THE ROLE OF CYTOSKELETAL PROTEINS IN THE MECHANISM OF INSULIN RELEASE

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Thesis submitted for the degree of Doctor of Philosophy

The University of Aston in Birmingham

September 1988

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Summary of thesis submitted for the degree
of Doctor of Philosophy

The role of cytoskeletal proteins in the mechanism of insulin release

This thesis is concerned with the role of β-cell cytoskeletal proteins in the mechanism of insulin release from islets of experimental animals, the Aston obese diabetic hyperglycaemic (ob/ob) mouse and their lean littermates and the cultured insulin secreting β-cell lines, HIT-TI5 and RINm5F.

Investigations were carried out into the glucose induced insulin response of the lean and obese mouse islets and HIT-TI5 cells and the D-glyceraldehyde response of RINm5F cells using a static incubation system. Colchicine was found to inhibit insulin release from both lean and obese mouse islets more significantly than cultured HIT-TI5 and RINm5F cells. (Colchicine pre-treatment also inhibited the second phase of insulin release from perifused lean mouse islets and HIT-TI5 cells). Cytochalasin B, used to investigate the role of the microfilamentous system in the mechanism of insulin release enhanced insulin release from both lean and obese mouse islets to a significantly greater degree than that from cultured HIT-TI5 and RINm5F cells.

Pre-treatment of isolated lean and obese mouse islets and cultured β-cells with a combination of colchicine and cytochalasin B significantly reduced the insulin response of the HIT-TI5 and RINm5F cells compared with the control values suggesting that intact microtubules are more important for the sustained release of insulin than the microfilamentous system. However, the response was not so clearly defined with the lean and obese mouse islets.

Tubulin was separated from the extracts of lean mouse islets and the HIT-TI5 and RINm5F cells and actin was separated from all of the cell types including the obese mouse islets by SDS-polyacrylamide electrophoresis.

A tubulin radioimmunoassay and a colchicine binding assay were developed to measure the tubulin content of lean and obese mouse islets, and the shift between the proportions of tubulin dimers and polymerized tubulin under stimulatory and non-stimulatory conditions. The assay methods developed were not prone to be accurate, sensitive and precise but gave some indication of the shift from unpolymerized to polymerized tubulin during glucose stimulated insulin release.

These studies show that microtubules do play a fundamental role in the mechanism of insulin release from both islets and cultured HIT-TI5 and RINm5F cells.

Key words:- Tubulin, microtubules, microfilaments insulin release, islets, insulin releasing cultured cell lines.

This thesis is dedicated to the most important people in my life, to the memory of my father Stanley, to my mother Jenny and mother-in-law Sylvia for their support, to my husband Neil for his love and encouragement throughout my work and to our baby - due to make its entry into this world any day, for making it all worthwhile.

ACKNOWLEDGEMENTS

Grateful thanks go to Dr R.F. Santerre of Eli Lilly Ltd. USA for providing samples of HIT-TI5 cells and to Dr S.J.H. Ashcroft from the John Radcliffe Infirmary, Oxford for providing samples of RINm5F cells.

My sincere thanks are extended to Mr Kevin Hughes for his help in procuring pieces of apparatus and assistance with the computer. My thanks also go to my colleagues in the laboratory for creating a happy atmosphere in which to work.

My thanks also go to my husband Neil for his help with the word processor and the production of this thesis. Thanks Neil !

My final thanks go to my supervisor Dr T.W. Atkins for giving me the opportunity to carry out this work, and for his invaluable guidance in the experimental work and the preparation of this thesis. Thanks Terry !

Financial support from the University of Aston is gratefully acknowledged.

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CHAPTER 1

INTRODUCTION

The mechanism of glucose stimulated insulin release from the β-cells of the islets of Langerhans is a complicated process which is not clearly understood. It is thought that the cytoskeletal protein tubulin associated with microtubules might play an important role in the release mechanism, particularly the movement of β-granules.

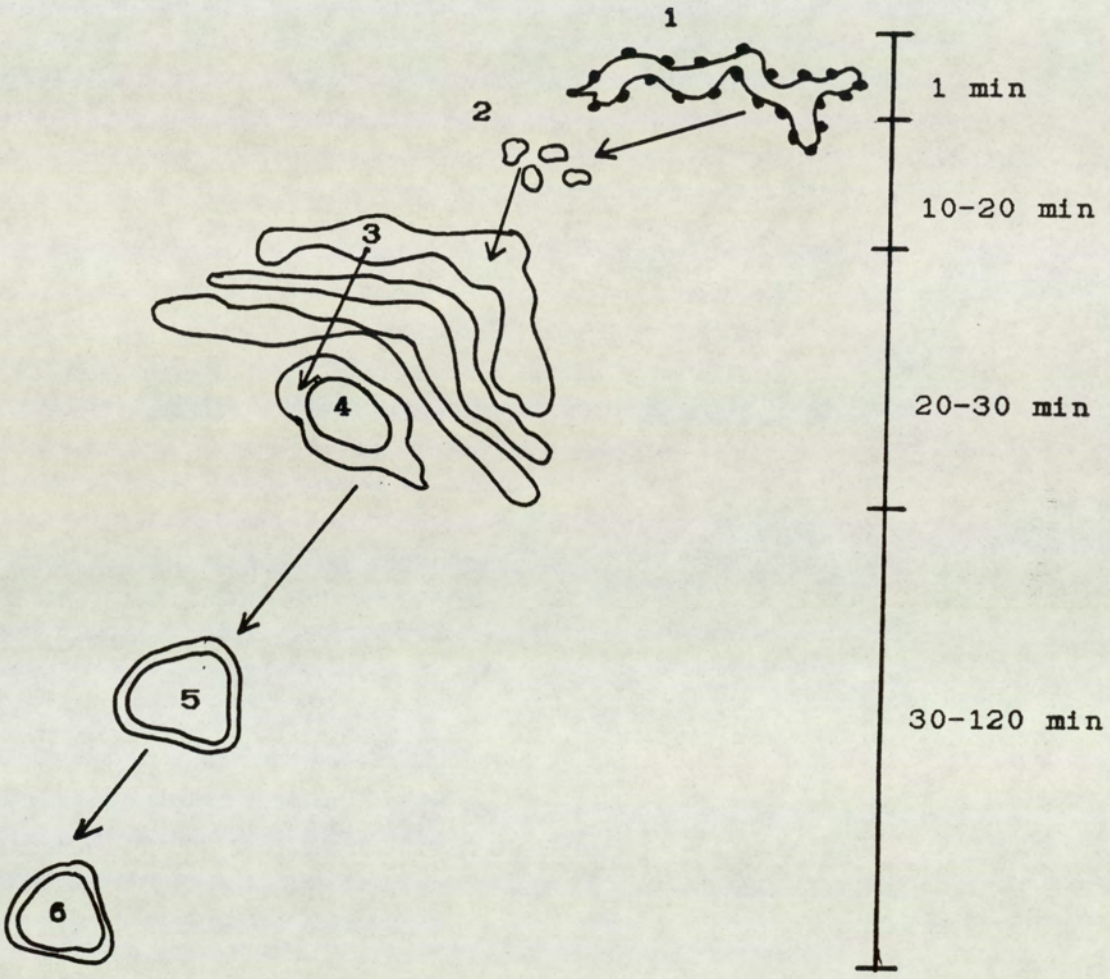
The suggestion that microtubules may play a fundamental role in secretory processes was first made by Slautterbeck (1). Seven years later Lacy and coworkers were able to demonstrate an inhibition of insulin release from the β-cells of the islets of Langerhans by agents known to affect microtubules, ie. colchicine, vinblastine, vincristine and deuterium oxide, (2). It was assumed that the inhibitory action of these drugs on insulin secretion was the specific result of their interference with the secretory mechanism of the β-cell. In general, it is thought that the inhibitory effect of these drugs is indeed specific for the secretory process and these agents do not cause a general alteration of the β-cell metabolism, (3).

The insulin secretion mechanism begins with the synthesis of hormone precursors after stimulation of the cell with a suitable blood borne stimulus, (in the case of β-cells the stimulus may be a rise in the blood glucose concentration above the fasting level of 4-5

mmol/l). The synthesis of insulin begins with the formation of preproinsulin a single chain polypeptide (11.5 kDa) on ribosomes associated with the rough endoplasmic reticulum, (4). The hydrophobic nature of the pre-region of preproinsulin promotes interaction with the lipid - rich endoplasmic reticulum membrane and ensures the vectorial discharge of preproinsulin through the endoplasmic reticulum membrane into the cisternal space, (4). Here preproinsulin rapidly undergoes proteolytic cleavage to produce proinsulin which itself undergoes rapid peptide chain folding and sulphydryl oxidation, (9 kDa), (4). The proinsulin is transported in microvesicles to the Golgi appparatus, (which takes about 20 minutes), where it is packaged into secretory granules. The β-granule membranes are formed by evagination of the Golgi membranes and the granules leave the Golgi via the trans or maturing face, Figure 1.1, (4).

During the formation of secretory granules, proinsulin is converted to insulin. This is a relatively slow process, (30 - 120 minutes), involving the sequential action of specific proteases with trypsin-like and carboxypeptidase like activities located in the β-granule membrane. The proteases cleave the proinsulin into insulin (6kDa) and C-peptide (3kDa). The cleavage of proinsulin leads to the decreased solubility of the insulin molecule, which in the presence of zinc (which is concentrated in the storage granule), leads to the precipitation of insulin as microcrystals of zinc-insulin

Figure 1.1 **Biosynthetic organisation of the β-cell (4)**



1 - Rough endoplasmic reticulum studded with ribosomes
2 - Transfer microvesicles
3 - Golgi apparatus
4 - Packaging into storage granule
5 - Maturation of storage granule - conversion of proinsulin into insulin
6 - Mature storage granule containing insulin and C-peptide

The time scale of these events is shown on the right of the diagram.

hexamers. The precipitation of insulin permits storage in its most concentrated form and renders it resistant to further enzymic degradation by the converting enzymes. The conversion of proinsulin into insulin continues within the newly formed granules as the granule matures and moves away from the maturing face of the Golgi apparatus and into the general cytoplasm of the β-cell to await the activation of the secretory process, (4).

It is the means by which secretory granules move to the β-cell plasma membrane which is of particular interest in this study. The movement of the the insulin secretory granules towards the β-cell membrane has been observed using cinemamicrography and found to be unidirectional and associated with track-like structures, possibly microtubules, (5,6).

The release of insulin from the β-cell involves the passage of the β-granules through a peripheral microfilamentous web of actin prior to fusion with the β-cell plasma membrane, (7). The time taken for the β-granule to move from the Golgi apparatus through the β-cell cytoplasm and microfilamentous web to the periphery probably accounts for the biphasic release of insulin in response to glucose. The first rapid or early phase corresponds to the rapid release of the β-granules lying close to the plasma membrane of the β-cell and the slower second phase would be the product of the transport of β-granules from the Golgi to the β-cell periphery prior to release, (8).

The insulin response of the β-cell to all physiological stimuli is rapid, (responding within a few minutes) and depends upon the presence of a functional microtubule / microfilamentous system within the cell, the maintenance of intracellular ATP and cyclic AMP concentrations, protein phosphorylation and the presence of extracellular calcium, (4). All these factors are extremely important and play crucial roles in coupling the stimulation of insulin synthesis with insulin secretion from the β-cell.

The stimulation of insulin release by concentrations of glucose above the fasting level of 4-5 mmol/l has been shown to be accompanied by a net influx of Ca^{2+} into the β-cell, (9). Primary stimuli such as glucose, glyceraldehyde and leucine, alter the resting potential of the β-cell membrane which is normally maintained between -50 and -70mv by K^+ permeability. Glucose reduces the K^+ permeability of the membrane, thus causing a rapid depolarization of the membrane and an opening of voltage dependent Ca^{2+} channels allowing Ca^{2+} to enter the β-cell, (9). Secondary stimuli such as glucagon, gastrin and also acetylcholine enhance the response to a primary stimulus such as glucose by redistributing intracellular Ca^{2+} stores by translocation from the endoplasmic reticulum and the inner surface of the β-cell membrane, (10).

The movement of Ca^{2+} within the cell has been followed by calcium pyroantimonate precipitation. This

technique has shown that ionised Ca^{2+} from the extracellular fluid is accumulated to form highly mobile reservoirs in the plasma membrane and mitochondria, (10,11). In addition Lenzen has shown that Ca^{2+} must be taken both from the extracellular space and from the intracellular stores to initiate insulin release, (10). Work with the sulphonylurea tolbutamide, which unlike glucose releases Ca^{2+} from intracellular stores without permitting the entry of extracellular Ca^{2+}, has confirmed that these intracellular stores must be replenished by extracellular Ca^{2+} to maintain insulin secretion, (10). Tolbutamide is believed to act via the activation of the β-cell adenylate cyclase system increasing the intracellular concentration of cAMP, (9). It is well established that the pancreatic β-cell contains all the components of the adenylate cyclase system including cAMP, plasma membrane adenylate cyclase, soluble and particulate cAMP phosphodiesterase activities and cAMP protein kinases, (4).

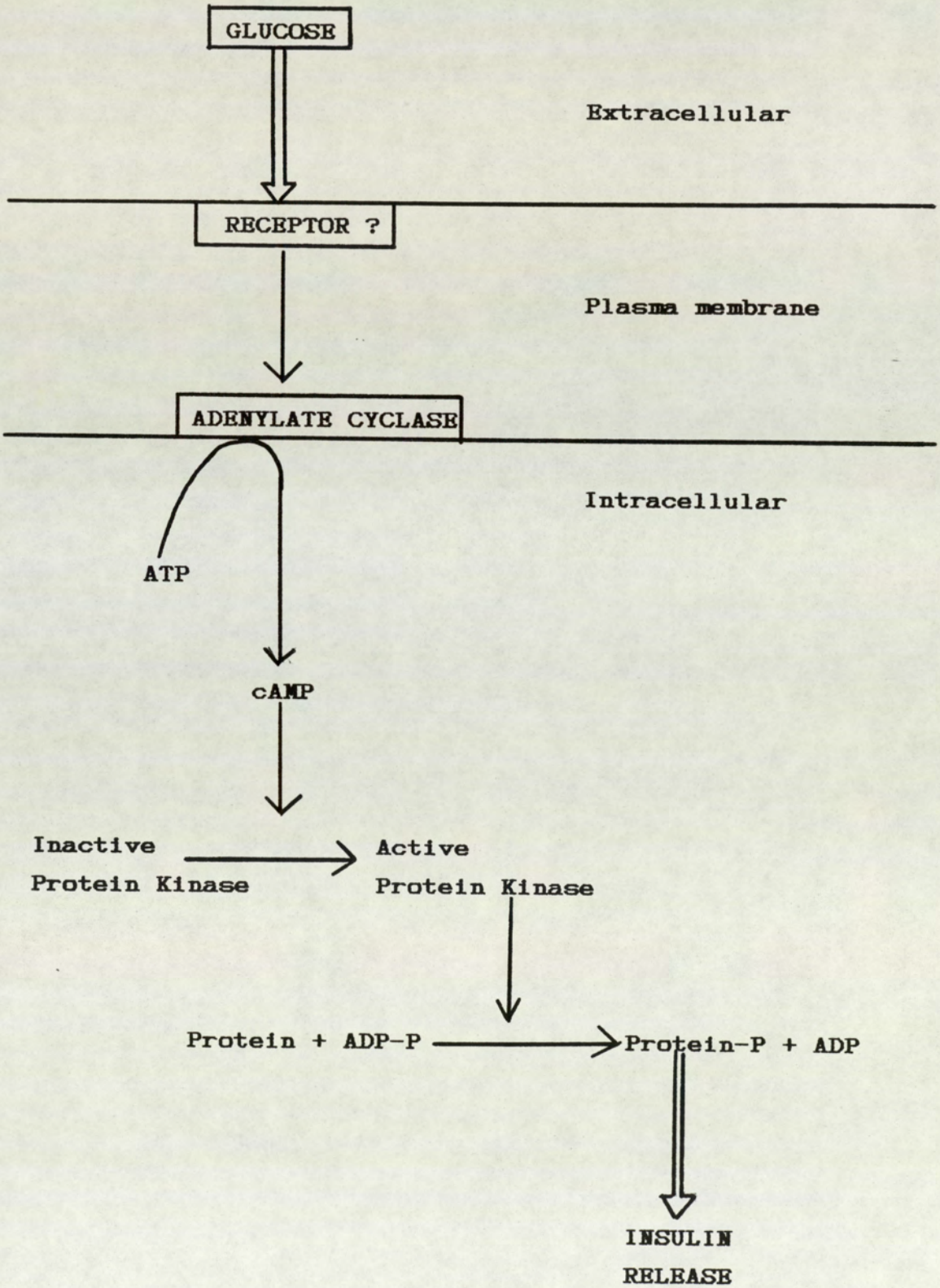
In 1964 Cerasi and Luft postulated that glucose in addition to serving as a metabolizable substrate, acted in the β-cell on a specific membrane receptor leading to an activation of adenylate cyclase, an increase in cAMP production and stimulation of insulin release, (12). cAMP was considered to be the intracellular second messenger involved in insulin release. The metabolism of glucose was suggested to be the means for increasing the availability of ATP for adenylate cyclase. Adenylate

cyclase activity has been determined in the β-cell membrane by Howell, (13). However, Malaisse and co-workers, (14), have proposed that cAMP should be considered to be a modulator of the metabolic and secretory response to insulin secretagogues and not as a signal for insulin release. Indeed even today after extensive work this difference in opinion has yet to be satisfactorily resolved, (9,15,16).

An increase in cAMP concentration within the β-cell is largely the product of increased adenylate cyclase activity or a decrease in cAMP phosphodiesterase activity. Inhibition of the latter with theophylline or sulphonylurea increases β-cell cAMP levels whereas extra-cellular glucose and glucose-6-phosphate have no effect on cAMP levels, (16). Increased β-cell cAMP levels lead to the activation of a cAMP dependent protein kinase, subsequent protein phosphorylation and the eventual release of insulin. A possible sequence of events is summarised in Figure 1.2. Indeed cAMP responsive protein kinase activity has been comprehensively reported in islet homogenates, (17- 23).

The exact role of cAMP and its possible association with intra β-cell Ca^{2+} levels remains to be understood. Henquin (24) has suggested that cAMP affects the permeability of Ca^{2+} channels facilitating Ca^{2+} influx, while Brisson has suggested that cAMP causes intra-cellular Ca^{2+} redistribution, rather than net uptake, (23). Certainly work using radioactive ^{45}Ca has

Figure 1.2 The role of the adenylate cyclase system in the mechanism of insulin release



confirmed that the subcellular distribution of ^{45}Ca in the β-cell is indeed affected by cAMP, (25,26,27).

In addition it has also been noted that cAMP only stimulates insulin release in the presence of secretagogues such as glucose, (28). Agents which suppress cAMP phosphodiesterase activity such as IBMX, theophylline and sulphonylureas increase intracellular levels of cAMP but do not induce insulin secretion in the absence of glucose, although they potentiate insulin release at low glucose concentrations, (28). When islets are exposed to 2.8 mmol/l glucose the cellular content of ATP is sufficient to maintain normal cAMP levels in the islet cells, (29), and enhance insulin release evoked by non-metabolizable secretagogues such as theophylline (30), although the cellular cAMP level does not rise sufficiently to cause any sizeable increase in insulin release. Even in the presence of high glucose concentrations the cAMP level in each β-cell is no greater than twice the basal value, (16). This might suggest that cAMP is not the only intracellular mediator of insulin release. Valverde has suggested that the elevation of islet cAMP induced by glucose might be mediated by Ca^{2+}/ calmodulin and the elevation would be secondary to an increase in cytosolic Ca^{2+} concentration, (31). This view is supported by the observation that the stimulant action of metabolizable secretagogues upon cAMP production in intact islets is suppressed when the islets are incubated in the absence of Ca^{2+}, (32,33).

Sugden, Christie and Ashcroft (34) together with Valverde, Sener and Malaisse (35) in 1979 independently reported the presence of calmodulin a Ca^{2+}-dependent regulatory protein, in rat pancreatic islets. Later work by Thams, Capito and Hedeskov in 1982 (36), estimated the concentration of calmodulin in mouse islets to be of the order of 0.03 pmoles/islet or 0.04 pmoles/μg islet protein. In the cloned rat insulinoma cell line, RINm5F, the concentration of calmodulin has been estimated to be in the region of 0.09 pmoles/μg protein (37).

There is considerable evidence to suggest that calmodulin might influence cAMP turnover in a variety of cells including islet β-cells, (31,34,35). The activation of adenylate cyclase by calmodulin has been confirmed in rat islets by Sharp, Wiedenteller, Kaelin, Siegel and Wollheim (34) and Thams, Capito and Hedeskov (36), and this observation is thought to have two important implications. Firstly, it is known that although endogenous cAMP *per se* cannot initiate insulin release, it enhances secretion induced by a variety of secretagogues such as glucose and leucine. Since these secretagogues are known to increase the β-cell cytosolic concentration of Ca^{2+}, the activation of adenylate cyclase by Ca^{2+}-calmodulin would provide a device for the amplification of the secretory response. Secondly, the activation of adenylate cyclase by Ca^{2+}-calmodulin could also account for the fact that several secretagogues, which do not exert any direct effect upon adenylate

cyclase activity in subcellular fractions, increase cAMP production in intact islets, (38).

There is also evidence to suggest that Ca^{2+}-calmodulin may cause a modest activation of cAMP phosphodiesterase in rat islets, (39). However, since Ca^{2+}-calmodulin also increases adenylate cyclase activity in islet cells, the overall effect of this regulatory protein upon cAMP metabolism may be viewed as an increase in the rate of turnover of the cyclic nucleotide in the β-cell, (38). The increase in cyclic cAMP production is thought to lead to a subsequent increase in cAMP responsive protein kinase activity (17-23) and protein phosphorylation. Thus Ca^{2+}-calmodulin might perhaps be said to modulate cAMP-dependent protein phosphorylation, (19).

Ca^{2+}-calmodulin-responsive protein kinase activity has been demonstrated in hamster insulinoma cells by Schubart, Erlichman and Fleischer (40,41) and in rat islets by Harrison and Ashcroft (19) and Gagliardino, Harrison, Christie, Gagliardino and Ashcroft, (42). Harrison, Gagliardino and co-workers showed that the major substrate for Ca^{2+}-calmodulin-dependent protein kinase activity was a protein of molecular weight 53-55 kDa. Brocklehurst and Hutton (43), using similar electrophoretic/autoradiographic techniques separated another protein kinase of 57 kDa, which was similarly dependent on Ca^{2+} and endogenous calmodulin from a rat islet cell tumour. It has been suggested that these

proteins of molecular weight 54 and 57 kDa might be the α and β subunits of tubulin (43). This idea was generated from work involving the pretreatment of an islet cell microsomal fraction with antitubulin antibody prior to the assay of protein kinase activity. This manoeuvre markedly decreased the subsequent Ca^{2+} and calmodulin-dependent protein phosphorylation of both the 57 kDa and 54 kDa proteins. On the other hand, Kowluru and MacDonald (45), said that the two proteins were not the same on the grounds that the phosphorylation of the 57 kDa protein was preferentially stimulated by Ca^{2+} and that of the 54 kDa by cAMP in the absence of Ca^{2+}. Kowluru and MacDonald also found that the two proteins differed from each other by their pH on two dimensional polyacrylamide gels, the 57 kDa protein being much more basic (pH 7.5 - 8.0), than the 54 kDa protein (pH 5.0 - 5.5.) These workers concluded that the 57 kDa protein could not be identified with the tubulin subunit (45).

Ca^{2+}-calmodulin has also been suggested to catalyze the phosphorylation of myosin light chains via a calmodulin sensitive myosin light chain kinase, (45). This in turn would permit myosin to interact with actin and allow the contraction of microfilaments in the cytoplasmic cell web of the β-cell, (46).

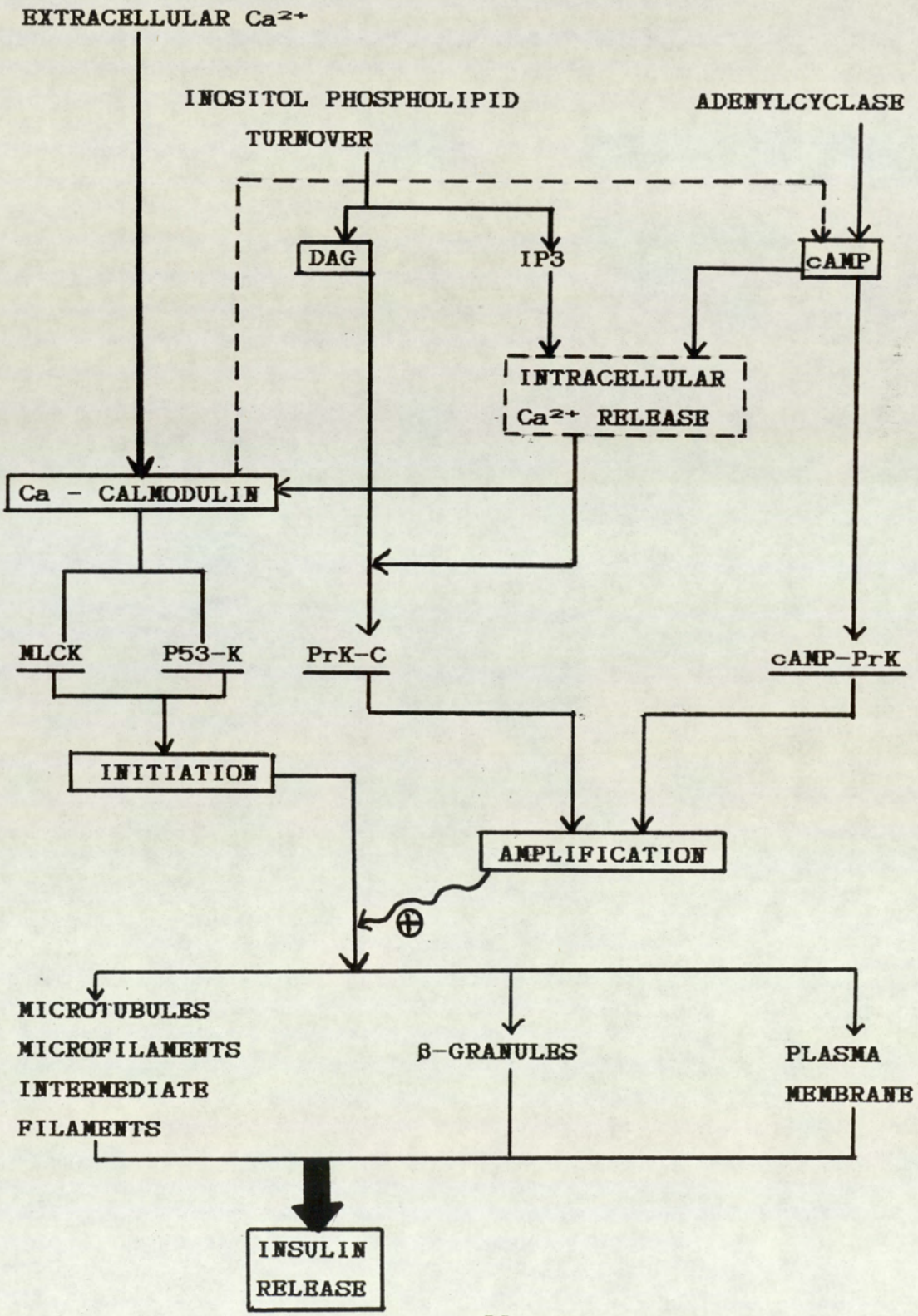
The possible role played by the protein phosphorylating activity of cAMP and Ca^{2+}-calmodulin in the mechanism of insulin release and its relationship with the turnover of inositol phospholipid turnover in the β-cell is summarised in Figure 1.3.

In 1953, Hokin and Hokin (47) observed that cholinergic stimulation of the exocrine pancreas resulted in a marked increase in ^{32}P incorporation into phospholipids, chiefly phosphoinositides and phosphatidic acid, suggesting enhanced phospholipid turnover. Increased ^{32}P incorporation into pancreatic islet phospholipids on exposure to high glucose concentrations was first demonstrated by Fex and Lernmark (48). Later Freinkel and colleagues demonstrated ^{32}P labelling of phosphatidic acid, phosphatidylinositol and phoshatidylethanolamine in glucose stimulated rat islets (49).

An alternative approach to ^{32}P labelling has been used by Clements and Rhoten, (50,51), in their studies, islet phospholipids were pre-labelled with ^{3}H-inositol and a fall in the labelled lipid phosphatidylinositol, occurred on exposure to glucose. This effect was accompanied by an increased production of water soluble derivatives of ^{3}H-inositol and suggested that glucose provoked a breakdown of inositol phospholipids in islets.

The turnover of β-cell phosphatidylinositol has been shown to be enhanced by many insulin secretagogues including glucose, mannose, leucine, α-ketoisocaproate, acetylcholine and carbamylcholine (52,53). Stimulation

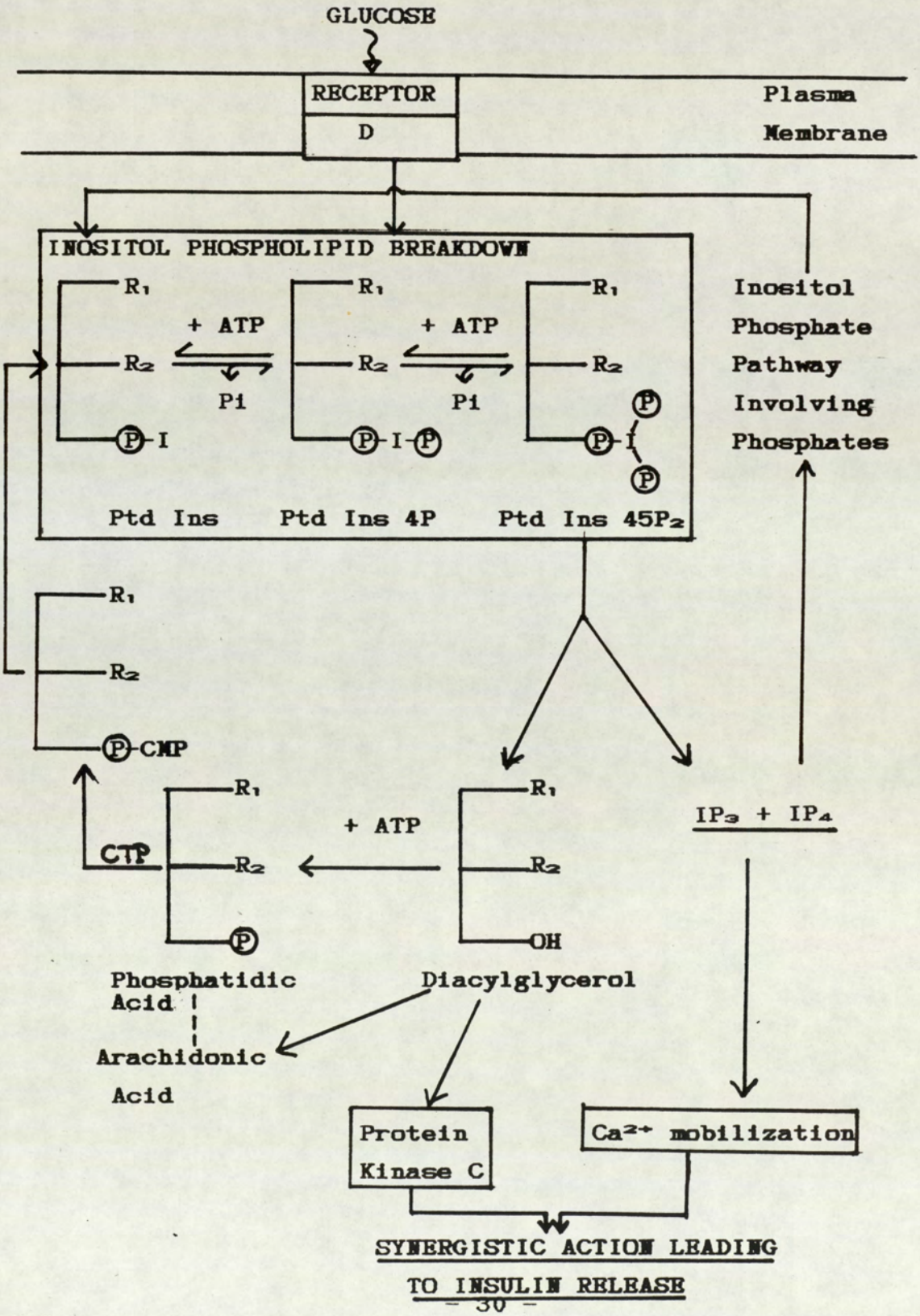
Figure 1.3. The possible involvement of protein phosphorylation in the mechanism of insulin release. (63).



of insulin release with these secretogues is thought to begin with the activation of phospholipase C, which leads to phophatidylinositol breakdown and the subsequent production of diacylglycerol, (DAG) and inositol 1,4,5-triphosphate, (IP_3), (33), Figure 1.4. IP_3 is believed to play an important role in the molbilization of intracellular Ca^{2+}. The addition of IP^3 to RINm5F cells, was shown to cause the release of Ca^{2+} from a non-mitochondrial pool in these cells, which was later shown to be the endoplasmic reticulum, (54-56). Inositol phospholipid hydrolysis in β-cells is a Ca^{2+} dependent process but it does not itself occur as a result of increased Ca^{2+} mobilization, (57).

The other breakdown product of phophatidylinositol, diacylglycerol (DAG), Figure 1.4, is believed to play a key role in a number of subcellular reactions, (58). An increased concentration of DAG in pancreatic islet cell membranes is thought to have a profound effect on membrane fluidity and fusion. Increased pancreatic islet cell membrane fluidity has been demonstrated in response to glucose, (59). Another responsive intracellular target for DAG has been identified as a Ca^{2+}-phospholipid dependent protein kinase, or protein kinase C, (58-60). This protein kinase has been isolated and characterized from rat pancreatic islets and SV40 transformed hamster β-cells, HIT-TI5 cells (60). Activation of protein kinase C initiates the phosphorylation of peptides of molecular weight 38, 35,29,15 11 kDa and in addition it

Figure 1.4 Phospholipid turnover and signal transduction (54,64).



Key to Figure 1.4

Ptd Ins	- Phosphatidylinositol
Ptd Ins 4P	- Phosphatidylinositol-4-phosphate
Ptd Ins 4,5,P_2	- Phosphatidlyinositol-4,5-biphosphate
R_1 + R_2	- Fatty acyl groups
I	- Inositol
P	- Phosphoryl group
D	- Phosphodiesterase
IP_3	- Inositol 1,4,5-trisphosphate
IP_4	- Inositol 1,3,4,5-trisphosphate

tetrakis

is thought to phosphorylate myosin light chain (20kDa), (60,61). The role of protein kinase C activation is not clearly understood but is thought to sustain the action of Ca^{2+}-calmodulin under the influence of cAMP (62,64), Figure 1.3.

The possible involvment of microtubular cytoskeletal proteins, particularly tubulin, in the release of insulin granules from β-cells was first suggested by Lacy, Howell, Young and Fink, (65). Their suggestion was supported by the observed inhibition of insulin release from isolated islets by the plant alkaloid colchicine. This compound has been shown to inhibit microtubule formation, (66). Microtubules have been suggested to facilitate the transport of insulin containing β-granules from the cytoplasm to the plasma membrane of the β-cell, (65). Indeed electron microscopic studies have demonstrated secretory granules isolated from Anglerfish *Lophius americanus* pancreas lying in very close association with microtubules isolated from chick brain homogenates, (67). In order to understand the mechanism by which microtubules might facilitate the movement of β-granules towards the plasma membrane and the possible involvement of cytoskeletal proteins phosphorylation in the mechanism of insulin release closer, investigation must be made of the structure and assembly of microtubules.

Microtubules may generally be defined as proteinaceous organelles. They are present in nearly all

eukaryotic cells and are composed of subunits of tubulin molecules assembled in long tubular structures. Tubulin exists in aqueous solution e.g. the cytoplasm of any cell, as a dimer of molecular weight 110,000 - 120,000 with a sedimentation coefficient of 6S, (66). The dimer can be split by denaturation with guanidine hydrochloride to produce two dissimilar monomers which are chemically distinct, (66). Electrophoresis of tubulin on alkaline gels containing urea sodium dodecyl sulphate, has demonstrated two bands designated α and β, present in a ratio of 1:1, (68).

Tubulin dimers polymerize under favourable conditions to form microtubules usually about 24 nm in diameter, with a 5 nm thick wall and a hollow central core of about 15nm in diameter, (69). The length of microtubules can vary considerably as a result of linear growth by the addition of consecutive dimers, and is usually measured in micrometers, (70). Microtubules have been assembled *in vitro* and this technique forms the basis of the tubulin isolation procedure, (71). Tubulin does not polymerize into microtubules at a concentration of less than 0.2 mg/ml of protein. This concentration is the 'critical *in vitro* concentration' and is possibly the lowest concentration at which dimers will merge to form a structure capable of nucleating the growth of a microtubule, (72). When this concentration is exceeded the number of polymers formed increases rather slowly with increasing subunit concentration, saturation

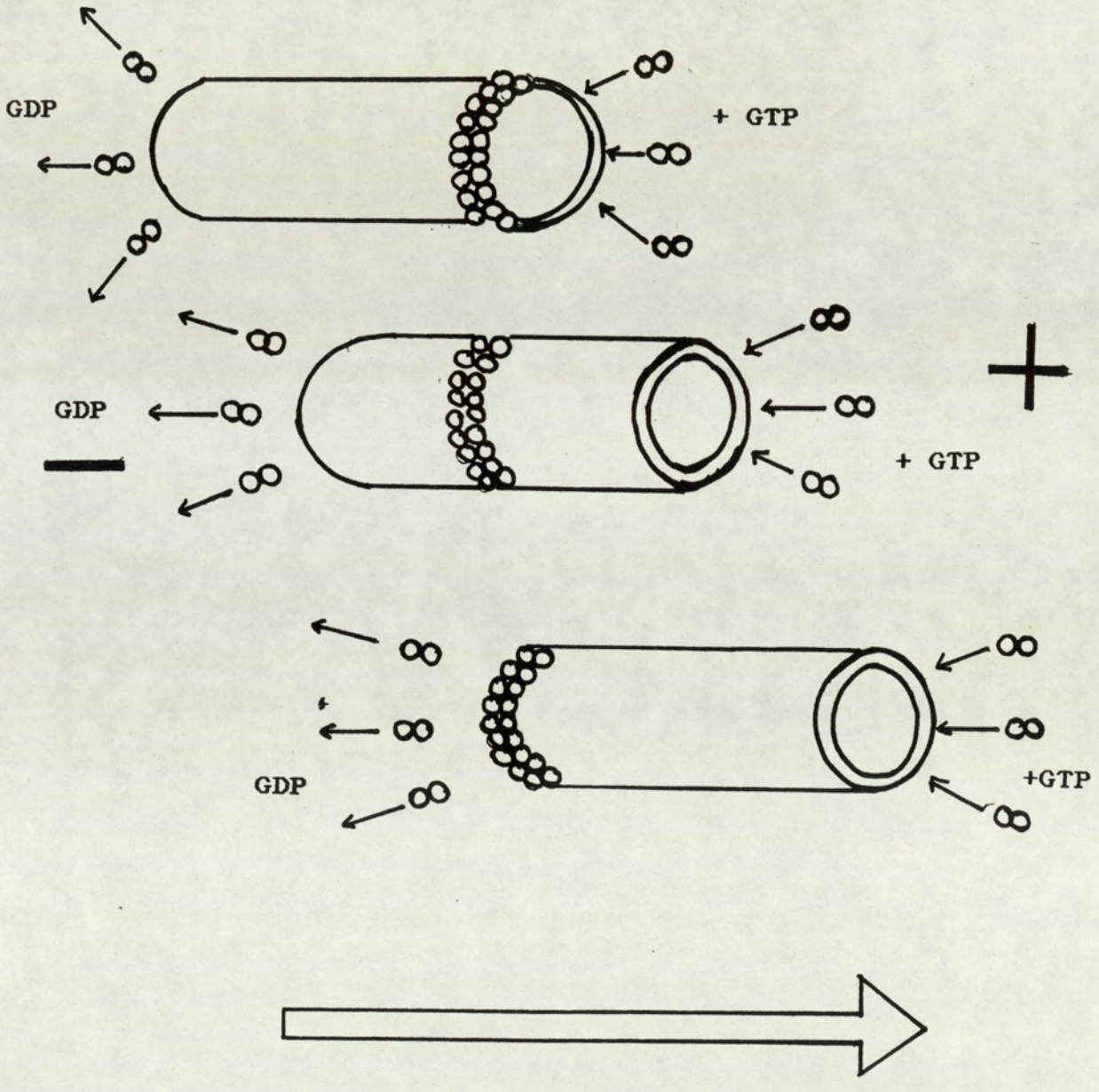
occurring at about five times the critical concentration of tubulin dimers, (1mg/ml).

Mitchison and Kirschner, (73) have proposed an alternative method of tubule elongation based upon the attachment of GTP and its subsequent hydrolysis to GDP. It is important to note that single microtubules do not span the whole distance from the nucleus to the cytoplasm, but would appear to remain as one continuous length moving closer to the plasma membrane, Figure 1.5. It is thought that tubulin dimers become detached from one end of the microtubule and attach themselves to the other end, that is the microtubule disassembles at one end and assembles at the other. This in turn causes unidirectional growth towards the plasma membrane by a process of 'treadmilling', Figure 1.5, (74).

It is well known that the guanosine nucleotides GTP and GDP are required for *in vitro* microtubule assembly, (75). Each tubulin dimer is capable of binding two moles of nucleotide, one loosely (GTP) at the exchangeable, or E site of the dimer, the other tightly at the non-exchangeable, or N site. Polymerization is associated with the hydrolysis of GTP to GDP at the N site by a transphosphorylase enzyme using ATP or GTP as the phosphate donor, (76).

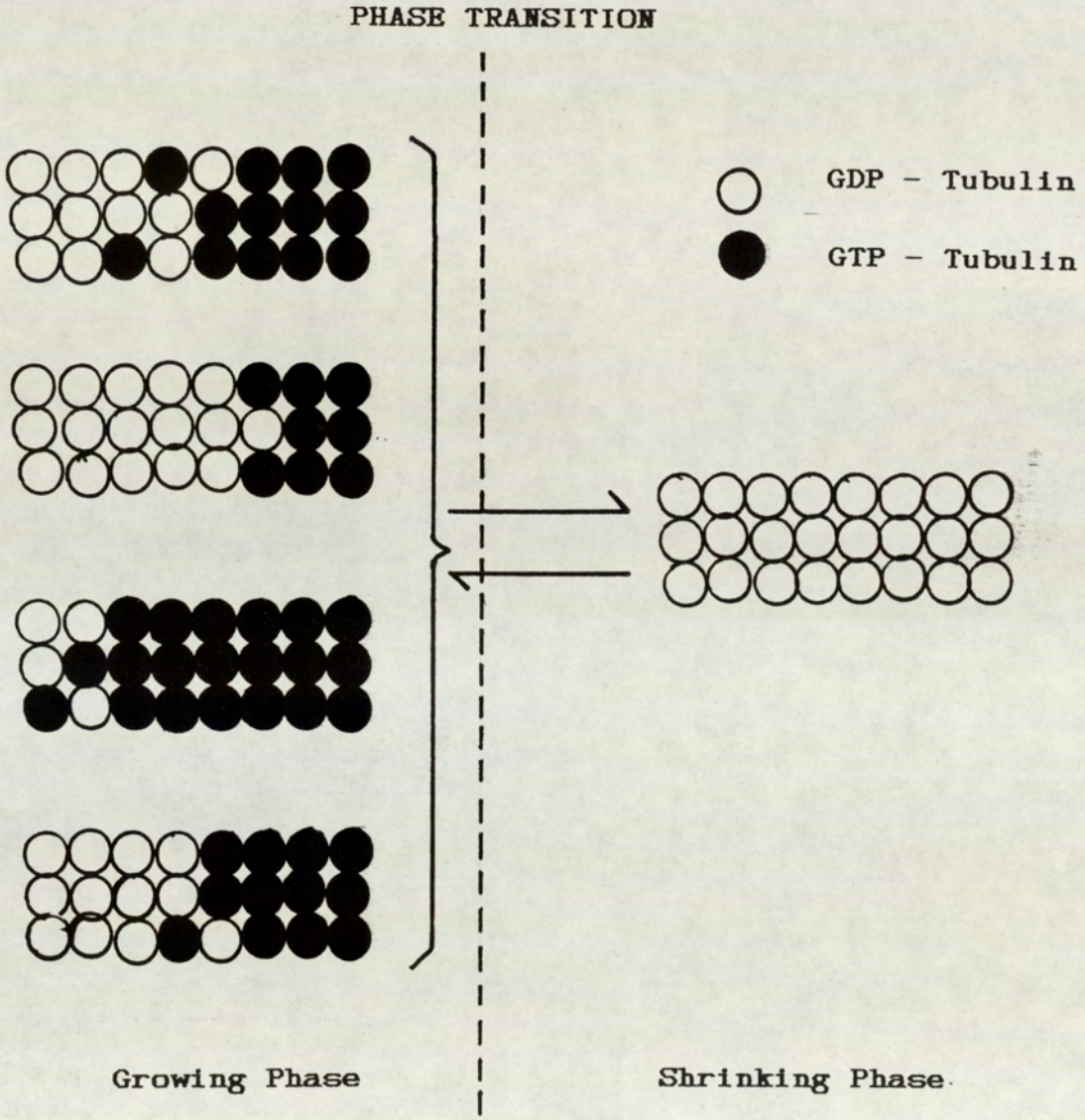
GTP binding to tubulin has been investigated by Mitchison and Kirschner and they have proposed a model known as the GTP capping model Figure 1.6, which is based on the hydrolysis of GTP to GDP during tubulin assembly,

Figure 1.5 GTP Capping Model - Treadmilling. (74)



Unidirectional growth towards plasma membrane
by disassembly and assembly

Figure 1.6 GTP Capping Model. (73)



The Growing Phase of the microtubule is stabilized by a GTP-tublulin cap which increases in length with increasing free tubulin concentration. The Shrinking Phase has lost its GTP-cap. Interconversion between the phases is rare.

but this hydrolysis is not necessarily simultaneous with tubulin dimer addition. The delay in hydrolysis causes a cap of GTP-tubulin to form, the length of which depends upon the rate of polymerization, (73). This would suggest that polymer growth is best represented as a balance between the rate of addition and release of GTP-tubulin, Figure 1.6. However, if GTP hydrolysis was to catch up with subunit addition then the situation would change. The GTP tubulin cap would then be converted to a GDP-tubulin end and new polymerization reactions would become relevant. The rate at which GDP-tubulin detaches from a microtubule exceeds the rate for GTP binding to tubulin by two to three orders of magnitude, leading to the rapid dissassembly of any microtubule with has an exposed GDP-tubulin end, Figure 1.6, (73).

For microtubule assembly *in vitro*, Ca^{2+} must be removed from the vicinity of dimer polymerization with a strong chelating agent such as EGTA which does not remove Mg^{2+}. Ca^{2+} is thought to play a regulatory role in microtubule assembly *in vitro* via the protein calmodulin. Calmodulin 'sensitizes' microtubules to Ca^{2+} and decreases the concentration of Ca^{2+} required for microtubule polymerization by two orders of magnitude, (77). However, *in vitro*, Ca^{2+} concentrations greater than millimolar, inhibit microtubule polymerization, but this concentration is far greater than the (total) Ca^{2+} concentration normally found in the cell (10^{-7}M), (77). The significance of the Ca^{2+} concentration *in vitro* will

be discussed later, but the Ca^{2+} concentration is an important consideration when preparing tubulin from brain tissue, (71). Magnesium at concentrations below 20 mmol/l are also required for tubulin polymerization, (71).

Optimal growth of microtubules *in vitro*, that is microtubule assembly, takes place at 37° C, (71). There is an intitial short lag phase which may represent the formation of nucleating structures. This is then followed by a rapid elongation of microtubules which is completed in 20-30 minutes. Optimal assembly of microtubules is seen at pH 6.9 as tubulin is a marginally acidic protein.

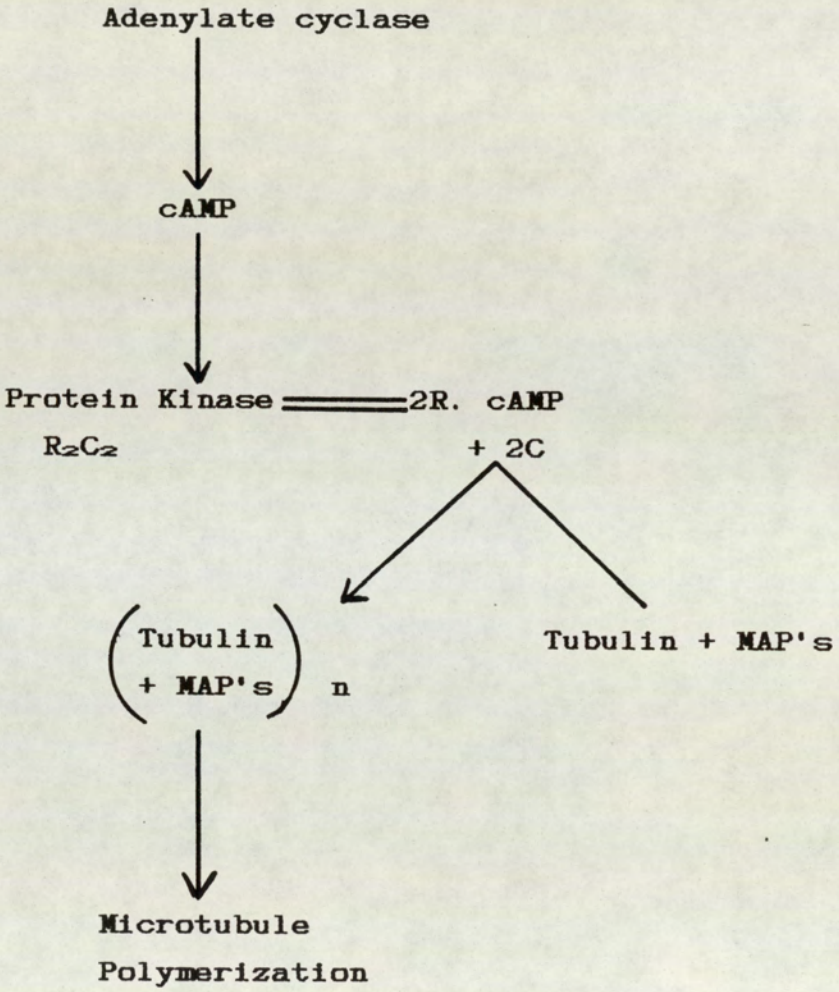
Using these optimised conditions, microtubules can be assembled *in vitro*, and their creation forms the basis of the purification method by Weisenberg, (71). In this process, microtubules are polymerized and depolymerized at least twice, (two cycles), so that any impurities such as blood or fat, present in the starting material, (porcine brain), will not polymerize along with the microtubules and can be discarded after cold centrifugation. However, besides these optimal requirements for microtubule assembly, there are several other factors which are equally necessary for polymerization. When microtubules are isolated by Weisenberg's method (75), electron microscopic examination has demonstrated the presence of small side arm's projecting laterally from each microtubule, (79).

These side arms consist of 'microtubule associated proteins' MAP1 and MAP2 and Tau proteins, having molecular weights of 350, 300, and 55-62 kDa respectively. All of these proteins appear necessary for microtubule assembly. On removal of these microtubular associated proteins by affinity chromatography, tubulin dimers can not longer polymerize, (80). However, the subsequent addition of dimethysulphoxide (DMSO) will restore the ability of tubulin to polymerize, (81). All these factors are relevant to the preparation and promotion of microtubule assembly *in vitro*. However, the importance of these different factors to microtubule assembly and β-granule movement in β-cells remains largely unknown.

Glucose stimulation of the β-cell leads to an increase in cytosolic Ca^{2+} concentration leading to increased protein phosphorylation by cAMP dependent protein kinases, calmodulin dependent protein kinases and protein kinase C, (63). Since tubulin *per se* does not appear to have an intrinsic protein kinase, the sites of action for protein phosphorylation are thought to be MAP's 1 and 2 and Tau protein, (82-84). However, phosphorylation of these microtubule associated proteins, particularly with Ca^{2+}-calmodulin has shown tubulin polymerization to diminish, (84,85). Howell has suggested that cAMP may be the most important microtubule regulator in that agents that increase intracellular cAMP levels also bring about microtubule phosphorylation,

(86), Figure 1.7. Yamamoto has suggested that MAP's 1 and 2 phosphorylation may be regulated by a dual control system of Ca^{2+}-calmodulin and cAMP, (84). Since Ca^{2+}-calmodulin has also been reported to increase adenylate cyclase activity in β-cells (38), Ca^{2+}-calmodulin may either enhance the phosphorylating activity of cAMP or act independently of it, reducing protein polymerization. Either way the phosphorylation of MAP's 1 and 2 and the process of microtubule polymerization are still not clearly understood.

Figure 1.7 The phosphorylation of tubulin and microtubule associated proteins by cAMP-dependent protein kinases. (86)



Cyclic AMP asserts its effect by activation of protein kinase, causing it to dissociate into receptor (R) and catalytic subunits (C). The dissociated catalytic subunit effects the phosphorylation of microtubule associated proteins (MAP's) and/or microtubules, which in turn promotes microtubule poymerization.

The movement of insulin containing β-granules within β-cells in monolayer culture has been studied using cinemicrography. Time lapse filming has shown that β-granules move in a discontinuous way by jumps or saltatory movements along an orientated, but wholly invisible pathway, (5,6). The speed of movement observed was somewhere in the range of 0.8-1.5 μm/s and increased with the addition of glucose. Speed was reduced however, with the omission of Ca^{2+} from the incubation medium or the addition of microtubule inhibiting agents such as vinblastine, (5,6). This implied that the disruption of the microtubules prevented saltatory movement.

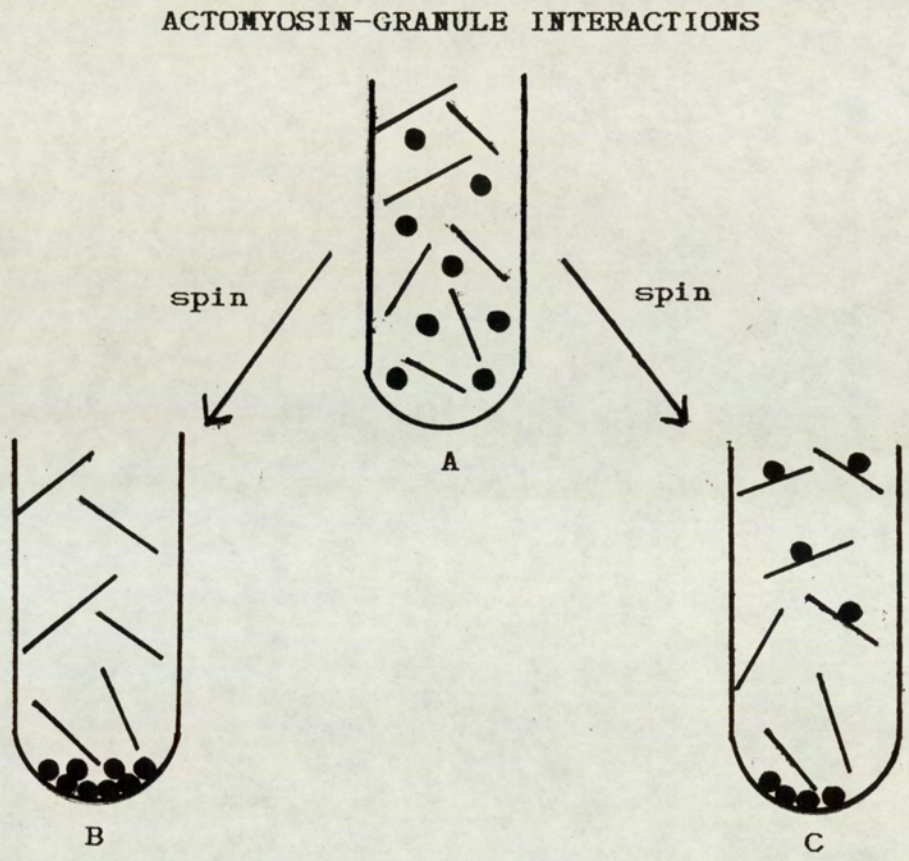
However, cytochalasin B did not affect glucose induced saltatory movements either in the presence of 8.3 or 16.7 mmol/l glucose, (6). Lacy has suggested that the presence of cytochalasin B enhanced β-granule movement at these glucose concentrations, (5). The reason for this observation is not clear but an effect for cytochalasin B, a microfilament inhibitor, on β-granule movement implies the involvement of microfilaments in addition to microtubules in the movement of granules in the process of insulin release. Somers and coworkers have also reported effects for cytochalasin B on the β-cell boundary, presumably disrupting the β-cell web, (6).

Attempts have been made to elucidate possible interactions between β-granules and the contractile proteins, actin, myosin and actomyosin, using an in vitro model system, (87), composed of isolated β-granules and a

suspension of contractile proteins. Interaction or binding of the β-granules to the contractile proteins was registered as an alteration in the sedimentation characteristics of the granules, Figure 1.8. Actomyosin appeared to be much more effective in promoting granule binding than actin, whilst very little effect was seen with myosin, (86). The presence of cellular levels of ATP (2 mmol/l) enhanced these interactions whilst high concentrations of Ca^{2+} had little effect, (88, 89). Further investigation revealed ultrastructural contacts between β-granule membranes and actomyosin, (89). It has been established that granules move in saltatory movements along microtubules in a predetermined direction towards the plasma membrane of the β-cell, (5,6) and that microfilaments, particularly actin and actomyosin are involved. It would seem useful to know how protein phosphorylation in the β-cell is linked to granule movement and where the energy comes from to drive the process.

Certainly calmodulin facilitates protein phosphorylation, the substrate in rat islets has been suggested to be a protein of M.Wt 53 kDa, P53, (19,42). This together with actomyosin ATP-ase activity and a possible role for calmodulin in microtubule assembly/disassembly suggests that calmodulin might form the link between increasing cytosolic Ca^{2+} concentrations and the initiation of granule movement, (90).

Figure 1.8 Schematic diagram showing an *in vitro* system to examine the interaction of isolated insulin storage granules with cytoskeletal components. (87).



After incubation of the two components at 37°C (A) the tube is centrifuged. If no interaction with cytoskeletal components occurs, the granules will sediment preferentially (B), leaving a low insulin content in the supernatant. If interactions occur however, the insulin concentration in the supernatant will be increased (C) in proportion to the degree of interaction observed.

If the actin-myosin complex, (actomyosin), rather than actin is the active microfilament (contractile protein), in the microtubule-microfilamenteous system, then a possible link between cytosolic Ca^{2+} concentration and microfilament activation can be postulated as shown in Figure 1.9, (90). Calmodulin is a constituent subunit of myosin light chain kinase (87) and calcium activation of this kinase and the subsequent phosphorylation of myosin would promote both the ATP splitting activity and contractile activity of myosin in association with actin. The possible roles played by Ca^{2+}, microtubules and microfilaments in the mechanisim of β-granule movement have been summarised in Figure 1.10, (87).

Figure 1.9 The action of myosin kinase as a direct link between cytosolic calcium concentrations and microfilament contraction in β-cells (90)

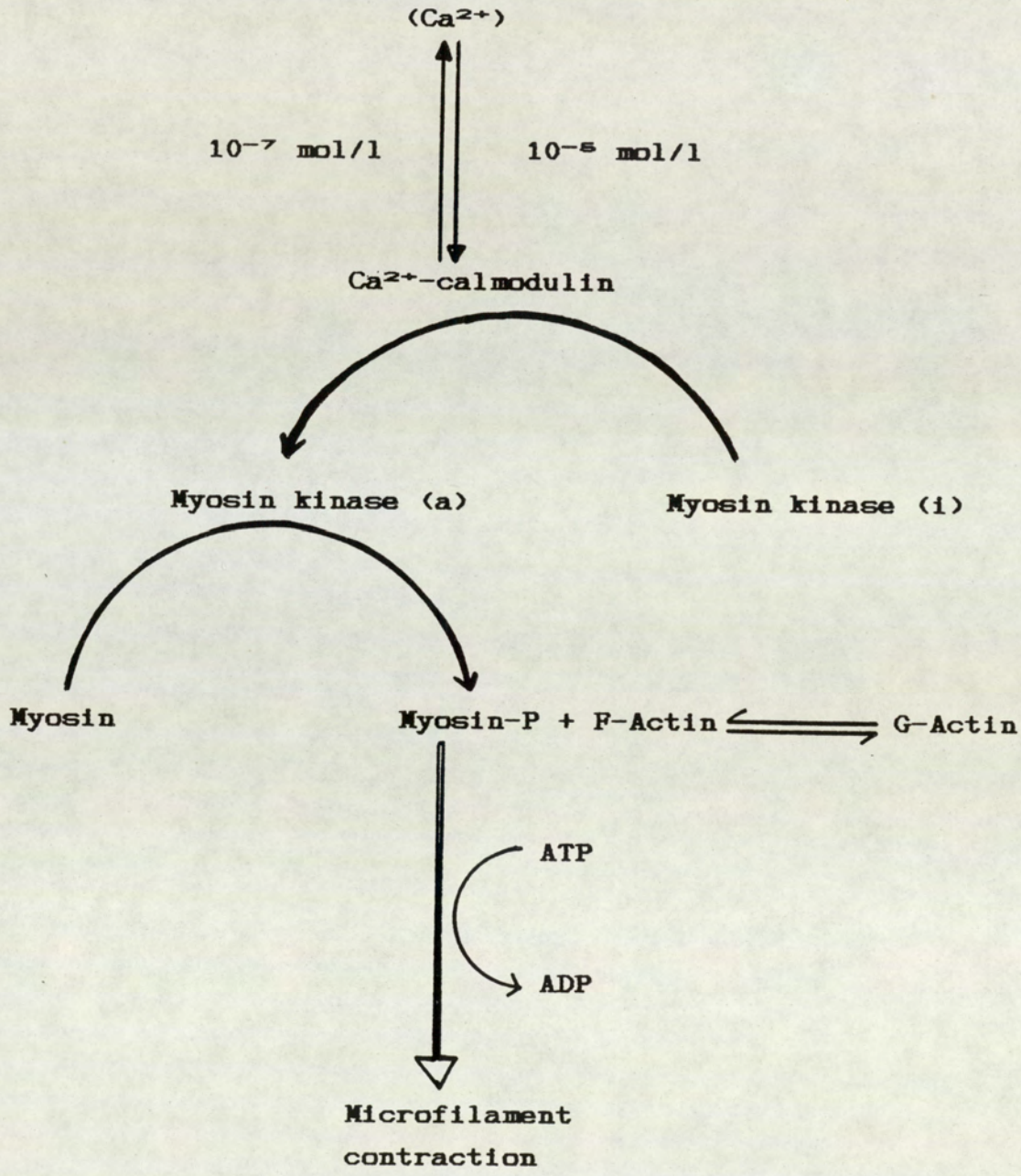
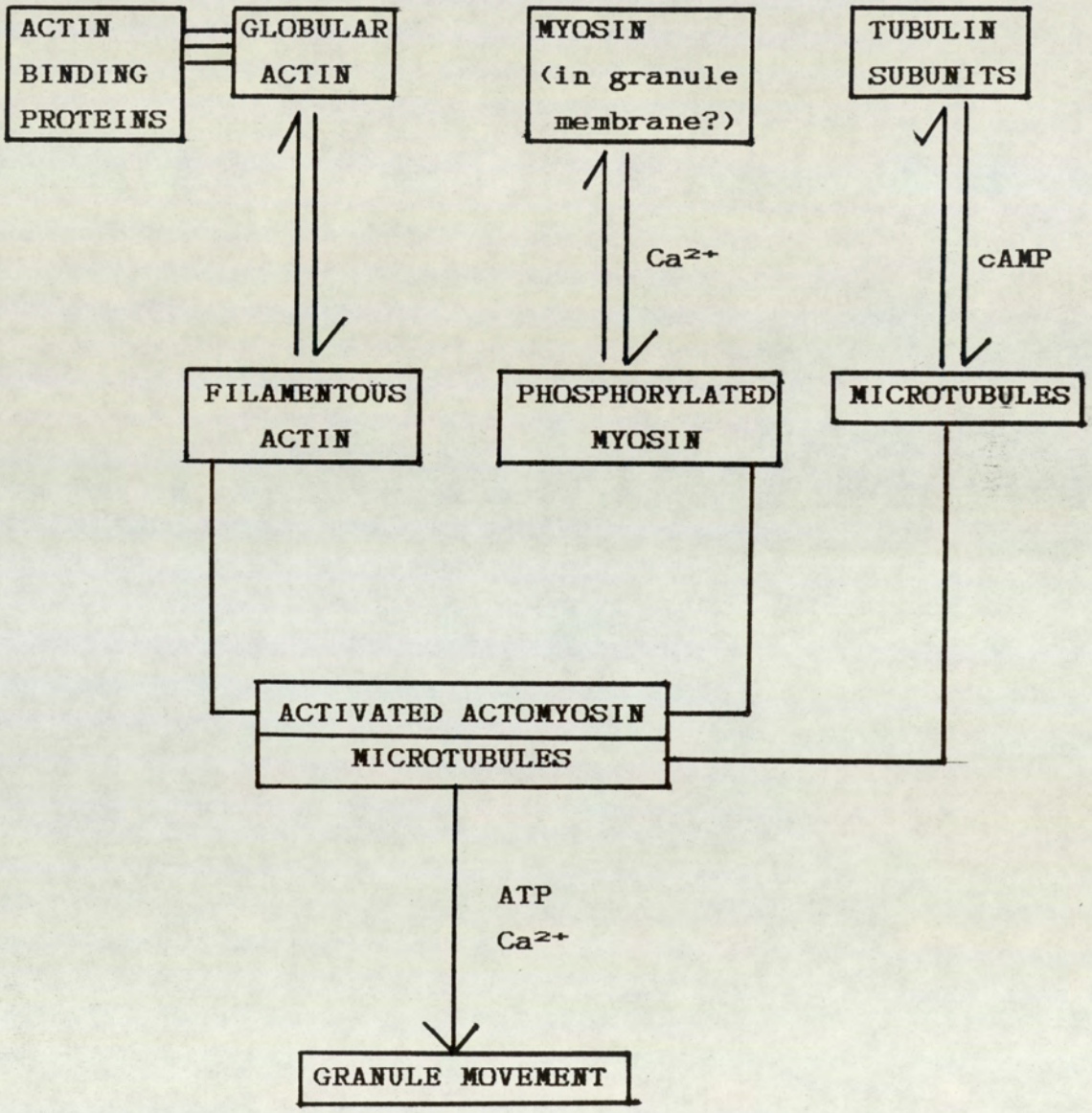


Figure 1.10. Possible interactions between components of the insulin secretion mechanism.



The microfilamentous web lies directly beneath the β-cell plasma membrane and consists predominantly of actin and myosin, the former constituting 1-2% and the latter 0.5-1% of the total β-cell protein, (91). Actin concentrations within isolated islets have been measured using an assay which depends upon the specific and irreversible binding of globular G-actin to DNAase -1 and the subsequent inactivation of this enzyme, (92). Use of this assay has shown that the stimulation of insulin release by glucose is accompanied by an increase in the proportion of filamentous F-actin from approximately 40% to 70% and that this increase is dependent upon the presence of ATP and not Ca^{2+}, (92,93,94). Thus an increase in microfilament polymerization in β-cells appears to be associated with insulin release and was not just a consequence of insulin release. This polymerization has also been observed in the absence of Ca^{2+}, (86).

In addition to assisting the movement of β-granules along microtubules, the microfilaments of the β-cell web appear to have another important function, (91). Cytochalasin B, an agent which disrupts the microfilamentous web, potentiates insulin release from islets, (94). This suggests the possibility that the microfilamentous web may have a dual role to play in insulin secretion. When insulin release is stimulated by an increase in blood glucose above the fasting level of 4-5 mmol/l, the β-granule is moved towards the β-cell

plasma membrane by microtubular activity under the influence of actomyosin followed by the contraction of microfilaments lying directly below the plasmsa membrane (86). However, under non-stimulatory conditions such as fasting blood glucose levels, the microfilaments do not contract which prevents the β-granule from reaching the β-cell membrane. Thus the microfilamentous web serves to regulate insulin release by either helping or preventing the movement of β-granules towards the membrane, depending upon whether or not insulin release has been stimulated, (91). Under stimulatory conditions the β-granule membrane is presented very close to the β-cell plasma membrane, the distance between the two being determined by the electrostatic repulsion between the granule membrane and the plasma membrane and Van der Waal's attractive force (94,95). The final stages of β-granule membrane fusion with the β-cell plasma membrane and the subsequent release of insulin into the extracellular space is thought to be independently regulated perhaps by calmodulin, and that granule lysis is mediated in part through alterations in cAMP stimulated protein kinase activity, (96).

The effect of microtubule inhibitors and microfilament disruptors on insulin release

A variety of microtubule inhibitors and microfilament disruptors have been used to confirm a role for microtubules and/or microfilaments in the mechanism of insulin release. These drugs and their effects are summarized in Table 1.1. The three drugs selected for the present study, colchicine, vinblastine and cytochalasin B were chosen on the basis of their potentially different modes of action on microtubules and microfilaments. Both colchicine and vinbastine inhibit microtubule elongation, the former prevent the addition of tubulin subunits to the microtubule and the latter creates large paracrystalline aggregates of tubulin. Both these drugs have the effect of inhibiting insulin release. Cytochalasin B appears to stimulate insulin release by preventing the formation of actin filaments. This causes a subsequent break down of the micro-filamentous web which ususally acts as a barrier between the β-granule and the β-cell plasma membrane, (86).

Colchicine

Colchicine is a plant alkaloid extracted from the meadow saffron or *Colchicum Autumnale*, and was first purified in 1883. A detailed stereochemical analysis of its structure was first published in 1974, (97,98). The

Table 1.1 Compounds used to study the role of microtubules and microfilaments in the mechanism of insulin release. (86)

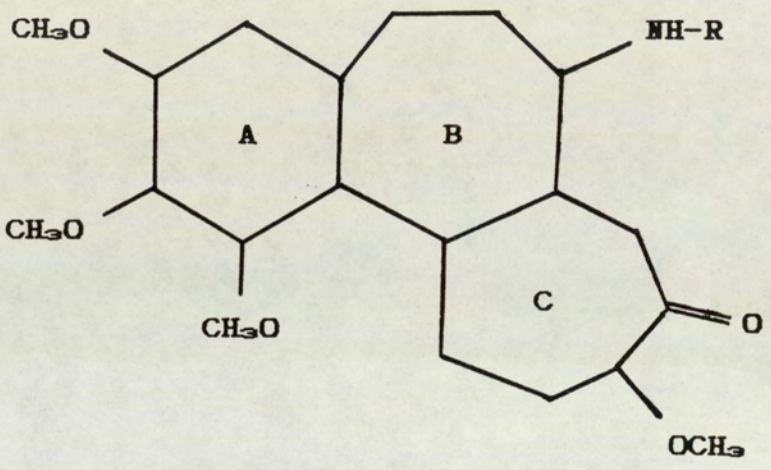
	Mechanism of Action	Effect on Secretion
Microtubules		
Colchicine	Inhibits addition of tubulin dimers to microtubules resulting in depolymerization	Inhibition
Vinblastine	Causes formation of para-crystalline tubulin aggregates	Inhibition
Nocodazole	Induces microtubule depolymerization	Inhibition
Taxol	Causes tubulin to assemble into microtubules	Inhibition
Deuterium Oxide	Stabilizes polymerized microtubules	Inhibition
Microfilaments		
Cytochalasin -B	Binds to actin filaments preventing addition of further actin	Stimulation
Phalloidin	Stabilizes actin filaments	* Stimulation

* in permeabilized cells

compound is a tropolene derivative composed of three fused rings: an aromatic ring (A), a twisted 7 carbon ring (B) and a 7 carbon tropolene ring (C). Figure 1.11 (97,99).

When islets are stimulated by glucose, insulin is released in a biphasic fashion. The first rapid phase, or spike reponse occurs within the first five minutes of glucose stimulation and is thought to correspond to the release of β-granules already in close association with β-cell plasma membrane. The second slower phase may contain a biosynthetic component and additionally represents the transport of centrally generated β-granules to the β-cell periphery which may take 30 to 120 minutes. This time period (lag phase) may be the reason why microtubule inhibitors such as colchicine and vinblastine take a period of about 90 minutes before they induce a significant inhibition of insulin release. The effect of colchicine on the first and second phases of glucose-induced insulin release from perifused rat islets is shown in Figure 1.12, (8). Colchicine specifically inhibits the second phase of glucose induced insulin release from perifused rat islets supporting the view that a component of the second phase of glucose stimulated insulin release is represented by the movement of β-granules to the cell periphery, (8). Indeed radioautographic work by Malaisse and coworkers (100), have demonstrated that the exposure of isolated rat islets to 0.01 mmol/l colchicine progressively decreases

Figure 1.11 The structure of colchicine. (97,99)

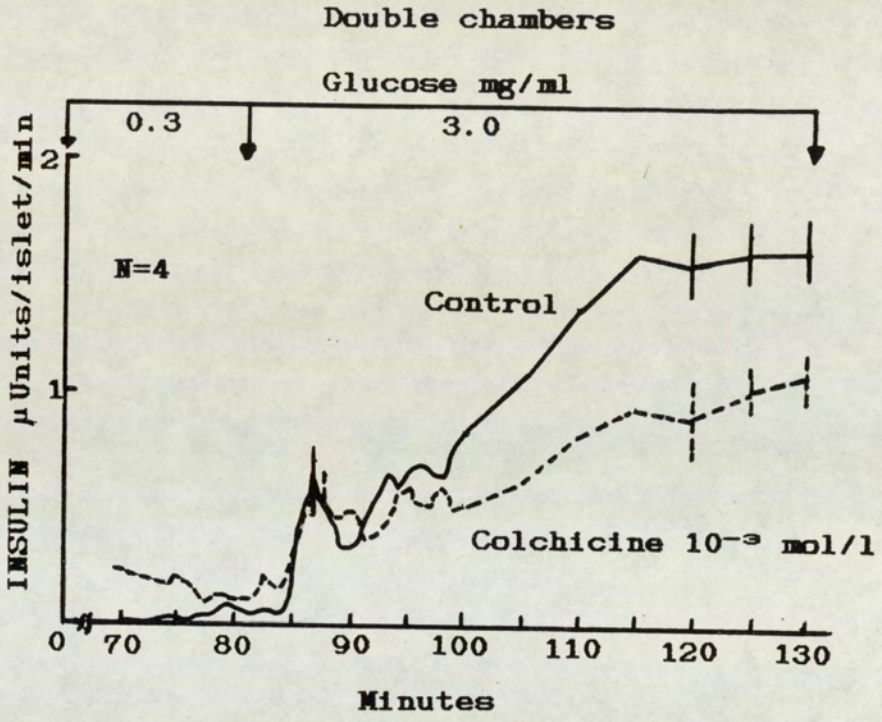


A = Aromatic ring

B = Twisted 7 carbon ring

C = Tropolene ring

Figure 1.12 Effect of colchicine on the first and second phases of glucose stimulated insulin release from perifused rat islets using the double chamber technique. (8)



Colchicine (10^{-3} mol/l) produced a significant inhibition of the second phase of secretion but did not inhibit the first phase.

the release of preformed insulin and after 2 hours retards the transport of newly synthesised proinsulin from the endoplasmic reticulum through the Golgi apparatus and its subsequent packaging into β-granules.

The actual mechanism by which colchicine binds to tubulin and initiates an inhibition of microtubule assembly is poorly understood. Early measurements of positive entropy and enthalpy changes suggested that colchicine became bound in a hydrophobic or non-polar pocket within the tubulin molecule, (101). More recent studies on the fluorescence lifetime of colchicine in combination with tubulin (about 1.2 ns) indicated that the alkaloid was extensively buried in the tubulin molecule. The gradually decreasing colchicine binding capacity of aging tubulin appears to be associated with the loss of this colchicine binding site, (102). The binding of colchicine is relatively slow, (about 10^{2} m^{-1} s^{-1}), (103), and the colchicine-tubulin assembly is unstable with a half-life of 36 hours, (104,105).

Two conflicting theories have been proposed about the way colchicine binding might affect microtubule assembly. The first proposed by Wilson and coworkers, (106-108) was initially based on studies using podophyllotoxin and vinblastine and extrapolated to cover colchicine. As colchicine and podophyllotoxin bind to the same site on tubulin, the general conclusion was that "the binding of just a few colchicine/podophyllotoxin molecules to the growing ends could prevent any

additional tubulin molecules from adding on", (108). In a later study using colchicine and beef brain tubulin, fragments of microtubules were exposed to tubulin in the presence and absence of colchicine, (106). The presence of the alkaloid alone was not sufficient to block microtubule assembly, but together with tubulin dimers, the tubulin-colchicine complex blocked more than half of the microtubule from further assembly. There was no apparent microtubule disassembly under these conditions, (106). It was assumed that disassembly could not have taken place at the end where colchicine was attached, for this would have suppressed the 'poisoning' effect of colchicine. In order to explain the experimental observation that microtubules disassembled in beef brain tubulin preparations treated with colchcine, it was suggested that disassembly took place at the end of the microtubule not "capped" by colchicine, (106).

The major criticism of this theory is that if 'end poisoning' is a fact, then the number of growing ends should progressively decrease and the shape of the assembly curve, which is normally exponential, should be modified. This does not appear to be the case and the microtubule ends remain assembly competent in the presence of the colchicine-tubulin complex, the number of assembly-competent ends not being modified. Apparently, colchicine decreases the affinity constant of microtubules for tubulin , while the disassembly constant does not appear to be modified. This is exactly the

opposite to the action of MAP's 1 and 2, which do not affect the assembly constant but decrease the value of the constant for disassembly, (109). Using different experimental conditions Wilson used tubulin dimers in the presence of heparin to prevent the formation of tubulin rings and to remove MAP's 1 and 2, (108). Thus an alternative theory that might be used to explain the inhibition of tubulin assembly by colchicine binding would suggest that the colchicine-tubulin dimer interferes with microtubule assembly and has no specific preference for either end of the microtubules, (108,110).

While colchicine acts to prevent the assembly of microtubules, the production of tubulin-colchicine (1:1) complexes is possible. This leads to the formation of tubulin filaments, double rings and paracrystalline aggregates, the assembly of which requires similar conditions to those of tubulin. These are critical tubulin concentration, a requirement for GTP or non-hydrolysable analogs, the presence of Mg^{2+}, a large positive entropy, enthalpy changes and exponential assembly with a lag period. These polymers (rings and filaments), are cold sensistive and display low GTPase activity which is not the result of any contamination. It has been concluded that colchicine does not affect the assembly of tubulin but only it's geometry and colchicine appears to bind to the lateral area of β-tubulin, (111-113).

Colchicine also binds to the 36S double tubulin rings containing Tau factors. It may be that this modified double ring would be incapable of polymerising further into a microtubule and this might explain the substoichiometrical action of colchicine which binds to all dimers of the 36S ring, (114).

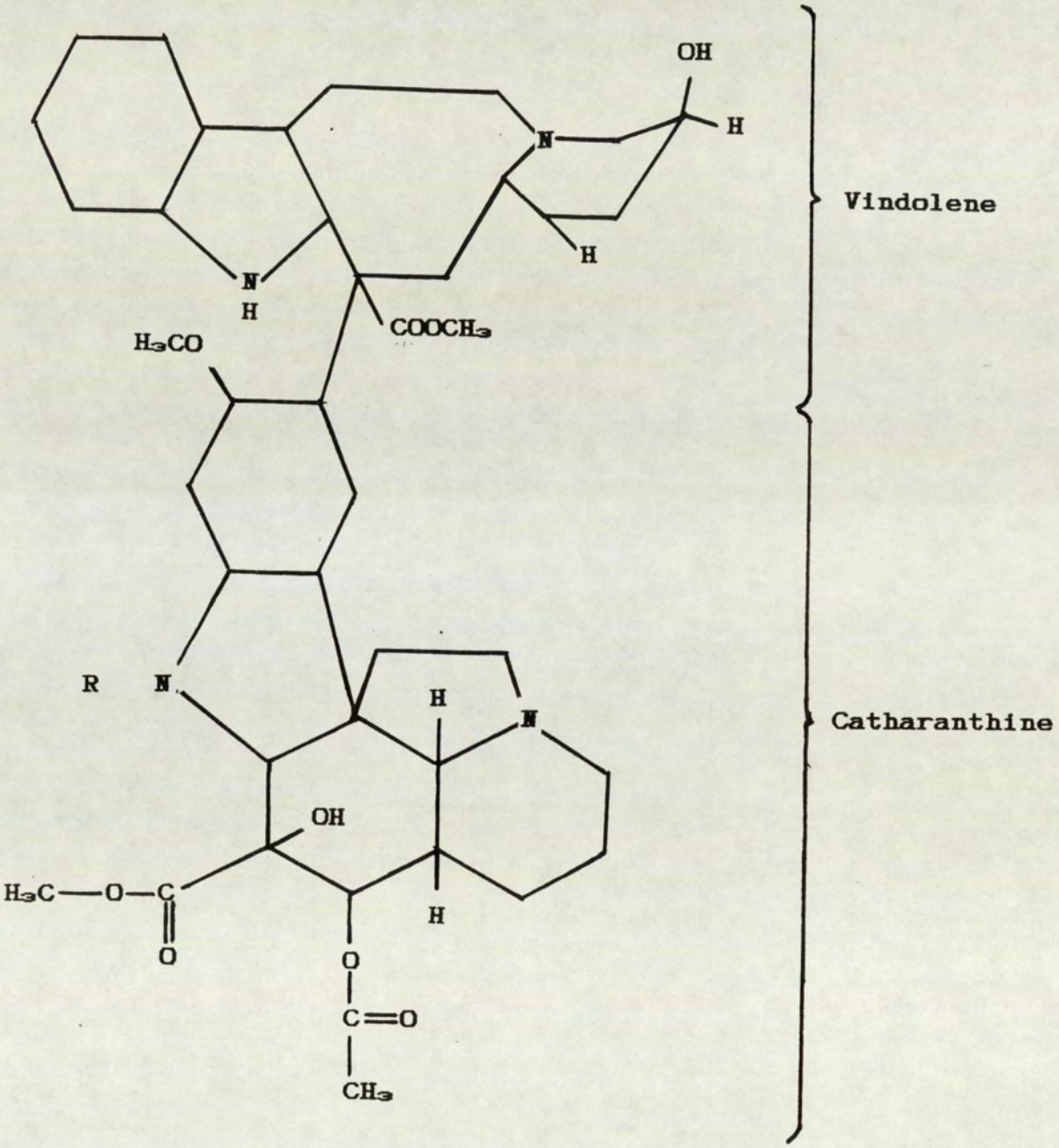
MAP's 1 and 2 , high molecular weight proteins and Tau proteins compete for tubulin binding regions close to, or identical to those which bind colchicine, (115).

Vinblastine

The vinca alkaloids were first extracted from the Madagascan plant *Catharanthus roseus (Vinca rosea L)*, (99). Vinblastine has been shown to suppress the bone marrow by acting as a mitotic poison causing spindle disruption in a manner similar to colchicine, (116,117). The property of mitotic suppression has led to vinblastine and two other vinca alkaloids, vincristine and vindesin being used as cancer chemotherapeutic agents. However, these drugs are not without distressing side-effects causing the disruption of axonal transport leading to the impaired action of limbs. The alkaloids are usually administered in a 'cocktail' with other drugs for the treatment of cancer to help reduce the incidence of the side effects.

The chemical structure of the vinca alkaloids differs greatly from that of colchicine and consists of two domains of fused catharantine and vindolene rings, Figure 1.13, (99). The structure and biological actions of vinblastine and vincristine are very similar. Vincristine *in vitro* has been shown to increase the release of insulin if the treatment lasts for less than 30 minutes but demonstrates an inhibitory action when treatment reaches 2 hours, (118). This may suggest a role for microtubules in the second slow phase of insulin release. Similarly, studies with intact rats have demonstrated an increase in insulin release for a short

Figure 1.13 The structure of vinblastine and vincristine. (99)



Vinblastine: R = CH_3

Vincristine: R = COH

period after vincristine injection which subsequently falls off after 1 hour, (119-121).

The vinca alkaloids bind to tubulin at a different site from colchicine and podophyllotoxin and it has been suggested that rat brain tubulin may have two binding sites for vinblastine, one showing high affinity and the other low affinity. The first high affinity binding site has a binding constant which corresponds to the half-maximal concentration of vinblastine needed to prevent microtubule assembly *in vitro*. The binding constant of the second low affinity binding site corresponds to the aggregation of tubulin. Both sites approach a 1:1 stochiometry, (122).

Spiral structures composed of tubulin protofilaments have been observed in the presence of vinblastine, which suggests that vinca alkaloids act principally on the lateral linkages of tubulin subunits, (123). These spirals are stable if MAP's 1 and 2 are present, but in the absence of MAP's, microtubules are completely disassembled, (123). Tau proteins are believed to be the principal microtubule asociated proteins involved in spiral formation, (124). The disassembly of microtubules into spirals and proto-filaments takes only a few minutes at low vinca alkaloid doses, (1 - 10 $x10^{-6}$ mol/l) and the resulting spirals are resistant to cold and Ca^{2+}, and can be disassembled in a 0.25 mol/l solution of NaCl. These results suggest that the spirals form from assembled microtubules and not necessarily from the tubulin subunit

pool, (125). The spiral filaments formed by vinblastine in the presence of Tau proteins unlike pre-formed microtubules bind avidly with colchicine and podophyllotoxin. They also react with N,N'-ethylene-bis iodoacetamide to generate cross links involving SH groups in β-tubulin, preventing the assembly of microtubules, by causing the formation of an abnormal β-tubulin, (126). Colchicine binding by the tubulin spirals might suggest that the colchicine binding site is on the lateral surface of the tubulin dimer and buried within the microtubule, (102,126).

In addition to the formation of spirals, vinca alkaloids also produce 'vinca crystals' or macrotubules. These have been observed in L-strain fibroblasts and human leukocytes after treatment with low concentrations of vinblastine and vincristine, (127). The macrotubules were 8μm long and constructed of hexagonally packed tubules with a diameter of 27-28nm, (127). The crystals formed were birefringent (128) and assembled from either modified microtubules or pools of unassembled tubulin. The formation of these macrocrystals provides a means for the purification of tubulin from cells by extracting the crystals which are very stable even in the presence of divalent cations, (129,130). The binding of vinblastine to macrocrystals is not affected by either colchicine, GTP, griseofulvin, temperature or Ca^{2+}, (131) and the binding of one molecule of vinblastine has been shown to induce the process of crystallisation. Macrocrystals

have been grown at 4°C in the absence of both MAP's 1 and 2 and Tau proteins, (129).

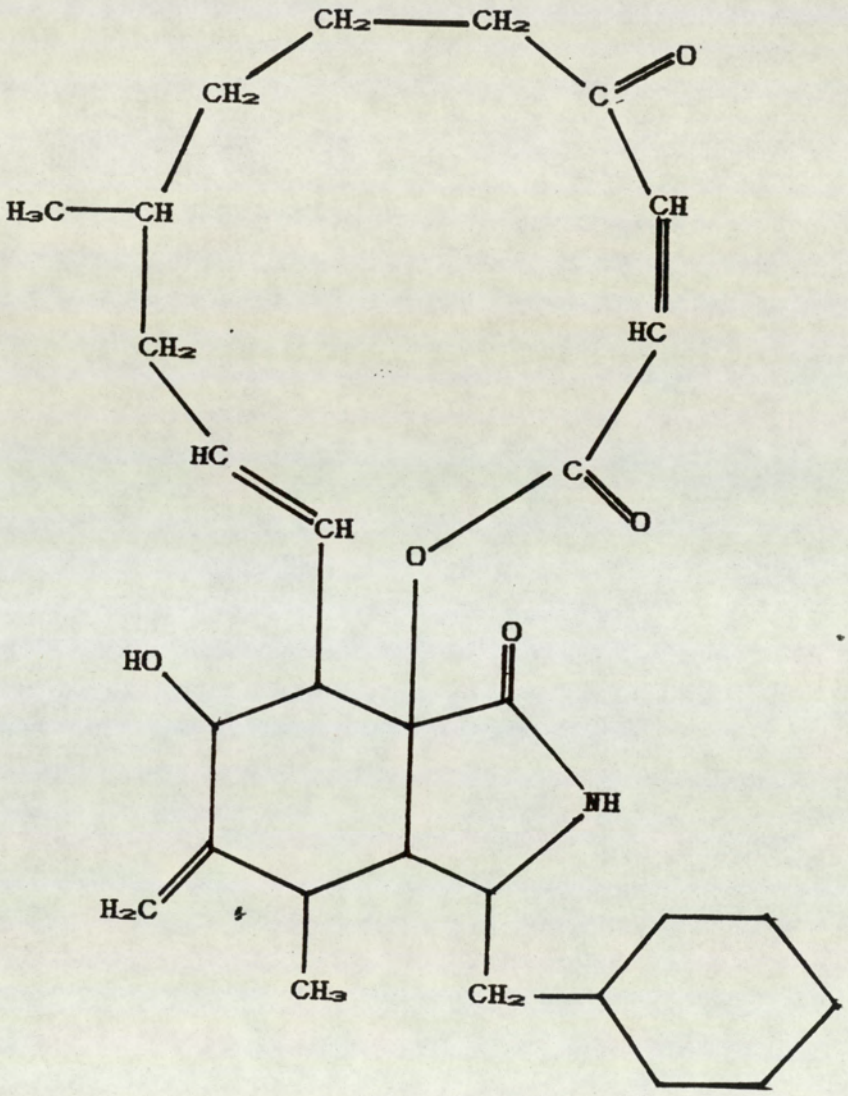
Both vinblastine and vincristine have the abililty to inactivate calmodulin-dependent phosphodiesterase activty associated with microtubules in bovine brain preparations, (132). Vinblastine (10 µmol/l) caused a 50% inhibition of calmodulin-dependent phosphodiesterase activity. This appears to involve the catharanthine moiety of the molecule. Vinblastine has been shown to bind to calmodulin in a Ca^{2+}-dependent manner and cause inhibition of the calmodulin dependent step leading to phosphodiesterase activation. A similar activity for vinblastine has been demonstrated with β-cell calmodulin dependent phosphodiesterase, (133). Such an inhibition might in turn increase the β-cell level of cAMP and facilitate the activation of insulin release.

Cytochalasin B

Cytochalasin B has been used by many workers to investigate the role of the microfilamentous system, particularly the protein actin, in the mechanism of insulin release, (134-136). Cytochalasin B is a metabolite of the fungus *Helminthosporium dermatoideum* and has the structure shown in Figure 1.14, (137).

Orci and coworkers have demonstrated a web of microfilaments 500 - 3000Å thick and composed of filaments 50 -70Å in diameter lying just beneath the plasma membrane of β-cells from isolated rat islets, (134,135). Following exposure to 10 μg/ml cytochalasin B for 90 minutes in the absence of glucose, the web was transformed into large heterogeneous masses of tightly packed filamentous and granular material which extended far into the cytoplasm. Numerous β-granules were found immediately adjacent to the plasma membrane and emiocytosis of β-granules was observed, (134). The same workers also demonstrated that cytochalasin B potentiated 16.7 mmol/l glucose induced insulin release and suggested that the band of microfilaments served as a potential barrier to the release of β-granules by emiocytosis. Upon removal of the barrier the granules could more easily approach the cell membrane and release their contents into the extracellular space. Thus cytochalasin B potentiated insulin release.

Figure 1.14 The structure of cytochalasin B (137)



Cytochalasin B is believed to bind to actin filaments preventing the addition of further actin and upsetting the equilibrium between G-actin and F-actin. This leads to the subsequent disruption of the microfilamentous web, (86) and removal of the 'barrier' preventing β-granules from reaching the β-cell plasma membrane.

Malaisse and coworkers noted that the enhancing effect of cytochalasin B on glucose stimulated insulin release increased as the concentation of cytochalasin B was increased, but when this concentration was exceeded the enhancing action was replaced by a more deleterious action on β-cells, (135).

The enhancing action of cytochalasin B on glucose stimulated insulin release has also been shown to be reversible, (134,135). The retrieval of isolated islets from media containing a stimulatory concentration of glucose (16.7 mmol/l) and cytochalasin B and subsequent transfer to fresh 16.7 mmol/l glucose medium in the absence of cytochalasin B leads to a reduction in the insulin response of the islets, (134,135).

The failure of cytochalasin B to modify glucose induced Ca^{2+} uptake also suggests that cytochalasin B might affect a late step in the secretory sequence, at or beyond the site of action of Ca^{2+} in the β-cell, that is the activation of protein kinases, (134-136).

Perifusion of rat islets by Lacy and coworkers with 5 μg/ml cytochalasin B in the presence of 16.7 mmol/l

glucose after a 30 minute pre-perifusion period with the same cytochalasin B concentration and basal glucose, (1.8 mmol/l), showed that cytochalasin B enhanced both the first and second phases of insulin release compared with controls perifused in the absence of cytochalasin B, (136). The same study showed the reversibility of the effect of cytochalasin B on glucose induced insulin release since its removal during the second phase of secretion resulted in a rapid decrease in the insulin secretion rate 2-3 minutes after the agent had been cleared from the perifusion system, (136).

A similar investigation involving the perifusion of islets with vinblastine, (10^{-4} mol/l) and cytochalasin B, (5 μg/ml) in the presence of 16.7 mmol/l glucose, demonstrated that the inhibitory action of vinblastine on the first and second phases of insulin release prevailed, that is the inhibition of both the first and second phase of insulin secretion, (136). This observation has lead to the conclusion that an intact microtubule system is required for granule movement and insulin release and that the action of the microfilaments in the regulation of emiocytosisis is secondary to β-granule movement and microtubule function.

β-cell models used to study the role of microtubules and microfilaments in the mechanism of insulin release.

Islets of Langerhans of lean and genetically obese diabetic (ob/ob) mice.

The genetically obese diabetic mouse (ob/ob) is a frequently used animal model of obesity and type II diabetes or non-insulin dependent diabetes mellitus, (NIDDM) in man, (138, 139). The animal presents a variety of abnormalities which appear at various stages in the development of the syndrome (139, 140) and any study involving islets from these mice needs to take into account the age of the animal and the metabolic conditions prevailing at the time of study.

The obesity of the animal is inherited on chromosome 6 as an autosomal recessive mutation, (141), which has been maintained within an inbred stock on the C57BL/6J background at Aston for almost 20 years. The mouse is characterized by severe obesity, hyperglycaemia and insulin resistance acompanied by moderate hyperphagia, hyperinsulinaemia, hyperglucagonaemia, hypercellularity of adipose tissue and enlarged islets of Langerhans, (139).

Obesity *per se*, from whatever cause, represents an imbalance between energy intake and energy expenditure. However, in the case of the obese mouse, hyperphagia does not develop immediately, (142), but only becomes apparent

when the mice reach 35 days of age, (143). Indeed, before weaning, the milk uptake of the obese mouse (ob/ob) parallels that of its normal lean litter mate (Ho), (143).

The earliest indication of the obese syndrome in the mice is a reduction in core temperature and oxygen consumption within 10 - 14 days, (144,145). However, impaired thermogenesis can only partly account for the increase in fat cell size seen after 10 - 20 days and it is increased liver and adipose lipogenesis that is the main cause of the increase in body weight seen between days 17-21, (146-148). An increase in the proportion of body fat at 10 - 12 days has been demonstrated in the Aston (ob/ob) mouse, (149), but obesity does not become clearly visible until 25 - 28 days, (146).

At day 17 - 21 there is a slight increase in serum insulin levels accompanied by hypoglycaemia, (150). Insulin levels then continue to rise rapidly and insulin resistance gradually appears and is represented by hyperglycaemia in the presence of a raised serum insulin concentration. The increase in insulin levels coupled with insulin resistance and hyperglycaemia reflect the development of type II diabetes seen in Man, (and may represent the link between obesity and diabetes).

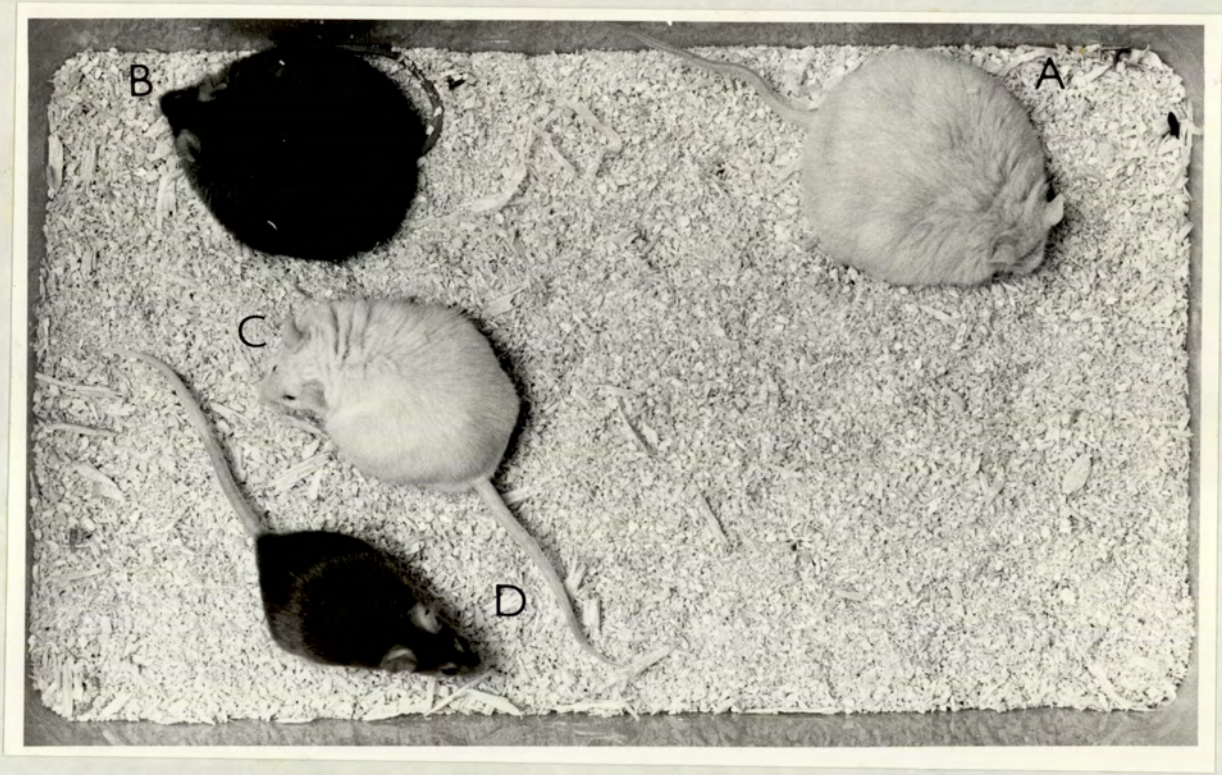
The increase in carcass fat, which appears early in the syndrome is accompanied by a decreased protein deposition which is first evident at 21 - 28 days and thus occurs before the appearance of overt insulin

resistance, (143,149,151). The obese mouse gains weight rapidly from day 35 and hyperinsulinaemia, hyperglycaemia and hyperphagia increase during this dynamic phase of the syndrome, with the concomitant development of progressive glucose intolerance, fasting hyperglycaemia and a resistance to exogenous insulin. With advancing age, (25 weeks and over), weight gain slows or stops and a loss of weight in surviving aged obese mice has been reported, (152,153). At 40 weeks of age plasma insulin concentrations are lowered and insulin resistance is reduced, (153), but the longevity of obese animals is reduced and death usually occurs when this age is reached. The dynamic phase of the syndrome is clearly demonstrated in Plate 1.1

The lean mice (Ho) which may come from the same litters as the obese mice (ob/ob) gain weight slowly between 5 - 40 weeks, but food intake and plasma glucose and insulin concentration do not significantly alter, (154). Thus for experimental purposes lean mice can be legitimately used as controls.

Experimental work described in this study has employed isolated islets from both lean and obese mice, using islets from lean mice as controls. The islets of obese mice show β-cell hyperplasia at 5 weeks (three times greater in size than lean mice of the same age), together with β-cell hypertrophy and heavy vascularisation, (154).

Plate 1.1 Genetically obese diabetic mice (ob/ob) pictured together with a normal lean (Ho) mouse



Mouse	Genotype	Coat colour	Age (weeks)	Weight(g)
A	ob/ob	white	21	108
B	ob/ob	brown	18	82
C	ob/ob	white	12	73
D	Ho	brown	13	37

Hyperplasia and hypertrophy continues until the mice reach 25 weeks of age when the β-cell population in the obese mouse islet has increased to several times that of the lean mouse, (154). After 25 weeks of age the β-cell population decreases and the weight gain of the obese mouse declines. While the β-cell population increases prior to 25 weeks, the glucagon secreting α-cell population declines until there is a significant increase in the β to α cell ratio, although the number of α-cells is always considerably greater than in lean mouse islets.

At 15 - 20 weeks of age some of the larger islets contain small intercellular vacuoles which occupy less than 5% of the area of islets at this stage, (154). Adter 25 weeks the size and number of vacuoles increases in the larger islets, whilst the smaller islets show little or no vacuolation. In some cases, enlarging islets coalesce and become surrounded by a distinct cellular layer giving a duct-like appearance. By 40 weeks vacuoles account for some 29% of the area of the islets, (154).

After 25 weeks peripheral α-cells become dispersed throughout the interior of the islet and by the time the animal reaches 40 weeks of age capsular and intra-islet fibrosis has appeared. The vacuolation is thought to be equivalent to the insulin hyalinosis seen in the islets of elderly human diabetics, (155,156) and the invasion of obese mouse islets with acinar like cells with age might result from endocrine-exocrine cell transformation.

The underlying cause of the changes in islet morphology, insulin resistance and obesity is not understood. The controversy as to whether hyperinsulinaemia is a primary defect preceeding hyperphagia and obesity, or if hyperinsulinaemia is a consequence of the two latter abnormalities, brings to light the possibilty that the ventromedial nucleus of the hypothalmus may be one of the underlying factors involved in hyperinsulinaemia. Lesions in this area , induced in weanling rats have been shown to increase circulatory insulin levels although the rats were neither hyperphagic or obese, (157). Lesioned rats on a diet restriction, which are not able to express an obese phenotype, showed islet β-cell degranulation and islet enlargement, (158). Hypersecretion of insulin relates to a central nervous system disorder and it may be said that the hyper-secretion of insulin is responsible for initially inducing obesity and that provided hyperinsulinaemia is present, hyperphagia is not necessary but acts only as an amplification factor, (159).

The use of cultured β-cell lines for studies on the mechanism of insulin release.

The use of isolated islets of Langerhans, both rodent and human, has contributed greatly towards an understanding of the mechanism of insulin release from β-cells. However, there are several problems associated with the use of either islets or primary cultures of islet cells for such studies. The major problem revolves around the difficulty in generating large numbers of viable isolated islets which, coupled with the cellular and hormonal heterogeneity within islets and loss of insulin production *in vitro*, exerts limitations on their legitimate use in experimental studies. If a convenient and homogeneous source of β-cells could be produced which would survive well in culture and release insulin in response to glucose without possible paracrine interference from other islet cells, more specific valid information could be obtained about the mechanism of insulin release and the involvement of the β-cell cytoskeleton in the process.

Indeed many attempts have been made to produce an immortal line of cultured β-cells and two of the most successful cell lines have been utilised in the present study, namely the clonal cell line of Simian Virus 40 - transformed hamster pancreatic β-cells, HIT-TI5, (160), and an insulin secreting cell line established from a

radiation induced transplantable rat islet cell tumour, RINm5F, (161,162).

The HIT-TI5 cell line is a continuous β-cell line established from SV40 transformed Syrian hamster islet β-cells and which has been shown to have modest glucose sensitivity responding maximally to a glucose concentration of 7.5 mmol/l, (160). HIT-TI5 cells also release insulin in response to other secretagogues such as IBMX, glucagon, the amino acids L-leucine and L-arginine and sulphonylureas, (160,163-165). However, the capacity of HIT-TI5 cells to release insulin in response to glucose decreases with increasing passage number, although glucose consistently doubles the basal insulin secretory rate, (161,163-165). This passage effect is believed to be the result of a progressive reduction in the available insulin secretory pool and total insulin content, (160).

The RINm5F cell line exhibits a slight passage effect and shows either no glucose sensitivity or a reduced response to glucose compared with HIT-TI5 cells and islet β-cells, (162). RINm5F cells also contain small amounts of radioimmunoreactive glucagon and somatostatin but these are believed to be 5-6 orders of magnitude less than that found in islet α or δ-cells, (166-167). Since these levels are low it is doubtful if they exert any paracrine influence on the release of insulin. RINm5F cells respond poorly or not at all to glucose, (9,15). Some stimulation of insulin release has

been demonstrated from RINm5F cells in response to low glucose concentrations, (2.8 mmol/l), so it would appear that these cells are not totally glucose insensitive, (167).

It has been suggested that the phosphorylation of glucose by β-cell glucokinase might be the rate limiting step for glucose metabolism in β-cells and thus glucose stimulated insulin release, (15, 168, 169). Glucokinase may be low or absent in RINm5F cells and such a defect might be responsible for the lack of glucose sensitivity. If this were the case substrates distal to glucose phosphorylation such as glyceraldehyde would stimulate insulin release. In the present study RINm5F cells have been shown to be responsive to D-glyceraldehydeand this sugar has been used to stimulate insulin release in experimental studies (170).

It is clear from the literature presented so far that the role of microtubules in the mechanism of glucose stimulated insulin release is not clearly understood. One of the major factors hampering the elucidation of the insulin release mechanism has been the difficulty in obtaining large quantities of β-cells. The present study makes use of the cultured β-cell lines HIT-TI5 and RINm5F in addition to the isolated islets of lean and genetically obese diabetic (ob/ob) mice to overcome the problem of β-cell number in order to study the role of the microtubules and the microfilaments in the mechanism of insulin release.

In the first section the insulin response of the islets and HIT-TI5 cells will be compared with each other by statically incubating each cell type in a range of glucose concentrations. Similarly the insulin response of RINm5F cells to D-glyceraldehyde will be investigated.

The role of the microtubules in the mechanism of insulin release of lean and obese mouse islets, HIT-TI5 and RINm5F cells will be studied by statically incubating isolated islets or cultured cells with the specific microtubule inhibitors colchicine and vinblastine prior to stimulating the release of insulin from each cell type with stimulatory concentrations of glucose or D-glyceraldehyde.

Similarly the role of the microfilaments in the mechanism of insulin release of lean and obese mouse islets and HIT-TI5 and RINm5F cells will be investigated

by statically incubating islets and cultured cells with the microfilament disruptor cytochalasin B prior to stimulating insulin release with stimulatory concentrations of glucose or D-glyceraldehyde.

Combined colchicine and cytochalasin B pre-treatment prior to stimulation of insulin release with stimulatory concentrations of glucose or D-glyceraldehyde will be carried out to investigate whether the microtubules or the microfilaments exert the greatest influence on glucose stimulated insulin release from lean and obese mouse islets, HIT-TI5 and RINm5F cells.

The effect of microtubule disruption on the biphasic response of lean mouse islets and HIT-TI5 cells to glucose will be carried out by perifusing both the islets and HIT-TI5 cells with stimulatory concentrations of glucose after pre-treatment with colchicine.

In order to separate the cytoskeletal proteins present in glucose stimulated islet and HIT-TI5 cells and D-glyceraldehyde stimulated RINm5F cells, each cell type will be subjected to Triton X-100 extraction followed by separation by SDS-polyacrylamide electrophoresis. Attempts will be made to identify the cytoskeletal proteins by means of their molecular weight by direct comparison with a series of commercially available protein markers of known molecular weight run simultaneously.

In the final section attempts will be made to measure the tubulin concentration in glucose stimulated

lean and obese mouse islets. Attempts will be made to examine the shift in the dynamic equilibrium between the pool of tubulin subunits (dimers) and polymerized tubulin, ie. microtubules, on the stimulation of insulin release with stimulatory concentrations of glucose. In order to measure the tubulin concentration of lean and obese mouse islets a tubulin radioimmunoassay will be developed using tubulin antiserum raised in rabbits and anti rabbit IgG. Tubulin will be isolated and purified from pig brains to provide a tubulin radioimmunoassay standard and be iodinated with ^{125}I to form ^{125}I-tubulin for use in the subsequent radioimmunoassay.

A colchicine binding assay will be developed using ^{3}H-colchicine, in addition to the tubulin radio-immunoassay, to provide comparative data for the tubulin content of lean and obese mouse islets.

CHAPTER 2

INSULIN SECRETION STUDIES WITH ISLETS AND CULTURED CELLS

GENERAL MATERIALS AND METHODS

Sources of chemicals

Reagents of analytical grade and double distilled water were used throughout. The chemicals and their sources were as follows. N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid (HEPES), D-glucose, D-glyceraldehyde, colchicine, vinblastine sulphate, cytochalasin B, dimethylsulphoxide (DMSO), glutathione (oxidised), powdered RPMI 1640, Ham's F12, Triton X-100, Guanosine triphosphate (GTP), Sodium dodecylsulphate (SDS), Tetramethylethylenediamine (TEMED), PAGE blue and the SDS Molecular Weight Markers MW-SDS-200 kit were purchased from Sigma Chemical Co. Ltd, UK.

2-mercaptoethanol, ammonium persulphate, acrylamide, acrylaide, methylene bis acrylamide (acrylamide BIS), TRIS base, Bromophenol blue, glycine glacial acetic acid, methanol, glycerol, sodium carbonate, sodium hydroxide, hydrochloric acid, sodium potassium tartrate, copper suphate (anhydrous) and Folin Ciocalteau reagent, thiomersalate and selenous acid were purchased from BDH Chemicals Ltd, UK.

Sodium metabisulphite and chloramine-T were purchased from Hopkins and Williams UK. Porcine

monocomponent insulin was purchased from Novo Research Institute, Bagsvaerd, Denmark. Worthington Collagense Class IV (145 U/mg) was purchased from Cooper Biomedical Ltd, UK. Bovine serum albumin fraction V was obtained from Miles Laboratories Ltd, UK. Human insulin standard RD13 and binding reagent RD12 were purchased from Wellcome Diagnostic Reagents Lyd, UK. $Na^{125}I$ (IMS30, 100 mCi/ml) were obtained from Amersham International, UK.

Foetal calf serum, horse serum, penicillin/ streptomycin (50 x concentrate) and Trypsin/ EDTA were obtained from Flow Laboratories Ltd, UK. All tissue culture plastic ware was purchased from either Costar, Cambridge, Mass, USA, Gibco, UK or Sterilin UK. All other reagents were purchased from Fisons Scientific Apparatus, UK.

Maintenance of the animal colony

Islets were isolated from genetically obese diabetic (ob/ob) and lean homozygous (Ho) mice from the Aston colony which has been maintained at Aston University for 20 years as an inbred stock on the C57BL/6J background. The mice were housed in an air-conditioned room at 22 ± 2°C, with a lighting schedule of 9.5 hours light (0800 - 1730) and 14.4 hours dark and allowed tap water and standard 3/8" pellets, (modified rat/mouse breeding diet cube, Heygate and Sons Ltd., Northampton), *ad libitum*.

Obese mice were selected from litters of known heterozygous monogamous pairs, which routinely generated litters consisting of 1 obese homozygous (ob/ob), 2 lean heterozygous (ob/+) and 1 lean homozygous (+/+). Lean homozygous (Ho) mice were used as the control for obese mice. All mice were used at an age of between 17 - 20 weeks, an age which corresponded to the maximum or most comprehensive expression of the obese hyperglycaemic syndrome.

Isolation of islet tissue by collagenase digestion.

Islets were isolated from the excised pancreata of lean and obese mice using a collagenase method based on that of Lacy and Kostianovsky, (166). Overnight fasted lean and obese mice aged 17 - 20 weeks were culled by cervical dislocation. The abdomen of each animal was then swabbed with alcohol prior to incision to reduce contamination from loose hairs and to minimise the risk of infection. The small intestine of each animal was displaced to the left and the spleen extended to the right to reveal the head and tail of the pancreas.

The pancreas was distended *in situ* with oxygenated Krebs-Hepes buffer, pH 7.4, containing 5.56 mmol/l glucose at room temperature, with the aid of an 18 guage hypodermic needle and a 1 ml syringe. Pancreas distension was carried out to ensure subsequent uniform collagenase activity and present the maximum tissue surface area to the enzyme. The pancreas was then excised carefully cutting away the connective tissue connecting the pancreas to the spleen, stomach and small intestine. Care was taken to remove all of the pancreas as the islets tended to be located in greater numbers at the head of the pancreas close to the major blood vessels rather than the tail. The pancreas was then washed in fresh buffer containing 5.56 mmol/l glucose and the blood vessels and fat were carefully removed with fine scissors. Washed pancreata were pooled in a small

ceramic crucible containing 10 ml of fresh Krebs-Hepes buffer, pH 7.4 supplemented with glucose (5.56mmol/l) and minced finely with round ended scissors. Droplets of fat which floated to the surface of the buffer were removed with a pasteur pipette. The mincing process was repeated several times, especially with obese mouse pancreata to remove as much fat as possibe and reduce the pancreas to 1 mm pieces.

This finely minced pancreas was then transferred to a screw topped flask containing Worthington Class IV collagenase, (4-5 mg per pancreas), and the reaction volume was made up to 15 ml with fresh Krebs-Hepes buffer, pH 7.4 containing 5.56mmol/l glucose. The flask was then immersed in a water bath at 37°C and shaken vigorously for 20 minutes at 100 cp/s. A further 5 - 10 ml of buffer was added at the end of the incubation period to rinse the homogenate off the side walls of the flask and shaking was continued for a further 5 - 10 minutes until sedimented islets could be observed completely free of acinar tissue at the bottom of the bottle. At this stage the digestion was assumed to be complete.

The homogenate was then transferred to a 10 ml centrifuge tube (Sterilin) and spun at 1,000 rpm for 4 minutes in a M.S.E. Chilspin centrifuge. The supernatant containing collagenase and digested acinar tissue was aspirated off and fresh buffer added. The pellet containing the islets was resuspended, washed and

recentrifuged. The supernatant was again removed to ensure complete removal of collagenase and the last remnants of acinar tissue.

The pellet was resuspended in fresh Krebs-hepes buffer with 5.56 mmol/l glucose and the contents of the tube poured out into a shallow petri dish. Islets were then picked out under a stereomicroscope with a drawn out pasteur pipette. Frequently 'clumping' occurred between the islets and the remnants of exocrine tissue fragments, (capillaries and connective tissue fibres) and to overcome this, 0.5 ml of DNAase (20 µg/ml) was added to the dish and the homogenate agitated to disaggregate the contents. The islets were then transferred to a black watch glass containing fresh Krebs -Hepes buffer containing 5.56 mmol/l glucose. The islets were then used in subsequent experimental work.

50 to 100 islets per pancreas could be isolated by this procedure from a single lean mouse pancreas while 100 to 150 islets could be obtained from each obese mouse pancreas depending on the age of the animal used. The islets of obese mice were 3 to 4 times larger than those of the lean mice but in older obese mice the islets were more fragile and translucent areas could be identified at their centres. Very large islets with translucent centres were discarded.

Maintenance of HIT-TI5 and RINm5F cells in culture

HIT-TI5 cells, an SV40 virus transformed cloned hamster β-cell line, passage 47, were a kind gift from Dr R Santerre of Eli Lilly Ltd, (160). The cells were derived from Syrian Hamster islets, which had been isolated using the collagenase technique of Lacy and Kostianovsky, (166) and separated by equilibrium sedimentation in metrizamide gradients. Isolated islets were dissociated to a single cell suspension by digestion in trypsin/EDTA and monolayers were maintained in Ham's F12 culture medium containing horse serum (15%), foetal calf serum (2.5%), penicillin (100 IU/ml), streptomyocin (100 μg/ml), selenous acid (0.1 μmol/l) and glutathione (10 μg/ml in standard 250 ml culture flasks, (Costar, Mass, USA).

Unscheduled DNA synthesis was induced in four day cultures with methanesulphonate (500 μg/ml) and afterwards the cells were resuspended with Simian Virus 40 and incubated for 1 hour at 37°C. During this period the SV-40 tumour antigen, (T-antigen), that is the part of the viral DNA which confers the rapid mulitplication of the virus, is inserted into the hamster β-cells enabling them to multiply for many generations in culture. After several washings to remove free SV-40 virus, the islet cells were resuspended in culture medium containing 0.5% Seaplaque agar, overlaid with Ham's F12 culture medium and maintained in a 5% CO_2 and humidified

air incubator until colonies of transformed cells could be identified by the eighth week. The cells were aneuploid with a modal chromosome number of 69. Light and electron microscopy show HIT-TI5 cells to contain a modest number of membrane bound secretory granules which are similar to mature secretory granules in normal hamster β-cells. The insulin content was found to be 0.3 - 2.5 μg/mg protein compared with 56.3 μg/mg protein of hamster islets, (160).

RINm5F cells, passage 90, were kindly donated by Dr S.J. Ashcroft of the John Radcliffe Hospital, Oxford. RIN cells were derived originally from an islet cell tumour induced by high-dose irradiation of an inbred NEDH rat, (161). The tumour was maintained by serial transplantation in NEDH rats and on reaching the tenth transplant the tumour was successfully hetero-transplanted into athymic nude mice with the BALB/c background, (162). Two cell lines were then derived, - one from the original rat transplants RIN-r and the other from the hetero-transplant into the nude mice RIN-m. The cell line RINm5F is the fifth clone of the original RIN-m cells. There were also several other sublines derived which were related to RINm5F but their insulin producing capacity fluctuated in time with culture, (199,200). The RINm5F cells are capable of producing a constant level of immunoreactive insulin over more than a year in culture but the cellular content of immunoreactive insulin is

only 19 pg/cell, which is only 1% of that normally found in native rat β-cells, (167).

Stock cultures of RINm5F cells were maintained in re-constituted powdered RPMI 1640 medium supplemented with 10% foetal calf serum, in 250 ml costar flasks.

Both the HIT-TI5 and RINm5F cells were maintained at 37°C in an atmosphere of 5% CO_2 in humidified air in a LTE Qualitemp 80 MI CO_2 incubator. The costar flasks were seeded initially with 5 x 10^6 HIT-TI5 cells or 2 x 10^6 RINm5F cells. At regular 3 day intervals when the cells were firmly attached to the bottom of the flask the spent media was aspirated off with a pasteur pipette and replaced with fresh medium. Care was taken to maintain aseptic conditions at all times.

HIT-TI5 cell cultures had an epithelioid appearance and formed large multi-layered islet-like structures without reaching confluence. The doubling time during log phase growth varied between 20-24 hours and the plating efficiency was 85 ± 2%, (160,164). The RINm5F cells also had an epithelioid appearance but the doubling time was 70-80 hours and the plating efficiency 76 ± 4%, (164).

Once per week the cultured β-cells were removed from each flask and split either to set up new cultures or to carry out static incubation experiments in 24 well clusterplates, (Costar). After the removal of the spent culture medium, 3 ml of trypsin/EDTA at 37°C was added to each flask and quickly washed over the surface of the

cultured β-cells for 2-3 seconds to remove dead or loosely attached cells. The solution containing the dead and damaged cells was apirated off and the remaining healthy cells were detached by exposure to a further 2-3 ml of trypsin/EDTA for 6-7 minutes at 37°C. After the trypsinisation, gentle rocking of the flask was used to confirm that the cells had separated from the flask surface and were present in suspension. Fresh medium at 37°C was added to the flask to dilute the trypsin/EDTA solution, increasing the contained fluid/cell volume to approximately 10 ml. The flask contents was then poured into sterile centrifuge tubes, (Sterilin), sealed and centrifuged at 1,000 rpm for 3 minutes in a MSE Chilspin centrifuge. The supernatant medium containing trypsin/EDTA was then aspirated off and the cell pellet carefully resuspended in 2-3 ml of fresh medium. Care was taken to ensure that all the supernatant had been removed to prevent residual trypsin causing further damage to the β-cells.

The cell number was estimated with the aid of a haemocytometer and 5 x 10^5 HIT-TI5 cells or 2 x 10^5 RINm5F cells were seeded into new 250 ml Costar flasks containing 20 ml of fresh medium, either Ham's F12 or RPMI 1640 repectively. For static incubation experiments 2 x 10^5 cells were transferred directly into each well of a costar 24 well clusterplate, each well containing 1 ml of fresh medium, either Ham's F12 or RPMI 1640.

Methods for the static incubation of isolated lean and obese mouse islets and cultured β-cells.

(i) Static incubation of lean and obese mouse islets in a range of glucose concentrations.

Islets of Langerhans were isolated from the excised pancreatata of overnight fasted lean and genetically obese diabetic ob/ob mice using collagenase digestion, page 84 (166). Islets were maintained in oxygenated Krebs-Hepes buffer, pH 7.4 containing 5.56 mmol/l glucose at room temperature. Groups of 8-10 islets were transferred to each well of a 24 well clusterplate (Costar), containing 1 ml of Krebs-Hepes buffer pH 7.4, supplemented with 5.56 mmol/l glucose at 37°C. After replacing the lid the whole plate was pre-incubated at 37°C for 30 minutes in an atmosphere of 95% air and 5% CO_2. The 30 minute pre-incubation in 5.56 mmol/l glucose was used to simulate the normal blood glucose level *in vivo* and produce a base line release of insulin release from isolated mouse islets.

After the pre-incubation period the culture medium was removed very carefully with a pasteur pipette and replaced with 1 ml of test medium consisting of fresh oxygenated Krebs-Hepes buffer pH 7.4 at 37°C containing glucose at concentrations of between 0 to 28 mmol/l. The multiwell clusterplates were then returned to the incubator for a further 60 minutes test incubation.

At the end of the test incubation period the supernatant from each well was transferred to an LP3 tube and centrifuged at 1,000 rpm for 3 minutes in an MSE Chilspin centrifuge to separate any free islets or cell fragments. 0.5 ml of supernatant was removed and frozen for insulin assay as described on page 101 . Insulin release was expressed as μU insulin/60 minutes/islet.

(ii) Static incubation of HIT-TI5 cells in a range of glucose concentrations.

2 x 10^5 HIT-TI5 cells from freshly trypsinised stocks were transferred to each well of a 24 well Costar clusterplate containing 1 ml of supplementd Ham's F12 culture medium. The plates were incubated at 37°C in 5% CO_2/air for 48 hours to allow adhesion and proliferation. Afterwards the media was replaced with 1 ml of fresh Krebs-Hepes buffer pH 7.4 without glucose. Glucose was omitted from the pre-incubation medium in order to induce a greater insulin response, (the optimum insulin release from HIT-TI5 cells is thought to be stimulated by about 7.5 mmol/l glucose), (184). After replacing the lid the whole apparatus was pre-incubated for 30 minutes at 37°C in an atmosphere of 95% air and 5% CO_2.

After the pre-incubation period the cultures were then washed in Krebs-Hepes buffer, pH 7.4, containing BSA -V 0.2 g/l and subsequently incubated for 90 minutes in 1 ml of fresh buffer containing glucose (0 - 28 mmol/l). At the end of the test incubation period the supernatant

was removed from each well, transferred to an LP3 tube and centrifuged at 1,000 rpm for 3 minutes. 0.5 ml of the mildly agitated supernatant was removed and frozen for insulin assay. In order to determine the mean number of cells present in each well during the experiment, a wells/plate were trypsinised and enumerated and the value was used for the quantification of insulin release, in terms of μU insulin/10^5 cells/90 minutes.

(iii) Static incubation of RINm5F cells in a range of D-glyceraldehyde concentrations.

3 x 10^5RINm5F cells were cultured for 48 hours in wells of a multiwell clusterplate, each well containing 1 ml of RPMI 1640 medium. When the cells had adhered to the bottom of the wells the medium was replaced with 1 ml of oxygenated Krebs-Hepes buffer pH 7.4 without glucose and pre-incubated for 30 minutes.

After the 30 minute pre-incubation period the medium was removed from each well and substituted with 1 ml of test medium consisting of Krebs-Hepes buffer pH 7.4 containing a range of D-glyceraldehyde concentrations from 0 to 20 mmol/l. The cells were then test incubated for a further 90 minutes.

At the end of the test incubation period the supernatant from each well was transferred to an LP3 tube and centrifuged at 1,000 rpm for 3 minutes and a 0.5 ml aliquot of supernatant was removed and frozen for insulin assay. The RINm5F cell number was confirmed at the end

of each experiment by trypsining several wells to remove cells and then counting with the aid of a haemocytometer. Insulin release was expressed as $\mu U/10^5$ cells/90 minutes.

(iv) Static incubation of lean and obese mouse islets in the presence of colchicine, vinblastine and cytochalasin B.

Islets of Langerhans were isolated from the pancreata of lean and genetically obese diabetic (ob/ob) mice using collagenase digestion as described earlier, page 84 . Groups of 8-10 islets were transferred to each well of a 24 well clusterplate (Costar), containing 1 ml of oxygenated Krebs-Hepes buffer pH 7.4 supplemented with 5.56 mmol/l glucose and either colchicine, (1 μmol/l, 0.1 mmol/l and 10 mmol/l), vinblastine (1,10 and 100 μmol/l), or cytochalasin B, (10 μg/ml) at 37°C. Colchicine and cytochalasin B were also used in combination, (10 mmol/l and 10 μg/ml respectively), to establish which effect on glucose stimulated insulin release predominated.

The plate was pre-incubated for 2 hours at 37°C in an atmosphere of 95% air and 5% CO_2. This period of pre-incubation period was thought necessary to ensure complete bindng of the cytotoxic drugs, particularly the binding of colchicine to tubulin dimers which has been reported to take 2 hours to reach saturation, (103).

Control and basal release islets were pre-incubated for 2 hours in Krebs-Hepes buffer pH 7.4 containing 5.56

mmol/l glucose alone to establish the normal insulin response of islets over this period of pre-incubation.

After the pre-incubation period the medium was removed from the control and drug treated wells with a pasteur pipette and replaced with 1 ml of fresh oxygenated Krebs-Hepes buffer pH 7.4 at 37°C containing stimulatory glucose (16.7 mmol/l), except for the basal release wells which received fresh buffer with 5.56 mmol/l glucose. The multiwell clusterplate was then returned to the incubator for a further 30 or 90 minutes test incubation.

At the end of the 30 or 90 minute test incubation the medium from each well was transferred to an LP3 tube and centrifuged at 1,000 rpm for 3 minutes in an MSE Chilspin centrifuge to separate any free islets or cell fragments. 0.5 ml of supernatant was removed and frozen for insulin assay, page 101. Insulin release was expressed as μU insulin/islet/30 or 90 minutes.

(v) Static incubation of HIT-TI5 cells in the presence of colchicine vinblastine and cytochalasin B.

2 x 10^5 HIT-TI5 cells were cultured for 48 hours in wells of a 24 well clusterplate, each well containing 1 ml of supplemented Ham's F12 culture medium. When the cells had adhered to the bottom of each well the media was replaced with 1 ml of fresh oxygenated Krebs-Hepes buffer pH 7.4 without glucose but containing either colchicine, (1 μmol/l, 0.1 mmol/l and 10 mmol/l),

vinblastine (1,10 and 100 μmol/l), cytochalasin B (10 μg/ml) or colchicine and cytochalasin B together, (10 mmol/l and 10μg respectively). Control and basal release cells were cultured like the test cells but the culture medium was replaced with 1 ml of Krebs-Hepes buffer pH 7.4 without glucose or any of the cytotoxic drugs. After replacing the lid the the drug treated cells, the controls and the basal release cells were pre-incubated for 2 hours at 37°C in an atmosphere of 95% air and 5% CO_2.

After the pre-incubation period the medium from the control and drug treated wells was removed and replaced with 1 ml of fresh oxygenated Krebs-Hepes buffer pH 7.4 containing a stimulatory glucose concentration of (7.5 mmol/l) except for the basal release cells which received Krebs-Hepes buffer without glucose. The multiwell clusterplate was then returned to the incubator for a further test incubation period of either 30 or 90 minutes at 37°C.

At the end of the 30 or 90 minute test incubation periods the medium was removed from each well, transferred to an LP3 tube and centrifuged at 1,000 rpm for 3 minutes. 0.5 ml of the supernatant was removed and frozen for insulin asssay. The HIT-TI5 cell number was confirmed at the end of the experiment by trypsinising several wells and counting the cells with the aid of a haemocytometer. Insulin release was expressed as μU insulin/1 x 10^5cells/30 or 90 minutes.

(vi) Static incubation of RINm5F cells in the presence of colchicine and cytochalasin B.

3 x 10^5 RINm5F cells were cultured for 48 hours in wells of a multiwell clusterplate, each well containing 1 ml of RPMI 1640 medium. When the cells had adhered to the bottom of the wells the medium was replaced with 1 ml of oxygenated Krebs-Hepes buffer pH 7.4 at 37°C without D-glyceraldehyde but containing either colchicine (10 mmol/l), or cytochalasin B (10 µg/ml) or colchicine and cytochalasin B together (10 mmol/l and 10 µg/ml). Control and basal release cells were cultured like the test cells but the culture medium was replaced with 1 ml of Krebs-Hepes buffer pH 7.4 without D-glyceraldehyde or any of the cytotoxic drugs. After replacing the lid the plate was pre-incubated for 2 hours at 37°C in an atmosphere of 95% air and 5% CO_2.

After the pre-incubation period the medium was removed and replaced with 1 ml of fresh oxygenated Krebs-Hepes buffer pH 7.4 containing stimulatory D-glyceraldehyde (10 mmol/l) except for the basal cells which received Krebs-Hepes buffer without D-glyceraldehyde. The multiwell clusterplate was then returned to the incubator for a further test incubation period of 30 or 90 minutes at 37°C.

At the end of the 30 or 90 minute test incubation periods the medium was removed from each well, transferred to an LP3 tube and centrifuged at 1,000 rpm for 3 minutes. 0.5 ml of the supernatant was removed and

frozen for insulin assay and the RInm5F cell number was enumerated at the end of the experiment by trypsinising several wells and counting the cells with the aid of a haemocytometer. Insulin release was expressed as $\mu U/10^5$ cells/30 or 90 minutes.

Method for the perifusion of isolated lean mouse islets and HIT-TI5 cells.

A double-chamber technique was used to determine the effect of colchicine (1mmol/l) on glucose stimulated insulin release from both isolated lean mouse islets and HIT-TI5 cells, the second chamber acting as a control.

(i) Perifusion of lean mouse islets.

Islets of Langerhans were isolated from the excised pancreata of lean mice using collagenase digestion, page 84 (166). Islets were maintained in oxygenated in Krebs-Hepes buffer pH 7.4 containing 5.56 mmol/l glucose at room temperature. 20 islets were dropped with the aid of a pasteur pipette onto the surface of a 5μm millipore filter supported by a Sericol plastic mesh inside a 25 mm swinnex filter chamber (Millipore UK). The chamber was closed and the islets pre-perifused with either Krebs-Hepes buffer pH 7.4 containing 5.56mmol/l glucose alone or Krebs-Hepes buffer containing 5.56 mmol/l glucose and colchicine (1 mmol/l) pH 7.4 for 90 minutes at 37°C.

This pre-treatment was carried out to ensure binding of colchicine to the microtubules and to determine the baseline level of insulin release.

The islets in both chambers were then test perifused for 90 minutes with Kres-Hepes buffer pH 7.4 containing a stimulatory concentration of glucose (16.7 mmol/l).

The perifusion experiments were carried out in a room at 37°C using a system which consisted of a Gilson minipuls 2 pump (Anachem Ltd, UK) and two LKB 2212 helirac fraction collectors fitted with number 1 cassettes (LKB Instruments, Bromma, Sweden). 1 ml fractions were collected throughout in LP4 tubes. The perifusion system had a dead volume of 2.5 ml, which at a flow rate of 1 ml/minute gave a response time of 2.5 minutes. Insulin release was measured by radio-immunoassay page [101] and expressed as μU/islet/minute.

(ii) Perifusion of HIT-TI5 cells.

5 x 10^5 HIT-TI5 cells were seeded into individual wells of a multiwell clusterplate each containing a 15 mm round Thermanox coverslip (Miles Laboratories Ltd, UK.) and incubated at 37°C in 95% air 5% CO_2 for 48 hours to allow adhesion and proliferation.

At the end of this period the coverslips carrying 3 x 10^5 cells were transferred to 25 mm swinnex filter chambers (Millipore Ltd, UK.). The chambers were then sealed and filled with either Krebs-Hepes buffer pH 7.4

for the controls or Krebs-Hepes buffer pH 7.4 containing colchicine (1 mmol/l), in the absence of glucose at 37°C.

The HIT-TI5 cells were pre-perifused for 90 minutes with Krebs-Hepes buffer pH 7.4 at 37°C with or without colchicine (1 mmol/l) in the absence of glucose, in order to establish a baseline level of insulin release. The cells were then test perifused for 90 minutes with Krebs-Hepes buffer containing a stimulatory concentration of glucose (7.5 mmol/l).

The perifusion system was identical to that used for isolated islets and consisted of a Gilson minipuls 2 pump (Anachem Ltd, UK) and two LKB 2212 helirac fraction collectors fitted with number 1 cassettes (LKB Instruments, Bromma, Sweden). 1 ml fractions were collected throughout in LP4 tubes. The perifusion system had a dead volume of 2.5 ml, which at a flow rate of 1 ml/minute gave a response time of 2.5 minutes. Insulin release was measured by insulin radioimmunoassay page 101 and expressed as μU/plate/minute. The cell number used in each experiment was enumerated by trypsinising four wells in every clusterplate and counting the cells with the aid of a haemocytometer. Insulin release was expressed as μU insulin/minute/1 x 10^5 cells.

In perifusion experiments statistical significance of the difference between secretory profiles was computed using the areas under the curves.

THE RADIOIMMUNOASSAY OF INSULIN

Radioimmunoassay of a peptide antigen, for example insulin or tubulin is based upon competition between labelled and unlabelled antigen (Ag), for bindng sites on an appropriate specific antibody (Ab), (171). Consequently, the more unlabelled antigen present in the sample, the less labelled antigen will bind with the antibody and there will be a subsequent drop in radioactivity associated with the antigen - antibody complex, (171). The concentration of unlabelled antigen in the original sample can be estimated by comparing the bound radioactivity in the antigen - antibody complex with that produced by standard solutions of known concentrations of unlabelled antigen, (201).

The double antibody radioimmunoassay method makes use of a second antibody to separate the complex of antigen and antibody from the unbound antigen free in the reaction mixture. With the addition of a second antibody directed against the first, a larger, heavier complex is formed which can be separated from the free antigen by centrifugation.

The insulin antibody or commercially available binding reagent (RD12) consists of a single pre-precipitated antibody prepared in bulk and is re-constituted with double distilled water when required. Using this 'Binding Reagent' it was possible to use the precipitation technique first described by Quabbe (202)

as a modification of the original Hales and Randle double antibody technique (201), in which the precipitated antigen - antibody complex was separated from the free labelled antigen by centrifugation and aspiration of the supernatant.

RADIOIMMUNOASSAY REAGENTS

a) Insulin Binding Reagent

The insulin binding reagent was reconstituted with 8 ml of de-ioniseed water to give a mixture of guinea pig anti insulin serum and rabbit anti guinea pig globulin serum in a buffer of sodium phosphate - 0.04 mol/l, EDTA - 0.02 mol/l, sodium azide - 0.1%, and bovine serum albumin - 0.5%, pH 7.4. After reconstitution the binding agent was stored at 4°C for a week or longer at -20°C without significant loss of activity.

b) Radio-iodinated Insulin (^{125}I-Insulin)

Crystalline porcine insulin was iodinated using the Chloramine-T Method as described on page 174 using Na^{125}I (1.149 mCI Na^{125}I, 3 days after synthesis). The iodinations were carried out in the standard conical reaction vial provided. The composition of the reagents used in the iodination are given in Appendix 3.

100 µl of 0.5 mol/l phosphate buffer, pH 7.4, 11.49 µl of porcine insulin (0.25 mg/ml) and 22.98 µl of chloramine-T (0.25 mg/ml), were added to the reaction vial which was then promptly sealed and inverted once. After 20 seconds reaction time 20 µl of sodium metabisulphite (0.5 mg/ml), was added to the reaction vial to stop the iodination reaction. After a further 15 seconds stabilization time 200 µl of 0.05 mol/l phosphate buffer, pH 7.4 containing 2.5% BSA was added. As described in the tubulin iodination for the tubulin radioimmunoassay, page 180 , the timing of the iodination reaction is crucial with regards to damage induced by over-exposure of the insulin or tubulin to chloramine-T, (182).

The labelled insulin was separated from the unreacted iodine and damaged insulin fragments by gel filtration on Sephadex G-50 fine. The column was purged with 8 ml of column primer to remove the material remaining from the previous iodination and to prevent the adsorption of freshly generated labelled insulin onto the column surface itself. A 10 µl aliquot was removed from the reaction vial at the end of the reaction for the assessment of the percentage of ^{125}I incorporated into insulin. The remaining reaction mixture was then transferred to the column and eluted at a flow rate of approximately 1 ml/minute. Sixty, one-minute fractions were collected in LP3 tubes on a Serva linear 2 fraction

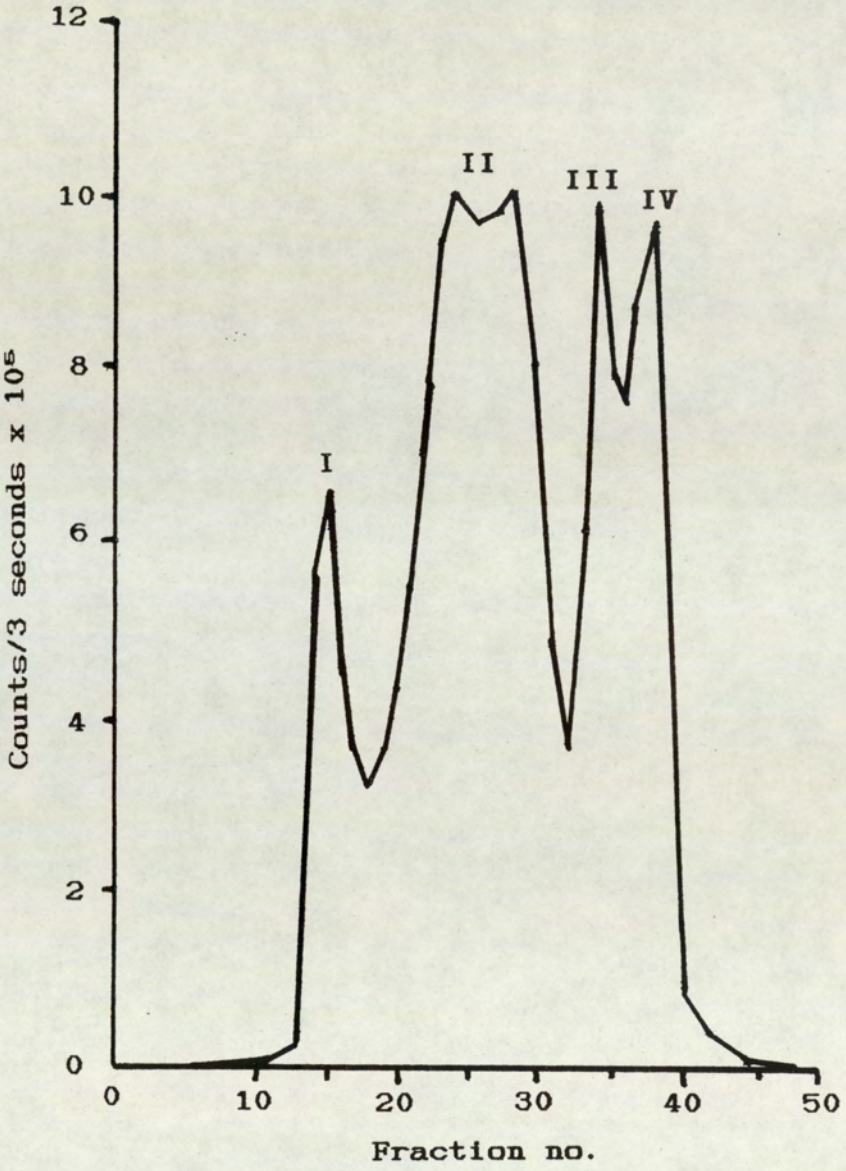
collector and each counted for 3 seconds using a gamma counter (ICN, Tracerlab).

Four well defined peaks could be identified in the elution profile, Figure 2.1. Peak I, (fractions 14-18) represented the high molecular weight fraction probably containing insulin aggregates, (201). Peak II, (fractions 19-30), contained monoiodinated insulin and demonstrated maximum activity associated with insulin. Peak III, (fractions 31-36) represented the free unreacted ^{125}I mixed with small damaged insulin fragments. Peak IV, (fractions 37-40), contained free unreacted ^{125}.

The specific activity, percentage incorporation of ^{125}I into insulin and the percentage iodination damage were calculated using trichloroacetic acid (TCA) precipitation of a 10 µl aliquot removed from the reaction vial at the end of the iodination reaction, as described in Appendix 3, page 267.

A typical iodination generated about 40 tubes each containing 50 µl aliquots of ^{125}I-insulin (≈2-3 µCi/tube), with a specific activity of 250.83 ± 14.99, µi/µg, n=6, a typical percentage of undamaged insulin of 94.35 ± 1.22 % and a typical percentage incorporation of ^{125}I into insulin of 62.85 ± 3.76%.

Figure 2.1 Typical elution profile of iodinated fractions separated on Sephadex G50 fine from the chloramine-T iodination of monocomponent porcine insulin



c) Preparation of Insulin Standard Solutions

Human insulin standard (RD 10), was reconstituted from its freeze dried form with radioimmunoassay buffer to yield an insulin concentration of 1 m unit/ml. Stocks consisting of 200 µl aliquots of this solution were frozen at -20°C in LP3 tubes. On the day of the assay, one 200 µl aliquot was thawed and diluted with 0.8 ml of radioimmunoassay buffer, (sodium phosphate 0.04 mol/l containing sodium chloride 0.9%, BSA 0.5% and sodium azide 0.1%, pH 7.4), to yield an insulin concentration of 200 µU/ml. Serial dilution of this stock was carried out to give a range of standard insulin concentrations of 6.25 to 200 µU/ml, Figure 2.2.

Assay Procedure

The sequence of reagent additions in the assay procedure is summarized in Table 2.1. 50 µl of each standard insulin concentration was transferred to an LP3 tube in triplicate. A 50 µl aliquot of radioimmunoassay buffer was added to three other LP3 tubes designated 'blanks'. These tubes represented the amount of radioactivity remaining on the walls of the radioimmunoassay tubes after washing. The unknown samples were assayed in duplicate in 50 µl aliquots as shown in Table 2.1.

Figure 2.2 The serial dilution of the insulin standard for the radioimmunoassay of insulin.

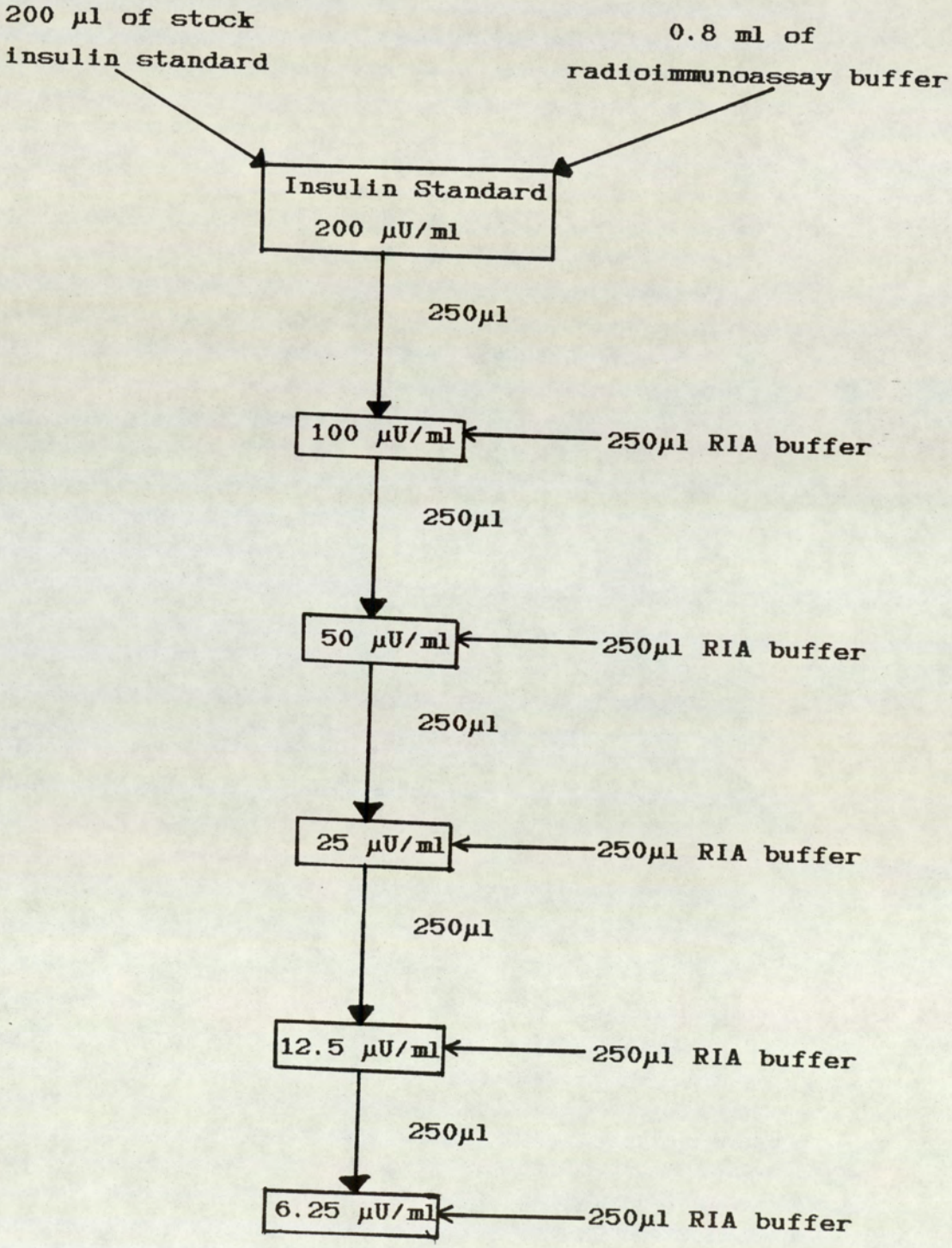


Table 2.1 Protocol for the addition of reagents in the insulin radioimmunoassay

TUBE NO.	INITIAL REACTANT (50 µl)	BINDNG REAGENT (50 µl)	^{125}I-INSULIN (50 µl)	BUFFER WASH (0.5ml)
Totals 1-3	-	-	+	-
Blanks 4-6	Buffer	Buffer	+	+
Insulin 7-9	6.25 µU/ml	+	+	+
Standards 10-12	12.5 µU/ml	+	+	+
13-15	25.0 µU/ml	+	+	+
16-18	50.0 µU/ml	+	+	+
19-21	100 µU/ml	+	+	+
22-24	200 µU/ml	+	+	+
Samples 25-26	+	+	+	+
27-28	+	+	+	+
29-30	+	+	+	+
31-				

50 µl of reconstituted binding reagent was added to all of the tubes except the blanks which each received a further 50 µl of radioimmunoassay buffer. The contents of each tube was then mixed and the rack of tubes covered with cling film and placed in the refrigerator at 4°C for a minimum of 4 hours. During this initial incubation period the ^{125}I-insulin was diluted to give a final activity of 11,000 cpm per 50 µl with a Specific Activity of 50 µCi/µg. At the end of the 4 hour incubation period, 50 µl of ^{125}I-insulin was added to all tubes and to three empty tubes marked 'Totals', Table 2.1. The latter represented the total amount of radioactive ^{125}I-insulin added to each tube. All the tubes were then vortexed vigorously and returned to the refrigerator for a further 18 hours incubation.

After incubation, 0.5 ml of radioimmunoassay buffer was added to each tube except the 'Totals' and the contents were mixed and centrifuged at 3,000 rpm for 30 minutes in a MSE Bench Centrifuge. The addition of buffer served to wash unbound ^{125}I-insulin from the walls of the tube. The contents of each tube (except the 'Totals'), was decanted and the last drop aspirated away with a pasteur pipette. Complete removal of the supernatant was essential as the presence of counts associated with unbound ^{125}I-insulin could cause a dilution of the insulin content of the samples when reference is made to the standard curve. The tubes were finally allowed to dry inverted over tissue paper for 1 -

2 hours and then counted for 3 minutes in the LKB Wallac Compugamma Counter.

The construction of standard curves and the computation of results was performed using a radio-immunoassay soft ware package asociated with the gamma counter. Log_{10} of the standard insulin concentration was plotted against counts per minute in the bound fraction, (spline plot), and the sample unknown insulin concentration was determined automatically by direct extrapolation from the graph. A typical standard curve is illustrated in Figure 2.3.

Assays were considered to be satisfactory if,

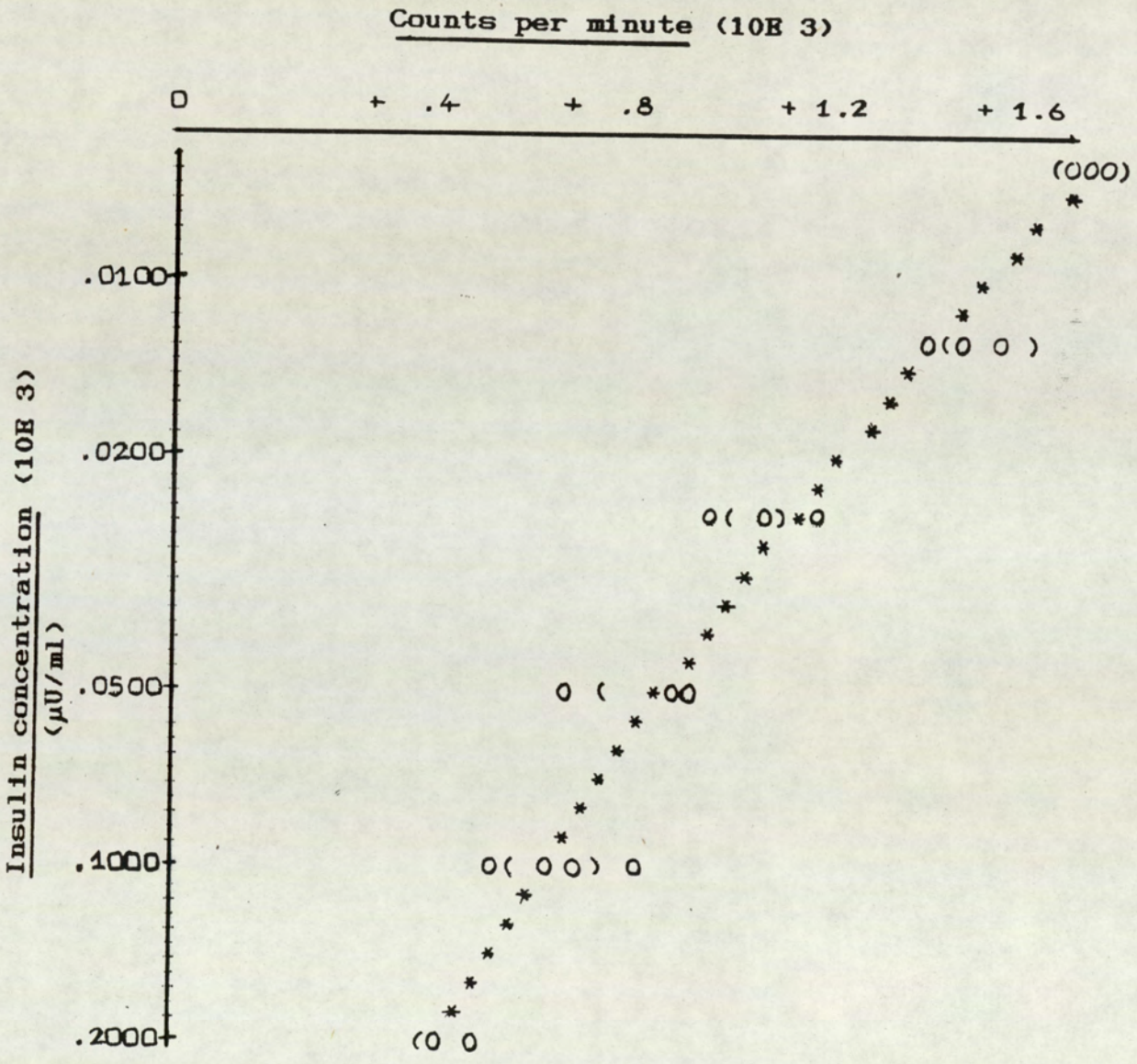
(a) there was good agreement between replicates,

(b) the blank value was less than 0 - 8% of the 'Total' counts

(c) the fitting factor F was less than 0.250, Figure 2.3.

The insulin radioimmunoassay had an intra- and interassay coefficient of variation of 4.95 and 9.37% respectively.

Figure 2.3 Typical standard curve for the insulin radioimmunoassay.



METHOD FOR THE SEPARATION OF CYTOSKELETAL PROTEINS FROM THE ISLETS OF LEAN AND OBESE MICE AND CULTURED HIT-TI5 AND RINm5F CELLS BY SDS-POLYACRYLAMIDE ELECTROPHORESIS.

Electrophoresis is a useful technique for the characterization of proteins and involves a dynamic separation of proteins according to electric charge at a particular pH and according to size if the medium has a sieving effect, (203). The procedure is rapid and requires only microgram quantities of protein, (204).

Cytoskeletal proteins were extracted from both lean and obese mouse islets HIT-TI5 and RINm5F cells prior to electrophoresis using the non-ionic detergent Triton X-100, (205). Triton X-100 extraction has been used to define the cytoskeletal proteins in cells in terms of the inter-connected proteins that remain after a cell has been treated with the detergent, (206). To date non-ionic detergents such as Triton X-100 have been comprehensively used in the extraction of proteins from a variety of cells and cell membranes such as HeLa cells and human erythrocyte membranes, (206-210). Triton X-100 has the disadvantage of also solubilising the nuclear proteins in the isolated islets, HIT-TI5 and RINm5F cells. The effects the presence of these nuclear proteins have on the separation of cytoskeletal proteins is uncertain.

After extraction the samples of islet, HIT-TI5 or RINm5F cell proteins were prepared for electrophoresis by

heating them to 100°C in a buffer containing mercaptoethanol and sodium dodecyl sulphate, (SDS). The heating process causes denaturation and the mercaptoethanol reduces the constituent disulphide bonds allowing the polypeptide chains to unfold, (211). Subsequent binding of sodium dodecylsulphate (SDS) to the poylpeptides dissociates the protein into subunits and completely unfolds each polypeptide to form long rod-like SDS-polypeptide complexes. The polypeptide chains in these complexes become coated with a layer of SDS molecules in such a way that their hydrocarbon chains are in tight association with the polypeptide chain and the charged sulphate groups of the detergent are exposed to the aqueous medium, (212). Hence mercaptoethanol and heat denaturation facilitate the optimum binding of SDS to the protein thereby increasing its electrophoretic mobility, (213-215).

The molecular weights of the constitutent SDS proteins are determined by comparing their electrophoretic mobilities on SDS-acrylamide gels to the mobiliies of well characterized marker proteins of known molecular weight, (205). The marker proteins used in this study were combined in a high molecular weight lyophilised mixture and contained carbonic anhydrase (from bovine erythrocytes) (29.0 KDa), egg albumin (45.0 KDa), bovine albumin (66.0 KDa), phosphorylase b (from rabbit muscle) (97.4 KDa), β-Galactosidase (from

Escherichia Coli) (116.0 KDa) and myosin (from rabbit muscle) (205.0 KDa).

Preparation of the separating gel.

The separating gel is the gel in which the mixture of SDS-proteins extracted from islets or cultured cells are separated from one another according to their molecular weight. The directions for making up the stock solutions prior to making up the gel are given in Appendix 4. Before making the gel two detergent washed glass plates were clamped vertical and level and held 0.75 mm apart using a rubber dam.

A 10% acrylamide/acrylaide gel was prepared by adding 5 ml of 1.5 mol/l Tris, pH 8.8, 0.2 ml of 10% sodium dodecyl sulphate (SDS), and 6.25 ml of a 32% acrylamide/acrylaide stock solution to 8.45 ml of water. The whole mixture was then stirred vigorously with a magnetic stirrer. To the continuously stirred mixture was added 100 µl of freshly prepared ammonium persulphate (0.1 g/ml), followed by 7.5 µl of TEMED, (tetramethylethylene-diamine). It was important to add the ammonium persulphate and TEMED last and to make sure that the gel was thorougly mixed because when these two are combined the release of free oxygen radicles from the persulphate reduces sufficient monomer to the free radicle state to initiates polymerization. In the absence of excess oxygen the reaction continues until

gelation is complete, (203). The final concentrations in the components of the separating gel are summarized in Table 2.2.

The gel was dispensed between the two glass plates to a height of approximately 12 cm with the aid of a piece of fine polythene tubing attached to a hypodermic needle on the end of a 20 ml syringe, Extreme caution was exercised during the preparation and pouring of the gel because acrylamide is known to be a potent neurotoxin, (216). An outer covering layer of 50% methanol/water was added to prevent the air gel interface from drying and cracking. The upper edges of the glass plate assembly were then covered with parafilm and the whole left to stand for 2-3 hours at room temperature until the gel had set. A small amount of spare gel was taken up into a pasteur pipette and this was used to monitor the gelling time.

Preparation of the stacking gel

The stacking gel is the gel in which the SDS-proteins are 'stacked' or concentrated in a specific position before they enter the separating gel. The stacking gel was prepared by mixing 2.5 ml of 0.5 mol/l Tris pH 6.8, with 100 µl of 10% SDS, 6.35 ml of water and 1 ml of 30% acrylamide containig 0.8% BIS (methylenebis-acrylamide). These components were mixed thoroughly prior to the addition of 50 µl of fresh ammonium

Table 2.2. Composition of the separating and stacking gels.

	Separating gel	Stacking gel
Tris base, pH 8.8	0.375 mol/l	-
Tris base, pH 6.8	-	0.125 mol/l
SDS	0.1 %	0.1 %
Acrylamide/Acrylaide	10 %	-
Acrylamide/BIS	-	3.0 %
Ammonium persulphate	0.5 mg/ml	0.5 mg/ml
TEMED	7.5 µl	5.0 µl
Gelling time	2-3 hours	1-2 hours

persulphate (0.1 g/ml) and 5 µl TEMED (tetramethyl-ethylene-diamine). The concentration of BIS determined the number of cross linkages and pore size of the gel. Increasing the BIS concentration increases the number of cross linkages and reduces the pore size. A low BIS concentration of 3%, Table 2.2, produces a gel with a relatively large pore size and the ability to concentrate the protein samples prior to separation.

The methanol/water overlay on the surface of the separating gel was aspirated off very carefully and the stacking gel was applied with a 20 ml syringe and a hypodermic needle connected to a fine piece of polythene tubing. The stacking gel was evenly distributed above the separating gel within a few mm of the top of the glass plates. A plastic comb was carefully inserted into the top of the stacking gel avoiding the creation of air bubbles. The stacking gel was then covered with a layer of 50% methanol/water and allowed to gel at room temperature.

After 1-2 hours the methanol/water layer was aspirated off and the comb carefully removed. The indentations made by the teeth of the comb in the gel formed the sample wells. Approximately 0.5 ml of a solution of 0.125 mol/l Tris and 0.1% SDS was then added to each of the sample wells. The upper edge of the plates assembly was then covered with parafilm and the whole placed in a refrigerator at 4°C overnight.

Preparation of cytoskeletal protein samples from isolated islets of lean and obese mice and cultured HIT-TI5 and RINm5F cells.

Stock cultures of HIT-TI5 and RINm5F cells were trypsinized, washed and enumerated with a haemocytometer. Approximately 1 ml of culture medium containing 15 x 10^6 cells was transferred to a 20 ml petri dish and the volume of medium made up to 10 ml with oxygenated Krebs-Hepes buffer, pH 7.4, containing either 7.5 mmol/l glucose or 10 mmol/l D-glyceraldehyde to stimulate insulin release from the HIT-TI5 and RINm5F cells respectively.

Islets from lean and obese mice were isolated using collagenase (page 84). Batches of 200-500 islets were incubated in 20 ml petri dishes containing 10 ml of oxygenated Krebs-Hepes buffer, pH 7.4, containing 16.7 mmol/l glucose. Both islets and cells were then incubated in an atmosphere of 5% CO_2 in humidified air at 37°C for 90 minutes. At the end of this incubation period the HIT-TI5, RINm5F cells and islets were each transferred to 10 ml sterilin centrifuge tubes and subsequently centrifuged at 1,000 rpm for 2 minutes. The supernatant was aspirated off with a pasteur pipette and replaced with 1 ml of extraction buffer containing Triton X-100, Appendix 4. The Triton X-100 insoluble pellet was resuspended in this buffer by agitation and retained at 4°C for 30 minutes.

After this time the cell debris was transferred to 2 ml Eppendorf microfuge tubes and centrifuged in a Beckman microfuge for 1 minute. The supernatant containing cytosolic proteins was transferred to another Eppendorf tube and frozen at -20°C until required. The surface of the pellet containing insoluble cytoskeletal proteins was washed with 100 µl of fresh extraction buffer without resuspension to remove any traces of residual soluble proteins. The washing solution was then added to the original supernatant prior to freezing. The surface of the pellet was then carefully dried with filter paper.

110 µl of sample buffer containing 0.5 mmol/l Tris-HCl, pH 6.8, 20% glycerol, 20% SDS, 1.43 mol/l 2-mercaptoethanol and 0.5 mg/ml bromophenol blue was diluted 1:1 with water and added to the pellet. The latter was dispersed with a needle and the suspension heated to 100°C for 10-15 minutes until the pellet had completely dissolved. This solution was stored at -20°C until required for electrophoresis.

Estimation of sample protein concentration

Prior to the incubation of islets in 16.7 mmol/l glucose, HIT-TI5 cells in 7.5 mmol/l glucose and RINm5F cells in 10 mmol/l D-glydceraldehyde, either 25 islets or 3.33×10^5 HIT-TI5 and RINm5F cells in 200 µl of the incubation medium and 0.8 ml of water were sonicated for 3 cycles at 560 W in an MSE Soniprep 150 sonicator. The

sonicates were then used to determine the total protein content per islet or per 10^6 RINm5F cells by the Folin Lowry Method (176), Appendix 2.

Dilution of islet, HIT-TI5 and RINm5F protein samples for electrophoresis

It proved difficult to assess the total protein concentration present in either the islet, HIT-TI5 or RINm5F cell pellets or the supernatant fractions, chiefly because the efficiency of the extraction procedure was not known and the presence of SDS and mercaptoethanol in the sample buffer (agents which denature protein) interfered with the Folin-Lowry method of estimating protein concentration, (176). Based on data obtained from the estimation of the total protein concentration in islets, HIT-TI5 and RINm5F cells, Table 2.3, it was decided to use 400 lean or obese mouse islets per sample and 15 x 10^6 HIT-TI5 or RINm5F cells/ml and process them accordingly. The supernatant and pellet fractions were diluted 1:1 with pre-diluted (1:1) sample buffer prior to gel application. The purpose of making these dilutions was to ensure that the protein concentration of the samples added to the gel did not exceed 1 mg/ml. Too high a protein concentration impairs the protein separation within the gel, (204).

Table 2.3. Summary of protein concentrations used with islets, HIT-TI5 and RINm5F cells.

CELL TYPE	AGE (weeks)	TOTAL PROTEIN CONTENT (µg/islet)	400 ISLETS (µg protein)
Lean mouse islets	15-20	7.18	2872
	31	4.54	1816
Obese mouse islets	14	3.83	1530
	24	5.30	2120
	31	4.25	1700
	PASSAGE	TOTAL PROTEIN CONTENT ($\mu g/10^6$ cells)	15×10^6 cells (μg protein)
RINm5F cells	95	201.90	3028
HIT-TI5 cells	59	322.10	4831

Dilution of marker protein standards for electrophoresis

The standard marker proteins were combined in a high molecular weight lyophilised mixture and contained:

	M. Wt.
Carbonic anhydrase (from bovine erythrocytes)	29.0 KDa
Egg albumin	45.0 KDa
Bovine albumin	66.0 KDa
Phosphorylase b (from rabbit muscle)	97.4 KDa
β-Galactosidase (from Escherichia Coli)	116.0 KDa
Myosin (from rabbit muscle)	205.0 KDa

The marker proteins were diluted (1:1) with pre-diluted sample buffer, (final SDS concentration of 4%), to give a stock standard marker protein concentration of 1 mg/ml.

Sample application and gel development

Prior to running the gel the solution of 0.124 mol/l Tris and 0.1% SDS was aspirated from the sample wells and replaced with running buffer containing 0.025 mol/l Tris, 0.192 mol/l glycine and 0.5% SDS, pH 8.3. The upper buffer reservoir was mounted on the gel frame and the raised channel positioned directly above the gel filled with running buffer.

25 µl of pre-diluted standard marker proteins and 25 µl of sample protein (total protein concentration of 1 mg/ml), was added to each well with the aid of a Hamilton Syringe. The presence of bromophenol blue in the sample buffer acted as an indicator of the migration position of the sample. The gel frame was then detached from the casting stand and with the upper reservoir still attached was inserted into the tank of running buffer maintained at 10°C. The upper reservoir immediately above the gel was then filled with the remaining running buffer and the lid and electrodes positioned in place.

The gel was developed using a current of 2.5 mA (500 V Max), until the samples had migrated to the bottom of the gel, (about 1½ hours).

Fixing and staining the developed gel.

After running, the developed gel was removed from the glass plates and positioned flat on a stainless steel

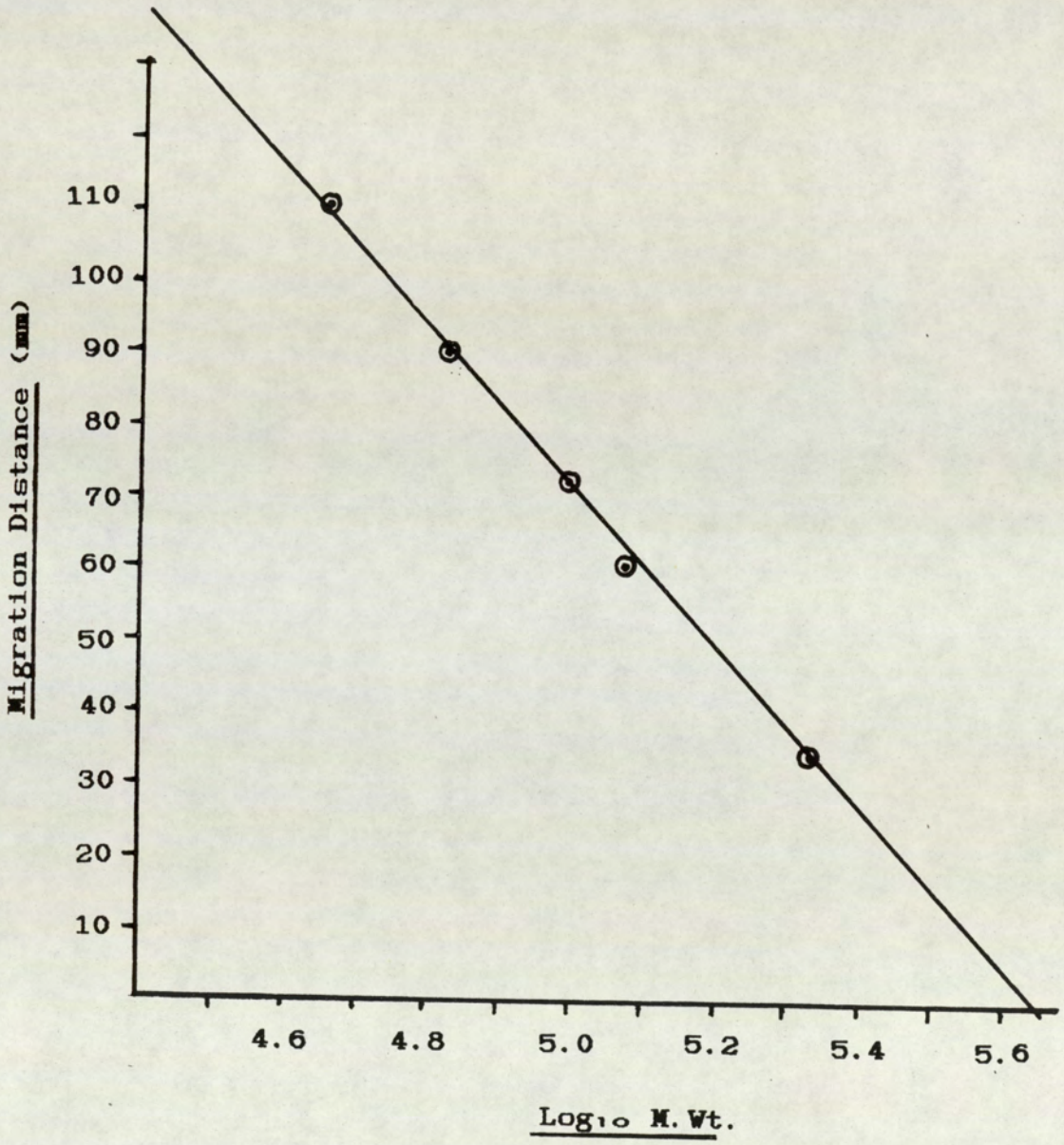
grid. The latter was lowered into a dish containing 0.25% PAGE blue'83 stain in water and glacial acetic acid with methanol as fixative, (5:1:4, v/v) and gently rocked into stain for about 1 hour. After this time the gel was destained in a mixture of water : acetic acid : methanol, (5:1:5. v/v) for 1 hour and finally destained for a further 55 minutes in fresh destaining medium containing 5% glycerol. The gel was then carefully sandwiched between thin layers of polythene and allowed to air dry overnight.

Characterization of islet, HIT-TI5 and RINm5F proteins in terms of molecular weight.

The mobility of each unknown band was established in mm (migration distance) and the molecular weight determined by reference to a standard curve constructed using the migration distance in mm of each standard protein and the $\log_{10}$ of known molecular weight, Figure 2.4. The molecular weight of unknown protein bands was established by extrapolation.

In addition to characterising each protein band in terms of its apparent molecular weight, quantification of each electrophoretogram was carried out on a LKB 2202 Ultroscan Laser Densitometer. This provided density profiles for each protein sample, see Figures 3.6, 3.7 and 3.8. The area under each peak provided a measure of

Figure 2.4 Standard curve for the estimation of molecular weights of unknown proteins



Correlation Coefficient r = 0.998

the concentration of a particular protein present, relative to the concentrations of the marker proteins.

Data presentation and statistical analysis.

The data are expressed throughout as means ± SEM together with the number of determinations, (n). Student's t-test was used for statistical comparisons and the differences were considered significant if $P<0.05$. In the perifusion experiments statistical significance of the differences between secretory profiles was computed using an analysis of the areas under the curves. Appendix 5, page 275.

Synposis of experiments

Experiment 1.

The insulin response of lean and genetically obese (ob/ob) mouse islets to a range of glucose concentrations

The isolated islets of lean and obese mice were pre-incubated with Krebs-Hepes buffer pH 7.4 containing 5.56 mmol/l glucose for 30 minutes in a static incubation system prior to 60 minutes test incubation in a range of glucose concentrations, (0 - 28 mmol/l), in Krebs-Hepes buffer pH 7.4.

Experiment 2

The insulin response of HIT-TI5 cells to a range of glucose concentrations.

HIT-TI5 cells were pre-incubated for 30 minutes with Krebs-Hepes buffer pH 7.4, in the absence of glucose, (in order to induce a greater insulin response), in a static incubation system. Afterwards the cells were test incubated for 60 minutes with a range of glucose concentrations (0 - 28 mmol/l)

Experiment 3

The insulin response of RINm5F cells to a range of D-glyceraldehyde concentrations

RINm5F cells were pre-incubated for 30 minutes with Krebs-Hepes buffer pH 7.4, in the absence of D-glyceraldehyde, (in order to induce a greater insulin

response), in a static incubation system. Afterwards the cells were test incubated for 60 minutes in a range of D-glyceraldehyde concentrations, (0 -20 mmol/l) in Krebs-Hepes buffer, pH 7.4.

Experiment 4

The effect of cytotoxic drugs on glucose stimulated insulin release from lean and obese mouse islets.

Both lean and obese mouse islets were statically incubated for 30 or 90 minutes with a stimulatory glucose concentration (16.7 mmol/l) after a 2 hour pretreatment period with either colchicine (1 µmol/l, 0.1 mmol/l and 10 mmol/l), vinblastine (1, 10 and 100 µmol/l), cytochalasin B (10 µg/ml), or colchicine and cytochalasin B together (10 mmol/l and 10 µg/ml repectively), in the presence of 5.56 mmol/l glucose.

Experiment 5

The effect of cytotoxic drugs on glucose stimulated insulin release from HIT-TI5 cells.

HIT-TI5 cells were challenged with a stimulatory glucose concentration (7.5 mmol/l) for 30 or 90 minutes in a static incubation system, after a 2 hour pre-treatment period with colchicine (1 µmol/l, 0.1 mmol/l and 10 mmol/l), vinblastine (1, 10 and 100 µmol/l), cytochalasin B (10 µg/ml) and colchicine and cytochalasin B together, (10 mmol/l and 10 µg/ml respectively), in the absence of glucose.

Experiment 6

The effect of cytotoxic drugs on D-glyceraldehyde stimulated insulin release from RINm5F cells.

RINm5F cells were challenged with a stimulatory concentration of D-glyceraldehyde (10 mmol/l) for 30 or 90 minutes in a static incubation system, after a 2 hour pre-treatment period with colchicine (10 mmol/l), cytochalasin B (10 µg/ml) or colchicine and cytochalasin B in combination (10 mmol/l and 10 µg/ml respectively), in the absence of D-glyceraldehyde.

Experiment 7

The effect of colchicine on the biphasic release of insulin from lean mouse islets.

Isolated lean mouse islets were perifused for 90 minutes with a stimulatory concentration of glucose, (16.7 mmol/l), after a 90 minute pre-perifusion with Krebs-Hepes buffer, pH 7.4 containing 5.56 mmol/l glucose in the presence or absence of colchicine (1 mmol/l).

Experiment 8

The effect of colchicine on the biphasic release of insulin from HIT-TI5 cells.

HIT-TI5 cells were perifused for 90 minutes with a stimulatory glucose concentration (7.5 mmol/l), after 90 minutes pre-perifusion with Krebs-Hepes buffer, pH 7.4, without glucose, in the presence or absence of colchicine (1 mmol/l).

The cytoskeletal proteins were extracted from isolated lean and obese mouse islets, HIT-TI5 and RINm5F cells with the the non-ionic detergent Triton X-100 and then separated by SDS-polyacrylamide electrophoresis. The molecular weights of each of the proteins were calculated by comparison with standard proteins of known molecular weight run simultaneously.

CHAPTER 3

INSULIN SECRETION STUDIES WITH ISLETS AND CULTURED CELLS

RESULTS

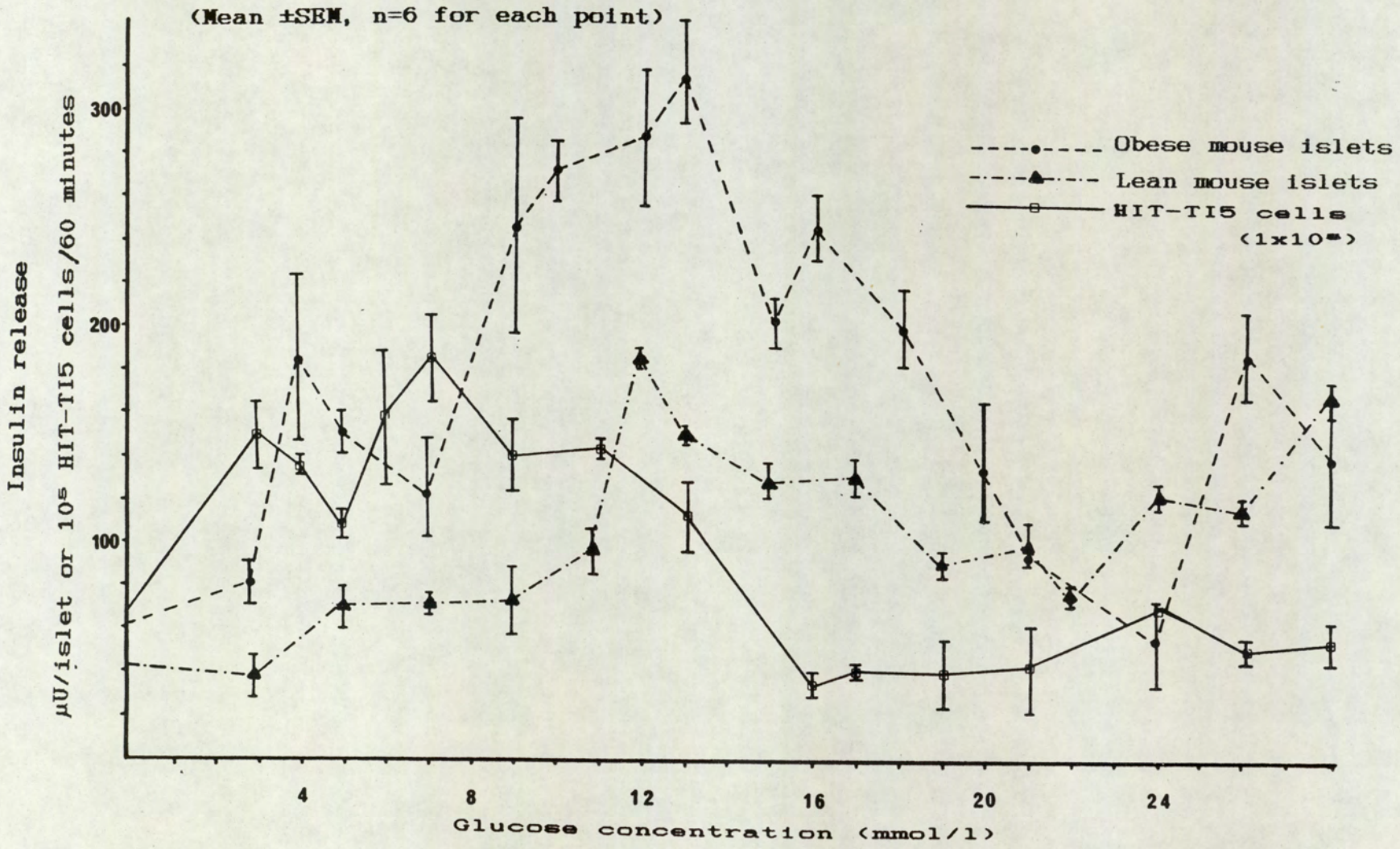
Experiment 1.

The insulin response of isolated lean and genetically diabetic obese (ob/ob) mouse islets to a range of glucose concentrations.

Statically incubated lean mouse islets produced a maximum insulin response of 189.5 ±3.2 μU /islet/60 minutes when incubated with 12 mmol/l glucose. The total area under the mean glucose stimulated profile was 11.1 x 10^3 ±574 μU/islet/60 minutes, Figure 3.1. (The computer program for calculating area under the curve is given in Appendix 5, page 275)

Statically incubated obese mouse islets produced a maximum insulin response of 312.3 ±28.9 μU/islet/60 minutes when incubated in the presence of 13 mmol/l glucose. The total insulin released over the whole glucose concentration range was 20 x 10^3 ±2.5 x 10^3 μU/islet/60 minutes, which was significantly higher than that generated by lean mouse islets. The lean and obese mouse islets appear to be more sensitive to glucose concentrations in the range of 7 - 20 mmol/l. Even so at these concentrations obese mouse islets secreted significantly greater concentrations of insulin than lean mouse islets.

Figure 3.1 Insulin response of lean and obese mouse islets and cultured HIT-TI5 cells to glucose.



Experiment 2.

The insulin response of HIT-TI5 cells to a range of glucose concentrations

Maximum insulin release from cultured HIT-TI5 cells occurred in response to 7.5 mmol/l glucose, Figure 3.1 and 3.2, whether incubated for 60 or 90 minutes. The total insulin released by the HIT-TI5 cells in response to 0 - 28 mmol/l glucose, (10.76 x 10^3 ±1.5 x 10^3 μU/1 x 10^5/60 minutes), was significantly less than that released by the obese mouse islets (20 x 10^3 ± 2.5 x 10^3 μU/islet/60 minutes), but not significantly different from that released by lean mouse islets, (11.1 x 10^3 ±574 μU/islet/60 minutes), Figure 3.1.

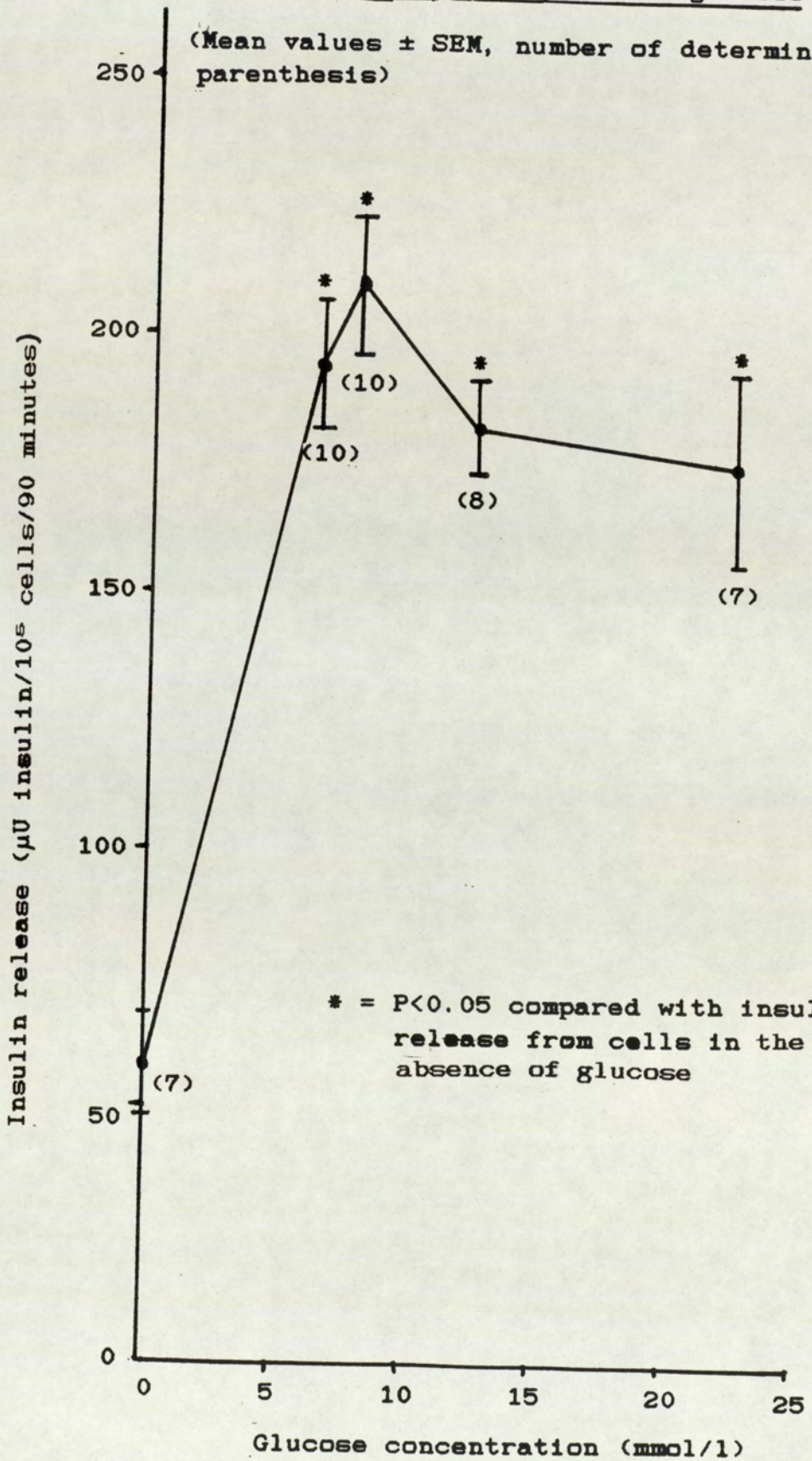
However, HIT-TI5 cells appeared to be more sensitive to glucose concentrations between 0 - 3 mmol/l and showed a sustained but lower response to higher glucose concentrations.

The insulin response of HIT-TI5 cells incubated in the presence of glucose was significantly higher than the insulin response of HIT-TI5 cells incubated in the absence of glucose, Figure 3.2.

Figure 3.2

Insulin release from statically incubated HIT-T15 cells in response to glucose

(Mean values ± SEM, number of determinations in parenthesis)



Experiment 3.

The insulin response of RINm5F cells to a range of D-glyceraldehyde concentrations.

D-glyceraldehyde stimulated insulin release from statically incubated RINm5F cells almost dose dependently. However, values were much reduced compared with isolated islets and HIT-TI5 cells, Figure 3.3. 10 mmol/l glyceraldehyde was used in subsequent experiments for the stimulation of insulin release.

Experiment 4.

The effect of colchicine, vinblastine and cytochalasin B pre-treatment on glucose stimulated insulin release from isolated lean and genetically obese (ob/ob) mouse islets.

a) Lean mouse islets

Increasing the glucose concentration from 5.56 to 16.7 mmol/l for both 30 and 90 minutes of incubation significantly increased the release of insulin from lean mouse islets, Table 3.1.

Pre-treatment of lean mouse islets with colchicine (1 μmol/l, 0.1 mmol/l and 10 mmol/l) significantly reduced subsequent glucose stimulated insulin release over the 30 and 90 minute test periods but these values remained significantly greater than basal values obtained in the presence of 5.56 mmol/l glucose alone. Clearly

Figure 3.3

Insulin release from statically incubated RINm5F cells in response to D-glyceraldehyde

(Mean values ± SEM, number of determinations in parenthesis)

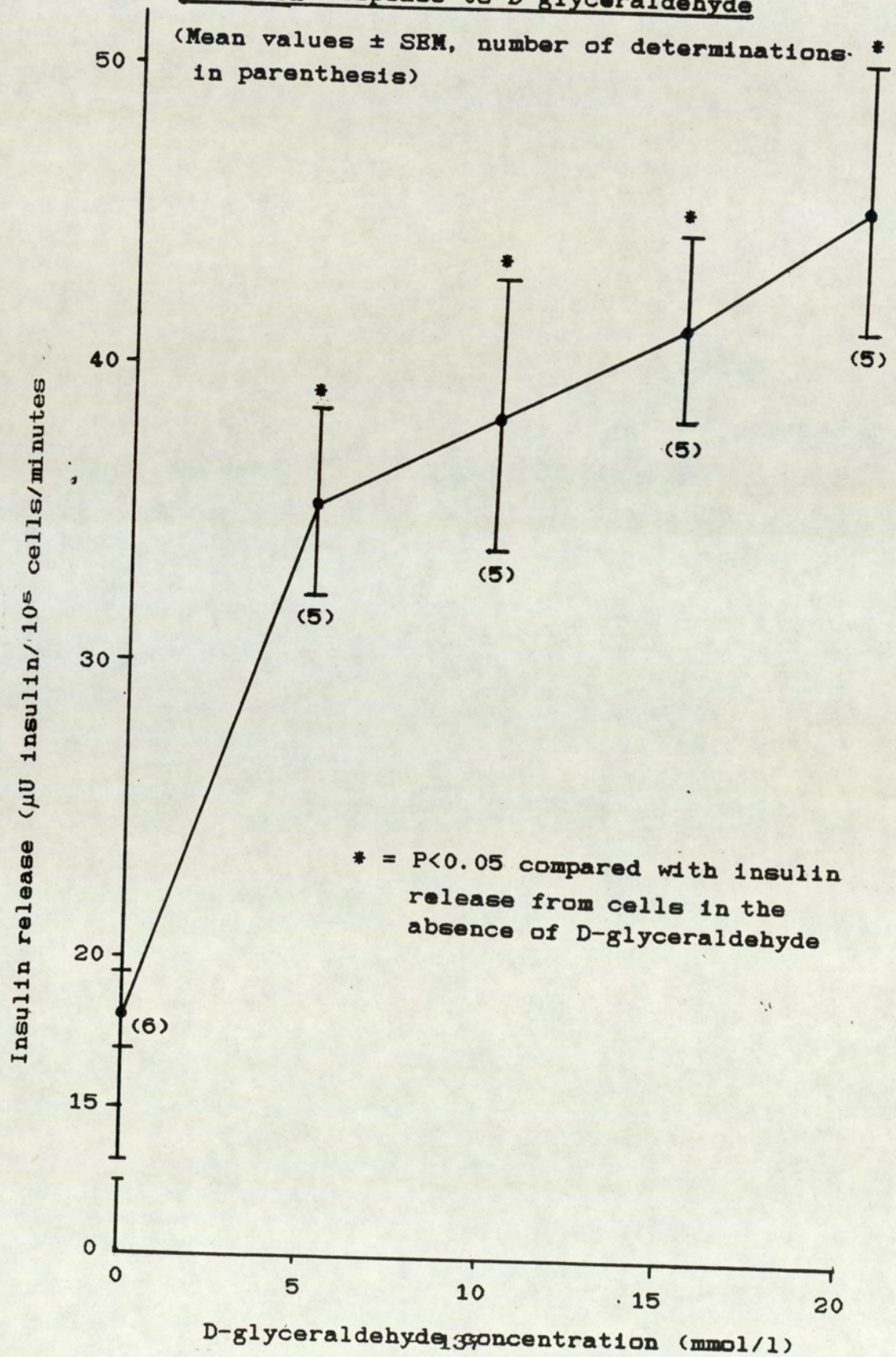


Table 3.1 Effect of colchicine, vinblastine and cytochalasin B on glucose stimulated insulin release from isolated islets of lean mice. (Mean values ± SEM)

PRE-TREATMENT	Insulin released during test period (μU insulin/islet)			
	n	30 minutes	n	90 minutes
BASAL - 5.56 mmol/l glucose	6	7.63 ± 0.77*	6	11.44 ± 0.57*
CONTROL - 16.7 mmol/l glucose	19	61.99 ± 8.3 λ	19	88.10 ± 9.7 λ
16.7 mmol/l glucose + 1μmol/l colchicine	6	26.61 ± 5.6 *λ	6	28.99 ± 2.5 *λ
16.7 mmol/l glucose + 0.1mmol/l colchicine	6	24.99 ± 2.9 *λ	6	27.70 ± 4.0 *λ
16.7 mmol/l glucose + 10mmol/l colchicine	6	17.76 ± 0.9 *λ	6	22.20 ± 2.2 *λ
16.7 mmol/l glucose + 1μmol/l vinblastine	10	82.90 ± 10.4 λ	7	106.0 ± 14.9 λ
16.7 mmol/l glucose + 10μmol/l vinblastine	10	71.90 ± 15.0 λ	6	96.80 ± 9.7 λ
16.7 mmol/l glucose + 100μmol/l vinblastine	10	52.00 ± 3.7 λ	11	83.90 ± 10.2 λ
16.7 mmol/l glucose + 10μg/ml cytochalasin B	9	105.90 ± 6.9 *λ†	6	121.60 ± 18.9 λ†
16.7 mmol/l glucose + 10mmol/l colchicine + 10μg/ml cytochalasin B	9	91.10 ± 3.6 *λ†θ	8	99.70 ± 15.5 λ†

* Significantly increased or decreased P<0.05 compared with control value
λ Significantly increased P<0.05 compared with basal value
† Significantly increased P<0.05 compared with 16.7 mmol/l glucose + 10 mmo/l/ colchicine
θ Significantly decreased P<0.05 compared with 16.7 mmol/l glucose +10 μg/ml cytochalasin B

colchicine did not completely prevent glucose stimulated insulin release and it is possible that transferring the islets to buffer containing 16.7 mmol/l glucose after colchicine pre-treatment in the presence of 5.56 mmol/l glucose caused the release of β-granules close to the β-cell membrane while colchicine treatment inhibited any subsequent microtubule facilitated sustained insulin release.

Vinblastine, (1 μmol/l, 10 μmol/l and 100 μmol/l) did not significantly inhibit insulin release from lean mouse islets.

Cytochalasin B (10 μg/ml) pre-treatment produced a significant increase in insulin release from lean mouse islets compared with the control value after the 30 minute test period but not after the 90 minute test period. The insulin released during both test periods was significantly higher than the corresponding basal values, Table 3.1.

Pre-treatment with cytochalasin B (10 μg/ml) and colchicine (10 mmol/l) in combination caused a significant increase in insulin release compared with control values obtained after the 30 minute test period and values obtained in the presence of 10 mmol/l colchicine after both 30 and 90 minute test incubation. However, insulin release from lean mouse islets in response to the combination was significantly lower than that obtained for cytochalasin B alone after 30 minutes test incubation. The stimulatory effect of cytochalasin

B compared with control values was only significant after the 30 minute test incubation period, suggesting that the cytochalasin B induced disruption of microtubules facilitated the release of β-granules lying close to the β-cell membrane when the islets were transferred to the stimulatory concentration of glucose (16.7 mmol/l). However, it would seem that the disruptive action of cytochalasin B exceeded the inhibitory action of colchicine resulting in the release of peripheral β-granules and elevated insulin release

b) Obese mouse islets.

Increasing the glucose concentration to 16.7 mmol/l significantly increased insulin release from obese mouse islets after both 30 and 90 minutes test incubation. All three concentrations of colchicine (1 μmol/l, 0.1 mmol/l and 10 mmol/l) significantly reduced glucose stimulated insulin release from obese mouse islets compared with controls after both the 30 and 90 minutes incubation, Table 3.2. 1 μmol/l vinblastine significantly reduced insulin release from obese mouse islets after the 30 minute test period while 100 μmol/l vinblastine significantly reduced insulin release from obese mouse islets after the 90 minute test period compared with the control values. Vinblastine would appear to have a more significant inhibitory action on glucose stimulated insulin release from obese mouse islets than on lean mouse islets, Table 3.1.

Table 3.2 Effect of colchicine, vinblastine and cytochalasin B on glucose stimulated insulin release from isolated islets of genetically obese diabetic (ob/ob) mice. (Mean values ± SEM)

PRE-TREATMENT	Insulin released during test period (μU insulin/islet)			
	n	30 minutes	n	90 minutes
BASAL - 5.56 mmol/l glucose	6	22.16 ± 1.74*	6	24.99 ± 2.34*
CONTROL - 16.7 mmol/l glucose	22	239.81 ± 20.6 λ	22	274.77 ± 23.4 λ
16.7 mmol/l glucose + 1μmol/l colchicine	12	70.40 ± 12.1 *λ	12	72.50 ± 8.3 *λ
16.7 mmol/l glucose + 0.1mmol/l colchicine	11	95.30 ± 12.6 *λ	11	84.10 ± 11.7 *λ
16.7 mmol/l glucose + 10mmol/l colchicine	12	86.50 ± 8.7 *λ	12	105.90 ± 11.4 *λ
16.7 mmol/l glucose + 1μmol/l vinblastine	10	120.00 ± 20.6 *λ	12	206.20 ± 16.3 λ
16.7 mmol/l glucose + 10μmol/l vinblastine	11	205.50 ± 28.5 λ	12	227.20 ± 27.0 λ
16.7 mmol/l glucose + 100μmol/l vinblastine	10	182.20 ± 24.2 λ	12	165.20 ± 29.6 *λ
16.7 mmol/l glucose + 10μg/ml cytochalasin B	9	295.40 ± 21.3 λ†	9	336.30 ± 23.4 λ†
16.7 mmol/l glucose + 10mmol/l colchicine + 10μg/ml cytochalasin B	9	243.80 ± 18.0 λ†	9	244.50 ± 24.0 λ†θ

* Significantly decreased P<0.05 compared with control value
λ Significantly increased P<0.05 compared with basal value
† Significantly increased P<0.05 compared with 16.7 mmol/l glucose + 10 mmol/l colchicine
θ Significantly decreased P<0.05 compared with 16.7 mmol/l glucose + 10 μg/ml cytochalasin B

Cytochalasin B (10 μg/ml) pre-treatment did not significantly enhance the insulin release from obese mouse islets after either the 30 or 90 minute test periods compared with control values but was always significantly greater than basal release values.

Obese mouse islets pre-treated with cytochalasin B (10 μg/ml) and colchicine (10 mmol/l) in combination released significantly more insulin than the 10 mmol/l colchicine pre-treated islets but not more than the controls treated with 16.7 mmol/l glucose alone. However, the rate of insulin release from obese mouse islets in response to the combination was significantly lower than the rate of insulin release in the presence of cytochalasin B alone after 90 minutes test incubation. This might suggest that the inhibitory action of colchicine on obese mouse islet β-cell microtubules might to some extent override the disruptive action of cytochalasin B on the microfilamentous web and the subsequent release of β-granules. ie. that colchicine prevents the newly synthesised β-granules reaching the periphery of the β-cell web.

Experiment 5.

The effect of colchicine, vinblastine and cytochalasin B on glucose stimulated insulin release from HIT-TI5 cells.

7.5 mmol/l glucose significantly stimulated the release of insulin from cultured HIT-TI5 cells after both 30 and 90 minutes test incubation, Table 3.3.

Only after 90 minutes test incubation did the highest concentration of colchicine (10 mmol/l) significantly reduce the insulin release from HIT-TI5 cells. However, the rate of insulin release from HIT-TI5 cells after pre-treatment with all concentrations of colchicine was always greater than the rate of basal release, Table 3.3. Only pre-treatment with the maximum concentration of vinblastine (100 μmol/l) showed a reduction in the rate of insulin release after the 90 minute test period compared with controls.

Cytochalasin B (10 μg/ml) did not significantly enhance glucose stimulated insulin release from HIT-TI5 cells above the control value, indeed after 30 minutes test incubation the rate of insulin release was lower than the control value.

Pre-treatment of HIT-TI5 cells with colchicine (10 mmol/l) and cytochalasin B (10 μg/ml) in combination significantly reduced the glucose stimulated insulin release compared with control values and values obtained after pre-treatment with cytochalasin B alone after both the 30 and 90 minute test periods. Also the rate of

Table 3.3 Effect of colchicine, vinblastine and cytochalasin B on glucose stimulated insulin release from HIT-TI5 cells.

(Mean values ± SEM)

PRE-TREATMENT	Insulin released during test period (μU insulin/10^5cells)			
	n	30 minutes	n	90 minutes
BASAL - Krebs-Hepes buffer only	6	14.30 ± 1.1 *	6	15.90 ± 3.2 *
CONTROL - 7.5 mmol/l glucose	38	59.68 ± 4.9 λ	39	89.90 ± 7.0 λ
7.5 mmol/l glucose				
+ 1μmol/l colchicine	12	66.20 ± 12.6 λ	12	88.70 ± 24.6 λ
+ 0.1mmol/l colchicine	14	67.90 ± 13.8 λ	14	77.80 ± 14.3 λ
+ 10mmol/l colchicine	15	49.40 ± 9.8 λ	14	51.50 ± 11.0 *λ
7.5 mmol/l glucose				
+ 1μmol/l vinblastine	12	65.50 ± 8.3 λ	6	89.40 ± 18.3 λ
+ 10μmol/l vinblastine	12	55.20 ± 9.6 λ	6	84.60 ± 7.0 λ
+ 100μmol/l vinblastine	10	49.20 ± 5.9 λ	12	50.00 ± 8.6 *λ
7.5 mmol/l glucose				
+ 10μg/ml cytochalasin B	6	50.70 ± 2.2 λ	6	100.70 ± 5.7 λ
7.5 mmol/l glucose + 10mmol/l colchicine + 10μg/ml cytochalasin B	6	26.10 ± 3.1 *λ†θ	6	37.80 ± 5.4 *λθ

* Significantly decreased $P<0.05$ compared with control value
λ Significantly increased $P<0.05$ compared with basal value
† Significantly decreased $P<0.05$ compared with 16.7 mmol/l glucose + 10 mmol/l colchicine
θ Significantly decreased $P<0.05$ compared with 16.7 mmol/l glucose + 10 μg/ml cytochalasin B

insulin release from HIT-TI5 cells pre-treated with the combination was significantly less than that released after pre-treatment with 10 mmol/l colchicine alone, at least after 30 minutes of test incubation. This might suggest that in combination colchicine and cytochalasin B act in unison to reduce glucose stimulated insulin release from HIT-TI5 cells, or that the inhibitory effect of colchicine on the microtubules exceeds the disruptive action of cytochalasin B on the micro-filamentous web.

Experiment 6.

The effect of colchicine and cytochalasin B on glyceraldehyde stimulated insulin release from RINm5F cells.

10 mmol/l glyceraldehyde significantly stimulated the rate of insulin release from RINm5F cells after both the 30 and 90 minute test incubation periods, Table 3.4.

Pre-treatment of RINm5F cells with 10 mmol/l colchicine caused a significant reduction in glyceraldehyde induced insulin release compared with controls.

Cytochalasin B (10 μg/ml) did not significantly enhance insulin release from RINm5F cells compared with controls but the rate of insulin release after cytochalasin B pre-treatment was always significantly

Table 3.4 Effect of colchicine and cytochalasin B on glyceraldehyde stimulated insulin release from RINm5F cells.

(Mean values ± SEM)

PRE-TREATMENT	Insulin released during test period (μU insulin/10^5cells)			
	n	30 minutes	n	90 minutes
BASAL Krebs - Hepes buffer only	6	15.10 ± 2.9 *	6	25.06 ± 5.8 *
CONTROL 10 mmol/l glyceraldehyde	12	31.10 ± 1.5 λ	10	35.60 ± 2.5 λ
10 mmol/l glyceraldeyde + 10mmol/l colchicine	12	20.60 ± 1.1 *	12	19.20 ± 8.3 *
10 mmol/l glyceraldehyde + 10μg/ml cytochalasin B	12	28.70 ± 2.6 λ	12	33.50 ± 2.1 λ
10 mmol/l glyceraldehyde + 10mmol/l colchicine 10μg/ml cytochalasin B	11	21.80 ± 2.0 *λθ	12	14.60 ± 1.4 *θ

* Significantly decreased P<0.05 compared with control value
λ Significantly increased P<0.05 compared with basal value
θ Significantly decreased P<0.05 compared with 10 mmol/l glyceraldehyde + 10 μg/ml cytochalasin B

higher than the basal release after both 30 and 90 minute test incubation periods.

The pre-treatment of RINm5F cells with colchicine (10 mmol/l) and cytochalasin B in combination produced a significant reduction in the rate of glyceraldehyde stimulated insulin release compared with control values and the rate of insulin release obtained after pre-treatment with cytochalasin B alone. These data might suggest that cytochalasin B does not have a significant effect on the microfilamentous system of the RINm5F cell, whereas colchicine (10 mmol/l) acting at the level of the microtubules significantly inhibits insulin release.

Experiment 7.

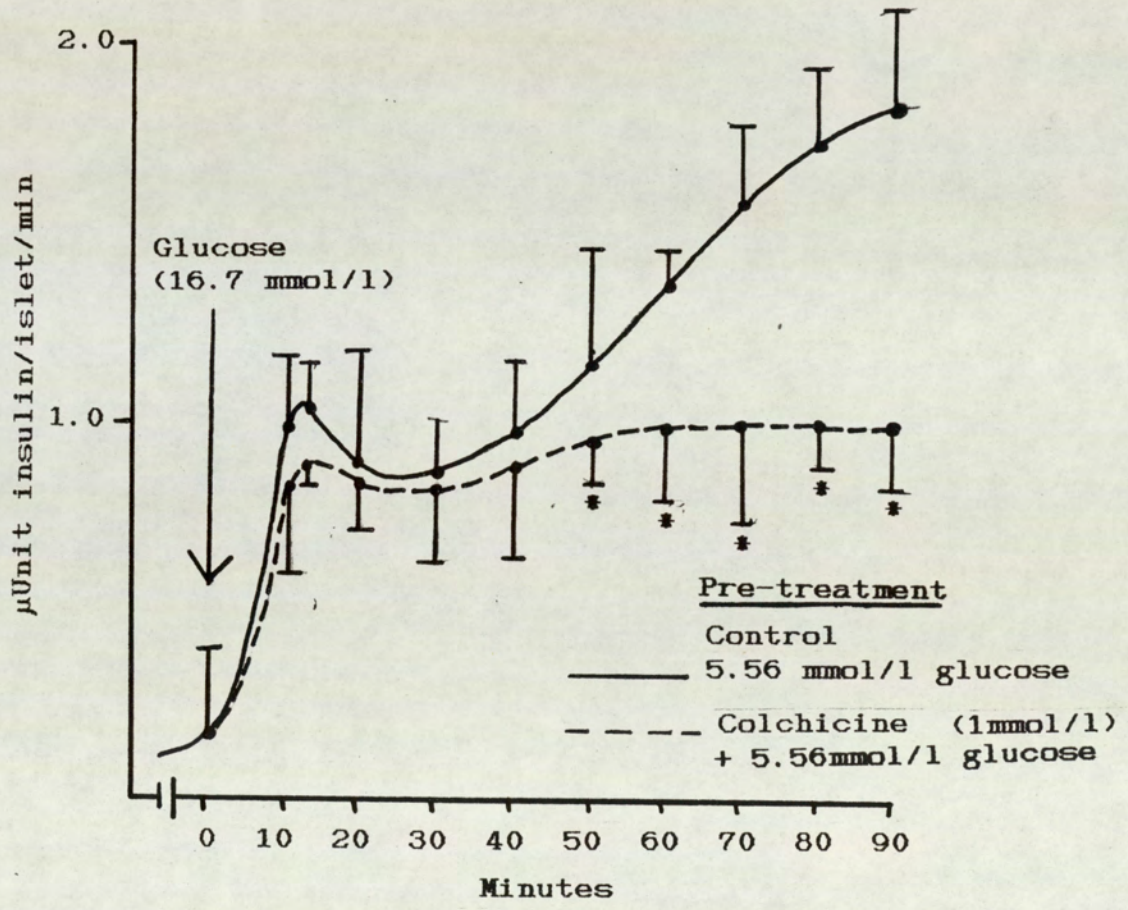
The effect of colchicine (1mmol/l) on the biphasic release of insulin from isolated lean mouse islets.

The dynamic release of insulin from perifused lean mouse islets revealed a reproducible biphasic profile, Figure 3.4. The total area under the mean control profile in response to 16.7 mmol/l glucose was 58.95 ±8.1 μU/islet/90 minutes, which was significantly higher than the insulin released by the islets pre-treated with 1 mmol/l colchicine of 44.1 μU/islet/90 minutes.

Pre-perifusion of lean mouse islets with 1 mmol/l colchicine for 90 minutes before perifusion with a stimulatory concentration of glucose, (16.7 mmol/l),

Figure 3.4 Effect of colchicine on the first and second phases of glucose stimulated insulin release from lean mouse islets.

(Each point is the mean of 6 determinations ± SEM)



* Significantly reduced $P<0.05$ compared with the profile obtained with untreated (control) islets.

caused a significant inhibition of the second phase of, insulin release. The area under the control profile over the period of 50 to 90 minutes (31.0 ±3.8 μU/islet/40 minutes) was significantly higher than the insulin released by colchicine pre-treated islets over this period, (20.0 ±3.4 μU/islet/40 minutes).

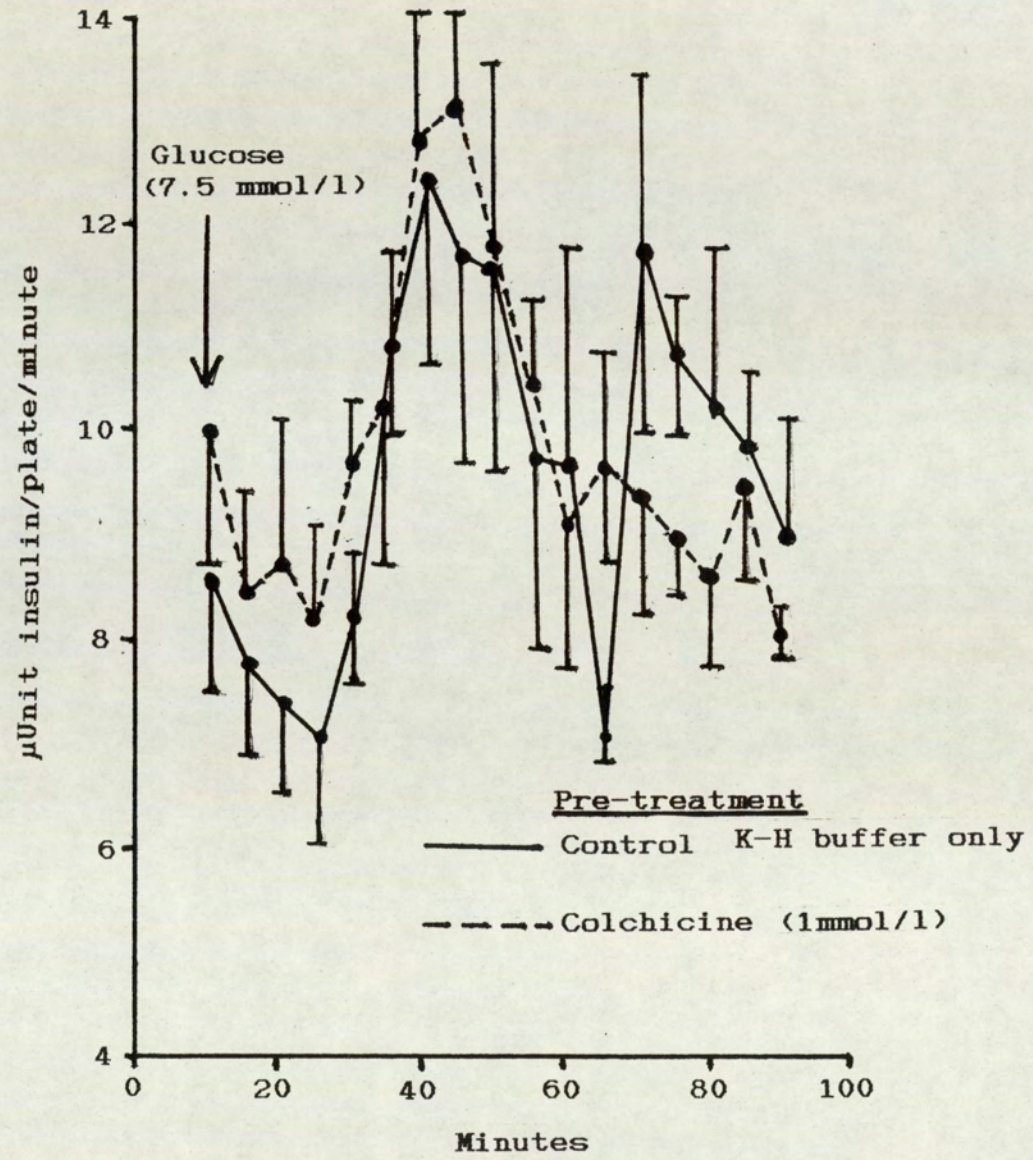
Experiment 8.

The effect of colchicine (1 mmol/l) on the biphasic release of insulin from HIT-TI5 cells.

Dynamic insulin release from perifused HIT-TI5 cells in response to 7.5 mmol/l glucose revealed a reproducible biphasic profile, Figure 3.5. The total area under the control profile obtained in response to 7.5 mmol/l glucose was 620 ±40 μU/plate/60 minutes compared with the insulin response of 567 ±44 μU/plate/60 minutes obtained when the HIT-TI5 cells were pre-perifused with 1 mmol/l colchicine. Pre-perifusion of HIT-TI5 cells with 1 mmol/l colchicine did not affect the first phase of insulin release but reduced the second phase of release, (between 60 and 90 minutes) from 173.2 ±33.9 to 153.8 ±27.0 μU/plate/30 minutes.

Figure 3.5 Effect of colchicine on glucose stimulated insulin release from perifused HIT-TI5 cells.

(Each point is the mean of 11 determinations ± SEM)



Separation and identification of cytoskeletal proteins in lean and obese mouse islets using SDS-electrophoresis.

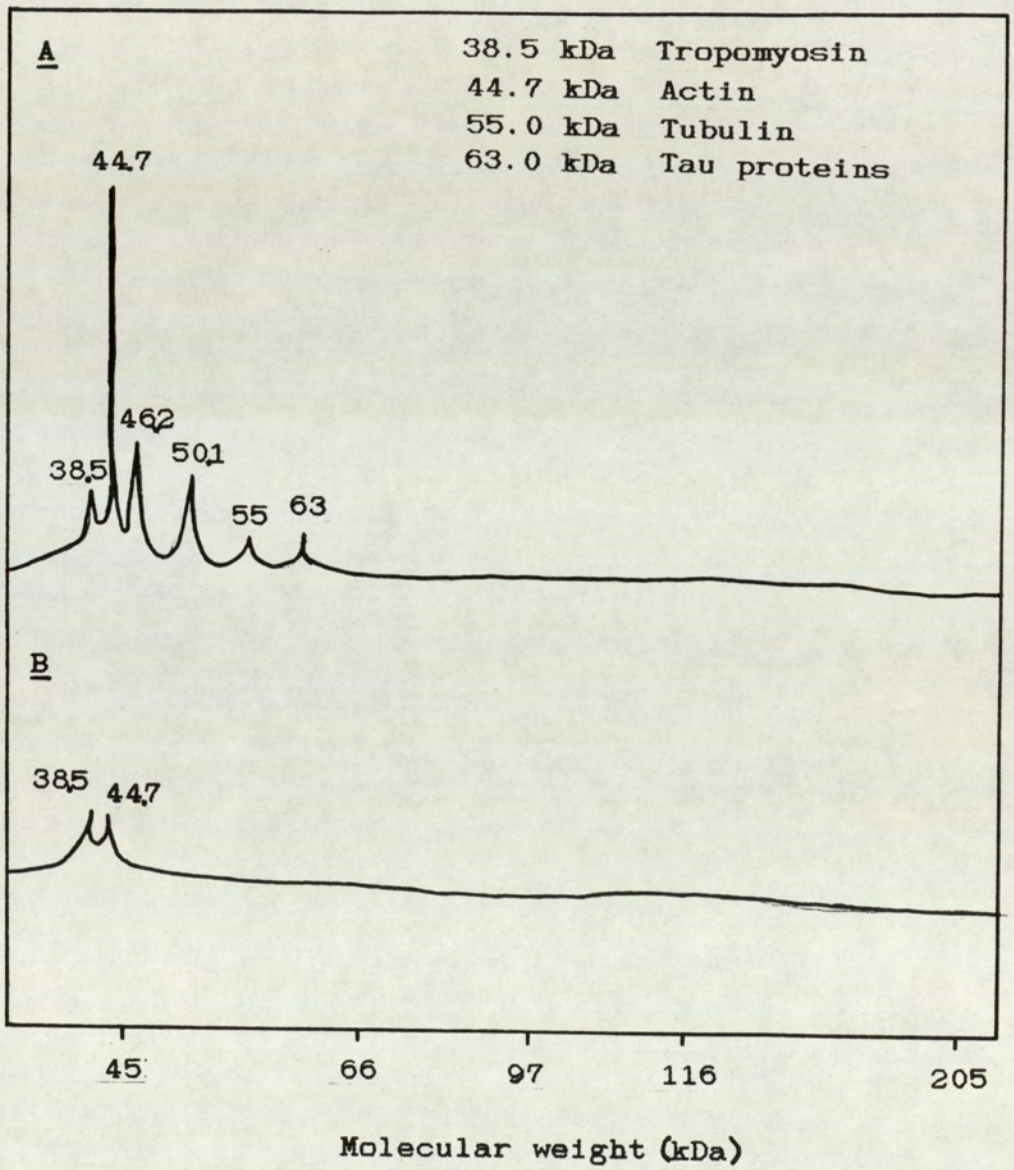
A 44.7 kDa protein was observed in both lean and obese mouse Triton X-100 insoluble fractions which corresponded with the 43 kDa protein actin, Figure 3.6. Actin is believed to be closely associated with the β-cell cytoskeleton and may be involved in the process of insulin release, (92,93), In association with this actin-like protein in lean mouse islet extracts was a 46.2 kDa protein which may correspond to the actin binding protein fragmin (43 kDa), a Ca^{2+} dependent modulator of filamentous actin (F-actin), (217,218). Positive identification was not possible in the absence of pure actin and fragmin markers.

A 55 kDa protein was also present in the extracts of lean mouse islets which almost certainly corresponds with the 55 kDa microtubule protein, monomeric tubulin. The latter is the major component of the cytoskeleton in the polymerized form, (74). It would therefore seem likely that the 63 kDa protein also found in lean mouse islets, Figure 3.6 was one of the Tau proteins (55-62 kDa), which are believed to be responsible for the assembly of microtubules from tubulin dimers, (80).

The 38.5 kDa protein present in both the lean and obese mouse islet extracts may be the 37 kDa protein tropomyosin, a regulator of microlfilament contraction, (219).

Figure 3.6

Intensity profiles of cytoskeletal proteins from isolated islets of lean and obese (ob/ob) mice by Triton X-100 extraction and run on 10% SDS-acrylamide gels



A = Triton X-100 insoluble lean mouse islet proteins

B = Triton X-100 insoluble obese (ob/ob) mouse islet proteins

Separation and identification of cytoskeletal proteins in HIT-TI5 cell extracts using SDS-electophoresis.

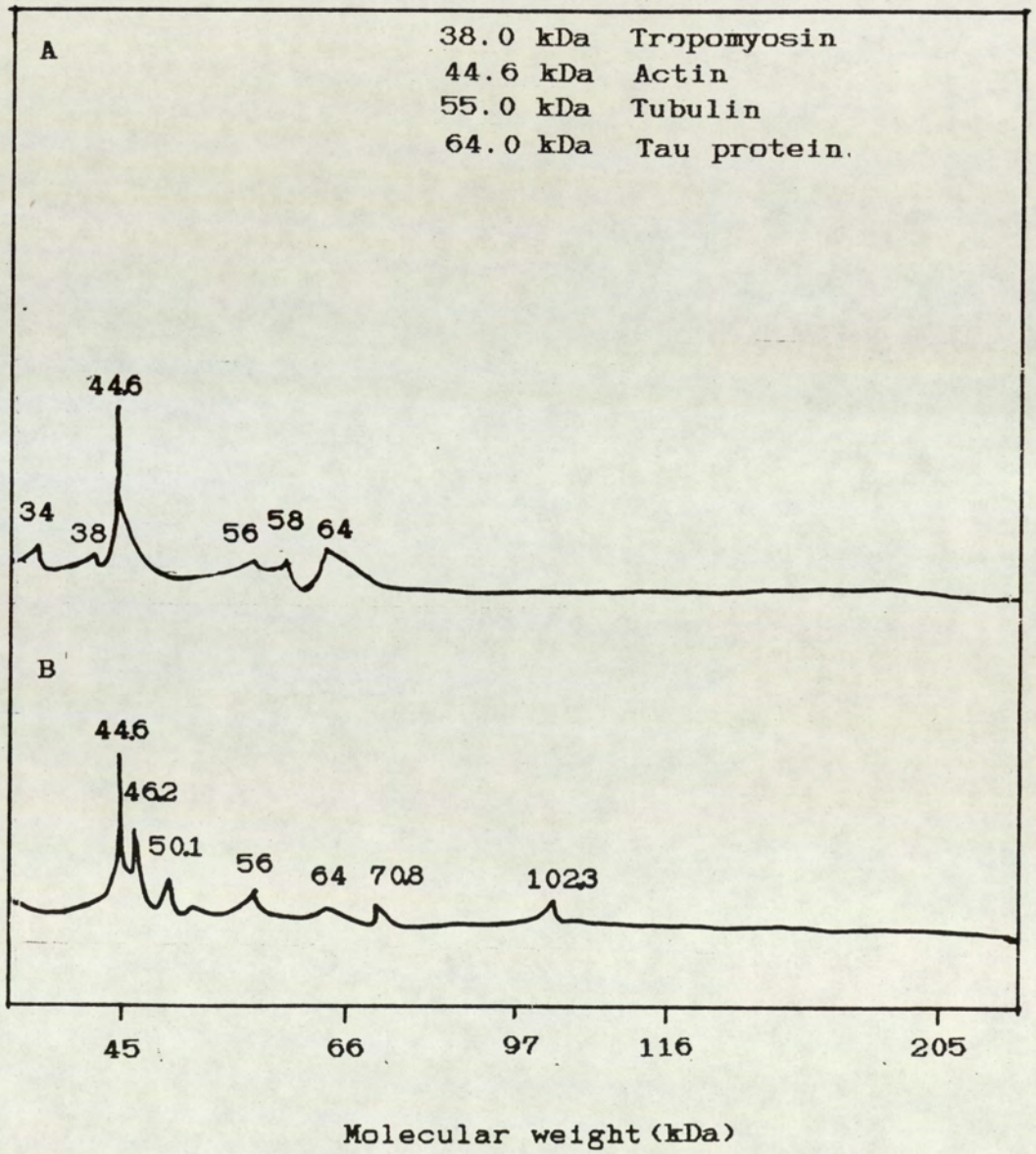
The 44.6 kDa protein present in both the Triton X-100 soluble and insoluble fractions from HIT-TI5 cells, Figure 3.7, was probably actin (43 kDa) and the 46.2 kDa protein found in the Triton X-100 soluble fraction probably fragmin, (43kDa).

The 56 kDa protein was probably tubulin (55 kDa) while the (55-62 kDa) proteins present in both the Triton X-100 soluble and insoluble fractions probably corresponded to the Tau proteins. The presence of tubulin in the Triton X-100 soluble fraction might suggest over extraction of the cytoskeletal proteins since Branston and coworkers have indicated that tubulin should only be present in the insoluble fraction, (90).

A 38 kDa protein, probably tropomyosin (37 kDa) was present in the Triton X-100 soluble fraction of HIT-TI5 cells, Figure 3.7, while the 102.3 kDa protein in the soluble fraction may correspond to the 100 kDa protein α-actinin, an actin cross linker, (220).

Figure 3.7

Intensity profiles of HIT-TI5 cell cytoskeletal proteins extracted with Triton X-100 and run on SDS-acrylamide gels



A = Triton X-100 insoluble proteins

B = Triton X-100 soluble proteins

Separation and identification of cytoskeletal proteins in RINm5F cell extracts using SDS-electrophoresis.

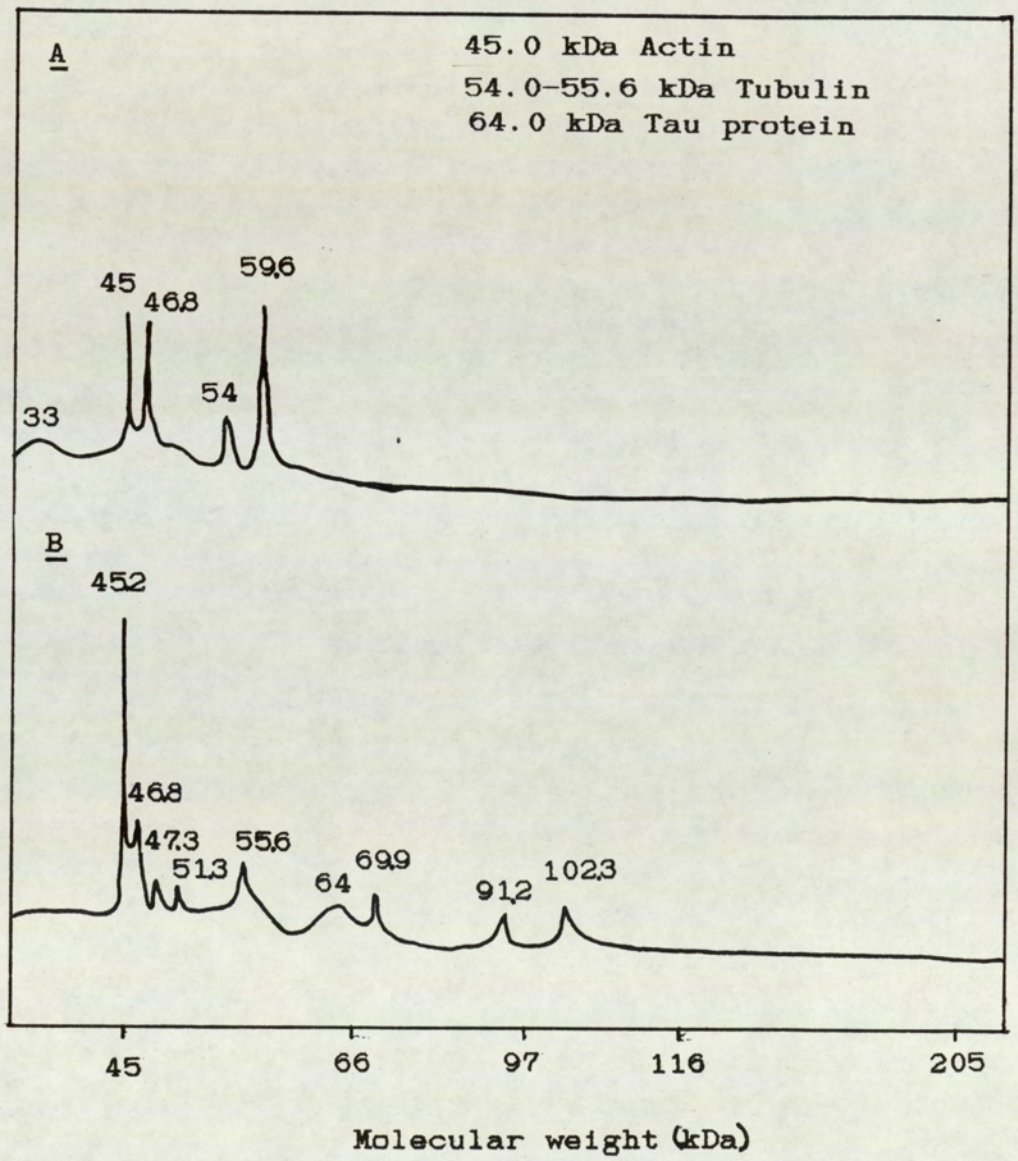
Actin appears to be present in both the Triton X-100 insoluble and soluble fractions of RINm5F cells as the 45-45.2 kDa protein, Figure 3.8. The 46.8 kDa protein found in both fractions may be actin-associated fragmin, (43 kDa).

The 54 and 55.6 kDa proteins found in both the Triton X-100 soluble and insoluble fractions are probably tubulin. The 59.6 kDa protein found in the insoluble fraction and the 64 kDa protein in the soluble fraction are probably both Tau proteins, (55-62 kDa).

The Triton X-100 soluble extracts of RINm5F cells also contained a 102.3 kDa protein which may be α-actinin. In addition, the soluble RINm5F cell fraction also contained a 91.2 kDa protein which may correspond to either gelsolin (91 kDa) or villin (95 kDa), both of which are Ca^{2+}-dependent modulators of F-actin, (220).

Figure 3.8

Intensity profiles of RINm5F cell cytoskeletal proteins extracted with Triton X-100 and run on SDS-acrylamide gels



A = Triton X-100 insoluble proteins

B = Triton X-100 soluble proteins

CHAPTER 4

DEVELOPMENT OF A RADIOIMMUNOASSAY METHOD AND A COLCHICINE BINDING METHOD FOR THE MEASUREMENT OF ISLET TUBULIN CONCENTRATION.

A. THE DEVELOPMENT OF A TUBULIN RADIOIMMUNOASSAY.

Since the introduction in 1960 of insulin radioimmunoassy by Yalow and Berson as a sensitive and reproducible method for the measurement of plasma insulin (171), radioimmunoassay has become the method of choice for determining minute quantities of such compounds as hormones, drugs and enzymes in plasma and other body fluids.

Most radioimmunoassays depend upon competition between an unlabelled protein (Ag), and a radioiodinated protein (Ag*), for binding sites on a limited amount of specific antibody (Ab). This Ag reduces the amount of free Ab, thus decreasing the availability of Ab to Ag*.

$$Ag.Ab \rightleftharpoons Ag + Ab + Ag^* \rightleftharpoons Ag^*.Ab$$

In performing the assay, Ag* and the Ab are incubated in the presence and absence of samples containing Ag. After reaching or approaching equilibrium, free Ag* and antibody-bound Ag* are separated and either one or the other is determined by radioactive counting. The protein concentration in the

unknown is measured by comparing the diminuition of Ag* binding produced by Ag in the unknown sample, to that of a standard curve obtained by adding graded, known concentrations of Ag to the assay system.

In the present study attempts were made to develop a radioimmunoasay for tubulin on the basis of a double antibody radioimmunoassay, in which the first antibody, antitubulin serum raised in rabbits, becomes saturated with tubulin (Ag) and radio-labelled tubulin (Ag*) forming a soluble complex. The second antibody, anti rabbit IgG, binds with the rabbit antitubulin to form a larger, heavier complex which can be separated from the free unbound Ag* by centrifugation.

The development of a radioimmunoassay for tubulin requires the preparation of a purified source of tubulin for the peparation of a range of standards used to construct the standard curve which will subsequently be used to estimate the unknown concentration of sample tubulin. A source of purified tubulin is also required for the preparation of radio-iodinated tubulin (Ag*).

I. ISOLATION OF TUBULIN FROM PIG BRAIN FOR USE AS A RADIOIMMUNOASSAY STANDARD.

Tubulin was isolated from pig brain by a modification of Weisenberg's original assembly - disassembly method, (71) according to Shelanski, Cantor and Taylor, (78). The assembly - disassembly method has the major advantage in that only those sub-units capable of polymerization into microtubules are separated, eliminating the presence of damaged tubulin aggregates in the preparation.

Three pigs brains, obtained from a local abattoir (A. Thompson Butcher's, Lichfield Road, Aston, Birmingham), were homogenised using a MSE motorised, steel homogeniser at 37°C in an equal volume of 100 mmol/l MES isolation buffer at pH 6.8, Appendix 1, page 252. The homogenate was placed on ice for 1 hour at 4°C and subsequently centrifuged at 75,000 g (33,000 rpm) for 1 hour at 4°C in an MSE Superspeed 50 centrifuge. The low temperature favoured the disassembly of the microtubules into the lighter tubulin sub-units and tubulin aggregates which remained in solution (78), so that the pellet of particulate waste material formed during centrifugation could be discarded.

Approximately 1 ml of a 50mmol/l GTP stock solution was added to the collected tubulin supernatants to give a final GTP concentration of 0.25 mmol/l and the supernatant was incubated at 37°C for 45 minutes. This

concentration of GTP was required to accelerate the rate of microtubule polymerization and to maintain the structure of microtubules formed at 37°C, (78). After incubation the crude tubulin preparation was centrifuged (75,000 g), for 45 minutes at 30°C. The supernatant was then aspirated off and discarded and the tubulin pellet frozen in liquid nitrogen and stored overnight at -20°C. This completed the first disassembly - assembly cycle.

The second disassembly - assembly cycle was initiated the following day when the crude tubulin pellet was thawed at room temperature and re-suspended in isolation buffer, (Appendix 1), containing 0.25 mmol/l GTP, pH 6.8 and the whole maintained in ice for 1 hour. The suspension was centrifuged at 75,000 g for 45 minutes at 4°C to remove impurities and the supernatant was separated and retained whilst the pellet of waste materials was discarded. This supernatant was again incubated at 37°C with 0.25 mmol/l GTP and 8 mmol/l glycerol and subsequently centrifuged (75,000 g) for 45 minutes at 30°C. This step completed the second cycle of disassembly - assembly and the sample was again frozen in liquid nitrogen and stored at -20°C.

The tubulin concentration produced by this method was usually in excess of 5 mg/ml (estimated by measuring the absorbance obtained at 280 nm, see Appendix 2 page 261 and consisted of 95% pure tubulin, (78). Table 4.1.

When purified tubulin or crude supernatant was incubated without GTP at 37°C, no microtubule assembly

Table 4.1. Percentage purity of tubulin (78).

CONDITIONS	Electron Microscopy *	Protein in Pellet % **	Purity of Tubulin % ***
Crude supernatants			
In Isolation Buffer			
(Appendix 1 page 252)	++	4	60
+ sucrose (1 mol/l)	+++	8 - 10	70
+ glycerol (4 mol/l)	++++	11 - 15	75 - 85
Partially purified			
In Isolation Buffer			
(Appendix 1 page 252)	+++	35 - 55	95 +
+ sucrose (1 mol/l)	++++	65 - 70	95 +
+ glycerol (4 mol/l)	++++	80	95 +

* Stained with 1 % uranyl acetate and examined on a 400-mesh grid square. 10 squares counted on each grid.

\+ = less than 5 tubules/square
++++ = more than 500 tubules/square.

** Protein content measured by Folin-Lowry Method (Appendix 2, page 261), (176).

*** Tubulin content measured by electrophoresis in sodium dodecyl sulphate - 8M urea polyacrylamide gels stained with fast green.

was observed even after 12 hours, (78). However, when GTP-free incubations are performed in the presence of glycerol or sucrose, a few microtubules were observed after 30 minutes and the majority appeared after 5 hours, (78,), Table 4.2.

Glycerol is usually preferred to sucrose because of the reduced risk of attack from micro-organisms, Table 4.2. and glycerol is thought to promote microtubule assembly via a general thermodynamic interaction with the protein (172,173). This interaction involves the release of water molecules which takes place during microtubule formation. Glycerol does not change the critical concentration for tubulin assembly - 0.2 mg/ml, (71), but at high concentrations stabilizes the microtubules by depressing their rates of assembly and disassembly, (174). However, the microtubules formed in the presence of glycerol may not represent the state of microtubules *in vivo*. 'C' shaped microtubules are often observed which probably represent sheets of open protofilaments, (175).

Table 4.2. Effect of GTP and Glycerol on Tubulin Polymerization (78).

CONDITIONS	30 minute incubation	4 hour incubation
Tubulin + GTP (1 mmol/l)		
In Isolation Buffer (Appendix 1)	+++	+++
+ sucrose (1 mol/l)	++++	++++
+ glycerol (4 mol/l)	++++	++++
Tubulin - GTP		
In Isolation Buffer (Appendix 1)	0	0
+ sucrose (1 mol/l)	+	+++
+ glycerol (4 mol/l)	+	++++

Tubulin samples were stained for electron microscopy with 1 % uranyl acetate after 30 minute and 4 hour incubations The samples were then examined on a 400- mesh grid square. 10 squares were counted on each of 2 grids.

\+ = less than 5 tubules/square
++++ = more than 500 tubules/square

II PURIFICATION OF PORCINE BRAIN TUBULIN AND ASSOCIATED PROTEINS.

The tubulin pellet containing microtubules and their associated proteins, MAP's and Tau, prepared and stored as described in section I, (page 165), is stable at -20°C for several weeks until required. The half life of decay, as measured by the ability to reassemble, is 30 days, (78). In order to separate pure tubulin from the microtubule associated proteins, the pellet was resuspended in an approximately equal volume of isolation buffer, (see Appendix 1, page 252). The pellet was homogenised in a Jencons hand operated homogenizer with a Teflon pestle, retained on ice for 1 hour and subsequently centrifuged (75,000g) for 1 hour at 4°C.

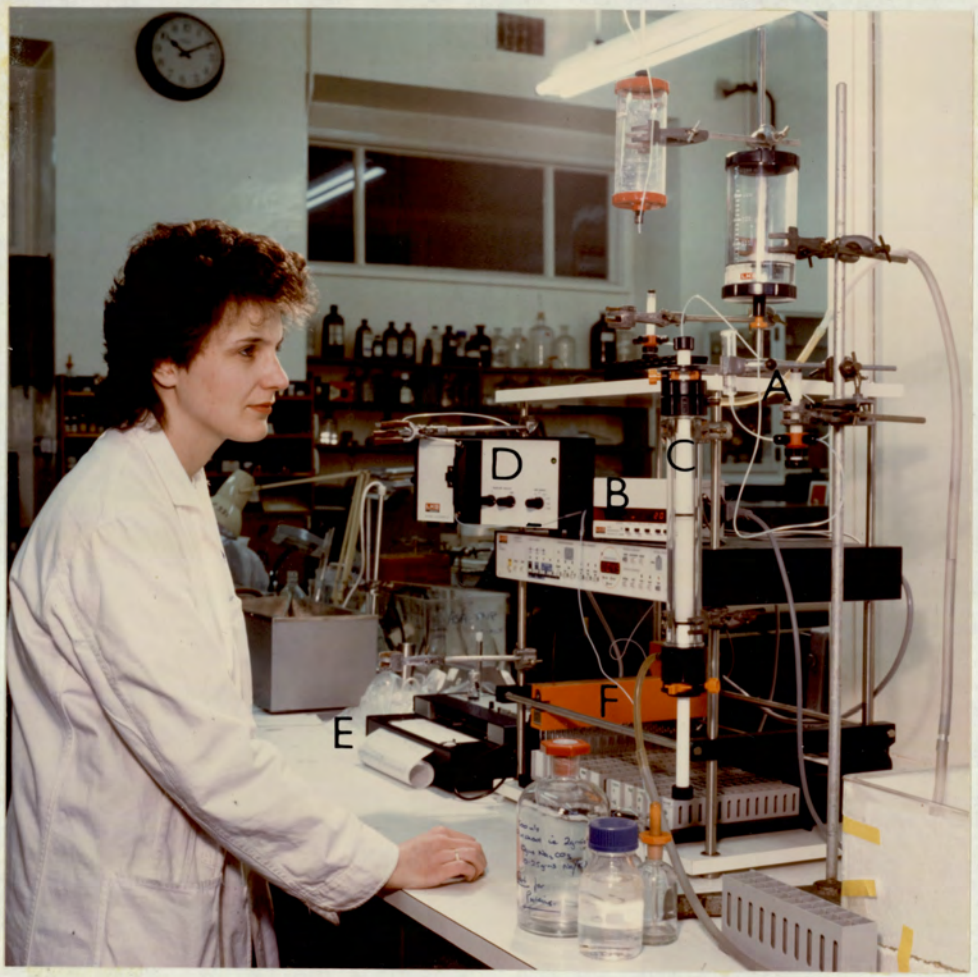
In order to transfer the protein to a lower MES concentration and to remove most of the glycerol (effectively reducing the viscosity of the tubulin solution), the supernatant (approximately 10 ml), was passed through a Sephadex G25 column (void volume = 25ml), equilibrated with 35 mmol/l MES-EDTA purification buffer pH 6.8, (the exact composition is given in Appendix 1, page 252). The crude tubulin was eluted in three fractions and monitored at 280nm using a LKB Ultraspec 2080 spectrophotometer, as described in Appendix 2, page 261). The fractions were pooled and concentrated in disposable Centricon protein concentrators (Amicon). Each concentrator contained a

filter which permitted the separation of molecules with molecular weights less than 30KDa. Centrifugation of the concentrators was carried out using a bench centrifuge, with a fixed head rotor at a 30° angle for 1 hour. This procedure had the effect of concentrating the tubulin sample, maximising the concentration of the tubulin yield prior to separation of tubulin and associated proteins, (MAP's and Tau proteins).

The concentrated crude tubulin fraction was then eluted through a phosphocellulose cation - exchange column, pH 6.8, to separate tubulin from MAP's and Tau proteins. Prior to use the phosphocellulose was stirred and washed sequentially for 30 minutes with 50% ethanol, NaOH 0.5 mol/l, distilled water, HC1 0.5 mol/l, and distilled water again until the pH remained constant at pH 6.8. Care was taken at each stage to prevent the phosphocellulose from drying out. After washing, the phosphocellulose was equilibrated in ten times normal strength MES-EDTA purification buffer, pH 6.8, and then poured as a slurry into an LKB column (1.6 cm x 20 cm). The phosphocellulose was then re-equilibrated by elution with at least three volumes of normal strength MES-EDTA purification buffer, (3 x 40 ml).

The concentrated tubulin sample was applied to the column via a 10 ml syringe which acted as a sample reservoir. A three way tap directly connected the sample reservoir and a buffer reservoir with the column as shown in Plate 2.1 The tap was positioned in such a way that

Plate 2.1. Apparatus used for the purification of tubulin



A - Three way tap
B - Pump
C - Phosphosphocellulose column
D - Spectrophotometer
E - Recorder
F - Fraction collector

Flow rate = 0.3 ml/minute
Fraction size = 1.5 ml/5 minutes

the tubulin sample was eluted through the column first, immediately followed by the elution/purification buffer (MES-EDTA pH 6.8). The crude tubulin sample was then eluted through the phosphocellulose column and 1.5 ml fractions collected, (flow rate = 0.3 ml/min). The absorbance of the eluate was monitored at 280 nm and a peak corresponding to tubulin obtained. A typical trace is shown in Figure 4.1.

Fractions of purified tubulin in 25 mmol/l MES-EDTA, were pooled and eluted through a Sephadex G25 column with 0.5 mol/l phosphate buffer pH 7.4 effectively changing the ambient buffer prior to radio-iodination of the tubulin. Fractions (0.5 ml) from the G25 column were pooled and concentrated in Centricon concentrators to give approximately 1-2 mg/ml of tubulin when estimated using Folin- Lowry Appendix 2, page 261 (176). The tubulin concentrations at each purification step and separation stage for three tubulin preparations A, B and C, have been summarised in Table 4.3.

Tubulin processed through phosphocellulose can no longer polymerize to form microtubules. This is because 5% of the total microtubule protein applied to the column is retained. This 5% is responsible for the initiation of microtubule polymerization and is composed of the proteins MAP 1, MAP 2 and Tau proteins, (174). These microtubule associated proteins copurify with tubulin for as many as 6 cycles before passing through phospho-cellulose. In addition, the larger 36S aggregates of

Figure 4.1 Elution of crude tubulin on phosphocellulose at pH 6.8

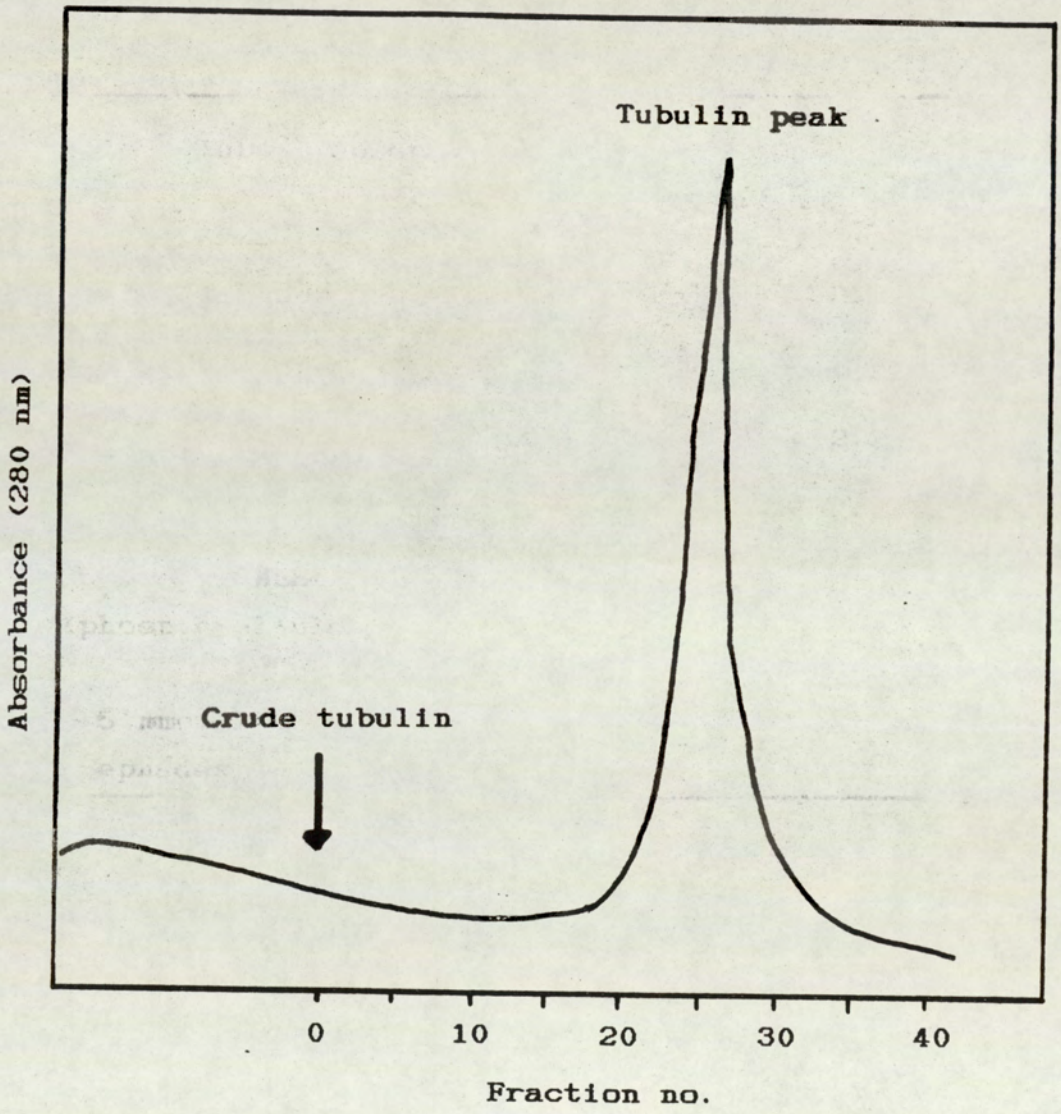


Chart speed = 0.5 mm/minute
Flow rate = 0.3 ml/minute
Fraction size = 1.5 ml/5 minutes.

Table 4.3 Protein concentrations at each stage in the purification and separation stages.

Protein concentration was measured by Folin Lowry (176).

	Tubulin preparation	A (mg/ml)	B (mg/ml)	C (mg/ml)
1.	Isolation buffer 100 mmol/l MES	-	2.5	10.0
2.	25 mmol/l MES-EDTA buffer (Sephadex G25 column)	7.4	2.2	5.3
3.	25 mmol/l MES-EDTA buffer (phosphocellulose column)	4.4	-	3.7
4.	0.5 mmol/l Phospate buffer (Sephadex G25 column)	0.88	1 .15	1.9

tubulin are retained in the phosphocellulose column, accounting for the loss of the characteristic light scattering pattern of tubulin (174). The tubulin eluted from the column is composed of 6S dimers which are incapable of forming the 36S aggregates required for microtubule formation in the absence of microtubule associated proteins 1 and 2 and Tau proteins, (174). Microtubule associated proteins and Tau proteins can be eluted from the phosphocellulose column using 25 mmol/l MES-EDTA buffer containing 1 mmol/l NaCl. If MAP's 1 and 2 and Tau proteins are added to the purified tubulin, microtubules will again form. However, once the polymerization of tubulin has been initiated by these proteins it will continue in their absence, (174).

Since only unpolymerized (dimeric) tubulin was required for radioimmunoassay purposes the MAP's 1 and 2 and Tau proteins were retained in the phosphocellulose column and the separated tubulin fractions were pooled and frozen at -20°C prior to use.

III PREPARATION OF RADIOIMMUNOASSAY COMPONENTS

a) PREPARATION OF PORCINE TUBULIN RADIOIMMUNOASSAY STANDARDS

The tubulin concentration in samples collected from the final purification stage using 0.5 mol/l phosphate buffer pH 7.4 was determined using the Folin-Lowry Method described in Appendix 2, page 261, (176). Tubulin concentrations at the end of the purification stage for three typical tubulin preparations A, B and C are given in Table 4.3. Having determined the tubulin concentration in the final purification sample, 1 ml of the tubulin sample was diluted with radioimmunoassay buffer A, 0.1 mol/l phosphate pH 7.6, Appendix 1, page 252 to give a final tubulin concentration of 4,0000 ng/ml. This stock tubulin concentration represented the highest radioimmunoassay standard. 800 µl aliquots of this tubulin standard (4,000 ng/ml) were transferred to LP3, stoppered and stored at -20°C until required for radioimmunoassay. The remaining undiluted tubulin from the purification process was frozen at -20°C in 100 µl aliquots to be used for iodination.

Prior to radioimmunoassay, one of the 800 µl aliquots, (4,000 ng/ml), was allowed to thaw at room temperature and subsequently vortex mixed. This stock standard was serially diluted with radioimmunoassay buffer A to give a range of standard tubulin

concentrations between 1 - 4,000 ng/ml. 100 µl aliquots were then dispensed in triplicate for use in radio-immunoassay.

b) PREPARATION OF IODINATED PORCINE TUBULIN

Purified Porcine tubulin was labelled with ^{125}I in in preference to ^{131}I because of the former's longer half life, (60 days) and greater stability.

Peptides can be radioiodinated if they possess a tyrosyl or histidyl residue in their amino acid sequence. ^{125}I undergoes electrophilic substitution with one of the hydrogen atoms in the aromatic ring of the residue by the following stochiometric reation. (177)

(i) $^{125}I \longrightarrow {}^{125}I^{+} + 2e$

(ii) $^{125}I^{+}$ + H-Tyr-Tubulin $\longrightarrow$ ^{125}I-Tyr-Tubulin + H^{+}

The incorporation of ^{125}I into the tyrosine residue requires an electron acceptor, the most commonly used oxidising agent is chloramine-T. In addition to the use of chloramine-T, there are several alternative methods which can be used for the incorporation of ^{125}I into proteins. These are the use of N-bromosuccinimide (177), iodine monochloride (178), lactoperoxidase (179), 1,3,4,6-tetrachloro-3α, and 6α-diphenyl glycouril (Iodo-

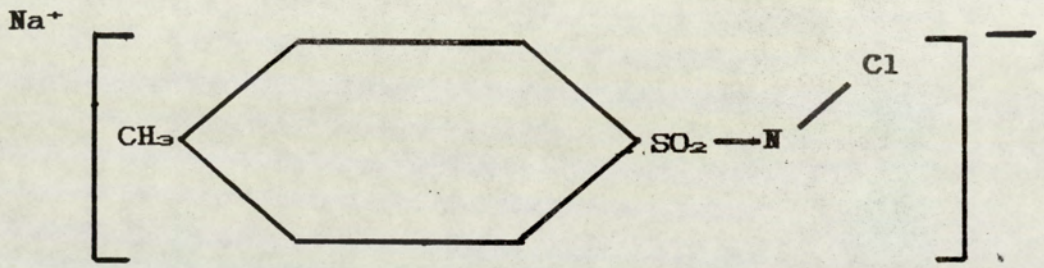
Gen) coated tubes (180,181). These methods incorporate much smaller amounts of ^{125}I into the protein, generating labelled species with lower specific activities and less iodination damage. Specific activity is defined as the unit radioactivity per unit mass and is usually expressed in terms of $\mu Ci/\mu g$, whilst damage refers to the loss of biological activity which occurs when compounds are iodinated, generally to a high specific activity).

(i) The Chloramine-T Method for the Iodination of Tubulin

This method has the advantages of being simple, rapid, gives a high incorporation of ^{125}I into the tyrosyl or histidyl residues of proteins and a high specific activity. Although problems are encountered with protein degradation, the chloramine-T method is widely used for radiolabelling proteins. It was first used to iodinate growth hormone (182) and later to iodinate insulin (183).

Chloramine-T, the sodium salt of the n-monochloro derivative of p-toluene sulphonamide oxidises $Na^{125}I$ by slowly breaking down in aqueous solution to form hypochlorous acid, - a mild oxidising agent, Figure 4.2. The ^{125}I produced on oxidation is then incorporated into the tyrosyl or histidyl residue of the protein required. Excess chloramine T is reduced by the addition of sodium

Figure 4.2 The structure of chloramine-T. (185)



metabisulphite. The latter when dissolved in water forms sodium bisulphite ($NaHSO_3$) which stops the oxidation of iodine and its subsequent incorporation into the protein.

Procedure for the iodination of purified tubulin using the Chloramine-T method.

The procedure used was a combination of that used by Le Guern, Pradelles and Dray (184), modified in our laboratory on the basis of experience with the iodination of insulin. A 50 ml chromatography column containing Sephadex G75 fine was prepared using 0.5 mol/l phosphate buffer (PBS) pH 7.4, as eluent containing 1 mmol/l calcium chloride and 0.6 mmol/l magnesium chloride, Appendix 1, page 252. The column was primed with eluent containing 2.5% BSA to reduce tubulin adsorption to the sephadex and the column wall.

The ^{125}Iodine, was purchased from Amersham in the form of sodium iodide (S.A. = 13.4 mCi/µg) in sodium hydroxide solution 2mCi/10 µl batches. The contents of the reaction vial was divided into two 5 µl volumes and 5 µl was transferred to a second reaction vial. One of the vials was stored in the refrigerator at 4°C for use as required, whilst the contents in the other (^{125}Iodine) was made up to 30 µl by adding 25 µl of 0.5 mol/l phosphate buffer, pH 7.4, containing 0.15 mol/l NaCl, (1 mCi/30 µl). The vial was stoppered and inverted several

times. 6.25 µg of purified tubulin in 0.5 mol/l phosphate buffer, pH 7.4, (14 µl of a 0.44 mg/ml purified tubulin solution) was then added to the reaction vial and the latter was mixed by inversion.

125 µl of fresh chloramine-T solution, (400 mg/100ml), was then added to the vial and the contents allowed to react for 1 minute at room temperature. (The total reaction volume was 169 µl).

After 1 minute, 250 µl of sodium metabisulphite, (2.5 mg/ml), was added to reduce the excess chloramine-T and free iodine to iodide. 200 µl BSA V (2.5%) in 0.5 mol/l phosphate buffer, pH 7.4, was then added to the reaction mixture to act as a carrier for the labelled tubulin. 10µl of the reaction mixture was removed and placed in a separate LP3 tube for the estimation of specific activity and percentage damage.

Iodination damage is defined as the loss of biological activity which occurs when compounds are iodinated to high specific activity, (185). Introduction of ^{125}I into the protein itself can alter the structure of the protein. Other possibe causes of damage are radiation damage and chemical damage which can be caused by either the oxidising agent (chloramine-T) or the reducing agent (sodium metabisulphite).

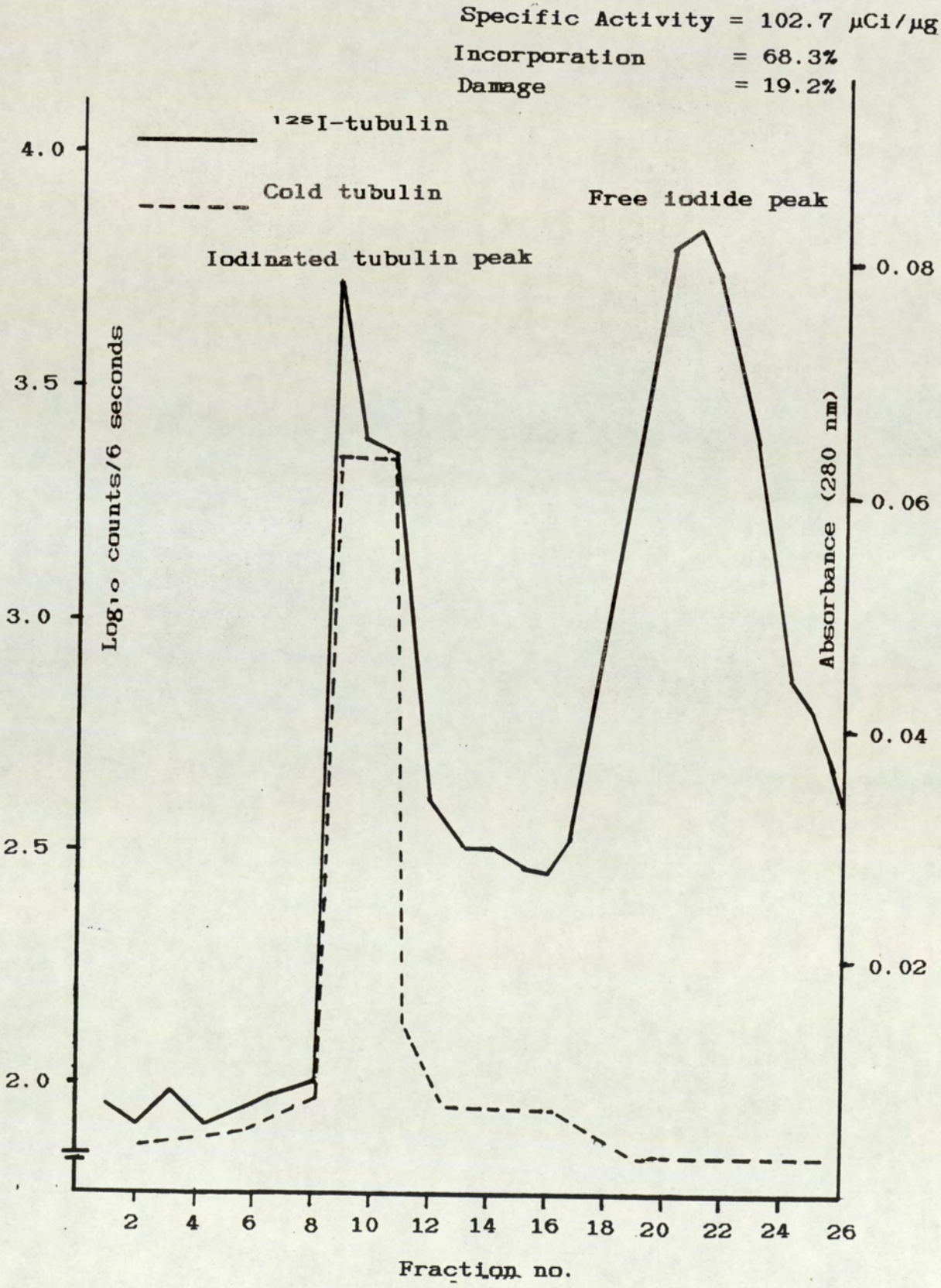
The total reaction mixture (less 10µl) was added to the top of the Sephadex G75 column and the labelled species separated by elution using 0.5 mol/l phosphate buffer, pH 7.4, (flow rate = 0.75 ml/minute) after

allowing for void volume . 0.75 ml fractions were collected in LP3 tubes which were then stoppered and counted for 3 seconds in a gamma counter, (ICN Tracerlab). The elution profile for labelled species obtained by plotting the radioactivity in each fraction versus fraction number, Figure 4.3 indicated two distinct peaks, - the first corresponded to ^{125}-tubulin, (confirmed by passing unlabelled tubulin through the column and measuring absorbance at 280 nm) and the second free iodide.

In an attempt to absorb more of the free iodide (^{125}I), KI-tyrosine in 0.5 mol/l phosphate buffer, pH 7.4, was substituted for 2.5% BSA after stopping the reation with sodium metabisulphate. The purpose of this modification was to reduce the amount of iodination damage to the tubulin and reduce the specific activity of ^{125}I tubulin generated, (185).

Table 4.4 summarises the results obtained using chloramine-T iodination of tubulin which shows that the addition of KI-tyrosine effectively reduced the percentage damage and specific activity. Sample methods used for calculating typical values of percentage incorporation, specific activity and percentage damage are given in Appendix 3, page 267 .

Figure 4.3 Typical elution profile of reaction products from a chloramine-T iodination of purified porcine tubulin separated on Sephadex G75



Flow rate = 0.75 ml/minute Void volume = 6.0 ml

Problems encountered using the Chloramine-T method of tubulin iodination.

The specific activities for ^{125}I-tubulin obtained using chloramine-T iodination were reasonably satisfactory, Table 4.4. The exception to this is the specific activity obtained when KI-tyrosine was added at the end of the iodination reaction to absorb excess iodide. The low incorporation and specific activity suggested that KI-tyrosine absorbed much more free iodide than BSA in the previous iodinations and may even have absorbed the ^{125}I bound to tubulin.

However, the percentage damage in all cases was high, (the mean value was 22.6% ± 2.1, n=4, for the iodinations carried out in the absence of KI-tyrosine). Ideally one would find values of 2-6% to be acceptable. This high percentage damage could have resulted from;

1. The length of the reaction period might have been too long. However, when iodinations were carried out using a reaction time of 45 seconds there was no significant change in the percentage damage.
2. The high percentage damage might have resulted from an inadequate separation of the ^{125}I-tubulin from the iodinated fragments and free ^{125}iodide by column chromatography.

Table 4.4. Chloramine-T iodination of purified porcine tubulin.

Sephadex	Reaction Time (seconds)	Incorporation (%)	Damage (%)	Specific Activity (μCi/μg)
G75	60	69.0	21.4	89.3
G75	60	88.3	20.9	132.8
G75	60	68.3	19.2	102.7
G75	45	37.8	28.8	53.1
Mean n=4 (±SEM)		65.8 (±10.4)	22.6 (±2.1)	94.5 (±16.5)
G75	60	58.5*	20.2*	10.3*

* KI-tyrosine added to reaction in 0.5 mol/l phosphate buffer, pH 7.4.

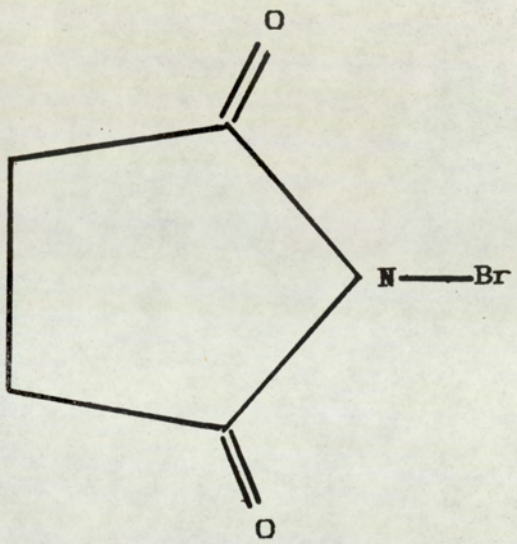
In an attempt to reduce iodination damage during the labelling of tubulin, it was decided to investigate the use of bromosuccinimide, a much more gentle method of iodination, (177). The structure of bromsuccinmide is given in Figure 4.4.

The bromosuccinimide method of iodination employed the same reaction mechanism as the chloramine-T method, but the former is a much milder oxidising agent and is routinely used to iodinate human prolactin, follicle stimulating hormone (FSH), luteinsing hormone (LH), thyroid stimulating hormone (TSH) and growth hormone, (177).

(ii) Procedure for the iodination of purified tubulin dimers using bromosuccininmide.

10µl of $Na^{125}I$ in NaOH (S.A. = 13.4 mCi/µg), was diluted with 15 µl of 10 mmol/l phosphate buffer, pH 7.4, Appendix 1, page 252 , to generate a specific activity of 1 mCi/25 µl. 7.5 µl of this solution containing approximately 300 µCi of ^{125}I was then transferred to a glass reaction vial containing 6 µg/ml of purified tubulin. 5 µl of bromosuccinimide, (0.2 mmol/l) was added to the vial to initiate the reaction. The contents were quickly vortex mixed and allowed to stand at room temperature (20°C) for 30 seconds. After this time 75 µl of 10 mmol/l phosphate buffer, pH 7.4, containing 1

Figure 4.4 The structure of bromosuccinimide (185)

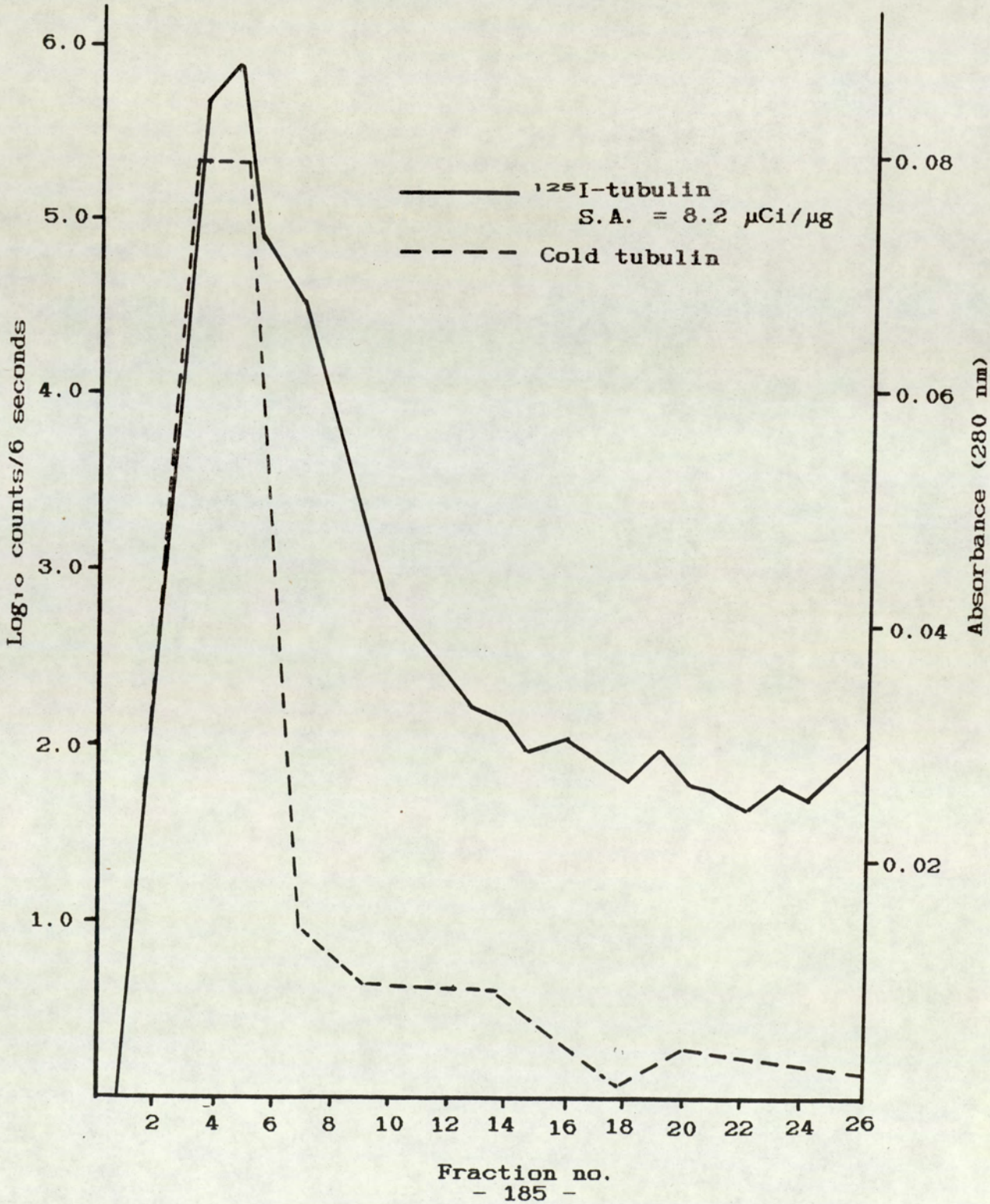


mmol/l potassium iodide and 1 mmol/l tyrosine, Appendix 1, page 252, was added to stop the reaction. The tyrosine was used to mop up excess iodide, which at this pH is very slightly ionised to the anion. The entire reaction mixture was then counted and applied together with several washings of 10 mmol/l phosphate buffer, ph 7.4, containing 0.5% BSA and 10 mmol/l EDTA, to the top of a Sephadex G35 column.

Elution was effected with 0.1 mol/l phosphate buffer, pH 7.4 containing 0.9% NaCl and 0.5% BSA and 250 µl fractions were collected and their radioctivity counted for 6 seconds in a gamma counter. The three fractions containing the highest activity of ^{125}I-tubulin were pooled and retained for later passage through a Sephadex G75 column for more efficient separation of free iodide and identification of ^{125}I-tubulin in these fractions.

A sample of purified tubulin dimers at the same concentration as that used in iodination was eluted through the Sephadex G75 column using the same phosphate 0.1 mol/l buffer, pH 7.4. The absorbance of each of the 0.5 ml fractions was monitored at 280 nm using an LKB Ultrospec 2080 Spectrophotometer. The three iodinated tubulin fractions were then pooled and eluted through the G75 column and the radioactivity of the 0.5 ml fractions measured in a gamma counter. Elution profiles for cold and labelled tubulin are shown in Figure 4.5. The distribution of the peaks in the profiles confirmed the

Figure 4.5 Elution profiles for ^{125}I-tubulin and cold purified porcine tubulin to confirm the integrity of ^{125}I-tubulin



production of ^{125}I-tubulin. The results of representative tubulin iodinations using bromosuccinimide have been summarised in Table 4.5. In several iodinations the KI-tyrosine buffer was replaced with 30 mmol/l phosphate buffer, pH 7.4, containing 10 mmol/l EDTA and 0.5% Tween-20, (polyoxyethylene sorbitan monlaurate), with the result that the specific activities obtained were significantly reduced.

In addition iodinations were also carried out using 30 mmol/l phosphate buffer 30mmol/l, containing 10 mmol/l EDTA and 0.5% Tween-20, pH 7.4, with the reaction carried out over ice, in an attempt to maintain the integrity of the dimeric tubulin, Table 4.5. This manoeuvre did not significantly improve the specific activity.

Table 4.5. Bromosuccinimide iodination of purified tubulin.

Sephadex	Bromo-succinimide (mmol/l)	Reaction Time (seconds)	Incorporation (%)	Specific Activity (μCi/μg)
G75	0.2	30	63.3	63.0
G75	0.1	15	38.4*	15.4*
G75	0.1	15	32.5*	8.6*
G75	0.1	15	33.6†	8.9†
Mean n=4 (±SEM)			42.0 (±7.2)	24.0 (±13.1)

* KI-tyrosine introduced in 0.01 mol/l phosphate buffer, pH 7.4.

† Iodination reaction carried out over ice.

IV THE PRODUCTION OF ANTIBODY BINDING CURVES TO ESTABLISH THE OPTIMUM ANTIBODY TITRE FOR RADIOIMMUNOASSAY

A binding curve was constructed by first carrying out a serial dilution of the first antibody, used in the tubulin radioimmunoassay, rabbit antitubulin, (purchased from Miles Yeda Ltd., Batch A0311. The antitubulin had been raised in rabbits against purified chick brain tubulin isolated from 13 day old chick embryos. The purity and homogeneity of the chick brain tubulin had been previously determined by sodium dodecyl sulphate - polyacrylamide gel electrophoresis, (SDS-PAGE).

The rabbit antitubulin was diluted with phosphate buffer A, pH 7.6, see Appendix 1 page 252, to provide antibody titres over the range 1:5 x 10^2 to 1:5 x 10^5.

100 µl of each antitubulin titre/concentration was transferred to LP3 tubes in triplicate. 50 µl of radioimmunoassay buffer A (0.1 mol/l phosphate buffer pH 7.4) was aliquoted into 3 separate LP3 tubes to act as blanks. 50 µl of ^{125}I-tubulin of known specific activity, (see Tables 4.4 and 4.5), was diluted with buffer A to give a total count of 1 x 10^4 cpm per 50 µl aliquot. 50 µl of this label was then added to every tube including the blanks and to three empty LP3 tubes to provide a measure of the total amount of radioactivity added (Totals). All the tubes except the 'Totals' were vortex mixed vigorously, covered with a strip of Nesco-

film and allowed to stand at room temperature for 24 hours. This time period was considered sufficiently long for the antitubulin titres and ^{125}I-tubulin to reach equilibrium (186).

After this time, 100 µl of the second antibody, anti-rabbit IgG, (Miles Yeda Ltd., Batch R2113), was diluted with buffer B, (buffer A containing 0.05 mmol/l EDTA, see Appendix 1, page 252), to give a final antibody titre of 1:16. 100 µl of buffer B was added to all the tubes except the totals and the blanks and the tubes were then vortex mixed, re-covered with Nescofilm and allowed to incubate for a further 24 hours at room temperature. This allowed the anti-rabbit IgG to bind and preciptiate the complex formed by the antitubulin and ^{125}I-tubulin.

After incubation all the tubes except the totals were centrifuged at 3,000 rpm for 1 hour at room temperature in an MSE centrifuge. The supernatants were decanted off and the last drops aspirated with the aid of a drawn out pasteur pipette. All the tubes except the totals were inverted over absorbant paper and allowed to dry for approximately 2 hours prior to counting for 1 minute in a gamma counter. The protocol for the addition of reaction components is summarised in Table A.6.

The first attempts at generating a binding curve using this protocol were not successful since problems were encountered with the stability of the antibody complex pellet formed after centrifugation. The pellet had a tendency to slip down the tube upon inversion. The

Table 4.6. Protocol for the addition of reaction components inorder to produce an antibody binding curve.

Initial Reactant (100 µl)	^{125}I-tubulin (11,000 cpm) (50 µl)	anti rabbit IgG diluted 1:16 in buffer B (50 µl)
Antitubulin titres		
1 : 5 x 10^{-2}	+	+
1 : 1 x 10^{-3}	+	+
1 : 2 x 10^{-3}	+	+
" "	+	+
" "	+	+
1 : 5 x 10^{-5}	+	+
Blank Buffer A	+	Buffer B
Total -	+	-

bound radioactivity associated with the final pellets after decantation was very low suggesting that little binding of cold tubulin and ^{125}I-tubulin with antitubulin had actually occurred, regardless of the antibody titre used. It remained to be established whether the reduced binding was due to an inadequate binding of ^{125}I-tubulin with antitubulin in the first instance or whether it was due to poor separation of the antitubulin - ^{125}I-tubulin complex from free unbound ^{125}I-tubulin. The following manouevres were carried out in an attempt to improve the final separation stage.

(i) <u>The use of potato starch and centrifugation to improve the separation of antitubulin - ^{125}I-tubulin complex from free unbound ^{125}I-tubulin.</u>

1 ml of potato starch (purchased from Sigma) suspended (0.5%) in buffer B Appendix 1 page 252, was added to each of the tubes used in the protocol for the formation of a binding curve after the second 24 hour incubation period. The tubes were then centrifuged and drained as described earlier but the pellets, although clearly visible as white precipitates at the bottom of the tubes, still collapsed when the tubes were inverted to expel supernatant. Centrifuging the tubes at higher speeds up to 4,000 rpm and for longer periods up to 2 hours, did not prevent the loss of the pellets.

Separation using potato starch was therefore discontinued.

(ii) Separation of antibody bound and free ^{125}I-tubulin using Protein A.

Protein A (Miles Laboratories UK. Ltd.) is a bacterial adsorbent formed by formalin fixation and heat inactivation of Staphylococcus Aureus Cowan 1 strain bearing Protein A on its surface. Protein A has been used successfully in the assay of Ferritin and Digoxin (187,188). Separation of radioactive label bound to antibody and unbound radioactive label works upon the principle that Protein A has a high affinity constant for the Fc portion of IgG of the antibody to which the 'bound' radioactive label is attached. Therefore, Protein A should bind directly with the IgG portion of antitubulin and replace the second antibody, anti rabbit IgG, as a precipitating agent in the separation stage.

40 µl of a 10% suspension of Protein A in buffer A (Appendix 1 page 252) was added to each tube except the totals 24 hours after the addition of antitubulin. The tubes were then vortex mixed and left for a further 40 minutes at room temperature to allow Protein A binding to the Fc portion of antitubulin. At the end of this period the tubes were centrifuged at 3,000 rpm for 15 minutes to form a pellet of Protein A bound antitubulin - ^{125}I-

tubulin complex. The unbound ^{125}I-tubulin in the supernatant was then decanted off. However, problems were still encountered with the pellet which had a tendency to collapse.

Modifications of the Protein A separation method.

a) The effect of increasing centrifugation speed and duration on the separation of unbound ^{125}I-tubulin.

In order to produce a firmer pellet of Protein A bound antitubulin - ^{125}I-tubulin complex, the centrifugation speed was increased from 3,000 to 4,000 rpm, together with an increase in the centrifugation time from 15 minutes to 60 minutes. In addition 1 ml of buffer A (Appendix 1 page 252) containing 0.5% BSA and 10 mmol/l EDTA was added, to each tube except the totals immediately prior to centrifugation, to act as a buffer wash to remove any ^{125}I-tubulin sticking to the walls of the LP3 tubes. The 10 mmol/l EDTA was added to reduce possible interference by Ca^{2+} ions in Protein A antitubulin - ^{125}I-tubulin binding.

The binding curve created for antitubulin and ^{125}I-tubulin using Protein A in the separation stage and a buffer A wash containing 0.5% BSA and 10 mmol/l EDTA, together with 60 minutes centrifugation at 4,000 rpm gave low percentage antibody-label binding values, with a maximum of 28.5% ± 3.2% n=5. The whole procedure

described above was repeated using fresh label, (SA = 63 μCi/μl), but this did not significantly improve the percentage binding values for antibody bound ^{125}I-tubulin. A representative binding curve is shown in Figure 4.6.

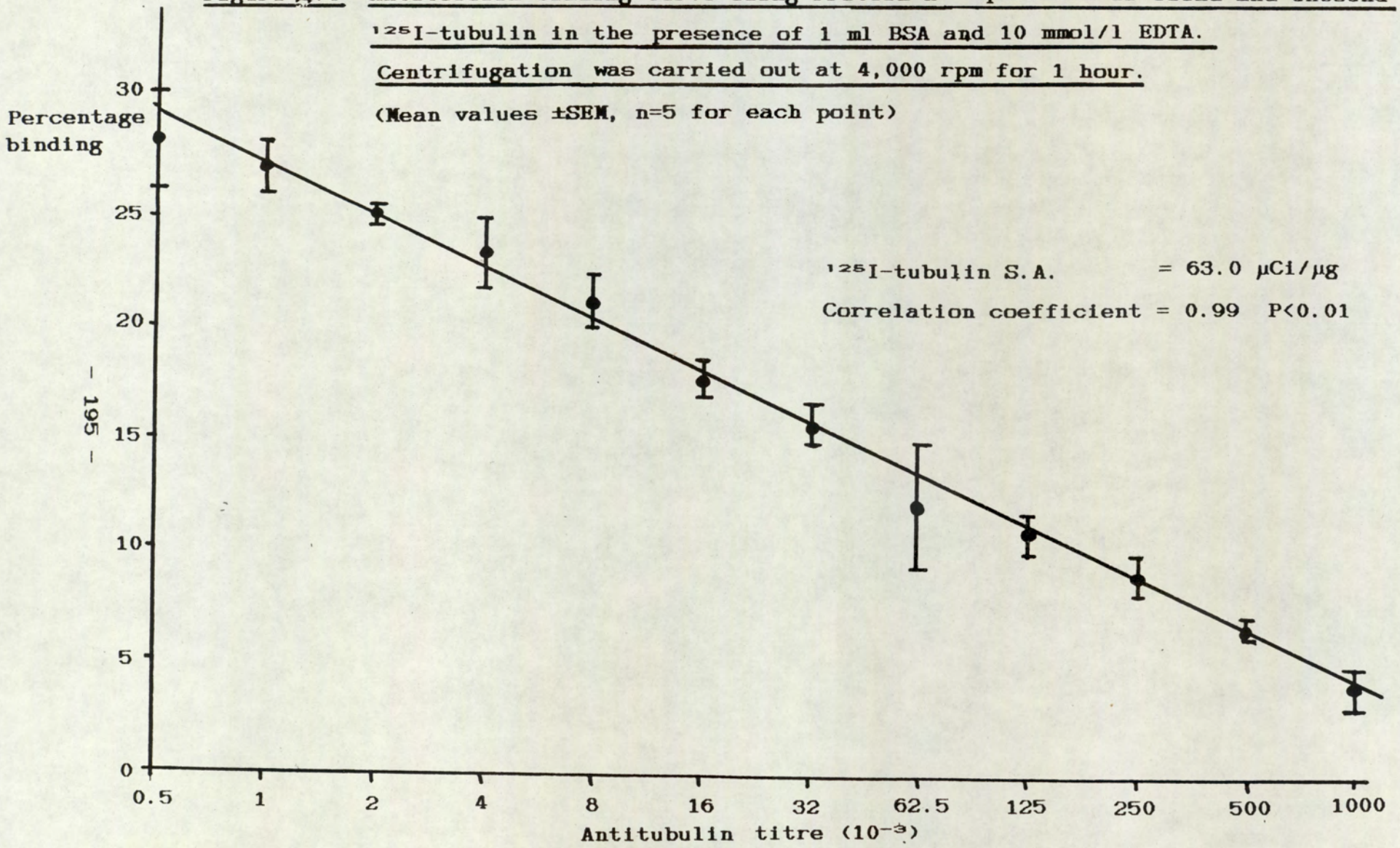
b) The effect of washing the tubes with buffer A containing 0.5% BSA and 10 mmol/l EDTA immediately prior to the addition of Protein A and subsequent centrifugation.

A further iodination (SA = 15.37 μCi/μg) was carried out to investigate the effect of washing with 1 ml of buffer A Appendix 1 page 252 containing 0.5% BSA and 10 mmol/l EDTA prior to the addition of Protein A. On this occasion, the binding curve assays were carried out in duplicate using a range of antitubulin dilutions from 1: 4 x 10^{-3} to 1:5 x 10^{-2}. Two sets of LP3 tubes were set up in duplicate, each set containing identical anti-tubulin titres, totals and blanks. 50 μl of ^{125}I-tubulin (11,000 cpm) was added to each tube and after vortex mixing, both sets of tubes were allowed to stand at room temperature for 24 hours, allowing the antitubulin and ^{125}I-tubulin to reach equilibrium binding as before.

At the end of this incubation period, the two sets of tubes were divided to form two identical assays, each containing the same range of antibody titres and ^{125}I-tubulin. 40 μl of buffer A containing 10% Protein A was

Figure 4.6 Antitubulin binding curve using Protein A separation of bound and unbound ^{125}I-tubulin in the presence of 1 ml BSA and 10 mmol/l EDTA. Centrifugation was carried out at 4,000 rpm for 1 hour.

(Mean values ±SEM, n=5 for each point)



added to the first set of tubes (all except the totals) and these were then incubated at room temperature for 40 minutes and subsequently centrifuged at 4,000 rpm for 60 minutes in an MSE bench centrifuge. The second set of tubes each received 40 µl of Protein A in buffer A and were incubated for 40 minutes at room temperature (like the first set of assay tubes), but 1 ml of buffer A (Appendix 1 page 252) containing 0.5% BSA and 10 mmol/l EDTA was added to all the tubes except the totals just prior to centrifugation. Each tube was then vortex mixed vigorously and centrifuged at 4,000 rpm for 60 minutes.

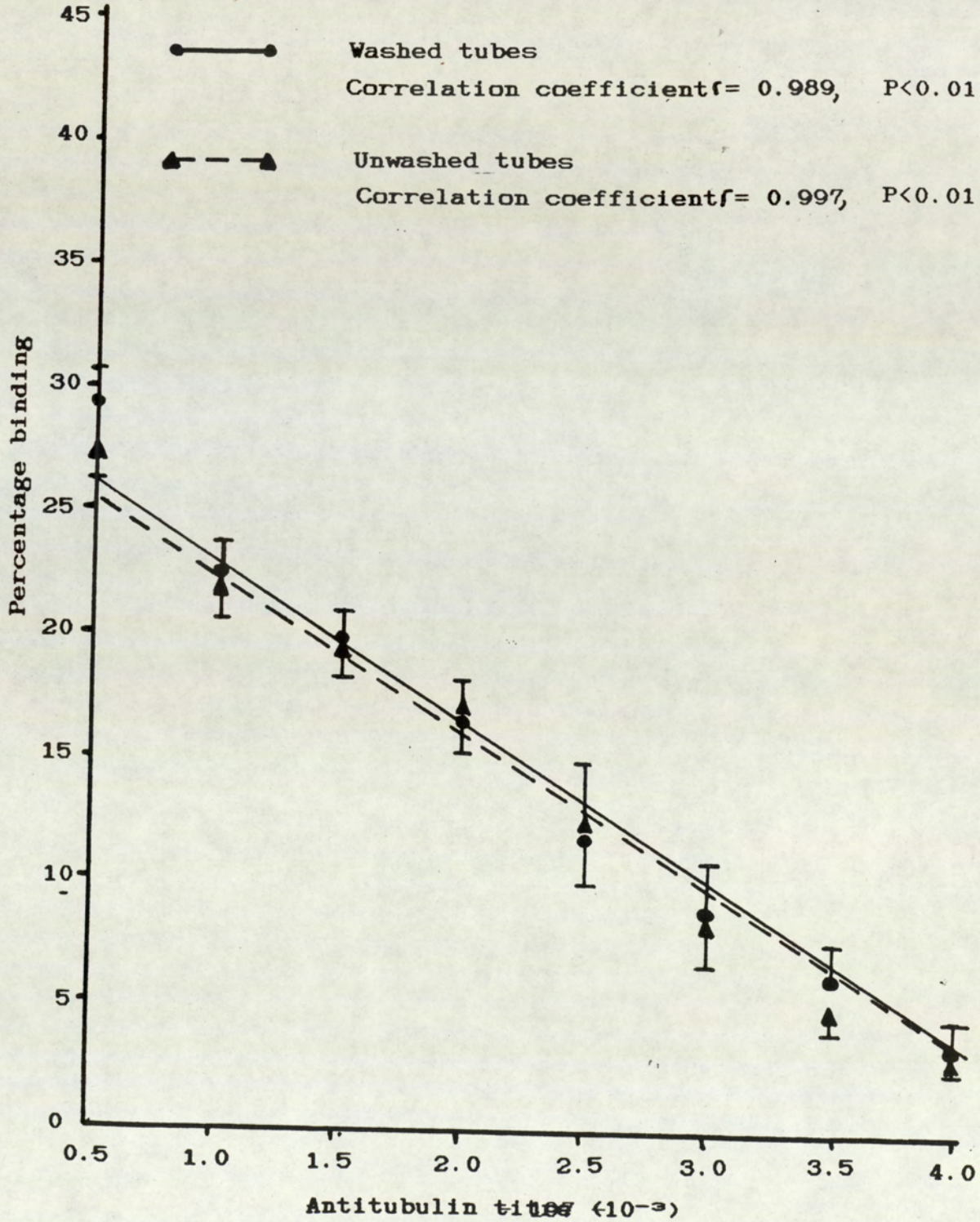
The effect of washing with buffer A containing 0.5% BSA and 10 mmol/l EDTA on the antitubulin binding curve is shown in Figure A.7. This washing manouevre had no significant effect on the percentage of ^{125}I-tubulin bound at any of the antitubulin titres used. This would suggest that very little ^{125}I-tubulin was bound to the walls of the LP3 tube and that the interference of complement with Protein A and antitubulin binding was negligible.

c) The effect of increasing the volume of 10% Protein A on the separation of antibody bound ^{125}I-tubulin and free ^{125}-tubulin.

The basic binding assay procedure was repeated in duplicate using antitubulin titres $1{:}4 \times 10^{-3}$ and $1{:}5 \times 10^{-2}$. 50 µl of buffer A containing 10% Protein A

Figure 4.7 The effect of washing with buffer A containing 0.5% BSA and 10 mmol/l EDTA on the antitubulin binding curve.

(Mean values ±SEM, n=5 for each point)



(Appendix 1 page252 was added to one set of tubes inorder to precipitate the antibody-label complex, whilst 25 µl of Protein A was added to the other set.

The binding curves obtained with the two volumes of Protein A are shown in Figure 4.8. The addition of 50 µl of 10% Protein A significantly increased the percentage binding of antibody bound label.

Finalised protocol for the generation of a tubulin - ^{125}I-tubulin binding curve

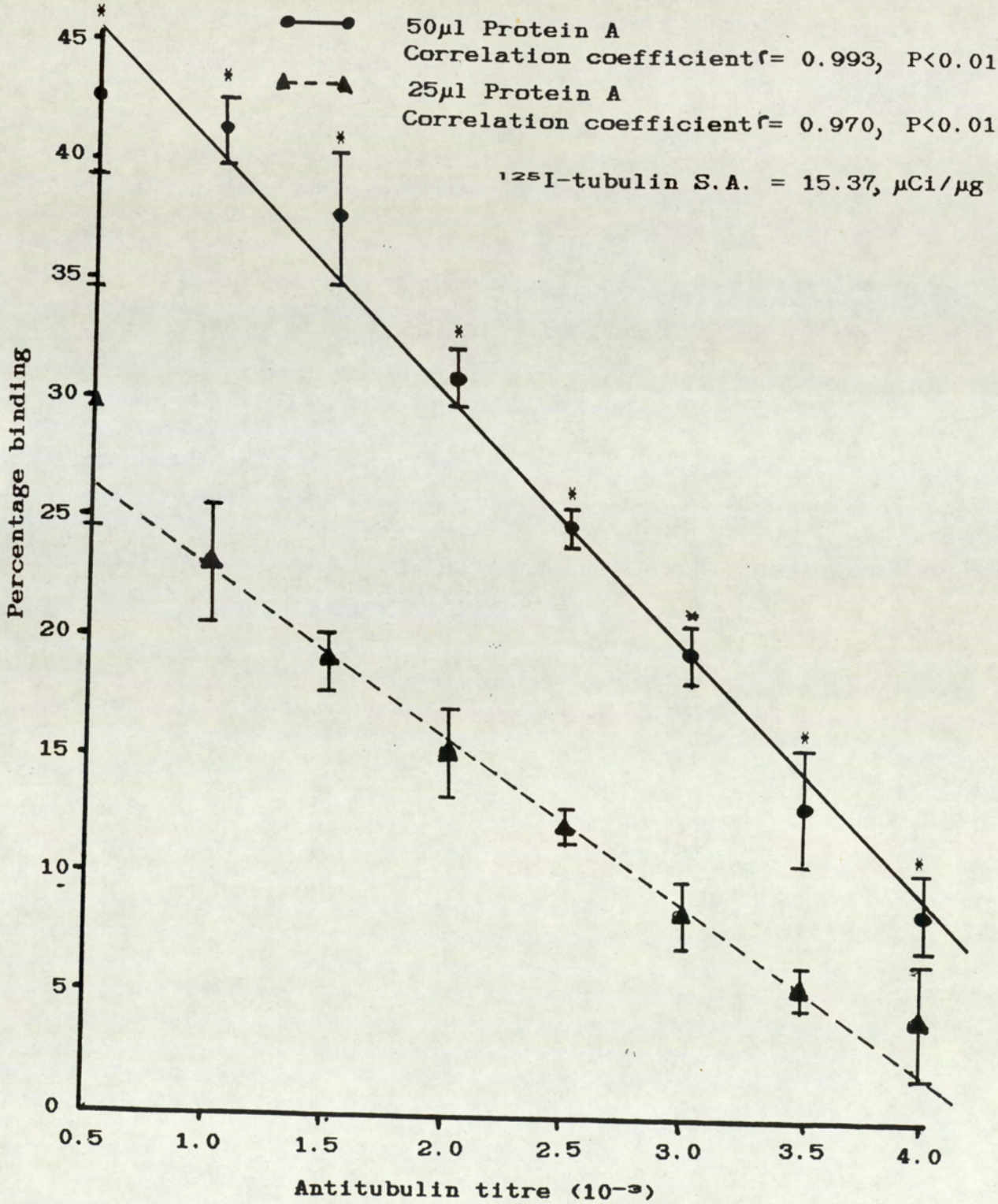
As a of result the previous attempts to improve the separation of antitubulin bound ^{125}I-tubulin from free unbound ^{125}I-tubulin and subsequently increase the percentage binding of the antitubulin bound ^{125}I-tubulin, it was decided to adopt the use of a 50 µl aliquot (Figure 4.8) of 10% Protein A in buffer B (Appendix 1 page 252), containing 10 mmol/l EDTA as the precipitating agent instead of the second antibody, anti rabbit IgG. In addition the second 24 hour incubation period was reduced to 40 minutes.

The centrifugation speed for separation of antitubulin bound ^{125}I-tubulin and free ^{125}I-tubulin was maintained at 4,000 rpm for 60 minutes and the supernatant containing unbound ^{125}I-tubulin was aspirated off with the aid of a pasteur pipette. The LP3 tubes were finally inverted over absorbant paper to drain. All

Figure 4.8 The effect of increasing the volume of Protein A on the antitubulin binding curve.

(Mean values ±SEM, n=5 for each point)

* = Significantly higher ^{125}I-tubulin antitubulin binding observed using 50 µl of Protein A (P<0.025)



the tubes were then counted in an ICN Tracerlab gamma counter for 1 minute as usual.

A finalised protocol for the addition of components required for the construction of the antitubulin - ^{125}I-tubulin binding curve is given in Table 4.7 and the binding curve obtained is shown in Figure 4.9. An antitubulin titre of 1:1 x 10^{-3} was selected for use in the subsequent tubulin radioimmunoassays, since this titre gave marginally higher percentage binding value compared with the value for 50% of the maximum percentage binding obtained, Figure 4.9. Since the maximum value for the percentage binding Figure 4.9 was low (42.6%), it was considered unwise to select the antitubulin titre which gave 50% of this value ie. 21% binding, since this would confer low sensitivity on the tubulin radioimmunoassay, (189).

The production of a competitive binding curve for the binding of ^{125}I-tubulin and cold tubulin with antitubulin.

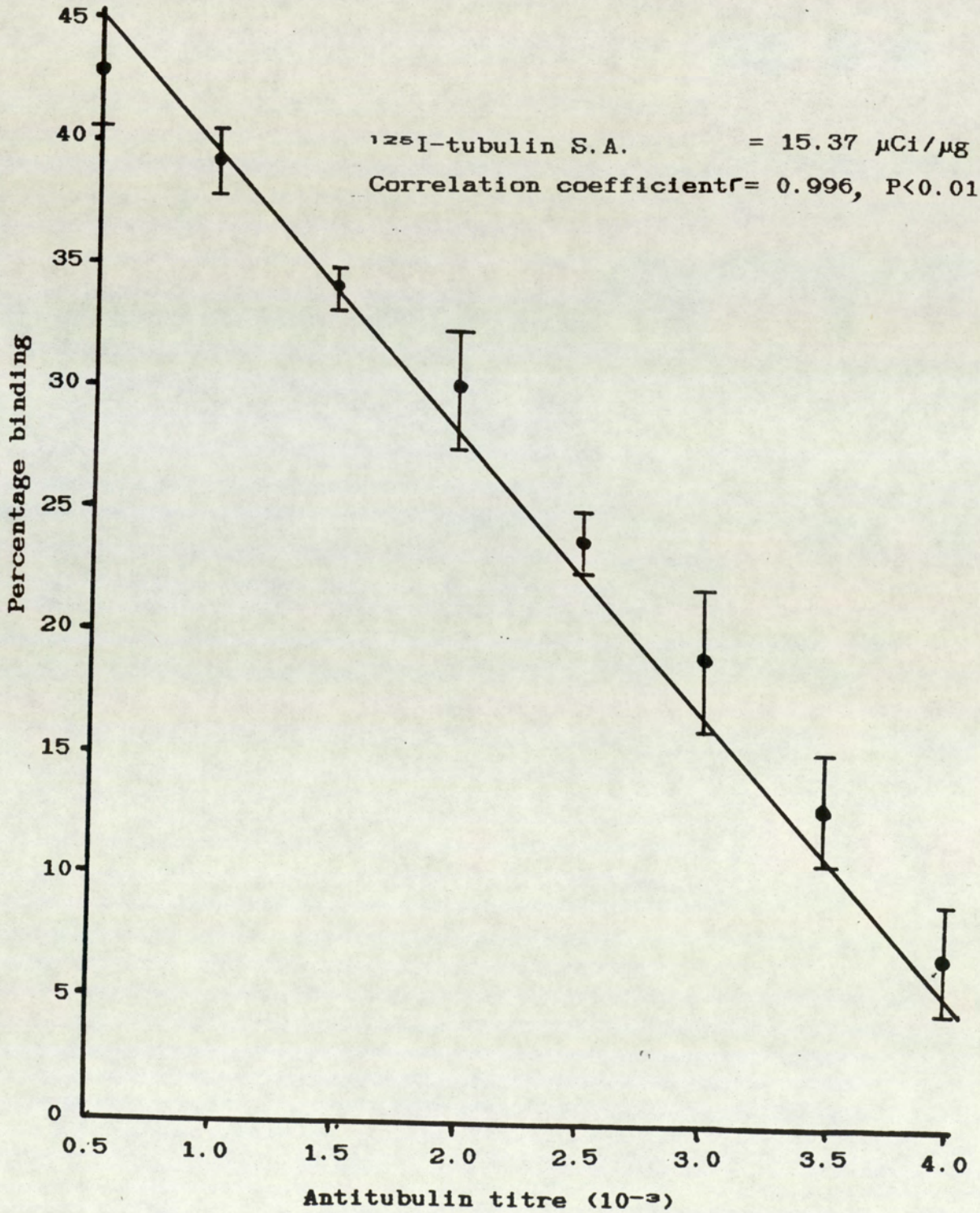
The competitive binding curve is the product of the competition between ^{125}I-tubulin and standard tubulin concentrations for binding sites on the antitubulin molecule. This competition curve forms the basis of the standard curve in subsequent tubulin radioimmunoassays.

Table 4.7 Finalised protocol for the addition of components required for the construction of the antitubulin - ^{125}I-tubulin binding curve

Initial reactant (100µl)	Label (11,000 cpm) (50µl)	10% Protein A in buffer B+ 10 mmol/l EDTA (50µl)
Antitubulin titres		
1 : 5.0×10^{-2}	+	+
1 : 1.0×10^{-3}	+	+
1 : 1.5×10^{-3}	+	+
1 : 2.0×10^{-3}	+	+
1 : 2.5×10^{-3}	+	+
1 : 3.0×10^{-3}	+	+
1 : 3.5×10^{-3}	+	+
1 : 4.0×10^{-3}	+	+
Blank Buffer A	+	+
Total -	+	+

Figure 4.9 Finalised antitubulin-^{125}I-tubulin binding curve.

(Mean values ±SEM, n=5 for each point)



The antitubulin titre selected was 1 x 10^{-3} from the tubulin - ^{125}I-tubulin binding curve, Figure 4.9 and the tubulin standards were prepared in buffer A (Appendix 1 page 252) to provide a range of tubulin concentrations between 1 and 4,000 ng/ml.

100 µl of the tubulin standards was placed in LP3 tubes in triplicate. To each was added 100 µl of antitubulin diluted 1 x 10^{-3} in buffer A (Appendix 1 page 252). Blank tubes received 200 µl of buffer A only. 50 µl of prediluted label (11,000 cpm /50µl, SA = 15.37 µCi/µl) was added to every tube including three empty tubes. The latter tubes indicated the total radioactivity added to each tube - 'totals'. All the tubes were then vortex mixed vigorously, covered with Nescofilm and allowed to stand for 24 hours at room temperature.

At the end of this period, the separation of unbound label was carried out by adding 50 µl of 10% Protein A in buffer B containing 10 mmol/l EDTA (Appendix 1 page 252) and incubating for 40 minutes at room temperature. This was followed by centrifugation at 4,000 rpm for 60 minutes. The supernatant was decanted and the tubes inverted over tissue paper for 2 hours to dry. The radioactivity in each tube was then counted for 10 minutes in a gamma counter. A protocol for the addition of the components for the generation of the competitive binding curve is shown in Table 4.8 and the curve generated is shown in Figure 4.10.

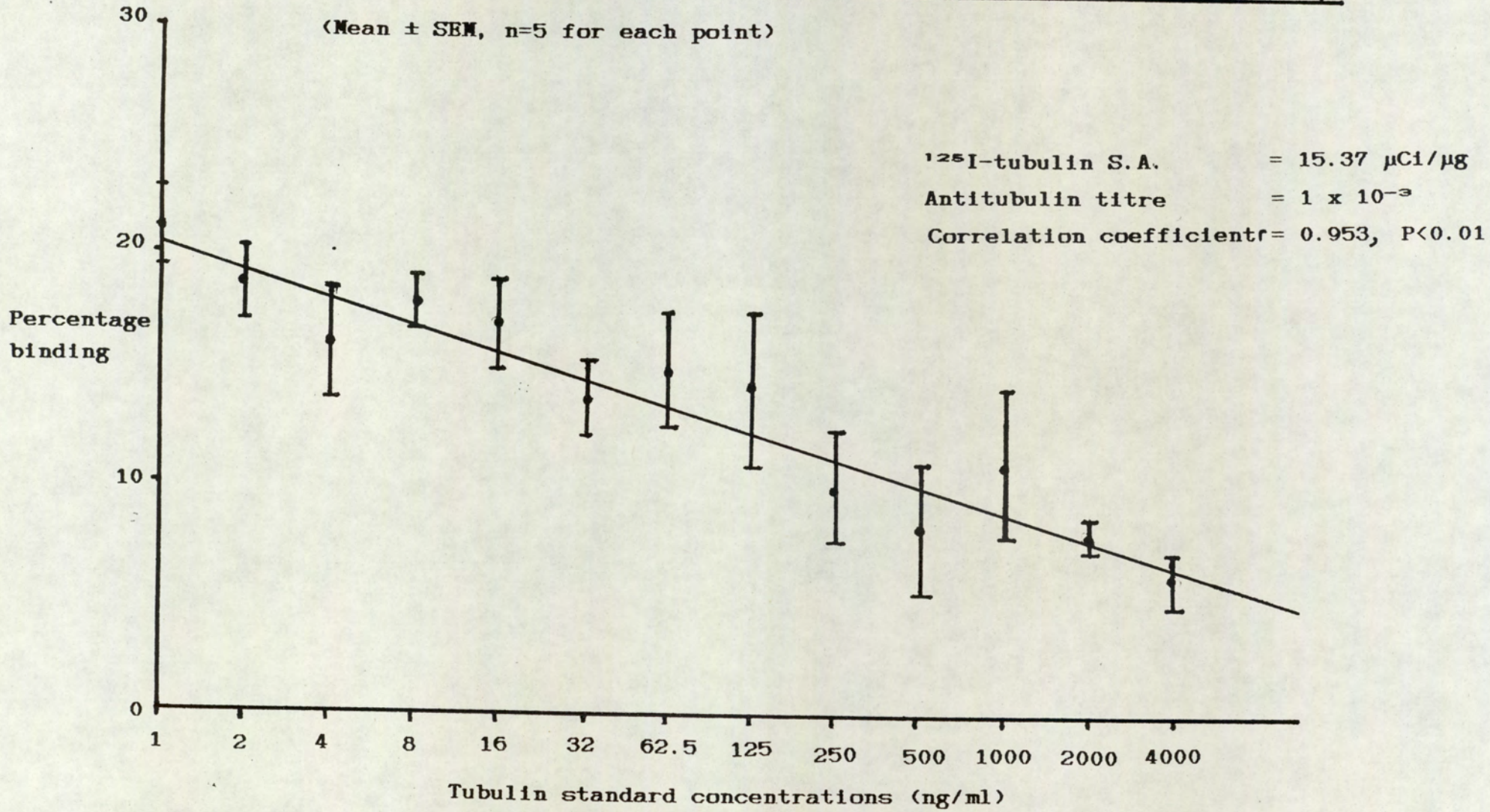
The gradient of the line was very low, suggesting poor sensitivity for the subsequent tubulin radio-immunoassay. Large differences in the standard tubulin concentration did not produce correspondingly large differences in the percentage radioactivity bound.

Table 4.8. **Protocol for the addition of components in the generation of a competitive binding curve, (standard curve).**

Initial Reactant (100µl)	Antitubulin 1×10^{-3} (100µl)	^{125}I-tubulin (11,000cpm) (50µl)	Protein A (50µl)
Tubulin Standards			
4,000 ng/ml	+	+	+
2,000 ng/ml	+	+	+
" "	+	+	+
1 ng/ml	+	+	+
Blank Buffer A	Buffer A	+	+
Total –	–	+	–

Figure 4.10 Competitive binding curve (standard curve) for tubulin radioimmunoassay

(Mean ± SEM, n=5 for each point)



V ESTIMATION OF THE TUBULIN CONTENT OF ISLETS FROM LEAN AND OBESE MICE. THE EFFECT OF GLUCOSE STIMULATION.

Islets were processed for tubulin radioimmunoassay according to the method of Pipeleers and coworkers, (190). 250 lean mouse islets and the same number of obese mouse islets were isolated by collagenase digestion (page 84) from overnight fasted mice. Each batch of islets was incubated in 7.5 ml of oxygenated Krebs-Hepes buffer pH 7.4 containing either 5.56 mmol/l or 16.7 mmol/l glucose for 60 minutes at 37°C in a 10 ml flask. Incubation in 5.56 mmol/l glucose was carried out to give some indication of basal (non-stimulatory) insulin release. Incubation of islets with 16.7 mmol/l glucose was carried out inorder to ensure that the β-cells were actively secreting insulin and conceivably might bring about a subsequent increase in the number of microtubules formed within the β-cell cytosol.

After incubation the islets were transferred to a 10 ml Sterilin centrifuge tube and pelleted by light centifugation at 1,000 rpm for 2 minutes. The supernatant was aspirated off and discarded, whilst the islet pellet was re-suspended in 150 µl of isolation buffer containing 5% DMSO, 8 mmol/l glycerol and 0.25 mmol/l GTP, pH 6.8, Appendix 1, page 252 , at room temperature, to preserve the microtubulules formed during the 16.7 mmol/l glucose incubation. The islets were

homogenised using a Jencons hand operated glass homogenizer with a Teflon pestle and the homogenate was subsequently centrifuged at 75,000g in a MSE Superspeed 50 centrifuge at room temperature for 30 minutes.

The supernatant (SN-1) was removed with a Pasteur pipette and 25 µl aliquots were placed in 1ml microfuge tubes (Beckman) and frozen at -20°C until required. The islet pellet containing microtubules was resuspended and homogenized with the Jencons hand operated glass homogeniser with a Teflon pestle in ice cold isolation buffer Appendix 1, page 252, containing 0.25 mmol/l GTP and 8 mmol/l glycerol but in the absence of DMSO. The suspension was then centrifuged at a speed of 75,000g for 30 minutes at 4°C. The supernatant (SN-2) was decanted and collected, aliquoted and frozen at -20°C. The second remaining pellet containing the residual islet proteins was discarded.

The first supernatant (SN-1) represented the pool of unpolymerized tubulin dimers present in the β-cells, whilst the second supernatant (SN-2) represented the tubulin polymerized in the form of microtubules during insulin secretion. The latter were subsequently depolymerized at 4°C to form tubulin dimers. The last stage in the isolation process separated tubulin polymerized in the form of microtubules from the residual islet cell nuclear and membrane proteins to provide tubulin in the dimeric form required for radioimmunoæsay.

Prior to tubulin radioimmunoassay, the supernatants were removed from the freezer and allowed to thaw at room temperature. 75 µl of radioimmunoassay buffer A Appendix 1, page 252 was then added to each sample (total volume = 100 µl) and vortex mixed.

Samples were then assayed for tubulin using the radioimmunoassay procedure described previously, page 200 Tubulin standards were prepared by dilution of tubulin (prepared in the isolation procedure, page 160), with buffer A Appendix 1, page 252, to provide a range of tubulin concentrations between 1 and 4,000 ng/ml.

100 µl of each of the tubulin standards were transferred to LP3 tubes in triplicate and together with the islet samples (100 µl of either SN-1 or SN-2), each received 100 µl of antitubulin diluted 1 x 10^{-3} in buffer A, Appendix 1, page 252. Blank tubes each received 200 µl of buffer A only. 50 µl of pre-diluted label, (11,000 cpm/50 µl, S.A.= 15.37 µCi/µl) was added to every tube including three empty tubes - 'totals'. All the tubes were then vigorously vortex mixed, covered with Nescofilm and allowed to stand for 24 hours at room temperature. The protocol for the addition of the tubulin radioimmunoassay components is summarised in Table A.9.

At the end of this period, the separation of unbound ^{125}I-tubulin was carried out by adding 50 µl of 10% Protein A in buffer B containing 10 mmol/l EDTA (Appendix 1, page 252) and incubating for 40 minutes at room temperature. This was followed by centrifugation at

Table 4.9. Protocol for the addition of components in the tubulin radioimmunoassay

Initial Reactant (100 µl)	Antitubulin 1 x 10^3 (100 µl)	^{125}I-tubulin (11,000 cpm) (50 µl)	Protein A (50 µl)
Tubulin Standards			
4,000 ng/ml	+	+	+
2,000 ng/ml	+	+	+
" "	+	+	+
1 ng/ml	+	+	+
Samples			
SN-1	+	+	+
SN-2	+	+	+
Blank Buffer A	Buffer A	+	+
Total –	–	+	–

4,000 rpm for 60 minutes. The supernatant was decanted and the tubes inverterted over tissue paper for 2 hours to dry. When dry, the radioactivity in each tube was counted for 10 minutes in a gamma counter (ICN Tracerlab). Finally a standard curve was constructed and the tubulin content of the samples estimated by reference to the standard curve. The tubulin contents of both lean and obese mouse islets incubated with basal 5.56 mmol/l glucose or stimulatory 16.7 mmol/l glucose are shown in Table 4.10.

Table 4.10. The effect of increasing the glucose concentration on the tubulin content of lean and genetically obese mice estimated by the tubulin radioimmunoassay

Islet Type	Glucose concn. (mmol/l)	Fraction	n	Tubulin concn Mean (± SEM) (ng/100 islets)	Total Tubulin (%)
Lean	5.56	SN-1	5	18.53 ± 3.21	85
		SN-2	5	3.27 ± 0.91	15
		SN-1+2	5	21.80 ± 2.69	100
Lean	16.7	SN-1	5	4.64 ± 0.85*	20
		SN-2	5	18.56 ± 2.90**	80
		SN-1+2	5	23.20 ± 3.21	100
Obese	5.56	SN-1	5	19.76 ± 2.11	82
		SN-2	5	4.34 ± 0.78	18
		SN-1+2	5	24.10 ± 3.31	100
Obese	16.7	SN-1	5	4.10 ± 0.89*	15
		SN-2	5	23.21 ± 2.85**	85
		SN-1+2	5	27.30 ± 2.69	100

SN-1 pool of unpolymerized tubulin dimers
SN-2 polymerized tubulin dimers, ie. microtubules
SN-1+2 total tublin content

* Significantly reduced ($P<0.05$) compared with values obtained in the presence of 5.56 mmol/l glucose

** Significantly increased ($P<0.05$) compared with values obtained in the presence of 5.56 mmol/l glucose

Results.

The tubulin contents of lean and obese mouse islets after incubation with a basal non-stimulatory (5.56 mmol/l) and a stimulatory (16.7 mmol/l) glucose concentration are shown in Table 4.10. The stimulation of insulin release with 16.7 mmol/l glucose caused a significant increase in the propotion of polymerised microtubules in islets from both lean and obese diabetic (ob/ob) mice. The sum of SN-1 and SN-2 make up the total tubulin content of the islets. Under basal, non-stimulatory conditions with 5.56 mmol/l glucose, the depolymerized tubulin subunit (dimer) pool (SN-1) composed some 85% and 82% of the total tubulin content of lean and obese mouse islets respectively. Increasing the glucose concentration in the incubation medium to 16.7 mmol/l caused a significant reduction in the size of the depolymerized tubulin fraction SN-1 to 20% and 15% respectively for lean and obese mouse islets, concomitant with a significant increase in the size of the polymerized tubulin fraction SN-2 to 80% and 85% respectively. Thus these data suggest that an increase in insulin release from islets stimulated by glucose concentrations in excess of the basal level is accompanied by the polymerization of tubulin dimers from the β-cell subunit pool to form microtubules.

The total tubulin content of both the lean and obese mouse islets incubated with 5.56 and 16.7 mmol/l glucose, Table 4.10 was considerably lower than values previously

obtained by Pipeleers and colleagues who used a similar method of tubulin extraction from rat islets but a colchicine binding assay for the estimation of tubulin content, (191). These workers obtained levels of 295.3 ±19.5 ng/100 islets in response to glucose (16.7 mmol/l) stimulation. Of this 151 ±3.92 ng/100 islets (51%) was in the polymerized form.

Discussion

The poor sensitivity of the radioimmunoassay is the product of there being little difference between the percentage of ^{125}I-tubulin bound to antitubulin and the percentage of (cold) tubulin bound to antitubulin. In addition the low percentage incorporation of ^{125}I into tubulin obtained during iodination with either chloramine-T or bromosuccinimide and the difficulty in separating bound ^{125}I-tubulin from free ^{125}I-tubulin gave high coefficients of variation for the assay, ie. the inter assay coefficient of variation was 16% ±2.3 and the intra assay coefficient of variation was 15% ±1.9.

The root of the problem may rest in the poor avidity of the antitubulin, giving poor antibody binding. Indeed the maximum percentage binding of ^{125}I-tubulin to antitubulin was 45% approximately, Figure 4.9, which would suggest that the strength of binding was rather weak. There are only a limited number of tubulin antisera available on the commercial market at the moment

and these are generally antisera of low titre and avidity suitable only for the immunofluorescent detection and identification of tubulin rather than its quantitative measurement.

On the basis of the poor sensitivity of the tubulin radioimmunoassay it was decided to compare it with an alternative method of measuring tubulin, namely the colchicine binding assay. This method has been used successfully by Montague and Howell who utilised the initial method of Weisenberg and Borisy, (192,193), to measure tubulin dimers and polymerized microtubules.

B. THE DEVELOPMENT OF A ^{3}H-COLCHICINE BINDNG ASSAY FOR THE ESTIMATION TUBULIN CONTENT OF LEAN AND OBESE MOUSE ISLETS.

An alternative means for the estimation of tubulin concentration involves the use of the plant alkaloid colchicine. This agent has long been known to cause the breakdown of mitotic spindles by depolymerizing spindle microtubules (194). Colchicine has the unique property of binding specifically, to tubulin preventing the aggregation of tubulin dimers into microtubules, (193,195). It is this unique property which forms the basis of the colchicine binding assay for tubulin, (194).

In the present work tubulin was estimated using the method of Pipeleers (190,191) which is a modification of the original colchicine binding assay developed by Weisenberg and Borisy in 1968 (193). These workers used the assay to estimate the tubulin content of a variety of rat tissues such as liver, kidney, muscle, adrenal glands and spleen, (196). Tritiated colchicine is used in the assay and the unbound ^{3}H-colchicine remaining after saturation of the sample tubulin is removed with charcoal. The tubulin concentration is calculated on the assumption that at saturation, 1 mole of colchicine is bound per mole of tubulin.

The estimation of depolymerized tubulin involves the use of tubulin stabilizing buffer (TS) pH 6.95, (Appendix 1 page 252) containing sucrose (0.25 mol/l). This buffer

has been reported to preserve colchicine binding activity for at least 2 weeks, (197). The incubation of tritated colchicine and sample tubulin is carried out over ice to ensure the integrity of the dimeric tubulin in the sample for maximum tritiated colchicine binding.

a). Procedure for the ^{3}H-colchicine binding assay using purified tubulin standards of known concentration.

A range of tubulin standard concentrations of 4.5 x 10^{-3} nmol/l to 4.5 x 10^{2} nmol/l was prepared by the serial dilution of a stock purified tubulin of known concentration (for preparation see page 165), with ice-cold tubulin stabilizing buffer (TS) pH 6.95, Appendix 1, page 252. 100 µl aliquots of each tubulin standard were placed in LP3 tubes in triplicate. Triplicate blank and total tubes were prepared containing 100 µl of TS buffer alone according to the protocol summarised in Table 4.11. The blanks represented background binding of ^{3}H-colchicine to the walls of the tubes and the charcoal whilst the totals represented the total radioactivity added to each tube.

100 µl of ice-cold Ring-A-4-^{3}H-colchicne, (Specific Activity = 12.8 mCi/mg), was added to 400µl of ice-cold TS buffer Appendix 1 page 252 containing 0.11 mmol/l unlabelled colchicine and 5 mg BSA V, (0.05% BSA V in each assay vial). 10 µl of ^{3}H- colchicine solution was

Table 4.11 Protocol for the addition of reagents in the colchicine binding assay for tubulin.

Initial reactant (100 µl)		^{3}H-colchicine (10 µl)	activated charcoal/water (1 ml)
BLANK	Buffer	+	charcoal
TOTALS	Buffer	+	water
SAMPLES	Standard tubulin (ng/ml)		
	4.95×10^4	+	charcoal
	" "	+	charcoal
	0.495	+	charcoal

added to each tube prior to incubation for 30 minutes over ice.

After incubation either 1ml of double distilled water or 1 ml of an activated charcoal suspension (3 mg/ml) was added to the tubes as indicated in Table 4.11. All tubes were then retained over ice for a further 10 minutes prior to centrifugation at 1,500 rpm for 10 minutes at 4°C. The activated charcoal served to absorb all the free cold colchicine not bound to tubulin. It was essential that the charcoal concentration did not exceed 3 mg/ml. Since higher concentrations have been shown to remove ^{3}H-colchicine-tubulin complexes from the mixture, (198).

0.5 ml of the supernatant containing ^{3}H-colchicine-tubulin was transferred to 10 ml plastic inserts within glass scintillation vials. 4.5 ml of Micellar NE 260 scintillant (N.E.N. Ltd. UK.) was then added to the insert and the vials counted immediately in a Packard Tri-Carb 2660 Beta counter for 5 minutes. The vials were then stored in a cool dark place for 1 week and subsequently recounted to account for chemiluminescence.

The counts per minute (cpm) obtained for each sample were converted to disintegrations per minute (dpm) with the aid of a quench curve prepared previously using chloroform as the quenching agent, Appendix 1, page 252.

Based on the assumption that at saturation, 1 mole of colchicine is bound per mole of tubulin and that the molecular weight of tubulin dimers is 1.1 kDa, (196), the

tubulin concentration in each sample was calculated as follows:

$$\text{n moles of tubulin} = \frac{\text{sample (dpm)} - \text{blank (dpm)}}{\text{total (dpm)} - \text{Blank (dpm)}} \times 1.1$$

Problems encountered using the colchicine binding assay to estimate the tubulin concentration of tubulin standards.

The tubulin concentrations of the tubulin standards as measured by the colchicine binding assay are summarised together with the protein concentration of each sample as measured by Folin-Lowry (176) in Table 4.12. Folin-Lowry is normally used as a means of estimating the total protein content of a sample but if the sample is exclusively purified tubulin then it may be assumed that Folin-Lowry is measuring the total tubulin content, ie. tubulin being the only protein present in the sample.

The data shown in Table 4.12 indicate that there is no statistical correlation between the estimation of tubulin content of the standards using the colchicine binding assay and the Folin-Lowry method (176). The tubulin content of the original stock sample was initially determined by the Folin-Lowry Method, and the sample was diluted 1:9 in a serial dilution as mentioned

Table 4.12. Comparison of the tubulin content of standards measured by Folin Lowry (176) and the ^{3}H-colchicine binding assay.

n	Tubulin concentration measured by Folin - Lowry Mean ±SEM (ng/ml)	^{3}H-colchicine binding assay Mean ±SEM (ng/ml)
5	$4.95 \times 10^4 \pm 3.31 \times 10^3$	2.816 ± 0.95*
5	$4.95 \times 10^3 \pm 5.21 \times 10^2$	1.914 ± 0.70*
5	$4.95 \times 10^2 \pm 45.30$	1.320 ± 0.81*
5	49.5 ± 4.29	0.374 ± 0.50*
5	4.95 ± 0.24	0.198 ± 0.40*
5	0.495 $\pm 5.6 \times 10^{-2}$	0.099 ± 0.20*

* Significantly lower standard $P<0.05$ tubulin concentrations when measured by the ^{3}H-colchicine binding assay,

earlier, to provide the standards. However, this 10-fold dilution pattern was not seen in the tubulin concentrations were estimated by the colchicine binding assay. This in turn might suggest that the colchicine binding assay lacks discrimination/sensitivity since it was unable to detect a 10 fold difference in concentration.

There are two possible reasons for this lack of sensitivity, the first may be an inadequate binding of the ^{3}H-colchicine to the sample tubulin in the first instance. The second cause may be the inadequate separation of free ^{3}H-colchicine from the tubulin bound ^{3}H-colchicine in the final stages of the assay. The low number of radioactive counts found in the supernatant after centrifugation suggested that a significant portion of both ^{3}H-colchicine, - tubulin bound and free were retained in the activated charcoal pellet.

A series of investigations was therefore carried out in an attempt to improve the sensitivity of the colchicine binding assay for tubulin.

(i) <u>The effect of increasing the incubation period of ^{3}H-colchicine with the tubulin standards from 30 to 60 minutes.</u>

The incubation of ^{3}H-colchicine with the sample was increased from 30 to 60 minutes in tubulin stabilising (TS) buffer pH 6.95, <u>Appendix 1</u>, page 252 over ice.

This was carried out in order to increase the binding of ^{3}H-colchicine to tubulin despite Pipeleers' suggestion that a 30 minute incubation period was sufficient for optimum binding, (190).

The data obtained is shown in Table 4.13. Increasing the incubation period for ^{3}H-colchicine binding with the tubulin sample did not significantly increase the estimate of tubulin content or improve the discrimination/sensitivity.

(ii) The effect of substituting dextran coated charcoal for activated charcoal in the colchicine binding assay.

Dextran coated charcoal was substituted for activated charcoal (3mg/ml) in an attempt to reduce the non-specific adsorption of ^{3}H-colchicine bound tubulin. The data obtained is shown in Table 4.14.

Neither increasing the incubation period for ^{3}H-colchicine binding with tubulin samples or substituting dextran coated charcoal for activated charcoal had any statistically significant effect on either tubulin concentrations or the sensitivity of the colchicine binding assay, Tables 4.13 and 4.14. It was therefore decided to continue using the shorter incubation period of 30 minutes and activated charcoal consistent with Pipeleers' (190) and Sherline's (196) methods.

Table 4.13. The effect of increasing the ^{3}H-colchicine binding assay incubation period from 30 minutes to 60 minutes on the measurement of standard tubulin concentration.

n	Tubulin concentration measured by Folin - Lowry Mean ± SEM (ng/ml)	^{3}H-colchicine binding assay Mean ± SEM (ng/ml)
5	$4.95 \times 10^{4} \pm 3.31 \times 10^{3}$	2.60 ± 0.85*
5	$4.95 \times 10^{3} \pm 5.21 \times 10^{2}$	2.00 ± 0.69*
5	$4.95 \times 10^{2} \pm 45.3$	1.41 ± 0.72*
5	49.5 ± 4.29	0.98 ± 0.60*
5	4.95 ± 0.24	0.23 ± 0.59*
5	0.495 $\pm 5.6 \times 10^{-2}$	0.11 ± 0.49*

* Significantly reduced $P<0.05$ compared with Folin-Lowry.

Table 4.14. The effect of substituting dextran coated charcoal for activated charcoal in the ^{3}H-colchicine binding assay.

n	Tubulin concentration measured by Folin - Lowry Mean ± SEM (ng/ml)	^{3}H-colchicine binding assay Mean ± SEM (ng/ml)
5	$4.95 \times 10^{4} \pm 3.31 \times 10^{3}$	2.54 ± 0.92*
5	$4.95 \times 10^{3} \pm 5.21 \times 10^{2}$	2.10 ± 0.80*
5	$4.95 \times 10^{2} \pm 45.30$	1.56 ± 0.75*
5	49.5 ± 4.29	1.01 ± 0.62*
5	4.95 ± 0.24	0.59 ± 0.54*
5	0.495 $\pm 5.6 \times 10^{-2}$	0.13 ± 0.41*

* Significantly reduced $P<0.05$ compared with Folin-Lowry

(iii) The effect of increasing the concentration of cold colchicine in the colchicine binding assay.

The dilution of ^{3}H-colchicine with cold colchicine was increased from 1:4 to 1:9 inorder to saturate some of the charcoal binding sites with unlabelled colchicine. Increasing the cold colchicine concentration marginally improved the sensitivity/discrimination of the colchicine binding assay but not significantly so, Table 4.15.

(iv) The effect of increasing the BSA concentration in the colchicine binding assay.

The BSA concentration in the cold colchicine solution (0.11 mmol/l colchicine in ice-cold TS buffer) was increased to give a final BSA concentration of 0.1% in each assay tube. This had the effect of only marginally improving the sensitivity of the ^{3}H-colchicine binding assay, Table 4.16.

In a further attempt to improve the sensitivity of the colchicine binding assay, the higher concentrations of cold colchicine and BSA were still retained but instead the concentration of activated charcoal in water was reduced from 3mg/ml to 2mg/ml. Under these circumstances the sensitivity (discrimantion) of the assay did not significantly improve.

An alternative means of separating free and tubulin bound ^{3}H-colchicine was carried out using DEAE cellulose

Table 4.15. The effect of increasing the concentration of cold colchicine in the ^{3}H-colchicine binding assay.

n	Tubulin concentration measured by Folin - Lowry Mean ± SEM (ng/ml)	^{3}H-colchicine binding assay Mean ± SEM (ng/ml)
5	$4.95 \times 10^{4} \pm 3.31 \times 10^{3}$	3.19 ± 1.00*
5	$4.95 \times 10^{3} \pm 5.21 \times 10^{2}$	2.04 ± 0.92*
5	$4.95 \times 10^{2} \pm 45.30$	1.21 ± 0.85*
5	49.5 ± 4.29	0.53 ± 0.71*
5	4.95 ± 0.24	0.35 ± 0.55*
5	$0.495 \pm 5.6 \times 10^{-2}$	0.13 ± 0.41*

* Significantly reduced $P<0.05$ compared with Folin-Lowry

Table 4.16. The effect of increasing the bovine serum albumin concentration in the reaction mixture of the ^{3}H-colchicine binding assay.

n	Tubulin concentration measured by Folin - Lowry Mean ± SEM (ng/ml)	^{3}H-colchicine binding assay Mean ± SEM (ng/ml)
5	$4.95 \times 10^{4} \pm 3.31 \times 10^{3}$	4.01 ± 1.10*
5	$4.95 \times 10^{3} \pm 5.21 \times 10^{2}$	3.15 ± 0.99*
5	$4.95 \times 10^{2} \pm 45.30$	1.95 ± 0.85*
5	49.5 ± 4.29	0.96 ± 0.76*
5	4.95 ± 0.24	0.21 ± 0.62*
5	0.495 $\pm 5.6 \times 10^{-2}$	0.09 ± 0.30*

* Significantly reduced $P<0.05$ compared with Folin-Lowry.

paper discs (Whatman DE 81) as described by Borisy (198) and Montague, Howell and Green, (192).

(v) The effect of using DEAE filter paper discs to separate free and tubulin bound ^{3}H-colchicine in the colchicine binding assay.

Four discs, 2.5 cm in diameter, were cut out of DEAE-cellulose paper, (Whatman DE81), and wetted with filter eluent buffer containing 0.067 mol/l Na phosphate and 0.1 mol/l KCl, pH 6.8, Appendix 1, page 252. The discs were then stacked on top of each other in a Millipore microanalysis filter holder to which 5 ml of filter buffer had been added. 100 µl of the reaction mixture from one of the assay tubes containing free and tubulin bound ^{3}H-colchicine was added to the filter holder together with a further 4.9 ml of filter buffer giving a final volume of 10 ml. Suction was applied to the holder via a water pump and the filter stack washed four times with 10 ml volumes of filter buffer at room temperature. The disc stack was allowed to run dry after the last wash to ensure that all the free ^{3}H-colchicine had eluted from the filter stack. The filter stack was then carefully removed from the holder with forceps and placed in a scintillation vial containing 10 ml of scintillant, (Triton, toluene and PPO, 30:70:0.5, v:v:w. (192)). The whole was then counted for 5 minutes in the

beta counter (Packard Tri-carb 2660) as described previously.

Since the half life of tubulin bound ^{3}H-colchicine has been estimated to be about 36 hours (104,105), it was considered unlikely that the ^{3}H-colchicine - tubulin complexes would dissociate either before or during the filtration process.

The effect of using DEAE filters in the assay of standard tubulin concentrations is shown in Table 4.17. A 10 fold dilution of the standard sample did not produce a 10 fold dilution of the tubulin content, ie. discrimination/sensivity had not improved markedly but the filter method of separation did provide an assay capable of detecting and discriminating between higher tubulin concentrations.

The interassay and intraassay variation was rather high, (21.5 and 15% respectively). Increasing both the number of discs in the filter stack and the number of washings with filter buffer did not bring about a significant improvement.

Table 4.17. The effect of using DEAE cellulose filter paper discs instead of activated charcoal on the separation of unbound ^{3}H-colchicine from tubulin bound ^{3}H-colchicine.

n	Tubulin concentration measured by Folin - Lowry Mean ± SEM (ng/ml)	^{3}H-colchicine binding assay Mean ± SEM (ng/ml)
5	$4.95 \times 10^4 \pm 3.31 \times 10^3$	83.40 ± 19.98*
5	$4.95 \times 10^3 \pm 5.21 \times 10^2$	40.97 ± 15.8*
5	$4.95 \times 10^2 \pm 45.3$	23.25 ± 9.70*
5	49.5 ± 4.29	12.81 ± 6.30*
5	4.95 ± 0.24	8.20 ± 4.80*
5	0.495 ± 5.6×10^{-2}	2.36 ± 1.10*

* Significant difference $P<0.05$ compared with Folin-Lowry.

The estimation of the tubulin content of lean and obese mouse islets after incubation with basal (5.56 mmol/l) and stimulatory (16.7 mmol/l) concentration of glucose using the ^{3}H-colchicine binding assay.

25 µl aliquots of islet extract as previously prepared for tubulin radioimmunoassay page 206 , were used in the ^{3}H-colchicine binding assay. The first supernatant (SN1) separated after centrifugation at 75,000g at room temperature for 30 minutes, represented the pool of unpolymerized tubulin dimers present in the ß-cells during insulin release. The second supernatant separated after centrifugation at 4°C for 30 minutes (SN2), represented the tubulin polymerized in the form of microtubules.

The 25 µl aliquots were thawed and diluted with 75 µl of ice-cold TS buffer, pH 6.95, Appendix 1 page 252 . Triplicate blank and total tubes were prepared containing 100 µl of TS buffer alone. 100 µl of ice-cold Ring -A-4-^{3}H-colchicine (S.A.= 12.8 mCi/mg) was added to 900 µl of ice cold TS buffer containing 0.11 mmol/l cold colchicine and 25 mg BSA V (0.1% BSA V in each assay vial). After adding 10 µl of the ^{3}H-colchicine solution to each tube they were incubated for 30 minutes over ice.

After incubation 100 µl from each assay vial was applied to a filter stack consisting of four paper discs of Whatman E81 Paper in a Millipore holder containing 5 ml of filter buffer, pH 6.8. 4.9 ml of filter buffer

(Appendix 1, page 252) was added to the filter stack and suction applied. Each filter stack was washed four times with 10 ml volumes of filter buffer, pH 6.8, and allowed to run dry. Each filter stack was then carefully removed from the holder and transferred to a scintillation vial containing 10 ml of scintillant. (triton, toluene and PPO 30:70:0.5 v:v:w (192)) and the whole counted in a beta counter for 5 minutes (Packard Tri-carb 2660). The vials were then stored in a cool dark place for 1 week and subsequently recounted to eliminate chemiluminescence.

The tubulin concentration in each sample was calculated on the assumption that at saturation, 1 mole of colchicine is bound per mole of tubulin.

Results.

The estimation of the tubulin content of lean and obese mouse islets after incubation with basal (5.56 mmol/l) and stimulatory (16.7 mmol/l) concentrations of glucose using the ^{3}H-colchicine binding assay.

The stimulation of insulin release from both lean and obese mouse islets with 16.7 mmol/l glucose caused a significant increase in the proportion of islet polymerized tubulin dimers (microtubules), Table 4.18.. During basal glucose stimulation (5.56 mmol/l), the depolymerized tubulin subunit (dimer) pool (SN-1) amounted to 70% and 65% of the total tubulin content of lean and obese mouse islets respectively. Increasing the glucose concentration in the incubation medium to 16.7 mmol/l caused a significant reduction ($P<0.05$) in the size of the depolymerized tubulin fraction SN-1 to 25% and 30% respectively for lean and obese mouse islets, concomitant with a significant increase ($P<0.05$) in the proportion of polymerized tubulin SN-2 to 75% and 70% respectively. Thus these data suggest that the stimulation of insulin release by elevated glucose concentrations is accompanied by the polymerization of tubulin dimers from the β-cell subunit pool to form microtubules.

Table 4.18. The effect of increasing the glucose concentration on the tubulin content of lean and genetically obese mice estimated by the ^{3}H-colchicine binding assay.

Islet Type	Glucose concn. (mmol/l)	Fraction	n	Tubulin concn Mean (± SEM) (ng/100 islets)	Total Tubulin (%)
Lean	5.56	SN-1	5	43.64 ± 8.91	70
		SN-2	5	18.70 ± 4.20	30
		SN-1+2	5	62.34 ± 10.31	100
Lean	16.7	SN-1	5	16.47 ± 4.15*	25
		SN-2	5	49.42 ± 8.59**	75
		SN-1+2	5	65.89 ± 9.98	100
Obese	5.56	SN-1	5	38.90 ± 5.21	65
		SN-2	5	20.95 ± 3.30	35
		SN-1+2	5	59.85 ± 8.45	100
Obese	16.7	SN-1	5	18.98 ± 3.95*	30
		SN-2	5	44.29 ± 6.21**	70
		SN-1+2	5	63.27 ± 9.85	100

SN-1 pool of unpolymerized tubulin dimers
SN-2 polymerized tubulin dimers, ie. microtubules
SN-1+2 total tubulin content

* Significantly reduced ($P<0.05$) compared with values obtained in the presence of 5.56 mmol/l glucose

** Significantly increased ($P<0.05$) compared with values obtained in the presence of 5.56 mmol/l glucose

Discussion.

The tubulin concentration in lean and obese mouse islets measured by the ^{3}H-colchicine binding assay was considerably less than the values obtained by Pipeleers and coworkers (191) using a similar ^{3}H-colchicine binding assay with rat islets, (295.3 ±19.5 ng/100 islets in reponse to 16.7 mmol/l glucose). The ^{3}H-colchicine binding assay developed in this study also had large inter and intraassay coefficients of variation, 18.3% (±2.4) and 16.5% (±2.1) respectively. The lower tubulin concentrations and high coefficients of variations may be the result of problems encountered with the intial binding of ^{3}H-colchicine to tubulin in the first instance or the problems associated with the separation of free ^{3}H-colchicine from tubulin bound ^{3}H-colchicine.

The estimation of the tubulin content of lean and obese mouse islets using both the tubulin radioimmunoassay and the ^{3}H-colchicine binding assay.

A comparison of the data obtained using both the tubulin radioimmunoassay and the ^{3}H-colchicine binding assay for the measurment of the tubulin content of lean and obese mouse islets exposed to increased glucose concentrations is summarized in Table 4.19.

The tubulin concentrations in each sample were always significantly greater ($P<0.05$) when measured by the ^{3}H-colchicine binding assay compared with the tubulin radio-immunoassay, although the proportions of the various extracts did not change significantly. Despite this neither assay method provided tubulin concentrations of the same order as those described by Pipeleers *et al* for rat islets (191).

Discussion.

The higher tubulin concentrations detected by the ^{3}H-colchicine binding assay compared with the tubulin radioimmunoassay suggests that the ^{3}H-colchicine binding assay is the more sensitive assay of the two. However, the inability of the colchicine binding assay to detect very small tubulin concentrations (less than 5 ng) might suggest significant non-specific ^{3}H-colchicine binding creating artificially high tubulin concentrations in the samples.

Table 4.19. The tubulin content of lean and genetically obese mice estimated by the tubulin radioimmunoassay and the ^{3}H-colchicine binding assay.

			Radioimmunoassay		^{3}H-Colchicine Binding Assay	
Islet Type	Glucose concn. (mmol/l)	Fraction	Tubulin concn. (ng/100 islets)	Total Tubulin (%)	Tubulin concn (ng/100 islets)	Total Tubulin (%)
Lean	5.56	SN-1	18.53	85	43.64 *	70
		SN-2	3.27	15	18.70 *	30
		SN-1+2	21.80	100	62.34 *	100
Lean	16.7	SN-1	4.64	20	16.47 *	25
		SN-2	18.56	80	49.42 *	75
		SN-1+2	23.20	100	65.89 *	100
Obese	5.56	SN-1	19.76	82	38.90 *	65
		SN-2	4.34	18	20.95 *	35
		SN-1+2	24.10	100	59.85 *	100
Obese	16.7	SN-1	4.10	15	18.98 *	30
		SN-2	23.21	85	44.29 *	70
		SN-1+2	27.30	100	63.27 *	100

SN-1 pool of unpolymerized tubulin dimers
SN-2 polymerized tubulin dimers, ie. microtubules
SN-1+2 total tublin content

* Tubulin content (mean of 5 determinations) was always significantly higher ($P<0.05$) when measured by ^{3}H-colchicine binding assay.

In both assays problems associated with the initial binding of the radioactive label to tubulin and the separation of free and bound label led to very high inter and intraassay coeficients of variation, (16% ±2.3 and 15% ±1.9 for the tubulin radioimmunoassay and 18.3 ±2.4 and 16.5% ±2.1 for the ^{3}H-colchicine binding assay).

This developmental work suggests that neither of the assays proved 'reliable' for the accurate determination of the tubulin concentration in mouse islets but may be useful to provide some indication of the changes in the size of the tubulin subunit pool within islets upon glucose stimulation. The tubulin radioimmunoassay might provide a better means for this purpose since it was capable of detecting lower tubulin concentrations and possibly more subtle changes in the size of the subunit pool.

Neither the tubulin radioimmunoassay nor the ^{3}H-colchicine binding assay were able to detect tubulin of the same order as values obtained by Pipeleers *et al* using a ^{3}H-colchicine binding assay for the tubulin content of rat islets (191). That there may be significant differences in the total tubulin and tubulin fractions of rat and mouse islets is unlikely and the disparity between tubulin values obtained by Pipeleers *et al* (191) and the data obtained in the present work is more likely to be a product of the numerous problems encountered.

CHAPTER 5

DISCUSSION

The use of isolated islets of Langerhans as an experimental tool has greatly advanced our understanding of the function of the pancreatic β-cell, (9,15,221). However, for the characterization of the mechanism and regulation of insulin and biosynthesis and release at the molecular level, large quantities of cellular material are required. This is not practical using isolated islets or primary cultures of islet cells, since they display only limited proliferative capacity, (221).

The cultured SV 40 virus transformed β-cells HIT-TI5 (160,163-165), and the radiation induced tumour derived RINm5F cells (161,162,167) are being increasingly utilised in insulin secretion studies and have been used in the present work together with isolated islets from lean and genetically obese diabetic (ob/ob) mice to investigate the role of cytoskeletal proteins in the mechanism of insulin release.

Studies on the stimulation of insulin release from lean and obese mouse islets and HIT-TI5 cells using a range of glucose concentrations in a static incubation system have demonstrated that HIT-TI5 cells are more sensitive to lower glucose concentrations than either lean or obese mouse islets but their glucose response is only modest in comparison, cofirming the work of Santerre *et al* (160), and more recently Ashcroft *et al* (221), while the lean and obese mouse islets responded

maximally to glucose concentrations of 12-13 mmol/l. The maximum secretory response of isolated islets was expected to occur at higher glucose concentrations in the region of 16.7 - 22.2 mmol/l (224). This reduced response may in some way be due to the type of incubation system used. It is possible that the lack of agitation of the clusterwell plates during the long incubation periods may have led to stagnation and reduced viability even though the incubation buffer was well aerated.

Obese mouse islets released significantly greater amounts of insulin compared with lean mouse islets over the whole range of glucose concentrations, (3-28 mmol/l), and may be attributed to hyperplasia of the β-cells in the large obese mouse islets, (154).

However, when considering the insulin response of cultured HIT-TI5 cells to glucose some account has to be made of the phenomenon known as the 'passage effect', whereby the amount of insulin released by HIT-TI5 cells declines steadily with time in culture with increasing passage number, (160,223). The passage effect is believed to be the result of a progressive reduction in the insulin secreted and the total insulin content with increasing passage number. In order to reduce the passage effect as much as possible the same passage number was used where possible for controls and test incubations in experimental work, (160,223).

In our hands the RINm5F cell line gives an unreliable insulin response to glucose (223), although a

slight but significant release of insulin has been reported in response to 2.8 mmol/l glucose, (225). The lack of a reproducible and physiologically meaningful insulin response to glucose by RINm5F cells may reflect abnormal glucose metabolism in these cells (226), or posibly the lack of specific membrane bound glucoreceptors, (227). It is generally thought that the genetic overexpression of hexokinase 2 may explain the unreliability of the insulin response to glucose by RINm5F cells, (228). However, the stimulation of insulin release from RINm5F cells in this study using D-glyceraldehyde is consistent with that previously reported by Praz and colleagues, (167).

The biphasic insulin response of the β-cell to a stimulatory concentration of glucose is well established, (8,229) and this was confirmed in the present work with the perifusion of lean mouse islets exposed to 16.7 mmol/l glucose. A similar but more exaggerated biphasic response was also observed with HIT-TI5 cells perifused with 7.5 mmol/l glucose. Compared with the usual biphasic insulin release profiles of lean mouse islets in response to elevated glucose, the first phase of insulin release from HIT-TI5 cells was reduced and of slower onset. This observation might reflect a difference in the size and distribution of secretory granule pools in HIT-TI5 cells compared with normal β-cells, (9).

The pre-treatment of lean mouse islets with 1 mmol/l colchicine prior to perifusion with a stimulatory

concentration of glucose (16.7 mmol/l) caused a significant reduction in the second phase of insulin release. This observation is consistent with the inhibition of the second phase of insulin release from rat islets demonstrated by Lacy and colleagues, (8), and suggests that microtubules are involved in the sustained release of insulin during the second phase of release.

Pre-treatment of HIT-TI5 cells with colchicine (1 mmol/l) prior to perifusion with a stimulatory concentration of glucose also reduced the second phase of insulin release but not significantly. This might suggest that microtubules do not play such a significant role in the release of insulin from HIT-TI5 cells. On the other hand the HIT-TI5 cell cytoskeleton may not be so well developed as it is in islets.

Colchicine pre-treatment had a significant inhibitory effect on glucose or glyceraldehyde stimulated insulin release from statically incubated lean and obese mouse islets and HIT-TI5 and RINm5F cells. Certainly lean and obese mouse islets were more sensitive than cultured β-cells to the inhibitory effect of colchicine pre-treatment since all concentrations of colchicine used induced subsequent insulin release irrespective of the period of incubation, ie. 30 or 90 minutes. However, only pre-treatment with the highest concentration of colchicine brought about a significant reduction in insulin release from HIT-TI5 cells and RINm5F cells after 90 minutes test incubation.

Vinblastine, another microtubule inhibitor, (116,117), did not significantly effect glucose stimulated insulin release from lean mouse islets but it did significantlly inhibit insulin release from obese mouse islets and HIT-TI5 cells after a 90 minute test incubation period but only after pre-treatment with the highest vinblastine concentration, (100 μmol/l). These observations with vinblastine and colchicine confirm that disruption of microtubules impairs glucose stimulated insulin release.

Cytochalasin B is thought to enhance insulin release by disruption of the β-cell microfilamentous web permitting the free random release of β-granules, (136). In the present study cytochalasin B pre-treatment generally enhanced glucose stimulated insulin release from lean and obese mouse islets and HIT-TI5 cells, but did not significantly influence insulin release from RINm5F cells.

The effect of pre-treating islets with a combination of colchicine (10 mmol/l) and cytochalasin B (10 μg/ml) brought about a significant stimulation of insulin release from lean mouse islets after 30 minutes test incubation but did not significantly influence glucose stimulated insulin release from obese mouse islets. This would suggest that neither colchicine nor cytochalasin B pre-treatment influenced glucose stimulated insulin release from obese mouse islets or that colchicine inhibition was effectively cancelled out by the

stimulatory effect of cytochalasin B, ie. the inhibitory action of colchicine and enhancing action of cytochalasin B compensate for one another.

Pre-treatment with the combination of colchicine and cytochalasin B significantly reduced glucose stimulated insulin release from HIT-TI5 cells and D-glyceraldehyde stimulated insulin release from RINm5F cells suggesting that the inhibitory action of colchicine dominated over the stimulatory action of cytochalasin B. This in turn might suggest that the microtubules play a more significant role in the mechanism of insulin release from cultured HIT-TI5 and RINm5F cells than the micro-filamentous web.

SDS-polyacrylamide gel electrophoresis of Triton X-100 soluble and insoluble fractions of lean mouse islets, HIT-TI5 cells and RINm5F cells revealed the presence of a protein of molecular weight 55-56 kDa which corresponded to monomeric tubulin and in addition the presence of 55-62 kDa proteins which corresponded to the Tau proteins. However, because of the unavailability of Tau protein markers it proved difficult to identify these proteins definitively.

A 43 kDa protein, probably actin was also found in Triton X-100 soluble and insoluble fractions of both lean and obese mouse islets, HIT-TI5 and RINm5F cells. Myosin (205 kDa) could not be detected in any of the islet or cell extracts, but a 38-38.5 kDa protein probably tropomyosin, a regulator of microfilament contraction

(219), was detected in extracts of lean and obese mouse islets and HIT-TI5 cells. HIT-TI5 cells and RINm5F cells also contained a 102 kDa protein, possibly α-actinin. In addition RINm5F cells also contained 91.2 kDa protein which might be either gelsolin (91 kDa) or villin (95 kDa), both of which are Ca^{2+}-dependent modulators of F-actin, (220). Since the inhibitory effect of colchicine on insulin release from HIT-TI5 and RINm5F cells seemed to predominate over the stimulatory action of cytochalasin B, it may be that actin and actin binding proteins found in the cultured cells may be more concerned with the motility and the anchorage of the cells themselves rather than their secretory processes.

The inabiltity to detect tubulin in extracts of obese mouse islets, which secreted large amounts of insulin both basally and in response to glucose stimulation might have been due to the condition of the islets. The latter were digested from the pancreata of old obese mice and there is evidence to suggest that such islets show an impaired secretion of insulin and contain large amounts of amyloid-like material, (154).

Although in the present work the separation and detection of cytoskeletal proteins by SDS polyacrylamide electrophoresis and the inhibition of glucose stimulated insulin release by colchicine have confirmed the presence of tubulin in the islets of lean and obese mice and also the cultured insulin releasing cell lines HIT-TI5 and RINm5F, the actual quantitative measurement of the

tubulin content of islets and cultured cells proved to be a much more difficult problem. It had been hoped to develop a tubulin radioimmunoassay method closely modelled on that described by of Le Guern, Pradelles and Dray (184), however considerable difficulties were encountered in obtaining suitable tubulin antisera. In addition problems were encountered in assessing ^{125}I-tubulin-antitubulin-tubulin binding and separating free from antibody bound ^{125}I-tubulin.

The finalised tubulin radioimmunoassay procedure produced an assay of relatively poor sensitivity and precision but it did provide a means for monitoring the dynamic changes within the tubulin subunit (dimers) pool and the microtubule pool on glucose stimulated insulin release from lean and obese mouse islets. Tubulin radioimmunoassay showed that the tubulin subunit (dimer) pool comprised 85% and 82% of the total tubulin content of lean and obese mouse islets respectively under basal stimulatory conditions with a non-stimulatory concentration of glucose (5.56 mmol/l . When the glucose concentration was increased to 16.7 mmol/l, the tubulin subunit pool was reduced to 20% and 15% of the total tubulin content of lean and obese mouse islets respectively. This observation confirms the suggestion made by Pipeleers (191) that stimulation of insulin release causes a dynamic shift from tubulin dimers in the subunit pool to polymerized tubulin, ie. microtubules within the cell.

There was no significant difference between the total tubulin content of lean and obese mouse islets, which is surprising in view of the increased size and insulin output of the obese mouse islets compared with lean. In addition, values obtained in the present study for total tubulin content of both lean and obese mouse islets were some 10-fold less than values obtained by Pipeleers and coworkers for isolated rat isletsusing a ^{3}H-colchcicine binding assay to measure the total tubulin content, (191).

There was a suspicion, however, that ^{3}H-colchicine binding assays produced artificially high values for tubulin measurement and on this basis a ^{3}H-colchicine binding assay was developed to provide comparative tubulin data. The major problem encountered with the ^{3}H-colchicine binding assay was the separation of free unbound ^{3}H-colchicine from tubulin bound ^{3}H-colchicine. Despite this the ^{3}H-colchicine binding assay gave significantly higher tubulin concentrations for lean and obese mouse islets than the tubulin radioimmunoassay, although the proportions of tubulin in either the dimeric or polymerized form were quite similar. However, Pipeleers values for the total tubulin content of rat islets (191) were still some 5 times higher than values obtained for mouse islets using the ^{3}H-colchicine binding assay developed in the present study.

Neither the tubulin radioimmunoassay nor the ^{3}H-colchicine binding assay developed in this study had

sufficient sensitivity and precision to accurately determine the absolute cellular tubulin concentrations in islets of lean and obese mice but the assays proved useful to monitor the dynamic conversion of tubulin dimers into polymerized microtubules during glucose stimulated insulin release.

This dynamic conversion between the tubulin subunit pool (dimers) and microtubules may be an important factor in type II, non-insulin dependent diabetes mellitus (NIDDM), for which the genetically obese diabetic mouse is often used as an experimental model. It is possible that in these animals there may be a higher proportion of polymerized tubulin in islets forming perhaps longer microtubules or more numerous microtubules to facilitate increased insulin release while the total tubulin concentration remains constant.

The tubulin radioimmunoassay developed in this study could be significantly improved by the availability of tubulin antibody with greater avidity and affinity, coupled with better separation of the free ^{125}I-tubulin from antibody bound tubulin.

On the other hand, impaired insulin release as seen in insulin dependent diabetes (IDD) may be the product of impaired microtubule formation from tubulin dimers. This may be caused by some error in the structural configuration of tubulin itself or by the impaired action of Tau proteins and microtubule associated proteins, or perhaps some breakdown in the link between protein

kinases and the microtubule microfilamentous system in the release mechanism, ie. stimulus secretion coupling. Impaired insulin release may also be experienced if the association of the β-granules with the microtubules and their subsequent movement towards the β-cell plasma membrane is at fault.

Further investigations are required to elucidate the role of microtubules and microtubule associated proteins and their interactions in microtubule formation and propulsion of β-granules in the mechanism of glucose stimulated insulin release.

This study has made a contribution towards the understanding of the role of the cytoskeleton in the mechanism of insulin release, in that changes in the β-cell cytoskeleton may play a fundamental role in the break down of the β-cell insulin release mechanism and the development of insulin dependent diabetes (IDD).

APPENDICES

APPENDIX 1

BUFFERS USED IN THE ISOLATION, PURIFICATION, RADIOIODINATION AND RADIOIMMUNOASSAY OF TUBULIN

I. Tubulin Isolation Buffer

The following was dissolved in 1 litre of double distilled water;

9.25 g of 2(N-Morpholino) ethane sulphonic acid (MES) - (100mmol/l),

380 mg of Ethylene glycol bis (B-aminoethylether) N,N,N',N', - tetracetic acid (EGTA) - (1 mmol/l),

101.6 mg of $MgCl_2$ - (0.5 mmol/l), and the pH adjusted to 6.8 with 1 mol/l HCl

GTP was stored at -20°C in a 50 mmol/l solution in Isolation Buffer, pH 6.8.

II. Tubulin Purification Buffer

The following was dissolved in 1 litre of double distilled water;

4.88g of 2(N-Morpholino) ethane sulphonic acid (MES) - (25 mmol/l),

6.9 µl of 1 mol/l 2-Mercaptoethanol - (0.1 mmol/l),

37.2 mg of Diaminoethane tetra-acetic acid, disodium salt (EDTA) - (0.1 mmol/l), and the pH adjusted to 6.8 with 1 mol/l HCl.

III. Tubulin Iodination Buffers

Chloramine-T method for the Iodination of Tubulin (183)

Phosphate Buffer (0.5 mol/l)

A stock solution was made up by dissolving 4.45g Disodium Hydrogen Phosphate in 500 ml of double distilled water. 90 ml of the stock was removed and added to 702 mg Sodium Dihydrogen Phosphate.

The buffer was made up to 500 ml with double distilled water and the pH adjusted to 7.4 with 1 mol/l HCl.

Phosphate Buffer containing 0.15 mol/l NaCl

50 ml of 0.5 mol/l phosphate buffer was added 438 mg NaCl.

Column Eluent

The following was added to 200 ml of 0.5 mol/l phosphate buffer;:

29.4 mg $CaCl_2$ - (1 mmol/l)

24.4 mg $MgCl_2$ - (0.6 mmol/l)

1g Bovine Serum Albumin BSA V - (0.5%), and the pH adjusted to 7.4 with 1 mol/l HCl .

2.5% BSA in 0.5 mol/l Phosphate Buffer

2.5 g BSA dissolved in 100 ml of 0.5 mol/l phosphate buffer, pH 7.4.

Chloramine-T (4 mg/ml)

400 mg of chloramine-T was added to an LP3 tube wrapped in foil to exclude light, (183). Immediately prior to iodination the chloramine-T was diluted to 100 ml with 0.5 mol/l phosphate buffer.

Sodium Metabisulphite (2.5 mg/ml)

250 mg Sodium Metabisulphite was added to 100 ml of 0.5 mol/l phosphate buffer.

Bromosuccinimide Method of Tubulin Iodination

Phosphate Buffer (10 mmol/l)

1.78g of Disodium Hydrogen Phosphate was dissolved in 1 litre of double distilled water and the pH adjusted to 7.4

Bromosuccinimide Stock Solution (10 mmol/l)

17.8 mg Bromosuccinimide was added to 10 ml of 10 mmol/l phosphate buffer- (10 mmol/l), pH 7.4.

Phosphate Buffer used to stop iodination reaction.

16.6 mg potassium iodide - (1 mmol/l)

18.1 mg tyrosine - (1 mmol/l)

was added to 100 ml of 10 mmol/l phosphate buffer and the pH adjusted to 7.4 with 1 mol/l HCl.

Washing Buffer

372.25 mg Diamnoethane tetra-acetic acid (EDTA) was added to 100 ml of 10 mmol/l phosphate buffer and the pH adjusted to 7.4 with 1 mol/l HCl.

Column Eluent

The following was added to 500 ml double distilled water;

8.9 g Disodium Hydrogen Phosphate - (0.1 mol/l)

4.9 g NaCl - (0.9 %)

2.5 g BSA - (0.5 %)

and the pH adjusted to 7.4 with 1 mol/l HCl.

Phosphate Buffer (30mmol/l)

2.67 g Disodium Hydrogen Phosphate was dissolved in 500ml of double distilled water and the pH adjusted to 7.4 with 1 mol/l HCl.

IV. Tubulin Radioimmunoassay Buffers.

Buffer A.

The following reagents were added to 1 litre of double distilled water;-

1.78 g Disodium Hydrogen Phosphate - (0.1 mol/l)

9.0 g NaCl - (0.9 %)

5.0 g BSA - (0.5 %)

0.5 ml Triton X-100 - (0.05 %)

and the pH adjusted to 7.6 with 1 mol/l HCl.

Buffer B

372.25 mg Diaminoethane tetra-acetic acid (EDTA) -(10 mmol/l) was added to 100 ml of Buffer A, pH 7.6.

The preparation of a quench curve for the ^{3}H-colchicine binding assay of tubulin.

This technique is used for the measurement of beta emitting nuclides such as ^{3}H and ^{14}C. The sample containing the radionuclide (in this case ^{3}H-colchicine) was dissolved in scintillant (NE 260). The scintillant consists of an aromatic organic solvent containing a fluor, - a compound that converted molecular excitation energy into light photons, together with a detergent to make the whole solution miscible when aqueous samples were being counted. The energy of the emitted beta-particles is transferred via the solvent to the fluor which emits the energy as light photons. These photons were accurately counted using a photomultiplier. However, only a small proportion of the available energy is liberated as light, the residue being dissipated as vibrational and rotational energy in the solvent.

The quenching agent chloroform was used to produce a quench curve which was required to minimize background counts. Background counts may be produced by the scintillation vial itself or by natural radiation, eg. ^{3}H and ^{14}C in the liquid scintillant components and the surrounding atmosphere.

A quench curve was prepared by counting a set of quenched standards of known efficiencies as shown in Table 5.1 with two counting channels to obtain a channel ratio. (One channel counted the low energy portion of the

emission spectrum and the other counted the high energy portion). The channels ratio was then plotted against the counting efficiency of each standard to obtain a correlation curve from which the efficiency of the unknown sample was determined, Figure 5.1.

Having determined the efficiency, which is defined as the ratio of the observed counts per minute (cpm) to the distintegrations per minute (dpm);-

Efficiency = E = cpm / dpm

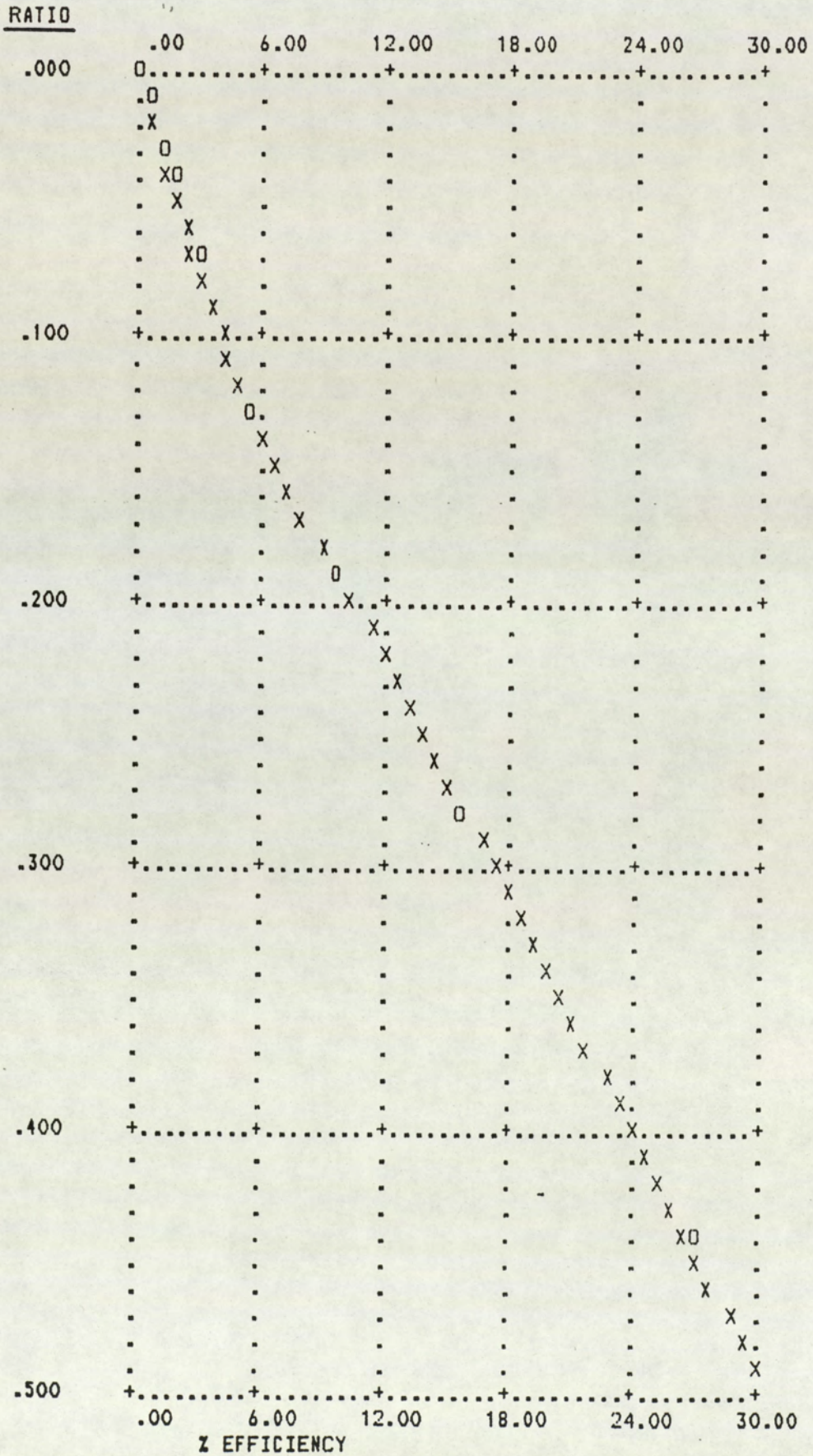
It was then possible to calculate the dpm, which gave a more accurate reading of the radioactivity contained within each reaction vial with the removal of background radiation.

Table 5.1 Protocol for the addition of components in the construction of a quench curve.

Tube No.	Scintillant NE 260 (5 ml)	Chloroform (ml)	Radioactivity 1.1×10^6 cpm (50 µl)
0(Bkg)	+	–	–
1	+	–	+
2	+	0.1	+
3	+	0.2	+
4	+	0.3	+
5	+	0.4	+
6	+	0.5	+
7	+	0.6	+
8	+	0.7	+
9	+	0.8	+
10	+	0.9	+
11	+	1.0	+

Each vial was counted for 5 minutes.

Figure 5.1 Quench curve for the ^{3}H-colchicine binding assay using chloroform as the quenching agent



APPENDIX 2

ESTIMATION OF PROTEIN CONCENTRATION

FOLIN-LOWRY METHOD (176)

This method of protein measurement involves the use of three reagents;- Alkaline Tartrate reagent, Alkaline Copper reagent and Folin's Ciocalteau reagent.

The first two reagents, when mixed in specific proportions and added to protein, (as described in the following procedure), is known to produce an interaction between the protein and Cu^{2+} in alkali. The Cu-protein complex reduces the phosphotungstic and phosphomolybdic acid of the Folin's Ciocalteau reagent to produce molybdenum blue and tungsten blue. The concentration of the protein in the sample is thus almost proportional to the amount of Cu-complex formed with its subsequent reducing activity, that is the amount of blue coloration in the liquid. The concentration of protein in the sample can then be estimated by measuring absorbance at 750 nm and comparing it to the absorbance of standard protein concentrations.

MATERIALS

Solution A - 2% anhydrous Na_2CO_3 in 0.1N NaOH, containing 0.02% sodium potassium tartrate.

Solution B - 0.5% $CuSO_4 5H_2O$

Solution C - prepared as required by mixing the two solutions A and B, 45ml:5ml respectively.

Folin's Ciocalteau reagent was purchased from BDH Ltd; bovine serum albumin (fraction V) was used as a reference standard. A stock solution of 1 mg/ml in water was diluted appropriately to give a range of protein concentrations from 0.01 mg/ml to 0.5 mg/ml.

An LKB Ultrospec 2208 Spectrophotometer was used to measure the colour developed by reading absorption of light at 750nm in a 3ml glass cuvette.

METHOD

Protein samples to be tested were thawed and 200 µl aliquots removed for protein estimation, (many samples required dilution with water to ensure that the protein concentration was within the range of the standards). 1ml of solution C was then added to each tube, including the standard tubes which contained 200µl of the

appropriate albumin solution and the blank tubes which contained 200µl of the buffer containing the dissolved protein samples. The tubes were each vortex mixed and allowed to stand at room temperature for 10 minutes. 100µl aliquots of dilute Folin's Ciocalteau reagent (1:1 with water), were then added very rapidly to each tube and mixed immediately to obtain maximum colour. After 30 minutes the colour development in each tube had reach its optimum value and was measured at 750 nm in the LKB 2208 Spectrophotometer. From the readings thus obtained, a standard curve was plotted as shown in Figure 5.2

The procedure was repeated 8 times with duplicate tubulin standards in 25 mmol/l MES-EDTA buffer pH 6.8, and again in 0.5 mol/l PBS pH 7.4, Figure 5.2, to produce standard curves which were then used in all subsequent estimations of tubulin concentration.

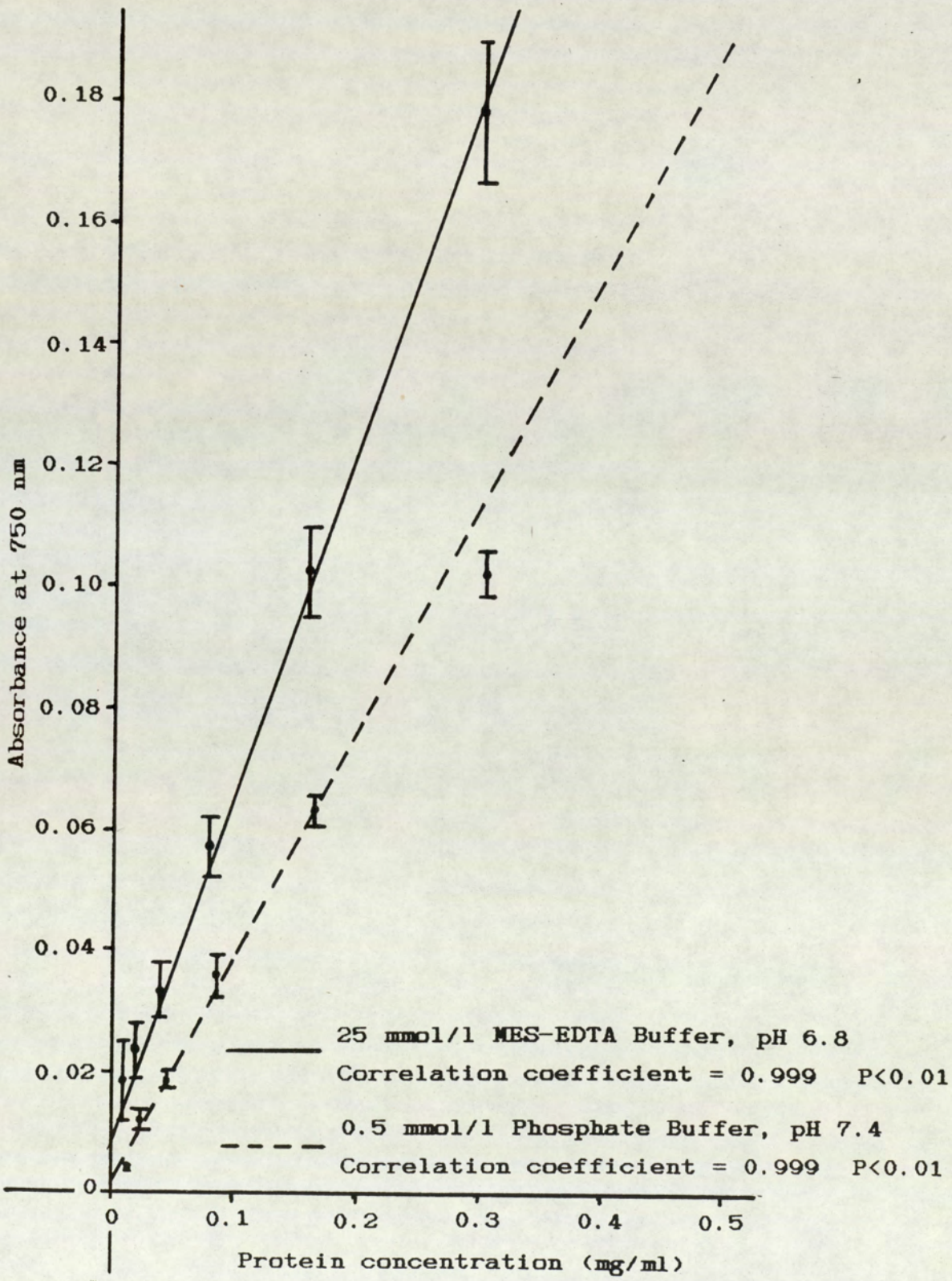
ESTIMATION OF PROTEIN CONCENTRATION BY DIRECT MEASUREMENT OF ABSORBANCE AT 280nm

This is essentially a much quicker method than the Folin-Lowry method but is not so sensitive. However it is particularly useful when only a quick estimate of the protein concentration is required.

Standard curves were produced for protein in both the 250 mmol/l MES-EDTA pH 6.8 buffer and the 0.5 mol/l PBS buffer pH 7.4 by preparing 5 mg/ml stock solutions of

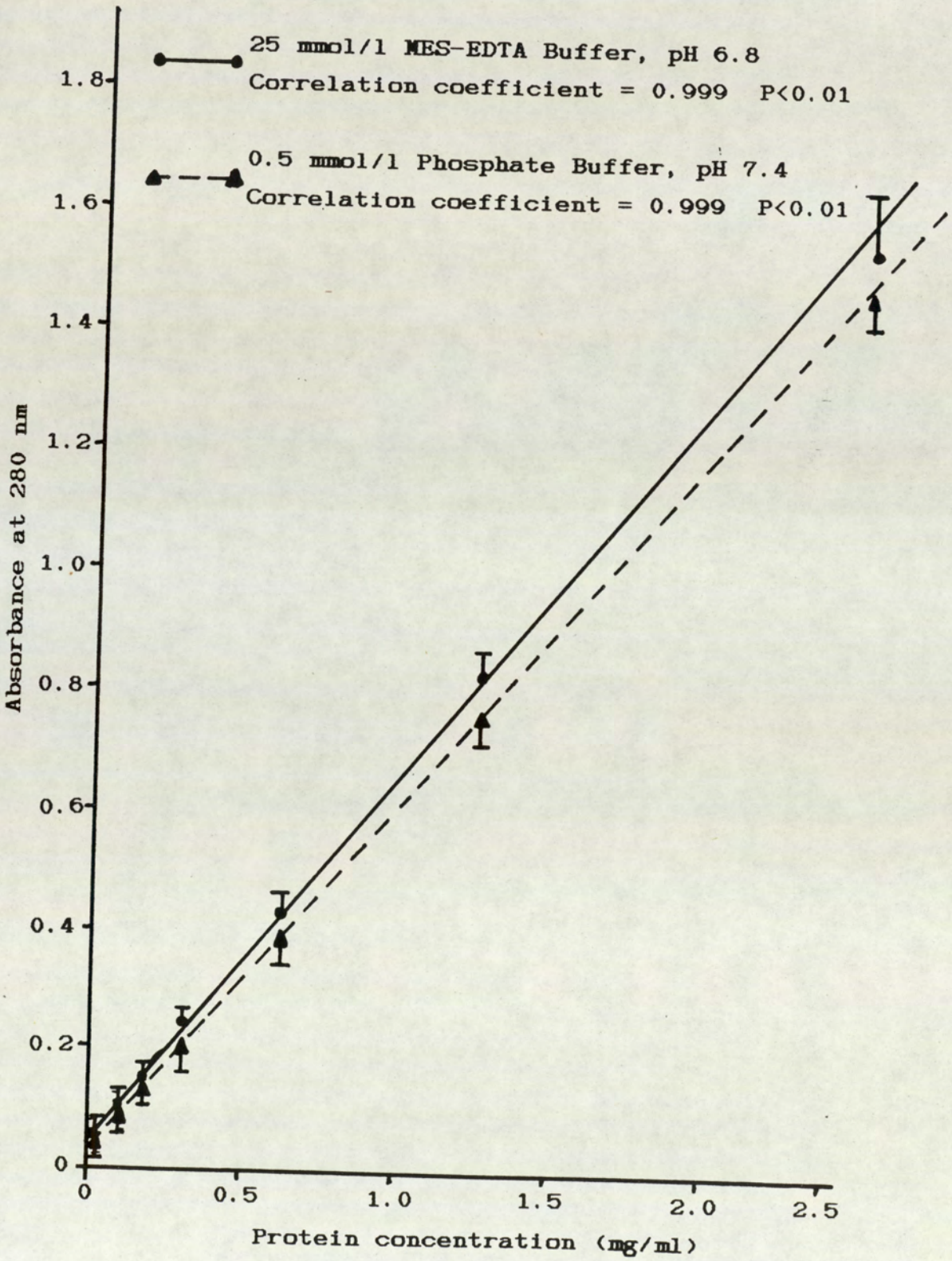
Figure 5.2 Standard curves used to estimate protein concentration by Folin-Lowry. (176)

(Mean values ±SEM, n=8 for each point)



bovine serum albumin in both these buffers which were then serially diluted. The absorbance of the standard solutions was measured at 280 nm in an LKB 2208 Ultraspec and a standard curve plotted for standards in each buffer, Figure 5.3. This was repeated several times so that for protein samples in either buffer the protein concentration could be estimated by measuring their absorbance directly and referring directly to the standard curve.

Figure 5.3 Standard curves used to estimate protein concentration by measuring absorbance at 280 nm (Mean values ± SEM, n=8 for each point)



APPENDIX 3

I. Reagents for the radioiodination of insulin using the Chloramine -T method (185)

Reagents

Phosphate Buffer (0.5 mol/l)

73g Na_2HPO_4 combined with 14.04 g $NaH_2PO_4 2H_2O$ and made up to 1 litre with double distilled water. pH adjusted to 7.4 with 1 mol/l NaOH.

Phosphate Buffer (0.05 mol/l)

7.3 g Na_2HPO_4 combined with 1.404g $NaH_2PO_4 2H_2O$ and made up to 1 litre with double distilled water, pH adjusted to 7.4 with 1 mol/l NaOH.

Column Eluent

0.5% BSA and 0.1% sodium azide (as a preservative) were dissolved in 0.05 mol/l phosphate buffer, pH 7.4.

Column Primer

2.5% BSA dissolved in 0.05 mol/l phosphate buffer, pH 7.4

Porcine Insulin (0.25 mg/ml)

0.25 mg of porcine monocomponent insulin was dissolved in 1 ml of 0.05 mol/l phosphate buffer, pH 7.4 and aliquoted into 25 microfuge tubes, which were stored frozen at

-15°C until required. A fresh tube of insulin was used for each iodination.

Chloramine-T (0.25 mg/ml)

25 mg of chloramine-T was added to an LP3 tube wrapped in foil to exclude light, (186). Immediately prior to iodination the chloramine-T was diluted to 100 ml with 0.05 mol/l phosphate buffer, pH 7.4

Sodium Metabisulphite (0.5 mg/ml)

50 mg of sodium metabisulphite was added to 100 ml of 0.05mol/l phosphate buffer, pH 7.4.

$Na^{125}I$ (1 mCi)

10 µl of Na^{125} was supplied by Amersham Iinternational (UK) in a small reaction vial containing dilute sodium hydroxide, pH 7-11. 100 µl of 0.5 mol/l phosphate buffer was added to the reaction vial to bring the pH close to 7.4, the optimum pH required for the chloramine-T reaction, (185).

II. Calculation of the Specific Activity of ^{125}I-insulin and percentage Damage.

Procedure

Trichloroacetic acid (TCA) precipitation - 1

10 µl from the iodination reaction vial was combined with 0.5 ml of eluent and 0.5 ml of 10% TCA and counted for 3 seconds.

919807 counts/3 secs (i)

The mixture was centrifuged at 2,000 rpm in an MSE bench centrifuge at room temperature for 10 minutes. The supernatant was then dicarded and the pellet counted for 3 seconds

615148 counts/3 secs (ii)

TCA precipitation - 2

^{125}I-insulin fractions 36 -41, representing the monoiodinated peak from the sephadex G 50 column, (page), were pooled and a 10 µl aliquot removed and added to 0.5 ml of eluent and 0.5 ml of 10% TCA. The mixture was then counted for 3 seconds.

61635 counts/3 secs (iii)

The mixture was centrifuged at 2,000 rpm for 10 minutes. The supernatant was then discarded and the pellet counted for 3 seconds

60209 counts/3 secs (iv)

Calculation of Specfic Activity of ^{125}I-insulin

On the day of iodination there was 1.149 mCi of ^{125}I in the vial. 11.49 µl of cold porcine insulin was added to the ^{125}I in the vial from a stock solution of 0.25 mg/ml.

Therefore, 2.8725 µg of insulin was present.

INCORPORATION - of ^{125}I into insulin

$$= (ii)/(i) \times 100 = 615148/919807 \times 100$$

$$= 66.9\ \%$$

66.9/100 x 1.149 mCi was incorporated by 2.8735 µg insulin

= 0.769 mCi ^{125}I/2.8725 µg insulin

= 769 µCi ^{125}I/2.8725 µg insulin

SPECIFIC ACTIVITY = 268 µCi/µg insulin

$$\%\ \text{DAMAGE} = \frac{((iii)-(iv))}{(iii)} \times 100 = \frac{(61635 - 60209)}{61635} \times 100$$

$$= 2.3\ \%$$

APPENDIX 4

ELECTROPHORESIS BUFFERS AND STOCK SOLUTIONS

1. Acrylamide/Acrylaide Stock (32% Acrylamide), used for separating gel.

0.5g acrylaide and 25ml of double distilled water were stirred vigorously for 1 hour. 16g of acrylamide was added and allowed to dissolve and was then made up to 50ml. The solution was then filtered and stored in the refrigerator in darkness.

N.B. This solution does not polymerize after more than 4 weeks in storage.

2. Acrylamide/Bis acrylamide Stock (30% Acrylamide, 0.8 % Bis), used for stacking gel.

0.4g Bis (methylenebis-acrylamide) and 15g acrylamide were dissolved in double distilled water and made up to 50 ml. The solution was stored at 4°C and made up fresh each month.

3. 10% Sodiumdodecyl sulphate, (SDS).

10g of SDS was dissolved in 100 ml of double distilled water and stored at room temperature.

4. 1.5 mol/l Tris pH 8.8

45.43g Tris was dissolved in 250 ml of double distilled water and stored at 4°C.

5. 0.5 mol/l Tris pH 6.8

15.14g Tris was dissolved in 250 ml of double distilled water and stored at 4°C.

6. 0.125 mol/l Tris + 0.1 % SDS

25ml of the 0.5 M Tris was added to 95.5 ml of double distilled water together with 0.5 ml of 20% SDS.

7. Bromophenol Blue

5mg of Bromophenol Blue was dissolved in 10 ml of double distilled water.

8. Running Buffer (0.025 mol/l Tris, 0.192 mol/l Glycine + 0.5% SDS) used during electrophoresis

15.15g Tris Base, 72g Glycine and 5g SDS were added to 5 litres of double distilled water, pH 8.3.

9. Stain and Fixer

500 ml methanol, 500ml double distilled water and 100 ml of glacial acetic acid were mixed together and 2.75g PAGE Blue added. The solution was then stored at 4°C. Staining activity decreases with time so the solution was discarded after 1 week.

10. Destain

Solution A - 400 ml methanol, 100ml glacial acetic acid, 1500 ml of double distilled water were mixed together.

Solution B - 25 ml of glycerol was added to 500 ml of Solution A

11. Extraction Buffer

The following were dissolved in 99 ml of double distilled water;-

242 mg Tris (320 mmol/l)

816 mg Sodium Chloride (0.14 mol/l)

20.33 mg Magnesium Chloride (1 mmol/l)

380.4 mg EGTA (10 mmol/l)

17.42 mg PMSF (1 mmol/l)

1 ml Triton X-100 (1%)

and the pH adjusted to 7.4 with 1 mol/l HCl

12. Sample Buffer

The following were mixed together;-

1.0 ml, 0.5 mol/l Tris pH 6.8

0.8 ml, Glycerol

1.6 ml, 10% SDS

0.4 ml, 2-Mercaptoethanol

0.2 ml, Bromophenol Blue 0.5 mg/ml

and the pH adjusted to 7.4 with 1 mol/l HCl.

N.B. The sample buffer was diluted 1:1 with water before being added to solid samples. When samples were aqueous 1 volume of sample buffer was added to 1 volume of aqueous sample.

APPENDIX 5

Computer program to calculate the area under a curve and perform t-tests.

```
*FX6,10

>L.
   10CLS:PRINT:PRINT:
   20PRINT CHR$(141);"THIS PROGRAM WAS WRITTEN BY K.HUGHES"
   30PRINT CHR$(141);"THIS PROGRAM WAS WRITTEN BY K.HUGHES"
   40PRINT CHR$(141);TAB(12)"NOVEMBER 1985"
   50PRINT CHR$(141);TAB(12)"NOVEMBER 1985"
   60INPUT TAB(7,20)"PRESS RETURN WHEN READY ";A$:IFA$=INKEY$(-74)  THEN 70 ELSE
10
   70DIM A(100):DIM BX(100)
   80CLS:PROCinput:PROCstimulated:PROCbasal:PROCtest:PROCprint1:
   90INPUT"       DO YOU WANT TO PRINT                          OUT OF THESE RESULTS ";
Y$
  100IF Y$="YES" OR Y$="Y" THEN PROCprint2 ELSE 110
  110PRINT:PRINT:
  120INPUT"DO YOU WANT TO ENTER MORE DATA ";D$:IF D$="YES" OR D$="Y" THEN 80 ELS
E PRINT:PRINT:PRINT"END OF THE PROGRAM"
  130PRINT:PRINT:END
  140DEF PROCinput:
  150INPUT"INPUT MOLECULE";M$
  160INPUT"INPUT TIME";T2
  170INPUT"INPUT UNITS";U$
  175ENDPROC
  180DEF PROCstimulated
  190CLS
  200PRINT:PRINT:
  210INPUT"INPUT NUMBER OF STIMULUS EXPT'S ";N
  220IF N<2 THEN PROCerror1
  230PRINT:PRINT:
  240A=0:B=0
  250FOR I=1 TO N
  260AREA=0
  270PRINT "      TIME (DAYS)";TAB(20);M$;TAB(34);U$
  280FOR T=1 TO T2 STEP1
  290PRINT T;
  300INPUT TAB(20)"INPUT VALUE ";BX(T)
  310IF T=1 THEN NEXT T
  320AREA=AREA+((BX(T-1)+BX(T)))*2.5
  330NEXT T
  340CLS
  350 PRINT "DATA FOR EXPERIMENT ";I;" NOW BEING STORED!"
  360PRINT:PRINT:
  370LET A(I)=AREA
  380A=A+A(I)
  390B=B+A(I)^2
  400NEXT I
  410C=A/N
  420V=(B-(C*A))/(N-1)
  430D=SQR(V)
  440FORX=1TO1000
  450NEXT X
  460ENDPROC
  470DEF PROCbasal
  480CLS
  490PRINT:PRINT:
  500INPUT"INPUT NUMBER OF BASALS ";N1
  510IF N1<2 THEN PROCerror2
  520PRINT:PRINT:
  530A=0:B=0
  540FOR I=1 TO N1
  550AREA=0
  560PRINT "      TIME (DAYS)";TAB(20);M$;TAB(34);U$
  570FOR T=1 TO T2 STEP1
  580PRINT T;
  590INPUT TAB(20)"INPUT VALUE ";BX(T)
  600IF T=1 THEN NEXT T
  610AREA=AREA+((BX(T-1)+BX(T)))*2.5
  620NEXT T
  630CLS
  640 PRINT "DATA FOR EXPERIMENT ";I;" NOW BEING STORED!"
  650PRINT:PRINT:
```

```
650PRINT:PRINT:
660LET A(I)=AREA
670A=A+A(I)
680B=B+A(I)^2
690NEXT I
700C1=A/N1
710V=(B-(C1*A))/(N1-1)
720D1=SQR(V)
730FORX=1TO1000
740NEXT X
750ENDPROC
760DEF PROCtest
770T=(C-C1)/SQR(D^2/N+D1^2/N1)
780ENDPROC
790DEF PROCprint1
800CLS
810PRINT:PRINT:
820PRINT"MEAN FOR STIMULATED  = ";C
830PRINT
840PRINT"S.D. FOR STIMULATED  = ";D
850PRINT
860PRINT"NO. OF STIMULATED    = ";N
870PRINT
880PRINT"MEAN FOR BASAL       = ";C1
890PRINT
900PRINT"S.D. FOR BASAL       = ";D1
910PRINT
920PRINT"NO. OF BASAL         = ";N1
930PRINT
940PRINT"RESULT OF T TEST     = ";T
950PRINT
960PRINT"DEGREES OF FREEDOM   = ";(N+N1)-2
970PRINT:PRINT:
980ENDPROC
990DEF PROCprint2
1000CLS
1010PRINT:PRINT:
1020INPUT"ENTER EXPERIMENTAL TITLE ";EX$
1030VDU2
1040*FX6
1050VDU21
1060PRINT:PRINT
1070PRINT TAB(20);EX$
1080PRINT:PRINT:
1090PRINT TAB(15);"MEAN FOR BASAL      = ";C1
1100PRINT TAB(15);"S.D. FOR BASAL      = ";D1
1110PRINT TAB(15);"NUMBER OF BASAL     = ";N1
1120PRINT:PRINT
1130PRINT TAB(15);"MEAN FOR STIMULUS   = ";C
1140PRINT TAB(15);"S.D. FOR STIMULUS   = ";D
1150PRINT TAB(15);"NUMBER OF STIMULUS  = ";N
1160PRINT:PRINT
1170PRINT TAB(15);"RESULT OF T.TEST    = ";T
1180PRINT TAB(15);"DEGREES OF FREEDOM  = ";(N+N1)-2
1190VDU6
1200VDU3
1210ENDPROC
1220DEF PROCerror1
1230PRINT:PRINT
1240VDU7
1250PRINT"YOU MUST ENTER AT LEAST TWO EXPERIMENTAL RESULTS AT A TIME. THE COMPU
TER CAN'T  DIVIDE BY ZERO !":PRINT:PRINT
1260INPUT"PRESS RETURN WHEN READY ";X$:IF X$=INKEY$(-74) THEN CLS: GOTO 200
1270ENDPROC
1280DEF PROCerror2
1290PRINT:PRINT
1300VDU7
1310PRINT"YOU MUST ENTER AT LEAST TWO BASAL        RESULTS AT A TIME. THE COMPUT
ER CAN'T   DIVIDE BY ZERO !":PRINT:PRINT
1320INPUT"PRESS RETURN WHEN READY ";X$:IF X$=INKEY$(-74) THEN CLS:GOTO 490
1330ENDPROC
>
```

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REFERENCES

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