# THE USE OF ANTIBODIES IN A STUDY OF THE STRUCTURE, FUNCTION AND DISTRIBUTION OF GLUCOSE TRANSPORT PROTEINS

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## ABSTRACT

The use of antibodies in a study of the structure, function and distribution of glucose transport proteins

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Antibodies were raised against synthetic peptides corresponding to most of the regions of the human erythrocyte glucose transporter predicted to be extramembranous in a model proposed by Mueckler et al. (1985) Science 229, 941-945 for the arrangement of the membrane-bound protein. The antibodies were used as probes to investigate the topology and to identify functionally important regions of this glucose transport protein. In addition, the antibodies were used in a study of the distribution of homologous glucose transporters in other tissues both of mammalian and non-mammalian origin.

Of the 21 antibodies produced all but two recognized the denatured glucose transporter, whilst only 7 recognized the native, functionally-active glycoprotein, even after its extensive deglycosylation. Use of these 7 antibodies as structural probes demonstrated that a large, central hydrophilic region of the sequence (residues 217-272) and the C-terminal region (residues 450-492) were both exposed at the cytoplasmic face of the membrane.

The anti-peptide antibodies were used to identify the sequence location of fragments of the protein produced by partial proteolytic digestion. This led to the identification of the exofacial site of glycosylation as being located in the N-terminal half of the protein, and the site of photolabelling by cytochalasin B as lying in the C-terminal half of the protein. The location of the sites of glycosylation and of the C-terminus of the protein were in agreement with the model of Mueckler et al. (1985), and disproved an alternative model proposed by Shanahan and D'Artel-Ellis (1984). Furthermore, identification of the sequence locations of proteolytic fragments of the protein enabled the epitopes of eight monoclonal antibodies against the transporter to be located. All proved to be within the central or C-terminal hydrophilic regions of the sequence, on the cytoplasmic surface of the membrane.

Antibodies against the central hydrophilic region of the sequence, but not against the C-terminal region, were found to inhibit cytochalasin B binding to the protein. Furthermore, the binding of antibodies to the central region was affected by the presence of bound substrate. These findings suggested that the central region of the sequence is important for transporter function, and is involved in the conformational changes that accompany substrate binding.

Proteins homologous to the human erythrocyte glucose transporter were found to be widely distributed in mammalian tissues. An apparently homologous transporter was even detected in the erythrocytes of the hagfish, the most primitive living vertebrate. The anti-peptide antibodies were also used to begin to probe the subcellular distribution of glucose transporters in mammalian tissues. Evidence was obtained that glucose transporters are present in the Golgi membranes of lactating rat mammary gland epithelial cells. They probably function here to transport glucose across the membranes to the intralumenal site of lactose synthesis. This finding is one of the first demonstrations that intracellular glucose transporters may be functionally important in some mammalian cells.

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CONTENTS

LIST OF DIAGRAMS

## LIST OF TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AChase | Acetylcholinesterase |
| a.m.u | Atomic mass units |
| ASA-BMPA | [2-N-(4-azidosalicoyl)-1-3-bis(D-mannos-4'-xyloxy) propyl-2-amine |
| β-NAD | β-nicotinamide adenine dinucleotide |
| BSA | Bovine serum albumin |
| cpm | Counts per minute |
| CNBD | Chloronitrobenzoxadiazole |
| DEAE- | Diethylaminoethyl- |
| DMF | Dimethylformamide |
| DMSO | Dimethylsulphoxide |
| DTNB | 5,5'-dithiobis-(2-nitrobenzoic acid) |
| DTT | Dithiothreitol |
| EDT | 1,2-ethanedithiol |
| EDTA | Ethylenediaminetetra-acetic acid |
| E-64 | Trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane |
| ELISA | Enzyme-linked immunosorbent assay |
| FDNB | 1-fluoro-2,4-dinitrobenzene |
| Fmoc- | Fluorenylmethoxycarbonyl- |
| G-3-PD | Glyceraldehyde-3-phosphate dehydrogenase |
| KLH | Keyhole limpet haemocyanin |
| MBS | m-maleimidobenzoyl-N-hydroxysuccinimide ester |
| S-MBS | Sulfo-m-maleimidobenzoyl-N-hydroxysuccinimide ester |
| MES | 2-[N-Morpholino]ethanesulphonic acid |
| NBMPR | Nitrobenzylthioinosine |
| NBTGR | Nitrobenzylthioguanosine |
| PBS | Phosphate-buffered saline |
| PBSA | Phosphate-buffered saline containing 0.02% (w/v) sodium azide |
| PBSA-T | Phosphate-buffered saline containing 0.05% (v/v) Tween 20 |
| 0.5P8 | 0.5mM sodium phosphate, pH 8.0 |
| 5P8 | 5mM sodium phosphate, pH 8.0 |
| PMSF | Phenylmethylsulphonyl fluoride |
| p.s.i | Pounds per square inch |
| SDS | Sodium dodecyl sulphate |
| TBS | Tris-buffered saline |
| TTBS | Tris-buffered saline containing 0.05% (v/v) Tween 20 |
| TEMED | N,N,N',N'-tetramethylenediamine |
| TFA | Trifluoroacetic acid |
| TLCK | 1-chloro-3-tosylamido-7-amino-2-heptanone |
| Tris | Tris(hydroxymethyl)aminoethane |

# CHAPTER 1 INTRODUCTION

## 1.1 INTRODUCTION

D-glucose is a major metabolic substrate for most mammalian cells. Although certain tissues have a preference for non-carbohydrate substrates (for instance fatty acids and ketone bodies in the case of muscle, Andres et al., 1956), most are able to metabolise glucose and some (such as brain), utilize it almost exclusively (Elbrink and Bihler, 1975).

The mammalian cell is surrounded by a thin layer of lipid and protein molecules that constitutes the plasma membrane. The structure and composition of this membrane are governed by the metabolic activities of the particular cell. The lipid, in the form of a bilayer, presents a hydrophobic barrier to the entry and exit of hydrophilic substances. It has been known for many years that simple diffusion of most such molecules across the lipid bilayer would be several orders of magnitude too slow to account for their rapid movement into cells. A comparison of D-glucose transport rates across artificial lipid bilayers, with rates across the human erythrocyte membrane illustrates this discrepancy; permeability coefficients of $10^{-9}$-$10^{-10}$cm/s and $10^{-4}$cm/s respectively have been determined (Jung, 1971a,b). Consequently, the conclusion was made that components present in biological membranes, but not in artificial lipid bilayers, assist the movement of hydrophilic molecules across the membrane - a concept described as carrier-mediated transport.

Membrane transport processes can be divided into two categories on the basis of their energy requirements. Where energy (derived from an ionic gradient or ATP hydrolysis) is required to drive a solute molecule against its concentration gradient, the transport process is

said to be active. In contrast, the movement of solute molecules down their electrochemical gradients, a process requiring no energy input, is described as passive transport. In this respect, passive transport is a similar process to simple diffusion, though much faster rates are achieved through mediation of a carrier molecule. As a result, passive transport is often referred to as facilitated diffusion. Active transport of glucose does occur in the cells of the intestinal and renal tubular epithelium (Crane, 1968; Schultz and Curran, 1970). In these cells glucose is driven against its concentration gradient using the energy derived from a transmembrane gradient of sodium ions. However, the uptake of glucose by most other mammalian cells, including the human erythrocyte, is by the passive process of facilitated diffusion.

## 1.2 THE HUMAN ERYTHROCYTE

Glucose transport in the human erythrocyte is the best studied example of facilitated diffusion. One reason for this is that erythrocytes from adult humans and other primates possess an unusually high capacity facilitated diffusion system for glucose. Although most mammals in the foetal stage possess erythrocytes with a high glucose transport capacity, in the majority of species the erythrocytes of the adult are much less permeable to glucose (Widdas, 1955).

The energy derived from glucose is required by the erythrocyte to maintain the reduced state of protein thiol groups and the $Fe^{2+}$ ion in haemoglobin, to maintain the structural integrity of the cytoskeleton and to maintain various ionic concentration gradients across the plasma membrane. The erythrocyte lacks mitochondria and so cannot produce ATP by oxidative phosphorylation. However, its energy requirement is comparatively low, and so can be met by anaerobic

metabolism. Glycolysis accounts for 90% of glucose utilization and the pentose phosphate pathway for the remaining 10% (Jacquez, 1984).

The relative structural and metabolic simplicity of the human erythrocyte has made it amenable to detailed kinetic study. The low level of metabolic activity relative to transport allows transport experiments to be carried out without complications arising from the rapid metabolism of the sugar. The absence of organelles ensures that most of the cell's internal volume is available for equilibration of the sugar. Consequently, from the point of view of purification, availability, the presence of only one membrane fraction and the high number of transport proteins in this fraction, the human erythrocyte is a convenient source of the glucose transport protein.

## 1.3 KINETICS OF GLUCOSE TRANSPORT IN THE ERYTHROCYTE

The human erythrocyte glucose transporter is the prototype of a facilitated diffusion system with a single substrate. Glucose transport across the erythrocyte membrane is stereospecific: the membrane is several orders of magnitude more permeable to D- than to L-glucose. Transport can be competitively inhibited by many other monosaccharides and it exhibits saturation kinetics (LeFevre and Davies, 1951; Widdas, 1954). These features indicate that in order to be transported, glucose must first bind to one of a limited number of sites, that is the glucose 'carriers' in the membrane.

Despite many investigations, the kinetics of the transport process remain controversial and there is still disagreement about whether any of the kinetic models yet proposed can account for all of the experimental features of glucose transport in the erythrocyte (Lowe and Walmsley, 1986; Wheeler and Whelan, 1988; Carruthers and Melchior, 1985; Naftalin, 1988; Helgerson and Carruthers, 1989).

A simple symmetrical model for transport was initially proposed (Sen and Widdas, 1962; Mawe and Hempling, 1965; Levine and Stein, 1966, Fig.1.1), where the steps associated with the binding of glucose (a,b,e and f in Fig.1.1) were considered diffusion-limited. Therefore it was assumed that the rate constants for these steps were very high relative to those for the translocation steps c,d,g and h. In addition, it was assumed that the binding constants at each face of the membrane were equal and that the rate constants associated with translocation were also all equal. In this model the maximum rate of glucose transport ($V_{max}$) and concentration of glucose required for half-maximal transport ($K_m$) are predicted to be independent of the direction in which transport is occuring. However, this was found experimentally not to be the case. $V_{max}$ for glucose entry into glucose-free cells (zero-trans influx) was determined as 23mM in cell water $min^{-1}$, much lower than that of 163mM in cell water $min^{-1}$, determined for glucose exit into glucose-free medium (zero trans efflux) (Levine and Stein, 1966). Also, the measured $K_m$ values for influx and efflux differed (Lieb and Stein, 1972; Bloch, 1974). In equilibrium-exchange experiments where glucose concentrations inside and outside the cells were the same and the transfer of glucose was monitored by the addition of radiolabelled glucose, a $V_{max}$ of 300mM in cell water $min^{-1}$ was determined (Lieb and Stein, 1972; Bloch, 1974). Consequently the simple symmetric model of glucose transport was found unsatisfactory. Additionally, another feature of glucose transport, namely exchange-diffusion (Levine et al., 1965) was incompatible with the simple symmetric model. When erythrocytes were loaded with a high concentration of glucose and resuspended in media containing varying concentrations of glucose (zero up to the internal concentration), efflux was much more rapid at higher medium concentrations. This observation suggested that the loaded carrier (CG) translocated much

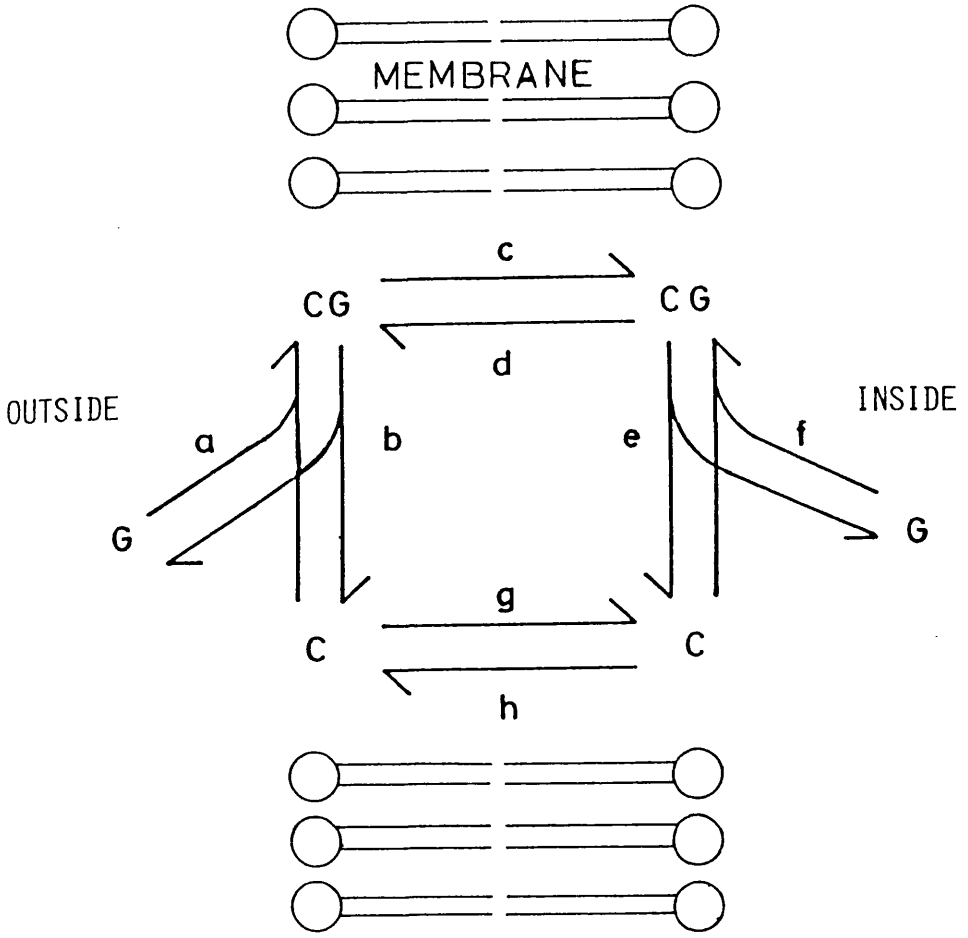



Fig.1.1 Kinetic scheme for the carrier-mediated transport of glucose across the membrane bilayer

Glucose (G) binds to the carrier (C) in the membrane and is transported across the bilayer as a glucose-carrier complex (CG). The rate constants for the individual steps are given by the letters a-h. See text for further details.

more rapidly than the unloaded carrier (C).

The above inconsistencies led Geck (1971) to propose the simple asymmetric carrier model. In this model, the dissociation constants for glucose binding at each face of the membrane are different, and the rate constants for translocation of the loaded and unloaded carriers also differ. However, this model also could not explain the results from some kinetic experiments. In Sen-Widdas experiments (Sen and Widdas, 1962), glucose transport was followed both into (infinite-trans), and out of (infinite-cis), an already saturated cytosol. The data obtained suggested both high and low affinity sites for D-glucose on the internal surface of the erythrocyte. To account for these sites Ginsburg (1978) and Holman (1980) put forward alternative kinetic models. Ginsburg's model suggested that transport occured by an asymmetric anti-parallel pair of carriers. In contrast, Holman proposed an allosteric pore where the binding site could accept one glucose molecule at a regulatory site, which then allowed two further molecules to bind, one at either face. Both models made predictions which could not be verified in later studies (Weiser et al., 1983).

Utilizing rapid reaction methods, Lowe and Walmsley (1986) have determined rates at $0^{o}C$ for the translocation steps. The results obtained were found to be consistent with the simple asymmetrical carrier model (Fig.1.1). The rate constant for reaction c was more than ten times that of reaction d. Similarly, the rate constant for reaction g was found to be more than ten times that of reaction h, and both of these rate constants (g and h), which represent the translocation of the unloaded carrier, were more than 100-fold lower than the corresponding rate constants for the loaded carrier. Additionally, Wheeler and Whelan (1988) utilizing initial rate kinetics, have recently reassessed the claim that the $K_m$ for

infinite-cis uptake of glucose into human erythrocytes is so low that the carrier model for transport must be rejected (Stein, 1986; Helgerson and Carruthers, 1987). The $K_m$ values obtained (15mM for fresh blood at 0°C, 39mM for outdated blood at 0°C and 11mM for outdated blood at 25°C), are considerably higher than the values of 2-3mM obtained previously (Hankin et al., 1972; Dustin et al., 1984), and are fully compatible with the asymmetric carrier model. This model is kinetically equivalent to a model involving conformational change in the transporter that exposes a single binding site alternately to the two sides of the membrane, which is the basis of the asymmetric one-site (mobile or alternating conformer) carrier model (Widdas, 1952; Vidaver, 1966; Barnett et al., 1975; Baldwin and Lienhard, 1981). Evidence for such a model is discussed in Section 1.8.2.

It was the major discrepancy in the $K_m$ for the infinite-cis uptake of glucose into erythrocytes together with other minor differences which gave rise to the proposal of more complex models to describe the experimental kinetic results. These include the asymmetric two-site (linear or simultaneous) carrier model (Baker and Widdas, 1973), the symmetric two-site carrier rate limited by intracellular water and sugar complexation (Naftalin and Holman, 1977; Naftalin et al., 1985), and the two-site allosteric carrier model proposed by Carruthers and colleagues on the basis of fluorescence quenching (Carruthers, 1986a,b), and ligand binding (Helgerson and Carruthers, 1987) studies. The latter model suggests that two substrate binding sites are simultaneously present, one exposed at each face of the membrane. Recently, Helgerson and Carruthers (1989) have proposed that the kinetics of 3-O-methylglucose transport in rat erythrocytes are compatible with the predictions of a symmetric two-site carrier demonstrating negative cooperativity between substrate binding sites, and reject the alternating conformation carrier model

in this transport system.

A model for transport fully compatible with all the kinetic data will necessitate further study although the evidence for a single-site alternating conformation carrier mechanism is now strong (Section 1.8.2). Possible reasons for the misinterpretation of much of the kinetic data obtained for glucose transport may have arisen from the reported demonstration that transport rates are regulated by ATP levels (Jacquez, 1983). For instance, in zero-trans influx experiments, the glucose-depleted erythrocyte might possess a much lower ATP level than the erythrocyte in infinite-cis, infinite-trans, equilibrium exchange or zero-trans efflux experiments. Each of these procedures may lead to undesired effects on ATP levels and consequently ambiguous kinetic interpretation. Alternatively, several workers have proposed that the apparent complexity of erythrocyte glucose transport results more from the technical difficulties of the kinetics experiments rather than an inherent complexity of the transport process itself (Lowe and Walmsley, 1986; Wheeler, 1986; Naftalin, 1988).

## 1.4 INHIBITORS OF GLUCOSE TRANSPORT IN THE ERYTHROCYTE

The inhibitors of facilitated glucose transport in the human erythrocyte can be divided into two main categories. The first are the the protein-modifying reagents, for example 1-fluoro-2,4-dinitrobenzene (Lienhard et al., 1977) and glutathione maleimide (Batt et al., 1976). Compounds in this group are mainly reagents which react with protein amino and sulphydryl groups. Consequently, they are relatively non-specific reagents reacting with a number of erythrocyte membrane components which include the glucose transporter. The use of these compounds allowed the first tentative identification of the

glucose transporter as a protein.

The second category of inhibitors do not form covalent bonds with proteins and includes sugars or sugar analogues. For many pairs of transported sugars it can be shown that the presence of one sugar inhibits the transport of the other, where the extent of inhibition depends on the relative affinities of the individual sugars for the transport protein. If the sugars are competing for the same binding site, this dependence on affinity would be expected. The disaccharides maltose and cellobiose, though not transported, compete with monosaccharides for the transporter binding site due to their structural homology with the latter. Similarly, n-propyl-β-D-glucopyranoside and 6-O-propyl-D-glucose inhibit monosaccharide transport, yet they too cannot be transported as a consequence of the propyl group attached to the sugar moiety (Barnett et al., 1975).

The fungal metabolite, cytochalasin B, is amongst the most potent competitive reversible inhibitors of glucose transport (Deves and Krupka, 1978). Cytochalasin B, especially in radioactive form, has proven invaluable in the study of the glucose transporter. Though bearing little obvious structural similarity to D-glucose it does appear to bind to the substrate binding site. More recently, an X-ray crystallographic comparison of the D-glucose and cytochalasin B molecules (Griffin et al., 1982) has suggested that the spatial arrangement of three potential hydrogen bond acceptor or donator groups in the cytochalasin B molecule mimics the spatial arrangement of hydroxyl groups present on carbon atoms 1,3 and 6 of β-D-glucose. These hydroxyl groups are thought to be involved in the formation of hydrogen bonds between D-glucose and polar groups present at the substrate binding site on the transporter (Barnett et al., 1973, Section 1.8.1). Cytochalasin B binds to D-glucose-sensitive sites on the erythrocyte membrane with an apparent dissociation constant ($K_d$)

of $3\text{-}5\times10^{-7}$M (Jung and Rampal, 1977). These sites constitute approximately two-thirds of the total erythrocyte membrane cytochalasin B binding sites at 200pmol sites/mg dry membrane. The remaining D-glucose-insensitive sites with a $K_d$ of $1\text{-}1.25\times10^{-7}$M for cytochalasin B binding, are cytochalasin E-inhibitable and are believed to be associated with cytoskeletal components of the erythrocyte membrane, most probably actin (Lin and Snyder, 1977).

While searching for a specific affinity probe for the transporter, two groups (Shanahan, 1982; Carter-Su et al., 1982) found that cytochalasin B could bind covalently to the transporter in human erythrocyte ghosts upon exposure to ultraviolet radiation. The main peak of label was found associated with erythrocyte band 4.5 proteins (nomenclature according to Steck, 1974) upon SDS/polyacrylamide gel electrophoresis, in agreement with findings utilizing protein-modifying reagents (Batt et al., 1976; Lienhard et al., 1977). Photoincorporation was inhibited in the presence of D-glucose, a finding consistent with the transporter being the site of labelling.

Other inhibitors in this category include phloretin and [2-N-(4-azidosalicoyl)-1-3-bis(D-mannos-4'-xyloxy) propyl-2-amine (ASA-BMPA), both of which are competitive inhibitors of glucose transport which specifically bind to an exofacial site(s) on the transporter (Krupka, 1985; Holman and Rees, 1987).

## 1.5 PURIFICATION AND CHARACTERIZATION OF THE GLUCOSE TRANSPORTER

The human erythrocyte membrane represents an ideal starting material for isolation of a glucose transport protein, because the transporter constitutes almost 5% by weight of the total membrane protein (Allard and Lienhard, 1985). Identification of the transporter was initially attempted by Kasahara and Hinkle (1976) using

reconstitution methods. These workers extracted proteins from human erythrocyte membranes using the non-ionic detergents Triton X-100 and octyl glucoside and reconstituted them into soybean phospholipid liposomes using freeze-thaw sonication. Specific cytochalasin B and $Hg^{2+}$-inhibitable D-glucose uptake into the liposomes was observed, although transport activity was low. Similarly, Zala and Kahlenberg (1976) extracted erythrocyte membranes with dimethyl maleic anhydride to remove peripheral proteins and reconstituted the detergent-solubilized residual integral proteins into erythrocyte lipid liposomes. These liposomes were also capable of inhibitor-sensitive D-glucose transport. In addition, SDS/polyacrylamide gel electrophoresis demonstrated that the major components of the integral membrane protein extract were bands 3, 7 and 4.5 (nomenclature according to Steck, 1974). This work was extended by both groups (Kasahara and Hinkle, 1977; Kahlenberg and Zala, 1978) with the introduction of a chromatography step. The detergent extract of integral membrane proteins produced above was chromatographed on a DEAE-cellulose column, and the resultant eluate reconstituted into liposomes. D-glucose transport activity in these liposomes was 20-fold greater than found previously and analysis by SDS/polyacrylamide gel electrophoresis demonstrated that the extract was enriched in band 4.5 proteins.

The reconstituted proteins showed specific transport of D-glucose, but at only 1% of the transport activity expected from that found in the intact erythrocyte (Kasahara and Hinkle, 1977; Zoccoli et al., 1978). That this low activity was attributable to inefficient reconstitution methods rather than the presence of transport proteins as an impurity of the integral membrane protein preparation was confirmed using reversible D-glucose-sensitive cytochalasin B binding as a stoichiometric assay for glucose transporters (Zoccoli et al.,

1978), a method which avoided the necessity to reconstitute the protein into sealed vesicles. Using this procedure, it was found that salt washing (Zoccoli et al., 1978), or alkali treatment (Baldwin et al., 1979) of human erythrocyte membranes led to the loss from the membrane of all cytochalasin B binding sites except those sensitive to D-glucose. This D-glucose-sensitive cytochalasin B binding component was subsequently isolated from alkali-treated erythrocyte membranes solubilized in Triton X-100 and subjected to ion-exchange chromatography. The isolated component migrated as a broad band, of average apparent $M_r$ 55,000 in the band 4.5 region on SDS/polyacrylamide gels, and demonstrated both the same affinity for cytochalasin B ($K_d = 1.2 \times 10^{-7}$M), and the same $K_i$ of 32mM for D-glucose inhibition of cytochalasin B binding as the transporter in the intact erythrocyte. It bound approximately 0.4 moles of cytochalasin B per mole of protein, assuming an $M_r$ of 55,000 (Baldwin et al., 1979). These features indicated that the band 4.5 proteins contained the glucose transport component of the human erythrocyte.

Baldwin et al. (1981) developed a method of reconstitution which preferentially incorporated one glucose transporter polypeptide per vesicle and used this in conjunction with the cytochalasin B binding assay of Zoccoli et al. (1978) to show that every cytochalasin B binding site was capable of transporting D-glucose, but at only 5% of the functional rate found in the intact erythrocyte. This study confirmed that the low transport activities obtained previously were probably due to poor reconstitution conditions and provided further evidence that the glucose transporter was a component of the erythrocyte band 4.5 proteins.

Subsequently, an improved procedure for the preparation of the glucose transporter was developed (Baldwin et al., 1982). Octyl glucoside was substituted for Triton X-100 as it was capable of

solubilizing 90% of erythrocyte membrane protein compared to the 24% for Triton X-100. Additionally, octyl glucoside is readily removed from the DEAE-cellulose column eluate (containing purified transporter and erythrocyte lipids) by dialysis against a suitable buffer, whereupon the transporter reconstitutes into membrane-like layers of phospholipid. In this state the transporter retains much of its cytochalasin B binding activity. Both the yield and specific activity (moles of cytochalasin B bound/mg of protein) were improved. Approximately 0.7 moles of cytochalasin B were bound per mole of transporter. This procedure was improved further by Cairns et al. (1984) who slightly increased the ionic strength of the buffers used for the DEAE-cellulose chromatography step. This modification improved the yield of transporter by 40% and increased the stoichiometry of cytochalasin B binding to almost one mole per mole of protein. The final preparation is known to be contaminated with the nucleoside transporter (Jarvis and Young, 1981; Wu et al., 1983), though this protein is present at only 3-5% of the level of the glucose transporter.

The isolated glucose transporter is a glycoprotein (Kasahara and Hinkle, 1977), and bears oligosaccharides (15% by weight) of the poly(N-acetyl-lactosamine) type on that part of the protein exposed to the extracellular surface of the membrane (Sogin and Hinkle, 1978; Gorga et al., 1979). Heterogeneity of glycosylation causes the protein to run as a broad band of apparent $M_r$ 43,000-74,000 with a peak at $M_r$ 55,000 on SDS/polyacrylamide gel electrophoresis (Sogin and Hinkle, 1978; Gorga et al., 1979). The transporter can be partially deglycosylated by treatment with endo-β-galactosidase from Bacteroides fragilis (Scudder et al., 1983) which specifically cleaves internal β-galactosidic linkages of oligosaccharides of the poly(N-acetyl-lactosamine) series (Fukuda and Matsumura, 1976). Complete

deglycosylation can be achieved by treatment with endoglycosidase F from Flavobacterium meningosepticum, which cleaves the glycopeptide bond of N-acetylglucosamine linked to asparagine (Elder and Alexander, 1982; Lienhard et al., 1984). The totally deglycosylated transporter migrates on SDS/polyacrylamide gel electrophoresis as a much sharper band of apparent $M_r$ 46,000 or 38,000 for unheated or boiled gel samples, respectively (Lienhard et al., 1984; Haspel et al., 1985).

The amino acid composition of the transporter has been determined by two groups (Sogin and Hinkle, 1978; Baldwin et al.,1982). Five cysteine residues per polypeptide chain were identified which agreed with the value obtained colorimetrically with 5,5'-dithiobis-(2-nitrobenzoic acid) on SDS-solubilized transporter (without prior reduction), indicating that the protein does not contain disulphide bridges (Baldwin et al., 1982).

Shelton and Langdon (1983) suggested that in the intact erythrocyte the transporter exists as a polypeptide of $M_r$ 100,000 and that the $M_r$ 46,000 polypeptide is the product of a proteolytic cleavage occuring during the purification. However, three lines of evidence have disproved this; (1) antibodies raised to the purified transporter do not react with polypeptides of higher $M_r$ in the erythrocyte membrane (Baldwin and Lienhard, 1980; Sogin and Hinkle, 1980), (2) photoaffinity labelling of the transporter polypeptide in intact erythrocytes with [$^3$H]cytochalasin B tags the $M_r$ 46,000 polypeptide rather than the one of $M_r$ 100,000 (Carter Su et al., 1982) and (3), when the purification was carried out from freshly obtained erythrocytes in the presence of protease inhibitors, the $M_r$ 46,000 species was still obtained (Baldwin et al., 1982).

## 1.6 SIMILARITIES TO GLUCOSE TRANSPORTERS IN OTHER TISSUES

Antibody and cytochalasin B labelling have been used to detect glucose transport proteins present in other cell and tissue-types which demonstrate structural and/or kinetic similarities to the human erythrocyte protein. These studies are described in the introduction section to Chapter 6 of this thesis, wherein anti-peptide antibodies were used as specific probes to study the tissue and subcellular distribution of such proteins.

## 1.7 MEMBRANE TOPOLOGY OF THE GLUCOSE TRANSPORTER

### 1.7.1 Proteolytic and chemical dissection of the glucose transporter

Baldwin et al. (1980) examined the susceptibility of the extracellular and cytoplasmic domains of the transporter to trypsin. Treatment of intact erythrocytes had no effect upon transport activity, nor upon the binding of cytochalasin B. In contrast, treatment of 'inside-out' vesicles (vesicles of erythrocyte membranes with the cytoplasmic face of the glucose transporter facing outwards), resulted in the loss of transport activity and of high-affinity transporter-specific cytochalasin B binding sites. Consequently, as the transporter possesses a carbohydrate-bearing extracellular domain (Sogin and Hinkle, 1978; Gorga et al., 1979) and a trypsin-sensitive cytoplasmic domain, it must therefore be a transmembrane protein.

Proteolytic and chemical dissection of the transporter has been used extensively in an attempt to elucidate its arrangement in the erythrocyte membrane. Trypsin digestion of the purified, reconstituted glucose transporter produces two large, membrane-bound fragments (Cairns et al., 1984; Deziel and Rothstein, 1984). The smaller of the membrane-bound fragments, of apparent $M_r$ 18,000, migrates as a sharp

band on SDS/polyacrylamide gel electrophoresis. When the intact transporter was photoaffinity labelled with [$^3$H]cytochalasin B prior to tryptic digestion, this fragment became radiolabelled and so probably encompasses part of the inhibitor binding site, which is known to lie on the cytoplasmic face of the erythrocyte membrane (Deves and Krupka, 1978). In contrast, the larger fragment was not radiolabelled, and migrated as a diffuse band of apparent $M_r$ 23,000-42,000 on electrophoresis. Treatment of this fragment with endo-β-galactosidase resulted in a sharper band of apparent $M_r$ 23,000 (Cairns et al., 1984) indicating that this fragment bears one or more sites at which a oligosaccharide chain is attached. Since this fragment is glycosylated and is produced by tryptic cleavage of the cytoplasmic domain of the protein, then it must span the membrane at least once. In addition, comparison of its apparent $M_r$ of 23,000 after endoglycosidase treatment with that of the intact protein ($M_r$ 46,000), indicated that a trypsin-sensitive site must lie close to the middle of the transporter polypeptide. The other half of the polypeptide was represented by fragments of apparent $M_r$ 25,500 and 23,500 seen on SDS/polyacrylamide gels after short times of tryptic digestion (Cairns et al., 1984). These fragments migrated as sharp bands even without endoglycosidase treatment. In addition, these fragments were also photolabelled by cytochalasin B and so are probably precursors to the $M_r$ 18,000 tryptic fragment (Cairns et al., 1984).

Cleavage of the intact, endo-β-galactosidase-treated, cytochalasin B-photolabelled transporter at its cysteine residues with 2-nitro-5-thiocyanobenzoic acid (Cairns et al., 1984) yielded a major unlabelled fragment of apparent $M_r$ 38,000 and several smaller fragments as seen on SDS/polyacrylamide gel electrophoresis. When endoglycosidase treatment was omitted the sharp band of the $M_r$ 38,000 fragment was replaced by a broad band of apparent $M_r$ 36,000-48,000,

and so it is likely that this fragment carries one or more sites of glycosylation of the transporter. The radiolabel was associated predominantly with a fragment of apparent $M_r$ 15,500. The latter result suggested that the site(s) labelled by [$^3$H]cytochalasin B lies within the N-terminal or C-terminal third of the intact transporter polypeptide.

An alternative approach was taken by Shanahan and D'Artel-Ellis (1984) who utilized *in situ* labelling and proteolytic dissection of the transporter in order to study its membrane topology. The transporter present in both sealed and unsealed human erythrocyte ghosts was photolabelled with [$^3$H]cytochalasin B and then subjected to enzymic dissection with proteases and glycosidases. Trypsin treatment of the transporter in sealed ghosts, as expected, did not affect the $M_r$ of the transporter. Treatment of unsealed ghosts yielded a major radiolabelled fragment of $M_r$ 21,500. Thermolysin also had no effect on the transporter in sealed ghosts but led to the complete loss of label from unsealed ghosts. Two possible explanations put forward to explain the latter finding were that thermolytic cleavage at the cytoplasmic surface of the membrane resulted either in the complete release of bound [$^3$H]cytochalasin B or in the generation of membrane-bound fragments too small to be detected on the electrophoresis system used. Chymotrypsin treatment resulted in the generation of a single radiolabelled fragment of $M_r$ 18,400 in both sealed and unsealed ghosts, indicating that its action occurs at the extracellular surface. Carboxypeptidase and aminopeptidase digestion indicated that the C-terminus of the transporter is located at the extracellular surface of the membrane and the N-terminus at the cytoplasmic surface. Treatment of the transporter in sealed ghosts with endo-β-galactosidase shifted the mobility of the transporter to a lower $M_r$ of 49,000. On the basis of these results, Shanahan and D'Artel-Ellis

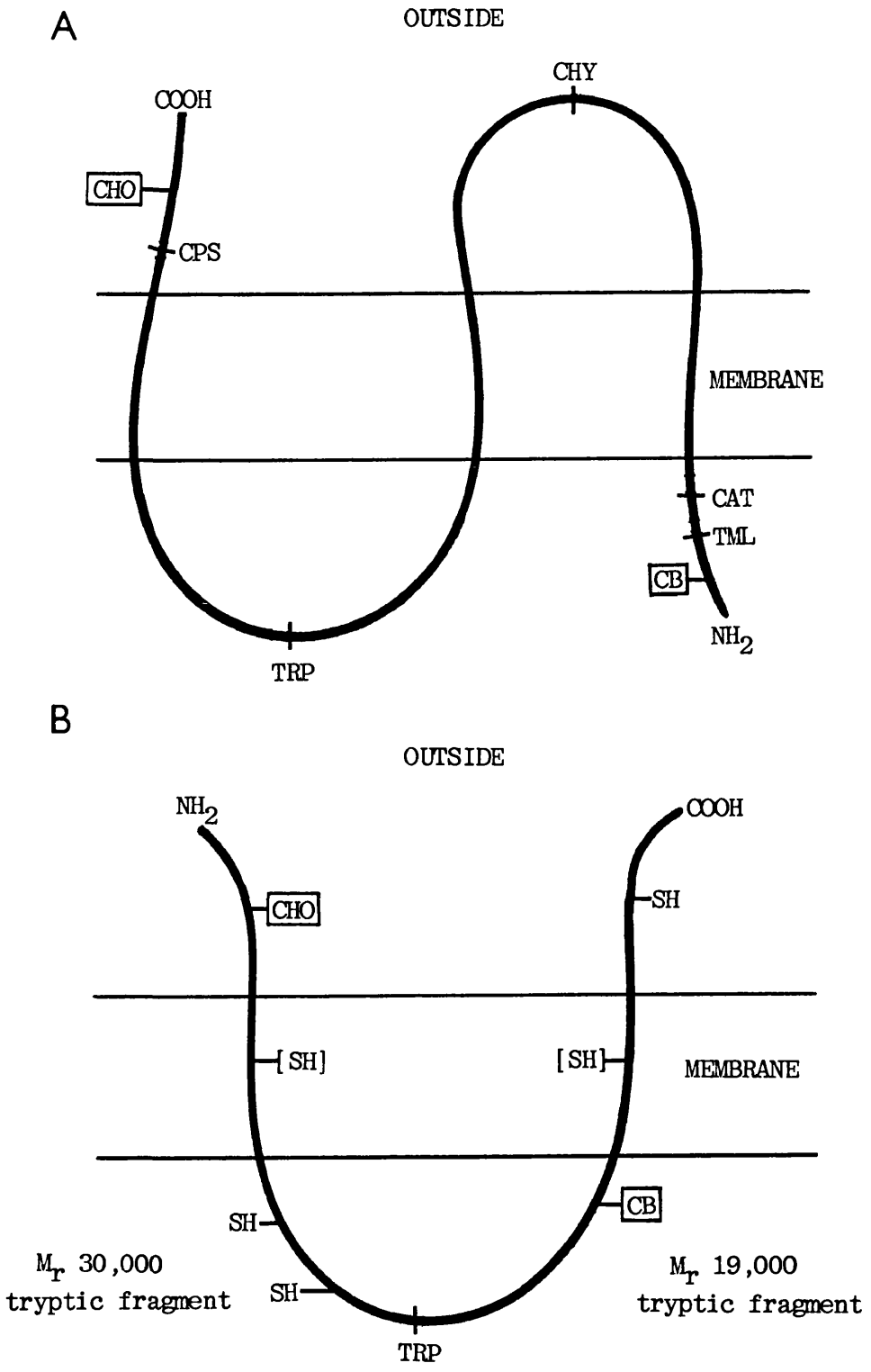



Fig.1.2 Proposed arrangements for the glucose transporter polypeptide in the human erythrocyte membrane.

(A), after Shanahan and D'Artel-Ellis (1984), and (B), after Deziel et al., (1985). The abbreviations are: CHO, oligosaccharide attachment site; CB, cytochalasin B binding site; TRP, trypsin cleavage sites; CHY, chymotrypsin cleavage site; CPS, carboxypeptidase cleavage site; CAT, cathepsin cleavage site; TML, thermolysin cleavage site; SH, location of reactive cysteine residues; [SH], cryptic cysteine residues. See text for further details.

suggested that the C-terminal region of the protein is exposed at the extracellular surface of the membrane, that the glycosylation site is located near the C-terminus and that the cytochalasin B binding site is located near the cytoplasmically-exposed N-terminus (Fig.1.2(A).

Deziel et al. (1985) proposed a model for the membrane disposition of the transporter polypeptide based on the location of reactive cysteine residues in the protein. A preparation of band 4.5 proteins was labelled with the sulphydryl reagent N-ethyl-[$^{14}$C]maleimide. In preparations of transporter denatured in SDS, all five known cysteine residues (Baldwin et al., 1982) were labelled with the reagent. When the transporter was reconstituted into artificial lipid vesicles, treated with trypsin and then denatured in SDS, three sulphydryl groups were labelled in a tryptic fragment of $M_r$ 30,000 which also bears the site of glycosylation of the transporter (Deziel and Rothstein, 1984), and two sulphydryl groups were labelled in a fragment of $M_r$ 19,000 which also carries the site of photolabelling with [$^{3}$H]cytochalasin B (Deziel and Rothstein, 1984). In the 'native' erythrocyte lipid-bound transporter only three of the five cysteine residues were accessible to the reagent, two in the $M_r$ 30,000 fragment and one in the $M_r$ 19,000 fragment. Therefore one cysteine residue in each fragment must be cryptic and inaccessible to N-ethyl-[$^{14}$C]maleimide. In intact erythrocytes, the single reactive sulphydryl group of the $M_r$ 19,000 fragment can be protected against reaction with N-ethylmaleimide by the impermeant sulphydryl reagent p-chloromercuribenzene sulphonate (PCMBS) (Rothstein, 1981). Therefore this cysteine residue is probably exposed at the extracellular face of the membrane. In contrast, the two cysteine residues in the $M_r$ 30,000 fragment are not protected by PCMBS, and therefore are unlikely to be exposed at the extracellular face of the transporter. Cytochalasin B affords temporary protection of the exofacial cysteine residue of the

$M_r$ 19,000 fragment, and longer term protection of the cysteine residues in the $M_r$ 30,000 fragment (LeFevre et al., 1975; Batt et al, 1976; Roberts et al., 1982; Deziel et al., 1985). On the basis of these findings, Deziel and co-workers concluded that both proteolytic fragments must cross the membrane bilayer, one of the three reactive cysteine residues is exofacial, two may be cytoplasmic, and the two cryptic residues inaccessible to N-ethylmaleimide may be located within the bilayer (Fig.1.2(B).

### 1.7.2 Cloning and sequencing of the glucose transporter

Recently the complete amino acid sequence of a glucose transport protein from human HepG2 hepatoma cells was deduced from the nucleotide sequence of a complementary DNA (cDNA) clone (Mueckler et al., 1985). Antibodies raised to the purified, intact human erythrocyte transporter were used to detect the relevant cDNA clone (Lienhard et al., 1982). The cDNA coded for a 492 residue polypeptide of corresponding $M_r$ 54,117. Structural analysis of the purified human erythrocyte glucose transporter by fast atom bombardment mass spectrometry and gas phase sequencing confirmed the identity of the clone and demonstrated that the HepG2 and erythrocyte transporters are very similar if not identical, and that neither the N-terminal or C-terminal sequences of the proteins were proteolytically processed after synthesis (Mueckler et al., 1985). Examination of the hepatoma sequence for potential membrane-spanning hydrophobic segments by the procedures of Kyte and Doolittle (1982), and of Eisenberg et al. (1984), together with the results of vectorial proteolytic digestion experiments, enabled a model to be proposed for the arrangement of the transporter in the membrane (Mueckler et al., 1985).

1.7.3 Proposed model for the glucose transporter in the membrane

In the model of Mueckler et al., (1985) (Fig.1.3), the polypeptide spans the membrane twelve times in the form of largely hydrophobic α-helices. The N-terminal region (residues 1-12), the C-terminal region (residues 451-492), together with a large, hydrophilic segment near the middle of the sequence (residues 207-271), are located on the cytoplasmic surface of the membrane, and the extracellular site of glycosylation is predicted to be $Asn_{45}$ in the N-terminal half of the protein. The site at which the protein is photolabelled with cytochalasin B lies within the C-terminal half of the protein (Cairns et al., 1984; Deziel and Rothstein, 1984). Additionally, the model predicts that several of the membrane-spanning domains (helices 3,5,7,8 and 11, Fig.1.3), may form amphipathic α-helices and contain abundant hydroxyl and amide side chains that could participate in glucose binding, or line a transmembrane pore through which the sugar moves.

Direct evidence for the location of the site of glycosylation has since been obtained (Mueckler and Lodish, 1986). These workers showed that when a fragment of the glucose transporter corresponding to the N-terminal 340 amino acids is synthesized in an in vitro translation system in the presence of pancreatic microsomes it becomes incorporated into the membrane and is glycosylated. Since the fragment lacks the only other potential site for N-linked glycosylation in the intact transporter ($Asn_{411}$), then $Asn_{45}$ must be the site of glycosylation. It follows then that the large, glycosylated tryptic fragment of $M_r$ 23,000-42,000 (Cairns et al., 1984; Deziel and Rothstein, 1984) must be derived from the N-terminal half of the transporter, and that the large, non-glycosylated fragment of $M_r$ 18,000, that bears the site of photolabelling by cytochalasin B (Cairns et al., 1984; Deziel and Rothstein, 1984), must be derived

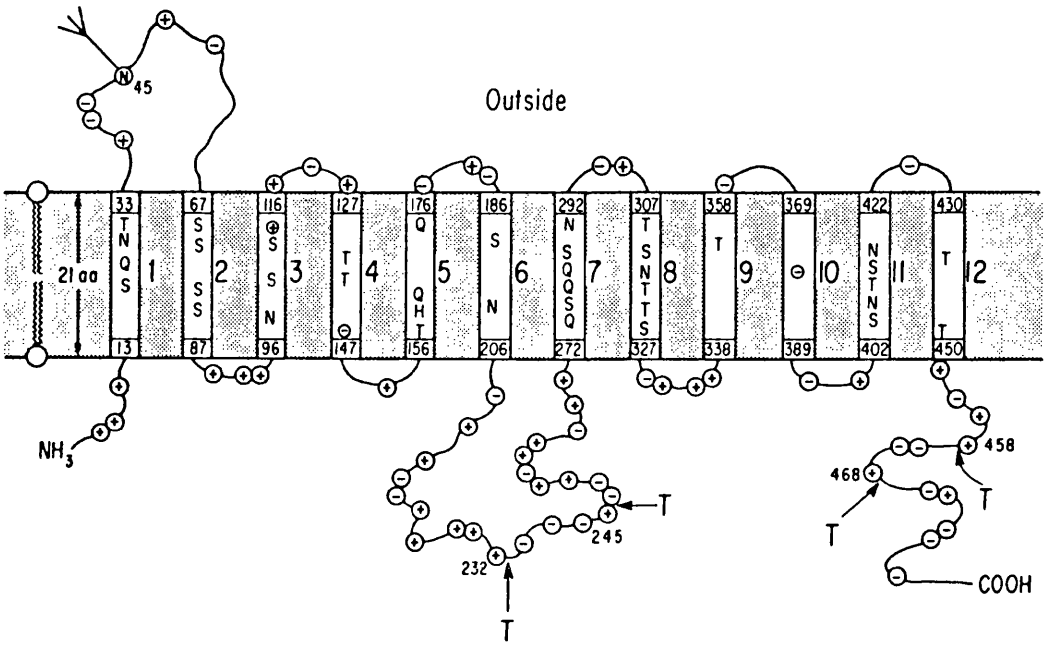



Fig.1.3 Proposed model for the arrangement of the glucose transporter polypeptide in the human erythrocyte membrane after Mueckler et al., (1985)

The twelve putative membrane-spanning regions are numbered and shown as rectangles. The relative positions of acidic (Glu, Asp) and basic (Lys, Arg) amino acid residues are indicated by circled (-) and (+) signs, respectively. Uncharged polar residues within the membrane-spanning regions are indicated by their single letter abbreviations: S, serine; T, threonine; H, histidine; N, asparagine; Q, glutamine. The predicted position of the N-linked oligosaccharide at $Asn_{45}$ is shown. The arrows point to positions of known tryptic cleavage sites in the native, membrane-bound erythrocyte glucose transporter. (Taken from Mueckler et al., 1985).

from the C-terminal half. This conflicts strongly with the model proposed by Shanahan and D'Artel-Ellis (1984) (Section 1.7.1), who proposed that the C-terminus of the protein is extracellular, that the site of glycosylation is near the C-terminus, and that the site of photolabelling by cytochalasin B is near the N-terminus.

This was the situation when I began my experimental work, little additional evidence in support of either model being available. Several concurrent lines of investigation during the course of my study have since provided evidence in support of the model of Mueckler et al. (1985). One such investigation by Cairns et al. (1987) utilized a combination of HPLC, N-terminal and amino acid analysis and fast atom bombardment mass spectrometry to identify soluble peptide fragments derived from vectorial tryptic digestion of the native glucose transporter, an extension of the initial studies of Mueckler et al. (1985). The findings of this work will be described in Chapter 4 (Section 4.3) of this thesis, in the light of the results of my studies on the membrane topology of the transporter using anti-peptide antibodies.

Most other (indirect) evidence in support of this model has been derived using physico-chemical methods. Circular dichroism studies (Chin et al., 1987; Pawagi and Deber, 1987), and infrared spectroscopic studies (Chin et al., 1986; Alvarez et al., 1987; Cairns et al., 1987), have confirmed that the transporter is largely composed of $\alpha$-helices, although some random coil and $\beta$-turn structure is present. Conflicting results on the presence of $\beta$-strands have been reported (Alvarez et al., 1987; Chin et al., 1987). In addition, these studies also indicated that the extramembranous regions of the transporter contain some $\alpha$-helical structure. Infrared spectroscopic analysis of the trypsin-cleaved transporter has confirmed this latter observation (Cairns et al., 1987); both the central hydrophilic and

C-terminal regions (residues 207-271 and 457-492, respectively, Fig.1.3), appear to contain α-helical structure interspersed with short stretches of β-turn and random coil structure. Deuterium and tritium exchange studies (Alvarez et al., 1987; Chin et al., 1986) have shown that a large proportion of the protein is readily accessible to the aqueous environment, possibly indicating that the α-helices are arranged into a water-filled channel. If the model of Mueckler et al. (1985) is correct, then the possible arrangement of the twelve helices must be constrained by the apparent shortness of the loops that connect them (Fig.1.3). Consequently, the helices adjacent in the sequence are likely to be adjacent in the tertiary structure of the transporter.

### 1.7.4 Location of the substrate binding site

The region of the transporter involved in sugar binding is presently unclear, although some advances have been made with the use of cytochalasin B, which appears to bind to the substrate site (Deves and Krupka, 1978; Griffin et al., 1982). Tryptic digestion of the transporter which removes the large, hydrophilic regions exposed at the cytoplasmic side of the membrane (Cairns et al., 1987), destroys its ability to transport glucose (Baldwin et al., 1980). However, trypsin treatment of the transporter does not abolish cytochalasin B binding, but rather reduces its affinity for the ligand (Cairns et al., 1984). In addition, cytochalasin B binding to the trypsinized transporter is inhibited by D-glucose (Cairns et al., 1987; Karim et al., 1987). The latter result indicates both that the substrate binding site is retained, at least in part, within the membrane-spanning regions of the protein, and that these regions of the protein are not greatly altered by trypsin treatment.

Further evidence for the location of the substrate binding

site(s) has come from tryptic digestion of the [$^3$H]cytochalasin B photolabelled transporter; the label appeared in a membrane-bound fragment of apparent $M_r$ 18,000, comprising residues 270-456 of the protein (Cairns et al., 1984, 1987; Deziel and Rothstein, 1984, Section 1.7.1). Thus, at least part of the binding site lies within the intramembranous C-terminal half of the protein. Experiments using 2-nitro-5-thiocyanobenzoic acid to cleave the cytochalasin B-photolabelled transporter at cysteine residues and N-bromosuccinimide to cleave at tryptophan residues yielded fragmentation patterns consistent with a labelling site somewhere between $Phe_{389}$ (at the cytoplasmic end of helix 10) and $Trp_{412}$ (in the middle of helix 11) (Cairns et al., 1984; Holman and Rees, 1987, Fig.1.4). Photolabelling of the transporter with cytochalasin B probably results from photoactivation of an aromatic amino acid residue in the protein rather than activation of the inhibitor (Deziel et al., 1984). The binding of ligands to the transporter has been shown to quench the intrinsic fluorescence of the protein, whilst causing a slight blue shift in the wavelength of maximum fluorescence emission (Gorga and Lienhard, 1982). These effects are indicative of a conformational change in the transporter such that one or more tryptophan residues move from a hydrophilic to a hydrophobic environment. This may be attributable to ligand binding near to either $Trp_{363}$, $Trp_{388}$ or $Trp_{412}$, all of which are located in the vicinity of the proposed substrate binding site (Fig.1.4). Interestingly, the only aromatic residue conserved in both bacterial and mammalian transporters (which can be photolabelled with cytochalasin B), in this region of the sequence is $Trp_{412}$ (Baldwin and Henderson, 1989) which may, therefore, be the site of labelling.

Cytochalasin B binds to, and thus photolabels exclusively, a cytoplasmically exposed substrate site in the transporter (Deves and

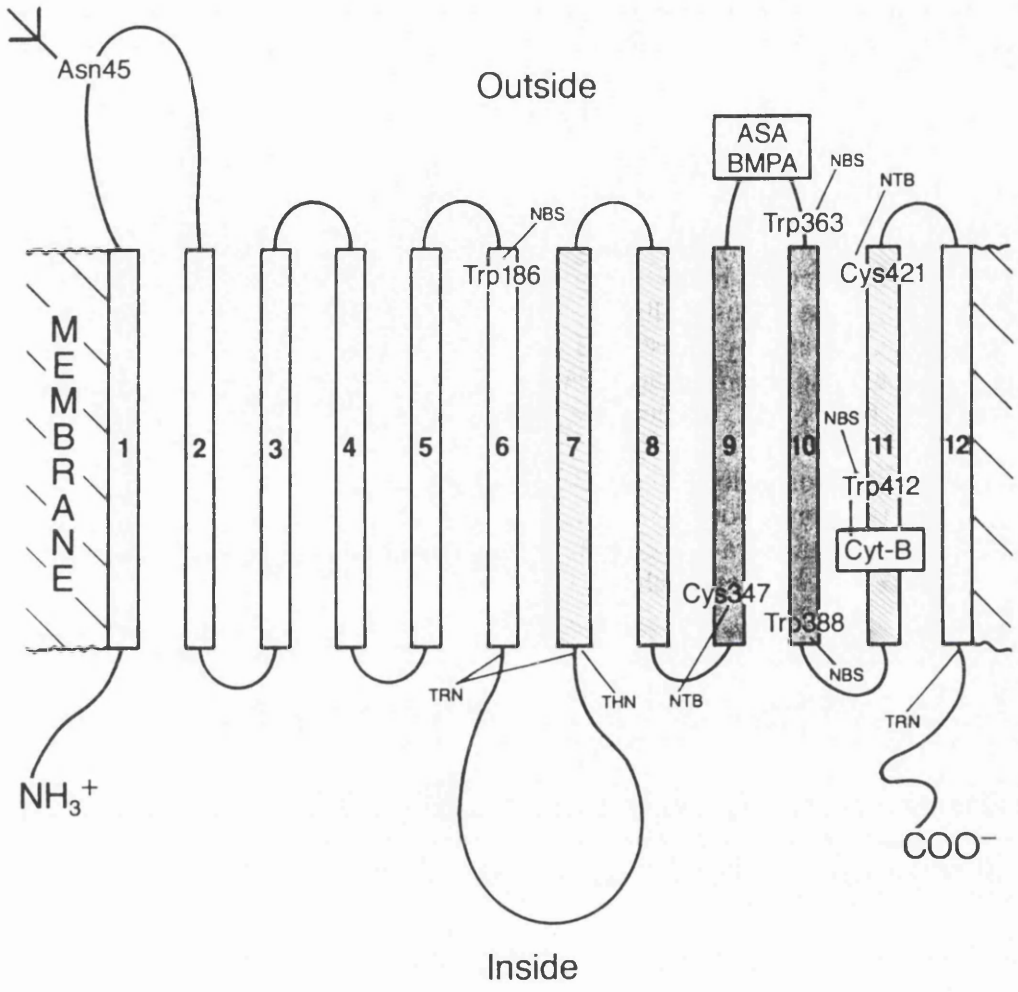



Fig.1.4 A diagrammatic representation of the proposed membrane disposition of the glucose transporter adapted from Fig.1.3.

The helices putatively assigned as the hydrophilic channel and those of the hydrophobic cleft are shaded light and dark, respectively. Trypsin (TRN), thermolysin (THN), 2-nitro-5-thiocyano-benzoic acid (NTB) and N-bromosuccinimide (NBS) cleavage sites are indicated, as are the proposed cytochalasin B (Cyt-B) and ASA-BMPA binding sites. (Taken from Walmsley, 1988). See text for further details.

Krupka, 1978). In contrast, an outward-facing substrate binding site (or the substrate binding site in its outward-facing conformation), has been photolabelled using a membrane-impermeant bis-mannose derivative ASA-BMPA (Holman and Rees, 1987, Section 1.4). Proteolytic and chemical cleavage of the transporter after labelling with this reagent yields fragmentation patterns consistent with a binding site between $Cys_{347}$ (in helix 9) and $Trp_{363}$ (in the extracellular region connecting helices 9 and 10, Fig.1.4). Therefore, it is likely that the region containing helices 9, 10 and 11 forms a part of the substrate binding site.

## 1.8. MOLECULAR MECHANISM OF GLUCOSE TRANSPORT

The kinetic models discussed in Section 1.3 do not explain the events occuring at the molecular level which give rise to the specific transport of D-glucose across the erythrocyte membrane. Kinetic analysis did demonstrate that all transported sugars displayed similar $V_{max}$ values for transport but differed greatly in their $K_m$ values. This demonstrated that the same transport site was used by all sugars, but that some sugars had higher affinities for the site than others. Cytochalasin B and other inhibitors of transport, disaccharides and some sugar analogues have high affinities for the transport site, as demonstrated by their competitive inhibition of glucose transport, yet they are not transported themselves. Consequently, these molecules must bind to the transport site but be unable to cross the membrane. Therefore two separate events occur during transport, namely binding and translocation.

### 1.8.1 Binding of glucose to the site of transport

Binding to the transport site must involve recognition of

specific atoms in the sugar and their spatial arrangement. Analysis of all the available transport data by LeFevre (1961) indicated that aldoses were generally transported more rapidly than ketoses especially where the pyranose ring form was very stable, and that the affinity of the sugar for the transport site positively correlated with the stability of the sugar in the $^4C_1$ chair conformation (LeFevre, 1961). Barnett et al. (1973) studied the kinetics of transport inhibition by deoxy and fluoride-substituted sugar derivatives in an attempt to determine which atoms in the glucose molecule were necessary for high affinity binding. Using this methodology, it was found that the hydroxyl groups at C-1, C-3 and C-4 positions of the sugar pyranose ring were important for high affinity binding to the transporter. Consequently, hydrogen bonding between these hydroxyl groups on the sugar and corresponding groups on the transporter was implicated in the binding process (Fig.1.5(A).

### 1.8.2 Translocation of the bound sugar

The detailed mechanism involved in the translocation of the bound sugar is unclear. A mobile carrier, diffusing across the bilayer and releasing its bound sugar was suggested as a possibility (Jung, 1975). Such a 'ferry-boat' mechanism has been accepted for the ionophore valinomycin, where alternating amide and ester linkages within a cage of D- and L-valine, D-hydroxyisovaleric acid and L-lactic acid holds the $K^+$ ion in a hydrophilic environment whilst exposing a hydrophobic surface to the lipid bilayer. However, Jung (1975) found that modeling of such a carrier around a sugar molecule resulted in a structure too large to account for the recorded rates of glucose transport if it is assumed that the cage diffuses across the membrane. As mentioned in Section 1.3, Vidaver (1966) had shown that the same kinetics explained by the mobile carrier hypothesis could also be explained by a protein

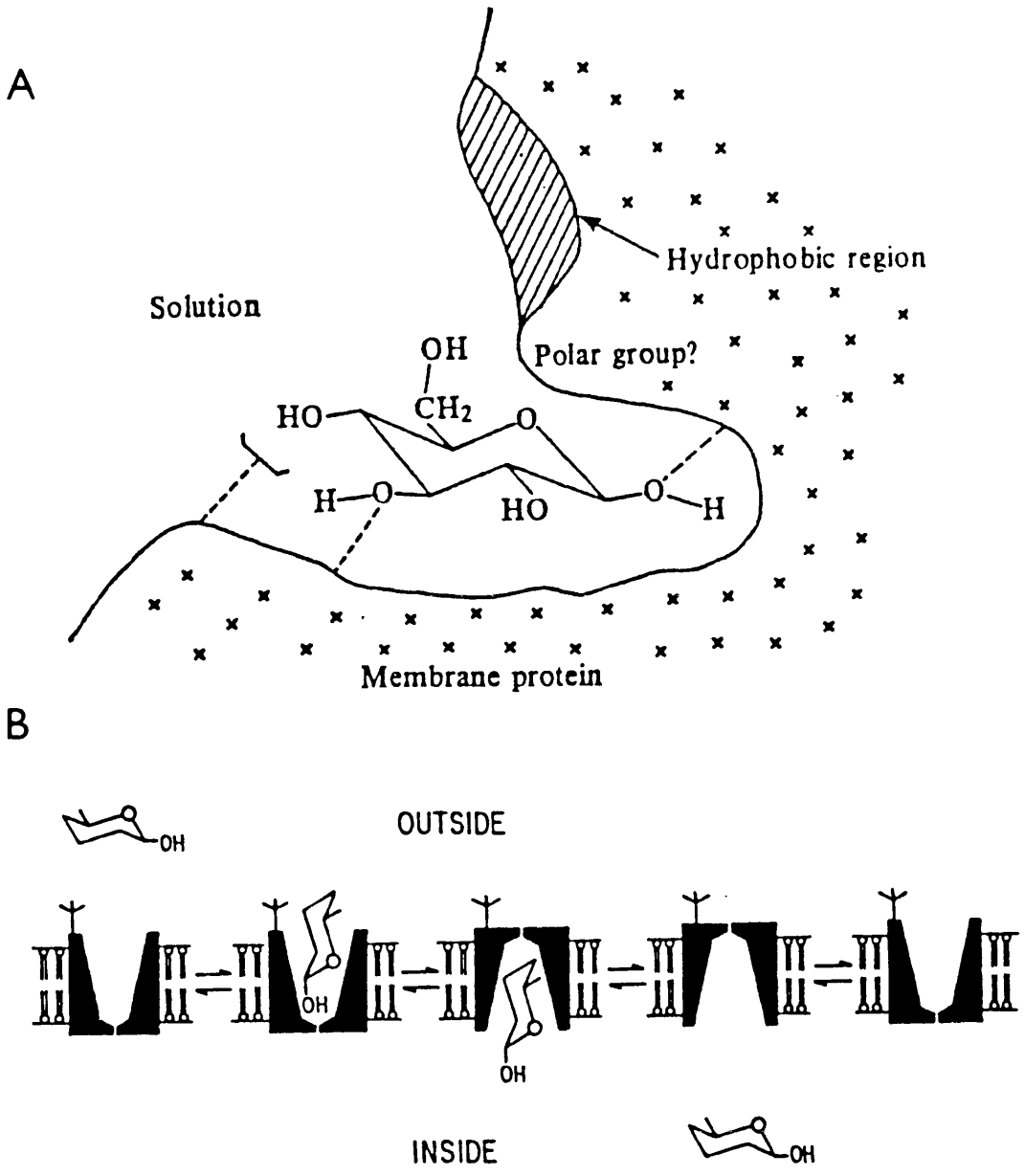



Fig.1.5 Schematic representation of glucose binding and translocation across the erythrocyte membrane

Fig.1.5(A) shows a model of the glucose-carrier complex at the outer face of the erythrocyte membrane (diagram taken from Barnett et al., 1973). Fig.1.5(B) depicts the alternating conformation model for glucose transport as proposed by Barnett et al., (1975). (Diagram taken from Baldwin and Lienhard, 1981).

undergoing conformational change. Barnett et al. (1975) provided evidence for two conformations of the transporter by examining the inhibitory effect of sugar analogues on hexose transport both into and out of human erythrocytes. These workers found that 6-O-n-propyl-D-glucose inhibited glucose transport when present outside the cell but not when present inside the cell. The fact that the presence of a bulky group at the C-6 position did not prevent competition of glucose transport (and therefore of glucose binding), suggested that this carbon atom of the sugar was not essential for interaction with the binding site on the extracellular face of the membrane. However, lack of competition at the cytoplasmic side of the membrane suggested that the C-6 hydroxyl was required for interaction with the sugar binding site facing the cytoplasm. The converse situation was found for n-propyl-β-D-glucopyranose, where the bulky group is attached to the C-1 carbon. The increasing ability of 6-O-propyl, 6-O-pentyl and 6-O-benzyl derivatives of D-glucose to compete with D-glucose outside the cell suggested that not only hydrophilic but also hydrophobic interactions were involved in the outward-facing conformation of the binding site (Barnett et al., 1975). On the basis of their findings, Barnett et al. (1975) proposed a structural model for glucose transport (Fig.1.5(B). In this model, transport occurs by binding of sugar in a fixed orientation to a transmembrane protein. Occupancy of the binding site by sugar is followed by a conformational change which translocates the sugar, still in its fixed orientation, across the membrane.

Several lines of evidence now support many features of this model. Firstly, the model suggests that the substrate binding site exists alternately at the cytoplasmic and extracellular faces of the membrane, as a result of conformational change, and therefore predicts that no complex should be identified which contains sugar bound at

both faces simultaneously. In agreement with this prediction, and therefore with the proposed model, Gorga and Lienhard (1981) using cytochalasin B and n-propyl-β-D-glucose (both of which bind to the inward-facing conformation of the protein, Deves and Krupka, 1978; Barnett et al., 1975), and 4,6-O-ethylidene-D-glucose (which binds to the outward-facing conformation, Baker and Widdas, 1973), were unable to detect any ternary complex between transporter, cytochalasin B and ethylidene-D-glucose, between transporter, cytochalasin B and propyl-β-D-glucose, or between transporter, cytochalasin B and D-glucose. Other evidence to support the lack of a ternary complex derives from more recent $^{1}H$ NMR studies by Wang et al. (1986). In this study it was shown that treatment of erythrocyte ghosts with cytochalasin B prevented D-glucose from binding to either side of the membrane.

These findings were supplemented by detection and measurement of a half-turnover of the transporter from its outward- and inward-facing forms using rapid kinetic methods (Appleman and Lienhard, 1985). In this study, a decrease in the intrinsic fluorescence resulting from the binding of ethylidene-D-glucose to the transporter was determined. This fluorescence was the result of the transporter changing conformation from its inward-facing to its outward-facing conformation. Three properties of this transient indicated that it represented a half turnover of the transporter: the first order rate constant decreased as the concentration of ethylidene-D-glucose increased; the value of the rate constant for the process is similar to that expected from steady-state kinetic studies of transport in the erythrocyte and D-glucose at a low concentration increased the rate of reaction.

Cytochalasin B itself is thought to bind the inward-facing conformation of the transporter since it is a competitive inhibitor of glucose efflux, but a non-competitive inhibitor of glucose influx

(Deves and Krupka, 1978). The inward- and outward-facing conformations are thought to be mutually exclusive since binding of ligands (such as 4,6-O-ethylidene-D-glucose), to the exofacial surface of the transporter precludes cytochalasin B binding (Deves and Krupka, 1978; Gorga and Lienhard, 1981). In contrast, ASA-BMPA (Sections 1.4 and 1.7.4), which is thought to label the outward-facing conformation of the transporter, (Holman and Rees, 1987) is an impermeable competitive inhibitor of glucose influx. Additionally, thermolysin cleavage of the transporter is promoted by cytochalasin B binding but retarded by ASA-BMPA binding (Holman and Rees, 1987, Section 1.8.3). A possible interpretation of these effects is that cytochalasin B sequesters the transporter in an inward-facing conformation which is more susceptible to thermolysin cleavage than the outward-facing conformation stabilized by ASA-BMPA. This indicates that the transporter can adopt inward- and outward-facing conformations, but not both simultaneously.

Studies involving the inactivation of the transporter with irreversible inhibitors (mainly thiol-specific reagents), also support this view of mutually exclusive conformations. Inactivation of the transporter with tetrathionate is accelerated by maltose and phloretin (binding to the outward-facing conformation), whilst cytochalasin B retards inactivation (Krupka, 1985). Reaction of an exofacial cysteine residue with an impermeant maleimide, glutathione-maleimide-I (May, 1988) irreversibly inhibited hexose entry into erythrocytes, an effect which was potentiated by phloretin and maltose binding to the outward-facing form of the transporter, but decreased by cytochalasin B binding to the inward-facing form. This indicated that in the one-site model, the accessibility of the external cysteine residue is increased in the outward-facing conformation of the carrier but that it is not actually located within the exofacial substrate binding

site. Additionally, reversible cytochalasin B binding was decreased in ghosts prepared from glutathione-maleimide-I-treated erythrocytes. Therefore stabilization of an outward-facing conformation of the transporter with this reagent caused the endofacial binding site to disappear. Many other examples of cytochalasin B protecting an exofacial thiol group have been reported (Batt et al., 1976; Roberts et al., 1982; Deziel et al., 1985).

### 1.8.3 Identification of regions of the glucose transporter sequence involved in conformational changes of the protein upon ligand binding

Although the evidence in support of a single-site alternating mechanism for glucose transport is strong, other models of greater complexity such as the two-site allosteric model (Helgerson and Carruthers, 1987) cannot be ruled out. The models are fundamentally different in their kinetics, but both imply that conformational changes are central to the molecular mechanism of transport. Although evidence of conformational changes in the mechanism of transport is also strong, identity of those regions of the protein that are affected by the changes remains largely unknown.

Inactivation of the transporter by 1-fluoro-2,4-dinitrobenzene is accelerated by transported sugars, but ligands which stabilize either conformation of the protein protect against inactivation (Krupka, 1971). This finding suggests that as the transporter changes conformation, a FDNB-sensitive site is exposed. A cysteine residue has been implicated at this site, since both chloronitrobenzoxadiazole (CNBD) and N-ethyl maleimide inactivation of the transporter are also accelerated by transported sugars (Rampal and Jung, 1987). The cysteine residue exposed during the conformational change might be at positions 347 or 421 since these are located in the areas of the protein designated as putative binding sites (located towards the

middle of helix 9 and the exofacial end of helix 11, respectively, Fig.1.4). However, exposure of $Cys_{347}$ seems more likely since the fluorescence emission spectrum of the CNBD-alkylated transporter indicates that the cysteine residue involved is located in a hydrophobic environment (Rampal and Jung, 1987). From these and other results Rampal and Jung concluded that the substrate-induced conformational changes occur primarily within the membrane-spanning regions of the transporter, while the hydrophilic regions outside remain largely unaffected. However, in a recent study, Gibbs et al. (1988) showed that the hydrophilic, cytoplasmic regions of the transporter do appear to be involved in transport-related conformational changes. This work took advantage of the finding that trypsin cleaves the transporter solely at the cytoplasmic face of the membrane (Lienhard et al., 1984; Deziel and Rothstein, 1984). It was found that in the presence of 4,6-O-ethylidene-D-glucose and phloretin, two inhibitors of D-glucose transport, the rate of tryptic cleavage at the cytoplasmic face of the transporter was slowed. Because these inhibitors bind preferentially at the extracellular face of the transporter (Baker and Widdas, 1973; Krupka, 1985), their effects must result from conformational changes within the cytoplasmic regions of the protein, rather than from steric hindrance. Such conformational changes must result in these cytoplasmic regions becoming less accessible to the enzyme. In contrast, binding of the physiological substrate, D-glucose, was found to enhance the rate of tryptic cleavage of the protein. This must reflect the presence of a conformer of the transporter in which the trypsin-sensitive cytoplasmic regions become more accessible to the enzyme. These workers concluded that the extramembranous, cytoplasmic regions encompassing residues 213-269 and 457-492 of the transporter sequence (Fig.1.3), are involved in these ligand-induced conformational

changes. Karim et al. (1987) have reported that photolabelling of the transporter with cytochalasin B (specific for a cytoplasmically-exposed site of the transporter, Deves and Krupka, 1978) increases its susceptibility to cleavage by thermolysin. However, it was not reported whether non-covalently bound cytochalasin B also has this affect. Kurokawa et al. (1986) have found that the covalent, but not the non-covalent, binding of cytochalasin B to the transporter leads to the increased exposure of an anionic domain and thus an alteration in chromatographic behaviour of the protein. Similarly, transporter photolabelled at its extracellular surface with the transport inhibitor ASA-BMPA, has been reported to be resistant to thermolytic cleavage, whereas protein labelled with cytochalasin B is readily cleaved (Holman and Rees, 1987). This result was interpreted by these workers to indicate a change in conformation of the transporter when an external ligand is bound such that the inside of the hydrogen bonding transmembrane helices 7 and 8, containing the proposed thermolytic cleavage site (Fig.1.4), are withdrawn from the cytosolic surface. Consequently, the adjacent cytoplasmic region connecting helices 6 and 7 might also be withdrawn in part and therefore become less accessible to macromolecules at the cytoplasmic surface of the membrane. This may provide a possible explanation for the above findings of Gibbs et al. (1988) that the cytoplasmic regions of the transporter are less susceptible to tryptic cleavage in the presence of bound exofacial ligands. However, Holman and Rees (1987) did not report whether the non-covalent association of cytochalasin B and ASA-BMPA with the transporter had any effect on the rate of thermolytic cleavage.

## 1.9 THE USE OF ANTIBODIES TO STUDY THE STRUCTURE AND FUNCTION OF MEMBRANE PROTEINS

Antibodies provide an alternative means to protein-chemical and physical methods in the study of membrane protein topology and function. Monoclonal antibodies (Kohler and Milstein, 1975) which bind specifically to a single protein determinant (epitope), have been used to describe the subunit organization of oligomeric complexes, topology of membrane proteins, the validity of secondary structural models and to identify regions associated with particular protein functions. Description of the subunit assembly of the nicotinic acetylcholine receptor was confirmed using monoclonal antibodies (Fairclough *et al.*, 1983). Identification of amino acid residues essential for the interaction of monoclonal antibodies with the bacteriorhodopsin of *Halobacterium halobium* (Ovchinnikov *et al.*, 1985) allowed refinements to be made to a proposed model describing the membrane topology of this chromoprotein. The location of proteolytic cleavage sites and stilbene-disulphonate-binding lysine residues (Jennings *et al.*, 1986) and the C-terminal region (Wainwright *et al.*, 1989) of the human erythrocyte anion transporter have been determined using monoclonal antibodies as structural probes.

Similarly, the production of antibodies to synthetic peptides has enabled the transmembrane topology of membrane proteins to be addressed. Vectorial binding studies using these site-specific, membrane-impermeable probes offers a means to assess protein structure predictions based on sequence data. Numerous studies have employed such an approach, for example; the lactose permease of *Escherichia coli* (Seckler *et al.*, 1983, 1986; Carrasco *et al.*, 1986), the nicotinic acetylcholine receptor (Ratnam *et al.*, 1986), the bovine heart ADP/ATP carrier (Brandolin *et al.*, 1989), the melibiose/$Na^+$

symporter of E. coli (Botfield and Wilson, 1989), the hamster $\beta_2$-adrenergic receptor (Wang et al., 1989) and synaptophysin, a major integral membrane protein of small synaptic vesicles (Johnston et al., 1989).

Detection of gold-labelled antibody on electron micrographs identified the C-terminus of the nicotinic acetylcholine receptor $\alpha$- and $\beta$-subunits as cytoplasmic (Ratnam et al., 1986). This observation highlighted inaccuracies in earlier models (Noda et al., 1983; Guy, 1983; Criado et al., 1985). Employing vesicles of known orientation and peptide antibodies specific for the C-terminus of the lactose permease of E. coli, Seckler et al. (1983) were able to describe a cytoplasmic location for this terminus, in full agreement with the proposed structural model based on sequential hydropathy analysis (Foster et al., 1983). Several later studies with peptide antibodies further support this model (Seckler et al., 1986; Carrasco et al., 1986). The model for lactose permease describes a protein consisting of twelve membrane-spanning domains and cytoplasmically-located N- and C-termini, very similar to features of the model proposed by Mueckler et al. (1985) for the human erythrocyte glucose transporter. Similarly, using identical methodology, Botfield and Wilson (1989) have recently demonstrated that the C-terminus of the E. coli melibiose/$Na^+$ symporter is located at the cytoplasmic surface of the membrane. This finding supports a topological model for the protein with ten transmembrane domains and cytoplasmic orientations for both termini (Yazyu et al., 1984) and rules out a model suggesting eleven transmembrane domains and a periplasmic location for the C-terminus (Botfield and Wilson, 1988).

Several studies have employed peptide antibodies in conjunction with proteases and impermeant chemical labels to study membrane protein topology. For example, this approach has provided further

evidence for the correctness of the model proposed by Foster et al., (1983) for the lactose permease of E. coli (Seckler et al., 1986). Labelling with the membrane impermeant probe fluorescein isothiocyanate and immunoprecipitation with solid-phase-bound peptide antibodies enabled Kyte et al. (1987) to identify a lysine residue important to the cytoplasmically-located MgATP binding site of the $\alpha$-polypeptide of the renal $Na^+/K^+$ ATPase.

In terms of membrane protein function, relatively few studies have been reported utilizing antibodies. Morgan and Roth (1986) used monoclonal antibodies as probes to study the structural basis of the insulin receptor function. Few antibodies were found to recognize the extracellular portion of the receptor, but two such examples were able to prevent insulin binding to the $\alpha$-subunit of the protein. All of the antibodies were found to cause some inhibition of tyrosine kinase activity, whilst several caused complete inhibition of both auto phosphorylation and kinase activity. Wu and Lever (1987) investigated conformation-dependant fluorescein isothiocyanate (FITC) labelling of the porcine renal $Na^+$/glucose symporter with monoclonal antibodies. FITC labelling of the symporter was enhanced in the presence of substrate and/or the monoclonal antibodies. These and other findings were consistent with a model in which the antibodies stabilize a $Na^+$-induced conformer of the protein.

Although to date no studies utilizing peptide antibodies to investigate membrane protein function have been reported, such an approach has been used to study conformational changes in a number of soluble proteins. Gariepy et al. (1986) monitored local changes in conformation between the calcium-free and calcium-saturated forms of calmodulin using peptide antibodies raised to the latter form of the protein. Binding of $Ca^{2+}$ ions was found to result in a significant (40%) decrease in immunoreactivity of calmodulin with the antibodies,

even though the $Ca^{2+}$-binding domains in both forms of the protein were known to be freely accessible to antibodies. The apparent preference of peptide antibodies for one conformer of the protein suggested that changes in the conformation of calmodulin occur in cognate sequences that are transformed by calcium from antigenic, flexible structures to less antigenic, relatively helical structures. Similarly, Tsapakos et al. (1985) used peptide antibodies in conjunction with proteases to study conformational changes in the tryptophan (trp) repressor protein of E. coli. Binding of L-tryptophan to the trp repressor protein was associated with a marked decrease in antibody reactivity that presumably accompanied a conformational change in this protein to a state with strong affinity for trp operator-bearing DNA.

### 1.9.1 Antibodies to the glucose transporter

To date, little use has been made of antibodies to study the structure and function of the glucose transporter. Polyclonal antibodies to the intact, purified human erythrocyte glucose transporter have been raised by a number of groups (Baldwin and Lienhard, 1980; Lienhard et al., 1982; Wheeler et al., 1982; Haspel et al., 1985). These antibodies were produced primarily to complement reversible and covalent [$^3$H]cytochalasin B labelling studies as a means of locating proteins homologous to the human erythrocyte transporter in other tissue types. These studies are described in Chapter 6 (Section 6.1) of this thesis. Additionally, polyclonal antibodies have been used to screen cDNA libraries for glucose transporter gene products (Mueckler et al., 1985) and in the immunoaffinity purification of transporters from other tissues, for example the adipocyte transport protein (Schroer et al., 1986).

Two groups, (Allard and Lienhard, 1985; Boyle et al., 1985), have produced monoclonal antibodies to the intact protein. Using these

antibodies, Allard and Lienhard (1985) were able to demonstrate conclusively that the glucose transporter is a protein of average apparent $M_r$ 55,000 and not of $M_r$ 100,000. A protein with the latter $M_r$, corresponding to erythrocyte 'band 3' proteins had been identified as the glucose transporter by Mullins and Langdon (1980a,b) and Shelton and Langdon (1983) on the basis of band 3 protein labelling and reconstitution studies, and by Langdon and Holman (1988) using antibodies to a preparation of band 3 proteins.

Both groups that had raised monoclonal antibodies against the glucose transporter found that their antibodies were specific for epitopes exposed at the cytoplasmic surface of the human erythrocyte membrane. Additionally, Boyle et al. (1985) showed that the antibodies did not label the large membrane-embedded fragments of $M_r$ 23,000-42,000 and $M_r$ 18,000 produced after extensive tryptic digestion of the native transporter (Cairns et al., 1984), suggesting that their epitopes are accessible to, and destroyed by, trypsin. Most of the antibodies were also found to alter the affinity of the transporter for cytochalasin B, thereby supporting other lines of evidence that this ligand binds to a site on the transporter exposed at the cytoplasmic surface of the membrane (Deves and Krupka, 1978; Baldwin et al., 1980). As a number of the monoclonal antibodies were found to inhibit cytochalasin B binding to the transporter, it was reasonable to assume that their cytoplasmic epitopes were located close to, or constitute part of, the ligand (and therefore the glucose?) binding site. The location and functional significance of the binding sites for these monoclonal antibodies is the subject of Chapter 5 of this thesis, wherein the antibodies will be discussed in detail.

## 1.10 AIMS OF THE STUDY

The objectives of the study described in this thesis were to use site-directed anti-peptide antibodies as probes to investigate the topology and to identify functionally important regions of the human erythrocyte glucose transporter. Models for the arrangement of this protein in the membrane proposed by other workers before my study began (Shanahan and D'Artel-Ellis, 1984; Deziel *et al.*, 1985; Mueckler *et al.*, 1985, Sections 1.7.1 and 1.7.3), were based largely upon *in situ* proteolytic cleavage of the native transporter or hydropathic analysis of its amino acid sequence, and little *direct* evidence for the arrangements had been published. In addition, little was known about which regions of the transport protein were important for its function. To this end, I proposed to synthesize peptides corresponding to most of the hydrophilic regions of the transporter sequence, to raise polyclonal antibodies against them, and then to use these as membrane-impermeant probes of structure and function. In addition I hoped using both synthetic peptides and other approaches, to identify the epitopes for a number of monoclonal antibodies which had already been raised against the intact transporter. It was known to which side of the membrane these antibodies bound, and the effects of some of them on transporter function had been investigated. Finally, I hoped to be able to use the anti-peptide antibodies to investigate how widely distributed the erythrocyte-type glucose transporter is in mammalian tissues. The findings of such a study would indicate how significant my studies on the structure and function of the erythrocyte transporter were likely to be for glucose uptake into mammalian cells in general.

# CHAPTER 2 MATERIALS AND METHODS

All chemicals and biochemicals were obtained from the Sigma Chemical Company (Poole, Dorset, U.K.) or BDH Chemicals (Poole, Dorset, U.K.) unless otherwise stated.

## 2.1 PREPARATION OF MEMBRANES

### 2.1.1 Erythrocyte membrane ghosts

All operations were performed at 4°C. Erythrocytes from 1 unit of outdated blood (obtained from the blood bank of the Royal Free Hospital), were washed free of plasma components by diluting to 1200ml in phosphate-buffered saline (PBS, 5mM sodium phosphate, 145mM NaCl, pH 8.0), and centrifuging at 4,500 rev/min for 10 minutes using a 6x250ml GSA rotor in a Sorvall RC-5B centrifuge. The resulting supernatant was discarded and the erythrocytes resuspended in more PBS, pH 8.0. Two or three further wash cycles were carried out, the layer of white cells on top of the pellet being removed by aspiration after each spin.

Erythrocyte membrane ghosts were prepared from the washed cells by the method of Steck and Kant (1974). Erythrocytes were lysed by suspension in 20 volumes of 5mM sodium phosphate, pH 8.0 (5P8). The membrane ghosts were sedimented by centrifugation at 11,500 rev/min for 20 minutes and the supernatant discarded. By a process of resuspension and centrifugation under these conditions a preparation of erythrocyte membranes almost free of haemoglobin was obtained. Any white cells, which had not been removed at the cell washing step, formed an easily removed pellet during this stage. The membranes were assayed for protein by the method of Lowry *et al.* (1951).

Alternatively, washed erythrocytes were lysed in 20 volumes of

5P8, containing 1mM EDTA and 0.11 mM phenylmethylsulphonyl fluoride (PMSF) and freed from haemoglobin by tangential flow ultrafiltration through a Pellicon cassette system (Millipore Corp., Bedford, M.A., U.S.A.), using a 0.4μm (coarse screen) durapore cassette filter at a pressure of 400 p.s.i. The resultant ghosts were concentrated by centrifugation at 11,500 rev/min for 30 minutes and any visible pellet of white cells was removed by aspiration. The membranes were assayed for protein as above.

2.1.2 'Right-side-out' and 'inside-out' erythrocyte membrane vesicles

All operations were performed at 4°C. Erythrocyte membrane ghosts were prepared as described in Section 2.1.1. Impermeable erythrocyte membrane vesicles of known orientation were prepared from the ghosts as described by Steck (1974) and Steck and Kant (1974).

For the preparation of 'right-side-out' vesicles, samples of ghosts (at 3mg protein/ml), were diluted in 40 volumes of 0.5mM sodium phosphate, pH 8.0 (0.5P8), and incubated on ice for 60 minutes. The ghost samples were then made 0.1mM in $MgSO_4$ and centrifuged at 15,000 rev/min for 30 minutes in a Sorvall SS-34 rotor. The supernatants were aspirated and the pellets resuspended to 1ml with 0.5P8, 0.1mM $MgSO_4$ by vortex mixing. Each sample was homogenized by gentle passage four times through a 27 gauge needle and stored in a cold room overnight. The samples were then combined and diluted with 0.5P8 to give a protein concentration of about 1mg/ml.

For the preparation of 'inside-out' vesicles, samples of ghosts as above were diluted in 40 volumes of 0.5P8 and incubated on ice for 30 minutes. The ghosts were centrifuged as above, the supernatants aspirated and the pellets stored moist in the cold room overnight. Each pellet was then resuspended to 1ml with 0.5P8, homogenized as above and diluted in 0.5P8 to about 1mg protein/ml.

The sealed 'right-side-out' and 'inside-out' vesicles were then separated from unsealed membrane fragments on a density gradient of Dextran T70 (Pharmacia Fine Chemicals, Hounslow, Middx., U.K.). Samples (5ml), of the vesicle preparations were layered onto the surface of 8ml 8% (w/v) Dextran T70 (density = 1.028g/ml), in 15ml centrifuge tubes. The samples were centrifuged for 2.5 hours at 25,000 rev/min (approx. 75,000xg, $r_{av}$.8.4cm), using a 6x15ml swing-out rotor in a MSE Prepspin 75 ultracentrifuge. The material floating on the interface between the two layers was collected. The vesicles were freed of dextran by 2 cycles of washing with 0.5P8 and centrifugation at 15,000 rev/min for 30 minutes. They were then assayed for protein by the method of Lowry et al. (1951).

The sidedness of the vesicle preparations was assessed by measuring the accessibility of acetylcholinesterase (AChase) as an extracellular surface marker and glyceraldehyde-3-phosphate dehydrogenase (G-3-PD), as a cytoplasmic surface marker, as described by Steck and Kant (1974). For AChase assay, 0.1ml samples of vesicles containing 2.5-5µg of protein were diluted to 0.2ml with either 5P8, or 5P8 containing 0.2% (v/v) Triton X-100 in 1ml glass cuvettes. The following reagents were then added sequentially to the cuvettes; 1.3ml 100mM sodium phosphate, pH 7.5, 0.1ml 10mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.1ml 12.5mM acetylthiocholine chloride, and the reaction of liberated thiol groups with DTNB followed spectrophotometrically at 412nm using a Pye Unicam SP-1800 U.V./visible spectrophotometer and chart recorder. The % enzyme accessibility was calculated from the change in $A_{412nm}$-detergent/$A_{412nm}$+detergent.

For G-3-PD assay, 0.1ml samples of vesicles containing 50µg protein were diluted to 0.2ml with 5P8 or 5P8 containing 0.2% (v/v) Triton X-100 in 1ml glass cuvettes. The following reagents were then

added sequentially to the cuvettes; 0.62ml 30mM sodium pyrophosphate, pH 8.4, 30µl 0.4M sodium arsenate, 50µl 20mM β-nicotinamide adenine dinucleotide (β-NAD), 0.1ml 15mM glyceraldehyde-3-phosphate, pH 7.0, (freshly prepared from DL-glyceraldehyde-3-phosphate diethylacetal-monobarium salt treated with Dowex 50 hydrogen-form resin), and the reduction of NAD to NADH followed spectrophotometrically at 340nm using the spectrophotometer described above. The % enzyme accessibility was calculated from the change in $A_{340nm}$-detergent/$A_{340nm}$+detergent.

2.1.3 Protein-depleted erythrocyte membranes

All operations were performed at 4°C. Erythrocyte membrane ghosts were stripped of their peripheral proteins as described by Gorga and Lienhard (1981). Erythrocyte ghosts (45ml at 4mg protein/ml in 5P8), were added to 240ml of an ice-cold solution containing 2mM EDTA, 15.4mM sodium hydroxide and 0.2mM dithiothreitol (DTT), which had been purged with nitrogen for 20 minutes. After 10 minutes in the resultant pH 12 conditions, the peripheral proteins were separated from the membranes by repeated centrifugation at 18,000 rev/min for 20 minutes. The membranes were resuspended between each spin with 50mM Tris-HCl, pH 6.8, and finally homogenized and assayed for protein by the method of Lowry et al. (1951).

2.1.4 Purification of the glucose transporter

The purified glucose transport protein was prepared by the method of Cairns et al. (1984). Octyl-β-D-glucopyranoside (octyl glucoside, Calbiochem-Behring, La Jolla, C.A., U.S.A.), was added to protein-depleted erythrocyte membranes giving a mixture containing 2mg/ml protein in 46.5mM Tris-HCl, pH 6.8, containing 2mM DTT and 1.35% (w/v) octyl glucoside. After shaking on ice for 20 minutes, the soluble

extract was separated from the insoluble membranous components by centrifugation at 45,000 rev/min for 1 hour at 4°C using a MSE 8x25ml rotor and Prepspin 75 ultracentrifuge. The extract was made 25mM in NaCl before subjection to chromatography on a column of DEAE-cellulose (DE-52, Whatman Chemical Separation Ltd., Maidstone, Kent, U.K.), equilibrated with 47.5mM Tris-HCl, pH 6.8, containing 2mM DTT, 25mM NaCl and 1% (w/v) octyl glucoside. Eluted protein fractions were detected by their absorbance at 280nm (using a Pye Unicam SP6-500 U.V./visible spectrophotometer), pooled and reconstituted by extensive dialysis against 50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH 7.4. The final product was assayed for protein by the method of Lowry et al. (1951), checked for purity by SDS/polyacrylamide gel electrophoresis (Section 2.3) and cytochalasin B binding activity measured (Section 2.5.1).

### 2.1.5 Intact erythrocytes

Erythrocytes were washed in PBS, pH 8.0 as described in Section 2.1.1 and cell numbers determined using a haemocytometer or by automatic counting of a 1:100,000 dilution of packed erythrocytes in PBS, pH 8.0 using an Accucomp C-1000 Coulter counter (Coulter Electronics Ltd., Luton, Bedfordshire, U.K.).

## 2.2 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA plates (96-well, Nunc Maxisorp, Nunc, Kamstrup, Denmark), were coated with purified glucose transporter (600ng per well) in 50mM sodium carbonate buffer, pH 9.6, overnight at room temperature. Synthetic peptides (1mg/ml in dimethylsulphoxide (DMSO), were diluted to a working concentration of 200ng/ml in 50mM sodium carbonate, pH 9.6 and 0.1ml aliquots were added to the wells of an ELISA plate

(giving 20ng peptide per well). The peptide-containing plates were dried in vacuo overnight over sodium hydroxide pellets before use. The wells were then washed 5 times with a buffer comprising 136mM NaCl, 2.7mM KCl, 1.6mM $Na_2HPO_4$, 1.2mM $KH_2PO_4$, 0.02% (w/v) sodium azide and 0.05% (v/v) Tween-20, pH 7.2 (PBSA-T). Unoccupied protein-binding sites were blocked by the addition of 0.2ml blocking buffer (PBSA-T + 1% (w/v) bovine serum albumin (BSA)) with incubation for 2 hours at room temperature. Wells were again washed 5 times with PBSA-T, after which 0.1ml aliquots of 2 or 3-fold serial dilutions of antisera or supernatants from competitive ELISA experiments (Section 3.3.1.3), all in PBSA + 1% BSA without Tween-20, were added to the wells and incubated overnight at room temperature. After washing five times with PBSA-T, 0.1ml of a 1:3000 dilution in PBSA-T of goat anti-rabbit or rabbit anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad Laboratories, Watford, Herts., U.K.), was added and incubated for 2 hours at room temperature. After washing the wells with PBSA-T, the substrate p-nitrophenyl phosphate (Sigma 104 phosphatase substrate) at 1mg/ml in 10mM diethanolamine buffer, pH 9.8, containing 1mM $MgCl_2$, was added at 0.1ml/well. The plates were then incubated at room temperature until a yellow colour had formed which was measured at 405nm using a Bio-Rad model 2550 EIA plate reader. The readings were repeated at several time points until a distinct absorbance range could be seen for each group of dilutions.

## 2.3 SDS/POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecylsulphate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970), using 1.5 or 3mm-thick 10 and 12% acrylamide slab gels. All materials were 'Electran' grade from BDH or electrophoresis purity reagents from

Bio-Rad.

Protein samples were solubilized in a sample buffer (40mM Tris-HCl, pH 6.8, 0.8mM EDTA, 0.8% (w/v) SDS, 4mM DTT, 10% (v/v) glycerol and 0.12% (w/v) pyronin Y) and incubated for 1 hour at room temperature before storage at -20°C. When protein samples contained Triton X-100 the amount of SDS used in the sample buffer was increased to 2% (w/v). Aliquots of the protein samples (10-125µl containing 5-50µg protein for 1.5mm thick-gels and 50-250µl containing 50-120µg protein for 3mm-thick gels), were loaded onto slab gels consisting of a 2cm stacking gel (3% acrylamide/0.08% bisacrylamide in a buffer of 125mM Tris-HCl, pH 6.8 plus 0.1% (w/v) SDS, polymerised by 0.1% (w/v) ammonium persulphate and 0.05% (v/v) tetramethylethylenediamine (TEMED)), and a 10cm separating gel (10 or 12% acrylamide/0.27 or 0.32% bisacrylamide in a buffer of 375mM Tris-HCl, pH 8.8 plus 0.1% (w/v) SDS, polymerised by 0.1% (w/v) ammonium persulphate and 0.016% (v/v) TEMED). Molecular weight markers used were a low molecular weight range preparation ($M_r$ range 14,400-97,400) from Bio-Rad, a Coomassie blue-prestained marker preparation ($M_r$ range 17,000-130,000) also from Bio-Rad, used when samples from the gel were to be transferred onto nitrocellulose (Section 2.4), or a marker mix prepared by dissolving equal amounts of BSA ($M_r$ 66,000), ovalbumin ($M_r$ 45,000), G-3-PD ($M_r$ 36,000), carbonic anhydrase ($M_r$ 29,000), soybean trypsin inhibitor ($M_r$ 20,100) and cytochrome c ($M_r$ 12,400), in gel sample buffer. The electrode buffer comprised 25mM Tris, 190mM glycine and 0.1% (w/v) SDS, pH 8.3. Electrophoresis was performed at 15mA (constant current) through the stacking gel and 30mA through the separating gel for 1.5mm gels and twice these values for 3mm gels, using a Bio-Rad Protean Mk I electrophoresis cell and Pharmacia EPS 500/400 power supply. The gels were run until the pyronin Y marker had migrated to within 0.5cm of the gel base or until the dye front was

9cm from the start of the separating gel if the gels were to be used in electrotransfer experiments.

For Coomassie blue staining of the proteins, the gels were soaked overnight in 10% acetic acid/25% isopropanol, soaked for 12 hours in 10% acetic acid/25% isopropanol/0.025% Coomassie Page Blue 83 (BDH), a further 12 hours in 10% acetic acid/10% isopropanol/0.0025% Coomassie Page Blue 83 and destained in 10% acetic acid. Weakly-stained Coomassie blue gels were restained by the more sensitive silver staining method of Morrisey (1981). The gel, in 10% acetic acid, was rinsed with distilled water and soaked in 10% glutaraldehyde for 30 minutes. It was rinsed again with distilled water and then soaked in 5µg/ml DTT for 30 minutes. The DTT was aspirated and the gel soaked in 0.1% (w/v) silver nitrate in water for 30 minutes. The silver nitrate was aspirated, and then the gel rinsed with water followed by a small volume of developing solution (0.3% (w/v) sodium carbonate containing 0.02% formaldehyde). The gel was developed in this solution until the protein bands had reached the required intensity when 2.3M citric acid was added to prevent further colour development. Gels were photographed (by the Dept. of Medical Illustration, Royal Free Hospital) and scanned as required at 530nm (Coomassie Blue-stained gels), or 600nm (silver-stained gels), using a Chromoscan 3 gel scanner (Joyce Loebl, Gateshead, Tyne and Wear, U.K.).

## 2.4 ELECTROTRANSFER AND IMMUNOBLOTTING

Proteins were transferred to nitrocellulose paper essentially as described by Towbin *et al.* (1979). SDS/polyacrylamide gel electrophoresis was performed as described in Section 2.3. The gels were removed from the glass plate supports and equilibrated in transfer buffer (38mM glycine, 48mM Tris, 0.0375% (w/v) SDS and 20%

(v/v) methanol) at room temperature for 20 minutes. Each gel was laid on top of a 15x9.2cm sheet of nitrocellulose paper (0.45nm pore size, Bio-Rad) avoiding trapped air. This was then sandwiched between filter papers soaked in transfer buffer arranged between the electrode plates of a LKB NovaBlot electrophoretic transfer apparatus set up within a LKB Multiphor II electrophoresis tank. Electrotransfer was performed using a constant current of 1.6mA per $cm^2$ of gel from a LKB Macrodrive I power supply at room temperature for 1 hour. Following transfer, protein was detected on appropriate pieces of nitrocellulose with 0.1% (w/v) amido black, 25% isopropanol, 10% acetic acid and destained with 10% acetic acid, to ensure that transfer had occurred and to locate marker proteins when necessary. For immunoblotting, each sheet of nitrocellulose was placed in a separate glass tank and incubated with shaking in Tris-buffered saline (TBS, 20mM Tris-HCl, 500mM NaCl, pH 7.5), for 10 minutes. The TBS was aspirated and the nitrocellulose was then incubated in blocking buffer (TBS containing 5% low fat dried milk powder and 0.2% sodium azide) for 1 hour at room temperature to block remaining protein binding sites on the membranes. The blocking buffer was aspirated and the nitrocellulose washed twice in TBS containing 0.2% (v/v) Tween-20 (TTBS). Each sheet of nitrocellulose was then incubated overnight at room temperature with one of the following primary antibodies diluted in antibody buffer (TBS containing 1% low fat dried milk powder and 0.2% sodium azide); anti-transporter/anti-peptide sera (1:500 dilution), monoclonal ascitic fluid (1:1000-1:100,000 dilution), affinity purified anti-transporter/anti-peptide antibodies/ anti-transporter monoclonal antibodies (2-10µg/ml). The antibodies were aspirated and the nitrocellulose washed twice with TTBS and once with TBS. Bound primary antibody was then detected with a second antibody conjugated to either alkaline phosphatase or $^{125}I$. For the alkaline phosphatase method, the

nitrocellulose was incubated with a 1:3000 dilution of goat anti-rabbit or rabbit anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad) in antibody buffer for 2 hours at room temperature. The nitrocellulose was then washed twice with TTBS and once with TBS. Substrate (0.37mM nitro-blue tetrazolium, 0.35mM 5-bromo-4-chloro-3-indolyl phosphate in 0.1M sodium bicarbonate, 1mM $MgCl_2$, pH 9.8), was next added and colour allowed to develop until strong purple bands were present. Development was halted by aspiration of the substrate solution and repeated washing of the nitrocellulose in distilled water. For the $^{125}I$ method, the nitrocellulose was incubated with 0.25μCi/ml of donkey anti-rabbit IgG $^{125}I$-$F(ab')_2$ conjugate (Amersham, Bucks., U.K.) in antibody buffer for 2 hours at room temperature. The nitrocellulose was then washed three times for 15 minutes in TTBS and then allowed to dry overnight between filter papers. The dried nitrocellulose was mounted on 3MM filter paper and wrapped in cling film. The mounted nitrocellulose was placed on top of a 13x18cm sheet of Kodak X-Omat S film in a light-resistant film cassette containing a Hi-speed X intensifying screen and the film exposed to the nitrocellulose for 48 hours at -70°C. The resultant autoradiogram was developed using facilities in the X-ray Dept. of the Royal Free Hospital.

## 2.5 LIGAND BINDING ASSAYS

### 2.5.1 Cytochalasin B

The binding of cytochalasin B to the purified glucose transporter and protein-depleted erythrocyte membranes was measured in triplicate by equilibrium dialysis using [4-$^3H$]cytochalasin B (New England Nuclear, Southampton, Hants., U.K.), essentially as described by Zoccoli *et al.* (1978).

The binding of cytochalasin B to the glucose transporter can be expressed as:-

$$R + L \rightleftharpoons RL \qquad \text{Eq. 2.1}$$

$$K_d = [R]\,[L]/[RL] \qquad \text{Eq. 2.2}$$

where [R], [L] and [RL] are the equilibrium concentrations of free glucose transporter, free ligand (cytochalasin B) and bound ligand respectively and $K_d$ is the dissociation constant. Therefore, in a mixture of the glucose transporter and cytochalasin B at equilibrium, the bound to free ligand ratio can be given as:-

$$\text{bound/free} = [RL]/[L] = [R]/K_d \qquad \text{Eq. 2.3}$$

[R], the free glucose transporter concentration, is related to the total glucose transporter concentration by:-

$$[R]_T = [R] + [RL] \qquad \text{Eq. 2.4}$$

where $[R]_T$ is the total glucose transporter concentration and [RL] the bound concentration. Substituting into Equation 2.3 therefore yields Equation 2.5:-

$$B/F = [RL]/[L] = [R]_T/K_d - [RL]/K_d \qquad \text{Eq. 2.5}$$

If the B/F ratio is measured using cytochalasin B at $4\times10^{-8}$M, which is less than the $K_d$ of 0.12μM (Zoccoli *et al.*, 1978), [RL] can never be greater than $0.25[R]_T$ and so as an approximation Equation 2.5 becomes:-

$$B/F = [R]_T/K_d \qquad \text{Eq. 2.6}$$

Therefore the ratio of bound to free cytochalasin B measured using low cytochalasin B concentrations is a good measure of the concentration of ligand binding sites.

The apparatus used for the cytochalasin B binding assay was similar to that described by Uhlenbeck (1972) and Zoccoli et al. (1978). Equilibrium dialysis racks consisted of two pieces of perspex clamped together by bolts and wing nuts. Each half of the rack had six circular chambers of 50µl capacity which were aligned with the other half when the apparatus was sealed. Between the two halves, a small piece of dialysis membrane separated the solutions in each chamber.

[4-$^3$H]Cytochalasin B (specific radioactivity 7-15Ci/mmol), was diluted to $8\times10^{-6}$M with ethanol and stored at -20°C. For binding assays, the $8\times10^{-6}$M stock of cytochalasin B was diluted to $8\times10^{-8}$M with the same buffer as the protein samples to be assayed. Into one side of each dialysis rack chamber was loaded 40µl $8\times10^{-8}$M [4-$^3$H]cytochalasin B. Into the other side of the chamber was loaded 40µl of protein sample. The top of the chamber was sealed to prevent evaporation and the racks shaken for 18 hours at room temperature on a rotary shaker. A 25µl sample was then removed from each chamber and added to 4ml of Cocktail T (BDH) for liquid scintillation counting. To determine non-specific binding of cytochalasin B, protein samples were heated to 100°C for 5 minutes before assaying.

Calculation of bound to free ratios for cytochalasin B were performed as follows:-

Let X be the radioactivity (in cpm) measured by sampling the half of the dialysis chamber containing protein - this was contributed by both bound and free ligand.

$$X = RL + L$$

Let Y be the radioactivity (in cpm) measured by sampling the other half of the chamber - this was contributed by free ligand alone, and since these are equilibrium conditions, it must be identical to the radioactivity contributed by the free ligand in the protein-containing half of the chamber.

$$Y = L$$

In order to convert cpm's to concentrations, the total added cytochalasin B has to be taken into consideration.

$$[RL] + [L] = [\text{cytochalasin B}] \cdot X/(X + Y) \qquad \text{Eq. 2.7}$$

$$\text{and } [L] = [\text{cytochalasin B}] \cdot Y/(X + Y) \qquad \text{Eq. 2.8}$$

Substituting Equation 2.8 into Equation 2.7:-

$$[RL] = [\text{cytochalasin B}] \cdot (X - Y)/(X + Y) \qquad \text{Eq. 2.9}$$

Since bound/free = [RL]/[L]

$$\text{then, bound/free} = (X - Y)/Y = X/Y - 1 \qquad \text{Eq. 2.10}$$

From Equation 2.10 it can be seen that a measure of the concentration of cytochalasin B binding sites (i.e. B/F) can be calculated by dividing the cpm on the side of the protein by the cpm on the ligand side and subtracting 1.

For routine cytochalasin B binding assay, binding was carried out

at a single, low concentration of [4-$^{3}$H]cytochalasin B ($4x10^{-8}$M). In order to keep compositions of the solutions on either side of the dialysis membrane similar, cytochalasin B was loaded into only one half of the dialysis chamber and allowed to diffuse across giving final concentrations of $4x10^{-8}$M for cytochalasin B and 0.5% (v/v) for ethanol. Assays carried out on erythrocyte ghost membranes included, in addition, $1.25x10^{-5}$M cytochalasin E to inhibit cytochalasin B binding to cytoskeletal sites not associated with glucose transport.

In order accurately to determine the concentration of cytochalasin B binding sites and measure the dissociation constant for binding to glucose transporter and erythrocyte membranes, binding assays were carried out over a range of cytochalasin B concentrations ($0.5x10^{-7}$M - $75.5x10^{-7}$M) and the results analysed by the method of Scatchard (1949). If a single set of binding sites exists, it follows from Equation 2.5 that a plot of [RL]/[L], or bound/free, against [RL], (bound ligand), will be a straight line. From the gradient of the line ($-1/K_d$) the $k_d$ for binding can be obtained and the intercept on the abscissa yields a value for the total concentration of binding sites, $[R]_T$. To construct such plots, [RL]/[L] and [RL] were determined from the measured radioactivities in each half of the dialysis chamber using Equations 2.10 and 2.9 respectively. Non-specific binding of cytochalasin B was again determined by heating samples to 100$^{o}$C for 5 minutes before assay. The data was analysed using the LIGAND curve-fitting method of Munson and Rodbard (1980).

### 2.5.2 Nitrobenzylthioinosine (NBMPR)

The binding of NBMPR to the nucleoside transporter component of the purified glucose transporter preparation was measured in triplicate by equilibrium dialysis using [Benzyl-$^{3}$H]NBMPR essentially as described by Jarvis and Young (1981).

[Benzyl-$^{3}$H]NBMPR (26Ci/mmol) was obtained from New England Nuclear and diluted to give a stock solution of 3.8μM with methanol. A single, saturating concentration (60nM) of NBMPR was used for binding measurements using the equilibrium dialysis methodology described in Section 2.5.1. To correct for non-specific binding, parallel experiments were performed in the presence of 20μM nitrobenzylthioguanosine (NBTGR). NBMPR binding activity (pmol/ml) was determined in the following way:-

a) In the absence of NBTGR.

Let X = radioactivity (in cpm) on protein side of dialysis chamber and let Y = cpm's on the ligand side of the chamber.

Then X = bound [NBMPR] + free [NBMPR]

Y = free [NBMPR]

(X-Y) = bound [NBMPR] Eq. 2.11

Some of this bound NBMPR will be non-specifically bound, especially to the membrane lipids, and this non-specific binding is known to be a non-saturable linear function of the concentration of free NBMPR (Y). It can therefore be represented as a constant (P) multiplied by the [free NBMPR], i.e. PY. Thus:-

Specific bound = (X-Y) - PY Eq. 2.12

The value of P can be determined by measuring the binding of NBMPR in the presence of a saturating concentration of the competing, unlabelled, high affinity ligand NBTGR, as described below.

b) In the presence of NBTGR.

Let W = radioactivity (in cpm) on protein side of the dialysis chamber (bound [NBMPR] + free [NBMPR]) and let Z = cpm's on the ligand side of the chamber (free [NBMPR]).

$$(W-Z) = \text{bound [NBMPR] (non-specific)} \qquad \text{Eq. 2.13}$$

As described above, this non-specific binding is a linear function of [free NBMPR], i.e.:-

$$(W-Z) = P\,[\text{free}] = PZ \qquad \text{Eq. 2.14}$$

Therefore,

$$P = (W-Z)/Z \qquad \text{Eq. 2.15}$$

Substituting Equation 2.15 into Equation 2.12, we get:-

$$\text{Specific bound cpm} = (X-Y) - (W-Z)(Y/Z) \qquad \text{Eq. 2.16}$$

Since (X+Y) = initial NBMPR concentration = 60nM (or 60pmol/ml)

$$\text{Specific bound NBMPR (pmol/ml)} = \frac{[(X-Y) - (W-Z)(Y/Z)]}{(X+Y)} \cdot 60 \qquad \text{Eq. 2.17}$$

## 2.6 ENZYME DIGESTIONS

### 2.6.1 Trypsin and Chymotrypsin

Solutions of trypsin and chymotrypsin in 1mM HCl were freshly prepared just before use. Incubations were carried out in a shaking water bath. Purified glucose transporter at a protein concentration of 100-250μg/ml in 50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH 7.4 was incubated at 25°C with 1% (w/w) diphenylcarbamyl chloride-treated trypsin for various periods of time up to 3 hours. Digestion was halted by addition of 2% (w/w) bovine lung aprotinin and samples prepared for SDS/polyacrylamide gel electrophoresis (Section 2.3).

More extensive digestion of the glucose transporter was achieved by incubation at 37°C with 5% (w/w) diphenylcarbamyl chloride-treated trypsin or 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK)-treated chymotrypsin for 7 hours and digestion halted by addition of 1mM TLCK or 0.1mM PMSF respectively.

### 2.6.2 Endo-β-galactosidase/Endoglycosidase F

Endo-β-galactosidase from *Bacteroides fragilis* (Scudder *et al.*, 1983, 1984), was kindly provided by Dr P. Scudder (Clinical Research Centre, Harrow, Middx., U.K.). The enzyme preparation had been assayed for activity using bovine corneal keratin sulphate (Scudder *et al.*, 1983). It had an activity of 5.6 units/ml, where 1 unit is that amount of enzyme catalysing the release of 1μmol of reducing power as galactose per min under the conditions of assay (Fukuda and Matsumura, 1976). Endoglycosidase F (Endo-β-N-acetylglucosaminidase F, EC 3.2.1.96) from *Flavobacterium meningosepticum* (Elder and Alexander, 1982), was obtained from Boehringer Mannheim (Lewes, Sussex, U.K.). The enzyme had a specific activity of 600 units/mg enzyme protein, where 1 unit is the enzyme activity which hydrolyses 1μmol of dansyl-

Asn-$(GlcNAc)_2(Man)_5$ within 60 minutes at 37°C.

Purified glucose transporter and protein-depleted erythrocyte membranes at a protein concentration of 2mg/ml in 100mM sodium phosphate, 50mM EDTA, 75mM 2-mercaptoethanol, pH 6.1, were incubated simultaneously with 0.3units/ml of endo-β-galactosidase and 4units/ml of endoglycosidase F in the absence of detergent at 25°C for 24 hours. Control samples of glucose transporter and membranes were treated under identical conditions but the enzymes were omitted. Digestion was halted by the addition of denaturing buffer (85mM Tris, pH 6.8, 1mM EDTA, 5% SDS) to samples of the digest used for SDS/polyacrylamide gel electrophoresis, but the enzymes were not inactivated in samples of transporter and membranes to be used in ELISA experiments, as the latter were required in as near a native, functionally-active form as possible. Samples of the digests were dialysed into 50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH 7.4 prior to cytochalasin B binding assay and ELISA.

## 2.7 PROTEIN DETERMINATION

Protein concentrations were determined by the method of Lowry et al. (1951) or the precipitation Lowry method of Peterson (1977), when substances interfering with the standard Lowry were present.

Solutions used in the standard Lowry were; (1) Solution A : 2% (w/v) sodium carbonate in 0.1M sodium hydroxide, (2) Solution B : 1% (w/v) copper sulphate (pentahydrate) in water, (3) Solution B* : 2% (w/v) sodium/potassium tartrate in water, (4) Solution C : 0.2ml solution B and 0.2ml solution B* added to 20ml of solution A containing 0.5% (v/v) SDS, and (5) Solution D : Folin's reagent diluted 1:1 with water. 1ml of solution C was added to 0.2ml of either

samples or protein standards containing 0-50µg protein. The samples were vortexed and incubated at room temperature for 15 minutes. Solution D (0.1ml), was then added to each sample with immediate vortexing. After 30 minutes the absorbances of the solutions at 750nm were measured. The amount of protein in the unknown samples was determined by comparison of the absorbance with a standard curve constructed using duplicate samples of 0, 12.5, 25, 37.5, and 50µg of BSA.

Solutions used in the precipitation Lowry were; (1) Solution A, 10% (w/v) sodium carbonate, 0.2% (w/v) potassium tartrate, 0.1% (w/v) copper sulphate in water, (2) Solution B : 10% (w/v) SDS, (3) Solution C : 0.8M sodium hydroxide, (4) Solution D : prepared from equal volumes of solutions A-C and water, and (5) Solution E : Folin's reagent diluted 1:1 with water.

For the precipitation step samples containing 5-50µg protein were adjusted to a volume of 1ml with water and solubilized with 0.1ml 0.15% (w/v) sodium deoxycholate for 10 minutes at room temperature. 0.1ml of 72% (w/v) trichloroacetic acid was added and the samples centrifuged at top speed in a bench top centrifuge for 15 minutes. The supernatants were immediately decanted and the tubes inverted over absorbant paper. Each protein-containing pellet and a series of protein standards (as above) were resuspended to 1ml with distilled water. Solution D (1ml), was then added and the samples were vortexed and incubated for 10 minutes at room temperature. Solution E (0.5ml), was then added to each sample with immediate vortexing. After 30 minutes the absorbance at 750nm was measured and protein determined from comparison with a standard curve constructed using samples of 0-50µg BSA.

## 2.8 COLORIMETRIC ASSAY FOR SULPHYDRYL GROUPS (Ellman, 1959)

10mM DTNB was prepared in 100mM sodium phosphate buffer, pH 7.5, containing 18mM sodium bicarbonate. 67µl of 10mM DTNB were mixed with 0.6ml 100mM sodium phosphate, pH 7.5 and then water and the SH-containing sample to be assayed were added so that the final sample volume was 1ml. After 10 minutes the absorbance at 412nm was measured. The value for a blank solution (water only added to the DTNB/phosphate mixture) was subtracted from the test $A_{412nm}$ measurements. Under these conditions 0.05µmol of SH groups yielded an absorbance of 0.705.

# CHAPTER 3 PRODUCTION, PURIFICATION AND CHARACTERIZATION OF ANTI-PEPTIDE ANTIBODIES TO THE GLUCOSE TRANSPORTER

## 3.1 INTRODUCTION

Antibodies are central to the humoral defence mechanism of vertebrates and are secreted by B-lymphocytes upon stimulation by an appropriate antigen. Individual B-lymphocytes have the capacity to synthesize and secrete a single species of immunoglobulin of predetermined specificity (Burnet, 1957; Nossal and Lederberg, 1958).

Five classes of immunoglobulin (IgG, IgM, IgA, IgD, IgE) exist, of which IgG and IgM predominate in the circulation. The structure of an IgG molecule is shown schematically in Fig.3.1. It is comprised of two identical polypeptides of $M_r$ 53,000 (heavy chains) and two polypeptides of $M_r$ 23,000 (light chains). Each IgG molecule has two antigen binding sites (paratopes, of identical specificity) located in the hypervariable domain of the heavy and light chains ($V_H$ and $V_L$ in Fig.3.1).

The production of antibodies to a foreign protein involves the cooperation of three cell types, antigen presenting cells (e.g. macrophages), T-lymphocytes and B-lymphocytes. B-lymphocytes secrete the antibody, but require the interaction of the other cell types to regulate proliferation and maturation to antibody-secreting status. Intact or denatured foreign protein can be recognized in free solution by macrophages and B-lymphocytes. Both cell types ingest and degrade the protein, presenting small fragments on their cell membrane in association with class Ia HLA molecules (proteins of the major histocompatibility complex). T-lymphocytes recognize the protein fragments in association with Ia, and proliferate. Subsequent interaction of T-lymphocytes with B-lymphocytes bearing the same

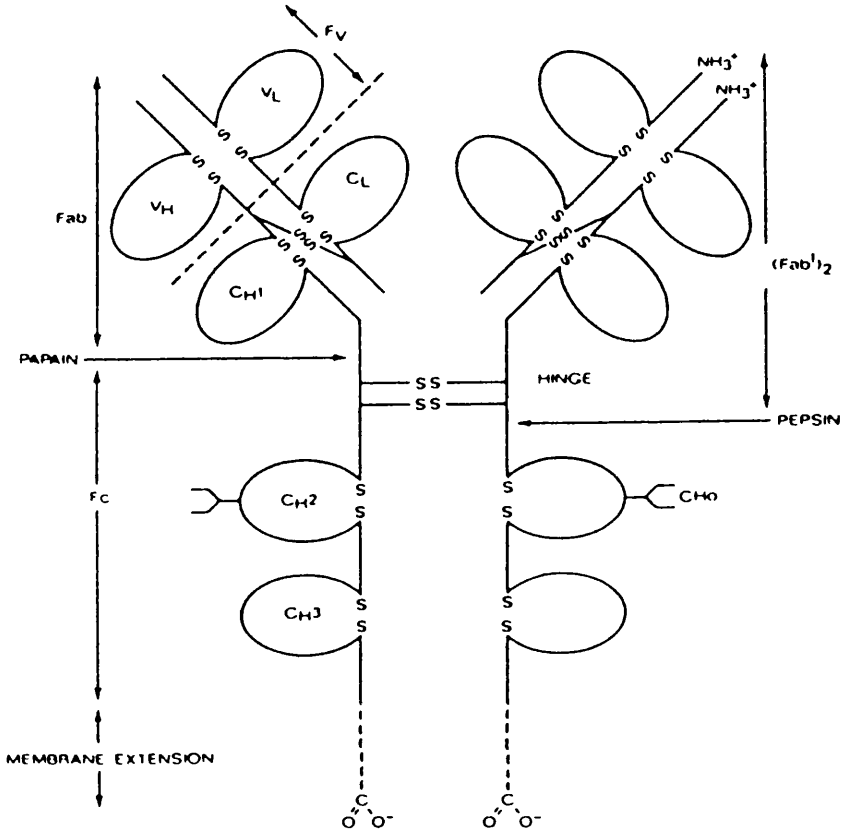



Fig.3.1 Structure of an Immunoglobulin G molecule

The immunoglobulin G molecule is comprised of two identical heavy (H) and two identical light chains (L) joined by disulphide bridges. Each contains a number of constant (C) and variable (V) regions. Antigen binding sites reside within the hypervariable region of $V_H$ and $V_L$. Sites of proteolytic digestion and product nomenclature are indicated. CHO, carbohydrate. Secretory immunoglobulins are synthesized without the C-terminal extension.

antigen-Ia combination initiates the proliferation and maturation of these B-cells into antibody-secreting B-lymphocytes (Schwartz, 1987).

Immunogenic polypeptides usually contain several antigenic determinants, one to interact with the B-cell antibody receptor and others to associate with Ia molecules (Golub, 1987). Immunization with short peptides (of 10 or less amino acid residues), requires prior coupling of the peptide to a carrier protein, as the peptide alone may possess insufficient antigenic determinants for interaction with Ia molecules and the T- and B-cell receptors, which may not recognize the same feature. In the peptide-carrier situation B-lymphocytes responding to the peptide will ingest and process peptide plus carrier and will present fragments of carrier (or peptide-carrier) in association with Ia molecules. T-lymphocytes primed by macrophages to this antigen-Ia combination will cause proliferation of the B-lymphocytes concerned with the generation of anti-peptide antibodies.

### 3.1.1 Anti-peptide antibody production

The site-directed nature and pre-determined specificity of anti-peptide antibodies are the essential features which distinguish them from all other antibodies (reviewed by Lerner, 1982, 1984). The generation of anti-peptide antibodies is most readily achieved by immunization with synthetic peptides bound to carrier proteins as discussed above. However, good immune responses have also been obtained by injecting free peptides of less than 15 residues long without any carrier (Young et al., 1983a; Atassi and Webster, 1983).

In an attempt to produce anti-peptide antibodies which are cross-reactive with the native protein of interest, methods which claim to predict suitable immunogenic sequences in a protein have been developed. Correlations between secondary structure (Chou and Fasman, 1978; Niman et al., 1983; Dyson et al., 1985, 1988; Wright et al.,

1988), atomic (segmental) mobility (Artymiuk et al., 1979; Moore and Williams, 1980; Williams and Moore, 1985; Lerner, 1984; Westhof et al., 1984; Tainer et al., 1984, 1985; Hirayama et al., 1985), sequence accessibility/polarity (Hopp and Woods, 1981; Stevens, 1986) and the generation of anti-peptide antibodies which recognize the native protein have been made. However, although these criteria have been used to aid the selection of protein sequences suitable for antibody production, their reliability has not been fully proven. Thus, in a review of over 100 peptide studies, Palfreyman et al. (1984) failed to assign statistical significance to any of the predictive algorithms used, but showed that the success rate (anti-peptide antibodies recognizing the native protein) was maximised by a peptide length of at least 10 residues containing a significant number of hydrophilic residues. The generation of antibodies to peptides from terminal sequences was successful in all cases, and this has been attributed to greater conformational freedom within these sequences (Walter, 1986).

The intriguing question which arises from these studies is: how does an antibody to a short synthetic peptide, assumed to be in a highly unordered state in solution, react with the more ordered version of the cognate sequence in the native protein? This phenomenon is difficult to explain unless the peptide is able to adopt a highly preferred conformation, either free in solution or on the B-cell receptor, approaching that which it adopts in the native protein, or that the protein itself approaches disorder through conformational fluctuations (Tainer et al., 1984, 1985; Westhof et al., 1984; Dyson et al., (1988).

## 3.2 PRODUCTION AND PURIFICATION OF ANTI-PEPTIDE ANTIBODIES TO THE GLUCOSE TRANSPORTER

In this study, the choice of which synthetic peptides (and corresponding anti-peptide antibodies) to prepare, was based solely on those regions of the glucose transporter polypeptide predicted to be extramembranous in the model of Mueckler et al. (1985) (Section 1.7.3). Fig.3.2 is a diagrammatical representation of this model, which shows the location of the predicted extramembranous regions of the transporter polypeptide to which corresponding peptides were synthesized. These regions of the protein are both relatively hydrophilic and carry many charged amino acid residues, which, as discussed in Section 3.1.1, may favour the production of anti-peptide antibodies which are cross-reactive with the native glucose transporter.

### 3.2.1 Peptide synthesis

Peptides corresponding to residues 1-15, 84-98, 144-158, 231-246, 240-255, 256-272, 326-340, 389-403, 450-467, 460-477 and 477-492 of the human erythrocyte glucose transporter (Fig.3.2), and a peptide corresponding to residues 231-246 of the rat brain glucose transporter (Birnbaum et al., 1986) were synthesized by the $N^{\alpha}$-fluorenylmethoxy-carbonyl-polyamide solid-phase method (Atherton and Sheppard, 1985) using a Mk 1 Cambridge Research Biochemicals Pepsynthesizer and $N^{\alpha}$-fluorenylmethoxycarbonyl-amino acid pentafluorophenyl esters (Fmoc-amino acid OPfp's) or $N^{\alpha}$-fluorenylmethoxycarbonyl-amino acid oxo-benzotriazine esters (Fmoc-amino acid ODhbt's).

#### 3.2.1.1 Solvents and reagents

N,N-dimethylformamide (DMF, 'Analar', BDH) was freed of

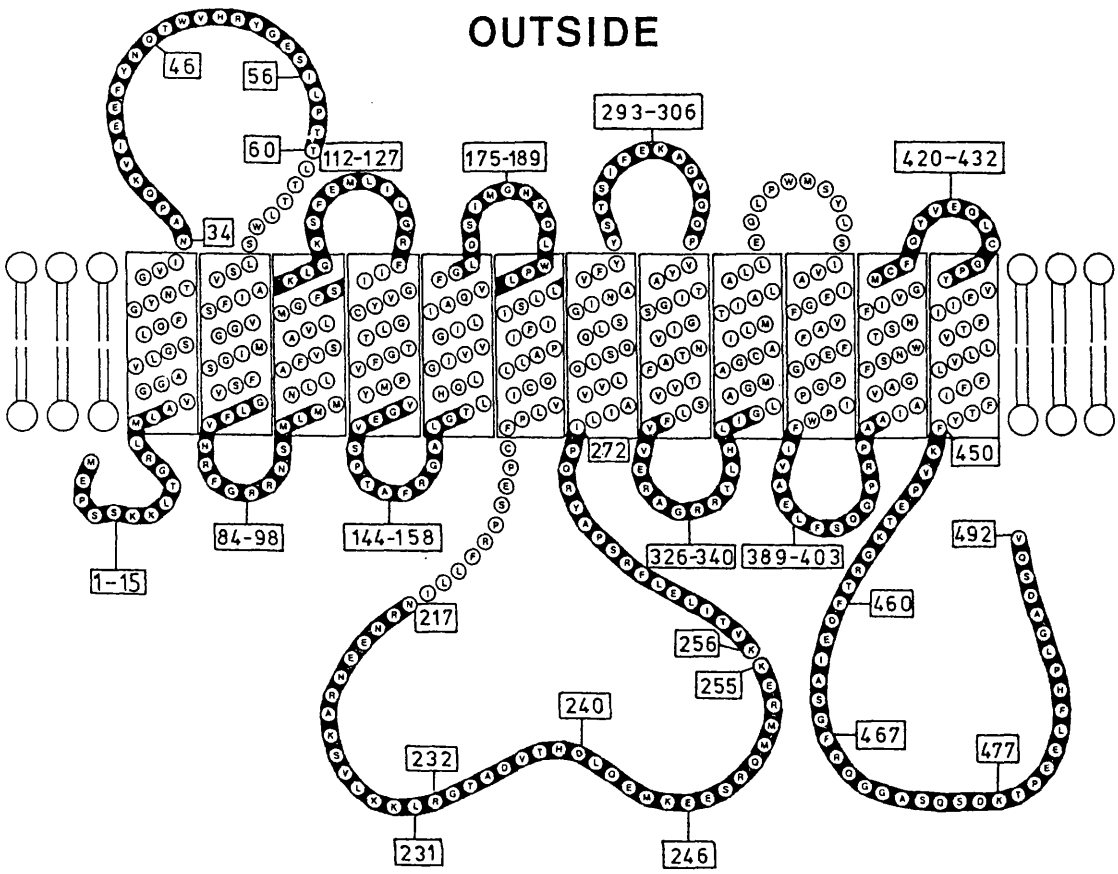



Fig.3.2 Location of chemically-synthesized peptides within the glucose transporter sequence

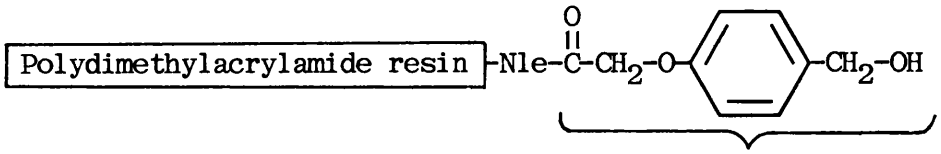
Regions of the sequence corresponding to synthetic peptides are outlined in black. Numbers refer to the sequence location of the N- and C-terminal residues of each peptide. The proposed model for the arrangement of the transporter in the membrane is taken from Mueckler et al., (1985).

contaminating dimethylamine by distillation under vacuum (15mm Hg) at 43°C. Alternatively, pre-treatment of DMF with a molecular sieve (sodium aluminosilicate-pore diameter 4Å, Sigma), at 4°C for at least 6 weeks prior to use, proved successful. Before use in peptide synthesis, molecular sieve-treated DMF was filtered through Whatman No. 1 filter paper to remove any sieve fines. In either case, treated DMF was not used in peptide synthesis for longer than 5 days without re-treatment. The removal of dimethylamine was confirmed by testing treated DMF for free $2^{o}$ amine with 1-fluoro-2,4-dinitrobenzene (FDNB). Equal volumes of FDNB solution (1mg/ml in 95% ethanol) and DMF were mixed and left to stand for 30 minutes before reading the absorbance of the solution at 381nm. A 'blank' solution (0.5mg/ml FDNB in 95% ethanol), gives a $A_{381nm}$ of about 0.2. The DMF under test was usable if it had a net $A_{381nm}$ no more than 0.1-0.15 higher than the blank. The removal of dimethylamine was necessary as its presence would produce untimely cleavage of the base-labile Fmoc group during peptide synthesis.

Dichloromethane ('Analar', BDH), was freed of contaminating HCl by passage through a 20x2cm column of activated, basic alumina (Aldrich, Gillingham, Dorset, U.K.), immediately before use. Piperidine (99%) and 1,2-ethanedithiol, (EDT, Aldrich), anhydrous trifluoroacetic acid (TFA, sequenal grade, Fluka, Glossop, Derbyshire, U.K.), and diethyl ether ('Analar', BDH), were used without further treatment.

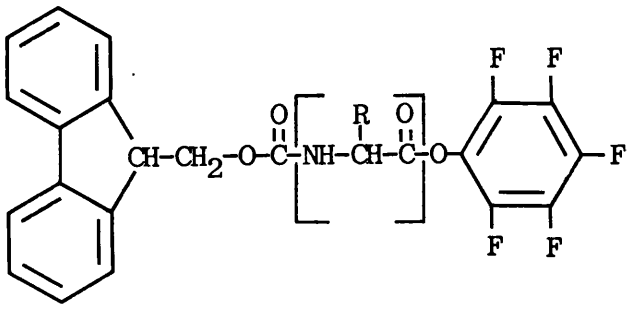
The resin used was Pepsyn KA (Milligen, Bedford, M.A., U.S.A.), which is freely permeable to dipolar, aprotic solvents such as DMF, and consists of a polar polydimethylacrylamide (polyamide) gel held within the pores of an inert, macroporous rigid kieselguhr matrix, containing 4-hydroxymethylphenoxyacetic acid as the peptide-linkage agent (Fig.3.3(A). The resin typically contained 0.09-0.1mmol

A



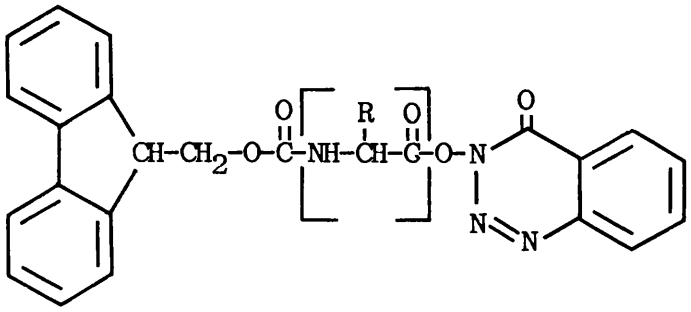

4-hydroxymethylphenoxyacetic acid

B




Fmoc moiety OPfp moiety

C




ODhbt moiety

Fig.3.3 Reagents employed in Fmoc-polyamide peptide synthesis

(A) Structure of Pepsyn KA resin. Nle is norleucine, (B) a Fmoc-amino acid pentafluorophenyl ester, (C) a Fmoc-amino acid oxo-benzotriazine ester.

equivalents of peptide linkage groups per gram and contains norleucine as an internal reference standard for amino acid analysis. Pepsyn KA is suitable for the synthesis of peptides with a C-terminal carboxylic acid.

The Fmoc-amino acid pentafluorophenyl esters (Fig.3.3(B), and Fmoc-amino acids required for the synthesis of symmetrical anhydrides were obtained from Milligen. Serine and threonine were supplied as Fmoc-amino acid oxo-benzotriazine esters (Fig.3.3(C). Dicyclohexylcarbodiimide for symmetrical anhydride synthesis and the catalysts 4-dimethylaminopyridine and 1-hydroxybenzotriazole came from Milligen or Aldrich. All other reagents were obtained from BDH, Sigma or Aldrich and were the highest grade available.

### 3.2.1.2 Assembly of the peptides

Peptide synthesis was performed on a scale utilizing 1 gram of Pepsyn KA resin which would produce a maximum theoretical yield of 0.09-0.1mmol of peptide. The first residue of the peptide (the C-terminal residue), was coupled to the resin as a symmetrical anhydride (an 'active' form of the amino acid). Symmetrical anhydrides of the amino acids were prepared immediately before use in the following way; The Fmoc-amino acid (1mmol) was dissolved in dichloromethane and dicyclohexylcarbodiimide (0.5mmol) was added. The mixture was stirred for 10 minutes in which time dicyclohexylurea (DCU), a white suspension, appears (Fig.3.4). The DCU was removed by filtration and washed with dichloromethane. The filtrate containing the symmetrical anhydride was evaporated to dryness under vacuum and the residue dissolved in 1ml of DMF for the coupling step. A four-fold molar excess of symmetrical anhydride over resin OH groups (0.4mmol) was applied to the resin in the presence of 0.04mmol of 4-dimethylaminopyridine to catalyse ester bond formation between the

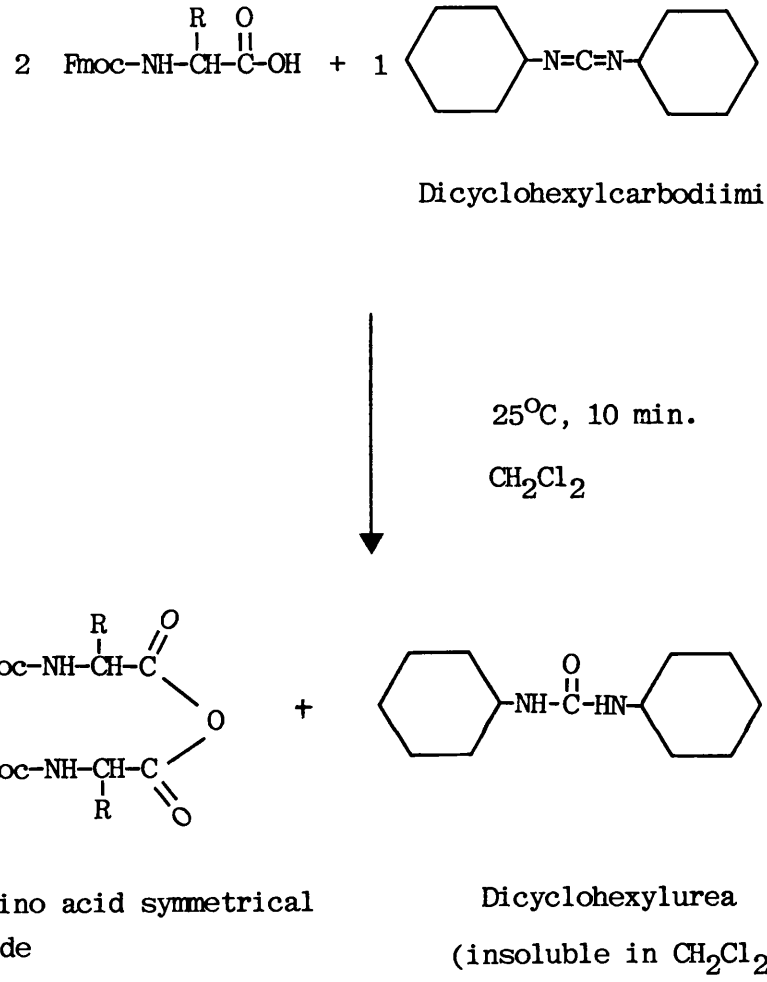



Fig.3.4 Synthesis of Fmoc-amino acid symmetrical anhydrides

amino acid and resin OH groups. Coupling was allowed to proceed for 60 minutes, after which time, excess symmetrical anhydride was removed by thorough washing of the resin with DMF. At this point, samples of resin (0.5mg) were removed for amino acid analysis to determine the extent of coupling of the C-terminal amino acid with reference to the resin-bound norleucine internal standard.

Having verified that coupling of the first amino acid had occurred, the next amino acid could be introduced. The resin was washed with 20% piperidine in DMF to deprotect the amino group of the amino acid by removal of the base-labile Fmoc moiety. Confirmation that deprotection had occured was achieved by detection of free amino groups by the Kaiser ninhydrin test (Kaiser et al., 1970). A sample (10-20 beads) of resin was washed with diethyl ether, dried under nitrogen and then two drops each of the following reagents were added; 280mM ninhydrin in ethanol, 80% phenol in ethanol and 50μM KCN in pyridine. The samples were heated for 5 minutes at 100°C. The presence of free amino groups gave a deep blue colour. Following successful deprotection, the next Fmoc-amino acid was introduced as the pentafluorophenyl or oxo-benzotriazine ester. A three-fold molar excess of the Fmoc-amino acid ester (0.3mmol) was dissolved in a minimum volume of DMF (containing 0.1mmol of 1-hydroxybenzotriazole to optimise coupling efficiency), and loaded onto the resin. Coupling was allowed to proceed for 60 minutes. After washing away excess amino acid with DMF, a check for completion of coupling was made using the Kaiser ninhydrin test. Complete coupling gave a yellow colour indicative of no free amino groups. The cycle of deprotection and coupling was then repeated for each amino acid until the peptide was completed. The resin was then washed with dichloromethane and diethyl ether, dried in vacuo overnight and stored at -20°C.

On completion of synthesis, side chain deprotection and cleavage

of each peptide from the resin was performed by treatment with anhydrous TFA, containing 5-10% scavenging agents (to prevent side reactions of the peptide with reactive species generated during the cleavage). The cleavage time and scavengers used varied depending on the amino acid composition of each peptide, but cleavage for six hours or overnight in a solution consisting of 95% TFA, 5% EDT was most commonly used. Each peptide-resin was treated with the cleavage solution in a side-arm sinter funnel and the liberated peptide collected in a round-bottomed flask, rotary-evaporated to dryness under vacuum and washed repeatedly with diethyl ether. Each peptide was finally dried under nitrogen, then under vacuum overnight and stored at -20$^{o}$C

Peptides corresponding to residues 34-60, 112-127, 175-189, 293-306 and 420-432 of the human erythrocyte glucose transporter, were synthesized by Dr T.C. Ciardelli in collaboration with Dr G.E. Lienhard in the Department of Biochemistry, Dartmouth Medical School, Hanover, U.S.A., by the solid phase methodology of Barany and Merrifield (1980) using tertiary butyloxycarbonyl (Boc) amino acids and a Biosearch Model 9500 peptide synthesizer. The peptides were radiolabelled (1000-40,000 cpm/mg of [$^{3}$H]), having at their N-terminus the sequence Cys-[$^{3}$H]gly- and an amide group at their C-terminus, (Table 3.1(A).

### 3.2.1.3 Analysis of the peptides

The amino acid compositions of the synthetic peptides prepared by the Fmoc-polyamide method were confirmed by amino acid analysis and fast atom bombardment (FAB) mass spectrometry, whilst their purity was assessed by reverse-phase high performance liquid chromatography.

FAB mass spectrometry of the peptides was kindly performed by Dr M. Panico of the Dept. of Biochemistry, Imperial College, London, U.K.

The mass of the quasi-molecular ion ($[M+H]^+$) for each peptide was determined using a V.G. ZAB high field mass spectrometer equipped with a M-SCAN fast atom bombardment gun. The gun was operated using xenon gas at a beam current of 20μA at 8kV. Samples of the peptides were dissolved in 5% (v/v) acetic acid and 2-5μl aliquots were loaded onto the fast atom bombardment target, which had been pre-coated with glycerol. Mass spectra were recorded up to a mass of 3000a.m.u. at full accelerating voltage.

For amino acid analysis, 100-250μg samples of the dry peptides were hydrolysed in 6M HCl ('Aristar', BDH) containing 0.04% (v/v) 2-mercaptoethanol and 0.04% (w/v) phenol at 110°C *in vacuo* for 24 hours. Following hydrolysis, the samples were dried down *in vacuo* over sodium hydroxide pellets. The dried samples were dissolved in a suitable volume of loading buffer (0.2M sodium citrate, pH 2.2, Pharmacia-LKB, Milton Keynes, Bucks., U.K.), and filtered through 0.22μm nylon filters (Anachem, Luton, Beds., U.K.), before analysis.

Amino acid analysis was performed using a LKB Bromma 4151 Alpha Plus analyser and data analysis performed using a Trilab 2000 multi-channel chromatography system (Trivector Systems International, Sandy, Beds., U.K.). Amino acids were separated by ion exchange chromatography and the peaks obtained integrated with respect to a calibration mixture of amino acids (Pharamacia-LKB). The quantity of each amino acid was determined automatically by computer using the integrated peak area values.

The sequences of the peptides and their quasi-molecular ion masses determined from FAB mass spectrometry are summarized in Table 3.1(A) together with the sequences of the peptides synthesized at the Dartmouth Medical School. The amino acid composition found for each peptide is given in Table 3.1(B).

Reverse-phase high performance liquid chromatography (HPLC)

Table 3.1A Chemically synthesized peptides of the glucose transporter

Glucose transporter residue numbers are given in superscript. The mass of the quasi-molecular ion ($[M+H]^+$) for each peptide determined by FAB-mass spectrometry is given.

$^{1}$Met-Glu-Pro-Ser-Ser-Lys-Lys-Leu-Thr-Gly-Arg-Leu-Met-Leu-$^{15}$Ala-[Cys]

$[M+H]^+$ 1764

$^{84}$Gly-Leu-Phe-Val-Asn-Arg-Phe-Gly-Arg-Arg-Asn-Ser-Met-Leu-$^{98}$Met-[Cys-Gly]

$[M+H]^+$ 1957

$^{144}$Val-Gly-Glu-Val-Ser-Pro-Thr-Ala-Phe-Arg-Gly-Ala-Leu-Gly-$^{158}$Thr-[Cys-Gly]

$[M+H]^+$ 1621

$^{231}$Leu-Arg-Gly-Thr-Ala-Asp-Val-Thr-His-Asp-Leu-Gln-Glu-Met-Lys-$^{246}$Glu-[Cys]

$[M+H]^+$ 1945

$^{240}$Asp-Leu-Gln-Glu-Met-Lys-Glu-Glu-Ser-Arg-Gln-Met-Met-Arg-Glu-$^{255}$Lys-[Cys]

$[M+H]^+$ 2170

$^{256}$Lys-Val-Thr-Ile-Leu-Glu-Leu-Phe-Arg-Ser-Pro-Ala-Tyr-Arg-Gln-Pro-$^{272}$Ile-[Cys]

$[M+H]^+$ 2133

$^{326}$Phe-Val-Val-Glu-Arg-Ala-Gly-Arg-Arg-Thr-Leu-His-Leu-Ile-$^{340}$Gly-[Cys]

$[M+H]^+$ 1826

$^{389}$Phe-Ile-Val-Ala-Glu-Leu-Phe-Ser-Gln-Gly-Pro-Arg-Pro-Ala-$^{403}$Ala-[Cys]

$[M+H]^+$ 1705

$^{450}$Phe-Lys-Val-Pro-Glu-Thr-Lys-Gly-Arg-Thr-Phe-Asp-Glu-Ile-Ala-Ser-Gly-$^{467}$Phe-[Cys-Gly]

$[M+H]^+$ 2188

Table 3.1A (continued)

$^{460}$Phe-Asp-Glu-Ile-Ala-Ser-Gly-Phe-Arg-Gln-Gly-Gly-Ala-Ser-Gln-Ser-Asp-$^{477}$Lys-[Cys]

$[M+H]^+$ 2002

[Cys]-$^{477}$Lys-Thr-Pro-Glu-Glu-Leu-Phe-His-Pro-Leu-Gly-Ala-Asp-Ser-Gln-$^{492}$Val

$[M+H]^+$ 1870

Peptides synthesized at the Dartmouth Medical School.

[Cys-[$^3$H]Gly]-$^{34}$Asn-Ala-Pro-Gln-Lys-Val-Ile-Glu-Glu-Phe-Tyr-Asn-Gln-Thr-Trp-Val-His-Arg-Tyr-Gly-Glu-Ser-Ile-Leu-Pro-Thr-$^{60}$Thr

[Cys-[$^3$H]Gly]-$^{112}$Phe-Ser-Lys-Leu-Gly-Lys-Ser-Phe-Glu-Met-Leu-Ile-Leu-Gly-Arg-$^{127}$Phe

[Cys-[$^3$H]Gly]-$^{175}$Gly-Leu-Asp-Ser-Ile-Met-Gly-Asn-Lys-Asp-Leu-Trp-Pro-Leu-$^{189}$Leu

[Cys-[$^3$H]Gly]-$^{217}$Asn-Arg-Asn-Glu-Glu-Asn-Arg-Ala-Lys-Ser-Val-Leu-Lys-Lys-Leu-$^{232}$Arg

[Cys-[$^3$H]Gly]-$^{293}$Tyr-Ser-Thr-Ser-Ile-Phe-Glu-Lys-Ala-Gly-Val-Gln-Gln-$^{306}$Pro

[Cys-[$^3$H]Gly]-$^{420}$Met-Cys-Phe-Gln-Tyr-Val-Glu-Gln-Leu-Cys-Gly-Pro-$^{432}$Tyr

Rat brain glucose transporter.

$^{231}$Leu-Arg-Gly-Thr-Ala-Asp-Val-Thr-*Arg-Asp-Leu-Gln-Glu-Met-Lys-$^{246}$Glu-[Cys]

$[M+H]^+$ 1964

* non-conserved residue in human erythrocyte and rat brain transporter sequences

Table 3.1B Amino acid analysis of the synthetic peptides

The amino acid compositions of peptides synthesized using the Fmoc-polyamide methodology are listed below. Residues given in brackets in Table 3.1A are excluded. The expected number of residues are given in brackets.

Peptide 1-15
Thr 0.9 (1), Ser 1.6 (2), Glx 0.9 (1), Pro 0.9 (1), Gly 1.1 (1), Ala 1.1 (1), Met 1.8 (2), Leu 3.2 (3), Lys 1.8 (2), Arg 1.1 (1)

Peptide 84-98
Asx 2.0 (2), Ser 0.9 (1), Gly 2.0 (2), Val 1.0 (1), Met 1.9 (2), Leu 2.1 (2), Phe 2.0 (2), Arg 3.0 (3)

Peptide 144-158
Thr 1.6 (2), Ser 0.8 (1), Glx 1.1 (1), Gly 3.2 (3), Ala 2.0 (2), Val 2.0 (2), Leu 1.1 (1), Phe 1.1 (1), Arg 1.0 (1)

Peptide 231-246
Asx 2.0 (2), Thr 2.0 (2), Glx 3.1 (3), Gly 1.0 (1), Ala 1.0 (1), Val 1.0 (1), Met 0.9 (1), Leu 2.1 (2), His 0.9 (1), Lys 1.0 (1), Arg 1.0 (1),

Peptide 240-255
Asx 1.1 (1), Ser 0.9 (1), Glx 5.9 (6), Met 2.9 (3), Leu 1.2 (1), Lys 2.0 (2), Arg 2.0 (2)

Peptide 256-272
Thr 0.9 (1), Ser 0.8 (1), Glx 2.4 (2), Pro 2.0 (2), Ala 1.0 (1), Val 1.0 (1), Leu 2.1 (2), Ile 2.0 (2), Phe 1.0 (1), Tyr 1.1 (1), Lys 0.9 (1), Arg 1.9 (2)

Peptide 326-340
Thr 0.8 (1), Glx 0.9 (1), Gly 2.1 (2), Ala 0.9 (1), Val 1.8 (2), Leu 2.5 (2), Phe 0.8 (1), His 1.2 (1), Arg 2.4 (2)

Peptide 389-403
Ser 0.7 (1), Glx 1.9 (2), Pro 2.4 (2), Gly 1.0 (1), Ala 3.1 (3), Val 1.1 (1), Leu 1.0 (1), Ile 0.9 (1), Phe 1.9 (2), Arg 1.0 (1)

Peptide 450-467
Asx 1.0 (1), Thr 1.9 (2), Ser 0.7 (1), Glx 2.3 (2), Pro 1.0 (1), Gly 2.2 (2), Ala 1.0 (1), Val 0.9 (1), Ile 1.0 (1), Phe 3.0 (3), Lys 2.0 (2), Arg 1.0 (1)

Peptide 460-477
Asx 1.9 (2), Ser 2.2 (2), Glx 3.2 (3), Gly 3.3 (3), Ala 2.1 (2), Ile 1.0 (1), Phe 2.0 (2), Lys 1.1 (1), Arg 1.1 (1)

Peptide 477-492
Asx 1.0 (1), Thr 1.0 (1), Ser 0.9 (1), Glx 3.1 (3), Pro 2.0 (2), Gly 1.0 (1), Ala 1.0 (1), Val 1.0 (1), Leu 2.0 (2), Phe 1.0 (1), His 1.0 (1), Lys 1.0 (1)

Rat peptide 231-246
Asx 2.0 (2), Thr 1.9 (2), Glx 3.3 (3), Gly 1.0 (1), Ala 1.0 (1), Val 0.9 (1), Met 0.9 (1), Leu 2.1 (2), Lys 1.0 (1), Arg 1.9 (2)

analysis of the peptides was performed on a system consisting of 2 Altex 110A pumps controlled by an Altex model 420 system controller programmer/ERC-3310 solvent degasser (Erma Optical Works Ltd., Tokyo)/Rheodyne model 7120 syringe loading and sample injector with 100µl loop/Pye Unicam PU4020 U.V. detector/Rikadenki R-OX series, 2 channel chart recorder and Pharmacia FRAC-100 fraction collector. Latterly, a Varian 5000 liquid chromatograph and Linear 1200 chart recorder were used (Varian Instrument Group, Palo Alto, California, U.S.A.), together with a Rheodyne model 7125 syringe loading and sample injector with 100µl loop.

Peptides were chromatographed on an Aquapore RP-300 column (4.6mm I.D. x 25cm), a $C_{18}$ column of 300Å pore size (Brownlee Labs, Santa Clara, C.A., U.S.A.), protected with the appropriate guard column. The peptides were fractionated by a gradient of acetonitrile (0-90%) in 0.06% (v/v) TFA. Solvent A (0.1% TFA), was prepared by the addition of TFA to double-distilled water which had been filtered through Sterivex-GS, 0.22µm filter units (Millipore). Solvent B (acetonitrile in 0.06% (v/v) TFA), was filtered in the same way prior to use. A flow rate of 1ml/min was used throughout and peaks were detected at 220nm. Where necessary, peaks in the eluate from the HPLC column were collected manually after calculating the time gap between the U.V. detector and collection tubes. These samples were dried down on a Speedy Vac Concentrator connected via a refridgerated condensation trap (Savant Instruments Inc. Hicksville, N.Y., U.S.A.) to a rotary piston vacuum pump. The dried samples were prepared for amino acid analysis or stored at -20°C.

HPLC showed that the peptides were at least 80% pure, as illustrated by the profile obtained for C-terminal peptide 477-492 in Fig.3.5. When significant (5-10% of total peak area), peaks were seen in the HPLC profiles of the peptides, analysis showed most of them to

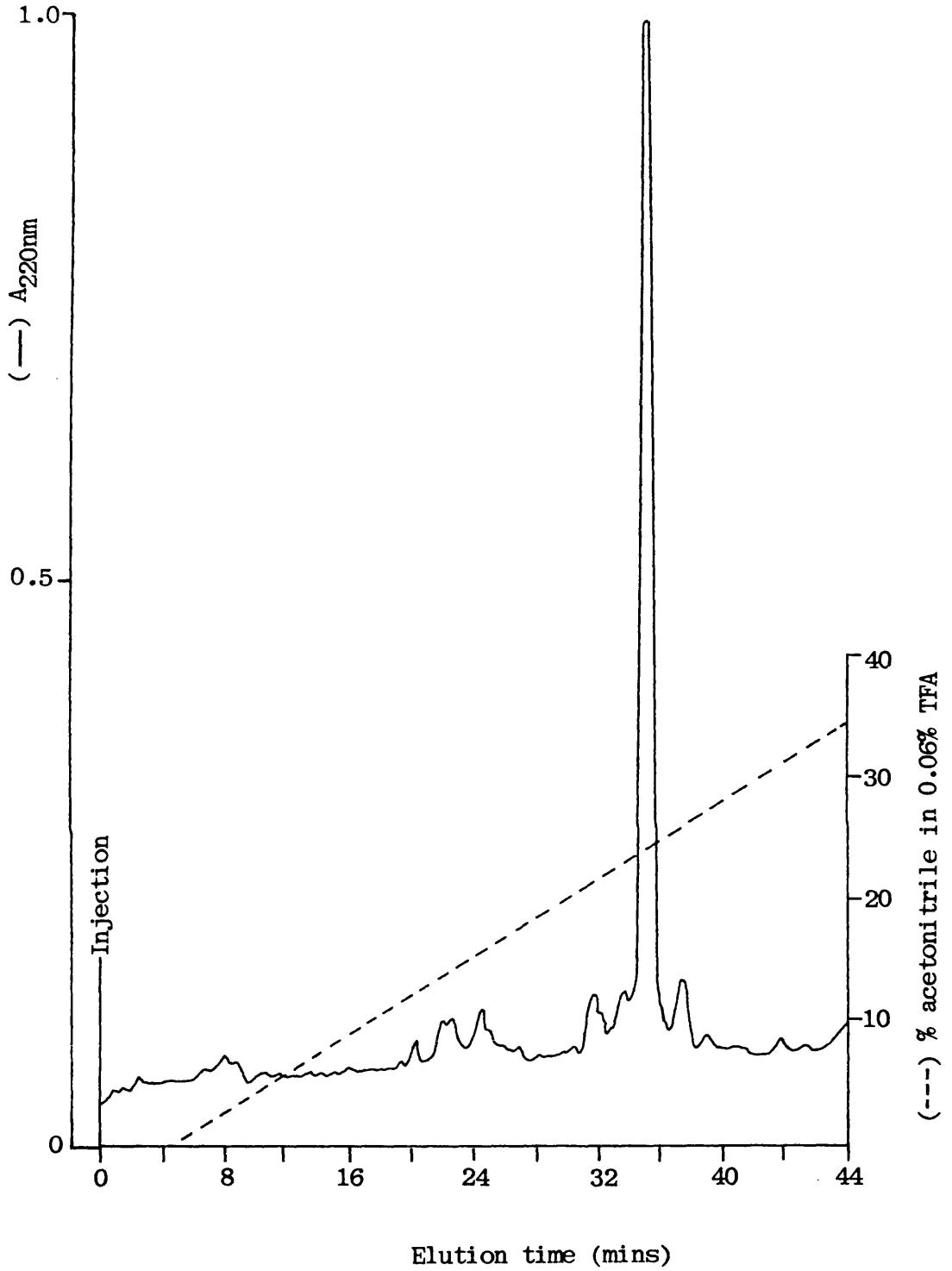



Fig.3.5 HPLC analysis of C-terminal peptide 477-492

A sample of C-terminal peptide 477-492 (50µl of a 1mg/ml solution in 0.1% TFA), was loaded onto an Aquapore RP300 HPLC column equilibrated with 0.1% TFA. A gradient of 0-90% acetonitrile in 0.06% TFA was applied over 60 minutes following a 5 minute wash. The major peptide peak was eluted at 25% acetonitrile, detected at 220nm.

have amino acid compositions very similar to the main peaks (results not shown). This finding suggested that the minor peaks represent either dimeric forms of the peptides or peptides whose amino acid side groups had not been fully deprotected during cleavage. As no suitable preparative HPLC column was available at the time this work was carried out, purification of the peptides on the scale required for conjugation to carrier proteins for immunization was not possible, and consequently they were used without further purification.

### 3.2.2 Conjugation of peptides to carrier proteins

Peptides were conjugated to keyhole limpet haemocyanin (KLH, Sigma), using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, Pierce, Chester, Cheshire, U.K.), essentially as described by LaRochelle *et al.* (1985), or sulfo-m-maleimidobenzoyl-N-hydroxysuccinimide ester (S-MBS, Pierce), a water-soluble derivative of MBS. An estimation of thiol groups was performed prior to coupling (Section 2.8) and peptides were reduced with dithiothreitol (DTT) as necessary. Peptides which were poorly soluble in aqueous buffers in the absence of up to 30% (v/v) DMF or DMSO, or peptides lacking lysine residues, were conjugated to bovine thyroglobulin (Sigma) using glutaraldehyde essentially as described by Carrasco *et al.* (1986).

#### 3.2.2.1 Conjugation of peptides to KLH

KLH (10mg in 0.625ml 10mM sodium phosphate, 145mM NaCl, pH 7.2), was dialysed overnight at 4°C against this buffer. Duplicate samples (80µg), were taken for amino acid analysis. 0.5ml (8mg), of the dialysed KLH was treated with 10µl of 50mM N-ethylmaleimide (final concentration 0.98mM) and incubated at 25°C for 10 minutes to block protein SH groups. The KLH was then reacted with 4mg MBS (20mg/ml in DMF), or 4mg S-MBS (20mg/ml in water), at 25°C for 30 minutes. KLH-MBS

was separated from unreacted MBS by gel exclusion chromatography on a 20x1cm column of Sephadex G50 (Pharmacia-LKB). The eluate was monitored at 280nm and peak protein-containing (void volume) fractions pooled.

Peptide (8mg), was dissolved in 0.5ml 50mM sodium phosphate, pH 7.2, and reacted with 100mM DTT for 10 minutes. The reduced peptide was separated from DTT and EDT by gel exclusion chromatography on a 20x1cm column of Sephadex G10 (Pharmacia-LKB). The eluate was monitored at 230nm and peak peptide-containing (void volume) fractions were pooled.

The pooled KLH-MBS and peptide samples were combined and incubated at 25$^{o}$C for 4 hours. Uncoupled peptide was removed by gel filtration chromatography on a second 20x1cm column of Sephadex G50 and the conjugate dialysed overnight against 2x2 litres of PBS, pH 7.2. Duplicate 80µg samples of the conjugate were taken for amino acid analysis. The success of peptide coupling was assessed by the difference in amino acid composition of KLH and the peptide-KLH conjugates determined by amino acid analysis (Section 3.2.1), and results expressed as the number of moles of peptide bound per 100,000g of protein. KLH conjugates were prepared for peptides corresponding to residues 1-15, 144-158, 231-246, 240-255, 256-272, 450-467, 460-477 and 477-492 of the human erythrocyte glucose transporter and residues 231-246 of the rat brain glucose transporter. The number of moles of peptide bound per 100,000g of KLH ranged from 8-25 (approximately 16-50% by weight if the average $M_r$ of a 16 residue peptide is taken to be 2000).

3.2.2.2 Conjugation of peptides to thyroglobulin

Bovine thyroglobulin (35mg in 2ml PBS, pH 7.2), was dialysed overnight against this buffer. Duplicate samples (80µg) were taken for

amino acid analysis. Peptide (2μmol), was dissolved in 0.1ml of dimethylsulphoxide (DMSO,'Analar', BDH) and added to 1.5ml (26.3mg;40nmol) of the dialysed thyroglobulin. Glutaraldehyde (45μmol;20μl of a specially purified 25% solution in water, Sigma), was added to the thyroglobulin/peptide mixture and incubated overnight at 4°C. Uncoupled peptide and DMSO were removed by gel exclusion chromatography on a 20x1cm column of Sephadex G75 (Pharmacia-LKB) and the conjugate dialysed against 2x2 litres of PBS, pH 7.2. Duplicate 80μg samples of the conjugate were taken for amino acid analysis. The degree of peptide coupling was assessed as described in Section 3.2.2.1. Thyroglobulin conjugates were prepared for peptides corresponding to residues 84-98, 326-340 and 389-403 of the human erythrocyte glucose transporter. The number of moles of peptide bound per 100,000g of thyroglobulin ranged from 15-27 (approximately 30-54% by weight).

### 3.2.3 Immunization procedure

Antisera against the conjugates and against the purified human erythrocyte glucose transporter were raised in male New Zealand White rabbits. The conjugates or transporter (200μg protein) in 0.5ml of PBS, pH 7.2, were emulsified with 1.5ml of complete Freund's adjuvant and then injected either intradermally at multiple sites along the back or intramuscularly into the rear leg. A booster injection of antigen (100μg protein) in incomplete Freund's adjuvant was made after 4 weeks, and then the animals were bled after another 2 weeks. Booster injections of antigen were continued every 4 weeks for as long as was required. Control sera were obtained from the rabbits before the first injection.

### 3.2.4 Isolation and treatment of antisera

Rabbits were bled via the ear vein for routine blood-taking or 'bled out' by cardiac puncture under anaesthesia before being killed. Rabbit blood was allowed to clot around a glass rod for 1 hour at 37°C and the serum was removed following sedimentation of the erythrocytes by centrifugation (3000 rev/min for 10 minutes). Antisera were treated at 56°C for 30 minutes to inactivate complement and then stored at -70°C.

### 3.2.5 Purification of anti-peptide antibodies

Two methods were used to purify glucose transporter-specific anti-peptide antibodies from rabbit serum. The first utilized a column containing immobilized synthetic C-terminal peptide as an affinity matrix and the second involved the adsorption and acid-elution of antibodies from protein-depleted erythrocyte membranes essentially as described by Schroer et al. (1986).

#### 3.2.5.1 Affinity purification of anti-peptide antibodies using immobilized synthetic peptides

This method of antibody purification was a two stage process. Total serum IgG was first isolated on a protein A-Sepharose CL4B column and the purified IgG fraction was then applied to the peptide affinity column.

An affinity column containing immobilized C-terminal peptide (corresponding to residues 477-492 of the glucose transporter), was prepared using Affigel-10 (Bio-Rad), which contains a N-hydroxysuccinimide ester as the active linkage agent capable of binding peptide amino groups. The column was prepared in the following way; 10mg of peptide were dissolved in 1ml of 50mM 2-[N-morpholino]ethanesulphonic acid (MES) buffer, pH 5.5, containing 10%

(v/v) DMF. Excess EDT was removed from the peptide by passage through a 20x1cm column of Sephadex G10 (as described in Section 3.2.2), using 50mM MES, pH 5.5, as the eluting buffer. Peak peptide-containing fractions (determined from measurement of $A_{230nm}$), were pooled and samples (60µl) taken for thiol assay (Section 2.8), to determine the initial amount of peptide present (as peptide-bound cysteine). The pooled peptide-containing fraction (3ml) was diluted to 6ml with the MES buffer and incubated with 3.5ml of washed Affigel-10 for 6 hours at 4°C. Following incubation, the Affigel-10 was allowed to settle and samples (60µl) of the supernatant were taken for thiol assay in order to assess the extent of peptide coupling to the gel. Typically, 60-70% of the peptide was coupled in this way. Remaining active esters on the gel were then blocked by the addition of 0.3ml of 1M ethanolamine. The gel was loaded into a 5x1ml Econo-column (Bio Rad), washed extensively with PBS, pH 7.2, and stored in PBS, pH 7.2, containing 0.02% (w/v) sodium azide before use.

Protein A-Sepharose CL4B (Pharmacia-LKB), was hydrated (1g swells to 3.5 ml gel), washed in PBS, pH 7.2, and 5ml packed into a 10x1cm Econo-column. This column had a binding capacity of 10-20mg IgG per ml, but in order to ensure complete removal of IgG from sera, the column capacity was taken as 15mg IgG per ml. Rabbit antisera was taken to contain 20mg total IgG per ml. On this basis, 3.75ml of anti-C-terminal peptide serum were loaded onto the protein A-Sepharose column and recirculated through the column five times at a flow rate of 30ml/hour. Following recirculation, the column was washed with PBS, pH 7.2, 2.5ml fractions of the wash material were collected and their absorbance at 280nm measured. Washing was continued until $A_{280nm}$ readings fell to near zero (Fig.3.6(A) and then bound IgG was eluted with a buffer containing 0.1M acetic acid, 145mM NaCl, pH 2.4. Acid-eluted IgG-containing fractions were detected by their $A_{280nm}$

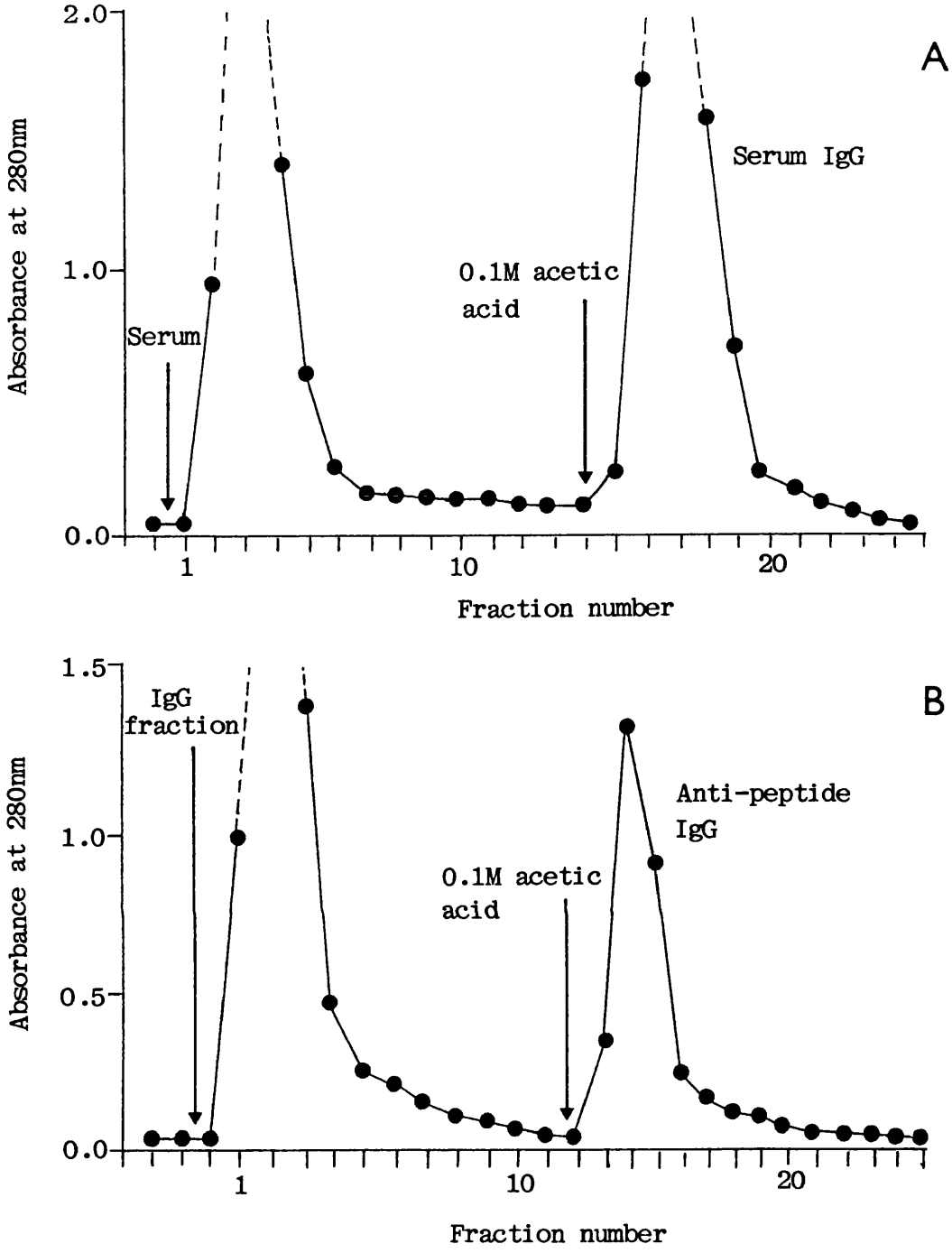



Fig.3.6 Affinity purification of C-terminal peptide antibodies

Antiserum to C-terminal peptide 477-492 was applied to a column of protein A-Sepharose CL4B. Non-immunoglobulins were eluted in PBS, pH 7.2, and the IgG fraction with 0.1M acetic acid, 145mM NaCl, pH 2.4. The elution profile for the column is shown in (A). The isolated IgG fraction was applied to a column of immobilized C-terminal peptide. Unbound material was washed away with PBS, 800mM NaCl, pH 7.2, and anti-peptide IgG was eluted with 0.1M acetic acid, 145mM NaCl, pH 2.4. The elution profile for the peptide column is shown in (B).

(Fig.3.6(A) and neutralized (to pH 7.2) with 2M Tris. The pooled peak IgG-containing fractions were then loaded onto the peptide affinity column and allowed to recirculate through the column for 2 hours at a flow rate of 30ml/hour. Following recirculation, non-specific IgG was eluted with 10mM sodium phosphate, 800mM NaCl, pH 7.2 (PBS*, pH 7.2), and washing continued until the $A_{280nm}$ of the collected 2ml wash fractions fell to near zero (Fig.3.6(B). Bound anti-peptide IgG was then eluted in 0.1M acetic acid, 145mM NaCl, pH 2.4. The peak $A_{280nm}$ fractions were collected (Fig.3.6(B) and neutralized immediately with 2M Tris before extensive dialysis against 2x2 litres of PBS, pH 7.2. Samples (10µg), of the protein A-Sepharose IgG fraction, crude antiserum and anti-peptide IgG fraction from the peptide column, were run on a SDS/12% polyacrylamide gel and Coomassie blue-stained (Fig.3.7). The purified samples were found to contain only IgG light and heavy chains (Fig.3.7(C). The anti-peptide and anti-glucose transporter activity of the purified antibodies were determined by ELISA with C-terminal peptide or purified glucose transporter as plate-bound antigens (Section 3.3.1.1). Using this method of purification, it was possible to isolate 0.3-0.5mg anti-C-terminal peptide IgG from each ml of antiserum.

However, this method of anti-peptide antibody purification although effective, was not widely used for the following reasons; (a) anti-peptide antibodies which were cross-reactive with the native glucose transporter could be purified more easily and in greater yield by adsorption onto protein-depleted erythrocyte membranes (Section 3.2.5.2), (b) recent work by Wilchek and Miron (1987) showed that significant leakage of immobilized ligands bound to a solid-phase support via N-hydroxysuccinimide esters occurs at extremes of pH. Consequently, considerable leakage of peptide from the column may occur during acidic elution of antibodies. The purification of anti-

peptide antibodies which did not recognize the native glucose transporter would still have necessitated the use of peptide affinity columns. However, a more reliable method of peptide immobilization would have been required. To this end, it may in the future be possible to use other active linkage agents such as Thiopropyl-Sepharose 6B (Pharmacia-LKB), in an attempt to couple the peptides to a solid-phase via their cysteine residues. However, no attempt was made during this project to purify antibodies using such columns.

3.2.5.2 Affinity purification of anti-peptide antibodies by adsorption onto protein-depleted erythrocyte membranes

The preparation of protein-depleted erythrocyte membranes was described in Section 2.1.3. Rabbit antiserum and protein-depleted erythrocyte membranes (10mg of membrane protein per ml of antiserum - the ratio of membranes to antisera required for complete removal of specific IgG as quantified by initial small scale ELISA experiments), in PBS, pH 7.2, were incubated together in 50ml Sorvall centrifuge bottles for 2 hours at $4^{o}C$ with constant gentle mixing on a rotary mixer. The membranes were then sedimented by centrifugation at 20,000 rev/min for 20 minutes at $4^{o}C$. The supernatants were kept in order to establish complete removal of anti-glucose transporter IgG by ELISA, and the membrane pellets were washed by homogenization in (PBS*, pH 7.2), to remove non-specifically-bound serum proteins. The membranes were centrifuged as above and the supernatant discarded. The wash step in PBS*, pH 7.2 was repeated twice more. Bound antibodies were then eluted from the membranes by homogenization in 0.2M glycine-HCl, pH 2.4, followed by immediate centrifugation as above. The antibody-containing supernatants were rapidly neutralized (to pH 7.2) with 2M Tris before extensive dialysis against 2x2 litres of PBS, pH 7.2. The anti-peptide and anti-glucose transporter activity of the purified

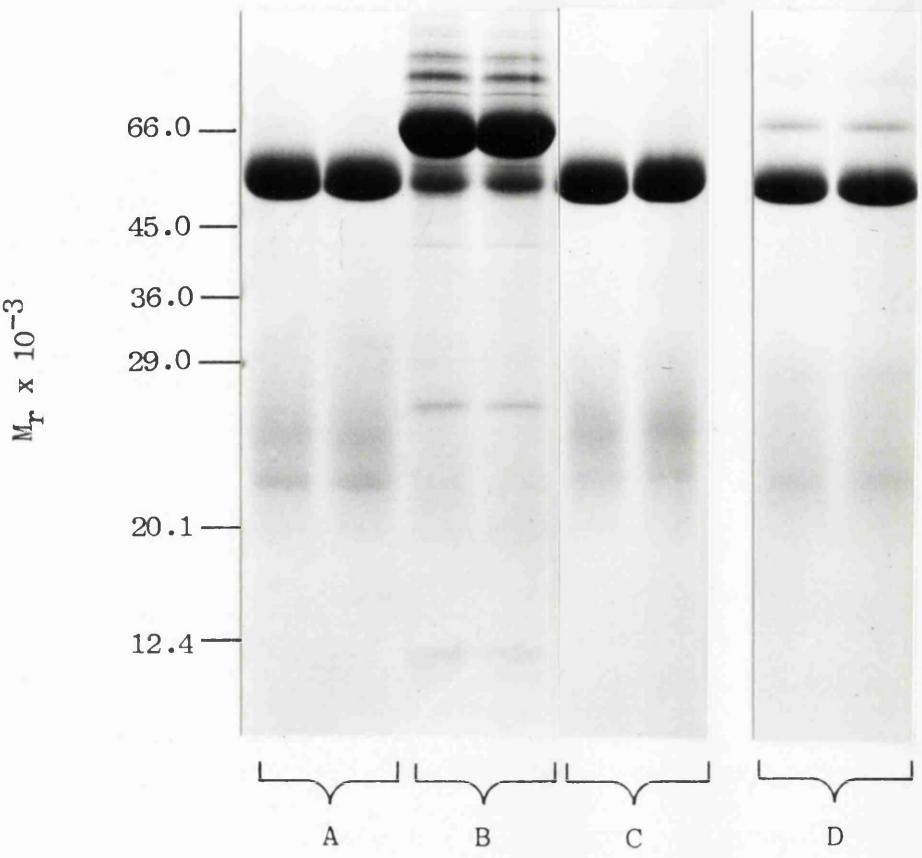



Fig.3.7 Analysis of the products of anti-peptide antibody affinity purification by SDS/polyacrylamide gel electrophoresis

Samples (10μg), of an IgG fraction isolated from antiserum to C-terminal peptide 477-492 on a protein A-Sepharose column (A), crude C-terminal peptide antiserum (B), an IgG fraction isolated from the C-terminal peptide affinity column (C) and IgG isolated from antiserum to C-terminal peptide 477-492 following adsorption and acid elution from protein-depleted erythrocyte membranes (D), were electrophoresed on a SDS/12% polyacrylamide gel and stained with Coomassie blue. The positions of $M_r$ markers are indicated.

antibodies was established by ELISA using the relevant synthetic peptide or purified glucose transporter as plate-bound antigens (Section 3.3.1.1). Samples (10μg), of the purified IgG fractions were run on SDS/12% polyacrylamide gels and Coomassie blue stained. The purified samples contained predominantly IgG light and heavy chain with traces of serum albumin, as illustrated by the result obtained for antibodies to C-terminal peptide 477-492 in Fig.3.7(D). Where necessary, purified antibodies were concentrated by ultrafiltration using a sample concentrator cell under nitrogen pressure or centrifugal micro concentrator units both fitted with YM10 membranes having a molecular weight cut-off of 10,000 (Amicon Ltd., Gloucs., U.K.). Typical yields of anti-peptide IgG were 0.6-1.5mg per ml of antiserum.

An attempt was made to affinity-purify antibodies to the N-terminal peptide, which do not appear to recognize the native glucose transporter (Section 3.3.1.1), using protein-depleted erythrocyte membranes in which the glucose transporter had been denatured by heating to 100°C for 10 minutes. The recovery of specific IgG was very low and consequently further purifications of antibodies which did not recognize the native transporter were not attempted using this method.

## 3.3 CHARACTERIZATION OF ANTI-PEPTIDE ANTIBODIES TO THE GLUCOSE TRANSPORTER

The antibodies prepared in Section 3.2 were characterized in terms of their anti-peptide and anti-glucose transporter activity, ability to recognize the native glucose transporter, and specificity for the glucose transporter polypeptide. Characterization was restricted to antibodies of the IgG class.

## 3.3.1 Assay of anti-peptide and anti-glucose transporter activity

### 3.3.1.1 ELISA

Antibodies in the serum of rabbits immunized with purified glucose transporter or peptide-KLH/thyroglobulin conjugates were assayed for anti-peptide/anti-glucose transporter activity by ELISA using plates coated with 600ng of purified glucose transporter, or 20ng of synthetic peptide per well (prepared as described in Section 2.2). Serial 2-fold or 3-fold dilutions of the antisera were prepared in PBSA containing 1% (w/v) BSA, and triplicate 0.1ml samples of each dilution were loaded onto the plates. The ELISA assays were performed as described in Section 2.2. Pre-immune rabbit sera were used in control experiments. ELISA plates from which either bound antigen or primary antibody had been omitted were used as 'blanks', the absorbance values from which were subtracted from the experimental values.

All of the antisera recognised the corresponding synthetic peptides in ELISA, as illustrated by antibodies to C-terminal peptide 477-492 and the N-terminal peptide in Fig.3.8. In addition, a high antibody titre to peptides 217-232, 231-246, 240-255, 256-272, 450-467, 460-477 and 477-492, corresponding to regions of the central hydrophilic and C-terminal regions of the transporter (Fig.3.2), was also evident in the antiserum produced by immunization of rabbits with purified glucose transporter (illustrated by the result with C-terminal peptide 477-492 in Fig.3.8(A)). This indicates that these regions of the transporter polypeptide are also immunogenic in the native protein. In contrast, no other peptide was recognized by antibodies in the anti-transporter serum, as illustrated by the result with the N-terminal peptide (Fig.3.8(B)).

However, only the antisera raised against peptides 217-232, 231-

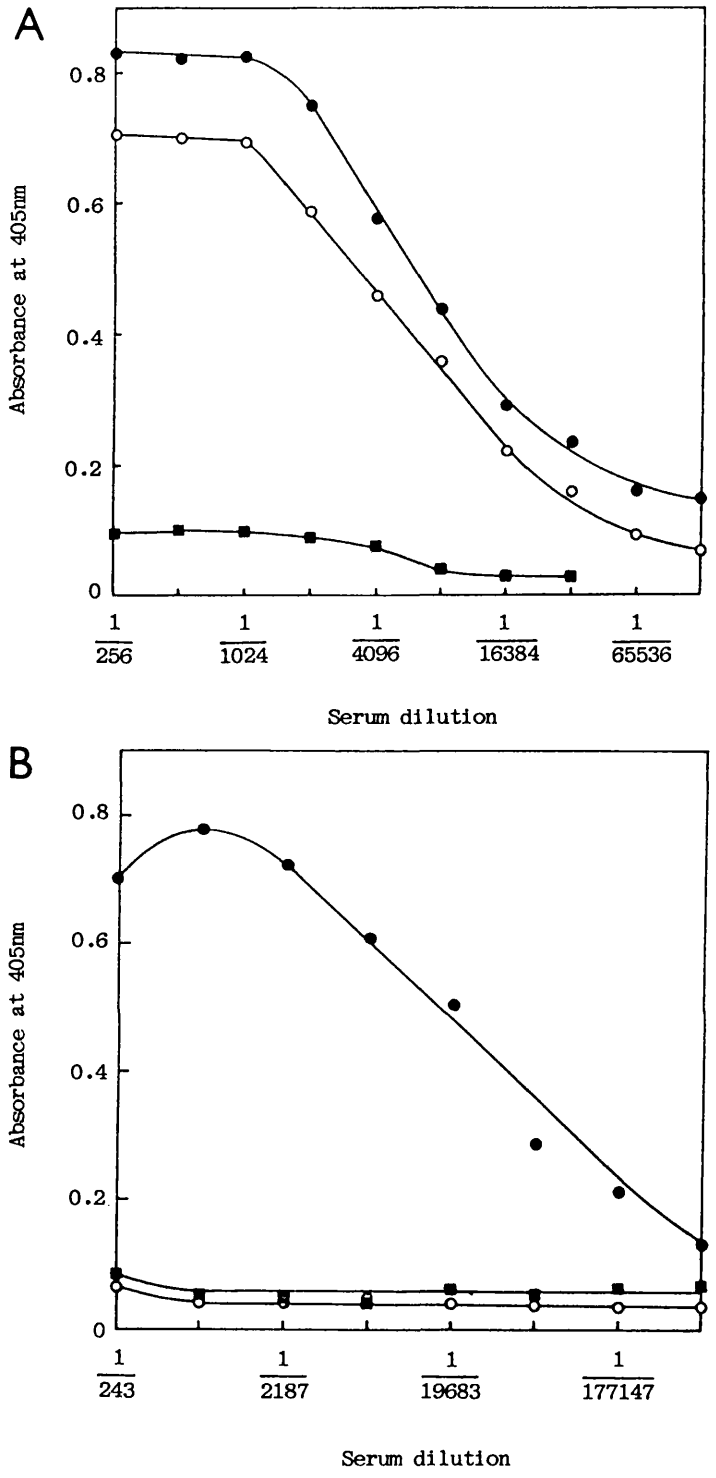



Fig.3.8 Recognition of synthetic peptides by anti-peptide and anti-transporter antibodies in ELISA

Microtitre plates were coated with 20ng per well of C-terminal peptide 477-492 (A), or N-terminal peptide (B). They were then incubated with serial 2-fold (A), or 3-fold (B) dilutions of antisera followed by alkaline phosphatase-linked second antibody and p-nitrophenyl phosphate as chromogenic substrate as described in section 2.2. Each point is the mean absorbance at 405nm of triplicate samples. (A), anti-C-terminal peptide serum (●), anti-transporter serum (O) and pre-immune serum (■). (B), anti-N-terminal peptide serum (●), anti-transporter serum (O) and pre-immune serum (■).

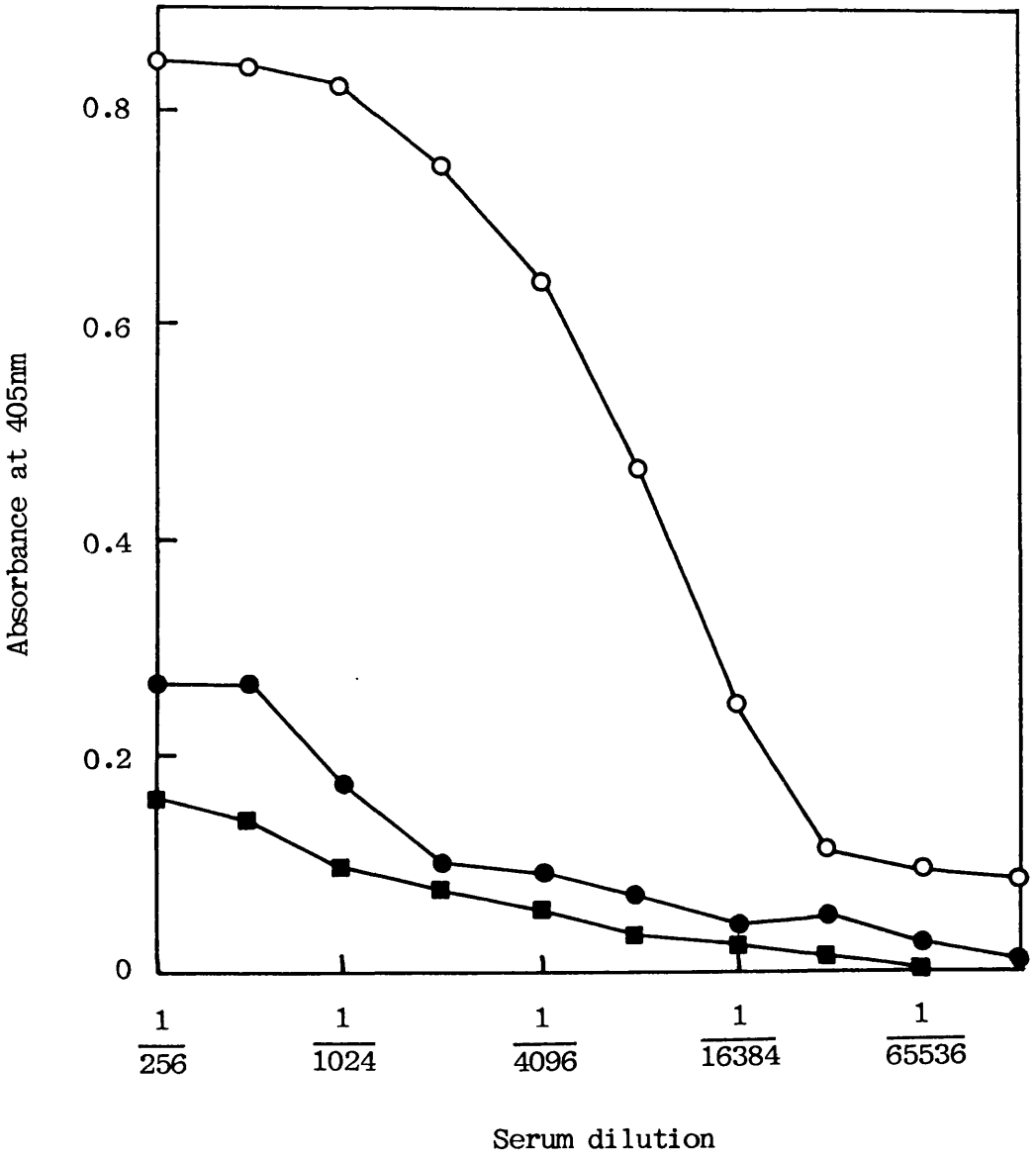



Fig.3.9 Recognition of the glucose transporter by anti-peptide antibodies in ELISA

Microtitre plates were coated with 600ng per well of purified glucose transporter. They were then incubated with serial 2-fold dilutions of antiserum to peptide 34-60 (●), antiserum to peptide 231-346 (○) or pre-immune serum (■). Bound antibody was detected as described in the legend to Fig.3.8. Each point is the mean absorbance at 405nm of triplicate samples.

246, 240-255, 256-272, 450-467, 460-477 and 477-492 strongly recognized plate-bound glucose transporter in ELISA assays. This is illustrated for antisera to peptide 231-246 in Fig.3.9. The antibody raised to residues 231-246 of the rat brain glucose transporter also strongly recognized the human erythrocyte protein on ELISA plates (result not shown). This is despite a single amino acid substitution within this sequence of the two proteins (Table 3.1(A). Antisera to the remaining peptides recognized the plate-bound transporter either weakly at low dilutions, (N-terminal peptide and peptides 34-60 and 293-306), or not at all, as illustrated for antisera to peptide 34-60 in Fig.3.9.

### 3.3.1.2 Western blotting

Despite the failure of several of the anti-peptide antibodies to recognize the plate-bound transporter, all except those against peptides 112-127 and 420-432 labelled the denatured transporter on Western blots of the purified protein (Section 2.4). The antibodies labelled a band of average apparent $M_r$ 55,000 corresponding to the intact glucose transporter (Sogin and Hinkle, 1978; Gorga et al., 1979), as illustrated for antibodies to peptides 231-245 and 450-467 in Fig.3.10. No labelling was seen with pre-immune sera (results not shown).

### 3.3.1.3 Competitive ELISA

The results of Western blotting experiments described above indicated that most of the anti-peptide antibodies recognized the denatured transporter. ELISA experiments showed that several of them also recognized the plate-bound transporter. However, it is likely that the latter is at least partially denatured. In order to determine whether the antisera recognized the transporter in its native

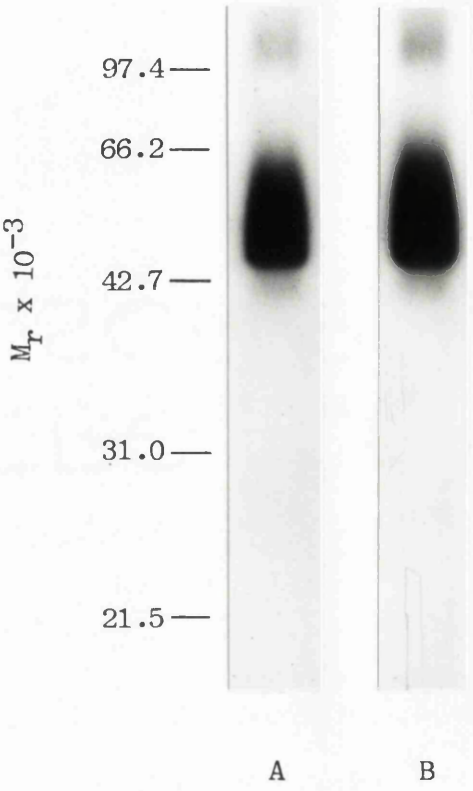



Fig.3.10 Recognition of the denatured glucose transporter by anti-peptide antibodies on Western blots

Samples (5µg), of the purified glucose transporter were electrophoresed on a SDS/10% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and immunolabelled with antiserum to peptide 231-246 (A), or antiserum to peptide 450-467 (B). Bound anti-peptide antibody was detected with goat anti-rabbit IgG-alkaline phosphatase conjugate as described in section 2.4. The positions of $M_r$ markers are indicated.

conformation, competitive ELISA experiments were performed using microtitre plates coated with purified glucose transporter, and protein-depleted erythrocyte membranes as the competing antigen. The membranes were used because they are known to be unsealed (Gorga and Lienhard, 1981), so that macromolecules have access to both sides of the membrane. The glucose transporter comprises about 10% of the protein in these membranes (Allard and Lienhard, 1985).

Samples (0.1ml), of a 1:2000 dilution of peptide antisera in PBSA, 1% BSA, were pre-incubated in eppendorf tubes for 2 hours at 25°C with various amounts of protein-depleted erythrocyte membranes (0 up to 220µg membrane protein), in a final volume of 0.2ml. Following incubation, bound and free antibody were separated by centrifugation at 11,500 rev/min for 15 minutes at 4°C using a MSE microcentaur centrifuge, and 0.1ml samples of the supernatants were added to the wells of an ELISA plate coated with purified glucose transporter (600ng per well). ELISA was then performed as described in Section 2.2. Values of $A_{405nm}$ for 'blank' incubations (from which antisera had been omitted), were subtracted from the experimental values. The % inhibition of antibody binding to plate-bound glucose transporter was calculated by comparing the absorbance in the presence of membranes with that of two standards, one with no membranes (giving 0% inhibition), and the other with no antibody (100% inhibition).

Antibodies in the sera to peptides 217-232, 231-246, 240-255, 256-272, 450-467, 460-477, 477-492 and rat peptide 231-246, all of which strongly recognized the plate-bound transporter, were inhibited in their binding to the glucose transporter by prior treatment with protein-depleted erythrocyte membranes. This is illustrated in Fig.3.11, which shows results obtained with antisera to the C-terminal peptide 477-492, where antibody binding to plates was inhibited by 80% at the highest amount of membranes used (25µg, containing

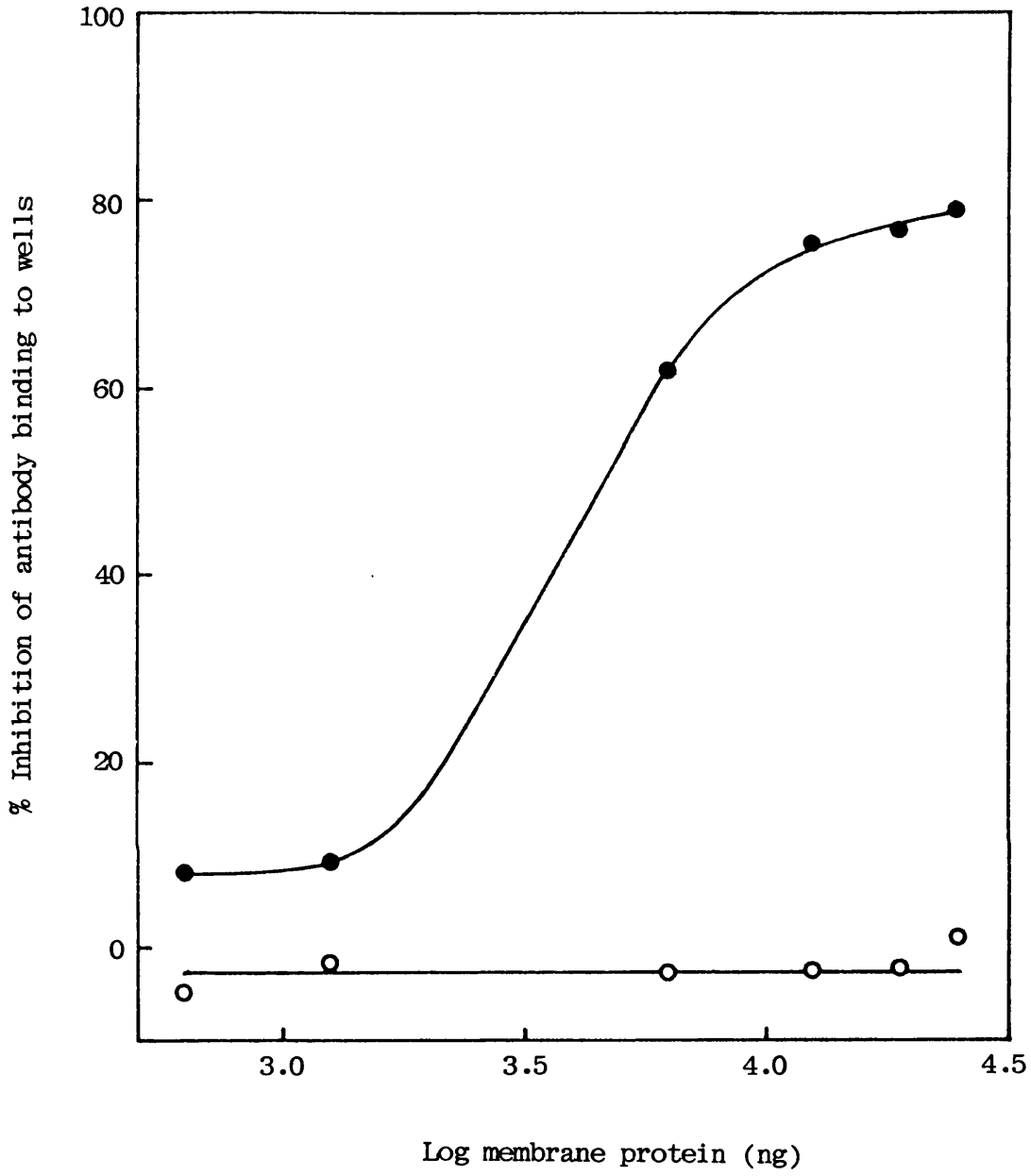



Fig.3.11 Competitive ELISA

The ability of protein-depleted erythrocyte membranes to compete for a limiting amount of antibody with 600ng of glucose transporter bound to the surface of a microtitre well. Samples of a 1 in 2000 dilution of antiserum were incubated with the amount of erythrocyte membrane protein indicated and then the amount of free antibody remaining in the supernatant after centrifugation was assessed by ELISA. Each point is the mean for triplicate samples. ●, anti-C-terminal peptide 477-492 serum; o, anti-N-terminal peptide serum.

approximately 2.5μg of glucose transporter). These results indicate that all the antibodies that strongly recognized the ELISA plate-bound glucose transporter also recognized the protein in its native conformation. In contrast, antibodies to the N-terminal peptide (residues 1-15), which only weakly recognized the plate-bound transporter, were not inhibited in their binding to the latter by 25μg of membranes (Fig.3.11), or up to 220μg of membranes (results not shown). Identical findings were made for the anti-peptide sera that weakly recognized the plate-bound protein, when either glucose transporter or the relevant synthetic peptide was used as the plate-bound antigen (results not shown). Therefore the latter antibodies did not recognize the transporter in its native state.

The experiment was repeated with antibodies to peptides 217-232, 231-246, 240-255, 256-272, 450-467, 460-477 and 477-492 which had been affinity-purified by adsorption onto protein-depleted erythrocyte membranes (Section 3.2.5.2), the results of which are shown in Fig.3.12. As expected, each antibody was inhibited in its binding to plate-bound glucose transporter following treatment with protein-depleted erythrocyte membranes (up to 220μg membrane protein). Interestingly, whilst antibodies to the three peptides derived from the C-terminal sequence 450-492 of the transporter were totally inhibited in their binding to glucose transporter by 25μg of membranes, an approximate 10-fold increase in the amount of membranes (220μg), was required to inhibit antibodies to peptides 217-232, 231-246 and 240-255 to the same extent, and antibodies to peptide 256-272 by 70% (Fig.3.12). These results may reflect a difference in the affinity of antibodies to the C-terminal and central hydrophilic regions of the transporter (Fig.3.2). Such differences in affinity might possibly stem from differences in the degree of segmental mobility in the two regions of the protein (discussed in Section 3.4).

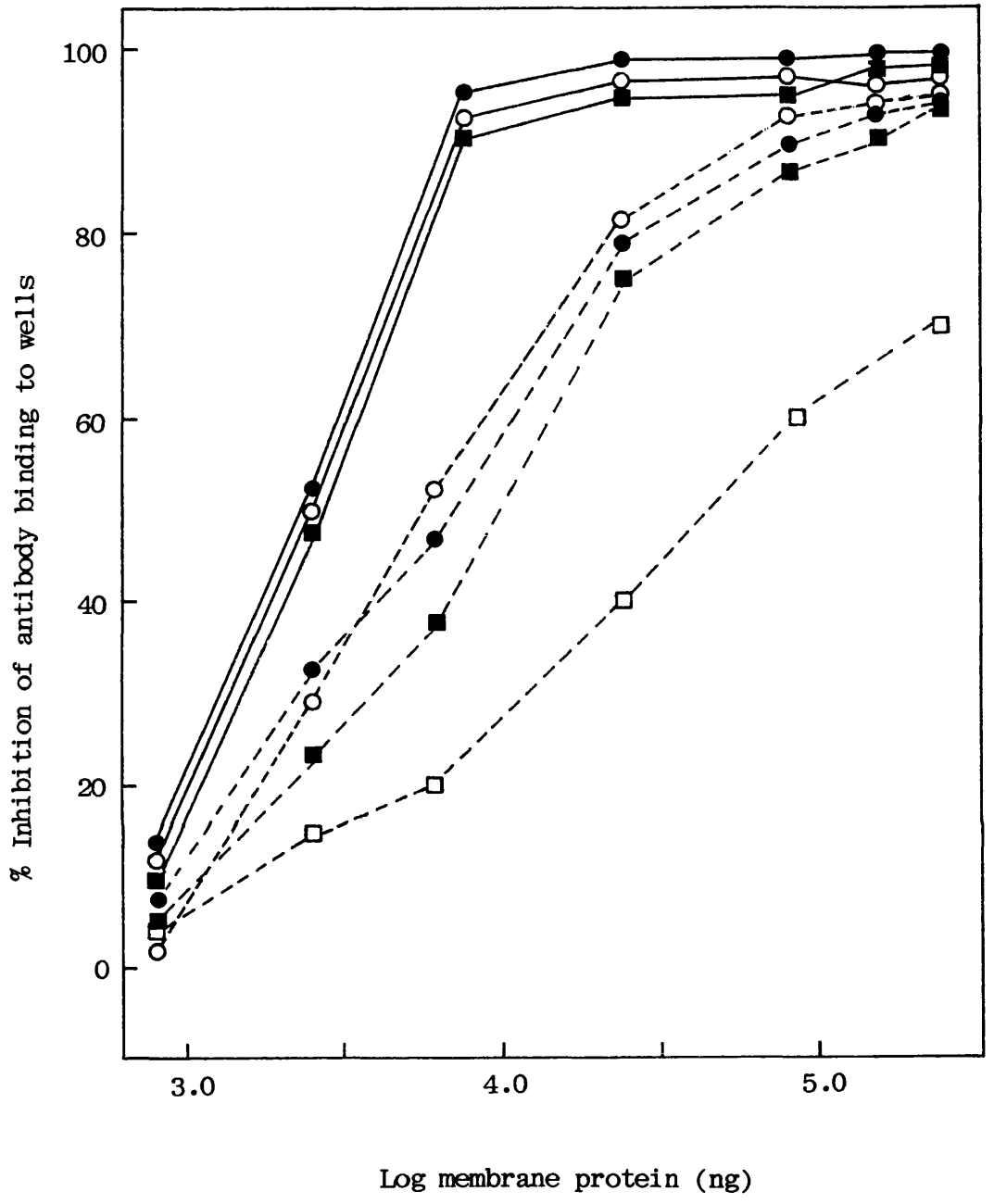



Fig.3.12 Competitive ELISA

The ability of protein-depleted erythrocyte membranes to compete for a limiting amount of antibody with 600ng of glucose transporter bound to the surface of a microtitre well. Samples of 1µg/ml solutions of affinity-purified antibodies against peptides 217-232 (■-■), 231-246 (●-●), 240-255 (○-○), 256-272 (□-□), 450-467 (■-■), 460-477 (○-○) and 477-492 (●-●), were treated with protein-depleted erythrocyte membranes as described in the legend to Fig.3.11. Each point is the mean for triplicate samples.

3.3.1.4 Immunoadsorption assay

The affinity-purified antibodies were used in further experiments to demonstrate not only that they recognized the native glucose transporter, but that they were specific for the latter. Such experiments were necessary because both the glucose and nucleoside transport proteins migrate as broad bands of identical $M_r$ on SDS/polyacrylamide gels (Kwong et al., 1986). Because of their similarities in size, kinetics and function, it has been suggested that the two proteins may in fact be homologous in sequence (Young et al., 1983b). Furthermore, both are present not only in protein-depleted erythrocyte membranes but also in the 'purified' glucose transporter preparation (Jarvis and Young, 1981; Baldwin et al., 1982). Western blotting and ELISA assays cannot therefore unambiguously establish to which species the antibodies bind. However, the two transporters do differ in that the glucose transporter specifically binds the inhibitor cytochalasin B whilst the nucleoside transporter specifically binds the inhibitor NBMPR (Lin and Spudich, 1974; Jarvis and Young, 1981, Section 2.5). Assay of the binding of these two ligands was therefore used to quantify the ability of immobilized anti-peptide antibodies to remove each transporter from a detergent-solubilized mixture of the two proteins.

To this end, immunoadsorption experiments were performed in the following way; purified glucose transporter (52µg/ml) in 50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH 7.4, was solubilized with 1% (w/v) octylglucoside at 4°C. Samples (1ml), were then incubated with 0.2ml of protein A-Sepharose CL4B to which 1.2mg of non-immune rabbit IgG (Sigma), or affinity-purified antibodies to peptides 231-246, 240-255, 256-272, 450-467, 460-477 and 477-492 were bound. Following incubation for 1 hour at 4°C with constant mixing, the samples were centrifuged (11,500 rev/min for 10 minutes at 4°C), the supernatants

| Immunoadsorbant | % Ligand binding activity remaining in solution | |
|---|---|---|
| | Cytochalasin B | NBMPR |
| Non-immune rabbit IgG | 100 | 100 |
| Anti-peptide 231-246 | 0 | 83 |
| Anti-peptide 240-255 | 0 | 93 |
| Anti-peptide 256-272 | 5 | 91 |
| Anti-peptide 450-467 | 4 | 95 |
| Anti-peptide 460-477 | 4 | 89 |
| Anti-peptide 477-492 | 12 | 91 |

Table 3.2 Immunoadsorption of solubilized glucose and nucleoside transporters

Removal of cytochalasin B and NBMPR binding activities from a detergent-solubilized preparation of purified glucose transporter by immobilized anti-peptide antibodies or non-immune rabbit IgG.

made 2mM in DTT, and then dialysed against 3x2 litres of 50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH 7.4, to remove detergent. The reconstituted supernatants were then assayed for cytochalasin B and NBMPR binding activity as described in Section 2.5. The residual cytochalasin B and NBMPR binding activities of the reconstituted supernatants are shown in Table 3.2. Each of the anti-peptide antibodies was found to remove greater than 85% of the cytochalasin B binding sites from the solution relative to the control, but only 5-17% of the NBMPR binding sites. (Insufficient antibody to peptide 217-232 was available for this experiment). The failure of some antibodies to absorb all the glucose transporter, even using this 8-fold molar excess of antibody, probably stemmed from hindrance of transporter access to all the bound immunoglobulin by the Sepharose matrix. Not only did these results demonstrate the specificity of the antibodies for the glucose transporter, but they also confirmed the ability of the antibodies to recognize the native protein, because only the latter is detectable in ligand-binding assays. In addition, it would appear that the nucleoside transporter shares little if any primary sequence homology with residues 231-272 and 450-492 of the glucose transporter polypeptide.

### 3.3.2 Immunoaffinity purification of the glucose transporter from a preparation of erythrocyte band 4.5 proteins.

As discussed in Sections 1.5 and 3.3.1.4, the 'purified' glucose transporter preparation is actually a mixture of erythrocyte band 4.5 proteins. The preparation consists largely of the glucose transporter (about 95%), with the nucleoside transporter present as a minor component (up to 5%). Immunoaffinity chromatography, using a column of immobilized anti-peptide antibody to isolate the glucose transporter from a preparation of band 4.5 proteins, has provided further evidence

for the specificity of the antibodies and a means of purifying the glucose transporter to homogeneity. To this end, a preparation of detergent-solubilized band 4.5 proteins was applied to a column containing immobilized antibodies to C-terminal peptide 477-492, and the products of chromatography characterized by ligand binding and Western blotting.

3.3.2.1 Preparation of the immunoaffinity column.

Affinity-purified antibodies to the C-terminal peptide 477-492 (12mg in 6ml of PBS, pH 7.2), were adsorbed on to 2ml of washed protein A-Sepharose CL4B by incubation with constant mixing at for 1 hour at 4°C. The incubation was centrifuged at 500 rev/min for 5 minutes at 4°C, and the supernatant assayed for protein to ensure complete binding of antibody to the protein A-Sepharose. The affinity adsorbant was washed in excess PBS, pH 7.2, followed by 10ml of 0.2M triethanolamine-HCl, pH 8.2, for 5 minutes at 25°C. Covalent cross-linking was then achieved by incubation of the antibody-protein A-Sepharose complex for 45 minutes at 25°C with 50ml of 20mM dimethylpimelimidate in 200mM triethanolamine, pH 8.2, essentially as described by Schneider et al. (1982). The resulting affinity adsorbant was packed into a column, washed thoroughly with PBS, pH 7.2 and stored in the same buffer containing 0.02% sodium azide. A control column containing 12mg of non-immune rabbit IgG (Sigma), was also prepared in the same way.

3.3.2.2 Immunoaffinity chromatography.

Human erythrocyte band 4.5 proteins were prepared as described in Section 2.1.4. The proteins (0.133mg/ml), were solubilized at 4°C in 50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH 7.4 containing 1% (w/v) octylglucoside, and samples (15.8ml;2mg), were applied to the

affinity and control columns which had been equilibrated with the detergent-containing buffer. The eluates were recirculated through the columns five times at a flow rate of 20 column volumes/hour, and the unbound material collected. The eluates were made 2mM in DTT before removal of detergent by dialysis against 3x2 litres of 50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH 7.4. A control sample of solubilized, but unfractionated band 4.5 proteins was dialysed in parallel. The columns were washed in 5ml of the octylglucoside-containing buffer and then equilibrated in 5ml of 50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH 7.4 containing 0.5% (v/v) Triton X-100. Bound glucose transporter was eluted from the columns in a buffer of 0.2M glycine-HCl, pH 2.4, containing 100mM NaCl, 1mM EDTA and 0.5% Triton X-100, and 1ml fractions were collected into polypropylene tubes containing 1M $Na_2HPO_4$ to bring the pH to neutrality. The resultant glucose transporter-containing fractions were reconstituted by removal of Triton X-100 with Biobeads (Sigma). Before re-use any bound glucose transporter remaining on the columns was removed by washing with 10ml of 50mM diethylamine-HCl, pH 11, containing 0.5% (w/v) sodium deoxycholate. The regenerated column was stored at $4^{o}C$ in PBS, pH 7.2, containing 0.02% sodium azide.

Cytochalasin B and NBMPR binding activities of the dialysed eluates, unfractionated band 4.5 proteins and Biobead-treated glucose transporter-containing fractions were determined by equilibrium dialysis (Section 2.5). The protein concentrations of all samples were estimated by the method of Peterson (1977). The results of such an experiment are shown in Table 3.3. The C-terminal peptide antibody column was found to bind most of the applied protein relative to the control column, about 6% of the total protein was collected in the unbound fraction. In addition, cytochalasin B binding assay indicated the complete removal of functionally-active glucose transporters from

| | Solubilized, unfractionated, band 4.5 proteins | Control protein-A-Sepharose IgG column | Immunoaffinity column eluate | Acid-eluted fraction |
|---|---|---|---|---|
| Protein (mg) | 2.0 | 2.0 | 0.12 | 1.1 |
| Cytochalasin B binding sites | | | | |
| Amount (nmol) | 11.70 | 10.98 | 0.0 | — |
| Yield (%) | 100 | 94 | 0.0 | — |
| Specific binding activity (nmol/mg protein) | 5.85 (6.38) | 5.49 | 0.0 | — |
| NBMPR binding sites | | | | |
| Amount (pmol) | 544 | 359 | 321 | — |
| Yield (%) | 100 | 66 | 59 | — |
| Specific binding activity (nmol/mg protein) | 0.27 (0.31) | 0.180 | 2.68 | — |

Table 3.3 Cytochalasin B and NBMPR binding activities of the products of immunoaffinity purification of the glucose transporter from a preparation of erythrocyte band 4.5 proteins

The results of three separate experiments are shown using the same preparation of band 4.5 proteins. NBMPR binding was performed at a single saturating ligand concentration (60nM). Values are corrected for non-specific NBMPR binding, measured in the presence of 20μM NBTGR. The concentrations of cytochalasin B binding sites were estimated from bound to free ratios measured at a ligand concentration of 40nM assuming a $K_d$ of 0.12 μM (Zoccoli et al., 1978). Values are corrected for non-specific cytochalasin B binding, measured after heating samples to 100°C for 5 minutes. Figures in brackets are values obtained for preparations prior to detergent solubilization.

the preparation. About 55% of the total protein applied to the column was recovered following treatment of the column with Triton X-100 under acidic conditions. However, this method of elution yielded functionally-inactive glucose transporter, which was most probably a consequence of the extreme conditions of pH. The use of acidic elution conditions was necessary, as earlier attempts to elute the active protein at neutral pH with a chaotropic agent (3M KSCN), or a 200-fold molar excess of synthetic C-terminal peptide in the presence of detergent, had failed (results not shown). In future experiments, it might be possible to recover the transporter in a functionally-active state by the inclusion of supplementary phospholipids in the washing and elution buffers, or through the use of milder elution conditions. However, no attempt was made to isolate the pure, functionally-active transporter during the course of this project.

NBMPR binding assay showed that the unbound fraction from the affinity column contained 89% and 59% of the functionally-active nucleoside transporters present in the control column eluate and solubilized, unfractionated band 4.5 protein samples respectively. The loss of NBMPR binding activity during chromatography may have resulted from denaturation of the nucleoside transporter in detergent, which is known to occur for the glucose transporter (Baldwin et al., 1982).

In order to assess the purity of the acid-eluted glucose transporter-containing fractions, the material was subjected to Western blot analysis. Samples (10µg), of the peak acid-eluted transporter-containing fractions and the unbound fraction from the affinity column, were prepared for SDS/polyacrylamide gel electrophoresis in a modified gel sample buffer in which the final concentration of SDS was increased from 0.8 to 2% (w/v) (Section 2.3), because of the possible presence of residual Triton X-100 in the acid-eluted fractions. The samples were electrophoresed on a SDS/12%

polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and immunolabelled with the relevant antibodies. The antibodies used were GTPR1, a mouse monoclonal antibody specific for an epitope in the central hydrophilic region of the glucose transporter (Boyle et al., 1985 and Chapter 5 of this thesis), a rabbit polyclonal antibody raised against the purified human nucleoside transporter (Kwong et al., 1989) and antibodies to the glucose transporter C-terminal peptide 477-492, each of which had been purified by adsorption onto protein-depleted erythrocyte membranes. The Western blot results are shown in Fig.3.13. The C-terminal peptide antibody (Fig.3.13(A)), labelled the intact glucose transporter ($M_r$ 55,000) in the acid-eluted fractions (lanes 2-4), together with two fragments derived from endogenous proteolysis of the transporter which must contain the intact C-terminal sequence of the protein. No labelling of material in the unbound fraction from the affinity column was seen (lane 5). Monoclonal antibody GTPR1 (Fig.3.12(B), labelled the acid-eluted glucose transporter and a single proteolytic fragment (lanes 7-9). In addition, it showed weak labelling of the unbound fraction (lane 10). The nucleoside transporter-specific antibody (Fig.3.12(C), labelled none of the acid-eluted fractions, but did label the unbound fraction (lane 15), and the control glucose transporter sample (lane 11). Therefore, the acid-eluted fragments appear to contain only glucose transporter, whilst the unbound fraction contains predominantly nucleoside transporter with traces of functionally-inactive glucose transporter which, as it did not bind to the affinity column, must be devoid of the intact C-terminal sequence of the protein. Consequently, the specificity of the anti-peptide antibodies for the glucose transporter has been further demonstrated and the transporter has been purified to probable homogeneity from other erythrocyte band 4.5 proteins, albeit in a functionally-inactive form.

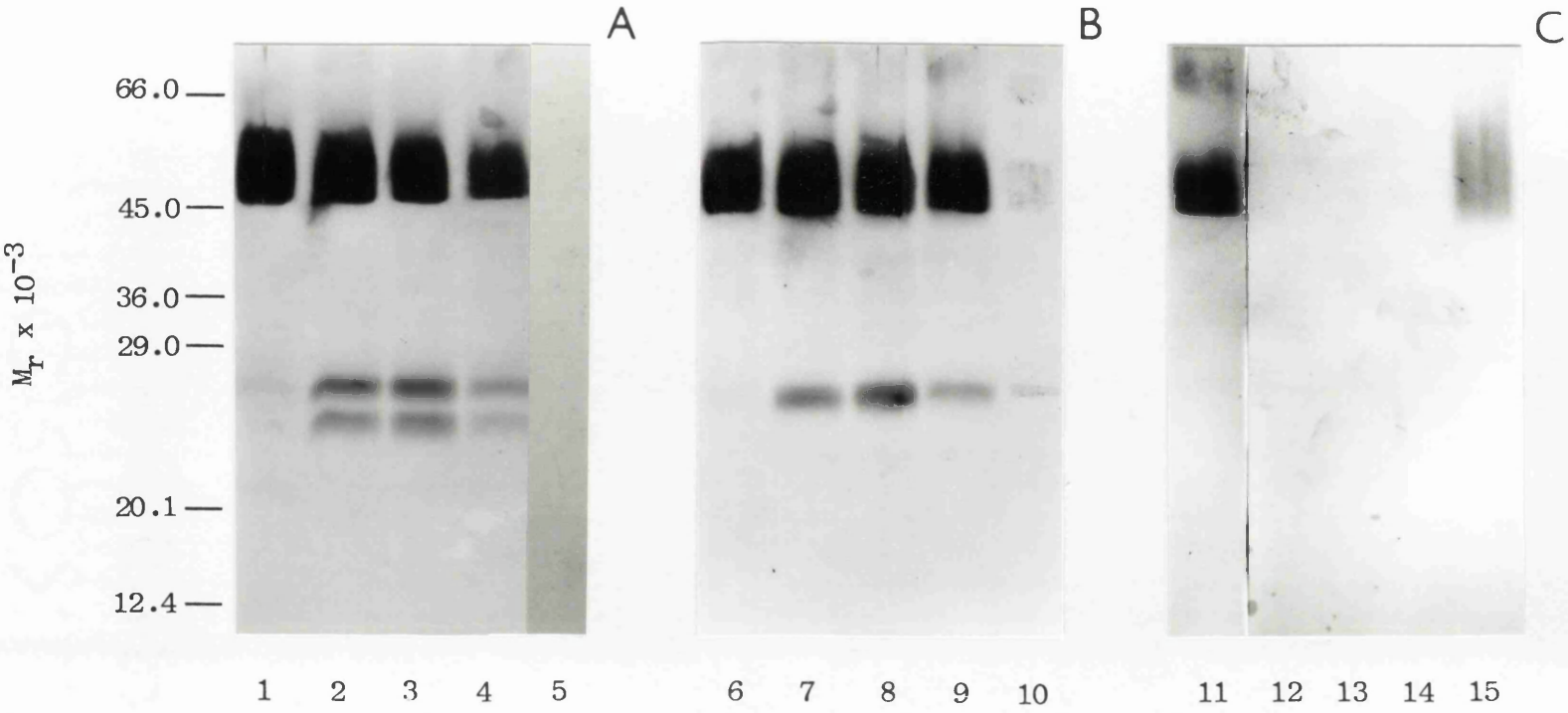



Fig.3.13 Western blot analysis of the products of immunoaffinity purification of the glucose transporter

Samples (10µg), of solubilized, but unfractionated erythrocyte band 4.5 proteins (lanes 1, 6 and 11), peak acid-eluted column fractions (lanes 2-4, 7-9 and 12-14), and unbound column material (lanes 5, 10 and 15), were electrophoresed on a SDS/12% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and immunolabelled with antibodies to C-terminal peptide 477-492 (A), monoclonal antibody GTPR1 (B), or anti-nucleoside transporter antibodies (C). The positions of $M_r$ markers are indicated.

## 3.4 DISCUSSION.

Seventeen antibodies were raised to peptides corresponding in sequence to regions of the human erythrocyte glucose transporter polypeptide predicted to be extramembranous in the model of Mueckler et al. (1985). Of these, seven were found to recognize the native, functionally-active protein as assessed by competitive ELISA and immunoadsorption assay, whilst all but two were found to recognize the denatured transporter on Western blots.

Antibody binding to the native protein was confined to the regions comprising residues 217-272 and 450-492, which constitute most of the predicted extramembranous central hydrophilic and C-terminal regions of the transporter (Mueckler et al., 1985). These regions also appear to be the main immunogenic regions of the native protein, because polyclonal antibodies raised against the intact, purified glucose transporter recognized in ELISA only those synthetic peptides corresponding to residues 217-272 and 450-492 of the transporter polypeptide. Furthermore the epitopes for a number of monoclonal antibodies raised against the intact protein also were all found to lie within these two regions (discussed in Chapter 5 of this thesis).

The lack of binding to the native protein of any of the antibodies raised against putative extracellular regions of the transporter, against the predicted short loops connecting transmembrane sequences at the cytoplasmic surface of the membrane, and against the N-terminal sequence may have several explanations. In the case of the extracellular regions, it may stem from the fact that there are very few sequence differences between these regions in the rabbit and human glucose transporters (Asano et al., 1988), or that the extracellularly-attached oligosaccharide chain of the transporter might sterically hinder the access of IgG molecules. (The latter

possibility will be addressed in Section 4.2.3). In addition, the prediction of the model that certain regions of the sequence lie outside the membrane may be incorrect or they may be constrained in conformations too different from those available to the peptide immunogens. Studies on water-soluble proteins have found that anti-peptide antibodies against highly mobile regions tend to react strongly with the native protein whereas anti-peptide antibodies against well-ordered regions do not (Tainer et al., 1984). (This may also explain why antibodies to the C-terminal region appeared to show a higher affinity for the native glucose transporter in competitive ELISA than antibodies to the central cytoplasmic region). Alternatively, the short loops may be too closely associated with the phospholipid head groups or other parts of the protein to allow antibody access. This is supported further by the observation that although many of these short loops, (including the N-terminal region), contain potential sites for tryptic cleavage (Mueckler et al., 1985), they are in fact resistant to proteolysis (Lienhard et al., 1984; Cairns et al., 1987). However, if the digestion is performed in the presence of 0.1% Triton X-100, further fragmentation of the transporter is obtained, indicating that at least some of these potential sites of tryptic cleavage are not intrinsically resistant to trypsin (Cairns et al., 1987).

Using a column of immobilized anti-peptide antibodies, it has been possible to purify the human erythrocyte glucose transporter to homogeneity, but in a functionally-inactive state. Such an approach could be used to isolate homologous glucose transport proteins from other tissue types. With the use of appropriate conditions (e.g. mild elution conditions and supplementation with phospholipids), it might be possible to isolate these proteins in a functionally-active form.

# CHAPTER 4 ANTI-PEPTIDE ANTIBODIES AS PROBES OF THE TRANSMEMBRANE TOPOLOGY AND FUNCTION OF THE GLUCOSE TRANSPORTER IN THE HUMAN ERYTHROCYTE MEMBRANE

## 4.1 INTRODUCTION

In order fully to understand the mechanism of glucose transport across cell membranes, it will ultimately be necessary to determine the 3-dimensional structure of the glucose transport protein by high resolution X-ray or electron diffraction studies. However, this approach awaits the successful crystallization of the functionally-active protein. As a prerequisite to crystallization, delipidation of the glucose transporter preparation would probably be necessary. However, removal of associated lipid results in a marked decrease in the functional activity of the transporter as a result of its denaturation (Sogin and Hinkle, 1978). Procedures suitable for delipidation of the transporter, whilst maintaining the protein in its native, functionally-active state are currently being sought. In the meantime, information on the arrangement of the glucose transporter polypeptide in the membrane must be obtained by other means. In this chapter, I will describe the use of a panel of anti-peptide antibodies raised against the glucose transporter (prepared and characterized as described in Chapter 3), as powerful specific probes to study the structure and function of this membrane-bound protein.

### 4.1.1 Transmembrane topology of the glucose transporter

The model for the transmembrane topology of the glucose transporter was proposed in 1985 by Mueckler *et al.* (Section 1.7.3), is shown schematically in Fig.4.1(A). This model was based largely upon hydropathic analysis of the recently determined amino acid

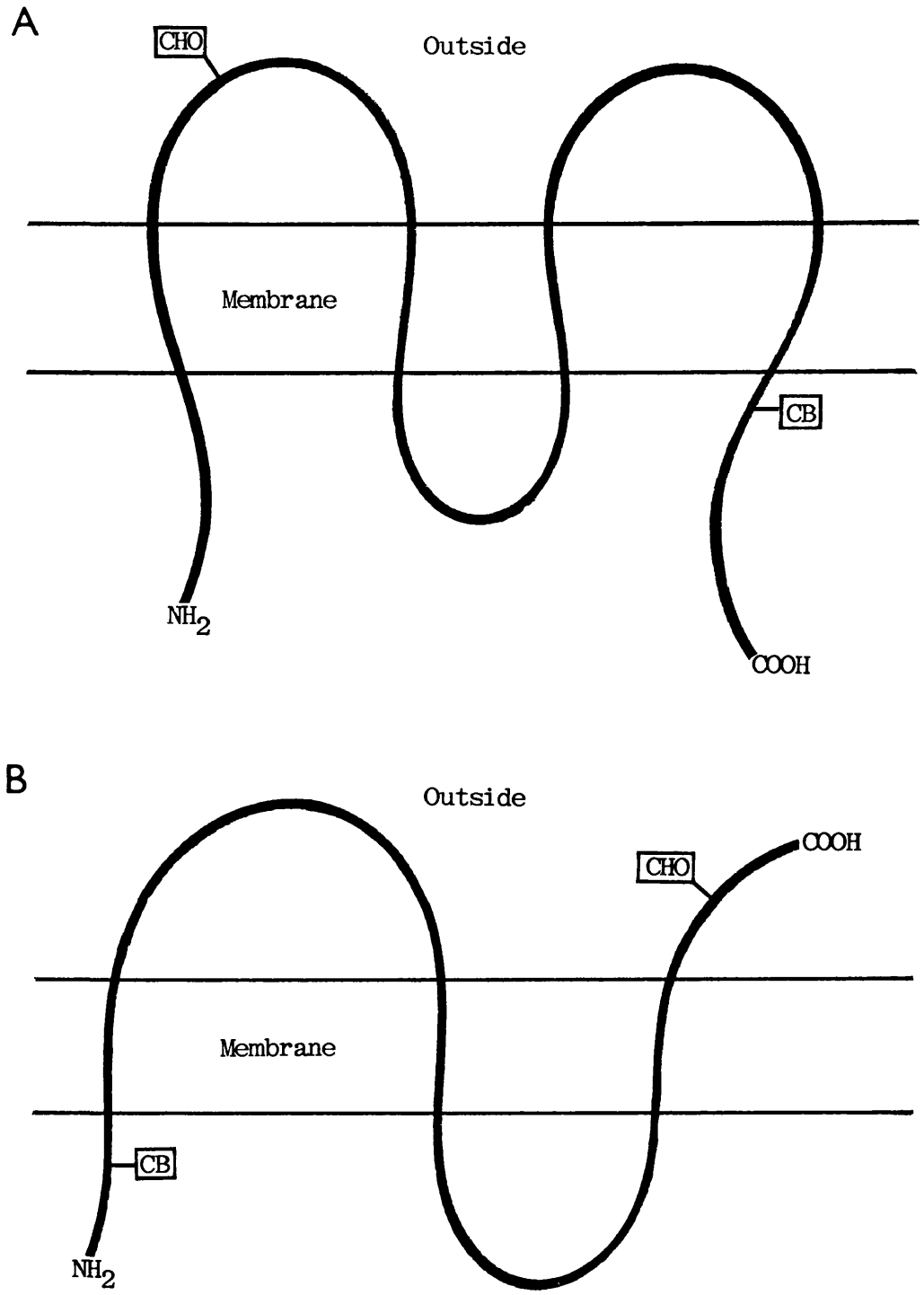



Fig.4.1 Conflicting models for the arrangement of the glucose transporter polypeptide in the erythrocyte membrane

A few only of the putative membrane-spanning regions are shown. (A) model based on hydropathy analysis, after Mueckler et al., (1985), (B) model based on in situ enzymic digestion of the transporter, after Shanahan and D'Artel-Ellis (1984). The predicted sites of oligosaccharide attachment are indicated by CHO, CB indicates the cytochalasin B binding sites.

sequence, although certain of its features were supported by the results of vectorial proteolytic digestion experiments (Mueckler et al., 1985). Because of the tentative nature of this model, in the current project site-specific anti-peptide antibodies were used in an attempt to provide direct evidence for the transmembrane topology of the protein. It was felt that such an approach was of particular importance given that the model of Mueckler et al. conflicted in many ways with one proposed by Shanahan and D'Artel-Ellis (1984) based on enzymic dissection experiments on the transporter in situ (Sections 1.7.1and 1.7.3). The salient features of the latter model are shown schematically in Fig.4.1(B). In the work described in this chapter, anti-peptide antibodies were used not only to resolve the conflict between the two models with regard to the location of the C-terminus and site of glycosylation of the protein, but also to investigate the locations of other regions of the protein with respect to the plane of the bilayer.

### 4.1.2 Function of the glucose transporter

As discussed in Section 1.8, little is known about the mechanism by which the glucose transport protein facilitates the passage of glucose across the erythrocyte membrane. Evidence for the involvement of conformational changes in the mechanism of transport is strong, but the precise identity of those regions of the protein that are affected by, or involved in, the changes remains largely unknown (Sections 1.8.2 and 1.8.3). Previous studies have shown that removal of the large, cytoplasmic regions of the transporter by tryptic digestion destroys the ability of the protein to transport hexose, and lowers its affinity for cytochalasin B (Baldwin et al., 1980; Cairns et al., 1984, Section 1.7.4). Additionally, these regions of the transporter have been implicated in conformational changes in the protein as a

result of ligand binding (Gibbs et al., 1988, Section 1.8.3). However, in these studies, it was not possible to examine the roles of each of these regions of the sequence in transporter function independently. This was made possible in the present study with the use of anti-peptide antibodies as probes of transporter function. Using antibodies, an attempt was made to identify more precisely those extramembranous regions of the glucose transporter polypeptide which may be functionally important in the protein. To this end, a study was made of the ability of antibodies, raised against known regions of the transporter sequence, to affect the reversible cytochalasin B binding activity of the protein. In addition, the effect of transporter-bound ligands on antibody binding to the transporter was examined as a means of identifying extramembranous regions of the protein which may be involved in conformational changes associated with the binding of a particular ligand.

## 4.2 ANTI-PEPTIDE ANTIBODIES AS PROBES OF THE TRANSMEMBRANE TOPOLOGY OF THE GLUCOSE TRANSPORTER POLYPEPTIDE

### 4.2.1 Location of the C-terminus of the glucose transporter

In Sections 3.3.1.3 and 3.3.1.4, three anti-peptide antibodies directed against the C-terminal region of the glucose transporter were found to recognize the native protein in competitive ELISA and immunoadsorption assays. It was therefore possible to use these antibodies to establish at which surface of the erythrocyte membrane the C-terminal region of the glucose transporter is exposed. To this end, competitive ELISA experiments were performed using the anti-peptide antibodies in conjunction with erythrocyte membrane vesicles of known sidedness as competing antigens. The preparation and characterization of these vesicles is described in Section 2.1.2. Marker enzyme assays indicated that the preparation of 'right-side-out' vesicles used in these experiments contained 92% sealed, right-side-out vesicles contaminated with 8% inside-out vesicles, whilst the 'inside-out' vesicle preparation contained 80% sealed, inside-out vesicles and 20% right-side-out vesicles. Both types of vesicle are known to contain equal amounts of glucose transporter per mg of membrane protein (Boyle et al., 1985).

Competitive ELISA was performed as described in Section 3.3.1.3. Initial experiments were performed using antiserum against the peptide corresponding to residues 477-492 at the extreme C-terminal end of the transporter polypeptide. Samples (0.1ml), of a 1:2000 dilution of the antiserum were incubated with a varying amount of 'right-side-out' or 'inside-out' vesicle membrane protein (up to 25µg/incubation). Following centrifugation of the vesicle-containing samples, the amount of free antibody remaining in the supernatant was determined by ELISA with 600ng of purified glucose transporter as the plate-bound antigen.

The results of such an experiment are shown in Fig.4.2(A). The 'inside-out' vesicle preparation was about 10-fold more effective than the 'right-side-out' vesicle preparation in competing with plate-bound glucose transporter for antibody binding. This result paralleled the relative concentrations of 'inside-out' vesicles in the two preparations and suggested that the C-terminus of the protein is exposed at the cytoplasmic surface of the erythrocyte membrane.

Ideally, such competitive ELISA experiments should have been performed using vesicle preparations containing 100% of either 'right-side-out' or 'inside-out' membranes. However, it was not possible to generate vesicle preparations of this purity. Therefore, to provide additional evidence for the location of the protein C-terminus, the ability of intact erythrocytes and unsealed, protein-depleted erythrocyte membranes to bind the antibody was compared in another competitive ELISA experiment. Protein-depleted erythrocyte membranes and intact erythrocytes were prepared as described in Sections 2.1.3 and 2.1.5 respectively. The results of such an experiment are shown in Fig.4.2(B). Protein-depleted membranes inhibited the binding of antibody to plate-bound glucose transporter by almost 85% at the highest amount used (25μg/incubation). Since the glucose transporter comprises about 10% of protein in these membranes (Allard and Lienhard, 1985), then the highest amount of membranes used would have contained about 2.5μg of glucose transporter. In contrast, the intact erythrocytes caused negligible inhibition even at the highest number used ($1.25 \times 10^8$ cells/incubation). Since an erythrocyte contains about $8 \times 10^{-8}$mg of membrane protein (Zoccoli and Lienhard, 1977), and the glucose transporter comprises 5% of total membrane protein (Allard and Lienhard, 1985), then this number of cells should contain about 5μg of glucose transporter. These results therefore firmly support the conclusion that the C-terminus of the transporter

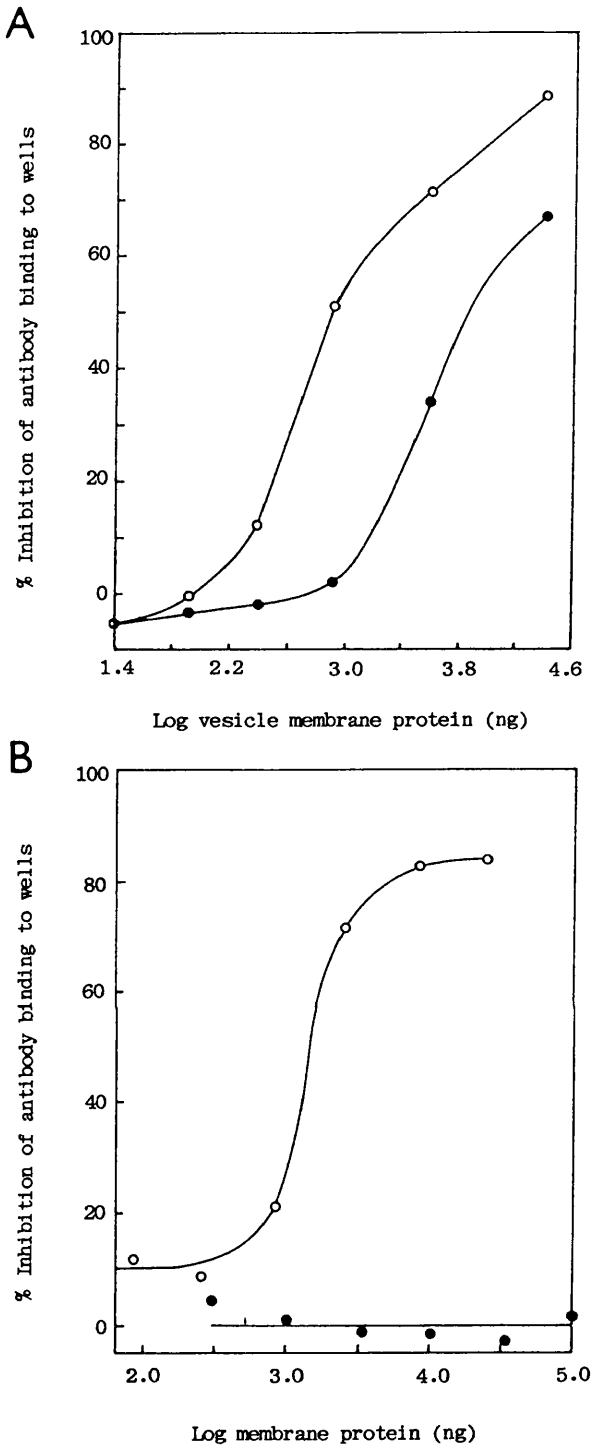



Fig.4.2 Establishment of the sidedness of antibody binding by competitive ELISA

The ability of (A) inside-out (○) and right-side-out (●) erythrocyte membrane vesicles, and (B) protein-depleted erythrocyte membranes (○) and intact erythrocytes (●), to compete for a limiting amount of antibodies to C-terminal peptide 477-492 with 600ng of glucose transporter bound to the surface of a microtitre well. Samples (0.1ml), of a 1 in 2000 dilution of antiserum were incubated with the amount of erythrocyte membrane protein indicated and then the amount of free antibody remaining in the supernatant after centrifugation was assessed by ELISA. Each point is the mean for triplicate samples.

is exposed at the cytoplasmic surface of the erythrocyte membrane.

A similar approach was used to establish the location of C-terminal residues 450 to 477 of the transporter polypeptide. The ability of intact erythrocytes and protein-depleted erythrocyte membranes to bind affinity-purified antibodies against peptides corresponding to residues 450-467 and 460-477 of the transporter (Fig. 3.2), were compared. Affinity-purified antibodies to peptide 477-492 were also included for comparison. The results of these competitive ELISA experiments are shown in Fig.4.3. Under the experimental conditions used, essentially complete inhibition of the binding of 100ng of each C-terminal peptide antibody to plate-bound glucose transporter was produced following incubation with 25μg of protein-depleted erythrocyte membrane protein. In contrast, intact erythrocytes containing equivalent amounts of glucose transporter had no effect on antibody binding to plate-bound glucose transporter. Taken together, these findings suggest that most of residues 450-492 of the transporter polypeptide are located at the cytoplasmic surface of the membrane and must be readily accessible to macromolecules.

### 4.2.2 Location of residues 217 to 272 of the glucose transporter

In Sections 3.3.1.3 and 3.3.1.4, four anti-peptide antibodies directed against residues 217 to 272 of the transporter polypeptide, which, in the model of Mueckler et al. (1985), are predicted to constitute most of a large, cytoplasmically-located hydrophilic region towards the centre of the protein (Fig. 3.2), were found to recognize the native transporter in competitive ELISA and immunoadsorption assays. Consequently, these antibodies could also be used to establish at which surface of the erythrocyte membrane this region of the transporter polypeptide is exposed. Again, competitive ELISA using intact erythrocytes and protein-depleted erythrocyte membranes as

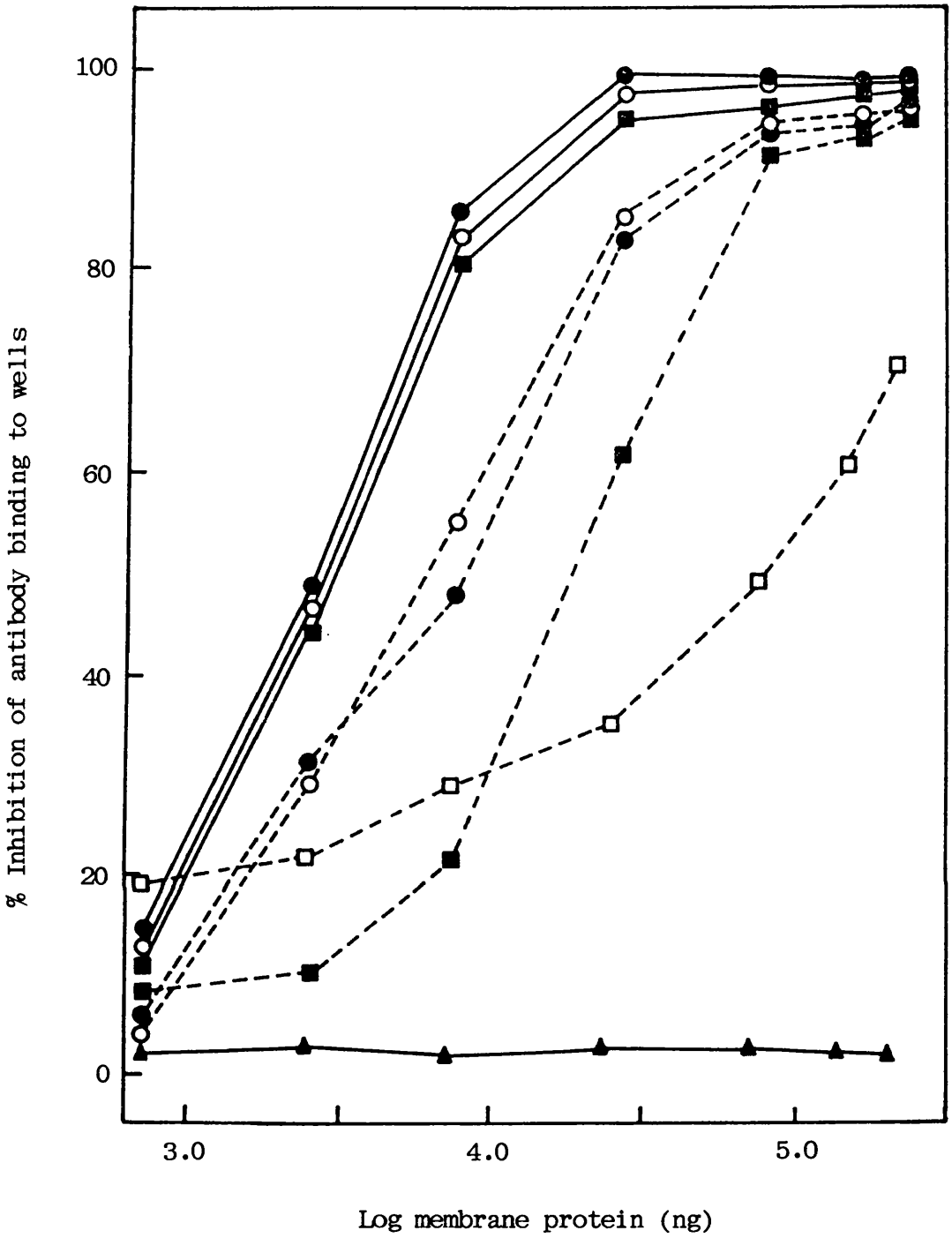



Fig.4.3 Establishment of the sidedness of antibody binding by competitive ELISA

The ability of intact erythrocytes (▲), and protein-depleted erythrocyte membranes (○ ● □ ■), to compete for a limiting amount of antibody with 600ng of glucose transporter bound to the surface of a microtitre well. Samples (0.1ml), of 1µg/ml solutions of affinity-purified antibodies against peptides 217-232 ( ■-■ ), 231-246 (●-●), 240-255 (○-○), 256-272 ( □-□ ), 450-467 ( ■—■ ), 460-477 (○—○) and 477-492 (●—●), were incubated with the amount of erythrocyte membrane protein indicated. Remaining free antibody was determined as described in the legend to Fig.4.2. For incubations with protein-depleted erythrocyte membranes, each point is the mean for triplicate samples. The values yielded by intact erythrocytes did not differ significantly for each antibody, and so are given as the mean for all 7 antibodies.

competing antigens was employed as described in Section 4.2.1. The results of these experiments are given in Fig.4.3. Under the experimental conditions used, almost complete inhibition (>90%) of the binding of 100ng of affinity-purified antibodies to peptides 217-232, 231-246 and 240-255 to plate-bound glucose transporter was produced, when up to 220μg of protein-depleted erythrocyte membrane protein was used as competing antigen. Inhibition of the binding of antibodies to peptide 256-272 was substantial (up to 70%), but was not complete at the highest concentration of membranes used in these experiments. In contrast, intact erythrocytes containing equivalent amounts of glucose transporter had no effect on the binding of any of the antibodies to plate-bound glucose transporter (Fig.4.3). Therefore, much of the glucose transporter polypeptide encompassing residues 217-272 must indeed be exposed at the cytoplasmic surface of the erythrocyte membrane. The ability of IgG molecules to bind to most of this region indicates that it is readily accessible to the aqueous interior of the cell.

### 4.2.3 Location of regions of the glucose transporter sequence predicted to be exposed at the extracellular surface of the erythrocyte membrane

Anti-peptide antibodies raised to five regions of the transporter sequence predicted to be extramembranous in the model of Mueckler *et al*. (1985) (residues 34-60, 112-127, 175-189, 293-306 and 420-432, Fig.3.2), were all found to demonstrate high anti-peptide activity in ELISA (Section 3.3.1.1). Except for antibodies against peptides 112-127 and 420-432, these antibodies also all recognized the denatured glucose transporter on Western blots (Section 3.3.1.2). However, they recognized the plate-bound transporter either very weakly (antibodies to peptides 34-60 and 293-306), or not at all (antibodies to peptides

112-127, 175-189 and 420-432), (Section 3.3.1.1). It is possible that the glucose transporter bound to plastic ELISA plates in this way (and incubated in the presence of a coating buffer of high pH), is at least partially denatured. Therefore the weak recognition of the plate-bound transporter seen with antisera against peptides 34-60 and 293-306 may in fact constitute recognition of the denatured protein. This conclusion is supported by the inability of protein-depleted erythrocyte membranes (in which the transporter is both native and functionally-active), to inhibit the binding of the latter antibodies to their respective plate-bound synthetic peptides in competitive ELISA (Section 3.3.1.3).

As discussed in Section 3.4, a possible explanation for the lack of anti-native glucose transporter activity of these antibodies could be that the oligosaccharide bound to the extracellular surface of the transporter may sterically hinder the access of IgG molecules to any extracellularly-exposed regions of the polypeptide, particularly for those antibodies directed against the region of oligosaccharide attachment. In order to investigate this possibility, the ELISA experiments were repeated with deglycosylated glucose transporter as the plate-bound antigen. If this approach resulted in improved binding of the antibodies to the transporter, then the deglycosylated protein could be employed as competing antigen in competitive ELISA. Deglycosylation of the purified glucose transporter was performed using a mixture of endo-β-galactosidase and endoglycosidase F as described in Section 2.6.2. Deglycosylation of membrane proteins with endoglycosidase F usually requires that the proteins first be denatured with SDS and a non-ionic detergent such as Triton X-100 (Elder and Alexander, 1982; Lienhard *et al.*, 1984). However, simultaneous treatment of the transporter with both enzymes circumvented the need for detergent. Following digestion in the

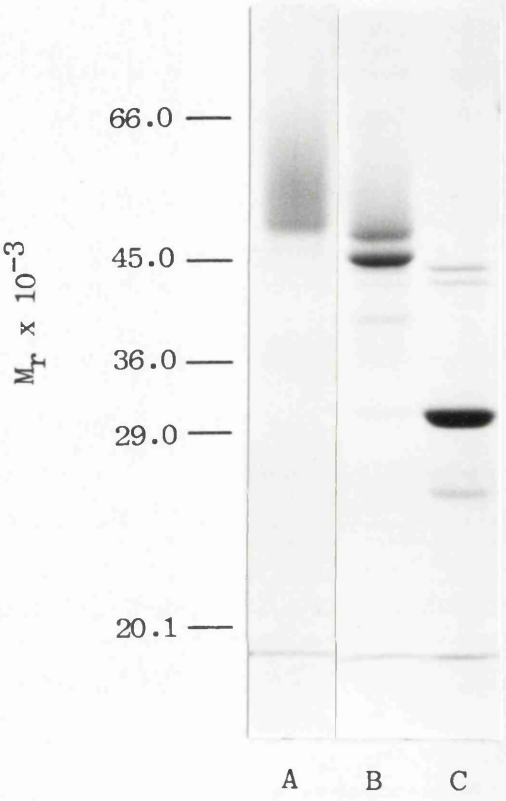



Fig.4.4 Analysis of the products of endo-β-galactosidase/endoglycosidase F digestion of the glucose transporter by SDS/polyacrylamide gel electrophoresis

Samples (10μg), of intact glucose transporter (A), deglycosylated glucose transporter (B) and endo-β-galactosidase/endoglycosidase F mixture alone (C), were electrophoresed on a SDS/10% polyacrylamide gel and then stained with Coomassie blue. The positions of $M_r$ markers are indicated.

absence of detergent, nearly 100% of the original transporter activity (as determined by reversible cytochalasin B binding), was retained by the preparation, indicating that much of the transporter remained in its native conformation (results not shown). The extent of deglycosylation of the transporter was determined to be >80% by spectrophotometric scanning at 530nm of the Coomassie blue-stained SDS/10% polyacrylamide gel shown in Fig.4.4. The deglycosylated transporter preparation exhibited primarily a sharp band of apparent $M_r$ 46,000 on gels, corresponding to the band previously identified by Lienhard et al. (1984) as the fully deglycosylated protein. A second, minor product of digestion migrated as a sharp band of apparent $M_r$ 48,000 and probably corresponded to a partially deglycosylated species (Fig.4.4(B). Both bands were labelled on Western blots by antibodies to peptides 34-60, 175-189 and 293-306, but not by those against peptides 112-127 and 420-432 (Fig.4.5). The lack of reactivity of the latter antibodies towards the native and denatured forms of the glucose transporter is therefore unlikely to have stemmed from steric hindrance by the oligosaccharide chain. Because of this lack of reactivity, these antibodies were not used in further experiments.

When ELISA experiments were repeated using antibodies to peptides 34-60, 175-189 and 293-306, none of the antibodies reacted any more strongly with the plate-bound, deglycosylated transporter than with the fully glycosylated transporter (results not shown). Therefore the inability of antibodies to bind to extracellular sites on the native glucose transporter polypeptide is unlikely to stem from steric hindrance by the oligosaccharide chains.

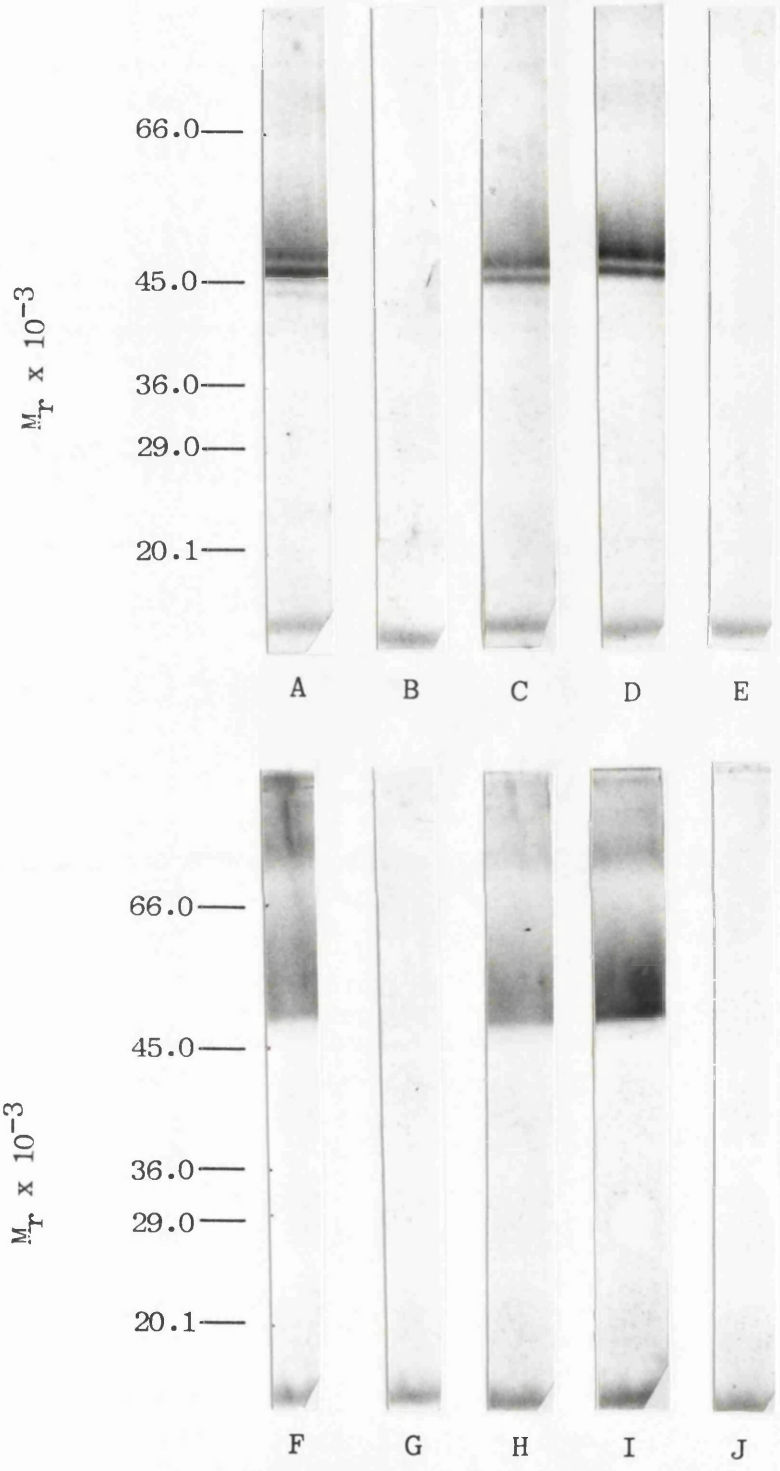



Fig.4.5 Recognition of the denatured glucose transporter before and after deglycosylation by anti-peptide antibodies on Western blots

Samples (10μg), of glucose transporter after (A-E), and before (F-J) enzymic deglycosylation, were electrophoresed on a SDS/10% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and then immunolabelled with antibodies to peptides 34-60 (A and F), 112-127 (B and G), 175-189 (C and H), 293-306 (D and I) and 420-432 (E and J). The positions of $M_r$ markers are indicated.

4.2.4 The sequence location of the large, membrane-bound fragments produced by prolonged proteolytic digestion of the native glucose transporter

Anti-peptide antibodies corresponding to N-terminal residues 1-15 and C-terminal residues 477-492 of the glucose transporter (Fig.4.6), were used to locate within the protein sequence the two large fragments of $M_r$ 23,000-42,000 and 18,000 produced by prolonged tryptic or chymotryptic cleavage of the native, membrane-bound transporter (Cairns et al., 1984; Deziel and Rothstein, 1984, Section 1.7.1). Digestion of the transporter with trypsin or chymotrypsin was performed as described in Section 2.6.1. Samples of the digests were electrophoresed on SDS/12% polyacrylamide gels, electrophoretically transferred to nitrocellulose paper, and then immunolabelled with the relevant antibody.

Antibodies to the N-terminal peptide labelled a broad band on Western blots of both tryptic and chymotryptic digests of the transporter, corresponding to the glycosylated fragment of $M_r$ 23,000-42,000 (Fig.4.7(A). This finding supports $Asn_{45}$ as the site of glycosylation of the transporter (Mueckler et al., 1985; Mueckler and Lodish, 1986, Section 1.7.3). Labelling of the chymotryptic fragment was expected because there are no likely sites for cleavage by this enzyme within the first 15 residues of the transporter sequence (Mueckler et al., 1985, Fig.4.6). However there are potential sites of tryptic cleavage at residues 6, 7 and 11 (Fig.4.6). The fact that the N-terminal peptide antibodies recognized this tryptic fragment suggested that whether or not it contains residues 1-7 of the transporter polypeptide, it almost certainly contains residues 8 and beyond. The lack of labelling of the glycosylated tryptic fragment by antibodies raised to the purified, intact glucose transporter on Western blots (Fig.4.7(B), confirmed the finding from ELISA

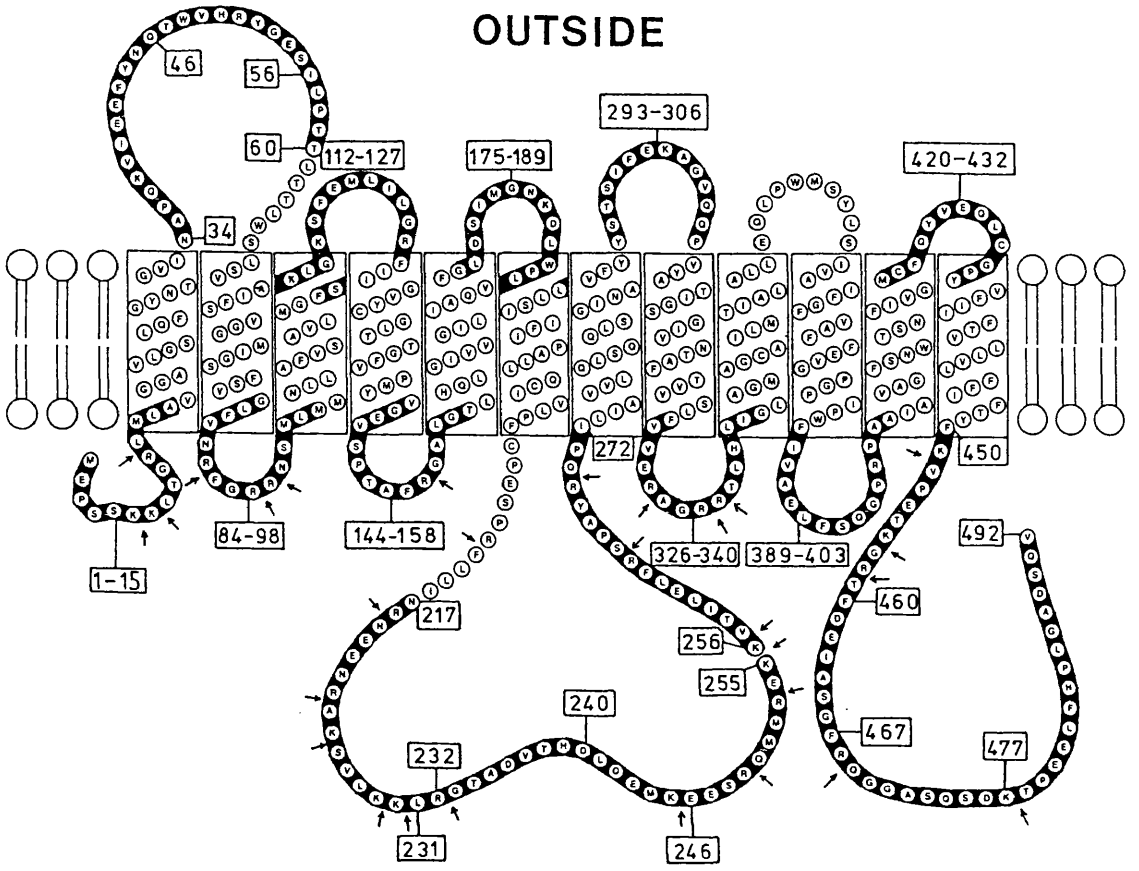



Fig.4.6 Location of chemically-synthesized peptides and tryptic cleavage sites within the glucose transporter sequence

Regions of the sequence corresponding to synthetic peptides are outlined in black. Numbers refer to the sequence location of the N- and C-terminal residues of each peptide. Arrows indicate the location of potential tryptic cleavage sites.

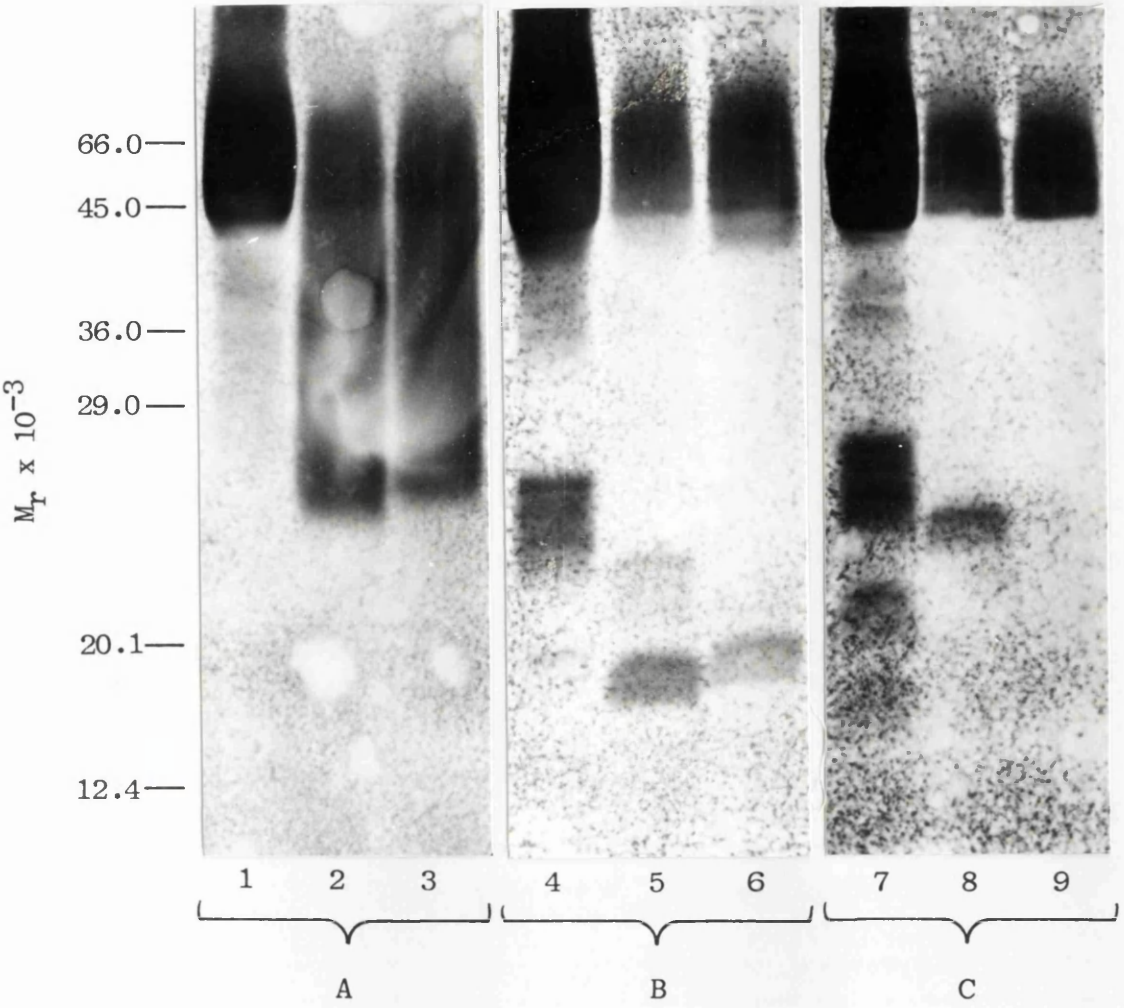



Fig.4.7 Binding of anti-peptide antibodies to the glucose transporter and its proteolytic fragments on Western blots

Samples (10μg), of intact glucose transporter (lanes 1, 4 and 7) and of glucose transporter extensively digested with trypsin (lanes 3, 5 and 8), or chymotrypsin (lanes 2, 6 and 9), were electrophoresed on a SDS/12% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and then immunolabelled with antibodies to the N-terminal peptide (A), antibodies to the intact glucose transporter (B), or antibodies to C-terminal peptide 477-492 (C). The positions of $M_r$ markers are indicated.

experiments that the N-terminal region is not immunogenic in the native protein (Sections 3.3.1.1 and 3.3.1.3).

The fragments of approximate $M_r$ 18,000-20,000 generated by prolonged tryptic or chymotryptic digestion of the transporter were labelled by anti-glucose transporter antibodies (Fig.4.7(B) and Fig.4.8(B), but not by antibodies to C-terminal peptide 477-492 (Fig.4.7(C) and Fig.4.8(A) on Western blots, indicating that they do not contain the C-terminus of the intact protein. However, fragments of higher $M_r$ seen predominantly after short times of tryptic digestion were labelled by the C-terminal peptide antibodies (Fig.4.8(A). These fragments, of approximate $M_r$ 25,500 and 23,500, are also photolabelled by cytochalasin B, and are probably precursors to the $M_r$ 18,000 fragment (Cairns et al., 1984). It follows that the $M_r$ 18,000 fragment, and its associated cytochalasin B binding site, are derived from the C-terminal half of the protein.

Although the $M_r$ 25,500 and 23,500 tryptic fragments must contain the C-terminus of the protein, the anomalous mobility of hydrophobic peptides on SDS/polyacrylamide gel electrophoresis precludes accurate determination of their true $M_r$ (Baldwin et al., 1982). For instance, the deglycosylated transporter migrates as a sharp band of apparent $M_r$ 46,000 on SDS/polyacrylamide gels (Lienhard et al., 1984, Section 4.2.3), although its true $M_r$ is 54,117 (Mueckler et al., 1985). It was therefore not possible to locate the N-termini of the tryptic fragments within the transporter sequence from apparent $M_r$ values. However, the pattern of labelling of these fragments by anti-peptide antibodies to other regions of the transporter polypeptide did allow reasonable identification of their origin from within the protein sequence, as described in the next section. The order of cleavages produced by trypsin appeared not to be obligatory: a fragment of slightly higher mobility than the intact transporter (apparent $M_r$

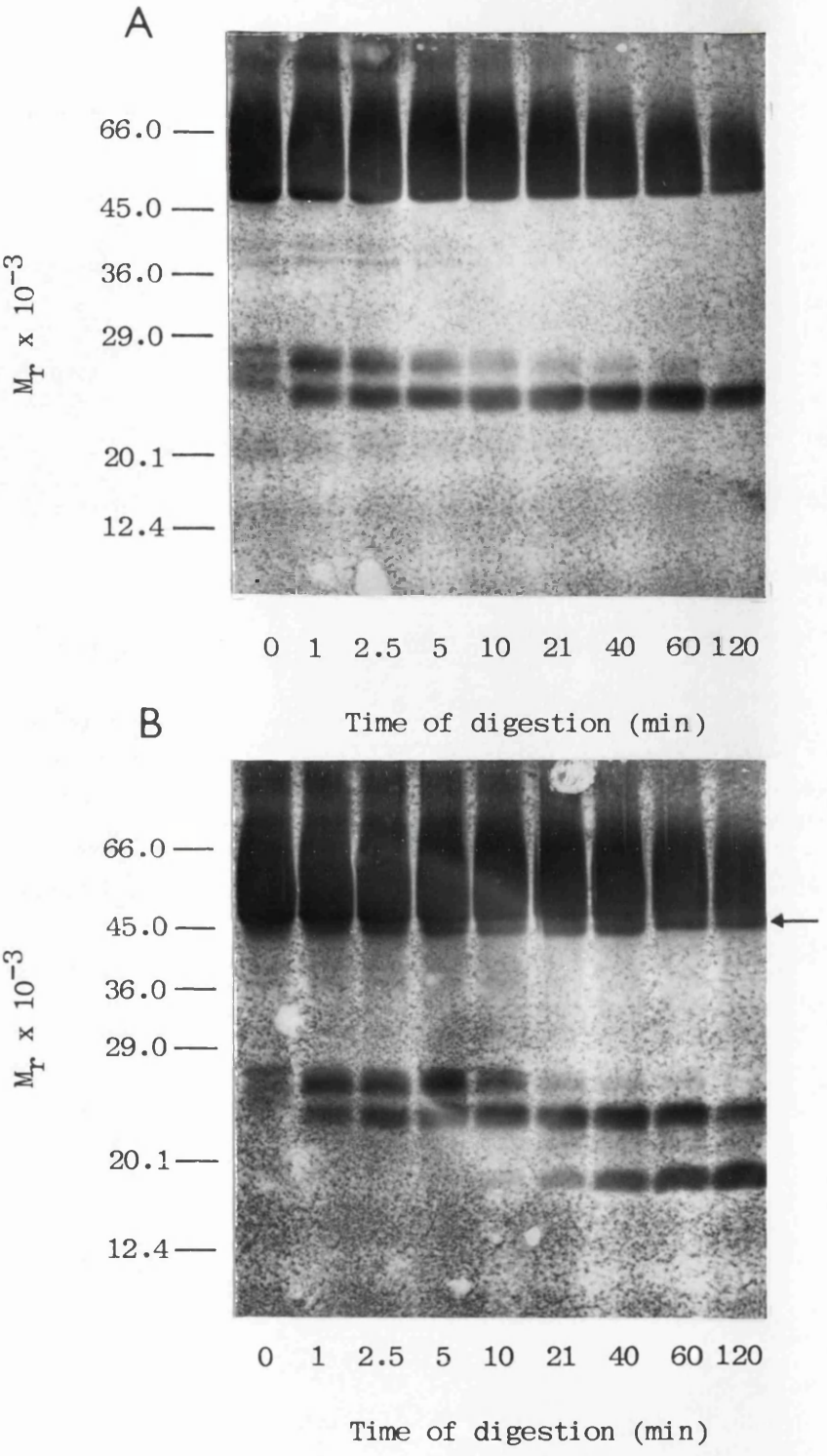



Fig.4.8 The time course of the appearance of immunologically-reactive fragments during tryptic digestion of the glucose transporter

Samples (10μg), of glucose transporter digested with trypsin for various times up to 2 hours were electrophoresed on a SDS/12% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and then immunolabelled with antibodies to C-terminal peptide 477-492 (A), or antibodies to the intact glucose transporter (B). The positions of $M_r$ markers are indicated.

45,000), seen after short times of digestion, was labelled by antibodies against the intact glucose transporter on Western blots (Fig.4.8(B) arrow), and by antibodies to the N-terminal peptide (result not shown), but not by antibodies to C-terminal peptide 477-492 (Fig.4.8(A). This fragment must therefore arise by initial cleavage of the protein near its C-terminus. The possible order of tryptic cleavage of the transporter is discussed in the next section.

4.2.5 Further identification of the major membrane-bound tryptic fragments of the glucose transporter by peptide mapping using anti-peptide antibodies

Extensive tryptic digestion of the native, membrane-embedded glucose transporter gives rise to a glycosylated fragment of apparent $M_r$ 23,000-42,000 on SDS/polyacrylamide gels (Cairns et al., 1984; Deziel and Rothstein, 1984) which was shown in the previous section to be derived from the N-terminal half of the protein. The latter finding is consistent with the amino acid composition of the isolated fragment, which corresponds reasonably well with that expected for residues 1-212 of the transporter (Cairns et al., 1987, Fig.4.6). A fragment of $M_r$ 18,000 is also produced, which is non-glycosylated and bears the site of photolabelling by cytochalasin B (Cairns et al., 1984; Deziel and Rothstein, 1984). The experiments described in the previous section showed that this fragment is derived from the C-terminal half of the transporter, but does not carry the intact C-terminal sequence of the protein. This finding is consistent with the amino acid composition of the fragment, which corresponds reasonably well with that expected for residues 270-456 of the transporter (Cairns et al., 1987, Fig.4.6). Shorter times of digestion by trypsin yield the glycosylated fragment together with a number of non-glycosylated fragments. The latter also carry the site of

photolabelling by cytochalasin B (Cairns et al., 1984) and so are probably precursors to the $M_r$ 18,000 fragment. Two of these non-glycosylated fragments of $M_r$ 25,500 and 23,500 were shown in the previous section to be labelled by antibodies to C-terminal peptide 477-492, but not by antibodies to the N-terminal peptide, indicating that they are derived from the C-terminal half of the protein.

As discussed in the previous section, the anomalous mobility of these very hydrophobic tryptic fragments on SDS/polyacrylamide gels has up to now made identification of their precise locations within the transporter sequence very difficult. Therefore, using Western blots of the products of limited tryptic digestion of the transporter in conjunction with anti-peptide antibodies, an attempt was made to map the major glycosylated and non-glycosylated tryptic fragments by determining what peptide sequences they contained.

Limited tryptic digestion of the transporter was performed as described in Section 2.6.1. Fig.4.9 shows a silver-stained SDS/12% polyacrylamide gel of the products of limited tryptic digestion of the transporter over a time course of up to 3 hours. At t=0, a band corresponding to the intact glucose transporter of apparent average $M_r$ 55,000 is seen. As the digestion proceeds, the broad, glycosylated fragment of $M_r$ 23,000-42,000 is generated together with non-glycosylated fragments (labelled 1-4 in Fig.4.9). The apparent $M_r$ values for the latter fragments were determined from a calibration curve produced from a spectrophotometric scan at 600nm of marker proteins also run on the gel. Bands 1,2 and 4 correspond to the previously described tryptic fragments of $M_r$ 25,500, 23,500 and 18,000 respectively, whilst band 3 is a less intensely stained fragment of apparent $M_r$ 21,000 which had not been previously noted by Cairns et al. (1984).

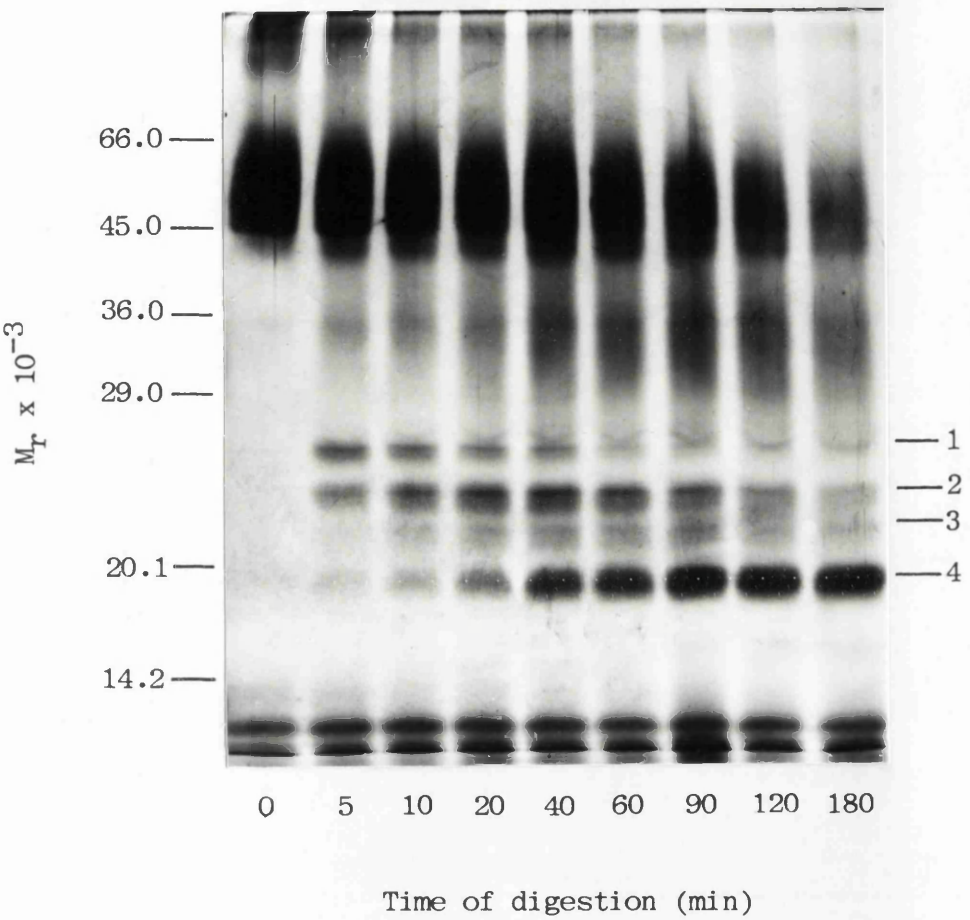



Fig.4.9 Analysis of the products of limited tryptic digestion of the glucose transporter by SDS/polyacrylamide gel electrophoresis

Samples (20µg), of glucose transporter digested with trypsin for various times up to 3 hours were electrophoresed on a SDS/12% polyacrylamide gel and then silver stained. The bands labelled 1-4 correspond to the non-glycosylated tryptic fragments of apparent $M_r$ 25,500, 23,500, 21,000 and 18,000 respectively. The positions of $M_r$ markers are indicated.

4.2.5.1 Sequence location of the glycosylated tryptic fragment

Antibodies to the N-terminal sequence of the transporter labelled only the intact protein and the glycosylated tryptic fragment of $M_r$ 23,000-42,000 (Section 4.2.4). Antibodies to peptides 34-60, 84-98, 144-158 and 175-189 (Fig.4.6), also recognized the intact transporter on Western blots (Sections 3.3.1.2 and 4.2.3), and the glycosylated tryptic fragment of $M_r$ 23,000-42,000, as illustrated by the results obtained for antibodies to peptide 175-189 in Fig.4.10 and the N-terminal peptide in Fig.4.7(A). These antibodies labelled none of the non-glycosylated fragments (Fig.4.10 and Fig.4.7(A). These findings confirm that the glycosylated tryptic fragment contains at least the first five putative membrane-spanning helices of the transporter (Mueckler et al., 1985, Fig.4.6). Because this fragment is produced by tryptic cleavage at the cytoplasmic surface of the membrane, it is likely also to contain the sixth membrane-spanning sequence (Fig.4.6). However, it was not labelled on Western blots by antibodies against peptide 217-232 (result not shown), a finding consistent with its formation by cleavage at $Arg_{212}$ (Cairns et al., 1987, Fig.4.6). No fragments of smaller $M_r$ were recognized by these antibodies, even after prolonged digestion (Fig.4.10). Therefore, the glycosylated tryptic fragment appears to be stable to further proteolysis, even though potential cleavage sites are present in the predicted cytoplasmic regions encompassing residues 84-98 and 144-158 (Fig.4.6). This is consistent with the inaccessibility of these cytoplasmic regions and the N-terminus of the native transporter to antibodies (Sections 3.3.1.1 and 3.3.1.3).

4.2.5.2 Sequence locations of the non-glycosylated tryptic fragments

Since the order in which different sites in the transporter sequence are cleaved by trypsin appears not to be obligatory (Section

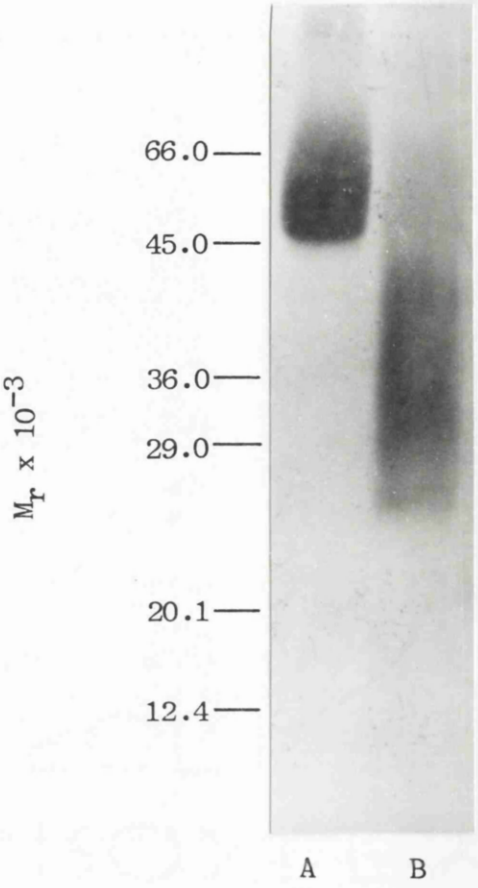



Fig.4.10 Binding of anti-peptide antibodies to the glycosylated fragment derived from extensive tryptic digestion of the glucose transporter

Samples (20μg), of intact glucose transporter (A), or glucose transporter extensively digested with trypsin (B), were electrophoresed on a SDS/12% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and then immunolabelled with antibodies against peptide 175-189. The positions of $M_r$ markers are indicated.

4.2.4), the non-glycosylated fragments visible on silver-stained gels of tryptic digests may not correspond to a single species of peptide. Rather, they may comprise a number of fragments of very similar $M_r$, which are not resolved under the conditions of SDS/polyacrylamide gel electrophoresis used in this study. Indeed, the fragments labelled 2 and 3 in Fig.4.9 do show signs of comprising multiple, overlapping bands. Consequently, the pattern of labelling of these fragments by anti-peptide antibodies requires careful interpretation. With this in mind, reasonable identification of the major species of peptide in each fragment has been possible, as described below.

Several distinct patterns were seen for the labelling of the non-glycosylated tryptic fragments (bands 1-4 in Fig.4.9) by antibodies directed against the cytoplasmic central and C-terminal hydrophilic regions of the transporter sequence (residues 217-272 and 450-492 respectively, Fig.4.6). Examples are illustrated in Fig.4.11 for antibodies to peptides 240-255 and 450-467. The results of these findings are summarized in Table 4.1.

The $M_r$ 25,500 fragment, which is also labelled by antibodies against C-terminal peptide 477-492 (Section 4.2.4), was strongly labelled by antibodies against peptide 231-246, but weakly, if at all by antibodies against peptide 217-232 (results not shown). From the distribution of tryptic cleavage sites in the central cytoplasmic region of the transporter (Fig.4.6), it appears that this fragment must contain residues 233-492. It may contain some residues N-terminal to residue 233, but in the light of the lack of reactivity of antibodies to peptide 217-232 towards this fragment, its N-terminus cannot precede $Ala_{224}$ (Fig.4.6). Antibodies against peptide 217-232 did faintly label an apparent precursor to the glycosylated $M_r$ 23,000-42,000 tryptic fragment seen after short times of digestion (result not shown), and so it is likely that one or more of the

| Antibody | Labelling of tryptic fragment | | | |
|---|---|---|---|---|
| | $M_r$ 25,500 | $M_r$ 23,500 | $M_r$ 21,000 | $M_r$ 18,000 |
| Anti-peptide 217-232 | - | - | - | - |
| Anti-peptide 231-246 | + | - | + | - |
| Anti-peptide 240-255 | + | - | + | - |
| Anti-peptide 256-272 | + | + | + | + |
| Anti-peptide 450-467 | + | + | - | + |
| Anti-peptide 460-477 | + | + | - | - |
| Anti-peptide 477-492 | + | + | - | - |

Table 4.1 Patterns of reactivity of anti-peptide antibodies with tryptic fragments of the glucose transporter on Western blots

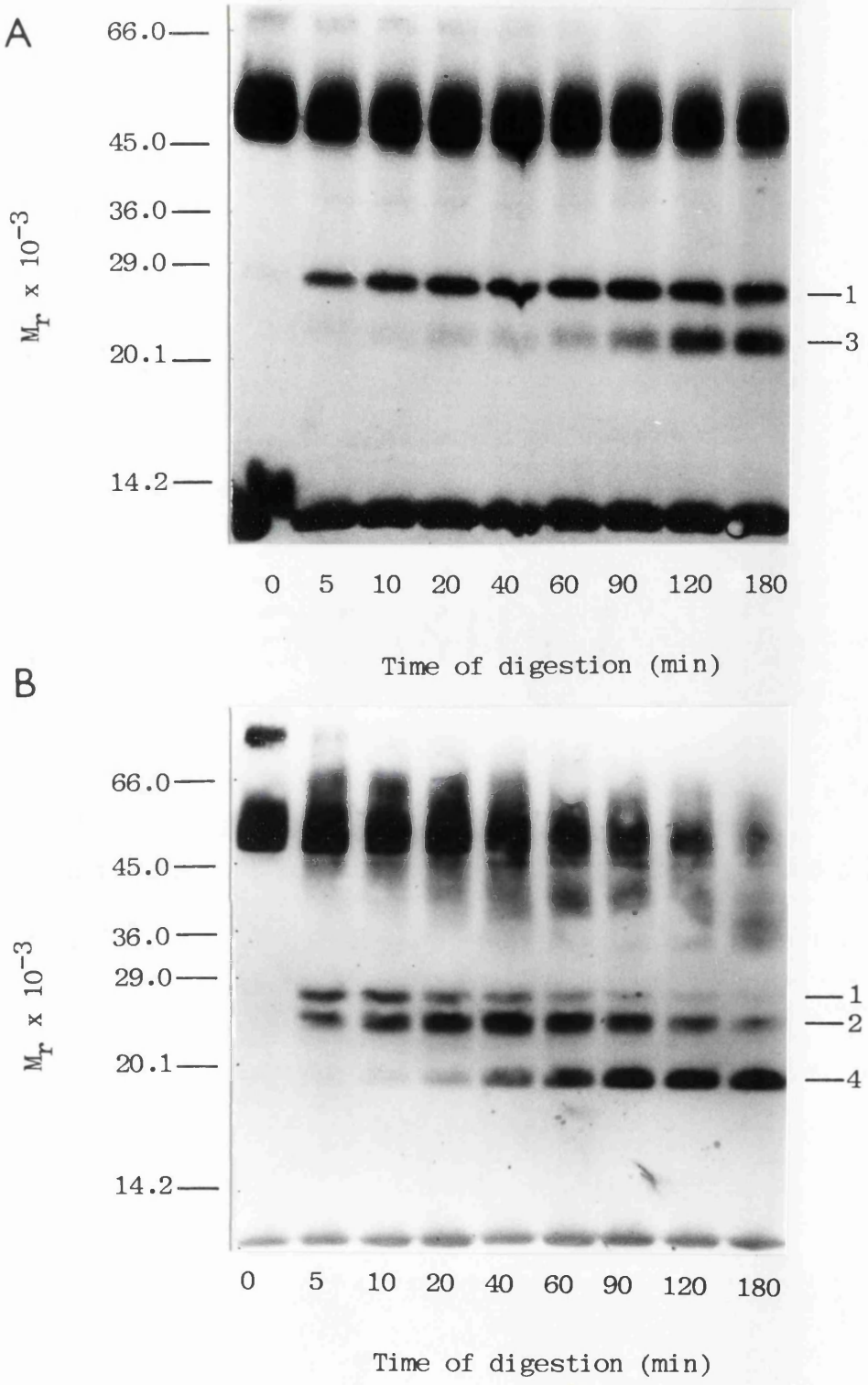



Fig.4.11 Western blot analysis of anti-peptide antibody binding to the non-glycosylated fragments derived from limited tryptic digestion of the glucose transporter

Samples (10µg), of glucose transporter digested with trypsin for various times up to 3 hours were electrophoresed on a SDS/12% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and then immunolabelled with antibodies to peptide 240-255 (A), or antibodies to peptide 450-467 (B). Labelled non-glycosylated tryptic fragments are numbered 1-4. The positions of $M_r$ markers are indicated.

cluster of five basic amino acid residues between $Arg_{223}$ and $Arg_{232}$ (Fig.4.6), represents a particularly sensitive site for tryptic cleavage of the transporter. The fragment of $M_r$ 23,500, which is also labelled by antibodies against C-terminal peptide 477-492 (Section 4.2.4), was not labelled by antibodies to peptide 240-255 (Fig.4.11(A), although it was labelled by antibodies against peptide 256-272 (result not shown). From the distribution of potential tryptic cleavage sites in the central cytoplasmic region of the transporter sequence, it must contain residues 265-492 (Fig.4.6). It may contain residues N-terminal to residue 265, but its N-terminus cannot precede $Glu_{246}$ (Fig.4.6). A likely candidate for the N-terminal residue of this fragment is $Val_{257}$ (Fig.4.6). The fragment of $M_r$ 21,000 was not labelled by antibodies against peptide 450-467, unlike the $M_r$ 18,000 fragment (Fig.4.11(B). The latter fragment is known to terminate at either $Lys_{456}$ or $Arg_{458}$ (see below), and so the $M_r$ 21,000 fragment must terminate either at $Lys_{456}$ or, more likely at $Lys_{451}$ (Fig.4.6). The fragment resembles the $M_r$ 25,500 fragment in being strongly labelled by antibodies against peptide 231-246 and 240-255 (Fig.4.11(A), but not against peptide 217-232 (result not shown). It therefore probably shares the same N-terminus as the latter fragment, that is somewhere between residues 224 and 233, and therefore contains a substantial portion of the central cytoplasmic domain. Perhaps surprisingly, this fragment is most abundant after 3 hours of digestion and so must represent a fairly stable product of digestion. The fragment of $M_r$ 18,000 was labelled by antibodies against peptide 450-467 (Fig.4.11(B), but not against peptide 460-477 (result not shown). In conjunction with the finding of Cairns *et al.* (1987) that a peptide corresponding to residues 459-468 is released from the membrane upon prolonged digestion of the transporter with trypsin (Section 4.4), this observation indicates that the $M_r$ 18,000 fragment

must terminate at $Lys_{456}$ or $Arg_{458}$ (Fig.4.6). In addition, labelling of this fragment with antibodies against peptide 256-272 suggests that (a component of) the fragment contains residues N-terminal to $Gln_{270}$ (Fig.4.6). The latter was suggested to be the likely N-terminus of the fragment from a determination of its amino acid composition by Cairns et al. (1987). However, fragments containing an additional 5 or even 13 residues at the N-terminus, and which are recognized by antibodies against peptide 256-272, might not be resolved from a fragment comprising residues 270-456/458 on SDS/polyacrylamide gels.

## 4.3 ANTI-PEPTIDE ANTIBODIES AS PROBES OF GLUCOSE TRANSPORTER FUNCTION

### 4.3.1 Effects of anti-peptide antibodies on the cytochalasin B binding activity of the glucose transporter

The ability of anti-peptide antibodies to affect the reversible cytochalasin B binding activity of the glucose transporter present in protein-depleted erythrocyte membranes was investigated. As only those antibodies against the cytoplasmically-exposed central hydrophilic and C-terminal regions bind the native transporter (Sections 3.3.1.1, 3.3.1.3, 4.2.1 and 4.2.2), the study was limited to antibodies against these regions (residues 231-246 and 450-492 respectively).

Protein-depleted erythrocyte membranes at a protein concentration of 0.25mg/ml in PBS, pH 7.2, were incubated for 2 hours at room temperature with various concentrations (up to 0.4mg/ml) of non-immune rabbit IgG, or affinity-purified antibodies against peptides 231-246, 240-255, 450-467, 460-477 and 477-492. Cytochalasin B binding activity was then measured by equilibrium dialysis as described in Section 2.5.1.

Antibodies against C-terminal peptides 450-467, 460-477 and 477-492 had no effect on cytochalasin B binding to the transporter

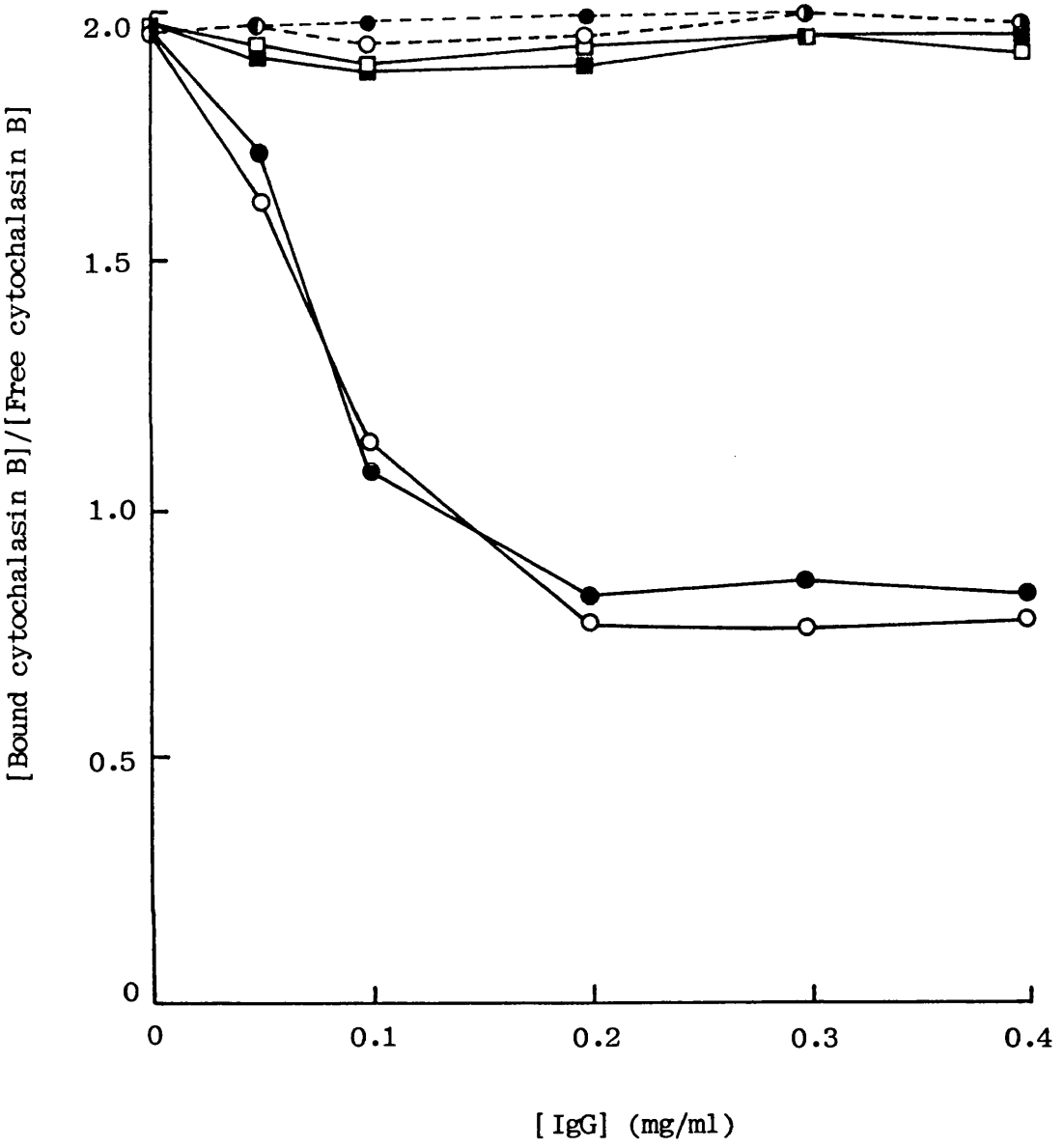



Fig.4.12 Inhibition of the cytochalasin B binding activity of protein-depleted erythrocyte membranes by anti-peptide antibodies

Protein-depleted erythrocyte membranes at a concentration of 0.25mg/ml in PBS, pH 7.2, were incubated with various concentrations of non-immune rabbit IgG (O-O), or affinity-purified antibodies against peptides 231-246 (●—●), 240-255 (○—○), 450-467 (□—□), 460-477 (■—■) and 477-492 (●--●). Cytochalasin B binding activity was then measured as described in Section 2.5.1. Each point is the mean of triplicate determinations.

(Fig.4.12). However, antibodies to peptides 231-246 and 240-255 did inhibit the binding as measured using a fixed, low concentration of cytochalasin B ($4x10^{-8}$M). A maximum inhibition of about 60% was seen when membranes at a concentration of 0.25mg protein/ml were incubated with 0.2mg IgG/ml (Fig.4.12). This concentration corresponds to about a 3-fold molar excess of antibody over glucose transporter. No inhibition was seen using a corresponding concentration of non-immune IgG (Fig.4.12). Protein-depleted membranes were used in preference to the purified transporter in these experiments because they are completely unsealed (Gorga and Lienhard, 1981). However, similar results were obtained using purified glucose transporter (results not shown).

The mechanism of this inhibition was examined further by measuring the effect of a maximally inhibitory concentration of IgG (0.4mg/ml), on cytochalasin B binding over a range of cytochalasin B concentrations. The results are shown in the form of Scatchard plots in Fig.4.13. In such plots, the ordinate is the ratio of bound to free ligand and the abscissa is the bound ligand. The slope of each line is $-1/K_d$ or $K_a$, the association constant for ligand binding to the receptor, and the intercept on the abscissa represents the concentration of binding sites. Analysis of the results by the LIGAND procedure (Munson and Rodbard, 1980) showed that neither antibody had a significant effect on the concentration of cytochalasin B binding sites when compared to the non-immune IgG control ($0.8 \pm 0.012\mu M$, $0.73 \pm 0.029\mu M$ and $0.82 \pm 0.034\mu M$ for non-immune IgG, anti-peptide 231-246 antibodies and anti-peptide 240-255 antibodies respectively, Fig.4.13). Instead, antibody binding decreased the $K_a$ for cytochalasin B binding from $8.4 \pm 0.9\mu M^{-1}$ to $3.4 \pm 0.6\mu M^{-1}$ for antibodies against peptide 231-246 and $3.5 \pm 0.4\mu M^{-1}$ for antibodies against peptide 240-255.

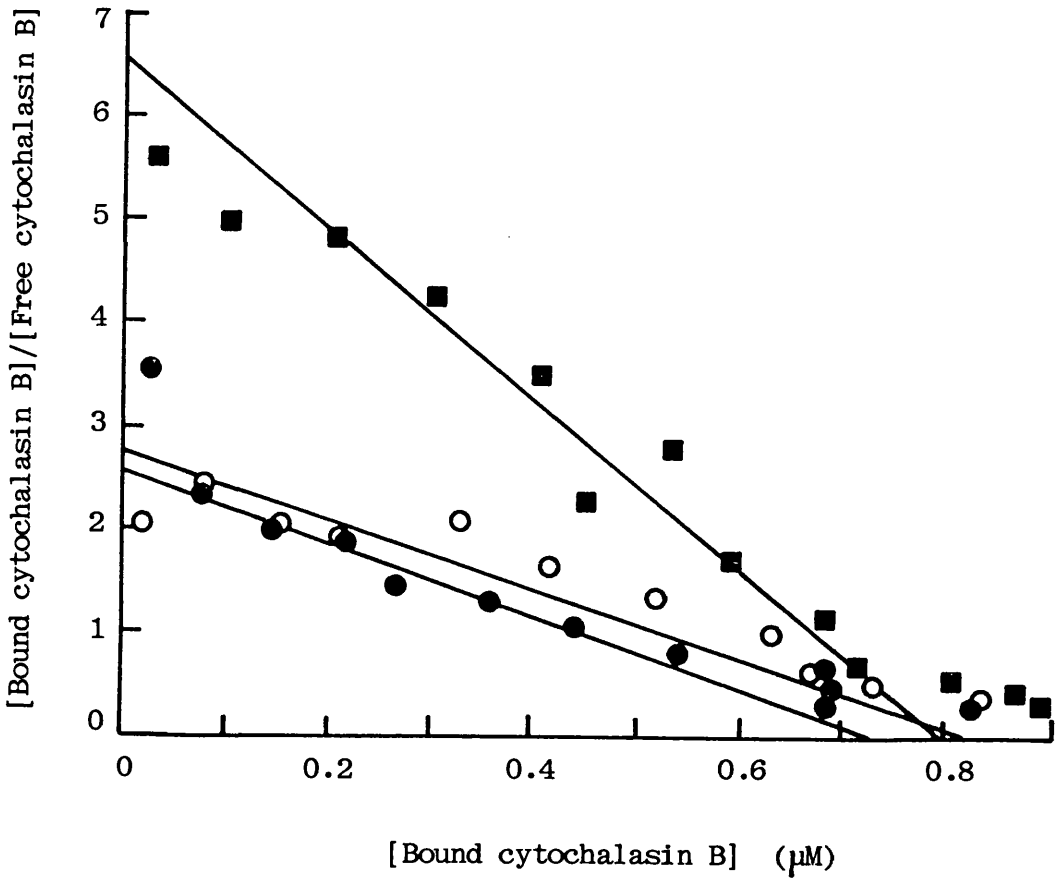



Fig.4.13 Scatchard plot analysis of cytochalasin B binding to protein-depleted erythrocyte membranes in the presence of control or anti-peptide antibodies

Protein-depleted erythrocyte membranes (0.5mg/ml in PBS, pH 7.2), were incubated with non-immune rabbit IgG (■, 0.4mg/ml), or with affinity-purified antibodies (0.4mg/ml) against peptides 231-246 (●) and 240-255 (○). Cytochalasin B binding was measured over the concentration range 0.5-75.5x$10^{-7}$M as described in Section 2.5.1. Each point is the mean of triplicate determinations. The straight lines are computerised best fits determined by the LIGAND procedure (Munson and Rodbard, 1980).

4.3.2 The effects of ligands on antibody binding to the cytoplasmic central and C-terminal regions of the glucose transporter

In the previous section, anti-peptide antibodies directed against certain regions of the glucose transporter were shown to affect the latter's ability to bind cytochalasin B. It was not possible to extend this approach to other ligands, because they were either not available in radiolabelled form, or bound with too low an affinity for their binding characteristics to be measured by equilibrium dialysis. Therefore in the experiments described in this section, an attempt was made to probe the function of the cytoplasmic central and C-terminal regions of the protein by examining whether cytochalasin B, or other ligands, affected the binding of specific antibodies to these regions of the sequence. The experiments were done by competitive ELISA using protein-depleted erythrocyte membranes as competing antigen, and synthetic peptides as plate-bound antigens.

Varying amounts of protein-depleted erythrocyte membranes (up to 0.2mg membrane protein/incubation), in PBS, pH 7.2, were preincubated for 2 hours with either (a) buffer alone, (b) 100μM phloretin + 0.6% ethanol, (c) 37.5μM cytochalasin B + 0.6% ethanol, (d) 0.6% ethanol control (phloretin and cytochalasin B stock solutions are in ethanol), (e) 200mM D-glucose, or (e) 200mM L-glucose. Affinity-purified antibodies to peptides 231-246, 240-255, 450-467, 460-477 and 477-492 (all at 1μg/ml in PBS, pH 7.2), were added to the membrane-ligand preincubations. Following incubation for a further 2 hours at room temperature, the membranes were sedimented by centrifugation, and 0.1ml samples of the supernatants were loaded into microtitre wells coated with 20ng per well of the relevant synthetic peptide. The % inhibition of antibody binding to wells by the erythrocyte membranes in the presence and absence of ligands was determined by ELISA (Section 3.3.1.1).

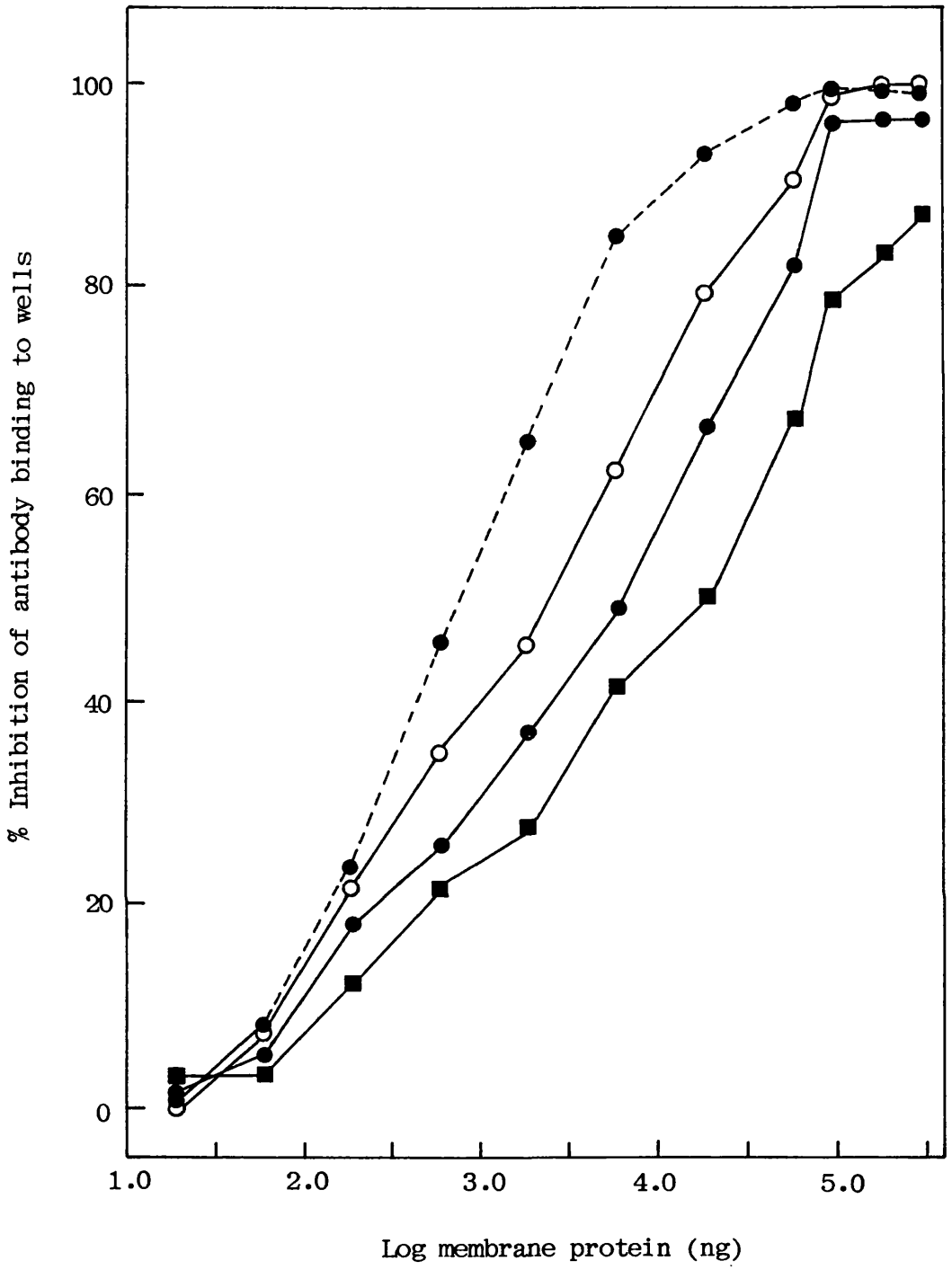



Fig.4.14 The effect of ligands on anti-peptide antibody binding to protein-depleted erythrocyte membranes in competitive ELISA

The ability of protein-depleted erythrocyte membranes to compete for a limiting amount of antibody against peptide 231-246 in the presence of various ligands, with peptide 231-246 bound to the surface of a microtitre well. Antibody binding in the presence of no ligand (●—●), 100μM phloretin (■—■), 20μM cytochalasin B (○—○) or 200mM D-glucose (●--●). Each point is the mean for triplicate determinations.

In the competitive ELISA, none of the antibodies were affected in their binding to plate-bound synthetic peptides by 0.6% ethanol or 200mM L-glucose when compared to the ligand-free controls (results not shown). In addition, phloretin, cytochalasin B and 200mM D-glucose had no effect on the binding of antibodies to peptides 450-467, 460-477 and 477-492 in ELISA (results not shown). In contrast, the % inhibition of binding to plate-bound synthetic peptides was attenuated for antibodies to peptides 231-246 and 240-255 in the presence of saturating amounts of D-glucose and cytochalasin B, but increased in the presence of a saturating amount of phloretin relative to the no-ligand controls, as illustrated by the results obtained for antibodies to peptide 231-246 in Fig.4.14. Therefore, the binding of anti-peptide 231-246 and 240-255 antibodies to the glucose transporter present in protein-depleted erythrocyte membranes, appeared to be increased in the presence of D-glucose and cytochalasin B, but decreased in the presence of phloretin. The implications of these findings are discussed in Section 4.4.

## 4.4 DISCUSSION

In this chapter, anti-peptide antibodies raised against the human erythrocyte glucose transporter were employed in a study of the transmembrane topology and function of this membrane-bound protein.

### Transmembrane topology of the glucose transporter

Antibodies against synthetic peptides corresponding to residues 217-232, 231-246, 240-255, 256-272, 450-467, 460-477 and 477-492 of the glucose transporter polypeptide, all of which recognised the native protein (Sections 3.3.1.1 and 3.3.1.3), were found to bind at the cytoplasmic surface of the erythrocyte membrane. These findings are consistent with the prediction of the model of Mueckler *et al.* (1985) that a hydrophilic region towards the middle of the transporter sequence, encompassing residues 207 to 271, and the C-terminal region of the sequence from residues 451 to 492 are located at the cytoplasmic side of the erythrocyte membrane. Additionally, these findings are consistent with those of Cairns *et al.* (1987) who in concurrent vectorial tryptic dissection experiments on the native transporter (Section 1.7.3), isolated and characterized peptide fragments corresponding to residues 213-269 and 457-492 of the protein. Since trypsin cleaves the transporter solely at its cytoplasmic surface (Lienhard *et al.*, 1984; Deziel and Rothstein, 1984), then these regions of the protein must be exposed at the cytoplasmic face of the erythrocyte membrane. The cytoplasmic location of the C-terminal region has subsequently been verified by Haspel *et al.* (1988a) using anti-peptide antibodies and by Andersson and Lundahl (1988) using monoclonal antibodies to the intact transporter in conjunction with synthetic peptides.

In contrast, antibodies against the N-terminal sequence (residues

1-15), against the predicted short loops connecting transmembrane sequences at the cytoplasmic surface (encompassing residues 84-98, 144-158, 326-340 and 389-403), and antibodies raised against the putative extracellular regions of the transporter polypeptide (encompassing residues 34-60, 112-127, 175-189, 293-306 and 420-432), failed to recognise the native protein (Sections 3.3.1.1 and 3.3.1.3). The possibility that the lack of recognition of the native transporter by the antibodies to the extracellular regions in ELISA assays might be due, at least in part, to steric hindrance from the oligosaccharide chain attached at $Asn_{45}$, has been excluded: the antibodies failed to react any more strongly with the plate-bound, deglycosylated, but functionally-active transporter than with the fully glycosylated transporter in ELISA. Similarly, steric hindrance by the oligosaccharide may not be the reason for the resistance of the extracellular regions of the transporter to tryptic cleavage (Lienhard et al., 1984; Deziel and Rothstein, 1984). Interestingly, Carrasco et al. (1986) have found that anti-peptide antibodies raised to the putative extracytoplasmic regions of the non-glycosylated lactose permease of E. coli (which shares a very similar predicted membrane topology with the human erythrocyte glucose transporter), also failed to recognise the native protein. This suggests that little exposure of the polypeptide to the extracellular face of the membrane may be a common structural feature of these transport proteins. However, the lack of antibody binding to the deglycosylated extracellular region of the transporter which normally bears the site of oligosaccharide attachment (residues 34-66), is more intriguing. This region, of some 32 amino acid residues, is considerably larger than any other predicted extracellular region of the polypeptide, and the lack of reactivity of antibodies directed against it may not be explained so readily in terms of an association with other membrane components.

Consequently, this region of the polypeptide may be at least partially buried within the tertiary structure of the protein. In direct contrast to the latter findings, Burdett and Klip (1988) have recently reported the production of polyclonal antibodies raised to the purified, intact glucose transporter which specifically recognize epitopes on the exofacial surface of both the native and deglycosylated forms of the protein present in intact human erythrocytes. Therefore some discrepancy exists as regards the accessibility of the putative extracellular regions of the transporter polypeptide to IgG molecules.

The conflict in the proposed models of Shanahan and D'Artel-Ellis (1984) and of Mueckler *et al.* (1985) regarding the membrane orientation of the transporter polypeptide was resolved using anti-peptide antibodies to identify the sequence location of fragments of the protein produced by extensive tryptic and chymotryptic digestion. Using this approach, the tryptic fragment of $M_r$ 23,000-42,000 bearing the site of glycosylation and the fragment of $M_r$ 18,000 bearing the site of photolabelling by cytochalasin B (Cairns *et al.*, 1984; Deziel and Rothstein, 1984), were found to be derived from the *N*- and *C*-terminal halves of the protein respectively. This result confirmed that $Asn_{45}$ in the *N*-terminal half of the protein is the extracellular site of glycosylation, and that the site photolabelled by cytochalasin B is situated towards the *C*-terminal end of the protein. Taken in conjunction with the cytoplasmic location for the *C*-terminal region, these results are consistent with the orientation of the transporter polypeptide in the erythrocyte membrane proposed by Mueckler *et al.* (1985) and discount the model proposed by Shanahan and D'Artel-Ellis (1984) which was based on data from enzymic dissection of the transporter *in situ*.

Anti-peptide antibodies were used to characterize further the

major products of trypsin digestion of the native transporter produced over short times of digestion. It appears that the order of tryptic cleavage of the transporter is not obligatory, but it is likely that early cleavage in the central cytoplasmic region of the native transporter polypeptide gives rise to the glycosylated fragment of $M_r$ 23,000-42,000, which is apparently stable to further proteolysis, and a non-glycosylated of $M_r$ 25,500, which is further degraded at its N- and C-termini ultimately to yield a fragment of $M_r$ 18,000, via precursor fragments of $M_r$ 23,500 and 21,000. The pattern of labelling of these fragments by anti-peptide antibodies lends more support to the model for the transporter of Mueckler et al. (1985), where most of residues 1-212 and 270-458 are membrane-bound and so are inaccessible to trypsin and antibodies, whilst most of residues 217-232 and 450-467 constituting the cytoplasmic central cytoplasmic and C-terminal regions of the protein respectively, are accessible to these macromolecules, and so must be extramembranous. These findings have, in addition, made possible the identification of peptide sequences constituting all or part of the epitopes for a number of monoclonal antibodies raised to the intact glucose transporter. This work is described in Chapter 5 of this thesis.

Function of the glucose transporter

Using anti-peptide antibodies as probes, it has been possible to make some assessment of the involvement of individual extramembranous, cytoplasmic regions of the transporter polypeptide in the (ligand binding) function of the protein.

None of the anti-peptide antibodies which bound to the C-terminal region of the protein had any effect on reversible cytochalasin B binding, suggesting that this region is not intimately involved in this function. In contrast, antibodies against peptides 231-246 and

240-255 in the central cytoplasmic region of the transporter did inhibit cytochalasin B binding. It is therefore likely that the central cytoplasmic region of the protein is important for transporter function. This inhibitory effect may reflect steric hindrance of cytochalasin B binding to the transporter by antibodies stemming from the close proximity of the amino acid sequence 231-255 to the binding site; stabilization of the protein in a conformation unfavourable for cytochalasin B binding as a result of antibody binding, or that the central cytoplasmic region constitutes an extramembranous component of the cytochalasin B binding site.

Anti-peptide antibody binding to the C-terminal region of the transporter was unaffected by pre-treatment of the protein with saturating amounts of cytochalasin B, phloretin and D-glucose. In contrast, the binding of antibodies to the central cytoplasmic region of the transporter appeared to be increased in the presence of saturating amounts of D-glucose and cytochalasin B, but decreased in the presence of phloretin. These findings suggest the following; binding of phloretin, (specific for an exofacial site on the transporter, Krupka, 1985), results in a change in conformation of the central cytoplasmic region (or that part of the region encompassing residues 231-255), which is less favourable for antibody binding, or causes a change in the conformation of this, or other regions of the protein that decreases the accessibility of the central region to antibodies at the cytoplasmic surface of the erythrocyte membrane. This is consistent with the findings of Gibbs et al. (1988) who found that the binding of phloretin slowed the rate of tryptic cleavage of the transporter, presumably as a result of the cytoplasmic regions of the protein becoming less accessible to the enzyme. These results could be interpreted in terms of a single site, alternating conformation model for glucose transport (Sections 1.3 and 1.8.2), in

which the transporter adopts a relatively trypsin-resistant (and antibody inaccessible?) 'outward-facing' conformation which is stabilized by phloretin, and a trypsin-sensitive (antibody accessible?) 'inward-facing' conformation. In contrast, the increased binding of antibodies to the amino acid sequence 231-255 of the central cytoplasmic region of the transporter in the presence of D-glucose might reflect either a change in conformation of the latter, favouring antibody binding, or a change in conformation of this or other regions of the protein that increases the accessibility of the central cytoplasmic region to antibodies. The latter possibility is consistent with the finding of Gibbs et al. (1988) that the rate of tryptic cleavage at the cytoplasmic surface of the transporter is increased in the presence of D-glucose. Consequently, under these conditions the transporter may exist in a relatively trypsin-sensitive (antibody accessible?) 'inward-facing' conformation. Similarly, the increased binding of antibodies to the transporter in the presence of cytochalasin B (which is specific for a cytoplasmically-exposed binding site on the protein, Deves and Krupka, 1978), may also be a consequence of the increased exposure of the central cytoplasmic region to macromolecules as a result of the binding of this ligand. Cytochalasin B does not appear to inhibit the binding of antibodies to residues 231-255 of the central cytoplasmic region of the transporter, whereas antibodies to this sequence do decrease the affinity of the protein for cytochalasin B (see above). The reason for this discrepancy remains unclear, but it may reflect steric hindrance by the antibodies of cytochalasin B access to its binding site, rather than a competition between cytochalasin B and antibody for overlapping binding sites.

# CHAPTER 5 CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER

## 5.1 INTRODUCTION

In the work described in this chapter, further information about the topology and function of the glucose transporter was sought using a number of monoclonal antibodies raised against the intact protein by other workers. Because of their unique specificity, monoclonal antibodies can be extremely powerful tools for use in such studies, particularly if the sequence location(s) of their epitopes are known. Although the antibodies used in the present study had previously been partially characterized, the regions of the transporter to which they bound had not been identified. Thus determination of the precise location(s) of their epitopes within the transporter sequence was an important goal of this work.

The monoclonal antibodies in question were GTPR1, GTPR2, GTPR3 and GTPR4 raised in collaboration with Dr J.M. Boyle at the Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Manchester, U.K. and G1, G2, G3 and G4, a gift from Dr G.E. Lienhard, Dept. Biochemistry, Dartmouth Medical School, Hanover, New Hampshire, U.S.A. Initial characterization of monoclonal antibodies GTPR1-4 (Meeran, 1985; Boyle *et al.*, 1985) and G1-4 (Allard and Lienhard, 1985) had demonstrated their specificity for the glucose transporter polypeptide by a number of criteria, including their ability to label the purified glucose transporter on Western blots and to immunoadsorb the cytochalasin B binding activity from detergent-solubilized glucose transporter preparations. Competitive ELISA assays using erythrocyte membrane vesicles of known sidedness or protein-depleted erythrocyte membranes and intact erythrocytes as competing

antigen, had shown that all the antibodies recognized epitopes exposed at the cytoplasmic surface of the erythrocyte membrane. In addition, GTPR1, GTPR2 and GTPR3 (the latter two of which were produced from subclones and are therefore probably the same antibody), were found to reduce the affinity of the glucose transporter for cytochalasin B by greater than two-fold, whilst antibodies G1-G4 were found to have a range of effects on the binding of this ligand to the transporter from slight enhancement (G2), to partial inhibition (G1 and G4), to almost complete inhibition (G3). The range of effects on cytochalasin B binding suggested that the antibodies recognized a number of different epitopes on the transporter. GTPR1-4 were found by Western blot analysis not to recognize the large, membrane-bound fragments of $M_r$ 23,000-42,000 and $M_r$ 18,000 produced by extensive tryptic cleavage of the native glucose transporter (Section 4.2.4), suggesting that their cytoplasmic epitopes were destroyed by proteolytic digestion, or were contained within water-soluble peptide fragments too small to be detected on Western blots. In the preliminary study of Allard and Lienhard (1985) antibodies G1-G4 were reported to be specific for human glucose transporters: on Western blots they recognized transporters from a number of human cell types, but were apparently not cross-reactive with the transporters of rodent or avian cells. It is therefore likely that at least part of the epitopes recognized by the monoclonal antibodies correspond to regions of sequence difference between the transporters from these different species.

In the present study, the locations of the cytoplasmic epitopes for these monoclonal antibodies were investigated using a number of approaches. First, Western blotting was used to identify fragments produced by partial tryptic digestion of the human erythrocyte glucose transporter which could be recognized by the antibodies: the sequence locations of these fragments had previously been determined using

anti-peptide antibodies (Section 4.2.5). Second, their ability to bind synthetic peptides corresponding to different regions of the glucose transporter sequence was studied. Such an approach has been successfully employed in the location of epitopes for monoclonal antibodies in a number of proteins including bovine rhodopsin (MacKenzie et al., 1984), and Torpedo acetylcholine receptor gamma-subunit (LaRochelle et al., 1985). Third, clues to the sequence location of the epitopes were sought by comparing the reactivity of the antibodies towards the human erythrocyte and rat brain transporters, which differ only very slightly in sequence (Birnbaum et al., 1986). Establishment of these sequence locations in conjunction with a study of the effects of the antibodies on cytochalasin B binding to the transporter shed some light not only on the transporter topology, but also upon the functional importance of different regions of the protein sequence.

## 5.2 LOCATION OF EPITOPES FOR MONOCLONAL ANTIBODIES TO THE GLUCOSE TRANSPORTER

### 5.2.1 Monoclonal antibody labelling of fragments produced by limited tryptic digestion of the native glucose transporter

As discussed in Sections 4.2.4 and 4.2.5, digestion of the native glucose transporter with low concentrations of trypsin for short periods of time, gives rise to a glycosylated fragment(s) of apparent $M_r$ 23,000-42,000 derived from the N-terminal half of the protein (Fig.5.1), together with non-glycosylated fragments of apparent $M_r$ 25,500, 23,500, 21,000 and 18,000 derived fron the C-terminal half of the protein. Using anti-peptide antibodies it was possible to determine approximately from which regions of the transporter sequence the non-glycosylated tryptic fragments originated (Section 4.2.5). In order to locate the epitopes recognized by the monoclonal antibodies, the reactivity of each of the latter towards the non-glycosylated tryptic fragments of the transporter on Western blots was assessed.

Samples (20µg protein), of purified glucose transporter, prepared as described in Section 2.1.4 and then digested with trypsin for various times, were electrophoresed on SDS/12% polyacrylamide gels and electrophoretically transferred to nitrocellulose paper. The resultant blots were probed with the relevant monoclonal antibodies which had been affinity-purified using protein-depleted erythrocyte membranes as described in Section 3.2.5.2. Bound monoclonal antibodies were detected with rabbit anti-mouse IgG-alkaline phosphatase conjugate (Section 2.4).

None of the monoclonal antibodies were found to label the glycosylated tryptic fragment of $M_r$ 23,000-42,000 or the non-glycosylated fragment of $M_r$ 18,000 which resulted from extensive digestion of the transporter (Fig.5.2). Some of the antibodies faintly

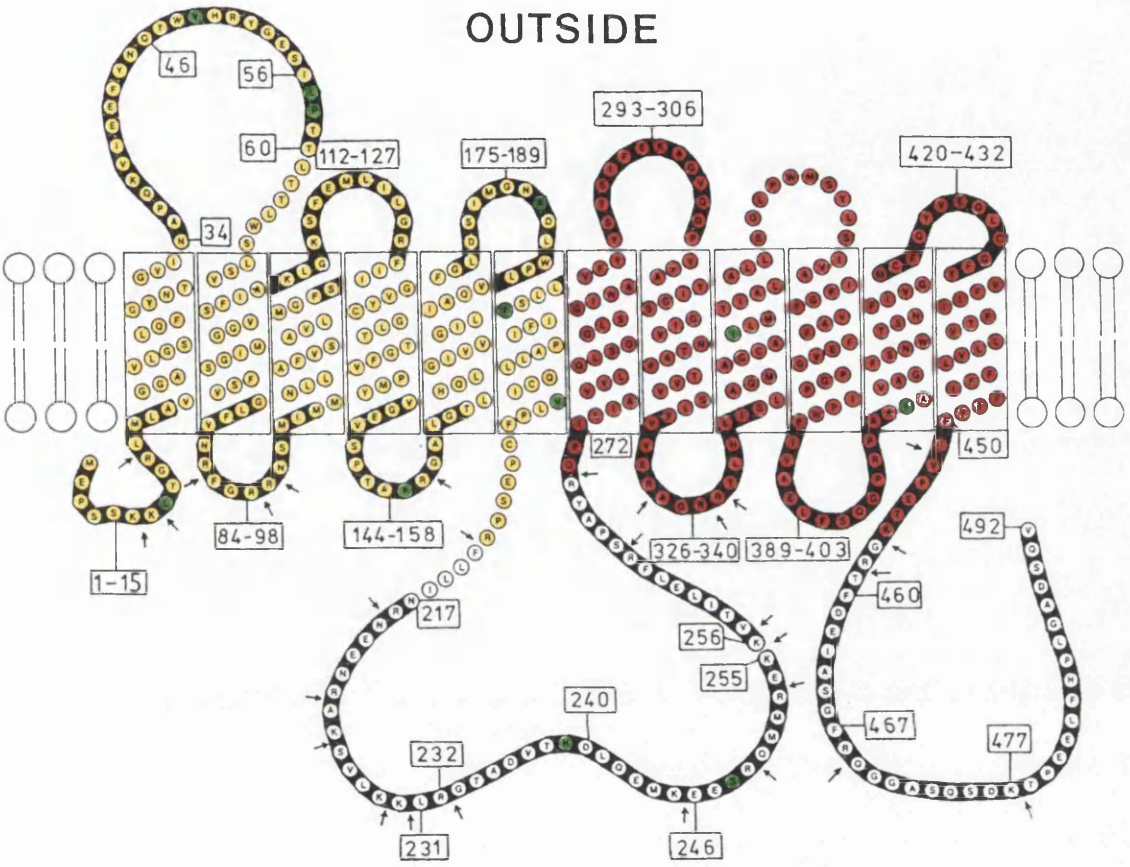



Fig.5.1 Location of chemically-synthesized peptides and membrane-bound tryptic fragments within the glucose transporter sequence

Regions of the sequence corresponding to synthetic peptides are outlined in black. Numbers refer to the sequence location of the N- and C-terminal residues of each peptide. Arrows indicate the location of potential tryptic cleavage sites. Residues comprising the tryptic fragments of $M_r$ 23,000-42,000 and 18,000 are coloured yellow and red, respectively. Non-conserved residues in the human erythrocyte and rat brain glucose transporter sequences are coloured green.

labelled apparent precursors to the glycosylated fragment which appeared after short times of digestion of the transporter, as seen for monoclonal antibody GTPR1 in Fig.5.2(A). This finding suggests that the epitopes recognized by these antibodies (GTPR1, GTPR2, GTPR3, G1 and G3) lie within the central cytoplasmic region of the transporter polypeptide. However, more conclusive evidence for the location of epitopes was provided by the much stronger labelling of the non-glycosylated fragments of the transporter by the monoclonal antibodies. The results of this study are summarized in Table 5.1. Five of the antibodies (GTPR1, GTPR2, GTPR3, G1 and G3), labelled the tryptic fragments of $M_r$ 25,500 and 21,000 but not those of 23,500 or 18,000, as illustrated by the result obtained for GTPR1 in Fig. 5.2(A). This pattern of labelling was identical to that found for polyclonal antibodies raised against peptides 231-246 and 240-255 (Section 4.2.5.2 and Table 4.1). Failure to recognize the $M_r$ 23,500 fragment, which contains the intact C-terminus of the transporter (Section 4.2.4), indicates that the epitopes for these antibodies cannot be within the C-terminal hydrophilic region of the transporter sequence. Lack of labelling of the $M_r$ 18,000 fragment, the N-terminus of which either $Gln_{270}$ or precedes this residue in the sequence (Section 4.2.5.2), indicates that the epitopes must lie within the central hydrophilic region of the transporter sequence, probably between $Ala_{224}$ and $Arg_{269}$ (Fig.5.1). In contrast, monoclonal antibodies GTPR4, G2 and G4 labelled only those fragments, of $M_r$ 25,500 and 23,500, which contain the intact C-terminus of the transporter (Section 4.2.5.2), as illustrated by the result obtained for GTPR4 in Fig.5.2(B). Lack of labelling of the $M_r$ 21,000 fragment indicates that the epitopes for these antibodies do not lie within the central hydrophilic region of the transporter sequence: the $M_r$ 21,000 and 25,500 fragments probably share the same N-terminal sequence which

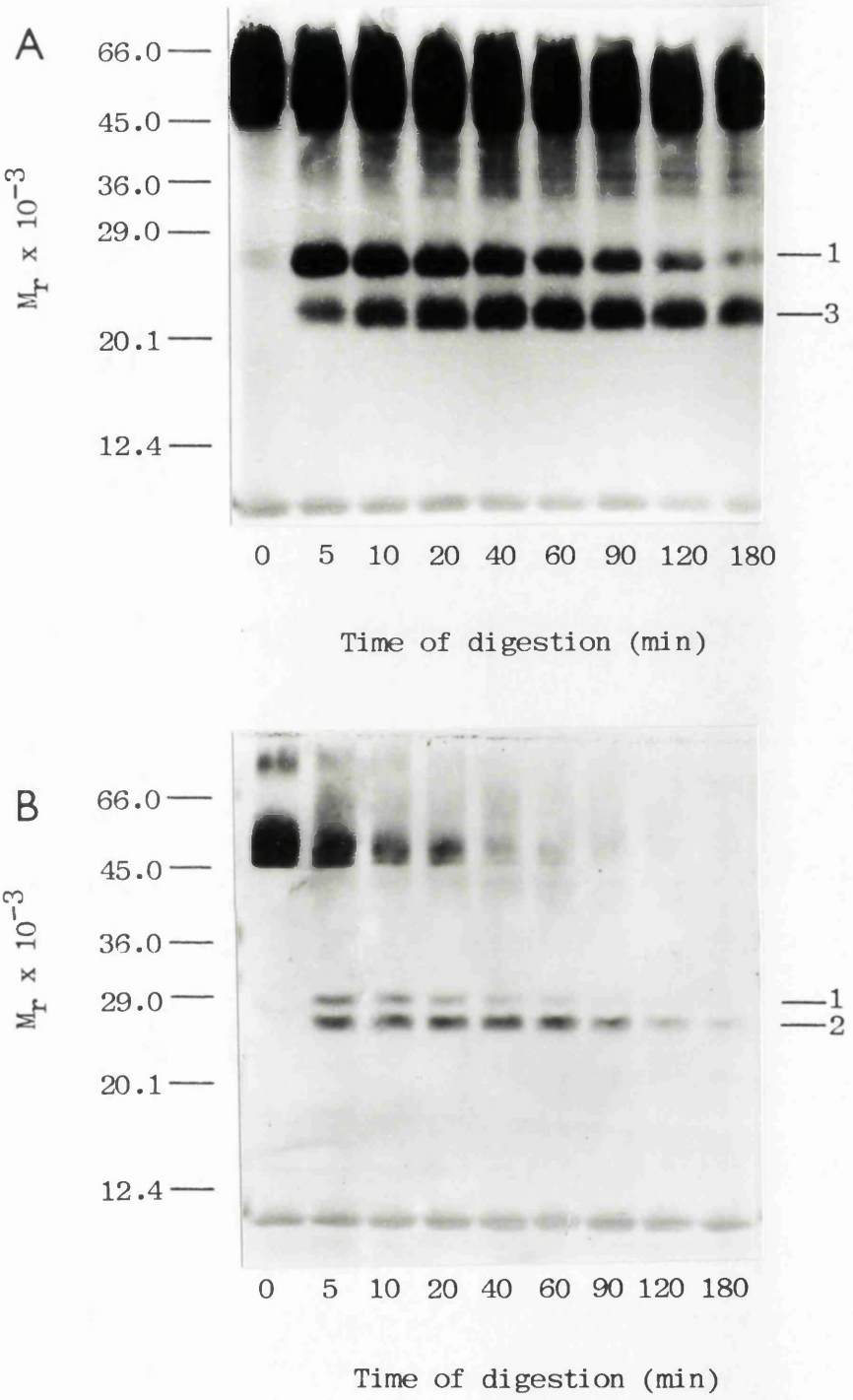



Fig.5.2 Binding of monoclonal antibodies to tryptic fragments of the glucose transporter on Western blots

Samples (20μg), of glucose transporter digested with trypsin for various times up to 3 hours were electrophoresed on a SDS/12% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and then immunolabelled with monoclonal antibody GTPR1 (A) or GTPR4 (B). The bands labelled 1-3 correspond to the non-glycosylated tryptic fragments of apparent $M_r$ 25,500, 23,500 and 21,000 (Section 4.2.5). The positions of $M_r$ markers are indicated.

| Monoclonal Antibody | Labelling of tryptic fragment | | | |
|---|---|---|---|---|
| | $M_r$ 25,500 | $M_r$ 23,500 | $M_r$ 21,000 | $M_r$ 18,000 |
| GTPR1 | + | - | + | - |
| GTPR2 | + | - | + | - |
| GTPR3 | + | - | + | - |
| GTPR4 | + | + | - | - |
| G1 | + | - | + | - |
| G2 | + | + | - | - |
| G3 | + | - | + | - |
| G4 | + | + | - | - |

Table 5.1 Patterns of reactivity of monoclonal antibodies with tryptic fragments of the glucose transporter on Western blots

includes much of this region (Section 4.2.5.2). In conjunction with the lack of labelling of the $M_r$ 18,000 fragment these findings indicate that the epitopes recognized by antibodies GTPR4, G2 and G4 lie within the C-terminal region of the transporter sequence between residues 459 and 492 (Fig.5.1).

## 5.2.2 Monoclonal antibody recognition of synthetic peptides corresponding in sequence to the cytoplasmic regions of the glucose transporter polypeptide

A more precise location for the epitopes recognized by the monoclonal antibodies was sought by investigating their ability to bind synthetic peptides in ELISA assays. The peptides tested corresponded in sequence to residues 1-15, 84-98, 144-158, 217-232, 231-246, 240-255, 256-272, 326-340, 389-403, 450-467, 460-477 and 477-492 of the predicted cytoplasmically-exposed regions of the transporter polypeptide (Mueckler et al., 1985, Fig.5.1). In addition, a peptide corresponding to residues 231-246 of the rat brain transporter sequence (Birnbaum et al., 1986) was also used.

### 5.2.2.1 Monoclonal antibody recognition of ELISA plate-bound peptides

ELISA plates were coated with synthetic peptides (20ng per well) as described in Section 2.2. After the blocking step, affinity-purified monoclonal antibodies were added to the relevant wells of the ELISA plates (9.46 to 946ng per well). Following overnight incubation at room temperature, bound monoclonal antibody was detected with rabbit anti-mouse IgG-alkaline phosphatase conjugate (Section 2.2). ELISA plates containing no peptides were used in control experiments.

Monoclonal antibodies GTPR1, GTPR2, GTPR3, G1 and G3 recognized only one plate-bound peptide, that corresponding to residues 231-246 of the human erythrocyte glucose transporter sequence (Fig.5.3(A).

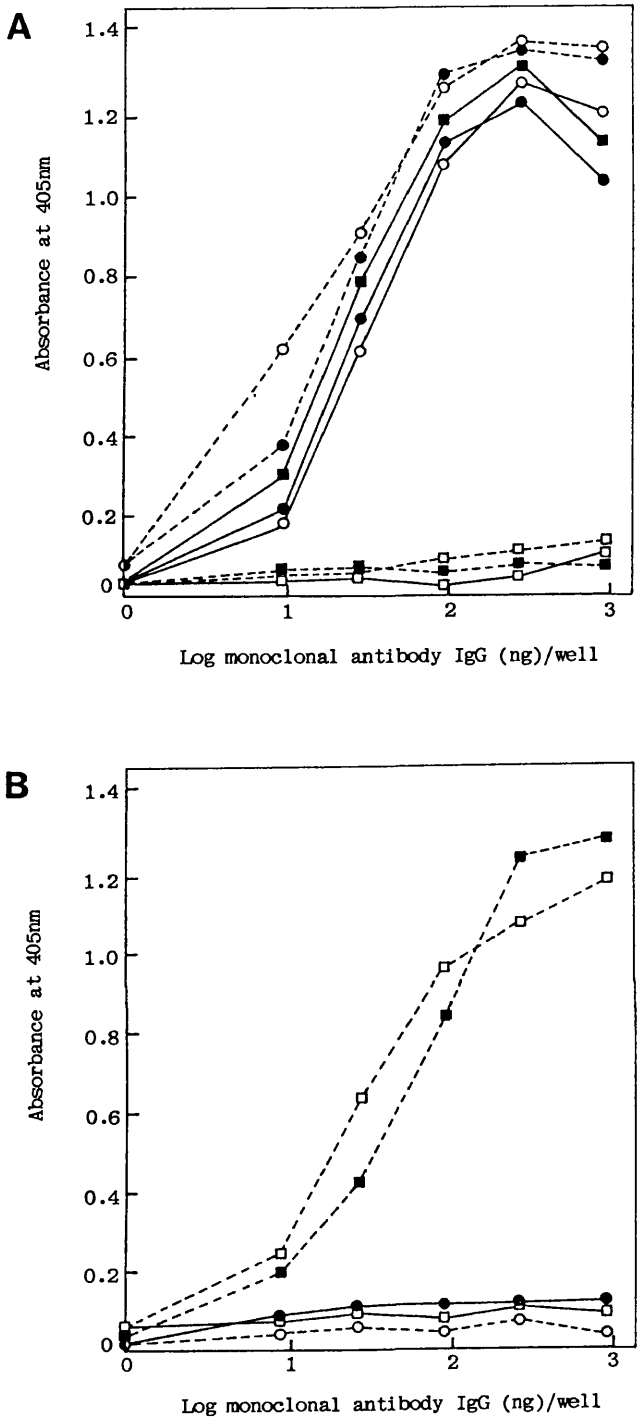



Fig.5.3 The ability of anti-glucose transporter monoclonal antibodies to recognize plate-bound synthetic peptides in ELISA

Microtitre plates were coated with 20ng per well of synthetic peptides corresponding to residues 231-246 (A) or residues 477-492 (B) of the glucose transporter. Plates were then incubated with the amounts of monoclonal antibody shown, followed by alkaline phosphatase-linked second antibody and p-nitrophenyl phosphate as chromogenic substrate. Each point is the mean of triplicate samples. Monoclonal antibodies used were GTPR1 (●—●), GTPR2 (○—○), GTPR3 (■—■), GTPR4 (□—□), G1 (○-○), G2 (■-■), G3 (●-●) and G4 (□-□).

However they did not recognize the peptide corresponding to residues 231-246 of the rat brain transporter sequence in ELISA (results not shown). The latter peptide differs in sequence from the human only by replacement of $His_{239}$ with an arginine (Table 3.1(A), and so it is likely that this residue forms part of or is very close to the epitope. Monoclonal antibodies G2 and G4 also recognized only one plate-bound peptide, that corresponding to residues 477-492 of the glucose transporter (Fig.5.3(B), indicating that at least part of the epitope(s) for these antibodies is located within the C-terminal 16 residues of the protein. These results are consistent with the pattern of labelling of glucose transporter tryptic fragments by the antibodies (Section 5.2). In contrast, monoclonal antibody GTPR4 did not recognize any plate-bound peptide (Fig.5.3).

5.2.2.2 Monoclonal antibody recognition of solution-phase peptides

Further experiments were performed in order to confirm that the binding of the monoclonal antibodies to ELISA plate-bound peptides, described in the previous section, was not artifactual but stemmed from these peptides forming (a part of) the epitopes in the intact protein. In these experiments solution-phase synthetic peptides were used to compete with plate-bound glucose transporter for monoclonal antibody binding.

Monoclonal antibodies at 3µg/ml in 0.2ml PBSA, pH 7.2, containing 1% BSA were incubated for 2 hours at room temperature with an equal volume of the same buffer containing various concentrations (0 to 120µM or 0 to 240µM) of the relevant synthetic peptide. The resultant 0.4ml incubations contained antibody at 1.5µg/ml (10nM) and 0 to 60µM or 0 to 120µM peptide. 0.1ml aliquots of each monoclonal antibody-peptide incubation (containing about 1pmol monoclonal IgG), were added to ELISA plates coated with 600ng (11pmol) per well of purified

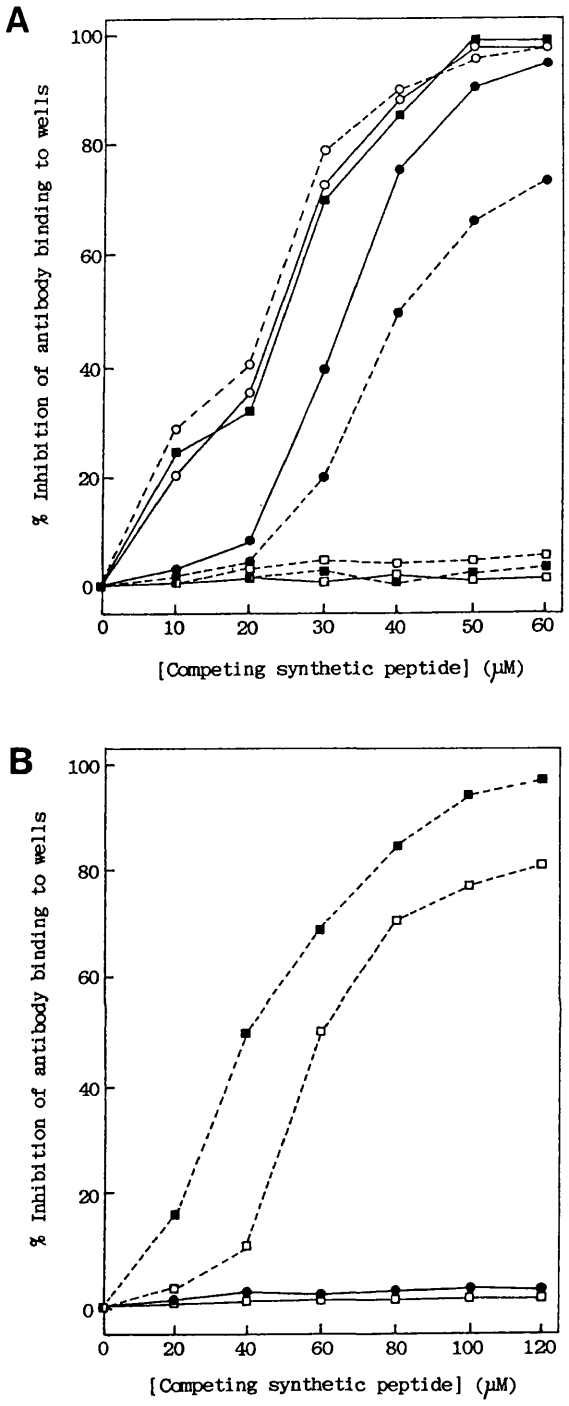



Fig.5.4 Competitive ELISA assay of the ability of solution-phase synthetic peptides to bind monoclonal antibodies

The ability of synthetic peptides corresponding to (A) residues 231-246 and (B) residues 477-492 of the glucose transporter to compete for a limiting amount of monoclonal antibody with 600ng of glucose transporter bound to the surface of a microtitre well. Samples of the monoclonal antibodies were incubated for 2 hours at room temperature with the concentrations of peptides indicated. The amount of free antibody was then measured by ELISA. Each point is the mean of triplicate samples. Monoclonal antibodies used were GTPR1 (●—●), GTPR2 (○—○), GTPR3 (■—■), GTPR4 (□—□), G1 (○--○), G2 (■--■), G3 (●--●) and G4 (□--□).

glucose transporter. The concentration of monoclonal antibody used in these experiments was determined not to be quite saturating from an ELISA curve derived from preliminary experiments using the antibodies with ELISA plate-bound glucose transporter (results not shown). Following overnight incubation, plate-bound monoclonal antibody was detected with rabbit anti-mouse IgG-alkaline phosphatase conjugate. Values of $A_{405nm}$ for 'blank' incubations (from which the antibodies were omitted) were subtracted from the experimental values. The % inhibition of monoclonal antibody binding to plate-bound glucose transporter was determined from a comparison of $A_{405nm}$ readings in the presence and absence of competing peptide.

Consistent with the findings in the previous section, the peptide corresponding to residues 231-246 of the human erythrocyte glucose transporter sequence, but no other peptide, competed with plate-bound glucose transporter for the binding of monoclonal antibodies GTPR1, GTPR2, GTPR3, G1 and G3 in competitive ELISA assay (Fig.5.4(A). However in order to inhibit binding by 50%, a 200- to 300-fold molar excess of peptide over glucose transporter was required. Similarly, monoclonal antibodies GTPR4, G2 and G4 were inhibited in their binding to plate-bound glucose transporter by the peptide corresponding to C-terminal residues 477-492 of the transporter, but by no other peptide (Fig.5.4(B). A 50% inhibition of antibody binding to the transporter required a 400- to 600-fold molar excess of peptide. None of the peptides competed with glucose transporter to bind monoclonal antibody GTPR4, as illustrated by the results obtained with peptides 231-246 and 477-492 in Fig.5.4.

### 5.2.3 Cross-reactivity of monoclonal antibodies with the rat brain glucose transporter

Additional support for the proposed locations of epitopes was

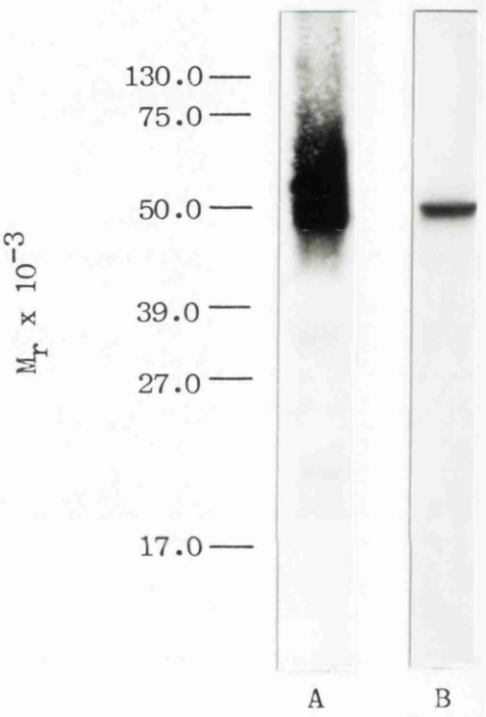



Fig.5.5 Cross-reaction of anti-human glucose transporter monoclonal antibodies with the rat brain glucose transporter

Samples of purified human erythrocyte glucose transporter (A) and rat brain microsomal membranes (B), were electrophoresed on a SDS/12% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and then immunolabelled with affinity-purified monoclonal antibody GTPR4. The positions of $M_r$ markers are indicated.

provided by examining the ability of the antibodies to recognize the rat brain transporter on Western blots. Samples (25μg protein), of rat brain microsomes, prepared as described in Section 6.2.1, and purified human erythrocyte glucose transporter (5μg protein), were electrophoresed on SDS/12% polyacrylamide gels, electrophoretically transferred to nitrocellulose paper and then probed with each of the affinity-purified monoclonal antibodies (5μg/ml), or a control monoclonal antibody (anti-O-6-deoxymethylguanosine, Boyle et al., 1985).

Monoclonal antibodies GTPR1, GTPR2, GTPR3, G1 and G3, all of which recognized the human erythrocyte transporter on Western blots, did not label any protein bands on Western blots of rat brain membranes (results not shown). This species-specificity is explicable if the epitope(s) contains, or is close to, $His_{239}$ in the human sequence because it is replaced by arginine in the rat brain (Mueckler et al., 1985; Birnbaum et al., 1986). In contrast, antibodies GTPR4, G2 and G4 did label a band of apparent $M_r$ 50,000 on Western blots of rat brain membranes, as illustrated by the result obtained for GTPR4 in Fig.5.5. No labelling was seen with the control monoclonal antibody (results not shown). This finding is consistent with the proposed locations of the epitopes for these antibodies within the hydrophilic C-terminal region of the transporter: the amino acid sequence of this region is identical in the human erythrocyte and rat brain glucose transporters (Mueckler et al., 1985; Birnbaum et al., 1986).

## 5.3 EFFECTS OF MONOCLONAL ANTIBODIES ON THE CYTOCHALASIN B BINDING ACTIVITY OF THE GLUCOSE TRANSPORTER

As discussed in Section 5.1, the monoclonal antibodies used in the present study were found previously to have a range of effects on

cytochalasin B binding to the glucose transporter (Allard and Lienhard, 1985; Boyle et al., 1985). Most of the antibodies (GTPR1, GTPR2, GTPR3, G1, G3 and G4 inhibited the binding of this ligand to the transporter to various extents, whilst antibody G2 produces slight enhancement of binding. The effect of GTPR4 was not established in these earlier studies. Therefore in the present study, the effects of GTPR4 on cytochalasin B binding to the transporter were determined. With the effect of each antibody then known, it would be possible to assess the involvement of those regions of the transporter sequence bearing epitopes for the monoclonal antibodies in the ligand-binding function of the protein.

Protein-depleted human erythrocyte membranes at a protein concentration of 0.25mg/ml in PBS, pH 7.2, were incubated for 2 hours at room temperature with various concentrations (up to 0.8mg/ml) of control monoclonal antibody (Section 5.2.2.2), or affinity-purified monoclonal antibody GTPR4. Antibodies GTPR1, G2 and G3 were included for comparison. Cytochalasin B binding activity was measured by equilibrium dialysis as described in Section 2.5.1.

Monoclonal antibodies GTPR4 and G2 had no apparent effect on cytochalasin B binding to the glucose transporter present in protein-depleted erythrocyte membranes (Fig.5.6). In contrast GTPR1 and G3 did inhibit the binding as measured using a fixed, low concentration of cytochalasin B ($4x10^{-8}$M), consistent with the previous findings of Allard and Lienhard (1985) and Boyle et al. (1985). A maximum inhibition of about 85 and 90% was seen when membranes at a concentration of 0.25mg protein/ml were incubated with 0.3mg GTPR1/ml or 0.5mg G3/ml, respectively (Fig.5.6). These concentrations correspond to about a 4.5- and 7.5-fold molar excess of antibodies GTPR1 and G3 respectively, over glucose transporter. No inhibition was seen using corresponding concentrations of control monoclonal antibody

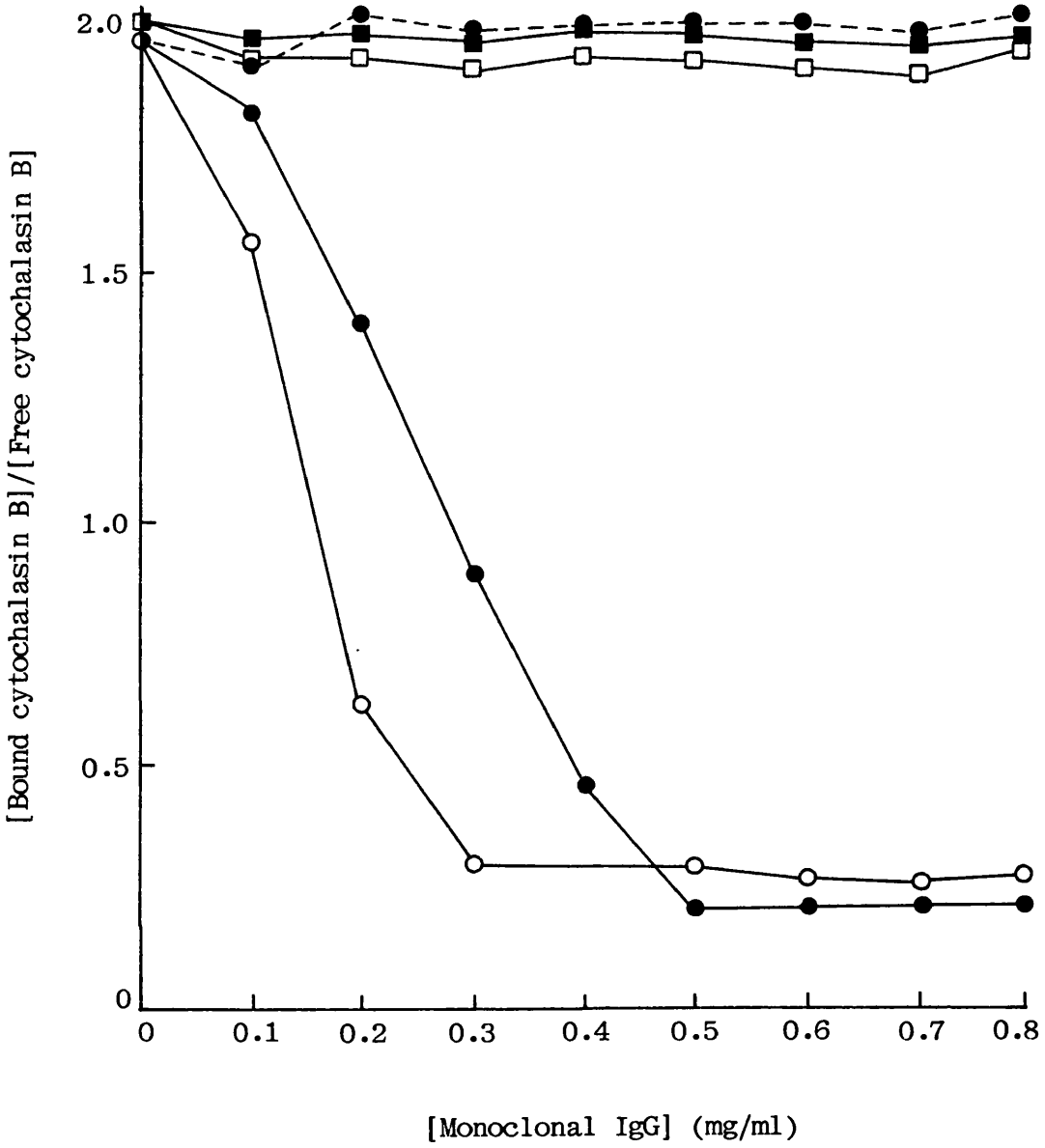



Fig.5.6 Inhibition of the cytochalasin B binding activity of protein-depleted erythrocyte membranes by monoclonal antibodies

Protein-depleted erythrocyte membranes at a concentration of 0.25mg/ml in PBS, pH 7.2, were incubated with various concentrations of control monoclonal antibody (●-●), or affinity-purified monoclonal antibodies GTPR1 (○—○), GTPR4 (■—■), G2 (□—□) and G3 (●—●). Cytochalasin B binding activity was then measured as described in Section 2.5.1. Each point is the mean of triplicate determinations.

(Fig.5.6). The mechanism of inhibition of cytochalasin B binding to the transporter by GTPR1 and G3 was not investigated further in the present study.

## 5.4 DISCUSSION

In the work described in this chapter, an attempt was made to locate the epitopes for eight monoclonal antibodies previously raised against the purified human erythrocyte glucose transporter (Allard and Lienhard, 1985; Boyle *et al.*, 1985). None of the antibodies labelled the membrane-bound products of extensive tryptic digestion of the native transporter and the antibodies are therefore likely to recognize epitopes within the hydrophilic, cytoplasmic regions of the protein cleaved off by trypsin. These findings are consistent with the antibodies all being directed against the cytoplasmic surface of the erythrocyte membrane (Allard and Lienhard, 1985; Boyle *et al.*, 1985). Polyclonal antibodies raised against the purified glucose transporter also primarily recognized these regions of the protein, although some reactivity to the membrane-bound tryptic fragment of $M_r$ 18,000 was also evident (Section 4.2.4). Therefore the hydrophilic, cytoplasmic regions of the native transporter polypeptide appear to be the predominant immunogenic regions of the protein.

The approximate locations of the epitopes for the antibodies were deduced from their pattern of labelling on Western blots of fragments produced by limited tryptic cleavage of the glucose transporter. The origin of these tryptic fragments from within the transporter sequence was determined previously using anti-peptide antibodies (Section 4.2.4). On the basis of these results, five of the monoclonal antibodies (GTPR1, GTPR2, GTPR3, G1 and G3) appeared to recognize epitopes in the central hydrophilic region (residues 213-272) of the transporter sequence, whilst GTPR4, G2 and G4 were directed against the C-terminal region (residues 450-492) of the protein (Fig.5.1). However, the exact locations of the epitopes were determined by examining the ability of the monoclonal antibodies to bind synthetic

peptides in ELISA assays corresponding in sequence to short segments of the transporter polypeptide predicted to be exposed at the cytoplasmic surface of the erythrocyte membrane (Mueckler et al., 1985). GTPR1, GTPR2, GTPR3, G1 and G3 all recognized a peptide corresponding to residues 231-246 of the human erythrocyte transporter sequence, whilst G2 and G4 recognized a peptide corresponding to C-terminal residues 477-492 of the protein. These findings were consistent with the locations of the epitopes predicted from reactivity of the antibodies with tryptic fragments of the transporter (see above). In addition, these peptides were found to compete with glucose transporter for the binding of the relevant monoclonal antibody in competitive ELISA assays, although a 200- to 600-fold molar excess of peptide over glucose transporter was required to inhibit antibody binding to the latter. These findings suggested that the antibodies have a much greater affinity for the intact transporter than for corresponding short segments of the polypeptide. This may reflect the different conformations of these molecules and/or the liklihood that additional residues make up the complete epitopes in the folded protein.

Further support for the locations of the epitopes was provided from a study of the cross-reactivity of the antibodies with the rat brain transporter. Antibodies GTPR1, GTPR2, GTPR3, G1 and G3 failed to recognize a peptide corresponding to residues 231-246 of the rat brain transporter, which differs from the equivalent human erythrocyte transporter sequence by a single amino acid substitution at residue 239 (arginine replaces histidine, Birnbaum et al., 1986, Fig.5.1). These findings suggested that the epitopes for the latter antibodies contain, or are close to, $His_{239}$ in the human transporter sequence. Consistent with this prediction, GTPR1, GTPR2, GTPR3, G1 and G3 also failed to label the rat brain transporter on Western blots. This

sequence difference may also account for the lack of reactivity of antibodies G1 and G3 towards other rodent tissues reported by Allard and Lienhard, 1985. Although a second amino acid substitution exists at residue 248 in the central hydrophilic regions of the rat and human transporters (Birnbaum et al., 1986, Fig.5.1), the fact that peptide 240-255, which encompassess this residue, was not recognized by the monoclonal antibodies, suggests that this site of substitution is not responsible for conferring the species-specificity of the antibodies. In contrast, antibodies GTPR4, G2 and G4 did label the rat brain transporter on Western blots. This was expected if they were indeed directed against the C-terminal region of the transporter: the amino acid sequence of this region is identical in the human and rat proteins (Mueckler et al., 1985; Birnbaum et al., 1986). The reactivity of antibodies G2 and G4 towards rodent transporters conflicted with the earlier findings of Allard and Lienhard (1985). However, the rodent tissue samples used in their study (from mouse and rat adipocytes) are now known to contain predominantly the immunologically-distinct insulin-sensitive glucose transporter (James et al., 1989; Kaestner et al., 1989). Therefore the problem may have been one of sensitivity of detection of glucose transporters homologous to the human erythrocyte and rat brain transporters in the rodent cell types.

In contrast to the other monoclonal antibodies, GTPR4 recognized the rat and human transporters on Western blots but failed to bind any plate-bound peptide. The epitope for this antibody was predicted to be located within the C-terminal region of the transporter from the pattern of labelling of membrane-bound tryptic fragments (see above). Since the epitope was lost upon extensive tryptic cleavage of the protein, it must lie either near a cleavage site or be located upon a small, water-soluble peptide liberated from the C-terminal region upon

digestion. Failure of this antibody to recognize synthetic peptides 450-467, 460-477 or 477-492 indicates that the epitope is not located wholly within peptides 459-468, 469-477 or 478-492 which are liberated by tryptic digestion of the transporter (Cairns et al., 1987). Lack of recognition of synthetic peptide 460-477 would not be expected if the epitope were destroyed by cleavage at $Arg_{467}$ (Fig.5.1). Similarly, lack of recognition of synthetic peptide 450-467 would not be expected if the epitope were destroyed by cleavage at $Lys_{456}$ or $Arg_{458}$ (Fig.5.1). It follows that the epitope is probably located close to the only other tryptic cleavage site in the C-terminal region of the transporter, $Lys_{477}$, although further studies will be required to confirm this.

Topological studies of the glucose transporter polypeptide using anti-peptide antibodies (Sections 4.2.1 and 4.2.2), yielded findings consistent with the predictions of the model of Mueckler et al. (1985) that the central hydrophilic region of the transporter sequence, encompassing residues 207 to 271, and the C-terminal region of the sequence from residues 451 to 492, are located at the cytoplasmic side of the erythrocyte membrane. That the cytoplasmic epitopes for the monoclonal antibodies also all lie within these regions, lends further support to this topological model. In a similar study by Andersson and Lundahl (1988), another monoclonal antibody that bound to a C-terminal peptide (residues 478-492) of the transporter also bound to the cytoplasmic surface of the erythrocyte membrane (see below).

Monoclonal antibodies to the purified human erythrocyte glucose transporter have been produced by several other groups. Tai and Carter-Su (1988) have recently described a monoclonal antibody they claim is specific for the human glucose transporter and uniquely labels the $M_r$ 18,000 tryptic fragment of the protein. Unfortunately these workers only investigated cross-reactivity of the antibody with

rodent cell types in which the amount of transporter is known to be low (rat erythrocytes) or in which the insulin-sensitive transporter is likely to be the major species present (adipocytes). They did not examine cross-reactivity towards rat brain membranes in which the 'HepG2/erythrocyte-type' transporter is abundant and so any cross-reactivity may have been missed. If their antibody is truly non-cross-reactive but labels the $M_r$ 18,000 tryptic fragment, then a sequence substitution in this region of the transporter (residues 270-456/458, Fig.5.1), must be involved in the epitope. The only differences in this region of the two proteins are at positions 348 and 404 which lie within putative membrane-spanning helices 9 and 11 (Birnbaum et al., 1986, Fig.5.1). If the transporter model is correct, it would be surprising if these residues were readily accessible to antibody molecules. On the other hand, if this antibody is cross-reactive then likely epitopes within the $M_r$ 18,000 fragment might be the sequence 451-458 remaining from cleavage of the C-terminal region of the transporter (Section 4.2.5, Fig.5.1). In addition, the observation that this antibody does not affect cytochalasin B binding to the transporter, suggested that its epitope is located within a region of the transporter polypeptide not involved in the binding of this ligand, such as the C-terminal region (see below and Section 4.3.1).

Andersson and Lundahl (1988), examined the reactivity of monoclonal antibodies raised against the intact transporter towards synthetic peptides corresponding to short segments of the transporter polypeptide. The antibodies recognized a peptide corresponding to C-terminal residues 478-492 (see above), but none recognized peptides corresponding to extracellular residues 51-64 and 292-307, or to residues 218-232 of the central hydrophilic region of the transporter (Fig.5.1). Interestingly, they observed that peptide 218-232 enhanced

the binding of monoclonal antibodies to the C-terminus of the transporter. This suggested that the cytoplasmic central and C-terminal regions of the transporter may interact in the folded protein. However, in the present study, monoclonal antibodies GTPR4, G2 and G4 were not affected in their binding to the C-terminus of the glucose transporter by peptides 217-232, 231-246, 240-255 and 256-272 in competitive ELISA assays (results not shown). These conflicting findings could be explained by different structural epitopes for the antibodies in the transporter; or that the finding of Andersson and Lundahl (1988) was artifactual.

The effects of anti-peptide antibodies on cytochalasin B binding to the glucose transporter (Section 4.3.1), were largely paralleled by the effects of monoclonal antibodies which bound to the same regions of the sequence. Thus monoclonal antibody GTPR4, which appears to bind to the C-terminal region of the transporter, had no effect on the cytochalasin B binding activity of the protein. Monoclonal antibody G2, which binds to the C-terminal peptide comprising residues 477-492 had, if anything, a slight stimulatory effect on binding (Allard and Lienhard, 1985: this study Section 5.3). However, antibody G4, which also binds to the C-terminus, inhibited binding (Allard and Lienhard, 1985). Such differences between the effects of monoclonal antibodies which appear to bind to the same region of the transporter sequence may of course stem from their epitopes being partially discontinous and so involving more than one region of the transporter sequence. Monoclonal antibodies GTPR1, GTPR2, GTPR3, G1 and G3, all of which bound to the central hydrophilic region of the transporter sequence, also inhibited cytochalasin B binding (Allard and Lienhard, 1985; Boyle et al., 1985; this study and, for GTPR3, Meeran, K. and Baldwin, S.A., unpublished observations). The mechanism of inhibition of cytochalasin B binding to the transporter previously determined for

GTPR1 (Boyle et al., 1985) also closely paralleled the effects of anti-peptide antibodies: namely a reduction in the affinity of the transporter for the ligand, without a reduction in the total number of binding sites (Section 4.3.1, and discussed in Section 4.4). Therefore consistent with the findings from studies with anti-peptide antibodies, the pattern of inhibition of cytochalasin B binding to the transporter by monoclonal antibodies indicate that the central hydrophilic region, but not the C-terminal region of the transporter is involved in the cytochalasin B binding function of the protein.

Useful future experiments would include an examination of the effects of the monoclonal antibodies on the glucose transport function of the protein. Such an approach was mentioned in Section 4.4 for anti-peptide antibodies. These studies should yield important structural information regarding the role of the extramembranous, cytoplasmic regions of the transporter polypeptide in sugar translocation. Further studies on the mechanism of inhibition by antibodies might also provide significant information regarding the structure/function relationships of the protein.

# CHAPTER 6 IDENTIFICATION OF PROTEINS HOMOLOGOUS TO THE HUMAN HEPG2/ERYTHROCYTE GLUCOSE TRANSPORTER IN MAMMALIAN AND NON-MAMMALIAN TISSUES

## 6.1 INTRODUCTION

Most animal cells have transport proteins which facilitate the movement of D-glucose across the plasma membrane. Methods originally employed in the characterization of the human erythrocyte glucose transporter, such as measurement of reversible D-glucose-inhibitable cytochalasin B binding and covalent photolabelling with [$^3$H]cytochalasin B, have more recently been used to characterize and identify D-glucose transporters in many different cell types.

Measurement of cytochalasin B binding to a variety of cell types in the presence and absence of D-glucose and of other cytochalasins has been used to demonstrate the existence of D-glucose-sensitive sites in cells such as the rat adipocyte (Wardzala et al., 1978) and chick embryo fibroblasts (Salter and Weber, 1979). The affinity constants derived from Scatchard analysis of cytochalasin B binding (Scatchard, 1949) were similar to that found for the human erythrocyte. An exception was the liver transporter which demonstrated a $K_d$ for cytochalasin B binding and $K_i$ for cytochalasin B inhibition of glucose transport which were both approximately one order of magnitude higher than those of any other cell type studied (Axelrod and Pilch, 1983, Table 6.1). Photoaffinity-labelling with [$^3$H]cytochalasin B has been used in numerous studies to identify glucose transporters, including those of rat adipocytes (Shanahan et al., 1982), skeletal muscle cells (Klip et al., 1983), human placenta microvillous membranes (Ingerman et al., 1983) and chick embryo fibroblasts (Shanahan et al., 1982; Pessin et al., 1982). In contrast,

Table 6.1 Biochemical and kinetic properties of some facilitated glucose transport proteins

| Tissue | $M_r$ | Insulin sensitivity | $K_m$ D-glucose (mM) | $K_i$ cytochalasin B (uM) | Refs* |
|---|---|---|---|---|---|
| Rat adipose | 42,000-47,000 | + | 5 | 0.1 | 1,2 |
| Rat skeletal muscle | 45,000-50,000 | + | 2.5 | - | 3 |
| Chick embryo fibroblast | 41,000 | + | 1.1-5.3 | 0.1 | 4 |
| Rat liver | 45,000-50,000 | - | 17-30 | 1.9 | 5,6,7,8 |
| Rat/ovine brain | 53,000-55,000 | - | 9 | 0.64 | 9,10,11 |
| Human erythrocyte | 55,000 | - | 1.6 | 0.66 | 12,13,14 |
| Rat small intestine (basolateral membrane) | 50,000-60,000 | - | 20-28 | 0.11 | 15 |
| Rat placenta | 45,000-68,000 | - | - | 0.78 | 16,17 |

*
1. Simpson and Cushman, (1986), 2. Horuk et al., (1986), 3. Wardzala and Jeanrenaud, (1981),
4. Salter and Weber, (1979), 5. Baur and Heldt, (1977), 6. Elliott and Craik, (1982),
7. Axelrod and Pilch, (1983), 8. Ciaraldi et al., (1986), 9. Pardridge, (1983),
10. Matthaei et al., (1987), 11. Baldwin et al., (1985), 12. Lin and Spudich, (1974),
13. Sogin and Hinkle, (1978), 14. Carruthers and Melchior, (1984), 15. Maenz and Cheeseman, (1987),
16. Johnson and Smith, (1982), 17. Wessling and Pilch, (1984).

the rat liver transporter, which has kinetic properties that differ substantially from those seen in most other cell types could not be directly covalently labelled with cytochalasin B (Axelrod and Pilch, 1983). This observation suggested that there are structural differences between the liver transporter and the transporters of other cell types.

These and other glucose transport proteins identified have been classified according to the mechanism by which they function, and fall into two major groups: (a) transporters which require the co-transport of cations such as $Na^+$ and $H^+$, represented by the mammalian kidney and intestinal brush border membrane and Leishmania donovani transporters; and (b) transporters that operate by facilitated diffusion, represented by the erythrocyte, liver, intestinal basolateral membrane, adipocyte, muscle, brain and fibroblast transporters (Table 6.1). The facilitated transporters can be further classified in terms of their responsiveness (adipocyte and muscle), or non-responsiveness (most other tissues), to insulin (Table 6.1).

The cation co-transport proteins listed above are both structurally and kinetically distinct from the facilitated glucose transport proteins (Crane 1977; Turner, 1986; Zilberstein et al., 1986). Indeed, the $Na^+$/glucose co-transporter from rabbit intestinal brush border membranes has recently been cloned and sequenced (Hediger et al., 1987) and shown to demonstrate no homology with the human erythrocyte glucose transporter. As the work described in this chapter is primarily concerned with the facilitated glucose transporters, further discussion regarding co-transport proteins will be confined to the relevant sections of the study.

The physiological significance of the variety of transport proteins is not absolutely clear, although this may reflect an adaptation of the cell to its extracellular environment. For instance,

the adipocyte transporter has an apparent affinity for glucose near the blood sugar concentration during starvation. Thus, glucose entry into adipocytes is regulated by the ability of insulin to increase the $V_{max}$ for transport (Wardzala et al., 1978). In contrast, in the liver, where the primary role of the organ is the storage and production of glucose (Newsholme and Start, 1973), glucose uptake increases in direct proportion to increases in blood sugar; the glucose concentration approaching the $K_m$ value of liver transport (17-30mM) only at very high concentrations (Ciaraldi et al., 1986). The higher $K_m$ of the liver transporter also ensures that at physiological glucose concentrations, uptake into the periphery, and not the liver, will be the primary route of glucose disposal. Such a role in glucose homeostasis could account for the noted kinetic and structural differences of the hepatic transporter (Axelrod and Pilch, 1983). Consequently, the biochemical properties and regulation of glucose transporters in different cells appear to reflect the biological role of the cell in glucose metabolism.

The similarities in $M_r$, affinity for glucose and inhibition of glucose transport by cytochalasin B, for the facilitated glucose transport proteins in most cell types (with the exception of liver), suggested the presence of a family of homologous proteins (Table 6.1). Studies utilizing polyclonal antibodies raised to the purified human erythrocyte glucose transporter provided further information regarding the structural similarities or differences in the transport proteins of various tissues. These antibodies were found to cross-react with components of rat adipocyte plasma membranes (Lienhard et al., 1982; Wheeler et al., 1982), virus-transformed fibroblasts (Lienhard et al., 1982), HeLa cells (Lienhard et al., 1982) and 3T3-L1 preadipocytes (Haspel et al., 1985), but not with rat liver membranes (Birnbaum et al., 1986).

Although cytochalasin B labelling procedures and the use of anti-transporter antibodies had shown that homologous transport proteins were probably present in a wide variety of tissues, the degree of similarity between the transporters of different tissues remained largely unclear. Subsequent cDNA cloning studies in fact later revealed that a family of at least four facilitated glucose transporters is found in mammalian tissues (Section 6.4). One problem in using a polyclonal anti-transporter serum raised against the intact erythrocyte protein to study transporter distribution is that it contains antibodies directed against many regions of the sequence. Some of these may recognize homologous transporters that are not the product of the HepG2/erythrocyte transporter gene. In addition, the erythrocyte glucose transporter preparation is not completely pure, and so the antiserum may contain antibodies directed against other proteins, such as the nucleoside transporter (Section 3.3.1.4). For these reasons, anti-peptide antibodies were used in the study described in this chapter to investigate the distribution of glucose transporters in different mammalian and non-mammalian tissues. From their reactivity towards different anti-peptide antibodies, an attempt was made to investigate their sequence similarities to the human HepG2/erythrocyte glucose transporter.

## 6.2 IDENTIFICATION OF GLUCOSE TRANSPORTERS IN MAMMALIAN AND NON-MAMMALIAN TISSUES.

In this section, anti-peptide antibodies were used as probes to identify proteins homologous to the human HepG2/erythrocyte glucose transporter in a number of tissues of mammalian origin. Such a study would yield information regarding how widely distributed these transport proteins are. The study was then extended in an attempt to identify homologous proteins in lower vertebrate and invertebrate organisms.

### 6.2.1 Preparation of membranes

Brain microsomal membranes were prepared essentially as described by Sweadner (1978). Briefly, 10g of excised rat brain was cut into small pieces and homogenized with a hand-held homogenizer in 25ml 0.1M sodium phosphate, pH 7.5, containing 0.25M sucrose, 1mM EDTA, 1mM sodium tetrathionate and protease inhibitors (2mM iodoacetamide, 0.2mM PMSF, 10µg/ml pepstatin). The homogenate was centrifuged at 3000 rev/min for 20 minutes to pellet unbroken cells, mitochondria and nuclei. The supernatant was recentrifuged at 45,000 rev/min for 60 minutes and the resulting pellet washed once with the homogenization buffer and finally resuspended to 7mg protein/ml in the same buffer.

Adipocyte plasma membranes were prepared by Mr Wayne Bowen of the Hannah Research Institute, Ayr, Scotland. Perirenal and parametrial fat pads were removed from virgin rats and adipocytes prepared by the collagenase digestion method of Rodbell (1964). Adipocytes were lysed in hypotonic buffer and plasma membranes isolated by a modification of the method of McKeel and Jarrett (1970).

Liver cell membranes was prepared by Miss Debbie Panton of the Hannah Research Institute, Ayr, Scotland. Briefly, diced rat liver was

homogenized in 20mM Tris-HCl, pH 7.4, containing 20mM sucrose. Following centrifugation at 3000 rev/min for 10 minutes, the resultant supernatant was centrifuged at 13,000 rev/min for 30 minutes, and the supernatant from this spin was centrifuged at 25,000 rev/min for 60 minutes. The final pellet was then resuspended and homogenized in 20mM Tris-HCl, pH 7.4, containing 20mM $CaCl_2$.

Rat testis Leydig cells were isolated and purified by Dr M.H.F. Sullivan of the Dept. Biochemistry, Royal Free Hospital School of Medicine, London, U.K., using the methods of Aldred and Cooke (1982) and Dix and Cooke (1981). Leydig cell membranes were prepared by hypotonic lysis and repeated washes by centrifugation in 5mM sodium phosphate buffer containing 0.1mM PMSF.

Isolated CHO-K1 cells were supplied by Drs M. Morgan and P. Faik of the Dept. Biochemistry, United Dental and Medical Schools of Guy's and St. Thomas' Hospital, London, U.K. These cells are a subclone derived from a proline-requiring ($CHO/Pro^-$) cell line (Kao and Puck, 1968). The parental $CHO/Pro^-$ cells were cultured from a biopsy of whole ovary from an inbred strain of the chinese hamster Cricetulus griseus (Puck et al., 1958). A membrane fraction from the CHO-K1 cells was prepared by hypotonic lysis. Cell pellets (containing $1-2x10^7$ cells), were lysed in 5mM sodium phosphate buffer, pH 7.4, containing 0.1mM PMSF. The treated cells were rapidly frozen in ethanol/dry ice and thawed (in an attempt to maximize the degree of lysis), and centrifuged at 11,500 rev/min. The supernatants were discarded, the membranous pellets resuspended in the same buffer and the freeze-thaw cycle repeated twice more. The final pellets were resuspended in 5mM sodium phosphate, pH 7.4.

Plasma membrane- and Golgi vesicle membrane-enriched fractions from the excised mammary glands of day 10 lactating rats were isolated by Dr R. Madon of the Hannah Research Institute, Ayr, Scotland, using

the methods of Clegg (1981) and West (1981). The purity of the preparations was assessed by marker enzyme assays, namely, 5'-nucleotidase for the plasma membrane preparation and galactosyltransferase (a component of lactose synthetase) for the Golgi vesicle preparation. 5'-nucleotidase assay revealed that the Golgi vesicle preparation used carried only 10% contamination with plasma membranes, whilst exhibiting a 20-fold enrichment with galactosyltransferase.

FRTL-5 thyroid cell plasma membranes were a gift of Dr S. Filetti of the Dept. Endocrinology and Clinical Medicine, University of Catania, Catania, Italy. The membranes were prepared by discontinuous sucrose gradient centrifugation (Cushman and Wardzala, 1980) and resuspended in 20mM HEPES buffer, pH 7.4, containing 1mM EDTA and 1mM PMSF.

Preparations of basolateral and brush border membrane vesicles from rabbit duodenum were a gift of Dr S.P. Shirazi-Beechey of the Dept. Biochemistry and Agricultural Biochemistry, The University College of Wales, Aberystwyth, Dyfed, U.K. The brush border membrane vesicles were prepared as described by Shirazi-Beechey *et al.* (1988), using a modification of the methods of Kessler *et al.* (1978) and Biber *et al.* (1981). The basolateral membrane vesicles were prepared as described by Shirazi-Beechey *et al.* (1986), by a modification of the method of Mircheff *et al.* (1980). Confirmation that the basolateral membrane preparations were not contaminated by brush border membranes was established by a glucose transport assay carried out in the presence and absence of 250μM phlorizin, whilst establishment of a basolateral membrane-free preparation of brush border membranes was made by glucose transport assays performed in the presence or absence of 100μM phloretin or 100mM NaSCN (Wright and Van Os, 1980).

Hagfish erythrocyte membranes were a gift of Dr J.D. Young of the

Dept. Biochemistry, The Chinese University of Hong Kong, Shatin, NT, Hong Kong. The membranes were prepared from hagfish erythrocytes by hypotonic lysis, essentially as described in Section 2.1.1.

A preparation of isolated surface membranes from the promastigote form of L. donovani were a gift of Dr A.K. Allen and Miss Louise Clarke of the Dept. Biochemistry, Charing Cross and Westminster Medical School, London, U.K. The membranes were prepared and characterized essentially as described by Zilberstein et al. (1986).

Samples of the membrane preparations (50-100µg protein), were solubilized in gel sample buffer (Section 2.3). The samples were electrophoresed on SDS/10% or 12% polyacrylamide gels, electrophoretically transferred to nitrocellulose paper (Section 2.4) and then probed with the relevant anti-peptide antibodies. Antibodies to peptides 217-232, 231-246, 240-255, 256-272, 450-467, 460-477, and 477-492 were used in affinity-purified form. Bound antibodies were detected with either donkey anti-rabbit IgG $F(ab')_2$-$^{125}I$ conjugate (liver, intestine, thyroid and hagfish membranes), or goat anti-rabbit IgG-alkaline phosphatase conjugate (Section 2.4).

## 6.2.2 Identification of proteins homologous to the human HepG2/erythrocyte glucose transporter in a range of mammalian tissues

### 6.2.2.1 Rat brain

It is now fairly well established that glucose is transported across the blood-brain barrier by a facilitated carrier mechanism that is not energy-dependant and not influenced by $Na^+$ or insulin (Pardridge, 1983). Some kinetic and molecular properties of the glucose transport proteins present in microvessel membrane fractions derived from rodent, ovine and porcine brain are given in Table 6.1. In terms of $K_m$ for D-glucose, $K_i$ for cytochalasin B inhibition of D-

glucose uptake and $M_r$, the brain transporter is very similar to that of the human erythrocyte. These similarities suggest the presence of a protein(s) homologous to the human HepG2/erythrocyte glucose transporter in brain microvessel membranes which may also be present in other regions of the brain. In order to demonstrate any sequence homology between the proteins, anti-peptide antibodies to the human erythrocyte glucose transporter were used to probe for immunologically-reactive components present in a microsomal membrane fraction prepared from whole rat brain.

Samples (50μg protein), of rat brain microsomes, prepared as described in Section 6.2.1, were electrophoretically transferred to nitrocellulose paper and then probed with antibodies against peptides 231-246, 240-255, 450-467. 460-477 and 477-492. Each of the anti-peptide antibodies labelled a component present in the brain microvessel membranes which migrated as a sharp band of apparent $M_r$ 50,000 on Western blots, as illustrated by the result obtained for antibodies to peptide 477-492 in Fig.6.1(B). No labelling was seen with non-immune rabbit IgG (result not shown).

6.2.2.2 Rat adipocyte

The adipocyte (and skeletal muscle) glucose transporters are of great interest because of their regulation by insulin (Crofford and Renold, 1965). The transporter is present in the adipocyte plasma membrane and within an intracellular pool (the so-called 'low density microsomes'), the latter transporters being mobilized and translocated to the plasma membrane in response to insulin stimulation (Wardzala et al., 1978; Suzuki and Kono, 1980; Cushman and Wardzala, 1980; Wheeler et al., 1982). The structural properties of the rat adipocyte transporter have been investigated by photolabelling studies with [$^3$H]cytochalasin B (Shanahan et al., 1982; Carter-Su et al., 1982;

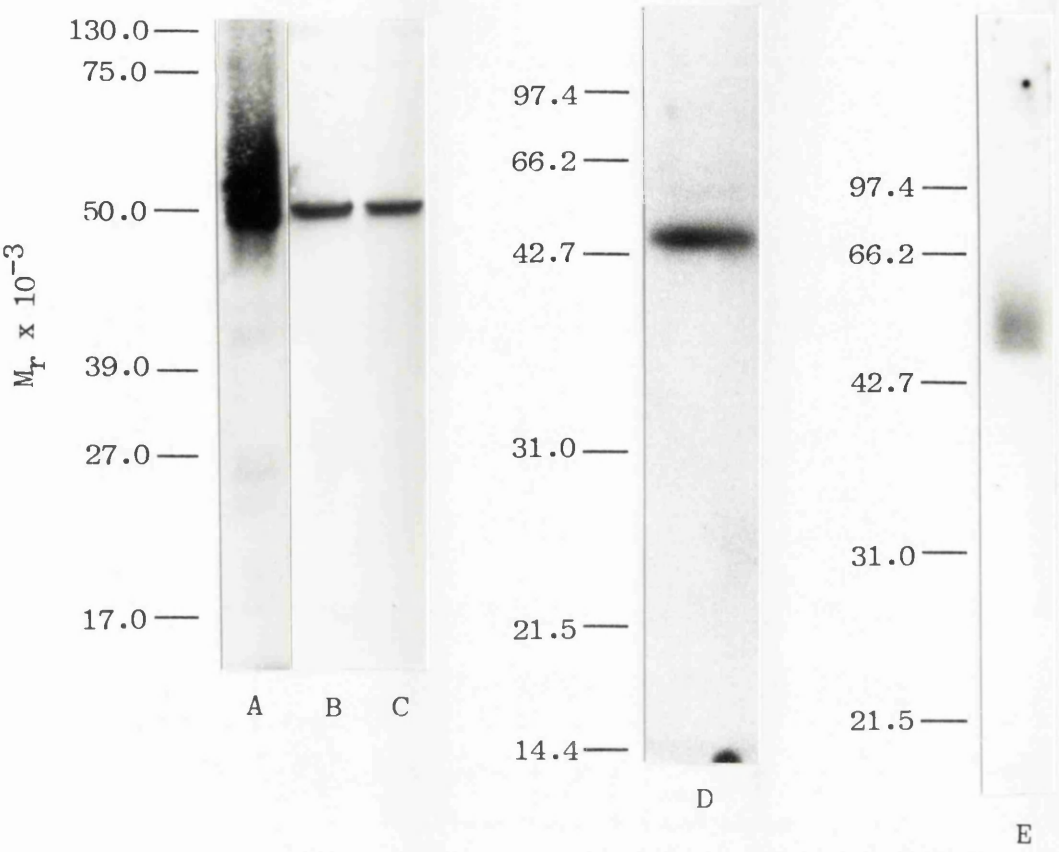



Fig.6.1 Detection of immunologically-reactive proteins present in various rat tissues

Samples of purified human erythrocyte glucose transporter (A), (1μg protein), rat brain microsomal membranes (B), rat testis Leydig cell membranes (C), rat adipocyte plasma membranes (D) and rat liver cell membranes (E), (each of 50μg protein), were electrophoresed on SDS/10% or 12% polyacrylamide gels, electrophoretically transferred to nitrocellulose paper and then immunolabelled with antibodies to C-terminal residues 477-492 (A, B, C and D), or 450-467 (E), of the human erythrocyte glucose transporter. The positions of $M_r$ markers are indicated.

Horuk et al., 1983). The photolabelled transporter migrated on SDS/polyacrylamide gels with an apparent $M_r$ 42,000-47,000. Although no difference in $M_r$ between the plasma membrane and intracellular transporters could be detected on gels, the proteins do exhibit differences in charge properties (Horuk et al., 1986). In addition the rat adipocyte transporter demonstrates kinetics very similar to those of the human erythrocyte protein (Table 6.1). Polyclonal antibodies raised to the purified, intact human erythrocyte glucose transporter have been used previously to study the adipocyte transporter (Wheeler et al., 1982; Lienhard et al., 1982). The antibodies labelled a protein of apparent $M_r$ 45,000-50,000 in both the adipocyte plasma membrane and low density microsomal membrane fractions. Although these antibodies were cross-reactive with the rat adipocyte transporter, it was not clear to what extent the two proteins were similar in structure. Indeed, cross-reactivity might possibly have been the result of antibodies directed against the oligosaccharide chains rather than against the polypeptide moieties of the proteins. Therefore in order to ascertain whether or not the polypeptide component of the proteins were at all homologous, anti-peptide antibodies were used to probe for immunologically-reactive species present in a preparation of rat adipocyte plasma membranes.

Samples (50μg protein), of rat adipocyte plasma membranes, prepared as described in Section 6.2.1, were electrophoretically transferred to nitrocellulose paper and then probed with the anti-peptide antibodies listed in Section 6.2.2. Each of the anti-peptide antibodies labelled a component present in rat adipocyte plasma membranes which migrated as a sharp band of apparent $M_r$ 45,000 on Western blots, as illustrated by the result obtained for antibodies to peptide 477-492 in Fig.6.1(D). No labelling was seen with non-immune rabbit IgG (results not shown).

6.2.2.3 Rat liver

As discussed in Section 6.1, the properties of the glucose transporters in the liver differ from those passive transporters present in most other cells. Although the liver transporter cannot be directly photolabelled with [$^3$H]cytochalasin B (Axelrod and Pilch, 1983), it can be labelled by photochemical cross-linking to [$^3$H]cytochalasin B via hydroxysuccinimidyl-4-azidobenzoate. Using this method, Ciaraldi *et al.* (1986) reported a $M_r$ of 40,000-50,000 for the liver transporter. The protein was detected in both liver plasma membrane and low density microsomal membrane fractions. Because of the significant kinetic and molecular differences of the liver glucose transporter, I was interested in using the available panel of anti-peptide antibodies to ascertain whether or not the rat liver transporter(s) was in any way structurally homologous to that of the human HepG2/erythrocyte protein. Consequently the antibodies were used to probe for immunologically-reactive species in a crude preparation of membranes isolated from whole liver.

Samples (50μg protein), of the liver membranes, prepared as described in Section 6.2.1, were electrophoretically transferred to nitrocellulose paper and then probed with antibodies to peptides 1-15, 84-98, 144-158, 217-232, 231-246, 240-255, 256-272, 450-467, 460-477 and 477-492, or antibodies to the purified human erythrocyte glucose transporter. Of the anti-peptide antibodies used, only one, corresponding to C-terminal residues 450-467 of the human erythrocyte glucose transporter, gave weak labelling of a component in rat liver membranes of apparent $M_r$ 50,000-55,000 on Western blots (Fig.6.1(E). A high concentration of anti-peptide antibody (25μg/ml) was required to achieve the intensity of labelling shown. No labelling was seen with antibodies to the purified human erythrocyte glucose transporter or with non-immune rabbit IgG (results not shown).

6.2.2.4 Rat testis Leydig cells

In a recent study into the relationship between glucose uptake and steroidogenesis in rat testis and tumor Leydig cells, Amrolia et al. (1988) showed that the characteristics of glucose transport in both cell types resembled those mediated by facilitated glucose transport proteins present in most other mammalian cells. In particular, Leydig cells were found to transport 2-deoxyglucose but not L-glucose, and uptake was inhibitable by cytochalasin B and forskolin. Additionally, in the presence of luteinizing hormone, the rate of 2-deoxyglucose uptake was increased by about 50%. Consequently, anti-peptide antibodies were used to determine whether proteins homologous to the human erythrocyte glucose transporter are present in rat Leydig cell plasma membranes, and therefore involved in glucose uptake into these cells.

Samples (50µg protein), of rat testis Leydig cell plasma membranes, prepared as described in Section 6.2.1, were electrophoretically transferred to nitrocellulose paper and then probed with antibodies to peptides 231-246 and 477-492. Both antibodies labelled a component in the Leydig cell membranes of apparent $M_r$ 50,000 on Western blots, as illustrated by the result obtained for antibodies to peptide 477-492 in (Fig.6.1(C). No labelling was seen with non-immune rabbit IgG (results not shown).

6.2.2.5 Chinese hamster ovary (CHO) cells

Faik et al. (1989) have previously demonstrated the presence of a phloretin-sensitive transport system for 2-deoxyglucose and 3-O-methylglucose in CHO-K1 cells. In order to establish whether glucose transporters structurally homologous to the human HepG2/erythrocyte protein are present in these cells, anti-peptide antibodies were used to probe for immunologically-reactive species in a crude membrane

preparation derived from CHO-K1 cells.

Samples (50μg protein), of CHO-K1 cell membranes, prepared as described in Section 6.2.1, were electrophoretically transferred to nitrocellulose paper and then probed with antibodies to peptides 231-246 and 477-492. Both antibodies labelled a component of apparent $M_r$ 50,000-55,000 present in these membranes on Western blots, as illustrated by the result obtained for antibodies to peptide 477-492 in Fig.6.2(D). No labelling was seen with non-immune rabbit IgG (results not shown).

6.2.2.6 Lactating rat mammary gland

Anti-peptide antibodies were used in conjunction with cytochalasin B binding assays to demonstrate the presence of glucose transporters in preparations of plasma membrane- and Golgi vesicle membrane-enriched fractions from lactating rat mammary gland epithelial cells. The concentrations of glucose transporters in the rat mammary membrane fractions and in preparations of human erythrocyte ghost membranes were determined by Dr R. Madon (Hannah Research Institute, Ayr, Scotland), using a centrifugal cytochalasin B binding assay. Scatchard analysis of D-glucose-inhibitable binding revealed that cytochalasin B bound to a maximum of 19, 55 and 657pmol of sites per mg of membrane protein, with $K_d$'s of 2.6, 5.4 and $3.3 \times 10^{-7}$M, in rat mammary plasma membranes, Golgi vesicle membranes and human erythrocyte ghost membranes respectively. The membrane fractions were then subjected to Western blot analysis. Samples of each rat mammary membrane preparation (50μg protein), and purified human erythrocyte glucose transporter (1μg protein), were electrophoretically transferred to nitrocellulose paper and then probed with antibodies against peptides corresponding to residues 231-246, 240-255, 450-467, 460-477 and 477-492 of the human

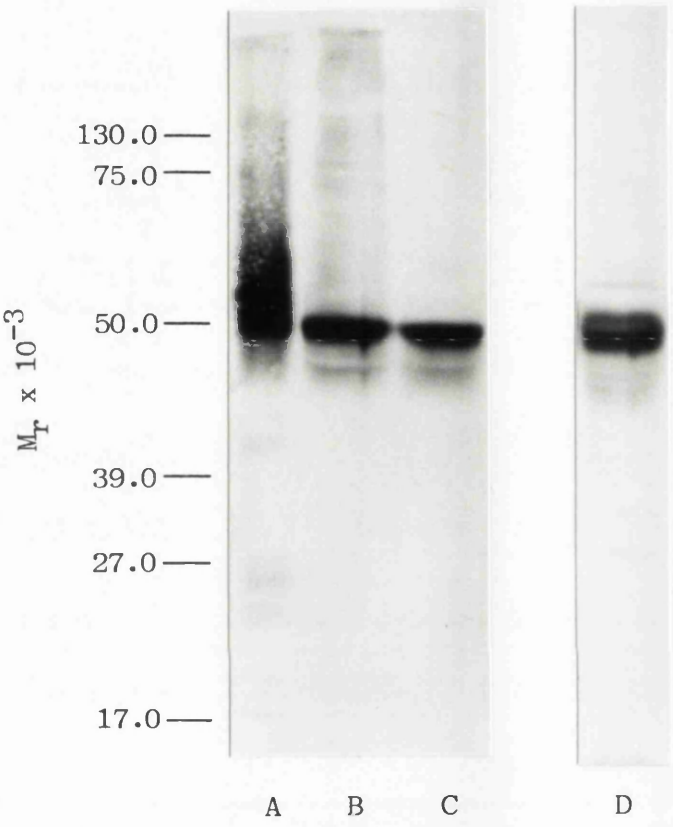



Fig.6.2 Detection of immunologically-reactive proteins present in Chinese hamster ovary cells and membrane fractions from lactating rat mammary gland

Samples of purified human erythrocyte glucose transporter (A), (1μg protein), plasma membrane-enriched fraction from lactating rat mammary gland (B), Golgi vesicle membrane-enriched fraction from lactating rat mammary gland (C) and Chinese hamster ovary cell membranes (D), (each of 50μg protein), were electrophoresed on a SDS/10% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and then immunolabelled with antibodies to C-terminal residues 477-492 of the human erythrocyte glucose transporter. The positions of $M_r$ markers are indicated.

erythrocyte glucose transporter and antibodies to a peptide corresponding to residues 231-246 of the rat brain glucose transporter (Birnbaum et al., 1986). Each of the anti-peptide antibodies labelled a sharp band of apparent $M_r$ 50,000 on Western blots of rat plasma and Golgi vesicle membranes, as illustrated by the result obtained for antibodies against peptide 477-492 shown in Fig.6.2(B and C), compared to a broad band of average apparent $M_r$ 55,000 for the human erythrocyte membranes (Fig.6.2(A). No labelling was seen with non-immune rabbit IgG (results not shown). Confirmation that the bands recognized by the antibodies were glucose transporters was provided by photoaffinity-labelling and immunoprecipitation experiments which are described in Section 6.3.

6.2.2.7 Rat thyroid gland

Recently, Filetti et al. (1987) demonstrated that glucose transport in FRTL-5 cells, a rat thyroid cell line, was thyroid stimulating hormone (TSH)-dependant. TSH is thought to stimulate glucose uptake by recruitment of functional glucose transporters to the thyroid plasma membrane from an intracellular pool, analogous to the action of insulin in adipose and skeletal muscle tissue. These workers showed that the transport system was sensitive to phloretin and cytochalasin B, was stereospecific for D-glucose and had a $K_m$ of 5.3mM for glucose, very similar to that of the human erythrocyte protein. In addition, whilst studying the expression of the glucose transporter gene in FRTL-5 cells, these workers observed a high level of transporter mRNA in hybridization studies using human HepG2 glucose transporter cDNA (S. Filetti, personal communication). These findings strongly suggested the presence of a glucose transport protein(s) homologous to the human HepG2/erythrocyte transporter. In order to verify any structural homology between the proteins, anti-peptide

antibodies were used to probe for immunologically-reactive species in a preparation of FRTL-5 thyroid cell plasma membranes.

Samples (50-100μg protein), of rat thyroid cell plasma membranes, prepared as described in Section 6.2.1, were electrophoretically transferred to nitrocellulose paper and then probed with the whole panel of available anti-peptide antibodies (Section 3.2), or antibodies to the purified, human erythrocyte glucose transporter. None of the anti-peptide antibodies used were found to label any component of the thyroid cell plasma membrane preparation when up to 100μg of membrane protein was present on the nitrocellulose (results not shown). However, antibodies against the intact erythrocyte glucose transporter, when used at 20μg/ml, did give weak labelling of a species of apparent $M_r$ 45,000-50,000 (result not shown).

6.2.2.8 Rabbit small intestine

The transport of D-glucose from the intestinal lumen to the bloodstream across an intestinal epithelial cell is a two stage process. Uptake and concentration of glucose from the lumen into the epithelial cell occurs via a phlorizin-sensitive $Na^+$/glucose co-transporter present at the brush border apical surface membrane (Hediger et al., 1987), which exhibits no sequence homology to the facilitated human erythrocyte glucose transporter. In contrast, glucose then leaves the cell to enter the bloodstream via a phloretin-sensitive glucose transporter of the facilitated diffusion-type, located at the basolateral membrane, which demonstrates kinetic dissimilarities to the human erythrocyte protein (Maenz and Cheeseman, 1987). I was interested to establish whether or not a protein homologous to the human HepG2/erythrocyte glucose transporter constituted at least one population of the facilitated transport proteins present at the intestinal basolateral membrane, and to

confirm the absence of such glucose transporters in the small intestinal brush border membrane. Consequently, anti-peptide antibodies were used to probe for immunologically-reactive species in preparations of rabbit small intestinal brush border and basolateral membrane vesicles.

Samples (100μg protein), of rabbit small intestinal basolateral and brush border membrane vesicles, prepared as described in Section 6.2.1, were electrophoretically transferred to nitrocellulose paper and the probed with antibodies to peptides 231-246, 240-255, 450-467, 460-477 and 477-492. No labelling of components in either intestinal membrane preparation was seen with any of the antibodies used, even at antibody concentrations of up to 30μg/ml (results not shown).

### 6.2.3 Identification of proteins homologous to the human HepG2/erythrocyte glucose transporter in lower vertebrate and invertebrate organisms

In Section 6.2.2, anti-peptide antibodies were used with some success to examine the distribution of proteins immunologically related to the human HepG2/erythrocyte glucose transporter in mammalian tissues. It was of interest therefore to extend this study to tissues of non-mammalian origin. Recently, the glucose transporter of chick embryo fibroblasts was found to be >65% homologous to the human HepG2 protein (White and Weber, 1989). Consequently, an attempt was made to identify homologous proteins in a much more distantly-related vertebrate, the hagfish. The study was then extended further to an invertebrate organism, *Leishmania donovani*.

#### 6.2.3.1 Hagfish erythrocytes

The hagfish is generally regarded as the most primitive living vertebrate. Membranes prepared from hagfish erythrocytes have been

found to be deficient in band 3 (anion transport) proteins, (J.D. Young, unpublished results), and may also lack cytoskeletal proteins. The uptake of D-glucose into intact hagfish erythrocytes or sealed erythrocyte membrane vesicles has been shown to be cytochalasin B-sensitive, saturable and stereospecific (J.D. Young and C.M. Tse, unpublished results). Additionally, the membranes contain a protein which can be photolabelled with [$^3$H]cytochalasin B in a D-glucose-inhibitable manner (J.D. Young, unpublished results). The photolabelled protein migrates on SDS/polyacrylamide gels as a sharper band which is also of higher apparent $M_r$ (55,000-60,000), than the human erythrocyte glucose transporter. Little else is known of this glucose transport protein. Therefore, anti-peptide antibodies were used to probe for immunologically-reactive species in a preparation of hagfish erythrocyte membranes, and to identify any sequence homology between this protein and the human erythrocyte glucose transporter.

Samples (50μg protein), of hagfish erythrocyte membranes, prepared as described in Section 6.2.1, were electrophoretically transferred to nitrocellulose paper and then probed with antibodies to peptides 1-15, 84-98, 144-158, 217-232, 231-246, 240-255, 256-272, 450-467, 460-477 and 477-492. Of the antibodies used two, corresponding to residues 240-255 and 450-467 of the human HepG2/erythrocyte transporter sequence, labelled a component of hagfish membranes which migrated with an apparent $M_r$ of 50,000-55,000 on Western blots (Fig.6.3). This band was also labelled by antibodies to the intact human erythrocyte glucose transporter (results not shown). No labelling was seen with non-immune rabbit IgG (results not shown).

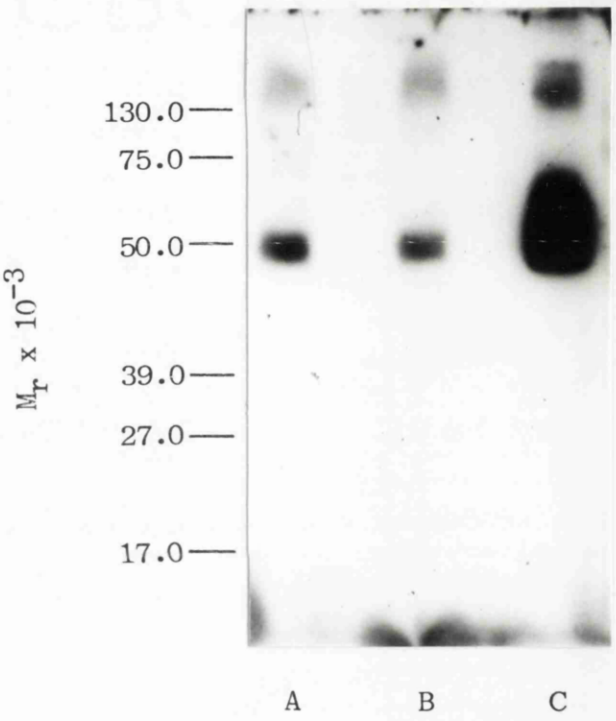



Fig.6.3 Detection of immunologically-reactive proteins present in hagfish erythrocyte membranes

Samples of hagfish erythrocyte membranes (A and B), (each of 50μg protein), or purified human erythrocyte glucose transporter (C), (1μg protein), were electrophoresed on a SDS/10% polyacrylamide gel, electrophoretically transferred to nitrocellulose paper and then immunolabelled with antibodies against residues 240-255 (A), or 450-467 (B and C), of the human erythrocyte glucose transporter. The positions of $M_r$ markers are indicated.

6.2.3.2 Attempted characterization of the glucose transport protein of Leishmania donovani

Since proteins homologous to the human HepG2/erythrocyte glucose transporter were detected in hagfish, the most primitive vertebrate organism, then a logical extension of this study was to move to an invertebrate species. For this purpose Leishmania donovani, an obligate intracellular protozoan parasite of humans was chosen.

Recently, Zilberstein et al. (1986) identified a glucose transport protein present in preparations of plasma membrane-derived vesicles from L. donovani. This and earlier work (Zilberstein and Dwyer, 1984, 1985), demonstrated that glucose uptake into this organism was carrier-mediated and required the co-transport of protons. The transporter was found to be stereospecific and to have a very high affinity for glucose ($K_m$ = 0.024mM). Cytochalasin B, but not cytochalasin E, inhibited the transport of 2-deoxyglucose into the extracellular promastigote form of L. donovani. In addition, the transporter present in isolated surface membranes and plasma membrane vesicles obtained from the promastigote form of this organism could be photochemically cross-linked (via hydroxysuccinimidyl-4-azido benzoate) to [$^3$H]cytochalasin B. This labelling was D-glucose-sensitive. Subsequent SDS/polyacrylamide gel electrophoresis showed that the covalently labelled transporter migrated with an $M_r$ of 20,000-30,000, considerably lower than the $M_r$ values reported for mammalian glucose transport proteins. The transporter was shown to be a mannose-containing glycoprotein. Since the transporter of L. donovani shares some features with those of mammalian cells (substrate specificity and inhibition by cytochalasin B), then there may be some evolutionary conservation of the substrate-binding site(s) in these proteins. However, the higher affinity of the L. donovani transporter for D-glucose and differences in molecular properties, may reflect an

adaptation of this transporter to cope with changes in its host's cellular environment. Consequently, anti-peptide antibodies were used to probe for immunologically-reactive species in a preparation of isolated surface membranes from the promastigote form of L. donovani.

Samples (100µg protein), of isolated surface membranes from L. donovani, prepared as described in Section 6.2.1, were electrophoretically transferred to nitrocellulose paper and then probed with all of the anti-peptide antibodies available (Section 3.2). None of the antibodies labelled any component of the L. donovani membranes when antibody concentrations of up to 30µg/ml were used (results not shown).

## 6.3 ANTI-PEPTIDE ANTIBODIES AS PROBES OF THE SUBCELLULAR DISTRIBUTION OF GLUCOSE TRANSPORT PROTEINS IN THE LACTATING RAT MAMMARY GLAND EPITHELIAL CELL

The transport of glucose in the epithelial cells of the lactating rat mammary gland is of great interest, as D-glucose is the major substrate for lactose production. Lactose, a disaccharide, is the primary carbohydrate of mammalian milk and is produced exclusively in animals by the Golgi apparatus of the mammary epithelial cells, from which it is secreted into the milk. It is produced from D-glucose and UDP-galactose (the latter itself is produced from glucose) in the lumen of the Golgi apparatus by the membrane-bound complex, lactose synthetase, consisting of galactosyltransferase and α-lactalbumin. The rate of glucose transport into the epithelial cell is known to be rate limiting for lactose production (Threadgold and Kuhn, 1984; Faulkner and Peaker, 1987).

As a consequence, within the mammary epithelial cell there are two membrane sites across which the transport of glucose is of

interest, the cell plasma membrane and the membranes of the Golgi apparatus. Threadgold et al. (1982) showed that monosaccharide transport into lactating rat mammary gland acini occured by a specific, carrier-mediated process. In mice a facilitative D-glucose transport system has also been described (Prosser and Topper, 1986; Prosser, 1988). In these animals the rate of sugar uptake into epithelial cells increases up to 40-fold as they progress from the virgin to the mid-lactating state (Prosser and Topper, 1986). However, the mechanism by which glucose crosses the membranes of the Golgi apparatus to date remains the subject of much controversy. Earlier studies have provided evidence for the presence of a non-specific size exclusion pore as opposed to a specific facilitated monosaccharide transporter in the Golgi membranes (White et al., 1980). In the present study, an attempt was made to clarify this situation by identifying and determining the subcellular distribution of glucose transporters in the lactating rat mammary epithelial cell.

In Section 6.2.2.6, anti-peptide antibodies were used to detect proteins homologous to the human HepG2/erythrocyte glucose transporter on Western blots of plasma membrane- and Golgi vesicle membrane-enriched fractions derived from the epithelial cells of the lactating rat mammary gland. In view of the fact that other workers had described the uptake of D-glucose into the Golgi apparatus as non-stereospecific (White et al., 1980), it was essential to establish that the antibodies were in fact recognizing glucose transport proteins in the Golgi vesicle membrane fraction. To achieve this, an attempt was made to covalently label glucose transporters in the rat mammary Golgi vesicle membranes with [$^{3}$H]cytochalasin B, and then to immunoprecipitate the photolabelled proteins from detergent-solubilized membranes using immobilized anti-peptide antibodies. Rat mammary plasma membranes were included for comparison and human

erythrocyte ghost membranes used in control experiments.

### 6.3.1 Photoaffinity labelling of membranes

Photoaffinity-labelling of the rat mammary and human erythrocyte membranes with [$^3$H]-cytochalasin B was performed using the method of Kasanicki *et al*. (1987). Samples of the membranes at 1mg protein/ml in 50mM sodium phosphate, pH 7.4, containing 100mM NaCl, 1mM EDTA and 500mM D- or L-glucose, were equilibrated with 0.51μM [4-$^3$H]-cytochalasin B on ice for 30 minutes. Cytochalasin E (10μM) was also included in each sample to reduce cytochalasin B binding to sites not associated with glucose transport. The samples were then transferred to 1ml quartz cuvettes (1cm light path). After flushing with nitrogen and stoppering, the cuvettes were irradiated on ice for 10 minutes with a 100W Mineralight model R-52 (Ultraviolet Products Inc., San Gabriel, C.A., U.S.A.) at a distance of 10cm. All of the following procedures were carried out at 4$^o$C. The irradiated samples were transferred to eppendorf tubes and non-covalently-bound [$^3$H]-cytochalasin B was removed from the membranes by two rounds of washing with 50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH 7.4, containing 20μM unlabelled cytochalasin B, followed by centrifugation at 11,500 rev/min for 10 minutes, discarding the supernatant each time.

### 6.3.2 Immunoprecipitation of [$^3$H]cytochalasin B-photolabelled proteins

The washed, photolabelled membranes prepared in Section 6.3.1 were resuspended in 50mM sodium phosphate, 100mM NaCl, 1mM EDTA. pH 7.4, and the protease inhibitors PMSF, E-64 and pepstatin A were added to final concentrations of 1mM, 0.1mM and 1.46μM respectively. The membrane samples were then solubilized in a mixture of 0.25% (w/v) SDS and 2.5% (v/v) Triton X-100 (high purity 'SurfactAmps' grade, Pierce), such that the final membrane protein concentration was 0.25mg/ml.

Where necessary, the solutions were clarified by centrifugation at 45,000 rev/min for 1 hour at 4°C.

Immunoadsorbants were prepared in the following way: 50mg of protein A-Sepharose was swollen and washed 5 times in PBS, pH 7.2, at 4°C by centrifugation to remove lactose and dextran. To the washed pellets was added 0.8mg of either affinity-purified antibodies to C-terminal peptide 477-492 or non-immune rabbit IgG, and the samples incubated for 1 hour at 4°C with gentle mixing. The samples were centrifuged and the supernatant discarded. The pellets were washed twice in PBS, pH 7.2 and finally resuspended in 0.2ml 50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH 7.4, to give about 2mg IgG/ml.

Each of the photolabelled, solubilized membrane samples was divided in two equal halves (1ml; 0.25mg protein) in eppendorf tubes. 25µl of the control or 'immune' immunoadsorbant suspension (50µg IgG), were added to each sample as appropriate and the mixtures incubated overnight at 4°C on a rotary platform. The protein A-Sepharose beads in each sample were then washed three times by centrifugation (3000 rev/min for 10 minutes at 4°C) with 50mM sodium phosphate, pH 7.4, containing 1% (v/v) Triton X-100 and 0.1% (w/v) SDS, and once with the same buffer containing 0.1% Triton X-100 and 0.01% SDS. Any absorbed polypeptides were then eluted in 0.1ml of a gel sample buffer containing 50mM Tris-HCl, pH 6.8, 1mM EDTA, 2% SDS, 10mM DTT, 6M urea and 10% glycerol. 75µl of each sample was then electrophoresed on a 3mm-thick, SDS/12% polyacrylamide gel. After the run, the gel was fixed, Coomassie blue stained and scanned spectrophotometrically at 530nm to locate the position of the molecular weight markers also run on the gel. Each sample-containing track was cut out to a width of 1cm using a long blade. Using a gel slicer (a device consisting of 70 razor blades separated by 2mm metal plate spacers), each track was cut into 2mm slices. These were placed in scintillation vials and allowed

to dry overnight in a fume cupboard together with four slices taken from a non-radioactive part of the gel to determine backgrounds. The gel slices were then solubilized by incubation with 0.4ml 30% (v/v) hydrogen peroxide for 8 hours at 50°C. After solubilization and cooling, 4ml of Cocktail T scintillant was added to each vial. The vials were shaken and counted on a Packard 3255 liquid scintillation spectrometer.

A major peak of D-glucose-inhibitable [$^3$H]cytochalasin B labelling, of apparent $M_r$ 50,000, was detected for both the rat mammary plasma membranes (Fig.6.4(A) and Golgi vesicle membranes (Fig.6.4(B), immunoprecipitated with anti-glucose transporter C-terminal peptide antibodies. These $M_r$ values agree with those for the species labelled on Western blots of each membrane fraction by anti-peptide antibodies (Section 6.2.2.6). Minor D-glucose-inhibitable peaks are probably attributable to fragments of the transport proteins arising from cleavage by endogenous proteases during membrane preparation. No peak of labelling was seen for the control immunoprecipitate (result not shown). A similar pattern was observed for human erythrocyte membranes, except that the peak of labelling was broader, and of apparent average $M_r$ 55,000 (Fig.6.4(C).

Fig.6.4 Electrophoretic profiles of photoaffinity-labelled glucose transporters immunoprecipitated from human erythrocyte and rat mammary epithelial cell membranes

Rat mammary Golgi vesicle membranes (A), rat mammary plasma membranes (B) and human erythrocyte membranes (C), labelled with [$^3$H]cytochalasin B in the presence of 500mM D-glucose (●) or 500mM L-glucose (○) were solubilized, incubated with Sepharose-bound antibodies to C-terminal residues 477-492 of the human erythrocyte glucose transporter, and the adsorbed proteins electrophoresed on a SDS/12% polyacrylamide gel. The radioactivity of 2mm slices was determined by liquid scintillation counting. Arrows indicate the positions of the $M_r$ markers.

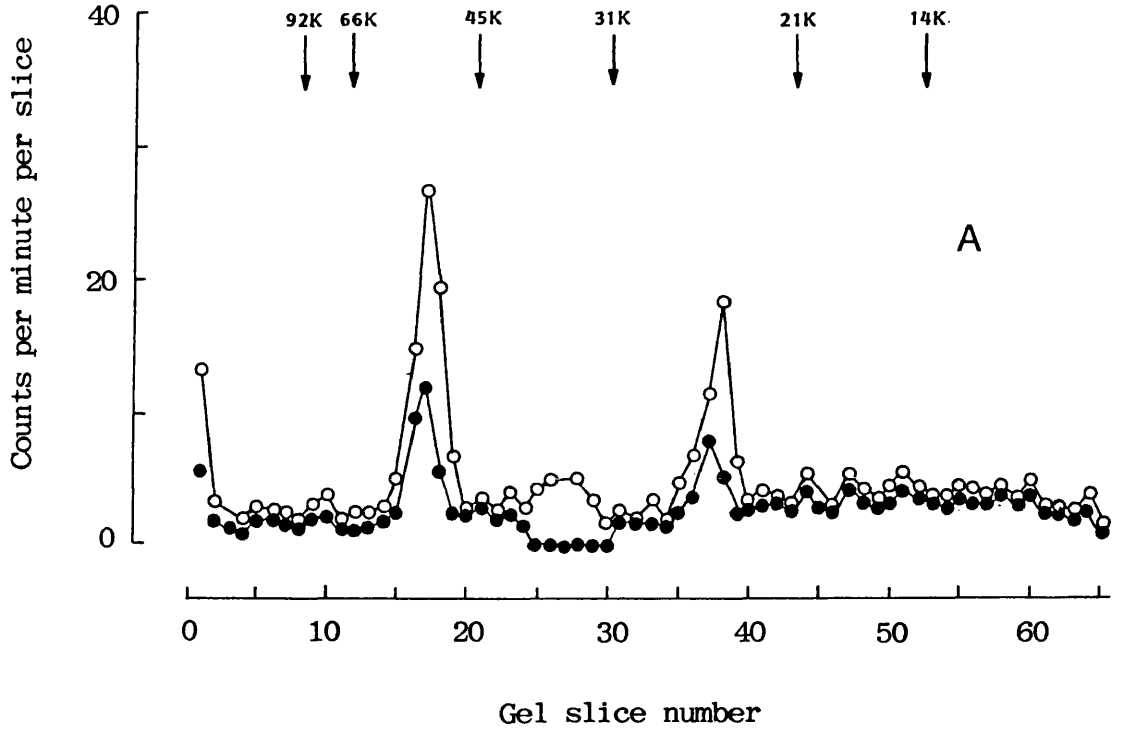
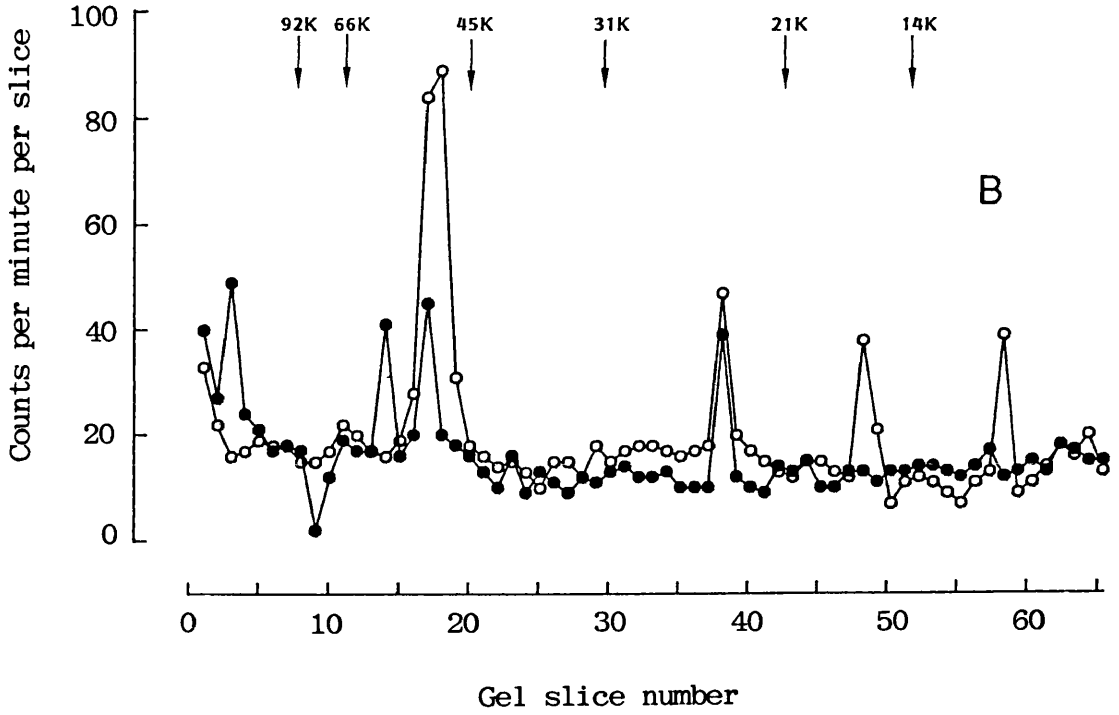
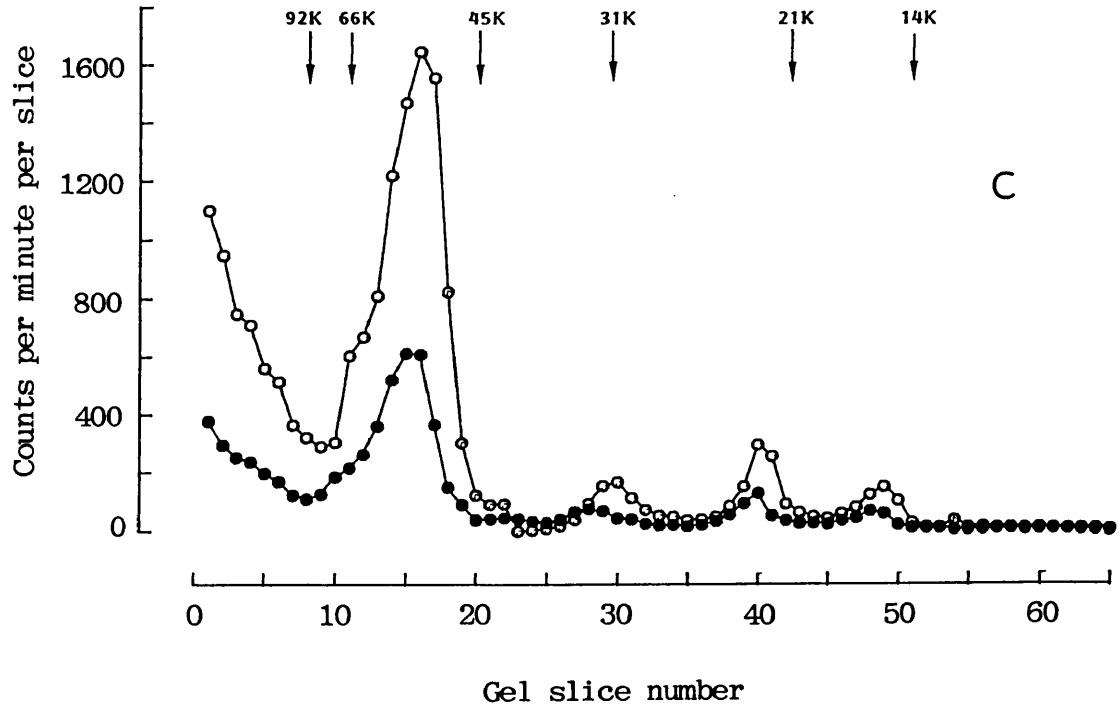


Fig.6.4 continued



## 6.4 DISCUSSION

Anti-peptide antibodies were used to detect proteins demonstrating sequence similarity to the human erythrocyte glucose transporter in various tissues of mammalian and non-mammalian origin. Such proteins were detected in membrane fractions from rodent brain, adipose tissue, liver, testis, ovary and lactating mammary gland, but not in membrane fractions from small intestine or thyroid gland. Some of the antibodies were also seen to cross-react with a protein present in the erythrocyte membrane of a primitive vertebrate, the hagfish. Ideally, confirmation that the labelled proteins in each membrane sample were indeed glucose transporters would be necessary using methods similar to those described for the rat mammary gland in Section 6.3. However, Western blotting and immunolabelling sufficed for the initial identification of glucose transport proteins in most of the membrane samples.

During the course of this study, the facilitative glucose transport proteins from a number of human and rodent tissues (and one from yeast), were cloned and sequenced. This has greatly clarified the situation as regards the tissue distribution of this family of proteins. The sequenced transporters and their distribution are listed in Table 6.2. The proteins show significant sequence homology with one another, and appear to fall into at least three major groups; (a) '(HepG2)/erythrocyte/brain-type', (b) 'liver-type', and (c) 'insulin-sensitive (adipocyte/muscle)-type'. A classification for the fetal muscle transporter is less clear, but it does appear to be a major species found in adult human brain tissue (Kayano *et al.*, 1988, Table 6.2). In contrast to the mammalian transporters of 492-509 amino acid residues and average $M_r$ 54,000-55,000, the glucose transporter of the yeast *Saccharomyces cerevisiae* is a protein of some 884 residues

Table 6.2 Features of the cloned and sequenced glucose transport proteins

| 'Type' of transporter | % identity with human HepG2/ erythrocyte (●) or rat brain (○) transporters | Chromosomal location of encoding gene | Tissue distribution* |
|---|---|---|---|
| Human HepG2/erythrocyte rodent brain-type | | | |
| Human HepG2/erythrocyte (Mueckler et al., 1985) | 97.6○ | 1 | brain+, heart, stomach, colon, gall bladder, placenta+, adipose tissue, skeletal muscle, erythrocyte+ |
| Rat brain (Birnbaum et al., 1986) | 97.6● | | |
| Rabbit brain (Asano et al., 1988) | 97.0●, 97.4○ | | |
| Mouse 3T3L1 adipocyte (Keastner et al., 1989) | 97.0●, 97.0○ | | |
| Liver-type | | | |
| Human liver (Fukumoto et al., 1988) | 55.5● | 3 | liver+, kidney+, β-pancreatic islets+, small intestine |
| Rat liver (Thorens et al., 1988) | 55.0○ | | |
| Human fetal muscle (Kayano et al., 1988) | 64.0● | 12 | brain+, kidney, placenta, gall bladder and traces in other adult tissues |

Table 6.2 continued

| | | | |
|---|---|---|---|
| Insulin-sensitive-type | | | |
| Rat adipocyte (James et al., 1989) | 65.0● | | |
| Rat skeletal muscle (Birnbaum, 1989) | 63.5○ | | |
| Human adipocyte (Fukumoto et al., 1989; Bell et al., 1989) | 65.3● | 17 | adipose+ tissue (brown and white), skeletal muscle+ (red and white), heart |
| Rat soleus muscle (Charron et al., 1989) | 65.0○ | | |
| Mouse 3T3L1 adipocyte (Kaestner et al., 1989) | 65.0○ | | |
| Yeast (S. cerevisiae) (Celenza et al., 1988) | 28.0● | | |
| Rabbit intestine $Na^+$/glucose transporter (Hediger et al., 1987) | none | | |

* From detection of mRNA transcripts or by immunoblotting with relevant antibody
+ Major transporter species in this tissue

($M_r$ 97,000), but still demonstrates a significant (28%) sequence homology to the human erythrocyte protein within part of its sequence (Celenza et al., 1988). A comparison of the HepG2/erythrocyte/brain, liver, adipocyte/muscle and fetal muscle transporter sequences shows little conservation within the last 25 or so C-terminal amino acid residues. Consequently, specific detection of glucose transporters showing homology to the human HepG2/erythrocyte protein in other tissues, using antibodies to the extreme C-terminal sequence of the latter, was a fortunate choice. Additionally, this study was of particular value in the light of findings that many tissues contain more than one type of glucose transport protein (Table 6.2).

Rat brain

Antibodies against the C-terminal sequence 450-492 and residues 231-255 from the central cytoplasmic region of the human erythrocyte glucose transporter, were found to label protein(s) of apparent $M_r$ 45,000-50,000 on Western blots of a membrane fraction isolated from whole rat brain. In addition, the labelled protein appeared as a sharp band on the blot when compared to the broad zone of labelling obtained for the human erythrocyte transporter. This suggests that the glycosylation state of the two proteins is different. These results are in reasonable agreement with the findings of Birnbaum et al. (1986) and Wang (1987), who detected a protein of $M_r$ 40,000-46,000 on Western blots of adult rat brain microsomes probed with antibodies to the intact human erythrocyte glucose transporter. Further evidence that rat brain tissue contains transporters of the human 'HepG2/erythrocyte-type' was obtained by Flier et al. (1987) who detected an mRNA transcript encoding the HepG2 transporter on Northern blots of total rat brain RNA probed with human HepG2 glucose transporter cDNA. These workers also found that the concentration of

HepG2 glucose transporter RNA was severalfold higher in brain microvessels than in extracts of whole brain. Using antibodies to C-terminal peptide 477-492, it has been possible for us to show that glucose transporters similar to the human HepG2/erythrocyte protein are abundant in the endothelial cells lining both human and bovine microvessels of the blood-brain barrier (Kasanicki et al., 1987, 1989). Similarly, the same antibody has been used in a detailed immunocytochemical study of the distribution of proteins homologous to the human HepG2/erythrocyte transporter in rat brain using immunogold methodology (Bagley et al., 1989). In this study the greatest density of glucose transporter-immunoreactivity was found to be associated with blood capillaries, choroid plexus and discrete regions of the neuropil, hippocampus and cerebellum.

The glucose transporter of rat brain cloned and sequenced by Birnbaum et al. (1986) was identified from a rat cDNA library using a polyclonal antibody to the human erythrocyte protein. Consequently, it was not perhaps surprising that the rat brain protein cloned was found to be similar to that of the erythrocyte, although the high sequence identity (97.6%) was very interesting. A similar finding was made for the glucose transporter of rabbit brain (Asano et al., 1988, Table 6.2). However, Oka et al. (1988) found that anti-peptide antibodies to the cytoplasmic C-terminal and central hydrophilic regions of the human erythrocyte glucose transporter were able to immunoprecipitate only 30% of total [$^3$H]cytochalasin B-photolabelled glucose transporters from a detergent-solubilized preparation of rat brain membranes. This suggested that the transporter cloned by Birnbaum et al. (1986), constituted only a proportion of total brain transporters; the remaining cytochalasin B binding sites being attributable to a protein immunologically-distinct from the human erythrocyte transporter.

Rat adipocyte

Antibodies against the cytoplasmic C-terminal and central hydrophilic regions of the human erythrocyte glucose transporter labelled a sharp band of apparent $M_r$ 45,000 on Western blots of a plasma membrane fraction prepared from rat adipocytes. This result is consistent with the findings of Wheeler et al. (1982), Lienhard et al. (1982) and Wang (1987), using antibodies to the intact human erythrocyte glucose transporter, and Haspel et al. (1988a) using a similar C-terminal peptide antibody, who demonstrated the presence of a homologous protein in the plasma membranes of resting and insulin-stimulated rat adipocytes. In addition, Flier et al. (1987) found significant amounts of mRNA encoding for a protein homologous to the human HepG2/erythrocyte transporter in both rat adipose and skeletal muscle tissue.

However, there is now considerable evidence that the 'HepG2/erythrocyte/brain-type' protein is not the major transporter species in these insulin-sensitive tissues. Wang (1987) demonstrated that antibodies to the human erythrocyte transporter labelled rat brain transporters 5-10 times more strongly than rat adipocyte or muscle transporters. Joost et al. (1988) showed that a ten-fold stimulation of rat adipocyte glucose transport activity in the presence of insulin was associated with only a two-fold increase in transporter immunoreactivity as assessed with antibodies to the human HepG2/erythrocyte transporter. Additionally, Oka et al. (1988) were able to immunoprecipitate only 3% of total [$^3$H]cytochalasin B-photolabelled glucose transporters from detergent-solubilized rat adipocyte membranes using anti-peptide antibodies to the human erythrocyte glucose transporter. James et al. (1988) have produced a monoclonal antibody specific for an insulin-sensitive glucose transporter present in rat adipocytes, which is not cross-reactive

with the human HepG2/erythrocyte or rodent brain transporters. Subsequently, a distinct insulin-sensitive transporter present in the adipocyte, which shares a 65% identity with the human erythrocyte and rat brain proteins was cloned and sequenced (James et al., 1989, Table 6.2). In addition, a similar protein has been characterized in several other insulin-sensitive tissues (Table 6.2). The 'HepG2/erythrocyte/brain-type' protein may therefore be present in insulin-sensitive tissues due to a basal expression of the encoding gene.

Rat liver

Antibodies to C-terminal peptide 477-492, and those to most of the predicted extramembranous regions of the human erythrocyte glucose transporter, failed to recognize the glucose transport protein present in a membrane preparation from whole rat liver. This result is consistent with the findings of Birnbaum et al. (1986) and Flier et al. (1987), who were unable to detect mRNA encoding the HepG2 transporter in rat liver, and of Wang (1987) and Haspel et al. (1988a), who were unable to label a transport protein in liver using antibodies to the purified human erythrocyte transporter and C-terminal peptide, respectively. In contrast, in the present study, an antibody directed against C-terminal residues 450-467 of the human erythrocyte transporter gave weak labelling of a species present in liver migrating with an apparent $M_r$ 50,000-55,000 on Western blots. This finding suggested some sequence similarity between this part of the C-termini of the two proteins. With the subsequent cloning and sequencing of the rat and human liver transporters (Thorens et al., 1988; Fukumoto et al., 1988, Table 6.2), it became clear that the liver and HepG2/erythrocyte transporters do indeed share a common stretch of C-terminal sequence (residues 480-487 and 450-457 of the

liver and HepG2/erythrocyte transporters respectively, are identical). The 'liver-type' transporter has been found to predominate in other tissues which demonstrate a high capacity for glucose transport (e.g. kidney and intestine), or tissues sensitive to changes in glucose concentrations (e.g. β-pancreatic islets, Thorens et al., 1988).

Rat testis Leydig cell

Antibodies to the C-terminal peptide 477-492 labelled a sharp band of apparent $M_r$ 50,000 in a preparation of plasma membranes isolated from rat testis Leydig cells. The presence of a facilitated glucose transporter is consistent with the kinetic characteristics of glucose uptake in these cells (Amrolia et al., 1988), and more recently with the finding that mRNA encoding the 'HepG2/erythrocyte/brain-type' transporters, but not the liver or insulin-sensitive transporters, was detected using the relevant cDNA probe on Northern blots of mRNA prepared from whole rat testis (Charron et al., 1989). Additionally, as it is now known that the insulin-sensitive, liver and HepG2/erythrocyte/brain transporters show no sequence homology within residues 477-492 of their C-termini, it is reasonable to assume that at least one population of glucose transporters present in Leydig cell membranes are of the 'HepG2/erythrocyte/brain-type', and that this protein may be sensitive to luteinizing hormone via a Leydig cell-specific mechanism.

Chinese hamster ovary cells

Antibodies to C-terminal peptide 477-492 labelled a species of apparent average $M_r$ 50,000 present in membrane preparations from CHO cells. Similarly, Haspel et al. (1988a,b) using an anti-peptide antibody to C-terminal residues 480-492 of the human erythrocyte glucose transporter labelled a transporter present in CHO cell

membranes. However, when compared to the result reported in the present study, the labelled protein ran as a fairly broad zone of apparent average $M_r$ 55,000 on SDS/polyacrylamide gels, similar to that seen for the human erythrocyte transporter.

Rat thyroid gland

Proteins homologous to the human HepG2/erythrocyte glucose transporter were not detectable in a plasma membrane fraction from rat thyroid gland using anti-peptide antibodies. This result was surprising in view of the high levels of transporter mRNA found in these cells. Whatever the explanation for this result, it suggests that caution must be used in drawing conclusions about the abundance of an expressed protein from studies of mRNA abundance.

Rabbit small intestine

Proteins homologous to the human HepG2/erythrocyte glucose transporter were not detectable in preparations of brush border or basolateral membranes from rabbit small intestine using anti-peptide antibodies. These results are consistent with the findings of Fukumoto et al. (1988) and Thorens et al. (1988), who, using cDNA probes for the HepG2 and liver transporters, were only able to detect mRNA encoding the latter protein in human and rat intestine. It appears then that mammalian intestine contains at least two distinct types of glucose transporter, namely the $Na^+$/glucose co-transporter present at the brush border membrane, and a facilitated transporter of the 'liver-type' located at the basolateral membrane. If the facilitated transporter of the basolateral membrane is indeed of the 'liver-type', then it might have been expected that antibodies to C-terminal peptide 450-467, which labelled the rat liver transporter (Section 6.2.2.3), might also label the protein in these membranes. However, the lack of

labelling could be explained by several factors. Firstly, the 'liver-type' transporter is 2 to 3-fold less abundant in intestine than liver (Fukumoto et al., 1988) and so the problem may be one of sensitivity of detection. Secondly, the rabbit 'liver-type' transporter may not contain the stretch of conserved C-terminal sequence common to the rat liver and human HepG2/erythrocyte transporters (see above). Ideally therefore, the study would best have been performed on membrane fractions from rat intestine. However these were not available at the time of the study.

Hagfish erythrocyte membranes

Evidence for a glucose transporter in hagfish erythrocyte membranes has come from several studies. Photolabelling of the membranes with [$^3$H]cytochalasin B yielded a labelled component of $M_r$ 55,000-60,000, and in the present study antibodies to the purified human erythrocyte glucose transporter labelled a component of essentially identical $M_r$ (50,000-55,000). The latter result suggested similarities between the hagfish and human HepG2/erythrocyte transporters. Similarities in their amino acid sequences was confirmed by the cross-reactivity of anti-peptide antibodies. Antibodies to residues 240-255 and 450-467 of the human erythrocyte transporter also labelled a component of $M_r$ 50,000-55,000 of hagfish erythrocyte membranes. The C-terminal peptide sequence 450-467 of the human erythrocyte protein, carries a short stretch of amino acid residues (450-457), which are well conserved in each type of mammalian glucose transporter, the transporter of the yeast Saccharomyces cerevisiae (Celenza et al., 1988) and partially within a number of proton-linked sugar transporters of Escherichia coli (Maiden et al., 1987). Consequently, this piece of sequence must be of some common structural or functional importance to these transport proteins. In contrast, the

sequence 240-255 from the central cytoplasmic region of the human erythrocyte protein, is not well conserved in any other transporter for which the primary sequence is known. The full extent homology between the two transporters awaits the cloning and sequencing of the hagfish protein.

Leishmania donovani glucose transporter.

Proteins homologous to the human HepG2/erythrocyte glucose transporter were not detectable in surface membranes derived from the promastigote form of L. donovani. This result suggested little homology between the extramembranous regions of the two transporters. However this study could not rule out sequence similarities within the membrane-spanning regions of proteins. Indeed, a putative glucose transport protein from the promastigote form of Leishmania enriettii has recently been cloned and sequenced (Cairns et al., 1989). Although the L. enriettii protein is 22% identical to the human erythrocyte glucose transporter, much of the identity exists within regions of the former predicted to be membrane-spanning.

Subcellular distribution of glucose transport proteins in the lactating rat mammary gland

Anti-peptide antibodies were used to study the subcellular distribution of glucose transport proteins in the epithelial cells of the lactating rat mammary gland. The apparent presence of glucose transporters in both plasma membrane- and Golgi vesicle membrane-enriched fractions was demonstrated. These glucose transporters cross-react with anti-peptide antibodies raised against the major cytoplasmic regions of the human erythrocyte transporter and residues 231-246 of the rat brain transporter, suggesting significant sequence similarities between the proteins. The different $K_d$ values for

cytochalasin B binding to the Golgi and plasma membrane fractions could be a reflection of structural and/or functional differences in the glucose transporters at the two sites. Therefore further comparison with the human erythrocyte glucose transporter would be necessary using a wider range of anti-peptide antibodies to the latter, in the hope of detecting any differences in immunogenicity, and hence sequence. In addition, the mammary gland transporters can also be photoaffinity-labelled with [$^3$H]cytochalasin B in a D-glucose-inhibitable manner and then specifically immunoprecipitated with immobilized anti-peptide antibodies to the C-terminus of the human erythrocyte transporter. The presence of a glucose transporter in the Golgi membrane fraction is interesting, as this finding conflicts with the results of earlier studies which indicated the presence of a non-stereospecific 'pore' for monosaccharides in the Golgi membrane (White et al., 1980). The results of the present study have led to some clarification as to how glucose, required for lactose synthesis, reaches the site of synthesis within the Golgi vesicle of the cell.

However the conclusion from this study, that mammary epithelial cell plasma membranes and Golgi membranes contain glucose transporters, depends upon both of these subcellular fractions being pure, and both being derived from the right cell type. The transporters present in the Golgi membrane fraction cannot arise from contamination with plasma membranes, as the Golgi membranes were shown to carry only 10% contamination with the latter. At the stage of lactation at which the glands are removed the predominant cell type is the secretory epithelial cell. It is conceivable however that other cell types, rich in glucose transporters, are contributing to the results. Therefore, an immunocytochemical study would be necessary in order to assess the transporter content of all the other cell types in

the gland. The results of such a study should indicate the likely degree of contamination of the epithelial cell membrane fractions with the membranes of other cell types. Hopefully this would then lead to immunocytochemistry at the electron microscope level in order to demonstrate directly the presence or absence of glucose transporters associated with the Golgi apparatus.

Other essential work for the future would include a demonstration that the glucose transporters in the Golgi membrane fraction are actually present on those vesicles involved in lactose synthesis. This could be achieved by immunoprecipitation of the Golgi vesicles using anti-peptide antibodies. If the immunoprecipitated vesicles contain galactosyltransferase and lactose synthetase activity, this will indicate that the glucose transporters in the Golgi membrane fraction are indeed located on the same vesicles involved in lactose synthesis, and will argue for a functional role of the glucose transporters at this site.

# CHAPTER 7 GENERAL DISCUSSION AND SUMMARY

## 7.1 Membrane topology of the glucose transporter

At the beginning of the present study several models had been proposed to describe the arrangement of the glucose transporter polypeptide in the erythrocyte membrane (Shanahan and D'Artel-Ellis, 1984; Deziel *et al.*, 1985; Mueckler *et al.*, 1985). These models were based largely upon *in situ* proteolytic cleavage of the native protein, or hydropathic analysis of the recently determined amino acid sequence of the human HepG2/erythrocyte glucose transporter. However, little *direct* evidence had been published for any of these models. Therefore in the present study such evidence was sought using anti-peptide antibodies as probes of the topology of the glucose transporter in the membrane. The production and characterization of these antibodies, which were directed against hydrophilic regions of the sequence likely to be extramembranous, were described in Chapter 3.

Using the anti-peptide antibody approach, the studies described in Chapter 4 shed considerable light on the arrangement of the polypeptide in the membrane. The *C*-terminal hydrophilic region of the transporter (residues 450-492) and the central hydrophilic region of the sequence (residues 217-272) were shown to be located at the cytoplasmic surface of the erythrocyte membrane. Further evidence for the cytoplasmic location of these regions of the protein was obtained in the work described in Chapter 5, where the sequence locations were identified for a number of monoclonal antibodies raised against the intact protein. All of these monoclonal antibodies had previously been shown to bind to the cytoplasmic surface of the membrane (Allard and Lienhard, 1985; Boyle *et al.*, 1985). Of the eight monoclonal antibodies investigated, the epitopes of five were shown to be located, at least in part, within residues 231-246 in the central

hydrophilic region of the sequence. This conclusion stemmed largely from the ability of these antibodies to recognize a synthetic peptide corresponding to this region of the sequence. Similarly, another two of the antibodies recognized a peptide corresponding to residues 477-492 of the sequence, which must therefore contain their epitopes, at least in part. The eighth monoclonal antibody was also shown to recognize the C-terminal region of the transporter, although the precise location of its epitope was not definitively established.

The topological findings from studies with anti-peptide antibodies and monoclonal anti-transporter antibodies were consistent with those of Cairns et al. (1987) who identified small peptides released from the cytoplasmic face of the native transporter by tryptic cleavage. They were also consistent with the predictions of the model of Mueckler et al. (1985) that the regions of the sequence comprising residues 207-271 and 451-492 are exposed at the cytoplasmic side of the membrane. Additional support for this model came from the use of anti-peptide antibodies to characterize the large, membrane-bound fragments produced by tryptic digestion of the native transporter. Such digestion was known to produce a glycosylated fragment of apparent $M_r$ 23,000-42,000, and a series of non-glycosylated fragments of $M_r$ 18,000-25,500 (Cairns et al., 1984). The larger non-glycosylated fragments were known to be precursors to that of $M_r$ 18,000, which had been shown to contain the site of photolabelling of the protein by cytochalasin B.

The sequence locations of the tryptic fragments of the transporter were established by examining their reactivity towards different anti-peptide antibodies on Western blots. These experiments, described in Chapter 4, showed that the glycosylated fragment is derived from the N-terminal half of the protein. This observation confirmed $Asn_{45}$ as the site of glycosylation and thus indicated that

this residue is located in an extracellular loop of the protein, as predicted by the model. The pattern of reactivity of the glycosylated fragment with anti-peptide antibodies was consistent with its comprising residues 1-212 of the sequence, and subsequent work by Cairns et al. (1987) has shown that its amino acid composition is also consistent with this sequence location. The fragment is resistant to further proteolysis, despite containing a number of potential cleavage sites in the native transporter.

After short periods of digestion with trypsin, a non-glycosylated fragment of the transporter of apparent $M_r$ 25,500 is produced. From the pattern of its reactivity with anti-peptide antibodies it was shown to contain most of the region comprising residues 224-492 of the transporter, although the precise location of its N-terminus is unclear. During longer periods of digestion this fragment was shown to be further cleaved in both its N-terminal and C-terminal hydrophilic regions eventually to yield a fragment of $M_r$ 18,000 via precursor fragments of 23,500 and 21,000. The pattern of labelling of the $M_r$ 18,000 fragment with anti-peptide antibodies on Western blots indicated that it contained residues 270-456/458 of the transporter, although the precise location of its N-terminus, which may have preceded residue 270 by up to 13 residues, was not established. This fragment was subsequently shown by Cairns et al. (1987) to have an amino acid composition consistent with its comprising residues 270-456/458 of the protein. Thus, the site of labelling of the transporter by cytochalasin B, which lies within the $M_r$ 18,000 fragment, must be located between residues 270 and 458 of the transporter. This location for the cytochalasin B labelling site, together with the location of the site of glycosylation and the cytoplasmic location of the protein C-terminus, all conflict with the predictions of the model of Shanahan and D'Artel-Ellis (1984), which must therefore be rejected.

Although much useful information about the transporter topology had been obtained using anti-peptide antibodies, a major limitation to their use as structural probes was that the antibodies raised against many of the predicted extramembranous regions of the transporter polypeptide failed to recognize the native, membrane-bound protein. Possible reasons for the lack of binding to the native transporter of antibodies raised against the putative extracellular regions of the protein, against the predicted short loops connecting transmembrane sequences at the cytoplasmic surface of the membrane and against the N-terminal sequence were discussed in Sections 3.4 and 4.4 and remain uncertain. However, the failure of antibodies to recognize the native transporter even after its deglycosylation seems to rule out steric hindrance by the oligosaccharide chain as an explanation for the lack of antibody binding. Therefore the precise arrangement of the extracellular and small cytoplasmic regions is likely to remain a subject of some speculation until the structure of the protein is determined by crystallographic means. The conservation in amino acid sequence seen within the small cytoplasmic regions encompassing residues 87-96, 147-156, 327-338 and 389-402 when the sequences of the human erythrocyte glucose transporter and bacterial proton-linked sugar transporters are compared (Maiden et al., 1987), suggests that these regions of the polypeptide are essential for maintenance of the structure and/or function of these transport proteins.

A more fundamental reason for the lack of antibody binding to these regions of the native transporter could stem from general inaccuracies in topological models for membrane proteins based almost exclusively on hydropathic analysis. Indeed, major discrepancies were found when hydropathic analysis and immunolocalization methods were used in conjunction to study the membrane topology of the acetylcholine receptor (McCrea et al., 1988). These workers concluded

that at least one of these approaches (probably hydropathic analysis used in isolation) has serious shortcomings.

The success of hydropathic analysis, which may utilize the predictive algorithms of Kyte and Doolittle (1982), Eisenberg et al. (1984) or Guy (1985), depends heavily on the assumption that 21 amino acids are the minimum that can span the membrane bilayer as an $\alpha$-helix. Segments of the polypeptide at least 21 amino acid residues in length which can form an $\alpha$-helix of sufficient hydrophobicity to span the membrane lipid bilayer are identified. Each helix can be composed of mainly hydrophobic residues or can be amphipathic with a non-polar face interacting with the membrane lipid and a polar face that might constitute part of a hydrophilic pore. These algorithms have in many cases predicted a large number of membrane-spanning $\alpha$-helices, for example 24 for the calcium channel (Tanabe et al., 1987; McCrea et al., 1987), 24 or 32 for the voltage-gated sodium channel (Noda et al., 1984; Salkoff et al.,1987) and 12-14 for the lactose permease of E. coli (Kaback, 1987). However, geometric considerations would indicate that not all of these predicted helices could directly contact the membrane lipid bilayer. Consequently, a number of more hydrophobic helices may pack on the outside of the protein and be in contact with the membrane lipids, whilst other helices carrying charged or polar residues are arranged within the outer ring of hydrophobic helices (Fig.7.1(A). These 'inner helices' may be less structurally constrained and constitute part of the hydrophilic channel through which the substrate is translocated. Lodish (1988) argues that if such an arrangement of helices exists, then there is no reason to suppose that the 'inner helices' are necessarily 21 amino acid residues in length (Fig.7.1(B), thereby raising questions as to the validity of the predictive algorithms used. He suggested that while the inner 'core' of helices may be $\alpha$-helical, some or all of

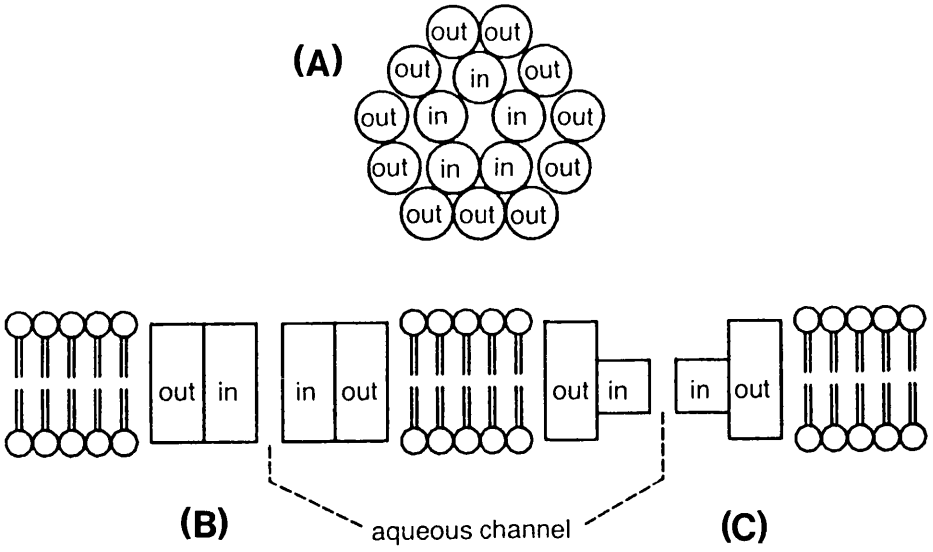



Fig.7.1 Schematic views of the packing of membrane-spanning α-helices in a multi-spanning membrane transport protein

(A) View perpendicular to the plane of the bilayer, showing inner (in) and outer (out) α-helices. Only the outer perimeter of the latter contact the fatty acyl core of the phospholipid bilayer. (B) Cross-sectional view of this multi-spanning protein in which both the inner and outer α-helices are at least 21 amino acids in length and span the entire bilayer. (C) Cross-sectional view of a membrane transport protein whose short inner helices would not be predicted by current algorithms. The outer ring of α-helices are at least 21 amino acids in length, and span the hydrophobic core of the phospholipid bilayer. The inner ring(s) of helices can be much shorter; it is if they form the polar channel itself. The hydrophilic segments connecting the membrane-imbedded segments are not drawn. Taken from Lodish (1988).

those helices may be short, for instance only 10 residues long. The hydrophilic channel through the protein would as a result be shorter than the distance across the lipid bilayer (Fig.7.1(C). Given as an example is the model of Guy and Seetharamulu (1986) for the voltage-gated sodium channel, the only model of a membrane protein which specifically includes short $\alpha$-helices within its membrane-spanning regions. It may also be possible that a predicted membrane-spanning 21 amino acid-containing segment could consist of two or more smaller folded domains. Such an arrangement would not be identified by the algorithms currently in use.

In the model of the human erythrocyte glucose transporter (Mueckler et al., 1985), putative membrane-spanning helices 3,5,7,8 and 11 may be amphipathic and constitute part of the hydrophilic 'core' of helices involved in glucose binding and translocation. If these helices were to exist as smaller, folded 'inner helices', or contain other secondary structure such as $\beta$-sheet as suggested by Lodish (1988), then their arrangement would not be predicted by the algorithms mentioned above. Consequently, a number of regions predicted to be extramembranous in the model of Mueckler et al. (1985), would in fact be located further within the tertiary structure of the transporter and as a result be inaccessible to IgG molecules.

However, even if all the membrane-spanning and extramembranous regions of the glucose transporter polypeptide could be identified by protein-chemical and immunological means, and the boundaries of these regions relative to the lipid bilayer were known, the structural analysis of the protein would still be incomplete. Topological information obtained in these ways is essentially two-dimensional. Crystallization of the transporter and X-ray crystallographic studies would be necessary for a three-dimensional picture of the protein. As discussed in Section 4.1, it is likely to be some time before crystals

of the transporter suitable for high resolution crystallographic studies become available. However, some advances are being made. For example molecular biological methods have been used to produce a soluble form of the lactose permease of E. coli (Roepe and Kaback, 1989), which can assume a non-denatured form in aqueous solution. Thus one of the first problems of membrane protein crystallization, namely removal of excess associated membrane lipid, whilst retaining functional activity, has been achieved. If such a form of the glucose transporter can be produced then crystallization of the protein may be possible in the not too distant future.

## 7.2 Function of the glucose transporter

Anti-peptide antibodies were used as structural probes to assess the involvement of the large, cytoplasmically-exposed hydrophilic regions of the transporter in the ligand-binding function of the protein. Antibodies to the protein C-terminus had no effect on cytochalasin B binding to the transporter. In contrast, antibodies against residues 231-255 in the central cytoplasmic region of the transporter were found to reduce its affinity for cytochalasin B. This effect was paralleled by that of a number of anti-glucose transporter monoclonal antibodies, the epitopes for which are located (at least in part) within the cytoplasmic central region of the transporter sequence. In each case, a reduction in the affinity of the transporter for this ligand was seen, but was associated with no loss of binding sites. These findings suggested the involvement of the latter region in cytochalasin B binding to the transporter. Since the binding sites for cytochalasin B and glucose probably overlap (Deves and Krupka, 1978), then this extramembranous region of the transporter may also be intimately involved in the binding (and translocation?) of the physiological substrate for the protein. The possible causes of this

antibody-induced inhibition of cytochalasin B binding to the transporter were discussed in Section 4.4. Further elucidation of the mechanism giving rise to this inhibitory effect will be required before any functional relationship between the central cytoplasmic region and the ligand/substrate binding site of the transporter can be established. However, cytochalasin B itself does not appear to inhibit binding of antibodies to residues 231-255 of the transporter. This suggests that the latter peptide sequence does not constitute part of a shared binding site for anti-peptide antibodies and cytochalasin B.

Additionally, a preliminary study into the effects of various transporter-bound ligands on the binding of antibodies to the cytoplasmic regions of the transporter was made. The binding of phloretin to an exofacial site on the transporter decreased the binding of antibodies to residues 231-255 of the central cytoplasmic region of the protein. This suggested that in the presence of an extracellularly-bound ligand the transporter adopts a 'outward-facing' conformation in which the central cytoplasmic region becomes less accessible to IgG molecules. In contrast D-glucose and cytochalasin B increased the binding of antibodies to this cytoplasmic region. This finding suggested that in the presence of these ligands the transporter adopts an 'inward-facing' conformation in which the central cytoplasmic region becomes more accessible to IgG molecules. These findings largely paralleled those of a similar study employing trypsin as a probe of conformational changes in the extramembranous cytoplasmic regions of the transporter (Gibbs et al., 1988).

The study of Gibbs et al. (1988) and the present study, suggest that the large cytoplasmic regions are involved in ligand/substrate-induced conformational changes in the transporter. However, the use of antibodies to study structural changes in the transporter polypeptide resulting from the binding of non-transported ligands is

very qualitative. Furthermore, it is likely that the conformational changes seen in the extramembranous regions of the transporter polypeptide accompany similar changes within its membrane-spanning regions. Additionally, little information can be gleaned as regards conformational changes which occur in the protein during the sugar translocation process. More useful information regarding the contribution of the extramembranous regions of the glucose transporter polypeptide might therefore come from a study of the effect of anti-peptide antibodies on the actual transport of D-glucose. To this end, measurement of [$^{14}$C]-D-glucose influx or efflux into sealed, 'right-side-out' erythrocyte ghost membranes containing antibodies directed against the cytoplasmic regions of the glucose transporter might be a feasible approach. These will be essential experiments for the future and should yield considerable information regarding the role of various regions of the transporter polypeptide in sugar transport.

## 7.3 Distribution of glucose transport proteins

Using anti-peptide antibodies it was possible to show that glucose transporters homologous to the human HepG2/erythrocyte protein are widely distributed in mammalian tissues, including brain, testis, ovary, adipose tissue and mammary gland. However, the facilitated transporters of liver and small intestine were clearly shown to be different. An apparently homologous transporter was even detected in erythrocytes of the hagfish, the most primitive living vertebrate. It is therefore likely that the findings concerning the structure and function of the erythrocyte protein in this thesis have a wide significance for glucose transport in general. Anti-peptide antibodies were also used to begin to probe the subcellular distribution of glucose transporters in mammalian tissues. Evidence was obtained from cytochalasin B photolabelling and immunoprecipitation experiments that

glucose transporters are present in the Golgi membranes of lactating rat mammary gland epithelial cells. They probably function here to transport glucose across the membranes to the intralumenal site of lactose synthesis. This finding is one of the first demonstrations that intracellular glucose transporters may be functionally important in some mammalian cells.

The anti-peptide antibodies used in this study have also proven useful in a number of collaborative studies. These include the identification and distribution of glucose transporters of the 'HepG2/erythrocyte/brain-type' at the mammalian blood/nerve barrier (Froenher et al., 1988), characterization of vesicles containing insulin-responsive intracellular glucose transporters isolated from 3T3 L1 adipocytes (Brown et al., 1988), heterogeneity of the glucose transporters in malignant and suppressed hybrid cells (Bramwell et al., 1989), immunocytochemical location of glucose transporters in rat brain (Bagley et al., 1989), identification and characterization of glucose transporters of the blood/brain barrier (Kasanicki et al., 1987) and the immunocytochemical location of glucose transporters in human and bovine brain microvessels (Kasanicki et al., 1988).

Finally, I envisage that the anti-peptide antibodies will be useful in other areas of research into the topology and function of the glucose transporter. Immobilized antibodies could be used in the isolation of fragments of the transporter following labelling and proteolytic or chemical cleavage. In particular, isolation of the fragment(s) of the transporter bearing the site of labelling by cytochalasin B or other ligands following cleavage of the detergent-solubilized protein, may allow the precise location of the ligand/substrate binding site of the protein to be determined. Such an approach was used by Rossie et al. (1987) to isolate and identify

cyanogen bromide-cleaved fragments of the sodium channel containing the cAMP-dependant phosphorylation sites in the protein. The antibodies may also prove useful for the affinity purification of homologous glucose transporters from other tissues and organisms.

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# Peptide-specific Antibodies as Probes of the Orientation of the Glucose Transporter in the Human Erythrocyte Membrane*

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**Antibodies were raised in rabbits against synthetic peptides corresponding to the N-terminal (residues 1–15) and the C-terminal (residues 477–492) regions of the human erythrocyte glucose transporter. The antisera recognized the intact transporter in enzyme-linked immunosorbent assays (ELISA) and Western blots. In addition, the anti-C-terminal peptide antibodies were demonstrated, by competitive ELISA and by immunoadsorption experiments, to bind to the native transporter. Competitive ELISA, using intact erythrocytes, unsealed erythrocyte membranes, or membrane vesicles of known sidedness as competing antigen, showed that these antibodies bound only to the cytoplasmic surface of the membrane, indicating that the C terminus of the protein is exposed to the cytoplasm.**

**On Western blots, the anti-N-terminal peptide antiserum labeled the glycosylated tryptic fragment of the transporter, of apparent $M_r$ = 23,000–42,000, showing that this originates from the N-terminal half of the protein. The anti-C-terminal peptide antiserum labeled higher $M_r$ precursors of the $M_r$ = 18,000 tryptic fragment, although not the fragment itself, indicating that the latter, with its associated cytochalasin B binding site, is derived from the C-terminal half of the protein. Antiserum against the intact transporter recognized the C-terminal peptide on ELISA, and the $M_r$ = 18,000 fragment but not the glycosylated tryptic fragment on Western blots.**

The human erythrocyte glucose transport protein is currently the best characterized example of a facilitated diffusion system. It has been purified to near homogeneity by a number of groups, and when reconstituted into the bilayers of artificial lipid vesicles catalyzes the transport of glucose with kinetics similar to those seen in the intact erythrocyte (1–4). The isolated protein is glycosylated, and migrates as a broad band of average apparent $M_r$ = 55,000 on SDS[1]-polyacrylamide gel electrophoresis (5, 6). It can be specifically photolabeled by UV irradiation in the presence of the potent inhibitor of transport, cytochalasin B (7, 8). Tryptic digestion of the native, membrane-embedded protein after photolabeling yields two large fragments, one of which bears the label, and a number of small peptides (9–11). The unlabeled fragment is glycosylated and migrates as a broad band of apparent $M_r$ = 23,000–42,000 on SDS-polyacrylamide gels. In contrast, the labeled fragment is not glycosylated and migrates as a much sharper band of apparent $M_r$ = 18,000.

In order fully to understand the molecular mechanism of transport, it will ultimately be necessary to determine the three-dimensional structure of the transporter by high resolution x-ray or electron diffraction studies. Such an approach awaits the successful crystallization of the protein. In the meantime, information on the arrangement of the transporter polypeptide in the membrane must be sought by other means. Recently the complete amino acid sequence of the glucose transporter from human HepG2 hepatoma cells was deduced from the nucleotide sequence of a cDNA clone (12). We showed, by fast atom bombardment-mass spectrometry and sequencing studies, that the erythrocyte protein is similar if not identical to the hepatoma transporter (12). In addition, the sequence of another glucose transporter, that from rat brain, has since been determined (13). The rat protein sequence is 97.6% identical to the human and evidence has been presented that in the rat there is probably only one gene that encodes the transporter found in most cell types (13).

Examination of the hepatoma protein sequence led Mueckler *et al.* (12), to propose a model for the arrangement of the transporter in the membrane in which the polypeptide crosses the lipid bilayer 12 times in the form of largely hydrophobic $\alpha$-helices. In this model, the N- and C-terminal regions of the protein and a large hydrophilic region near the middle of the sequence are exposed on the cytoplasmic side of the membrane. Evidence for such an arrangement comes from the identification of peptides derived from both the central region and the C-terminal region in tryptic digests of the native erythrocyte protein (11, 12). Trypsin appears to cleave the native transporter only when it has access to the cytoplasmic side of the erythrocyte membrane: tryptic digestion of intact erythrocytes does not alter the apparent $M_r$ of the transporter polypeptide as revealed by SDS-polyacrylamide gel electrophoresis (10, 14).

In the model $Asn^{45}$ was proposed to be the site of glycosylation of the transporter (12), and direct evidence for this proposition has since been reported (15). It follows that the large, glycosylated tryptic fragment of the transporter must be derived from the N-terminal half of the protein, and that the large, nonglycosylated fragment that bears the site of photolabeling by cytochalasin B must be derived from the C-terminal half. However, these conclusions conflict with a model proposed by Shanahan and D'Artel-Ellis (16) on the basis of *in situ* proteolytic dissection experiments. These

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; KLH, Keyhole limpet hemocyanin; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone.

workers suggest that the C-terminal region of the protein is exposed at the extracellular surface of the membrane rather than the cytoplasmic surface, that the glycosylation site is located near the C terminus, and that the cytochalasin B binding site is located near the N terminus. In order to resolve the conflict between these two models we have now raised antibodies to the N-terminal and C-terminal regions of the protein, in order to investigate the locations of these regions with respect to the plane of the bilayer, and to determine from which half of the intact transporter each of the two large tryptic fragments is derived. Similar approaches, employing peptide-specific antibodies, have recently been successfully applied to the study of several membrane proteins, including the lactose carrier of *Escherichia coli* (17). The results reported herein support the model originally proposed by Mueckler *et al.* (12).

## EXPERIMENTAL PROCEDURES

*Materials*—[4-$^3$H]Cytochalasin B (10.3 Ci/mmol) was obtained from New England Nuclear. Alkaline phosphatase-linked goat anti-rabbit IgG for ELISA was obtained from Miles Laboratories Ltd. (Slough, United Kingdom). Bio-Rad supplied an Immun-Blot assay kit (GAR-AP). Amino acid derivatives and resin for peptide synthesis were purchased from Cambridge Research Biochemicals (Cambridge, United Kingdom). All other reagents and proteins were from Sigma (Poole, Dorset, United Kingdom). Outdated human blood was provided by the blood bank of the Royal Free Hospital.

*Peptide Synthesis*—Peptides corresponding to residues 1–15 (N-terminal peptide) and to residues 477–492 (C-terminal peptide) of the human erythrocyte glucose transporter (12) were synthesized by the $N^{\alpha}$-fluorenylmethoxycarbonyl-polyamide method using a Cambridge Research Biochemicals Pepsynthesizer and $N^{\alpha}$-fluorenylmethoxycarbonyl-amino acid pentafluorophenyl esters (18). A cysteine residue was added to the C terminus of the N-terminal peptide and to the N terminus of the C-terminal peptide to facilitate coupling to the carrier protein. The peptides were cleaved from the support using trifluoroacetic acid containing 5% (v/v) ethanedithiol and then washed with ether. Fast atom bombardment-mass spectrometry, performed as described previously (12), yielded signals at the expected $[M + H]^+$ values of 1764 and 1870 for the N- and C-terminal peptides, respectively. High performance liquid chromatography analysis of the peptides on a $C_{18}$ column using gradients of acetonitrile in 0.1% (v/v) trifluoroacetic acid indicated that they were at least 80% pure, and so they were used without further purification. After hydrolysis in 6 M HCl at 110 °C for 24 h the N-terminal peptide yielded the amino acid composition $Thr_{0.9}$-$Ser_{1.6}$-$Glx_{0.9}$-$Pro_{0.9}$-$Gly_{1.1}$-$Ala_{1.1}$-$Met_{1.8}$-$Leu_{3.2}$-$Lys_{1.8}$-$Arg_{1.1}$ and the C-terminal peptide composition $Asx_{1.0}$-$Thr_{1.0}$-$Ser_{0.9}$-$Glx_{3.1}$-$Pro_{2.0}$-$Gly_{1.0}$-$Ala_{1.0}$-$Val_{1.0}$-$Leu_{2.0}$-$Phe_{1.0}$-$His_{1.0}$-$Lys_{1.0}$.

*Production of Antibodies*—Peptides were coupled to Keyhole limpet hemocyanin (KLH) using maleimidobenzoyl-*N*-hydroxysuccinimide ester, essentially as described by LaRochelle *et al.* (19). Comparison of the amino acid compositions of the peptide-KLH conjugates with that of KLH indicated that approximately 8 mol of C-terminal peptide and approximately 10 mol of N-terminal peptide were bound per 100,000 g of protein. Antisera against the conjugates and against the purified human erythrocyte glucose transporter were then raised in male New Zealand White rabbits. The conjugates or transporter (200 μg of protein) in 0.5 ml of 10 mM sodium phosphate, 150 mM NaCl, pH 7.2, were emulsified with 1.5 ml of complete Freund's adjuvant and then injected intradermally at multiple sites along the back. An additional injection of antigen (100 μg) in incomplete Freund's adjuvant was made after 4 weeks, and then the animals were bled after another 2 weeks. Antisera were treated at 56 °C for 30 min to inactivate complement and then stored at −70 °C. Control sera were obtained from the rabbits before the first injection. Antibody to the C-terminal peptide was affinity purified by adsorption from serum onto alkali-stripped erythrocyte membranes and released with acid, using a procedure similar to that developed by Schroer *et al.* (20) for affinity purification of antitransporter antibodies. In this procedure, 6 mg of alkali-stripped membranes were found to adsorb all the peptide-specific antibody from 1 ml of antiserum, as quantified by ELISA. Each milliliter of serum yielded about 0.9 mg of affinity purified antibody.

*ELISA*—For determination of antibody titers, ELISA was carried out essentially as previously described (21) except that 1% (w/v) bovine serum albumin was substituted for fetal calf serum as a blocking agent, and goat anti-rabbit IgG conjugated to alkaline phosphatase was used as the second antibody. Microtiter plates were coated with either 600 ng of purified glucose transporter or 20 ng of peptide/well. Competitive ELISA experiments were also performed as previously described, using a limiting dilution of antiserum (21). Both antisera and competing antigens were diluted in detergent-free buffer to avoid membrane lysis. After incubation of mixtures of antisera and antigens for 30 min at room temperature, bound and free antibody were separated by centrifugation at 4 °C for 15 min at 11,500 × *g* (for membranes) or at 2,900 × *g* (for erythrocytes). Free antibody remaining in the supernatant was then assayed by ELISA.

*Western Blot Analysis*—SDS-polyacrylamide gel electrophoresis was carried out by the procedure of Laemmli (22) using 12% acrylamide gels. Electrophoretic transfer to nitrocellulose paper and staining for protein were as previously described (21). The binding of antibody to the transporter and its proteolytic fragments on the nitrocellulose were detected using goat anti-rabbit IgG conjugated to alkaline phosphatase, with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium as chromagens, as described by the manufacturers of the Immun-Blot assay kit. Antisera were used at a dilution of 1 in 300.

*Preparation and Assay of Membranes*—Human erythrocyte membranes, alkali-stripped membranes, vesicles of known sidedness, and purified glucose transporter were prepared as previously described (9, 21). The sidedness of inside-out and right-side-out vesicles was assayed by measuring the accessibility of acetylcholinesterase and of glyceraldehyde-3-phosphate dehydrogenase, as described by Steck and Kant (23). Erythrocyte numbers were measured using a hemocytometer. The quantity of erythrocyte membrane protein used in competitive ELISA experiments with intact erythrocytes (*e.g.* in Fig. 3) was estimated from cell numbers on the assumption that an erythrocyte contains $8 \times 10^{-10}$ mg of membrane protein (24). Protein was measured by the procedure of Lowry *et al.* (25) except that 0.5% (w/v) SDS was included to solubilize membranous samples.

The binding of cytochalasin B to the purified transporter and to alkali-stripped membranes was measured in triplicate by equilibrium dialysis using $4 \times 10^{-6}$ M [4-$^3$H]cytochalasin B as described by Zoccoli *et al.* (26). Correction for nonspecific binding was made by making measurements after heating the membranes to 100 °C for 5 min. The corrected ratio of bound to free cytochalasin B obtained from these assays is approximately equal to the concentration of cytochalasin B binding sites divided by the dissociation constant for cytochalasin B, and is referred to as the cytochalasin B-binding activity.

*Immunoadsorption*—For immunoadsorption experiments, purified glucose transporter (52 μg/ml) in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4, was solubilized with 1% (w/v) octylglucoside at 4 °C. Samples (1 ml) were then incubated with 200 μl of protein A-Sepharose CL-4B to which 1.2 mg of either affinity purified anti-C-terminal peptide antibody or non-immune rabbit IgG was bound. After incubation at 4 °C for 1 h with constant mixing, the samples were centrifuged, the supernatants made 2 mM in dithiothreitol, and then dialyzed against 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4, to remove the detergent. The reconstituted supernatants were then assayed for cytochalasin B binding activity.

*Proteolytic Digestions*—Purified glucose transporter at a concentration of 250 μg/ml in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4, was incubated at 25 °C with 2.5 μg/ml diphenylcarbamyl chloride-treated trypsin for various periods of time. Digestion was halted by addition of 5 μg/ml bovine lung aprotinin. More extensive digestion of the transporter was achieved by incubation with 12.5 μg/ml diphenylcarbamyl chloride-treated trypsin or TLCK-treated chymotrypsin for 7 h at 37 °C. Digestion was halted by the addition of 1 mM TLCK or 0.1 mM phenylmethylsulfonyl fluoride, respectively.

## RESULTS AND DISCUSSION

*Characterization of Peptide-specific Antibodies*—Peptide-specific antibodies in the serum of rabbit immunized with peptide-KLH conjugates were assayed by ELISA against peptides bound to microtiter plates. Fig. 1*A* shows that antibodies to the C-terminal peptide were elicited in rabbits by the appropriate conjugate. A high anti-C-terminal peptide antibody titer was also evident in the antiserum produced by

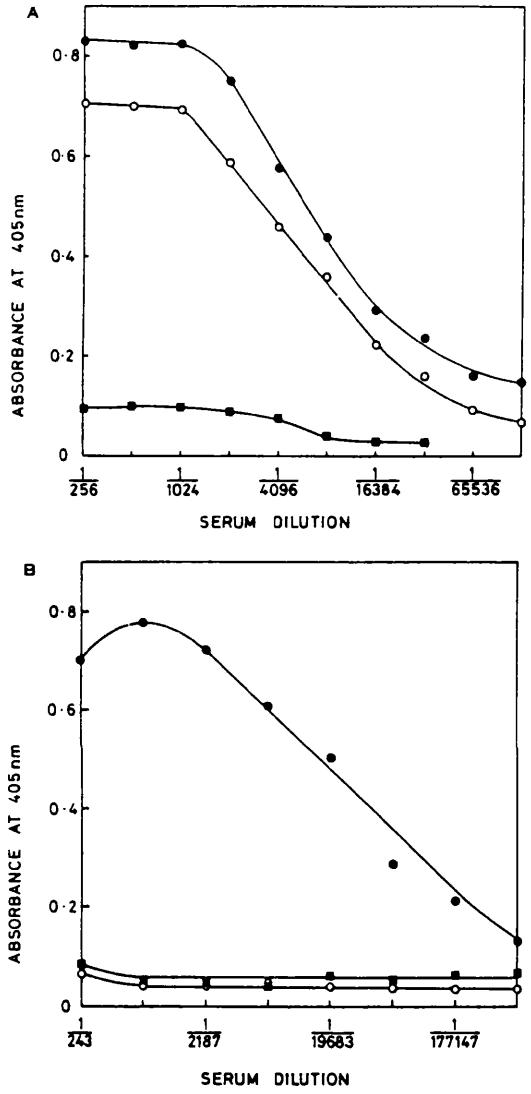


FIG. 1. **ELISA of antipeptide and of antitransporter antibodies.** Microtiter plates were coated with 20 ng/well of C-terminal peptide (*A*) or N-terminal peptide (*B*). They were then incubated with serial 2-fold (*A*) or 3-fold (*B*) dilutions of antisera followed by alkaline phosphatase-linked second antibody and *p*-nitrophenyl phosphate as chromogenic substrate, as described under "Experimental Procedures." Each point is the mean absorbance at 405 nm of triplicate samples. *A*, anti-C-terminal peptide serum (●), antitransporter serum (○), and pre-immune serum (■). *B*, anti-N-terminal peptide serum (●), antitransporter serum (○), and pre-immune serum (■).

immunization of rabbits with purified glucose transporter (Fig. 1*A*), indicating that the C terminus is also immunogenic in the intact protein. Fig. 1*B* shows that the N-terminal peptide-KLH conjugate also elicited peptide-specific antibodies, but that antitransporter serum did not contain antibodies that recognized this peptide.

Both of the antipeptide sera recognized the transporter on Western blots (Fig. 4, *A* and *C*): no labeling of the protein by preimmune serum was seen (data not shown). The sera also recognized the intact transporter bound to microtiter plates, giving results in ELISA very similar to those shown in Fig. 1 for binding to the immobilized peptides (data not shown). However, it is likely that the transporter bound to plastic in this way is at least partly denatured. In order to determine whether the antisera recognized the transporter in its native conformation, competitive ELISA experiments were carried out using microtiter plates coated with purified glucose transporter, and alkali-stripped erythrocyte membranes as competing antigen. The latter were used because they are known to be unsealed (27), so that macromolecules have access to both sides of the membrane. Fig. 2 shows that in these experiments prior treatment of the anti-C-terminal peptide serum with membranes inhibited antibody binding to the plates by about 80% at the highest membrane concentration used. In contrast, no inhibition of the binding of the anti-N-terminal peptide antibodies was seen, indicating that unlike the anti-C-terminal peptide antibodies, these antibodies do not recognize the transport protein in its native conformation.

Further evidence that the anti-C-terminal peptide antibody recognized the native conformation of the transporter was provided by immunoadsorption experiments. These employed antibody that had been affinity purified by adsorption to alkali-stripped erythrocyte membranes and elution with acid. The affinity purified antibody was shown by SDS-polyacrylamide gel electrophoresis to comprise almost entirely IgG, with traces of albumin. In the immunoadsorption experiments, detergent-solubilized transporter was incubated with an excess of antibody bound to protein A-Sepharose CL-4B. Transporter remaining unadsorbed in the supernatant was quantified, after removal of detergent by dialysis, by measure-

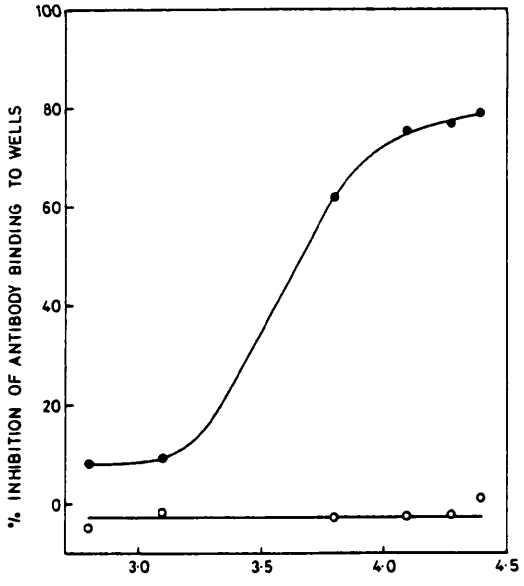


FIG. 2. **Competitive ELISA.** The ability of alkali-stripped erythrocyte membranes to compete for a limiting amount of antibody with 600 ng of glucose transporter bound to the surface of a microtiter well. Samples (100 μl) of a 1 in 2000 dilution of antiserum were incubated with the amount of erythrocyte membrane protein indicated and then the amount of free antibody remaining in the supernatant after centrifugation was assayed by ELISA. Each point is the mean for triplicate samples. ●, anti-C-terminal peptide serum; ○, anti-N-terminal peptide serum.

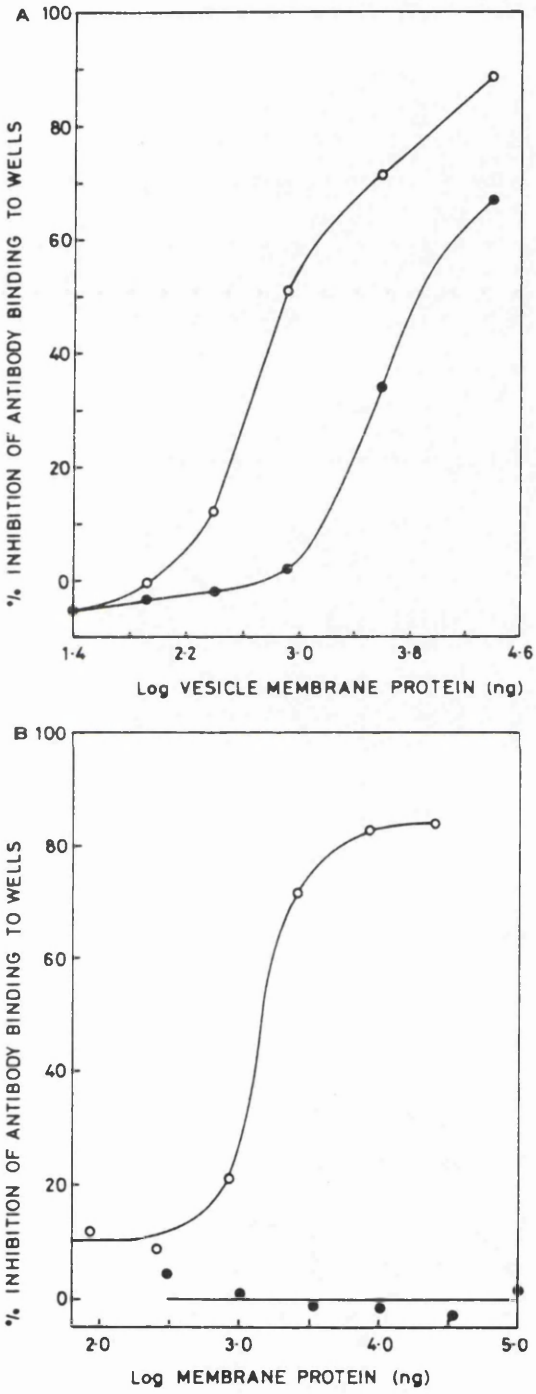


FIG. 3. **Competitive ELISA.** The ability of (*A*) inside-out (○) and right-side-out (●) erythrocyte membrane vesicles, and (*B*) alkali-stripped erythrocyte membranes (○) and intact erythrocytes (●), to compete for a limiting amount of anti-C-terminal peptide antibody with 600 ng of glucose transporter bound to the surface of a microtiter well. Experimental procedures were as described in the legend to Fig. 2 and in the text.

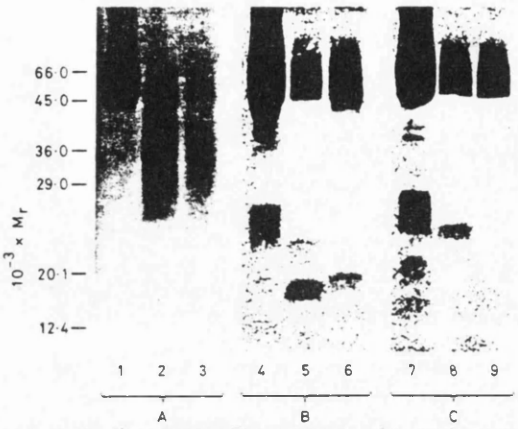


FIG. 4. **Binding of antibodies to the transporter and its proteolytic fragments on Western blots.** Samples (20 μg) of intact transporter (*lanes 1, 4,* and *7*) and of transporter extensively digested with trypsin (*lanes 3, 5,* and *8*) or with chymotrypsin (*lanes 2, 6,* and *9*) were electrophoresed on a 12% SDS-polyacrylamide gel, electrophoretically blotted onto nitrocellulose paper, and then immunologically stained with anti-N-terminal peptide serum (*A*), antitransporter serum (*B*), or anti-C-terminal peptide serum (*C*), as described under "Experimental Procedures." The following molecular weight markers were used: bovine serum albumin ($M_r$ = 66,000), ovalbumin ($M_r$ = 45,000), glyceraldehyde-3-phosphate dehydrogenase ($M_r$ = 36,000), carbonic anhydrase ($M_r$ = 29,000), soybean trypsin inhibitor ($M_r$ = 20,100), and cytochrome *c* ($M_r$ = 12,400).

ment of cytochalasin B binding activity. Incubation of 52 μg of transporter with 1.2 mg of immobilized antibody was found to remove 88% (mean of triplicate samples) of the cytochalasin B binding activity from the supernatant, compared to a control treated with an equivalent amount of non-immune IgG. Failure to adsorb all the transporter, even using this 8-fold molar excess of antibody, probably stemmed from hindrance of transporter access to all the bound immunoglobulin by the Sepharose matrix.

Although the results described above indicate that the anti-C-terminal peptide antibodies bound to the native, functionally active conformation(s) of the transporter, their binding did not appear to affect its ability to bind cytochalasin B: addition of antibody to unsealed, alkali-stripped membranes in an approximately 6-fold molar excess over transporter did not inhibit cytochalasin B binding activity at all. These results contrast with our previous findings that two monoclonal antibodies raised against the intact transporter did greatly inhibit its ability to bind cytochalasin B (21).

*Location of the C Terminus of the Glucose Transporter*—Since the anti-C-terminal peptide antibodies recognized the native transporter, it was possible to use them to establish at which side of the membrane the C terminus of the protein is exposed. For this purpose, competitive ELISA experiments were performed using erythrocyte membrane vesicles of known sidedness as competing antigen. Enzymatic assays indicated that the "right-side-out" vesicle preparation used contained 92% sealed, right-side-out vesicles contaminated with 8% inside-out vesicles. The "inside-out" vesicle preparation contained 80% sealed inside-out vesicles and 20% right-side-out vesicles. Both types of vesicle are known to contain equal amounts of glucose transporter per mg of membrane protein (21). Fig. 3*A* shows that the inside-out vesicle preparation was about 10-fold more effective than the right-side-out vesicle preparation in competing with solid-phase bound transporter for antibody binding. This finding suggests that

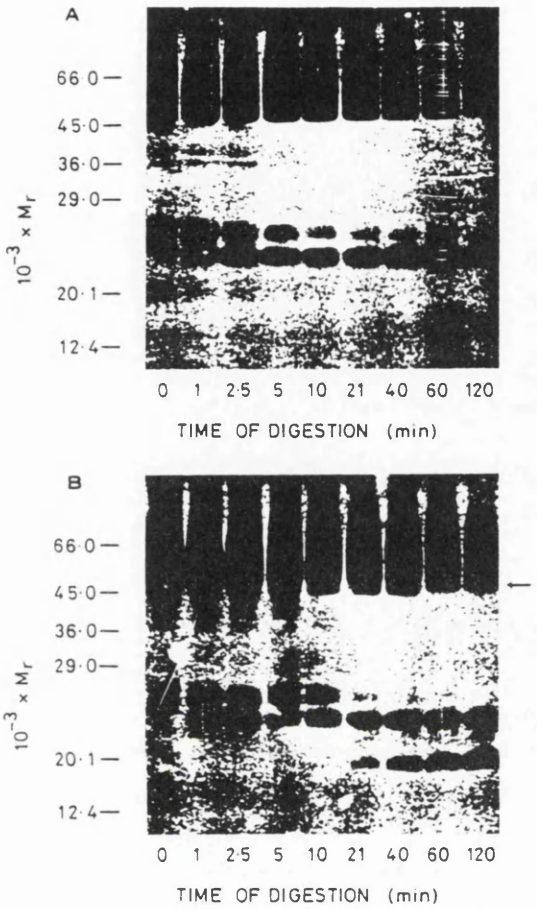


FIG. 5. **The time course of the appearance of immunologically reactive fragments during trypic digestion of the transporter.** Samples (10 μg) of transporter digested with trypsin for various times as described under "Experimental Procedures" were electrophoresed on an SDS-polyacrylamide gel and blotted onto nitrocellulose as described in the legend to Fig. 4. The blots were then immunologically stained with either (*A*) anti-C-terminal peptide antiserum or (*B*) antitransporter serum.

the C terminus of the protein is exposed on the cytoplasmic face of the erythrocyte membrane. Confirmation of this conclusion was obtained by comparing the ability of intact erythrocytes and unsealed, alkali-stripped membranes to bind antibody in a competitive ELISA experiment. Alkali-stripped membranes inhibited the binding of antibody to solid-phase bound transporter by almost 85% at the highest amount used (25 μg/well; Fig. 3*B*). Since the transporter comprises about 10% of the protein in these membranes (28), then the highest amount used would have contained about 2.5 μg of transporter. In contrast, intact erythrocytes caused negligible inhibition even at the highest number used ($1.25 \times 10^8$ cells/well; Fig. 3*B*). Since an erythrocyte is taken to contain about $8 \times 10^{-10}$ mg of membrane protein (24), and the glucose transporter comprises 5% of the total membrane protein (28), then this number of cells should contain about 5 μg of transporter.

*Location of the Large Tryptic Fragments in the Transporter Sequence*—The antipeptide sera were also used to locate within the protein sequence the two large fragments produced by tryptic digestion of the transporter (9, 10). The anti-N-terminal peptide serum labeled a broad zone on Western blots of both tryptic and chymotryptic digests of the protein, corresponding to the glycosylated fragment of apparent $M_r$ = 23,000–42,000 (9, 10; Fig. 4*A*). This finding supports the identification of $Asn^{45}$ as the site of glycosylation (12, 15). Labeling of the chymotryptic fragment was expected, because there are no likely sites for cleavage by this enzyme within the first 15 residues of the transporter sequence (12). However, there are potential sites of tryptic cleavage at residues 6, 7, and 11 (12). The fact that the antibodies recognized the tryptic fragment suggests that, whether or not it contains residues 1–7 of the transporter, it almost certainly contains residues 8 and beyond. Western blot analysis also confirmed the finding from ELISA experiments that the N-terminal region is not immunogenic in the intact protein: the antitransporter serum did not label the glycosylated tryptic fragment on Western blots (Fig. 4*B*).

The fragments of approximate $M_r$ = 18,000–20,000 produced by prolonged tryptic and chymotryptic digestion of the transporter were labeled by the antitransporter serum (Figs. 4*B* and 5*B*) but not by the anti-C-terminal peptide serum (Fig. 4*C*) on Western blots, indicating that they do not contain the C terminus of the intact protein. However, fragments of higher $M_r$ seen predominantly after short times of tryptic digestion were labeled by the anti-C-terminal peptide serum (Fig. 5*A*). These fragments, of approximate $M_r$ = 25,500 and 23,500, are also photolabeled by cytochalasin B and are probably precursors to the $M_r$ = 18,000 tryptic fragment (9). It follows that the latter, and its associated cytochalasin B binding site, are derived from the C-terminal half of the protein.

Although the $M_r$ = 25,500 and 23,500 fragments must contain the C terminus of the protein, the anomalous mobility of hydrophobic peptides on SDS-polyacrylamide gel electrophoresis precludes accurate estimation of their true $M_r$ (2). Thus, the deglycosylated transporter migrates as a sharp band of apparent $M_r$ = 46,000 on 10% SDS-polyacrylamide gels (14), although its true $M_r$ is 54,117 (12). It is therefore not possible to locate the N termini of the tryptic fragments within the transporter sequence with any certainty. However, a possible sequence of tryptic cleavages leading to the fragments would be first at $Lys^{256}$ and then $Arg^{269}$, yielding fragments of calculated $M_r$ = 25,845 and 24,298, respectively. Cleavage at $Arg^{458}$ would then yield a fragment of calculated $M_r$ = 20,693, lacking the C terminus of the protein. Whether or not these speculations prove to be correct, the order of cleavages produced by trypsin is not obligatory: a fragment of mobility slightly higher than the transporter, seen after short times of digestion, is labeled by antitransporter serum on Western blots (Fig. 5*B*, *arrow*) and by anti-N-terminal peptide serum (not shown), but not by anti-C-terminal peptide serum (Fig. 5*A*). This fragment must therefore arise by initial cleavage of the protein near its C terminus.

*Conclusions*—The use of peptide-specific antibodies described in this article has provided further evidence for the correctness of the model proposed by Mueckler *et al.* (12) for the arrangement of the transporter polypeptide in the erythrocyte membrane. The results show that the site of glycosylation is in the N-terminal half of the protein, that of photolabeling by cytochalasin B is in the C-terminal half, and that the C terminus of the protein is exposed at the cytoplasmic side of the membrane. We envisage that such antipeptide antibodies will now also prove useful in the affinity purification of the transporter from a variety of sources. It is known

that the "purified" preparations of transporter from human erythrocytes do in fact contain small amounts of other proteins, such as the nucleoside transporter (29), and immunoaffinity chromatography on antibody columns may enable these to be removed. The fact that the anti-C-terminal peptide antibodies gave a small amount of labeling of blots of the undigested transporter in the $M_r$ = 23,000–27,000 region (Figs. 4*C* and 5*A*) suggests, however, that at least one of the "contaminating" proteins, previously identified as band 7 (2), in fact results from slight endogenous proteolysis of the transporter polypeptide itself. The antibodies should also be very useful, specific probes for investigating the tissue and subcellular distribution of the transporter polypeptide at the light and electron microscope level. Work in these areas is currently in progress in our laboratory.

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# Site-specific antibodies as probes of the topology and function of the human erythrocyte glucose transporter

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Antibodies were raised against synthetic peptides corresponding to most of the regions of the human erythrocyte glucose transporter predicted to be extramembranous in the model of Mueckler, Caruso, Baldwin, Panico, Blench, Morris, Lienhard, Allard & Lodish [(1985) Science **229**, 941–945]. Most of the antibodies (17 out of a total of 19) recognized the intact denatured protein on Western blots. However, only seven of the antibodies recognized the native membrane-bound protein, even after its deglycosylation. These antibodies, against peptides encompassing residues 217–272 and 450–492 in the hydrophilic central and *C*-terminal regions of the transporter, bound to the cytoplasmic surface of the erythrocyte membrane. This finding is in agreement with the prediction of the model that these regions of the sequence are cytoplasmic. Antibodies against peptides from the central cytoplasmic loop of the transporter were found to inhibit the binding of cytochalasin B to the membrane-bound protein, whereas antibodies against the *C*-terminal region had no effect. The anti-peptide antibodies were then used to map the sequence locations of fragments of the transporter arising from tryptic digestion of the membrane-bound protein. This in turn enabled the epitopes for a number of anti-transporter monoclonal antibodies to be located within either the central cytoplasmic loop or the *C*-terminal region of the protein. Of those monoclonal antibodies which inhibited cytochalasin B binding to the protein, all but one were found to have epitopes within the central region of the sequence. In conjunction with the results of the anti-peptide antibody studies, these findings indicate the importance of this part of the protein for transporter function.

## INTRODUCTION

Most mammalian cells take up glucose by the passive process of facilitated diffusion [1]. The transport proteins responsible for this process in different tissues differ in their kinetic and regulatory properties [2,3]. However, the recent cloning of the passive transporters from several mammalian tissues has revealed that, despite their different properties, they are members of a family of related proteins, which also includes transporters from yeasts and bacteria [4–11].

A complete understanding of the mechanism of transport will require elucidation of the three-dimensional structure of at least one of these proteins at atomic resolution. However, the only member of the family that has so far been purified to near homogeneity and upon which direct structural studies are possible is the human erythrocyte glucose transporter [12,13]. Sequence studies have shown that this protein is probably identical to a transporter encoded by a cDNA clone from the human hepatocellular carcinoma cell line HepG2, and also very closely resembles a transporter from rat brain [4,5]. The predicted $M_r$ of the protein from its sequence is 54116, and the purified protein migrates as a broad band of average apparent $M_r$ 55000 on SDS/polyacrylamide-gel electrophoresis as a result of heterogeneous glycosylation [4,12–14].

Hydropathic analysis of the sequence, together with the knowledge from spectroscopic studies that the protein is predominantly α-helical [15–17], enabled us to propose a model for the arrangement of the polypeptide in the membrane [4]. In this model, the protein spans the bilayer 12 times in the form of hydrophobic or amphipathic α-helices. The *N*- and *C*-terminal regions of the protein (residues 1–12 and 451–492), together with a large hydrophilic region (residues 207–271) linking proposed transmembrane helices 6 and 7, are predicted to lie at the cytoplasmic face of the membrane. We have obtained some direct evidence for this arrangement by vectorial proteolytic digestion studies [4,17]. Similarly, we and others have confirmed the cytoplasmic location of the *C*-terminus of the protein using antibodies raised against a synthetic peptide corresponding to residues 477–492 [18,19]. In the present paper we extend this approach by using anti-peptide antibodies to other regions of the sequence as structural probes.

Abbreviations used: KLH, keyhole-limpet haemocyanin; MBS, maleimidobenzoyl-*N*-hydroxysuccinimide ester; NBMPR, nitrobenzylthioinosine; NBTGR, nitrobenzylthioguanosine.

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Tryptic digestion of the transporter has also provided information about the function of different regions of the protein. One or both of the central hydrophilic and *C*-terminal regions of the sequence are important for transporter function, because transport activity is abolished if they are removed from the membrane by tryptic digestion [20]. Similarly, the affinity of the protein for the inhibitor of transport, cytochalasin B, is greatly reduced by tryptic digestion [21]. However, low-affinity D-glucose-sensitive cytochalasin B binding to the protein is still seen after extensive digestion, indicating that the two large membrane-bound fragments which result from such digestion of the transporter retain the substrate-binding site, at least in part [17,21,22]. One of these fragments is glycosylated and migrates as a broad band of apparent $M_r$ 23000–42000 on SDS/polyacrylamide gels [21,23]. We have shown that it is derived from the *N*-terminal half of the protein, and probably comprises residues 1–212 [17,18]. The *C*-terminal half of the protein yields a fragment that migrates as a sharp band of apparent $M_r$ 18000 [21,23]. This fragment bears the label if the transporter is photoaffinity labelled either with [$^3$H]cytochalasin B or with bis-mannose derivatives before digestion [21–24]. These reagents are thought to bind to the substrate binding site(s) at the cytoplasmic and extracellular surfaces respectively of the membrane. It is therefore probable that the substrate binding site(s) of the transporter is located at least in part within this region of the sequence.

Another way of probing the functional importance of different regions of a protein is to examine the effect of site-specific antibodies on its activity. To this end, we have previously raised two panels of monoclonal antibodies against the transporter, some of which affected its function [25,26]. Unfortunately, the antibodies did not bind to the fragments yielded by prolonged tryptic digestion of the transporter, and so it was not possible to locate their epitopes. In the present study we have been able to examine the effects of anti-peptide antibodies in order to probe the function of specific regions of the transporter. In addition, these antibodies have enabled us to map the fragments produced by short periods of digestion of the transporter with trypsin. Because some of these fragments are also recognized by the monoclonal antibodies, it has been possible to locate the relevant epitopes and correlate these with the effects of the monoclonal antibodies on transporter function.

This work was presented, in part, at the 629th Meeting of the Biochemical Society held in December 1988 at the Royal Free Hospital School of Medicine, London.

## EXPERIMENTAL

### Materials

[4-$^3$H]Cytochalasin B (18.5 Ci/mmol) and [*benzyl*-$^3$H]nitrobenzylthioinosine (NBMPR; 26 Ci/mmol) were purchased from New England Nuclear (Stevenage, Herts., U.K.). Alkaline-phosphatase-linked goat anti-rabbit IgG for e.l.i.s.a. was obtained from Miles Laboratories (Slough, Berks., U.K.). Protein A–Sepharose CL-4B, octyl glucoside and nitrobenzylthioguanosine (NBTGR) were from Sigma (Poole, Dorset, U.K.). Nitrocellulose, $M_r$ standards for SDS/polyacrylamide-gel electrophoresis and Immuno-Blot assay kits (GAM-AP and GAR-AP) were obtained from Bio-Rad Laboratories (Watford, Herts., U.K.). Amino acid derivatives and resin for peptide synthesis by the $N^{\alpha}$-fluorenylmethoxycarbonyl-polyamide solid-phase method were obtained from Milligen (Watford, Herts., U.K.). Endoglycosidase F from *Flavobacterium meningosepticum* was obtained from Boehringer (Lewes, Sussex, U.K.) and endo-$\beta$-galactosidase from *Bacteroides fragilis* was kindly donated by Dr. P. Scudder (Clinical Research Centre, Harrow, Middx., U.K.). Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) and sulpho-MBS were purchased from Pierce (Chester, U.K.). All other chemicals were from Sigma or BDH (Poole, Dorset, U.K.) and were the highest grade available. Out-dated human blood was provided by the blood bank of the Royal Free Hospital.

### Peptide synthesis

All peptides corresponding to regions of the glucose transporter predicted to be exposed on the cytoplasmic face of the membrane [4] with the exception of peptide 217–232 were synthesized using the $N^{\alpha}$-fluorenylmethoxycarbonyl-polyamide solid-phase method [27] and characterized exactly as described previously [18]. A cysteine residue was added to the *C*-terminus of each peptide to facilitate coupling to a carrier protein. Characterization showed that the peptides were at least 80% pure, and so they were used without further purification. The other peptides were synthesized by the solid-phase method of Barany & Merrifield [28] using t-butyloxycarbonyl amino acids in a Biosearch Model 9500 peptide synthesizer, and were purified by h.p.l.c. before use. A cysteine–glycine sequence was attached to the *N*-terminus of the peptides to facilitate coupling to the carrier protein.

### Production of and characterization of antibodies

Peptides 84–98, 326–340 and 389–403, which contain no lysine residues, were coupled to bovine thyroglobulin using specially purified glutaraldehyde (grade I, Sigma), essentially as described by Carrasco *et al.* [29]. The other peptides were coupled to keyhole-limpet haemocyanin (KLH) with MBS as described previously [18], or with sulpho-MBS, a water-soluble analogue of MBS. The compositions of the KLH– and thyroglobulin–peptide conjugates were determined by amino acid analysis. Typically, between 10 and 25 mol of peptide was bound per 100000 g of carrier protein. Antisera were then raised against the peptide conjugates exactly as described previously [18], except that in some cases the conjugates were administered to the rabbits by intramuscular injection.

Antisera were processed and screened for their anti-peptide and anti-(glucose transporter) activity by e.l.i.s.a. and by competitive e.l.i.s.a. as described previously [18]. Western blotting was also performed as described previously [18,26], except that the buffer used to block protein-binding sites on the nitrocellulose contained 5% (w/v) low-fat dried milk powder rather than gelatin. Antibodies which bound to the native glucose transporter were affinity-purified by adsorption on to protein-depleted erythrocyte membranes [18]. Monoclonal antibodies GTPR3 and GTPR4 were raised against the intact glucose transporter exactly as described previously for antibodies GTPR1 and GTPR2 [26]. Production of the other monoclonal antibodies used has already been described elsewhere [25,26].

### Deglycosylation of the transporter

Deglycosylation of the transporter in the absence of detergents was achieved using a mixture of glycosidases [16]. Purified glucose transporter at a protein concentration of 2 mg/ml in 100 mM-sodium phosphate/50 mM-EDTA/75 mM-2-mercaptoethanol, pH 6.1, was incubated with 0.3 units of endo-$\beta$-galactosidase/ml and 4 units of endoglycosidase F/ml at 25 °C for 24 h. Control samples were incubated in the absence of enzymes. Samples of the digests were dialysed into 50 mM-sodium phosphate/100 mM-NaCl/1 mM-EDTA, pH 7.4, before assay of cytochalasin B-binding activity and e.l.i.s.a.

### Other procedures

The preparation of protein-depleted erythrocyte membranes and of the purified glucose transporter [21], immunoadsorption experiments [18,26], ligand-binding measurements [26] and other assays were all as described previously except where otherwise indicated in the text or Figure legends. Rat brain membranes were prepared by the method of Wang [30].

## RESULTS

### Recognition of the native and denatured transporter by anti-peptide antibodies

Antibodies were raised in rabbits against peptides corresponding to most of those regions of the transporter predicted to be extramembranous [4], as illustrated in Fig. 1. The peptides comprised residues 35–46, 46–56, 34–60, 84–98, 112–127, 144–158, 175–189, 217–232, 231–246, 240–255, 256–272, 293–306, 326–340, 389–403, 420–432, 450–467 and 460–477. (For the sake of completeness, Fig. 1 also illustrates the sequence location of peptides comprising residues 1–15 and 477–492 of the transporter. We reported the properties of antibodies raised against these regions of the sequence in a previous publication [18].) All of the antisera showed a high titre of anti-peptide antibodies when assayed by e.l.i.s.a. using the respective synthetic peptides as microtitre plate-bound antigens (results not shown). However, only the antisera raised against peptides 217–232, 231–246, 240–255, 256–272, 450–467 and 460–477 strongly recognized plate-bound purified glucose transporter in e.l.i.s.a. assays. The other antisera appeared to recognize the intact transporter weakly or not at all.

Despite the failure of several of the anti-peptide antibodies to recognize the plate-bound transporter, all except those against peptides 112–127 and 420–432 labelled the denatured transporter on Western blots. One possible explanation for the lack of recognition of the native protein in e.l.i.s.a. assays might be steric hindrance from the oligosaccharide chain attached at Asn-45, particularly for those antibodies directed against the region of carbohydrate attachment. In order to test this hypothesis, deglycosylated transporter was prepared as described in the Experimental section. Following treatment with glycosidases, the protein retained > 95% of its original cytochalasin B binding activity, indicating that it remained in its native conformation. The extent of deglycosylation was estimated to be > 80% by spectrophotometric scanning of Coomassie Blue-stained gels of the deglycosylated transporter. These showed primarily a sharp band of apparent $M_r$ 46000, cor-

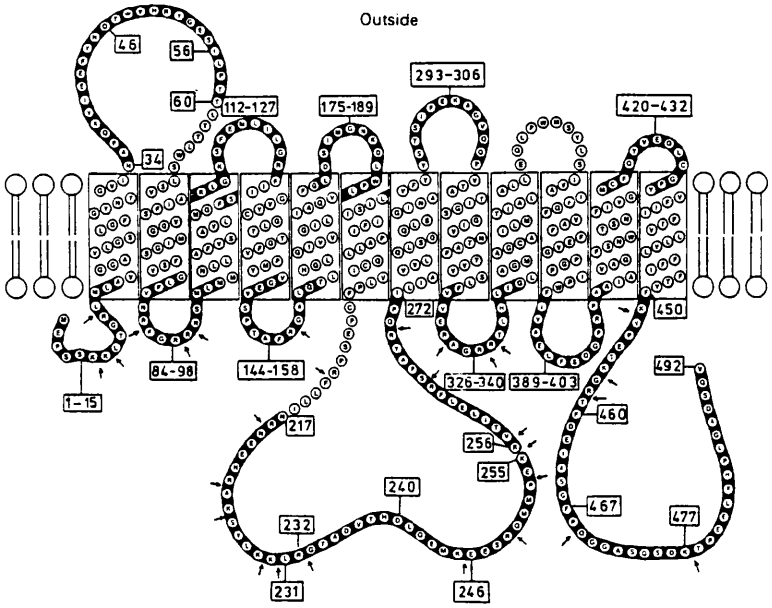


**Fig. 1. Location of chemically synthesized peptides within the glucose transporter sequence**

Regions of the sequence corresponding to synthetic peptides are outlined in black. Numbers refer to the sequence location of the *N*- and *C*-terminal residues of each peptide. Arrows indicate the location of potential tryptic cleavage sites. The proposed model for the arrangement of the transporter in the membrane is taken from [4].

responding to the band previously identified as the fully deglycosylated transporter [31]. A minor band of approximate $M_r$ 48000 probably corresponded to a partially deglycosylated species. Both bands were labelled on Western blots by all of the anti-peptide antibodies except those against peptides 112–127 and 420–432. However, none of the anti-peptide antibodies reacted any more strongly with the plate-bound deglycosylated transporter than with the fully glycosylated transporter in e.l.i.s.a. (results not shown).

Antisera raised against peptides 217–232, 231–246, 240–255, 256–272, 450–467 and 460–477 strongly recognized plate-bound purified glucose transporter in e.l.i.s.a. assays. This finding suggested that they could bind to the native protein, although it is possible that the transporter is at least partially denatured upon adsorption to plastic in an e.l.i.s.a. assay. Additional evidence that (a fraction of) the antibodies in each serum recognized the native transporter was provided by the fact that antibodies could be affinity-purified from the sera by adsorption on to erythrocyte membranes, as described in the Experimental section. Yields of IgG ranged from 0.25 mg/ml of serum to 0.8 mg/ml of serum.

The affinity-purified antibodies were used in further experiments to demonstrate not only that they recognized the native glucose transporter, but also that they were specific for the latter. Such experiments were necessary because both the glucose and nucleoside transport proteins of human erythrocyte membranes migrate as broad bands of identical $M_r$ on SDS/polyacrylamide gels [32]. Because of their similarities in size, kinetics and function, it has been suggested that the two proteins may in fact be related [33]. Furthermore, both are present not only in protein-depleted erythrocyte membranes but also in the 'purified' glucose transporter preparation [13,34]. Western blotting and e.l.i.s.a. assays cannot therefore unambiguously establish to which species the antibodies bind. However, the two transporters do differ in that the glucose transporter specifically binds the inhibitor cytochalasin B, whereas the nucleoside transporter specifically binds the inhibitor NBMPR [13,34]. Assay of the binding of these two ligands was therefore used to quantify the ability of immobilized antibodies to remove each transporter from a detergent-solubilized mixture of the two proteins, as described in the Experimental section. As shown in Table 1, when an 8-fold molar excess of antibody over glucose transporter was used, antibodies raised against peptides 231–246, 240–255, 256–272, 450–467 and 460–477 removed $\geqslant 95\%$ of the cytochalasin B binding sites from solution, as compared with $\leqslant 17\%$ of the NBMPR binding sites. (Insufficient antibody against peptide 217–232 was available for this experiment.) Not only did these results demonstrate the specificity of the antibodies, but they

**Table 1. Immunoadsorption of solubilized glucose and nucleoside transporters**

Samples (52 $\mu$g) of purified glucose transporter in 1 ml of 50 mM-sodium phosphate/100 mM-NaCl/1 mM-EDTA, pH 7.4, were solubilized by addition of 1% *n*-octyl-$\beta$-D-glucoside, then incubated for 1 h at 4 °C with 200 $\mu$l of Protein A–Sepharose CL-4B to which 1.2 mg of the relevant non-immune or affinity-purified IgG was bound. The ligand-binding activities of the supernatants were then measured, after removal of detergent, as described previously [26]. Binding measurements were performed in triplicate and differed from the mean by < 10%.

| Immunoadsorbant | Ligand··· | Ligand-binding activity remaining in solution (%) | |
|---|---|---|---|
| | | Cytochalasin B | NBMPR |
| Non-immune rabbit IgG | | 100 | 100 |
| Anti-peptide 231–246 | | 0 | 83 |
| Anti-peptide 240–255 | | 0 | 93 |
| Anti-peptide 256–272 | | 5 | 91 |
| Anti-peptide 450–467 | | 4 | 95 |
| Anti-peptide 460–477 | | 4 | 89 |

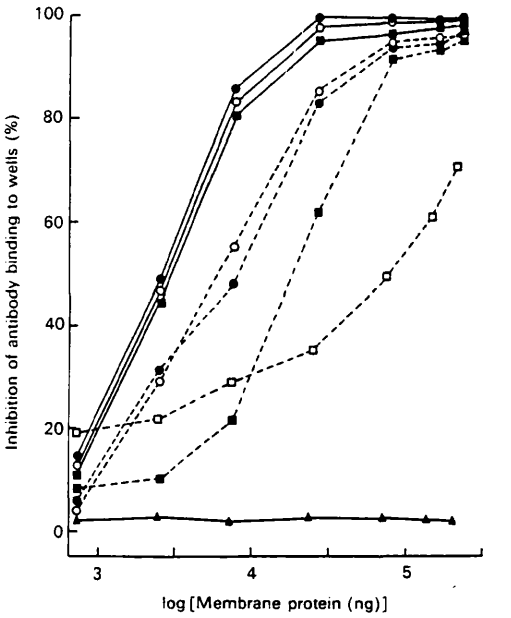


**Fig. 2. Establishment of the sidedness of antibody binding by competitive e.l.i.s.a.**

The ability of intact erythrocytes (▲) and unsealed erythrocyte membranes (○, ●, □, ■) to compete for a limiting amount of antibody with 600 ng of glucose transporter bound to the surface of a microtitre well was assessed. Samples (100 $\mu$l) of 1 $\mu$g/ml solutions of affinity-purified antibodies against peptides 217–232 (■– –■), 231–246 (●– –●), 240–255 (○– –○), 256–272 (□– –□), 450–467 (■—■), 460–477 (○—○) and 477–492 (●—●) were incubated with the amount of erythrocyte membrane protein indicated. The amount of free antibody remaining in the supernatant after centrifugation was then assessed by e.l.i.s.a. as described previously [26]. For incubations with unsealed erythrocyte membranes, each point is the mean of triplicate determinations. The values yielded by intact erythrocytes did not differ significantly for each antibody, and so are given as the mean for all seven antibodies.

also confirmed the ability of the antibodies to recognize the native protein, because only the latter is detectable in ligand-binding assays.

### Sidedness of anti-peptide antibody binding to erythrocyte membranes

In order to determine at which surface of the erythrocyte membrane their epitopes were exposed, the affinity-purified antibodies were tested in competitive e.l.i.s.a. experiments using unsealed erythrocyte membranes and intact erythrocytes as the competing antigen, as described previously for monoclonal antibodies [26]. Incubation of each of the six antibodies with protein-depleted membranes, which are unsealed [35], inhibited the subsequent binding of antibodies to the plate-bound transporter in an e.l.i.s.a. assay (Fig. 2). Under the experimental conditions used, essentially complete inhibition of the binding of 100 ng of anti-peptide antibodies against peptides 450–467 and 460–477 was produced by incubation with 25 $\mu$g of membranes. Almost identical results obtained with antibodies against peptide 477–492, which we described previously [18], are included in Fig. 2 for comparison. Almost complete inhibition ($> 90\%$) was also seen for antibodies against peptides 217–232, 231–246 and 240–255, although somewhat larger amounts of membranes were required to achieve this (Fig. 2). Inhibition of the binding of anti-(peptide 256–272) was substantial (up to $70\%$) but was not complete at the highest concentration of membranes used in the experiment.

In complete contrast with the results obtained with unsealed membranes, intact erythrocytes containing equivalent amounts of transporter had no effect on the binding of any of the antibodies to the plate-bound glucose transporter (Fig. 2). This finding indicates that the epitopes for all six antibodies are exposed at the cytoplasmic surface of the membrane. The observed recognition of the epitopes in unsealed membranes also provides additional confirmation of the ability of the antibodies to recognize the native membrane-bound conformation of the glucose transporter. The differences in the amounts of membranes required for inhibition of the different antibodies may reflect differences in antibody affinity.

### Effects of antibodies on cytochalasin B binding activity

The effect of the anti-peptide antibodies on the function of the glucose transporter was examined by investigating the effect of the six affinity-purified antibodies on cytochalasin B binding to protein-depleted erythrocyte membranes. The latter were used in preference to the purified transporter because they are completely unsealed [35]. Antibodies against peptides 450–467 and 460–477 had no effect on cytochalasin B binding (Fig. 3). However, antibodies to peptides 231–246 and 240–255 did inhibit the binding as measured using a fixed low concentration of cytochalasin B ($4 \times 10^{-8}$ M). A maximum inhibition of about $60\%$ was seen when membranes at a concentration of 0.25 mg of protein/ml were incubated with 0.2 mg of IgG/ml (Fig. 3). This concentration corresponds to about a 3-fold molar excess of antibody over glucose transporter. No inhibition was seen using a corresponding concentration of non-immune IgG (Fig. 3). The mechanism of the inhibition was examined further by measuring the effect of a maximally inhibitory concentration of IgG on cytochalasin B binding over a range

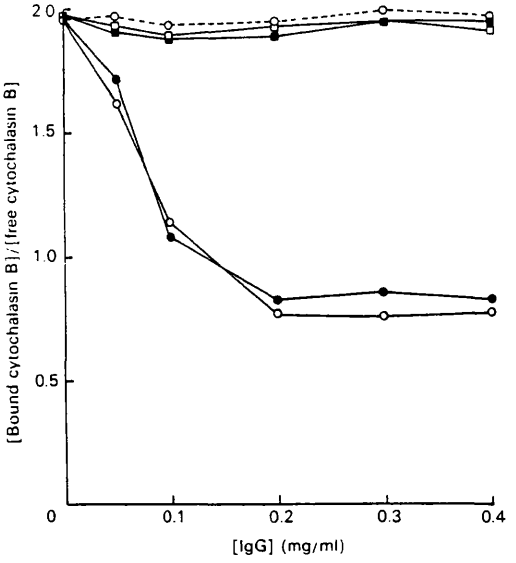


**Fig. 3. Inhibition of the cytochalasin B binding activity of protein-depleted erythrocyte membranes by anti-peptide antibodies**

Protein-depleted erythrocyte membranes at a concentration of 0.25 mg/ml in 10 mM-sodium phosphate/150 mM-NaCl, pH 7.2, were incubated with various concentrations of non-immune rabbit IgG (○--○), or affinity-purified antibodies against peptides 231–246 (●), 240–255 (○—○), 450–467 (□) and 460–477 (■). Cytochalasin B binding activity was then measured as described in the Experimental section. Each point is the mean of triplicate determinations.

of cytochalasin concentrations. The results are shown in the form of Scatchard plots in Fig. 4. Analysis of the results by the LIGAND procedure [36] showed that neither antibody had a significant effect on the concentration of cytochalasin B binding sites. Instead, antibody binding decreased the $K_a$ for cytochalasin B binding from $8.4 \pm 0.9\ \mu M^{-1}$ to $3.4 \pm 0.6\ \mu M^{-1}$ for antibody against peptide 231–246 and to $3.5 \pm 0.4\ \mu M^{-1}$ for antibody against peptide 240–255.

### Identification of the membrane-bound tryptic fragments of the glucose transporter

The availability of the anti-peptide antibodies allowed us to determine the sequence locations of the membrane-bound fragments produced by tryptic digestion of the glucose transporter more precisely than was previously possible. Tryptic digestion of the transporter yielded the same pattern of fragments as reported previously (results not shown), i.e. a glycosylated fragment of average apparent $M_r$ 35000, and a non-glycosylated fragment of apparent $M_r$ 18000 [18,21]. The $M_r$-18000 fragment was preceded at early times of digestion by precursors of apparent $M_r$ 25500, 23500 and 21000. The latter was formed in small amounts, and had not previously been noted by us [21].

In a previous study, we showed that the amino acid composition of the glycosylated fragment was consistent

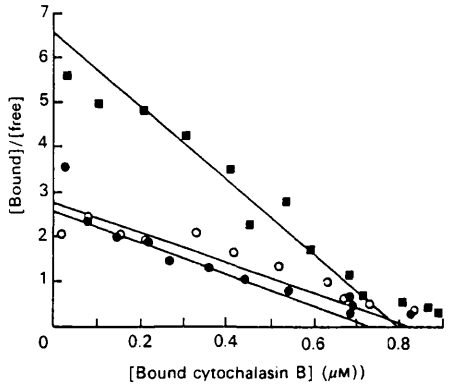


**Fig. 4. Scatchard plot analysis of cytochalasin B binding to protein-depleted erythrocyte membranes in the presence of control or anti-peptide antibodies**

Protein-depleted membranes (0.5 mg/ml in 10 mM-sodium phosphate/150 mM-NaCl, pH 7.2) were incubated with non-immune rabbit IgG (■, 0.4 mg/ml) or with affinity-purified IgG (0.4 mg/ml) against peptides 231–246 (●) and 240–255 (○). Cytochalasin B binding was measured over the concentration range $(0.5–75.5) \times 10^{-7}$ M, as described previously [13]. Each point is the mean of triplicate determinations. The straight lines are computerized best fits determined by the LIGAND procedure [36].

with its comprising residues 1–212 of the glucose transporter [17]. We also showed that it was labelled on Western blots by antibody against a peptide corresponding to residues 1–15 of the transporter [18]. In the present study the same result (not shown) was obtained with antibodies against sequences 34–60, 84–98, 144–158 and 175–189, confirming that this fragment contains the first five putative membrane-spanning helices of the transporter [4,17]. Because it is produced by tryptic cleavage at the cytoplasmic surface of the membrane, it is likely also to contain the sixth membrane-spanning sequence. However, it was not labelled on Western blots by antibodies against peptide 217–232, a finding consistent with its formation by tryptic cleavage at Arg-212 (results not shown).

Several distinct patterns were seen for the labelling of the non-glycosylated tryptic fragments by antibodies directed against the central and *C*-terminal hydrophilic regions of the transporter sequence. The results are summarized in Table 2. The $M_r$-25500 fragment, which is also labelled by antibodies against peptide 477–492 [18], was strongly labelled by antibodies against peptide 231–246, but weakly labelled, if at all, by antibodies against peptide 217–232. From the distribution of tryptic cleavage sites in the central cytoplasmic region of the transporter sequence, it is clear that this fragment must contain residues 233–492. It may contain some residues which are *N*-terminal to residue 233, but its *N*-terminus cannot precede Ala-224. Antibodies against peptide 217–232 did faintly stain an apparent precursor to the glycosylated fragment seen after short times of digestion (results not shown) and so it is likely that one or more of the cluster of five basic residues between Arg-223 and

**Table 2. Patterns of reactivity of anti-peptide and monoclonal antibodies with tryptic fragments of the glucose transporter on Western blots**

| | Labelling of tryptic fragment | | | |
|---|---|---|---|---|
| Antibody | $M_r$-25500 | $M_r$-23500 | $M_r$-21000 | $M_r$-18000 |
| Anti-peptides: | | | | |
| 217–232 | − | − | − | − |
| 231–246 | + | − | + | − |
| 240–255 | + | − | + | − |
| 256–272 | + | + | + | + |
| 450–467 | + | + | − | + |
| 460–477 | + | + | − | − |
| Monoclonals: | | | | |
| GTPR1 | + | − | + | − |
| GTPR2 | + | − | + | − |
| GTPR3 | + | − | + | − |
| GTPR4 | + | + | − | − |
| G1 | + | − | + | − |
| G2 | + | + | − | − |
| G3 | + | − | + | − |
| G4 | + | + | − | − |

Arg-232 represents a particularly sensitive site for tryptic cleavage of the transporter. The fragment of $M_r$ 23500, which is also labelled by antibodies against peptide 477–492 [18], was not labelled by antibodies against peptide 240–255, although it was labelled by those against peptide 256–272. From the distribution of potential tryptic cleavage sites in the central region of the transporter sequence, it must contain residues 265–492. It may contain residues *N*-terminal to residue 265, but its *N*-terminus cannot precede Glu-246. A likely candidate for its *N*-terminal residue is Val-257 (Fig. 1). The $M_r$-21000 fragment was not labelled by antibodies against peptide 450–467, unlike the $M_r$-18000 fragment. The latter is known to terminate at either Lys-456 or Arg-458 (see below), and so the $M_r$-21000 fragment must terminate either at Lys-456 or, more likely, at Lys-451. The fragment resembles the $M_r$-25500 fragment in being strongly labelled by antibodies against peptide 231–246 but not by those against peptide 217–232. It therefore probably shares the same *N*-terminus as the latter fragment, i.e. somewhere between residues 224 and 233. The $M_r$-18000 fragment was labelled by antibodies against peptide 450–467, but not by those against peptide 460–477. In conjunction with our previous finding [17] that a peptide corresponding to residues 459–468 is released from the membrane upon prolonged digestion with trypsin, this observation indicates that the $M_r$-18000 fragment must terminate at Lys-456 or Arg-458. In addition, labelling of this fragment with antibodies against peptide 256–272 suggests that (a component of) the fragment contains residues *N*-terminal to Gln-270. The latter was suggested to be the likely *N*-terminus of the fragment from a determination of its amino acid composition by Cairns *et al.* [17]. However, fragments containing an additional five or even 13 residues at the *N*-terminus, and which might therefore be recognized by antibodies against peptide 256–272, might not be resolved from a fragment comprising residues 270–456/458 on SDS/polyacrylamide gels.

### Location of epitopes for monoclonal antibodies against the glucose transporter

We have previously reported the sidedness of binding to erythrocyte membranes of a number of different monoclonal antibodies raised against the glucose transporter [25,26]. All bound to the cytoplasmic face of the erythrocyte membrane. The two additional antibodies used in the present study, GTPR3 and GTPR4, also bound to the cytoplasmic face of the membrane (J. M. Boyle & K. Meeran, unpublished work). In order to locate the epitopes for all of these antibodies, we investigated their ability to recognize tryptic fragments of the glucose transporter on Western blots. The results are summarized in Table 2. Five of the antibodies (GTPR1, GTPR2, GTPR3, G1 and G3) labelled the fragments of $M_r$ 25500 and 21000 but not those of $M_r$ 23500 or 18000. This pattern of labelling was identical to that found for antibodies against peptides 231–246 and 240–255 (Table 2). Failure to recognize the $M_r$-23500 fragment, which contains the intact *C*-terminus of the transporter, indicates that the epitopes for these antibodies cannot be within the *C*-terminal hydrophilic region of the transporter sequence. Lack of labelling of the $M_r$-18000 fragment, whose *N*-terminus is either Gln-270 or precedes this residue in the sequence, indicates that the epitopes must lie within the central hydrophilic region of the transporter sequence, probably between Ala-224 and Arg-269. In contrast, monoclonal antibodies GTPR4, G2 and G4 labelled only those fragments, of $M_r$-25500 and -23500, which contained the intact *C*-terminus of the transporter (Table 2). Lack of labelling of the $M_r$-21000 fragment indicates that the epitopes do not lie within the central hydrophilic region of the transporter sequence: the $M_r$-21000 and -25500 fragments probably share the same *N*-terminal sequence which includes much of this region (see above). In conjunction with the lack of labelling of the $M_r$-18000 fragment, these findings indicate that the epitopes recognized by antibodies GTPR4, G2 and G4 lie within the *C*-terminal region of the transporter sequence, between residues 459 and 492.

A more precise location for the epitopes recognized by the monoclonal antibodies was sought by investigating their ability to recognize synthetic peptides in e.l.i.s.a. assays. The peptides tested corresponded in sequence to residues 217–232, 231–246, 240–255 and 256–272 in the central hydrophilic region of the transporter and to residues 450–467, 460–477 and 477–492 in the *C*-terminal hydrophilic region of the transporter. Monoclonal antibodies GTPR1, GTPR2, GTPR3, G1 and G3 recognized only one plate-bound peptide, that corresponding to residues 231–246 of the transporter sequence (Fig. 5*a*). In addition, they did not recognize a synthetic peptide corresponding to residues 231–246 of the rat brain glucose transporter in e.l.i.s.a. (results not shown). This peptide differs in sequence from that of the human only by replacement of His-239 with an arginine, and so it is likely that this residue forms part of, or is very close to, the epitope. The human peptide, but not the rat peptide, also competed with plate-bound glucose transporter for monoclonal antibody binding in a competitive e.l.i.s.a. assay (Fig. 6*a*). However, in order to inhibit binding by 50%, a 200–300-fold molar excess of peptide over glucose transporter was required, suggesting that the antibodies have a much greater affinity for the intact transporter than for corresponding short segments of the polypeptide. This finding may reflect the different conformations of these molecules and/or the likelihood that additional residues make up the complete epitope in the folded protein.

Monoclonal antibodies G2 and G4 also recognized only one plate-bound peptide, that corresponding to residues 477–492 of the glucose transporter (Fig. 5*b*).

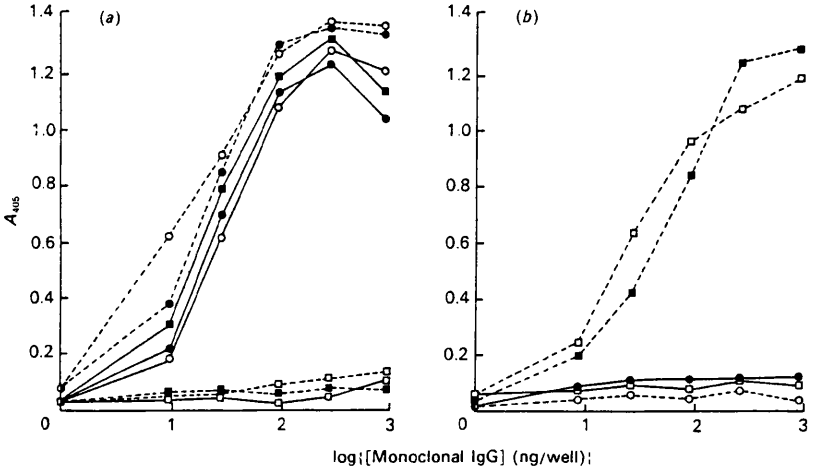


**Fig. 5. Ability of anti-(glucose transporter) monoclonal antibodies to recognize plate-bound synthetic peptides in e.l.i.s.a. assays**

Microtitre plates were coated with 20 ng of synthetic peptides/well, corresponding to residues 231–246 (*a*) or residues 477–492 (*b*) of the glucose transporter. Plates were then incubated with the amounts of monoclonal antibody shown, followed by alkaline-phosphatase-linked second antibody and *p*-nitrophenyl phosphate as chromogenic substrate. Each point is the mean of triplicate measurements. Monoclonal antibodies used were GTPR1 (●—●), GTPR2 (○—○), GTPR3 (■—■), GTPR4 (□—□), G1 (○- -○), G2 (■- -■), G3 (●- -●) and G4 (□- -□).

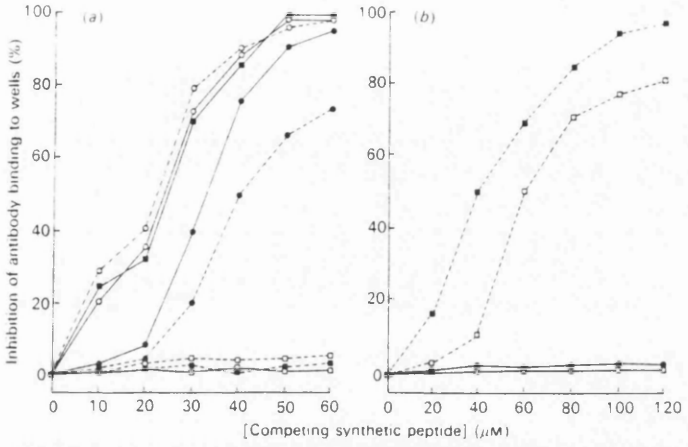


**Fig. 6. Competitive e.l.i.s.a. assay of the ability of solution-phase peptides to bind monoclonal antibodies**

The ability of synthetic peptides corresponding to residues 231–246 (*a*) and residues 477–492 (*b*) to compete for a limiting amount of monoclonal antibody with 600 ng of glucose transporter bound to the surface of a microtitre well was measured. Samples (100 μl) of the monoclonal antibodies (3 μg/ml) were incubated for 2 h at 25 °C with the concentrations of peptides indicated. The amount of free antibody was then measured by e.l.i.s.a., as described in the Experimental section. Monoclonal antibodies used were GTPR1 (●—●), GTPR2 (○—○), GTPR3 (■—■), GTPR4 (□—□), G1 (○- -○), G2 (■- -■), G3 (●- -●) and G4 (□- -□).

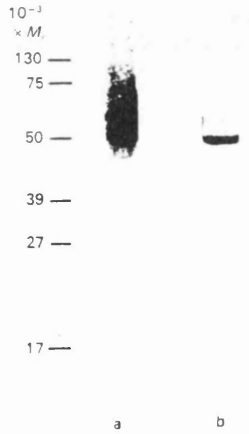


**Fig. 7. Cross-reaction of anti-(human glucose transporter) monoclonal antibody with rat brain glucose transporter**

Samples of purified human erythrocyte glucose transporter (*a*) and rat brain membranes (*b*) were electrophoresed on an SDS/12% polyacrylamide gel, transferred electrophoretically to nitrocellulose paper and then immunologically stained with monoclonal antibody GTPR4 as described in the Experimental section. The positions of $M_r$ markers are indicated.

This peptide also competed with plate-bound transporter for antibody binding in a competitive e.l.i.s.a. assay (Fig. 6*b*), indicating that the epitope(s) for these antibodies is located within the *C*-terminal 16 residues of the protein. In contrast, monoclonal antibody GTPR4 did not recognize any plate-bound peptide. The epitope for this antibody had been predicted to be located within the *C*-terminal hydrophilic region of the transporter from the pattern of labelling of membrane-bound tryptic fragments (see above). Since it was lost upon extensive tryptic cleavage of the protein, it must either lie near a cleavage site or be located upon a small water-soluble peptide liberated from the *C*-terminal region upon digestion. Failure of the antibody to recognize synthetic peptides 450–467, 460–477 or 477–492 indicates that the epitope is not located wholly within the peptides 459–468, 469–477 or 478–492 which are liberated by tryptic digestion of the transporter [17]. Lack of recognition of synthetic peptide 460–477 would not be expected if the epitope were destroyed by cleavage at Arg-467. Similarly, lack of recognition of synthetic peptide 450–467 would not be expected if the epitope were destroyed by cleavage at Lys-456 or Arg-458. It follows that the epitope is probably located close to the only other tryptic cleavage site in the *C*-terminal region of the transporter, Lys-477, although further studies will be required to confirm this.

Additional support for the proposed locations of epitopes was provided by examining the ability of the antibodies to recognize the rat brain glucose transporter on Western blots. Monoclonal antibodies GTPR1, GTPR2, GTPR3, G1 and G3, all of which recognize the human erythrocyte glucose transporter on Western blots, did not label any protein bands on Western blots of rat brain membranes (results not shown). This species specificity is explicable if the epitope contains, or is close to, His-239 in the human sequence, because this is replaced by arginine in the rat [4,5]. In contrast, monoclonal antibodies GTPR4, G2 and G4 did label a sharp band of apparent $M_r$ 50000 on Western blots of rat brain membranes (illustrated for GTPR4 in Fig. 7). Other workers have identified the rat brain glucose transporter as a sharp band on Western blots stained with polyclonal antibodies against the human erythrocyte

glucose transporter [30]. The sharpness of the labelled band in comparison with the diffuse nature of that seen for the human erythrocyte glucose transporter (Fig. 7) probably stems from a lesser degree of heterogeneity in glycosylation of the brain protein. The finding in the present study that monoclonal antibodies GTPR4, G2 and G4 recognized the rat brain transporter is consistent with the proposed locations of the epitopes for these antibodies within the hydrophilic *C*-terminal region of the transporter: the amino acid sequence of this region is identical in rat brain and human erythrocyte glucose transporters [4,5].

## DISCUSSION

Antibodies against synthetic peptides corresponding to residues 217–232, 231–246, 240–255, 256–272, 450–467 and 460–477 were all found to recognize the native glucose transporter and to bind at the cytoplasmic face of the erythrocyte membrane. Our previous study reported identical results for an antibody against residues 477–492 [18]. These findings are consistent with the prediction of our model [4] that the central hydrophilic region of the transporter sequence, encompassing residues 207 to 271, and the *C*-terminal region of the sequence from residues 451 to 492 are located at the cytoplasmic side of the erythrocyte membrane. The cytoplasmic epitopes for a number of monoclonal antibodies, which we were able to locate in the present study, also all lay within these two regions. In a similar study by Andersson & Lundahl [37], another monoclonal antibody that bound to the *C*-terminal peptide (residues 478–492) of the transporter also bound to the cytoplasmic surface of the membrane. The lack of binding to the native protein of any of the antibodies raised against putative extracellular regions of the transporter, against the predicted short loops connecting transmembrane sequences at the cytoplasmic surface of the membrane, and against the *N*-terminal sequence [18] may have several explanations. In the case of the extracellular regions, it may stem from the fact that there are very few sequence differences between these regions in the rabbit and human glucose transporters [38]. In addition, the prediction of the model that certain regions of the sequence lie outside the membrane may be incorrect or they may be constrained in conformations too different from those available to the peptide immunogens. Studies on water-soluble proteins have found that anti-peptide antibodies against highly mobile regions tend to react strongly with the native protein, whereas anti-peptide antibodies against well-ordered regions do not [39]. Alternatively, the short loops may be too closely associated with the phospholipid head groups or with other parts of the protein to allow antibody access. Similar findings have been reported for the lactose transporter of *Escherichia coli* [29,40].

In previous studies, we have shown that removal of the central hydrophilic and *C*-terminal regions of the transporter by tryptic digestion destroys the ability of the protein to transport hexose, and lowers its affinity for cytochalasin B [20,21]. However, it was not possible to examine the roles of each of these regions of the sequence in transporter function independently. This has now become possible using anti-peptide antibodies. None of the anti-peptide antibodies which bound to the *C*-terminal region of the protein had any effect on cytochalasin B binding, suggesting that this region is not intimately involved in this function. In contrast, antibodies against peptides 231–246 and 240–255 in the central cytoplasmic region of the transporter did inhibit cytochalasin B binding. It is therefore likely that the central cytoplasmic region of the protein is important for transporter function.

The effects of the anti-peptide antibodies on transporter function were largely paralleled by the effects of monoclonal antibodies which bound to the same regions of the sequence. Thus monoclonal antibody GTPR4, which appears to bind to the *C*-terminal region of the transporter, had no effect on the cytochalasin B binding activity of the protein (A. Davies, unpublished work). Monoclonal antibody G2, which binds to the *C*-terminal peptide comprising residues 477–492, had, if anything, a slight stimulatory effect on binding [25]. However, antibody G4, which also binds to the *C*-terminus, inhibited binding [25]. Such differences between the effects of monoclonal antibodies which appear to bind to the same region of the transporter sequence may of course stem from their epitopes being partially discontinuous and so involving more than one region of the transporter sequence. Monoclonal antibodies GTPR1, GTPR2, GTPR3, G1 and G3, all of which bound to the central hydrophilic region of the transporter sequence, also inhibited cytochalasin B binding ([25,26]; for GTPR3, K. Meeran & S. A. Baldwin, unpublished work).

The site-specific polyclonal and monoclonal antibodies characterized in the present study have enabled direct evidence to be obtained for several features of the transporter structure. They have also enabled us to begin to dissect out the function of different regions of the protein. We envisage that they will be powerful tools for the future investigation of the structure, function and regulation of mammalian sugar transport proteins.

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Table 2. *Inhibition of $Na^+$-dependent uridine transport in rat renal brush-border membrane vesicles by various compounds*

The uptake of uridine (2 s) was initiated by addition of brush-border membrane vesicles to medium containing (final concentrations) 5 μM-[$^3$H]uridine, 100 mM-$NaNO_3$ and test compound. For nitrobenzylthioinosine and dipyridamole, vesicles were preincubated with these inhibitors before addition of [$^3$H]uridine. Values are taken from [14].

| Inhibitors | Apparent $K_i$ (μM) |
|---|---|
| Thymidine | 5 ± 1 |
| 2′-Deoxyuridine | 8 ± 1 |
| Adenosine | 4 ± 0.5 |
| 2′-Deoxyadenosine | 3.5 ± 0.1 |
| 2-Chloroadenosine | 7 ± 1 |
| Inosine | 300 ± 44 |
| 2′-Deoxyinosine | 320 ± 30 |
| Guanosine | 90 ± 3 |
| 2′-Deoxyguanosine | 110 ± 6 |
| Uracil | a |
| D-Glucose | a |
| Nitrobenzylthioinosine | b |
| Dipyridamole | b |

[a] No inhibition at 1 mM.
[b] No inhibition at 30 μM.

midine nucleoside- and adenosine-specific transport system in rat renal proximal tubules. Preliminary results using rabbit and bovine vesicles suggest a similar inhibition pattern ([17]; T. C. Williams & S. M. Jarvis, unpublished work). However, it is interesting to note that the $K_i$ values for inosine and guanosine as inhibitors of $Na^+$-dependent uridine transport (300 and 90 μM) were significantly different from the $K_m$ values for influx (2 and 3.5 μM, respectively) [18]. Moreover, the $K_i$ values for inosine and guanosine as inhibitors of $Na^+$-dependent adenosine transport by rat renal brush-border membrane vesicles were similar to their $K_m$ values [18]. These studies would suggest the existence of another active $Na^+$-co-transporter for nucleosides with a high affinity for purine nucleosides. Few studies have been performed on the detailed kinetics of this second active nucleoside carrier.

In conclusion, the renal proximal tubule reabsorbs filtered nucleosides by $Na^+$-dependent systems. The kinetics of one of these systems have been investigated using uridine. Uridine is taken up by a high-affinity saturable route with a defined specificity that differs from the broad specificity of the facilitated diffusion nucleoside systems. The $Na^+$:uridine coupling stoichiometry is 1:1. In addition, rat renal brush-border membrane vesicles also appear to possess a $K^+$-dependent uridine transporter, the physiological significance of which is unknown.

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## Identification of glucose transporters in plasma membrane and Golgi vesicle fractions prepared from lactating rat mammary gland

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The importance of glucose transport as the rate-limiting step in rodent milk production has been well documented (Threadgold & Kuhn, 1984; Faulkner & Peaker, 1987), and a facilitated diffusion system for glucose in mouse mammary plasma membranes has been described (Prosser, 1988). However, there is controversy about the mechanism by which glucose, required for lactose synthesis, crosses the membranes of Golgi vesicles. Some studies have yielded evidence for the presence here of a non-stereospecific pore, rather than a transporter (White *et al.*, 1980). In the present study we sought to clarify this situation by identifying and determining the subcellular distribution of glucose transporters in the lactating rat mammary epithelial cell.

Mammary glands were removed from day-10 lactating rats and plasma membrane- and Golgi vesicle-enriched fractions isolated as described previously (Clegg, 1981; West, 1981). Human erythrocyte membranes were prepared by hypotonic lysis. Affinity-purified polyclonal antibodies against the *C*-terminus (residues 477–492) of the human erythrocyte glucose transporter were as previously described (Davies *et al.*, 1987).

The concentrations of glucose transporters in membrane fractions were determined using a centrifugal cytochalasin B-binding assay. Scatchard analysis of D-glucose-inhibitable

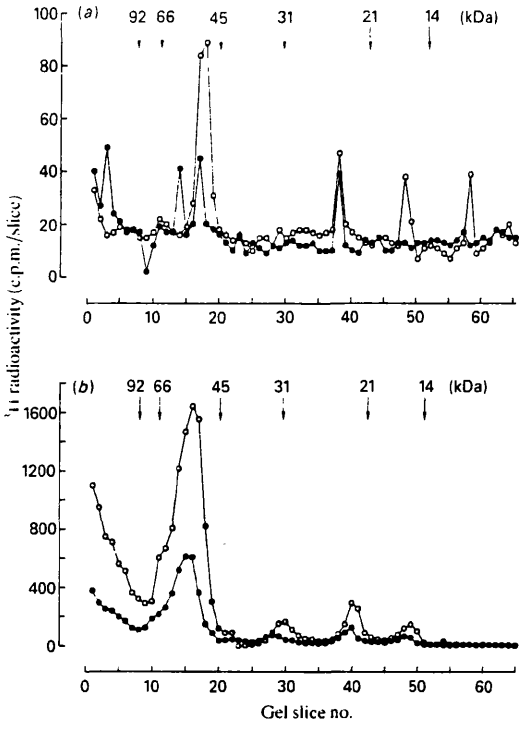


Fig. 1. *Electrophoretic profiles of photoaffinity-labelled glucose transporters immunoprecipitated from rat mammary (a) and human erythrocyte (b) plasma membranes*

Membranes labelled with [$^3$H]cytochalasin B in the presence of 500 mM-L-glucose (○) or 500 mM-D-glucose (●) were solubilized, incubated with Sepharose-bound anti-(glucose transporter) antibodies, and the adsorbed proteins electrophoresed on an SDS/12% (w/v) polyacrylamide gel. The radioactivity of 2 mm gel slices was determined by liquid scintillation counting. Arrows indicate the positions of $M_r$ markers.

binding revealed that cytochalasin B bound to a maximum of 19, 55 and 657 pmol sites/mg protein, with $K_d$ values of 2.6, 5.4 and $3.3 \times 10^{-7}$ M, in rat mammary plasma membranes, Golgi membranes and human erythrocyte membranes, respectively.

The anti-transporter antibodies labelled a sharp band of apparent $M_r$ 50000 on Western blots of rat plasma and Golgi vesicle membranes, compared to a broad band of average apparent $M_r$ 55000 for the human erythrocyte membranes. No staining was seen with non-immune IgG. Confirmation that the band recognized by the antibodies was a glucose transporter was provided by photoaffinity-labelling experiments. Membranes were photolabelled with [$^3$H]cytochalasin B in the presence of either 500 mM-D- or -L-glucose, by the procedure of Kasanicki *et al.* (1987). Photolabelled membranes were solubilized in a mixture of SDS and Triton X-100, before incubation with Sepharose-bound anti-transporter antibodies or control IgG. After extensive washing, polypeptides bound to the immunoadsorbant were released by treatment with urea/SDS and then electrophoresed on SDS/12% (w/v) polyacrylamide gels (Laemmli, 1970), which were subsequently sliced and subjected to liquid scintillation counting. A major peak of D-glucose-inhibitable labelling, of apparent $M_r$ 50000, was detected for both the plasma membranes (Fig. 1) and the Golgi vesicles (not shown), immunoprecipitated with anti-transporter antibodies. No peak of labelling was seen for the control immunoprecipitate. A similar pattern was seen for human erythrocyte membranes, except that the peak of labelling was broader, and of average apparent $M_r$ 55000 (Fig. 1).

These data indicate that glucose transporters homologous to the human erythrocyte protein are present in both the plasma membrane and Golgi vesicle fractions of lactating rat mammary epithelial cells. The sharpness of the bands seen upon Western blotting and photoaffinity labelling suggests that the mammary proteins may be less heterogeneously glycosylated than the erythrocyte transporter. The presence of transporters in the Golgi vesicle fraction is intriguing, given the results of studies which indicate the presence of a non-stereospecific monosaccharide channel in Golgi membranes (White *et al.*, 1980). These transporters cannot be accounted for by contamination with plasma membranes, because 5'-nucleotidase assays revealed that the Golgi vesicles carried only 10% contamination with the latter, while exhibiting a 20-fold enrichment with galactosyl transferase. In addition, the transporters appeared to bind cytochalasin B with lower affinity than those in the plasma membrane fraction. However, it remains to be demonstrated whether the transporters in the Golgi fraction are actually present on those vesicles involved in lactose synthesis.

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