY

Various extracellular stimuli provoke the entry of normally quiescent rat thymic lymphocytes into the cell division cycle. This study has investigated the events which follow signal presentation and which culminate in cell cycle entry. The initial experiments examined mitotic activity in cultured thymocytes exposed to mitogenic and anti-mitogenic compounds. Like stimuli for secretion and contraction in other tissues, mitogens show an intimate dependency upon the extracellular cationic environment for their activity. Subsequent work therefore investigated the redistribution of various cations across the plasma membrane initiated by mitogenic signals.

Thirty minutes after an elevation of the extracellular calcium concentration, which is believed to be a primary mitogenic signal *in vivo*, cultured thymocytes were committed to divide. Stimulation was only evident when free access of ionized calcium to the cytosol was unimpeded. A three-fold increase in extracellular calcium increased intracellular calcium approximately one hundred-fold and was associated with heightened potassium exit. An enhanced potassium efflux has been observed in a variety of tissues consequent upon an elevation in the free cytosolic calcium concentration. Via this indicator, several hormonal and non-hormonal mitogens were found to increase ionized calcium in the thymocyte cytosol although they failed to promote calcium influx.

Those mitogens which required an extracellular supply of either calcium or magnesium ions, all raised intracellular calcium concentrations. In the case of those mitogens which interfered primarily with sodium metabolism, this was achieved by an inhibition of calcium extrusion.

Thus a rise in cytosolic ionised calcium, which is known to couple extracellular signals to metabolic events such as secretion and contraction in various tissues, now appears to play a key role in stimulus-mitosis coupling. The increased free calcium concentrations created by enhanced entry, impaired exit or mobilization of intracellular stores provides the link between extracellular mitogenic signals and the intracellular cell cycle regulatory mechanisms.

Key words: Stimulus-mitosis coupling; calcium; rat thymocyte.

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1.1. Introduction.

A co-ordinated system of extracellular signalling regulates the growth and development of mammalian tissues in vivo. To achieve the necessary close control, the external environment must be able to rapidly interact with those cytosolic processes that regulate the progress of individual cells through the division cycle. In order to reach these key intracellular mechanisms any extracellular signal (the primary messenger) must first recognise the target cell, and of course, be recognised as an appropriate signal by the target cell. Initial recognition must not be exclusively at the outer membrane surface, although stereospecific receptors for many primary messengers are membrane-bound. The second event, following mutual recognition, must be transmission of the signal to the appropriate cellular mechanism. This may occur without alteration of the primary messenger where the first signal is in a suitable form. Alternatively, the first signal may be translated into a second (or third) messenger which is then able to interact with the necessary processes. At this stage an amplification step may occur to ensure a response to a weak extracellular signal. The ultimate step will involve the final messenger (be it primary, secondary etc.) interacting with the target molecules within the cytosol. The present study is intended to investigate such a signal-transfer phenomenon which will occur when quiescent rat thymic lymphocytes are recruited into the cell cycle by a mitogenic signal.

An increased free calcium concentration is implicated in the activation of many cell types following the reception of an acceptable stimulus. Such a change in calcium is also indicated when thymocytes are recruited. Therefore the subsequent chapters review evidence suggesting a general role for calcium in cellular activation

and detail those processes whereby the external mitotic signal can be received and transmitted by the cell. Appropriately the cellular metabolism of calcium is discussed in the first Section before a consideration of the other major intracellular regulatory molecules, namely the cyclic nucleotides. The second Section reviews other examples where the external and internal environment interact to produce a controlled cell activation. As monovalent cations play a key role in transmitting extracellular messengers their cellular metabolism is discussed in the first part of Section 2. The remaining parts of this Section review the processes of secretion and contraction, where a rise in cytoplasmic calcium is known to couple external signal with the cellular response. The third Section of the Introduction returns to the model of cell proliferation and first considers the basic unit of proliferation, the cell cycle. The regulation, both internal and external, of this cycle is then discussed in the remaining part. The fourth and final Section reviews the specific experimental model, the rat thymocyte, and details the known evidence indicating how proliferation is regulated in this cell type.

1.2 The intracellular metabolism of calcium.

It seems probable that the ability to maintain an intracellular calcium concentration below that of the surrounding environment predates the eukaryotic state; indeed several prokaryotes maintain reduced intracellular levels (Silvers,Toth & Schribner,1975). Such reductions of internal calcium would prevent precipitation of primitive phosphate energy sources (Kretsinger,1976), and facilitate regulation of magnesium sensitive enzymes by decreasing divalent cation competition (Williams,1974). It would also allow evolution of complex molecules such as tubulin and DNA, both of which are inactivated by calcium levels above 10^{-5}M (Margulis,1975; Williams,1976). The role of calcium in triggering, signal effect coupling, cell motility and proliferation suggests that, in addition to these evolutionary benefits, the inward calcium gradient provides a rapid intracellular signalling system (Heilbrunn, 1956). Calcium is uniquely suited to this role as it forms rapidly reversible cross linkages with protein molecules (Williams,1976) and its high coordination number and irregular geometry make it a "flexible" molecule (Williams,1976). Basal intracellular calcium concentrations of 10^{-7}M (DiPolo, Requenza, Mullins, Brinley, Scarpa & Tiffert,1976) and target protein calcium affinities of 10^{-6}M (Urry, Long, Ohnishi, Jacobs & Mitchell,1975) give a signal to target ratio of 1:10. Assuming that a hundredfold increase is necessary to avoid false message generation a calcium concentration change of less than ten micromolar would elevate intracellular levels to 10^{-5}M, giving the desired signal to target ratio of 10:1 (Kretsinger,1976). Sophisticated mechanisms capable of creating elevations and subsequently restoring normal levels must therefore exist.

Despite membrane discrimination restricting calcium entry the large chemical gradient causes passive influx of ions into the cell. In squid

axons this is sufficient to double total intra-axonal calcium within 24 hours (Baker & Crawford,1972). Membrane potential directly bars calcium entry, consequently depolarization permits influx (Suarez-Kurtz & Reuben, 1975). Thus membrane depolarizing agents (e.g. neurotransmitters) remove the barrier to calcium entry (Kometiani,1978). Depolarization itself may carry calcium ions into excitable cells, either by the fast sodium entry channel or by the slow calcium channel (Hodgkin & Huxley,1952 ; Kohlhardt, Bauer, Krause & Fleckenstein,1972). Calcium may also enter cells by the direct ionophoretic action of certain molecules such as A23187 (Reed & Lardy,1972; Reed,1972). Pressman has defined an ionophore as a compound facilitating the transport of an ion through a natural or artificial lipid membrane, from one aqueous phase to another (Pressman, Harris, Jagger & Johnson,1967). Figure 1:1 represents a general schema for cellular calcium homeostasis; passive, potential dependent and ionophoretic calcium entry is depicted as (a), (b) & (c) respectively. These processes will either continually or spasmodically elevate intracellular calcium. To restore basal levels free intracellular calcium must be reduced. One mechanism is the sequestration of calcium into non-ionized pools (Baker & Crawford,1972; Hodgkin & Keynes,1957). Some of this buffering capacity resides in cytosolic components having a limited, high affinity capacity for calcium binding (Baker & Schlaepfer,1975; Baker, Hodgkin & Ridgeway, 1971). This may be particularly important in the restoration of levels following action potentials. The majority of sequestered calcium resides in mitochondrial stores (Carafoli & Crompton,1976). These organelles are capable of concentrating calcium from the surrounding environment (Baker & Schlaepfer,1975), via an ATP - dependent process (Baker, Hodgkin & Ridgeway, 1971). Cellular pH (Gear, Rossi, Reynafarje & Lehninger,1967), and the mitochondrial membrane potential (Rottenberg & Scarpa,1974; Mitchell,1966)

all influence this process. Their almost unlimited capacity is due to precipitation of stored calcium as rapidly exchangeable phosphate salts (Brierley & Slautterbach, 1964). It is estimated that mitochondrial buffering alone may maintain internal calcium at micromolar concentrations (Drahota, Carafoli, Rossi, Gamble & Lehninger, 1965). Indirect evidence suggests the sink capacity is reversible (Crawford, 1975; Fleckenstein, 1977), with release of calcium from mitochondria able to elevate intracellular calcium. One possible mechanism suggests that in isolated heart mitochondria extra-mitochondrial sodium provokes calcium release by reducing mitochondrial membrane potential (Carafoli, Tiozzo, Lugli, Cravetti & Kratzing, 1975). Half maximal release is stimulated by 8 mM sodium (Crompton, Kunzi & Carafoli, 1977; Carafoli & Crompton, 1976). This effect may be restricted to excitable tissue as liver mitochondria do not respond to sodium ions. Cyclic AMP at 10^{-6}M releases calcium from isolated renal mitochondria (Borle, 1974). This remains a contentious issue, other laboratories fail to reproduce the effect (Carafoli & Crompton, 1966) whilst Borle consistently observes the effect at even lower nucleotide levels (Borle & Uchikawa, 1978).

The endoplasmic and sarcoplasmic reticula fulfil a similar storage function, demonstrating energy-dependent calcium accumulation (Moore, Chen, Knapp & Landon, 1975; Moore & Pastan, 1977; Ford & Podolsky,1972). In the model of calcium homeostasis (Figure 1:1) mitochondrial,cytosolic and microsomal storage is shown as (d), (e) and (f) respectively. Although such storage may regulate calcium ion activity over short periods they are not infinitely expandable. The cell must therefore balance influx by extruding calcium against the electrochemical gradient. Two such processes exist. One links calcium exit to sodium influx as a coupled exchange (Reuter & Seitz,1968;Blaustein,1974),the other is an uncoupled

efflux directly utilising energy in the form of ATP (Lew,1978; Lew,1971). Coupled exchange, however, is not entirely energy-independent as ATP is required to maintain the sodium gradient (see section 2.1). Abolition of this gradient, by removal of extracellular sodium or by increasing intracellular sodium, prevents calcium exit and consequently raises intracellular calcium levels (Fleckenstein,1977; Cooke & Robinson,1971; Blaustein,1974; Judah & Ahmed,1964). The experimental demonstration of coupled exchange is complicated as only a small percentage of inward sodium movement is coupled to calcium exit (Blaustein & Russel,1975). Using cardiac membrane vesicles the exchange of tracer Na^{+} and Ca^{++} may be detected (Reeves & Sutkho,1979). In purified sarcolemma the exchange occurs with an electrogenic ratio of 3 Na^{+} for each Ca^{++} (Pitts,1979). Vesicular pumping may be run in the reverse direction by manipulating the sodium gradient.

In the red blood cell ATP dependent extrusion of calcium is the sole mechanism for the reduction of the intracellular calcium (Lew,1971). Squid axon possesses both coupled and uncoupled devices. Here removal of external sodium or depletion of ATP reduces calcium efflux (Baker & McNaughton,1978; DiPolo, 1978; Dipolo & Beague, 1979). The calcium sensitivity of the two systems suggests that at low intracellular calcium the ATP-dependent extrusion dominates, but at high internal calcium levels coupled exchange predominates (DiPolo & Beague,1979). Using vanadate as a selective inhibitor of ATP-ase activity the axonal and red cell uncoupled exchange may be prevented, (DiPolo & Beague,1979; Bond & Hudgins, 1978). This directly implicates the calcium-stimulated ATP-ase in the export of calcium ions. Whilst of undoubted significance in these two cases the contribution of calcium ATP-ases in other systems remains uncertain. Calcium extrusion may be shown to be partly ATP dependent in cultured cells (Lindsay,1976;

Cittadini, Bossi, Rosi, Wolf & Terranova,1977). Although these investigators demonstrate calcium-stimulated hydrolysis of ATP in homogenates (often at non-physiological levels) others cannot detect calcium-stimulated activity (Chambault, Leroy-Pecker, Feldmann & Hanoune, 1974). This may be due to the masking effect of large Mg-stimulated ATP-ase activity in plasma membrane fractions. In the calcium homeostatic model (Figure 1:1) coupled efflux and Ca-ATP-ase activity are denoted as (g) & (h) respectively.

The contribution of plasma membrane calcium to intracellular pools may also be significant. Calcium reversibly associates with cell surface muco-polysaccharides and carboxylic acid or phosphatidyl serine residues (Langer Serena & Nudd,1974; McDonald, Bruns & Jarrett,1976). A variety of stimulants displace this membrane bound calcium pool (Tupper, DelRosso, Hazelton & Zorgniotti,1979; McDonald, Bruns & Jarrett,1976). This pool is shown in Figure 1:1 as (k). The preceding studies demonstrate the compartmentalisation of intracellular calcium and the processes by which it is regulated. It must be remembered that thymic lymphocytes, employed in this study clearly differ in both structure and function from many of the cells detailed above. It is important therefore to note that Figure 1:1 represents a generalised model, only parts of which may apply in the thymic lymphocyte.

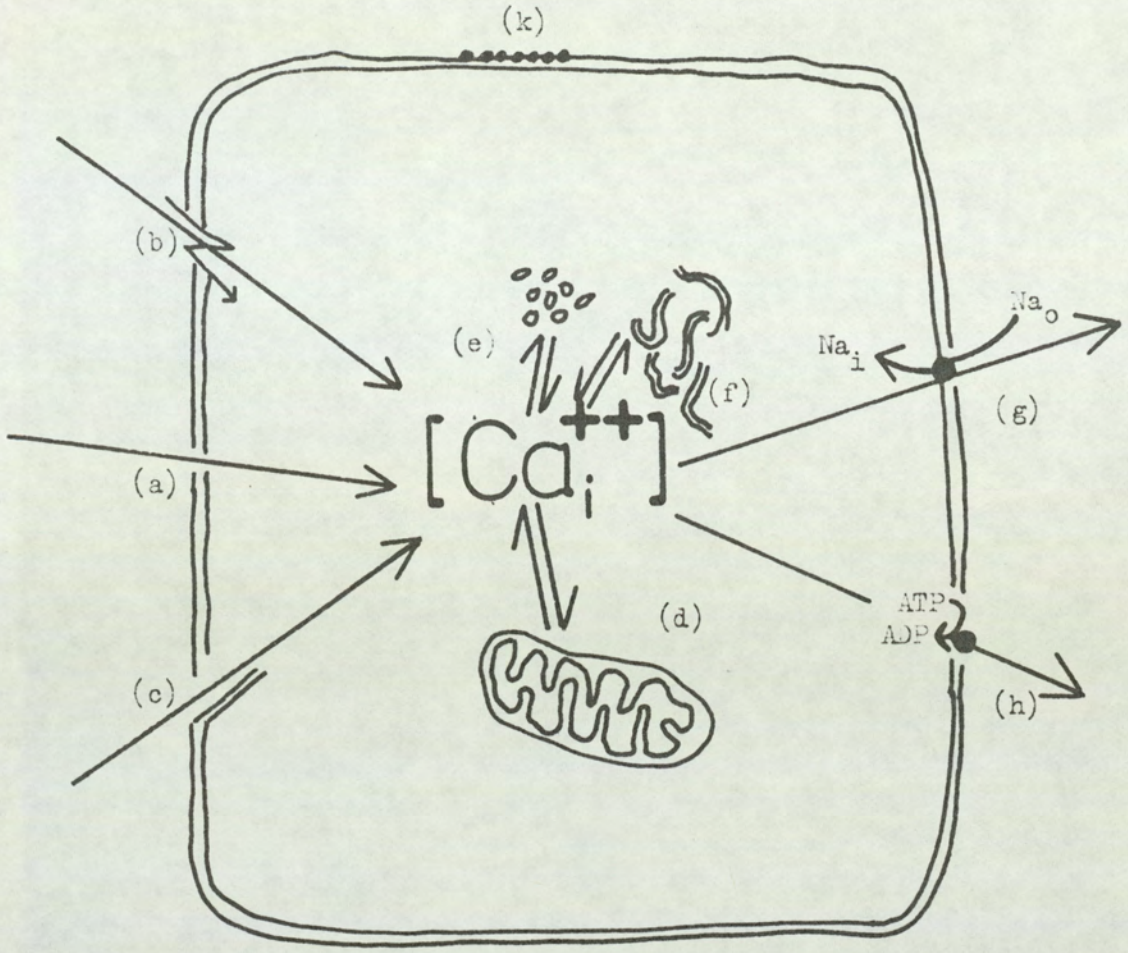


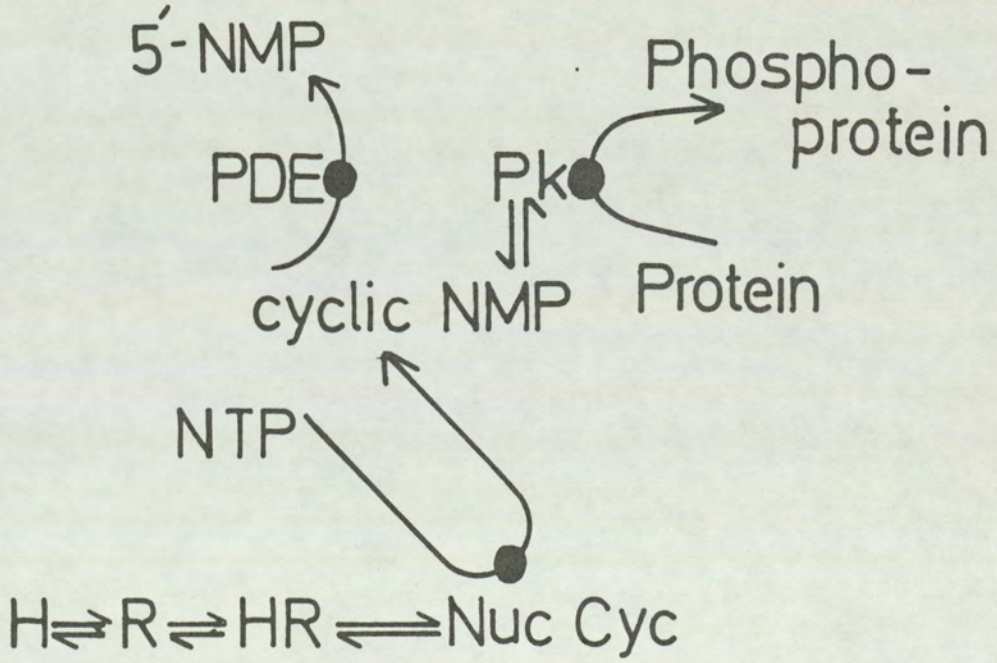
Figure 1.1 Intracellular calcium homeostasis

a) Passive influx
b) Potential dependent influx
c) Ionophoretic or hormone induced entry
d) Mitochondrial buffering
e) Cytosolic buffering
f) Microsomal buffering
g) Sodium/Calcium exchange
h) Calcium ion activated ATP-ase
k) Membrane bound calcium

1.3 The intracellular metabolism of cyclic nucleotides

In addition to mitogenically significant fluctuations in the intracellular calcium concentration it appears that alterations in cyclic nucleotide metabolism are also important in mitotic events. The next section therefore reviews the mechanisms governing their genesis, action and distribution. The major cyclic nucleotides, cyclic 3'5' AMP and cyclic 3'5' GMP, which enjoy almost universal cellular distribution are generated within the cytosol in response to hormonal stimulii. Specific enzymes catalyse their formation from ATP and GTP respectively. Their action as secondary messengers is the result of an activation of specific protein kinase molecules, which themselves catalyse specific protein phosphorylation. The cyclic nucleotide molecules are de-activated by the hydrolytic action of cyclic nucleotide phosphodiesterases, which convert them to inactive 5'-nucleotide monophosphates. The overall metabolism of cyclic nucleotides is summarised in figure 1:2.

Fig. 1.2. Generalized reaction scheme of cyclic nucleotide metabolism



H - Hormone; R - Receptor; Nuc. Cyc. - Specific hormone activated nucleotide cyclase; NTP - nucleotide triphosphate; cyclic NMP - cyclic 3'5' nucleotide monophosphate; pK - cyclic nucleotide activated protein kinase; PDE - phosphodiesterase; 5'-NMP ; nucleotide 5' monophosphate

Formation of intracelluar cyclic 3'5' AMP

In response to hormonal occupation of stereospecific receptors the enzyme adenyl cyclase catalyses the formation of cyclic AMP (Rall & Sutherland,1962). With the exception of soluble enzyme in rat testis (Braun & Dodds,1975) the cyclase activity is membrane bound and faces the cell interior (Wolff & Jones,1971). As the enzyme is resistant to purification, information on its component parts must be inferred from chemically treated preparations. These studies reveal that functional cyclase consists of at least three components; receptor, catalytic & guanyl nucleotide binding units (Cuatrecasas,1974; Birnbaumer,1973; Rodbell,1978). Other features of this complex, such as divalent cation and fluoride binding, may be associated with either the complete holoenzyme or its constituent parts.

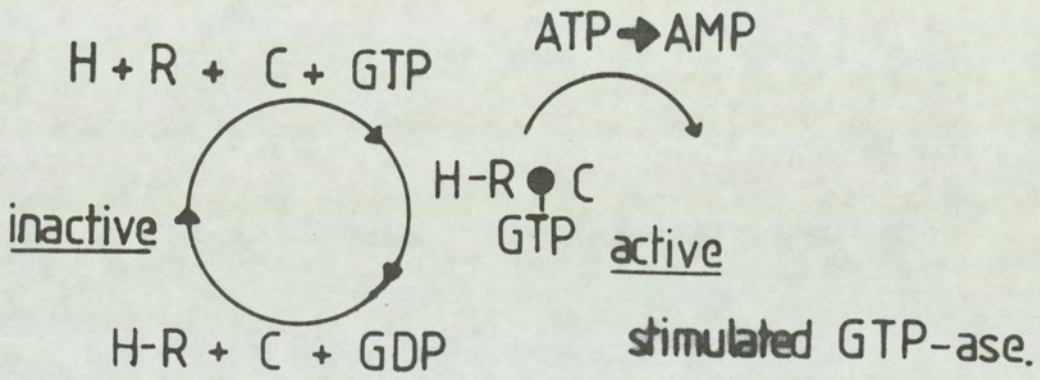
Cell hybridization experiments reveal that hormone receptors, free to move in the plane of the outer lipid phase (Rodbell, Birnbaumer & Rohl,1970) exist separately from the catalytic units (Orly & Schramm, 1976). Receptors for different hormones may interact with a single catalytic unit (Schramm,1979; Schramm, Orly, Eimerl & Korner,1977).

Enzyme activity is modified by guanyl nucleotide binding sites, present on both receptor and catalytic components (Ross, Haga, Howlett, Schwarzmeier, Schleifer & Gilman,1979; Rodbell,1978). GDP binding maintains the basal catalytic activity whereas GTP activates the enzyme. Depletion of GTP from whole cell preparations or its removal from homogenates abolishes hormone responsiveness (Ross et al,1979). Micromolar levels of GTP partially activate catalytic activity in the absence of hormone (Londos, Lin, Welton, Lad & Rodbell,1977). Hormone binding promotes GTP displacement of GDP at the catalytic regulatory site thereby activating cyclase activity (Iyengar & Birnbaumer,1979).

The inability of GTP alone to stimulate maximally is due to intrinsic GTP hydrolysis, converting bound GTP to GDP and thereby deactivating the enzyme. The non-hydrolysable analogue Gpp NHp fully and irreversibly activates cyclase (Londos, Lin, Welton, Lad & Rodbell,1977; Rodbell, Lin, Salomon, Harwood, Martin, Rendell & Berman,1975). Thus enzyme deactivation is directed by the GTP-ase activity. The promotion of GTP binding and its subsequent hydrolysis which occurs in intact cells (Rendell, Rodbell & Berman,1977) suggests that activation or deactivation may both be directed by hormone binding (Terasaki, Brooker, de Vellis, Inglish, Hsu & Moylan, 1977). The following mechanism is suggested for GTP activation of catalytic a activity (Iyengar & Birnbaumer,1979).

Figure (1:3)

GTP activation of catalytic activity



Hormone receptor interaction is also regulated by GTP (Lad, Welton & Rodbell,1977). Receptor occupancy promotes GTP binding which in turn diminishes receptor affinity for hormone, thereby deactivating the hormone-receptor complex (Rodbell, Krans, Pohl & Birnbaumer,1971; Welton, et al,1977). The existence of two guanyl nucleotide binding activities in the small fragments of the cyclase (Pfeuffer & Helmreich,1975) suggests that the regulatory sites are linked in some way to the major components. This does not indicate the nature of the physical coupling between occupied

receptors and functional catalytic units. One possible linkage may be provided by a soluble factor extractable from competent enzyme. This factor restores activity to a S49 lymphoma cell line possessing functional receptor and catalytic units but lacking hormonal responsiveness (Haga, Ross, Anderson, Gilman, 1977; Ross, et al,1979). The coupling of hormone receptor and catalytic unit will also be influenced by the membrane lipid fluidity. It is therefore significant that the β -adrenergic agonists activate phosphatidyl inositol turnover, thereby increasing fluidity and promoting sub-unit coupling by facilitating lateral movement of sub-units (Hirata, Strittmatter & Axelrod,1979).

The intracellular calcium concentration also regulates adenyl cycalse activity. Calcium may either stimulate or inhibit the formation of cyclic AMP. Thus in homogenates of rat parotid and adrenal medulla 1mM calcium reduces cyclase activity (Rasmussen & Goodman,1975), whereas in the rat anterior pituitary apparent elevations of the intracellular calcium, induced by the ionophore A23187, promote cyclic AMP formation (Zonefrati, Tanini, Rotella & Toccafondi,1978). In brain tissue two populations of adenyl cyclase exist (Brostrom, Brostrom & Wolff,1977). Using the calcium binding protein Calmodulin (see section 1.4) as an affinity ligand these may be chromatographically resolved. The resultant activity comprises 80% Calmodulin insensitive and 20% sensitive enzyme (Westcott, LaPorte & Storm, 1979). The insensitive enzyme is stimulated by magnesium over a range of 0.5 to 10mM. and is inhibited by elevations of calcium ion (Birnbaumer, 1973; Brostrom, et al,1977). In contrast the calmodulin sensitive enzyme is stimulated by micromolar levels of calcium and is inhibited by magnesium (Brostrom, Brostrom & Wolff,1977). Significantly this enzyme is also inhibited at high (millimolar) levels of calcium. At high magnesium to calcium ratios the insensitive enzyme will be active; the sensitive

enzyme is activated at high calcium to magnesium ratios. At higher calcium levels both enzymes are inactive. This ionic dualism may apply uniquely to brain; calmodulin sensitive cyclase systems exist in other tissues but their ionic dependencies have not been defined.

Thus adenyl cyclase activity is clearly maintained by guanyl nucleotides and modified by the ambient divalent cation environment. Production of the second major cyclic nucleotide is not, however, as clearly defined.

Formation of intracellular cyclic 3'5' GMP

Guanyl cyclase catalyses the production of cyclic 3'5' GMP from metal complexed GTP in a manganese-dependent reaction, (Hardman & Sutherland,1969; White, Aurbach & Carlson,1969). The enzyme occurs in both particulate and soluble fractions from most cells (Kimura & Murad, 1974). The ratio of soluble to particulate enzyme is a function of the cell type or its metabolic state (Sulakhe, Sulakhe, Leung, St. Louis & Hickie,1976; Kimura & Murad,1975; Gordius & Reutter,1975; Ichihara & Murad,1979). Particulate and soluble enzymes are not identical since the latter fails to react with antibody prepared against particulate enzymes (Garbers, Chrisman & Hardman,1978). Although a variety of agents elevate cyclic GMP levels in the intact cells these same compounds have no effect on guanyl cyclase activity in homogenates (Kimura & Murad,1974). This suggests hormone receptor interaction indirectly activates guanyl cyclase. It is evident that soluble and particulate enzymes are independently regulated. A variety of mechanisms may serve to couple receptor binding to the activation of both cyclase enzymes (Murad, Mittal, Arnold, Ichihara, Braughter & El-Zayat,1978). The inhibition of soluble activity by physiological levels of ATP suggests that the particulate activity is the major cellular enzyme (Kimura & Murad,1974). Figure 1:4 lists a range of treatments capable of elevating intracellular cyclic GMP levels, all these agents require the presence of extracellular calcium to exert this effect.

Figure (1:4)

Agents capable of elevating intracellular cyclic GMP

Stimulant	Reference
Cholinergic agonists	Schultz & Hardman,1975.
α-Adrenergic agonists	Schultz & Hardman,1974.
Histamine	Van Sande, Decoster & Dumont,1975.
Potassium induced depolarisation	Butcher,1975.
Calcium ionophore,A23187	Ferrendelli, Rubin & Kinscherf,1976.

Increments in the extracellular calcium concentration itself may elevate cyclic GMP (Clyman, Blacksin, Sandler, Manganiello & Vaughan,1975). The agents listed in Figure 1:4 are all believed to induce cell activation by elevation of intracellular calcium and this may be the coupling mechanism between stimulant and cyclase, (Schultz, Hardman, Schultz, Baird & Sutherland,1973; Schultz, Hardman, Hurwitz & Sutherland,1973). Soluble guanyl cyclase activity is also stimulated by elevated ionized calcium. concentrations in the presence of sub-saturating manganese concentrations. Calcium may replace manganese in the metal-GTP substrate complexes liberating free manganese ions which would then activate the enzyme (Garbers, Chrisman & Hardman,1978). However, the high concentrations of calcium required suggest it is not a normal regulatory mechanism. Particulate enzyme activity at this high calcium concentration appears to be inhibited (Wallace & Pastan,1976), although at physiologically significant micromolar levels this enzyme is stimulated in a variety of tissues (Wallace & Pastan,1976; Böhme, Graf & Schultz,1978; Bartalfi, Breakfield & Greenyard, 1978). Powerful oxidising agents, which may generate either nitrous oxide or hydroxyl free radical, reversibly activate both forms of guanyl cyclase (Murad, Mittal, Arnold, Ichihara, Braughler & El-Zayat,1978). Free radical activation of guanyl cyclase reverses the stimulatory effect of calcium on the enzyme, which become magnesium sensitive (Bartalfie et al, 1978; Murad, et al,1978). Physiological production of free radicals is a significant feature of fatty acid metabolism (Egan, Paxton & Kuehl,1976), and certainly fatty acids can elevate guanyl cyclase activity in homogenates (Garbers, et al,1978), or in intact cells (Wallace & Pastan,1976). Thus in addition to calcium ions free radicals may also serve as a second link in cyclase activation. To date no role of intracellular calcium in free radical generation has been described, although any effect of calcium upon

the redox potential of the cell may influence radical activity (Goldberg, Graff, Haddox, Stephenson, Glass & Moser,1978). Thus calcium and free radicals seem to be the most likely candidates for the physiological control of cyclase activity, but whether they act in concert or independently is still in question.

Intracellular actions of cyclic nucleotides

Alterations in protein activity by the enzymatic transfer of terminal phosphates from ATP to specific amino acid residues is mediated by three protein kinase systems. One is cyclic nucleotide independent and may be activated by intracellular calcium, either directly or via the calmodulin activator (Joos & Anderer,1979; Nishizuka, Takai, Kishimoto, Hashimoto, Inoue, Yamamoto, Criss & Kuroda, 1978). The remaining two kinases are cyclic AMP or cyclic GMP dependent (Kuo & Greengard,1969). The cyclic AMP dependent kinase (A-Kinase) can be detected in cytosolic, nuclear and plasma membrane fractions (Uno, Ueda & Greengard,1976; Palmer, Castagna & Walsh,1974; Grant, Breithaupt & Cunningham,1979). It occurs in two isoenzyme forms, Peaks I & II signifying their elution positions (Reimann, Walsh & Krebs,1971; Corbin, Keely & Park,1975). Both isoenzyme forms contain two regulatory (R) and two catalytic (C) sub-units giving the general formula R_2C_2 (Brostrom, Reimann, Walsh & Krebs,1970; Gill & Garren, 1970). The two isoenzymes respond to physiological levels of adenyl cyclase stimulants (Lee, Radloff, Schweppe & Jungmann,1976; Corbin, Keely, Soderling & Park,1975). Regulatory sub-units contain cyclic AMP bindings sites whilst catalytic units contain both protein recognition and phosphorylation sites (Brostrom, Corbin, King & Krebs,1971; Armstrong, Kondo & Kaiser,1979). The holoenzyme possesses little catalytic activity but cyclic AMP binding to the regulatory sub-units reveals latent activity by dissociating the regulatory and catalytic sub-units (Brostrom, et al, 1971).

Figure (1 5)

General reaction for cyclic AMP activation of protein kinase activity

$$R_2C_2 + 2\ {}^{c}AMP \qquad R_2{}^{c}AMP_2 + 2C$$

The free catalytic units are reported to translocate within the cell but this is disputed (Zick, Cesta & Shaltiel,1979). Although both isoenzyme catalytic sub-units are identical the two species of regulatory sub-units fail to cross react to specific antisera (Corbin, Keely & Park,1975; Rubin, Ehrlichman & Rosen,1972; Beavo, Bechtel & Krebs,1975). The intracellular levels of isoenzyme forms are therefore dependent upon the ratio of regulatory sub-units (Yamamura, Nishiyamoto & Shimomura,1973). The action of free catalytic fragments is minimised by the binding of an inhibitory protein to them. (Walsh, Ashby, Gonzales, Calkins, Fisher & Krebs,1971). Although catalytic units complexed to inhibitor protein are not detected in intact cells sufficient inhibitor protein is present to remove all basal activity (Ashby & Walsh,1972; Beavo, Bechtel & Krebs,1974). This ensures maximum sensitivity to small increments in cyclic AMP when adenyl cyclase is stimulated (Beavo, Bechtel & Krebs,1974). Cellular content of inhibitor protein is flexible, and in some cell types may regulate the level of kinase activity (Ashby & Walsh,1972; Costa,1978).

Under basal, non-stimulated, conditions dissociation of the Peak I enzyme is reduced by the binding of MgATP to allosteric sites on the holoenzyme. This decreases the affinity of the receptor units for cyclic AMP (Bechtel & Beavo,1974; Haddox, Newton, Hartle & Goldberg,1972). In adipose tissue activation of the Peak I enzyme can be caused by levels of adrenaline which provoke increases in cyclic AMP undetectable by modern techniques, testifying to the enhanced sensitivity conferred by inhibitor protein and MgATP (Corbin, Keely, Soderling & Park,1975). Because the Peak II enzyme contains different regulatory sub-units the response to MgATP also differs. Thus binding of MgATP induces an autophosphorylation which in turn increases the affinity for cyclic AMP (Rosen & Ehrlichman,1975; Ehrlichman, Rosenfield & Rosen,1974; Maeno, Reyes,

Ueda, Rudolph & Greengard, 1974). Two different cyclic AMP affinities are observed in normal Peak II enzyme, depending on whether MgATP is bound (Knight, 1975). These responses of Peak I and Peak II to MgATP create differential sensitivity to elevations in cyclic AMP.

Both isoenzymes are activated at a time which coincides with the initiation of DNA and histone synthesis during the cell cycle in a variety of tissues (Johnson & Hadden, 1975; Cross & Ord, 1971; Ord & Stocken, 1969; Russell,1978). In synchronised cells the relative levels of Peaks I and II vary throughout the cycle suggesting they activate different processes (Costa, 1978; Russell,1978). Protein phosphorylation also plays an important role in the regulation of cellular cation content. Kinase sensitive intrinsic membrane proteins regulate cation permeability in rat sarcolemma and endoplasmic reticulum (Tada, Ohmori, Yamada, & Abe, 1979; Ferguson & Twite, 1974; Tada, Kirchberger & Katz, 1975), and the monovalent ion transport system of avian erythrocytes (Gardner, Klaevemann, Bilezikian & Aurbach, 1974; Gardner, Mensch, Kiino & Aurbach, 1975). The significance of this action in cell activation is discussed in Section 2.

The second cyclic nucleotide dependent protein kinase, cyclic GMP (or G-Kinase), is also widely distributed (Kuo & Greengard, 1970;Donnelly, Kuo, Miyamoto & Greengard, 1973; Kuo & Kuo, 1977). G-Kinase resembles A-Kinase in that it contains two regulatory and catalytic sub-units,however,cyclic GMP binding does not induce dissociation of the holoenzyme (Gill, Walton, & Sperry, 1977). G-Kinase activity appears to depend upon the additional presence of a modulator protein in most tissues (Kuo, Kuo, Shoji, Davis, Seery & Donnelly, 1976). In bovine brain the enzyme appears independent of modulator but the purity of the preparation is in doubt (Takai, Nishiyama, Yamamura & Nishizuka, 1975). The concentration of modulator is increased in alloxan diabetic rats (Kuo,1975) which may serve to increase the .....

sensitivity of the kinase to cyclic GMP. The extent of protein phosphorylation induced by the kinase enzymes will be limited by dephosphorylation mechanisms and by reductions in cyclic nucleotide concentrations. This may occur either by cyclic nucleotide extrusion or by catabolic processes within the cell.

Cyclic nucleotide extrusion makes a significant contribution to the intracellular homeostasis of these modecules in lower phyla (Makman & Sutherland,1965; Konijn,1972). Mammalian cells also extrude substantial quantities of cyclic AMP and GMP (Davoren & Sutherland,1963; Franklin & Foster,1973; Broadus, Hardman. Kaminsky, Bull Sutherland & Liddle,1971). Their extrusion may be maintained against a concentration gradient, and for cyclic AMP at least, a specific carrier is indicated (Clark, Su, Ortmann Cubeddu, Johnson & Perkins,1975; De Vellis, Inglish & Brooker,1974). Extrusion is directly proportional to the intracellular content, consequently adenyl cyclase stimulants promote extrusion in a variety of tissues (Hardman, Davis & Sutherland,1969; Kowal & Harano,1974). Thus the increase in cyclic AMP formation which follows PGE_1 stimulation of WI38 fibroblasts is accompanied by increased cyclic nucleotide extrusion. Long after the restoration of basal cyclic AMP levels the enhanced extrusion of cyclic AMP continues (Kelly & Butcher,1974). Thus extrusion, along with refractoriness, receptor density regulation, and catabolism helps to regulate the size and duration of the second messenger response.

The only known catabolic pathway for cyclic nucleotides is irreversible hydrolysis to their respective 5' - nucleotide monophosphates by selective phosphodiesterase enzymes (Butcher & Sutherland,1962; Russell, Thompson, Schneider & Appleman,1972; Thompson, Little & Williams,1973). In most mammalian tissues hydrolytic activity is the result of multiple particulate and soluble enzyme forms. Each isoenzyme possesses unique physical and

chemical properties. Several isoenzyme forms have been isolated, however, some undoubtedly arise as preparative artifacts (Sheppard & Tsien,1975; Goren & Rosen,1972). It is suggested that interconversions of a single enzyme species could account for the various physico-chemical characteristics (Pichard & Cheung,1976). However, three consistently isolated partly-purified enzymes retain different kinetic behaviours and substrate affinities (Wells & Hardman,1977). Their in vitro properties are discussed below, but it is important to note that these do not necessarily indicate their in vivo function.

In the particulate fraction of cellular homogenates cyclic AMP hydrolysis is predominantly caused by a high affinity (low Km) enzyme. Although cyclic GMP at high concentrations can serve as substrate the enzyme is specific for cyclic AMP at normal nucleotide levels (Thompson & Appleman,1971; Russel, Terasaki & Appleman,1973). In the majority of mammalian tissues neither cyclic GMP nor calcium regulate this enzyme. However, enzyme from rat kidney is activated by both agents (Van Inwegen & Pledger,1976:, Van Inwegen, Swafford, Strado & Thompson,1977). The proximity of this high affinity cyclic AMP phosphodiesterase to adenyl cyclase suggests it may be the primary regulator of cyclic AMP concentration Indeed in many circumstances where hormones increase adenyl cyclase activity the high affinity phosphodiesterase activity also increases (Thompson & Strada,1978). Some agents which have no association with adenyl cyclase may also stimulate this low Km enzyme suggesting that elevation of cyclic AMP is not the sole stimulus for phosphodiesterase activity (Fain,1975). In fat cells both lipolytic and antilipolytic agents increase phosphodiesterase activity but only the lipolytic stimulants elevate cyclic AMP (Allan & Sneyd,1975; Pawlson, Lowell-Smith, Manganiello & Vaughan,1974; Loten & Sneyd,1970; Desai, Marian & Appleman,

1976). In some instances where cyclic AMP is not involved in phosphodiesterase activation new protein synthesis is a necessary prelude to activation (Thompson & Strada,1978). However, examples also exist of steroid hormone inhibition of phosphodiesterase which also involves new protein formation (Gardner, Thompson & Stancel,1976). Thus the particulate form of phosphodiesterase not only appears to restore elevated cyclic AMP levels to normal but may also reduce basal levels.

The hydrolytic activity in the soluble fraction is the product of two isoenzymes. One form has a low affinity for both nucleotides (Beavo, Hardman & Sutherland,1971). Nevertheless in crude homogenates it may hydrolyse significant quantities of both nucleotides and thus limit elevations in their concentrations. Physiological levels of cyclic GMP may direct this enzyme towards cyclic AMP hydrolysis (Beavo, Hardman & Sutherland,1971;,Terasaki & Appleman,1975). It is possible that the elevation of intracellular cyclic GMP could further increase the cyclic GMP / cyclic AMP ratio by this effect. The second soluble isoenzyme is activated by micromolar concentrations of intracellular calcium in the presence of the calcium binding activator calmodulin (Cheung,1970; Kakiuchi, Yamazaki & Nakajima,1970; Teo, Wang & Wang,1973; Teo & Wang, 1973). In whole cells this effect may be mimicked by large extracellular calcium concentrations; the stimulation is proportional to the extracellular calcium content (Namm, Mayer & Maltbie,1968; Harary, Renaud, Sato & Wallace,1976). In guinea pig brain cyclic AMP hydrolysis is inhibited by the EGTA chelation of calcium concentrations (Schultz,1975). These abnormal treatments of intact cells suggest that intracellular calcium is a primary regulator of cyclic AMP hydrolysis. However, partly purified soluble enzyme preparations indicate that cyclic GMP may be the preferred substrate. Although the enzyme possesses a higher Vmax for

cyclic AMP (Goren & Rosen,1972; Ho, Wirch, Stevens & Wang,1977; Brostrom & Wolff,1976; Morrill,Thompson & Stellwagen,1979), the reported affinity for cyclic GMP is approximately ten times greater (Uzunov, Gnegy, Revuelta & Costa,1976; Ho, Wirch, Stevens & Wang,1977). Indeed intracellular cyclic GMP concentrations seem to be inversely correlated with the activity of the soluble diesterase enzyme (Weiss & Geenberg,1975). This suggests that under physiological conditions cyclic GMP is the preferred substrate. The similar cytosolic distribution of both guanyl cyclase and the phosphodiesterase would tend to confirm this. Whatever the physiological consequences of the regulatory mechanisms proposed above the role of calcium in activating some cyclase and phosphodiesterase activities suggests the ion may play a significant role in ensuring that hormonally induced elevations in cyclic nucleotides are self regulating. Since these actions of calcium are in part mediated by the calcium binding protein calmodulin its actions are discussed below.

1.4. The calcium-dependent regulatory protein, Calmodulin

The activity of a variety of intracellular enzymes is modified by changes in cytosolic calcium. The recent discovery of a widely distributed calcium-binding protein, Calmodulin, able to interact with many calcium-sensitive proteins has shown how it is possible for calcium to influence so many diverse enzyme activities (Figure 1.6; Means & Dedman, 1980). Calmodulin has an affinity for calcium approaching the physiological free calcium concentration of cytosol (Dedman, Potter, Jackson, Johnson & Means, 1977). Thus more properly it may be considered as a cytosolic "calcium receptor".

Fig.1.6. Calmodulin and calcium-sensitive cell processes

Adenyl cyclase	Brostrom, Huang, Breckenridge & Wolff,1975
Soluble cyclic nucleotide phosphodiesterase	Brostrom et al., 1975; Cheung, 1970
Skeletal muscle actomyosin ATPase	Amphlett, Vanaman & Perry, 1976
Myosin light kinase	Teshima & Kakiuchi, 1974
Phosphorylase kinase	Andersen, Osborn & Weber, 1978
Synaptic membrane phosphorylation	Grab, Berzins, Cohen & Siekevitz, 1979
Microtubule assembly	Welsch, Dedman, Brinkley & Means, 1978
Erythrocyte membrane Ca/Mg activated ATPase	Gopinath & Vincenzi, 1977
Pre-synaptic nerve terminal phosphorylation	Grab et al., 1979
Glycogen synthetase phosphorylation	Srivastava, Waisman, Brostrom & Soderling, 1979

Physico-chemical analysis of highly purified Calmodulin has revealed three equivalent and one non-equivalent binding sites for calcium or magnesium (Vanaman & Watterson, 1976; Yerna, Hartshorne & Goldman, 1979). The different divalent cation affinities of these two classes of binding sites ensures that all four sites are occupied by magnesium at basal intracellular calcium and magnesium levels. In such a Mg_3Mg_1

configuration the protein is inactive. A micromolar elevation in the ambient calcium concentration will substitute calcium for magnesium at one or more of the three lower affinity sites (Brostrom, Brostrom, Breckenridge & Wolff, 1979). Such calcium/magnesium-bound Calmodulin configurations are active and will interact with the Calmodulin-binding portion of sensitive enzymes. This interaction will serve to activate the enzyme in a calcium and calmodulin dependent manner. A further increase in calcium within the cell will result in a Ca_3Ca_1 configuration which is inactive. Thus Calmodulin serves as a protein activator only in response to physiological elevations in calcium (Wang & Desai, 1976). Thus it is apparent that the calcium/calmodulin complex will be able to modify intracellular processes directly (or indirectly via cyclic nucleotide changes). This will ensure the co-ordinated transfer of extracellular information to a variety of extracellular processes.

Although calcium metabolism has been considered in isolation other cations are important for the maintenance, and indeed manipulation, of the cytosolic calcium content. Perturbation of any single species will induce complementary changes in the other members of an inter-linked ionic network. Thus changes in cytosolic calcium will be induced by, or accompanied by, altered transmembrane distributions of monovalent cations. Indeed, in a variety of tissues, stimulation of differentiated cellular function due to a rise in cytosolic calcium is associated with such changes in monovalent ion balance. In muscular contraction and cellular secretion it is evident that calcium furnishes the

link between stimulus and activation. The present study is intended to establish potential similarities between these stimulus-activation events and the mitotic recruitment. To enable such a comparison, the next section first reviews intracellular monovalent cation metabolism and then considers stimulus-secretion and excitation-contraction coupled events.

2.1. Intracellular homeostasis of monovalent cations

The sodium potassium ion activated ATPase

The high potassium, low sodium, interior of mammalian cells is partitioned from the low potassium, high sodium, external environment by the semi-permeable plasma membrane. This unequal distribution, maintained by the expenditure of metabolic energy, is essential for sustaining the transmembrane potential, for the functioning of potassium-dependent cytosolic processes and for the maintenance of a low concentration of free calcium ions in the cytosol (Suelter, 1970 and see Section 1). Because the existing transmembrane monovalent cationic gradients produce passive ionic traffic, considerable active transport in the reverse direction is essential to maintain the necessary internal environment. The mechanism of the active transport is the sodium and potassium ion-activated ATPase (EC 3.6.1.3)(Na/K ATPase) present in the plasma membrane (Skou, 1957). This enzymic ion pump exchanges intracellular sodium for extracellular potassium. Under normal conditions this is a magnesium-dependent reaction resulting from the hydrolysis of ATP (Skou, 1957; Glynn, 1962). Evidence linking the ion pumping mechanism with the hydrolytic reaction may be summarized thus:-

1. Hydrolysis and pump activity exhibit identical ionic requirements (Abeles, 1969).
2. Optimal substrate and cation concentrations produce maximal hydrolytic and pump activity (Post, Merritt, Kinsoluing & Albright, 1960; Glynn, 1962).
3. Cardiac glycosides demonstrate identical structural and ionic requirements for the inhibition of both pump and ATPase hydrolytic activity (Abeles, 1969; Lindenmayer, 1976).

4. Both activities may be donated to artificial lipid vesicles by the addition of partially purified ATPase protein (Hilden & Hokin, 1975). In such restored vesicles three sodium ions are exchanged for two potassium ions when each ATP molecule is hydrolyzed (Goldin & Tong, 1975).

The overall reaction may be summarized thus:-

$$ATP + 3Na^+_i + 2K^+_o \xrightleftharpoons{Mg^{++}} 3Na^+_o + 2K^+_i + ADP \qquad (2.1)$$

o = outside cell membrane

i = inside cell membrane

During the ion-pumping process the major of the two constituent pump proteins becomes phosphorylated at a specific aspartic acid residue under the influence of magnesium and sodium ions (Nishigaki, Chen & Hokin, 1974). The second, smaller protein, is associated with the pump activity; antibody directed against this protein inhibits pumping (Jørgensen, 1974; Jean, Albers & Koval, 1975). Photoaffinity labelling with extracellular ouabain and binding with anti-catalytic antibody reveals that the enzyme does indeed span the membrane (Dahl & Hokin, 1974; Kyte, 1974; Ruoho & Kyte, 1974; Garay & Garrahan, 1973). The precise mechanism of ion exchange is unknown but phosphorylation, resulting in an active phospho-enzyme complex, initiates the reaction (Post, Kumes, Tobin, Orcutt & Sen, 1969).

$$E + P \xrightleftharpoons{Na^+\ Mg^{++}} E\ \ P \qquad (2.2)$$

This phosphorylation reaction accompanies the binding of sodium ions to each of three identical intracellular cation binding sites (Garay & Garrahan, 1973). Although these are selective for sodium the high internal potassium content ensures some competitive inhibition at each of these sites (Glynn, 1962; Simons, 1974). Thus, under basal

conditions active (3 Na^+ bound) and inactive (less than 3 Na^+ bound) forms of the enzyme are in equilibrium.

$$[E \cdot 3K^+] \rightleftharpoons [E \cdot 2K^+ Na^+] \rightleftharpoons [E \cdot K^+ 2Na^+] \rightleftharpoons [E \cdot 3Na^+] \text{ active} \quad (2.3)$$

Any change in the intracellular monovalent cation content will alter the proportion of active and inactive enzymes. Increased cytosolic sodium will serve to stimulate pumping whereas a raised concentration of potassium will reduce active ion transport.

The kinetics of ATP hydrolysis suggest the existence of a second phosphoenzyme complex, induced by potassium binding to two non-identical external cation binding sites (Post, Sen & Rosenthal, 1965; Lindenmayer & Schwartz, 1975). Although sodium may compete for these sites there does not appear to be a regulatory role per-se at the external face (Lindenmayer, Schwartz & Thompson, 1974). This may be because the level of extracellular sodium is not prone to wide fluctuations. The overall reaction may be summarized thus:-

$$E \overset{Na^+ Mg^{++}}{\rightleftharpoons} E \cdot Na_3 \rightharpoonup E_1P \overset{K^+}{\rightleftharpoons} E_2P \longrightarrow E + P \quad (2.4)$$

(After Fahn, Koval & Albers, 1966)

It must be noted that this represents only one of a series of proposed models for the pump function. It is however the only model accounting for the stoichiometry of the in-vitro enzyme.

The number and activity of pump enzyme units present at the cell membrane will also regulate ion pumping. Thus expression of latent enzyme, synthesis of new active enzyme, and the recycling of denatured or inhibited enzyme will all contribute to overall pumping capacity (Cook, Will, Proctor & Brake, 1976; Averdunk & Lauf, 1975; Vaughan & Cook, 1972). Intracellular ionized calcium is able to modify the activity of the pump when present in broken membrane preparations.

Millimolar calcium levels inhibit activity by competing for the magnesium activation sites present on the catalytic protein (Tobin, Akera, Baskin & Brody, 1976). Even allowing for an over-estimation of the free calcium content due to non-specific binding, this cannot be considered a physiological effect. Indeed, the dissociation constant for calcium at this binding site is higher than the free cytosolic calcium concentration (Lindenmayer & Schwartz, 1975). At physiological calcium levels calcium can still inhibit the enzyme but this action is due to an elevation in the sodium threshold necessary to activate the phosphorylation of the enzyme. This is believed to be due to competitive inhibition between sodium and calcium at the cation binding sites (Lindenmayer & Schwartz, 1975). At physiological calcium levels approximately one per cent of the pump sites would be inhibited. Therefore a slight increase in cytoplasmic calcium would be sufficient to perturb pump function. Eventually the new sodium threshold for activation of the pump would be exceeded due to the unrestricted passive sodium influx.

Although membrane lipids are required for normal enzyme activity, they probably non-specifically maintain the conformation of active sites (Hilden & Hokin, 1976; Grisham & Barnett, 1972). Pumping may be secondarily modified due to lipid-mediated changes in membrane permeability (Kimelberg & Paphadjopoulais, 1974). Lipid ratios favouring membrane fluidity support a higher enzyme activity due to enhanced passive sodium and potassium movements (Chen, Heiniger & Kandutsch, 1978; Bakker Grunwald & Sinenisky, 1979). Conversely a high sterol content reduces passive permeability and lowers pump action (Chen et al., 1978).

A number of hormones are also known to influence pump activity although their precise action is unknown. Activation after cholinergic stimulation of intact cells has been variously attributed to a direct effect of the hormone (Kometiani, 1978) or to cyclic GMP induced by the cholinergic receptor (Stewart, Sax, Funk & Sen, 1979). Certainly beta-adrenergic induced activation is not mediated by cyclic AMP (Flatman & Clausen, 1979). Indeed, although cyclic AMP directed protein kinase phosphorylation of the pump is observed (Dowd & Schwartz, 1975) other investigators find exogenous cyclic-AMP, albeit at high concentrations, inhibits the enzyme (Braughler & Corder, 1978; Limas, Notargiacomo & Cohn, 1973). In addition to direct hormone enhancement the pump may be activated as a result of hormone induced changes in membrane permeability (Putney & Parod, 1978). The oestradiol provoked increases in ATPase activity of rat hypophysis and vascular smooth muscle is due to an initial change in membrane permeability (Knudsen, 1976; Harder & Coulson, 1979).

2.2. Regulation of membrane permeability

The large transmembrane monovalent ion gradients ensure that any chemically or electrically provoked alterations in membrane potential will result in the rapid movement of monovalent cations. The speed of these potential dependent transmembrane currents, their activation and inactivation, and their cation selectivity cannot be accounted for by simple phase-partition, carrier-mediation or displacement of intramembrane ions (Armstrong, 1974; Narahashi, 1974; Parsegian, 1969; Kramer, 1976). Studies of the electrically excitable squid axon suggest that a series of transmembrane, hydrophilic, ion-selective channels exist to carry the ions (Verveen, De Felice,1974; Keynes, Bezanilla, Rojas, Taylor,1975).

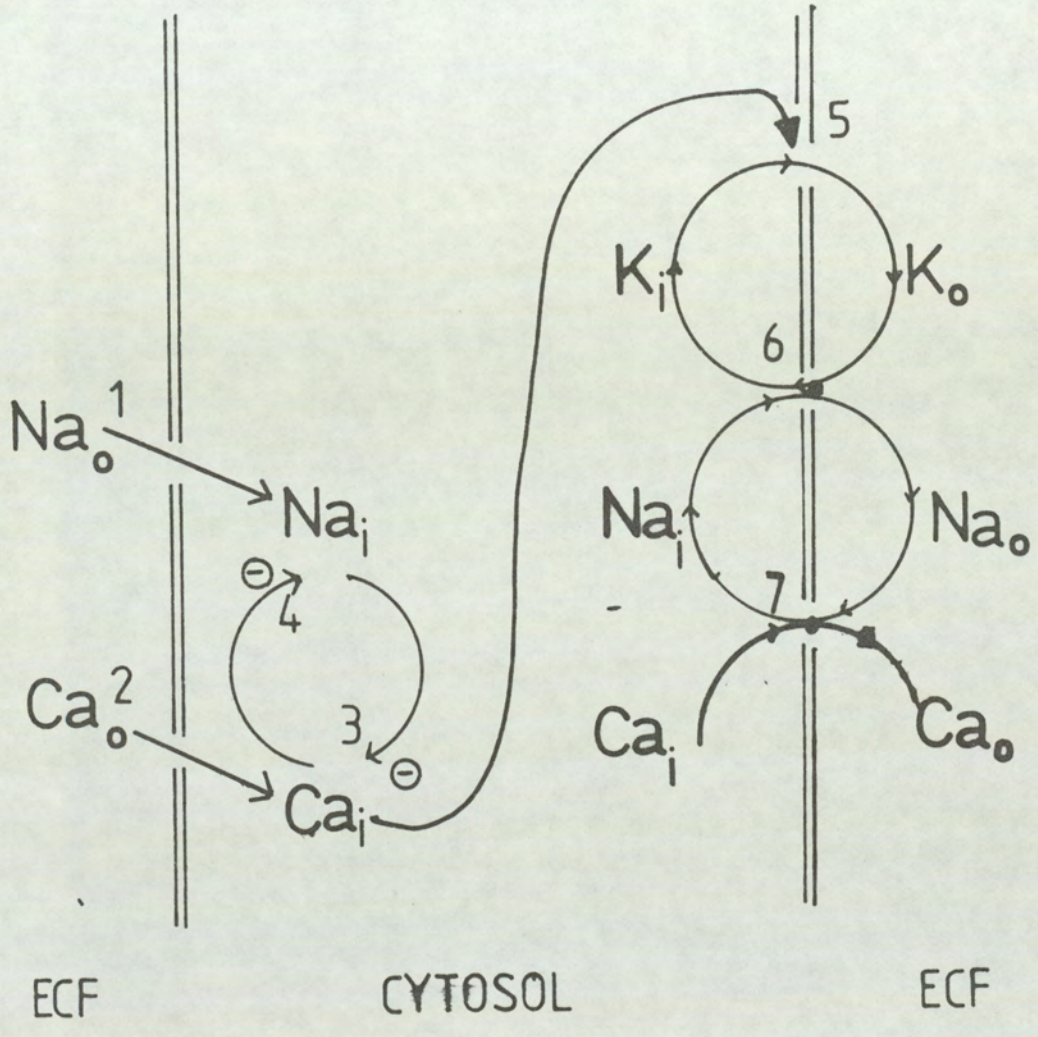
The number of channels is believed to be constant, alterations in ionic fluxes being dependent upon the assembly of channel subunits or the gating of hitherto inactive, preformed channels (Ehrenstein, Lecar & Latorre, 1978). As hydrolytic enzymes selectively destroy an apparent gating function of treated membranes, the latter model is favoured (Rojas & Armstrong, 1971).

The action potential, and associated monovalent ion movements, induced by excitation of squid axon is believed to represent a universal system of potential-dependent membrane permeability regulation. The initial, and major, depolarizing current is carried into the axoplasm by a very fast, self-inactivating movement of sodium ions (INa), travelling through potential dependent sodium channels. The sodium current is followed by a slow, secondary, inward current (Isi). This is carried predominantly by calcium ions (Hodgkin & Huxley, 1952). Some sodium may enter via these latter channels but it is of little significance to the depolarization (Connor, 1977). In some excitable tissue Isi is a major depolarizing current. Prevention of calcium entry by antagonists such as lanthanum and cobalt prohibits the depolarization (Meech 1974; Hagiwara, 1973). In squid axon, and many other tissues so far studied, the two inward currents are succeeded by an outwardly directed current (Io) carried by intracellular potassium ions (Krnjevic & Lisiewicz, 1972; Hodgkin & Huxley, 1952). This Io current causes membrane hyperpolarization which terminates the excitability created by the initial depolarization. The resting membrane potential is subsequently restored by the membrane Na/K ATPase (see above). Induction of Io appears to be related directly to a rise in cytosolic calcium ions (Meech & Sturmwasser, 1970; Meech, 1972). It has been suggested that the passage of calcium through the slow channels converts them

into a potassium selective configuration (Hofmeier & Lux,1979). However, during potassium egress the slow calcium channels remain functional (DiFrancesco & McNaughton, 1979). Moreover, direct injection (iontophoresis) of calcium can elicit potassium conductivity without prior use of the slow calcium channels (Isenberg 1975; Meech & Sturmwasser 1970). The general applicability of calcium induced potassium conductance has recently been reviewed by Meech who cites nine excitable tissues where the effect occurs (Meech 1976).

In non-excitable tissues the calcium-activated efflux of potassium is also well established. Passive elevation of calcium within erythrocytes induces an outward potassium current (Romero & Whittam 1971; Lew & Ferreria, 1976) resulting in membrane hyperpolarization (Lassen, Pape & Vestergaard-Bogind 1976). It would appear that this widely distributed ionic phenomenon is essential for any rapid intracellular signalling system employing an increased cytosolic calcium concentration as a secondary messenger. Irrespective of the initial signal the stimulatory calcium concentration may be restored by potassium efflux induced hyperpolarization of the membrane and the subsequent Na/K ATPase activation (Fig.2.1). Mechanisms such as these are believed to act in both secretory and contractile tissues, where intracellular calcium provides the link between extracellular stimuli and the cell activation. These events are discussed in detail in the following section.

Fig. 2.1 Ionic changes following cellular excitation



The initial stimulus induces either membrane depolarization (1) or direct calcium influx (2). The former will inhibit calcium extrusion by restricting Ca/Na exchange (3), whilst the latter restricts sodium extrusion via the ion pump (4). The resultant increase in free calcium is halted by initiation of the potassium conductance (5). The reduced cytosolic potassium will allow the Na/K ATPase to function. (6) This will result in restoration of the transmembrane sodium gradient which will in turn reduce the calcium content through Na/Ca exchange (7).

2.3. Stimulus-secretion coupling.

Considerable evidence indicates that intracellular calcium pools provide the necessary coupling between secretagogue and secretion in exocrine and endocrine tissues (Douglas, 1968; Del Castillo & Stark, 1952). Both vesicular and non-vesicular secretions are enhanced by a rise in the cytosolic calcium content.

Exocrine parotid

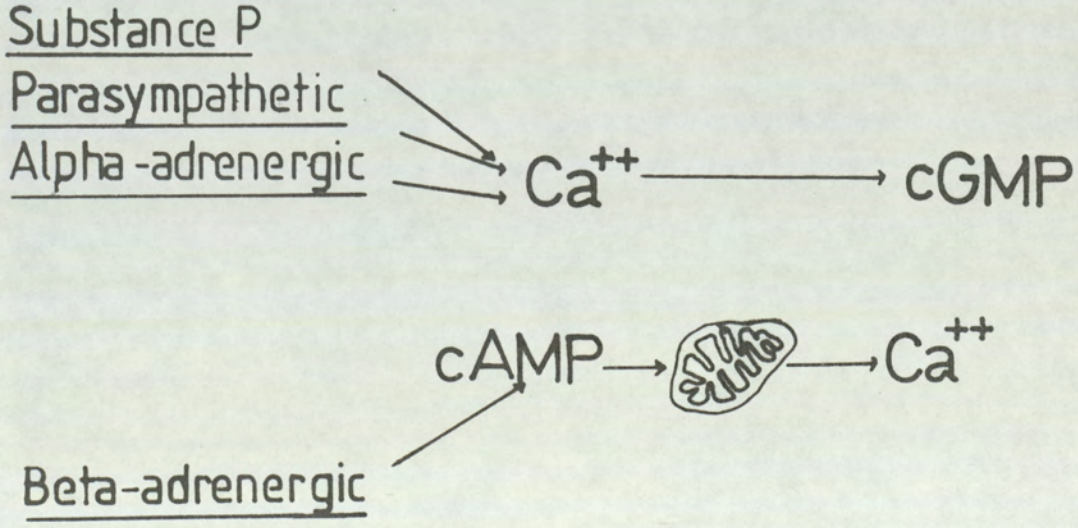
Ion and fluid release from the parotid is enhanced by a rise in cytosolic calcium which acts directly upon the membrane to modify its permeability. The secretory response is evoked by substance P, parasympathetic and alpha-adrenergic sympathetic stimulation (Rudich & Butcher, 1976; Selinger, Batzri, Eimerl & Schramm, 1973; Douglas & Poisner, 1962). All these agents furnish the required calcium ions by increasing calcium entry from the extracellular fluid (Douglas & Poisner, 1962; Putney, 1976; Butcher, Rudich, Emler & Nemerovski, 1976; Butcher, 1978). As predicted above, one consequence of this calcium influx is an enhanced potassium exit, this being the major electrolyte released from the parotid (Putney, 1976; Kanagasuntheram & Randle, 1976). Two phases of potassium efflux are observed from the hormone stimulated parotid. The first is a rapidly inactivated flux, independent of exogenous calcium. The second is dependent upon extracellular calcium and is a sustained efflux providing the majority of the released ions (Pedersen & Petersen, 1973; Peterson & Pedersen, 1974). The initial, essentially extracellular calcium-independent, efflux is believed to be mediated by secretagogue induced transfer of calcium from the membrane to the cytosol (Putney, 1977). All three classes of secretory hormones have been shown to displace this pool by a receptor-mediated event (Marier, Putney & Van de Walle, 1978). Once calcium has entered the

cell from this membrane pool it must be replaced before a further such secretion can be induced (Putney, 1977). The sustained efflux of potassium corresponds with the sustained calcium influx, which is presumed to occur via calcium channels (Putney, 1977; Marier et al. 1978). Secretagogue action is also associated with the entry of extracellular sodium ions into the parotid (Putney & Parod, 1978). In the rat lacrimal gland, whose response appears to be identical to the parotid, the sodium influx occurs through what are thought to be potential-dependent channels (Tangkrisananinont & Pholpramool,1979). The rise in cytosolic sodium may account for the calcium influx, which in turn will initiate potassium efflux and the subsequent Na/K ATPase activation (Putney & Parod, 1978; Roberts & Petersen, 1978). Cyclic GMP has been proposed as a link in the secretory stimulation, indeed alpha-adrenergic and cholinergic agents enhance the formation of parotid cyclic GMP (Butcher, McBride & Rudich, 1976; Rudich & Butcher, 1976). However, substance P does not alter cellular cyclic GMP whilst activating secretion (Rudich & Butcher, 1976). Significantly, inhibition of parotid phosphodiesterase, leading to a cyclic GMP rise, without any influence on calcium, fails to initiate secretion (Butcher et al. 1976). It is reasonable to assume that coupling between stimulus and secretion is provided simply by calcium and that cyclic GMP may or may not influence the coupling.

Although the release of parotid amylase does not depend upon the influx of extracellular calcium the ion is required in the external environment to maintain sufficient cytosolic calcium for the beta-adrenergic induced vesicular secretion (Butcher, 1978; Guidotti & Costa, 1973). The cyclic AMP generated by beta-adrenergic agonist-receptor interaction induces secretion by mobilizing internal calcium

reservoirs, presumably mitochondrial or endoplasmic reticulum (Kanagausuntheren & Randle, 1976). Other adenylate-cyclase stimulants, or even exogenous cyclic AMP can substitute for the beta-adrenergic stimulus (Schramm & Selinger, 1975). It would appear that the cyclic AMP so generated, as well as the calcium released, is essential for the release of enzyme, as ionophoretic elevations of cytosolic calcium only submaximally release enzyme in the absence of cyclic AMP. Upon the addition of cyclic AMP producing stimulants the enzyme release due to ionophore was greatly potentiated (Selinger, Batzri, Eimerl & Schramm, 1973; Butcher, 1978). This partial requirement for cyclic AMP may provide a necessary intracellular device, allowing the intracellular discrimination between secretory stimulii requiring vesicular or non-vesicular responses (fluid and electrolyte). The two responses are, however, separate in their calcium source and their cyclic AMP dependency. The following model may be applied to such a system whereby a common coupling factor can independently activate two systems (Figure 2.2).

Figure 2.2. Stimulus secretion coupling of the exocrine parotid.



Exocrine pancreas.

Since cholinergic and alpha-adrenergic stimulation is coupled to the vesicular secretion of pancreatic enzymes, rather than fluid release as in the parotid, a different mechanism must govern pancreatic fluid release. Evidence suggests that the release of fluid, rich in sodium and bicarbonate ions from the pancreatic ductal cells is evoked by secretin, acting specifically through adenylate cyclase (Rutten, De Pont & Bonting, 1972). Thus, the action of secretin is reproduced by artifically increased cyclic AMP concentrations (Smith & Case, 1975; Kempen, DePont & Bonting, 1975). The precise mechanism of cyclic AMP mediated fluid release is unknown. However, when pancreatic enzymes are released, the raised cytosolic calcium is also associated with fluid secretion (Schulz, Milutinovic & Heil, 1978; Ueda & Petersen, 1977; Folsh & Wormsley, 1973). The raised cytosolic calcium concentration, probably provoked by the cyclic AMP generated by secretin, may well be inducing the fluid release (Schulz et al. 1978). In the duck salt gland the induced sodium influx has been attributed to a cyclic GMP activation of the Na/K ATPase (Stewart et al. 1979). Thus, it is possible that calcium activates guanylate cyclase, which in turn acts as a quaternary messenger. A similar, four-step mechanism (stimulant-cyclic AMP-calcium-cyclic GMP), may also account for the mitogenic response of parotid tissue to a beta-adrenergic stimulus (see Section 3).

As mentioned above, pancreatic enzyme release may be mediated by three groups of hormones, namely cholinergic agonists, cholecystokinin/pancreozymin and related hormones, and by the bombesin family of peptides. Each of these groups use a different receptor system to activate pancreatic enzyme release (Hokin, 1966; Kanno, 1972; Petersen & Philpott, 1975; Ueda & Petersen, 1977; Philpott & Petersen, 1979). Although

all these agonists require extracellular calcium to stimulate secretion, evidence suggesting this calcium enters the cell is conflicting (see Kanno, 1972; Philpott & Petersen, 1979). Whilst no secretagogue stimulated calcium influx is demonstrable in pancreatic slices (Case & Clausen, 1973), preparations containing only hormone responsive cells do show enhanced calcium uptake (Schulz, 1975; Schulz Milutinovic & Heil, 1978). The actual stimulatory role of calcium is indisputable, an increase in the extracellular calcium concentration of the perfusate provokes calcium uptake and stimulates secretion by pancreatic slices (Schulz, 1975). The mechanism by which calcium activates enzyme release appears to be ion-promoted fusion of vesicles and the secretory apparatus (Creutz, Pazoles Pollard 1979). Thus, although both mechanisms in the pancreas are stimulated by calcium, they are associated with different calcium reservoirs. Enzyme release is dependent upon external ions whilst fluid release is believed to be mediated through cytosolic calcium. It would appear that two mechanisms of secretory stimulation exist, one using cyclic AMP and cytosolic calcium, whilst the other is provoked by cholinergic, alpha-adrenergic and similar stimulations and uses extracellular calcium. These two pathways may be coupled to either vesicular or non-vesicular secretions, depending upon the tissue.

The chromaffin cells of the rat adrenal medulla release catecholamines in response to cholinergic stimulation. The response requires extracellular calcium ions, which can be seen to enter the stimulated cells (Douglas & Rubin, 1961; Douglas & Poisner, 1962; Kidokoro, Ritchie & Hagiwara, 1979). That the raised cytosolic calcium is the secretory coupling factor can be seen when ionophoretic increases in cytosolic calcium trigger secretion (Lastowecka & Trifaro, 1974). The mechanism by which cholinergic agonists increase calcium uptake is through a potential dependent calcium influx (Stallcup, 1979)

1979; Kidokoro et al. 1979). It has been suggested that extracellular cyclic AMP may influence secretion, however, such effects are only observed at grossly elevated cyclic AMP concentrations (Peach, 1972; Poisner, 1973; Rahwan, Borowitz & Miya, 1973). There is a secretagogue induced increase in cyclic AMP content under normal conditions (Jaanus & Rubin, 1974). This increase does not coincide with secretion and merely serves to replenish depleted catecholamine stores by promoting new hormone synthesis (Guidotti, Kursowara, Chuang & Costa, 1975). This model of secretory action follows the general model proposed above, where cholinergic-extracellular calcium-intracellular calcium produce stimulation.

Unlike the above secretory events, protein release from mononuclear leucocytes is influenced by both cyclic nucleotides. Upon antigen addition to sensitized mast cells there is a rapid increase in calcium uptake, which proves to be the coupling agent necessary for the subsequent exocytosis (Mongar & Schild, 1958; Foreman & Mongar, 1972; Foreman, Mongar,& Gomperts, 1973). Similar changes occur when neutrophils encounter phagocytosable material (Ignarro, 1978; Smith, 1978). The rise in cytosolic calcium serves to promote cyclic GMP formation (Smith & Ignarro, 1975). This cyclic GMP may be essential for secretion as the addition of exogenous cyclic GMP stimulates secretion (Ignarro & George, 1974). In both mast cells and neutrophils exogenous cyclic AMP, or adenylate cyclase stimulants are able to inhibit calcium uptake and prevent secretion (Foreman, Hallet & Mongar, 1977; Ignarro, 1978). It is possible that cyclic GMP promotes, and that cyclic AMP inhibits, the stimulatory calcium influx, such a mechanism also exists in the lectin stimulated lymphocyte (Freedman, 1979). This dualistic action of cyclic nucleotides may be specific for mononuclear leucocytes.

However, in all the tissues detailed above the response to the secretagogue is coupled by a change in cytosolic calcium. In the exocrine pancreas and parotid the two secretions from each tissue are provoked from different calcium reservoirs, and may be associated with one or other cyclic nucleotide. In the mononuclear leucocyte the secretion is modified by both cyclic nucleotides acting in opposite directions.

2.4. Excitation- contraction coupling.

Unlike secretory tissues, which lack direct electrical excitability, muscular tissue is able to respond to both electrical and chemical stimulation. Contraction is initiated in all three classical muscle types by a rise in cytosolic calcium (Blinks, Prendergast & Allen, 1976; Solaro, Wise, Shiner & Briggs, 1974; Ruegg, 1971). By binding to troponin/tropomyosin units calcium de-represses the contraction-producing interaction between actin and myosin (Ebashi, Nonomura, Toyo-oka & Katayama, 1976). Thus, an increased cytosolic calcium concentration activates the contractile apparatus (Ebashi, 1972). As in secretory tissue, the coupling calcium may originate from either the extracellular or intracellular calcium stores. In skeletal muscle, the coupling calcium is provided exclusively from internal sources, whilst cardiac and smooth muscles are able to employ both reservoirs (Sandow, Krishna, Pagala & Sphicas, 1975; Ebashi & Endo, 1968; Langer, 1973).

Smooth muscle.

The heterogeneous nature of smooth muscle types prohibits generalisations concerning the coupling mechanisms. Depending upon the species and the location of the muscle either extracellular or intracellular calcium may provide the coupling between stimulus and contraction (Greenberg, Long & Diecke, 1973). As a rule of thumb, the calcium content of muscle is dependent upon the sarcoplasmic reticulum. Thus the

quantity of sarcoplasmic reticulum, and hence calcium, determines which pool of calcium is utilized (Devine, Somlyo & Somlyo, 1972). Smooth muscles such as guinea pig taenia coli, lacking a well defined sarcoplasmic reticulum, depend almost exclusively upon external calcium (Bülbring & Tomita, 1970). In this case the coupling calcium enters the sarcoplasm as the Isi current of depolarization (Anderson, Ramon, Snyder 1971). As no internal calcium store is available repeated action potentials may be necessary to activate contraction through calcium influx (Casteels & Raeymaekers, 1979).

In contrast to such forms of smooth muscle those possessing extensive sarcoplasmic reticulum do not exhibit significant Isi calcium currents (Keatinge, 1968; Mangel, Nelson, Connor & Prosser, 1979). Calcium necessary for the coupling is provided by potential-induced release of internal sarcoplasmic reticulum calcium stores (Mangel et al., 1979). In the extracellular calcium dependent form of smooth muscle the relaxation appears to require re-export of the triggering calcium via sodium/calcium exchange (Blaustein, 1977). For this to occur membrane hyperpolarization is necessary to re-establish the potential gradients essential for this exchange (Burgen & Spero, 1968). This hyperpolarization is caused by the calcium activated potassium conductance discussed in Section 2.2 above. In the somewhat unique guinea pig taenia coli the alpha-adrenergic induced relaxation is mediated by this calcium-induced hyperpolarization (Bülbring & Tomita, 1970). Whether cyclic GMP is involved in this process in unclear but there does appear to be activation of guanylate cyclase (Diamond, 1978). In the intracellular calcium-dependent smooth muscle the sarcoplasmic reticulum store of calcium, released to induce contraction, is rebound by the action of a cylcic AMP stimulatable calcium pump (Hurwitz, Fitzpatrick, Debbas, Landon, 1973). However, the reported contraction-inducing action

of the E-series prostaglandins is associated with a rise in cyclic AMP (Harbon, Vesin, Khac & Leiber, 1978). In both of the smooth muscle forms the role of calcium is quite convincingly demonstrated and follows the external and internal calcium dependencies observed with the secretory tissue.

Skeletal muscle.

It is still uncertain if the well established calcium component of the skeletal muscle action potential contributes to the coupling calcium (Beaty & Stefani, 1976; Blinks, Prendergast & Allen, 1976). Contraction can still occur long after the removal of external calcium (Armstrong, Bezanilla & Horowicz, 1972;Andersson & Edman, 1974), and in the presence of calcium antagonists (Dörrscheidt-Kafer, 1977) or chelating agents (Andersson & Edman, 1974). The quantity of calcium entering via the Isi current is itself not sufficient to initiate contraction (Taylor & Godt, 1976; Reuter, 1974). Thus it is evident that an intracellular pool must be utilized to provide sufficient calcium. Studies of the membrane depolarization reveals that the sarcolemmal trabecular system is also depolarized (Constantin, 1970). This system is physically very close to the sarcoplasmic reticulum and there is evidence suggesting that their depolarization can trigger potential gating of sarcoplasmic reticulum stores of calcium (Ebashi & Endo, 1968). The suggestion that the trabecular system releases calcium, which induces a further sarcoplasmic calcium release, has been questioned because the effect was not observed at physiological levels of magnesium ions (Polimeni & Page, 1970). As calcium is released from internal stores it must be returned there during relaxation. Adrenaline mediated relaxation is associated with a rise in sarcoplasmic cyclic AMP, which promotes return of the activating calcium through a cyclic AMP activated calcium pump (Katz, Tada & Kirchberger, 1975).

Cardiac muscle.

The reliance of skeletal muscle upon internal calcium allows only an all-or-none contractile response. In cardiac muscle a more graded contraction is necessary. The importance of calcium in regulating the contraction and relaxation phases of cardiac muscle is indicated by the complexity of the coupling mechanism linking excitation and contraction. Two inward currents are again responsible for the action potential induced depolarization of cardiac muscle. Following potential-dependent arrest of the fast sodium current (INa) the plateau phase of the action potential is established by the secondary calcium current (Isi)(Ulbricht, 1977; Beeler & Reuter, 1970). This depolarization-induced increase in cytosolic calcium instigates a chain of events culminating in contraction (Langer, 1973). In the frog heart the Isi alone provides sufficient calcium to initiate contraction (Kavaler, Anderson & Fisher, 1978; Fabiato & Fabiato, 1977). However, in mammalian hearts the increase in calcium is not sufficient to provoke contraction and must be supplemented from internal stores (Solaro & Briggs, 1974). The mechanism for the calcium release is not certain although it has been suggested that a potential-dependent release, similar to that occurring in skeletal muscle occurs (Langer, 1976). However, as calcium influx is critical for contraction, and the sarcoplasmic reticulum stores may be released by the direct addition of physiological sarcoplasmic calcium concentrations it is also possible that a calcium-induced calcium release occurs (Fabiato & Fabiato, 1979). Interestingly the rate of increase in cytosolic calcium directly influences the rate of release of stored calcium. As in skeletal muscle there is a well defined sarcoplasmic reticulum calcium pump (Katz et al. 1975). This can only restore the second phase of cyto-

plasmic calcium and another process must be responsible for removing the initial calcium that enters from the external environment. This is accomplished by the sodium/calcium exchange (Langer, 1976; Tillisch, Fung, Ham & Langer, 1979).

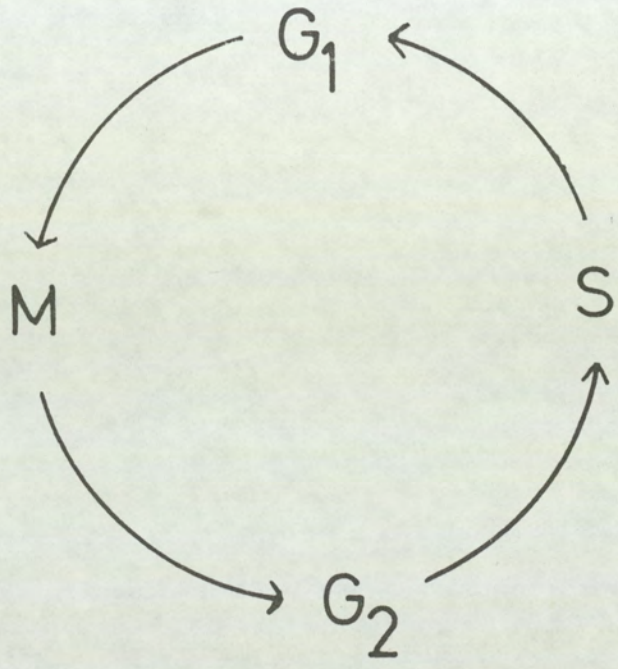
The more specialised function of muscle tissue has resulted in the unique calcium storage organ - the sarcoplasmic reticulum. This may contribute all the calcium required for the coupling or may supplement the initial transmembrane fluxes. Whether such a calcium reservoir exists in the intracellular calcium-dependent secretory tissue is uncertain although both mitochondria and endoplasmic reticulum may store calcium. It is evident that both external and internal calcium ions may link the extracellular stimulus with cell activation. The third section is devoted to a discussion of the possible role of such a coupling process in mitotic activation.

3.1. The cell cycle

The fundamental unit of cell proliferation is the mitotic, or division cycle. This is the period between the production of a cell by the mitosis of its parent cell, and the formation of daughter cells by its own mitosis (Mitchison, 1971). The cycle, stretching between successive mitosis, may be divided into four unequal and biochemically distinguishable phases, each based on a temporal relationship to the period of DNA synthesis (Howard & Pelc, 1953; Petersen, Tobey & Anderson, 1969). After the initial mitotic phase (M), there is a pre-synthetic pause (or gap) designated G_1, which precedes the incorporation of nucleotides into DNA. Following the subsequent DNA synthetic phase (S), the cell enters the second pause, or post-synthetic gap (G_2). The G_2 phase is terminated by mitosis which marks both the initiation and completion of a cell cycle. Mitosis itself is characterized by condensation and separation of the chromatin and by the ultimate cytokinesis (Mitchison, 1971). Such a four phase cycle (G_1 S G_2 M) is presented in figure 3.1.

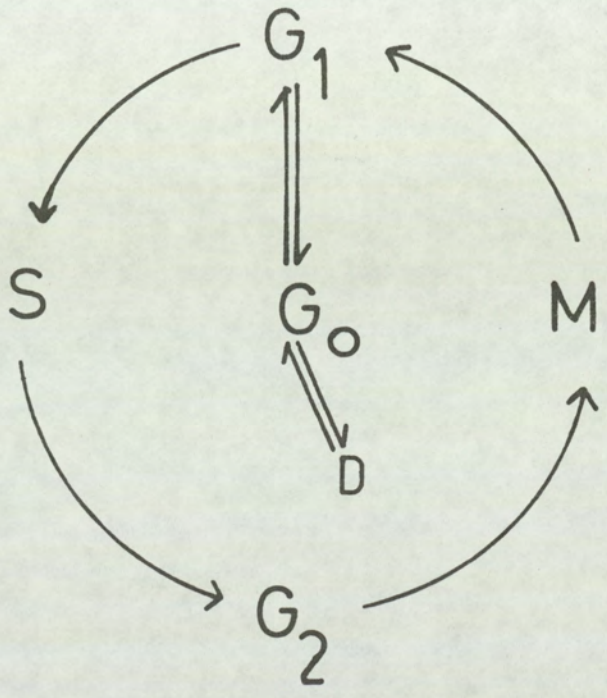
After dividing, newly formed cells may embark immediately upon the next division cycle by entering G_1 directly from M. Alternatively, they may reversibly de-cycle and enter a non-dividing phase termed G_o (Lajtha, 1963). The G_o, or resting phase, is kinetically and biochemically distinguishable from the G_1 phase; G_o cells take longer to reach S than immediately post-mitotic cells (Epifanova, Abuladze & Zosimovskaya, 1975) and exhibit reduced macromolecular synthetic activity (Martin & Stein, 1976). This fifth compartment is presented in figure 3.2.

Figure 3.1. The four compartment cell cycle (after Howard & Pelc, 1953)



The G_o phase may be exited either to provide terminally-differentiated cells (figure 3.2a)) or to allow quiescent cells to re-enter the division cycle (figure 3.2.b)). It is evident that G_o is a broad term encompassing a wide range of metabolic states. One such G_o compartment will contain cells, such as neurones, incapable of re-entering the cycle whilst a second cohort of G_o cells will be able to rejoin the cycle in response to an appropriate stimulus (e.g. peripheral lymphocytes and the hepatocyte). These responsive cells will be termed G/D to distinguish them from non-differentiated cells residing in a quiescent state. Such a cell is the rat thymic lymphocyte, or the burst forming unit of haematopoietic tissue, which can rapidly divide to produce differentiated cells. These quiescent

Figure 3.2. The five compartment cell cycle (after Lajtha, 1963)

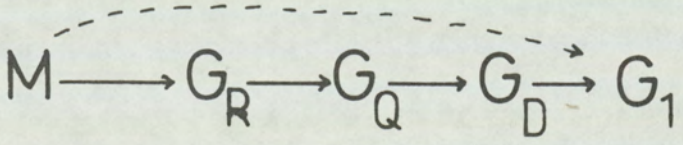


(a) Exit from G_o producing differentiated cells.
(b) Exit from G_o to the division cycle.

cells are $G_o/_Q$ in the terminology used here. A further population of cells residing in G_o will contain cells temporarily restricted from entering G_1 by the lack of an essential nutrient or growth factor, or by a hostile external environment. Restriction due to the lack of alanine, serum or a correct temperature have all been observed (Naha, Meyer & Hewi, 1975; Talvare & Basilico, 1978; Hartwell, 1976). These restrictions due to sub-optimal growth conditions may be distributed throughout the G_1 phase of the cycle, with cells capable of passing from one restriction G_o to another (Talvare &

Basilico, 1978). For convenience these separate restriction points are grouped together as $G_o/_R$ and are presented in figure 3.3. in conjunction with other G_o stages. The lack of extracellular calcium is also capable of causing restriction in 3T3 fibroblasts (Paul & Ristow, 1979), although some confusion arises over whether this calcium restriction occurs in cells already committed to divide (Whitfield, Boynton, MacManus, Sikorska & Tsang, 1979). Such an uncertainty illustrates the nebulous nature of the junction between the G_o and G_1 phases. If exchange does occur between the two phases, it must be before the cell is irrevocably committed to the events in G_1 (see Frankfurt, 1968). This has led to a second model comprising a two phase cell cycle. One phase, termed the B phase, encompasses the obligatory and sequential events after commitment and will include part of G_1 S G_2 and M. The second, (A), phase contains G_o and the non-committed portion of G_1 (Smith & Martin, 1973).

Figure 3.3 Components of the G_o and G_1 phases of the division cycle



Broken line illustrates direct transit of a cycling cell from M to G. The events of G_o are depicted in a linear sequence for convenience.

The two-phase and five phase cycle models both predict that under the appropriate intracellular and extracellular environments the G_o cell will be stimulated to re-enter the cycle. This commitment appears to be due to the synthesis of a cytosolic protein(s), that initiate the cycling and replicatory machinery (Taylor, 1965). Such cytoplasmic factors are detectable and will induce precocious DNA synthesis in quiescent nuclei implanted into S phase cells (Prescott & Goldstein, 1967; Rao & Johnson, 1970). This signalling is also evident from the synchronous behaviour of nuclei in multinucleate cells (Church, 1967). Whether there is one, or a series of commitment events is unknown but it is evident that their effect is exerted late in G_1 when a whole series of DNA replicative enzymes become active or are synthesized. Some of these are listed in figure 3.4 - as can be seen, these may run into the S phase, which illustrates the arbitrary designation of the model.

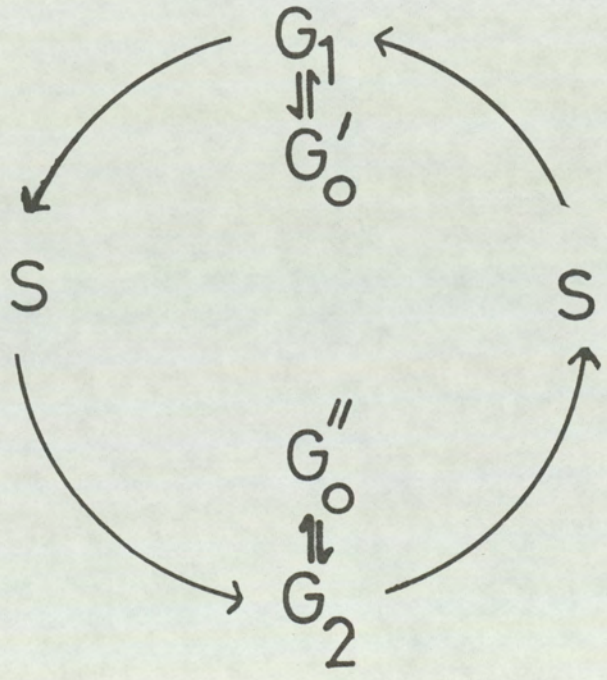
Fig. 3.4 Replicative enzymes produced prior to DNA synthesis in lymphoid cells.

Enzyme	Cycle phase	Reference
Histone kinase	late G_1	Cross & Ord, 1971
Histone phosphorylase	late G_1	Cross & Ord, 1971
RNA directed DNA polymerase	G_1/S	Penner, Cohen & Loeb, 1971
Thymidine kinase	G_1/S	Wilms & Wilmanns, 1969
Thymidylate synthetase	G_1/S	Youdale & MacManus, 1975
RNA polymerase II	S	Handmaker & Graef, 1970

After completing DNA replication the short and relatively invariable G_2 phase is entered (Prescott, 1976). The G_2 phase appears to prepare the cell for the imminent mitosis, indeed protein synthesis is necessary for G_2 transit (Doida & Okada, 1969). There is what appears to

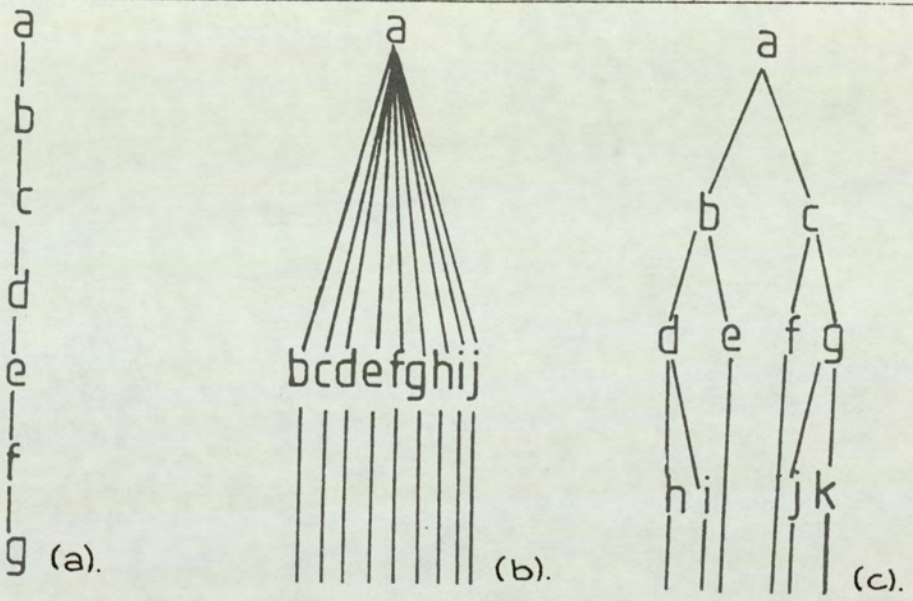
be a restriction point in G_2 as high extracellular cyclic AMP concentrations reversibly inhibit the G_2 progress of certain epithelial cells (Voorhees, Colburn, Stawiski, Duell, Haddox & Goldberg, 1974). This G_2 restriction would result in the arrest of cells containing double the normal DNA content, such non-dividing 4n cells have been identified in certain tissues (Gelfant, 1962). This second non-dividing compartment has been designated G_o'' in some models (fig.3.5).

Fig.3.5. The six compartment cell division cycle.



Obviously, the normal passage of a cell through the various stages of the cycle will depend upon the ordered expression of the metabolic steps necessary for each stage. Many theories exist to describe the nature of these steps. They may be sequentially expressed, with each requiring the completion of its predecessor (Figure 3.6a)); they may each proceed individually after the initial event (figure 3.6b)); or they may be a mixture of the two (figure 3.6c)).

Fig. 3.6. Models for the expression of events throughout the cell cycle



For the initial induction, (and possibly the continuation), of these events, some system of intracellular regulators is essential. Intracellular cyclic nucleotides and the calmodulin protein are prime contenders for this regulatory role, indeed cyclic nucleotides and calmodulin induce activation of many intracellular enzymes and processes (see Sections 1 and 2).

Dissection of potentially significant changes in cyclic nucleotide content during the cell cycle, almost exclusively in-vitro studies, is fraught with many difficulties.

1. Low internal concentrations, imprecise assay conditions, and the lack of non-invasive cell synchrony techniques lead to

many conflicting observations in the literature.

2. Effects attributed to cyclic nucleotides which are inferred from studies using exogenously added nucleotides or phosphodiesterase inhibitors may not be attributable to cyclic nucleotide changes (Smith, Steiner & Parker, 1971).

3. Inconsistencies could also result from changes in the effectors of cyclic nucleotides, notably protein kinases and their substrates which may change during the cycle (Russell, 1978).

The cyclic nucleotide changes associated with the initiation of cell replication are discussed below.

3.2. Cyclic nucleotide fluctuations during the cell cycle.

G_o and early G_1

Figure 3.3. predicts that a cycling cell may be immediately post-mitotic, or a hitherto quiescent cell recruited by external stimulation or even a cell released from a temporary restriction. As cyclic nucleotides have been measured in all three cases some attempt has been made to correlate the immediate history of the cell with the reported cyclic nucleotide changes.

1. Cells released from restriction. Generally these are cultured cells grown in serum free or serum poor conditions, and consequently are arrested in a non-dividing state ($G_{o/R}$). Upon the addition of serum or certain growth factor and hormone combinations these cells rapidly re-enter the division cycle. Unless otherwise indicated, the studies below are all performed on the 3T3 murine fibroblast model. Serum addition, and consequently G_o exit, has been associated with a rapid decrease in the intracellular cyclic AMP content (Burger, Bombick, Breckenridge & Sheppard, 1972; Siefert & Rudland, 1974; Oey, Vogel & Pollack, 1974). Similar mitotic and cyclic nucleotide responses also follow the application of fibroblast growth factor

(Rudland, Gospadorowicz & Siefert, 1974) insulin (Goldberg, Haddox, Dunham, Lopez & Hadden, 1974) and non-serum mitogens such as phorbol esters and trypsin (Burger et al., 1972; Rochette-Egly & Castagna, 1979). The cyclic AMP fall has not been observed by some investigators using apparently identical procedures (Coffey, Hadden, Lopez & Hadden, 1978). In the regenerating rat liver in vivo, cyclic AMP levels rise rather than fall, during early G_1 (Tsang, Rixon & Whitfield, 1980). Moreover, prevention of the cyclic AMP drop by adding exogenous cyclic AMP did not prohibit the normal mitotic response to serum (Rechler, Bruni, Podskalny, Warner & Carchman, 1977). It is quite possible that this cyclic AMP fall is not a universal event and is merely associated with the metabolic changes of the recruitment of restricted cells.

Upon entering a restricted G_o, caused by nutrient depletion at cell confluency or high density, the cyclic AMP content of 3T3 cells rises (Otten, Johnson & Pastan, 1971). Application of exogenous cyclic AMP inhibits the growth of many cell types, moving them into a G_o state. These cyclic AMP inhibitable cells include murine diploid fibroblasts (Froelich & Rachmeler, 1974; Heidrick & Ryan, 1970), lymphoma (Coffino, Gray & Tomkins, 1975), and neuroblastoma (Gilman & Nirenberg, 1971). Human lymphocytes are also sensitive to such high cyclic AMP levels (Parker, 1976). This phenomenon has led to a general theory that **cyclic AMP negatively regulates proliferation (Coffino, Gray & Tomkins, 1975)** and therefore a fall in cyclic AMP would coincide with the end of growth restriction. Bearing in mind the pharmacological doses required for growth inhibition and the unreproducibility of the cyclic AMP fall this attractive hypothesis would appear to be an oversimplification.

Findings concerning cyclic GMP changes following activation of serum starved cells are equally contradictory. Many investigators have ob-

served a cyclic GMP rise during the early stages of release from restrictions (Friedman, Johnson & Zeilig, 1976; Siefert & Rudland, 1974; Estensen, Hadden, Hadden, Touraine, Touraine, Haddox & Goldberg, 1974). However, other studies detect the opposite change (Miller, Lovelace & Pastan, 1975; MacManus, Boynton & Whitfield, 1978), or no alteration (Zeilig & Goldberg, 1977).

2. Recruited quiescent cell. The commonly employed model for these investigations is the human peripheral blood lymphocyte. Recruitment is initiated by the application of plant lectins or other suitable polyclonal activators, the mitotic consequences of which are discussed later. As in other systems, the cyclic nucleotide changes reported by different groups are contradictory. Different cyclic GMP measurement techniques may account for some inconsistencies (Coffey, Hadden, Lopez & Hadden, 1978). A rise in lymphocyte cyclic GMP has been observed following recruitment of human lymphocytes (Hadden, Coffey, Hadden & Haddox, 1977). This rise appears to be associated with the subsequent cell division as both the cyclic GMP surge and mitosis are mitogen concentration dependent, are dependent upon extracellular calcium ions and can both be provoked by A23187-induced calcium ionophoresis (Coffey et al. 1978; Hadden et al. 1977). A second group of investigators consistently fail to confirm these findings, using a system of proven sensitivity and the same cell model (Parker, Sullivan & Wedner, 1974; Parker, 1978; Wedner, Dankner & Parker, 1975). In contrast they observe a cyclic AMP surge during lectin treatment (Parker, Sullivan & Wedner, 1974). As this cyclic AMP rise also occurred at lectin concentrations that inhibit cell division, and even with non-mitogenic lectins (Parker, 1978) the physiological significance of the cyclic AMP surge is uncertain.

Perhaps the most convincing evidence for the cyclic GMP surge reported in many cycling, recruited and restriction released cells is the ability of added cyclic GMP to activate DNA and RNA synthetic machinery in isolated lymphocyte nuclei. This occurs at a cyclic GMP concentration approaching that of the stimulated cell (Johnson & Hadden, 1975), and may signify a physiological role for the mitogen induced cyclic GMP rise.

3. Synchronously dividing cells. These cells, although artificially synchronized, approximate to immediately post-mitotic cells embarking on a round of division. The cyclic nucleotide levels have been quantified for a variety of cell types, synchronized by many different techniques. These cells include foetal rat hepatocytes (George, Rodgers & White, 1978), Chinese hamster fibroblasts and human lymphoid cells (Millis, Forrest & Pious, 1974). In all three types the cyclic AMP fall and the cyclic GMP rise were detected. In summary, it appears that the cycic AMP change is of doubtful significance and that cyclic GMP changes, whilst controversial, could signal the start of the cell cycle.

Late G_1 and S

After the initial recruitment cells committed to divide exhibit a rise in their cyclic AMP content during the late G_1 and early S phase. Figure 3.7 lists several examples of cell types where this increase is observed.

Fig.3.7. Cultured cells exhibiting a rise in cyclic AMP during late G_1 and S.

Foetal rat liver	George et al. 1978
Murine lymphoblasts	Millis, Forrest & Pious, 1974
Novikoff hepatoma	Goldberg et al. 1974
Synchronized hamster fibroblasts	Goldberg et al. 1974
Murine 3T3 fibroblasts	MacManus, Boynton & Whitfield,1978.

This surge in cyclic AMP is essential for the further progress of cycling cells. By inhibiting the increase in vivo regenerating liver cells are prevented from dividing (MacManus, Braceland, Youdale & Whitfield, 1973; Tsang et al. 1980). The late G_1 rise in cyclic AMP may be an intracycle 'cue', serving to regulate further cycle progression by initiating the events necessary for cycle transit. The nucleotide changes observed during the M and G_2 stages of the cycle may also reflect such mechanisms (Zeilig, Johnson,Friedman & Sutherland 1972; George et al. 1978). If indeed cyclic nucleotides regulate recruitment and cycle progression they must be responding to the extracellular signals that regulate growth. The following paragraphs discuss these external messengers in more detail, demonstrating the integrated action of extra- and intracellular messengers in correlating cell division with the demands for increased cell production. As with cyclic nucleotides, the majority of the evidence for growth regulating substances is obtained from in vitro studies.

3.4. Extracellular growth regulatory substance

The positive regulation of cell growth by extracellular factors.

Many components normally present in the extracellular environment are able to sustain or stimulate cell proliferation when added to cells in culture (Baserga, Rovera & Farber, 1971). Amongst these agents are a series of growth stimulating peptides, not yet promoted to the ranks of true hormones. Although the physiological function of many of these 'growth factors' remains obscure, some in vivo growth potentiation has been detected. Broadly speaking, growth factors are divisible into those enhancing mitosis with little cell selectivity, and those promoting tissue-specific division and differentiation. Some may not directly promote growth, merely aiding the passage of cells through the

cycle or postponing cell death (Nishawaki, Armelin & Sato, 1975). Rather than consider these widely different peptides as a group, the subsequent paragraphs illustrate similarities and differences between selected factors.

Nerve growth factor (NGF).

Although initially identified in snake venom and murine salivary glands, the principle target of this peptide is neural tissue (Levi-Montalcini, Meyer & Hamburger, 1954). In vitro it has been found to support only the proliferation and development of neuronal cells (Levi-Montalcini & Angeletti, 1963). The factor is required in vivo for the formation of a competent nervous system, its removal using anti-NGF antiserum severely impairs neural development (Levi-Montalcini, 1964). NGF has a well characterized plasma membrane receptor (Frazier, Boyd & Szutowicz, 1974), but the consequences of receptor occupancy remain obscure. One report documents rapid receptor-ligand internalization (Yankner & Shooter, 1979), whilst a second shows immobilized peptide retaining full biological activity (Frazier, Boyd & Bradshaw, 1973). Membrane depolarization appears to result from NGF binding, indeed transient depolarization and subsequent calcium influx can substitute for NGF (Schubert, La Corbiere, Whitlock & Stallcup, 1978).

Epidermal growth factor (EGF).

After the initial purification of murine epidermal growth factor the isolation of human EGF revealed a protein apparently identical to urogastrone, a potent inhibitor of gastric acid secretion (Gregory, 1975; Carpenter & Cohen, 1976; Hirata, Moore, Bertagna & Orth, 1980). Unlike NGF the cell specifity of EGF is very broad, responsive cells including fibroblast, epithelial and mammary cells (Cohen & Taylor, 1974; Cohen & Savage, 1974; Rheinwald & Green, 1977; Lechner & Kaighn, 1979). EGF internalization after membrane receptor binding

has been observed (Fox & Das, 1979). The addition of the factor to tissue culture lowers the amount of extracellular calcium necessary to support continued and enhanced proliferation (McKeehan & McKeehan, 1979), which suggests that EGF can, like NGF, manipulate cellular calcium metabolism. Indeed, the reported cyclic AMP fall after EGF (Hollenberg & Cuatrecasas, 1975) may be due to calcium/calmodulin action on cyclic nucleotide metabolism.

Fibroblast growth factor (FGF)

This again is a broad spectrum growth factor, influencing a wide range of responsive cells of endodermal and mesodermal origin (Canalis & Raisz, 1980; Gospodarowicz, 1974). Unlike the previously discussed factors the action of FGF requires additional compounds normally present in serum (Holley & Kiernan, 1974). The high concentration of FGF in the supernatant of cultures containing large quantities of platelets has led investigators to suggest that FGF is identical to the platelet produced growth factor (Antoniades, Stathakos & Scher, 1975). Immunological cross reactivity has been detected between FGF of pituitary origin and the platelet factor (Antoniades & Scher, 1977). The platelet factor (PDGF) is released by platelets at sites of tissue damage (Rutherford & Ross, 1976) and may assist in the healing response (Ross Clomset, Kariya & Harker, 1974). These two factors FGF/PDGF may act as progression factors, assisting other more selective growth promoting factors to stimulate the proliferation of a specific group of cells (Vogel, Raines, Kariya, Rivest & Ross, 1979). A similar role has been proposed for the E and F series prostaglandins which may also act as short-range 'homing signals' for mitogenic factors (O'Farrell, Clingon, Rudland & Jiminez de Azua, 1979). The ability of FGF to elevate fibroblast cyclic GMP (Rudland, Gospadorowicz & Siefert, 1976) suggests the FGF/PDGF action may be to recruit cells from G_o.

Growth hormone dependent growth factors (The somatomedins)

The ability of growth hormone (GH) to stimulate the division of a multitude of cell types is the result of an intermediary series of mitogenic growth factors (Salmon & Daughaday, 1957; Salmon & Hosse, 1971). Five such factors are identified GH mediators ; these are two insulin-like growth factors (ILA I and II), Multiplication Stimulating Activity (MSA), and Somatomedin A and C (Phillips & Vassilopoulou-Sellin, 1979). These compounds stimulate cartilage and fibroblast cell proliferation (Salmon & Hosse, 1971, Pierson & Temin, 1972). All of them bear remarkably similar structures and exhibit parallel growth promoting activities, moreover all can substitute weakly for insulin in activating normal insulin-like metabolic changes (Zapf, Rinderknecht, Humbel & Froesch 1978). Insulin itself stimulates a variety of cells to grow (Zapf et al. 1978). This parallel and cross-reactivity has been partly resolved by the high degree of structural homology (Rechler, Zapf, Nissley, Froesch, Moses, Podskalny, Schilling & Fryklund, 1977) which allows the two groups (GH factors and insulin) to cross-react with each other's receptors (King, Kahn, Rechler & Nissley, 1980).

It is apparent that these circulating factors may contribute to growth and are responsible for many of the growth promoting activities of serum. However, it is unclear if their circulating levels fluctuate within the plasma in response to physiological demands. The additive action of many hormones and factors would allow hormone level changes to control proliferation without a necessary change in systemic GF levels. The final paragraph discusses the mitogenic potential of hormones both in vivo and in vitro.

Hormonal mitogens

Many hormones, both steroid and peptide, are able to modify cell

proliferation. Perhaps the most obvious of such hormones are those responsible for secondary sex organ development. Thus testosterone, oestradiol and progesterone are essential for the continued mitotic activity in these developing tissues in vivo (Chatterton, 1971, Allison, Appleton, Hanwell & Wright, 1976). Although oestradiol has been shown to promote division in vitro (King, Cambray & Robinson, 1976, Pietras & Szego 1979), it is thought to act through an inducible growth factor (Sirbasku, 1978). Amongst other trophic hormones are ACTH, acting upon the adrenal cortex (Gospadorowicz, Ill & Birdwell,1977) TSH upon thyroid gland (Westermark, Karlsson & Wolinder, 1979) and FSH/LH for granulosa and luteal cells (Gospadorowicz & Moran, 1976). The actual mechanism of these hormonal stimulations is not clear. The disappointing lack of direct effects of pure hormone preparations upon target tissue in vitro, in the absence of serum, suggests that growth factors mediate hormone action (Nandi, Yang, Richards, Guzman, Rodrigues & Imagawa, 1980).

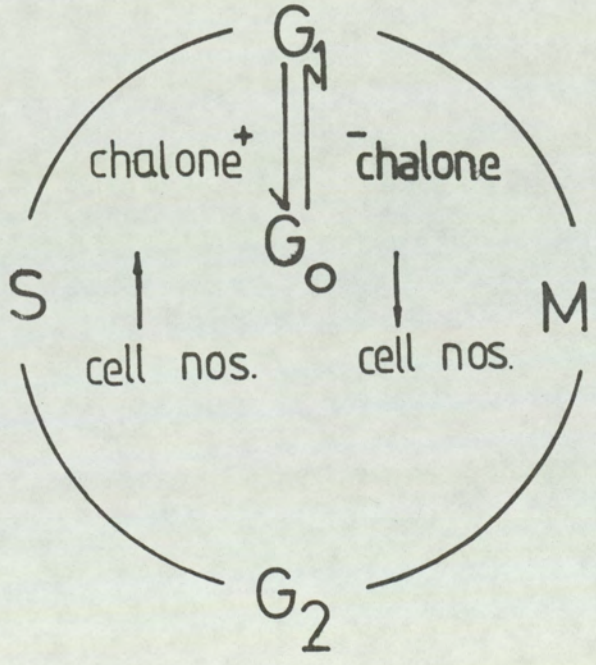
When hormonal mitogens are added to cultured cells in the presence of serum, ionic changes reminiscent of those associated with stimulus-activation coupling in Section 2 are observed. Thus vasopressin, a mitogen of 3T3 fibroblasts (Rozengurt, Legg & Pettican, 1979), promotes a rapid sodium entry, reminiscent of depolarization (Smith & Rozengurt, 1978). This sodium influx may account for the calcium changes observed under similar circumstances (Chen, Babock & Lardy,1978). Serum itself is associated with the rapid entry of calcium into stimulated fibroblasts (Tupper, Del Rosso, Hazelton & Zorgniotti, 1978). This limited consideration of ionic events during mitotic recruitment is continued in the discussion.

Negative regulation of cell growth

At this juncture it is worthwhile considering the existence of negative, or inhibitory, growth regulators which would counteract the

positive factors discussed above. The principle negative factors are undoubtedly the elusive chalones. These compounds would be produced continually by differentiated cells within an organ or tissue. When the local extracellular level attains a pre-determined concentration, further growth of the tissue is prohibited by a mitotic blockade, cells being placed into a G_o state (Bullough, 1972). This model of chalone action predicts that removal of the chalone producing (differentiated) cells will automatically reduce the external chalone concentration and allow restoration of cell division until the inhibitory chalone level is restored (Bullough, 1975) (Figure 3.8).

Fig.3.8. Proposed action of chalones on the cell cycle



Although, to date, over twenty water soluble cellular extracts have been shown to reversibly inhibit proliferation of their native tissue (Attallah & Houck, 1976), the very existence of chalones has been challenged by many investigators. Criticism stems largely from the lack of pure, or even partly characterized chalone preparations, and from the often demonstrated lack of tissue specificity. Evidence for the existence of a lymphoid chalone, which would be particularly pertinent for thymocyte proliferation, is reviewed below.

Lymphocytes may be characterized as either thymus-dependent (T) or bone marrow dependent (B) cells (see Section 4). Soluble extracts from lymph node, spleen and thymus caused the inhibition of lectin stimulated T cells (Garcia-Giralt, Diaz-Rubio & Rappaport, 1975). To be designated a chalone, this putative T cell chalone would have no influence on B cell proliferation. However, similar extracts were later shown to inhibit lipopolysaccharide (LPS) induced B cell proliferation (Attallah,Sunshine,Hunt & Houck,1975).It is possible that this cell product exerts a general suppressive effect on lymphocyte proliferation. Such a factor would be a candidate for the T-suppressor cell product which would have the same capabilities. A second T cell extract, suppressing allograft rejection and graft versus host reaction has been shown to be highly cytotoxic, and a non specific immuno-suppressant (Garcia-Giralt et al. 1975; Kiger, Florentin & Mathe, 1975; Attallah & Houck, 1977). It is reasonable to conclude that at present there is no firm evidence for the existence or otherwise of a lymphoid chalone.

A variety of serum components are able to inhibit cell proliferation in vitro by directly interacting with mitotic cells. These include the vitamin A-derivatives, retinols, which show a broad range of cell specifity (Sporn, Dunlop, Newton & Smith, 1976; Sporn & Newton, 1979).

Prostaglandins of the E series, which show selectivity for connective tissue (Korn, Halushka & LeRoy, 1980) and myeloid precursor cells, are also inhibitory (Kurland, Hadden & Moore, 1977). The mechanism by which the levels of these negative factors are modulated, and their role in shaping overall cell proliferation, is unknown.

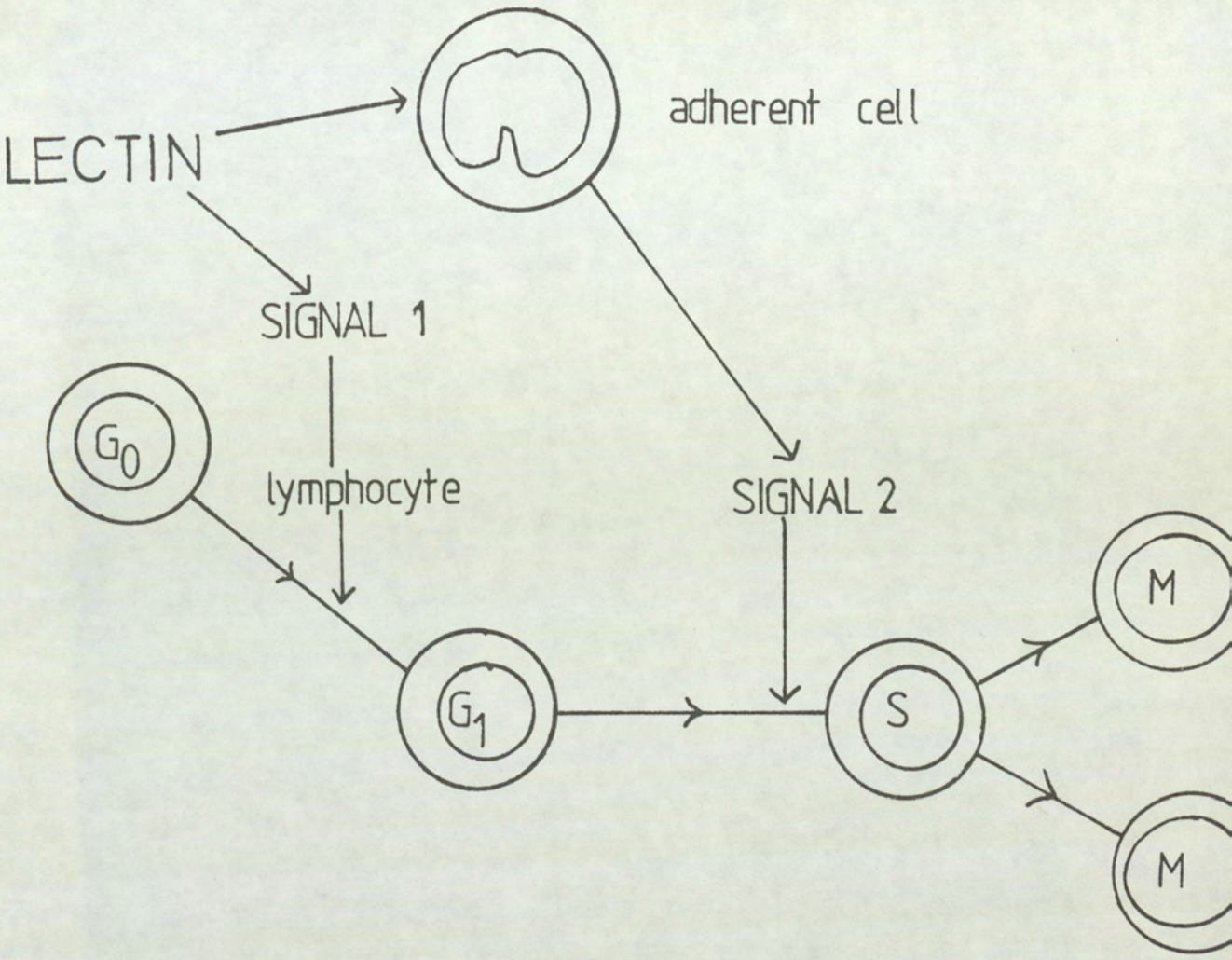
Before discussing the thymocyte cell model it is worth discussing in detail an example of mitotic activation that supports the concept of stimulus-mitosis coupling (Berridge, 1976).

3.5. Lectin induced lymphocyte proliferation - an example of stimulus-mitosis coupling?

The normal response of quiescent lymphocytes to an appropriate antigen is the division of a restricted clone of cells (Ling & Kay,1975). Lectins seem to mimic and amplify this process by acting as polyclonal mitogens, stimulating up to 70% of the mitotically competent population (Loeb, 1975). This permits biochemical analysis of events associated with the recruitment of a well synchronized population. The earliest event of triggering is the lectin-mediated cross linkage of glycoproteins present in the membrane (Greaves & Janossy, 1972; Wedner & Parker, 1976). This 'receptor' binding is followed by a plethora of metabolic changes associated with the increased activity necessary for division (Resch & Ferber, 1972; Van den Berg & Betel, 1973), the majority of which are not causally related to recruitment (Kaplan, 1978).

Activation itself is not a single inductive event, lectin presence is necessary for both the initiation of G_o exit (signal I) and an event late in G_1/S (signal II)(Kay, 1970). Recent evidence suggests that signal II is provided by a factor released from adherent cells (Rosenstreich, 1976) which may be identical to those released during mixed lymphocyte reactions (Alvarez, Silva & de Landazuri, 1979)(Figure 3.9).

Fig. 3.9. Hierarchy of lectin induced mitogenic signals



The initial signal, I, is of interest to us as it is the recruiting stimulus that activates the quiescent cells. One of the earliest events following lectin stimulation is a rise in the influx of calcium from the external environment (Allwood, Asherson, Davey & Goodford, 1971; Ozato, Huang & Ebert, 1978; Freedman, Raff & Gomperts, 1975). This rise in cytosolic calcium has biological significance as calcium-free extracellular environments do not support stimulation (Allwood et al. 1971; Whitney & Sutherland, 1973). Furthermore, the calcium ionophore A23187 initiates identical proliferative responses whilst promoting calcium influx (Maino, Green & Crumpton, 1974; Jensen, Winger,

Rasmussen & Nowell, 1977). This suggests that a rise in cytosolic calcium is the triggering stimulus in lectin-induced lymphocyte proliferation. This has many similarities with cell activation as described in Section 2.

The subsequent events of activation are also in accord with a general model of cell activation. Thus a rapid efflux of cytosolic potassium occurs from the triggered lymphocytes (Aull, Nachbar & Oppenheim, 1977; Iversen, 1976). This may be provoked by the rise in cytosolic calcium or by the change in membrane lipid composition (Crumpton, Auger, Greene & Maino, 1976). However, the net effect of potassium efflux is controversial as an equal and opposite potassium influx is detectable (Averdunk & Lauf, 1975). This K^+ influx is through the sodium-potassium ATPase and may be restorative, like the potassium efflux and recapture cycle in other systems (see Section 2). A second possibility, that the futile K^+ cycle lowers cytosol ATP content, is discounted as there was no decrease in ATP after stimulation (Segel, Androphy & Lichtman, 1978).

The preceding chapter suggests that the extracellular environment directly influences cell proliferation, acting through intracellular regulator processes that govern the cell cycle. The link between the external signal and the internal response has not been considered, although evidence suggesting that calcium fulfills this role in other cell processes has been presented in Section 2. The final introductory chapter now considers the specific cell model employed in the current investigation, and attempts to provide some insight into the coupling between mitogen and mitogenesis.

4.1. The control of thymic lymphocyte proliferation in vivo.

There are two functionally distinct lymphocyte populations within the mammalian immune system. One, composed of antibody-secreting cell precursors (the so-called B lymphocyte), derives directly from the bone marrow (Greaves, Owen & Raff, 1973; Nossals & Makela, 1962; Miller & Mitchell, 1967). The second population, which mediates cellular immune reactions and exerts a regulatory influence over the antibody response, also originates from bone marrow stem cells (Davies, Leuchars, Wallis & Doenhoff, 1971; Metcalf, 1970; Ford & Micklem, 1963). These cells, which emigrate from the marrow and mature within the thymus are termed T-lymphocytes (Clanmann & Mosier, 1972; Miller & Osoba, 1967). In addition to this seeding the thymus, particularly in early life, also contains a self-renewing stem cell population (Cläesson & Hartmann, 1976; Röpke & Everett, 1974).

The end product of the intrathymic division and differentiation of T lymphocyte precursors is the small, non-cycling, terminally differentiated cell which comprises some 75% of the total thymic lymphoid pool in the rat (Craddock, Nakai, Fukuta & Vansleyer, 1964). Although the thymus contains such a high proportion of non-dividing cells the entire murine lymphoid compartment is renewed within four days (Metcalf & Wiadrowski, 1966). This self-renewal is due to the high mitotic activity of the remaining 15% of the lymphoid population. These active cells primarily distinguishable by their size and stage of differentiation (Saint-Marie & Leblond, 1965; Metcalf & Wiadrowski, 1966), give rise to the end cell through a series of reduction divisions (Craddock et al. 1964). For the thymic mass to remain stable the very high rate of proliferation must be balanced by large scale intrathymic death of self-reactive cells and by a smaller scale cell emigration (Joel, Chanana, Cottier, Crankite & Laissue, 1977;

Claesson & Hartmann, 1976).

Although proliferative events within the thymus were originally considered to be autonomous (Miller & Osoba, 1967), thymocyte production in vivo is now known to be influenced by both the external and internal environment. Initial development and maintenance of the tissue is dependent upon both growth hormone (GH) and corticotropin (ACTH). Hypophysectomy or anti-GH antiserum administration both induce thymic atrophy in young animals (Pierpaoli & Sorkin, 1972). Indeed, both a direct effect of GH on thymocyte mitosis, and an indirect effect on the nutritive epithelium have been observed in vitro (Whitfield, MacManus & Rixon, 1971; Comsa, Leonhardt & Ozminski, 1979). ACTH may also influence the epithelial tissue, which responds to ACTH injection in adrenalectomized mice (Brink-Johnson & Dougherty, 1965). A further pituitary-thymus axis is indicated by the TSH-induced restoration of thymic growth in congenitally hypopituitary mice (Pandian & Talwar, 1971; Pierpaoli, Baroni, Fabris & Sorkin, 1969). The TSH effect may be mediated by T_4, which can itself restore growth (Pierpaoli et al. 1969). Parathyroid hormone can also positively influence intrathymic cell production. The thymus gland atrophies in surgically hypoparathyroid adult rats (Rixon & Whitfield, 1972). A variety of PTH dependent mitotic responses in vivo are discussed in more detail below.

Reduction of plasma testosterone, oestradiol and corticosterone following castration or adrenalectomy results in enhanced thymic growth, suggesting that the steroid hormones exert a negative influence over thymic proliferation (Dougherty, 1952; Dougherty, Berliner, Scheebeli & Berliner, 1964). Adrenalectomy may both remove lymphotoxic corticosteroids (Kinoshita, Kimura & Fukamizu, 1974) and enhance circulating levels of the trophic ACTH. The latter poss-

ibility may be more valid. Although cortisol will lyse thymic lymphocytes at high concentrations, more physiological levels actually promote division (Whitfield, MacManus & Rixon, 1970). The inhibitory role of sex-steroids is more precisely defined, their removal by castration increases thymus size, whilst their administration produces marked thymic atrophy (Sobhon & Jivasattham, 1974; Ito & Hoshino, 1963; Scheiff & Haumont, 1979).

In addition to this external influence there is considerable evidence for intrathymic self regulatory processes. Several products released by the thymic epithelium are able to exert a stimulatory influence over lymphocyte development. Both T-cell immuno-competence and overall cell numbers in athymic mice can be restored by thymic epithelial tissue grafted into the recipient within a cell impenetrable container (Bach, 1976; Metcalf, Sparrow, Nakamura & Ishidate, 1961). A polypeptide isolated from bovine thymus is capable of modifying T-cell activity in vitro (Goldstein, Asanuma, Battisto, Hardy, Quint & White, 1970). This peptide, thymosin, also stimulates thymocyte proliferation and differentiation in vivo (Goldstein, Slater & White, 1966; Wara & Ammann, 1975). A second peptide, thymopoietin, has also been identified. This appears to influence the same processes as thymosin and its precise in vivo role is unclear (Goldstein, 1975). In addition to these two factors the thymus and T lymphocytes themselves produce a variety of active substances during an immune reaction. The majority of these influence the immune response and are not considered here. In vivo production of thymosin is particularly susceptible to hormonal modulation. Those extrathymic hormones reported to stimulate or inhibit thymocyte proliferation influence thymic hormone production in the same fashion. Increased thymosin production follows castration, whilst hypophysectomy decreases its production. Both of these effects

are reversed by restoration of the appropriate hormone (Comsa, Leonhardt & Ozminski, 1979).

A rapidly responding population is responsible for the increased thymic lymphocyte proliferation associated with physiological demands for lymphocyte production in the rat, and presumably other species (Whitfield, Rixon, Perris & Youdale, 1969; Miller & Osoba, 1967). As mentioned above proliferation of the rat thymic lymphocyte is closely linked to parathyroid hormone levels. Closer investigation has revealed that parathyroid dependent changes in the divalent cation climate of the external fluid modulate thymic mitotic activity in vivo. A similar relation between PTH divalent cations and mitosis is observed in rat bone marrow and regenerating liver (Perris, 1971). Artificially elevated divalent cation levels in vivo, produced by calcium or magnesium ion injections, were followed by the recruitment of the rapidly responding cell population (Perris & Whitfield, 1967). This "unphysiological" effect has close physiological parallels. The circadian rhythm in thymocyte recruitment closely follows changes in plasma calcium content, peaks in both are coincident (Hunt & Perris, 1974). During the cellular immune response the plasma calcium concentration rises at the same time as thymic proliferation is increased (Edwards, Mekori, Atkinson & Perris, 1976). Surgical removal of the parathyroid glands results in a hypocalcaemia and hypoplasia of the thymus and other parathyroid dependent tissues (Perris, 1971; Rixon & Whitfield, 1972). This reduced proliferative activity delays the cell mediated immune response and abolishes the hypercalcaemic proliferative episode in the thymus (Atkinson, 1976). Calcium may be the ultimate mediator of PTH dependent thymic proliferation as normocalcaemic aparathyroid rats do not exhibit thymic aplasia (Edwards **pers. comm.**). These effects of cal-

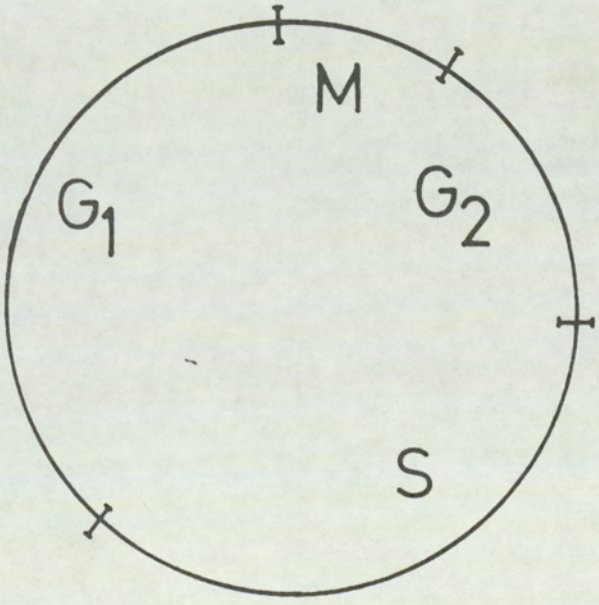
cium appear to be confined to the male rat (Dawson & Perris, 1974). This is due to the presence of the anti-proliferative sex-steroid, oestradiol in the female (Smith, Gurson, Riddell & Perris, 1975; Morgan & Perris, 1974). During the oestrus cycle decline in oestradiol, the calcium stimulatable response may reappear, as a marked increase in both calcium and thymic mitosis is evident (Smith et al. 1975). The present investigation has revealed that oestradiol may potentiate some aspects of calcium-induced proliferation in vitro. Thus it may be that in the female the presence of oestradiol lowers the calcium level required to trigger the quiescent population in the same way EGF acts upon mammalian fibroblasts (McKeehan & McKeehan, 1979). Consequently the hypercalcaemia inducing proliferation may not be readily apparent in vivo.

Whilst it may be argued that the mitotic response to calcium ions could be induced by an intermediary active mitogenic agent in vivo the many in vitro observations favour a more direct role for calcium. Such studies, using short-term serum free cultures, do not reproduce the precise intrathymic environment and may not reflect the coordinated response occurring in vivo. However, the intracellular regulatory mechanisms will remain essentially intact, providing a powerful tool for the investigation of in vivo mitotic regulation. The mitogenic capacity of magnesium illustrates this point. Although injections of magnesium ions, or their addition to cultured cells, provokes mitosis there does not appear to be a physiological counterpart to the stimulatory in vivo hypercalcaemia (Whitfield, Perris & Rixon, 1969; Perris, Whitfield & Rixon, 1967). Even though this is the case the magnesium-ion activated thymocyte has provided valuable information regarding the recruitment mechanism.

4.2. Mitogenic stimulation of rat thymocytes in vitro.

In vitro studies have provided a great insight into mitotic activation of the rat thymocyte. Four hours after the addition of mitogenic agents in vitro (and in vivo) the stimulated production of mitotic figures becomes evident (Whitfield, Rixon, Perris & Youdale, 1969; Perris, 1971). The quiescent, recruitable population, has been identified as a pre-S"G_o" population. Both S phase blockade and inhibitors of DNA synthesis prevent the recruitment response, which itself involves DNA synthesis (Morgan, 1976; Youdale & MacManus, 1975; Whitfield, Perris & Youdale, 1969). From comparisons with murine thymocyte cell cycle times it is possible to predict that the mitotic cells originate in the G_1 period (Metcalf, 1966)(Figure 4.1).

Fig.4.1. Murine thymocyte cell cycle.



Total cycle time Tc 8 hours. G_1 = 3 hrs, S = 3 hrs. G_2 = 1 hr. M = 50 min.

Such a G_1 entry point would agree with the observed cyclic AMP surge occurring thirty minutes after stimulation (MacManus & Whitfield, 1971). This cyclic AMP rise would then be equivalent to that observed as other cells pass the G_1/S boundary (see Section 3). This places the entry time approximately thirty minutes before the onset of the S phase.

The quiescent population may be recruited in vitro by a temporarily increased extracellular divalent cation concentration as stated above. Although this produces a smaller recruitment than the in vivo response the lower basal rate in vitro means that the same proportion of mitotic cells are produced in both cases. In addition to these two mitogens a variety of stimulatory compounds have been identified. Table 4.1 lists many of the agents so far shown to initiate recruitment in vitro.

Table 4.1. Mitogens acting upon cultured thymocytes.

Cortisol	Whitfield, MacManus & Rixon, 1970
Bradykinin	Perris & Whitfield, 1969
Adrenaline	MacManus, Whitfield & Youdale, 1971
Isoprenaline	Morgan, Hall & Perris, 1975
Growth hormone	Whitfield, Perris & Youdale, 1969
Oxytocin	Whitfield, Perris & Youdale, 1969
Prolactin	Whitfield, Perris & Youdale, 1969
PTH	Whitfield, Perris & Youdale, 1969
ADH	Whitfield, Perris & Youdale, 1969
Histamine	Morgan & Perris, 1975
Acetylcholine	Morgan, Hall & Perris, 1975
Insulin	Morgan, Hall & Perris, 1975

Table 4.1. (continued)

Dopamine	Morgan, Hall & Perris, 1975
Glucagon	Morgan, Hall & Perris, 1975
Concanavalin A.	Whitfield, MacManus, Boynton, Gillan and Isaacs, 1974.

The absence of extracellular calcium renders several of these compounds mitogenically impotent, the remainder being fully active in the absence of calcium ions. This calcium-independent group are without exception dependent upon the extracellular presence of magnesium ions. Thus thymocyte mitogens may be divided into either calcium or magnesium-dependent axes (Morgan, Hall & Perris, 1975). As the cations are mitogenic in their own right, it is quite probable that the mitogens act ultimately through their respective divalent cation. This concept of two discrete mitogenic axes has been considerably reinforced by the observation that sex-steroids exert an inhibitory influence in vitro strictly related to the cation dependency of the mitogen. As noted earlier oestradiol prohibits calcium stimulated proliferation. This inhibitory action is extended to all calcium-dependent mitogens (Morgan, Hall & Perris, 1975; Morgan & Perris, 1975). Furthermore testosterone addition abolishes selectively magnesium and magnesium-dependent mitogen action (Morgan & Perris, 1975). The calcium dependent axis may be re-defined as a calcium-dependent, oestradiol inhibitable, limb whilst the second axis may be termed magnesium-dependent and testosterone inhibitable.

The list of hormonal mitogens in table 4.1. includes hormones believed to exert their normal action through different cyclic nucleotide second messengers. Interestingly, the two members of each pair

(Insulin:Glucagon) and (Beta-adrenergic:Cholinergic or Alpha-adrenergic) act through separate mitogenic axes (Table 4.2.) Moreover, it can be seen that the two groups of calcium-dependent hormones act normally through cyclic GMP and that magnesium dependent mitogens classically use cyclic AMP (see Section 2).

Table 4.2. Cationic dependency of antagonistic hormone pairs (after Morgan and Perris, 1975).

Ca dependent	Mg dependent
Insulin	Glucagon
Cholinergic/Alpha-adrenergic	Beta-adrenergic

This dichotomy suggests that the two cyclic nucleotides are influencing separate mechanisms for inducing mitosis, in the same way as they can promote different secretory responses within one cell type.

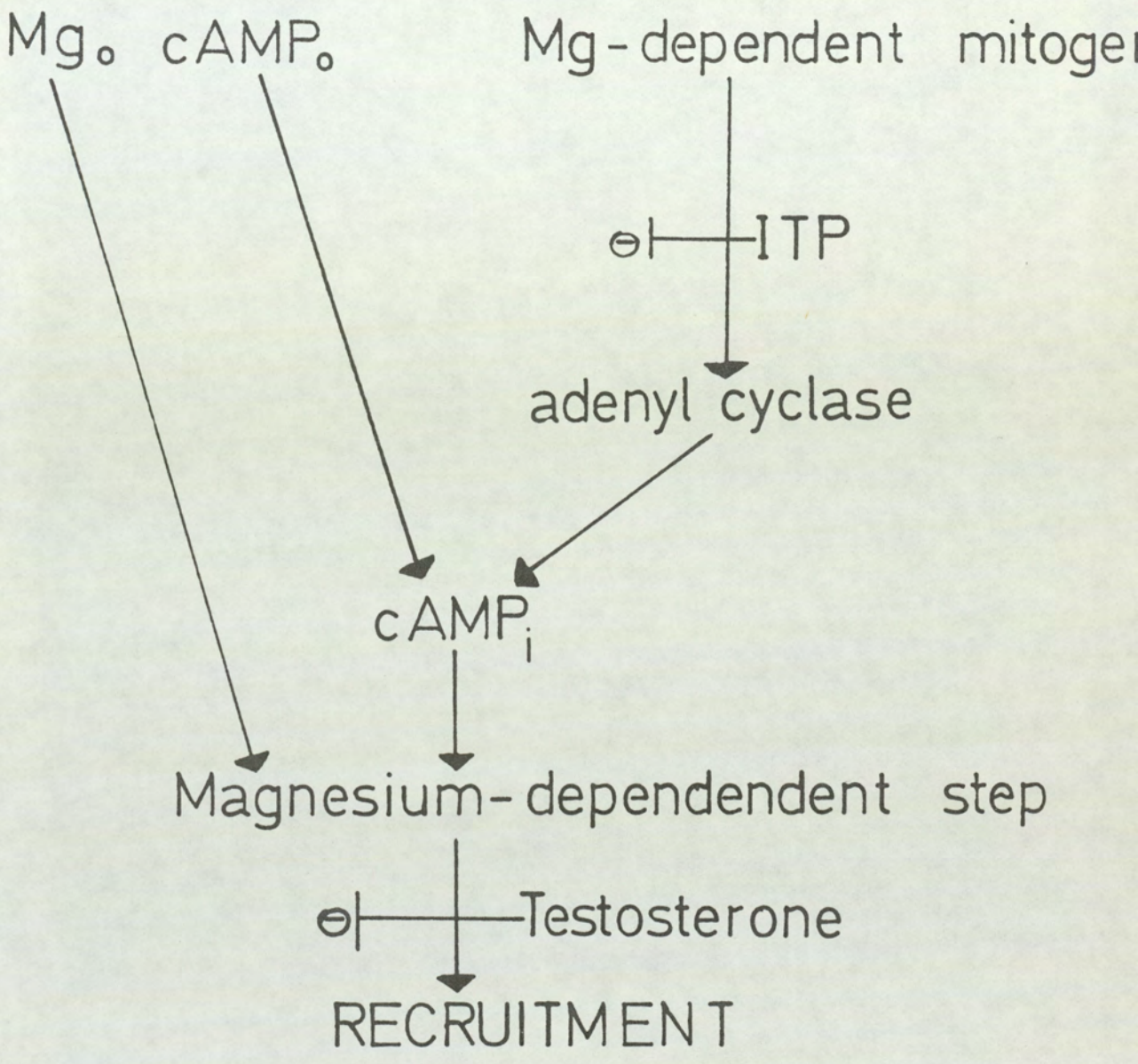
The two principle regulatory actions of cyclic nucleotides have been discussed in Sections 2 and 3. These are (1) to provide the calcium for stimulus-response coupling and (2) to regulate cell cycle progression. Thus any interaction between exogenously added cyclic nucleotides and the mitotic processes may be the result of several different reactions. It is important to recognise that mitogenic studies using such exogenous additions of cyclic nucleotides are not intended to represent faithfully in vivo mitogenic events. They have been performed solely to elucidate the mechanisms governing recruitment.

Addition of exogenous cyclic AMP or cyclic GMP will stimulate mitosis when at high extracellular concentrations (10^{-7}M). At such a level both cyclic AMP and GMP are magnesium-dependent and testosterone inhibitable (Morgan, Hall & Perris, 1977). Conversely, when added at low (10^{-12}M) concentrations both nucleotides will stimulate

mitosis in a calcium-dependent oestradiol sensitive manner (Morgan et al. 1977). As cyclic GMP can inhibit the thymocyte phosphodiesterase at 10^{-7} molar concentrations, and thereby elevate cyclic AMP within the cell, it is possible that high concentrations of both compounds raise intracellular cyclic AMP, which is itself believed to be involved in magnesium-dependent hormonal mitogenesis (Whitfield, MacManus, Franks, Gillan & Youdale, 1971; Morgan et al. 1977). The low cyclic nucleotide concentrations may both enhance cytosolic cyclic GMP levels but this is uncertain. If they did it would conveniently explain the calcium dependency of their action.

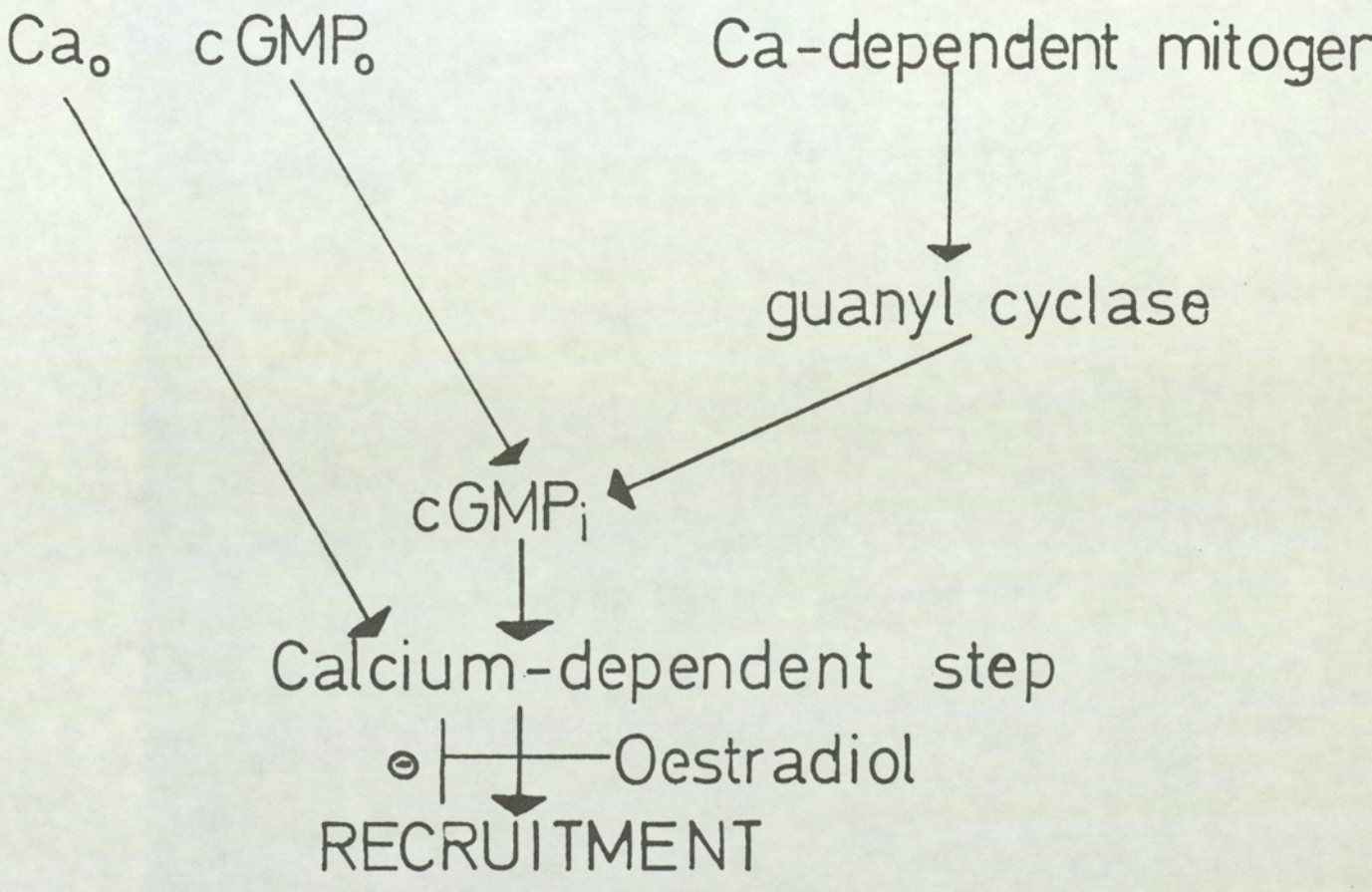
The addition of the adenylate cyclase activators Guanosine triphosphate (GTP) and Guanyl imido triphosphate (Gppnhp) will increase cytosolic cyclic AMP formation (Rodbell, Lin & Salomon,1974; Rodbell, Lin, Salomon, Londos, Harwood, Martin, Rendell & Berman, 1975). Both of these agents stimulate mitosis and require external magnesium ions (Perris & Morgan, 1975). Thus the magnesium-dependent step lies after cyclic AMP formation. This is confirmed by the use of inosine triphosphate (ITP) which will prevent mitogen induced cyclic AMP formation by inhibiting adenylate cyclase (Bilezikan & Aurbach, 1974). ITP addition inhibits only those mitogens believed to act via cyclic AMP formation (Perris & Morgan, 1975). The action of magnesium and external cyclic AMP themselves is unabated. This suggests the following model for thymic lymphocyte activation through an ultimate magnesium-dependent step. (Figure 4.2).

Fig. 4.2. Magnesium dependent mechanism of thymocyte recruitment



By analogy with this model the following figure (Figure 4.3) has been constructed suggesting a possible calcium and cyclic GMP interaction to evoke calcium dependent mitogenesis.

Fig. 4.3. Calcium dependent mechanism of thymocyte recruitment



The connection of two different nucleotide mechanisms within one cell type has already been illustrated in detail in Section 2. Indeed the dichotomy of the mitotic response between Mg/ cAMP and Ca/ cGMP axes has several parallels with other cell activation events. Most notable of these is the secretory response to an excitatory stimulus and the contractile response of muscle tissue. In both of these cases the ultimate link between external signal and the cellular response is a change in the internal calcium ion content. If such changes occur within the thymocyte they would directly implicate calcium ions in stimulus-mitosis coupling. The following experimental observations

have been made with a view to investigating the events occurring within the thymic lymphocyte cationic homeostatic system upon stimulation with the ultimate intention of identifying the precise mechanisms by which calcium (and magnesium) ions mediate the action of extracellular growth-promoting substances and influence the mitotic activity of quiescent cells.

5 Methods and Materials

Unlike the majority of continuously cultured cells the mitotic behaviour of thymic lymphocytes, in short term suspension cultures, closely follows that observed in the intact animal (Perris, 1971). The regular appearance of mitotic cells *in vivo* and in culture is the result of comparable basal proliferative activities (Hunt, 1974). Moreover a series of mitogenic agents exhibit the same capacity to stimulate the proliferation of a normally quiescent (G_o) cell population *in vivo* and *in vitro* (Morgan & Perris, 1974; Whitfield, Rixon, MacManus and Balk, 1972). This parallel reactivity provides an almost unique insight into mitotic events closely related to *in vivo* proliferative responses of the native tissue. The technique of short-term suspension culturing is itself remarkably suited to the study of *in vitro* mitotic activity as detailed below:-

1. The availability of a responsive, naturally quiescent, cell population avoids the need to induce cell synchrony or quiescence by using artificial procedures.
2. The short incubation period avoids the necessity to supply serum for growth supplementation, thereby avoiding complex interactions between mitogenic and serum factors. Furthermore the simple salt solution permits experimental manipulation of both organic and ionic components of the extracellular medium.
3. Unlike cultured adherent cells the entire thymocyte surface is exposed to the culture medium, and consequently added mitogens. Apart from providing a physiological environment this enables clear examination of such parameters as nutrient uptake or receptor binding.
4. Density dependent growth inhibition and nutrient depletion are avoided due to the short culture period, moreover constant agitation

avoids the local accumulation of potentially toxic metabolites.

5. The rapid and simple preparation of the cell suspensions avoids the metabolic duresses incurred during the preparation of cell cultures from already established cell lines and intact tissue.

Thymocyte cultures were prepared using a modification of the technique developed by Whitfield and co-workers (Whitfield, Brohée and Youdale, 1964). Albino male Wistar rats[Footnote 1] weighing under 200 grammes were used exclusively, avoiding fluctuations in mitotic activity due to age-related thymic involution or oestrus cycle endocrine periodicity (Perris, 1971, Smith, Gurson, Riddle and Perris, 1975). Animals were housed in a controlled-temperature environment with free access to food and water. Lighting was partly regulated, with electric illumination being available from 08.00 to 17.00 hours. Animals were sacrificed between 08.30 and 09.30 hours, thereby minimising changes due to the inherent circadian rhythm in rat thymocyte mitotic activity (Hunt and Perris, 1974). Despite these precautions a slight seasonal drift in basal activity was observed. This may reflect seasonal changes as it correlates with changes in the plasma calcium content (Edwards and Atkinson, unpublished observations).

Thymus glands were rapidly excised from animals anaesthetized with a weak ether/air mixture, barbiturates were not employed as they have a reported antimitotic action (Baserga and Weiss, 1971). Recently it has become evident that stress-induced changes in rat plasma hormone levels are produced by a variety of sacrifice techniques (Wong, personal comm.) However the time course of these changes only coincides with the later stages of gland excision so the washing procedure after removal should adequately remove the stress-hormones before they can influence mitotic

Footnote 1. Bantin & Kingman Ltd.

activity. Indeed the relatively unstressful decapitation technique does not alter basal mitotic activity. After removal of the thymic tissue the entire organ was rapidly rinsed in three changes of 0.9% saline, which removes superficial debris and blood. Thymocytes were freed from the reticulum by thorough mincing in culture medium, cell aggregates and the reticulum were then removed by filtration through four-ply moistened muslin. The resultant cell suspension was assessed using a Coulter ZB electronic particle counter[Footnote 1]. After adjusting the concentration to approximately 5×10^7 cells per millilitre aliquots (1 ml) were placed into sterile plastic culture tubes and incubated at $37^{\circ}C$ in a rotating roller drum assembly for six hours. During this period the number of viable cells (as assessed by the Trypan blue exclusion) only dropped by two to four per cent from 92 - 96% initial to 90 - 94%. This drop is accounted for by a reduction in the small-cell population which are mitotically inert.

Although thymocytes appear to proliferate normally in a simple salts solution a more complete pre-prepared culture medium was used. Medium 199[Footnote 2] (Morgan, Morton & Parker, 1950) was used throughout, avoiding the possibility of mitogen-induced nutrient requirements causing G1 restriction. The medium 199 was manufactured nominally free of added calcium and magnesium ions, having no detectable calcium present (detection limit 3×10^{-5}M). EGTA buffers were not used to regulate the free calcium concentration as the chelator may perturb cell membranes or chelate other vital metal ions. Where desired calcium and magnesium ions were added from stock-solutions of their respective chloride and sulphate salts. The various ionic environments will be referred to by the designation provided under Figure M1 (column 1) which also lists the final

Footnote 1. Coulter Electronics Ltd.

Footnote 2. Wellcome Ltd.

media composition (column 2). Addition of putative mitogenic or anti-mitotic agents was performed as desired in 10 microlitre aliquots. An equal volume of the diluent used (0.9% NaCl or 0.9% NaCl Ethanol) was also added to the control cultures.

Figure M1

Divalent cation content of culture medium 199 employed in mitotic investigations.

Treatment	Ionic content (mM)		Volume of stock solutions added per ml. of culture (µl)	
	Ca	Mg		
Control	0.6	1.0	10	10
High Ca	1.8	1.0	30	10
High Mg	0.6	2.5	10	25
OCa	-	1.0	-	10
OMg	0.6	-	10	-

As the mitotic activity of cultured cells is markedly pH sensitive (Eagle 1973) precise buffering is essential, particularly in long term cultures. For these short-term investigations adequate buffering was provided by a simple bicarbonate-carbon dioxide equilibrium. This was established between the CO_2 evolved by the cells and $NaHCO_3$ added at 2.4 g/l of medium. The cell concentration employed ensures sufficient CO_2 is produced, provided the vessels remain sealed, to maintain a pH of 7.2 ±0.2 throughout the incubation. Where appropriate the metaphase arresting agent colchicine [Footnote 1] was added to the culture medium to give a final concentration of 0.6×10^{-4}M, this being the minimum required to provoke spindle disruption without influencing mitotic activity (Whitfield, MacManus & Gillan, 1973). After six hours of incubation two drops of the culture were removed and placed on a microscope slide and mixed with one drop of a binding agent (calf serum). After drying in a

Footnote 1. Ciba Ltd.

stream of warm air the cells were fixed in neutral formalin and stained in Delafield's haematoxylin according to established procedures (Whitfield, Brohee, & Youdale, 1964). Mitotic activity was determined by scoring the cell population for the percentage of cells arrested in quasi-metaphase configurations. One thousand cells from duplicate cultures were examined under oil immersion at 1250 x magnification by two observers. The 4000 + cells counted will provide an adequate sample of the culture; previous studies have shown that trained observers rarely differ by more than 0.5% using this technique. Where such differences occurred the slides were recounted. Using this technique with a basal ionic composition (see figure M1) approximately 4% of the cells entered mitosis over the six hour period. When either cation was elevated approximately 6% of the cells reached mitosis over the same period (figure M2). The substitution of the inorganic buffer HEPES did not alter the measured mitotic activity.

Figure M2. Mitotic activity (% colchicine metaphase) observed with altered ionic environments and buffer system.

Culture conditions	Buffer system	
	Bicarbonate/CO_2	Hepes
Control	4.1±0.1 (N = 65)	3.9±0.2 (N = 6)
High Ca	6.3±0.1 (N = 55)	6.1±0.2 (N = 6)
High Mg	6.0±0.2 (N = 15)	6.1±0.2 (N = 6)
O Ca	3.9±0.1 (N = 25)	
O Mg	3.9±0.1 (N = 25)	

Although the rate of proliferation is nearly doubled during mitotic stimulation (see above) the actual increase is only two per cent of the entire population. Clearly the use of a subjective technique to measure such small changes is potentially hazardous, and was only attempted by trained observers. Despite the laborious and somewhat subjective nature

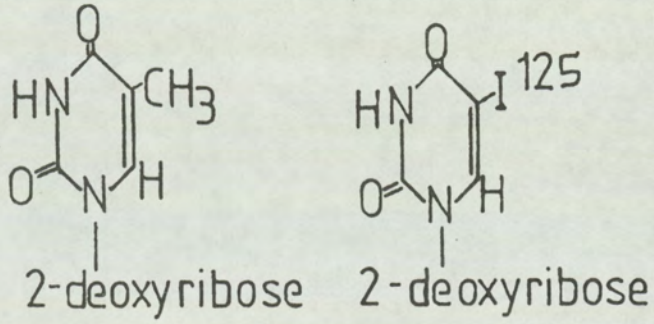
of the colchicine metaphase technique it has proven superior to alternative methods. A brief review of these is now included, demonstrating the unsuitability of other subjective and objective techniques for the measurement of rat thymocyte mitotic activity. Conveniently these may be grouped under direct and indirect observation of the dividing cell.

Direct. The simplest such estimate would be the measurement of the progressive increase in cell numbers as proliferation proceeds. Unfortunately over a short incubation insufficient cells would divide to make such measurements valid. During mitosis the dividing cells in the culture will become morphologically distinguishable. A count of the percentage of mitotic cells in the entire population (mitotic index) would then give a measure of proliferation. If a given treatment recruited additional cells it would be supposed that the proportion of cells in distinct phases of the cell cycle would increase correspondingly, giving a higher mitotic index. If however, the agent altered the duration of mitosis, or indeed other phases of the cell cycle, this would result in a spurious alteration in the mitotic index. Furthermore, as thymocytes are recruited in a semi synchronous fashion the number of mitotic figures would only be elevated for a short period, making such measurements hazardous. The laborious and highly subjective nature of direct estimates has influenced the development of indirect techniques.

Indirect. Most such techniques attempt to measure DNA synthesis, assuming this to be directly related to mitotic activity. Autoradiographic analysis of cells which have incorporated the radioactive precursor ^{3}HThymidine (^{3}HTdR) will reveal that portion of the population synthesizing DNA. However, DNA synthesis by non-mitotic cells, tracer reutilization or changes in the length of the cell cycle may spuriously

alter the number of labelled cells.To counter these problems the proportion of labelled cells present in recognisable mitotic configurations must be determined (per cent labelled mitoses). This is extremely sensitive but again is laborious and somewhat subjective. The simplest method for quantifying DNA synthesis would appear to be radiometric quantification of labelled precursor incorporation into newly synthesized DNA. This requires that the precursor pool remains relatively constant during the synthetic phase of the cell cycle. Unfortunately recent experiments have revealed that the thymidine pool of cultured cells is highly labile (Simnett & Fischer 1973; Youdale and MacManus 1975). Therefore it is essential to establish whether the thymocyte precursor pool remains constant. Iodine labelled deoxyuridine (^{125}IUdR)[Footnote 1] which is incorporated into DNA as a thymidine analogue was employed (see Figure M3).

Figure M3. Chemical structure of Thymidine and ^{125}IUdR.



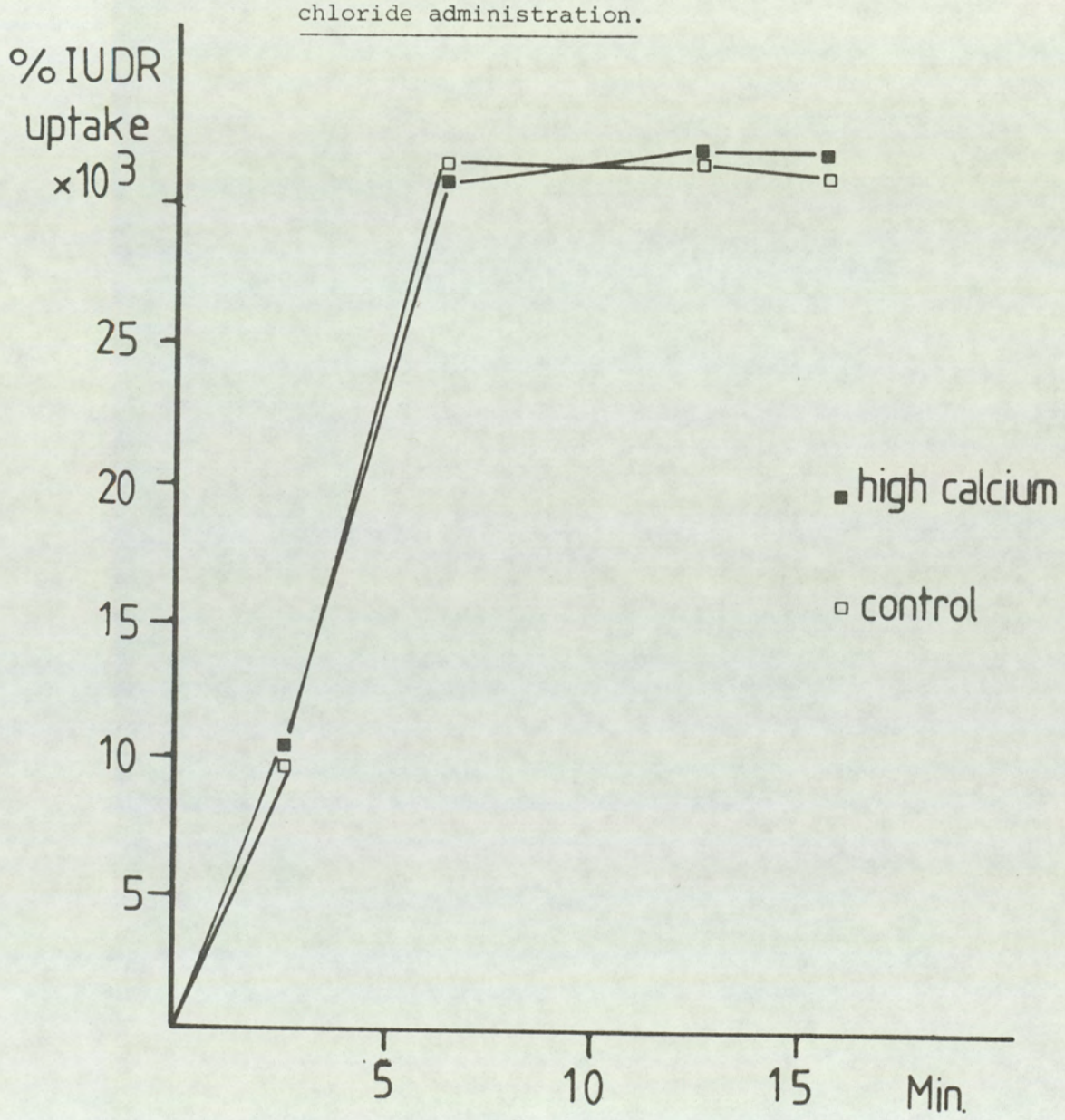
This analogue is highly stable in comparison with ^{3}HTdR, moreover in contrast to thymidine, the iodinated derivative is only poorly incorporated into DNA. This ensures that reutilization of incorporated isotope is negligible (Feinendeegen, 1973).

The initial experiment was performed to determine if mitotic stimulation by high calcium influenced the rate of uptake of ^{125}IUdR into the cells. Using the isotope tracer uptake technique described

Footnote 1. Amersham Ltd.

below the incorporation of ^{125}I into thymocytes was found to be identical in both control and stimulated cells. Discrimination is not exerted at the entry stage in the metabolism of nucleotide (Fig.M4). Moreover, the transmembrane equilibration of the tracer was extremely rapid, thereby minimizing the pre-incubation time required to saturate internal nucleotide pools.

Figure M4 ^{125}IUdR uptake into thymic lymphocytes following calcium chloride administration.



Cells were incubated with tracer for the desired times and then separated from the incubation medium by centrifuging through a water-impermeable oil. The uptake is expressed as percentage radio-activity taken up by the cells.

When the effect of known thymocyte mitogens was investigated upon the incorporation of ^{125}IUdR into ethanol insoluble (presumably DNA bound) pools (Pritchard & Micklem 1972) it was apparent that DNA synthesis did not correspond with mitotic activity (Fig.M5)

Figure M5. Effect of mitogen addition upon ^{125}IUdR incorporation into DNA

Treatment	% of control incorporation	Mitotic activity
Zero calcium	102	Basal
High calcium	85	+++
High Magnesium	87	+++
Concanavalin A 5µg/ml*	89	+++
Concanavalin A/Zero Calcium+	102	Basal
Adrenaline 5 x 10^{-5}M*	83	+++
Adrenaline/Zero Mg ‡	105	Basal

* Maximum mitogenic dose
\+ Ca dependent mitogen
‡ Mg dependent mitogen

Cells were pre-incubated with tracer at a concentration of 2µCi/ml for 30 minutes. Mitogens were then added and cells harvested after 30 minutes by centrifugation with 70% ethanol. After three ethanolic extractions the remaining radioactivity was measured. The results are expressed as % of control incorporation and are from three separate experiments.

From figure M5 it is evident that enhanced proliferation was always associated with a reduced isotope incorporation. Furthermore, mitogens in the absence of their required divalent cations did not depress incorporation in the absence of stimulated mitosis. It is probable that the tracer incorporation is lowered in stimulated cells due to activation of endogenous thymidine synthesis. This would be mediated through thymidylate synthetase and thymidine kinase (producing de novo thymidine and converting other nucleotides (Weber 1975).)

As the use of indirect techniques is somewhat inaccurate the assay

of mitotic activity chosen for these experiments was the direct, colchicine induced, metaphase arrest estimation. Whilst somewhat subjective this assay is the most convenient and simple to perform, being the most suitable direct technique available. By using the minimum stathmokinetic concentration the side effects of tubulin disaggregation will be lessened, if not avoided. Indeed, colchicine treatment reveals the same number of dividing cells as the PLM technique. Moreover at the concentration used colchicine did not influence glucose, leucine, calcium, ^{3}Hthymidine or ^{125}IUdR passage across the cell membrane in unstimulated cultures (Morgan, 1976, Atkinson, unpublished data).

It was suggested above that the coupling between stimulation and proliferation is provided by changes in the transmembrane ion distribution, linked ultimately to an increased intracellular calcium concentration. In large cells such as squid axon or amphiuma red cells the free calcium concentration of the internal fluid may be measured by calcium induced fluorescence of an appropriate chromophobe dye introduced into the cytosol. Such a technique is impractical with the thymocyte as its size precludes the micro-injection of fluorescent dyes. Direct measurement is also inappropriate as the ratio of free to bound calcium is very small and would change during measurement. To reveal the mitogen induced alterations in cytosolic calcium an indirect measurement technique was employed. This quantifies the transient transmembrane fluxes of tracer molecules during stimulation. To be effective the tracer must satisfy the following criteria first proposed by Ussing.

1. The tracer must have the same thermodynamic properties as the molecule it replaces e.g. molar volume and activity coefficient.
2. It must not alter the system chemically, nor must its transport

cause a shift in the centre of mass of the system. The latter would only be appropriate when the mass of the tracer greatly exceeds that of the substituent, i.e. tracer/substituent mass ratio must be close to unity.

The tracer chosen to represent calcium was the β-emitting isotope Calcium-45[Footnote 1]. Being the isotopic form of the natural molecule this will fulfil all conditions. When added at a concentration of 1 μCi/ml the calcium content of the tracer (50 μg/ml) present in the 10 μl aliquot will not significantly alter the external calcium concentration. Potassium isotopes suitable for tracer investigation are very costly and extremely short lived. Consequently the widely used periodic relative Rubidium-86 [Footnote 1] was chosen. This γ-emitting isotope was chosen to substitute for potassium, which it can represent in a variety of potassium-dependent processes. The β radiation from calcium^{45} was detected using liquid scintillation spectroscopy in a Packard tri-carb counter, Model 2660. Rubidium^{86} was measured using a Tracerlab gamma-set 500 pre-calibrated for the rubidium photopeak. In both cases internal standards were used to correct for decay, the efficiency of β detectors was corrected by using an online data handling system.

It proved necessary to modify the culture technique to enable measurement of the tracer redistributions, the changes made are detailed below.

1. As continual sampling from the culture during incubation would abolish the carbon dioxide/bicarbonate equilibrium, and consequently alter the pH, a more suitable buffer system was adopted. Hepes[Footnote 2] (N-2 Hydroxyethyl piperazine N-2 ethan sulfonic acid) was added

Footnote 1. Amersham Ltd.

Footnote 2. Flow Laboratories Ltd.

to the culture medium at a final concentration of 1 mM. Following the manufacturer's recommendation the bicarbonate content of the medium was reduced to 1g/l, allowing an adequate metabolic pool. This buffer system supported normal and stimulated mitotic activity to the same extent as the CO_2/bicarbonate (Figure M2)

2. A thirty minute pre-incubation period, allowing recovery from preparative procedures, was used for all ionic uptake studies, whilst a sixty minute period was employed for efflux investigations. Such pre-incubations did not influence the mitotic competence ; responsiveness to mitogens remained constant during a one-hour preincubation. After a series of preliminary experiments, the cell concentration was raised two-fold. This permitted reproducible sampling of the cell and supernatant radioactivity. The adjusted cell concentration of 10^8 cells/ml did not influence mitotic capacity of the cells.

The major problem of tracer incorporation measurements is the efficient removal of the unincorporated isotope present in the extracellular fluid. Washing of the cells after incubation has proven inadequate, not only due to tracer exchange between cells and washing medium but also because the cell membrane permeability is drastically altered by certain mitogens (Segel,Lichtman,Hollander, Gordon& Klemperer, 1976). Consequently a modification of the centrifugal technique developed by Danon & Marikovsky was used (Danon & Marikovsky,1959; Funder & Weith,1967; Freedman, Raff & Gomperts, 1975). This method completely avoids the use of washing procedures.

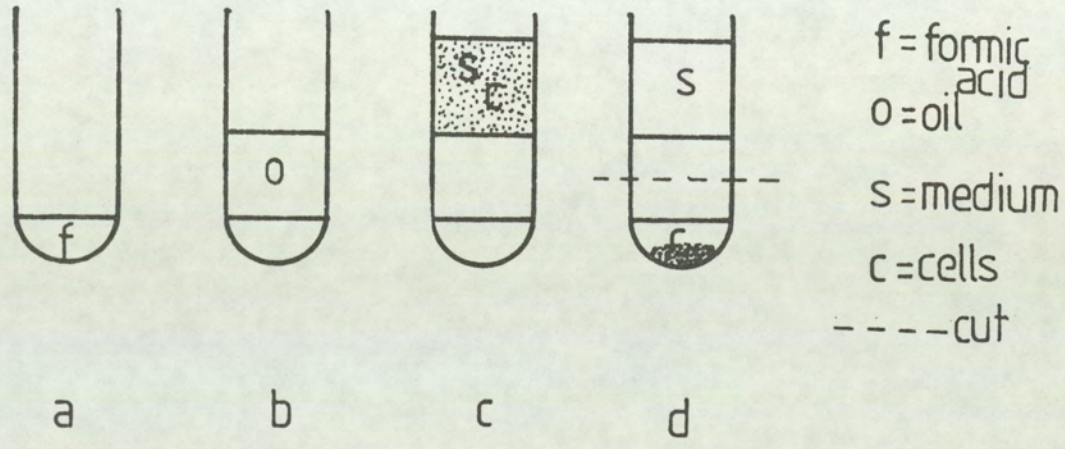
After the preincubation aliquots of the thymocyte cultures were withdrawn and added to pre-warmed tubes containing tracer and any desired compound. At different times thereafter 200 µl aliquots, containing 2×10^7 cells,were removed for the measurement of tracer distribution. All such assays were performed in triplicate. The cells

were separated from the unincorporated tracer by centrifugation through a water impermeable oil barrier. This was achieved by layering 100 µl aliquots of oil above 98% formic acid in 400 µl microcentrifuge tubes Footnote 1 (Figure M6a). At the desired time 200 µl of medium and cells were layered above the oil (Figure M5b) forming a three-tier gradient. The sealed tubes were rapidly centrifuged at over 8500 g for two minutes in a Beckmann microcentrifuge Model B Footnote 1. This resulted in the isotope laden cells passing through the oil layer into the acid layer (Figure M6c). The incubation time for each tube was taken from the time of addition to isotope to the start of the centrifuge run. This is approximate but as the centrifuge achieves maximum force within five seconds it is an adequate approximation. After centrifugation the tubes were immersed in liquid nitrogen which solidified the contents. The supernatant and cell pellet were separated by a lateral cut through the oil layer (Fig. M6d).

Three factors, all dependent upon inherent properties of the oil layer, will determine the sensitivity of this technique. The first priority is for the oil to be chemically inert, secondly it must be highly water impenetrable, finally maximum cell passage should occur with the minimal carry-over of the supernatant. Preliminary experiments revealed that a minimum oil volume of 75 µl was essential to prevent mixing of the acid and cell suspension during pipetting. Thus a standard 100 µl oil barrier was adopted throughout. An initial screening for water impermeability, using an aqueous $Na^{125}I$ solution, revealed that many oils were insufficiently resistant. The passage of Chromium -51 labelled cells through suitably water resistant oils was determined

Footnote 1. Beckmann Ltd.

Fig.M6 The three layer density gradient for the separation of radio-labelled cells and unincorporated isotope.



by comparing the Cr 51 remaining in the supernatant after centrifugation with that present in the cell pellet (Figure M7). This revealed that the silicone oil Ms550 proved to be superior in allowing maximum cell passage.

As cells pass through the silicone oil layer some of the extracellular fluid will be trapped within the interstitial spaces. This fluid, containing significant quantities of isotope, may be carried completely into the formic acid, where it can readily be quantified using an extracellular space marker. Alternatively, as the cells traverse the hydrophobic region the trapped fluid may be lost into the oil layer. This would generate an isotopic gradient across the

Fig. M7. Passage of Chromium-51 labelled thymocytes through 100 µl aliquots of water-impermeable oils.

Oil	% passage
Silicone oil Ms 550 Footnote 1	98
nDibutyl Phthalate Footnote 2	94
nDibutyl Phthalate/Corn oil (10:3)	95
Silicone oil Ms550/Corn oil (10:2)	95

Cells prelabelled with $NaCr^{51}O_3$ for 30 minutes at 10^8 cells/ml. Washed in culture medium 3 times. 200 µl aliquots placed in triplicate tubes above the oil barrier and centrifuged. Results from the separate experiments are mean % activity in cell pellet.

oil, leading to considerable inaccuracies in the separation of incorporated and non-incorporated isotope. The isotope content of the silicone MS550 oil was determined after centrifugation of prelabelled (Calcium^{45}) Footnote 3 cells or cells added to a labelled (3H-inulin) Footnote 3 solution and rapidly centrifuged. Thus any transfer of label between cells and oil or supernatant and oil will be detected. The isotope content of the oil layer after centrifugation was determined by isolating it from both supernatant and formic acid layers. This was achieved by adding 50 µl of an oil denser than Ms 550 to pre-centrifuged tubes (Figure M8a). A second centrifugation placed this oil between the Ms550 and the cell pellet (Figure M8b). A third oil, lighter than Ms550 was placed at the oil/supernatant junction by the same procedure (Figure M8c). After freezing in liquid nitrogen the Ms550 layer was completely removed by cutting through both

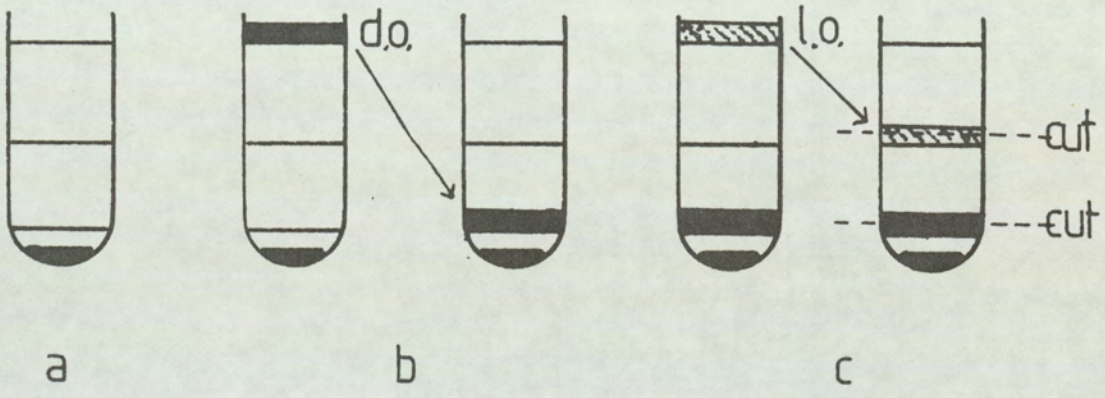
Footnote 1. Dow Corning Ltd.

Footnote 2. B.D.H. Ltd.

Footnote 3. Amersham Ltd.

additional oil barriers (Figure M8c). There was no detectable radioactivity present in the silicone barrier, indicating that no exchange of tracer takes place between cells or the trapped extracellular fluid and the oil barrier. Thus any fluid between cells as they enter the oil is carried into the formic acid during centrifugation. By measuring this carry over the activity in the formic acid may be corrected to represent cell-associated isotope.

Fig.M.8 Isolation and separation of impermeable silicone oil barrier after centrifugation of tracer containing cells or supernatant.



Both EGTA complex Calcium 45 and 3H-inulin were used as extracellular space markers, allowing measurement of the carry over. When incubated with the thymocytes for 20 minutes neither tracer entered the thymocytes, as indicated by a constant value for carry over with time.

Furthermore,the amount of trapped isotope increased in proportion to increased cell numbers. Both tracers may therefore be considered inert. Under basal conditions the carry over of the extracellular fluid represented a constant 0.13% ±0.003 (n=35) of the supernatant. This contributes a considerable amount of radioactivity to the cell pellet and any change in this value provoked by experimental treatment of the thymocytes will artificially change the isotope "associated" with the cells. Thus a range of mitogens and other agents used in the subsequent studies were added to cultures to determine their effect, if any, on the carry over. In the presence of mitogenic concentrations of calcium, magnesium, adrenaline or ouabain,alone or in conjunction with inhibitory concentrations of oestradiol or testosterone,failed to influence the carry over to any extent. Thus the amount of calcium entering the thymocytes can be derived from the following equation, all values being pre-corrected for counting efficiency and background radioactivity.

$$(1)\quad \text{Uptake}_{(\text{isotope + cold})} = \text{Specific activity of tracer in external medium} \times \left(\text{dpm}_{(\text{cell pellet})} - \text{dpm}_{(\text{carry over})}\right)$$

The movement of the rubidium tracer into the cytosol must be expressed as a distribution ratio between cells and medium as the specific activity of rubidium in biological fluids is a meaningless value. As the true specific activity of the isotope within the cytosol is unknown due to the many subdivisions of the internal distribution the efflux data is presented differently (see below).

$$(2)\quad \text{Tracer movement} = \frac{\text{dpm}_{(\text{cell pellet})} - \text{dpm}_{(\text{carry over})}}{\text{dpm}_{(\text{supernatant + cell pellet})}}$$

Any change in the isotope content of the cells may be due to three different processes. Firstly if the rate of tracer uptake is changed, secondly if the efflux component changes without a change in uptake and thirdly if the cellular volume changes without an actual increase in internal ion concentration. A series of experiments was performed to establish if indeed the cellular volume was changed during mitotic activation. Using a Coulter ZBI cell counter coupled with a channelyzer pulse height analyser [Footnote 1] a distribution profile of cellular volumes was obtained under different experimental conditions. To compare the volume profiles after different treatment an arbitrary index of the profile was generated from each curve. The cell volume corresponding to half the peak cell numbers was extrapolated and assigned the value R (see Figure M9).

Any change in the volume profile will shift the curve, and hence R, accordingly. The new value of R may be compared with the control value and the volume shift quantified. If no shift occurs, the value of treated cells R_T will equal that of control cells R_C and the ratio of R_T/R_C will be unity. An increase in the cell volume will increase the value of R_T and the ratio R_T/R_C will exceed one. (Figure M10). Conversely cell shrinkage will decrease the value of R_T/R_C.

Figure M11 lists the ratios obtained for R_T/R_C after several experimental manipulations. The observations presented were made at 10 minutes although the same values were still measurable after 30 minutes and 1 hour. It is evident from Figure M.11 that no detectable change in cell volume occurs during the activation. This is to be expected as the recruitment phenomenon results in the division of medium to large thymic lymphocytes to produce small daughter cells.

Footnote 1. Coulter Electronics Ltd.

Fig. M.9. The volume distribution profile of thymocytes showing the extrapolation of the volume index R.

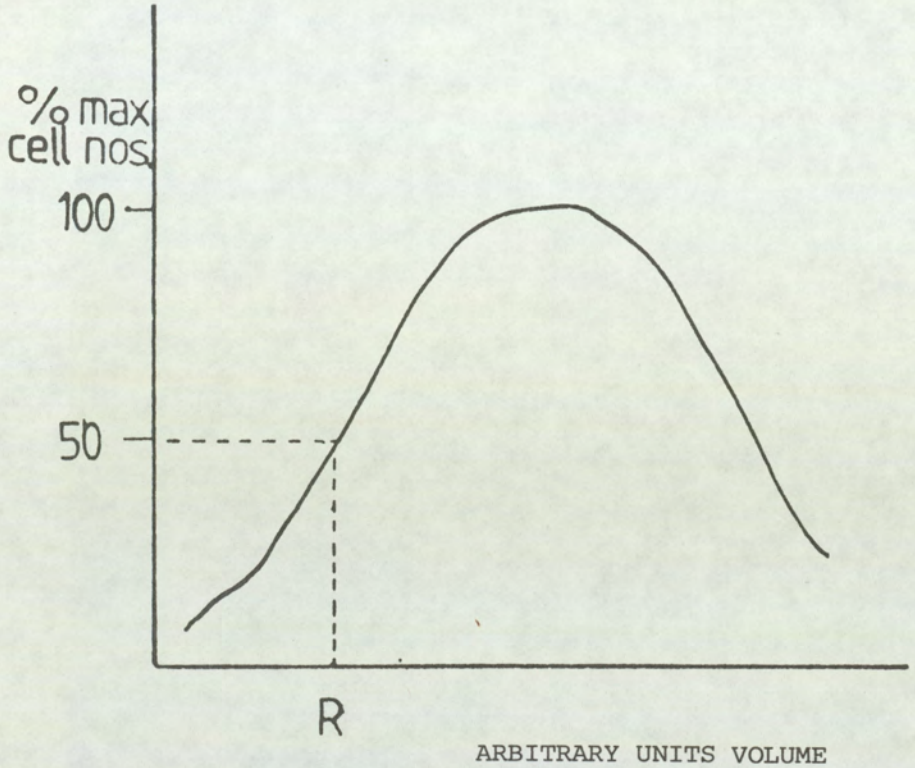
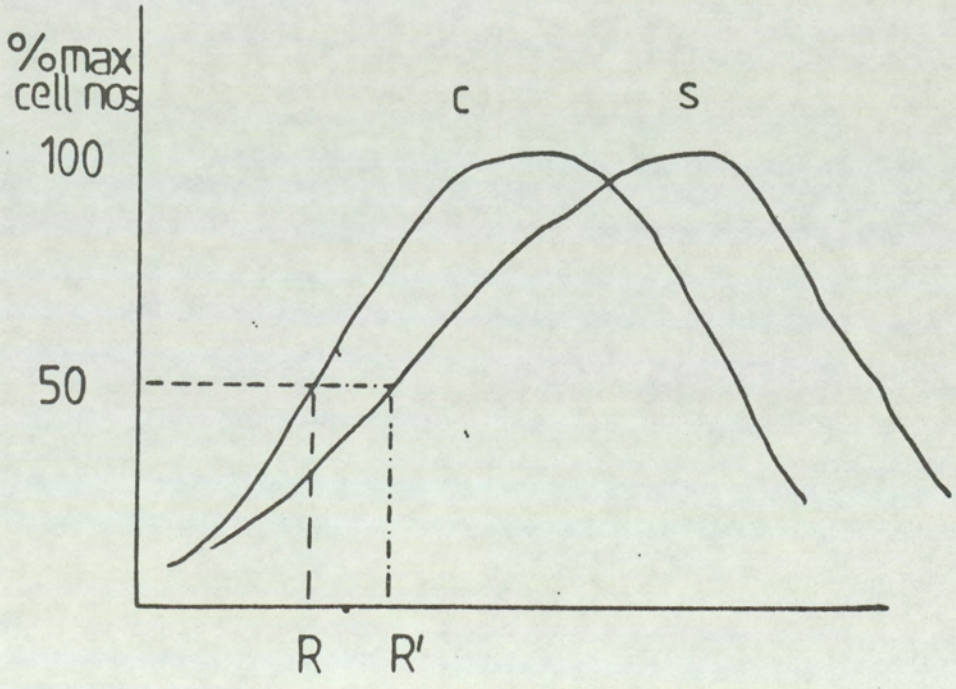


Fig. M.10 Increased cell volume resulting from a 15% osmotic shock



Two cultures of thymocytes were established as described. To the test culture of 1 ml a 150 µl aliquot of distilled H_2O was added. The control culture received 150 µl of medium. After 10 minutes the volume profiles were measured.

Fig. M.11. The volume indices obtained after treating thymocyte cultures with mitogenic and antimitotic agents

Control	R = 1.00
High Ca	1.02
High Mg	1.01
Oestradiol (Oe)	1.03
Testesterone (To)	0.97
High Ca + Oe	1.04
High Mg + To	1.00
Adrenaline 5×10^{-5} M	0.96

Having proven that the uptake of isotope can be measured and that cell volume is fixed it becomes essential to determine the final variable, efflux. Ideally this would be measured in parallel with the uptake experiments, using a different isotope for each directional flux. However, unidirectional measurements are permissible provided tracer movement itself is measured under near identical conditions in both uptake and efflux studies. To measure unidirectional efflux the intracellular ion pool(s) must be prelabelled with tracer, ideally to equilibrium. This entails a further modification to the experimental procedure. Cells were pre-equilibrated with 1μCi/ml of tracer for one hour, after which time excess tracer in the supernatant was removed by three washes in prewarmed medium. This treatment did not influence the mitotic responsiveness of the cultures, nor does it deplete internal ionic stores as efflux follows an identical pattern to unwashed cells. Although all the internal ion pools are not fully saturated during this pre-incubation the flattening of the uptake curves for both calcium and rubidium after 1 - 2 hours suggests the internal pools are becoming so. After the washing procedure the cells were handled in the same way as for uptake experiments. However, the washing period itself proved to be of variable time and therefore small variations in the starting isotope content between experiments was unavoidable. To overcome this the efflux of isotope was expressed as the percentage of the initial isotope content (3).

$$(3) \quad \text{Efflux at time (t)} = \frac{(\text{dpm (cells)} - \text{dpm (carry over)})_{t_t}}{(\text{dpm (cells)} - \text{dpm (carry over)})_{t_o}}$$

6 Results

Rat thymocytes continued to enter mitosis and accumulate as recognisable mitotic configurations when suspended in colchicine-supplemented culture medium 199. When the calcium and magnesium composition of the external medium was adjusted to reflect the ambient levels of normal rat blood, some four percent of the cells entered mitosis over a six-hour period (Fig. R1). This basal proliferative activity was not influenced by the exclusion of either, or both, of the divalent cations (Fig. R1). Thus it would appear that neither calcium nor magnesium are essential for the completion of the mitotic cycle by cells already committed to divide prior to removal from the rat. In accord with previous studies (see Introduction, section 4) an approximately three-fold elevation in the extracellular concentration of either calcium or magnesium initiated the recruitment, into the mitotic cycle, of a normally quiescent cell population. This additional cohort of cells served to elevate the proportion of mitotic cells within the culture population to six percent (Fig. R1).

Fig. R1. The mitotic activity of rat thymocytes cultured in vitro under different external divalent cation environments.

Treatment	Divalent cation content (mM)		% of cells in mitosis after 6 hrs. (Mean ± SEM)	
	$CaCl_2$	$MgSO_4$		
Control	0.6	1.0	4.1±0.1	n = 20
Zero calcium	0	1.0	3.9±0.1	n = 10
Zero magnesium	0.6	0	4.1±0.1	n = 10
Zero calcium & magnesium	0	0	3.7±0.2	n = 5
High calcium	1.8	1.0	6.3±0.2	n = 20***
High magnesium	0.6	2.5	6.0±0.2	n = 20***

*** Significantly different from corresponding control cultures ($P<0.001$)

If the quiescent population of thymocytes is first recruited into the mitotic cycle, via an in vivo hypercalcaemic episode, a subsequent exposure in vitro to elevated calcium or magnesium levels no longer induces additional cells to divide (Fig.R2). Thus it may be concluded that both calcium and magnesium ions act upon the same population of recruitable cells.

Fig.R2. The influence of divalent cations upon the division of thymocytes previously recruited by an in vivo immune challenge of sheep red blood cells (SRBC)

Treatment of animals three days prior to an invitro culture of thymocytes	In vivo plasma calcium concentration at day three (mg%)	Mitotic activity (% cells in mitosis after six hours)		
		Control	High calcium	High magnesium
Control (Saline injection n = 5)	10.1±0.2	3.2±0.2	6.1±0.3	6.0±0.2
Stimulated (SRBC injection n = 5)	10.7±0.2**	6.6±0.3***	6.5±0.3	6.1±0.2

** Significantly different from control cultures $P < 0.005$

*** Significantly different from control cultures $P < 0.001$

The stimulated rats received an intra-peritoneal injection of approximately 2×10^8 washed SRBC suspended in 0.9% saline. After three days the animals were sacrificed and thymocyte cultures prepared in the usual manner. The immunisation-induced hypercalcaemia and thymic proliferation observed by Edwards et al. 1976 were confirmed. The calcium content of the heparin treated plasma was analysed using a Corning automatic calcium titrator.

The sudden elevation in the extracellular divalent cation concentrations used to provoke mitosis would probably overwhelm internal cation homeostatic processes. This would, temporarily at least, raise the the intracellular divalent cation content. Such an increased availability of free divalent cations has been shown to activate cellular responses in a variety of cell types (see Introduction, section 2). Indeed, the di-

valent cation ionophore A23187 and plant lectins, both of which increase intracellular divalent cation concentrations, are potent stimulators of human lymphocyte proliferation (Jensen, Winger, Rasmussen & Nowell, 1977; Allwood, Asherson, Davey & Goodford, 1971). Thus there appears to be a prima facie case for an intracellular action of the stimulatory divalent cations during the recruitment of rat thymocytes.

The monovalent cations play a significant role in modulating the intracellular content of divalent cations in both excitable and non-excitable tissues (see Introduction, sections 1 & 2). Therefore we reasoned that thymocyte proliferation could be influenced by experimental manipulation of the transmembrane monovalent cation equilibria, which would then secondarily influence the intracellular calcium content. Thymocytes were exposed to the cardiac glycoside ouabain, which will specifically inhibit the plasma membrane Na/K ATPase activity (Lindenmayer, 1976). Pump inhibition, and the consequent reduction in the active transport of sodium and potassium ions, would directly elevate cytosolic sodium. This, as section 2 predicts, will lead to an inhibition of sodium-calcium exchange and hence elevate cytosolic calcium. It was first necessary to establish the efficiency of thymocyte Na/K ATP-ase inhibition by ouabain. The "pump activity" in a broken membrane preparation was assessed by measuring the inorganic phosphate (Pi) released from ATP by the hydrolytic activity of the pump (Palmer et al. 1966; Schwartz, 1971). Figure R3 shows that the enzyme activity revealed by this assay is highly sensitive to ouabain inhibition. In the presence of 10^{-4} Molar ouabain the inorganic phosphate production was reduced to approximately 15% of the initial activity. Very little dose-dependency was detectable, lower ouabain levels failed to influence this non-ideal system. The degree of sensitivity to the high ouabain concentration is in close agree-

ment with that reported for rat thymocytes (Segal, Hollander, Gordon, Klemperer & Lichtman, 1975) and human peripheral blood lymphocytes (Lichtman, Segel & Lichtman, 1979).

Fig. R3. The influence of ouabain upon the ATP hydrolytic activity of a crude thymocyte membrane preparation.

Total inorganic Pi release without addition	0.27±0.03 µMpi/mg/protein/hr
Release in presence of 10^{-4} Ouabain	0.04±0.01** " " " "
Release in presence of 10^{-6} Ouabain	0.23±0.05 " " " "
Release in presence of 10^{-8} Ouabain	0.24±0.06 " " " "

** Significantly different from untreated control ($P<0.005$ $n=3$)

Thymocytes were homogenized in 30 mM Tris buffer containing 5 mM $MgCl_2$ pH 7.6. Crude membranes were prepared from the 1500 g sediment. This pellet was suspended in assay buffer (according to Schwartz, 1971). Phosphate released from the added ATP was measured colorimetrically by the method of Fiske & Subba Row, 1925.

It must be remembered that this assay of pump activity does not reflect the true ATPase activity of intact cells as the absence of normal transmembrane ionic gradients will influence the expressed activity. Other investigations reported below show that the pump is indeed sensitive to far lower ouabain concentrations.

Many previous studies have shown that ouabain, when added to cultured cells at a high concentration reduced the long term rate of cell division (Mayhew & Levinson, 1968; McDonald, Sachs & Ebert, 1972; Quastel & Kaplan, 1968). Contrary to these observations, ouabain added to the rat thymocyte cultures at 10^{-4} Molar concentrations did not prevent previously committed cells from dividing normally (Figure R4a). When added to thymocytes in conjunction with a mitogenic concentration of either calcium or magnesium ions the same 10^{-4} Molar concentration completely prevented the additional mitotic activity due to the mitogenic divalent cations (Figure R4b). As calcium and magnesium are

Fig. 4a The inability of a high Ouabain concentration to inhibit the entry of committed cells into mitosis

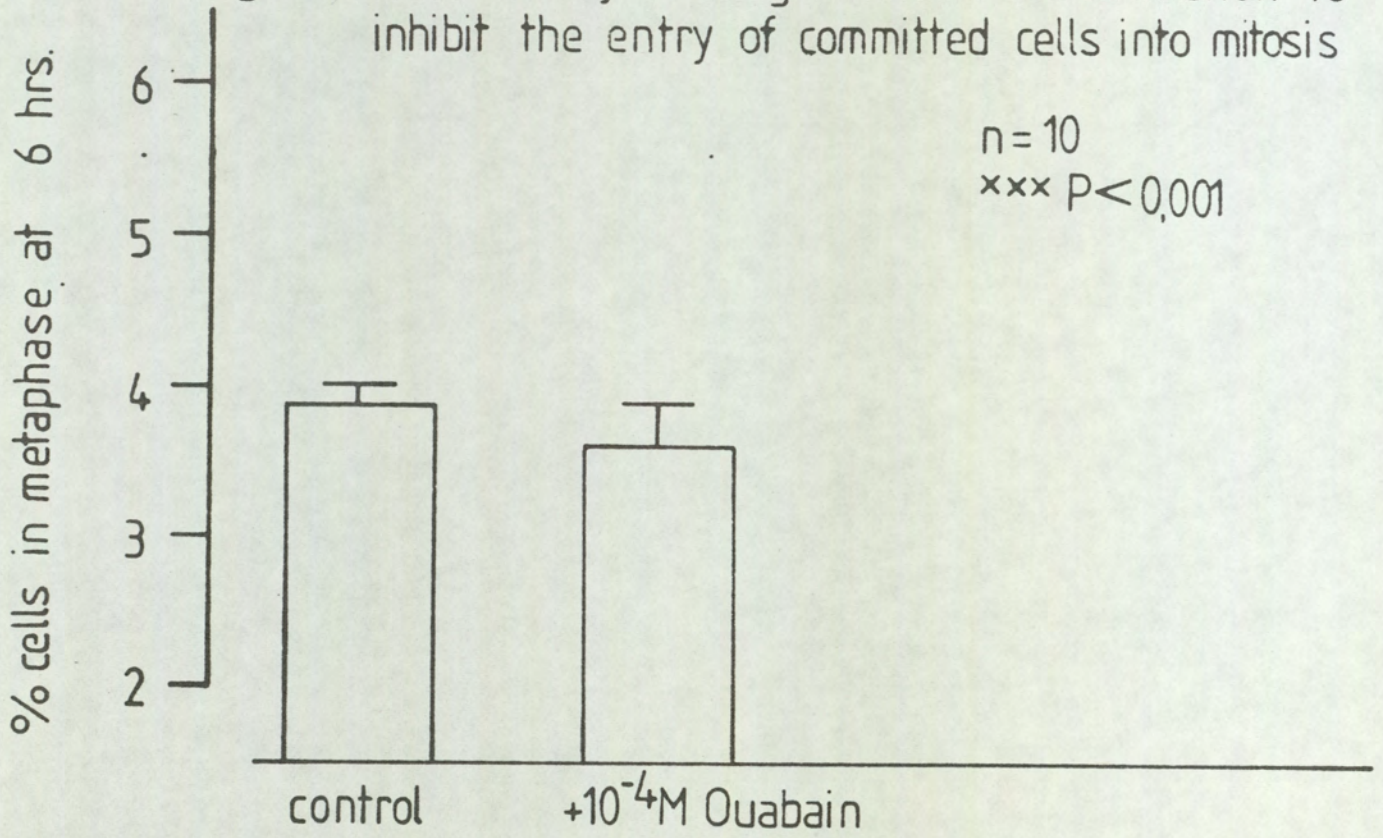
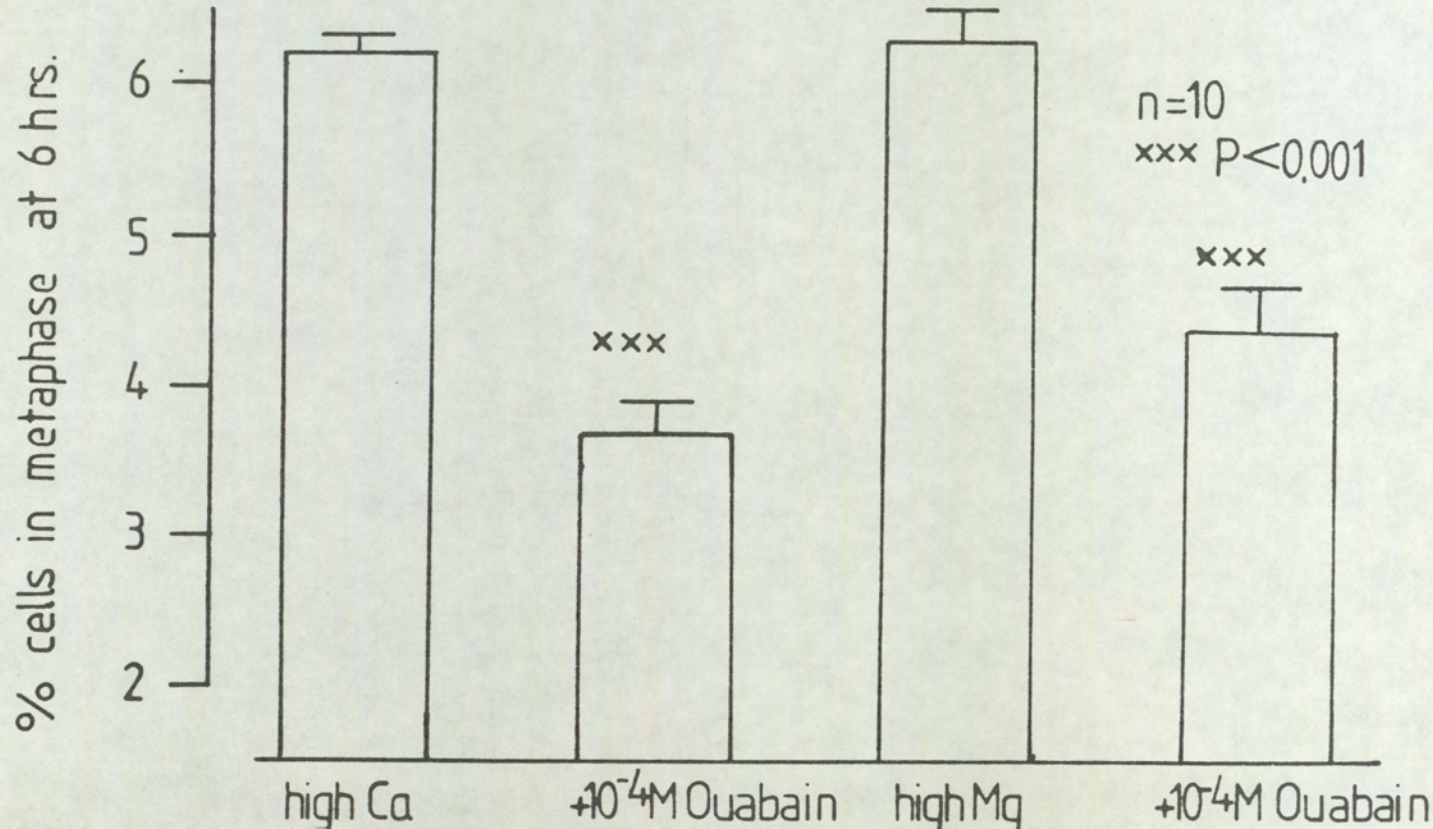


Fig.R4b The action of a high Ouabin concentration upon recruitment of thymocytes by calcium and magnesium ions



believed to stimulate recruitment through different mechanisms (Morgan & Perris, 1974) and both prove to be ouabain-sensitive, the glycoside must act at a common point, presumably subsequent to recruitment. One possibility is that the reduced cellular potassium content, produced by pump inhibition, would prohibit the mitogen-induced increase in cellular protein synthesis. Some of these newly synthesized proteins may be essential for the traverse of the G_1 cell cycle phase (Ledbetter & Lubin, 1977, see also Introduction, Section 4). When the inhibitory ouabain concentration was added to thymocytes, neither mitogen-stimulated nor basal incorporation of radiolabelled leucine (^{3}H leucine) into newly synthesized proteins was influenced by the glycoside (Figure R5).

Fig.R5. Influence of an elevated calcium content and ouabain addition upon thymic lymphocyte protein synthesis.

Treatment	^{3}H leucine incorporation into TCA insoluble protein (expressed as dpm/10^7 cells)
Control	204±14
High calcium	257±15**
Control + 10^{-4} Ouabain	208±12
High calcium + 10^{-4} Ouabain	258± 8**

** Significantly increased above untreated culture ($P < 0.005$ $n = 3$)

Thymocytes were cultured in the presence of 0.1 µCi/ml of ^{3}H leucine for thirty minutes prior to their exposure to mitogen and/or ouabain at 10^6 cells/ml. The incorporated and unincorporated label was separated by TCA precipitation after a four hour incubation, at which point the cells begin to enter mitosis.

The positive inotropism induced in cardiac tissue by ouabain, and by inference the change in intracellular cations, is strictly dose-dependent (Biedert, Barry & Smith, 1979, Lamb & McCall, 1975). Therefore we reasoned that at reduced concentrations ouabain would exert less of an inhibitory influence upon thymocyte mitosis. When the glycoside concen-

tration was reduced to under 10^{-6}M both calcium and magnesium were able to resume their normal mitogenic action (Figure R6). When these non-inhibitory concentrations of ouabain, which presumably inhibit the Na/K ATPase to a lesser extent than 10^{-4}M ouabain, were added to un-stimulated thymocytes, there was a stimulation of proliferation (Figure R7). This ouabain stimulation of thymocyte mitosis was expressed over two distinct and separate concentrations ranges. Such biphasic responses have previously been noted when cyclic nucleotides were added to cultured rat thymocytes (Morgan, Hall & Perris, 1977). In this instance it was shown that the two peaks of mitotic activity were the result of the same mitogen inducing recruitment through either the calcium or magnesium dependent mitotic axes. This biphasic response was dependent upon the mitogen concentration in the extracellular fluid (see Introduction, section 4). The mitotic response to ouabain revealed a similar dichotomy when the divalent cation dependency was assessed. In the absence of extracellular calcium the mitotic potential of 10^{-7}M ouabain was abolished, whilst the stimulation at 10^{-11}M remained undiminished (Figure R8).

Thus the high, 10^{-7}M ouabain concentration, may be categorised as a calcium-dependent mitogen. In common with all other such mitogens this high (10^{-7}M) concentration proved to be sensitive to the presence of oestradiol (Figure R9). Only the high concentration had this sensitivity to oestradiol, the calcium-independent stimulation provoked by 10^{-11}M ouabain was not affected (Figure R9). This low peak of ouabain stimulation, uninfluenced by the absence of calcium or the addition of oestradiol, proved to be highly sensitive to the removal of extracellular magnesium ions (Figure R10). Previously identified magnesium-dependent mitogens have been shown to be testosterone-

Fig.R6 Dose-dependency of Ouabain-inhibition of thymocytes recruited by divalent cations

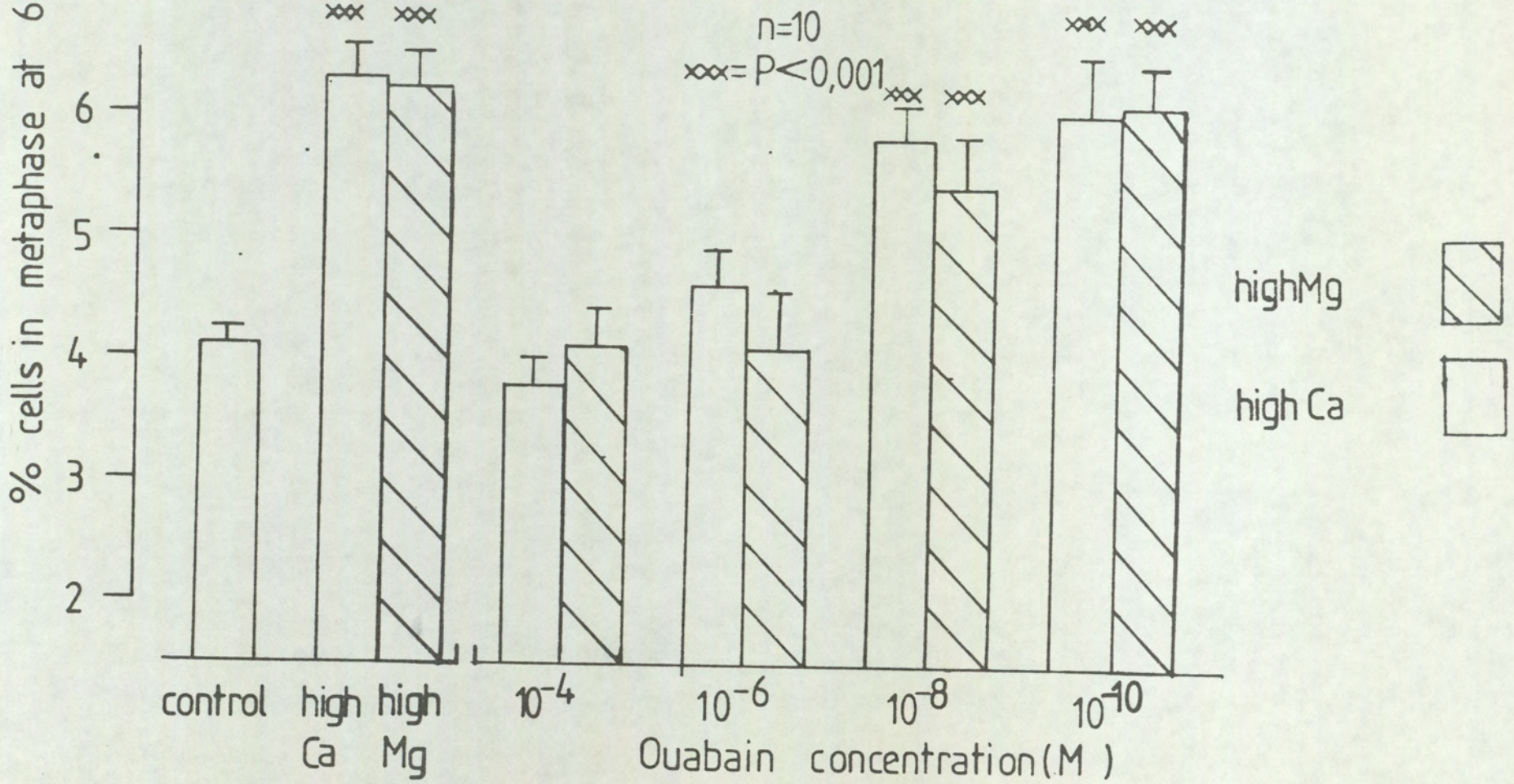


Fig.R7 The biphasic mitotic stimulation provoked by a range of ouabain concentrations

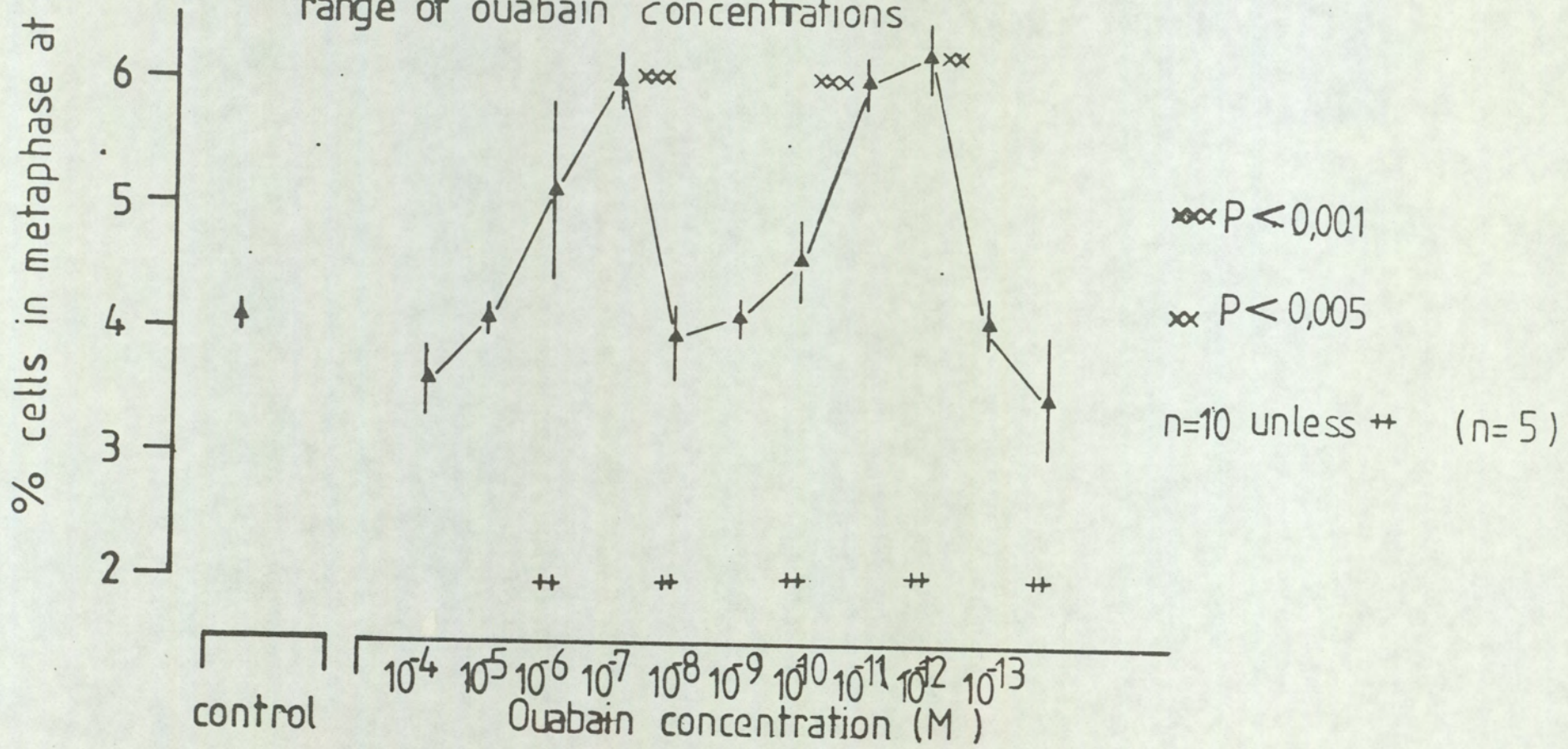


Fig.R8 The extracellular calcium-dependency of the mitogenic action of Ouabain (zero calcium)

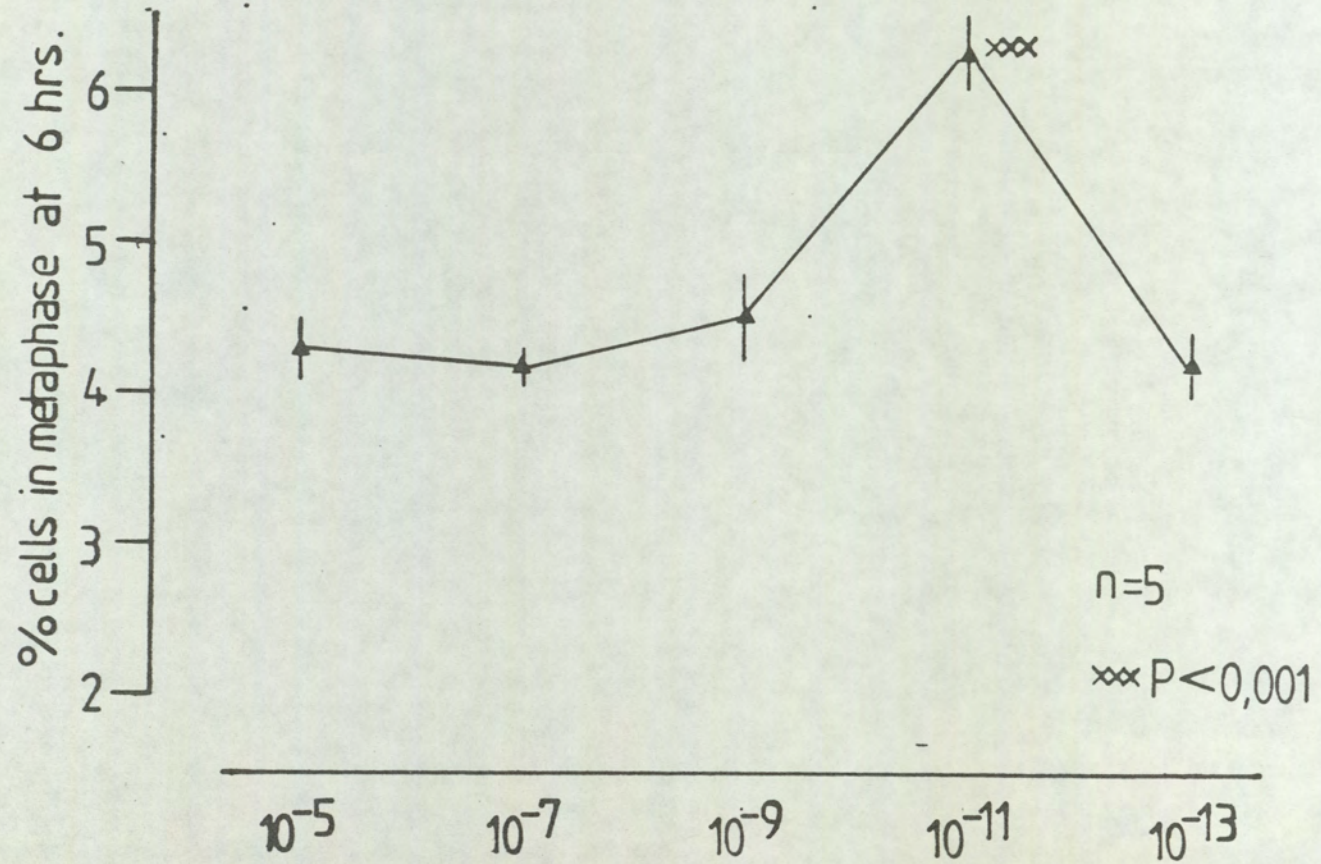


Fig. R9 The influence of 0,1µg Oestradiol benzoate on Ouabain induced mitosis

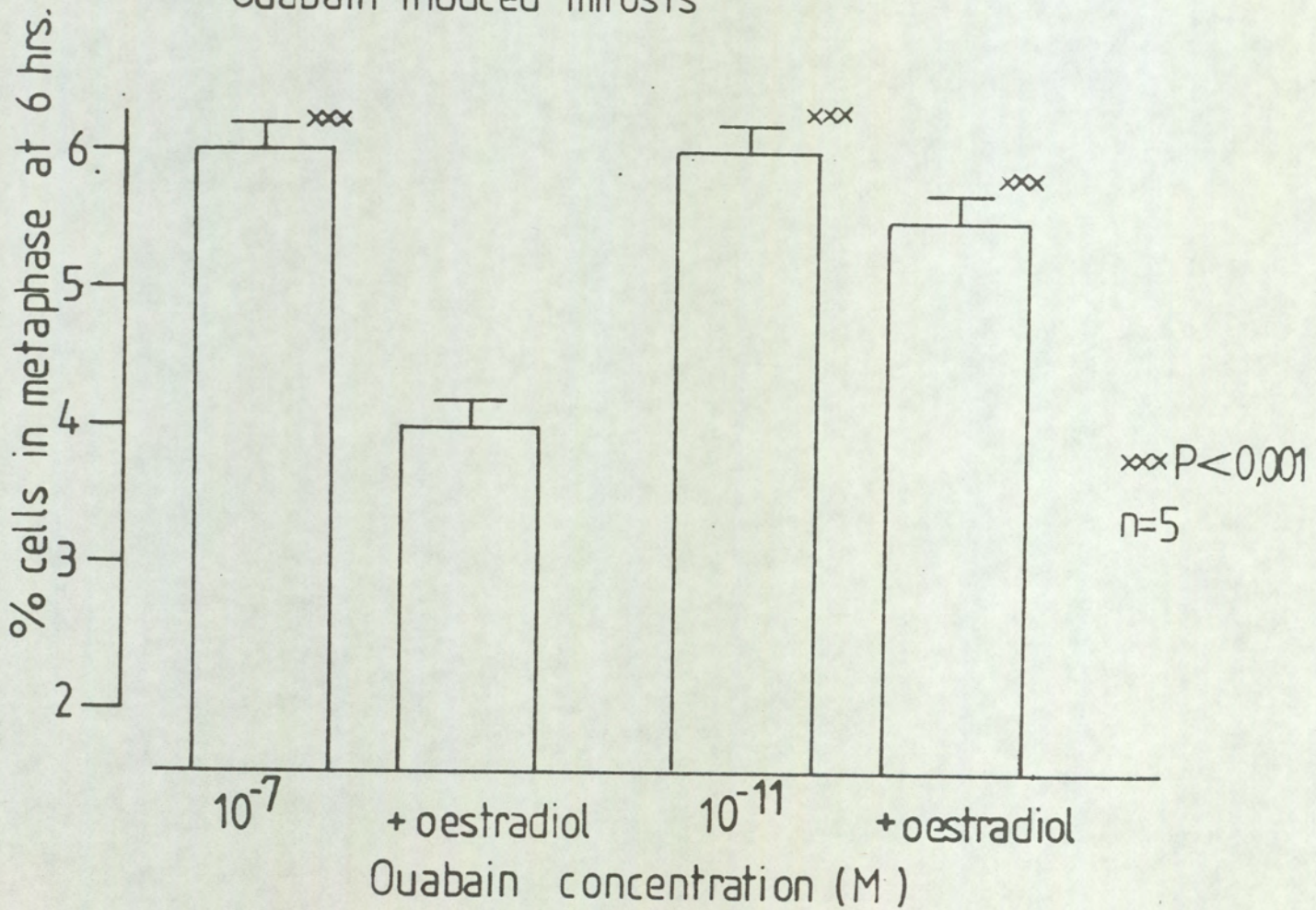
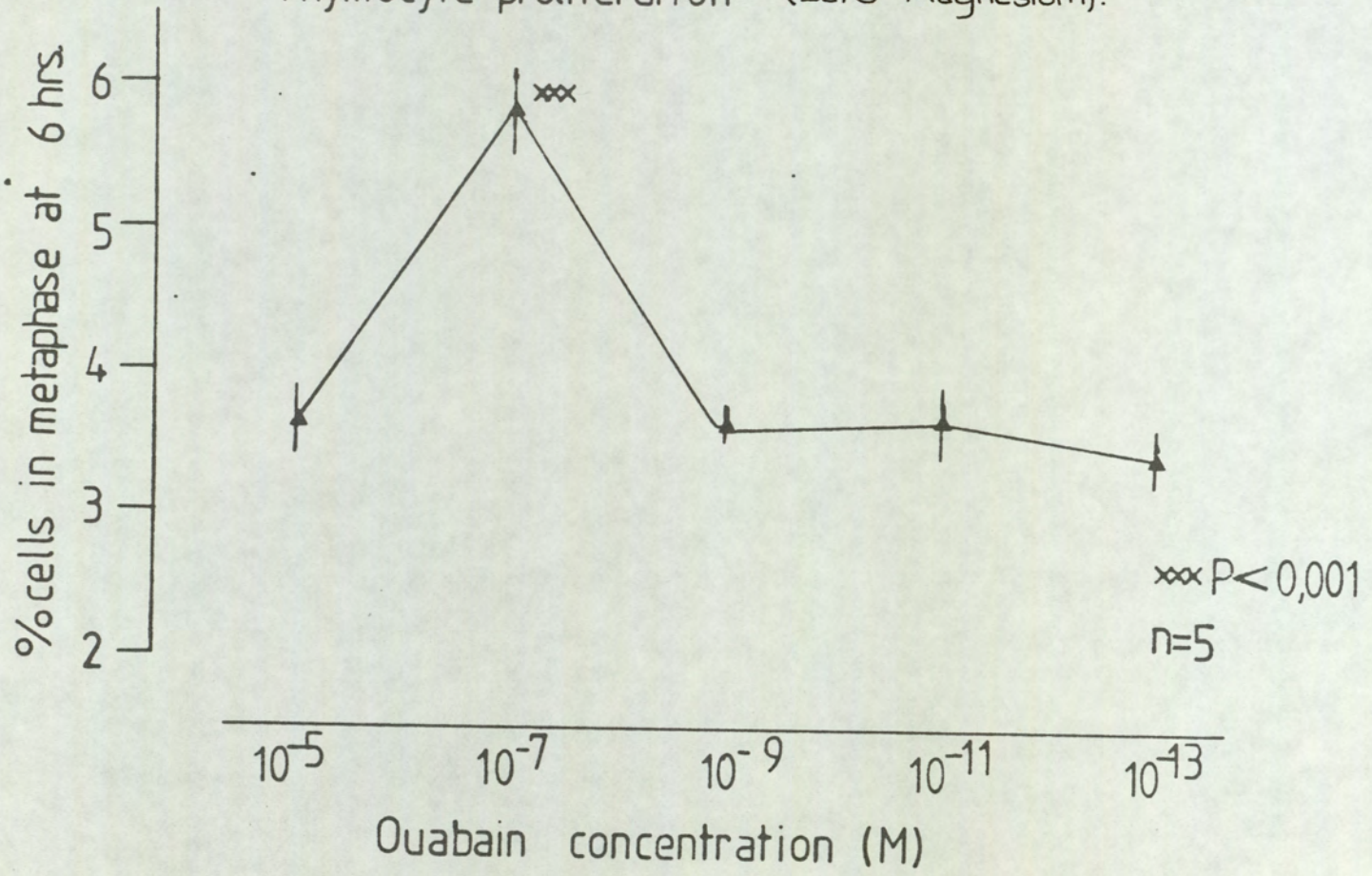


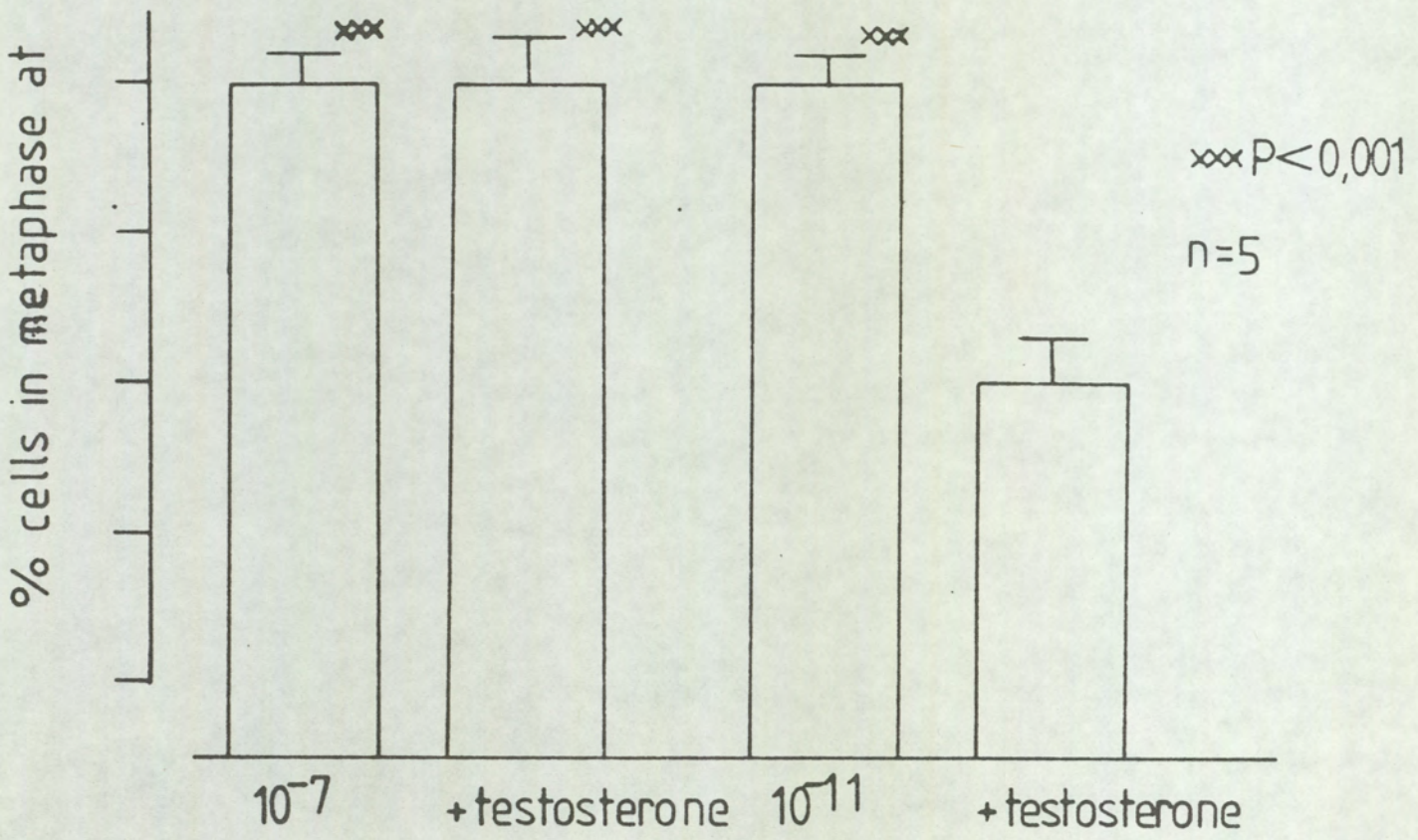
Fig.R10 The magnesium-dependency of Ouabain-induced thymocyte proliferation (zero magnesium).



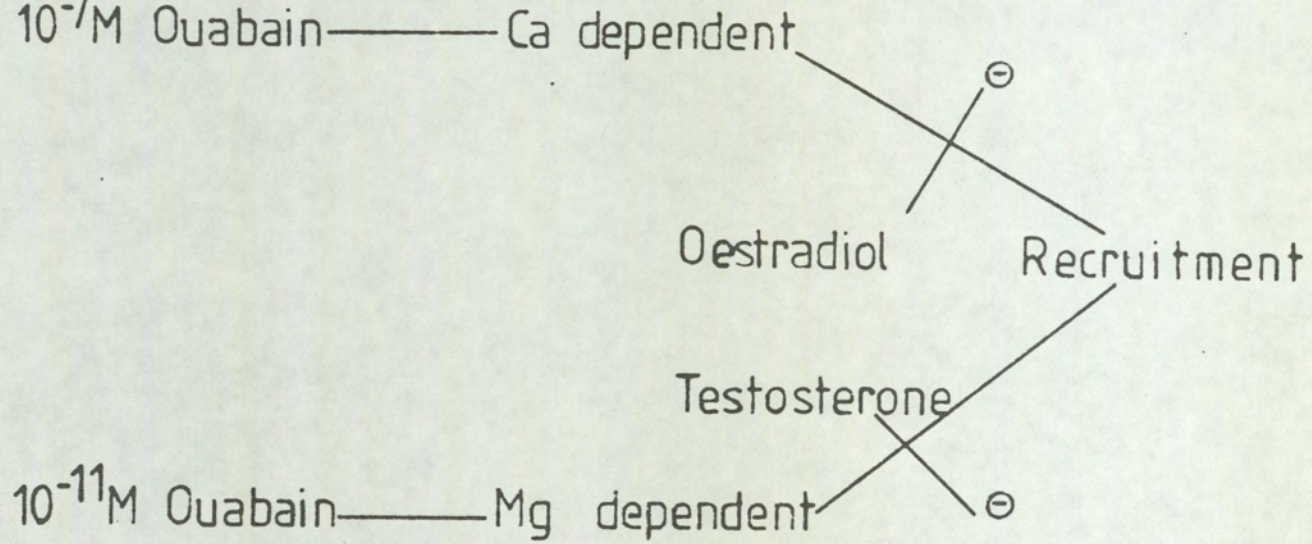
inhibitable, and as anticipated the low(10^{-11}M) ouabain concentration was no exception. Figure R11 shows that testosterone abolishes only the mitotic potential of the magnesium-dependent glycoside concentration, the calcium-dependent 10^{-7} concentration was unaffected. This glycoside treatment further confirms the existence of the two discrete mitogenic axes (Perris & Morgan, 1975). Figure R12 summarises the various dependencies of ouabain stimulation and shows that the glycoside acts as either a calcium-dependent, oestradiol-inhibitable, or a magnesium-dependent, testosterone-inhibitable mitogen.

If the glycoside-induced proliferation is indeed due to changes in the intracellular cation content, brought about by pump inhibition, then ouabain, at mitogenic concentrations, should influence Na/K ATPase activity. The previous ATPase assay would not be suitable for such a sensitive measurement. Consequently the technique developed by Shank & Smith was adopted to detect ouabain-mediated pump inhibition under more physiological intact-cell conditions (Shank & Smith, 1978). The influence of a range of ouabain concentrations from 10^{-5} to 10^{-15} Molar was tested upon the volume recovery response of osmotically stressed cells (see Methods). The resultant changes in cell volumes are shown in Figure R13. A reduced recovery rate indicates, albeit indirectly, that the function of the pump is restricted (Tosteson, 1964). As can be seen in Figure R13 the recovery of cells treated with mitogenic concentrations of ouabain is drastically reduced. The technique did not allow discrimination between different glycoside concentrations but confirms that the lowest mitotically active ouabain concentration (10^{-11}M) is still able to inhibit the pump. This suggests that the mitotic (and anti-mitotic) potential of ouabain is related to pump inhibition and the subsequent changes in the internal cationic environment.

Fig.R11 0,1 µg Testosterone blockade of Ouabain-induced thymocyte proliferation



FigR12 Summary of Ouabain-stimulated mitosis



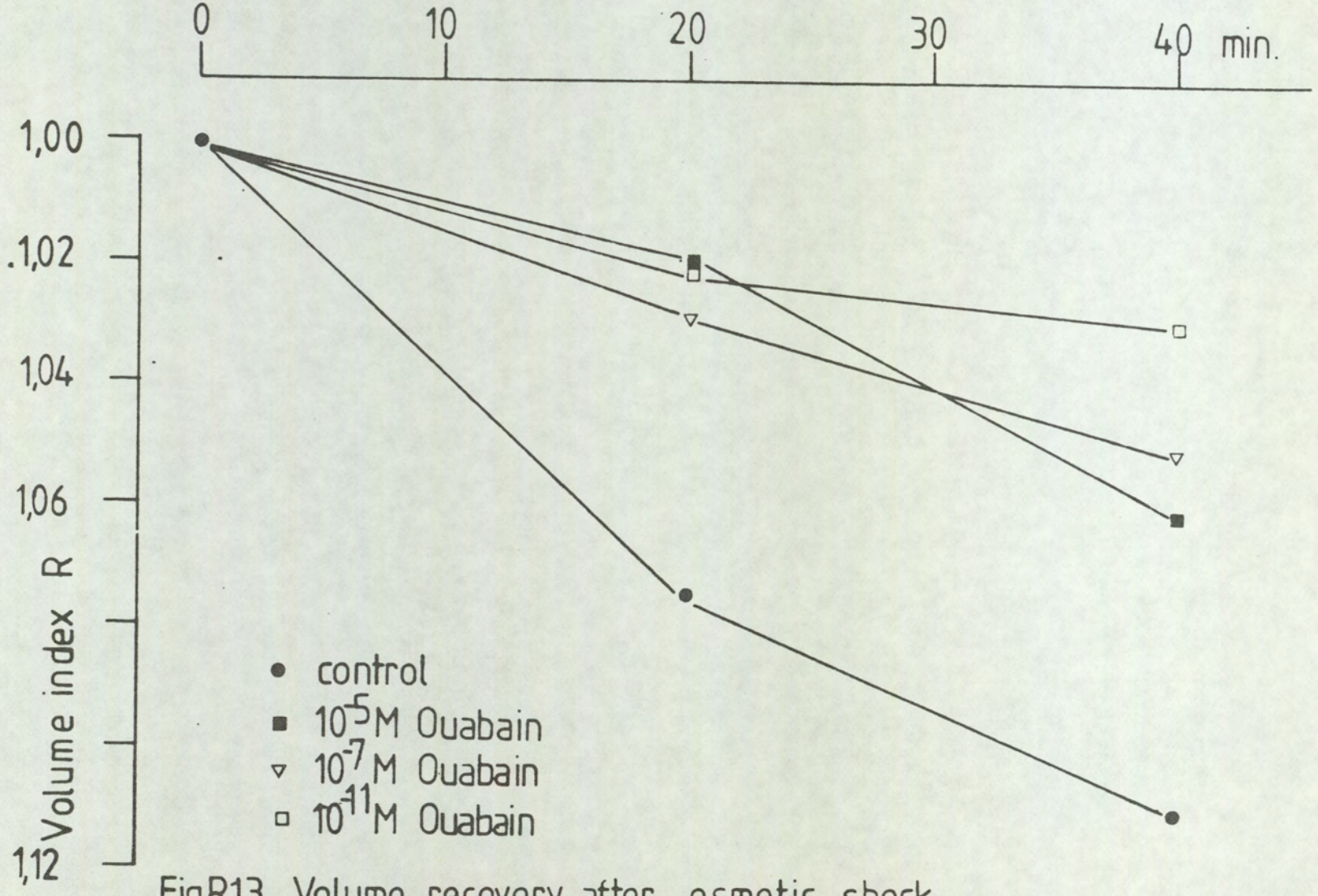


Fig.R13 Volume recovery after osmotic shock

It has been suggested that ouabain can exert metabolic effects other than pump inhibition during its incubation with cultured epithelioid cells (Lelievre, Paraf, Charlemagne & Sheppard, 1977). Whilst in all probability these changes are a consequence of pump inhibition, it was imperative to employ other Na/K ATPase inhibitors to exclude such a possibility. Extracellular fluoride ions have been shown to inhibit the functioning of membrane monovalent cation pumps in other mammalian cells (Yoshida, Nagai, Kamei & Nakagawa, 1968; Kirschner, 1964). Like ouabain, high concentrations of fluoride inhibited both calcium and magnesium induced mitogenesis (Figure R14). This could not be due to the removal of divalent cations by fluoride-metal precipitation for simple stoichiometric reasons. In a manner comparable with ouabain, a reduced fluoride ion concentration allowed both calcium- and magnesium-stimulated proliferation (Figure R15), which suggests a dose-dependent inhibition of the ATPase. The ability of extracellular fluoride to stimulate thymocyte proliferation was then determined to see if a second (purported) pump inhibitor could promote mitotic activity. As Figure R16 shows fluoride treatment indeed provoked mitosis. Unlike ouabain the stimulation appeared only over a single concentration range.

In a third series of experiments the indirect subversion of pump activity was attempted. As a high proportion of cellular ATP production is directed towards pump function any interference with the cellular energy supply would reduce pump activity to some extent. The metabolic inhibitor 2.4 DNP was added to cultures in an attempt to uncouple mitochondrial respiration and lower cellular ATP levels. It was first necessary to establish that this treatment was not influencing normal proliferative activity due to a general cytotoxic effect. Figure R17 demonstrates that, at a range of concentrations, 2.4 DNP permitted calcium-stimulated mitotic activity. The higher concentrations did prove to be mitogenic in their own right (Figure R18).

Fig.R14 The antimitotic action of high Fluoride concentrations on thymocyte mitosis

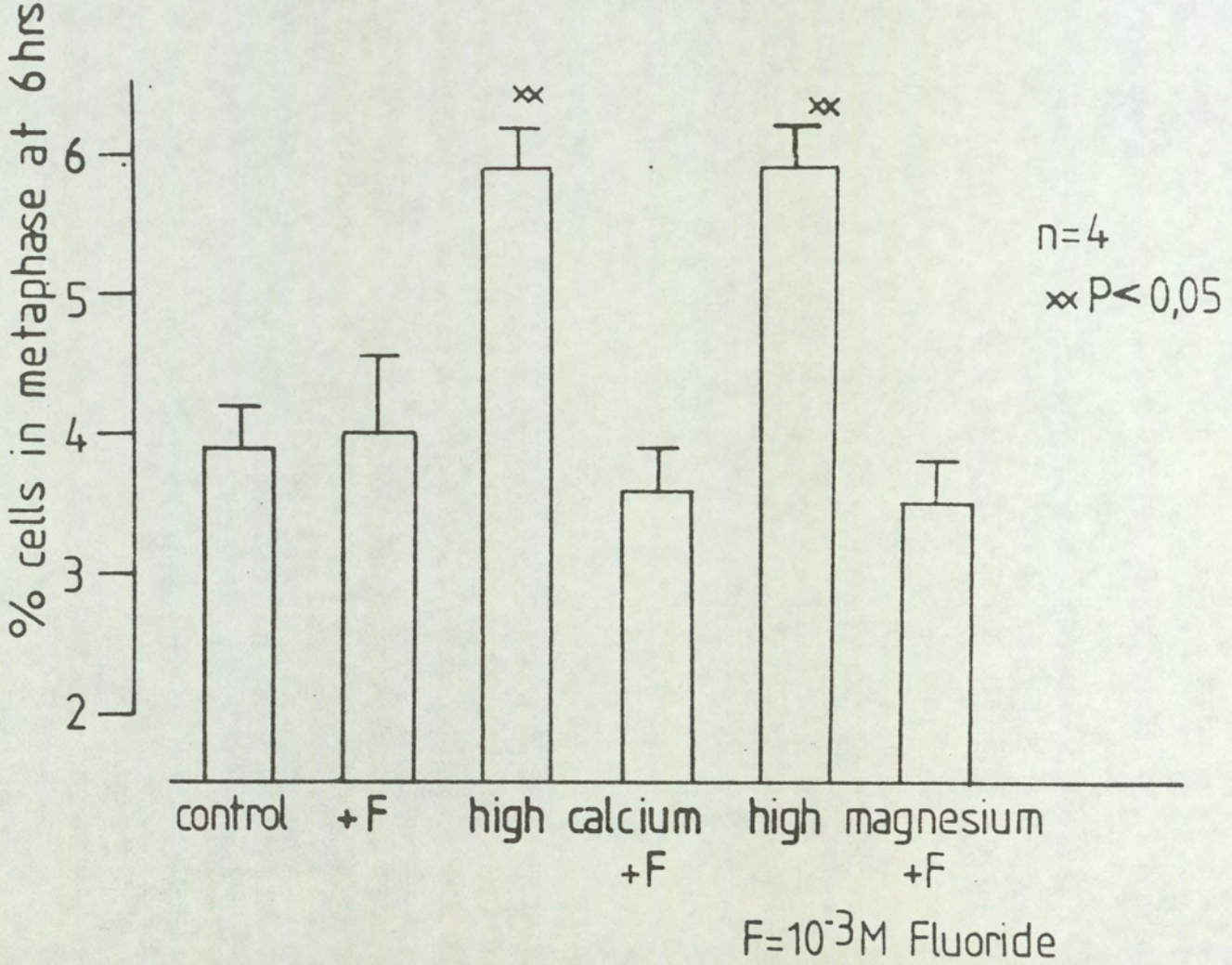
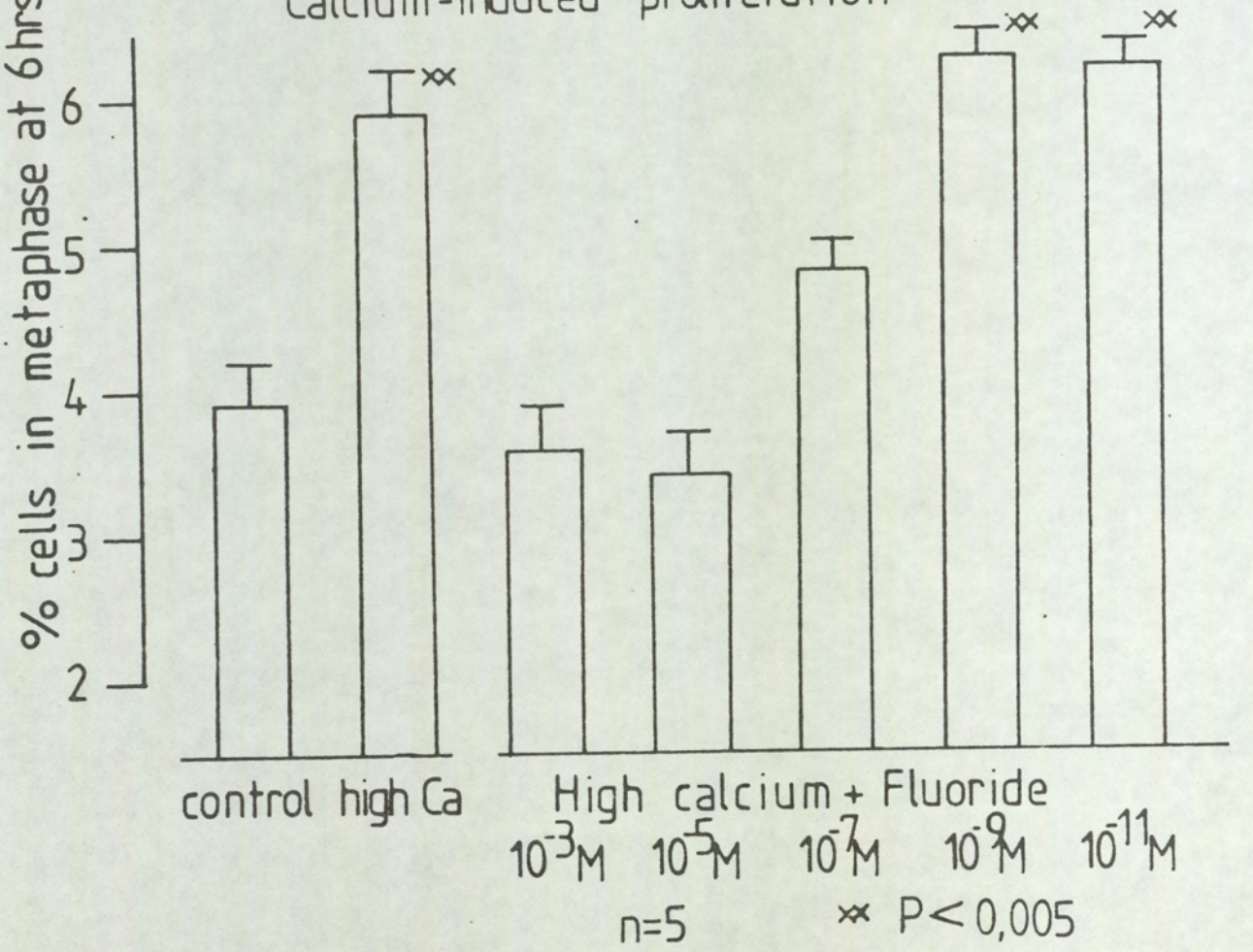


Fig.R15 Dose dependency of Fluoride inhibition of Calcium-induced proliferation‡



‡ Data for magnesium as for calcium.

Fig. R16 The Fluoride stimulation of thymocyte mitosis

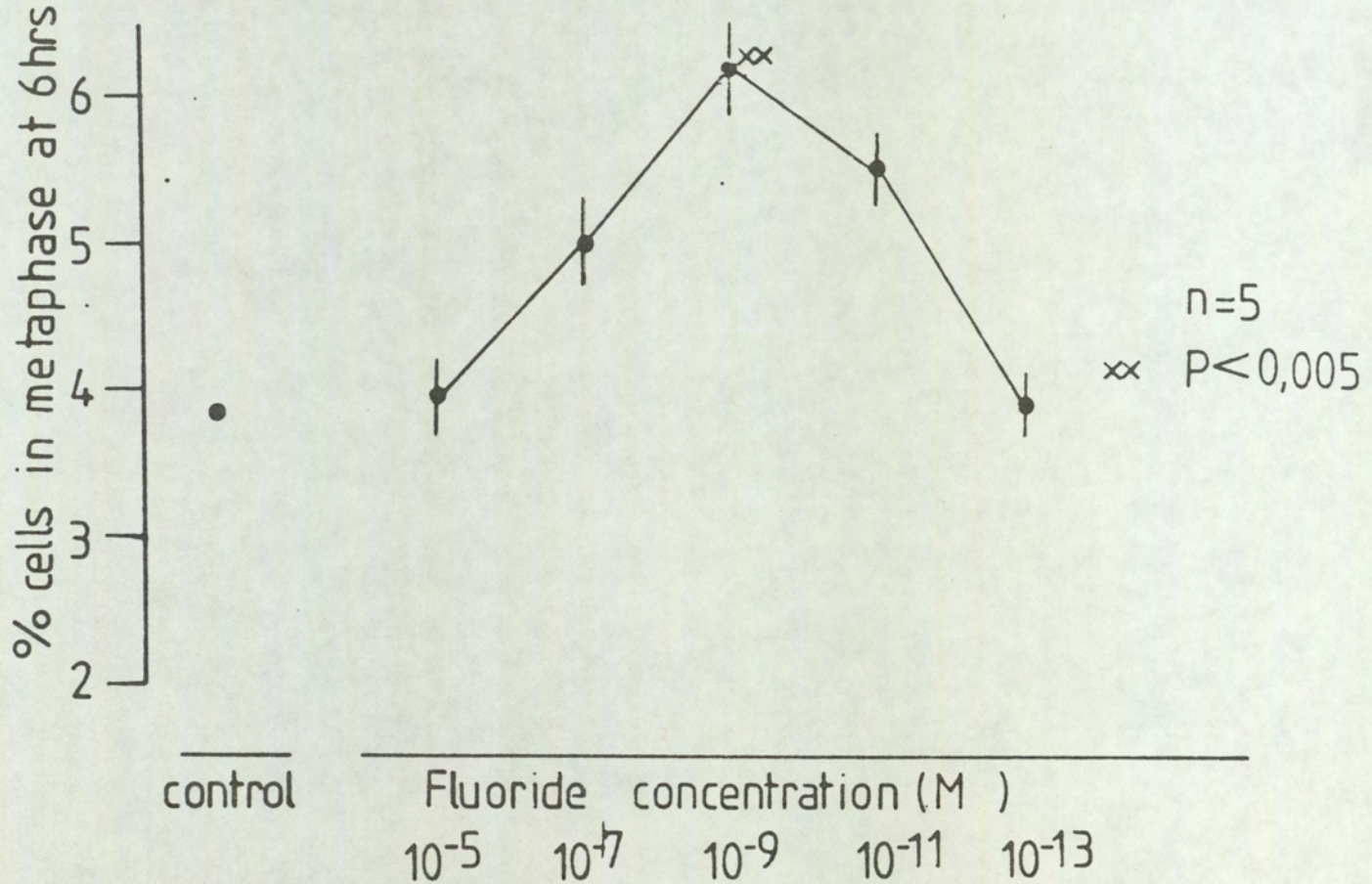


Fig.R17 2,4 DNP effect upon calcium-stimulated mitosis

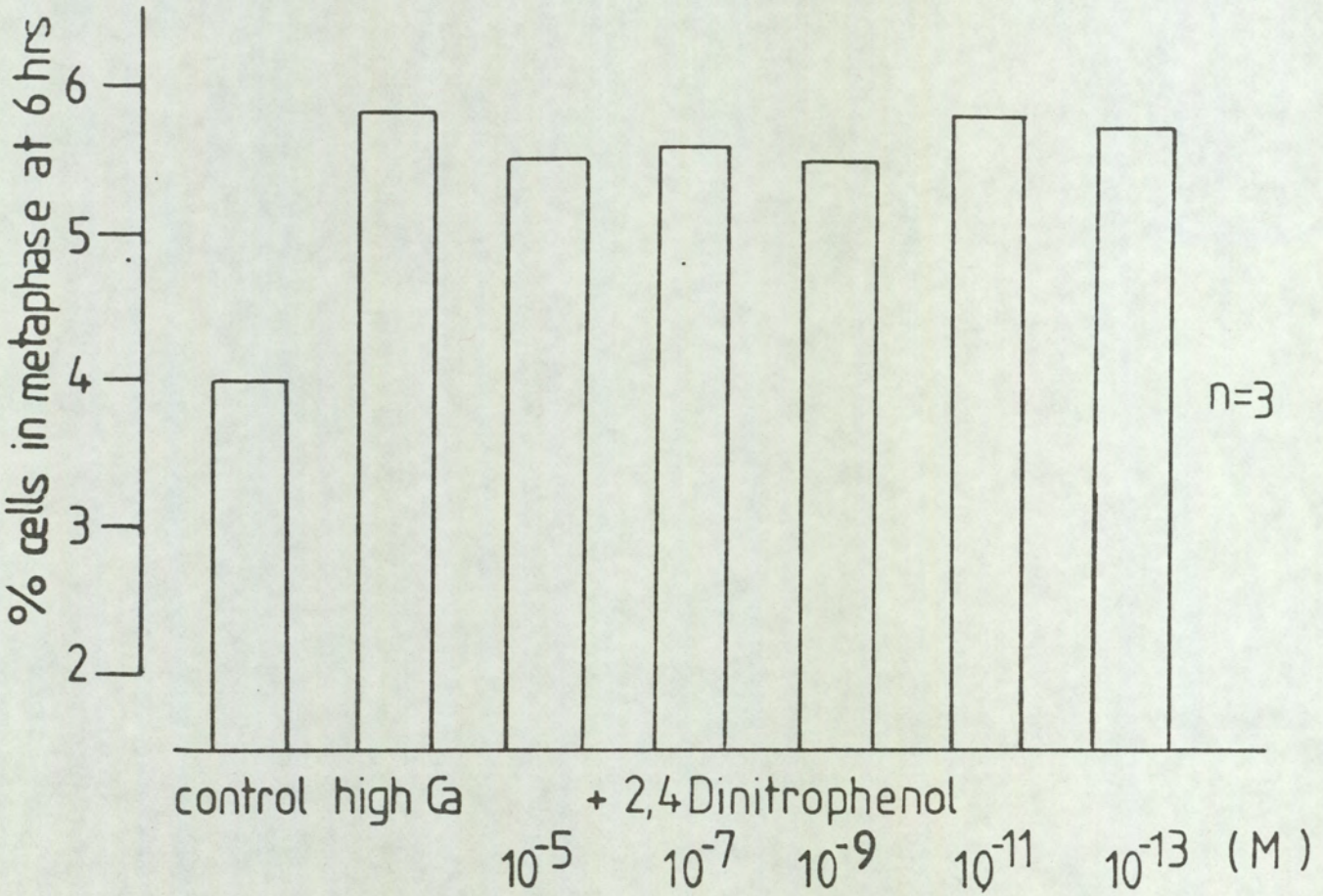
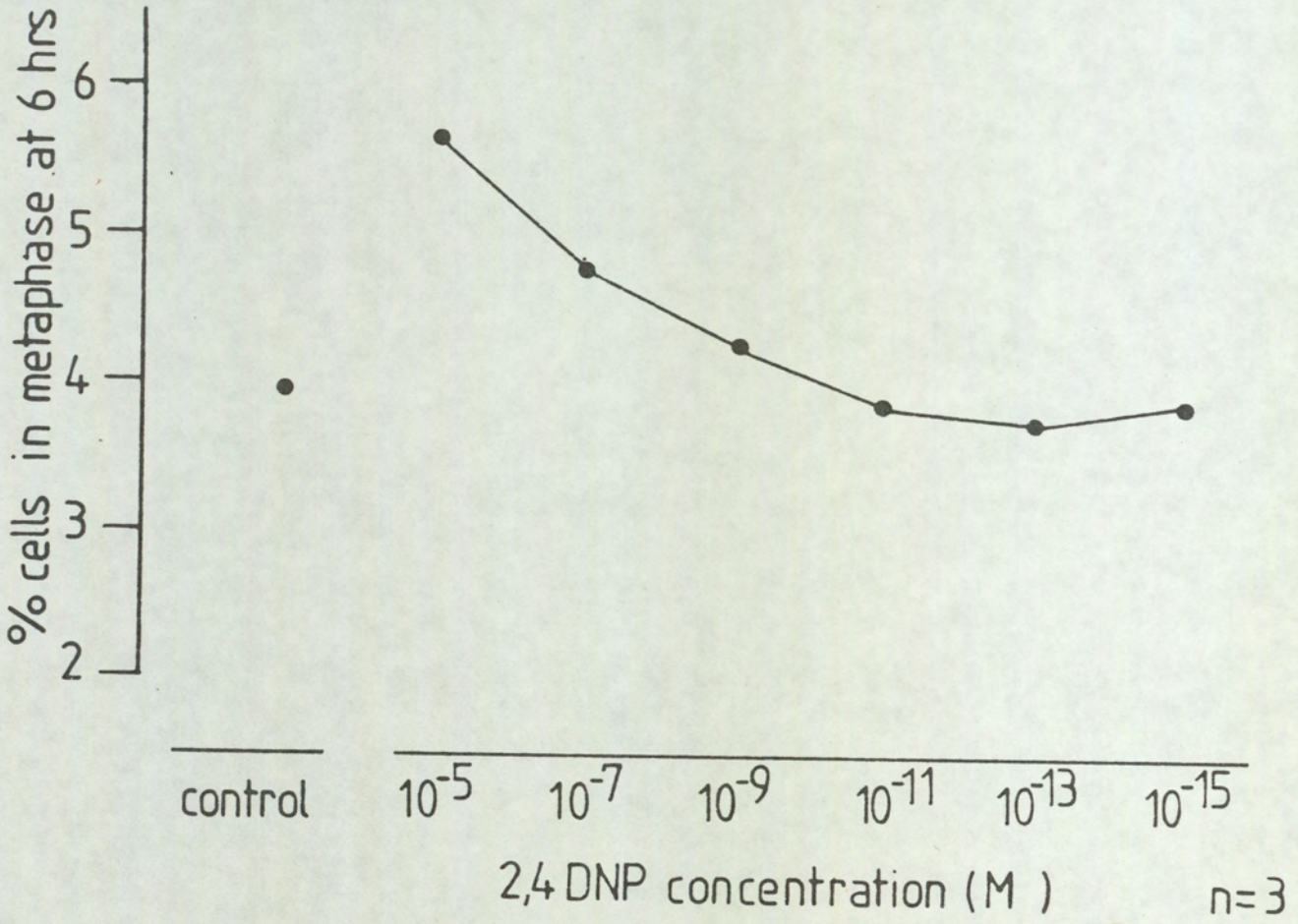


Fig.R18 Mitotic stimulation induced by 2,4 DNP



Although these treatments are all believed to cause alterations in intracellular cation concentrations it was necessary to directly influence the cation balance without recourse to pharmacological treatments. As intracellular sodium ions are normally furnished from the extracellular reservoir by transmembrane diffusion an increased transmembrane sodium gradient would be expected to increase the intracellular sodium concentration. When the external sodium concentration was raised by as little as a five millimolar increase (from the normal 145 mM to 150 mM), without osmotic compensation, the quiescent rat thymocytes were recruited into the cell cycle (Figure R19). This proliferative response to increased extracellular sodium proved to be dose-dependent; above a 30 mM increment there was no evident stimulation of mitosis (Figure R19). The increased external osmotic pressure, or chloride ion content were not responsible for the mitotic triggering as both lithium and potassium chloride increments proved to be mitotically inert (Figure R20 and Figure R21). The increased internal sodium ion content, produced by either pump inhibition or enhanced sodium entry, will interfere with the transmembrane calcium exchange. The effect of the removal of extracellular calcium upon sodium-stimulated mitosis was tested. This calcium depletion would prevent an elevation of cytosolic calcium caused by the increased internal sodium ion content. In the absence of the extracellular calcium ions a 10 mM increment in extracellular sodium was no longer able to provoke a mitotic response (Figure R22). The stimulation due to higher sodium concentrations was not influenced by such calcium deprivations (Figure R22). The calcium-independent stimulation due to a 20 mM sodium increase was abolished by the removal of extracellular magnesium ions, which did not influence the lower (10 mM) mitotic sodium concentration (Figure R23). Again this dichotomy confirms the two-axis hypothesis of thymocyte activation. Moreover, these obser-

Fig.R19 The mitotic response to extracellular Sodium

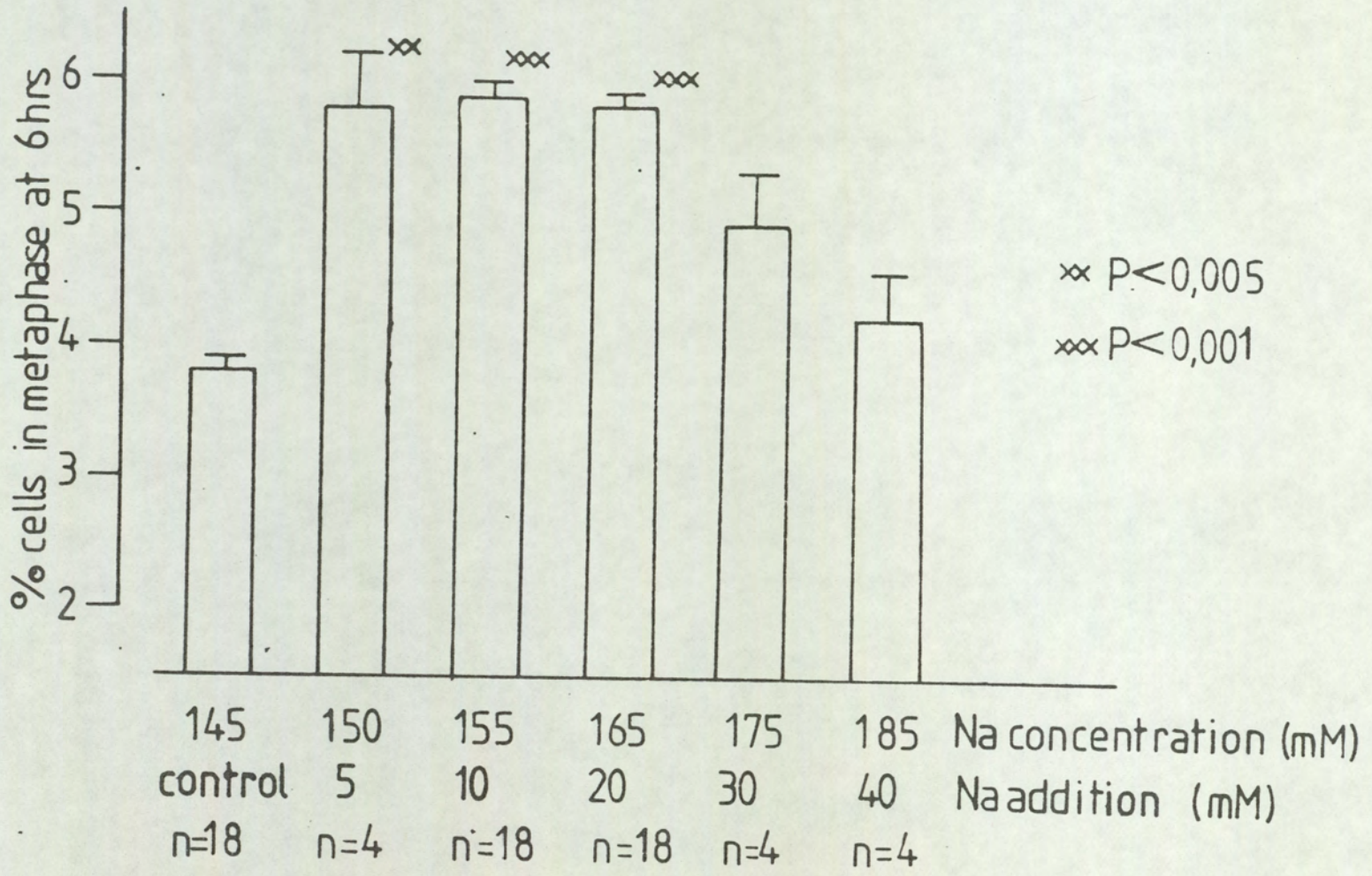


Fig.R20 Mitotic effect of Lithium chloride addition to thymus cultures

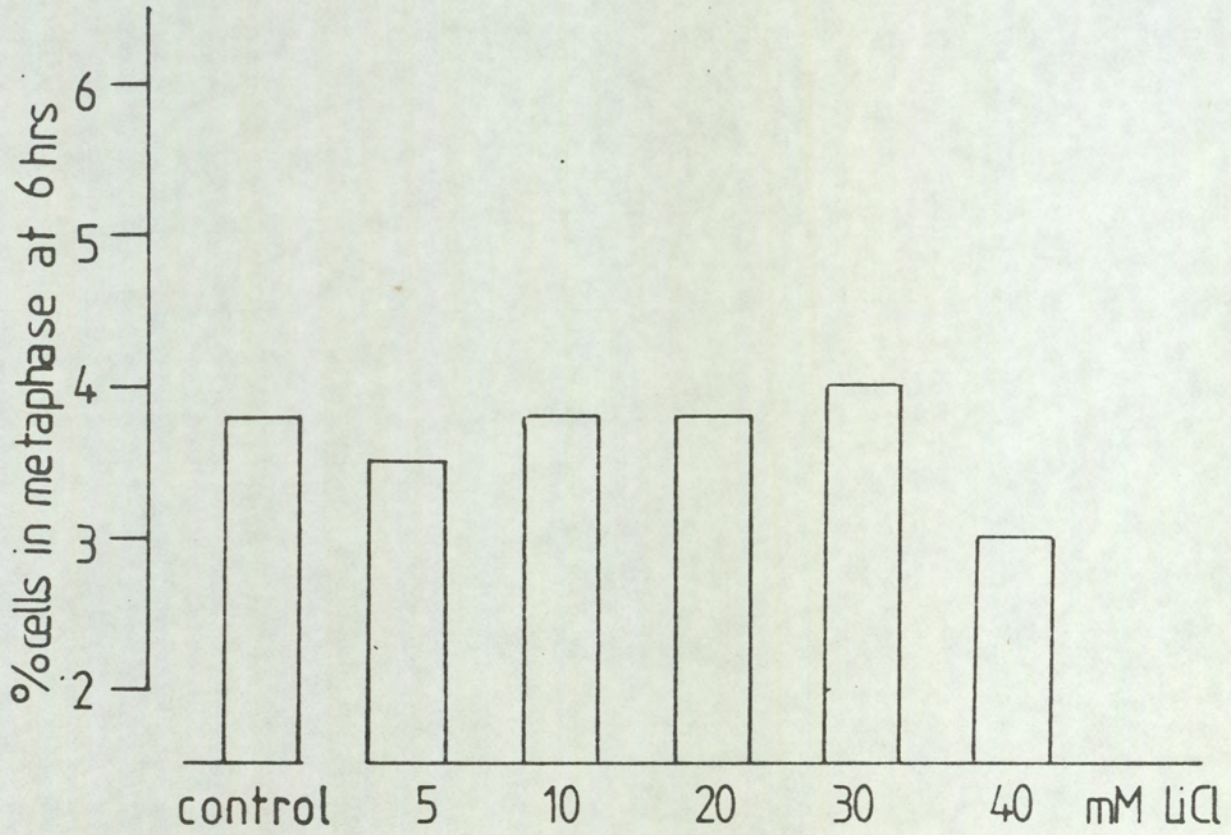


Fig. R21 The effect of potassium addition upon thymocyte mitosis

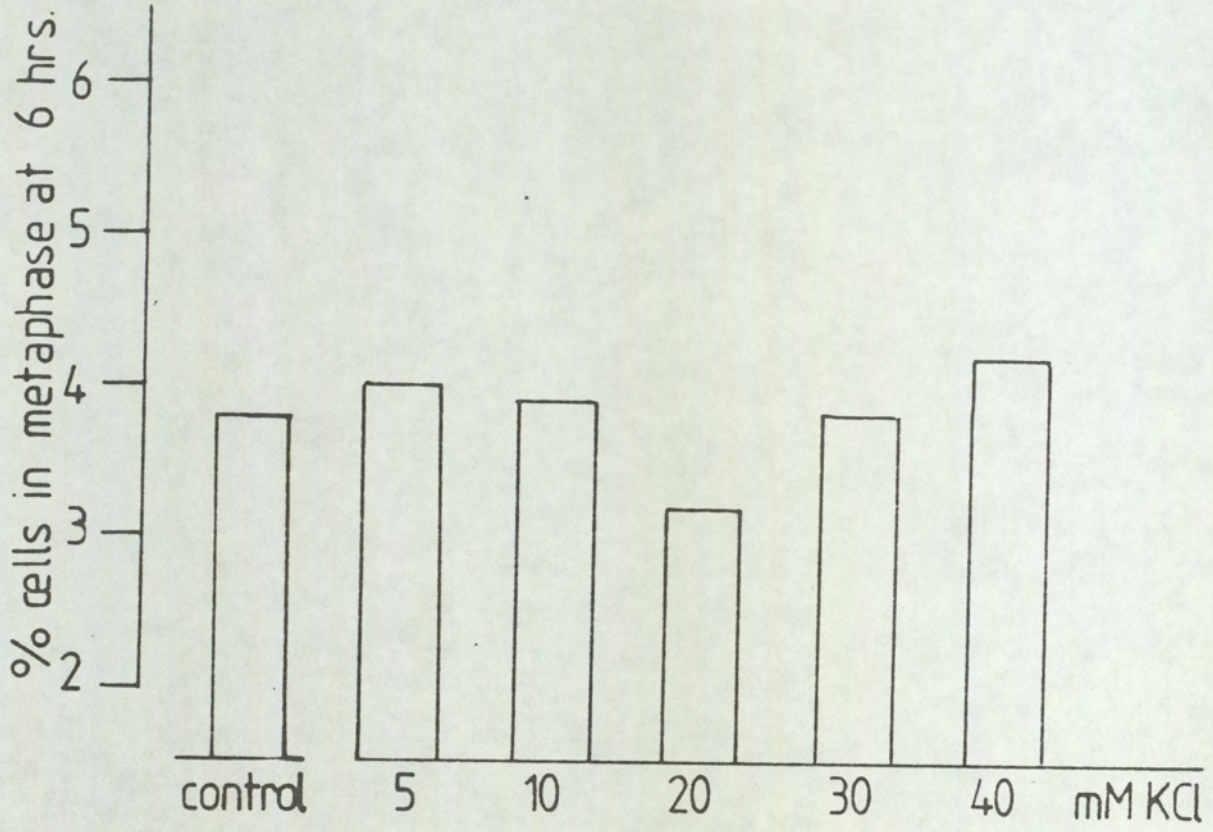


Fig.R22 The calcium dependency of sodium-induced thymocyte mitosis

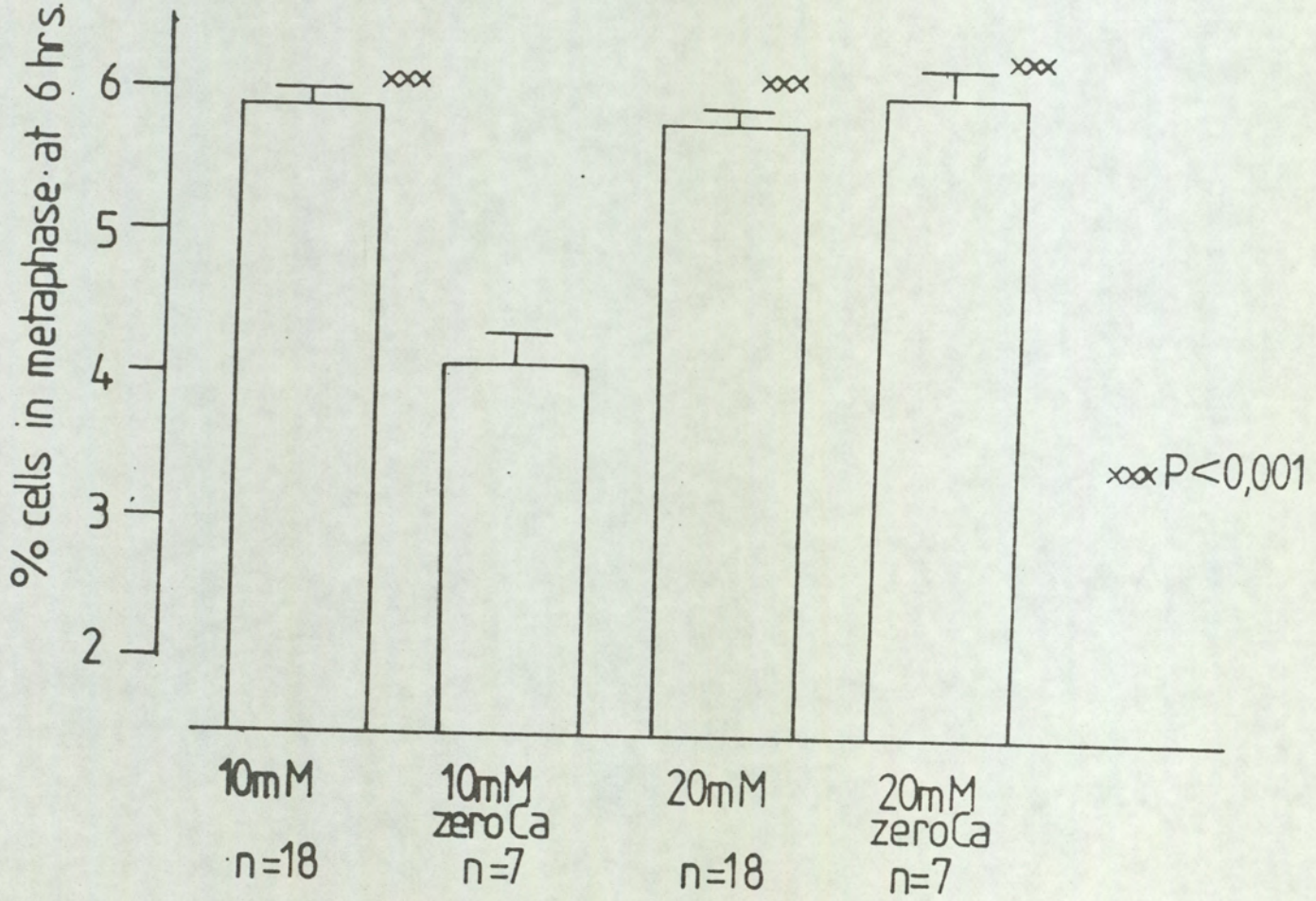
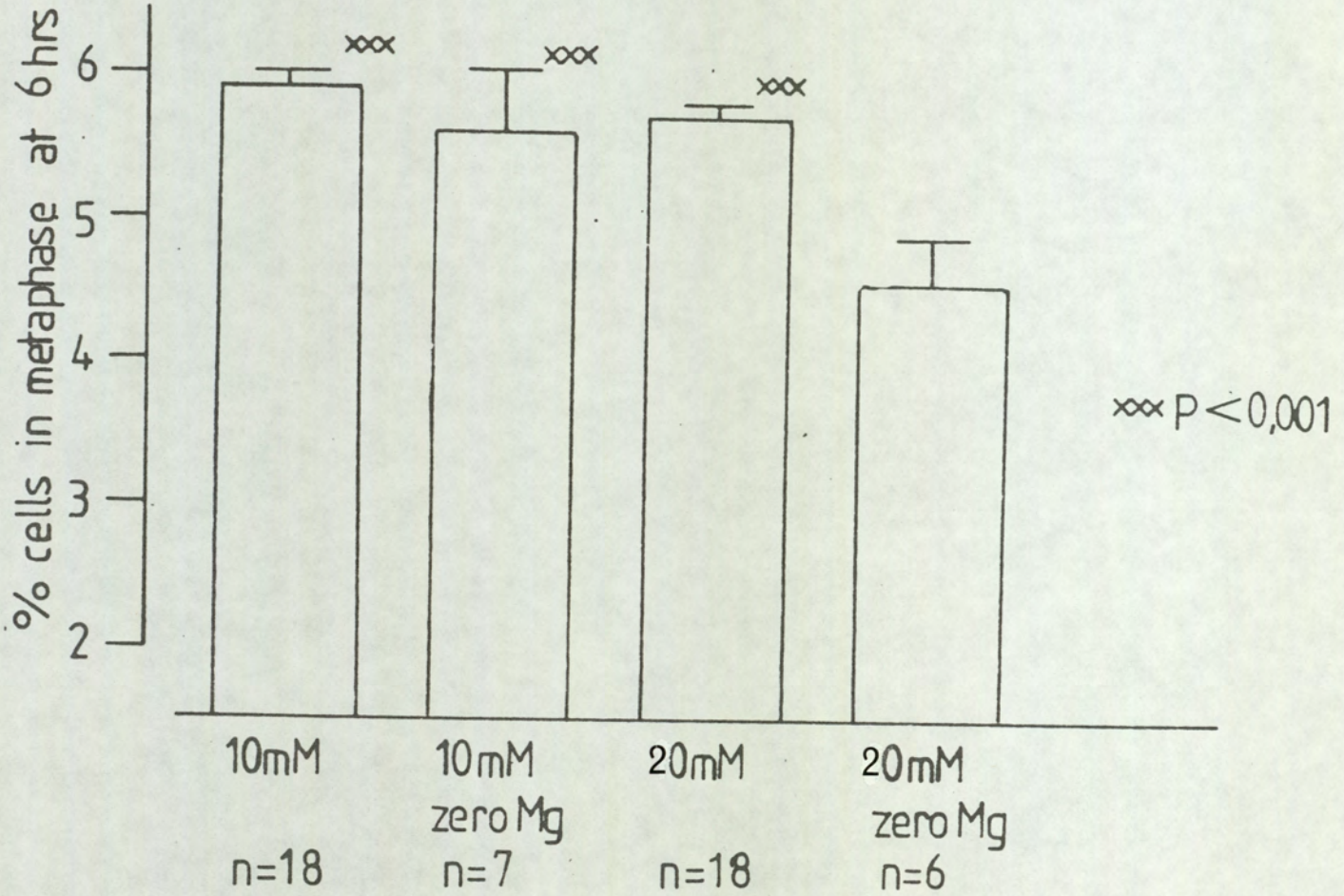


Fig.R23 The magnesium-dependency of the sodium induced thymocyte mitosis



vations confirm that part of the mitotic action attributable to ouabain can indeed be due to an increased intracellular sodium concentration.

The dependency upon external calcium ions exhibited by many thymocyte mitogens attests to the importance of the extracellular calcium reservoir. How this external calcium contributes to mitotic stimulation by a variety of different mitogens is uncertain. One possibility is that extracellular calcium directly increases the intracellular calcium pool following its transmembrane movement. Alternatively, the extracellular calcium may be necessary solely for the maintenance of a labile intracellular or membrane-bound calcium pool. Whatever the mechanism by which intracellular processes depend upon external calcium it may be anticipated that isolation of the cytosol from the external environment would incapacitate all calcium-dependent mitogens. To achieve such an isolation a calcium-specific agonist was employed. Verapamil is one such agent and will inhibit Ca entry by blockading calcium-selective channels in the plasma membrane (Fleckenstein, 1977; Maier, Antonczyk, Schindler & Heidland, 1979). If, as suggested, verapamil does prevent calcium movement into thymocytes then the calcium-induced stimulation would be compromised. In an initial series of experiments verapamil, at concentrations above 5 x 10^{-5} g/l proved highly cytotoxic. At lower, non-toxic concentrations, verapamil did not influence basal mitotic activity (Figure R24). When verapamil was added prior to calcium stimulation of thymocytes, the mitotic response normally produced by 1.8 mM calcium was blocked by the antagonist in a dose-dependent manner (Figure R24). Significantly, concentrations that inhibited calcium-induced mitogenesis failed to prevent magnesium-stimulated proliferation (Figure R24). This suggests a calcium selective, non-cytotoxic action for verapamil. Furthermore, the magnesium-stimulated proliferation which does not require extracellular

calcium is not acting through a calcium-channel movement of magnesium.

Fig. R.24. The action of a calcium selective antagonist, verapamil upon divalent cation induced thymocyte proliferation

Treatment	No Verapamil	5×10^{-6} g/l Verapamil	5×10^{-9} g/l Verapamil
Control	4.1±0.1 (n=6)	4.2±0.3 (n=6)	4.4±0.2 (n=6)
High calcium	5.8±0.3 (n=6)***	3.4±0.4 (n=4)	5.4±0.2 (n=4)**
High magnesium	5.6±0.2 (n=4)**	5.4±0.3 (n=4)**	5.2±0.3 (n=4)**

*** Significantly different from control cultures ($P<0.001$)

** Significantly different from control cultures ($P<0.005$)

A second calcium-selective antagonist, lanthanum, also proved capable of blocking calcium stimulated proliferation (Figure R.25).

Fig. R.25. The influence of lanthanum upon calcium-induced mitosis.

Treatment	% cells in mitosis at 6 hours
Control	3.7±0.2
High Ca	5.9±0.3**
Control + 3mMLa Cl_3	4.1±0.3
High Ca + 3mMLa Cl_3	3.6±0.2

** Significantly different from control cultures ($P<0.005$ n = 3)

The addition of the inhibitory verapamil concentration in conjunction with the calcium-dependent ouabain concentration also resulted in the abolition of ouabain-mediated mitosis (Figure R.26). The magnesium-dependent concentration of ouabain was not influenced by verapamil (Figure R26).

Fig. R.26. The verapamil sensitivity of ouabain stimulated mitosis.

	No Verapamil	5×10^{-6}M Verapamil	5×10^{-9}M Verapamil
Control	3.7±0.2	3.9±0.2	4.7±0.3
10^{-7}M Ouabain	5.9±0.3**	4.3±0.3	6.1±0.2**
10^{-11}M Ouabain	6.1±0.3**	5.9±0.2**	6.3±0.3**

** Significantly different from control cultures ($P<0.005$ n=3).

The extracellular calcium, which apparently must be allowed to enter the cytosol, was only able to stimulate mitosis when present outside the cell in a freely diffusible form (Figure R27). Thus the addition of citrate ions which act as calcium chelators, was tested upon calcium-stimulated mitosis.

Fig. R.27. The effect of calcium chelation upon calcium-stimulated mitosis.

Treatment.	% Cells in mitosis at 6 hrs.
Control	3.7±0.3
High calcium	6.1±0.3**
Control + 3mM sodium citrate	3.4±0.3
High calcium + 3 mM sodium citrate	3.4±0.2

**Significantly different from control cultures ($P<0.005$ $n = 3$)

Thus it may be assumed, that for an increase in the extracellular calcium concentration to stimulate mitosis, the ion must pass in an ionic form into the cytosol. This suggests that the trigger for mitosis, in this instance at least, is a rise in the free ionic calcium content of the cytosol. This is also the conclusion that can be drawn from the mitotic potential and cation requirement exhibited by other agents that either directly, or indirectly, influence intracellular calcium homeostasis.

Before considering direct evidence for altered calcium levels within the cytosol, it would be useful to summarise the preceding experimental observations.

1. A single population of quiescent thymocytes was recruited by elevations in the external concentration of either calcium or magnesium ions.
2. Inhibition of the plasma-membrane Na/K ATPase by ouabain, fluoride and 2.4 DNP at very high concentrations prevented recruitment.

3. Lower concentrations of ouabain were not anti-mitotic, and provoked mitosis in a biphasic manner. A high ouabain concentration proved to be calcium-dependent whilst the lower stimulatory concentration was magnesium-dependent. Only the calcium-dependent concentration required unrestricted calcium entry as verapamil was able to inhibit its effect.

4. Fluoride and 2.4 DNP also stimulated division, but in a monophasic manner.

5. Thymocytes were stimulated by an increased transmembrane sodium gradient, which provoked either calcium- or magnesium-dependent mitosis in a concentration-related fashion.

6. Calcium ions will only stimulate proliferation when added to thymocytes in a freely diffusible form. Calcium entry into the cytosol is necessary for the mitotic effect, as both verapamil and lanthanum inhibit selectively the calcium-induced mitosis.

The period of time which elapses between mitogen addition and the commitment of responsive cells to divide is the crucial period for mitotic activation. This time will see the external "triggering stimulus" translated and coupled to the intracellular cell cycle regulatory machinery. The preceding experiments have shown that intracellular cations participate in mitogen-induced thymocyte activation. Therefore an investigation of the ionic rearrangements, if any, occurring during this initial activation period will shed light upon the important coupling process. Previous studies have shown that mitogen-stimulated thymocytes begin to enter the cell cycle, as indicated by the onset of DNA synthesis, within one hour of stimulation (Morgan, 1976; Whitfield, MacManus, Boynton, Gillan & Isaacs, 1974). This activation period was

further investigated by withdrawing the mitogenic extracellular calcium environment at different times during the incubation. If the stimulatory calcium level was maintained for only thirty minutes the quiescent thymic lymphocytes became irreversibly committed to enter the cell cycle (Figure R28). Thus, any crucial ionic rearrangements serving to trigger mitosis must be completed during this period. Consequently the following ion-flux investigations almost exclusively relate to this thirty minute period.

Alteration of the cytosolic calcium content appears to be a common factor linking stimulus to response in both secretory and contractile cells. Similar calcium changes are also implicated in the mitotic recruitment of thymocytes (see Figure R1 and Introduction, section 4). Consequently the transmembrane movement of calcium was monitored during the recruitment of quiescent thymocytes. Calcium uptake by the thymocytes was observed by substituting the normal culture medium for one containing approximately 1μCi/ml of calcium-45 (Ca^{45}). The final specific activity of this medium, which may properly be considered an infinite and uniform reservoir, was 40μCi/mg. Under basal, unstimulated conditions, the uptake of calcium, more properly termed cell-associated calcium, indicated the existence of a rapidly equilibrating, relatively small, reservoir (Figure R.29). This pool has a half-time of under thirty seconds and is probably analogous to the glycocalyx or membrane bound reservoir observed in other cell types. It is certainly not an intracellular reservoir as it was unaffected by the presence of verapamil, which prevented the entry of calcium into the cytosol (Figure R.30). Once this extracellular reservoir became saturated, a constant (linear) uptake of calcium was observed (Figure R.29). Presumably this corresponds to a transmembrane movement of calcium as it was highly sensitive to verapamil (Figure R.30). Over

Fig R28 Requirement for continued presence of mitogen

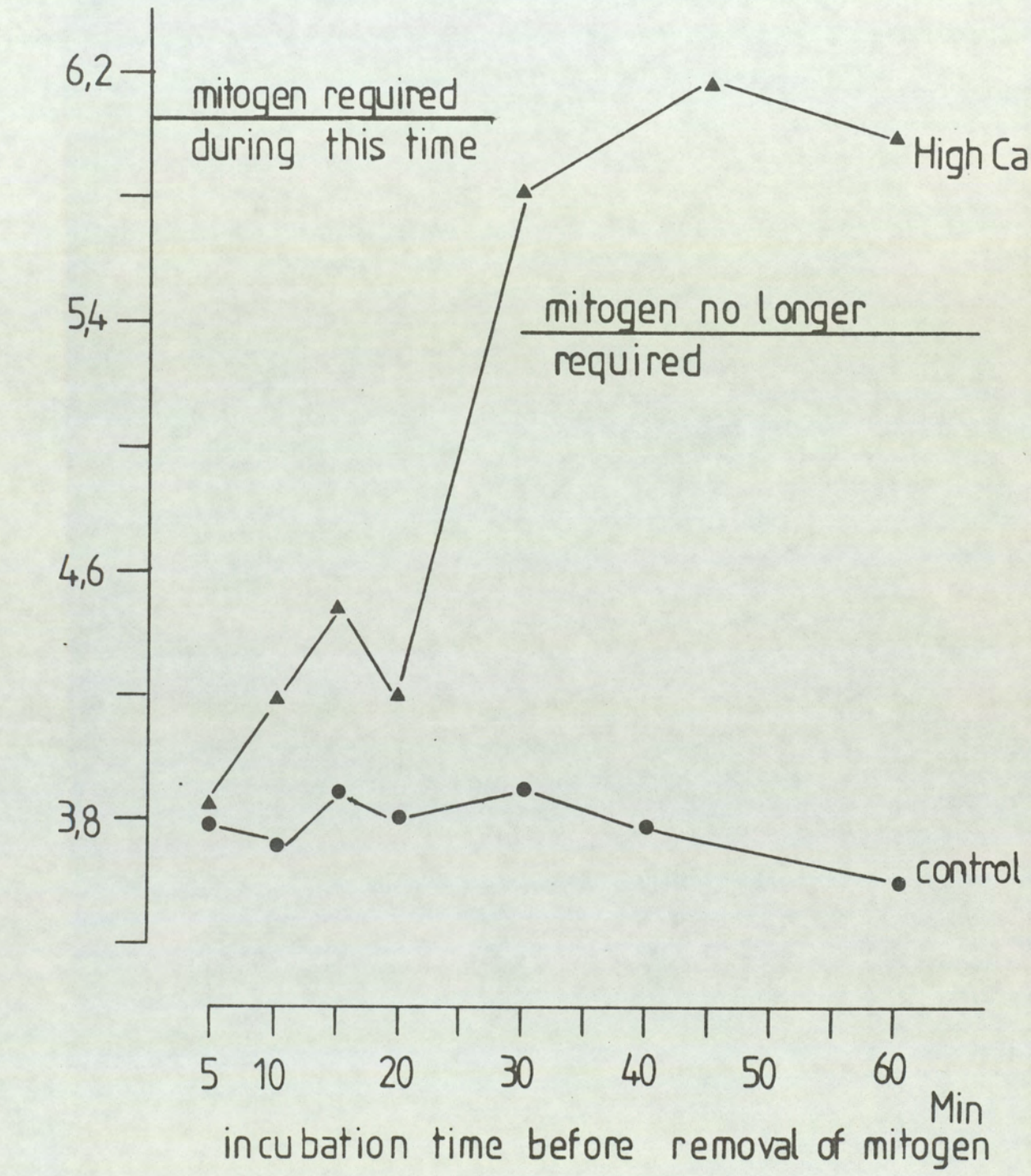


Fig. R29 Calcium uptake revealed by Ca-45

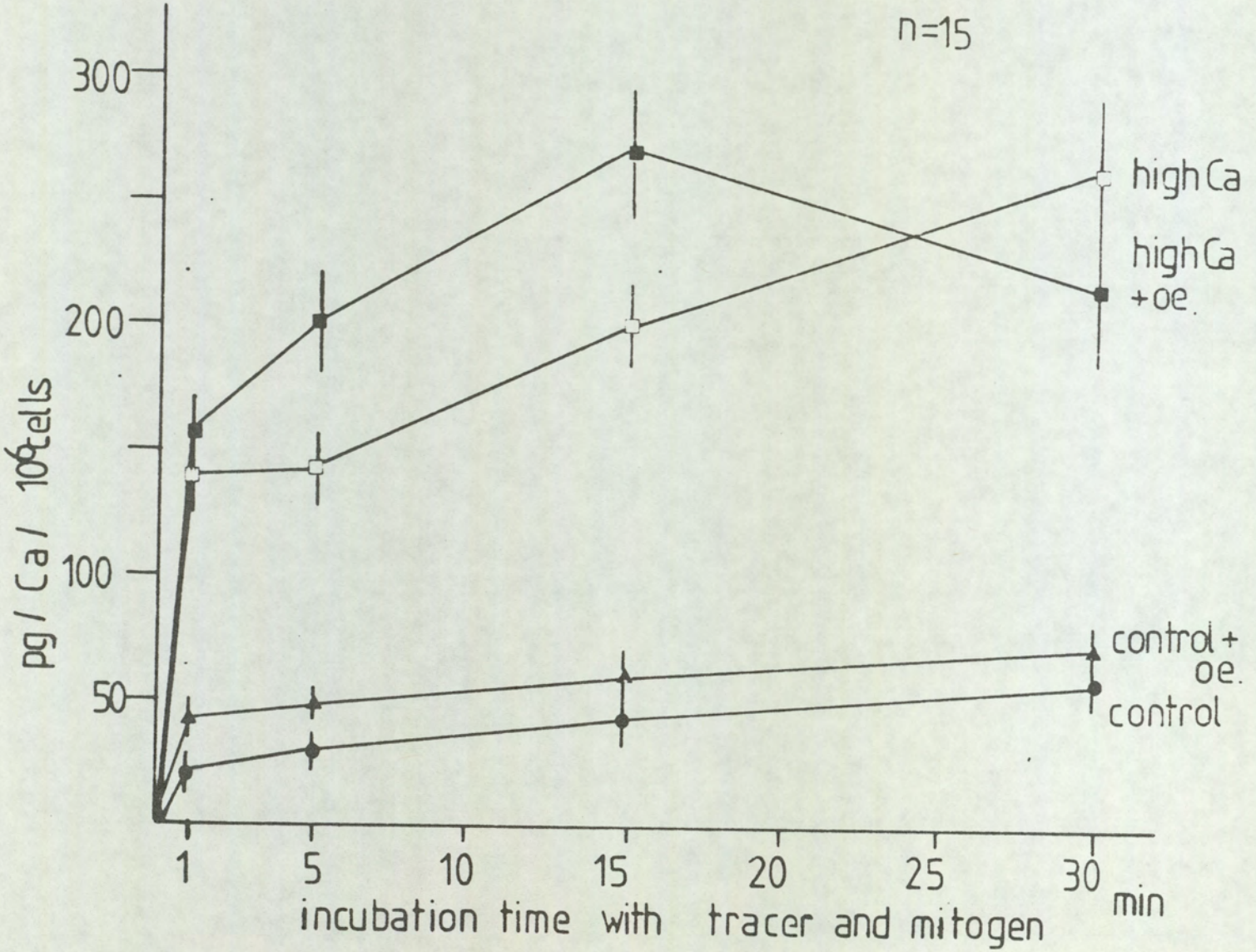
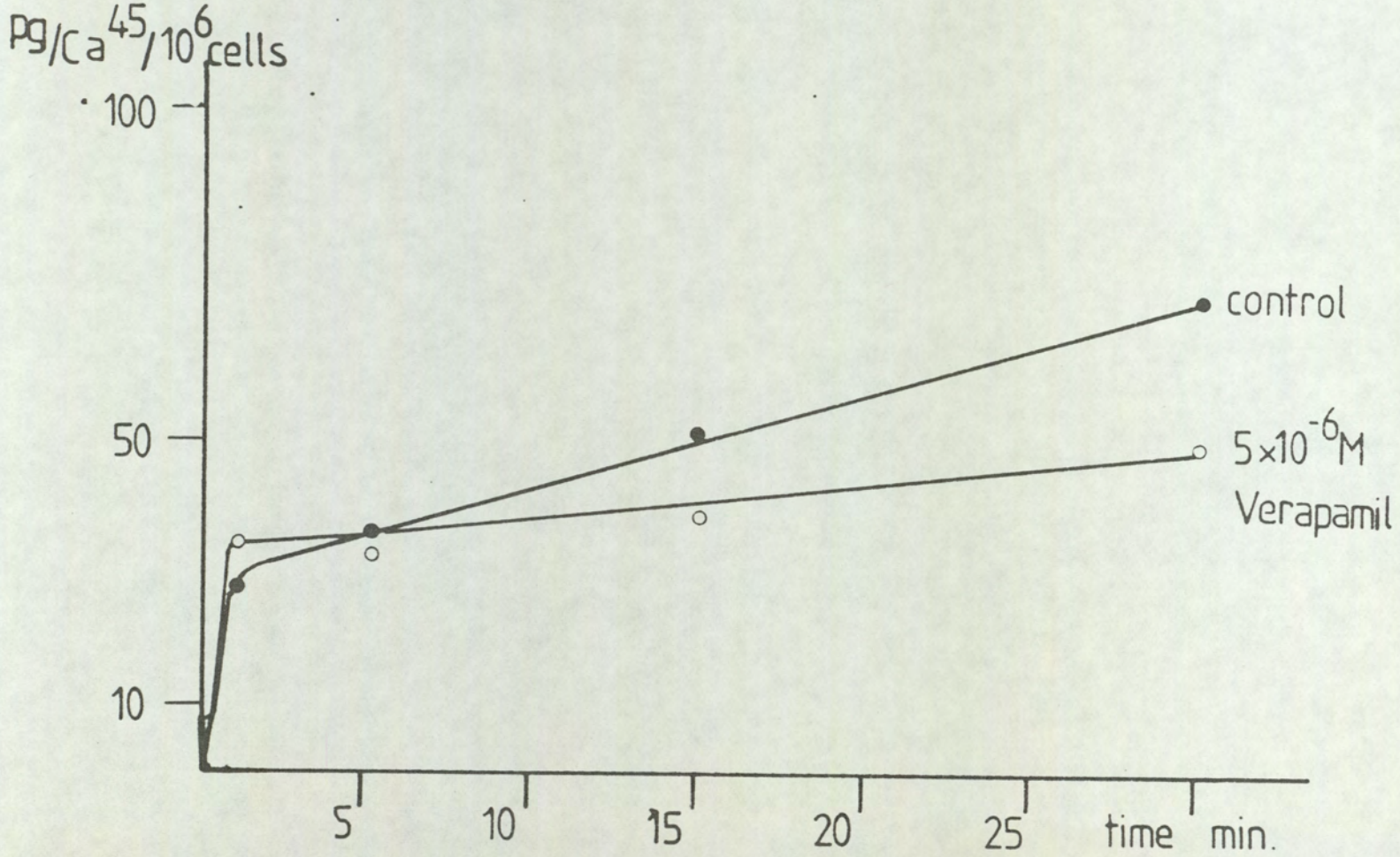


Fig. 30 The influence of Verapamil on the basal rate of Calcium-45 uptake into rat thymocytes



a thirty minute period this constant influx approximated to a net calcium uptake of 1.4 pg/min/10^6 cells. This value is approximately one third of the rate previously observed for rat thymocytes (Hume, 1978). This earlier report may include the membrane bound pool in the influx calculation. The constant rate of the uptake suggests that the simultaneous efflux of calcium, which under "steady state" conditions would be equal and opposite to the uptake, is occurring from a calcium pool not significantly saturated with calcium-45.

The effect of the sudden, mitosis provoking, elevation of extracellular calcium upon calcium uptake was then investigated. Although the specific activity of the culture medium was altered by the increased calcium content, the results are expressed in terms of total calcium movement and therefore automatically compensate for a changed specific activity. As the external calcium content was suddenly raised to 1.8 mM one would expect the calcium homeostatic system to be thrown out of equilibrium. Indeed, this became evident from the dramatic five-fold expansion in membrane-associated calcium occurring in the presence of 1.8 mM extracellular calcium (Figure R29). This change in calcium binding may be the result of simple electrostatic attraction between calcium and the cell surface. After this initial change calcium uptake reached a steady rate of approximately 4 pg/min/10^6 cells, and as such represents a three-fold increase over the basal rate (Figure R29). This approximate stoichiometry between calcium uptake and the external ion concentration was also observed in human lymphocytes (Lichtman et al. 1979). The first conclusion to be drawn from this initial study, presented in Figure R29, is that both the membrane bound and intracellular calcium pools may be increased following an elevation of the extracellular calcium content.

When unstimulated cells were incubated with an anti-mitotic concentration of oestradiol there was no significant alteration in the rate of calcium entry over the initial thirty minute period. The oestradiol-treated basal cells exhibited an uptake of 1.5 pg/min/10^6 cells (Figure R29). Oestradiol treatment did however slightly raise the calcium content of the thymocytes within five minutes. At still higher oestradiol concentrations this elevation became greater and resulted in a dose-dependent increase in calcium uptake (Figure R31).

Fig. R.31. The dose-dependency of the oestradiol influence on transmembrane calcium transport.

Treatment	Calcium uptake (pg/min/10^6 cells)
Control	1.4
0.1µg/ml Oestradiol	1.5
0.2µg/ml Oestradiol	1.7
0.5µg/ml Oestradiol	4.5

When the usual inhibitory 0.1µg/ml concentration of oestradiol was added to thymocytes simultaneously with the normally mitogenic 1.8 mM calcium concentration, the enhanced calcium uptake due to the calcium elevation was further increased (Figure R.29). The rate of oestradiol + high calcium treated cells became 7.1 pg/min/10^6 cells compared with 4 pg/min/10^6 cells in calcium-stimulated cells. Although this new rate of uptake resulted in a higher movement of calcium into the cell the membrane bound pool is saturated. After a fifteen minute period, the enhanced calcium entry provoked by oestradiol in conjunction with high calcium suddenly disappeared. The rate of calcium influx decreased sharply and reached the rate in oestradiol-untreated thymocytes at thirty minutes. This decline may be due to a mixture of enhanced calcium extrusion and an inactivation of calcium entry. It

may not be directly related to the steroid-mediated inhibition of cell division as the inhibitory effect is exerted well before fifteen minutes (Morgan & Bramhall personal communication).

When cells were exposed to high, mitogenic concentrations, of extracellular magnesium ions neither the influx of calcium nor its binding to the cell surface were altered (Figure R.32). Similarly, when thymocytes were incubated with the calcium-dependent mitogen insulin, or the calcium-independent mitogen, adrenaline, there was no detectable change in calcium entry (Figure R.33). The addition of 'low' (under 10^{-5}M) concentrations of ouabain, which were capable of stimulating thymocyte proliferation (Figure R.7) were also without influence on calcium uptake (Figure R.34). High, antimitotic concentrations of ouabain did however enhance influx (Figure R.34) which may be related to its inhibitory action. Thus, mitogenic triggering per-se does not require extra influx of calcium from the external environment.

Although, contrary to our expectations, the calcium-dependent mitogen insulin did not enhance calcium influx it is still possible that other calcium-dependent agents, e.g. acetylcholine or alpha-adrenergic agonists will modify calcium transport. The inability of magnesium and the magnesium-dependent mitogen, adrenaline to influence calcium transport was, however, anticipated. These agents are already known to act independently of the extracellular calcium concentration (see Introduction, section 4). Ouabain-induced mitosis is a 'special case' and it is anticipated that the primary action of ouabain will be upon calcium exit and not calcium uptake. The ability of the inhibitory concentrations of both oestradiol and ouabain to increase the influx of calcium suggests that this effect is in some way related to the inhibitory process itself.

Fig. R 32 The influence of Magnesium on calcium entry

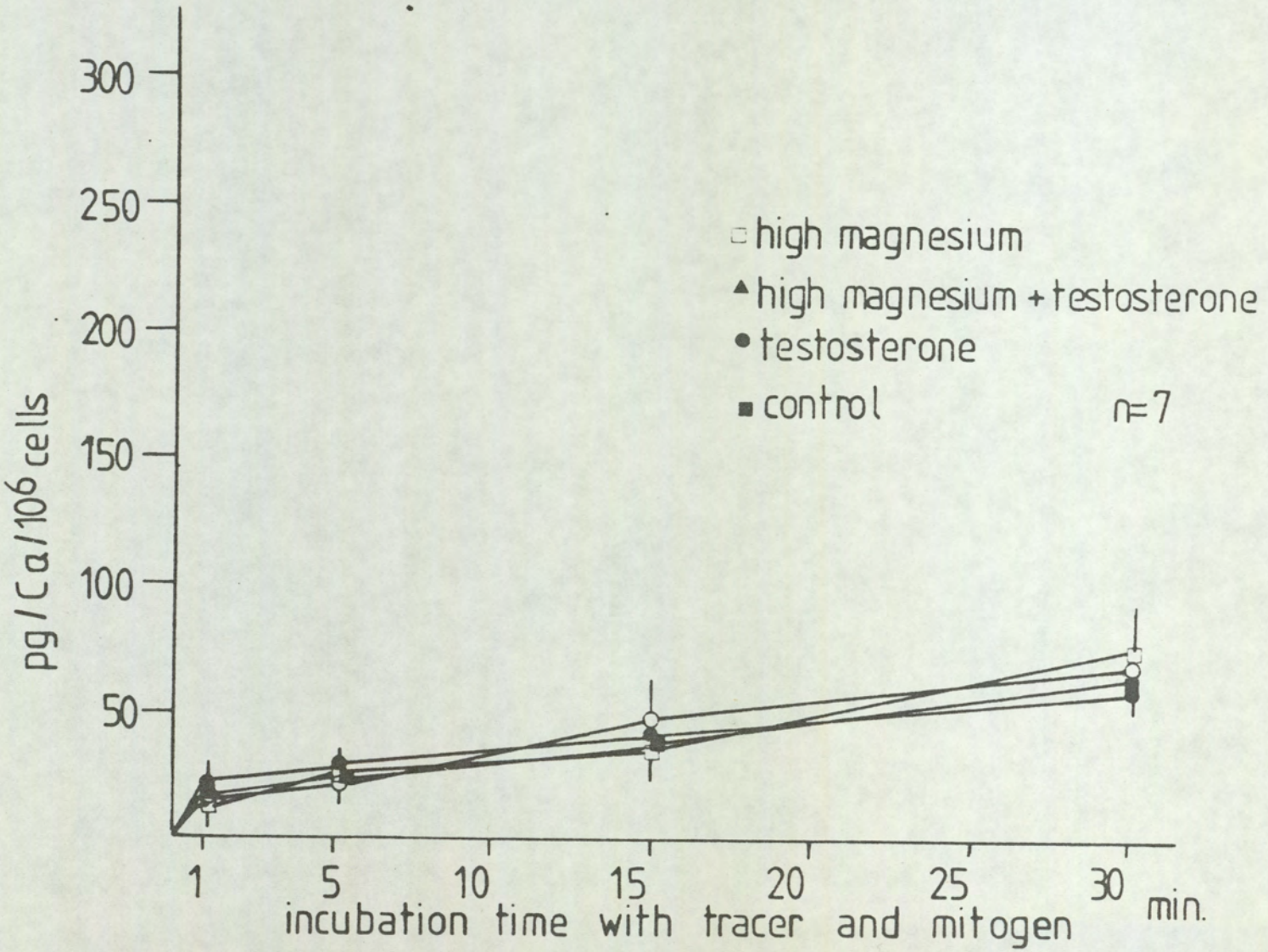


Fig R.33 Effect of Adrenaline and Insulin on calcium entry

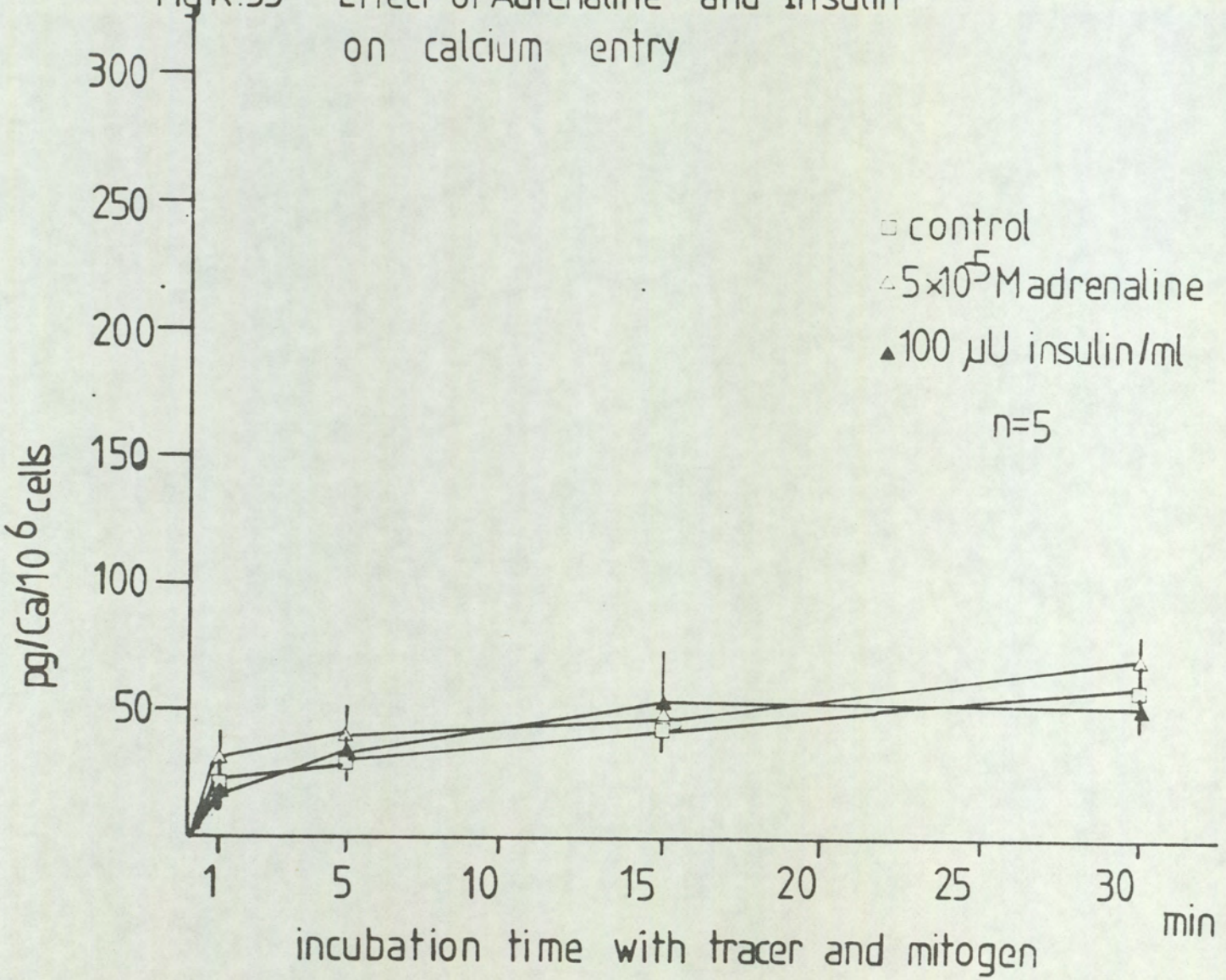
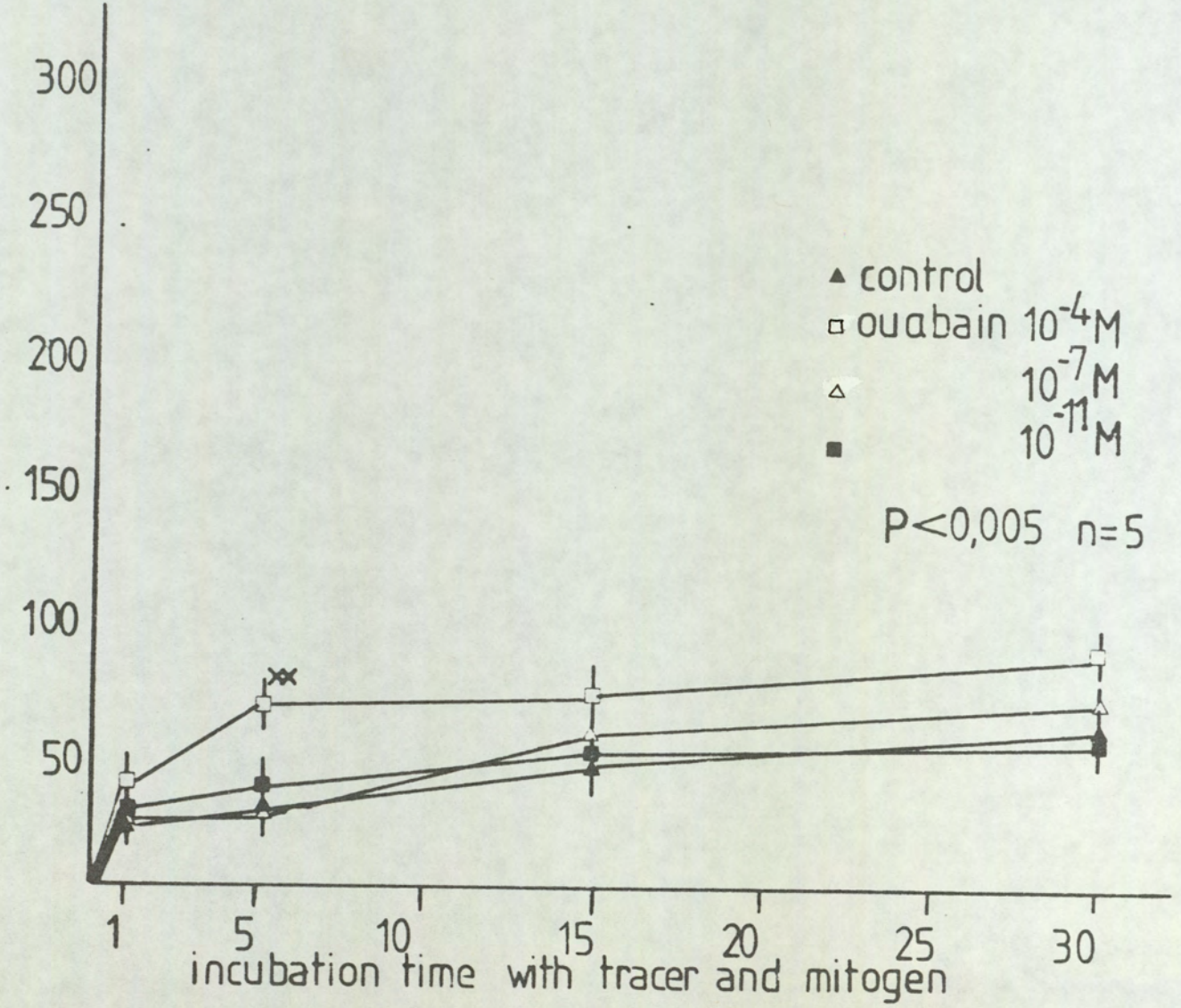


Fig. R34 The effect of Ouabain on calcium uptake



Although not all the anticipated calcium-transport alterations were observed it is remotely possible that the technique used is insufficiently sensitive to detect enhanced calcium uptake. Furthermore, the increased entry of calcium into activated cells may not necessarily result in an alteration in the biologically-active free intracellular calcium pool. Therefore it became necessary to gain some impression of the changes, (if any), in this calcium pool. Direct measurement is not appropriate in these cells for the reasons stated in the Methods section. From the various studies reported in section 2 of the Introduction it appears that measurement of changes in the transmembrane potassium conductance might be a valid alternative to direct calcium measurement. This is assuming that the release of cell potassium will be increased following a rise in the free cytosolic calcium content.

As has already been explained above, the tracer Rubidium-86 (Rb^{86}) was used to represent potassium ions in all experiments to measure potassium fluxes. Although this substituent is a valid tracer molecule and behaves like potassium in a variety of systems (Kimelberg, 1974; Bernstein & Israel, 1970; Mills & Tupper, 1975) it was essential to prove that Rb^{86} was also acceptable in the thymocyte. As Figure R.35 demonstrates, the entry of Rb^{86} into both control and stimulated cells was linear over the thirty minute activation period. Thus, the internal potassium reservoir was not saturated during this time, and tracer re-export would be minimal. The Rb^{86} influx proved to be sensitive to the Na/K ATPase inhibitor ouabain, the reduction in uptake being noticeable within ten minutes (Figure R.36). This figure also demonstrates that the very low (10^{-11}M) mitogenic concentration of ouabain influences monovalent cation balance, and by inference Na/K ATPase function. This confirms the earlier observ-

Fig R 35 Rubidium uptake into thymocytes

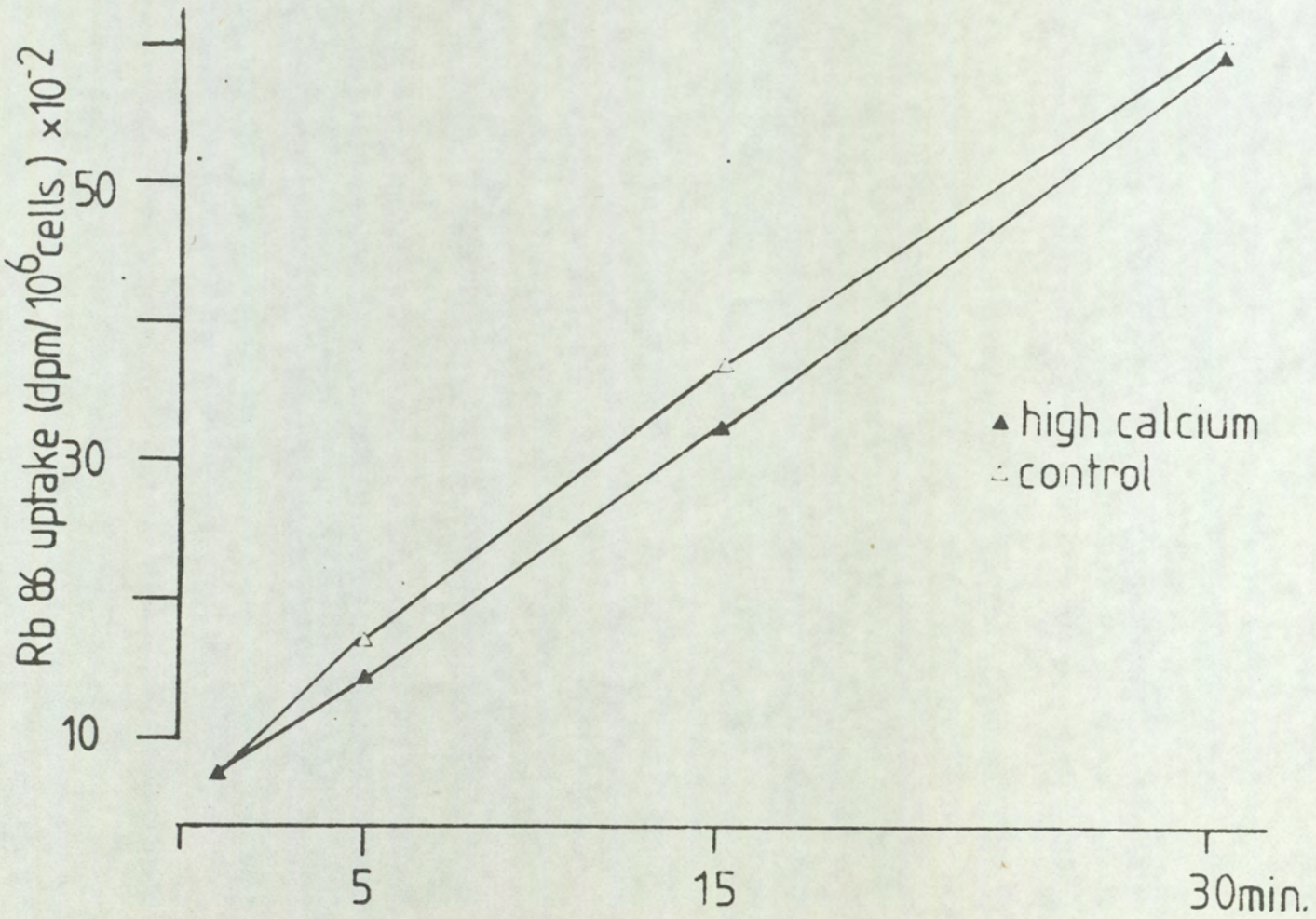
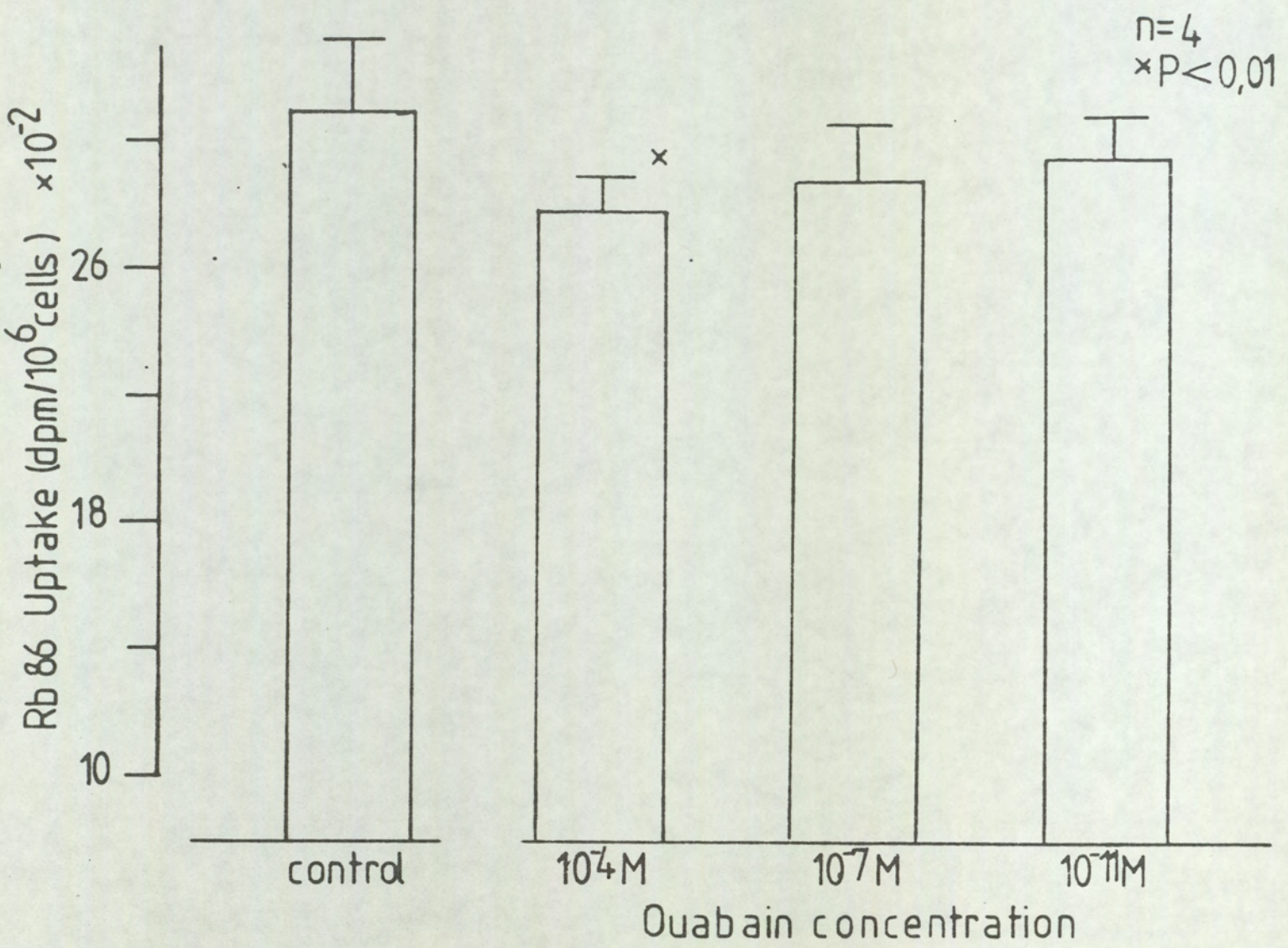


Fig. R 36 Effect of Ouabain on Rb 86 uptake after 15min.



ations made upon both membrane homogenates and whole cells. The ouabain-dependency further indicates that the Rb^{86} molecule is acting as a suitable potassium substitute.

Whilst a stimulatory calcium concentration depressed Rb^{86} uptake noticeably within fifteen minutes, this was not statistically significant (Figure R.37). The addition of oestradiol to the calcium stimulated cultures produced a further depression in rubidium transport, which again was not significant (Figure R.37). Parenthetically it should be noted that a mitogenic extracellular concentration of magnesium, insulin or adrenaline all failed to influence rubidium incorporation. From this study it must be concluded that rubidium uptake,and presumably Na/K ATPase function, is not significantly altered during the early period of stimulation. Whilst it is possible that the enhanced rubidium exit reported below is causing the lowered rubidium uptake, it is also possible that the action of these two treatments may be related to an inhibitory action of intracellular calcium upon the Na/K ATPase system.

Having established the constancy of the inward rubidium movement, it was possible to investigate mitogen-mediated changes in the outward transmembrane potassium current. Cells pre-labelled with rubidium for sixty minutes were washed to remove all extracellular tracer. The rubidium loaded cells were then placed into fresh medium and the release of intracellular isotope studied. The initial efflux was extremely rapid, but decayed away in an apparently exponential manner (Figure R.38). On a very simple level this may be considered to indicate two intracellular potassium pools in contact with the external environment. A similar model has been described for the

Fig R 37 Rubidium entry after 15 min.

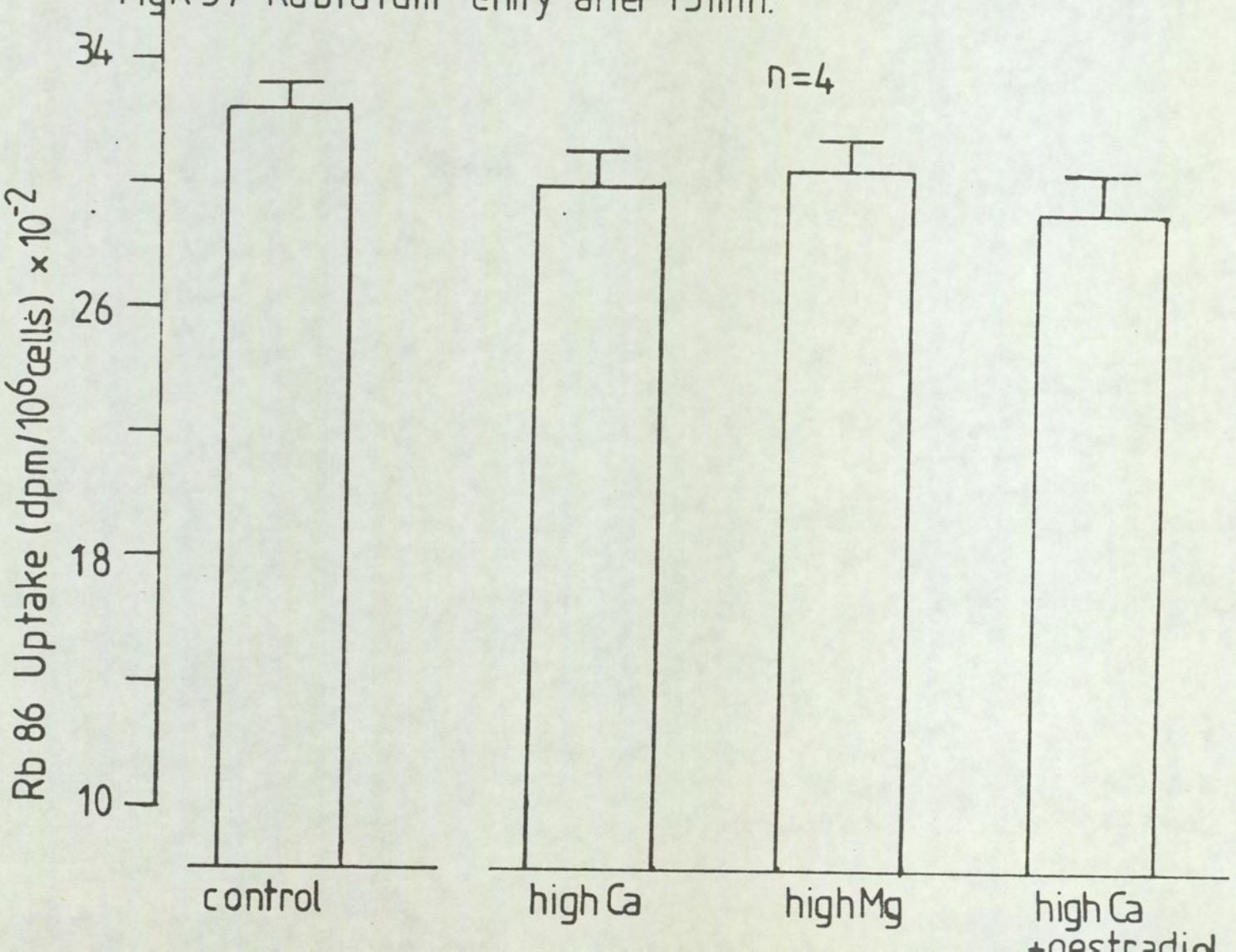
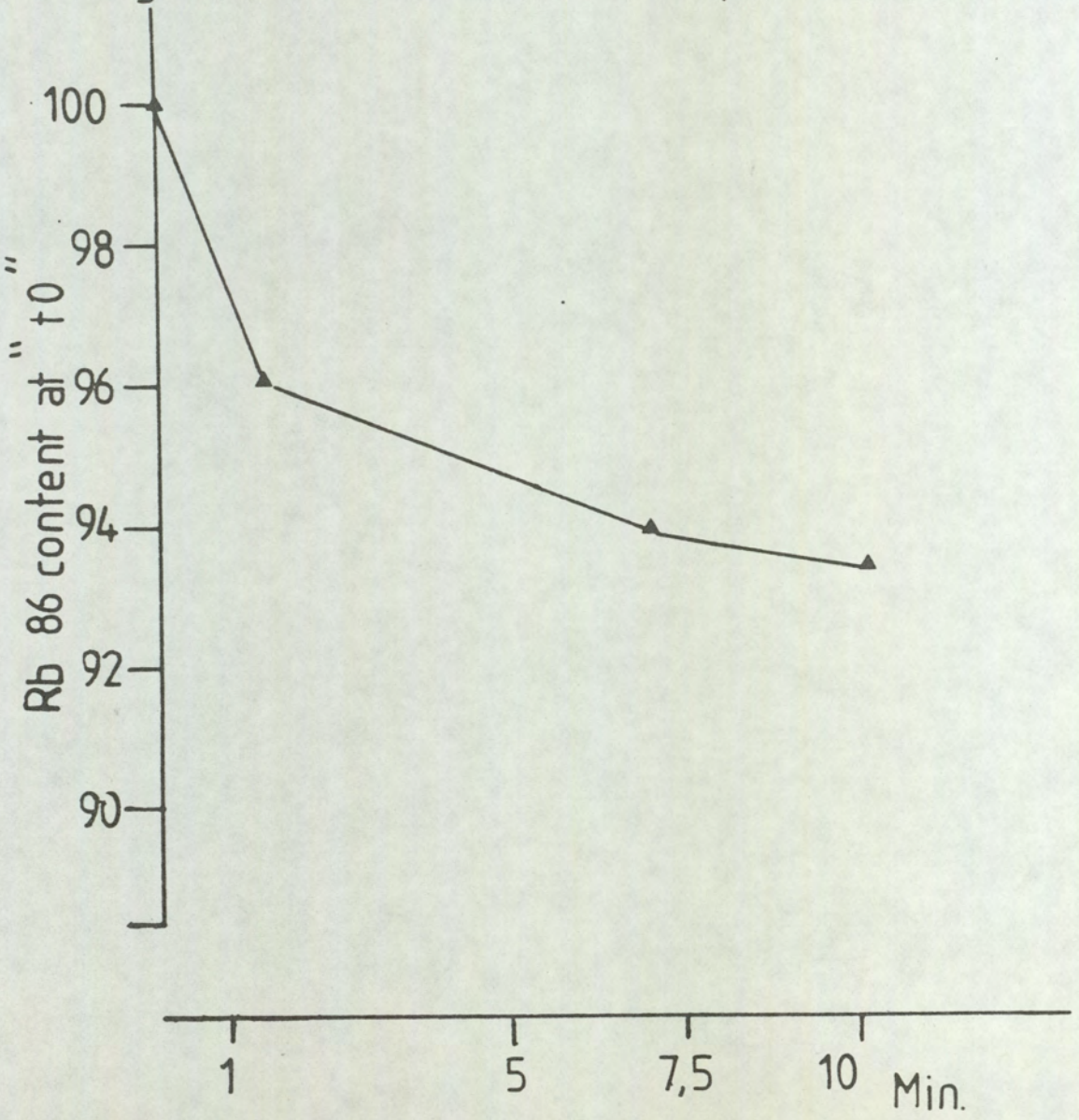


Fig.R 38 Efflux of Rb 86 from pre-labelled cells



human lymphocyte (Negendank & Shaller, 1979). Ten minutes after the addition of 1.8 mM calcium the rubidium efflux by the first, fast, component of efflux was enhanced. This suggests that the rapidly exchangeable intracellular potassium pool is sensitive to an increased internal calcium concentration (Figure R.39). Indeed, the prevention of calcium entry by the inhibitory agent verapamil, resulted in the abolition of the enhanced rubidium efflux (Figure R.40). This is taken as confirmation that the rise in calcium does indeed trigger rubidium efflux.

The addition of oestradiol to unstimulated thymocytes increased the basal rubidium efflux (Figure R.41) confirming that the oestradiol promoted calcium uptake into basal cells raised the cytosolic calcium content. Oestradiol did not influence the rubidium release provoked by high calcium (Figure R.41). Thus, although oestradiol and 1.8 mM calcium are additive in promoting calcium entry the rubidium exit appears to be maximally activated by calcium alone.

When the calcium-dependent mitogen, insulin or the magnesium-dependent mitogen, glucagon, were added to rubidium loaded thymocytes both were found to increase the efflux of the tracer (Figure R.42). The effect of the magnesium-dependent mitogen was not expected as such agents consistently failed to alter transmembrane calcium influx. The validity of the adrenaline phenomenon was explored by adding magnesium ions themselves. This treatment again provoked the increased rubidium conductance (Figure R.43). Interestingly testosterone treatment, which will only inhibit magnesium-dependent mitogens, did not alter rubidium exit (Figure R.43). However, when testosterone was added in the presence of a stimulatory magnesium ion concentration the Rb^{86} conductance was stimulated in an additive manner (Figure R.43). This phenomenon may be related to the anti-mitotic action of

Fig.R 39 Calcium stimulation of Rb 86 efflux

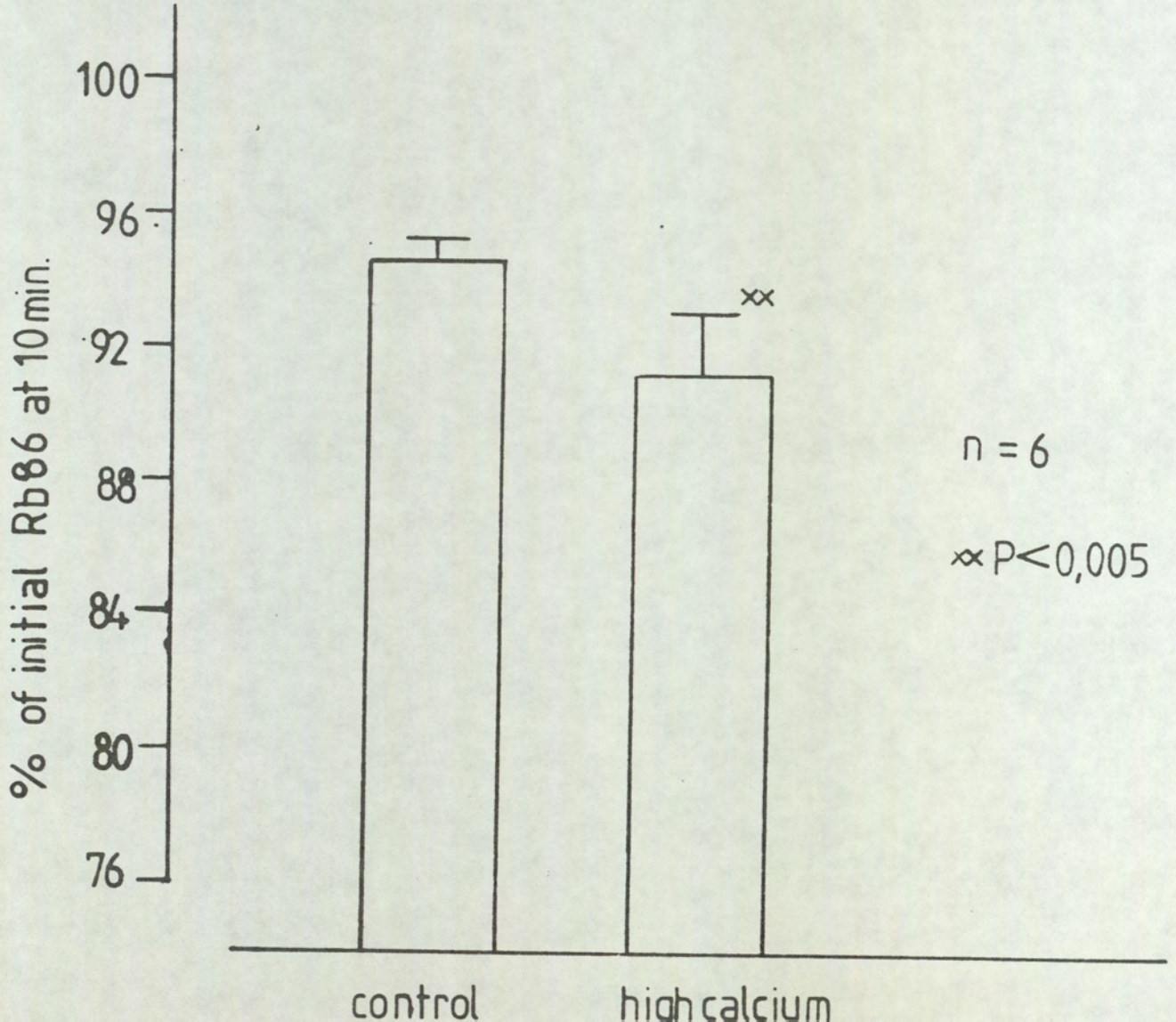


Fig.R40 Verapamil blockade of calcium-induced Rb-86 efflux

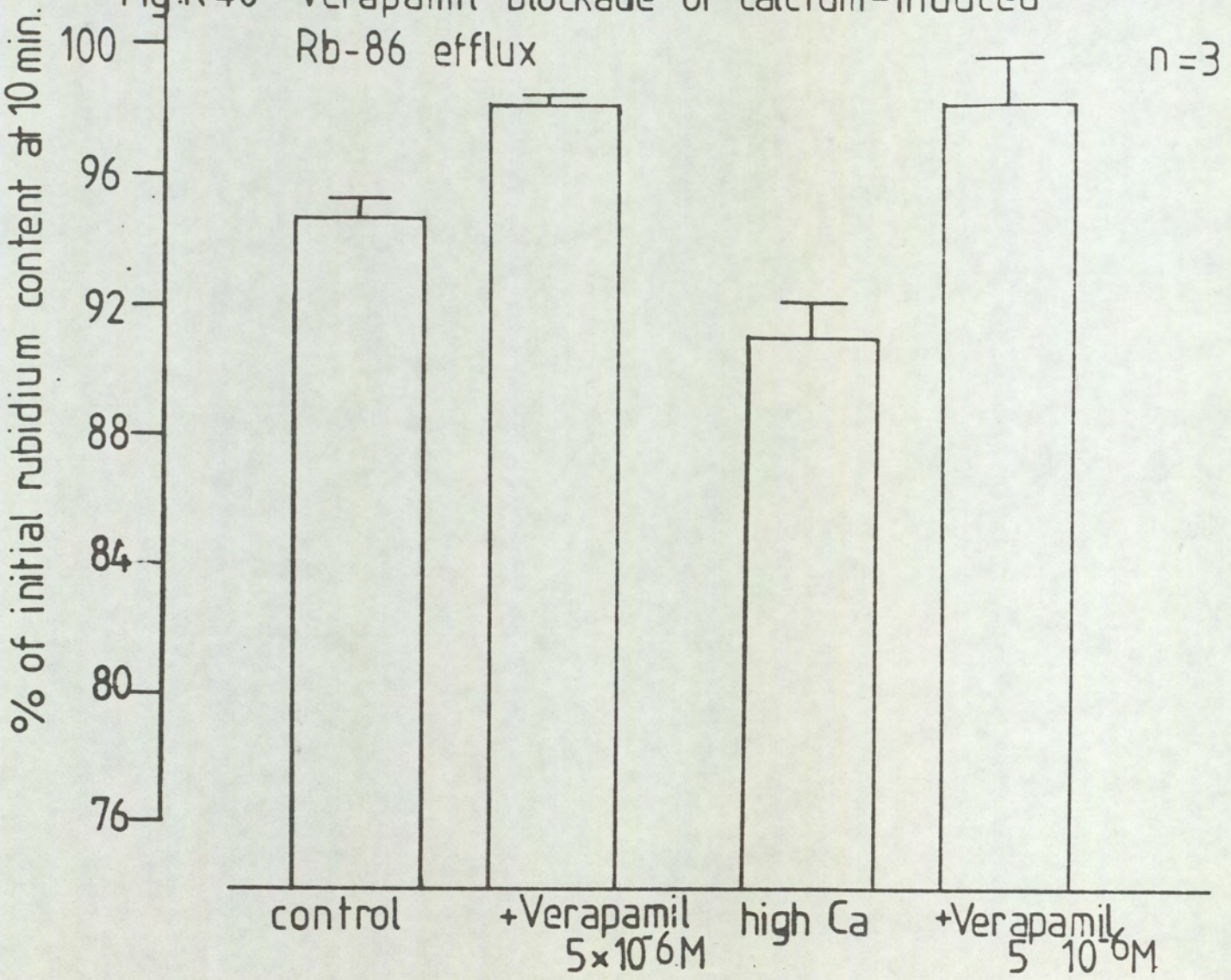


Fig 41 Oestradiol effect on rubidium release

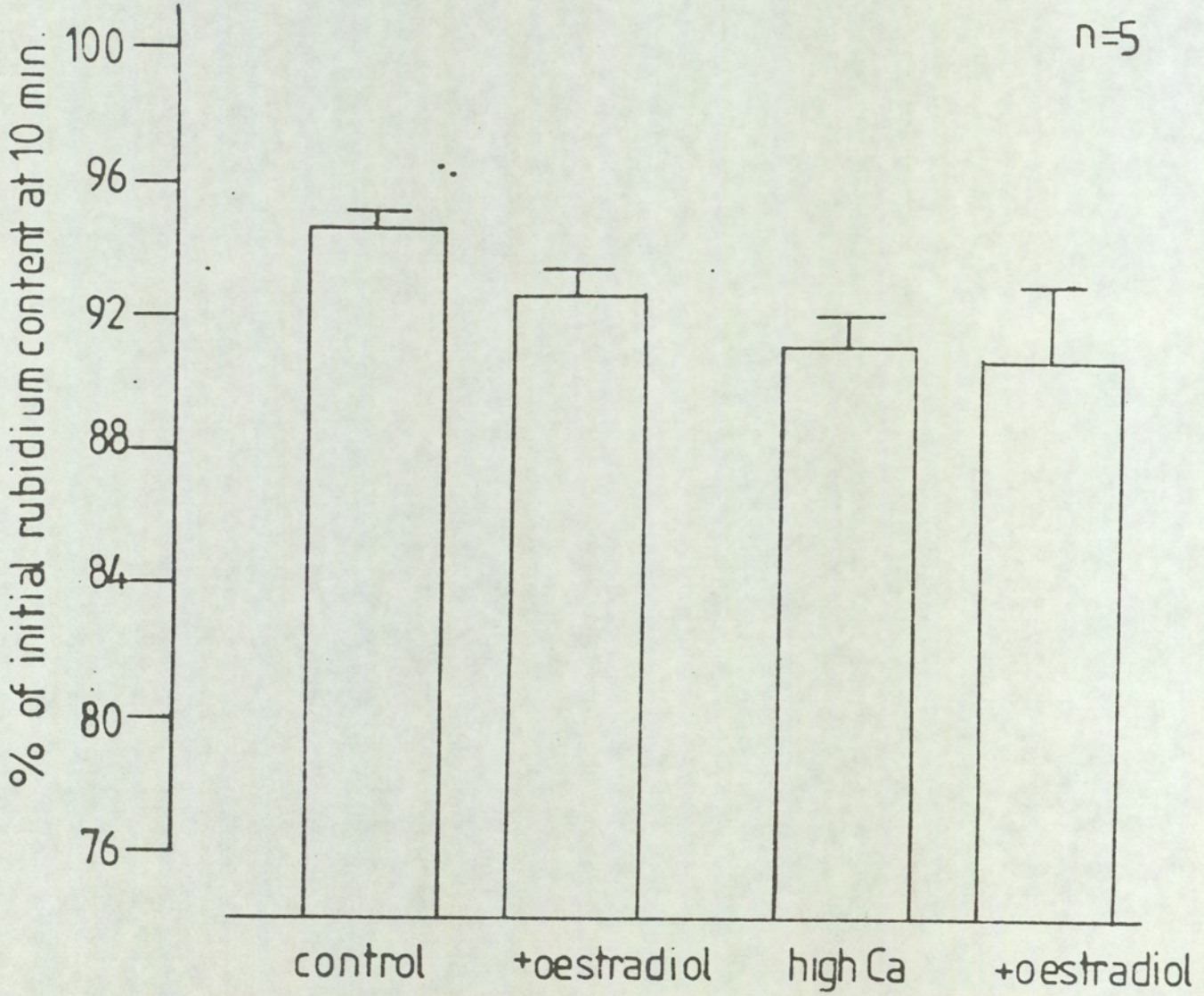


Fig R42 Action of hormonal mitogens upon rubidium efflux

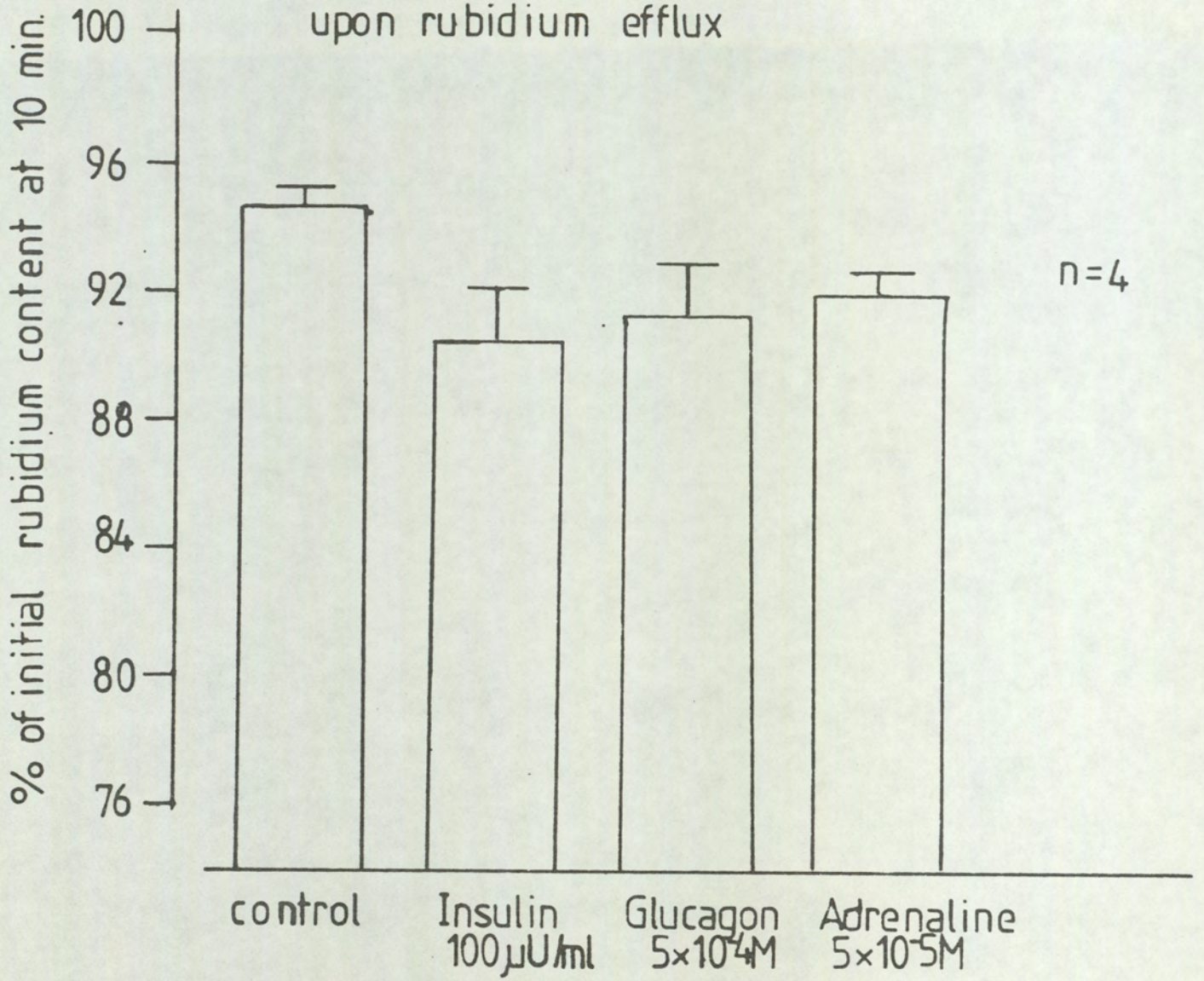
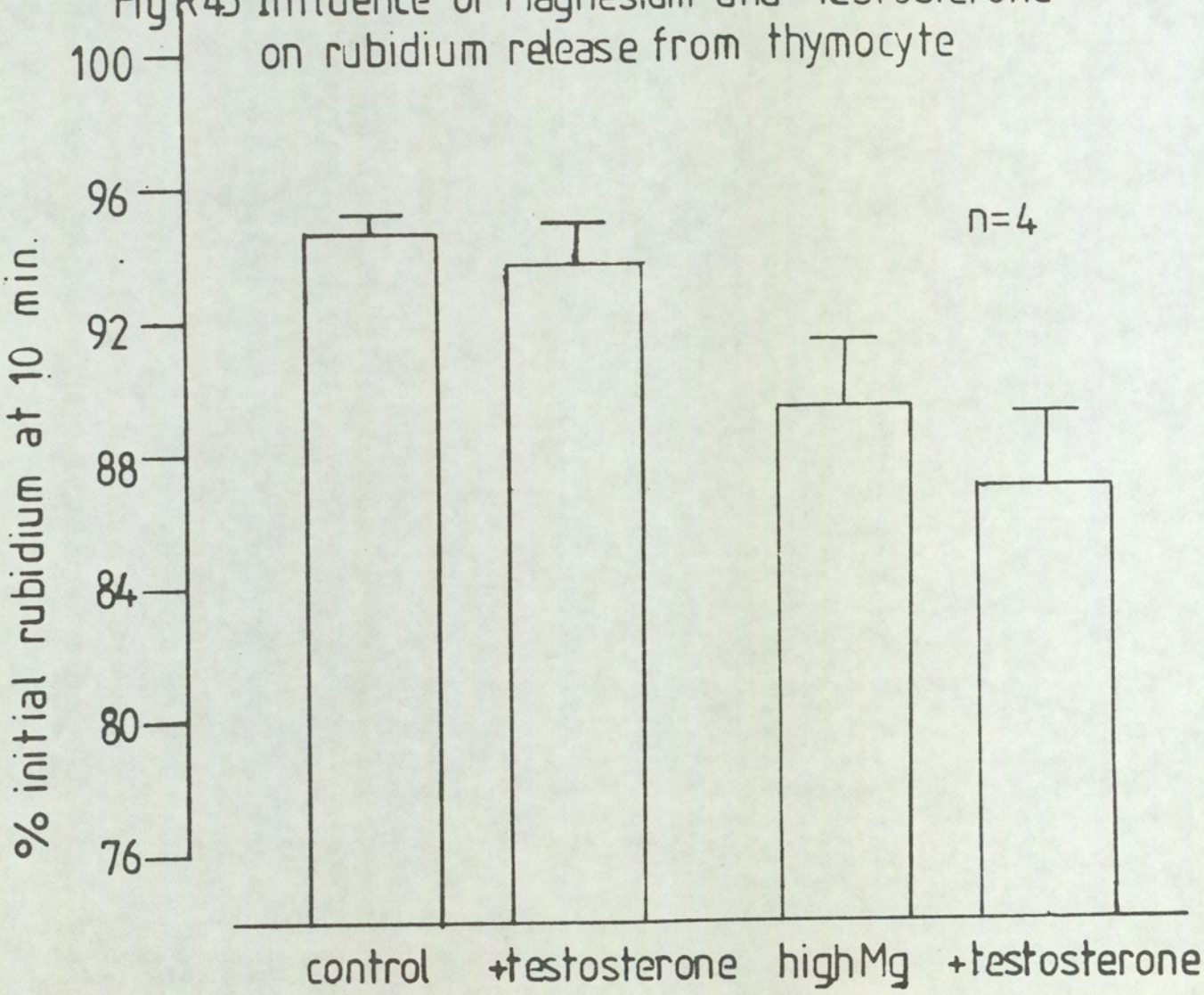


Fig R43 Influence of Magnesium and Testosterone on rubidium release from thymocyte



the steroid.

The concentration dependency of two thymocyte mitogens was tested to confirm that the rubidium conductance was provoked only at mitogenically active concentrations of stimulatory compounds. Both histamine and exogenous cyclic GMP exhibit a marked concentration dependency in their stimulation of mitosis (Morgan, Hall & Perris, 1977; Morgan & Perris, 1975). Figures R.44 and R.46 show the rubidium conductivity response to these agents. Cyclic GMP addition provokes a biphasic stimulation of thymocyte proliferation over two concentration ranges, each requiring a different extracellular divalent cation (Morgan, Hall & Perris, 1977). At both the 10^{-6} and 5×10^{-11}M levels the nucleotide stimulated rubidium exit (Figure R.46). These concentrations correspond to the mitotically active cyclic GMP levels. Histamine promotes thymocyte mitosis over an extremely wide concentration range of 10^{-9} to 10^{-13}. The effect of these concentrations upon the rubidium efflux is displayed in Figure R.44. It is evident that an approximate relationship exists between the mitotic potential and rubidium efflux stimulated by Histamine.

The two mitogenic concentrations of either ouabain or sodium both activated rubidium conductance (Figure R.48 and Figure R.49). These agents were able to provoke rubidium efflux at either their calcium- or magnesium-dependent concentrations, whereas the mitotically inactive 10^{-9} concentration did not influence conductivity. The mechanism of these two mitogens is believed to be mediated through an inhibition of intracellular calcium exit (see above). Consequently a series of experiments were performed to determine if they did actually restrict calcium exit. Thymocytes were pre-labelled with calcium-45 in the same way as for rubidium. In an initial experiment (Figure R.50) it was demonstrated that the addition of 1.8 mM

Fig R44 Histamine alteration of rubidium content

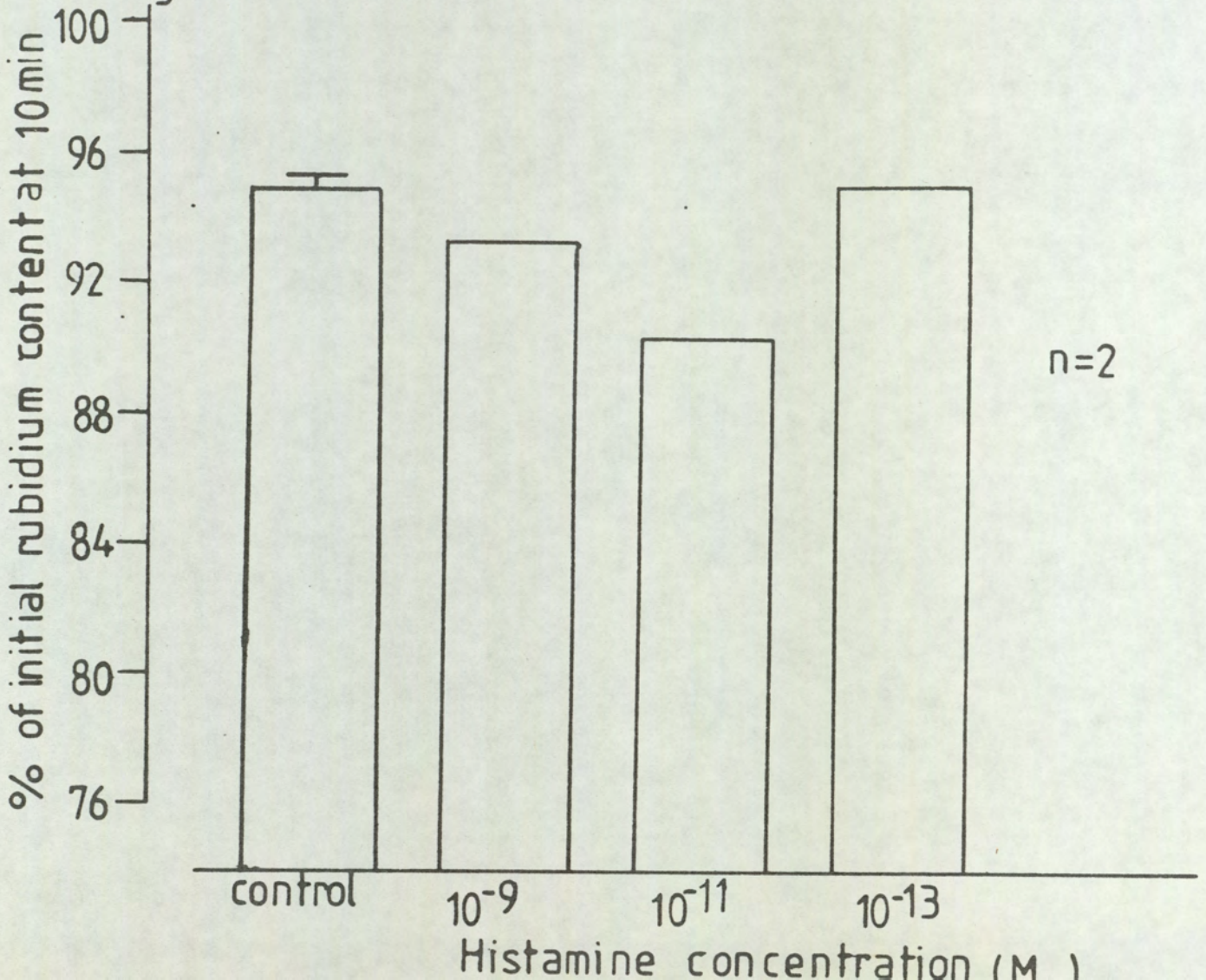


Fig R46 Influence of Cyclic GMP on Rb 86 content

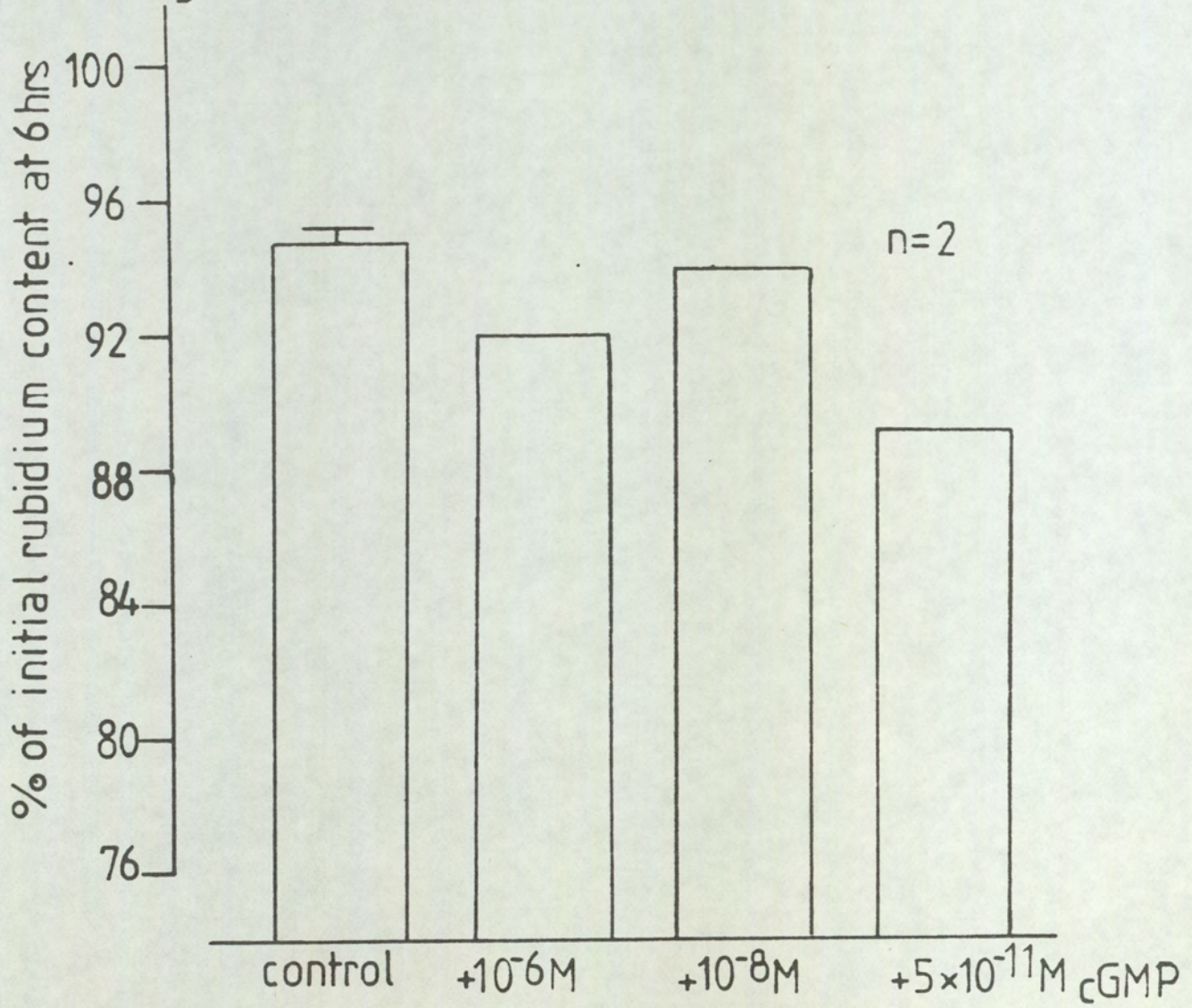


Fig.R 48 Ouabain alteration of rubidium exit

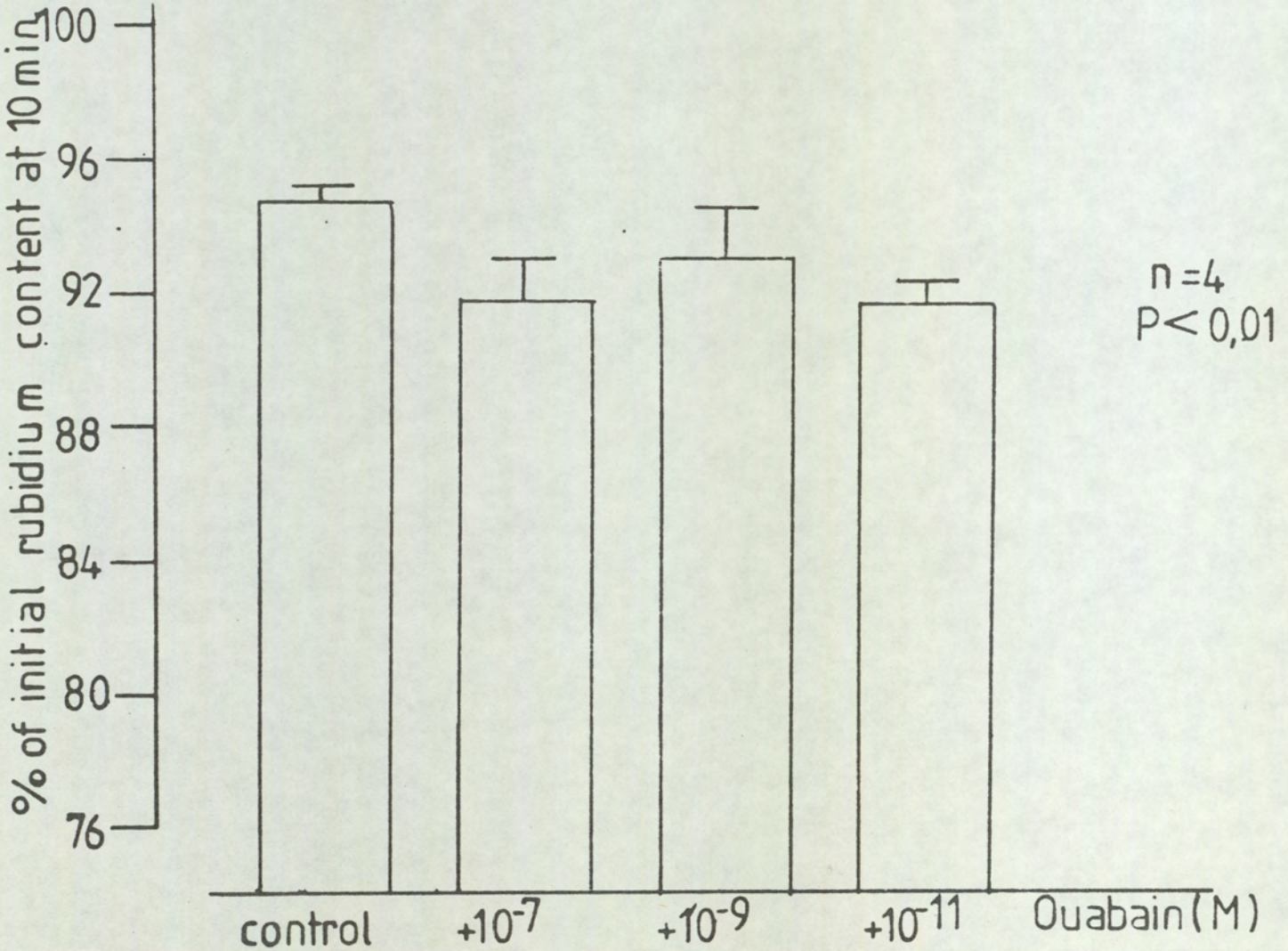
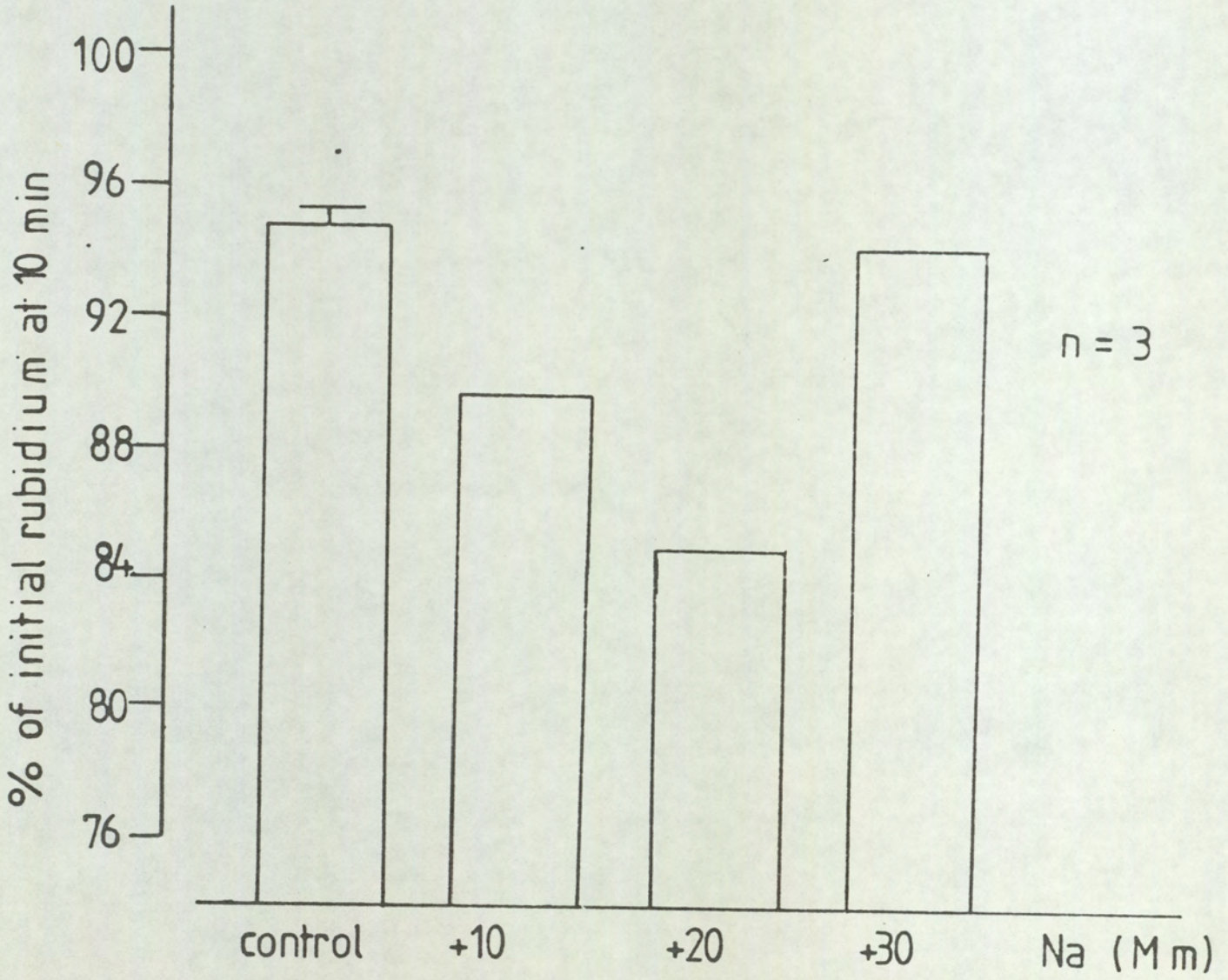
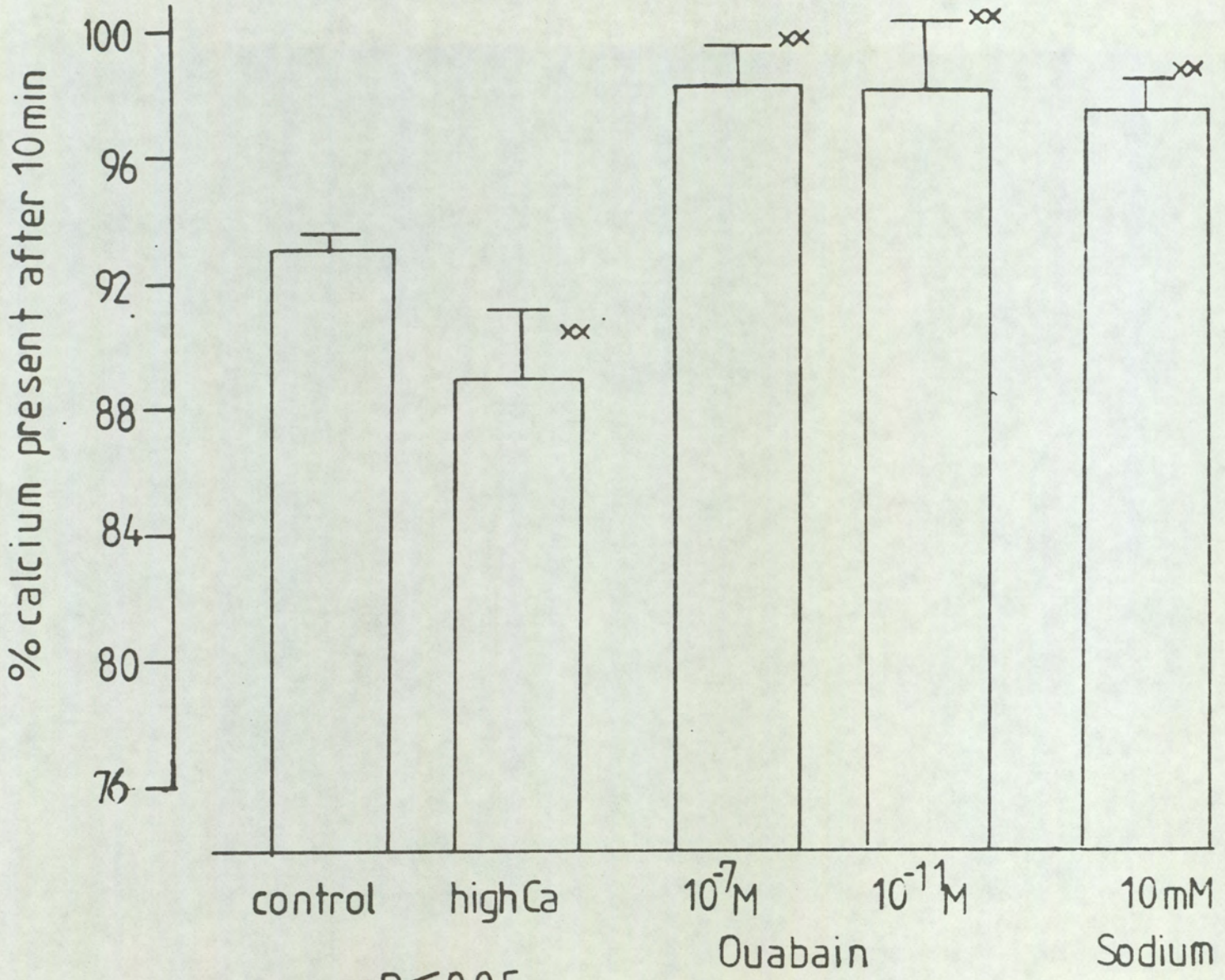


Fig R49 Sodium induced rubidium efflux



extracellular calcium provoked a rapid exit of cellular calcium. Whilst calcium uptake was increased over three-fold the efflux was increased less than two-fold (Figure R.50). Ouabain treatment at the calcium-dependent (10^{-7}M) concentration almost completely blocked the efflux of calcium ions (Figure R.50). Significantly the calcium-dependent 10 mM sodium concentration was also able to inhibit calcium exit (Figure R.50). Ouabain at the magnesium-dependent (10^{-11}M) concentration also inhibited calcium exit. This last result is of tremendous significance for not only does this show that a magnesium-dependent agent interferes with calcium metabolism it also shows that the rubidium conductance promoted by magnesium-dependent agents can be due to an increased cytosolic calcium. Thus it is now evident that both calcium and magnesium, and their respective dependent mitogens can all influence rubidium exit. This phenomenon, dependent upon a rise in cytosolic calcium, demonstrates that all thymocyte mitogens have the potential to elevate intracellular calcium ion concentration. If this is indeed so, then changes in the intracellular calcium content will provide the essential coupling factor between thymocyte mitogen and the mitotic apparatus. Before considering this stimulus-mitosis event in the light of the experimental results it will be useful to summarise briefly the evidence presented in the preceding section.

Fig R50 Calcium efflux from thymocytes



xx P < 0,05

n = 4

SUMMARY

An elevated calcium or magnesium concentration provoked the recruitment of a single, quiescent, population of thymocytes into the mitotic cycle. The calcium-induced stimulation required the unrestricted access of the divalent cation to the cell interior. Upon elevating the extracellular concentration of this ion its entry into the cell was increased. The rate of uptake was higher than the rate of efflux suggesting that the internal calcium content would be increased. This was confirmed by the activation of rubidium exit following the application of 1.8 mM extracellular calcium. The rubidium efflux, dependent on the entry of calcium ions, was also observed when the glycoside, ouabain or extracellular sodium was used to provoke mitosis. Both of these agents interfere with extracellular calcium homeostasis and serve to inhibit calcium efflux from the thymocytes. Thus calcium, from either the external or internal environment, may provoke recruitment. When calcium-dependent mitogens were employed there was no increase in calcium entry but the rubidium conductance still appeared. Rubidium exit provoked by the calcium-dependent concentration of ouabain was sensitive to the calcium antagonist, verapamil. Thus, although these agents do not increase calcium entry the continual basal influx is necessary for stimulation. Neither magnesium nor magnesium-dependent mitogens altered calcium uptake although they all provoked rubidium efflux, without requiring the entry of extracellular calcium. It is possible that magnesium itself could activate rubidium exit but the ability of the magnesium-dependent concentration of ouabain to inhibit calcium extrusion suggests that their mitotic action is also mediated through an alteration in calcium homeostasis. The intracellular calcium environment may also participate in the anti-mitotic action of the steroids testosterone and oestradiol.

Both of these compounds, and indeed ouabain, promoted an increase in the intracellular calcium concentration, although this was not associated with an increased cell division.

It is now apparent that increased extracellular calcium, which will provoke mitosis, resulted in an elevated internal free-calcium content. Calcium- and magnesium-dependent mitogens did not alter calcium entry but still elevate intracellular calcium, presumably to a stimulatory level. As ouabain and sodium can promote mitosis by modifying intracellular calcium homeostasis it is possible that these other mitogens also promote mitosis by providing intracellular calcium from within the cell. The entire concept of stimulus-mitosis coupling by an altered intracellular calcium concentration, and the mechanisms by which external mitogens can provide such a calcium change, are discussed below in the light of these experimental findings.

7 Discussion

Cellular functions as diverse as contraction and secretion are modified by a transient alteration in the free intracellular calcium concentration (Putney, 1979; Berridge, 1975). Calcium ions can also influence the cell cycle entry and progression of mammalian cells. Hence, the proliferation rates of rat, mouse, human and chick fibroblasts, and murine lymphoblasts are all reversibly slowed by a long-term reduction in the calcium content of their culture medium (Frank, 1973; Gail, Boone & Thompson, 1973; Boynton & Whitfield, 1976; Hazelton & Tupper, 1978; Moscatelli, Sanui & Rubin, 1979; Brennan & Lichtman, 1973). Such sustained calcium deprivation may non-specifically restrict mitosis by interfering with the many intracellular processes dependent upon calcium (Balk, Whitfield, Youdale & Braun, 1973). However, a short-term reduction of intracellular calcium also induces a temporary arrest of normally cycling fibroblasts, these cells being halted at specific calcium-sensitive points with the G_1 phase of the cell cycle (Paul & Ristow, 1979). Such a calcium-dependent restriction also appears to occur in vivo. Proliferatively committed rat hepatocytes will execute many pre-replicative steps before arriving at a G_1 stage that requires a normal external calcium level for their cell cycle progress (Rixon & Whitfield, 1975; Whitfield, MacManus, Rixon, Boynton, Youdale & Swierenga, 1976). To some extent this mechanism would account for the hypoplasia produced within the rat bone marrow and thymus by a persistent hypocalcaemia (Rixon & Whitfield, 1972; Perris, Weiss & Whitfield, 1970).

Whilst this permissive role of calcium may be a universal feature of dividing cells, the ion is also able to positively regulate the proliferation of certain cells. The close relationship between an increased extracellular calcium concentration and stimulated proliferative activity within various rat tissues in vivo has already been

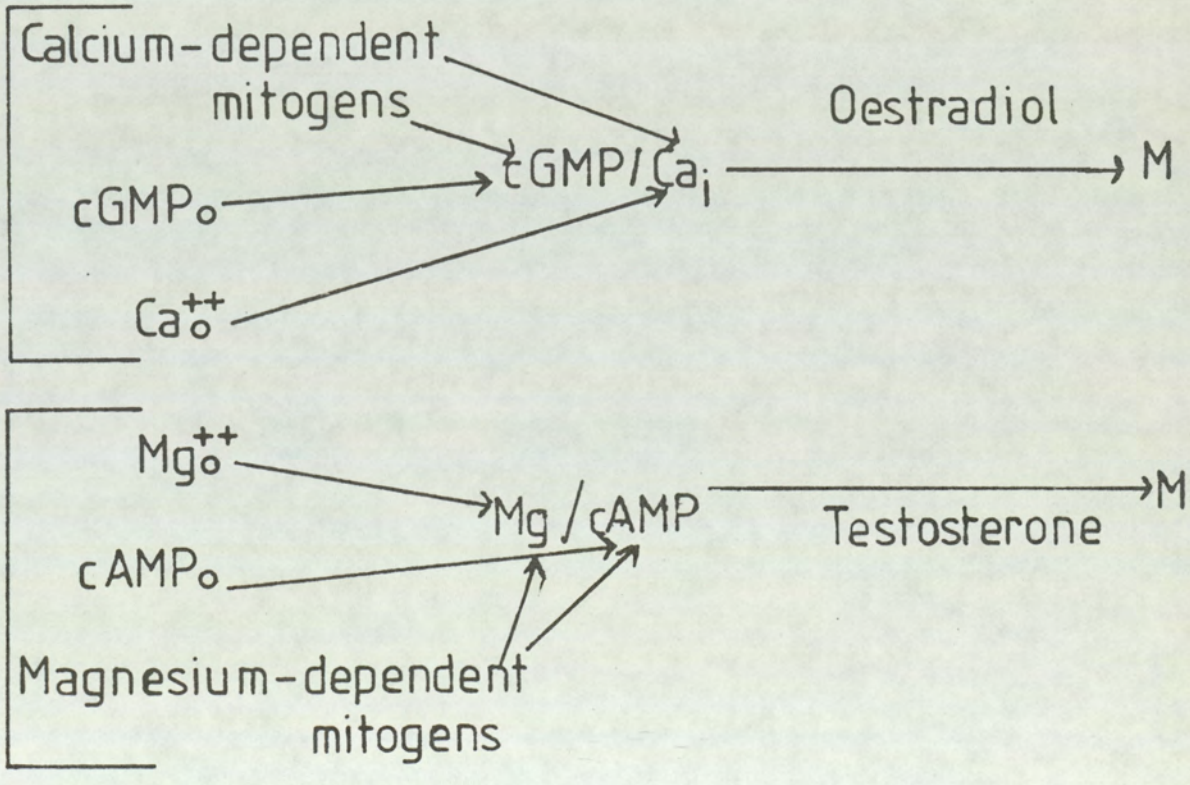
considered in the Introduction (Section 4). It is important to note, that under a variety of physiological and artificial conditions, hypercalcaemia is invariably associated with an increase in the number of mitotic cells within these tissues (Perris, 1971; Whitfield et al. 1976). Cells prepared from the thymus or bone marrow will also demonstrate this mitotic response to hypercalcaemia in vitro (Perris, Whitfield & Rixon, 1967; Morton, 1968). Likewise, an increased extracellular calcium concentration is also able to raise the mitotic activity of cultured human fibroblasts and murine bone marrow, fibroblasts and lymphoblasts (Dulbecco & Elkington, 1975; Gallien-Lartigue, 1976; Brennan & Lichtman, 1975; Boynton, Whitfield, Isaacs & Tremblay, 1977a; Boynton, Whitfield, Isaacs & Tremblay, 1977b). This calcium-induced mitosis is believed to represent one aspect of the stimulus-mitosis coupling phenomenon (Berridge, 1976). The present study therefore investigates those mechanisms by which external calcium ions, and other mitogenic stimuli, activate processes that culminate in the mitotic recruitment of quiescent thymic lymphocytes.

A sudden elevation in the extracellular content of either calcium or magnesium ions was able to induce the rapid mitotic recruitment in vitro of thymocytes belonging to a single quiescent cell population (Figure R1 and R2). The stimulatory process was completed within thirty minutes, after which the triggering stimulus was no longer required (Figure R28). The processes involved in mediating this rapid cell activation are not fully understood. One recent hypothesis suggests that the two mitogenic divalent cations act through discrete axes, ultimately influencing cellular cyclic nucleotide concentrations (Perris & Morgan, 1975). Magnesium, and hormones requiring extracellular magnesium, are thought to exert their mitotic influence via a stimulation

of adenylate cyclase. In contrast calcium and calcium-dependent mitogens may act via guanylate cyclase stimulation. Both of these contentions are supported to some extent by the reported divalent cation sensitivities of the respective nucleotide-cyclase enzyme systems (Schultz, Hardman, Hurwitz & Sutherland, 1973; Birnbaumer, Pohl, Michiel, Krans, Ralbell, 1970; Rasmussen & Goodman, 1977 (Introduction, Section 1). Although these two mechanisms of recruitment are believed to be independent they must ultimately interact with a common series of processes to instigate recruitment. The most likely candidates are cyclic-nucleotide changes which would be able to specifically regulate cell cycle progression (Figure D.1).

Fig. D.1. Hypothetical model of thymic lymphocyte activation via two discrete axes

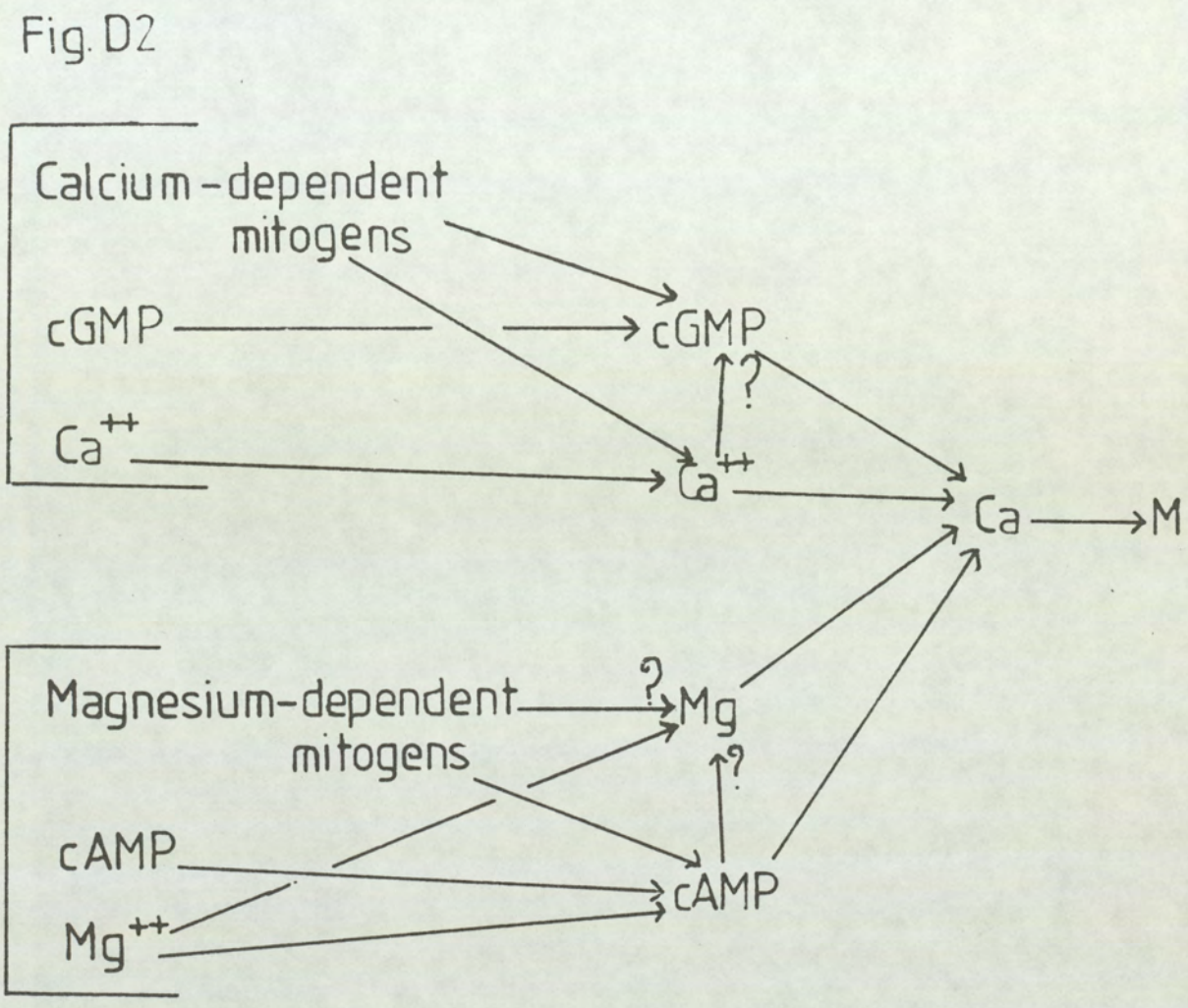
Fig D1.



Extracellular calcium ions will only stimulate division in the rat thymocyte or chick fibroblast when available in a freely diffusible, non-chelated form (Figure R27, Moscatelli, Sanui & Rubin, 1979). That calcium must also have unrestricted access to the cytosol (Figure R24 and R25) suggests that the free ion itself controls mitosis from within the cytosol. The role of magnesium is more speculative, indeed it has been demonstrated that calcium ions are more suited to the role of intracellular messenger than are magnesium ions (Introduction, Section 1). Furthermore, there is a marked scarcity of observations relating magnesium to cell-activation processes. These points, and the relative insensitivity of intracellular magnesium to changes in the external magnesium concentrations (Kretsinger, 1976; Page & Polumeni, 1972; Brennan & Lichtman, 1973) suggests that changes in magnesium within the cytosol are not the ultimate process by which external magnesium induces proliferation. An alternative model to that in Figure D.1 is for all activation processes to ultimately revolve around alteration of the cytosolic calcium content. (Figure D.2).

Although many aspects of cellular calcium metabolism remain concealed during calcium-45 tracer investigations, several conclusions may be drawn from such studies. Following a three-fold increase in the external calcium concentration, to the mitogenic 1.8 mM level, the membrane-associated calcium pool expanded five-fold from approximately 30 to 150 pg/10^6 cells (Figure R29). This increase is probably due to the displacement, by calcium, of sodium ions bound to the negative phospholipid residues present in the plasma membrane (Hauser, Finer & Darke, 1977). Interestingly, the sodium permeability of synthetic phosphatidyl-serine membranes show a marked calcium dependency (Paphadjopoulos, Vail, Newton, Nir, Jacobson, Poste &

Fig.D.2. Hypothetical model for thymic lymphoblast activation via a single "calcium" axis.



Lazo, 1977). However, the precise relation of the membrane binding phenomenon to mitotic activation is unclear. Mitotically stimulated rat liver cells show a rapid expansion of their membrane bound calcium pool (Yamagami & Terayama, 1979) whereas serum or insulin stimulation of quiescent 3T3 fibroblasts is associated with a drop in the membrane bound calcium (Tupper, Del Rosso, Hazelton & Zorgniotti, 1978; McDonald, Burns & Jarrett, 1976). The expanded membrane-bound calcium reservoir of the thymocyte may not contribute calcium directly to the cytosol. Kinetic studies performed in Hela cells and rat kidney

suggest that the membrane bound pool is not in direct equilibrium with the cytosol (Ichikawa & Borle, 1978; Borle, 1972; Borle, 1975). In cardiac tissue a similar controversy over the role of membrane-bound calcium also exists (Issenberg & Klockner, 1980). The precise role of calcium bound to cell membranes in any subsequent activation remains to be determined.

The movement of calcium-45 following the sudden calcium elevation shows that the influx rate of all calcium ions increased from the basal 1.4 pg/min/10^6 cells to 4 pg/min/10^6 cells. Over the entire thirty minute activation period this would represent a total influx of 120 pg/10^6 cells (Figure R.29). Efflux of calcium from the pre-labelled thymocytes was only increased two-fold following treatment with 1.8 mM calcium (Figure R50). Assuming that the basal efflux and uptake are balanced, at 1.4 pg/min/10^6 cells in either direction, the new efflux rate would be approximately 2.4 pg/min/10^6 cells. Also assuming a cell volume (cytosol only) of 100μm^3 the net increase in cytosolic calcium would be approximately 10μM. This corresponds well with the 50 μM increase suggested for lectin-activated human lymphocytes (Hesketh, Smith, Houslay, Warren & Metcalf, 1977) but not with the value of 970μM reported for similarly treated murine splenocytes (Freedman, 1979). A 10 μM increase in cell calcium will not appreciably alter the total cell calcium content but if all the 10 μM entered the free calcium pool it would greatly increase the basal 10^{-7}M free ion concentration. As free intracellular calcium is subjected to strict homeostatic control, namely cytosolic and mitochondrial buffering, it is essential to show the increased influx of calcium results in a rise in the active calcium pool.

In many excitable and non-excitable cell types, the release of cytosolic potassium is a consequence of a rise in the free calcium concentration (Meech, 1976; Smith & Vernon, 1979). Using rubidium-86 as a tracer for calcium the occurrence of this effect was observed in the thymic lymphocyte. The Rb^{86} content of thymocytes was significantly decreased within ten minutes of calcium stimulation (Figure R39). The increased rubidium exit causing this fall was blocked by the addition of the antimitotic compound verapamil (Figure R40) which also prevented the increased entry of calcium (Figure R30). This shows that the increased rubidium release is indeed due to a rise in free calcium within the cell. More importantly it also proves that the enhanced mitosis caused by exposure to high calcium is the result of a rise in the free cytosolic calcium concentration.

The mechanism by which an increased extracellular magnesium content induces proliferation is less certain. Extracellular magnesium is in a simple equilibrium with the free intracellular ion (Gilbert, 1960; Ling, Walton & Ling, 1979). Thus any rise in the external ion content would be transmitted to the cytosol and result in a rise in cellular magnesium. Such an effect has been observed in rat liver slices, chick embryo fibroblasts and frog skeletal muscle (Van Rossum, 1970; Sanui & Rubin, 1977; Ling, Walton & Ling, 1979). It is possible that this increase in cytosolic magnesium may itself induce proliferation. However, the mitogenic magnesium treatment was able to stimulate thymocyte rubidium conductance to the same extent as a 1.8 mM calcium concentration (Figure R43). A direct effect of magnesium upon rubidium conductance would seem unlikely as an ionophoretic increase in neutrophil magnesium content did not alter the cellular potassium equilibrium in the absence of extracellular calcium ions (Gallin, 1980). A magnesium ion-induced increase in cytosolic

calcium within the thymocyte would be one possible explanation for the activation of rubidium conductance by extracellular magnesium increments. As a direct increase in calcium entry, caused by the high magnesium content did not occur (Figure R32) any such rise in cytosolic magnesium may secondarily elevate calcium levels by releasing intracellular calcium ions. A brief consideration of intracellular magnesium homeostasis reveals that this is indeed possible.

The majority of intracellular magnesium ions are reversibly bound to cytosolic components such as adenosine nucleotides, internal membranes and magnesium-dependent enzymes (Page & Polimeni, 1972; Polimeni & Page, 1973; Sanui, 1970; Sordahl, 1975; Rose, 1968). There is evidence suggesting that calcium competes with magnesium at some of these sites (Carvaltho, Sanui & Pace, 1963; Wolff & Brostrom, 1979). Thus any rise in the intracellular magnesium content, provoked by raising the external concentration, may displace calcium ions from these internal binding sites, particularly the endoplasmic reticulum (Moore & Pastan, 1977). A further process, whereby a rise in intracellular magnesium can alter the free calcium content, is by a magnesium-induced perturbation of mitochondrial calcium metabolism. Magnesium may inhibit calcium uptake into, and promote calcium extrusion from, isolated mitochondria (Nicholls, 1978; Jacobus, Flozzo, Lugli, Lehninger & Corafoli, 1975). Thus the net result of a rise in intracellular magnesium could be a change in the calcium buffering capacity of the mitochondria and other cellular components. Although the total intracellular contents of calcium and magnesium ions are approximately equal, the relative concentration of the free ions are not (Kretzinger, 1976; Polimeni & Page, 1973). Thus a small increase in the intracellular magnesium level may displace sufficient calcium ions to considerably elevate the free calcium concentration.

A weak contraction of glycerinated smooth muscle, which is normally a calcium-induced event, can be elicited by the addition of a large magnesium concentration (Nakahta, 1979). This again suggests that magnesium is able to release intracellular calcium. This same phenomenon is also indicated in the rat thymocyte by the extracellular magnesium ion dependency exhibited at sub-optimal mitogenic calcium concentrations. If the minimum (1.2 mM) stimulatory calcium concentration is used, thymocytes are only recruited when magnesium ions are present in the culture medium (Riddell, 1971). If we assume that a 1.2 mM external calcium concentration only just increases the intracellular calcium level above the stimulatory threshold then the removal of external magnesium will reveal internal calcium binding sites and lower the free calcium concentration below the required threshold. A similar phenomenon is also observed in chick embryo fibroblasts where the lowest mitogenic calcium concentration is dependent upon external magnesium (Rubin, 1975; Rubin, Terasaki & Sanui, 1978). This hypothesis, of a sustaining influence of intracellular magnesium upon cytosolic calcium, requires that removal of external magnesium ions would reduce the internal magnesium content. Magnesium exchange across mammalian cell membranes has originally been considered a slow process (Gilbert, 1960). However a recent report suggests that equilibration can occur within five minutes (Ling, Walton & Ling, 1979). Removal of external magnesium does indeed lower the magnesium content of chick embryo fibroblasts and murine lymphoblasts (Brennan & Lichtman, 1973; Sanui & Rubin, 1977). Thus it is possible for a drop in intracellular magnesium to make divalent cation binding sites available to calcium and therefore remove free calcium ions from the cytosolic pool. Such a mechanism may then be applied to explain the magnesium-dependency of thymocyte mitogens. These agents do not require the presence of extracellular calcium ions

to exert their mitotic effect. Thus, although 2.5 mM magnesium and adrenaline were both able to provoke rubidium exit from the rat thymocyte (Figure R43 and R42), neither influenced calcium entry (Figure R32 and Figure R33). The prevailing transmembrane magnesium gradient would make it unlikely that the entry of magnesium itself was stimulated by the non-ionic mitogens (Page & Polimeni, 1972; Gilbert, 1960). Thus if these agents initiate proliferation by elevating the intracellular calcium concentration the source of the triggering ions must be the cytosolic calcium stores.

No calcium-dependent mitogens so far identified require extracellular magnesium ions, instead all need the basal level of extracellular calcium (MacManus, Boynton & Whitfield,1978). Whether calcium removal inhibits mitogen-induced calcium influx or prevents calcium accumulation in the cytosol is uncertain. It is clear that due to the calcium buffering system the basal level of calcium within the cell will not be drastically reduced. The inhibitory action of calcium removal must therefore reside in a prohibition of mitogen-mediated increases in free calcium. When such a calcium-dependent mitogen (Insulin) was tested calcium influx was not altered (Figure R33). However, insulin treatment did lead to an increase in rubidium conductance which we assume is characteristic of a rise in free calcium within the cell (Figure R42). The failure to detect a mitogen-induced stimulation of calcium uptake does not exclude the possibility that some mitogens act in this manner. Indeed, the calcium influxes associated with secretory and contractile stimulation suggest this is highly probable. The observations made so far in this study of cationic rearrangements are summarised in Figure D3.

Fig. D3. Cation induced mitogenesis and the associated transmembrane ionic movements

1. External calcium or magnesium ions can induce proliferation when their concentrations are sufficiently elevated.
2. Calcium will only stimulate when it is present in a freely diffusible form and when its transmembrane passage is unimpaired.
3. Following a mitogenic increase in the external calcium concentration the membrane calcium pool expands five-fold.
4. The same calcium stimulation increases influx three-fold and efflux two-fold, giving a net cellular calcium elevation of approximately 10 μM.
5. The calcium entry itself stimulates rubidium efflux from the thymocytes.
6. Rubidium efflux is also provoked by calcium-dependent mitogens, although insulin did not alter calcium uptake.
7. Magnesium and magnesium-dependent mitogens, whilst not altering calcium entry, also stimulated rubidium exit.

It is now established that an increased cytosolic calcium is provoked by raising the transmembrane calcium (and possibly magnesium) gradient, or by the addition of either calcium or magnesium-dependent mitogens. It is essential to test the hypothesis that recruitment is indeed mediated by this increased cytosolic calcium.

Intracellular calcium is under close homeostatic control, and is closely linked to monovalent cation metabolism in a cytosolic cationic network. (Rasmussen & Goodman, 1977). Intracellular calcium may thus be altered by a shift in the transmembrane distribution of any of the ionic species contributing to this ionic net. Such a redistribution would be articifially provoked by disruption of the membrane bound Na/K ATPase ion pump. However, the pump inhibitory glycoside, ouabain, is

able to inhibit the proliferation of a variety of cell types (Lubin 1967; Quastel & Kaplan, 1968; Mayhew & Levinson, 1968; McDonald, Sachs, Orr, Ebert, 1972). This antiproliferative action of high glycoside levels is probably due to a reduced cytosolic potassium content as it can be overcome by an elevated extracellular (and by inference intracellular) potassium concentration (Kaplan, 1978). A reduced potassium content, produced by either the potassium ionophore, valinomycin, or by an alteration in the membrane lipid composition, will also inhibit mitosis in other tissues (Danielle & Hollian, 1976; Chen, Heiniger & Kandutsch, 1978). The cellular process that is sensitive to reduced potassium would appear to be protein synthesis (Ledbetter & Lubin, 1977; Shank & Smith, 1976). However, when the concentration of ouabain (10^{-4}M) which inhibited thymocyte recruitment (Figure R4a) was tested, neither basal nor mitogen-stimulated protein synthesis was altered (Figure R5). Thus in the rat thymocyte, ouabain prevents recruitment, but not basal proliferation, without depressing protein synthesis. One possible reason for this may be, paradoxically, an increased cytosolic calcium. The 10^{-4}M concentration of ouabain promoted calcium influx, although not to an extent comparable with the influx promoted by a high calcium treatment (Figure R34). It is possible that a combination of increased calcium uptake and reduced cytosolic potassium serve to prevent recruitment whilst leaving the later stages of cell cycle transit unaffected.

Although lower concentrations of ouabain did not influence calcium uptake they were able to provoke mitosis, the effect being evident over two discrete concentration ranges (Figure R7). Those ouabain concentrations that stimulated mitosis were shown to inhibit the functioning of the Na/K ATPase (Figure R3, R13, R36). Unfortunately the techniques employed to demonstrate this inhibition do not directly

quantify pump activity. However, the influx of rubidium, which will be mostly through the pump, was reduced in proportion to the concentration of ouabain added to the thymocytes (Figure R36). Thus it is reasonable to assume that the higher (10^{-7}M) ouabain concentration inhibits the pump to a greater extent than the 10^{-11}M concentration. The ability of ouabain to increase cardiac contractility is believed to be indirectly due to a rise in the cardiac sodium content following pump inhibition. This increase in cytosolic sodium, caused by its unopposed entry, will secondarily elevate intracellular calcium by preventing calcium extrusion through the Na/Ca exchange mechanism (Muller, 1965; Blaustein, 1977; Akera, Larson & Brody, 1970; Horwicz & Gerber, 1965). Such a glycoside-induced increase in cardiac calcium has been observed (Thyrum, 1974; Wood & Schwartz, 1978; Biedert, Barry & Smith, 1979). Non-cardiac tissue has also proven susceptible to ouabain-induced alterations in cytosolic calcium transport (Lamb & McCall, 1972). In addition to ouabain, a second pump inhibitor, adriamycin (Gosalvez & Blanco, 1975), increased the calcium content of HeLa cells (Dasdia, Di Marco, Goffredi, Minghetti & Necco, 1979). As we have predicted that such a rise in cytosolic calcium will trigger thymic lymphocyte mitosis it is no surprise to find that ouabain is able to promote division in other tissues. This is summarised in Figure D4; it is perhaps no coincidence that all of the tissues listed are capable of rapid cell division in vivo.

Fig. D4. Ouabain stimulation of proliferative activity.

Tissue	Species	Reference
Embryonic neural retina	Chick	Kaplowitz & Moscona, 1976
Embryonic spinal cord	Dog	Stilwell, Cone & Cone, 1973
Splenic lymphocyte	Mouse	Ryser & Politoff, 1977
Bone marrow CFU-E	Mouse	Spivak, Misiti, Stuart, Sharkis & Sensenbrenner, 1978

The ability of only the calcium-dependent and magnesium-dependent mitogenic concentrations of ouabain to stimulate thymocyte rubidium release suggests that the effect is closely associated with mitotic recruitment and not merely a consequence of pump-inhibition. That both mitogenic glycoside concentrations are activating rubidium efflux through an elevation in cytosolic calcium was confirmed by the observed changes in calcium transport. Neither 10^{-7} nor 10^{-11} ouabain increased the entry of calcium (Figure R34), but both concentrations almost completely prevented the efflux of calcium (Figure R50). This action is in fact the predicted consequence of pump inhibition and suggests that calcium exit from the thymocyte is almost exclusively dependent on the transmembrane sodium gradient. In the case of 10^{-7}M ouabain, the inhibition of calcium extrusion would account for the required calcium presence outside the cell. If the basal calcium influx of 1.4 pg/min/10^{6} cells which continues in the presence of 10^{-7}M ouabain was not inhibited by removing extracellular calcium the cytosolic calcium would rise by 3 μM over a thirty minute period. The sensitivity of this concentration to Verapamil confirms the role that calcium entry must play at this glycoside level. The independence of 10^{-11} ouabain from extracellular calcium suggests that its action is exerted at some intracellular site and is not dependent upon continual calcium influx. The inhibition of calcium extrusion at this 10^{-11}M concentration, and the magnesium dependency, would then be explicable if we assume that magnesium is again essential to maintain the stimulatory calcium content produced by the glycoside.

One mechanism by which 10^{-11}M ouabain could elevate cytosolic calcium would be by stimulating the release of mitochondrial calcium. This could be due to the rise in cytosolic sodium ions produced by pump inhibition. A small (.5 mM) increase in the extra-mitochondrial

sodium content is indeed able to release calcium from isolated mitochondria (Nicholls, 1978; Haworth, Hunter & Berkoff, 1980; Crompton, Capano & Carafoli, 1976). The inhibition of calcium extrusion would then contribute to the triggering increase in calcium.

In summary we may say:-

1. Ouabain stimulates mitosis at two distinct concentrations.
2. These mitogenic concentrations both inhibit the Na/K ATPase.
3. Both inhibit calcium exit and provoke rubidium release.
4. The 10^{-7}M concentration proved to be calcium-dependent, and hence to rely on a continued calcium influx to sustain an increase in cytosolic calcium of 3μM.
5. The 10^{-11}M concentration was magnesium-dependent and may well be releasing intracellular calcium stores as the efflux of calcium was inhibited and rubidium conductance activated at this concentration.

If the mitotic activity of ouabain is due to ATPase inhibition alone then other pump inhibitors would be expected to provoke mitosis. Fluoride ions are known to inhibit pump-mediated cation movements and hence may be assumed to inhibit the pump (Yoshida, Nagai, Kamei & Nakagawa, 1968; Kirschner, 1964). A rise in cytosolic calcium, due to fluoride inhibition of the ATPase, is indicated in both pig erythrocytes and isolated rat uteri, where an increased potassium efflux resulted from fluoride treatment (Wilbrandt, 1940; Daniel, 1963). Furthermore fluoride was able to stimulate calcium activated Histamine secretion by mast-cells in place of the normal secretagogue (Patkar, Kazimierczak & Diamant, 1977). This all indicates that fluoride mediated pump inhibition is accompanied by a rise in cytosolic calcium. As would be expected from the ouabain study, high fluoride concentrations were able to inhibit calcium-induced thymocyte proliferation (Figure R14).

Similar anti-proliferative actions have been reported when HeLa cells L929, mouse and human fibroblasts were exposed to millimolar fluoride concentrations (Carlson & Suttie, 1967; Langnes, Schors, Prange & Lescow, 1978; Albright, 1964). Lower, non-inhibitory fluoride concentrations were able to produce the anticipated mitotic stimulation of the thymocytes (Figure R16). It should be noted that this stimulatory fluoride concentration is several orders of magnitude below that required to stimulate adenylate cyclase in membrane preparations. This demonstration of a second ATPase inhibitor which provokes mitosis (and a third if one includes 2.4 DNP which increases the calcium content of pancreatic β cells (Hellman, 1979)(Figure R18), indicates that pump inhibition is responsible for ouabain-stimulated cell division. The proposed mechanism for this stimulation will then be a rise in calcium caused by an initial increase in sodium.

Although pump inhibition and the prevention of calcium extrusion are coincident it is still possible that another pump-related event is activating the thymocytes (LeLievre, Paraf, Charlemagne & Sheppard, 1977); it can be answered by direct manipulation of the cytosolic sodium without resource to pump inhibitors. It has been calculated that a rise in the cytosolic sodium of as little as 0.5 mM would be able to raise the calcium content of most cells by over 15% (Blaustein, 1977). When the extracellular sodium concentration was raised by as little as a 5 or 10 mM increase, the thymocyte proliferation was stimulated (Figure R19). This was coincident with an inhibition of calcium efflux and the provocation of rubidium release (Figure R49, R50). A higher (20 mM) sodium increment also provoked mitosis and rubidium efflux (Figure R19, R49). At a 30 mM increase there was no alteration of basal mitosis or rubidium conductance (Figure R19, R49).

The 10 mM increase in sodium proved to be calcium-dependent (Figure R22), therefore by the criteria mentioned above we can assume

this to indicate a rise in cytosolic calcium, dependent upon the continued influx of calcium. This sequence of events is the same as those indicated when 10^{-7}M ouabain stimulated mitosis. Thus it is reasonable to conclude, if sodium is indeed inhibiting calcium exit, that the increment in cytosolic sodium produced by a 10 mM external sodium elevation will be of the same magnitude as that provoked by 10^{-7}M ouabain. The 20 mM mitogenic sodium increment was magnesium-dependent (Figure R23) and may well act through the same processes as 10^{-11}M ouabain. If this were so then the increase in intracellular sodium, caused by a 20 mM external elevation, must be equal to that caused by the lower (10^{-11}M) ouabain concentration. One would have expected that the higher concentrations of ouabain and sodium would both elevate cytosolic sodium to a greater extent than the lower ouabain and sodium concentrations. This apparent anomaly may be satisfactorily explained in terms of Na/K ATPase activation. In human lymphocytes a cytosolic sodium increase of 5 mM was able to activate the pump two-fold (Segel, Simon & Lichtman, 1979). Thus the inability of a 30 mM extracellular sodium increment to stimulate either mitosis or rubidium conductance may be due to a stimulation of the ion-pump by the extra sodium ions entering the cell. This model requires that an intracellular balance exists between elevated sodium and pump activity. Significantly (in Introduction, Section 2), we have shown that calcium can modify pump-sensitivity to sodium ions. Thus it is not inconceivable that a 10 mM external sodium increment will raise intracellular sodium to a greater level than a 20 mM external increase. Two other thymocyte mitogens, histamine and cyclic GMP (exogenous), were also only able to provoke rubidium efflux at their mitogenic concentrations (Fig.R44,R46). Thus a direct relationship must exist between increased intracellular calcium, however, provoked, rubidium conductance and mitotic activation. The key concept in this system is the direct

action of intracellular calcium in initiating recruitment into the cell cycle. Similar calcium involvement is indicated in the recruitment of other quiescent cell types.

Polyclonal activation of lymphocyte proliferation is by plant lectins and is dependent upon the presence of extracellular calcium (Allwood, Asherson, Davey & Goodford, 1971; Whitney & Sutherland, 1972). Mitogenic lectin treatment is able to rapidly increase the unidirectional influx of calcium into these lymphocytes, and indeed rat thymic lymphocytes (Freedman, Raff & Gomperts, 1975; Ozato, Huang & Ebert, 1977; Whitney & Sutherland, 1972). The influx of calcium, and presumably the resultant rise in cytosolic calcium, is followed by profound rearrangements of cellular monovalent cations (Kaplan, 1978). However, the background noise created by lectin-induced alterations in membrane permeability make interpretation of these changes, and their chronological sequencing difficult (Averdunk & Lauf, 1975; Negendank & Collier, 1976). The fertilization of sea-urchin eggs is accompanied by more defined cationic rearrangements. The transition of the ovum from G_o to G_1 is also dependent upon extracellular calcium and is accompanied by a fertilization induced calcium influx (Clothier & Timourian, 1972). Free calcium, measured indirectly by calcium-induced protein fluorescence, is elevated by this calcium influx (Nakamura & Yasumasu, 1974). Most significantly the increase in cytosolic calcium is accompanied by a large increase in the exit of potassium from the cytosol (Ito & Yoshioka, 1973). This same sequence of events also occurs if the divalent cation ionophore A23187 is used to "fertilize" the eggs by directly providing the increased calcium content (Steinhardt & Epel, 1974).

Mitotic triggering of many different cells is accompanied by other changes in the transmembrane ionic flow. It is essential to distinguish between early and late ionic changes as the latter may not be essential for recruitment, although they are undoubtedly required at a later stage. Events such as alterations in the activity of the membrane Na/K ATPase occurring after stimulation, probably reflect a compensatory mechanism, restoring the original cation content of the activated cells (Segel, Kovach & Lichtman, 1979). Indeed a large number of mitogenic compounds are known to elevate membrane Na/K ATPase activity (Averdunk, 1972; Tupper, 1976; Wiesmann, Sinha & Klahs, 1977; Rozengurt & Heppel, 1975; Sanui & Rubin, 1978; Sivak, 1977).

The early monovalent ion fluxes associated with recruitment are largely transient changes, occurring close to the initial activation event. Of the many mitogenic substances present in serum, two, insulin and vasopressin, are known to provoke sodium influx into mammalian cells (Wiesmann et al. 1977; Smith & Rozengurt, 1978). It is not known if calcium metabolism is influenced under these circumstances, but both insulin and vasopressin are able to stimulate thymocyte mitosis (Morgan, Hall & Perris, 1975; Whitfield, Perris & Youdale, 1969; Perris personal communication.) Epidermal growth factor, which lowers the extracellular calcium requirement for fibroblast proliferation is also believed to alter sodium influx (McKeehan & McKeehan, 1979). Any influx of sodium caused by these compounds may alter the intracellular calcium content by preventing calcium exit, as do both oubain and sodium in the rat thymocyte (Figure R50). Alternatively the sodium influx may provoke mitochondrial calcium release (Haworth, Hunter & Berkoff, 1980). Interestingly, which mechanism occurs, may well depend upon the concentration of the agonist. Angiotensin, vasopressin and alpha-adrenergic agonists, which are

all able to stimulate division in sensitive cells (Miller, Hussain, Svenson & Lohim, 1977; Gill, Ill & Simonian, 1977; Morgan, 1976) are able to release mitochondrial calcium (Blackmore, Dehaye & Exton, 1979; Chen, Babcock & Lardy, 1978) and promote transmembrane calcium entry (Michell, Kirk & Billah, 1979) in isolated liver cells.

In summary, it is apparent that the mitosis-inducing mechanisms employed by ouabain and sodium are by no means restricted to these compounds. The widespread interrelation between sodium and calcium movements during stimulated mitosis suggests that it is an important mechanism for the induction of proliferation. The ability of ouabain and sodium to provoke magnesium-dependent mitosis testifies to the occurrence of two distinct mitogenic processes in the thymocyte.

In the past the use of steroid mitotic inhibitors has provided considerable information about the two axes serving the recruitment process. Oestradiol will inhibit only calcium-dependent mitosis whilst testosterone selectively prevents magnesium-dependent division. This specificity suggests that the steroid blockade stage occurs before the ultimate common step that commits a cell to recruitment. Sex-steroid treatment in conjunction with the addition of ouabain was able to prevent the glycoside-induced mitosis. Oestradiol prevented only 10^{-7}M (calcium-dependent) induced mitosis (Figure R10) whilst testosterone blocked the 10^{-11}M (magnesium-dependent) action (Figure R11). It does appear that oestradiol action is not directed through classical 17-beta oestrogen receptors as the alpha form of the steroid is equally anti-mitotic (Bramhall, Morgan, Britten & Perris, 1976). The normal steroid-genome interaction is also unlikely as the inhibition is extremely rapid, occurring within ten minutes (Morgan, 1976). Indeed oestradiol is able to inhibit the proliferation of 3T3 fibroblasts lacking oestradiol receptors of any type (Breslow, Epstein, Forbes & Fontaine, 1979).

A general role for oestradiol in inhibiting mitosis is also apparent in vivo, where the calcium-provoked mitosis of both the rat bone marrow and thymus is sensitive to oestradiol (Smith, Gurson, Ridell & Perris, 1975). This inhibition of proliferation in vivo is also extended to many forms of lymphoid cells and results in a suppressive effect. This includes inhibition of the MLR responder and the natural killer cell populations (Pavia, Siiteri, Perlman & Stites, 1979; Seaman, Gindhert, Greenspan, Blackman & Talal, 1979). Thus it is no surprise that oestrogen-treated animals exhibit delayed skin graft rejection, thymic atrophy and a diminished response to polyclonal activators (Siiteri, Febres, Clemens, Chang, Gondos & Stites, 1977; Scheiff & Haumont, 1979; Ablin, Bruns, Guinan & Bush, 1974; Waltham, Burde & Berrios, 1971). Non lymphoid tissue is also susceptible to oestradiol. The mitotic activity of the 3T3 fibroblast mentioned above and the murine colonic epithelium is inhibited by oestrogen (Hoff & Chang, 1979; Breslow et al., 1979).

In sharp contrast to these inhibitory actions, high concentrations of oestradiol, usually in the presence of serum, are able to positively regulate cell division. In vivo cells of the adrenal cortex and adenohypophysis are mitotically stimulated by oestradiol (Pappritz, Keazor & Ueberberg, 1977; Le Guellec, Keviret, Guellaen, Valotaire & Duval, 1978). In vitro the proliferation of rat hepatocytes having 17-beta oestradiol receptors and of splenic CFU-E cells is promoted by oestradiol (Pietras & Szego, 1979; Anagnostou, Zander, Barone & Fried, 1976). This positive action of oestradiol is of course in addition to its general trophic action in promoting secondary sex gland development (Jensen & Jacobson, 1962; Lippmann, Bolan & Huff, 1976; Sonnenschein & Sato, 1980).

Having shown that oestradiol is capable of both stimulating and inhibiting mitosis it becomes essential to identify any interactions between the steroid and the ionic rearrangements provoked by thymocyte mitogens. Long term changes in monovalent cation metabolism are observed in the hypophysis of oestradiol treated rats and also after oestrogen addition to isolated rat uteri. This would be consistent with an activation of the plasma membrane Na/K ATPase (Knudsen, 1976; Fujimoto & Morrill, 1978). The reason for such a pump stimulation may well be a prior steroid-induced alteration in the intracellular monovalent cation concentrations. Indeed diethyl-stilbestrol causes a rapid potassium exit and transient hyperpolarization in vascular smooth muscle (Harder & Coulson, 1979). A similar rapid exit of rubidium was provoked by oestradiol addition to the rat thymocyte, suggesting a steroid-induced rise in cytosolic calcium (Figure R41). Significantly, although oestradiol could stimulate rubidium exit from unactivated cells, the enhanced efflux due to high calcium treatment was not further influenced (Figure R41). This indicates that rubidium exit is already maximally activated by 1.8 mM calcium. When the effect of oestradiol on calcium homeostasis was investigated it became apparent that the steroid alone did indeed enhance calcium entry. Figure R29 shows that oestradiol does not influence membrane bound calcium but rather promotes calcium uptake. There is a marked dose-dependency between the steroid concentration and calcium uptake (Figure R31). This leads us to predict that at a high oestradiol concentration, the thymocytes may become mitotically activated, which may explain the positive and negative effects of oestradiol on growth. The antimitotic action of oestradiol may be explained as being due to an excessive increase in cytosolic calcium. If thymocytes are exposed to external calcium concentrations above 3 mM,

they are not activated. Thus if calcium entry is indeed proportional to the external calcium content this suggests a maximum stimulatory internal calcium concentration. The large increase in calcium uptake provoked by oestradiol into thymocytes already stimulated by 1.8 mM calcium may well exceed this threshold and inhibit mitosis (Figure R29). The occurrence of increased potassium conductance in the absence of stimulated division shows that the effect is not merely associated with a cell cycle stage.

The observations concerning the influence of testosterone on cation homeostasis reveals again that cationic process may mediate the anti-proliferative steroid action. Antimitotic concentrations of testosterone, which inhibit only magnesium and magnesium-dependent mitogens, did not alter the transmembrane movement of calcium. Testosterone did however provoke a rubidium exit in addition to that already stimulated by high magnesium (Figure R43). This suggests that the steroid, acting in conjunction with a magnesium-dependent mitogen that sub-maximally elevates both intracellular calcium and rubidium exit, is further elevating the cytosolic calcium, albeit to an inhibitory level.

The ability of oestradiol and testosterone to block mitosis after the initial elevation of extracellular calcium, produced by either hormonal or non-hormonal mitogens, places the blockade at a point between calcium elevation and some common recruitment process. As the steroids inhibit separate mitogenic axes it is probable that the inhibitory steroid action is exerted very close to the calcium elevation.

Previous studies of the membrane potential changes occurring in rat thymocytes after mitogen treatment do not consistently confirm the changes in ionic transport detailed above. The electrical potential of the plasma-membrane was studied using a potential-sensitive dye that partitions into fluorescent and non-fluorescent forms under the influence of the membrane potential (Davilla, Salzberg, Cohen, 1973; Hladky & Rink, 1976; Morgan, Bramhall, Britten & Perris, 1976). The addition of extracellular calcium ions to thymic lymphocytes induced rapid and concentration-dependent membrane hyperpolarization (Morgan, Bramhall, Britten & Perris, 1976). Such a hyperpolarization would be consistent with the calcium ion induced rubidium efflux observed above. Oestrogenic treatment, which normally provoked rubidium exit (Figure R41) was able to hypopolarize the thymocytes (Morgan et al. 1976). Whilst an initial calcium influx may transiently produce this effect, the rubidium efflux provoked by the steroid would be expected to ultimately hyperpolarize the membrane. Indeed a similar treatment has been seen to do so in another tissue (Harder & Coulson, 1979). Oestrogens which do not interfere with mitosis were also able to hypopolarize the cells in the potential study (Bramhall, Morgan, Britten & Perris, 1976). This would suggest that hypopolarization is not itself related to the steroid effect upon mitosis. The membrane potential measurement technique has recently been criticised (Simons, 1976; Hladky & Rink, 1976). Consequently where a conflict exists between the observed ionic movements and the measured change in membrane potential it is advisable to assume that other processes may be interfering with the potential measurement.

It is evident from the experiments reported above that the monovalent and divalent cation rearrangements leading to an elevated intracellular calcium content, are intimately associated with the induction of thymocyte mitosis. Stimulus-secretion and stimulus-contraction coupling also revolve around a final activation of the cell by an increase in the free cytosolic calcium concentration. The two mechanisms of stimulation in these tissues, dependent upon either external or internal calcium ions, appear to have their parallels in the mitotic activation of the thymocyte.

One series of thymocyte mitogens, both hormonal and non-hormonal, are now known to be entirely dependent upon the continued presence of extracellular calcium ions. In having this strict calcium requirement they are similar to the group of compounds promoting secretion only when calcium is available outside the secretory cell. Under appropriate conditions the contraction of certain forms of muscle tissue is also dependent on the external calcium concentration (Introduction, Section 2). In both of these latter cases enhanced calcium entry is often observed. Similar increases in calcium entry also occur during the calcium-dependent mitotic activation of some cell types, including lectin-activated lymphocytes and fertilised sea-urchin eggs (see above). In common with the secretory and contraction responses discussed above the thymocytes universally exhibited a rise in cytosolic calcium, as indicated by rubidium release when they were mitotically activated. Thus calcium-dependent concentrations of Insulin, Histamine, Cyclic GMP, ouabain and sodium and extracellular 1.8 mM calcium itself, all stimulate mitosis in association with an elevated intracellular calcium concentration. Although these compounds all have the same ultimate action in ele-

vating cytosolic calcium the mechanisms through which the stimulatory, or coupling, calcium increase is furnished appear to be somewhat different.

The first sub-group of calcium-dependent thymocyte mitogens would contain those that alter transmembrane calcium influx (Figure D5a). Principal amongst these is extracellular calcium, which was proven to increase free intracellular calcium by promoting calcium entry into the thymocytes. Also included in this group would be concanavalin A (Ozato, Huang & Ebert, 1977;Phorbol ester (PMA)(Whitfield, MacManus & Gillan, 1973; Schimmel & Hallam, 1979) and possibly the divalent cation ionophore A23187 (Morgan, 1976; Jensen, Winger, Rasmussen & Nowell, 1977), all of which are known thymocyte mitogens. Speculatively we could also include oestradiol; if a sufficiently high concentration was employed the increased basal influx due to the steroid would be able to stimulate mitosis. It is not inconceivable that cholinergic or alpha-adrenergic agonists which promote calcium entry into other tissues, and also provoke recruitment of the thymocytes, would do so through alterations in calcium influx. Whilst calcium, concanavalin A, PMA and A23187 would increase entry of calcium through normal calcium channels or specific mitogen-induced passages the hormonal mitogens would have to employ classical hormone-receptor interactions.

As the majority of calcium-dependent mitogens are believed to act ultimately via cyclic GMP formation they would be expected to interact with guanylate cyclase. In the rat thymocyte the addition of Thymosin V provokes cyclic GMP production which, significantly, is dependent upon extracellular calcium (Naylor, Thurman & Goldstein, 1980). Thus, by direct analogy with the cyclic GMP alterations

provoked during stimulus-activation coupling, we can predict that calcium-dependent hormonal mitogen action is not primarily through cyclic GMP. The calcium dependency of both mitogenesis and cyclic GMP formation suggests that the alteration in calcium influx would be the link between hormone and recruitment (Figure D5b). Alterations in membrane fluidity subsequent to receptor binding by a variety of hormones may explain how calcium-dependent hormones can initiate recruitment via an alteration in calcium permeability (Figure D5a). Many recent studies have shown that a hormone receptor-induced increase in membrane lipid turnover occurs (Michell, 1975; Michell, Kirk & Billah,1979). This increased metabolism results in an increase in the phosphatidic acid levels within the membrane and hence an increased permeability to calcium (Putney, Weiss, Van de Walle & Haddas, 1980). This concept has recently been challenged by the observation that calcium entry appears to precede increased lipid metabolism in rabbit neutrophils (Cockcroft, Bennet & Gomperts,1980). However, the precise hierarchy of events may have been overlooked as small local increases in lipid turnover may rapidly initiate calcium influx, which then would be amplified by subsequent alterations. Significantly, hormones stimulating secretion from the rat parotid that require extracellular calcium alter lipid metabolism whilst those secretagogues that initially act upon cyclic AMP, and are independent of extracellular calcium, do not alter lipid turnover (Jones & Michell, 1974; Michell et al. 1979).

The second calcium-dependent sub-group consisting of agents that can indirectly influence calcium uptake, includes those mitogens that alter monovalent cationic distributions. We have seen how ouabain or sodium by a postulated increase in intracell-

ular sodium are able to trigger mitosis by preventing calcium efflux (Figure D5C). The extracellular calcium requirement would be necessary to sustain the rate of calcium entry and thereby increase free calcium concentration. Other substances that increase sodium entry into the cells would also be expected to stimulate division. Possible thymocyte mitogens would then include those serum components, discussed above, that are able to alter sodium influx into quiescent fibroblasts.

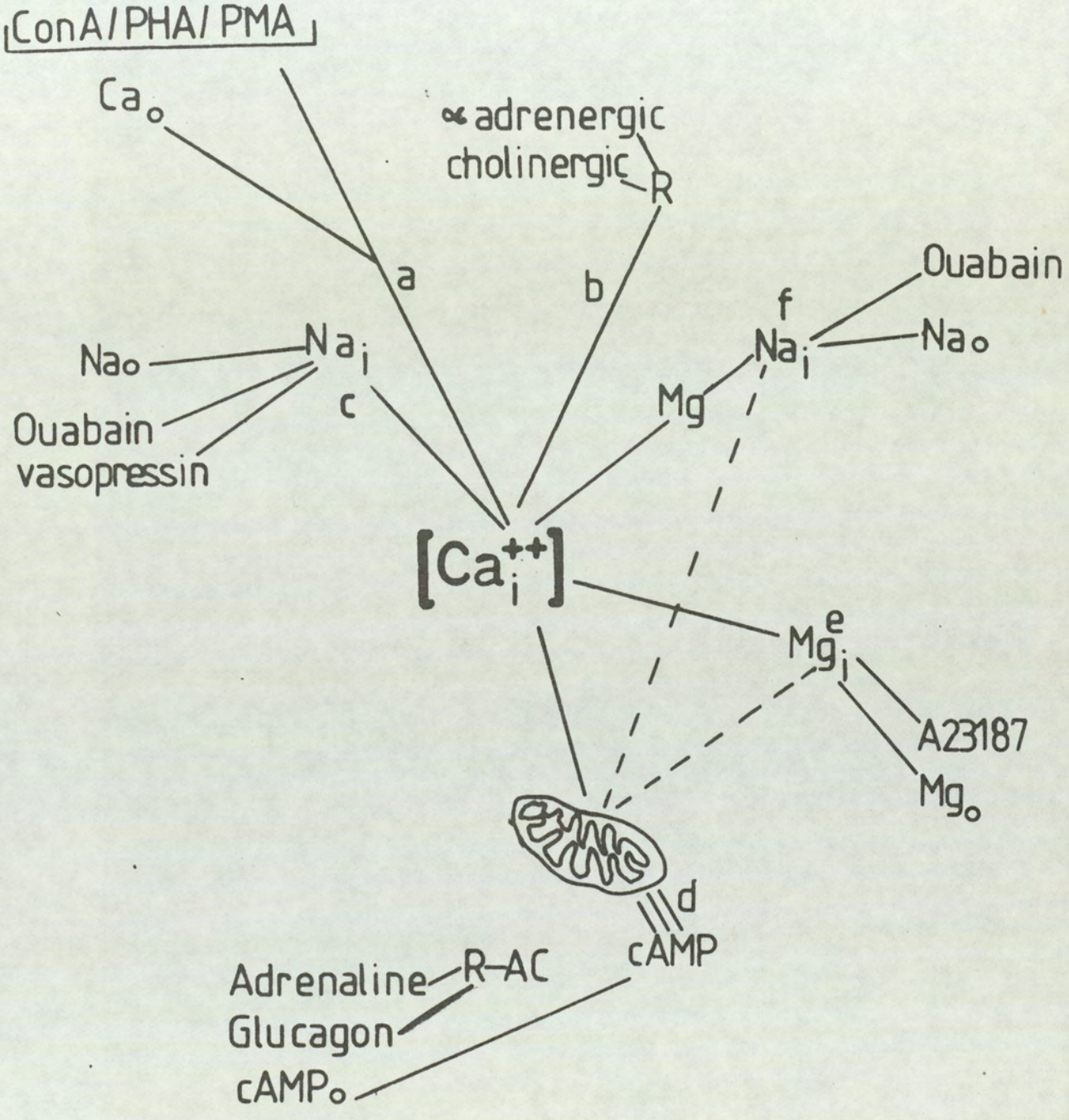
Before considering magnesium-dependent mitogens it is worth noting that calcium-dependent hormones are characteristically active at low concentrations and that an increased intracellular calcium is able to stimulate many other cell processes. Moreover the ability of alterations in extracellular calcium-induced physiological pressures in vivo to stimulate mitosis suggests this is indeed the major mitogenic axis. Those agents acting via a magnesium-dependent step may represent compounds that fortuitously are able to interfere with intracellular calcium homeostasis and hence provoke mitosis. The non-physiological levels required for magnesium-dependent hormonal stimulation and their probable dependence upon an ultimate increased intracellular calcium content all favour this contention.

Magnesium-dependent hormonal mitogens are able to promote mitosis in the total absence of extracellular calcium ions. Thus although an increased intracellular calcium content is implied by the activation of rubidium conductance, the independence of external calcium suggests that these mitogens influence intracellular calcium. We have demonstrated above that the magnesium requirement can be explained if one assumes magnesium assists in maintaining the free cytosolic calcium content. How then would

a magnesium-dependent hormonal mitogen produce a rise in intracellular calcium? One possibility is that a hormonal induced increase in cyclic AMP would be able to release calcium from intracellular stores. Indeed several magnesium-dependent mitogens are known to act in other tissues through cyclic AMP which unlike cyclic GMP production would be the first event after hormone-receptor binding. The release of calcium from isolated mitochondria by cyclic AMP has been demonstrated and recently confirmed (Juzu & Holdsworth, 1980)
Thus this mechanism is open to those hormonal mitogens acting via cyclic AMP (Figure D5d). This cyclic AMP/intracellular calcium axis corresponds closely with a similar mechanism in secretory tissue. Certainly where two secretory possibilities exist in a single tissue the external calcium/cyclic GMP and internal calcium/cyclic AMP axes can act independently. A similar response would be possible in the rat thymocyte.

Magnesium-dependent mitogens like their calcium-dependent counterparts, are a heterogenous collection of compounds. Those that interact with cyclic AMP have been considered but a second group of non-hormonal mitogens would not be expected to act initially with cyclic AMP. The ability of elevated external magnesium ion concentration to provoke mitosis suggests that a rise in the internal magnesium content will induce mitosis, albeit indirectly via altered calcium levels. Thus agents able to increase intracellular magnesium would constitute a second sub-group of magnesium-dependent mitogens. Theoretically the ionophore A23187 is a contender for this effect but this remains to be proven. This pathway is shown in Figure D5e. It must be remembered that the increased magnesium content would alter both mitochondrial and extramitochondrial calcium stores (Nicholls, 1978).

Fig. D5 Recruitment of rat thymic lymphocytes



A third series of magnesium-dependent mitogens may be acting via a sodium-mediated alteration in calcium metabolism (Figure D5f). The magnesium-dependent concentrations of ouabain were able to inhibit calcium exit from the cells. This stimulation did not require continued calcium entry consequently free calcium must be provided from the cytosol. This may be via sodium-mediated mitochondrial release. A direct effect of ouabain on cellular magnesium content appears to be unlikely (Van Rossum, 1970).

Although the two mitogenic axes exhibit contrasting external divalent cation dependencies, and are inhibitable by different sex-steroids, the current evidence suggests that calcium ions play a common role in both pathways. Thus an increased cytosolic calcium concentration is indicated when either calcium or magnesium-dependent mitogens stimulate mitosis. This ability of calcium to couple an external signal to the internal regulatory machinery confirms the existence of a stimulus-mitosis coupling event in the rat thymic lymphocyte.

Figure D5 summarises a hypothetical model for the recruitment of rat thymic lymphocytes that has been compiled in the light of the present experimental observations. From this figure it can be seen that, although evolution has provided the rat thymocyte with the ability to respond to a variety of extracellular signals demanding an increased mitotic activity, all of these processes ultimately converge upon the free intracellular calcium ion.

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