HUMAN PROINSULIN AND INSULIN : ANTIBODY PRODUCTION, ASSAY DEVELOPMENT AND CLINICAL APPLICATION

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DECLARATION

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PUBLICATIONS

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ABBREVIATIONS

Ab	antibody
Ag	antigen
AI/G	amended insulin/glucose ratio
APC	antigen presenting cell
ANOVA	analysis of variance
β	beta
B	bound
BMI	body mass index
Bq	Becquerels
$^\circ$C	degrees Celsius
c	centi - , $x10^2$
CP	C-peptide
cpm	counts per minute
CV	coefficient of variation
DEAE	diethylamino ethyl
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EPPS	N-(2-hydroxyethyl)-piperazine-N'-(3-propane-sulfonic acid)
F	free
F_1	first generation
FCA	Freunds complete adjuvant
FCS.	foetal calf serum
FIA	Freunds incomplete adjuvant
γ	gamm
g	gram **or** gravity **or** gauge
GIP	gastric inhibitory polypeptide
^{125}I-	radioisotope of iodine

h	hour
HAT	hypoxanthine, aminopterin, thymidine
HEPES	N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid)
HFS	hormone-free serum
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HPLC	high performance liquid chromatography
Ia	I-region associated (antigen)
IDDM	insulin-dependent diabetes mellitus
IgG	immunoglobulin G
IGT	impaired glucose tolerance
Ins	insulin
Ir	immune response (gene)
IRMA	immunoradiometric assay
ITT	insulin tolerance test
iv	intravenous
k_a	association constant
k_d	dissociation constant
K_{eq}	equilibrium constant
l	litre
M	mega - , $\times 10^6$
m	metre **or** milli - , $\times 10^{-3}$
McAb	monoclonal antibody
MDP	muramyl dipeptide
MHC	major histocompatibility complex
min	minute
MLSO	Medical Laboratory Scientific Officer
mol	mole
MOPC	mineral oil induced plasmacytoma

n	number **or** nano - , $\times10^{-9}$
NIBSC	National Institute for Biological Standards and Control
NIDDM	non-insulin-dependent diabetes mellitus
NS	not significant
OGTT	oral glucose tolerance test
p	probability **or** pico - , $\times10^{-12}$
PBS	phosphate buffered saline
PEG	polyethylene glycol
PI	proinsulin
QC	quality control
r	correlation coefficient
RIA	radioimmunoassay
RPMI	Roswell Park Memorial Institute
SAPU	Scottish Antibody Production Unit
SD	standard deviation
SP	solid-phase
TEA	triethylamine
TFA	trifluoroacetic acid
T_H	helper T lymphocyte
T_S	suppressor T lymphocyte
U	units
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organisation
yr	year
μ	micro -, $\times10^{-9}$

DEDICATED TO DAD AND MUM

Great God, I ask thee for no meaner pelf

Than that I may not disappoint myself,

That in my action I may soar as high,

As I can now discern with this clear eye.

HENRY DAVID THOREAU

SUMMARY

Production of high titre, high avidity, polyclonal antisera to insulin was attempted in a number of animal species but was only successful in guinea-pigs. In particular, the use of Strain 2 guinea-pigs resulted in a 100% success rate (Chapter 3).

Ten murine monoclonal antibodies (McAbs) were produced using porcine insulin and human proinsulin as immunogens. Six reacted with insulin and proinsulin, one with C-peptide and proinsulin, and three with proinsulin only (Chapter 4).

The detection of McAbs specific for proinsulin required the preparation of monoiodinated ^{125}I-proinsulin. This involved the development of a reverse-phase high performance liquid chromatography (HPLC) technique to separate the various iodinated species of ^{125}I-proinsulin (Chapter 5, Section 1.2).

A two-site immunoradiometric assay (IRMA) for human insulin was developed using the guinea-pig anti-insulin in conjunction with an insulin McAb raised using porcine insulin as immunogen. Proinsulin cross-reacted in the assay to a degree dependent upon the form being measured and its concentration (Chapter 5, Section 2).

Initial evaluation suggested that the avidity of the C-peptide McAb was too low to provide a proinsulin IRMA of the required sensitivity (Chapter 5, Section 4). Subsequent reassessment with the previously unavailable NIBSC proinsulin standard and a higher avidity insulin McAb suggested that a potentially useful assay could be developed (Chapter 5, Section 6.3).

The highest avidity proinsulin-specific and insulin McAbs, raised using human proinsulin as immunogen, were used in a two-site IRMA to measure human proinsulin. The presence of insulin interfered with the initial assay developed (Chapter 5, Section 5) and the protocol was modified to abolish this effect

(Chapter 5, Section 6). The assay measured intact, 65-66 split and des 64-65 proinsulin.

Clinical validation of the assays developed was performed by measuring insulin and proinsulin concentrations in serum from normal adult subjects under a variety of conditions (Chapter 6, Section 1). The release of proinsulin relative to insulin was found to be delayed in response to an increase in blood glucose concentrations.

Serum proinsulin and insulin concentrations were also measured in patients in various states of altered or patho-physiology. Measurement of fasting proinsulin compared to insulin concentrations provided improved discrimination in the diagnosis of insulinoma (Chapter 6, Section 2.3).

The reported association between cirrhosis of the liver and glucose intolerance, hyperinsulinaemia and hyperproinsulinaemia was confirmed although the data suggested that only hyperinsulinaemia was truly secondary to the development of cirrhosis. The hyperinsulinaemia appeared to be due to reduced degradation rather than hypersecretion (Chapter 6, Section 2.5).

Data is presented which supports the role of insulin resistance in the development of impaired glucose tolerance in pregnancy (Chapter 6, Section 2.2) and the high circulating concentration of insulin in a boy with Mendenhall's Syndrome (Chapter 6, Section 2.4). However, an abnormal pattern of insulin secretion in the basal state also appears to contribute to the hyperinsulinaemia in Mendenhall's Syndrome.

The glucose intolerance has also been shown to be due to an abnormal pattern of insulin release in response to increased blood glucose concentrations in patients with non-insulin dependent diabetes mellitus (NIDDM) (Chapter 6, Section 2.1) and cirrhosis of the liver (Chapter 6, Section 2.5).

From the clinical data presented in this thesis, it is hypothesised that two pools of insulin exist, only one of which contains intact proinsulin. It is hypothesised that the pattern of release of insulin from these pools varies in the basal and stimulated states, and that various pathophysiological conditions, including insulin and non-insulin dependent diabetes mellitus, exhibit altered patterns of secretion which are characteristic of the underlying disease mechanism.

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CHAPTER 1

INTRODUCTION

1 PROINSULIN, INSULIN AND C-PEPTIDE

1.1 Historical Background

The Islets of Langerhans were first described in 1869 (1), approximately fifty years before the discovery of insulin (2). In 1889, von Mering and Minkowski showed that pancreatectomy produced diabetes in dogs (3), but it was not until 1921 that insulin was isolated from the pancreas of dogs in a relatively pure form by Banting and Best (2).

Since its discovery, research involving insulin has resulted in an impressive list of firsts. It was the first protein to be crystallised (4), sequenced (5), quantitated by radioimmunoassay (RIA) (6), synthesised by chemical techniques (7), shown to be synthesised as a larger precursor molecule (8), quantitated by immunoradiometric assay (IRMA) (9) and prepared for commercial use by recombinant DNA technology (10).

1.2 Biosynthesis

The processes involved in the biosynthesis of insulin have been reviewed in detail (11) and a summary is given here.

Insulin is initially synthesised as pre-proinsulin on the rough endoplasmic reticulum of the β cells in the pancreas. The signal sequence is then removed prior to transfer of the proinsulin molecule to the Golgi apparatus where it is packaged into clathrin-coated secretory granules. These granules provide the environment for the conversion of proinsulin to insulin and C-peptide by converting enzymes. The conversion process is accompanied by the shedding of the clathrin coat such that non-coated granules are formed which contain equimolar amounts of insulin and C-peptide as well as a residual amount (approximately 3% in molar terms) of proinsulin (12). It is in these mature secretory granules that insulin is stored until it is released in response to an appropriate stimulus.

1.3 Amino Acid Sequence and Species Variation

The amino acid sequence of proinsulin (13) is shown in Figure 1.1. It is converted to insulin and C-peptide by removal of the two dibasic linking residues which connect the A and B chains of insulin to C-peptide. The species variation in amino acid sequence of proinsulin is a reflection of that observed in insulin and in particular C-peptide.

The amino acid sequences of twenty-eight insulins (14) including human (15) have been determined. The most highly variable residues are A8-A10 and B30 (Table 1.1). Changes in these residues do not appear to affect the tertiary structure of insulin where they are located in close proximity on the surface of the molecule and affect properties such as solubility and crystallisation but not receptor binding (14).

In contrast to insulin which is a highly conserved molecule in evolutionary terms, C-peptide molecules from different species are very heterogeneous, both in length and amino acid sequence (Table 1.2) (16). However, there are a number of conserved residues and these are proximal to residues in insulin that are also conserved, suggesting that interactions between these residues are highly probable. The residues in insulin that interact with C-peptide are principally those thought to be important for the biological activity of the insulin molecule (16).

1.4 Secretion

Insulin and C-peptide are released from the β cell in equimolar amounts together with approximately 3% proinsulin in molar terms (12). However, in the peripheral circulation the ratio of C-peptide to insulin is much higher and proinsulin may account for as much as 20% immunoreactive insulin (17). This is due to the marked differences in the hepatic extraction of these peptides.

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Figure 1.1

Structure of human proinsulin.
(In: Edwards CRW ed. Endocrinology London : W Heinemann Medical Books Ltd. 1986).

TABLE 1.1

AMINO ACID SEQUENCE OF INSULIN IN VARIOUS SPECIES AT
RESIDUES A8-A10 AND B30

Species insulin	A8	A9	A10	B30	Other residues*
Human	Thr	Ser	Ile	Thr	-
Porcine	Thr	Ser	Ile	Ala	-
Rabbit	Thr	Ser	Ile	Ser	-
Equine	Thr	Gly	Ile	Ala	-
Bovine	Ala	Ser	Val	Ala	-
Ovine	Ala	Gly	Val	Ala	-
Chicken	His	Asn	Thr	Ala	B1, B2, B27
Guinea-pig	Thr	Gly	Thr	Asp	A4, A12, A13, A14, A17, A18, B3, B4, B10, B14, B17, B20, B21, B22, B27

* Other residues different from human insulin.

TABLE 1.2
AMINO ACID SEQUENCE OF C-PEPTIDE IN VARIOUS SPECIES

C-peptide	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Human	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser
Porcine				Asn	Pro		Ala		Ala							Leu	-	-		Gly
Equine					Pro				Glu									Leu		Gly
Bovine/Ovine		Val		Gly	Pro				Ala	Leu			Ala							Gly
Guinea-pig		Leu			Pro			Glu	Thr					Met		Leu				Gly

C-peptide	21	22	23	24	25	26	27	28	29	30	31
Human	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
Porcine			Ala						Pro	Pro	
Equine							Ala		Gln		
Bovine		-		-	-	-	-		Pro	Pro	
Guinea-pig			-		-	-		Gln	Ala		

Only differences in amino acid structure from human are marked. Deletions are marked as -, and those amino acids present have been correlated to give maximum sequence homology.

The major physiological stimulus for insulin secretion is glucose. Other molecules also affect secretion and these can be divided into three main groups: substrates, hormones and neurotransmitters. A summary of the main substances in each group thought to be important physiologically, is given in Table 1.3 (18).

1.5 Physiological Effects

The biological activity of proinsulin is qualitatively similar to insulin but considerably less potent. The exact percentage activity attributed to proinsulin relative to insulin varies with the assay system used and has been quoted as ranging from approximately 5-25% (19). It has also been shown that the split and des-amino forms of proinsulin exhibit greater biological activity than the intact molecule (19, 20).

C-peptide does not appear to have any intrinsic biological activity although it does inhibit the biological activity of proinsulin by interacting with a number of the residues in the insulin moiety which are involved with receptor binding (16). The main function of C-peptide however, appears to be in facilitating the correct folding of the A and B chains of insulin such that the disulphide bridges are correctly formed (21).

Insulin regulates carbohydrate, protein and lipid metabolism to promote an anabolic state. The major target organs are the liver, muscle and adipose tissue, and a summary of the metabolic effects of insulin on these tissues is given in Table 1.4 (22).

1.6 Metabolism

It has been estimated that the half-lives of proinsulin, insulin and C-peptide, as measured by the disappearance of these hormones in the serum of patients following pancreatectomy, are 17.2, 4.8 and 11.1 minutes respectively (17, 23). Similar

TABLE 1.3

THE MAJOR PHYSIOLOGICAL FACTORS REGULATING INSULIN
SECRETION

Group	Factor	Action on insulin secretion
Substrates	Glucose	Stimulate
	Amino acids	Stimulate
	Free fatty acids	Stimulate
Hormones	Glucagon	Stimulate
	Gastric inhibitory polypeptide (GIP)	Stimulate
	Somatostatin	Inhibit
Neurotransmitters	Cholinergic	Stimulate
	α-adrenergic*	Inhibit
	β-adrenergic*	Stimulate

*The overall effect of sympathetic (adrenergic) innervation *in vivo* is to
depress insulin release.

TABLE 1.4

METABOLIC EFFECTS OF INSULIN IN THE MAJOR TARGET ORGANS

Target organ	Anabolic effects	Anti-catabolic effects
Liver	Increased glycogen synthesis	Decreased glycogenolysis
	Increased lipogenesis	Decreased gluconeogenesis
Muscle	Increased amino acid uptake	Decreased protein degradation
	Increased protein synthesis	
	Increased glucose uptake	
	Increased glycogen synthesis	
Adipose Tissue	Increased lipid assimilation	Decreased lipolysis
	Increased lipogenesis	
	Increased glucose uptake	

estimates were obtained following suppression of endogenous insulin and C-peptide secretion by somatostatin. The half-lives measured were 3.9 minutes for insulin and 10.2 minutes for C-peptide (24).

The primary organs involved in the clearance of insulin from the circulation and degradation of the hormone are, in order of importance, the liver, kidney and muscle (25). The liver is the predominant organ, extracting 50-75% of insulin after its release into the portal vein prior to its appearance in the peripheral circulation, and a further 40-60% of all the insulin which reaches the periphery (26). The greater part of the insulin remaining is metabolised by the kidney (27).

In contrast, the kidney appears to be the main organ responsible for the clearance of both proinsulin and C-peptide from the circulation (28). Proinsulin is also metabolised by the liver to an extent which reflects its biological activity in this organ, but C-peptide does not appear to be extracted by the liver (26).

1.7 Assays

Measurement of proinsulin, insulin and C-peptide by immunoassay techniques is complicated by the structural similarities between proinsulin and both insulin and C-peptide. Polyclonal antisera raised against all three hormones contain antibodies which react with one or both of the other hormones. Insulin antisera generally cross-react with proinsulin 50-90% on a molar basis while C-peptide antisera usually cross-react to a lesser extent (29). In contrast, although proinsulin antisera cross-react with both insulin and C-peptide, it is possible to absorb out the antibodies with these specificities such that only antibodies specific for proinsulin remain (30, 31). Radioimmunoassays developed using these antisera inevitably show the same patterns of cross-reaction.

The introduction of both monoclonal antibody technology and two-site IRMA techniques has overcome some of the problems mentioned above, and the assay methodology available at the present time for each hormone is detailed below.

C-Peptide

The measurement of C-peptide has been unaffected by the recent advances described above. No McAbs have been produced which are specific for C-peptide and as yet the development of a two-site IRMA for this analyte has not been reported. However, measurement of C-peptide is least affected by cross-reaction as proinsulin reacts with the best available polyclonal antiserum at a level of only 30% in molar terms and it is present in serum at a much lower molar concentration than C-peptide in the majority of conditions (29). In cases where high concentrations of proinsulin are suspected or proven, a separation step to remove proinsulin from the sample prior to C-peptide assay should be performed.

Insulin

Monoclonal antibodies have facilitated the assay of insulin by two-site IRMA techniques. This has resulted in a reduction of the percentage cross-reaction of proinsulin in the measurement of insulin when compared to RIA's. Recently however, a major advance in the assay of insulin has been made with the production of a McAb specific for insulin and its use in a two-site IRMA which measures insulin without cross-reaction with proinsulin (32).

Proinsulin

The original assays developed for the measurement of proinsulin utilised polyclonal antisera to insulin and C-peptide in RIA (33) and IRMA (34) techniques. However, their use was limited due to the shortage of a well-defined human proinsulin standard. The availability of biosynthetic human proinsulin resulted in the more

widespread development of RIA's for proinsulin which utilised either insulin and C-peptide (35, 36) or proinsulin-specific polyclonal antisera (30, 31, 37, 38). A two-site IRMA based on proinsulin-specific and insulin McAbs has also been reported (39).

The main problem remaining in the assay of proinsulin is the fact that it is present in more than one form in human serum (40). Depending upon the assay, the individual conversion intermediates of proinsulin are measured to various degrees or not at all (30, 35, 37, 39). A recent major advance has been the development of two-site IRMA's, utilising McAbs, which allow the measurement of intact proinsulin and each of the conversion intermediates (32). The application of these assays should provide valuable information on the physiology and pathophysiology of the different forms of proinsulin.

2 POLYCLONAL ANTIBODY PRODUCTION

2.1 Introduction

The production of both polyclonal and monoclonal antibodies for use as reagents in immunoassay techniques exploits the humoral immune response which forms part of the natural protection of vertebrates against invading organisms. This protection is conferred by the immune system which functions by discriminating self from non-self, and comprises various mechanisms to eliminate foreign cells, viruses and macromolecules. A basic understanding of the components of the immune system and their functions is required therefore, to successfully manipulate this natural defence mechanism and produce antibodies with defined characteristics.

2.2 The Immune System

The vertebrate immune system is capable of mediating immunity in both a non-specific and specific manner. Non-specific, innate, immunity is mediated by

physical and chemical barriers (skin, mucus and lysozyme), as well as cellular components which are collectively referred to as the reticuloendothelial system. This type of immunity is not enhanced by repeated exposure to antigen but does play an important role in the development of acquired immunity (41).

Specific, acquired, immunity is enhanced by repeated exposure to antigen and is mediated by a group of cells called lymphocytes. Lymphocytes are part of the lymphoid system and can be divided into two groups based on the primary lymphoid organs in which they are produced (42). In mammals, B cells originate from the foetal liver and bone marrow while T cells originate from the thymus. It is in this environment that the lymphocytes acquire their repertoire of specific antigen receptors and therefore become committed to a single antigenic specificity for their life-span (43). They also learn to discriminate between self and non-self. Mature T and B cells migrate to the secondary lymphoid organs which include the spleen and lymph nodes, and it is in this environment that they interact with macrophages and each other to mount an immune response. It has recently been shown that cells other than macrophages are capable of interacting, in a similar manner, with T lymphocytes in the course of an immune response. Collectively these cells are known as antigen-presenting cells (APC's) (44).

2.3 The Humoral Immune Response

A humoral immune response is one which results in the production of antibodies by B lymphocytes. The activity of the B lymphocytes is regulated directly or indirectly by two sub-sets of T lymphocytes known as T-helper (T_H) and T-suppressor (T_S) cells (45, 46).

The initial stages in an antibody response involve transport of the antigen from the periphery, via the lymphatics, to the local lymph node. At some stage of this journey the antigen will be engulfed non-specifically by macrophages. Once internalised it is partially degraded (47, 48) and fragments of it appear on the cell

surface complexed with I region associated (Ia) antigens (49) which are coded for by the I region of the major histocompatibility complex (MHC) (50). These antigen fragment-Ia antigen complexes then interact with receptors, on the surface of T_H cells, which are specific for both components of the complex (51). The bound T_H cells are thus stimulated to proliferate resulting in the clonal expansion of the selected T_H cells. These are now primed and will only interact with B cells which present simultaneously antigen and Ia antigen in the same format as originally presented on the macrophage (52). This phenomenon is known as MHC restriction and appears to be antigen-specific.

Antigen is also taken up by B cells, but unlike macrophages this process is specific and is mediated by immunoglobulin on the B cell surface (53). Free antigen is bound by the surface immunoglobulin and the complex internalised. Here the antigen is degraded (54, 55) before fragments of it appear on the cell surface complexed to Ia antigens (52). The antigen fragment-Ia antigen complexes on B cells are identical to the analogous complexes on the macrophages involved in the priming of the appropriate T_H cells (56). This interaction between T_H and B lymphocytes provides the stimulus that leads to the differentiation of B cells into either plasma cells, which are short-lived and devoted to the production of antibody, or memory cells, which are long-lived and do not secrete antibody.

2.4 Primary and Secondary Responses

The first time a particular foreign antigen is encountered by the immune system, the response observed is weak and slow compared to subsequent encounters. This is because only a small number of the total lymphocyte population will be capable of interacting with the antigen. As explained in the previous section, after the initial contact, both T and B lymphocytes are subject to clonal selection and expansion such that on re-exposure to the same antigen there are relatively more reactive lymphocytes present resulting in a quicker and stronger response.

2.5 Genetic Control of the Murine Immune Response

Autosomal dominant genes controlling the immune response to various antigens, particularly those involving synthetic polypeptides and 'weak' antigens closely related to self, have been reported (57). These immune response (Ir) genes have been mapped to the I region of the MHC (H-2 in the mouse) (58). The Ia antigens, which also map to the I region of the MHC, are the products of these genes (59) and are expressed predominantly on B lymphocytes and macrophages (60) where they effect the antigen-specific MHC restriction of T cells. A number of different theories to account for the antigen-specific effects of Ia antigens on the immune response have been proposed, but it is unclear which one(s) are correct (59).

2.6 Immunogenicity

The immunogenicity of a molecule refers to the ability to induce an immune response, ie, produce antibodies, whereas antigenicity relates only to the ability to combine with antibody.

In general, the greater the phylogenetic distance between the immunogen and the recipient the more vigorous the immune response. Responses against highly conserved proteins tend to be weak as they are limited by the structural differences between the immunogen and the host's self-proteins and therefore by host regulatory mechanisms (61).

In addition, to be immunogenic a molecule must possess two distinct epitopes (62): one which can combine with surface antibody on B cells and is therefore displayed on the intact antigen, and another, which after degradation of the immunogen inside the B cell/macrophage can bind simultaneously to both the Ia antigens on the B cell/macrophage surface and the T cell receptor (63). The former epitope determines the specificity of the antibodies produced and is an absolute requirement in eliciting an antibody response.

These requirements for immunogenicity can be divided into two categories : those properties which are intrinsic to the molecule and those which are extrinsic (64). The intrinsic properties relate to the epitope which binds to the B cell and include accessibility and hydrophilicity. The extrinsic properties relate to the Ia antigen-T cell receptor binding site, recognition of which is restricted by a number of variables all of which are a function of the host and therefore change depending on the species or individual chosen for immunisation.

Antigens tend not to be immunogenic for one of two reasons (for explanation see above).

(i)   They are too small to possess an Ia antigen-T cell receptor binding site in addition to an epitope recognised by B lymphocytes and as such are classified as haptens.

(ii)   They share a high degree of homology with a recipient molecule and as such are not recognised as foreign due to MHC restriction of the immune response.

Both of these problems can be circumvented by conjugating the antigen to an immunogenic carrier molecule. This provides the Ia antigen-T cell receptor binding site which was not present in case (i) and is an alternative to the one present, but not recognised as foreign, in case (ii). In the latter case, immunisation of a number of different inbred strains of animal may be an additional or alternative solution.

2.7 Immunisation

There are no generally accepted guidelines for the production of antisera, therefore the immunisation schedule adopted tends to reflect personal preferences. The variables which should be considered prior to starting an immunisation programme are as follows.

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(i)   **The immunogen.** Is the antigen immunogenic or does it need to be coupled to an immunogenic carrier protein?

(ii)   **The choice of animal species and strain.** This will be dependent on the immunogen to be used ie, differences in sequence/structure between it and various species molecules; the volume of antiserum required; and practical considerations such as facilities for housing animals.

(iii)   **The quantity of immunogen to inject.** Doses have been recommended which range from as little as 1-50 µg for mice (65) up to several mg for larger animals (66). In the majority of cases however, doses of 100-500 µg should suffice for larger animals.

(iv)   **The route of immunisation and number of sites.** The most common routes are intramuscular, subcutaneous, intraperitoneal and intradermal. It is commonly accepted practice to use several sites for each route of inoculation except for intraperitoneal where one site is usually preferred.

(v)   **The use of adjuvant.** This is the only generally accepted practice. The most commonly used adjuvant is Freunds which comes in two forms, complete (FCA) and incomplete (FIA). Freunds complete adjuvant contains *Mycobacterium tuberculosis* of which the active component is muramyl dipeptide (MDP) (67). The mechanisms of action of adjuvants are complex but most incorporate two components. Firstly, they form a depot which protects the antigen from catabolism and releases it slowly over an extended period of time. Secondly, they stimulate the immune response in a non-specific manner. For FCA this involves local formation of granulomas which are rich in macrophages and immunocompetent cells (68). Normally FCA is used for the primary immunisation with FIA being used for subsequent booster injections.

(vi)   **Timing of injections and bleeding.** A four weekly injection regime is recommended with bleeds being taken seven to fourteen days after a booster injection (66). The protocols used in this thesis (Chapter 2, Section 6) were based on those conditions found to be optimal by the Scottish Antibody Production Unit (SAPU) (69, 70).

3 MONOCLONAL ANTIBODY PRODUCTION

3.1 Introduction

Polyclonal antibodies are complex mixtures of different antibodies of varying specificity and avidity. The relative amounts of a single antibody within this mixture are subject to change at different times during an immunisation schedule for any one animal, and comparisons of antisera raised in different animals even of the same species may show the presence or absence of particular subsets of antibodies. This results in practical difficulties in providing a continuous supply of polyclonal antibodies as a standard reagent for use in immunoassay techniques.

However, Kohler and Milstein (71) developed a technique which allows the growth of clonal populations of cells *in vitro* which secrete antibodies of a defined specificity and avidity. This allows the continuous supply of standard homogeneous reagents known as monoclonal antibodies. The technique involves isolating antibody-secreting plasma cells from immunised animals (these cannot normally be grown *in vitro*) and fusing them with a myeloma cell-line. The resulting hybrid cells, or hybridomas, retain the capacity of the myeloma cell to grow indefinitely *in vitro* together with the ability of the plasma cell to secrete antibody.

3.2 Immunisation

The choice of mouse strain for immunisation is determined by the responsiveness of a particular strain for a given antigen. If there is no good reason for choosing

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otherwise, BALB/c mice are preferable as all available myeloma cell lines are derived from BALB/c mice and this therefore allows the resulting hybridomas to be grown in this strain. Immunisation of a different strain requires the use of F_1 hybrid animals to grow the hybridomas *in vivo*.

The serum titre in the immunised animal is related to the number of McAbs eventually produced and the range of avidities and specificities observed reflects those present in the serum prior to fusion. Therefore if McAbs of particular specificities are desired, it may be advantageous to screen for the presence of these antibodies in the serum of a number of immunised animals such that the most suitable may be chosen for fusion.

3.3 Plasma Cells

The plasma cells used for fusion are isolated from a lymphoid organ, usually the spleen, of an immunised animal. The larger spleen cells which have recently undergone antigen stimulation and blast transformation appear to fuse preferentially with myeloma cells (72). This can be achieved by giving large doses of antigen intravenously without adjuvant, to an animal which has been immunised by conventional means, on each of the 3-4 days preceding fusion (73). Other workers have found that a single boost 3-4 days prior to fusion results in the greatest number of positive hybrids (74).

3.4 Myeloma Cells

Myelomas are malignantly transformed antibody-secreting cells and can be induced in a few strains of mice by injecting mineral oil into the peritoneum. The most commonly used fusion partners today are derived from myelomas isolated from BALC/c mice (75). However, these early myelomas secreted antibody and subsequent cell lines have been selected which are non-functional in this respect. A further refinement has been the selection of cell lines which are selectively killed

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when grown in medium containing hypoxanthine, aminopterin and thymidine (HAT). Normal cells have two pathways for the synthesis of nucleotides, the de novo and salvage pathways. Cells lacking one of the enzymes involved in the salvage pathway, hypoxanthine-guanine phosphoribosyl transferase (HGPRT), can be selected for with 8-azaguanine. The addition of HAT to medium blocks the de novo synthesis pathway (aminopterin) and forces cells to use the salvage pathway (utilising HT) which requires the presence of HGPRT. Therefore, the use of a myeloma cell line lacking this enzyme allows unfused myeloma cells to be selectively killed by growing the fusion products in HAT medium. The myeloma cells which have fused with plasma cells (ie, hybridomas) will not be affected as the salvage pathway will be contributed by the plasma cell (76). The ability to kill selectively myeloma cells is necessary because fusion efficiency is low. Therefore hybrid cells are greatly outnumbered by unfused myeloma cells which if not killed will result in the death of the hybridomas due to overgrowth. The cell line used in the work presented in this thesis, X63. Ag 8. 653 (77) lacks the enzyme HGPRT and is non-functional with respect to antibody synthesis.

3.5 Fusion Procedure

Most fusions designed to produce hybridomas use polyethylene glycol (PEG) as fusogen. The optimum concentration is in the region of 35-50%, but there is some variation with cell line (78). The mechanism of fusion is complex and involves cell agglutination, membrane fusion and cell swelling (79).

3.6 Cell Growth and Cloning

The fusion products are grown in medium containing HAT to kill selectively unfused myeloma cells (Section 3.4). Unfused plasma cells will not survive in culture for more than a few days. The remaining hybridomas are a heterogeneous mixture of which only a small number may be producing antibody of the desired specificity. It is therefore necessary to clone cells as soon as possible after the

detection of positive cultures. Cloning is also necessary as non-producer variants may arise due to chromosome loss, particularly during the early stages of hybrid growth. At least two cloning steps should be performed to ensure that a monoclonal cell line has been obtained. There are two main cloning techniques, that of limiting dilution or cloning in soft agar. The former method is more frequently used.

Cloning by limiting dilution is based on the Poisson distribution:

$$f(0) = e^{-\lambda}$$

where $f(0)$ is the fraction of wells with no growth and λ is the average number of clones per well. If $\lambda = 1$, then $f(0) = 0.37$, ie, if at least 37% of wells show no growth then the probability is that those that do show growth contain true monoclonal hybridomas (80).

3.7 Expansion of Cloned Hybridomas

Cloned hybridomas can either be grown *in vitro* producing antibody as culture supernatant or *in vivo* producing antibody as ascitic fluid.

The latter technique makes use of the fact that mice primed with mineral oils are more susceptible to the development of spontaneous mineral oil induced plasmacytomas (MOPC's) and this is used to encourage hybridoma growth *in vivo* (75). The mice are primed with an intraperitoneal injection of pristane (2, 6, 10, 14-tetramethyl pentadecane) prior to the intraperitoneal inoculation of hybridoma cells. The optimal interval between priming and inoculation to maximise the amount of antibody produced and also result in a high percentage of responding mice, has been reported to range from 10 days (81) to 3-4 weeks (82).

3.8 Screening Assays

The assays used for both the evaluation of serum in the immunised animal and screening of the fusion products should be as similar as possible to the type of assay

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in which the McAb will ultimately be used (83). Apart from this the assay chosen should be sensitive enough to detect the concentrations of antibody found in culture supernatant (<1 μg/ml). It should also be simple and rapid, such that large numbers of samples can be handled at one time.

If a fusion yields large numbers of positive cultures it may be necessary to assign priorities. This can be achieved by screening not only for the presence of antibody, but also for particular specificities or avidities. It should also be noted that coupling an antigen to solid-phase or labelling with enzymes or radioactive molecules may destroy or make inaccessible particular epitopes. Whereas this may not be noticed with a polyclonal antiserum, it may lead to the preferential selection of McAbs of particular specificities which may not necessarily correlate with the desired specificities.

3.9 Advantages and Disadvantages of Monoclonal Antibodies

Advantages

Once a stable cell line has been established it is possible to produce a continuous supply of a homogeneous reagent with defined characteristics. Unlike polyclonal antiserum which contain 15-20 mg/ml immunoglobulin of which as little as 1% may be specific for the immunising antigen (74), McAbs can be produced *in vitro* or *in vivo* such that the majority of immunoglobulin present is specific for the immunising antigen. This usually circumvents the requirement for affinity purification of antibody prior to labelling for use in immunometric assay techniques.

The specificity of McAbs ie, they react with a single epitope on an antigen, can be advantageous in assays where the measurement of a particular antigen is complicated by the presence of closely related molecules.

Disadvantages

Monoclonal antibodies tend to be low avidity compared to polyclonal antibodies. The production of such antibodies reflects their preponderance in the serum of immunised animals. Also, the avidity of polyclonal antisera in part depends on co-operative effects between antibodies of differing specificities and as such is greater than would be anticipated from the individual avidity of each antibody present.

In certain situations the specificity of McAbs may not be advantageous. This arises from the same property that makes them particularly useful, ie, that they can distinguish between different forms of the same antigen. Thus problems may occur when attempting to measure the concentration of molecules which are genetically polymorphic or heterogeneous.

Finally, individual McAbs may differ markedly in their stability to procedures such as freezing and thawing, in their susceptibility to changes dependent on procedures such as labelling with enzymes or radioactive molecules and coupling to solid-phase, and the optimum conditions for their use.

Conclusion

The use of McAbs in immunoassay techniques does offer distinct advantages over polyclonal antibodies in particular situations. However, it should be realised that they do have their disadvantages and therefore their use should be viewed as complementary to, rather than as a replacement for, polyclonal antisera.

4 IMMUNOASSAY METHODOLOGY

4.1 Introduction

The basic principle underlying immunoassay techniques is the Law of Mass Action. Assuming a reversible reaction, this can be represented by the equation:

$$Ag + Ab \underset{k_d}{\overset{k_a}{\rightleftharpoons}} Ag\,Ab \qquad ...\ (1)$$
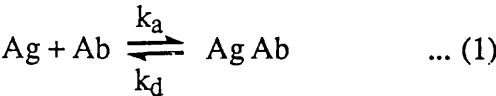
where k_a and k_d are the association and dissociation constants respectively. The equilibrium constant K can then be expressed as:

$$K_{eq} = \frac{k_a}{k_d} = \frac{[AgAb]}{[Ag][Ab]} \qquad ...\ (2)$$

where [Ag], [Ab] and [AgAb] represent the concentrations of free and complexed antigen and antibody in the final equilibrium mixture (84).

From equation (1) it can be seen that if either of the two reactants is present at limiting concentration then the other distributes between the free and bound states. If the non-limiting reactant is labelled, its distribution can be measured. Radioimmunoassay (6) employs radioactively labelled antigens whereas the immunoradiometric (9) and two-site immunoradiometric assays (85) employ radioactively labelled antibodies. A simplified representation of the three assay techniques is shown in Figure 1.2.

4.2 Radioimmunoassay

Purified antigen is required for radioactive labelling, which when labelled, should be recognised by the antibody as identical or very similar to the analyte. Labelled antigen is incubated with a limiting amount of antibody, such that at equilibrium all the antibody, but only a percentage of the labelled antigen, reacts to form the bound

A. RADIOIMMUNOASSAY

$$Ag + {}^*Ag + Ab \rightleftharpoons Ag\,Ab + {}^*Ag\,Ab$$

B. IMMUNORADIOMETRIC ASSAY

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

C. TWO-SITE IMMUNORADIOMETRIC ASSAY

$$Ag + Ab^* + SP\text{-}Ab \rightleftharpoons SP\text{-}Ab\,Ag\,Ab^*$$
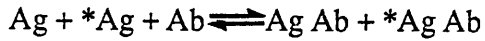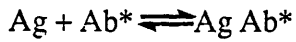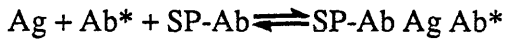
Figure 1.2

A simplified representation of the three immunoassay techniques employing radioactive labels.

Ag = antigen; *Ag = iodinated antigen; Ab = antibody; Ab* = iodinated antibody;

SP-Ab = solid-phase coupled antibody.

fraction. Addition of unlabelled antigen reduces the proportion of antibody-bound labelled antigen in a dose dependent fashion. Thus separation of the antibody-bound from the free labelled antigen, followed by measurement of the bound fraction, yields a dose-response curve where the percentage bound has an inverse relationship to the amount of unlabelled antigen added (Figure 1.3A).

The sensitivity of an RIA is governed by four factors (86):

(i)   The equilibrium constant of the antigen-antibody interaction ie, the avidity of the antiserum.

(ii)   The experimental error involved in the measurement of antibody-bound and free antigen.

(iii)   The specific activity of the labelled antigen.

(iv)   The misclassification of the bound and free antigen.

In practice, factors (i) and (ii) impose the major constraints on assay sensitivity (86) while factor (iv) is a major cause of experimental imprecision and bias (87).

4.3 Immunoradiometric Assay

The immunoradiometric assay differs from the radioimmunoassay in that it employs labelled antibodies rather than antigens, and requires the use of excess rather than limiting amounts of antibody.

Antigen is incubated with excess labelled antibody such that at equilibrium, with a high avidity antibody, most of the antigen, but only a percentage of the labelled antibody, reacts to form the bound fraction. Free labelled antibody is separated by the addition of solid-phase coupled antigen. The amount of labelled antibody bound to antigen (not coupled to solid-phase) is directly proportional to the amount of antigen present (Figure 1.3B).
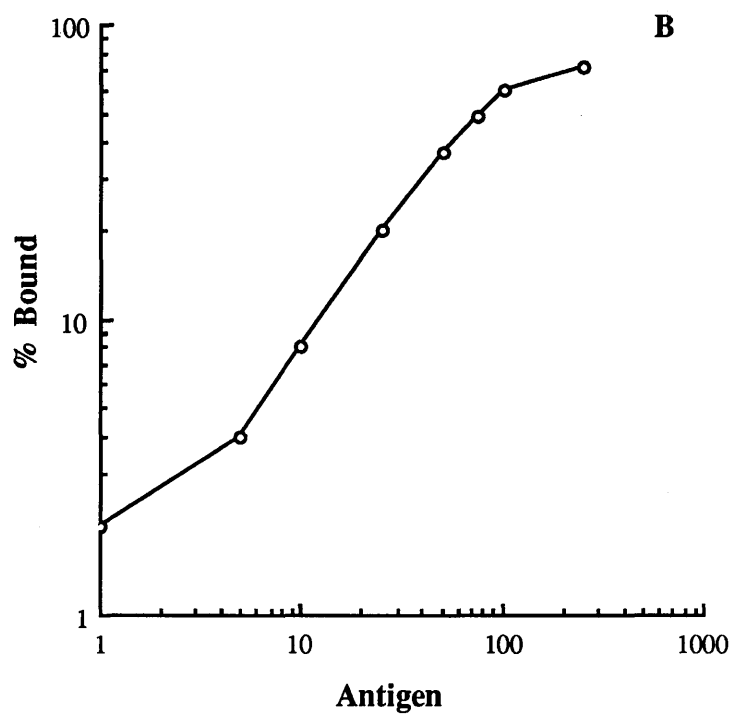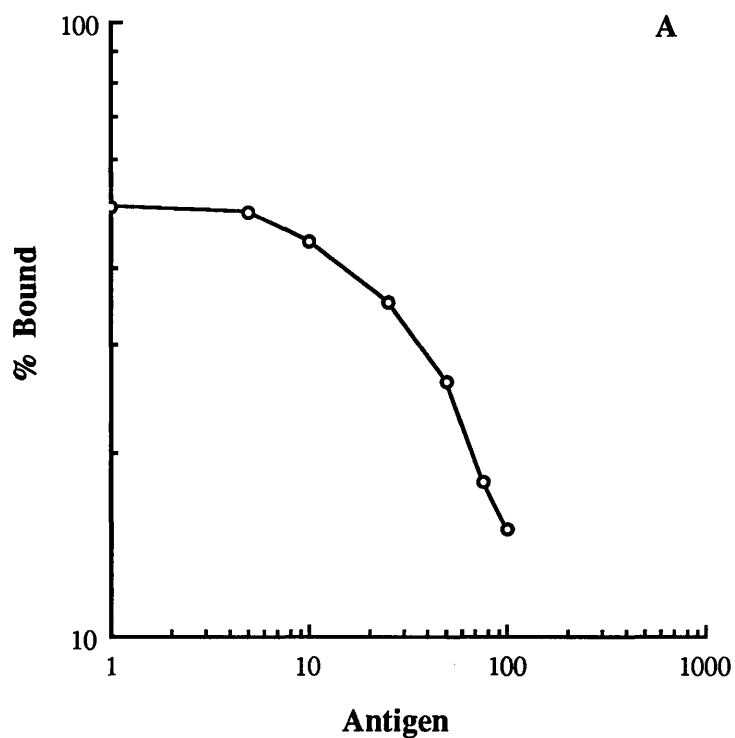
Figure 1.3

Representative dose-response curves for a radioimmunoassay (A) and an immunoradiometric assay (B).

It has been suggested that the potential sensitivity of IRMA's is less dependent on the antibody avidity than RIA (86) but it has been shown, with the use of monoclonal antibodies, that while this is true, the avidity of the antibody is still a major determinant in the final sensitivity achieved (88, 89). The ultimate sensitivity of IRMA's however, derives more from the specific activity of the moiety used for labelling the antibody and the zero dose binding levels which can be achieved (86).

The disadvantages of this technique are the requirement for large amounts of antigen, and specific antibody which requires immunoaffinity purification (90, 91). This technique also fails to achieve the low zero dose binding levels that are required to maximise the sensitivity of IRMA's (86). This is due to the presence, in any labelled preparation, of radioactive material which is not immunoreactive, and thus in this case cannot be removed by the solid-phase coupled antigen (90). This unbindable fraction of the labelled antibody increases the zero dose binding count against which specific antibody binding is measured thus limiting the sensitivity which can be obtained.

4.4 Two-Site Immunoradiometric Assay

This modification of the conventional IRMA, described above, replaces the solid-phase coupled antigen with a solid-phase coupled antibody capable of binding to the antigen to be measured simultaneously with the labelled antibody. As with the conventional IRMA, the amount of labelled antibody bound to the antigen is directly proportional to the amount of antigen present (Figure 1.3B).

IRMA techniques have a number of advantages compared with RIA. They are faster, have a wider working range and are potentially more sensitive (74). The two-site IRMA's also have the potential for improved specificity due to the recognition by antibodies of two distinct epitopes on the molecule (90).

While the two-site IRMA still requires large amounts of antibody, one of which requires immunoaffinity purification, there is no longer the requirement for antigen in the separation step (90).

4.5 Polyclonal or Monoclonal Antibodies

Radioimmunoassay

While monoclonal antibodies offer the potential advantages of increased specificity and an unlimited supply of a standardised reagent (89) they are, in general, of lower avidity than the polyclonal antisera which can be raised to the same antigen (89, 92). Such antibodies produce RIA's of poor sensitivity. High avidity McAbs may approach but do not significantly improve on, the sensitivity of assays achieved with polyclonal antisera (89).

Two-Site Immunoradiometric Assay

The major contribution of McAbs has been in the wider application of the two-site IRMA and in the realisation of the full potential of such techniques (74). Until the introduction of McAbs, there was a lack of evidence to support the theoretical and practical advantages which had been proposed for IRMA techniques (88).

Polyclonal antisera have been employed to develop IRMA's for a number of hormones and proteins (93) but their application has been relatively restricted due to a number of factors (74). The main disadvantages of polyclonal antibodies have been the requirements for large quantities of immunoaffinity purified material, and for two antibodies capable of binding simultaneously to the antigen to be measured (polyclonal antisera usually exhibit substantial overlap in their epitope reactivity) (74).

Conclusion

While polyclonal antisera remain the reagents of choice for the majority of RIA's, the use of McAbs alone or in conjunction with polyclonal antisera have proved essential for the realisation of the advantages proposed for IRMA techniques.

5 OBJECTIVES

The objectives of this project were:

(i)   To raise high avidity, high titre polyclonal antisera to insulin.

(ii)   To raise high avidity, high titre polyclonal antisera to C-peptide.

(iii)   To produce high avidity monoclonal antibodies reactive with insulin, proinsulin and C-peptide.

(iv)   To develop a two-site IRMA for human insulin.

(v)   To develop a two-site IRMA for human proinsulin.

(vi)   To develop either an RIA or two-site IRMA for human C-peptide.

(vii)   To establish proinsulin concentrations under physiological conditions in the fasting state and during a variety of dynamic function tests.

(viii)   To investigate proinsulin concentrations under pathophysiological conditions in the fasting state and during a variety of dynamic function tests.

CHAPTER 2

MATERIALS AND METHODS

1 MATERIALS

All chemicals used were analytical grade unless otherwise stated.

Anti-immunoglobulin and animal sera	SAPU
BSA standard	Pierce Chemical Co.
Cell culture media and reagents	Flow Laboratories
Charcoal (activated)	Sigma Chemical Co.
DMSO	Sigma Chemical Co.
FCA, FIA	Difco Laboratories
Folin-Ciocalteu reagent	Sigma Chemical Co.
GelBond^R film	Sigma Chemical Co.
Glutaraldehyde (electron microscopy grade)	Sigma Chemical Co.
Insulin (various species)	Sigma Chemical Co.
Ovalbumin	Sigma Chemical Co.
PEG 1500	BDH
Proinsulin (intact and intermediates)	Gift from Dr B.H.Frank, Eli Lilly Research Laboratories
Rosil^R (C18, 3 μm)	Alltech Associates/Applied Science
Sephacryl^R S300 (unmodified)	Pharmacia Fine Chemicals
Sephadex^R G50 and G75	Pharmacia Fine Chemicals
Sepharose^R 6B and CL-4B	Pharmacia Fine Chemicals
Strain 2 guinea-pigs	Animal Unit, Southampton General Hospital
TSK DEAE 5 PW column	Anachem

2 BUFFERS

2.1 Charcoal Reagent

Used as described in Section 5.5 of this chapter.

Buffer:	$NaH_2PO_4 . 2H_2O$	3.1 g
	Thiomersal	0.12 g
	BSA	2.5 g

The above chemicals were dissolved in less than 500 ml distilled water and the pH adjusted to 7.4 prior to the addition of further distilled water to give a final volume of 500 ml.

Charcoal reagent:	10 ml buffer
	10 ml donkey serum
	1 g activated charcoal

The above reagents were mixed together and stirred for at least 20 minutes before use.

2.2 EPPS Buffer

Assay buffer for immunoradiometric assays.

EPPS	25.23 g
Sodium azide	0.5 g
Sheep serum	5 ml
Tween 20	2 ml

The above reagents were dissolved in less than 1 litre distilled water and the pH adjusted to 8.0 prior to the addition of further distilled water to give a final volume of 1 litre. Due to problems with heterophilic antibodies (Chapter 6, Section 1.5) it was subsequently necessary to add 1% (v/v) mouse serum to this buffer.

2.3 1% TEATFA pH 3.0

Column buffer for reverse-phase HPLC of ^{125}I-proinsulin (Section 5.2 of this chapter).

A 1% (v/v) solution of trifluoroacetic acid (TFA) in distilled water was prepared and the pH adjusted to 3.0 with triethylamine (TEA).

3. GENERAL METHODS

3.1 Protein Estimation

A modification of the Lowry protein assay (94) was used to measure protein in solution or coupled to solid-phase.

Reagents

(i)   Alkaline copper reagent was prepared by mixing 10 g sodium carbonate in 50 ml 1 mol/l sodium hydroxide with 100 mg sodium potassium tartrate dissolved in 2-3 ml distilled water and 50 mg cupric sulphate similarly dissolved in 2-3 ml distilled water. The volume was then adjusted to 100 ml with distilled water.

(ii)   Stock Folin-Ciocalteu reagent was diluted 1:25 with distilled water to provide a working solution.

(iii)   Bovine serum albumin (BSA) standard (Pierce) was diluted in the appropriate buffer to give standards of 0.5, 0.4, 0.3, 0.2 and 0.1 mg/ml.

Method

Protein estimations were carried out in duplicate by mixing 200 µl protein standard or sample with 200 µl alkaline copper reagent, vortexing and incubating at room temperature for 10 minutes. 1 ml working strength Folin-Ciocalteu reagent was then

added, the tubes vortexed and placed in a water bath at 55°C for 5 minutes, after which rapid cooling was achieved by placing the tests in cold water for 1-2 minutes. The absorbance was measured at a wavelength of 650 nm within 30 minutes. A standard curve was constructed and used to determine the protein content of the samples.

3.2 Immunogen Preparation

Immunogens were prepared either as water-in-oil (95) or multiple (96) emulsions.

Water-in-Oil Emulsions

One volume antigen and nine volumes adjuvant, in separate glass syringes, were emulsified by repeated passage through a 19 g needle fitted with twin Luer hubs.

Multiple Emulsions

Antigen and adjuvant (1:1, v/v) were homogenised, using a Silverston laboratory mixer, to give a water-in-oil emulsion. An equal volume of 2% Tween 80/0.9% saline was then added and the mixture rehomogenised to form a multiple emulsion.

3.3 Serum Separation

Human blood was allowed to clot for 30 minutes at room temperature before centrifugation (1000 g, 10 min, 20°C). Blood from all other species was incubated either overnight at 4°C or 2 hours at 37°C prior to centrifugation as above. The serum was then decanted, divided into aliquots where appropriate and stored at -20°C.

3.4 Conjugation of Insulin to Carrier Proteins

Insulin was conjugated to either human immunoglobulin G (IgG) or ovalbumin using a two-step glutaraldehyde technique (97), followed by size-exclusion chromatography on Sephadex G75 to remove free insulin.

Method

Porcine insulin (2 mg in 200 μl 0.01 mol/l hydrochloric acid) was diluted with 750 μl 6.7 mmol/l phosphate buffered saline (PBS) pH 7.3 prior to the addition of glutaraldehyde to a final concentration of 1.25%. The mixture was then rolled overnight at room temperature. The activated insulin was diluted 1:4 with 0.1 mol/l sodium carbonate/0.5 mol/l sodium chloride pH 9.2 (carbonate buffer) and dialysed against the same overnight at room temperature. Human IgG (10 mg) or ovalbumin (6 mg) in carbonate buffer (1ml) were then added to the activated insulin and the mixture rolled overnight at room temperature. The reaction was terminated by the addition of 1 ml 1 mol/l lysine in carbonate buffer.

Unconjugated insulin was removed by size-exclusion chromatography on a Sephadex G75 column (30 x 1 cm) equilibrated and eluted in carbonate buffer. The conjugate eluted first followed by unreacted insulin. The conjugate was assessed for human IgG or ovalbumin reactivity by immunodiffusion (Section 4.1 of this chapter) using the appropriate antisera and the quantities of conjugated and unconjugated insulin were measured by RIA. The ratio of insulin:carrier protein was calculated assuming that all the carrier protein was conjugated.

3.5 Ion-Exchange HPLC of Murine Ascitic Fluid

The purity of McAbs obtained from ascitic fluid by caprylic acid fractionation was assessed by anion-exchange HPLC on a TSK DEAE 5PW column (7.5 x 0.75 cm). The column was equilibrated in 20 mmol/l Tris-HCl pH 7.7 and samples were dialysed against the same before application to the column. Elution was performed at a flow rate of 1ml/min using a gradient of 0.5 mol/l sodium chloride/20 mmol/l Tris-HCl pH 7.7 from 0-30% over 40 min, increasing to 100% from 40-50 min and returning to 0% at 60 min. Eluted protein was measured using a flow-through UV absorbance monitor (280 nm) which was connected to a chart recorder.

4. IMMUNOCHEMICAL METHODS

4.1 Immunodiffusion

Immunodiffusion was performed according to the method of Ouchterlony (98). Agarose (1% w/v) in Tris-HCl (0.05 mol/l, pH 8.6) containing 1% w/v PEG 4000 was melted, poured into plastic Petri dishes to a depth of 2-3 mm and left to set at room temperature. A pattern consisting of one central well and six peripheral wells was cut out of the gel and the wells filled with antigen or antiserum as appropriate. The plates were then left in a moist chamber in the dark for 24-48 hours before examination.

To stain, gels were washed for a minimum of 48 hours in 0.15 mol/l sodium chloride, press-dried on to GelBond^R film, air or oven-dried and stained with Crowle's double stain (99). To destain, the plates were soaked in 3% v/v acetic acid until all excess stain was removed, washed under running tap water and air or oven-dried.

4.2 Immunoadsorbent Preparation

Immunoadsorbents were prepared according to the method of Wright & Hunter (100). The required volume of Sephacryl^R S300 (unmodified), measured after the slurry had settled out in a measuring cylinder, was washed thoroughly with distilled water before resuspension in 4.5 volumes 5 mmol/l sodium meta-periodate / 0.1 mol/l sodium acetate pH 5.0 and mixed on a roller for one hour at room temperature in a closed vessel. Glycerol (10% v/v) was then added and the gel mixed for a further hour. The activated Sephacryl^R was washed with either excess 0.1 mol/l sodium bicarbonate pH 9.0 if coupling was to proceed immediately, or excess 0.02 mol/l sodium phosphate pH 6.2 containing 0.1% sodium azide if the activated gel was to be stored before use.

Protein (10 mg/ml in 0.1 mol/l carbonate pH 9.0) and activated gel were mixed at equivalent volumes for 16 hours at room temperature. Uncoupled protein was removed by filtration at negative pressure on a sintered glass funnel and kept for future use. The immunoadsorbent was resuspended in two volumes 6.7 mmol/l PBS pH 7.3 and allowed to settle for 30 minutes before decanting the supernatant to remove fines. The solid-phase was resuspended in a final volume seven times that of the settled gel and solid sodium borohydride added (132 mmol/l). After 30 minutes the gel was transferred to a sintered glass funnel and washed with PBS pH 7.3 followed by 0.02 mol/l sodium phosphate pH 6.2 containing 0.1% sodium azide. The solid-phase was stored in the latter buffer at 4°C.

4.3 Immunoglobulin Fractionation

The IgG fraction of antisera and ascitic fluid from all species, except guinea-pig, was prepared by caprylic acid precipitation (101). Guinea-pig IgG was isolated by affinity chromatography using anti-guinea-pig IgG coupled to Sephacryl S300 (Section 4.2 of this chapter). Chicken egg yolk IgG was purified according to the method of Jensenius et al (102).

Caprylic Acid Precipitation

One volume serum was mixed with two volumes 0.06 mol/l sodium acetate pH 4.0 and 7.5 ml caprylic acid added for every 100 ml original serum volume. The caprylic acid was added dropwise with vigorous stirring and the preparation mixed for 30 minutes before centrifugation (1000 g, 30 min, 20°C). The supernatant was filtered through Whatman grade 4, qualitative filter paper and dialysed against several changes of a buffer appropriate to further studies. The resulting IgG fraction was concentrated by ultrafiltration in an Amicon stirred cell using a PM30 membrane.

Affinity Chromatography

Antiserum and solid-phase coupled anti-guinea-pig IgG (v/v) were mixed for 2 hours at room temperature. The slurry was then poured into a column and unbound protein washed through with 0.02 mol/l sodium phosphate pH 6.2, followed by elution of non-specifically bound protein with 2 mol/l sodium chloride in the same buffer. Guinea-pig IgG was eluted with 0.1 mol/l glycine pH 2.6 and collected in an equal volume of 1 mol/l sodium phosphate pH 6.2. Protein was concentrated by ultrafiltration as described above.

Purification of Egg Yolk IgG

The egg yolk was separated from the white, the membrane cut open and the yolk poured into a measuring cylinder. Nine volumes of distilled water were added and after mixing, the pH of the diluted egg yolk was adjusted to 7 with 0.1 mol/l sodium hydroxide prior to freezing at -20°C. The egg yolk was then thawed at 37°C and precipitated lipid removed by centrifugation (1000 g, 30 min, 20°C). The IgG was precipitated by the addition of solid ammonium sulphate to a concentration of 2 mol/l and the mixture stirred for 30 minutes followed by centrifugation (1000 g, 30 min, 20°C) to recover the IgG. The precipitate (IgG) was dissolved in 0.5 volumes 6.7 mmol/l PBS pH 7.3 and stored at -20°C until required.

5 IMMUNOASSAY TECHNIQUES

5.1 Preparation of Hormone-Free Serum

The relevant hormones (ie, proinsulin, insulin and C-peptide) were removed from pooled normal human serum by absorption with agarose coated charcoal.

Charcoal (activated Norit PN5), was coated with agarose by heating a 5% (w/v) agarose solution to 70°C and adding 20 g activated charcoal per 100 ml agarose. The slurry was allowed to cool to 50°C before being poured into 200 ml acetone, in

a fume hood, with vigorous stirring. The granular precipitate formed was filtered through Whatman grade 4, qualitative filter paper and air-dried overnight.

Serum was rendered hormone-free by mixing 10 g agarose-coated charcoal with 100 ml serum overnight at room temperature. Hormone-free serum was recovered by centrifugation (1600 g, 15 min, 20°C) and stored in suitable aliquots at -20°C.

5.2 Protein Iodination

Antigens and antibodies were labelled with ^{125}I using a solid-phase lactoperoxidase technique (103). Iodinated protein was separated from unreacted iodide by either gel filtration chromatography or reverse-phase high performance liquid chromatography.

Iodination Protocol

Antigens (5-10 μg in 10 μl) or antibodies (20-40 μg in 10 μl) were iodinated by the addition of:

10 μl	0.5 mol/l sodium phosphate pH 7.4
10 μl	(excess) solid-phase lactoperoxidase (prepared locally)
5 or 10 μl	^{125}I, carrier-free (18.5 or 37 MBq respectively)
10 μl	hydrogen peroxide (30% w/v stock diluted 1:20,000 with distilled water)

The mixture was vortexed and incubated at room temperature for 30 minutes, with a second 10 μl aliquot of hydrogen peroxide being added after the first 15 minutes. The reaction was terminated by the addition of 200 μl column buffer.

Gel Filtration Chromatography

Labelled antibodies and antigens were purified on Sepharose^R 6B (25 x 1.5 cm) or Sephadex^R G50 (30 x 1 cm) columns respectively. Both columns were equilibrated and eluted with 0.1 mol/l sodium phosphate/0.154 mol/l sodium chloride pH 7.4

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containing 1% BSA and 0.1% sodium azide, at a flow rate of 10 ml/hour. 1 ml fractions were collected and radioactivity measured in a gamma counter.

Small amounts of aggregated material eluted in the column void volume, followed by iodinated protein and then unreacted iodide (Figure 2.1). After counting, the relevant fractions were pooled, diluted in column buffer, divided into aliquots and stored at 4°C or -20°C.

Reverse-Phase HPLC

Proinsulin was the only protein which required this method of purification to provide a suitable tracer.

After the iodination reaction had been terminated, the mixture was centrifuged (1000 g, 5 min, 20°C), to remove the solid-phase lactoperoxidase. A stainless steel column (4.6 x 250 mm) packed with Rosil^R (C18, 3 μm) was equilibrated and eluted with 1% TEATFA pH 3.0 (Section 2.3 of this chapter) containing 34% acetonitrile, at a flow rate of 1 ml/minute and a back pressure of 3500 psi. 100 μl iodination mixture was injected on to the column per run with 1 ml fractions being collected and counted on a gamma counter.

After each run the column was regenerated by running 1% TEATFA pH 3.0 containing 95% acetonitrile until a stable baseline was achieved. Re-equilibration was carried out by washing through with at least three column volumes equilibration/elution buffer.

The last major peak on the chromatogram (Figure 2.2, peak 6) was pooled, diluted with an equal volume of 0.1 mol/l sodium phosphate/0.154 mol/l sodium chloride pH 7.4 containing 1% BSA and freeze-dried to remove the acetonitrile and trifluoroacetic acid. The tracer was reconstituted to the original pool volume with distilled water, divided into aliquots and stored at -20°C.
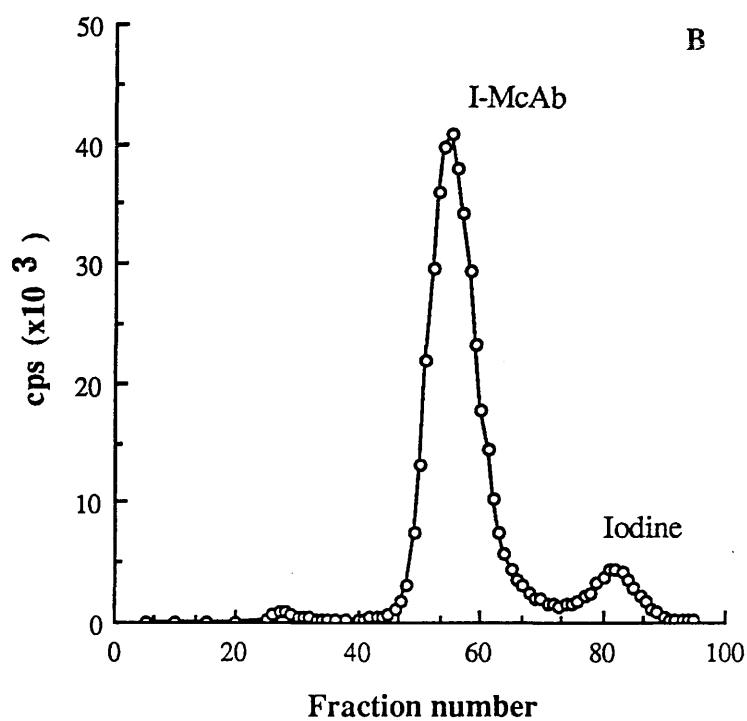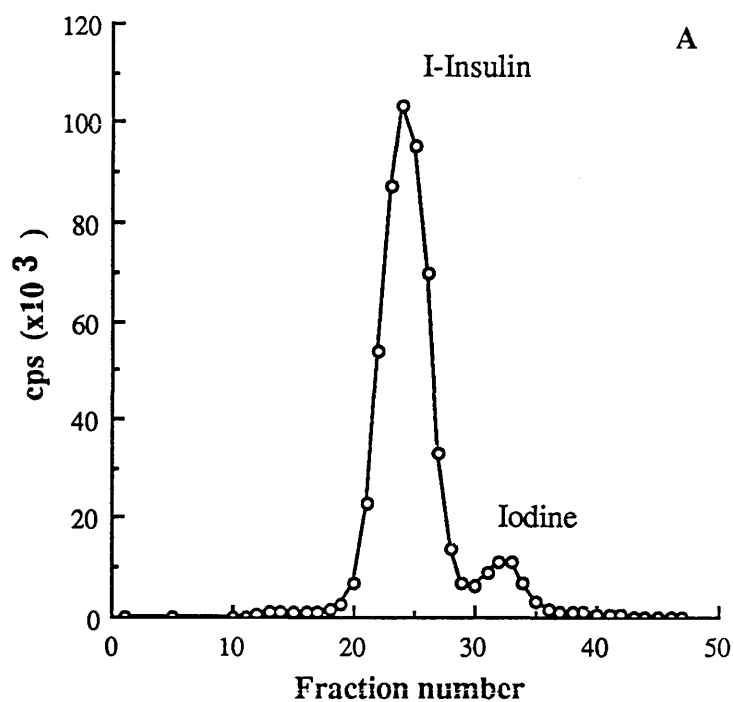
Figure 2.1

Representative elution profiles for ^{125}I-insulin (A) and ^{125}I-monoclonal antibody (B).
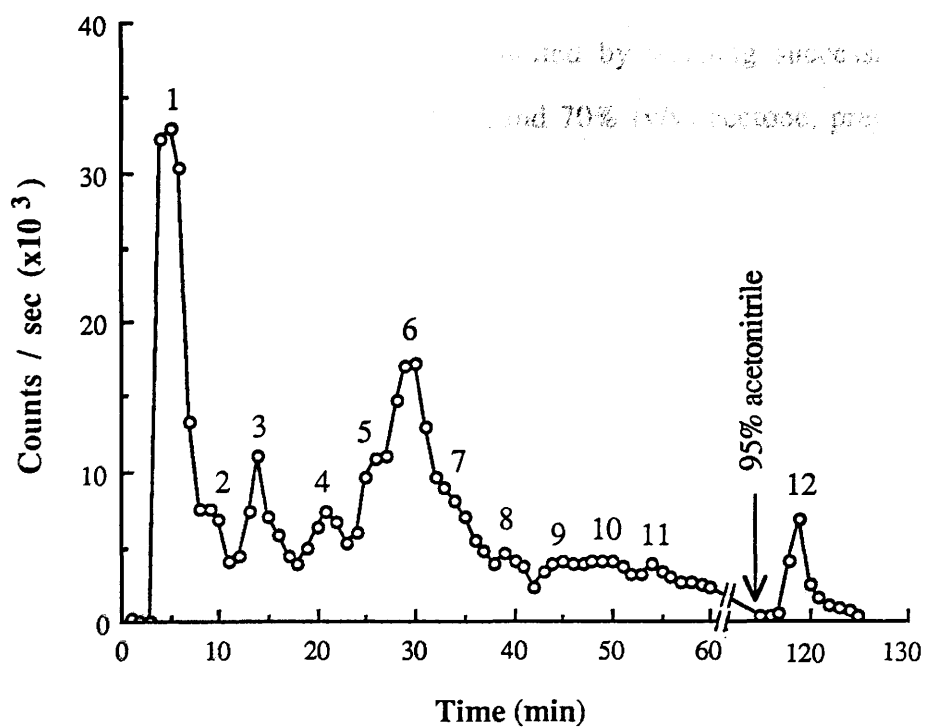
Figure 2.2

Representative elution profile for ^{125}I-proinsulin prepared by reverse-phase HPLC.

5.3 Solid-Phase Preparation

Sepharose^R CL-4B was activated by a carbodiimide technique (104) for use in RIA and IRMA.

Sepharose^R CL-4B was calibrated by pouring the gel into a measuring cylinder, allowing it to settle overnight and adding sufficient distilled water to give a 50% v/v suspension.

One volume calibrated gel was dehydrated by washing successively with 2.5 volumes distilled water; 30%, 50% and 70% (v/v) acetone, prepared with distilled water; 100% acetone in a sintered glass filter funnel under negative pressure. The gel was not allowed to dry out at any stage as this resulted in a tendency to flocculate.

The dehydrated Sepharose^R was transferred, in acetone, to a conical flask and further acetone added to make up the original calibrated volume. Activation was accomplished by adding carbonyldiimidazole to 0.15 mol/l, sealing the flask and mixing the contents for one hour. Activated gel was rehydrated in the same manner as before by successive washes with 2.5 volumes 100% acetone; 70%, 50% and 30% (v/v) acetone, prepared with distilled water; 0.1 mol/l EPPS pH 8.0 (coupling buffer). The gel was transferred to a polycarbonate bottle containing the protein to be coupled (in coupling buffer) and further coupling buffer added to give the original calibrated volume. Coupling was achieved by gentle mixing for 18 to 24 hours at room temperature.

Unbound protein was recovered by centrifugation (1200 g, 15 min, 20°C) and used again for future solid-phase preparations. The gel was then extensively washed with various buffers (v/v) and recovered by centrifugation (1200 g, 15 min, 20°C) between each wash cycle as follows:

(1) 0.5 mol/l sodium bicarbonate pH 8.0, rotate 20 minutes; (2) repeat; (3) 0.1 mol/l sodium acetate pH 4.0, rotate 60 minutes; (4) 0.1 mol/l sodium acetate pH 4.0, rotate 16-20 hours; (5) 0.154 mol/l sodium chloride, rotate 20 minutes; (6) repeat.

The solid-phase was then adjusted to the original calibrated volume with 0.154 mol/l sodium chloride containing 0.1% sodium azide and stored at 4°C.

Sepharose^R coupled anti-immunoglobulin was prepared using 25 ml neat antiserum per 200 ml calibrated gel, whereas primary antibodies were coupled as IgG fractions (Section 4.3). Polyclonal anti-insulin and monoclonal anti-proinsulin were coupled at ratios of 0.05 mg and 0.024 mg IgG per mg gel respectively.

5.4 Evaluation of Anti-Immunoglobulin Solid-Phase for RIA

Various solid-phase coupled anti-immunoglobulins (ie, anti-rabbit IgG, anti-mouse-γ-globulin, anti-guinea-pig IgG and anti-sheep/goat IgG) were evaluated for use as separating reagents in RIA utilising primary antibodies to a variety of antigens raised in the appropriate species. The first incubation conditions were held constant (ie, dilution of antiserum, mass of label, incubation time and temperature) while the effects of varying the mass of solid-phase coupled second antibody added and the incubation time were observed. The solid-phase was added at 0.25, 0.5, 1.0 and 2.0 mg/tube while the incubation times were 15, 30, 60, 90 and 120 minutes.

Usually a 60 minute incubation with 1 mg solid-phase/tube gave optimum binding as can be seen in Figure 2.3 which shows the pattern of results normally obtained.

5.5 RIA for Antibody Detection and Evaluation

Polyclonal and monoclonal antibody production were monitored by RIA. A solid-phase second antibody separation technique was used except for chicken and donkey test bleed evaluation which employed a charcoal separation technique.
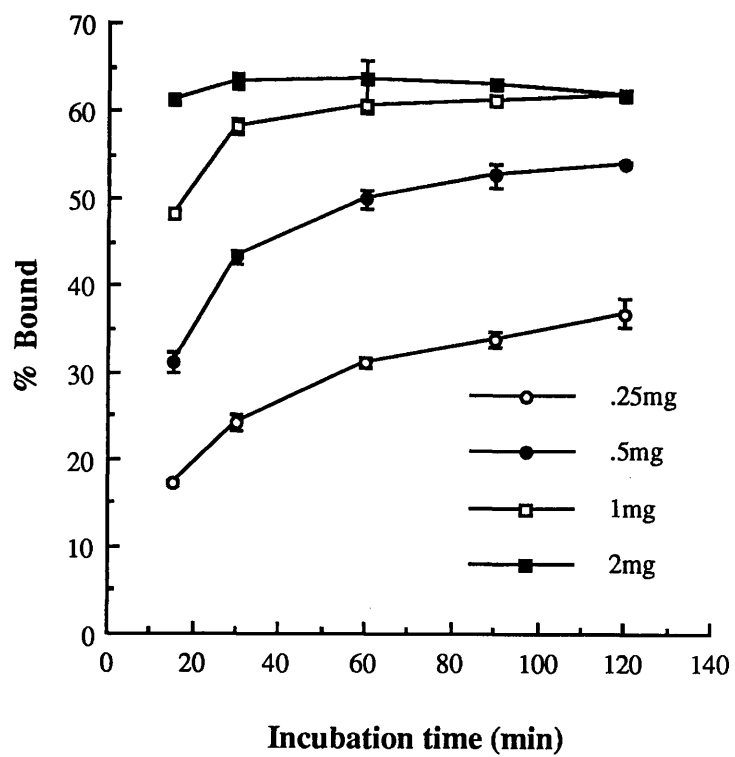
Figure 2.3

Determination of optimum mass and incubation time for Sepharose^R CL-4B coupled second antibody.

Polyclonal Antiserum Evaluation

Antibody dilution curves were constructed for all test bleeds and donations (Figure 2.4). The test system was as outlined below:

100 µl hormone-free serum

+

100 µl antiserum (various dilutions)

+

100 µl ^{125}I-antigen (5 000 or 10 000 cpm)

incubated (3 h, room temperature)

All species except chicken and donkeys	**Chicken and donkeys**
Added 200 µl (1 mg) Sepharose^R CL-4B coupled anti-immunoglobulin	Added 400 µl charcoal reagent and mixed
incubated (1 h, continuous mixing)	incubated (10 min, room temperature)
Added 3 ml 0.2% Tween 20/ 0.9% sodium chloride	Centrifuged (1000 g, 4°C, 30 min)
Centrifuged (1000 g, 20°C, 1 min) Repeated twice	Removed 400 µl supernatant into clean tubes
Aspirated supernatant	

Counted on gamma counter

Figure 2.4

A representative antiserum dilution curve.

Monoclonal Antibody Detection

Monoclonal antibodies were detected in culture supernatants according to the following protocol:

50 μl culture supernatant

+

200 μl ^{125}I-antigen (10 000 cpm)

⎮ incubated (overnight, room temperature)
▼

Added 200 μl (1 mg) Sepharose^R CL-4B coupled anti-mouse-γ-globulin

⎮ incubated (1 h, continuous mixing)
▼

Washed solid-phase as described for polyclonal antibody evaluation

⎮
▼

Counted on gamma counter

Culture supernatants were considered to be 'antibody-positive' if they bound more than 10% of the added label.

Ascitic fluid containing monoclonal antibody was evaluated either as above or as described for polyclonal antisera except that hormone-free serum was not added to the primary incubation.

5.6 Displacement Studies

Single Dose Displacement Curves

Single dose displacement curves were constructed for both polyclonal and monoclonal antibodies prior to Scatchard analysis and specificity determinations.

The method was as outlined below:

100 µl antibody (various dilutions)

+

100 µl buffer **or** standard (at appropriate concentration)

+

100 µl ^{125}I-antigen (5 000 or 10 000 cpm)

| incubated (3 h, room temperature)
▼

Added 200 µl (1 mg) Sepharose^R CL-4B coupled anti-immunoglobulin

| incubated (1 h, continuous mixing)
▼

Washed as described for polyclonal antibody evaluation

▼

Counted on gamma counter.

Results were calculated as described for polyclonal antiserum evaluation (Section 5.5) and antibody dilution curves in the presence and absence of cold antigen plotted together (Figure 2.5).

Further studies were performed using the dilution of antibody which exhibited maximum displacement with between 30 and 50% of the added label bound in the absence of standard (i.e. $50\% > B_0 > 30\%$).

Specificity Determination

The specificity of monoclonal antibodies reactive to insulin was determined using various species insulins while those reactive to proinsulin and C-peptide were evaluated with different forms of split and des-amino proinsulins. The same general assay protocol was used for all specificity determinations and is described over.
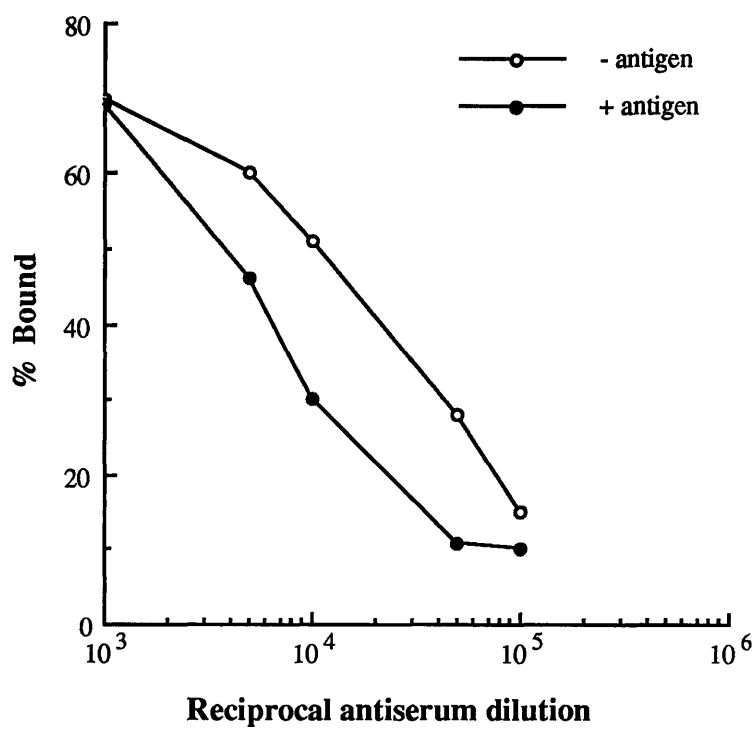
Figure 2.5

A representative single dose displacement curve.

100 μl antibody (B_0 = 30-50%)

+

100 μl buffer **or** standard (range of concentrations)

+

100 μl ^{125}I-antigen (5 000 or 10 000 cpm)
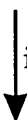
incubated (3 h, room temperature)

Solid-phase added, incubated, washed and counted
as described in previous sections.

Results were calculated in terms of label bound in the presence of standard
expressed as a percentage of label bound in the absence of standard (% B/B_0) and
plotted against the corresponding standard concentration. The displacement curves
generated for each antibody using various antigens were plotted as a single graph
(Chapter 4, Sections 2.2 and 2.3).

Determination of ^{125}I-Antigen Specific Activity

The specific activity of ^{125}I-labelled antigens was measured by self-displacement in
an RIA as described below:

100 μl antibody (B_0 = 30-50%)

+

100 μl ^{125}I-antigen (various masses)

OR

100 μl antigen (various concentrations)

+

100 μl ^{125}I-antigen (5 000 cpm)

incubated (3 h, room temperature)

Solid-phase added, incubated, washed and counted as described previously.

Results were calculated in terms of ^{125}I-antigen bound expressed as a percentage of the total amount added (% bound), and the displacement curves in the presence of labelled and unlabelled antigen plotted together. Representative curves for ^{125}I-labelled proinsulin and insulin are shown in Figure 2.6 parts A and B respectively. The doses of labelled and unlabelled antigen required to achieve half-maximal displacement were determined from these curves and the specific activity of the labelled antigen calculated. The specific activities of the ^{125}I-labelled proinsulin and insulin shown in Figure 2.6 were 11.8 MBq/μg and 5.3 MBq/μg respectively.

Scatchard Analysis

Scatchard analysis was performed according to the protocol outlined in the previous section with various masses of unlabelled antigen and a known mass of ^{125}I-labelled antigen added to a limiting amount of the antibody under evaluation. Results were plotted as the ratio of bound/free ^{125}I-antigen (B/F) at each antigen concentration ([H]). The avidity constant of the antibody was then calculated according to the method of Scatchard (105).

5.7 Immunoradiometric Assay of Human Proinsulin

Standards and serum samples (200 μl) were incubated with 200 μl Sepharose^R CL-4B coupled PH5/B5 (1 mg) for 30 minutes at room temperature. The solid-phase was then washed six times with assay buffer (0.1 mmol/l EPPS pH 8) prior to the addition of 100 μl ^{125}I-PH4/B1 (0.37-0.56 MBq/μg; 200 000 cpm) and a further incubation of 1 hour at room temperature. After a further six washes with 0.2% Tween 20/0.9% sodium chloride, the bound radioactivity was quantitated on a gamma counter. Both incubations were performed on an orbital mixer. Results were calculated as the amount of ^{125}I-PH4/B1 bound expressed as a percentage of the total amount added. A standard curve was constructed and used to determine the proinsulin concentration of the samples.
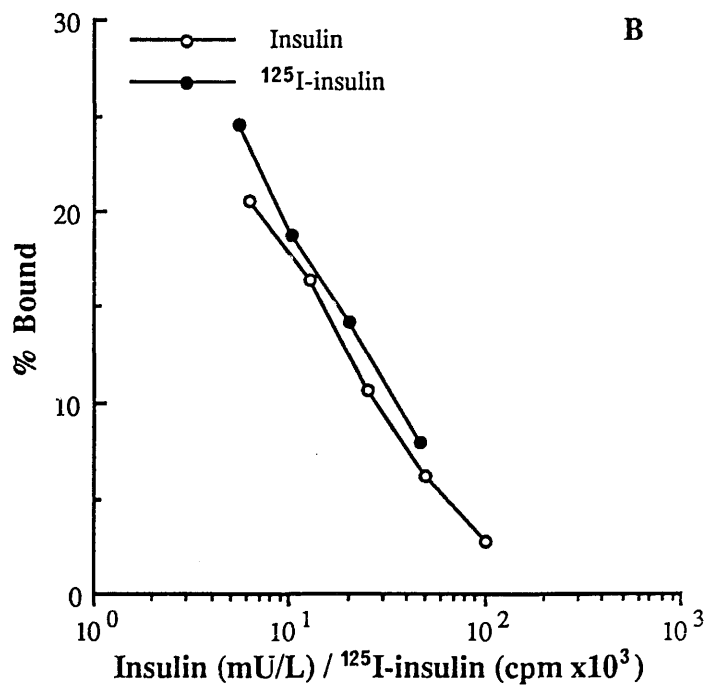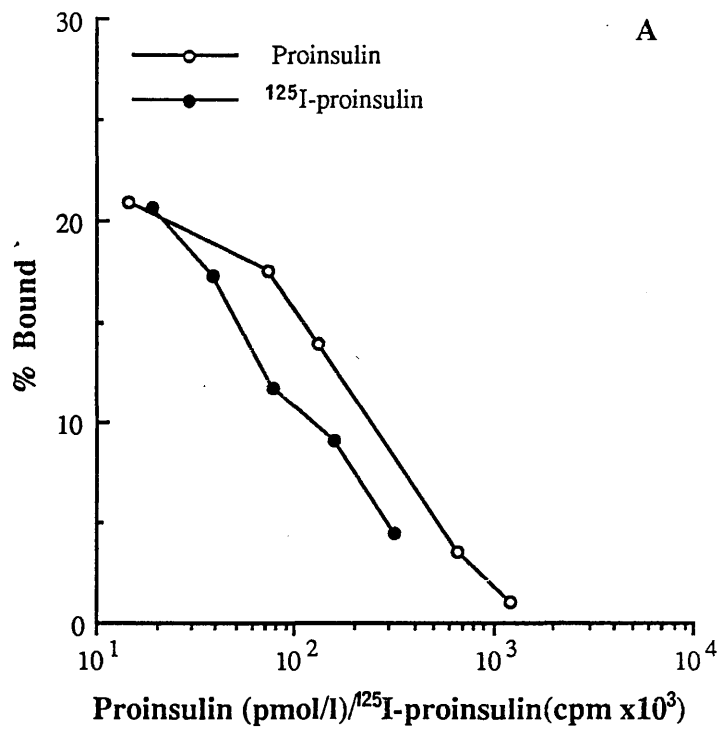
Figure 2.6

Determination of the specific activity of ^{125}I-proinsulin (A) and
[125]I-insulin (B).

5.8 Immunoradiometric Assay of Human Insulin

Standards and serum (100 µl) were incubated with 100 µl ^{125}I-ID1/C10 (0.37-0.56 MBq/µg; 200 000 cpm) for two hours at room temperature prior to the addition of 200 µl Sepharose^R CL-4B coupled guinea-pig anti-insulin and a further incubation of 1 hour at room temperature. The solid-phase was then washed four times with 0.2% Tween 20/0.9% saline and the bound radioactivity quantitated on a gamma counter. The incubation in which solid-phase was present was performed on an orbital mixer. Results were calculated as described in Section 5.7 of this chapter.

6 POLYCLONAL ANTIBODY PRODUCTION

6.1 General Guidelines

The following guidelines for polyclonal antibody production were followed except where stated otherwise.

(i)   A multiple emulsion technique was used for immunogen preparation.

(ii)   FCA was used for immunisation with FIA being used for subsequent boosts.

(iii)   Boosts were given at four-weekly intervals.

(iv)   Test bleeds and blood donations were taken seven days after boosting.

(v)   Test bleeds and blood donations were evaluated by RIA.

6.2 Guinea-Pigs

Strain 2 guinea-pigs over three months of age (ie, immunologically competent), were immunised and boosted with porcine insulin. Each animal received a dose of 50 µg in a final volume of 1 ml evenly distributed over two intramuscular and two subcutaneous sites. Test bleeds were obtained by spotting blood from an ear vein on to filter paper. The paper was air-dried, a 3 mm diameter circle punched out and eluted in 1 ml isotonic saline overnight at room temperature. The sample eluted from this size of disc was equivalent to 2 µl of serum (Chapter 3, Section 1.2).

When a serum titre similar to that of the commercially available Wellcome anti-insulin was achieved, animals were exsanguinated by cardiac puncture. Serum was separated (Section 3.3 of this chapter) and stored frozen for future use.

Ascitic fluid containing specific antibody was produced in two immunised animals by weekly intraperitoneal injection of either 0.5 ml FCA with 50 µg porcine insulin as a water-in-oil emulsion, or 0.5 ml FCA alone (106). Animals were weighed weekly and ascitic fluid collected, using a 5 ml syringe fitted with a 19 g needle, when present, (ie, an increase in weight suggested the presence of ascitic fluid). After ascites production had ceased, these guinea-pigs were also exsanguinated.

6.3 Rabbits

High responder semi-lop New Zealand White rabbits were immunised and boosted with a porcine insulin-human IgG conjugate (4:1). Each animal received 100 µg conjugate in a final volume of 1 ml, evenly distributed over two intramuscular and two subcutaneous sites. Test bleeds were taken from the marginal ear vein, processed as described in Section 3.3 of this chapter, and tested by immunodiffusion, to allow the detection of antibodies to human IgG, and by RIA to determine the specific anti-insulin titre.

6.4 Sheep

Sheep were immunised and boosted with either human insulin or a human insulin-ovalbumin conjugate (16:1). Each animal received 100 µg protein (5 ml), evenly distributed over six intramuscular and four subcutaneous sites. Test bleeds were taken from the jugular vein, the serum separated and tested by immunodiffusion to detect antibodies to ovalbumin, and by RIA to measure specific insulin antibody.

6.5 Mice

Mice were immunised and boosted intraperitoneally with 6-10 μg antigen prepared as a water-in-oil emulsion. Test bleeds were taken from the tail vein on to filter paper and prepared for assay as described in Section 6.2 of this chapter. When a stable serum titre was achieved, mice were rested until required as spleen donors for the production of monoclonal antibodies (Section 7.5 of this chapter). Three days prior to hybridisation, 6-10 μg antigen (in 0.1 ml saline) was administered intravenously via the tail vein.

6.6 Chickens

Chickens were immunised and boosted with porcine insulin prepared as a water-in-oil emulsion. Each animal received 50 μg in a final volume of 1 ml, evenly distributed between two sites in the breast muscle. Test bleeds were taken from a wing vein and the plasma separated and tested by RIA to determine the anti-insulin titre.

Chickens were injected and bled by Dr P Sharp, Institute of Animal Physiology and Genetics Research, Roslin, Edinburgh.

6.7 Donkeys

Donkeys were immunised and boosted initially with a human insulin-ovalbumin conjugate (16:1), followed by several booster injections with porcine insulin. Each animal received 100 μg protein in a final volume of 5 ml, evenly distributed over six intramuscular and four subcutaneous sites. Test bleeds were taken from the jugular vein and the serum separated and tested by RIA to determine the specific anti-insulin titre.

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7 MONOCLONAL ANTIBODY PRODUCTION

Murine McAbs were produced by the fusion (hybridisation) of spleen cells from immunised BALB/c mice with the myeloma cell line X63.Ag 8.653, according to local adaptations of the method of Kohler and Milstein (71).

7.1 Media

HEPES buffered RPMI 1640 was used throughout. Myeloma cells were grown in 1 x RPMI 1640 containing 20 mmol/l HEPES, 2 mmol/l glutamine, fungizone (2.5 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% foetal calf serum (FCS). Hybridomas were maintained in the same medium except that HAT (100, 0.4 and 16 μmol/l respectively) was added and the FCS was raised to 20%. Mouse peritoneal macrophages were used as feeder cells as required (e.g. fusion and clone plates).

7.2 Preparation of Mouse Peritoneal Macrophages

Peritoneal macrophages were collected from stock mice which had been killed by administration of excess diethyl ether. This was the method used routinely for killing mice.

The peritoneal cavity of each mouse was rinsed with 5 ml 1 x RPMI containing 20 mmol/l HEPES (referred to as medium in future), using a syringe and 19 g needle. Washes from individual mice were pooled, centrifuged (800 g, 10 min, 20°C) and the pellet resuspended in complete medium (ie, medium containing glutamine, fungizone, penicillin/streptomycin, FCS and HAT) at 3×10^4 cells/ml.

Unless otherwise stated, cells were always centrifuged at 800 g for 10 minutes at 20°C.

7.3 Maintenance of Myeloma Cells

The murine myeloma cell line X63. Ag 8.653 was maintained in logarithmic growth at a viability of greater than 95% for a minimum of 7 days before hybridisation. Cell counts were performed in a Neubauer haemocytometer using trypan blue (0.5% in 0.85% sodium chloride;v/v, dye:cell suspension) to assess cell viability.

7.4 PEG Preparation

Polyethylene glycol (M.W. 1500) was melted in a water bath at 55°C and medium added to give a 50% solution (w/v). The pH was adjusted to approximately 7 with 0.3 mol/l sodium hydroxide using the phenol red in the RPMI as an indicator and the solution filtered through a sterile 0.2 μm filter unit (Millipore).

7.5 Preparation of Spleen Cells

Immediately prior to hybridisation, an immunised mouse, which had been boosted intravenously three days previously (Section 6.5 of this chapter), was killed and the spleen removed using aseptic techniques. All further manipulations were carried out in a sterile laminar flow cabinet.

In a small petri dish containing fresh medium, the spleen was dissected free from fatty tissue, cut in half and the cells teased from the capsule with scalpel blades. Clumps of cells were dispersed by pipetting up and down with a 10 ml pipette before transfer to a 50 ml centrifuge tube. After debris had settled, the spleen cell suspension was decanted and centrifuged. The pellet was washed twice with medium, resuspended in 10 ml of the same and an aliquot taken for counting as described for myeloma cells (Section 7.3 of this chapter).

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7.6 Hybridisation Protocol

The day before hybridisation, complete medium containing peritoneal macrophages was dispensed into fifteen 96-well flat-bottomed microtitre plates (100 μl/well).

Immediately prior to hybridisation myeloma cells were counted, washed once with medium and mixed with spleen cells (1:1). This suspension was divided into three aliquots, each of which was used in a separate hybridisation experiment.

The myeloma/spleen cell suspensions were centrifuged and the supernatants discarded. To each pellet, 800 μl 50% PEG was added over one minute with stirring, and the mixture stirred for another 90 seconds prior to the addition of 10 ml medium over 5 minutes. After centrifugation, the cells were resuspended in 50 ml complete medium and plated out over five 96-well plates (100 μl/well) containing a feeder layer as described above. Three hybridisations from each mouse spleen provided a total of fifteen 96-well plates. The plates were sealed with sellotape, placed in a 37°C incubator and left untouched, except to monitor cell growth, until the initial screen 10-14 days later (Section 5.5 of this chapter).

7.7 Cloning Procedure

The cells from each antibody-positive well on the original fusion plates were cloned at least twice before attempting the production of antibody *in vivo* (ie, ascitic fluid). Cells were routinely cloned from 96-well plates, either the original fusion plate or a previous clone plate, by the method of limiting dilution.

The cells from one well of a 96-well plate were diluted in 1.5 ml complete medium in a 2 ml well. This cell suspension was then dispensed into the first column (8 wells, 100 μl/well) of a 96-well plate previously seeded with 100 μl/well complete medium containing peritoneal macrophage feeders. Using a multichannel pipette the cells were double diluted across the plate, and complete medium

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(100 µl/well) added to columns 1-11. Complete medium (800 µl) was also added to the cells remaining in the 2 ml well. These cells were expanded and frozen to insure against cloning failure. Each clone plate was sealed with sellotape, placed in a 37°C incubator and left untouched, except to monitor cell growth, until screening 1-3 weeks later (Section 5.5 of this chapter).

Hybrids from three wells in the last one or two columns in which all wells supported cell growth, were further expanded and/or cloned.

7.8 Screening Protocols for Antibody Detection

In an initial screening procedure, monoclonal antibodies to insulin were detected by simple binding of ^{125}I-porcine insulin and separation with solid-phase coupled anti-mouse-γ-globulin. Displacement studies were then performed with human insulin to discriminate high and low avidity antibody producing clones. For proinsulin monoclonal antibodies the screening protocol used ^{125}I-human proinsulin in the primary screen. Positive supernatants were retested with ^{125}I-porcine insulin while supernatants from all hybridomas which proved stable through two cloning steps were tested for reactivity with ^{125}I-labelled insulin and proinsulin as well as ^{125}I-C-peptide.

7.9 Expansion of Hybridomas *in vitro*

Hybridomas were routinely expanded from fusion and clone plates to provide safeguard frozen stocks, and from second clone plates to provide sufficient cell numbers for the production of antibody *in vivo*.

Expansion was achieved by the transfer of cells sequentially to 2 ml wells, 25 cm^2 flasks and 75 cm^2 flasks, with feeder cells being added at each transfer step. The cell density was maintained at 10^5-10^6 cells/ml.

7.10 Expansion of Hybridomas *in vivo*

BALB/c mice were primed by the intraperitoneal injection of 0.5 ml pristane (2, 6, 10, 14-tetramethylpentadecane) ten days before the injection (intraperitoneal) of 10^6 hybridoma cells (500 μl, washed and resuspended in medium) as described by N. Hoogenraad et al. (81).

The time interval between the injection of hybridoma cells and the production of ascitic fluid was variable (2-6 weeks). When present, ascites was collected (tapped) from mice anaesthetised with diethyl ether, using a 19 g needle, allowing the ascitic fluid to drain under gravity. Mice were tapped several times over a period of days or weeks, after which they were killed and any remaining ascitic fluid removed.

7.11 Storage and Recovery of Cells

All cell lines were stored frozen in liquid nitrogen. Cells were centrifuged and resuspended at $1-5 \times 10^6$ cells/ml in 10% DMSO/90% FCS. 1 ml aliquots were dispensed into cryovials and frozen slowly in a polystyrene box at -70°C. After a minimum of twelve hours, usually overnight, the vials were transferred to liquid nitrogen for long-term storage.

Recovery of cells was achieved by rapid thawing at 37°C, after which the contents of the cryovial were immediately diluted in 20 ml medium and centrifuged. The pellet was resuspended in 1 ml complete medium and transferred to a 25 cm^2 flask containing 4 ml complete medium previously seeded with peritoneal macrophages.

CHAPTER 3

POLYCLONAL ANTIBODY PRODUCTION

1 GUINEA-PIGS

1.1 Comparison of Outbred and Inbred Animals

A group of twelve outbred guinea-pigs was immunised and boosted with porcine insulin. Two animals died after the primary immunisation (200 μg insulin) from suspected hypoglycaemia and the dose was lowered to 100 μg for subsequent boosts. One animal died after the first boost but no cause of death was established. Test bleeds were taken by cardiac puncture which resulted in two further deaths. After three boosts, antiserum dilution curves from the remaining seven guinea-pigs were compared with a commercially available antiserum (Wellcome) and the results shown in Figure 3.1. The highest titre antiserum raised in outbred guinea-pigs was a hundred-fold lower than the titre of the commercial antiserum, with two animals showing no serum antibody response.

An inbred strain of guinea-pigs, Strain 2, was then purchased and the animals immunised as described in Chapter 2, Section 6.2. Figure 3.2 shows the range of responses observed in comparison with the outbred group after the same number of boosts. The lowest responders in the inbred group produced similar antiserum titres to the highest responders in the outbred group. Subsequently, inbred guinea-pigs were adopted for immunisation.

The avidity of a pool of insulin antiserum raised in Strain 2 guinea-pigs was compared to that of the Wellcome antiserum by displacement of ^{125}I-porcine insulin with human insulin standards (Figure 3.3). Although the antiserum raised in Strain 2 guinea-pigs was of a lower titre than the commercial one (1:20 000 vs 1:60 000 initial dilution) the avidity was comparable. To date, sixty Strain 2 guinea-pigs have been immunised with insulin and all have produced high titre, high avidity antisera suitable for use in RIA and IRMA techniques.
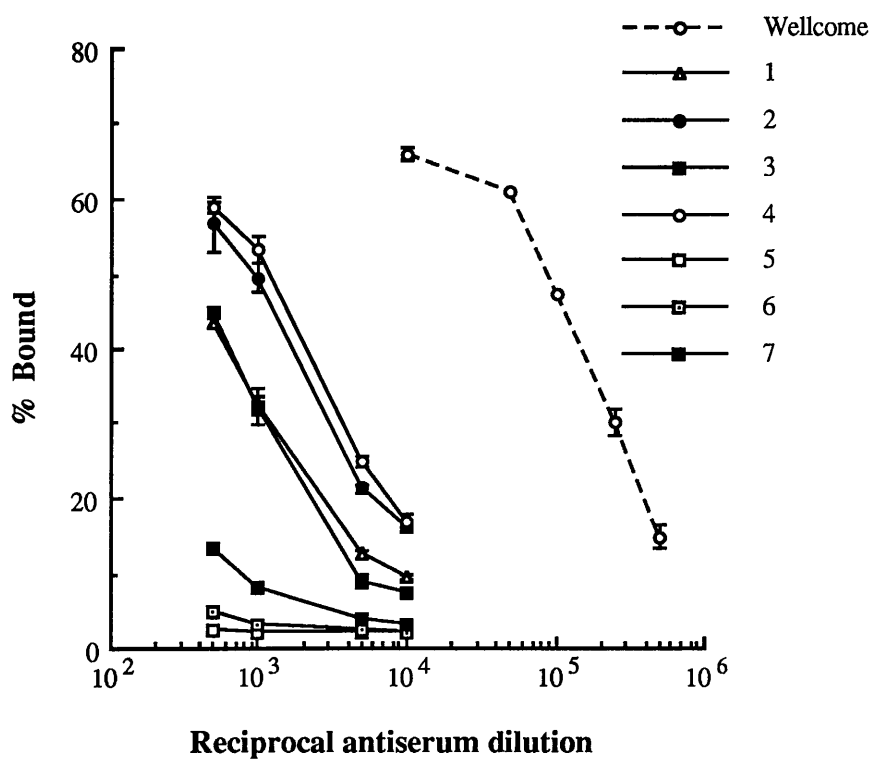
Figure 3.1

Comparison between insulin antisera raised in outbred guinea-pigs and a commercially available antiserum (Wellcome).
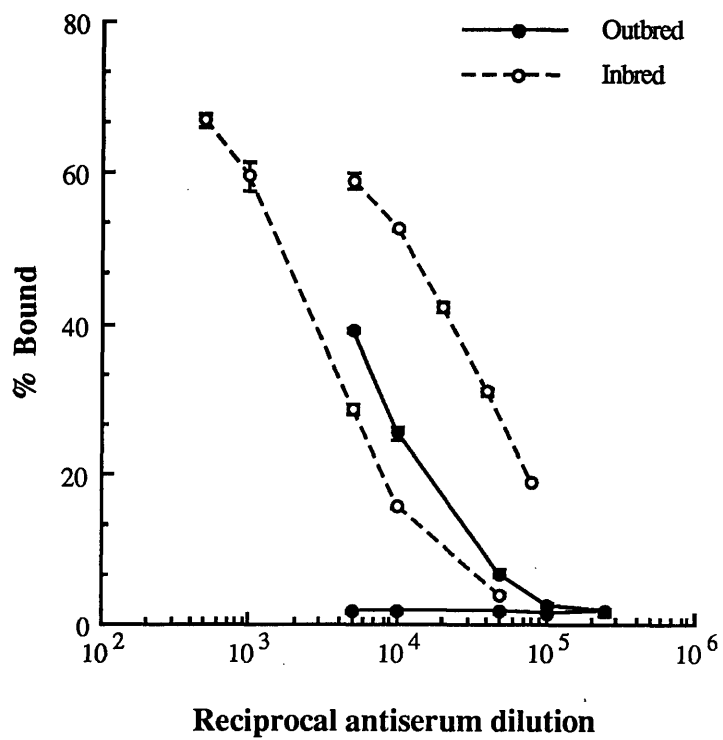
Figure 3.2

Comparison of the range of insulin antibody responses observed in outbred and inbred guinea-pigs.
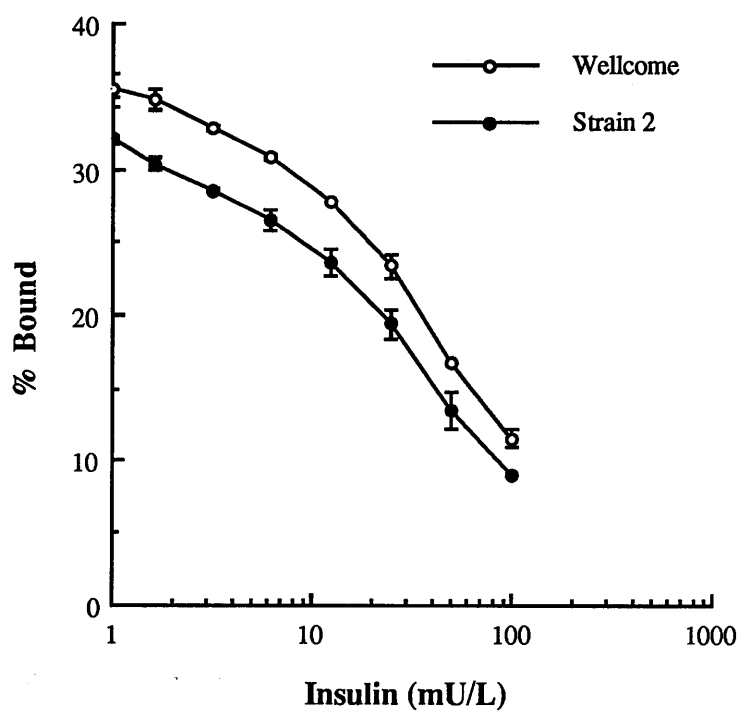
Figure 3.3

Insulin RIA standard curves constructed with Wellcome or Strain 2 guinea-pig antisera.

1.2 Evaluation of Blood Spots for Test Bleeding Guinea-Pigs

The high risk of mortality associated with taking test bleeds by cardiac puncture was unacceptable and a safer method, that of collecting small volumes of blood (<100 μl) from a peripheral vein on to filter paper, was assessed.

Six blood spots were taken from each of two guinea-pigs immediately before exsanguination and eluted in 2 ml saline as described in Chapter 2, Section 6.2. Samples eluted from blood spots were arbitrarily designated as 'neat'. The results from each series of blood spot dilution curves were pooled and plotted, as mean and standard deviation, together with the corresponding serum dilution curve (Figure 3.4). Similar percentage binding was achieved with a serum dilution approximately 1000 times greater than the arbitrary dilution assigned to the blood spots. Therefore, the sample volume eluted from the original blood spots into 2 ml saline was probably 2 μl to result in a 1:1000 dilution.

This method of test bleeding has been used successfully to monitor accurately serum antibody levels in both guinea-pigs and mice.

1.3 Avidity Constants of Antiserum for Insulin and Proinsulin

The avidity constants of the insulin antiserum, raised in Strain 2 guinea-pigs, for insulin and proinsulin were 1.6×10^{11} - 1.0×10^{12} l/mol and 1.1×10^{10} l/mol respectively. The relevant Scatchard plots are shown in Figure 3.5.

1.4 Ascites Production

Production of ascitic fluid containing specific antibody was attempted (Chapter 2, Section 6.2) in two guinea-pigs which exhibited a good serum titre of anti-insulin following a series of ten boosts. One of the animals was humanely killed prior to any ascitic fluid production. The second guinea-pig produced approximately 40 ml ascites over a one month period after which it was exsanguinated (Table 3.1).

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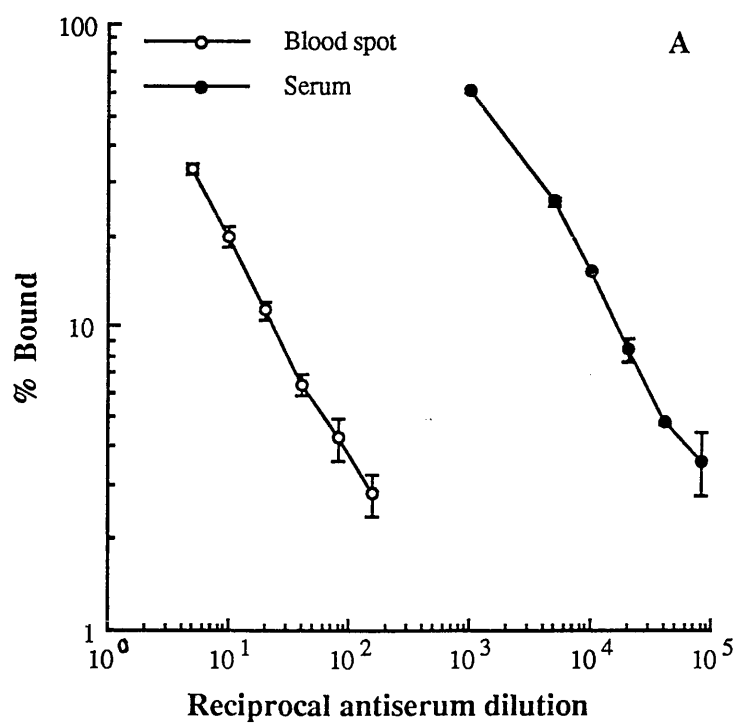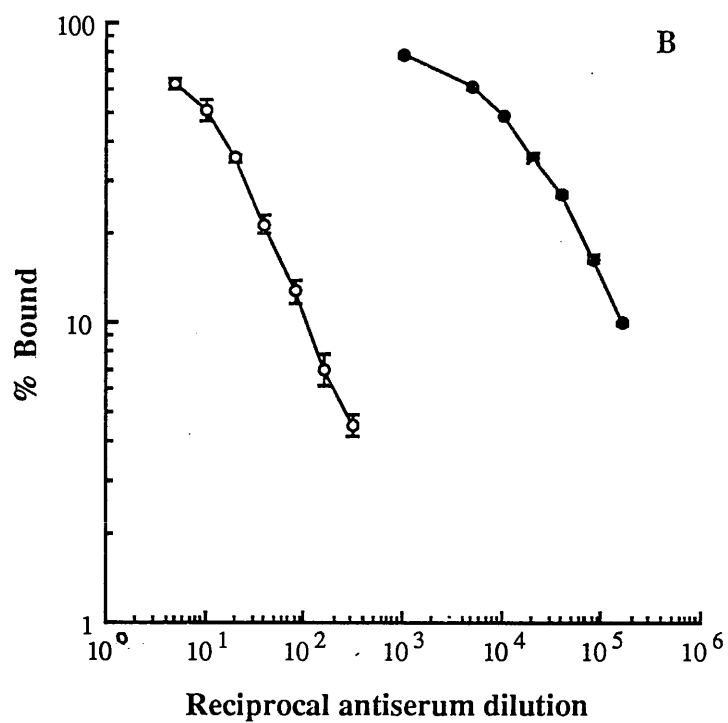
Figure 3.4

Antiserum dilution curves prepared from blood spots and serum
from each of two guinea-pigs.
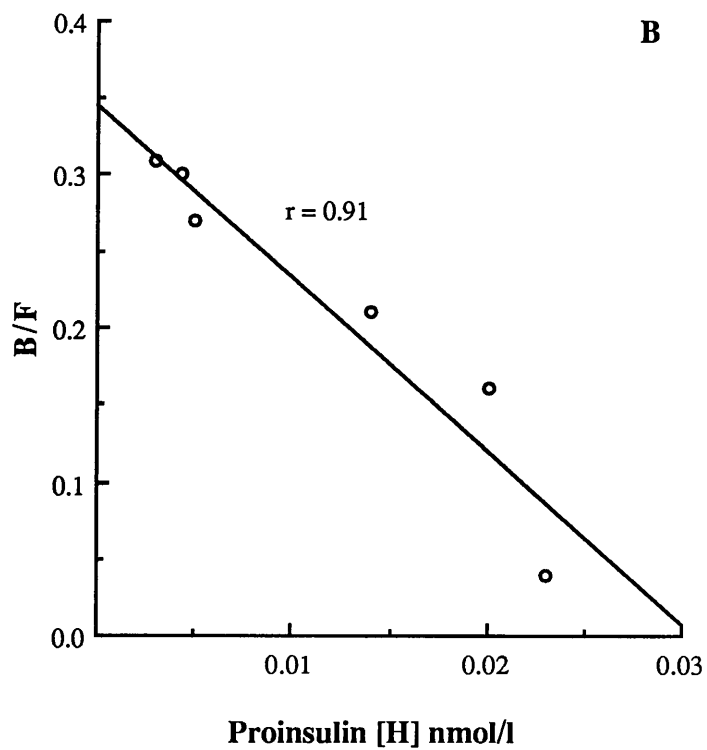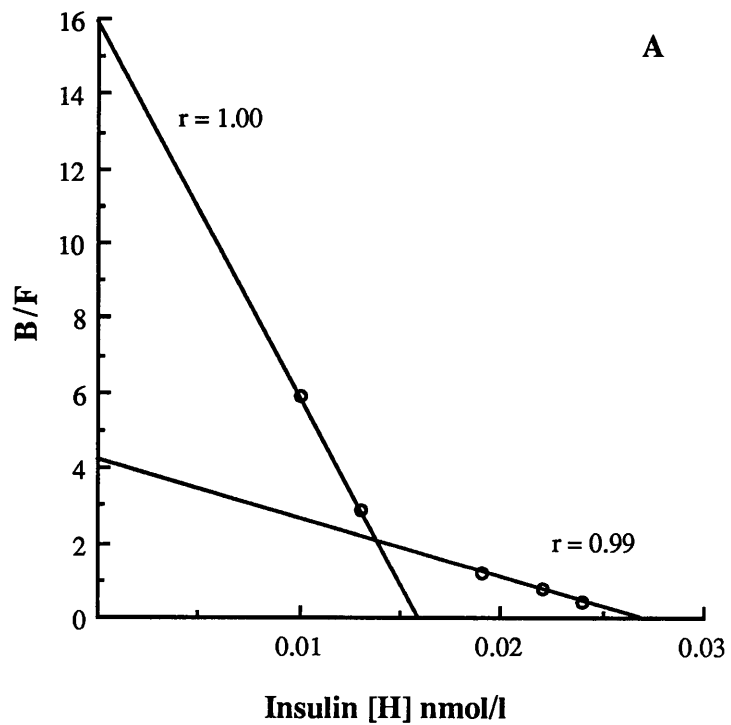
Figure 3.5

Scatchard plots of the insulin antiserum raised in Strain 2 guinea-pigs with insulin (A) and proinsulin (B) standards.

TABLE 3.1

IMMUNISATION SCHEDULE FOR THE PRODUCTION OF ASCITIC FLUID IN GUINEA-PIGS

Date	Treatment	Guinea-pig A			Guinea-pig B		
		Weight (g)		Volume of ascites (ml)	Weight (g)		Volume of ascites (ml)
		Pre*	Post*		Pre	Post	
10.6.86	FCA/Insulin	-	-	-	-	-	-
17.6.86	FCA/Insulin	-	-	-	-	-	-
24.6.86	FCA	820.3	-	-	770.6	-	-
1.7.86	FCA	794.2	-	-	769.3	-	-
8.7.86	FCA	690.2	-	-	764.5	-	-
15.7.86	FCA/Insulin	652.8	-	-	722.1	-	-
22.7.86	FCA	501.5	-	-	723.8	709.9	15
		Animal humanely killed					
28.7.86	-	-	-	-	733.6	729.4	5
29.7.86	FCA/Insulin	-	-	-	734.5	732.6	2
31.7.86	-	-	-	-	679.4	669.5	10
5.8.86	FCA	-	-	-	675.7	672.9	3
8.8.86	-	-	-	-	660.3	-	5
					Animal exsanguinated		

*Pre and post refer to the weights of the guinea-pigs before and after ascitic fluid was removed.

The ascitic fluid obtained from this guinea-pig was pooled and assessed in conjunction with the corresponding serum collected at exsanguination. The antibody titre was lower in ascites than serum (Figure 3.6) and the higher avidity antibodies appeared to be missing as indicated by the higher doses of insulin required to achieve the same displacement of ^{125}I-insulin from the antibody present in ascitic fluid compared with serum (Figure 3.7).

Post-mortem examinations performed on both animals showed a marked inflammatory reaction which involved the whole of the peritoneum in guinea-pig B, but was localised to the area surrounding, but not involving, the pancreas in guinea-pig A (Figure 3.8A). The inflammatory cell infiltrate comprised abundant epithelioid macrophages and lymphocytes (Figure 3.8B). Examination of the peritoneum revealed a dense infiltrate of immunoglobulin-secreting plasma cells deep to the peritoneal surface (Figure 3.8C).

1.5 Discussion

Guinea-pigs have been the species of choice for the production of high avidity antisera to insulin due to the large number of amino acid differences in the primary structure of guinea-pig and mammalian insulins (14). However, the immune response of guinea-pigs to insulin has been shown to be under the control of immune response genes (108) and therefore only animals of the appropriate haplotype will respond. The data presented in Figure 3.2 support this observation as only some of the outbred compared with all of the inbred guinea-pigs immunised with insulin responded. The use of Strain 2 guinea-pigs has resulted in the reproducible production of anti-insulin with all the animals immunised producing antisera suitable for use in immunoassay techniques.

The T cells of Strain 2 guinea-pigs are known to recognise the A-chain loop determinant on the insulin molecule (108). The data presented here suggest that the
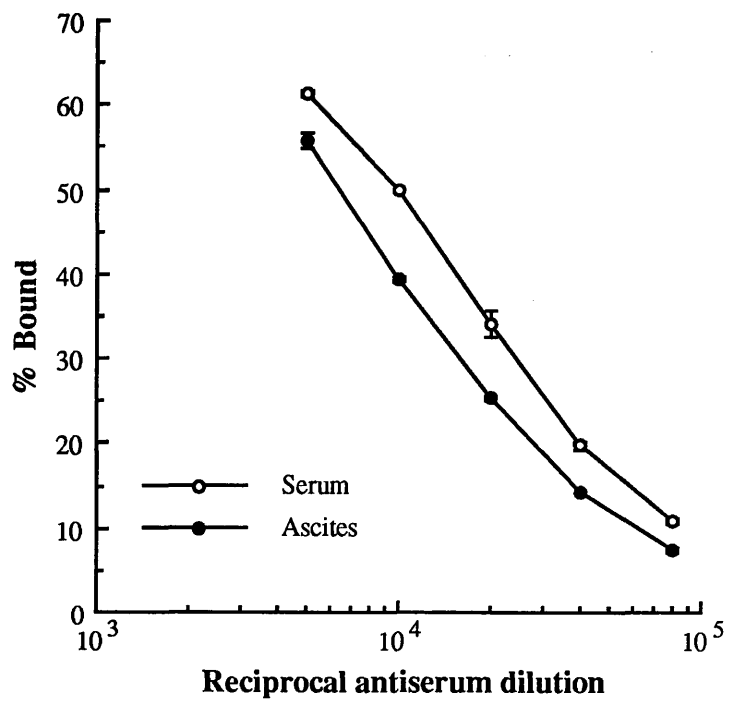
Figure 3.6

Comparison of serum and ascites anti-insulin dilution curves.
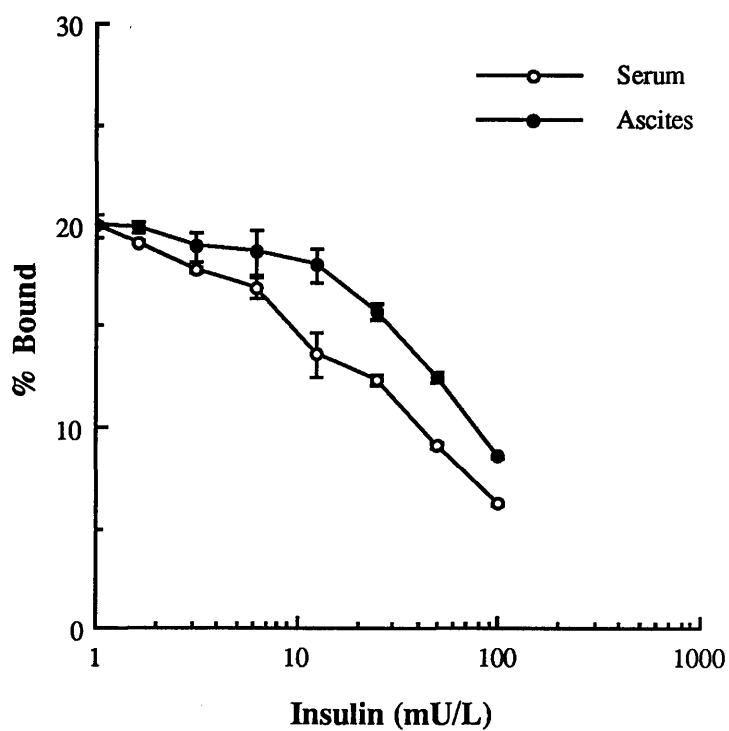
Figure 3.7

Comparison of insulin RIA standard curves prepared with anti-insulin present in serum and ascitic fluid.

Figure 3.8

Effect of intraperitoneal injection of Freunds complete adjuvant in guinea-pigs.

A   Guinea-pig A. The pancreas on the left is normal. Omental fat on the right contains a marked inflammatory cell infiltrate.

    Haematoxylin and eosin; x 25

B   Guinea-pig A. High power of the inflammatory cell infiltrate. There are abundant epithelioid macrophages and lymphocytes, and a Langhan's giant cell is present. This reaction is typical of a response to mycobacteria (107).

    Haematoxylin and eosin; x 400

C   Guinea-pig B. High power of the peritoneum. Immediately deep to the peritoneal surface there is a dense infiltrate of immunoglobulin-secreting plasma cells.

    Haematoxylin and eosin; x 500
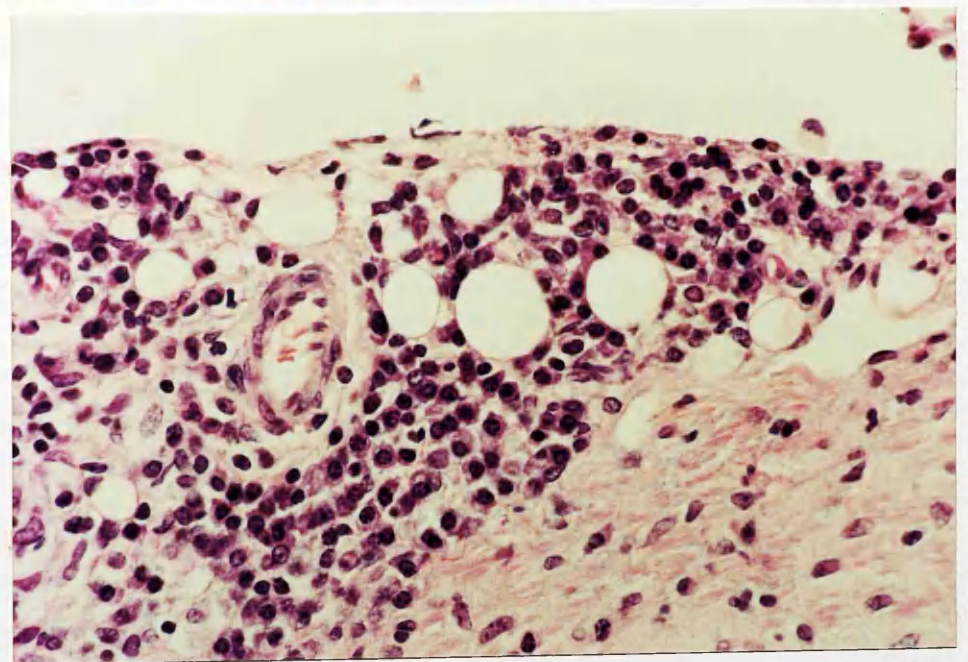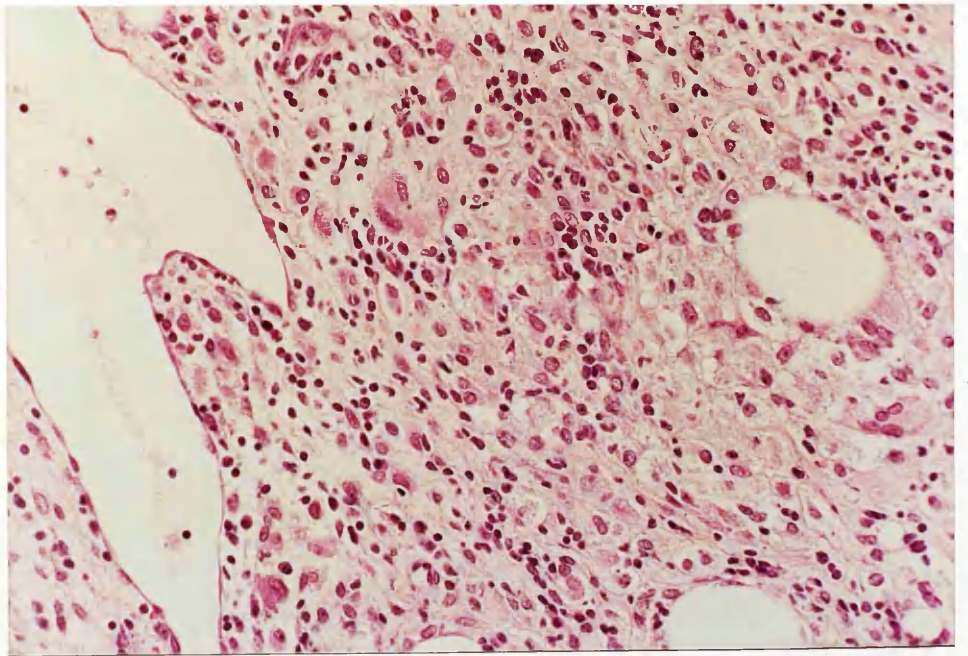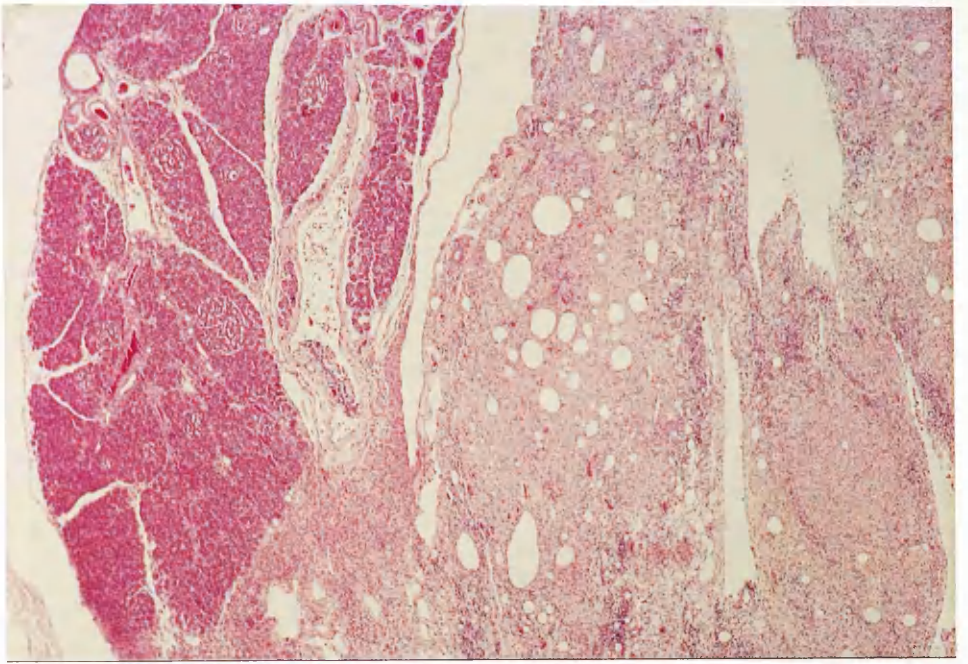
antibody response may also reflect the immunodominance of this epitope over others on the insulin molecule in this strain of guinea-pig. Scatchard analysis of the anti-insulin raised in Strain 2 guinea-pigs resulted in a typical polyclonal curve against insulin but gave a straight line, characteristic of a McAb, when evaluated with proinsulin (Figure 3.5). This suggests that the antiserum reacts with a single epitope on the proinsulin molecule. Evidence that this epitope may be the A-chain loop or an epitope in close proximity to this is presented in Chapter 5, Figure 5.13. Two McAbs to insulin, one of which reacted with the A-chain loop (PH4/B1), were assessed for use in an insulin IRMA in conjunction with the guinea-pig anti-insulin. No binding was observed over the standard curve when the McAb recognising the A-chain loop was used, thus suggesting that both antibodies recognised the same or spatially close epitopes.

The attempt to increase the yield of antiserum obtained per guinea-pig by the production of ascitic fluid in pre-immunised animals was successful in terms of the volume of reagent harvested. However, the avidity of the anti-insulin in the ascitic fluid was lower than that in the serum and therefore this technique was not pursued.

2 RABBITS

Three rabbits were immunised and boosted once with a porcine insulin-human IgG conjugate (Chapter 2, Section 3.4). The resulting antiserum had a strong anti-human IgG reactivity but no detectable insulin antibody. Due to the strength of the immune response to human IgG it was decided that further boosting would be unproductive and the experiment was terminated.
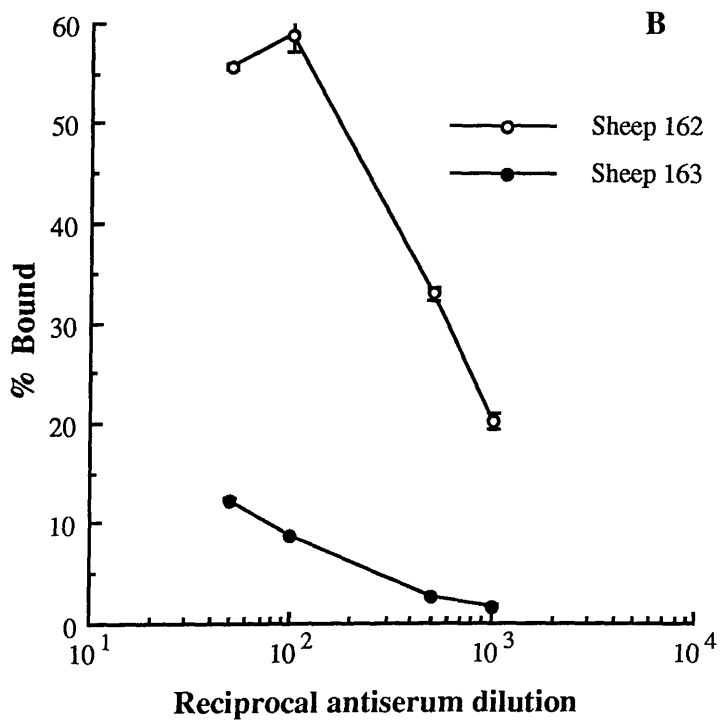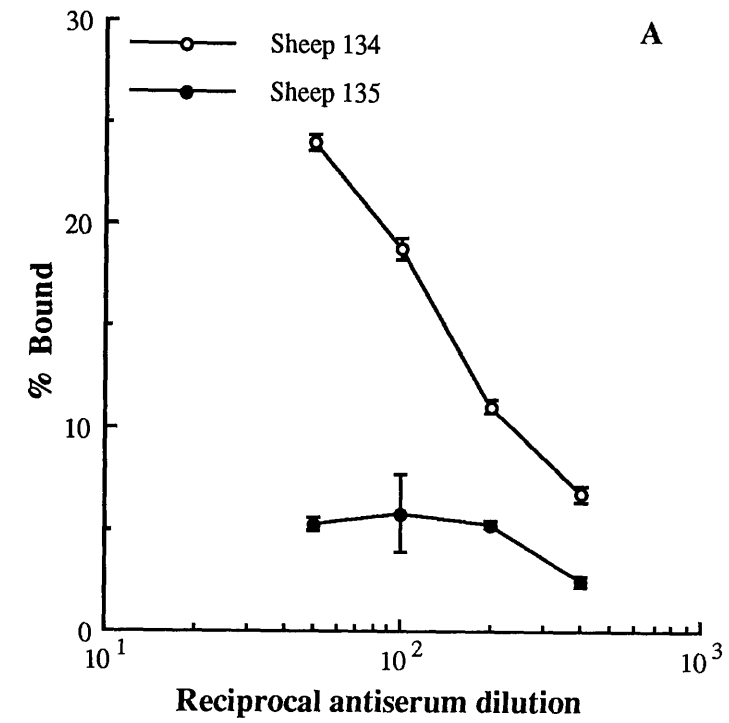
Figure 3.9

Antiserum dilution curves prepared from test bleeds taken from sheep immunised with human insulin (A) or a human insulin-ovalbumin conjugate (B).
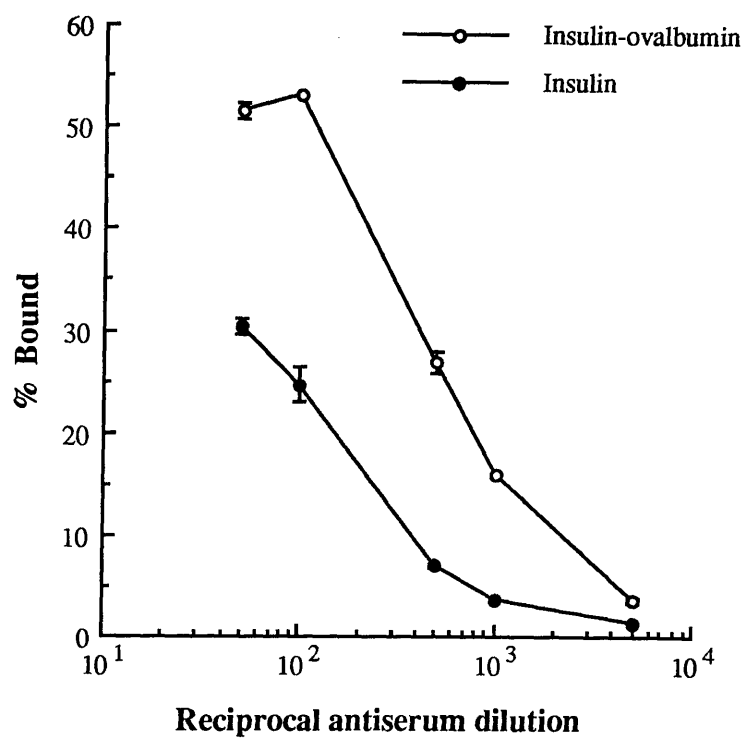
Figure 3.10

Comparison of test bleeds taken from a sheep boosted with a human insulin-ovalbumin conjugate and one month later with human insulin.
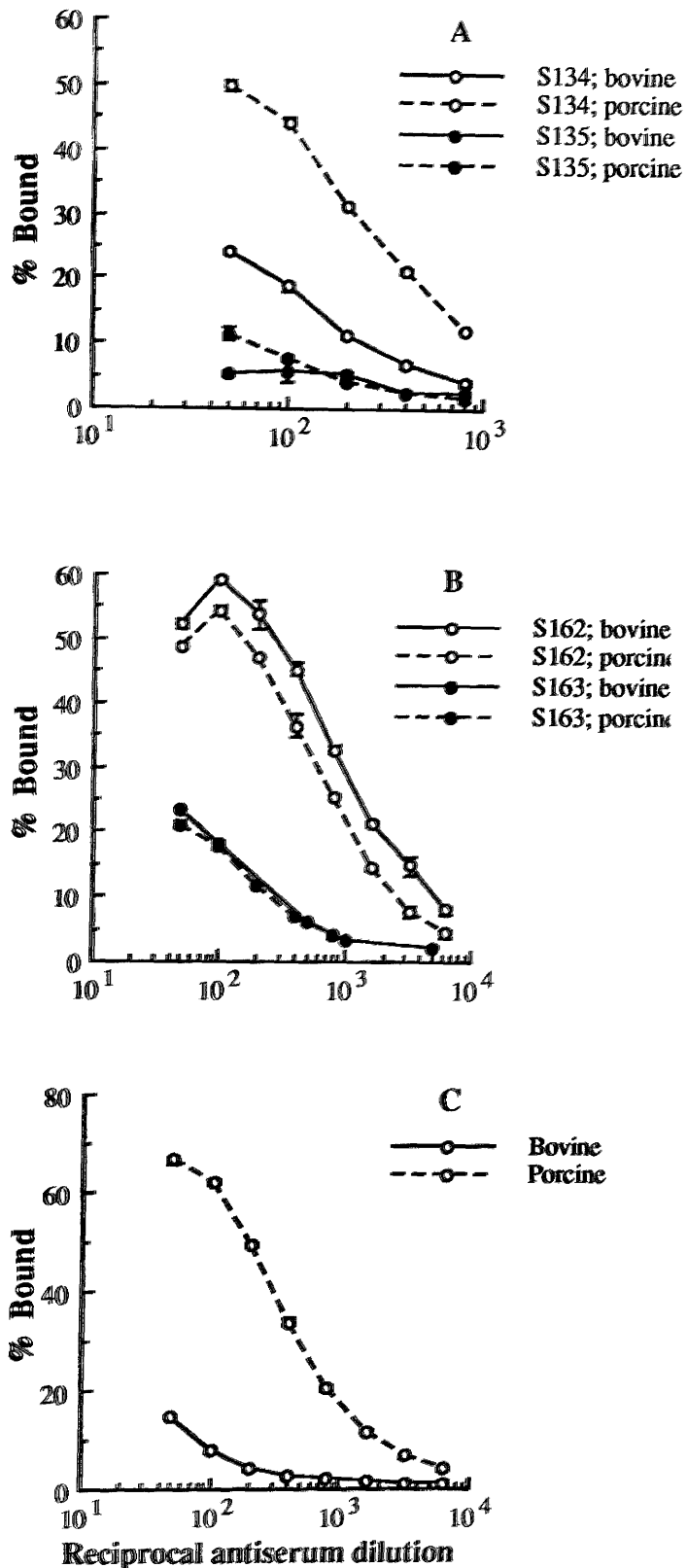
Figure 3.11

Comparison of antiserum dilution curves prepared with ^{125}I-labelled bovine and porcine insulins, from sheep immunised with either human insulin (A) or a human insulin-ovalbumin conjugate (B) and a commercial sheep anti-insulin (C).

3 SHEEP

3.1 Comparison of Immunisation with Human Insulin or Human Insulin-Ovalbumin Conjugate

Two groups of two sheep were immunised and boosted three times with either human insulin (Humulin) or a human insulin-ovalbumin conjugate (Chapter 2, Section 3.4). In both experiments a single sheep responded to produce a significant titre of insulin antibody (Figure 3.9). However, both sheep immunised with the conjugate showed a strong response to ovalbumin. The two sheep immunised with the conjugate were given a final boost with human insulin alone in an attempt to improve the anti-insulin titre by specifically stimulating the appropriate B lymphocytes. Unfortunately, a lower titre of insulin antibody was observed in the test bleeds taken after this boost compared to those taken after the previous boost with conjugate (Figure 3.10).

3.2 Epitope Specificity of Antisera

A commercial sheep anti-insulin (The Binding Site) was evaluated in conjunction with the above antisera in terms of antiserum dilution curves performed with both porcine and bovine iodinated insulins (Figure 3.11). The commercial antiserum and the one raised using human insulin as immunogen exhibited a preference for ^{125}I-porcine insulin. Since the insulin in these two species differ only by two amino acids, at the A-chain loop (amino acids 8-10 on the A chain), this pattern of binding suggests that these antisera are directed, at least primarily, against this epitope. Human and porcine insulins are identical at these positions. In contrast, the antiserum raised using the conjugate as immunogen, does not distinguish between ^{125}I-labelled porcine and bovine insulins suggesting that it reacts with an epitope outside the A-chain loop.

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3.3 Discussion

It is probable that the immune response of sheep to insulin is under the control of Ir genes, for whilst both sheep showed a strong response to ovalbumin, only one exhibited a significant response to insulin (Figure 3.9).

The apparent difference in specificity of the insulin antisera raised using either insulin or an insulin-ovalbumin conjugate (Figure 3.11) suggest that the A-chain loop determinant of the insulin molecule is immunodominant in sheep. The data also suggest that the conjugation of insulin to ovalbumin masked this epitope, resulting in the immune response being directed against a different epitope. In this respect, it would have been interesting to evaluate in a similar manner the tests bleeds, taken from a single sheep, after a booster injection with an insulin-ovalbumin conjugate one month and with insulin the following month (Figure 3.10). The lower titre of the test bleed taken after the booster injection with insulin may reflect the production of a previously unstimulated population of antibodies.

4 MICE

4.1 Immunisation with Porcine Insulin

Initially, a group of four BALB/c and New Zealand Black F_1 hybrid mice was immunised with porcine insulin (Chapter 2, Section 6.5). After three boosts there was no detectable serum antibody response and the experiment was terminated. Five BALB/c mice were then immunised according to the same schedule and all responded. This difference in response was due to differences in the major histocompatibility complex between each group of mice (Chapter 1, Section 2.5). The antiserum dilution curves obtained after two boosts are shown in Figure 3.12. Mouse number 5 was used as the spleen cell donor for the fusion which generated
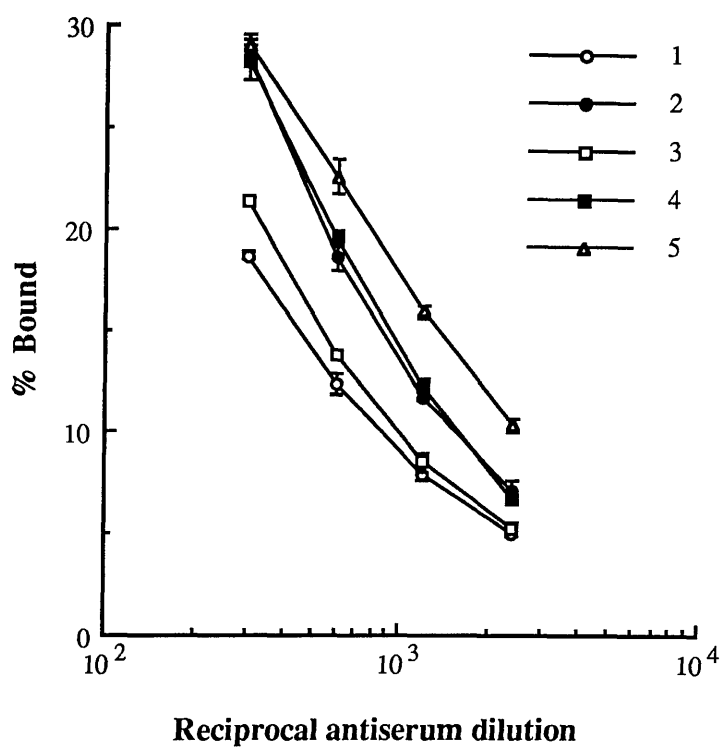
Figure 3.12

Antiserum dilution curves prepared from test bleeds taken from mice immunised with porcine insulin.

McAbs ID1/C10, ID4/E5 and ID1/A4 (Chapter 4). Monoclonal antibody ID1/C10 was used to develop the insulin IRMA in conjunction with the polyclonal anti-insulin raised in Strain 2 guinea-pigs (Chapter 5, Section 2).

4.2 Immunisation with Human Proinsulin

Four BALB/c mice were immunised and boosted once with human proinsulin before test bleeds were taken and antiserum dilution curves constructed (Figure 3.13). The serum response remained unchanged after two further boosts and the best responder (mouse 4) was used as the spleen cell donor for the hybridisation experiment performed on 19.7.85, which produced no stable antibody-secreting hybridomas (Chapter 4, Table 4.1).

The above experiments were performed using a heterogeneous ^{125}I-human proinsulin preparation purified by gel filtration chromatography. Proinsulin contains four tyrosine residues, each of which is available for iodination, and one of these (B26) lies very close to the immunodominant epitope recognised by McAbs specific for proinsulin (the B-C chain junction). Iodination of proinsulin at this position could possibly inhibit the binding of McAbs with the desired specificity. It has been shown that iodination of proinsulin at the A14 tyrosine residue does not inhibit the binding of proinsulin-specific antibodies (109) and a method for separating the various iodinated species of proinsulin was developed using reverse-phase HPLC (Chapter 5, Section 1.2).

The original test bleeds from the four BALB/c mice were then retested with ^{125}I-proinsulin prepared by reverse-phase HPLC (Figure 3.14). The results indicated that four of the five mice had responded to the immunisation schedule confirming that the original iodinated proinsulin, purified by gel filtration chromatography, could not bind to all the antibodies present in the serum of these animals.
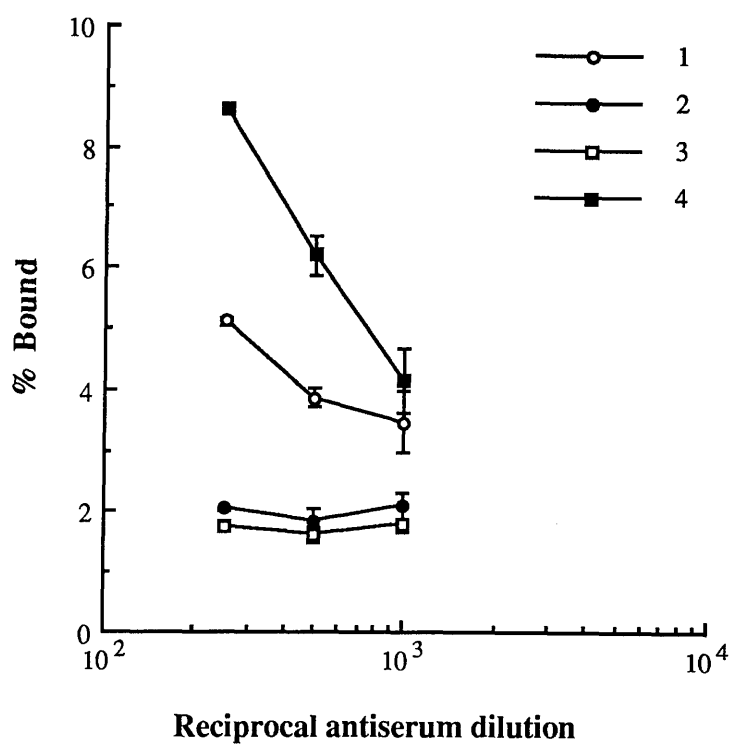
Figure 3.13

Serum antibody response of mice immunised with human proinsulin (assessed using ^{125}I-proinsulin purified by gel filtration chromatography).
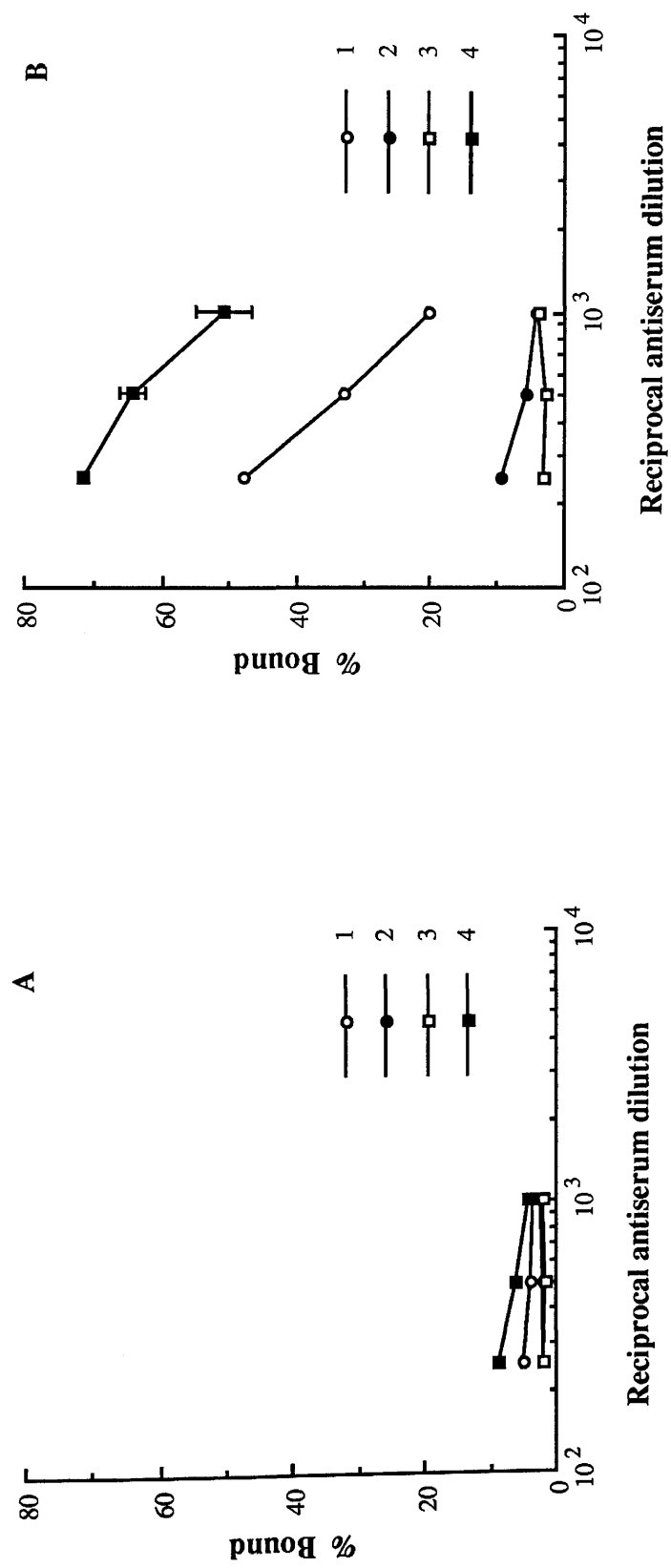
Figure 3.14

Comparison of the serum antibody response of mice immunised with human proinsulin (assessed using ^{125}I- proinsulin purified by either gel filtration chromatography (A) or reverse-phase HPLC (B)).

Mouse 1, which had been rested for 11 months, was then boosted, a test bleed taken and antiserum dilution curves constructed with both ^{125}I-labelled proinsulin and insulin (Figure 3.15). This animal was sacrificed and its spleen removed for the fusion experiment performed on 6.6.86, which yielded one McAb reactive against the C-peptide moiety of proinsulin (PD4/H4) and two reactive to both insulin and proinsulin (PF1/B9 and PF2/B5). The McAb PD4/H4 proved to be of inadequate avidity for the development of a sensitive proinsulin IRMA (Chapter 5, Section 4).

A subsequent group of eight BALB/c mice was immunised with human proinsulin in an attempt to produce higher avidity proinsulin-specific McAbs from a short immunisation schedule. Test bleeds were taken after two boosts and antiserum dilution curves constructed with iodinated proinsulin and insulin. Representative results from two mice are shown in Figure 3.16. The mouse giving the results shown in Figure 3.16A was used as the spleen cell donor for the fusion which generated three proinsulin-specific McAbs (PH5/B5, PI2/G4 and PI3/B10) and one which reacted with insulin and proinsulin (PH4/B1). Monoclonal antibodies PH5/B5 and PH4/B1 were used to develop the final proinsulin IRMA (Chapter 5, Section 6).

4.3 Discussion

The immune response of mice to insulin is known to be under the control of Ir genes (110, 111, 112) and this explains the non-responder status of the first group of mice immunised.

The difference in the observed serum antibody response of a group of mice immunised with human proinsulin, when tested with two different ^{125}I-proinsulin preparations (Figure 3.14) emphasises the importance of establishing a suitable screening system for the evaluation of mouse serum prior to fusion and the subsequent detection of McAbs (Chapter 4, Section 3).
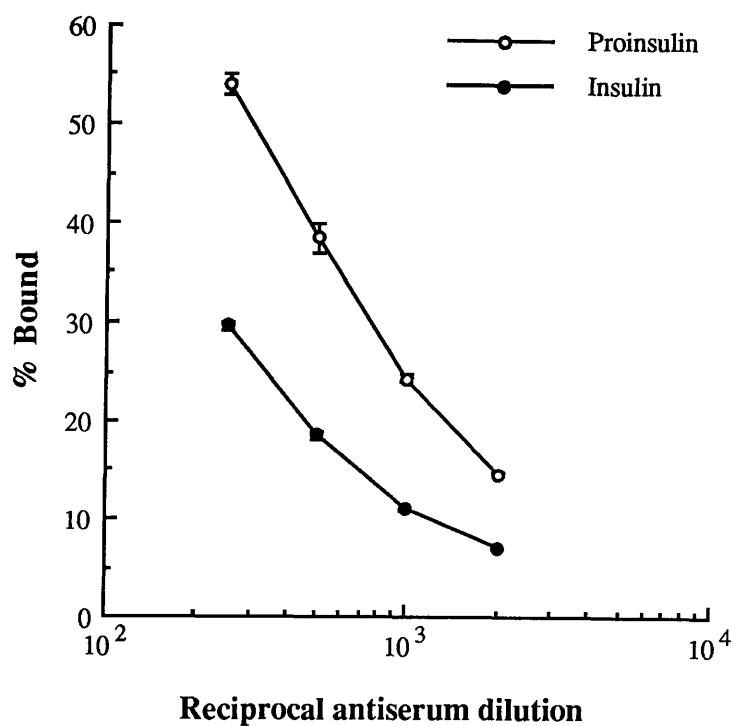
Figure 3.15

Serum antibody response to proinsulin and insulin of the mouse
used as spleen cell donor for the fusion which produced McAbs
PD4/H4, PFI/B9 and PF2/B5.
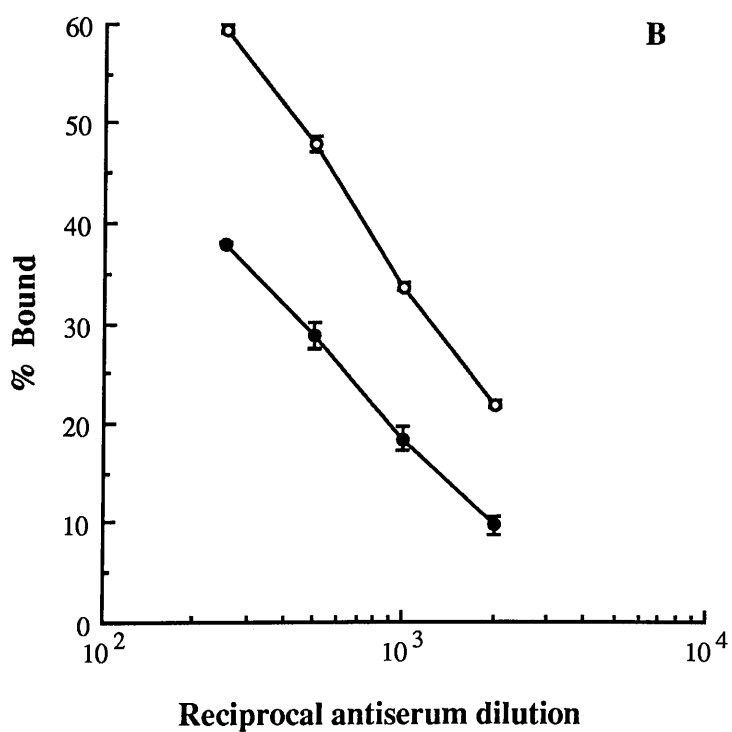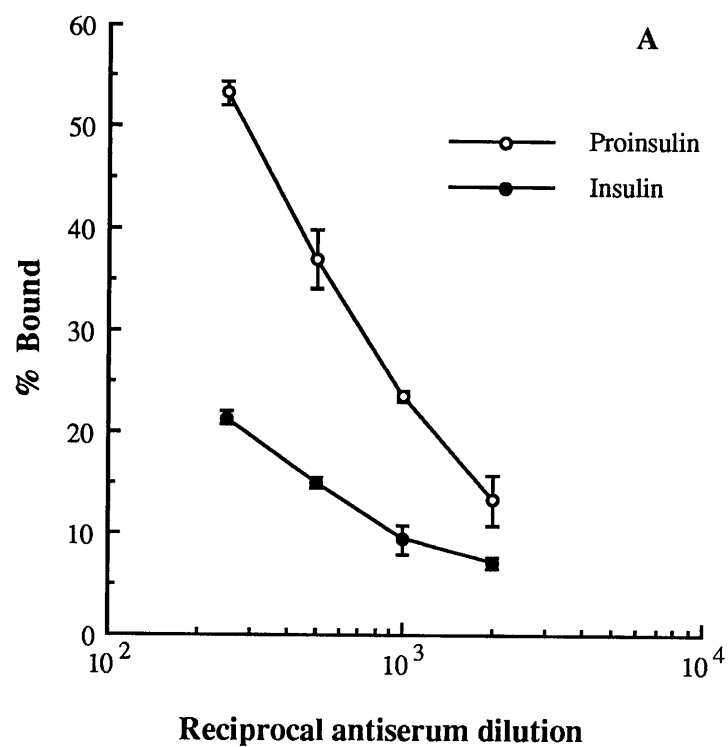
Figure 3.16

Representative serum antibody responses to proinsulin and insulin from two mice immunised with human proinsulin.

The production of high avidity proinsulin-specific McAbs from a short immunisation schedule appeared to be successful. This approach was based on the assumption that the dose of proinsulin used for immunisation was not small enough to only stimulate the highest avidity clones of plasma cells secreting antibody of the desired specificity. To have proved the validity of this argument, a considerable amount of work would have been required which was not directly relevant to the aims of this thesis.

5 CHICKENS

5.1 Immunisation of Two Strains of Chicken with Porcine Insulin

Five ISA Brown chickens were immunised and boosted with porcine insulin. Two birds died after the first boost and a post-mortem examination revealed streptococcal infection of the liver. Test bleeds taken from the remaining three birds after four boosts were compared with the antiserum raised in Strain 2 guinea-pigs (Figure 3.17). A different strain of chicken, Dwarf Broilers, was then immunised according to the same protocol. Representative dilution curves from five of the ten birds immunised are shown in Figure 3.18 together with those of the highest and lowest responders from the ISA Brown group. The titre of the antisera raised in both strains was similar but 100-1000 fold lower than that of the guinea-pig antiserum. The avidities of the antisera from each of the chickens was assessed in conjunction with the guinea-pig antiserum by performing single dose displacement studies (200 mU/l insulin) at three serum dilutions (Table 3.2). Chicken 28/4 produced the highest avidity antiserum and was later evaluated for use as a solid-phase coupled reagent in an IRMA to measure insulin (Section 5.3).

5.2 Antibody Levels in the Eggs of an Immunised Chicken

The antibody titre present in the yolk of eggs laid by an immunised chicken (28/5) was approximately half that present in the plasma of the same bird (Figure 3.19).

This titre was maintained for four weeks after a boost. This was assessed by the binding of ^{125}I-porcine insulin by egg yolk antibody, at two dilutions, from eggs collected from a single chicken (28/5) over this period (Figure 3.20).

5.3 Evaluation of Antiserum in an IRMA to Measure Insulin

Purified IgG was prepared from the egg yolks of eggs laid by chicken 28/4 which produced the highest avidity antiserum (Chapter 2, Section 4.3). The IgG was coupled to Sepharose^R CL-4B and compared with solid-phase coupled guinea-pig anti-insulin for use in an IRMA to measure insulin, in conjunction with two ^{125}I-labelled McAbs reactive with insulin but with different epitope specificities. The chicken antiserum did not bind insulin over the range of concentrations tested, suggesting poor avidity.

5.4 Discussion

The immune response of chickens is known to be under the control of Ir genes (113). Although particular strains of chicken were used in this project, they were not inbred with respect to Ir genes and it was not surprising therefore, that a range of antibody titres and avidities was observed in the birds immunised with insulin.

At the outset, the use of chickens for the production of antisera to insulin was thought likely to succeed due to the relatively large number of amino acid differences between avian and mammalian insulins. However, as was shown with guinea-pigs (Section 1.5 of this chapter) success probably depends upon immunising the appropriate inbred strain. If this problem could be overcome, the use of chickens for immunisation with insulin would provide a feasible alternative to guinea-pigs especially as large quantities of specific antibody can be obtained from the yolks of eggs laid by immunised chickens. This is an important consideration in the development of immunometric assay techniques which require the use of excess antibody reagents.
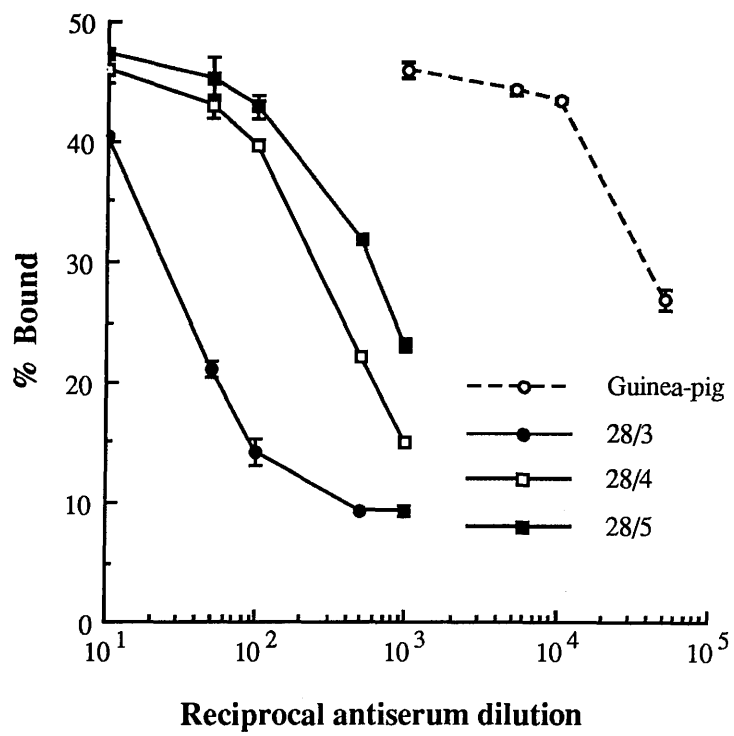
Figure 3.17

Comparison of insulin antisera raised in ISA Brown chickens and Strain 2 guinea-pigs.
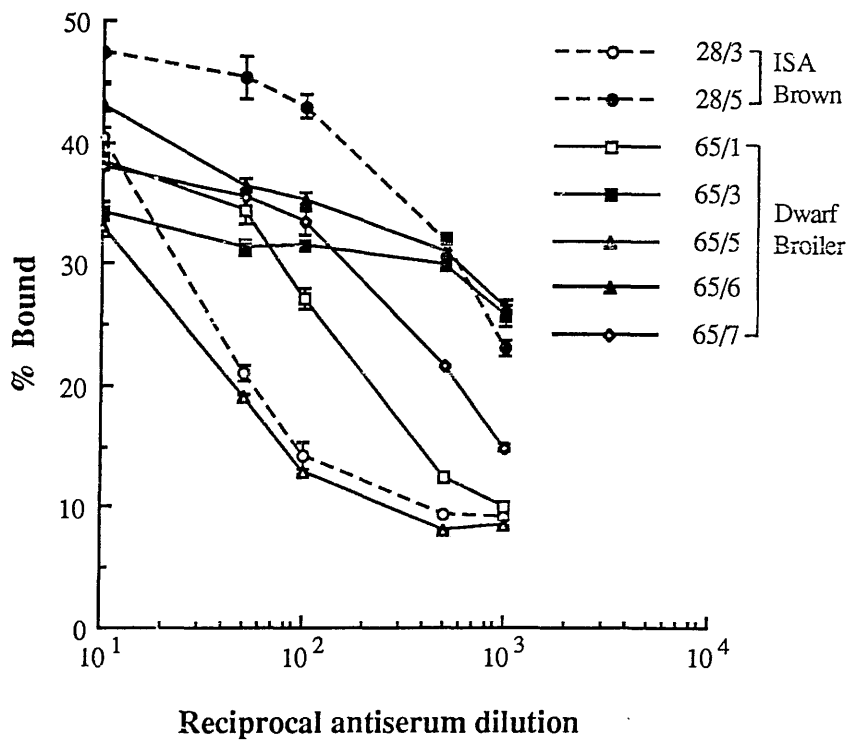
Figure 3.18

Comparison of responses of ISA Brown and Dwarf Broiler
chickens to immunisation with porcine insulin.

TABLE 3.2

COMPARISON OF AVIDITIES OF CHICKEN ANTISERA IN RELATION
TO A GUINEA-PIG ANTISERUM

Chicken identification	% Displacement ± SD		
	Dilution of antisera		
	1:50	1:100	1:500
28/3	0	7.2±7.1	1.4±1.9
28/4	36.5±2.1	55.1±0.1	38.6±0.1
28/5	0	6.6±1.8	21.5±30.4
65/1	27.4±6.6	41.4±2.3	33.6±6.5
65/2	28.4±0.8	16.0±5.4	0
65/3	0	18.0±4.2	32.3±1.3
65/4	5.1±1.6	0	0
65/5	6.9±0.2	12.9±1.6	0
65/6	0	3.6±2.5	7.0±0.2
65/7	15.5±1.7	29.9±8.8	26.8±4.0
65/8	18.4±1.3	13.9±3.0	26.7±1.3
65/9	15.8±21.5	0	4.4±0.8
65/10	0	4.7±2.8	0
Guinea-pig*	55.4±0.8	66.1±1.4	49.5±1.8

*Multiply dilutions by 10^3
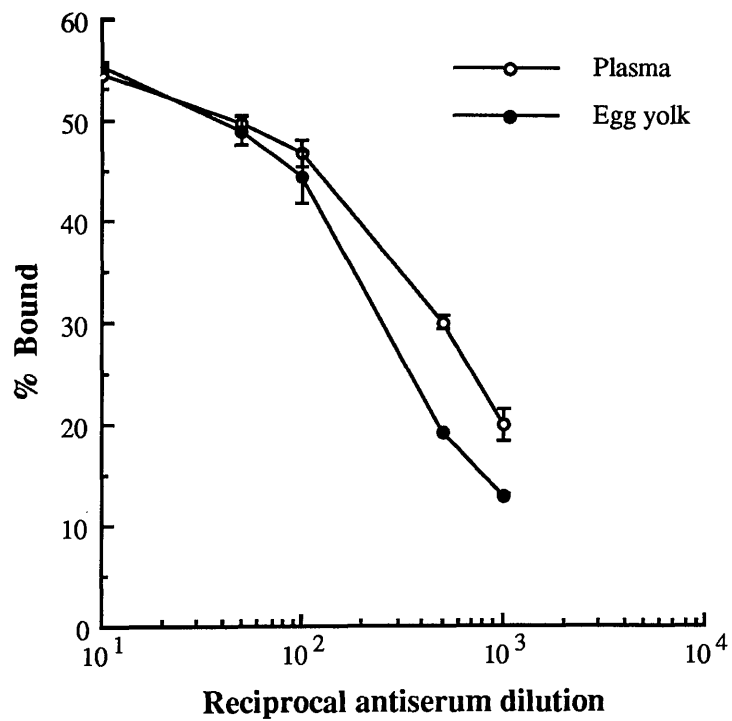
Figure 3.19

Comparison of antibody titre in the plasma and egg yolk of an ISA Brown chicken (28/5) immunised with porcine insulin.
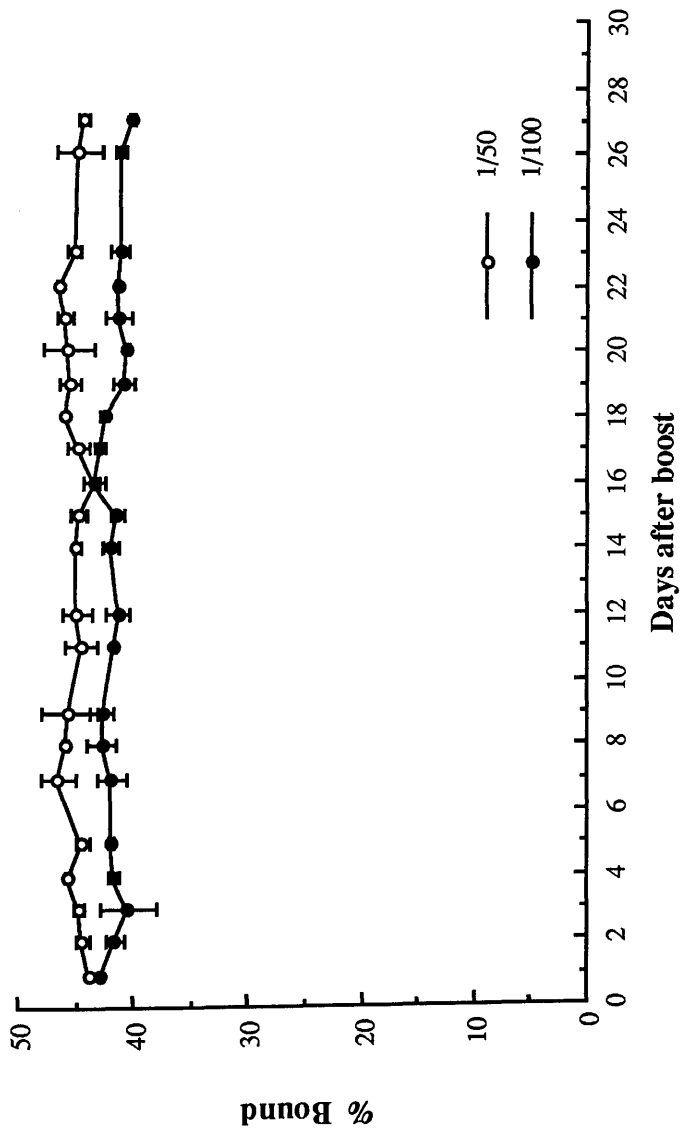
Figure 3.20

Antibody levels in the egg yolks of an immunised chicken (28/5) over a one month period.

6 DONKEYS

Two donkeys were immunised and boosted three times with a human insulin-ovalbumin conjugate (Chapter 2, Section 3.4) before test bleeds were taken and compared with the guinea-pig antiserum (Figure 3.21A). No response was obtained. The animals were boosted three times with porcine insulin and further test bleeds taken. The antisera produced were of low titre (Figure 3.21B) and low avidity as indicated by the percentage displacements achieved with 200 mU/l human insulin (Table 3.3). The IgG fraction of the antiserum raised in donkey 42 was coupled to Sepharose^R CL-4B and assessed in an IRMA to measure insulin as described for the chicken antiserum (Section 5.3). Similar results were obtained, ie, no binding of insulin over the range of insulin concentrations tested.

The data presented in Figure 3.21 suggest that the epitope required for an antibody response to insulin in donkeys is masked by conjugation to ovalbumin. From the observations made in Section 3.3 of this chapter, it would appear that, as with sheep, the A-chain loop is immunodominant in donkeys.
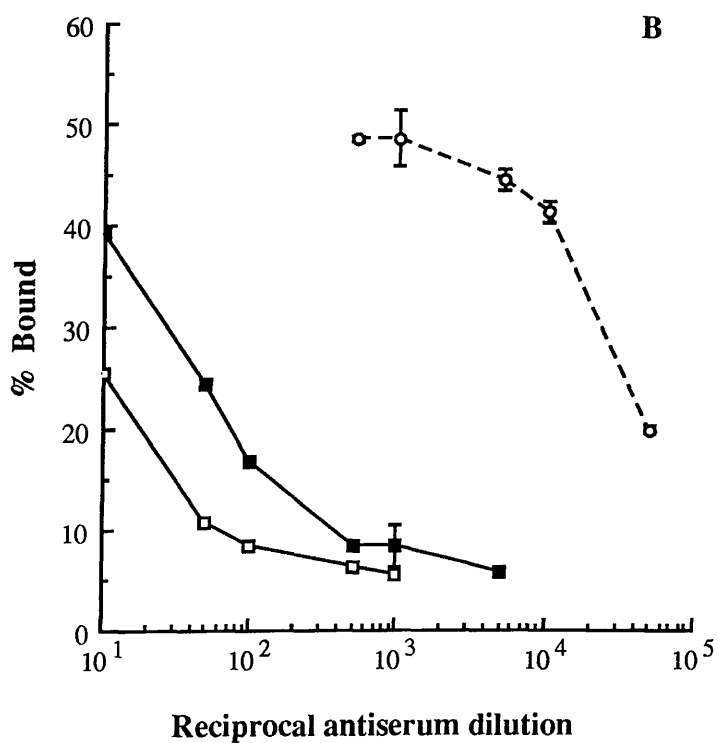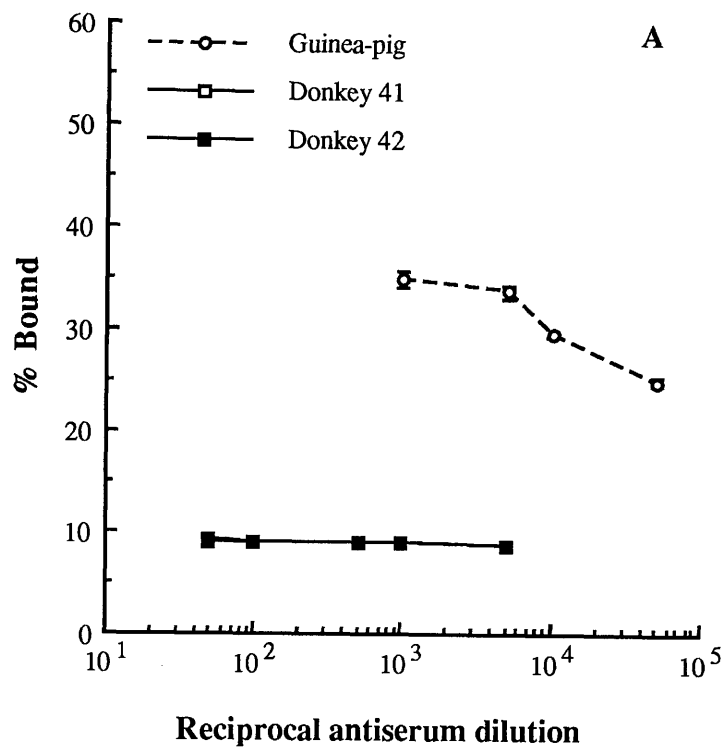
Figure 3.21

Comparison of insulin antisera raised in Strain 2 guinea-pigs, and donkeys after three boosts with a human insulin-ovalbumin conjugate (A) and three further boosts with porcine insulin (B).

TABLE 3.3

COMPARISON OF AVIDITIES OF DONKEY ANTISERA IN
RELATION TO A GUINEA-PIG ANTISERUM

| Donkey number | % Displacement ±SD | | |
| | Dilution of antisera | | |
	1:10	1:50	1:100
41	0	0	0
42	0.8±0.3	11.5±9.7	24.2±4.7
Guinea-pig*	NA	55.4±0.8	66.1±1.4

NA = not assessed   *Multiply dilutions by 10^3

CHAPTER 4

PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES

1 PRODUCTION OF MONOCLONAL ANTIBODIES

Monoclonal antibodies were produced as described in Chapter 2, Section 7.

Table 4.1 summarises the relevant details of four hybridisation experiments which generated a total of ten antibody secreting cell lines, all of which have maintained antibody production both in culture and in regenerated frozen stocks to date. More than 60% of hybridomas detected in the initial screen were lost prior to and at the primary cloning step due to either cell death or loss of antibody production. No hybridomas were lost at or after the second cloning.

Six monoclonal antibodies reactive to both insulin and proinsulin were produced using either porcine insulin (3 McAbs) or human proinsulin (3 McAbs) as immunogen. Three of the remaining four antibodies generated using human proinsulin as immunogen, were specific for proinsulin and one was directed against the C-peptide moiety of proinsulin.

The initial hybridisation experiment with human proinsulin as immunogen did not yield any antibody-secreting hybridomas which were stable through two cloning steps. This failure was attributed in part to the poor quality ^{125}I-human proinsulin, purified by gel filtration, used for screening. This label was unstable when stored at 4°C (Chapter 5, Section 1.2) and shown to be incapable of binding to all the antibodies present in the serum of mice immunised with proinsulin (Chapter 3, Section 4.2). The remaining two fusions were screened using ^{125}I-proinsulin which had been purified by reverse-phase HPLC.

The results from the first of the three proinsulin fusions also differed in the pattern of epitope specificity observed in the antibody-positive supernatants. The apparent low level of antibodies reactive to the insulin moiety of proinsulin was almost certainly due to the inadvertent use of iodinated bovine rather than porcine insulin in the screening assays. The commercial source of this preparation provided random batches of either porcine or bovine insulin without prior notification. This could

TABLE 4.1

PRODUCTION OF MONOCLONAL ANTIBODIES

Fusion date	Immunogen	Antibody-positive supernatants on initial screen[1]	Epitope specificity of supernatants[2]		Hybridomas cloned[3]		Hybridoma/antibody	Antigen specificity[4] identification
			Insulin	Proinsulin/C-peptide	x1	x2		
7.12.84	Porcine insulin	9	NA		7	3	ID1/C10* ID4/E5 ID1/A4	Insulin/Proinsulin -
19.7.85	Human proinsulin	9	1	8	2	0	-	-
6.6.86	Human proinsulin	12	7	5	6	3	PD4/H4 PF1/B9 PF2/B5	C-peptide/Proinsulin Insulin/Proinsulin
10.4.87	Human proinsulin	14	10	4	4	4	PH5/B5** PI2/G4 PI3/B10 PH4/B1**	Proinsulin Insulin/Proinsulin

NA = not applicable   *used in insulin IRMA   **used in proinsulin IRMA

[1] The total number of supernatants screened per fusion experiment was 1440.

[2] Epitope specificity refers to the region of the proinsulin molecule bound by a particular antibody, ie insulin, C-peptide or proinsulin itself.

[3] Hybridomas refers to cells contained in individual wells from which antibody-positive supernatants were aspirated.

[4] Antigen specificity refers to the hormones bound by the monoclonal antibodies secreted from 'stable' cell lines.

have caused the misclassification of some antibodies as unreactive to insulin because the amino acid differences between porcine and bovine insulin occur in the region where anti-insulin McAbs, raised using proinsulin as immunogen, would be likely to bind (Section 2.2 of this chapter).

2 CHARACTERISATION OF MONOCLONAL ANTIBODIES

2.1 General

Monoclonal antibodies were characterised for antigen binding specificity by RIA with ^{125}I-labelled human proinsulin, porcine insulin and human C-peptide (Table 4.2). Six antibodies bound both insulin and proinsulin, three proinsulin only and one C-peptide and proinsulin. Antibodies within each group have been classified as anti-insulin, anti-proinsulin and anti-C-peptide respectively throughout the remainder of this thesis.

Details of the isotypes of all the antibodies and the avidity constants of four are shown in Table 4.3. Eight of the antibodies were of the IgG_1 and two of the IgG_{2a} subclass. The avidity constants, calculated from the Scatchard plots shown in Figures 4.1 and 4.2, ranged from 1.2×10^8 to 2.1×10^9 l/mol.

2.2 Anti-Insulin Monoclonal Antibodies

Displacement curves with porcine insulin were performed to determine the relative avidities of the six insulin McAbs (Figure 4.3). Two groups of three antibodies were clearly distinguished and the division correlated with the hormone used for immunisation:porcine insulin resulted in the higher avidity (approximately 10^9 l/mol) and human proinsulin in the lower avidity (approximately 10^8 l/mol) groups.

This same division of the antibodies was apparent when species specificity studies were carried out to assess epitope recognition. Figure 4.4 shows the pattern of results obtained for the three McAbs raised using porcine insulin. Porcine, human,

TABLE 4.2

BINDING OF MONOCLONAL ANTIBODIES TO ^{125}I-LABELLED
PROINSULIN, INSULIN AND C-PEPTIDE

Antibody	% Bound ± SD		
	^{125}I-Proinsulin	^{125}I-Insulin	^{125}I-C-peptide
ID1/C10*	65.6±0.5	48.8±0.5	NA
ID4/E5	78.3±0.7	58.2±0.5	NA
ID1/A4	75.2±0.6	59.7±0.3	NA
PD4/H4	31.6±0.9	1.1±0.1	16.6±0.5
PF1/B9	53.3±0.5	42.3±0.6	1.1±0.0
PF2/B5	49.4±0.3	39.8±0.7	1.1±0.1
PH5/B5**	74.2±0.0	0.9±0.0	0.6±0.0
PI2/G4	30.0±2.1	0.7±0.0	0.7±0.1
PI3/B10	58.2±1.8	0.9±0.1	0.7±0.0
PH4/B1**	81.2±0.6	61.2±0.4	0.6±0.1
NSB	1.6±0.0	1.1±0.1	0.7±0.1

NA = not assessed   *used in insulin IRMA   **used in proinsulin IRMA

^{125}I-labelled proinsulin and insulin were prepared as described in Chapter 2, Section 5.2. ^{125}I-C-peptide was obtained from Novo. Details of the method are given in Chapter 2, Section 5.5.

TABLE 4.3

ISOTYPES AND AVIDITY CONSTANTS OF MONOCLONAL ANTIBODIES PRODUCED

Antibody code	Antigen specificity	Isotype	Avidity constant (l/mol)
ID1/C10*		IgG_1	2.1×10^9
ID4/E5		IgG_1	2.1×10^9
ID1/A4		IgG_1	NA
PF1/B9	Insulin/Proinsulin	IgG_1	NA
PF2/B5		IgG_1	NA
PH4/B1**		IgG_{2a}	1.2×10^8
PI2/G4		IgG_1	NA
PI3/B10	Proinsulin	IgG_{2a}	NA
PH5/B5**		IgG_1	1.3×10^8
PD4/H4	C-peptide/Proinsulin	IgG_1	NA

NA = not assessed   *used in insulin IRMA   ** used in proinsulin IRMA

Isotyping was performed by the Ouchterlony double diffusion technique (Chapter 2, Section 4.1) with sheep anti-mouse class and subclass specific antisera (Serotec). Affinity constants were determined by Scatchard Analysis (Chapter 2, Section 5.6).
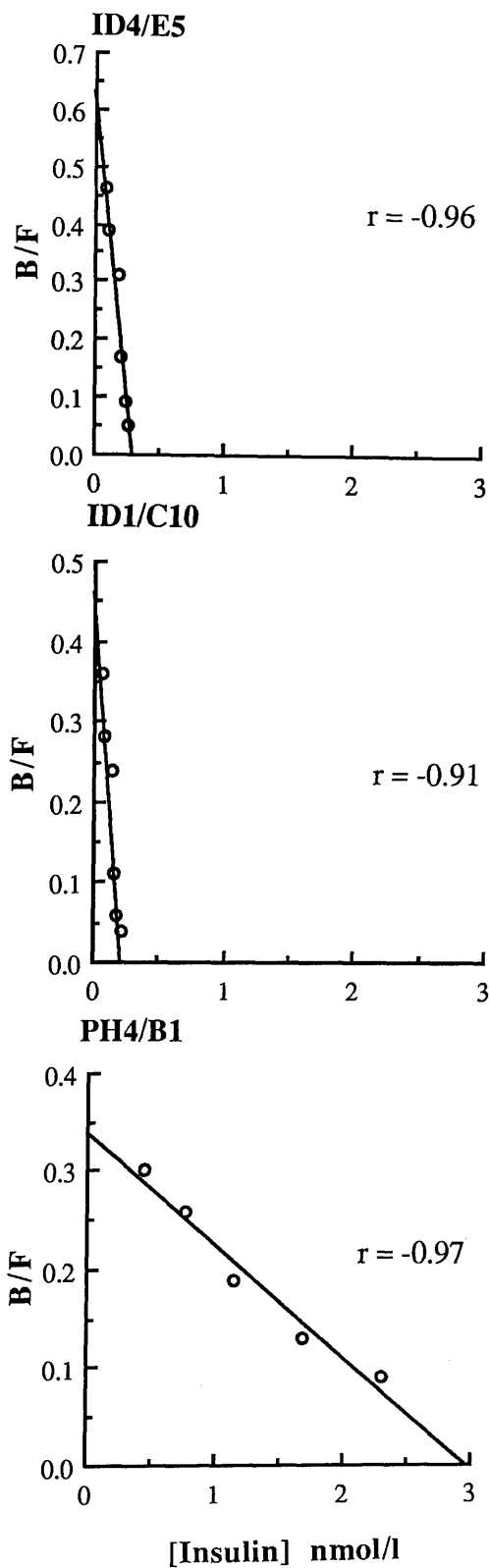
Figure 4.1

Scatchard plots of three anti-insulin monoclonal antibodies.

Scatchard analysis was performed as described in Chapter 2,
Section 5.6
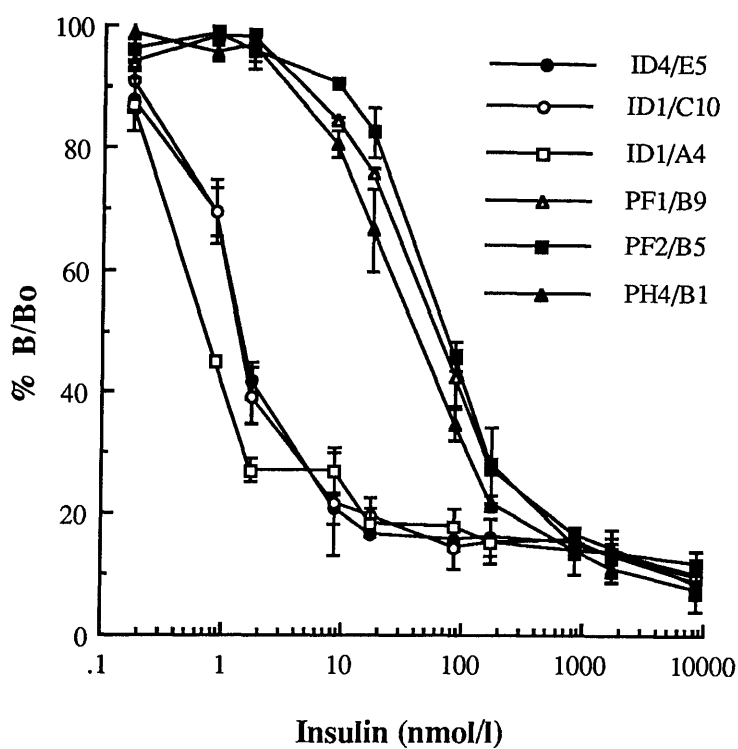
Figure 4.3

Relative avidities of the anti-insulin monoclonal antibodies for porcine insulin.

Details of the method are outlined in Chapter 2, Section 5.6
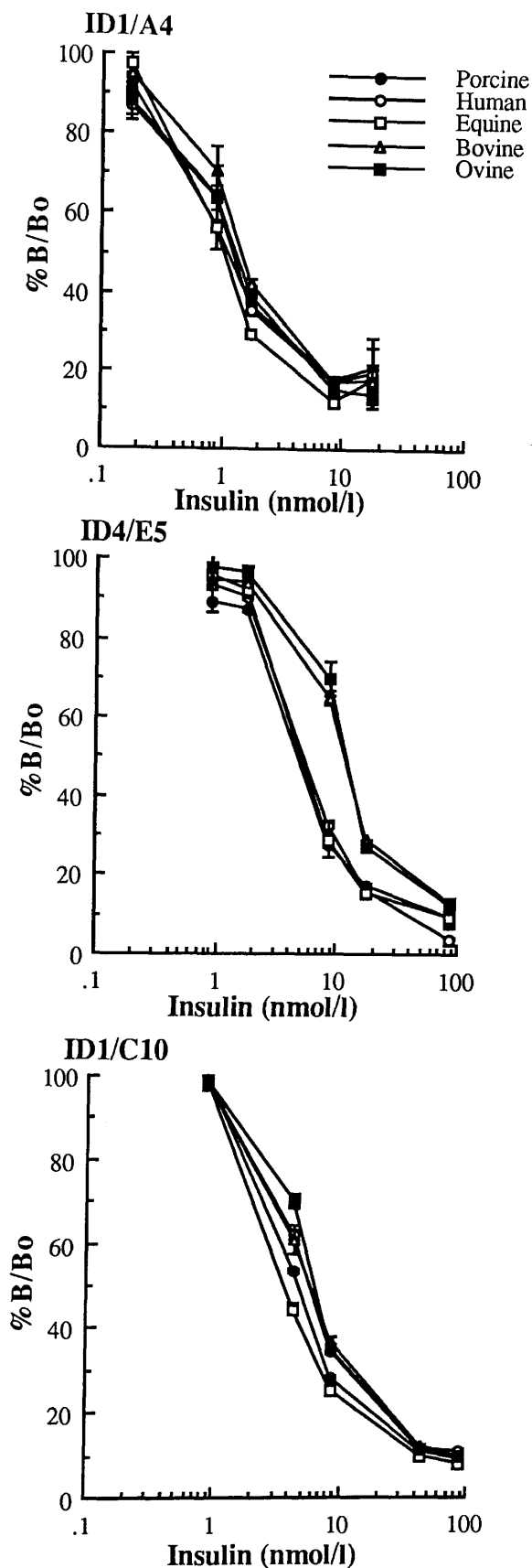
Figure 4.4

Species inhibition curves of the three anti-insulin monoclonal antibodies raised using porcine insulin as immunogen.

Details of the method are outlined in Chapter 2, Section 5.6.

equine, bovine and ovine insulins were equally effective in displacing ^{125}I-porcine insulin from two of the three antibodies. Monoclonal antibody ID4/E5 required slightly higher doses of bovine and ovine insulins to cause a similar level of displacement to the other three insulins. As equine, bovine and ovine insulins differ from porcine at the A chain loop (ie, amino acid positions 8-10 on the A chain) by 1, 2 and 3 amino acids respectively, these antibodies do not react with this epitope. There may be some interaction of ID4/E5 with this portion of the insulin molecule but later results (Chapter 5, Section 2.2) suggest that this is not the complete binding site for this antibody. The carboxyl terminal of the B chain also appears not to be involved in epitope recognition as porcine and human insulin differ by one amino acid at position B30.

In contrast, evaluation of two of the three McAbs raised using human proinsulin as immunogen resulted in a pattern of binding suggesting that they were directed against the A chain loop (Figure 4.5). Increasing concentrations of porcine and human, equine, bovine and ovine insulin were required to cause the same displacement of ^{125}I-porcine insulin from these antibodies.

An important feature of all the species specificity curves is the parallelism observed between the curves for any one antibody. This suggests that the antibody under evaluation was binding to the same site on each of the different species insulin.

2.3 Anti-Proinsulin/C-Peptide Monoclonal Antibodies

The relative avidities of the four McAbs in this group, for intact human proinsulin, are shown in Figure 4.6. The doses of proinsulin required to achieve displacement are of the correct order of magnitude (ie, nmol) but are not accurate due to the use of a secondary standard material prior to the acquisition of the primary proinsulin standard WHO 84/611. The assay was not repeated when the NIBSC standard became available since comparative information could be extracted from the results as presented.

PH4/B1

PF1/B9

Legend:
- □ Porcine
- ■ Human
- ○ Equine
- ● Bovine
- △ Ovine
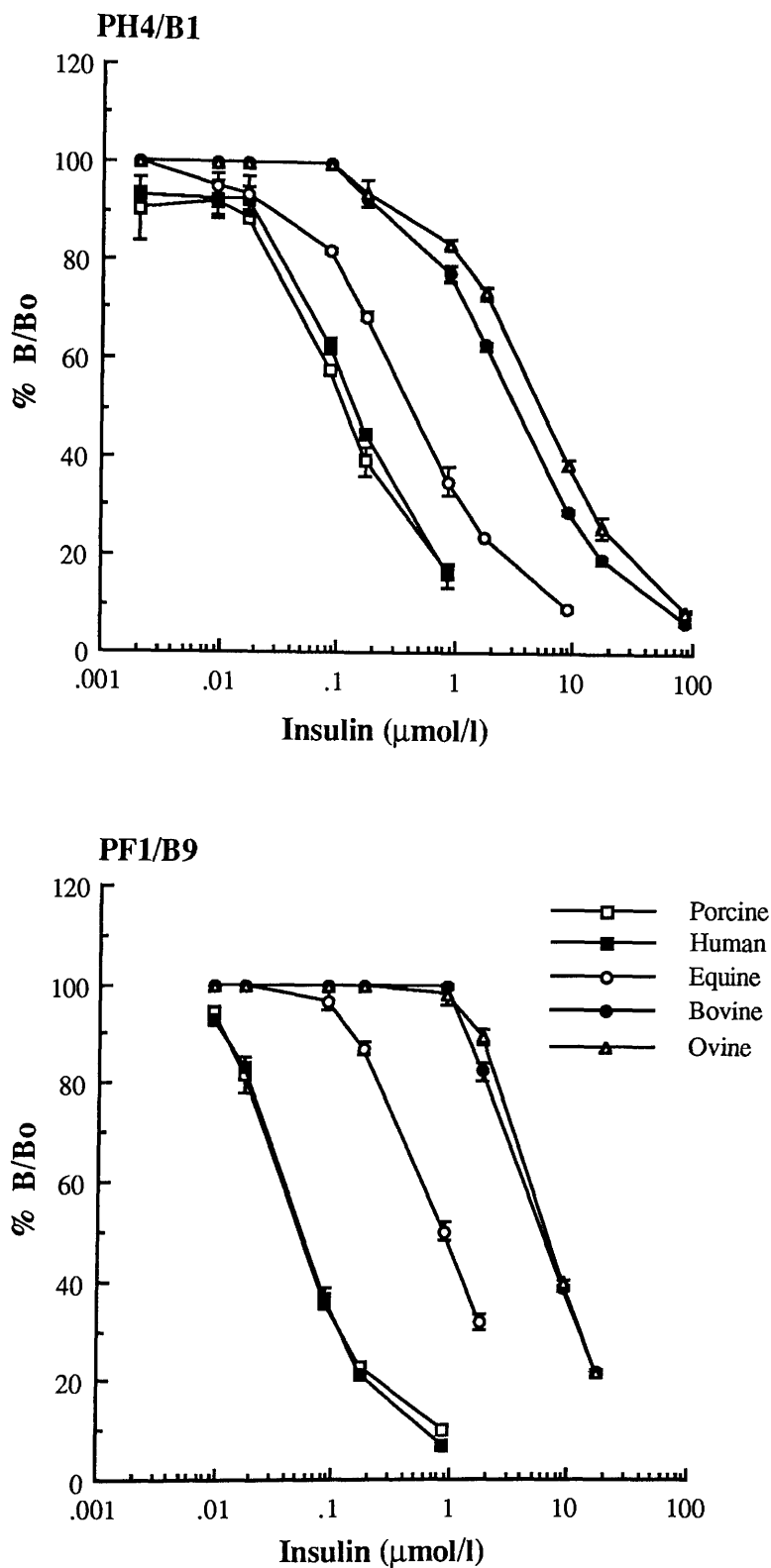
Figure 4.5

Species inhibition curves of two of the three anti-insulin monoclonal antibodies raised using human proinsulin as immunogen.

Details of the method are outlined in Chapter 2, Section 5.6
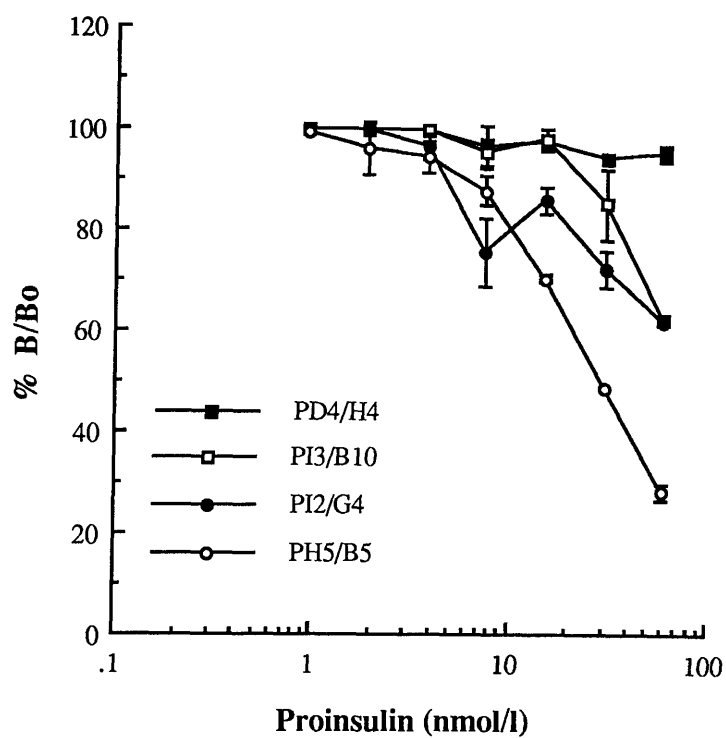
Figure 4.6

Relative avidities of the three anti-proinsulin and one anti-C-peptide monoclonal antibodies for intact human proinsulin.

Details of the method are outlined in Chapter 2, Section 5.6.

The displacement of ^{125}I-intact human proinsulin from antibody PH5/B5 by intact, split and des-amino proinsulins is shown in Figure 4.7. Complete displacement curves were not achieved due to the scarcity of these materials. However, it is possible to detect an emerging pattern of specificity since only the 32-33 split and des 31-32 forms failed to cause displacement, suggesting specificity at the B-C chain junction, ie, B chain of insulin and C-peptide. This observation was confirmed during specificity studies on the final proinsulin IRMA, developed using this antibody (Chapter 5, Figure 5.51 and Table 5.15). Epitope studies were not attempted on the other three low avidity antibodies.

Iodinated human proinsulin and C-peptide were not displaced from the C-peptide McAb (PD4/H4) with doses of cold proinsulin and C-peptide up to 60 nmol/l and 5 nmol/l respectively.

3 DISCUSSION

The hybridisation experiments performed resulted in small numbers of antigen-specific antibody-producing hybridomas being detected at the initial screen, and a subsequent low yield of stable hybridomas following cloning. This observation is in agreement with the reported yields of other groups raising McAbs to insulin and proinsulin (39, 114, 115, 116, 117).

Within each fusion, the antibodies produced exhibited very similar specificities for insulin, proinsulin or C-peptide. This suggests that the antibody response of mice to small molecules such as insulin and proinsulin may in practice be limited to a single or small number of epitopes. A similar observation was made in Chapter 3 following the immunisation of larger animals with insulin. It was also noted that in the two fusions in which proinsulin was used as immunogen, either C-peptide or proinsulin-specific McAbs were obtained. It is suggested that this might reflect subtle differences in the antibody response of individual mice to a particular
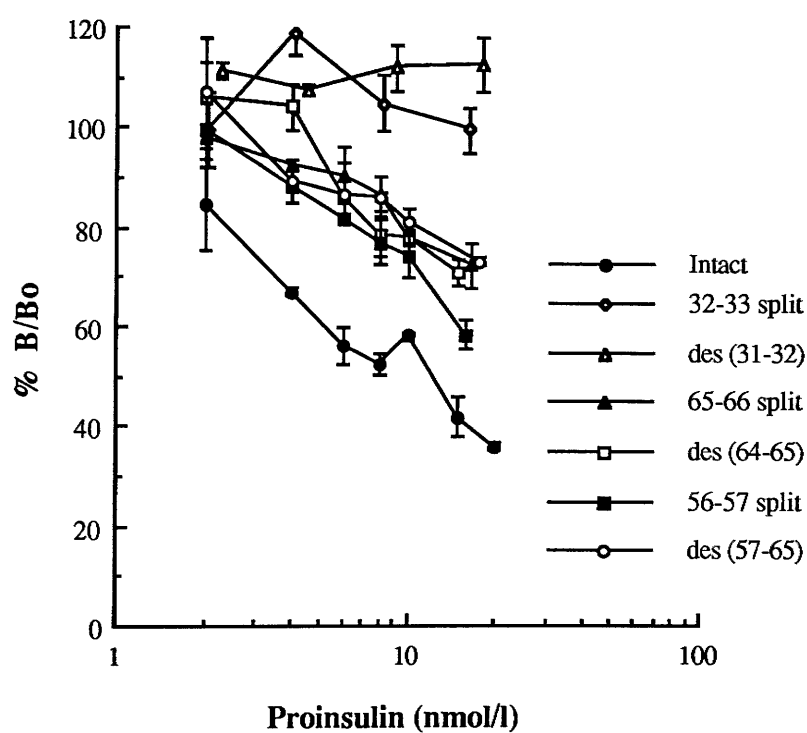
Figure 4.7

Specificity of monoclonal antibody PH5/B5 for split and des-amino proinsulins.

immunogen, even within an inbred strain. If this was the case, screening of mice prior to fusion to ascertain the presence of particular subsets of antibodies, as has been advocated previously (74), would be advantageous.

The importance of establishing a screening procedure which is capable of detecting the antibodies of interest is highlighted by the failure of the initial hybridisation experiment which was attributed to the use of an unsuitable ^{125}I-proinsulin preparation (Chapter 3, Section 4.3).

CHAPTER 5

ASSAY DEVELOPMENT

1 REAGENT PRODUCTION

1.1 Immunoglobulin Fractionation

Monoclonal antibodies were routinely purified from ascitic fluid by caprylic acid fractionation (Chapter 2, Section 4.3) prior to iodination or coupling to Sepharose[R] CL-4B. The purity of these antibodies was assessed by anion-exchange HPLC (Chapter 2, Section 3.5). Representative elution profiles of ascitic fluid before and after fractionation are shown in Figure 5.1 A and B respectively. All ascitic fluid evaluated contained transferrin and albumin in addition to the McAb, but the ratio of the three proteins varied. The McAb shown in part B was estimated to be greater than 90% pure.

1.2 Iodination of Proinsulin

Gel Filtration Chromatography

Initially, ^{125}I-labelled proinsulin was purified by gel filtration chromatography on Sephadex G50 (Chapter 2, Section 5.2). A typical elution profile is shown in Figure 5.2. The immunoreactivity of this preparation was evaluated by constructing dilution curves with a polyclonal (guinea-pig) and monoclonal (ID4/E5) anti-insulin and comparing them with a dilution curve constructed using ^{125}I-insulin and the monoclonal anti-insulin (Figure 5.3). The maximum binding of ^{125}I-proinsulin purified in this way was 30% (polyclonal) and 25% (monoclonal) compared with 60% (monoclonal) for the ^{125}I-insulin. Aliquots of this preparation were stored at 4°C or -20°C for one week and re-evaluated with the two insulin antibodies as described above (Figure 5.4). The dilution curves obtained indicated that the aliquot stored at 4°C had lost all immunoreactivity after one week and therefore storage at -20°C was recommended.
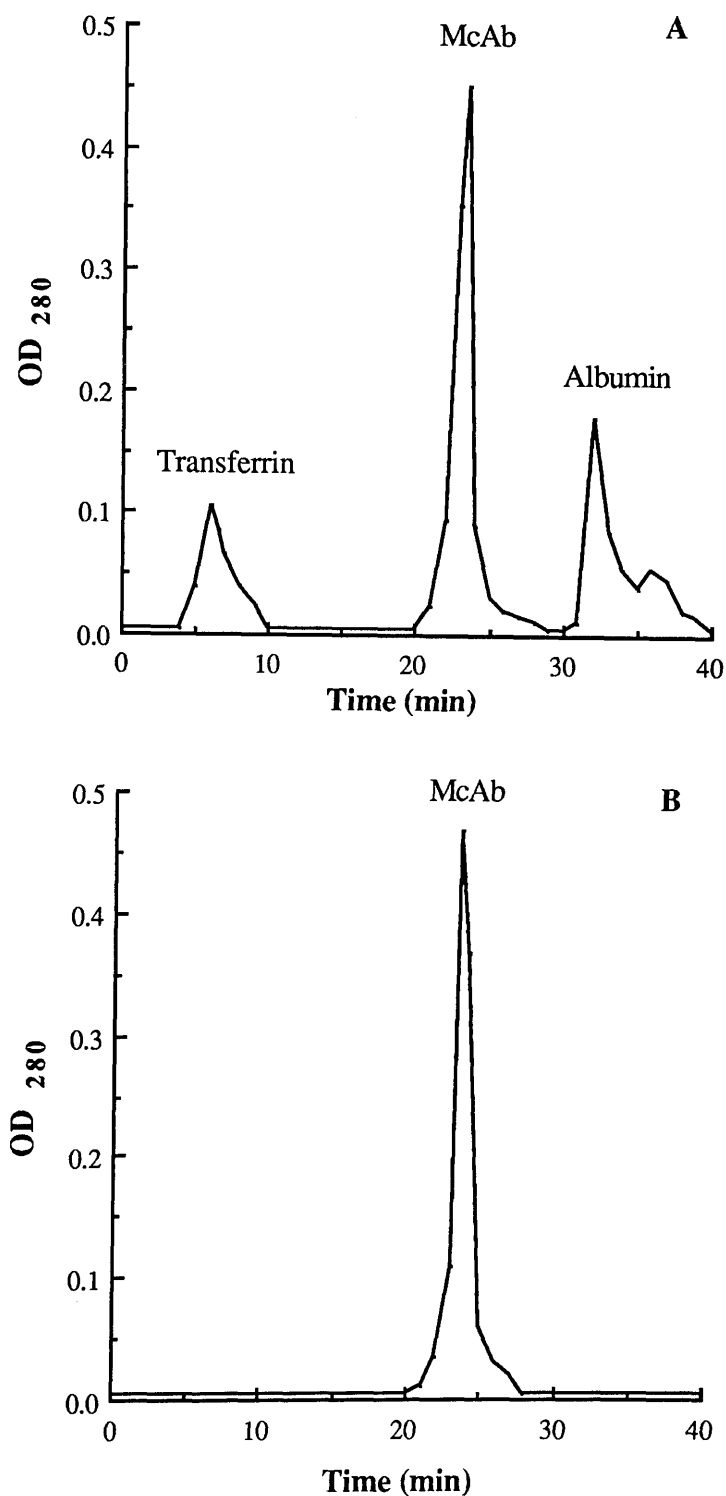
Figure 5.1

Elution profiles, from HPLC on an anion-exchange column, of ascitic fluid containing monoclonal anti-insulin before (A) and after (B) caprylic acid fractionation.
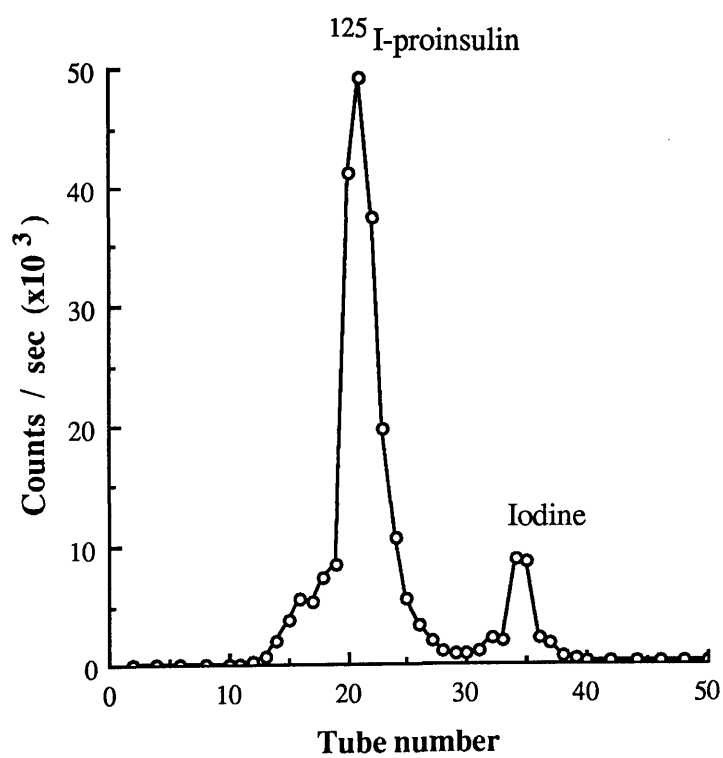
Figure 5.2

Representative elution profile of ^{125}I-proinsulin on Sephadex G50.
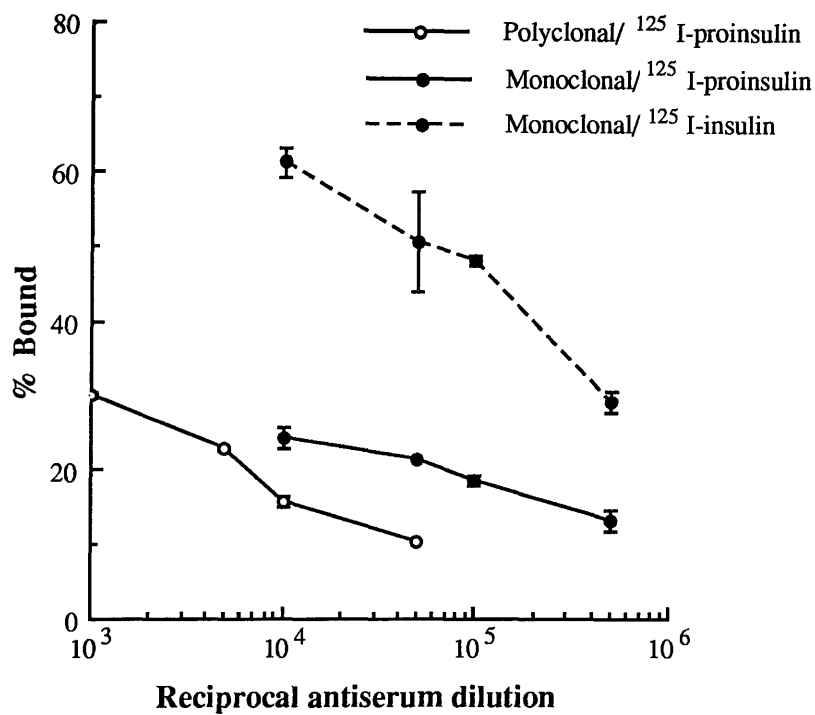
Figure 5.3

Assessment of ^{125}I-proinsulin, purified by gel filtration chromatography, using a polyclonal and monoclonal anti-insulin.
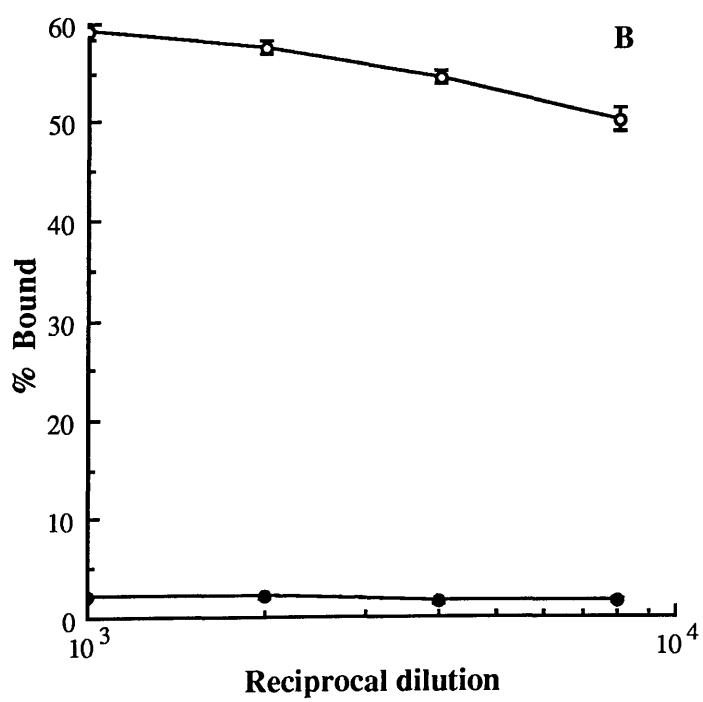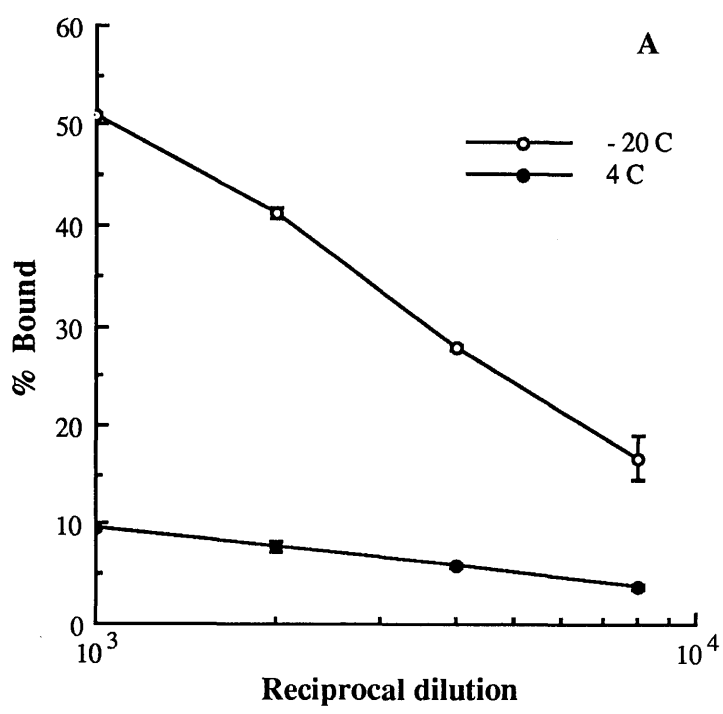
Figure 5.4

Dilution curves prepared with a polyclonal (A) and monoclonal (B) anti-insulin and ^{125}I-proinsulin stored at 4°C or -20°C.

^{125}I-proinsulin, prepared as described above, was found to be unsuitable for use as a screening reagent for the detection of antibodies reactive to proinsulin in the serum of mice immunised with proinsulin, (as described in Chapter 3, Section 4.2), and also for the detection of proinsulin-specific McAbs in hybridisation experiments (as described in Chapter 4, Section 1). Therefore, an alternative method of purification, reverse-phase HPLC, was investigated.

Reverse-Phase HPLC

Starting conditions for the purification of ^{125}I-proinsulin by reverse-phase HPLC were based on published results of similar work on ^{125}I-insulin (118). Details of the column and elution buffers used are given in Chapter 2, Section 5.2. Fractions of the column eluant were collected at one minute intervals and the appropriate peak fractions pooled and tested for proinsulin immunoreactivity. The results are presented in tabular form as the percentage bound of each pool at a single dilution of polyclonal (guinea-pig) and monoclonal (ID4/E5) anti-insulin. The percentage of iodinated insulin bound by both antibodies is also shown for reference purposes. The only factor changed between experiments was the concentration of acetonitrile.

Initially, a concentration of 31.5% acetonitrile was used as this was between the concentrations, 31.2% and 32.5%, previously shown to elute split and intact proinsulins respectively from a similar C18 column (119). The elution profile obtained is shown in Figure 5.5. From Table 5.1, it can be seen that peaks 1-3 did not show any proinsulin reactivity and these were assumed to contain unreacted iodine. Pools 4-9, collected from 60-230 minutes at 31.5% acetonitrile, all showed proinsulin immunoreactivity as did peaks 10 and 11 collected while cleaning the column with 95% acetonitrile.

The concentration of acetonitrile was raised to 32.5% and a freshly iodinated aliquot of ^{125}I-proinsulin applied (Figure 5.6 and Table 5.2). Peaks 1-5 appeared to
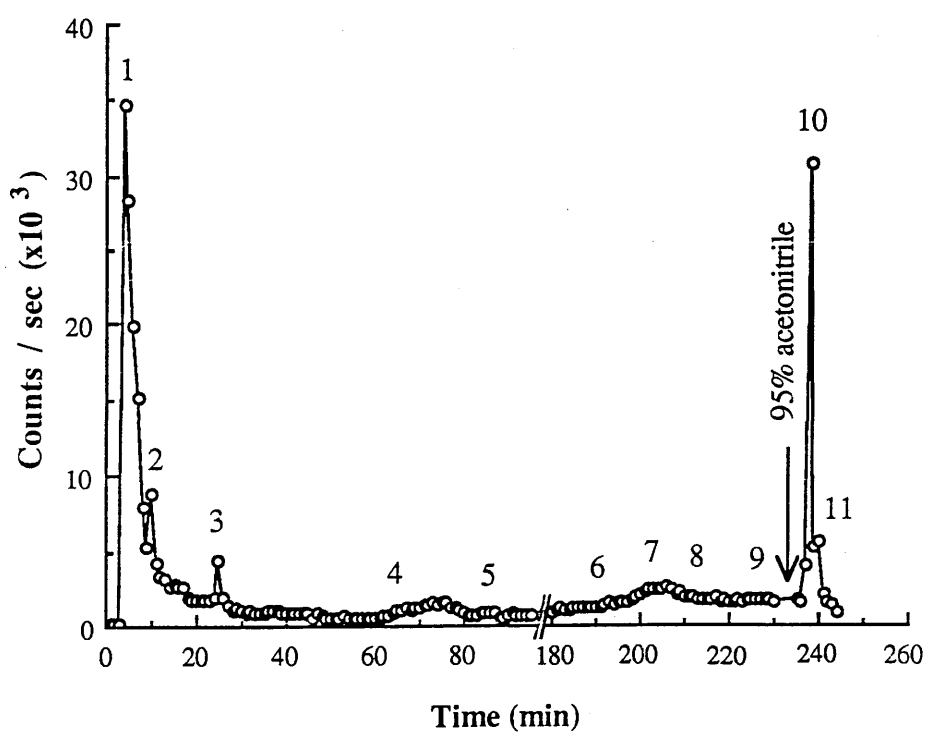
Figure 5.5

Purification of ^{125}I-proinsulin by reverse-phase HPLC at 31.5% acetonitrile.

TABLE 5.1

ASSESSMENT OF ^{125}I-PROINSULIN PREPARED BY REVERSE-PHASE
HPLC AT 31.5% ACETONITRILE

Proinsulin pool	% Bound±SD	
	Polyclonal anti-insulin	Monoclonal anti-insulin
Insulin*	58.3±0.8	40.2±0.4
1	0.5±0.1	0.6±0.0
2	0.7±0.1	0.7±0.1
3	1.2±0.1	1.0±0.0
4	47.8±0.2	56.6±0.7
5	33.0±0.5	37.4±1.7
6	51.0±3.8	63.9±0.9
7	68.0±1.0	80.0±0.2
8	60.2±1.9	70.0±0.8
9	60.8±0.3	69.7±0.3
10	62.9±0.9	70.6±2.7
11	22.1±0.5	24.1±0.9

*[125]I-labelled porcine insulin prepared as described in Chapter 2, Section 5.2
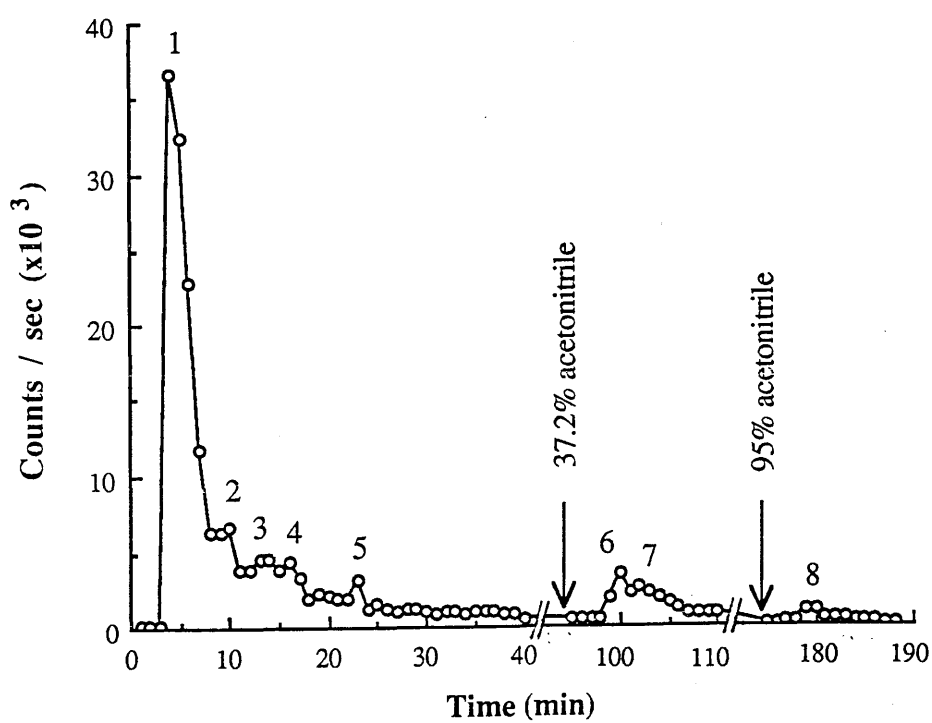
Figure 5.6

Purification of ^{125}I-proinsulin by reverse-phase HPLC at 32.5% acetonitrile.

TABLE 5.2

ASSESSMENT OF ^{125}I-PROINSULIN PREPARED BY REVERSE-PHASE
HPLC AT 32.5% ACETONITRILE

Proinsulin pool	% Bound±SD	
	Polyclonal anti-insulin	Monoclonal anti-insulin
Insulin*	66.4±0.1	52.0±0.7
1	0.6±0.1	0.6±0.0
2	0.7±0.0	0.8±0.0
3	1.1±0.0	1.1±0.1
4	1.1±0.1	1.1±0.3
5	1.2±0.0	1.0±0.1
6	1.2±0.0	1.0±0.1
7	59.2±1.0	65.7±0.1
8	54.3±0.3	56.4±1.0

*^{125}I-labelled porcine insulin prepared as described in Chapter 2, Section 5.2

contain unreacted iodine. No further peaks were detected up to 95 minutes at 32.5% acetonitrile and the concentration was increased to 37.2%. Two peaks eluted, the first of which appeared to be unreacted iodine (peak 6) while the second (peak 7) contained ^{125}I-proinsulin. A final peak of ^{125}I-proinsulin was eluted in 95% acetonitrile. The lower recovery of the ^{125}I-proinsulin from the column during this run compared with the previous run (31.5% acetonitrile) suggested that the iodination had been unsuccessful, with poor incorporation of ^{125}I into the proinsulin. However, the time taken to elute the unreacted iodine from the column appeared to be similar for both runs suggesting that a higher concentration of acetonitrile was necessary to elute the ^{125}I-labelled proinsulin in a shorter time.

A further aliquot of proinsulin was iodinated and the concentration of acetonitrile adjusted to 33%. Peaks 1 and 2 appeared to contain unreacted iodine while peaks 3-8 showed proinsulin reactivity (Figure 5.7 and Table 5.3). The peak eluted in 95% acetonitrile was not collected.

Increasing the acetonitrile concentration from 31.5% to 33% appeared to decrease the number of peaks containing unreacted iodine from three to two and also appeared to result in most of the ^{125}I-labelled proinsulin being eluted by 70 minutes as opposed to 230 minutes. Therefore, the acetonitrile concentration was increased further to 34%. This resulted in the first elution profile in which distinct peaks were observed (Figure 5.8). The pools of ^{125}I-proinsulin collected in this experiment were evaluated using a proinsulin-specific McAb (Dr K Siddle, Cambridge), in addition to the two insulin antibodies. Peaks 1 and 2 contained unreacted iodine while peaks 3-12 contained ^{125}I-proinsulin (Table 5.4). The majority of the ^{125}I-labelled proinsulin was eluted by 60 minutes. Peak 6 exhibited the highest percentage binding with all three antibodies and these fractions were collected in subsequent runs to provide a ^{125}I-proinsulin preparation suitable for use in screening procedures for the detection of antibodies reactive to proinsulin (Chapter 3, Section 4.2 and Chapter 4, Section 1).
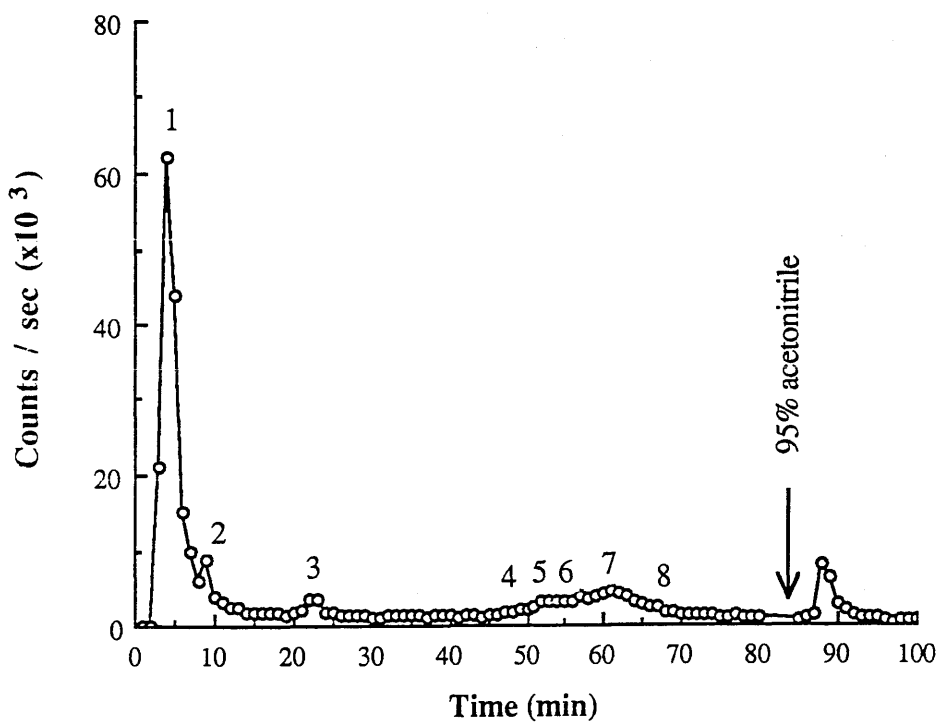
Figure 5.7

Purification of ^{125}I-proinsulin by reverse-phase HPLC at 33% acetonitrile.

TABLE 5.3

ASSESSMENT OF ^{125}I-PROINSULIN PREPARED BY REVERSE-PHASE
HPLC AT 33% ACETONITRILE

Proinsulin pool	% Bound± S D	
	Polyclonal anti-insulin	Monoclonal anti-insulin
Insulin*	64.2±0.9	43.2±1.5
1	0.4±0.0	0.4±0.1
2	0.5±0.3	0.5±0.1
3	30.4±1.3	35.7±0.8
4	56.7±0.9	67.6±1.0
5	58.0±0.7	70.2±0.1
6	61.6±0.7	73.4±0.8
7	63.1±0.2	74.5±1.5
8	53.3±1.0	61.2±1.5

*^{125}I-labelled porcine insulin prepared as described in Chapter 2, Section 5.2
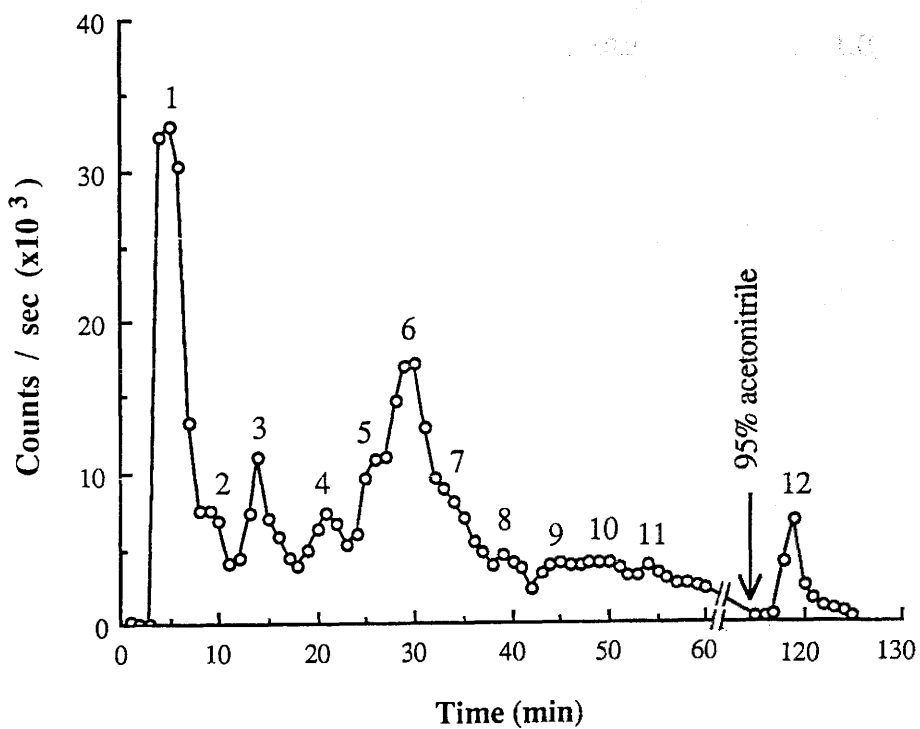
Figure 5.8

Purification of ^{125}I-proinsulin by reverse-phase HPLC at 34% acetonitrile.

TABLE 5.4

ASSESSMENT OF ^{125}I-PROINSULIN PREPARED BY REVERSE-PHASE
HPLC AT 34% ACETONITRILE

Proinsulin pool	% Bound±SD		
	Polyclonal anti-insulin	Monoclonal anti-insulin	Monoclonal anti-proinsulin
Insulin*	65.9±0.4	58.2±0.5	1.1±0.2
1	1.0±0.1	1.1±0.2	1.0±0.3
2	2.6±0.0	2.8±0.1	2.3±0.2
3	44.0±1.1	51.5±0.7	43.2±0.2
4	47.4±0.1	54.9±0.1	44.9±0.7
5	58.3±0.4	68.2±1.3	55.1±0.5
6	66.6±1.1	78.3±0.7	61.8±1.2
7	58.3±1.1	69.0±0.4	61.5±0.6
8	48.1±0.1	54.4±2.0	44.9±0.6
9	53.6±1.0	64.9±0.2	55.3±2.3
10	57.1±0.3	65.4±1.1	56.9±2.1
11	54.3±0.4	62.3±1.0	53.3±1.4
12	27.4±0.1	30.4±0.5	33.1±0.0

*[125]I-labelled porcine insulin prepared as described in Chapter 2, Section 5.2

The elution profile obtained with 34% acetonitrile appeared to be similar to that presented in the only report in the literature for the preparation of ^{125}I-labelled proinsulin purified by reverse-phase HPLC (120). This paper was unavailable at the time these experiments were started. The column packing described was similar to the one used above (C18) but the buffer system and concentration of acetonitrile differed. The elution buffer was 29.5% acetonitrile in 0.2 mol/l ammonium acetate pH 5.5 compared with the above elution buffer of 34% acetonitrile in 1% trifluoroacetic acid with the pH adjusted to 3 with triethylamine (1% TEATFA pH 3).

The elution profile of ^{125}I-proinsulin, from the column used for the previous experiments, using 29.5% acetonitrile in 0.2 mol/l ammonium acetate pH 5.5 (ie, the conditions described in reference 120) is shown in Figure 5.9 with the relevant results shown in Table 5.5. Peaks 1 and 2 appeared to contain unreacted iodine while pools 3-8 contained ^{125}I-proinsulin. Under these conditions proinsulin was not eluted from the column as distinct peaks. Similar percentage binding of the various pools of ^{125}I-proinsulin was achieved using this separation system as with that shown in Figure 5.8, but the maximum binding was observed at 60 minutes compared to 30 minutes respectively. There did not appear to be any obvious advantage in adopting the buffer system described in reference 120 as this would have necessitated re-optimisation of the concentration of acetonitrile. Therefore, an elution buffer of 34% acetonitrile in 1% TEATFA pH 3 was adopted.

1.3 Evaluation of Anti-Insulin Ascites for Use as Labelled Antibody

Ascitic fluid was produced as described in Chapter 2, Section 7.10 for each hybridoma. Taps of ascitic fluid from individual mice at the same stage were pooled to yield taps 1, 2, 3 etc. The antibody content of each pool was evaluated by constructing dilution curves with ^{125}I-insulin and the results for two anti-insulin hybridomas, ID1/C10 and ID4/E5, are shown in Figure 5.10 parts A and B
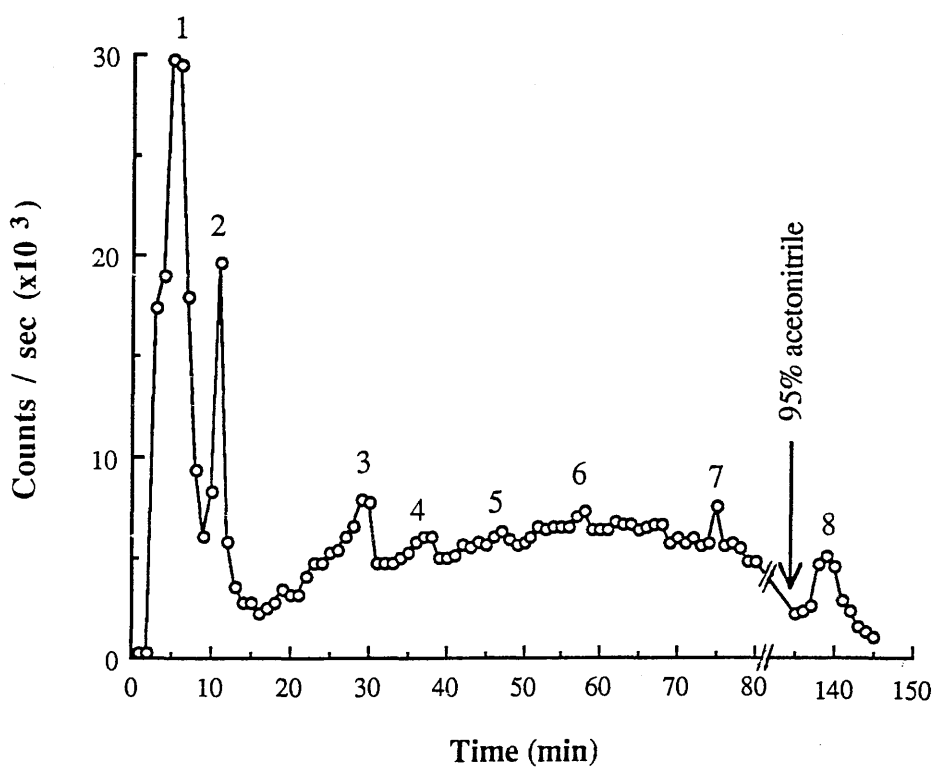
Figure 5.9

Purification of ^{125}I-proinsulin by reverse-phase HPLC at 29.5% acetonitrile in 0.2 mol/l ammonium acetate pH 5.5.

TABLE 5.5

ASSESSMENT OF ^{125}I-PROINSULIN PREPARED BY REVERSE-PHASE HPLC USING 0.2 MOL/L SODIUM ACETATE pH 5.5 CONTAINING 29.5% ACETONITRILE

Proinsulin pool	% Bound± S D	
	Polyclonal anti-insulin	Monoclonal anti-insulin
Insulin*	56.4±0.9	43.1±0.3
1	0.5±0.0	0.5±0.1
2	0.9±0.2	0.6±0.1
3	34.0±0.5	39.0±0.3
4	60.5±0.3	71.3±1.1
5	68.5±1.0	78.8±0.5
6	69.1±0.7	80.8±0.7
7	68.8±0.1	77.6±1.4
8	27.6±1.2	29.8±0.2

*^{125}I-labelled porcine insulin prepared as described in Chapter 2, Section 5.2
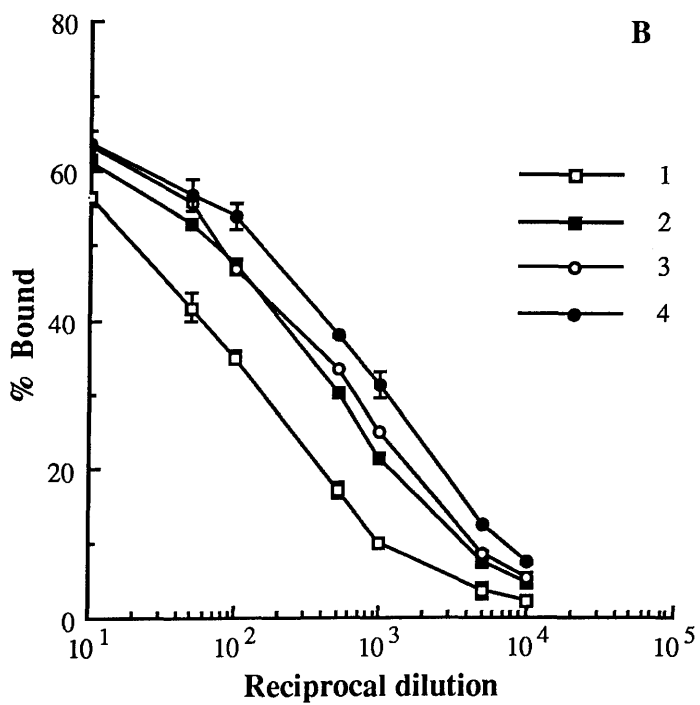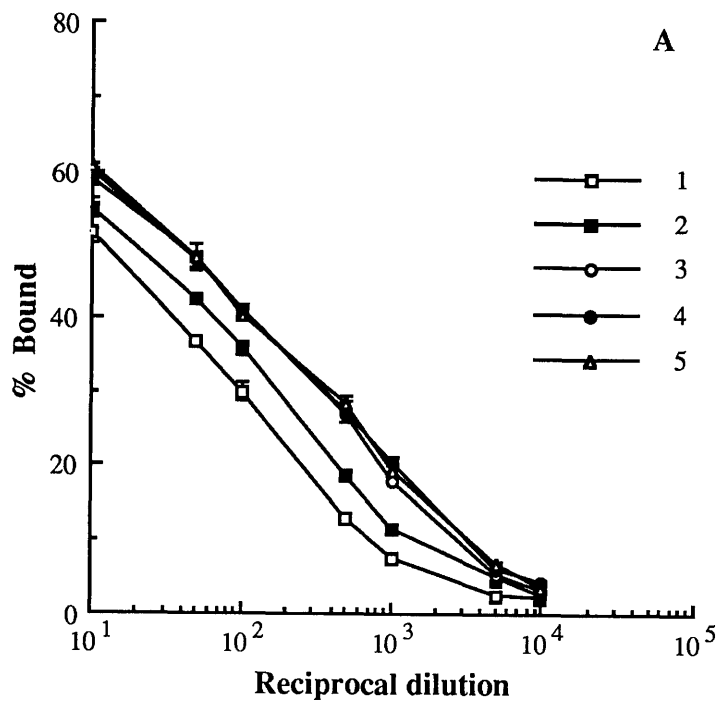
Figure 5.10

Dilution curves of sequential ascitic fluid taps from two anti-insulin hybridomas.

A - ID1/C10   B - ID4/E5

respectively. Ascitic fluid produced by hybridoma ID1/C10 appeared to show an increase in antibody content up to tap 3 after which there did not seem to be any further increase. In contrast, the antibody content of the ascites produced by hybridoma ID4/E5 appeared to increase up to the fourth tap. Aliquots of each of these ascites taps for each antibody were fractionated using caprylic acid and the protein content of the supernatants (>90% McAb as shown in Section 1.1 of this chapter) measured by a modified Lowry protein assay (Chapter 2, Section 3.1). The pattern of results obtained from this experiment (Table 5.6) were similar to those shown in Figure 5.10.

Aliquots of the purified McAb from each of the four taps of ascitic fluid produced by hybridoma ID4/E5 were iodinated and assessed in an immunoradiometric assay in conjunction with solid-phase coupled polyclonal anti-insulin (Figure 5.11, details as for Figure 5.1, Section 2.2 of this chapter). The specific radioactivities of the iodinated taps 1-4 were 0.65, 0.35, 0.37 and 0.32 MBq/μg respectively. A lower maximum binding was achieved with tap 1 (20%) compared with the subsequent three taps (40%). This could either be due to iodination damage to the antibody or the presence of non-specific host IgG in the ascitic fluid. The second, third and fourth taps all resulted in maximum binding levels of approximately 40%, but the rate of change in binding of the standard curve obtained with the second tap was less than that achieved with the third and fourth taps which were similar. This suggests the presence of non-specific IgG in tap 2. Therefore, subsequent experiments made use of tap number 3 onwards for the production of ^{125}I-labelled monoclonal antibodies.

1.4 Discussion

The antibody content (Table 5.6) and antibody titre (Figure 5.10) increased in sequential taps of ascitic fluid obtained from two hybridomas. This is in agreement with published work (82). When the taps from one hybridoma (ID4/E5) were

TABLE 5.6

MONOCLONAL ANTIBODY CONCENTRATION IN SEQUENTIAL
TAPS OF ASCITIC FLUID PRODUCED BY TWO ANTI-INSULIN
HYBRIDOMAS

Tap number	Protein concentration (mg/ml)	
	ID1/C10	ID4/E5
1	1.68	2.04
2	2.28	4.44
3	3.96	5.76
4	3.96	7.92
5	4.20	-
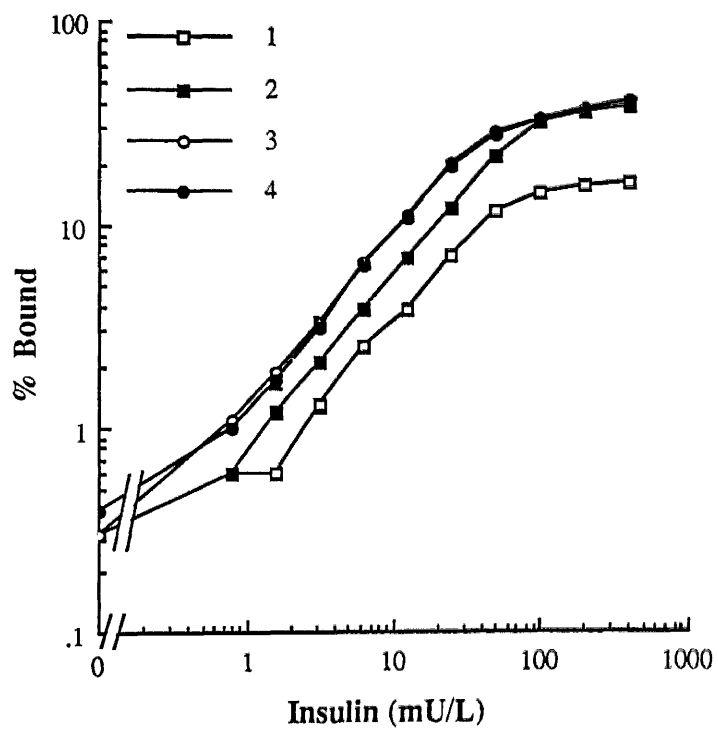
Figure 5.11

Insulin IRMA standard curves prepared with iodinated sequential taps of anti-insulin (ID4/E5).

iodinated and evaluated in an IRMA to measure insulin, the maximum binding increased with successive taps but the rate of rise of the standard curve at the lower end also increased even after the maximum possible binding had been achieved. Thus, the maximum binding achieved was not the only factor important in deciding which taps were suitable for use in IRMA techniques as has previously been suggested (88).

It was hoped that the results obtained in Figure 5.11, the comparison of iodinated sequential taps of insulin McAb ID4/E5, would correlate with either the protein concentration (>90% IgG) recovered by caprylic acid fractionation (Table 5.6) or the dilution curves performed (Figure 5.10B). However, neither set of results suggested that taps 3 and 4 would yield identical standard curves in the insulin IRMA and therefore were of no predictive value.

Due to the time which would be involved in iodinating IgG from each tap of ascitic fluid from each hybridoma to be assessed, it was subsequently assumed that IgG prepared from ascitic fluid collected at or after a third tapping, would result in optimal labelled antibody preparations.

2 INSULIN IRMA

2.1 General Guidelines on IRMA Development

The following points are common to all the assays discussed in this chapter unless otherwise stated.

(i)   Monoclonal antibodies were labelled with ^{125}I to a specific activity of 0.37-0.56 MBq/µg.

(ii)   The solid-phase matrix used was Sepharose[R] CL-4B.

(iii)   Solid-phase was added to assay tubes in a volume of 200 µl.

(iv)   Incubation steps in the presence of solid-phase were mixed continuously on an orbital shaker (Denley).

(v)   All incubations were at room temperature.

(vi) ^{125}I-labelled antibody was added to standard or sample in a primary incubation step prior to a secondary incubation with solid-phase coupled antibody.

(vii) Prior to counting, the solid-phase was washed three times with 0.2% Tween 20/0.9% sodium chloride as described in Chapter 2, Section 5.5.

(viii) Radioactivity was quantitated using a Nuclear Enterprises gamma counter (NE1600).

(ix) Results were calculated as the amount of label bound expressed as a percentage of the total label added (% bound).

2.2 Evaluation of Monoclonal Antibodies for Use in an Insulin IRMA

Separate aliquots of the six anti-insulin McAbs were radiolabelled or coupled to solid-phase. Each antibody was then paired, either as label or solid-phase, with each of the other five antibodies to determine those McAbs compatible for a two-site immunoradiometric assay (Table 5.7). No two antibodies were capable of binding to the insulin molecule simultaneously. Therefore, all subsequent experiments compare the various McAbs in conjunction with a solid-phase polyclonal anti-insulin raised in guinea-pigs (Chapter 3, Section 1).

Figure 5.12 shows the standard curves obtained with ^{125}I-labelled ID1/C10 and ID4/E5. Both antibodies gave similar results but ID1/C10 resulted in a slightly wider linear working range. The third McAb (ID1/A4) in this group (raised using porcine insulin as immunogen) gave poor incorporation of ^{125}I, and was not evaluated further. Monoclonal antibody ID1/C10 was also compared with PH4/B1, one of the three McAbs raised using human proinsulin as immunogen. These two antibodies have different avidity constants (2.1×10^9 and 1.2×10^8 l/mol respectively) and bind to different regions of the insulin molecule. No binding of ^{125}I-PH4/B1 was observed over the range of insulin concentrations tested (Figure 5.13). This suggested that either the avidity of this antibody was inappropriately low for use in

TABLE 5.7

EVALUATION OF INSULIN MONOCLONAL ANTIBODIES FOR USE IN A TWO-SITE IRMA

				125I-McAb				
		ID1/C10	ID4/E5	ID1/A4	PF1/B9	PF2/B5	PH4/B1
	ID1/C10	-*	-	-	-	-	-
	ID4/E5	-	-	-	-	-	-
Solid-phase	ID1/A4	-	-	-	-	-	-
McAb	PF1/B9	-	-	-	-	-	-
	PF2/B5	-	-	-	-	-	-
	PH4/B1	-	-	-	-	-	-

* - indicates no binding observed with insulin at 100 mU/l.
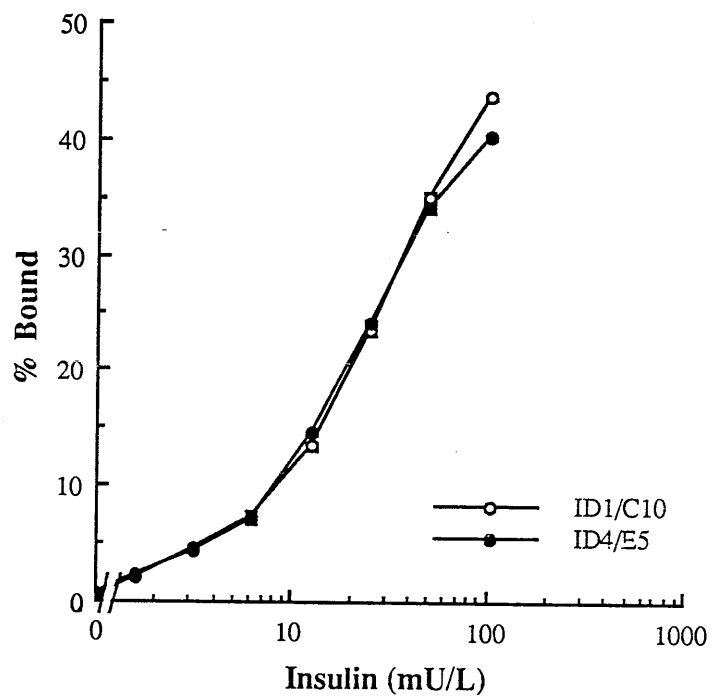
Figure 5.12

Comparison of two anti-insulin monoclonal antibodies in an insulin IRMA.

Standard(200 µl) and ^{125}I-McAb (100 000 cpm in 100 µl) were incubated for 2 h followed by a 1 h incubation with solid-phase coupled polyclonal anti-insulin (1 mg).
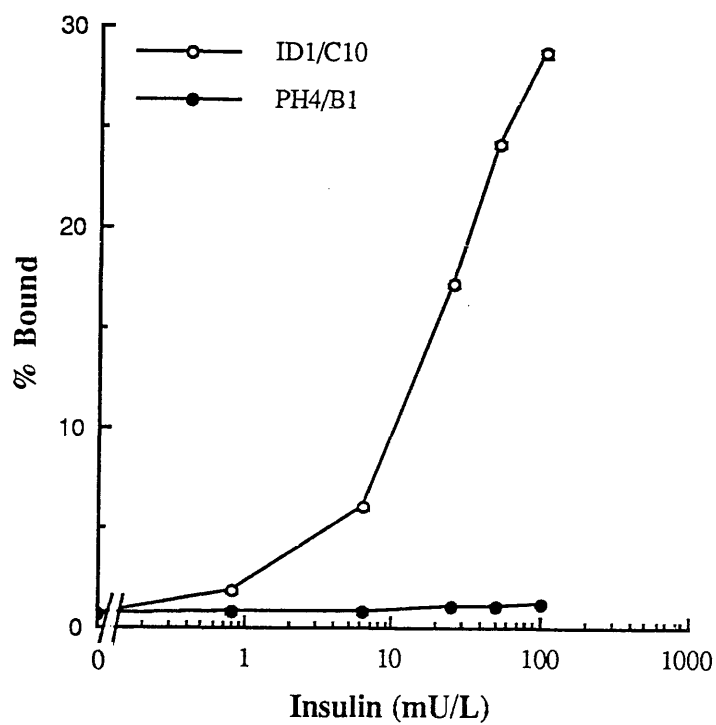
Figure 5.13

Evaluation of a third anti-insulin monoclonal antibody in an insulin IRMA.

Details as for Figure 5.12

the development of a clinically useful IRMA or the epitope recognised was similar to that of the polyclonal antiserum. Further development of the insulin IRMA was performed therefore, with ^{125}I-ID1/C10 and solid-phase coupled guinea-pig anti-insulin as described below.

2.3 Assay Optimisation

The optimum conditions for various parameters of the insulin IRMA were determined as follows.

Incubation Times

The primary incubation involved mixing 100 µl aliquots of standard and ^{125}I-labelled ID1/C10 (100 000 cpm) with the addition of solid-phase coupled polyclonal anti-insulin (1 mg) for the second incubation. The optimum reaction times for the primary and secondary incubation steps were 2 h and 1 h respectively (Figure 5.14).

In subsequent graphs the rate of change in the percentage bound at the lower end of the standard curve is less. This is due to the use of a pool of antiserum from several animals which exhibited a range of avidities in their individual sera. The overall avidity of this pool was lower than that of the antiserum from the single guinea-pig which had been used previously.

Solid-Phase Coupled Antibody

Five solid-phases were prepared at different coupling ratios of IgG:gel. The amount of IgG coupled per mg gel increased as the amount offered increased, but the overall percentage uptake of IgG by the gel decreased at first and appeared to reach a plateau (Table 5.8). These solid-phases were then evaluated in the insulin IRMA as described above, using the optimum incubation times. Assay sensitivity, arbitrarily calculated as the dose of insulin resulting in a percentage bound twice that of the zero dose, was optimal at a coupling ratio of 0.05 mg IgG/mg gel (Figure 5.15A).

105
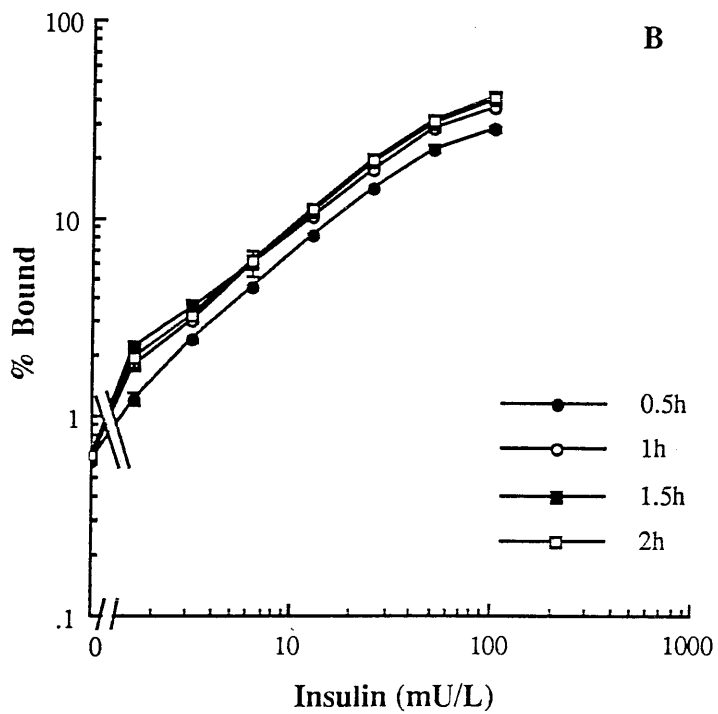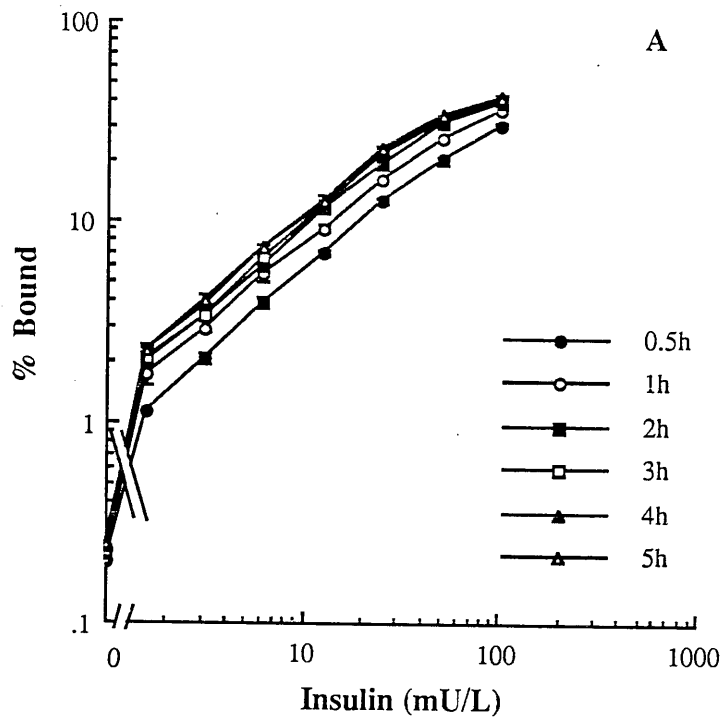
Figure 5.14

Optimisation of the primary (A) and secondary (B) incubation times of the insulin IRMA.

During optimisation of the primary incubation (A), the secondary incubation was held at 1 h and during optimisation of the secondary incubation (B), the primary incubation was held at 2 h.

TABLE 5.8

COUPLING OF POLYCLONAL ANTI-INSULIN TO SEPHAROSE^R CL-4B

IgG offered (mg)*	0.025	0.050	0.100	0.150	0.200
IgG coupled (mg)*	0.014	0.021	0.030	0.044	0.056
% uptake of IgG	56	42	30	29	28

*mg IgG per mg gel.

For details of coupling reaction see Chapter 2, Section 5.3.
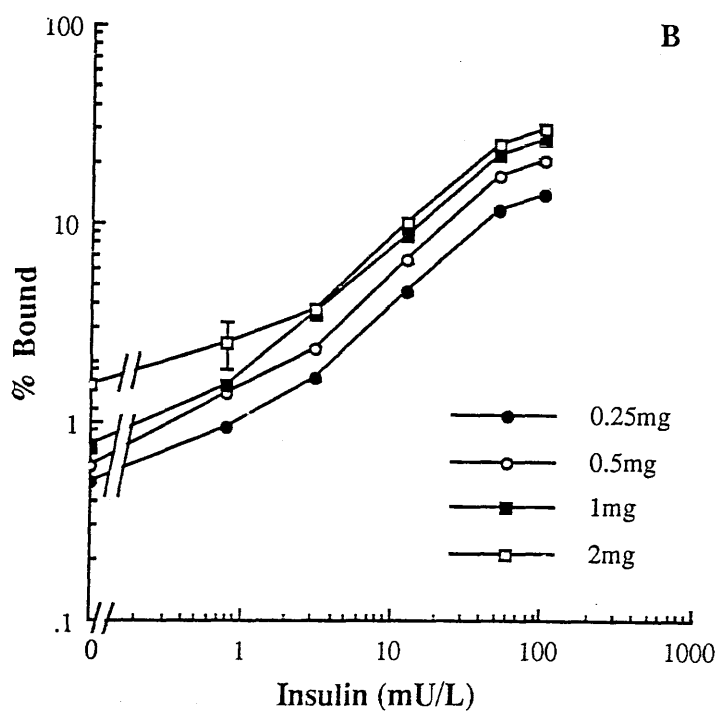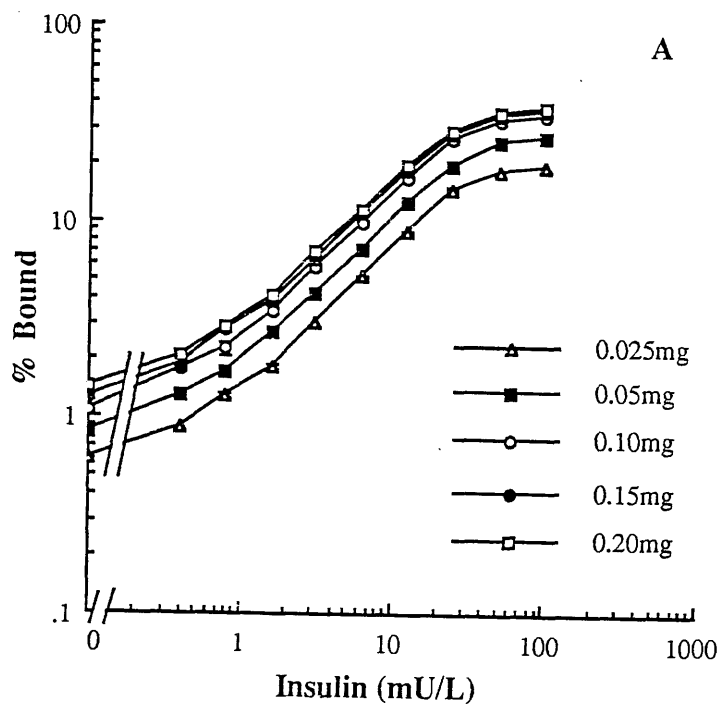
Figure 5.15

Optimisation of the coupling ratio of IgG:solid-phase (A) and the mass of solid-phase added per tube (B) in the insulin IRMA.

A - 1 mg solid-phase/tube
B - solid-phase prepared with 0.1 mg IgG/mg gel.

The effect of varying the mass of solid-phase added per tube was also examined. Similar assay sensitivity was achieved with either 0.5 mg or 1 mg solid-phase (Figure 5.15B). A mass of 0.5 mg was chosen since the data suggested that this was likely to result in consistently lower zero dose binding levels and had the added advantage of greater economy of solid-phase.

Wash Cycles

100 µl aliquots of standards (0-100 mU/l) and ^{125}I-ID1/C10 (100 000 cpm) were incubated for 2 h followed by a second incubation with solid-phase coupled polyclonal anti-insulin (0.5 mg) for 1 h prior to washing and counting. After wash cycles 3,4,5 and 6 the assay was counted and standard curves constructed (Figure 5.16). Four wash sequences were required in order to attain maximum sensitivity without reducing the assay range.

^{125}I-Labelled Antibody

The effect of varying the mass of ^{125}I-labelled antibody added per tube was assessed by incubating standard and ^{125}I-ID1/C10 (100 µl) for 2 h prior to the addition of 0.5 mg solid-phase coupled polyclonal anti-insulin and a further incubation of 1 h followed by washing (x4) and counting. The results are shown in Figure 5.17. The specific activity of the ^{125}I-ID1/C10 used was 0.44 MBq/µg, which was equivalent to 4.9 ng antibody/100 000 cpm in subsequent experiments. Compared with the use of 100 000 cpm in the previous experiments, the addition of 50 000 cpm resulted in improved sensitivity with no apparent change in the assay range. Addition of 200 000 cpm allowed measurement of insulin over a wider range of concentrations (up to 100 mU/l) with a slight reduction in sensitivity.

Since clinical utility required that the assay measure insulin up to 100 mU/l, the effect of adding 100 000 cpm or 200 000 cpm was re-evaluated in conjunction with sample volumes of 50 µl and 100 µl. The assay was performed as described above.
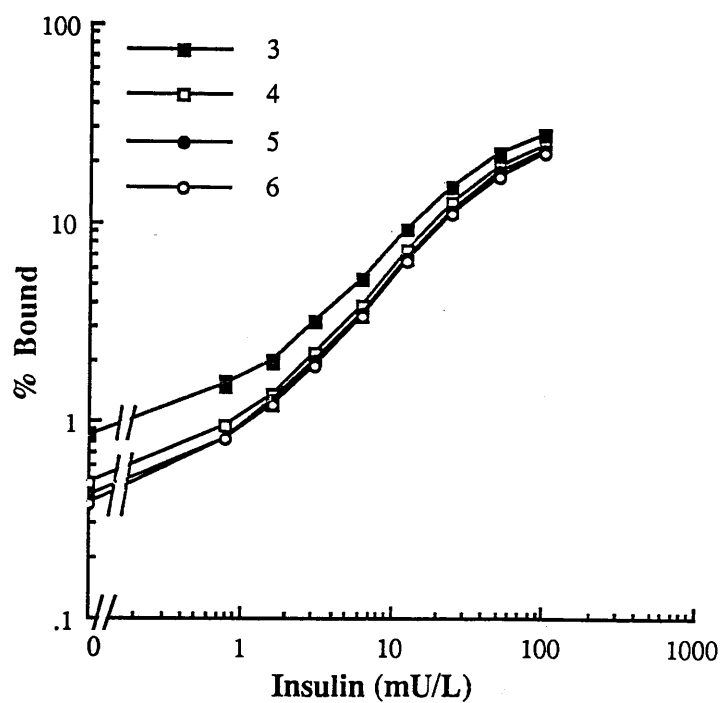
Figure 5.16

Optimisation of number of washes required, prior to counting, in
the insulin IRMA.
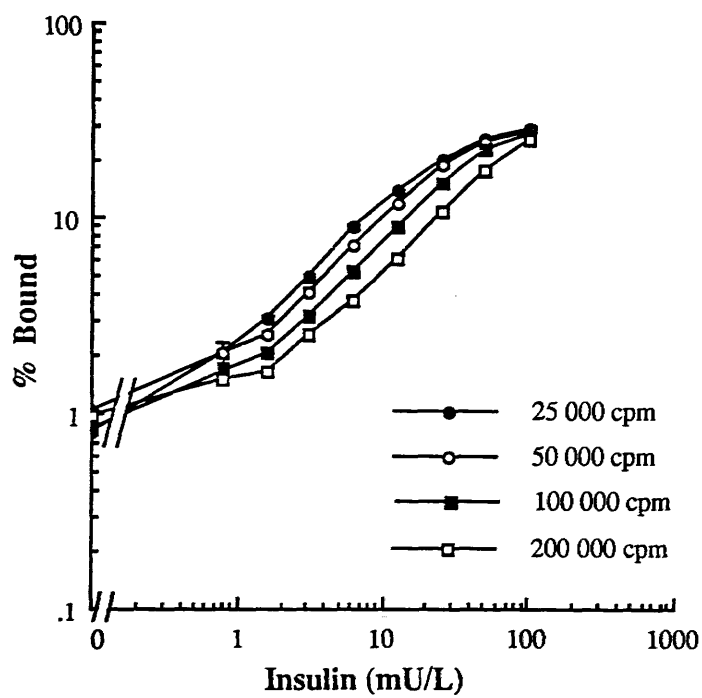
Figure 5.17

Optimisation of the mass of ^{125}I-ID1/C10 added per tube in the insulin IRMA.

Sensitivity of less than 1 mU/l combined with a range up to 100 mU/l was achieved with either 100 µl sample and 200 000 cpm ^{125}I-ID1/C10 or 50 µl sample and 100 000 cpm ^{125}I-ID1/C10 (Figure 5.18).The former combination was preferred as greater precision should be possible when pipetting larger volumes (100 µl vs 50 µl) and quantitating higher count rates (200 000 cpm vs 100 000 cpm).

2.4 Assay Validation

Matrix Effects

To establish the effect of different matrices on the insulin IRMA, blood samples from three volunteers (A, B and C) were collected as serum, plasma-heparin (2 mg/10 ml blood) and plasma-EDTA (10-20 mg/10 ml blood), all with and without Trasylol (4 000 U/10 ml blood). Insulin standard curves were constructed in each of the above, together with hormone-free serum (HFS) and assay buffer (Figure 5.19).

The pattern of curves obtained from standards diluted in each matrix were indistinguishable between individuals. No difference was observed between serum and plasma in either the presence or absence of Trasylol and serum was chosen as the sample matrix. Similarly, there was no detectable difference in the standard curves whether standards were diluted in buffer or HFS. Standards were therefore prepared in assay buffer and stored at -70°C for future use.

Stability of Insulin in Whole Blood and Serum

To determine the stability of insulin in whole blood and serum, blood samples were collected from five volunteers and treated as follows:

(i) A reference sample, designated zero time, was allowed to stand at room temperature for 30 minutes prior to separation and immediate freezing of the serum.
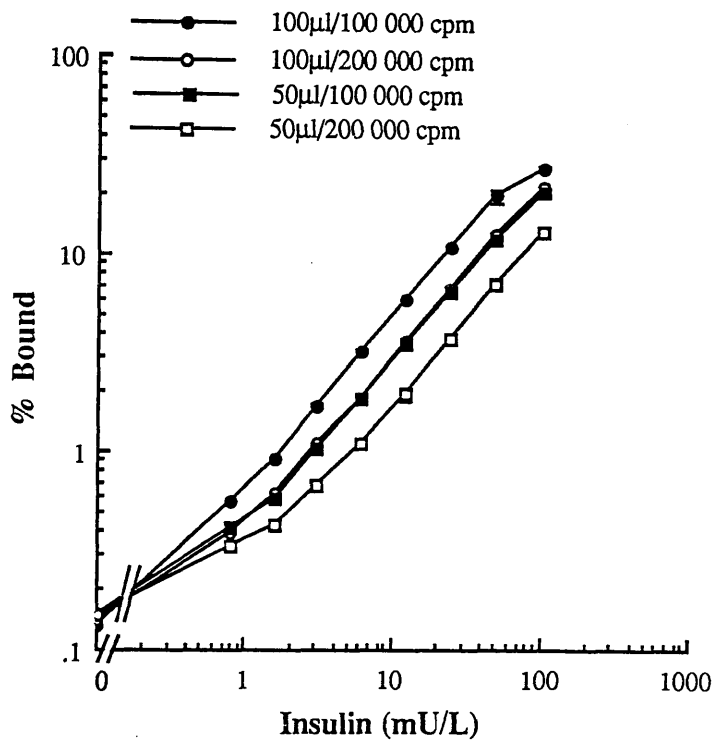
Figure 5.18

Evaluation of two sample volumes (50 μl and 100 μl) in conjunction with two masses of ^{125}I-ID1/C10 (100 000 cpm and 200 000 cpm) in the insulin IRMA.
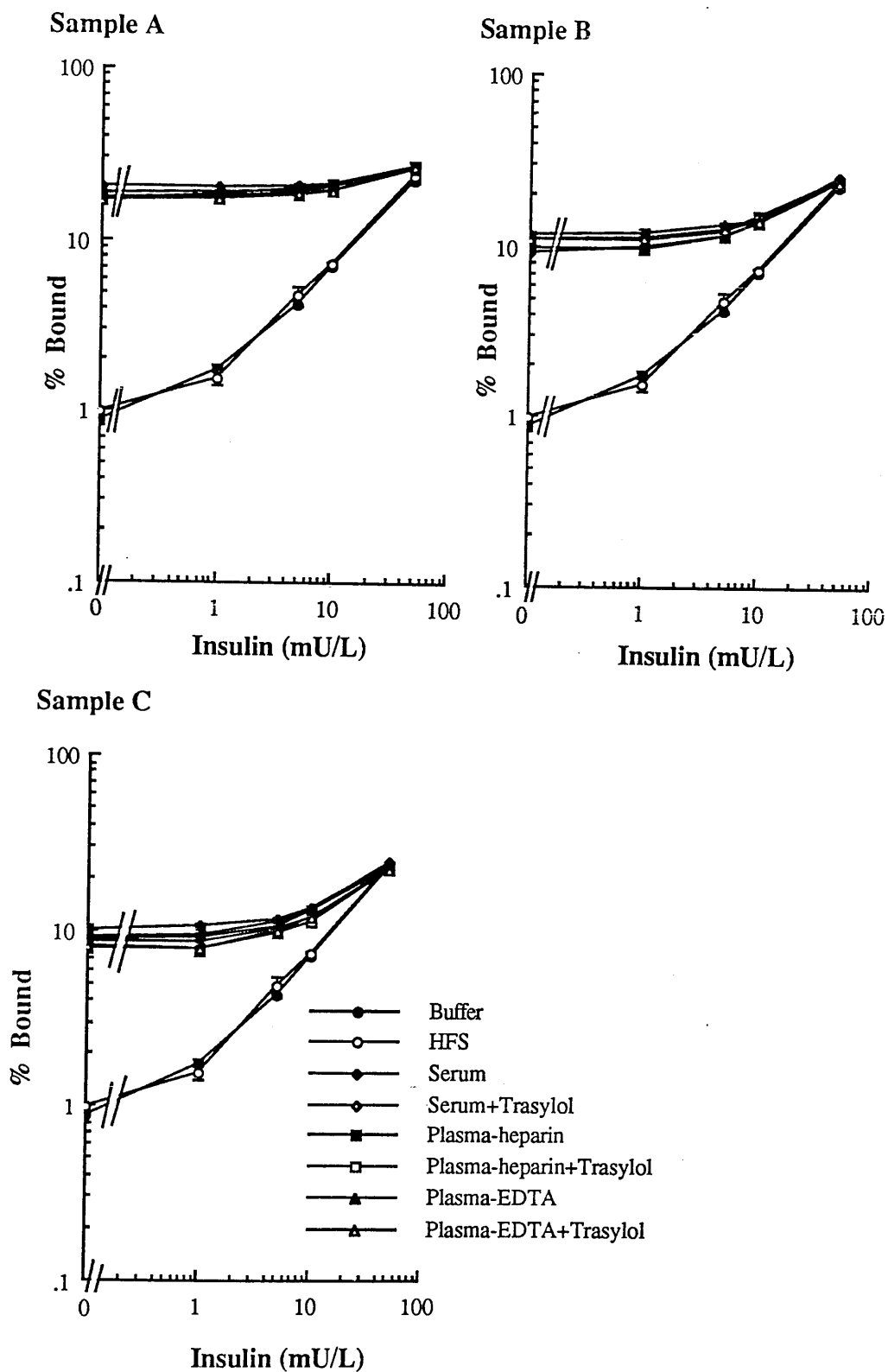
Figure 5.19

The effect of sample matrix on the insulin IRMA.

(ii) A second sample of blood was treated as described in (i) but the serum was divided into five aliquots which were allowed to stand at room temperature for a further 0.5, 1, 2, 4 or 6 hours before freezing.

(iii) Five further aliquots of whole blood were allowed to stand at room temperature for 0.5, 1, 2, 4 or 6 hours (in addition to the 30 minutes required by the reference sample) prior to separation and immediate freezing of the serum.

These experimental samples were assayed simultaneously and the results calculated as the concentration of insulin measured at each time point expressed as a percentage of the concentration of insulin measured at zero time (ie, % insulin remaining). The results for each of the five volunteers are shown separately with the median values at each time point being connected (Figure 5.20). Two-way analysis of variance (ANOVA) supported the view that no significant degradation of insulin occurred in whole blood or serum up to six hours storage at room temperature.

Recovery of Insulin from Serum

The recovery of insulin from serum was evaluated by adding standard insulin to each of four individual sera. Ten replicates were performed for each dose level in each individual serum and the percentage recovery calculated from the equation:

$$\% \text{ Recovery} \quad = \quad \frac{\text{Measured Insulin - Serum Insulin}}{\text{Added Insulin}} \times 100$$

The results obtained are summarised in Table 5.9 and indicate that insulin was recovered quantitatively over the working range of the assay.

Parallelism Between Standards and Endogenous Serum Insulin

Serum samples from four patients with cirrhosis of the liver were diluted with assay buffer and measured in the insulin IRMA. The results from each individual (A-D), plotted together with the standard curve, are shown in Figure 5.21. Sequential
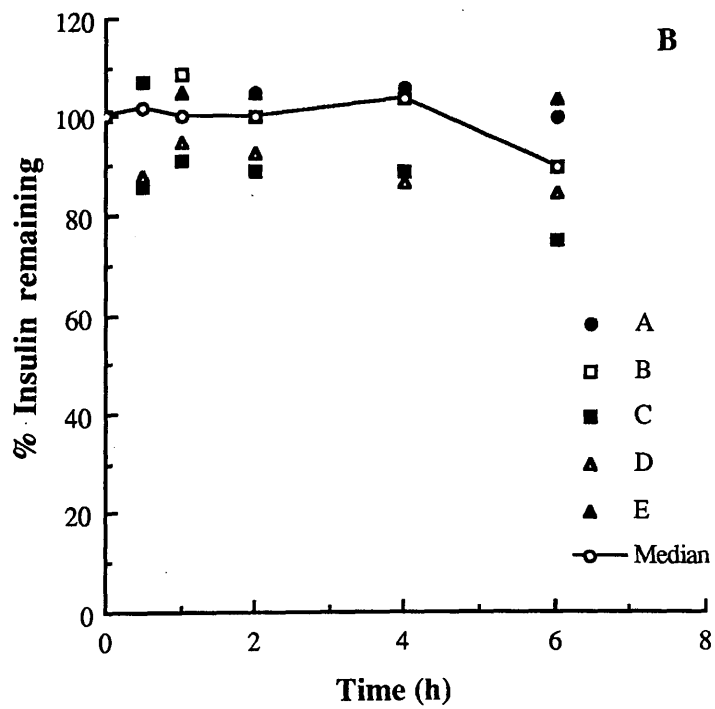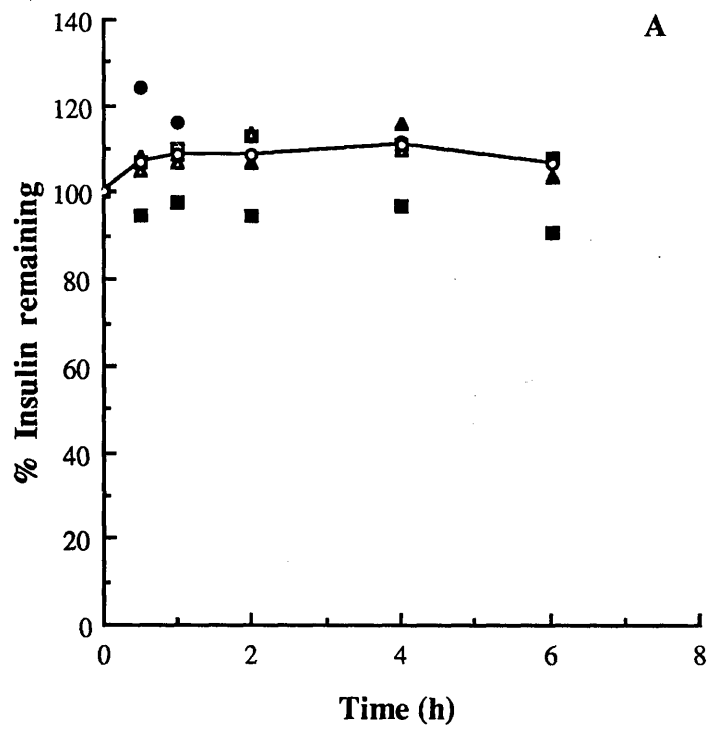
Figure 5.20

Stability of insulin in whole blood (A) and serum (B).

TABLE 5.9

INSULIN IRMA RECOVERIES

Insulin added (mU/l)	% Recovery ±SD			
	A*	B	C	D
10	86.6±3.5	101.0±2.8	104.5±1.9	97.5±4.5
25	91.8±3.0	101.8±2.3	95.6±2.9	95.4±2.6
50	98.3±2.4	94.5±4.1	98.0±2.5	96.7±3.9
100	109.4±2.3	98.5±1.5	105.7±1.2	110.7±4.0

*A-D = individual sera.
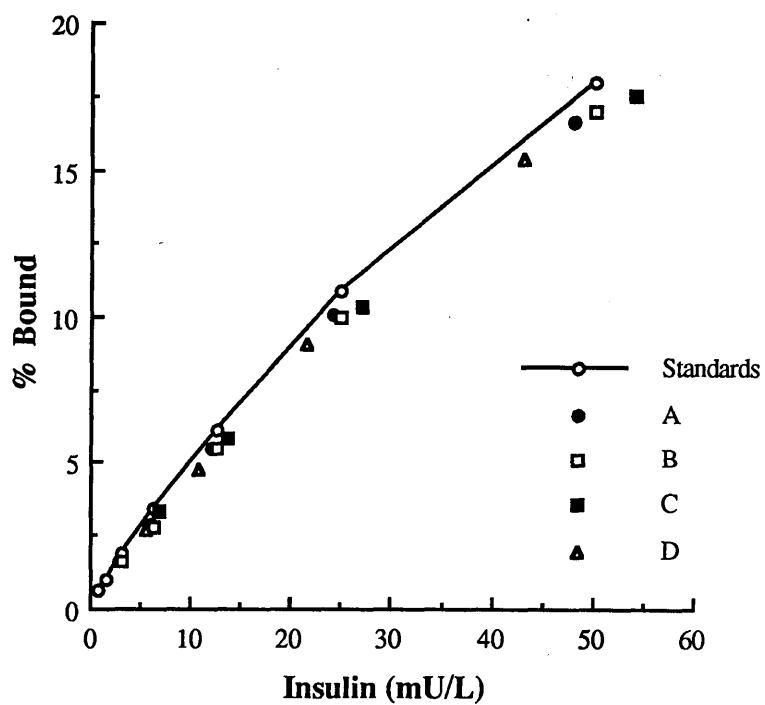
Figure 5.21

Parallelism between standards and endogenous serum insulin in four patients (A-D).

dilution of all four patient samples produced results which were parallel to the standard curve over its working range.

Stability of Insulin to Repeated Freeze-Thaw Cycles

Insulin was added to pooled human serum at four dose levels (25, 50, 70 and 90 mU/l) and each resulting pool was divided into aliquots and frozen at -30°C. Separate aliquots of each pool were then subjected to 0, 1, 2, 3 or 4 freeze-thaw cycles prior to assay in the insulin IRMA. The results (Figure 5.22), evaluated by two-way analysis of variance indicated a significant difference ($p<0.05$) in measured insulin concentrations between freeze-thaw cycles. However, the coefficient of variation (CV) between the values for each dose level were lower than the corresponding intra-assay coefficients of variation suggesting no difference between measured insulin concentrations in serum samples after four freeze-thaw cycles.

Stability of Insulin in Frozen Samples

Blood samples were collected from five volunteers and the serum separated as described in Chapter 2, Section 3.3. The serum from each subject was divided into aliquots and stored at -30°C until required for assay. Single aliquots from each volunteer were thawed and assayed at various time intervals over a six month period (Figure 5.23). Two-way analysis of variance indicated that there was no significant degradation of insulin in frozen samples stored at -30°C for up to six months.

Cross-Reaction of Intact, Split and Des-Amino Proinsulins in the Insulin IRMA

The pattern of cross-reaction for intact, split and des-amino proinsulins is shown in Figure 5.24. The percentage cross-reaction of each form at two dose levels (10 and 300 pmol/l) is shown in Table 5.10.
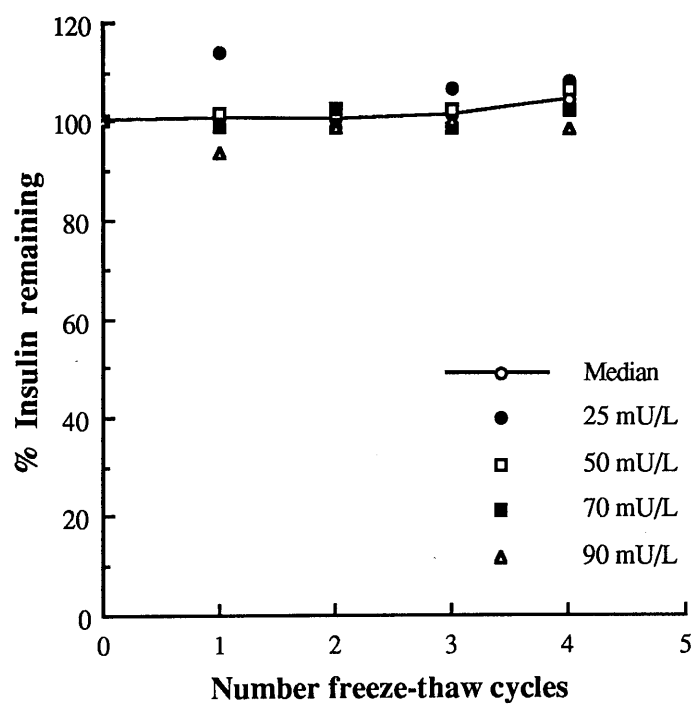
Figure 5.22

Stability of insulin to repeated freeze-thaw cycles.
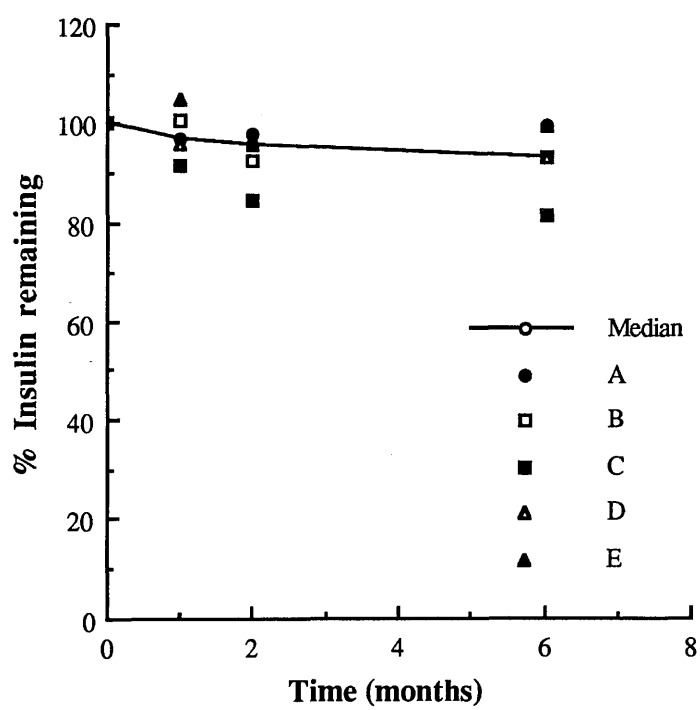
Figure 5.23

Stability of insulin in frozen samples.
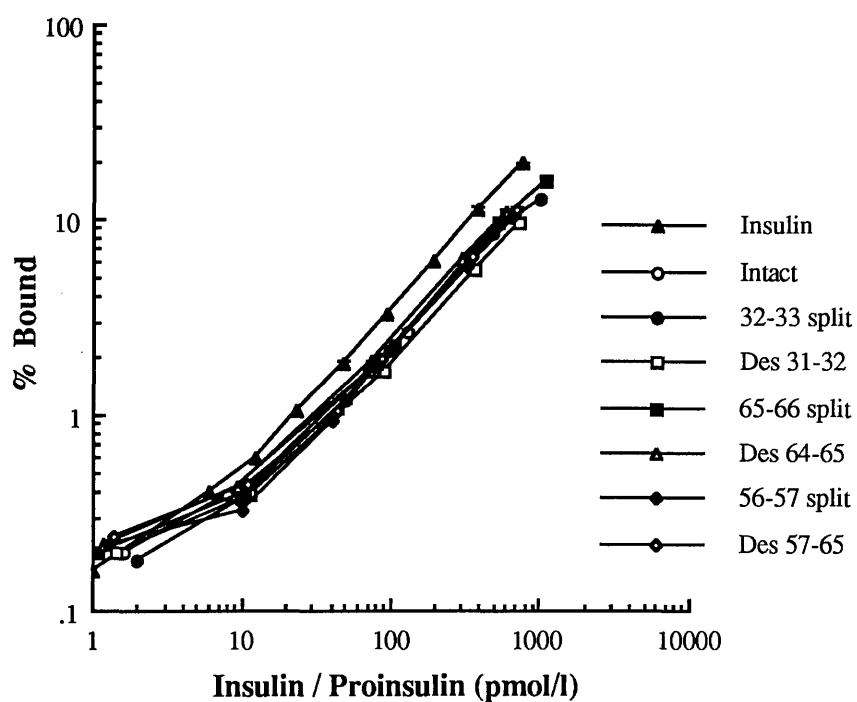
Figure 5.24

Cross-reaction of intact, split and des-amino forms of proinsulin
in the insulin IRMA.

TABLE 5.10

PERCENTAGE CROSS-REACTION OF INTACT, SPLIT AND DES-
AMINO PROINSULINS IN THE INSULIN IRMA

| | % Cross-reaction | |
| | Proinsulin (pmol/l) | |
Proinsulin form	10	300
Intact	85.2	57.7
32-33 split	70.4	60.8
Des 31-32	68.5	49.5
65-66 split	73.5	61.9
Des 64-65	83.3	66.9
56-57 split	61.1	57.7
Des 57-65	79.6	59.8

The Final Insulin IRMA

Figure 5.25 shows a typical standard curve and within assay precision profile for the optimised insulin IRMA. The sensitivity of the assay was 0.3 mU/l (CV 22%) (121) with a range of 0.8->100 mU/l (CV <10%). The inter-assay coefficients of variation were calculated using quality control (QC) samples prepared at five dose levels and the results are shown in Table 5.11.

2.5 Correlation of Insulin RIA and IRMA

The insulin IRMA, developed as described above, was compared with the routine RIA in the Institute of Biochemistry at Glasgow Royal Infirmary. The RIA involved the incubation of sample and anti-insulin (raised in Strain 2 guinea-pigs as described in Chapter 3, Section 1) overnight at 4°C followed by the addition of ^{125}I-insulin and a further incubation of 3 hours at room temperature. Sepharose^R CL-4B coupled anti-guinea-pig IgG was then added and the assay mixed continuously on an orbital shaker for 30 minutes prior to three washes as described in Section 2.1 of this chapter. The RIA was performed by MLSO staff in the above department.

Cross-Reaction of Intact, Split and Des-Amino Proinsulins in the Insulin RIA

The pattern of cross-reaction of intact, split and des-amino proinsulins in the insulin RIA is shown in Figure 5.26. The percentage cross-reaction of each form at two dose levels (10 and 300 pmol/l) is shown in Table 5.12.

Correlation of One Hundred Patient Samples Measured in Both the RIA and IRMA

One hundred patient samples measured in the routine RIA, at Glasgow Royal Infirmary, covering a range of insulin concentrations from 2.4 to 97.2 mU/l were also measured in the IRMA. There was a strong positive correlation between the
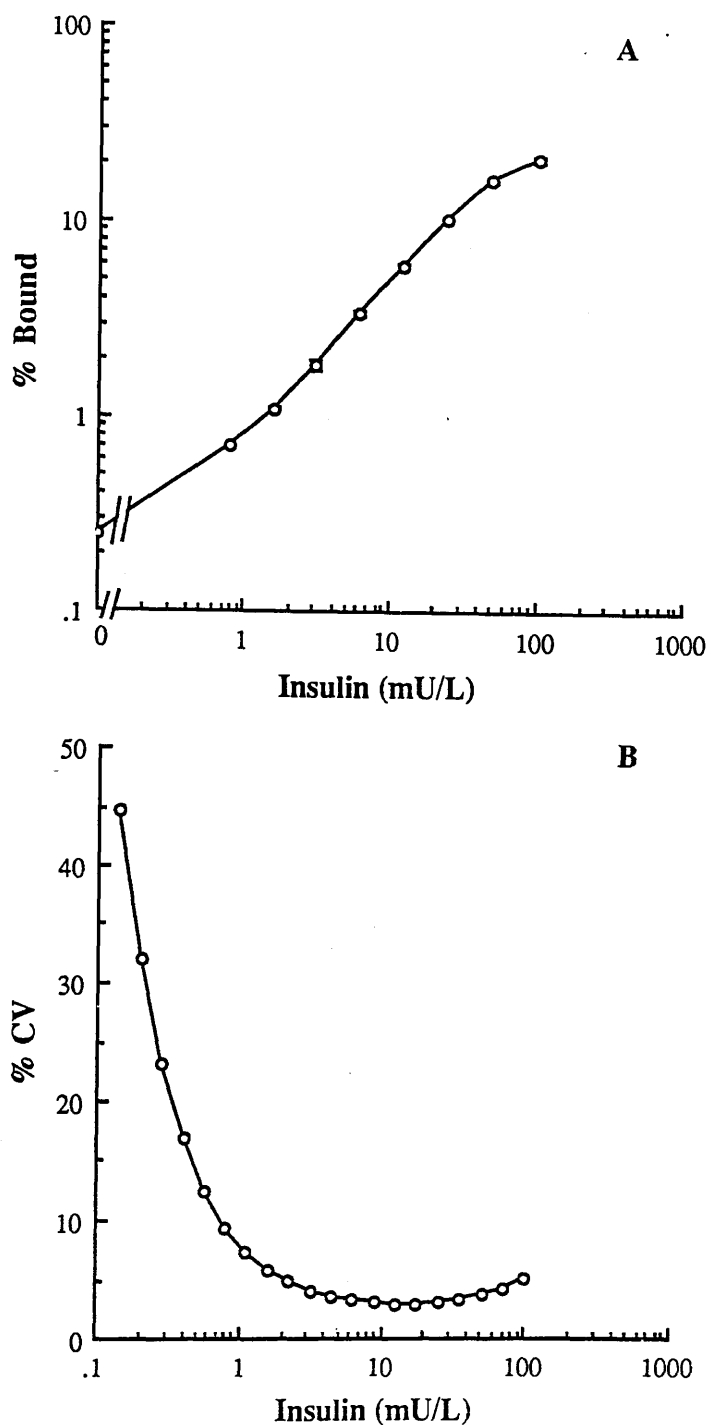
Figure 5.25

A representative standard curve (A) and within assay precision
profile (B) for the optimised insulin IRMA.

TABLE 5.11

INTER-ASSAY COEFFICIENTS OF VARIATION FOR THE INSULIN IRMA

Insulin (mU/l)	%CV	Number of samples
13	1.8	4
25	3.9	60
50	4.6	60
70	3.7	60
90	5.6	60
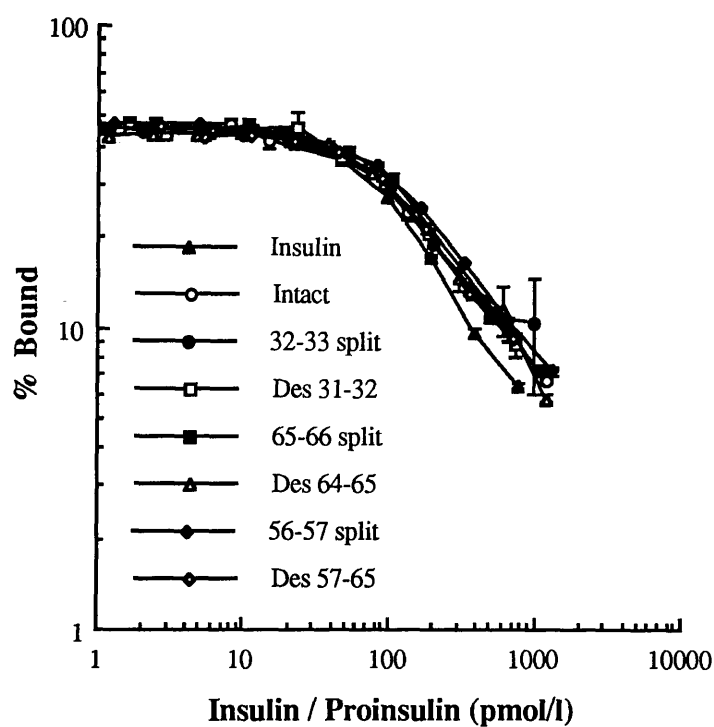
Figure 5.26

Cross-reaction of intact, split and des-amino forms of proinsulin in the insulin RIA.

TABLE 5.12

PERCENTAGE CROSS-REACTION OF INTACT, SPLIT AND DES-AMINO PROINSULINS IN THE INSULIN RADIOIMMUNOASSAY

	% Cross-reaction	
	Proinsulin (pmol/l)	
Proinsulin form	10	300
Intact	107.4	86.2
32-33 split	-	90.1
Des 31-32	-	90.1
65-66 split	-	80.1
Des 64-65	-	92.1
56-57 split	136.8	85.1
Des 57-65	-	91.6

results from each assay (r=0.96) and the slope of the regression line was calculated to be 0.87, indicating that the IRMA results were 13% lower than those from the RIA (Figure 5.27). This difference could be accounted for in part by the lower cross-reaction of the various forms of proinsulin in the IRMA compared with the RIA (Tables 5.10 and 5.12 respectively).

3 DEVELOPMENT OF A PROINSULIN IRMA

3.1 Production of Monoclonal Antibodies for Use in a Proinsulin IRMA

In an attempt to produce McAbs specific for proinsulin three fusions were performed with proinsulin as immunogen (Chapter 4, Table 4.1). The initial fusion, performed on 19.7.85, did not yield any stable antibody-secreting hybridomas. This failure was attributed in part to the poor quality ^{125}I-proinsulin, purified by gel filtration, used for screening (Chapter 4, Section 1). An alternative method of purifying ^{125}I-proinsulin, reverse-phase HPLC, was developed (Section 1.2 of this chapter) and two further hybridisation experiments performed. The first, performed on 6.6.86, yielded one McAb reactive with C-peptide and proinsulin (PD4/H4) and two reactive with insulin and proinsulin (PF1/B9 and PF2/B5). A second fusion, performed on 10.4.87, yielded three McAbs specific for proinsulin (PH5/B5, PI2/G4 and PI3/B10) and one which reacted with insulin and proinsulin (PH4/B1).

3.2 Evaluation of Monoclonal Antibodies for Use in a Proinsulin IRMA

The C-peptide and proinsulin McAbs were evaluated for use in a proinsulin IRMA in conjunction with a polyclonal guinea-pig anti-insulin and the insulin McAbs available at the time of their production. The following three sections in this chapter deal with the proinsulin assays developed with the above McAbs as they were produced. A short summary of each section is outlined on the following page.
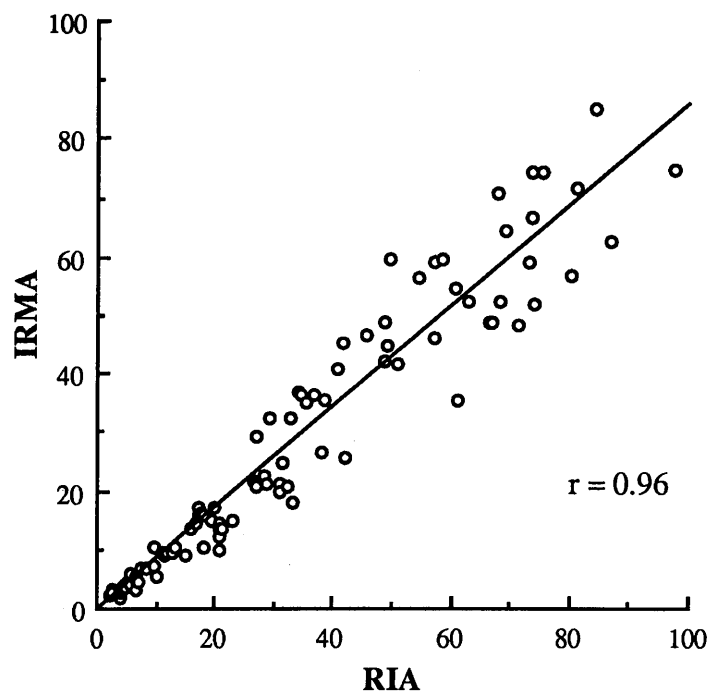
Figure 5.27

Correlation of one hundred patient samples analysed in both the insulin RIA and IRMA.

(i) Section 4   **Proinsulin IRMA with monoclonal anti-C-peptide.**

This section describes the evaluation of the McAb reactive with C-peptide in conjunction with a polyclonal guinea-pig anti-insulin and various insulin McAbs produced in the same and earlier fusions. The desired assay sensitivity was not achieved with any combination of antibodies and the factors investigated in an unsuccessful attempt to improve the sensitivity are outlined.

(ii) Section 5   **Proinsulin IRMA-1 with proinsulin-specific monoclonal antibody.**

This section describes the evaluation of the highest avidity McAb specific for proinsulin, PH5/B5, in conjunction with a polyclonal guinea-pig anti-insulin and the various insulin McAbs produced previously, in addition to the one produced in the same fusion experiment (PH4/B1). The optimisation of a proinsulin IRMA utilising the proinsulin-specific McAb (PH5/B5) coupled to solid-phase and the insulin McAb (PH4/B1) labelled with ^{125}I is described. The assay involved a primary incubation of ^{125}I-anti-insulin with sample followed by a secondary incubation with solid-phase coupled anti-proinsulin. The interference of insulin in this assay, which rendered it unsuitable for clinical use, is also outlined.

(iii) Section 6   **Proinsulin IRMA-2 with proinsulin-specific monoclonal antibody.**

The assay described in this section utilises the same McAbs as the one described in Section 5, but the order of addition of the reagents was reversed. This resulted in an assay in which proinsulin was immunoextracted from the sample by solid-phase

coupled anti-proinsulin prior to the addition of the ^{125}I-labelled anti-insulin. This attempt to abolish the interference of insulin was successful and the clinical validation of this assay is outlined.

4 PROINSULIN IRMA WITH MONOCLONAL ANTI-C-PEPTIDE

4.1 Evaluation of Monoclonal Antibodies for Use in a Proinsulin IRMA

The C-peptide McAb (PD4/H4), produced in the fusion performed on 6.6.86, was evaluated in conjunction with the two insulin McAbs (PF1/B9 and PF2/B5) produced in the same fusion as well as a polyclonal guinea-pig anti-insulin and one of the three insulin McAbs produced in a previous hybridisation experiment in which insulin was used as immunogen.

Comparison of Anti-C-Peptide Coupled to Solid-Phase or Labelled with ^{125}I

The C-peptide McAb (PD4/H4) was either labelled with ^{125}I or coupled to solid-phase and evaluated in conjunction with the insulin McAb PF1/B9 similarly coupled to solid-phase or labelled with ^{125}I. The use of the C-peptide McAb labelled with ^{125}I appeared to result in a more sensitive assay (Figure 5.28). Identical results were obtained with McAb PF2/B5 which has a similar avidity and epitope specificity to PF1/B9. Subsequent experiments were performed with McAb PD4/H4 labelled with ^{125}I rather than coupled to solid-phase.

Comparison of Polyclonal or Monoclonal Anti-Insulins Coupled to Solid-Phase

Monoclonal antibody PD4/H4 was labelled with ^{125}I and assessed in conjunction with three different insulin antibodies coupled to Sepharose^R CL-4B. The
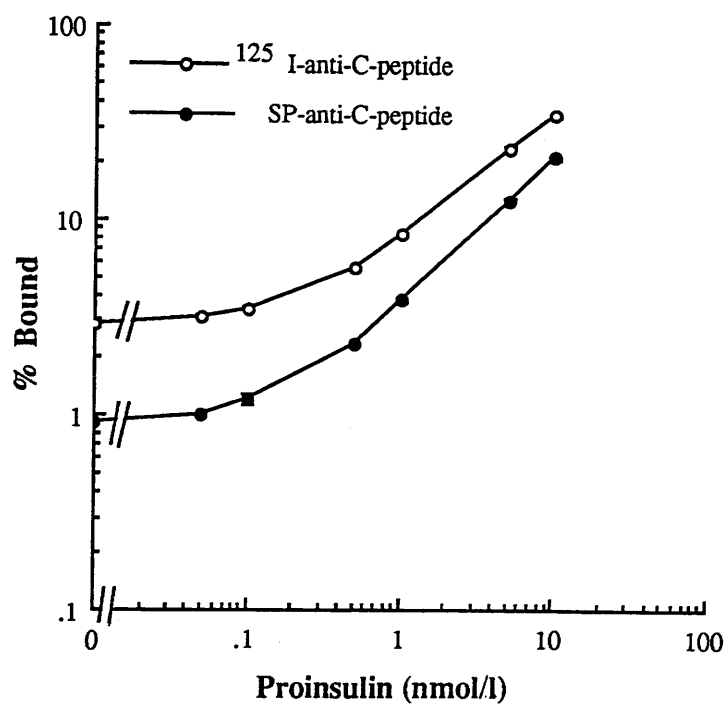
Figure 5.28

Comparison of monoclonal anti-C-peptide iodinated or coupled to solid-phase. Standard (200 μl) and ^{125}I-labelled McAb/100 000 cpm in 100 μl were incubated for 24 h at 4°C followed by a second incubation with solid-phase coupled McAb for 3 h at room temperature.

antibodies used were:

(i)   A polyclonal guinea-pig anti-insulin

(ii)   Anti-insulin McAb PF1/B9 produced in the same fusion. This antibody reacts with the insulin A-chain loop and has an avidity constant of $<1.2 \times 10^8$ l/mol.

(iii)   Anti-insulin McAb ID4/E5 produced using insulin as immunogen. This antibody reacts with an epitope outside the A-chain loop and has an avidity constant of 2.1×10^9 l/mol.

The results (Figure 5.29) indicated that the polyclonal solid-phase was superior to either monoclonal solid-phase in terms of assay sensitivity. The lower avidity McAb (PF1/B9) appeared to result in a more sensitive assay than the higher avidity ID4/E5. This suggests that the epitopes recognised by antibodies PD4/H4 and ID4/E5 lie close together spatially on the proinsulin molecule or that once bound, the PD4/H4 McAb alters the conformation of the epitope recognised by McAb ID4/E5.

Subsequent experiments were performed using ^{125}I-labelled anti-C-peptide (PD4/H4) in conjunction with solid-phase coupled polyclonal anti-insulin.

4.2 Assay Optimisation

Clinical use required that the assay measure fasting levels of proinsulin (<10 pmol/l) (33) and therefore various parameters were examined in an attempt to maximise the sensitivity of the assay.

Sample Volume

The effect of increasing the sample volume from 100 µl to 200 µl was evaluated by incubating standard (100 µl or 200 µl) and ^{125}I-PD4/H4 (100 000 cpm in 100 µl) for 24 h prior to a second incubation with 1 mg solid-phase coupled polyclonal anti-insulin for 3 h.   A sample volume of 200 µl appeared to improve the assay sensitivity (Figure 5.30) and was adopted for subsequent experiments.
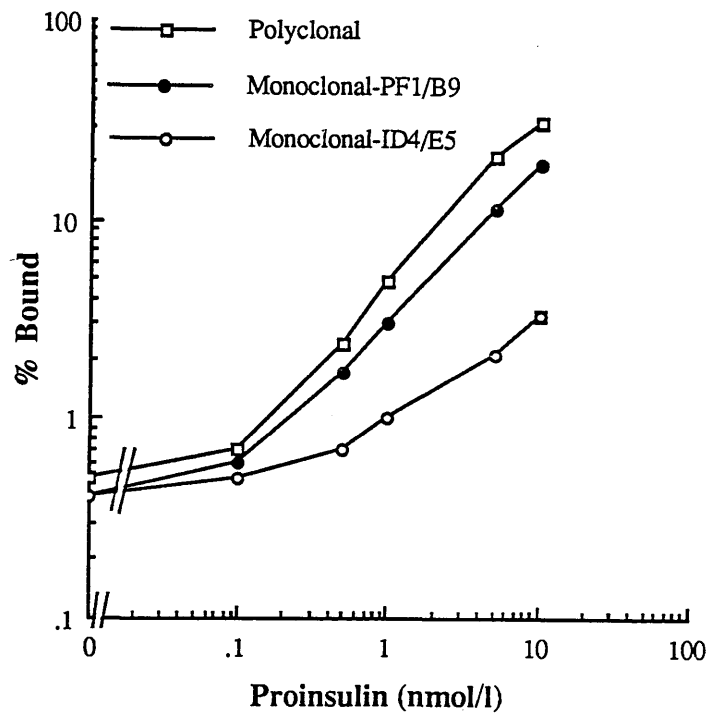
Figure 5.29

Evaluation of ^{125}I-anti-C-peptide monoclonal in conjunction with polyclonal and monoclonal anti-insulins in an IRMA to measure proinsulin.

100 μl aliquots of standard and ^{125}I-PD4/H4 (100 000 cpm) were incubated for 3.5 h prior to a second incubation with solid-phase coupled anti-insulin (1 mg) for 2 h.
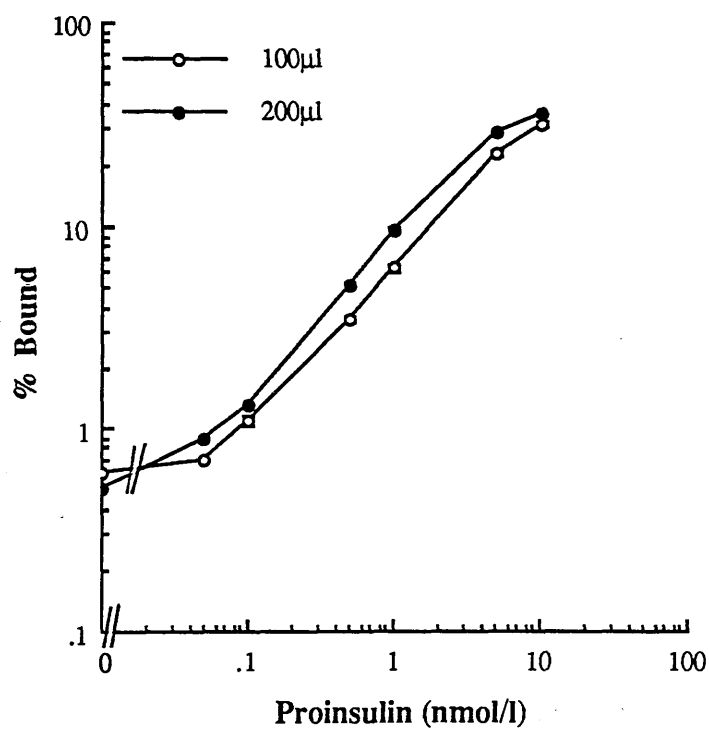
Figure 5.30

Comparison of two sample volumes (100 μl and 200 μl) in the proinsulin IRMA.

^{125}I-Labelled Antibody

The effect of adding various masses of ^{125}I-labelled antibody per tube was assessed using two preparations with different specific radioactivities (approximately 0.44 and 0.93 MBq/μg). The assay was performed as described in the previous section using a sample volume of 200 μl. The same pattern of results was obtained with both preparations (Figure 5.31) but the antibody labelled to a specific activity of 0.44 MBq/μg appeared to result in improved sensitivity.

Calibration of Proinsulin Standard Material

The standards used in the above experiments were prepared from stock material reconstituted for use as an immunogen and consequently lacking in accuracy. Therefore, intact proinsulin standards obtained from Professor C N Hales, were used as a reference for secondary calibration of the above material. The assay was performed as described in previous sections except that the sample volume was reduced to 100 μl to maximise use of the reference material. The value originally assigned to the 'in-house' standards required to be multiplied by 0.56 to give the comparative proinsulin concentration to the reference (Figure 5.32). The standard recalibration led to an improvement in the assay sensitivity by a factor of 2.

Comparison of Intact and Split Proinsulin Standards

32-33 split and 65-66 split proinsulin standards, also obtained from Professor C N Hales, were assayed in parallel with the intact proinsulin standard to evaluate differences in immunoreactivity between the three forms. It has been shown previously that in an immunometric assay system using anti-C-peptide antisera, intact proinsulin immunoreactivity was approximately one hundred times lower than that of the two split forms (39). However, in the assay described here intact proinsulin exhibited greater immunoreactivity than either split form (Figure 5.33).
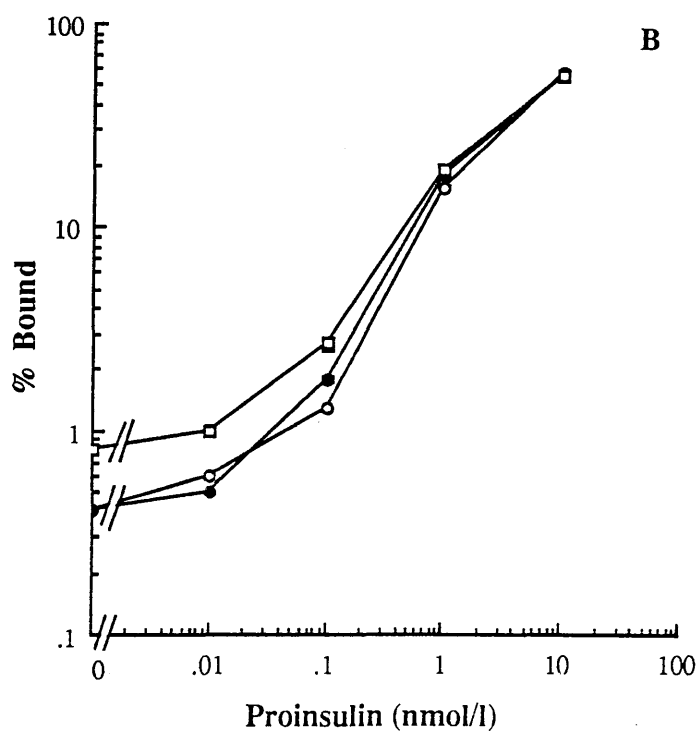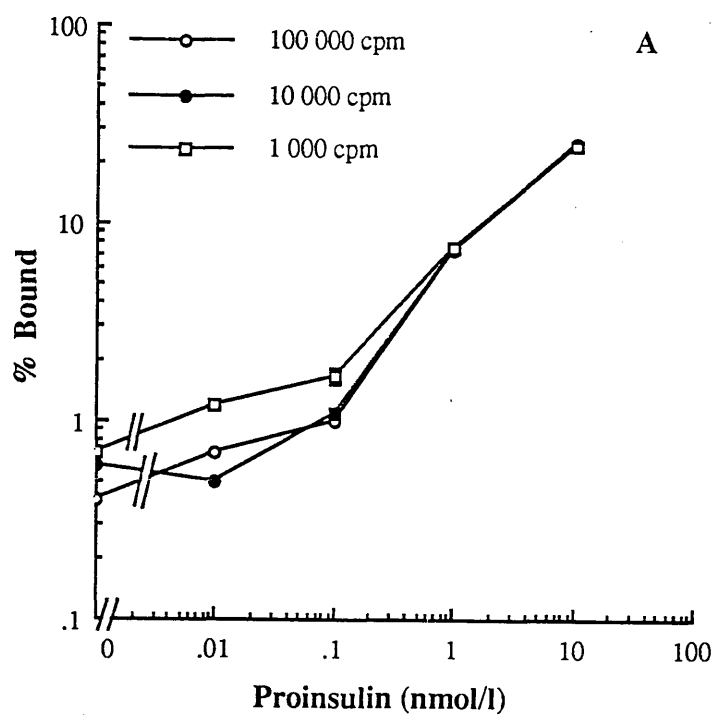
Figure 5.31

Effect of adding different masses of ^{125}I-PD4/H4 per tube.

A - specific activity = 0.93 mBq/μg
B - specific activity = 0.44 mBq
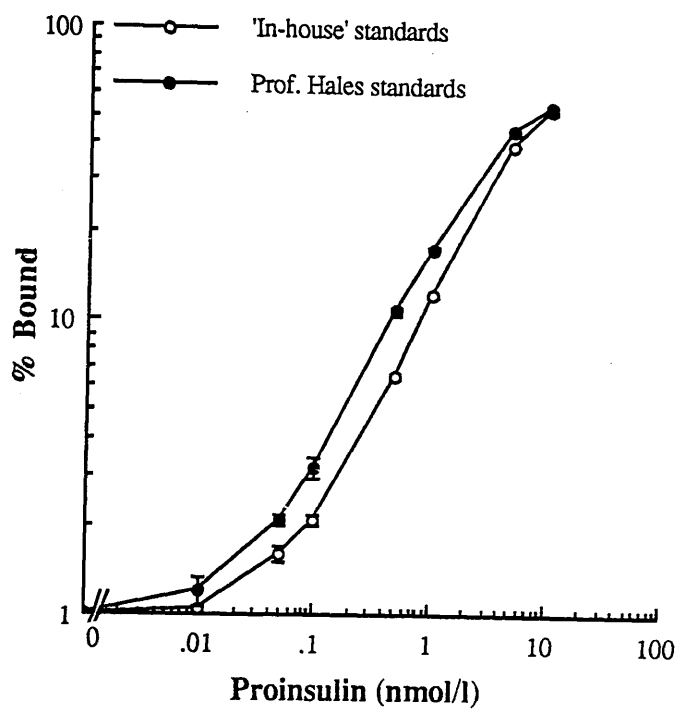
Figure 5.32

Calibration of 'in-house' proinsulin standards against those
obtained from Professor C N Hales.
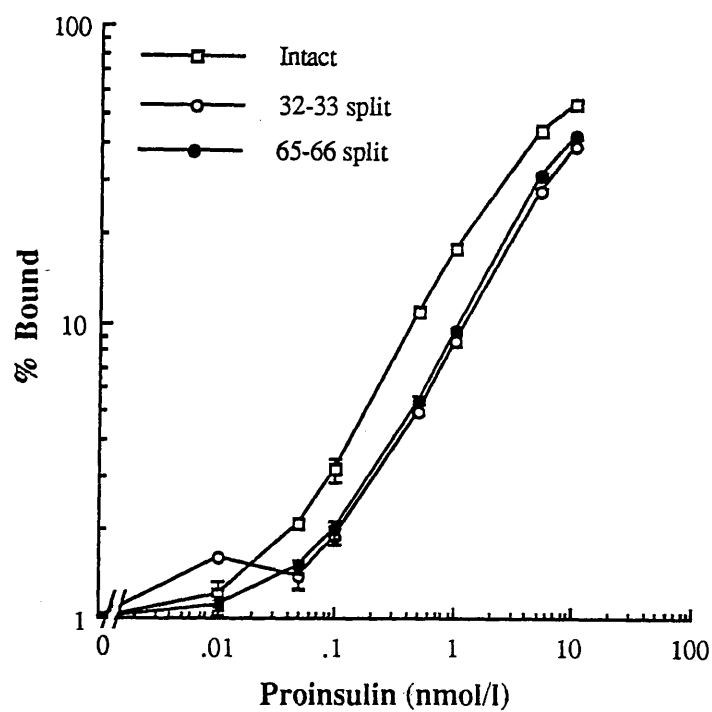
Figure 5.33

Immunoreactivity of intact, 32-33 split and 65-66 split
proinsulins in the proinsulin IRMA.

The above data show that the maximum sensitivity achieved using the C-peptide McAb (PD4/H4) to measure proinsulin, was approximately 20-40 pmol/l. This suggested that the development of a more sensitive proinsulin assay required a McAb of higher avidity.

5 PROINSULIN IRMA-1 WITH PROINSULIN-SPECIFIC MONOCLONAL ANTIBODY

The third hybridisation experiment performed using proinsulin as immunogen produced four McAbs, all of which were of higher avidity than the antibodies produced in the previous fusion (ie, those assessed in Section 4 of this chapter). Three of the antibodies were specific for proinsulin (PH5/B5, PI2/G4 and PI3/B10) and one reacted with insulin and proinsulin (PH4/B1).

5.1 Evaluation of Monoclonal Antibodies for Use in a Proinsulin IRMA

The highest avidity proinsulin-specific McAb PH5/B5 (Chapter 4, Section 2.3) was iodinated and evaluated in conjunction with solid-phase coupled polyclonal (guinea-pig; 0.03 mg IgG/mg gel) and monoclonal (PH4/B1; 0.022 mg IgG/mg gel) anti-insulin. The assay involved incubating standard (200 µl) and ^{125}I-PH5/B5 (100 000 cpm in 100 µl) for 24 h followed by a second incubation with solid-phase coupled anti-insulin (1 mg) for 3.5 h prior to washing (x6) and counting. The solid-phase coupled monoclonal antibody resulted in a more sensitive assay and was used in preference to the solid-phase coupled polyclonal antiserum (Figure 5.34).

Similar assay sensitivities were achieved when the anti-proinsulin monoclonal was iodinated or coupled to solid-phase, although the use of this antibody as a solid-phase reagent resulted in a narrower working range (Figure 5.35). However, practical considerations dictated that the anti-proinsulin monoclonal be used as the solid-phase reagent as the hybridoma secreting the anti-insulin monoclonal (PH4/B1) did not grow well *in vivo*. Therefore, all subsequent experiments were
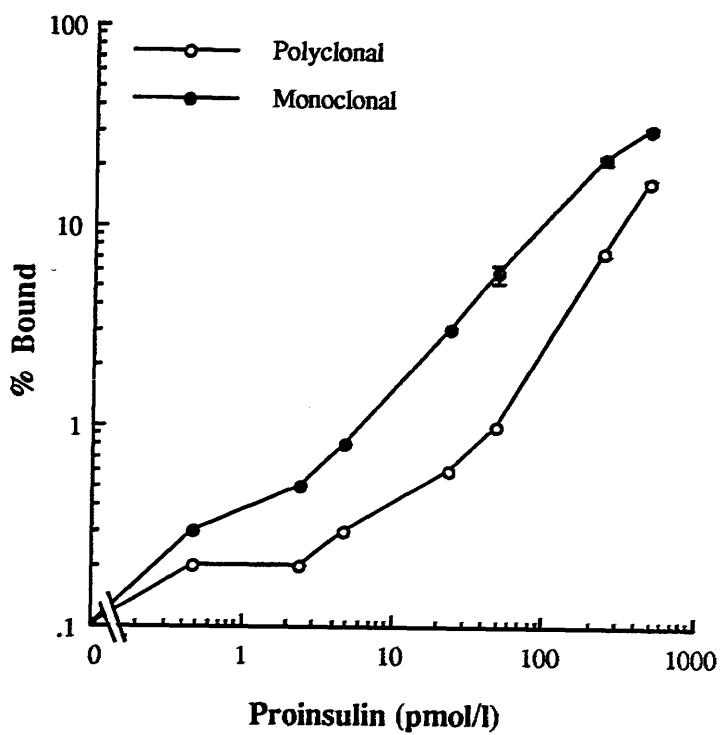
Figure 5.34

Evaluation of ^{125}I-labelled monoclonal anti-proinsulin, in conjunction with a polyclonal and monoclonal anti-insulin, in an IRMA to measure proinsulin.
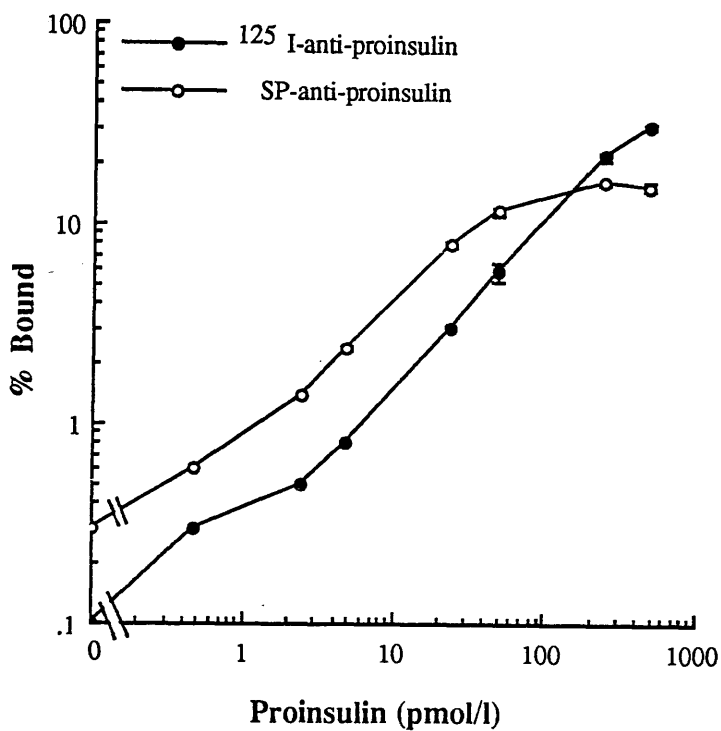
Figure 5.35

Evaluation of monoclonal anti-proinsulin iodinated or coupled to solid-phase (SP).

Assay details as in text for Figure 5.1 with the monoclonal anti-insulin being used as partner antibody.

anti-insulin solid-phase   = 0.022 mg IgG/mg gel
anti-proinsulin solid-phase = 0.007 mg IgG/mg gel

performed with the combination ^{125}I-labelled anti-insulin (PH4/B1) and solid-phase coupled anti-proinsulin (PH5/B5).

5.2 Assay Optimisation

^{125}I-Labelled Anti-Insulin

Monoclonal antibody PH4/B1 was iodinated to three specific radioactivities (1.00, 0.44 and 0.26 MBq/µg) and assessed in the proinsulin IRMA performed as described in Figure 5.29 with 200 µl aliquots of standard. The standard curves obtained were similar, with the antibody labelled to a specific radioactivity of 0.44 MBq/µg giving slightly better sensitivity (Figure 5.36A). ^{125}I-PH4/B1 (0.44 MBq/µg) was then added at masses equivalent to 25 000, 50 000, 100 000 and 200 000 cpm per tube. A mass equivalent to 50 000 cpm appeared to result in a slight improvement in sensitivity in comparison with 100 000 cpm used previously (Figure 5.36B).

Solid-Phase Coupled Anti-Proinsulin

Four solid-phase antibody reagents were prepared at different coupling ratios of IgG:gel. The quantity of IgG coupled per mg gel increased as the quantity offered increased, but the overall percentage uptake of IgG by the gel decreased before reaching an apparent plateau (Table 5.13). This pattern of results was similar to that observed for the insulin assay (Section 2, Table 5.8). When these solid-phases were evaluated in the proinsulin IRMA as described in the section above (using 50 000 cpm instead of 100 000 cpm), a coupling ratio of 0.024 mg IgG per mg gel was found to be optimal (Figure 5.37).

Incubation Times

The assay involved a primary incubation with 200 µl standard and 100 µl ^{125}I-PH4/B1 (50 000 cpm) followed by a secondary incubation with solid-phase coupled

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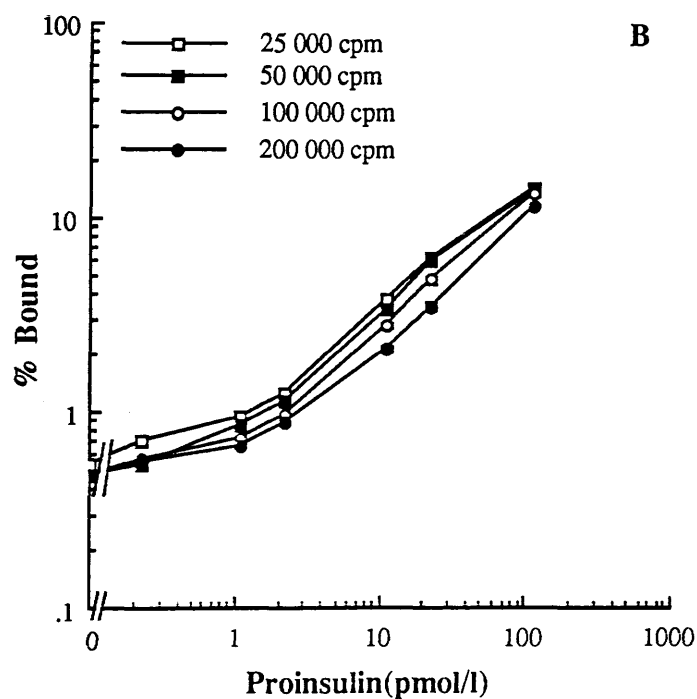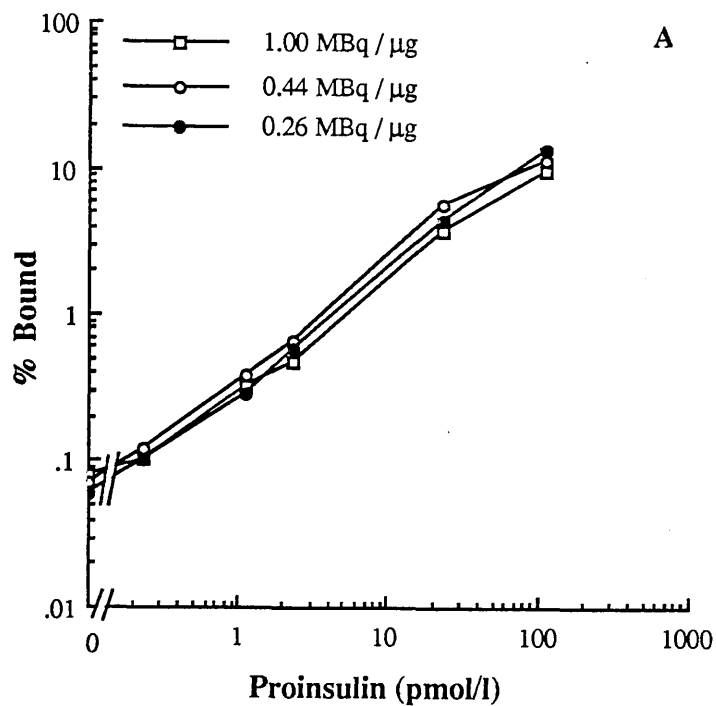
Figure 5.36

Evaluation of anti-insulin iodinated to three specific activities
(A) and the effect of varying the mass of one of the preparations
(0.44 mBq) added per tube (B).

TABLE 5.13

COUPLING OF MONOCLONAL ANTI-PROINSULIN TO
SEPHAROSE^R CL-4B

IgG offered (mg)*	0.012	0.024	0.050	0.100
IgG coupled (mg)*	0.002	0.003	0.005	0.006
% uptake of IgG	16.7	12.5	10	10

*mg IgG per mg gel.
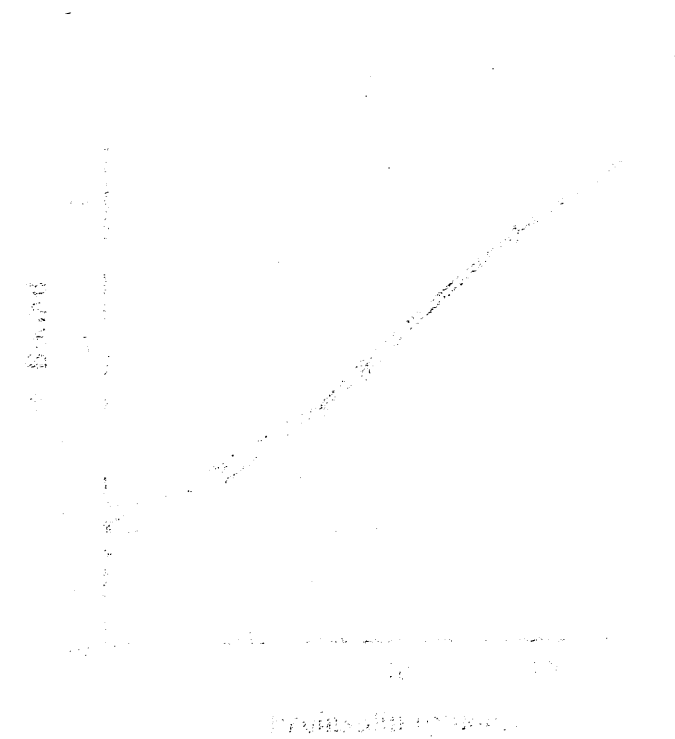
For details of coupling reaction see Chapter 2, Section 5.3.
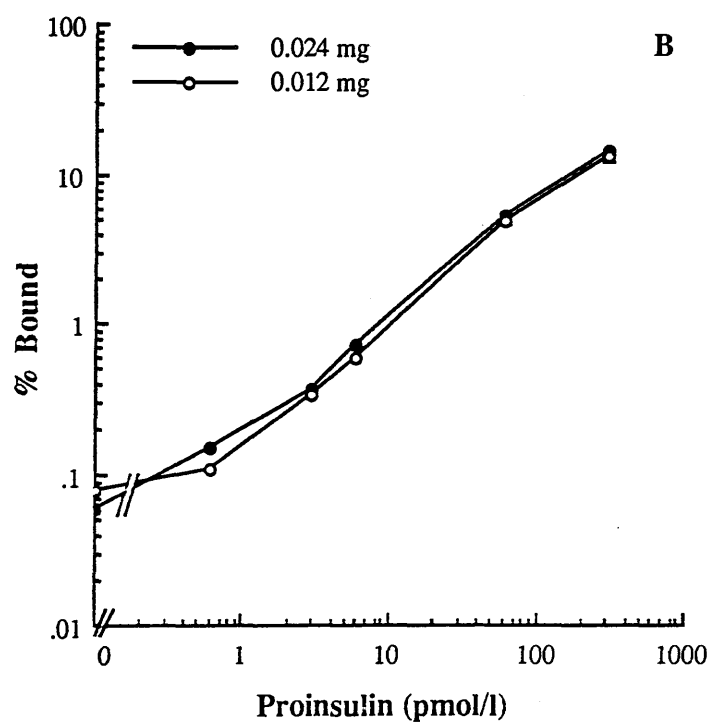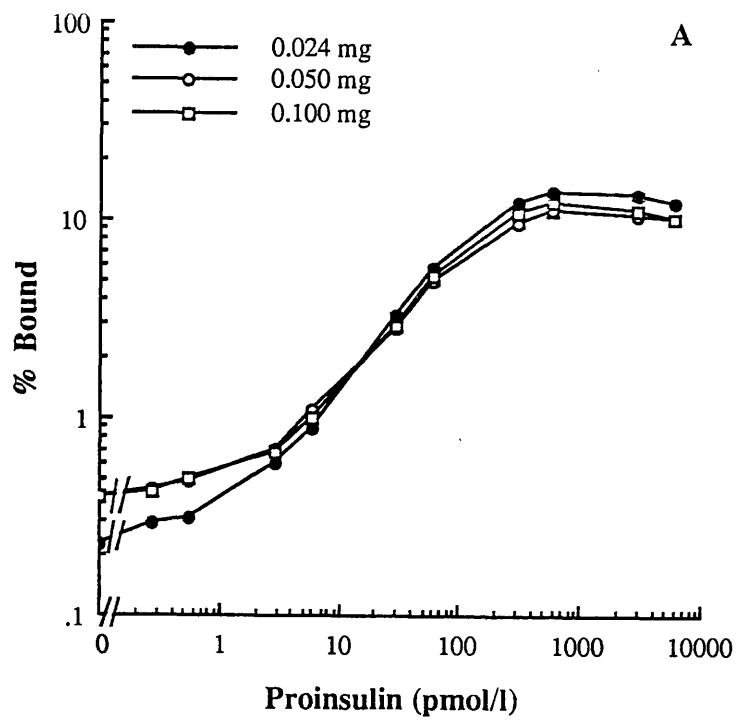
Figure 5.37

Optimisation of the coupling ratio of IgG:solid-phase.

PH5/B5 (1 mg) prior to washing (x6) and counting. Initially, optimisation of the primary incubation was performed at 3, 6, 16 and 24 hours (Figure 5.38). The sensitivity and range of the assay did not appear to change from 3 to 24 hours and seven incubation times between 0.5 and 6 hours were evaluated (Figure 5.38 B and C). A reaction time of 1 h appeared optimal in terms of assay sensitivity. Evaluation of the second incubation between 0.5 and 4 hours indicated that maximum sensitivity was achieved after 0.5 h (Figure 5.39).

One or Two Incubation Steps

The two-step assay protocol described above (1 h + 0.5 h) was compared with a one-step assay in which all the reagents were added simultaneously and incubated for 1.5 h (Figure 5.40). The latter technique markedly reduced the assay sensitivity and the two-step protocol was subsequently adopted.

Wash Cycles

200 μl aliquots of standards (0-660 pmol/l) and 100 μl ^{125}I-PH4/B1 (50 000 cpm) were incubated for 1 h followed by a second incubation with solid-phase coupled PH5/B5 (1 mg) for 0.5 h prior to washing and counting. The assay was counted after each wash cycle (1-8) and standard curves constructed (Figure 5.41). For maximum sensitivity to be achieved, six wash cycles were mandatory.

5.3 Assay Validation

Matrix Effects

To establish the effect of different matrices on the proinsulin IRMA, blood samples from three volunteers (A, B and C) were collected as serum, plasma-heparin (2 mg/10 ml blood) and plasma-EDTA (10-20 mg/10 ml blood), all with and without Traysylol (4 000 U/10 ml blood). Standard curves were constructed with
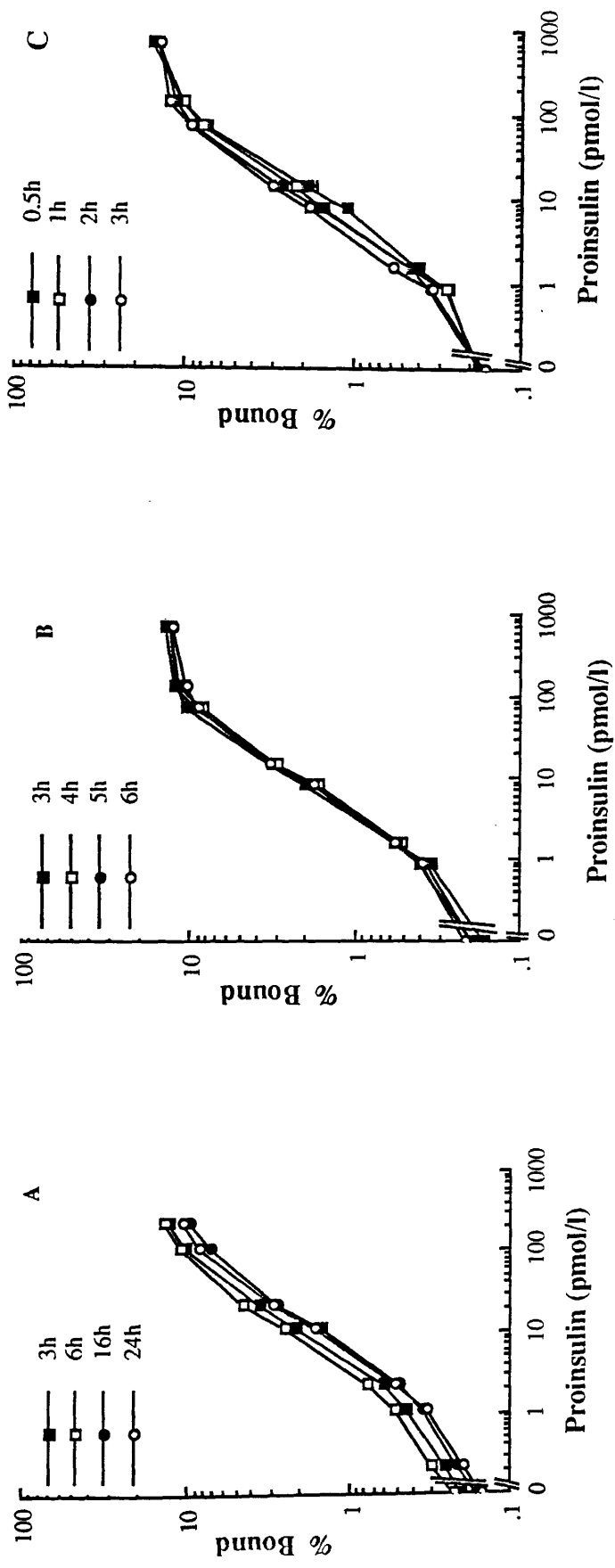
Figure 5.38

Optimisation of the primary incubation step of the proinsulin IRMA-1.
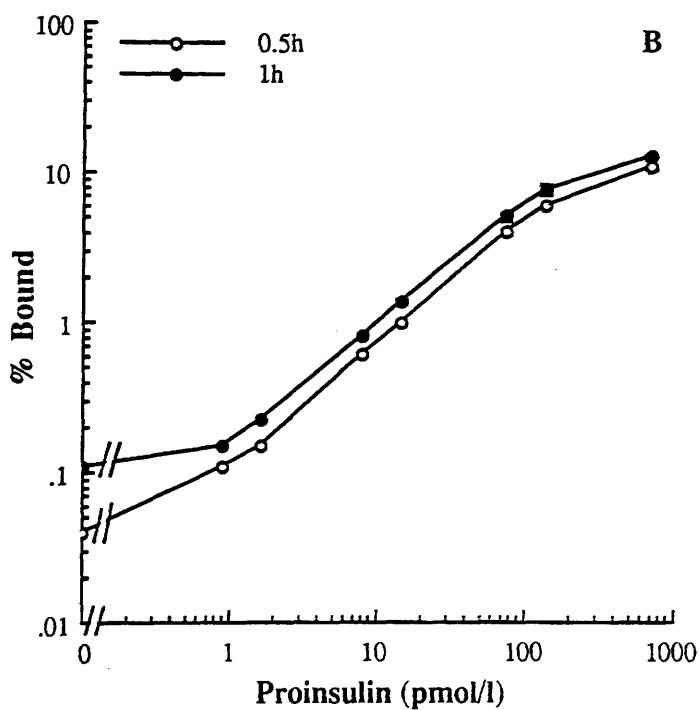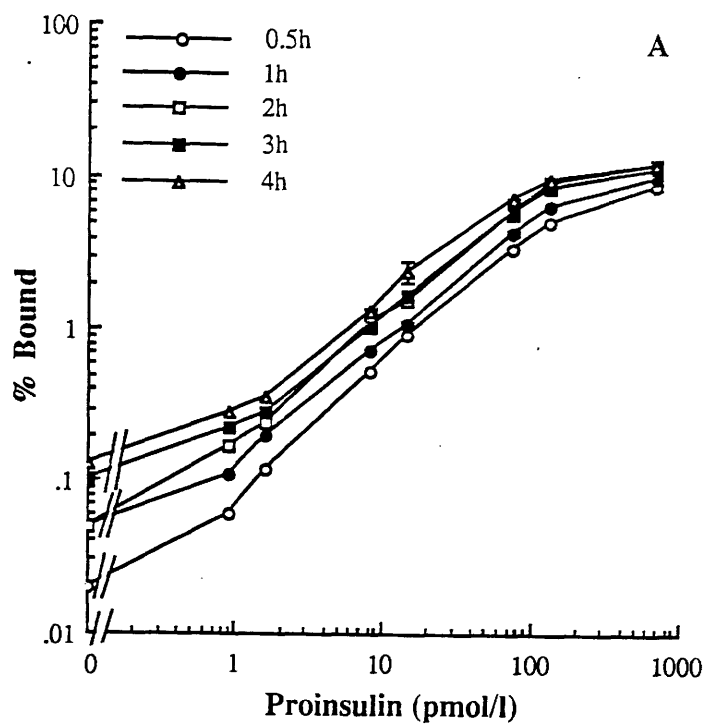
Figure 5.39

Optimisation of the secondary incubation of the proinsulin
IRMA-1.
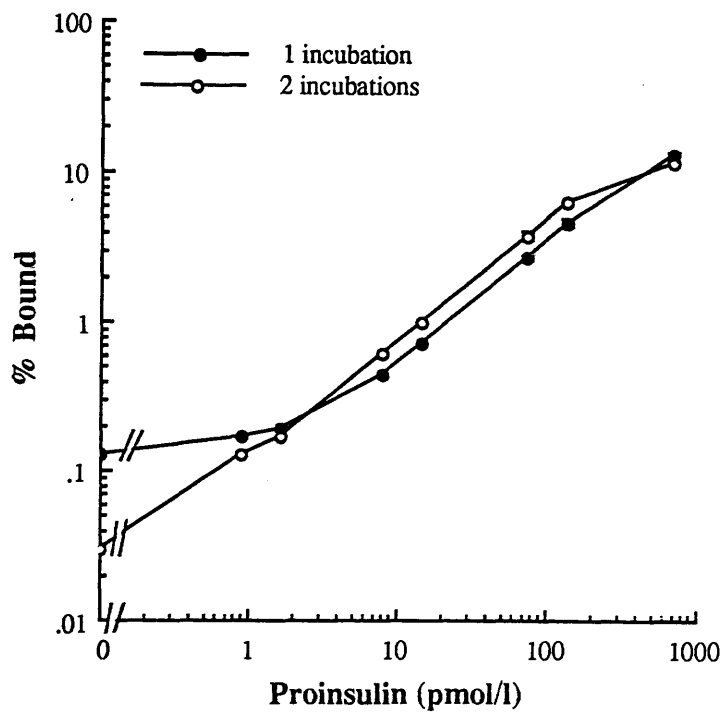
Figure 5.40

Comparison of a one-step or two-step assay protocol.
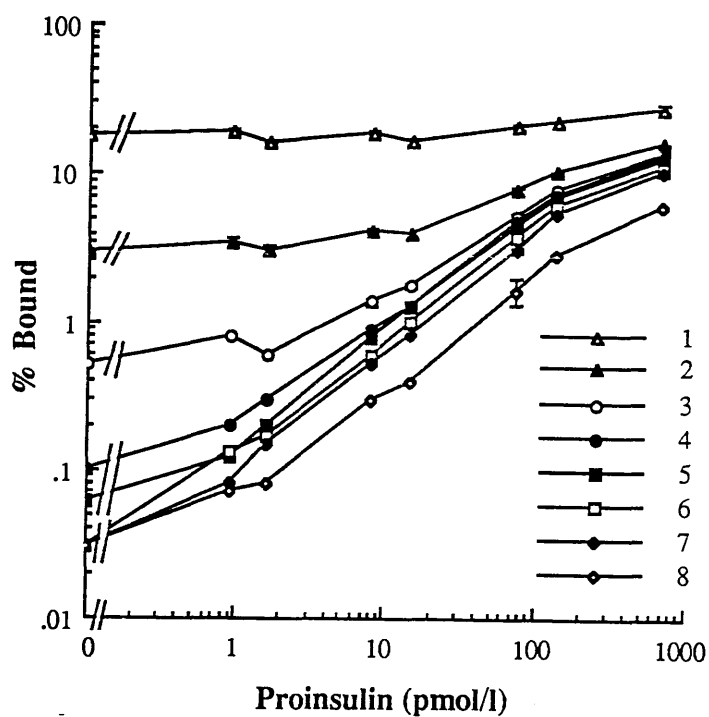
Figure 5.41

Optimisation of the number of washes required, prior to counting, in the proinsulin IRMA-1.

proinsulin standards diluted in assay buffer, HFS and each of the above test sera (Figure 5.42). The pattern of curves obtained from standards diluted in each matrix were similar for all three individuals with no difference observed between serum and plasma in either the presence or absence or Trasylol. Therefore, serum was chosen as the sample matrix. There was also no difference in the standard curves obtained when standards were diluted in buffer or HFS. Standards were subsequently prepared in assay buffer and stored at -70°C for future use.

Stability of Proinsulin in Whole Blood and Serum

To determine the stability of proinsulin in whole blood and serum, blood samples were collected from five volunteers and treated as described in the corresponding part of Section 2.4. The samples were assayed simultaneously and the results calculated as the concentration of proinsulin measured at each time point expressed as a percentage of the concentration of proinsulin measured at zero time (% proinsulin remaining). The results from each of the five volunteers are shown separately with the median values at each time point being connected (Figure 5.43). Two-way analysis of variance showed that there was a significant degradation of proinsulin in whole blood ($p<0.0001$) and serum ($p<0.001$). Analysis of the data by the Students paired t-test indicated that proinsulin levels in whole blood decreased significantly after 2 hours at room temperature ($p<0.01$) and after 4 hours in serum at room temperature ($p<0.01$). Therefore, it was recommended that blood samples were allowed to clot at room temperature for 30 minutes prior to separation and the serum be frozen within a further 30 minutes if proinsulin was to be measured.

Recovery of Proinsulin from Serum

Recovery of proinsulin from serum was evaluated by adding known quantities of standard proinsulin to each of three individual sera and one pool of human serum. Two replicates were performed for each dose level in each serum and the percentage
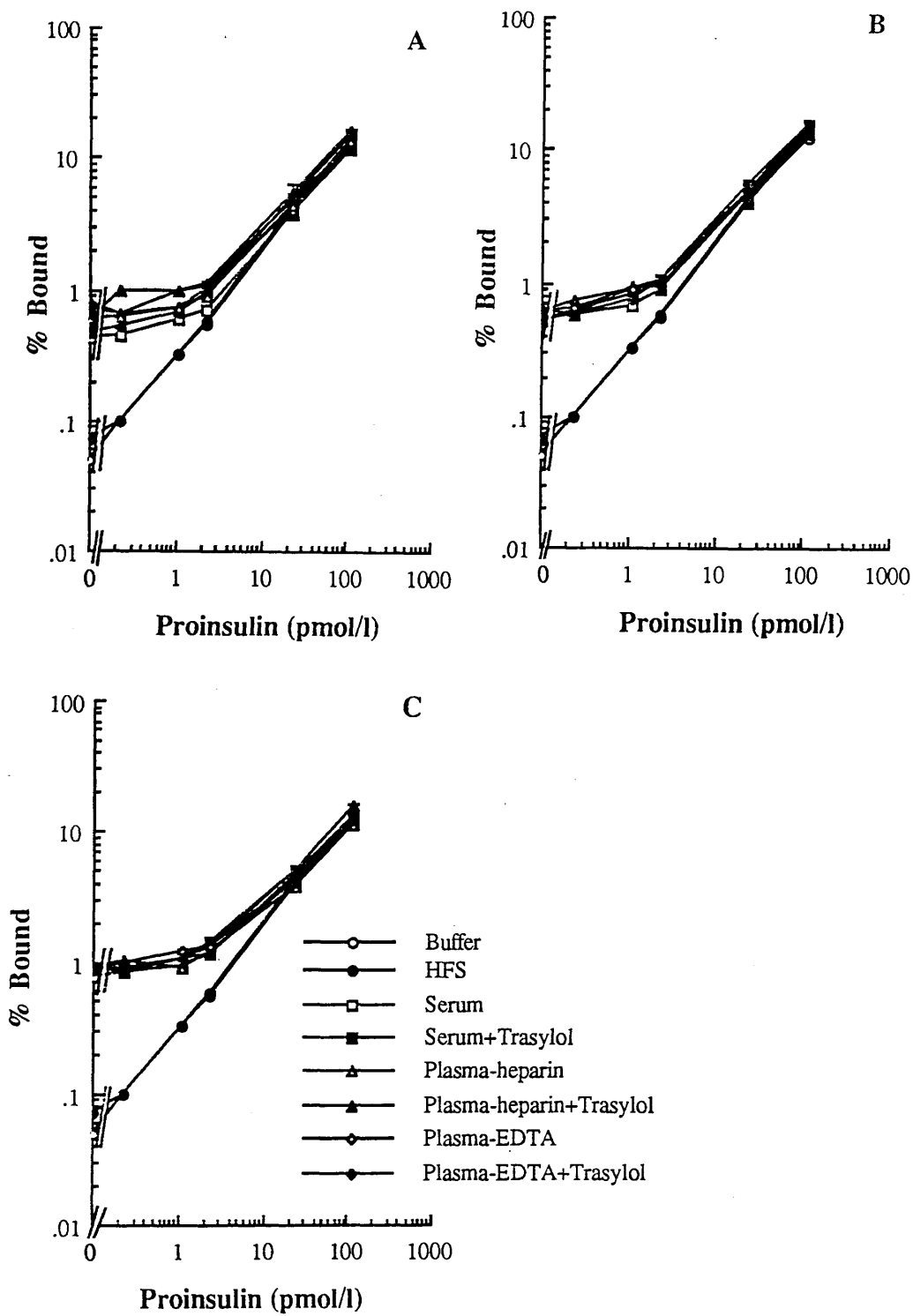
Figure 5.42

Effect of different matrices on the proinsulin IRMA-1.
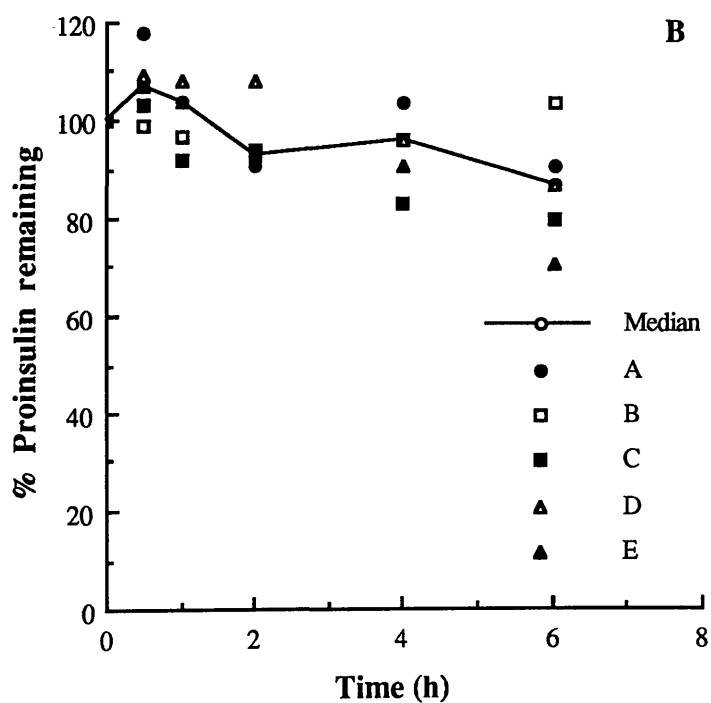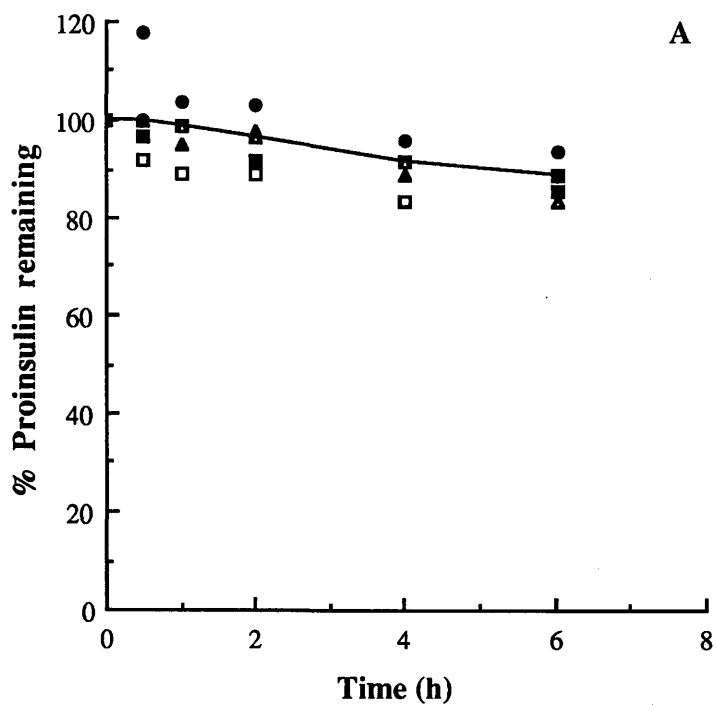
Figure 5.43

Stability of proinsulin in whole blood (A) and serum (B).

recovery calculated from the equation:

$$\% \text{ Recovery} = \frac{\text{Measured proinsulin - Serum proinsulin}}{\text{Added Proinsulin}} \times 100$$

The results obtained are summarised in Table 5.14 and indicate that proinsulin was recovered quantitatively over the working range of the assay.

The Effect of Insulin on the Proinsulin IRMA

The effect of insulin on the proinsulin IRMA was investigated by diluting proinsulin standards with buffer or insulin standards. Standard curves were constructed in the presence of different concentrations of insulin (Figure 5.44). The effect of adding 25 and 50 mU/l insulin was to shift the standard curve to the right suggesting that the insulin was competing with proinsulin for antibody binding sites. The monoclonal anti-proinsulin does not react with insulin (Chapter 4, Table 4.2), therefore the insulin must be competing with proinsulin for binding to the ^{125}I-labelled monoclonal anti-insulin. Insulin interference was not abolished by increasing the mass of ^{125}I-anti-insulin added per tube (Figure 5.45). Therefore this assay was considered to be unsuitable for use with clinical samples.

6   PROINSULIN IRMA-2 WITH PROINSULIN-SPECIFIC MONOCLONAL ANTIBODY

In an attempt to abolish the interference of insulin in the proinsulin IRMA, the assay was reversed, ie, proinsulin was immunoextracted from standards or samples by incubation with solid-phase coupled monoclonal anti-proinsulin prior to washing (to remove insulin) and a second incubation with ^{125}I-anti-insulin. All subsequent assays were performed using the WHO proinsulin standard, code 84/611, which only became available during this study.

TABLE 5.14

RECOVERY OF PROINSULIN FROM SERUM

Proinsulin added (pmol/l)	% Recovery ±SD			
	A	B	C	D
7.3	107.1±10.8	104.1±8.1	132.2±29.4	72.5±4.7
13.2	101.6±5.5	107.8±5.8	113.1±10.0	93.0±2.6
66.0	104.9±15.3	119.9±0.1	108.4±5.4	100.2±13.9
120.0	97.6±22.8	127.1±2.5	88.4±2.8	92.4±6.2

*A-C = individual sera   D = pooled human serum
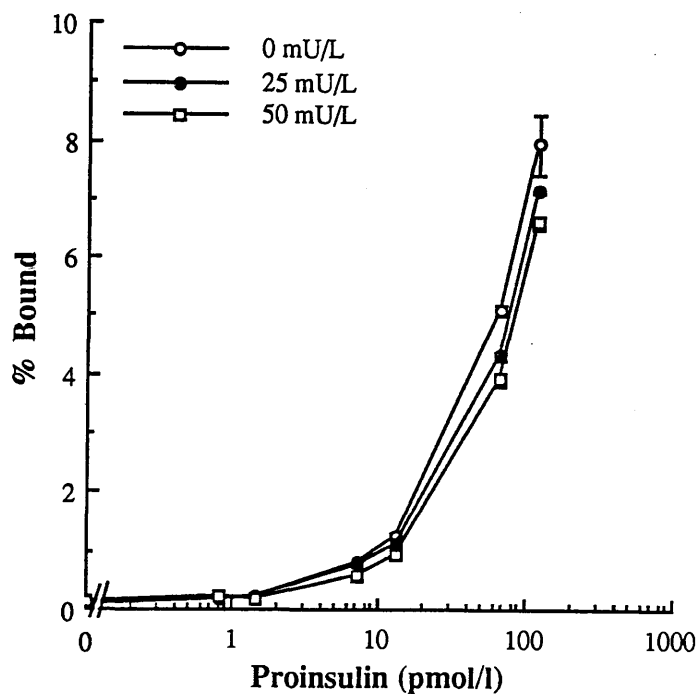
Figure 5.44

Effect of insulin (25 and 50 mU/l) on the proinsulin IRMA-1.

200 μl proinsulin standard (diluted in buffer or insulin) and 100 μl ^{125}I-PH4/B1 (50 000 cpm) were incubated for an hour followed by a second incubation with solid-phase coupled PH5/B5 (1 mg) for 0.5 h. The assay was washed six times prior to counting.
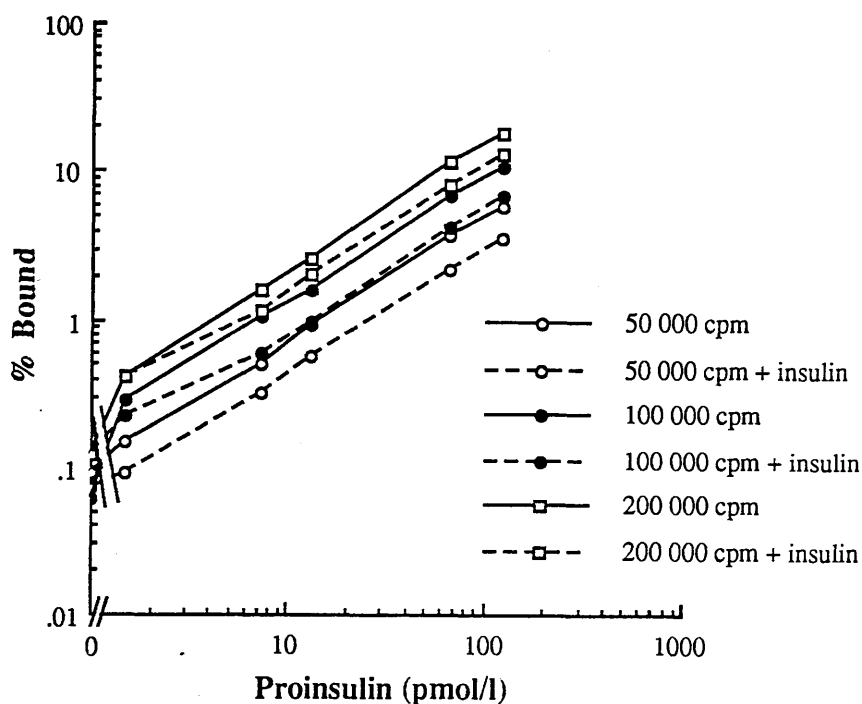
Figure 5.45

Effect of insulin on the proinsulin IRMA-1 : varying the mass of ^{125}I-PH4/B1 added per tube.

Assay details as for Figure 5.44 but 100 mU/l insulin was used to dilute proinsulin standards.

6.1 Assay Optimisation

Incubation Times

The primary incubation involved mixing 200 µl aliquots of standard and solid-phase coupled PH5/B5 (1 mg) for 0.5 h followed by three washes with assay buffer. ^{125}I-PH4/B1 (50 000 cpm in 100 µl) was then added and incubated for 1 h prior to six washes with 0.2% Tween 20/0.9% saline. The primary incubation was complete after 0.5 h while the second incubation was optimal at 1 h (Figure 5.46). Increasing the length of the second incubation beyond 1 h caused a sharp rise in the zero dose binding resulting in decreased assay sensitivity.

Solid-Phase Coupled Anti-Proinsulin

The effect of varying the amount of solid-phase added per tube was evaluated at four masses, 0.25, 0.5, 1 and 2 mg. The assay was performed as described above. A mass of 0.5 mg was optimal in terms of assay sensitivity (Figure 5.47A).

^{125}I-Labelled Anti-Insulin

The effect of adding masses equivalent to 25 000, 50 000, 100 000 or 200 000 cpm ^{125}I-PH4/B1 per tube was evaluated and the results are shown in Figure 5.47B. The standard curves obtained were superimposable and a mass of 200 000 cpm was chosen for subsequent experiments as this was likely to result in greater accuracy in the quantitation of radioactivity.

Wash Cycles

The number of wash sequences required after the second incubation prior to counting, was evaluated in the presence and absence of insulin (100 mU/l). Proinsulin standards were diluted in buffer or buffer containing insulin, and 200 µl aliquots incubated with solid-phase coupled PH5/B5 (0.5 mg) for 0.5 h prior to six
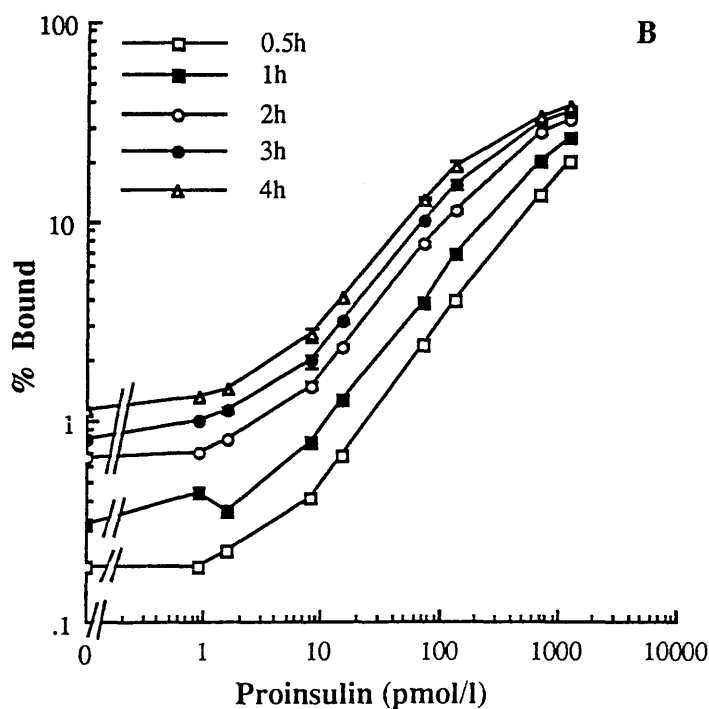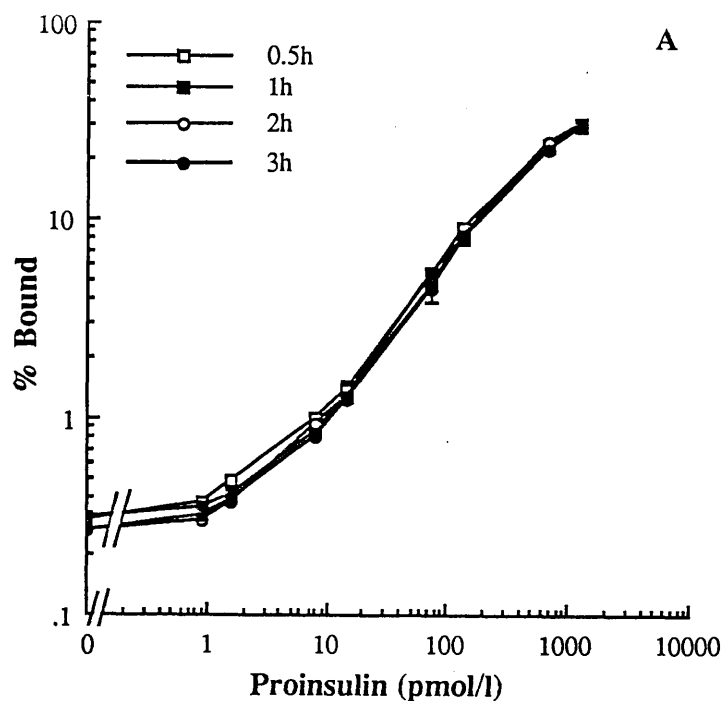
Figure 5.46

Optimisation of the primary (A) and secondary (B) incubation steps in the proinsulin IRMA-2.

During optimisation of the primary incubation (A), the secondary incubation was held at 1 h and during optimisation of the secondary incubation (B), the primary incubation was held at 0.5 h.
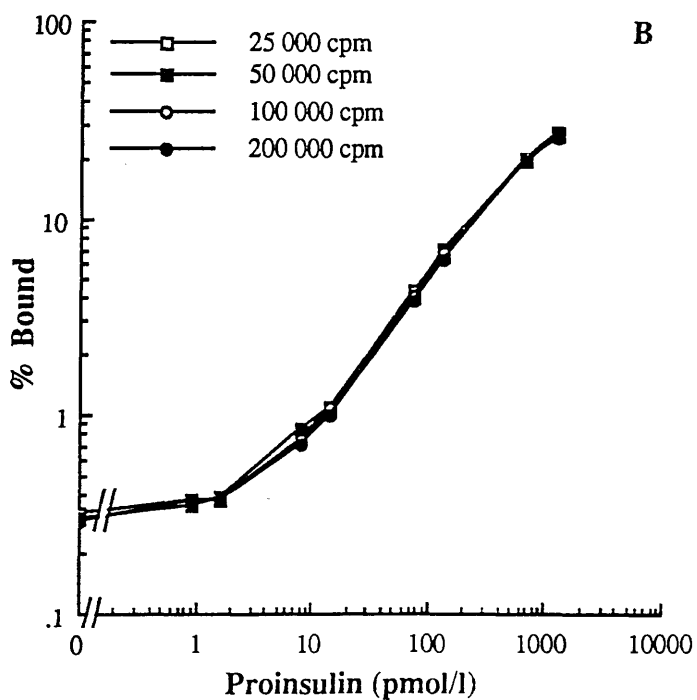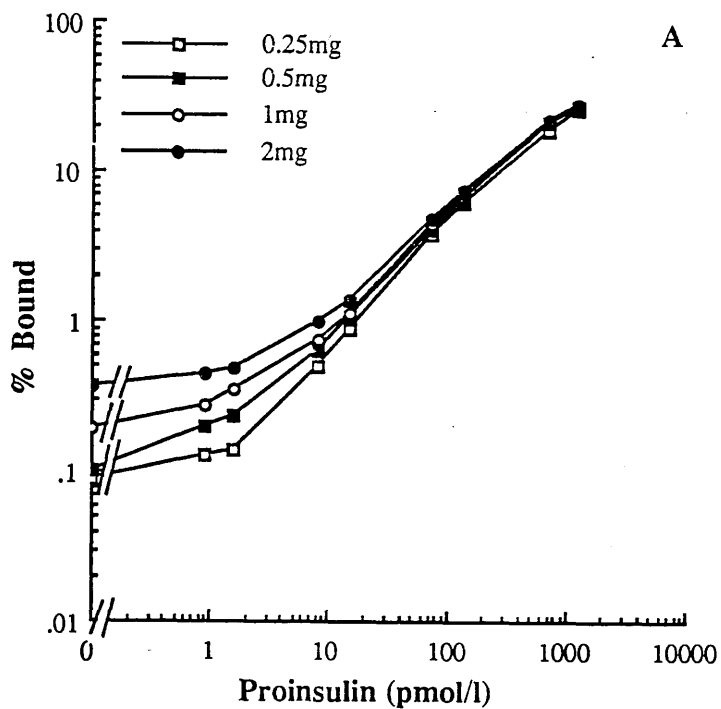
Figure 5.47

Optimisation of the mass of solid-phase (A) and ^{125}I-labelled antibody (B) added per tube in the proinsulin IRMA-2.

A - ^{125}I-PH4/B1 was 50 000 cpm.
B - solid-phase coupled PH5/B5 was 1 mg.

washes with assay buffer. ^{125}I-PH4/B1 (200 000 cpm in 100 µl) was then added and incubated for 1 h prior to three or six washes with 0.2% Tween 20/0.9% saline and counting. Assay tubes were only counted after three and six washes as previous experiments had shown six to be optimal (Section 5, Figure 5.41). The assay sensitivity was improved from approximately 10 pmol/l to less than 2 pmol/l by increasing the number of wash cycles from three to six respectively (Figure 5.48).

The effect of four or six wash cycles between the primary and secondary incubation steps was evaluated as described above using undiluted proinsulin standards and six wash cycles after the second incubation. There was no difference in the standard curves obtained (Figure 5.49) and six wash cycles was adopted as this was likely to result in consistently lower zero dose binding levels.

6.2 Assay Validation

The effect of insulin (1000 mU/l) on the redeveloped proinsulin IRMA is shown in Figure 5.50A. An increase in the percent of ^{125}I-anti-insulin bound was observed at the lower end of the standard curve in the presence of insulin, in contrast to the reduction seen in the previous assay (Section 5, Figure 5.45). The WHO insulin standard (66/304) is known to contain a trace amount of proinsulin as determined by gel filtration chromatography. Therefore 4 ml insulin standard (1000 mU/l) was absorbed with 60 mg Sepharose^R CL-4B coupled monoclonal anti-proinsulin (2 h at room temperature) and used, in parallel with buffer and unabsorbed insulin (1000 mU/l), to dilute proinsulin standards (Figure 5.50B). Absorption of the insulin standard with the proinsulin-specific McAb markedly reduced the observed effect of insulin on the assay, as shown by the decrease in the percent of ^{125}I-anti-insulin bound at the lower end of the standard curve. This corresponded to an apparent reduction in the concentration of proinsulin contamination from approximately 10 pmol/l (pre-absorption) to approximately 1 pmol/l (post-absorption).
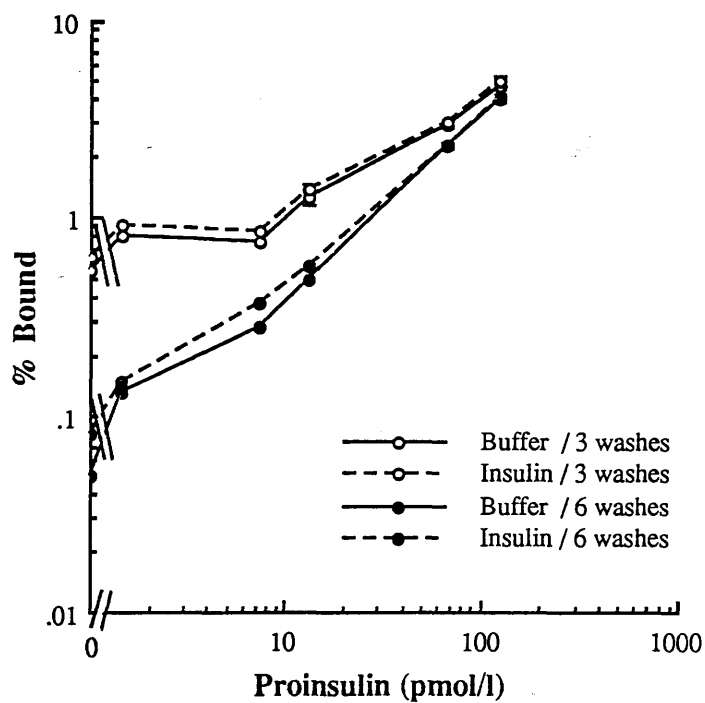
Figure 5.48

Optimisation of number of wash sequences required after the
second incubation of the proinsulin IRMA-2 prior to counting.
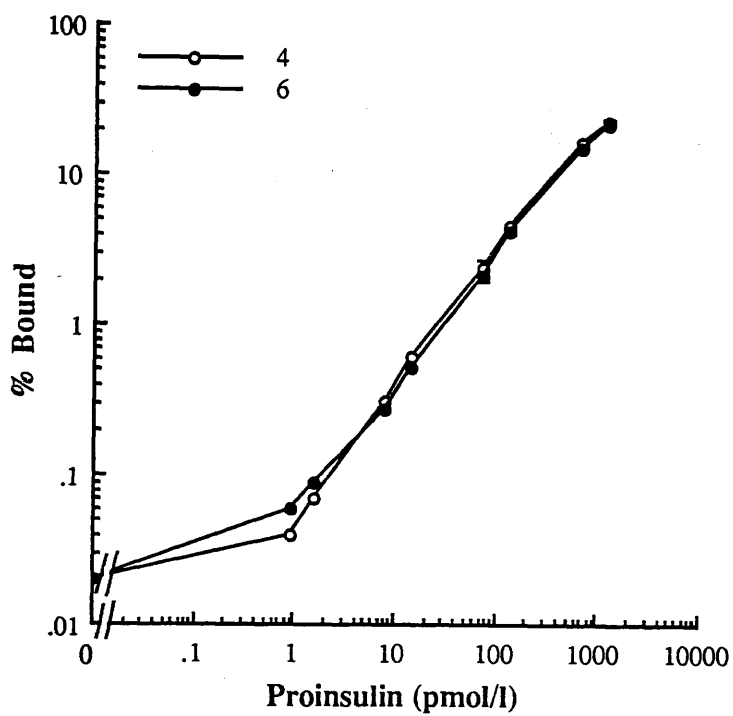
Figure 5.49

Optimisation of the number of wash sequences required between
the first and second incubations of the proinsulin IRMA-2.
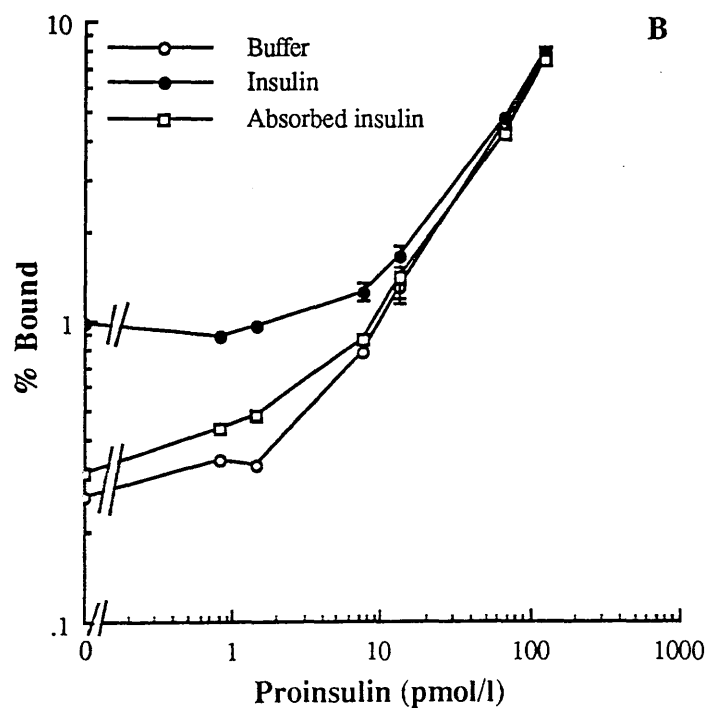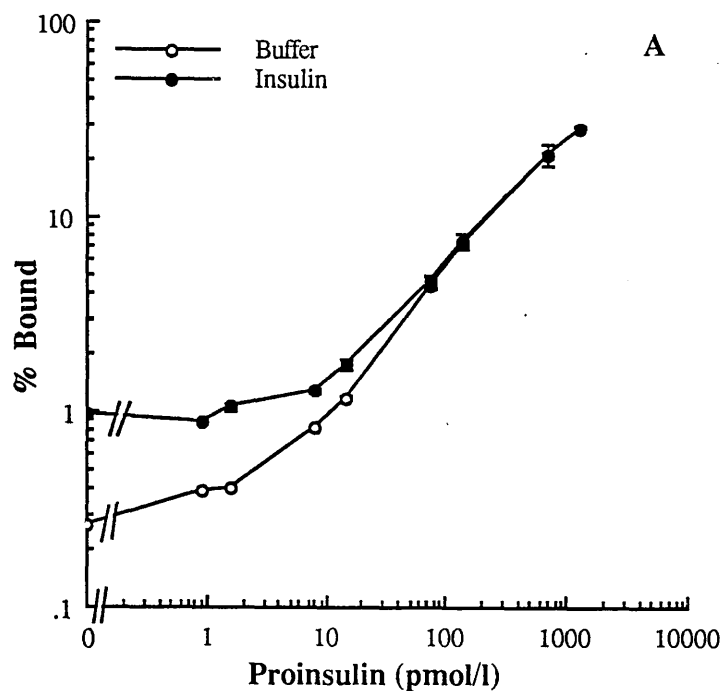
Figure 5.50

Effect of insulin on the proinsulin IRMA-2 before (A) and after (B) absorption with a solid-phase coupled proinsulin-specific monoclonal antibody.

Reactivity of Split and Des-Amino Proinsulins in the Proinsulin IRMA

The pattern of reactivity of split and des-amino proinsulins in the proinsulin IRMA is shown in Figure 5.51. The percentage reactivity of each form at two dose levels is shown in Table 5.15. The assay described does not react with 32-33 split or des 31-32 proinsulins except when they are present in extremely high concentrations (>1 nmol/l). 56-57 split proinsulin showed the greatest reactivity relative to intact proinsulin (70.8 to 75.0%). The other three forms exhibited similar reactivities relative to intact proinsulin: des 57-65 (54.2-68.8%), des 64-65 (53.3-64.6%) and 65-66 split (54.2-55.0%). As the two forms 56-57 split and des 57-65 do not occur physiologically (40, 118) the proinsulin assay effectively measures intact proinsulin, 65-66 split and des 64-65 proinsulins.

Recovery of Intact Proinsulin from Serum

Recovery of intact proinsulin from serum was evaluated by adding known quantities of standard proinsulin to each of four individual sera. Ten replicates were performed for each dose level in each individual serum and the percentage recovery calculated from the equation:

$$\% \text{ Recovery} = \frac{\text{Measured proinsulin - Serum proinsulin}}{\text{Added proinsulin}} \times 100$$

The results obtained are summarised in Table 5.16 and indicate that intact proinsulin was recovered quantitatively from serum.

Recovery of Split and Des-Amino Proinsulins from Serum

Recovery of the split and des-amino forms of proinsulin were evaluated in one serum at two dose levels. Ten replicates were performed at each dose level for each

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form of proinsulin. The percentage recovery was calculated as described for intact proinsulin and the results summarised in Table 5.17.

Parallelism Between Intact Proinsulin Standards and Endogenous Serum Proinsulin

Serum samples from four patients, selected because they had proinsulin concentrations greater than 70 pmol/l, were diluted with assay buffer and measured in the proinsulin IRMA. The results from each patient (A-D) were plotted together with the standard curve (Figure 5.52). All four samples diluted in parallel with the standards.

Stability of Proinsulin to Repeated Freeze-Thaw Cycles

Intact proinsulin was added to pooled human serum at four dose levels (15, 35, 105 and 305 pmol/l) and each resulting pool was divided into aliquots and frozen at -30°C. Separate aliquots of each pool were then subjected to 0, 1, 2, 3 or 4 freeze-thaw cycles prior to assay in the proinsulin IRMA. The results (Figure 5.53) were subjected to two-way analysis of variance which indicated that there was no significant difference in proinsulin concentrations after four freeze-thaw cycles.

Stability of Proinsulin in Frozen Samples

Blood samples were collected from five volunteers and the serum separated as described in Chapter 2, Section 3.3. The serum from each subject was divided into aliquots and stored at -30°C until required for assay. Single aliquots from each volunteer were thawed and assayed at various time intervals over a five month period (Figure 5.54). Two-way analysis of variance indicated that there was no significant degradation of proinsulin in frozen samples stored at -30°C up to five months.

TABLE 5.17

RECOVERY OF SPLIT AND DES-AMINO PROINSULINS FROM SERUM

Proinsulin form	Proinsulin added (pmol/l)	% Recovery ±SD
Intact	7.3	98.6±6.2
	66.0	110.6±8.7
65-66 split	5.3	96.6±5.2
	52.6	93.4±7.7
Des 64-65	7.5	104.5±10.7
	60.0	95.2±8.7
56-57 split	8.0	97.1±6.6
	64.0	94.2±8.2
Des 57-65	8.7	101.5±11.6
	69.9	90.7±6.4
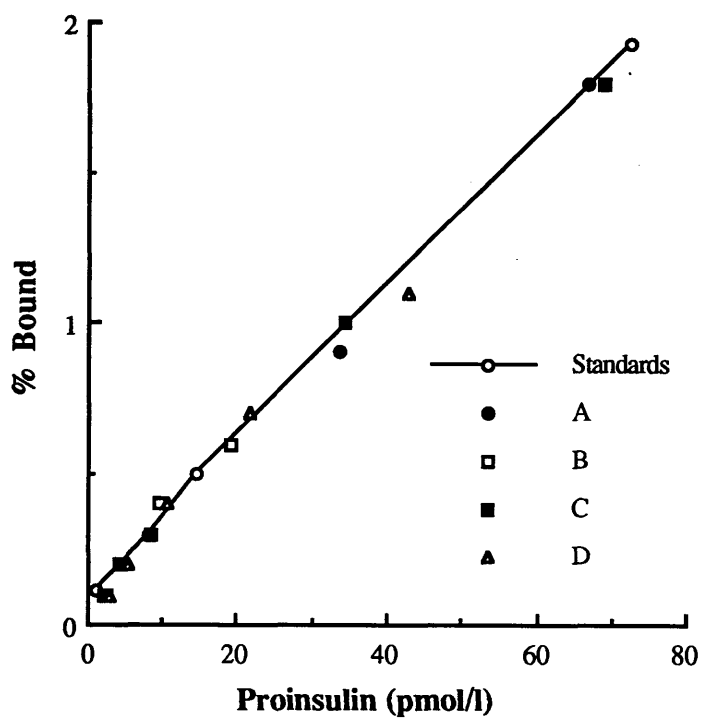
Figure 5.52

Parallelism between intact proinsulin standards and endogenous serum proinsulin in four patients (A-D).
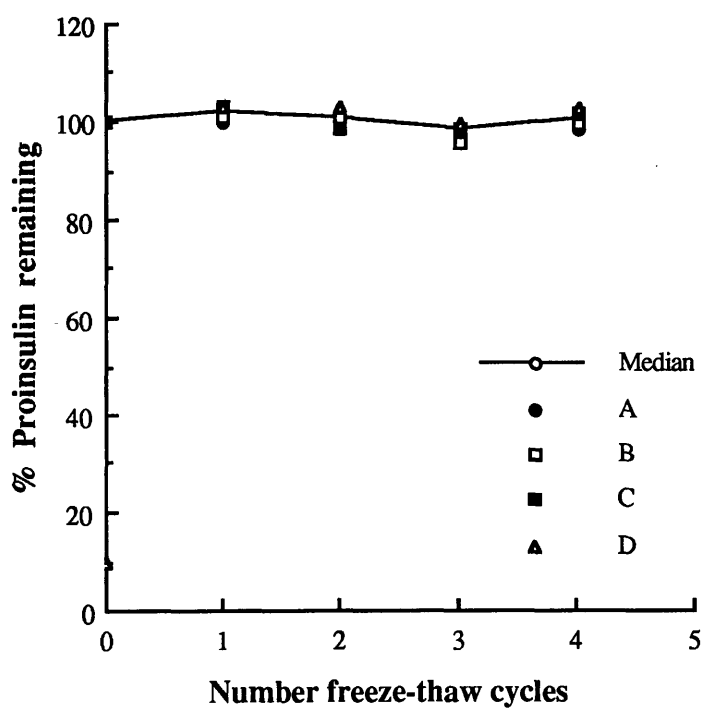
Figure 5.53

Stability of proinsulin to repeated freeze-thaw cycles.
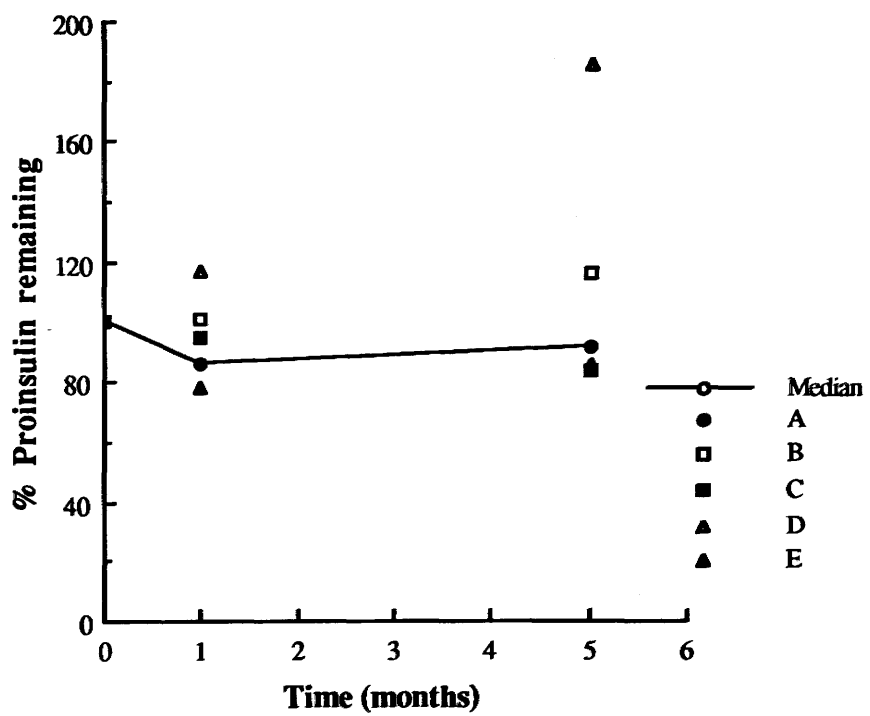
Figure 5.54

Stability of proinsulin in frozen samples.

The Final Proinsulin IRMA

Figure 5.55 shows a typical standard curve and within assay precision profile for the optimised proinsulin IRMA. The sensitivity of the assay was 1.2 pmol/l (CV 22%) with a range of 3 to >1200 pmol/l (CV<10%). The inter-assay coefficients of variation were calculated using QC samples prepared at five dose levels and the results are shown in Table 5.18.

6.3 Comparison of the Three Proinsulin IRMA's Developed

The final proinsulin IRMA was compared with those described in Sections 5 and 4 (Figures 5.56 parts A and B respectively). The assay protocols are outlined in Table 5.19.

The assay described in Section 5 (^{125}I + SP) resulted in better sensitivity than the final assay apparently due to the lower zero dose binding achieved. This cannot be a function of the solid-phase or ^{125}I-labelled antibody as the same preparations were used in both assays. The only difference between the assays is the order of addition of the reagents and therefore the length of time the solid-phase is incubated with ^{125}I-labelled antibody, ie, one hour in the final assay compared with 30 minutes in the other. When this incubation was reduced to 30 minutes in the final assay (Section 6.1, Figure 5.46), the zero dose binding was lowered but the binding levels at each dose of proinsulin were lowered by the same factor, resulting in no increase in sensitivity. Therefore, the most likely reason for the reduced sensitivity of the final proinsulin IRMA is that the proinsulin, once bound to the solid-phase, is either altered structurally such that it has a lower affinity for the ^{125}I-labelled antibody or a proportion of the bound proinsulin is unavailable to bind to the iodinated antibody. However, the more sensitive assay was not clinically useful as the presence of insulin interfered with the assay causing a shift of the standard curve to the right (Section 5, Figure 5.44-5.45).
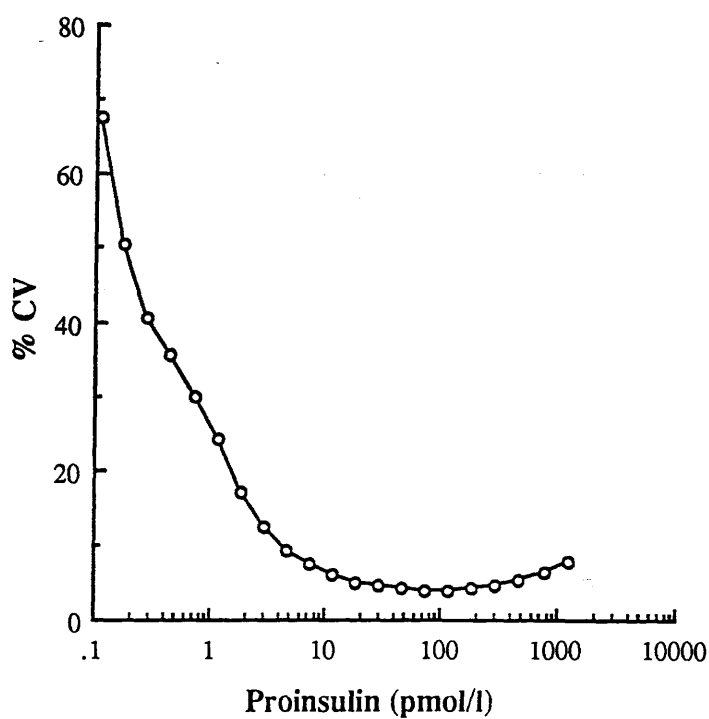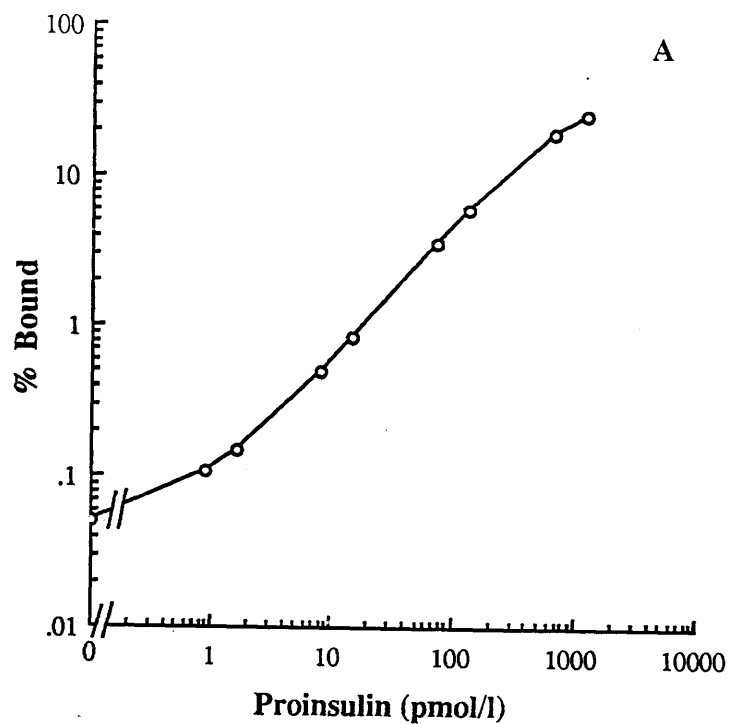
Figure 5.55

A representative standard curve (A) and within assay precision
profile (B) for the optimised proinsulin IRMA-2.

TABLE 5.18

INTER-ASSAY COEFFICIENTS OF VARIATION FOR THE PROINSULIN
IRMA-2

Proinsulin	% CV	n
3	12.1	5
15	12.5	52
35	11.5	52
105	7.5	52
305	7.9	52
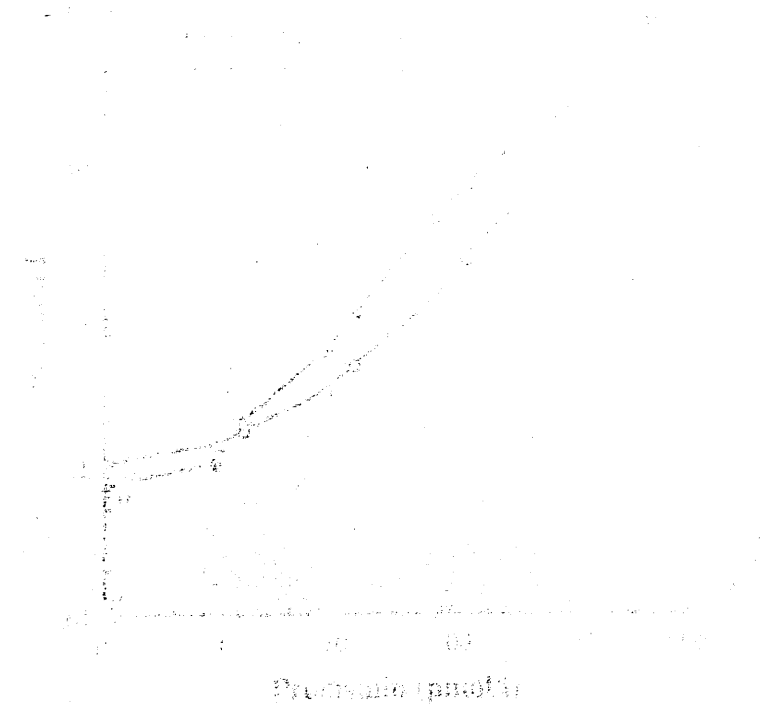
Figure 5.56

Comparison of the final proinsulin IRMA with those described
in Section 5 (A) and Section 4 (B). Details of assay in text.

TABLE 5.19

PROTOCOLS FOR THE ASSAYS COMPARED IN FIGURE 5.56

Anti-proinsulin[1] (SP + ^{125}I)	Anti-proinsulin[2] (^{125}I + SP)	Anti-C-peptide[3]
200 µl standard	200 µl standard	200 µl standard
+	+	+
200 µl SP-PH5 (0.5 mg)	100 µl ^{125}I-PH4/B1 (200 000 cpm)	200 µl SP-PD4/H4
↓ 30 min		↓ 30 min
Wash x 6 with assay buffer	↓ 1 h	Wash x 6 with assay buffer
+ 100 µl ^{125}I-PH4/B1 (200 000 cpm)	+ 200 µl SP-PH5 (0.5 mg)	+ 100 µl ^{125}I-PH4/B1 (200 000 cpm)
↓ 1 h	↓ 30 min	↓ 1 h
Wash x6 with 0.2% Tween 20/0.9% saline	Wash x6 with 0.2% Tween 20/0.9% saline	Wash x6 with 0.2% Tween 20/0.9% saline

[1]Assay described in Section 6.

[2]Assay described in Section 5.

[3]Assay using antibody evaluated in Section 4.

As the final assay described here, using the solid-phase anti-proinsulin, does not measure 32-33 split proinsulin, it was considered advantageous to re-evaluate the C-peptide McAb (PD4/H4) which had previously been shown to be capable of detecting intact, 65-66 split and 32-33 split proinsulins (Section 4, Figure 5.33). Therefore the C-peptide McAb was coupled to solid-phase and re-assessed using the primary proinsulin standard WHO 84/611 and the ^{125}I-labelled monoclonal anti-insulin (PH4/B1), both of which were unavailable when the C-peptide McAb was originally assessed. The sensitivity of the original assay described in Section 4 was approximately 20-40 pmol/l. The use of the above reagents improved the sensitivity to approximately 3-10 pmol/l. As 32-33 split proinsulin has been shown to be the major circulating form of this hormone (32), an assay with a sensitivity in this range should be capable of measuring this form in the fasting state.

In conclusion, it would appear that there is some potential for the development of a proinsulin IRMA using the monoclonal anti-C-peptide (PD4/H4) as described above, but this would require further optimisation and clinical validation studies to be performed.

7 DISCUSSION

The availability of biosynthetic human proinsulin has resulted in the more widespread development of assays for proinsulin. Comparison of the proinsulin IRMA described in this chapter with other assays utilising ^{125}I as a label (30, 31, 32, 35, 36, 37, 38) shows that it is as good as the best assays available (32) in terms of sensitivity but has the advantage of being the only assay which can be performed within one working day.

The data presented in Section 2 of this chapter confirm that two-site IRMA techniques can offer the advantages of improved specificity and sensitivity, coupled with wider working ranges and shorter incubation times when compared with RIA

techniques. Furthermore, the ultimate sensitivity achieved in an IRMA compared with an RIA is less dependent on the avidity of an antibody, for when two McAbs PD4/H4 and PH5/B5 were compared in an IRMA to measure proinsulin, in conjunction with the same partner antibody, the difference in sensitivity of the resulting assays was of the order of 10 pmol/l (Figure 5.56B). In contrast, when the same two antibodies were compared in an RIA, McAb PH5/B5 showed displacement of ^{125}I-proinsulin with cold proinsulin at doses of approximately 10 nmol/l whereas McAb PD4/H4 did not exhibit displacement of ^{125}I-proinsulin with doses of cold proinsulin up to 60 nmol/l (Chapter 4, Figure 4.6).

It is also clear from the data in this chapter that not only does a two-site IRMA require the availability of two antibodies which bind to different epitopes on the molecule to be measured, but that these antibodies must be capable of binding to the molecule simultaneously (Section 2.2, Table 5.7).

Finally, it was shown that the epitope specificity of a McAb may be as important as the avidity in determining its usefulness in a two-site IRMA technique.

CHAPTER 6

CLINICAL APPLICATION

1 NORMAL PHYSIOLOGY

1.1 Introduction

Clinical validation of the insulin and proinsulin IRMA's was performed by measuring concentrations of insulin and proinsulin in serum from normal adult subjects under a variety of conditions. Glucose was measured by the hexokinase method and C-peptide using the commercially available Medgenix kit. Measurement of glucose and C-peptide was performed by MLSO staff in the Institute of Biochemistry at Glasgow Royal Infirmary. Unless otherwise stated, results are expressed as median values with range. Statistical analyses were performed using the Wilcoxon Signed Rank and Mann-Whitney U Tests. To convert insulin concentrations from pmol/l to mU/l divide by 7.57 (calculated assuming a molecular weight of 5734 for insulin).

1.2 Twenty-Four Hour Profiles

Methods

Serum samples were collected from six male volunteers at 30 minute intervals from 1400 h one day to 1330 h on the following day. The content and timing of meals was standardised such that all subjects ate the same quantity of food at the same time.

The secretion rates of insulin and proinsulin into the peripheral circulation were calculated by deconvolution (122). The half-lives and distribution volumes of insulin and proinsulin used in the calculation were 3.9 minutes (24) and 17.2 minutes (23), and 12.5 l (122) and 9.3 l (123) respectively. The insulin and proinsulin serum concentration profiles were also analysed by cross-correlation (124). These analyses were performed by Dr D R Matthews at the Diabetes Research Laboratories, Radcliffe Infirmary, Oxford.

Results

Details of the six volunteers are shown in Table 6.1. All results are arranged in order of increasing subject age and body mass index (BMI). The glucose profiles are shown in Figure 6.1. The corresponding profiles of serum insulin and proinsulin concentrations and secretion rates, together with the analogous proinsulin/insulin ratios expressed in percentage terms, are shown in Figure 6.2.

Glucose, insulin and proinsulin concentrations rose in response to feeding (Figures 6.1 and 6.2). The number of glucose and insulin peaks observed varied between individuals, but for any one patient the pattern of insulin peaks mirrored that observed for glucose. The proinsulin profile was similar to insulin but individual peaks were not as distinct and there appeared to be a delay in the increase of proinsulin concentrations relative to insulin following the ingestion of food. This was confirmed by cross-correlation (Figure 6.3) which indicated that proinsulin concentrations increased later than insulin with a lag time of approximately 30 minutes. This estimation may in part be an artefact of the 30 minute sampling intervals adopted. More correctly the lag time should be reported as being between 1 and 30 minutes.

The sum of the glucose concentrations over the 24 hour period was similar for all six patients and ranged from 239-263 nmol/ l /24 h. However, the total amount of insulin secreted over the same period was highest in patients 1 and 2 (140 and 138 nmol respectively) and decreased progressively (117, 104, 87 and 87 nmol) in patients 4, 5, 6 and 3 respectively (Table 6.2). This decrease in total insulin secretion was, in general, reflected in the post-prandial peak insulin concentrations achieved. The total amount of proinsulin secreted did not vary in parallel with insulin (Table 6.2). Under basal conditions, from 0200-0700 h, the total quantity of insulin and proinsulin released gave a completely different pattern of results from the total 24 h secretion data (Table 6.2).

TABLE 6.1

TWENTY-FOUR HOUR PROFILE PATIENT DETAILS

Patient number	Age (years)	BMI
1	29	22.9
4	33	22.9
2	33	26.0
5	33	27.0
6	37	27.2
3	40	28.1

Patients have been listed in order of increasing age and BMI (kg/m^2).

Figure 6.1

Twenty-four hour glucose profiles.

Patient 1

Patient 4

Patient 2

Patient 5

Patient 6

Patient 3
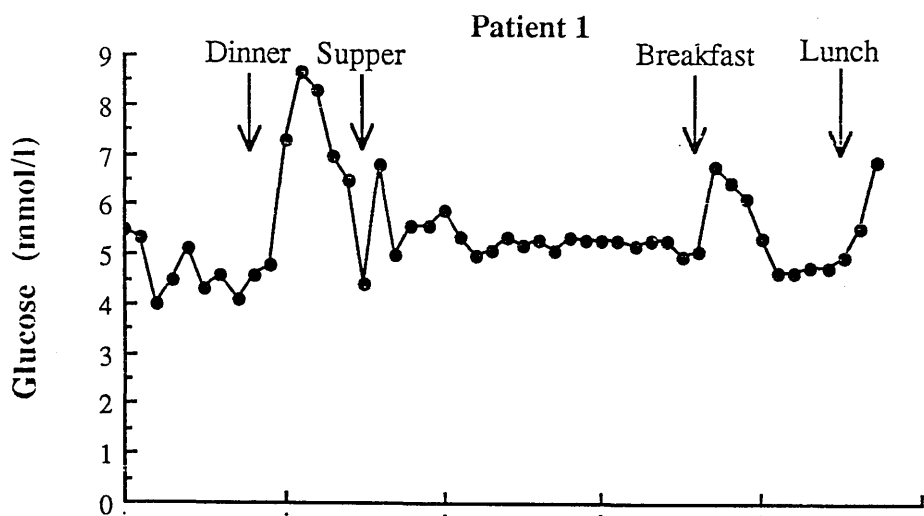
Figure 6.2

Twenty-four hour profiles of insulin and proinsulin serum concentrations and secretion rates together with the corresponding proinsulin/insulin ratios expressed in percentage terms.

Ins = insulin   PI = proinsulin   pm/L = pmol/l
PI/I % = proinsulin/insulin ratio expressed as a percentage.
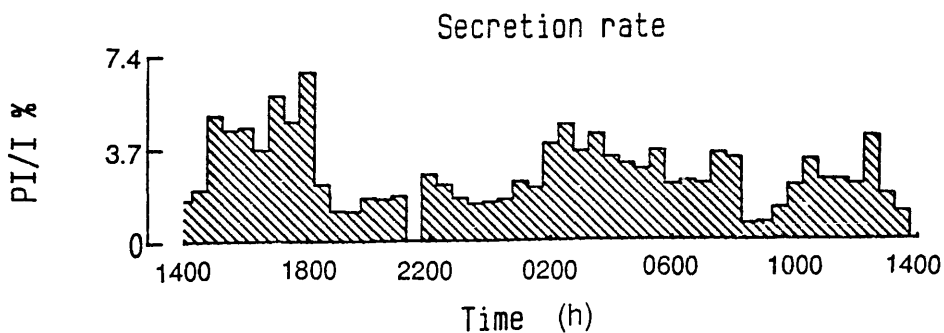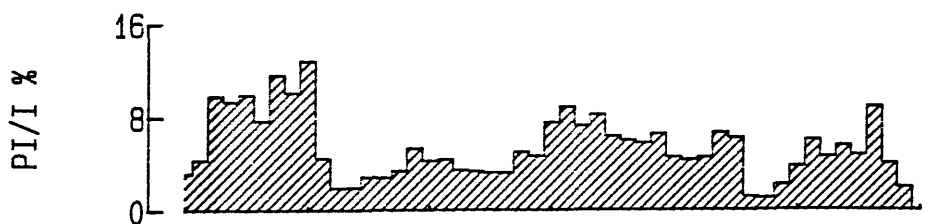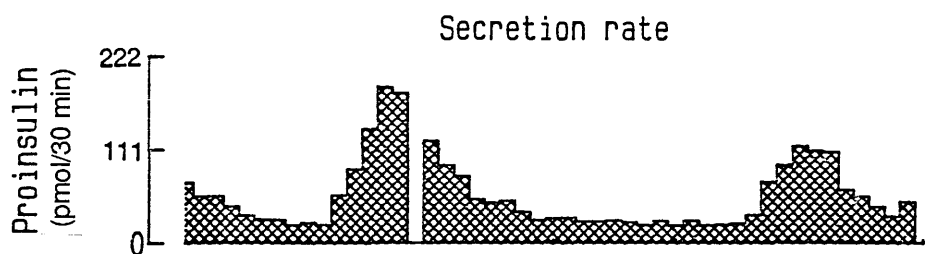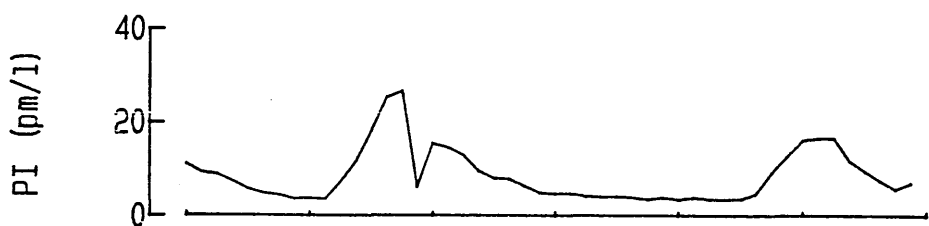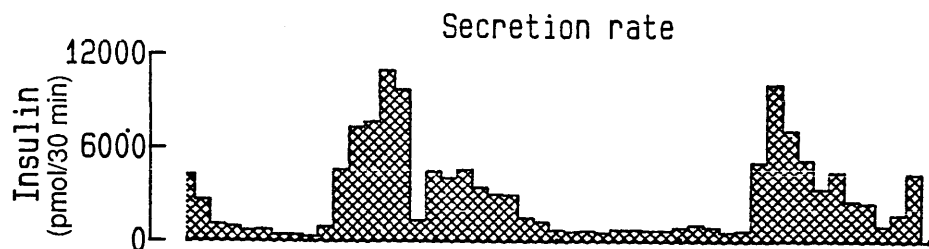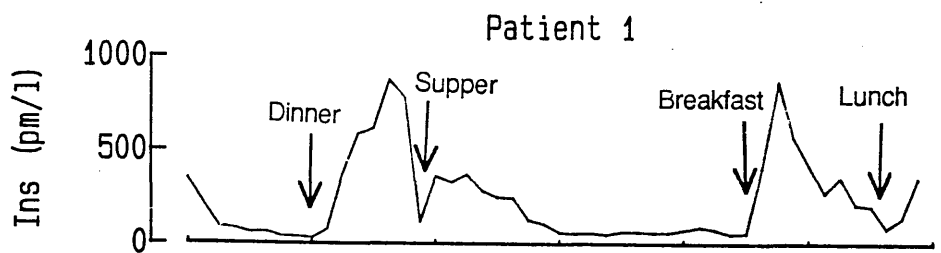
Patient 1
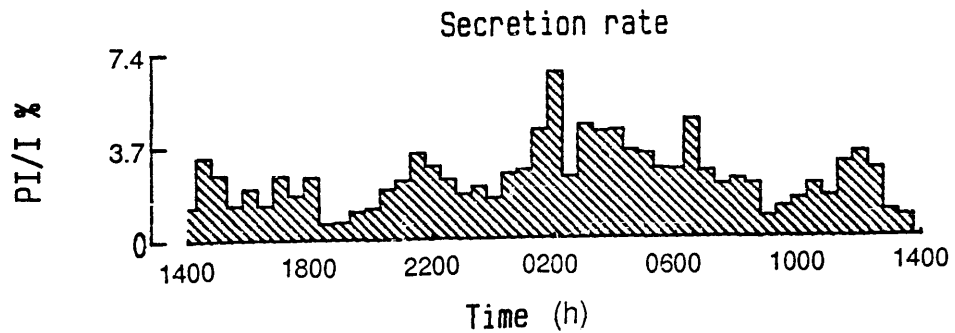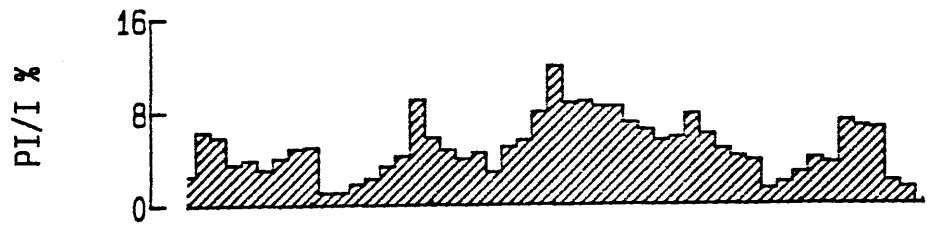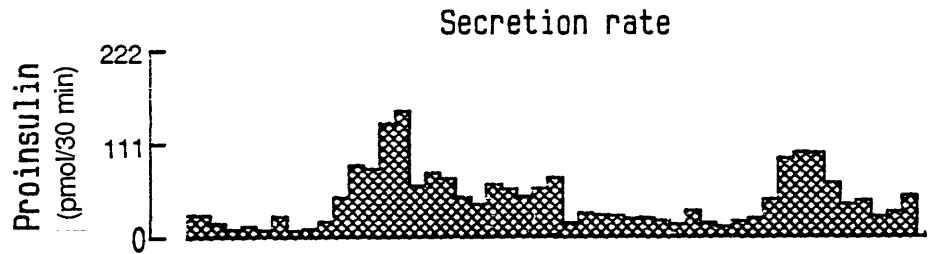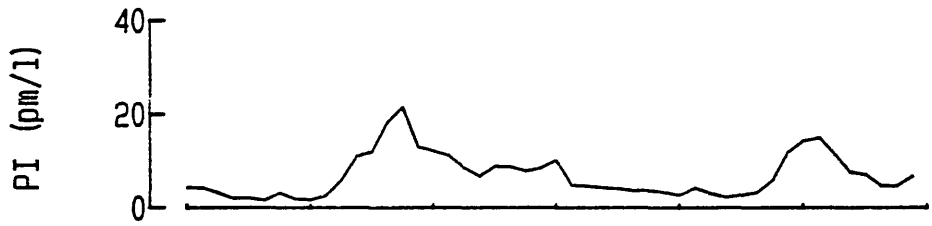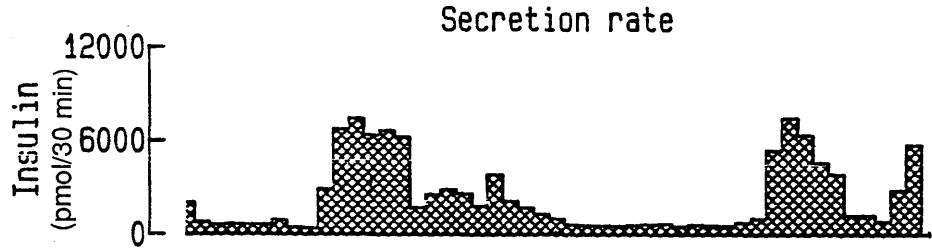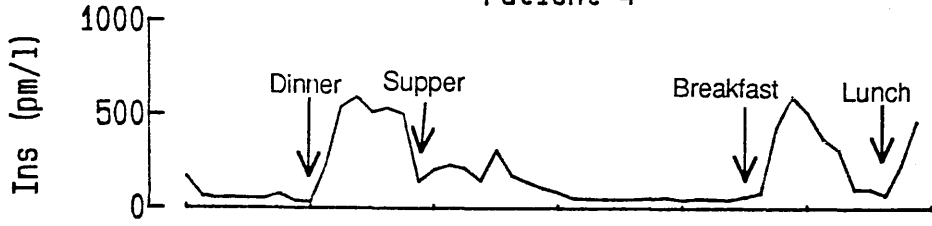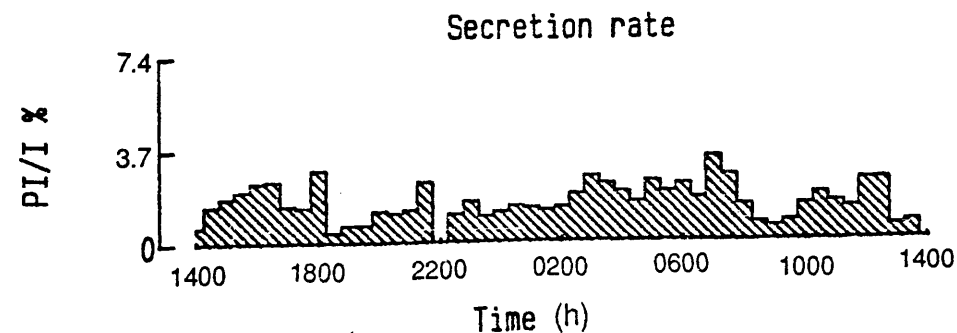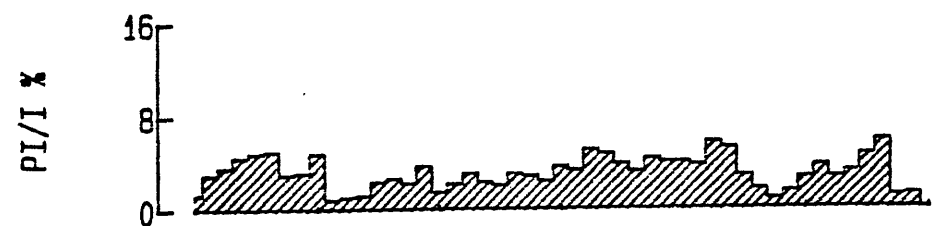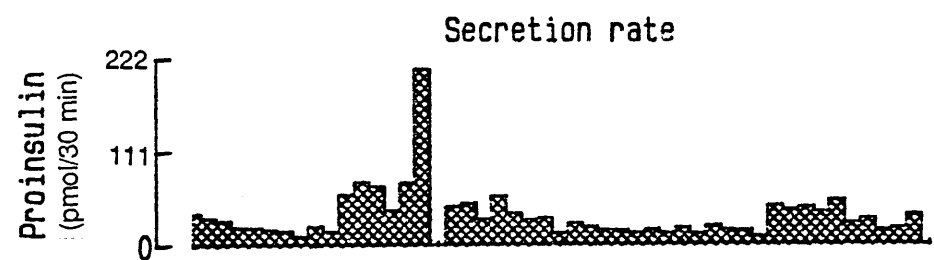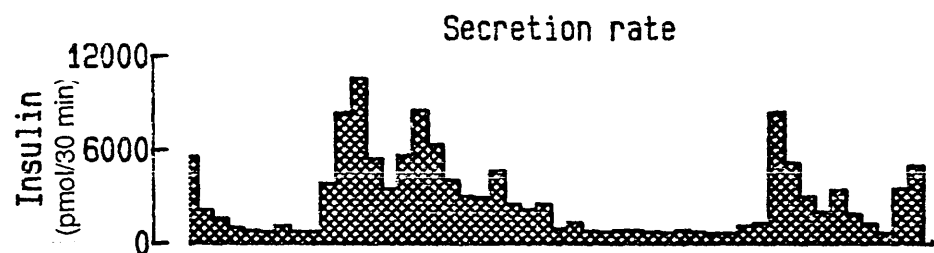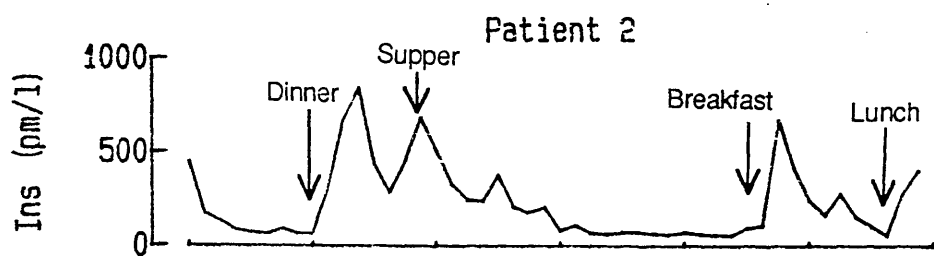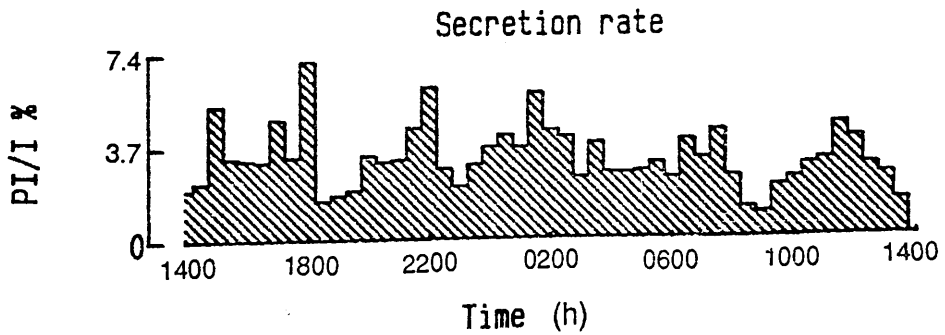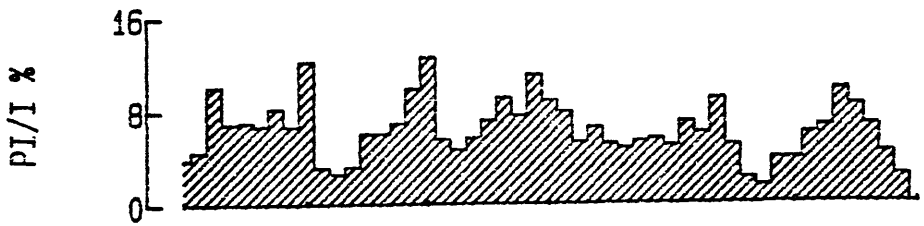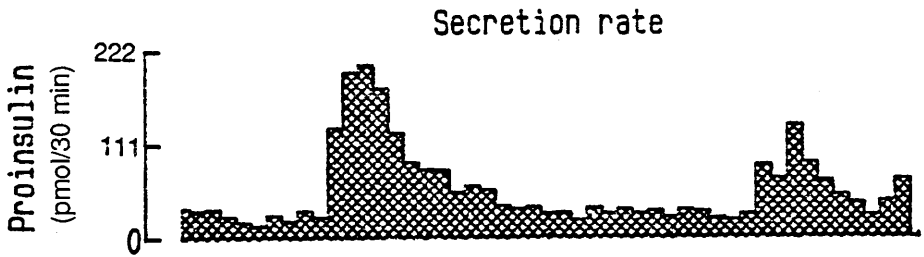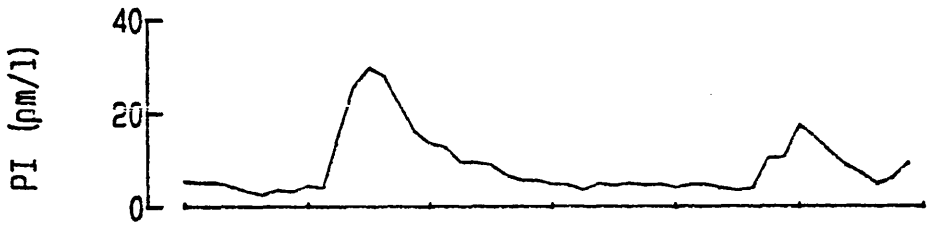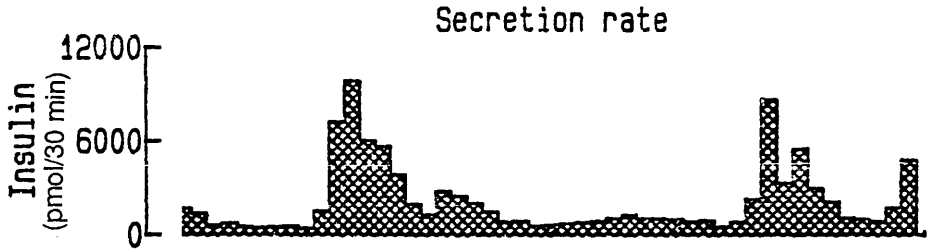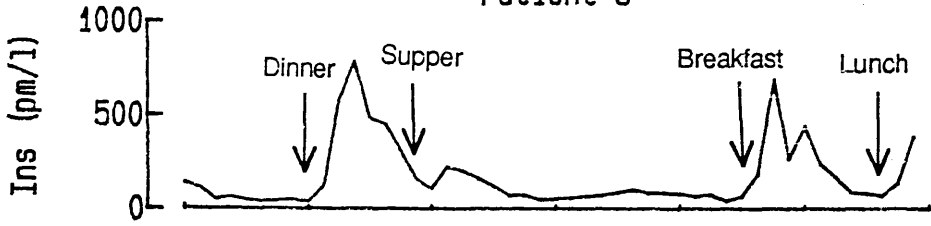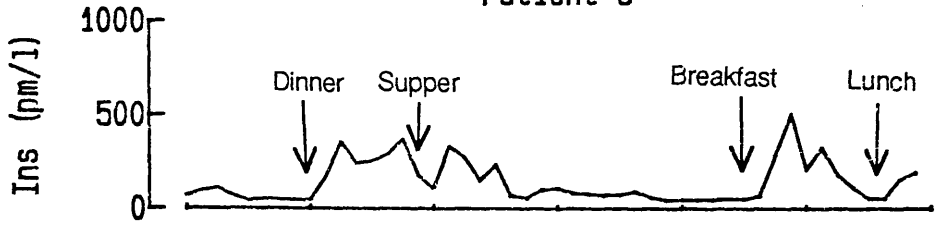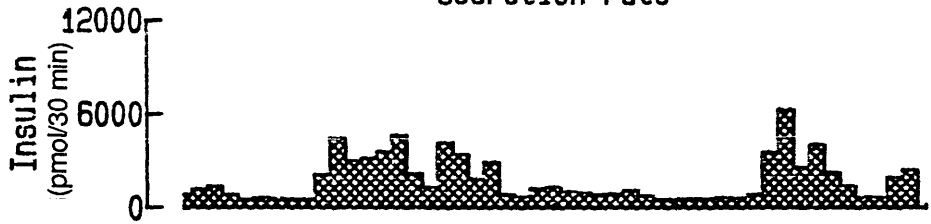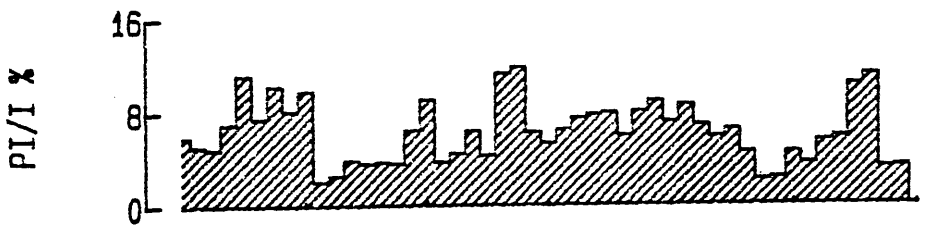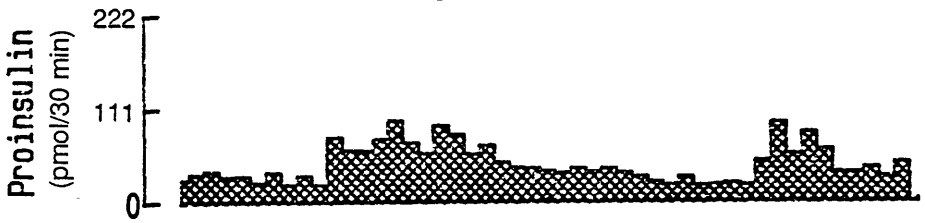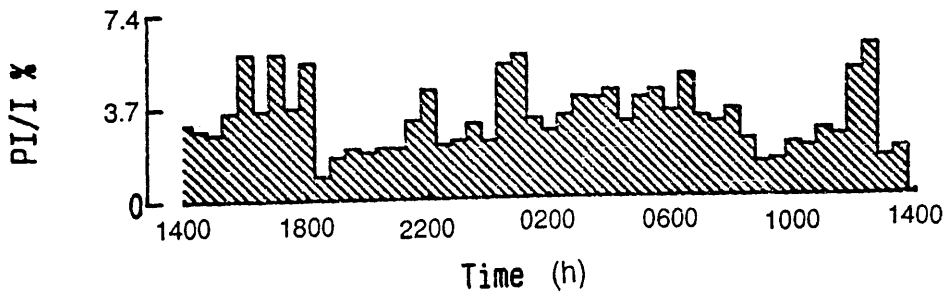
Patient 4

Patient 2

Patient 5

Patient 6

Patient 3
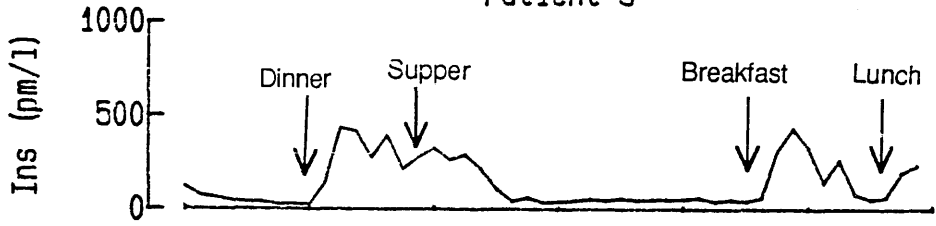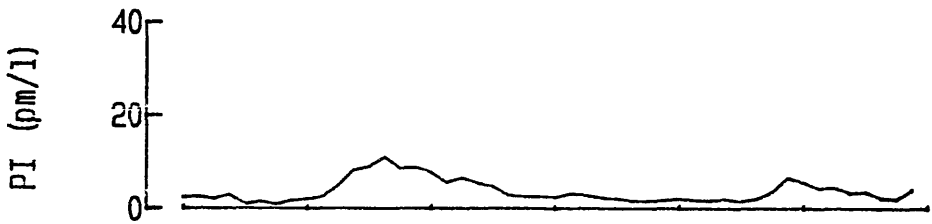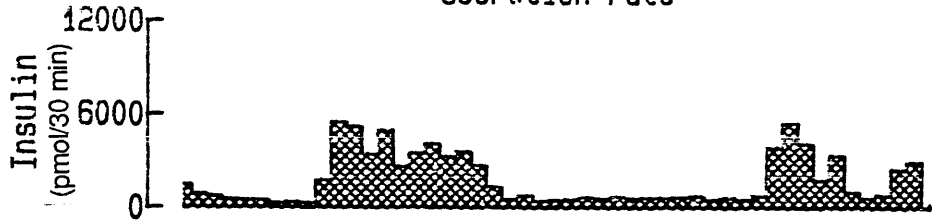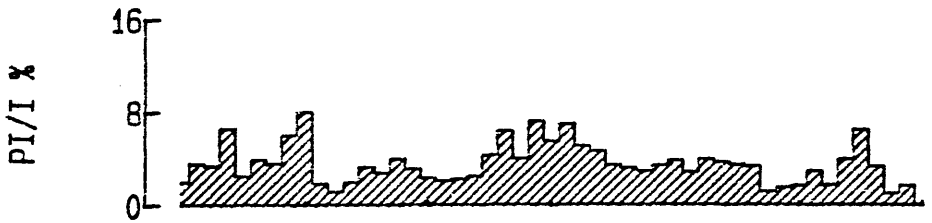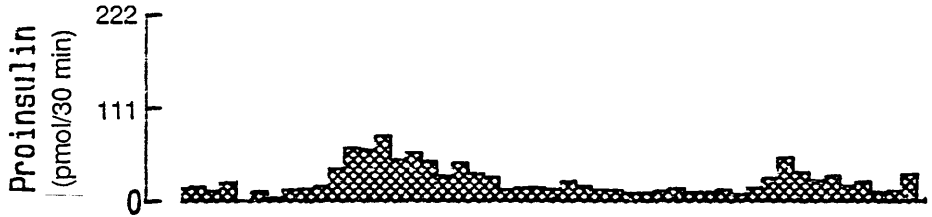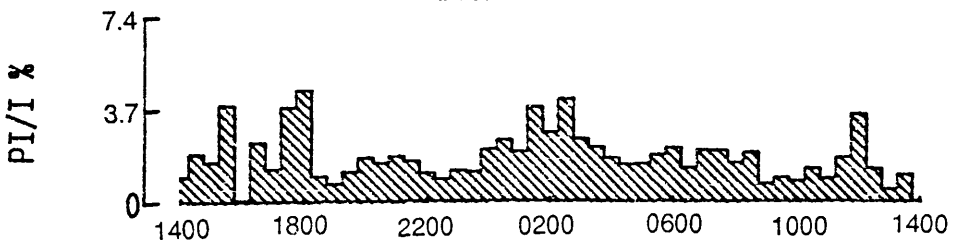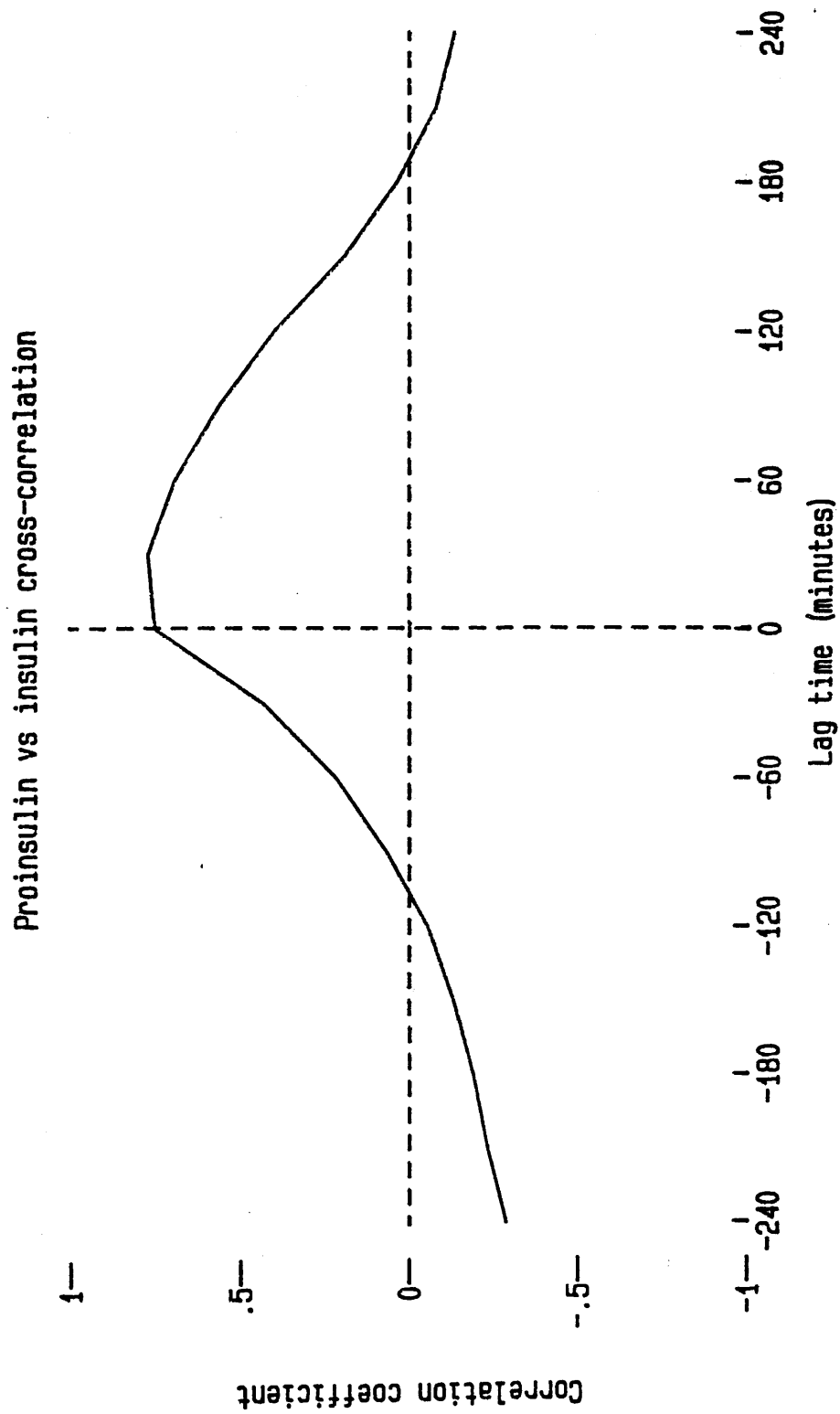
Figure 6.3

Cross-correlation of proinsulin and insulin secretion in response to feeding.

TABLE 6.2

SUM OF GLUCOSE CONCENTRATIONS, INSULIN AND PROINSULIN SECRETION AND THE ANALOGOUS
PROINSULIN/INSULIN PERCENTAGE RATIOS OVER TWENTY-FOUR HOURS AND DURING BASAL CONDITIONS

| Patient number | Twenty-four hours | | | | Basal* | | |
	Sum of glucose concentrations (mmol/l)	Insulin secretion (nmol)	Proinsulin secretion (nmol)	Proinsulin secretion % Insulin secretion	Insulin secretion (nmol)	Proinsulin secretion (nmol)	Proinsulin Secretion % Insulin secretion
1	263	140	2.7	1.9	9.0	0.29	3.2
4	252	117	2.1	1.8	7.2	0.25	3.5
2	250	138	1.7	1.2	9.8	0.21	2.1
5	241	104	2.7	2.6	10.8	0.34	3.1
6	239	87	2.2	2.5	9.7	0.36	3.7
3	262	87	1.1	1.3	7.3	0.16	2.2

*Basal conditions were from 0200-0700 h.

The ratio of proinsulin/insulin secretion expressed as a percentage (PI/Ins %) was calculated for the total secretion over 24 hr and in the basal state. In each of the six individuals the ratio was higher in the basal state, although the magnitude of the increase varied between patients. The PI/Ins % ratios were also calculated for each time point of the study using both the concentration and secretion rate data (Figure 6.2). In both series of data the PI/Ins % ratio decreased significantly in response to feeding (Table 6.3). The ratios then gradually increased to pre-meal values, but even under basal conditions, 0200 - 0700 h, there were fluctuations in the observed ratios (Figure 6.2).

Discussion

The data presented here compare well with previously published material in two respects. Firstly, the observation that more than one glucose peak occurs following the ingestion of food and the exact number varies between individuals. Secondly, the synchronous nature of post-prandial glucose and insulin pulses (125, 126). The reported 13 minute oscillations of serum insulin during basal conditions (127) could not be observed due to the 30 minute sampling interval employed in this study.

1.3 Fasting Data

Methods

Serum samples were collected from healthy laboratory personnel after an overnight fast of at least ten hours.

Results

Serum glucose, insulin, proinsulin and C-peptide concentrations were compared between non-obese males (n = 17) and both non-obese females (n = 17) and obese (n = 6) males. The data is presented in Table 6.4 and Figure 6.4.

TABLE 6.3

SERUM GLUCOSE CONCENTRATIONS AND PROINSULIN/INSULIN
PERCENTAGE RATIOS BEFORE AND AFTER FEEDING DURING THE
TWENTY-FOUR HOUR STUDY

		Patient Number					
		1	4	2	5	6	3
BREAKFAST	Pre	5.1	5.1	4.7	5.0	4.8	5.3
Glucose (mmol/l)	Post	6.8	5.6	4.9	5.8	5.2	7.4
				$p = 0.05$			
PI/Ins %	Pre	6.3	3.9	2.9	5.1	6.6	3.4
(concentration)	Post	1.2	1.4	1.7	2.2	4.6	1.2
				$p = 0.05$			
PI/Ins %	Pre	3.3	2.1	1.5	2.4	3.6	2.0
(secretion)	Post	0.7	0.8	0.7	1.2	2.3	0.7
				$p = 0.05$			
LUNCH	Pre	5.0	5.3	4.5	4.9	4.7	4.9
Glucose (mmol/l)	Post	5.6	4.9	5.4	5.5	5.8	7.0
				NS			
PI/Ins %	Pre	9.0	6.7	6.0	6.8	11.3	3.4
(concentration)	Post	4.1	2.0	1.1	4.4	3.3	1.0
				$p = 0.05$			
PI/Ins %	Pre	4.2	2.7	2.4	2.9	6.1	1.3
(secretion)	Post	1.9	1.0	0.6	2.5	1.6	0.5
				$p = 0.05$			
DINNER	Pre	4.6	4.6	4.7	4.9	4.6	4.5
Glucose (mmol/l)	Post	4.8	5.4	5.0	4.2	5.0	5.1
				NS			
PI/Ins %	Pre	2.9	5.1	4.9	12.3	9.9	8.1
(concentration)	Post	4.5	1.1	0.9	3.2	2.1	1.8
				$p = 0.05$			
PI/Ins %	Pre	6.8	2.5	2.9	7.2	5.5	4.5
(secretion)	Post	2.3	0.7	0.4	1.6	1.0	1.0
				$p = 0.05$			

TABLE 6.4

FASTING CONCENTRATIONS OF GLUCOSE, INSULIN, PROINSULIN AND C-PEPTIDE IN NON-OBESE FEMALES AND MALES AND OBESE MALES

	Non-obese females (n = 17)	Non-obese males (n = 17)	Obese males (n = 6)
Age (years)	32 (21-47)	33 (26-57)	40.5* (37-44)
BMI	21.7** (18.5-25.6)	23.8 (19.9-26.3)	28.3*** (27.0-31.4)
Glucose (mmol/l)	4.7 (4.1-6.2)	4.7 (4.0-6.0)	5.0 (2.6-6.2)
Insulin (pmol/l)	36 (17-75)	39 (19-102)	93** (41-195)
Proinsulin (pmol/l)	1.56** (1.23-3.00)	2.34 (1.40-5.73)	3.86 (1.03-6.96)
C-peptide (pmol/l)	410 (120-750)	410 (210-760)	680 (270-1110)

*$p = 0.05$ ** $p < 0.05$ *** $p < 0.01$

Results are expressed as median values with the range shown in brackets. The non-obese males acted as a control group against which the obese males and non-obese females were compared.

Figure 6.4

Fasting concentrations of glucose, insulin, proinsulin and C-peptide in non-obese females and males and obese males.

The non-obese males acted as a control group against which the non-obese females and obese males were compared. See Table 6.4 for statistical analysis.

- represents the median
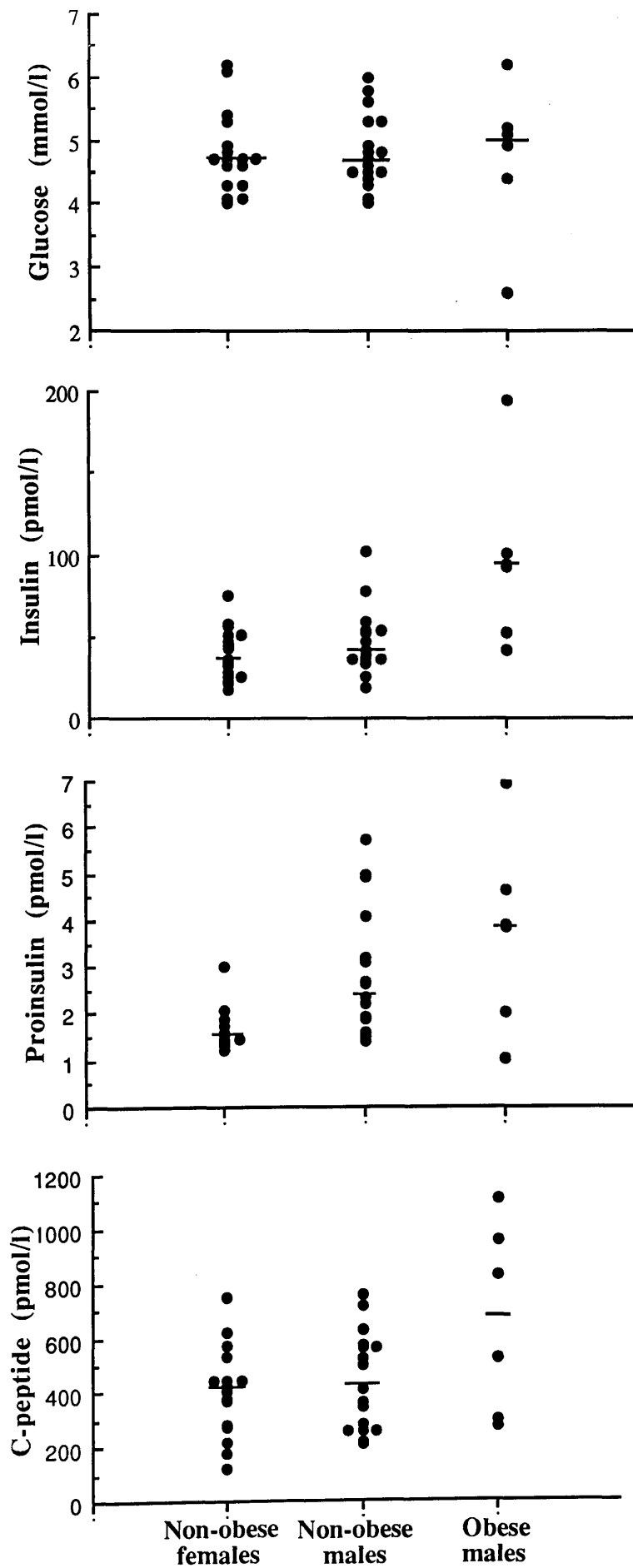
The ages of the non-obese male and female groups were similar and although the BMI of the two groups was significantly different the ranges were similar (Table 6.4). Proinsulin was the only analyte to show a significant difference in concentration between the two groups (Table 6.4 and Figure 6.4).

The non-obese and obese males differed significantly in BMI and although the ages of the two groups were significantly different at the 5% level of probability, the range of the obese group (37-44 yr) was encompassed within that of the non-obese group (26-57 yr). The median values of glucose, proinsulin and C-peptide were higher in the obese group but did not reach statistical significance. However, the fasting insulin concentrations of the obese males were significantly increased above those of the non-obese males (Table 6.4 and Figure 6.4).

The non-obese male and female groups were each subdivided into two groups ($\leq$35 yr and >35 yr) which were significantly different with respect to age but not BMI (Table 6.5). The glucose, insulin, proinsulin and C-peptide concentrations for each group are shown in Table 6.5 and Figures 6.5 and 6.6.

No significant differences were observed between the non-obese male sub-groups. However, the non-obese females over 35 years of age showed a significant decrease in fasting insulin and C-peptide concentrations but no difference in proinsulin or glucose concentrations.

Discussion

The lower fasting serum proinsulin concentrations measured in non-obese females compared with non-obese males have not been reported previously. However, a larger population requires to be studied to establish the true significance of this observation.

TABLE 6.5

FASTING CONCENTRATIONS OF GLUCOSE, INSULIN, PROINSULIN AND C-PEPTIDE IN NON-OBESE MALES AND FEMALES ACCORDING TO AGE

	Male		Female	
	≤35 (n = 10)	>35 (n = 7)	≤35 (n = 10)	>35 (n = 7)
Age (years)	31 (26-34)	39** (36-57)	28 (21-35)	38** (36-47)
BMI	23.7 (19.9-26.0)	24.9 (20.5-26.3)	21.9 (18.5-25.6)	21.4 (20.8-23.8)
Glucose (mmol/l)	4.8 (4.0-5.8)	4.5 (4.3-6.0)	4.8 (4.3-6.2)	4.6 (4.1-5.4)
Insulin (pmol/l)	45 (19-102)	39 (34-52)	49 (17-75)	28* (21-36)
Proinsulin (pmol/l)	2.29 (1.50-5.73)	2.63 (1.40-4.98)	1.57 (1.23-3.00)	1.46 (1.36-2.09)
C-peptide (pmol/l)	460 (220-760)	410 (210-560)	440 (280-750)	270** (120-410)

* $p < 0.005$ ** $p < 0.01$

Results are expressed as median values with the range shown in brackets. Within the male and female groups the >35 year sub-group was compared with the analogous <35 year sub-group.

Figure 6.5

Comparison of fasting concentrations of glucose, insulin, proinsulin and C-peptide in non-obese males ≤35 years and >35 years.
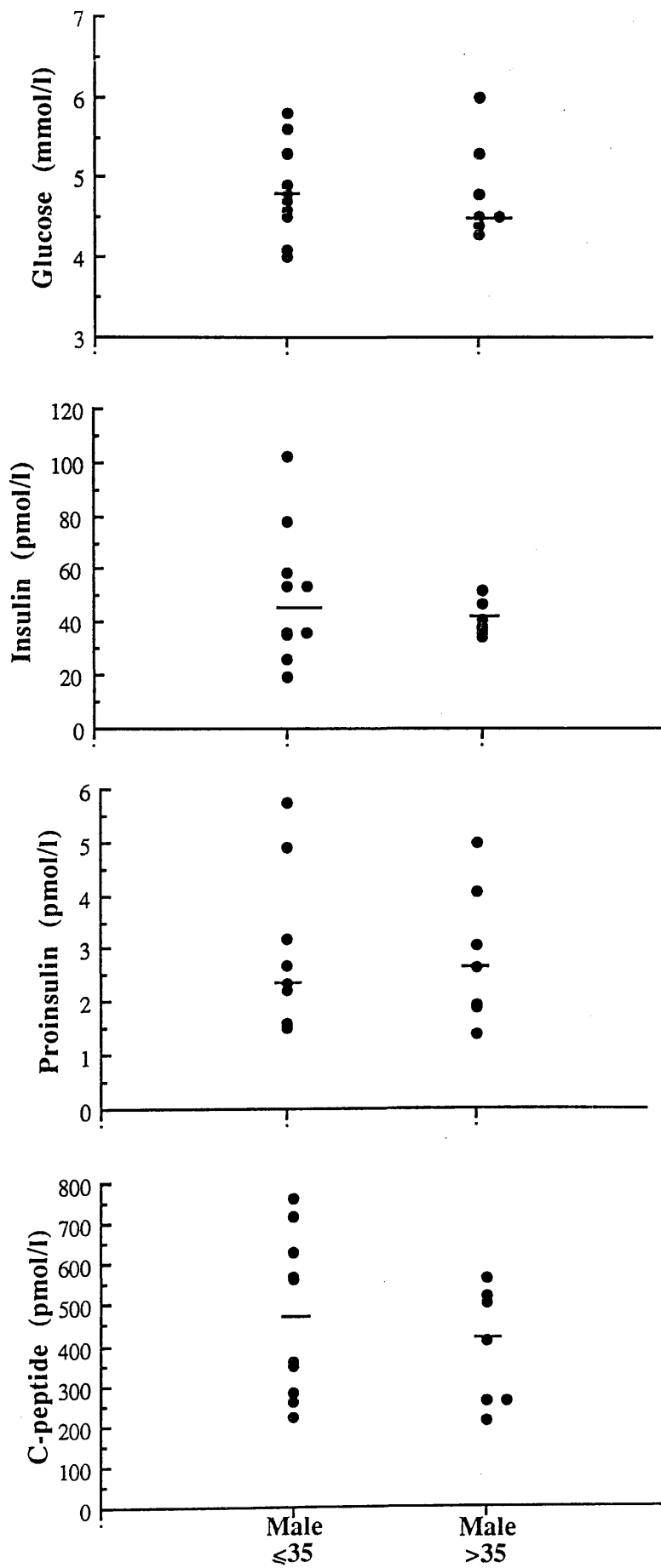
Figure 6.6

Comparison of fasting concentrations of glucose, insulin, proinsulin and C-peptide in non-obese females ≤35 years and >35 years.
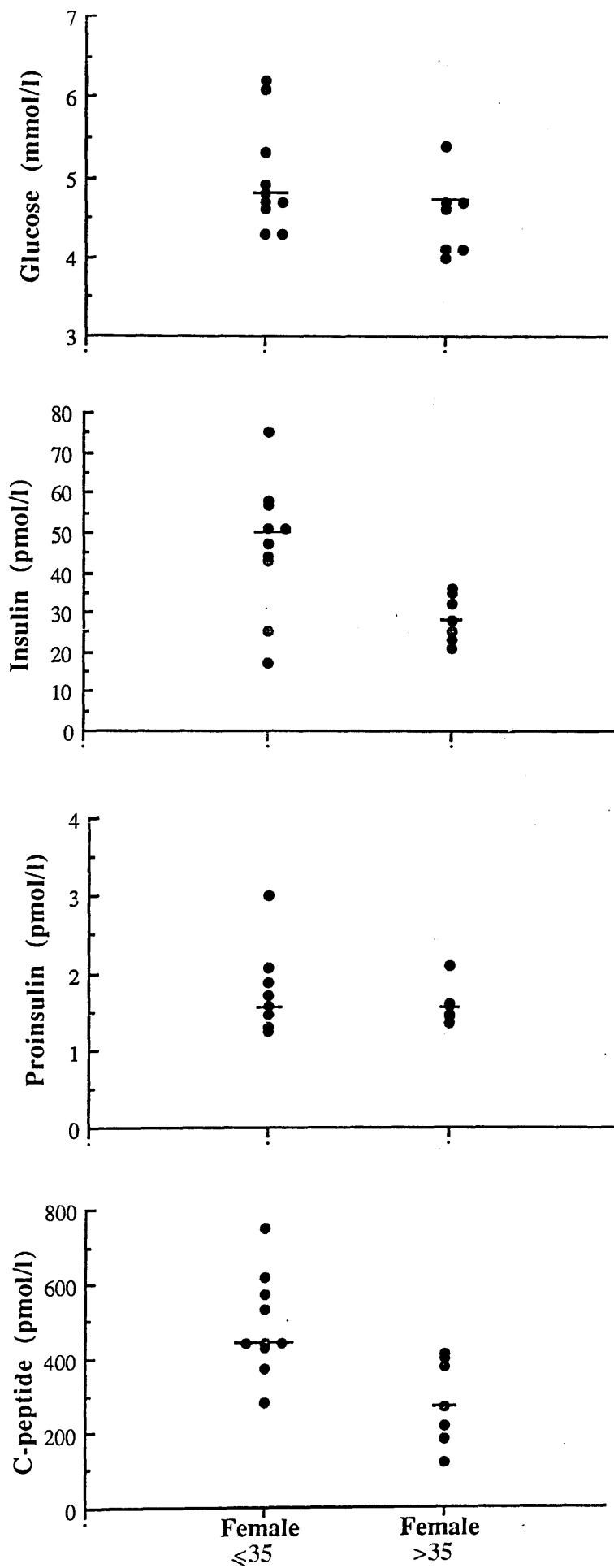
Hyperinsulinaemia, both in the basal state and under conditions which stimulate insulin secretion, is a common finding in obesity. The mechanisms involved appear to be a combination of increased insulin secretion and decreased hepatic extraction (128, 129, 130, 131, 132). The results obtained in this study did show a significant increase in basal serum insulin concentrations in obese compared with non-obese males. The serum proinsulin and C-peptide concentrations were also elevated but not to an equivalent degree and did not reach statistical significance. This data is consistent with the dual mechanism of hyperinsulinaemia in obesity as described above.

Glucose tolerance tends to deteriorate with increasing age (133, 134) but the effect on fasting glucose concentrations is small (135). Also, it has been suggested that factors other than age contribute to the glucose intolerance of the elderly (135, 136, 137). However, in general, fasting and stimulated plasma insulin concentrations have not been found to decline with age and if anything tend to increase (133, 134, 135, 136, 137). Therefore, the finding in this study of decreased fasting insulin and C-peptide concentrations in females over 35 years compared to those under 35 years of age is contrary to previous observations. The explanation for the lower serum concentrations of insulin is probably due to decreased secretion as the serum C-peptide concentrations are decreased by an equivalent factor. Again, a larger population covering a wider age range would be required to substantiate this finding. It should be noted that the corresponding male group exhibited similar trends to the females, although they did not reach statistical significance.

1.4 Oral Glucose Tolerance Test

Methods

Healthy laboratory personnel undertook a 75 g oral glucose tolerance test (OGTT) after an overnight fast of at least ten hours. Two basal serum samples were taken at

-15 and 0 minutes after which 75 g glucose, in 300 ml water, was drunk within five minutes, and a further four serum samples collected at 30, 60, 90 and 120 minutes.

Results

Glucose, insulin, proinsulin and C-peptide concentrations, at all time points of the OGTT, were compared within and between the two groups studied: non-obese males and females. The median age of the non-obese male group was 33 years, range 29 - 57 years, and the median BMI was 24.4, range 20.5 - 26.3. The non-obese female group did not differ significantly from this with a median age of 36 years, range 27 - 42 years, and a median BMI of 21.5, range 20.8 - 25.6. The results are summarised in Table 6.6 and Figures 6.7 and 6.8.

In both groups glucose and insulin concentrations rose to give maximum values 30 minutes after ingestion of the glucose load. Serum C-peptide concentrations peaked at 60 minutes in both groups while proinsulin concentrations peaked at 90 minutes in the male group and were highest at 120 minutes in the female group, although the concentrations at 90 and 120 minutes were not significantly different (Table 6.6). Only the glucose concentrations returned to basal values within the time course of the OGTT showing no significant difference from 90 minutes onwards.

The PI/Ins % ratio in both groups fell to a nadir at 30 minutes and returned to basal levels at 90 minutes (Table 6.7). Thus the PI/Ins % ratio profile mirrored the glucose profile throughout the time course of the OGTT.

The median proinsulin concentrations in the female group were lower than the male group at all time points of the OGTT but only reached statistical significance at -15, 0 and 30 minutes. Glucose and C-peptide concentrations were not significantly different between the groups at any time point of the OGTT, and insulin exhibited a similar pattern except at 120 minutes at which point the insulin concentration was

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TABLE 6.6

SERUM CONCENTRATIONS OF GLUCOSE, INSULIN, PROINSULIN AND C-PEPTIDE DURING A 75 g ORAL GLUCOSE TOLERANCE TEST IN NON-OBESE MALES AND FEMALES

			Time (mins)				
	-15	0	30	60	90	120	
Glucose (mmol/l)							
Male (n=10)	4.5 (4.0-5.3)	4.6 (4.0-5.3)	7.3+++ (4.3-9.0)	5.6+ (3.8-7.2)	4.7 (3.3-6.1)	4.3 (2.9-5.5)	
Female (n=9)	4.3 (4.2-4.7)	4.3 (4.0-4.8)	7.3+++ (5.3-9.3)	5.8++ (4.0-9.6)	4.2 (3.8-7.5)	4.5 (3.7-5.8)	
Insulin (pmol/l)							
Male (n=10)	48+ (33-122)	40 (26-102)	436+++ (254-802)	403+++ (170-600)	243+++ (53-497)	151+++ (30-288)	
Female (n=9)	36 (19-97)	35 (23-57)	331+++ (144-452)	281+++ (161-554)	281+++ (102-642)	198*+++ (97-559)	

TABLE 6.6 (cont)

			Time (mins)			
	-15	0	30	60	90	120
Proinsulin (pmol/l)						
Male (n=10)	2.57 (1.46-5.64)	2.14 (1.50-5.73)	6.98+++ (3.00-13.74)	10.17+++ (4.08-16.86)	11.68+++ (4.15-22.11)	10.27+++ (3.32-29.27)
Female (n=9)	1.54*** (1.16-2.13)	1.47*** (1.23-2.09)	3.67** (1.99-5.43)	6.04+++ (6.80-10.71)	7.89+++ (5.68-18.69)	8.18+++ (4.64-16.18)
C-peptide (pmol/l)						
Male (n=10)	515 (290-860)	450 (300-610)	2000+++ (1160-2200)	2140+++ (140-3510)	1670+++ (1030-2480)	1200+++ (840-1810)
Female (n=9)	320 (190-840)	310 (180-720)	1280+++ (500-2970)	2080+++ (1410-3130)	1680+++ (1040-2770)	1650+++ (930-2390)

* p = 0.05   ** p <0.05   *** p<0.01   for between group comparisons.
+ p <0.05   ++ p = 0.01   +++ p <0.01   for within group comparisons against 0 time.

Results are expressed as median values with the range shown in brackets.
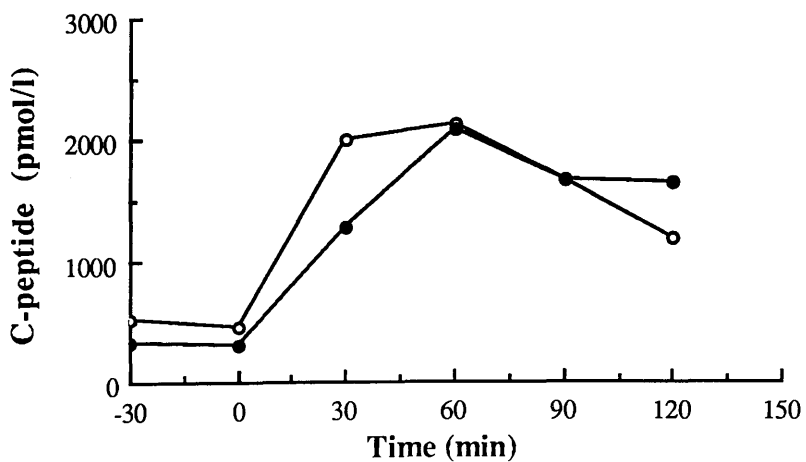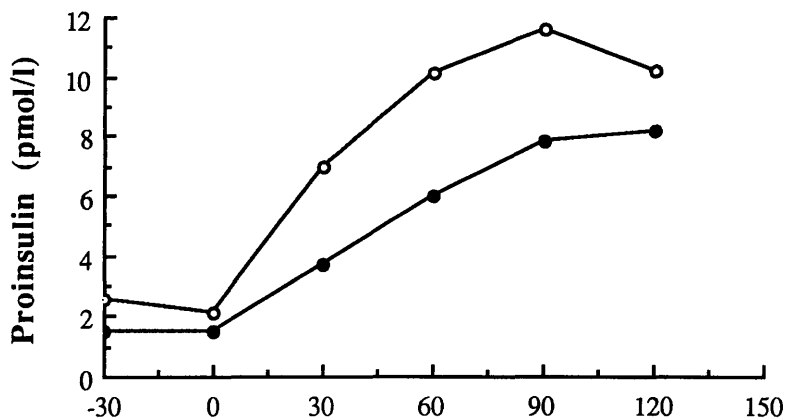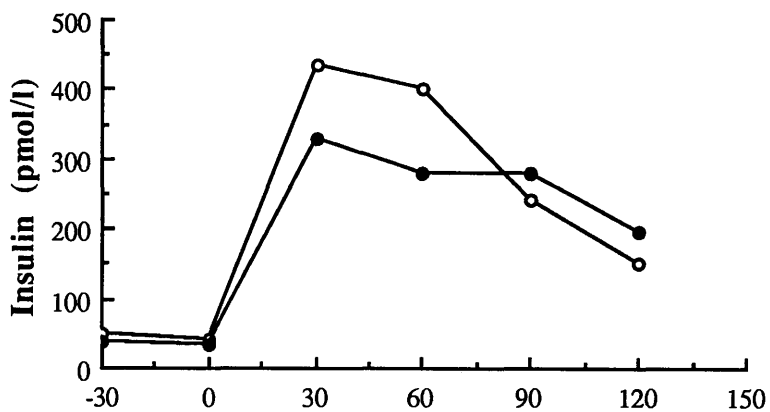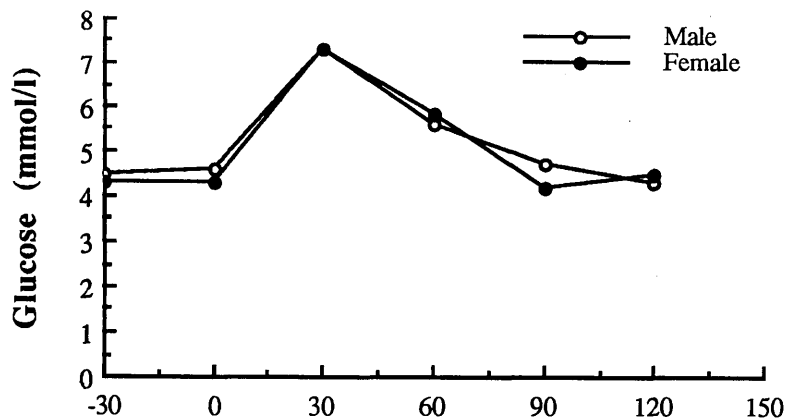
TABLE 6.7

PROINSULIN/INSULIN PERCENTAGE RATIOS DURING A 75 g ORAL GLUCOSE TOLERANCE TEST IN NON-OBESE MALES AND FEMALES

	Time (mins)					
	-15	0	30	60	90	120
Males (n=10)	4.2 (1.5-17.1)	5.6 (2.3-15.9)	1.6+ (0.5-3.1)	2.8+ (0.7-6.1)	5.4 (1.7-17.7)	7.6 (3.7-31.3)
Females (n=9)	4.7 (1.6-8.2)	5.2 (2.4-6.4)	1.4+ (0.8-3.2)	1.9+ (1.1-3.8)	3.9 (1.8-7.9)	4.1 (2.2-8.3)

+ p <0.01 for within group comparison against 0 time. Results are expressed as median values with the range shown in brackets.

significantly higher in the female group (Table 6.6). There was no significant difference between the PI/Ins % ratios of the two groups at any time point.

Discussion

The lower proinsulin concentrations observed in females compared with males at the -15, 0 and 30 minute time points of the OGTT are in agreement with the fasting data results (Table 6.4).

The decrease in the PI/Ins % ratio during the initial stage of the OGTT followed by a gradual increase to values higher than those observed in the basal state has been reported previously (138, 139, 140).

1.5 Glucagon Stimulation Test

Methods

The samples analysed were those collected previously as part of a study to compare the oral glucose load and intravenous (iv) glucagon injection as stimuli to C-peptide secretion in normal men (141). In summary, ten healthy men were fasted overnight and a basal serum sample taken prior to a slow 1 mg iv injection of glucagon. Six minutes later a second serum sample was taken. Three weeks later each subject underwent a 75 g OGTT with serum samples collected before and 30, 60, 90 and 120 minutes after glucose ingestion. Plasma glucose was determined by the glucose oxidase method and C-peptide by an RIA using reagents supplied by Novo Laboratories Ltd.

Results

One subject was excluded from the calculation of results as he exhibited abnormally high fasting and stimulated concentrations of proinsulin. All other analytes measured on this individual were within the normal ranges. Therefore, one sample from his series of specimens was assayed at a number of dilutions and also in the

presence of 1% (v/v) mouse serum. The measured proinsulin concentrations did not dilute in a linear fashion, and the addition of mouse serum reduced the concentration of measured proinsulin to the lower end of the fasting range (Table 6.8), consistent with the presence of heterophilic antibodies. The results from the remainder of the group are presented in Table 6.9 and Figures 6.9 and 6.10.

During the OGTT, glucose concentrations peaked 30 minutes after the ingestion of the glucose load and returned to basal values by 60 minutes. Insulin concentrations also peaked at 30 minutes but did not return to basal values by the end of the test. Proinsulin concentrations peaked at 60 minutes and remained elevated until the end of the test (Table 6.9 and Figure 6.9).

Glucose, insulin and proinsulin concentrations rose significantly in response to the intravenous injection of glucagon (Table 6.9 and Figure 6.10).

The PI/Ins % ratios were calculated for the OGTT and the glucagon stimulation test and the results are shown in Table 6.9 and Figures 6.9 and 6.10 respectively. During the OGTT the PI/Ins % ratio reached a trough at 30 minutes and returned to basal values by 90 minutes. A similar fall in the PI/Ins % ratio was observed after the intravenous injection of glucagon.

Discussion

The results obtained from the subject with abnormally high proinsulin concentrations but normal insulin, glucose and C-peptide concentrations suggest the presence of antibodies to murine immunoglobulin in the serum of this individual. These antibodies would only interfere with the proinsulin assay as it entails the use of two murine McAbs. The insulin assay utilises one murine McAb and a guinea-pig antiserum. The presence of these antibodies in the serum of normal individuals has been reported (142, 143) and mouse serum was subsequently incorporated in the proinsulin assay diluent in order to prevent a repeat of this phenomenon.

TABLE 6.8

INVESTIGATION OF ABNORMALLY HIGH MEASURED SERUM PROINSULIN CONCENTRATIONS IN A SINGLE SUBJECT

Sample dilution	Proinsulin concentration (pmol/l)	
	Assay diluent	Assay diluent + 1% (v/v) mouse serum
neat	19.17	0.9
1:2	9.94	-
1:4	2.91	-
1:8	0.83	-
1:16	UD	-

UD = undetectable

TABLE 6.9

SERUM GLUCOSE, INSULIN AND PROINSULIN CONCENTRATIONS AND PROINSULIN/INSULIN PERCENTAGE RATIO DURING A 75 g ORAL GLUCOSE TOLERANCE TEST AND GLUCOSE STIMULATION TEST IN HEALTHY ADULT MALES

	OGTT/Time (mins)					Glucagon stimulation test	
	0 (n=9)	30 (n=8)	60 (n=8)	90 (n=8)	120 (n=7)	Pre (n=9)	Post (n=9)
Glucose (mmol/l)	4.3 (3.9-4.8)	6.0*** (4.6-9.2)	4.0 (3.1-6.3)	3.7 (2.8-6.1)	3.8 (2.2-5.5)	4.2 (3.8-4.6)	5.6** (4.5-6.4)
		n=9	n=9	n=9	n=9		
Insulin (pmol/l)	34 (21-67)	389** (128-703)	140** (100-354)	107** (43-357)	96 (31-235)	47 (33-83)	298*** (245-632)
Proinsulin (pmol/l)	1.32 (1.01-2.87)	3.77** (2.27-9.78)	4.50** (2.83-12.25)	3.50** (2.17-12.67)	5.48** (1.82-12.02)	1.10 (0.97-2.53)	2.64*** (1.34-4.44)
PI/Ins %	4.3 (1.6-7.4)	1.1** (0.6-3.2)	3.4* (0.9-5.2)	3.3 (1.4-7.5)	5.6 (2.6-14.5)	2.8 (1.2-3.9)	1.1*** (0.4-1.6)

* $p = 0.05$ ** $p = 0.01$ *** $p < 0.01$ for within group comparisons against 0 time for OGTT, and comparison of pre and post in the glucagon stimulation test. Results are expressed as median values with the range shown in brackets.

Figure 6.9

Serum glucose, insulin and proinsulin concentrations and proinsulin/insulin percentage ratios during a 75 g glucose tolerance test.

Time (min)
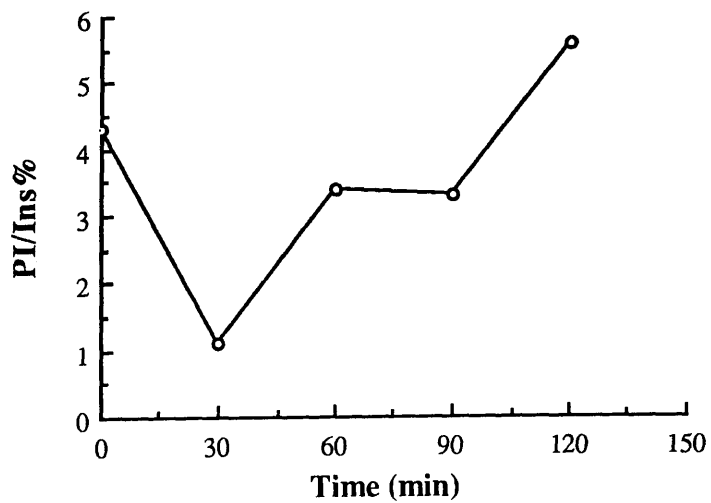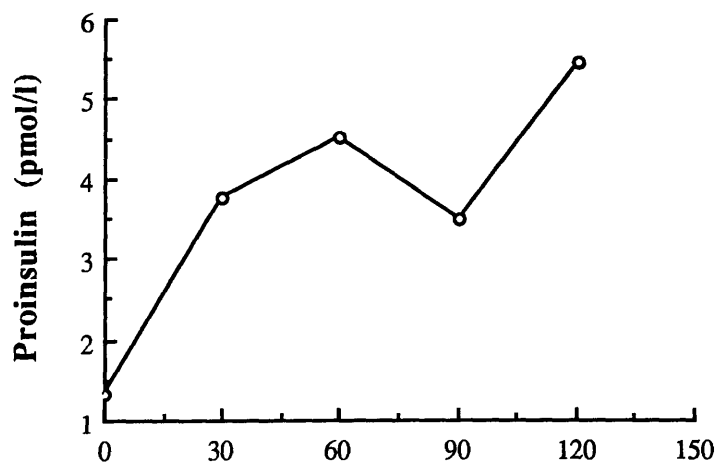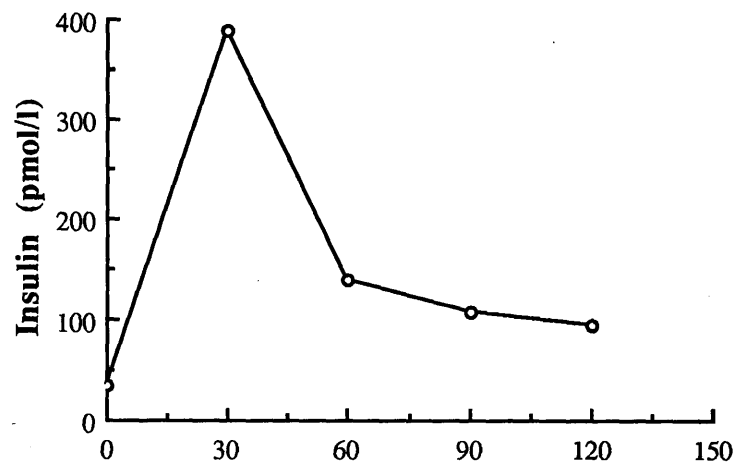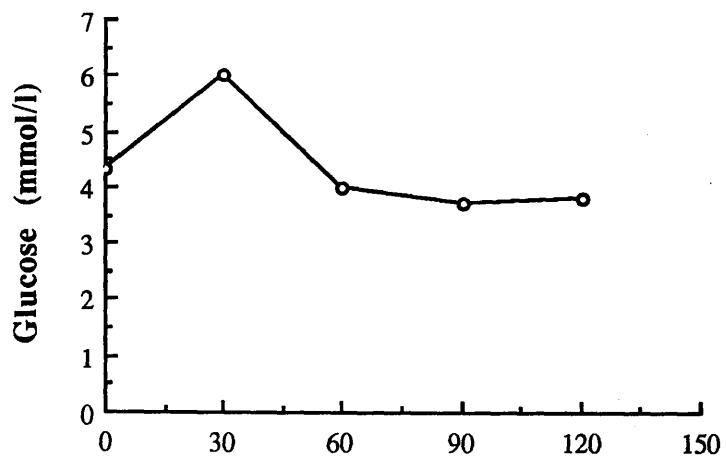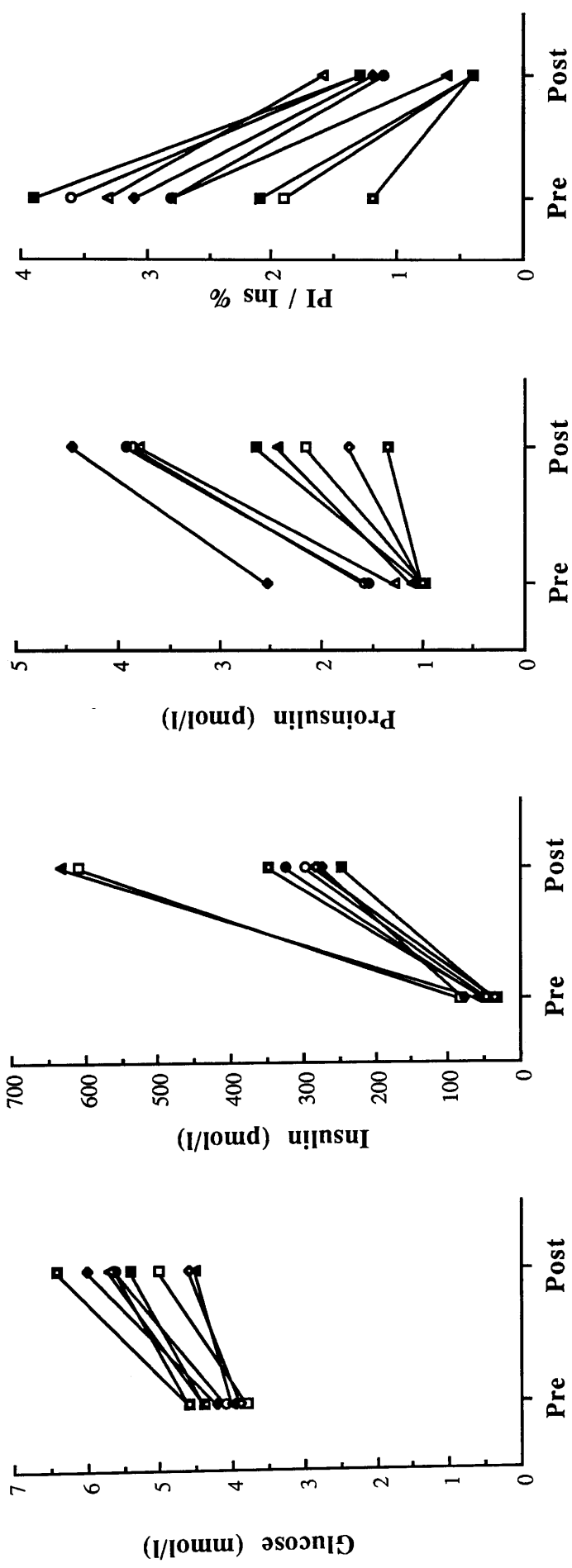
Figure 6.10

Serum glucose, insulin and proinsulin concentrations and proinsulin/insulin percentage ratios before and after an intravenous injection of glucagon.

Lower concentrations of proinsulin were observed during the 75 g oral glucose tolerance test in this group of individuals compared with the normal data presented in Table 6.6. In Chapter 5, Figure 5.43, it was shown that proinsulin concentrations were decreased in serum which had been incubated at room temperature for more than 4 h. It is possible that the samples in this study had been subjected to such conditions during the performance of the initial assays for insulin and C-peptide, and therefore some degradation of proinsulin may have occurred.

1.6 Insulin Tolerance Test

Method

Proinsulin and insulin concentrations were measured in serum samples taken during an insulin tolerance test (ITT) from seven patients investigated for possible pituitary insufficiency but who were shown subsequently to have normal anterior pituitary function. Human insulin (0.15 U/kg body weight) was injected intravenously and serum samples collected before and 30, 45, 60, 90 and 120 minutes after injection.

Results

The results are summarised in Table 6.10 and Figure 6.11. Insulin concentrations rose to a peak 30 minutes after the intravenous injection of insulin and returned to basal values at 120 minutes. The glucose concentrations fell to a nadir at 30 minutes in response to the rise in serum insulin, and subsequently increased but did not return to fasting values by the end of the test. Proinsulin concentrations reached a nadir at 45 minutes and were not significantly different from basal values by 120 minutes.

Discussion

The observed suppression of proinsulin secretion by administration of exogenous insulin is consistent with previous publications (144, 145). It has also been

TABLE 6.10

SERUM GLUCOSE, INSULIN AND PROINSULIN CONCENTRATIONS DURING AN INSULIN TOLERANCE TEST

	Time (mins)						
	0	30	45	60	90	120
Glucose (mmol/l)	4.6 (4.3-5.3)	1.4** (1.1-1.9)	2.0** (1.6-3.0)	2.6** (1.9-3.2)	3.3** (2.2-4.0)	3.4** (2.9-4.5)
Insulin (pmol/l)	46 (14-55)	1290** (65-1905)	387** (134-1684)	213** (78-966)	89** (61-186)	54 (19-970)
Proinsulin (pmol/l)	1.21 (0.39-2.99)	1.00 (0.39-2.60)	0.52* (0.30-2.26)	0.57* (0.21-1.99)	0.59* (0.33-1.77)	0.63 (0.45-1.57)

* p <0.05 ** p = 0.02 for within group comparisons against 0 time.   Results are expressed as median values with the range shown in brackets.
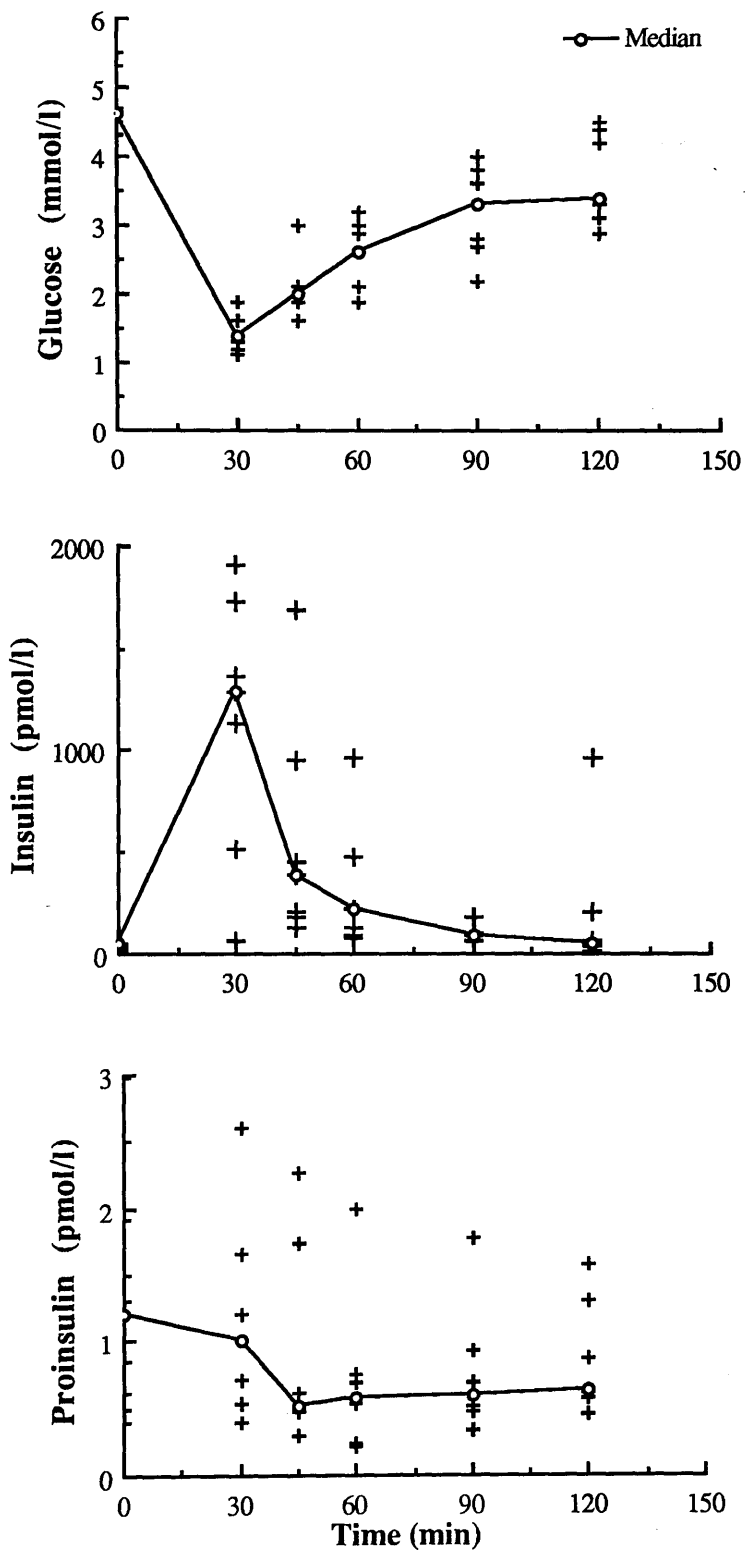
Figure 6.11

Serum glucose, insulin and proinsulin concentrations during an insulin tolerance test.

The individual values for each patient are shown together with the median values.

suggested that in the diagnosis of insulinoma, the suppression of proinsulin rather than C-peptide by exogenous insulin gives better discrimination between patients with insulinoma and control subjects (146).

1.7 General Discussion

The data presented in the previous sections confirm that serum insulin and proinsulin concentrations, measured by the assays described in this thesis, conform to the expected pattern in normal adult subjects under the majority of conditions evaluated.

An interesting observation is that the release of proinsulin is delayed relative to insulin in response to the ingestion of food (Section 1.2, Figure 6.3), and consequently the ratio of PI/Ins % falls rapidly in response to the initial rise in blood glucose concentrations after feeding (Table 6.3). The latter phenomenon was also observed during the OGTT (Section 1.4, Table 6.7) and glucagon stimulation test (Section 1.5, Table 6.9).

These data suggest the presence of two pools of insulin in the β cell, only one of which contains proinsulin. Under basal conditions the proinsulin-containing insulin pool ('Pool PI-Ins') is released, but in response to glucose the non-proinsulin insulin pool ('Pool Ins') is released preferentially initially, thus causing a fall in the PI/Ins % ratio. The basic concept of two pools of insulin, one of which is important in the fasting state and the other after feeding has been suggested previously (147).

During the OGTT (Table 6.7) and following feeding in the 24 h study (Table 6.3), the PI/Ins % ratio gradually increases again after the initial fall. This would suggest that the release of 'Pool Ins' occurs with a finite time after which release of 'Pool PI-Ins' once again becomes predominant. This would correlate with the first and second-phase release of insulin in response to glucose (148).

From the 24 h profiles of the PI/Ins % ratio (both concentration and secretion rate in Figure 6.2) it can be seen that even under basal conditions the ratio fluctuates suggesting intermittent release of insulin from 'Pool Ins'. This could be accounted for by the pulsatile nature of insulin release observed under basal conditions (127).

Further evidence for the existence of two pools of insulin comes from the relatively recent discovery that pancreastatin inhibits the first-phase insulin secretory response but has little or no effect on the more prolonged second phase (149). Pancreastatin could therefore inhibit the release of 'Pool Ins' but not 'Pool PI-Ins'. In addition it has been shown that pancreastatin co-localises to the secretory granules of insulin (150) and it has been suggested that chromogranin A may be a precursor of pancreastatin (151). It could be hypothesised that the conversion of chromogranin A to pancreastatin only occurs in the secretory granules which form 'Pool Ins' and not in those forming 'Pool PI-Ins'. This would provide a mechanism for the changeover from first to second phase responses. An increase in blood glucose concentrations would stimulate the release of 'Pool Ins' which contains pancreastatin, and the latter on reaching a certain threshold, would inhibit the further release of insulin from 'Pool Ins' thereby causing the glucose to stimulate release of insulin from 'Pool PI-Ins'. As 'Pool PI-Ins' is hypothesised to contain chromogranin A rather than pancreastatin, the concentration of pancreastatin would fall below the threshold value and leave the first phase response intact for stimulation by further rapid increases in blood glucose concentrations.

If the above hypothesis proves to be substantiated, then the presence of proinsulin in only one of the two existing pools of insulin will provide a valuable marker for the investigation of the roles of each pool under physiological conditions and the possible defects which occur prior to and during pathophysiological changes.

2 ALTERED PHYSIOLOGY AND PATHOPHYSIOLOGY

2.1 Non-Insulin Dependent Diabetes Mellitus

Method

Serum samples were collected from two patients with non-insulin dependent diabetes mellitus (NIDDM) during the course of a 75 g OGTT.

Results

Details of the two patients are shown in Table 6.11. Only one of the two patients was receiving an oral hypoglycaemic agent for control of blood glucose concentrations at the time of study (patient MW). The serum glucose, insulin and proinsulin concentrations as well as the PI/Ins % ratios during the OGTT are shown in Table 6.12.

Both patients had high fasting blood glucose concentrations which remained elevated throughout the OGTT and peaked later than normal (Table 6.6). Fasting concentrations of insulin and proinsulin were above the normal range (Table 6.6) and insulin concentrations peaked later than normal. The concentrations of insulin at all time points of the OGTT were within or slightly above the normal ranges observed in this thesis (Table 6.6). In contrast, proinsulin concentrations were higher than the normal range (Table 6.6) at all time points except the 90 minute sample from patient MW. The PI/Ins % ratios were similar except at 30 and 60 minutes when they were above the normal range (Table 6.7). The expected fall in the PI/Ins % ratio in response to an increase in blood glucose concentrations was impaired in both patients.

Discussion

The recent report in the literature of uniformly decreased insulin concentrations at the 30 minute sampling period in patients with NIDDM (139) was established utilising

TABLE 6.11

DETAILS OF NON-INSULIN DEPENDENT DIABETIC PATIENTS

Patient initials	Age	BMI	Duration of NIDDM (years)	Medication
SD	61	30.4	3	Nifedipine (10 mg tds)
MW	59	32.7	5	Metformin (850 mg/day)

TABLE 6.12

SERUM GLUCOSE, INSULIN AND PROINSULIN CONCENTRATIONS AND PROINSULIN/INSULIN PERCENTAGE   RATIOS IN TWO PATIENTS WITH NIDDM DURING A 75 g ORAL GLUCOSE TOLERANCE TEST

Patient initials	Time (mins)				
	0	**30**	**60**	**90**	**120**
Glucose (mmol/l)					
SD	6.8	10.0	14.1	-	13.6
MW	8.0	9.5	13.6	15.3	14.2
Insulin (pmol/l)					
SD	184	351	549	684	582
MW	145	192	260	415	379
Proinsulin (pmol/l)					
SD	12.21	15.04	27.20	37.89	48.56
MW	7.42	10.34	12.66	16.76	20.52
PI/Ins %					
SD	6.6	4.3	5.0	5.5	8.3
MW	5.1	5.4	4.9	4.0	5.4

an insulin IRMA which showed no cross-reactivity with proinsulin. The insulin IRMA which was developed in this thesis exhibits 60-85% cross-reaction of proinsulin depending on the dose and form measured (Table 5.9) and this may be a contributory cause of the discrepancy in results.

The impairment in the fall of the PI/Ins % ratio in response to increased blood glucose concentrations correlates with the known loss of first-phase insulin response in NIDDM patients (152) and could be explained by an abnormality in the release of insulin from 'Pool Ins' as discussed in Section 1.7 of this chapter. It is interesting to note that the patient on metformin did not have a PI/Ins % profile more similar to the normal pattern (Table 6.7) than the patient not receiving medication. In this respect it would be interesting to evaluate NIDDM patients before and after starting drug therapy.

2.2 Oral Glucose Tolerance Test in Pregnancy

Methods

Plasma samples were obtained from fifteen pregnant females, in the second or third trimester, who underwent a 75 g OGTT at the Metabolic Unit, Western General Hospital, Edinburgh. The patients had been referred to the Unit for a variety of reasons, for evaluation of glucose tolerance. Plasma glucose was measured by the glucose oxidase method by MLSO staff in the Metabolic Unit. The non-obese pregnant female group comprised healthy laboratory personnel whose results have been presented in Section 1.4 of this chapter.

Results

Details of the patients included in this study are shown in Table 6.13. The pregnant females were divided into groups on the basis of glucose tolerance and their BMI prior to pregnancy. Two patients were not included in these groups: one exhibited normal glucose tolerance but had been classified as having gestational diabetes in a

TABLE 6.13

ORAL GLUCOSE TOLERANCE TEST IN PREGNANCY : PATIENT DETAILS

Group	Patient initials	Age	BMI	Weeks gestation
Non-pregnant, normal glucose tolerance, non-obese	RR	32	22.1	NA
	AC	28	21.5	NA
	DB	37	23.1	NA
	AMcL	27	25.6	NA
	JE	38	21.1	NA
	HC	42	21.0	NA
	VN	29	23.3	NA
	AK	36	20.8	NA
	CG	41	21.4	NA
Pregnant, normal glucose tolerance, non-obese	JF	28	19.9	UK
	AC	32	25.3	22
	MW	26	25.6	32
	PS	35	19.4	UK
	MM	18	21.8	29
	KD	24	24.9	33
Pregnant, normal glucose tolerance, obese	LA	31	27.3	35
	CT	31	30.2	UK
	KF	27	36.2	33
	SD	22	27.2	31
Pregnant, impaired glucose tolerance, non-obese/obese	PC	32	27.6	28
	NN	40	34.6	32
	EH	23	19.3	UK
Previous gestational diabetic	CB	27	21.6	30
Placental tumour	LMcK	27	23.3	30

NA = not applicable   UK = unknown

previous pregnancy, and the other was discovered to have a benign placental tumour following caesarian section performed pre-term because of foetal distress.

Individual patient results for glucose, insulin and proinsulin concentrations, as well as PI/Ins % ratios are presented in Tables 6.14-6.17 respectively. Due to insufficient patient numbers, only two comparisons could be performed: non-obese pregnant vs non-pregnant females, and non-obese vs obese pregnant females. The results of these statistical analyses are shown in Tables 6.18 and 6.19 respectively.

Pregnant non-obese females had higher fasting glucose concentrations than non-pregnant non-obese females, and during the OGTT their plasma glucose and insulin concentrations peaked at 60 minutes instead of 30 minutes (Tables 6.14, 6.15 and 6.18).

In non-obese and obese pregnant females, plasma glucose and insulin concentrations were not significantly different at any time during the OGTT (Tables 6.14, 6.15 and 6.19). However, the obese group exhibited higher fasting concentrations of proinsulin which remained significantly elevated until 90 minutes after ingestion of the glucose load (Table 6.16 and 6.19). The PI/Ins % ratio was also increased in the obese group but only reached statistical significance at 0, 120 and 180 minutes (Tables 6.17 and 6.19).

One of the three patients with impaired glucose tolerance required exogenous insulin (patient PC, Table 6.14). The plasma insulin concentrations in this patient were lower than the other two patients with impaired glucose tolerance from 30-120 minutes of the OGTT. However, the concentrations were comparable to those of the pregnant females with normal glucose tolerance (Table 6.15). The plasma proinsulin concentrations in patient PC were lower throughout the OGTT, than the other two patients with impaired glucose tolerance and were below (0-60 min) or at the lower end (90-180 min) of the range observed in the pregnant females with normal glucose tolerance. The PI/Ins % ratio of patient PC in the fasting state was

TABLE 6.14

SERUM/PLASMA GLUCOSE CONCENTRATIONS IN PREGNANT AND NON-PREGNANT FEMALES DURING A 75 g ORAL GLUCOSE TOLERANCE TEST

Group	Patient initials	Time (mins)						
		0	30	60	90	120	150	180
Glucose (mmol/l)								
Non-pregnant, normal glucose tolerance, non-obese	RR	4.3	7.9	7.3	7.0	5.8	-	-
	AC	4.7	7.3	5.2	4.2	5.0	-	-
	DB	4.7	8.1	8.0	7.5	5.2	-	-
	AMcL	4.3	6.7	5.8	4.2	4.5	-	-
	JE	4.7	7.5	7.8	5.2	4.0	-	-
	HC	4.0	5.3	4.4	3.8	4.1	-	-
	VN	4.8	9.3	9.6	7.1	5.8	-	-
	AK	4.1	5.4	5.0	4.2	3.7	-	-
	CG	4.1	6.1	4.0	3.9	4.5	-	-
Pregnant, normal glucose tolerance, non-obese	JF	5.0	8.3	9.7	7.6	6.3	5.5	5.5
	AC	4.7	6.1	6.8	6.4	5.7	5.2	4.7
	MW	4.7	7.9	7.9	7.5	7.3	5.8	4.9
	PS	4.8	6.8	5.3	5.3	5.4	5.6	5.3
	MM	4.8	6.5	7.6	5.6	4.0	3.6	4.1
	KD	5.3	7.5	7.7	7.2	7.4	6.8	4.5
Pregnant, normal glucose tolerance, obese	LA	4.5	10.3	7.3	9.5	7.2	5.3	3.7
	CT	5.2	7.9	8.4	6.8	7.2	6.6	4.7
	KF	4.4	8.0	8.1	7.4	7.0	5.0	4.0
	SD	4.8	8.7	9.8	7.4	4.3	4.8	4.3
Pregnant, impaired glucose tolerance, non-obese/obese	PC*	5.6	9.4	10.9	10.4	9.0	8.1	6.6
	NN	5.8	11.7	13.5	11.1	9.4	6.9	5.3
	EH	4.8	7.8	9.3	8.6	8.0	6.7	4.8
Previous gestational diabetic	CB	4.5	8.2	9.4	6.9	7.0	8.6	9.4
Placental tumour	LMcK	4.3	8.7	10.1	9.5	8.4	5.6	3.9

*Patient required insulin

TABLE 6.15

SERUM/PLASMA INSULIN CONCENTRATIONS IN PREGNANT AND NON-PREGNANT FEMALES DURING A 75 g ORAL GLUCOSE TOLERANCE TEST

Group	Patient initials	Time (mins)						
		0	30	60	90	120	150	180
Insulin (pmol/l)								
Non-pregnant,	RR	51	452	444	366	251	-	-
normal glucose	AC	25	331	277	102	103	-	-
tolerance, non-obese	DB	28	249	406	464	221	-	-
	AMcL	47	429	526	281	291	-	-
	JE	57	136	251	145	97	-	-
	HC	25	187	270	177	183	-	-
	VN	23	448	554	642	559	-	-
	AK	35	114	161	282	196	-	-
	CG	36	379	281	202	198	-	-
Pregnant, normal	JF	26	207	330	377	353	250	232
glucose tolerance,	AC	40	363	740	580	290	127	94
non-obese	MW	52	588	414	410	384	158	48
	PS	36	476	141	124	258	134	60
	MM	61	356	581	222	75	55	66
	KD	78	525	541	418	497	367	68
Pregnant, normal	LA	39	1060	364	581	453	191	94
glucose tolerance,	CT	68	374	467	328	463	378	131
obese	KF	97	620	1073	855	611	302	129
	SD	76	721	1108	560	138	202	60
Pregnant, impaired	PC*	76	472	472	500	529	502	300
glucose tolerance,	NN	94	1012	101	1180	1025	436	201
non-obese/obese	EH	88	977	977	922	1079	600	236
Previous gestational diabetic	CB	40	528	612	389	321	451	590
Placental tumour	LMcK	102	921	1876	1782	1794	545	251

*Patient required insulin

TABLE 6.16

SERUM/PLASMA PROINSULIN CONCENTRATIONS IN PREGNANT AND NON-PREGNANT FEMALES DURING A 75g ORAL GLUCOSE TOLERANCE TEST

Group	Patient initials	Time (mins)						
		0	30	60	90	120	150	180

Proinsulin (pmol/l)

Group	Patient initials	0	30	60	90	120	150	180
Non-pregnant, normal glucose tolerance, non-obese	RR	1.23	4.53	5.41	6.50	5.99	-	-
	AC	1.31	4.59	9.82	8.06	8.57	-	-
	DB	1.46	3.62	10.71	18.69	16.18	-	-
	McL	1.56	3.33	5.85	5.88	6.39	-	-
	JE	1.61	1.99	4.80	5.68	4.64	-	-
	HC	1.58	3.48	5.14	7.89	7.24	-	-
	VN	1.47	4.93	8.77	14.33	15.26	-	-
	AK	1.43	3.67	6.04	11.42	10.99	-	-
	CG	2.09	5.43	8.82	7.18	8.18	-	-
Pregnant, normal glucose tolerance, non-obese	JF	-	2.62	5.93	9.32	12.01	10.25	8.57
	AC	1.24	2.83	5.86	10.59	8.40	7.50	5.77
	MW	2.85	7.35	8.44	9.44	11.79	8.08	3.60
	PS	0.89	5.23	4.36	4.77	5.50	3.23	2.80
	MM	1.79	3.18	7.76	8.80	5.03	4.09	4.00
	KD	2.47	6.93	14.59	16.20	18.72	15.64	9.70
Pregnant, normal glucose tolerance, obese	LA	2.18	25.27	23.44	45.92	57.70	33.61	18.14
	CT	4.34	13.09	22.96	27.08	40.11	39.96	25.58
	KF	5.68	23.23	39.15	51.32	66.47	43.28	25.07
	SD	3.16	6.74	19.04	17.70	10.43	7.22	4.12
Pregnant, impaired glucose tolerance, non-obese/obese	PC*	0.80	1.14	3.51	7.13	6.87	7.11	5.86
	NN	3.47	6.28	13.88	22.71	28.25	21.56	15.09
	EH	5.19	7.20	13.83	24.32	36.63	33.57	23.94
Previous gestational diabetic	CB	0.66	6.23	13.72	15.94	13.40	17.18	20.00
Placental tumour	LMcK	3.98	28.70	81.11	153.11	203.11	161.51	85.54

*Patient required insulin

TABLE 6.17

PROINSULIN/INSULIN PERCENTAGE RATIOS IN PREGNANT AND NON-PREGNANT FEMALES DURING A 75 g ORAL GLUCOSE TOLERANCE TEST

Group	Patient initials	Time (mins)						
		0	30	60	90	120	150	180
Proinsulin/Insulin %								
Non-pregnant, normal glucose tolerance, non-obese	RR	2.4	1.0	1.2	1.8	2.4	-	-
	AC	5.2	1.4	3.5	7.9	8.3	-	-
	DB	5.2	1.5	2.6	4.0	7.3	-	-
	AMcL	3.3	0.8	1.1	2.1	2.2	-	-
	JE	2.8	1.5	1.9	3.9	4.8	-	-
	HC	6.3	1.9	1.9	4.5	4.0	-	-
	VN	6.4	1.1	1.6	2.2	2.7	-	-
	AK	4.1	3.2	3.8	4.0	5.6	-	-
	CG	5.8	1.4	3.1	3.6	4.1	-	-
Pregnant, normal glucose tolerance, non-obese	JF	-	1.3	1.8	2.5	3.4	4.1	3.7
	AC	3.1	0.8	0.8	1.8	2.9	5.9	6.1
	MW	5.5	1.3	2.0	2.3	3.1	5.1	7.5
	PS	2.5	1.1	3.1	3.8	2.1	2.4	4.7
	MM	2.9	0.9	1.3	4.0	6.7	7.4	6.1
	KD	3.2	1.3	2.7	3.9	3.8	4.3	14.3
Pregnant, normal glucose tolerance, obese	LA	5.6	2.4	6.4	7.9	12.7	17.6	19.3
	CT	6.4	3.5	4.9	8.3	8.7	10.6	19.5
	KF	5.9	3.7	3.6	6.0	10.9	14.3	19.4
	SD	4.2	0.9	1.7	3.2	7.6	3.6	6.9
Pregnant, impaired glucose tolerance, non-obese/obese	PC*	1.3	0.4	0.7	1.4	1.3	1.4	2.0
	NN	3.7	1.1	1.4	1.9	2.8	4.9	7.5
	EH	5.9	1.2	1.4	2.6	3.4	5.6	10.1
Previous gestational diabetic	CB	1.7	1.2	2.2	4.1	4.2	3.8	3.4
Placental tumour	LMcK	3.9	3.1	4.3	8.6	11.3	29.6	34.1

*Patient required insulin

TABLE 6.18

STATISTICAL ANALYSIS OF SERUM/PLASMA GLUCOSE AND HORMONE CONCENTRATIONS IN NON-OBESE PREGNANT COMPARED WITH NON-PREGNANT FEMALES, WITH NORMAL GLUCOSE TOLERANCE, DURING A 75 g ORAL GLUCOSE TOLERANCE TEST

	Time (mins)				
	0	**30**	**60**	**90**	**120**
Glucose (mmol/l)					
Non-pregnant	4.3	7.3	5.8	4.2	4.5
Pregnant	4.8	7.2	7.7	6.8	6.5
p	<0.01	NS	NS	NS	NS
Insulin (pmol/l)					
Non-pregnant	35	331	281	281	198
Pregnant	46	420	478	394	322
p	NS	NS	NS	NS	NS
Proinsulin (pmol/l)					
Non-pregnant	1.47	3.67	6.04	7.89	8.18
Pregnant	1.79	4.20	6.85	9.38	10.10
p	NS	NS	NS	NS	NS
PI/Ins %					
Non-pregnant	5.2	1.4	1.9	3.9	4.1
Pregnant	3.0	1.2	1.9	3.2	3.3
p	NS	NS	NS	NS	NS

NS = not significant

Results are expressed as median values. The ranges are not shown, as individual concentrations are presented in Tables 6.12 - 6.15.

TABLE 6.19

STATISTICAL ANALYSIS OF SERUM/PLASMA GLUCOSE AND HORMONE CONCENTRATIONS IN NON-OBESE COMPARED WITH OBESE PREGNANT FEMALES, WITH NORMAL GLUCOSE TOLERANCE, DURING A 75g ORAL GLUCOSE TOLERANCE TEST

	Time (mins)						
	0	30	60	90	120	150	180
Glucose (mmol/l)							
Non-obese	4.8	7.2	7.7	6.8	6.5	5.6	4.8
Obese	4.7	8.4	8.3	7.4	7.1	5.2	4.2
p	NS	NS	NS	NS	NS	NS	NS
Insulin (pmol/l)							
Non-obese	46	420	478	394	322	146	67
Obese	72	672	770	571	458	252	107
p	NS	NS	NS	NS	NS	NS	NS
Proinsulin (pmol/l)							
Non-obese	1.79	4.20	6.85	9.38	10.10	7.79	4.89
Obese	3.75	18.16	23.20	36.10	48.91	36.79	21.61
p	=0.05	=0.05	=0.01	=0.01	NS	NS	NS
PI/Ins %							
Non-obese	3.0	1.2	1.9	3.2	3.3	4.7	6.1
Obese	5.8	3.0	4.3	7.0	9.8	12.5	19.4
p	=0.05	NS	NS	NS	=0.01	NS	=0.05

NS = not significant.

Results are expressed as median values. The ranges are not shown as individual concentrations are presented in Tables 6.12 - 6.15.

the lowest measured in this study, but followed the expected pattern during the OGTT, falling to a nadir at 30 minutes and gradually rising to fasting values (Table 6.17).

The other two patients with impaired glucose tolerance had higher plasma insulin concentrations at all time points of the OGTT (Table 6.15) and increased plasma proinsulin concentrations from 90-180 minutes (Table 6.16) when compared with the non-obese pregnant females. The PI/Ins % ratios were similar to the non-obese pregnant females with normal glucose tolerance throughout.

Patient CB who exhibited normal glucose tolerance at the time of testing, but was known to have had gestational diabetes during a previous pregnancy, appeared to have similar plasma insulin and proinsulin concentrations to the non-obese pregnant females with normal glucose tolerance from 0-120 minutes. At 150 and 180 minutes the plasma glucose concentrations started to rise again and a corresponding increase in insulin and proinsulin concentrations was observed (Tables 6.14 - 6.16). This patient exhibited a low fasting PI/Ins % ratio which fell in response to an increase in plasma glucose concentrations at 30, 150 and 180 minutes thus following the expected pattern (Tables 6.14 and 6.17).

Patient LMcK had impaired glucose tolerance and was subsequently found to have a benign placental tumour. The plasma insulin and proinsulin concentrations, throughout the OGTT, were higher than the non-obese pregnant females with normal glucose tolerance and were increased from 60-120 minutes (insulin) and 30-180 minutes (proinsulin) when compared with the two patients (NN and EH) with impaired glucose tolerance (Tables 6.15 and 6.16). The PI/Ins % ratios were increased at all time points of the OGTT except 0 when compared with either the non-obese pregnant females with normal glucose tolerance or those with impaired glucose tolerance. The decrease in the PI/Ins % ratio at 30 minutes was not as marked in this patient (Table 6.17).

Discussion

Glucose tolerance is known to decrease in normal human pregnancy with a corresponding increase in plasma insulin concentrations (153, 154). While these trends were apparent in the data presented here, they did not reach statistical significance possibly due to the small number of patients studied.

It has also been shown that although the absolute concentration of plasma proinsulin is increased in late normal and gestational diabetic pregnancy, this does not account for the glucose intolerance as the PI/Ins % ratio is unchanged (153, 155). This observation was confirmed with the data presented above. The only group to show an increase in the PI/Ins % ratio was the obese pregnant female group who had normal glucose tolerance.

The results presented in Table 6.17, the PI/Ins % ratios, exhibit a number of interesting observations which merit discussion. The PI/Ins % ratio was significantly higher in the obese compared with the non-obese pregnant females. In three of the four patients in this group the fall in the ratio at 30 minutes was not as great as that of either non-obese pregnant or non-pregnant females. This profile is similar to that observed for the two patients with NIDDM (Table 6.12) although the impairment of the decrease at 30 minutes is not so great. Therefore these three obese pregnant females are similar to the two patients with NIDDM both in their obesity and PI/Ins % ratio profiles during an OGTT. Obesity is already known to be a common factor in patients with NIDDM (156) and as an impaired PI/Ins % ratio profile may prove to be another common factor it would be interesting to establish whether the profile in these patients returned to normal post-partum, thus indicating whether pregnancy, which is known to be diabetogenic (153) had revealed this trait.

Two of the patients with impaired glucose tolerance (NN and EH) showed a normal PI/Ins % ratio profile during the OGTT while the absolute plasma concentrations of

both these hormones were raised. This is consistent with impaired glucose tolerance in pregnancy being due to insulin resistance (153) rather than a deficiency or abnormality of insulin secretion.

Finally, the patient with impaired glucose tolerance who required administration of exogenous insulin (patient PC) exhibited a PI/Ins % ratio profile which followed the expected pattern, but the absolute ratios were lower at all time points. This profile differs from those observed for patients with NIDDM or the obese pregnant female group. In Section 1.7 of this chapter it was hypothesised that two different pools of insulin existed, 'Pool Ins' and 'Pool PI-Ins'. Taking this hypothesis a step further, if two different types of β cell existed which corresponded to the two pools of insulin, then it could be possible that 'Pool Ins' β cells are involved in the pathogenesis of NIDDM while 'Pool PI-Ins' β cells are those initially involved in the pathogenesis of insulin-dependent diabetes mellitus (IDDM).

2.3 Insulinoma

Methods

Archive samples from three patients investigated for insulinoma, and subsequently proven positive following surgery, were assayed for insulin and proinsulin.

Results

Plasma glucose, insulin, proinsulin and C-peptide concentrations as well as the PI/Ins % ratios of a number of fasting samples from each patient are shown in Table 6.20. All patients were hypoglycaemic at the time of sampling and C-peptide concentrations (when assayed) were within the normal range. Only patient WT exhibited clearly raised fasting concentrations of insulin whereas all three patients had fasting proinsulin concentrations outwith the normal range, although in patient NK the concentration was only marginally elevated. Patient GS was the only one with an abnormal PI/Ins % ratio.

TABLE 6.20

PLASMA GLUCOSE, INSULIN, PROINSULIN AND C-PEPTIDE CONCENTRATIONS AND THE PROINSULIN/INSULIN PERCENTAGE RATIOS IN FASTING SAMPLES FROM PATIENTS WITH INSULINOMA

Patient initials	Sex	Glucose (mmol/l)	Insulin (pmol/l)	Proinsulin (pmol/l)	C-peptide (pmol/l)	PI/Ins %
GS	Female	-	95	87.44	350	92.0
		1.9	102	84.50	390	82.8
WT	Female	1.5	255	12.69	-	5.0
		-	204	11.92	-	5.8
NK	Male	1.9	81	6.81	300	8.4
		1.7	53	7.59	300	14.3
		1.1	121	6.51	550	5.4
		1.4	82	7.90	400	9.6
Normal* range	Female	4.1-6.2	17-75	1.23-3.00	120-750	1.2-6.9
	Male	4.0-6.0	19-102	1.40-5.73	210-760	2.3-16.7

*Established from the fasting and OGTT data presented in this thesis.

However, the diagnosis of insulinoma is not made on the basis of a raised fasting insulin concentration. It is the fact that the insulin concentration is inappropriate in relation to the degree of hypoglycaemia which is important. This can be expressed as an amended insulin/glucose ratio (AI/G) and is calculated from the equation (157, 158):

$$AI/G = \frac{insulin\ (mU/l)}{glucose\ (mmol/l) - 1.7}$$

An AI/G of greater than 30 is consistent with insulinoma.

Plasma proinsulin and insulin concentrations were measured in samples taken from patient GS during an insulin suppression test. These results, together with the previously available glucose and C-peptide data are presented in Table 6.21. There was incomplete suppression of both plasma proinsulin and C-peptide concentrations following administration of exogenous insulin.

The insulin and proinsulin concentrations, together with the PI/Ins % ratios in samples taken from patient WT during pancreatic portal venous sampling, are shown in Table 6.22. Unfortunately, there was not a sufficient volume of the sample measured previously as having the highest insulin concentration to permit measurement of proinsulin. However, in the remaining samples the one with the highest insulin concentration also had the highest proinsulin concentration although the ratio of PI/Ins % was lowest. It is also interesting to note that the PI/Ins % ratio was less than 50% of that observed in the periphery in all the samples taken during this procedure except for the sample collected from the right gastric vein.

Discussion

It is generally accepted that measurement of fasting proinsulin concentrations is useful in the diagnosis of insulinomas, especially in those cases where patients

TABLE 6.21

**PLASMA GLUCOSE, INSULIN, PROINSULIN AND C-PEPTIDE
CONCENTRATIONS IN A PATIENT WITH INSULINOMA
DURING AN INSULIN SUPPRESSION TEST**

Time (min)	Glucose (mmol/l)	Insulin (pmol/l)	Proinsulin (pmol/l)	C-peptide (pmol/l)
0	3.1	129	41.59	430
30	1.2	12550	49.56	470
45	0.5	5280	39.13	380
60	0.9	924	35.26	350
90	1.6	681	32.46	280
120	2.0	285	35.37	330

The patient was GS.

TABLE 6.22

PLASMA INSULIN AND PROINSULIN CONCENTRATIONS IN A PATIENT
DURING PANCREATIC PORTAL VENOUS SAMPLING

Sample number	Location	Insulin (pmol/l)	Proinsulin (pmol/l)	PI/Ins %
1	Splenic vein	563	12.31	2.2
3	Splenic vein	684	13.57	2.0
4	Splenic vein	637	14.59	2.3
5	Portal vein	1195	14.39	1.2
6	Portal vein	1191	17.20	1.4
7	Portal vein	1030	18.32	1.8
9	Superior/common pancreatic vein	725	12.75	1.8
10	Left gastric vein	1759	17.01	1.0
11	Superior mesenteric vein	2160	19.72	1.0
12*	Right colic vein	>7570	-	-
13	Gastro-epiploic vein	5674	31.02	0.5
14	Right gastric vein	328	14.04	4.3
15	Superior pancreatico-duodenal vein	1528	19.94	1.3
	Peripheral vein	204	11.92	5.8

*There was not sufficient sample remaining to re-measure the insulin concentration
or measure the proinsulin and C-peptide concentrations. The patient was WT.

exhibit low plasma insulin concentrations during fasting (19, 146, 159, 160, 161). When inappropriately high concentrations of insulin and/or proinsulin in the presence of hypoglycaemia, after an overnight or more prolonged fast, have not been clearly demonstrated additional test can confirm the diagnosis of insulinoma. One of these tests, the insulin suppression test, involves administration of exogenous insulin to induce hypoglycaemia and simultaneous measurement of serum C-peptide or proinsulin concentrations. Normal subjects show marked suppression of both C-peptide and proinsulin concentrations whereas insulinoma patients show diminished or absent suppression (145, 157). The data shown in Table 6.21 is consistent with the diagnosis of insulinoma.

A technique to enhance the localisation of insulinomas has been developed which involves catheterisation of the portal and splenic veins and venous sampling for measurement of insulin. In this way, the tumour can be located by the presence of higher concentrations of insulin in the veins draining the tumour than in the veins draining uninvolved pancreas. This technique has been used successfully for tumours which otherwise proved difficult to locate although some problems have been encountered (162). This technique was used to locate the tumour in patient WT (Table 6.20).

The higher PI/Ins % ratio found in the sample taken from the right gastric vein is due to a decreased concentration of insulin. This is probably a result of enzymatic degradation of insulin due to the fact that this vein drains the lesser curvature of the stomach and the pyloris.

2.4 Mendenhall's Syndrome

Methods

Archive fasting and OGTT samples from a 14 year old boy with Mendenhall's Syndrome were assayed for insulin and proinsulin.

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Results

The clinical and biochemical features of this patient have been described previously (163).

The fasting plasma sample had a glucose concentration of 17.8 mmol/l with insulin and proinsulin concentrations of 1997 pmol/l and 27.04 pmol/l respectively. The sample was assayed at several dilutions in the insulin assay and was found to dilute in parallel with the standards (Table 6.23).

The plasma glucose, insulin, proinsulin and C-peptide concentrations together with the PI/INS % and PI/CP % (proinsulin/C-peptide) ratios during the OGTT are shown in Table 6.24. Plasma glucose, insulin and C-peptide concentrations peaked at 30 minutes with proinsulin showing a peak at 60 minutes. The PI/Ins % and PI/CP % ratios showed the expected decrease at 30 minutes followed by a gradual rise. However, while the PI/CP % ratios were within the normal range, the PI/Ins % ratios were markedly lower.

Discussion

Mendenhall's Syndrome is known to be a state of insulin resistance (163, 164, 165) and this accounts in part for the high circulating concentrations of insulin due to decreased degradation and clearance. An increase in insulin secretion in the basal state would also appear to contribute to the high circulating insulin concentrations as evidenced by the increased fasting C-peptide concentrations observed. This in turn would account for the decreased PI/Ins % ratios while the PI/CP % ratios are within the normal range, although they are at the lower end. The marginally elevated proinsulin concentrations are probably due to decreased clearance caused by the insulin resistance. The first-phase insulin secretion appears to be intact as the PI/Ins % ratio falls as expected at 30 minutes during the OGTT and thereafter rises as the insulin concentrations fall and the proinsulin concentrations increase.

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TABLE 6.23

MEASUREMENT OF INSULIN CONCENTRATION IN SERIALLY DILUTED
PLASMA FROM A BOY WITH MENDENHALL'S SYNDROME

Sample dilution	Measured insulin concentration (pmol/l)
1:5	408
1:10	205
1:20	98
1:40	48

TABLE 6.24

SERUM GLUCOSE, INSULIN, PROINSULIN AND C-PEPTIDE CONCEN-
TRATIONS AND PROINSULIN/INSULIN AND PROINSULIN/C-PEPTIDE
PERCENTAGE RATIOS IN A BOY WITH MENDENHALL'S SYNDROME
DURING A 75 g ORAL GLUCOSE TOLERANCE TEST

Time (min)	Glucose (mmol/l)	Insulin (pmol/l)	Proinsulin (pmol/l)	C-peptide (pmol/l)	PI/Ins %	PI/CP %
0	11.7	2410	8.04	2 300	0.33	0.35
5	18.9	2873	7.48	5 500	0.26	0.14
30	30.1	9093	13.33	10 200	0.15	0.13
60	29.9	7753	21.61	4 100	0.28	0.53
90	24.4	5516	15.09	3 900	0.27	0.39

It is hypothesised that the increased insulin secretion observed in this patient is due to release of insulin from 'Pool Ins' rather than 'Pool PI-Ins' as the basal concentrations of proinsulin are only marginally elevated in comparison to the C-peptide concentrations. This would result from the continuous glucose challenge resulting from the observed hyperglycaemia. The constant hyperinsulinaemia could then result in a down-regulation of receptors which in turn would aggravate the existing situation. It is not known whether a continuous glucose challenge in normal individuals would result in the same pattern of secretion and therefore no conclusions can be made concerning a possible defect of insulin secretion in this syndrome.

In view of the recent description of a third-phase of insulin release, observed in intact islet or whole pancreas preparations in response to a continuous glucose challenge (166, 167), it is interesting to speculate whether this syndrome exhibits an *in vivo* expression of this phenomenon and if so, whether the insulin response observed is abnormal.

2.5 Oral Glucose Tolerance Test in Cirrhosis

Methods

Three groups of patients underwent a standard 75 g OGTT. The normal control group comprised healthy laboratory personnel whose results have been presented in Section 1.4 of this chapter. The inpatient control group were selected randomly from patients admitted to the Department of Surgery at Glasgow Royal Infirmary, for procedures unrelated to hepatic, renal and gastro-intestinal disease. Typically they were receiving treatment for varicose veins and hernias. The cirrhotic patients were all biopsy-proven.

Results

Details of the three patient groups are shown in Table 6.25. The cirrhotic and inpatient control groups were matched for age, and both were older than the normal control group. All of the normal control group exhibited normal glucose tolerance whereas in the inpatient control group four (44%) had impaired glucose tolerance and two (22%) were classified as diabetic. A similar distribution was observed in the cirrhotic group where seven patients (50%) had impaired glucose tolerance and three (21%) were diabetic.

The glucose, insulin, proinsulin and C-peptide concentrations of the three groups during a 75 g OGTT are shown in Table 6.26 and Figure 6.12. The glucose, proinsulin and C-peptide profiles of the age-matched inpatient control and cirrhotic groups were similar at fasting and at all time points of the OGTT. However, compared to the younger normal control group, both groups exhibited significantly increased concentrations of glucose and proinsulin from 30 to 120 minutes during the OGTT and significantly decreased concentrations of C-peptide at 30 minutes (cirrhotic group) and 30 and 60 minutes (inpatient controls).

Insulin concentrations at fasting and during the OGTT were similar in the inpatient control and cirrhotic groups except at 90 minutes when the cirrhotic patients had a significantly increased plasma insulin concentration. The inpatient and normal controls only differed at 120 minutes when the former group had significantly higher insulin concentrations. In contrast, the cirrhotic group exhibited fasting hyperinsulinaemia and also showed significantly increased insulin concentrations at 60, 90 and 120 minutes during the OGTT when compared with the normal control group.

The PI/Ins % and PI/CP % (proinsulin/C-peptide) ratios for the three groups during the OGTT are shown in Table 6.27. The PI/Ins % ratios are interesting for they

TABLE 6.25

DETAILS OF PATIENT GROUPS IN CIRRHOSIS STUDY

| | n | | Age | Glucose profile | | |
	Male	Female	Median (range)	Normal	Impaired	Diabetic
Normal controls	10	9	33 (27-57)	19	0	0
Inpatient controls	6	3	65 (46-73)	3	4	2
Cirrhotics	9	5	62 (30-83)	4	7	3

TABLE 6.26

PLASMA GLUCOSE, INSULIN, PROINSULIN AND C-PEPTIDE CONCENTRATIONS IN PATIENTS WITH CIRRHOSIS DURING A 75 g ORAL GLUCOSE TOLERANCE TEST

			Time (mins)			
	-15	0	30	60	90	120
Glucose (mmol/l)						
Normal controls (n=19)	4.4 (4.0-5.3)	4.5 (4.0-5.3)	7.3 (4.3-9.3)	5.7 (3.8-9.6)	4.6 (3.3-7.5)	4.5 (2.9-5.8)
Inpatient controls (n=9)	4.8 (4.1-7.0)	4.7 (4.2-7.8)	9.1*** (6.2-13.8)	10.8*** (7.8-18.9)	10.2*** (5.0-20.8)	8.9*** (3.5-21.3)
Cirrhotics (n=14)	4.9 (4.4-8.6)	4.9 (4.3-8.5)	9.0^{+++} (6.8-12.7)	11.3^{+++} (8.1-16.3)	10.8^{+++} (7.2-18.4)	9.2^{+++} (5.5-19.1)
Insulin (pmol/l)						
Normal controls (n=19)	45 (19-122)	36 (23-102)	403 (114-802)	397 (161-600)	262 (53-642)	177 (30-559)
Inpatient controls (n=9)	57 (28-79)	48 (17-101)	285 (188-1310)	403 (233-1079)	262 (137-1174)	430** (51-732)
Cirrhotics (n=14)	85^{+} (14-441)	72^{+++} (11-504)	396 (160-1091)	700^{+++} (215-1496)	643^{+++}xx (263-1627)	598^{+++} (286-2146)

TABLE 6.26 (cont)

	Time (mins)					
	-15	0	30	60	90	120
Proinsulin (pmol/l)						
Normal controls (n=19)	1.84 (1.16-5.64)	1.59 (1.23-5.73)	4.71 (1.99-13.74)	8.77 (4.08-16.86)	9.36 (4.15-22.11)	9.30 (3.32-29.27)
Inpatient controls (n=9)	2.09 (1.48-7.24) n=7	3.39 (1.54-6.04)	8.66*** (5.87-19.56)	17.11*** (11.13-38.70)	23.63*** (10.48-46.83)	36.18*** (7.73-47.85)
Cirrhotics (n=14)	3.88 (0.50-36.60) n=13	4.76++ (0.77-31.77) n=13	8.52+++ (2.35-41.92)	15.16+++ (3.23-49.74)	21.82+++ (7.86-66.35)	25.16+++ (10.83-55.42)

TABLE 6.26 (cont)

C-peptide (pmol/l)

	Time (mins)						
	-15	0	30	60	90	120
Normal controls (n=19)	430 (190-860)	400 (180-720)	1520 (500-2970)	2100 (1140-3510)	1680 (1030-2770)	1510 (840-2390)
Inpatient controls (n=9)	370 (160-650)	400 (220-400)	740** (250-2600)	1300 (550-4000)	1000 (650-4900)	1000 (540-3900)
Cirrhotics (n=14)	445 (270-840)	435 (290-1000)	870+++ (580-1900)	1250++ (750-3200)	1850 (780-2600)	1950 (200-4100)

* = normal vs inpatient controls   + = normal controls vs cirrhotics   x = inpatient controls vs cirrhotics

*, +, x   p = 0.05   **, ++, xx   p <0.05   ***, +++, xxx   p p <0.01

Results are expressed as median values with the range in parenthesis.

Figure 6.12

Plasma giucose, insulin, proinsulin and C-peptide concentrations in patients with cirrhosis during a 75 g oral glucose tolerance test.

—□— normal controls

—○— inpatient controls

—●— cirrhotics
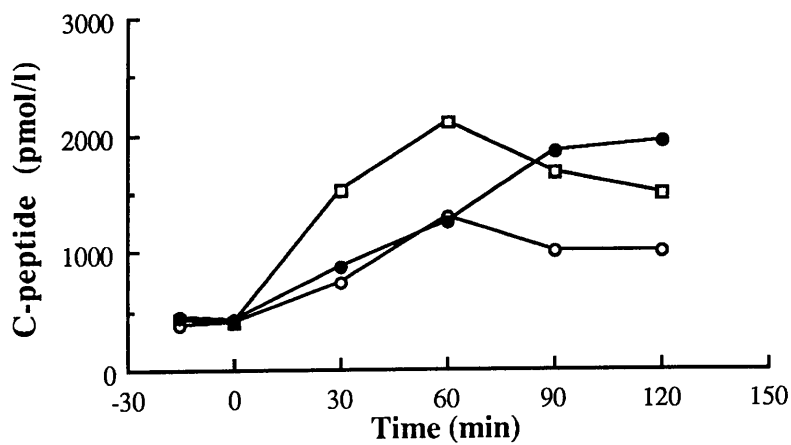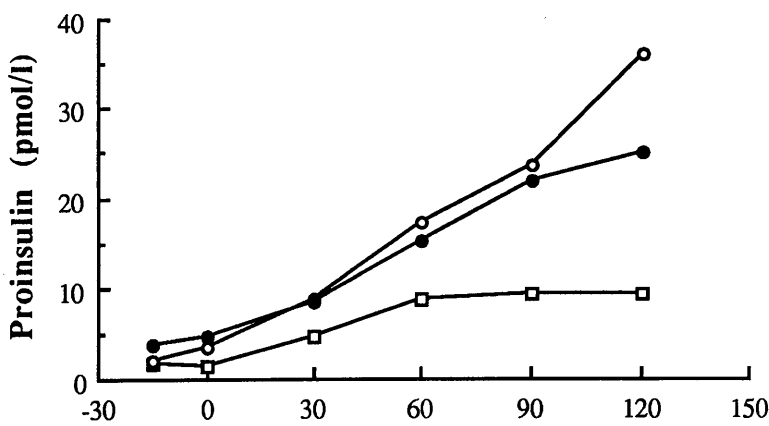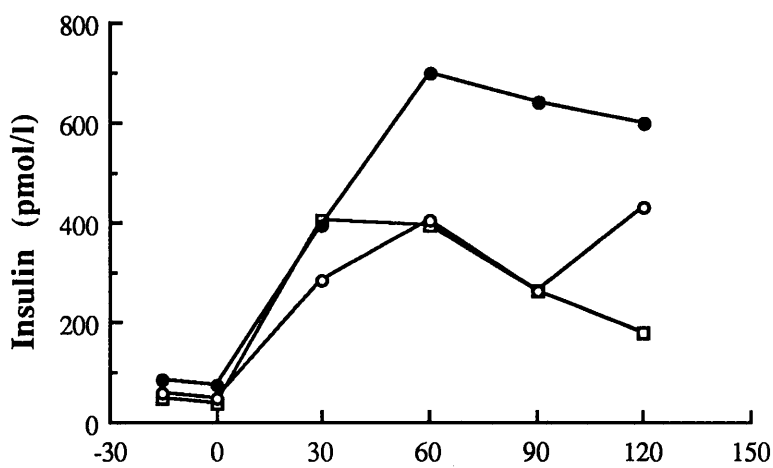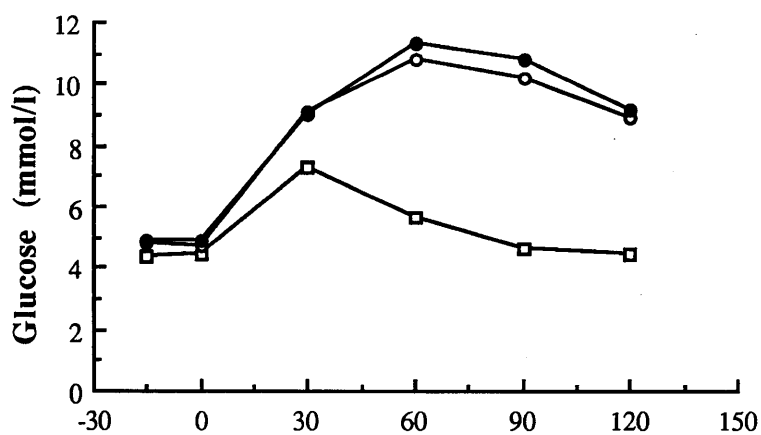
TABLE 6.27

PROINSULIN/INSULIN AND PROINSULIN/C-PEPTIDE PERCENTAGE RATIOS IN PATIENTS WITH CIRRHOSIS DURING A 75 g ORAL GLUCOSE TOLERANCE TEST

PI/Ins %

			Time (mins)				
	-15	0	30	60	90	120
Normal controls (n=19)	4.2 (1.5-17.1)	5.2 (2.3-15.9)	1.5 (0.5-4.3)	2.6 (0.7-6.1)	4.0 (1.7-17.7)	5.6 (2.2-31.3)
Inpatient controls (n=9)	6.1 (2.0-9.2) n=7	6.3 (2.2-19.9)	3.0** (1.2-6.3)	4.8* (2.1-6.9)	7.4** (4.0-14.9)	8.6 (5.5-18.9)
Cirrhotics (n=14)	3.6 (1.5-33.6)	4.0 (2.0-47.2) n=13	1.5 (1.1-19.1)	2.1 (1.1-15.6)	3.1 (1.6-25.2)	3.7 (1.8-18.9)

TABLE 6.27 (cont)

		Time (mins)				
	-15	**0**	**30**	**60**	**90**	**120**
PI/CP %						
Normal controls (n=19)	0.51 (0.17-1.44)	0.50 (0.14-1.36)	0.32 (0.14-1.18)	0.42 (0.17-1.48)	0.53 (0.25-1.84)	0.64 (0.27-2.87)
Inpatient controls (n=9)	0.89 (0.24-1.24) n=7	0.85 (0.38-1.47)	1.18*** (0.38-3.08)	1.42*** (0.58-3.53)	1.91*** (0.61-5.42)	1.79*** (0.95-3.85)
Cirrhotics (n=14)	0.92 (0.23-5.30) n=13	0.87 (0.23-4.07) n=13	0.83+++ (0.39-5.24)	1.17+++ (0.37-4.83)	1.39+++ (0.39-3.37)	1.08+++ (0.60-4.03)

* normal vs inpatient controls   + normal controls vs cirrhotics   x inpatient controls vs cirrhotics

*, +, x p = 0.05   **, ++, xx p <0.05   ***, +++, xxx p <0.01

Results are expressed as median values with the range in parenthesis.

only detect the inpatient control group as being different from the normal controls. At first sight, the cirrhotic group would appear to have a normal PI/Ins % profile, but this results from increases in both insulin and proinsulin concentrations. A more accurate reflection of actual events is given by the PI/CP % ratios which detect both the inpatient and cirrhotic groups as being similar and different from the normal control group. Both groups showed an increased fasting PI/CP % ratio but this did not reach statistical significance. The increased ratios observed during the OGTT however, were significant. The normal controls exhibited a decrease in the PI/CP % ratio at 30 minutes ($p < 0.01$) and a return to basal values at 60 minutes until the end of the test. Both the inpatient and cirrhotic group failed to show the decrease at 30 minutes and exhibited raised PI/CP % ratios from 60 minutes until the end of the test ($p < 0.05$).

Discussion

The observed prevalence of abnormal glucose tolerance in the cirrhotic patients in this study was higher than has been reported previously (168). However, the similar prevalence found in the inpatient control group suggests that other factors may also have contributed to the higher incidence observed in this study.

The hyperinsulinaemia of the cirrhotic group, when compared with the normal control group, both at fasting and following ingestion of a glucose load is in agreement with other studies (140, 169, 170, 171, 172, 173, 174). Similarly hyperproinsulinaemia at fasting and after a glucose load has been reported (171). The data presented here is consistent with hyperproinsulinaemia following a glucose load, but although the cirrhotic group showed median fasting proinsulin concentrations twofold greater than the normal control group (171) this was not statistically significant. One group did not find any difference in serum proinsulin concentrations at fasting and following a glucose load (140) and it was suggested that this was due to the fact that their assay did not measure 32-33 split and

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des 31-32 proinsulin. As their assay, although an RIA, exhibited similar specificity to the one used in this study, their explanation for not detecting hyperproinsulinaemia in cirrhotic patients does not appear to be valid.

The majority of investigations have reported C-peptide concentrations at fasting and/or following a glucose load to be normal (170, 171) or increased (140, 169, 171, 172, 173, 176). The results obtained in this study are in accordance with those that found normal fasting C-peptide concentrations (170), but differ from all previous investigations in finding that the C-peptide concentrations during an OGTT are decreased in cirrhotic patients compared with normal controls.

All previous studies have only compared cirrhotic patients with normal healthy control subjects who in many instances were laboratory personnel (170, 171, 172, 173) or not specified (140, 176). Only one group stated that the control as well as the cirrhotic group were hospitalised during the study (172). In addition to the comparison of the cirrhotic group with a normal control group, this study also compared them with an equivalent inpatient population selected at random.

When the inpatient and normal control groups were compared, the inpatient group, like the cirrhotic patients, exhibited glucose intolerance, increased proinsulin concentrations and decreased C-peptide concentrations during an OGTT. They only differed from the control group with respect to insulin concentrations at 120 minutes of the OGTT. Therefore, in comparison with the inpatient control group, the cirrhotic patients only appeared to be abnormal in having fasting hyperinsulinaemia and increased insulin concentrations at 60 and 90 minutes during the OGTT.

The hyperinsulinaemia cannot be explained by increased secretion as fasting C-peptide concentrations are normal and are decreased in response to an OGTT compared to normal controls. A decrease in the insulin response to glucose has also been shown by Greco et al (174) who measured insulin in portal and peripheral blood following a glucose load. Whereas peripheral hyperinsulinaemia was

observed, the concentrations of insulin in portal blood were lower than in control subjects. Johnston et al (177) also suggested the presence of mild β cell deficiency in cirrhosis. Therefore the hyperinsulinaemia observed must be due to decreased degradation rather than increased secretion. This is substantiated by other groups who have shown a decreased fractional uptake of insulin by human cirrhotic liver (178) and a longer half-life for insulin in cirrhotic rat liver (179). A number of other studies also concluded that the hyperinsulinaemia of cirrhosis was due to decreased degradation rather than increased secretion (170, 177, 180) but the majority of studies have concluded that both hypersecretion and diminished degradation are responsible (140, 169, 171, 172, 173, 176). It has also been suggested that hyperproinsulinaemia contributes substantially to the increased concentrations of insulin (171). This study found increased concentrations of proinsulin in patients with cirrhosis but conclude that it does not contribute to the increased insulin concentrations as the inpatient group which showed an equivalent increase in proinsulin did not exhibit the same extent of hyperinsulinaemia.

It has been reported that patients with cirrhosis of the liver have an impaired first-phase but not second-phase, insulin response (181). The data presented here is consistent with this as the PI/CP % ratios did not show the expected decrease at 30 minutes during an OGTT. This also correlates with the prevalence of IGT and diabetes observed.

In conclusion, the data in this study suggest that cirrhosis of the liver is associated with glucose intolerance, hyperinsulinaemia and hyperproinsulinaemia. The hyperinsulinaemia is attributable to diminished degradation. However, an inpatient population exhibited the same characteristic features as the cirrhotic patients except for the hyperinsulinaemia. Therefore, it is suggested that only the hyperinsulinaemia is truly secondary to development of cirrhosis of the liver and other factors may be involved in causing the glucose intolerance, hyperproinsulinaemia and diminished insulin response during an OGTT.

CHAPTER 7

GENERAL DISCUSSION

When the aims of this thesis were originally proposed, the main difficulty envisaged was the production of insulin and C-peptide antibodies of sufficiently high avidity to permit the development of a two-site IRMA capable of measuring the low concentrations of proinsulin found in human serum in the basal state. It was hoped that production of a proinsulin-specific McAb would be possible and that use of such an antibody in an assay to measure proinsulin would overcome the problems associated with cross-reaction from insulin and C-peptide.

It soon became apparent that these views were rather simplistic. Although it had been suggested that proinsulin in human serum was heterogeneous (40), it was not known to what extent this reflected intact proinsulin and the various conversion intermediates. Also, the shortage of well-defined proinsulin standards did not permit characterisation of the proinsulin assays which had been developed (33, 34) and it was assumed that all the forms of proinsulin were measured equally. It was only with the introduction of biosynthetic human proinsulin that the situation was clarified and previous extracted pancreatic proinsulin standards were shown to behave differently to the biosynthetic proinsulin standards in the assays available (182, 183). The extracted pancreatic standards appeared to comprise partially hydrolysed rather than intact proinsulin and this led to the suggestion that the major circulating form of proinsulin was possibly one or both of the conversion intermediates rather than the intact molecule (183). At about the same time it was also shown that polyclonal and monoclonal antibodies specific for proinsulin reacted with an epitope which involved the B-C chain junction, and therefore these antibodies could not be used in an assay to measure the 32-33 split/des 31-32 forms of proinsulin.

It was therefore decided that the assays developed in this thesis would reflect the specificity of the antibodies developed as there was no indication as to which form of proinsulin should be measured. The final assay developed measured intact and

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65-66 split/des 64-65 proinsulin and was sufficiently sensitive to measure fasting serum concentrations of proinsulin.

A two-site insulin IRMA was also developed, using a polyclonal and monoclonal anti-insulin, and was shown to cross-react to a lesser extent with all forms of proinsulin than the locally established RIA.

During the work performed to produce antibodies to insulin and proinsulin and the subsequent development of the assays, a number of interesting observations were made. It would appear that the polyclonal antibody response to a molecule the size of insulin may not be as heterogeneous as expected. The data presented in Chapter 3 indicated that the antibodies raised to insulin in a number of animal species appeared to be restricted to a single, or small number, of epitopes and that certain epitopes on the molecule were immunodominant. When the immunodominant epitope was masked due to conjugation to a carrier protein, the immune response either failed or was directed against a different epitope. Similar evidence was obtained from hybridisation experiments where it was noted that the McAbs produced in any one fusion appeared to be restricted with respect to the epitope recognised. The fusion using insulin as immunogen resulted in three McAbs of apparently identical specificity. Likewise, the two fusions with proinsulin produced either McAbs against C-peptide or a proinsulin-specific determinant but not both.

The practical constraints of the above finding were observed when the polyclonal anti-insulin was used to develop a two-site IRMA for insulin as only one of the two specificities of McAb produced could be used as a partner antibody.

Practical difficulties were also encountered when developing the proinsulin IRMA due to the presence of high molar concentrations of insulin compared to proinsulin in clinical samples. This required that proinsulin be reacted with a solid-phase

coupled proinsulin-specific McAb, to permit the removal of insulin, prior to the addition of the radiolabelled insulin McAb thus abolishing possible interference.

It would appear that the sensitivity of the proinsulin assay cannot be further improved with the use of ^{125}I-labelled antibodies, unless an amplification step was included, as the zero dose binding tubes gave a count rate which was only 200-300 cpm above the background count rate.

The aim of this thesis which was not met was the production of suitable antibodies to permit the development of an immunoassay for C-peptide. The C-peptide McAb produced was shown to be of too low avidity for use in RIA techniques to measure either proinsulin or C-peptide, although latterly it was shown to have potential for the development of a proinsulin IRMA.

C-peptides are known to be poor immunogens (184, 185, 186) and require conjugation to a carrier protein prior to immunisation. Even then, the production of high avidity antisera is difficult (185). The cost of human C-peptide restricted the quantity which could be purchased and required that conjugation techniques be evaluated in a model system. Although some initial groundwork was performed, due to the limited time available, this work was not completed.

If the original aims of this thesis were to be reassessed at this point in time with respect to antibody production and assay development, they would probably reflect the recent achievement by W J Sobey et al (32) in developing IRMA's for each of the forms of proinsulin as well as an insulin-specific IRMA. However, in view of the recent reports in the literature of antibodies specific for the A-C junction of the proinsulin molecule (187) and the B30 residue of human insulin (188), it may be possible to develop an improved IRMA which would be specific for 32-33 split/ des 31-32 proinsulin. In addition, it would be interesting to establish whether it is possible to develop a two-site assay for a molecule the size of C-peptide. It would also be proposed that alternative detection systems be evaluated in an attempt to

improve the sensitivity of the various proinsulin assays, thus allowing more accurate and precise measurement of proinsulin in the basal state.

The final two aims of this thesis were related to the clinical application of the assays developed. Proinsulin and insulin concentrations were measured during fasting and various dynamic function tests under both physiological and pathophysiological conditions. From the data collected, a number of interesting observations were made which merit further investigation.

The finding that females have lower concentrations of intact proinsulin than males at fasting and during an OGTT requires the investigation of larger numbers to determine the true significance of this observation. It would also be interesting to measure the concentration of 32-33 split/des 31-32 proinsulin in the same groups to assess whether a similar pattern resulted. If the differences were found to be significant they would have major implications for studies involving the measurement of proinsulin as male and female groups would have to be investigated separately.

The most interesting finding was that insulin appeared to be released from two pools, only one of which appeared to contain intact proinsulin. It would be useful to ascertain whether the same apparent distribution occurs for the proinsulin conversion intermediates. However, in the studies performed in this thesis, the above observation enabled the use of PI/Ins % and PI/CP % ratios, in conjunction with the raw insulin, proinsulin and C-peptide concentration data, to detect abnormal patterns of insulin release from one or both pools.

It is suggested that the future aims of this work might encompass the following proposals:

(i)   Observation of the PI/Ins % and PI/CP % ratio profiles, from normal volunteers and patients with NIDDM, over the first 30 minutes of an OGTT using various sampling intervals. It should then be possible to determine the maximum sampling interval from which a clinically useful profile can be obtained.

(ii)   Using the conditions determined above, establishment of normal PI/Ins % and PI/CP % ratio profiles in a large group of normal volunteers.

(iii)   Investigation of larger numbers of patients with IGT, NIDDM and IDDM during an OGTT to evaluate whether characteristic profiles can be determined for each group.

(iv)   Study of a group of people known to be at risk of developing NIDDM and/or IDDM to ascertain whether characteristic changes occur in the PI/Ins % and/or PI/CP % profiles over the years preceding the clinical manifestations of diabetes.

(v)   Study of newly diagnosed NIDDM patients with respect to their PI/Ins % and PI/CP % profiles during an OGTT. Investigation of whether any changes occur as they proceed from treatment by diet alone, to drug therapy and if necessary, administration of exogenous insulin.

From the results of the above studies it would be hoped that it may be possible to detect those people who are at risk of developing insulin or non-insulin dependent diabetes mellitus. This might be useful in the future if prophylactic treatment for the

prevention of diabetes became available. It also might be possible to determine guidelines to evaluate which patients with NIDDM can be successfully treated by diet, drugs and/or insulin and to assess when and if these requirements change.

It would also be interesting to investigate the PI/Ins % and PI/CP % ratios in response to insulin secretagogues other than glucose to determine if differences occur in the pattern of insulin secretion depending upon the stimulus received. Again investigation of normal volunteers, patients with NIDDM/IDDM and those in the prediabetic period may possibly provide useful markers for those at risk of developing diabetes.

Finally, the possible role of pancreastatin in the regulation of insulin secretion and the clinical relevance of the third-phase of insulin secretion, especially in the context of the boy with Mendenhall's Syndrome, would be an interesting topic of investigation.

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