Studies on the development of assay methods for the measurement of proinsulin.

A Thesis presented for the

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by

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph D is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work

Signed _Veronica J. ___   Date _8/6/93_

Date _10/9/93_

Dedication

To the Memory of my Father

Acknowledgement

to college   that is!!! Many thanks to my friend John, who's word of wisdom at the early stages of my career have obviously had an impact I would especially like to acknowledge my uncle Monsignor T J , thanks for always believing in me, your generosity knows no bounds I would like to pay a special tribute to my uncle Fr Mick (R I P), a great friend and a great listener Finally, to two very special people who have been with me every step of the way Agnes and Evelyn

ABSTRACT

Studies on the development of assay methods for the measurement of proinsulin.

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The recent availability of synthetic human proinsulin through recombinant DNA technology has permitted detailed studies of its actions on metabolism to be undertaken, as well as giving researchers the opportunity to develop methods for measurement of proinsulin in various metabolic conditions Initally, this project focussed on the use of rat hepatocytes as a model system for investigating the effect of proinsulin on 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate limiting enzyme controlling endogenous cholesterol synthesis An 'in vitro' bioassay technique, based on the effect of proinsulin on enzyme activity, was developed to compare the level of proinsulin in serum from non-diabetic subjects with that from a group of diabetic patients Higher levels of serum proinsulin were observed in the latter group Subsequent work investigated the contribution of biologically active proinsulin to total immunoreactive proinsulin in these patients A two-site enzyme linked immunosorbent assay was developed using commercial anti-insulin and anti C-peptide immunoglobulins to determine serum proinsulin The level of immunoreactive proinsulin in diabetic patients was shown to be approximately three-fold higher than the level of bioactive proinsulin as obtained by the bioassay technique The advantages and disadvantages of both these methods will be discussed

ABBREVIATIONS.

BSA	Bovine serum albumin
D AMP	3-(2,4-dinitroaniliano)-3' amino-N-methyldipropylamine
DTT	Dithiotreitol
EDTA	Ethylene-diamine-tetra acetic acid disodium salt dihydrate
HBSS	Hanks balanced salt solution
HRP	Horse radish peroxidase
OPD	O-phenylenediamine
PBS	Phosphate buffered saline
p-NPP	p-Nitrophenylphosphate
ELISA	Enzyme linked immunosorbent assay
EMIT	Enzyme multiplied immunoassay technique
HPLC	High performance liquid chromatography
RP-HPLC	Reverse phase - High performance liquid chromatography
IEMA	Immunoenzymometric assay
RIA	Radioimmunoassay
IRMA	Immunoradiometric assay
IRI	Immunoreactive insulin
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IgG	Immunoglobulin
HDL	High density lipoproteins
LDL	Low density lipoproteins
VLDL	Very low density lipoproteins
HPI.	Human proinsulin
PLM	Proinsulin like material
PLC·	Proinsulin like components

NIDDM	Non-insulin-dependent diabetes mellitus
OD	Optical density
SRP	Signal recognition particle
RER	Rough endoplasmic reticulum

CHAPTER 1

INTRODUCTION

Interest in the study of proinsulin stems from the potential importance of this peptide in the pathophysiology and treatment of diabetes Originally, proinsulin was seen as a contaminant in insulin preparations with little useful action Indeed, great efforts were undertaken to remove this substance from beef- and pork-insulin preparations Now, that it is available in large enough quantities through recombinant DNA technology, it is possible to study and compare its metabolic effects with those of insulin

While several studies have shown short term effects of proinsulin on aspects of carbohydrate metabolism relatively few have shown effects on lipid metabolism and, in particular, on cholesterol metabolism A possible relationship between proinsulin and cholesterol metabolism was raised by a recent study showing a link between increased concentrations of circulating proinsulin-like material and cardiovascular risk factors such as total cholesterol, high density lipoprotein cholesterol and triglyceride levels in non-insulin-dependent diabetic patients (Nagi et al, 1990)

Most of the proinsulin assays that have been developed to date have had to await the development of immunoradiometric assays However, their sensitivity is barely adequate for the accurate determination of intact proinsulin in fasting normal subjects. Moreover, assays based on the use of ^{125}I as an antibody label and cellulose solid phase are not ideal for the analysis of large numbers of samples In order to avoid the frequent iodinations necessitated by the use of ^{125}I-labelled reagents, enzyme-linked immunosorbent assays were developed The first such assay was however noticeably lengthy and suffered from the disadvantage of not detecting proinsulin in fasting type 1 diabetic subjects (Hartling et al,

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1986) One of the aims of this study was to determine the effect of proinsulin on 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase, the rate-limiting enzyme controlling cholesterol biosynthesis. Isolated rat hepatocytes were chosen as a useful model for examining the effect of proinsulin on this enzyme. In view of the difficulty of measuring circulating proinsulin, the possibility of being able to measure proinsulin in serum by making use of its biological activity was considered worthy of investigation. Hence this study set out to develop a bioassay for intact proinsulin based on its effect on the activity of HMG CoA reductase. Bioassays were constructed to determine the concentration of intact proinsulin in serum samples obtained from non-diabetic and diabetic patients

It has been known for some years that circulating proinsulin concentrations are elevated in non-insulin-dependent diabetes and it has been proposed that this finding may be used as a marker of ß cell injury. Whether increased proinsulin levels in diabetic patients reflect abnormal proinsulin processing or enhanced synthesis is not yet fully understood. Furthermore, whether elevated levels of immunoreactive proinsulin also reflect increased levels of biologically active intact proinsulin has not been studied before. Hence, an investigation of the proportion of intact proinsulin to total immunoreactive proinsulin in non-diabetic and type 2 (non-insulin-dependent) diabetic patients formed the third major aim of this study

Thus, a study of the effect of proinsulin on HMG CoA reductase activity in isolated rat hepatocytes, construction of a bioassay based on its metabolic effect, comparison of the concentrations of biologically active intact proinsulin in serum samples from fasting non-diabetic and type 2

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diabetic patients and development of a rapid and sensitive two site ELISA suitable for immunoreactive proinsulin measurement in serum form the basis for the work described in this thesis

1 1 Proinsulin biosynthesis, trafficking and processing in the pancreatic β-cell.

The discovery of proinsulin in 1967 by Steiner and Oyer was one of the most important milestones in the study of peptide biosynthesis A great deal of subsequent work dealing with the formation of many bioactive peptides eventually led to the recognition that the biosynthetic pathway of insulin might serve as a model for the processing of secretory polypeptides in general.

Insulin is synthesised as a precursor preproinsulin, in the rough endoplasmic reticulum of the pancreatic β cell The biosynthesis of insulin is typical of a general mechanism whereby larger, inactive precursors undergo successive limited proteolysis in three major stages.

i)   Prepropeptides are synthesised on the ribosomes through translation of their respective messenger RNA`s

ii) The prepropeptides are transformed into propeptides via loss of a signal peptide

iii) Propeptides are sequentially converted to active peptides via proteolytic cleavage, to be stored in secretory granules until released

Analogous synthetic schemes have been shown for parathyroid hormone, corticotrophin and other protein hormones Thus, hormone release from a secretory cell in response to a secretagogue is but the last in a cascade of events leading from transcription of the gene to exocytosis of secretory granule contents These events must be well regulated and coordinated to ensure appropriate cellular function Clearly, the functional status of the insulin-producing β cell in the

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pancreas is central to the well being of an individual, as exemplified by the profound metabolic dysregulation and long term complications of diabetes mellitus. The first section of this introduction will review the synthesis, trafficking and processing of proinsulin in the pancreatic ß cell.

1.1.1   The human insulin gene

The ß-cell response to physiological stimuli is fundamentally related to the insulin gene and the intranuclear events regulating its expression (Bell *et al*, 1980). Detailed knowledge of these processes could conceivably chronicle the events causing both qualitative and quantitative defects in insulin secretion in diabetes. This knowledge might allow the development of genetic interventions to correct these defects or to restore the process of insulin synthesis in patients who are insulin deficient.

The human insulin gene is now known to be located within the short arm of chromosome 11(Owerbach *et al*, 1981). Containing 1789 base pairs, it is considerably longer than would be necessary simply for the production of the cytoplasmic mRNA coding for the precursor peptides of insulin. The unexpected length is at least partly accounted for by two intervening nucleic acid sequences not represented in the mature mRNA. These sequences interrupt the 5' untranslated segment of the gene, as well as the region coding for the C-peptide (Fig 1.1). The two introns (179 and 786 base pairs in length, respectively) indicate that newly transcribed mRNA must undergo modification with removal of the intervening segments and ligation of the nucleic acid sequences represented in the mature mRNA. Base pairs at the beginning and end of the sequences- common to other mammalian genes with such sequences-are probably markers of the junctional areas, pinpointing the regions to be 'cut ' and

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Figure 1.1: The human insulin gene and the intranuclear events regulating its expression

NUCLEUS
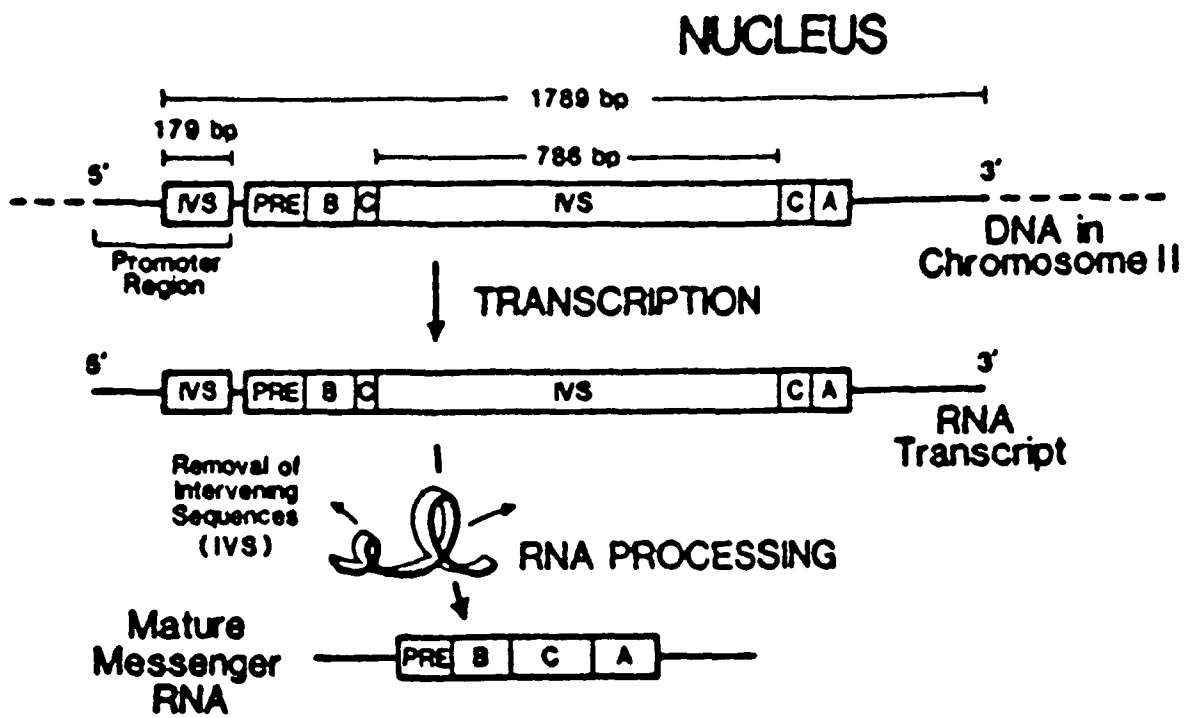
'spliced' (Kruger *et al*, 1982) Thus, the complementary, overlapping bases at the ends of the loops must identify the segments that will be spliced together The removal of the sequences and splicing of the RNA base segments may then occur as a concerted process

After the removal of the sequences from the proinsulin mRNA precursor, the mature form is transported from the nucleus to the cytoplasm The mature mRNA contains a 5',7-methylguanosine 'cap' and an extended polyadenylated 'tail' This completely processed mRNA then binds to the ribosomes of the rough endoplasmic reticulum and directs preproinsulin biosynthesis

1 1 2   Biosynthesis of preproinsulin

Translation of mature mRNA is initiated by interaction between a signal recognition particle (SRP), a polypeptide -RNA complex and ribosomes involved in the synthesis of secretory proteins (Walter *et al*, 1981) The ribosome-bound SRP then arrests the elongation of the nascent polypeptide, presumably via linkage to the signal sequence of the emerging chain (Walter and Blobel,1981) The initial product of translation of insulin mRNA is a 12000-dalton preproinsulin (a peptide ordered as prepeptide, B chain, C peptide, A chain) of which the first 20 to 25 ammo acids constitute the signal or leader sequence (Halban, 1990). This region is characteristically rich in hydrophobic residues and is responsible for directing the nascent polypeptide through the membrane of the rough endoplasmic reticulum (RER)

The preproinsulin-RNA-SRP complex binds to a receptor on the rough endoplasmic reticulum membrane, termed 'docking protein', thus lifting the inhibition of chain elongation Polypeptide synthesis will now be completed on the RER membrane At the same time, the SRP dissociates

and recycles into the cytosol (Walter and Blobel, 1981)

1 1 3 Synthesis of proinsulin.

Once this sequence has penetrated the RER membrane, it is rapidly cleaved off to form proinsulin (the insulin A -and - B chains linked by C peptide) This occurs for the most part before translation of the entire preproinsulin molecule has been completed Once within the lumen of the RER, proinsulin and its conversion products (insulin and C peptide) will always be enveloped by a limiting membrane until released from the ß cell or degraded within it

1 1 4 The regulation of proinsulin synthesis

Glucose is, as for secretion, the primary stimulus The short term effects of glucose on preproinsulin synthesis are restricted to a stimulation of translation, and this occurs within minutes of raising ambient glucose (Welsh *et al,* 1986) Glucose increases the rate of initiation and elongation of the preproinsulin chain and facilitates interaction between the SRP and the docking protein thereby allowing completion of the polypeptide Over a longer time period, glucose is thought to stimulate transcription (Giddings *et al,* 1985) and to stabilise mRNA (Meyer *et al,* 1982) Acting together, these mechanisms may result in a more than 20-fold rise in the rate of insulin biosynthesis.

1 1 5 From the Rough endoplasmic reticulum to the Golgi complex

Proinsulin is transported from the RER to the cis-elements of the Golgi complex in smooth microvesicles Although poorly understood, the mechanism of transport is ATP-dependent Both GTP, and cytosolic factors, including a recently characterised N-ethylmaleimide-sensitive fusion protein are believed to be involved also (Beckers *et al,* 1989)

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Passage of proinsulin from one Golgi stack to the next is assured by a discrete population of vesicles typified by the presence of a protein coat on the cytosolic face of their limiting membrane. The coat has not yet been

characterised, but is known not to be clathrin. The movement of vesicles from one stack to the next is well orchestrated, and must involve a targetting system to ensure delivery of a vesicle to its correct destination. As for RER to Golgi transport, both the N-ethylmaleimide-sensitive fusion protein and GTP are again required for inter-Golgi stack transport (Orci *et al*, 1989). The non-clathrin coat is lost just before the vesicles fuse with a Golgi membrane (Orci *et al*, 1989).

1.1.6   Regulated versus constitutive release

It has recently been suggested that there are two pathways for the release of a secretory product, namely the regulated and constitutive pathways (Kelly, 1985). The constitutive pathway is probably common to most cells in the body. This pathway involves transport of products from the trans-Golgi in smooth vesicles followed by fusion with the plasma membrane, with a transit time of about 10 minutes. The rate of transit and fusion are not influenced by the environment and there is no major storage compartment (Csorba, 1991) (Fig 1.2).

Highly specialised secretory cells such as the ß cell have not only the constitutive pathway but also a regulated pathway that is the hallmark of their differentiated status. The regulated pathway involves packaging products into secretory granules, conversion of a proprotein to the native protein where appropriate, and release by exocytosis in response to a stimulus. The secretory granules represent a well-defined storage

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Figure 1.2 : Proinsulin trafficking and processing events in the pathway of insulin production by the pancreatic ß cell (Csorba,1991).
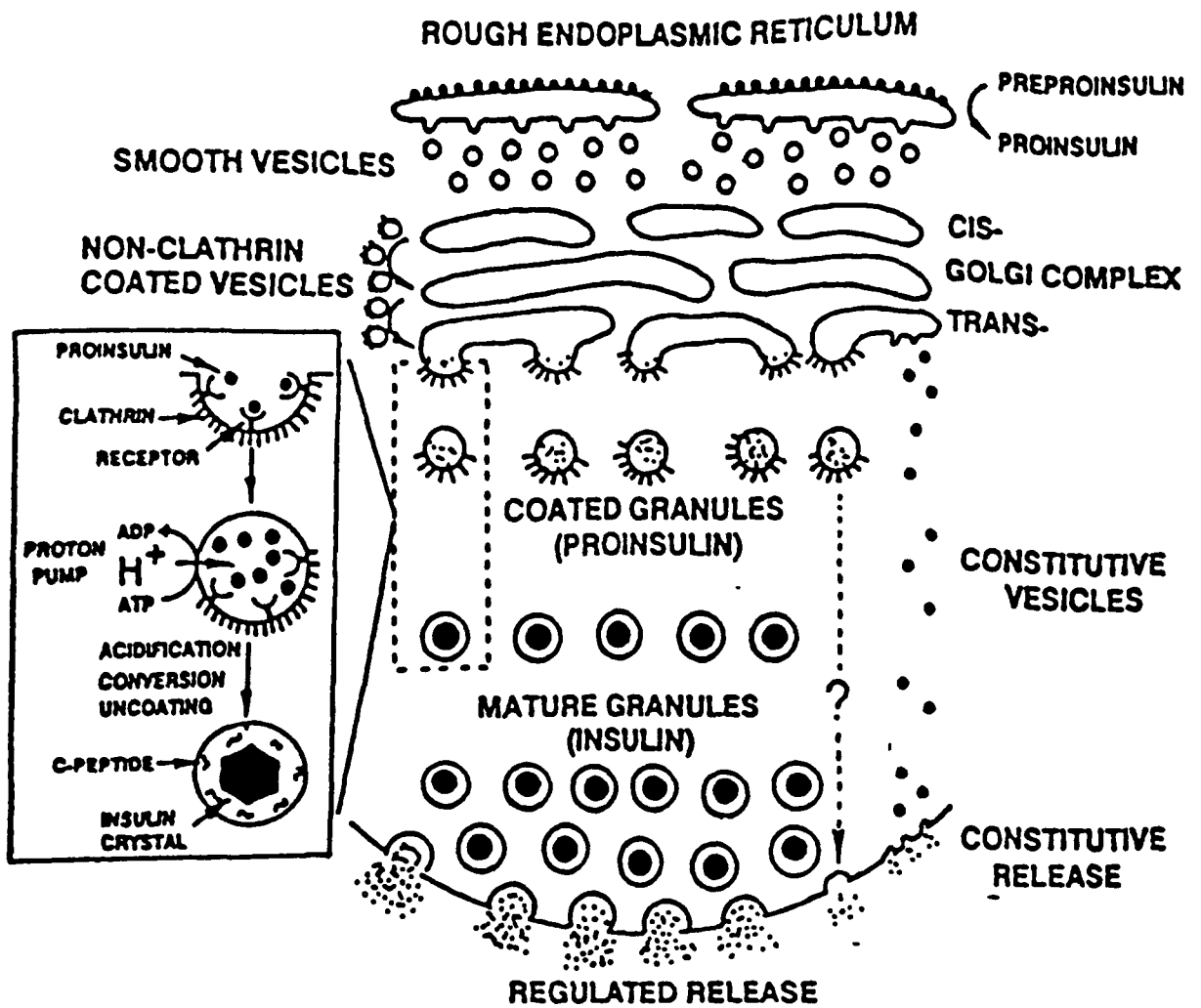
compartment, and, in marked contrast to products employing the constitutive pathway, their contents can spend hours or even days in transit between the Golgi complex and the plasma membrane (Fig 1 2)

1 1 7   Targetting of proinsulin to secretory granules.

The trans-most cisternae of the Golgi complex are the site for sorting of products destined for the constitutive or the regulated secretory pathway (Orci *et al*, 1987) In the latter case, the products must be directed towards nascent secretory granules Proinsulin was shown to be intimately associated with the inner face of membrane domains of the Golgi complex, probably by receptors (Orci ,1982 and Orci *et al*, 1985) Although these receptors are not yet characterised, it is now thought that the mechanism of proinsulin targetting to secretory granules may be receptor- mediated and may be similar to the mannose 6-phospate receptor -mediated pathway involved in the targetting of enzymes fom the Golgi complex to the lysosome

Whatever the mechanism, it is remarkably efficient, since >99% of newly synthesised proinsulin in normal ß cells is directed towards the regulated pathway (Rhodes and Halban, 1987). Therefore, very little proinsulin is released via the constitutive pathway under normal circumstances

The earliest detectable form of the secretory granule carries a partial clathrin coat and contains proinsulin (Orci ,1982 and Orci *et al*, 1985). This is the immature or coated granule It is formed by ATP-dependent pinocytosis of the clathrin-coated domains of the trans Golgi. The immature granule is itself mildly acidic, acidification having been initiated within the trans- Golgi

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1 1 8   Proinsulin conversion to insulin.

Three events occur in parallel in the coated granule, underlying the central role of this organelle in insulin production (Fig 1 2 inset) These events are

i) progressive acidification of the granule

ii) proinsulin to insulin conversion

iii) clathrin uncoating

Granule acidification is due to to the action of an ATP-dependent proton pump Although the pH of immature granules is mildly acidic, mature granules are markedly so (Orci et al, 1985) This progressive acidification appears to be critical for proinsulin to insulin conversion It has been reported that by using a pH probe 3-(2,4-dinitroanilino)-3'amino-N-methyldipropylamine (DAMP), the generation of immunoreactive insulin occurs in conjunction with the acidification of coated secretory vesicles (Anderson et al, 1984) This study suggests that there is a critical pH required for the conversion of proinsulin to insulin, estimated to be pH5 5 (Orci et al, 1986) This is in good agreement with the earlier work which showed that the pH optimum for the enzymes thought to process proinsulin is approximately pH5 5 (Docherty et al, 1982)

The enzymes responsible for conversion have been studied for over 20 years, but identified and partially characterised only recently Processing of human proinsulin is believed to occur by endoproteolysis at the junctions of amino acids 32 and 33 and 65 and 66 to yield respectively 32-33 and 65-66 split proinsulin Removal of the pairs of basic amino acids thereby exposed at the new C-terminal positions is thought to be carried out by a carboxypeptidase and produces respectively des-31-32- and des-64-65-proinsulin (Fig 1 3)

Figure 1.3 : The enzymes involved in the
pathway of conversion of proinsulin to insulin:
Endopeptidase I and II and Carboxypeptidase
(CP) (Hutton,1989).
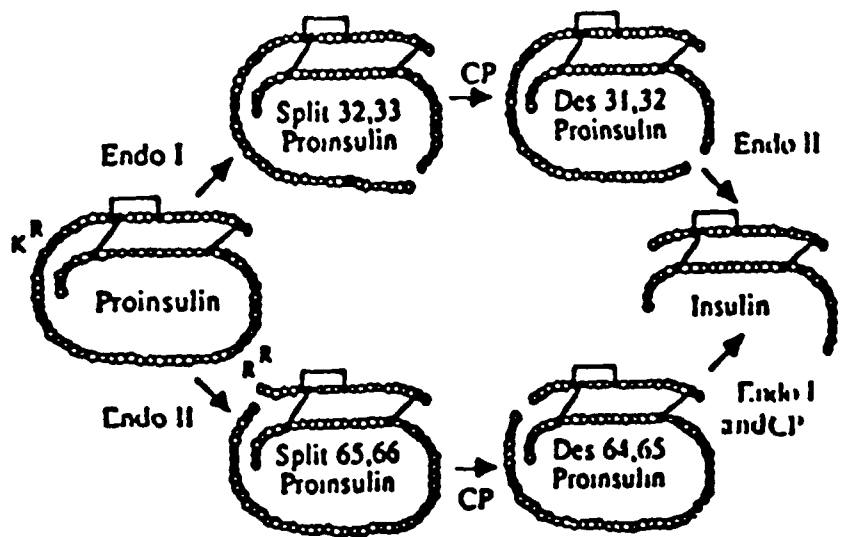
The difficulty in characterising these enzymes over the years was in part related to the findings that endopeptidases are very fastidious in regard to their ionic and pH requirements and are rapidly inactivated by conditions previously used to extract tissue Also, in most preparations used to date, their activity is obscured by the presence of non-specific proteases of lysosomal origin This is where, the availability of highly purified subcellular fractions from insulinoma tissue has proved invaluable Assays for

processing activity in subcellular fractions prepared from insulinoma tissue have demonstrated that the secretory granule compartment is a major intracellular site of concentration of the converting enzymes, (Davidson *et al*, 1987)

Proinsulin is converted to insulin by the excision of the Arg Arg sequence at positions 31,32 and the Lys Arg sequence at positions 64,65 in the molecule (Fig 1 3) Conversion appears to involve initial attack by endoproteases on the C-terminal side of these dibasic sequences followed by the action of an exopeptidase specific for C-terminal basic amino acids Reaction products include both insulin and the intermediates des 31,32 proinsulin and des 64,65 proinsulin which are formed by cleavage at one or other of the basic sites followed by carboxypeptidase H action These intermediates are also produced in intact ß cells and correspond to the major circulating forms of proinsulin immunoreactivity (Given *et al*, 1985)

Further analysis reveals that at least three different catalytic activities are involved in the conversion process These are designated type 1 endopeptidase, type 2 endopeptidase and carboxypeptidase H (Davidson *et al*, 1988) Type 1 endopeptidase cleaves exclusively after

the Arg31-Arg32 sequence at the B-C chain junction, type 2 cleaves preferentially after the Lys64-Arg65 sequence of the A-C chain junction Carboxypeptidase H works equally well with substrates extended C terminally by Lys or Arg residues albeit at different rates (Davidson and Hutton 1987) The activities of these enzymes appear to be regulated by compartmental pH and Ca^{++}, which provides a simple mechanism whereby different dibasic sites within the one protein can be cleaved in different subcellular compartments (Davidson *et al*, 1988) Modulation in intragranular pH in the range of 4 to 7 could provide a means of regulating their activities Type1 activity (Arg Arg-specific) is virtually abolished at pH7, and the type2 enzyme (Lys Arg -specific) retains about thirty percent of its activity The carboxypeptidase enzyme is markedly reduced at neutral pH, however, even at five percent of its maximal activity, it still vastly exceeds the maximal rate of endoproteolytic cleavage (Hutton, 1989) The enzymes involved in proinsulin processing require metal ions for activity The carboxypeptidase is a classic Zn metalloenzyme, while the endopeptidases are activated by Ca^{++} the type1 enzyme requires calcium in the millimolar range for half-maximal activation, type2 requires an approximate 25-fold lower concentration However, the concentration of calcium in the compartments through which proinsulin passes prior to reaching the granule is unlikely to reach the levels that support type1 activity This is because, firstly, the overall content of calcium is lower and secondly, more calcium will be in a complexed form due to a more alkaline pH of these compartments This dual control exerted by calcium and pH ensure that the final conversion of the single chain precursor does not occur until it reaches the granule compartment This is important since insulin is considerably less soluble than proinsulin (Grant *et al,* 1972) Another unusual property of the

endopeptidases particularly the type2 enzyme, is that they are irreversibly inhibited by monovalent anions. The activity of the endopeptidases is probably the rate limiting step in the overall process of conversion and, thus, the regulatory effects of pH, calcium and anions documented *in vitro* may have relevance in the intact cell. Pulse chase labelling experiments in which insulin-related peptides are first immunoprecipitated and then analysed by HPLC show that the principal product of type1 endopeptidase cleavage, des 31,32 proinsulin, appears in parallel with insulin after a thirty minute delay (Davidson *et al*, 1988). This coincides with the transfer from newly coated granule to uncoated granule compartment and its acidification (Fig 1.2). The des 64,65 intermediate, however, appears earlier, at a point in time when the precursor is distributed within the trans Golgi network and in the newly formed coated vesicles. This is consistent with the findings that the type2 enzyme activity can operate at a more neutral pH and at lower calcium concentrations than type1.

Whereas the relationship between granule acidification and conversion is apparant, that between these two events and clathrin uncoating is more obscure. Nevertheless, Halban (1990) reported a series of experiments that did suggest a relationship between conversion and uncoating. In this study, ß cells were incubated with analogues of arginine and lysine. These became incorporated into newly synthesised proinsulin and prevented conversion to insulin since the sites for endopeptidase cleavage no longer contained pairs of the native amino acids. However, in addition to blocking conversion, it was found that the modified proinsulin accumulated in coated granules. This suggests that granule uncoating is dependent on conversion.

1 1 9   Role of proinsulin structural domains in targetting to granules and conversion to insulin

It is apparent that two central events in insulin production are the binding of proinsulin to the 'regulated pathway targetting receptor' in the trans Golgi and cleavage by the proinsulin conversion enzymes Specific proinsulin structural domains involved in these events have now been characterised by Halban (1990) by the use of recombinant DNA technology The approach has been to modify the insulin gene by site-directed mutagenesis and to then transfect the mutant gene into transformed secretory cells equipped with the regulated pathway and to see whether there is regulated or constitutive release of proinsulin or insulin The cell line of choice was AtT20, which consists of transformed pituitary corticotrophs. Fig 1 4 is a model proposed by Halban (1990) to predict the consequences of changes to the structural domains of proinsulin that are critical for targetting to granules and conversion to insulin It is presumed that the 'targetting' and 'conversion' domains are separate Native proinsulin is targetted to granules by interaction with a putative trans-Golgi receptor Conversion to insulin occurs within granules following cleavage by converting enzymes. The net result is the regulated release of insulin In Mutant A, the structural domain recognised by the Golgi receptor has been modified, leading to constitutive release of the mutant proinsulin (Fig 1 4) An example of such a mutant was demonstrated in one case of familial hyperproinsulinaemia in which the histidine at position 10 of the insulin B-chain was replaced by an aspartate This led to partial diversion of the mutant proinsulin from the regulated to the constitutive pathway In Mutant B, a change has occurred that enables targetting to granules but prevents conversion (Fig 1 4) This mutant proinsulin will be released by the regulated pathway Most other cases of familial hyperproinsulinaemia in which one basic

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Figure 1.4: A model predicting the consequence of changes to the structural domains of proinsulin that are critical for targetting to granules and its conversion to insulin (Halban,1990).
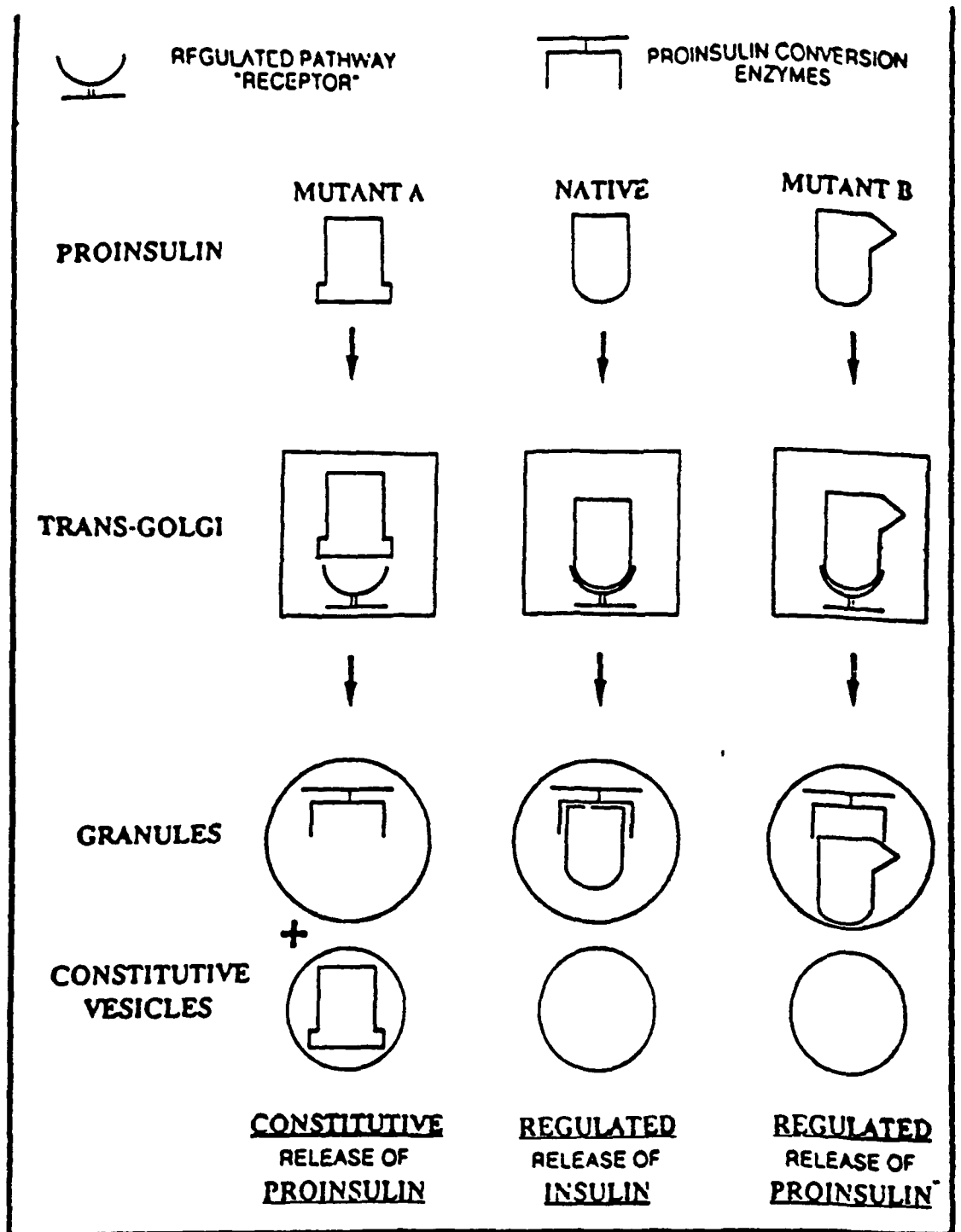
amino acid at one of the cleavage sites has been replaced as a result of a mutation fall into this category

1 1 10   The mature granule and the insulin crystal.

The removal of C peptide from proinsulin during its conversion to insulin results in profound physicochemical alterations (Emdin *et al*, 1980) The C peptide contains a relatively high proportion of charged amino acids Proinsulin, like insulin, can exist as a hexamer in the presence of Zn^{++} It is currently assumed that proinsulin exists as the Zn hexamer in immature granules, with conversion resulting in mature granules Thus, the mature granule contains crystalline insulin, an equimolar amount of C peptide in soluble form, and, some residual, non converted proinsulin Once within the mature granule, insulin can be stored, released by exocytosis, or degraded by fusion with lysosomes (Orci *et al*, 1985)

1 1.11   Biological activity of proinsulin

Effects on carbohydrate metabolism

While the major fate of proinsulin is to be processed to insulin in the pancreatic ß cell, much effort has indeed gone into investigating the metabolic effects of the proinsulin portion that is not processed. The recent successful application of recombinant-DNA techniques to the manufacture of human proinsulin has made the hormone available in pure form for investigations of its biological activity.

Studies of its effect on carbohydrate metabolism showed that it could suppress glucose output from the liver in preference to stimulating peripheral tissue uptake of glucose (Revers *et al*, 1984) The suppression of glucose output from the liver by proinsulin was found to be long-lasting and reflected the reduced clearance and longer half-life of

proinsulin as compared with insulin. The glucose- lowering effect of proinsulin was due mostly to its suppression of hepatic glucose output. Subsequent studies showed that proinsulin could inhibit glycogenolysis and glycogen phosphorylase in rat hepatocyte cultures.The half-maximal effective concentration was shown to be ~26 times greater than insulin (Hartmann *et al*, 1987) (Fig 1.5). Other studies on the effect of proinsulin on intermediary carbohydrate metabolism have shown that proinsulin can stimulate both ^{14}C-glucose incorporation into glycogen and glycogen synthase activity but with a relative biological potency of 3% compared with that of insulin (Ciaraldi and Brady, 1989). The biological activity of proinsulin is probably related to the compound`s structural similarities to insulin, allowing some specific but weak receptor binding. A recent study showed that proinsulin had 3% (on a molar basis) of the potency of porcine insulin for displacing radiolabelled insulin from receptors (Ciaraldi and Brady, 1989). Thus, it would appear that if the actions of proinsulin are to be compared with insulin, far greater amounts of proinsulin, on a molar basis must be used. More recently, a proinsulin effect different to that of insulin, was demonstrated on lactate and pyruvate metabolism in man (Davis *et al*, 1991).The net decrease in blood lactate levels during proinsulin infusion was attributed to a greater rate of lactate utilization compared with glycolytic production.

Effects on lipid metabolism

One of the earliest studies on the effect of proinsulin on lipid metabolism in type 2 diabetes showed a significant reduction in plasma triglycerides and HDL_3 cholesterol and an increase in HDL_2 cholesterol compared with insulin- treated patients (Drexel *et al*, 1988). A later study also showed that proinsulin therapy lowered triglycerides and VLDL-cholesterol in type 2 diabetic patients to a greater extent than insulin

Figure 1.5: Inhibition of glucagon-activated glycogenolysis by insulin and proinsulin (Hartmann et al, 1987).
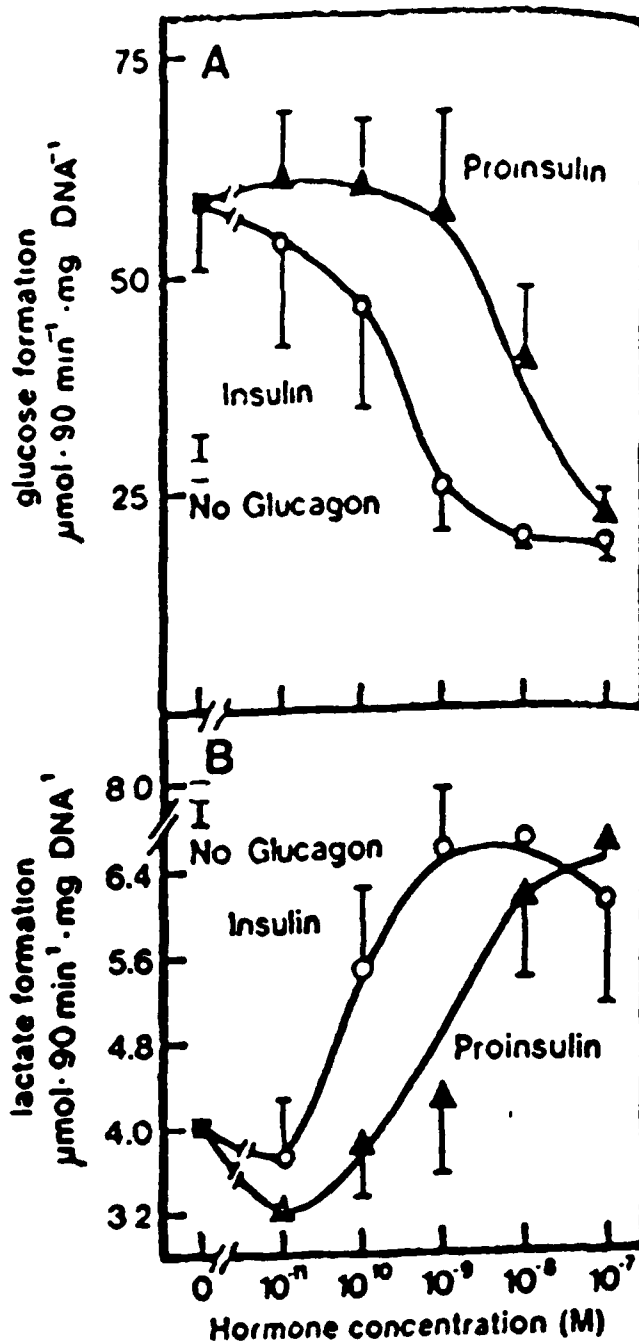
(Winocour *et al*, 1991). However, the reduction in triglycerides was balanced by an increase in LDL-cholesterol concentration. In one third of the patients with hyperlipidemia, postprandial triglyceridemia not only decreased, but fasting triglyceridemia and VLDL cholesterol concentrations were lower and LDL cholesterol concentrations were higher after HPI treatment than after insulin treatment. A possible explanation for this is that proinsulin may preferentially suppress the production of triglyceride-rich lipoproteins in type 2 diabetes. A lower VLDL cholesterol level may arise if proinsulin altered the ratio of protein to lipid in the VLDL particle, thereby affecting its catabolism to the extent that it was cleared more quickly from the circulation. It was suggested that VLDL clearance may occur as a result of enhanced hepatic lipase activity rather than enhanced lipoprotein lipase activity.

To conclude, studies In normal individuals have shown that proinsulin is an intermediate acting, soluble insulin agonist with a potency varying between 5-20% that of insulin. It has a longer half-life than that of insulin and therefore a slower metabolic clearance rate. It suppresses hepatic glucose production more than it stimulates peripheral glucose disposal, and has an hepatic specific effect. Proinsulin exhibits metabolic control in patients with diabetes by lowering blood glucose and plasma triglycerides and may be more effective than insulin in normalising the lipid profiles of patients with non insulin-dependent diabetes mellitus.

1.2 Regulation of hydroxymethylglutaryl Coenzyme A (HMG CoA) reductase.

The enzyme HMG CoA reductase catalyses the rate controlling step in the biosynthesis of cholesterol. Specifically, it catalyses the reductive deacylation of S-HMG CoA to R-mevalonate by two equivalents of

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NADPH (Rodwell *et al,* 1976) (Fig 1.6). HMG CoA reductase is an integral glycoprotein of the smooth endoplasmic reticulum (Liscum *et al,* 1983). Functionally, the enzyme is composed of two non-covalently linked subunits of Mr= 98KDa (Edwards *et al,* 1985). Each subunit can be divided into two domains: a catalytic cytoplasmic domain and a membranous domain. The cytoplasmic domain can be released by the action of a leupeptin sensitive protease as a soluble active enzyme with Mr = 50-55 KDa (Liscum *et al,* 1983).The membranous domain with seven membrane spanning regions projects into the lumen of the endoplasmic reticulum and plays a key role in the regulation of enzyme degradation (Liscum *et al* , 1985). The amino acid sequence of HMG CoA reductase obtained from a Chinese hamster ovary cell line indicates a polypeptide of 887 amino acids.The N-terminal region (residues 1-267) is extremely rich in hydrophobic amino acids and is therefore thought to anchor HMG CoA reductase to the microsomal membrane (Chin *et al,* 1984). The enzyme's active site is presumed to be located in the more hydrophilic portion of the enzyme nearer to the C - terminus (Chin *et al,* 1984).

1.2.1   Enzyme release.

Microsomal membranes subjected to a slow freeze-thaw cycle release their HMG CoA reductase activity with high efficiency (Brown *et al,* 1978).This release results from the freeze-fracture of the lysosomes that contaminate most microsomal preparations.The freed lysosomal proteases liberate from the microsomal membrane a soluble catalytically active albeit proteolytically degraded lower molecular weight fragment of the enzyme. Recently the undegraded native form of the HMG CoA reductase has been solubilised in the presence of proteolytic inhibitors using non ionic detergents e.g. polyoxyethylene ether Type W-1

Figure 1.6: The reaction catalysed by HMG Co A reductase
(Rodwell *et al*, 1976)
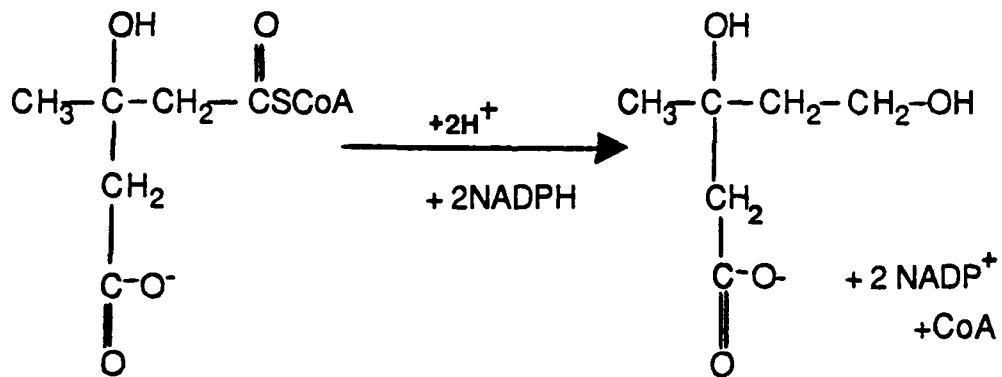
HMG Co A   Mevalonate

(Kennelly *et al*, 1983).

1.2.2   Structure and Kinetic Properties.

The enzyme in chinese hamster ovary cells is a glycoprotein with a subunit size of 97kDa. By applying radiation inactivation of both microsomal and primary hepatocyte enzymes, it was determined that the *in situ* form of the enzyme had a molecular mass of 200kDa (Edwards *et al*, 1985). They concluded that rat liver HMG CoA reductase *in situ* is a non-covalently linked dimer of the 97kDa subunit. The Km for DL-HMG CoA is between $4\text{-}5\times10^{-5}$M. The molecular weight of the *in situ* enzyme is 2.3×10^5. For the particulate form, the pH optimum was within pH 7.3-7.7, whereas that of the partially purified enzyme was lower, at pH 7.0. Activity was rapidly lost at temperatures above 40°C.

1.2.3   Thiol Dependence.

Rat liver microsomal HMG CoA reductase is an allosteric enzyme undergoing conformational changes in response to alterations in thiol concentrations. Several reports have shown an increase of the activity of this enzyme in the presence of thiols, especially glutathione and dithiothreitol (Roitelman and Shechter, 1984). The latter has reported that rat hepatic thiol-deficient microsomal HMG CoA reductase exists in a latent inactive form that can be easily activated by the addition of thiols. Thus, reduction of the enzyme by thiols is essential for reaction. At low thiol concentrations microsomal HMG CoA reductase displays sigmoidal NADPH-dependent kinetics indicating positive cooperativity in binding NADPH to the enzyme's catalytic site (Roitelman and Shechter, 1984). At high thiol concentrations the sigmoidal NADPH-dependent kinetics gradually shift toward classical Michaelis-Menten hyperbola. It is of

interest that these phenomena were not observed with the freeze-thaw solubilised reductase which displayed classical Michaelis-type NADPH-dependent kinetics regardless of the thiol concentration used for it's activation The kinetic studies involving physiological concentrations of NADPH and thiols showed for the first time that cholesterol synthesis as determined by the activity of HMG CoA reductase is tightly coupled to the "reductive state" of the cell

1 2.4   Other Mechanisms Underlying Enzyme regulation.

Apart from thiol dependent modulation of activity, further mechanisms of control have been proposed These involve phosphorylation (inactivation) and dephosphorylation (activation) of the reductase protein, (Ingebritsen *et al*, 1979), the effects of membrane lipids (Orci *et al*, 1984) and changes in the rates of enzyme synthesis (Clarke *et al*, 1984) and degradation (Marrero *et al*, 1986)

i)   The Effects Of Membrane Lipids.

Our knowledge of the structural organisation of HMG CoA reductase in the endoplasmic reticulum membrane allows an appreciation of how adjacent lipids and proteins may affect the activity of the enzyme An increase in the cholesterol content of the membranes decreases the fluidity and tends to displace proteins toward the surface of the membrane. The carboxy terminal domain siting the active site of HMG CoA reductase is projected further into the cytosolic space so that it is more exposed to proteases and other enzymes that modulate enzyme activity An increase in degradation of HMG CoA reductase in UT-1 cells (a cell line of chinese hamster ovary cells that has been chronically starved of cholesterol as a result of growth in the presence of compactin, an inhibitor of reductase) was shown to be correlated with endocytosis of

LDL and selective aquisition of cholesterol by endoplasmic reticulum (Orci *et al*, 1984). HMG CoA reductase activity was also found to be diminished in the liver microsomal fraction of rats fed a diet supplemented with cholesterol. Moreover, the activity of the enzyme varied as a function of incubation temperature in a manner quite distinct from rats fed a normal diet. The experimental results were attributed to an increase in the concentration of cholesterol in the vicinity of the enzyme.

ii) Diurnal Rhythmn.

In addition to the regulation exerted by diet and drugs, rat liver reductase exhibits a diurnal rhythm of activity, the peak occuring at the middle of the dark cycle (Clarke *et al*, 1984). Studies into the control mechanisms involved in the activity cycle have suggested that reductase mRNA production and enzyme synthesis are required for the expression of the diurnal rhythm.This theory is further supported by reports showing that the diurnal rhythm of reductase mass and activity is closely paralleled by the level of its mRNA in rat liver (Clarke *et al*, 1984). In intact animals the diurnal rise in HMG CoA reductase activity during feeding is attributed principally to an increased rate of synthesis of reductase (elevated mRNA coding for reductase) and a diminished rate of degradation of the enzyme. This pattern is reversed as starvation ensues. However, the diurnal feeding cycle is also under endocrine influence as depicted by changes in the phosphorylation state of HMG CoA reductase (Easom and Zammit, 1985). The mechanism of HMG CoA reductase degradation is not simply an impairment of reductase synthesis. Mevalonate has been shown to be an effective repressor of HMG CoA reductase in several cell lines and whole rat liver (Brown and Goldstein, 1980). This metabolite, together with LDL completely represses the expression of the gene of HMG CoA reductase in cells treated with compactin, an inhibitor, of

enzyme activity. However a faster-acting mechanism of enhanced decrease of enzyme activity was suggested by Marrero *et al* (1986). These workers proposed that phosphorylation of HMG CoA reductase induced by mevalonate accelerated its rate of degradation in isolated rat hepatocytes. Thus, it would appear that an increase in phosphorylation of HMG CoA reductase heralds degradation of the enzyme.

iii) Reversible Phosphorylation - Dephosphorylation.

HMG CoA reductase exists in interconvertible active and inactive forms. This interconversion of HMG CoA reductase is accomplished by covalent phosphorylation and dephosphorylation of the enzyme. Fig 1.7 illustrates a bicyclic kinase/phosphatase system controlling HMG CoA reductase. This system *in vivo* is a physiologically important mechanism of regulatory control. The appropriate use of protein kinase and protein phosphatase inhibitors and of HMG CoA reductase phosphatases permits measurements of both the quantity of HMG CoA reductase molecules present and the proportion of these that are in the active, dephosphorylated form. This methodology was investigated to examine whether insulin and glucagon, hormones known to regulate sterol biosynthesis do so by altering the phosphorylation state of HMG CoA reductase *in vivo* (Ingebritsen *et al*, 1979). They observed that rapid alterations in the fraction of enzyme in the active form occured after treatment of rat hepatocytes with these hormones. A rapid response to physiological concentrations of these hormones thus precluded a change in the total activity of HMG CoA reductase. It has been proposed that phosphorylation-dephosphorylation of HMG CoA reductase allows cells to rapidly adjust their rate of cholesterol synthesis often prior to a subsequent adjustment in the quantity of enzyme protein present - a major mechanism of long term regulatory control. It is of interest that two

Figure 1.7: Bicyclic system for modulation of HMG Co A reductase through reversible phosphorylation.
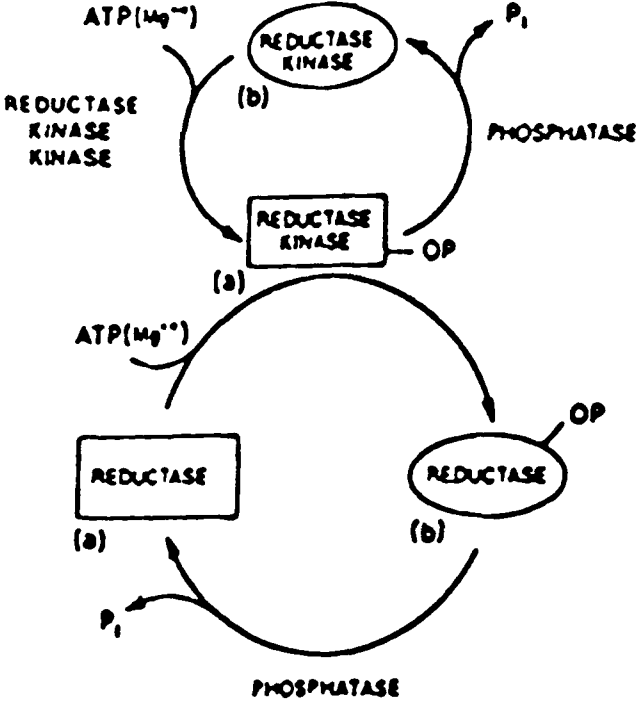
Key: (a) Active enzyme
       (b) Inactive enzyme.

other regulatory enzymes that participate in cellular cholesterol homeostasis, acyl-CoA: cholesterol-o-acyltransferase and cholesterol 7-alpha hydroxylase appear to be regulated by phosphorylation-dephosphorylation mechanisms (Scallen and Sanghvi, 1983). This suggests that coordinate regulation of these enzymes by phosphorylation-dephosphorylation may play an important role in maintaining appropriate levels of unesterified cholesterol. A physiological role for changes in the phosphorylation state of hepatic HMG CoA reductase *in vivo* has been established through demonstration that this parameter varies markedly in response to the diurnal feeding cycle of the rat (Easom and Zammit, 1984). It has been suggested that diurnal variations in the circulating insulin concentrations may be involved in the generation of the diurnal cycles observed for both total HMG CoA reductase activity and the expressed / total activity ratio of the enzyme (Easom and Zammit, 1985).

1.2.5   The effect of insulin and proinsulin on HMG CoA reductase.

An important role for insulin in the regulation of the activity of HMG CoA in the liver has been evident for a considerable length of time. It has been demonstrated that administration of insulin to normal or diabetic rats resulted in an increase in hepatic HMG CoA reductase activity and rate of cholesterogenesis even if the treatment was performed in the middle of the light period and in animals that did not have access to food (Lakshmanan *et al*, 1973). Activity was maximal after 2h and decreased to pretreatment levels by 6h. Although reductase activity was low in diabetic rats, injection of insulin produced maximal reductase activities which were comparable to those in normal animals (Lakshmanan *et al*, 1973). In effect, diabetes appears to abolish the diurnal rhythm. Daily injections of a slowly released form of insulin restores essentially normal

peak and nadir reductase activity and hence a normal rhythm Administration of a rapidly released insulin to a 7-day diabetic rat, restored activity to about the levels anticipated in a normal rat at that time of day (Nepokroeff et al, 1974) Similarly it has been established that streptozotocin-induced diabetes in rats results in a marked decrease in the total activity of HMG CoA reductase in the liver (Lakshmanan et al, 1973 and Young et al, 1982) Studies have been undertaken to investigate the possible role of insulin in modulating the fraction of HMG CoA reductase in the active form i e in altering the phosphorylation state of the enzyme It has been reported that in isolated hepatocytes the expressed / total HMG CoA reductase activity ratio was increased by the inclusion of insulin in the incubation medium (Ingebritsen et al, 1979) The effect of diabetes on the expressed and total activities of HMG CoA reductase in rat liver in vivo was also investigated Streptozotocin-induced diabetes resulted in a marked decrease in total activity of HMG CoA reductase and in the fraction of the enzyme in the active form. Intravenous infusion of insulin into diabetic rats resulted in a rapid and total dephosphorylation of the enzyme in vivo without any change in total activity However long term (4h) treatment with insulin produced a rapid increase in expressed/total HMG CoA reductase activity to approximately 90% followed, after a lag of 2-3h, by a 5-6 fold increase in total activity (Easom and Zammit, 1985) The stimulation of reductase by insulin is partially blocked by glucagon and is also blocked by dietary cholesterol

1 3   Measurement in pharmacology.

It is necessary to have reliable methods for measuring drug effects ,in order to be able to compare quantitatively the effects of a given substance under different circumstances . It is also necessary to be able to measure the concentration of drugs and other substances in, say,

blood or other body fluids The first of these requirements is met by the techniques of bioassay, the second requirement may be met by highly specific and sensitive biological or chemical assay techniques Although bioassays are steadily being displaced by quicker and more accurate chemical assays, it is of interest to note that The British Pharmacopoeia (1980) still lays down bioassay as the official method for estimating the activity of a number of substances, such as corticotrophin, insulin and heparin The purpose of this section is to discuss the principles underlying the main types of bioassay that are particularly useful in pharmacological studies

1.3 1 Quantitative bioassay.

Bioassay is defined as the estimation of the concentration or potency of a substance by measurement of the biological response that it produces There are five main uses of bioassay in pharmacology

i) To measure the pharmacological activity of new or chemically undefined substances

ii) To measure the concentration of known substances.

iii) To investigate the function of endogenous mediators

iv) To measure the clinical effectiveness of a form of drug treatment

v) To measure drug toxicity

It is an essential technique in the development of new drugs. The first stage in assessment of any new compound is usually to compare its biological activity in various test systems with that of known compounds The choice of suitable test systems for this preliminary bioassay is important and not always easy Many assays are carried out on whole animals which can throw up false positive or negative results, unlike assays on isolated systems which can have better predictive value Parallel bioassays are used to identify unknown mediators in a

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biological system. For example, if the biological activity of a sample is thought to be due to serotonin, then measurements of the relative potency of the sample, assayed against authentic serotonin, ought to give the same result, irrespective of what assay system is used. If a range of assay systems is used, and the relative potency of serotonin and the unknown substance is the same in all of them, then it is likely that the activity is due to serotonin. If, on the other hand, in one or more assay systems the relative potency is not found to be the same, it must be concluded that the biological activity is not wholly due to serotonin, but partly at least to other substances.

1.3.2   *In Vivo* Assays.

This classical technique, is now less widely used. Because a whole animal contains such a complexity of tissue, the results gained from such an assay are often subject to interference from various sources present in circulation or in tissue fluids of the animal. For example, the response recorded is dependent not only on the bioactivity of the hormone, but also on the rate of absorption of the hormone from the site of administration and the half life of the hormone in circulation. The definition of a standard organism to any extent is impossible, and therefore the difference between individuals in a bioassay must be taken into account. Despite the inherent variability involved, *in vivo* bioassays are still the method of choice of the WHO Expert Committee on biological standardisation for defining a standard hormone preparation (1982). For instance, various insulin preparations are assayed by estimation of the decrease in blood sugar levels caused by subcutaneous injection of the preparation into intact rabbits, and are thus standardised. In addition to the quantitative assay described, qualitataive identification is also required by the Food and Drug Administration (FDA) regulation. This

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assay involves demonstration that convulsions induced in rabbits by subcutaneous injection of high doses of the preparation are relieved by the intravenous injection of dextrose solution (Rossi, 1985) Accuracy is very important in standardisation procedures, as for example, in the case of insulin, discrepencies in the order of 10% from the required dose may result in severe adverse reactions in the diabetic patient (Rossi, 1985) Although *in vitro* techniques have been shown to give more accurate results in general than *in vivo* assays, their use has not to date been accepted for standardisation

1 3 3 Tissue Preparation.

Many bioassays have been developed using a preparation of the target tissue of the hormone as a test system. This minimises the interference with hormone bioactivity which may occur in circulation en route from the site of administration to its specific receptors on the target tissue One of the first insulin assays was developed using the rat diaphragm (Vallance-Owen and Hurlock, 1954) The response was measured in terms of diaphragm dry weight which represented insulin -induced glucose uptake and conversion to gycogen

Dispersed cells:

Cell dispersion is brought about by treatment with enzymes such as trypsin and/or collagenase, or chelating agents (EDTA) The use of fresh dispersed cells in bioassays is one way of overcoming the problems caused by variation between tissues of different animals. The response measured is generally secretion of a biologically important product Dispersed cell bioassays are in general more sensitive than methods described previously but there is the possibility of damage being caused to cells by enzymic dispersion However bioactivity of damaged,

dispersed cells can be maintained if cultured in 5%CO_2 at 37°C for 1-2 days. Cells will remain active in suspension or attached to a solid surface for up to two weeks (Talbot *et al*, 1987). Permanent or immortalised cell lines (often from tumours) can be maintained for very much longer periods, considerably reducing the use of whole animals.

1.3.4   Cytochemical Bioassay.

This perhaps is the most sensitive bioassay, and has been developed for relatively few hormones to date. It relies on hormone induced changes in some intracellular event for example, activation of an enzyme. The sensitivity and precision of this system are higher than the methods mentioned previously, but lacks technical simplicity and high sample throughput. In some cases however, this is the only means of examining low physiological levels of biologically active hormone, for example parathyroid hormone, in unextracted plasma (Klee *et al*, 1988).

1.3.5.   Design of bioassays:

A bioassay can estimate the dose of an unknown test sample (T) that will produce the same biological effect as that of a known dose of a standard (S). There are two types of bioassay, namely direct and indirect. The aim of the direct bioassay is to determine the dose of standard and unknown that produce the same response. For example, the concentration of digitalis in an unknown test sample may be determined by injecting a standard preparation of digitalis into each of a group of frogs and determining the dose required to stop the heart. A similar experiment on another group of frogs is carried out with the unknown. Calculation of the ED_{50} values (i.e the dose required to produce a response in 50% of the subjects tested) enables the potency ratio, M, of T to S to be given by:

$$M = \frac{ED_{50} \text{ standard}}{ED_{50} \text{ unknown test}}$$

In an indirect bioassay, no attempt is made to achieve exactly matching responses to standard and unknown Instead, comparisons are based on analysis of dose-response curves and the matching doses of standard and unknown are calculated rather than measured directly Such calculations become much simpler if the dose response curves are linear In many cases this can be achieved by using a logarithmic dose scale The amount of unknown preparation required to produce an effect equal to that produced by a certain amount of standard will be inversely proportional to their relative potencies (Rossi, 1980 and 1985) Their relative potency is determined by the distance between the two curves (Fig 1 8) It is worth noting that a comparison of the magnitude of the effects produced by equal doses of S and T does not provide an estimate of M, because the ratio of the effects produced by S and T will vary according to the dose chosen

A convenient design is a 2+2 assay in which two doses of unknown and two doses of standard are examined If all the activity recorded in the unknown sample is due to the same substance in the standard solution, then the dose response lines would be perfectly parallel and the potency ratio of standard to unknown would simply equal the horizontal distance (M) between the lines (Fig 1.8) (Crossland,1980) However, in practice, the points rarely fall on two parallel lines and therefore, the following calculations are employed

The log of the activity ratio, M is given by

M=v/m

where v = vertical separation of the two curves

Figure 1.8: Data for a 2+2 Bioassay
(Crossland,1980)
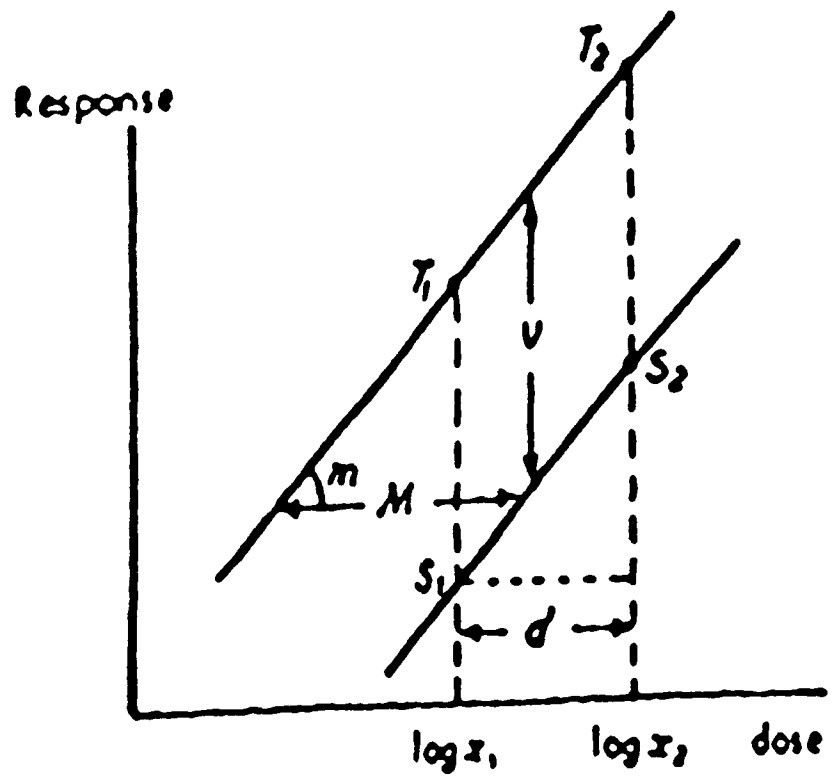
m = mean slope of lines

v is given by

$$v = 1/2 \, [(T_1\text{-}S_1) + (T_2\text{-}S_2)]$$

m is given by

$$m = [(S_2\text{-}S_1) + (T_2\text{-}T_1)] / 2d$$

where d = the differences between the log (dose)'s of standard and

test preparation

i e $d = \log X_2 - \log X_1$

Hence M = $\dfrac{[(T_1\text{-}S_1) + (T_2\text{-}S_2)]d}{(S_2\text{-}S_1) + (T_2\text{-}T_1)}$ Equation 1

If the doses for the unknowns and the standards is not the same, the above equation (1) becomes

$$M = \dfrac{[(T_1\text{-}S_1) + (T_2\text{-}S_2)]d}{(S_2\text{-}S_1) + (T_2\text{-}T_1)} \quad \dfrac{S_1}{T_1}$$

These simple calculations allow more accurate interpretation of bioassay data, and minimization of human error involved in drawing the best-fit lines If the lines are not parallel, it is not possible to define the relative potencies of S and T unambiguously in terms of a simple ratio

An example of a typical 2+2 bioassay has been illustrated by Rang and Dale (1987) (Fig 1 9) This is a 2+2 bioassay of an unknown test sample extract versus a standard pituitary extract on an isolated rat uterus. Two doses of unknown (T_1 and T_2) and two doses of standard (S_1 and S_2) were tested They were chosen to give responses of similar magnitude,

Figure 1.9: An example of a 2+2 bioassay of
an unknown versus a standard pituitary extract
on an isolated rat uterus (adapted from Rang
and Dale,1987)
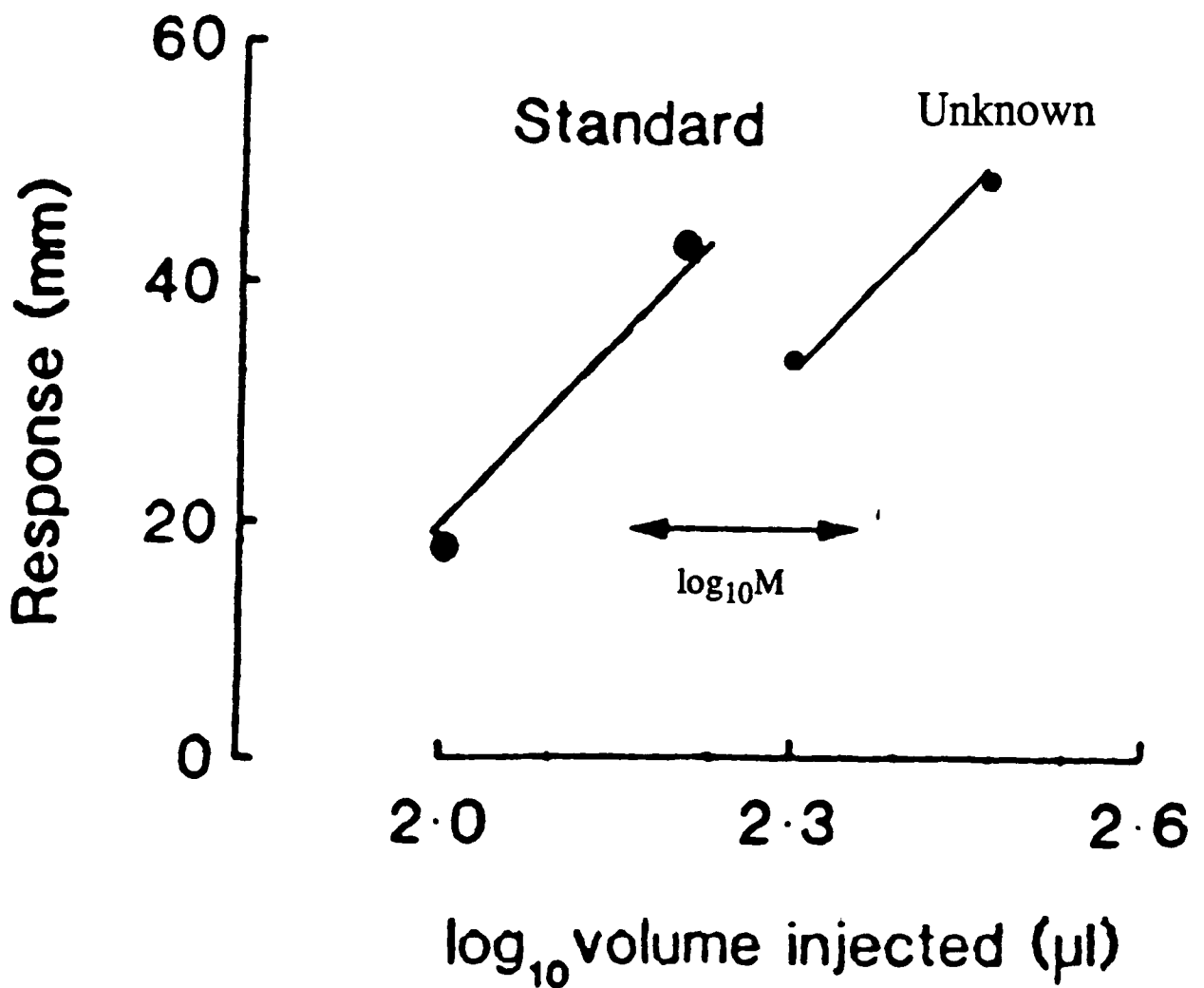
and with T_1/T_2 equal to S_1/S_2 for convenience in analysis In this assay, $\log_{10} M = 0.20$, $M = 1.58$ Since the standard contained 400mU/ml, the unknown was estimated as 400 /1.58 =252mU/ml The precision of the bioassay depends both on the inherent variability of the test system and on the steepness of the log-dose response curve The steeper the curve and the lower the variability of the test system, the more precise will the assay be

1.4   Alternative methods for measurement of hormone concentration

In the 1960's, the development of the principle of enzyme immunoassay and methods for the labelling of antibodies or antigens with enzymes provided a whole new surge of interest in immunoassay procedures

There are various ways in which immunoassays systems can be classified One simple way is to divide enzyme immunoassays into two categories, heterogenous (ELISA) and homogenous (EMIT). In the heterogenous assay antigen-antibody complexes are physically separated from free antigen and antibody using some type of solid phase system In homogenous enzyme-immunoassay no such separation is necessary and the elimination of this step is an advantage Here the binding reaction of antigen and antibody modifies the activity of the enzyme label The extent of this modification is proportional to the amount of binding and hence the concentration of material being measured.

Recently ELISA techniques have been developed for the measurement of human proinsulin (HPI) in serum each varying in sensitivity and the type of capturing or signalling antibody employed Prior to reviewing the methods that have been used for the measurement of proinsulin, this

section will address the design of ELISA assays, including the solid phase support and enzyme-labelled detection.

1.4.1 The competitive ELISA method

Enzyme-linked immunosorbent assays may be classified into two main types, competitive and non-competitive assays. Competitive assays as their name implies measure competition in binding to antibody between a fixed amount of labelled antigen and an unknown quantity of unlabelled antigen, 'the sample'. Fig 1.10 shows a competitive ELISA for measuring antigen concentration. There are many variants and the assay can, of course, be reversed and used to measure the competition of labelled and unlabelled antibody for antigen. Competitive techniques are more demanding in terms of accuracy with which the different reagents need to be dispensed and purity of the labelled ligand. They are easier to quantitate and can be less influenced by contaminants. There are several types of competitive ELISA assays, competitive ELISA for measurement of antigen as described above (Fig 1.10) and competitive ELISA using enzyme labelled antibody to measure antibody and antigen levels (Fig 1.11).This type of assay involves the immobilization of the antigen onto the solid phase. Enzyme labelled antibody and antibody in the sample then compete for binding to the antigen. This assay measures the level of specific antibody present in the sample, and has been used for detecting antibodies to disease-causing organisms e.g. HIV virus in AIDS (Felber *et al*, 1988). As in competitive enzyme-labelled antigen ELISA tests, the amount of product formed is inversely proportional to the concentration of standard or sample antibody (Fig 1.11). A variation that is also extensively used involves firstly the immobilisation of the antigen onto a solid phase. A solution of enzyme-labelled antibody and samples containing standard or unknown antigen concentrations are then added. The greater the level of antigen in the added sample the smaller the

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Figure 1.10: Competitive ELISA for measuring antigen concentration (O`Kennedy *et al*, 1990).

(1) Attach antibody (Y) to solid phase

(2) Incubate with enzyme-labelled antigen (◁Ⓔ) with or without unknown sample antigen (◁)

(3) Antigen binds and unbound antigen is removed by washing

(4) Incubate with enzyme substrate and measure enzyme product formation
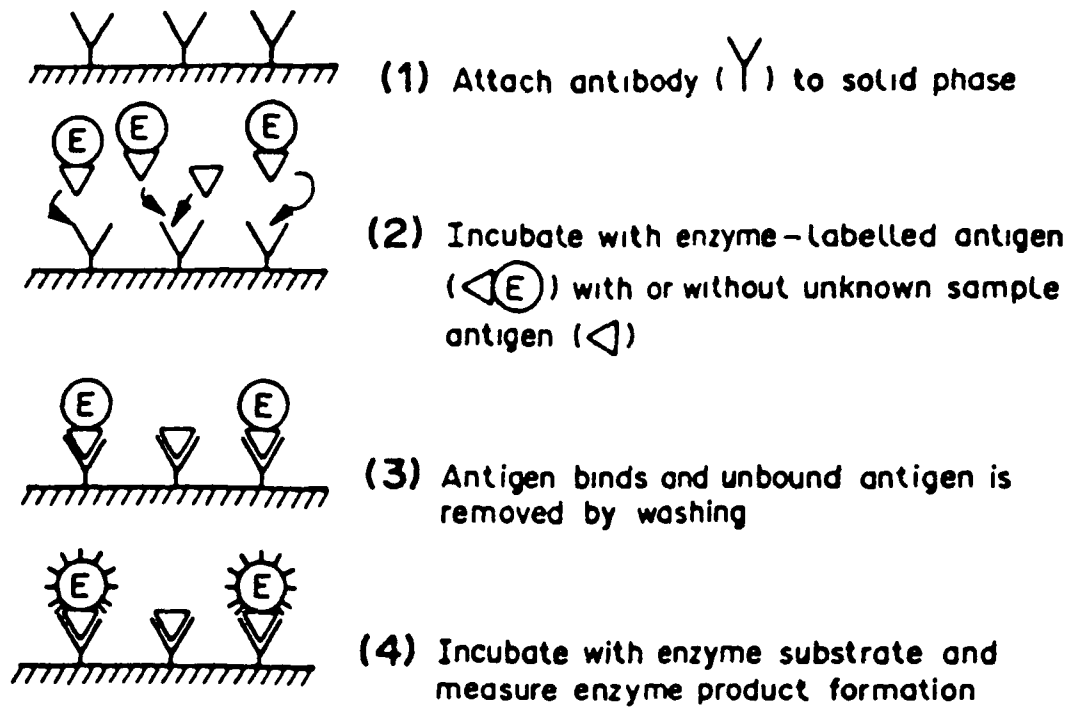
Figure 1.11. Competitive ELISA using immobilised antigen and enzyme- labelled antibody (O`Kennedy *et al*, 1990)

(1) Immobilise antigen on solid phase and wash

(2) Incubate with enzyme-labelled and unlabelled antibody

(3) Antibodies bind to antigen and unbound antibodies are removed by washing

(4) Following addition of substrate the amount of coloured product formed is inversely proportional to standard and test sample antibody levels
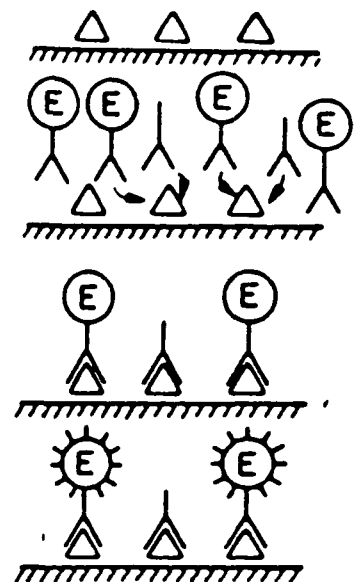
number of enzyme-labelled antibodies available for binding to the immobilised antigen. The amount of enzyme product formed is inversely proportional to the concentration of antigen in the standard or test solutions (Fig 1 12)

Many of the above systems employ a two-step assay using a second enzyme-labelled anti-immunoglobulin antibody (Fig 1 13) The second antibody is raised in a different species from the first antibody The advantages of this are that one labelled second antibody will bind to the primary antigen-specific antibody This will amplify the signal produced The other main advantage is that the use of the second antibody removes any problems associated with the production of a range of labelled antigen-specific antibodies Although it does introduce an extra step in the assay, the extra sensitivity achieved more than compensates for this

1.4 2   Non competitive assays

In non-competitive assays only one component, the sample, is present at a limiting concentration Thus errors in dispensing other reagents have little or no effect on the result. This type of assay will normally be easier to control and yield acurate results but is more likely to be influenced by cross reactions and non-specific binding (Kemeny,1991) Non-competitive assays involve the use of either immobilised antibody or antigen.

(i) Immobilised antibody

In these assays the antibody is immobilised onto the solid phase Following washing , the antigen solution (standard and test samples) is added. Labelled antibody is then added After the substrate is added the level of colour product formation is then measured and this is proportional to the amount of antigen present in the standard and test

Figure 1.12: Determination of antigen
concentration using a competitive binding
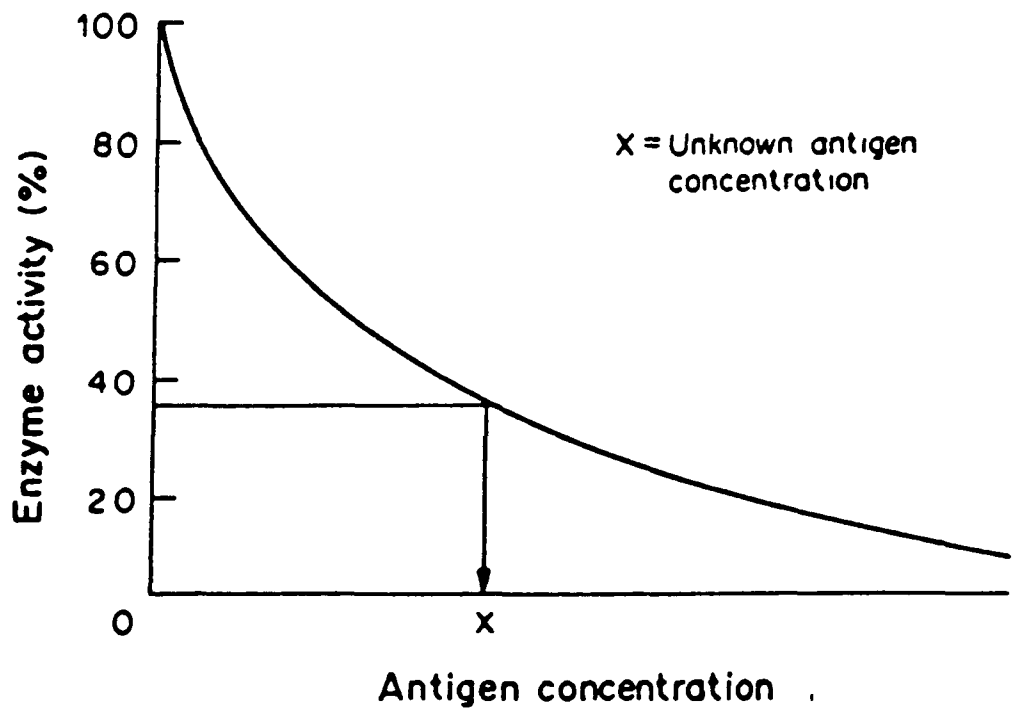antigen-enzyme-labelled assay
(O'Kennedy *et al*, 1990)

Figure 1.13: Use of enzyme-labelled anti-immunoglobulin antibody (O`Kennedy *et al*, 1990)

Enzyme-labelled
anti-immunoglobulin antibody ⟶

Antibody to antigen ⟶

Immobilised antigen ⟶
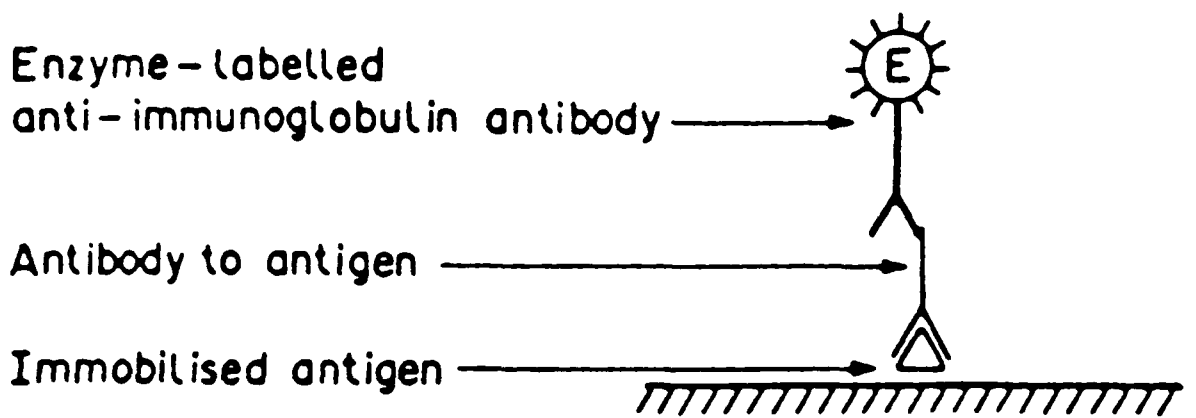
samples. These assays are often called sandwich assays or two-site ELISA's due to the use of immobilised antibody, antigen and labelled antibody (Fig 1.14(a)). An amplification system, involving the use of enzyme-labelled antibody may be used.

(ii) Immobilised antigen.

It is also possible to have the antigen immobilised on the solid phase. Any antibody specific for that antigen, in the standards or test solutions, will bind to it. A second enzyme-labelled antibody specific to this antibody is then added. The amount of enzyme activity is again directly proportional to the antibody levels in the samples. This type of assay is of great importance in relation to the screening of hybridomas for antibody production to specific antigens (Fig 1.14(b)).

1.4.3   Immobilisation of antibodies and antigens to solid phases for enzymeimmunoassay.

Before setting out to develop any immunoassay system, it is necessary to be fully aware of the advantages and disadvantages of the various phases and methods of immobilisation that can be used. Equally, it is very important for each new assay to optimise parameters such as the concentration of antigen or antibody immobilised, concentrations of detergents/washing solutions, number of washing steps etc.The solid phase chosen needs to bind the reactants with high capacity, give reproducible binding characteristics, have little dissociation on washing etc, orientate the antibody with the Fab portion available for binding and be flexible in its use and configuration forms (e.g. plastics which are available as plates, strips, tubes, beads or sticks; nitrocellulose sheets; glass etc). By far the most popular solid phase is plastic with plates, either polystyrene or polyvinyl chloride (PVC), being very widely used.

Figure 1.14 (a): Non competitive enzyme assay using immobilised antibody (O`Kennedy *et al*, 1990)

(1)   Antibody is immobilised on solid phase
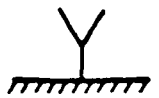
(2)   Antigen (◁▢)-containing solutions are then added
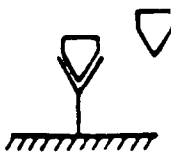
(3)   A second enzyme-labelled antibody to the antigen is added. This antibody reacts with a different epitope to the first antibody
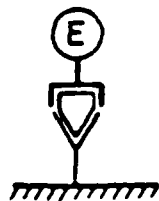
(4)   Enzyme activity is measured following addition of substrate
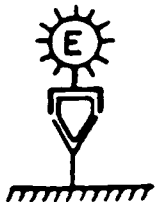
Enzyme-labelled antibody against second antibody

Second antigen-specific antibody

Antigen

Antibody specific to antigen immobilised on solid phase
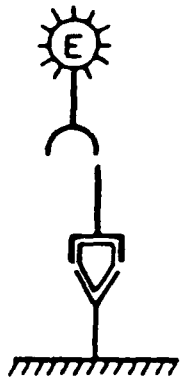
Proteins can adsorb directly onto plastic, at high pH, though the exact mechanism is not fully clear and depends on the nature of the protein, the plastic, the buffer (pH, ionic strength), temperature, time of incubation, and concentration of protein used To optimise and standardise immobilisation, it is necessary to use the appropriate concentration of antigen, standardise assay conditions and wash extensively (often at least six times between assay steps to remove loosely bound immunoreactants) The reliability and sensitivity of ELISA is dependent upon the concentration and stability of the adsorbed protein, as for example in screening hybridomas for the production of specific antibody Too high concentrations of antigen may produce weak protein-protein interactions Too low a concentration of antigen will give poor sensitivity and low affinity antibodies may not be detected To determine the optimum amount of antigen for coating, chequer board titration studies and peroxidase saturation studies are necessary In order to prevent non-specific binding of antibodies to antigens and to plates, mainly by electrostatic and hydrophobic interactions, it is generally necessary to use a blocking solution such as 0 1-1% (w/v) bovine serum albumin, gelatin or other protein solution Antigen coated plates are incubated with this in solution for approximately 1 hour at 37°C or overnight at 4°C. It is also useful to include a mild detergent such as Tween-20 (0 05-0 1%v/v) in all wash solutions and dilutents to further reduce non-specific binding of antibodies This detergent does not affect antigen -antibody binding but recent reports have suggested that it may remove antigens from nitrocellulose during washing procedures in western blotting It is, therefore, recommended that the use of any detergent be carefully monitored and tested in all immunoassay procedures

Detergents such as Tween and Triton X-100 should never be used

during initial antigen coating procedures as they compete with protein for binding to the solid phase. In relation to all enzyme immunoassays involving plates, it is essential to have a quality control procedure that identifies intrinsic errors in plates that lead to lack of reproducibility in assays. These include "edge effects", whereby the wells at the edges of the plates have different binding characteristics and well-to-well variation in optical properties and binding. Some of these effects are due to surface characteristics of the plastic, manufacturing methods, and temperature gradients during coating. To design an assay with optimal sensitivity, it is essential therefore to choose an antiserum of highest possible affinity, to use incubation times that allow an equilibrium between antigen and antibody, use lower concentrations of antigen and antibody and longer times for colour development.

Therefore with these factors taken into account the sensitivity of the ELISA assay is comparable to that of radioimmunoassay. It can be concluded that, except for very low affinity antibody, ELISA is a capacity assay unlike a radiobinding assay which is influenced by both antibody concentration and affinity. The former assay is thus best suited to detecting low affinity antibody whereas the latter is more efficient in the presence of low levels of high affinity antibody (Sodoyez *et al*, 1991).

1.4.4   Selection and conjugation of enzymes.

i) Choices of enzymes.

There is no one enzyme with all the ideal properties for enzyme immunoassay. The ideal properties of an enzyme label for immunoassay are the following: high turnover number, high stability, low cost, easy detection, high purity, ease of conjugation, no interfering compounds/conditions in samples (e.g. enzyme inhibitors, extremes of

pH etc) and no endogenous enzyme activity in samples. This would give high background effects. Those that possess many of these properties include glucose oxidase, ß-galactosidase, alkaline phosphatase, peroxidase and urease. These five are by far the most utilised, with alkaline phosphatase and peroxidase being the most popular. Peroxidase, alkaline phosphatase and glucose oxidase are very widely used in immunohistochemistry and have a variety of different substrates available. This is important, because substrates that give soluble and insoluble enzyme reaction products are required, the former for solution assays and the latter for histochemistry. The substrate p-nitrophenyl phosphate (p - NPP) is used as a substrate for alkaline phosphatase. It is easy to use and produces linear colour development with time. This substrate is stable, safe and available commercially in convenient tablet form. Enzyme activity doubles between 25°C-37°C but hydrolysis of the substrate occurs at temperatures above 30°C. The rate of colour development with alkaline phosphatase can be increased by using an amplified substrate system. In this technique, the labelling enzyme first reacts with a substrate such that the product of this reaction then starts a second enzyme-mediated cycling reaction, resulting in the accumulation of coloured product.

O-phenylene diamine (OPD) is the most widely used chromagen for horse radish peroxidase (HRP). However, the rate of generation of colour is not always linear, usually if an incorrect amount of HRP substrate has been used. The pH too is critical and this can change when the chromagen salt (OPD) is dissolved in substrate buffer. The hydrogen peroxide concentration is also critical- too much and the enzyme product is inactivated, too little and the sensitivity is lost. Substrate should be made up no earlier than 10 minutes before use and the peroxide added

just prior to use. OPD is also light sensitive. If samples do not possess endogenous peroxidase activity then peroxidase is a very cheap, easy and useful enzyme for conjugation.

ii) Conjugation of Enzymes.

The conjugation of antibody or antigen (hapten) to the enzyme is fundamental to enzyme immunoassay. The ideal conjugation process would give very high yields of conjugate, be cheap, easy to perform, and produce little loss in enzyme activity or immunoreactivity. Generally for homogenous assays, a 1:1 ratio (1 enzyme molecule per antibody or antigen) conjugate is required. However, for other assay systems the idea is to maximise the amount of enzyme conjugated while retaining full activity of the antibody and the enzyme. By far the most common form of conjugation involves chemical linkage. If this is the chosen method it is important to take into consideration the theoretical and practical considerations that will affect the conjugation step such as the concentrations of reactants, pH, ionic strength, concentration of coupling agent, purity of buffers, protection of active groups with biological activity, site of conjugation and formation of polymers.

Glutaraldehyde has been used extensively as a homobifunctional crosslinking reagent because of its ease of use, low cost, and effectiveness with certain proteins. It is useful for alkaline phosphatase conjugations but less so for glucose oxidase or peroxidase.

For the conjugation of glycoproteins, periodate ($NaIO_4$) is a very useful reagent. There are a number of periodate methods. The original method was devised by Nakane and Kawaoi (1974) for conjugating HRP to antibodies, but more recent methods give better results in terms of ease, extent of conjugation and activity of conjugates (Tijssen and Kurstak,

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1984) The carbohydrates (about 20%) of horse radish peroxidase are oxidised with sodium periodate, producing aldehyde and carboxyl groups. The aldehyde groups then form Schiff bases with the amino groups of the added antibody or antigen (Fig 1 15)

There is a range of other bifunctional reagents that can be used for conjugation e g p-benzoquinone for peroxidase The use of heterobifunctional reagents for conjugation may provide better conjugates in the long term N-succinimidyl-3-(2-pyridyldithio)-proprionate (SPDP) has been used for conjugation although the results obtained were not always satisfactory for immunoassays

In all cases conjugates need to be purified, the removal of conjugating agents, hapten free enzymes and in particular, free antibody being essential to maximise enzyme immunoassay sensitivity Generally this is achieved by a combination of precipitation, gel filtration and affinity chromatographic techniques The purity of the preparation may be tested by gel filtration, SDS-PAGE or HPLC which provides an ideal method for fast analysis of the purity of conjugate preparations such as enzyme-linked antibodies. Conjugate preparations need to be stored carefully and the conditions (e g buffers, ionic strength etc) are dependent on the nature of the enzyme, antibody or hapten Peroxidase is sensitive to microorganisms and to antimicrobial agents such as sodium azide and methanol and can be inactivated by plastic, although the latter can be prevented by the addition of Tween 20 to the dilutent Peroxidase conjugate should therefore be stored at -20°C in 75% glycerol and all buffers and wash solutions should be free of sodium azide Alkaline phosphatase conjugates are normally stored at 4°C in buffer containing

Figure 1.15: Original NaIO$_4$ method of direct conjugation of peroxidase to antibody (IgG)
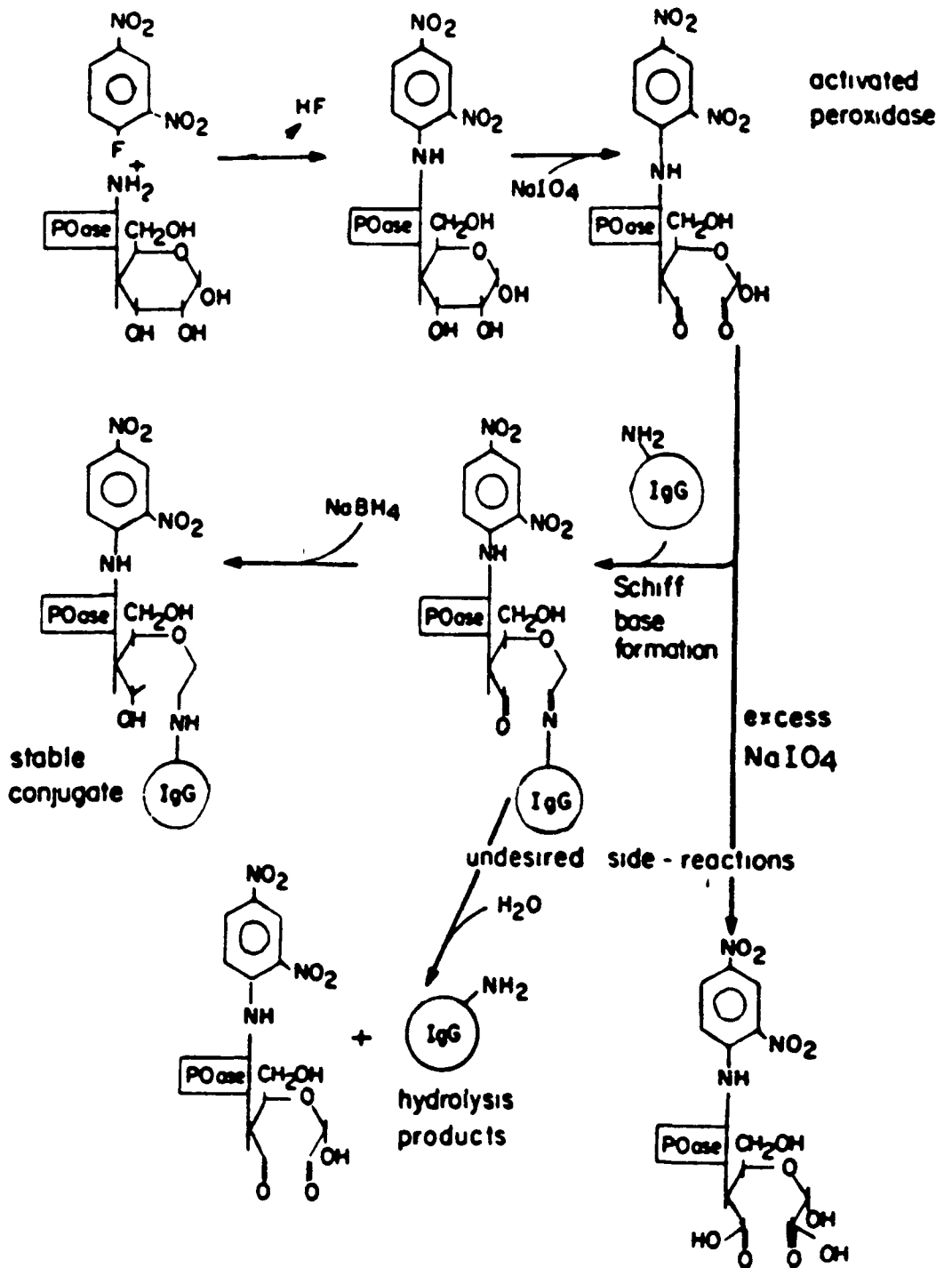
1-2% w/v protein, 75% glycerol and azide and can, under these conditions, be stored at -20°C (Kemeny, 1991). Enzymes may also be conjugated to antibodies or other molecules using biotin-avidin or biotin-streptavidin bridges. This may allow the conjugation of a very large number of enzyme molecules per molecule of immunoglobin thus giving far greater sensitivity in the assay.

1.4.5 Advantages of enzyme immunoassays

The two-site assay exemplifies some of the best features of ELISA. The main advantages are (i) high sensitivity, (ii) high specificity, (iii) assays are relatively cheap and require small amounts of reagents, (iv) assays are rapid and can give both qualitative and quantitative results, (v) detection is either performed by using special readers or visually, (vi) results are reproducible, (vii) automated, high throughput and manual methods are available, (viii) very versatile (ix) no problems with radiation or disposal of waste and (x) both polyclonal and monoclonal antibodies can be used.

One of the earliest ELISA assays developed for monitoring circulating proinsulin was among the most sensitive but was still not sufficiently sensitive enough to measure concentrations expected from diabetic subjects in the fasting state (Hartling et al, 1986). In spite of this the method proved useful in characterising ß cell function in stimulated situations, as well as in the diagnosis of insulinoma. The method varies from radioimmunoassays in several aspects: (i) the use of stable enzyme labelled antibody, (ii) the low demand for sample volume, (iii) high sensitivity and (iv) large capacity without previous separation of proinsulin from other plasma components. More recent methods have attempted to improve the sensitivity of this ELISA. An amplified ELISA for

proinsulin in serum has been described using a polyclonal antibody raised in guinea pig against intact proinsulin. A sandwich was formed with proinsulin using a monoclonal antibody against C-peptide labelled with alkaline phosphatase (Dhahir *et al*, 1992). This demonstrated considerable improvement in sensitivity. It enabled the estimation of the low concentration of proinsulin and its partially processed intermediates in fasting normal individuals, some diabetics and in other pathological conditions. A sensitive immunoenzymometric (IEMA) assay for insulin and proinsulin using an alkaline phosphatase-labelled monoclonal antibody and an amplification system for measurement of the label was recently developed which achieved greater sensitivity than that observed in immunoradiometric assays (Alpha *et al*, 1992).

1.4.6   A review of methods used for the measurement of proinsulin.

Assay methods initially developed to measure proinsulin-like compounds (PLC) in serum or plasma were based on an indirect approach. Soon after its discovery, proinsulin was demonstrated in plasma by taking advantage of both its higher molecular weight and cross reactivity with insulin. On fractionating plasma samples by gel chromatography, proinsulin eluted prior to insulin as shown by the distribution of its immunoreactivity (IRI) (Roth *et al*, 1968). Another approach was the use of an insulin-specific protease which destroyed insulin but not proinsulin. After enzymatic destruction of proinsulin, significantly higher levels of PLC were observed in type II diabetic patients (Duckworth and Kitabachi, 1972). Immunoprecipitation methods were later used in an indirect approach to quantify proinsulin in serum by measuring the C-peptide activity of precipitates produced after treatment of sample with insulin antibodies, (Heding, 1977). Whereas C-peptide activity in the supernatant is due to free C-peptide,that in the precipitate corresponded

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to PLC. A similar approach was taken by Ward *et al* (1986) who extracted plasma proinsulin with antiserum to human C-peptide. Subsequently, proinsulin was dissociated from the immunoprecipitate using antiserum to insulin and assayed for IRI, using proinsulin standards. Unfortunately, any contamination of the immunoprecipitate with free C-peptide would result in falsely elevated proinsulin levels. It has been reported that this type of assay measures proinsulin much less well than proinsulin conversion intermediates (Gray *et al,* 1984). Cohen *et al,* (1985) were the first to use biosynthetic human proinsulin in a direct assay. Using affinity chromatography, they extracted a large plasma sample for proinsulin-like material, then quantitated it with a polyclonal antibody against the BC junction. This method measured intact proinsulin and its Arg65Gly66-split derivative. When antibodies against both the BC and AC junctions were used in a simultaneous assay, both Arg32Gly33 and Arg65Gly66-split derivatives were detected, (Cohen *et al* , 1986). Deacon and Conlon (1985) attempted to simplify the procedure by extracting only 1ml of serum with ethanol. Their assay was capable of detecting 4pM human proinsulin. Yoshioka *et al* (1988) also used a specific RIA to study serum proinsulin levels. Although they were only able to get proinsulin-specific antiserum in one guinea pig among six immunised animals, the antiserum did not recognise human insulin nor C-peptide, but cross reacted with derivatives of human proinsulin. In order to improve the assay sensitivity, they incubated serum samples and antiserum for 3 days before adding labelled proinsulin and attained a sensitivity of 2pM.

Side by side with the developments of proinsulin radioimmunoassay methods, a new methodology was being developed to take cognisance of some of the deficiencies of the RIA. The discovery of proinsulin, the

demonstration that PLM exist in human plasma and the realisation that proinsulin reacts like insulin in many insulin radioimmunoassays served to question the specificity and validity of plasma insulin radioimmunoassays The problem of specificity is unlikely to be serious in the assay of insulin in plasma from normal subjects, in which it has been estimated that PLM accounts for only 10-20% of the immunoreactive insulin in plasma However, in plasma where PLM are present in higher concentrations, their presence may interfere with the interpretation of RIA measurements of insulin The principle of immunoradiometry, the new methodology devised by Miles and Hales (1968) to measure insulin, is that the compound to be measured is assayed directly in combination with specific labelled antibodies in excess With antibody excess, all the unlabelled antigen will be bound, unlike in RIA where an antigen must compete with a labelled antigen for a limited number of antibodies Hence the IRMA will be more sensitive than the RIA Moreover, while low molecular weight polypeptides often cannot be labelled to high specificity, the large molecular size of antibodies allows the preparation of relatively undamaged purified radiotracers with high specific actvity Increased specificity was achieved with the development of a two-site IRMA, where the antigen is extracted with a specific capturing antibody, coupled to a solid phase The bound antigen is next reacted with another labelled signalling antibody directed against a second epitope Since the resulting product reflects antigen uptake by the immunosorbent or solid phase in the first reaction, radioactivity attached to this complex during the second incubation will be a measure of antigen concentration Synthesis of intact and split proinsulins by recombinant DNA technology (Frank *et al* 1981, 1984) in combination with the monoclonal antibody technique (Kohler and Milstein, 1975) has allowed unlimited production of highly specific

antibodies directed against insulin, proinsulin, 65-66 split and 32-33 split proinsulins This permitted the development of a direct two-site IRMA by Sobey *et al* (1989) which superceded the originally developed indirect two-site IRMA (Beck and Hales,1975) Novel methods utilizing a series of monoclonal antibody- based two site immunoradiometric assays for insulin, proinsulin and two split proinsulins have now become available as a result of the work carried out by Gray *et al* (1987) and Sobey *et al* (1989) The more recent assays were sensitive enough to detect the 32-33 split proinsulin as one of the major PLM present in serum Thus, IRMA may enable the specific assay of insulin and of the PLM for the first time and be of value in determining the true insulin status of individuals and in the search for abnormalities of insulin synthesis and processing

While several immunological methods for measuring PLM in serum samples have now been published, there have been few reports of these methods being capable of distinguishing between the split- and des- forms, probably because it is difficult to develop site-specific immunoassays that can distinguish between the small structural differences in the various proinsulin conversion products Recently however, a reverse-phase high-performance-liquid chromatographic (RP-HPLC) method capable of separating insulin, proinsulin and its four major intermediates was reported (Linde *et al*, 1991) The method required an evaluation of several stationary-mobile phase combinations in order to achieve the desired separation It was possible to separate all six components using Nucleosil $300-5C_4$ as the stationary phase and 0 1% trifluoracetic acid-acetonitrile as the mobile phase Serum samples from normal subjects and patients with non insulin-dependent diabetes mellitus which were immunopurified prior to chromatography and analysed by an ELISA were shown to contain both intact proinsulin and

des-proinsulin intermediates (Linde *et al*, 1991) The inability of current immunological methods to distinguish between the split- and des-forms may account for 32-33 split proinsulin being perceived as the major proinsulin intermediate detected in human serum Furthermore, work has shown that transformations of split forms to the corresponding des forms did not occur during chromatography

As mentioned previously, possible mechanisms for the increase of proinsulin in serum in diabetic patients may be enhanced synthesis and secretion from ß cells or defective proteolytic conversion of proinsulin to insulin in storage granules One method for determining if proteolytic conversion of proinsulin is defective in diabetes might be to determine the ratios of intact proinsulin to total proinsulin in diabetic patients and non-diabetic patients Thus, the objective of this project was to determine the proportion of total immunoreactive proinsulin (intact, split and des form intermediates) that was biologically active in fasting serum samples from non -diabetic and type 2 (non-insulin-dependent) diabetic patients This was achieved by determining the concentrations of biologically active intact proinsulin by cytochemical bioassay Measurement of intact proinsulin levels depended on examining its effect on HMG CoA reductase activity and comparing the doses of unknown serum that gave the same response as standard intact proinsulin doses In addition, a novel two-site enzyme-linked immunosorbent assay based on the sequential use of anti C-peptide IgG and anti-insulin IgG was investigated for monitoring total immunoreactive proinsulin

CHAPTER 2

MATERIALS AND METHODS

2 1 <u>Chemicals</u>

The following materials were purchased from Riedel de Haen Chemical Company (IRL) Sodium carbonate, sodium bicarbonate, sodium tartarate, copper sulphate pentahydrate, tris, hydrated magnesium chloride, potassium chloride, ethanol, ethylene-diamine-tetracetic acid di-sodium salt dihydrate (EDTA), potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, sodium hydroxide, sulphuric acid, benzene and acetone

The following were purchased from Sigma Chemical Company Ltd (Poole, Dorset, UK) ß-nicotinamide adenine dinucleotide phosphate (B-NADP), glucose-6-phosphate monosodium salt, glucose-6-phosphate dehydrogenase (Torula Yeast), collagenase, bovine serum albumin, tricine, Hank's Balanced Salt solution (HBSS), Swim's S-77 medium powder, horseradish peroxidase, sephadex G25, sodium borohydride, dialysis tubing, pig-Insulin IgG antiserum, anti-insulin IgG commercial conjugate and o-phenylenediamine (OPD)

Glucose and Folin's Ciocalteau phenol reagent, were obtained from Koch-Light Ltd (Suffolk, UK) Dithiothreitol (DTT), from Aldrich Chemical Company Human biosynthetic proinsulin, was obtained from the Lilly Research Centre Ltd (Surrey,UK) Anti-Human C-peptide guinea pig serum and human insulin were obtained from Novo Nordisk, Dublin Sodium azide was obtained from Merck Phosphate buffered Saline tablets was obtained from Oxoid 4ml capacity reservoirs, fnts (20μm pore size for 4ml reservoirs, 3/8 diameter), and unbonded silica gel were obtained from Jones Chromatography (UK) Silica gel thin layer chromatography plates 60A LK6D were obtained from Whatman (US)

Ready Value scintillation cocktail was obtained from Beckman (Ireland)

Sodium Periodate, Acetic acid, sodium salt, [2-^{14}C], 3-HMG CoA, DL-3 [glutaryl-3-^{14}C] [54 2 mCi/mmol) and [^{3}H]-Mevalonic Acid (25Ci/mmol) from Du Pont NEN Research Products (Boston, Mass 02118) All solvents used were BDH laboratory grade

2 1 1 Equipment

A Waters Protein PAK 300SW-10μm HPLC column, 7 8x300mm, was supplied by Waters, Chromatographic division, Millipore Corporation, Milford, Mass 01757 The system used a Shimadzu, UV-vis spectrometer ELISA absorbances were read on a Titertek TwinreaderPlus

Sorvall RC-5B Centrifuge (Du-Pont Instruments)

L8-70M Ultracentrifuge (Beckman)

Labofuge 6000 (Hereus Christ)

LS-7500 Liquid Scintillation Counter (Beckman)

Labsonic U Soniprep (Braun 2000)

2.1 2 Animals

Male Wistar rats (200-300g) were used in all experiments The rats were fed *ad libitum* and were housed at 21°C in windowless rooms under reversed lighting conditions (lights on 4pm-4am, lights off 4am-4pm) Rats were killed at least 6h after beginning of dark cycle

2 2 Methods

2.2 1 Rat hepatocyte preparation.

Rats were killed by cervical dislocation An incision was made in the abdominal region and continued to the thorax Intestines were moved to one side before perfusion of the liver with collagenase by a modification of the method of Berry and Friend (1969)

Initially, 600ml modified Hank's buffer, pH7 4 containing 25mM sodium bicarbonate (7 5% w/v) and 5 56mM glucose, was perfused via a cannula through the hepatic portal vein and then discarded, in order to remove blood and Ca^{2+} ions from the liver Bicarbonate-free modified Swim's S-77 medium pH 7 4 containing 1 5% (w/v) bovine serum albumin, 50mM tricine and 22 2mM glucose was then perfused to ensure complete removal of the Hank's buffer from the liver Collagenase (30mg) dissolved in 150ml bicarbonate-free modified Swim's S-77 medium was then perfused through the liver for 10-15 mins The liver was removed and cut into 5mm slices (approx) with scissors and suspended in the Swim's S-77 medium containing collagenase After incubation for 15 mins at 37°C on a shaking water bath S8-16 (Techne) at mark 5, the suspension was left to settle for a further 10 mins The liver was then filtered through muslin cloth to gently separate the cells from connective tissue Gravity sedimentation on ice for 10 mins was carried out three times to yield a suspension of parenchymal cells

2 2.2 Preparation of microsomes from hepatocytes.

Hepatocytes were pelleted by centrifugation at 1000g for 5 mins and the buffer discarded 3ml of hypotonic medium pH 7 4 containing 1 5mM Tris, 10mM KCl,10mM $MgCl_2$ $6H_2O$ and 18mM EDTA were added and the cells disrupted by sonication on ice using a Mason Soniprep 1500 MSE

for 2 mins, with a 15 sec break between each 30 sec sonication 7ml

fractionation buffer, pH7 2, containing 100mM sucrose, 50mM KCl, 40mM

KH_2PO_4 and 30mM EDTA was added and mitochondria and cell debris

removed by two centrifugations at 800g for 10 mins followed by 15000g

for 20 mins at 4°C, using a Sorval RC-5B centrifuge (Du-Pont

Instruments) The supernatant was then ultracentrifuged at 104000g for

60 min at 4°C using L8-70M Ultracentrifuge (Beckman) to pellet the

microsomes The pellets were frozen at -70°C until assayed

2 2 3   Determination of cell viability and cell count.

Cells were diluted to 1 5 with modified Swim's S-77 buffer containing

1 5% (w/v) bovine albumin, and suspended in an equal volume of trypan

blue (0 05%v/v) solution When viewed under a light microscope, those

cells that excluded trypan blue were considered viable Cell counts were

carried out using a haemocytometer

2 2 4   Hepatocyte incubation.

Four 9ml aliquots of cells were incubated with 1ml aliquots of either

0 154M NaCl solution or human biosynthetic proinsulin at the following

concentrations 2 7-270 pM

In the case of the bioassay four 9ml aliquots of cells were incubated with

one of the following

i)   0 5ml of 12pM human proinsulin

ii) 1.0ml of 12pM human proinsulin

iii) 0 5ml of human test serum

iv) 1 0ml of human test serum

Incubations were in an atmosphere of 5%CO_2 at 37°C for 2h followed by

a viability check and cell count

Serum was obtained from 7 Control (non-diabetic) subjects (fasting

blood sugar range 4 22-6 67mM) and 14 type 2 (non-insulin dependent) diabetic subjects (fasting blood sugar range 7 0-16 9mM) respectively

2 2 5   Preparation of microsomes from rat liver.

Rat liver was homogenised in 50mM potassium-phosphate buffer, pH7 4, containing 30mM EDTA, 250mM CaCl$_2$ and 1 0mM DTT, at 0°C The microsomes were pelleted by three successive centrifugations as described above The pellets were frozen at -70°C until assayed

2.2.6   HMG CoA reductase assay

The assay for HMG-CoA reductase activity was a modification of the method described by Shapiro *et al*, (1974) Incubations were performed in plastic eppendorf tubes in a total volume of 148μl Microsomal pellets were resuspended in assay buffer pH 7 4, (50mM phosphate buffer containing 30mM EDTA, 250mM CaCl$_2$ and 1 0mM DTT) and 100μl transferred to each tube After 5 min preincubation at 37°C, 40μl of cofactor solution was added (450 nmoles of NADP$^+$, 4 5 μmoles of glucose-6-phosphate, 0.3 I U of glucose-6-phosphate dehydrogenase) and a further 5 min incubation followed The reaction was initiated by the addition of substrate, 52nmoles of DL-hydroxymethyl [3-^{14}C]-glutaryl CoA (specific activity 3000 dpm/nmole) and incubated at 37°C for 30 mins, covered and shaken at mark 5 on a shaking bath S8-16 (Techne) 25μl of 10M HCl was then added to terminate the reaction, followed by 4 μl of [5-^{3}H] mevalonic acid (specific activity 19684 dpm/pmole) as internal standard A further incubation at 37°C for 30 mins allowed the mevalonic acid to lactonise The denatured protein was sedimented by a 5 min centrifugation on a labofuge 6000 (Hereus Christ). The mevalonate product was resolved by either of two chromatographic techniques

described below

2 2 7 Enzyme blank assay.

The assay procedure used for examining enzyme blanks is similar to the
one described in section 2 2.6 above Microsomal pellets were
resuspended in assay buffer pH 7 4 and 100μl transferred in triplicate to
eppendorf tubes After 5min preincubation at 37°C, 40μl cofactor was
added to the tubes These were incubated for 5min at 37°C 25μl of 10M
HCL was then added to the tubes to terminate the reaction followed by a
further 5min incubation at 37°C. 8μl of substrate as described previously
was then added and the tubes were incubated for 30min at 37°C
followed by the addition of 4μl of [5-^{3}H] mevalonic acid as an internal
standard The mevalonate product was resolved by solid phase
extraction The enzyme blank was shown to represent 18-25% of
mevalonate produced in an active microsomal preparation This may be
attributed to either non-enzymatic hydrolysis of the ^{14}C -labelled HMG
CoA, resulting in degradation product of similar polarity to the
^{3}H mevalonate It could also be due to suboptimal performance of the
column resulting in elution of a fraction of ^{14}C -labelled substrate along
with ^{3}H -labelled internal standard

2.2 8 Substrate blank assay.

Microsomal pellets were resuspended in assay buffer pH 7 4 as
described previously and 100μl transferred to eppendorf tubes in
triplicate After 5min incubation at 37°C, 40μl of cofactor was added to the
tubes which were then incubated for 5min at 37°C followed by the
addition of 8μl of H$_2$O instead of substrate The tubes were then
incubated for 30min at 37°C 25μl of 10M HCL was then added to

terminate the reaction, followed by the addition of 4µl of [5-^{3}H] mevalonic acid as internal standard The mevalonate product was resolved by solid phase extraction The substrate blank was shown to represent 4-5% of the mevalonate produced in an active microsomal preparation

2 2 9   Separation of mevalonate

i) by thin layer chromatography (TLC)

120µl of the supernatant solution was applied to activated silica gel thin layer chromatography plates (60 A LK6D, Whatman) and developed in benzene acetone (1 1) (v/v) until the solvent front reached a mark 14 1cm from the base The plate was then scored horizontally at lengths equivalent to R_f values of 0 2, 0 34, 0 5, 0 64, 0 78 and 1 0 (Fig 2 1) Each section was scraped using a razor blade into mini-scintillation vials with 5ml Ready-Value scintillation cocktail (Beckman)

ii) by solid phase extraction.

Columns were set up using 4ml capacity reservoirs (Jones Chromatography, UK) A frit (20 µm pore size, 3/8" diameter) was placed in the column The column was then filled with 2g of unbonded silica gel (Jones Chromatography) Another frit was then placed on top of the gel The column was then placed over a mini-scintillation vial (Beckman) and pre-equilibrated with 1ml toluene acetone (3 1) (v/v) 120µl of the supernatant solution was then applied to the columns A small amount of pressure was applied using a 1ml syringe to load the samples onto the column This was left for 30-45 minutes to ensure that the sample has entered the column 2ml of toluene acetone (3 1) (v/v) was then added and collected as it ran through the column The 2ml fraction collected was designated as the first vial The column was then transferred to a second mini-scintillation vial and 1ml of toluene acetone (3 1) (v/v) was

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Figure 2.1 TLC chromatogram illustrating
the method of plate scoring after
development in benzene acetone (1 1, v/v)

SOLVENT FRONT

(14.4 cm)

R_f

1.0

0 78

0 64

0.50

0 34

0 20

0

ORIGIN
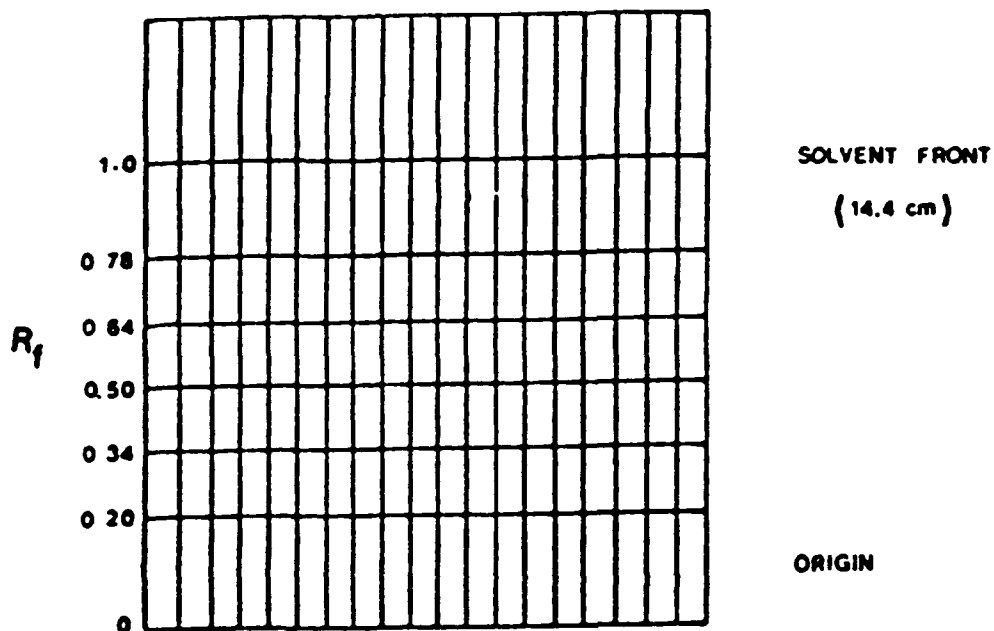
added This was allowed to run through into the second vial The column

was then transferred to a third mini-scintillation vial and 4ml of

toluene.acetone (3 1) (v/v) was added This was allowed to run into the

third vial The column was then transferred to a fourth mini-scintillation

vial and 2ml of toluene acetone (3 1) (v/v) was added This was allowed

to run into the fourth vial The vials were dried down to a volume of 300μl

using a Haake Buchler Vortex Evaporator (Gallon-Kamp, UK) Each vial

was then filled with 5ml of Ready-Value scintillation cocktail

Counting was performed using an LS 7500 liquid scintillation counter

(Beckman) The amount of product produced was calculated using the

following equation

nmoles mevalonate produced per 30 mins per 100μl protein=

$$\frac{\text{dpm }[^{14}\text{C}]\text{mevalonate recovered}}{\text{specific activity }[^{14}\text{C[HMG] CoA}} \quad \times \quad \frac{\text{dpm }[^{3}\text{H] mevalonate added}}{\text{dpm }[^{3}\text{H] mevalonate recovered}}$$
(3000dpm/nmole)

2 2 10 Estimation of microsomal protein content

Microsomal pellets were assayed by the method of Lowry et al, (1951).

Triplicate aliquots of microsomal fractions were diluted to 1ml with

distilled water. Protein standards containing 0-1 mg/ml bovine serum

albumen and a control containing 1ml distilled water were also set up in

triplicate 5ml of fresh reagent containing 50ml of 2% (w/v) sodium

carbonate in 0 1M sodium hydroxide, 0 5ml of 0 5% (w/v) copper

sulphate pentahydrate and 0 5ml of 1% (w/v) potassium tartarate were

added to each tube The tubes were mixed and left in the dark room for

10 min 0 25ml of Folin Ciocalteau phenol reagent (1 3) (v/v) was added

and the tubes left in the dark for a further 25min. Absorbance was read at

600nm and the protein concentration of the microsomal fractions was

read from the standard curve Standard curves were found to be linear within the range used (Fig 2 2)

2 3 Development of ELISA for proinsulin.

This involved characterisation of two antibodies (anti C-peptide IgG, and anti insulin IgG) with respect to (a) the determination of their working dilutions, (b) the determination of their cross reactivities and (c) the determination of optimum incubation times for each step in the assay The following buffers, stocks and substrates were used

1 Carbonate buffer 0 05M Na_2CO_3 , pH9 6

2. Blocking buffer 1% BSA in carbonate buffer.

3. Wash buffer 0 05% Tween 20 in PBS

4 HRP substrate 40mg O-Phenylenediamine per 100ml citrate phosphate buffer, pH5 0 and 0 003% H_2O_2

5 Human proinsulin stock, 25mg/ml

6. Anti-insulin IgG, 3 1mg/ml

7 Anti C-peptide IgG, 5mg/ml

2.3.1 Conjugation of anti-insulin IgG to Horse Radish Peroxidase (HRP) using the periodate method.

Anti-insulin IgG was conjugated to horse radish peroxidase (HRP) using the periodate method as described by (Tijssen and Kurstak, 1984) (Fig 1 15) 2mg of HRP was dissolved in 0.5ml of 0.1M Na_2CO_3 and placed in a foiled covered scintillation vial. 0 5ml of 8mM sodium periodate was added and the mixture left for 2h at room temperature 5mg of the purified commercial IgG, in sodium carbonate buffer following dialysis was added, along with a small amount of Sephadex G25 (3mg approx), and the mixture left for 3h at room temperature After this period, the conjugate was eluted from the Sephadex by passing it through a glass

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Figure 2 2 Standard curve of protein concentration (mg/ml) as a function of absorbance 600nm
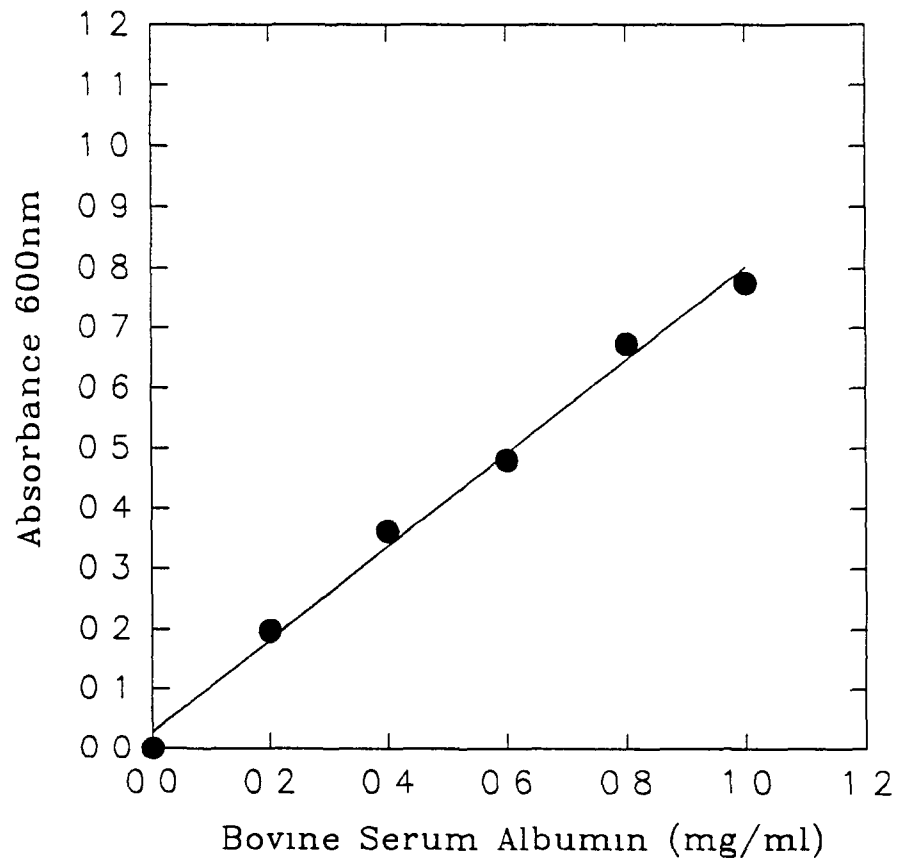
pasteur pipette packed with glass wool, and was immediately stabilised with 1/20 volume of sodium borohydride (5mg/ml in 0.1mM NaOH, freshly prepared). After 30 min, 3/20 volumes of sodium borohydride was added and the conjugate left for one hour or stored at -20°C. The conjugate was subsequently characterised by HPLC.

2.3.2   Characterisation of the conjugate.

20μl samples of the prepared conjugate, 0.1M HRP, and anti insulin IgG were applied to a Waters Protein Pak 300 SW column for analysis. The flowrate was 0.5ml/minute and the running buffer was 0.1M sodium phosphate, particle and gas-free. A Shimadzu uv-vis spectrophotometer was used to detect absorbance at 280nm (Fig 's 2.3(a) and 2.3(b)). These figures demonstrate that when 20μl of HRP solution was injected it had a retention time of 18.10 min. When 20μl of both anti-insulin IgG and HRP were injected the retention time for anti-insulin IgG was shown to be 16.93 min and that for HRP was 18.04 min. When 20μl of the prepared conjugate and anti-insulin IgG were injected the retention times for the conjugate and anti-insulin IgG were shown to be 14.53 and 16.82 minutes respectively. The conjugate has a shorter retention time (14.45 minutes) than that of anti-insulin IgG or HRP thus demonstrating that the procedure for conjugation was successful (Fig 2.3(a)).

2.3.3   Determination of the protein concentration of the capturing (anti-C-peptide IgG) and signalling (anti-insulin IgG conjugate) antibodies by the Bio Rad assay

Standards in the range 0 to 0.05mg/ml were prepared using phosphate buffered saline (PBS) as diluent and BSA as protein standard. 200μl of diluted Bradford reagent was dispensed into a series of wells in a

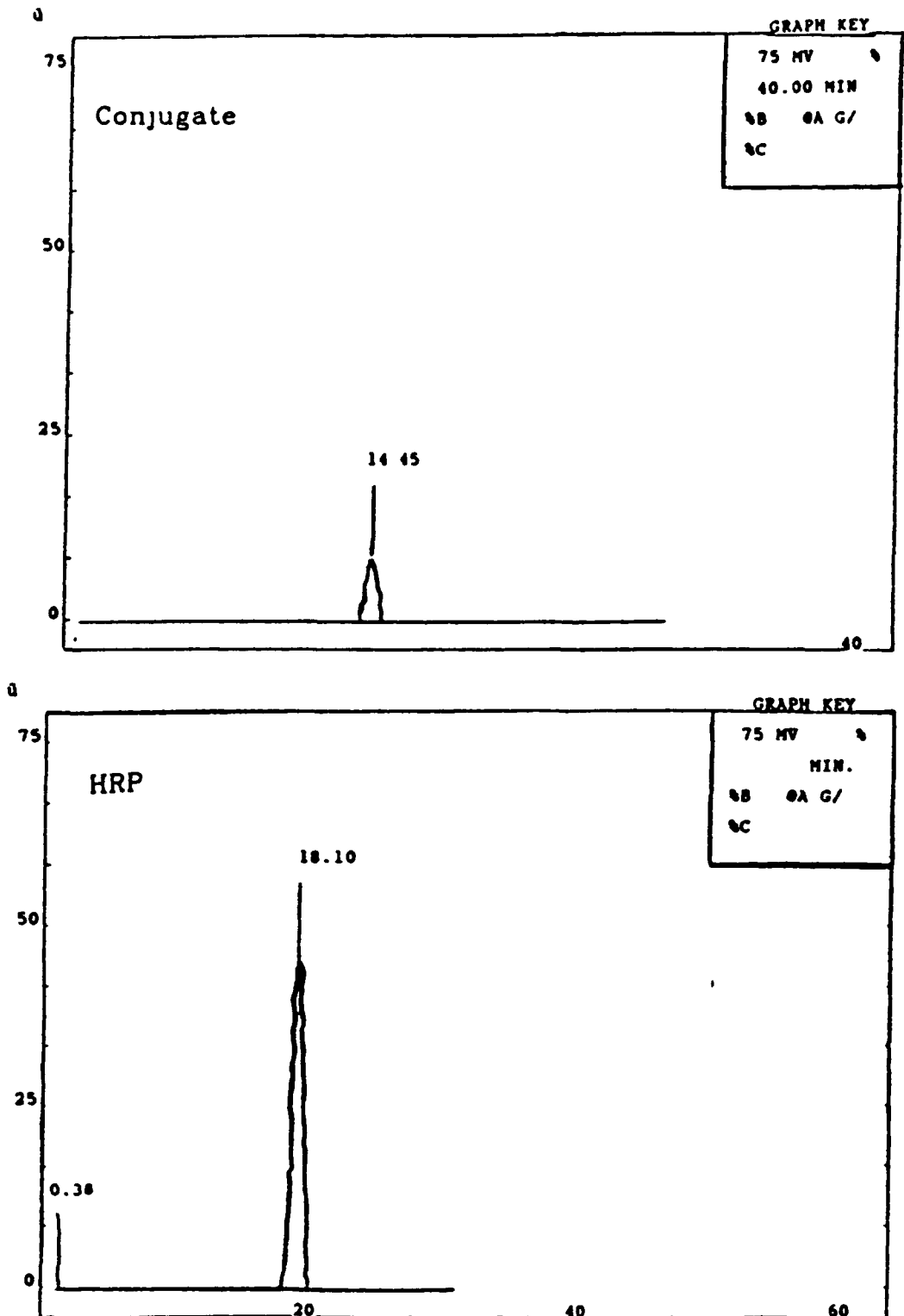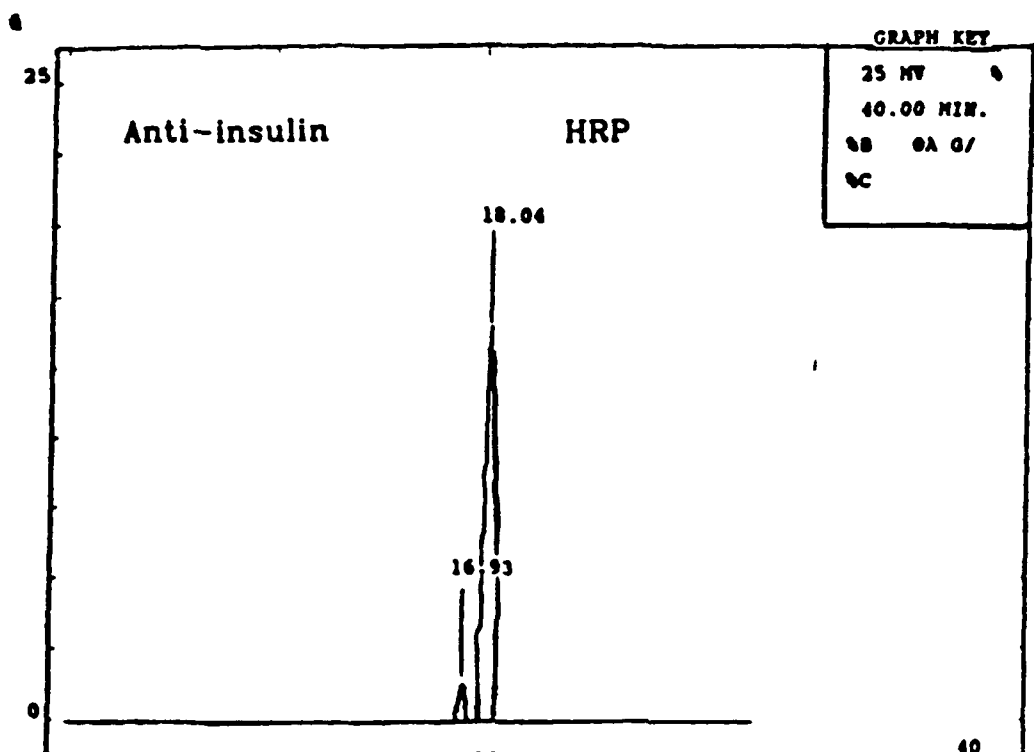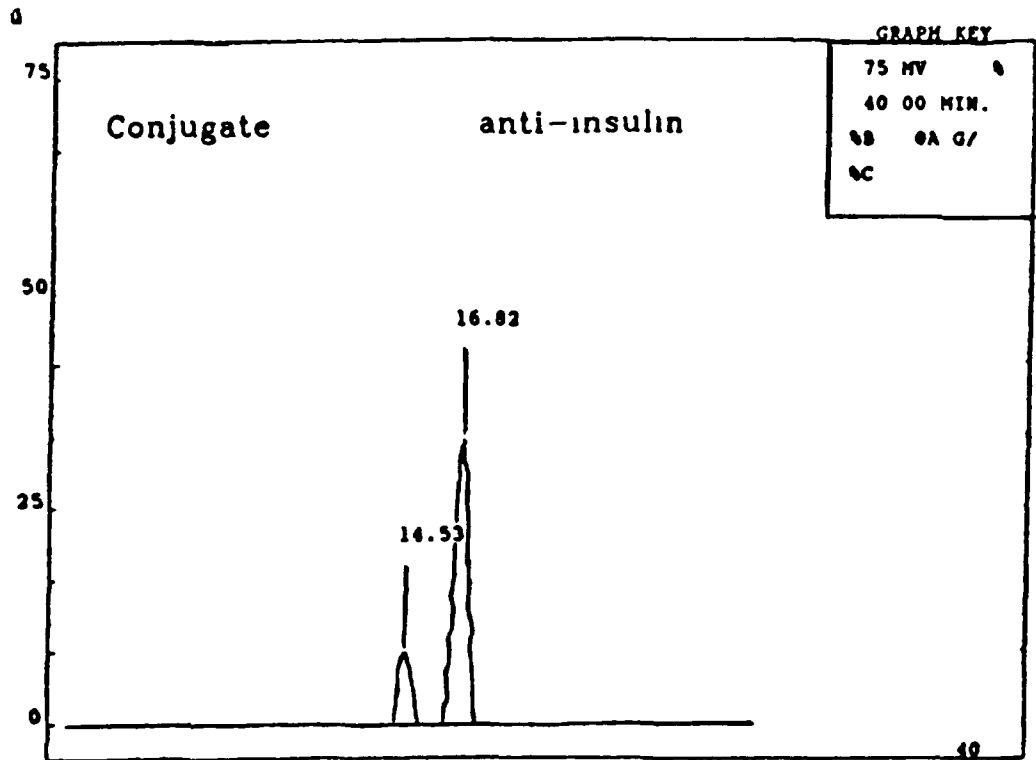Figure 2.3 (a): HPLC chromatogram of anti-insulin IgG -HRP conjugate and HRP

Figure 2.3 (b): HPLC chromatogram of anti-insulin IgG and HRP conjugate and anti-insulin IgG and HRP.

microtitre plate followed by 20μl of each sample or 20μl of each standard in duplicate Some fractions were diluted first using PBS to bring them within the range of the standard curve A cover was placed over the plate and the contents of each well mixed thoroughly by moving the plate backwards and forwards and from right to left on the bench. Between 5 and 60 minutes after mixing, the absorbance of each well was recorded at 595nm using PBS as a blank (Fig 2 4)

2 3 4   Characterisation of the antibodies.

a) Determination of the working dilution of the antibodies
A checkerboard titration was performed, wherein serial dilutions of proinsulin were immobilised on a plate, and serial dilutions of the purified antibodies were reacted against the immobilised antigen to give a 2-dimensional result (Fig 2 5 and 2 6) One plate was performed for the anti-C-peptide IgG, and one for the anti-insulin IgG-HRP conjugate The former required the inclusion of a third (commercial conjugate) for detection, while the latter did not. 200μl of the proinsulin dilutions were added in duplicate to 2 microtitre plates, which were incubated at 37°C for 2h, washed (x3) in wash buffer, and blocked (250μl of blocking buffer added to wells), followed by incubation at 37°C for 2h and washing (x3) in wash buffer The anti C-peptide IgG dilutions were added in duplicate to each of the proinsulin concentrations on one plate, and the anti-insulin IgG-HRP dilutions to the other Both plates were incubated for 2h at 37°C, following which, the wells were washed (x3) in wash buffer To the plate containing the anti-C-peptide IgG 100μl of commercial conjugate was added at its working dilution This plate was then incubated for 2h at 37°C, and washed (x5). 100μl of fresh substrate was added, and the plate incubated for 30min at 37°C The reaction was stopped by the

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Figure 2.4: Standard curve of protein concentration (µg/ml) as a function of absorbance 595nm.

Figure 2.5: A checkerboard titration to determine the working dilution of anti C-peptide IgG.

Wells Coated With Proinsulin : ● 2.5µg/ml ⊛ 0.25µg/ml ◯ 0.025µg/ml

A Blank
B
C 1/100
D 1/250
E 1/500
F 1/1000
G 1/2000

* Working Dilutions Of Anti C-Peptide : Dilutions Indicated at the Left Of Each Row
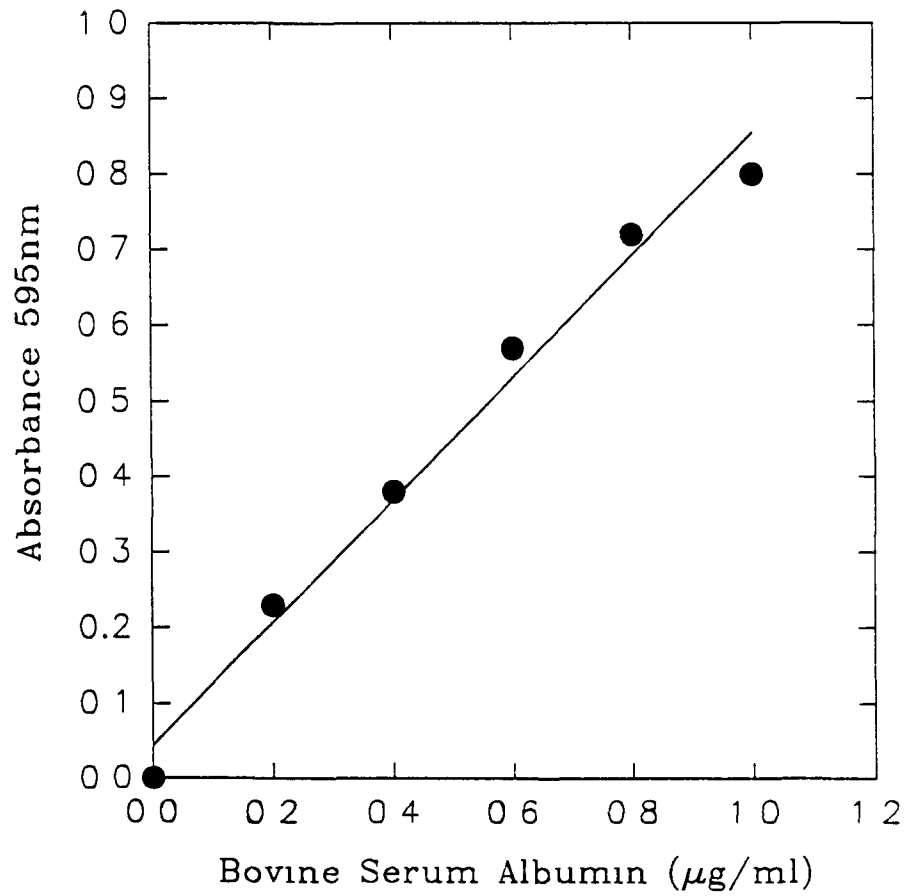
Figure 2.6: A checkerboard titration to determine the working dilution of anti-insulin IgG-HRP conjugate.

Wells Coated With Proinsulin :   ● 2.5μg/ml   ⊛ 0.25μg/ml   ○ 0.025μg/ml

	Blank	1/5	1/10	1/15	1/20	1/25
A						
B						
C						
D						
E						
F						
G						

* Working Dilutions of Anti-Insulin IgG : Dilutions Indicated at the Left Of Each Row

78
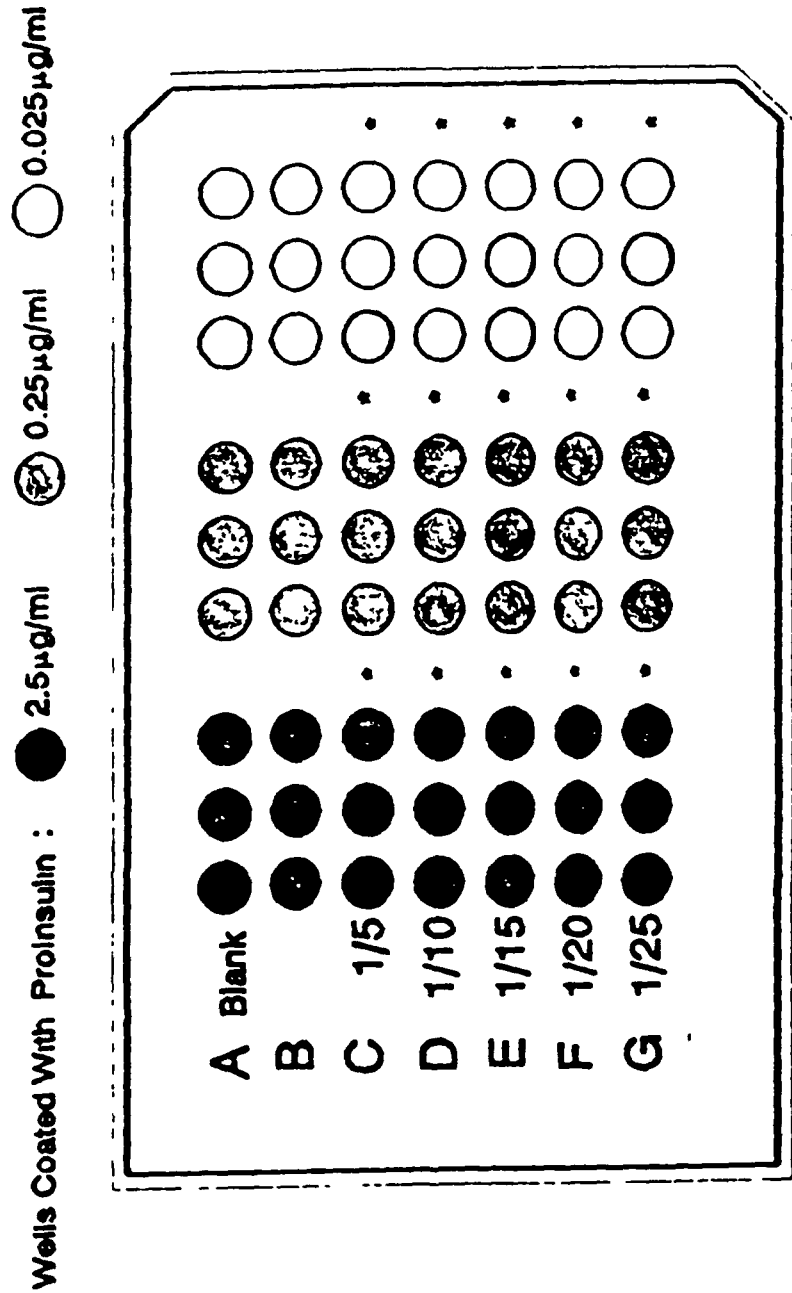
addition of 50µl 2M sulphuric acid and the absorbances read at 492nm
The plate containing the anti insulin IgG-HRP conjugate did not receive
the commercial conjugate but had substrate added directly

b) Determination of the cross-reactivities of each antibody

ı) <u>Cross-reactivity of anti-C-peptide IgG with human insulin</u>

Human proinsulin and human insulin stock solutions were each diluted
from 0 1mg/ml serially to 0 1ng/ml 200µl of each dilution was added to
the wells of a microtitre plate, followed by incubation at 37°C for 2h and
washing (x3) The plate was subsequently blocked as described, and
100µl of the anti-C-peptide IgG added at its working dilution, followed by
2h incubation at 37°C and washing (x3) 200µl of commercial conjugate
at its working dilution was then added to each well and the plate
incubated at 37°C for 2h, followed by washing (x5), substrate addition,
and the reading of absorbances at 492nm

ıı) <u>Cross reactivity of anti insulin IgG with C-peptide.</u>

This assay was performed exactly as was (1) above, except that instead
of the commercial conjugate, the prepared anti-insulin IgG-HRP
conjugate was added This was incubated at 4°C overnight in order to
mimic better the conditions in the completed assay (see below) The
cross reactivities of insulin (1-1000ng/ml) and C-peptide (0 01-20µg/ml)
using these concentration ranges were also examined in the complete
assay

(c) *Determination of the optimum incubation times for each step in the*
   assay

1 <u>Adsorption of anti-C-peptide IgG to the wells.</u>

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100μl of anti-C-peptide IgG at its working dilution was added to the wells of 5 microtitre plates, which were incubated at 37°C for 1,2,6,8 and 10 hours respectively The plates were subsequently blocked, and 100μl of commercial conjugate at its working dilution added, followed by incubation for 2h at 37°C, addition of 100μl of fresh substrate, and the reading of the absorbances at 492nm

2 Binding of proinsulin by anti C-peptide IgG

Proinsulin stock was diluted to 10ng/ml in carbonate buffer and 100μl added to the wells of 3 microtitre plates, which were then incubated at 37°C for 2h, and were subsequently washed (x3) and blocked 100μl of anti C-peptide IgG diluted to its working dilution in blocking buffer was added to each well, and the plates were incubated for 1,2 and 3h, respectively, at 37°C, following which they were each washed (x3) and 100μl of commercial conjugate at its working dilution was added to each well The plates were incubated for 2h at 37°C, washed (x5), and 100μl of substrate was added to each well, following which the absorbances at 492nm were read

3 Binding of proinsulin by anti-insulin IgG

This study was performed exactly as was (2) above, except that incubation times of 2,4,6,8 and 10 hours for the conjugate were performed, and the addition of the commercial conjugate was not required

2 3 5 The completed assay- standard curves, cross reactivities, and analysis of patient serum samples

100μl of anti C-peptide IgG diluted to its working concentration in

carbonate buffer was added to the wells of a microtitre plate, and the plate incubated at 37°C for 2h, following which the wells were washed (x3), and the plate blocked as described previously 100µl of appropriately diluted proinsulin stock (for standard curves), insulin and C-peptide stock (for cross reactivity assays), or patient serum samples, were added in triplicate to the wells, followed by incubation at 37°C for 2h The wells were subsequently washed (x3), and 100µl of anti-insulin IgG-HRP conjugate was added, and the plate incubated overnight at 4°C Following incubation, the wells were washed (x5) in wash buffer, 100µl of freshly prepared substrate was added to each well, and the plate incubated for 30 min at 37°C The reaction was then stopped with 50µl of 2M sulphuric acid, and the absorbances at 492nm read after a further 5 min at room temperature

Summary of ELISA method for proinsulin.

1 Add 100µl anti C-peptide IgG to wells

2 Incubate at 37°C, 2h

3 Wash, block

4 Add 100µl serum, or proinsulin standards to wells

5. Incubate 37°C, 2h

6 Wash

7. Add 100µl anti-insulin IgG-HRP conjugate

8 Incubate 4°C overnight

9. Wash

10. Add 100µl substrate

11. Incubate 30min at 37°C

12 Stop rxn with H_2SO_4

13. Read absorbance 492nm

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CHAPTER 3

CHARACTERISATION OF HEPATOCYTES AND HEPATOCYTE
REDUCTASE ASSAY

Prior to the use of isolated rat hepatocytes for the study of the direct effect of proinsulin, it was necessary to characterise both the test system and the biological response being examined

Therefore suitable criteria for characterising the method of hepatocyte preparation and their viability were determined Anticipating that each bioassay would require the assay of four cell aliquots in triplicate for reductase activity and protein, it was decided to determine the optimal conditions for the assay of HMG CoA reductase activity and, in view of the number of samples to be assayed at any one time, the most suitable method of product isolation

3.1   Hepatocyte yield and viability

The hepatocyte yield from rats weighing on average 250-300g was typically $3\text{-}4\text{x}10^8$, the approximate wet weight of cells after liver perfusion being 5-7g. This allowed for multiple enzyme assays to be carried out on each cell preparation The bioassay format, as described in Chapter 2 required that the total cell yield be divided into four equal aliquots prior to incubation with either hormone or serum. Therefore, for every enzyme determination, 10ml cell suspensions containing approximately $7\,5\text{x}10^7$ cells were harvested after incubation for 2h to yield microsomal fractions The average protein yield from each microsomal pellet was $3\,6\pm0\,09$ mg/ml (mean $\pm$SD)

The viability of hepatocytes as determined by the trypan blue exclusion test ranged between 80-90% before and after 2h incubation period at $37^\circ C$ in 5% CO_2

3 2   Optimization of standard assay conditions

Prior to the estimation of HMG CoA reductase activity in microsomal fractions of rat hepatocytes, assay conditions for the measurement of the

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enzyme with respect to the concentration of substrate required, length of incubation time with substrate and concentration of protein were established In these experiments, ^{14}C mevalonate was separated from reactants by direct application of the deproteinised, acidified incubation mixture to thin layer chromatography plates

Mevalonate synthesis as a function of incubation time is shown in Fig 3 1 100µl aliquots of a suitably diluted rat hepatocytes and intact rat liver fractions, containing 830µg and 430µg protein respectively were incubated with 8µl of [^{14}C] HMG CoA (corresponding to 52nmoles per assay) for varying lengths of time between 0 and 60min at 37°C Although microsomal enzyme activity after 30min was markedly lower in hepatocytes (3 7 nmol/mg) relative to intact liver (17 5 nmol/mg), activity was linear with time of incubation up to 60min for hepatocytes and 30min for the intact rat liver

Fig 3 2 depicts mevalonate synthesis as a function of intact liver and hepatocyte microsomal protein concentration 100µl aliquots of a suitably diluted hepatocyte and rat liver microsomal fraction containing between 0 and 800µg and 0 and 400µg of protein respectively were assayed for HMG CoA reductase Although reductase activity from rat hepatocytes and intact rat liver was observed to be linear with protein concentration within the range tested it is apparent that the specific activity of HMG CoA reductase in hepatocytes (100 pmol min^{-1} mg^{-1}) was approximately six fold lower than that observed in intact liver microsomes (639 pmol min^{-1} mg^{-1})

Having established the linearity of the assay with regard to protein and time, the enzyme as expressed in liver and hepatocytes was then studied vis a vis its kinetic parameters The assay conditions with respect to protein concentration per 100µl and length of incubation time with

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Figure 3 1 Activity of HMG CoA reductase
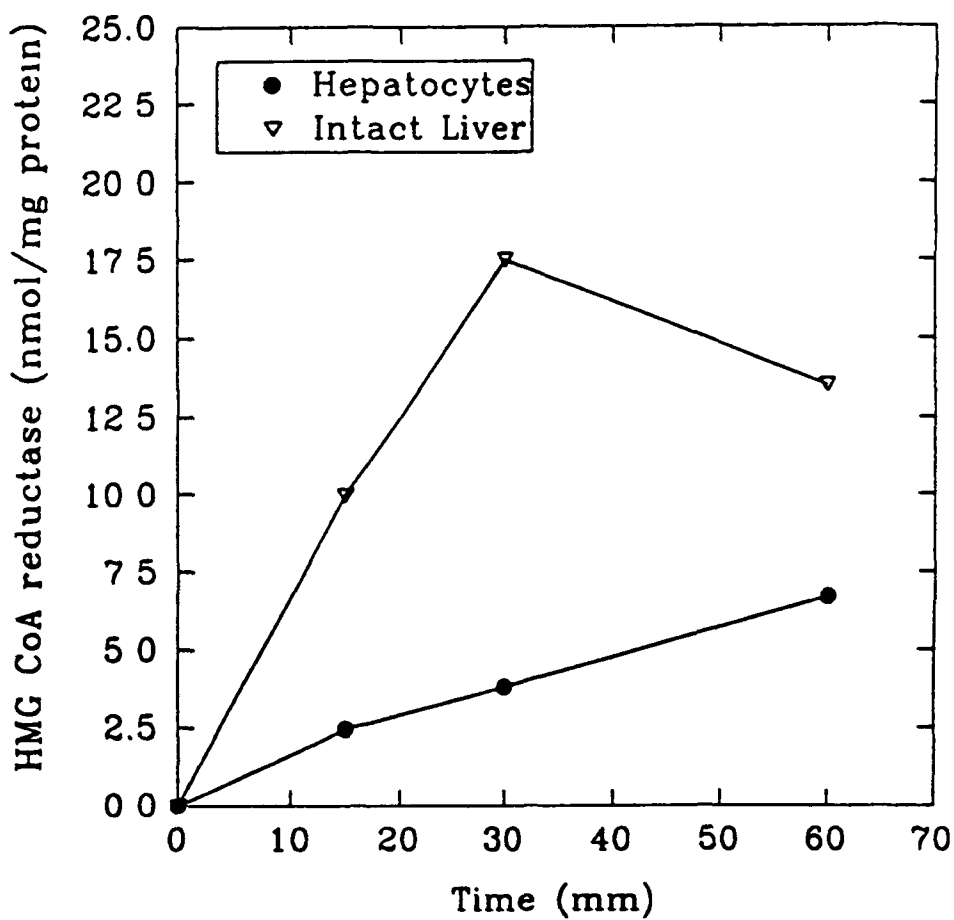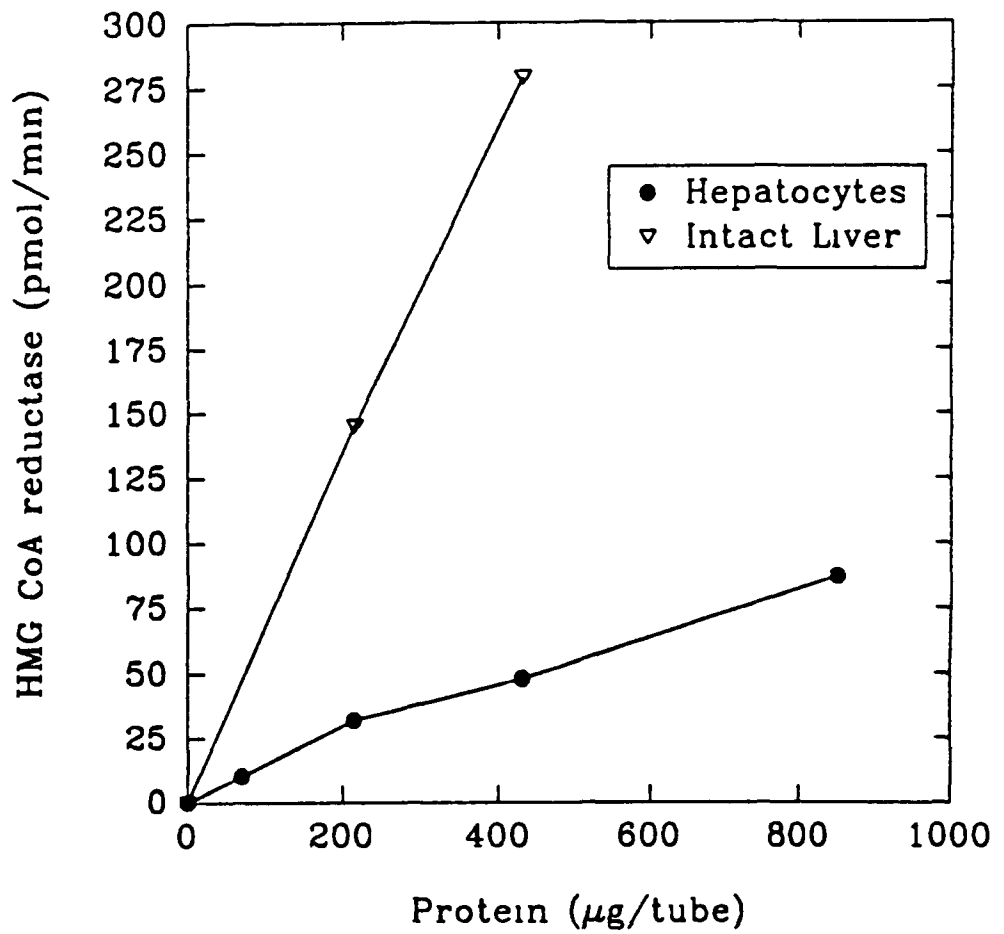(nmol/mg) as a function of time (min)

Figure 3 2 Activity of HMG CoA reductase (pmol/min) as a function of protein concentration (μg/tube)

substrate that were adopted for the determination of Km and Vmax of HMG CoA reductase were 0.43 mg and 60min respectively at 37°C in rat hepatocytes and 0.4mg and 30min at 37°C in rat liver. Fig 3.3 shows the specific activity of HMG CoA reductase (pmol. min^{-1}. mg $protein^{-1}$) as a function of substrate concentration (nmol hydroxymethylglutaryl CoA per assay). Analysis was by the standard method except for the indicated changes in the concentration of substrate (specific activity 3000 d.p.m. /nmol). Maximal enzyme activity in both fractions was obtained using 52 nmoles of substrate per assay. Increasing the substrate concentration by a factor of 2 did not greatly alter the specific activity of HMG CoA reductase in rat liver (272 pmol. min^{-1}. mg protein $^{-1}$). It is apparent however that halving the substrate concentration reduced the specific activity of the enzyme in rat liver by approximately 20% (Table 3.1). The values of Km and Vmax for HMG CoA reductase activity in rat hepatocytes and intact liver are shown in Table 3.2. The data were extrapolated from Lineweaver Burk plots as shown in Fig 3.4. The concentration of DL-hydroxymethylglutaryl CoA used (52 nmoles / assay or 3.466 $\times 10^{-4}$M) exceeded the Km values by a factor of 5 in hepatocytes and 3.8 in rat liver. It is however apparent that the specific activity of the enzyme in rat liver microsomal pellet fraction had decreased relative to that observed in the same rat liver pellet when assayed fresh for determining the time and protein courses of the enzyme assay. This reflected the lability of the enzyme after storing at - 20°C and subsequent thawing of a microsomal pellet suspension.

3.3 Comparison of solid phase extraction and direct thin layer chromatography as methods for separation of mevalonic acid.

Fig 3.5 shows that the specific activity of HMG CoA reductase determined

Figure 3 3 Specific activity of HMG CoA reductase
$(pmol\ min^{-1}\ mg^{-1})$ as a function of substrate
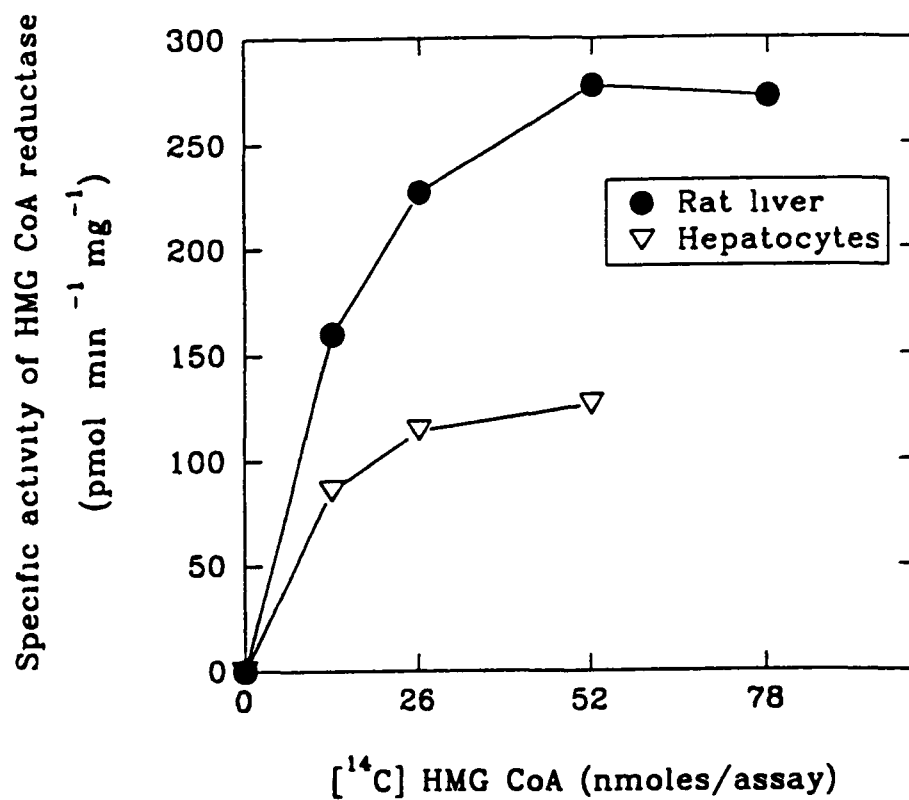Concentration (nmoles/assay)

TABLE 3.1

The effect of hydroxymethylglutaryl Co A concentration on reductase activity

[HMG CoA] (nmoles/assay)	Specific activity of HMG CoA reductase (pmol min^{-1} mg^{-1})	
	Intact liver	Hepatocytes
13	162 0	86
26	226 5	11 4
52	277 6	126
78	272 0	

TABLE 3 2

Kinetic constants of HMG CoA reductase in microsomal fractions of rat liver and hepatocytes.

	HMG CoA reductase	
	Km (mM)	Vmax (pmol min^{-1} mg^{-1})
Intact liver	0 090	334 3
Hepatocytes	0 067	153 0

Figure 3.4: Lineweaver-Burk plot of $1/V$ of HMG Co A reductase (pmol.min^{-1}.mg^{-1}) versus $1/S$ (mM) in liver and hepatocytes.
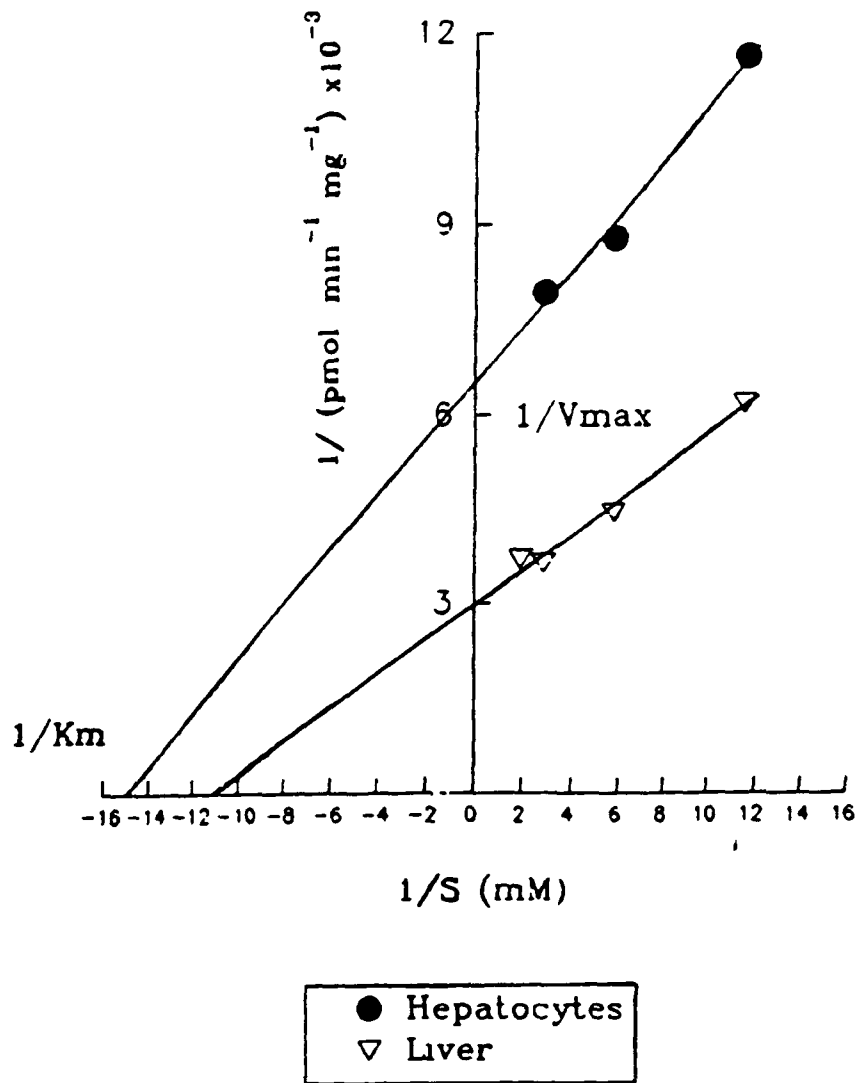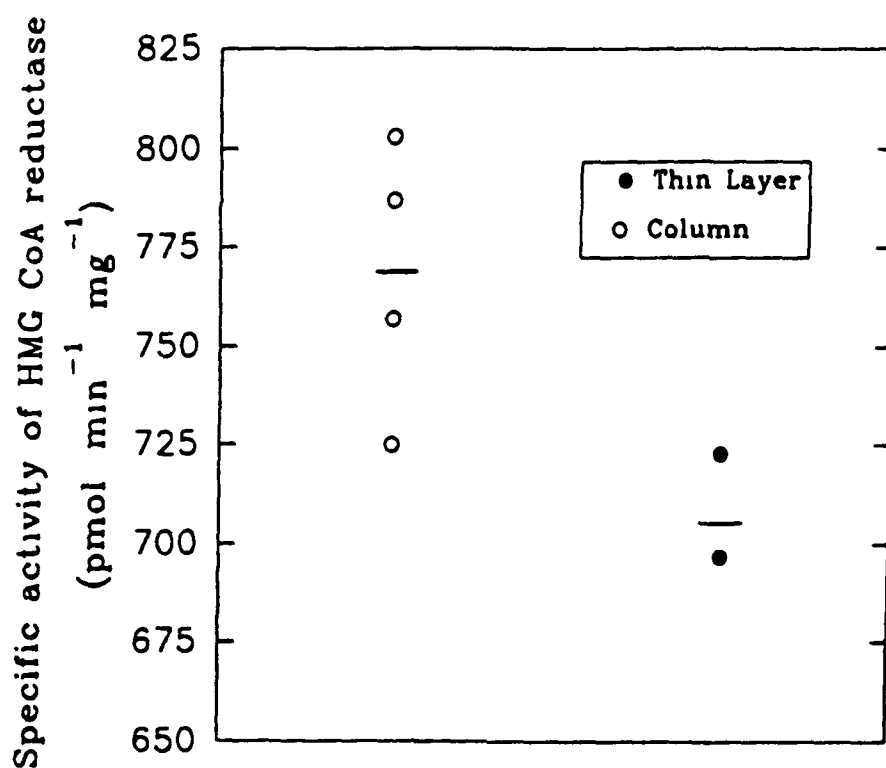
Figure 3 5 Comparison of the specific activity of HMG CoA reductase obtained when using column chromatography or thin layer chromatography

after solid phase extraction (768 45±17 1 pmol min^{-1} mg protein^{-1}) was not markedly different to that observed following direct thin layer chromatography (705 5±17 4 pmol min^{-1} mg $^{-1}$) The reproducibility of the solid phase extraction method is also evident, the coefficient of variation being 4 9% This was the method chosen for all subsequent work as it proved less tedious and resulted in a greater recovery of mevalonate The percentage recovery of mevalonate for solid phase extraction was 62±2 2% (n=21) and for thin layer chromatography 55±8 88% (n=6) (mean±SD)

Table 3 3 demonstrates the repeatability of the assay in freshly prepared hepatocytes using solid phase extraction as assessed by determining reductase activity in three equal aliquots of cells prepared by a single liver perfusion Specific activity ranged between 223 and 266 8 pmol min^{-1} mg protein^{-1}, the % coefficient of variation (CV) being 8 9%

3 4   Lability of HMG CoA reductase.

Table 3 4 shows the decrease in specific activity of HMG CoA reductase observed after storage of microsomal fractions of rat liver at -20°C for three days Specific activity of reductase in unfrozen freshly prepared microsomal fractions was approximately 5.4 times higher than that observed in frozen fractions Anticipating a practical difficulty with measuring the specific activity of the enzyme in microsomal pellets obtained from rat hepatocytes on the same day as their isolation, all microsomal pellets were frozen at -70°C, as an unsuspended fraction prior to enzymatic assay

To conclude, the assay procedure described in Chapter 2 permitted analysis of microsomal preparations with widely different reductase activities as expressed by hepatocytes and intact liver

TABLE 3.3

Specific activity of HMG CoA reductase in a rat hepatocyte microsomal fraction obtained from a rat killed in the middle of the dark cycle. HMG CoA reductase activity was measured three times.

	HMG CoA reductase. $(pmol . min^{-1} . mg^{-1})$
First assay	266.8
Second assay	249.8
Third assay	223.0

TABLE 3.4

Specific activity of hepatic HMG CoA reductase $(pmol. min^{-1}. mg^{-1})$ (Mean $\pm$ SD, n=2) as obtained from intact rat.

| Storage period (days)	HMG CoA reductase $(pmol. min^{-1} . mg^{-1})$	Coefficient of Variation (%)
0	810$\pm$55 (2)	6.7
3	149$\pm$26 (2)	17.5

CHAPTER 4

EFFECT OF PROINSULIN ON HMG CoA REDUCTASE ACTIVITY

It has been known since the early 70's that hepatic cholesterol production in the rat increases following injection of pharmacological doses of insulin However it was not until some time later when simplified *in vitro* systems had been established that it could be ascertained whether changes in the activity of HMG CoA reductase were mediated directly by fluctuations in pancreatic hormone concentrations To date, insulin and glucagon are the only pancreatic hormones that have been shown to directly affect the activity of the rat liver enzyme Since proinsulin constitutes 10-20% of the measurable circulating immunoreactive insulin in humans, the relative biological activity of proinsulin on reductase activity is of obvious interest This chapter describes the effect of proinsulin on the activity of HMG CoA reductase in freshly prepared hepatocytes

4 1   A comparison of the effects of proinsulin and insulin on HMG CoA reductase activity

Hepatocytes obtained from rats, killed in the middle of the dark period were incubated at $37^{\circ}C$ in standard medium either alone or in the presence of insulin (72nM) or proinsulin (50nM) The dose of insulin that was chosen represented the concentration shown previously to produce a significant increase in reductase activity (Gibbons *et al*,1984) Fig 4 1 depicts the specific activity (pmol min^{-1} mg protein $^{-1}$) of HMG CoA reductase (mean±SD) obtained from cells incubated with insulin (199±17 92 (3)), proinsulin (217±17 34(3)) and saline (control) (125±7 51(3)) Insulin stimulated reductase activity by 59% while proinsulin produced an even greater stimulation 73% (Fig 4 1)

Thus it is apparent that proinsulin, like insulin can exert short term hormonal control of hepatic HMG CoA reductase activity in the rat

Figure 4.1: Specific activity of HMG Co A reductase ($pmol.min^{-1}$ mg^{-1}) in isolated hepatocytes maintained in culture with saline, insulin (72nM) and proinsulin (50nM)
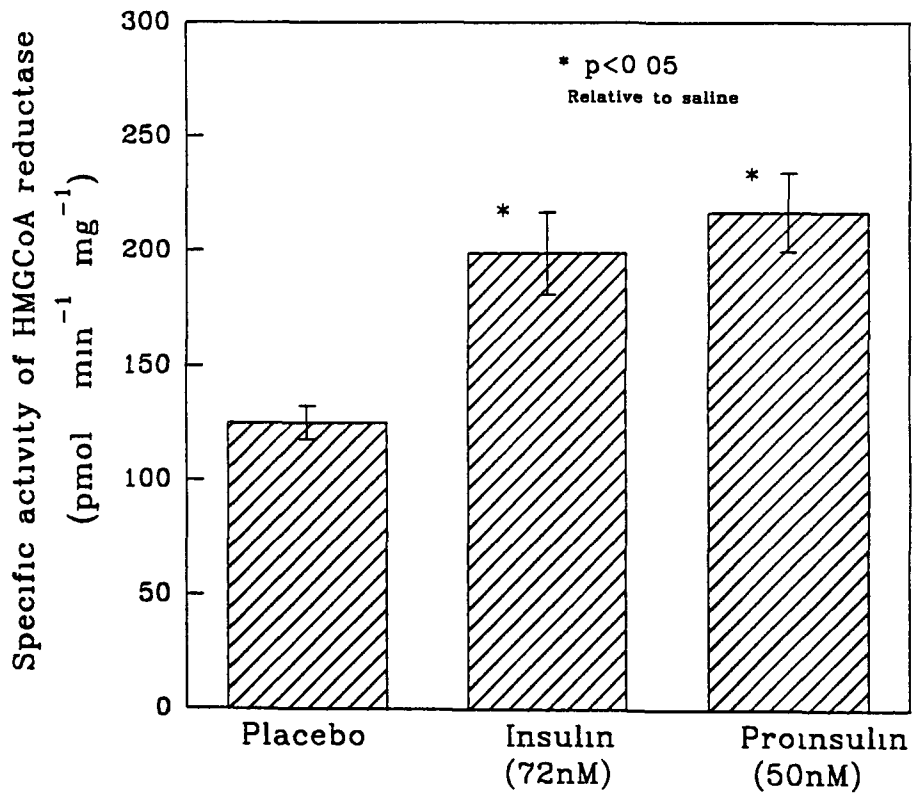
4 2   Establishment of a dose-response curve for proinsulin

In an attempt to determine if proinsulin concentration in biological

samples could be measured by a bioassay technique based on the effect

of proinsulin on HMG CoA reductase activity, it was necessary to

examine the proinsulin response over a range of concentrations likely to

be expected.

Hepatocytes were incubated for 2h in the presence of various

concentrations of proinsulin (2 7-270pM) Fig 4 2 demonstrates a

standard curve relating specific activity of HMG CoA reductase with $\log_{10}$

proinsulin concentration Reductase activity was observed to increase

approximately 68 8% as the concentration of proinsulin increased 100

fold from 2 7pm to 270pM Specific activity of HMG CoA reductase

(pmol min^{-1} mg protein $^{-1}$) in cells incubated with 1ml of 2 7pM, 27pM

and 270pM proinsulin were 114±9, 140±10 5 and 191±5 respectively

Thus a biological response was observed over a proinsulin

concentration range that closely approximated to that observed for serum

proinsulin in different physiological and pathological conditions

4 3   The effects of serum and insulin on the biological response of
proinsulin

The effects of serum and insulin on the proinsulin response were

subsequently investigated by incubating hepatocytes with 2 7pM

proinsulin alone and with either insulin (100pM) or 10% (v/v) human

serum Fig 4 3 shows that the specific activity of HMG CoA reductase was

significantly elevated (p<0 05) when serum was present with proinsulin

in the medium compared with when proinsulin alone was present

(714±251 pmol min^{-1} mg protein $^{-1}$ vs 160±4 98 pmol min^{-1} mg

protein $^{-1}$) Substitution of serum with insulin produced a 157% increase

in reductase activity (252±44 11 pmol min^{-1} mg protein^{1}) though not as

Figure 4 2 Specific activity of HMG CoA reductase as a function of Log_{10} human proinsulin concentration (pM) (mean ± SEM, n=2)
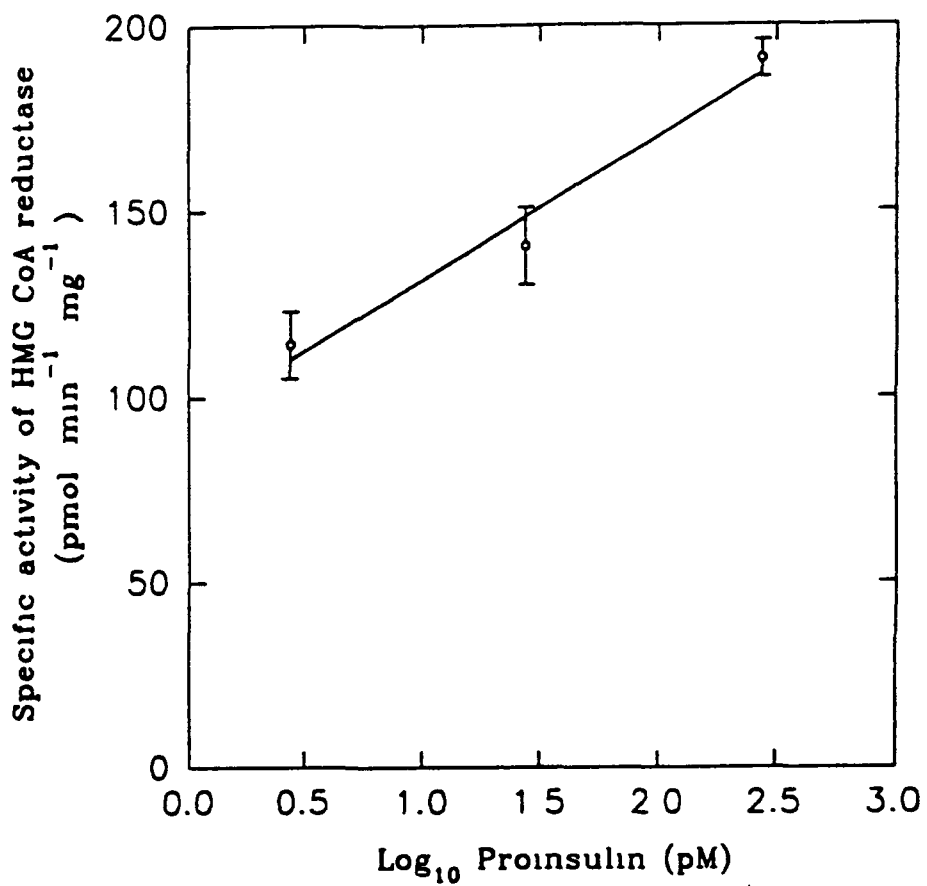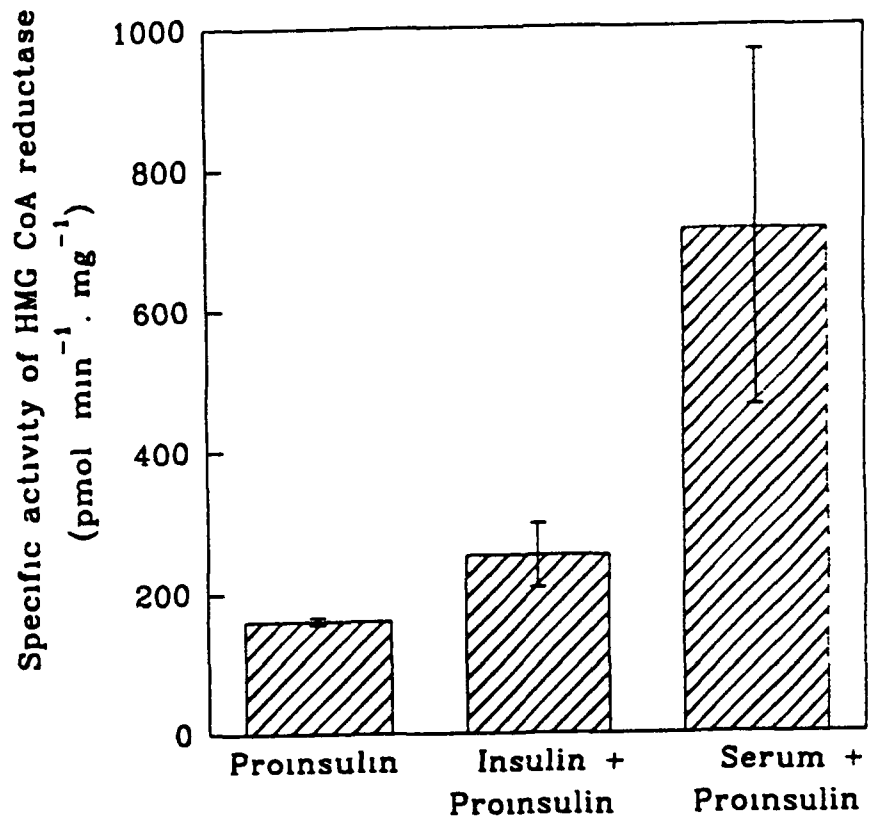
Figure 4.3: Specific activity of HMG Co A reductase after adding serum and insulin(100pM) to incubation medium containing proinsulin (2.7pM) (Mean±SD,n=3).

great as the serum effect (446%) (Fig 4 3)

It was apparent from this study that the biological activity of proinsulin at a concentration as low as 2 7pM could be detected However, its biological activity was lower than that in the presence of either insulin or serum

4 4   Establishment of suitable volumes for a proinsulin bioassay.

In order to construct a proinsulin bioassay it was necessary to determine the most suitable volumes of hormone to add to cells Hepatocytes were incubated for 2h in the presence of different volumes of a 12pM proinsulin standard solution, a concentration chosen apropos it being close to the maximum level of circulating proinsulin that has been observed in humans under normal physiological conditions and also because it was on the linear portion of the graph showing the relationship between specific activity and $\log_{10}$ proinsulin concentration (Fig 4 2)

Fig 4 4 depicts the specific activity of HMG CoA reductase as a function of volume of 12pM standard Although a higher level of activity was obtained when 2ml of standard was added to cell medium, the extent of the increase did not match the 1 7-1 8 fold increase reported on adding 0 25ml, 0.5ml and 1ml of proinsulin standard The magnitude of this biological response was thought to be suitable for a bioassay and hence the volumes of standard chosen for the construction of a 2+2 bioassay were 0 5ml and 1 0ml This experiment did however suggest that there may be a critical volume of Swim`s S-77 medium required for cell maintenance over 2h since addition of 8ml of cell medium represents at least a 12 5% decrease in concentration of cell medium components relative to other test hepatocyte incubations

Figure 4 4 Quantitative effect of proinsulin (12pM standard) on the specific activity of the enzyme HMG CoA reductase (pmol min^{-1} mg^{-1})
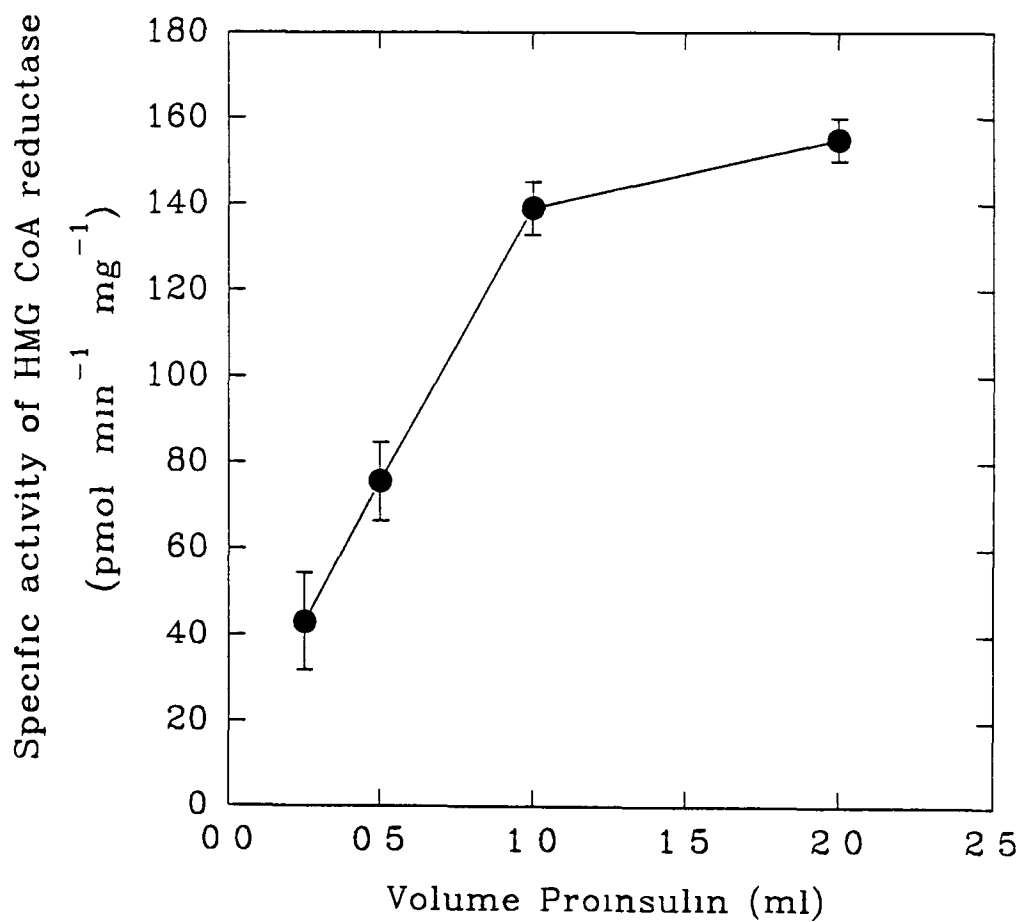
4 5 2+2 Bioassay for proinsulin.

The previous section reported on the effect of incubating proinsulin with hepatocytes. A linear biological response with respect to its effect on hepatocyte HMG CoA reductase was observed over a wide concentration range of proinsulin. This constituted the first step in the development of a proinsulin bioassay. This section reports on the results obtained after construction of a 2+2 bioassay for serum samples obtained from 7 non-diabetic subjects and 14 diabetic patients. Hepatocytes were prepared by perfusion of rat liver with collagenase as described in Chapter 2. Four 9ml aliquots of cells were incubated as described previously with one of the following 1ml of proinsulin standard (12pM), 0 5ml of proinsulin standard (12pM), 1ml of serum or 0 5ml of serum. After harvesting, microsomal fractions were prepared which were subsequently assayed for HMG CoA reductase activity.

Fig 4 5 is an example of a 2+2 bioassay of an unknown control serum sample versus standard proinsulin on isolated rat hepatocytes (Bioassay No 1) Equal doses of sera (T_1 and T_2) and standards (S_1 and S_2) were chosen for convenience in analysis. Fig 4 5 demonstrates the specific activity of HMG Co A reductase (pmol min^{-1} mg protein^{-1}) as a function of $\log_{10}$ proinsulin in microlitres. Specific activity after incubation of cells with 0 5ml (S_1) and 1.0ml (S_2) of proinsulin standard increased 38% from 71±11 pmol min^{-1} mg protein^{-1} to 98±6 4 pmol min^{-1} mg protein^{-1} respectively. Doubling the dose of unknown resulted in a 24% increase in reductase specific activity. However, it is apparent from Fig 4 5 that the two lines in this bioassay are of similar slope (the difference in slopes of these lines was not significant) suggesting that the response to altering the volume of serum sample in the medium was similar to that of altering the volume of standard proinsulin preparation. In this bioassay, M, the potency ratio of standard to unknown, and a parameter that is dependent

Figure 4 5 Two-plus-two bioassay of an unknown (control) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3).
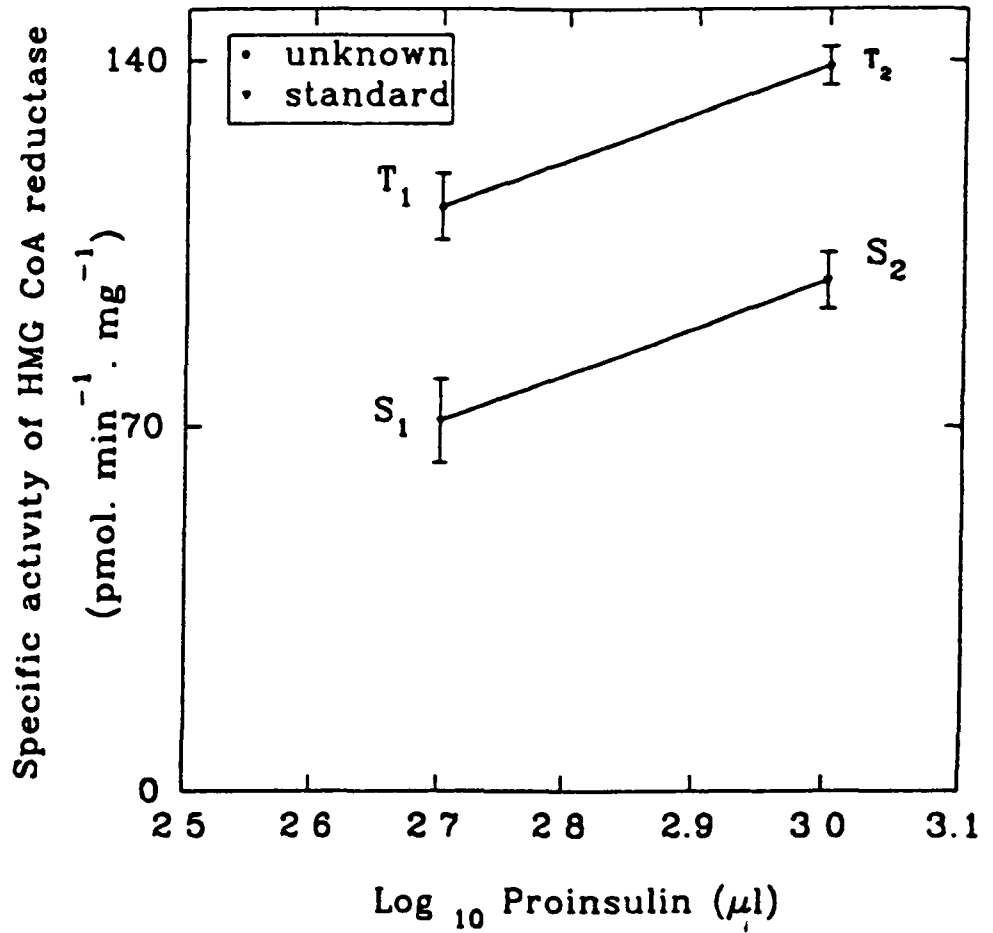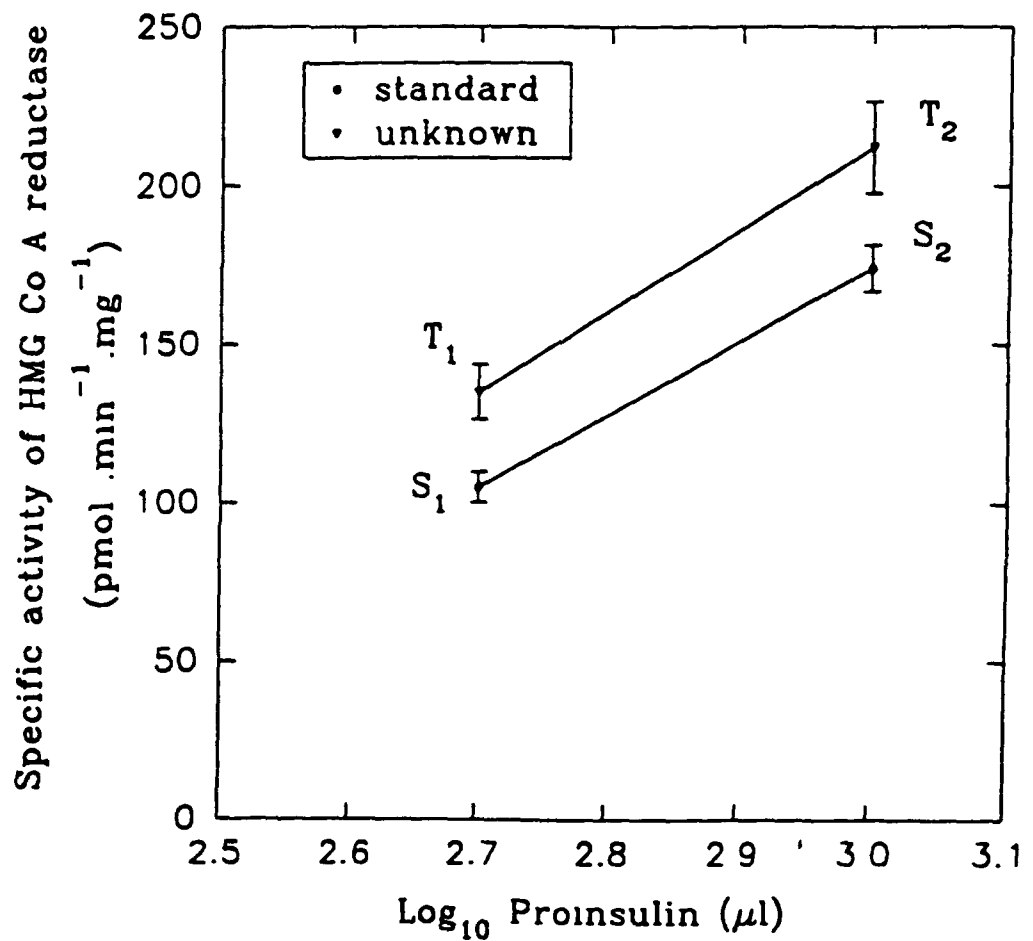

In this bioassay $Log_{10}M$ = 0.456; M = 2 85
Since the standard was 12pM the unknown
was estimated as 12/2.85 = 4.21pM

on the vertical distance between the lines and the mean slope of the

lines, was calculated to be 2 85 using equation 1 (Chapter 1) Since the

standard hormone concentration was 12pM, the concentration of

proinsulin in the unknown serum sample was estimated as 4 21pM, i e

the concentration of standard divided by the potency ratio, M

Fig 4 6 is an example of a bioassay obtained for an unknown serum

sample from a diabetic patient versus standard proinsulin (Bioassay

No 16) The variation between batches of hepatocytes prepared at

different times is immediately apparent Enzyme specific activity after

incubation of cells with 0 5ml (S_1) and 1 0ml (S_2) standard was 105±8 4

and 174±12 7 pmol min^{-1} mg protein^{-1} respectively Doubling the dose

of unknown and standard resulted in a 57% and 66% increase

respectively in reductase activity The potency ratio, M in this bioassay

was calculated to be 1 37 Since the concentration of standard was

12pM, the concentration of proinsulin in the serum sample was

calculated to be 8 76pM

Data relating to the values of S_1, S_2, T_1 and T_2 and the calculated

potency ratio obtained for each bioassay is shown in Table A in the

Appendix Five of the twenty one bioassays (Bioassays 2,3,4,7 and 8)

set up were considered unsuitable for calculation of serum proinsulin

due to an absence of effect on enzyme activity by incubating cells with

proinsulin These bioassays (along with Bioassays 6,10,11,12,15,18

and19) showed a significant difference in the mean slope of the lines for

standard and unknown and so were omitted from the final calculations

Although the rest of this section describes nine 2+2 parrallel bioassays

which yielded a value for proinsulin, data is given which highlights

specific and undesirable characteristics of the bioassay work which

render validation extremely difficult Chapter 6 of this thesis will attempt to

Figure 4.6 Two-plus-two bioassay of an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)

In this bioassay, $Log_{10}M = 0\ 136$, $M = 1.37$ Since the standard was 12pM, the unknown was estimated to as $12/1\ 37 = 8\ 76$ pM

evaluate these shortcomings and those which are most likely to have contributed to the failure of the remaining bioassays

Fig 4 7 shows the mean specific activity of HMG CoA reductase obtained when hepatocytes in 9 bioassays were incubated with 0 5ml and 1 0ml of proinsulin standard Mean specific activity increased significantly $(p<0\ 05)$ from 69 44$\pm$8 13 pmol min^{-1} mg $protein^{-1}$ to 103 11$\pm$12 54 pmol min^{-1} mg $protein^{-1}$ (Mean $\pm$SEM), (Fig 4 7) However, the %increase in specific activity obtained upon doubling the volume of proinsulin in any individual bioassay ranged between 16% and 70% (Mean= 43%) (Table A, appendix)

Fig 4 8 shows the specific activity of microsomal HMG CoA reductase obtained when cells were incubated with serum from control and diabetic patients Specific activity in cells incubated with 0 5ml serum was similar in both groups (93 5$\pm$18 5 (2) vs 94 85$\pm$18 (7) pmol min^{-1} mg $protein^{-1}$ (Mean$\pm$SEM) Doubling the dose of diabetic serum added to hepatocytes increased reductase activity by 39% from 94 85$\pm$18 pmol min^{-1} mg $protein^{-1}(T_1)$ to 131 71$\pm$16 19 pmol min^{-1} mg $protein^{-1}$ (T_2) The % increase when the volume of control serum was doubled approximated to 35% from 93.5$\pm$18.5 pmol min^{-1} mg $protein^{-1}$ (T_1) to 126$\pm$13 pmol min^{-1} mg $protein^{-1}(T_2)$ (mean$\pm$SEM)

Fig 4 9 shows that serum proinsulin concentration as measured by bioassay correlated very significantly with M, the potency ratio of standard hormone preparation to the unknown test sample, r=-0 97, $p<0\ 001$ The vertical distance (v) between lines correlated significantly with serum proinsulin concentration Fig 4 10 shows a negative correlation between (v) and proinsulin concentration, r= -0 689, $p<0\ 02$ A positive correlation though not significant existed between the mean

Figure 4.7: Average specific activity of
microsomal HMG Co A reductase obtained in
9 bioassays when cells were incubated with
0.5ml (S_1) and 1.0ml (S_2) of human proinsulin
standard (Mean $\pm$SEM, n=9)

Figure 4 8   Average specific activity of microsomal HMG CoA
reductase obtained in 7 bioassays when cells were
incubated with 0 5ml(T_1) and 1 0ml(T_2) of human
serum (NIDDM) (mean ± SEM, n=7)

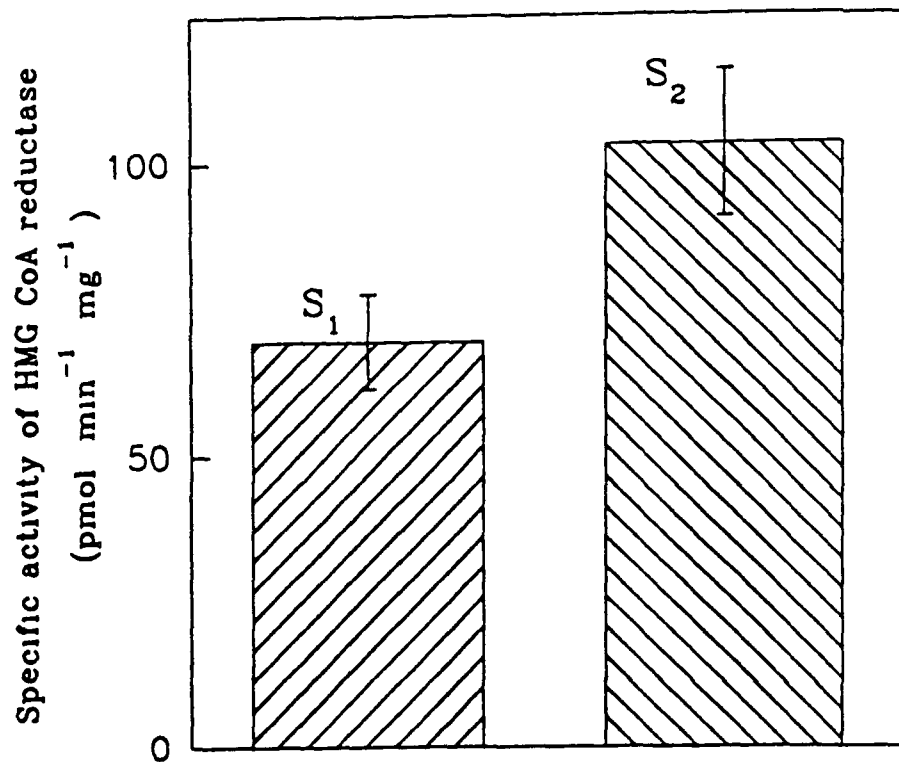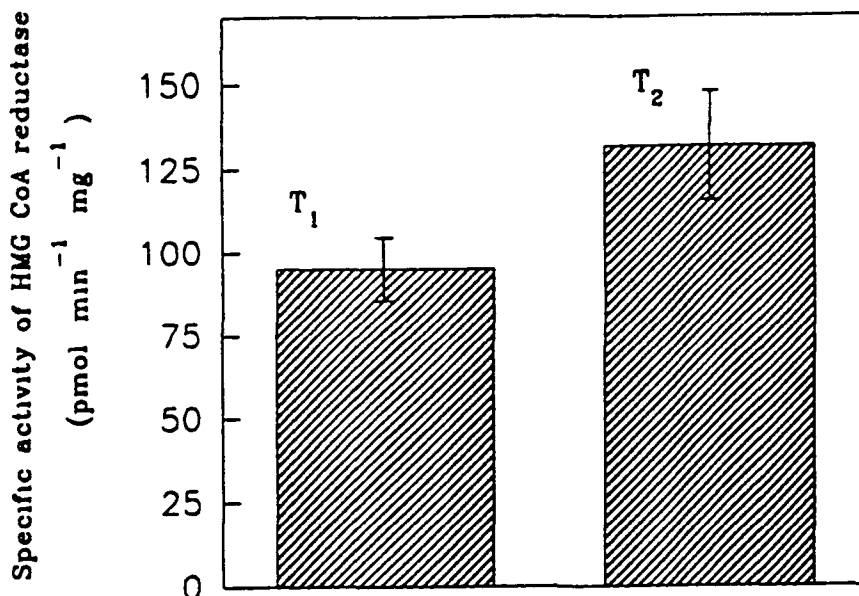Average specific activity of microsomal HMG Co A
reductase obtained in 2 bioassays when cells were
incubate with 0 5ml(T_1) and 1 0ml (T_2) of human
serum (control) (mean±SEM, n=2)
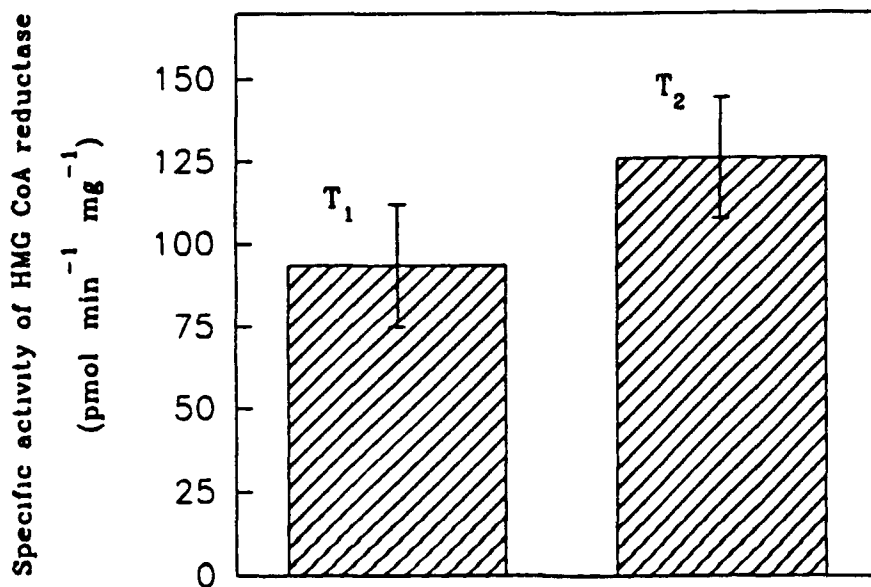
Figure 4 9 Graph of the potency ratio (M) as a
function of serum proinsulin concentration (pM),
(n=9).
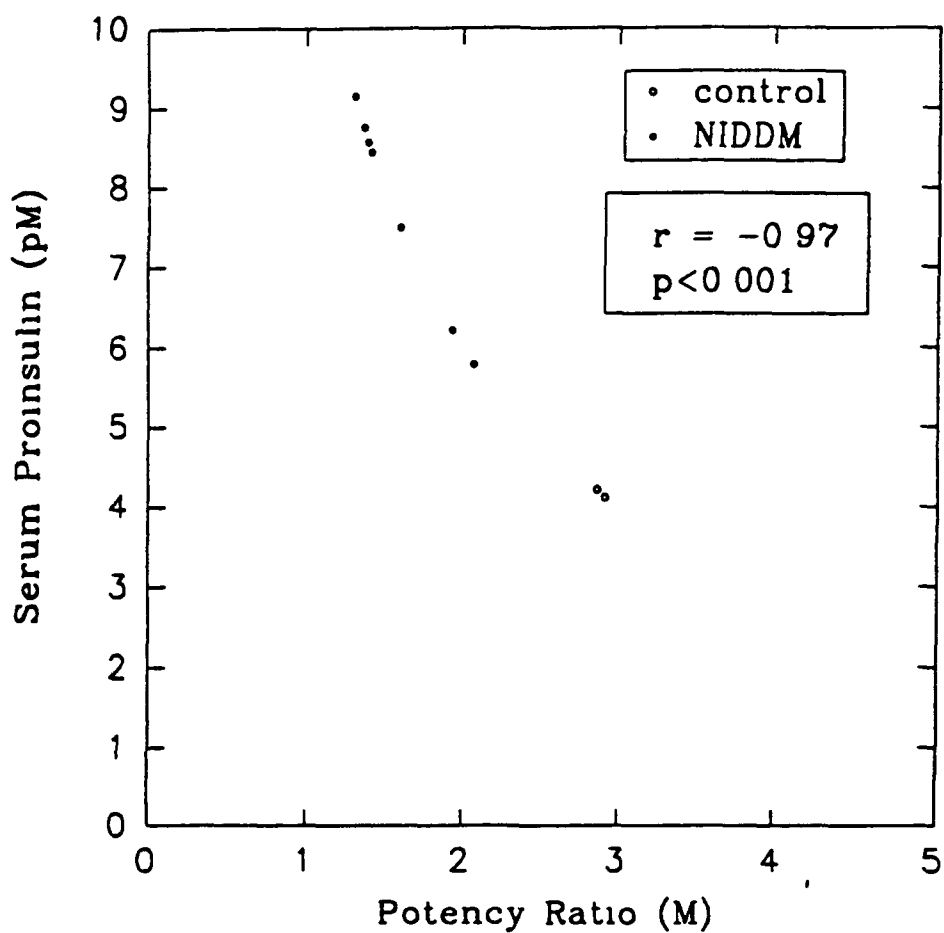
Figure 4 10. Graph of (v) the vertical distance as a function of serum proinsulin concentration (pM) (n=9).
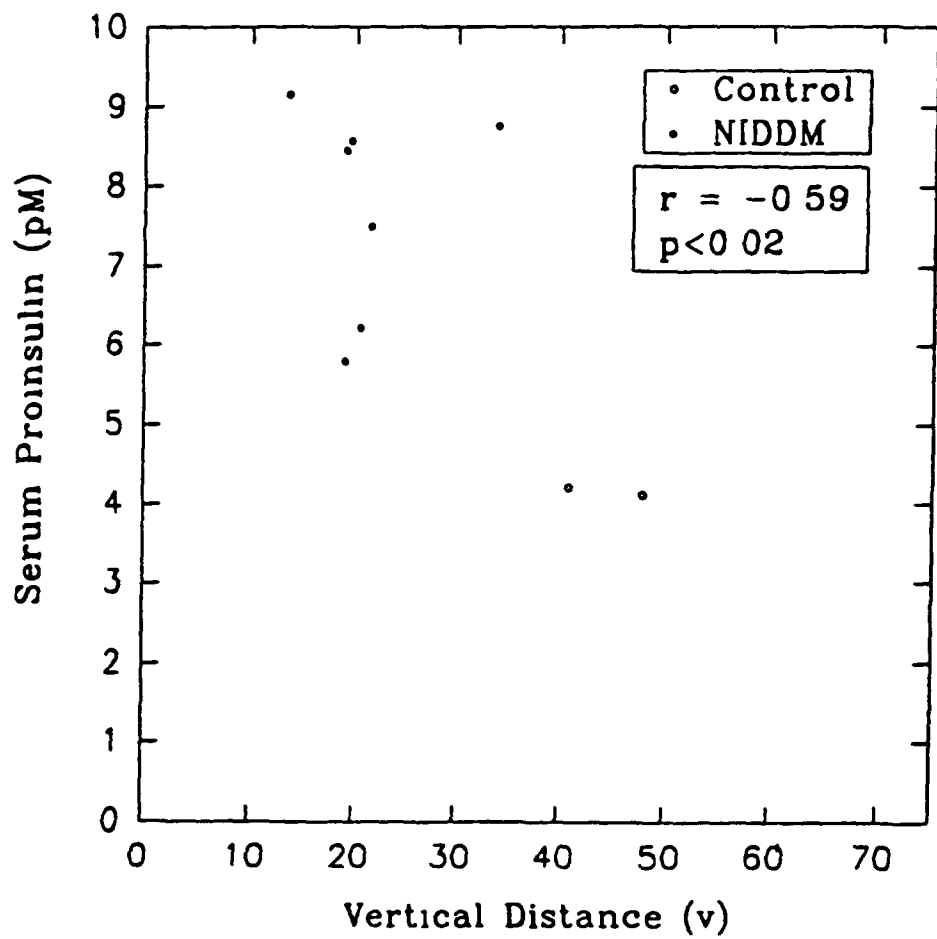
slope of the lines and proinsulin concentration, r= 0 55, p<0 06 (Fig 4 11)

Fig 4 12 demonstrates the results obtained using this approach to measure the proinsulin concentration in serum obtained from 2 non-diabetic subjects and 7 type 2 non insulin dependent diabetic patients A higher level of biologically active proinsulin was observed in serum from diabetic patients (7 77± 0 5 (7) pM) compared with control non-diabetic subjects (4 16±0 05 (2) pM) (Mean ±SEM (n))

Figure 4 11 Graph of the mean slopes of the line (m) as a function of serum proinsulin concentration (pM) (n=9).

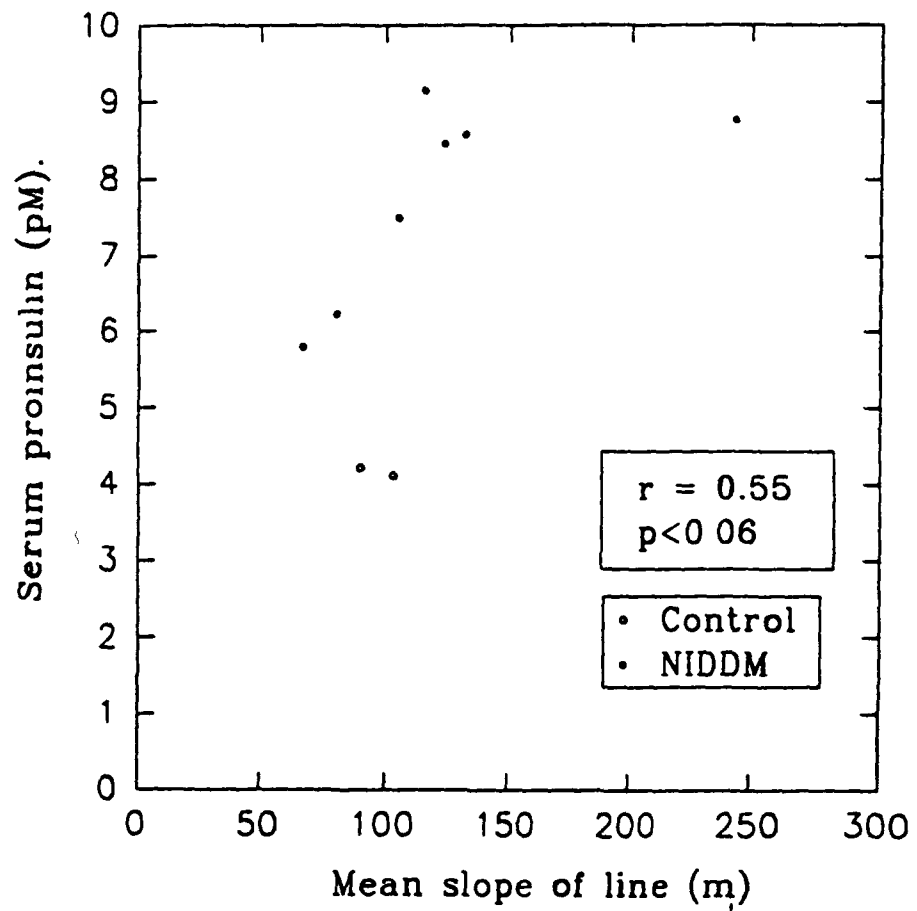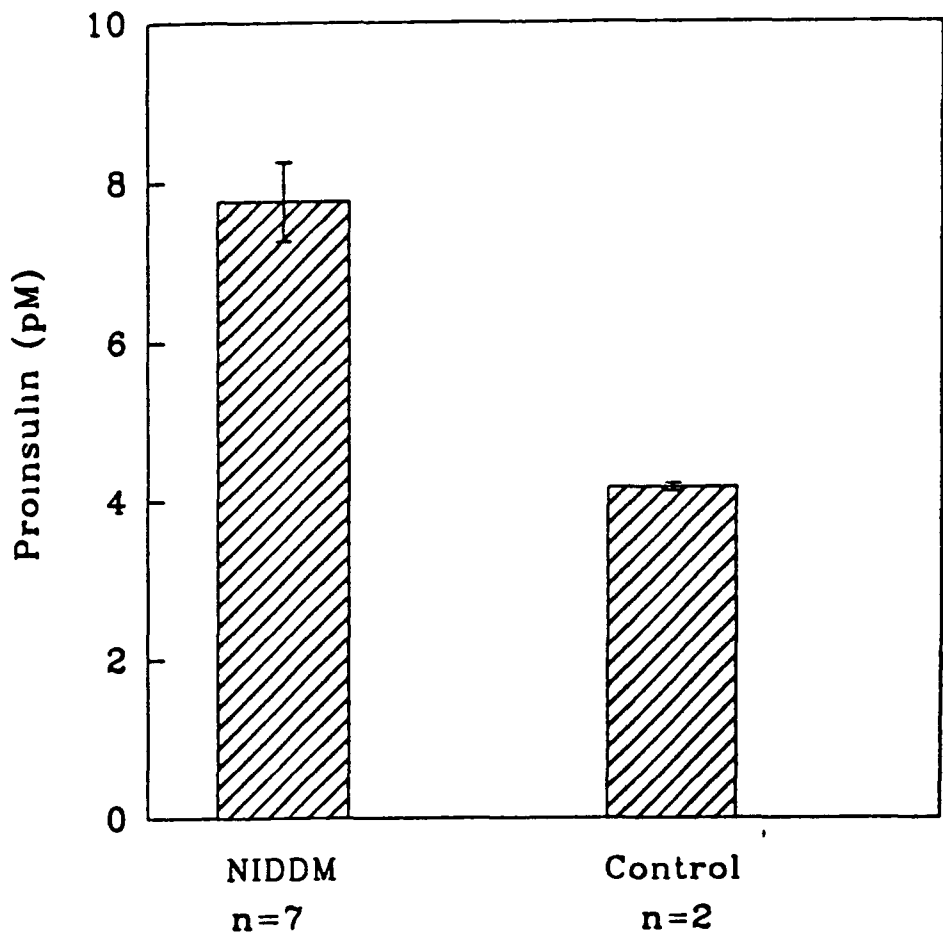Figure 4 12. Serum Proinsulin Concentration (pM) in
2 control non diabetic subjects and 7 NIDDM Patients
(mean±SEM)

CHAPTER 5

DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR PROINSULIN

The latter half of the 80's saw a resurgence of research interest in immunoassay procedures for proinsulin determination This arose largely as a result of the availability of biosynthetic human proinsulin by recombinant DNA technology

This chapter describes progress made in the development of a non-competitive sandwich assay using two antibodies, an anti-C-peptide IgG and an anti-insulin IgG The former antibody provided the lower layer in a sandwich immunoassay, the upper layer being composed of an anti insulin IgG-horse radish peroxidase conjugate

5 1 <u>Determination of the working dilutions of the commercial antibodies</u>

A checkerboard titration was performed wherein serial dilutions of proinsulin were immobilised on a plate and serial dilutions of the antibodies were reacted against the immobilised antigen to give a 2-dimensional result. The working dilution of the anti-insulin IgG-HRP conjugate prepared as described in Chapter2 was determined using three different coating concentrations of proinsulin, 0 025μg/ml, 0 25μg/ml and 2.5μg/ml The concentration ranges chosen for optimisation and characterisation studies had previously been investigated using purified antibodies in this laboratory (O`Farrell and Devery, 1992) Fig 5 1 indicates that the appropriate working dilution of the anti-insulin IgG-HRP conjugate was in the order of 1 10 Although a slightly higher titre may have been satisfactory for detection of proinsulin concentration greater than or equal to 2 5μg/ml, it was decided however that, for the range of concentration the assay was expected to detect that a 1 10 dilution was the maximum which would be effective

The working dilution of the anti-C -peptide IgG to be used in the ELISA was determined by adding three different concentrations of proinsulin,

Figure 5.1. Working dilution of Anti-insulin IgG

conjugate Versus OD 492nm

Dilution factor of Anti-Insulin IgG Conjugate
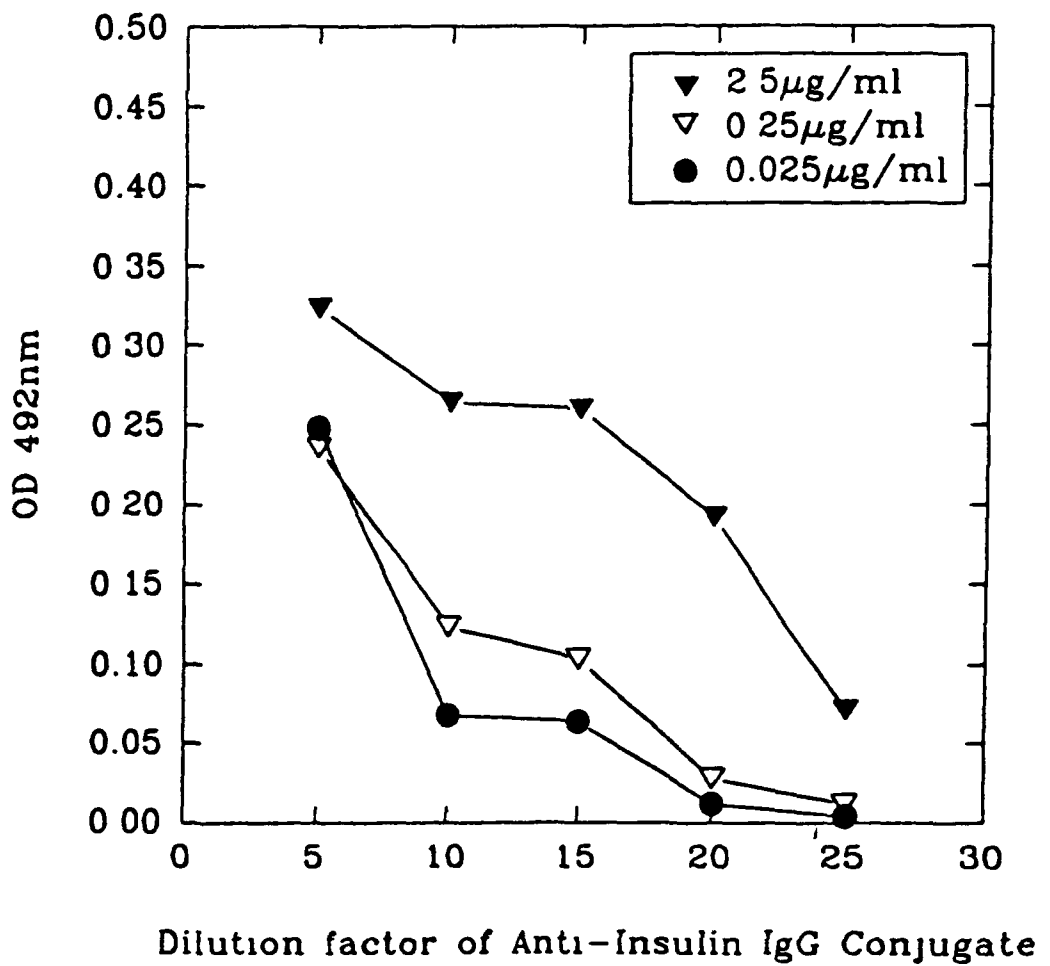
0.025µg/ml, 0.25µg/ml and 2.5µg/ml to microtitre plates. After incubation, washing and blocking as described previously in Chapter 2, anti-C-peptide IgG dilutions (1:100 to 1:2000) were added to the immobilised proinsulin. Fig 5.2 shows that a working dilution of between 1:100 and 1:1000 would be effective for the anti C-peptide IgG. The 1:2000 dilution gave an absorbance reading that did not differ from that obtained by the 1:1000 dilution. The 1:250 dilution did not produce a significantly greater response than the 1:500 dilution to justify its use. On account of the scarcity of the anti-C-peptide IgG, it was decided to use a 1:500 dilution. This is the equivalent of a 10µg/ml solution for the signalling antibody which is within the standard range for coating antibodies.

5.2   Determination of the optimum incubation times for each step in the assay.

The following three graphs show time courses of :

i) anti C-peptide IgG adsorption to a microtitre plate(Fig 5.3),

ii) anti C-peptide IgG binding to immobilised proinsulin (Fig 5.4)

iii) anti-insulin IgG binding to immobilised proinsulin (Fig 5.5).

100µl antiC-peptide IgG diluted 1:500 was added to the wells of microtitre plates for varying lengths of time as described in Chapter 2. It is apparent from Fig 5.3 that an incubation time of 2h at 37°C is optimal for the adherance of the anti C-peptide to the wells of a microtitre plate.

The binding of proinsulin (10ng/ml) by anti C-peptide IgG was investigated as described in Chapter 2. Fig 5.4 shows that binding was completed after a 2h incubation at 37°C. However, a much longer incubation time of 8h at the same temperature was required for the binding of proinsulin to anti insulin IgG conjugate (Fig 5.5). This requirement was verified by the performance of assays after 2h, 4h and 6h. In each of these assays absorbance readings did not rise significantly

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Figure 5 2 Working dilution of Anti C-peptide IgG

Versus

OD 492nm

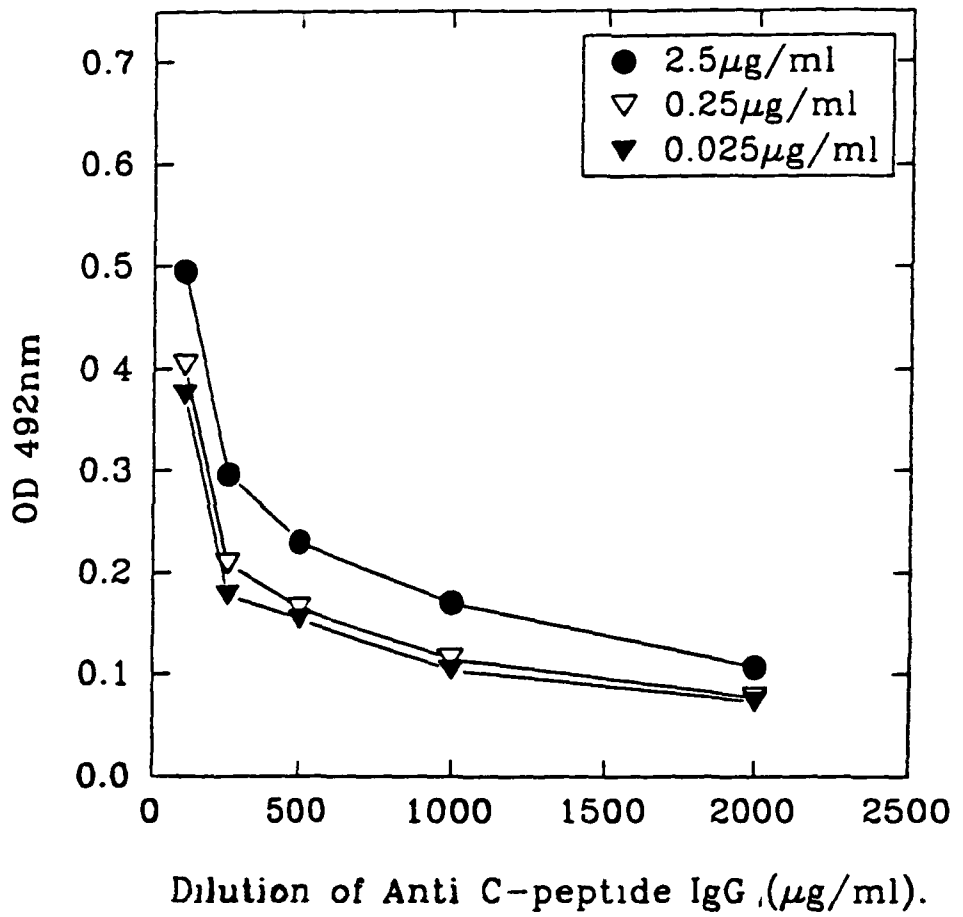Dilution of Anti C-peptide IgG (μg/ml).

Figure 5 3: Adsorption of Anti C-peptide IgG
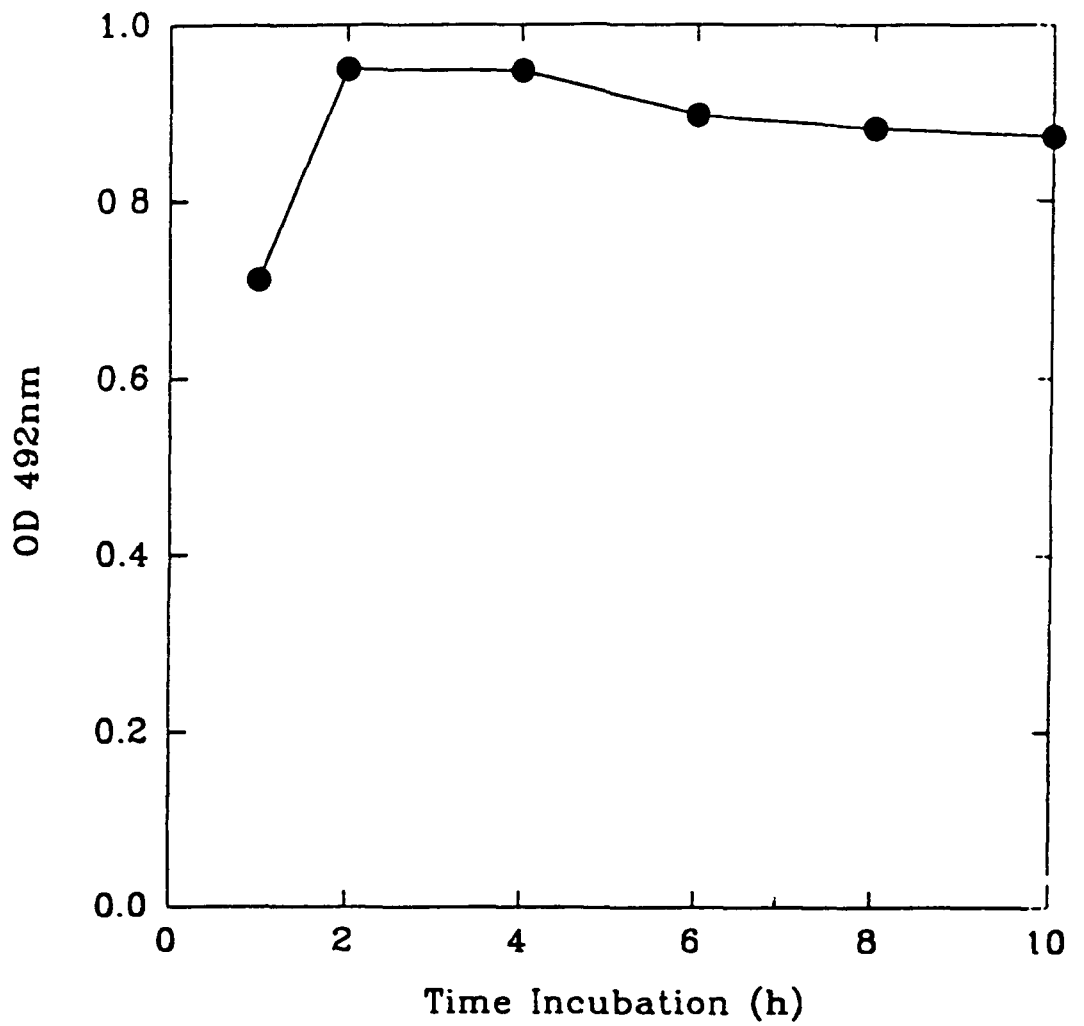to plate Time (h) Versus OD 492nm

Figure 5.4: Time course of anti C-peptide IgG
binding to immobilised proinsulin.
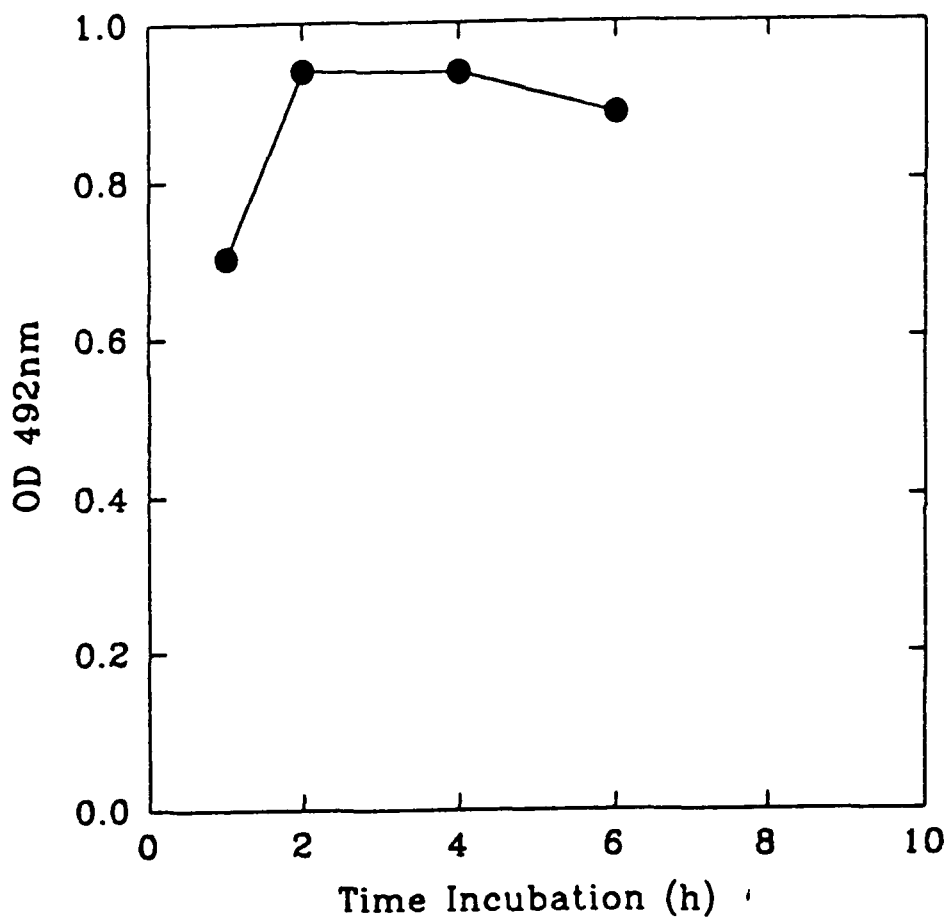Time(h) versus OD 492nm.

Figure 5.5: Time course of anti Insulin IgG conjugate binding to immobilised proinsulin. Time (h) versus OD 492nm.

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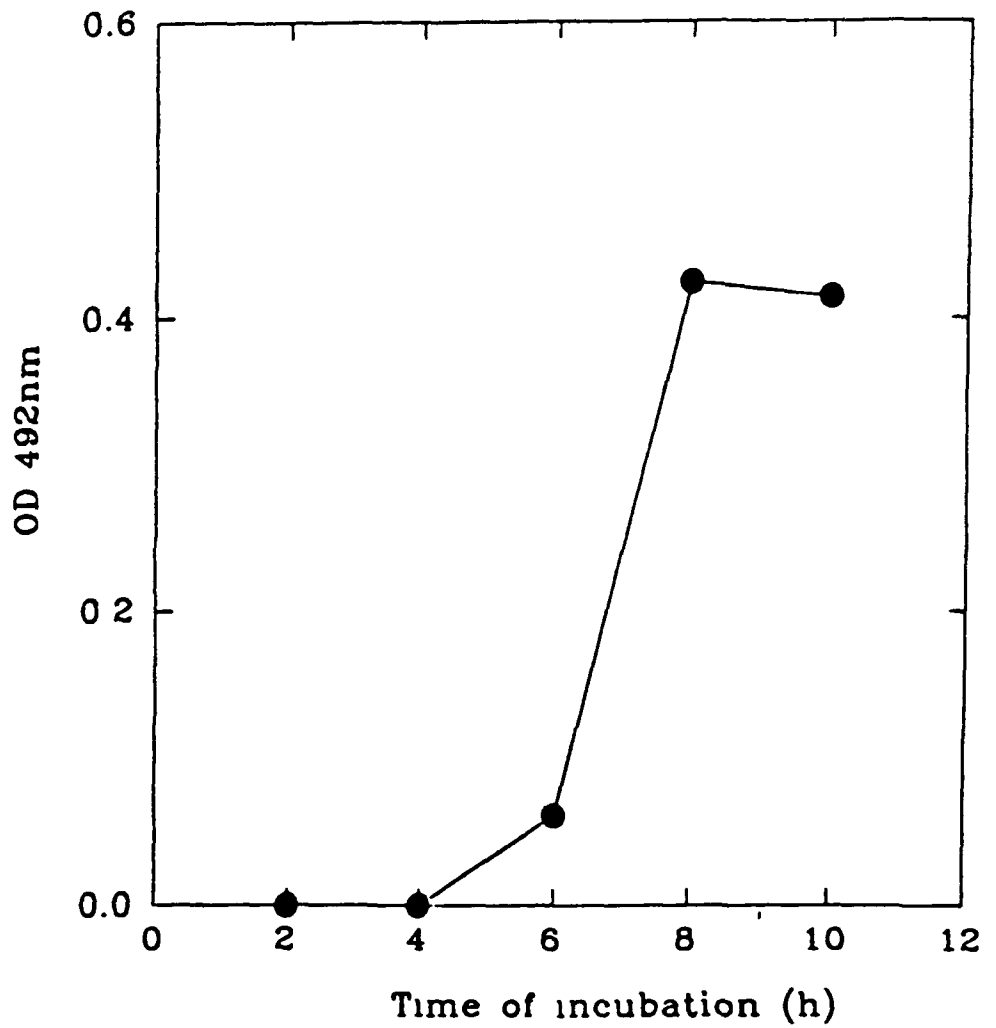
above background for the proinsulin concentration (10ng/ml) examined

The longer incubation at 37°C was thought to affect the stability of the

conjugate Binding of an equivalent amount of proinsulin (10ng/ml) to

anti-insulin IgG followed by overnight incubation at 4°C produced similar

absorbance readings (absorbance=0 449±0 03) to that obtained after 8h

at 37°C (absorbance=0 424±0 01)

5 3   Optimisation of ELISA

To ensure the reproducibility of the ELISA a series of positive and

negative controls were examined All combinations of antigens and

antibodies (primary and secondary) were assayed as described in

Chapter 2 Table 5 1 shows the absorbance values at 492nm in a series

of positive control assays that were set up to optimise the assay In assay

no 1, all the ELISA steps as outlined in the protocol in Chapter 2 were

carried out, i e in the presence of primary antibody, antigen (proinsulin),

secondary antibody, and substrate, absorbance was 0 526±0 008 In

assay no 2, in which the plate was coated with C-peptide (10µg/ml), and

blocked prior to addition of anti C-peptide IgG and commercial conjugate

the absorbance was 0 516 ±0 02 In assay no 3, the plate was coated

with insulin (10µg/ml), and blocked prior to the addition of anti-insulin

IgG, prepared conjugate and substrate This yielded an absorbance of

0 359±0 025 In assay no 4, after coating the plate with proinsulin

(10µg/ml), and blocking the plate with BSA prior to addition of anti-insulin

IgG-HRP conjugate and substrate, the absorbance reading was

0 243±0 043 Table 5 2 shows the absorbance values at 492nm in a

series of negative control assays that were set up to optimise the assay

In the absence of antigen and anti C-peptide IgG (assay no 1), anti

insulin IgG-HRP conjugate (assay no. 2), substrate (assay no 3),

conjugate and substrate (assay no 4) or antigen, conjugate and

TABLE 5.1

Series of positive controls for optimisation of ELISA

	Assay Number			
	1	2	3	4
Primary Antibody Anti C Peptide IgG	+	+		
Block BSA	+	+	+	+
Proinsulin	+			+
Secondary Antibody Anti-Insulin IgG HRP-Conjugate	+		+	+
Anti-Insulin IgG			+	
Insulin			+	
Commercial Conjugate Goat Anti-Guinea-Pig IgG HRP-Conjugate		+		
C-Peptide		+		
Substrate	+	+	+	+
O.D. @ 492nm Mean ± S.D.	0 526 ± 0.008	0 516 ± 0 02	0 359 ± 0.0251	0 243 ± 0 043

TABLE 5.2

Series of negative controls for optimisation of ELISA

	Assay Number				
	1	2	3	4	5
Primary Antibody Anti C-peptide IgG	-	+	+	+	+
Block BSA	+	+	+	+	-
Antigen: Proinsulin	-	+	+	+	-
Secondary Antigen Anti Insulin IgG-HRP Conjugate	+	-	+	-	-
Substrate	+	+	-	-	-
O.D. @ 492nm Mean ± S.D.	0 001 ± 0 0001	0 002 ± 0 0001	0 002 ± 0 0001	-0 003	0 000

substrate (assay no 5), absorbance at 492nm was negligible The data clearly indicate that each step in the ELISA is necessary for colour development and that absence of either primary or secondary antibodies, antigen or substrate gave near zero readings These results demonstrate that all of the steps for the ELISA as outlined in the protocol are necessary to obtain absorbance values within a workable range The data demonstrate the reactivity of the conjugate, the specificity of the antibodies towards C-peptide, insulin and proinsulin Although the absorbance values obtained when a plate was coated with insulin (assay no 3) were higher than when the plate was coated with proinsulin (assay no 4), the absorbance readings in both assays were lower than those obtained using the complete assay protocol Overall, the values obtained for the positive controls are 300-500 fold higher than those obtained for the negative controls

5 4   Cross reactivity with structurally related peptides

The basis of the sandwich immunological assay of proinsulin described above is the recognition of both an insulin and a C-peptide antigenic epitope by signalling and capturing antibodies The extent to which these antibodies were capable of distinguishing between C-peptide, insulin and proinsulin was investigated by determining the cross reactivities of each antibody as described in Chapter 2 The absorbance readings at 492nm obtained upon the incubation of anti-insulin-IgG with proinsulin (0 1-12.5μg/ml) and with C-peptide (0 1-12 5μg/ml) are shown in Tables 5 3 and 5 4 respectively From these data, it can be seen that human C-peptide cross reacted with anti insulin IgG only at relatively high concentrations of the C-peptide Cross reactivity of 37 71% occurred in the presence of 12.5μg/ml C-peptide , equivalent to 4.1μM (Table 5.5) However this decreased to 1 71% when C-peptide concentration

TABLE 5.3

Table of OD values obtained using anti-insulin IgG (300μg/ml) against a range of proinsulin concentrations (12 5μg/ml - 0 1μg/ml).

Proinsulin concentration	OD 492nm
12.5μg/ml	0 297±0.004
5μg/ml	0.212±0 003
1μg/ml	0.197±0 004
0.1μg/ml	0 175±0 002

TABLE 5.4

Table of OD values obtained using anti-insulin IgG (300μg/ml) against a range of C-peptide concentrations.

C-peptide concentration	OD 492nm
12.5μg/ml	0.112±0.004
5μg/ml	0.073±0 002
1μg/ml	0.017±0 003
0 1μg/ml	0 003±0.0001

TABLE 5.5

Table of cross reactivities obtained between anti-insulin (300µg/ml) and C-peptide (12 5µg/ml - 0 1µg/ml)

C-peptide concentration	%Cross reactivity
12.5µg/ml	37 71
5µg/ml	34 43
1µg/ml	8 63
0.1µg/ml	1 71

decreased to 0.1μg/ml equivalent to 0.033μM. Thus it can be seen that even a supraphysiological concentration of C-peptide is not measured in the assay. The effect of insulin, normally present in much greater concentration than proinsulin was subsequently investigated.

Tables 5.6 and 5.7 demonstrate the absorbance readings at 492nm obtained upon the incubation of anti C-peptide IgG with proinsulin (0.1ng/ml to 10μg/ml) and with insulin (0.1ng/ml to 10μg/ml) respectively. From their relative absorbances, it can be seen that cross reactivity between insulin and anti -C-peptide decreased from 65.2% to 0.5% as the concentration of insulin decreased 10^5 fold. However at insulin concentrations lower than 1ng/ml, cross reactivity with the anti C-peptide IgG was less than 3.6% (Table 5.8).

Table 5.9(a) and 5.9(b) show the cross reactivities for insulin and C-peptide in the complete assay. Insulin and C-peptide demonstrated less than 10% cross reactivity at levels below 50ng/ml for insulin and 10μg/ml for C-peptide respectively.

5.5   Assay characteristics.

A standard curve for human proinsulin constructed under these conditions is shown in Fig 5.6. The data represent the composite standard curve obtained from six separate assays relating $\log_{10}$ proinsulin concentration (pg/ml) with absorbance at 492nm. The lowest detectable absorbance value above the mean zero response was 0.08±0.02. The detection limit of the assay was 1pg/ml (0.11pM), as assessed by the response representing the mean plus three times the standard deviation of the lowest detectable absorbance (absorbance reading =0.08+3(0.02) =0.14). The assay was linear up to 500pg/ml (55.5pM) which provided a suitable working range for detecting

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TABLE 5.6

Table of OD values obtained using anti C-peptide (10μg/ml) against a range of proinsulin concentrations (10 μg/ml - 0 1ng/ml)

Proinsulin concentration	OD 492nm
10μg/ml	0 305±0 009
1μg/ml	0 274±0.003
0.1μg/ml	0 229±0 001
10ng/ml	0.228±0 005
1ng/ml	0.223±0 004
0 1ng/ml	0.191±0.005

TABLE 5.7

Table of OD values obtained using anti C-peptide IgG (10μg/ml) against a range of insulin concentration (10μg/ml - 0 1ng/ml)

Insulin concentration	OD 492nm
10μg/ml	0 199±0.007
1μg/ml	0.121±0.004
0.1μg/ml	0 067±0 001
10ng/ml	0 021±0 001
1ng/ml	0 008±0.0001
0.1ng/ml	0 001±0 0001

TABLE 5.8

Table of % cross reactivities obtained between anti C-peptide IgG (10μg/ml)
and insulin (10μg/ml - 0.1ng/ml)

Insulin concentration	% Cross reactivity
10μg/ml	65 24
1μg/ml	44 26
0 1μg/ml	29 35
10ng/ml	9.21
1ng/ml	3.58
0.1ng/ml	0 50

TABLE 5.9(a)

Table of cross reactivities obtained for insulin (ng/ml) for the complete assay (mean±SD, n=3)

Insulin (ng/ml)	OD 492nm	% Cross reactivity
1000	0.140±0 004	40
50	0 024±0 007	9 5
20	—	
10	—	
5	—	
1	—	

TABLE 5 9(b)

Table of cross reactivities obtained for C-peptide (ng/ml) for the complete assay (mean±SD, n=3)

C-peptide (ng/ml)	OD 492nm	% Cross reactivity
20,000	0 098±0 003	38 8
10,000	0 011±0.005	4.3
1,000	—	
100	—	
10	—	

Figure 5 6 Standard Curve of OD 492nm
Versus
Proinsulin Concentration (pg/ml) (n=6)
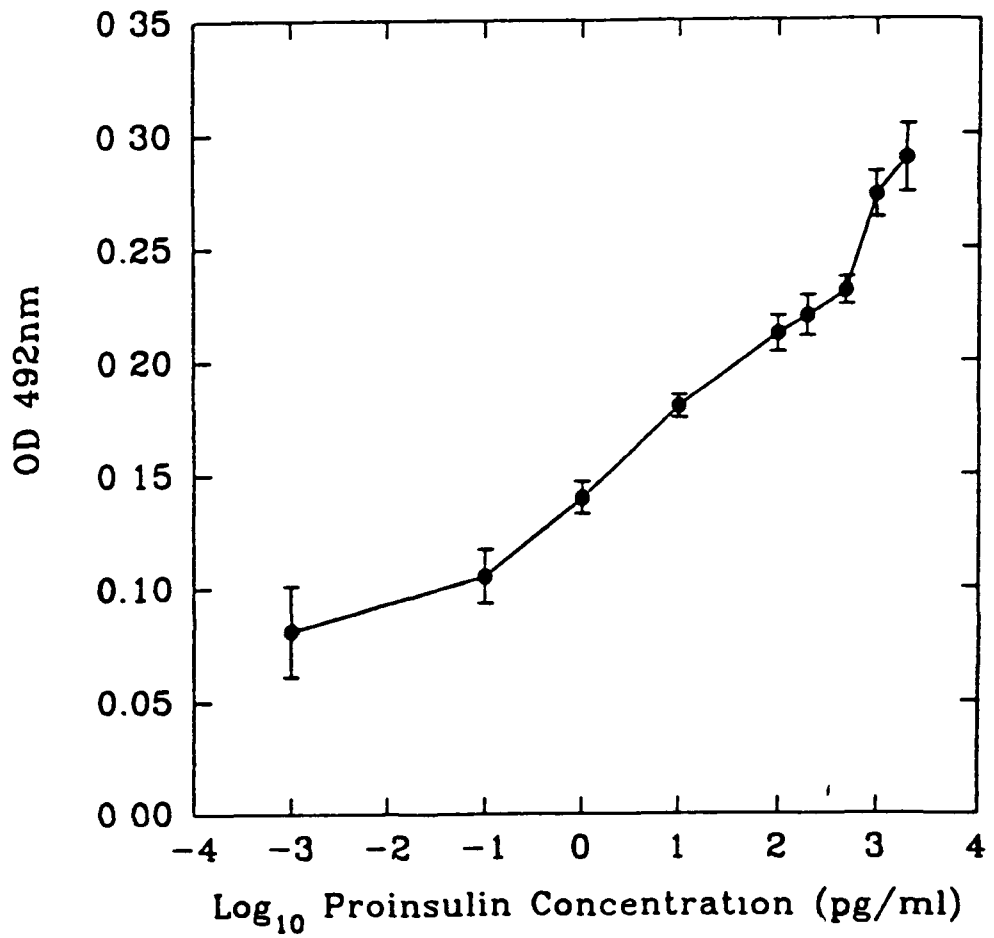
proinsulin in human serum The interassay coefficient of variation (CV) was determined from the mean of triplicate estimations in a limited number of assays (n=6) Interassay CV was 3 6% at a proinsulin concentration of 10pg/ml, 4 1% at a proinsulin concentration of 250pg/ml and 2 6% at a proinsulin concentration of 500pg/ml

5 6   Analysis of serum samples.

Sera from 8 non-insulin-dependent diabetic (NIDDM) patients and 4 non-diabetic control subjects were assayed in triplicate Serum samples had been stored at -20°C prior to assay and each sample was assayed in undiluted form Serum proinsulin was within the assay range for all samples In healthy control subjects, the serum proinsulin concentration (Mean±SD) was 13 67±1 58 pM It was significantly elevated in patients with diabetes (23 21±1 14pM, p<0 01) (Fig 5 7)

Table 5 10 demonstrates the reproducibility of results obtained for serum proinsulin concentration from 2 non-insulin-dependent diabetics and 2 control subjects In each case the values obtained were shown to vary between 2-3% indicating good reproducibility.

Table 5 11 demonstrates the relative accuracy of the assay system for proinsulin measurement A series of known concentrations were examined. The results obtained indicate that the assay returned values which were within 5% of those expected

Significantly higher levels of proinsulin were recorded for both controls and type 2 diabetic subjects using the ELISA technique as compared with the values obtained employing the bioassay method (Fig 5 8)

Figure 5 7 Serum proinsulin concentration (pM) in 4 control non diabetic subjects and 8 NIDDM patients
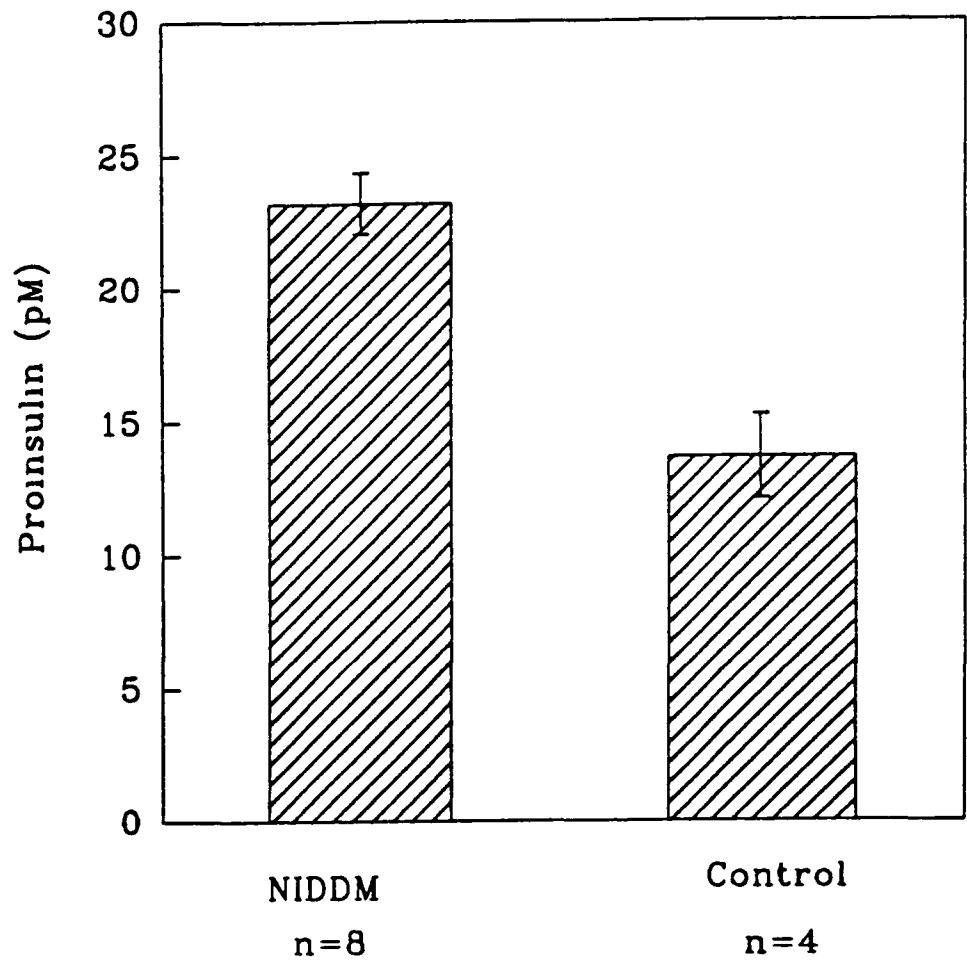
TABLE 5 10

Reproducibility of results obtained for serum proinsulin (pM) measurement in two diabetic patients (J W and M H) and two non-diabetic subjects (J D and M M)

Patient	Assay 1	Assay2	Mean ±SD	% Variation
J W	23 9	24 9	24 4±0 73	2 9
M H	27 1	25 6	26 3±1 10	4 1
J D	10 9	11 6	11 2±0 47	4 1
M M	16 5	17 3	16 8±0 53	3 1

TABLE 5 11

Relative accuracy of results obtained for proinsulin measurement

Expected Concentration (pg/ml)	Concentration Calculated (pg/ml)	% Deviation from expected result
220	209 4	4 82
550	560 6	1 89
2100	2210 9	5 02

Figure 5 8 Comparison of ELISA and Bioassay technique for the measurement of serum proinsulin concentration (pM) in control and NIDDM subjects
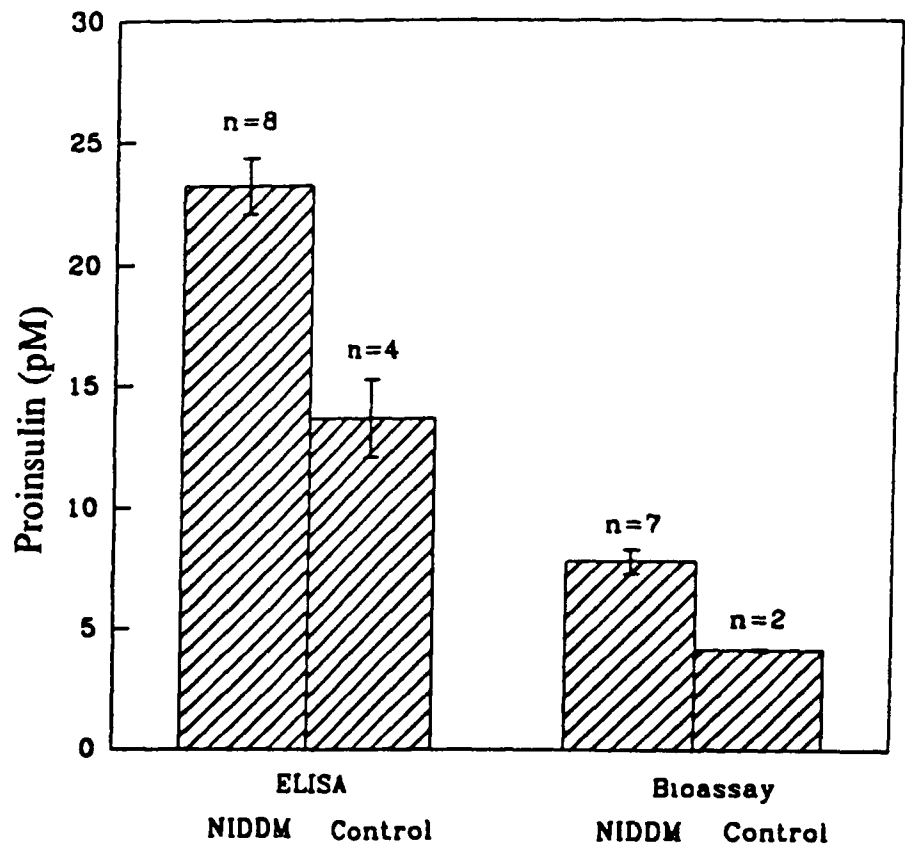
CHAPTER 6

DISCUSSION

This study dealt with two types of assay procedure, namely bioassay and immunoassay for the measurement of proinsulin in serum. While several problems were encountered with the former, I shall nevertheless discuss the rationale for the work described above and attempt to explain the difficulties experienced with establishing a reliable proinsulin bioassay. The bioassay involved quantitating the response which followed the addition of proinsulin to rat hepatocyte culture medium and comparing it with the response produced by addition of test serum sample to an equivalent aliquot of hepatocytes. Freshly prepared isolated rat hepatocytes were chosen as the biological system largely as a result of proinsulin having been shown by Revers *et al* (1984) to be preferentially taken up by the liver. The response was the change seen in the specific activity of HMG CoA reductase, an enzyme located in the endoplasmic reticulum of liver cells. Since all bioassays are comparative, experimental design had to ensure that variation in the biological system was minimised.

The first approach taken to minimise biological variation was to select groups of rats which were matched as closely as possible with regard to gender, weight and age. It was apparent from Chapter 3 that the hepatocyte yield from male Wistar rats weighing on average 250-300g was typically $3-4 \times 10^8$ per animal, the approximate wet weight of cells after liver perfusion being 5-7g. The ability to control the cellular environment is an obvious major advantage in investigations with isolated cell systems compared to studies with intact animals. The ability to perform numerous comparative studies on hepatocytes derived from one liver is also advantageous due to variations between different animals. The viability of hepatocytes as determined by the trypan blue exclusion test ranged between 80-90% before and after 2h incubation

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period at 37°C in 5% CO_2, demonstrating relatively low contamination with disrupted cells The high cell number and the % viability that was obtained as a result of collagenase perfusion and hepatocyte preparation/purification suggested that hepatocytes maybe a suitable choice of test system for studying hepatic metabolism As an average of $3\text{-}4\times10^8$ cells was obtained from each rat, one animal could be used for a number of assays, reducing costs and making possible a comparison of the effects of proinsulin on a standard test system over a number of experiments. Therefore, for every enzyme determination, 10ml cell suspensions containing approximately $7\,5\times10^7$ cells were incubated for 2h prior to harvesting for enzyme assay

Another condition that was considered important in the design of the bioassay for minimising biological variation was the length of time the animals were kept under reverse lighting conditions HMG CoA reductase activity is known to vary under widely diverse physiological conditions. Low activities were observed in rats killed in the middle of the light cycle, while higher activities were observed in rats killed in the middle of the dark cycle (Clarke *et al*,1984) This diurnal rhythm of enzyme activity has previously been explained as the result of diurnal modulation by insulin of both total activity of HMG CoA reductase and the ratio of expressed to total enzyme *in vivo* (Easom and Zammit, 1985) The latter workers also showed that the peak in the diurnal cycle in circulating insulin concentrations *in vivo* coincided with that of the diurnal rhythm of dephosphorylated/total HMG CoA reductase In view of several previous reports showing that HMG CoA reductase exhibits a circadian rhythm with maximum levels at midnight (middle of the dark period) and basal levels during much of the light period, all animals used in this study were maintained under a reversed light -dark cycle for a

minimum of 21 days prior to killing in the middle of the dark cycle.

A further restriction in design of the bioassay was the establishment of controlled optimal assay conditions for measuring the specific activity of HMG CoA reductase The determination of activity in hepatocytes required characterising the substrate requirements of the assay, the length of time for substrate incubation and the optimum amount of protein concentration per assay tube The substrate concentration used in the assay (52nmoles) was approximately five fold higher than the value of Km (0 067mM) that was obtained for the hepatocyte enzyme (Table 3 2) Increasing the substrate concentration by a factor of 1 5 or halving the concentration of the substrate did not greatly alter the activity of the microsomal HMG CoA reductase observed (Table 3 1). In the case of the time course experiment, enzyme activity was shown to be linear up to 60min for rat hepatocytes and 30min for intact rat liver at which times HMGCoA reductase activity was found to be 6 7nmoles/mg protein and 17 5 nmoles/mg protein respectively (Fig3 1) The activity profile for rat liver is similar to that of Shapiro *et al* (1974) who found enzyme activity to be linear up to 30min, at which point HMG CoA reductase activity was found to be15-25 nmoles/mg protein Enzyme activity was shown to be linear up to 830ug of protein per assay tube for rat hepatocytes and 400µg of protein per assay tube for intact rat liver (Fig3 2) Reductase activity in rat liver is slightly lower than that obtained by Shapiro *et al* (1974) who showed enzyme activity to be linear up to 600ug of protein per assay tube.

Having established optimal conditions for the assay, attempts to improve the assay in terms of time were investigated Chapter 3 described an assay method for HMG CoA reductase activity, that was a modification of that described by Shapiro *et al* (1974) Of late, the latter was the most

widely used assay to measure reductase activity. Separation of mevalonate as the mevalonolactone involved direct application of the deproteinised reaction mixture to TLC plates. This in itself was less tedious and resulted in a greater recovery of mevalonate and therefore assay sensitivity than many previously reported methods that involved extraction of radioactive mevalonolactone from the incubation mixture into ether (Brown *et al*, 1973), before separation by thin layer chromatography (TLC). The modified assay described here used a solid phase extraction step to separate the more polar HMG CoA from mevalonate. The [^{14}C]-labelled HMG CoA and the HMG CoA breakdown products formed during incubations are sufficiently polar so that they are quantitatively retained on the solid phase and are therefore not detected on scintillation counting. Fig 3.5 compares separation of mevalonate from HMG CoA by TLC on silica gel plates and by solid phase extraction. The silica gel plates were developed in benzene acetone (1:1) (v/v) as described in chapter 2, and the mevalonate was found to have an R_f value between 0.5 and 1.0 which was sufficiently resolved from HMG CoA which had an R_f of beween 0 and 0.2 as described in chapter 2 (Fig 2.1).This study demonstrated that the TLC method was quite a prolonged procedure and limited the separation on one plate to a maximum of five samples in triplicate and one blank. Variability in recovery can be attributed to the liklihood of loss of silica powder by scraping of the plate, and during transfer of the scraped silica to scintillation vials. Loss of mevalonate can also occur during the spotting procedure if sufficient time is not allowed for each spot to dry. In an attempt to circumvent the above limitations of TLC the use of mini-columns containing silica to resolve the mevalonate product was investigated. Using the same separation principle as TLC mevalonate was preferentially eluted from the columns using a low polarity

toluene : acetone mixture (3:1 v/v). The number of steps prone to operator error are reduced, and the tedious extraction of mevalonate by scraping is removed. The solid phase extraction method resulted in a specific activity of 768.45±17.1 pmol.min^{-1}.mg^{-1}. The above mentioned limitations of the TLC procedure may account for the slightly lower specific activity of 705.5± 17.4 (mean ± SEM) (n=2) found in rat liver microsomes (Fig 3.5). The coefficient of variation was found to be 4.9% using solid phase extraction and 3.4% using the TLC procedure (Fig 3.5).These values, being less than 5% are still comparable to those obtained by Shapiro *et al* (1974) using the TLC procedure. These results serve to illustrate the reproducibility of the assay method as they represent results of independent determinations carried out on a microsomal fraction using two different methods of product separation.Table 3.3 demonstrates the repeatability of the assay method for measuring HMG CoA reductase in hepatocytes. Specific activity was measured in microsomal fractions from three batches of hepatocytes obtained from a single rat. The mean ± SD value of activity was observed to be 246.5±22 pmol.min^{-1}.mg^{-1}. The % coefficient of variation was 8.9%. The activity of HMG CoA reductase was significantly reduced after 3 days of freezing (Table3.4). Furthermore, on the day of isolation, the variation encountered was only 6.7% from the mean (811± 55 pmol.min^{-1}mg^{-1}). However the variation was greater after 3 days freezing at -20°C at 17% (149±26 pmol . min^{-1}.mg^{-1}). This may be due to proteolysis of the enzyme as encountered before with freeze - thaw solubilisation procedures (Roitelman and Shechter, 1984).

The time, protein and substrate profiles of enzyme activity in rat liver microsomal extracts show that the assay permitted reliable quantitation of HMG CoA reductase. However a wide range of activity was found

depending on whether the microsomal pellet was prepared from hepatocytes or intact liver and on how it was stored prior to assay HMG CoA reductase activity in microsomal preparations from whole livers was comparable with those previously reported by others using similar assay techniques However, in freshly isolated hepatocytes the measured activity of this enzyme was much lower than the activity found in the intact liver Furthermore, the data reported in Chapter 5 show that isolated hepatocytes that were incubated for two hours showed even a greater loss of enzyme activity This makes accurate measurements very difficult This loss of activity is a very commonly observed phenomenon and may reflect the method of preparation and isolation of hepatocytes (Prof M. Berry, personal communication EuroHUG Meeting `93, York) It would appear from this study that there is a time scale over which enzyme activity is lost, the earliest detectable time point being approximately 70min when the entire cell isolation and washing procedure had been completed The liver is a complex three dimensional organ composed of more than one cell type As a result of liver perfusion and the method of hepatocyte preparation the liver is dissociated into single cells that are devoid of polarity The importance of cell-cell contact to the expression of many hepatic functions is in evidence from work carried out by Shephard et al (1993) Induction of cytochrome P450 monooxygenase system, a clearly defined differentiated function in liver, does not occur in isolated rat hepatocytes unless they are cocultured with rat epithelial cells which divide until contact with hepatocytes is made in vitro It is conceivable that the method of isolation could be a trigger that underlies the altered expression of many hepatic functions. The method of cell isolation and maintenance as described here may cause oxidative stress, thereby altering cell-cell communication systems Although not investigated it is possible that the decreased HMG CoA reductase activity

in hepatocytes as measured throughout this study could be a down stream measure of an initial event which may be an alteration of the signal transduction mechanism between cells involving protein kinase activity The sole criterion used for assessing cell viability in this study was the degree of trypan blue exclusion by cells An assessment of the metabolic integrity of the cells would have yielded more valuable information with a view to assessing the biochemical function capacity of the cells. Measurement of gluconeogenesis, glycolysis or the capacity of the cells to synthesise urea, would have afforded suitable tests for assessing cytoplasmic and mitochondrial function of isolated cells Furthermore, it is plausible that changes in either redox potential or the phosphorylation potential of cells markedly affect HMG CoA reductase activity. Earlier work by Kennelly and Rodwell, (1985) showed that HMG CoA reductase undergoes conformational changes in response to alterations in the reductive state If the reductive state is high, the enzyme becomes activated showing Michaelis -Menten kinetics whereas if the environment is in a low reductive state, the enzyme is in a latent inactive form. Furthermore, changes in the concentration of ATP, ADP, P_i and even intracellular pH are quite likely to have happened if the metabolic status of the cells has been altered. This could influence the actual free energy charge for ATP hydrolysis Thus, the phosphorylation potential of hepatocytes prepared as described in Chapter 2 may have altered from that of intact liver cells and this may significantly affect the degree of phosphorylation of enzymes such as HMG CoA reductase whose rate of degradation is increased by covalent phosphorylation. Clearly, this study has shown that additional studies are needed to optimise the experimental conditions, to characterise isolated cell systems and to explore more fully the potential of hepatocyte suspensions and or primary cultures

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An important role for insulin in the regulation of the activity of HMG CoA reductase in the liver has been evident for a considerable time. Insulin has been shown to increase the activity of HMGCoA reductase in liver *in vivo* and in the isolated rat hepatocyte (Ingebritsen *et al*, 1979, Feingold *et al*, 1982 and Easom and Zammit, 1985). However, no information is available on the role of its precursor, proinsulin or its intermediate forms which might also have a biological effect on reductase activity. The recent availability of synthetic human proinsulin through recombinant DNA technology has allowed detailed studies of its actions on metabolism to be undertaken and given researchers the opportunity to develop methods for the measurement of proinsulin under various conditions.

Having characterised the hepatocyte test system and the conditions for the assay of reductase activity, the effect of proinsulin could be determined. It is apparent from Fig 4.1 that proinsulin, like insulin can exert short term hormonal control of hepatic HMG CoA reductase activity in the rat. HMG CoA reductase activity was significantly higher ($p < 0.05$) in cells incubated with insulin [199 ± 17.92 (3) pmol. min. $^{-1}$. mg $^{-1}$] and proinsulin [217 ± 17.34 (3) pmol. min $^{-1}$. mg $^{-1}$] compared with placebo [125 ± 7.51 (3) pmol. min $^{-1}$. mg $^{-1}$]. The results of this study, showing a 59% increase in HMG CoA reductase activity in hepatocytes maintained in culture for 2h with insulin (72nM) are consistent with previously reported work showing that insulin (at a concentration equivalent to $10^4 \mu U/ml$ i.e. 72nM) produced an approximate 40% increase in HMG CoA reductase in hepatocytes (Gibbons *et al*, 1984). The study has also shown that proinsulin at a concentration of 50nM produced a 73%

stimulatory effect on the rate limiting enzyme of cholesterol biosynthesis in rat hepatocytes (Fig 4 1) A possible explanation for this striking result may be that binding of proinsulin to the insulin receptor is a concentration-dependent process A previous study investigating the inhibitory action of insulin and proinsulin on basal and glucagon-activated glycogenolysis in cultured rat hepatocytes showed that the amount of glucose formed by hepatocytes that were incubated with either 100nM insulin or proinsulin was quite similar unlike when cells were incubated with 1nM of each hormone (Hartmann *et al*, 1987) However, that study did report an approximate 3.3 fold higher half-maximal effective concentrations of proinsulin (5nM) relative to insulin (0 15nM) in inhibiting glyogen phosphorylase activity Similar differences in potency were observed for these hormones in the release of glucose and lactate from radiolabelled glycogen in hepatocytes (Hartmann *et al*, 1987) Thus, it appears that a 50nM proinsulin concentration may bind more effectively and mediate effects on target enzymes more effectively than a ten-fold lower concentration By contrast with the number of studies showing an effect of proinsulin on carbohydrate metabolism, similar type studies have not heretofore been carried out to determine the relative effects of proinsulin and insulin on lipid metabolising enzymes.

Having obtained a clearly measurable response, its relationship to the proinsulin dose had to be established Fig 4 2 shows the log dose-response of proinsulin on specific activity of hepatocyte HMG CoA reductase activity Both the slope of the line and its position on the X-axis were used as indices of the sensitivity and the detection range of the assay respectively It is apparent from Fig 4 2 that rather wide detection limits characterised the response The lower limit of 2.7pM corresponded with that found in other radioimmunoassay methods, the upper limit approached levels previously reported in insulinoma patients Ideally, the

detection limits should not be so wide as to restrict the sensitivity Activity was shown to increase 68 8% over the 100-fold concentration range Since a bioassay is an indirect approach to the measurement of potency, it required analysis of the log dose -response curves for both standard and test sample to calculate the dose of unknown that matched the standard For convenience in analysis, the volumes of serum to be used in the assay should match those of the proinsulin standard which showed a measurable response Fig 4 4 showed that addition of 250μl, 500μl and 1000μl of 12pM standard to the incubation medium resulted in an approximate doubling of enzyme activity (1 7-1 8 fold) Therefore, the volumes of serum that were chosen for testing were 0 5ml and 1 0ml The ratio between the two doses was the same for standard and unknown

Prior to constructing a bioassay to measure the level of proinsulin in serum, an examination of the effect of serum and insulin at a single physiological concentration on reductase activity was carried out It is apparent from Fig 4 3 that a concentration of at least 100pM insulin can stimulate HMG CoA reductase while incubation with serum resulted in a massive 440% increase in activity That the presence of both insulin (100pM) and proinsulin (2 7pM) in the incubation medium produced a greater response in hepatocytes than proinsulin alone suggests that insulin at that concentration is more biologically active than proinsulin. It should however be borne in mind that the absence of a control study in this experiment hinders the interpretation of the proinsulin effect. The presence of analytes in serum capable of stimulating reductase activity need not necessarily affect the result of a parrallel proinsulin bioassay since cells are incubated with two doses of intact proinsulin standard. By comparing the relative responses to doubling of the volume of standard and of serum, a measure, though indirect, of the potency of intact proinsulin in serum can be obtained

A total of twenty one bioassays was constructed *in toto* However five

(Bioassay No `s 2,3,4,7 and 8) see Appendix, showed negligible

response to proinsulin standard A possible explanation for this might

reside in the possibility of structural damage to these cells as a result of

the perfusion technique The digestion of the proteins on the surface of

hepatocytes could conceivably occur in the presence of impure

collagenase Perfusion with impure collagenase (purity was not assesed)

could have markedly affected the structural integrity of cell surface

receptors such as the insulin receptor resulting in a diminished biological

response of isolated rat hepatocytes The remaining sixteen bioassays

demonstrated the relative potency of the serum to proinsulin standard by

altering HMG CoA reductase activity Each two-plus-two bioassay was

set up as follows· four 9ml aliquots (0 5x10^7 cells /9ml) were incubated at

37°C in an atmosphere of 5% CO_2 with one of the following, 0 5ml or

1 0ml of 12pM human proinsulin standard, 0 5 or 1 0ml of control

/diabetic human serum After 2h the cells were harvested for microsomal

preparation and for assay of HMG CoA reductase activity as descnbed in

Chapter 2. In general, the response in the sixteen bioassays was

positive, associated with increased activity of the stimulus. While enzyme

activity was very variable between hepatocyte batches, the doses chosen

gave responses in almost all bioassays lying on the linear part of the

dose response curve (Fig 4 4), i e between 44 and 140 pmol.min^{-1} mg

protein^{-1} Of the sixteen bioassays that showed a response to the

proinsulin standards, nine were parallel type bioassays i e the log dose-

response curves for both standard and unknown were of similar slope

(as attested to by the Student t-test), indicating that the activity in the

unknown sample is due to the same substance as the standard solution

Microsomal enzyme activity was approximately 48% higher in cells that

were incubated with 1ml standard hormone preparation (12pM) compared to cells incubated with 0 5ml hormone preparation (Fig 4 7) It is apparent that incubation of cells with 0 5ml human serum from either the control (93 5±18 5 pmol min $^{-1}$ mg protein $^{-1}$) or diabetic patient (94 85±18 pmol min $^{-1}$ mg protein $^{-1}$) groups resulted in higher microsomal enzyme activity than incubation of cells with 0 5ml standard alone (69 44±8 13 pmol min $^{-1}$ mg protein $^{-1}$) (Fig 4 8) The remaining seven bioassays, in which the plots of the log-test sample at the two doses were not parallel to the plots of the standards at the same volumes were those in which a greater increase in specific activity was found on adding 1ml of serum to the cell incubation medium suggesting an element of crossreactivity As mentioned previously, both insulin and serum can cause marked increases in enzyme activity The possibility that insulin or even biologicaly active proinsulin-like intermediate forms may be present in these sera must await similar 2+2 bioassay studies using insulin, split-or des-intermediate standards Fig 4 9 shows the correlation between serum proinsulin as measured in nine bioassays with the value M, the potency ratio. A strong correlation was observed (r=-0.97, p<0 001) between the potency ratio and the serum proinsulin concentration that was calculated. This correlation was also reflected in the two parameters on which M is dependent , i e v, the vertical distance between the two lines and m, the mean slope of the two lines It is apparent from the strong correlation (r=-0 689, p<0.02)) between v and serum proinsulin concentration, that as the vertical distance between the lines decreases the potency of the unknown serum sample increased (Fig 4 10) and that as the mean slope of the lines increased that serum proinsulin concentration also increased (r=+0 55, p<0 06) (Fig 4 11) The latter however did not reach statistical significance

Fig 4.5 is a example of a two - plus - two parrallel bioassay of a control subject versus a standard human proinsulin on isolated rat hepatocytes. Two doses of unknown (T_1 & T_2) and two doses of standard (S_1 & S_2) were tested. They were chosen to give responses of similar magnitude, and with T_1/T_2 equal to S_1/S_2 for convenience in analysis. This figure illustrates the specific activity of HMG CoA reductase (pmol. $min^{-1}.mg^{-1}$) as a function of log_{10} proinsulin volume (μl). The potency ratio, $log_{10} M$ (which is equivalent to the ratio of the activity of proinsulin in the unknown sample to the standards) is dependent on two parameters, (v) the vertical distance between the curves and (m) the mean slopes of the lines. In this bioassay $log_{10} M=0.47$; M=2.85. Since the standard was 12pM, the unknown proinsulin concentration was estimated as 12/2.86 = 4.21pM. Fig 4.6 is an example of a two - plus - two bioassay for a diabetic subject. In this case however the potency ratio ($log_{10} M$) of unknown versus the standard was calculated to be =1.37 and therefore the proinsulin concentration was estimated to be 8.76pM. Cross reactivity with insulin or derivatives of proinsulin did not occur since the two lines in each case are of similar slope. This is in contrast to previous findings demonstrating cross reaction in radioimmunological assays, although not with insulin, but with derivatives of proinsulin (Cohen *et al*, 1985, Yoshioka *et al*, 1988).

The results of this study showed that a higher level of biologically active proinsulin was observed in serum from type 2 (non-insulin- dependent diabetic) patients (Fig 4.12). However, there would be little difficulty in concluding that the levels of proinsulin were significantly higher in type 2 diabetics relative to the controls if there were assurances that metabolically active cells expressing measurable, reproducible amounts of HMG CoA reductase had been prepared. Furthermore, the extent of

cross reactivity between proinsulin and insulin as suggested in the non parrallel bioassays poses serious difficulties to the validation of the bioassay as a suitable method for proinsulin measurement The values observed for proinsulin concentration in control (4 16±0 05pM, n=2) and diabetic (7 77±0 5pM, n=7) subjects using the bioassay method are however within the physiological concentration ranges previously recorded by radioimmunoassay, [5.8±3 3 pM (controls) and 12 6±7 5 pM (NIDDM)] (Yoshioka et al, 1988) The main advantage of a reliable bioassay over methods used previously to measure proinsulin is that it determines the biological activity of proinsulin As mentioned in Chapter1, widely used radioimunoassay procedures are in themselves limiting due to cross reactivity of structurally similar components A reliable bioassay for proinsulin would have several important applications For example, proinsulin is often regarded as a contaminant in the production of human insulin by the proinsulin route (Wetzel et al, 1981) Inefficient processing of the prohormone may lead to higher levels of proinsulin in human insulin preparations. This would be particularly undesirable in view of recent findings by Nagi et al (1991) who demonstrated that the elevated concentrations of intact proinsulin and 32-33 split proinsulin in subjects with type 2 diabetes are associated with deleterious changes in levels of recognised cardiovascular risk factors. The level of contamination could be assessed using a reliable bioassay. More importantly a bioassay for measurement of human proinsulin levels in fasting and post-prandial serum would be of great importance in the treatment and study of diabetes, insulinoma and other disorders involving abnormalities of ß cell dysfunction, insulin production and secretion

The next step in this study was to develop an immunometric assay for proinsulin that would permit comparison of the relative concentrations of

serum immunoreactive proinsulin in non-diabetic and type 2 diabetic patients and which, in combination with the bioassay method, would allow an assessment of the proportion of total immunoreactive proinsulin that is biologically active in these patients.

Measuring proinsulin accurately has been difficult, in part because its low concentration in the circulation requires a very sensitive assay and in part because most antisera raised against proinsulin cross react with insulin and C-peptide. Since single-site immunoassays for proinsulin can cross - react with both insulin and C-peptide, and proinsulin metabolites also known as split proinsulins, can cross - react in assays for proinsulin, it was necessary to establish a two - site ELISA (enzyme-linked-immunosorbent assay).

In the process of establishing the configuration of this assay it was necessary to consider the very wide range of concentrations of insulin and its related peptides and C-peptide in serum. A solid phase antibody preparation that expressed a very high binding capacity should be used to avoid its saturation by potentially cross reacting materials. A method is described here wherein proinsulin was conveniently estimated by a 2-site technique using a capturing antibody that recognised some part of the C-peptide moiety of proinsulin. The sandwich was completed with an enzyme-labelled antibody specific for the insulin moiety of the proinsulin such that the only possibility for the sandwich formation was when the intact proinsulin molecule and/or one or both of its split forms was present. Even if in the assay proinsulin conversion fragments did cross react with the antibodies this would not be considered a major liability because such products are probably secreted under biological conditions similar to those in which intact proinsulin is secreted. Enzyme activity was measured following the addition of substrate (OPD), the

amount of colour developed being proportional to the amount of proinsulin in the sample. Conjugation of anti-insulin IgG to horse radish peroxidase (HRP) was carried out using periodate and the prepared conjugate was characterised using HPLC as previously described in chapter 2. The conjugation performed on the anti-insulin IgG was successful as attested to by HPLC (Chapter 2) and by subsequent assays performed, which indicated that the conjugate had retained biological activity.

In order to perform an ELISA the optimum working conditions of the antibodies in question had to be first determined. Characterisation of the antibodies involved determination of the working dilutions, cross reactivities and the optimum incubation times for each step in the assay as previously described in Chapter 2. Fig 5.1 indicated that the working dilution of the anti-insulin IgG conjugate was in the order of 1:10 corresponding to 300µg/ml. Fig 5.2 illustrated that the optimum working dilution for anti-C-peptide that would be effective was a 1:500 (10µg/ml), which is within the acceptable coating concentration for a capturing antibody (Kemeny, 1991). Fig 5.3 showed that an incubation time of 2h at 37°C was optimal for the adherence of the anti C-peptide IgG to wells of a microtitre plate. Similarily incubation for 2h at 37°C was also sufficient for anti C-peptide to bind immobilised proinsulin (Fig 5.4). Initally the optimum incubation time for anti-insulin IgG HRP conjugate at 1:10 dilution was shown to be 8h (Fig 5.5). However it was thought that the long incubation time of 8h at this high temperature (37°C) is likely to have adverse effects on stability, in particular on the conjugate, which in turn could adversely affect the sensitivity of the assay. We therefore examined the effect on assay sensitivity of a longer incubation time at a lower temperature (24hours at 4°C) (Abs492nm=0.449±0.03 n=3). Since

incubation at 4° C overnight gave similar absorbance readings as those at 37° C for 8h (Abs 492nm = 0 424 ±0 01) employing the same assay conditions as described in Chapter 2, we choose to carry out all subsequent assays using the longer incubation time at the lower temperature to prevent any likely diminishment of stability

Fig 5 6 demonstrates the composite standard curve from six separate assays, relating proinsulin concentration ($\log_{10}$ pg/ml) with absorbance 492nm The standard curve was linear in the range (1-500 pg/ml), i e (0.1 - 55 55 pM) The 0 1pM sensitivity for proinsulin combined with a wide range of detection makes this method comparable with the enzyme amplification technique described by Dhahir et al (1992) The linear range of the assay included the range of concentration of serum proinsulin reported previously using immunoradiometric assays Values ranging from 5 8pM (control) subjects to 12 6pM (type 2 non- insulin - dependent) diabetic patients were reported for the radioimmunoassay described by Yoshioka et al (1988) Another radioimmunoassay making sequential use of antisera to C-peptide and insulin returned a value of 15pM as the fasting proinsulin concentration in non diabetic control subjects and 32pM in patients with type 2(non-insulin-dependent) diabetes mellitus (Ward et al ,1987) More recently a mean value for intact proinsulin in sera from 25 type 2 diabetic subjects was noted as 23 pM with values ranging from (2 4-52 pM) (Nagi et al, 1990) A reference range between 1 2-13 pmol/l with a median of 4 1 pmol/l was found in 38 healthy fasting subjects using the ELISA method developed by Hartling et al, (1986) However a wider operating range (between 0-160 pmol/l) was reported for this latter method So it would be useful to extend the operating range of the ELISA described here so as to incorporate values from patients with insulinomas or islet cell tumours where values for

proinsulin may be as high as 263 pmol/l

Since proinsulin is composed of both insulin and C-peptide moieties one would expect that antibodies that react with proinsulin will also cross react with insulin and C-peptide However cross reactivity was observed in this assay but only at high concentrations with negligible cross reactivity at the lower concentrations Interference in this assay by C-peptide even in supraphysiological amounts is negligible The high binding capacity of the antibody-coated solid phase as shown in Fig 5 4 suggests that C-peptide concentration in normal subjects and in almost all known pathological conditions would not be so greatly in excess of the proinsulin concentration as to have any marked effect on the proinsulin concentration By incubating the serum sample in the coated well and then removing it and washing the well before adding the labelled anti-insulin IgG, interference by binding of insulin in the serum sample to the labelled antibody is precluded Insulin and C-peptide did not cross react in this assay at levels below 1ng/ml and 0 1µg/ml respectively. This result correlates well with those obtained from the study on cross reactivity in the complete assay, where it was demonstrated that human insulin and C-peptide did not cross react at levels below 50ng/ml and 10µg/ml respectively (Table 5 9(a) and 5.9(b)) Different assay conditions (i e. immobilised proinsulin and the involvement of only one antibody type) may account for the slightly higher cross reactivities observed for the separate antibodies relative to those values obtained for cross reactivities from the complete assay. However higher cross reactivities have been reported in the literature The ELISA assay described by Hartling et al (1986) showed that insulin in samples did not interfere at concentrations below 400 pM while human C-peptide in the amplified ELISA described by Dhahir et al (1992) did not cross react

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even at 10,000 pM

The reproducibility of the assay system for proinsulin measurement was determined by assaying serum from two fasting NIDDM patients and two control subjects in triplicate on two separate occasions The serum samples had been stored at -20°C prior to assay, and each sample was assayed in undiluted form on both occasions (Table 5 10) The results demonstrated good reproducibility with the values varying by 2-3% A series of known proinsulin concentrations were also assayed to give an indication of the relative accuracy of the results obtained for the unknowns Table5 11 demonstrates the expected concentrations and the results actually obtained The results show that the assay returned values which were within 5% of those expected

The assay described here, a two-site (non competitive) ELISA using antisera to C-peptide and insulin, represented an improvement in sensitivity for the assay of proinsulin by 10- fold over previous techniques (Hartling *et al*, 1986 and Sobey *et al*, 1989) and yet was as sensitive as the amplified enzyme-linked immunoassay described by Dhahir *et al* (1992) The method enabled the estimation of the low concentration of proinsulin expected in fasting normal individuals In addition, it was sensitive enough to detect circulating proinsulin levels in type 2 (non-insulin-dependent) diabetic patients Serum from 8 fasting diabetic patients and 4 control subjects was assayed in triplicate The samples were stored at -20°C prior to assay and were assayed in undiluted form Fig 5 7 demonstrates that the level of proinsulin concentration in type2 non- insulin- dependent diabetic patients [23 21 ± 1 14 pM (8), mean ± SEM (n)] was significantly higher (p<0 01) relative to the control subjects [13 67 ± 1 58pM (4)]

In summary, the assay described is a relatively simple and specific micro - ELISA showing no cross reactivity with physiological levels of insulin and C-peptide The assay required low sample volume (100µl serum) frozen at -20°C It is faster than other immunoassays (1 5 days vs 7 days) and employs an enzyme -labelled antibody that is stable

The bioassay and ELISA techniques discussed may offer researchers the opportunity to measure both the biological activity of circulating proinsulin and also the immunoreactive proinsulin levels using anti C-peptide and anti-insulin immunoglobulins Both methods demonstrated similar results in so far as higher levels of serum proinsulin were observed in the serum of diabetic patients compared with non-diabetic subjects. However Fig 5 8 demonstrates that the values obtained using the ELISA technique are higher than those obtained using the bioassay technique for both control [13 67 ± 1 58 pM (4) Vs 4 16±0.05 pM (2), mean ± SEM (n)] and the diabetic patients [23.21 ± 1 14 pM (8) vs 7 77±0 5 pM (7); mean±SEM (n)] The lower values obtained in the bioassay compared with the ELISA are possibly a result of the fact that only bioactive intact proinsulin is measured in the bioassay, while in the case of the ELISA all immunoreactive proinsulin is detected In addition, assuming that the bioassay measured total intact proinsulin, then 27% of the total proinsulin-like material in control serum was made up of intact proinsulin Though not significant, intact proinsulin represented a slightly higher percentage (31%) of total immunoreactive proinsulin in diabetic serum than control serum The fact that a complete match of data for sera assayed by the bioassay and ELISA was not possible (due to the difficulties associated with the bioassay) does render however these figures somewhat abstract since they are based on the means of the two

groups studied.

In conclusion, two techniques, an *in vitro* bioassay and an immunological ELISA technique were investigated with respect to their suitability for measuring intact proinsulin and total immunoreactive proisulin. Both were sensitive enough to detect serum proinsulin concentrations at physiological levels for both fasting non diabetic subjects and type 2 (non-insulin-dependent) diabetic patients. However, of the two techniques, the bioassay was a less robust assay to perform than the ELISA, largely as a result of it lacking technical simplicity and the inter bioassay variation in the biological response of isolated rat hepatocytes. Until the extent of biological variation is minimised further, there seems to be little justification for developing a bioassay based on the effect of proinsulin on HMG CoA reductase. Moreover, a more pronounced biological response that is unique to proinsulin would greatly facilitate the setting up of a more reliable bioassay. However, if a range of cytochemical bioassay systems is used and the relative potency of standard proinsulin and unknown serum is the same in all of them, then it is likely that the activity is due to proinsulin. If, on the other hand, in one or more assay systems the relative potency is not the same, it must be concluded that the biological activity is not wholly due to proinsulin, but partly at least to other substances. By contrast, the ELISA was a more user- friendly procedure to employ. Although the cross reactivities of des- and split proinsulins in this ELISA were not determined, the assay did however exhibit remarkably low cross reactivity with both human insulin and C-peptide. Using the ELISA system several unknown serum samples can be assayed at the same time, where as in the case of the bioassay only one sample can be examined per assay.

As to the mechanism of the increase in serum proinsulin in Type 2 diabetes the most plausible possibility is that relatively more proinsulin as compared to insulin is secreted from the β cells in this condition It is conceivable that one mechanism to explain an increase in proinsulin secretion would be enhanced stimulation of β cells by glucose This would accelerate synthesis and release of insulin which may result in mobilisation of younger secretory granules that have insufficient time to undergo full maturation and are consequently richer in proinsulin The release of immature secretory granules containing relatively more biologically active proinsulin than mature granules may account for the higher amount of proinsulin observed in non-insulin dependent diabetic patients Measurement of proinsulin in post-prandial samples might have provided useful information with respect to insulin proinsulin secretion patterns Another mechanism of an increase in proinsulin could be a decreased conversion of proinsulin to insulin by some unknown mechanism during metabolic derangement of diabetes (Saad *et al,* 1990) Notwithstanding that data from the bioassay could only produce a result on 2 serum samples from the non- diabetic patient group and on 7 from the type 2 diabetic group, and that the bioassay did not return values on all the sera samples assayed by the ELISA, the somewhat similar proportions of intact proinsulin to total proinsulin as determined from the means of both these groups could suggest that enhanced synthesis and secretion and not decreased conversion as a result of defective proteolysis accounts for the higher amount of proinsulin observed in diabetic patients Clearly, an alternative approach involving either HPLC as reported by Linde *et al* (1991) or a series of monoclonal antibody-based assays specific for insulin, intact proinsulin, split and des proinsulins is needed to investigate the mechanism of proinsulin secretion in type 2 (non-insulin-dependent) diabetes

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APPENDIX

TABLE A

Summary of values obtained for each parameter
in the development of a bioassay for serum proinsulin
measurement (pM) in control subjects (bioassay No 1-7)
and Type 2 diabetics (bioassay No 8-21)

| Bioassay No	S_1	S_2	T_1	T_2	m	v	(Protein) mg/100ml	M	(Proinsulin) pM
* 1	71±11	88±6 36	112±14	139±9 2	90	41.00	0 42±0 32	2.85	4.21
2	56±8 03	62±2 82	64±4 53	68±0 50	16 66	7.00	0 35±0 02	2.63	4.56
3	92±1.35	104±11.3	134±9 53	157±9.89	53 33	47.50	0 45±0 00	6.52	1.84
4	162±13 4	189±24.7	222±6 36	234±10 6	65 00	52.50	0 36±0 03	6 42	1.87
* 5	34±2.82	58±9 79	75±10.4	113±0 70	103 3	48.00	0 21±0 01	2.91	4.11
6	71±2 00	90±0.40	132±4 24	170±7 50	108 3	66.50	0 40±0 00	4.11	2.92
7	55±1 85	60±1.13	80±0 66	102±9.19	45 00	33.50	0 44± 02	5.55	2.16
8	114±5.65	136±4.94	129±6 36	171±7.77	106 6	25.00	0.38±0.06	1.71	7.01
* 9	64±2 82	103±19 8	85±8 44	120±2 12	123 3	19.00	0 36±0.05	1.42	8.45
10	92±3 83	159±7 07	111±4 94	236±11 3	203 3	37.00	0.41±0 04	1.35	8.88
11	32±4 94	58±5 16	59±1 14	74±1.61	88 33	21.50	0 53±0 06	2.06	5.82
12	90±11.11	156±13 2	116±0.63	203±4.59	255 0	36.50	0.35±0 01	1.39	8.63
* 13	60±8 48	94±5.48	84±7 77	113±2.12	105 0	21.50	0 44±0 00	1.60	7.50
* 14	84±3.95	127±1.41	107±3 53	145±4 94	131.4	19.50	0 43±0 04	1.40	8.57
15	105±8 46	176±12.7	186±2 12	232±10 2	191 6	69.50	0 21±0 05	2.30	5.22
* 16	105±8 46	174±12.7	135±15 1	212±24 7	243 3	34.00	0 22±0 06	1.37	8.76
* 17	63±11 31	82±0 71	81±22 62	105±5 65	80 00	20.50	0 36±0 05	1.93	6.22
18	55±0 848	77±7.78	80±2 33	114±8.20	93.33	31.00	0.36±0 04	2.14	5.60
19	26±2.61	35±4.24	32±2 12	46±2 22	38 33	8.50	0 38±0 06	1.66	7.23
* 20	41±21.92	57±5.51	58±16 26	78±11.31	64 46	19.00	0.37±0 01	2.07	5.79
* 21	101±5.65	135±17 6	114±11 8	149±22.6	115.0	13.50	0.25±0 02	1.31	9.15

* Parallel Bioassay

Two-plus-two bioassay of an unknown (control) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)
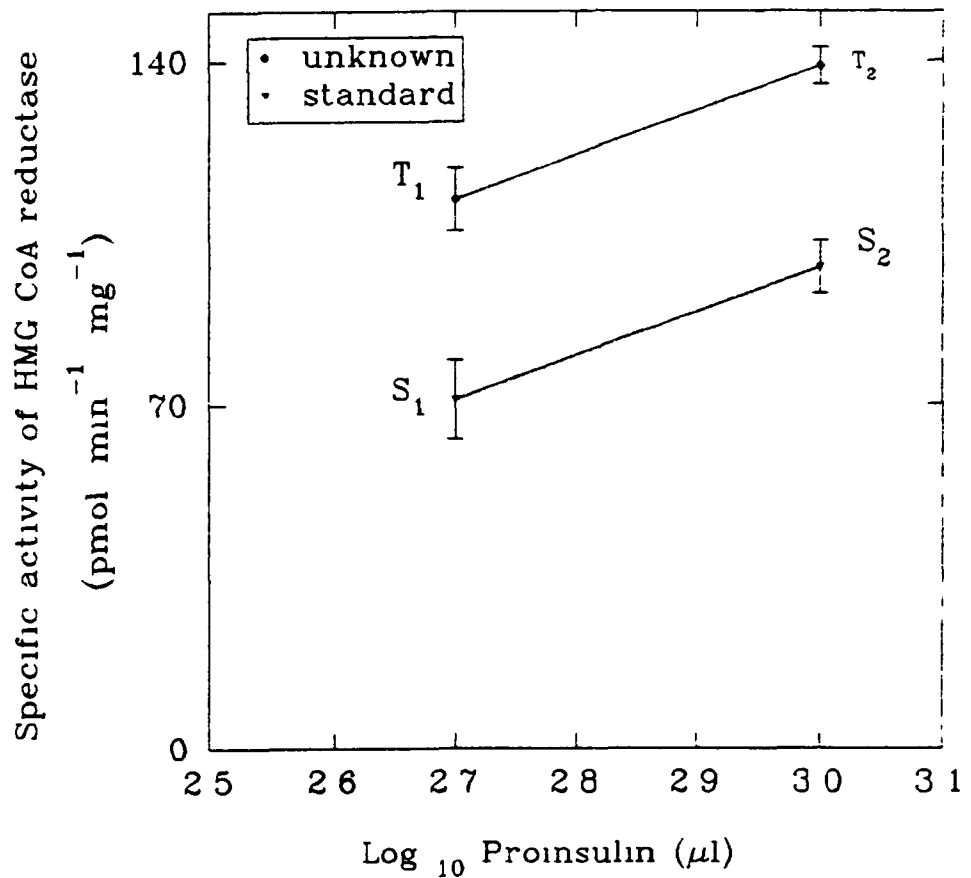
In this bioassay $Log_{10}M = 0\ 456$, $M = 2\ 86$
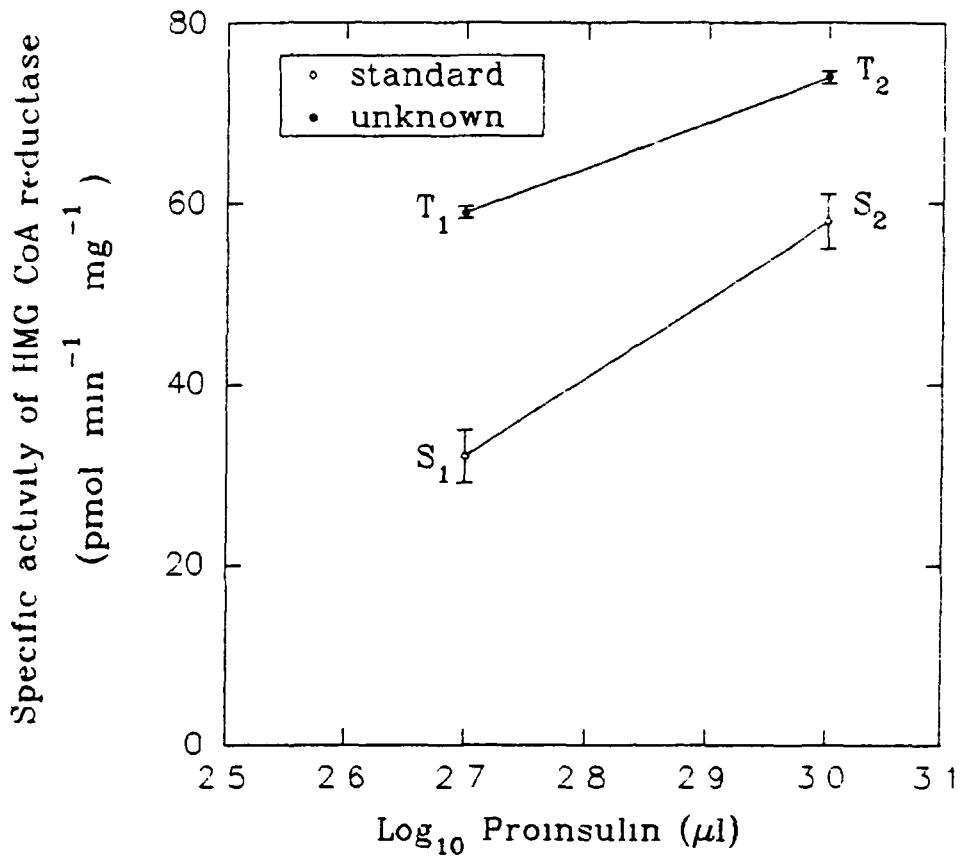Since the standard was 12pM the unknown
was estimated as $12/2\ 86 = 4\ 21pM$

Two-plus-two bioassay of an unknown (control) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)
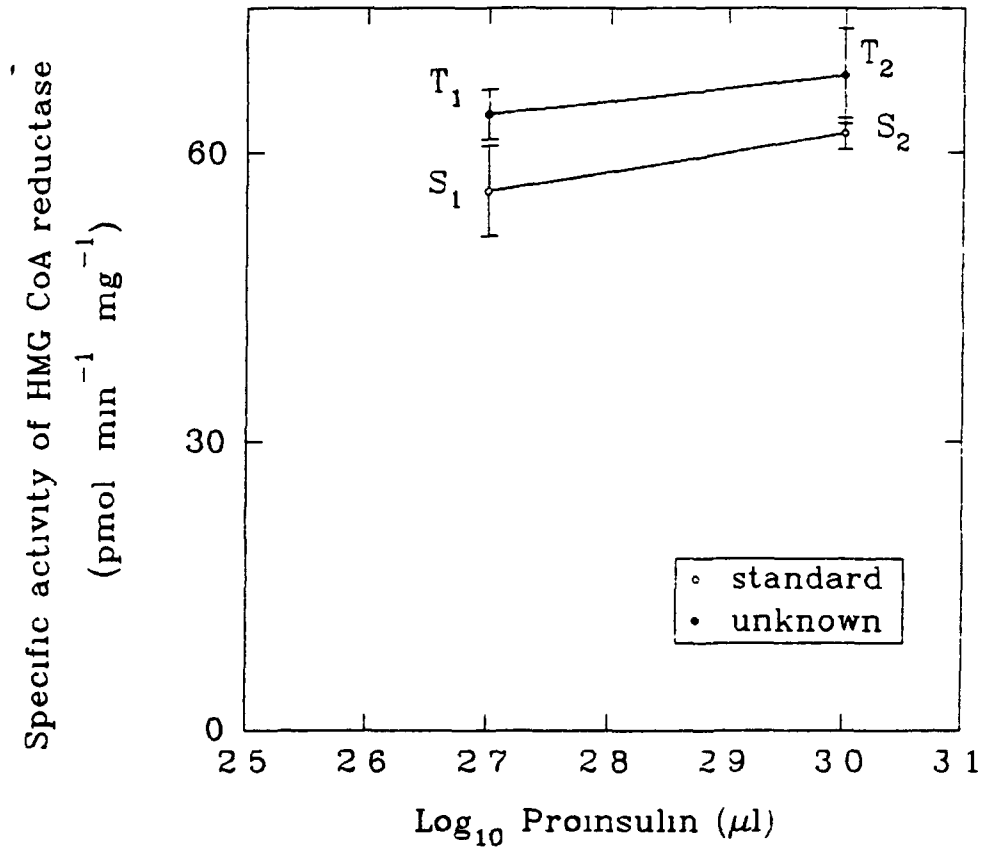
In this bioassay $Log_{10}M = 0\,419$, $M= 2\,63$ since the standard was 12pM, the unknown was estimated as $12/2\,63 = 4\,56$pM

Two–plus–two bioassay of an unknown (control) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)
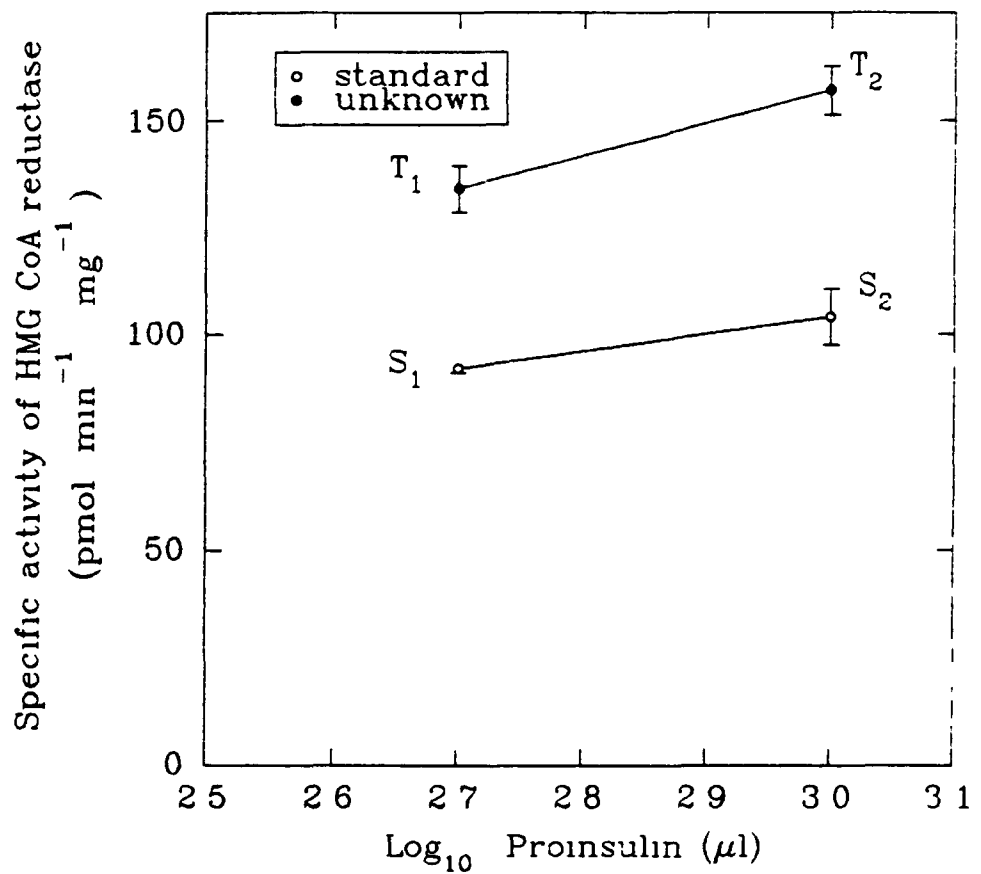
In this bioassay Log_{10} = 0 814, M = 6 52
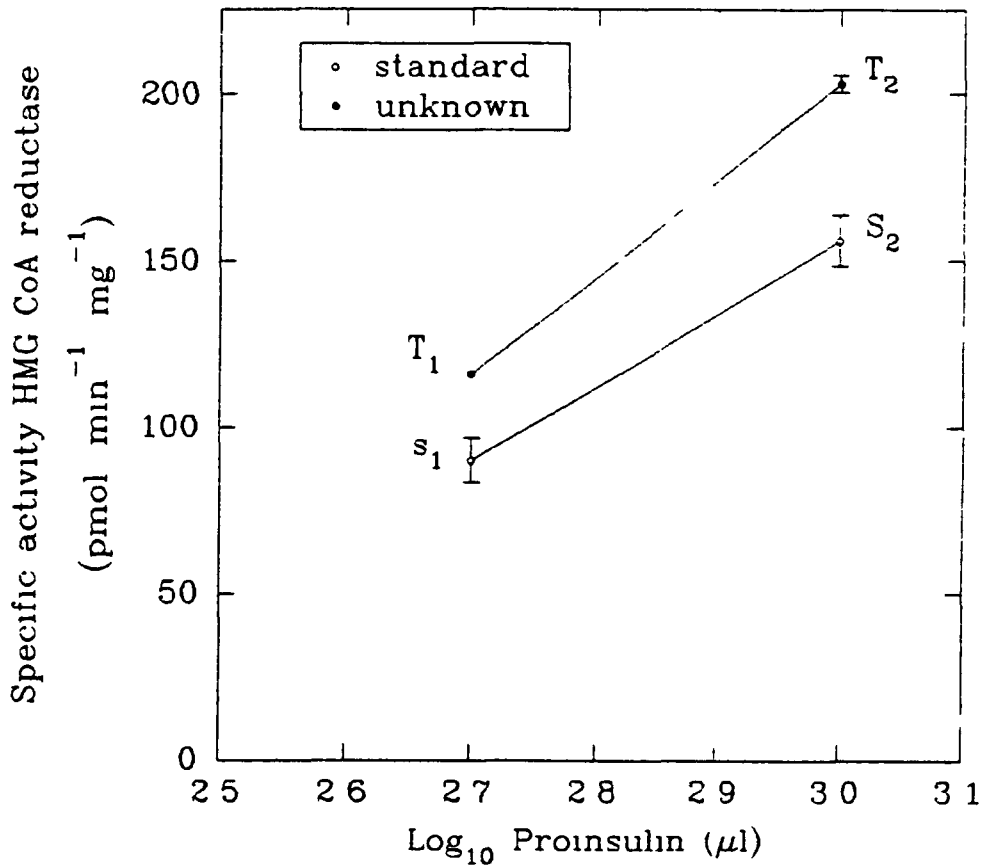Since the standard was 12pM, the unknown was estimated as 12/6 52 = 1 84pM

Two−plus−two bioassav of an unknown (control) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)
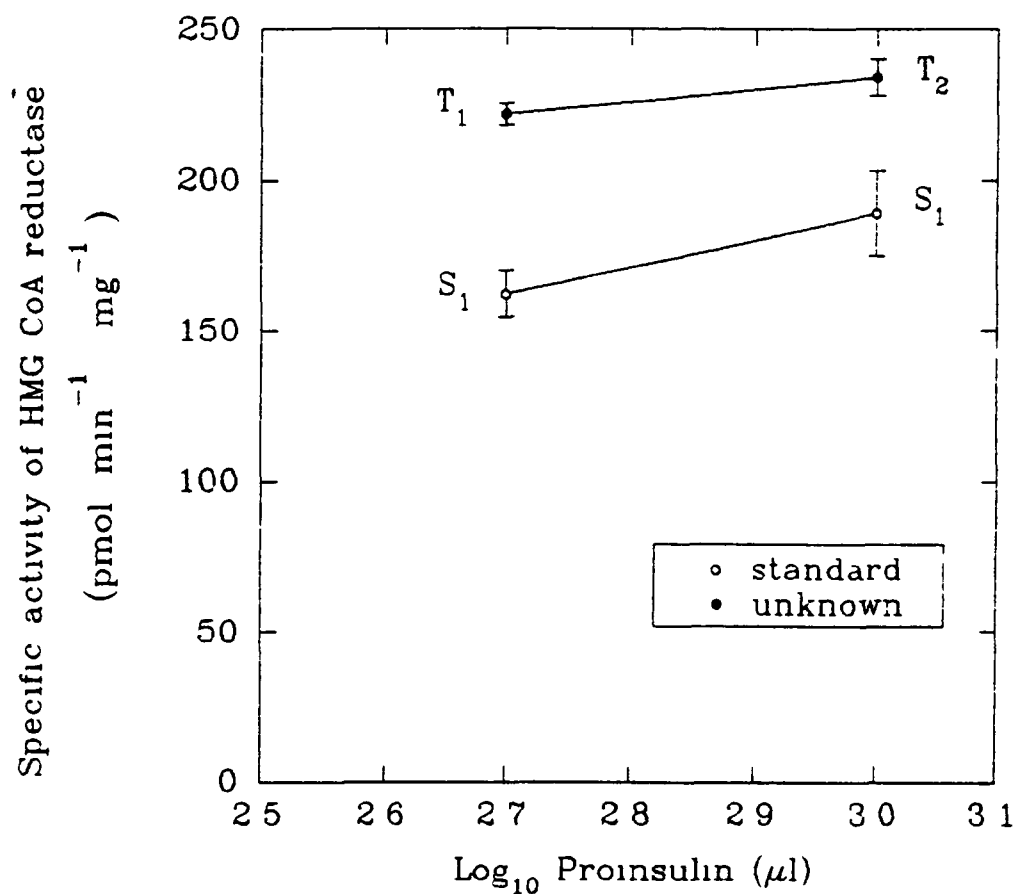
In this bioassay $Log_{10}M = 0 807$, $M = 6 42$
Since the standard was 12pM, the unknown was estimated as 12/6 42 = 1 87pM

Two-plus-two bioassay of an unknown (control) versus a
standard human proinsulin on isolated rat hepatocytes
(mean ± SEM, n=3)
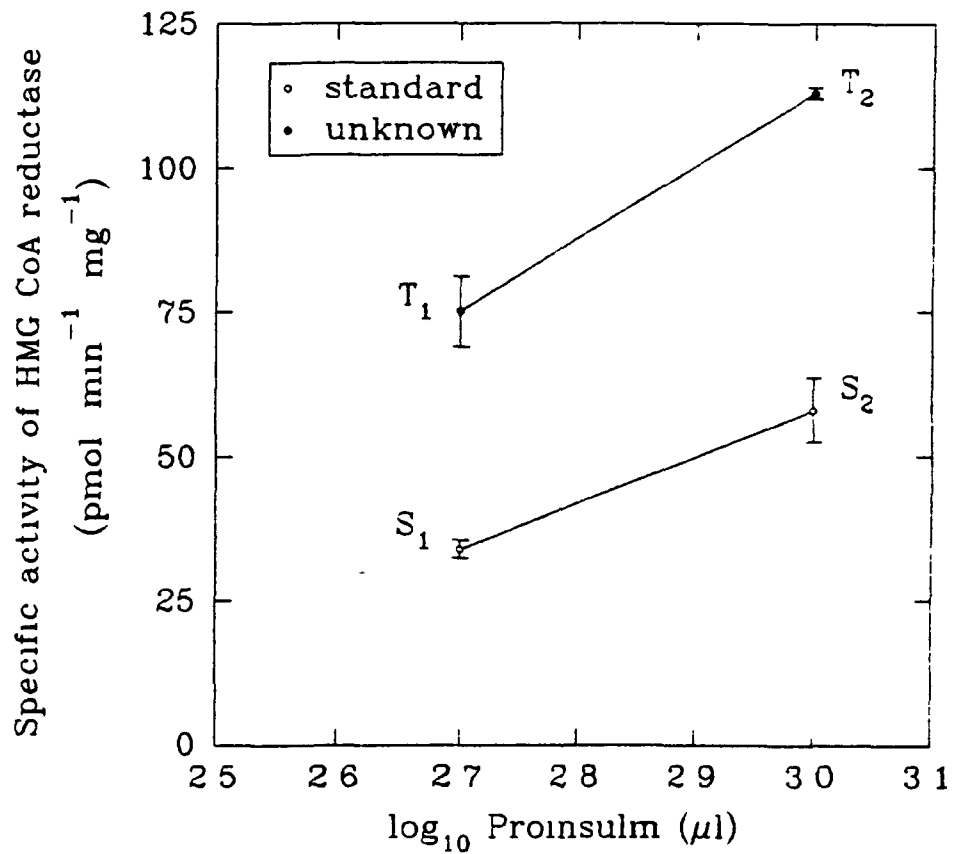
In this bioassay $Log_{10}M = 0.463$, $M = 2.91$
since the standard was 12pM, the unknown
was estimated as $12/2.91 = 4.11$pM
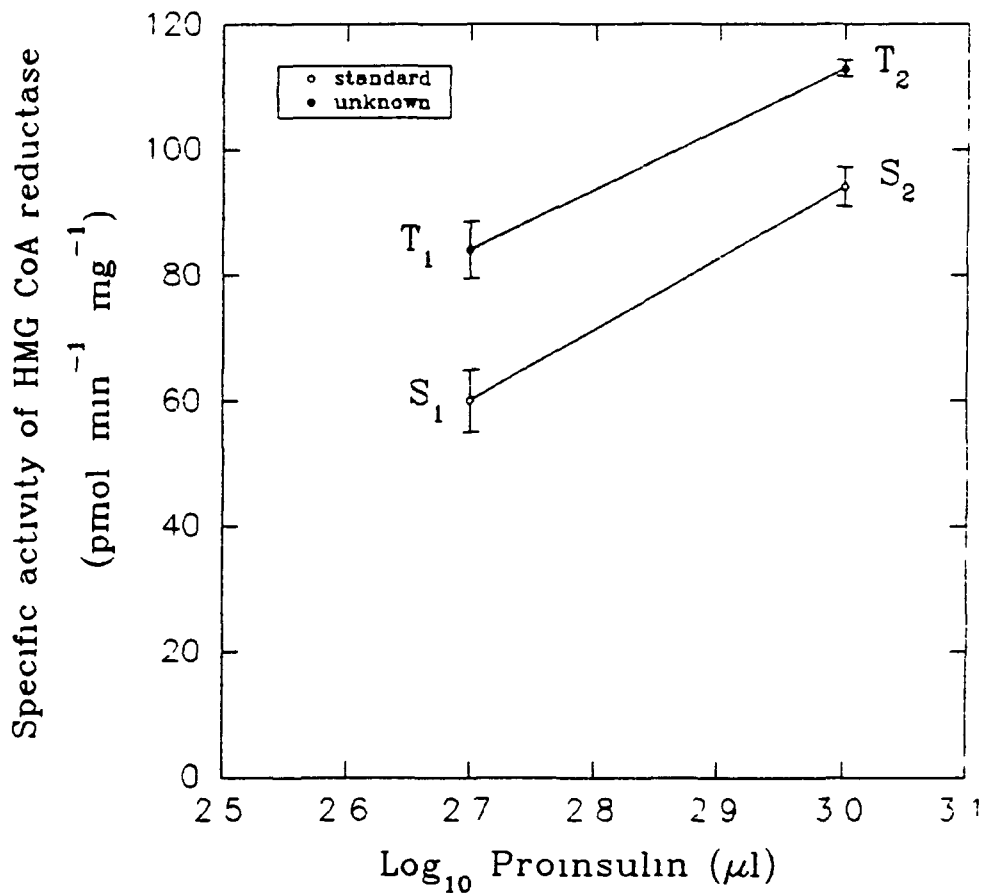
Two-plus two bioassay of an unknown (control) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)
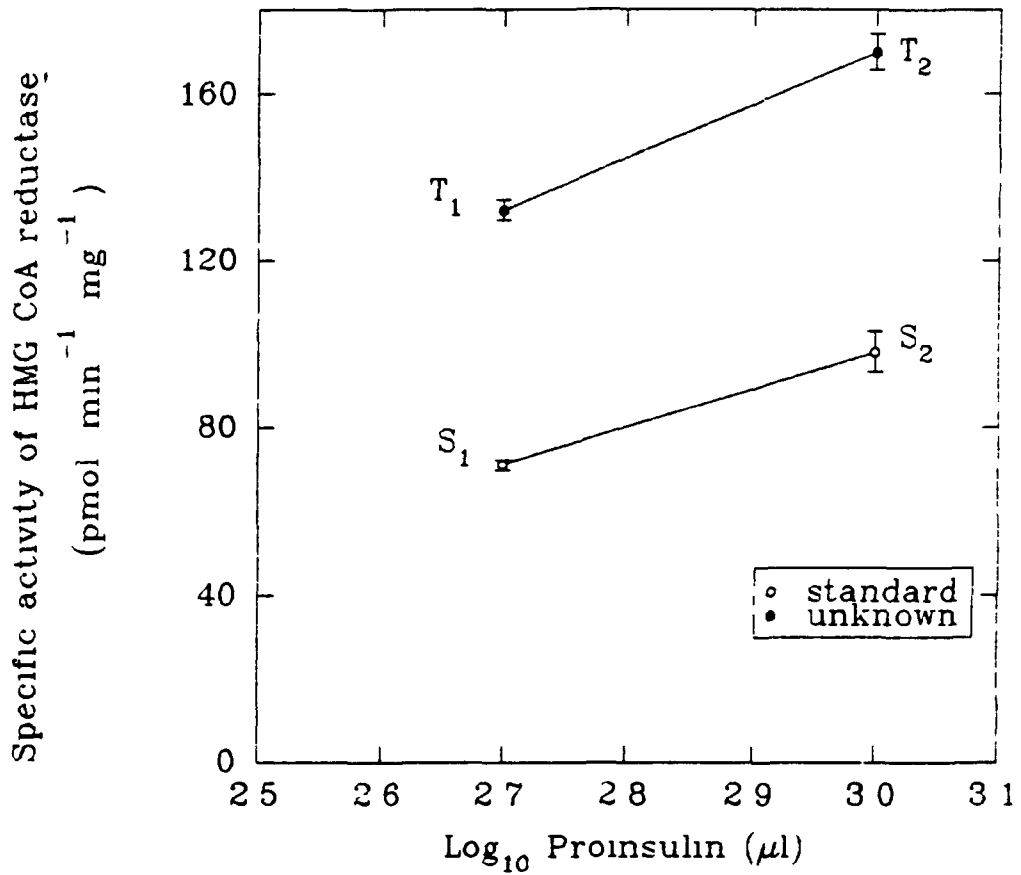
In this bioassay $Log_{10}M = 0.613$, $M = 4.11$
Since the standard was 12pM, the unknown was estimated as $12/4.11 = 2.92$pM

Two−plus−two bioassay of an unknown (control) versus a
standard human proinsulin on isolated rat hepatocytes
(mean ± SEM, n=3)
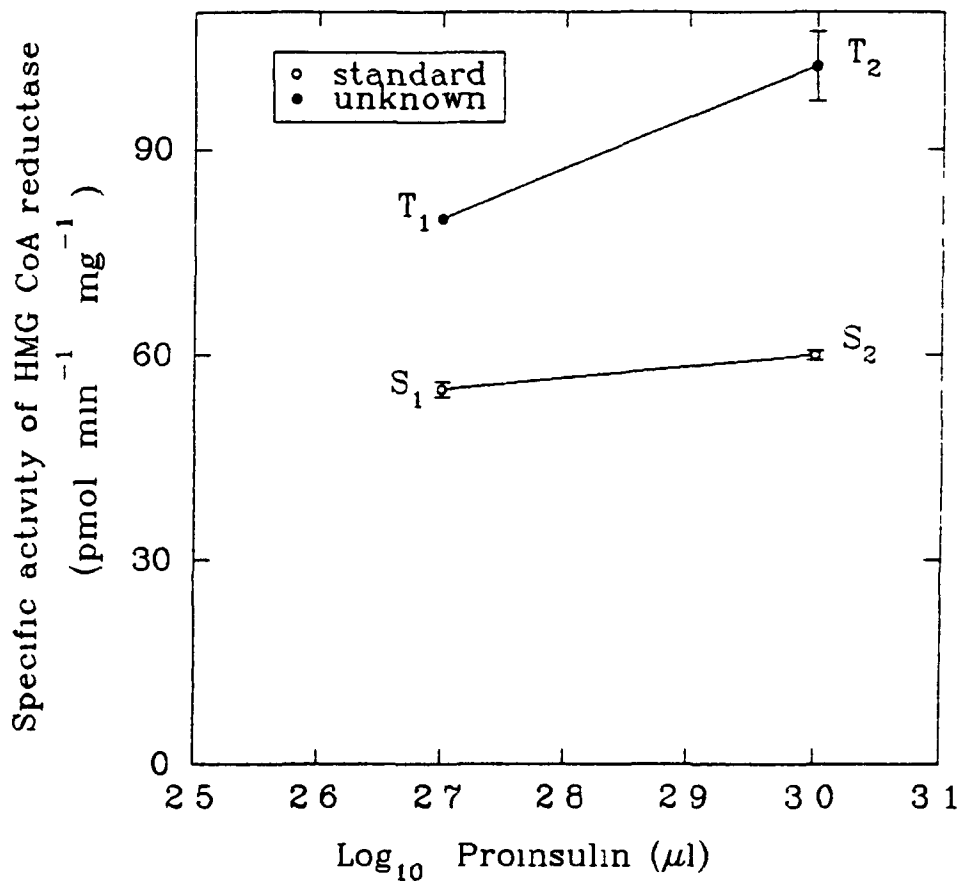
In this bioassay $Log_{10}M = 0.744$, M = 5.55
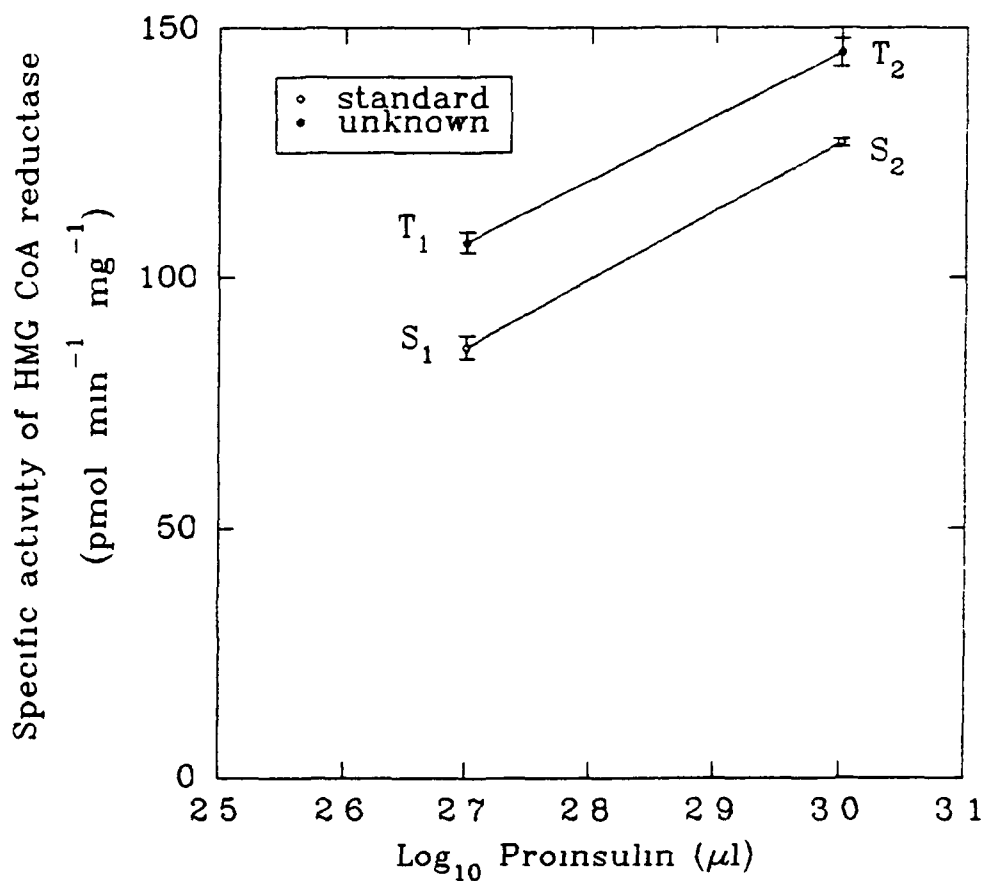Since the standard was 12pM, the unknown
was estimated as 12/5.55 = 2.16pM

Two-plus-two bioassay of an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)
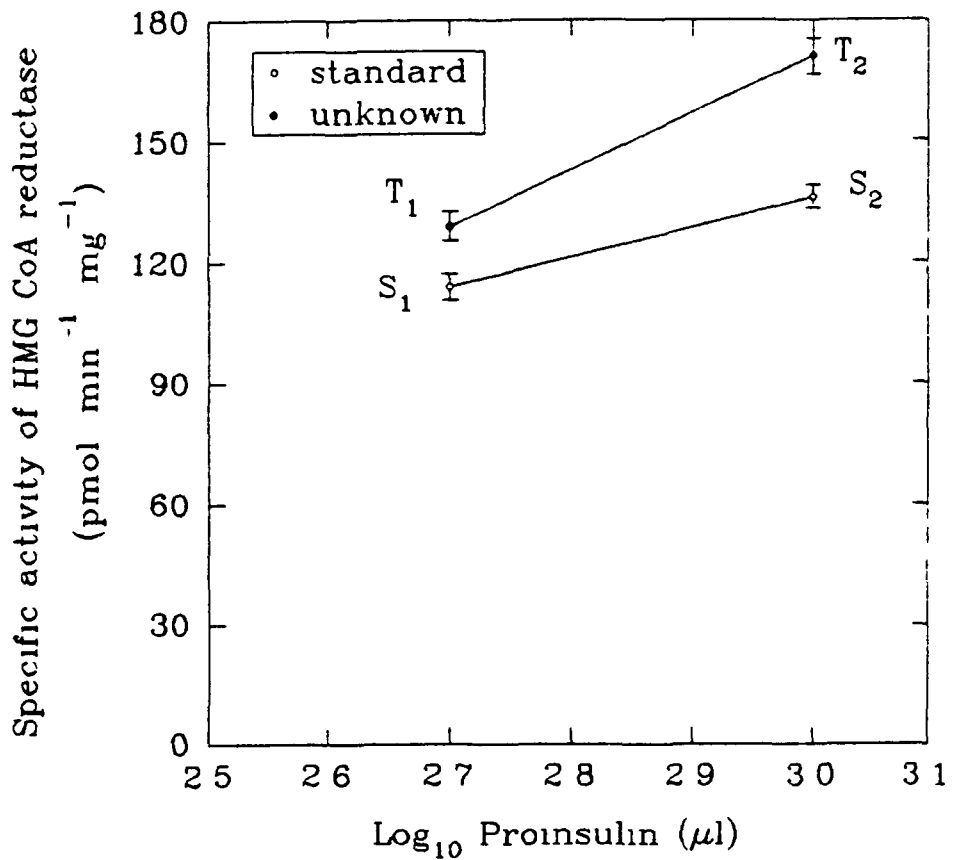
In this bioassay $Log_{10}M = 0\,232$, $M = 1\,71$ since the standard was 12pM, the unknown was estimated as $12/1\,71 = 7\,01pM$
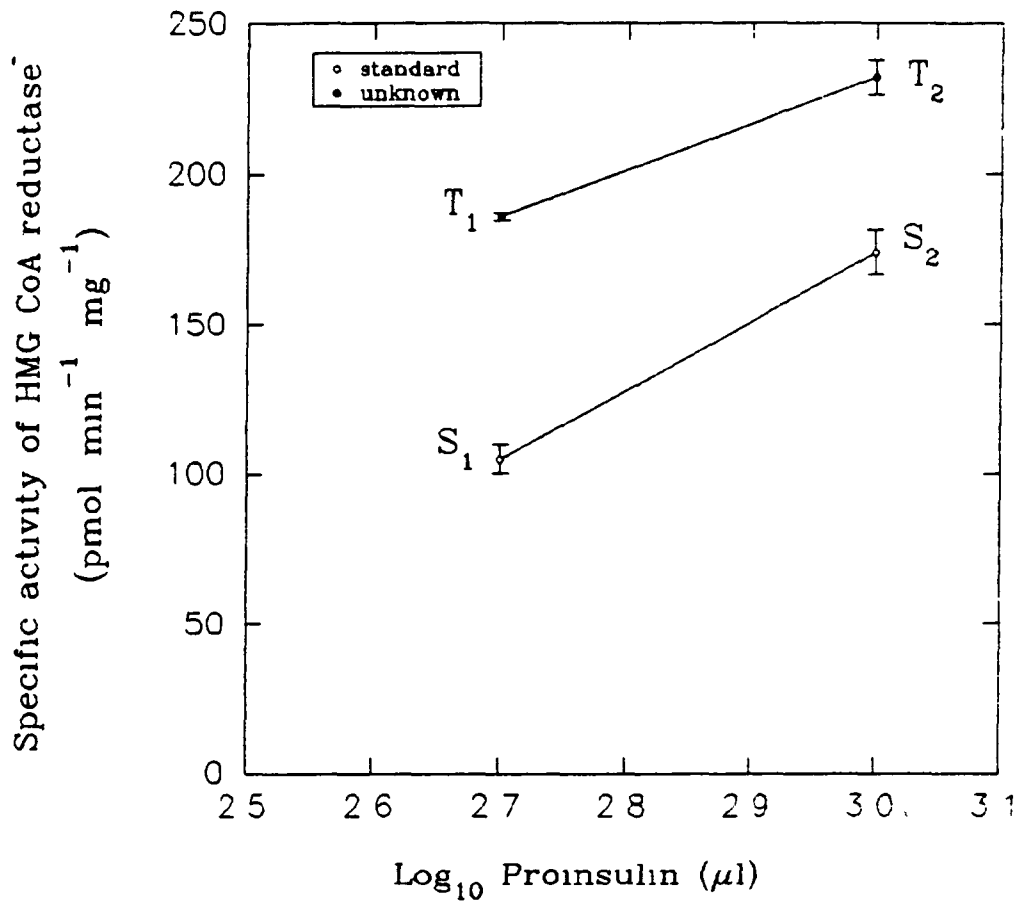
Two–plus–two bioassay of an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)
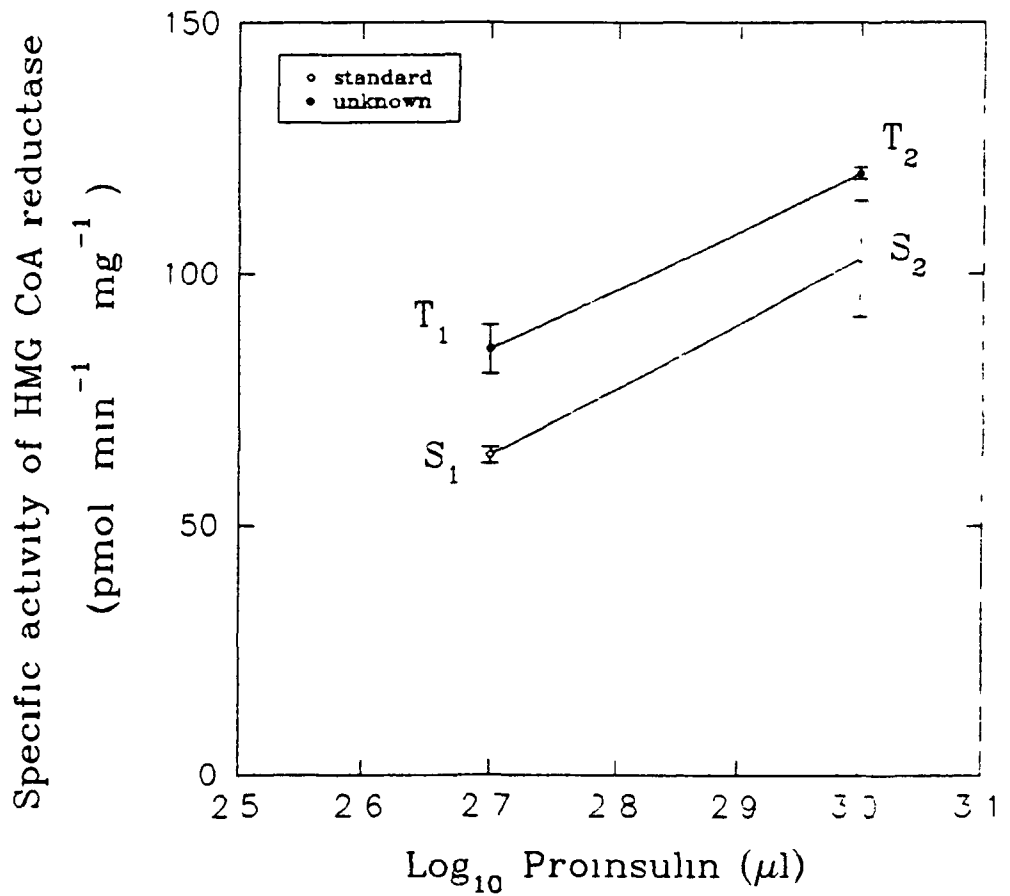
In this bioassay Log_{10} M = 0 152, M =1 42
Since the standard was 12pM, the unknown
was estimated as 12/1 42 = 8 45pM

x
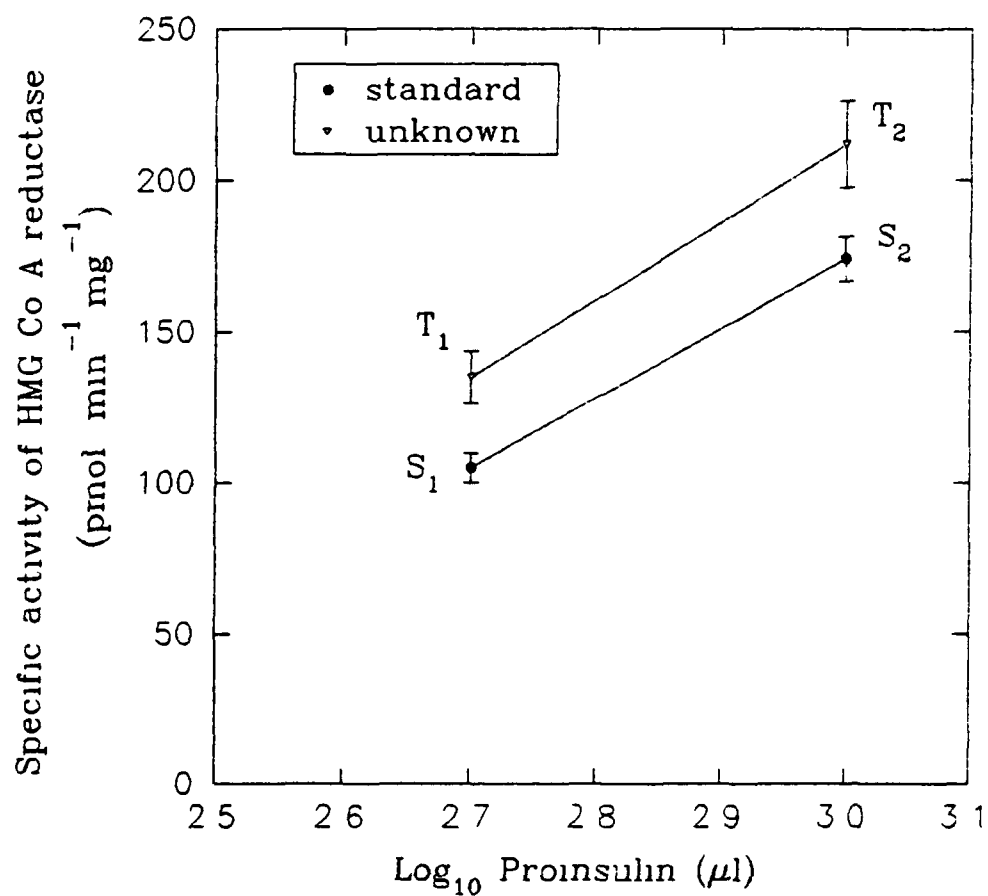
Two−plus−two bioassay of an unknown(NIDDM) versus a
standard human proinsulin on isolated rat hepatocytes
(mean ± SEM, n=3)
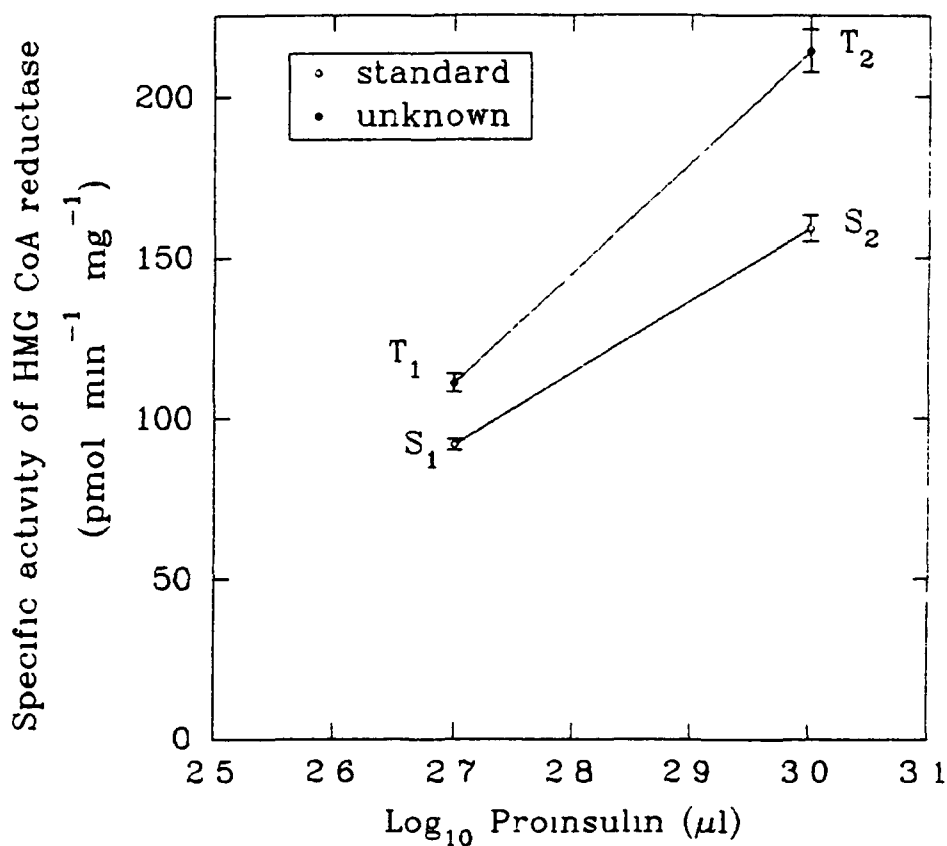
In this bioassay $Log_{10}M = 0\ 130$, $M = 1\ 35$
Since the standard was 12pM, the unknown
was estimated as 12/1 35 = 8 88pM

Two-plus-two bioassayof an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean± SEM, n=3)

In this bioassay $Log_{10}M = 0313$, $M = 206$
Since the standard was 12pM, the unknown was estimated as $12/206 = 582pM$

Two–plus–two bioassay of an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)

In this bioassay Log_{10} M = 0 143, M = 1 39
Since the standard was 12pM, the unknown was estimated as 12/1 39 = 8 63
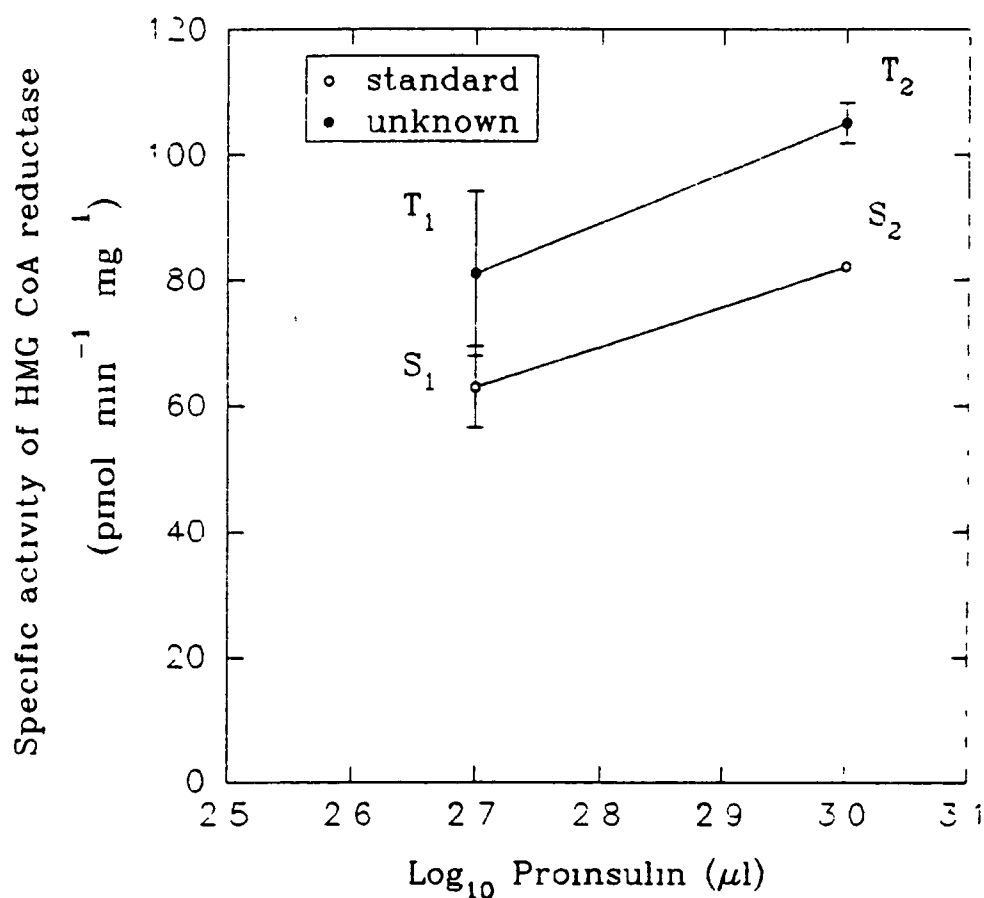
Two−plus−two bioassay of an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)

In this bioassay $Log_{10}M$ = 0 204; M = 1 60
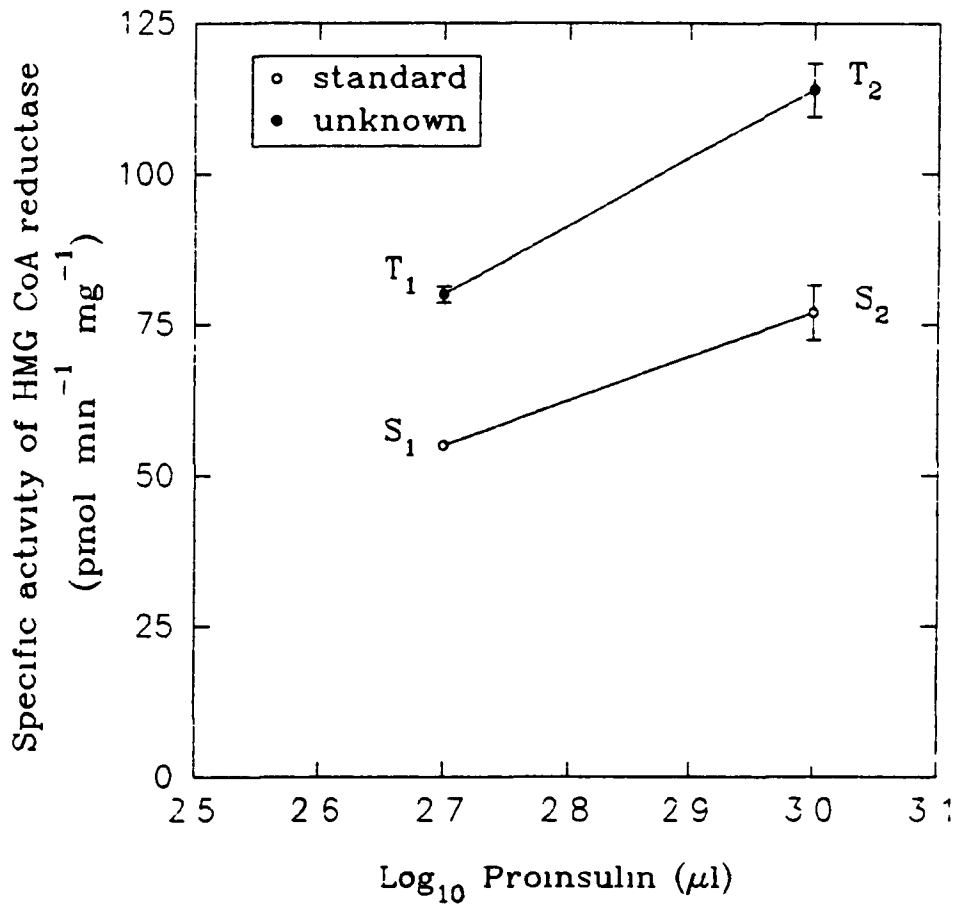Since the standard was 12pM, the unknown
was estimated as 12/1 60 = 7 50pM

Two–plus–two bioassay of an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)

In this bioassay $Log_{10}M = 0\ 146$, $M = 1\ 40$
Since the standard was 12pm, the unknown was estimated as $12/1\ 40 = 8\ 57pM$

Two–plus–two bioassay of an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)

In this bioassay $Log_{10}M$ = 0 361, M = 2 30
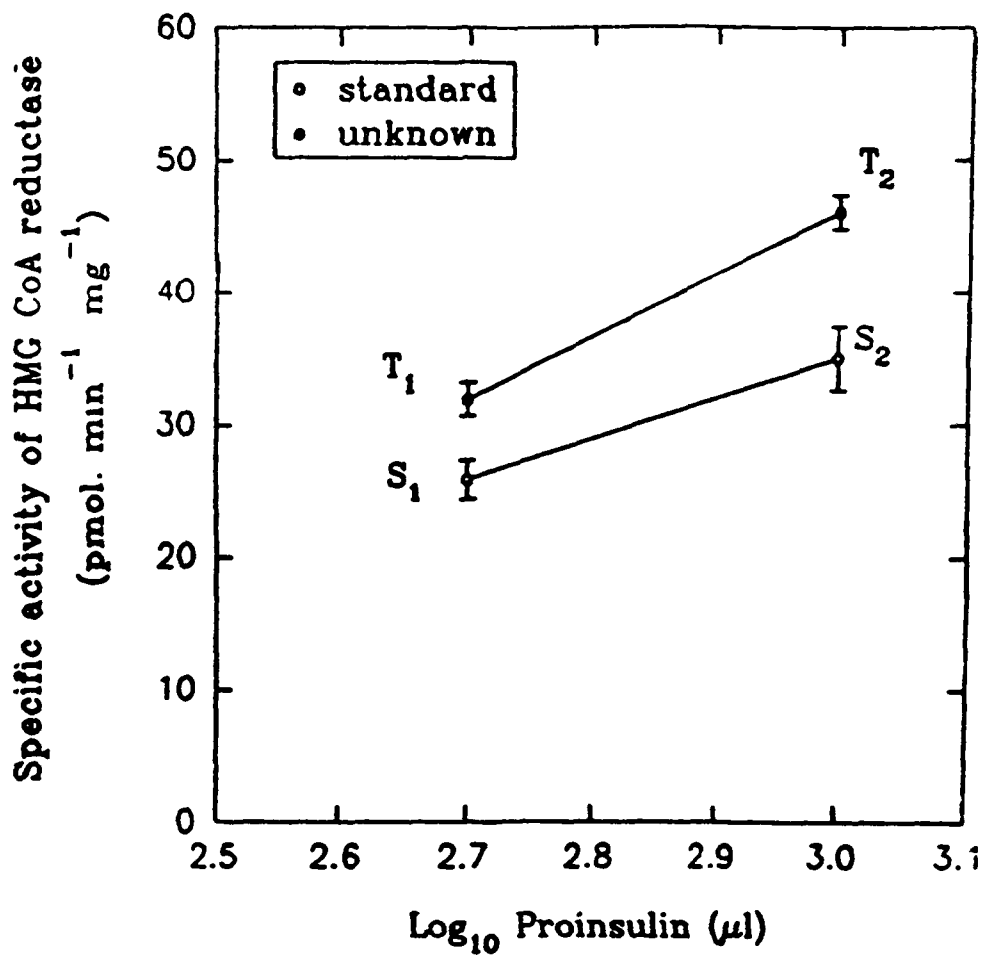Since the standard was 12pM, the unknown was estimated as 12/2 3 = 5 22pM

Two–plus–two bioassay of an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)

In this bioassay, $Log_{10}M = 0\ 136$, $M = 1\ 37$
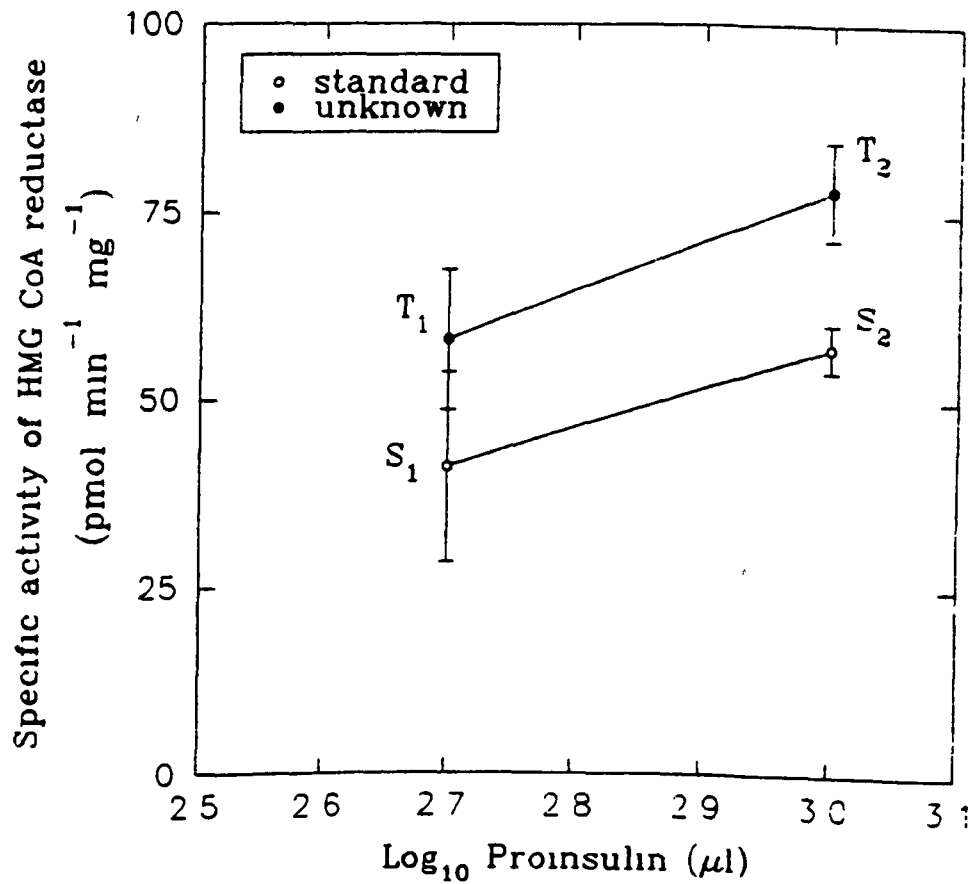Since the standard was 12pM, the unknown was estimated to as $12/1\ 37 = 8\ 76$ pM

Two-plus-two bioassay of an unknown (NIDDM) versus a
standard human proinsulin on isolated rat hepatocytes
(mean ± SEM, n=3)

In this bioassay $Log_{10}M = 0.285$, $M = 1.93$
Since the standard was 12pM, the unknown
was estimated as $12/1.93 = 6.22pM$

Two-plus-two bioassay of an unknown (NIDDM) versus a
standard human proinsulin on isolated rat hepatocytes
(mean ± SEM, n=3)

In this bioassay $Log_{10}M$ = 0 33, M = 2 14
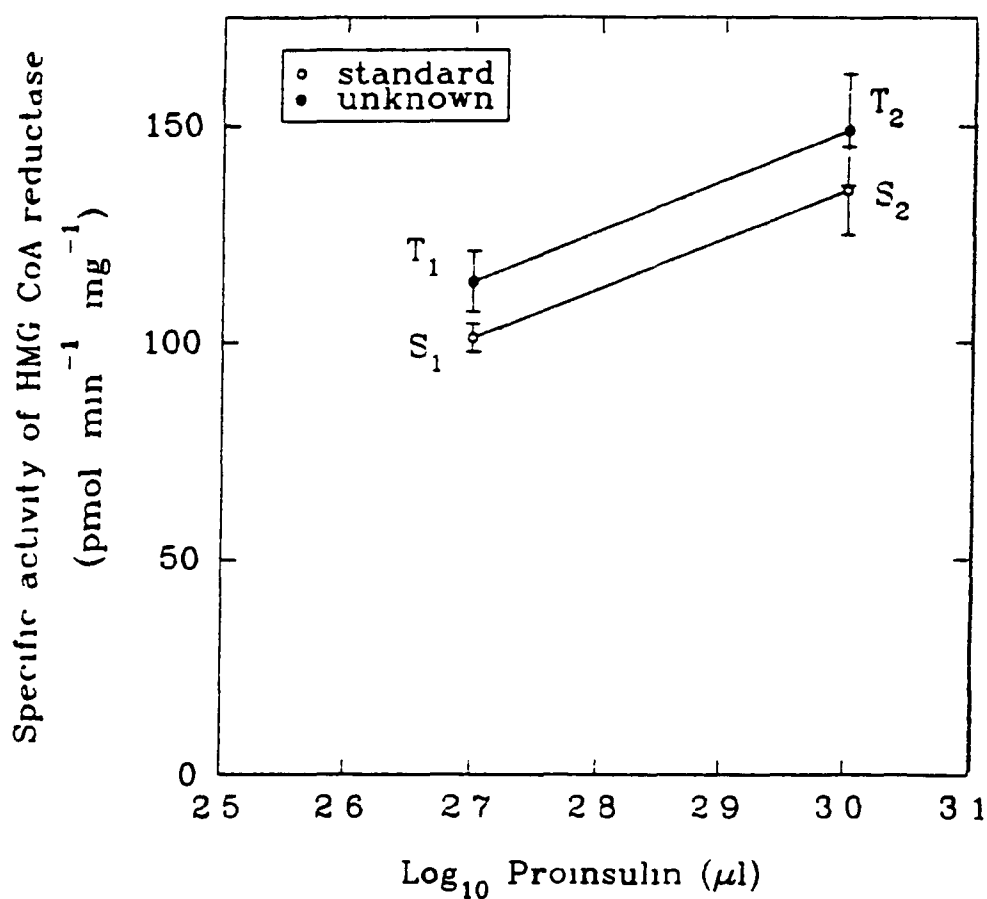Since the standard was 12pM, the unknown
was estimated as 12/2 14 = 5 60pM

Two-plus-two bioassay of an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM. n=3)

In this bioassay $Log_{10}M = 0.220$; $M = 1.66$.
Since the standard was 12pM, the unknown was estimated as $12/1.66 = 7.23pM$.

Two-plus-two bioassay of an unknown (NIDDM) versus a standard human proinsulin on isolated rat hepatocytes (mean ± SEM, n=3)

In this bioassay $Log_{10}M = 0\,315$, $M = 2\,07$
Since the standard was 12pM, the unknown was estimated as $12/2\,07 = 5\,79$pM

Two-plus two bioassay of an unknown (NIDDM) versus a
standard human proinsulin on isolated rat hepatocytes
(mean ± SEM, n=3)

In this bioassay $Log_{10}M = 0.117$, $M = 1.31$
Since the standard was 12pM, the unknown
was estimated as $12/1.31 = 9.15pM$