The Role of Insulin and the Insulin-Like Growth Factors in the Proliferation of the Rat Thymic Lymphocyte.

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Concanavalin A, provoked a 35-fold increase in the rate of proliferation of rat thymocytes. Insulin (10^{-6}M), and insulin-like growth factor I (10^{-10}M) approximately doubled the rate of DNA synthesis. Both of these structurally related molecules acted through the type I insulin-like growth factor receptor. The sequential addition of Concanavalin A and insulin, promoted a much greater proliferative response than to either of the two agonists alone.

Insulin also increased the uptake of glucose and amino acids by the cells. Glucose uptake was enhanced at insulin concentrations of 10^{-6}M and 10^{-10}M. Amino acid uptake was more strongly affected at the higher concentration. Insulin-like growth factor I (10^{-11}M) also enhanced amino acid uptake. The effects of insulin on metabolism were mediated by both insulin and type I insulin-like growth factor receptors. These effects were greatly enhanced after a pre-treatment with Concanavalin A.

Concanavalin A provided a primary mitogenic signal to the cells. Amongst the responses was an increased expression of insulin and/or type I insulin-like growth factor receptors. The consequent enhanced cellular sensitivity to these agonists, enabled them to facilitate the passage of the cells through the cell cycle by: i) providing a secondary mitogenic signal, and ii) promoting the uptake of raw materials and energy substrates. The initiation of DNA synthesis and passage through the cell cycle was thus punctuated by the sequential expression of various cell surface receptors. This regulated cellular sensitivity, enabling them to react in a precisely orchestrated fashion to hormones and other molecules in their environment.

The intracellular mechanism of insulin action remains an enigma. Although the presence of extracellular calcium was essential for insulin stimulation of amino acid uptake and DNA synthesis, the cation did not subserve a direct mediator function. Insulin promoted an increase in intracellular pH, which was mediated by the Na^+/H^+ antiport. Other mechanisms were probably also involved in mediating the full cellular response to insulin.

Key words: Rat Thymic Lymphocytes — Insulin
Insulin-like Growth Factors — Lymphocyte proliferation

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I. INTRODUCTION.

I.1. Cell Growth Control.

I.1.1 The Cell Cycle.

The cell cycle is the period between the division of a cell, and the subsequent division of its daughter progeny. Implicit in this concept is the idea of progression through a regularly recurring sequence of events, leading to division. The cell cycle is generally characterised by two types of metabolic activity: growth, the doubling of cellular constituents between subsequent divisions, and DNA replication and division. These two parallel sets of activities, the growth cycle and the DNA division or nuclear cycle (Baserga, 1984), can be uncoupled under some circumstances, and this results in unbalanced growth (Ross,1983; Zetterberg and Engstrom,1983). However, under physiological conditions they remain closely linked, and for most practical purposes the proliferative continuity of a clone of cells can be defined by such things as bursts of synthesis of certain proteins, fluctuations in the concentrations of some small molecules and ions, changes in susceptibility to certain drugs, as well as periods of DNA synthesis and division. Based on measurements of such parameters, the cell cycle has been divided into four consecutive phases: G_1, preparation of cells for DNA replication, S, replication of genetic material, G_2, preparation of cells for mitosis, and M, mitosis. (Howard and Pelc, 1953; Pardee et al., 1978; Wille and Scott, 1984; Ronot and Adolphe, 1986; Rabinovitch et. al., 1988).

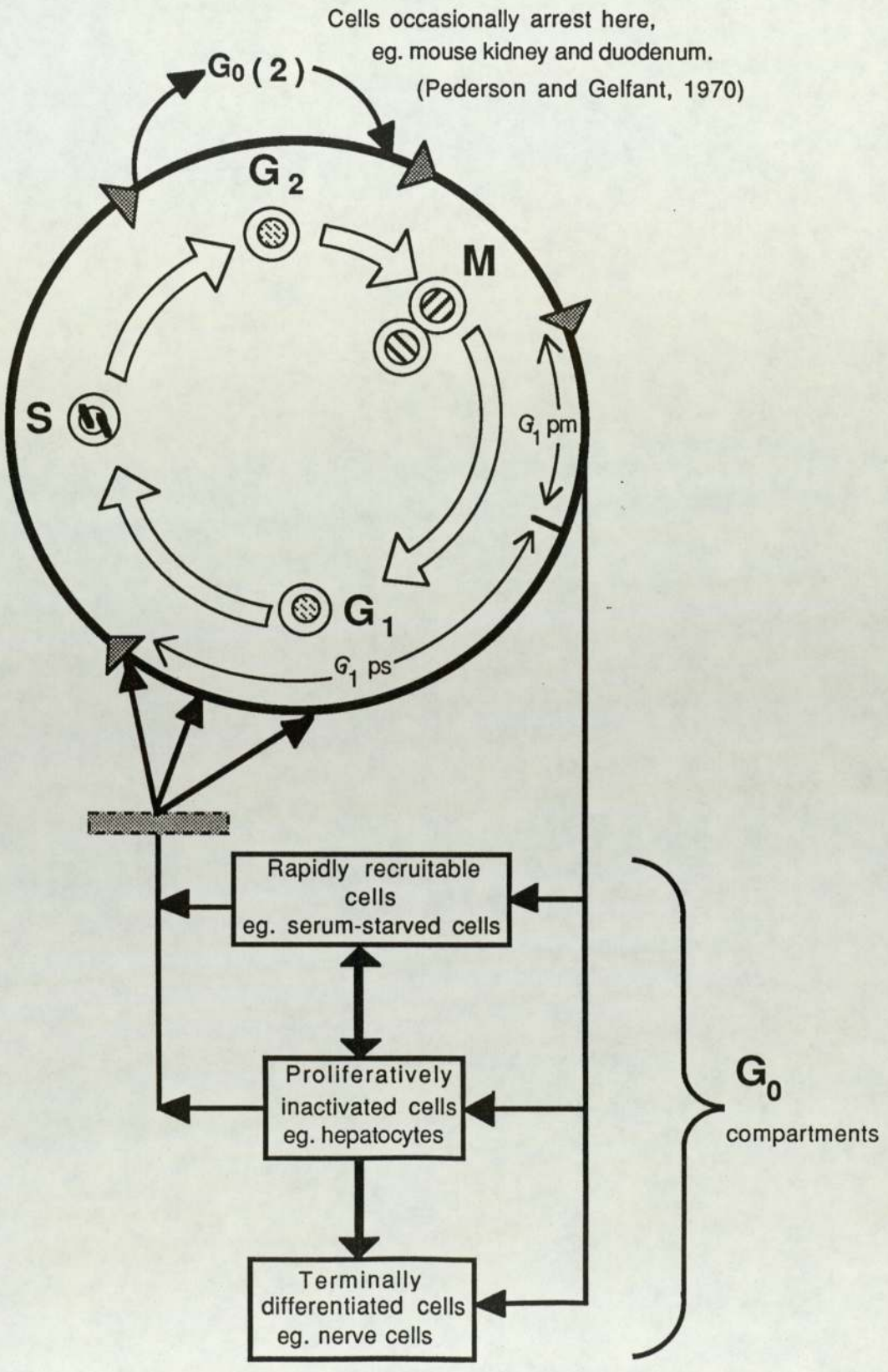
After mitosis some cells permanently leave the cell cycle and undergo maturation and differentiation. Other cells may temporarily leave the proliferative cycle and enter into a quiescent state termed G_0. Conditions which induce cells to arrest in G_0 *in vitro* include serum starvation (Martin and Stein, 1976; Larrson et al., 1986) and nutrient deprivation

(Holley and Kiernan, 1973). When optimal conditions are restored to such cultures, the cells re-enter the cycle after a certain "lag" period (Baserga, 1976; Larrson et al., 1985). Differences in the duration of the "lag" phase, and the possible necessity for a different group of stimuli to reinitiate division after long term residence in G_0 (Whitfield, 1982), have led to the suggestion that cells held for progressively longer times in G_0 fall into progressively deeper states of arrest (Baserga, 1976; Wille and Scott, 1982). (See Figure 1).

There is considerable variability in the transit times of different cell types through a cell cycle. Most of the asynchrony, however, appears to be generated during the G_1 phase (Prescott, 1968). This fact, coupled with the observation that G_0 arrested cells re-enter the cycle via G_1, forms the basis for the conclusion that events occuring in G_1 determine the regulation of proliferation and the control of the initiation of DNA synthesis (Baserga, 1976; Whitfield, 1982; Larsson et al., 1985, Pardee, 1987). It has been proposed that the decision whether to divide, is made at a single point in G_1, termed the restriction point (Pardee, 1974). Other workers however, claim that a number of distinct G_1 arrest states exist dependent on what method is used to induce cessation of growth (Wille and Scott, 1984).

In Swiss 3T3 cells, Zetterberg and Larsson (1985) have found that the intermitotic period can be extended by 8 - 12 hours after only 1 hour's exposure to serum free medium. The delay, which possibly represents a transient entry into G_0 , was only observed in cells younger than 3.5 hours, measured from the last mitosis. The authors thus divide G_1 into two parts: a G_1pm (post-mitotic) phase, during which a cell is susceptible to growth factors and may, in their absence, enter G_0, and a G_1ps (pre-S) phase where a cell is growth factor independent and goes on to divide even if incubated in serum free medium. The G_1ps phase is, however, highly variable in length, and differences in its duration are responsible

Figure 1. The Cell Cycle and Some Associated Groups of Proliferatively Arrested Cells.



for most of the variability in transit times of the G_1 phase, and also of the whole cell cycle.

The proposal for the existence of exclusive control point(s) in G_1 is not however without challengers. Cooper (1987) suggests that the relevant regulatory events occur continuously during the period between successive S phases. His proposed "Law of Cell Age Invariance" states that no batch treatment of cells can reverse their order of division. This is at variance with the inference from Zetterberg and Larsson's work (1985), since exposure to low serum conditions for 1 hour would delay division, by 8 - 12 hours, of only that subset of cells in the G_1pm phase of the cycle. Cells in other phases would remain unaffected, and progress through the cycle with no delay, hence catching up with, or overtaking their G_0 arrested counterparts.

Clearly, ordered, controlled growth is essential for the development and maintenance of a normal animal, and an understanding of the processes involved is a prerequisite for the understanding of abnormal growth, of which the various types of cancer are the most common examples. It therefore becomes pertinent to ask what regulates the extent of cellular proliferation? Although there is, as yet, no definitive answer to this question, it seems probable that it depends on a) the environmental signals, and b) the genes and gene products that interact with and respond to these signals. The following sections are a brief review of these aspects of cellular growth control.

I.1.2 Growth Factors.

The cells of many tissues and organs in the adult, where there is no net growth, retain the capacity to respond to extracellular signals such as hormones, growth factors and antigens, by re-entering the cell cycle and increasing their rate of proliferation. In this way, due to the selective

response of different cell types to different factors, cycling of individual cells is regulated according to the needs of the whole organism. The mechanisms regulating proliferation of cells *in vivo* are very complex in order to balance the growth and differentiation states of the various cell types in an animal. Cell culture techniques are therefore employed to study aspects of proliferation in relatively well defined conditions.

Early investigations showed that in order for normal cells to proliferate *in vitro* , not only must temperature, pH and nutrient conditions mimic those *in vivo* , but the presence of whole serum was also required (Todaro et. al., 1965; Holley and Kiernan, 1968; Holley, 1975). Later experiments, using nutrient media and purified growth factors, have elucidated those components of serum which are necessary to support growth for some specific cell types (Rozengurt, 1980; Shipley and Ham, 1981 and 1983), and shown that these specific requirements are relaxed in the case of neoplastic cell proliferation (Holley, 1975; Paul, 1985; Wahl and Carpenter, 1987).

The proliferation of normal cells *in vitro* can be regulated, positively or negatively, by a large number of physiological and pharmacological agents, some examples of which are given in Table 1. Even in this small selection, it can be seen that a wide range of chemically diverse factors may contribute to the control of proliferative processes. Clearly, a number of these are unlikely to be relevant under most physiological circumstances, although they may be useful in defining the biochemical mechanisms taking place *in vitro* . It seems likely that, *in vivo* , a subtle interplay between numerous agents determines the proliferative status of a given cell or cell type.

Very little is known about negative regulators of cell growth, though some have been purified from normal tissue (Assoian et. al., 1983), and from the conditioned medium from cultured cells (Holley et. al., 1980).

Table 1: Examples of Growth-Regulating Agents.

Agent	Positive or Negative Regulator	Reference
Growth Factors:		
Platelet Derived Growth Factor (PDGF)	+ve	Stiles, 1983
Interleukin 2 (IL-2)	+ve	Robb, 1984
Erythropoietin	+ve	Stanley and Jubinsky, 1984
Insulin-Like Growth Factors (IGFs)	+ve	Froesch et. al., 1985
Epidermal Growth Factor (EGF)	+ve	Cohen, 1986
Fibroblast Growth Factor (FGF)	+ve	Gospodarowicz et. al., 1987
Transforming Growth Factor β (TGF-β)	-ve/+ve	Moses et. al., 1987
Fibroblast Growth Regulator (FGR)	-ve	Wang and Hsu, 1986
Hormones and Regulatory Agents:		
Insulin	+ve	Straus, 1984
Bombesin	+ve	Zachary et. al.,1985
Prostaglandins	-ve	Baker et. al., 1981
Interferons	-ve	Taylor-Papadimitriou et. al., 1985
Microtubule-Disrupting Agents:		
Colchicine	-ve/+ve	Friedkin and Crawford, 1983
Tumour Promotors:		
Phorbol Esters	+ve	Isakov et. al., 1985
Neurotransmitters:		
Acetylcholine	+ve	Morgan et. al., 1984
Plant Lectins:		
Concanavalin A (Con A)	+ve	Metcalfe et. al., 1985
Phytohaemagglutinin (PHA)	+ve	Serke et. al., 1987

Factors that appear to inhibit proliferation of certain cell types include Interferon-β (Wang and Hsu, 1986), Fibroblast Growth Regulator (FGR) (Steck et. al., 1982) and Transforming Growth Factor-β (Sporn and Roberts, 1985).

In contrast much more progress has been made in our understanding of the structure and activities of growth stimulatory factors. Some molecules are required for division of all cell types, and may be regarded as permissive in nature. These include insulin and transferrin (Barnes and Sato, 1980), which have been found to be ubiquitous requirements for cell proliferation in serum-free medium (Gospodarowicz and Moran, 1976). Others are more specific and are required for growth of particular cell types. These include the polypeptide growth factors such as Epidermal Growth Factor (EGF) and Platelet-Derived Growth Factor (PDGF). Most of the polypeptide growth factors act in a paracrine manner *in vivo* , being manufactured in a specific cell type(s), and travelling very short distances to their target cells by diffusion. There are however important exceptions such as the Insulin-Like Growth Factors (IGFs), which are conveyed in the blood (endocrine), and Interleukin-2 and Transforming Growth Factors, which stimulate the cells in which they are produced (autocrine) (James and Bradshaw, 1984; Deuel, 1987).

Structural information is now available for a number of growth factors, and it has become clear that they may be divided into a series of "families" on the basis of amino acid sequence. For example, a high degree of sequence homology exists between insulin, IGFs I and II, Nerve Growth Factor (NGF) and relaxin (Blundell and Humbel, 1980), and also between EGF and several Tranforming Growth Factors (TGFs) (Marquardt et. al., 1983). The degree of structural relatedness between members of each "family" probably indicates derivation from a common ancestor molecule, by gene duplication and subsequent evolutionary divergence.

Some oncogenic products also share structural similarities with growth factors. The transforming protein of the simian sarcoma virus, $p28^{sis}$, is, for example, virtually identical to the B-chain of PDGF (Doolittle et. al., 1983).

A striking aspect of the effect of growth factors on cultured cells is the existence of synergism between agents, such that specific combinations of factors can produce a proliferative response similar to that of whole serum (Rozengurt, 1980 and 1983; Conover et. al., 1983; Roger et. al., 1987). One implication of these observations is the possible activation of different intracellular signalling pathways by different growth factors, which may, when acting in concert, provoke a maximum proliferative response. Comparisons of the temporal aspects of re-entry into the cell cycle from quiescence, also favour a hypothesis involving different roles for different growth factors (Richmond et. al., 1980; Sand and Christoffersen, 1987).

Growth factors have been divided into two groups on the basis of their ability to induce 'competence' in quiescent cells or regulate 'progression' to S phase of the cell cycle (O'Keefe and Pledger, 1983). These two types of factor are proposed to regulate two discrete sets of events in the re-initiation of DNA synthesis, which may be associated with passage through the G_1pm and G_1ps phases of the cell cycle respectively. Competence factors 'prime' G_0 arrested cells, initiating a set of responses, probably including synthesis of new mRNAs and protein production, which enable the cells to respond to certain other progression factors. For example, PDGF has been found to be a competence factor for BALB/c3T3 cells, whereas IGFs, insulin and EGF are progression factors for the same cells (Stiles et. al., 1979; O'Keefe and Pledger, 1983; Deuel, 1987).

There is however, some controversy surrounding the competence/ progression factor model. It has been shown that PDGF alone promotes

the growth of human glial cells in the nutritionally balanced medium MCDB - 105, in the absence of progression factors (Heldin et. al., 1980). However, the possibility that the cells produced an IGF which was responsible for autocrine growth stimulation was not ruled out. Generally, either insulin or an IGF is required for optimum growth in serum free medium (Straus, 1984; King and Kahn, 1985).

I.1.3 Growth Factor Receptors and Transmembrane Signalling.

Studies with many radiolabelled growth-promoting molecules have shown that the initial step in their interaction with target cells is to bind to specific, saturable, glycoprotein receptors which protrude from the external leaflet of the plasma membrane (James and Bradshaw, 1984; Carpenter, 1987). However, there are notable exceptions to this rule in the case of steroid hormones, which generally exert their effects by binding to intracellular receptors (Jensen et. al., 1982; Smith et. al., 1986).

In culture systems, an element of cross-reactivity is sometimes observed between a ligand and receptors for a structurally related molecule. Hence at high concentrations insulin may bind to IGF receptors (King and Kahn, 1985), hystricomorph insulin may bind to PDGF receptors (King et. al., 1983), and the various types of TGF-β interact with all types of TGF-β receptors (Massague et. al., 1987). Although cellular resposes are induced in all these cases, only the last seems to be of physiological importance, except perhaps in conditions of ligand overproduction.

The sensitivity of a cell to a given growth factor is largely determined by the number and affinity of its receptors which bind that factor, and both these parameters can be altered in response to a number of stimuli. Scatchard plot analyses (Scatchard, 1949) allow the estimation of affinity and receptor concentration on a given cell type. Some growth factors, including insulin and EGF, yield non-linear Scatchard plots, which have

been interpreted in two ways by different workers. Firstly, it has been suggested that this is due to two subclasses of receptor for the same ligand, of 'high' and 'low' affinity (Krupp and Livingston, 1978; King and Cuatrecasas, 1982). The evidence in favour of this possibility is strong in those cases where the affinity constants for 'high' and 'low' affinity sites differs by a large amount, and the subtypes can be manipulated biochemically (Greene, 1986). It is also possible however, that the deviations from linearity of Scatchard plots for some ligands reflects co-operative interactions. Here, partial occupancy of a cell's complement of a receptor type, produces either greater (positive co-operativity) or lesser (negative co-operativity) affinity of the remaining receptors of that type (Cuatrecasas and Hollenberg, 1975; DeMeyts et. al., 1976; Helmerhorst, 1987).

Alterations of receptor affinity can also be brought about by treatment with heterologous ligands, a process known as transmodulation. Thus treatment of Swiss 3T3 cells with PDGF (Collins et. al., 1983), phorbol esters (Brown et. al., 1979) and vasopressin (Rozengurt et. al., 1981) decreases the affinity of EGF receptors for EGF. This may be brought about by at least two mechanisms, one of which appears to rely on Protein Kinase C activation (Carpenter, 1987; Friedman and Rosner, 1987). Conversely an increase in Type II IGF receptor affinity is observed in adipocytes treated with insulin (Oppenheimer et. al., 1983).

Mechanisms for altering the number of a receptor type on the cell membrane also exist. Following ligand binding, down regulation may take place, whereby occupied receptor complexes aggregate and are then internalised by endocytosis. This process has been well documented for insulin and EGF (Schlessinger et. al., 1978; Carpenter, 1987). Not only are numbers of receptors reduced in this way, but the cell surface ligands are also removed for lysosomal degradation, or possible intracellular

involvement in cellular response (Posner et. al., 1981; James and Bradshaw, 1984). Heterologous down-modulations in receptor number have also been observed between different Colony Stimulating Factors (CSFs)acting on bone marrow cells (Nicola, 1987).

The final example of a situation in which receptor numbers can be altered concerns the induction of expression of a receptor type, after treatment with a heterologous ligand. This may involve insertion into the cell membrane of pre-formed receptors, as in the case of glucose transporters after adipocyte exposure to insulin (Cushman and Wardzala, 1980). In other circumstances, induction of receptor expression involves transcription of new mRNA and protein synthesis, as when IL-2 receptors (Robb, 1984), and transferrin receptors (Neckers and Cossman, 1983), are expressed by T lymphocytes after mitogenic or antigenic stimulation.

Receptors not only serve to recognise the extracellar signals impinging on a cell, but they also participate in the generation of intracellular signals leading to metabolic responses. Recent advances have suggested that there are at least four functional classes of receptor, based on how they fulfil the function of transmitting the extracellular message across the plasma membrane. They may be categorised as: receptors which act as ligand-gated ion channels, receptors which mediate the internalisation of ligands, receptors with intrinsic tyrosine kinase activity, and receptors which catalyse the exchange of guanine nucleotides by G proteins. These categories are by no means exclusive, and some receptor types appear to mediate more than one of these functions. Nevertheless, in terms of an overall study of growth control, it may be more enlightening to note general aspects, than to discuss individually each of the receptor types which may be involved.

Receptors which act as ion channels, or receptor operated channels (ROCs) are membrane spanning glycoproteins in which the receptor and

the channel exist in one or two closely adjacent molecules. They are therefore distinct from ion channels which open in response to changes in potential difference across the cell membrane (VOCs) and from second messenger operated ion channels (SMOCs) (See Section I.1.4.2.). They function because of the steep electrochemical gradients which exist across the plasma membrane for certain ions. Hence when a ligand binds to such a receptor, a conformational change in the protein takes place which opens the ion channel. This allows a flux of the ion(s) into or out of the cell, altering their concentration(s) in the cytoplasm, and having diverse effects on cellular metabolism. The nicotinic acetylcholine receptor has been considered a prototype Ca^{++} ROC, opening on ligand binding to allow the passage of Ca^{++} into the cell (Meldolesi and Pozzan, 1987). It does however, also allow the passage of Na^{+} into and K^{+} out of the cell.

The significance of ROCs in growth control is difficult to assess. For example, even where a source of extracellular Ca^{++} is essential for proliferation, it is difficult to differentiate between Ca^{++} entry through SMOCs and ROCs. However, using monoclonal antibodies to different epitopes on the T-lymphocyte antigen receptor, and measuring the resultant changes in Ca^{++} conductance of lipid bilayers prepared from the plasma membranes of T-lymphocytes, Alcover et. al. (1987) have shown that a) although all the antibodies used induced receptor aggregation, only those specific for certain epitopes enhanced Ca^{++} conductance, and b) the same antibodies which increase Ca^{++} conductance, also cause a $[Ca^{++}]_i$ rise in intact cells. This suggests that Ca^{++} ROCs may open in response to antigen binding, and that the $[Ca^{++}]_i$ rise which precedes activation of the cells may be at least partially dependent on this process.

Receptors which mediate the internalisation of ligands are numerous, and the phenomenon of receptor-mediated endocytosis has already been discussed in relation to down-regulation of receptors. The extent to which

this process may contribute to intracellular signalling is however controversial (James and Bradshaw, 1984; Carpenter, 1987). In some cases it does, nevertheless, perform an important function in mediating the transport of substances into the cell. Substances transported in this way include iron, via the transferrin receptor (Hedley, 1987) and low density lipoprotein (Geisow, 1986).

Another important group of receptors, termed carriers or transporters, mediate entry of other lipid-insoluble molecules. Operationally this is a three step process including binding of the ligand on the outside of the plasma membrane, translocation across the membrane, and release of the solute into the cytoplasm. Some receptors of this type are energy dependent and carry solutes up a concentration gradient. The energy requirement may be coupled to influx of Na^+ down its concentration gradient. These include the glucose transporters of epithelial cells of the small intestine and the kidney (Elbrink and Bihler, 1975), and also some amino acid transporter types, (Guidotti et. al., 1978). Others are energy-independent and mediate facilitated diffusion, allowing the ligand into or out of the cell, depending on its concentration gradient. These include the glucose carriers of most cells, such as adipocytes (Crofford and Renold, 1965; Garvey et. al., 1986).

Receptors with intrinsic tyrosine kinase activity have only recently been detected, but have special relevance to growth control, since they seem to be exclusively growth factor receptors. The first tyrosine kinase activities to be detected were associated with the transforming proteins of the oncogenic retroviruses (Eckhart et. al., 1979). Although virally coded, these proteins have been found to be products of co-opted cellular genes, and hence their provenance is ultimately cellular (Bishop, 1983). The best characterised of the retroviral tyrosine kinases is $pp60^{v\text{-}src}$, the transforming protein of Rous sarcoma virus (Hunter and Cooper, 1985).

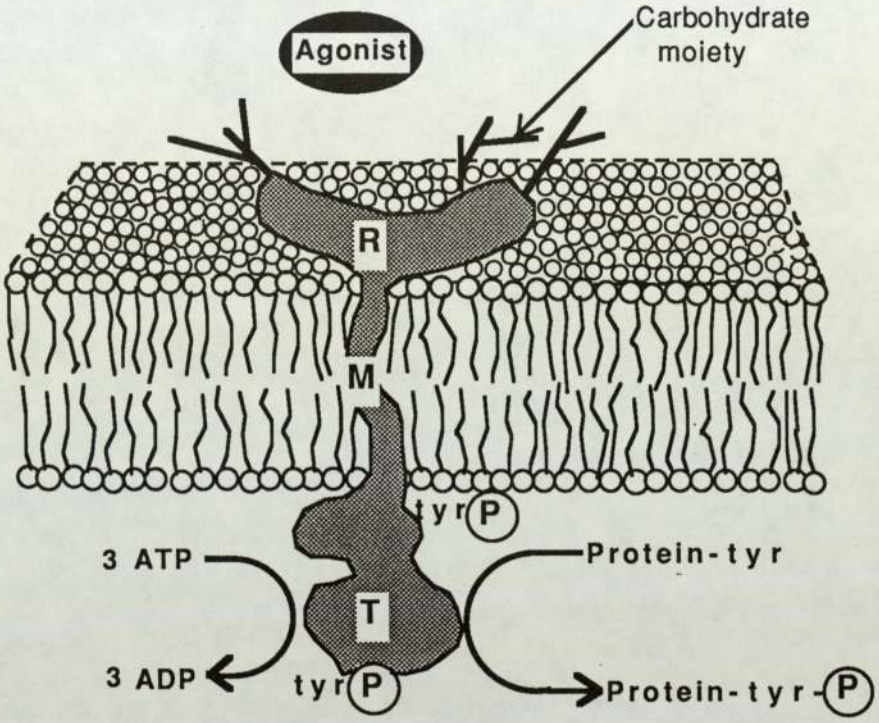
Another type of tyrosine kinase activity was however detected in association with growth factor receptors, firstly with the EGF receptor (Ushiro and Cohen, 1980), and subsequently with the receptors for PDGF (Ek et. al., 1982), insulin (Kasuga et. al., 1981), IGF I (Jacobs et. al., 1983) and CSF I (Coussens et. al., 1986). The tyrosine kinase activity is associated in all cases, with the intracellular domain of these large, membrane-spanning receptors. Ligand binding leads to autophosphorylation of specific tyrosine residues, within this intracellular domain (Downward et. al., 1984a; Cordera et. al., 1985) (See Figure 2).

In the case of the EGF receptor, the degree of tyrosine phosphorylation on ligand binding, can be decreased by protein kinase C phosphorylation of threonine residues on the receptor, and there is also evidence that other cellular kinases may have regulatory roles (Bertics et. al., 1985; Carpenter, 1987). Other cellular proteins may be phosphorylated including the glucocorticoid receptor (Rao and Fox, 1987) and the progesterone receptor (Woo et. al., 1986). Other less well defined proteins have also been shown to be phosphorylated (Cooper and Hunter, 1985; Sefton, 1985; Deuel, 1987), but the significance of these phosphorylations has yet to be established.

Evidence is now accumulating which suggests that the tyrosine kinase activity of these receptors is required, though it may not be sufficient alone, in order for the growth promoting signals of the respective ligands to be propogated into the cell (Sefton, 1985; Chen et. al., 1987; Chou et. al., 1987). It is of interest to note that a number of oncogene products share homologies with this type of growth factor receptor. Thus, for example, $gp65^{erbB}$, product of the erbB oncogene of avian erythroblastosis virus, is a truncated EGF receptor (Downward et. al., 1984b) and the feline c-fms proto-oncogene product is related to the receptor for Mononuclear Phagocyte Growth Factor (CSF 1) (Sherr et. al., 1985). Both these receptors,

Figure 2. Hypothetical Growth Factor Receptor with Tyrosine Kinase Activity.

Autophosphorylation of the receptor, and phosphorylation of exogenous substrates are shown.



Key:

R - Extracellular domain, recognises and binds to agonist.

M - Hydrophobic membrane-spanning domain.

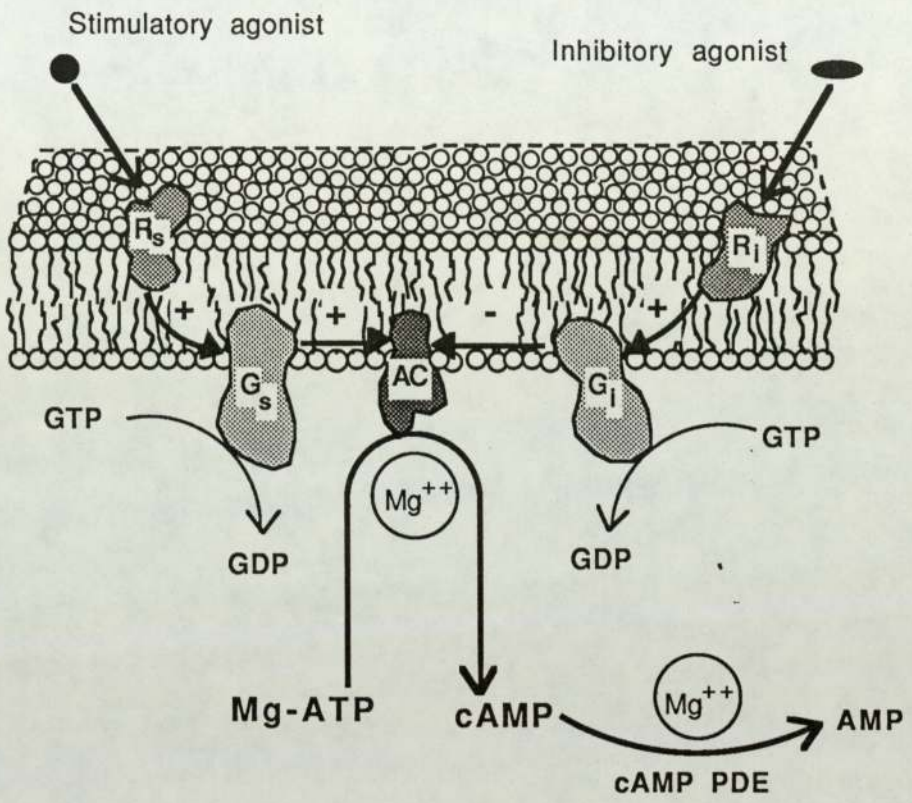
T - Intracellular domain: displays tyrosine kinase activity when ligand is bound to R

and the associated oncogene products are tyrosine kinases. The v-erb-A oncogene product shares homologies with the glucocorticoid receptor (Weinberger et. al., 1985), which is a substrate for tyrosine kinase activity. It seems then, that tyrosine kinase activity is closely associated with normal growth control as well as with many cases of neoplastic growth (Hunter and Cooper, 1983; Heldin and Westermark, 1984).

The fourth receptor subtype are those which catalyse the exchange of guanine nucleotides by G proteins. The G proteins function as intermediaries in transmembrane signalling pathways that consist of three structures; the specific receptor, the G protein(s), and effector(s), which are usually enzymes. There are a large number of receptor types which are known to be coupled to G proteins (Dohlman et. al., 1987), and a number of these may be associated with growth control. These include putative G proteins involved in protein kinase C (PKC) activation (Huang et. al., 1987), phospholipase A2 activation (Burgoyne et. al., 1987), and the opening of various ion channels (Gomperts, 1983; Gill, 1986; Houslay, 1987). The best characterised of these systems linked to growth control, are however the various receptors and G proteins which regulate the membrane-bound adenylate cyclase (Spiegel et. al., 1981; Smigel et. al., 1982; Houslay, 1986), and phospholipase C (Smith et. al., 1986; Cockcroft, 1987; Lo and Hughes, 1987; Wang et. al., 1987) enzymes.

Adenylate cyclase (AC), converts ATP to 3',5'-cyclic AMP (cAMP), an important intracellular second messenger which controls a variety of cellular functions including aspects of the proliferative response (Rozengurt, 1985; Whitfield et. al., 1987) (See Section I.1.4.3.). The activity of the AC enzyme is controlled by a multi-component system comprising distinct stimulatory and inhibitory receptors and G proteins (Spiegel et. al., 1981; Houslay, 1986) (See Figure 3). Growth regulatory agents may interact with this system in a variety of ways, either directly through the

Figure 3. The Adenylate Cyclase/cAMP System, Showing the Role Played by Receptors and G proteins in Membrane Transduction.



Key:

R_s - Receptor for stimulatory agonist

R_i - Receptor for inhibitory agonist

G_s - G Protein which stimulates adenylate cyclase

G_i - G Protein which inhibits adenylate cyclase

AC - Adenylate cyclase enzyme

cAMP PDE - cAMP dependent phosphodiesterase

membrane receptors, or indirectly by modulating a) the intracellular metabolism of cAMP, and b) the activity of other system components. (Rozengurt, 1985 and 1986; Segal et. al., 1986).

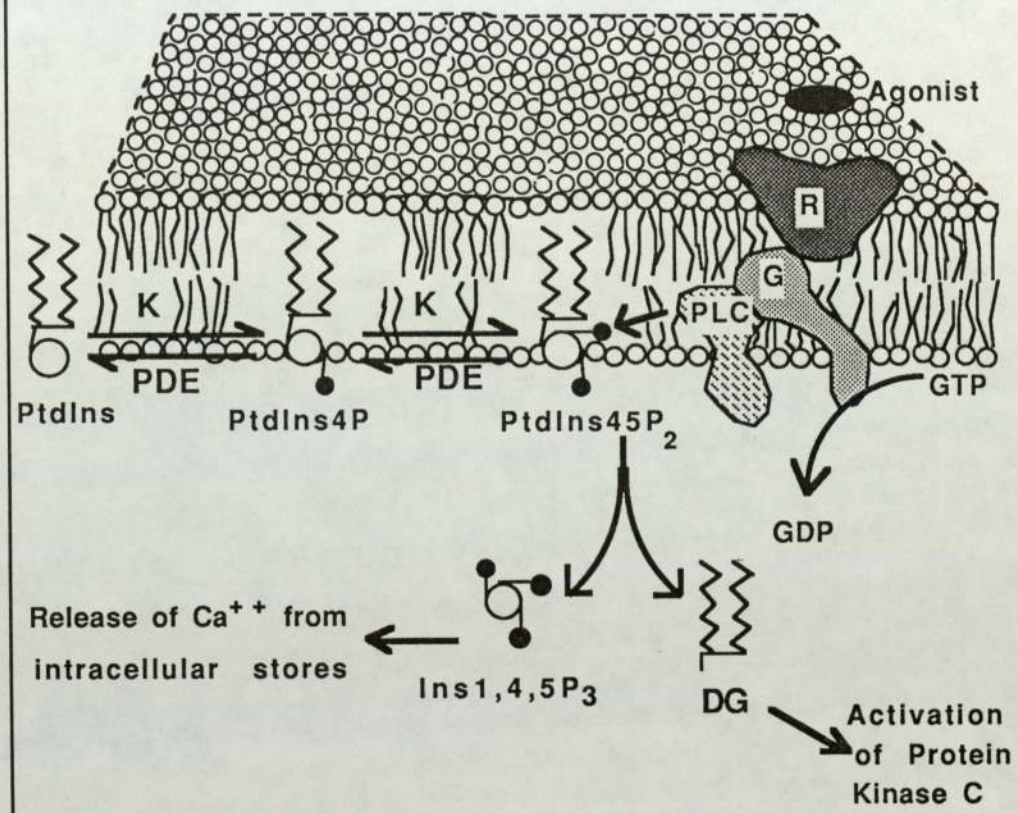
A number of growth factor receptors are coupled to the G protein which activates the enzyme phospholipase C (PLC), including those for thrombin (Paris et. al., 1987) and PDGF (Vicentini and Villereal, 1986). The substrate for PLC is a minor membrane inositol lipid, phosphatidylinositol 4,5-bisphosphate (Ptd-Ins4,5P_2). The parent molecule is a more abundant membrane lipid, phosphatidylinositol (PtdIns), which may be phosphorylated by a specific kinase to form phosphatidylinositol 4-phosphate (PtdIns4P), which is in turn phosphorylated by another specific kinase to form Ptd-Ins4,5P_2 (Berridge, 1984; Berridge and Irvine, 1984). A dynamic equilibrium is sustained between these three membrane lipids by the action of the two kinases, and two corresponding phosphodiesterases (Parthasarathy and Eisenberg, 1986). (See Figure 4).

The key event in signal transduction is the cleaving of Ptd-Ins4,5P_2, by PLC, to form two products: inositol1,4,5-trisphosphate (Ins1,4,5P_3), and diacylglycerol (DG). Both these molecules are postulated as having second messenger functions within the cell, Ins1,4,5P_3 in promoting Ca^{++} release from intracellular stores, and DG in activating protein kinase C. (Nishizuka et. al., 1984; Exton, 1985; Berridge, 1987a). These aspects are discussed in more detail in sections I.1.4.1. and I.1.4.2. It is of interest to note however, that proliferation in response to some growth factors can be abolished by microinjection of antibody to Ptd-Ins4,5P_2 (Matuoka et. al., 1988).

All the G proteins involved in these processes are heterotrimers, consisting of α, β and γ subunits in order of decreasing mass. The α subunits appear to be unique in each case, but β and γ subunits may be shared among a number of α subunits to make up the specific oligomers

Figure 4. The Phospholipase C/Inositol Lipid System.

The role of receptor, G protein, phospholipase C, and membrane inositol lipids in signal transduction.



Key:

R - Receptor

G - G Protein

K - Kinase

PDE - Phosphodiesterase

Other abbreviations as in text

(Houslay, 1986; Gilman, 1987). The term G protein is derived from their regulation by GTP. A cyclic process is initiated by the binding of GTP to the α subunit, which activates the pathway. The subsequent hydrolysis of GTP and dissociation of GDP causes deactivation (Dohlman, 1987; Vaughan, 1987). There is also evidence that a cycle of association and dissociation of the subunits themselves may be superimposed on this regulatory GTPase cycle (Gilman, 1987; Wessling-Resnick et. al., 1987). Other characteristics shared by some G proteins include activation by aluminium plus fluoride ions, and ADP-ribosylation of the α subunit by bacterial toxins including cholera and pertussis toxins (Janssens, 1987; Vaughan, 1987).

Oncogenic products may also be involved in several steps of the phosphoinositide cycle. For example, the products of the ros and src genes phosphorylate PtdIns and both PtdIns and PtdIns4P respectively (Sugimoto et. al., 1984; Sekar and Hokin, 1986), and the ras gene product, p21, is a G protein which may be involved in receptor/PLC coupling in certain transformed cells (Berridge and Irvine, 1984; Lo and Hughes, 1987). In its activated form, often caused by point mutation, the ras oncogene codes for a G protein with reduced GTPase activity. Hence the activation step, binding to GTP, occurs normally, but deactivation, hydrolysis and release of GDP, is slower than normal, and the duration of PLC activity is extended (Hoshino et. al., 1987; Yu et. al., 1988).

In summary, the receptor types which have been most specifically associated with cellular proliferation are the tyrosine kinase receptors, and those linked via G proteins to polyphosphoinositide turnover and adenylate cyclase activation. The roles played by these receptor types in membrane transduction may represent the starting points of alternative or co-operating intracellular signalling pathways which lead ultimately to DNA replication and cell division (Berridge, 1987b; Chambard et. al.,

1987). Although our understanding of these signalling mechanisms is far from complete, some of the major aspects are discussed in the following sections.

I.1.4. Intracellular Signalling and the Generation of the Growth Response.

It has been noted that DNA synthesis is a 'late' event, taking place at least 8 hours after the initial growth stimulatory event (Rozengurt, 1980; Zetterburg and Larsson, 1985). A complex array of biochemical reactions respond co-ordinately prior to DNA synthesis, and this regulatory programme has been called the "pleiotypic response"(Hershko et. al., 1971). It includes a large number of processes including RNA synthesis, protein synthesis, nucleic acid precursor uptake, glucose uptake, amino acid uptake and various ionic fluxes. The relationships between these interlinked processes is far from well understood, but some inroads have been made by attempting to look at the temporal relationships between the various events. It is assumed that when a growth factor interacts with its receptor on the cell surface, some primary mitogenic response(s) takes place which is as a direct result of this interaction (Metcalfe et. al., 1985). Secondary responses are then triggered which result in a complex network of biochemical reactions, leading ultimately to DNA synthesis and cell division. Another way of classifying these responses is as 'regulatory signals' or 'obligatory events'(Rozengurt, 1986). Regulatory signals, the early growth-factor induced events, are dependent on the particular growth factor used to elicit the response. They are crucial for the action of the specific growth factor, but may be bypassed by other groups of factors. Obligatory events are molecular processes that must take place in order for proliferation to occur, regardless of the initial regulatory signals used to activate the cell.

Most success in unravelling the mechanism of growth stimulatory activity has been achieved in studies of the early, regulatory processes associated with growth factor action. This is probably largely due to their inherent simplicity compared to later events, and also their close temporal relationship with growth factor/receptor binding. In the following sections some of the commonly observed early mitogenic responses are described, and possible relationships to later events are proposed, where information is available.

I.1.4.1. The Role of Monovalent Cation Fluxes and Changes in pH of the Cytoplasm.

Amiloride-sensitive sodium ion (Na^+) influxes have been observed in virtually all quiescent animal cell systems when they are stimulated to divide. They occur within seconds of mitogenic stimulation, and persist for at least 2 - 60 minutes in the presence of mitogen (Leffert and Koch, 1985; Rozengurt, 1985; Moolenaar et.al., 1986; Prasad et. al., 1987). It has been proposed that changes in intracellular Na^+ concentration ($[Na^+]_i$) may affect the DNA synthetic process by decreasing membrane potential (Kiefer et. al., 1980; Felber and Brand, 1983), and triggering specific protein phosphorylation (Pouyssegur et.al., 1982). However, an additional effect of mitogen-induced Na^+ influx is to decrease H^+ content of the cell since it is coupled to H^+ export via an integral membrane protein, the Na^+/H^+antiport (Grinstein and Rothstein, 1986; Moolenaar, 1986; Reuss et.al., 1986; Villereal et. al., 1986). Also, the rise in $[Na^+]_i$ activates the plasma membrane Na^+/K^+ ATPase, which exports Na^+ from the cell in exchange for K^+. Hence, over time, $[Na^+]_i$ may remain relatively constant while $[H^+]_i$ decreases and $[K^+]_i$ increases. Rozengurt (1985) has suggested that there may be a $[K^+]_i$ level below which cells fail to initiate DNA synthesis, but it is the increase in intracellular pH due to activation of the

Na^+/H^+ antiport that has come to be regarded as possibly representing an obligatory event (Pouyssegur et.al., 1986; L'Allemain et. al., 1986; Vicentini and Villereal, 1986; Baliga et. al., 1987; Musgrove et. al., 1987; Madshus, 1988).

Although cytoplasmic alkalinisation due to Na^+/H^+ antiport activation occurs in numerous cell types when stimulated with a variety of mitogenic agents (see Table 2), it is unlikely that it is always a consequence of the same mechanism (Frelin et. al., 1986; Reuss et. al., 1986; Rozengurt and Mendoza, 1986). The most well known activator of the Na^+/H^+ antiport is the phospholipid and calcium-dependent enzyme, protein kinase C (PKC) (Nishizuka, 1984 and 1986; Coussens et. al., 1986; Parker and Ullrich, 1987). The physiological activator of PKC is thought to be diacylglycerol (DG), which is produced in the cell membrane during the signal-induced turnover of inositol phospholipids (Bell, 1986; Isakov et. al., 1987) (see Section I.1.3.). It is therefore possible, by this mechanism, to propose a direct link between mitogenic agents with receptors linked to PLC, and cytoplasmic alkalinisation after mitogenic stimulation. Interest in this pathway has been further stimulated by the observation that tumour-promoting agents such as phorbol esters, cause direct activation of PKC (Sekar and Hokin, 1986; Castagna, 1987; Isakov and Altman, 1987; Stabel et. al., 1987).

Evidence from other sources, however, indicates that involvement of PKC is not necessary in order for some growth factors to activate the Na^+/H^+ antiport (Vara and Rozengurt, 1985). Most notably, one PKC-independent pathway seems to involve tyrosine kinase activity (Cassel et. al., 1986; Grinstein and Rothstein, 1986), perhaps linking cytoplasmic alkalinisation with another group of growth factor receptors. Other mechanisms may involve calcium mobilisation (Muldoon et. al., 1987; Ober and Pardee, 1987), including the participation of the intracellular

Table 2. Examples of Growth Stimulatory Agents which Provoke Cytoplasmic Alkalinisation in Various Cell Types.

Agent	Cell Type	Reference
Thrombin and Insulin	ChineseHamster Lung Fibroblasts	Pouyssegur et. al., 1982
Epidermal Growth Factor (EGF)	Human Fibroblasts	Moolenaar et. al., 1983
Phorbol-12-myristate 13-acetate (PMA)	Rat Thymocytes	Grinstein et. al., 1985
Interleukin-2	Murine T-Cells	Mills et. al., 1985
Insulin and EGF	Swiss 3T3 Cells	Vara and Rozengurt, 1985
Platelet-Derived Growth Factor (PDGF)	NR6 Fibroblasts	Cassel et. al., 1986
Bombesin	Swiss 3T3 Cells	Mendoza et. al., 1986
12-0-tetradecanoylphorbol -13-acetate (TPA)	Human Fibroblasts	Muldoon et. al., 1987
Serum	Human Amelanotic Melanoma Cells	Musgrove et. al., 1987
EGF and Thrombin	Chinese Hamster Embryo Fibroblasts	Ober and Pardee, 1987

calcium-binding protein calmodulin (Jamieson and Villereal, 1987), cyclic AMP (cAMP) (Grinstein et. al., 1987) and osmotic effects (Grinstein et. al., 1986). (See Figure 5).

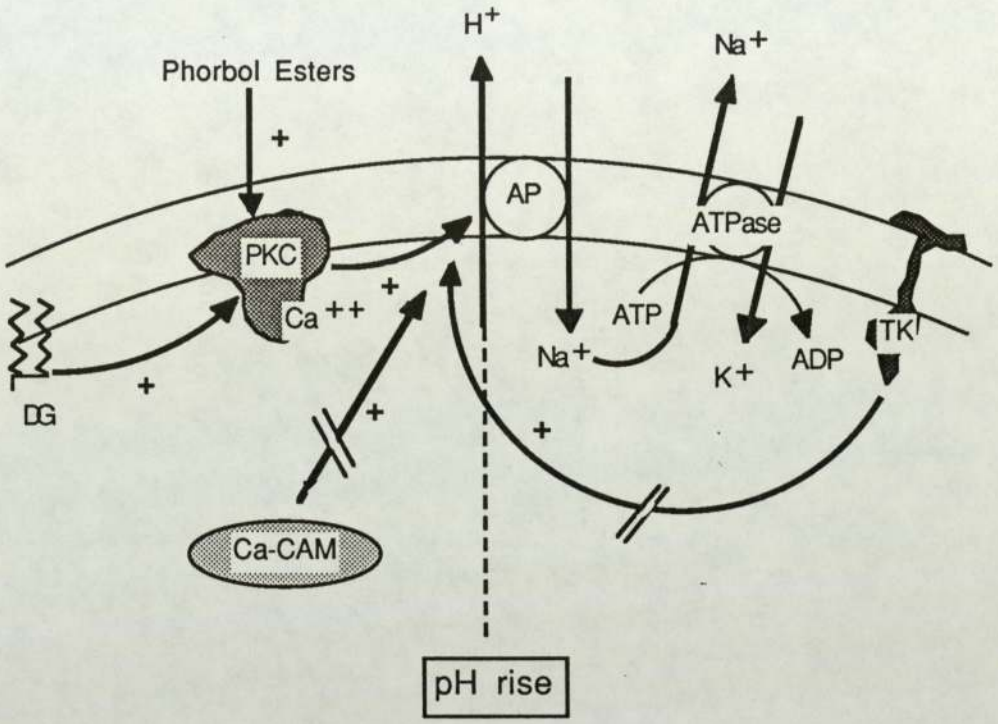
It is well known that a number of enzymes are sensitive to changes in pH, and may be activated or deactivated by changes in the pH of the environment of the order of magnitude observed after mitogen stimulation (0.15 - 0.25 pH units) (Metcalfe et. al., 1985; Chambard and Pouyssegur, 1986). Indeed, the key rate limiting enzyme of the glycolytic pathway, phosphofructokinase, is extremely pH sensitive (Trivedi and Danforth, 1966), and it has been suggested that the increased rate of glycolysis after growth factor stimulation may be caused by the rise in pH activating this enzyme (Moolenaar et. al., 1986). Intracellular pH may also control ribosomal protein S6 phosphorylation and protein synthesis (Chambard and Pouyssegur, 1986).

Although a rise in cytoplasmic pH seems to represent an obligatory event in the initiation of DNA synthesis, it is not sufficient alone for a maximum proliferative response to be generated (Vicentini and Villereal, 1986; Grinstein and Cohen, 1987). Other signals must act in concert to provide the appropriate triggers and optimum conditions for DNA replication and mitosis.

I.1.4.2. The Role of the Divalent Cations, Calcium and Magnesium.

Calcium ions (Ca^{++}) are recognised as important intracellular messengers, required by many cell types to regulate numerous physiological processess in response to various stimuli (Rasmussen and Barrett, 1984; Denton and McCormack, 1985; Alkon and Rasmussen, 1988). Under normal circumstances, the free calcium concentration of the extracellular environment ($[Ca^{++}]_e$) is about 10^4 times greater than that of the cytosol ($[Ca^{++}]_i$). There is thus a large inwardly directed

Figure 5. Possible Mechanisms of Activation of the Na^+ /H^+ Antiport, Leading to Cytoplasmic Alkalinisation after Mitogenic Stimulation.



Key:

Ca-CAM - Calcicalmodulin

AP - Antiport

TK - Tyrosine kinase (receptor)

\+ - Stimulation

Other Abbreviations as in Text

electrochemical gradient for Ca^{++}. In accordance with its function as a second messenger, to avoid inappropriate triggering of responses, and also because high $[Ca^{++}]_i$ is lethal for all cells (Farber, 1981; Schanne et. al., 1979), a number of mechanisms have evolved in order to maintain $[Ca^{++}]_i$ within narrow limits.

In order to mediate its second messenger function, Ca^{++} may enter the cytoplasm through plasma membrane channels; receptor operated channels (ROCs) (see section I.1.3.), voltage operated channels (VOCs) and second messenger operated channels (SMOCs). VOCs are particularly associated with electrically excitable cells, but have been found to be present in many other cell types (Carafoli, 1987; Meldolesi and Pozzan, 1987). Plasma membrane SMOCs are less well characterised, but their existence is inferred from experiments in which the Ca^{++} signal generated by an agent cannot be accounted for by ROCs, VOCs or intracellular redistribution of Ca^{++} (Putney, 1986; Meldolesi and Pozzan, 1987). The most well studied example of this phenomenon is linked to the metabolism of Ptd-Ins4,5P_2 (see section I.1.3.). It has been proposed that either phosphatidic acid (Putney et. al., 1981), which results from DG phosphorylation, or inositol 1,3,4,5-tetrakisphosphate (Ins1,3,4,5P_4) (Irvine and Moor, 1986; Zilberman et. al., 1987), derived from Ins 1,4,5P_3, are the second messengers linked to SMOC openning.

In addition to influx of Ca^{++} across the plasma membrane, its concentration in the cytoplasm can be increased by efflux from intracellular organelles, particularly the endoplasmic reticulum in non-muscle cells. Ca^{++} release from endoplasmic reticulum is thought to be brought about by the action of Ins 1,4,5P_3 (Streb et. al., 1983; Burgess et. al., 1984; Prentki et. al., 1984; Whitaker and Irvine, 1984).

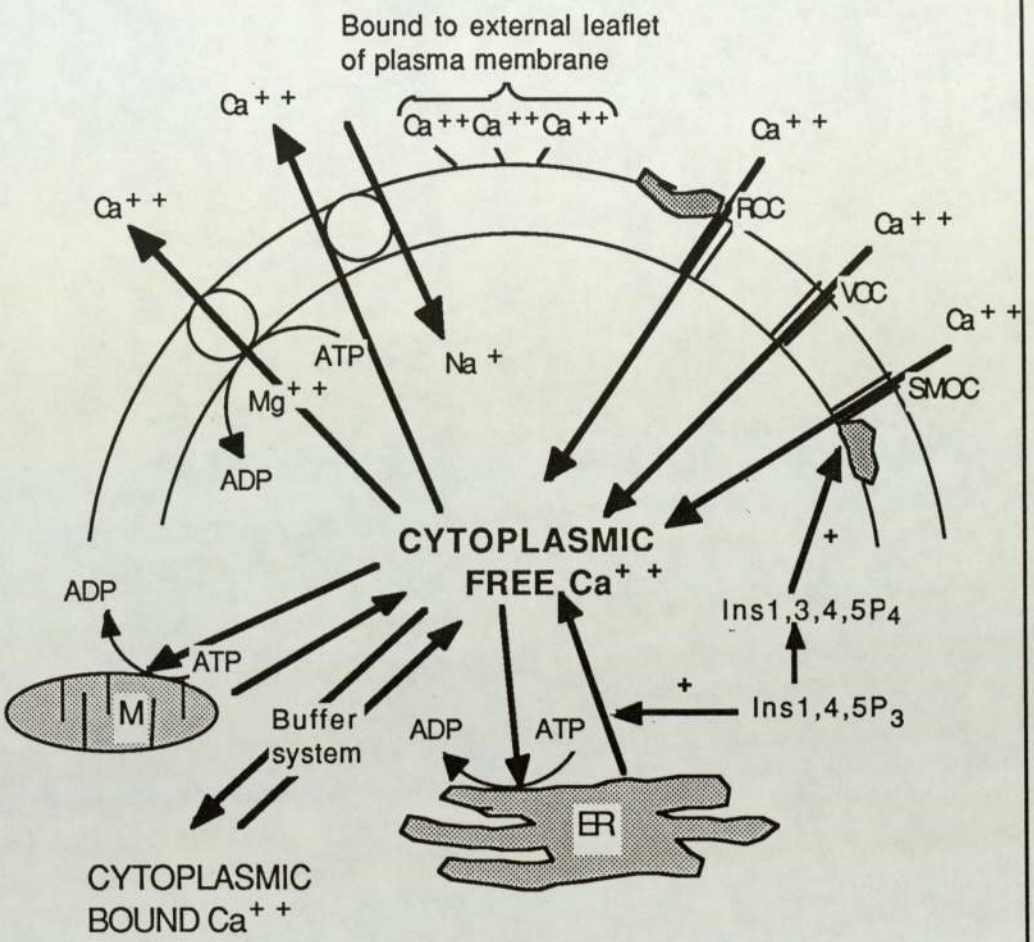
To balance the influx of Ca^{++}, there are other mechanisms which act to lower the $[Ca^{++}]_i$. The plasma membranes of most cells contain

electrogenic Ca^{++}/Na^{+} exchangers (Blaustein, 1974; Carafoli, 1987), whose directionality is governed by membrane potential and $[Na^{+}]_i$. There is also an ubiqitous, $[Ca^{++}]_i$-sensitive, calmodulin-activated, Mg^{++}-dependent plasma membrane Ca^{++}ATPase enzyme (Rasmussen and Barrett, 1984; Carafoli, 1987). Ca^{++} may also be sequestrated into the endoplasmic reticulum by an active process (Somlyo, 1984; Carafoli, 1987).

The role of mitochondria in maintenance of intracellular Ca^{++} homeostasis is controversial. Although they may both release and sequestrate Ca^{++} by a number of mechanisms (Carafoli, 1974; Denton and McCormack, 1985), it seems that this generally serves the purpose of maintaining their own special intraorganelle requirements, rather than being directly involved in regulation of $[Ca^{++}]_i$ (Somlyo, 1984; Denton and McCormack, 1985; Carafoli, 1987). $[Ca^{++}]_i$ may also depend to some extent on the buffering capacity of various intracellular Ca^{++}-binding proteins (Carafoli, 1987), and proportions of intracellular and externally bound cations (Sanui and Rubin, 1982; Vidair and Rubin, 1982). It may also be noted that local changes in $[Ca^{++}]$ in specific regions of the cytoplasm may be sufficient to generate responses even when overall $[Ca^{++}]_i$ remains unchanged. Figure 6 shows a model of mechanisms involved in the maintenance of intracellular Ca^{++} homeostasis.

There is a large body of evidence supporting the involvement of Ca^{++} signals in control of proliferation both *in vivo* and *in vitro* . The onset of proliferation is preceded by an obligatory requirement for Ca^{++} in many cell types including hepatocytes (Whitfield et. al., 1980), 3T3 cells (Paul and Ristow, 1979) and murine B lymphocytes (Dennis et. al., 1987). Generally, drastic reduction in $[Ca^{++}]_e$ arrests normal cells at the G_1/S boundary of the cell cycle (Paul and Ristow, 1979; Tupper et. al., 1980; Whitfield et. al., 1980), providing strong circumstantial evidence of a role for Ca^{++} in the proliferative response. Interestingly, this requirement is relaxed in many

Figure 6. A Model of Cellular Calcium Homeostasis Showing Main Calcium Compartments and Routes of Calcium Fluxes.



Key:

M - Mitochondion

ER - Endoplasmic reticulum

Other abbreviations as in text

cases of neoplastic growth (Boynton and Whitfield, 1976; Swierenga et. al., 1980). Studies using radioactively labelled $^{45}Ca^{++}$ and fluorescent Ca^{++} indicator dyes show Ca^{++} fluxes across cell membranes, and a sustained rise in $[Ca^{++}]_i$ after mitogenic stimulation in numerous cell types including 3T3 cells (Morris et. al., 1984; Pandiella et. al., 1987), human peripheral blood lymphocytes (Deutsch and Price, 1982; Komada et. al., 1987), human fibroblasts (Mix et. al., 1984; Moolenaar et. al., 1984), and T lymphocytes (Kimoto et. al., 1983; Gukovskaya et. al., 1986; Imboden and Weiss, 1987). These effects are observed after stimulation with many mitogenic agents acting on appropriate cell types including EGF (Moolenaar et. al., 1986), PDGF (Frantz, 1985), interleukins (Rossio et. al., 1986) and vasopressin (Mendoza et. al., 1986), and may represent an obligatory event in the proliferative process. Although it seems that there are two sources of trigger Ca^{++}, the endoplasmic reticulum and the extracellular environment (Metcalfe et. al., 1985; Moolenaar et. al., 1986; Lopez-Rivas et. al., 1987), it is unlikely that the same mechanism of Ca^{++} mobilisation is utilised in all cases. Some agents, such as PDGF (Rozengurt and Mendoza, 1986) appear to act via $Ins1,4,5P_3$ generation and the inositol lipid pathway, whereas others, such as EGF, acheive the same result by an unknown mechanism which does not involve the hydrolysis of phosphoinositides (Moolenaar et. al., 1986; Pandiella et. al., 1987).

It seems that at least part of Ca^{++}'s involvement in the proliferative process may be mediated by the Ca^{++}-binding protein calmodulin (CAM) (Klee et. al., 1980). There are a number of different Ca^{++}-binding proteins synthesised in different cell types (Dedman, 1986; Mathew et. al., 1986), but unlike others, CAM seems ubiquitous, lacking species or tissue specificity (Wallace et. al., 1982). Its functions are wide ranging, and include direct activation of effector systems such as Ca^{++}ATPase (Means

and Dedman, 1980) and indirect effects on regulatory systems via its interactions with protein kinases and phosphodiesterases (Cheung, 1980). Studies using CAM inhibitors (Durkin et. al., 1983) and measurement of CAM levels through the cell cycle (Criss and Kakiuchi, 1982; Rasmussen and Means, 1987), indicate an involvement of CAM in the initiation of DNA synthesis.

There is no plasma membrane chemical gradient for magnesium ions (Mg^{++}), as there is for Ca^{++}. They are however required as cofactors by a large number of membrane-bound and cytoplasmic enzymes, including Ca^{++}ATPase and AC (see this section and section I.1.3.), and are an absolute requirement for cell proliferation (Günther, 1981 and 1986; Walker, 1986). It has been proposed that small fluctuations in $[Mg^{++}]_i$, which may occur after mitogen stimulation, can alter the kinetics of many enzymes which collectively determine the overall rate of cell proliferation (Rubin, 1975; Terasaki and Rubin, 1985). However, although it has been shown that elevated $[Mg^{++}]_i$ is needed to support high rates of normal cell proliferation (Cameron et. al., 1980), it is difficult to determine a critical threshold level of $[Mg^{++}]_i$ necessary for growth.

Although the rate of proliferation of various cell types is dependent on the availability of extracellular Mg^{++} (Sanui and Rubin, 1976; Rubin, 1982; Walker, 1986), it is more sensitive to the availability of extracellular Ca^{++} (Abboud et. al., 1985). Furthermore, the reduced lectin stimulation of lymphocytes from Mg^{++}-deficient rats (Günther and Averdunk, 1979), has been shown to result from altered Ca^{++} metabolism in a low Mg^{++} environment (Averdunk and Günther, 1985; Vormann and Günther, 1987). It therefore seems probable that although adequate Mg^{++} is a prerequisite for normal proliferative control, it subserves a 'housekeeping', rather than a 'triggering' function.

I.1.4.3. The Role of the Cyclic Nucleotides cAMP and cGMP.

cAMP is an important intracellular messenger, mediating the functions of numerous hormones and other agents (Robison et. al., 1971; Rasmussen and Barrett, 1984). (See Figure 3, section I.1.3., for its mechanism of formation, and aspects of its metabolism).

For some time cAMP was considered to be an inhibitory agent in the passage of G_0 cells into the cell cycle (Kran et. al., 1973; Pastan et. al., 1975; Parker, 1976). More recently, however, this view has been challenged, and cAMP is now considered to be a stimulatory agent in some systems such as hepatocytes (Swierenga et. al., 1980; Boynton et. al., 1981) and Swiss 3T3 cells (Rozengurt et. al., 1981; O'Neill et. al., 1985; Yamashita et. al., 1986). Indeed, Rozengurt and co-workers (1985 and 1986) propose a central role for cAMP in their unified hypothesis of growth control in 3T3 cells. The authors divide agents which provoke entry into the cell cycle of quiescent 3T3 cells, into two groups. One group acts through PKC and initiates a series of ion fluxes, and the other group elevates [cAMP]i. Only when both pathways are activated is a potent mitogenic effect observed. Some agents, such as PDGF, activate both pathways, and provoke a maximum response in the absence of other agents. Synergistic interactions are observed however, when an agent which activates PKC is added to cultures in combination with an agent which elevates [cAMP]i. It seems therefore, that cAMP plays an important role in the proliferation of 3T3 cells, and perhaps of other fibroblasts.

The role of cAMP in lymphocyte proliferation is, however, more controversial. Addition of cAMP analogues, or agents which raise $[cAMP]_i$, have been shown to have an inhibitory effect on the proliferation of B-lymphocytes (Blomhoff et. al., 1987) and human blood lymphocytes (Kaever and Resch, 1985). Other evidence, however, suggests that dibutryl cAMP may synergise with other growth stimulatory agents

in activating guinea pig lymphocytes (Otani et. al., 1987). However, these results are not directly comparable, since different agents were used at different concentrations. Another approach is to measure [cAMP]i, after stimulation with various agonists. T-lymphocyte treatment with interleukin-2 (IL-2), has been shown to decrease (Beckner and Farrar, 1986), or increase (Wickremasinghe et. al., 1987) [cAMP]i, in mouse and human cells respectively and the T-lymphocyte mitogen concanavalin A (ConA), produces a modest increase in [cAMP]i. However, high doses of ConA, which may inhibit the proliferative process, produce the greatest enhancement of [cAMP]i (Hadden et. al., 1979), and succinyl ConA stimulates the cells without raising [cAMP]i (Kaever and Resch, 1985).

Clearly, further work is necessary before a definitive statement can be made on the role of cAMP in the proliferation of lymphocytes and other cell types. Nevertheless, some of this conflicting evidence may be explained by the suggestion that cAMP may serve both stimulatory and inhibitory functions, at different phases of the cell cycle (O'Keefe and Pledger, 1983; Smets and Van Rooy, 1987). For example it seems to be positively involved in the acquisition of competence (G_0/G_1 transition), but antagonistic during the progression phase (G_1/S transition) (Smets and Van Rooy, 1987).

cAMP and cGMP often have reciprocal effects on controlling cell function, and it has been suggested that they act in a 'yin-yang' fashion, where the ratio of the two molecules is of importance (Pastan et. al., 1975). More recent evidence, however, implies that the situation may be rather more complicated (Houslay, 1985). For example, phorbol esters have been shown to increase $[cGMP]_i$ in a variety of cell types (Zwiller et. al., 1985), but may either increase or decrease $[cAMP]_i$ depending on the tissue (Heyworth et. al., 1985; Sugden et. al., 1985).

The role of cGMP in cellular proliferation is even more difficult to interpret than that of cAMP. Some studies indicate that an increase in $[cGMP]_i$ is associated with the initiation of DNA synthesis (Seifert and Rudland, 1974; Armato et. al., 1981), others imply a reduction in $[cGMP]_i$ is correlated with the same event (Macmanus et. al., 1978), and yet others indicate that it has no effect (Kaever and Resch, 1985). However, on balance, at least for the lymphocyte model, it may be that initiation of DNA synthesis is associated with a rise in $[cGMP]_i$ (Hadden et. al., 1979) and that the cGMP-dependent protein kinase is involved in the proliferative process (Largen and Votta, 1983), though the precise roles of these agents remains somewhat obscure.

I.1.4.4. Integration of Proliferative Signals and Summary.

Although each proposed second messenger has been treated separately, this is entirely for simplicity of presentation. Physiologically, the intracellular signalling pathways are functionally interlinked at all levels (see Lichtstein and Rodbard, 1987). Hence growth factor receptors linked to PLC activate both the PKC and Ca^{++}-CAM pathways (Berridge, 1987a. and b.), and the cAMP and Ca^{++} second messenger systems are intimately intertwined in various ways. For example, a rise in $[Ca^{++}]_i$, acting through CAM-associated phosphorylations, may increase or decrease $[cAMP]_i$ in different cells, by activating AC, and either increasing or decreasing cAMP-phosphodiesterase activity, depending on the cell type and/or other prevailing circumstances (Rasmussen and Barrett, 1984; Erneux et. al., 1985; Alkon and Rasmussen, 1988). In addition, arachidonic acid release is associated with DG metabolism (Berridge, 1984), and this molecule may a) activate guanylate cyclase (Takai, et. al., 1982) or b) serve as a precursor of prostaglandin E_1, which may function in an autostimulatory manner to increase AC activity (Rozengurt et. al., 1983).

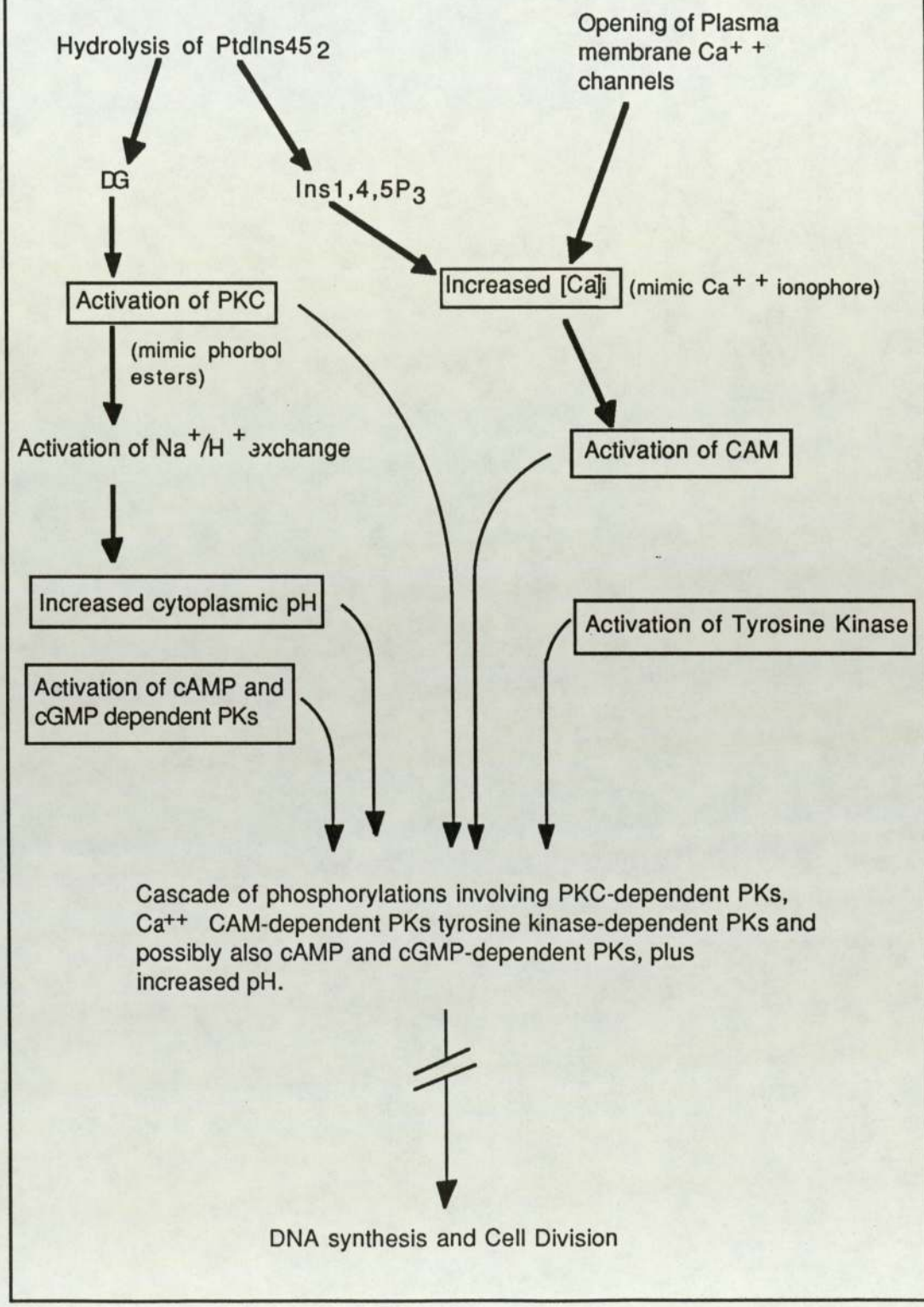
Also, PKC has been shown in different systems to both inhibit (Katada et. al., 1985; Williams et. al., 1987) and to enhance (Rozengurt et. al., 1987; Yoshimasa et. al., 1987) intracellular cAMP accumulation. There are many other examples of 'cross-talk' between cellular signalling pathways.

The networks of responses may also overlap at the level of effector molecules further along the pathway(s) to DNA synthesis. Phosphorylation/ dephosphorylation of proteins has long been established as a major mechanism for cellular regulation (Krebs, 1985; Espinal, 1986; Taylor, 1987), and growth factor activated pathways can be traced to the activation of a number of protein kinases (PKs) including the serine/threonine kinases PKC, cAMP and cGMP-dependent PKs and Ca^{++}CAM-dependent PKs, and the tyrosine kinases associated with a number of growth factor receptors (see section I.1.3.). It is increasingly recognised that a complex cellular response, such as the initiation of proliferation, requires the synergistic interaction of complementary signals and sequences of events at a number of levels which probably include protein phosphorylation (Rozengurt and Mendoza, 1986; Lockwood et. al., 1987; Whitfield et. al., 1987).

A definitive model of the processes involved in the initiation of DNA synthesis is as yet impossible to construct, but Figure 7 summarises some of the major mechanisms which current evidence implies may be important in this respect. All mechanisms are not neccesarily activated by any given agonist, and synergy between growth factors may be generated by the triggering of different pathways.

Generally, growth factor action seems to be associated with increases in $[Ca^{++}]_i$ and the activation of CAM, increases in cytoplasmic pH, sometimes associated with PKC activation, tyrosine kinase activity, and in some cases, surges of cyclic nucleotides.

Figure 7. Some Important Events in the Initiation of DNA Synthesis in Normal Cells.



Later in the process, 15 -20 minutes after the initial stimulation, many authors have noted an increase in mRNA transcription of the c-fos (Greenberg and Ziff, 1984; Morgan and Curran, 1986; Marx, 1987) and c-myc (Kaibuchi et. al., 1986; Bravo et. al., 1987) proto-oncogenes. Evidence is accumulating that the products of these genes may be involved in proliferation (Greenberg and Ziff, 1984; Alitalo et. al., 1987; Marcu, 1987). However, they may be regulated by separate signalling mechanisms (Kumagai et. al., 1987; McCaffrey et. al., 1987), and it has been suggested that their expression may be a regulatory signal associated with growth factor pathways which act via PKC, rather than an obligatory event in the intiiation of DNA synthesis (Rozengurt and Sinnett-Smith, 1987).

I.2. The Role of Insulin and the Insulin-Like Growth Factors in Growth Control.

I.2.1. Insulin and Its Role in Metabolism.

Insulin is a polypeptide hormone of 51 amino acids secreted by the β cells of the islets of Langerhans in the pancreas. It consists of two disulphide-linked peptide chains, the A and B chains. It is best known for its role as the 'anabolic hormone', since it stimulates synthetic pathways while inhibiting degradative ones, and has diverse effects on carbohydrate, protein and fat metabolism (Denton, 1986). Liver, muscle and fat tissue are probably the major physiological targets of insulin (Cahill, 1971), but most other cell types also exhibit some responsiveness to insulin.

Cellular responses to insulin are sometimes divided into acute metabolic and chronic growth promoting effects. The time of onset of acute metabolic effects is rapid (seconds to minutes after insulin binding) and is observed at low insulin concentrations (0.1 - 1.0 nM) (Coppock et. al., 1980; King et. al., 1980). Examples of acute metabolic effects include activations of transport systems including those for glucose, amino acids and ions, activations of enzyme systems including glycogen synthase, pyruvate dehydrogenase and acetyl CoA carboxylase and inhibitions of enzyme systems such as phosphorylase kinase and triacylglycerol lipase. (For reviews see Cahill, 1971; Czech, 1980; Montague, 1983; Denton, 1986). At the molecular level, many of the enzyme systems modulated by insulin undergo phosphorylation/dephosphorylation in response to the agonist. For example, acetyl CoA carboxylase (Brownsey and Denton, 1982) and ATP-citrate lyase (Alexander et. al., 1982) are phosphorylated in response to insulin, whereas glycogen synthase (Sheorain et. al., 1982) and pyruvate dehydrogenase (Hughes et. al., 1980) are

dephosphorylated. It can thus be seen that insulin has a wide spectrum of acute metabolic effects, and probably also a variety of mechanisms of action. For the purposes of this study, however, only the enhancement of the transport of glucose and amino acids need be briefly analysed a little further.

It has been known for nearly 40 years that insulin increases glucose transport into cells (Levine et. al., 1949). In the target tissues muscle and fat, insulin can lead, within minutes, to up to 15 fold increases in the rate of glucose uptake (Czech, 1980; Hom and Goodner, 1984; Klip et. al., 1986). The glucose transport systems which respond to insulin are of the facilitated diffusion type while Na^+-dependent/active glucose transporters appear not to be sensitive to insulin (Oka and Czech, 1985; Simpson and Cushman, 1986). Most analyses of the phenomenon have been undertaken using the adipocyte model, but glucose uptake is enhanced in other tissues including muscle cells, (Hom and Goodner, 1984), lymphocytes (Helderman, 1981) and fibroblasts (Germinario and Oliveira, 1979; Tupper and Smith, 1985).

The stimulatory action of insulin occurs through a change in maximum rate (V_{max}) of glucose transport, in the absence of a significant change in the apparent affinity (K_m) of the transporter for glucose (Czech, 1980; Glieman and Rees, 1983; Toyoda et. al., 1987). Kinetic analysis alone cannot, however, distinguish between an increased number of transporters in the cell membranes of insulin-stimulated cells, or an increased activity of the same number of transporters. Independent observations in two laboratories, using two different methods have suggested that an increase in the number of glucose transporters in insulin-treated cells is of prime importance.

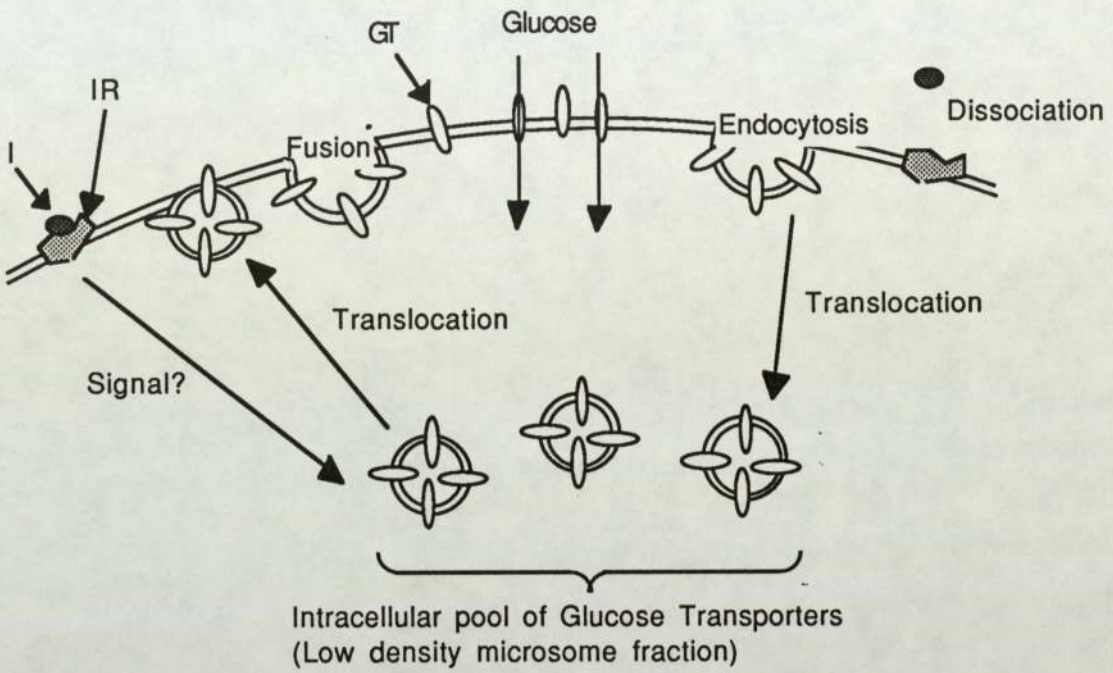
Firstly, Cushman and Wardzala (1980) developed a technique for assaying glucose transporter concentration in plasma membranes, and

other subcellular fractions of control and insulin-treated cells, using cytochalasin B. Cytochalasin B is a potent competitive inhibitor of glucose transport, which binds specifically to glucose transporters. Its binding to the different subcellular fractions obtained from control and insulin-treated cells, quantitatively reflects the distribution of glucose transporters. Secondly, Kono and colleagues (Suzuki and Kono, 1980; Kono, 1984), refined the technique for solubilising glucose transporters and then assayed their activities by following glucose transport after reconstitution of the transporters into lecithin liposomes. Results from both these and other sources have led to the proposal that insulin action results in the mobilisation or recruitment of intracellular transporters from a low density microsome fraction to the plasma membrane, where they operate to transport hexoses (Czech, 1984; Kono, 1984; Simpson and Cushman, 1986; Baly and Horuk, 1987). (See Figure 8).

In addition to enhancing glucose uptake, insulin also increases the rate of amino acid uptake into cells. A number of techniques have shown that there are distinct transporters in the plasma membrane for different amino acid subtypes (Christensen, 1978; Guidotti et. al., 1978). Substrate recognition by receptor types of the various systems is however incomplete, and a specific amino acid may be transported into the same cell by two or three types of transporter (Christensen, 1978). A stimulatory effect of insulin on amino acid uptake has been reported for a number of cell types including fibroblasts (Knight et. al., 1981; Longo et. al., 1985; Tupper and Smith, 1985), lymphoid cells (Goldfine et. al., 1972; Kwock et. al., 1976), fat cells (Touabi and Jeanrenaud, 1969), muscle cells (Guidotti et. al., 1976) and hepatocytes (Kletzien et. al., 1976). These studies indicate that insulin regulation is predominantly or entirely due to its effect on the activity of the so-called transport

Figure 8. The Translocation Hypothesis for the Insertion of Glucose Transporters into the Plasma Membrane in Response to Insulin.

(Modified from Simpson and Cushman, 1986)



Key:

I - Insulin

IR - Insulin Receptor

GT - Glucose Transporter

system A. System A is Na^+-dependent and sensitive to metabolic inhibitors and pH. It can generate steep concentration gradients between the intracellular and extracellular compartments, and is at least partially energised by the electrochemical gradient for Na^+ (Guidotti, 1978). Under normal conditions, it is system A which transports the amino acid analogue α-aminoisobutyric acid (AIB).

Insulin interaction with its receptor has been found to be temporally separated from its maximal effect on amino acid transport (Goldfine et. al., 1972; Hollenberg and Cuatrecasas, 1975), and it has been suggested that insulin has a dual action on system A (Elsas et. al., 1971; Guidotti et. al., 1974). These authors have proposed that insulin may act relatively quickly at the level of the cell membrane to protect transport proteins from degradation or inactivation, and more slowly to enhance the rate of synthesis of these proteins, and their insertion into the plasma membrane.

Although insulin acts to increase the numbers of membrane transporters for both glucose and amino acids, the mechanisms whereby these effects are achieved are quite different. The number of glucose transporters is increased by insertion into the membrane of preformed transporters from an intracellular pool. The number of amino acid system A transporters is increased by protecting existing transporters from degradation, and more slowly, by synthesizing more transporters.

The effects of insulin on hexose and amino acid transport in classical target tissues are well established, and can account for many of its effects on metabolism. A possible explanation of insulin's growth stimulatory action is that it stimulates these, and other anabolic processes, and that this metabolic enhancement has a positive effect on growth. This 'Metabolic Regulation Hypothesis', however, is unlikely

to be the total explanation for the action of insulin on cell division, largely because the dose response curves for metabolic and growth effects generally display very different properties, even in the same cell type (King and Kahn, 1985; Straus, 1981). This will be explained more fully after a brief description of the growth-promoting effects of insulin.

I.2.2. Insulin and Control of Growth.

Several lines of evidence implicate insulin as a regulator of growth *in vivo* , especially during foetal development. Infants born to diabetic mothers with hyperglycaemia often exhibit excessive body weight and size. This has been attributed to the growth promoting effects of the high levels of insulin in the foetal circulation, which arise in response to the elevated blood glucose levels of the mother (Susa et. al., 1979). The reverse of this syndrome also occurs in infants with insulin deficiency. They have low birth weights, decreased fat deposition and deficient muscle development (Hill, 1978).

Other effects of insulin on normal growth and development are primarily surmised from abnormalities associated with hyperinsulinaemia. Hence numbers of insulin receptors, affinity of receptors and post-receptor defects may contribute to the heterogenous syndrome of leprechaunism (Kobayashi et. al., 1978; D'Ercole et. al., 1979; Taylor et. al., 1982; Podskalny and Kahn, 1983). Studies of the effects of any factor *in vivo* , are however, always difficult to interpret because of the simultaneous presence of numerous other factors which may interact in various ways. More precise descriptions of the possible role of insulin in growth control can therefore be ascertained from *in vitro* studies.

Gey and Thalhimer (1924) first showed the ability of insulin to promote growth of chick embryo fibroblasts in culture. Subsequently,

insulin has been shown to stimulate DNA synthesis and proliferation *in vitro* of many cell types under a variety of experimental conditions (See Table 3). Insulin acts on G_0/G_1 arrested cells, limited for serum (Holley and Kiernan, 1974) or essential nutrients (Kamely and Rudland, 1976), and is required for normal growth of most cell types in culture (Barnes and Sato, 1980).

Although insulin alone can stimulate DNA synthesis, it has generally been found to have a rather weak effect compared to the effect of whole serum (Temin, 1967; Griffiths, 1972; Dicker and Rozengurt, 1978). The presence of insulin has however been shown to decrease the concentration of serum (Temin, 1967), or other growth factors (Jimenez de Asua et. al., 1977; Dicker and Rozengurt, 1978), required to achieve maximum stimulation of proliferation. This 'serum-sparing' effect of insulin and its ability to synergise with other growth factors has been observed in numerous systems (See Straus, 1984 and King and Kahn, 1985), but it has been most extensively studied in 3T3 cell lines. The two major unified hypotheses of growth control in these cells indicate important possible roles for insulin.

Firstly, Rozengurt and colleagues have proposed a central role for insulin in their theory of the early signals leading to proliferation in Swiss mouse 3T3 cells (see Section I.1.4.3.). Briefly, it is proposed that the synergistic effects of specific combinations of growth factors is a consequence of their membership of one of two classes of factors. One class share the ability to increase $[cAMP]_i$, and the other class activates PKC and enhances various ion fluxes. Any member of the first class in combination with any member of the second class, produces maximal stimulation of DNA synthesis. A few substances, including PDGF and FDGF, activate both pathways, and achieve maximal stimulation in the absence of other agents. Insulin however, produces maximal

Table 3. Examples of Cell Types in which Insulin has been shown to Exert a Growth-Stimulatory Effect.

Cell Type	Reference
Murine T Lymphocytes	Snow et. al., 1980
Rat myoblasts	Crace et. al., 1984
Rat Epithelial Cells	McKeehan et. al., 1984
AKR-2B Cells (derived from mouse embryos)	Shipley et. al., 1984
Mouse Mammary Gland	Zwierzchowski et. al., 1984
Human Fibroblasts	Conover et. al., 1985
Swiss 3T3 Cells	Talha and Harel, 1985
Hepatoma Cells	Lauris et. al., 1986
Transformed BHK21/C13 Cells	Kamei, 1987
Mouse Lens Epithelial and 3T3 Cells	Reid and Reid, 1987
Dog Thyrocytes	Roger et. al., 1987
Rat Hepatocytes	Sand and Christoffersen, 1987

stimulation when added to cultures in combination with a member of either class of factors. Rozengurt and colleagues offer no explanation for this unique, and superficially anomalous ability of insulin, to replace either agents which raise $[cAMP]_i$ or agents which activate PKC, in their model. If insulin activated both pathways, it would be expected to promote maximal stimulation of DNA synthesis when added to cultures alone, and it fails to achieve this effect. However, the mechanism of insulin action, and the identity of the intracellular signal(s) produced on insulin binding remain largely unknown (see Section I.2.5.). It is possible that insulin activates neither of the pathways specified in the 'dual control' model of Rozengurt and colleagues, but nevertheless provokes cellular responses which bypass each of them, under specific circumstances. For example, although insulin does not activate PKC, it does provoke the redistribution of various ions across the plasma membrane (see Section I.2.5.1.). Provided $[cAMP]_i$ is elevated by a synergistic factor, this effect may be sufficient to 'tip the balance' between a cell which remains quiescent and a cell which enters the cell cycle.

Secondly, a role for insulin is also specified in the competence/progression theory for BALB/c 3T3 cells, of Pledger and colleagues. (see Section I.1.2.). Briefly, this scheme also classifies growth factors into two groups, and a member from each group, added in combination or specific temporal sequence, produces synergistic effects on proliferation. The first class of factors, competence factors, are required for entry of G_0 arrested cells into G_1. The second group, progression factors, which includes insulin, are required for the passage of competent cells through G_1 and into S phase of the cell cycle.

In contrast to the acute metabolic effects of insulin, which are observed at 'low' (0.1 - 1.0nM) insulin concentrations, these growth

promoting effects are generally observed at high (0.1 - 1.0μM) insulin concentrations, and require hours or days to be manifested (Straus, 1984; Kahn, 1985; King and Kahn, 1985).The requirement for supraphysiological insulin concentrations has raised a number of possibilities. Of these, it has been convincingly shown that the growth promoting actions of insulin are not as a result of any contaminants of insulin preparations (Petrides and Böhlen, 1980), nor is insulin degraded sufficiently during the course of experiments to account for the 100 - 1000 fold discrepancy in concentration required to exert its growth-promoting and metabolic properties (King and Kahn, 1981). It has been suggested that different portions of the molecule may be involved in mediating the growth-promoting and metabolic functions of insulin (King and Kahn, 1981), and that it may exert its growth-promoting effects by binding with low affinity to a receptor for another growth factor (Straus, 1984 ; King and Kahn, 1985).

However, before examining this last possibility in greater detail in a subsequent section, it is of note that there are important exceptions, where insulin stimulates DNA synthesis at low, physiological concentrations. Examples where this is the case include a rat hepatoma cell line (Koontz and Iwahashi, 1981), normal pericytes from bovine retinal capillaries (King et. al., 1983b) and human mammary tumour cells (Osborne et. al., 1978).

I.2.3. The Insulin-Like Growth Factors and Growth Control.

The Insulin-like growth factors (IGFs) form two groups of polypeptides with insulin-like biological activity (Hall and Sara, 1983; Perdue, 1984; Froesch et. al., 1985). Three separate accounts of their discovery are available, from workers pursuing different lines of research. Firstly Salmon and Daughaday (1957) made the observation that growth hormone cannot stimulate the incorporation of ^{35}S into

incubated cartilage *in vitro* . They found that its ability to do so *in vivo* is due to its capacity to induce the production of other mediator factors, the somatomedins. Secondly, Temin and colleagues (Pierson and Temin, 1972), were investigating the macromolecular constituents of serum which are required for cell growth in culture. They found that a material extracted from medium conditioned by rat liver cells had both insulin-like and somatomedin-like activity (Dulak and Temin, 1973). They called this factor Multiplication Stimulating Activity (MSA). The third route to the discovery of IGFs stems from an entirely different observation. Froesch and colleagues were analysing the ability of serum, in which insulin had been inactivated by anti-insulin antibodies, to mediate glucose oxidation and lipogenesis (Froesch et. al., 1963). This is the so called non-suppressible insulin-like activity (NSILA). Purified NSILAs have since been shown to consist of two biologically active peptides IGF-I and IGF-II (Rinderknecht and Humbel, 1976).

The three types of factor; somatomedins, MSAs and NSILAs have been found to represent the same two molecules, or homologues of these molecules in different species (Herington and Kuffer, 1983; Spencer et. al., 1983; Zapf, 1983). I shall call these factors insulin-like growth factors I and II (IGF I and IGF II).

The genes coding for both IGF I and IGF II have been located (Tricoli et. al., 1984), and the processes, including mRNA production and splicing and proteolytic cleavage, leading to production of IGF I have been studied (Jansen et. al., 1983; Rotwein, 1986; Rotwein et. al., 1986). The chemical structures of IGF I and IGF II have been elucidated by Rinderknecht and Humbel (1978 a and b). They contain 70 and 67 amino acids respectively and are 62% homologous in primary amino acid structure. They both also display sequence homologies with the

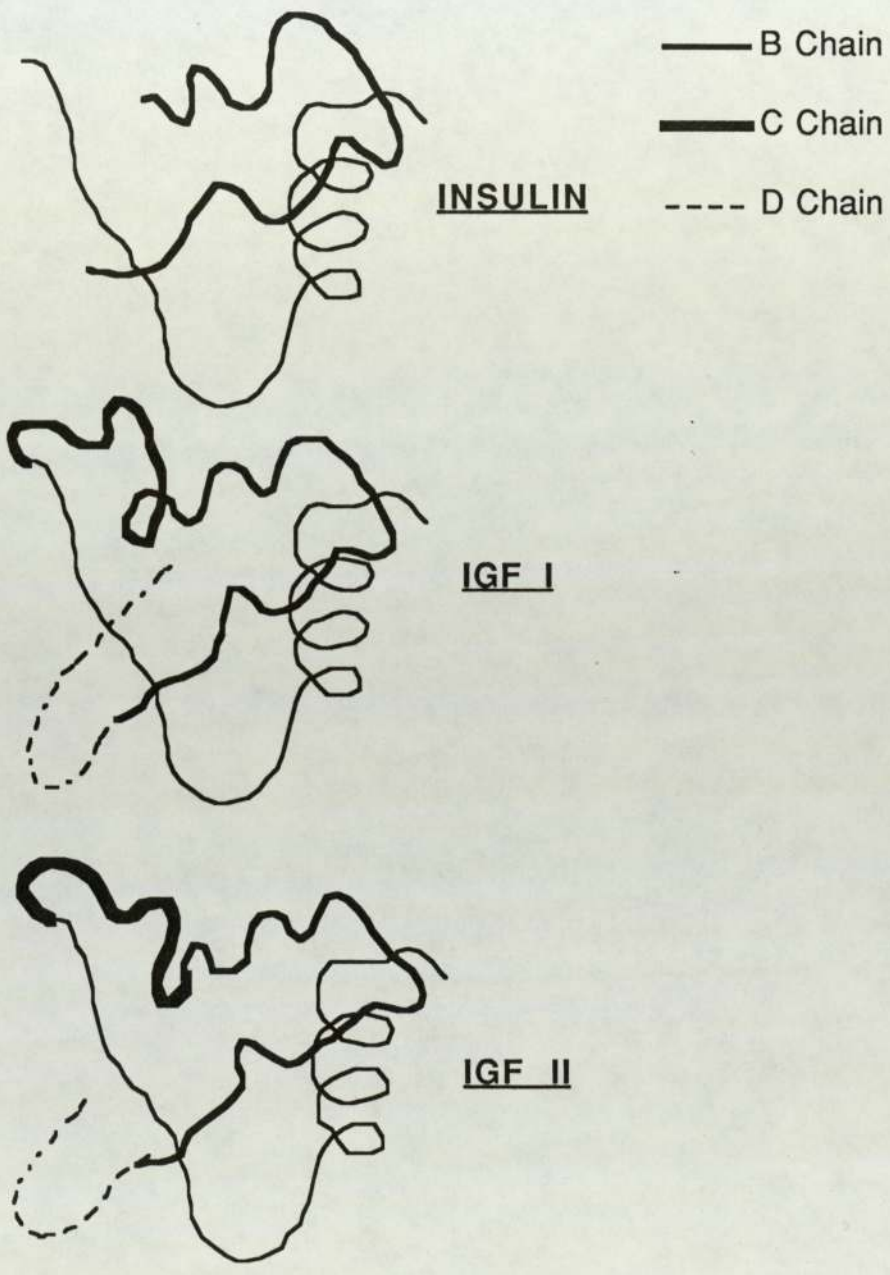
insulin molecule, but are sufficiently different in structure to be separable with certain antibodies (Rinderknecht and Humbel, 1976; Van Wyk and Underwood, 1978). Both IGF I and IGF II have A and B chains, but in contrast to insulin, they retain a connecting C peptide. They also have an additional D chain on the carbon terminal of the A chain (See Figure 9).

In the adult, circulating IGFs are produced largely in the liver, but may originate from other tissues to a lesser degree (Zapf et. al., 1981). In the foetus, all cells may produce IGFs (Hall and Sara, 1983), and autocrine stimulation of growth by both IGF I and IGF II has been observed in rat medullary carcinoma cells (Höppener et. al., 1987). It seems that they may act as autocrine, paracrine (eg. in nervous tissue, Hossenlopp et. al., 1986), and endocrine hormones in different circumstances.

Hepatic production of IGF I seems to be largely controlled by the level of growth hormone (GH), which acts by regulating the expression of the IGF I gene (Froesch et. al., 1985; Mathews et. al., 1986). Other hormones may also have regulatory roles however, including somatostatin, which decreases IGF I production (Bass et. al., 1986), thyroid hormones, which stimulate production and sex steroids, whose effects are stimulatory or inhibitory depending on the dose (Furlanetto, 1983). Unlike the situation for most other polypeptide hormones, including insulin, very little or no preformed IGFs are stored in the cells where they are produced (Schwander et. al., 1983). Instead their synthesis and rapid secretion parallel those of albumin from the same liver cells (Froesch et. al., 1985). Circulating concentrations of IGFs can reach levels of over 10^{-7}M (Hall and Sara, 1983), but less than 1% of this seems to be free in the circulation, the remainder being bound to specific IGF binding-proteins (Rinderknecht and Humbel, 1976; Hintz

Figure 9. Schematic Diagrams of Insulin, IGF I and IGF II to show the Structural Relationships.

(Modified from Honegger and Blundell, 1983)



and Liu, 1983; Carlsson-Skwirut et. al., 1987). The source of the binding proteins is also the liver (Schwander et. al., 1983), and it has been suggested that under some circumstances, their presence may enhance biological responses to IGF I (Elgin et. al., 1987).

It has been shown that IGFs are capable of mimicking all of the biological activities of insulin on metabolism and growth (Zapf et. al., 1978; Froesch et. al., 1985). The major difference between IGFs and insulin is in the dose response curves for the various processes. Hence, generally, IGFs stimulate DNA synthesis at low concentrations, whereas the concentrations required for metabolic enhancement are high (King and Kahn, 1981). As discussed previously, the reverse is generally true for insulin. Also, as for insulin, there are exceptions, where low concentrations of IGFs may enhance metabolic processes (Parkes et. al., 1986).

A role for IGFs as growth regulatory factors *in vivo* is supported by various lines of evidence. Hence acromegalic patients have high IGF I levels (Zapf et. al., 1981), and pituitary dwarfs have low IGF I levels which rise to normal on treatment with GH (Merimee et. al., 1982). The levels of IGF I parallel growth in these cases. In order to define the roles of GH and IGFs, the hypophysectomised rat is the experimental animal most often used. Most evidence supports the 'somatomedin hypothesis' alluded to previously, whereby IGFs act as mediators of the growth promoting action of GH. IGF I, however, being more potent than IGF II in this respect (Fryklund et. al., 1974; Clemmons and Van Wyk, 1981; Froesch et. al., 1985; Schoenle et. al., 1985). The finding by Isaksson et. al., (1982) that local injection of GH into the cartilage plates of hypophysectomised rats increases cartilage proliferation does not necessarily contradict this hypothesis since there may be local production of IGFs in response to GH (Skottner et al., 1986), or other

'priming' effects (Zezulak and Green, 1986). Another convincing item of evidence indicating that IGF I rather than GH is directly related to growth, is the finding that the pygmies of Central Africa do not synthesize normal amounts of IGF I despite normal levels of active GH (Froesch et. al., 1985).

The physiological role of IGF II is more enigmatic than that of IGF I. Its synthesis is less dependent on GH, and the evidence linking it to mediation of GH function is more tenuous (See Hall and Sara, 1983; Froesch et. al., 1985). The available evidence seems to indicate however, that it may serve an important function in foetal growth and development (Underwood et. al., 1983; Perdue, 1984; D'Ercole and Underwood, 1986).

IGFs stimulate RNA and DNA synthesis and cell division in numerous cell types *in vitro* (See Table 4). They act synergistically with other factors in a manner similar to that described for insulin, and have been given similar roles in growth promotion. They have been designated progression factors in the competence/progression theory (Stiles et. al., 1979; Bockus et. al., 1983), and may substitute either for agents which raise $[cAMP]_i$ or agents which activate PKC, in Rozengurt's scheme for 3T3 cells (Rozengurt, 1985 and 1986).

It has been noted that dose response analyses of the growth promoting and metabolic effects of IGFs and insulin display unusual properties. Generally, low doses of IGFs and high doses of insulin are required for maximal growth promotion, whereas high doses of IGFs and low doses of insulin stimulate maximal acute metabolic effects. These observations have led to the proposal that IGFs and insulin may have low affinities for each other's receptors. Before further discussing this possibility, it is appropriate to describe some features of the various receptor types which may bind insulin and the IGFs.

Table 4. Examples of Cell Types in which Insulin-Like Growth Factors have been shown to Promote Growth.

Cell Type	Factor	Reference
Chick Embryo Fibroblasts	IGFs I & II	Zapf et. al., 1978
Human Fibroblasts	IGF I	Clemmons and Van Wyk, 1981
Bone Cells	IGFs I & II	Schmid et. al., 1983
Chinese Hamster Fibroblasts	IGFs I & II	Van Obberghen-Schilling and Pouyssegur, 1983
Aortic Smooth Muscle Cells	IGFs I & II	King et. al., 1985
L6 Myoblasts	IGFs I & II	Ballard et. al., 1986
SH-SY5Y Human Neuroblastoma Cells	IGFs I & II	Mattsson et. al., 1986
HumanFoetal Fibroblasts	IGF II	Conover et. al., 1987
Balb/c-3T3 Cells	IGF I	Olashaw et. al., 1987
K562,Human Erythroblastoid Cells	IGF II	Tally et. al., 1987

I.2.4. Receptors for Insulin and the Insulin-Like Growth Factors.

As with other peptide hormones, insulin and the IGFs bind to specific proteins located on the plasma membranes of sensitive cells. These receptors serve the dual function of recognising the hormone and transmitting a transmembrane signal to the cellular interior.

The insulin receptor protein was first isolated from rat liver and adipocyte membranes with the aid of nonionic detergents (Cuatrecasas, 1972). It was suggested that the receptor was a glycoprotein with an apparent molecular weight of 350 kilodaltons (kd). Subsequent studies have shown that it consists of two different types of glycosylated subunit (α and β), which are arranged in a β–α–α–β complex, linked by disulphide bonds (Czech, 1985; Houslay, 1985; Kahn, 1985; Denton, 1986). The α-subunit, which has no membrane spanning region, has a molecular weight of about 135kd, and contains the major part of the insulin binding site (Massague et. al., 1980; Grunfeld et. al., 1985). The β-subunit has a molecular weight of approximately 95kd and both extracellular and intracellular domains. This subunit exhibits insulin-stimulated tyrosine kinase activity and undergoes autophosphorylation primarily on tyrosine residues in the cytoplasmic domain (Kasuga et. al., 1982; Yu and Czech, 1986; Ballotti et. al., 1987). The nucleotide sequence of the human insulin receptor DNA has been determined (Ebina et. al., 1985), and it is apparent that a precursor polypeptide containing one α and one β subunit is initially translated (Hedo and Gorden, 1985). This is subsequently processed, cleaved and glycosylated, the subunit structure assembled, and the complex inserted in the plasma membrane (Houslay, 1985).

The kinetics of insulin binding to its receptors is complex and yields curvilinear Scatchard plots (Kahn, 1976; Forsayeth et. al., 1987). It is

suggested that either there are sub-populations of insulin receptors with different affinities for the insulin molecule, or that the insulin receptor complex exhibits negative co-operativity. Some recent evidence has tended to cast doubts on the latter possibility (Czech, 1985; Houslay, 1985; Helmerhorst, 1987), but others maintain that insulin binding may induce interactions between receptors which result in conformational changes which reduce receptor affinity (Gu et. al., 1988). Alternatively, the two binding sites on each receptor molecule may have different affinities for insulin (Pang and Shafer, 1984).

After insulin binding, receptors have been shown to aggregate and become internalised by receptor-mediated endocytosis (Jaret and Smith, 1974; Schlessinger et. al., 1978). This process leads to a reduction in the number of insulin receptors on the cell surface, ie. insulin-induced down-regulation (Baldwin et. al., 1980; Rouiller and Gorden, 1987). Recent evidence suggests that phosphorylation of the receptor by PKC may stimulate this process (Hachiya et. al., 1987). The internalised insulin molecules may be degraded by fusion of the vesicles with lysosomes (Duckworth and Kitabchi, 1981; Shimizu et. al., 1981). Alternatively, intracellular insulin may play a role in mediating some of the molecule's effects (Goldfine et. al., 1977; Goldfine, 1978; Smith and Jaret, 1987). This possibility will be explored more fully in section I.2.5.4. The internalised receptors may either be recycled back to the plasma membrane, or degraded in the cell (Duckworth and Kitabchi, 1981; Marshall, 1985).

In intact cells insulin stimulates the phosphorylation of its receptor β subunit on tyrosine residues (Kasuga et. al., 1982; Gammeltoft and Van Obberghen, 1986), and it has been shown that the insulin receptor itself is a tyrosine kinase (Czech, 1985; Kahn, 1985; Houslay, 1986). Opinions vary on whether the phosphorylation of the receptor is necessary in

order for kinase activity towards exogenous substrates to be exhibited. Some evidence indicates that the intramolecular autophosphorylation must take place before the kinase is activated (Kwok et. al., 1986), but other evidence suggests that kinase activity is unaffected by absence of β subunit autophosphorylation (Goren et. al., 1987; Morrison and Pessin, 1987).

Studies of the kinase activity of insulin receptors have been undertaken either in intact cells or in cell-free receptor preparations. A consistent finding is that in cell-free systems only tyrosine phosphorylations are observed in response to ligand binding, but in whole cells both the receptor and other proteins are also phosphorylated on serine and threonine residues in response to insulin (Denton, 1986; Gammeltoft and Van Obberghen, 1986). This has led to the proposal that the insulin receptor may interact with another cellular serine or threonine kinase. Most evidence seems to identify this molecule as the CaM-dependent kinase which is phosphorylated on tyrosine residues by the insulin receptor tyrosine kinase (McDonald et. al., 1983; Haring et. al., 1985; Colca et. al., 1987). Whether this alters the function of the CAM-dependent kinase is unknown, but insulin may also increase $[Ca^{++}]_i$ which may activate CAM (see Section I.2.5.1.).

The two most important factors regulating insulin receptor kinase activity are insulin, which increases V_{max}, and Mn^{++}, which decreases K_m of the enzyme (White et. al., 1984). Other agents may also play more minor roles however. These include a dependence on Ca^{++} (Plehwe et. al., 1983), and phosphorylation of receptor serine and threonine residues by both cAMP-dependent PK (Tanti et. al., 1987) and PKC (Bollag et. al., 1986; Häring et. al., 1986). The effect of these phosphorylations is to decrease tyrosine kinase activity by increasing

the K_m of the enzyme. Hydrogen peroxide increases receptor tyrosine kinase activity (Koshio et. al., 1988).

Evidence is accumulating which suggests that the tyrosine kinase activity of the insulin receptor is required for at least some of the effects of insulin on cell metabolism to be expressed (Malchoff et. al., 1985; Kadota et. al., 1987; Sale et. al., 1987). Hence monoclonal antibodies which inhibit the kinase activity block the ability of insulin to stimulate *Xenopus* oocyte maturation (Morgan et. al., 1986), glycogen synthesis in hepatoma cells, and glucose uptake in a number of cell types (Morgan and Roth, 1987). Mutant insulin receptors which lack tyrosine kinase activity also fail to mediate post-receptor effects of insulin (Chou et. al., 1987; McClain et. al., 1987), and it has been proposed that defects in tyrosine kinase activity may be one cause of cellular insulin resistance in type I diabetes mellitus (Kadowaki et. al., 1984; Häring et. al., 1987).

The expression of tyrosine kinase activity by the insulin receptor seems to link it to other proteins concerned in growth control (see Section I.1.3.). Indeed there are immunological similarities between the insulin receptor and src oncogene product (Perrotti et. al., 1986), and the human insulin receptor gene may have transforming potential under some circumstances (Wang et. al., 1987).

The type I IGF receptor is structurally very similar to the insulin receptor. It is a heteromeric glycoprotein made up from 2 α (M_r = 130kd) and 2 β (M_r = 92kd) subunits, linked by disulphide bonds (Perdue, 1984; Rechler and Nissley, 1985). It is synthesised from a high molecular weight precursor polypeptide in a manner similar to the insulin receptor (Jacobs et. al., 1983). Long term exposure to IGF I produces a decrease in cellular sensitivity to the molecule, but this may be as a result of a decrease in receptor affinity for IGF I rather than

receptor mediated endocytosis of the ligand/receptor complex (DeVroede et. al., 1984).

In common with insulin, IGF I stimulates the phosphorylation of the β subunit of the type I IGF receptor and exogenous proteins on tyrosine residues (Jacobs et. al., 1983; Zick et. al., 1984; Yu et. al., 1986), although the substrate specificity of the two receptor kinases may be distinct (Sahal et. al., 1988). PKC may also play a role in the regulation of type I IGF receptor kinase activity by stimulating the phosphorylation of serine/threonine residues on the receptor (Jacobs et. al., 1983; Jacobs and Cuatrecasas, 1986).

The type II IGF receptor is very different in structure. It seems to be a monomeric species of molecular weight 250kd (Perdue, 1984; Rechler and Nissley, 1985). It is synthesised originally as a 245kd precursor, and subsequent glycosylation is essential for the acquisition of IGF II binding activity (MacDonald and Czech, 1985; MacDonald et. al., 1988).

Regulation of the number of type II IGF receptors on the plasma membranes of various cell types has been found to be linked to growth status (Polychronakos and Posner, 1985) and cell density (Scott and Baxter, 1987), but perhaps most importantly, in response to insulin. Insulin activates the appearance of more type II IGF receptors on the adipocyte cell surface (Oka et. al., 1984), and also on pancreatic acini (Potau et. al., 1984). The mechanism appears to involve a redistribution of receptors between an intracellular pool and the plasma membrane, analogous with the effect of insulin on glucose transporters (Wardzala et. al., 1984; Oka and Czech, 1985) (See Section I. 2.1.).

The type II IGF receptor has no intrinsic kinase activity, but it is phosphorylated on serine/threonine (Haskell et. al., 1985) and tyrosine (Corvera et. al., 1986) residues in intact cells. The physiological

significance of this is not known, but insulin decreases both types of phosphorylation (Corvera and Czech, 1985; Corvera et. al., 1988).

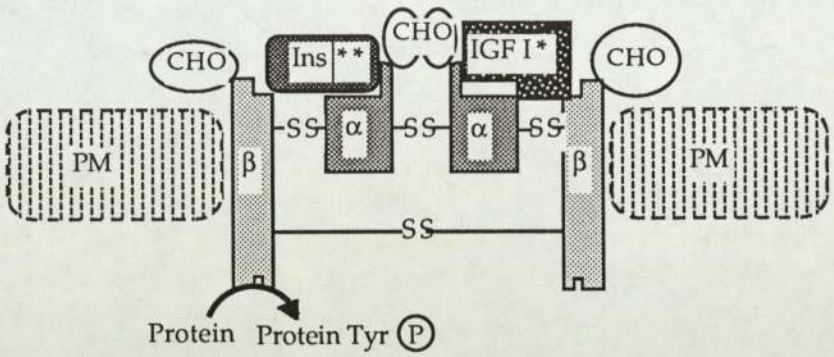
Most cell types express insulin receptors, although the number per cell is very variable both between cell types and on the same cell type under different circumstances. Some cells, such as rat adipocytes also possess type II IGF receptors, but not type I IGF receptors (King et. al., 1980). Chick embryo fibroblasts possess insulin and type I IGF receptors, but not type II IGF receptors (Kasuga et. al., 1982), but many cell types, such as human fibroblasts, possess all three types of receptor (Nissley and Rechler, 1978). Also, as hinted previously, the three ligands may bind to the different receptors with varying affinities. Insulin binds with high affinity to its own receptor, with low affinity to the type I IGF receptor, but does not bind the type II IGF receptor even at high concentrations (Van Obberghen-Schilling and Pouysségur, 1983; King and Kahn, 1985; Pepe et. al., 1987). IGF I binds to the type I IGF receptor with high affinity, and to the insulin and type II IGF receptors with low affinity (Barenton et. al., 1987; Ewton et. al., 1987; Pepe et. al., 1987). IGF II binds to the type II IGF receptor with high affinity, the type I receptor with low affinity, but does not bind to the insulin receptor (Ballard et. al., 1986; Conover et. al., 1987). (See Figure 10).

Since most cells possess more than one type of receptor, and because of the cross-reactivity of ligands for the three receptor types, it is frequently difficult to determine which receptor is mediating a particular response. Attempts to clarify this situation have included determination of competitive binding affinities of the three peptides, dose response analyses, and most directly, use of Fab fragments of anti-receptor antibodies (See Rechler and Nissley, 1985; Taylor et. al., 1987).

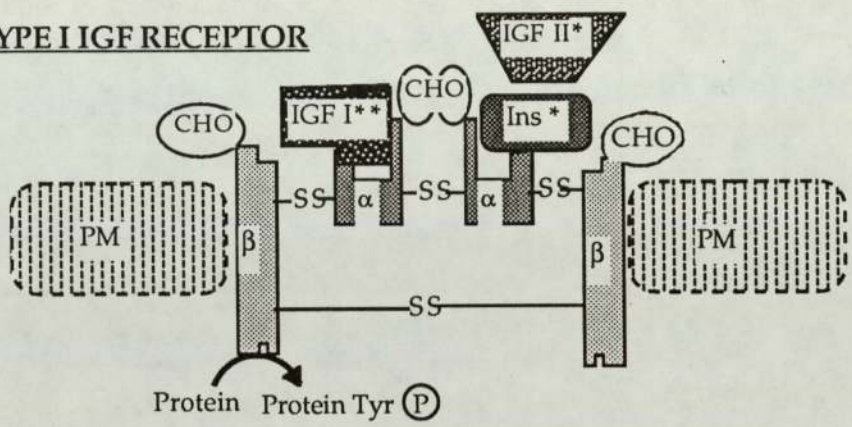
The necessity for supraphysiological concentrations of insulin in order to promote growth in a number of cases, and also the low affinity

Figure 10. Schematic Diagram of the Structures of the Insulin and Type I and Type II IGF Receptors, Indicating Ligand Affinities.

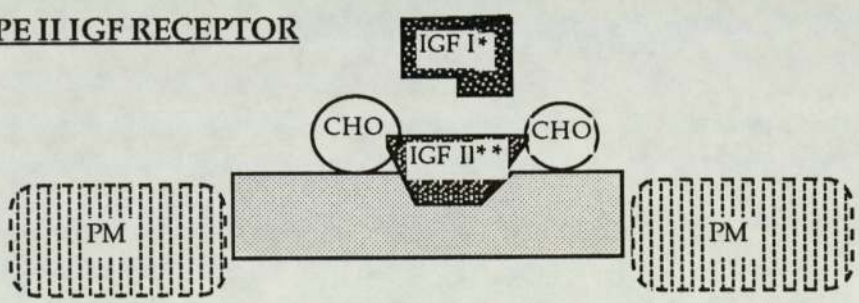
INSULIN RECEPTOR



TYPE I IGF RECEPTOR



TYPE II IGF RECEPTOR



Key:

PM - Plasma membrane

CHO - Carbohydrate moiety

SS - Disulphide bond

* - Low affinity binding

** - High affinity binding

of the insulin molecule for the type I IGF receptor have been noted. The proposal that both insulin and IGF I exert their growth-promoting effects via the type I IGF receptor is therefore logical, and indeed borne out in many cell types such as rat adipocytes (King et. al., 1980), aortic smooth muscle cells (King et. al., 1985) and chinese hamster fibroblasts (Van Obberghen-Schilling and Pouysségur, 1983). However, in other cell types insulin also seems to mediate its proliferative potential through insulin receptors. Examples include human skin fibroblasts (Flier et. al., 1986), pericytes from bovine retinal capillaries (King and Kahn, 1985) and a hepatoma cell line (Koontz, 1980). The growth-promoting effects of IGF II may also be mediated by the type II IGF receptor (Tally et. al., 1987), but more commonly through the type I IGF receptor (Mottola and Czech, 1984; Conover et. al., 1987; Ewton et. al., 1987; Furlanetto et. al., 1987).

Since high concentrations of IGF I are required for acute metabolic responses, and IGF I has low affinity for insulin receptors, similar reasoning to that above leads to the proposal that both insulin and IGF I exert their metabolic effects through the insulin receptor. Again, this appears to be the case in some cell types such as rat adipocytes (King et. al., 1980). In other cell types however IGF I appears to mediate glucose transport and other metabolic effects through the type I IGF receptor (Knight et. al., 1981; Cascieri et. al., 1986). The metabolic effects of IGF II may also be mediated by either type I IGF receptors (Conover et. al., 1987; Ewton et. al., 1987; Kiess et. al., 1987), or through type II IGF receptors (Hari et. al., 1987).

Although some responses in some tissues may be mediated solely by one receptor type, it can be seen that it is not uncommon for the same response to be produced by insulin binding the insulin receptor, and IGFs binding IGF receptors. This suggests that there may be

convergence in post-receptor signalling pathways. The steps which occur after insulin or IGF binding are poorly understood, and a unified, well-documented concept of cellular signalling remains to be established. Details of some parts of the insulin effector system are however available, and the most important of these are examined in the following section, with respect to known growth-promoting signals. IGF mechanisms of action are less well studied, but these are noted where information is available.

I.2.5. The Search for Second Messengers for Insulin and the IGFs.

Any model put forward as a potential mechanism of insulin action must account for the diversity of cellular processes known to be modulated by insulin. None of the selection of currently popular ideas reviewed here can yet be shown to fulfil this prerequisite of a 'mediator', at least exclusively. It is possible that two or more independent chemical signals are generated simultaneously in response to insulin binding, or that the elusive species, so actively sought, is at present obscure or unknown. Nevertheless, current lines of research may provide important insights, and the following sections briefly summarise prevailing ideas with respect to the mechanisms by which insulin and IGFs may carry out their growth-promoting functions.

I.2.5.1. The Role of Ion Fluxes.

The ability of insulin to regulate ionic events is perhaps less well recognised than its effects on organic molecules. Nevertheless, it is well established that insulin does effect ion fluxes across the cell membrane (Czech, 1977; Moore, 1983), and these may be important events in the intiation of DNA synthesis, as previously discussed. It is, of course,

impossible to alter the flux of only one species of ion across the cell membrane, but for convenience of presentation each effect is considered separately.

One of the most prominent clinical responses to insulin is decreased serum $[K^+]_i$, and this is mainly the result of net K^+ tranfer from plasma and interstitial fluid to the intracellular space (Zierler, 1985). This is achieved by an insulin-induced stimulation of the Na^+/K^+ATPase (Moore, 1983). An increase in the outward electric current generated by this enzyme may be partially responsible for the hyperpolarisation of the plasma membrane which also occurs in response to insulin treatment (Davis and Martin, 1985). This effect has been proposed as a mechanism whereby insulin could mediate some of its effects, such as glucose transport (Zierler, 1985; Marunaka, 1986), but as a general signal it seems untenable. For example, although insulin increases glycogen synthase activity in muscle, fat and liver, hyperpolarisation does not occur in liver. Indeed, in liver, hyperpolarisation occurs in response to glucagon, which antagonises insulin in its effects on glycogen synthesis (Czech, 1977).

Insulin also stimulates the activity of the Na^+/H^+ antiport, and leads to cytoplasmic alkalinisation (Moore, 1983 and 1986). This stimulation bypasses the PKC system (Vara and Rozengurt, 1985). The possibility that changes in $[pH]_i$ could alter activities of various enzymes has been discussed along with its putative role in initiation of DNA synthesis (see Section I.1.4.1.). Some evidence implies that cytoplasmic alkalinisation secondary to an increase in Na^+/H^+ antiport activity is involved in insulin-induced stimulation of glucose transport (Kitagawa et. al., 1987), glycolysis (Moore, 1985 and 1986) and protein and DNA synthesis (Busa and Nuccitelli, 1984). A role for a rise in $[pH]_i$ in insulin action would explain the ability of phorbol esters to mimic

some of the effects of insulin, since they also raise $[pH]_i$, but via activation of PKC.

Insulin also has effects on Ca^{++} fluxes, the best documented being an inhibition of the Ca^{++}ATPase (Moore, 1983; Pershadsingh and MacDonald, 1984). This effect, coupled with an alleged ability of insulin to mobilise Ca^{++} from binding sites on the inner surface of the plasma membrane, has lead to the proposal that insulin may act by increasing $[Ca^{++}]_i$ (Kissebah et. al., 1975; Clausen, 1977). Although a role for Ca^{++} in insulin-induced glucose transport is supported by some evidence (Schudt et. al., 1976; Clausen, 1980; Draznin et. al., 1987; Pershadsingh et. al., 1987), other authors disagree (Klip and Ramlal, 1987). With reference to growth control, however, Ca^{++} may be involved in insulin-induced stimulation of DNA synthesis in mouse mammary gland (Zwierzchowski et. al., 1984). However, a rise in $[Ca^{++}]_i$ would oppose some other actions of insulin, such as its inactivation of phosphorylase (Khoo, 1976), and its inhibition of the stimulatory effects of phenylephrine on glycogenolysis and gluconeogenesis that are themselves considered to be mediated by a rise in $[Ca^{++}]_i$ (Blackmore et. al., 1979).

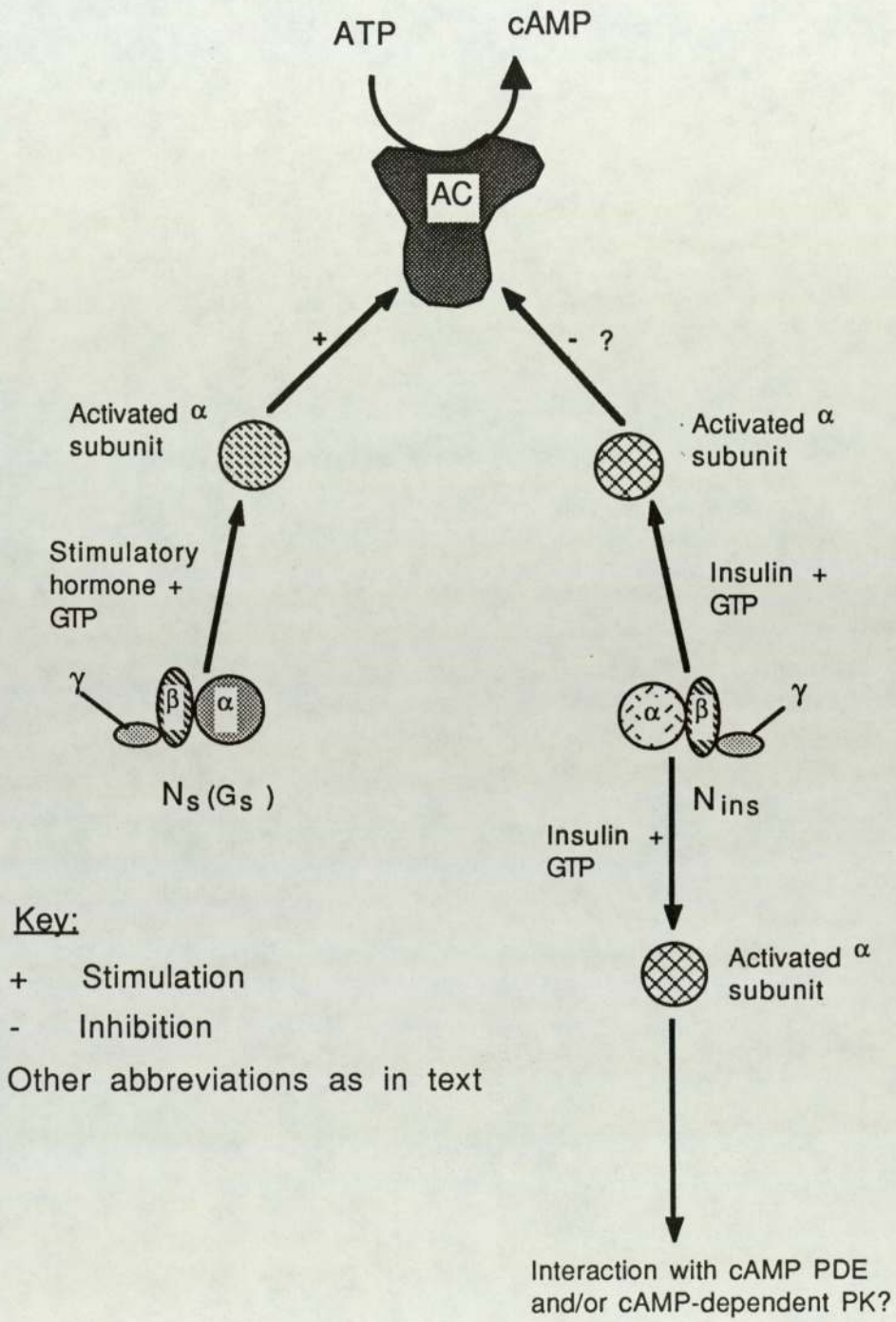
Although it cannot be considered as a second messenger exclusively responsible for all insulin actions, Ca^{++} may nevertheless play a role in some of the actions of insulin. Its role in altering ion fluxes may be particularly relevant in the synergistic actions of insulin with other growth factors. Notably in Rozengurt's model as described in section I.2.2. Although there is little specific information concerning the role of IGFs, IGF II also stimulates a Ca^{++} influx in BALB/c 3T3 cells primed with EGF (Nishimoto et. al., 1987), and this may be associated with its role as a progression factor for these cells.

I.2.5.2. The Role of cAMP and the Putative G-Protein, N_{ins}.

It is well established that under appropriate conditions, insulin can antagonise actions of hormones, such as glucagon, which act by raising $[cAMP]_i$ (Denton et. al., 1981; Simpson and Cushman, 1986). However, although insulin does reduce $[cAMP]_i$ in liver and fat cells, which have previously been exposed to another hormone which increases $[cAMP]_i$, it has little or no effect under basal conditions, even though many effects of insulin are manifested (Cheng and Larner, 1985). Actions of insulin which have been dissociated from changes in $[cAMP]_i$ include stimulation of amino acid transport (Goldfine and Sherline, 1972), glycogen synthase stimulation (Chiasson et. al., 1980) and antilipolysis (Fain and Rosenberg, 1972).

Nevertheless, some effects of insulin may involve decreases in $[cAMP]_i$, and recent evidence suggests that it may also produce a decrease in sensitivity of the cAMP-dependent PK (Ciudad et. al., 1987). Direct inhibition of AC of liver plasma membranes has also been observed, but only in the presence of non-saturating concentrations of glucagon, moderate concentrations of Mg^{++}, and most importantly, an adequate concentration of GTP (Heyworth and Houslay, 1983). Activation of PDEs with low affinity for cAMP may also occur following insulin stimulation of fat and liver cells, a response which is mimicked by guanine nucleotides (Houslay, 1985). These observations have lead Houslay and colleagues to propose an elegant model for the control of liver $[cAMP]_i$ by insulin and glucagon, whereby a novel G-protein, N_{ins}, may interact with both PDEs and the AC system in the plasma membrane (Houslay, 1985 and 1986a and b) (See Figure 3, Section I.1.3. for the AC system, and Figure 11 for the possible interaction of N_{ins} with this system). Little is known about the identity of N_{ins}, and the system remains speculative, but N_{ins} is suggested to

Figure 11. Mechanisms whereby the Putative N_{ins} may Interact with the Adenylate Cyclase System.
(Modified form Houslay, 1986a)



Key:

\+ Stimulation

\- Inhibition

Other abbreviations as in text

have a 25kd α-subunit, which is a substrate for cholera toxin ADP-ribosylation (Houslay, 1985). Some evidence links G-protein activation with glucose transport in adipocytes (Lönnroth et. al., 1987), and experimentally induced type I diabetes leads to the loss of expression of a G-protein (Gawler et. al., 1987).

Any proposed roles for N_{ins} in the aspects of insulin action involving growth control are speculative, and its effect in reducing $[cAMP]_i$ might be considered antagonistic to initiation of DNA synthesis. It has been suggested however, that N_{ins} may be a cellular form of the protein p21, coded by the ras family of oncogenes (Cooper and Lane, 1984; Kamata et. al., 1987) (See Section I.1.3). The EGF receptor, which shares a number of similarities with the insulin receptor, has been shown to enhance the GTP-dependent phosphorylation of p21 on serine and threonine residues (Kamata and Feramisco, 1984). The enhanced phosphorylation of p21 resulting from interaction with the occupied EGF receptor leads to an increase in the ability of p21 to bind guanine nucleotides. Also, p21 has been shown to express kinase activity. It has been suggested that the occupied insulin receptor may interact with N_{ins} in a similar manner, and if N_{ins} also expresses kinase activity, this may be the source of the serine/threonine kinase activity associated with the insulin receptor (Houslay, 1986).

I.2.5.3. The Role of Novel 'Mediators'.

The second messenger concept of hormone action, and the roles of cAMP, Ca^{++}, PtdIns4,5P_2 hydrolysis etc. have been described (see Sections I.1.3. and I.1.4.). There is considerable evidence for linking these mediators into a number of cellular responses to hormonal stimulation, and since none of them appear to mediate all the effects of

insulin, the idea that insulin may work through the production of a unique second messenger is attractive.

Larner and colleagues were the first to provide evidence that such a substance might exist (Larner et. al., 1974 and 1979). The mediator(s) has never been isolated and purified in sufficiently large and uncontaminated quantities to be fully characterised. It has been proposed, however, that the mediator (or family of mediators) is an oligopeptide or glycopeptide, perhaps cleaved from a protein associated with the insulin receptor after activation of its kinase capacity (Jarett and Kiechle, 1984; Cheng and Larner, 1985; Gottschalk and Jarett, 1985). Alternatively, other evidence suggests that insulin stimulates the activity of a phospholipase which selectively hydrolyses a novel membrane phosphatidylinositol glycan, leading to production of a carbohydrate mediator as well as a unique species of DG (Sale et. al., 1986; Saltiel et. al., 1986 and 1987; Gottschalk and Jarett, 1988). The similarities between this system and the bifurcating PtdIns4,5P_2 pathway are evident, and it would be of great interest to ascertain whether N_{ins} might play a role in this system analogous to the G-protein mediation of PLC activation.

No specific material is available concerning the role of mediator(s) in the growth-promoting actions of insulin. However, in cell-free systems, effects of the mediator(s), as opposed to effects of insulin itself, have been proposed in enhancing cAMP PDE activity (Kiechle and Jarett, 1981; Saltiel et. al., 1986) and antagonising hormone stimulated AC activity (Saltiel et. al., 1982). These effects indicate a reduction in $[cAMP]_i$, in contrast to the proposed rise associated with initiation of DNA synthesis. Also, mediators may inhibit cAMP-dependent PK activity (Villalba et. al., 1988). More consistent with the role of insulin as a mitogen, is the observation that mediator(s) may stimulate nuclear

RNA synthesis (Horvat, 1980). However, an increase in Ca^{++}ATPase has also been demonstrated in response to the mediator (MacDonald et. al., 1981), in contrast to the effect of insulin, which inhibits this enzyme.

The 'mediator hypothesis' exhibits a number of anomalies which still require rigorous exploration. Most serious perhaps, is the lack of full chemical characterisation for the proposed mediator molecule(s). Without a source of 'pure' mediator, the possibility remains that contaminants of the crude material are responsible for the observed effects, rather than the mediator itself. Nevertheless, the concept of the production of a novel mediator remains a promising approach towards understanding the mechanism of insulin action.

I.2.5.4.The Role of Internalisation of Insulin/Receptor Complexes.

It has been noted that insulin binding initiates the aggregation of its receptors, and the internalisation of ligand/receptor complexes by specific receptor-mediated endocytosis. Although not sufficiently rapid to account for all the effects of insulin, some evidence seems to imply that this process may serve a function other than in regulation of receptor number and degradation. It has been suggested that that this mechanism provides a route for delivering insulin to specific binding sites located on the nuclear membrane and other intracellular sites, and that this process is associated with long-term actions of insulin, including growth control (Goldfine et. al., 1977; Goldfine, 1978; Smith and Jarett, 1987). However, microinjection of insulin into fibroblasts has been shown to have no effect on DNA synthesis, where the presence of extracellular insulin stimulates the process (King and Kahn, 1985).

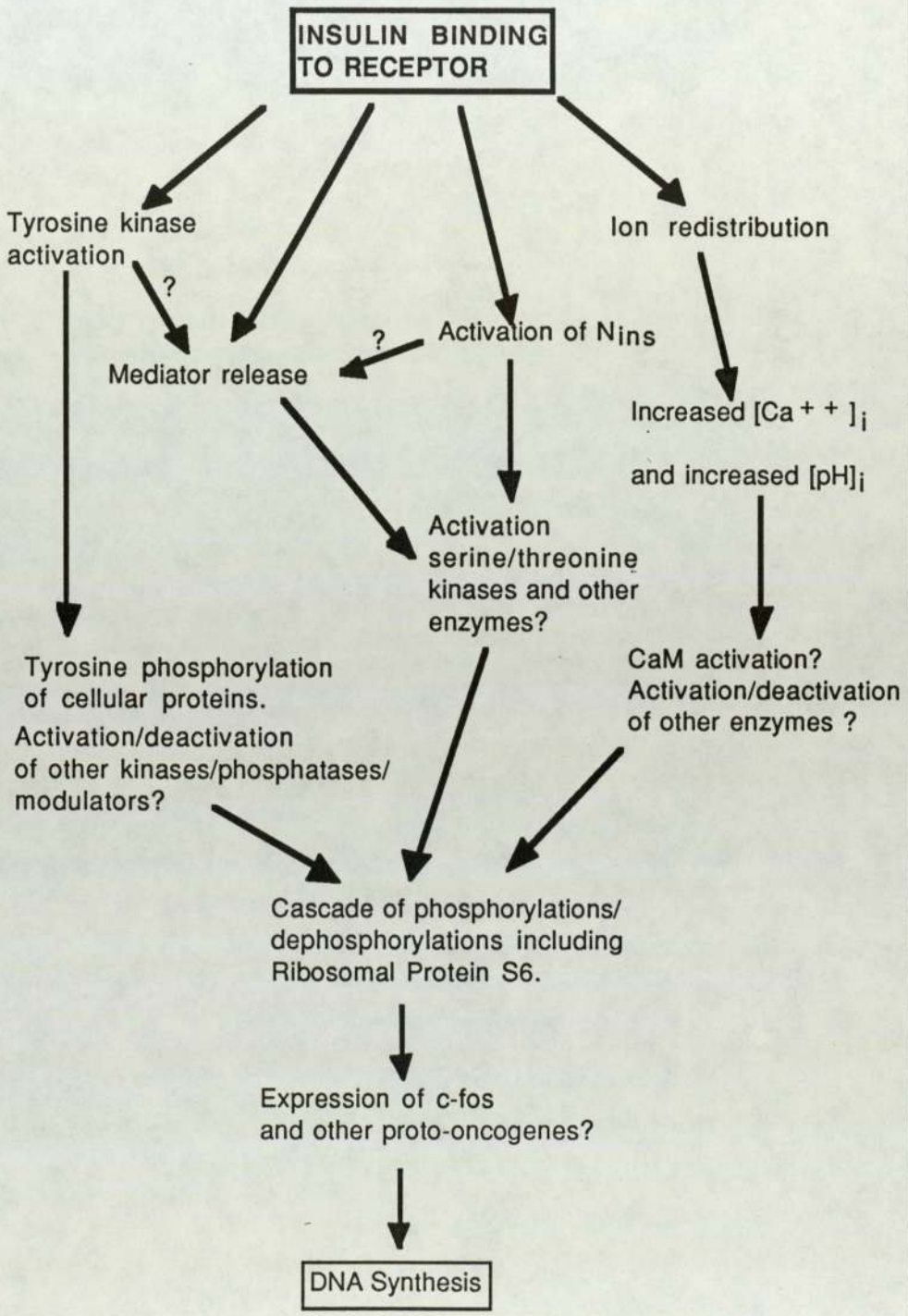
However, it is of interest to note that internalised vesicles would be expected to have activated β-subunits on their outer surfaces, and hence would provide a vehicle for transporting tyrosine and possibly other associated kinases to other regions of the cell where they can act on intracellular substrates. The significance of this, if any, has not been determined.

I.2.5.5. Summary and Integration.

The mechanism via which insulin exerts its growth-promoting effects is still unclear. Many investigations are however suggesting the possible involvement of a complex array of processes which may include roles for novel mediator(s) and G-proteins, as well as the more familiar systems of ion redistributions associated with growth factor activation. It is clear, however, that the control of cellular kinases and phosphatases is of importance in the actions of insulin. Whether control is brought about directly or indirectly by the receptor TK, N_{ins}, a mediator(s) or any combination of the three, remains to be established. Figure 12 represents a summary of some of the important mechanisms by which insulin may control DNA synthesis and cell division. It may be noted however, that the involvement of IGF receptors in growth control, and the likelihood of post-receptor convergence in IGF and insulin signalling pathways, makes the real situation more complicated than Figure 12 implies.

A logical point at which to start an investigation of insulin-induced phosphorylations, of importance to the regulation of growth, is the TK activity of the insulin receptor, since this is a property shared by a number of other growth factor receptors. The scarcity of [^{32}P]tyrosine-containing proteins has impeded efforts to identify potentially relevant substrates for this activity, but some tyrosine phosphorylations have

Figure 12. Summary of some Important Mechanisms by which Insulin may Exert its Effects on Cell Growth.



been observed in response to insulin binding. These include a 15kd membrane protein (Bernier et. al., 1987), a 120kd glycoprotein (Perrotti et. al., 1987) the holomeric forms of the G-proteins N_i (AC system) and N_o (unknown function) (O'Brien et. al., 1987), and various others (Cooper and Hunter, 1985; Caro et. al., 1987; Häring et. al., 1987). The significance of these phosphorylations is however, unknown. Both insulin and IGF I stimulate tyrosine phosphorylation of a 185kd substrate through their respective receptors in intact cells (White et. al., 1985; Izumi et. al., 1987; Machicao et. al., 1987). Although the significance of this protein is unknown, it could be a substrate common to both insulin and IGF I intracellular signalling pathways which represents a convergence point. The existence of such a convergence point is predicted by the existence, under certain circumstances, of an identical subset of cellular responses which may be produced by insulin binding to the insulin receptor, and IGF I binding to the type I IGF receptor (see Section I.2.4.). A set of cellular proteins which are phosphorylated on tyrosine residues in response to a number of growth factors, including insulin and IGF I, has also been determined (Kadowaki et. al., 1987). It is tempting to suggest that these proteins may be involved in growth control, and their phosphorylation is an obligatory event in the initiation of DNA synthesis, but no evidence to this effect exists as yet.

It has been proposed that the signal generated by the insulin receptor tyrosine kinase is amplified and transmitted by other serine/threonine kinases and phosphatases, and/or modulatory proteins of these enzymes (Avruch et. al., 1985; Yu et. al., 1987). Hence, for example, insulin activates an S6 kinase which phosphorylates ribosomal protein S6 on serine residues (Tabarini et. al., 1985; Maller et. al., 1986). Ribosomal protein S6 phosphorylation is associated with activation by

a number of growth factors, and is considered to be an important event involved in the enhanced protein synthesis which precedes DNA synthesis (Martin-Pérez et. al., 1984). Later on in the pathway leading to DNA synthesis, insulin has also been shown to induce expression of the c-fos gene by a PKC independent mechanism (Stumpo and Blackshear, 1986; Stumpo et. al., 1988). Enhanced transcription of this gene is also associated with the prelude to DNA synthesis (see Section I.1.4.4.).

I.3. Rationale for this Study.

I.3.1. The Mammalian Immune System.

During mammalian development, primordial haematolymph precursor cells migrate from the anterior end of the embryo successively to the blood islands of the yolk sac, the liver, and finally to the bone marrow. Throughout the remainder of the life of the organism, the bone marrow produces haematopoietic stem cells, which have lymphocyte precursors among their progeny (See Sharma et. al., 1987).

The lymphoid system in the adult is composed of the primary lymphoid organs; the bone marrow and the thymus, and a collection of secondary lymphoid organs, including the spleen and numerous lymph nodes. Two circulatory networks, the blood and the lymphatic system, interconnect the lymphoid organs, allowing the traffic of lymphocytes between them. Lymphocytes make up 20 - 80% of the nucleated cells in blood.

The chief function of the immune system is to protect the body from invasion by pathogenic micro-organisms and other substances, and to achieve this, the system specifically recognises and selectively eliminates foreign invaders. It is beyond the scope of this summary to describe, in detail, the functioning of this complex system. However, in order to convey the physiological significance of the role of cell division in the immune response, and the central importance of the thymus, two concepts are briefly described below; duality in the immune system, and clonal expansion (See Weissman et. al., 1978; Roitt, 1980).

The immune system recognises foreign entities by their molecular features, termed antigens. When antigen enters the body, two different types of adaptive immunological reaction may occur; a 'humoral' and

a 'cell-mediated' response. The duality of the immune system results from two populations of lymphoid cells; the B and T lymphocytes, which provide distinct but overlapping protection mechanisms in conjunction with other accessory cell types.

B-lymphocytes undergo the first part of their maturation in the bone marrow, and subsequently migrate to the secondary lymphoid organs. Each cell has a specific type of receptor protein displayed on its cell membrane, which enables it to recognise a specific antigen. When the B-lymphocyte comes into contact with that antigen, antigen/receptor binding takes place, and initiates the process of clonal expansion. Hence, in the presence of antigen, and other factors, B-lymphocytes undergo a number of successive divisions, and eventually differentiate into plasma cells, which secrete antibodies. Antibodies, or immunoglobulins, are serum proteins which bind specifically to the antigen which triggered the original B-lymphocyte activation, and initiate a variety of elimination responses, including cell lysis and phagocytosis by macrophages, which constitute the humoral immune response.

T-lymphocytes also originate in the bone marrow, but undergo further maturation in the thymus, where multiple distinct T-lymphocyte lineages are established. T-lymphocytes also migrate to secondary lymphoid organs, and in response to appropriate stimulation, divide and differentiate into cells with a range of effector functions. T-killer cells mediate the lysis of cells displaying 'foreign' antigenic determinants, such as virally infected or cancer cells. T-helper cells secrete a number of factors, collectively called lymphokines, which are required for the successful functioning of both humoral and cell-mediated immune responses. T-suppressor cells are also involved in immunoregulation. The cell-mediated branch of the immune response

is also responsible for graft rejection and the delayed hypersensitivity reaction.

A full discussion of the immune response and the roles of B and T lymphocytes is inappropriate in this context, but more detailed descriptions of the processes of maturation and activation of T-lymphocytes are given in the following sections.

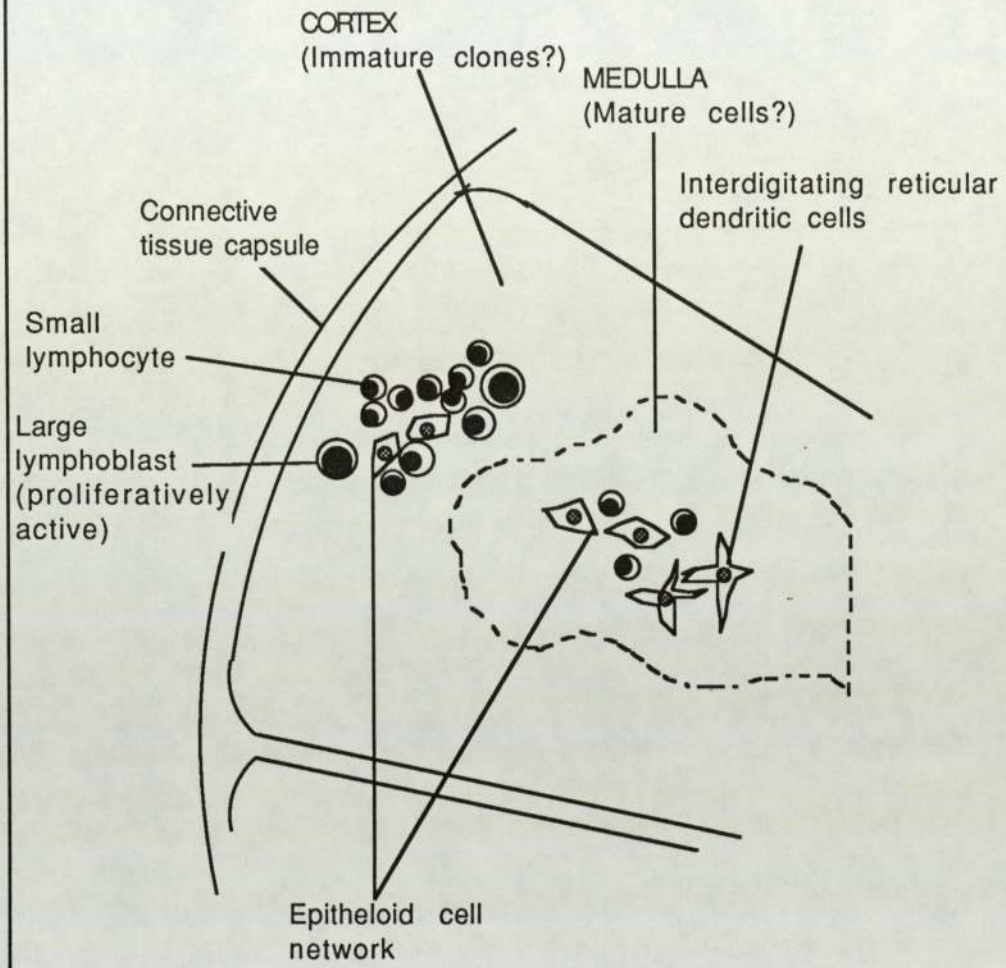
I.3.1.1. Cell Division in the Thymus.

The thymus is a bilobed structure lying ventral to the heart, and is the exclusive site of generation of T-lymphocytes. The gland is organised into a series of lobules made up of a meshwork of epithelial cells and interspersed aggregates of lymphocytes. The outer cortical area is densely populated with actively mitotic lymphocytes, and surrounds an inner medullary zone with more reticular dendritic and epitheloid cells, but fewer lymphocytes. (See Figure 13).

There are at least four distinct events during the maturation of the T-lymphocyte lineage; embryological establishment of the thymic micro-environment, seeding by haematopoietic T-lymphocyte precursors, intrathymic proliferation and differentiation to immunocompetence, and migration to peripheral lymphoid tissues. Although seeding of the thymus occurs at a low level throughout life, intrathymic proliferation and differentiation are of major importance here, since they provide the heterogenous set of lymphocytes, thymocytes, which are the object of this investigation.

It is known that the thymus is a major site of lymphocyte generation, that many cells die within the organ, and only a relatively small proportion leave to become peripheral T-lymphocytes (Claësson and Hartmann, 1976; Scollay et. al., 1980). Studies analysing cell surface markers and kinetics have shown that there are a number of distinct sub-populations of thymocytes which can be divided into several

Figure 13. Regions and Cell types within a Thymus Lobule.



developmental streams (Shortman and Jackson, 1974; Fathman et. al., 1975; Scollay and Shortman, 1983; Pardoll et. al., 1987). However, the precise sequence of events in each developmental stream, their interconnections, relationship to the expression of various cell surface differentiation antigens, and the eventual emergence of immunocompetent sub-populations, remain poorly understood (See Robinson, 1980; Scollay and Shortman, 1983; Marrack and Kappler, 1986; El Rouby and Papiernik, 1987). Nevertheless, some classifications of thymic sub-populations can be made.

The anatomical sub-division of the thymus into cortex and medulla has its parallel in a series of cell surface markers believed to define 'cortical' and 'medullary' thymocytes. 'Cortical' thymocytes are immunologically non-functional, and their set of cell surface markers represent phenotypes unique to the thymus. 'Medullary' thymocytes are functional, and have the phenotypes of peripheral T-lymphocytes (Scollay and Shortman, 1983). This has been taken to imply that the subsets represent immature and mature thymocytes respectively. However, the relationship between the groups is unlikely to be simply that of progenitor/daughter cells, since although some 'cortical' thymocytes migrate to the medulla, others are exported directly to the periphery (Reichert et. al., 1984), and the fates of medullary thymocytes are diverse (Roitt, 1980; Scollay and Shortman, 1983).

Another system of thymocyte classification, which makes no attempt to define functional sub-population, describes only proliferative potential. Hence 10 - 15% of thymocytes are large, actively dividing blasts, which give rise to the bulk of small non-proliferating thymocytes. The small thymocytes may be subdivided into two groups depending on their ability to re-initiate DNA synthesis and division on receipt of an appropriate signal. Hence 80% of this group are

proliferatively inactivated, but the remaining cells may respond rapidly (within 5 - 7 hours) to mitogenic stimuli (Whitfield et. al., 1979). These 'rapidly recruitable' cells represent a G_0 subgroup, very close to the G_1/S boundary.

Early experiments on the partial restitution of immunocompetence in thymectomised females during pregnancy, imply that a soluble product of the thymus (from the foetal thymuses) is, at least in part, responsible for the influence of the thymus on T-lymphocyte development (See Roitt, 1980). Since then, a number of factors, including various thymosin fractions, thymopoietin II (TP), facteur thymique serique (FTS) and thymic humoral factor (THF) have been isolated from thymus tissue and blood (Goldstein et. al., 1981). Four of these factors; thymosin α_1 and β_4, TP and FTS, have been sequenced, and are chemically unrelated. The thymosin fractions are synthesised in the thymic epithelial cells (Van Den Tweel et. al., 1979), and different thymosin peptides may be specifically associated with different intrathymic regions (Goldstein et. al., 1981).

In addition to these 'thymic hormones', a number of other factors, products of either the lymphocytes themselves, or accessory cells of the immune system, such as macrophages, are involved in growth and differentiation of thymocytes within the thymus. Little is known about the roles these factors may play in sequential steps in the maturation processes of intrathymic lineages. Factors which may be important in the evolution of immunocompetent T-lymphocyte lineages include interleukin 2 (IL-2) (Habu, 1986; Jenkinson et. al., 1987; Shimonkevitz et. al., 1987), interleukin 4 (IL-4) (Palacios et. al., 1987) and thymocyte growth peptide (TGP) (Ernström et. al., 1987).

Although these processes continue throughout life, there is a decline in the activity of thymus-derived lymphocytes after the onset of sexual

maturity in several species, including the rat (Segal et. al., 1985). The thymus decreases in mass, and lymphocyte content (Kay, 1979), and other activities are also affected. The major age-associated defect is a decrease in the ability to undergo blastogenesis in response to mitogenic or antigenic challenge (Kay and Makinodan, 1976), but this may result from underlying changes in the developmental procedures within the thymus. Effects of sex can be differentiated from those of age, and it has been proposed that the higher circulating concentrations of sex steroids, after puberty, may play an important role in the decrease in thymic function (Oosterom and Kater, 1981; Segal et. al., 1985).

In contrast to the early developmental stages of T-lymphocyte growth and differentiation, the antigen-dependent phase of growth, which generally takes place in the secondary lymphoid organs, is better understood. Some of the factors and events associated with T-lymphocyte activation, clonal expansion and differentiation into effector cells are discussed in the next section.

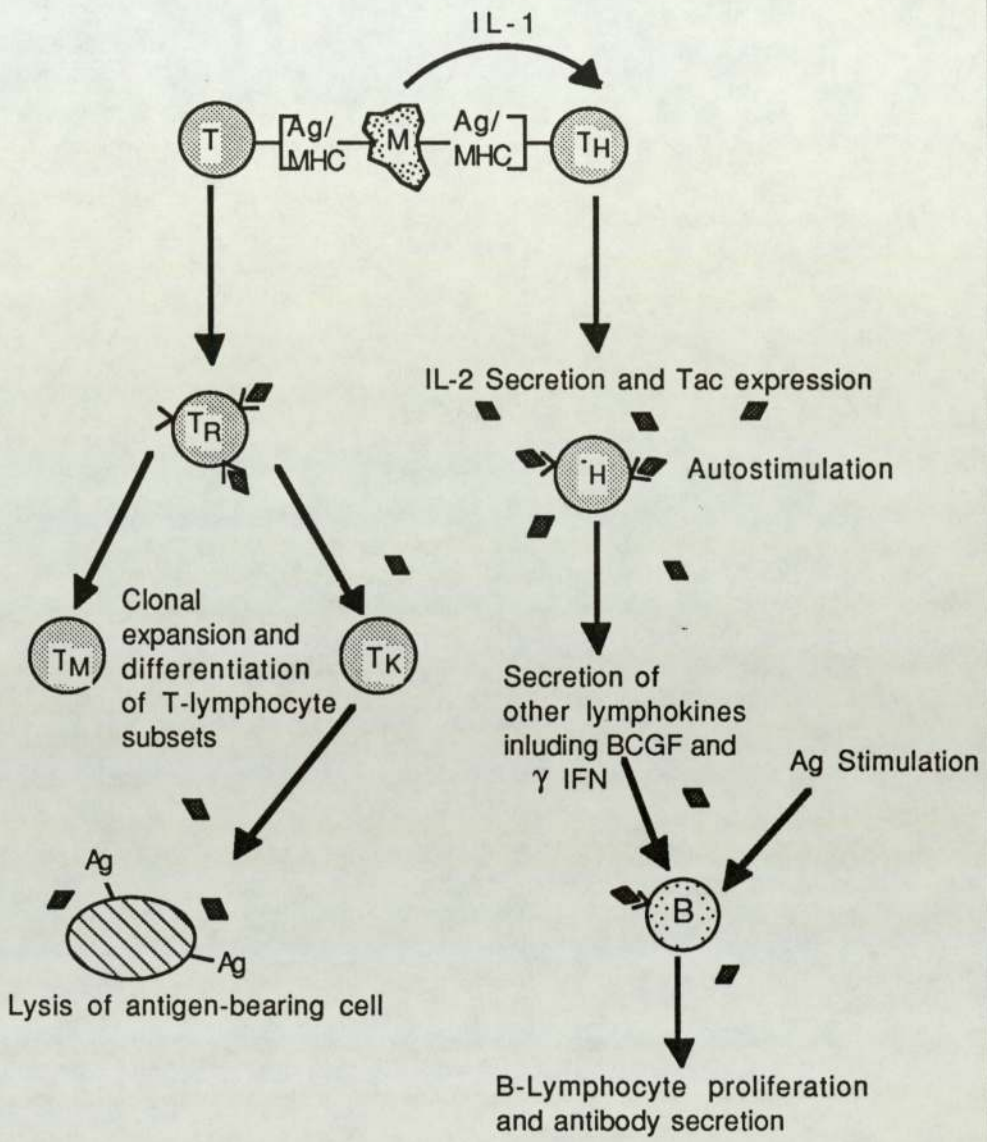
I.3.1.2. T-Lymphocyte Growth Factors and Activation.

During antigenic stimulation, the primary event in T-lymphocyte activation is thought to be the presentation, by macrophages, of antigenic determinants in combination with certain cell surface products of the major histocompatibility complex (Robb, 1984; Abraham et. al., 1987). This process binds and cross-links T-lymphocyte antigen receptor complexes, and can be mimicked by plant lectins such as concanavalin A (ConA) (Sharon and Lis, 1987), and monoclonal antibodies to the T-lymphocyte antigen receptor (Alcover et. al., 1987). The activated macrophage also secretes a factor, interleukin 1 (IL-1), which 'primes' the resting T-lymphocyte, rendering it responsive to receptor triggering (Williams et. al., 1985; Abraham et. al., 1987).

Whether antigens, lectins or antibodies are used as the initial trigger, an obligatory event in the activation of T-lymphocytes, is the induction of expression of two genes; those coding for the best characterised of the T-lymphocyte growth factors, IL-2, and its receptor, Tac (Krönke et. al., 1985; Alcover et. al., 1987; McGuire and Rothenberg, 1987). Under appropriate conditions, all classes of T-lymphocyte synthesize and release IL-2, but T-helper cells are probably the major source. Once released, IL-2 stimulates all classes of T-lymphocyte regardless of subclass or antigen specificity (Robb, 1984). The specificity of the immune system is maintained at the level of Tac expression. The receptor is only expressed by 'primed' cells, already activated by antigen-receptor triggering (Cantrell and Smith, 1983; Waldmann, 1986). (See Figure 14).

The early molecular events which regulate the process of T-lymphocyte activation have been shown to contain elements similar to those described for growth stimulation in other cell types (See Section I.1.4.). Hence, on stimulation with IL-2, antigens, or mitogens, rapid alterations in critical cation fluxes, including Ca^{++} (Finkel et. al., 1987; Imboden and Weiss, 1987; Komada et. al., 1987) and H^{+} (Rogers et. al., 1983; Mills et. al., 1985; Gelfand et. al., 1987) have been observed. This has lead to the suggestion that activation of the inositol phospholipid signal transduction pathway may be an important element in T-lymphocyte activation (Taylor et. al., 1984; Isakov et. al., 1987). In contrast, IL-2 profoundly inhibits both basal and hormone stimulated AC activity (Beckner and Farrar, 1986; Farrar et. al., 1986), although the role of cAMP in T-lymphocyte activation is no less enigmatic than in proliferation of other cell types (Farrar et. al., 1986; Otani et. al., 1987; Wickremasinghe et. al., 1987). Tyrosine phosphorylation is also associated with T-lymphocyte growth (Casnellie and Lamberts, 1986;

Figure 14. T-Lymphocyte Activation by Antigen and the Role of Interleukin 2 in the Immune Response.



Key:

M - Macrophage

T_H - T-Helper cell

T_M - T-Memory cell

T_K - T-Killer cell

Ag - Antigen

T_R - IL-2 Receptor-bearing Cell

▰ - IL-2

Other abbreviations as in text

Hall et. al., 1987). The T-lymphocyte antigen receptor (Klausner et. al., 1987; Patel et. al., 1987) and the IL-2 receptor (Ogawa et. al., 1987) are substrates for tyrosine kinase activity. There is also evidence for alternative, discrete signal transduction pathways activated by different ligands (Grinstein et. al., 1987; Mire-Sluis et. al., 1987; Imboden, 1988), which may synergise in producing the biological response. For example, some agents activate PKC, whereas others appear not to do so, but achieve a similar effect through some other mechanism. Multiple kinase involvement in signal transduction (Patel et. al., 1987) also supports the concept of the integrated participation of a number of second messenger pathways in initiating T-lymphocyte activation and proliferation.

Subsequent to IL-2 stimulation, T-lymphocyte clones have been shown to acquire other growth-associated proteins through enhanced gene transcription. These include receptors for other growth factors such as insulin and transferrin (Krönke et. al., 1985; Snow, 1985), and the product of the proto-oncogene c-myc (Kelly et. al., 1983; Pompidou et. al., 1987). Activated T-lymphocytes may also secrete other lymphokines including γ-interferon (IFN-γ) and B-lymphocyte growth factor I (BCGF I) (Robb, 1984; Waldmann, 1986), which are involved in regulating other aspects of the immune response. (See Figure 14).

It thus appears that T-lymphocyte proliferation occurs as a series of precisely orchestrated events. Lymphocyte-specific growth factors such as IL-2 are primarily associated with this process, but other agents are also involved in modulating the biological response. Insulin is among the factors which contribute to T-lymphocyte activity, and a discussion of the role this molecule may play in T-lymphocyte function, and the associated aims and objectives of this study, is undertaken in the next section.

I.3.2. The Possible Role of Insulin in T-Lymphocyte Growth, Metabolism and Function.

A number of lines of evidence indicate that insulin may be involved in immunoregulation. Alterations in normal insulin concentrations *in vivo* may arise in diabetes mellitus and obesity, and both conditions may be experimentally induced (see Bailey and Flatt, 1986). Nutritional status has a complex series of relationships with circulating insulin concentrations, insulin-sensitivity of various cell types and functioning of the immune system (Hambreaus et. al., 1977; Chandra, 1980). T-lymphocyte insulin receptors have similar characteristics to those of traditional target tissues (Gavin et. al., 1973), and exhibit similar deviations from normal during experimental perturbations. Hence high circulating insulin concentrations lead to a decrease in cell sensitivity to insulin due to down-regulation of the receptors (Soll et. al., 1974; Helderman and Raskin, 1980). This may explain, at least in part, the immunosuppresive effects of high concentrations of insulin (Hunt and Eardley, 1986). Conversely, low insulin concentrations enhance insulin sensitivity of the cells (Gavin et. al., 1974; Goldfine, 1975), and there is an initial period of hyperresponsiveness of the glucose transport system to insulin treatment in streptozotocin diabetic rats (Kahn and Cushman, 1987).

Patients with diabetes mellitus have been found to have a decreased number of circulating T-lymphocytes, and it has been suggested that this is largely due to a reduction in the number of circulating T-helper cells (Galluzzo et. al., 1984). The extent to which total T-lymphocyte number is decreased below normal correlates well with the degree of metabolic control of the patients (Selam et. al., 1979). Other activities that have been reported to be abnormal in diabetes mellitus include

lymphocyte proliferation (Kurtz et. al., 1985; Drell and Notkins, 1987), suppressor cell activity (Lederman et. al., 1981) and cytotoxic killer-cell function (Pozzilli et. al., 1979). Experimentally induced diabetes also has effects on immune function, which can be restored by insulin treatment. These include reduced cellularity of the lymphoid organs and inability to reject allogeneic skin grafts (Pavelic et. al., 1978), reduced ConA responsiveness and impaired delayed type hypersensitivity reactions (Gaulton et. al., 1985).

The mechanism whereby insulin exerts these immunoregulatory effects is unknown, but some insight has been obtained by characterising the receptor subtypes borne by the cells at different stages of their ontogeny. The resting peripheral T-lymphocyte bears on its surface a large number of antigen receptors, but few for endocrine peptides such as insulin (Alcover et. al., 1987). However, after antigen receptor binding and cross-linking, but before initiation of DNA synthesis, the variety and number of receptors increases dramatically (Plaut, 1987). Among the receptor types to emerge on freshly activated peripheral T-lymphocytes are those for insulin (Krug et. al., 1972; Helderman and Strom, 1978), IGF I (Rosenfeld and Hintz, 1980) and IGF II (Brown et. al., 1985). It has been suggested that the possession of insulin receptors may be a universal marker for dividing/activated cells of T-lymphocyte lineage (Helderman et. al., 1978). However, other experiments have failed to detect insulin receptors on clones of cytotoxic killer cells (Braciale et. al., 1982), and although some sub-populations of thymocytes bear insulin receptors (Goldfine, 1975), there is no evidence to link this property with activation/division of the cells. In this study an attempt is therefore made to establish the relationship between the expression of insulin receptors, and proliferative activity of rat thymocytes.

In initial attempts to demonstrate a functional role for insulin in peripheral T-lymphocyte activation, serum-free culture systems have been used. Under these conditions, the mitogenic response to ConA is severely impaired, but is restored in the presence of high concentrations of insulin (Snow et. al., 1980). A similar 'serum-sparing' effect of insulin is observed in antigen-driven T-lymphocyte growth (Snow et. al., 1981). Insulin may also act synergistically with transferrin in augmenting these processes (Strom and Bangs, 1982). In all these cases, however, a supraphysiological concentration of insulin is required in order for the effects to be observed. The possibility that these synergistic and 'serum-sparing' effects of insulin might be analogous with its action in enhancing growth stimulation in combination with other agents in other cell types is therefore investigated. The ability of insulin to promote growth of rat thymocytes in culture is analysed, and compared with that of the polyclonal T-lymphocyte activator, concanavalin A. Synergistic interactions between the mitogens are also investigated in order to ascertain whether insulin might act as a progression factor in this system. The requirement for high insulin concentrations suggests the possibility of its interaction with type I IGF receptors. The growth response of rat thymocytes to insulin is therefore also compared with that to IGF I.

As previously mentioned, the mechanisms by which insulin exerts its effects on T-lymphocyte growth are unknown, but it may affect the intermediary metabolism of activated peripheral T-lymphocytes. In these cells insulin has been shown to enhance amino acid transport and incorporation into proteins (Goldfine et. al., 1972; Kwock et. al., 1976). It may also enhance glucose uptake and metabolism (Boyett and Hofert, 1972; Helderman, 1981) although others have not observed this

effect (Goldfine et. al., 1972). The effects of insulin on the uptake of amino acids and glucose into rat thymocytes are therefore further analysed. An attempt is also made to correlate and compare these metabolic effects with growth-promoting effects, in order to establish their putative relationship, and to define the role of insulin in the 'pleiotypic response' in rat thymocytes.

A preliminary investigation of some possible intracellular signals which may mediate the insulin response is also undertaken, and comparisons made with signals associated with the response to ConA, and other growth-promoting agents.

II. MATERIALS AND METHODS.

Lymphocytes are generally well-suited to growth in culture, and being anchorage-independent, are easy to manipulate and readily accessible to various treatments. A number of T-lymphocyte cell lines are available, but are generally highly adapted to *in vitro* conditions, and therefore may not accurately reflect physiological responses (Kaczmarek et. al., 1987). In order to combine the advantages of an *in vitro* system, where conditions can be well controlled, with maximum physiological relevence, experiments were carried out on freshly isolated thymocytes in primary culture. Heterogenous cultures are obtained which contain a mixture of cells; some may be actively growing, others in G_0 phases of the cell cycle, and in various stages of differentiation and maturity.

II.1.Assessment of Proliferation.

There are two readily distinguishable phases of the cell cycle where proliferative activity can be easily assessed. Some methods rely on observation of the division process itself, and thus measure mitosis directly. Other methods make use of the synthesis of DNA which takes place in the S phase. Certain molecules labelled with fluorescent or radioactive 'tags' may be added to cultures, and their incorporation into newly synthesized DNA observed and measured. Three methods of assessing proliferation in rat thymocytes were undertaken; one measuring mitosis, and two measuring DNA synthesis.

II.1.1. Mitosis in the "Rapidly Recruitable" sub-set of Thymocytes.

Cells may be arrested in metaphase of mitosis by agents which interfere with the formation of the 'spindle'; a formation of microtubules essential for the successful separation of chromosomes which accompanies cell division. Cells thus arrested are visually distinguishable from others by their large size and characteristic 'raspberry-shaped' nucleus which stains

heavily with haematoxylin. The metaphase arresting agent colchicine was therefore used to assess the proliferative activity of the 'rapidly recruitable' sub-set of thymocytes.

Thymus glands from young (100-150g), male, albino rats of the Wistar strain were rapidly excised under ether anaesthesia. The glands were rinsed twice in tissue culture medium, and then minced in approximately 1.0ml of medium. The resultant cell concentrate was filtered through four layers of moistened cheesecloth to remove cell aggregates and reticulum fragments. The cell density was then adjusted to 5×10^7 cells/ml, by dilution with medium.

The basal medium was calcium and magnesium free Medium 199 (Wellcome Ltd.), buffered to pH 7.2 with 13mM sodium bicarbonate and 20mM Hepes (Flow Labs. Plc.). The metaphase arresting agent colchicine (Ciba Ltd.) was added to a final concentration of 6.2×10^{-5}M. This is sufficient to cause spindle disruption without effecting entry into mitosis. (Whitfield et al, 1974). $CaCl_2$ and $MgSO_4$ were added to cultures, where appropriate, from concentrated stock solutions. The concentration of the stocks were such that addition of a 10µl aliquot to a 1.0ml culture gave a final Ca^{++} concentration of 0.6mM, and a final Mg^{++} concentration of 1.0mM, chosen to mimic the concentration of these ions in rat blood (Perris et al, 1971). Addition of human recombinant insulin (Actrapid, Novo industri A/S) was also in 10µl aliquots from dilutions of a 10^{-4}M stock solution. Preliminary studies showed that the preservative present in the insulin solution had no effect on mitosis in these cells.

1.0ml aliquots of cell suspension, plus any appropriate 10µl aliquots of insulin/ions, were incubated in 10ml sterile culture tubes (Sterilin Ltd.) at 37°C. These were rotated about their long axes at 30 revs/minute for 6 hours.

After incubation, three drops from each culture were placed on a microscope slide with one drop of newborn bovine serum (Gibco Ltd.) to

act as a binding agent. The serum/culture mix was smeared over the slide and dried in a warm air stream. The slides were then fixed in neutral (pH 7.4) phosphate buffered formalin for 20 minutes, and stained with Delafield's haematoxylin (BDH Chemicals Ltd.), using a procedure established by Whitfield, Brohée and Youdale (1969) (See Figure 15). The slides were then mounted in DPX (BDH Chemicals Ltd.), and the proportion of cells arrested in colchicine metaphase determined by microscopic examination.

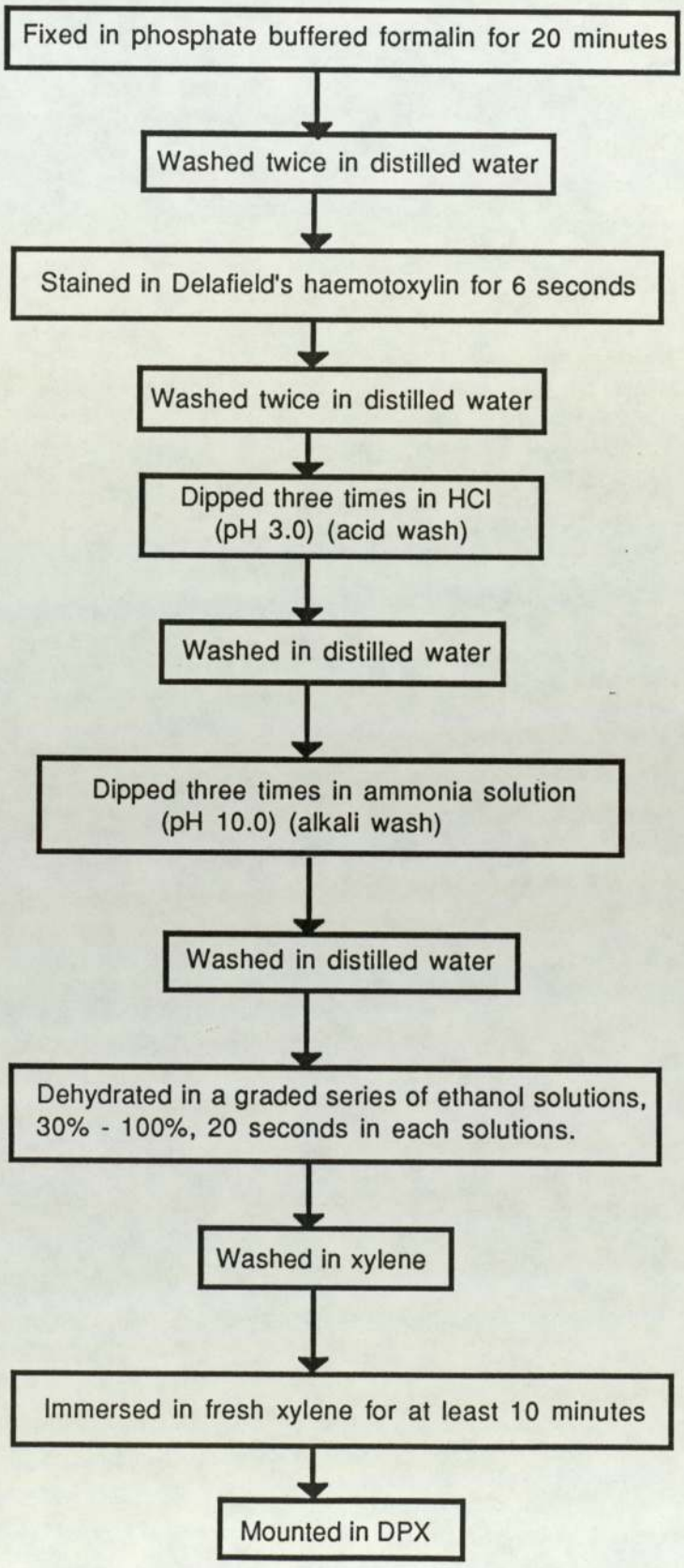
Each slide was scored by two individuals who each counted at least 1000 cells per slide. At basal ion concentrations approximately 4% of cells are arrested in colchicine metaphase, whereas when the external Ca^{++} concentration is raised to 1.8mM, this proportion rises to 6% (Perris et. al., 1985). These were therefore used as internal controls in all experiments.

II.1.2. DNA Synthesis.

II.1.2.1. [^{14}C]-Formate Uptake in the "Rapidly Recruitable" Sub-set of Thymocytes.

Measurement of the incorporation of tritiated thymidine (^{3}HTdR) into newly synthesised DNA has been found to be a successful method of assessing proliferation in numerous cell types (Rozengurt, 1982; Abboud et al, 1985; King et al, 1985). However, problems have been encountered, especially when using this method with certain lymphocyte types (Oliveira et al, 1974; Bodycote and Wolff, 1986). Youdale and Macmanus (1975) have studied the response of "rapidly recruitable" thymocytes, and have suggested that incorporation of ^{3}HTdR fails to reflect their proliferative activity. They suggest that mitogenic stimulation enhances the activity of the thymidylate synthetase enzyme which catalyses *de novo* thymidine synthesis, and this is incorporated into new DNA in preference to exogenous thymidine. They further suggest that

Figure 15. Flow Chart to Show Haematoxylin Staining Procedure.



incorporation of ^{14}C-formate into DNA is a more reliable method of assessing DNA synthesis in these cells.

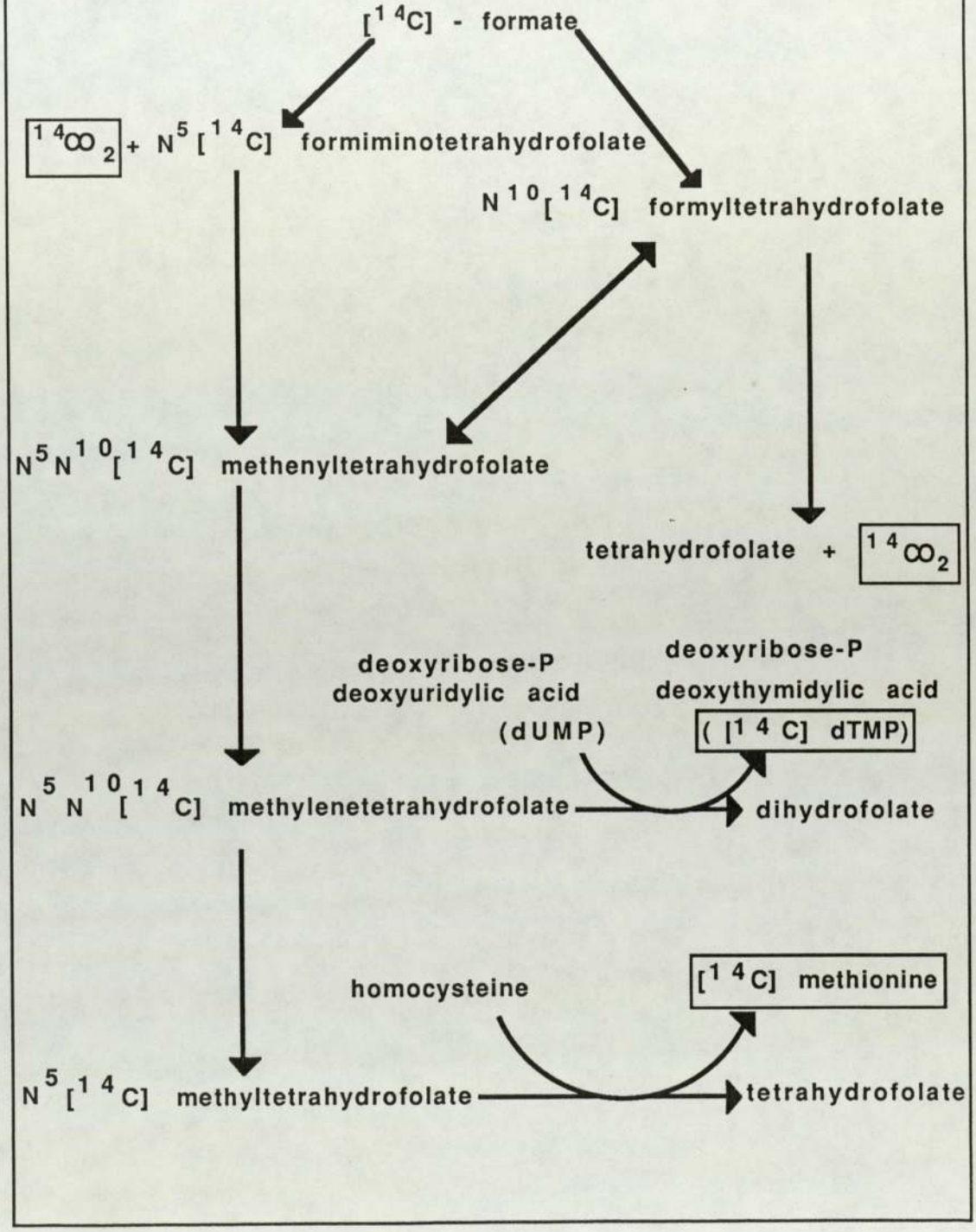
Formate uptake has previously been used as a measure of DNA synthesis in the study of blood precursor cells and lymphocytes. It has been used in methods which count total radioactive incorporation (Totter, 1954; Thomas and Lochte, 1957; Scott, 1962), and also autoradiographically (Osogoe and Yanagi, 1982). In none of these examples however, was the DNA extracted, or a DNAase enzyme used to ensure only incorporation of ^{14}C into DNA was being measured.

^{14}C-formate may be metabolised by cells in a number of ways as shown in Figure 16. After incubation, the ^{14}C may be located in $^{14}CO_2$, [^{14}C]methionine or [^{14}C]dTMP. The $^{14}CO_2$ will diffuse out of cells in suspension culture, and any remaining in the cytoplasm will be small in quantity, and constant despite variations in incubation times. However, assessment of total radioactive incorporation represents a measure of both DNA and protein synthesis. Nevertheless, radioactive incorporation of [^{14}C]-formate into the cells was investigated, and an attempt made to determine what proportion of radioactivity was associated with DNA.

Thymocytes were isolated from 100-150g albino, male, Wistar rats as previously described, and their density in medium 199, with normal [Ca^{++}] and [Mg^{++}], adjusted to 2×10^8 cells/ml. Cell suspensions were placed in 10ml sterile culture tubes (Sterilin Ltd.), and rotated about their long axes at 30 revs/min at 37°C for 20 minutes to allow the cells to recover from the trauma of isolation. An equal volume of medium containing [^{14}C]-formate (New England Nuclear) at 2μCi/ml was then added to the cultures.

To measure total radioactive incorporation into the cells, three 200μl aliquots were removed from the incubation tubes at appropriate times, and layered above 175μl of MS 550 silcone fluid (BDH Chemicals Ltd.) in 400μl microcentrifuge tubes (Beckman Ltd.). The tubes were then spun at

Figure 16. Cellular Metabolism of [^{14}C]Formate.
End products containing ^{14}C are boxed.

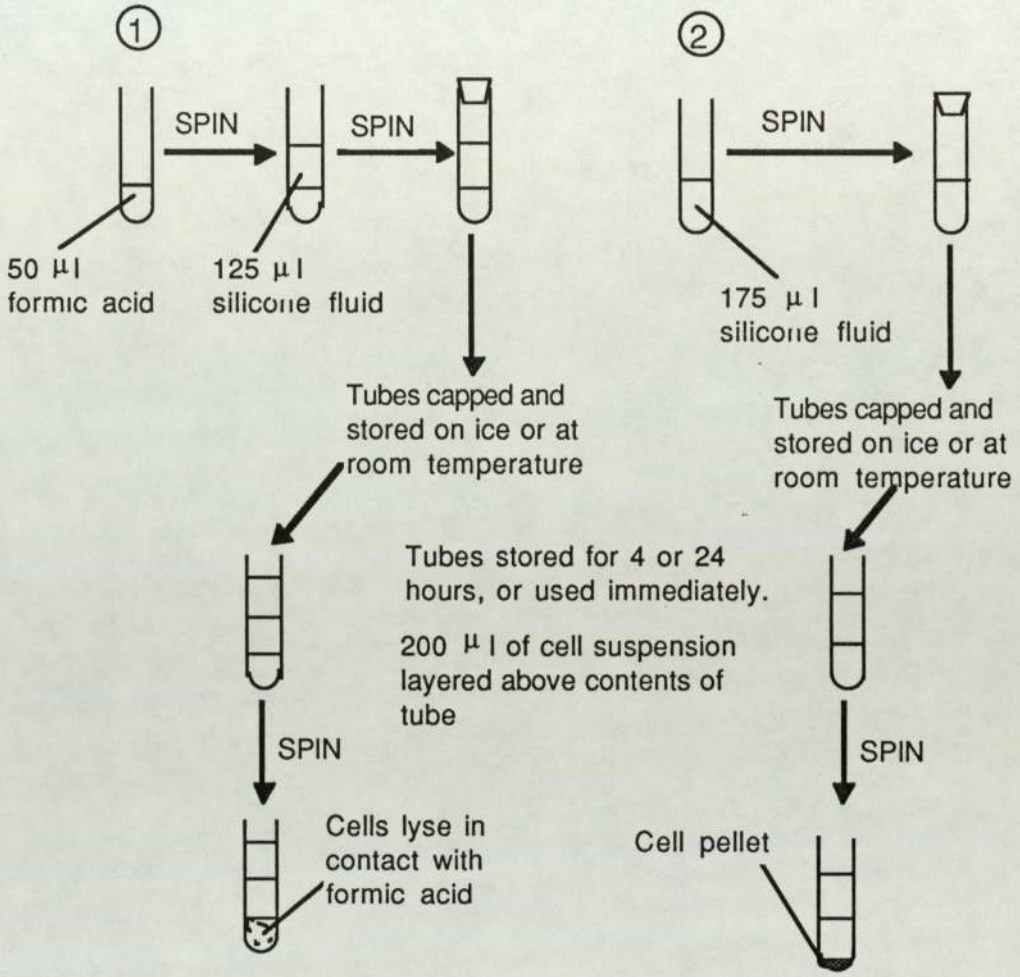


1000 revs/min for 1.5 minutes in a microcentrifuge (Beckman Ltd.). Of the methods tested, this treatment most effectively separated cells from medium; the cells passed through the silicone fluid to form a pellet at the bottom of the tube, while the medium remained above the silicone fluid (See Figure 17). The tubes were then frozen in liquid nitrogen, and the ends containing the cell pellets cut off and placed in scintillation vials. 3ml of scintillant NE260 (Nuclear Enterprises Ltd.) was added to each vial, and the radioactivity counted using a Packard Liquid Scintillation System.

In each experiment at least three tubes were layered with medium containing the radioactive isotope, but no cells. These tubes were treated as above, and absence of radioactivity in the cut ends was assumed to denote no passage of medium through the silicone fluid layer. Also, for each treatment, three 200µl aliquots of cell suspension were removed from the incubation tube immediately after addition of the [^{14}C]-formate, and treated as above. Counts generated by these samples were considered to represent formate bound to the cell membranes rather than incorporated into macromolecules. The mean of this triplicate was therefore subtracted from other figures prior to analysis.

To assess the proportion of radioactivity associated with DNA, aliquots were removed from the incubation tubes at appropriate times and added to 100% ethanol, to give a final ethanol concentration of 70%, in order to fix the cells (Gray and Coffino, 1974). Tests showed that this treatment did not cause clumping of the cells, provided cells were added to ethanol with constant agitation. The cells were then washed twice, and resuspended in physiological saline (PS) (see Appendix 1). The resultant cell suspensions were divided into two equal parts, and DNAase I from bovine pancreas (Sigma Chemical Co.) was added to one of each pair of tubes, to a final concentration of 30 Kunitz units/ml. Since Mg^{++} is required as a co-factor in DNAase action, the [Mg^{++}] of all suspensions was raised to 4.2mM by

Figure 17. Test of Methods of Preparing and Storing Microcentrifuge Tubes in Order to Obtain the best Separation of Cells and Medium from an Incubation with a Radioactive Isotope.



Irrespective of time or temperature of storage, on occasions there is contamination of the lysate by medium containing radioisotope

METHOD REJECTED

Consistent counts obtained, and remain unchanged if pellet solubilised with protosol prior to counting (New England Nuclear). Changes in time and temperature of storage have no effect.

METHOD ADOPTED (without solubiliser)

addition of an appropriate amount of stock $MgSO_4$. The tubes were then incubated overnight at 37°C, rolling about their long axes at 30 revs/min.

The cells were then washed three times in PS, and the cell density adjusted to 10^8 cells/ml. 200µl aliquots from each tube were added to 3ml NE260 in scintillation vials, and radioactivity counted as described above.

II.1.2.2. Tritiated Thymidine (^{3}HTdR) Incorporation in Longer-Term Cultures.

In addition to the 'rapidly recruitable' subset of thymocytes, another group of G_0 cells may respond to mitogenic stimulation over a longer time scale. It was found that measuring incorporation of ^{3}HTdR was a successful method of assessing proliferation of thymocytes over 48 - 96 hours in culture. Thymidine is the only nucleotide exclusive to DNA. Hence tracing its incorporation into cellular macromolecules, specifically indicates the extent to which cells are synthesising DNA.

A number of problems are associated with long-term tissue culture, which are less apparent in the short-term cultures already described, since they do not become manifest within 6 hours. A low degree of contamination of cultures by micro-organisms, for example, may be ignored in short-term culture, but in longer-term cultures measures must be taken to avoid contamination. Bacteria and fungi have much shorter cell cycles than animal cell types, and will grow rapidly, using up the nutrients in the medium, secreting toxic waste products and exerting other effects deleterious to the growth of other cells. Aseptic technique is therefore essential in setting up and manipulating the cultures, and they must be kept in a sterile environment. Hence all equipment used in handling animals and cultures was either pre-sterilised by the manufacturer and suitably packed for transport and storage, or autoclaved on site. Any solutions added to cultures were filtered through a 0.2µm micropore filter (Flow Labs Ltd.). Manipulations of cells and cultures was

undertaken in a Hepaire laminar air-flow tissue culture hood, and as a final precaution against bacterial contamination, medium was supplemented with the antibiotics streptomycin (100IU/ml) and penicillin (100µg/ml) (Flow Labs Ltd.).

Although cells may grow and be maintained in nutrient media for some hours, it was observed early in the development of tissue culture systems that addition of serum greatly enhanced the growth potential and viability of cells over longer time scales. Preliminary experiments showed that in order to maintain >90% viability (measured by trypan blue exclusion, see Appendix 2) of thymocytes over 72 hours, the addition of at least 5% foetal calf serum (FCS) to the basic medium was necessary. All cultures were therefore grown in medium 199, buffered as described in section II.1.1., and supplemented with antibiotics and 5% FCS (Gibco Ltd.).

Thymocytes were isolated as described in section II.1.1., except that aseptic technique was observed. The cells were washed in medium, and the cell density adjusted such that after addition of all solutions, the final cell density would be 5×10^6 cells/ml. The cell suspension was then aliquoted into 96 well microtitre plates (Flow Labs Ltd.), 50µl of cell suspension/well. Solutions of mitogens, concanavalin A (ConA) (Sigma), human recombinant insulin (Actrapid Novo industri A/S) and synthetic insulin-like growth factor I (IGF I) (24 - 41) (Peninsula Labs Inc.), diluted in medium, were then added to the cultures in the wells, to make a final volume of 100µl in each well. Triplicate cultures were set up for each set of conditions in each experiment. The cultures were then incubated in a sterile incubator at 37°C in an atmosphere of 95% air/5% carbon dioxide.

The half-life of circulating insulin *in vivo* is 2 - 5 minutes (Duckworth and Kitabchi, 1981). Most degradation of insulin however involves insulin binding and internalisation, and takes place in the liver and kidneys. Nevertheless, there may be insulinases in the tissue culture system which significantly reduce the concentration of insulin over 48 -

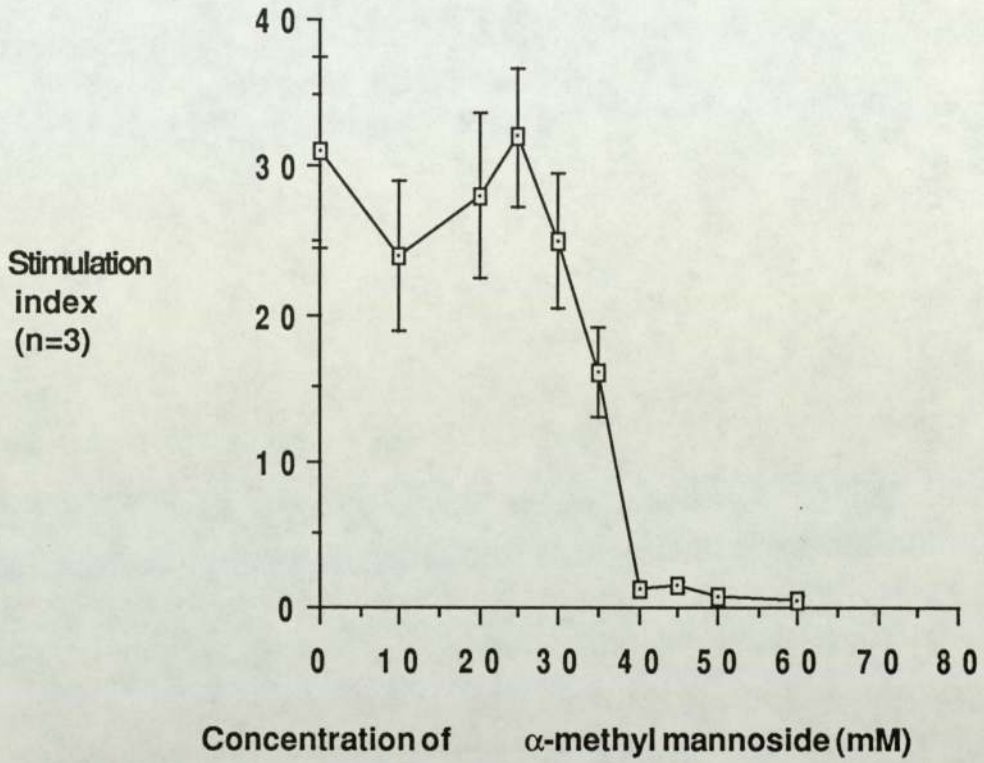
96 hours. The concentration of insulin in cultures of different ages was therefore measured by radioimmunoassay. This procedure was kindly undertaken by Dr. David Lambert, and the half-life of insulin in these thymocyte cultures found to be about 18 hours. Fresh solutions of insulin were therefore added to cultures periodically to maintain its concentration at an appropriate level throughout the experiment.

Four hours before the end of the incubation 1μCi of ^{3}HTdR (Amersham International Plc) diluted with medium to a volume of 50μl, was added to each well of the microtitre plate. At the end of the incubation the cells from each well were harvested onto individual filter mats (Skatron A/S) using a Titertek Cell Harvester (Flow Labs Ltd.). The filter mats were dried, and each section, with the adherent harvested cells, was placed in a scintillation vial containing 5ml of a toluene based scintillant (See Appendix 1). Radioactivity was then counted using a Packard Liquid Scintillation System.

Where cultures were Ca^{++}-free, the specific Ca^{++} complexing agent, 1,2-Di(2-aminoethoxy)ethane-N,N,N',N'-tetra-acetic acid (EGTA) was used to chelate out the ion. A Corning EEL Calcium Analyser was used to find the exact amount of EGTA required to just chelate out all the Ca^{++} in a specific volume of the medium. Free Ca^{++} was replaced in EGTA treated medium by addition of an appropriate volume of concentrated $CaCl_2$ solution.

On some occasions cultures were treated sequentially with ConA and then another putative mitogen or other agent. ConA treated cultures were first set up in 50ml tissue culture flasks (Flow Labs Ltd.), and before addition of the second mitogen, the cells were washed twice with fresh medium, and the ConA-complexing agent α-methyl-mannoside (Sigma) added to a concentration of 40mM (see Figure 18). The cells were again washed twice, and the cell density re-adjusted before cultures were set up with the second mitogen, in microtitre plate wells as described above.

Figure 18. Calculation of the Concentration of α-methyl mannoside Required to Inhibit the Mitogenic Activity of 0.75µg /ml ConA on Rat Thymocytes.



The stimulation index represents the number of counts generated by a treatment, as a multiple of the number of counts generated under control conditions (ie. in the absence of ConA). Hence control stimulation index is always 1.0.

From the graph it can be seen that 40mM α-methyl mannoside is the lowest concentration which completely inhibits the mitogenic action of 0.75µg/ml ConA. This concentration of α-methyl mannoside was therefore used, where necessary, to remove ConA from cultures.

II.2. Measurement of Metabolite Uptake.

In many systems insulin and other growth factors have been shown to have a number of effects on cellular carbohydrate and protein metabolism. In order to study these effects and their relationship with growth control, glucose and amino acid uptake into cells were chosen as representative phenomena associated with carbohydrate and protein metabolism respectively.

Uptake of radioactively labelled analogues of these precursor molecules, which are transported into cells through the same mechanism as their physiological counterparts was measured. The amount of radioactivity associated with the cells after incubation with the radioactive analogue, allows an estimation to be made of the amount of glucose/amino acid uptake which had taken place during that incubation. To avoid problems which may occur through further metabolism of the radioactively labelled molecules, analogues were chosen which are taken up into the cells, but are not further metabolised.

II.2.1. Glucose Uptake.

A radioactively labelled glucose analogue, 3-ortho-methyl-D-[U-^{14}C]glucose (^{14}C3-OMG), was used to trace the uptake of glucose into the cells. This molecule is taken up by cells via the same carrier as D-glucose, but is not further metabolised (Whitesell and Gliemann, 1978).

Rat thymocytes were isolated as previously described, but instead of medium 199, physiological saline (See Appendix 1), buffered to pH 7.4 and with the glucose substituted by 1.0mM unlabelled 3-OMG, was used as a culture medium. The use of medium 199 was inappropriate because D-glucose, present in the culture medium, has a much higher affinity for the membrane carrier molecule than 3-OMG. D-glucose is thus taken up in preference to 3-OMG, and at most concentrations, effectively prevents

uptake of 3-OMG into cells. Viability tests using trypan blue exclusion (See Appendix 2), indicated >90% viability of rat thymocytes after 4 hours maintenance in physiological saline. Nevertheless, medium 199 was used as the culture medium in pre-incubations, and cells were washed twice and resuspended in physiological saline, prior to testing ^{14}C3-OMG uptake.

A method similar to that described for ^{14}C-formate incorporation was employed to measure ^{14}C3-OMG uptake. Incubation tubes (10ml, Sterilin Ltd.) were set up containing 10^8 cells/ml and 1.5μCi/ml ^{14}C3-OMG (Amersham International Plc) in physiological saline. At appropriate times triplicate 200μl aliquots were removed and layered above 175μl MS 550 silicone fluid in microcentrifuge tubes. The tubes were spun, and the radioactivity associated with the cell pellet counted in the same way as described for ^{14}C-formate.

In each experiment at least three tubes were layered with medium containing the radioactive isotope, but no cells. These tubes were treated as above, and absence of radioactivity in the cut ends was assumed to denote no passage of medium through the silicone fluid layer. Also, for each treatment, three 200μl aliquots of cell suspension were removed from the incubation tube immediately after addition of the [^{14}C]3-OMG, and treated as above. Counts generated by these samples were considered to represent [^{14}C]3-OMG bound to the cell membranes. The mean of this triplicate was therefore subtracted from other figures prior to analysis. In addition, radioactivity associated with 200μl of the medium containing a known amount of 3-OMG and ^{14}C3-OMG was also measured, in order to calibrate the results.

Insulin was added to cultures as small volumes from dilutions of a 10^{-4}M stock solution prepared from human recombinant insulin. Where pre-incubations with insulin were undertaken, the concentration of

insulin was adjusted to remain at the stated concentration throughout the pre-incubation and uptake test.

II.2.2. Amino Acid Uptake.

Amino acid uptake was assessed in a similar way, using the radioactively labelled amino acid analogue 2-amino [1-^{14}C] isobutyric acid (^{14}CAIB). This molecule is transported into cells via the insulin-sensitive, amino acid transport system 'A', but is not incorporated into proteins (Woods and Dandona, 1984).

Isolation of cells, and incubation media were as described for ^{14}C3-OMG; pre-incubations taking place in medium 199, and uptake tests in physiological saline. However, glucose (5mM) and AIB were added to physiological saline for use in these experiments. The final concentration of AIB in cultures was 1mM unless otherwise stated.

Insulin, ConA and IGF I were added to cultures as small volumes from dilutions of concentrated stock solutions. Pre-incubations of over 4 hours duration were set up aseptically in 50ml tissue culture flasks (Flow Labs Ltd.), using medium 199 with 5% foetal bovine serum as described in section II.1.2.2. At appropriate times, samples were withdrawn from these cultures, the cells washed twice and resuspended in either physiological saline or medium 199 without serum, depending on whether any further pre-incubation was required. ConA was removed from cultures using α-methyl mannoside as described in section II.1.2.2.

To measure ^{14}CAIB uptake, incubation tubes (10ml, Sterilin Ltd.) were set up containing 10^8 cells/ml and 0.5μCi/ml ^{14}CAIB (Amersham International Plc.) in physiological saline. Triplicate 200μl aliquots were withdrawn at appropriate times, and treated as described for [^{14}C]-formate and ^{14}C3-OMG.

Where cultures were pre-incubated with insulin, ConA or IGF I, an appropriate concentration of the agonist was maintained throughout all

stages of the experiment unless otherwise stated. Ca^{++} and Mg^{++} free cultures were pre-incubated in Ca^{++} and Mg^{++} free medium 199 (Wellcome Ltd.), and tested in physiological saline made up without these ions. Where appropriate, the ions were added to the medium/physiological saline from concentrated solutions as described in section II.1.1.

In each experiment, samples were tested for passage of isotope through the oil layer, membrane bound isotope and calibration as for ^{14}C3-OMG.

II.3. Enzyme-Linked Immunological Techniques for the Detection of Insulin Receptors.

Immunological reactions are frequently used for assays or detection procedures because they can give high levels of specificity and sensitivity. Generally, antibodies specific for the molecular species to be detected, are labelled with an easily distinguishable marker. Fluorescent and radioactive markers have been used extensively, and a number of methods for detection, quantitation and localisation of substances have been developed. Although these methods are very successful, they have some disadvantages. Immunofluorescence, for example, is time consuming, difficult to automate, requires special equipment to read the results, and is relatively insensitive. Reagents for radioimmunoassay are expensive, have a short shelf life, and special safety measures must be observed in their handling and disposal.

Some of these disadvantages can be overcome by using enzymes conjugated to antibody molecules. The specificity of the method depends on the discriminating properties of the antibody, while detection is acheived through the properties of the enzyme. The enzyme molecules cannot be detected directly, but they can be used to catalyse reactions which involve the conversion of a colourless substrate into a coloured product. The coloured product forms the basis for measurement, and can

be assessed visually or spectrophotometrically. (See Schuurs and Van Weemen, 1977; Malvano, 1980).

Two methods were developed which take advantage of these principles. Both were designed to detect insulin receptors, in order to evaluate previous evidence which suggests that their expression may be associated with activation of T-lymphocytes. Both procedures make use of similar reagents and sequential binding steps. Immuno-peroxidase staining allows the visualisation of receptors, whereas the enzyme-linked immunosorbent assay (ELISA) allows more objective assessment of the number of receptors.

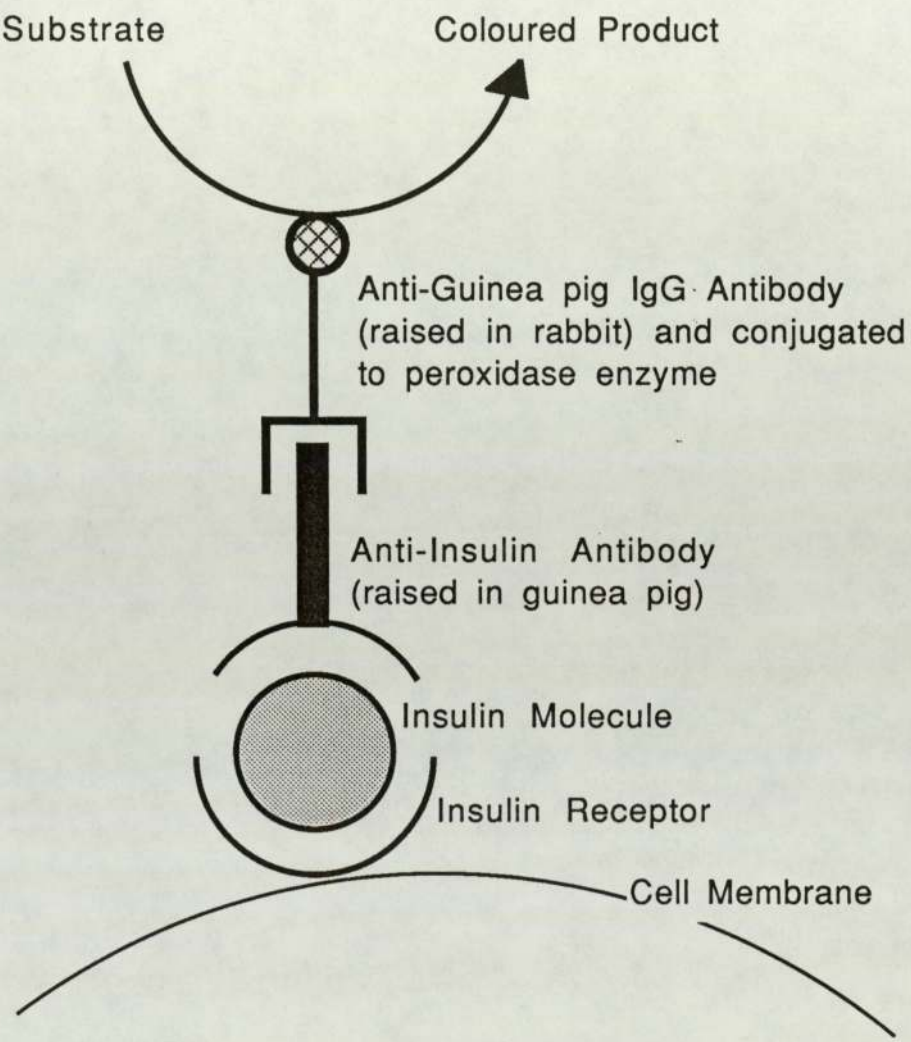
II.3.1. Immuno-peroxidase Staining of Insulin Receptors.

The main objective of the method was to link insulin receptors, present on the cell surface, with peroxidase molecules, so that after substrate conversion, presence of the coloured product would specifically indicate presence of insulin receptors. The link was acheived indirectly, using three agents which are characterised by their ability to bind specific proteins. Insulin, which binds specifically to the insulin receptor, an anti-insulin antibody, which binds insulin, and a second antibody which binds the first antibody. The second antibody was conjugated to a peroxidase molecule in such a way that neither the specific binding capacity nor the enzymic function was impaired in the hybrid molecule. Sequential addition of these three agents to cell preparations provided the required link between insulin receptors and peroxidase molecules. (See Figure 19).

By permitting indirect visualisation of insulin receptors, the method allowed the presence and location of the receptors on thymocyte cell membranes to be ascertained. Since the intensity of staining depends on the number of bound enzyme molecules, a subjective assessment of the number of insulin receptors present can also be made.

Figure 19. Principle of Detection of Insulin Receptors Using Indirect Immunoperoxidase Techniques.

The principle is the same for staining and Elisa methods



Rat thymocytes were isolated aseptically, and cultured in 50ml tissue culture flasks (Flow Labs Ltd.) in medium 199 with 5% serum, with or without 0.75μg/ml ConA , as described in section II.1.2.2.

After 24 hours incubation, the cells were washed twice in tris buffered saline (TBS) (See Appendix 1). Cultures were treated with α-methyl mannoside to remove ConA, as described in section II.1.2.2., and the cells were resuspended in TBS at a density of 10^7 cells/ml.

Samples were taken from each culture, and four drops smeared onto each of a number of microscope slides. The smears were dried in a warm air stream, and fixed by immersion in formol buffered acetone (See Appendix 1), for 10 minutes. The slides were then rinsed in distilled water and immersed in TBS for 5 minutes.

The slides were subsequently immersed in a bath containing 300ml absolute methanol and 75ml of 3% hydrogen peroxide, for 20 minutes. This was made up immediately before use, and serves to eliminate any endogenous peroxidase activity. The slides were then rinsed in distilled water and immersed in TBS for 5 minutes.

After the hydrogen peroxide treatment, the slides were laid flat in a humidity chamber, and 4 - 6 drops of non-immune foetal bovine serum, diluted 1:40 with TBS, were pipetted over each smear. The slides were left in the humidity chamber for 20 minutes. The proteins in the serum attach to highly charged sites on the cell membranes which would bind any protein. This non-specific 'serum pre-treatment', before addition of any reagent which binds to a specific protein, therefore helps to reduce non-specific, background staining.

The excess serum was carefully drained off, but the slides were not washed. The slides were again laid flat, and 4 - 6 drops of insulin (10^{-6}M) were pipetted over each smear. The slides were left in the humidity chamber for 20 minutes, during which time insulin molecules bind to any insulin receptors on the cell membranes. The slides were rinsed twice

in distilled water, once in TBS, and then immersed in TBS for 5 minutes. This 'full' washing procedure was designed to remove any insulin which was not bound to specific receptors.

The slides washed were next pre-treated with serum, as described, and 4 - 6 drops of guinea pig anti-insulin antibody (Dako Ltd.), diluted 1:200 in TBS, pipetted over each smear. The slides were laid flat in a humidity chamber for 20 minutes, during which time the anti-insulin antibody binds to the bound insulin molecules. The slides were then fully washed.

After serum pre-treatment, 4 - 6 drops of peroxidase conjugated rabbit anti-guinea pig IgG (Dako Ltd.), diluted 1:40 with TBS, was pipetted over each smear. The slides were laid flat in a humidity chamber for 20 minutes, during which time the anti-IgG antibody binds to the anti-insulin antibody. The slides were then fully washed. The optimal dilutions of the antibodies for use in this system, were determined by checkerboard titration (see Figure 20).

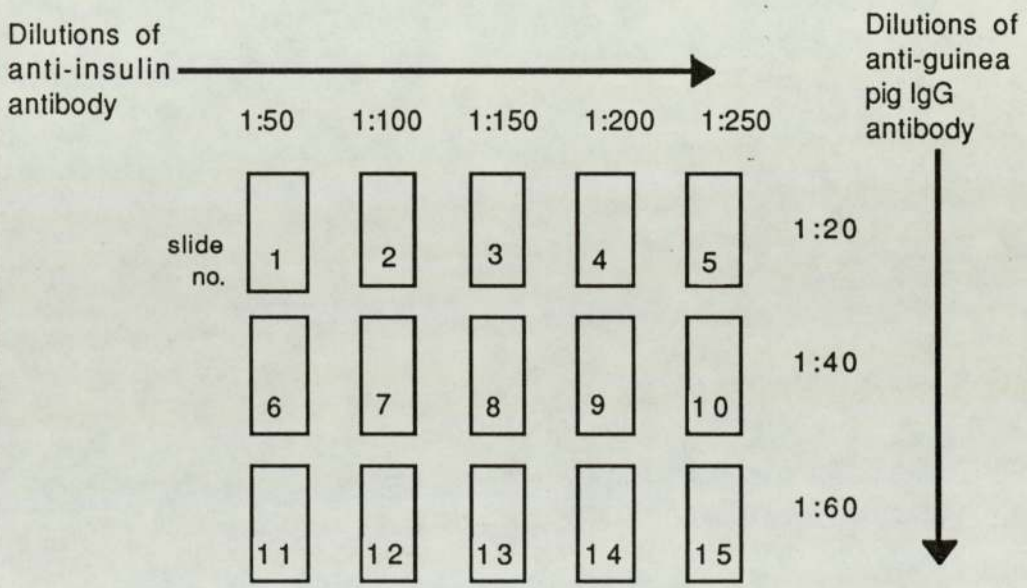
The final stage of this initial staining was to provide a substrate for the enzyme, horseradish peroxidase, which was indirectly bound to the cell membrane insulin receptors. 4 - 6 drops of 3,3-diaminobenzidine tetrahydrochloride (DAB) (Dakopatts A/S) substrate solution (See Appendix 1) were pipetted over each smear, and the brown colour allowed to develop. Each bound enzyme may catalyse the conversion of a large number of substrate molecules, hence the colour becomes more intense with time. The reaction was followed by observing a representative slide under a microscope. When a suitable colour had developed on the sample slide, after a few minutes, the reaction was terminated by immersion of all the slides in distilled water.

The smears were counterstained with Harris's haematoxylin, and mounted in DPX as described in section II.1.1. The purple counterstain provides a contrast with the brown product of peroxidase activity, allowing easy identification of positive and negative specimens.

Figure 20. Setting up of Checkerboard Titration to Find Optimal Dilution of Antibodies for Immuno-Peroxidase Staining.

To acheive meaningful results in the immuno-peroxidase staining technique, optimal dilutions of antibodies must be used. The dilution for any antibody depends on a number of factors including the antibody titre in the original solution, presence of contaminating proteins, the choice of diluent and processing protocol, and the time of incubation. Although higher dilutions of antibodies can generally be used if the length of incubation is increased, practical considerations limited incubation times here to twenty minutes.

Slides of known positive samples were prepared as described in the text, but with dilutions of antibodies as follows:



The optimal dilutions of the two antibodies are represented by the slide with the most intense specific stain, and the least amount of background staining. Slide no. 9 best fulfilled these requirements, so dilutions of antibodies were chosen:
anti-insulin 1:200, anti-guinea pig IgG 1:40.

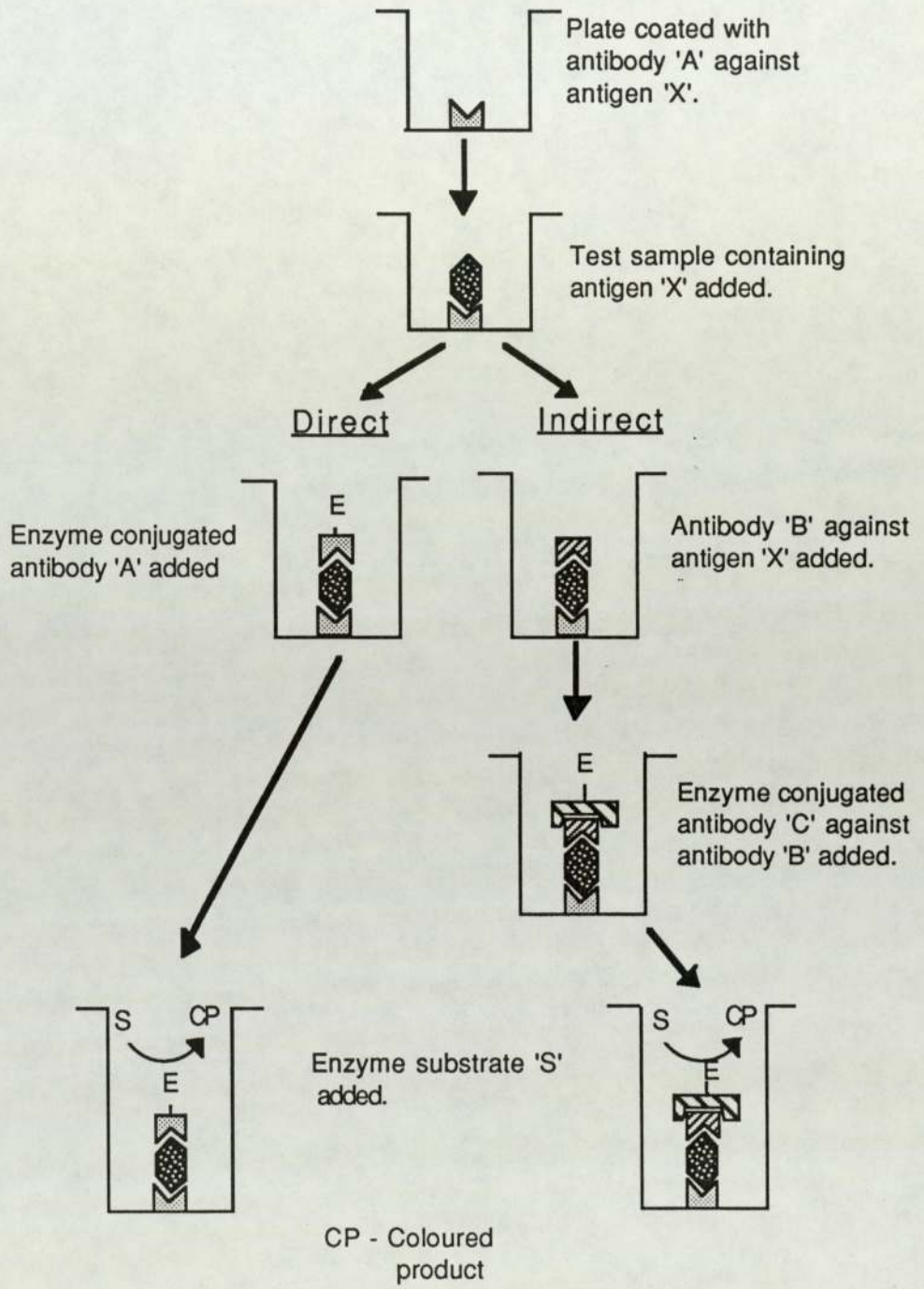
To ensure the specificity of the procedure, slides were made where treatment with one of the specific binding reagents was substituted with treatment with foetal bovine serum. It was found that if either the insulin, anti-insulin antibody, or peroxidase conjugated anti-IgG antibody binding stages were substituted in this way, the chain of specific molecules, bound as shown in Figure 19 was broken, and no specific peroxidase staining took place.

II.3.2. Enzyme-Linked Immunosorbent Assay (ELISA) for the Detection of Insulin Receptors.

Most ELISA procedures for the assay of antigen involve the adsorption of antibody molecules onto the surface of wells in microtitre plates (WHO, 1976; Voller et. al., 1979). Samples suspected of containing antigen are then placed in the antibody-coated wells, and any antigen present will be bound to the solid phase. Detection and quantification of the bound antigen can then be undertaken, directly or indirectly, using an enzyme labelled antibody (see Figure 21). However, some peculiarities specific to the system under discussion, and other practical considerations, necessitated some modifications to this procedure.

Since neither an anti-insulin receptor antibody nor an enzyme-conjugated anti-insulin antibody was available, it was not possible to use the direct method. The indirect method, as shown in Figure 19, requires two different antibodies against the same antigen. Anti-insulin antibodies however, are difficult to produce, since the molecule is very similar in most mammalian species. It therefore requires prolonged exposure to 'foreign' insulin before any immune response is initiated, and the antibody titre may be very low. The only known exception is the guinea pig, where a molecule closely related to PDGF has evolved to subserve the functions normally associated with insulin in other mammals. The guinea pig therefore mounts a high titre antibody response to insulin, and

Figure 21. Direct and Indirect ELISA Techniques for Antigen Assay, Using Antibody-Coated Wells.



all commercially available anti-insulin antibodies are produced in the guinea pig. Although it is theoretically possible to isolate clones of monoclonal antibodies directed towards different epitopes on the insulin molecule, it was considered simpler, less time consuming and less expensive, to modify the technique in order to make use of more readily available reagents.

The method adopted made use of the same principles and reagents as the immunoperoxidase staining method. The wells of microtitre plates were coated with a thin layer of cells, and the other binding reagents added sequentially to form links as shown in Figure 19. A different substrate for the peroxidase enzyme was used which formed a soluble orange coloured product. The intensity of the colour in the final solution in the wells is proportional to the number of insulin receptors available for binding in the cell layer, and was measured spectrophotometrically.

Cells were isolated aseptically and cultured in medium 199 with 5% foetal bovine serum in 50ml tissue culture flasks as described in section II.1.2.2. ConA (0.75µg/ml) was added to cultures as a small volumes (10µl/ml of culture) from concentrated stock solutions.

At appropriate times during incubation, samples of cells were withdrawn from the cultures, washed twice and resuspended in TBS (see Appendix 1) at a density of 10^5 cells/ml. ConA was removed using α-methyl mannoside as described in section II.1.2.2. The cell suspension was pipetted into flat-bottomed wells in 96 well tissue culture treated microtitre plates (Flow Labs Ltd.), 50µl/well. The plates, without lids, were placed overnight in a hot room at 37°C, allowing the liquid to evaporate to leave cell-coated wells.

Formol buffered acetone (see Appendix 1) (100µl) was pipetted into each well, and the plate placed in a humidity chamber for 15 minutes. This process fixes the cells. The plate was then washed by filling all the wells with Tween buffer (see Appendix 1), leaving the plate in a humidity

chamber for 5 minutes, then inverting and tapping the bottom of the plate, to eject all the liquid. This process was repeated three times between addition of each agent to ensure that all except tightly bound molecules were removed.

A freshly made up solution of 4 volumes of absolute methanol: 1 volume of 3% hydrogen peroxide, was added to the plate, 100µl/well, and the plate placed in a humidity chamber for 20 minutes. This process eliminates any endogenous peroxidase activity. The plate was then washed three times.

Insulin (10^{-6}M), anti-insulin antibody diluted 1:500, and anti-guinea pig IgG diluted 1:250, all diluted in Tween buffer (see Appendix 1) were added sequentially, 100µl to each well. Each reagent was incubated in a humidity chamber for 20 minutes, and the plate was washed three times between addition of each reagent.

The plate was washed three further times before addition of the substrate solution. The substrate chosen was orthophenylenediamine (OPD) (Dako Ltd.), which produces a yellow/orange colour on incubation with peroxidase. The OPD solution (see Appendix 1) was prepared shortly before use, and allowed to stand for 5 minutes, to ensure it was free from contamination with peroxidase. Presence of peroxidase in the OPD solution is indicated by a yellow colouration after a few minutes in daylight. 100µl of OPD solution was added to each well, and the plate placed in a humidity chamber away from light. After a few minutes, when a sufficient colour had developed, the reaction was stopped by adding 150µl of 1M sulphuric acid to each well.

It was important to ensure that each well was incubated with OPD solution for the same length of time. Intensity of colour is only proportional to the number of insulin receptors in the cell layer if all the bound enzymes have been in contact with substrate for the same time. Therefore when OPD solution and 1M sulphuric acid were added to the

wells, a multichannel pipette was used to ensure all wells were filled in the shortest time possible, and care was taken to ensure that substrate and stopping solution were added to wells in the same order.

The intensity of colour in each well was then determined by reading the plate on a Titertek Multikan Plus ELISA reader (Flow Labs Ltd.), using a 492nM monochromatic filter.

Optimal dilutions of antibody solutions were determined using a checkerboard titration method analogous to that described in Figure 20. The object was to determine the concentrations of the two antibodies where a maximum colour difference was generated between positive and negative samples. Isolated hepatocytes and erythrocytes were used as known insulin receptor-positive and negative cell types respectively (see Appendix 3 for details of isolation). Alternate rows of wells were layered with hepatocytes, erythrocytes and thymocytes, the plates were treated as described above but antibodies added such that each cell type was treated, in duplicate, with each dilution of both antibodies. Antibody dilutions were chosen where absorbance in the 'hepatocyte' wells was about ten times greater than that in 'erythrocyte' wells. 'Thymocyte' wells showed absorbances between these two extremes. Samples of freshly isolated hepatocytes and erythrocytes were also tested as reference samples, along with thymocytes in each experiment.

There are a large number of steps in this ELISA procedure, and hence there are a large number of potential areas where error could be generated. Since the sequential binding of the reagents is not necessarily 1:1, small differences in numbers of bound molecules, particularly in early stages, may be amplified. To test the replicability of the method, the 96 wells of one plate were coated with thymocytes from the same culture, and all treated identically as above. Theoretically the absorbance should be the same in each well of such a plate. It was found that the difference between the highest and lowest absorbance thus generated was 14%.

When the process was repeated, using different cultures to coat the wells of three further plates, errors of 19%, 15% and 14% were generated. This relatively high margin of error was accomodated partly by taking the mean of eight replicates for each sample in each experiment. Nevertheless, for any treatment to differ significantly from controls, values must differ by >20%.

II.4. Calcium and pH Measurements.

Although the mechanism of insulin action is unknown, a number of factors have been associated with its action in different systems. As a preliminary step towards identifying cellullar mechanisms which may play a role in transducing the insulin signal, its effects on cellular Ca^{++} and pH were investigated. These parameters were of particular interest because alterations in ion fluxes are associated with insulin action in a number of systems, and with growth control in general. The effects of insulin were therefore compared with the effects of ConA in this system.

Two methods of studying alterations in cellular Ca^{++} were undertaken. Firstly, its influx from the extracellular environment was investigated by tracing its uptake using the radioactive isotope $^{45}Ca^{++}$. Secondly $[Ca^{++}]_i$ was measured using the intracellular fluorescent Ca^{++} indicator, Fura 2. $[pH]_i$ was measured in a similar way using the indicator bis(carboxyethyl) carboxyfluorescein (BCECF).

II.4.1. Measurement of Calcium Influx.

The method is similar in principle and practice to those used for investigating the influx of metabolites (see section II.2.). During experiments $^{45}Ca^{++}$ was present as a fixed proportion of the total Ca^{++} in the medium. The amount of the isotope taken up by the cells during incubation, is therefore proportional to the total Ca^{++} uptake.

Rat thymocytes were isolated as described in section II.1.1., and their density in medium 199 without serum, but with normal Ca^{++} and Mg^{++}, adjusted to 2×10^8 cells/ml. Cell suspensions were placed in 10ml sterile culture tubes (Sterilin Ltd.), and rotated about their long axes at 30 revs/minute, at 37°C for 20 minutes, to recover from the trauma of isolation.

At the start of the uptake test, an equal volume of medium, containing 20μCi/ml $^{45}Ca^{++}$ was added to the cell suspensions in the culture tubes. Insulin and ConA, at appropriate concentrations, were also added to the cultures in the same solution as the isotope.

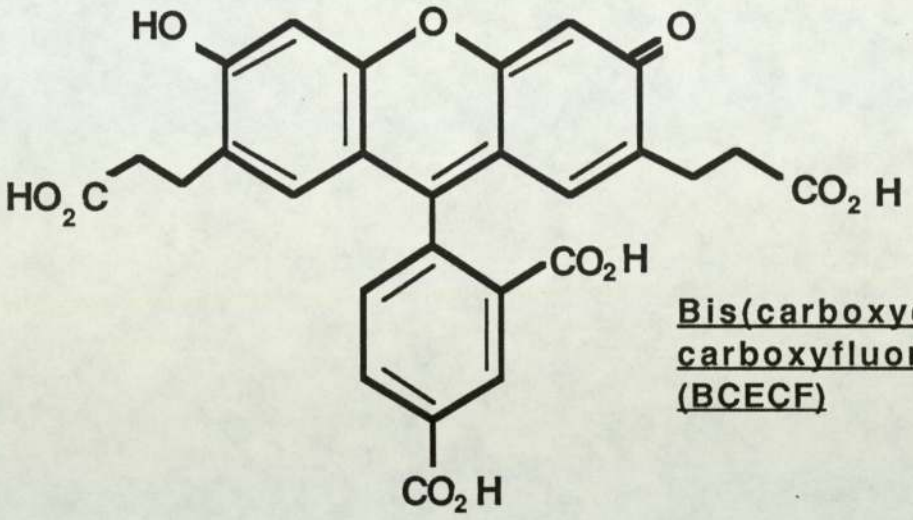
At appropriate times three 200μl aliquots were withdrawn from each tube, and treated as described for ^{14}C-formate in section II.1.2.1. Similar samples were also tested to determine that complete separation of cell sample from medium was acheived, and to ascertain the amount of radioactivity associated with isotope bound to the cell membranes. Calibration of the results was acheived by measuring the number of radioactive counts associated with a known amount of Ca^{++} and $^{45}Ca^{++}$.

II.4.2. Measurement of $[Ca^{++}]_i$ Using Fura 2.

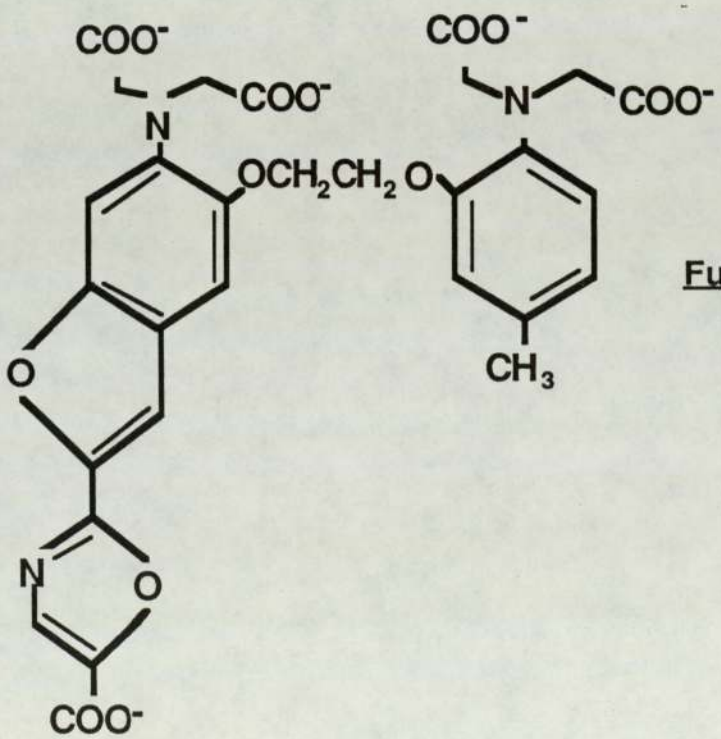
Although measuring the uptake of $^{45}Ca^{++}$ generates information about influx of Ca^{++} across the cell membrane, an increase in Ca^{++} influx does not necessarily indicate an increase in $[Ca^{++}]_i$. Ca^{++} efflux may also be enhanced, and intracellular redistributions of the ion may be stimulated. Use of intracellular Ca^{++} indicators however, gives direct measurements of $[Ca^{++}]_i$.

A number of indicators have been developed which may be trapped intracellularly, and which fluoresce in combination with Ca^{++} (Tsein et. al., 1984; Cobbold and Rink, 1987), but the most readily available are Quin 2 and Fura 2. Fura 2 (see Figure 22) was chosen because it fluoresces 30 times more brightly than Quin 2, allowing much lower loading

Figure 22. Structure of the Fluorescent Indicator Dyes BCECF and Fura 2.



Bis(carboxyethyl) carboxyfluorescein (BCECF)



Fura 2.

concentrations to be used. Fura 2 also has better Ca^{++}/Mg^{++} selectivity than Quin 2, and its higher dissociation constant (K_d), of 224nm means that at the normal resting $[Ca^{++}]_i$ for most cells, the dye is less than 50% saturated, which allows greater accuracy of measurements in the range 1 - 10 μM.

The most accurate $[Ca^{++}]_i$ measurements made with Fura 2 utilise dual excitation wavelengths (Tsein and Poenie, 1986; Cobbold and Rink, 1987). When the dye complexes with Ca^{++}, excitation efficiency at 340nm increases, while that at 380nm decreases. By measuring the signal at both wavelengths, a ratio can be obtained which is uniquely determined by the ratio of free and bound dye, and hence also $[Ca^{++}]_i$. This makes the method very accurate and sensitive, and eliminates most of the variation caused by instrumental fluctuations and changes in dye content which may occur through leakage during the course of an experiment.

However, the complex instrumentation required for fluorescence ratio imaging was unavailable. The fluorescent signal was therefore measured at only one wavelength, using a procedure similar to that routinely used with Quin 2 (Tsein et. al., 1982).

Rat thymocytes were isolated as described in section II.1.1., and their density in medium 199 with normal Ca^{++} and Mg^{++} and no serum, adjusted to 6×10^7 cells/ml. Cell suspensions were placed in 10ml sterile culture tubes and rotated about their long axes at 30 revs/minute at 37°C. The acetoxymethyl ester of Fura 2 (Fura 2 AM) (Calbiochem.), was added to a concentration of 5μM, from a concentrated stock solution in dimethylsulphoxide. The acid form of the dye is unable to permeate the cell membrane, so in order to acheive loading into the cytoplasm, cells are incubated with Fura 2 AM. Once inside the cells, non-specific esterases cleave the Fura 2 AM, generating the free dye, now trapped inside the cell.

After 15 minutes loading at 5μM Fura 2 AM, the cultures were diluted with medium 199, 1:10, and incubated for a further 30 minutes. 1.5ml

aliquots of the cell suspensions were withdrawn and spun in a Beckman microcentrifuge. The cells were resuspended in 3ml of physiological saline (see Appendix 1), and placed in a quartz cuvette.

Fluorescence was measured using a Perkin Elmer LS-5 Luminescence Spectrometer with excitation 340nm and emission 510nm. Cell suspensions were kept at 37°C by an integral heating system, and were constantly agitated by a magnetic stirrer during fluorescence measurements. Agents were added to the cell suspensions in the cuvette as small volumes from concentrated stock solutions.

At the end of each experiment 40µl of 10% triton X was added to the cell suspension in the cuvette. This lyses the cells, liberating the Fura 2, which then becomes saturated by the relatively high [Ca^{++}] of the extracellular medium, and the fluorescence signal increases to a maximum value (Fmax). 50µl of 0.5M EGTA, and 25µl of 10% (w/v) NaOH were then added. Under these conditions Ca^{++} is bound to EGTA in preference to Fura 2, and the fluorescence signal decreases to a minimum value (Fmin). This procedure enables the calibration
of the results using the formula:

$$[Ca^{++}]_i = \frac{F - Fmin}{Fmax - F} \times K_d\ (224nM)$$

where F = Fluorescence signal from cells under test,
and K_d = The dissociation constant.

II.4.3. Measurement of $[pH]_i$ Using Bis(carboxyethyl)carboxyfluorescein (BCECF).

In order to investigate the effects of agonists on $[pH]_i$ the fluorescent dye BCECF was used (see Figure 22). This allows direct measurement of $[pH]_i$ in a manner analogous to that described for Fura 2. The fluorescence signal from cells loaded with BCECF increases almost linearly from pH 6.5 to pH 8.0 (Madshus, 1988).

Rat thymocytes were isolated as described in section II.1.1., and their density in medium 199 with normal Ca^{++} and Mg^{++} and no serum, adjusted to 10^7 cells/ml. Cell suspensions were placed in 10ml sterile culture tubes and rotated about their long axes at 30 revs/minute at 37°C.

The acetoxymethyl ester of BCECF (BCECF AM) (Calbiochem.), was added to a concentration of 5μM, from a concentrated stock solution in dimethylsulphoxide. Loading of BCECF into the cytoplasm takes place as for Fura 2, the membrane permeable ester being trapped intracellularly by the action of cytoplasmic esterases. Loading with BCECF was continued for 30 - 40 minutes.

Aliquots were then withdrawn from the incubation tubes, centrifuged, and resuspended in the same volume of physiological saline (see Appendix 1). Fluorescence was measured as described for Fura 2, but with excitation wavelength set to 500nm, and emission wavelength set to 520nm.

To calibrate the samples, aliquots of cells loaded with BCECF were resuspended in high K^+ buffers (pH 6 - 8), containing the proton ionophore nigericin (10^{-5}M) (Sigma). Under these conditions, where intracellular and extracellular K^+ is approximately equal, nigericin allows equilibration of intracellular and extracellular pH.

II.5. Statistical Analysis.

Results were analysed using a Macintosh Plus microcomputer, and the programme StatView (Brainpower Inc.). One way analysis of variance (ANOVAR) was used to detect general trends in the data. Specific comparisons between individual items of data were made using independent paired or unpaired T tests.

Where appropriate, in the 'Results' (Section III.), the following notations are used to show significance:

* - $p > 0.1$

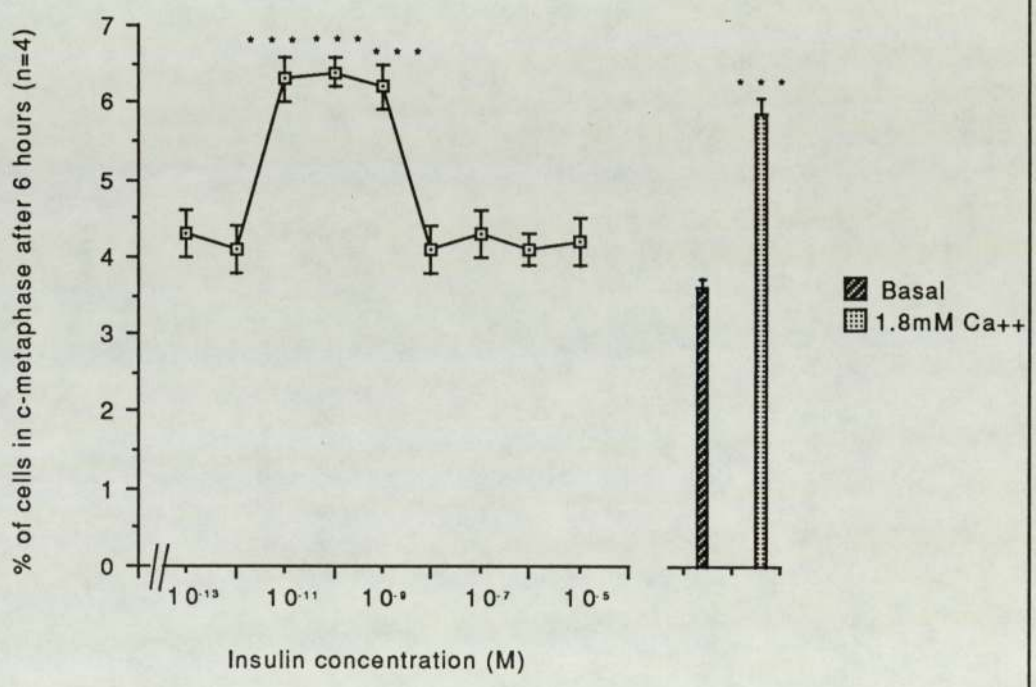
** - $p > 0.05$

*** - $p > 0.01$

III. RESULTS.

Using the 'colchicine metaphase arrest' technique, about 4% of rat thymocytes were found to be trapped in the metaphase configuration after 6 hours in culture. Raising the extracellular Ca^{++} concentration from the basal 0.6mM to 1.8mM, increased this proportion to about 6% (see Figure 24), confirming the observations of Morgan (1976), Cade (1985), Perris et. al. (1985), and Wadwha (1986). Incubation with insulin (10^{-9} - 10^{-11}M), similarly increased the proportion of cells undergoing mitosis in 6 six hours from 4% to 6% (Figure 24), reaffirming earlier studies by Morgan (1976).

Figure 24. Percentage of Rat Thymocytes in Colchicine Metaphase Arrest after Six Hours Incubation with Various Concentrations of Insulin.

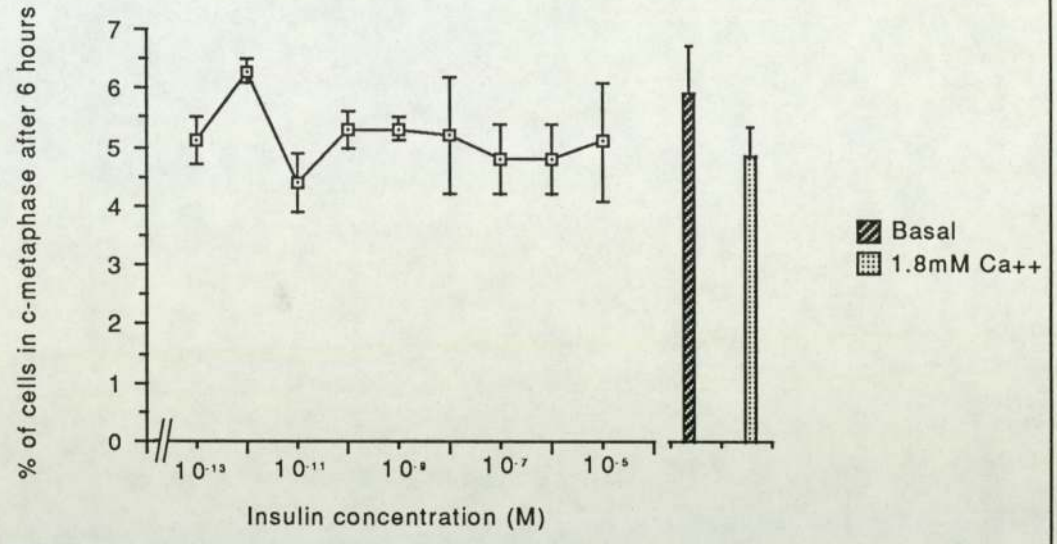


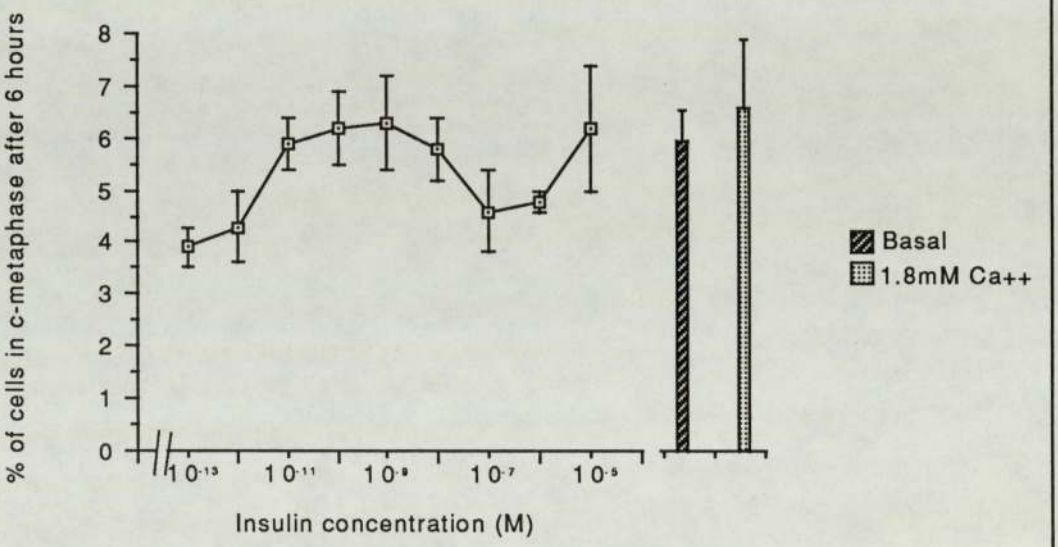
As indicated in section II.1.1., the mean values from each of the four separate experiments represented in Figure 24, were obtained by 'scoring' the slides by two individuals. A total of at least 1000 cells/slide were counted by each individual on each occasion, and where discrepancies occured, the slides were 're-scored' until concordance was achieved between the 'scores' from each individual. The percentage of cells in colchicine metaphase under basal conditions was about 4%, and only rose to about 6% after stimulation. Hence only a very small difference in the number of 'c-metaphase' cells, (about 2%), was measurable between basal and 'stimulated' cultures. On occasions, some difficulty was encountered in achieving concordance between the counts from two individuals. Therefore, in order to test the objectivity of the counting procedure, two further identical experiments were carried out. For each experiment, cultures were set up containing various insulin concentrations, and the internal controls, with basal (0.6mM) and stimulatory (1.8mM) calcium concentrations. The procedure was followed as outlined in the methods section, but the identity of the slides was masked before counting. The results from these two experiments are shown in Figures 25a and b, which should be compared with Figure 24.

When the identity of the slides was unknown, no significant difference was detected between basal cultures and those incubated with high (1.8mM) Ca^{++}. Previously, the mitogenic potential of 1.8mM Ca^{++} had been considered well established, and was routinely used to check on the normal responsiveness of the cultures (Morgan, 1976; Cade, 1985, Perris et. al., 1985; Wadhwa, 1986). There was also no detectable significant difference between basal cultures, and those stimulated with insulin at any concentration, in contrast to the results shown in Figure 24, and those previously obtained by Morgan (1976). Furthermore, 'scores' of the same slide, by different individuals, differed by as much as 106%.

It is clear that the method used for 'scoring' the number of cells in colchicine metaphase was highly subjective. Even experienced observers

Figures 25a and b. Percentage of Rat Thymocytes in Colchicine Metaphase Arrest in Response to Insulin when the Identity of the Slides was Unknown, Examples 1 and 2.



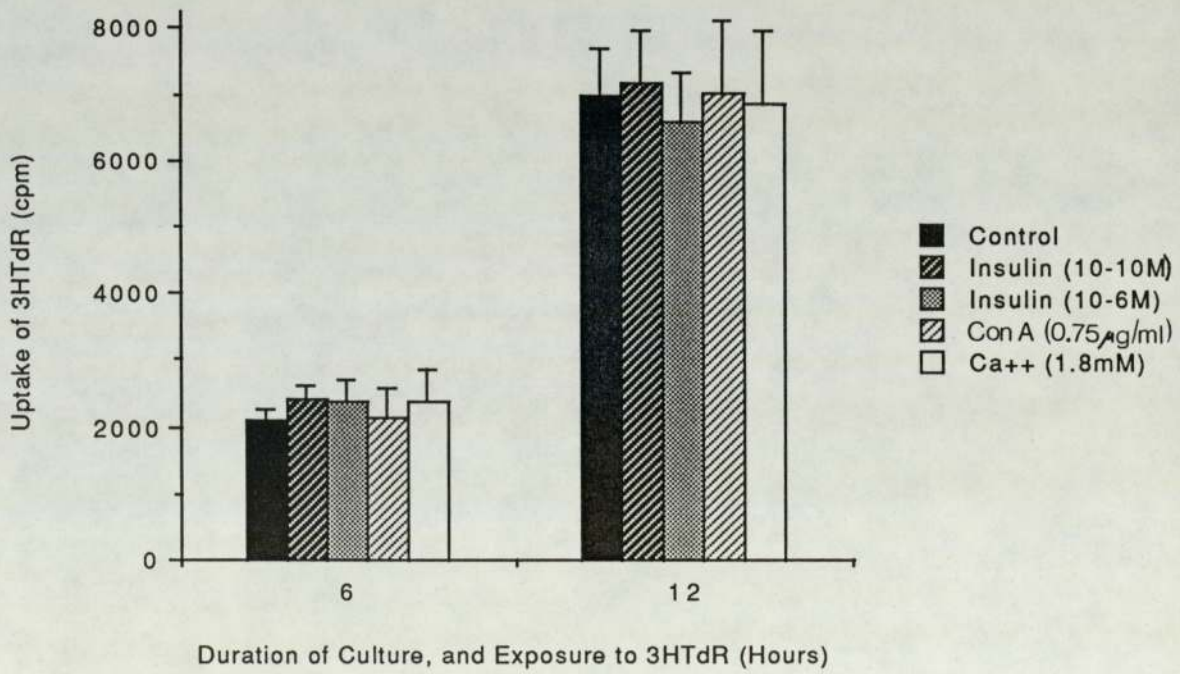


Each graph represents the results from one experiment. The SEMS are derived from three separate 'scores' of the same slides. The slides were 'scored' on three occasions, by individuals who were familiar with and experienced in using the technique.

could therefore be inadvertently influenced by preconceived views of the outcome of an experiment. Although there may be differences between the various treatments, a thoroughly objective alternative methodology was therefore required, whereby entry into the cell cycle of the 'rapidly recruitable' subset of rat thymocytes could be examined unequivocally.

As discussed in section II.1.2.1., scintillometric measurement of the uptake of tritiated thymidine (^{3}HTdR), has previously been found to be unsuccessful in reflecting the extent of DNA synthesis in the 'rapidly recruitable' subset of rat thymocytes (Youdale and Macmanus, 1975). We were likewise unable to detect any significant increase in ^{3}HTdR incorporation above control levels, after either 6 or 12 hour incubations, with insulin (10^{-10}M and 10^{-6}M), 1.8mM Ca^{++},or the plant lectin Concanavalin A (Con A, 0.75µg/ml) (Figure 26).

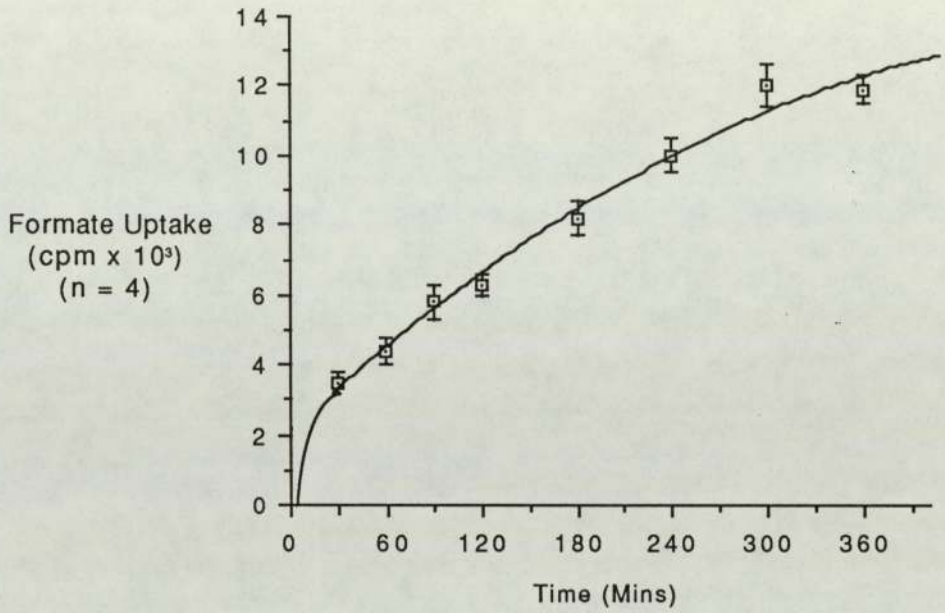
Figure 26. Cumulative uptake of Tritiated Thymidine by Control, High Calcium, Insulin-Stimulated and Concanavalin A-Stimulated Cultures of Rat Thymocytes.



The treatments were therefore either unable to harness 'rapidly recruitable' thymocytes and prompt them to initiate DNA synthesis over these time periods, or the technique was incapable of measuring such recruitment.

Another objective and quantitative method of assessing DNA synthesis involves measuring the incorporation of ^{14}C-formate into the cells, as discussed in section II.1.2.1. The total cumulative uptake of ^{14}C-formate into rat thymocytes was measured initially, under basal conditions (Figure 27).

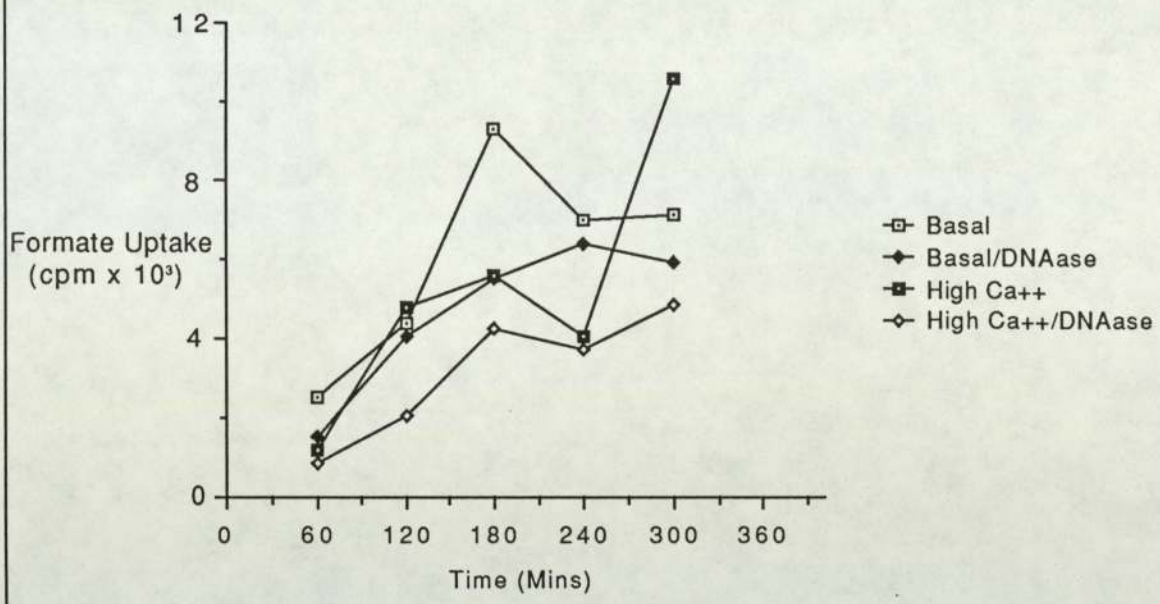
Figure 27. Cumulative Uptake of ^{14}C-Formate by Rat Thymocytes Under Basal Conditions



After an initial rapid uptake over thirty minutes, which may represent binding to the cell membrane and accumulation of $^{14}CO_2$ in the cytoplasm, a steady uptake was noted over a 6 hour period. Presumably, this was largely the sum of ^{14}C-formate incorporation into both protein and DNA.

To ascertain what proportion of ^{14}C-formate uptake was associated with DNA synthesis, cultures exposed to formate for various times were treated with DNAase. Enzymic treatment certainly removed a fraction of the incorporated ^{14}C on all occasions, but it proved to be a highly variable and inconsistent fraction. This was true of cultures maintained under both basal and high calcium conditions (Figure 28).

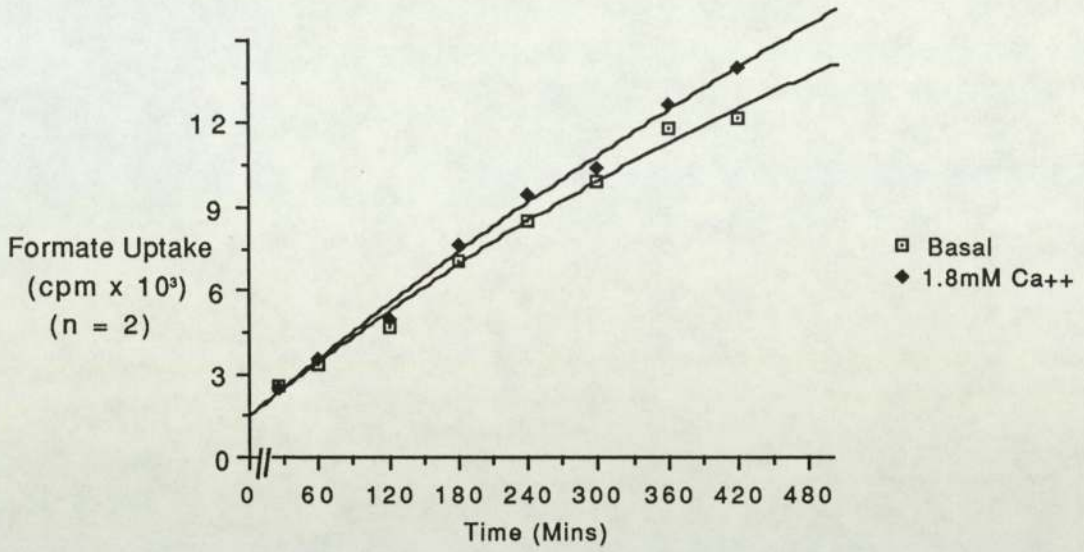
Figure 28. Cumulative Uptake of ^{14}C-Formate by Rat Thymocytes in Basal and 'High Calcium' Medium, Before and After Enzymatic Digestion of DNA.



The smooth uptake pattern seen in Figure 27 was no longer evident, and this probably reflected unpredictable cell losses associated with the several digestion and washing stages in the method (section II.1.2.1.). For these cultures therefore, the DNAase digestion technique seemed quite inadequate to differentiate between formate incorporation into protein and DNA.

As a prelude to division, it seemed most likely that both the protein and DNA synthetic pathways would be enhanced. It is almost inconceivable that increased formate incorporation into DNA would be accompanied by an equal and opposite diminished incorporation into protein. Total ^{14}C-formate incorporation may therefore allow a distinction, in a crude way, between basal and premitotic DNA and protein synthesis. When ^{14}C-formate incorporation into cultures maintained under basal and high calcium conditions were compared however, no consistent differences in ^{14}C-formate content could be discerned over 8 hours (Figure 29).

Figure 29. Cumulative Uptake of ^{14}C-Formate by Rat Thymocytes Under Basal and High Calcium Conditions.



Using the ^{14}C-formate incorporation technique it must therefore be concluded that the so-called mitogenic calcium concentration (1.8mM) does not enhance DNA synthesis.

Neither the colchicine metaphase arrest technique, nor measurement of the incorporation of ^{3}HTdR or ^{14}C-formate into DNA, were successful in detecting any difference in proliferative potential between basal and 'stimulated' cultures of rat thymocytes over 6 hours. Indeed, the results cast considerable doubt on the existence of an apparently well documented sub-set of 'rapidly recruitable' rat thymocytes, which enter the cell cycle from G_0, within 6 hours of mitogenic stimulation (Youdale and Macmanus, 1975; Perris et. al., 1985; Whitfield et. al., 1987).

However, there was little doubt that in long term cultures, ^{3}HTdR incorporation could be used to detect alterations in the rate of DNA synthesis between basal, insulin-treated and Con A-treated cells (Figures 30 a and b).

Figure 30a. Cumulative Uptake of Tritiated Thymidine by Rat Thymocytes over 96 Hours Under Basal, Insulin Treated and ConA Treated Conditions.

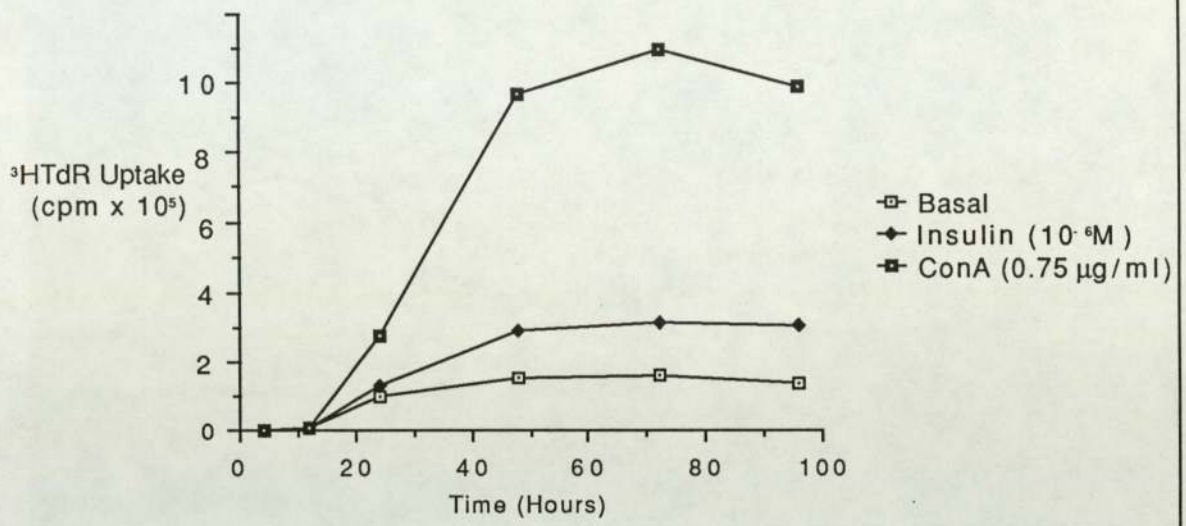


Figure 30b. Uptake of Tritiated Thymidine Pulsed for the Final 4 Hours of Incubation in Control, Insulin Treated and ConA Treated Rat Thymocytes.

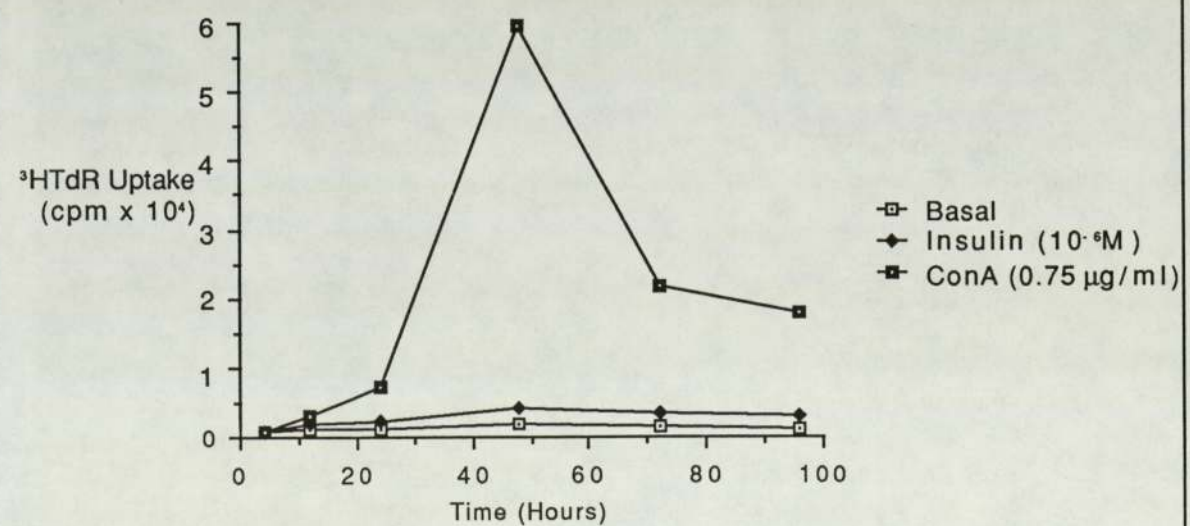
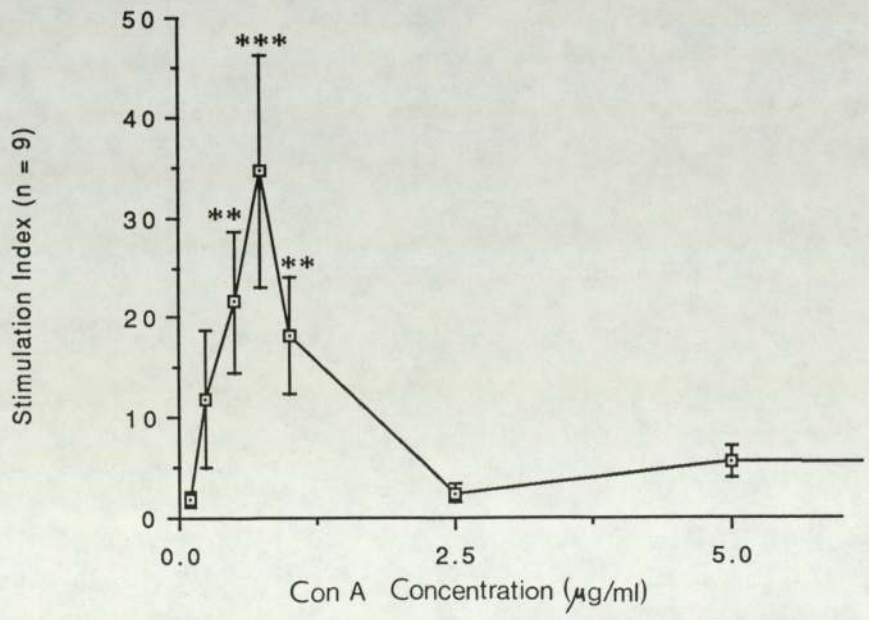


Figure 30a shows the cumulative uptake of ^{3}HTdR, where the isotope was added to cultures at the start of the experiment. Although there was little uptake initially, there was significant ^{3}HTdR uptake after 24 hours. After 48 hours, significant differences in the amount of ^{3}HTdR incorporated into the DNA of control, ConA-treated and insulin-treated cultures were distiguishable. These observations were partially corroborated by the data shown in Figure 30b, where ^{3}HTdR was added to cultures 4 hours before the end of the experiment. DNA synthesis was enhanced in ConA and to a much smaller extent, in insulin-treated cultures after 24-48 hours. The peak response to both agonists was observed after 48 hours of exposure, but enhanced DNA synthesis occured up to at least 96 hours after the start of the experiment. The technique of scintillometric measurement of the incorporation of ^{3}HTdR into DNA was therefore adopted as a simple, well established method which could be used routinely to assess the proliferation of cells over a longer timescale.

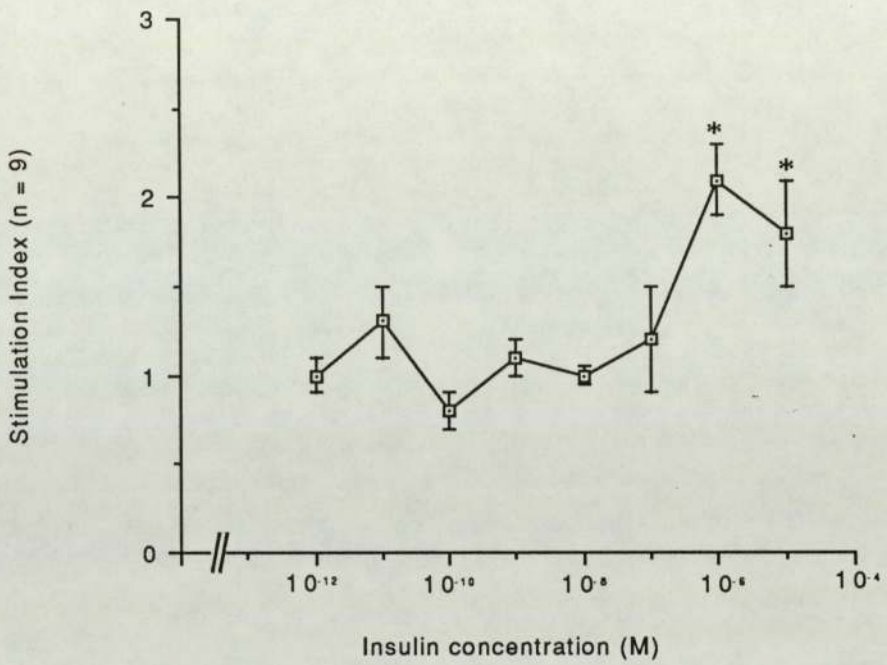
Cells were exposed to the polyclonal activator Con A for 48 hours, and ^{3}HTdR added for the final 4 hours. When compared to control cultures, 0.75µg/ml Con A caused an approximately 35 fold increase in DNA synthesis (Figure 31).

Figure 31. Incorporation of Tritiated Thymidine into DNA in Response to Con A, in Rat Thymocytes.



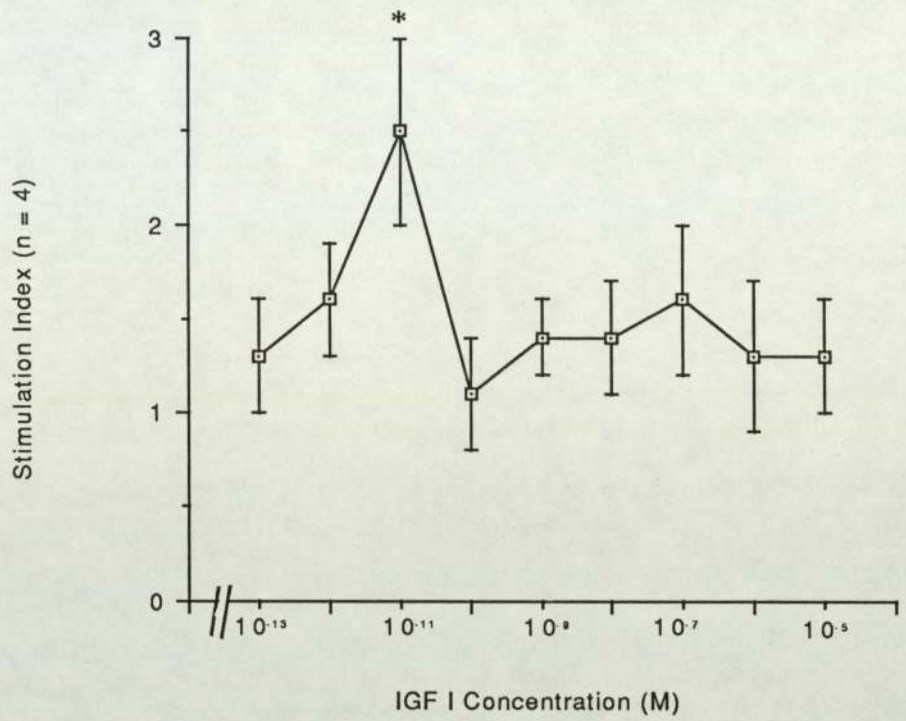
Insulin also increased ^{3}HTdR uptake, but in contrast to the dramatic enhancement observed with Con A treatment, the maximum uptake was only about twice that occuring under control conditions. The response was observed at an insulin concentration of 10^{-6}M, very much higher than the physiological concentration of about 10^{-10}M (Figure 32).

Figure 32. Incorporation of Tritiated Thymidine into DNA over the Final Four Hours of a 48 Hour Culture of Rat Thymocytes, in Response to Insulin.



It seemed most probable that a much smaller sub-population of the cells were stimulated to divide in response to insulin, than were stimulated by the polyclonal activator, Con A. Nevertheless, a significant proportion of the cells did respond to high concentrations of insulin by entering S phase of the cell cycle. This requirement for high, supraphysiological concentrations of insulin implied that either thymocyte insulin receptors have reduced affinity for insulin, or the agonist may cross react with type I IGF receptors. If insulin exerted its growth promoting actions through type I IGF receptors in rat thymocytes, then insulin-like growth factor I (IGF I) would be expected to promote a similar response at low concentrations.

Figure 33. Incorporation of Tritiated Thymidine into DNA over the Final Four Hours of a 48 Hour Culture of Rat Thymocytes in Response to IGF I.



The magnitude of the response to IGF I (Figure 33) was similar to that for insulin, with a maximum stimulation index of 2.5 ± 0.5, but the optimal concentration of IGF I which provoked this response, 10^{-11}M, was much lower. These observations were consistent with the proposal that insulin and IGF I stimulated the proliferation of the same sub-population of rat thymocytes, both acting through the type I IGF receptor. Since a similar response was elicitedusing either insulin (10^{-6}M) or IGF I (10^{-11}M),the higher concentration of insulin was usedin a number of further experiments to mimic the response.

The ability of insulin to synergise with other growth factors has been noted (section I.2.2.). To investigate the possibility that it may synergise with Con A, cultures were set up containing a non-stimulatory concentration of Con A (0.1µg/ml), a marginally stimulatory concentration (0.25µg/ml), and the maximally stimulatory concentration (0.75µg/ml). In addition to Con A, some cultures contained insulin at concentrations of 10^{-7}M, 10^{-6}M or 10^{-5}M. (Figures 34a, b and c).

Figure 34a. Effect on ^{3}HTdR Incorporation of Simultaneous Addition of Insulin and ConA (0.1 mg/ml).

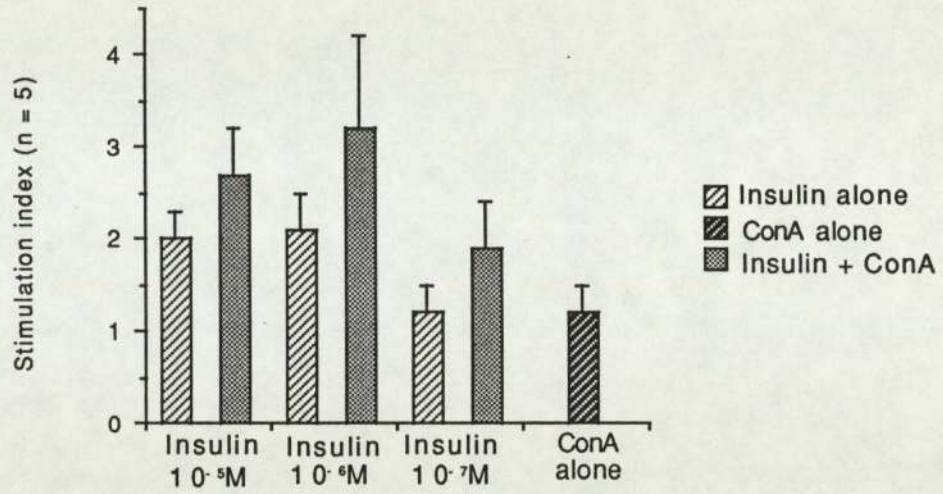


Figure 34b. Effect on ^{3}HTdR Incorporation of Simultaneous Addition of Insulin and ConA (0.25 mg/ml).

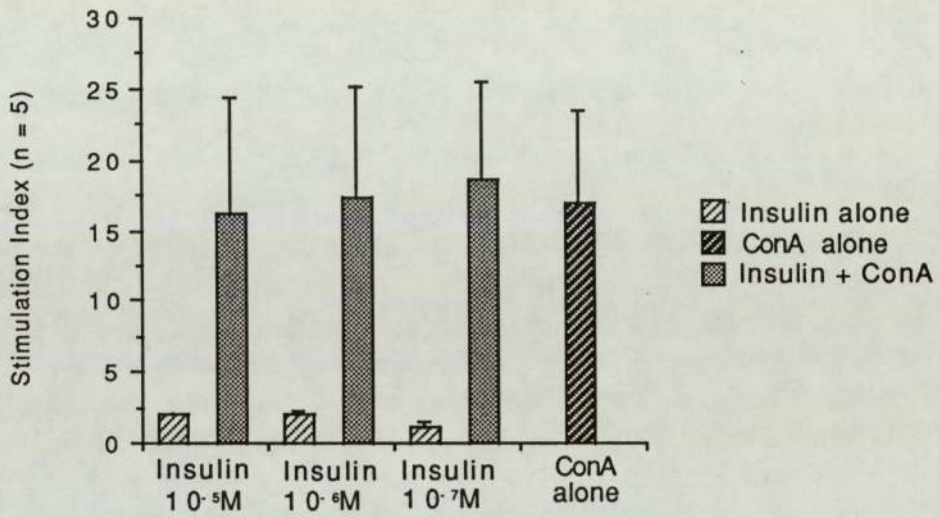
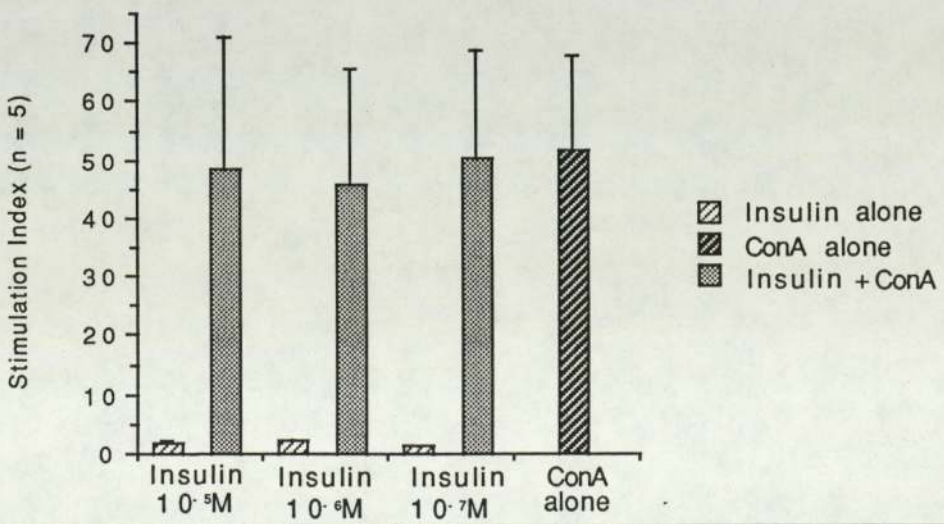


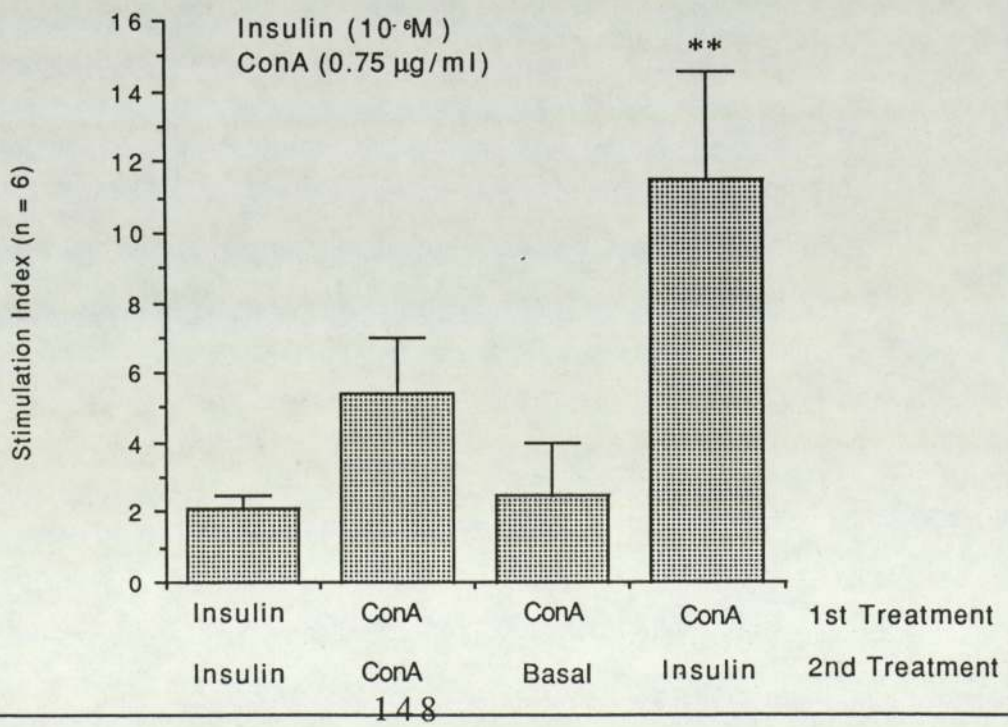
Figure 34c. Effect on ^{3}HTdR Incorporation of Simultaneous Addition of Insulin and ConA (0.75 mg/ml).



A significant additive or synergistic effect of insulin was not detected at any of the Con A concentrations tested. However, although the results were not statistically significant, there was some indication that the addition of 0.1μg/ml Con A to insulin at all concentrations tested, provoked a marginally greater proliferative response than either 0.1μg/ml Con A alone, or insulin alone (Figure 34a). With higher Con A concentrations the margins of error exceeded the magnitude of the insulin response, and hence any additive effect was impossible to detect (Figures 34b and c).

Whether or not Con A and insulin act synergistically when added simultaneously at the beginning of cultures, there may be different temporal requirements for the two agonists in the processes of entry into G_1, and subsequent passage through S phase of the cell cycle. Experiments were therefore undertaken to test the possibility that Con A may 'prime' the cells in some way, rendering them more responsive to a subsequent addition of insulin. Cultures were set up containing the maximally stimulatory concentrations of the agonists, and 48 hours after the start of the experiment the cells were washed and resuspended in fresh medium containing either the same or a different agonist. The total culture time was 96 hours (Figure 35).

Figure 35. Incorporation of ^{3}HTdR into Rat Thymocytes After Two Sequential 48 Hour Treatments.



When cells were incubated with insulin for the first 48 hours, and then washed and resuspended with insulin, the stimulation index was 2.1 ± 0.3, comparable to cultures incubated with insulin alone for 48 hours (Figure 32). If however, cells were incubated with Con A for both halves of the experiment, the stimulation index was 5.4 ± 1.6, significantly higher than 96 hour 'insulin' cultures, but significantly less than 48 hour 'Con A' cultures (Figure 31). However, although the maximum rate of DNA synthesis in response to Con A was observed after 48 hours, significant numbers of cells responded after 96 hours (Figure 30b). Removal of Con A after 48 hours reduced the stimulation index still further, unless it was replaced by insulin (Figure 35). When cells were incubated with Con A for 48 hours, and then with insulin for the next 48 hours, the stimulation index (11.5 ± 3.1), was approximately double that for Con A, and more than five times that obtained with insulin treatment alone, over the same time. This strongly suggested that the Con A treatment 'primed' the cells in some way, such that an enhanced proliferative response to insulin could subsequently take place.

Other cultures maintained for a total of 96 hours were 'split' and resuspended at either 24 or 72 hours after the start of the experiment. Unless Con A was present for more than the first 24 hours, DNA synthesis after 96 hours had declined to control levels in the absence of insulin. In the presence of insulin for the last 72 hours of the incubation, the proliferative response resembled that observed after 96 hours in the presence of insulin alone (Figure 36a). Either 24 hours is an insufficient length of time for the 'priming' effect of Con A to be exerted, or the response may have passed its peak, 72 hours after the addition of insulin.

When cultures were 'split' at 72 hours, pretreatment with Con A and subsequent treatment with insulin for the final 24 hours yielded a

stimulation index greater than that for insulin alone, but less than that for Con A alone over the same time (Figure 36b). Although a 24 hour treatment may not be sufficient for the full response to insulin as shown in Figure 35 to be realised, the presence of insulin for the final 24 hours of culture after the removal of ConA prevents the rapid decrease in proliferative potential seen in its absence (3rd and 4th columns of Figure 36b).

Figure 36a. Incorporation of Tritiated Thymidine into Rat Thymocytes After Two Sequential Treatments of 24 and 72 Hours.

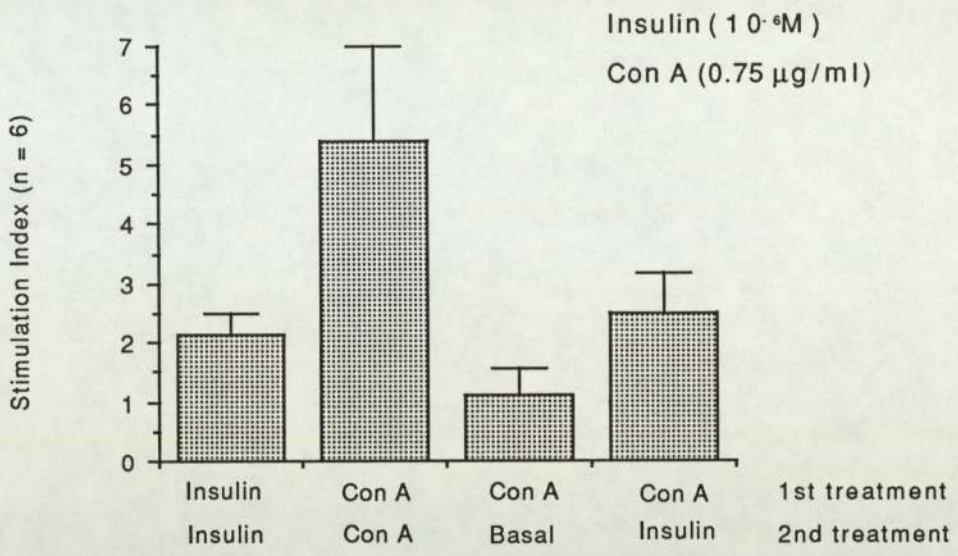
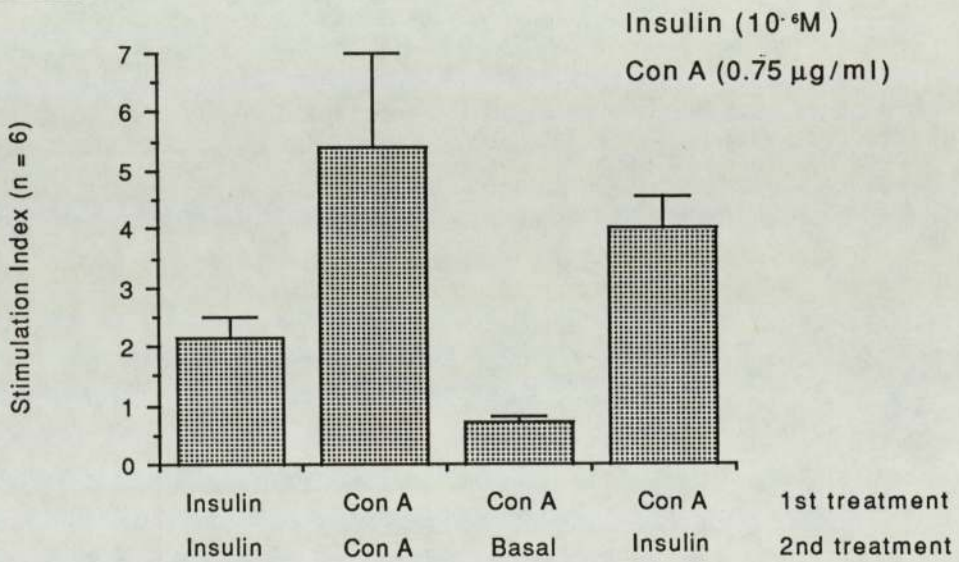


Figure 36b. Incorporation of Tritiated Thymidine into Rat Thymocytes After Two Sequential Treatments of 72 and 24 Hours.

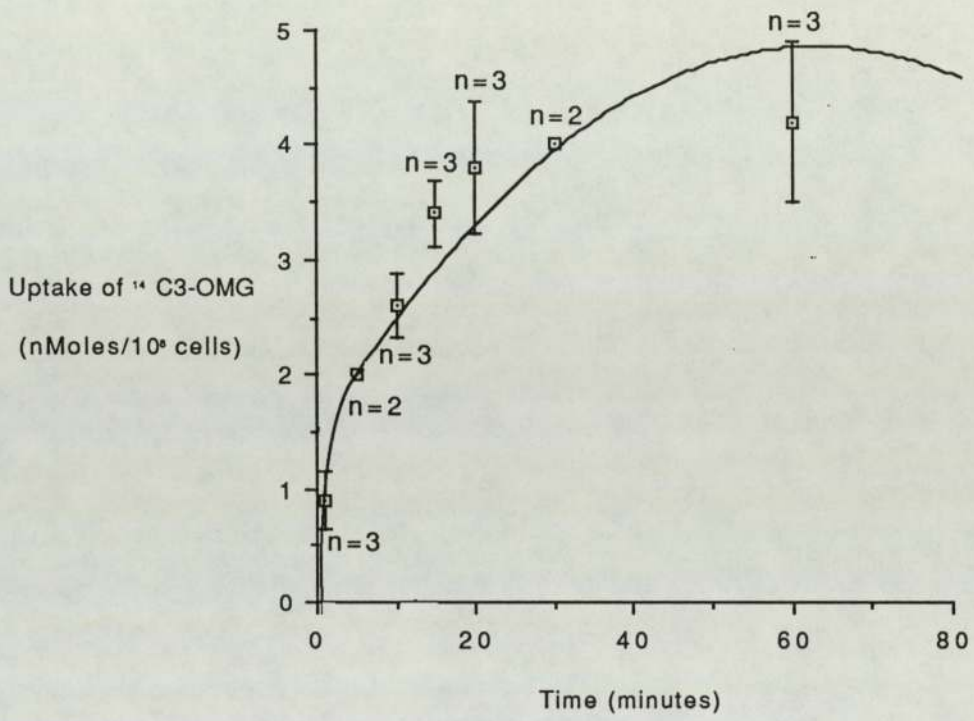


In summary, Con A, insulin and IGF I all stimulated DNA synthesis in rat thymocytes. The response to Con A was much greater than to the other agonists. The smaller proliferative responses to insulin and IGF I may both have been mediated by type I IGF receptors. No significant synergistic or additive effect between Con A and insulin was detected when the agonists were added simultaneously to cultures. However pretreatment with , Con A

'for 48 hours, primed' the cells so that the response to a subsequent addition of insulin was markedly enhanced.

Mitogenic stimulation is frequently accompanied by generally enhanced cellular metabolism, and since insulin has well established metabolic properties, its ability to promote glucose uptake into rat thymocytes was investigated. Under basal conditions, uptake of the glucose analogue, 3-ortho-methyl glucose (3-OMG), is initially rapid, but levels off to a plateau after about 30 minutes (Figure 37).

Figure 37. Cumulative Uptake of ^{14}C3-OMG by Rat Thymocytes Under Basal Conditions.



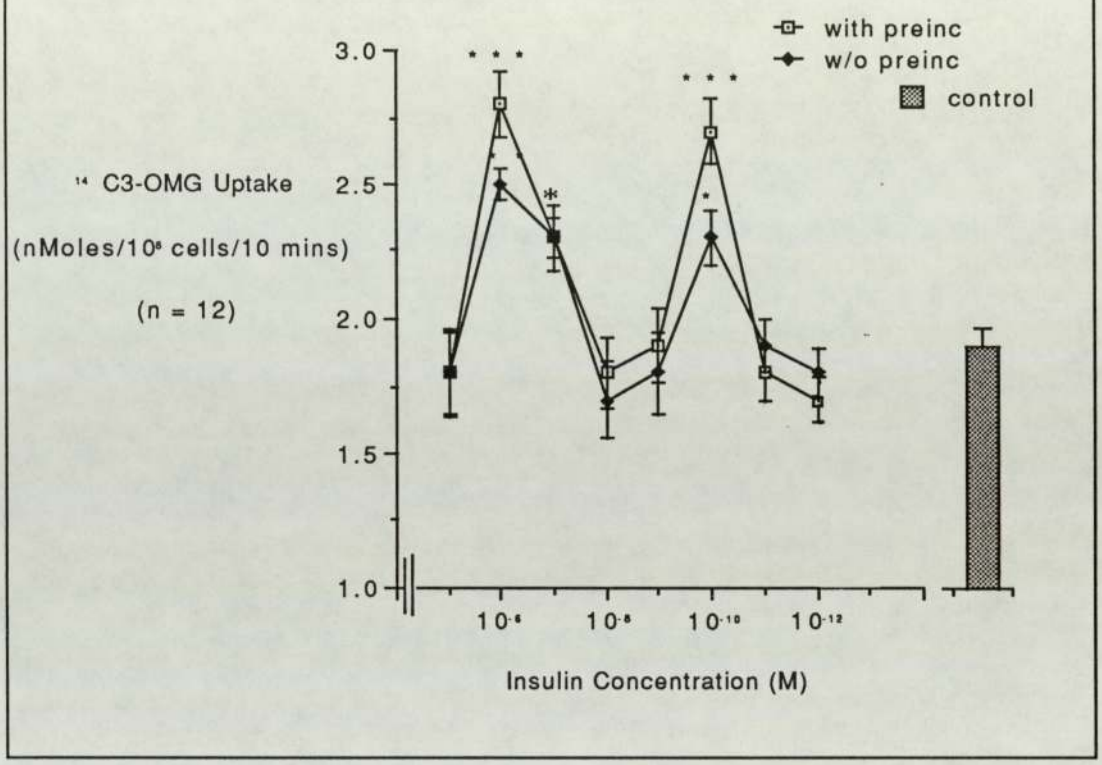
This type of curve is expected when a process of facilitated diffusion operates. The initial rapid uptake occured into cells which contained no 3-OMG. Later, as the intracellular concentration of this non-metabolisable molecule increased, uptake was balanced by its outward diffusion, and there was no net uptake.

The purpose of defining such a curve was to find a suitable time at which to observe any difference in uptake in the presence and absence of insulin. It

was decided to use uptake after 10 minutes for this purpose. At this time uptake was approximately half maximum. Sufficient ^{14}C-3-OMG had accumulated in the cells to yield high, reproducible radioactive counts (cpm), yet uptake was still increasing in a linear fashion.

Insulin significantly enhanced 3-OMG uptake into rat thymocytes at both its physiological concentration (10^{-10}M), and at higher concentrations (10^{-6} - 10^{-7}M) (Figure 38).

Figure 38. The Effect of Insulin on ^{14}C3-OMG Uptake by Rat Thymocytes.



The pattern of the biphasic response was similar whether uptake was measured immediately after isolation of the cells, or if cells were first pre-incubated for 2 hours with insulin. The magnitude of the response at both concentrations was significantly greater however, when a pre-incubation had been undertaken. Pre-incubation with insulin may provoke the translocation/activation of glucose transporters, which thus transport more 3-OMG in 10 minutes than those of freshly isolated cells.

These results are not in agreement with with Goldfine et. al. (1972), who found no increase in 3-OMG uptake into rat thymocytes after 1 hour's pre-

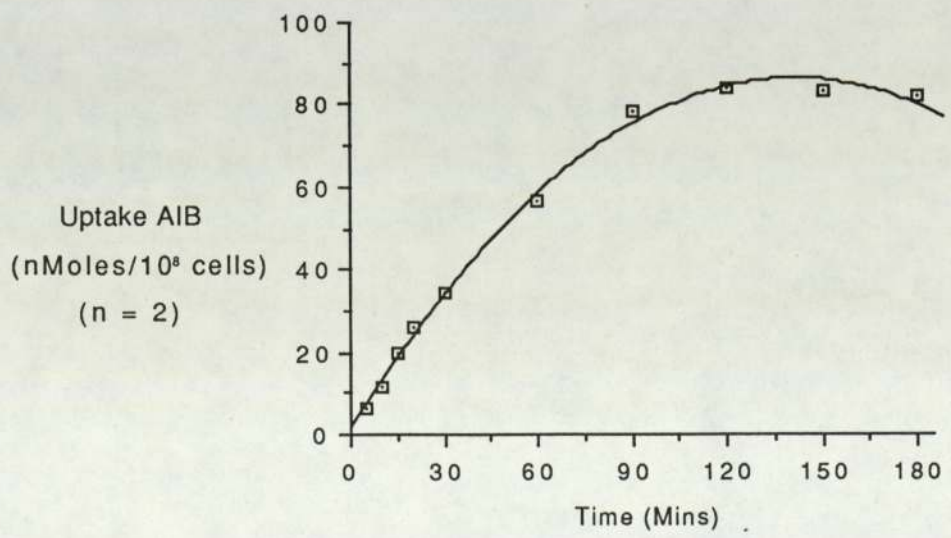
incubation with 3μM insulin. However, other studies have shown an increase in glucose uptake into these same cells in the presence of about 10^{-6}M insulin (Toma et. al., 1965; Boyett and Hofert, 1972), although no biphasic effect was observed.

In pre-incubated cells, 3-OMG transport was enhanced by 42% and 47% in the presence of 10^{-10} and 10^{-6}M insulin respectively (Figure 38). This biphasic response, occuring at the physiological insulin concentration, and at its 'growth promoting' concentration, may indicate a metabolic response which is mediated by both insulin and type I IGF receptors. These results, in combination with evidence from Figures 32 and 33, are consistent with the proposal that type I IGF receptors mediate both growth and metabolic effects, whereas insulin receptors promote only metabolic effects in these cells.

One drawback in the use of 3-OMG to mimic glucose movement, is that such uptakes must be carried out in the absence of glucose itself. It is possible that this may compromise the metabolic requirements of the cells, and hence affect the results. Therefore to further investigate the role of insulin and other agents in metabolic processes, amino acid uptake into the cells was studied in the presence of 5.0mM D-glucose.

Uptake of the amino acid analogue α-amino isobutyric acid (AIB) was measured over time to establish the point at which total uptake was approximately half maximal (Figure 39).

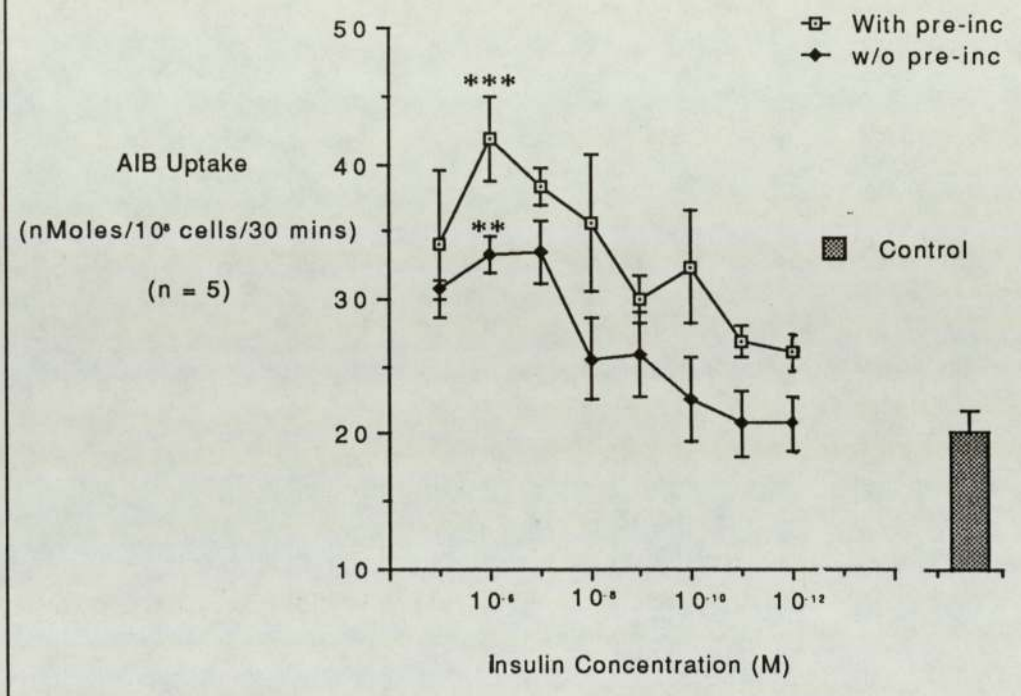
Figure 39. Cumulative Uptake of AIB by Rat Thymocytes Under Basal Conditions.



The amino acid transport system 'A' which transports AIB, is energy dependent, and can generate large concentration gradients between the extracellular and intracellular environments. A relatively high concentration of AIB was therefore built up in the cells over an extended timescale, before the plateau phase of the curve was reached. The effects of agonists on this process were therefore assessed after 30 minutes of AIB uptake.

Insulin increased AIB uptake into rat thymocytes with a peak response at an insulin concentration of 10^{-6}M. As with 3-OMG uptake, a 2 hour pre-incubation with insulin enhanced this effect, but unlike 3-OMG, the response was not biphasic (Figure 40).

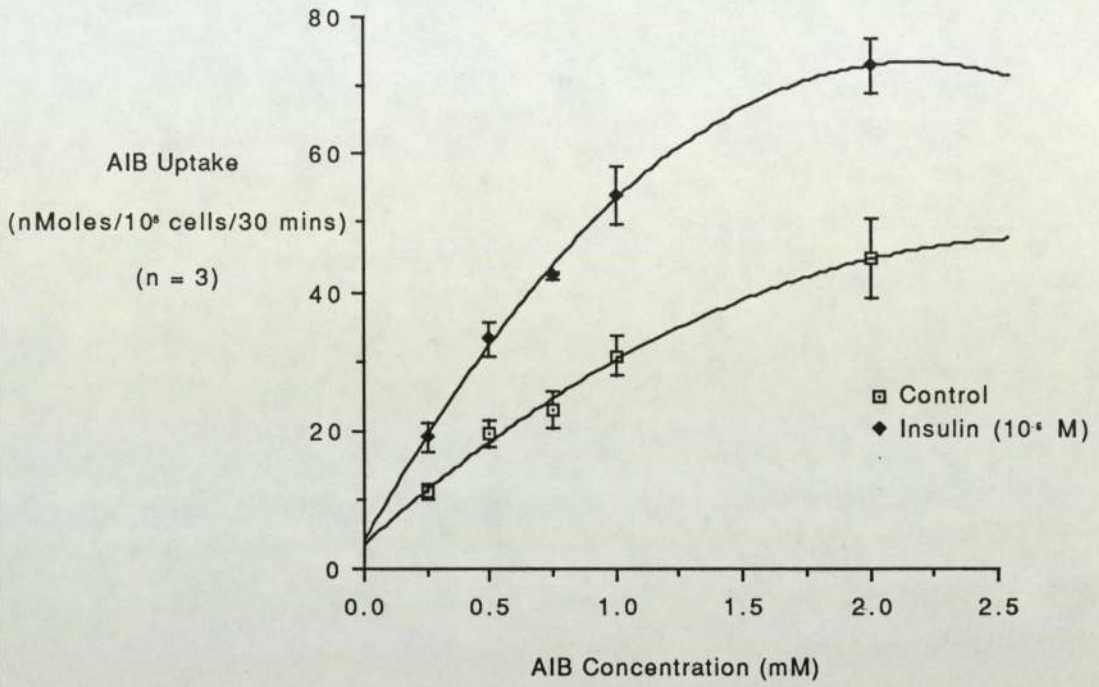
Figure 40. The Effect of Insulin on AIB Uptake by Rat Thymocytes.



For cells pre-incubated with 10^{-6}M insulin, AIB uptake was enhanced about 78% above control levels, confirming earlier findings by Goldfine et. al. (1972) and Kwock et. al. (1976). The requirement for high 'growth stimulatory' insulin concentrations suggests that the effect may be mediated by type I IGF receptors.

AIB influx into control and insulin-treated cells is dependent on the extracellular AIB concentration (Figure 41).

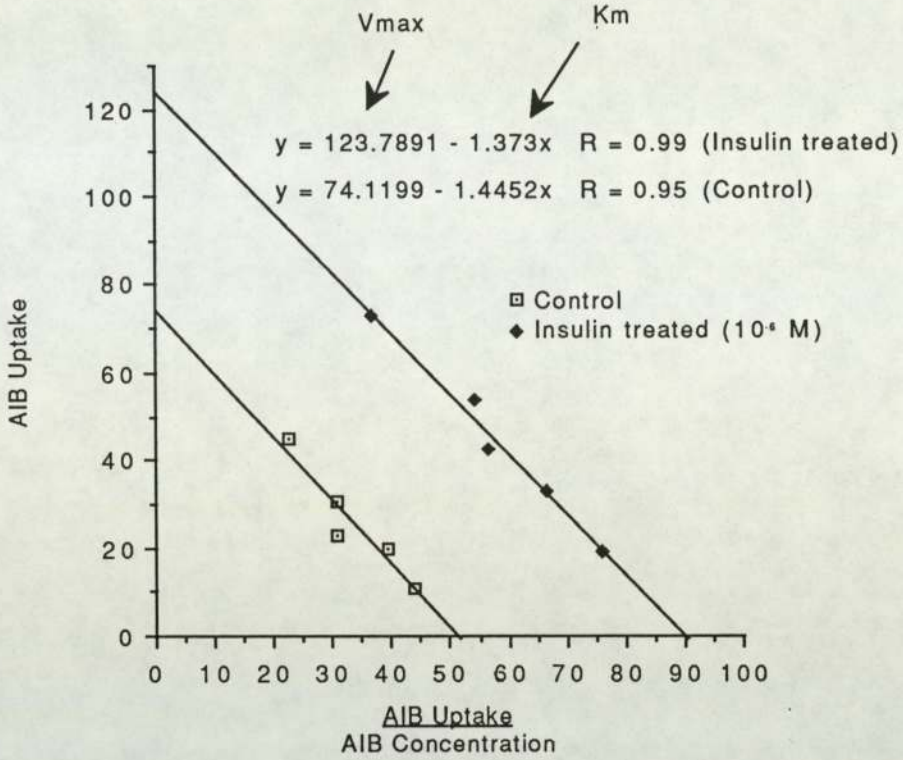
Figure 41. Uptake of AIB into Rat Thymocytes as a Function of Its Concentration in the Medium.



Below about 1mM, there was a roughly linear relationship between AIB uptake and extracellular AIB concentration. At higher AIB concentrations, the curve began to 'level off' as the transporters presumably became saturated. ie. they were transporting the amino acid at a rate approaching maximum. The maximum rate was higher in insulin treated than in control cells.

In order to ascertain the maximum rate of amino acid transport (Vmax), and also to obtain a measure of the affinity of the transporters for AIB (Km), in insulin treated and control cells, the data shown in Figure 41 were transformed, using the Michaelis-Menten equation, to give a Hofstee-Eadie plot (Figure 42).

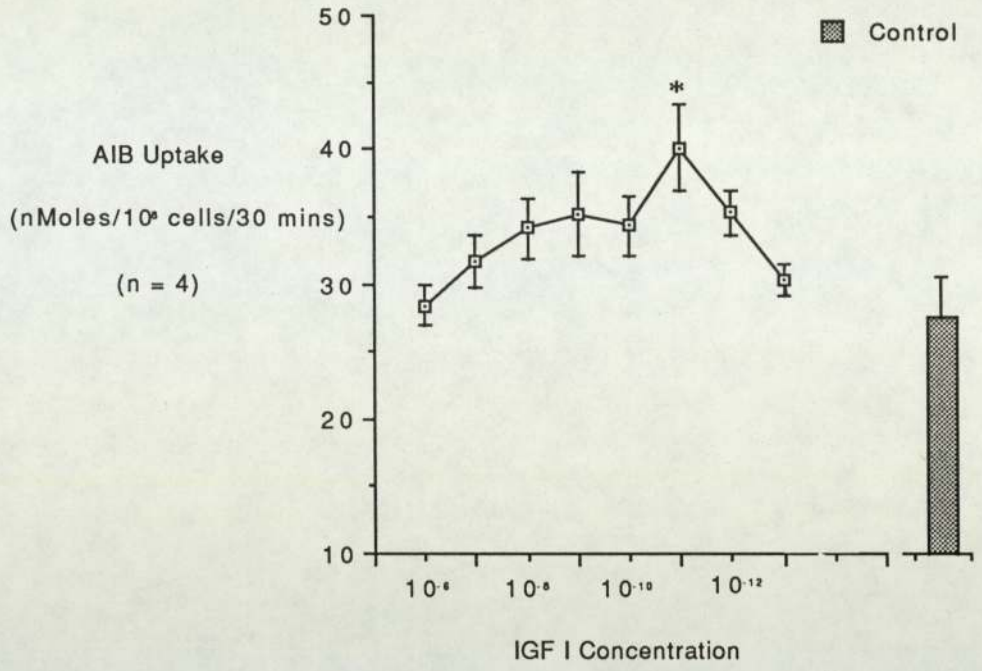
Figure 42. Transformation of Data Shown in Figure 41, to Give a Hofstee-Eadie Plot.



Using this derivation, a straight line was obtained for each set of points. An intercept of Vmax was obtained on the 'y' axis, and the slope of the graph was equal to -Km. From the equations for the graphs, it can be seen that there was no significant difference between the affinity (Km = about 1.4mM) of receptors in control and insulin treated cells, but Vmax was increased from about 74 to 124 nMoles/10^8 cells/30 minutes (68%). Insulin treatment leads to an increase in the number of system 'A' amino acid transporters in the membranes of rat adipocytes (see Section I.2.1.). Since an increased number of transporters would lead to a higher maximum rate of amino acid transport, without affecting transporter affinity, insulin may act similarly in rat thymocytes.

It has been suggested that the requirement for supraphysiological concentrations of insulin for maximum enhancement of AIB uptake, may indicate that this metabolic effect was mediated by type I IGF receptors. If this is the case, then a similar response to IGF I would be expected to occur at 'low' concentrations. A peak enhancement of AIB uptake in response to IGF I was indeed detected at a concentration of 10^{-11}M (Figure 43).

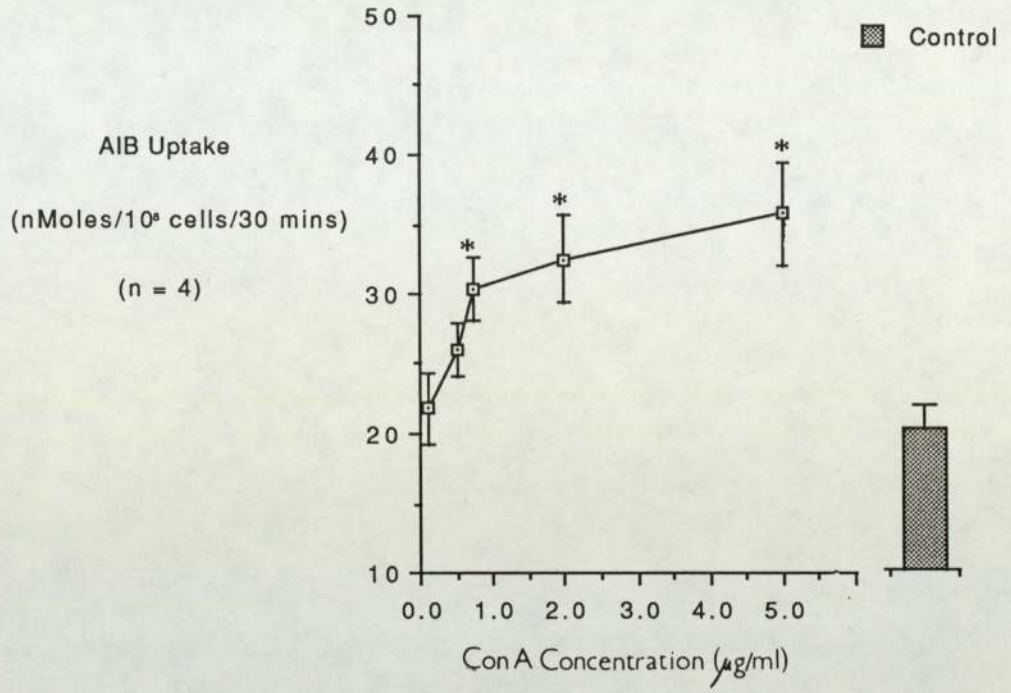
Figure 43. The Effect of IGF I on AIB Uptake by Rat Thymocytes.



A peak response to IGF I at this low concentration implies the participation of type I IGF receptors. However, the degree of enhancement of AIB uptake in response to IGF I was less than the enhancement in response to insulin. Also there was a small enhancement of AIB uptake in response to 10^{-10}M insulin (Figure 40). Hence it is possible that insulin may have a dual effect on this process. Part of the response may be be associated with a general metabolic enhancement, mediated by insulin receptors, and part may be specifically associated with growth promotion, mediated by type I IGF receptors.

Enhanced amino acid uptake is part of the 'pleiotypic response' which occurs after mitogenic stimulation of numerous cell types. To confirm a general association between cell division and enhanced amino acid uptake, the effect of Con A on AIB uptake was measured for comparison. At its maximally mitogenic concentration (0.75 µg/ml), Con A, like IGF I, increased AIB uptake by about 46% above control (Figure 44). Similar observations have been previously made for rat peripheral lymphocytes (Van Den Berg and Betel, 1973).

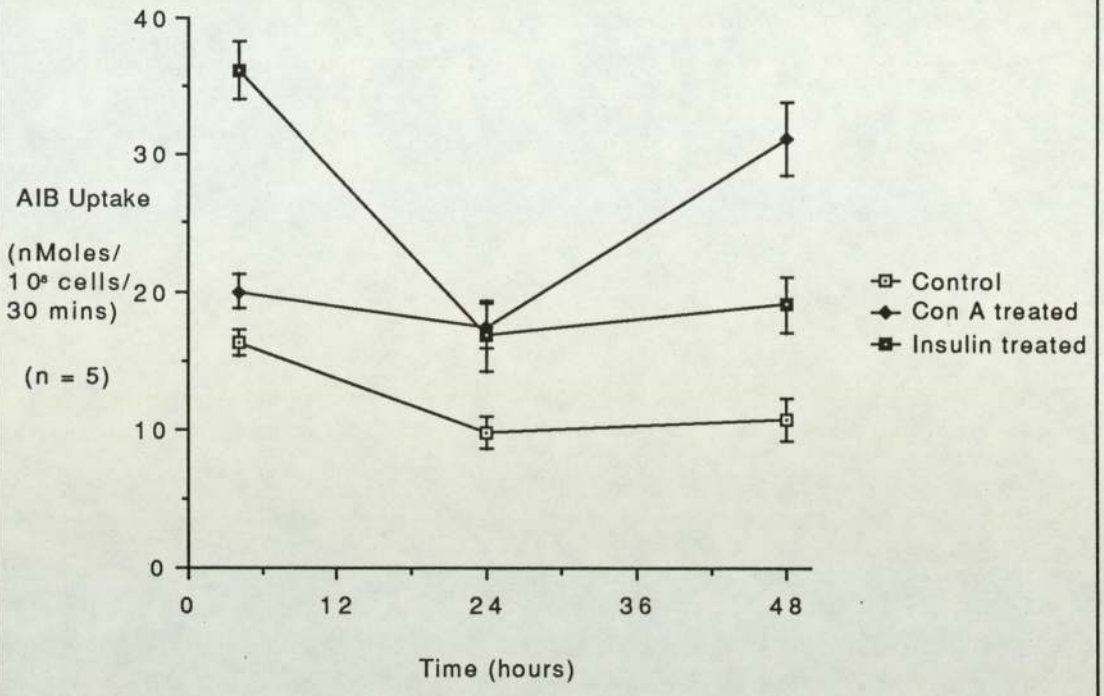
Figure 44. The Effect of Con A on AIB Uptake by Rat Thymocytes.



Although a significant enhancement of AIB uptake was observed at 0.75 μg/ml Con A, the effect was small compared to its dramatic ability to increase ^{3}HTdR incorporation 35 fold at the same concentration. If the premise is accepted that Con A stimulates a larger sub-population of rat thymocytes than insulin, then insulin must provoke a greater enhancement of AIB uptake per responsive cell than Con A. Alternatively, a larger number of cells may respond to insulin by increasing AIB uptake, than are mitogenically responsive.

However, since ^{3}HTdR incorporation was assessed after much longer preincubations, the results are not directly comparable with those measuring AIB uptake. In order to make a more accurate comparison between the sets of data, AIB uptake into control, Con A and insulin treated cells was measured at intervals over a 48 hour incubation period (Figure 45).

Figure 45. Effect of Con A (0.75 µg/ml) and Insulin (10^{-6}M), on AIB Uptake by Rat Thymocytes over 48 Hours in Culture.



For all cultures, total AIB uptake was reduced between 4 and 24 hours after the start of the experiment. This may be associated, in part, with cellular adaptation to culture conditions. Distortion of the results due to this effect can however be minimised by comparing stimulation indices (SI = $\frac{\text{Stimulated value}}{\text{Control value}}$) at each time interval.

Both insulin and Con A enhanced AIB uptake above control levels throughout the 48 hour incubation period. However, the peak response to insulin (SI = 2.2) occured early, after only 4 hours of exposure to the agonist, whereas the peak response to Con A (SI = 2.9) was delayed until 48 hours after the start of the experiment. Using the data from Figures 30b and 45, stimulatory effects of insulin and Con A on AIB uptake and ^{3}HTdR incorporation were compared (Table 5).

Table 5. Comparison between the Effects of ConA and Insulin on AIB Uptake and ^{3}HTdR Incorporation over 48 Hours.

Time of pre-incubation (hours)	Stimulation Index			
	ConA (0.75 μg/ml)		Insulin (10^{-6}M)	
	^{3}HTdR	AIB	^{3}HTdR	AIB
4	1.2	1.4	1.2	2.2
24	6.7	1.7	2.3	1.7
48	34.1	2.9	2.3	1.8

It can be seen that after 4 hours incubation there was a negligible effect of either agonist on ^{3}HTdR incorporation, whereas the effect of insulin on AIB uptake was at its peak at this time. After 24 hours, ^{3}HTdR incorporation was raised in response to both agents, but was already at its maximum level above control for insulin. AIB was raised to the same extent in response to both agents, but this represented an increase over 4 hour levels for ConA, and a decrease for insulin. After 48 hours ^{3}HTdR incorporation and AIB uptake in response to ConA were at maximum levels. The insulin responses were similar to those measured at 24 hours.

AIB uptake increased as an accompaniment to DNA synthesis in ConA stimulated cells. In insulin stimulated cells, the maximum AIB uptake preceded DNA synthesis, although it remained raised as DNA synthesis proceeded. This furnishes further evidence for a 'fast', metabolic component, and a slower 'growth stimulatory' component of insulin action in these cells.

The ability of ConA to 'prime' cells, rendering them more responsive to a subsequent addition of insulin, was shown for ^{3}HTdR incorporation (Figure 35). The possibility that the increased amino acid uptake, observed in response to insulin, could also be further enhanced by 'priming' with ConA was therefore investigated.

Control and ConA (0.75 μg/ml) containing cultures were set up, and at intervals over 48 hours, samples were withdrawn and pre-incubated for a further 2 hours with or without insulin. AIB uptake was then measured in the usual way. (Figures 46a, b and c).

Figure 46a. Effect of an 8 Hour Pre-incubation on Insulin Stimulated AIB Uptake into Rat Thymocytes.

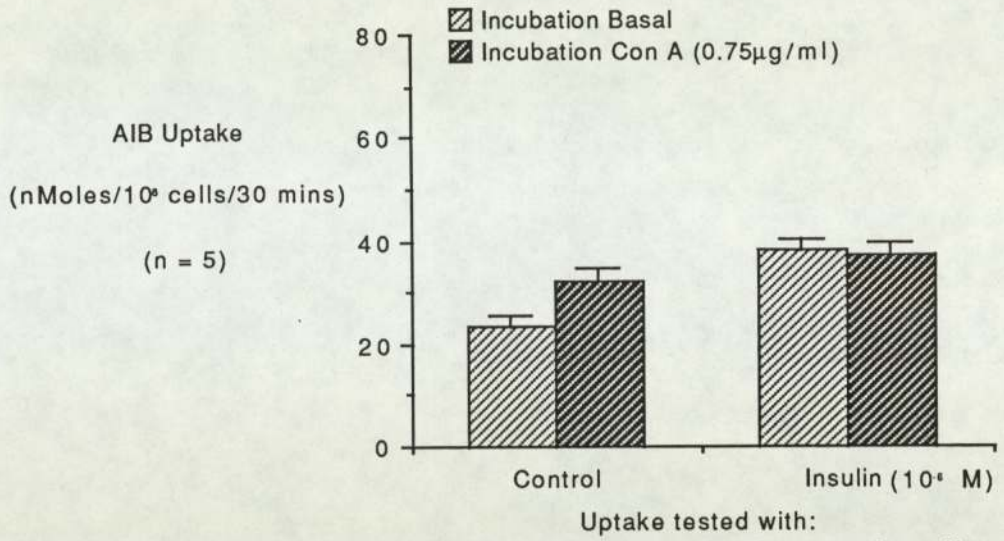


Figure 46b. Effect of a 24 Hour Pre-incubation on Insulin Stimulated AIB Uptake into Rat Thymocytes.

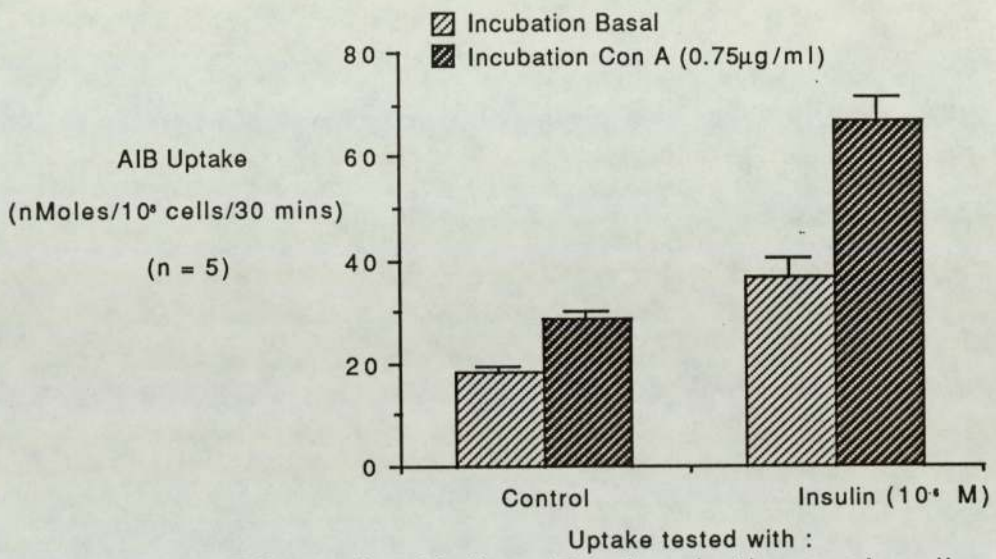
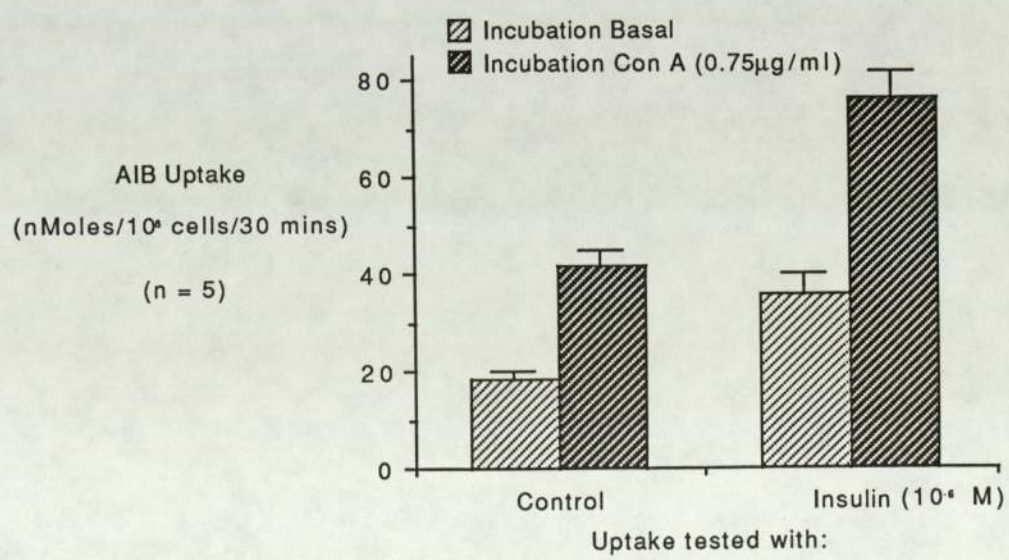


Figure 46c. Effect of a 48 Hour Pre-incubation on Insulin Stimulated AIB Uptake into Rat Thymocytes.



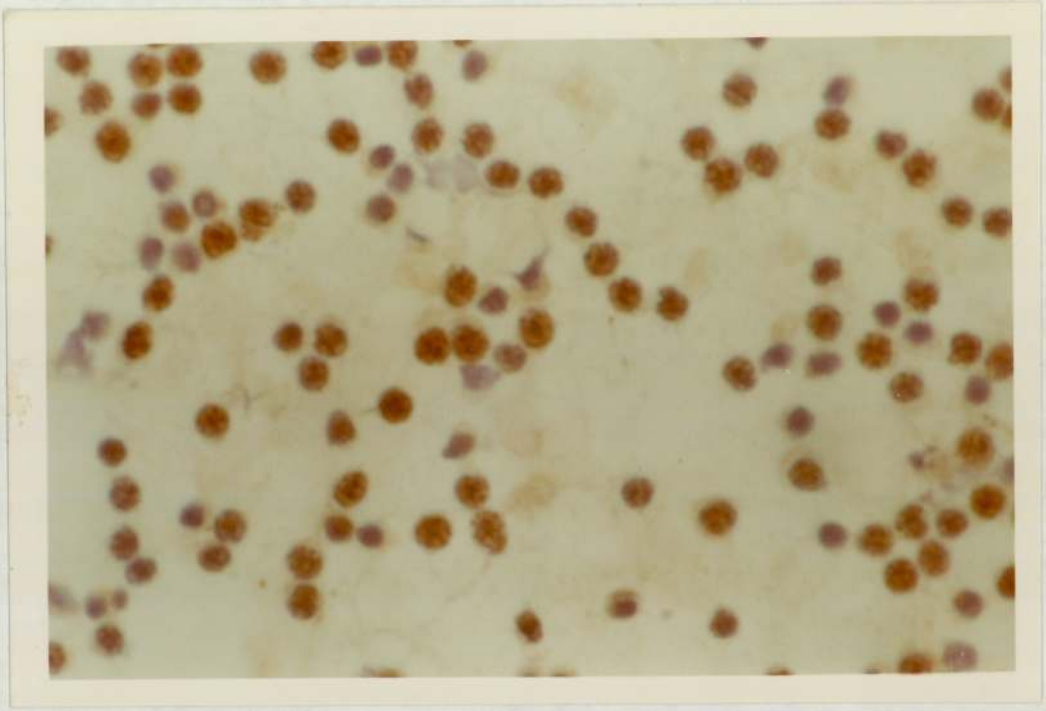
Comparing the lighter shaded columns in Figures 46a, b and c, it can be seen that the effect of 10^{-6}M insulin on cells incubated under basal conditions was to enhance AIB uptake, confirming earlier observations (Figures 40 and 45). Comparing the first two columns in each figure, it can be seen that incubation with 0.75μg/ml Con A also enhanced AIB uptake, again confirming previous observations (Figures 44 and 45). However, although both agonists provoked some degree of enhancement of AIB uptake at all times tested, the effect of an initial incubation with Con A, for at least 8 hours, and a subsequent uptake test in the presence of insulin, was to dramatically increase AIB uptake above that observed with either agonist alone. It seemed that some effect of Con A, which took between 8 and 24 hours to be manifested, rendered the cell subsequently more responsive to insulin.

It is clear that at least some rat thymocytes possess insulin and/or type I IGF receptors since binding of an agonist caused rapid metabolic effects on glucose and amino acid uptake, which may be prerequisites for later DNA synthesis and growth. Con A also promoted glucose and amino acid uptake, but more slowly than insulin. Ultimately however, a much larger fraction of the cells are induced to divide. The response to Con A may be due, in part, to an enhanced sensitivity to insulin or IGF I, which could be responsible for some of the increased metabolite uptake. One explanation for this could be that Con A provoked the expression of additional insulin or type I IGF receptors. To establish whether this might be the case, an attempt was made to visualise insulin receptors by immuno-peroxidase staining.

Most rat thymocytes incubated under basal conditions expressed insulin receptors (Figure 47). A small proportion of the cells showed only the blue/purple haematoxylin stain, indicating the absence of insulin receptors. However, the initial step of the binding procedure involved the

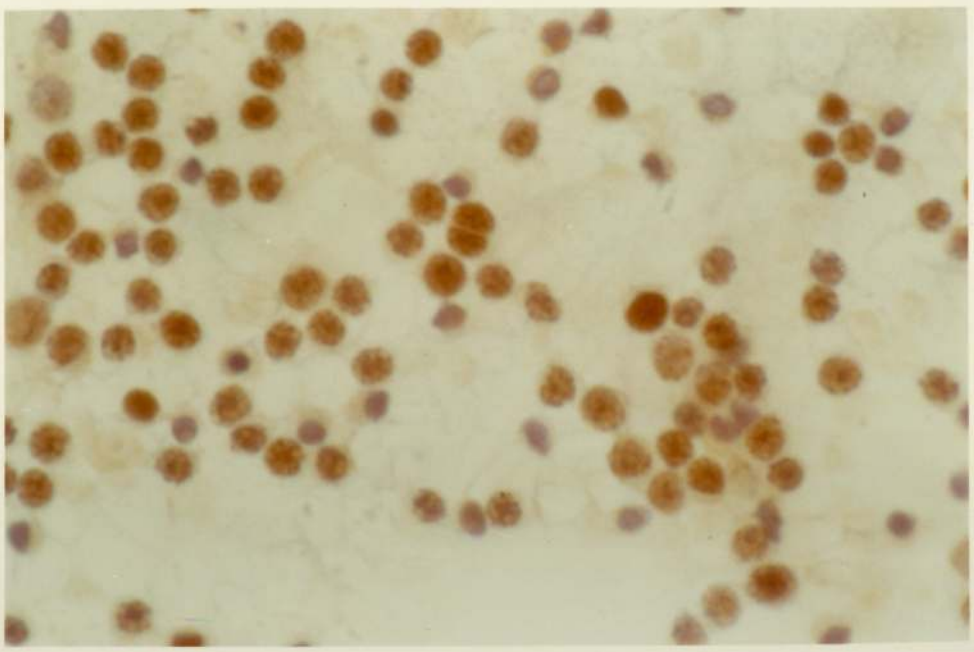
use of 10^{-6}M insulin. At this concentration, insulin may bind to both insulin and type I IGF receptors, and hence the brown colouration displayed by most cells in Figure 47 may indicate the possession of either or both types of receptor.

Figure 47. Immuno-Peroxidase Staining of Insulin Receptors on Rat Thymocytes - Cells Incubated Under Basal Conditions. Full Staining Procedure Undertaken.



Similarly, most rat thymocytes incubated with 0.75μg/ml Con A also possessed insulin and/or type I IGF receptors (Figure 48). Again a small proportion of the cells appeared to express neither receptor type.

Figure 48. Immuno-Peroxidase Staining of Insulin Receptors on Rat Thymocytes - Cells Incubated with 0.75µg/ml Con A. Full Staining Procedure Undertaken.



There was more evidence of proliferative activity in Con A treated cultures; the cells were larger (more blast-like), and unseparated cells, the daughters of recent mitoses were visible (Figures 48 and 49b). There was, however, no evidence of a difference in numbers of stained cells or intensity of brown staining, between cells incubated under basal conditions and those incubated with 0.75µg/ml Con A. The subjective impression from Figures 48 and 49 therefore implied that similar numbers of rat thymocytes possessed insulin and/or type I IGF receptors whether or not they had been incubated with Con A for 48 hours.

The relative numbers of a specific type of cell surface receptor can however be assessed with more objectivity and sensitivity using the ELISA method, where absorbance is proportional to receptor number (section II.3.2.).

Hepatocytes are among the traditional 'target' tissues for insulin, and hence would be expected to display large numbers of insulin receptors. Using the ELISA method, this supposition was verified, the absorbance values for hepatocyte coated wells being very high (Figure 50).

Figure 49a. Lack of Immuno-Peroxidase staining of Insulin Receptors on Rat Thymocytes - Cells Incubated Under Control Conditions. Anti-Insulin Antibody Replaced by Non-Immune Serum in Staining Procedure.

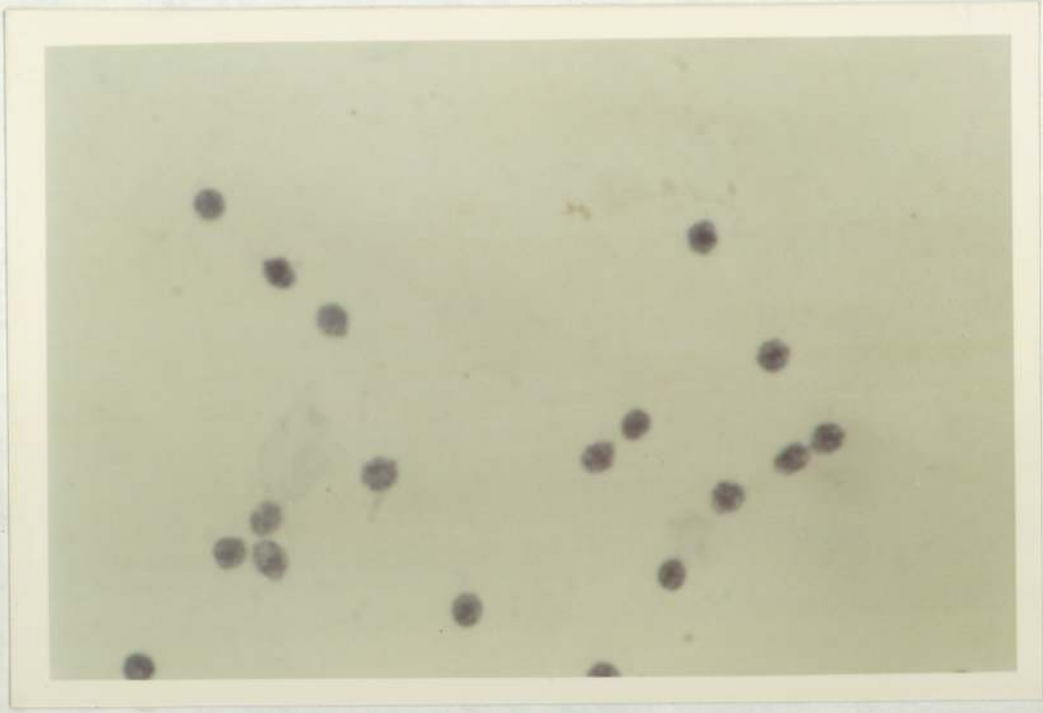


Figure 49b. Lack of Immuno-Peroxidase staining of Insulin Receptors on Rat Thymocytes - Cells Incubated with 0.75µg/ml Con A. Insulin Replaced by Non-Immune Serum in Staining Procedure.

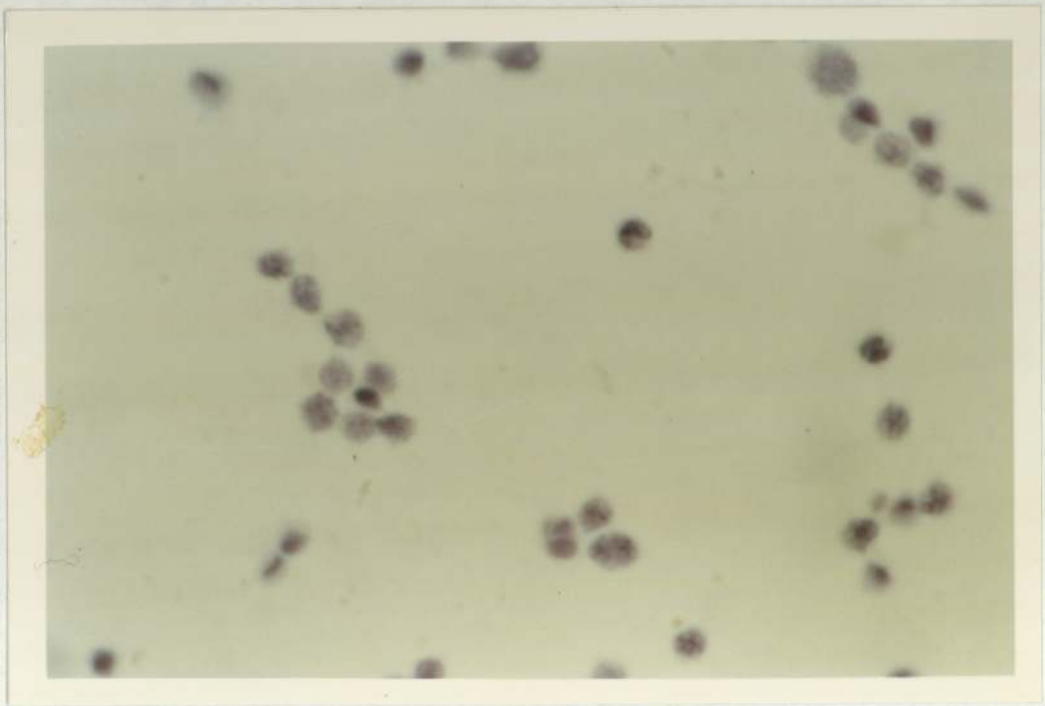
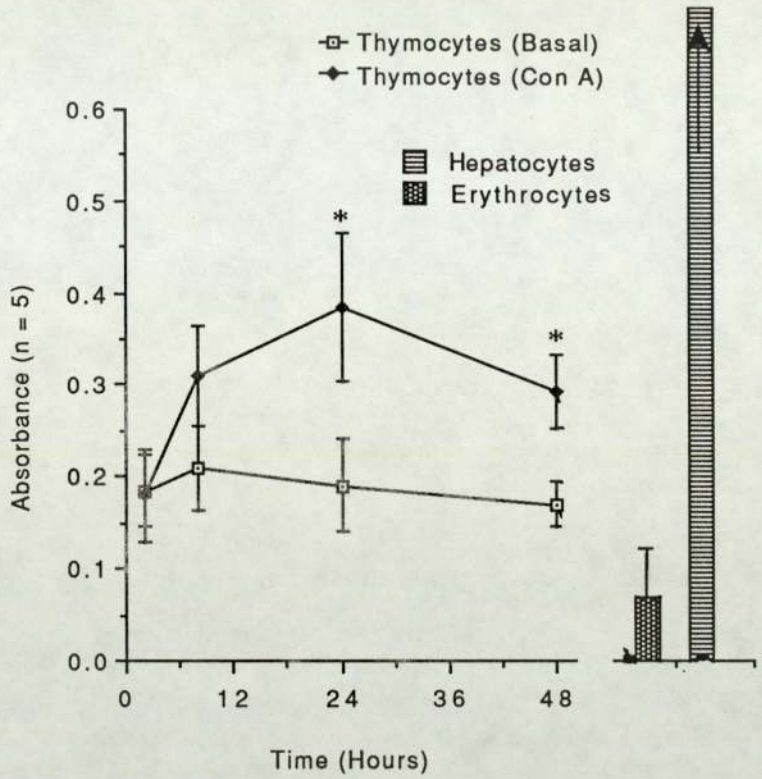


Figure 50. The Effect of Incubation with 0.75µg/ml Con A on the Number of Insulin Receptors on Rat Thymocytes.



Conversely, erythrocytes displayed very few cell surface insulin receptors. Under basal conditions, thymocytes expressed some number of insulin receptors which was intermediate between hepatocytes and erythrocytes. However, 24 hours exposure to Con A, provoked an approximate doubling in the number of such receptors expressed by rat thymocytes. Since 10^{-6}M insulin was used in the initial binding step, the method measured both insulin and type I IGF receptors. It was not possible to deduce whether more cells expressed the receptors, or whether more receptors were expressed per cell. The presence of more insulin and/or type I IGF receptors in the population as a whole, could nevertheless explain the increased responsiveness of the cells to 10^{-6}M insulin after Con A treatment.

As discussed in section I.1.3., the initial interaction between an agonist and a target cell involves binding, usually to specific receptors on the external leaflet of the plasma membrane. This binding process triggers a cascade of intracellular events which initiate the cellular adaptations which lead ulti-

mately to the cellular response to the agonist. A number of early signals have been associated with both lymphocyte proliferative control and insulin action, and preliminary investigations were made in an attempt to ascertain the roles played by two such signals; Ca^{++} and pH.

The involvement of Ca^{++} in the mitogenic response of various cell types, in response to a number of growth factors has been noted (section I.1.4.2.). Ca^{++} has also been tentatively associated with the mechanism of insulin action (section I.2.5.1.). A series of experiments were therefore undertaken to investigate whether Ca^{++} was involved in the responses of rat thymocytes to Con A and insulin.

In the absence of Ca^{++} in the extracellular environment, the proliferative response of the cells in response to Con A (Figure 51), insulin (Figure 52) and IGF I (Figure 53), was abolished.

Figure 51. The Effect of Removing Ca++ from the Extracellular Environment, on Tritiated Thymidine Incorporation into Rat Thymocytes in Response to Con A.

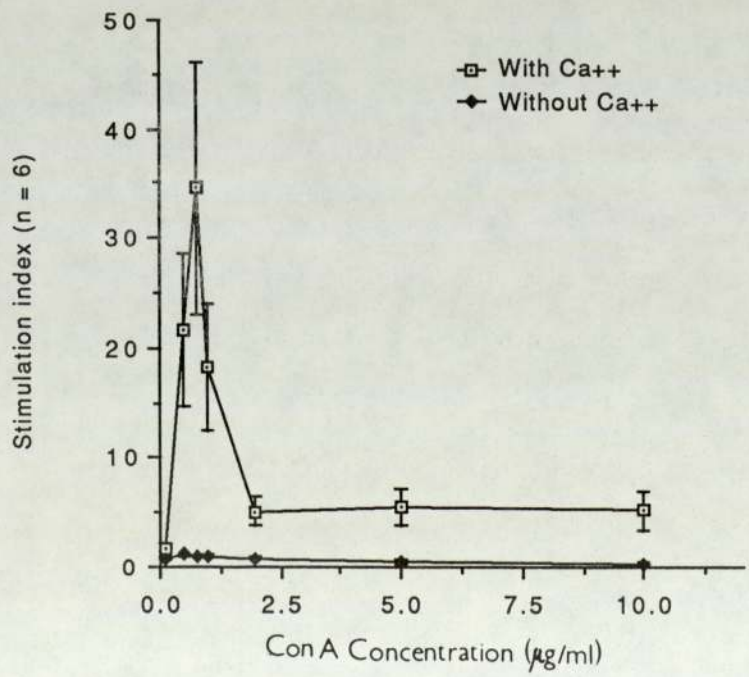


Figure 52. The Effect of Removing Ca++ from the Extracellular Environment, on Tritiated Thymidine Incorporation into Rat Thymocytes in Response to Insulin.

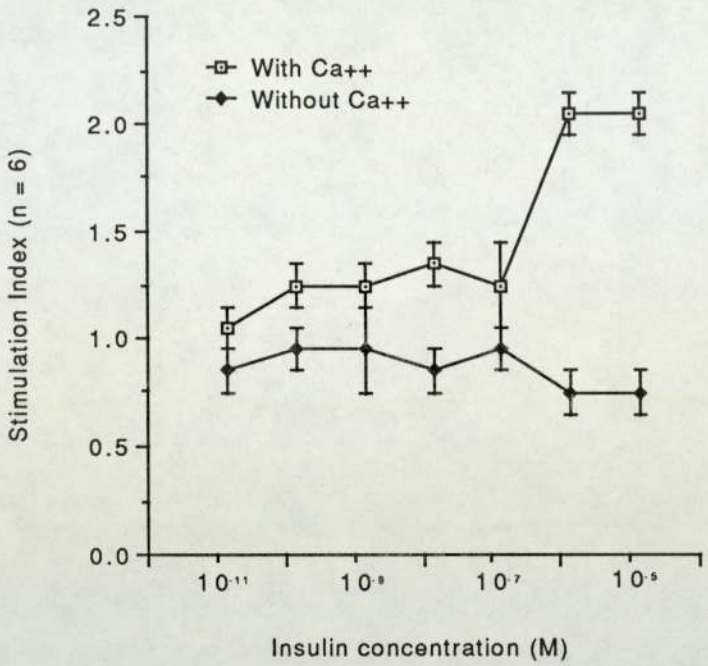
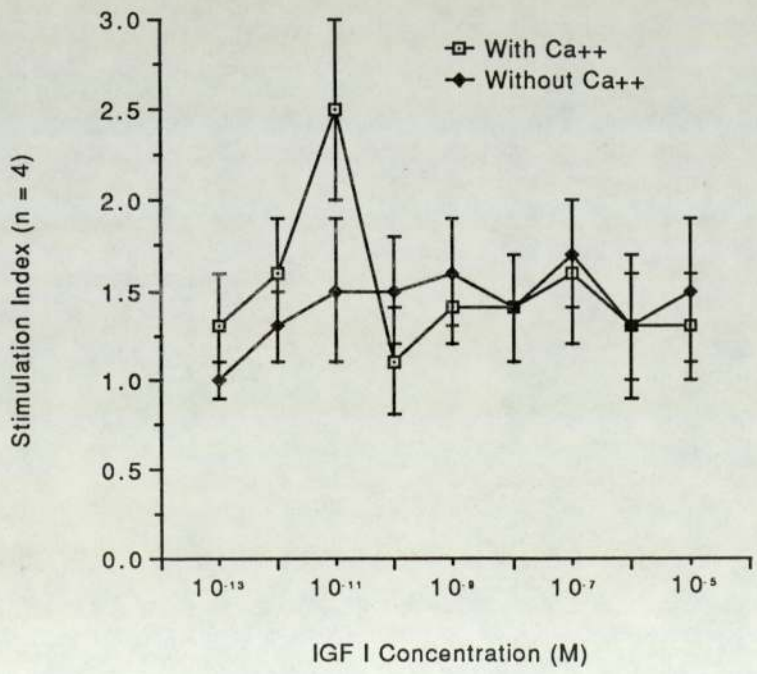


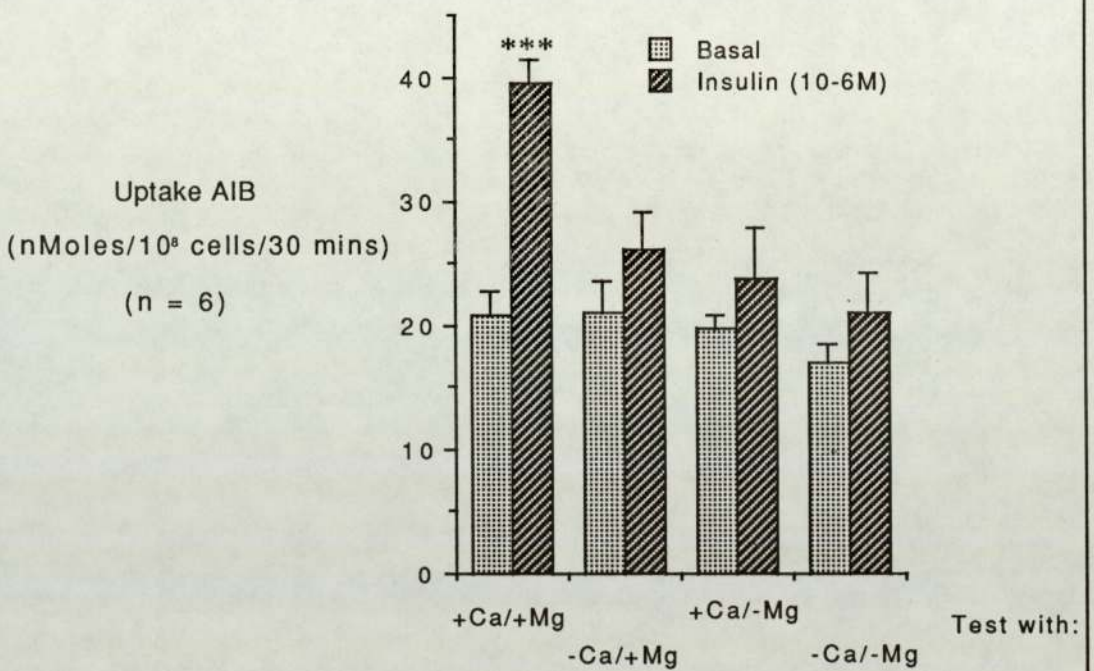
Figure 53. The Effect of Removing Ca++ from the Extracellular Environment, on Tritiated Thymidine Incorporation into Rat Thymocytes in Response to IGF I.



These results implied that Ca^{++} may be a general requirement in order for the processes of DNA synthesis to be accomplished, regardless of the agent used to provoke the growth response.

The effect on insulin-stimulated AIB uptake, of omitting the divalent cations Ca^{++} and Mg^{++}, was also studied (Figure 54).

Figure 54. Effect of the Absence of Divalent Cations in the Extracellular Environment, on Insulin Stimulated AIB Uptake into Rat Thymocytes.

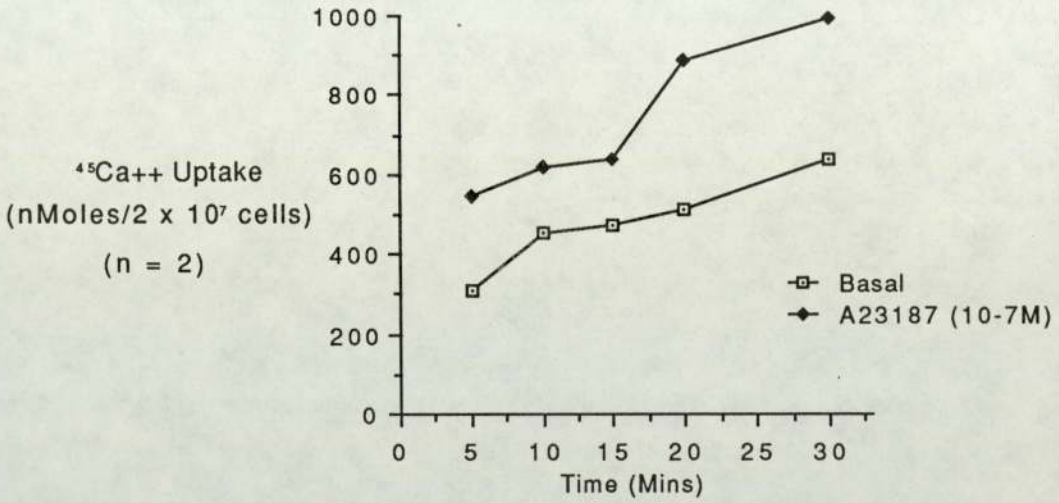


In the absence of insulin, there was no significant effect of omitting either or both cations. In the presence of insulin however, omission of either or both cations reduced AIB uptake to that observed under basal conditions. It seemed that the presence of both Ca^{++} and Mg^{++} was necessary in order for the insulin-stimulated enhancement of amino acid uptake to be observed.

Although the absence of Ca^{++} compromised the cellular growth response to Con A and insulin, and the enhancement of amino acid uptake in response to insulin, Ca^{++} may not necessarily have acted as a second messenger, mediating the response to the agonists. Its presence may have been permissive for numerous biochemical reactions which were required in order for the cellular response to take place. In an attempt to ascertain whether the extracellular

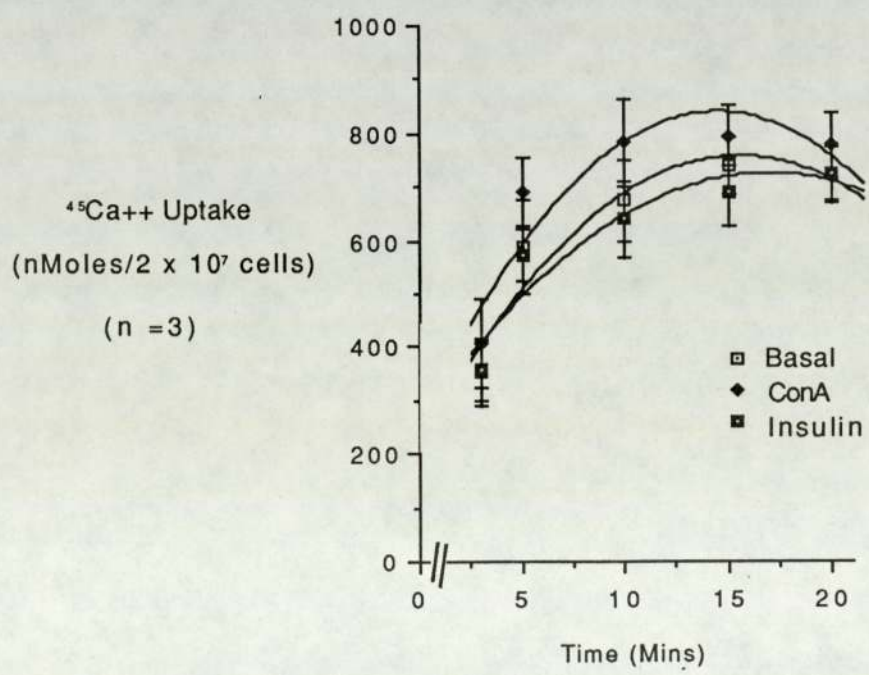
environment could act as a source of 'trigger' Ca^{++}, the influx of the radioactive isotope $^{45}Ca^{++}$ was measured. Initial tests using the Ca^{++} ionophore A23187, indicated that the method was sufficiently sensitive to show any differences in Ca^{++} uptake which might be provoked by the agonists (Figure 55).

Figure 55. Effect of the Calcium Ionophore A23187, on $^{45}Ca^{++}$ Uptake by Rat Thymocytes.



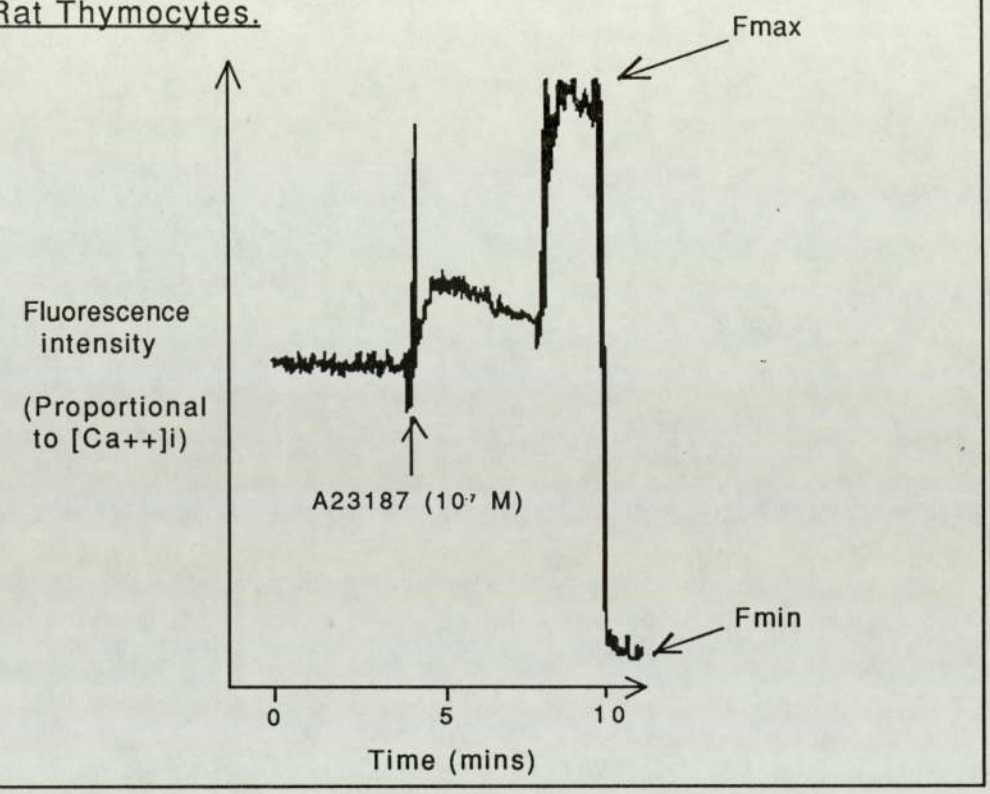
However, no significant difference in Ca^{++} uptake over 20 minutes could be detected between control, Con A-treated, and insulin-treated cells (Figure 56).

Figure 56. Effect of Insulin (10^{-6}M), and ConA (0.75 µg/ml), on $^{45}Ca^{++}$ Uptake by Rat Thymocytes.



Whether or not an influx of extracellular Ca^{++} was required to trigger the Con A and insulin induced responses, $[Ca^{++}]_i$ could also be increased by intracellular redistribution. The intracellular Ca^{++} indicator, Fura 2 was employed to test the possibility that $[Ca^{++}]_i$ may alter in response to the agonists. The Ca^{++} ionophore A23187 would be expected to raise $[Ca^{++}]_i$. This agent was again used therefore, to ensure that the system was responsive. As expected, addition of 10^{-7}M A23187 produced a rapid, large increase in $[Ca^{++}]_i$ (Figure 57 and Table 6).

Figure 57. Effect of the Calcium Ionophore A23187, on [Ca++]i in Rat Thymocytes.



Addition of 0.75µg/ml Con A also provoked a significant increase in $[Ca^{++}]_i$ (Figure 58 and Table 6). Con A has previously been shown to raise $[Ca^{++}]_i$ in other T-lymphocyte types (Metcalfe et. al., 1985; Imboden and Weiss, 1987). The response to Con A was slower to develop than the response to A23187, taking 7 - 8 minutes to raise $[Ca^{++}]_i$ from its resting level of 144nM to its peak of 227nM (values for experiment shown in Figure 58). However, addition of insulin (10^{-6}M or 10^{-5}M), produced no significant change in $[Ca^{++}]_i$ over the same time period (Figure 59 and Table 6).

Figure 58. Effect of ConA (0.75 µg/ml) on [Ca++]i in Rat Thymocytes.

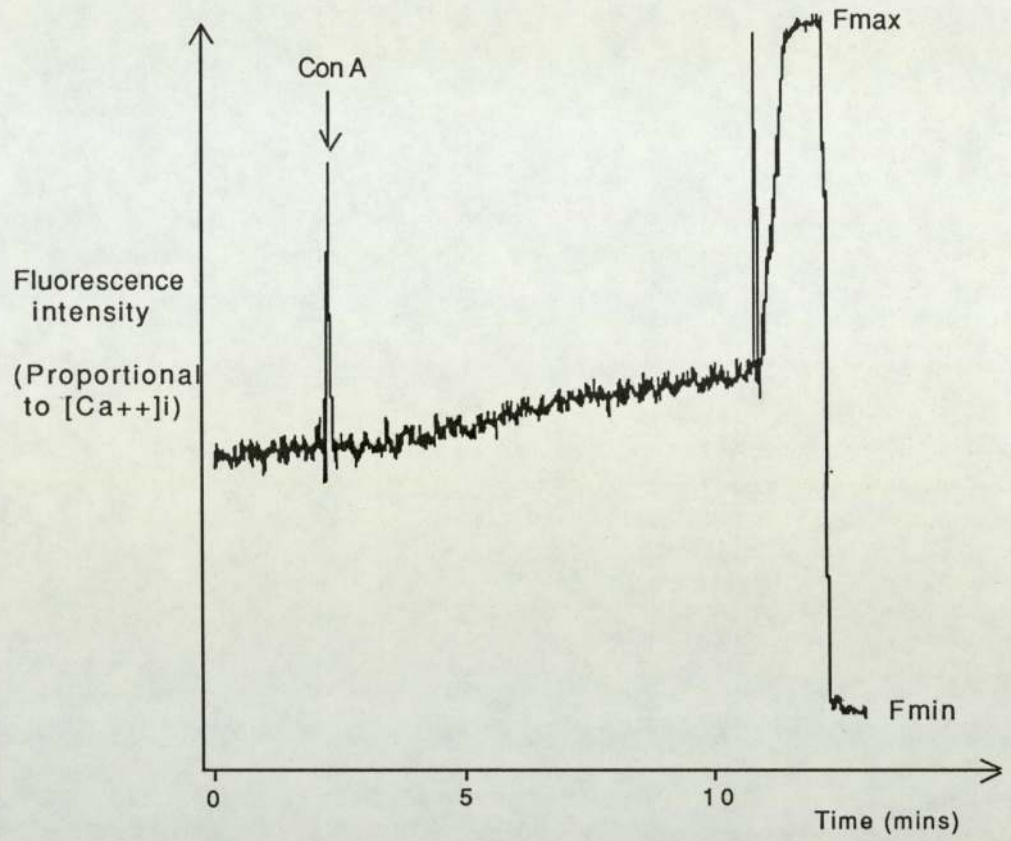
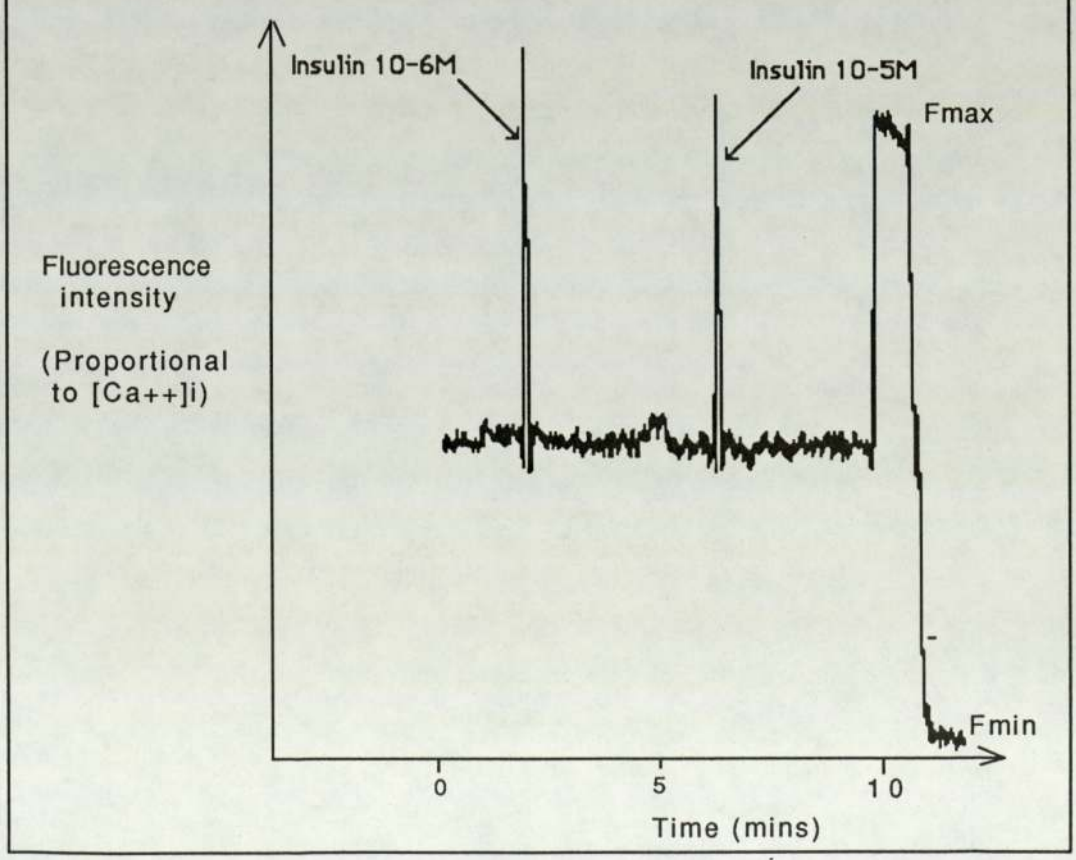


Figure 59. Effect of Insulin on [Ca++]i in Rat Thymocytes.



To ensure that the apparent rise in $[Ca^{++}]_i$ on addition of A23187 and Con A was caused neither by the addition of a small volume of liquid, nor by gradual dye leakage out of the cells, a trace was recorded showing the effect of adding a similar small volume of physiological saline to the cell suspension in the cuvette. No increase in fluorescence intensity was measured over the same time period (Figure 60).

Figure 60. Effect of the Addition of Small Volumes of Physiological Saline on [Ca++]i in Rat Thymocytes.

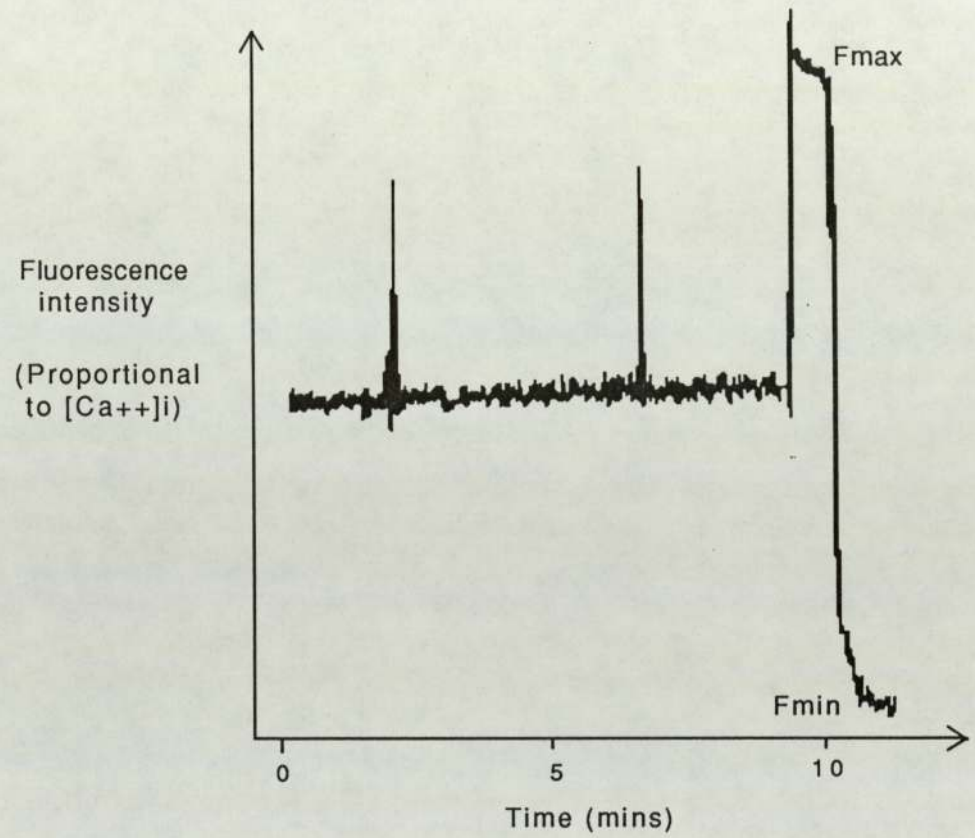


Table 6. The Effect of Agonists on [Ca++]i Levels after 8 Minutes in Freshly Isolated Rat Thymocytes.

Treatment	[Ca++]i (nM)	Significance compared to resting levels
Resting	155 ± 25 (n = 15)	-
Con A (0.75µg/ml)	239 ± 32 (n = 6)	**
A23187 (10-7M)	309 ± 40 (n = 4)	***
Insulin (10-6M)	170 ± 28 (n = 6)	ns

The changes in $[Ca^{++}]_i$ measured using this method were averages over the whole population of cells. Hence it was possible that insulin significantly increased $[Ca^{++}]_i$ in a small number of cells, the method being insufficiently sensitive to detect it. Alternatively, an increase in $[Ca^{++}]_i$ may play no part in insulin action in rat thymocytes. The requirement for its presence as shown in Figures 52 and 54, may be due to an involvement in binding or other indirectly related cellular reactions. The results are however consistent with the hypothesis that Ca^{++} may subserve a second messenger function in the action of Con A in rat thymocytes.

Another phenomenon which has been associated with both growth promotion (section I.1.4.1.) and insulin action (section I.2.5.1.) in a number of cell types, is an increase in intracellular pH. This parameter was therefore monitored using the intracellular pH indicator BCECF.

Both Con A (0.75 µg/ml) (Figure 61), and insulin (10^{-6}M) (Figure 62) provoked maximum increases of between 0.1 and 0.15 pH units in the intracellular pH of rat thymocytes .

Figure 61. Effect of ConA (0.75 µg/ml) on [pH]i in Rat Thymocytes.

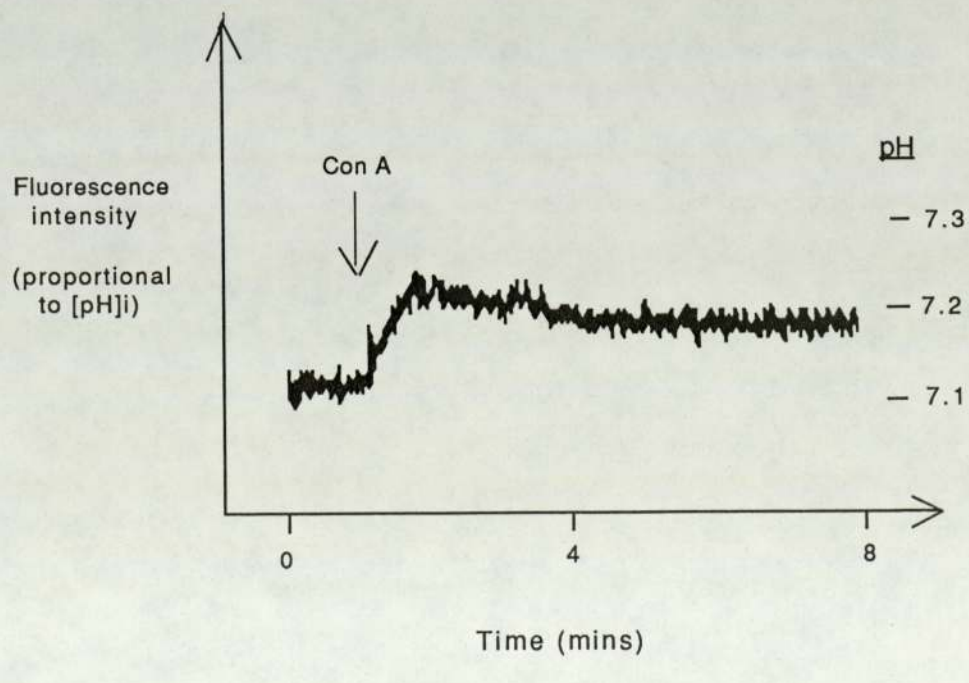
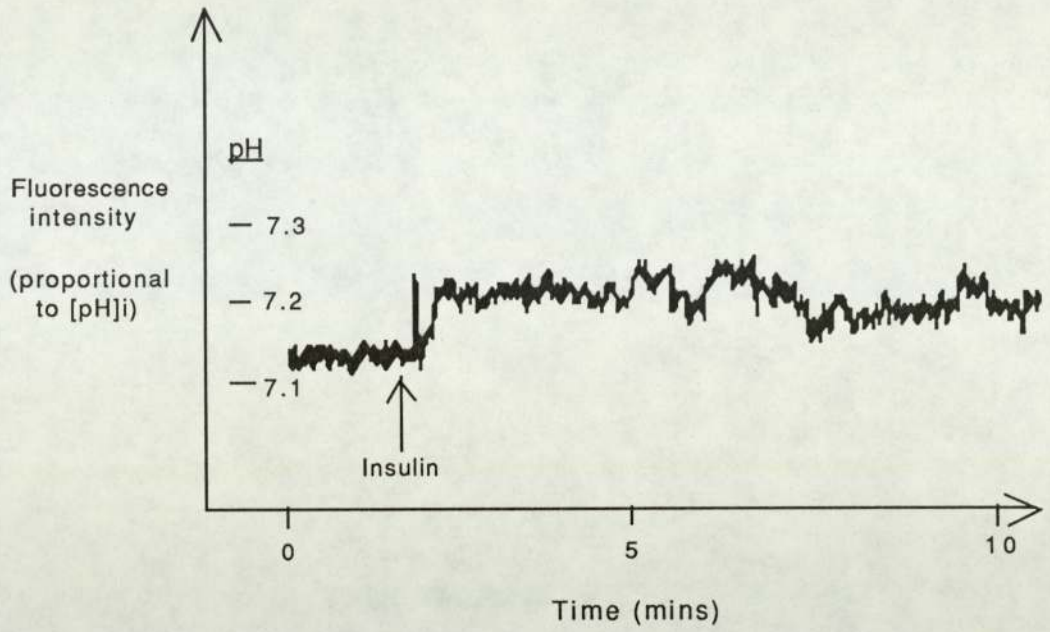


Figure 62. Effect of Insulin (10^{-6}M) on [pH]i in Rat Thymocytes.

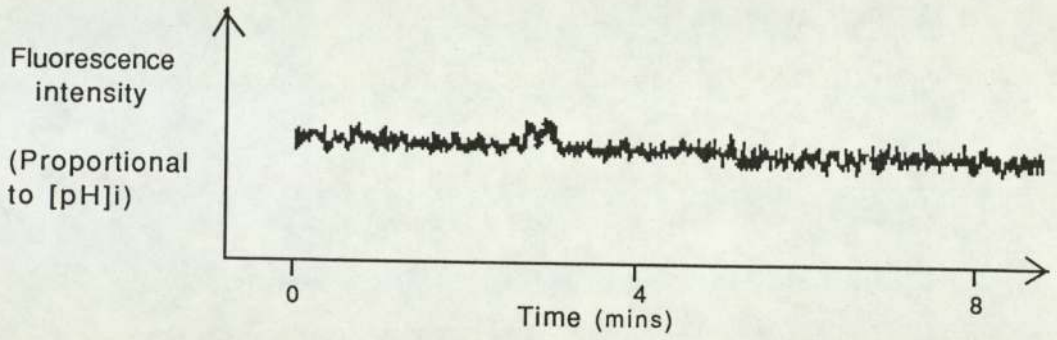


(The addition of small volumes of physiological saline to cell suspensions provoked no change in pH over the same time scale (results not shown)

The response to Con A was 'smoother' than the response to insulin. The initial, relatively rapid alkalinisation reached a peak about one minute after Con A addition, and then fell slightly to a sustained plateau about 0.1 pH unit above resting level. Homeostatic 'resetting' mechanisms may bring about the regulation of intracellular pH at a slightly higher level after Con A treatment. The response to insulin appeared to be less rigourously sustained, and more 'erratic' in profile. It may be that the response to insulin differed between different sub-populations of rat thymocytes, thus giving this appearance of irregularity, when pH was averaged over the whole population. Alternatively insulin may have initiated oscillatory changes in intracellular pH of the cells.

One mechanism whereby alterations in intracellular pH may be brought about is via activation of the Na^+/H^+ antiport (section I.1.4.1.). In order to ascertain whether this could be the mechanism of alkalinisation in these cells, monitoring of intracellular pH was undertaken in the same way, but in the presence of amiloride (100μM), a supposedly specific inhibitor of the antiport. In the presence of this drug the response to Con A (Figure 63) and insulin (not shown) was abolished,

Figure 63. Effect of Con A (0.75 µg/ml) on Intracellular pH of Rat Thymocytes in the Presence of Amiloride (100µ M).



These results are consistent with the proposal that the increase in cytoplasmic pH associated with both Con A and insulin treatment of rat thymocytes, is brought about by the activation of the Na^+/H^+ antiport.

At least part of the early response of rat thymocytes to the polyclonal activator Con A, involved an increase in $[Ca^{++}]_i$, which may act as a trigger of later events, in association with Na^+/H^+ antiport activation, raised $[pH]_i$, and possibly other factors. Amongst the later responses to Con A was an enhanced expression of insulin and/or type I IGF receptors, which took place 12 - 24 hours after initial exposure to the agonist. The binding of insulin to insulin /type I IGF receptors, also activated the Na^+/H^+ antiport, initiating a rise in $[pH]_i$. This may have effected cellular reactions, perhaps in combination with other factors, leading to enhanced metabolism, and facilitating passage through the cell cycle to division.

IV. DISCUSSION.

Assessment of the putative mitogenic potential of insulin using a subjective scoring method is clearly hazardous. Even experienced observers can inadvertently be influenced by preconceived views of the outcome of an experiment. It was established several years ago that elevated Ca^{++} concentrations would stimulate division of thymocytes *in vivo* and *in vitro* (Perris, 1971; Perris et. al., 1985). To supposedly check on the normal responsiveness of thymocyte cultures, a basal and a high Ca^{++} treatment were thus routinely included in experiments investigating responses to insulin. When the identity of these internal control slides was known, invariably two observers counting independently, found numbers of mitotically arrested cells of 4% and 6% respectively. Statistical considerations suggested that by counting 2000 cells/slide, such a difference should be detectable. However when the same slides were counted without the identities being known, these clear cut differences were eliminated (Figures 24, 25a and 25b).

The group of Whitfield et. al. have published extensively on a variety of mitogens (not including insulin) for short term thymic lymphocyte cultures (Whitfield et. al., 1969, 1979, 1980, Boynton et. al., 1981). Using specific pathogen free Sprague Dawley rats, their basal and stimulated mitotic activities over a 6 hour period were 5% and 10% respectively. Their autoradiographic procedures indicated that under basal conditions, about 11% of the population was synthesising DNA at the outset of incubation. Two hours after mitogen application 18% of the cells were to be found in S phase (Youdale and Macmanus, 1975). Although these differences are greater than those encountered in our laboratory, it is not clear whether in the counting of mitotic figures or labelled nuclei, the identities of the slides were known to the scorer(s). Furthermore, no

indication was given of the total number of cells/nuclei counted in each case, but if this was less than 1000/treatment/replicate, then their results may have been statistically invalid.

When Youdale and Macmanus (1975) measured the total radioactivity incorporated after exposure to ^{3}HTdR, raised extracellular [Ca^{++}] did not appear to enhance DNA synthesis. The discrepancy between this result and that obtained autoradiographically was ascribed to the ability of the mitogen to stimulate thymidylate synthetase. The resultant pool of unlabelled thymidine generated endogenously, would dilute out the radioactively labelled ^{3}HTdR, so that the latter's incorporation into DNA would be an inaccurate reflection of DNA synthesis. We were likewise unable to detect any enhanced incorporation of ^{3}HTdR by rat thymocytes over short term (6 - 12 hour) cultures, in response to the plant lectin Con A, insulin (10^{-6} and 10^{-10}M) or 1.8mM extracellular Ca^{++} (Figure 26).

In an attempt to demonstrate that raising extracellular [Ca^{++}] really did stimulate DNA synthesis, even though ^{3}HTdR uptake failed to show it, Youdale and Macmanus followed incorporation of radioactive formate into DNA. One hour after exposure to mitogenic Ca^{++} concentrations, formate incorporation was increased by almost 50%; the majority of the radioactivity was found in the thymine residues of the extracted DNA. However the extractions involved several steps, there was no indication of the efficiency of extraction from control and test cultures, and very high cell densities were needed to obtain measurable radioactive counts. Indeed values were only 1000 - 1500 cpm per 10^{10} cells, for a one hour incubation. The procedures were complex and protracted, and could not readily be used for routine assessment of DNA synthesis.

The approach to this problem in our laboratory was to follow total radioactive formate uptake into the cells, and subsequently to try and estimate what fraction of this might be attributable to DNA. Treatment of

fixed cells with DNAase should reveal what proportion of the formate had been incorporated into DNA, and what fraction into other materials, largely protein. Although, total incorporation measurements were refined so that consistent reproducible values were obtained (Figures 27 and 29), DNAase procedures were less successful (Figure 28). Once again, complex, protracted methods involving several centrifugation and resuspension steps may have resulted in inconsistent cell losses, and made the technique unsuitable for analysis of DNA synthesis. Nevertheless, even comparisons of total radioactive formate uptake in basal and mitogen treated cultures should have revealed any increase in DNA synthesis in mitogen treated cultures, since it is unlikely that the cellular reactions preceding mitosis would involve increased DNA synthesis and an equal but opposite decrease in protein synthesis. No such difference in total radioactive formate incorporation into basal and high Ca^{++} treated cultures was however detected (Figure 29).

Since no difference in proliferative potential over six hours between control and mitogen treated cultures was convincingly demonstrated using any of the methods chosen here, considerable doubt is cast on the existence of a subset of 'rapidly recruitable' rat thymocytes. Previous evidence of such a group of cells has largely been based on the use of methods which involve either 'counting' of mitotically arrested (Perris et. al., 1985), or autoradiographically labelled (Youdale and Macmanus, 1975) cells, or the incorporation of formate into DNA (Youdale and Macmanus, 1975). The inherent problems associated with both techniques have been highlighted in these studies.

Some authors claim to have observed an increase in scintillometrically measured ^{3}HTdR uptake into rat thymocytes after short-term culture with mitogens (Kimoto et. al., 1983 and Ernström et. al., 1987). However, the first group observed a dramatic increase in ^{3}HTdR incorporation into

both control and Con A treated cells after 6 hours of incubation. There was no significant difference between the radioactivity associated with control and mitogenically stimulated cells. The second group purported to show an enhanced recruitment of rat thymocytes into the S phase of the cell cycle 5 hours after treatment with a thymocyte specific growth peptide (TGP) extracted from the conditioned medium of murine thymic leukaemia cells. The authors used the response of freshly isolated rat thymocytes in culture as an assay system for their TGP. However, their results showed relatively low radioactive counts obtained from treated cultures (less than 4×10^3 cpm/culture), and they provided no evidence of the reproducibility of their results. It was also unclear to what extent uptake of ^{3}HTdR differed between control and TGP treated rat thymocytes.

It seems then, that further investigation is required before the existence of a subset of rat thymocytes, which initiate DNA synthesis within 6 hours of mitogenic stimulation, is unequivocally demonstrated. However, whether or not such cells exist, rat thymocytes are certainly responsive to mitogenic stimulation over longer time scales, and this response is detectable using scintillometric measurement of ^{3}HTdR incorporation into newly synthesised DNA; the response to the plant lectin Con A, for example, has frequently been studied (Metcalfe et. al., 1985, Sharon and Lis, 1987).

In our hands, Con A was also clearly a potent mitogen for these cells (Figure 31). However, although this response is of interest, and worthy of analysis since it may throw light on the mechanisms of T lymphocyte activation, it is not a physiological response. The much smaller proliferative responses to insulin and IGF I (Figures 32 and 33) may however have direct physiological relevance.

A comparison of the concentrations of insulin (10^{-6}M) and IGF I (10^{-11}M) required to elicit a mitogenic response, suggested that both agonists may act by binding to type I IGF receptors. As discussed in the introduction (section I.2.4.), the requirement for supraphysiological concentrations of insulin to promote growth in a number of cell types, has been found to be due to its cross reactivity at high concentrations with type I IGF receptors (Straus, 1984; King and Kahn, 1985). Rat thymocytes therefore probably represent another cell type in which the growth promoting aspects of insulin action are mediated by type I IGF receptors, high affinity insulin receptors playing no significant part in this process.

It was also noted in the introduction (sections I.2.2. and I.2.3.), that insulin and IGF I generally have a rather weak effect on DNA synthesis in fibroblasts when acting alone, but act synergistically with a variety of other agents (Dicker and Rozengurt, 1978; O'Keefe and Pledger, 1983; Rozengurt, 1986). The presence of serum in the cultures described here makes direct comparison between these systems and the serum free cultures used by Rozengurt's and Pledger's groups hazardous, but some parallels can be drawn. Firstly, insulin and IGF I were found to be relatively weak mitogens for rat thymocytes, as they were for fibroblasts. In contrast to the fibroblast model however, insulin did not appear to synergise with the low concentrations of growth factors presumably present in serum. It is possible that the amount of foetal calf serum used here (5% by volume) was too small to provide sufficient concentrations of other growth factors. However, a simpler explanation for the apparent lack of synergy between insulin and serum may be that T lymphocytes require different growth factor(s) to initiate the proliferative response. They certainly bear few, if any, receptors for the main serum growth factors, PDGF, EGF and FGF (Plaut, 1987, and section I.3.1.2.). The growth

factors present in serum may be mitogenic for fibroblasts, but not for thymocytes.

Certainly physiologically, the factors which initiate T lymphocyte growth are either restricted in their location, such as the 'thymic hormones' (section I.3.1.1.), or are very specific, such as antigens. The 'primary' stimulus, provided *in vivo* by these agents, was mimiced in the experiments described here by using the polyclonal activator, Con A. The ability of insulin to support greatly enhanced DNA synthesis after the initial 'primary' stimulus (Figure 35), suggests a parallel between its role in thymocyte growth, and its role as a progression factor in fibroblast proliferation. Con A may thus be regarded as a thymocyte 'competence' factor, priming the cells to respond to the subsequent addition of a 'progression' factor, insulin.

However, since Con A alone promotes DNA synthesis, it may be inaccurate to suggest that its role is strictly analogous to a competence factor. However, although Con A may superficially act as both a 'competence' and a 'progression' factor in this system, there is evidence to suggest that the physiological 'primary' stimulus which it is designed to mimic, is insufficient alone to promote full T lymphocyte activation (Robb, 1984; Plaut, 1987). Also, the receipt of the 'primary' signal initiates a set of responses in T lymphocytes which include the production and secretion of IL-2 and the expression of IL-2 receptors (section I.3.1.2. and Figure 14). In the cultures described here then, Con A may well have acted only as a 'competence' factor, and the subsequent passage through the S phase of the cell cycle may have been supported by autostimulation of the cells with IL-2, which acts as the 'progression' factor. The contribution of this IL-2 would, of course, have been diminished when cells were washed in the so-called 'split culture' experiments (Figures 35, 36a and 36b). Nevertheless, whether or not further IL-2 was secreted

when the cells were recultured, insulin, probably acting through type I IGF receptors, certainly and most significantly, acted as a 'minor' growth factor which potentiated the progression of rat thymocytes through the S phase of the cell cycle.

Results from metabolite uptake experiments gave an indication of some of the processes which may be altered in the presence of insulin, facilitating the passage of cells through DNA synthesis and mitosis. Insulin stimulated glucose transport into the cells at both its physiological concentration (10^{-10}M) and at its 'growth promoting' concentration (10^{-6}M) (Figure 38). It may be that in the heterogenous cell suspension, different sub-populations have insulin receptors differing in number or affinity. It seems more likely however, that enhancement of glucose uptake is a metabolic response of the cells which may be initiated by binding to both insulin and type I IGF receptors.

The more marked effects on glucose transport seen after a 2 hour pre-incubation with insulin, may be because insulin causes a gradual insertion of more glucose transporters into the plasma membrane (section I.2.1.). In adipocytes, new glucose transporter molecules only appear 10 minutes after insulin application (Simpson and Cushman, 1986). These results may also help to explain discrepancies in the literature; some authors having found a stimulatory effect of insulin on sugar transport in rat thymocytes (Toma et. al., 1965; Boyett and Hofert, 1972), whereas others have found no such effect (Goldfine et. al., 1972).

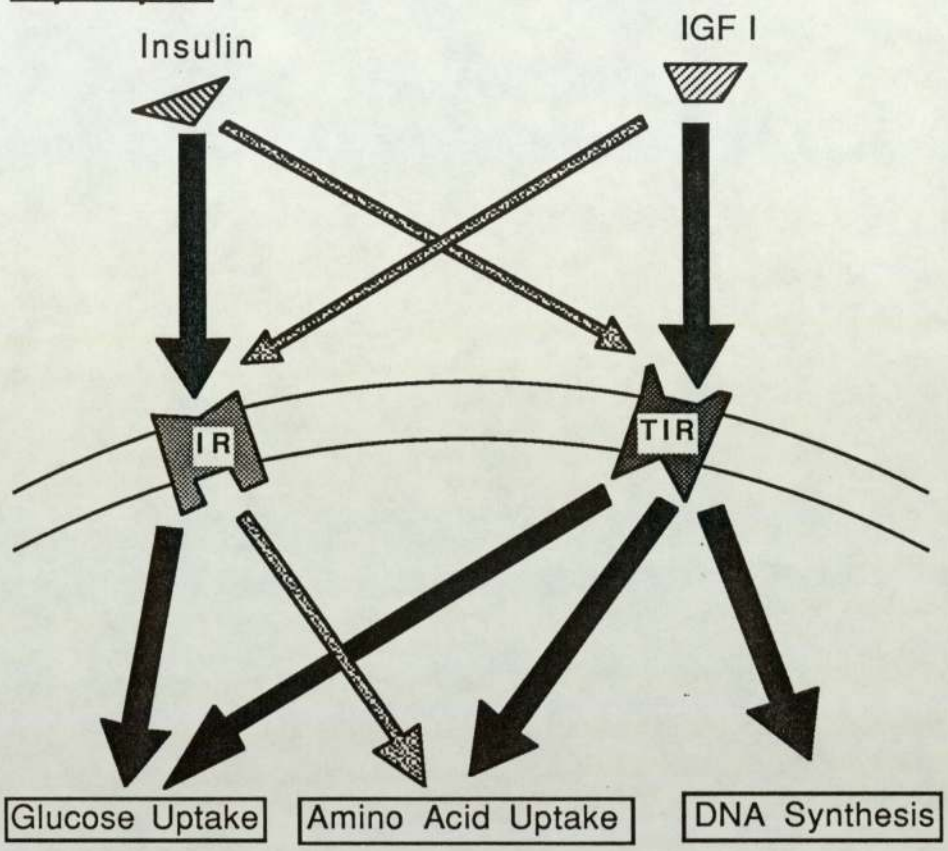
Insulin also promoted amino acid uptake into the cells (Figure 40). The greater response observed after a 2 hour pre-incubation with insulin indicated a temporal separation between insulin binding and maximum enhancement of amino acid uptake. The stimulatory action of insulin came about through an increase in the maximum rate of amino acid transport, in the absence of a change in the affinity of the system 'A'

transporters for α-aminoisobutyric acid (Figure 42). Taken together, these observations are consistent with the proposal mentioned in the introduction with respect to adipocytes (section I.2.1.), that insulin acts relatively quickly to protect amino acid system 'A' transport proteins from degradation, and more slowly and gradually to enhance the rate of synthesis of these proteins and their insertion in the plasma membrane.

The stimulatory effect of insulin on amino acid uptake was not biphasic, the peak being at the 'growth promoting' concentration of insulin (10^{-6}M). This implies that the response may be mediated by type I IGF receptors. However, although low concentrations (10^{-11}M) of IGF I provoked an increase in amino acid transport above control levels (Figure 43), it was less than the enhancement observed with 10^{-6}M insulin. Also, a small enhancement of amino acid uptake was observed in response to 10^{-10}M insulin (Figure 40). There is a possibility then, that insulin may enhance amino acid uptake by acting through both insulin and type I IGF receptors. The larger response may be associated with growth promotion, and takes place due to type I IGF receptor binding, the smaller response is mediated by insulin receptors, and may be associated with a more general metabolic enhancement in response to insulin.

Since IGF I stimulated both DNA synthesis and amino acid uptake, and insulin at both 'high' and 'low' concentrations stimulated glucose uptake in rat thymocytes, it may be that both metabolic and growth promoting actions of insulin are mediated by type I IGF receptors. Conversely, insulin receptor binding may only be linked to metabolic processes in these cells (Figure 64). However, since insulin, at low concentrations is present in all long term cultures studied here, it may be incorrect to assume that insulin, acting via its own receptor has no effect on growth processes, although any such effect is relatively weak.

Figure 64. The Control of Biological Responses by Insulin and IGF I, and Their Respective Receptors in Rat Thymocytes.



Metabolic effects:-

Growth effects:-

Key:-

IR - Insulin Receptor

TIR - Type I IGF Receptor

Dark arrows represent high affinity binding or strong stimulation of a process.
Shaded arrows represent weak binding affinity or weak stimulation of a process.

It is important to note that although the responses were sustained, the enhancement of both glucose and amino acid uptake in response to insulin, as described above, are initiated over relatively short time scales, in contrast to the growth response to the same agonist. There is also a small enhancement of amino acid uptake in response to Con A over a short time scale (Figure 44). A similar phenomenon has been observed in rabbit thymocytes (Schmidt-Ullrich et. al., 1976) and rat peripheral lymphocytes (Van den Berg and Betel, 1973), though its physiological significance is somewhat obscure. It seems reasonable to assume that these 'fast' responses are due to some action of Con A itself, possibly related to functional membrane alterations, since the increased expression of IL-2 receptors and secretion of IL-2, which may accompany and form part of the longer term growth promoting effects of Con A, are unlikely to be established within 2.5 hours of exposure to the agonist. Certainly over a longer a time scale (48 hours), a much greater enhancement of amino acid uptake in response to Con A is observed (Figure 45). This may, at least in part, be due to an effect of autostimulatory IL-2. Once again however, whether or not this is the case, insulin was able to potentiate the stimulatory effect of Con A/IL-2 on amino acid uptake, provided the time of exposure to Con A was sufficient (between 8 and 24 hours), presumably for some essential 'priming event' to be accomplished.

The nature of this 'priming event' may be the expression of new insulin and/or type I IGF receptors (Figure 50). The emergence of insulin receptors in response to primary activation of peripheral T lymphocytes has previously been observed (Helderman, 1981; Snow, 1985), and indeed the possession of such receptors has been proposed as being diagnostic of activation in these cells (Helderman et. al., 1978; Snow, 1985). However, the experimental methods cannot distinguish between insulin and type I

IGF receptors except by inference. Nevertheless, as previously discussed, it seems most likely that high affinity insulin receptors are involved in responses to 10^{-10}M insulin whereas where 10^{-6}M insulin elicits a response, it seems most likely that binding to type I IGF receptors is responsible. The immunoperoxidase methods detect both insulin and type I IGF receptors. The available evidence suggests that in peripheral T lymphocytes, the expression of type I IGF receptors is regulated in a manner similar to that for insulin receptors. Resting T lymphocytes appear to lack type I IGF receptors, but after 'primary' activation, they are amongst the receptor types which are newly expressed (Rosenfeld and Hintz, 1980; Plaut 1987).

The situation for thymocytes is clearly more complicated than for peripheral T lymphocytes, since the former possess some insulin and/or type I IGF receptors under basal conditions (Figures 48 and 50). It has been suggested that about 12 hours of exposure to the 'primary' agonist are required before the expression of new insulin receptors can be detected on peripheral T lymphocytes (Snow, 1985). Certainly, some event which takes place between 8 and 24 hours after exposure to Con A renders rat thymocytes even more responsive to insulin (Figures 46a, 46b and 46c). The timescale of this response correlates well with the appearance of more insulin receptors, 12 to 24 hours after exposure to Con A (Figure 50). It seems then that thymocytes possess some insulin/type I IGF receptors, and are responsive to insulin, prior to activation, and hence the possession of such receptors is by no means exclusively related to activation/proliferation in these cells. Although it may be noted that some freshly isolated rat thymocytes may have been activated *in vivo*. The series of responses to Con A may, in a sense, mirror the responses of peripheral T lymphocytes to activation, in that more insulin/type I IGF

receptors are expressed, and the responsiveness of the cells to insulin is enhanced.

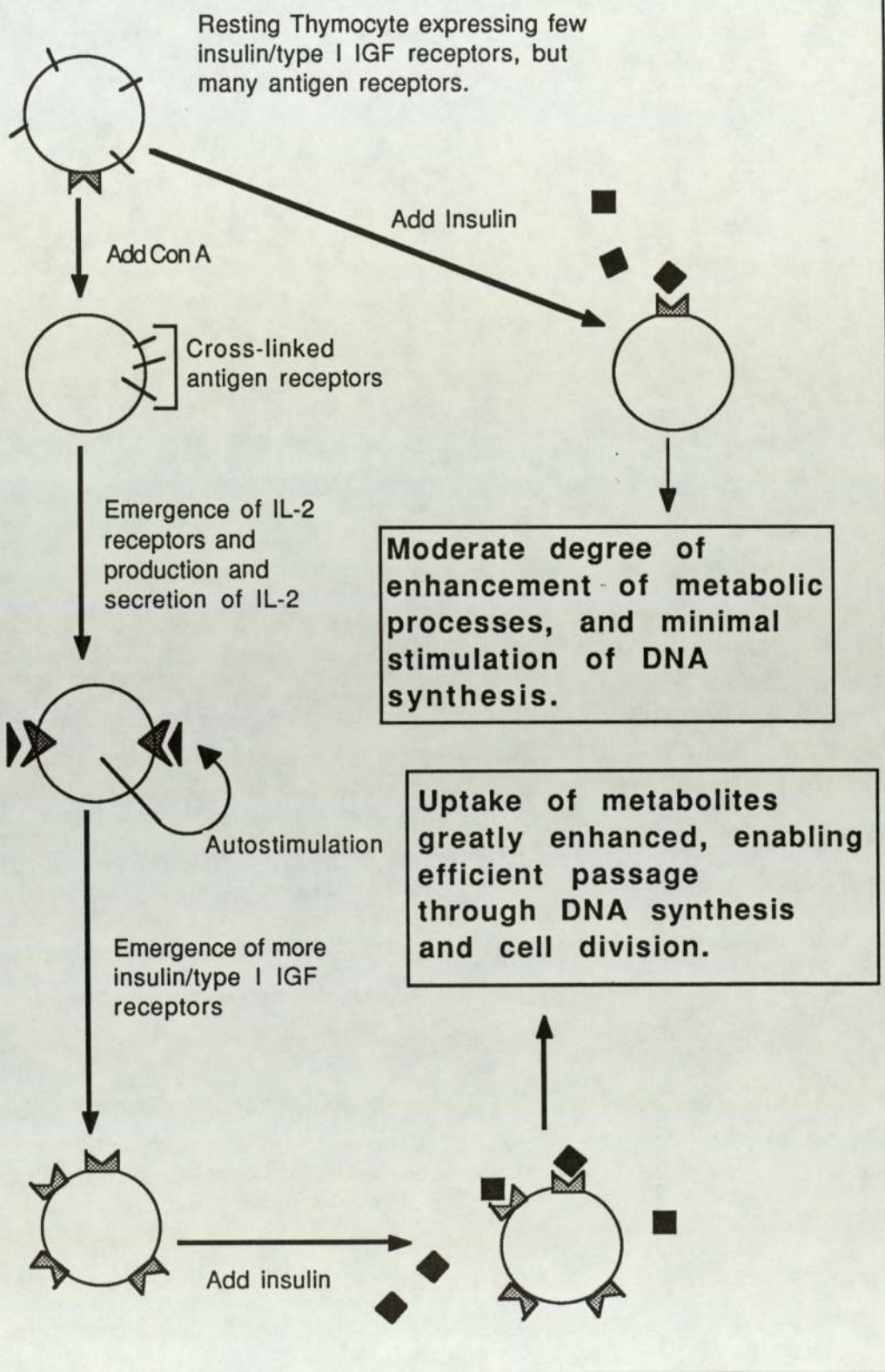
Teleologically it seems sensible to link metabolic enhancement with growth in these cells, and it has been noted that enhanced uptake of glucose and amino acids forms part of the 'pleiotypic response' to growth promoting agents in various cell types. The timescale over which Con A and insulin induce growth promotion and enhancement of amino acid transport have been compared (Table 5), and it has been noted that although enhanced amino acid uptake accompanies enhanced DNA synthesis after Con A treatment, it precedes the peak proliferative response to insulin. In freshly isolated rat thymocytes, which possess some insulin/type I IGF receptors, insulin induces an enhanced uptake of amino acids (and glucose). After 48 hours exposure to insulin, this response is reduced, possibly due to receptor down regulation (section I.1.3.). Although a growth response occurs in these cells in response to insulin, the magnitude of this response is relatively small. Therefore it seems unlikely that the increased uptake of nutrients acts as a 'trigger' except perhaps for a small proportion of cells which are biochemically 'poised on the brink' of entry into the cell cycle. However, Con A clearly provides a 'trigger' for the cells to initiate DNA synthesis which is not directly related to an enhanced nutrient uptake. The main increase in amino acid uptake occurs later, as the processes of DNA synthesis progress and demand for increased supplies of raw materials and energy substrates is enhanced. The evidence presented here provides an indication of a potentially pivotal role for insulin/IGF I in this process. Con A provides the primary stimulus to initiate DNA synthesis. After Con A treatment, rat thymocytes may express IL-2 receptors and secrete IL-2 into the medium, providing further (auto)stimulation. In response to IL-2 the cells may express greater numbers of a variety of receptors for

polypeptide hormones including insulin and IGF I. These may then act to enhance both metabolism and the supply of raw materials which are required to fuel the passage of the cells through DNA synthesis and mitosis (Figure 65).

The linear progression of rat thymocytes through this orderly series of physiological/biochemical states, the pathway to proliferation, illustrates an important concept in hormone action, and the regulation of cellular sensitivity to hormones. On the one hand, where the agonist is localised, such as the thymic hormones, or very specific, such as an antigen, cells may express receptors for such an agonist at most stages of their life history. The receptor is present, in a sense, to detect the presence of the stimulatory agent, as well as to participate in the generation of the primary intracellular response to it. On the other hand, where the presence of an agonist is more general, another set of cellular strategies must be adopted in order to avoid inappropriate triggering of responses. For example, adult plasma concentrations of IGF I are maintained at a relatively constant concentration (Hall and Sara, 1983; Froesch et. al., 1985), and plasma insulin concentrations are largely dependent on blood glucose levels. Clearly then, the sensitivity of lymphocytes to these agents must be regulated to avoid either a constant trophic effect in the presence of IGF I, or the initiation of a growth response in response to eating a meal, when a release of insulin would ensue. The expression of greater numbers of receptors for these agents after the 'primary' growth stimulus, provides an elegant answer to this problem of sensitivity regulation. Here, the receptor does not act as a 'detector', but it is the hormone itself, which awaits the appearance of the receptor, before a physiological response can be generated.

Some insight into the molecular nature of the early intracellular responses to Con A may also be deduced from the evidence presented

Figure 65. The Sequential Emergence of Various Receptor Types on Rat Thymocytes in Response to Mitogenic Stimulation and the Effect of Insulin.



here. Clearly, a source of extracellular Ca^{++} is required in order for the proliferative response to take place (Figure 51), and a rapid rise in intracellular $[Ca^{++}]_i$ occurs subsequent to Con A binding (Figure 58 and Table 6). The close association of this ion with cell growth (section I.1.4.2.), coupled with the evidence presented here, makes it seem likely that Ca^{++} acts as a second messenger in the proliferative response of rat thymocytes to Con A. However, it would be hazardous to conclude that the rise in $[Ca^{++}]_i$ is exclusively derived from the extracellular environment, since a 48 hour culture in Ca^{++}-free conditions would severely deplete intracellular Ca^{++} stores.

Con A also provokes a rapid rise in intracellular pH (Figure 61), another phenomenon which has repeatedly been found to be associated with growth (section I.1.4.1.). Since this response is abolished in the presence of amiloride (Figure 63), it is suggested that activation of the Na^+/H^+ antiport provides the mechanism of cytoplasmic alkalinisation.

Certain other agents which provoke this dual response of a) an increase in intracellular $[Ca^{++}]$, and b) an increase in intracellular pH, have been shown to act via the inositol phospholipid system (section I.1.3. and Figure 4). These results are entirely consistent with the proposal that Con A acts to promote proliferation in rat thymocytes via this pathway. Previous studies have indicated that this system also operates when Con A is used to provoke growth in murine thymocytes (Metcalfe et. al., 1985), and human peripheral T lymphocytes (Gelfand et. al., 1987).

Whatever the mechanism of insulin action it seems to be quite distinct. Although a source of extracellular Ca^{++} is necessary in order for the growth response to be evoked (Figure 52), and both Ca^{++} and Mg^{++} are required for insulin stimulation of amino acid uptake (Figure 54), the absence of a rise in intracellular Ca^{++} after insulin binding (Figure 59 and Table 6) suggests that it may not play a 'trigger' function. However, the

possibility remains that since there may be only a limited number of insulin-responsive cells in a culture of freshly isolated rat thymocytes, the method may be insufficiently sensitive to detect any response. Whether or not a rise in $[Ca^{++}]_i$ plays any role in the initiation of the growth response to insulin, both the divalent cations have essential roles in cellular metabolism (section I.1.4.2.), and may be involved in binding of various agonists to their receptors. Their precise role in insulin action is obscure (section I.2.5.1.), but there may be an optimal range of intracellular $[Ca^{++}]$ required in order for insulin stimulated processes to take place (Draznin et. al., 1987).

One rapid effect of insulin in rat thymocytes is to raise the cytoplasmic pH (Figure 62). Since this response is blocked in the presence of amilioride, it is suggested that activation of the Na^+/H^+ antiport mediates this alkalinisation. Similar increases in cytoplasmic pH in response to insulin have been observed in a variety of tissues (Section I.2.5.1. and Moore, 1986). Although it appears that the Na^+/H^+ antiport may be activated by some mechanism which bypasses protein kinase C, the nature of this mechanism is unknown (Vara and Rozengurt, 1985). Fluctuations in cytoplasmic pH have been associated with insulin-stimulated glucose transport (Klip et al., 1986), glycolysis (Moore, 1986) and protein synthesis (Busa and Nuccitelli, 1984). Since below a critical threshold value $[pH]_i$ becomes limiting for cell proliferation (Moolenaar,1986), this aspect of insulin action may also be of importance in its growth regulatory functions. However, for other lymphocyte types, a rise in pH alone has been found to be an insufficient stimulus for the generation of a proliferative response (Grinstein and Cohen, 1987). It seems probable then that other intracellular signals contribute in the initiation of DNA synthesis in rat thymocytes in response to insulin. It is tempting to suggest that N_{ins}, the insulin receptor tyrosine kinase and

changes in $[cAMP]_i$ (see section I.2.5.) may be among the factors which have contributory roles to play in this respect. Although such suggestions are largely speculatory as yet, they provide a useful starting point for further research.

In conclusion, it is clear that the growth promoting effects of insulin on rat thymic lymphocytes are complex. Although in culture, insulin can produce a number of biological effects, many of them require supraphysiological insulin concentrations, and for growth promotion, long incubation periods are necessary. At least two receptors, the insulin receptor and the type I IGF receptor, are involved in mediating these effects. Although insulin does not appear to act efficiently as a 'primary' growth factor, providing the cells with an initial positive stimulus to multiply, it has important secondary functions in enhancing nutrient uptake, and possibly promoting other intracellular reactions which facilitate the passage of cells through the cell cycle.

Insulin is the only growth factor known to be crucial for survival, and it is tempting to speculate that during its early evolutionary development, insulin subserved the function of a growth factor. Since growth is closely associated with the availability of metabolites, the amount of insulin produced may have become dependant on the concentration of these metabolites. As more complex organisms evolved, duplication and divergence of the original insulin gene may have taken place, which gave rise to insulin and the IGFs. Insulin retained its sensitivity to glucose and its metabolic effects became predominant, whereas the IGFs became more specialised for growth regulatory functions.

Clearly the functions, and the mechanisms of action of these molecules are not entirely distinct, and for lymphocytes generally metabolic enhancement is predominantly associated with proliferation. The

existence of a dual set of interlinked functions which may be controlled by insulin and IGFs may, at least in part, explain the necessity for a complex series of intracellular mediators, some of which may be unique, which are required to mediate its effects. Certainly the main unsolved problem relating to insulin action is the elucidation of the intracellular mechanisms that control its effects on metabolism and growth. Recent work in this field has however brought to light a number of new proposals (section I.2.5.) which open new avenues for future research. Hopefully such endeavours will soon lead to benefits for patients suffering from disorders of insulin/IGF metabolism.

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APPENDICES.

Appendix 1. Preparation of Solutions.

Physiological Saline.

NaCl	0.4238g
KCl	0.0186g
Na_2HPO_4	0.0070g
D-glucose	0.0450g
Hepes	0.5ml of 1M solution
$CaCl_2$	0.5ml of 60mM solution
$MgSO_4$	0.5ml of 100mM solution

Made up to 50ml with distilled water.

Toluene Based Scintillant.

Toluene	750ml	
2-methoxyethanol	250ml	
POPOP	0.1g	(1,4 -Di-2-(5-phenyloxazoyl)-benzene)
PPO	3.0g	(2,5-Diphenyloxazole)

Tris Buffered Saline (TBS).

Tris	60.55g
NaCl	85.20g

Dissolved in about 500ml distilled water. pH adjusted to 7.6 with about 370ml 1M HCl. Final volume adjusted to 1000ml.

Diluted 1:10 before use.

Formol Buffered Acetone.

Acetone	60ml

Formalin (40%)	33ml
Distilled water	38ml
1.5M Phosphate buffer	8.0ml

Prepared shortly before use.

1.5M Phosphate Buffer.

Na_2HPO_4	3.6g
KH_2PO_4	16.7g

Made up to 100ml with distilled water.

Diaminobenzidine (DAB)-Hydrogen Peroxide Solution.

6.0mg of DAB dissolved in 10ml of TBS.

3µl of 30% hydrogen peroxide added immediately before use.

Tween Buffer.

$NaH_2PO_4.H_2O$	0.345g
$NaH_2HPO_4.12H_2O$	2.680g
NaCl	8.474g

Made up to 1000ml with distilled water, and pH adjusted to 7.2 with concentrated NaOH.

1.0ml Tween 20 and 20.75g NaCl added immediately before use.

Orthophenylenediamine (OPD) Solution.

8.0mg of OPD dissolved in 12ml citric acid phosphate buffer.

5µl 30% (v/v) hydrogen peroxide added immediately before use.

Citric Acid Phosphate Buffer.

Citric acid	7.3g
Na_2HPO_4	23.87g

Made up to 1000ml with distilled water, and pH adjusted to 5.0.

Appendix 2. Procedure for the Determination of Lymphocyte Viability Using Trypan Blue Exclusion.

Immediately before use, trypan blue (0.2%) was mixed with 4.25% NaCl solution, 4:1 by volume. Equal volumes of this trypan blue solution and cell suspension, at a density between 10^6 and 10^7 cells/ml, were then mixed together in a test tube.

The tube was allowed to stand for at least 1 minute, but less than 3 minutes. Between these times, viable cells exclude the dye, while nonviable cells take up the dye.

The cell suspension was then loaded into a haemocytometer and the numbers of stained (nonviable) and unstained (viable) cells counted separately from a total of at least 200 cells.

The proportion of viable cells was then determined using the formula:

$$\%\text{viable cells} = \frac{\text{number of viable cells}}{\text{number of viable cells + number of dead cells}} \times 100\%$$

Appendix 3. Procedures for the Isolation of Erythrocytes and Hepatocytes.

Cells and tissues were removed from 100-150g, albino, male rats of the Wistar strain, anaesthetised with ether.

To obtain erythrocytes, 1ml of blood was removed by cardiac puncture, and placed in a 5ml heparinised tube. The cells were then centrifuged and the supernatant discarded. The cells were washed and resuspended in TBS, and the density adjusted to 10^5 cells/ml.

These 'erythrocyte' suspensions contain a small proportion of white blood cells, but these were ignored for the purposes described in section II.3.2.

To obtain hepatocytes, the following buffers were perfused sequentially into the liver via the hepatic portal vein, and drained from the hepatic vein: 50ml Ca^{++} free Krebs ringer bicarbonate (KRB), supplemented with 0.5mM EGTA, pH 7.4; 25ml Ca^{++} free KRB, pH 7.4; KRB with 1.25mM $CaCl_2$ supplemented with 500mg/l collagenase, pH 7.45, recirculated for 10 minutes.

The liver was then excised and dispersed in about 50ml KRB with 2.5mM $CaCl_2$.

The resultant cell concentrate was filtered through 4 layers of moistened cheesecloth, centrifuged, and resuspended in TBS, and the cell density adjusted to 10^5 cells/ml.