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**Studies on the liver and islet -cell glucose transporter GLUT2: the role of GLUT2 and other -cell proteins as possible autoantigens**

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**STUDIES ON THE LIVER AND ISLET  $\beta$ -CELL  
GLUCOSE TRANSPORTER GLUT2: THE ROLE OF  
GLUT2 AND OTHER  $\beta$ -CELL PROTEINS AS  
POSSIBLE AUTOANTIGENS.**

submitted by Nicola J. Jordan  
for the degree of Ph.D.  
of the University of Bath  
1992.

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## ABSTRACT

The glucose transporter isoform, GLUT2, has been investigated using the bis-D-mannose photolabel ATB-BMPA {2-N-[4-(1-azido-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine}. Labelled protein with an apparent molecular mass of 45-66 kDa was precipitated from both liver plasma membranes and islet tissue using anti-GLUT2 peptide antibodies. ATB-BMPA photolabelling of GLUT2 could be displaced by 4,6-O-ethylidene-D-glucose and D-glucose, which inhibited labelling by 92 and 57% respectively. Since GLUT2 was shown to have a similar affinity to GLUT1 and GLUT4 for the external ligand ATB-BMPA, it is suggested that the varying affinities for substrates shown by the different transporter isoforms, may arise from differences in their ability to bind hexoses at the internal binding site.

The identity of the glucose transporter isoforms expressed by cultured  $\beta$ -cell lines has been investigated by Western blotting and ATB-BMPA photolabelling. Protein labelled in a hexose-displaceable manner in HITm2.2, RINm5F and MIN 6 cells was precipitated by anti-GLUT1 but not GLUT2 C-terminal peptide antibodies.  $\beta$ -cell lines have been characterised in terms of their morphology, insulin secretion and 2-deoxyglucose uptake and their suitability as models of native  $\beta$ -cells has been discussed.

An ELISA has been developed which detects slightly higher levels of antibodies that bind to  $\beta$ -cell line plasma membranes in serum samples from children with recently diagnosed insulin-dependent diabetes mellitus (IDDM) compared with controls.  $\beta$ -cell surface autoantigens have also been investigated by Western blotting.

It has been attempted to use ATB-BMPA photolabelling and immunoprecipitation to identify putative anti-GLUT2 autoantibodies in IDDM serum (Johnson *et al.*, 1990a *N. Engl. J. Med.* **322**, 653-659). The antibodies that were detected were present in both

normal and diabetic serum and precipitated liver plasma membrane proteins labelled in a 4,6-0-ethylidene-D-glucose displaceable manner. It has been proposed that these antibodies recognise cytoplasmic epitopes of detergent-solubilised GLUT2. The putative diabetes-specific anti-GLUT2 autoantibodies could not be detected using this technique.

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## ABBREVIATIONS

AEC	3-amino-9-ethylcarbazole
AMP	adenosine monophosphate
ASA-BMPA	2-N-(4-azidosalicyl)-1,3-bis-(D-mannos-4-yloxy)-propyl-2-amine
ATB-BMPA	2-N-[4-(1-azi-2,2,2-trifluoroethyl)-benzoyl]-1,3-bis-(D-mannos-4-yloxy)-propyl-2-amine
BB-BMPA	2-N-(4-benzoyl)-benzoyl-1,3-bis-(D-mannos-4-yloxy)-propyl-2-amine
BB/W	Biobreeding/Wistar rat
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
B <sub>max</sub>	Total number of binding sites
BSA	Bovine serum albumin
C <sub>12</sub> E <sub>9</sub>	Nonaethylene glycol dodecyl ether
cyt B	Cytochalasin B
D	Deviation between sample sets in K-S test
DHS	Donor horse serum
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMSO	dimethylsulphoxide
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid) Ellman's reagent
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDT	Ethanedithiol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β aminoethyl)ether N,N,N',N',-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FCS	Foetal calf serum
FIA	Freund's incomplete adjuvant
FITC	Fluorescein isothiocyanate
Fmoc	9-fluoroenylmethoxycarbonyl
GAD	Glutamic acid decarboxylase
GLUT <sub>n</sub>	Glucose transporter isoform <sub>n</sub>
HBSS	Hanks balanced salts solution
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HI	Heat-inactivated
HIT	Hamster islet tumour β-cell line
HLA	Human leucocyte antigen (human MHC)

HPLC	High-performance liquid chromatography
HRP	Horse radish peroxidase
IAA	Insulin autoantibody
ICA	Islet cell antibody
ICCA	Islet cell cytoplasmic antibody
ICSA	Islet cell surface antibody
IDDM	Insulin-dependent diabetes mellitus
IgG	Immunoglobulin G
JDF	Juvenile diabetes foundation
$K_d$	Affinity constant
kDa	kilodalton
KLH	Keyhole limpet haemocyanin
KRB	Krebs-Ringer bicarbonate buffer
K-S	Kolmogorov-Smirnov statistical test
MBS	N-maleimidobenzoic acid-N-hydroxysuccinimide ester
MHC	Major histocompatibility complex
MIN	Mouse insulinoma $\beta$ -cell line
NBT	Nitroblue tetrazolium
NCS	Newborn calf serum
NEM	N-ethyl maleimide
NGS	Normal goat serum
NIDDM	Non-insulin-dependent diabetes mellitus
NK	Natural killer cell
NOD	Non-obese diabetic mouse
NP-40	Nonidet P-40
3-O-MG	3-O-methyl-D-glucose
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl fluoride
RIA	Radioimmunoassay
RIN	Rat insulinoma $\beta$ -cell line
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
$T_c$	Cytotoxic T lymphocyte
TEMED	N,N,N',N'-tetramethylethylene diamine
TES	Tris/EDTA/sucrose buffer
TFA	Trifluoroacetic acid
$T_H$	Helper T lymphocyte

<b>TMB</b>	<b>3,3, '5,5'</b> tetramethylbenzidine
<b>TRIS</b>	<b>Tris-(hydroxymethyl)-methylamine</b>
<b>T<sub>s</sub></b>	<b>Suppressor T lymphocyte</b>
<b>Tween-20</b>	<b>Polyoxyethylenesorbitan monolaurate</b>
<b>ZDF</b>	<b>Zucker diabetic fatty rat</b>

## 1.0 INTRODUCTION

### 1.1 GENERAL OUTLINE AND AIMS

Normal glucose homeostasis in mammals is achieved by the secretion of insulin from pancreatic  $\beta$ -cells, this is dependent on the ability of these cells to respond to fluctuations in blood glucose concentrations. This thesis examines the possibility that glucose sensing by  $\beta$ -cells is partly accomplished by the expression of GLUT2, a high capacity glucose transporter. GLUT2 is also expressed in the liver, another organ involved in glucose homeostasis (Thorens *et al.*, 1988), and is just one member of a family of structurally similar glucose transport proteins that have different transport and regulatory characteristics and are expressed in a tissue-specific manner (Gould & Bell, 1990). There is some experimental evidence which implies that a defect in the glucose transport capacity of pancreatic  $\beta$ -cells may render them unresponsive to hyperglycaemia and hence may be responsible for the development of the disease diabetes mellitus (Unger, 1991).

In non-insulin dependent diabetes mellitus (NIDDM) there is a decrease in GLUT2 expression and glucose transport in  $\beta$ -cells, the cause of which is currently unknown (Johnson *et al.*, 1990b; Orci *et al.*, 1990b; Thorens *et al.*, 1990e). A similar reduction in GLUT2 expression and glucose transport in the residual  $\beta$ -cells occurs at the onset of insulin-dependent diabetes mellitus (IDDM) (Tominaga *et al.*, 1986; Orci *et al.*, 1990a). In this type of diabetes the pathogenesis is almost certainly of an autoimmune nature. Circulating anti-islet cell surface antibodies (ICSA) have been detected which inhibit glucose-induced insulin secretion and glucose transport (Kanatsuna *et al.*, 1983; Alden *et al.*, 1984; Johnson *et al.*, 1990a; Kitagawa *et al.*, 1990). It has been proposed that the antibodies that inhibit hexose transport may be directed against the  $\beta$ -cell GLUT2 protein (Johnson *et al.*, 1990a).



Positive identification of  $\beta$ -cell autoantigens may be useful in the development of a screen and eventually a cure for IDDM, which at present is a chronic, incurable illness that impairs the health and shortens the life-span of sufferers to the extent that about one half of those diagnosed by the age of twenty-five will be dead before the age of forty (Keen, 1982).

The results of work undertaken for this thesis have been divided into three sections. Initially, an investigation of GLUT2 was carried out in liver tissue using specific anti-GLUT2 peptide antibodies to immunoprecipitate the GLUT2 protein after it had been radiolabelled using the photoaffinity probe ATB-BMPA {2-N-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)propyl-2-amine}. This photoprobe has previously been used to label other members of the glucose transporter family (Clark & Holman, 1990; Holman *et al.*, 1990). The aim was to compare the characteristics of GLUT2 with those of the other transporter isoforms, and to develop techniques which could then be used to label GLUT2 in other tissues and potentially to detect anti-GLUT2 autoantibodies in the serum of diabetic patients.

Part two of the results section describes the use of photolabelling and Western blotting to establish which transporter isoforms are expressed by three  $\beta$ -cell lines (HIT, RIN and MIN) and to assess the suitability of these cells for use in assays designed to screen for anti-islet cell surface or anti-GLUT2 antibodies.

Finally, as described in part three, it was attempted to develop a simple ELISA which could be used to detect ICSA. Western blotting was used to identify the target  $\beta$ -cell autoantigens and attempts were made to confirm the presence of anti-GLUT2 autoantibodies in IDDM patients by immunoprecipitation of proteins radiolabelled by the glucose transporter specific photoprobe, ATB-BMPA.

## 1.2 FACILITATIVE GLUCOSE TRANSPORT

Glucose transport in, and out of cells is required for the maintenance of glucose homeostasis. The realization that defects in glucose transport may be associated with diseases such as diabetes mellitus has recently led to an increased interest in the mechanisms involved in the uptake of this hexose. D-glucose is the major source of energy for most mammalian cells and since the plasma membrane is impermeable to polar molecules, the uptake of this sugar occurs via specific carrier proteins.

The facilitative glucose transporter family of proteins is responsible for the carrier-mediated passive diffusion of glucose across the cell membranes of the majority of cell types. Transport is bidirectional, but net transport occurs from a high to a low sugar concentration. The carrier is an integral membrane protein with a strong specificity for the D-stereoisomers of pentose and hexose monosaccharides adopting the chair formation of the pyranose ring (Carruthers, 1990).

Facilitative glucose transport has been most widely studied in human red blood cells. The protein responsible for transport activity has been purified and antibodies raised against it (Kasahara & Hinkle, 1977; Allard & Lienhard, 1985). This ultimately enabled a cDNA clone of the transporter to be isolated from a cell line, Hep G2 (Mueckler *et al.*, 1985). Further investigation revealed that this protein was not responsible for facilitative glucose transport in all tissues, but was just one member of a family of structurally related proteins which are the products of distinct genes found on different chromosomes (Bell *et al.*, 1990). Expression of the transporter isoforms is tissue-specific, although any one tissue may contain more than one isoform, such that the expression reflects the precise transport requirements of the tissue concerned.

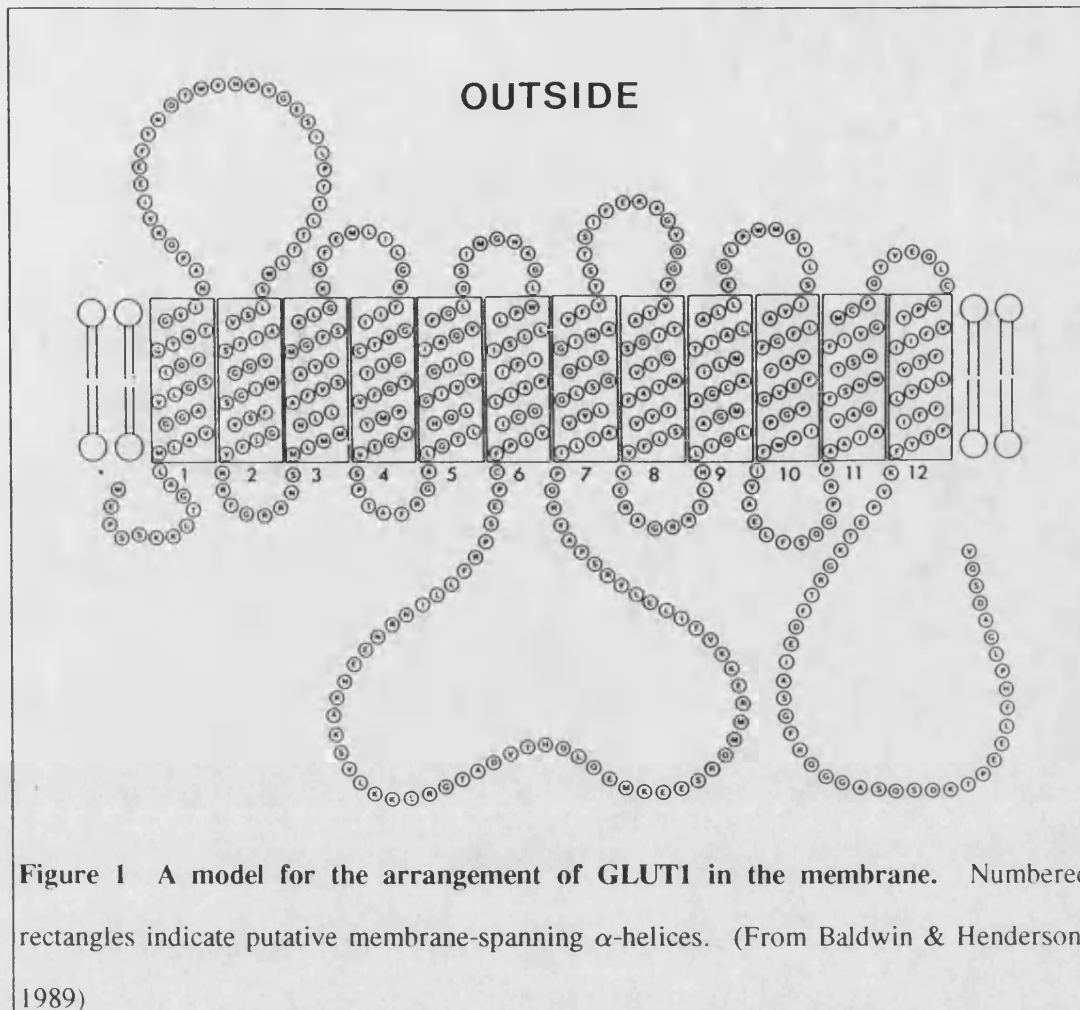
To date, seven genes for members of the facilitative glucose transporter family have been identified, they are named GLUT1-GLUT7. The numbers reflect the order in

which the proteins were identified. GLUT6 is a facilitative transporter-like sequence which cannot encode a functional protein (Kayano *et al.*, 1990). cDNA clones and sequences have been obtained for all the other transporters and the proteins have been functionally expressed and demonstrated to transport D-glucose (Kayano *et al.*, 1990; Gould *et al.*, 1991; Waddell *et al.*, 1992).

### 1.2.1 Models of glucose transporter structure

From a hydropathic profile of the predicted GLUT1 protein sequence, Mueckler *et al.* (1985) proposed a structure for the membrane topology of the protein made up of 12 membrane spanning hydrophobic regions (M1-M12) linked by short cytoplasmic loops of hydrophilic amino acids and long intracellular C- and N-terminal regions. Infrared spectroscopic analysis indicates that the native glucose transporter is predominantly  $\alpha$ -helical in structure, although some  $\beta$ -structure and random coil conformations are also present (Lienhard *et al.*, 1992). The membrane spanning regions are probably composed of  $\alpha$ -helices and, with the exception of the extracellular hydrophilic region between M1 and M2, which has a  $\beta$ -sheet structure, the remaining extramembraneous domains are also believed to be  $\alpha$ -helical as shown by tryptic digestion (Carruthers, 1990) and molecular modelling techniques (P.A. Hodgson - personal communication).

The basic model of GLUT1 shown in Figure 1 is also applicable to the other isoforms which have superimposable hydropathic plots and exhibit between 39% and 65% amino acid identity and 50-76% similarity (Gould *et al.*, 1991). The similarity between the same transporter isoform in different mammalian species is also high.



A study of the conserved regions of the glucose transporters is of interest, since these are likely to be the amino acids involved in the actual mechanism of transport. Regions that are significantly different are presumed to be responsible for the distinctive catalytic properties of each isoform and the differential susceptibility to physiological regulation by endocrine and metabolic factors.

Homology analyses have indicated that hydrophobic regions M5, M10 and M11 are >90% conserved between transporter isoforms, and M1, M2, M4, M7, M8 and M12 share >80% sequence homology. Less homology is seen in the hydrophobic regions M6 and M9 (>70%) and M3 (>60%) (Carruthers, 1990). Multiple sequence alignment data indicates that 4 of the 12 membrane spanning regions, M7, M8, M10 and M11 show a high degree of conservation of key hydrophilic and aromatic side chains. Membrane-spanning

regions M9 and M12 are less conserved and have fewer hydrophilic amino acids (P.A. Hodgson, D.J. Osguthorpe and G.D. Holman - unpublished work). This agrees with photoaffinity labelling data that it is the C-terminal half of the protein that is important in ligand binding.

A 3-dimensional atomic model for GLUT1 produced by Hodgson, Osguthorpe and Holman (unpublished work) proposes that the transporter consists of 2 rings of 6 membrane spanning helices, M1-M6 and M7-M12 respectively. Membrane spanning regions M7-M12 are proposed to form a hydrophilic channel or pore through which glucose can move. The model proposes that the channel is lined with helices M7, M8, M10 and M11, which due to the large number of serine and glutamine residues, are able to interact with glucose. The more hydrophobic regions M9 and M12 may be positioned so they are not involved in the formation of the channel, but maintain the ring shape of the domain structure. There is a region in M10 which is rich in glycine and proline residues and might be expected to impart flexibility to this region, which could be important in the putative conformational change which accompanies the transport of glucose (Gould & Bell, 1990). It has been proposed that the upward movement of helices M7 and M8 exposes the outer binding site, and when M10 and M11 move down in the membrane plane, an inner binding site is exposed (A.F. Davies, A. Davies, R.A.J. Preston, A.E. Clark, G.D. Holman and S.A. Baldwin - unpublished work).

Another model of the glucose transporter called the "pentagonal pore" has also been proposed (Lienhard *et al.*, 1992). The pore consists of the 5 transmembrane segments M3, M5, M7, M8 and M11, these are believed to take up an  $\alpha$ -helical formation such that all of the hydrophilic groups are located on one side of the helix and the hydrophobic groups are on the other side. If these helices bound together with their hydrophobic sides facing towards the remaining transmembrane segments and the lipid environment of the membrane, their hydrophilic surface could form a pore which could bind glucose.

These models are however speculative; in order to determine the real structure of the transporter proteins X-ray crystallographic studies are required which are dependent on the ability to form crystals from the glucose transporter proteins.

Several other regions of the transporter isoforms, in addition to the membrane-spanning domains, also have substantial sequence identity. For example, there is a conservation of charged amino acids in the short endofacial loops linking the transmembrane segments. It has been speculated that these regions may be responsible for ionic interactions with cytoplasmic proteins such as glucokinase in liver and  $\beta$ -cells (Newgard *et al.*, 1990).

Non-conserved regions of glucose transporter proteins include the large extracellular loop between M1 and M2, which varies greatly in length in different transporter isoforms and also contains a potential N-linked glycosylation site. The C- and N-termini are also very different in each of the isoforms. Other non-conserved regions of the transporters that may be responsible for the isoform-specific properties have not yet been identified.

### **1.2.2 Interaction of substrates and analogues**

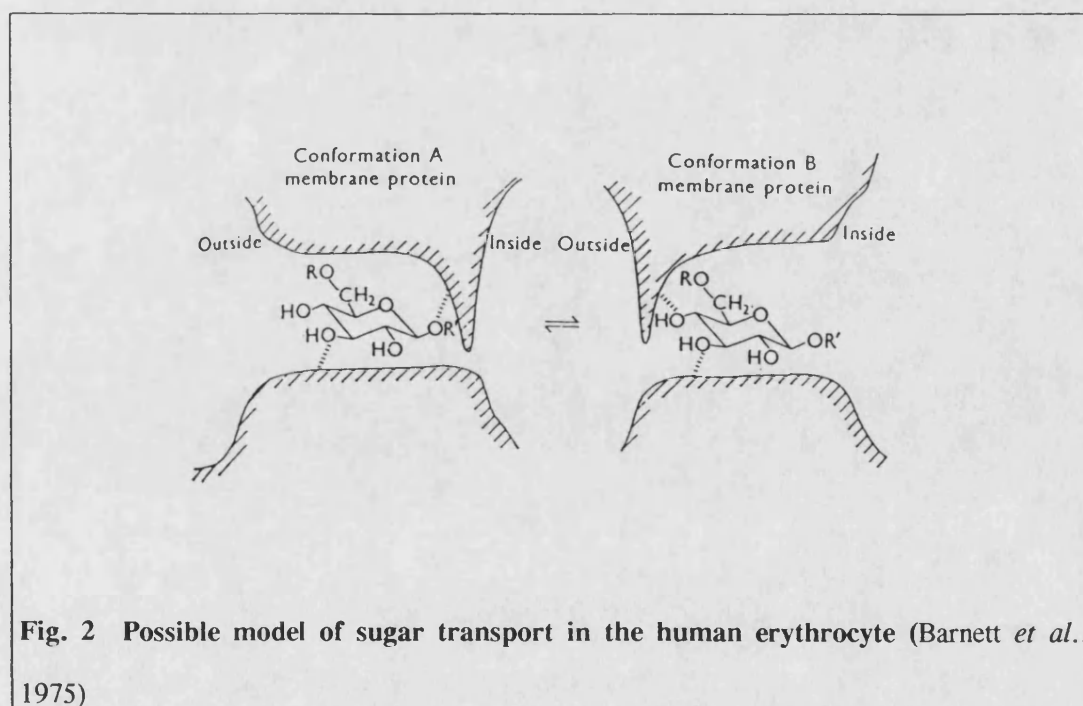
#### **i) Investigation using sugar analogues**

Since facilitative glucose transport is bidirectional, there must be binding sites for the hexose on both the endo- and exo-facial sides of the transporter protein. Hexoses have been shown to interact with the active site of the transporter by hydrogen-bonding, in which the hydroxyl (OH) groups on the sugar act as hydrogen (H) bond acceptors (Barnett *et al.*, 1973a).

Studies involving the binding of sugar analogues to the erythrocyte sugar transporter have indicated that there are significant differences between the sugar influx

and efflux binding sites, and have allowed a model to be proposed for the mechanism of transport. The spatial arrangement of the internal and external binding sites has been investigated by substituting bulky groups into specific positions in the sugar molecule. Hexose derivatives with a large substituent group at C1 cannot bind to the outer face of the transporter (Barnett *et al.*, 1975). Conversely, since 4,6-O-ethylidene D-glucose and 4-O-propyl glucose can inhibit glucose transport at the exofacial site, bulky groups at the C4 and C6 positions do not interfere with binding at the outside of the transporter. Therefore, binding at the exofacial site involves an interaction between OH groups at the C1 and C3 positions of the sugar (Barnett *et al.*, 1973a). The reverse occurs at the internal sugar binding site where C1-alkyl-substituted compounds bind well, but C4 and C6 substituted compounds do not (Barnett *et al.*, 1973b; Barnett *et al.*, 1975). A similar situation occurs in rat adipocytes (Holman *et al.*, 1981; Holman & Rees, 1982).

The conclusion from such studies is that the sugar enters the transporter from the outside with C1 first and C4/C6 trailing. The orientation of the sugar is maintained but a conformational change of the transporter occurs and thus the C4/C6 end is recognised first by the endofacial site. This is illustrated in Figure 2 .



**Fig. 2** Possible model of sugar transport in the human erythrocyte (Barnett *et al.*, 1975)

## ii) Investigation using photoaffinity labels.

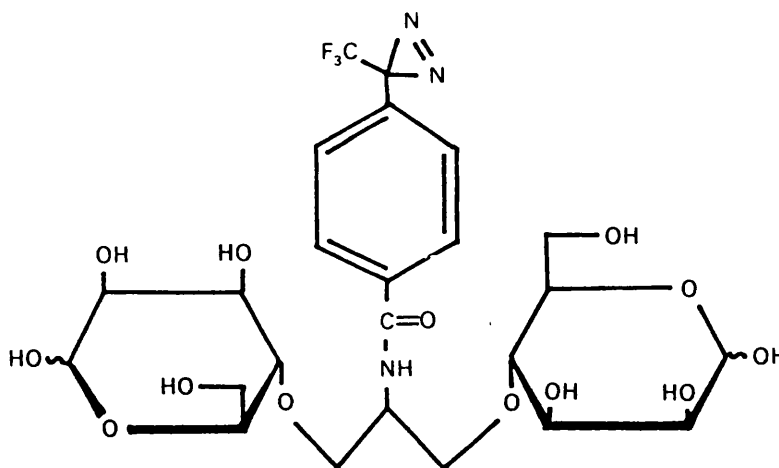
Since the deduced amino acid sequences of the glucose transporter proteins have been published, attempts have been made to establish which regions of the transporters are involved in interactions with sugar molecules. Photoaffinity labelling of the transporters has been used in such studies.

The fungal metabolite, cytochalasin B (cyt B), is a potent inhibitor of facilitative glucose transport (Jung & Rampal, 1977; Devés & Krupka, 1978; Axelrod & Pilch, 1983). When irradiated with high intensity ultra violet light, [<sup>3</sup>H] cyt B forms a covalent bond with a site on the cytoplasmic face of the erythrocyte transporter. Proteolytic cleavage of the erythrocyte transporter subsequent to covalent labelling with [<sup>3</sup>H] cyt B suggests that it specifically binds the endofacial surface of the transporter between Phe<sub>389</sub> and Trp<sub>412</sub>, that is, between membrane spanning regions M10 and M11 (Cairns *et al.*, 1984; Holman & Rees, 1987). This is not necessarily the endofacial glucose binding site, since although glucose can inhibit cyt B labelling, the substrate and inhibitor need not combine at the same site, as glucose could induce a long-range conformational change that reduces cyt B binding in a separate region of the protein (Devés & Krupka, 1978).

A series of photolabels, specific for the exofacial binding site, has also been developed (Midgley *et al.*, 1985; Holman, 1989). They are bis-D-mannose compounds in which the two sugars are cross-linked through the C4-hydroxyl group by a propyl-2-amine bridge. This gives an impermeant, non-transported molecule, 1,3-bis-(D-mannos-4-yloxy)-propyl-2-amine or BMPA, which can then be radiolabelled (Holman, 1989). The bulky photoactive moiety is introduced into the bridge, and since it is in the C4 position of the hexose it does not interfere with the hexose groups required for binding (Clark & Holman, 1990).



Since the bis-D-mannose compounds have a low affinity for the transporter, the photolabel substituent must be selective (Clark & Holman, 1990). The aryl azide, 2-N-(4-azidosalicyl)-1,3-bis-(D-mannos-4'-yloxy)-propyl-2-amine (ASA-BMPA), has been used to label the erythrocyte glucose transporter (Holman *et al.*, 1986), but it was found to be unsuitable for labelling adipocytes, in which the transporter was present in low abundance. In this situation ligands containing precursor groups such as benzophenones or (azitrifluorethyl)-benzoyl groups, which produce carbenes on irradiation, were found to be more effective (Holman *et al.*, 1988). The benzophenone derivative 2-N-(4-benzoyl)-benzoyl-BMPA (BB-BMPA) and 2-N-[4-(1-azi-2,2,2-trifluoroethyl)-benzoyl]-BMPA (ATB-BMPA), the structure of which is shown in Figure 3, both label the erythrocyte and adipocyte transporters (Holman *et al.*, 1988; Clark & Holman, 1990; Holman *et al.*, 1990). ATB-BMPA has the advantage that it is soluble in physiological buffers, requires short irradiation times and because of its improved selectivity can be used to label transporters in isolated membranes in addition to whole cells (Clark & Holman, 1990; Jordan & Holman, 1992). ASA-BMPA binds the exofacial surface of the transporter, probably between membrane-spanning regions M9 and M10 (Holman & Rees, 1987). ATB-BMPA has recently been shown to bind GLUT1 on the exofacial surface of transmembrane region M8 between Ala<sub>301</sub> and Arg<sub>330</sub> (A.F. Davies, A. Davies, R.A.J. Preston, A.E. Clark, G.D. Holman and S.A. Baldwin - unpublished work).



**Figure 3** Structure of ATB-BMPA. From Clark & Holman, 1990

Overall, the results obtained from photolabelling studies suggest that transmembrane segments M7-M10 are involved in ligand binding. That is, that the transport function is linked solely with the C-terminal half of the protein. Labelling has also provided information about the mechanism of transport, since it has shown that the internal and external binding sites cannot be occupied simultaneously (Holman *et al.*, 1986). This supports the alternating conformation model of glucose transport. Oka *et al.* (1990a) have also shown that the C-terminal 37 amino acids are essential for the catalytic function of GLUT1. Their role is to allow the formation of the outward facing conformation as indicated by the inability of a C-terminal mutant to be labelled by ATB-BMPA.

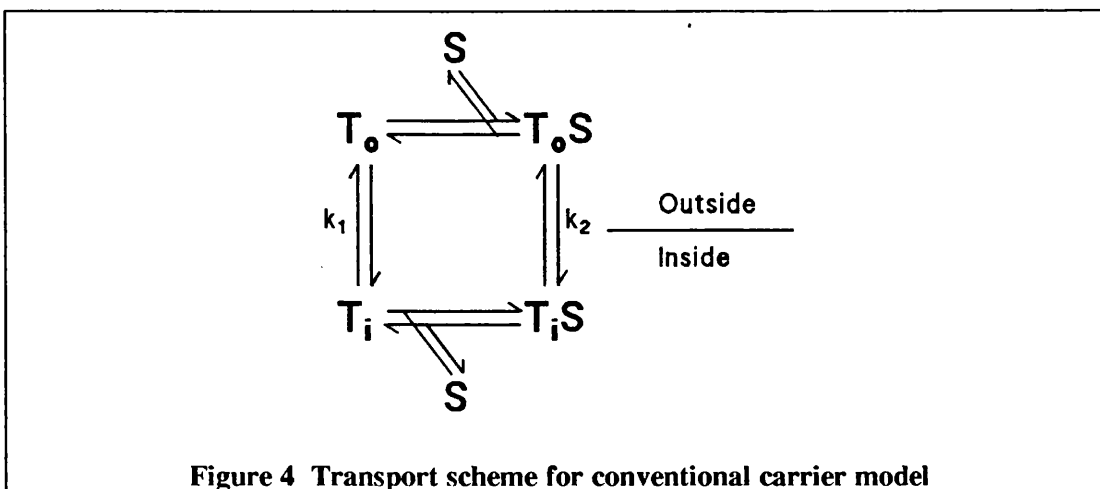
### 1.2.3 Proposed mechanism of transport.

Glucose is transported down its concentration gradient via a carrier protein which displays Michaelis-Menten saturation kinetics, is stereospecific and can be regulated and inhibited. As such, the interaction of hexoses with the transporter is similar to that of an enzyme with its substrate. Models developed to describe the proposed mechanism of glucose transport have been deduced from kinetic data, much of which has been obtained from measuring transport in the human erythrocyte.

The carrier model was proposed in 1952 by Widdas. Since hexose transport is bidirectional, there must be binding sites for D-glucose at both the endo- and exo-facial surfaces of the transporter. These sites are proposed to be mutually exclusive, that is, at any point in time a carrier is available for influx or efflux but not for both simultaneously (Carruthers, 1990).

Experimental evidence suggests that the transporter can exist in two forms, an inward facing conformation ( $T_i$ ) and an outward facing conformation ( $T_o$ ). In the absence of glucose, the transporter is believed to oscillate rapidly between the  $T_i$  and  $T_o$  isomers.

In the erythrocyte, isomerisation ( $k_1$ ) between the two states occurs 100 times/sec at 20°C (Lienhard *et al.*, 1992). The structures of the two conformations are not known but it seems probable that there is a channel open at one end and constricted at the other. Glucose would bind at the open end and be held by weak hydrogen bonds. It could be transported by a conformational change which would close the protein behind it and open the constricted end in front of it, releasing the hexose molecule on the opposite side of the membrane (Lienhard *et al.*, 1992).



As illustrated in Figure 4, hexose transport is believed to involve 4 steps. When there is a high concentration of sugar outside the cell it binds to a transporter in the exofacial ( $T_o$ ) conformation. The transporter/substrate complex ( $T_oS$ ) changes conformation ( $T_iS$ ) so that glucose now occupies the endofacial binding site. The substrate is then released into the cytoplasm. When net sugar transport from a high to a low concentration occurs, the transporter isomerises from  $T_i$  to  $T_o$  so that it is again available for sugar binding at the cell surface. During equilibration exchange, the step involving isomerisation in the absence of substrate is unnecessary and the  $T_i$  form binds a substrate molecule and transports it out of the cell. The isomerisation ( $k_2$ ) of the transporter/glucose complex occurs 900 times/sec. This is much more rapid than the isomerisation of the unloaded transporter, since the presence of the substrate lowers the energy barrier between the two conformations (Lienhard *et al.*, 1992; Walmsley, 1988). This can explain the

phenomenon of exchange acceleration observed in human erythrocytes, in which equilibrium exchange occurs faster than the uni-directional flux, since the conformational change of the empty transporter is rate limiting for net transport, this is known as the trans-effect (Wheeler & Hinkle, 1985; Carruthers, 1990). The alternating conformation model also explains the asymmetry of glucose transport in the human erythrocyte where the  $K_m$  and  $V_{max}$  for sugar exit are higher than for sugar entry. This can arise if inward and outward facing conformations have different affinities for D-glucose or if the unloaded carrier is asymmetrically distributed.

Experimental evidence has been obtained supporting the alternating conformation model, since it is consistent with the existence of two forms of the transporter, the presence of one conformation reducing the availability of the other. For example, when erythrocytes are incubated with maltose, which binds to the exofacial site but cannot be transported, a high proportion of the transporters are trapped in the outward-facing conformation. If the cells are then mixed with glucose a rapid initial surge in glucose uptake occurs due to the availability of outward facing transporters. This is followed by a slower steady state uptake of D-glucose which is limited by the slow isomerisation of the unloaded transporter (Lowe & Walmsley, 1987; Walmsley, 1988).

In summary, the alternating conformation model is widely accepted as being the best model currently available since it accounts for most of the observed kinetic properties.

#### **1.2.4 Characteristics of the transporter isoforms.**

Details of the tissue-specific glucose transporter isoforms are given in Table 1.

Name	Number of amino acids in human sequence	Calculated M <sub>r</sub> from amino acid sequence	Tissue expression	K <sub>m</sub> (mM) 3-O-MG for glucose transporters expressed in <i>Xenopus</i> oocytes	Regulatory factors	References
GLUT1 Erythrocyte Hep G2	492	54.1  (Apparent M <sub>r</sub> from SDS-PAGE 45-55)	Erythrocyte, Immortal cell lines, Brain. Minor expression in: Placenta, Adipocytes, Muscle	19.4 ± 1.94	Oncogenes, Tumour promoters, Growth factors, Fasting, Refeeding, Obesity, Insulin, Exercise, Glucose deprivation	Mueckler <i>et al.</i> (1985) Gould & Lienhard (1989) Gould <i>et al.</i> (1991) Keller <i>et al.</i> (1989)
GLUT2 Liver Pancreatic	524	57.5  (Apparent M <sub>r</sub> from SDS-PAGE 55-61)	Liver, Pancreatic β-cells, Kidney, Intestine	42.3 ± 4.1	Refeeding, Fasting, Diabetes	Thorens <i>et al.</i> (1988) Fukumoto <i>et al.</i> (1988) Gould <i>et al.</i> (1991)
GLUT3 Brain	496	53.9	Brain Minor expression in: Kidney, Gall bladder, Placenta, Foetal skeletal muscle	10.6 ± 1.3	?	Kayano <i>et al.</i> (1988) Gould <i>et al.</i> (1991)
GLUT4 Insulin regulatable	509	54.8  (Apparent M <sub>r</sub> from SDS-PAGE 45-55)	Muscle, Adipose tissue, Heart	1.8	Insulin, Fasting, Refeeding, Obesity, Exercise, Diabetes	James <i>et al.</i> (1989) Birnbaum (1989) Fukumoto <i>et al.</i> (1989) Charron <i>et al.</i> (1989) Kaestner <i>et al.</i> (1989) Keller <i>et al.</i> (1989)
GLUT5 Intestine	501	54.9	Small intestine Minor expression in: Kidney, Adipocytes, Brain, Skeletal muscle	Not determined	?	Kayano <i>et al.</i> (1990)
GLUT7 Liver microsome	528 (rat)	53	Liver microsomes	Not determined	?	Waddell <i>et al.</i> (1992)

**Table 1 The facilitative glucose transporter family**

#### 1.2.4.1 GLUT1 - The erythrocyte-type glucose transporter

GLUT1 was the first member of the glucose transporter family of proteins to be identified. A cDNA clone coding for 492 amino acids was obtained from a human cell line Hep G2 (Mueckler *et al.*, 1985). GLUT1 has since been cloned from a number of other tissues and species. There is a high degree of conservation between species, with a 98% identity between human and rat GLUT1 (Gould & Bell, 1990). The transporter is heterogeneously glycosylated giving it an apparent molecular mass which varies from 45-55 kDa.

GLUT1 is ubiquitous in its distribution and is often co-expressed with other tissue-specific glucose transporters. High amounts are expressed in erythrocytes and in the brain where it seems to be mostly associated with blood microvessels. It is also found in foetal tissues, including the placenta, and in most immortal cell lines. At low levels GLUT1 is expressed in adipose, muscle, kidney, colon and liver tissue (Gould & Bell, 1990; Holman & Gould, 1992). The expression of low levels of GLUT1 in most tissues suggests that it plays a role in supplying the basal glucose requirements of cells (Mueckler, 1990). Since GLUT1 has a high affinity for D-glucose the high expression of this isoform in erythrocytes and the brain allows these glucose-dependent tissues to take up glucose efficiently even when blood glucose concentrations are low.

The kinetics of isolated GLUT1 have been investigated by functional expression of the protein in *Xenopus* oocytes. Under equilibrium exchange conditions a  $K_m$  for 3-O-methyl-D-glucose (3-O-MG) of  $\approx 17$  mM has been obtained (Gould *et al.*, 1991). This agrees well with equilibrium exchange studies in human erythrocytes in which a range of  $K_m$  values from 17-38 mM for D-glucose have been measured (see Carruthers, 1990 and references therein). Human erythrocytes display asymmetry and exchange acceleration, therefore, under net flux conditions, much lower  $K_m$  values are measured. The  $K_m$  for D-glucose uptake is  $\approx 1.6$  mM and for efflux 4.6 mM (Lowe & Walmsley, 1986). The  $K_m$

for net D-glucose influx in most tissues expressing GLUT1 is  $\approx$  1-2 mM (Thorens *et al.*, 1990c).

The expression of GLUT1 is increased by any factors that increase cellular activity, such as oncogene infection, cell division and growth factors (Kahn & Flier, 1990; Mueckler, 1990). This explains its inappropriate expression in most cell lines where it appears to be an adaptation to enhanced rates of growth and cell division. GLUT1 expression can be increased in cultured cells starved of glucose. It has also been shown that a low insulin level in the blood, as occurs in diabetes, can increase expression of GLUT1 around the terminal hepatic venules in the liver (Tal *et al.*, 1991).

#### **1.2.4.2 GLUT2 - The liver/pancreatic-type glucose transporter**

GLUT2 has been cloned and sequenced from rat (Thorens *et al.*, 1988), mouse (Asano *et al.*, 1989) and human liver (Fukumoto *et al.*, 1988). The rat gene codes for a 522 amino acid protein which shares 82% sequence identity with the 524 amino acid human protein (Gould & Bell, 1990). GLUT2 has also been cloned from human pancreatic tissue (Permutt *et al.*, 1989), where it is found to be expressed solely in the  $\beta$ -cells. In addition to being present in liver and  $\beta$ -cells, GLUT2 mRNA has also been found in cells of the intestinal epithelium and proximal tubule cells of the kidney (Thorens *et al.*, 1988; Thorens *et al.*, 1990a; Thorens *et al.*, 1990b).

Analysis of the predicted primary structure of GLUT2 has indicated that the topological organisation of this isoform is identical to that predicted for GLUT1. The major differences between GLUT2 and the rest of the transporter family reside in the C-terminal 28 residues and the exofacial loop between M1 and M2 which is almost double the length in GLUT2. This loop contains a single asparagine-linked glycosylation site (Fukumoto *et al.*, 1988). GLUT2 also contains a potential cAMP-dependent protein kinase phosphorylation site on the cytoplasmic face of the protein (Fukumoto *et al.*, 1988).

Overall, the transmembrane segments of GLUT2 display a 55-65% sequence identity with GLUT1, although a greater degree of similarity is seen in M5 and M10 which are 75% and 80% sequence identical respectively (Thorens *et al.*, 1990c).

The apparent molecular weight of rat GLUT2 varies from 53-61 kDa depending on the tissue in which it is expressed. In liver it has an apparent molecular weight of 53 kDa. In islet  $\beta$ -cells its apparent molecular weight is 55 kDa, in the kidney it is 57 kDa and in the intestine 61 kDa (Thorens *et al.*, 1988). These differences in size may be due to tissue-specific post-translational modifications such as glycosylation, phosphorylation or proteolysis (Thorens *et al.*, 1990a; Thorens *et al.*, 1990c).

Transport in liver is symmetrical and is not accelerated under exchange conditions (Craik & Elliot, 1979). In rat hepatocytes a  $K_m$  of about 20 mM for 3-O-MG has been measured under both net flux and equilibrium exchange conditions at 20°C (Craik & Elliot, 1979). The  $K_m$  for D-glucose transport in isolated hepatocytes is very high, approximately 60 mM (Elliott & Craik, 1982; Granner & Pilkis, 1990). This is similar to the  $K_m$  of 50 mM measured for D-glucose uptake in pancreatic  $\beta$ -cells (Hellman *et al.*, 1971). When GLUT2 is expressed in *Xenopus* oocytes,  $K_m$  values of 42 mM and 66 mM measured under equilibrium exchange conditions have been obtained for 3-O-MG and D-glucose respectively (Gould *et al.*, 1991).

GLUT2 is distinct from the other transporter isoforms in that it has a high  $K_m$  and therefore a much lower affinity, but a much higher capacity for D-glucose. This means that in tissues expressing GLUT2, glucose transport is not limiting at high glucose concentrations. GLUT2 also differs from the other transporter isoforms by being able to transport D-fructose. This has been demonstrated in isolated rat hepatocytes (Okuno & Gliemann, 1986) and in *Xenopus* oocytes expressing GLUT2 (Gould *et al.*, 1991). It has also been shown that the affinity of cyt B for glucose sensitive binding sites in liver membranes, which are now known to be GLUT2 transporters, is one order of magnitude



lower than in adipocyte and erythrocyte membranes, now known to contain GLUT4 and GLUT1 respectively (Axelrod & Pilch, 1983).

#### **i) The role and regulation of GLUT2 in liver**

The glucose transporter proteins expressed in liver, in addition to supplying the glucose requirements of the hepatocytes themselves, play an important role in the glucose homeostasis of the whole organism. GLUT2 is the predominant transporter in liver tissue, although GLUT1 can be detected in a subset of hepatocytes that lie within one row of the terminal hepatic venules (Tal *et al.*, 1990; Tal *et al.*, 1991).

The presence of GLUT2 in hepatocytes is important for their gluconeogenic function. Under conditions favouring net glucose release, liver hexokinase activity decreases, glucose accumulates and is exported via GLUT2. The high  $K_m$  of this transporter ensures that intracellular glucose concentrations do not become rate limiting to efflux. During conditions favouring glucose uptake, the presence of large amounts of GLUT2 ensures that at high plasma glucose concentrations glucose transport does not become rate limiting for metabolism.

Although levels of GLUT2 mRNA are significantly altered by fasting and refeeding, levels of the corresponding protein remain unchanged (Chen *et al.*, 1990). This indicates that the regulation of GLUT2 is not related to a transcriptional change in gene expression, but rather to post-transcriptional changes including functional alteration of the protein (Kahn & Flier, 1990; Seino *et al.*, 1991). Contradictory results have been obtained on the effect of streptozotocin-induced diabetes on the level of GLUT2 in liver. Thorens *et al.* (1990d) found no alteration in liver GLUT2 in diabetic animals, whereas Oka *et al.* (1990c) found that diabetic animals had increased levels of liver GLUT2.

## ii) The role and regulation of GLUT2 in the kidney and intestine

GLUT2 is expressed in the kidney and intestine in association with other facilitative glucose transporter isoforms and the Na<sup>+</sup>-glucose symporter. GLUT2 is localised in regions of these tissues involved in trans-epithelial glucose transport, in particular, glucose efflux.

In the intestine, GLUT2 is present only in the differentiated epithelial cells of the jejunum, ileum and duodenum, where it is present in the basolateral membrane. It is not found on the brush border of the apical membrane which contains the Na<sup>+</sup>-glucose symporter (Thorens *et al.*, 1990a). Glucose is absorbed by the intestinal epithelium over the apical membrane by active transport. It is then passed into capillaries via the basolateral membrane facilitative glucose transporter. A high K<sub>m</sub> transporter such as GLUT2 is required to prevent glucose efflux being limited at high intracellular glucose concentrations.

In the kidney GLUT2 is present in cells where gluconeogenesis occurs, that is, in the basolateral membrane of cells from the proximal tubule. The remaining glycolytic regions of the kidney express GLUT1 (Thorens *et al.*, 1990b). The factors regulating GLUT2 expression in the kidney and intestine are unknown.

## iii) The role and regulation of GLUT2 in the pancreatic β-cell

GLUT2 is expressed in pancreatic β-cells, where it is found at a higher density in the plasma membrane of the microvilli facing adjacent endocrine cells than in the flat regions of the plasma membrane. This localization may be mediated via interactions with the underlying cytoskeleton (Orci *et al.*, 1989). The expression of the high K<sub>m</sub> GLUT2, which operates with pseudolinear kinetics over the physiological range of glucose concentrations, ensures that the intracellular β-cell D-glucose concentration rapidly

approaches that of the blood enabling the  $\beta$ -cell to fulfil a "glucose sensor" function. Any defect in GLUT2 which limits glucose uptake may be expected to impair the normal  $\beta$ -cell response to hyperglycaemia.

$\beta$ -cell GLUT2 can be down regulated by a low plasma glucose concentration, as occurs in hyperinsulinaemia (Chen *et al.*, 1990) and fasting (Koranyi *et al.*, 1990), it is also decreased in cells cultured in low concentrations of glucose (Seino *et al.*, 1991). The decreased GLUT2 expression in IDDM (Orci *et al.*, 1990a) and NIDDM (Johnson *et al.*, 1990b; Orci *et al.*, 1990b; Thorens *et al.*, 1990e; Unger, 1991) is unknown, but it is apparently not mediated by hyperglycaemia (Orci *et al.*, 1990b), since recent evidence has shown that in cultured islets GLUT2 expression is increased by high and decreased by low glucose concentrations (Yasuda *et al.* 1992).

#### 1.2.4.3 GLUT3 - The brain-type glucose transporter

GLUT3 was cloned from human foetal skeletal muscle (Kayano *et al.*, 1988). Its predominant site of expression is the brain, although it is also expressed at lower levels in fat, kidney, liver, muscle and placenta tissue. This may be due to the presence of fibroblasts and blood vessels, which contain GLUT3 mRNA, in all of these tissues. In rodent tissue GLUT3 expression seems to be restricted entirely to the brain (Yano *et al.*, 1991).

The human GLUT3 gene codes for a 496 amino acid protein which shares 64% identity with GLUT1. Under equilibrium exchange conditions a  $K_m$  for 3-O-MG uptake of  $\approx 10$  mM has been obtained for GLUT3 expressed in *Xenopus* oocytes (Gould *et al.*, 1991). The role of GLUT3 seems to be similar to that of GLUT1, that is, it is a low  $K_m$  transporter supplying the basal glucose requirements of cells, particularly neural cells which have an absolute requirement for glucose.

#### 1.2.4.4 GLUT4 - The insulin-responsive glucose transporter

The GLUT4 insulin-responsive glucose transporter isoform was first cloned and sequenced in 1989 (Birnbaum, 1989; Charron *et al.*, 1989; Fukumoto *et al.*, 1989; James *et al.*, 1989; Kaestner *et al.*, 1989). The rat gene encodes a 509 amino acid protein with an apparent molecular mass of 43 kDa, or when deglycosylated, 38 kDa (James *et al.*, 1989). GLUT4 has 65% sequence identity with GLUT1 and there is a 95% homology between the rat and human GLUT4 (Gould & Bell, 1990). When expressed in *Xenopus* oocytes GLUT4 has a  $K_m$  for 3-O-MG transport of 1.8 mM when measured under equilibrium exchange conditions (Keller *et al.*, 1989).

GLUT4 is expressed only in tissues in which glucose transport can be acutely regulated by insulin, that is, in adipose tissue and cardiac and skeletal muscle (Gould & Bell, 1990). In these tissues under basal conditions, that is, in the absence of insulin, the major glucose transporter isoform in the plasma membrane is GLUT1. The addition of insulin causes a 20-30 fold increase in the rate of glucose transport by increasing the  $V_{max}$  for uptake without altering the  $K_m$  for transport (Taylor & Holman, 1981). The acute action of insulin does not involve de novo protein synthesis but is mediated by the translocation of the GLUT4 glucose transporter isoform from intracellular vesicles to the plasma membrane. The structural property of GLUT4 that enables it to respond to glucose has not yet been identified. The proposed translocation of GLUT4 has become known as the "recruitment hypothesis" (Cushman & Wardzala, 1980; Suzuki & Kono, 1980). Insulin increases the cell-surface availability of GLUT4 by 15-20 fold, but the amount of GLUT1 only increases 3-5 fold indicating that it is the GLUT4 isoform that is responsible for mediating the insulin-stimulated increase in glucose transport (Holman *et al.*, 1990). There is some controversy over whether recruitment alone can quantitatively account for the insulin stimulation of glucose transport. This has led to the proposal that insulin also acts to increase the intrinsic activity of the recruited GLUT4 proteins (Suzuki, 1988).

In insulin-resistant states such as fasting, diabetes mellitus and obesity, there is a decreased expression of GLUT4 in adipocytes and muscle tissue which results in decreased glucose uptake in response to insulin. These changes can be corrected by refeeding, insulin therapy or weight reduction respectively (Kahn & Flier, 1990).

#### **1.2.4.5 GLUT5 - The small intestine glucose transporter**

GLUT5 was cloned from human small intestine (Kayano *et al.*, 1990). The gene encodes a 501 amino acid protein which has 41.7% identity with human GLUT1 and is thus the most divergent member of the glucose transporter family so far described.

GLUT5 mRNA has been detected in kidney, adipose and skeletal muscle tissue, although it is predominantly expressed in the small intestine, where it is localised on the apical brush border on the luminal side of the intestinal epithelial cells (Holman & Gould, 1992). While the protein can transport glucose in a cyt B-inhibitable manner, it has recently become evident that the major role of this isoform is actually as a high affinity fructose transporter (Holman & Gould, 1992).

#### **1.2.4.6 GLUT6 - A pseudogene**

A facilitative-glucose transporter-like cDNA sequence has been designated GLUT6 by Kayano *et al.* (1990). The gene has been identified in a number of tissues, but because of the presence of multiple stop codons and frame shifts the sequence cannot encode a functional transporter protein.

#### **1.2.4.7 GLUT7 - A hepatic microsomal glucose transporter**

The most recently identified member of the glucose transporter family, GLUT7 is found in the liver endoplasmic reticulum and nuclear envelope but not in the plasma

membrane (Waddell *et al.*, 1992). The cDNA clone for GLUT7 was obtained by screening a library with an antibody raised against a purified rat hepatic microsomal glucose transport protein (Waddell & Burchell, 1991).

GLUT7 codes for a 528 amino acid protein with a calculated mass of 53 kDa. GLUT7 is 68% identical to GLUT2, with  $\approx 100\%$  identity being observed in membrane-spanning regions M1-M4, M9 and M10. The loop between M1 and M2 is identical, but the C-terminus contains an extra 6 amino acids, which may function to retain the protein in the endoplasmic reticulum.

GLUT7 is believed to be the component T<sub>3</sub> of the hepatic microsomal glucose-6-phosphatase system. This is a complex located in the endoplasmic reticulum membrane which is made up of 3 transport proteins, T<sub>1</sub> (glucose-6-phosphate transporter), T<sub>2</sub> (phosphate transporter) and T<sub>3</sub> (glucose transporter), a Ca<sup>2+</sup> regulatory protein and the catalytic glucose-6-phosphatase protein itself. The enzyme is responsible for producing glucose as the terminal step of gluconeogenesis and glyconeolysis. The T<sub>3</sub> transporter (GLUT7) enables the glucose produced by the enzyme to leave the endoplasmic reticulum. It may therefore play a role in the regulation of the hepatic output of glucose.

### 1.3 GLUCOSE HOMOEOSTASIS

In higher animals it is essential that the blood glucose concentration is maintained within a narrow range. Sustained hypo- and hyper-glycaemia, if uncorrected, can both be rapidly fatal. Homoeostatic mechanisms are normally able to maintain blood glucose concentrations in humans within the safe range of 4-7 mM (Matschinsky, 1990). A key organ in glucose homoeostasis is the liver. After eating, it rapidly reduces blood glucose by converting it into the storage carbohydrate glycogen by the process of glyconeogenesis. In periods of starvation the liver can release glucose immediately by glycogen degradation, and over longer periods by gluconeogenesis. The liver accounts for 90% of the body's

gluconeogenesis, the kidney the remaining 10%. Important to the functioning of both of these tissues is the presence of the high  $K_m$  facilitative glucose transporter protein, GLUT2, which does not limit the rate of glucose influx or efflux even at high glucose concentrations (Thorens *et al.*, 1990a).

Glucose homeostasis is under hormonal control. The two most important hormones are glucagon and insulin which respectively raise and lower blood sugar concentrations by acting upon liver, muscle and adipose tissue. These hormones are produced by the endocrine tissue of the pancreas namely, distinct clusters of about 5000 cells known as the Islets of Langerhans, which make up 1-2% of the total weight of the pancreas (Montague, 1983). In the adult human, 60-70% of the cells within the islet are insulin-secreting  $\beta$ -cells (Montague, 1983). About 20% of the islet cells are  $\alpha$ -cells which secrete glucagon. The remaining cells are  $\delta$ -cells (10%) which secrete somatostatin, and PP cells (2%) which secrete pancreatic polypeptide hormone, the exact function of which is unknown (Montague, 1983).

### 1.3.1 Insulin secretion from normal pancreatic $\beta$ -cells

The only efficient means by which an organism can lower its blood glucose concentration is by the release of the hormone insulin from storage granules in the  $\beta$ -cell cytoplasm (Prentki & Matschinsky, 1987). The  $\beta$ -cell has a unique method of responding to its major agonists. The main  $\beta$ -cell secretagogues, which include the sugars D-glucose, D-mannose, the amino acids leucine, arginine and lysine and certain fatty acids share the common feature that they can all be metabolized by the  $\beta$ -cell to generate metabolic signals responsible for the initiation of the insulin secretory response. This is in contrast to the situation in other secretory tissues in which agonists generate signals via a receptor. Since D-glucose, the predominant physiological signal controlling insulin secretion, does not mediate its insulinotropic effect by interacting with a receptor at the cell surface, it is essential to the secretory function of the  $\beta$ -cell that uptake of D-glucose is not rate limiting

to the signal generating metabolic pathways. The expression of the high  $K_m$  glucose transporter GLUT2 ensures that  $\beta$ -cells are effectively freely permeable to glucose (Thorens *et al.*, 1988). Therefore, if a fairly high threshold level of intracellular glucose is required to initiate insulin-secretion, the expression of a high  $K_m$  transporter would be required to ensure that the intracellular glucose concentration reached this threshold.

Understanding the factors involved in the normal recognition of D-glucose by  $\beta$ -cells is important, since defects in these sites may be associated with the impaired ability of D-glucose to induce insulin secretion in the disease diabetes mellitus (Robertson & Porte, 1973; Malaisse, 1988). Although it need not actually be located at the cell surface, a sensor or receptor component is necessary in any stimulus response pathway. In both liver and islet cells in addition to the normal low  $K_m$  glucose phosphorylating enzyme hexokinase, there is also a unique high  $K_m$  phosphorylating enzyme called glucokinase (ATP:D-glucose 6-phosphotransferase [EC 2.7.1.2]), This enzyme is widely believed to play a pivotal role as the  $\beta$ -cell glucose sensor, since it is the activity of this enzyme that determines the rate of metabolic flux through the glycolytic pathway. (Bedoya *et al.*, 1986; Matschinsky, 1990). Inhibition of glucose phosphorylation by mannoheptulose blocks glucose-induced insulin secretion indicating that the phosphorylation step is important (Ashcroft & Crossley, 1975; Hedekov, 1980).

The 15 amino acids present at the amino terminus of islet glucokinase may, by virtue of their high number of positively charged residues, allow for an electrostatic interaction with islet GLUT2 (Newgard *et al.*, 1990). This association may effectively compartmentalize the  $\beta$ -cell so that phosphorylation is carried out by glucokinase rather than hexokinase (MacDonald, 1990). This putative interaction may enable the  $\beta$ -cell glucose transporter and glucokinase to function in tandem as the  $\beta$ -cell glucose sensor (Thorens *et al.*, 1988).



Although there is a considerable amount of experimental evidence supporting the hypothesis that most  $\beta$ -cell agonists are metabolized, the identity of these proximal signals remains elusive. Most evidence favours energy parameters rather than specific metabolites as signals. The signals are probably unique to each individual secretagogue but may include the  $\text{NAD}^+(\text{P}):\text{NAD(P)H}$  ratio, pH, phosphorylation potential, guanine nucleotides and acyl-co-A (Prentki & Matschinsky, 1987). Much more is known about the distal components of the signalling system which appear to operate the same in  $\beta$ -cells as in other secretory cells (MacDonald, 1990).

The proposed sequence of events that results in insulin secretion is as follows. The metabolism of glucose causes an increase in ATP which mediates the closure of  $\text{K}^+$  channels (Prentki & Matchinsky, 1987). This results in the depolarization of the  $\beta$ -cell causing voltage-gated  $\text{Ca}^{2+}$  channels to open and influx of  $\text{Ca}^{2+}$  to occur. This transient rise in  $\text{Ca}^{2+}$  is a prerequisite for glucose-induced insulin secretion as shown by the ability of  $\text{Ca}^{2+}$  channel antagonists to inhibit the sustained phase of glucose-induced insulin release.  $\text{Ca}^{2+}$  may mediate insulin secretion by a number of mechanisms including the activation of protein kinases which results in an alteration in the phosphorylation states of specific proteins which effect insulin secretion in an as yet unknown manner.

### 1.3.2 $\beta$ -cell lines as models of normal islet $\beta$ -cells

The study of the physiology of  $\beta$ -cells has been impeded by the fact that the normal method used to isolate them from pancreatic tissue is a time consuming and expensive procedure which results in the production of a small quantity of a heterogeneous population of islet cells. The development of  $\beta$ -cell lines, that can be maintained in tissue culture, has been of importance in studies of insulin secretion and  $\beta$ -cell antigenicity. Some of the similarities and major differences between  $\beta$ -cell lines and native  $\beta$ -cells are outlined below.

### 1.3.2.1 The RIN $\beta$ -cell line

The RIN or rat insulinoma cell line was established in culture from radiation-induced transplantable islet tumours in rats (Gazdar *et al.*, 1980). Several subclones have been established from the RIN cell line, including RINm5F a widely used clone.

RIN cells are abnormal  $\beta$ -cells in that they do not secrete insulin in response to glucose, although alternative secretagogues including leucine, glyceraldehyde and arginine are able to induce insulin secretion (Lambert & Atkins, 1987). The defect is glucose-specific, suggesting a deficiency in the normal glucose transport or glucose-phosphorylating activity of the cell line. RIN cells are also resistant to the cytotoxic action of the diabetogenic drugs alloxan and streptozotocin which affect native  $\beta$ -cells (Kawada *et al.*, 1987; Malaisse, 1988). Most studies have shown that there is a deficiency in RIN cells which results in a slow uptake of 3-O-MG. In RIN 1046-38 cells a  $K_m$  for D-glucose of 6 mM has been obtained (Powers *et al.*, 1990). This contrasts with the behaviour of pancreatic  $\beta$ -cells which have a high  $K_m$  for D-glucose and equilibrate sugar within 2-3 minutes (Meglasson *et al.*, 1986; Malaisse, 1988). The abnormal expression in RIN cells of the GLUT1 transporter isoform (Thorens *et al.*, 1988; Powers *et al.*, 1990; Brant *et al.*, 1992), may contribute to both the low rate of glucose transport in these cells and their abnormal glucose response (Thorens *et al.*, 1988). A relationship between a reduction in glucose transport and decreased insulin secretion, has also been demonstrated by Shibaski *et al.* (1990) in an experiment in which insulin secretion in RIN cells was increased by transfecting the cells with GLUT1 mRNA. This indicated that an increase in glucose flux, regardless of the transporter isoform through which it occurred could mediate insulin release from RIN cells.

It has been shown that RIN cells have defects in addition to abnormal glucose transporter expression. Glucose metabolism in this cell line is 8-10 fold higher than in normal  $\beta$ -cells, therefore the rate of glucose transport may be limiting to glucose phosphorylation (Halban *et al.*, 1983). It has also been shown that RIN cells do not

express glucokinase (Halban *et al.*, 1983; Shimizu *et al.*, 1988) although the hexokinase enzyme is 8 times more active than in normal islets (Halban *et al.*, 1983). How important the presence of glucokinase is to the normal glucose response is unclear, since although it has been shown that increasing the activity of RIN cell glucokinase does increase basal insulin secretion it does not make the cells responsive to glucose (Fernandez-Mejia & Davidson, 1990).

Owing to their failure to secrete insulin in response to glucose despite having a normal response to other insulinotropic agents, RIN cells have become important models in which to investigate the glucose sensor function of  $\beta$ -cells. The essential deficiency in RIN cells has yet to be identified.

### 1.3.2.2 The HIT $\beta$ -cell line

Another popular  $\beta$ -cell model, the HIT (hamster islet tumour) cell line was established by Simian virus 40 transformation of Syrian hamster pancreatic islet cells (Santerre *et al.*, 1981). The clone used in most studies is the HIT-T15 line. HIT cells are important models of the  $\beta$ -cell since they retain the ability to secrete insulin in response to glucose. Although stimulation of glucose-induced insulin secretion is 3.5 fold lower than in hamster islets, peak stimulation of HIT cells by glucose occurs at 7.5 mM which is similar to the response of normal hamster islets (Santerre *et al.*, 1981; Lambert *et al.*, 1986). HIT cells also be stimulated with leucine and arginine (Lambert *et al.*, 1986) although the general responsiveness decreases with increasing passage number (Zhang *et al.*, 1989).

HIT cells have been shown to contain glucokinase, but they differ from normal  $\beta$ -cells in their predominant usage of the low  $K_m$  hexokinase enzyme (Shimizu *et al.*, 1988). Since the glucose transport inhibitors cyt B and phloretin are able to block glucose-induced

insulin secretion but mannoheptulose, an inhibitor of glucose phosphorylation, is not, glucose metabolism in HIT cells is limited by the rate of entry of glucose into the cell (Ashcroft & Stubbs, 1987). Therefore in contrast to native  $\beta$ -cells, glucose transport is the rate determining step and the glucose transporter is the "glucose sensor" that confers concentration dependence and specificity on the HIT cell line.

Western and Northern blotting of HIT cells has confirmed that like RIN cells they abnormally express GLUT1 (Powers *et al.*, 1990; Brant *et al.*, 1992) and exhibit slow glucose transport in which equilibration between internal and external sugar is not attained (Meglasson *et al.*, 1986). Ashcroft & Stubbs (1987) have reported a  $K_m$  for D-glucose of 4.3 mM.

HIT cells have been used to investigate the regulation of  $\beta$ -cell glucose transporter expression, the advantage being that, using cells in culture, a single regulatory factor can be investigated in isolation. Unfortunately, results obtained with cultured cells have been contradictory. Using isolated rat  $\beta$ -cells cultured in high concentrations of glucose, Orci *et al.* (1990b) found a slight, but not significant, increase in the GLUT2 protein. Similarly, in HIT cells, although GLUT2 mRNA was found to increase 3 fold when glucose was increased from 2.8-22.2 mM, no change in the level of the GLUT2 protein was detected (Seino *et al.*, 1991). In contrast, in a study by Purrello *et al.* (1991) both GLUT1 and GLUT2 mRNA levels were shown to decrease when HIT cells were cultured in high glucose. This correlated with a decrease in glucose-induced insulin secretion and in the rate of 2-deoxyglucose transport.

HIT cells express the low  $K_m$  glucose transporter GLUT1, and have rates of glucose transport low enough to limit glucose metabolism, yet they can still secrete insulin in response to glucose. This implies that the ability of  $\beta$ -cells to respond to glucose is not simply dependent on a high rate of glucose transport or on exclusive expression of GLUT2 (Thorens *et al.*, 1988). Possibly of more importance, is the ratio of GLUT2 to

glucokinase. These two proteins may interact to form functional "glucose sensing units" which may be involved in the  $\beta$ -cell response to glucose (Newgard *et al.*, 1990; Unger, 1991).

### 1.3.2.3 The MIN $\beta$ -cell line

Two permanent cell lines have recently been developed which may help to solve the question of the importance of the GLUT2 transporter in glucose-induced insulin secretion. The MIN (mouse insulinoma) cell lines were established from insulinomas obtained by targeted expression of the Simian virus 40 T antigen gene in transgenic mice. MIN cells produce insulin and have the morphological characteristics of pancreatic  $\beta$ -cells (Miyazaki *et al.*, 1990). The MIN 6 cell line is reported to express a high level of GLUT2, but GLUT1 is said to be barely detectable. These cells exhibit glucose-induced insulin secretion comparable with that found in normal mouse islets. In contrast, the MIN 7 line, in addition to high levels of GLUT2, also expresses high levels of GLUT1 and does not respond to glucose.

MIN 6 cells are therefore a useful model of native  $\beta$ -cells being unusual in that they have reportedly been immortalized without the induction of GLUT1. The exclusive expression of high levels of GLUT2 may be the factor rendering these cells insulin responsive.

## 1.4 DIABETES MELLITUS

Diabetes mellitus is a chronic metabolic disorder affecting normal glucose homeostasis. It is characterised by hyperglycaemia, caused by a relative or absolute deficiency in insulin. Only recently was it realized that diabetes mellitus is not a single disease but a group of syndromes in which hyperglycaemia is the common feature. In

1979, the National Diabetes Data Group developed a nomenclature and classification system for diabetes mellitus which has now been universally accepted.

Type I diabetes is also known as insulin-dependent diabetes mellitus, abbreviated as IDDM. It was formerly called juvenile onset diabetes since it occurs mainly, but not exclusively in younger people, typically those less than 30 years old. The type I diabetic patient is absolutely dependent on an exogenous supply of insulin, required because the insulin secreting  $\beta$ -cells in the pancreas have been completely destroyed.

Type II diabetes is also known as non-insulin dependent diabetes mellitus (NIDDM). It was formerly called maturity or adult onset diabetes since it generally occurs in older people, although it is also known in the young. Approximately 70-80% of all diabetic patients have the non-insulin dependent form of diabetes and in many cases it is linked to obesity and insulin resistance in the peripheral tissues (Lefèbvre, 1988). In NIDDM patients,  $\beta$ -cells are still present, therefore these patients are not dependent on exogenous insulin. However, since the  $\beta$ -cell response to glucose is usually impaired (Robertson & Porte, 1973), insulin may be administered to relieve symptomatic hyperglycaemia. In many cases oral hypoglycaemic drugs, such as the sulphonylureas, can reduce hyperglycaemia by acting as alternative insulin secretagogues and by improving glucose uptake in peripheral tissues.

Important features of these two types of diabetes are illustrated in Table 2.

	Type I insulin dependent diabetes mellitus (IDDM)	Type II non-insulin dependent diabetes mellitus (NIDDM)
Age at onset	Usually < 30	Usually > 40
Appearance of symptoms	Usually rapid	Insidious
First functional abnormality	Reduced glucose induced insulin secretion	Reduced glucose induced insulin secretion
Ketoacidosis	Common	Uncommon
Obesity	Uncommon	Common
Concordance in monozygotic twins	≈ 50%	≈ 100%
HLA association	Present	Absent
Percentage of diabetic patients	≈ 20%	≈ 80%
Insulin resistance	Rare	Common
Initial pathology of the pancreas	Insulinitis	Usually none
Late pathology of the pancreas	Absence of $\beta$ -cells	$\beta$ -cell mass usually normal Amyloid deposition & fibrosis
Disease aetiology	Autoimmune destruction of $\beta$ -cells	Unknown
Anti-islet cell antibodies	Present at onset Reduction in $\beta$ -cell mass	Absent

Table 2 A comparison of the features of Type I and Type II diabetes mellitus.

#### 1.4.1 The pathogenesis of diabetes mellitus

In type I diabetes the absence of insulin means that glucose cannot be taken up by insulin-sensitive tissue. This causes an increase in the blood glucose concentration. When this exceeds the renal threshold of 10 mM, glycosuria occurs and with it, dehydration (Montague, 1983). This results in a decrease in the peripheral circulatory volume, tissue anoxia occurs and lactic acid is produced. If untreated, acidosis occurs and patients can become comatose and die. Since, in the absence of insulin, glucose cannot be utilized by adipose cells, fat depots are mobilized and taken up by the liver which produces acetoacetic acid and  $\beta$ -hydroxybutyric acid. This results in ketoacidosis and excretion of ketone bodies. Not long ago death from ketoacidosis at the onset of diabetes was very common, today it accounts for less than 1% of all diabetes related deaths (Terranova *et al.*, 1982). Symptoms at the onset of diabetes include extreme thirst, hunger, polyuria and weight loss.

Until 1921 when insulin was discovered, type I diabetes was rapidly fatal. Today diabetics can lead relatively normal lives, although insulin therapy cannot be considered a "cure" since the administration of exogenous insulin never exactly mimics normal insulin secretion. In an attempt to keep blood sugar within the normal range too much insulin can be administered, the resulting hypoglycaemic episode can lead to coma and death if untreated. Less intensive insulin therapy resulting in hyperglycaemia has been linked with the long term complications of diabetes. Current diabetic therapy therefore aims for a compromise between these two extremes.

Much of the mortality and morbidity associated with diabetes is caused by the chronic complications arising from long-term metabolic disturbances. After 3-15 years of diabetes 80-90% of all patients exhibit microangiopathy arising from a thickening of the capillary basement membrane (Williamson & Kilo, 1982). This change causes blood vessels to become blocked, oxygen exchange cannot occur and tissue hypoxia results. When microangiopathy occurs in the extremities of the limbs it can cause gangrene. In the kidney it can result in nephropathy and kidney failure, which is responsible for the death of a third of all patients with IDDM (Cullinan, 1982). When microangiopathy occurs in the eye it can cause retinopathy which is the commonest cause of blindness in people of working age in the U.K. (Connor & Boulton, 1989). Heart disease and neuropathy are also commonly associated with diabetes. The precise way in which diabetes causes such widespread organ damage is not known but the risks seem to be associated with sustained hyperglycaemia and increased duration of diabetes.

The complications of diabetes mellitus are therefore both numerous and serious. According to some estimates more than 60 million people today may be suffering from diabetes (Zimmet, 1991). This is a problem not only in terms of personal suffering but in the burden that it places on society in terms of expensive health care and lost working days. At present, diabetes affects about 5% of the population of the Western world (Lefebvre, 1988), but there is evidence that it is an increasing problem. It has even been



suggested that the rise in incidence of NIDDM in developing countries is best described as an epidemic (Zimmet, 1991).

### **1.4.2 The aetiology of NIDDM**

Type II diabetes is probably not a single disease but a collection of syndromes. Relatively little is known about the basic defects and even less about the underlying causes of the disease.

There is a strong hereditary component since concordance between monozygotic twins is almost 100% (Lefèbvre, 1988) and the risk for siblings of diabetic patients is 29%. In certain populations, for example the Pima Indians of Arizona, prevalence can reach 30-35%. NIDDM may be associated with poor diet, decreased physical activity, increased smoking, obesity and increased alcohol consumption.

Morphologically the pancreas of a type II diabetic appears almost normal. The  $\beta$ -cells however seem to have a specific functional deficiency, that is, a decreased ability to secrete insulin in response to stimulation with glucose. NIDDM is therefore characterised by abnormal insulin secretion from the pancreas, with the additional problem of insulin resistance in the peripheral tissues which in many cases is augmented by obesity. The precise biochemistry of the insulin resistance is unknown.

#### **1.4.2.1 Evidence for abnormal glucose transport in NIDDM**

Although in the type II diabetic pancreas D-glucose does not stimulate insulin secretion, alternative insulinotropic agents such as arginine and the sulphonylureas can still produce an insulin response. The abnormality must therefore be confined to the "glucose sensor" component of the  $\beta$ -cell insulin secretory pathway. A down-regulation of the high

$K_m$   $\beta$ -cell GLUT 2 could potentially cause this altered glucose sensitivity. This has been investigated in several animal models of NIDDM.

A reduction in  $\beta$ -cell GLUT2 has been detected in Zucker diabetic fatty (ZDF) rats (Johnson *et al.*, 1990b; Orci *et al.*, 1990b), in insulin-resistant diabetic Wistar Kyoto rats (Orci *et al.*, 1990b), in GK rats, which exhibit a selective inhibition of glucose-stimulated insulin secretion, (Orci *et al.*, 1990b) and in rats that developed NIDDM as a result of neonatal injection of streptozotocin (Thorens *et al.*, 1990e). In severely diabetic animals reductions in GLUT2 of 75% and 85% were found respectively in ZDF rats and in rats made diabetic with streptozotocin (Johnson *et al.*, 1990b; Thorens *et al.*, 1990e). In diabetic animals the transport of 3-O-MG was also reduced to less than 40% of that found in control animals (Johnson *et al.*, 1990b). Destruction of about 60% of the total  $\beta$ -cell population was shown to be sufficient to prevent the secretion of insulin in response to glucose, although it did not affect the arginine response (Johnson *et al.*, 1990b).

Orci *et al.* (1990b) investigated whether the reduction in GLUT2 could be caused by hyperglycaemia. The conclusion reached was that both in vitro and in vivo, exposure to high glucose could not induce a deficiency in  $\beta$ -cell GLUT2 nor could elimination of hyperglycaemia in prediabetic animals prevent the GLUT2 deficiency. Since it does not occur in response to diabetic hyperglycaemia, the decrease in the expression of GLUT2 may play a role in the development of NIDDM.

A scenario for the development of NIDDM could therefore be as follows. An unknown primary event, genetic or environmental, reduces  $\beta$ -cell GLUT2. This reduction causes a decrease in glucose transport and reduces glucose-induced insulin secretion which leads to hyperglycaemia. The condition is aggravated by the insulin resistance of the peripheral adipose and muscle tissues which are normally responsible for insulin-stimulated glucose disposal. This insulin resistance may be partially attributable to decreased expression of the GLUT4 glucose transporter in these tissues (Friedman *et al.*, 1991; Vogt

*et al.*, 1991). The level of blood glucose is not therefore reduced by uptake into these tissues, hyperglycaemia increases, the  $\beta$ -cell is unable to respond and NIDDM results (Unger, 1991).

### **1.4.3 Insulin dependent diabetes mellitus (IDDM)**

The disease IDDM arises from a specific necrosis of islet  $\beta$ -cells. Although the onset of the disease is generally sudden and occurs when the  $\beta$ -cell mass has decreased below 90%, the destruction process is insidious and can have started many years previously. During this pre-overt period patients often have an abnormal insulin response to an oral or intravenous glucose challenge.

The mediator of this destruction is the immune system, which attacks self antigens present on the islet  $\beta$ -cell. The antigenic target of the immune response has still to be unequivocally identified, as have the factors leading to the breakdown of normal self tolerance. The autoimmune process may be initiated by environmental factors or may be the result of a genetic defect.

#### **1.4.3.1 Environmental or genetic aetiology ?**

Although diabetes can be induced experimentally by chemicals such as alloxan and streptozotocin which are selectively toxic to  $\beta$ -cells, there is very little evidence that any specific chemical is associated with the normal aetiology of IDDM.

There is some evidence for an association between infection with certain viruses, including the mumps virus, rubella and Coxsackie B4, and the development of IDDM (Yoon, 1990). The evidence for viral involvement has stemmed largely from work on experimental animals. However, epidemiological studies have also shown that there is a seasonal variation in the clinical onset of IDDM with relatively more cases diagnosed

during the autumn and winter months, when viral infections are more common (Green, 1990). There is also a peak in incidence at the ages of 12-15 years and 5-6 years, the times when children start or change schools and thereby meet new viruses (Bottazzo, 1986). Viruses could act as primary injurious agents of  $\beta$ -cells or they could alter the structure or expression of normal  $\beta$ -cell proteins. The anti- $\beta$ -cell antibodies detected in diabetic patients could therefore be generated in response to antigens produced by the damaged or infected  $\beta$ -cells. Alternatively, a viral infection could indirectly trigger an autoimmune response against the  $\beta$ -cell if antigenic epitopes present on the virus were also contained in  $\beta$ -cell proteins. There is some evidence that an epitope on RIN  $\beta$ -cells, recognised by antibodies in diabetic serum, is shared with the rubella virus (Karounos *et al.* 1990).

Genetic factors also play a role in the development of IDDM, since first-degree relatives of diabetic patients have a risk of developing the disease that is about 10-20 fold higher than in the general population (Green, 1990). However, the fact that the concordance rate for monozygotic twins is only 50% means that an environmental factor is also likely to be involved. There is evidence that genes within the major histocompatibility complex (MHC) contribute to the development of IDDM, since there is an association with the HLA-D region which contains the DP, DQ, and DR genes that code for the class II molecules found on cells of the immune system (Bottazzo, 1986; Atkinson & Maclaren, 1990). The serologic types HLA-DR3 and -DR4 are positively associated with IDDM, since about 95% of Caucasian diabetics have DR3 or DR4 alleles compared with about 50% of the general population.

The inheritance of the DR3 or DR4 haplotype is a marker for about 100 genes that are in linkage disequilibrium with the DR3 and DR4 genes. Potentially, any one of these genes could confer susceptibility to IDDM. However, the observed association of other autoimmune diseases with the inheritance of certain HLA haplotypes means that the class II loci themselves must be considered the strongest candidates for the disease genes in autoimmune diabetes (Green, 1990). The genes encoding DR3 and DR4 probably do not

confer susceptibility themselves, but are linked to genes in the DQ region that do (Atkinson & Maclaren, 1990; Nepom, 1990).

It has been proposed that the presence of an aspartic acid residue at codon 57 in the  $\beta$ -chain of the protein encoded by HLA-DQ could help to prevent the development of IDDM (Todd *et al.*, 1987). This negatively charged residue seems to play a role in the formation of a salt bridge between the  $\alpha$  and  $\beta$  chains of the DQ molecule (Green, 1990). The substitution of valine, a non-charged amino acid, at codon 57 has been shown to cause susceptibility to diabetes. If this substitution affects the structure of the DQ molecule it may facilitate the autoimmune response that results in IDDM (Atkinson & Maclaren, 1990).

Since there are no unique class II sequences found only in diabetics, there are probably many genes both within and outside the MHC that contribute IDDM susceptibility.

#### **1.4.3.2 Autoimmune aetiology**

Although during development immunoreactive cells that recognise many different epitopes are generated randomly, the normal immune system is able to distinguish self from non-self with great precision, mounting immune responses only against foreign antigens. Self tolerance may be achieved by the elimination during early ontogeny of T-cell clones possessing receptors recognising self antigens. If potentially autoreactive clones leave the thymus they can be held in a state of long-term unresponsiveness known as clonal anergy. Alternatively, peripheral autoreactive T cells can be inactivated or inhibited by suppressor T ( $T_S$ ) cells.

There is evidence that the autoimmune response in IDDM occurs against a normal  $\beta$ -cell antigen, since autoantibodies from diabetic serum recognize antigens in normal

pancreas. This implies that the defect in IDDM that allows a response to occur against a self antigen is in the regulatory component of the immune system. A failure to correctly regulate other autoreactive clones could also explain the increased incidence of other autoimmune disorders, such as idiopathic Addison's disease and autoimmune thyroid disease, in diabetic patients.

Although an impaired regulatory response may predispose to IDDM susceptibility, an initiating factor is still required to trigger the immune attack against the  $\beta$ -cells. It has been shown that in pancreatic tissue taken from diabetics who died shortly after diagnosis of IDDM, some of the remaining  $\beta$ -cells within the islets abnormally express MHC class II molecules (Bottazzo *et al.*, 1985) and seem to have a hyper-expression of MHC class I molecules. Bottazzo *et al.* (1985) proposed that the abnormal expression of class II antigens could initiate an autoimmune response by cytotoxic T cells ( $T_C$ ) by enabling helper T ( $T_H$ ) cells to be directly activated by the islet  $\beta$ -cells, without the need for the processing of  $\beta$ -cell antigens by antigen presenting cells. Class II molecules have been shown to be induced by interferon (Bottazzo *et al.*, 1983; Bottazzo, 1986). When  $\beta$ -cells were made to over-express this cytokine, pancreatic inflammation and insulinitis occurred and  $\beta$ -cells increased their expression of MHC molecules (Sarvetnick, 1990). It is not however known whether or not the production of class II molecules on  $\beta$ -cells precedes insulinitis and therefore whether it is a consequence or cause of the autoimmune response against the  $\beta$ -cells.

Although the factor initiating the autoimmune attack on  $\beta$ -cells is unknown, the agents of destruction appear to be both humoral and cell mediated.

#### 1.4.3.2.1 Cell-mediated autoimmunity

In the Biobreeding (BB) rat and the non-obese diabetic (NOD) mouse, both models of autoimmune diabetes, as well as in 78% of human IDDM patients, insulinitis can be

detected in pancreatic islets at the onset of diabetes (Foulis & Bottazzo, 1988; Lo, 1990). The infiltrating cells are mononuclear lymphocytes the majority being activated T-cells of the CD8<sup>+</sup> type (T<sub>C</sub>). Small numbers of CD4<sup>+</sup> (T<sub>H</sub>) are also detected (Bottazzo *et al.*, 1985). There is evidence from animal studies that autoimmune diabetes is essentially T-cell mediated since neonatal thymectomy can stop development of the disease as can treatment with cyclosporin A, which affects the function of T cells. Diabetes can also be transferred from diabetic animals by adoptive transfer of CD4<sup>+</sup> and CD8<sup>+</sup> cells (Lo, 1990). Antibodies directed against these cell types can prevent disease onset (Cooke, 1990). The other major cell types that can be detected in the islet infiltrate are natural killer (NK) cells, macrophages and B lymphocytes. In animals, depletion of NK cells and inhibition of macrophages can prevent the development of diabetes (Cooke, 1990; Mandrup Poulsen *et al.*, 1990).

Although the specific destruction of  $\beta$ -cells is generally deemed to be effected by a specific class II-restricted immune response, it is also possible that non-specific immunological events are involved. The selective killing of the  $\beta$ -cells is possible because of the unusual susceptibility of this cell type. For example, the effect of cytokines on  $\beta$ -cells is dramatic. Active  $\beta$ -cells can readily be killed by interleukin 1 which causes cell lysis.  $\beta$ -cells are also susceptible to free oxygen radicals, produced by the respiratory burst of phagocytic cells, because of their low levels of superoxide dismutase and other scavengers (Mandrup Poulsen *et al.*, 1990).

The following scenario summarises some of the putative mechanisms involved in  $\beta$ -cell destruction. In individuals with a genetic predisposition to IDDM, a triggering factor causes inflammation in the islets. This factor may be a virus which cross-reacts with a  $\beta$ -cell autoantigen. Macrophages could present the foreign antigen in association with MHC class II molecules, thereby activating specific T<sub>H</sub> cells. These in turn could activate T<sub>C</sub> cells which would kill the islet  $\beta$ -cells expressing the cross reacting autoantigen. Alternatively, the  $\beta$ -cells themselves could be induced to express class II molecules and

could then initiate the autoimmune response by self-presentation of autoantigens. It is also possible that autoantigens released from injured  $\beta$ -cells could be presented by macrophages to generate specific T cell help. Once specific  $T_H$  cells are activated, B lymphocytes may also be activated. The presence of cytokines and antibodies would attract more macrophages and natural killer cells.  $\beta$ -cells could be killed specifically by  $T_C$  cells and antibody- or complement-mediated cytotoxicity, as well as by direct cytokine sensitivity.  $\beta$ -cell lysis would release autoantigens which would generate more autoantibodies thereby enhancing the ongoing  $\beta$ -cell destruction (Atkinson & Maclaren, 1990).

#### **1.4.3.2.2 Antibody-mediated autoimmunity**

At the onset of IDDM about 91% of patient have circulating antibodies reactive with components of islet cells (Lernmark *et al.*, 1981). Although these antibodies can be present in patient's serum many years before the onset of the clinical symptoms of diabetes, their relevance to the pathogenesis of IDDM remains a matter of debate, since experimental transfer of diabetic antibodies has never been shown to cause the disease. Similarly there are no transplacental effects on neonates born to diabetic mothers.

Islet cell specific antibodies found in the serum of patients with IDDM have traditionally been divided into two groups according to the method by which they are detected:

- i) ICCA - Islet cell cytoplasmic antibodies
- ii) ICSA - Islet cell surface antibodies

#### **i) Islet cell cytoplasmic antibodies (ICCA)**

Studies have shown that between 50-90% of patients with IDDM have ICCA (Sigurdsson & Baekkeskov, 1990). Initially, the lack of standardized methodology for the



ICCA assay made inter-laboratory comparisons of ICCA titres difficult. Recently, international workshops have established standardized protocols, which by use of standard sera enable titres to be reported in standard JDF (Juvenile Diabetes Foundation) units.

The conventional ICCA assay uses immunofluorescence to detect antibodies binding to cytoplasmic islet cell antigens in cryostat sections of human blood group O pancreas (Lernmark *et al.*, 1981; Marner *et al.*, 1984). The ICCA which are of the IgG class (Millward *et al.*, 1988) do not only recognise species-specific antigens, since they have also been shown to detect antigens present in rodent pancreas (Dib *et al.*, 1987). ICCA react with all of the four islet cell types and not specifically with  $\beta$ -cells (Doniach *et al.*, 1985). The target antigen of ICCA has neither been isolated nor fully identified, although it has been suggested that it may be a glycolipid with the characteristics of a sialoglycoconjugate (Nayak *et al.*, 1985; Marshall *et al.*, 1991). Living cells are not permeable to immunoglobulins and since ICCA recognise cytoplasmic antigens it seems likely that they were generated following the death of the  $\beta$ -cell and therefore that they do not play a major role in the aetiology of IDDM.

However, prospective studies have shown that ICCA can appear up to 8 years before diabetic symptoms (Srikanta *et al.*, 1983). About 80% of patients have ICCA at diagnosis, this figure drops to about 62% after the first year, 37% after 2-4 years and after 4 years only 11% of patients are still ICCA positive (Lernmark *et al.*, 1981; Bruining *et al.*, 1984). This decrease probably reflects the diminishing prevalence of  $\beta$ -cells and therefore a decrease in antigenic stimulation. Other studies have found ICCA to be more persistent with 62% of patients still having antibodies 3 years after diagnosis (Kolb *et al.*, 1988).

Although the prevalence of ICCA in the general population is low ( $\approx 0.4\%$ ), approximately 25% of first degree relatives of IDDM patients have persistent ICCA in their serum. This high prevalence questions the validity of using these antibodies as

predictors of IDDM, since only about a quarter of these ICCA-positive relatives will actually become diabetic (Lernmark, 1980). Although approximately 99% of relatives developing IDDM have ICCA up to 8 years previously, this still leaves 30-60% of relatives positive for ICCA who do not develop IDDM in an 8 year follow up period (Johnston *et al.*, 1989). Some studies have suggested that high titres of ICCA (> 80 JDF units) are predictive for IDDM development, whereas low titres (< 4 JDF units) are less so (Bonifacio *et al.*, 1989; Johnson *et al.*, 1989; Karjalainen, 1990). Other studies have however found no correlation with the ICCA titre and  $\beta$ -cell damage (Neifing *et al.*, 1990). In conclusion, the role of ICCA as markers of  $\beta$ -cell damage and IDDM pathogenesis remains controversial.

## ii) Islet cell surface antibodies (ICSA)

Islet cell surface antibodies are, by definition, antibodies which react with surface antigens on viable islet cells. They are non-species specific and traditionally have been detected in an indirect immunofluorescence assay using dispersed viable rat or mouse islets as the antigen (Lernmark *et al.*, 1978; Lernmark *et al.*, 1981; Marner *et al.*, 1984). Human pancreas has also been used to detect ICSA both in an indirect immunofluorescence assay using cultured foetal islets (Pujol-Borrell *et al.*, 1982) and in an assay using single human islet cells in which immunofluorescence is detected by flow cytometry (Peakman *et al.*, 1990; Vergani *et al.*, 1991). Quantification of ICSA titres has been improved by using  $^{125}\text{I}$  protein A to detect the bound antibodies (Lernmark *et al.*, 1980). ICSA have also been detected using rat plasma membrane in an ELISA which is reported to detect higher binding by diabetic compared with control sera (Peakman *et al.*, 1989). Cultured  $\beta$ -cells can be used to detect ICSA, for example, RIN cells (Lander *et al.*, 1989), human insulinoma cells (Maclaren *et al.*, 1975) and HIT cells (Matsuba *et al.*, 1987) have been used in indirect immunofluorescence and  $^{125}\text{I}$  protein-A binding assays to demonstrate the presence of higher levels of ICSA in diabetic compared with normal serum. RIN cells have also been successfully used to detect ICSA in a cell surface ELISA (Thomas *et al.*, 1987)

and in an assay which has shown that 94% of diabetic sera contain antibodies that can displace a specific monoclonal antibody from the surface of RIN cells (Keilacker *et al.*, 1988; Thomas *et al.*, 1990; McEvoy *et al.*, 1991).

As there is no standardizable assay for ICSA, reports of the prevalence of these antibodies have been variable. Between 30-80% of patients with new onset IDDM have ICSA (Lernmark *et al.*, 1978; Lernmark *et al.*, 1981; Pujoll-Borrell *et al.*, 1982). They are found independently of ICCA, with 49% of patients being discordant for the two types of antibody (Freedman *et al.*, 1980; Lernmark *et al.*, 1981). Using an indirect immunofluorescence assay on rat islet cells Lernmark *et al.* (1981) reported that 53% of seventeen young diabetic patients had ICSA at the onset of IDDM. This decreased to 30% after 6 months and to 20% after 18 months indicating the evanescent nature of these antibodies. Between 2-4% of the general population have ICSA (Lernmark *et al.*, 1978; Lernmark *et al.*, 1981). ICSA have been detected in 44% of children with glucose intolerance, which is often an indication that they will progress to overt diabetes, although the significance of ICSA to the pathogenesis of IDDM has not yet been established since ICSA titre at diagnosis does not seem to correlate with residual  $\beta$ -cell function.(Todd & Bottazzo, 1985).

In vitro, ICSA have been shown to kill  $\beta$ -cells by complement-dependent and cell-mediated cytotoxicity (Dobersen *et al.*, 1980; Maruyama *et al.*, 1984). As discussed in section 1.4.3.2.3 (iii) these antibodies may also affect  $\beta$ -cell function. Therefore, there is some evidence that ICSA are involved in the pathogenesis of human IDDM. Further experimental evidence is provided by the detection of ICSA in three animal models of diabetes. ICSA have been found in the BB rat (Dyrberg *et al.*, 1982), in mice in which an autoimmune form of diabetes was induced by multiple low doses of streptozotocin (Itoh *et al.*, 1984), and in the NOD mouse (Karounos & Thomas, 1990).

The antigens recognised by ICSA have not been identified, but since they are expressed on the surface of viable  $\beta$ -cells they are potentially accessible to attack by autoantibodies and are therefore of putative pathological significance in the aetiology of IDDM.

#### **1.4.3.2.3 Identification of putative autoantigens**

Only two target antigens of islet cell antibodies have been positively identified. The first is insulin, the second, a 64 kDa protein recently identified as glutamic acid decarboxylase (GAD) (Baekkeskov *et al.*, 1990). It has also been proposed that the  $\beta$ -cell glucose transporter protein GLUT2 may be an autoantigen although this has yet to be proven (Johnson *et al.*, 1990a). A number of other antigens have been identified by molecular mass only.

##### **i) Insulin as an autoantigen.**

Insulin autoantibodies (IAA) can be detected in some newly diagnosed diabetic patients prior to their treatment with exogenous insulin. IAA are especially prevalent in young children, but they do not seem to be important in the pathogenesis of IDDM, since their presence before diagnosis does not affect the clinical course of the disease (Karjalainen *et al.*, 1988).

##### **ii) The 64 kDa antigen.**

The best characterised islet cell autoantigen is a 64 kDa protein first described in 1982 by Baekkeskov and co-workers. It has been identified as the enzyme glutamic acid decarboxylase (GAD) (Baekkeskov *et al.*, 1990). This enzyme converts glutamic acid to the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). It is found in GABA secreting neurones of the central nervous system and in pancreatic  $\beta$ -cells where it has been

speculated that its role is as an endocrine transmitter involved in glucose-induced insulin secretion (Baekkeskov *et al.*, 1990). This is supported by the failure to detect GAD in insulinoma cell lines which have an abnormal response to glucose (Baekkeskov *et al.*, 1988).

The initial interest in this 64 kDa antigen was aroused by the discovery that between 70-80% of serum samples from patients with recently diagnosed IDDM, but not from control individuals, had IgG antibodies that immunoprecipitated a 64 kDa protein from crude detergent lysates of <sup>35</sup>S methionine labelled human islets (Baekkeskov *et al.*, 1982). The assay was later modified to enable rat islets to be used, by the inclusion of an additional detergent extraction step (Baekkeskov *et al.*, 1987; Christie *et al.*, 1988). Unfortunately, this method of detecting anti-64 kDa antibodies, although highly specific, is too expensive and of too low a capacity to be suitable for routine screening of serum samples.

The subcellular location of  $\beta$ -cell GAD has not been finally resolved although it has been shown to exist in a 65 kDa hydrophilic form and a 64 kDa hydrophobic form which can be both membrane bound and soluble (Baekkeskov *et al.*, 1990; Christgau *et al.*, 1991). Most evidence, including the inability to label the protein with the cell surface label <sup>125</sup>Iodide (Colman *et al.*, 1987), suggests that GAD is not expressed at the  $\beta$ -cell surface but is probably present in cytoplasmic, synaptic-like vesicles (Baekkeskov *et al.*, 1990; Reetz *et al.*, 1991). This does not preclude the involvement of anti-64 kDa antibodies in the pathogenesis of IDDM, since peptides derived from the GAD protein could be expressed on the  $\beta$ -cell surface where they could initiate an autoimmune response against the healthy  $\beta$ -cells.

The prevalence of anti-64 kDa antibodies amongst healthy control individuals is about 3% (Sigurdsson & Baekkeskov, 1990). At the onset of IDDM approximately 70% of diabetics have autoantibodies against both the 64 and 65 kDa GAD proteins. Antibodies

against the 64 kDa protein are also present in the pre-diabetic period as would be expected if they are of aetiological importance. In one familial study they have been detected in 79% of patients up to 8 years before the onset of IDDM (Baekkeskov *et al.*, 1987). The antibodies seem to be of higher predictive value than either IAA or ICCA which cannot always be detected when anti-64 kDa antibodies are present (Atkinson *et al.*, 1990). Anti-64 kDa antibodies have also been detected in animal models of autoimmune diabetes. In the BB rat a 64 kDa islet protein was immunoprecipitated by ICSA-positive serum from prediabetic animals (Baekkeskov *et al.*, 1984) and approximately 87% of newly diabetic NOD mice have also been found to have anti-64 kDa antibodies (Atkinson & Maclaren, 1988).

Most evidence suggests that anti-64 kDa antibodies are predictive for IDDM. The recent cloning of the protein should therefore facilitate the development of a screening test for pre-overt IDDM. If GAD turns out to be a major target of the autoimmune attack in IDDM it could also become a major tool for prevention of the disease.

### **iii) $\beta$ -cell GLUT2 as a putative autoantigen in IDDM.**

One of the earliest clinical symptoms of both type I and II diabetes, which can antedate the overt disease by 5-8 years, is a selective loss of the first phase insulin release in response to intravenous glucose challenge. In this pre-diabetic period before the occurrence of overt hyperglycaemia, the response to other secretagogues remains normal (Rabinowe & Eisenbarth, 1984). This suggests that although viable  $\beta$ -cells are present at this stage, they exhibit a functional defect in the glucose-specific component of the  $\beta$ -cell insulin secretion pathway.

Circulating  $\beta$ -cell autoantibodies can be detected prior to the onset of the glucose intolerance that will lead to overt type I diabetes. These antibodies have been demonstrated to affect  $\beta$ -cell function both *in vivo* and *in vitro*. ICSA-positive serum from

diabetic children has been shown to inhibit glucose-induced insulin secretion from perfused rat islets (Kanatsuna *et al.*, 1982; Kanatsuna *et al.*, 1983; Alden *et al.*, 1984; Kitagawa *et al.*, 1990). This inhibition occurred in the absence of complement and without  $\beta$ -cell lysis and was specific for glucose-induced insulin secretion with no effect on arginine- or tolbutamide-stimulated insulin release (Kitagawa *et al.*, 1990). ICSA positive sera were found to produce a similar effect *in vivo*. When serum from diabetic children was passively transferred into animals, in most cases it did not cause outright diabetes, it did however lead to a reduced insulin secretory response which was detected by experimental perfusion of the pancreases (Svenningsen *et al.*, 1983).

The complexity of the pathway that enables the  $\beta$ -cell to respond to glucose, means that antibodies directed against any of the components involved in this sequence of events could potentially have an inhibitory effect on insulin secretion. For example, a monoclonal antibody which bound to a glycolipid on the  $\beta$ -cell surface was able to block glucose-induced insulin secretion, possibly by covering the surface of the carbohydrate rich  $\beta$ -cell and blocking external signals (Ziegler *et al.*, 1988). However, one of the strongest candidates for the autoantigen is the  $\beta$ -cell glucose transporter GLUT2, the presence of which is believed to be essential for normal glucose-induced insulin secretion.

As previously discussed, down-regulation of GLUT2 has been proposed to play a role in the pathogenesis of NIDDM (Unger, 1991). There is some experimental evidence from both IDDM patients and animal models which supports the theory that the  $\beta$ -cell GLUT2 protein is also involved in the pathogenesis of autoimmune diabetes. In the first fortnight following onset of diabetes in BB/W rats the pancreas can still secrete insulin in response to arginine, but the glucose response is absent. It has been possible to show that this correlates temporally with a >90% reduction in the rate of  $\beta$ -cell 3-O-MG transport. The rate is significantly lower than in control animals even when corrected to account for the reduction in mass of the islet endocrine tissue (Tominaga *et al.*, 1986). More recent studies on the BB rat have shown that the reduction in glucose transport, that occurs in

diabetic animals, can be correlated with a halving in the quantity of  $\beta$ -cell GLUT2 (Orci *et al.*, 1990a). The observed  $\beta$ -cell insensitivity to glucose and reduction in the rate of glucose uptake probably arise as the result of a number of factors including an 80% reduction in the number of  $\beta$ -cells, a 50% decrease in GLUT2 expression and the action of putative inhibitory autoantibodies (Orci *et al.*, 1990a). The reduction in GLUT2 alone may be sufficient to account for the loss of glucose-induced insulin secretion even in the absence of inhibition of the high  $K_m$  glucose transport by immunoglobulins (Unger, 1991). The factors causing a reduction in GLUT2 remain unknown, but the reduction does not appear to be a consequence of hyperglycaemia (Orci *et al.*, 1990b). It could result from a specific autoimmune attack against GLUT2 itself or from a non-specific autoimmune injury of  $\beta$ -cells that affects their ability to express GLUT2.

It has yet to be established whether the reduction in  $\beta$ -cell GLUT2 observed in BB rats also occurs in human IDDM, but results consistent with the hypothesis that GLUT2 is a target of the autoimmune process in patients with IDDM have been obtained by Johnson *et al.* (1990a). It was demonstrated that 95% of IgG samples from patients with new onset IDDM, but not from NIDDM patients or control subjects, were able to halve the rate of 3-O-MG transport via the high  $K_m$  glucose transporter in rat islets. Transport in the liver was unaffected. The inhibition was completely abolished by incubation of the diabetic IgG with cells or cell membranes containing GLUT2, for example, hepatocytes or  $\beta$ -cells. Incubation with cells or membranes that did not express GLUT2, such as erythrocytes or kidney brush border cells, did not remove the inhibitory activity. These findings strongly suggest the presence of an antibody reaction against either GLUT2 or an as yet unidentified protein that influences  $\beta$ -cell function (Johnson *et al.*, 1990a).

The relevance of the putative GLUT2 antibodies to the pathogenesis of IDDM is not known. In particular it has not been established whether a 50% reduction in glucose transport would actually affect insulin secretion *in vivo* since glucose transport capacity normally exceeds by 4 fold the rate of glucose phosphorylation (Johnson *et al.*, 1990c). It



is therefore unknown whether anti-GLUT2 antibodies are an epiphenomenon or whether they initiate a chain of events that results in the loss of glucose-induced insulin secretion in vivo.

#### **iv) Other $\beta$ -cell autoantigens.**

Numerous  $\beta$ -cell autoantigens have been described but most have not been fully characterised. Many are probably produced as a response to antigens exposed following  $\beta$ -cell injury and therefore are unlikely to be relevant to the early pathogenesis of IDDM.

Monoclonal antibodies have been produced that react with both  $\beta$ -cell surface and cytoplasmic components, although it is not known whether or not these antigens are important in IDDM pathogenesis (Eisenbarth *et al.*, 1981; Srikanta *et al.*, 1986). Potentially of more relevance to the aetiology of autoimmune diabetes are the monoclonal antibodies that have been produced from lymphocytes of diabetic animals. Some monoclonal antibodies have been generated which recognise a 64 kDa  $\beta$ -cell antigen and are able to inhibit insulin release from islets ( Hari *et al.*, 1986; Zlobina *et al.*, 1989). Human monoclonal antibodies have also been produced from patients with new onset IDDM. The first report was of an antibody that reacted with a cytoplasmic antigen (Eisenbarth *et al.*, 1982). A human monoclonal antibody has also been produced that reacts with a  $\beta$ -cell surface monosialoganglioside (Spitalnik *et al.*, 1988). Western blotting has also enabled the identification of antigens in islets and  $\beta$ -cell lines that are specifically recognised by antibodies present in diabetic serum. The antigens are a heterogeneous group of proteins and glycoproteins for which a range of molecular weights have been reported (Karounos *et al.*, 1988; Buchs *et al.*, 1990).

### 1.4.3.3 Screening for IDDM

The interest in diabetes-specific autoantibodies, that appear in individuals many years before the symptoms of diabetes, has largely stemmed from their potential to be used as markers for ongoing autoimmune destruction of pancreatic  $\beta$ -cells enabling early identification of individuals with pre-clinical diabetes. At present, since the incidence of IDDM in the general population is low, screening of the whole population is not considered to be cost effective. The advantages of knowing that you are at risk of developing diabetes are currently minimal, because even early treatment cannot cure the disease nor produce any long-term improvements in metabolic control.

The most obvious reason for wanting to predict diabetes is the hope that if detected in the early stages, before complete  $\beta$ -cell destruction occurs, it may be possible to actually slow or halt the immune response against the  $\beta$ -cells. If treatments, which may have serious side effects are to be initiated before evidence of clinical symptoms, then it follows that the screening assay must be sufficiently sensitive and accurate so that individuals are neither inadvertently omitted from treatment nor treated unnecessarily.

The ideal screening assay would be cheap, the reagents would have a long shelf life, it would be standardizable, quantitative and suitable for screening large numbers of samples. None of the currently available immunofluorescence or radioactive assays fulfil these criteria. It is for this reason that much research has been dedicated to the identification and purification of  $\beta$ -cell autoantigens. These could then be used to develop the ideal assay for IDDM.

Identification of autoantigens may be useful in the short term for developing a screen for IDDM. In the long term the knowledge of  $\beta$ -cell autoantigens may be applied to the development of a specific therapy for IDDM. Diabetes is an autoimmune disease and therefore if detected early enough, before total  $\beta$ -cell destruction has occurred, it should

theoretically be possible to halt the disease process by the use of treatments that suppress the immune system. The results of this type of immunosuppressive therapy have however generally been disappointing (Lernmark, 1985). Even considering the serious nature of the complications that are likely to arise after several years of conventional insulin therapy this type of general immunosuppression is unjustifiable as a treatment for IDDM. What is required is a specific therapy that could block just the single autoimmune reaction responsible for initiating the disease and which is safe enough to be given to symptomless individuals before the onset of IDDM. Therapies that can be envisaged require the identification of the  $\beta$ -cell autoantigen or autoreactive lymphocyte clones responsible for initiating  $\beta$ -cell destruction. For example if the autoantigen could be identified, labelled with a toxin or radioactive isotope and administered to a patient, its interaction with autoreactive T or B lymphocytes could cause their death thereby preventing further  $\beta$ -cell destruction.

Identification and cloning of autoreactive T or B lymphocytes could enable anti-idiotypic antibodies to be raised against them. When administered to patients, these antibodies should specifically destroy the autoreactive lymphocyte clones. Antibodies against specific  $T_H$  cells may also prevent triggering of a specific anti- $\beta$ -cell immune response (Lernmark, 1985). One method of identifying anti- $\beta$ -cell lymphocytes is to isolate them using the antigen with which they interact (Bergman *et al.*, 1990; Sobel *et al.*, 1990; Elias *et al.*, 1991). It may then be possible to immunize against diabetes by using peptides derived from autoreactive T or B cell receptors to generate antibodies that react against the autoreactive lymphocyte clones. It has been demonstrated that a peptide recognised by T lymphocytes can be used as a vaccine to prevent development of diabetes in mice. Alternatively the autoreactive T-cells can be attenuated and themselves used as a therapeutic T-cell vaccine (Elias *et al.*, 1991). It is not yet known whether the same approach would work for human IDDM.

Many researchers now believe that IDDM will become a preventable disease. Exactly how this will be achieved is currently only a matter for speculation. What is certain is that until a diagnostic test which predicts IDDM with 100% certainty has been developed and autoantigens responsible for initiating the disease have been identified, then the research into  $\beta$ -cell autoantigens must continue.

## 1.5 AIMS

The major aims of the work described in this thesis are as listed below:-

- 1) To determine whether the exofacial photoaffinity probe ATB-BMPA, which has previously been used to label the glucose transporter isoforms GLUT1 and GLUT4 (Clark & Holman, 1990; Holman *et al.*, 1990) could be used to photolabel the GLUT2 transporter isoform in liver plasma membranes and thus provide an alternative to the endofacial label cyt B. To use ATB-BMPA to compare GLUT2 with the other transporter isoforms.
- 2) To identify the glucose transporter isoforms that are expressed in cultured  $\beta$ -cell lines by using Western blotting and ATB-BMPA photolabelling followed by immunoprecipitation with specific anti-GLUT1 and GLUT2 peptide antibodies.
- 3) To develop a simple  $\beta$ -cell line plasma membrane ELISA which could be used to screen for the presence of anti-islet cell surface antibodies (ICSA) in serum from children with recently diagnosed IDDM.
- 4) To investigate the targets of the autoantibodies present in the serum of patients with IDDM and to attempt to establish the presence of diabetes-specific autoantibodies directed against the GLUT2 glucose transporter isoform which is expressed in islet  $\beta$ -cells.

## 2.0 MATERIALS AND METHODS

### 2.1 GENERAL REAGENTS

Unless otherwise stated all standard laboratory reagents were obtained from Fisons plc (Loughborough, UK), BDH Laboratory Supplies (Poole, Dorset, UK), Sigma Chemical Co. (Poole, Dorset, UK) or Aldrich Chemical Co. Ltd (Gillingham, Dorset, UK).

### 2.2 BUFFERS AND SOLUTIONS

#### **Gey and Gey medium**      pH 7.4

120 mM NaCl  
27 mM NaHCO<sub>3</sub>  
1 mM Na<sub>2</sub>HPO<sub>4</sub>  
5 mM KCl  
0.2 mM KH<sub>2</sub>PO<sub>4</sub>  
1 mM MgCl<sub>2</sub>·6H<sub>2</sub>O  
0.3 mM MgSO<sub>4</sub>·7H<sub>2</sub>O  
4 mM D-glucose  
2 mM CaCl<sub>2</sub>·2H<sub>2</sub>O

Prepare just before use and aerate with 5% CO<sub>2</sub> 95% O<sub>2</sub>

#### **Hanks balanced salts solution (HBSS)**      pH 7.4

5.4 mM KCl  
0.44 mM KH<sub>2</sub>PO<sub>4</sub>  
0.813 mM MgSO<sub>4</sub>·7H<sub>2</sub>O  
138 mM NaCl  
4.17 mM NaHCO<sub>3</sub>  
0.35 mM Na<sub>2</sub>HPO<sub>4</sub>  
5.55 mM D-glucose  
1.27 mM CaCl<sub>2</sub>·2H<sub>2</sub>O - as required

#### **Hepes-buffered balanced salts solution**      pH 7.4

132 mM NaCl  
4.76 mM KCl  
1.24 mM MgSO<sub>4</sub>·7H<sub>2</sub>O  
1.95 mM CaCl<sub>2</sub>·2H<sub>2</sub>O  
2.19 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O  
10 mM Hepes

**Krebs Ringer Bicarbonate (KRB) pH 7.4**

119 mM NaCl  
5.94 mM KCl  
2.54 mM CaCl<sub>2</sub>  
1.19 mM MgSO<sub>4</sub>  
1.19 mM KH<sub>2</sub>PO<sub>4</sub>  
25 mM NaHCO<sub>3</sub>  
10 mM Hepes  
0.1-0.2% bovine serum albumin (BSA)

**Phosphate buffered saline (PBS) pH 7.3**

140 mM NaCl  
2.7 mM KCl  
1.5 mM KH<sub>2</sub>HPO<sub>4</sub>  
8.1 mM Na<sub>2</sub>HPO<sub>4</sub>

**Sodium phosphate buffer pH 7.4**

5 mM Na<sub>2</sub>HPO<sub>4</sub>

**TRIS-buffered saline (TBS) pH 7.4**

154 mM NaCl  
10 mM TRIS

**TRIS/EDTA/sucrose buffer pH 7.4**

10 mM TRIS  
1 mM EDTA  
250 mM sucrose

**Transfer buffers****For wet transfer pH 8.3**

25 mM TRIS  
192 mM glycine  
20% (v/v) methanol

**For semi-dry transfer**

39 mM glycine  
48 mM TRIS  
0.0375% (w/v) SDS  
20% (v/v) methanol  
In double distilled water

**Electrophoresis stock reagents****30% (w/v) Acrylamide Stock**

60 g acrylamide  
1.6 g N'N methylene bis acrylamide  
60 g sucrose  
Adjust volume to 200 ml with double distilled water

**Resolving gel buffer pH 8.8**

1.5 M TRIS  
0.4% (w/v) SDS

**Stacking gel buffer** pH 6.8  
0.5 M TRIS  
0.4% (w/v) SDS

**Electrophoresis buffer** pH 8.3  
25 mM TRIS  
192 mM glycine  
0.1% (w/v) SDS

### **2.3 HUMAN SERUM SAMPLES**

Serum samples designated "Set 1" were provided by Dr. Pennock (Pediatric Dept., Bristol Royal Infirmary). Diabetic serum samples were obtained from children admitted to the hospital with recently diagnosed IDDM, and control samples were obtained from non-diabetic children who had been admitted to the hospital for other reasons. Some of these samples were very cloudy and had to be centrifuged before use to remove a white precipitate.

Additional samples, designated "Set 2" were obtained from 21 children recently diagnosed as having IDDM. These samples were shown to contain ICCA by a standard immunofluorescence assay. Control samples were obtained from the non-diabetic siblings of diabetic children, these samples did not contain ICCA. Set 2 samples were kindly provided by Dr. P. Bingley (Dept. of Diabetes and Metabolism, St. Bartholomews Hospital Centre for Clinical Research, London). Preliminary studies were carried out using samples provided by Dr. J. Reckless (Royal United Hospital, Bath) from adults attending the outpatient's diabetic clinic. Serum samples from Pima Indians with NIDDM were obtained from Dr. C. Bogardus (Clinical Diabetes and Nutrition Section, National Institutes of Health, Phoenix, Arizona, USA).

A large volume of serum which was used as an internal standard in ELISAS was obtained from a healthy blood donor. All serum samples were received frozen. On receipt

they were defrosted and aliquoted into small working volumes. All samples were then stored at -20°C.

## 2.4 PROTEIN ESTIMATIONS

The concentration of membrane or total cell protein was estimated using either the Bio-Rad assay based on the Coomassie Brilliant Blue dye-binding method of Bradford (1976) or with a modification of the Lowry protein assay (Lowry *et al.*, 1951; Markwell *et al.*, 1981).

### 2.4.1 Modified Bio-Rad assay

The protein content of samples was estimated by comparison with a standard curve ranging from 0-15 µg of protein (BSA) in 775 µl of sodium phosphate buffer (pH 7.4). The addition of 25 µl of 0.1 M NaOH to all standards and test samples aided the solubilization of membrane proteins. 200 µl of Bio-Rad dye-binding reagent (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), were added and the samples were mixed. After 5 min the absorbance was measured at 595 nm. All samples and standards were measured in duplicate.

### 2.4.2 SDS-modified Lowry

The protein content of samples was estimated from a standard curve ranging from 0-100 µg of BSA adjusted to a volume of 200 µl with water. Protein was solubilized by a 10 min incubation at room temperature with 1 ml of a 100 fold dilution of solution B (4% (w/v) CuSO<sub>4</sub>·5H<sub>2</sub>O) in solution A (2% (w/v) Na<sub>2</sub>CO<sub>3</sub> in 0.4% (w/v) NaOH, 0.16% (w/v) KNa tartrate, 3% (w/v) SDS). Colour was developed over a 30 min period following the addition of 100 µl of a 1:1 dilution of Folin and Ciocalteu's phenol reagent (BDH) in



water. Absorbance was measured at 750 nm. All samples and standards were tested in duplicate.

## 2.5 CELL CULTURE

Four immortal pancreatic  $\beta$ -cell lines were cultured. The HIT-T15 cell line (Santerre *et al.*, 1981) was a gift from Dr. R.F. Santerre (Lily Research Laboratories, Indianapolis, USA). The HITm2.2 sub-clone (Edlund *et al.*, 1985) and RINm5F line were both kindly provided by Dr. D. Boam (Queen Elizabeth Hospital, Birmingham Medical School, UK). MIN 6 cells (Miyazaki *et al.*, 1990) were obtained from Dr. J-I. Miyazaki (Kumamoto University Medical School, Japan).

3T3-L1 fibroblasts, used as a control in some experiments, were cultured by Dr. J. Yang (Dept., of Biochemistry, University of Bath) as previously described (Frost & Lane, 1985).

Growth medium, serum, reagents and tissue culture plasticware were purchased from ICN Flow (High Wycombe, Bucks., UK) or Gibco Ltd (Paisley, Scotland). Serum was heat-inactivated (HI) at 56°C for 30 min before use. Dulbecco's A phosphate-buffered saline (PBS) was prepared from tablets (Oxoid). All reagents and equipment which were not purchased sterile were autoclaved at 20 lb/in<sup>2</sup> for 30 min.

### 2.5.1 HIT-T15 culture

HIT-T15 cells were cultured in HAMS-F12 medium containing 2 mM glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin which was supplemented with either 10% (v/v) HI-foetal calf serum (FCS) or 15% (v/v) HI-donor horse serum (DHS) plus 2.5% (v/v) HI-FCS. The cells were routinely seeded in 25 cm<sup>2</sup> flasks at a density of 2x10<sup>6</sup> cells per 8 ml of medium. If seeded at lower densities cell growth was poor. Within an hour of

plating out, the cells reaggregated into small clumps which reached a maximum size after 4-5 days growth. If left longer than this the cells started to detach. Cultures were maintained at 37°C in 5% CO<sub>2</sub> and 95 % air. The medium was changed every 2 days.

To subculture, the medium was removed and the cells were rinsed briefly with PBS. The cells were then incubated for about 8 min at 37°C in a film of 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in PBS until the cells just started to lift off the surface. The action of trypsin was inhibited by the addition of 5 ml of serum-supplemented medium and the cells were detached by gentle aspiration. The cells were washed by centrifugation for 3 min at  $\approx 200 g_{av}$ , the pellet was resuspended in growth medium and a count of viable cells was carried out by mixing an equal volume of resuspended cells with 0.02% Trypan Blue in PBS, dead cells stained blue. Under these conditions the cultures were split 1:2 or 1:3 after 4-7 days growth.

For storage, cells were resuspended at a density of  $1 \times 10^7$  cells/ml in cold lactalbumin hydrolysate medium containing 10% (v/v) HI-newborn calf serum (NCS) and 10% (v/v) dimethyl-sulphoxide (DMSO). After slow-cooling overnight in the vapour phase of liquid nitrogen, the cells, in cryotubes, were transferred to liquid nitrogen tanks for storage. When required again, the cells were rapidly defrosted at 37°C and washed immediately in 20 ml growth medium before plating out as usual.

### 2.5.2 MIN 6 culture

MIN 6 cells were cultured in DMEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 20% (v/v) HI-FCS and 7  $\mu$ l/L 2-mercaptoethanol.

These cells were grown at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air in 25 cm<sup>2</sup> flasks containing 5-8 ml of medium or in 9 cm diameter plates containing 10 ml of

medium. MIN 6 cells grew in small clusters, they were subcultured after 7-8 days growth. Trypsinization was carried out as described for HIT-T15 cells except that the incubation was at room temperature. It was important not to make a single cell suspension, but to leave small aggregates of several cells. The cells were routinely split 1:5 and the medium was changed every 3 days. MIN 6 cells were frozen in FCS containing 10% DMSO. Since recovery was relatively low, it was important to start the culture after thawing at a high cell density.

### **2.5.3 HITm2.2 and RINm5F culture**

Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and either 10% (v/v) HI-FCS or 5% (v/v) HI-FCS plus 5% (v/v) HI-NCS. RIN cells could also be grown in medium supplemented with 10% HI-NCS. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air. The cells were seeded in 9 cm dishes at a density of 1-1.5x10<sup>6</sup> cells per dish. RIN cells reached ≈90% confluence after about 7 days and HIT cells reached 70-80% confluence after 4-6 days. The cells were routinely split 1:7. The cells were fed every 2 days and subcultured as described for HIT-T15 cells. Cells were frozen in FCS containing 10% DMSO and stored in liquid nitrogen.

## **2.6 DETECTION OF INSULIN**

### **2.6.1 Indirect immunofluorescence**

HITm2.2, RINm5F and HIT-T15 cells were grown in 1 ml wells. The cells were washed briefly in warm PBS and fixed overnight at 4°C in a buffer containing 4% (v/v) formaldehyde, 46 mM Na<sub>2</sub>HPO<sub>4</sub> and 26 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O (pH 7.4). The cells were washed in PBS and blocked for 1 hour at room temperature (or 37°C) in PBS containing 5% (v/v) normal goat serum (NGS). The cells were then incubated for 2 hr at 37°C with

150  $\mu$ l of a pre-diluted monoclonal anti-insulin antibody (Amersham International, Amersham, UK). Control wells were incubated with PBS only. After 3 washes with PBS, 200  $\mu$ l of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG antibody, diluted 1:50 in PBS/5% (v/v) NGS, were added to each well and incubated for 1 hour at 37°C in the dark. The cells were washed in PBS, mounted in a PBS:glycerol (1:1) buffer and examined with a fluorescence microscope. Cytoplasmic fluorescence indicated the presence of insulin.

### **2.6.2 Insulin radioimmunoassay (RIA)**

HITm2.2 and RINm5F cells were grown in 1 ml wells. In order to determine the mean number of cells present per well, two wells per experiment were trypsinized and the cells counted. Following a brief wash with KRB buffer (see section 2.2), the cultures were incubated at 37°C for 60 min (RINm5F) or 90-130 min (HITm2.2) with 1 ml of fresh KRB to which the secretagogues D-glucose, L-arginine or L-D-isoleucine had been added at the indicated concentrations.

After incubation, the supernatant was removed, centrifuged to remove any cell debris and stored at -70°C until assayed with a commercial insulin RIA kit (Amersham International), which detected insulin over the range 0-160  $\mu$ U/ml. The protocol recommended by the manufacturers was followed and results were expressed in terms of  $\mu$ U insulin secreted per 60 min per  $2 \times 10^5$  cells.

## **2.7 MEASUREMENT OF 2-DEOXYGLUCOSE UPTAKE IN CULTURED $\beta$ -CELLS**

Uptake of 2-deoxyglucose, which is phosphorylated but not further metabolised, was measured in HITm2.2 and RINm5F cells. Cells were detached from culture plates by incubation at 37°C in PBS containing 1 mM EDTA. The cells were resuspended in a

Hepes-buffered balanced salts solution (see section 2.2), washed briefly by centrifugation and aliquoted into round bottomed plastic tubes. Each tube contained 200  $\mu$ l of a cell suspension containing  $5 \times 10^5$  HIT or  $1 \times 10^6$  RIN cells respectively. Transport assays were performed at 37°C. At time zero, 50  $\mu$ l of a cocktail containing 250  $\mu$ M 2-deoxyglucose and a tracer amount of  $^3\text{H}$  2-deoxyglucose (0.1  $\mu$ Ci/assay) were added to the cell suspension. The final concentration of 2-deoxyglucose was 50  $\mu$ M. Sugar uptake was stopped after the indicated intervals by the addition of 2 ml of stopping solution (0.1 mM phloretin in balanced salts solution). The cells were immediately pelleted by a brief low speed centrifugation and the washing step was repeated. The supernatant was removed by briskly inverting the tubes and traces of liquid around the rim were wiped away.

The cell pellet was solubilized in 0.1 M NaOH and a small aliquot was removed for estimation of protein using the Bio-Rad protein assay. The remainder of the sample was added to scintillation fluid and the incorporated radioactivity was determined. Results were expressed in terms of nmol 2-deoxyglucose transported/mg protein/min.

## 2.8 ISOLATION OF RAT ISLETS OF LANGERHANS

Islets were isolated using a modification of the collagenase digestion procedure first described by Lacy & Kostianovsky (1967). The present protocol was developed from the method used at Kings College Hospital, London (Dr. M. Peakman - personal communication). Islets were separated from exocrine tissue on a Ficoll (Sigma) gradient essentially as described by Lernmark (1976) and McDaniel *et al.* (1983).

4-6 Wistar rats (200-250 g) were stunned and killed by cervical dislocation, the pancreas was removed from each animal and placed in a petri-dish containing ice-cold Gey and Gey medium (see section 2.2). The exocrine tissue was disrupted by the injection of about 20 ml of ice-cold Gey and Gey medium into multiple sites under the capsule of the pancreas. After inflation, the organ was chopped into 1-2 mm pieces in a small volume of

medium and the pieces were allowed to settle. The supernatant, containing autolytic enzymes and fatty tissue, was removed by aspiration. The tissue pieces were washed three times in this way. Tissue from 2 pancreases was transferred to a plastic universal tube with 8 ml of medium containing 14 mg of Type XI collagenase (Sigma) or 10 ml of medium containing 10 mg of collagenase P (Boehringer Mannheim). The tissue was digested by vigorous manual shaking for 5-6 min in a 37°C water bath. When the mixture appeared homogeneous, digestion was terminated by the addition of ice-cold medium. The digested tissue was washed 3 times by centrifugation for 3 min at 300  $g_{av}$ .

After washing, the digested tissue from 2 pancreases was vortex-mixed with 4 ml of 27% (w/w) Ficoll in Gey and Gey medium at room temperature. 23%, 20.5% and 11% solutions of Ficoll in Gey and Gey medium were made by appropriate dilution of the 27% Ficoll stock. 4 ml of each were carefully layered onto the 27% layer in order of decreasing Ficoll concentration. The resulting gradient was centrifuged at room temperature (10 min, 800  $g_{av}$ ) which caused the islets to separate out at the interfaces of the 11% and 20.5% layers. The islets were hand-picked from the Ficoll with a fine-ended silicized pipette under the low magnification of a stereomicroscope. Islets stood out as solid "pearl-like" structures when illuminated from above against a dark background. Generally, the islets separated on Ficoll gradients were small, the average yield was about 100-200 islets per pancreas.

## 2.9 ISOLATION OF RAT HEPATOCYTES

Hepatocytes for use in photolabelling studies were isolated from rat liver essentially as described by Howard *et al.* (1967).

The liver of a freshly killed rat was perfused in situ via the hepatic portal vein with ice-cold  $Ca^{2+}$ -free HBSS (see section 2.2). Perfusion was continued until the liver was blanched. The liver was then removed and about 3 g of tissue were chopped finely with

scissors. The chopped tissue was distributed between 3 plastic capped pots containing 7 ml of digestion buffer ( $\text{Ca}^{2+}$ -free HBSS containing 0.15% collagenase (Type 1) and 0.15% hyaluronidase). The tubes were incubated at 37°C in a shaking water bath for 60 min. Large pieces of undigested tissue were removed by filtration through gauze. The resulting cell suspension was washed in ice-cold HBSS by centrifugation at 1000 rpm. The pelleted liver parenchymal cells were removed from the erythrocytes which remained in the supernatant. Washing was repeated three times. The viability of the parenchymal cells was assessed by Trypan Blue staining. Typically, viability was  $\approx 78\%$ .

## **2.10 PREPARATION OF CELL MEMBRANES**

### **2.10.1 Preparation of rat adipocyte plasma membranes**

Adipocyte plasma membranes were used as a control in some experiments. They were kindly prepared by A.E. Clark (Dept. of Biochemistry, University of Bath) according to the method described by Holman *et al.* (1988).

### **2.10.2 Preparation of human erythrocyte ghosts**

Human blood was washed with PBS by centrifugation for 10 min at 3000 g (4°C). The "buffy coat" of leukocytes and platelets that sedimented above the erythrocytes was removed by aspiration. Approximately 5 ml of washed erythrocytes were added to 250 ml ice-cold haemolysing buffer containing 5 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM EDTA and 1  $\mu\text{g/ml}$  PMSF (phenylmethylsulphonylfluoride). After 20 min on ice the membranes were pelleted by centrifugation for 20 min at 16,000  $g_{av}$  (4°C). The pellet was re-haemolysed and the centrifugation repeated. The resulting ghosts were disrupted by resuspending the membrane pellet in 5 mM sodium phosphate buffer (pH 7.4).

### 2.10.3 Preparation of rat liver membranes

#### 2.10.3.1 Method I - Liver plasma membranes

Liver plasma membranes for use in photolabelling experiments, Western blotting, ELISAS and GLUT2 purification were isolated on a Percoll (Pharmacia LKB, Uppsala, Sweden) gradient essentially as described by Prpic *et al.* (1984).

The liver was removed from one male 200 g Wistar rat and placed in 150 ml of ice-cold isolation medium (250 mM sucrose, 5 mM Hepes, 1 mM EGTA pH 7.4) containing the proteinase inhibitors aprotinin, antipain, leupeptin and pepstatin A (Sigma) each at a concentration of 3  $\mu\text{g/ml}$ . The liver was minced, then homogenised with 10 strokes of a loose-fitting Potter-Elvehjem homogeniser, followed by 4 strokes of a tight-fitting homogeniser. The homogenate was filtered through 2 layers of nylon mesh and centrifuged at 1464  $g_{av}$  (Sorval RC5B, SS-34 rotor, 3500 rpm) for 10 min at 4°C. The resulting pellet was resuspended in isolation medium and homogenised with 4 strokes of the tight-fitting homogeniser. The volume was adjusted to 124.5 ml with isolation buffer and the homogenate was mixed thoroughly with 16.8 ml Percoll and centrifuged at 34,540  $g_{av}$  (Sorval RC5B, SS-34 rotor, 17,000 rpm) for 35 min at 4°C. The plasma membrane was identified as a fluffy white layer close to the top of the gradient, lying just beneath a broader band containing nuclei, which was discarded. The plasma membrane was aspirated and washed by centrifugation at 100,000  $g_{av}$  (Beckman Ti60 rotor, 40,000 rpm) for 30 min at 4°C in 5 mM sodium phosphate buffer (pH 7.4), which prevented the formation of membrane vesicles. 2-4 mg of plasma membrane protein were obtained per rat liver. Membranes were stored at -70°C in small aliquots.



### **2.10.3.2 Method II - Crude liver membranes**

Liver membranes were obtained from rat liver essentially as described by Thorens *et al.* (1988). The liver was homogenized in 0.3 M sucrose and centrifuged for 10 min at 5900  $g_{av}$ . The pellet was discarded and the centrifugation was repeated. The resulting supernatant was centrifuged at 120,000  $g_{av}$  for 40 min to obtain the membrane pellet.

### **2.10.4 Preparation of $\beta$ -cell line and islet membranes**

#### **2.10.4.1 Method I - Preparation of $\beta$ -cell plasma membranes**

Plasma membranes were prepared from  $\beta$ -cell lines as described by Kunze *et al.* (1987) and as modified by Karounos & Thomas (1990).

Confluent cell cultures (5-25 dishes) were washed twice with warm PBS. The cells were then scraped into 4 ml of ice-cold buffer containing 5 mM TRIS, 2 mM EDTA and 1  $\mu$ g/ml PMSF. The cells were lysed on ice for 40 min, then homogenised by 20 strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 3000  $g_{av}$  to remove mitochondria and nuclei. The supernatant was centrifuged at 100,000  $g_{av}$  for 40 min at 4°C (Beckman Ti60 rotor, 40,000 rpm) to pellet the plasma membranes. Membranes were stored at -70°C in 10 mM TRIS, 100 mM NaCl and 2 mM EDTA (pH 7.4) containing the proteinase inhibitors aprotinin, antipain, leupeptin and pepstatin A (Sigma) each at a concentration of 3  $\mu$ g/ml.

#### **2.10.4.2 Method II - Preparation of $\beta$ -cell plasma membranes using a Ficoll gradient**

This method was based on a protocol for the isolation of adipocyte plasma membrane as described by McKeel & Jarett (1970) and as modified by Rees (1981).

Confluent cell cultures (10-20 dishes) were washed briefly with PBS. The cells were then detached by incubation at 37°C in PBS containing 1 mM EDTA and pelleted by centrifugation. The cell pellet was transferred to 10-20 ml of ice-cold TES buffer (see section 2.2). Cells were homogenised in a very tight-fitting Potter-Elvehjem homogeniser using 10 strokes of a motor driven pestle rotating at 1500 rpm. In order to minimize the clearance between the plunger and glass vessel, homogenization was carried out at room temperature.

The homogenate was centrifuged at 500  $g_{av}$  for 5 min to pellet unbroken cells. The supernatant was then centrifuged at 4°C for 20 min at 11,200  $g_{av}$  (Sorval RC5B, SS34 rotor, 11,000 rpm). The resulting pellet was resuspended in 0.5-1.0 ml TES by homogenization with 10-15 strokes of a loose-fitting homogenizer and 300-500  $\mu$ l of the homogenate were layered onto a 4 ml Ficoll gradient. The gradient was prepared by dissolving Ficoll in TES to a concentration of 18% (w/w). Before use, the Ficoll solution was filtered and cooled to 4°C. A plasma membrane-containing fraction was separated from the remaining nuclei and mitochondria by centrifugation of the gradient at 4°C for 45 min at 37,000  $g_{av}$  (Beckman, SW50.1 rotor, 20,000 rpm). The plasma membrane fraction was removed from the interface and washed in TES by centrifugation at 4°C for 60 min at 70,000  $g$  (Beckman, SW50.1, 27,000 rpm). The resulting pellet was resuspended in 5 mM sodium phosphate buffer and stored at -70 °C.

#### **2.10.4.3 Method III - Preparation of total $\beta$ -cell membranes**

Cultured cells were washed with PBS and detached from petri-dishes by incubation at 37°C for 5-10 min in PBS containing 1 mM EDTA. The cells or islets were then homogenized in ice-cold 5 mM sodium phosphate buffer or TES (see section 2.2). The homogenate was centrifuged at 100,000  $g_{av}$  for 40 min at 4°C and the pelleted membranes were resuspended in a small volume of 5 mM sodium phosphate buffer and stored at -70°C.

## 2.11 TRITON X-114 EXTRACTION OF $\beta$ -CELL MEMBRANE PROTEINS

The phase extraction of amphiphilic membrane proteins from HITm2.2 and RINm5F cells was based on the protocols described for the Triton X-114 extraction of the 64 kDa autoantigen from  $^{35}\text{S}$  methionine-labelled islets (Baekkeskov *et al.*, 1987; Christie *et al.*, 1988; Baekkeskov *et al.*, 1989).

HITm2.2 or RINm5F cells were detached from their culture dishes by incubation in PBS containing 1 mM EDTA. The cells were washed in PBS and approximately  $3 \times 10^7$  cells were homogenized with 10 strokes of a tight-fitting Potter-Elvehjem homogenizer in 0.25 M sucrose/10 mM Hepes (pH 7.4) containing 1  $\mu\text{g}/\text{ml}$  aprotinin, 0.1 mM p-chloromercuribenzenesulphonic acid and 10 mM benzamidine hydrochloride. The homogenate was centrifuged at 75,000  $g_{\text{av}}$  at 4°C for 30 min and the pelleted membranes were resuspended by homogenization in 1 ml of extraction buffer (20 mM TRIS, 150 mM NaCl, 1  $\mu\text{g}/\text{ml}$  aprotinin, 20 mM NEM, 0.1 mM Na vanadate, 5 mM EDTA, 5 mM NaF and 2% (v/v) Triton X-114. Proteins were solubilized for 2 hours on ice, then insoluble material was removed by centrifugation at 16,000  $g_{\text{av}}$  at 4°C for 20 min.

The supernatant was subjected to a phase separation procedure as described by Bordier (1981) and Christie *et al.* (1988). The Triton X-114 detergent had a cloud point of 20°C, therefore when warmed above this temperature the detergent separated into an aqueous and a detergent-enriched phase with the amphiphilic membrane proteins partitioning into the detergent phase. 150  $\mu\text{l}$  of the Triton X-114 extracted proteins were overlaid onto 250  $\mu\text{l}$  of a sucrose cushion which contained 6% (w/v) sucrose in the extraction buffer prepared without the Triton X-114 detergent. Tubes were incubated at 30°C for 3 min which caused the detergent extract to become cloudy. Aggregated detergent micelles and associated integral membrane proteins were pelleted by centrifugation at 1500  $g_{\text{av}}$  for 3 min at 25°C. A small cloudy droplet appeared below the sucrose cushion. The aqueous phase above the cushion was removed and Triton X-114

was added to a concentration of 2% (v/v). Proteins were re-extracted by incubation for 10 min on ice. The solubilized proteins were again overlaid on a sucrose cushion. This phase separation procedure was repeated twice.

The final detergent pellet was removed from beneath the sucrose cushion and was diluted in extraction buffer without added Triton X-114. Extracted proteins were solubilized by boiling for 1 min in sample buffer containing 80 mM TRIS, 3% (w/v) SDS, 15% (w/v) sucrose, 5% (v/v) 2-mercaptoethanol and 0.005 % (w/v) Bromophenol Blue. Proteins were analysed by SDS-PAGE and Western blotting. Approximately 20–40  $\mu\text{g}$  of protein (from  $2 \times 10^6$  cells ) were loaded per lane.

## 2.12 ASSAY OF 5'NUCLEOTIDASE ACTIVITY

The method used to measure 5' nucleotidase (EC 3.1.5.3) activity in membranes was modified from the protocol described by Newby *et al.* (1975). 50  $\mu\text{l}$  of a membrane suspension adjusted to 0.6 mg of protein/ml in 5 mM sodium phosphate buffer were added to 200  $\mu\text{l}$  of a solution containing 1 mM adenosine monophosphate (AMP), a tracer amount of [ $^{14}\text{C}$ ]AMP, 5 mM  $\text{MgSO}_4$  and 50 mM TRIS (pH 8.0). The reaction was initiated by incubation at 37°C for 30 min and terminated by the addition of 200  $\mu\text{l}$  of 0.15 M  $\text{ZnSO}_4$ . The subsequent addition of 200  $\mu\text{l}$  of 0.15 M  $\text{Ba(OH)}_2$  precipitated the charged AMP molecules leaving any radiolabelled adenosine, formed by the nucleotidase activity, in the supernatant. The precipitate was pelleted by centrifugation and samples of the supernatant were taken for liquid scintillation counting. Blanks were included in which membranes were added to the incubation mixture at the same time as the  $\text{ZnSO}_4$ . The specific activity was calculated from a control to which buffer was added instead of  $\text{ZnSO}_4$  and  $\text{Ba(OH)}_2$ . Results were expressed as  $\mu\text{mol}$  adenosine produced/mg protein/30 min.

## 2.13 PRODUCTION OF ANTI-GLUCOSE TRANSPORTER PEPTIDE ANTIBODIES

Table 3 gives details of the rabbit anti-glucose transporter peptide antibodies used in this study, these include antibodies raised against the GLUT1, GLUT2 and GLUT4 glucose transporter isoforms.

### 2.13.1 GLUT2 C-terminal peptides

The C-terminal sequence of GLUT2 is unique to this transporter isoform, therefore antibodies raised against it should be specific for GLUT2. Antisera have been used that were raised against three slightly different peptides from the C-terminal region of rat (Thorens *et al.*, 1988) and human GLUT2 (Fukumoto *et al.*, 1988). Sequences are shown in Table 3.

**Peptide A** - This peptide corresponds to the 13 C-terminal amino acids with an additional cysteine incorporated for the purpose of coupling to the protein carrier. This peptide was purchased from Multi-Peptide Systems.

**Peptide B** - This peptide has the same sequence as peptide A except that it is longer by 3 amino acids. 20 mg of the peptide were kindly provided by Dr. S. Baldwin.

**Peptide C** - This peptide corresponds to the upper C-terminal region of GLUT2, 18 amino acids away from the putative transmembrane region (M12). Antiserum raised against this peptide was kindly provided by Hoffmann La Roche.

**Peptide D** - This peptide corresponds to the 18 terminal amino acids of the human GLUT2 sequence. Antiserum raised against this peptide was kindly provided by Dr. S. Cushman.

Antiserum raised against peptide B was routinely used in all experiments unless otherwise stated.

Antiserum	Amino acids	Peptide sequence (* denotes amino acids incorporated for coupling to carrier protein)	Acknowledgements
Anti-human GLUT1 C-terminal peptide	480-492	* C E E L F H P L G A D S Q V Cys Glu Glu Leu Phe His Pro Leu Gly Ala Asp Ser Gln Val	Antiserum provided by A.E. Clark (Dept. of Biochemistry, University of Bath.)
Anti-rat GLUT2 C-terminal peptide (A)	510-522	* C T V Q M E F L G S S E T V Cys Thr Val Gln Met Glu Phe Leu Gly Ser Ser Glu Thr Val  Peptide A	
Anti-rat GLUT2 C-terminal peptide (B)	507-522	* C R K A T V Q M E F L G S S E T V Cys Arg Lys Ala Thr Val Gln Met Glu Phe Leu Gly Ser Ser Glu Thr Val  Peptide B	Peptide donated by Dr. S. Baldwin (Dept. of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, London.)
Anti-rat GLUT2 C-terminal peptide (C)	499-508	K K S G S A P P R K Lys Lys Ser Gly Ser Ala Pro Pro Arg Lys  Peptide C	Antiserum provided by Hoffmann La Roche, USA.
Anti-human GLUT2 C-terminal peptide (D)	507-524	H R P K A A V E M K F L G A T E T V His Arg Pro Lys Ala Ala Val Glu Met Lys Phe Leu Gly Ala Thr Glu Thr Val  Peptide D	Antiserum provided by Dr. S. Cushman (NIH, Bethesda USA.)
Anti-rat GLUT2 exofacial loop peptide	47-60	* C G V P L D D R R A T I N Y D Cys Gly Val Pro Leu Asp Asp Arg Arg Ala Thr Ile Asn Tyr Asp	
Anti rat GLUT4 C-terminal peptide	497-509	* * C G S T E L E Y L G P D E N D Cys Gly Ser Thr Glu Leu Glu Tyr Leu Gly Pro Asp Glu Asn Asp	Antiserum provided by A. E. Clark (Dept. of Biochemistry, University of Bath.)

**Table 3 Details of anti-glucose transporter peptide antibodies.**

### 2.13.2 Synthesis of anti-rat GLUT2 exofacial loop peptide

The exofacial loop peptide corresponded to part of the amino acid sequence predicted from the nucleotide sequence of the large exofacial loop between membrane spanning regions M1 and M2 of rat GLUT2. This sequence is found in GLUT2 and GLUT7, but is absent from the shorter extracellular loops found in the other transporter isoforms.

The peptide was synthesised under the guidance of Dr. R. Kinsman (Dept. of Chemistry, University of Bath) using a Cambridge Research Biochemicals Pepsynthesiser II. The synthesis was performed using the Fmoc (9-fluorenylmethoxycarbonyl) polyamide method of solid phase synthesis. The solvent used for all couplings was freshly distilled N,N-dimethylformamide (DMF). All reagents were used in a 4 fold excess.

The carboxy-terminal amino acid was coupled to 0.5 g of the supporting resin (Pepsyn KA) via an ester bond which was generated by the recirculation of the active ester-derivatised amino acid and the catalyst (4-dimethylaminopyridine in DMF) through the resin for 7 hours. A sample of the resin was washed and subjected to Fmoc analysis by cleavage of the Fmoc group in 20% piperidine in DMF. Free Fmoc groups were quantified by measuring the absorbance at 190-300 nm. This demonstrated that maximum loading of the resin had been achieved. The carboxy-terminal amino acid was "deprotected" by washing for 10 min in 20% piperidine in DMF followed by a 15 min wash in DMF. All subsequent amino acids were coupled stepwise by recirculation of the active ester derivative and the catalyst 1-hydroxybenzotriazole through the resin plus its growing peptide chain for 25 min. The resin was then washed in DMF for 25 min, deprotected, then the coupling cycle was repeated with the following amino acid.

In order to check for completion of the coupling reaction, Kaiser or Istatin tests were performed on small samples of resin following coupling. The resin was washed in

DMF, dichloromethane and diethyl ether then dried under a stream of nitrogen. For the Kaiser test, 6-10 beads of resin were heated at 100°C for 5 min in 2 drops of each of 5% ninhydrin in ethanol, 400% phenol in ethanol and 2% 0.001 M KCN in pyridine. Complete coupling, indicated by the absence of uncoupled primary amino acids was confirmed by a pale yellow colour. When coupling to a secondary amino acid (e.g. proline) the Kaiser test was modified by the addition of 2 drops of Istatin reagent (3.3% (w/v) Istatin in benzyl alcohol stirred for 2 hours at room temperature and filtered to remove insoluble Istatin). Complete coupling was confirmed by the absence of colour on the resin beads.

Following the coupling of the amino terminal residue, the resin was washed in DMF, tertiary amyl alcohol and glacial acetic acid to remove any by-products of the synthesis. This was followed by a wash with tertiary amyl alcohol, dichloromethane and diethyl ether to dry the resin. Cleavage of the peptide from the resin and removal of side-chain protecting groups were performed at the same time by incubating the resin in trifluoroacetic acid (TFA). 3% (w/v) phenol and 3% (v/v) ethanedithiol (EDT) were included as scavengers to prevent side-reactions occurring with the released peptide. The optimum time for cleavage and deprotection was determined by shaking a small sample of resin with the TFA, phenol and EDT reagent and removing samples of the supernatant after 1, 2, 4, 8 and 24 hours. The sample was added to diethyl ether on ice and the precipitate that formed was pelleted by centrifugation at 800  $g_{av}$  for 10 min. The precipitated peptide was washed 4 times in diethyl ether.

The peptide was subjected to analytical reverse phase high-performance liquid chromatography (HPLC) using a C<sub>18</sub> column. The sample components were fractionated by a gradient of acetonitrile (5-70%) in TFA:water and compounds were detected by measurement of their absorbance at 215 nm. The peptide peak reached a maximum size after incubation with the cleavage reagent for 8 hours, this time also minimised the generation of by-products. Large scale cleavage of the peptide was performed under



identical conditions. Approximately 200 mg of peptide were produced which were stored at -20°C under nitrogen. Analytical HPLC showed the peptide to be at least 80% pure, it was therefore used for immunization without further purification.

### **2.13.3 Coupling of peptides to the carrier protein**

#### **2.12.3.1 Ellman's test for thiol groups**

Cysteine groups were required for the coupling of the peptide to the carrier protein. The content of thiol groups in the cysteine residue of each peptide was determined by Ellman's method, as described by Davies (1990)

A stock solution of Ellman's reagent was prepared containing 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB) in 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 18 mM NaHCO<sub>3</sub> (pH 7.5). For each assay 67 µl of the DTNB stock were added to 600 µl of 100 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5) and the volume was adjusted to 1.0 ml with water and the thiol-containing standard or sample. A standard curve ranging from 0.01-0.33 µmol/ml of cysteine hydrochloride was prepared by dilution of a 1 mM stock. Each peptide was dissolved at a concentration of 8 mg/ml in a buffer found to maximise its solubility. For peptide A, which dissolved poorly in all buffers, solubilization was maximised in 0.1 M sodium borate (pH 9.0). The loop peptide was soluble in 100 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 7.2) and Peptide B was soluble in both of these buffers. 10 µl of each peptide solution were added to each assay tube. After a 10 min incubation at room temperature the absorbance of all samples was measured at 412 nm. The content of thiol groups in the peptide sample was then estimated from the standard curve.

Peptide A was poorly soluble and contained only 19% of the total number of expected thiol groups. Peptide B was estimated to have 79% of the expected number of thiol groups and the loop peptide had 100% of the thiol groups available. When the thiol

content was low, peptides were either coupled using the glutaraldehyde method (see section 2.13.3.2) or were reduced using dithiothreitol (DTT). To reduce the peptide, 8 mg were solubilized in 500  $\mu$ l of 50 mM  $\text{Na}_2\text{HPO}_4$  (pH 7.2), DTT was added to a concentration of 100 mM and the reaction was allowed to proceed at room temperature. The reaction mixture was separated by passing through a Sephadex G10 column equilibrated with 50 mM  $\text{Na}_2\text{HPO}_4$  (pH 7.2). The peptide-containing fractions, which had a high absorbance at 236 and 280 nm, were added immediately to KLH-MB as described in section 2.13.3.3.

### 2.13.3.2 Glutaraldehyde coupling of peptide and carrier protein

Glutaraldehyde was used to couple peptide A which had a low number of available thiol groups. The coupling procedure was a modification of the method described by Walter *et al.* (1980). 5 mg of peptide A in 200  $\mu$ l of 0.1 M sodium borate (pH 9.0) were mixed with 6 mg of Keyhole Limpet haemocyanin (KLH) dissolved in 400  $\mu$ l of 10 mM  $\text{Na}_2\text{HPO}_4$  (pH 7.4). 200  $\mu$ l of a 20 mM glutaraldehyde solution were added dropwise and the mixture was stirred at room temperature for 30 min. Unreacted products were separated on a Sephadex G25 column (20x1 cm) equilibrated with 50 mM sodium phosphate buffer (pH 6.0) at 4°C. Fractions emerging after the void volume contained the peptide-KLH conjugate as determined by turbidity and absorbance at 280 nm. The protein concentration of the conjugate was determined, it was aliquoted and stored at -20°C.

### 2.13.3.3 MBS coupling of peptide and carrier protein

The use of N-maleimidobenzoic acid-N-hydroxysuccinimide ester (MBS) to couple a peptide to a protein was first described by Liu *et al.* (1979). The protocol used in the present study has been adapted from Green *et al.* (1982) and Davies (1990).

A 16 mg/ml solution of KLH in 10 mM  $\text{Na}_2\text{HPO}_4$  (pH 7.2) was dialysed overnight at 4 °C against the same buffer. 500  $\mu$ l of the protein solution were added to

10  $\mu\text{l}$  of a 50 mM solution of N-ethyl maleimide (NEM) and the mixture was stirred at 25°C for 10 min in order to block any thiol groups on the KLH. The dialysis and blocking steps could be omitted with no obvious effect on the quality of the antiserum produced. 2 mg of MBS were solubilized in the minimum volume of DMF ( $\approx 50 \mu\text{l}$ ) and added dropwise to 250  $\mu\text{l}$  of the KLH solution. The reaction mixture was stirred for 30 min at 25°C. The products were passed down a Sephadex G25 or G50 column (1x20 cm) equilibrated at 4°C with 50 mM  $\text{Na}_2\text{HPO}_4$  (pH 6.0) or 10 mM  $\text{Na}_2\text{HPO}_4$ /145 mM NaCl (pH 7.4). Unreacted MBS was retained on the column, the KLH-MB conjugate emerged after the void volume. Turbid fractions with a high absorbance at 280 nm were pooled, giving a final volume of  $\approx 2$  ml. The KLH-MB was added to about 5 mg of the peptide dissolved in 1 ml of 50 mM  $\text{Na}_2\text{HPO}_4$  (pH 7.2) or 0.1 M sodium borate (pH 9.0). After adjustment of the pH to 7.0, the mixture was stirred at room temperature for three hours. The coupled peptide-KLH conjugate was dialysed overnight against PBS and the protein concentration was determined. The conjugate was stored in aliquots at -20°C.

#### 2.13.4 Immunisation protocol

Antisera were raised against the GLUT2 peptide-KLH conjugates in sandy half-lop-eared rabbits. Each animal was injected with 200-400  $\mu\text{g}$  of conjugate in 500  $\mu\text{l}$  of PBS emulsified with 0.5-1.5 ml of Freund's complete adjuvant (FCA). All injections were given intramuscularly into 2-4 sites in the thigh. This first injection was followed 2-3 weeks later with an injection of 200-400  $\mu\text{g}$  of conjugate in 500  $\mu\text{l}$  of PBS emulsified with 0.5-1.5 ml of Freund's incomplete adjuvant (FIA). 2-4 weeks later a boost injection was given consisting of 100-200  $\mu\text{g}$  of protein in 1 ml of PBS containing 6 mg of aluminium ammonium sulphate (alum). These boost injections were repeated every 4-6 weeks, or whenever the antibody titre fell. Small test bleeds were taken from an ear vein and the antibody titre was measured using an ELISA (see section 2.18.2). At 4-6 week intervals 30 ml of blood were obtained by cardiac puncture under general anaesthesia. Blood was allowed to clot in glass tubes at room temperature for  $\approx 4$  hours. The clot was loosened

and the sample was left at 4°C overnight. The serum was obtained by centrifugation at  $\approx 2000 g_{av}$  for 10 min and stored in small aliquots at -70°C.

### **2.13.5 Purification of antibodies from anti-GLUT2 peptide antiserum**

#### **2.13.5.1 Affinity Purification of anti-GLUT2 C-terminal peptide antibodies**

Rat C-terminal peptide was immobilized on a Reacti Gel 6X matrix of 6% cross-linked agarose, derivatised with 1-1'carbonyl-diimidazole carbonate (Pierce Chemical Company, Illinois, USA ). 2 ml of Reacti Gel slurry were washed in ice-cold buffer (0.9% (w/v) NaCl, 0.1 M boric acid, pH 8.3). The gel cake was then added to 4 mg of peptide solubilized in 8 ml of the same buffer and was stirred at 4°C for 44 hours. The gel was washed briefly, then incubated in 8 ml of 1 M ethanolamine (pH 9.0) for 3 hours in order to block remaining active groups. The gel was washed with water and packed into a column. Antiserum was purified following the method described by Oka *et al.* (1988).

The peptide column was equilibrated with PBS at room temperature, then 5-10 ml of antiserum were re-circulated through the column for  $\approx 2$  hours. The column was washed with  $\approx 20$  ml of PBS followed by 20 ml of 10 mM  $Na_2HPO_4$ /2 M NaCl (pH 7.4). Anti-peptide antibodies were eluted by washing the column with 10 ml of 3.5 M sodium thiocyanate in 10 mM  $Na_2HPO_4$  (pH 6.6). The antibody-containing fractions were dialysed against PBS and concentrated using polyethylene glycol 20,000 (PEG). All column washes were tested for the presence of anti-peptide antibody using an ELISA (see section 2.18). Antibody was only detected in the sodium thiocyanate wash. Between 50-100  $\mu g$  of pure antibody were obtained per ml of serum. The antibody was stored at -70°C.

### 2.13.5.2 Purification of IgG from anti-GLUT2 antiserum

IgG was purified from rabbit anti-GLUT2 C-terminal peptide (B) antiserum according to the method described by Johnstone & Thorpe (1987). 10 ml of serum were warmed to 25°C and 1.8 g Na<sub>2</sub>SO<sub>4</sub> were added to make an 18% (w/v) solution. The serum was stirred for 30 min at 25°C then centrifuged at 3000 g<sub>av</sub> for 30 min at 25°C. The supernatant was discarded and the volume of the precipitate was noted. The pellet was dissolved in water and the volume was adjusted to 5 ml. Sufficient Na<sub>2</sub>SO<sub>4</sub> was added to make a 14% (w/v) solution (accounting for the salt carried over in the first precipitate). The serum was stirred at 25°C for a further 30 min and the centrifugation step was repeated. The precipitate was redissolved in water to give a volume of 3 ml and dialysed overnight against PBS at 4°C. The protein yield was calculated from the absorbance at 280 nm in a 1 cm light path.

$$\text{IgG (mg / ml) in sample} = \frac{A_{280 \text{ nm}} \times 10}{13.6}$$

## 2.14 IMMUNOAFFINITY PURIFICATION OF LIVER GLUT2

### 2.14.1 Preparation of immunoaffinity columns

#### i) Coupling of IgG to Sepharose-4B

The cyanogen bromide activation of Sepharose-4B and subsequent coupling of antibody were performed as described by March *et al.* (1974). 10 ml of settled Sepharose-4B beads were washed with water then added to 20 ml of 1 M Na<sub>2</sub>CO<sub>3</sub> and stirred slowly. 1 ml of a 2 g/ml solution of cyanogen bromide in acetonitrile was added and the slurry was stirred vigorously for 2 min in a fume cupboard. The slurry was washed under a vacuum in a coarse sintered glass funnel with 200 ml of each of 0.1 M NaHCO<sub>3</sub> (pH 9.5), water and 0.2 M NaHCO<sub>3</sub> (pH 9.5). The moist cake of beads was rapidly transferred to a large

plastic tube containing 30 mg of IgG in 20 ml of 0.2 M NaHCO<sub>3</sub> (pH 9.5). IgG was prepared from anti-GLUT2 C-terminal peptide antiserum as described in section 2.13.5.2. Coupling was performed by a rotation for 20 hours at 4°C. It was calculated that ≈ 1.5 mg of IgG were coupled per ml of gel. Any remaining active sites on the gel were blocked by mixing the coupled gel cake with 2 M ethanolamine for ≈ 1.5 hours at room temperature. The coupled Sepharose-4B beads were washed with 200 ml of NaHCO<sub>3</sub> (pH 10.0) and rotated overnight at 4°C with 40 ml of 0.2 M NaHCO<sub>3</sub>/1% (w/v) casein. The beads were washed successively with 100 ml of 50 mM glycine/50 mM NaCl (pH 3.0), 10 mM sodium phosphate/50 mM NaCl (pH 7.4), 50 mM glycine/50 mM NaCl (pH 10.0) and 10 mM sodium phosphate/50 mM NaCl (pH 7.4). The immunosorbent was used to affinity purify GLUT2 using method I described in section 2.14.2.(i).

#### **ii) Coupling of purified anti-GLUT2 antibody to Reacti Gel 6X**

Affinity purified anti-GLUT2 C-terminal peptide (A) antibodies (see section 2.13.5.1) were coupled to Reacti Gel 6X as recommended by the manufacturers and as described in section 2.13.5.1 for the coupling of peptide with the following exceptions: 1 ml of gel was coupled to 3 mg of antibody in 4 ml of coupling buffer. The gel was then washed in 2 M TRIS (pH 8.0) to block remaining active sites. The efficiency of antibody coupling was about 70%, as estimated from an SDS-Lowry protein assay. The antibody loading was therefore about 2 mg/ml gel. The immunosorbent was used to affinity purify GLUT2 using method II described in section 2.14.2.(ii).

### **2.14.2 Affinity purification of GLUT2**

#### **i) Method I - (Momoi & Lennon, 1982)**

The method used was essentially as described by Momoi and Lennon (1982). 7-8 mg of liver plasma membrane, prepared as described in section 2.10.4.1, were solubilized

by a rotation for 3 hours at 4°C, in 40 ml of buffer containing 2% Triton X-100, 10 mM sodium phosphate and 50 mM NaCl (pH 7.4). Insoluble material was removed by centrifugation for 30 min at 20,000  $g_{av}$ . The solubilized liver plasma membrane proteins were mixed with the antibody-coupled Sepharose 4B beads which had been fully equilibrated with buffer A (0.5% Triton X-100, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM NaCl, pH 7.4). Following an overnight rotation at 4°C, the beads were packed into a column and the gel was washed sequentially with 400 ml of buffer A, 100 ml of buffer B (0.2% Na cholate, 10 mM TRIS, 100 mM NaCl, pH 7.4) and 50 ml of buffer B containing 0.25 M NaCl. The bound GLUT2 was recovered by alternating elutions with 25 ml volumes of 1 M NaCl and 2 M NaCl in buffer B which were repeated 4 times. The fractions were concentrated with PEG and dialysed extensively against 10 mM sodium phosphate buffer (pH 7.4). Proteins were analysed by SDS-PAGE and Western blotting. The column was washed with 50 ml of 50 mM glycine/50 mM NaCl containing 0.2% Na cholate (pH 3.0). This was followed by 50 ml of 50 mM glycine/50 mM NaCl containing 0.5% Triton X-100. The column was finally washed and stored in 10 mM Na<sub>2</sub>HPO<sub>4</sub>/50 mM NaCl buffer (pH 7.4).

## ii) Method II - (Davies, 1990)

The method used was essentially as described by Davies (1990). Before use the coupled Reacti Gel 6X was washed extensively with buffer A (50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4) and buffer B (0.2 M glycine, 1 mM EDTA, 100 mM NaCl, pH 2.4). The gel was then packed into a small glass column and equilibrated at 4°C with buffer A containing 1% n-octyl  $\beta$ -D-glucopyranoside.

2 mg of liver plasma membrane protein were solubilized for 1 hour on ice in 15 ml of buffer B containing 1% n-octyl  $\beta$ -D-glucopyranoside and proteinase inhibitors (details and concentrations are given in section 2.10.4.1). Insoluble material was removed by centrifugation for 20 min at 20,000  $g_{av}$ . The solubilized material was loaded onto the column and pumped slowly through until it had recirculated  $\approx$  5 times. The unbound

fraction was collected and the column was washed with 15 ml of equilibration buffer. The bound proteins were eluted by washing with  $\approx 2$  ml of buffer B containing 1% n-octyl  $\beta$ -D-glucopyranoside. 2 ml fractions were collected and neutralized immediately by the addition of 1 M sodium phosphate. The fractions were dialysed overnight against buffer A and after concentration with PEG were analysed by SDS-PAGE and Western blotting. The column was washed with PBS and 50 mM diethanolamine (pH 11.0) and was stored at 4°C in PBS containing 0.02% azide.

## **2.15 PHOTOLABELLING WITH ATB-BMPA**

### **2.15.1 Preparation of ATB-[2-<sup>3</sup>H]-BMPA and ATB-BMPA**

The [2-<sup>3</sup>H] and non-radioactive ATB-BMPA were prepared by Dr. G.D. Holman as previously described (Clark & Holman, 1990). ATB-[2-<sup>3</sup>H]-BMPA had a specific activity of 10 Ci/mmol) and was stored at -20°C in PBS in the dark at a concentration of 0.5 mM (5 mCi/ml).

### **2.15.2 Photolabelling of liver plasma membranes**

Suspensions containing 100  $\mu$ g of liver plasma membrane protein were mixed with 100  $\mu$ Ci of ATB-[2-<sup>3</sup>H]-BMPA and competing substrates and inhibitors as required. D-glucose, D-mannitol, D-fructose and 4,6-O-ethylidene-D-glucose were prepared as 1 M stock solutions in double distilled water. Phloretin and cytochalasin B (Sigma) were dissolved in ethanol as concentrated stock solutions. The final ethanol concentration in assays did not exceed 5%. These inhibitors were added to cells or membranes just prior to the addition of the photolabel to give the concentrations indicated in the figure legends. The final sample volume was adjusted to 100  $\mu$ l with 5 mM sodium phosphate buffer (pH 7.4). Samples were irradiated for 60s in polystyrene dishes using a Rayonet RPR-100 photoreactor with RPR-3000 lamps. The labelled membranes were washed 3 times by



centrifugation (20 min, 20,000  $g_{av}$  4°C) in 5 mM sodium phosphate buffer. Washed membranes were either analysed directly by SDS-PAGE or were used for immunoprecipitations.

### 2.15.3 Photolabelling of whole cells

The conditions used for photolabelling cells are described in detail in the figure legends. Just prior to labelling, cells were washed to remove any glucose-containing medium. Photolabelling was carried out at room temperature in a HEPES-buffered balanced salts solution pH 7.0 (see section 2.2), which was also used for the washing steps. Hexoses were added from concentrated stock solutions to give the concentrations indicated in the figure legends. When cell suspensions were labelled, between  $5 \times 10^6$  and  $2.5 \times 10^7$  cells in 500  $\mu$ l of buffer were irradiated in the presence of 100–400  $\mu$ Ci of ATB-BMPA. Cells were initially irradiated for 30 s, the suspension was then agitated and the irradiation continued for a further 30 s. Cells were washed  $\approx$  5 times by low speed centrifugation. Monolayers of cultured  $\beta$ -cell lines in 35 mm dishes were irradiated for 60s in 0.5 ml of buffer containing 100  $\mu$ Ci ATB-BMPA. The cell monolayers were also washed 5 times.

### 2.15.4 Immunoprecipitation of ATB-BMPA labelled proteins

Photolabelled proteins were immunoprecipitated from liver, islets and  $\beta$ -cells with rabbit antisera raised against GLUT2 or GLUT1 peptide sequences. Preimmune rabbit serum was used in control experiments. Unless otherwise stated, the GLUT2 antiserum used routinely for immunoprecipitations was anti-rat GLUT2 C-terminal peptide (B) antiserum. Serum samples from human diabetic patients or normal controls were also used to immunoprecipitate labelled proteins from liver plasma membranes.

For each immunoprecipitation, 5 mg of Protein A-Sepharose (Sigma) were conjugated to 50  $\mu$ l of serum. When the amount of Protein A-Sepharose was altered details

have been given in the figure legends. The Protein A-Sepharose was swollen for 30 min in PBS. It was then washed three times by low speed centrifugation to remove stabilizers. 50  $\mu$ l of human or rabbit serum were diluted in 500  $\mu$ l of PBS and mixed with the Protein A-Sepharose beads by rotation for 3-16 hours at 4°C. Excess antibody was removed by washing the beads four times in PBS.

The pellets of washed photolabelled cells or membranes were solubilized for 1 hour on ice in 0.5-1 ml of 5 mM sodium phosphate buffer containing 2% nonaethylene glycol dodecyl ether (C<sub>12</sub>E<sub>9</sub>) detergent (Boehringer Mannheim) and the proteinase inhibitors pepstatin A, leupeptin, antipain and aprotinin each at 5  $\mu$ g/ml. In some experiments, as indicated in the figure legends, the C<sub>12</sub>E<sub>9</sub> detergent was replaced by 1% n-octyl  $\beta$ -D-glucopyranoside (Sigma) or 4% Triton X-100 and 0.5% SDS. Any insoluble material was removed by centrifugation for 20 min at 20,000  $g_{av}$  at 4°C. The solubilized material was incubated with the Protein A-Sepharose antibody-complex for 2½-3 hours by a rotation at 4°C. The Protein A-Sepharose immunoprecipitates were pelleted by low speed centrifugation. In some experiments the supernatant was removed and added to another Protein A-Sepharose antibody complex for a second 2½ hour rotation.

The Protein A-Sepharose immunoprecipitates were washed five to seven times with 1 ml of ice cold 5 mM phosphate buffer containing 0.2% C<sub>12</sub>E<sub>9</sub>. Labelled proteins were released from the conjugate by incubation for 30 min at room temperature in electrophoresis sample buffer (see section 2.16). Following a low speed centrifugation to remove the Sepharose beads radiolabelled proteins were analysed by SDS-PAGE (see section 2.16).

As a control, identical immunoprecipitations were also performed using labelled material which was then analysed by Western blotting. To prevent interference between the  $\approx$ 55 kDa glucose transporter protein and the  $\approx$ 51 kDa IgG heavy chain polypeptide, SDS-PAGE was performed under non-reducing conditions on 8% polyacrylamide gels.

In some experiments the non-precipitated proteins in the supernatant were also saved for analysis by electrophoresis. An aliquot was either mixed directly with electrophoresis sample buffer and analysed by SDS-PAGE. Alternatively, the protein was removed from the detergent-containing buffer by precipitation with chloroform and methanol (Wessel & Flügge, 1984). A 1 ml sample of detergent-solubilized proteins was mixed with 3.6 ml of methanol and 900 $\mu$ l of chloroform were added. The sample was vortex-mixed and 2.7 ml water were added. The sample was mixed again and centrifuged at 9000  $g_{av}$  for 1 min. The upper phase was carefully removed, discarded and replaced with 2.7 ml of methanol in order to precipitate the protein from the lower chloroform phase and interphase. Following vortex mixing, the protein was pelleted by centrifugation for 2 min at 9000  $g_{av}$  and the supernatant was removed by aspiration. The precipitated proteins were solubilized in electrophoresis sample buffer and analysed by SDS-PAGE.

#### **2.15.5 Analysis of ATB-BMPA labelled samples**

Photolabelled samples were analysed by SDS-PAGE, the gels were stained briefly with Coomassie Blue, then destained and cut into 6.6 mm wide slices. Each slice was placed in a scintillation vial and dried at 80°C for 2 hours. Slices were then solubilized in capped vials for a further 1-2 hours at 80°C in 0.5 ml of hydrogen peroxide containing 2% (v/v) ammonia solution. 8 ml of scintillation fluid were added to each vial and the radioactivity was then counted. The position of the radiolabelled peak was compared with the molecular weight marker proteins. The size of the peak was quantified by summing the radioactivity present in each slice under the peak and subtracting a background value based on the average amount of radioactivity in the slices on either side of the peak (Calderhead & Lienhard, 1988).

### 2.15.6 Estimation of the binding constant of ATB-BMPA for liver GLUT 2

The binding constant of ATB-BMPA was determined by the displacement of radiolabelled ATB-[ $^3\text{H}$ ]-BMPA by non-labelled ATB-BMPA. Photolabelling of 100  $\mu\text{g}$  samples of liver plasma membrane protein using 100  $\mu\text{Ci}$  ATB-[ $^3\text{H}$ ]-BMPA was performed in the presence of a range of concentrations of unlabelled ATB-BMPA. The final ATB-BMPA concentrations used were 100  $\mu\text{M}$  (no added unlabelled ATB-BMPA), 300  $\mu\text{M}$ , 500  $\mu\text{M}$ , 700  $\mu\text{M}$  and 900  $\mu\text{M}$ . The radioactive and unlabelled ATB-BMPA compounds were mixed together in a final volume of 100  $\mu\text{l}$  of 5 mM phosphate buffer before the addition of membranes. Samples were then irradiated, immunoprecipitated and subjected to electrophoresis. The size of the GLUT 2 peak was calculated for each concentration of ATB-BMPA. A background value was then subtracted to take account of the binding and immunoprecipitation which was non-specific. The background value used was calculated from the average amount of radioactive protein precipitated by preimmune rabbit serum. Values for the binding constant ( $K_d$ ) and the total number of binding sites ( $B_{\text{max}}$ ) were obtained from non-linear regression (weighted for relative error) of the equation:

$$B = (B_{\text{max}}/K_d) \cdot F / (1 + F/K_d)$$

where B and F are the bound and free ATB-BMPA. The data were graphically presented as F/B against F plots which were used to calculate the binding constant  $K_d$ . The number of pmol of ATB-BMPA bound per 100  $\mu\text{g}$  of liver plasma membrane protein were calculated from the peak areas (in dpm) which, based on the known specific radioactivity of ATB-BMPA were converted into moles of ATB-BMPA bound.

### 2.16 SDS-PAGE

Proteins from whole cells, membrane preparations, affinity purifications or immunoprecipitates were analysed by SDS-PAGE using the Protean II gel system (Bio-Rad).

The samples were solubilized by incubation at room temperature for 30-60 min in sample buffer containing 10% (w/v) SDS, 6M urea, 0.05% (w/v) Bromophenol Blue and 10% (v/v) 2-mercaptoethanol. For analysis of Protein A-Sepharose immunoprecipitates  $\approx 100 \mu\text{l}$  of solubilization buffer were added directly to the washed beads. For analysis of membrane preparations, samples were diluted 2 fold in solubilization buffer such that there was at least  $1 \mu\text{l}$  of solubilization buffer per  $\mu\text{g}$  protein. When sample buffer was added directly to whole cells, solubilization was aided by passage of the samples through a fine needle. In some experiments, when the samples were analysed under non-reducing conditions, the 2-mercaptoethanol was omitted and replaced with 0.1 M iodoacetamide (Johnstone & Thorpe, 1987). Proteins extracted from HIT and RIN cells with the detergent Triton X-114 were prepared for electrophoresis as described in section 2.11.

SDS-PAGE was carried out essentially as described by Laemmli (1970). Gels were prepared from stock solutions, details of which are given in section 2.2. The resolving gel contained 8 or 10% acrylamide, 0.55 M TRIS (pH 8.8) and 0.15% (w/v) SDS. The gel was polymerised chemically by the addition of  $0.6 \mu\text{l/ml}$  N, N, N', N', tetramethylethylene-diamine (TEMED) and  $6 \mu\text{l/ml}$  of a 10% solution of ammonium persulphate. The poured gel was overlaid with a layer of water-saturated butanol and left to polymerize ( $\approx 1$  hour). Following removal of the butanol, the resolving gel was overlaid with a stacking gel containing 3.75% acrylamide, 125 mM TRIS (pH 6.8) and 0.1% SDS which was polymerized as described above.

Electrophoresis was generally carried out overnight at 25 mA for a 3 mm gel or at 12 mA for a 1.5 mm gel in buffer containing 25 mM TRIS, 192 mM glycine and 0.1% SDS (pH 8.3). Molecular mass protein standards (Sigma) were included on each gel. Myosin (205 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). Prestained markers with approximate molecular masses of  $\approx 116$  kDa ( $\beta$ -galactosidase) and  $\approx 29$  kDa (carbonic

anhydrase) were also added to some samples so that the positions of the lanes could be easily identified.

For analysis of proteins by Western blotting, samples were subjected to electrophoresis on 1.5 mm thick gels with 40-50  $\mu\text{g}$  of protein per lane. ATB-BMPA labelled samples were subjected to electrophoresis on 3 mm thick gels and following staining were processed as described in section 2.15.5.

### **2.16.1 Staining polyacrylamide gels for protein**

Gels were agitated at room temperature in 0.2% (w/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid, 30% (v/v) methanol and 60% (v/v) water for  $\approx 15$  min. Excess stain was removed by destaining in several changes of 10% (v/v) acetic acid, 30% (v/v) methanol and 60% (v/v) water.

## **2.17 WESTERN BLOTTING**

### **2.17.1 Electrophoretic transfer of proteins to nitrocellulose**

Proteins separated by electrophoresis on 1.5 mm thick polyacrylamide gels were transferred to nitrocellulose paper (Gelman Sciences pore size 0.4  $\mu\text{m}$ ) using one of two alternative electrophoretic transfer methods.

#### **i - Wet transfer**

The polyacrylamide gel was soaked for 10-15 min in transfer buffer (see section 2.2). Wearing gloves, a sandwich was then constructed in which the gel was overlaid with a piece of wet nitrocellulose paper, care was taken to exclude all air bubbles. This sandwich was surrounded on each side by a layer of filter paper and a nylon pad, both

soaked in transfer buffer. The whole sandwich was placed between two plastic plates which were taped together tightly and slotted into a transfer tank with the nitrocellulose paper facing the anode. The blotting apparatus used was the Bio-Rad "Trans-Blot" cell or a similar tank which had graphite plates as the electrodes. It contained  $\approx 1.5$  L of transfer buffer at 4°C. Electrophoretic transfer was carried out for 6 hours at 29 Volts at 4°C which optimized the transfer of proteins with molecular masses between 116 and 29 kDa.

## ii - Semi-dry transfer

Proteins were transferred to nitrocellulose paper using a semi-dry blotting apparatus (Novablot, Pharmacia LKB), and following the manufacturers instructions. Briefly, this involved layering a stack of buffer-soaked filter papers onto the anode plate, covering it with a piece of nitrocellulose paper followed by the polyacrylamide gel and a final stack of filter papers. All layers were cut to the same size and smoothed out to exclude any air bubbles. Once the cathode plate had been replaced, the gel was transferred at  $0.8 \text{ mA/cm}^2$  for 105-120 min.

### 2.17.2 Staining nitrocellulose filters for total protein

Proteins were visualised by staining the washed nitrocellulose paper by agitating it for  $\approx 2$  min in 0.1% (w/v) Ponceau S in 3% (w/v) trichloroacetic acid prepared in double distilled water. Background staining was removed by washing in double distilled water. Although relatively insensitive and having a tendency to fade, this stain was useful for marking the positions of molecular weight marker proteins, since it did not distort the nitrocellulose paper. It also confirmed that satisfactory transfer of proteins had occurred. Ponceau S staining could be removed completely by washing in several changes of PBS or TRIS-buffered saline (TBS) - see section 2.2.

Once the temporary stain was removed, identification of the position of transferred proteins was facilitated if pre-stained molecular weight marker proteins with weights of about 116 and 29 kDa were included on the polyacrylamide gel. These could be seen as blue bands on the nitrocellulose paper.

### **2.17.3 Immunoblotting of nitrocellulose-bound protein**

Nitrocellulose paper was cut either into blocks or single tracks which were placed (using forceps) in plastic dishes or trays divided into longitudinal wells. The nitrocellulose was blocked, and primary antibody added using either protocol I, II or III described below and as indicated in the figure legends.

Bound primary antibodies were detected by the addition of one of the secondary antibody conjugates listed below. In order to prolong their life, the antibody-enzyme conjugates were diluted 1:1 with glycerol and then stored at -20°C.

- i) Horse radish peroxidase (HRP)-conjugated goat-anti-human (or rabbit) IgG antibody (Sigma). Diluted 1:1000 in the appropriate blocking buffer and incubated for 2 hours at room temperature.
- ii) Alkaline phosphatase conjugated goat-anti-human (or rabbit) IgG antibody (Sigma) diluted 1:2000-1:4000 in blocking buffer and incubated at room temperature for 2 hours.
- iii) <sup>125</sup>I-labelled donkey anti-rabbit IgG antibody (Amersham International), diluted in blocking buffer to give a specific activity of 0.2-0.35  $\mu$ Ci/ml and incubated for 3 hours at room temperature.

#### **2.17.3.1 Blocking protocol I**

The protocol followed was modified from that described by Thorens *et al.* (1988). Nitrocellulose filters were washed for 20 min in TBS containing 0.1% Tween-20. This



was followed by an incubation for 30 min at 37°C in blocking buffer containing 5% (v/w) non-fat dried milk (Marvel) and 0.2% (v/v) Nonidet P-40 (NP-40 - Sigma). At this stage the blocked filters could be air-dried and stored at -20°C until required. The blocked filters were shaken with rabbit or human serum overnight at room temperature in blocking buffer to which 0.05% (w/v) thiomerosal was added to inhibit microbial growth. The serum was used at dilutions ranging from 1:20-1:5000, although 1:500 was the standard dilution used.

Blots were washed (2x20 min) at room temperature in TBS containing 0.2% NP-40 and 0.1% Tween-20 respectively. The filters were then reblocked in the blocking buffer for 30 min at 37°C. Following an incubation with one of the secondary antibody conjugates listed above, the nitrocellulose was washed for 3x10 min in TBS containing 0.2% NP-40 and for 3x10 min in TBS/0.1% Tween-20. The blots were then developed as described in Section 2.17.4.

#### **2.17.3.2 Blocking protocol II**

This method was based on the protocol used by Dr. S. Cushman and co-workers (personal communication). Nitrocellulose filters were blocked for 1 hr at 37°C with TBS containing 3% (w/v) BSA. The filters were then shaken for  $\approx$ 2-16 hr with antiserum diluted 1:50-1:5000 in blocking buffer. The filters were washed in TBS alone (2x5 min), followed by washes with TBS containing: 0.1% (v/v) NP-40 (5 min), 0.2 M NaCl (5 min), and 0.1% (v/v) NP-40 (5 min). This was followed by two final 5 min washes in TBS alone. Following incubation with one of the antibody conjugates listed above, the same series of washes was repeated and the blots were developed as described in section 2.17.4.

### **2.17.3.3 Blocking protocol III**

This blocking protocol was essentially as recommended by Amersham International for use with the enhanced chemiluminescence (ECL) detection system.

The nitrocellulose was blocked for 1 hour at room temperature in TBS containing 5% (w/v) non-fat dried milk and 0.2 % (v/v) Tween-20. Antisera diluted 1:8000 in the blocking buffer were incubated with the nitrocellulose for a further hour at room temperature. The nitrocellulose was washed with TBS/0.2% Tween-20 (2x10 min), TBS/0.2 M NaCl (5 min) and finally in TBS/0.2% Tween (20 min). Bound antibodies were detected by incubation for 1 hour at room temperature with alkaline phosphatase conjugated goat-anti-rabbit IgG antibodies (Sigma), diluted 1:4000 in blocking buffer. Following washing as described above, the blots were developed using the enhanced chemiluminescence system.

### **2.17.4 Development of immunoblots**

#### **2.17.4.1 <sup>125</sup>I-labelled anti-IgG**

Following washing, the nitrocellulose was air-dried, wrapped in cling-film and exposed to photographic film at room temperature for 2-5 days. When quantification was required, the nitrocellulose blots were aligned with the developed film and 5 mM slices were cut from the region of interest on the blot. Radioactivity was measured in a gamma-counter.

#### **2.17.4.2 HRP-conjugated anti-IgG**

The nitrocellulose blots were washed briefly with 50 mM sodium acetate (pH 5.0) in double-distilled water. The substrate was freshly prepared by a 20 fold dilution in

sodium acetate buffer of a stock of 0.4% 3-amino-9-ethylcarbazole (AEC) in DMF. Hydrogen peroxide was added to give a final concentration of 1  $\mu\text{g/ml}$ . The blots were then incubated with the substrate at room temperature for about 30 min or until the pink-coloured bands appeared. The developed blots were then washed thoroughly with water.

#### **2.17.4.3 Alkaline phosphatase conjugated anti-IgG**

Two stock solutions were prepared, which were stable for about 1 year at 4°C.

- i) 0.5 g of Nitroblue Tetrazolium (NBT - Sigma) in 10 ml of 70% DMF.
- ii) 0.5 g of Bromochloroindyl phosphate p-toluidene salt (BCIP - Sigma) in 10 ml of DMF.

Immediately prior to use 66  $\mu\text{l}$  of NBT stock, 33  $\mu\text{l}$  of BCIP stock and 20  $\mu\text{l}$  of a 2 M  $\text{MgCl}_2$  stock were added to 10 ml of a 10% (w/w) diethanolamine buffer (pH 9.5). The nitrocellulose blot was shaken in this solution until purple bands appeared, usually within 5-10 min. The blots were then washed thoroughly with water.

#### **2.17.4.4 Enhanced chemiluminescence (ECL)**

Blots were exposed to the chemiluminescent substrate for 1 min as recommended by the manufacturers (Amersham International). They were then wrapped in cling film and exposed to ECL film (Amersham International) for 10-30 s.

### **2.18 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)**

An ELISA was used to confirm the presence of anti-GLUT2 peptide antibodies in serum samples from immunized rabbits. ELISAS were also developed to screen serum samples from type I diabetic patients for the presence of anti- $\beta$ -cell surface antibodies.

### 2.18.1 Islet cell surface antibody ELISAS

Flat bottomed polystyrene microtitre plates (Titertek, Flow ICN) were coated with  $\beta$ -cell line plasma membrane antigens by the addition to each well of 100  $\mu$ l of a 10  $\mu$ g/ml membrane protein suspension in 50 mM NaHCO<sub>3</sub> buffer (pH 9.6). The plates were covered and incubated overnight at 4°C. Membranes were prepared from HITm2.2 cells using either method I or II described in sections 2.10.4.1 and 2.10.4.2 respectively; details are given in the figure legends. Membranes were prepared from MIN 6 cells using method I described in section 2.10.4.1. Some serum samples were also assayed against liver plasma membranes prepared as described in section 2.10.4.1.

Excess antigen was removed by sharply tapping the inverted plate. Each well was washed (3x10 min) with 200  $\mu$ l of PBS/0.1% (w/v) casein/0.05% Tween-20 (Kenna *et al.* 1982). Serum, diluted 1:100 in PBS/1% casein/0.05% Tween was added (100  $\mu$ l/well) and incubated for 2 hours at 37°C. Each sample was tested in triplicate on one plate and on 1-5 separate occasions. Samples were coded so that their identity was unknown at the time of assaying.

A standard serum serially diluted 1:12.5-1:12589 was used to obtain a standard curve which was included on every plate. This enabled absorbance values of test sera to be expressed as a percentage of the absorbance given by the standard serum, allowing comparisons to be made between results obtained on different occasions. A reference sample was also assayed on every plate and it was checked to see that its titre did not deviate greatly from the mean.

After incubation, the plate was washed as previously described and 100  $\mu$ l of HRP-conjugated goat-anti-human IgG antibodies were added to each well (diluted 1:1000 in PBS/1% casein/0.05% Tween). After incubation for 1-1.5 hours at 37°C the wells were washed 3 times with PBS/0.05% Tween and 100  $\mu$ l of substrate were added per well. The

substrate was prepared from TMB (3,5,3',5'-tetra-methylbenzidine) dissolved in DMSO to give a 1% (w/v) stock solution. This stock was then diluted 1:100 in 0.1 M sodium acetate/citrate buffer prepared in double-distilled water (pH 6.0) and hydrogen peroxide was added to a concentration of 0.25  $\mu$ l/ml.

The colour was allowed to develop for 10-30 min, then the reaction was stopped by the addition of 50  $\mu$ l of 2 M  $H_2SO_4$ . Absorbances were measured at 450 nm using a Titertek Multiskan MCC (Flow ICN) and were corrected for non-specific conjugate binding by measuring against a blank in which antiserum was omitted.

### **2.18.2 Anti-GLUT2 peptide antibody ELISAS**

The procedure was identical to that described above, except that the plates were coated either with 10  $\mu$ g/ml of liver plasma membranes or 0.2  $\mu$ g/ml of peptide. All washings and incubations were carried out using PBS/0.1% casein/0.05% Tween. Rabbit sera were typically tested in duplicate at dilutions from 1:50-1:6400. Binding was compared with that shown by the serum taken from the same animal prior to immunization. The second antibody used was HRP-conjugated goat anti-rabbit IgG at a 1:1000 dilution.

## **2.19 STATISTICAL ANALYSIS**

The Kolmogorov-Smirnov (K-S) two sample test (Siegel, 1956) was used to compare levels of antibodies in type I diabetic patients and non-diabetic controls. The K-S test is a non-parametric test which makes no assumptions about the distributions from which the samples were drawn (Sprent, 1981). It is also suitable for the analysis of a small number of samples (Siegel, 1956).

A cumulative frequency distribution was calculated for control and diabetic samples using the same intervals for both distributions. By subtraction of one step function

from the other, the two tailed K-S test identifies the part of the distribution in which the maximum difference ( $D_{\max}$ ) occurs. By comparison with a theoretical value of  $D$ , which varies depending on the number of samples, it could be determined whether there was any significant difference in the population distributions from which the samples were drawn.

Level of significance	Critical value of $D$	
$p = 0.1$	$1.22 \sqrt{\frac{n_1 + n_2}{n_1 \cdot n_2}}$	$n_1 = \#$ in sample 1 $n_2 = \#$ in sample 2
$p = 0.05$	$1.36 \sqrt{\frac{n_1 + n_2}{n_1 \cdot n_2}}$	
$p = 0.01$	$1.63 \sqrt{\frac{n_1 + n_2}{n_1 \cdot n_2}}$	

The null hypothesis that the 2 samples came from the same population was rejected if  $D_{\max}$  exceeded the theoretical value of  $D$  at the required level of significance.

### 3.0 RESULTS

#### **PART I CHARACTERIZATION OF LIVER GLUT2 USING ATB-BMPA AND ANTI-PEPTIDE ANTIBODIES**

It has previously been shown that the exofacial photoprobe ATB-BMPA {2-N-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)-propyl-2-amine} can be used to specifically label the glucose transporter isoforms GLUT1 (Clark & Holman, 1990) and GLUT4 (Holman *et al.*, 1990). The aim of the present investigation was to demonstrate that ATB-BMPA could be used to label GLUT2, thereby providing an alternative to the [<sup>3</sup>H]cytochalasin B photolabel, which has a low affinity for GLUT2 and the use of which is limited to studies of membranes rather than whole cells (Ciaraldi *et al.*, 1986; Hellwig & Joost, 1991).

This section of results describes the use of ATB-BMPA to label GLUT2 and the optimization of the labelling and immunoprecipitation conditions. The work involved the production of anti-GLUT2 peptide antibodies and the demonstration that they specifically recognised GLUT2 in liver plasma membranes.

The characteristics of GLUT2 have been compared with those of the other transporter isoforms and results produced and techniques developed which could potentially be used to detect GLUT2 in  $\beta$ -cells (Part II), and putative anti-GLUT2 autoantibodies in the serum of patients with IDDM (Part III).

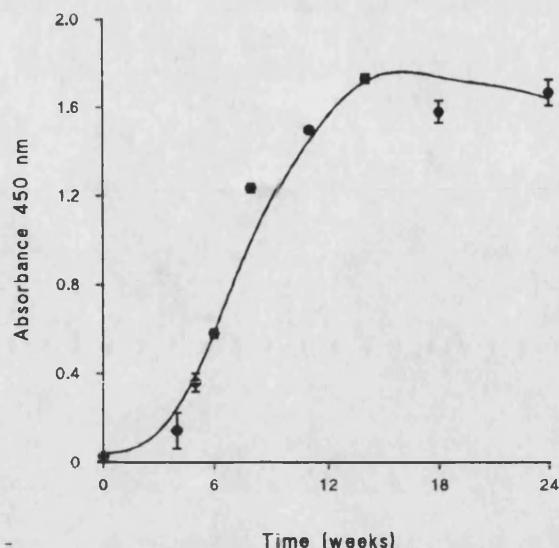
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### 3.1 PRODUCTION OF ANTI-GLUT2-PEPTIDE ANTIBODIES

The immune response of rabbits immunized with GLUT2 peptides was monitored by testing each serum sample in an ELISA using the immunising peptide as the antigen. A typical response is shown in Figure 5. The IgG titre increased rapidly up to about week 14 when a maximum response was attained. Regular boosting maintained the titre at this level. All antisera contained antibodies that bound strongly to the peptide against which they were raised, but not all peptides generated equally high titres of antibodies able to recognise the intact GLUT2 protein.

The levels of antibodies binding to the GLUT2 protein were estimated from ELISAS in which liver membranes were used as the antigen. Western blotting was then used to confirm that the membrane antigen recognised was GLUT2, and that the antisera were specific for this protein. The ability to detect anti-GLUT2 antibodies by ELISA and Western blotting was dependent on the method used to prepare the membranes. When crude liver membranes were used (section 2.10.3.2), no specific binding of any anti-rat GLUT2 peptide antiserum could be detected either in an ELISA or in a Western blot (results not shown). However, antibodies raised against the human-GLUT2-C-terminal peptide sequence did bind to a protein in the crude liver membranes, which had an apparent molecular mass of  $\approx 64-66$  kDa. This protein was not however GLUT2, since a band of the same molecular mass was also recognised by this serum in membranes from cells known not to contain GLUT2, for example, human erythrocytes and adipocytes (results not shown). The identity of the 64-66 kDa protein was not known, but it was not recognised when the anti-human GLUT2 antibody was tested against purified liver plasma membranes (Figure 6).

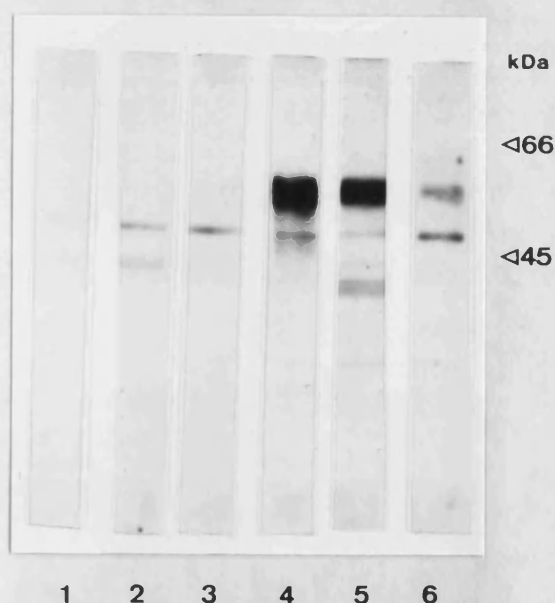




**Fig. 5** Typical rabbit IgG response to a GLUT2 C-terminal peptide-KLH conjugate administered with adjuvant.

Rabbit antiserum (1:100 dilution) was tested in an ELISA in which the peptide antigen was coated onto the plates at a concentration of 0.2  $\mu\text{g/ml}$ . The results are means obtained by assaying samples in triplicate. The error bars shown represent  $\pm$  S.E.M.

**Fig. 6** Immunochemical detection of GLUT2 in purified rat liver plasma membranes



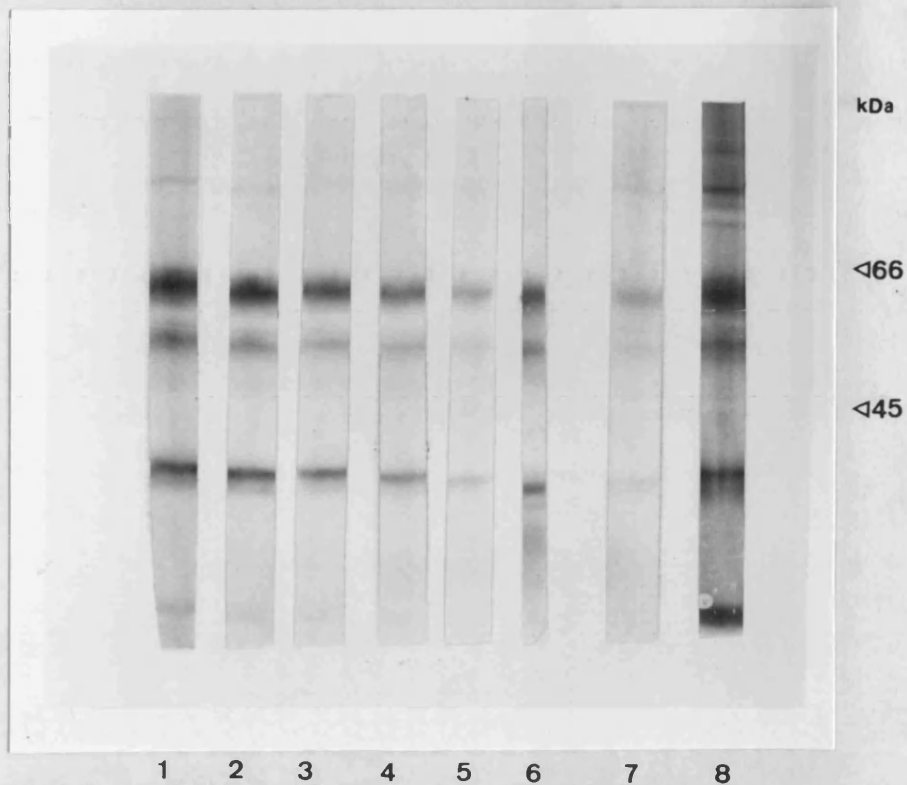
Proteins from rat liver plasma membranes were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted using blotting protocol III and the ECL detection system. The antisera used were:

1. preimmune serum
2. anti-rat GLUT1 C-terminal peptide
3. anti-rat GLUT2 C-terminal peptide (B) preabsorbed by incubation overnight with peptide (B)
4. anti-rat GLUT2 C-terminal peptide (B)
5. anti-rat GLUT2 exofacial-loop peptide
6. anti-human GLUT2 C-terminal peptide .

All anti-GLUT2 antisera detected a band with an apparent molecular mass of  $\approx 57$  kDa. This was recognised more strongly by antisera raised against the rat sequence than the human sequence. A non-specific band with an apparent molecular mass of  $\approx 51$  kDa was also recognised by all sera. In addition the anti-GLUT1 antibody and the anti-GLUT2 exofacial loop antibody detected non-specific bands with apparent molecular weights of 48 and 47 kDa respectively.

Liver plasma membranes were prepared as described in section 2.10.3.1. The 5' nucleotidase activity was consistent between different batches of membrane and did not decrease during the time that the membranes were stored at  $-70^{\circ}\text{C}$ . The value obtained for the activity of this enzyme at  $37^{\circ}\text{C}$  was  $6.15 \mu\text{mol}/\text{mg protein}/30 \text{ min}$ . Membranes prepared by this method did contain immunodetectable GLUT2 as demonstrated by the binding of anti-GLUT2 peptide antisera in ELISAS and Western blots.

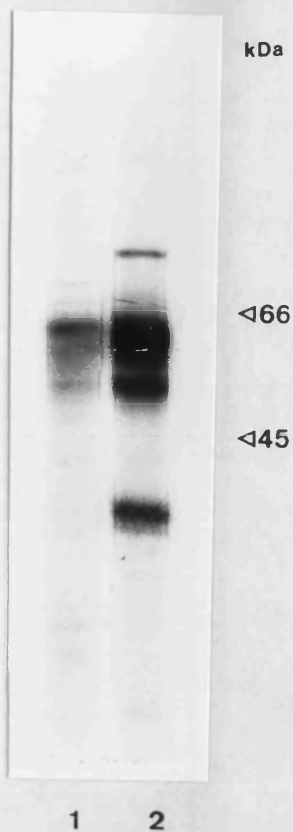
Figure 6 shows the results obtained by Western blotting liver plasma membranes with the antisera that were used in immunoprecipitation experiments. The major band recognised by both the anti-rat GLUT2 C-terminal peptide (B) antiserum and the anti-rat GLUT2 exofacial loop peptide antiserum had an apparent molecular mass of about 57 kDa. Antibodies directed against this protein could be removed by pre-absorption with the immunising peptide. The same protein was recognised, albeit less strongly, by the anti-human GLUT2 C-terminal peptide (D) antiserum. In addition to the major 57 kDa protein, anti-GLUT2 peptide antisera also detected other proteins in liver plasma membranes with apparent molecular weights of  $\approx 81, 51, \text{ and } 36 \text{ kDa}$ . As shown in Figure 7, this was evident particularly when high concentrations of serum were used for blotting. The 81 kDa band appeared to be recognised non-specifically since antibodies directed against it were not removed by pre-absorption with the immunizing peptide (results not shown). The other bands were recognised by antibodies directed specifically against the peptide, since they were detected by peptide-affinity-purified antibody (Figure 7, lane 6) and could largely be removed by pre-absorption with the immunising peptide. However, as shown in Figure 6 some of the binding to the 51 kDa band may have been non-specific since this protein was also recognised by anti-GLUT1 and preimmune serum. The nature of these minor bands was unknown. The strong 57 kDa band appeared to be the whole GLUT2 protein since a band of the same apparent molecular mass was also detected in rat islets (Figure 8).



**Fig. 7** Western blotting of liver plasma membrane with antisera raised against GLUT2 C-terminal peptides A, B and C.

Proteins from rat liver plasma membranes (section 2.10.3.1) were separated by SDS-PAGE in sample buffer containing 5 mM DTT instead of mercaptoethanol, transferred to nitrocellulose using a wet transfer method and immunoblotted using blotting protocol II (2.17.3.2). Blots were developed with an alkaline phosphatase conjugated anti-rabbit IgG antibody.

Lanes 1-5 were blotted with anti-rat GLUT2 C-terminal peptide (B) antiserum at dilutions of 1:100, 1:500, 1:1000, 1:2000 and 1:5000. Lane 6 was blotted with 2  $\mu\text{g/ml}$  of affinity purified C-terminal peptide antiserum. Lane 7 was blotted with a 1:500 dilution of anti-rat GLUT2 C-terminal peptide (A) antiserum, and lane 8 with a 1:500 dilution of anti-rat GLUT2 C-terminal peptide (C) antiserum. All sera were obtained from rabbits when the anti-peptide antibody titre was at its peak. All antisera detected three bands with apparent molecular weights of  $\approx 57$  kDa,  $\approx 51$  kDa and  $\approx 36$  kDa. The  $\approx 57$  kDa band was still detected at a 1:5000 dilution, the other bands were recognised less strongly at this dilution. In comparison with peptide A, peptides B and C produced higher titres of GLUT2-binding antibodies.



**Fig. 8 Comparison of the apparent molecular mass of GLUT2 in liver and islets.**

Proteins from rat liver plasma membranes (lane 2) and whole rat islets (lane 1) were solubilized in electrophoresis sample buffer containing 5 mM DTT instead of mercaptoethanol and were separated by SDS-PAGE then transferred to nitrocellulose using a wet transfer method. Proteins were immunoblotted with anti-GLUT2 C-terminal peptide (B) antiserum at a 1:500 dilution using blotting protocol II (methods section 2.17.3.2). Binding was detected by incubation with 0.2  $\mu$  Ci/ml  $^{125}$ I of anti-rabbit IgG conjugated antibody. A strong broad band was detected at  $\approx$ 57 kDa in both liver and islets. The serum also detected bands at  $\approx$ 35 kDa, 51 kDa and 81 kDa in liver plasma membranes.

	Anti-GLUT2 antiserum				
	Rat C-terminal peptide A	Rat C-terminal peptide B	Rat C-terminal peptide C	Human C-terminal peptide D	Rat loop peptide
Numbers of animals immunized	3	3	1	1	2
% increase in absorbance of test serum compared to a preimmune control	54, 60, 32 mean=49	113, 274, 78 mean=155	200	45	3, 17 mean=10

**Table 4 Comparison of the levels of anti-rat liver plasma membrane antibodies in four different anti-GLUT2 peptide antisera.**

Anti-GLUT2 peptide antisera were tested in an ELISA in which liver plasma membranes (10  $\mu$ g/ml) were used as the antigen. All sera were obtained from rabbits when the anti-peptide antibody titre was at its peak. Values for the percentage increase in antibody binding (measured as absorbance at 450 nm) of test serum (1:100 dilution) compared to a preimmune control (1:100 dilution) are shown for each individual animal and a mean value is also given for all antisera raised against the same immunogen. The results show variability in the response of individual animals to the same immunogen. It is also shown that antisera raised against the C-terminal peptides B and C produce antibodies that bind strongly to liver plasma membranes.

The anti-rat GLUT2 antisera were all specific for GLUT2 and did not bind to any proteins in 3T3-L1 adipocytes or human erythrocytes, therefore they did not recognise either the GLUT1 or GLUT4 transporter isoforms. This is illustrated in Figure 24a-d.

As shown in Table 4 not all of the rat GLUT2 peptides produced equally high levels of antibodies recognising the whole GLUT2 protein. The antiserum raised against the loop peptide reacted very weakly with liver plasma membranes in an ELISA and although antibodies were present which recognised the denatured GLUT2 transporter in a Western blot, a very sensitive ECL detection system was required to detect them (Figure 6). They could not be detected using an alkaline-phosphatase colour detection system (results not shown). Sera raised against the rat C-terminal peptides B and C (residues 507-522 and 499-508 respectively) produced the highest titres of antibodies binding to liver plasma membranes in an ELISA (Table 4). These antisera also bound strongly to GLUT2 in a Western blot (Figure 7). Peptide A (residues 510-522) appeared to be a poor immunogen, since it produced antiserum containing only low levels of antibodies that bound to GLUT2 in either ELISAS (Table 4) or Western blots (Figure 7).

The regions of the peptide sequences that appeared to be most important in forming immunogenic epitopes were identified using peptide ELISAS. As shown in Table 5, all antisera contained antibodies that bound strongly to the peptide against which they were raised, this is indicated by a >2000% increase in antibody binding in the test serum compared to a preimmune control. Although peptides A (510-522) and B (507-522) differed only in that peptide B contained an additional 3 residues, less than half of the antibodies that were raised against peptide A recognised peptide B and vice versa. Antiserum raised against peptide A also showed negligible binding to peptide D (human 507-524), although 8/13 residues were identical. Although peptides B and D shared only 10/16 amino acids, a high percentage of antibodies were present in antiserum B that recognised peptide D and vice versa.

		% increase in binding of test antiserum (1:100 dilution) to test peptide compared to a preimmune control (1:100). Mean $\pm$ S.E.M. (n).		
Peptide	Test peptide sequence	Antiserum to peptide A	Antiserum to peptide B	Antiserum to peptide D
A (Rat 510-522)	T V Q M E F L G S S E T V	2059 $\pm$ 371 (3)	1008 $\pm$ 518 (3)	100 $\pm$ 20 (3)
B (Rat 507-522)	R K A T V Q M E F L G S S E T V	553 $\pm$ 155 (3)	2621 $\pm$ 312 (3)	639 $\pm$ 205 (3)
D (Human 507-524)	H R P K A A V E M K F L G A T E T V * * * * * * * * * *	172 (1)	2207 (1)	3552 (1)

**Table 5 Comparison of the cross-reactivity of three GLUT2 anti-C-terminal peptide antisera with the three corresponding GLUT2 C-terminal peptides.**

Antisera were tested in an ELISA in which plates were coated with the appropriate peptide at a concentration of 0.2  $\mu$ g/ml. The number of samples tested from different animals is shown in parentheses. The \* indicates amino acids in the human sequence that are different from the corresponding residues in rat GLUT2. The values given show the average ( $\pm$ S.E.M) percentage increase in binding (measured as absorbance at 450 nm) given by a 1:100 dilution of the test serum compared to the same dilution of a preimmune control. Very high binding antisera gave values  $>2000\%$ , high binding sera gave values of  $\approx 500-1000\%$  and sera that gave values  $<200$  were considered to show negligible binding to the test peptide.

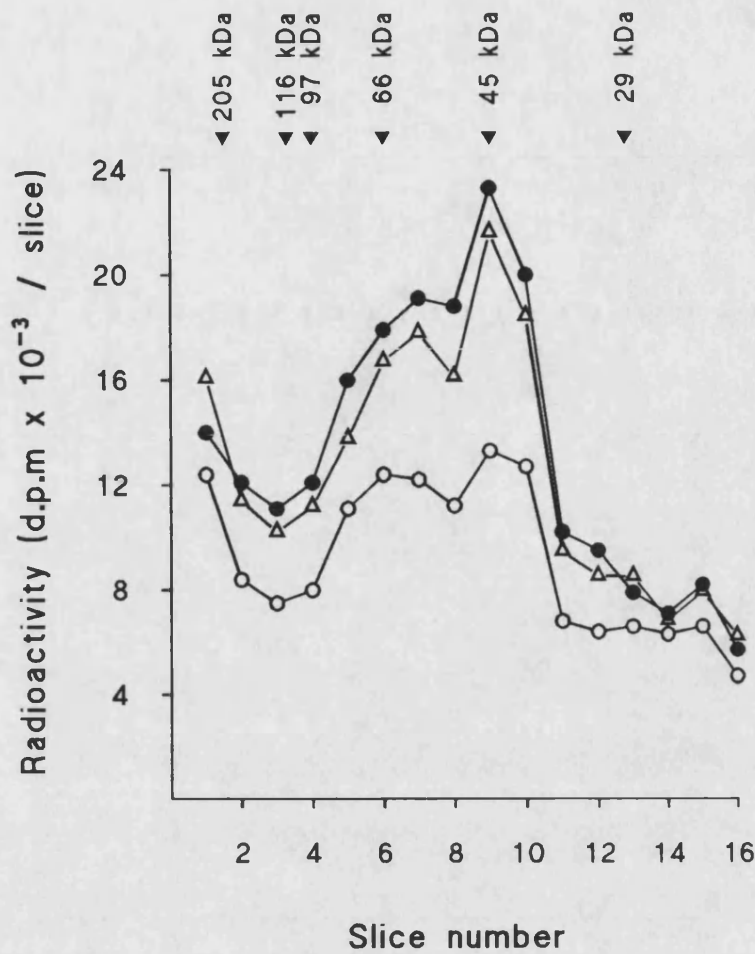
Neither of these sera bound as strongly to peptide A. The only residues common to peptides B and D not also shared with peptide A were the arginine and lysine residues at positions 507 and 508 (rat) and 510 and 511 (human). Therefore suggesting that these residues formed part of a strongly immunogenic region of the GLUT2 C-terminal peptide.

Since antibodies against rat-GLUT2 C-terminal peptide B produced a strong, relatively specific, response to the 57 kDa GLUT2 protein, this was the antiserum chosen for routine use in the following investigation of GLUT2.

### **3.2 ATB-BMPA PHOTOLABELLING OF RAT LIVER PROTEIN**

#### **3.2.1 Photolabelling of liver plasma membranes**

When ATB-[2-<sup>3</sup>H]-BMPA was irradiated in the presence of purified rat liver plasma membranes, analysis of the proteins by SDS-PAGE revealed that the photolabel was incorporated into a protein (or proteins) which migrated as a broad band with an apparent molecular mass of 45-66 kDa. This was usually detected as a single peak, occasionally as shown in Figure 9, as a double peak. This variability probably arose from the method used to slice the gels, which did not enable two very close peaks to be resolved all of the time. The second peak may have been due to labelling of the  $\approx 51$  kDa protein detected by anti-GLUT2 antibodies in Western blots of liver plasma membranes. ATB-BMPA could be displaced from liver plasma membranes by  $43 \pm 1.6\%$  (mean and S.E.M. from three independent experiments) when irradiation was carried out in the presence of 500 mM 4,6-0-ethylidene-D-glucose. From two independent experiments it was demonstrated that the addition of 500 mM D-glucose did not significantly reduce the incorporation of ATB-BMPA. 500 mM D-mannitol, included as a control, also caused no displacement of the photolabel. The result of a typical displacement experiment is shown in Figure 9.



**Fig. 9** Photolabelling of liver plasma membranes with ATB-BMPA.

Liver plasma membranes were photolabelled by irradiation in the presence of ATB-BMPA. After washing by centrifugation, labelled proteins were analysed by SDS-PAGE and a broad peak of labelled protein with an apparent molecular mass of  $\approx 45\text{--}66$  kDa was obtained. In the illustrated example labelled proteins appeared as a double peak, in replicate experiments a single peak has also been obtained. Arrowheads indicate the position of molecular weight marker proteins. Labelling in the presence of 500 mM D-mannitol (●), which was included as a control, or 500 mM D-glucose ( $\Delta$ ) caused no significant displacement of ATB-BMPA. Irradiation in the presence of 500 mM 4,6-O-ethylidene-D-glucose (○) inhibited ATB-BMPA binding to liver plasma membranes by 43%.



### 3.2.2 Immunoprecipitation of ATB-BMPA labelled proteins from liver plasma membranes

In order to identify the proteins labelled by ATB-BMPA in liver plasma membranes, immunoprecipitations were carried out using antibodies raised against short peptide sequences of GLUT1 and GLUT2. The specificity of these antisera was determined by Western blotting as described in Section 3.1.

When used to immunoprecipitate photolabelled liver plasma membrane proteins solubilized in buffer containing the detergent C<sub>12</sub>E<sub>9</sub> (nonaethylene glycol dodecyl ether), both the anti-rat-GLUT2 C-terminal peptide (B) and the anti-rat-GLUT2 exofacial loop peptide antisera, immunoprecipitated approximately equivalent amounts of a labelled protein with an apparent molecular weight of 45-66 kDa. Anti-human GLUT2 C-terminal peptide antiserum precipitated only about half of the amount of the same labelled protein that was precipitated by the rat GLUT2 antisera. These results are shown in Figure 10a.

In order to establish whether the 45-66 kDa precipitated protein was recognised specifically by anti-GLUT2 peptide antibodies, or non-specifically by antibodies present in normal rabbit serum, preimmune serum was used to immunoprecipitate ATB-BMPA labelled proteins from liver plasma membrane. As shown in Figure 10b, non-immune serum precipitated a small amount of labelled protein with a similar molecular mass to that of the protein precipitated by the anti-GLUT2 antiserum. On average, this non-specific immunoprecipitation, was calculated to account for  $32 \pm 9.2\%$  (mean and S.E.M. from six independent experiments) of the total amount of protein precipitated by anti-rat-GLUT2 C-terminal-peptide antiserum. All results were therefore corrected to account for this non-specific precipitation.

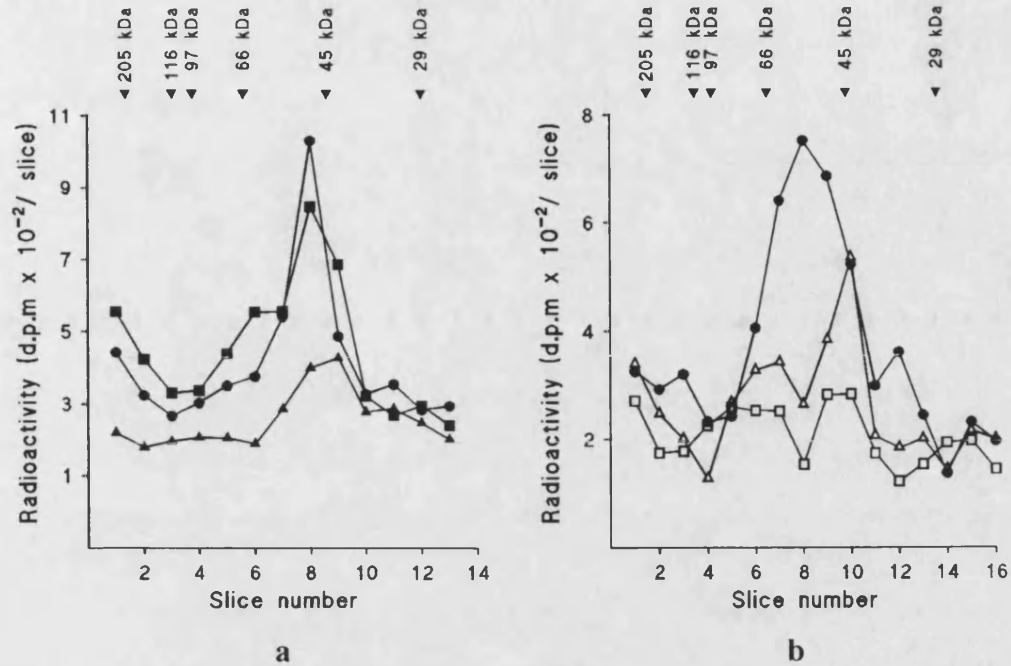


Fig. 10a-b Immunoprecipitation of photolabelled liver plasma membrane proteins.

Liver plasma membranes were photolabelled by irradiation in the presence of ATB-BMPA, washed then solubilized in buffer containing the detergent  $C_{12}E_9$ . Solubilized labelled proteins were immunoprecipitated with 5 mg of Protein A-Sepharose coupled to 50  $\mu$ l of the indicated anti-GLUT2 antisera: Labelled immunoprecipitates were analysed by SDS-PAGE. Arrowheads indicate the position of molecular weight marker proteins.

- a:** Anti-rat GLUT2 C-terminal peptide antiserum (●)  
 Anti-rat GLUT2 exofacial loop peptide antiserum (■)  
 Anti-human GLUT2 C-terminal peptide antiserum (▲)

Both of the anti rat-GLUT 2 antisera precipitated a similar amount of a protein with an apparent molecular weight of 45-66 kDa. The anti-human GLUT2 antiserum immunoprecipitated approximately 60% of the amount of the same labelled protein that was precipitated by the anti-rat GLUT2 antisera.

- b:** Anti-rat GLUT2 C-terminal peptide antiserum (●)  
 Anti-rat GLUT1 C-terminal peptide antiserum (Δ)  
 Preimmune serum (□)

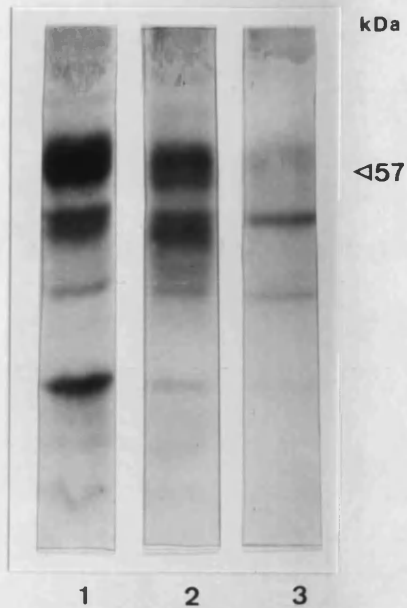
All sera precipitated some labelled protein with an apparent molecular mass of 45-66 kDa. In the illustrated experiment non-specific immunoprecipitation (as determined from the amount of protein precipitated with non-immune rabbit serum), accounted for  $\approx 27\%$  of the labelled protein precipitated by the anti-GLUT2 antiserum. When corrections were made to account for this non-specific precipitation, anti-GLUT2 serum precipitated  $\approx 3$  fold more labelled protein than anti-GLUT1 serum.

Anti-GLUT1 C-terminal peptide antiserum also immunoprecipitated radiolabelled liver protein with an apparent molecular weight of 45-66 kDa. The quantity precipitated was  $47 \pm 13.9\%$  (mean and S.E.M. from four independent experiments) of the amount precipitated by anti-GLUT2 antiserum. However, when corrected for non-specific immunoprecipitation only 15% of the protein precipitated by anti-GLUT1 antiserum was specific compared with 68% of that precipitated by anti-GLUT2 antiserum. The average ratio of immunoprecipitable GLUT2 to GLUT 1 was therefore approximately 5:1. This confirmed the results obtained by immunoblotting, that GLUT1 was not a major component of liver plasma membrane (Figure 6).

### 3.2.3 Quantification of the recovery of immunoprecipitated GLUT2

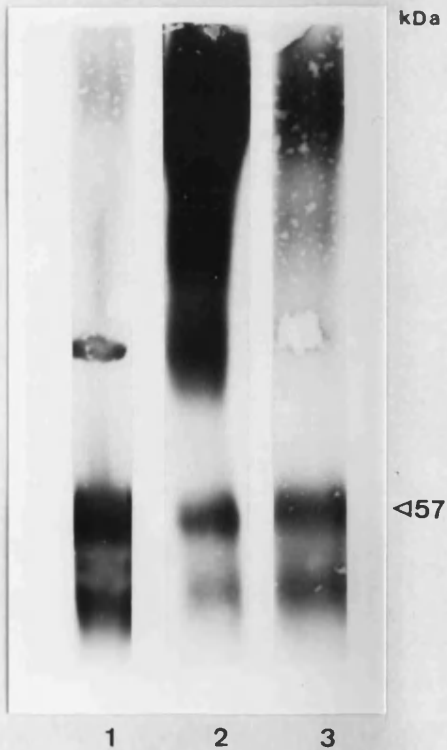
The amount of GLUT2 immunoprecipitated was low. On average only  $4.6 \pm 0.87\%$  (mean and S.E.M from five independent experiments) of the 45-66 kDa proteins labelled by ATB-BMPA in whole liver plasma membranes, were immunoprecipitated by anti-GLUT2 C-terminal peptide antiserum. Corrections were made to account for the fact that 32% of the labelled protein was also precipitated non-specifically by preimmune serum and that only 43% of the labelling was displaceable with 4,6-0-ethylidene-D-glucose. It was therefore calculated that approximately 7% of the proteins labelled in a 4,6-0-ethylidene-D-glucose displaceable manner were immunoprecipitated by the anti-GLUT2 C-terminal peptide antiserum. The above calculation assumes complete detergent solubilization of photolabelled GLUT2. The possibility was considered that the low precipitation of GLUT2 could have resulted from incomplete solubilization of GLUT2 in the C<sub>12</sub>E<sub>9</sub> detergent. However, as shown in Figure 11, when analysed by Western blotting, an approximately equivalent amount of GLUT2 was detected in both whole and detergent-solubilized liver plasma membranes. Solubilized proteins were precipitated with chloroform and methanol, which enabled them to be analysed by SDS-PAGE.

**Fig. 11 Detection of GLUT2 in detergent-solubilized liver plasma membranes.**



Liver plasma membranes (50  $\mu\text{g}$ ) were solubilized in 1 ml of buffer containing 2% of the detergent  $\text{C}_{12}\text{E}_9$ . The insoluble material was removed by centrifugation and analysed directly by SDS-PAGE (lane 3). The solubilized proteins were precipitated by treatment with chloroform and methanol (section 2.15.4) and analysed by SDS-PAGE (lane 2). As a control 50  $\mu\text{g}$  of liver plasma membranes were also analysed by SDS-PAGE (lane 1). Proteins were transferred electrophoretically to nitrocellulose and immunoblotted with anti-GLUT2 C-terminal peptide antiserum using the ECL method of detection. GLUT2 was detected as a 57 kDa band in liver plasma membranes and solubilized liver plasma membranes but was not present in the non-solubilized fraction.

**Fig. 12 Investigation by Western blotting of the efficiency of GLUT2 immunoprecipitation.**



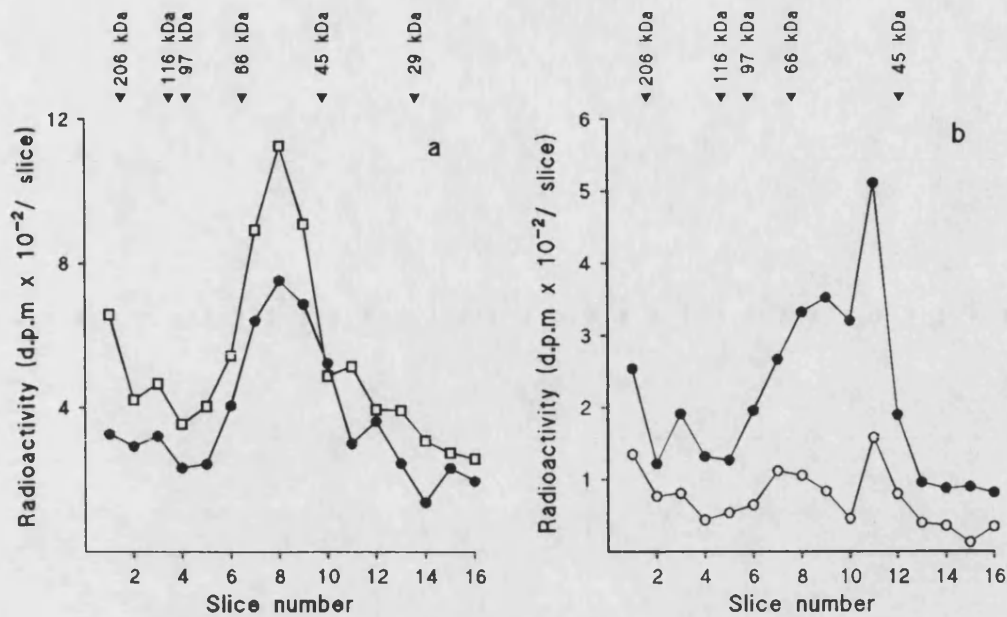
Liver plasma membranes were solubilized in buffer containing the detergents Triton X-100 (4%) and SDS (0.05%). Solubilized proteins were immunoprecipitated with 5 mg of Protein A-Sepharose coupled to 50  $\mu\text{l}$  of anti-GLUT2 C-terminal peptide antiserum. Liver plasma membrane (lane 1), immunoprecipitated proteins (lane 2) and supernatant (lane 3) were analysed by electrophoresis under non-reducing conditions on an 8% polyacrylamide gel. Following electrophoretic transfer of the proteins to nitrocellulose, samples were immunoblotted with anti-GLUT2 C-terminal peptide antiserum (1:500 dilution) following protocol II (section 2.17.3.2) and developed with alkaline phosphatase conjugated anti-rabbit IgG antibodies. GLUT2 was detected in both the immunoprecipitate and supernatant as a  $\approx 57$  and 51 kDa band. The colourimetric method of detection used did not allow quantification of the relative amounts of GLUT2 present in each sample.

GLUT 2 could not however be clearly detected in the non-solubilized protein fraction, indicating that under the conditions used, GLUT2 was almost completely solubilized by the detergent C<sub>12</sub>E<sub>9</sub>.

Using Western blotting it was shown that immunodetectable GLUT2 was immunoprecipitated by anti-GLUT2 C-terminal peptide antiserum, although GLUT2 could also be detected in the supernatant that remained following the immunoprecipitation. These results were obtained by electrophoresis under non-reducing conditions on 8% polyacrylamide gels, conditions that largely enabled the broad band of IgG to be separated from the lower molecular weight GLUT2 band. However, the presence of large quantities of IgG gave such a high background on Western blots developed with a <sup>125</sup>I detection system that it prevented the quantification of the total amount of GLUT2 precipitated. Although not quantitative, the results shown in Figure 12, which is a typical example of other similar experiments in which proteins were solubilized in buffers containing either 2% C<sub>12</sub>E<sub>9</sub> or 4% Triton X-100 and 0.05% SDS, clearly show that the immunoprecipitation of GLUT2 was incomplete. The same result was obtained whichever detergent was used.

Comparable results were obtained using ATB-BMPA photolabelling to quantify the amount of GLUT2 precipitated following solubilization of liver plasma membrane proteins in different detergents. There was no significant difference in the amount of GLUT2 precipitated when proteins were solubilized in 1% n-octyl β-D-glucopyranoside, 2% C<sub>12</sub>E<sub>9</sub> or 4% Triton X-100 and 0.05% SDS (results not shown).

It was also attempted to increase the amount of GLUT2 immunoprecipitated, by increasing the amount of precipitating antibody. However, a 4-fold increase in the amount of Protein A-Sepharose-antibody conjugate used, resulted in only a further 1.1 fold increase in the amount of labelled protein precipitated by anti-GLUT2 antiserum (Figure 13a).



**Fig. 13a-b Immunoprecipitation of ATB-BMPA labelled GLUT2 from liver plasma membranes: The effect of increasing the amount of anti-GLUT2 antiserum.**

Liver plasma membrane proteins were photolabelled with ATB-BMPA, washed then solubilized in buffer containing the detergent C<sub>12</sub>E<sub>9</sub>. Solubilized labelled proteins were immunoprecipitated and analysed by SDS-PAGE. Arrowheads indicate the position of molecular weight marker proteins.

**a:** Proteins were precipitated with:

5 mg of Protein A-Sepharose coupled to 50 μl of anti-GLUT2 C-terminal peptide antiserum (●)

20 mg Protein A-Sepharose coupled to 200 μl of anti-GLUT2 C-terminal peptide antiserum (□)

Increasing the amount of Protein A-Sepharose and immunoprecipitating antibody 4-fold resulted in a ≈ 10% increase in the amount of immunoprecipitated GLUT2 protein.

**b:** Solubilized labelled proteins were immunoprecipitated for 2.5 hours with 5 mg of Protein A-Sepharose coupled to 50 μl of anti-GLUT2 C-terminal peptide antiserum (●). The supernatant was then incubated for a further 2.5 hours with another 5 mg of Protein A-Sepharose coupled to GLUT2 antiserum (○). The anti-GLUT2 antibody removed 82% of the total immunoprecipitated labelled protein after a single treatment.

As illustrated in Figure 13b, most of the labelled protein was precipitated in a single treatment with 5 mg of Protein A-Sepharose coupled to 50  $\mu$ l of anti-GLUT2 antiserum. A second treatment with the same amount of antibody removed an additional quantity of labelled protein equivalent to 18% of that already precipitated.

ATB-BMPA was also used to label viable rat hepatocytes. A broad band of protein with an apparent molecular mass of 45-66 kDa was radiolabelled, solubilized in the detergent C<sub>12</sub>E<sub>9</sub> and immunoprecipitated with an anti-GLUT2 antibody. The maximum amount of GLUT2 precipitated was equivalent to about 2% of the total amount of solubilized labelled protein (results not shown).

#### **3.2.4 Displacement of ATB-BMPA from immunoprecipitated GLUT2**

As shown in Figure 14 and Table 6, ATB-BMPA binding to, and labelling of, GLUT2 could be reduced by using several inhibitors of glucose transport. When corrected for non-specific immunoprecipitation, 500 mM 4,6-O-ethylidene-D-glucose displaced 92% of the ATB-BMPA binding to precipitable GLUT2. 500 mM D-fructose, 100  $\mu$ M cytochalasin B and 500 mM D-glucose displaced 77%, 65% and 57% respectively of the ATB-BMPA binding to GLUT2. The amount of labelled protein precipitated when photolabelling was carried out in the presence of 100  $\mu$ M phloretin was not significantly different from the amount of labelled protein precipitated non-specifically by preimmune serum. Therefore, 100  $\mu$ M phloretin was able to displace all of the specific ATB-BMPA binding to liver GLUT2.

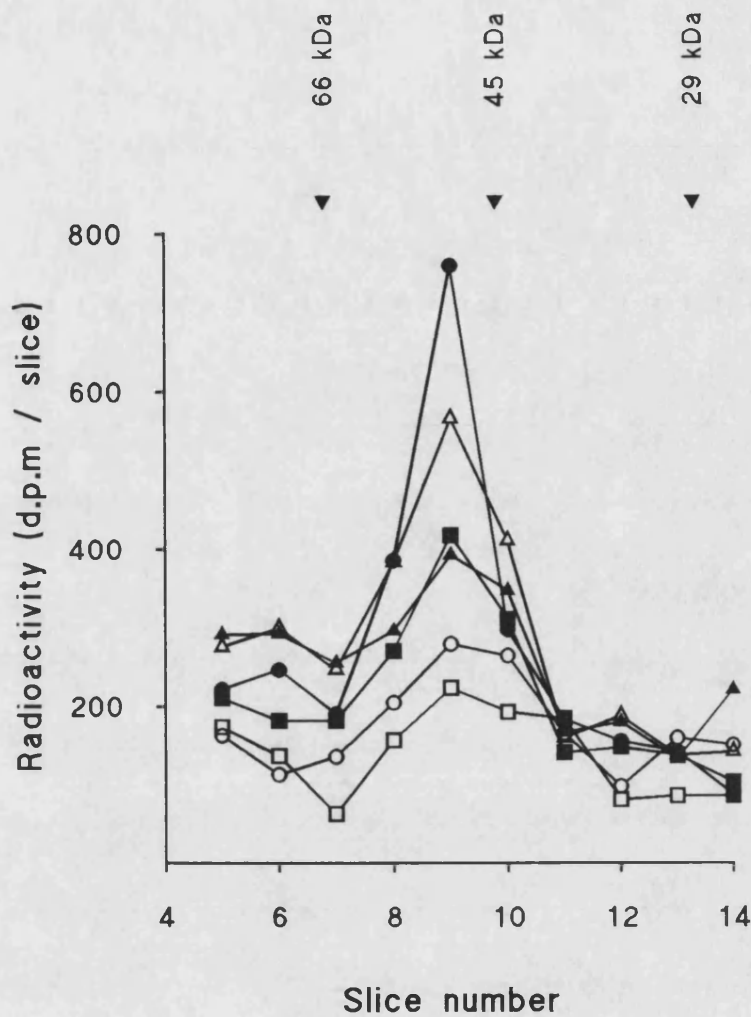


Fig. 14 Displacement of ATB-BMPA from immunoprecipitates of liver GLUT2.

Liver plasma membranes were irradiated in the presence of ATB-BMPA and the following compounds which inhibit glucose transport or bind to the transport protein. 500 mM D-glucose ( $\Delta$ ), 100  $\mu$ M cytochalasin B ( $\blacktriangle$ ), 500 mM D-fructose ( $\blacksquare$ ), 500 mM 4,6-O-ethylidene-D-glucose ( $\circ$ ) and 100  $\mu$ M phloretin ( $\square$ ). Peak areas were compared with that obtained in a control sample in which irradiation was carried out in the presence of 500 mM D-mannitol ( $\bullet$ ). After washing and solubilization in buffer containing  $C_{12}E_9$ , the labelled proteins were immunoprecipitated with 5 mg of Protein A-Sepharose conjugated to 50  $\mu$ l of anti-GLUT2 C-terminal peptide antiserum and analysed by SDS-PAGE. Arrowheads indicate the position of molecular weight marker proteins. For clarity, only the glucose transporter region of the gel is shown. There were no other labelled peaks in other regions of the gel.



Precipitating antiserum	Inhibitor	Inhibitor concentration [mM]	Peak area (% of control) [Mean $\pm$ S.E.M. (n)]	Inhibition corrected for non-specific precipitation (%)
Anti-(GLUT2 C-terminus)	D-Mannitol	500	100	0
	D-Glucose	500	75 $\pm$ 3.8 (3)	57
	Cytochalasin B	0.1	67 $\pm$ 10.7 (3)	65
	D-Fructose	500	55 $\pm$ (6.2 (4)	77
	4,6-O-Ethylidene			
	-D-glucose	500	40 $\pm$ 2.2 (9)	92
	Phloretin	0.1	30 $\pm$ 3.5 (3)	$\approx$ 100
Preimmune	D-Mannitol	500	32 $\pm$ 9.2 (6)	

**Table 6. ATB-BMPA photolabelling of liver GLUT2 in the presence of inhibitors of glucose transport.**

Photolabelling of liver plasma membranes was carried out in the presence of the indicated inhibitors as described in section 2.15.2. Labelled membranes were solubilized in C<sub>12</sub>E<sub>9</sub> detergent buffer and immunoprecipitated with 5 mg of Protein A-Sepharose conjugated to 50  $\mu$ l of anti-GLUT2 C-terminal peptide antiserum. Precipitated proteins were analysed by SDS-PAGE and a peak of labelled protein with an apparent molecular weight of between 45 and 66 kDa was obtained. The peak area was compared to that obtained in a control sample in which irradiation was carried out in the presence of D-mannitol. The level of non-specific immunoprecipitation was determined from the amount of photolabelled protein precipitated by preimmune serum. The peak areas obtained in the presence of inhibitors were calculated as a percentage of a control which contained D-mannitol and are means  $\pm$  S.E.M for three to nine independent experiments as indicated. In some experiments D-mannitol was omitted, but this did not alter the amount of ATB-BMPA binding (results not shown).

### 3.2.5 Determination of the binding constant of ATB-BMPA for liver GLUT2

The displacement of radiolabelled ATB-BMPA from liver plasma membranes by non-labelled ATB-BMPA was used to calculate the binding constant of ATB-BMPA for liver GLUT2. As shown in Figure 15a, the size of the labelled GLUT2 peak decreased as the concentration of unlabelled ATB-BMPA present during the irradiation was increased. Figure 15b shows a plot of F/B against F. The binding constant ( $K_d$ ) for ATB-BMPA binding to GLUT 2 was  $250 \pm 78 \mu\text{M}$  and the total number of binding sites ( $B_{\text{max}}$ ) was calculated as  $2.1 \pm 0.29 \text{ pmol/mg}$  of membrane protein. These values were based on mean F/B and F values from four separate experiments  $\pm$  S.E.M.

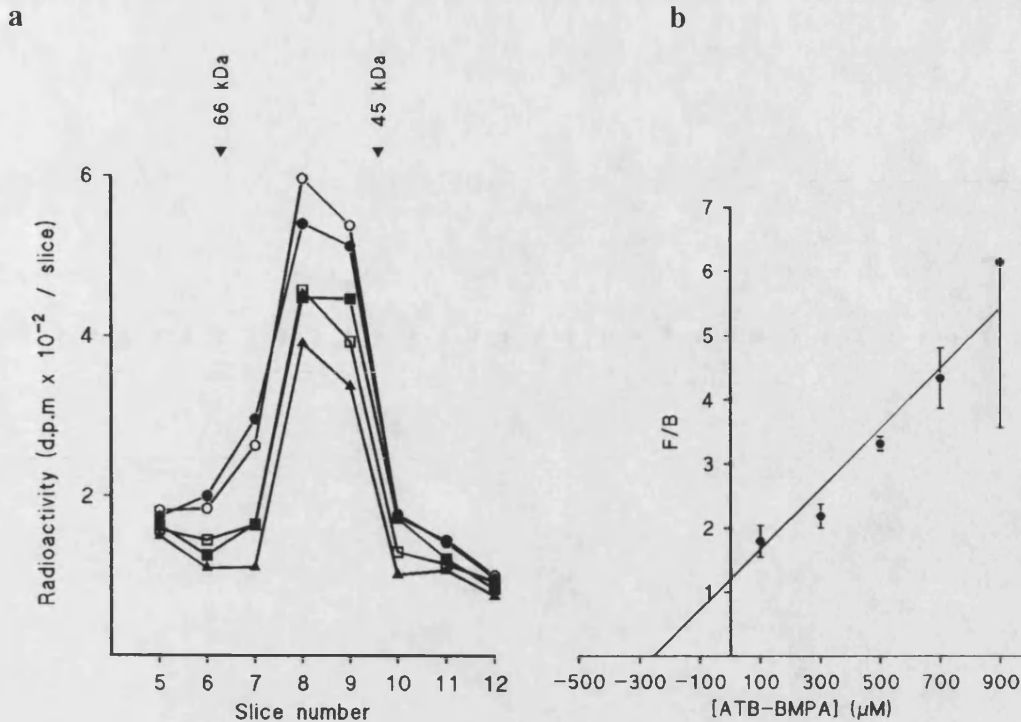


Fig. 15a-b Determination of the binding constant ( $K_d$ ) and the  $B_{max}$  for ATB-BMPA and GLUT2.

**a:** Liver plasma membranes were photolabelled with 100  $\mu$ Ci of ATB-[2- $^3$ H]-BMPA in the presence of a range of concentrations of unlabelled ATB-BMPA, such that the final concentrations were 100  $\mu$ M ( $\bullet$ ) -no added unlabelled ATB-BMPA, 300  $\mu$ M ( $\circ$ ), 500  $\mu$ M ( $\blacksquare$ ), 700  $\mu$ M ( $\square$ ) and 900  $\mu$ M ( $\blacktriangle$ ). Labelled membranes were washed, solubilized in buffer containing the detergent  $C_{12}E_9$  and immunoprecipitated with 5 mg of Protein A-Sepharose coupled to 50  $\mu$ l of anti-GLUT2 C-terminal peptide antiserum. Labelled proteins were analysed by SDS-PAGE. Arrowheads indicate the position of molecular weight marker proteins. The figure shows a decrease in the amount of labelled GLUT2 immunoprecipitated as the amount of unlabelled ATB-BMPA present during irradiation is increased.

**b:** The size of the GLUT2 peak was calculated for each concentration of ATB-BMPA shown in Figure 15b. A background value of 32% was then subtracted to take account of the binding and immunoprecipitation which was non-specific. Values for the binding constant ( $K_d$ ) and the total number of binding sites ( $B_{max}$ ) were obtained from non linear regression (weighted for relative error) of the equation  $B = (B_{max}/K_d) \cdot F / (1 + F/K_d)$  where B and F are the bound and free ATB-BMPA respectively. The data are presented as F/B against F plots. From this analysis  $B_{max}$  was calculated as  $2.1 \pm 0.29$  pmol/mg of liver plasma membrane protein and the binding constant ( $K_d$ ) was  $250 \pm 78$   $\mu$ M. The results are based on the mean F/B and F values from four separate experiments and the error bars shown represent  $\pm$  S.E.M.

## **PART II CHARACTERIZATION OF $\beta$ -CELL LINES AND INVESTIGATION OF THEIR GLUCOSE TRANSPORTER ISOFORM EXPRESSION**

Cell lines often behave very differently from the original cell type from which they were derived. In addition, changes may be brought about by mutations which are accumulated during prolonged continuous culture and result in the development of a cell line which displays characteristics significantly different from those of the original cell line.

The following section of results describes the features of four  $\beta$ -cell lines, including their morphology, growth rate and insulin secretion. One of the aims of this section of work was to compare the features of the cell lines used in this laboratory with the characteristics of the same cell lines reported from other laboratories. This information could be used to determine whether the cells were good models of native  $\beta$ -cells and therefore whether they were likely to be expressing similar antigenic proteins that would make them suitable for use in the detection of islet cell antibodies (ICA).

Since it has previously been reported that  $\beta$ -cell lines show abnormal glucose transporter expression, this has been investigated by ATB-BMPA photolabelling and Western blotting. One of the main aims was to determine whether  $\beta$ -cell lines expressed GLUT2, and therefore whether they could be used to screen for the presence of anti- $\beta$ -cell-GLUT2 autoantibodies (Part III).

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### 3.3 MORPHOLOGY AND GROWTH RATES OF B-CELL LINES

#### 3.3.1 HITm2.2

As illustrated in Figures 16a-f, the appearance of HITm2.2 cells varied in a passage dependent manner. At early passages, the cell population was heterogeneous. The majority of cells were rounded with "blebs" on the surface and many were poorly adherent, but approximately 10% of the cells had a more elongated or fibroblastic-type appearance (Figure 16a). A dramatic change occurred in the cells at passage 6 (p6), when about 70% of the cells detached and died, of those remaining, about 50% were elongated in shape, the rest were still rounded (Figure 16b). By p8 the number of elongated cells had increased to about 90% (Figure 16c). Over a prolonged period of continuous culture, the majority of the cells remained elongated and their morphology did not alter significantly (Figure 16d), although they grew increasingly rapidly and attained a higher degree of confluence (Figure 16e). At about p30, the cells again changed shape, becoming less elongated and more triangular in their appearance (Figure 16f). This shape was still predominant at p51.

This pattern of morphological changes was repeated on several occasions. The use of tissue culture dishes from a different manufacturer had no effect on the changes in cell shape. When defrosted, the cells from any given passage number behaved in the same way as they did prior to freezing.

The growth rate of HITm2.2 cells was dependent on the passage number. At p4-p5 the cells needed to be subcultured every 5 days. At p14, the cells took 4-5 days to reach confluence and had a doubling time of 25 hours (Figure 20a). At passage numbers higher than p18, the cells reached confluence in 3-4 days. HITm2.2 cells did not show contact inhibition of growth, but continued to divide and detach when fully confluent.

**Fig. 16a-f Light micrographs of HITm2.2 cultures: Demonstration of morphological changes from p5-p37.**

HITm2.2 cells were cultured in RPMI-1640 medium supplemented with 10% FCS as described in section 2.5.3.

**a: 3 day HITm2.2 culture (p5).** The majority of cells are rounded and flattened with "blebs" on the surface, some are growing in loosely attached clumps. Magnification x3000.

**b: 5 day HITm2.2 culture (p6).** The rounded cells are poorly adherent, approximately 50% of the cells have an elongated morphology. Magnification x3000.

**c: 3 day HITm2.2 culture (p8).** About 90% of cells are of the elongated shape and are firmly attached. Magnification x3000.

**d: 3 day HITm2.2 culture (p21).** Cells are firmly attached and elongated in shape. Magnification x1500.

**e: Confluent HITm2.2 culture.** Magnification x1500.

**f: 3 day HITm2.2 culture (p37).** Most cell are round or triangular in shape. They attach well and grow quickly. Magnification x1500.

**Fig. 17a-b Light micrographs of RINm5F cultures.**

Cells were cultured in RPMI-1640 medium with 10% FCS as described in section 2.5.3.

**a: 5 day RINm5F culture (p10).** Magnification x1000.

**b: 5 day RINm5F culture (p10).** Cells are irregularly shaped with extensive cytoplasmic projections. Magnification x1500.

**Fig. 18a-b Light micrograph of HIT-T15 cultures.**

Cells were cultured in HAMS-F12 medium supplemented with 15% DHS and 2.5% FCS as described in section 2.5.1.

**a: 6 day HIT-T15 culture (p51).** Cells grow in clumps and fail to form a true monolayer. Magnification x1500.

**b: 6 day HIT-T15 culture (p51).** Magnification x3000.

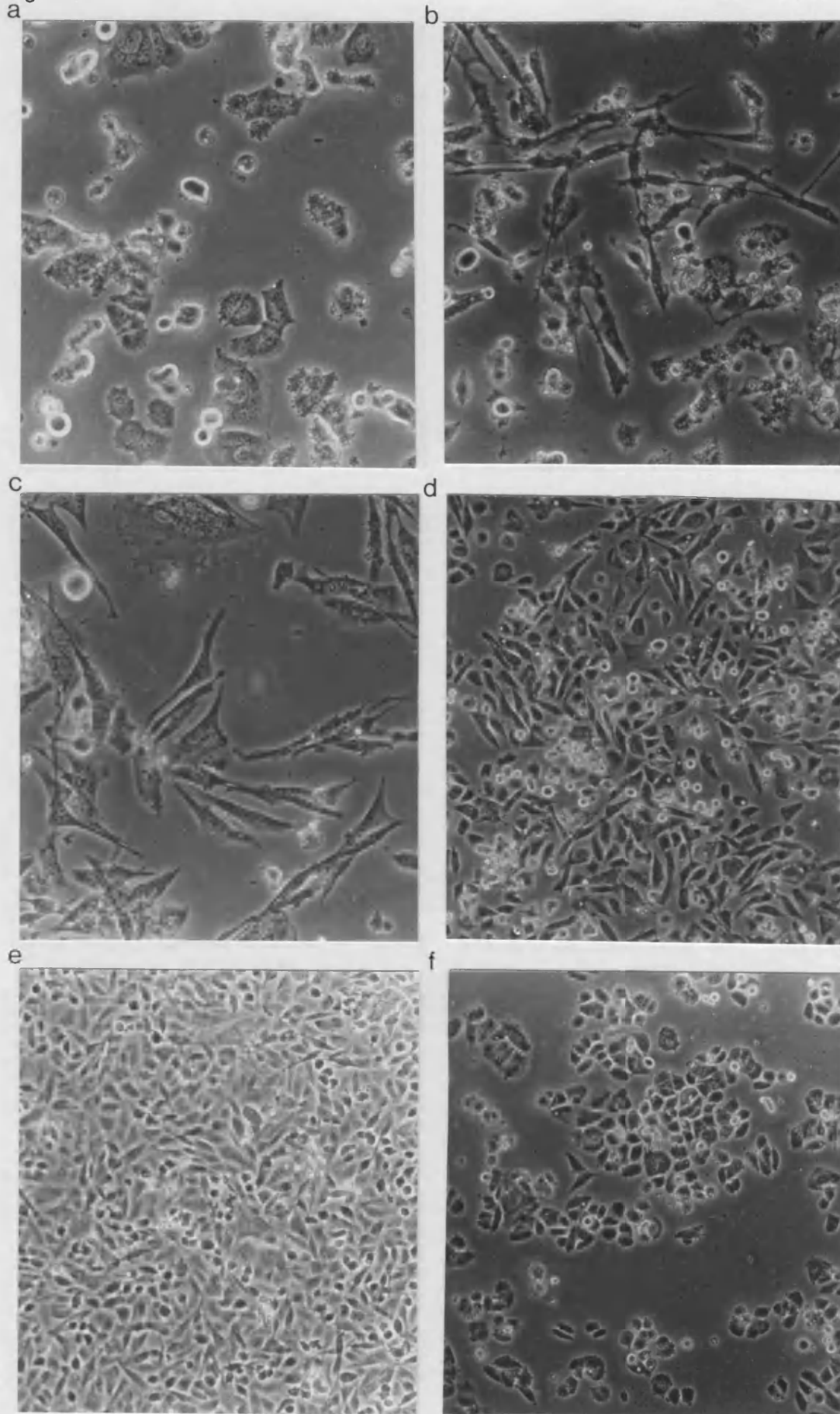
**Fig. 19a-b Light micrograph of MIN 6 cultures.**

Cells were cultured in DMEM supplemented with 10% FCS as described in section 2.5.2.

**a: 2 day MIN 6 culture (p14).** Cells are rounded or triangular in shape and are firmly attached to the culture surface. Magnification x1500.

**b: 2 day MIN 6 culture (p14).** Magnification x3000.

Fig 16a-f



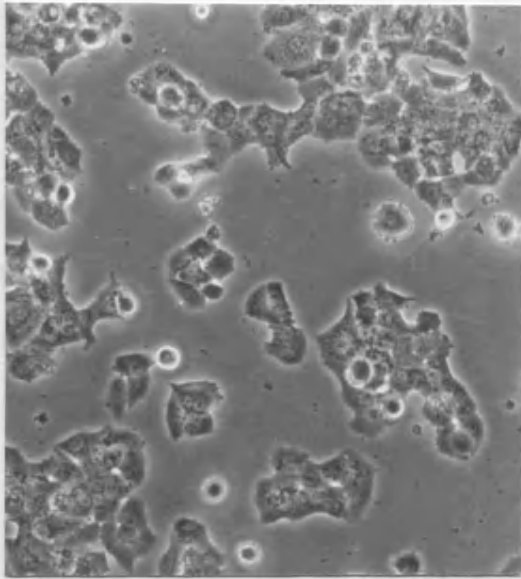
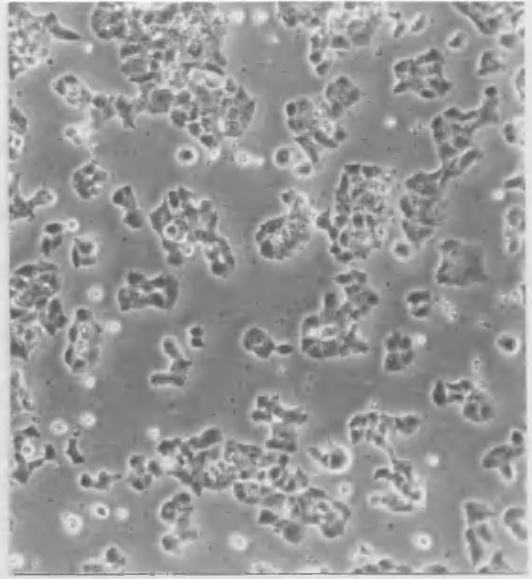


Fig 19



a b

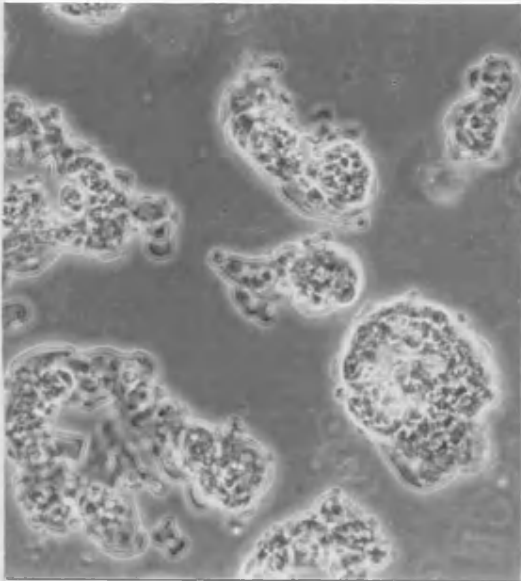
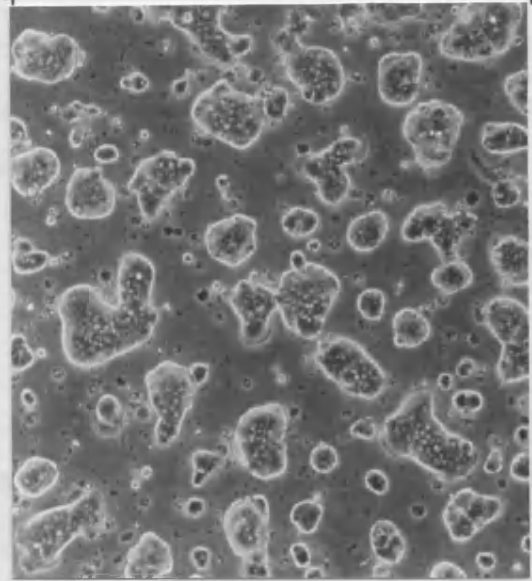


Fig 18



a b

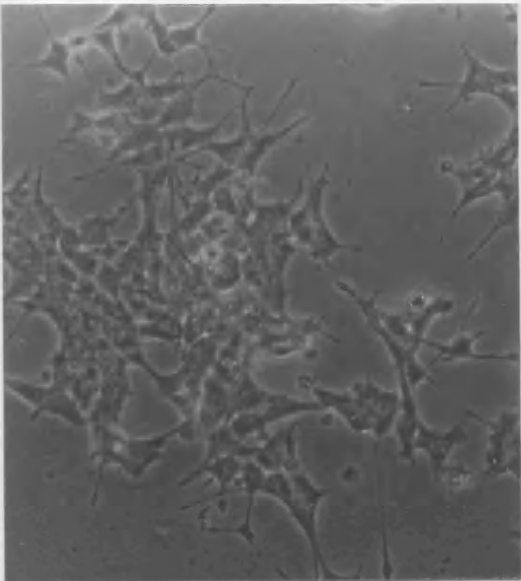
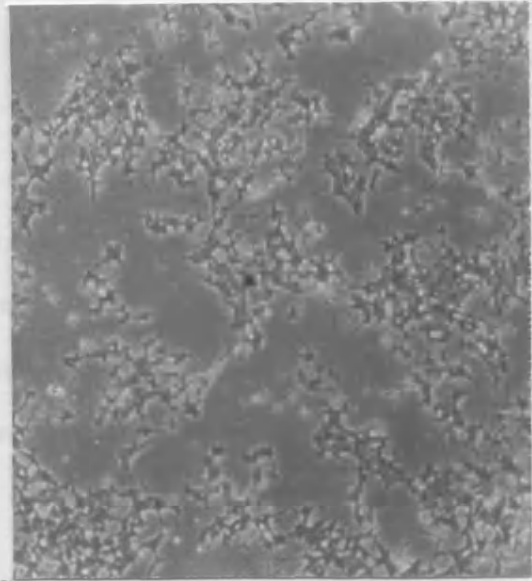


Fig 17



a b



### **3.3.2 RINm5F**

RINm5F were small flattened cells with irregularly shaped pseudopodia (Figures 17a-b). Even at high passage numbers no obvious change in morphology occurred. The cells grew to about 90% confluence and had a doubling time of  $\approx 26.5$  hours (Figure 20b), which remained fairly constant regardless of the passage number.

### **3.3.3 HIT-T15**

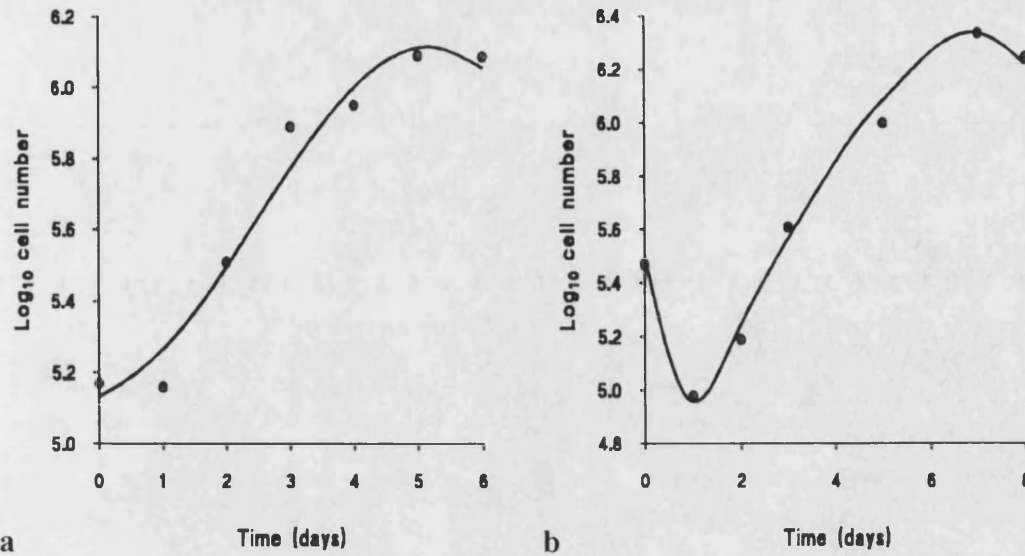
HIT-T15 cells tended to self-associate within an hour of subculturing. They did not form a monolayer but grew as large multi-layered "islet-like" structures, which reached a maximum size after 4-5 days growth (Figures 18a-b). At high confluency the cells rounded and detached from the colonies.

### **3.3.4 MIN 6**

MIN 6 cells were rounded or triangular in shape. They grew in small clumps and were firmly attached to the culture plates. The cells reached about 70% confluence after 7-8 days growth (Figures 19a-b).

## **3.4 INSULIN SECRETION**

Indirect immunofluorescence assays for insulin were performed on HITm2.2 cells with the aim of determining whether cells of different morphology contained different amounts of insulin. In HITm2.2 cultures at p11, 12, 14 and 16, all of which contained both elongated adherent and rounded poorly adherent cells, no cells could be detected which stained positively for insulin (results not shown).



**Fig. 20a-b Growth curve of HITm2.2 and RINm5F cells.**

Cells were cultured in 2 ml dishes in RPMI-1640 containing 10% FCS as described in section 2.5.3.

**a:** The population doubling time of HITm2.2 cells at p14 was 25 hours in the exponential phase.

**b:** The population doubling time of RINm5F cells at p6 was 26.5 hours in the exponential phase.

Treatment	Cell line and passage number	$\mu\text{U insulin /60 min}/2 \times 10^5$ cells (n)
Basal (KRB only)	HITm2.2 p49	0 (1)
2 mM D-glucose	HITm2.2 p49	0 (2)
11 mM D-glucose	HITm2.2 p6	$15 \pm 1.9$ (4)
	HITm2.2 p25	$5 \pm 0.2$ (4)
	HITm2.2 p49	$0.4 \pm 0.3$ (1)
20 mM D-glucose	HITm2.2 p49	$1.2 \pm 0.3$ (2)
15 mM arginine	HITm2.2 p49	0 (2)
15 mM leucine	HITm2.2 p49	0 (2)
Basal	RINm5F p17	$129 \pm 23$ (2)
2 mM D-glucose	RINm5F p17	$107 \pm 3$ (2)
11 mM D-glucose	RINm5F p17	116 (1)
20 mM D-glucose	RINm5F p17	$128 \pm 1.0$ (2)
15 mM arginine	RINm5F p17	$333 \pm 0$ (2)
15 mM leucine	RINm5F p17	$131 \pm 8.0$ (2)

**Table 7 Insulin secretion from HITm2.2 and RINm5F cells in static culture.**

Insulin secretion in the presence of secretagogues was measured by RIA as described in section 2.6.2. The results are expressed as means  $\pm$  S.E.M. for 1-4 independent samples as indicated.

However, HITm2.2 cells were demonstrated by RIA to secrete insulin and to be responsive to D-glucose. As shown in Table 6, at low passage numbers, HITm2.2 cells secreted 15  $\mu\text{U}$  of insulin/60 min/ $2 \times 10^5$  cells in response to 11 mM D-glucose. This response had decreased by p25 to 5  $\mu\text{U}$  of insulin/60 min/ $2 \times 10^5$  cells and by p49 to 0.4  $\mu\text{U}$  of insulin/60 min/ $2 \times 10^5$  cells. At this high passage number 1.2  $\mu\text{U}$  of insulin/60 min/ $2 \times 10^5$  cells were secreted in response to 20 mM D-glucose. Under basal conditions, that is, in the absence of D-glucose, insulin secretion was undetectable. At p49, HITm2.2 cells did not secrete insulin in response to arginine or leucine.

In contrast to HITm2.2 cells, the parent cell line HIT-T15, cultured in 11 mM D-glucose, did contain cytoplasmic insulin which could be detected in an indirect immunofluorescence assay (Figure 21a).

RINm5F cells contained very high levels of insulin which stained strongly in an indirect immunofluorescence assay and which was associated with cytoplasmic granules (Figure 21b). These cells secreted a very high basal level of insulin, approximately 130  $\mu\text{U}$  of insulin/60 min/ $2 \times 10^5$  cells. As illustrated in Table 6, RIN cells were unresponsive to D-glucose which caused no significant increase in insulin secretion. A 4.6 fold increase in insulin release was however observed in the presence of 15 mM arginine, but 15 mM leucine did not stimulate insulin secretion in this cell line.

### **3.5 MEASUREMENT OF 2-DEOXYGLUCOSE UPTAKE IN HITm2.2 AND RINm5F CELLS**

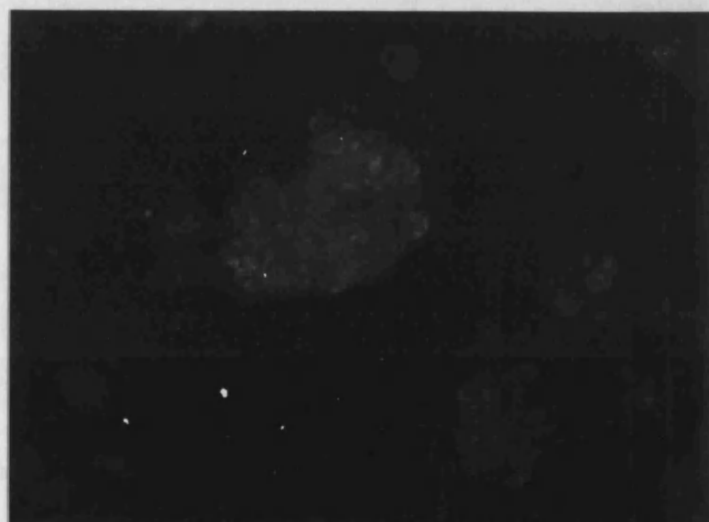
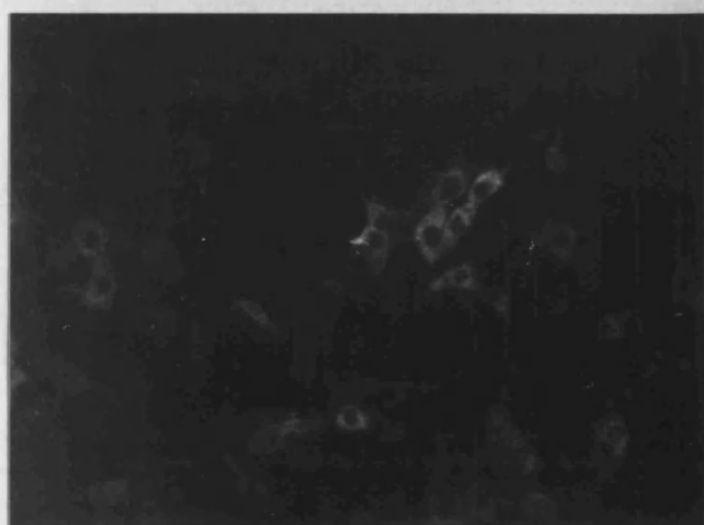
Under the experimental conditions used, 2-deoxyglucose transport in HITm2.2 cells was linear for 5 min, in RINm5F cells, transport was linear for 10 min. Both cell lines when cultured in 11 mM D-glucose had transport rates for 2-deoxyglucose of 0.08 nmol/mg protein/min measured at 50  $\mu\text{M}$  2-deoxyglucose. These results are illustrated in Figures 22a-b.

**Fig. 21** Detection of insulin by indirect immunofluorescence.

Cell cultures were fixed overnight in formaldehyde, then incubated with a mouse monoclonal antibody against insulin, binding was detected with an anti-mouse IgG conjugate. Insulin was located in granules in the cytoplasm.

**a:** HIT-T15 (p54) Magnification x3000.

**b:** RINm5F (p6) Magnification x 3000.

**a****b**

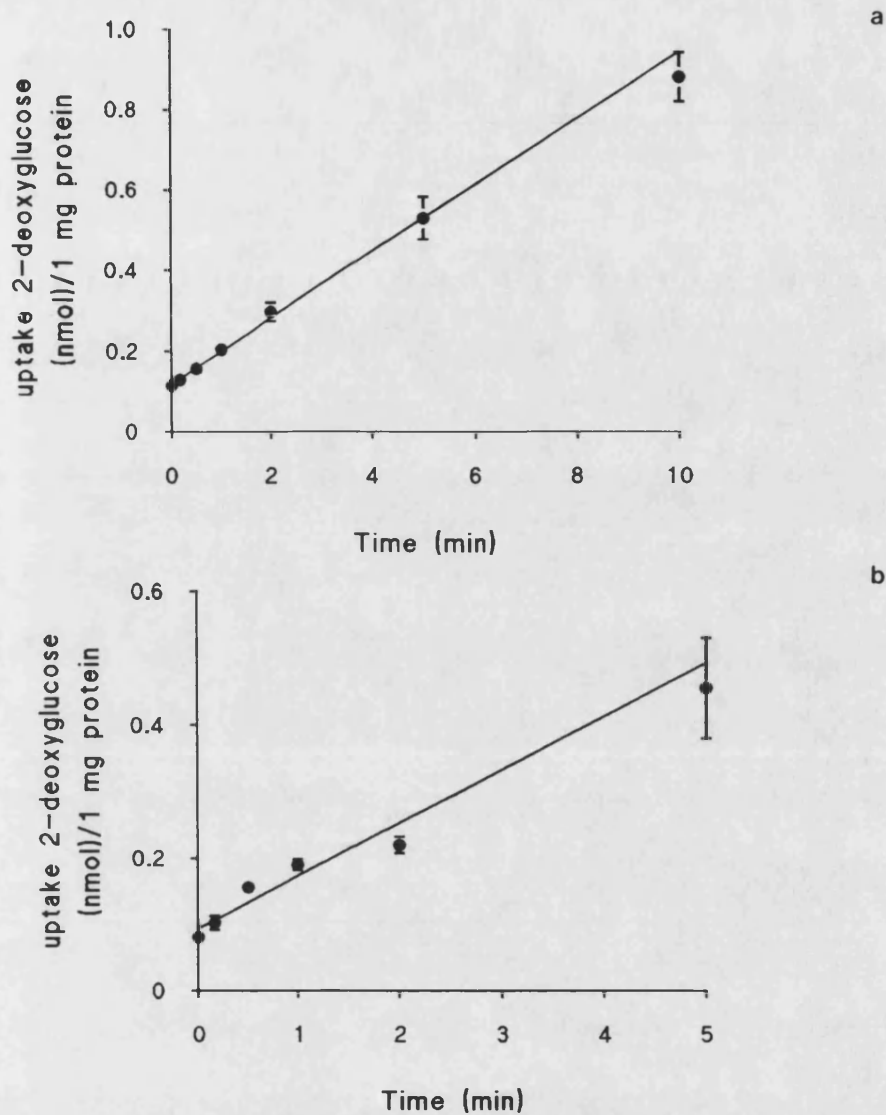


Fig. 22a-b Uptake of 2-deoxyglucose by HITm2.2 and RINm5F  $\beta$ -cell lines.

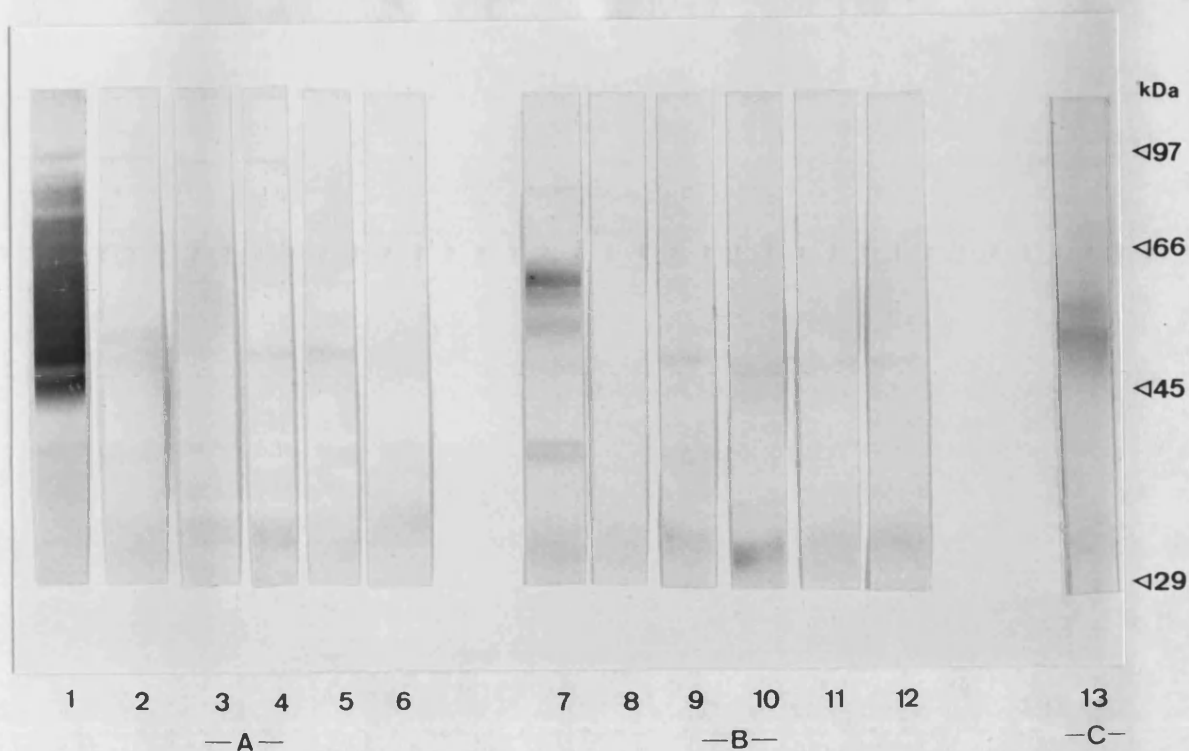
2-deoxyglucose uptake was measured in cells that had been grown in RPMI medium containing 11 mM D-glucose. The cells were brought into suspension by a brief incubation in PBS and 1 mM EDTA, washed and the uptake of 2-deoxyglucose (50  $\mu$ M) was measured. After the indicated time intervals, uptake was stopped by the addition of buffer containing phloretin and the cells were washed by centrifugation. Cell pellets were solubilized in 0.1 M NaOH and the incorporated radioactivity was counted. Counts were normalised to the protein content in each assay. Data are given as mean  $\pm$  S.E.M. of two determinations for RINm5F cells (a) and HIT m2.2 cells (b). For both cell lines 2-deoxyglucose uptake was 0.08 nmol/1 mg protein/min.

## 3.6 GLUCOSE TRANSPORTER EXPRESSION IN $\beta$ -CELLS

### 3.6.1 Investigation of glucose transporter expression by Western blotting

When plasma membranes (prepared using the method described in section 2.10.4.1) from HITm2.2, HIT-T15, RINm5F and MIN 6 cells were immunoblotted with anti-GLUT1 or anti-GLUT2 C-terminal peptide antiserum, neither GLUT1 nor GLUT2 was detected in any of these  $\beta$ -cell lines. This is illustrated in Figure 23, which is a typical result obtained from a Western blot developed with a colourimetric detection system. As positive controls, anti-GLUT1 and GLUT2 antisera were shown to detect proteins in erythrocyte and hepatocyte membranes which had molecular weights consistent with their being the glucose transport proteins GLUT1 (44-70 kDa) and GLUT2 ( $\approx$ 58 kDa) respectively. As an additional control, plasma membranes prepared from acutely insulin-stimulated 3T3-L1 adipocytes, (Palfreyman *et al.*, 1992) were blotted alongside the  $\beta$ -cell lines. GLUT4 was detected strongly as a  $\approx$ 51 kDa band, but although these cells have previously been shown to express equal amounts of the GLUT1 and GLUT4 transporter isoforms (Calderhead *et al.*, 1990), in this study anti-GLUT1 antiserum detected only a very faint band with an apparent molecular mass of  $\approx$ 50 kDa in the 3T3-L1 cells.

As shown in Figures 24a-d, similar results were also obtained using a more sensitive  $^{125}\text{I}$  labelled anti-IgG detection system, which enabled quantification of the glucose transporter expression. GLUT2 was only detected in liver, not in HITm2.2 or RIN cells. GLUT4 was the predominant transporter in 3T3-L1 cells and GLUT1 was detected in erythrocytes and a very small amount was present in 3T3-L1 cells. GLUT1 was not detected in HIT or RIN cells. This experiment also demonstrated that the anti-glucose transporter antibodies were specific for the isoform against which they were raised.



**Fig. 23 Investigation of glucose transporter isoform expression in  $\beta$ -cell lines by Western blotting.**

The  $\beta$ -cell lines were cultured as described in section 2.5 and plasma membranes were prepared using method I (section 2.10.4.1). 3T3-L1 adipocytes were cultured as described in section 2.5 and membranes were prepared according to method III (section 2.10.4.3). Erythrocyte ghosts and liver plasma membranes were also prepared as described in the methods sections 2.10.2 and 2.10.3.1 respectively. Proteins were separated by electrophoresis on 10% polyacrylamide gels, and following electrophoretic transfer to nitrocellulose were immunoblotted for two hours with anti-GLUT1 C-terminal peptide antiserum (A); anti-GLUT2 C-terminal peptide antiserum (B); or anti-GLUT4 C-terminal peptide antiserum (C) at a 1:1000 dilution using blocking protocol II. Bound antibodies were detected with alkaline phosphatase conjugated anti-rabbit IgG antibodies.

Lane 1 - human erythrocyte membranes

Lanes 2,8 & 13 - 3T3-L1 membranes

Lanes 3 & 9 - HITm2.2 membranes

Lanes 4 & 10 - HIT-T15 membranes

Lanes 5 & 11 - RINm5F membranes

Lanes 6 & 12 - MIN 6 membranes

Lane 7 - liver plasma membranes

GLUT1 detected a protein with an apparent molecular weight of 44-70 kDa in erythrocytes. GLUT2 detected a protein with an apparent molecular weight of  $\approx$ 58 kDa in liver. GLUT4 detected a protein with an apparent molecular weight of 51 kDa in 3T3-L1 cells. Neither anti-GLUT1 nor anti-GLUT2 antiserum detected any proteins in any of the  $\beta$ -cell lines.

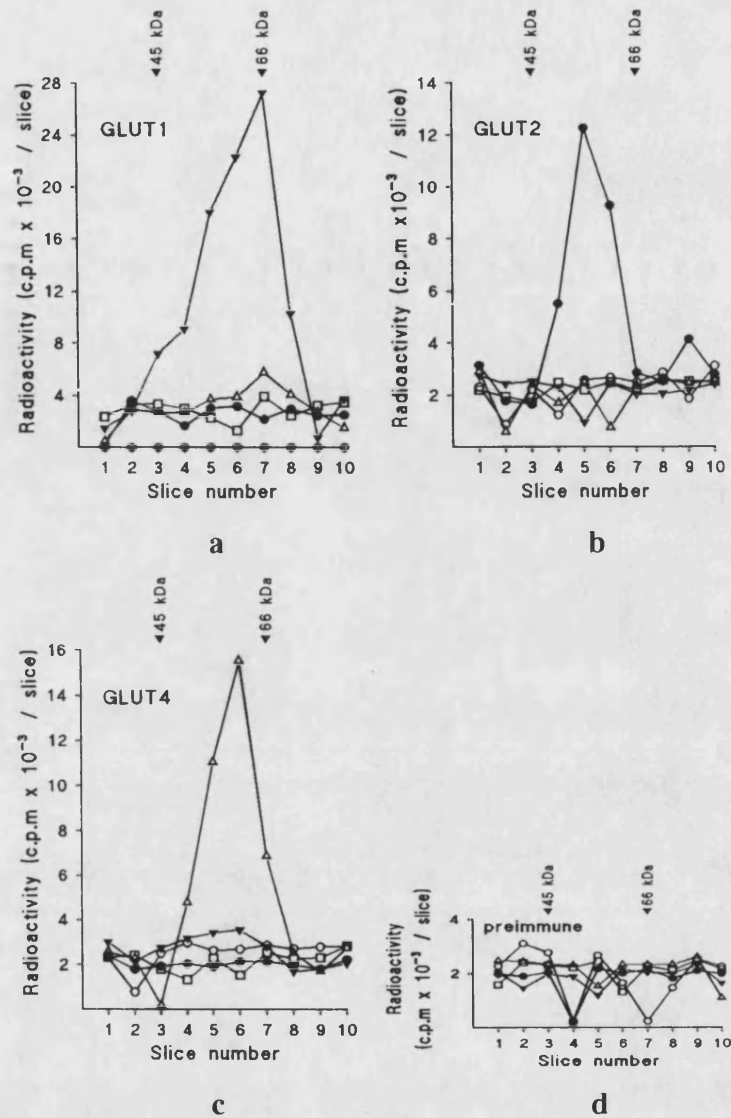


Fig. 24a-d Quantification of glucose transporter isoform expression.

50  $\mu$ g of protein from liver plasma membranes (●), erythrocyte ghosts (▼) and a total membrane fraction from HITm2.2 (○), RINm5F (□) and 3T3-L1 cells (Δ) were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose and incubated with a 1:500 dilution of anti-GLUT1 C-terminal peptide antiserum (a), anti-rat GLUT2 C-terminal peptide B antiserum (b), anti-GLUT4 C-terminal peptide antiserum (c) or preimmune serum (d). Blots were developed with <sup>125</sup>I-labelled anti-IgG and quantified by gamma counting. Glucose transporter isoforms were detected as bands of labelled proteins with apparent molecular weights of 45-66 kDa. GLUT4 was only detected in 3T3-L1 adipocytes (peak size 29223 cpm) and GLUT2 was detected only in liver plasma membranes (peak size 20439 cpm). GLUT1 was detected strongly in erythrocytes (peak size 81062 cpm) and a small amount of this transporter was detected in the 3T3-L1 cells (peak size 7865 cpm). No transporter isoform was detected in either the HITm2.2 or RINm5F  $\beta$ -cell lines.



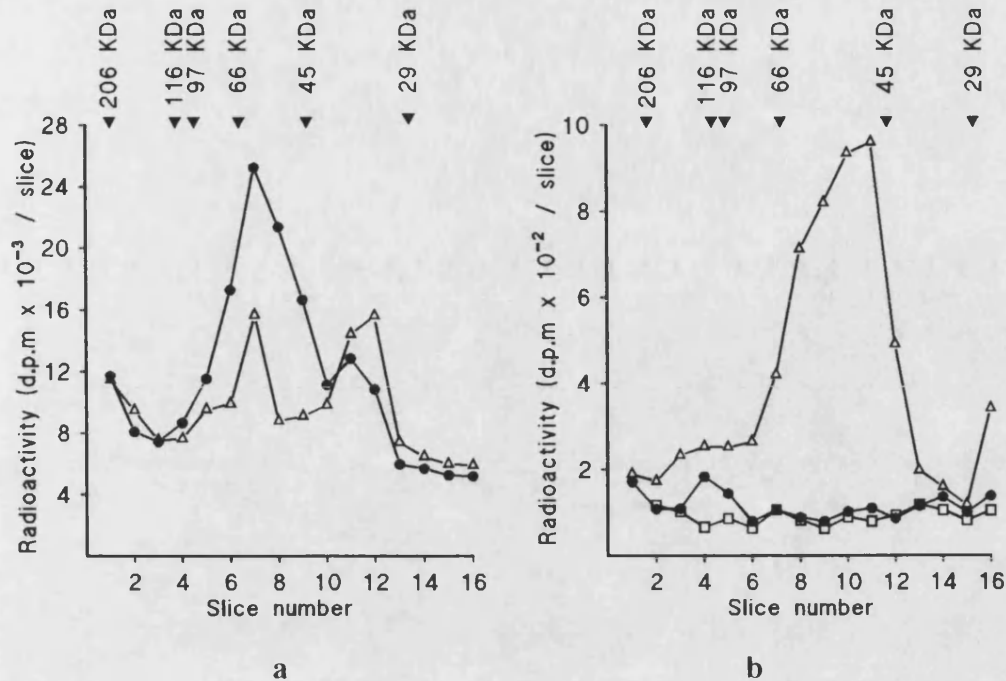
The use of whole solubilized cells, instead of plasma membranes and a very sensitive ECL detection system also failed to show the presence of either GLUT1 or GLUT2 in any of the  $\beta$ -cell lines tested (results not shown). Therefore, the transporter expressed in  $\beta$ -cell lines could not be identified by Western blotting. However, as shown in Figure 8,  $\beta$ -cell GLUT2 could be detected in whole rat islet cells when they were immunoblotted with anti-GLUT2 C-terminal peptide antiserum.

### **3.6.2 Investigation of glucose transporter expression by ATB-BMPA labelling**

#### **3.6.2.1 ATB-BMPA photolabelling of HITm2.2 cells**

Attempts to photolabel monolayers of HITm2.2 cells were unsuccessful owing to the relatively low density of cells and high dilution of the ATB-BMPA, which resulted in a negligible incorporation of the photolabel. However, as shown in Figure 25a, when ATB-BMPA was irradiated in the presence of a dense suspension of HITm2.2 cells, analysis of the radiolabelled proteins by SDS-PAGE revealed that ATB-BMPA was incorporated into a major peak with an apparent molecular weight of  $\approx 45$ -66 kDa. 750 mM D-glucose displaced the labelling of this major protein by  $\approx 66\%$ , but did not significantly displace the binding of the photolabel to a minor peak which ran on the gel between the 29 and 45 kDa markers.

In order to identify the protein that was photolabelled in a glucose-displaceable manner, immunoprecipitations using anti-C-terminal peptide antisera specific for the GLUT1 and GLUT2 transporter isoforms were carried out on the detergent solubilized photolabelled cells. As shown in Figure 25b, anti-GLUT1 antiserum immunoprecipitated a labelled protein with an apparent molecular weight of 45-66 kDa. Neither anti-GLUT2 antiserum nor preimmune serum precipitated any labelled proteins. The amount of labelled protein that was immunoprecipitated by GLUT1 was equal to approximately 9% of the total solubilized protein labelled in a D-glucose displaceable manner.



**Fig. 25a-b** Detection and immunoprecipitation of ATB-BMPA photolabelled proteins from HITm2.2 cells.

Suspensions of  $\approx 2 \times 10^7$  HITm2.2 cells (p17) in 0.5 ml HEPES-buffered balanced salts solution were photolabelled with 400  $\mu\text{Ci}$  of ATB-BMPA in the presence or absence of 750 mM D-glucose as indicated. After washing, the cells were homogenised and the pellet obtained by centrifugation at  $100,000 g_{av}$  was either analysed directly by SDS-PAGE (a), or solubilized in buffer containing the detergent  $C_{12}E_9$  and immunoprecipitated with 5 mg Protein A-Sepharose coupled to 50  $\mu\text{l}$  of the indicated antiserum (b). Arrowheads indicate the position of molecular weight marker proteins.

**a:** 750 mM D-glucose ( $\Delta$ )  
no glucose ( $\bullet$ )

ATB-BMPA labelled a protein with an apparent molecular weight of 45-66 kDa. Labelling was displaced by 66% by D-glucose. Labelling of another protein with an apparent molecular weight of 29-45 kDa was not displaced with D-glucose.

**b:** Preimmune ( $\square$ )  
Anti-GLUT2 C-terminal peptide ( $\bullet$ )  
Anti-GLUT1 C-terminal peptide ( $\Delta$ )

Only the GLUT1 antiserum precipitated a labelled protein with an apparent molecular weight of 45-66 kDa from HITm2.2 cells.

From labelling and immunoprecipitation experiments only the GLUT1 transporter isoform could be identified in HITm2.2 cells.

### **3.6.2.2 ATB-BMPA photolabelling of HIT-T15 cells**

When cultures of HIT-T15 cells growing in dishes were irradiated in the presence of ATB-BMPA, the radiolabel was incorporated into a protein which was soluble in buffer containing the detergent C<sub>12</sub>E<sub>9</sub> and which had an apparent molecular weight of  $\approx$  66 kDa. A typical gel profile is shown in Figure 25c. The identity of the labelled protein was unknown since it was not immunoprecipitated by either anti-GLUT1 or anti-GLUT2 C-terminal peptide antiserum.

### **3.6.2.3 ATB-BMPA photolabelling of RINm5F cells**

When a dense suspension of RINm5F cells was irradiated in the presence of ATB-BMPA, analysis of the proteins by SDS-PAGE revealed that the photolabel was incorporated into two bands of protein. The heavier migrated as a broad band between the 66 and 97 kDa molecular weight marker proteins. The second protein had an apparent molecular weight of approximately 45 kDa. A typical gel profile is shown in Figure 26a, which also shows that labelling in the presence of up to 930 mM D-glucose did not displace ATB-BMPA binding to either of these proteins. The identity of the 66-97 kDa protein was unknown, but as shown in Figure 26b, about 9% of the 45 kDa protein was immunoprecipitated by anti-GLUT1 C-terminal peptide antiserum. ATB-BMPA labelling of this precipitated protein was displaced when irradiation was carried out in the presence of 360 mM D-glucose.

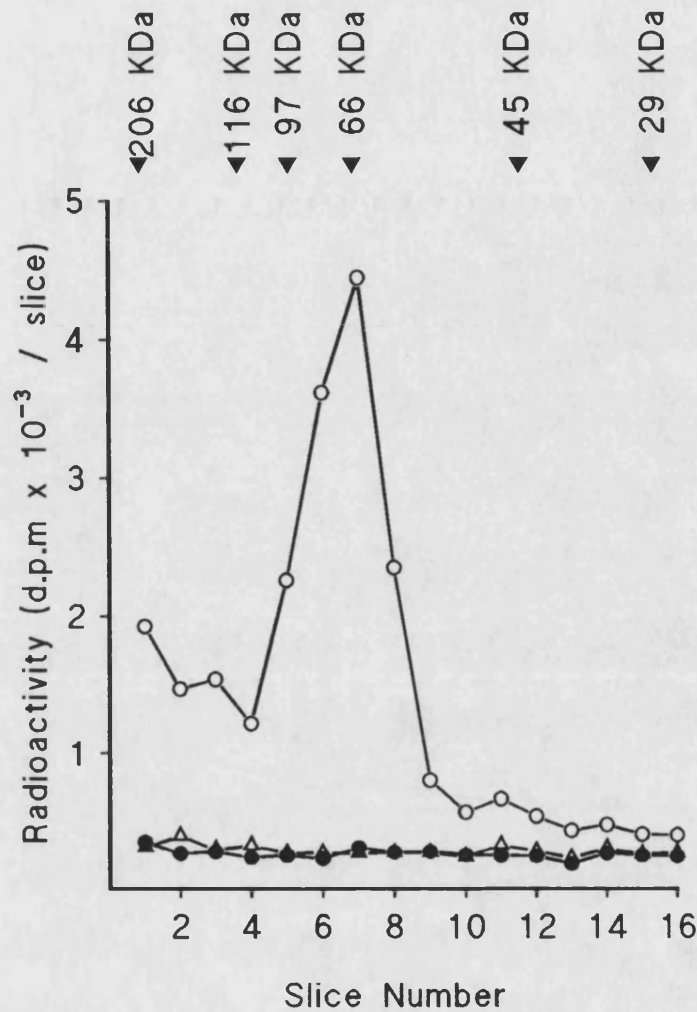
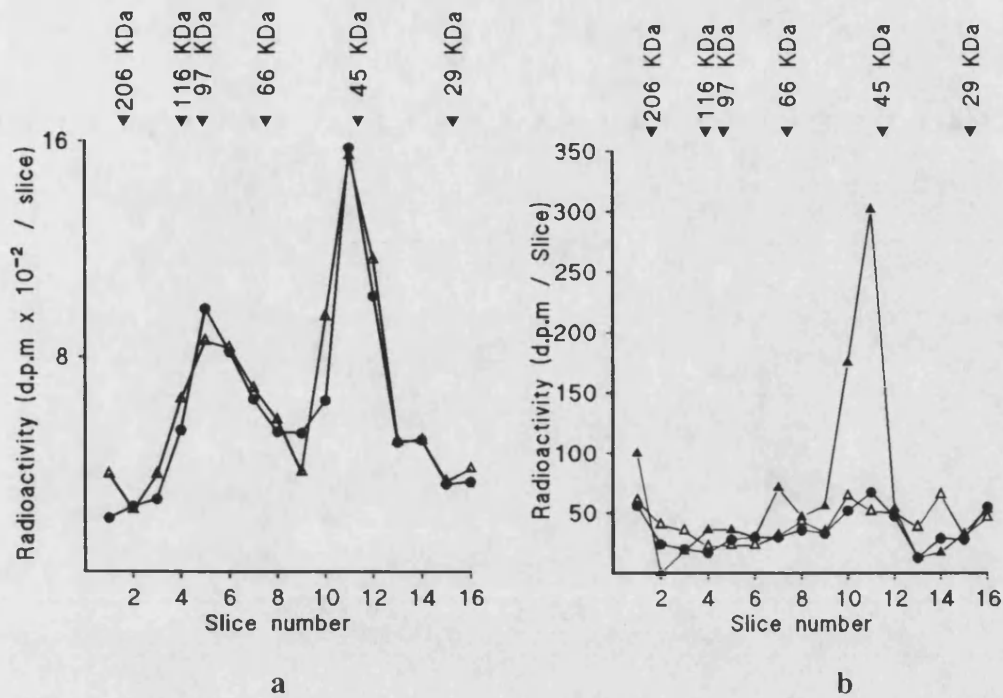


Fig. 25c ATB-BMPA photolabelling of HIT-T15 cells and attempted immunoprecipitation of labelled proteins.

Cultures of HIT-T15 cells in 35 mm tissue culture dishes were irradiated in the presence of 100  $\mu$ Ci of ATB-BMPA in a final volume of 0.5 ml of Heps-buffered balanced salts solution. After washing, the cells were solubilized in buffer containing the detergent C<sub>12</sub>E<sub>9</sub>. Solubilized labelled proteins were immunoprecipitated with 5 mg of Protein A-Sepharose coupled to 50  $\mu$ l of anti-GLUT1 ( $\Delta$ ) or anti-GLUT2 ( $\bullet$ ) C-terminal peptide antiserum. Immunoprecipitates and labelled proteins precipitated from the supernatant by the addition of chloroform and methanol were analysed by SDS-PAGE. An ATB-BMPA labelled protein with an apparent molecular weight of  $\approx$  66 kDa could be detected in the proteins solubilized from the labelled cells (O), but neither anti-GLUT2 nor GLUT1 antiserum precipitated any labelled protein from HIT-T15 cells.



**Fig. 26a-b** Detection and immunoprecipitation of ATB-BMPA photolabelled proteins from RINm5F cells.

Suspensions of  $9 \times 10^6$  RIN cells (p12) in 0.3 ml of HEPES-buffered salts solution were photolabelled with 200  $\mu\text{Ci}$  of ATB-BMPA in the presence or absence of 360 mM D-glucose. After washing, the cells were homogenised and the pellet obtained by centrifugation at 100,000  $g_{av}$  was solubilized in buffer containing the detergent  $C_{12}E_9$ . Solubilized proteins were rotated for 3 hours with 5 mg of Protein A-Sepharose coupled to either anti-GLUT2 or anti-GLUT1 C-terminal peptide antiserum. Immunoprecipitated proteins and soluble proteins remaining in the supernatant were analysed by SDS-PAGE.

**a:** In the absence of D-glucose (●) two bands with apparent molecular weights of 66-97 kDa and  $\approx 45$  kDa were detected in the supernatant containing the solubilized proteins which remained following the immunoprecipitation with anti-GLUT2 C-terminal peptide antiserum. Labelling was not displaced from either protein by performing the irradiation in the presence of 360 mM D-glucose ( $\Delta$ ).

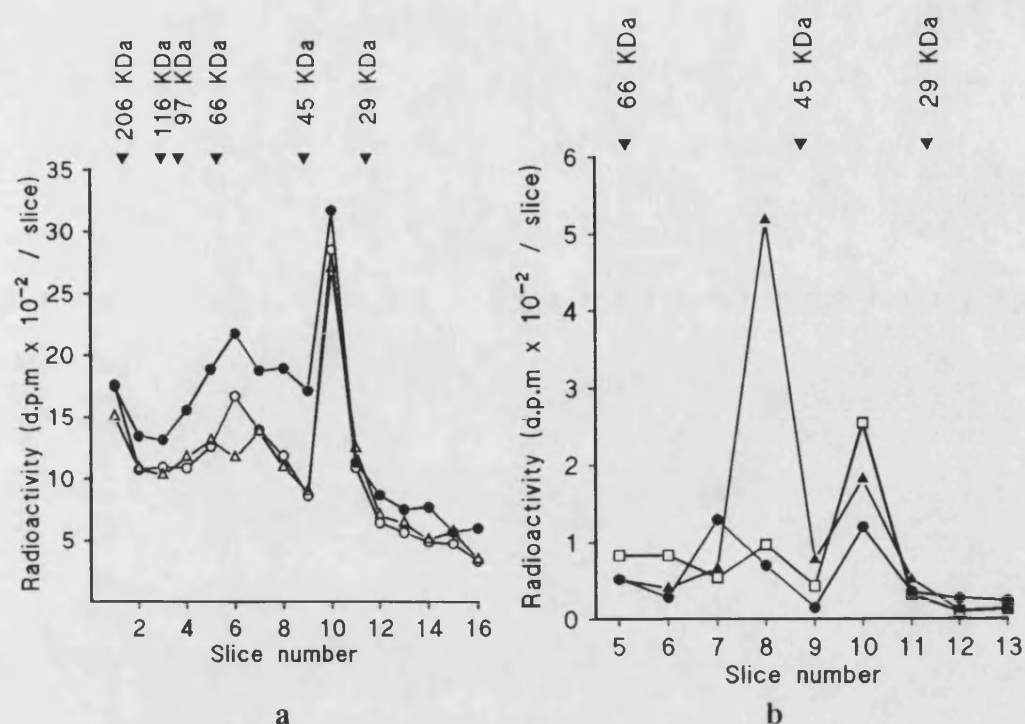
**b:** Anti-GLUT1 C-terminal peptide antiserum immunoprecipitated a photolabelled protein with an apparent molecular weight of  $\approx 45$  kDa from RINm5F cells ( $\blacktriangle$ ). No labelled GLUT1 was precipitated from cells labelled in the presence of D-glucose ( $\Delta$ ). Anti-GLUT2 C-terminal peptide antiserum did not immunoprecipitate any labelled protein from RIN cells labelled in the absence of D-glucose (●).

Anti-GLUT2 C-terminal peptide antiserum did not precipitate any labelled protein from ATB-BMPA labelled RINm5F cells (Figure 26b). Therefore, from immunoprecipitation and labelling experiments, only the GLUT1 transporter isoform which had an apparent molecular weight of  $\approx 45$  kDa could be detected in RIN cells.

#### 3.6.2.4 ATB-BMPA photolabelling of MIN 6 cells

When monolayers of MIN 6 cells were photolabelled by irradiation in the presence of ATB-BMPA, the photolabel was incorporated into proteins, which when analysed by SDS-PAGE appeared as 2 bands. The heavier band was broader and had an apparent molecular weight of  $\approx 45$ -97 kDa. The lighter protein ran as a narrow band between the 29 and 45 kDa marker proteins. As shown in Figure 27a, binding of ATB-BMPA to the 45-97 kDa protein could be displaced by 43% when irradiation was carried out in the presence of 150 mM 4,6-O-ethylidene D-glucose and by 63% by the addition of 150 mM D-glucose. Neither of these sugars displaced binding of the photolabel to the lower molecular weight protein. Similar results were obtained when either a suspension of MIN 6 cells or cell membranes were labelled (results not shown).

Anti-GLUT1 C-terminal peptide antiserum immunoprecipitated  $\approx 11\%$  of the total amount of the 45-97 kDa protein labelled in a hexose displaceable manner in MIN6 cells. This protein was not precipitated by either preimmune or anti-GLUT2 antiserum (Figure 27b). Anti-GLUT1, anti-GLUT2 and preimmune serum all non-specifically immunoprecipitated a labelled protein from MIN 6 cells, which had an apparent molecular weight of between 29-45 kDa. From its apparent molecular weight determined by SDS-PAGE analysis, this protein appeared to be identical to the protein labelled in whole MIN 6 cells in a non-hexose displaceable manner. Its identity was unknown. Therefore from labelling and immunoprecipitation experiments, only the GLUT1 transporter isoform which had an apparent molecular weight of  $\approx 50$  kDa could be detected in MIN 6 cells.



**Fig. 27a-b** Detection and immunoprecipitation of ATB-BMPA photolabelled proteins from MIN 6 cells.

MIN 6 cultures (p23) in 35 mm dishes were irradiated in the presence of 100  $\mu$ Ci of ATB-BMPA in 0.5 ml of Hepes-buffered salts solution and the indicated hexoses. Following washing, the cells were either analysed directly by SDS-PAGE (a) or solubilized in buffer containing the detergent C<sub>12</sub>E<sub>9</sub> and immunoprecipitated with 10 mg of Protein A-Sepharose coupled to 100  $\mu$ l of the indicated antisera (b). Arrowheads indicate the position of molecular weight marker proteins.

- a:**
- 150 mM D-mannitol (●)
  - 150 mM D-glucose (Δ)
  - 150 mM 4,6-O-ethylidene-D-glucose (○)

ATB-BMPA labelled a protein with an apparent molecular weight of  $\approx$ 45-66 kDa. Labelling was displaced by 63% by D-glucose and by 43% by 4,6-O-ethylidene-D-glucose. Labelling of a protein with a molecular weight of 29-45 kDa was not displaced by the addition of either of these hexoses.

- b:**
- Anti-GLUT1 C-terminal peptide antiserum (▲)
  - Anti-GLUT2 C-terminal peptide antiserum (●)
  - Preimmune serum (□)

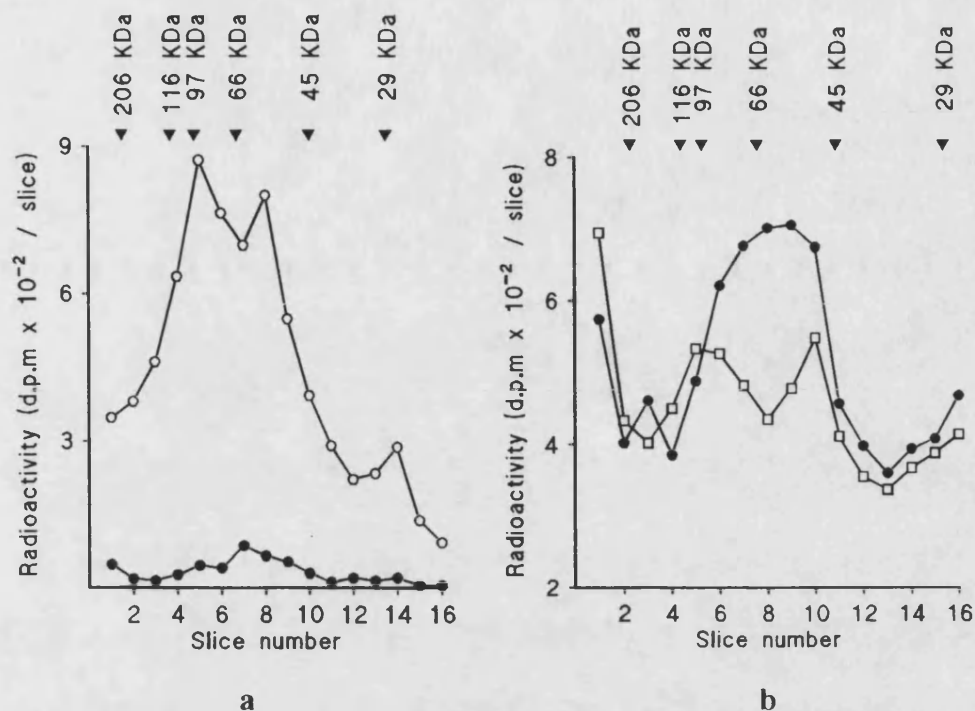
All sera precipitated a non-specifically labelled protein with an apparent molecular weight of 29-45 kDa. Only GLUT1 antiserum precipitated a protein with an apparent molecular weight of 45-66 kDa. Only the glucose transporter region of the gel is shown for clarity.

### 3.6.2.5 ATB-BMPA photolabelling of rat islets

Preliminary experiments were carried out in order to determine whether the GLUT2 protein in islets could be labelled with ATB-BMPA. Since islets could only be isolated in small quantities, the results of many experiments in which islets were irradiated in the presence of ATB-BMPA and analysed by SDS-PAGE were inconclusive, since the incorporation of photolabel was so low. However, as shown in Figure 28a, when a relatively large number of freshly isolated rat islets were irradiated in the presence of a high concentration of ATB-BMPA, radiolabel was incorporated into proteins, which when analysed by SDS-PAGE were found to migrate as a very broad band between the 45 and 116 kDa marker proteins. A similar result was obtained using a large number ( $\approx 950$ ) of frozen islets which were kindly supplied by Hoffmann La Roche (Nutley, New Jersey, U.S.A.) Labelling in the presence of 500 mM 4,6-O-ethylidene-D-glucose did not significantly displace the binding of the photolabel to these frozen islets (results not shown). Approximately 6% of the ATB-BMPA labelled protein in islets was immunoprecipitated with anti-GLUT2 C-terminal peptide antiserum. The precipitated protein had an apparent molecular weight of between 45-66 kDa. Owing to the low availability of islets, it could not be demonstrated whether ATB-BMPA binding to GLUT2 was displaced by labelling in the presence of an inhibitor such as D-glucose.

As shown in Figure 28b, not all of the labelled protein precipitated from islets by anti-GLUT2 antiserum was necessarily GLUT2, since 48% of the labelled protein was also precipitated non-specifically by preimmune rabbit serum. The identity of the labelled proteins that were not specifically precipitated by anti-GLUT2 antibodies was unknown. However, since whole islets, and not  $\beta$ -cells, were used, the GLUT1 transporter isoform may also have been present. Again, owing to the low availability of islet material the presence of this isoform could not be investigated.





**Fig. 28a-b** Detection and immunoprecipitation of ATB-BMPA labelled proteins from rat islets.

**a:** Islets freshly isolated from 6 rat pancreases were suspended in 200  $\mu$ l of a HEPES-buffered balanced salts solution and irradiated in the presence of 400  $\mu$ Ci of ATB-BMPA. After washing and solubilization of the whole islets in buffer containing the detergent C<sub>12</sub>E<sub>9</sub>, solubilized proteins were rotated for 3 hours with 5 mg of Protein A-Sepharose coupled to 50  $\mu$ l of anti-GLUT2 C-terminal peptide antiserum. Analysis of immunoprecipitated proteins (●) and labelled proteins remaining in the supernatant (○) was by SDS-PAGE. Anti-GLUT2 antiserum precipitated approximately 6% of the 45-66 kDa solubilized labelled protein.

**b:** Frozen islets were defrosted and centrifuged at 20,000  $g_{av}$  for 20 min to enable them to be transferred to 200  $\mu$ l of a D-glucose-free HEPES-buffered balanced salts solution. The islets were irradiated in the presence of 400  $\mu$ Ci of ATB-BMPA, washed, homogenised and a membrane pellet obtained by centrifugation at 100,000  $g_{av}$ . This pellet was solubilized in buffer containing the detergent C<sub>12</sub>E<sub>9</sub>. The solubilized, labelled proteins were immunoprecipitated with 10 mg of Protein A-Sepharose coupled to 100  $\mu$ l of anti-GLUT2 C-terminal peptide antiserum (●) or 100  $\mu$ l of preimmune serum (□). Precipitated proteins were analysed by SDS-PAGE. Compared with preimmune serum, GLUT2 C-terminal peptide antiserum precipitated double the amount of a protein with an apparent molecular weight of 45-66 kDa.

Attempts to photolabel crude membrane preparations from islet cells were unsuccessful. Similarly, the solubilization of labelled islets in a buffer containing the detergents Triton X-100 (4%) and SDS (0.05%) instead of C<sub>12</sub>E<sub>9</sub> did not increase the amount of GLUT2 immunoprecipitated. Increasing the amount of time that the ATB-BMPA was left in contact with the islets both before and during irradiation in order to ensure complete penetration of the label into the clumps of islet cells also failed to improve the ATB-BMPA photolabelling of islet tissue (results not shown).

### **PART III INVESTIGATION OF THE ANTIGENS RECOGNISED BY AUTOANTIBODIES PRESENT IN SERUM FROM CHILDREN WITH IDDM**

The anti- $\beta$ -cell surface antibodies present in the serum of patients with new onset IDDM are widely believed to play a role in the pathogenesis of this disease, which is characterised by a specific necrosis of the islet  $\beta$ -cells. Very few  $\beta$ -cell autoantigens have been positively identified. There is a need therefore, for the identification of  $\beta$ -cell autoantigens which may be involved in the initial pathology of IDDM. In the short term, this is important for the development of an assay to screen for "pre-diabetes". In the long term, it may result in the application of immunotherapy designed to arrest the autoimmune attack against a specific  $\beta$ -cell antigen, and therefore to cure IDDM.

This section of results describes the development of a  $\beta$ -cell line plasma membrane ELISA, designed to detect the presence of anti- $\beta$ -cell surface antibodies in serum from diabetic children. Attempts were made to use Western blotting to identify  $\beta$ -cell antigens recognised specifically by antibodies in diabetic serum.

Since, in the very early stages of autoimmune diabetes, GLUT2 expression and glucose transport are decreased (Tominaga *et al.*, 1986; Orci *et al.*, 1990a) and  $\beta$ -cells show a decreased glucose-induced insulin response, it is possible that anti-GLUT2 autoantibodies play a role in the pathogenesis of IDDM and that the GLUT2 protein is therefore a  $\beta$ -cell autoantigen. Evidence supporting this hypothesis has been obtained by Johnson *et al.* (1990). Using techniques established in and information obtained from the preliminary studies carried out on the GLUT2 protein (Part I), it has been attempted to confirm the existence of these putative anti-GLUT2 antibodies using ELISAS, Western blotting and immunoprecipitation of ATB-BMPA labelled proteins.

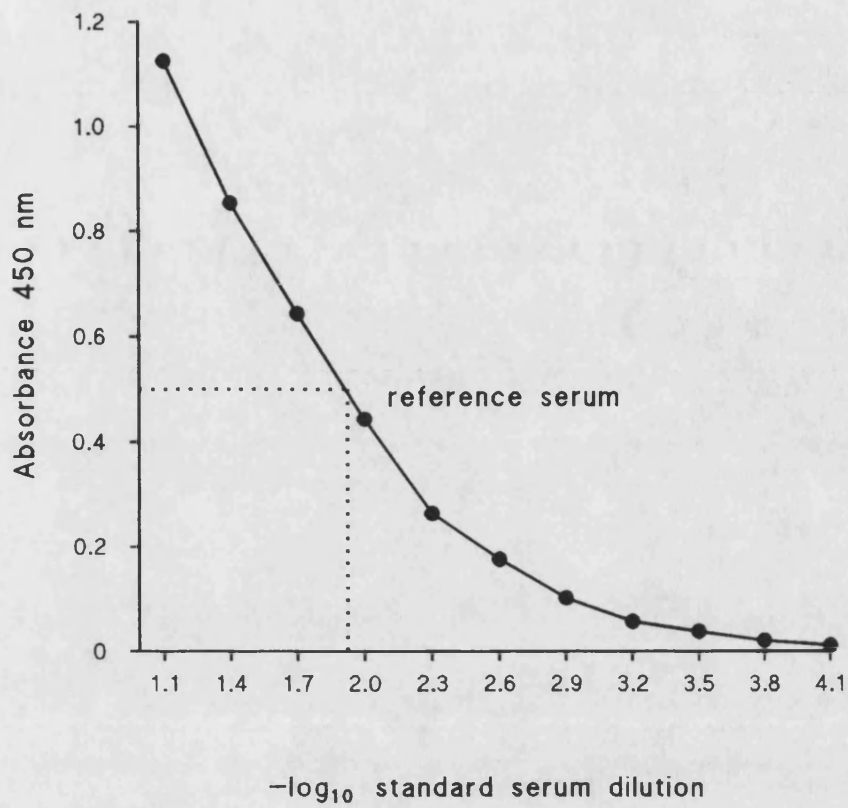
Ideally, the GLUT2 protein used in these studies should have been obtained from islet tissue. However, it was not technically feasible to obtain sufficient material from this source. Theoretically, a  $\beta$ -cell line should have been a good alternative, but as was shown in Part II these abnormal  $\beta$ -cells do not appear to express the GLUT2 protein. Therefore, liver plasma membranes have been used as a source of GLUT2. They were considered to be a suitable substitute since Johnson *et al.* (1990a) confirmed that antibodies able to inhibit  $\beta$ -cell glucose transport cross-reacted with GLUT2-containing liver plasma membranes.

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### 3.7 THE USE OF AN ELISA TO DETECT ANTI- $\beta$ -CELL MEMBRANE ANTIBODIES IN SERUM FROM DIABETIC AND CONTROL CHILDREN

Serum samples were screened for the presence of antibodies against the HITm2.2 and MIN 6  $\beta$ -cell lines. The HITm2.2 line was chosen because it grew rapidly and appeared to retain glucose-induced insulin-secretion. MIN 6 cells were used because they have been reported to exhibit glucose-inducible insulin secretion comparable with that found in normal islets and to contain GLUT2 mRNA (Miazaki *et al.*, 1990), although the GLUT2 protein was not detected in the present study.

Serum samples screened against these two cell lines were obtained from 2 sources designated set 1 and set 2 as detailed in section 2.3. Both sets contained samples taken from children with recently diagnosed IDDM and from normal children of a similar age. The serum samples in set 2, which were originally collected as part of an autoimmune diabetes study at St. Bartholomews Hospital Centre for Clinical Research, were considered to be a "better" set of experimental samples, since they had been collected and stored under controlled conditions. All IDDM samples in set 2 were known to be positive for ICCA tested by standard immunofluorescence assays and all control samples were ICCA negative. The samples in set 1 were originally drawn from patients for the purpose of analysis in hospital biochemistry or pathology laboratories. The conditions under which they were collected and stored were therefore not standardized. Many of the serum samples in set 1 contained high levels of lipid and some contained a white precipitate which had to be removed by centrifugation. The identity of the precipitate was unknown but it was found in both IDDM and control samples.



**Fig. 29** Typical standard curve used in MIN 6 ELISAS to convert absolute absorbance values of test sera to a percentage of the standard serum.

The reference serum has a value of  $86.4 \pm 6.44\%$  (mean of 10 determinations  $\pm$  S.E.M).

Since anti- $\beta$ -cell autoantibodies have previously been shown to be restricted to the IgG class of immunoglobulins (Lernmark *et al.*, 1978; Millward *et al.*, 1988), only this class of antibody was analysed in all ELISAS. Titres of membrane-binding antibodies (measured by absorbance at 450 nm), were expressed as a percentage of the absorbance given by the standard serum, using a standard curve like the one shown in Figure 29. The reference samples included on each plate did not differ significantly from each other in their absorbance values.

Samples from set 1 and set 2 have been assayed separately, since sera from these two sources were not available concurrently.

### **3.7.1 Comparison of the levels of anti- $\beta$ -cell line membrane antibodies in serum samples (set 1) from diabetic and control children**

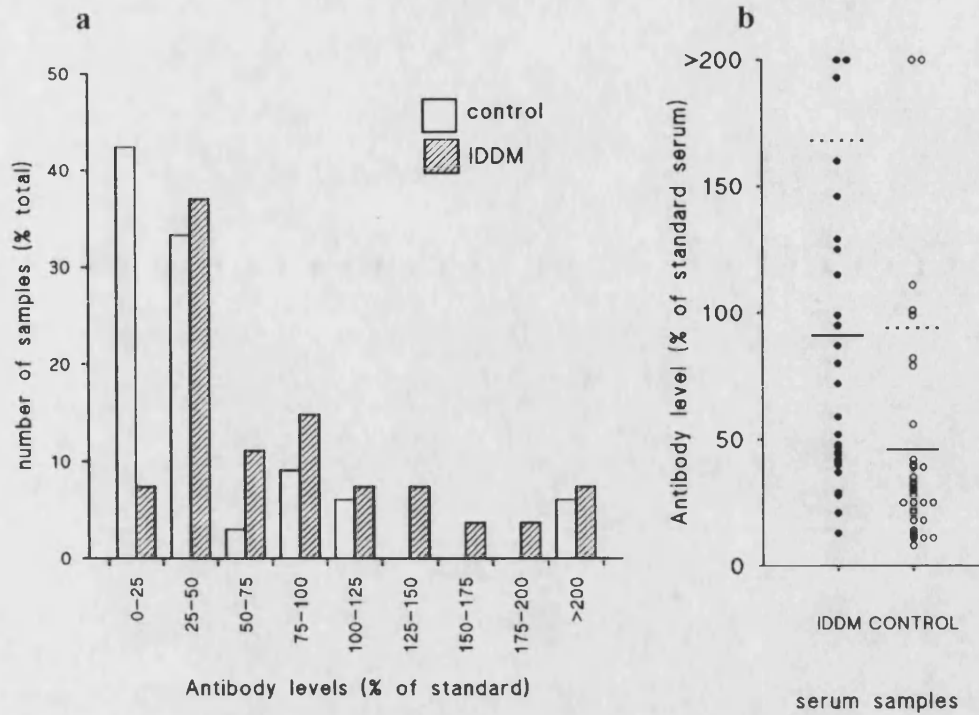
Titres of anti-HITm2.2 membrane antibodies were measured in serum samples from 27 children with recently diagnosed insulin-dependent diabetes and compared with those of 33 samples from control children (set 1). Each sample was assayed in triplicate. The average variation between triplicate measurements was  $\pm 13.6\%$  (S.E.M. expressed as a percentage of the mean titre). Any value that had a S.E.M. much higher than this average was rejected. Each sample was tested in 3-4 independent assays. The inter-assay variation was high,  $\pm 23\%$  for diabetic samples and  $\pm 20\%$  for control samples. Variability is expressed as the S.E.M. calculated as a percentage of the mean titre for each sample. The high intra- and inter-assay variation probably arose from uneven coating of the ELISA plates with the antigen, and from variations in the quality of the plasma membrane preparation used (see discussion section 4.4)

A cumulative frequency distribution was calculated from both sets of data, as shown in Figure 30a, and a Kolmogorov-Smirnov (K-S) analysis was performed on the results. The greatest difference between diabetic and control samples occurred in titres

above 25% of the standard serum. That is, 92.6% of IDDM samples and 57.6% of control samples had titres greater than 25% of the standard. The calculated K-S value of 0.350 was above the critical value of D of 0.316. This indicated that there were significantly more IDDM samples than control samples with binding higher than 25% of the standard. This difference was significant at  $p=0.1$ , and was nearly significant at  $p=0.05$ , where the critical value of 0.352 was not quite exceeded. The distribution of the titres of the anti-HITm2.2 membrane antibodies in samples from IDDM and control populations is more clearly shown in Figure 30b. More IDDM sera have higher titres of these antibodies, but there is an overlap between the two populations. Therefore, it was not possible to establish a cut-off point which could have been used to determine whether a sample had been obtained from a normal or a diabetic patient.

As a comparison, the same set of serum samples was screened against MIN 6 plasma membranes. In this assay the problems of high intra-and inter-assay variation were resolved by the use of plasma membranes prepared using Method I (section 2.10.4.1), which produced membranes of a consistent quality, and by the homogenization of the membranes in the coating buffer, which resulted in a more homogeneous coating of the ELISA plates. Using these methods the intra-assay variation was reduced to  $\pm 2.7\%$  (S.E.M. expressed as a percentage of the mean titre). Due to limited availability of serum samples, each sample was tested in triplicate in a single assay. Statistical analysis of the results using the K-S test revealed that the greatest difference between diabetic and control samples occurred with titres of anti-MIN 6 antibodies above 25% of the standard serum, that is, 74.2% of the control samples and 96.6% of the diabetic samples had titres  $> 25\%$  of the standard. These results were calculated from the data shown in Figure 31a. The calculated K-S value of 0.224 was below the critical value of D of 0.315 necessary for significance at  $p=0.1$ , therefore in this assay the higher binding of IDDM samples was not significantly different from that shown by the controls. The distribution of antibody levels in IDDM and control samples is shown in Figure 31b to be completely overlapping.



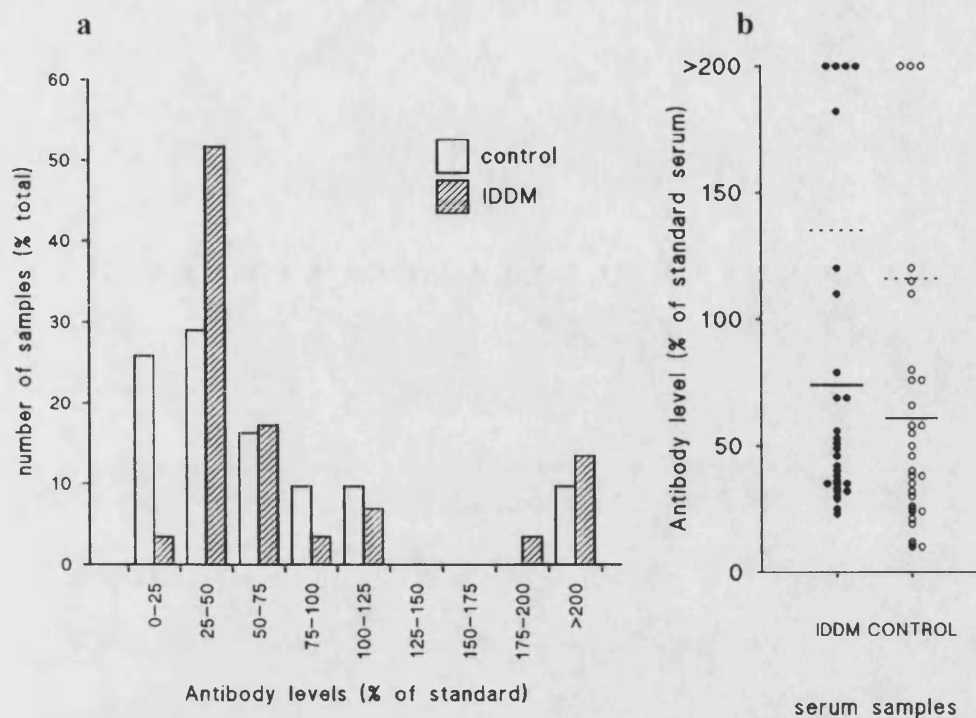


**Fig. 30a-b IgG binding to HITm2.2  $\beta$ -cell membranes. A comparison between antibody levels in IDDM (n=27) and control (n=33) serum samples from set 1.**

Using an ELISA, serum samples were assayed for antibodies against HITm2.2 plasma membranes (prepared using method II described in section 2.10.4.2). Samples, diluted 1:100 were assayed in triplicate on 3-4 occasions. The titres of IgG antibodies in each test sample (measured as absorbance at 450 nm) has been expressed as a percentage of the standard serum.

**a:** Results are presented as a histogram. Analysis with the K-S statistical test revealed that there were significantly ( $p=0.1$ ) more IDDM than control samples with antibody titres above 25% of the standard.

**b:** Results are presented as a scatter diagram. The solid lines depict the mean titre of each data set and the dashed lines + 1 standard deviation.



**Fig. 31a-b** IgG binding to MIN 6  $\beta$ -cell membranes. A comparison between antibody levels in IDDM ( $n=29$ ) and control ( $n=31$ ) samples from set 1.

Using an ELISA, serum samples were assayed for antibodies against MIN 6 plasma membranes, prepared using method I described in section 2.10.4.1. Samples diluted 1:100 were assayed in triplicate. The titre of IgG (measured as absorbance at 450 nm) in each sample has been expressed as a percentage of the standard serum.

**a:** Results are presented as a histogram. Although there were more higher binding IDDM samples than control samples, by K-S analysis the difference was shown not to be significant at  $p=0.1$ .

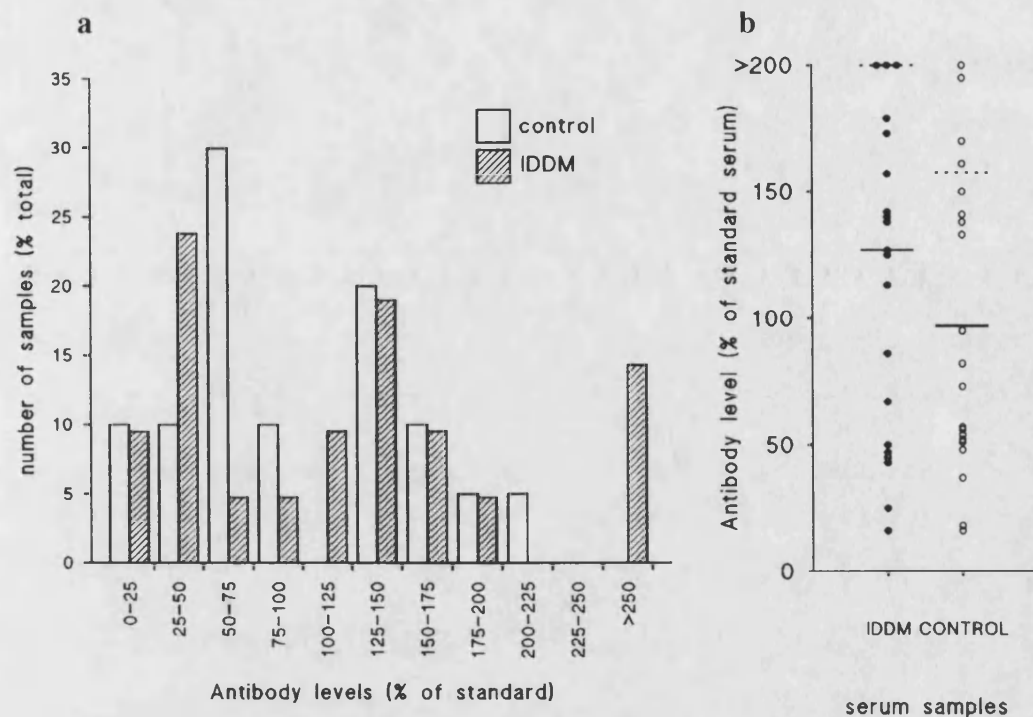
**b:** Results are expressed as a scatter diagram. The solid lines depict the mean titre of each data set, the dashed lines + 1 standard deviation.

The small volume of the serum samples in set 1 prevented them being re-assayed against HITm2.2 membranes using an ELISA in which variation was reduced by the methods described above for the MIN ELISA. The HIT and MIN ELISAS were therefore repeated using a second set of serum samples (set 2).

### **3.7.2 Comparison of the levels of anti- $\beta$ -cell line membrane antibodies in the serum of ICCA-positive diabetic children and ICCA-negative controls (set 2)**

In this second study, antibody levels in serum samples from 21 recently diagnosed ICCA-positive IDDM children and 20 ICCA-negative non-diabetic siblings were assayed against HITm2.2 and MIN 6 membranes which had been prepared using method I (section 2.10.4.1). The membranes were homogenized in the coating buffer to produce an even coating of the plates and consequently to reduce the assay variation. Each sample was tested in triplicate against MIN 6 membranes and the mean variation was  $\pm 2.6\%$  (S.E.M. expressed as a percentage of mean titre). Each sample was also tested in five independent experiments. The inter-assay variation was  $\pm 11.2\%$  (S.E.M. expressed as a percentage of mean titre) and a reference serum included on each plate had a value which was not significantly different from the mean shown in Figure 29.

A cumulative frequency distribution was calculated from the data shown in Figure 32a and a K-S analysis was performed on the results. The greatest difference between diabetic and control samples occurred with titres of anti-MIN 6 antibodies above 100% of the standard, that is, 40% of the controls and 57.2% of the diabetics had titres  $> 100\%$  of the standard. This difference gave a K-S value of 0.172 which was lower than the critical value of 0.381 ( $p=0.1$ ), therefore there was no significant difference between the level of antibodies in the IDDM and control samples. This is also illustrated in Figure 32b, which shows that the two data sets have overlapping distributions.



**Fig. 32a-b** IgG binding to MIN 6  $\beta$ -cell membranes. A comparison between antibody levels in IDDM ( $n=21$ ) and control ( $n=20$ ) serum samples from set 2.

Using an ELISA, serum samples diluted 1:100 were assayed for antibodies against MIN 6 plasma membranes prepared according to method I described in section 2.10.4.1. Samples were assayed in triplicate on five separate occasions. The titre of IgG in each test sample has been expressed as a percentage of the standard serum.

**a:** Results are presented as a histogram. By K-S analysis there was no significant ( $p=0.1$ ) difference in the levels of antibodies in the IDDM and control samples.

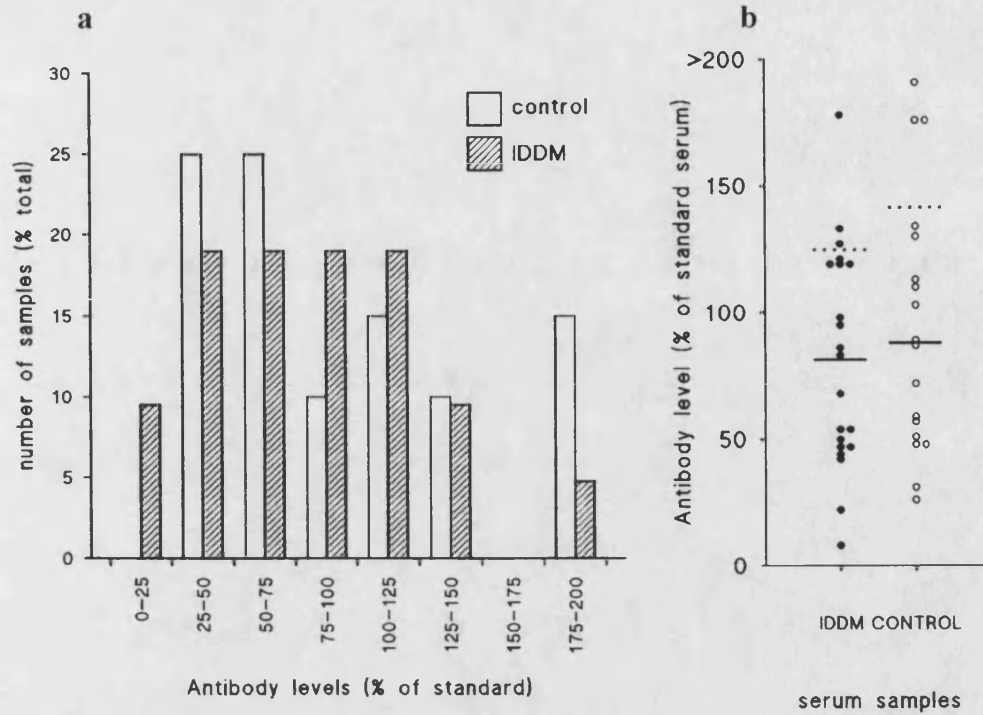
**b:** Results are presented as a scatter diagram. The solid lines depict the mean titre of each data set and the dashed line + 1 standard deviation.

IDDM and normal samples from set 2 were also assayed against HITm2.2 membranes which had been prepared using Method I (2.10.4.1). The variation between triplicate samples was  $\pm 2.98\%$  (S.E.M expressed as a percentage of mean sample titre). Each sample was tested on five separate occasions. Inter-assay variation was  $\pm 11.0\%$  (S.E.M. expressed as a percentage of mean sample titre).

When a K-S analysis was performed on the data shown in Figure 33a, the greatest difference between control and IDDM samples was found to occur with titres of anti-HITm2.2 antibodies above 150% of the standard, that is, 4.77% of IDDM samples and 15% of controls had titres  $> 150\%$  of the standard. The calculated K-S value of 0.106 was less than 0.381, the critical value of D at  $p=0.1$ . Therefore, there was no significant difference between anti-HITm2.2 antibody levels in control and IDDM samples. This is also illustrated in Figure 33b, in which both IDDM and control sera can be seen to have a wide range of titres which fall into an overlapping distribution.

### **3.7.3 Comparison of antibody binding to MIN 6 and HITm2.2 membrane antigens**

In approximately half of all the samples tested, from both set 1 and set 2, the value obtained for the titre of anti- $\beta$ -cell membrane antibodies was very similar whether it was obtained by screening against HITm2.2 or against MIN 6 membranes. As illustrated in Figures 34a-b this was mostly, though not exclusively, observed when the antibody titres were low. Approximately 55% of the samples from set 1 contained levels of antibodies that differed by more than  $\pm 20\%$  in their binding to MIN compared with HIT membrane antigens (Figure 34a). This difference in levels of antibodies recognising MIN and HIT antigens was also observed in the samples from set 2 (Figure 34b) in which  $\approx 46\%$  of the samples differed by more than  $\pm 20\%$  in their binding in the HIT compared with the MIN ELISA. Typically the titres of anti-MIN6 membrane antibodies were higher than those of anti-HITm2.2 antibodies.

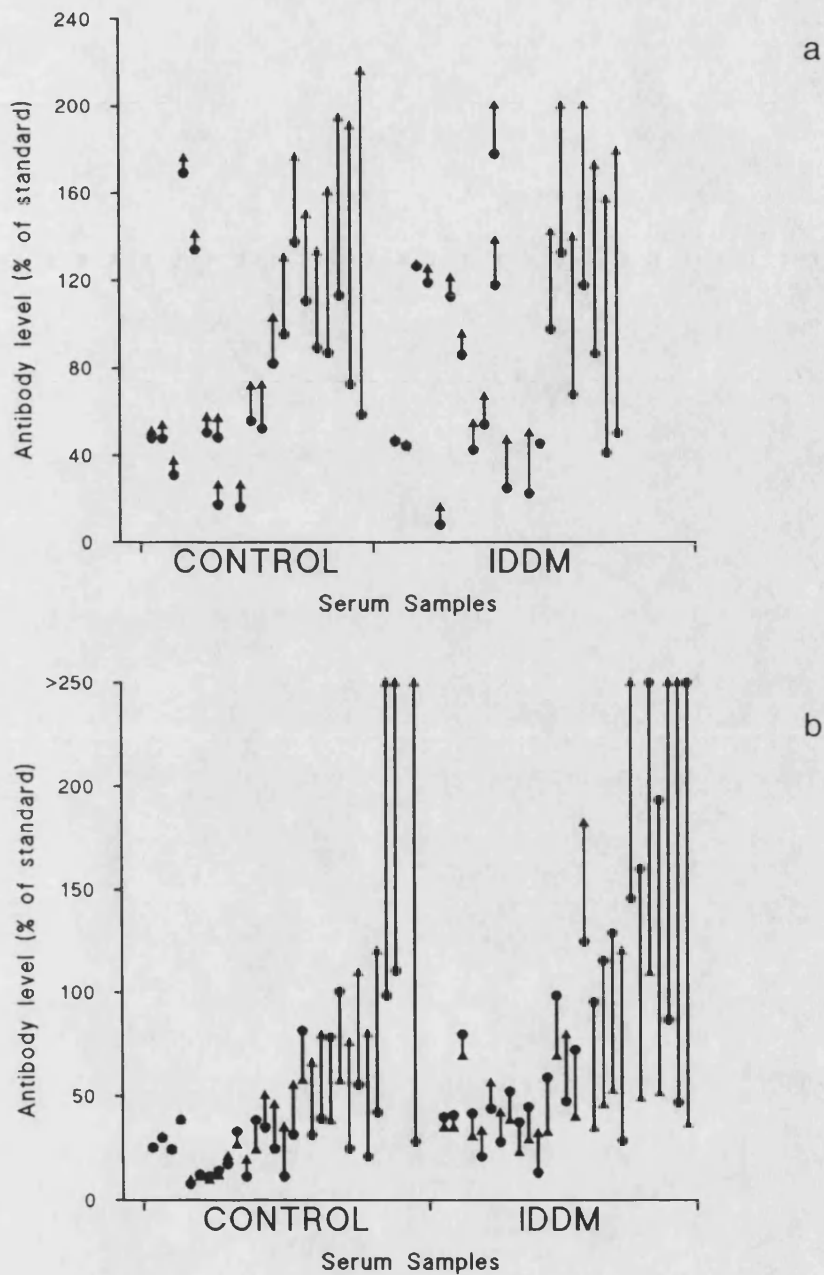


**Fig. 33a-b IgG binding to HITm2.2  $\beta$ -cell membranes. A comparison between antibody levels in IDDM (n=21) and control (n=20) serum samples from set 2.**

Using an ELISA, serum samples diluted 1:100 were assayed for antibodies against HITm2.2 plasma membranes, prepared according to method I described in section 2.10.4.1. Samples were assayed in triplicate on five separate occasions. The titre of IgG in each sample has been expressed as a percentage of the standard serum.

**a:** Results are presented as a histogram. By K-S analysis there was no significant ( $p=0.1$ ) difference in the levels of antibodies in the IDDM and control samples.

**b:** Results are presented as a scatter diagram. The solid lines depict the mean titre of each data set and the dashed line + 1 standard deviation.



**Fig. 34a-b** Comparison of the levels of antibodies in serum samples from recently diagnosed diabetic or control children that bind to MIN 6 and HITm2.2 membrane antigens.

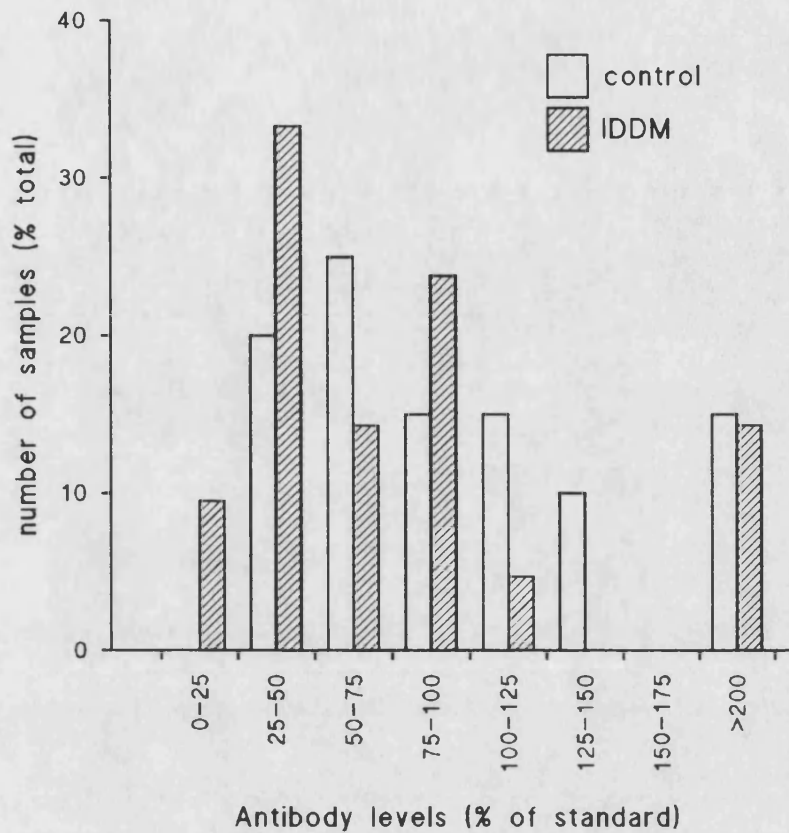
Diabetic and control serum samples from set 1 and set 2 (Fig 34a and 34b respectively) were assayed in MIN 6 (▲) or HITm2.2 (●) membrane ELISAS as described in sections 3.7.1 and 3.7.2. The diagram compares the value obtained for the mean antibody titre (expressed as a percentage of the standard serum) for each sample measured from a MIN 6 and a HITm2.2 ELISA. For clarity the samples have been displayed in order of increasing difference in the titre of antibodies measured in the two different ELISAS.

#### **3.7.4 Comparison of the levels of anti-rat liver plasma membrane antibodies in the serum of ICCA-positive diabetic children and ICCA-negative controls**

Serum samples from set 2 were also assayed against liver plasma membrane, since higher levels of anti-liver membrane antibodies in diabetic compared with control samples could have been indicative of the presence of anti-GLUT2 autoantibodies. However, when a K-S analysis was performed on the results shown in Figure 35, it was revealed that there was no significant difference between the levels of anti-liver antibodies in the control compared with the diabetic serum samples. The greatest difference between the data sets occurred above 50% of the standard serum, with 80% of the normal and 57% of the IDDM samples having titres > 50% of the standard. This gave a K-S value of 0.229 which did not exceed the critical value of D of 0.381 for significance at  $p=0.1$ . Each IDDM ( $n=21$ ) and control ( $n=20$ ) sample was assayed in triplicate and the S.E.M., expressed as a percentage of the mean titre, was  $\pm 3.6\%$ .

Therefore, serum samples from diabetic patients did not contain significantly higher levels of anti-liver plasma membrane antibodies than were present in normal serum. The ELISA was therefore not suitable for the detection of anti-GLUT2 autoantibodies. It was attempted to use affinity purified GLUT2 (see section 3.9) as the antigen in an ELISA, but in preliminary studies, binding of antibodies from IDDM serum could not be detected (results not shown). This was probably due to the low concentration of GLUT2 that was used, this was a consequence of the low availability of affinity-purified GLUT2.





**Fig. 35 IgG binding to rat liver plasma membranes. A comparison between antibody levels in IDDM (n=21) and control (n=20) serum samples from set 2.**

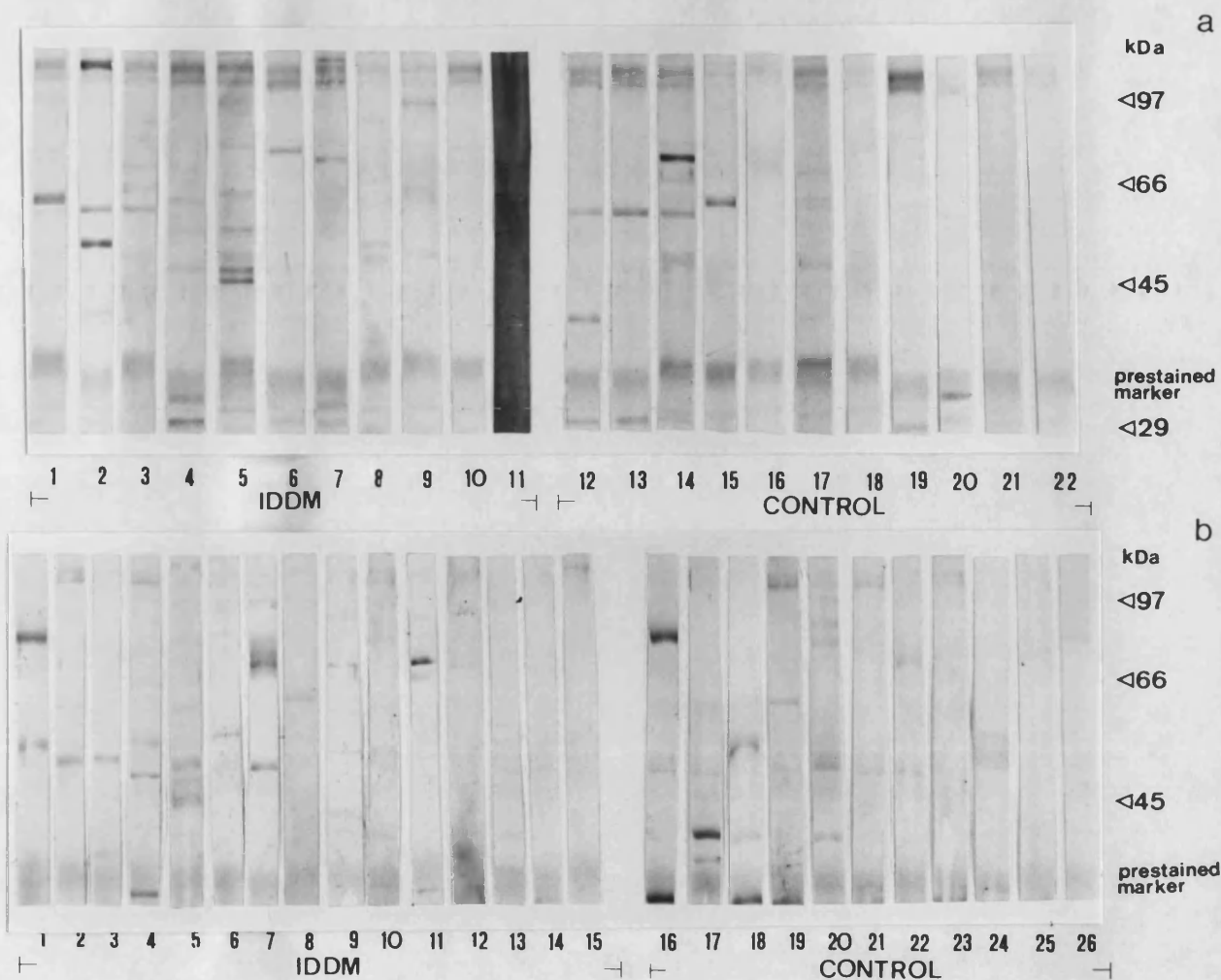
Using an ELISA, serum samples diluted 1:100 were assayed for antibodies against liver plasma membranes. Samples were assayed in triplicate. The titre of IgG in each test sample has been expressed as a percentage of the standard serum.

By K-S analysis there was no significant ( $p=0.1$ ) difference in the levels of antibodies in the IDDM and control samples.

### **3.8 THE USE OF WESTERN BLOTTING TO DETECT ANTIGENS RECOGNISED BY ANTIBODIES PRESENT IN THE SERUM OF IDDM PATIENTS**

Plasma membrane proteins prepared from HITm2.2, RINm5F and MIN 6  $\beta$ -cell lines have been immunoblotted with serum samples from children with recently diagnosed IDDM or from non-diabetic controls. Owing to the small volume of serum available, samples were not preabsorbed with rat liver powders before use.

Using the alkaline phosphatase colourimetric detection system a heterogeneous group of  $\beta$ -cell proteins were found to be recognised by antibodies present in serum samples from both normal and diabetic children. In MIN 6 membranes dominant bands with apparent molecular masses of  $\approx$  69, 58, 55, 49, 45, 39, 35 and 30 kDa were detected. A typical result obtained by immunoblotting MIN 6 membranes with a random selection of IDDM and control samples is shown in Figure 36a. None of the antigens detected was diabetes specific. As shown in Figure 36b dominant bands at  $\approx$  76, 66, 50, 47, 40 and 33 kDa were detected in HITm2.2 membranes. Most serum samples detected the same major antigen(s) in HITm2.2 membranes as they detected in RINm5F cells, indicating that these two cell lines were antigenically similar (results not shown). No antigen was detected in any of the  $\beta$ -cell lines tested by more than one IDDM sample without also being recognised by control samples. It was demonstrated that diabetic serum contained a wider range of antibodies that were able to recognise more antigens in  $\beta$ -cell lines compared with serum from control children. On average, each control sample recognised 1.8 bands in MIN6 plasma membranes. This was compared with the 3.1 bands recognised on average by each IDDM sample.



**Fig. 36a-b Immunoblotting of  $\beta$ -cell line plasma membranes with serum from diabetic and control children.**

Plasma membranes were prepared from MIN 6 and HITm2.2 cells according to method I as described in section 2.10.4.1. 50  $\mu$ g of protein were loaded per lane of a thin SDS-polyacrylamide gel and the proteins were separated by electrophoresis. Proteins were transferred electrophoretically to nitrocellulose. This was overlaid with human serum samples diluted 1:100 in TBS containing 3% BSA (blocking protocol II). Following an overnight incubation, the blots were washed, then developed with alkaline phosphatase conjugated anti-human IgG antibodies.

**a:** **IDDM** MIN 6 plasma membranes blotted with eleven IDDM serum samples from set 1

**Control** MIN 6 plasma membranes blotted with eleven normal serum samples from set 1

Both control and IDDM sera recognised a heterogeneous group of proteins in MIN plasma membranes. More bands were detected by the diabetic samples.

**b:** **IDDM** HITm2.2 plasma membranes blotted with fifteen IDDM serum samples from set 2

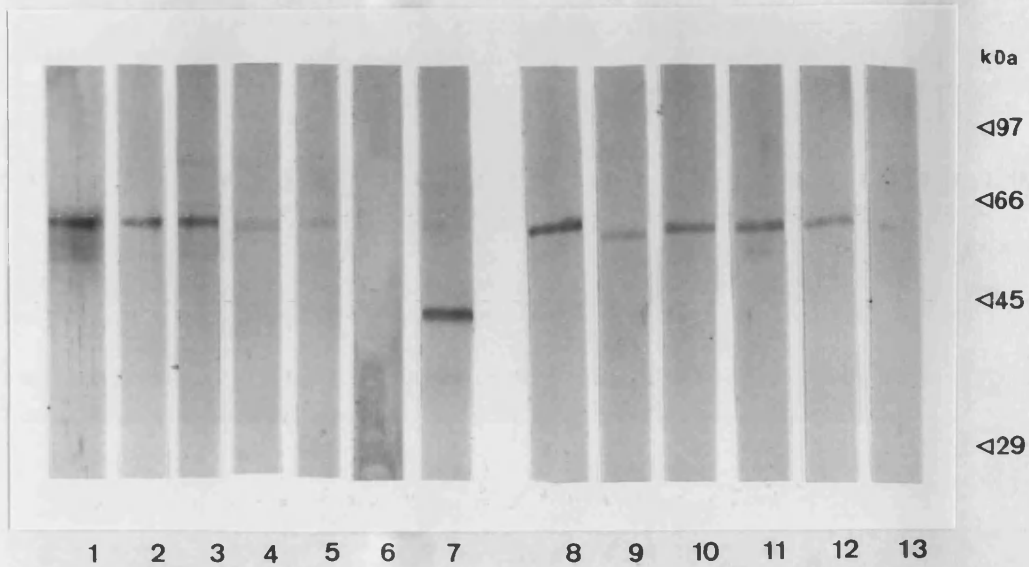
**Control** HITm2.2 plasma membranes blotted with eleven control serum samples from set 2

Both control and IDDM sera recognised a heterogeneous group of proteins in HIT plasma membranes. More bands were detected by the diabetic samples.

In an attempt to improve the sensitivity of this blotting technique, the detergent Triton X-114 was used to extract amphiphilic proteins from a membrane preparation of HITm2.2 and RINm5F cells. The extracted proteins were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted. As illustrated in Figure 37, immunoblotting of Triton X-114 extracts from HITm2.2 cells detected a protein with an apparent molecular mass of  $\approx 58$  kDa which was recognised very strongly by 2/6 IDDM samples and less strongly by a further 3/6 IDDM samples. This was not however a diabetes-specific antigen, since it was also recognised by 5/6 control samples. One IDDM sample also bound strongly to a protein with an apparent molecular mass of  $\approx 44$  kDa. The sera tested recognised exactly the same proteins in Triton X-114 extracts of RINm5F cells (results not shown).

The identity of the  $\approx 58$  kDa protein was not known, but a protein with the same apparent molecular mass was recognised in the Triton X-114 extract by anti-human GLUT2 C-terminal peptide antiserum. This serum had previously been shown to recognise other proteins in addition to GLUT2 including BSA and antigens present in erythrocyte and adipocyte membranes (see section 3.1). The 58 kDa protein was not recognised by the anti-rat-GLUT2 C-terminal peptide antiserum (results not shown). It was also demonstrated that the  $\approx 58$  kDa protein was not BSA, which could have been adsorbed onto the  $\beta$ -cell lines during culture in FCS-containing medium, since the human samples tested did not recognise BSA in a Western blot (results not shown).

It was also attempted to demonstrate the presence of anti-GLUT2 autoantibodies in serum samples from diabetic children by immunoblotting against GLUT2-containing liver plasma membranes. Some of the diabetic sera tested did contain antibodies that reacted with rat liver plasma membrane proteins, but no antigens were recognised that had an apparent molecular mass equivalent to that of the liver GLUT2 protein (results not shown).



**Fig. 37** Immunodetection of proteins extracted from HITm2.2 cells with the detergent Triton X-114.

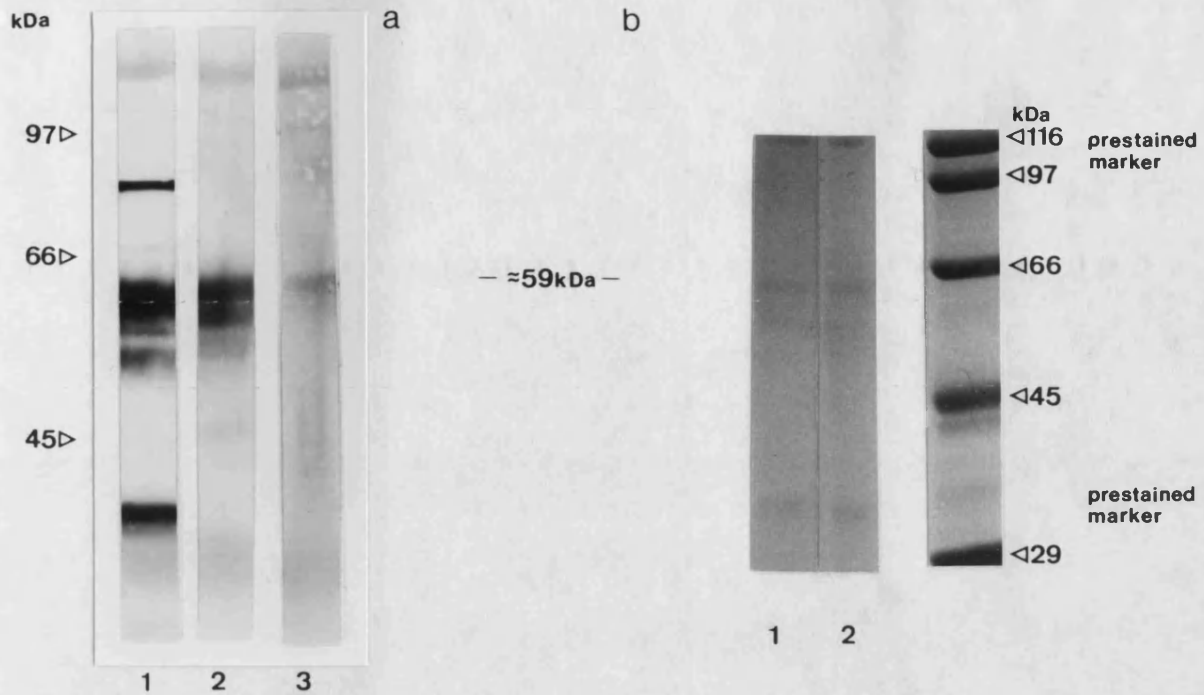
Proteins were extracted from a membrane fraction of HITm2.2 cells using the detergent Triton X-114 as described in section 2.11. The extracted proteins were analysed by SDS-PAGE and electrophoretically transferred to nitrocellulose. Blocking protocol I (section 2.17.3.1) was used to immunoblot the immobilised proteins with anti-human-GLUT2 C-terminal peptide antiserum (lane 1), serum from recently diagnosed insulin dependent diabetic children (lanes 2-7) and serum from non-diabetic control children (lanes 8-13). All sera were used at a 1:100 dilution. Bound IgG was detected by incubation with the appropriate anti-rabbit or anti-human horse-radish peroxidase conjugated anti-IgG. Anti-human GLUT2 antiserum detected a protein with an apparent molecular mass of  $\approx 58$  kDa. A protein with the same apparent molecular mass was strongly recognised by 2/6 serum samples from IDDM patients and less strongly by another 3/6 samples. In addition one IDDM sample detected a protein with an apparent molecular mass  $\approx 44$  kDa. The  $\approx 58$  kDa protein was also detected by 5/6 control samples.

### 3.9 AFFINITY PURIFICATION OF LIVER GLUT2

Two methods were used to immunoaffinity purify a protein from rat liver plasma membranes which was recognised by anti-GLUT2 antiserum in a Western blot and had a molecular weight on SDS-PAGE consistent with its identification as GLUT2.

Method I used a protocol previously described for the purification of an integral membrane glycoprotein, the nicotinic acetylcholine receptor (Momi & Lennon, 1982). From initial experiments, it was shown that some GLUT2 could be detected in the unbound fraction which washed off the column, this indicated that the binding affinity of the column was low and that the binding capacity of the column had been exceeded. The 0.25 M NaCl wash did not elute immunodetectable GLUT2 from the column. A protein with an apparent molecular weight of  $\approx 59$  kDa could be detected in the 1 M and 2 M NaCl washes when they were immunoblotted with anti-rat GLUT2 C-terminal peptide antiserum. As shown in Figure 38a, a protein present in the fractions eluted from the column with 2 M NaCl was recognised strongly by anti-human GLUT2 C-terminal peptide antiserum and less strongly by anti-rat GLUT2 C-terminal peptide antiserum. The protein recognised by these sera had a molecular weight identical to the protein recognised strongly by anti-GLUT2 antiserum in liver plasma membranes. No bands were detected when these blots were probed with preimmune serum (results not shown).

Proteins present in the 1 and 2 M washes were also detected by Coomassie Blue staining of SDS-polyacrylamide gels. Two proteins with apparent molecular masses of  $\approx 59$  and  $\approx 51$  kDa were detected very faintly as shown in Figure 38b. The  $\approx 59$  kDa band aligned with the protein detected by immunoblotting with anti-GLUT2 antiserum, the identity of the lower band was unknown, but it was possibly the lower band detected by immunoblotting liver plasma membranes with anti-GLUT2 antiserum. Since these were the only major proteins detected, albeit in very low quantities, this indicated a significant increase in the purification of the GLUT2 protein.



**Fig. 38a-b Immunoprecipitation of liver GLUT2 (method I).**

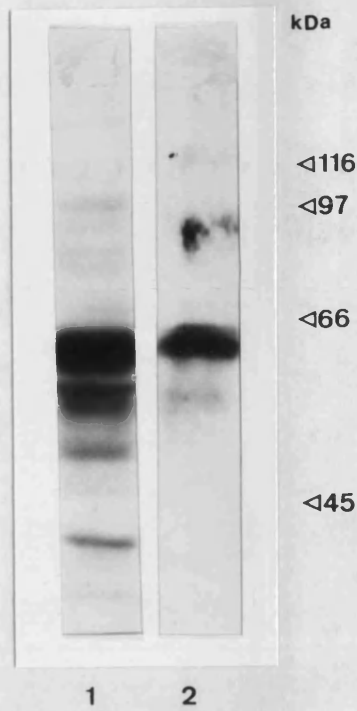
Liver plasma membrane proteins adsorbed to a GLUT2 immunoaffinity column were eluted in buffers containing 1 M and 2 M NaCl. Following concentration, the proteins were analysed by SDS-PAGE and stained with Coomassie Blue (b), or transferred electrophoretically to nitrocellulose (a) and immunoblotted with the indicated antiserum. Bound antibody was detected using goat anti-rabbit IgG alkaline phosphatase conjugate.

- a:**
- Lane 1 liver plasma membrane blotted with anti-rat GLUT2 C-terminal peptide antiserum
  - Lane 2 2 M NaCl wash blotted with anti-human GLUT2 C-terminal peptide antiserum
  - Lane 3 2 M NaCl wash blotted with anti-rat GLUT2 C-terminal peptide antiserum

GLUT2 in liver plasma membrane had an apparent molecular weight of  $\approx 59$ -60 kDa with a lower band at  $\approx 51$  kDa also detected. Anti-human GLUT2 serum detected a broad band with an apparent molecular weight of  $\approx 60$  kDa in the 2 M NaCl affinity column wash. A weaker band of the same apparent molecular weight was detected by anti-rat GLUT2 antiserum.

- b:**
- Lane 1 Coomassie Blue stain of the 2 M NaCl wash
  - Lane 2 Coomassie Blue stain of the 1 M NaCl wash

Two bands with apparent molecular masses of  $\approx 59$  and 51 kDa were detected in both the 1 M and the 2 M NaCl washes. These were the only major proteins detected.



**Fig. 39** Immunoaffinity purification of liver GLUT2 (method II).

Liver plasma membrane proteins adsorbed to a GLUT2 immunoaffinity column were eluted in a glycine buffer (pH 2.4) according to method II described in section 2.14.2. Fractions were pooled, concentrated and electrophoresed (lane 2) alongside liver plasma membrane (lane 1). Proteins were transferred electrophoretically to nitrocellulose and immunoblotted with anti-GLUT2 C-terminal peptide antiserum. Bound antibody was detected using the ECL detection system. A protein with an apparent molecular mass of  $\approx 60$  kDa was recognised in the eluant from the affinity column which aligned with the  $\approx 60$  kDa GLUT2 protein detected in the liver plasma membranes.



The second immunoaffinity purification method had previously been used for the purification of GLUT1 from human erythrocytes (Davies, 1990).

The amount of GLUT2 that bound to the column was again low, since large amounts of GLUT2 could be detected in the unbound fraction which was washed off the column. Some protein was however adsorbed, and could be eluted by washing in a glycine buffer at pH 2.4. When proteins present in the glycine eluant were analysed by Western blotting using a sensitive ECL detection system, a protein with an apparent molecular weight of  $\approx 59$  kDa was recognised by anti-rat GLUT2 antiserum. This protein aligned with the upper GLUT2 band detected by anti-GLUT2 antiserum in liver plasma membranes (Figure 39).

When the pH 2.4 wash was subjected to electrophoresis on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue, no proteins could be detected (results not shown). This indicated that only a very low amount of the protein detected by anti-GLUT2 antiserum was present, but it also showed that the preparation was free of high amounts of other contaminating proteins.

Neither of these methods enabled GLUT2 to be purified in sufficiently large quantities for it to be used in the detection of putative anti-GLUT2 autoantibodies in human diabetic serum.

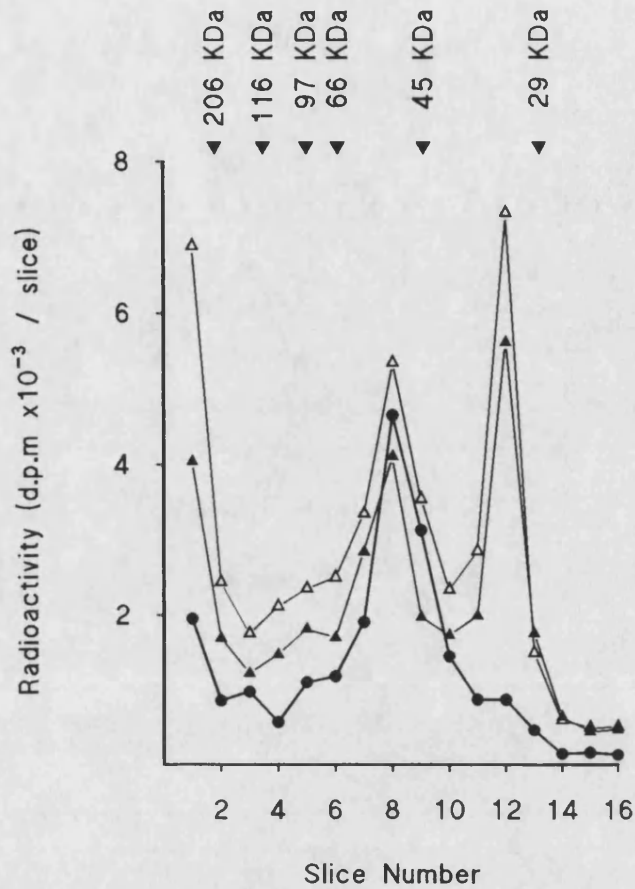
### **3.10 USE OF THE PHOTOLABEL ATB-BMPA TO INVESTIGATE THE PRESENCE OF ANTI-GLUT2 AUTOANTIBODIES IN THE SERUM OF DIABETIC CHILDREN**

Serum samples (100  $\mu$ l) from 4 mammalian species were incubated with 5 mg of Protein A-Sepharose. The antibody-Protein A-Sepharose complex was then rotated with ATB-BMPA photolabelled proteins from liver plasma membrane, which had been

solubilized in buffer containing the detergent C<sub>12</sub>E<sub>9</sub>. Immunoprecipitated proteins were analysed by SDS-PAGE. Normal rabbit, horse and sheep serum did not precipitate a significant amount of photolabelled protein (results not shown). Human serum from a child with IDDM however, contained Protein A-binding antibodies that did immunoprecipitate ATB-BMPA labelled proteins. When analysed by SDS-PAGE the labelled proteins appeared as a double peak, the heavier protein ran as a broad band with an apparent molecular weight of  $\approx 45\text{-}66$  kDa. The second peak was narrower but higher, and ran between the 29 and 45 kDa marker proteins. The ratio of the areas of the two peaks was  $1:1 \pm 0.23\%$  (mean and S.E.M. of four serum samples).

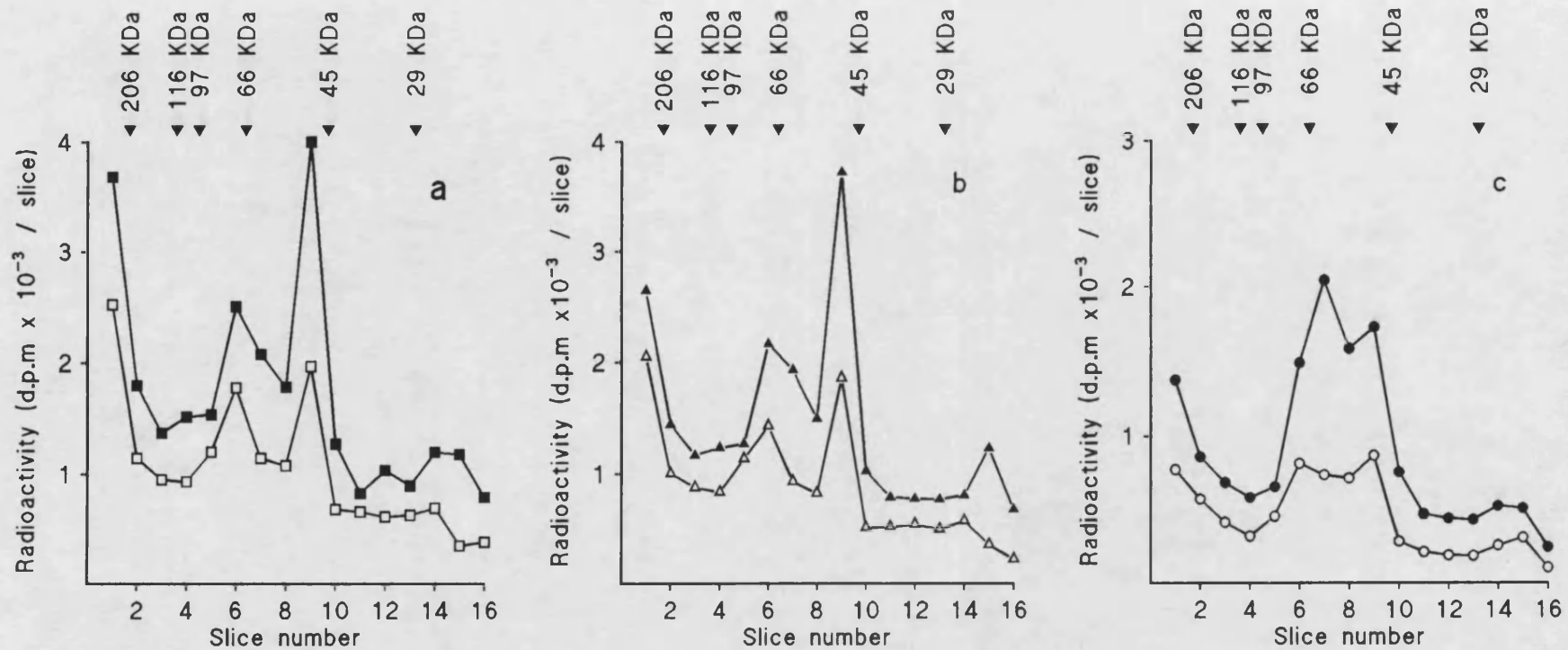
The ATB-BMPA labelled liver plasma membrane proteins precipitated by human diabetic serum were compared with the labelled proteins precipitated by anti-GLUT2 C-terminal peptide antiserum. When equivalent amounts of serum were used for the immunoprecipitations, human serum was found to precipitate 1.5 fold as much total labelled protein as the anti-GLUT2 antiserum. This is illustrated in Figure 40, in which the GLUT2 peak aligned with the broad 45-66 kDa peak precipitated by the human serum. In other experiments, as for example the one illustrated in Figure 41, in which immunoprecipitated GLUT2 was resolved into two separate labelled peaks (see section 3.2.1), these two bands aligned with the two peaks precipitated by the human serum. This immunoprecipitation was not specific to IDDM samples, all human sera tested, including samples from normal children, children with IDDM and samples from Pima Indians with NIDDM (results not shown), precipitated a similar amount of the same labelled protein. A typical example is shown in Figure 40.

As shown in section 3.2.4, the binding of ATB-BMPA to the photolabelled GLUT2 immunoprecipitated by C-terminal peptide antiserum, could be displaced by  $60 \pm 2.2\%$  (mean and S.E.M. of nine independent experiments) by photolabelling in the presence of 500 mM 4,6-0-ethylidene-D-glucose.



**Fig. 40** Immunoprecipitation of ATB-BMPA photolabelled liver plasma membrane proteins with normal and diabetic human serum and anti-GLUT2 peptide antiserum.

Liver plasma membranes (100  $\mu$ g) were photolabelled with 100  $\mu$ Ci of ATB-BMPA. Following washing, the membranes were solubilized in buffer containing the detergent C<sub>12</sub>E<sub>9</sub>. Solubilized labelled proteins were immunoprecipitated with 5 mg of Protein A-Sepharose coupled to 50  $\mu$ l of the following sera. Anti-GLUT2 C-terminal peptide (●), a serum sample from a diabetic child (▲) and a serum sample from a normal child (Δ). Immunoprecipitated proteins were analysed by SDS-PAGE. Anti-GLUT2 C-terminal peptide antiserum precipitated labelled protein with an apparent molecular weight of 45-66 kDa. Human serum immunoprecipitated labelled proteins with apparent molecular masses of 45-66 kDa and  $\approx$  35 kDa.



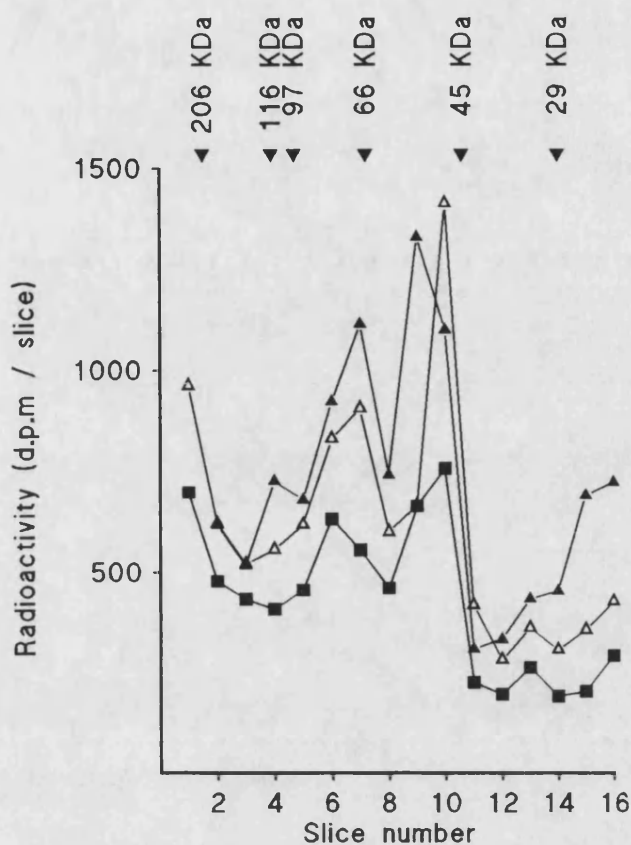
**Fig. 41a-c 4,6-0-ethylidene-D-glucose displacement of ATB-BMPA binding to the rat liver plasma membrane proteins immunoprecipitated with human serum and rabbit anti-GLUT2 antiserum.**

Liver plasma membranes (100  $\mu$ g) were photolabelled with ATB-BMPA in the presence (open symbols) or absence (solid symbols) of 500 mM 4,6-0-ethylidene-D-glucose. After washing and solubilization in buffer containing the detergent  $C_{12}E_9$ , solubilized proteins were immunoprecipitated with 5 mg of Protein A-Sepharose coupled to 50  $\mu$ l of normal human serum ( $\square, \blacksquare$ ) [Fig a], serum from an insulin-dependent diabetic patient ( $\Delta, \blacktriangle$ ) [Fig b], or anti-rat GLUT2 C-terminal peptide antiserum ( $\circ, \bullet$ ) [Fig c]. Immunoprecipitates were analysed by SDS-PAGE. In the illustrated example, 4,6-0-ethylidene-D-glucose inhibited the photolabelling of proteins immunoprecipitated with human sera by 46%. Photolabelling of GLUT2 was inhibited by 58%. In this example the protein immunoprecipitated by anti-GLUT2 antiserum was resolved as a double peak.

By comparison, it was shown that the immunoprecipitation of photolabelled proteins by immunoglobulin from control and diabetic children was reduced by  $49 \pm 3.4\%$  and  $51 \pm 2.4\%$  (means and S.E.M. from four normal and four diabetic samples respectively), when ATB-BMPA was irradiated in the presence of 500 mM 4,6-0-ethylidene-D-glucose.

Analysis of the peak areas also revealed that 4,6-0-ethylidene-D-glucose caused a similar displacement of labelling to both of the proteins precipitated by the human serum. On average, 4,6-0-ethylidene-D-glucose inhibited the binding of ATB-BMPA to the 45-66 kDa protein by  $47 \pm 7.2\%$  (mean and S.E.M. for four independently tested serum samples) and binding to the 29-45 kDa protein by  $41 \pm 7.5\%$  (mean and S.E.M. for four independently tested serum samples). There was no significant ( $p=0.05$ ) difference in the amount of 4,6-0-ethylidene-D-glucose-inhibition of ATB-BMPA labelling of proteins precipitated by the control compared with the diabetic serum samples tested (Student's T-Test, eight samples tested in two independent experiments). The result of a typical displacement experiment is shown in Figures 41a-c.

The immunoprecipitating antibodies in human serum were present in high titres, or had a high affinity or avidity for the ATB-BMPA labelled protein. As illustrated in Figure 42, a significant amount of labelled protein was precipitated using only 1 mg of Protein A-Sepharose coupled to 10  $\mu$ l of serum. Increasing the amount of Protein A-Sepharose and immunoprecipitating antibody from 1 mg to 2 mg, resulted in a  $2.6 \pm 0.61$  fold increase in the amount of labelled protein precipitated (mean and S.E.M. of four independently analysed serum samples). Increasing the amount of Protein A-Sepharose-antibody conjugate from 2 mg to 5 mg, resulted in only  $0.95 \pm 0.33$  (mean and S.E.M. of four independently analysed samples) fold more ATB-BMPA labelled protein being immunoprecipitated. This indicated that 5 mg of Protein A-Sepharose-antibody conjugate precipitated an approximately maximal amount of ATB-BMPA labelled protein from 100  $\mu$ g liver plasma membranes. Similar results were obtained using both diabetic and normal serum.



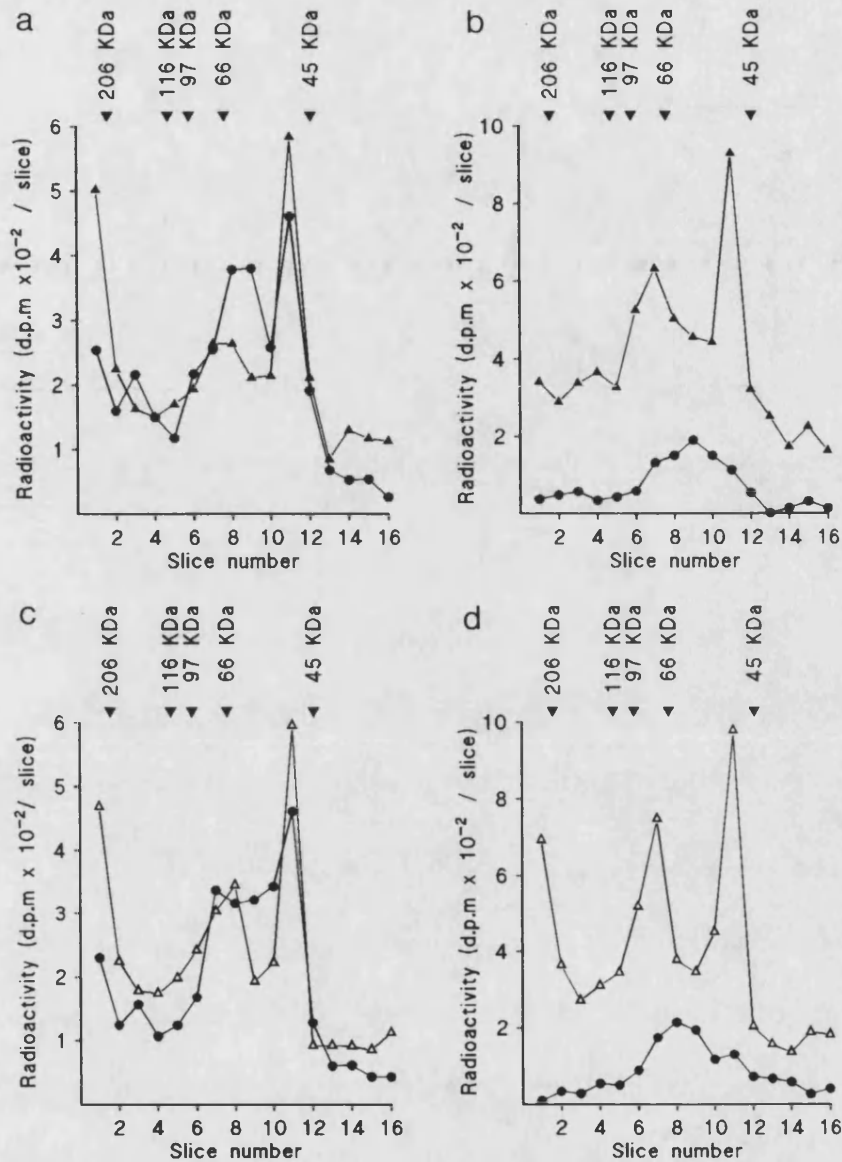
**Fig. 42 Immunoprecipitation of ATB-BMPA photolabelled liver plasma membrane proteins using a range of concentrations of normal human serum.**

Liver plasma membranes (100  $\mu\text{g}$ ) were photolabelled with ATB-BMPA, washed and solubilized in buffer containing the detergent  $\text{C}_{12}\text{E}_9$ . Solubilized labelled proteins were immunoprecipitated with 1, 2 or 5 mg of Protein A-Sepharose conjugated to 10, 20 or 50  $\mu\text{l}$  of normal human serum (■, Δ, ▲ respectively).

The illustrated example is representative of a number of normal and diabetic samples tested. The amount of labelled protein precipitated increased as the quantity of immunoprecipitating serum was increased. Maximum precipitation was obtained with  $\approx 5$  mg of Protein A-Sepharose conjugated to 50  $\mu\text{l}$  of human serum.

The preliminary experiments described above demonstrated that all human serum contains antibodies able to immunoprecipitate a rat liver plasma membrane protein labelled by the photoprobe ATB-BMPA in a 4,6-O-ethylidene-D-glucose displaceable manner. When analysed by SDS-PAGE, the labelled protein had an apparent molecular weight identical to that of immunoprecipitated liver GLUT2. The following experiments were carried out in an attempt to demonstrate whether any of these precipitating antibodies were present only in serum from diabetic patients and whether the protein precipitated by the human serum was liver GLUT2.

As illustrated previously in Figure 13b, it was possible to remove  $\approx 80\%$  of the total photolabelled immunoprecipitable GLUT2 from 100  $\mu\text{g}$  of liver plasma membrane by a single treatment with 5 mg of Protein A-Sepharose coupled to 50  $\mu\text{l}$  of anti-GLUT2 C-terminal peptide antiserum. By carrying out an initial immunoprecipitation of the solubilized ATB-BMPA labelled liver proteins by treatment with 5 mg of Protein A-Sepharose-anti-GLUT2 conjugate, it was possible to reduce the amount of labelled protein subsequently precipitated by 5 mg of Protein A-Sepharose coupled to 50  $\mu\text{l}$  of human serum. This is shown in Figures 43a-d. The amount of labelled protein precipitated by human serum was reduced to  $58.5 \pm 0.2\%$  (mean and S.E.M. for two samples) of the total amount precipitated from membranes not depleted of GLUT2. When the immunoprecipitation treatment was carried out in reverse order, that is, photolabelled proteins were precipitated by incubation with human serum followed by treatment with anti-GLUT2 antiserum, the amount of precipitated GLUT2 was reduced to  $41.8 \pm 4.1\%$  (mean and S.E.M. of two samples) of the quantity normally precipitated. Regardless of the order of immunoprecipitation, approximately the same amount of labelled protein was precipitated in total by the two immunoprecipitation treatments.



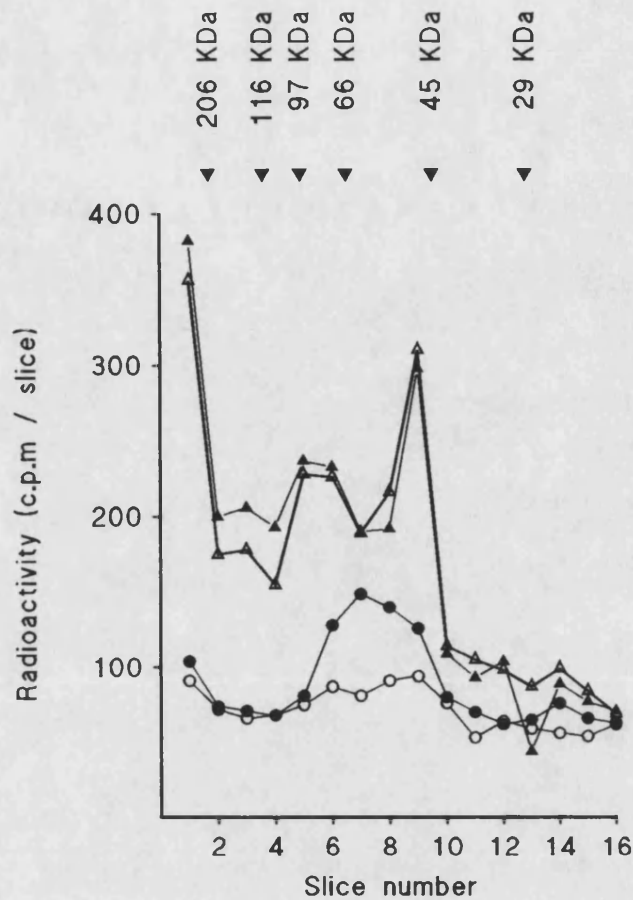
**Fig. 43a-d Competition between anti-GLUT2 peptide antibodies and antibodies in human serum for the immunoprecipitation of photolabelled liver proteins.**

Liver plasma membranes were photolabelled with ATB-BMPA, washed and solubilized in the detergent  $C_{12}E_9$ . Solubilized labelled proteins were subjected to a double immunoprecipitation with 5 mg of Protein A-Sepharose coupled to 50  $\mu$ l of anti-GLUT2 C-terminal peptide antiserum, and either a normal human serum sample or a serum sample from an IDDM patient. Immunoprecipitated proteins were analysed by SDS-PAGE.

Figure	1 <sup>st</sup> Precipitation	2 <sup>nd</sup> Precipitation
43a	GLUT2 antiserum (●)	Diabetic serum (▲)
43b	Diabetic serum (▲)	GLUT2 antiserum (●)
43c	GLUT2 antiserum (●)	Control serum (Δ)
43d	Control serum (Δ)	GLUT2 antiserum (●)
13b	GLUT2 antiserum (●)	GLUT2 antiserum (●)

When GLUT2 was removed from the liver plasma membranes, the human serum (Figs a and c) precipitated  $\approx 42\%$  less labelled protein. Attempts to precipitate GLUT2 following a precipitation with human serum resulted in  $\approx 58\%$  less GLUT2 being immunoprecipitated (Figs b and d).





**Fig. 44** Preabsorption of human and anti-GLUT2 sera with liver plasma membranes prior to their use in the immunoprecipitation of ATB-BMPA photolabelled proteins from liver plasma membranes.

Serum samples (50  $\mu$ l) from a recently diagnosed insulin-dependent diabetic ( $\Delta$ ) and rabbit anti-rat GLUT2 C-terminal peptide antiserum ( $\circ$ ) were incubated for 3 hours at 4°C with 2 mg of liver plasma membrane protein. The membranes were removed by centrifugation at 800  $g_{av}$  (2x5 min) and at 50,000  $g_{av}$  for 20 min (Johnson *et al.*, 1990a). The remaining supernatant was then coupled to 5 mg of Protein A-Sepharose and was used to immunoprecipitate solubilized photolabelled proteins from rat liver plasma membranes. The results obtained were compared with immunoprecipitations carried out using diabetic ( $\blacktriangle$ ) and anti-GLUT2 sera ( $\bullet$ ) which had not been preabsorbed. The immunoprecipitated proteins were analysed by SDS-PAGE. Preabsorption with liver plasma membranes reduced immunoprecipitation by GLUT2 antiserum but not by human serum. Arrowheads indicate the position of molecular weight marker proteins.

Although these experiments were only carried out on a small number of samples, the results suggested that between 42-58% of the antibodies in human and anti-GLUT2 C-terminal peptide antiserum were competing for the same antigen, namely GLUT2. Again, there was no significant difference between amounts of protein precipitated by the diabetic compared with the control samples. Attempts were made to immunoblot the human serum precipitated proteins using anti-GLUT2 antiserum but the results obtained were inconclusive. Although no GLUT2 was detected, it may have been present but at a level below the sensitivity of detection of the assay. The large amounts of human IgG also present may have contributed to the poor results obtained with this technique. Similar problems were encountered with the detection of the GLUT2 protein precipitated with anti-GLUT2 antiserum (see section 3.2.3).

As shown in Figure 44, the immunoprecipitating antibodies in human serum could not be removed by preabsorption with liver plasma membranes. This contrasted with the anti-GLUT2 peptide antiserum which immunoprecipitated 56% less labelled GLUT2 following preabsorption with liver plasma membranes. This suggested that the antibodies in human serum, which precipitated ATB-BMPA labelled proteins, only recognised these proteins under the conditions used for immunoprecipitations, that is, in detergent solubilized membranes.

## 4.0 DISCUSSION

### 4.1 INTERACTION OF THE GLUT2 PROTEIN WITH ANTI-GLUT2 PEPTIDE ANTIBODIES

It is well documented that antibodies raised against synthetic peptides conjugated to carrier proteins can recognise the proteins from which the peptides were derived (Fearney *et al.*, 1971; Maron *et al.*, 1971). The antigenic regions of native proteins often consist of only a few amino acids, usually polar residues located in hydrophilic regions on the surface of the protein. The C-terminus of a protein is an ideal region against which to raise anti-peptide antibodies since, in the native protein it is probably conformationally less restricted than other parts of the molecule. However, a short peptide can assume a number of different conformations that do not exist in the native protein, therefore only a proportion of the anti-peptide antibodies are likely to be able to recognise the peptide sequence as it exists in the native protein. Therefore, the more mobile the region of the protein chosen for the production of anti-peptide antibodies, the higher the number that are likely to bind to the native protein.

Immunisation with the purified erythrocyte glucose transporter protein generates antibodies that react with the C-terminal hydrophilic tail, therefore, this is naturally an immunogenic region of this protein (Davies, 1990). Investigation of the glucose transporter family has been aided by the fact that the C-terminal regions of the different isoforms, with the exception of GLUT2 and GLUT7 (Waddell *et al.*, 1992), are sufficiently different to enable antibodies raised against C-terminal peptides to be isoform specific. In the case of GLUT2, peptide sequences from the hydrophilic loop between M1 and M2 have also been used to produce anti-GLUT2 peptide antibodies (Thorens *et al.*, 1988; Orci *et al.*, 1989; Oka *et al.*, 1990b).

The present study has described the production of anti-GLUT2 C-terminal and exofacial-loop peptide antibodies which have been demonstrated to bind to the GLUT2 protein when it is immobilised on nitrocellulose paper or ELISA plates and when detergent-solubilized.

The quality of the anti-GLUT2 antibodies produced was dependent on the sequence of the immunising peptide. It was found that peptides that included the arginine and lysine residues at positions 507 and 508 (rat) and 510 and 511 (human) seemed to generate especially high titres of antibodies that cross-reacted with the GLUT2 protein. These residues may therefore form part of an important immunogenic epitope in the peptide, which is also present in the same conformation in the whole GLUT2 protein. Of the anti-rat GLUT2 C-terminal peptide antisera tested, those raised in response to rat C-terminal peptides B and C (residues 507-522 and 499-508 respectively), contained the highest titres of antibodies that recognised liver plasma membrane proteins in ELISAS and Western blots. It is therefore suggested that these sequences may produce "better" anti-GLUT2 antisera than the C-terminal decapeptide containing residues 513-522, which has previously been widely used to produce anti-GLUT2 antibodies (Thorens *et al.*, 1988; 1990a; 1990e; Orci *et al.*, 1989; 1990a).

Thorens and co-workers (1988; 1990a) have previously shown that an antibody raised against the rat GLUT2 C-terminal decapeptide detected a single 53 kDa protein in Western blots of crude liver membranes. In the present study these results could not be repeated using the same crude membrane preparation and antibodies raised against the C-terminal 13 or 16 amino acids of rat GLUT2, although the same anti-peptide antibodies successfully detected GLUT2 when purified liver plasma membranes were used. It is proposed that this discrepancy arose from two sources, first from the use by Thorens *et al.* (1988) of a more sensitive  $^{125}\text{I}$ -labelled IgG detection system, and secondly from the different concentration of GLUT2 in the membrane preparation.

The method used routinely in the present study to prepare liver plasma membranes, has previously been shown to produce a membrane fraction with a high 5' nucleotidase activity of 24  $\mu\text{mol/mg/30 min}$  (Prpic *et al.*, 1984). This method uses a Percoll gradient to purify plasma membranes from material pelleted at low speed (1500 g) from homogenised cells. This contrasts with the method used by Thorens *et al.* (1988) in which homogenised material pelleted at 6000 g is discarded. This is likely to result in the loss of a substantial amount of the total plasma membrane. The subsequent very high speed spin included in the Thorens protocol may also be expected to contaminate the membrane preparation with light microsomes. Therefore, the detection of GLUT2 with anti-C-terminal peptide antibodies in immunoblots either requires that a high concentration of GLUT2 is present (as in the membranes used in the present study), or that a sensitive detection system, as used by Thorens *et al.* (1988) is used. Similarly, in the present study it was found that the anti-exofacial loop peptide antibodies only detected GLUT2 in immunoblots of liver plasma membranes when a very sensitive ECL detection system was used.

These results suggest that the titre of anti-GLUT2 peptide antibodies that are actually able to recognise the whole GLUT2 protein is quite low. This situation could arise if the antigenic epitopes in the whole protein do not adopt the same conformation as in the peptide, or if they are not accessible to the antibodies. For example, the C-terminal region of GLUT2 may not project sufficiently from the protein to enable the antibodies to bind. It was not established why both the anti-loop and anti-C-terminal peptide antibodies had an equally low affinity for GLUT2. Fortunately, the detection problems caused by the low affinity for GLUT2 of the anti-peptide antibodies could largely be circumvented by the use of a plasma membrane preparation which contained a high concentration of this protein.

From Western blotting the apparent molecular mass of the major GLUT2 species present in liver plasma membranes was estimated to be about 57 kDa. A strong band of the same apparent molecular mass was also detected in islets. The size difference between

rat liver (53 kDa) and  $\beta$ -cell (55 kDa) GLUT2 previously noted by Thorens *et al.* (1988) was not observed in the present study. However, the reported apparent molecular mass assigned to the GLUT2 protein has been variable. Johnson *et al.* (1990b) detected  $\beta$ -cell GLUT2 as a 60 kDa protein and Oka *et al.* (1990b) detected liver GLUT2 as a 55 kDa protein. In the present study, in addition to the main 57 kDa band, other bands with apparent molecular weights of  $\approx 51$  and  $\approx 36$  kDa were also recognised specifically by anti-GLUT2 C-terminal peptide antibodies. Other studies have previously detected multiple bands in Western blots of GLUT2. A weak band of unknown identity has been detected below the main GLUT2 band in islets (Orci *et al.*, 1989; Johnson *et al.*, 1990b) and in liver (S. A. Baldwin-personal communication). Using anti-rat (509-522) and anti-human (511-522) GLUT2 C-terminal peptide antibodies, Brant *et al.* (1992) detected proteins with apparent molecular masses of 56, 50 and 33 kDa in oocytes expressing GLUT2, and proteins of 56, 42 and 31 kDa in a rat liver plasma membrane preparation very similar to the one used in the present study (Pilkis *et al.*, 1974; Marchmont *et al.*, 1981). These results suggest that the  $\approx 57$ , 51 and 33 kDa proteins recognised by the anti-GLUT2 antisera used in the present study were derived from the GLUT2 protein itself. It was not known how the minor bands differed from the main (57 kDa) GLUT2 species, but they were unlikely to be proteolysed fragments, since proteinase inhibitors were included at all stages of the membrane preparation and the C-terminus was still present.

The erythrocyte-type glucose transporter has previously been successfully affinity purified using anti-GLUT1 peptide antibodies (Davies, 1990). However, in the present study problems were encountered with the immunoaffinity purification of GLUT2, which were attributed to the low affinity of the anti-GLUT2 C-terminal peptide antibody for the solubilized GLUT2 protein. The binding capacity of the immunosorbent appeared to be very low, since most of the loaded GLUT2 did not bind to the column. However, despite the low amount of binding, a protein with an apparent molecular mass of  $\approx 59$  kDa, which was recognised by anti-GLUT2 C-terminal peptide antibodies in a Western blot, was eluted from the affinity matrix using either high salt (Momoi & Lennon, 1982) or low pH

conditions (Schroer *et al.*, 1986; Davies, 1990). Similar results were produced using both methods. Analysis of proteins was however facilitated by the use of n-octyl  $\beta$ -D-glucopyranoside as the solubilizing detergent since it was easily removed by dialysis. The drawback of this detergent was its cost. Solubilization in the detergent Triton X-100 had the disadvantage that it could not be readily removed.

Although the four different molecular mass protein species detected by immunoblotting with anti-GLUT2 antibodies were present in the original solubilized liver plasma membranes, it was mainly the major  $\approx 59$  kDa protein that was eluted from the column, presumably since this was the predominant species present.

Although these results have indicated that GLUT2 can be immunoaffinity-purified from liver plasma membranes, the yield was so low that it was insufficient to be used in assays to detect anti-GLUT2 autoantibodies. It will therefore be necessary to improve both the degree of purification and to increase the yield before purified GLUT2 can be used for antigenic studies.

## **4.2 PHOTOLABELLING OF GLUT2 WITH ATB-BMPA**

It has previously been shown that the bis-D-mannose compound ATB-BMPA can photolabel the glucose transporter isoforms GLUT1 (Clark & Holman, 1990) and GLUT4 (Holman *et al.*, 1990) in erythrocytes and adipocytes respectively. The photoactive group is selective enough for highly specific labelling of these glucose transport proteins to be obtained. The specificity is not simply a result of the impermeant nature of ATB-BMPA, since it has also been successfully used to label isolated membranes (Clark & Holman, 1990).

The results shown in part I of this thesis have demonstrated for the first time that ATB-BMPA can be used to photolabel the glucose transporter isoform GLUT2. Previously, the only available method of labelling the liver glucose transport protein was with the endofacial ligand cyt B, when used in conjunction with a photochemical cross-linking agent (Ciaraldi *et al.*, 1986). In the study by Ciaraldi and co-workers, approximately 50% of the cyt B binding was displaced by 500 mM D-glucose. A more recent study by Hellwig & Joost (1991) failed to detect any glucose-inhibitable binding of cyt B to liver plasma membranes. It was suggested that cyt B has a much lower affinity for GLUT2 than it does for the transporter isoforms GLUT1 and GLUT4, and that this indicated important structural differences between the isoforms in the specific domains responsible for cyt B binding. Since cyt B is an endofacial ligand (Basketter & Widdas, 1978; Devés & Krupka, 1978; Holman & Rees, 1987) this indicates that variations at the endofacial hexose binding site, reflected by differences in the affinity for cyt B, may be responsible for functional differences between the transporter isoforms. GLUT2 is different from the other members of the facilitative glucose transporter family, in that it has a particularly low affinity for D-glucose as well as having an approximately 10 fold lower affinity for cyt B (Axelrod & Pilch, 1983).

It is proposed that fewer differences are present at the exofacial than at the endofacial binding sites of the transporter isoforms. In the present study an ATB-BMPA binding constant of 250  $\mu\text{M}$  was measured for GLUT2 which is similar to the value of 150  $\mu\text{M}$  previously reported for the GLUT1 and GLUT4 isoforms (Palfreyman *et al.*, 1992). This suggests that the ATB-BMPA binding site, in contrast to the cyt B binding site, is conserved in all glucose-transporter isoforms. Recent studies have indicated that on irradiation ATB-BMPA labels the exofacial region of helix 8 and that helices 7, 8 and 9 are important in exofacial ligand binding (A.F. Davies, A. Davies, R.A.J. Preston, A.E. Clark, G.D. Holman and S.A. Baldwin - personal communication). Therefore, the ATB-BMPA photolabel and translocated substrates probably bind at the same exofacial site.



Since ATB-BMPA apparently has a similar affinity for all of the transporter isoforms, it has advantages over the cyt B label which has a much lower affinity for GLUT2.

Irradiation of ATB-BMPA in the presence of hepatocytes or liver plasma membranes resulted in the photolabelling of a broad band of protein with an apparent molecular mass of between 45 and 66 kDa. The broadness of the peak suggested that the specificity of ATB-BMPA labelling in liver tissue was not as high as in erythrocytes or adipocytes, that is, that ATB-BMPA was labelling some polypeptides unrelated to glucose transporters. In erythrocytes, ATB-BMPA labels a single band of protein which has an apparent molecular mass of  $\approx 45$ -66 kDa. It has been demonstrated that the label is specifically binding to a glucose transporter protein, namely GLUT1, by displacing ATB-BMPA labelling totally with 100 mM D-glucose (Clark & Holman, 1990). In adipocytes, the ATB-BMPA labelling of a 55 kDa protein, namely GLUT4, is also completely displaceable with D-glucose. In these fat cells a second protein with an apparent molecular mass of about 75 kDa is also labelled, but it can be demonstrated that this protein is not a glucose transporter protein since labelling is not displaced with D-glucose (Holman *et al.*, 1990). The increased amount of non-specific labelling observed in liver tissue in the current study may be partially due to the use of membranes instead of whole cells. It may also reflect the low abundance of GLUT2 in liver cells compared with the number of other proteins present. Photolabelling of liver plasma membrane proteins could be displaced by 43% by 4,6-O-ethylidene-D-glucose, a sugar which has an affinity for the exofacial glucose binding site (Barnett *et al.*, 1973b). The poor glucose displacement of ATB-BMPA photolabelling of membrane proteins was probably the result of a combination of the low affinity of GLUT2 for D-glucose (Elliott & Craik, 1982; Granner & Pilkis, 1990), in addition to the presence of some non-specific photolabelling.

It was demonstrated that some of the ATB-BMPA labelled protein in liver plasma membranes was GLUT2, since labelled protein with an apparent molecular mass of  $\approx 45$ -66 kDa was precipitated by anti-GLUT2 antiserum. 500 mM D-glucose was able to inhibit

the specific ATB-BMPA binding to GLUT2 by 57%. Since the  $K_m$  for D-glucose transport in hepatocytes is 60 mM (Granner & Pilkis, 1990), 500 mM D-glucose would be expected to inhibit ATB-BMPA labelling by more than 80%. Therefore the  $K_i$  for D-glucose displacement of ATB-BMPA from GLUT2 may be even higher than the  $K_m$  for glucose transport by hepatocytes.

Since the exofacial binding site of the transporter isoforms appears to be conserved, the observed differences in affinities of the different transporters for D-glucose are likely to arise from differences in the interaction of the substrate with the internal site of the transporter. The ability to bind a non-transported ligand such as ATB-BMPA or 4,6-O-ethylidene-D-glucose at the outside site is kinetically a simpler reaction than that involved in binding and transporting a substrate molecule. The apparent  $K_m$  for equilibrium sugar binding or exchange is dependent both on the rate constant for the membrane translocation step and on the affinity constant at the inside site. It is these parameters that may vary between the different transporter isoforms resulting in the observed different affinities for transported substrates.

Inhibition of ATB-BMPA labelling of immunoprecipitable GLUT2 was greatest when photolabelling was performed in the presence of non-transported ligands known to have an affinity for the exofacial binding site of the transporter. The hexose 4,6-O-ethylidene-D-glucose, which like the ATB-BMPA molecule has bulky groups at the C4 and C6 positions (Holman, 1989), has an affinity for the outside binding site but cannot be translocated, therefore the 92% inhibition produced by this sugar was a reflection of the affinity of binding at the exofacial hexose binding site only. Maximum inhibition ( $\approx 100\%$ ) of ATB-BMPA labelling of GLUT2 was produced by phloretin, which is also considered to bind at the extracellular binding site (Krupka, 1971; Krupka & Devés, 1980; Krupka & Devés, 1981), although other evidence does suggest that it may also bind to some inside-specific sites (Basketter & Widdas, 1978).

The previously reported low affinity binding of the endofacial ligand cyt B to liver membranes and GLUT2 (Axelrod & Pilch, 1983; Hellwig & Joost, 1991) has been confirmed in the present study. Cyt B inhibited the binding of ATB-BMPA to GLUT2 by 65%, this contrasts with its ability to totally displace photolabel from the GLUT1 and GLUT4 transporter isoforms (Clark & Holman, 1990; Holman *et al.*, 1990). According to the carrier model of glucose transport, the internal and external substrate binding sites are not available simultaneously (Devés & Krupka, 1978; Carruthers, 1990). By binding at an endofacial site, cyt B can therefore block the binding of the exofacial ligand ATB-BMPA by causing a conformational change which occludes the external substrate binding site (Devés & Krupka, 1978; Holman, 1989). The most likely explanation for the poor displacement by cyt B of ATB-BMPA binding to GLUT2, does not propose a fundamental difference in the mechanism of transport in this isoform, but simply that cyt B binds poorly to GLUT2 (Hellwig & Joost, 1991). It is not clear whether cyt B and glucose bind at exactly the same endofacial site, but this would seem unlikely owing to the very different structures of the two molecules (Devés & Krupka, 1978). However, it has also been suggested that the cyt B does actually bind to the same region of the glucose transporter that interacts with the transported hexose (Hellwig & Joost, 1991).

In summary, all of the evidence from inhibition studies suggests that the different kinetic properties of GLUT2 arise from differences on the endofacial surface of the protein.

D-fructose caused a 77% inhibition in ATB-BMPA binding to GLUT2. This is in accordance with the observations made by Okuno and Gliemann (1986) who demonstrated that D-fructose is transported with low affinity by the glucose transporter in hepatocytes and by Gould *et al.* (1991) who found that oocytes expressing GLUT2 can transport D-fructose.

The present study of GLUT2 was affected by difficulties with the specificity of ATB-BMPA labelling and poor immunoprecipitation efficiency which were not a problem in previous studies of GLUT1 and GLUT4 (Clark & Holman, 1990; Holman *et al.*, 1990). Both of these factors caused significant problems when it was attempted to photolabel GLUT2 in rat islet tissue. The problems were compounded by the fact that islets could only be isolated in very low numbers. ATB-BMPA did label a very broad band of protein in islets, but labelling was not displaced by 4,6-0-ethylidene-D-glucose. This strongly suggested that most of the photolabelling of whole islets was non-specific. However, some GLUT2 was shown to be photolabelled, since anti-GLUT2 antiserum specifically precipitated approximately 3% of the labelled protein from the islets. Further experiments on islet cells must await the resolution of the technical difficulties responsible for the poor recovery of photolabelled GLUT2. These difficulties can probably be more easily resolved using rat liver plasma membrane which is a plentiful and easily available source of GLUT2.

The amount of photolabelled GLUT2 that was immunoprecipitated from hepatocytes or liver plasma membranes was low compared with the total amount of labelling. Approximately 2% of the total amount of protein labelled in whole hepatocytes was precipitated by anti-GLUT2 antiserum. This value was increased slightly by the use of liver plasma membranes from which approximately 7% of the total proteins labelled in a 4,6-0-ethylidene-D-glucose displaceable manner were specifically immunoprecipitated by anti-GLUT2 antiserum. Although preimmune serum did not recognise any bands in Western blots of liver plasma membranes, when coupled to Protein A-Sepharose it did immunoprecipitate a peak of labelled protein of similar molecular mass to that precipitated by the anti-GLUT2 antiserum. On average, this non-specific precipitation accounted for about 32% of the total amount of precipitation by anti-GLUT2 antiserum. By Western blotting it was demonstrated that the labelled protein non-specifically precipitated by normal rabbit serum was not GLUT2, since anti-GLUT2 antiserum did not detect GLUT2 in preimmune serum immunoprecipitates. The identity of the protein was not known.

It was shown by Western blotting that the low recovery of labelled GLUT2 was partially caused by poor immunoprecipitation of the detergent solubilized protein. The conditions used prevented quantification of the immunoprecipitation efficiency, but did demonstrate that although some GLUT2 was precipitated, some also remained in the supernatant and was not precipitated. Therefore, the immunoprecipitation of GLUT2 appeared to be incomplete. However, the amount of precipitated GLUT2 could not be significantly enhanced either by increasing the amount of precipitating antiserum or by carrying out repeated rounds of immunoprecipitation.

It was considered possible that the anti-peptide antibodies had a particularly low affinity for the detergent-solubilized GLUT2 protein. This could possibly explain the poor immunoprecipitation efficiency for GLUT2 observed in this, and other studies (Dr. G. W. Gould - personal communication). However, this would not have been expected to affect both the anti-loop peptide and anti-C-terminal peptide antibodies to the same extent, although the results demonstrated that both antibodies precipitated equally low amounts of labelled GLUT2. The solubilization of membrane proteins in two alternative detergents was also shown not to significantly improve the recovery of labelled GLUT2. The proteins were solubilized in the anionic detergent SDS, which denatures proteins, in order to determine whether the anti-GLUT2 C-terminal peptide antibody was able to recognise the GLUT2 protein more efficiently when it was denatured, but no improvement in GLUT2 precipitation was observed. The low recovery of GLUT2 was also shown not to result from incomplete solubilization of the labelled transporter, since by Western blotting it was shown that GLUT2 was fully solubilized in the C<sub>12</sub>E<sub>9</sub> detergent used routinely.

Proteolytic activity, which could have cleaved the C-terminus of the transporter resulting in a loss of immunoreactivity, was prevented by the inclusion of proteinase inhibitors. Proteolysis was not thought to have been responsible for the poor recovery of GLUT2, since it would have been expected to have had a greater effect on precipitation by

the anti-C-terminal peptide than the anti-loop peptide antibody, whereas both precipitated a similar amount of GLUT2 protein.

Although the discrepancy between the amount of protein labelled in liver plasma membranes and the amount of GLUT2 immunoprecipitated was in part due to the incomplete precipitation of the GLUT2 protein, it was also necessary to consider whether the apparent low recovery of GLUT2 was in fact largely a result of non-specific labelling by ATB-BMPA of non-GLUT2 proteins. However, since 4,6-O-ethylidene-D-glucose displaced 43% of the ATB-BMPA binding to whole liver plasma membranes, this suggests that at least this amount of labelling was specific to a glucose-transport protein. The possibility that the non-GLUT2 precipitable but 4,6-O-ethylidene-D-glucose inhibitable binding of ATB-BMPA to liver plasma membranes was due to binding to another glucose transporter proteins has been considered.

Waddell and co-workers (1992) have recently described the cloning of another glucose transporter protein from liver. GLUT7, as it has been named, is present in liver microsomes and is virtually identical to GLUT2, the major region of variation being the C-terminus. The liver membranes photolabelled in the present study were believed to be a relatively pure preparation of plasma membranes uncontaminated with microsomes. However, since the 5' nucleotidase activity was lower than that measured by Prpic *et al.* (1984) for membranes prepared using the same method, it was possible that non-plasma membrane components of the liver cell were present. If GLUT7 had accounted for a substantial amount of the photolabelling in the liver membranes used, it would have been expected that the anti-loop peptide antibody would have precipitated more labelled protein than the anti-C-terminal peptide antibody, since the loop sequence is shared by GLUT2 and GLUT7 whereas the C-terminus is exclusive to GLUT2. Since both antibodies precipitated the same amount of labelled protein, unless the loop antibody was less efficient than the C-terminal peptide antibody, photolabelling of GLUT7 does not appear to account for the

non-GLUT2 precipitable, 4,6-O-ethylidene-D-glucose displaceable ATB-BMPA labelling of liver plasma membrane proteins.

The number of GLUT2 ATB-BMPA binding sites measured in liver plasma membranes was 2.1 pmol/mg of membrane protein. This is lower than the number of cyt B binding sites measured in earlier reports. The number of cyt B binding sites in liver plasma membranes has been measured as 130 pmol/mg of membrane protein and 42 pmol/mg by Axelrod and Pilch (1983) and Ciaraldi *et al.* (1986) respectively. In comparing the number of binding sites estimated either from ATB-BMPA or cyt B binding, several factors need to be considered. First, some variation may be due to the different methods used to prepare liver plasma membranes in each case. Secondly, in the present study ATB-BMPA labelling has been followed by specific immunoprecipitation of labelled GLUT2, whereas the binding of cyt B to liver plasma membranes has not been shown to be specific for GLUT2. In fact, Hellwig and Joost (1991) failed to detect a specific binding site for cyt B on GLUT2. However, the value of 2.1 pmol/mg is likely to be an underestimation of the number of GLUT2 binding sites, since, as discussed above, it is probable that the immunoprecipitation of GLUT2 was incomplete. Only  $\approx 7\%$  of the proteins labelled in liver plasma membranes in a 4,6-O-ethylidene-D-glucose displaceable manner could be precipitated by anti-GLUT2 antiserum. If an assumption is made that the remaining 93% of 4,6-O-ethylidene-D-glucose inhibitable binding is also to the GLUT2 protein (which, it is proposed was not precipitated due to low antibody affinity), then a value of 30 pmol/mg of membrane protein can be obtained for the number of binding sites. This is closer to the value obtained by Ciaraldi *et al.* (1986) for the number of cyt B binding sites in whole liver plasma membranes, but is based on a correction which assumes that all the 4,6-O-ethylidene-D-glucose displaceable ATB-BMPA labelling of liver plasma membrane is specific for GLUT2.

### 4.3 CONSIDERATION OF THE SUITABILITY OF $\beta$ -CELL LINES AS MODELS OF NATIVE ISLET $\beta$ -CELLS

It has recently become possible to prepare populations of islet cells enriched in  $\beta$ -cells using autofluorescence-activated cell sorting (Christie *et al.*, 1990), but it is a technically demanding, expensive and time-consuming procedure. For these reasons,  $\beta$ -cell lines that can be maintained in continuous culture have been widely used in studies of  $\beta$ -cell physiology and autoantigenicity. Although the cell lines are convenient to work with, they are derived from cells that have been immortalised by viral transformation (HIT and MIN cells - Santerre *et al.*, 1981; Miyazaki *et al.*, 1990) or irradiation (RIN cells - Gazdar *et al.*, 1980). This treatment, and the adaptation of cells to culture conditions, may cause the cell lines to have biochemical and physiological features very different from those displayed by the original cell type *in vivo*. An additional problem with cell lines is that their characteristic features are not necessarily permanent. Cell lines often exhibit morphological and physiological changes dependent on passage number and culture conditions.

The HIT-T15 cells used in the current study contained insulin and grew in poorly adherent clumps that resembled islets, these morphological characteristics were consistent with those previously described for this cell line (Santerre *et al.*, 1981; Hill & Boyd 1985). MIN 6 cells also grew in clusters that differed from those of the HIT-T15 cells by being smaller and more adherent. Since both cell lines were obtained directly from the laboratory in which they were developed, were cultured as recommended, and were used at early passage numbers it was assumed that their physiology was essentially as described by the original authors (Santerre *et al.*, 1981; Miyazaki *et al.*, 1990).

The use of HITm2.2 cells as models of  $\beta$ -cells has not been widely reported. This cell line was developed as a subclone of HIT-T15 cells showing increased stability of transfection competence (Edlund *et al.*, 1985). In the present study this stability was



shown not to extend to the morphological or insulin-secretory characteristics of the cells, since they exhibited passage-dependent changes. The original population of HITm2.2 cells was a mixture of adherent and non-adherent cells. The culture conditions used selected for the growth of the adherent cells which divided more rapidly. Gillard *et al.* (1989), have previously described a mixed population of RINm5F cells which, by selective harvesting, could be used to develop adherent and non-adherent RIN sub-clones. The RINm5F cells used in the present study were all of the rapidly dividing adherent type (Thomas *et al.*, 1987). Their doubling time in the exponential growth phase was 26.5 hours this was faster than the 30-40 hours doubling time previously observed (Lambert & Atkins, 1987). The adherent nature of the RINm5F and HITm2.2 cells made them easier to work with than the HIT-T15 and MIN 6 cells which detached more easily from culture plates and clumped together making enumeration difficult.

Cytoplasmic insulin could not be detected in HITm2.2 cells using an indirect immunofluorescence assay. Dr. D. Boam has also detected only very low levels of insulin in this cell line (personal communication). However, by RIA it was shown that HITm2.2 cells retained the ability to secrete insulin in response to glucose, a characteristic feature of the parent HIT-T15 cell line which makes it a good model of the native islet  $\beta$ -cell (Hill & Boyd, 1985; Lambert *et al.*, 1986). The glucose response was shown to decrease at high passage numbers, as has also been observed in HIT-T15 cells (Zhang *et al.*, 1989). HITm2.2 cells did not secrete insulin in response to arginine or leucine which contrasted with the observed stimulation by these amino acids in HIT-T15 cells (Lambert *et al.*, 1986). This discrepancy may have reflected a difference in the m2.2 and T15 lines, or may have been a result of a general decrease in insulin secretion at high passage number.

The RINm5F cells used in the present study secreted very high levels of insulin. The basal level of secretion was about five fold higher than that produced by the RINm5F cells used in a study by Lambert and Atkins (1987). Gazdar *et al.* (1980) have however reported wide fluctuations in the insulin-secretory capacity of the original RINm cell line.

Therefore, these differences may have been due to the use in the present study of a different RIN subclone to that used by Lambert and Atkins (1987). RINm5F cells have previously been shown to increase their insulin secretion 1.3 fold when stimulated with 15 mM arginine (Lambert & Atkins, 1987). In the present study 15 mM arginine caused a 2.5 fold increase in insulin release. However, despite their arginine response and high levels of basal insulin secretion, RINm5F cells are poor models of islet  $\beta$ -cells, as they are unable to secrete insulin in response to glucose.

Due to the presence of the high  $K_m$  glucose transporter GLUT2, the rate of glucose uptake by native islet  $\beta$ -cells is not limiting to its subsequent metabolism. This ability to equilibrate D-glucose rapidly across the cell membrane is believed to be important in enabling  $\beta$ -cells to secrete insulin in response to glucose (Thorens *et al.*, 1988). The defect in RINm5F cells that is responsible for their inability to respond to D-glucose is unknown. It has previously been reported that  $\beta$ -cell lines exhibit slow sugar uptake (Meglasson *et al.*, 1986), but since identical rates of 2-deoxyglucose uptake were measured in the present study in both the non-glucose responsive RINm5F line and the glucose responsive HITm2.2 cells, a low rate of sugar transport cannot be the only factor that prevents RIN cells from responding to D-glucose. The rate measured in the present study using cells cultured in 11 mM D-glucose was lower than the 2.3 nmol/0.1 mg/min measured previously for 1 mM 2-deoxyglucose uptake in HIT-T15 cells cultured in 16.7 mM D-glucose (Purrello *et al.*, 1991), whereas a reduction in glucose transport would have been expected at this higher glucose concentration (Purrello *et al.*, 1991; Seino *et al.*, 1991). The difference was attributed to the use in the present study of HITm2.2 rather than HIT-T15 cells. This illustrates one of the disadvantages of working with cell lines, that is, the variability that can arise from the use of different subclones. These may even have been produced inadvertently in different laboratories by the selective pressures imposed by slightly different culture conditions. Discrepancies in results are not uncommon, even between laboratories ostensibly using the same cell line. One example is in the measurement of 3-O-methyl-D-glucose uptake in RINm5F cells. Trautmann and

Wollheim (1987) demonstrated that these cells had a high  $K_m$  for this sugar which was equilibrated within 2 min, whereas Malaisse (1988) measured a low  $K_m$  of 2 mM for the same sugar.

The photolabel ATB-BMPA has been used in conjunction with anti-GLUT1 and anti-GLUT2 specific antibodies to identify the glucose transporter isoforms present in the  $\beta$ -cell lines. Since the aim was solely to establish whether the GLUT2 protein was present in these cells, it was not attempted to accurately quantify glucose transporter expression. However, it is proposed that ATB-BMPA could be of future use in investigations of the regulation of GLUT1 and GLUT2 expression in  $\beta$ -cells or islets cultured under different conditions.

In HITm2.2, RINm5F and MIN 6 cells, ATB-BMPA photolabelled protein with an apparent molecular mass of 45-66 kDa. Photolabelling in the presence of D-glucose inhibited the labelling of this protein by 66% and 63% in HITm2.2 and MIN 6 cells respectively. This strongly suggested that most of the photolabel was binding to a glucose transporter. The GLUT1 isoform was identified in RIN, MIN and HITm2.2 (but not HIT-T15) cells by the immunoprecipitation of an ATB-BMPA labelled protein using anti-GLUT1 antiserum. This confirmed previous reports that the normal tissue-specific pattern of glucose transporter isoform expression does not occur in immortal cell lines, which generally show a predominant, if not exclusive, expression of the GLUT1 glucose transporter isoform (Kahn & Flier, 1990; Mueckler, 1990). GLUT1 has been detected previously in MIN, RIN, HIT and  $\beta$ -TC cells both at the level of mRNA (Thorens *et al.*, 1988; Buchs *et al.*, 1990; Miyazaki *et al.*, 1990) and protein (Buchs *et al.*, 1990; Brant *et al.*, 1992).

The inability to detect GLUT2 in any  $\beta$ -cell line by immunoprecipitation of ATB-BMPA photolabelled proteins with anti-GLUT2 antiserum is at variance with the

previously reported detection of GLUT2 mRNA in RINm5F (Thorens *et al.*, 1988), HIT (Seino *et al.*, 1991) and MIN 6 cells (Miyazaki *et al.*, 1990). However, the detection of mRNA does not necessarily mean that large quantities of the protein are also present in the plasma membranes (Seino *et al.*, 1991). When the actual amount of GLUT2 protein has been measured in  $\beta$ -cell lines by immunoblotting, it has been shown that the GLUT2 protein is present in much lower amounts than the GLUT1 transporter isoform. Therefore, it is proposed that the amount of GLUT2 in RIN, HIT and MIN cells was low enough to be below the level of detection of the ATB-BMPA labelling and precipitation assay.

Only about 10% of the  $\beta$ -cell line proteins labelled in a hexose-displaceable manner were immunoprecipitated with anti-GLUT1 antiserum. The ability to displace the labelling with glucose implied that a glucose transporter protein was being photolabelled, therefore the low recovery of labelled transporter may have resulted from an incomplete immunoprecipitation. However, there was no evidence that GLUT1 immunoprecipitation was inefficient. The anti-GLUT1 antiserum used in the present study has previously been shown to efficiently precipitate photolabelled GLUT1 (Clark & Holman, 1990). If an assumption is made that these  $\beta$ -cell lines do not contain appreciably more GLUT1 than 3T3-L1 cells, the conditions used in the present study, which were comparable with those used in a study by Palfreyman *et al.* (1992), should have enabled at least 80% of the photolabelled GLUT1 to be precipitated.

From the studies with liver tissue it was known that the efficiency of GLUT2 precipitation was low. Therefore, the possibility must be considered that some of the hexose-displaceable binding of ATB-BMPA to  $\beta$ -cell proteins was to GLUT2, which for some unknown reason was not precipitated. However, since GLUT2 could be detected in hepatocytes and even in very small numbers of islets, the amount of the GLUT2 protein present in the  $\beta$ -cell lines would have to be very low for it not to be detected by ATB-BMPA photolabelling and immunoprecipitation. Therefore, the identity of the majority of the protein labelled in a hexose-displaceable manner, but not immunoprecipitated was

unknown. It is however possible that it could have been a different glucose transporter isoform, that is, neither GLUT1 nor GLUT2, since Brant *et al.* (1992) have demonstrated the presence of GLUT4 in the  $\beta$ -TC  $\beta$ -cell line. The presence of this isoform was not investigated in the present study.

It was also evident that ATB-BMPA was labelling non-glucose transporter proteins in all of the  $\beta$ -cell lines. In MIN 6 and HITm2.2 cells, a peak with an apparent molecular mass of 29-45 kDa was labelled in a non-hexose-displaceable manner, and in RINm5F cells a non-precipitated peak with an apparent molecular mass of 66-97 kDa was labelled. In HIT-T15 cells photolabelling of a 66 kDa protein appeared to be non-specific, since the labelling was not hexose displaceable. The high degree of non-specific photolabelling in these  $\beta$ -cell lines appears to be similar to the situation observed in liver tissue and may reflect the low abundance of glucose transporter protein compared with other membrane proteins. The photolabelling of HIT-T15 cells, which grew in large clumps, may have been poorly efficient due to the decreased penetration or activation of the photolabel.

In the present study the absence of a large amount of GLUT2 in the  $\beta$ -cell lines was confirmed by immunoblotting. The antibody used was known to recognise GLUT2 under Western blotting conditions, yet GLUT2 was not detected in  $\beta$ -cells using either whole cells or plasma membrane fractions and sensitive immunodetection systems (ECL and  $^{125}\text{I}$ -labelled anti-IgG). These results were at variance with the reported detection of GLUT2 in HIT and RIN cell lines by Brant *et al.* (1992). This discrepancy may be attributed to differences in the cell lines that were used in the two studies. However, some GLUT2 may have been present in the HITm2.2 and RINm5F cell lines used in the current study, since when membrane proteins were extracted with the detergent Triton X-114, a 58 kDa protein was detected with anti-human GLUT2 C-terminal peptide antiserum. This antiserum was known to show some non-specific binding, therefore it can only be concluded tentatively that the cells were expressing some GLUT2.

The inability to detect GLUT1 in  $\beta$ -cell lines by Western blotting, despite demonstrating its presence by ATB-BMPA labelling and immunoprecipitation, could be explained if the antibody used had a low affinity for the denatured GLUT1 protein. This may be expected to cause a problem when attempting to immunodetect relatively low amounts of GLUT1, as in the cell lines, but would not be a problem in the Western blotting of erythrocytes in which very high expression of GLUT1 occurs. Similar results have been obtained in 3T3-L1 cells from which labelled GLUT1 could be immunoprecipitated (Palfreyman *et al.*, 1992), although using the same antibody it could not be detected in the present study by Western blotting.

In summary, the presence of GLUT1 in MIN 6, HITm2.2 and RINm5F cells has been demonstrated by photolabelling and immunoprecipitation, although it could not be confirmed by Western blotting. The GLUT2 protein was either absent or expressed at such a low level that it could not be detected by ATB-BMPA photolabelling and immunoprecipitation or by normal Western blotting techniques. It must therefore be concluded that  $\beta$ -cell lines are not ideal models of native islet  $\beta$ -cells. The absence or low expression of GLUT2 precludes their use in assays designed to screen for the presence of anti- $\beta$ -cell GLUT2 autoantibodies. However, other evidence including the retention of insulin secretion, suggests that the cell lines do possess some  $\beta$ -cell-like properties and therefore that they may be used successfully to detect other anti- $\beta$ -cell autoantibodies.

#### **4.4 ASSAYING FOR ANTI- $\beta$ -CELL SURFACE ANTIBODIES IN HUMAN SERUM.**

ICSA, which may be of pathogenic significance and diagnostic importance in IDDM, have traditionally been detected in an assay which uses freshly isolated rat islets and detects bound human IgG using an FITC-conjugated second antibody (Lernmark *et al.*, 1978). This is not an assay that can be readily standardised nor quantified and it is too time-consuming and expensive to be of practical use in the screening of large numbers of

samples. Attempts to improve the detection of ICSA have largely focused on the replacement of rat islet cells with cultured  $\beta$ -cell lines. The potential disadvantage of using cell lines is that the antigens present on the cultured cells might not be present on the native cells *in vivo*, and vice versa. However, there have been a number of reports demonstrating that  $\beta$ -cell lines do express some of the same cell surface antigens as are present in pancreatic islets *in vivo* (Eisenbarth *et al.*, 1981; Karounos & Thomas, 1990) and that these antigens can be specifically recognised by antibodies present in serum from diabetic patients (Maclaren *et al.*, 1975; Marnier *et al.*, 1984; Thomas *et al.*, 1990). Although the use of cell lines can reduce the cost and improve the convenience and reproducibility of ICSA assays, most of those that have been described still use whole viable cells, making them cumbersome to use.

It was the aim of the present study to determine whether an assay could be developed to detect ICSA using  $\beta$ -cell line plasma membranes in an ELISA. A similar approach to ICSA detection has been described by Peakman *et al.* (1989), who developed an ELISA using rat islet plasma membranes that detected significantly higher binding of IgG from diabetic compared with normal serum. A plasma membrane ELISA offers advantages over traditional whole cell assays in terms of greater convenience and reproducibility, lower cost and increased number of samples that can be handled.

In the early stages of autoimmune diabetes, most patients have antibodies against both cytoplasmic (ICCA) and islet cell surface (ICSA) antigens (Lernmark *et al.*, 1978; 1981). It is the ICSA that are potentially of greater significance in the aetiology of the disease, since they are directed against antigens that are accessible in living cells. A possible drawback of a plasma membrane ELISA is therefore that, in addition to detecting antibodies directed against cell surface antigens, the assay may also be able to detect binding by ICCA to "cytoplasmic" epitopes. That is, to regions of proteins present in a plasma membrane preparation that would not be exposed to the immune system at the surface of a viable cell. However, the  $\beta$ -cell membrane ELISAS developed in the present

study did not appear to detect antibodies directed against cytoplasmic antigens, since the IDDM samples in set 2, which were known to be positive for ICCA, did not show significantly higher binding than the ICCA-negative control samples.

Initially, variation in the plasma membrane assay was found to be high. Intra-assay variation was successfully reduced from  $\pm 13.6\%$  to  $\pm 2.7\%$  by homogenisation of the membrane sample in the coating buffer which ensured an even coating of the plate. Two different methods were used to prepare plasma membranes from the  $\beta$ -cell lines. In the first method, cells were disrupted by vigorous homogenisation and plasma membranes were separated using a Ficoll gradient. The vigorous homogenisation necessary to disrupt these small cultured cells in a hypertonic buffer also tended to disrupt the nuclear membrane releasing DNA which caused clumping of cytoplasmic components and prevented the reproducible preparation of a pure plasma membrane fraction (Howell *et al.*, 1989). It appeared that the variability in the quality of the plasma membranes prepared by this method contributed to the high inter-assay variation ( $\pm 21.5\%$ ). Inter-assay variation was reduced to about  $\pm 11\%$  when membranes were prepared from cells that had been disrupted by lysis in a hypotonic buffer aided by gentle homogenisation. This method was chosen since it has previously been used by Karounos *et al.* (1990) to detect anti-RINm5F membrane antibodies in diabetic serum.

Although, at a significance level of  $p=0.05$ , there was no statistically significant difference in the levels of anti-MIN or anti-HIT antibodies in diabetic compared with control serum that were measured in any of the ELISAS developed in the present study, the trend was definitely for the diabetic samples to show higher levels of binding. This was particularly evident when the samples in set 1 were assayed against HITm2.2 membranes that had been prepared on a Ficoll gradient. In this assay IDDM samples showed an increased binding above that shown by control samples which was significant at  $p=0.1$ . The difference was almost significant at  $p=0.05$ , the standard acceptable level for significance testing.



It was apparent that a different distribution of antibody titres was present in the samples in set 1 compared to set 2. In set 1, the majority of control samples had very low levels of anti-MIN and anti-HIT membrane antibodies, that is, titres less than 25% of the standard serum, whereas the majority of IDDM samples had titres that were slightly higher, that is between 25-50% of the standard. In contrast, in set 2 there was no excess of control samples with very low antibody titres. In view of the difference in titres in the samples from set 1 compared to set 2, the possibility must be considered that the significantly ( $p=0.1$ ) higher binding of IDDM samples to HITm2.2 membranes was obtained using a non-representative set of serum samples. It is possible that the low binding of many of the control samples in set 1 was caused in some way by treatment to which they had been subjected before they were received in the laboratory. For example, the samples could have been left at room temperature for prolonged periods, although since IgG is a robust antibody (Johnstone & Thorpe, 1987) it should have been able to withstand this putative treatment. Additionally, there was no reason to believe that the control samples had been treated any differently from the IDDM samples. One known difference between the control samples in set 1 and 2 which could have influenced the results, was that the samples in set 2 were all from healthy children, whereas those in set 1 had all been hospitalised for some reason (not IDDM). Therefore in conclusion, there was no a priori reason to believe that set 1 serum samples were any less representative than the samples from set 2.

The ideal assay for IDDM marker antibodies should be able to detect all cases of IDDM and yet should be specific enough not to give a positive result for a normal sample. This is especially important if immunotherapeutic or immunosuppressive treatments are to be initiated based on the results of tests for the presence of marker antibodies. Even in the assay in which IDDM samples showed significantly ( $p=0.1$ ) higher binding, an overlap still existed in the levels of anti- $\beta$ -cell antibodies in the control and IDDM populations,

which would prevent the assay being used for screening purposes without some modification.

The nature of the antigens in the  $\beta$ -cell lines to which the human antibodies were binding was unknown. Approximately half of the sera tested showed a very similar amount of binding to both HIT (hamster origin) and MIN (mouse origin) membranes. This suggests that there are common  $\beta$ -cell antigens, recognised by human antibodies, that are shared by both of these cell lines and that they are not species-specific (Lernmark *et al.*, 1978). The remaining samples showed quite different levels of binding to the two cell lines, indicating that each cell line may also express unique antigens or different amounts of the same antigen. Such differences in antigen expression between different cell lines are to be expected since they have previously been observed in different subclones of the same  $\beta$ -cell line (Eisenbarth *et al.*, 1981; Gillard *et al.*, 1989).

Obviously these  $\beta$ -cell line membrane ELISAS require further refinement and evaluation by the testing of much larger numbers of samples (all of which should be collected under standardised conditions). However, the results were promising in view of the trend for higher binding, albeit not statistically significant, of the IDDM serum samples. Since the best results were obtained using HITm2.2 plasma membranes prepared on a Ficoll gradient, future work would need to investigate the possibility of improving the reproducibility of this method of membrane preparation. Due to limited availability of the sera that were used in the present study, they were not preabsorbed with rat or mouse tissue powders before being assayed. It was therefore possible that the high binding shown by some normal sera was due to non-specific binding to rodent antigens and that the assay may be improved if the sera could be preabsorbed. However, although some authors have previously claimed to get better results in ICA assays by preabsorbing sera with rodent tissue (Matsuba *et al.*, 1987) others have found that the preabsorption did not affect the results (Lernmark *et al.*, 1978; Lander *et al.*, 1989).

#### **4.5 THE ATTEMPTED IDENTIFICATION OF ANTIGENS IN $\beta$ -CELLS THAT ARE RECOGNISED BY AUTOANTIBODIES IN IDDM SERUM**

The first report of islet cell surface antibodies in IDDM patients was published in 1978 (Lernmark *et al.*, 1978). Since then numerous studies, including the present one, have confirmed the higher incidence of anti- $\beta$ -cell antibodies in IDDM compared to control serum, but to date the only positively identified  $\beta$ -cell autoantigen is a 64 kDa protein called glutamic acid decarboxylase (Baekkeskov *et al.*, 1990). It is not currently known whether autoantibodies against this antigen are actually involved in the aetiology or pathogenesis of IDDM. Therefore, it is important to identify as many other  $\beta$ -cell antigens as possible, the aim being to maximise the chances of identifying a novel diabetes-specific autoantigen that is involved in causing the disease. This antigen could potentially be used to develop a highly specific assay designed to detect the pre-overt stage of diabetes.

Many of the assays that have previously been applied to the detection of diabetes-specific autoantigens have employed complex methodology, such as detergent extractions and immunoprecipitations (Baekkeskov *et al.*, 1982). In the current study it has been attempted to identify diabetes-specific islet cell surface antigens using a simple Western blotting method.

When plasma membranes from MIN 6 and HITm2.2  $\beta$ -cells were immunoblotted with a selection of serum samples from control and diabetic children a heterogeneous group of antigens were detected. The main conclusion drawn from these results was that typically, the diabetic samples recognised a larger number of distinct  $\beta$ -cell antigens than the control sera. This suggested that the higher binding shown by the IDDM samples in the  $\beta$ -cell line ELISAS was a result of antibody binding to a large number of antigens, rather than being due to a high titre of antibodies against a single antigen. The majority of these antibodies were probably generated as a result of the exposure of the immune system to antigens liberated from  $\beta$ -cells which were damaged at the onset of diabetes. Therefore,

although possibly enhancing the rate of  $\beta$ -cell necrosis, these antibodies may not have played a role in the actual initiation of the disease.

The current Western blotting technique was not, however, sensitive enough to enable the positive identification of any diabetes-specific autoantigens in  $\beta$ -cell plasma membranes. This contrasts with earlier reports of the use of Western blotting to detect multiple diabetes-specific islet cell antigens (Karounos *et al.*, 1988; Buchs *et al.*, 1990). However, comparing results obtained from different laboratories is difficult, largely due to the use of different sources of  $\beta$ -cells and different methods of antigen extraction. In particular, earlier work has often used antigens solubilized in detergents such as NP-40 (Kuo *et al.*, 1991) or SDS. This treatment extracts both cytoplasmic and cell surface antigens (Karounos *et al.*, 1988; 1990), whereas in the present study purified plasma membranes were used, which is a method more suitable for the detection of cell surface antigens. The method of antigen preparation can make a large difference to the results obtained, as has been illustrated by Karounos and co-workers. Antigens with apparent molecular masses of 150, 84, 60, 49 and 35 kDa were detected by diabetic sera in detergent extracts from whole RIN cells (Karounos *et al.*, 1988), whereas completely different proteins with masses of 166, 136, 52 and 49 kDa were recognised in extracts from plasma membranes (Karounos & Thomas, 1990).

It is possible that whole plasma membranes may be used more successfully to detect diabetes-specific cell surface autoantigens if the non-specific background binding could be reduced. This may be achieved by the preabsorption of sera with rat tissue powders, which was not carried out in the present study due to the small volumes of serum available.

It was attempted to increase the sensitivity of the Western blotting procedure by the extraction of membrane proteins into the detergent Triton X-114. This technique has previously been used with rat islet cells and has reportedly been demonstrated to detect

antibodies in diabetic serum against the 64 kDa islet cell GAD protein (Herold *et al.*, 1988). The results obtained using this extraction procedure were very different from those obtained using whole plasma membranes from the same cells. Instead of multiple antigens, a single protein with an apparent molecular mass of about 58 kDa was detected by most samples. However the technique still failed to detect any diabetes-specific autoantigens in the HITm2.2 and RINm5F cells. The inability to detect the 64 kDa autoantigen confirms previous reports that this protein is not expressed in cultured  $\beta$ -cell lines (Baekkeskov *et al.*, 1988; 1990).

The identity of the 58 kDa protein was not known, although it showed some similarities to the protein that was photolabelled by ATB-BMPA in liver plasma membranes and immunoprecipitated with human serum. Both proteins had apparent molecular masses of between 45 and 66 kDa and were recognised in detergent-solubilized membrane proteins by antibodies present in most human serum samples. Since both proteins have an apparent molecular mass similar to that of the GLUT2 protein, it is suggested that this may have been the identity of the antigen that was recognised by the antibodies present in the human serum. This possibility is discussed in further detail below.

#### **4.6 IDENTIFICATION OF PUTATIVE ANTI-GLUT2 ANTIBODIES IN HUMAN SERUM**

At present, the evidence for the involvement of anti- $\beta$ -cell GLUT2 autoantibodies in the pathogenesis of autoimmune diabetes is largely circumstantial. The major evidence comes from animal models of IDDM in which  $\beta$ -cell glucose transport and expression of GLUT2 are reduced at the onset of diabetes (Tominaga *et al.*, 1986; Orci *et al.*, 1990a). Serum samples from recently diagnosed diabetic patients have also been shown to contain IgG antibodies that can both inhibit glucose-induced insulin secretion (Kanatsuna *et al.*, 1983) and glucose transport in islet  $\beta$ -cells (Johnson *et al.*, 1990a).

In this present study it has been attempted to confirm the existence of these putative anti-GLUT2 autoantibodies. Although the autoimmune response that occurs in diabetes is generally directed specifically against the  $\beta$ -cell (Lernmark *et al.*, 1978), it has been shown that human antibodies that inhibit  $\beta$ -cell glucose transport can be removed from diabetic serum by preabsorption with liver membranes (Johnson *et al.*, 1990a). Therefore, it is theoretically possible to detect anti- $\beta$ -cell GLUT2 autoantibodies using GLUT2-containing liver plasma membranes. Although it has been shown that liver plasma membranes can be used in ELISAS and Western blots to detect anti-GLUT2 peptide antibodies in rabbit immune serum, in the present study neither of these techniques enabled anti-GLUT2 autoantibodies to be detected in human serum.

The results obtained by assaying IDDM sera in a liver plasma membrane ELISA, confirmed that diabetic serum does not contain especially high levels of antibodies that react with any antigens in liver membranes, as there was no significant difference between antibody binding in control and IDDM samples. Similarly, the GLUT2 protein was not detected by any human serum sample when immunoblotted against liver plasma membranes. Therefore, if anti-GLUT2 autoantibodies were present, they were either at titres too low to be detected by these assays, or they did not recognise the antigen in the conformation it assumed when immobilised on an ELISA plate or nitrocellulose paper. Baekkeskov *et al.* (1990) have also previously been unable to demonstrate the presence of low levels of anti-64 kDa autoantibodies using Western blotting.

The possibility was considered that increasing the proportion of GLUT2 present in the antigen preparation used for either Western blotting or ELISAS could improve the sensitivity of these assays, thereby enabling low levels of anti-GLUT2 autoantibodies to be detected. However, it has not been possible to affinity purify sufficient GLUT2 to enable this theory to be tested.

Radiolabelling of islet cell proteins followed by an immunoprecipitation with human serum has previously been shown to be a technique that is sensitive enough to detect very low amounts of the 64 kDa  $\beta$ -cell autoantigen (Baekkeskov *et al.*, 1990). A similar strategy was applied in the current investigation. Instead of using  $^{35}\text{S}$  methionine to label all of the proteins in the islet tissue, ATB-BMPA was used to specifically photolabel the glucose transporter protein, GLUT2. It was then attempted to demonstrate the presence of anti-GLUT2 autoantibodies by immunoprecipitation of labelled protein with human diabetic and control serum samples. The antibodies previously detected in human serum, that inhibited  $\beta$ -cell glucose transport were of the IgG class (Johnson *et al.*, 1990a), so they should have a high affinity for Protein A-Sepharose (Richman *et al.*, 1982). Therefore, if present in sufficiently high titres they may be expected to be able to immunoprecipitate detectable quantities of labelled GLUT2.

However, this technique did not detect any antigens that were recognised exclusively by antibodies present in diabetic serum. In fact, all human serum samples tested were demonstrated to precipitate ATB-BMPA labelled proteins from liver plasma membranes, which had apparent molecular masses of about 66 kDa and 45-66 kDa. The similarity of the apparent molecular masses of the proteins precipitated by human serum and anti-GLUT2 C-terminal peptide antiserum, together with the observation that labelling of proteins precipitated by both sera could be displaced to a similar extent (50-60%) by 4,6-*O*-ethylidene-D-glucose, suggested that it was the same protein that was being precipitated by rabbit anti-GLUT2 peptide antibodies and by antibodies present in human serum.

Other evidence supporting the hypothesis that human IgG and rabbit anti-GLUT2 antibodies were precipitating the same labelled protein was obtained from competition studies. By performing a pre-immunoprecipitation with human serum, it was possible to reduce the amount of GLUT2 that could be subsequently precipitated by about 58%. A pre-immunoprecipitation with rabbit anti-GLUT2 antiserum resulted in a decrease of

approximately 41% in the amount of labelled protein subsequently precipitated by human serum. It is possible to account for the slight discrepancy that was observed in the percentage reduction depending on the order of precipitation, since the immunoprecipitation with anti-GLUT2 antiserum was of low efficiency (see section 4.1) and did not totally remove all of the GLUT2 protein, whereas more of the labelled GLUT2 appeared to be removed by precipitation with the human serum.

No accurate measurement was made of the maximum amount of labelled protein that could be precipitated by the human antibodies, although it was estimated that human serum precipitated approximately 1.5 fold more labelled protein than anti-GLUT2 antiserum. The possibility has been considered that the high amount of precipitation by the human serum could be due to the precipitating antibody being IgM and not IgG, since the polyvalent nature of IgM makes it a high avidity antibody (Roitt, 1984). However, it has previously been shown that although human IgM can bind to Protein A, it does not bind as well as IgG. Therefore the precipitating antibodies were likely to have been of the IgG class.

Although these results imply that all human serum contains antibodies that can immunoprecipitate GLUT2 from liver membranes, it does not seem likely that these antibodies bind to GLUT2 in whole cells, since this would contradict the results obtained by Johnson *et al.* (1990a), who found no evidence of antibodies inhibiting  $\beta$ -cell glucose transport in normal human serum samples. It is therefore proposed that antibodies (probably IgG but maybe IgM) are present universally in normal human serum which cross-react, not with GLUT2 epitopes accessible on the surface of living cells, but with epitopes on the GLUT2 protein only exposed under certain experimental conditions. For example, if the antibodies only bound to epitopes exposed in the detergent solubilized protein, they would not be expected to have any pathogenic effect *in vivo*. This could also explain why the precipitating antibodies could not be pre-absorbed with non-solubilized



liver plasma membranes, and why human serum did not detect any strong 45-66 kDa band in Western blots of liver plasma membrane proteins.

When human serum immunoprecipitates were immunoblotted with rabbit anti-GLUT2 peptide antiserum, GLUT2 could not be detected, this suggests either that human serum was not precipitating GLUT2 or, more probably, that ATB-BMPA labelling and immunoprecipitation provided a more sensitive method of GLUT2 detection compared to immunoblotting. Confirmation that the human serum does precipitate GLUT2 must await improvements in the sensitivity of GLUT2 detection by Western blotting.

In summary, it was shown that all of the small number of human serum samples tested contained antibodies able to precipitate an ATB-BMPA labelled protein from liver plasma membranes. It has been tentatively proposed that the protein is GLUT2 and that antibodies directed against it recognise cytoplasmic epitopes not normally accessible *in vivo*. The origin of these antibodies is not known, but they could have been formed against antigens that were released from damaged liver tissue during, for example, mild liver disease. Unless it can be demonstrated that the high level of background immunoprecipitation with control sera represents binding to a protein other than GLUT2, and a way can be found of removing it, it does not seem likely that ATB-BMPA photolabelling will be able to be used to detect the putative anti-GLUT2 diabetes-specific antibodies that inhibit  $\beta$ -cell glucose transport (Johnson *et al.*, 1990a).

None of the techniques described in this thesis was able to demonstrate the presence of anti-GLUT2 autoantibodies present exclusively in serum from diabetic patients. However, it cannot be assumed from these results that the anti-GLUT2 autoantibodies do not exist. Indeed, in view of the problems associated with the detection of the relatively abundant GLUT2 protein in liver tissue using a specific high titre antibody probe, it is hardly surprising that the anti- $\beta$ -cell GLUT2 autoantibodies have remained so elusive.

## 4.7 CONCLUSIONS

This thesis has described the first use of the exofacial probe, ATB-BMPA, to label the glucose transporter isoform GLUT2. The protein which was labelled in liver plasma membranes had an apparent molecular mass of  $\approx 45\text{-}66$  kDa, and could be specifically immunoprecipitated with an anti-GLUT2 C-terminal peptide antibody. Since only 43% of the ATB-BMPA binding to liver membrane proteins was inhibited by 4,6-O-ethylidene-D-glucose, this suggested that the ATB-BMPA labelling of liver tissue was less specific than that observed in erythrocytes (Clark & Holman, 1990) and adipocytes (Holman *et al.*, 1990). Nevertheless, it is suggested that ATB-BMPA provides a good alternative to the endofacial label cyt B. Since ATB-BMPA was shown to have a similar binding affinity ( $K_d$ ) for GLUT2 as for GLUT1 and GLUT4 (Palfreyman *et al.*, 1992), it has been proposed that these isoforms are all similar at the exofacial binding site and that the reported differences shown by GLUT2, such as a lower affinity for cyt B and D-glucose, may arise from differences at the endofacial binding site.

The inability to immunoprecipitate any more than 7% of the total liver plasma membrane protein labelled in a 4,6-O-ethylidene-D-glucose displaceable manner has been attributed to a low immunoprecipitation efficiency, probably resulting from a poor affinity of the anti-GLUT2 C-terminal peptide antibody for the detergent-solubilized GLUT2 protein. This putative weak interaction may also explain the inability to purify large amounts of GLUT2 from liver membranes by immunoaffinity chromatography. More work needs to be carried out in order to improve the efficiency of GLUT2 immunoprecipitation. This may also enable GLUT2 to be investigated in other tissue, such as islet  $\beta$ -cells, where the source material is limited. Immunoprecipitation may possibly be improved by the use of anti-peptide antibodies raised against different regions of the GLUT2 protein.

Using both Western blotting and ATB-BMPA photolabelling followed by immunoprecipitation, GLUT1 was the only glucose transporter isoform detected in the cultured  $\beta$ -cell lines HITm2.2, MIN6 and RINm5F. The normal  $\beta$ -cell isoform GLUT2 could not be detected. This confirmed the earlier reports of GLUT1 expression in cultured  $\beta$ -cells, but contrasted with the reported detection of GLUT2 mRNA (Thorens *et al.*, 1988; Miyazaki *et al.*, 1990). This may be attributed either to differences in the cell lines used, or it may indicate that the GLUT2 protein was being expressed at a level below the detection limits of the assays used in the present study. In view of the abnormal glucose transporter expression it is concluded that these  $\beta$ -cell lines are not ideal models of native islet  $\beta$ -cells.

Plasma membranes prepared from the HITm2.2 and MIN6  $\beta$ -cell lines have been used as the antigen in ELISAS in which serum from diabetic and control children was tested for the presence of anti- $\beta$ -cell surface antibodies. The largest difference between antibody titres in control and diabetic samples was obtained when serum samples were assayed against plasma membranes prepared from HITm2.2 cells using a Ficoll gradient, but the increased binding shown by the diabetic samples in this assay was not statistically significant. The aim of this work was to investigate the feasibility of using a simple membrane ELISA as a screening assay to aid early diagnosis of pre-diabetes. It is concluded that a  $\beta$ -cell line membrane ELISA is potentially suitable for the detection of diabetes-specific anti- $\beta$ -cell surface antibodies, although the assays presented in this thesis require further refinement. Larger numbers of serum samples need to be screened and assay variation needs to be reduced, possibly by the use of a different plasma membrane preparation.

Human serum has been shown to contain antibodies that can immunoprecipitate protein from liver plasma membranes labelled with ATB-BMPA in a 4,6-O-ethylidene-D-glucose displaceable manner. This protein has been tentatively identified as GLUT2. It has been proposed that the immunoprecipitating antibodies were directed against epitopes

of GLUT2 which were only recognised in the detergent solubilized protein, but were not accessible in living cells. The origin of these antibodies is unknown, but they are present in all human serum samples and are not therefore the same as the diabetes-specific anti-GLUT2 antibodies previously detected by Johnson *et al.* (1990a). The current work has not been able to confirm the presence of putative diabetes-specific anti-GLUT2 autoantibodies using either Western blotting, which proved unsuitable for the detection of any diabetes-specific anti- $\beta$ -cell autoantibodies, or ATB-BMPA photolabelling. It is proposed that a better technique for the investigation of these antibodies may be an ELISA which uses purified GLUT2 protein as the antigen. In view of the difficulties experienced in purifying GLUT2 from liver tissue, the most convenient source of GLUT2 may be the protein obtained from the cloned gene.

In conclusion, this thesis contributes to the basic information available about GLUT2, and should therefore be of relevance to future investigations of the putative role of this protein in the pathogenesis of both NIDDM and IDDM.

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