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A BIOCHEMICAL AND BIOPHYSICAL INVESTIGATION INTO THE STRUCTURE AND
FUNCTION OF THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORT PROTEIN

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ABSTRACT

A biochemical and biophysical investigation into the structure and function of the human erythrocyte glucose transport protein
by Angela F. Davies

At the onset of this study, much evidence existed for the occurrence of conformational changes in the human erythrocyte glucose transporter, but the regions of the protein affected by these changes were largely unknown. By investigation of the effects of the physiological substrate D-glucose and four inhibitors of glucose transport on the rate of cleavage of the transport protein by trypsin, two hydrophilic cytoplasmically-exposed regions of the polypeptide were implicated in a transport-related conformational change. Further evidence for the occurrence of a conformational change in the transporter in the presence of one of the transport-inhibitors was also obtained as a result of an investigation by X-ray solution scattering techniques.

In order to perform these X-ray solution scattering experiments, it was first necessary to solubilize the transporter in its native form in monodisperse solution. Maintenance of the solubilized transporter in a stable, native form will also be a pre-requisite for future X-ray crystallographic work. The transporter was known to be very unstable when solubilized in octyl glucoside at low temperatures (8°C) (Baldwin et al., 1982). Therefore a range of detergents were investigated for their suitability for solubilization of the glucose transporter. The non-ionic detergent octyl thioglucoside was found to be more efficient than octyl glucoside for solubilization and was also capable of selective solubilization of the glucose transporter as opposed to the nucleoside transporter. Glucose transporter solubilized in either detergent was found to be highly stable if kept at 0°C, more than 75% of the cytochalasin B-binding activity remaining after 50 hours. However, increasing the temperature of the incubation to 8°C dramatically reduced the stability of the octyl glucoside-solubilized material, only 3% of the cytochalasin B-binding activity remaining after 18 hours. As a result of this work, conditions were found which allowed the study by X-ray solution scattering to be carried out.

Identification of the regions of the amino-acid sequence involved in the cytoplasmic and extracellular sugar-binding sites of the glucose transporter would be an important advance in our understanding of the transport mechanism. In this study the glucose transporter was covalently radiolabelled at either the cytoplasmic- or extracellular-surface with \[^3H\]cytochalasin B or \[^3H\]2-N-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)-2-propylamine (\[^3H\]ATB-BMPA) respectively. Covalent labelling by each of these transport inhibitors is itself inhibitable by D-glucose, and therefore may involve covalent interactions in the vicinity of the sugar binding-sites at the cytoplasmic and extracellular surfaces respectively. However one must not dismiss the possibility that the D-glucose- and inhibitor-binding sites are linked allosterically. The radiolabelled protein was then cleaved by a number of enzymatic and chemical means in order to produce a range of fragments, which were identified by the use of antibodies raised against synthetic peptides corresponding to regions of the glucose transporter sequence (Davies et al., 1987, 1990). This resulted in the identification of two regions of the protein which are likely to be involved in the two sugar-binding sites.
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ABBREVIATIONS

ASA-EMPA [2-N-(4-azidosalicoyl)-1-3-bis(D-mannos-4'-yloxy)-2-propylamine]
ATB-EMPA 2-N-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)-2-propylamine
BSA Bovine serum albumin
CHAPS 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate
CHAPSO 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulphonate
CMC Critical micellar concentration
CMT Critical micellar temperature
cpm Counts per minute
DEAE- Deoxy Big
Diethylaminoethyl-
Deoxy Big
CHAP [N,N-bis-(3-D-glucanomidopropyl)-deoxycholamide]
dpm Disintegrations per minute
DTT Dithiothreitol
EDTA Ethylenediaminetetra-acetic acid
FDNB 1-fluoro-2,4-dinitrobenzene
GTS/OG/PL Glucose transporter/octyl glucoside/phospholipid mixed micelles
Mr Relative molecular mass
MEGA-10 Decanoyl-N-methylglucamide
NBS N-bromosuccinimide
NBMPR Nitrobenzylthionosine
NBTGR Nitrobenzylthioguanosine
OG/PL Octyl glucoside/phospholipid mixed micelles
5PS 5 mM-sodium phosphate, pH8
PBS Phosphate-buffered saline
PMSF Phenylmethylsulphonyl fluoride
p.s.i. Pounds per square inch
rpm Revolutions per minute
SDS Sodium dodecyl sulphate
TEMED N,N',N'-tetramethylethylenediamine
TBS Tris-buffered saline
TTBS Tris-buffered saline containing 0.02% (v/v) Tween-20
Tris Tris(hydroxymethyl)aminoethane
CHAPTER 1  INTRODUCTION

1.1 INTRODUCTION

Glucose is by far the most abundant of all the molecules of biological origin. The bulk of glucose in trees and vegetation is in the form of cellulose, a polymerised form of glucose which cannot be broken down by the digestive enzymes of the human intestinal tract. However, the other polymers of glucose (starches in plants and glycogen in animals) can be digested. Therefore for animals which derive their energy from that stored in plants it is not surprising that glucose should play a key role in their metabolism. Carbohydrates account for about 60% of the energy intake of a normal human diet, the remaining 40% being derived from fats and proteins. Some tissues such as the brain metabolize glucose almost exclusively (Elbrink & Bihler, 1974) whereas others have a preference for non-carbohydrate sources such as fatty acids and ketone bodies in the case of muscle (Andres et al., 1956).

Animal cells are surrounded by a thin layer of protein and lipid molecules that constitute the plasma membrane. The structure and composition of this membrane is governed by the metabolic activity of the particular cell. The lipid, in the form of a bilayer, presents a hydrophobic barrier to the entry and exit of hydrophilic substances, and it has been known for many years that simple diffusion could not account for the rapid movement of many such molecules into cells. For example, glucose was found to pass across artificial lipid bilayers with a permeability coefficient of $10^{-9}$-$10^{-10}$ cm/sec compared with that of $10^{-4}$ cm/sec for transport across the human erythrocyte membrane (Jung 1971, a,b). The conclusion drawn from such findings was that some components of biological membranes are responsible for the
transport of hydrophilic substances across the membrane; a concept
described as carrier-mediated transport. The discovery that
sulfhydryl-reagents could inhibit the transport of glucose across the
erthrocyte membrane led to the identification of the component
responsible as a protein (Smith & Ellman, 1973).

Most mammalian cells take up glucose by the process of passive
facilitated diffusion, a mediated transport process which requires no
energy expenditure. The transport proteins responsible for this vary
from tissue to tissue in both their kinetic and regulatory properties
(Simpson & Cushman, 1986). However, the recent cloning of the passive
glucose 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 yeast and
bacteria (see section 1.8). (Mueckler et al., 1985; Birnbaum et al.,
1986; Maiden et al., 1987; Celenza et al., 1988; Fukomoto et al.,
1988; Thorens et al., 1988; Birnbaum, 1989; James et al., 1989; Gould
& Bell, 1990).

1.2 GLUCOSE AND THE HUMAN ERYTHROCYTE

The glucose transporter of human erythrocytes is the best
characterized of all the facilitated diffusion systems. One reason for
this is that in these cells the capacity for glucose transport far
exceeds the rate of glucose metabolism and consequently glucose
rapidly equilibrates across the cell membrane. Most mammals in the
foetal stage possess erythrocytes with a high capacity glucose
transport system but this capacity decreases in all adult mammals
except the primates (Widdas, 1955). Secondly, the absence of organelles
means that most of the cell's internal volume is available for
equilibration with sugars, making these cells ideal for kinetic
studies of transport.

Once inside the erythrocyte glucose is converted to glucose 6-phosphate which is itself not transported across the membrane. Thus glucose can continue passively to enter the cell down its concentration gradient. Glucose metabolism is via the glycolytic pathway and the pentose phosphate pathway, which account for 90% and 10% of erythrocyte glucose utilisation respectively. The energy thus derived is utilized in maintaining the reduced state of protein thiol groups and the Fe$^{2+}$ ion in haemoglobin, in maintaining the structural integrity of the membrane by preventing fatty acid oxidation, and in maintaining various ionic concentration gradients across the plasma membrane.

The glucose transport protein accounts for 5% of the erythrocyte membrane protein by weight, and can be purified to near homogeneity in large quantities (Baldwin et al., 1982; Cairns et al., 1984). This purified material retains its ability to transport glucose with kinetics similar to those in the intact erythrocyte (Wheeler & Hinkle, 1981). These characteristics of the human erythrocyte and its glucose transporter have made it a convenient system for the study of both the kinetic and structural aspects of this protein.

### 1.3 MODELS FOR ERYTHROCYTE GLUCOSE TRANSPORT

#### 1.3.1 The kinetics of glucose transport

Several features indicate that glucose is transported across the erythrocyte membrane by a carrier component of the membrane. For example the transport mechanism is stereospecific, the transporter having a much higher affinity for D-glucose ($K_m$ 1.6mM) than L-glucose ($K_m$ 3M) (LeFevre, 1961). In addition, transport exhibits saturation kinetics and is competitively inhibited both by other sugars and sugar

Many kinetic studies have shown that glucose transport in the erythrocyte is markedly asymmetric, the $K_m$ and $V_{max}$ values for zero trans efflux (exit of glucose into sugar-free medium) being substantially greater than the corresponding values for influx, particularly at lower temperatures (the efflux values are approximately 12-fold greater than influx values at 0°C) (Lowe & Walmsley, 1986). In addition the $K_m$ and $V_{max}$ values measured under equilibrium exchange conditions (measurement of the flux of tracer, isotopically-labelled glucose when the concentration of unlabelled glucose is kept equal on both sides of the membrane) are even larger than those for zero trans efflux, again particularly at low temperature (the equilibrium exchange values are approximately 8-fold higher than than zero-trans efflux values at 0°C (Lowe & Walmsley, 1986). The first kinetic model that could account for these findings was the simple asymmetric carrier model outlined in Figure 1.1. This model, supported by several workers including Geck (1971) and Eilam & Stein (1974), stemmed from the earlier symmetric carrier model of Widdas (1952). Although the asymmetric carrier model originally envisaged a carrier protein moving from one side of the membrane to the other, acting as a 'ferry' for glucose (Regan & Morgan, 1964), identical kinetics would result from a model such as that proposed by Vidaver (1966), where a sugar-binding site is alternately exposed, via a conformational change, at the two sides of the membrane, binding glucose at one side of the membrane and releasing it at the other.

In the model, the asymmetry of transport arises from differences in the dissociation constants for sugar-binding at the two faces of the membrane, and from differences in the rate constants governing re-
Figure 1.1 Simple asymmetric carrier model for sugar transport.

$G_o$ and $G_i$ represent glucose outside and inside the cell and $T_o$ and $T_i$ represent the outward- and inward-facing conformations of the transporter respectively. (From Wheeler & Hinkle, 1985).
orientation of the loaded and unloaded sites. The explanation for the greater \( V_{\text{max}} \) observed under equilibrium exchange rather than zero-trans conditions would be that the rate constants governing re-orientation of the loaded site are greater than those for the re-orientation of the empty site (the rate-limiting step in zero-trans flux would be re-orientation of the empty site).

Despite the success of the asymmetric carrier model in explaining the asymmetry of transport and the phenomenon of trans-stimulation (that is the fact that \( V_{\text{max}} \) for equilibrium exchange is greater than that for net flux), many workers have rejected it on the basis of two lines of experimental evidence. The first of these was the evidence that the ratio of \( V_{\text{max}}/K_m \) differs for net influx, efflux and equilibrium exchange. Such a finding is inconsistent with the model. However, Krupka (1989) has shown that for any transport system which obeys Michaelis-Menten kinetics, and in which the equilibrium ratio of intracellular : extracellular substrate is 1, the ratio of \( V_{\text{max}}/K_m \) must be identical in the three types of kinetic experiment mentioned above. It follows not only that the reported experimental data cannot be used to reject the model, but that they must contain errors. A second observation that has been claimed to be inconsistent with the model is the finding of a low \( K_m \) value for the infinite-cis uptake of glucose by the erythrocyte (in infinite-cis experiments, the net flux of glucose from a limitingly high concentration on the cis face of the membrane is measured as a function of the concentration on the trans face) (Hankin et al., 1972). However these studies of infinite-cis kinetics used a time-course procedure for rate estimations. A more recent study in which initial rate measurements were used (Wheeler & Whelan, 1988) reported higher \( K_m \) values consistent with the simple asymmetric carrier model.

Despite this new evidence, controversy about the mechanism of
transport remains. In contrast to the simple model described above, in which a single sugar-binding site exists, a recent model proposed on the basis of fluorescence quenching (Carruthers, 1986 a,b) and ligand binding studies (Helgerson & Carruthers, 1987) suggests that two substrate-binding sites are simultaneously present, one exposed at each face of the membrane. It is this model and the simple alternating conformation model (Vidaver, 1966; Barnett et al., 1975; Baldwin & Lienhard, 1981) that are presently receiving the most interest. However there is still much dispute over which if either of these models is capable of explaining all of the experimental features of glucose transport (Barnett et al., 1975; Appleman & Lienhard, 1985; Lowe & Walmsley, 1986; Lowe & Walmsley, 1987; Helgerson & Carruthers, 1987; Naftalin, 1988; Wheeler & Whelan, 1988; Appleman & Lienhard, 1989).

1.3.2 Evidence for conformational changes in the glucose transporter

Although the single-site and two-site models are different in many respects, both imply that conformational changes in the protein are central to the transport process. The occurrence of such conformational changes has in fact been detected by a large variety of techniques: The first piece of evidence that the binding of D-glucose to the transporter induced a change in the conformation of the protein came from experiments with the irreversible inhibitor of glucose transport 1-fluoro-2,4-dinitrobenzene (FDNB). The rate of inactivation by this inhibitor was increased in the presence of D-glucose, 2-deoxyglucose and mannose (all readily transported by the glucose transporter) suggesting a conformational change in the protein upon interaction with the sugar which resulted in the exposure of the reactive groups of the protein to the reagent (Bowyer & Widdas, 1958). Similar results were also observed with another inhibitor of transport
N-ethylmaleimide (NEM) (Dawson & Widdas, 1963; Bloch, 1974).

The discovery that the binding of certain ligands to the glucose transporter (e.g. D-glucose, 4,6-O-ethylidene-D-glucose and cytochalasin B) elicited a quenching effect on the intrinsic fluorescence of the protein (Gorga & Lienhard, 1982) together with the use of stopped flow techniques led to the conclusion that during the binding of ethylidene glucose to the transporter, the protein undergoes a change in conformation. This change is such that the substrate binding site is exposed at the extracellular side of the membrane and a residue responsible for the intrinsic fluorescence is quenched probably due to a change in the environment surrounding the residue (Appleman & Lienhard, 1985 and 1989). Irreversible inactivation of the transporter by protein-modifying reagents is similarly affected by the orientation of the protein. For example the rate of inhibition of sugar transport by the impermeant maleimide glutathione-maleimide-I, which acts at an exofacial cysteine residue, is increased by phloretin binding to the outward-facing conformation of the transporter (Krupka, 1985) but decreased by cytochalasin B binding to the inward-facing conformation (May, 1988). This indicates that the involved cysteine residue is more accessible to the reagent when the transporter is in its outward-facing conformation, and also that the residue is not located within the sugar binding site.

Circular dichroism and Fourier transform infrared spectroscopy have both shown the protein to have a secondary structure consisting predominantly of $\alpha$-helix (approx. 80%) with some $\beta$-turn structure (approx. 10%) the remaining structure being random coil (Chin et al., 1986; Chin et al., 1987; Alvarez et al., 1987). However these two techniques provide conflicting results concerning the presence of $\beta$-strands (Chin et al., 1987, Alvarez et al., 1987). The circular dichroism studies demonstrated a change in the tilt-angle of the
α-helices of the glucose transporter relative to the plane of the membrane, in the presence and the absence of the substrate D-glucose, and Fourier transform infrared spectroscopy suggested a difference in the content of α-helical and β-strand structures of the glucose transporter dependent on whether the protein was largely in the inward-facing or the outward-facing conformation (according to the single-site alternating conformation model) (Alvarez et al., 1987).

Both techniques indicated the presence of α-helical structure in both the intramembranous and extramembranous regions of the protein. Such structure was demonstrated to be within both the central cytoplasmic loop linking helices 6 and 7 (residues 207-271) and the C-terminal region (residues 457-492) by infrared spectroscopic studies of the trypsin-cleaved transporter (Cairns et al., 1987).

1.4 INHIBITORS OF THE GLUCOSE TRANSPORTER

Inhibitors of the glucose transporter have been invaluable tools in the study of both the structure and the mechanism of the protein. They can be divided into two groups.

The first group comprises protein modifying reagents which covalently react with specific groups on proteins in general, and as such are not specific for the glucose transporter. For example FDNB reacts with protein amino- and thiol-groups, and NEM reacts with protein thiol-groups (Bowyer & Widdas, 1958; Dawson and Widdas, 1963; Bloch, 1974).

The second group includes sugars and sugar analogues which are bound non-covalently and are reversible inhibitors of glucose transport, for example cytochalasin B, phenylα-D-glucoside, 4,6-O-ethylidene-D-glucose, and phloretin. These inhibitors appear to compete with D-glucose for the sugar-binding site but are not
transported. Cytochalasin B and phloretin bind exclusively to sites at the cytoplasmic and extracellular side of the erythrocyte membrane respectively. However phenyl glucoside and ethylidene glucose bind with higher affinity, but not exclusively, to the cytoplasmic and the extracellular side of the membrane respectively (Devès & Krupka, 1978a; Barnett et al.; 1975 Baker & Widdas, 1973; Krupka, 1985). These competitive inhibitors will be further discussed in Chapter 3. Other inhibitors in this category are the two disaccharides of glucose, cellobiose and maltose which due to their second glucose moiety bind to the transporter but cannot be transported.

The structure of cytochalasin B bears no obvious structural similarity to D-glucose. However in a study of the X-ray crystallographically determined 3-dimensional structures of the cytochalasins and D-glucose, Griffin et al. (1982) proposed that cytochalasin B binds to the glucose transporter by hydrogen bonds at N2 (donates) O7 (accepts) and O23 (accepts) which bear a spatial analogy to O6, O3 and O1 respectively of D-glucose (Figure 1.2A). In addition the hydrophobic region from C13 to C19 appears to act as an anchor in a hydrophobic region of the protein. As well as being one of the most potent reversible inhibitors of the glucose transporter (Deves & Krupka, 1978 a,b) [3H]-cytochalasin B can be used covalently to label the transporter in the presence of ultraviolet light (Carter-Su et al., 1982; Shanahan, 1982). This covalent labelling is inhibitable by D-glucose, and can therefore be assumed to involve covalent interactions in the vicinity of the sugar binding-site at the cytoplasmic surface of the membrane.

Similarly, a bis(D-mannose) derivative, 2-N-(4-azidosalicyloyl)-1,3-bis(D-mannos-4'-yloxy)-2-propylamine (ASA-BMPA) (Fig 1.2B) inhibits the glucose transporter, at the exofacial surface of the erythrocyte membrane. This binding is inhibitable by D-glucose, ethylidene glucose
Figure 1.2  Inhibitors of the glucose transporter.

Cytochalasin B (A) and 2-N-(4-azidosalicyl)-1,3-bis(D-mannos-4'-yloxy)-2-propylamine (ASA-BMPA) (B) bind to the glucose transporter at the cytoplasmic and extracellular surfaces of the erythrocyte membrane respectively.
and cytochalasin B. In a similar manner to cytochalasin B this inhibitor can be used to photolabel the sugar binding-site using ultraviolet irradiation. Therefore it can be assumed that ASA-BMBA binds covalently to the extracellular binding site of the glucose transporter (Holman et al., 1986; Holman & Rees, 1987).

1.5 PURIFICATION AND CHARACTERIZATION OF THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER

1.5.1 Purification

The erythrocyte membrane is a rich source of the glucose transport protein which constitutes approximately 5% of the membrane protein by weight. The protein was first purified from the erythrocyte membrane by solubilization with the non-ionic detergent Triton X-100 and subsequent reconstitution into soybean phospholipid vesicles by freeze-thaw sonication (Kasahara & Hinkle, 1976). The preparation was found to transport D-glucose in a cytochalasin B-inhibitable manner but the transport activity was very low. With the introduction of an anion exchange chromatography step (DEAE-cellulose) before the reconstitution, the D-glucose transport activity of the preparation was increased 20-fold. Additionally SDS/polyacrylamide gel electrophoresis showed a corresponding enrichment of the band 4.5 proteins compared to bands 3 and 7 (nomenclature of Steck, 1974) (Kasahara & Hinkle, 1977). However the rate of net D-glucose uptake was still only approximately 0.5% of that expected for the purified protein. Baldwin et al. (1979) discovered that washing the erythrocyte membranes in an alkaline buffer (pH 12) prior to detergent solubilization led to a loss from the membrane of all cytochalasin B binding sites except those sensitive to D-glucose. Subsequent DEAE-chromatography and reconstitution resulted in a preparation which was...
seen to run as a broad band of apparent Mr 55000 in the band 4.5 region on SDS/polyacrylamide gels. The preparation exhibited the same affinity for cytochalasin B \( K_d = 1.2 \times 10^{-7} \text{ M} \) and the same inhibition constant for D-glucose inhibition of cytochalasin B binding \( K_i = 32 \text{ mM} \) as the glucose transport protein in the intact erythrocyte, and assuming the Mr of 55000 to be correct, bound cytochalasin B with a ratio of 1 cytochalasin B molecule per 2 glucose transporter molecules. This suggested that the glucose transporter could exist in the form of a dimer (Baldwin et al., 1979).

A further improvement in the procedure was introduced by Baldwin et al. (1982) with the use of another non-ionic detergent, octyl-\( \beta \)-D-glucopyranoside (octyl glucoside). This greatly increased the efficiency of solubilization and reconstitution of the glucose transporter from the alkali-stripped erythrocyte membranes. Triton X-100 led to the solubilization of only 24% of the glucose transport protein from alkali-stripped membranes (Baldwin et al., 1979) whereas octyl glucoside resulted in the solubilization and reconstitution of approximately 90%. In addition to this octyl glucoside seemed to be selective in its solubilization of the glucose transporter since there was a 1.5-fold increase in the specific activity of the preparation. Additionally, since octyl glucoside has a high critical micellar concentration, it was possible to remove the detergent from the anion-exchange column eluate by dialysis, allowing spontaneous reconstitution of the protein into membrane-like bilayers of erythrocyte phospholipids. Rapid reconstitution is essential because the transporter is unstable in its detergent-solubilized state. The use of octyl glucoside yielded a preparation of transporter that could bind approximately 0.7 moles of cytochalasin B per mole of protein. Further improvements were made to the purification protocol by Cairns et al. (1984), who increased the ionic strength of the buffer used
during fractionation of the detergent-solubilized erythrocyte membranes by anion-exchange chromatography. The improved protocol resulted in a preparation which could bind nearly 1 mole of cytochalasin B per mole of protein. The only known contaminant of this preparation of the glucose transporter is the nucleoside transporter which constitutes 3-5% of the total protein (Jarvis & Young, 1981).

1.5.2 Characterization

The purified glucose transporter is a glycoprotein (Kasahara & Hinkle, 1977) being glycosylated at the extracellular surface of the membrane by an oligosaccharide of the poly (N-acetyl lactosamine) type which constitutes 15% of the glycoprotein by weight (Sogin & Hinkle, 1978; Gorga et al., 1979). The heterogeneous nature of this oligosaccharide causes the transporter to migrate as a broad band of apparent Mr 43000 - 74000 with a peak at Mr 55000 on SDS/polyacrylamide gels (Sogin & Hinkle, 1978; Gorga et al., 1979). Complete deglycosylation of the transporter by a 1:1 mixture of endoglycosidase F: glycopeptidase F (both from Flavobacterium meningosepticum) results in the transporter migrating as a sharper band of apparent Mr 46000 (Lienhard et al., 1984). Endoglycosidase F cleaves the glycosidic bond between the two residues of the N,N'-diacetylchitobiose which attaches the oligosaccharide to the protein via an N-linkage to asparagine (GlcNAc\(^{\beta 1-\text{\textasciitilde}\text{\textasciitilde}}\text{4GlcNAc}\)). Glycopeptidase F cleaves the glycopeptide bond linking the N,N'-diacetylchitobiose to asparagine (GlcNAc-GlcNAc\(^{\text{\textasciitilde}\text{\textasciitilde}}\text{Asn}\))(Elder & Alexander, 1982; Plummer et al., 1984).

The suggestion that the Mr 46000 polypeptide is a product of proteolysis of a Mr 100000 polypeptide (Shelton & Langdon, 1983) has been largely dismissed due to the following evidence: Antibodies raised against the purified glucose transporter do not react with any
protein of higher molecular weight than $M_r$ 46000 in the human erythrocyte membrane (Baldwin & Lienhard, 1980; Sogin & Hinkle, 1980). Photoaffinity labelling of the transporter in human erythrocytes with $[^3H]$-cytochalasin B results in the labelling of a $M_r$ 46000 polypeptide but not one of $M_r$ 100000 (Carter-Su et al., 1982). Purification of the glucose transporter from freshly drawn blood in the presence of protease inhibitors does not result in the production of a $M_r$ 100000 product but of the $M_r$ 46000 product (Baldwin et al., 1982).

The amino-acid composition of the glucose transporter has been investigated by two groups whose results were mainly in agreement, although Baldwin et al. (1982) identified a higher content of both methionine and cysteine residues than Sogin & Hinkle (1978) and also identified the N-terminal amino acid as methionine as opposed to aspartate. The cysteine content was estimated to be five residues per polypeptide chain from amino-acid analysis of the reduced S-carboxamidomethylated protein. Interestingly, this value equalled the number of free sulfhydryl groups (4.7 per molecule) estimated from the reaction of the protein with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) indicating the absence of disulphide bonds in the native protein (Baldwin et al., 1982).

1.6 MEMBRANE TOPOLOGY OF THE ERYTHROCYTE GLUCOSE TRANSPORTER

1.6.1 Proteolytic and chemical dissection of the glucose transporter

In an attempt to elucidate the topology of the glucose transporter much use has been made of proteolytic and chemical cleavage of the protein either in situ in erythrocyte membranes or in its purified form. The results of this work have often been conflicting.
1.6.1.1 Dissection of the glucose transporter

Treatment of the glucose transporter in intact erythrocytes with trypsin was found to have no effect on transport activity or the binding of cytochalasin B to the protein. However if the glucose transporter was in a preparation of "inside-out" vesicles (that is sealed vesicles prepared from erythrocyte membranes such that the cytoplasmic surface of the membrane is facing outwards) then treatment with trypsin was found to result in an increase in the affinity of the transporter for D-glucose, complete loss of transport activity and a decrease in the capacity of the glucose transporter to bind cytochalasin B (Baldwin et al., 1980; Cairns et al., 1987). This evidence that part of the transporter is exposed cytoplasmically together with the previous finding that the carbohydrate moiety of the transporter is exposed extracellularly (Sogin & Hinkle, 1978; Gorga et al., 1979) provided the first evidence that the glucose transporter was a transmembrane protein.

Extensive digestion of the purified, reconstituted glucose transporter with trypsin produces two stable, large membrane-bound fragments (Cairns et al., 1984; Deziel & Rothstein, 1984). When visualised on SDS/polyacrylamide gels one of these fragments migrates as a broad band of apparent Mr 23000 - 42000 and the other as a sharp band of apparent Mr 18000. Following treatment with endo-β-galactosidase the Mr 23000-42000 fragment migrates as a sharp band of apparent Mr 23000 (Cairns et al., 1984). Endo-β-galactosidase cleaves internal β-galactosidic linkages of oligosaccharides of the poly(N-acetyl lactosamine) series (Fukudu & Matsumura, 1976). Therefore this finding and the fact that this fragment is stained on SDS/polyacrylamide gels by the periodate-Schiff reagent for carbohydrates (Deziel & Rothstein, 1984) indicated that it contained one or more sites of N-linked glycosylation. Since the transporter
polypeptide of \( M_r \) 46000 yields two fragments of \( M_r \) 23000 (after deglycosylation) and \( M_r \) 18000, cleavage by trypsin must occur close to the centre of the polypeptide. Furthermore, since the \( M_r \) 23000 - 42000 fragment is glycosylated extracellularly (Sogin & Hinkle, 1978; Gorga et al., 1979) and is a product of trypsin-cleavage which occurs at the cytoplasmic surface of the protein this fragment must cross the erythrocyte membrane at least once.

If the tryptic cleavage is performed on purified glucose transporter previously photoaffinity-labelled with \(^3\text{H}\)cytochalasin B, the \( M_r \) 18000 fragment is radiolabelled. Two other fragments of apparent \( M_r \) 25500 and \( M_r \) 23500 are visualised as sharp bands on SDS/polyacrylamide gels after short periods of digestion and are also radiolabelled. These are probably precursors of the \( M_r \) 18000 fragment (Cairns et al., 1984). Cleavage experiments with glucose transporter radiolabelled at its extracellular sugar-binding site with \(^3\text{H}\)ASA-EMPA have demonstrated that the \( M_r \) 18000 is labelled by both ASA-EMPA and cytochalasin B (Holman et al., 1986). Since this fragment is labelled both at the extracellular surface and the cytoplasmic surface this fragment too must cross the membrane at least once.

Cleavage of \(^3\text{H}\)cytochalasin B-labelled glucose transporter at cysteine residues, with 2-nitro-5-thiocyano-benzoic acid (NTCB), results in the production of an unlabelled fragment that migrates as a broad band of apparent \( M_r \) 36000 - 48000 and a number of smaller fragments one of which is radiolabelled and has an apparent \( M_r \) 15500 on SDS/polyacrylamide gels. If the cleavage is carried out using endo-\( \beta \)-galactosidase-treated protein the broad band at \( M_r \) 36000-48000 is replaced by a sharp band of apparent \( M_r \) 38000 (Cairns et al., 1984). Therefore this \( M_r \) 38000 fragment carries at least one site of glycosylation. Since the major radiolabelled fragment has an apparent \( M_r \) 15500 the site of labelling by cytochalasin B, which probably
corresponds to the cytoplasmic sugar-binding site (Deves & Krupka, 1978b), seems to be located in either the C-terminal or the N-terminal third of the polypeptide.

Deziel et al. (1985) investigated the reactivity of the five known cysteine residues (Baldwin et al., 1982) by examining their extents of labelling with N-ethyl [14C]maleimide. All five of the cysteine residues reacted with the reagent in SDS-denatured preparations of the intact transporter. Treatment of the SDS-denatured products of extensive trypsin-cleavage resulted in the labelling of three cysteine residues in the glycosylated fragment of apparent Mr 23000-42000 and two cysteine residues in the fragment of apparent Mr 19000. If the denaturation step was omitted only three cysteine residues of the intact transporter were labelled by N-ethyl [14C]maleimide, two being in the Mr 23000-42000 fragment and one in the Mr 18000 fragment. Therefore one cysteine residue in each fragment must be inaccessible to the label without denaturation, suggesting that it is "cryptic" and located within the bilayer. When the impermeant sulfhydryl reagent para-chloromercuribenzenzene sulphonate (PCMBS) was used to protect extracellular cysteine residues protection was only afforded to the non-cryptic cysteine residue of the Mr 19000 fragment suggesting that this residue is exposed extracellularly (Rothstein, 1981; Deziel et al., 1985).

In summary, cleavage of the glucose transporter with trypsin at the cytoplasmic surface of the protein and near to the centre of its polypeptide sequence results in the production of two fragments: One of these contains the site(s) of N-linked glycosylation, crosses the membrane at least once, and contains three cysteine residues, one cryptic and two exposed cytoplasmically. The other contains both a cytoplasmic and an extracellular sugar-binding site and again crosses the membrane at least once, bearing two cysteine residues, one cryptic.
and one exposed extracellularly. The cytoplasmic sugar-binding site is either in the N-terminal or the C-terminal third of the polypeptide sequence. This is summarized in Figure 1.3A.

1.6.1.2 Dissection of the glucose transporter in situ

Work concentrating solely on proteolytic dissection of the radiolabelled glucose transporter in situ in erythrocyte membranes has led to conflicting results. Shanahan & D'Artel-Ellis (1984) found, as was expected, that trypsin was not able to cleave the glucose transporter at the extracellular surface of the membrane but that cleavage at the cytoplasmic surface resulted in the production of a non-glycosylated fragment of apparent Mₚ 21500. However, this cleavage resulted in the loss of both cytochalasin B-binding activity and glucose transport-activity in contrast to the results of previous workers (Baldwin et al., 1980). Thermolysin (a protease of broad specificity) had no effect on the transporter in sealed ghosts but cleavage at the cytoplasmic surface was found to result in complete loss of the [³H]cytochalasin B label from photoaffinity-labelled membrane. No smaller labelled fragment was seen on SDS/polyacrylamide gels, suggesting that the label was cleaved from the transporter or that labelled fragments produced were too small to be seen on the electrophoresis system used. Chymotrypsin, again conflicting with previous results (Deziel & Rothstein, 1984), was found to cleave the transporter at the extracellular surface of the membrane producing a radiolabelled fragment of apparent Mₚ 18400. Cleavage of the C-terminus with carboxypeptidases and the N-terminus with aminopeptidases indicated that the termini are located at the extracellular and cytoplasmic surfaces respectively. Since these workers also found that endo-β-galactosidase acting extracellularly removed the carbohydrate moiety from the intact transporter and
Figure 1.3 Two proposed models for the arrangement of the glucose transporter polypeptide in erythrocyte membrane.

That of Deziel et al. (1985) (A) shows the proposed sites of carbohydrate attachment (CHO) and cytochalasin B-binding (CB) and the protein's cysteine residues (SH). Boxes indicate cryptic residues. That of Shanahan and D'Artel-Ellis (1984) (B) shows the proposed N- and C-terminal (N_t and C_t), the sites of carbohydrate attachment (CHO) and cytochalasin B-binding (CB) and sites of cleavage by Trypsin (TRP), chymotrypsin (CHY), thermolysin (TML), carboxypeptidase (CPS), and cathepsin (CAT).
decreased the apparent $M_r$ from 57000 to 49000, they proposed a model in which the site of glycosylation is near to the C-terminus which is located extracellularly, and the cytochalasin B binding site is located near to the cytoplasmically located N-terminus (Figure 1.3B).

1.6.2 Cloning and sequencing of the glucose transporter

Using rabbit antiserum raised against the purified human erythrocyte glucose transporter, Mueckler et al. (1985) screened a $\lambda$gt11 library prepared from the human hepatoma cell line HepG2 and were able to isolate a complementary DNA (cDNA) clone. The cDNA encoded a 492 residue polypeptide of $M_r$ 54117 whose deduced amino acid composition agreed well with that of the purified erythrocyte glucose transporter (Baldwin et al., 1982). Fast atom bombardment mass spectrometry and gas-phase sequencing of the erythrocyte glucose transporter led to the conclusion that the erythrocyte and HepG2 glucose transporters were very similar if not identical. The predicted sequences of the N- and C-termini were confirmed indicating that there was no proteolytic processing of either terminus after synthesis (Mueckler et al., 1985).

The deduced amino acid sequence was analysed for hydrophobicity by the methods of Kyte & Doolittle (1982) and Eisenberg et al., (1984). The resulting profile suggested the presence of 12 membrane-spanning regions and these data together with the results of proteolysis of the transporter (Cairns et al., 1984; Deziel & Rothstein, 1984; Lienhard et al., 1984; Shanahan & D'Artel-Ellis, 1984) were used to produce a model for the arrangement of the glucose transporter in the membrane as shown in Figure 1.4 (Mueckler et al., 1985).
Figure 1.4 Proposed model for the arrangement of the glucose transporter in the human erythrocyte membrane (Mueckler et al., 1985).

The twelve putative membrane-spanning domains 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 domains 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 Asn45 is shown. The arrows point to the positions of known tryptic cleavage sites in the native, membrane-bound, erythrocyte glucose transporter.
1.6.2.1 Proposed model of the glucose transporter in the membrane

In the model of Mueckler et al. (1985) the glucose transporter polypeptide crosses the membrane 12 times in the form of largely hydrophobic $\alpha$-helices, each composed of approximately 21 amino acid residues. Both the N-terminus (residues 1-12) the C-terminus (residues 451-492) and a large hydrophilic loop between helices 6 and 7 (residues 207-271) are situated on the cytoplasmic side of the membrane. The site of glycosylation believed to be Asn$^{45}$ is situated on the extracellular side of the membrane in the N-terminal half of the protein.

Helices 3, 5, 7, 8 and 11 seem likely to form amphipathic $\alpha$-helices since they contain several asparagine, glutamine, serine and threonine residues which would be localized to one face in each helix. The hydroxyl and amide side chains are likely candidates to line a transmembrane channel and bind to the hexoses in transit.

The two potential sites of glycosylation by the N-linked oligosaccharide were Asn$^{45}$ or Asn$^{411}$. In the model Asn$^{411}$ is within a membrane-spanning domain making its glycosylation unlikely. In agreement with this, cleavage of the polypeptide at cysteine residues produced a fragment composed of residues 133-421 or 133-428 which was not glycosylated (Cairns et al., 1984). Thus the site of glycosylation was assigned to Asn$^{45}$. Direct evidence for this site of glycosylation has since been obtained: Mueckler & Lodish (1986) demonstrated that when a fragment corresponding to residues 1-340 was synthesized in an in vitro translation system in the presence of pancreatic microsomes it was incorporated into the membrane and glycosylated. From this it follows that the non-glycosylated $M_r$ 18000 fragment of the transporter produced by trypsin cleavage, which contains the site of cytochalasin B- and ASA-EMPA-labelling, is derived from the C-terminal half of the transporter (Cairns et al., 1984; Deziel & Rothstein,
1.6.3 Evidence in support of the model of Mueckler et al.

The studies initiated by Mueckler et al. (1985) involving the use of fast atom bombardment mass spectrometry and gas-phase sequencing were taken a step further by Cairns et al. (1987) who used HPLC, N-terminal analysis and fast atom bombardment mass spectrometry to investigate the fragmentation of the glucose transporter during vectorial digestion experiments on the membrane-embedded protein using trypsin. Three major water-soluble fragments from the central hydrophilic loop were shown to be liberated from the cytoplasmic surface of the membrane upon digestion: they corresponded to residues 213-223, 226-253, and 257-269. In addition hydrophilic peptides were liberated from the region corresponding to residues 459-492 from the C-terminus of the protein. The two major membrane-bound fragments resulting from trypsin cleavage of the intact transporter, of apparent Mr 18000 and 23000 - 42000, were identified as comprising residues 270-456 and 1-212 respectively. These findings are compatible with the model proposed by Mueckler et al. (1985) where residues 207-271 and 450-492 are exposed on the cytoplasmic side of the membrane. None of the potential sites of trypsin cleavage at the N-terminus were cleaved indicating that the N-terminal region of the protein is inaccessible to proteases in the native protein. This is consistent with the finding that antibodies raised against a synthetic peptide corresponding to residues 1-15 cannot bind to the protein in its native conformation, but do recognise the denatured protein (Davies et al., 1987). Similarly there was no evidence for trypsin cleavage at potential sites of cleavage (Lys and Arg residues) between helices 2 and 3, 4 and 5, and 8 and 9 (Figure 1.4) consistent with the finding that antibodies raised against synthetic peptides in these regions
cannot bind to the protein in its native conformation but do recognize the denatured protein (Davies et al., 1990). The lack of cleavage by trypsin may result from steric hindrance between the phospholipid head-groups and the enzyme. Digestion in the presence of SDS does allow further fragmentation to occur (Section 6.2.2).

Using polyclonal antibodies raised against synthetic peptides corresponding to various regions of the human erythrocyte glucose transporter, Davies et al. (1987) demonstrated that the C-terminus of the protein is exposed on the cytoplasmic surface of the membrane and that the sites of cytochalasin B labelling and N-linked glycosylation are in the C-terminal and N-terminal half of the protein respectively. In addition, similar techniques using polyclonal and monoclonal antibodies (Davies et al., 1990) demonstrated that a large hydrophilic loop (residues 217-272) between postulated helices 6 and 7 is exposed at the cytoplasmic surface of the membrane.

Circular dichroism studies (Chin et al., 1987; Pawagi & Deber, 1987) and Fourier transform infrared spectroscopic studies (Chin et al., 1986; Alvarez et al., 1987; Cairns et al., 1987) have confirmed the presence of a large proportion of \( \alpha \)-helical structure in the transporter, in agreement with the model. By performing Fourier transform infrared spectroscopic studies on trypsin-treated transporter \( \alpha \)-helical structure has been localized in both the intramembranous part of the protein and in the cytoplasmic regions which are removed from the cytoplasmic surface by trypsin (Cairns et al., 1987).

Tritium and deuterium exchange studies (Jung et al., 1986; Alvarez et al., 1987) indicate the presence of an aqueous pore consistent with the model where helices 3, 5, 7, 8 and 11 are considered amphipathic in nature and might surround such a channel. Tritium exchange demonstrated that the substrate D-glucose had no effect on the number
of hydrogens available for exchange but decreased the rate of exchange, as opposed to cytochalasin B which decreased the number of available hydrogens (Jung et al., 1986). This suggests that whereas D-glucose occupies the channel transiently, cytochalasin B collapses part of the channel whilst binding to the inward-facing conformation of the transporter.

1.7 THE MECHANISM OF GLUCOSE TRANSPORT

Kinetic analyses have demonstrated that all transported sugars have similar $V_{\text{max}}$ values for transport by the glucose transporter but varying values for $K_m$ (Krupka, 1971). That is to say that they seem all to bind to the same binding site but with varying affinities. Transport inhibitors such as cytochalasin B probably bind to the same site as D-glucose, or to sites allosterically linked to this site, as is demonstrated by their ability competitively to inhibit binding of the substrate. However they are not themselves transported. This indicates two distinct processes involved in transport by the glucose transporter: binding and translocation.

1.7.1 The substrate binding site

The exact regions of the glucose transporter polypeptide involved in the binding site are unknown. However some insight has been gained by investigations involving the transport inhibitors cytochalasin B and ASA-BMPA (Section 1.4) which appear to bind at or close to the substrate binding site at the cytoplasmic and extracellular surfaces of the membrane respectively (Devés & Krupka, 1978; Holman et al., 1986; Holman & Rees, 1987).

Digestion of the transporter with trypsin results in cleavage of the cytoplasmically exposed C-terminus and the central hydrophilic
loop between helices 6 and 7. This results in loss of glucose transport activity, but not in the ability of the transporter to bind D-glucose and cytochalasin B (although the affinity of the transporter for these is increased and decreased respectively) (Baldwin et al., 1980; Cairns et al., 1984; Cairns et al., 1987). Indeed D-glucose is still able competitively to inhibit the binding of cytochalasin B to the transporter (Cairns et al., 1987). This indicates that the substrate binding site is affected by the removal of the two cytoplasmic regions of the transporter but is not lost. The decrease in affinity for the ligands may be due to a conformational change in the structure of the protein rather than loss of part of the binding site and such a conformational change could also explain the loss of transport activity.

Trypsin cleavage of the glucose transporter, previously photolabelled either at the cytoplasmic binding site with [³H]cytochalasin B, or at the extracellular binding site with [³H]ASA-BMPA, results in the production in both cases of a radiolabelled membrane-bound fragment of apparent Mₚ 18000 comprising residues 270-456 (Cairns et al., 1984, 1987; Deziel & Rothstein, 1984; Holman et al., 1986; Davies et al., 1990). Therefore either all or part of both the cytoplasmic and extracellular substrate binding sites lie within the C-terminal half of the protein.

Partial cleavage of the radiolabelled transporter at tryptophan residues with N-bromosuccinimide results in the production of a fragment of apparent Mₚ 32000 probably comprising residues 186-492 containing both the extracellular and cytoplasmic sites. More extensive cleavage results in the production of a fragment of apparent Mₚ 13000 probably comprising residues 388-492 which is labelled by cytochalasin B but not by ASA-BMPA. Further cleavage of the cytochalasin B-labelled fragment yields a labelled peptide of apparent
Mₜ 3000 possibly comprising residues 388-412. Thus the site labelled by cytochalasin B, which probably corresponds to a part of the cytoplasmic substrate-binding site, may lie between residues 388 and 412. The accessibility of this site to the cytoplasm further suggests that it may lie within the cytoplasmically exposed region linking helices 10 and 11 according to the model of Mueckler et al. (1985). The extracellular binding site may lie between residues 186 and 387 (Holman & Rees, 1987). In agreement with these conclusions, cleavage of the labelled protein at cysteine residues with 2-nitro-5-thiocyanobenzoic acid (NTCB) results in the production of a fragment of apparent Mₜ 5000 which may comprise residues 347-420, and which contains both binding sites (Cairns et al., 1984; Holman & Rees, 1987). Therefore the extracellular binding site may lie between residues 347 and 388 and has been assigned to the extracellular loop between helices 9 and 10 (Holman & Rees, 1987).

As mentioned above, cleavage of the transporter with trypsin at the cytoplasmic surface removes the cytoplasmically exposed hydrophilic loop between helices 6 and 7 and the C-terminal region. The consequent loss of glucose transport activity, increase in the affinity of the protein for D-glucose and decrease in the affinity for cytochalasin B (Baldwin et al., 1980, Cairns et al., 1987), suggest that these regions of the polypeptide may play an important role in maintaining the structure of the sugar-binding site via conformational changes in the protein. In agreement with this conclusion, antibodies raised against synthetic peptides corresponding to regions of the cytoplasmic hydrophilic loop of the glucose transporter between helices 6 and 7 were found to inhibit the binding of cytochalasin B to the binding site (Davies et al., 1990).

Therefore the evidence available suggests that the region of the polypeptide comprising helices 9, 10 and 11, and the cytoplasmically
Figure 1.5 The proposed membrane disposition of the glucose transporter (Mueckler et al., 1985) showing proposed sites of proteolytic and chemical cleavage, photolabelling and glycosylation (from Walmsley, 1988.)

The α-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.
located hydrophilic loop between helices 6 and 7, are involved either directly or indirectly in the structure of the substrate binding site (Figure 1.5).

1.7.2 Binding to the sugar binding site and translocation

An investigation of the binding of various derivatives of D-glucose to the glucose transporter has demonstrated the asymmetry of the transport system (Barnett et al., 1975). Sugars substituted at the C-1 position (for example n-propyl-β-D-glucopyranoside) were found to inhibit the binding of D-glucose only if present at the cytoplasmic side of the membrane and the substituted sugars themselves were not transported. Conversely sugars substituted at the C-6 or C-4 position (for example 6-O-propyl-D-glucose and 4,6-O-ethylidene-D-glucose) were only able to inhibit the binding of D-glucose when present at the extracellular surface and themselves were not transported. It therefore appears that the asymmetry of the sugar-transport system is such that hexose derivatives with large substituent groups at C-4 or C-6 can bind to the system on the outside of the cell but not on the inside, whereas sugars with large substituents at C-1 can only bind at the inside and not at the outer surface. Large substituents at either end of the molecule will prevent transport. A model consistent with these observations is shown in Figure 1.6, depicting hydrogen bonds between the hydroxyl groups at C-1, 4 and 6 and the protein. When entering the cell the sugar first binds to a site on the extracellular surface of the membrane via groups in the C-1 region of the molecule. The protein then undergoes a change in conformation such that the sugar is exposed at the cytoplasmic side of the membrane and binding to the protein is via the C-4 and C-6 regions of the sugar. This model can successfully explain the effect of a bulky group on a sugar molecule to prevent its transport thereby effectively blocking the
Figure 1.6 Possible model for sugar transport in the human erythrocyte (from Barnett et al., 1975).

6-O-Propyl-D-glucose (R = C₃H₇; R' = H) can bind to the transport system in conformation A, but cannot be transported for steric reasons. Similarly, propyl β-D-glucopyranoside (R = H; R' = C₃H₇) can bind to conformation B but cannot be transported. D-glucose can bind to both conformations, and if the transport site changes conformation from A to B, is effectively transported from outside (O) to inside (I) the erythrocyte. (Only some of the probable hydrogen bonds are shown.)
transporter and explains the asymmetry of the system as observed by Baker & Widdas (1973). Earlier models did not deal with the possibility of asymmetry (Kahlenberg & Dolansky, 1972; Barnett et al., 1973).

An investigation of the thermodynamics of glucose transport have shown that binding of D-glucose to the transporter at the cytoplasmic side of the erythrocyte membrane is slightly exothermic whereas binding of D-glucose to the transporter at the extracellular side is endothermic (Walmsley & Lowe, 1987). This has been attributed to the dissociation of water from both the binding site and glucose, as glucose effectively exchanges its hydrogen-bonds with water for a similar number of hydrogen-bonds with the transporter. By excluding water the binding site is stabilized thereby reducing the energy required for a change in conformation to occur.

Much evidence now exists for this model, as opposed to the two-site model in which two binding sites are exposed simultaneously at both sides of the membrane (Carruthers, 1986 a,b; Helgerson & Carruthers, 1987; Section 1.3.1). In this single-site model no ternary complexes of the glucose transporter, with ligand bound at both sides of the membrane, should exist since binding at each site is mutually exclusive. In agreement with this no evidence was found for ternary complexes during an investigation of the kinetics of binding of inhibitors to the glucose transporter (Gorga & Lienhard, 1981) using cytochalasin B and n-propyl-β-D-glucose which bind at the cytoplasmic surface (Devès & Krupka, 1978; Barnett et al., 1975) and 4,6-ethylidene-D-glucose which binds at the extracellular surface (Baker & Widdas, 1973). Additionally a more recent investigation using proton-NMR techniques found no evidence of a ternary complex (Wang et al., 1986).

Binding of ligands to the transporter quenches the intrinsic
fluorescence of the protein (Gorga & Lienhard, 1982). This has been exploited in pre-steady-state investigations of a single half-turnover of the transporter, either from its inward-facing to its outward-facing conformation due to the binding of 4,6-ethylidene D-glucose (Appleman & Lienhard, 1985; Appleman & Lienhard, 1989) or from its outward-facing to its inward-facing conformation due to the binding of phenyl D-glucoside (Appleman & Lienhard, 1989). The identification of this half-turnover step provides direct evidence for the alternating conformation model for transport.

1.8 THE FAMILY OF FACILITATIVE GLUCOSE TRANSPORTERS

The uptake of glucose into most eukaryotic cells is catalysed by members of two distinct gene-families (Baldwin & Henderson, 1989); the facilitated-diffusion glucose transporters which are expressed in most mammalian cells, and the Na+/glucose cotransporters which are restricted to selected epithelial cells such as the renal tubules and the small intestine. The facilitative glucose transporters allow passive equilibration of glucose across cell membranes with no energy expenditure, as opposed to the cotransporters which transport glucose against its concentration gradient using energy provided by the sodium ion gradient. The cotransporters are kinetically and structurally distinct from the facilitative transporters and will not be discussed in detail here.

Initial insights into the distribution of facilitative glucose transporters in tissues other than the erythrocyte were gained by their capacity for D-glucose-inhibitable cytochalasin B binding, for example in cells such as the rat adipocyte (Wardzala et al., 1978) and chick embro fibroblasts (Salter & Weber, 1979). The affinity constants for cytochalasin B binding were found to be comparable to those for
the human erythrocyte glucose transporter in these cases. However in the case of the rat liver transporter both the $K_d$ for cytochalasin B binding and the $K_i$ for cytochalasin B inhibition of glucose transport were found to be one order of magnitude higher than for any other cell type (Axelrod & Pilch, 1983). Photo-affinity labelling of the glucose transporter with $[^3H]$cytochalasin B has been used by a number of groups to identify glucose transporters in a variety of cell types, including rat adipocytes (Shanahan et al., 1982), skeletal muscle cells (Klip et al., 1983), human placenta microvillus membranes (Ingerman et al., 1983) and chick embryo fibroblasts (Shanahan et al., 1982; Pessin et al., 1982). However the rat liver glucose transporter was not photo-labelled by cytochalasin B suggesting that there are fewer structural similarities between this transporter and that in other cell types (Axelrod & Pilch, 1983). Of these facilitative transporters a further subdivision can be made depending on whether the tissue is sensitive to insulin, as is the case with adipocytes and skeletal muscle.

The isolation and sequencing of complementary DNA (cDNA) clones encoding many mammalian, yeast, bacterial, fungal and algal glucose transporters has allowed examination of amino-acid sequence homology and the division of the eukaryotic facilitative glucose transporters into five families, as described below (Mueckler et al., 1985; Birnbaum et al., 1986; Hediger et al., 1987; Asano et al., 1988; Celenza et al., 1988; Fukumoto et al., 1988; Kayano et al., 1988; Thorens et al., 1988; Bell et al., 1989; Charron et al., 1989; Birnbaum, 1989; Fukumoto et al., 1989; James et al., 1989; Kæstner et al., 1989; Gould & Bell, 1990; Mueckler, M. 1990). In addition, antibodies raised against synthetic peptides corresponding to sequences of the glucose transporters have been used for identifying and characterising glucose transporters in a number of tissues. For
example at the bovine blood/brain barrier (Kasanicki et al., 1987), at
the mammalian blood/nerve barrier (Froehner et al., 1988), and for
immunocytochemical localization of glucose transporters in rat brain
(Bagley et al., 1989).

It is reasonable to suppose that such a variety of glucose
transporters has arisen due to varying requirements of different
tissues and their environments. Nature has subtly modified a family of
proteins with the same basic function in order to regulate glucose
metabolism.

The family of mammalian glucose transporters is briefly summarised
here. GLUT is the symbol for the facilitative glucose transporter and
the numbering system is based purely on the chronological order of
publication of the cDNA sequences.

GLUT 1 - the erythrocyte-type transporter - has been identified as
the major transporter type in brain (Birnbaum et al., 1986; Flier et
al., 1987; Wang et al., 1987) placenta (Wessling & Pilch, 1974) and the
erthrocyte (Mueckler et al., 1985) and is also present in other
tissues (Table 1.1). A comparison of the predicted amino acid
sequences indicates that the sequence of GLUT 1 is highly conserved
there being 98% identity between the human and rat proteins.

GLUT 2 - the liver type transporter - is distinct from GLUT 1 in
that it has ten-fold higher values for both the binding constant for
cytochalasin B and the $K_m$ for glucose (Axelrod and Pilch, 1983). Glut
2 shows 55.5% identity with GLUT 1 and identity between human and rat
GLUT 2 is 82% (Fukumoto et al., 1988; Thorens et al., 1988). This
isoform is also expressed in pancreatic $\beta$-cells (Thorens et al.,
1988; Orci et al., 1989) and is under-expressed in the insulinoma
cell-line RIN5F, which does not secrete insulin in the normal manner
in response to increased external glucose levels. This suggests that
<table>
<thead>
<tr>
<th>Designation (common name)</th>
<th>Size (number of amino acids)</th>
<th>Major sites of expression</th>
<th>Chromosomal location</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT 1 (Erythrocyte, HepG2, brain)</td>
<td>492</td>
<td>Foetal tissues, brain, kidney and colon</td>
<td>1</td>
</tr>
<tr>
<td>GLUT 2 (Liver)</td>
<td>524</td>
<td>Liver, β-cell, kidney and small intestine</td>
<td>3</td>
</tr>
<tr>
<td>GLUT 3 (Foetal muscle)</td>
<td>496</td>
<td>Many tissues including brain, placenta and kidney</td>
<td>12</td>
</tr>
<tr>
<td>Glut 4 (Muscle/adipocyte insulin-regulatable)</td>
<td>509</td>
<td>Skeletal muscle, heart and adipocytes</td>
<td>17</td>
</tr>
<tr>
<td>Glut 5 (Small intestine)</td>
<td>501</td>
<td>Small intestine</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 1.1  The family of human facilitative glucose transporters.  
(From Gould & Bell, 1990)
GLUT 2 may be involved in the triggering of insulin secretion by these cells and a decrease in its expression could perhaps be responsible for the lack of secretion in type II diabetes.

GLUT 3 - the foetal skeletal muscle transporter - was identified from a human foetal skeletal muscle library and shown to have 64% and 52% identity with GLUT 1 and GLUT 2 respectively (Kayano et al., 1988). Analysis of the amino-acid sequence suggests similar membrane topology to GLUT 1. mRNA for this isoform of the transporter is found in all tissues but is low in adult muscle suggesting that is not the major protein responsible for glucose uptake in this tissue.

GLUT 4 - the insulin responsive transporter - is expressed in insulin sensitive tissues. In the basal state this isoform is localized in an intracellular pool but in response to insulin is transported to the plasma membrane. This transporter accounts for 90% of the glucose transporter protein in these cells (Oka et al., 1988; Zorzano et al., 1989). Rat, human and mouse cDNA clones have been isolated and characterised (Birnbaum, 1989; Charron et al., 1989; Fukumoto et al., 1989; James et al., 1989; Kaestner et al., 1989). GLUT 4 has 65%, 54% and 58% identity with GLUT 1,2 and 3 respectively, and there is 95% homology between human and rat proteins.

GLUT 5 - the small intestine transporter. A cDNA clone has been recently isolated and characterised, which is expressed at very high levels in the human small intestine and has 42%, 40%, 39% and 42% identity with GLUT 1,2,3 and 4 respectively (Kayano et al., 1990).

1.9 AIMS OF THIS STUDY

The aims of this study were to investigate further the structure and function of the human erythrocyte glucose transporter by both biochemical and biophysical means, and to elucidate the regions of the
protein involved in the conformational changes of the transporter and in the sugar binding site. Much evidence was available for the involvement of conformational changes in the mechanism of transport, but the identity of the regions of the protein affected by these changes was largely unknown. To this end I used tryptic digestion to investigate the involvement of the hydrophilic, cytoplasmic regions of the protein in these conformational changes and to complement this I also investigated the conformational changes of the glucose transporter by solution X-ray scattering techniques. In an attempt to elucidate the areas of the transporter polypeptide involved in both the extracellular and the cytoplasmic sugar-binding sites I radiolabelled the protein at the two sites with specific radiolabelled probes and carried out proteolytic and chemical fragmentation studies. The fragments produced were identified by the use of polyclonal antibodies raised against synthetic peptides corresponding to specific regions of the sequence.
All chemicals and biochemicals were obtained from the Sigma Chemical Company (Poole, Dorset, U.K.) or from BDH Chemicals (Poole, Dorset, U.K.) unless stated otherwise.

2.1 Preparation of Membranes

2.1.1 Erythrocyte membrane ghosts

All operations were carried out either on ice or at 4°C. One unit of human blood (freshly outdated and obtained from the blood-bank of the Royal Free Hospital, London) was diluted to 1200 ml with ice-cold phosphate-buffered saline (PBS, 5 mM sodium phosphate, 150 mM sodium chloride, pH 8.0) and the cells sedimented by centrifugation for 10 minutes at 4500 rpm in a 6x250 ml GSA rotor in a Sorvall RC-5B centrifuge. The layer of white cells on top of the pellet was removed by aspiration and the supernatant discarded. The cells thus obtained were resuspended and sedimented in the same manner two or three times more until the supernatant was clear and all plasma components were removed.

Erythrocyte membranes ("ghosts") were prepared by a development of the method of Steck & Kant (1974). The erythrocytes were lysed by dilution to 2000 ml in ice cold lysis-buffer (5P8) consisting of 5 mM sodium phosphate, 1 mM EDTA, pH 8.0, 0.11 mM phenylmethylsulphonyl fluoride (PMSF). The erythrocyte membranes were then harvested by filtration of the intracellular components through a Pellicon cassette system (Millipore Corp., Bedford, M.A., U.S.A.) using a 0.4 μm (coarse screen) durapore cassette filter at a pressure of 400 p.s.i. The ghosts were concentrated by centrifugation at 11500 rpm for 20 minutes and any remaining leukocytes were removed at this stage by aspiration.
of the hard white pellet beneath the pellet of ghosts. The ghosts were homogenized by three passes of a hand-held homogenizer and assayed for protein (Section 2.2).

2.1.2 Alkali-stripped erythrocyte membranes

Erythrocyte membrane ghosts were stripped of their peripheral proteins by the method of Gorga & Lienhard (1981): 45 ml of erythrocyte ghosts at 4 mg/ml were mixed with 240 ml of ice-cold 15.4 mM sodium hydroxide, 2 mM ethylenediaminetetra-acetic acid (EDTA), 0.2 mM dithiothreitol (DTT) which had been purged with nitrogen for 5 minutes immediately before use. After 10 minutes in the resulting pH12 conditions the membranes were collected by centrifugation at 18000 rpm for 20 minutes using a 8x50 ml SS34 rotor in a Sorvall RC-5B centrifuge. The supernatant was discarded and the pellet washed twice with 50 mM Tris-HCl, pH 6.8 and resuspended in approximately 10 ml of buffer. After homogenisation by three passes of a hand-held homogeniser the homogeneous preparation was assayed for protein (Section 2.2).

2.1.3 Purification of the glucose transporter

Purified human erythrocyte glucose transporter was prepared by the method of Cairns et al. (1984). All operations were carried out on ice or at 4°C. Alkali-stripped erythrocyte membranes (144 mg of protein) were solubilised with octyl-β-D-glucopyranoside (octyl glucoside, Calbiochem – Behring, La Jolla, C.A., U.S.A.) such that final concentrations were 2 mg/ml protein and 1.35% (w/v) octyl glucoside in 46.5 mM Tris-HCl, 2 mM DTT, pH 7.4. After shaking on ice for 20 minutes the soluble material was removed from the insoluble membranous material by centrifugation at 45000 rpm for 1 hour at 4 °C using a Kontron T70 ultracentrifuge and a Kontron 6x38.5 ml rotor
The supernatant was immediately removed to ice and made 25 mM in NaCl in readiness for anion exchange chromatography on DEAE-cellulose (DE-52, Whatman Chemical Separation Limited, Maidstone, Kent, U.K.). The anion exchange matrix was equilibrated with 47.5 mM Tris-HCl, 1% (w/v) octyl glucoside, 2 mM DTT, 25 mM NaCl, pH 7.4 and then loaded with 60 ml of supernatant. Elution was continued with 90 ml of 47.5 mM Tris-HCl, 1% (w/v) octyl glucoside, 2 mM DTT, 25 mM NaCl, pH 7.4. Fractions (15 ml) were collected on ice and assayed for protein by their absorbance at 280 nm using a Beckman DU65 U.V./visible spectrophotometer (Beckman Instruments, Fullerton, C.A., U.S.A.) and the peak fractions pooled. After the addition of NaCl and EDTA to final concentrations of 100 mM and 1 mM respectively the resulting preparation was reconstituted by dialysis against 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4 for 48 hours using four changes of 2 litres of buffer. After dialysis the reconstituted transporter was assayed for protein (Section 2.2), for functional glucose transporter by its ability to bind cytochalasin B (section 2.5.1) and for purity by SDS/polyacrylamide gel electrophoresis (section 2.6.1).

2.2 PROTEIN DETERMINATION

The protein content of samples was measured by the method of Lowry et al. (1951). Samples containing approximately 25 µg of protein were made up to 200 µl with water and a set of standards of 0, 12.5, 25, 37.5 and 50 µg was constructed in a similar fashion using a stock of standardised bovine serum albumin (BSA). Using the stocks listed below the following procedure was carried out for both standards and samples:

1 ml of solution C was added to each tube, vortexed and incubated at
room temperature for 20 min. 100 μl of solution D was added with immediate mixing and incubated at room temperature for 30 minutes. The absorbance was measured at 750 nm against air using an LKB NovoSpec 4040 Mark II visible spectrophotometer (LKB-Pharmacia, Watford, Herts, U.K.) and the mass of protein in the samples was determined by comparison with the standard curve.

Solution A. 2% (w/v) Na₂CO₃ in 0.1 M NaOH
Solution B. 1% (w/v) CuSO₄·5H₂O
Solution B*. 2% (w/v) Na/K tartrate
Solution C. Add 0.4 ml each of B and B* sequentially to a solution containing 39 ml of Solution A and 1 ml 20% (w/v) SDS immediately before use
Solution D. Add 2 ml of Folin & Ciocalteu reagent to 2 ml of H₂O immediately before use

2.3 PHOSPHOLIPID DETERMINATION

This involved extraction of the phospholipid by the method of Folch, Lees and Stanley (1957) and subsequent oxidation and spectrophotometric determination of free phosphate. All tubes, pipettes, pipette tips and glass marbles were washed in phosphate-free RES (Chemical Concentrates (RES) Ltd, Sittingbourne, Kent, U.K.) and rinsed in distilled water before use.

Phospholipid extraction:
1. Samples of approximately 100 μg of phospholipid were made up to 0.25 ml with H₂O in 10 ml glass tubes (RES-washed) and to them was added 5 ml chloroform:methanol 2:1 (v/v). They were then vortexed carefully ensuring no losses and after the addition of 1 ml 0.7% (w/v) NaCl were vortexed again.

2. Following centrifugation for 10 minutes at 3500 rpm in an MSE Centaur clinical centrifuge (MSE, Crawley, Sussex, U.K.) the top phase
was carefully removed and discarded.

3. 2.5 ml of upper phase (3% chloroform, 49% methanol, 0.34% NaCl) was added followed by vortexing, centrifugation and subsequent removal of the upper phase. This step was repeated three times.

4. The lower phases were removed to RBS-washed 13x100 mm borosilicate tubes. Two 1 ml volumes of chloroform:methanol 2:1 (v/v) were used to rinse the glass tubes and were added to the borosilicate tubes.

5. The chloroform was evaporated at 50°C in a water bath under a gentle stream of nitrogen.

Digestion and colorimetric test:

6. To each of the thoroughly dried down samples was added 0.5 ml 72% (w/v) perchloric acid. A glass marble was placed on top of each tube and they were heated at 180°C in a metal heating block for 20 minutes. When cool 3.5 ml of H₂O was added to each tube.

7. 0.5 ml ammonium molybdate (2.5% (w/v)) and 0.5 ml ascorbic acid (10% (w/v)) was added to each tube and vortexed. Colour was developed by heating at 37°C in a water bath for 10 minutes.

8. Absorbance was measured at 797 nm using an LKB visible spectrophotometer, and the mass of phosphorus in the samples was calculated by comparison with standards containing 0-5 µg potassium phosphate.

Since the molecular mass of the average phospholipid is 750, and the atomic mass of phosphorus is 31, mass of phospholipid was calculated from mass of phosphorus by multiplying by 24.2.

2.4 DETERMINATION OF SODIUM DODECYL SULPHATE (SDS)

SDS concentration was assayed by the method of Hayashi (1975), which relies on the partitioning of methylene blue between an organic phase and an aqueous phase containing the SDS.

Samples containing approximately 5 µg SDS were made up to 1 ml in
13x100 mm borosilicate tubes. To each tube was added 500 μl of methylene blue (0.005% in 0.7 mM sodium phosphate, pH 7.2) followed by 3 ml chloroform. The tubes were vortexed and chilled on ice for 15 min to aid in phase separation. They were then vortexed again and centrifuged at 3500 rpm for 5 min in an MSE Centaur clinical centrifuge. The absorbance of the lower phase was measured at 655 nm and SDS content was calculated from a standard curve prepared using samples containing 0–10 μg SDS.

2.5 LIGAND BINDING ASSAYS

Glucose transporter preparations were routinely assayed for cytochalasin B binding sites as a measure of the activity of the glucose transporter. When it was necessary to assay the glucose transporter preparation for contaminating nucleoside transporter, the preparation was assayed for nitrobenzylthioinosine (NEMPR) binding sites.

2.5.1 Cytochalasin B

Cytochalasin B binds to the glucose transporter at the cytoplasmic sugar-binding site (section 1.4). Therefore measuring the capacity of a glucose transporter preparation to bind $[^3H]$cytochalasin B is a good guide to the activity of the transporter in the preparation.

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

$$ R + L \rightleftharpoons RL $$

Therefore: $K_d = [R] [L] / [RL]$  \hspace{1cm} \text{Equation 2.1}$
where \( R, L \) and \( RL \) are the concentrations of free glucose transporter, free cytochalasin B and transporter/cytochalasin B complex at equilibrium respectively. Therefore at equilibrium the ratio of bound to free ligand \((B/F)\) is given by:

\[
B/F = \frac{[RL]}{[L]} = \frac{[R]}{K_d}
\]

Equation 2.2

The concentration of free glucose transporter \([R]\) is related to the total concentration of glucose transporter \([R]_T\) by:

\[
[R]_T = [R] + [RL]
\]

Equation 2.3

Therefore:

\[
B/F = \frac{[RL]}{[L]} = \left( \frac{[R]}{K_d} \right) - \left( \frac{[RL]}{K_d} \right)
\]

Equation 2.4

The fraction of binding sites occupied can be described as:

\[
\frac{[RL]}{[R]_T} = \frac{[RL]}{[R] + [RL]}
\]

Substituting from Equation 2.1:

\[
\frac{[RL]}{[R]_T} = \left( \frac{[R]}{K_d} \right) \left( \frac{[R] + [RL]}{K_d} \right)
\]

\[
= \frac{[L]}{[L] + K_d}
\]

Equation 2.5

If the \( B/F \) ratio is measured using cytochalasin B at a concentration of \( 4 \times 10^{-8} \) M which is less than the \( K_d \) value of 0.12 \( \mu M \) (Zoccoli et al., 1978) then Equation 2.5 indicates that the fraction of the binding sites occupied by cytochalasin B can never exceed 25%, a
maximal figure achieved when measurements are made using very low concentrations of sites. That is to say, \( R_L \) cannot exceed 25% of \([R]_T\) and so Equation 2.4 can be approximated to:

\[
B/F = \frac{[R]_T}{K_d}
\]

Equation 2.6

Therefore the ratio of bound to free cytochalasin B measured using cytochalasin B at a concentration of \( 4 \times 10^{-8} \) M is a good measure of the concentration of cytochalasin B binding sites and therefore the activity of the transporter.

The bound to free ratio was routinely measured by equilibrium dialysis using the apparatus described by Uhlenbeck (1972) and Zoccoli et al. (1978). This consisted of chambers of 50 \( \mu l \) drilled in perspex, pairs of which are separated by dialysis membrane with a cut-off of 12000 daltons (previously boiled once in 20 mM Na\(_2\)CO\(_3\), 1 mM EDTA and three times in water, to remove heavy metal ions). When clamped together one of each pair of chambers was filled with 40 \( \mu l \) of \( 8 \times 10^{-8} \)M \(^3\)Hcytochalasin B and the other filled with the glucose transporter sample. The \(^3\)Hcytochalasin B (New England Nuclear, Southampton, Hants, U.K.) was kept as a stock of \( 8 \times 10^{-6} \) M in ethanol and was diluted before use into the same buffer as that containing the protein sample. Each sample was assayed in triplicate and incubated on a shaker for 18 hours at room temperature, with the tops of the chambers sealed to prevent evaporation. To correct for non-specific binding to membrane lipids, samples previously boiled at 100°C for 5 minutes were assayed in parallel, again in triplicate. Samples (25 \( \mu l \)) were then removed from each chamber, mixed with 2 ml of Ultima Gold scintillation fluid (Canberra Packard Ltd, Pangbourne, Berkshire, U.K.) and assayed for radioactivity by liquid scintillation counting using a Beckman LS5000 liquid scintillation counter. Variation between
Triplicates was less than 10%.

Ratios of bound to free cytochalasin B were calculated as follows:-
The radioactivity measured in the chamber containing the protein sample represented bound plus free $[^3H]$cytochalasin B. Its value in counts per minute (cpm) was designated "x".
The radioactivity measured in the chamber containing no protein represented free $[^3H]$cytochalasin B and was designated "y" cpm. That is to say:-

\[ x = [HL] + [L] \quad \text{Equation 2.7} \]

and \[ y = [L] \quad \text{Equation 2.8} \]

Since \[ \frac{B}{F} = \frac{[HL]}{[L]} \]

\[ \frac{B}{F} = \frac{(x - y)}{y} = \frac{x}{y} - 1 \quad \text{Equation 2.9} \]

Therefore a simple calculation produced a measure of the total B/F value and subtraction of the non-specific B/F value obtained with boiled samples resulted in the value for specific B/F. In order to provide a measure of the relative activities of different transporter preparations this figure was divided through by the concentration of the glucose transporter protein to give the binding activity in units of B/F per mg/ml. A typical figure for a high activity preparation was 90 B/F per mg/ml.

2.5.2 Nitrobenzylthioinosine (NEMPR)

The binding of NEMPR was measured by equilibrium dialysis using [Benzyl-$^3$H]NEMPR as described by Jarvis & Young (1981). In contrast to
the assay for cytochalasin B-binding, this assay uses a saturating concentration of the ligand (30 nM) such that the measured concentration of specifically-bound [\(^3\)H]NBMPR equals the concentration of binding sites. [\(^3\)H]NBMPR (New England Nuclear) was kept as a stock of 3.8 \(\mu\)M in methanol and diluted to 60 nM in the appropriate buffer before use. To measure non-specific binding an assay was carried out in parallel for each sample in the presence of a very large excess (20 \(\mu\)M) of nitrobenzylthioguanosine (NBTGR). NBTGR is another high affinity ligand for the nucleoside transporter which competes with NBMPR for the binding sites. All samples were assayed in triplicate.

In the absence of NBTGR:-
The radioactivity measured in the chamber containing the protein sample represented the bound plus free [\(^3\)H]NBMPR and was designated "A" cpm.
The radioactivity measured in the chamber containing no protein represented the free [\(^3\)H]NBMPR and was designated "B" cpm.
Therefore:-

\[ A - B = \text{bound [NBMPR]} \]  
Equation 2.10

Some of this NBMPR was bound non-specifically to the membrane phospholipids in a manner which is non-saturable and is a function of the free NMBPR concentration. Thus:-

\[ \text{specifically bound [NBMPR]} = (A - B) - pB \quad \text{where } p \text{ is a constant} \]  
Equation 2.11

In the presence of NBTGR:-
The radioactivity measured in the chamber containing the protein sample represented non-specifically bound [\(^3\)H]NBMPR plus free
[\textsuperscript{3}H]NBMPR and was designated A' cpm.
The radioctivity measured in the chamber containing no protein represented free [\textsuperscript{3}H]NBMPR and was designated B' cpm.
Therefore:

\[ A' - B' = [\text{NBMPR}] \text{ bound non-specifically} \]

Equation 2.12

As described above, previous workers have shown that this non-specific binding is a linear function of the free [\textsuperscript{3}H]NBMPR:

\[ A' - B' = pB' \]

Equation 2.13

Therefore

\[ p = (A' - B') / B' \]

Equation 2.14

Substituting Equation 2.14 into Equation 2.11:

\[ \text{specifically bound cpm} = (A - B) - \frac{(A' - B')B}{B'} \]

Equation 2.15

In addition, we know that the total cpm present in any set of chambers, i.e. A + B, represents the original concentration of [\textsuperscript{3}H]NBMPR added, or 60nM (60 pmol/ml). Thus the concentration of specifically-bound [\textsuperscript{3}H]NBMPR could be calculated from the following equation:

\[ \text{specifically bound [NBMPR] (pmol/ml)} = \frac{(A-B) - (A' - B')B}{(A + B)} \cdot 60 \]

Equation 2.16
2.6 ELECTROPHORETIC TECHNIQUES

Electrophoretic techniques were employed both analytically, for example in the visualisation of cleavage fragments by SDS/polyacrylamide gel electrophoresis, and preparatively, for example during isolation of purified peptide from an SDS/polyacrylamide gel by electroelution. Electro transfer of peptides previously separated on a gel onto nitrocellulose membrane allowed subsequent identification of the peptides by immunolabelling.

2.6.1 SDS/polyacrylamide gel electrophoresis

SDS/polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970) using 1.5 mm or 3.0 mm thick 12% acrylamide slab gels, cast and run using the Protean I system from Bio-Rad (Bio-Rad, Richmond, C.A., U.S.A). All materials used were of electrophoresis grade (either BDH 'Electran' or Bio-Rad).

Protein samples of volume x were solubilised by the addition of x/10 volumes of denaturing buffer (500 mM Tris-HCl, 10 mM EDTA, 10% SDS, pH 6.8). To each solubilized sample was added x/20 volumes of 100 mM DTT, x/10 volumes of glycerol and x/100 volumes of Pyronin Y (12 mg/ml) as a tracker dye. The final gel samples were 39.7 mM Tris-HCl, 0.8 mM EDTA, 0.8% SDS, 4 mM DTT and were stored at -20°C.

The size of sample loaded was dependent on the thickness of the gel, a maximum of 125 µl or 50 µg of protein being run on a 1.5 mm thick gel and a maximum of 250 µl or 120 µg of protein being run on a 3 mm thick gel. Samples were loaded under the running buffer (25 mM Tris, 190 mM glycine, 0.1% (w/v) SDS, pH 8.3) into the wells of a 2 cm deep stacking gel (3% (w/v) acrylamide, 0.08% (w/v) bis-acrylamide, 125 mM Tris-HCl, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, 0.05% (v/v) tetramethylethlenediamine (TEMED)) and separated by
passage through a 10 cm deep separating gel (12% (w/v) acrylamide, 0.32% (w/v) bis-acrylamide, 375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, 0.016% (v/v) TEMED). Electrophoresis using 1.5 mm thick gels was carried out at 15mA per gel through the stacking gel and at 30 mA per gel through the separating gel and continued until the tracker dye was approximately 1 cm from the lower edge of the gel. Currents of twice this were employed for 3 mm thick gels. To aid in the estimation of the $M_r$ of protein bands, protein standards of known $M_r$ were also electrophoresed on each gel. The standards used were either a commercial preparation of low $M_r$ markers from Bio-Rad ($M_r$ 14400-97400) or a preparation made up in the laboratory containing equal amounts by mass of BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, soybean trypsin inhibitor, and cytochrome C, thus covering the range of $M_r$ 12400-66000. The latter mixture was prepared for electrophoresis as described above for protein samples in general. Estimates of polypeptide $M_r$ were made by comparison of distance migrated with the distance migrated by the standard proteins, using a plot of $\log_{10} M_r$ versus migration distance. In electroblotting experiments (section 2.6.4) Coomassie blue-prestained marker proteins of $M_r$ in the range 16000-110000 (Bio-Rad) were electrophoresed on the same gel as the samples of interest to facilitate estimation of the $M_r$ of transferred protein bands.

If the gels were to be stained to visualise protein they were subjected to Coomassie blue staining (section 2.7.1) or silver staining (section 2.7.2), depending on the sensitivity required. If they were to be used for electroelution (section 2.6.3) or electrotransfer (section 2.6.4) no staining procedure was carried out.
Tricine/SDS/polyacrylamide gel electrophoresis was carried out by the method of Schägger & von Jagow (1987) using 1.5mm or 3.0mm thick slab gels, cast and run using the Protean I system from Bio-Rad. Protein samples were prepared as described in Section 2.6.1 and loaded under the cathode electrode buffer (100 mM Tris-HCl, 100 mM tricine, 0.1% (w/v) SDS, pH 8.25) into the wells of a 2 cm deep stacking gel (3.9% (w/v) acrylamide, 0.12% (w/v) bis-acrylamide, 744 mM Tris-HCl, pH 8.45, 0.07% (w/v) SDS, 0.08% (w/v) ammonium persulphate, 0.08% (v/v) TEMED). Separation of the protein bands was then achieved by electrophoresis through a 3 cm deep spacer gel (9.7% (w/v) acrylamide, 0.3% (w/v) bis-acrylamide, 1 M Tris-HCl, pH 8.45, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate, 0.03% (v/v) TEMED) and a 7 cm deep separating gel (15.5% (w/v) acrylamide, 1.0% (w/v) bis-acrylamide, 1 M Tris-HCl, pH 8.4, 0.1% (w/v) SDS, 0.03% (w/v) ammonium persulphate, 0.03% (w/v) TEMED). The anode electrode buffer was 200 mM Tris-HCl, pH 8.9. These gels were electrophoresed at 30 volts until the samples had entered the stacking gel matrix (approximately 1 hour) and then at 95 volts for approximately 16 hours until the tracker dye was 1 cm from the lower edge of the gel. Due to the use of a constant voltage power supply these settings were adhered to whether one or two, 1.5 mm or 3.0 mm gels were run simultaneously.

Due to the increased range of Mr of proteins that could be separated by this electrophoresis method, two different preparations of Mr standards were used: the low Mr standard mix from Bio-Rad as described above (Mr 14400-97400) and 'very' low Mr standards from Sigma, produced by cleavage of apomyoglobin (from horse heart) with cyanogen bromide (Mr 2510-16950). These were combined in one gel sample so as to load 2 μg of each protein band per lane. Staining was as for SDS/polyacrylamide gels when required.
2.6.3 Electroelution and electrodialysis

Electroelution of proteins or peptides from SDS/polyacrylamide gels or Tricine/SDS/polyacrylamide gels was performed by the method of Jacobs & Clad (1986), using a Schleicher & Schuell Biotrap (Dassel, West Germany).

After electrophoresis of the sample two adjoining lanes of the gel comprising an Mr standard lane and a sample lane were removed from the gel and rapidly silver-stained (section 2.7.2). Meanwhile the rest of the gel was kept in a small volume of running buffer at 4°C. The silver-stained lanes were then used as a template for excision of the required band from the gel. Once excised the bands were cut into pieces of approximately 0.5 cm square and positioned in the elution chamber of the Biotrap, such that their shortest axes were running parallel with the eventual electric current. Care was taken not to block the flow of buffer in the chamber, the buffer being 25 mM Tris, 190 mM glycine, 0.025% (w/v) SDS, pH 8.3. Electroelutions were performed at 100 volts for approximately 16 hours followed by 200 volts for one hour. Before harvesting the sample (approximately 600-800 µl) the current was reversed for 12 seconds at 200 volts to remove any sample attached to the anodic membrane of the trap-chamber and the eluted material was then flushed up and down twice in a Pasteur pipette to wash the walls of the chamber. The electroeluate normally contained a very high concentration of SDS due to the nature of the membranes of the Biotrap. Therefore it was always necessary to electrodialyse after electroelution if the samples were to be used for proteolytic or chemical cleavage experiments.

Electrodialysis was carried out by the same method as electroelution, the sample being placed back into the trap-chamber, using fresh biotrap membranes, and being electrodialysed against 25 mM Tris, 190 mM glycine, pH 8.3 at 100 volts for 6-9 hours. The
subsequent decrease in SDS concentration was continually monitored by means of the SDS assay (Section 6.4), samples of 1 μl being taken with a micropipettor every 30 mins. The concentration of SDS was decreased to 1% at which point the sample was harvested.

2.6.4 Electrotransfer and Immunoblotting

Electrotransfer of proteins and peptides from SDS/polyacrylamide and Tricine/SDS/polyacrylamide gels was performed by a semi-dry method using an LKB Novoblot system within an LKB Multiphor II electrophoresis tank (LKB-Pharmacia). Immediately after completion of gel electrophoresis the gel to be blotted, neither fixed nor stained and with the stacking gel removed, was soaked in transfer buffer (39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, 20% (v/v) methanol) at room temperature for 20 minutes with gentle shaking. The blotting unit was then constructed as follows: 1 piece of nitrocellulose transfer membrane (0.45 nm pore size, Bio-Rad) was pre-wet with transfer buffer and laid onto a stack of 9 pieces of pre-wet filter paper on the wet anode plate of the apparatus. Care was taken to avoid air bubbles. The gel was then laid on top of this followed by a further 9 pieces of pre-wet filter paper and the wet cathode plate. The gel was electroblotted at 1.6 mA per cm$^2$ of gel for 1 hour, using an LKB Macrodrive I power supply.

For immunoblotting each sheet of nitrocellulose membrane was incubated in a plastic 100 ml screw-cap bottle on a tube-roller in Tris-buffered saline (TBS, 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) for 10 minutes. Each was then incubated with blocking buffer (TBS containing 5% (w/v) low-fat dried milk powder and 0.02% (w/v) sodium azide) for 1 hour at room temperature to block the remaining protein-binding sites on the transfer media. The membranes were then washed twice for 5 minutes in TTBS (TBS containing 0.02% (v/v) Tween-20) and incubated
overnight at room temperature with the required antibody at the appropriate concentration in antibody-buffer (TBS containing 1% (w/v) low-fat dried milk powder and 0.02% (w/v) sodium azide). Anti-peptide sera were used at dilutions in the range of 1:50 to 1:100 and affinity purified anti-peptide and anti-transporter antibodies were used at concentrations of 5-10 μg/ml. Following this incubation the membranes were washed twice in TTBS and once in TBS. The bound primary antibodies (all raised in rabbits) were then detected by reaction with secondary antibody conjugated to alkaline-phosphatase, in this case goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad). The reaction was achieved by incubation of the membranes with a 1:3000 dilution of the antibody-conjugate in antibody buffer for 2 hours at room temperature. After two washes in TTBS and one wash in TBS (to remove Tween-20 which could otherwise form a precipitate when in contact with the colour development reagents) the membranes were transferred to glass dishes and incubated in alkaline phosphatase-substrate (0.37 mM nitro-blue tetrazolium, 0.35 mM 5-bromo-4-chloro-3-indoyl phosphate in 0.1 M sodium bicarbonate, 1 mM magnesium chloride, pH 9.8) until the desired amount of colour had developed. The reaction was terminated by three washes of the membranes in distilled water.

2.7 STAINING OF SDS/POLYACRYLAMIDE GELS

The technique chosen for staining SDS/polyacrylamide gels was largely dependent on the amount of protein to be detected. Coomassie-blue staining could detect as little as 1 μg of protein whereas silver staining could detect as little as 1 ng. Both techniques described here were used for staining both SDS/polyacrylamide and Tricine/SDS/polyacrylamide gels.
2.7.1 Coomassie-blue staining

The gel was fixed overnight by soaking in 10% acetic acid, 25% isopropanol with constant gentle shaking. This also served to remove excess SDS from the gel which would otherwise hinder staining. The gel was then soaked for 8-10 hours in stain 1 (10% (v/v) acetic acid, 25% (v/v) isopropanol, 0.025% (w/v) Page Blue 83 (BDH)) and for 15 hours in stain 2 (10% (v/v) acetic acid, 10% (v/v) isopropanol, 0.0025% (w/v) Page Blue 83). Finally the gel was destained for 24-72 hours in 10% (v/v) acetic acid. The latter also served as a short-term storage solution for stained gels.

2.7.2 Silver staining

This was performed by the method of Morrisey (1981). The gel was soaked in 5% (v/v) methanol, 7% (v/v) acetic acid for 15 minutes to remove excess SDS and then fixed by soaking in 10% (w/v) glutaraldehyde for 30 minutes with gentle shaking. The glutaraldehyde was thoroughly removed by washing in many changes of water for 2 hours or overnight. The gel was then soaked in 5 μg/ml (DTT) for 30 minutes and 0.1% (w/v) silver nitrate for 30 minutes, always with continual shaking. After rinsing once in distilled water and once in a small amount of developer (0.05% (v/v) formalin, 3% (w/v) sodium carbonate) the gel was soaked in developer with continual shaking until the desired degree of staining was achieved. The reaction was stopped by the addition of citric acid to 74 mM and incubation for 10 minutes. After rinsing in a large volume of distilled water the stained gel was stored in 0.03% (w/v) sodium carbonate.
2.8 PHOTOLABELLING OF THE GLUCOSE TRANSPORTER

The glucose transporter was routinely photolabelled in a D-glucose-inhibitable manner either at the extracellular surface by $[^3H]$ATB-BMPA or at the cytoplasmic surface by $[^3H]$cytochalasin B.

2.8.1 Cytochalasin B

$[^3H]$Cytochalasin B (New England Nuclear) was dried down from a solution in ethanol, resuspended in 5 μl of ethanol and combined with glucose transporter and L-glucose such that the resulting solution contained 1 μM $[^3H]$cytochalasin B, 0.139 mg/ml glucose transporter and 0.495 M L-glucose in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4. The mixture was vortexed briefly, incubated on ice for 5 minutes, and introduced into scrupulously clean stoppered quartz cuvettes previously flushed with nitrogen. The cuvettes were positioned on a petri-dish supported on ice and irradiated for 10 minutes with high intensity ultraviolet light from a 100 W lamp (Mineralight model R-52, Ultraviolet products Inc., San Gabriel, C.A., U.S.A.) positioned 10 cm from the upper surface of the cuvettes. Following irradiation the photolabelled material was freed of unbound $[^3H]$cytochalasin B by suspending in twice the volume of (50 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, 20 μM unlabelled cytochalasin B, pH 7.4) followed by centrifugation at 55000 rpm for 1 hour in a Beckman TL-100 micro-ultracentrifuge. The pellet was resuspended in the same buffer and the wash step was repeated. The final pellet was resuspended in 500 μl of (50 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, pH 7.4).
2.8.2 ATB-BMPA

$[^{3}H]$ATB-BMPA (4 mCi at a specific activity of 10 Ci/mmol) was mixed with glucose transporter in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4, resulting in a solution of 4.5 ml containing approximately 8.5 mg of protein. The solution was placed into plastic petri dishes and irradiated at 300 nm in a Rayonet Mini-reactor for three x 30 seconds with agitation between each treatment. The photolabelled glucose transporter was washed free of unbound $[^{3}H]$ATB-BMPA by suspending to 35 ml with (50 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, pH 7.4) and centrifuging at 45000 rpm at 4°C for 1 hour in a Prepspin 65 ultracentrifuge (MSE). The final pellet was resuspended to 4.5 ml in the same buffer.

2.8.3 Radioactivity profiles of the photolabelled glucose transporter determined by scintillation counting

Photolabelling of the glucose transporter and its fragments was investigated routinely by obtaining a profile of radioactivity from SDS/polyacrylamide or Tricine/SDS/polyacrylamide gels (sections 2.6.1 and 2.6.2). After staining, the required gel tracks were excised as 1 cm wide strips and sliced into 2 mm slices down the length of each track working from top to bottom. Each slice was placed into a numbered vial and the radioactivity was eluted by one of the following methods: Either (1) the slices were dehydrated by air-drying for 24-48 hours and then dissolved by heating to 60°C for 8 hours in 400 µl of hydrogen peroxide solution (30% (v/v) in water) or (2) they were treated with 1 ml of 50% Solvable (New England Nuclear) and heated to 50°C for 3 hours which serves to swell the polyacrylamide slices and allow elution of the protein into the solution. The radioactivity was then counted following the addition of Ultima Gold scintillation
cocktail (Canberra Packard Ltd.) using 2 ml per vial following protocol (1) or 4 ml per vial following protocol (2). Scintillation counting was performed using a Beckman LS5000 Liquid Scintillation Counter.
CHAPTER 3 PROTEOLYTIC DISSECTION AS A PROBE OF CONFORMATIONAL CHANGES IN THE HUMAN ERYTHROCYTE GLUTOSE TRANSPORTER.

3.1 INTRODUCTION.

Two main types of model have been proposed to account for the features observed during the transport of D-glucose across the human erythrocyte membrane by the glucose transport protein (Section 1.3.1). Although the two models are fundamentally different in many respects, both the single-site alternating conformation model (Vidaver, 1966; Barnett et al., 1975; Baldwin & Lienhard, 1981) and the more complex two-site model (Carruthers, 1986a; Carruthers, 1986b; Helgerson & Carruthers, 1987) imply that conformational changes in the transport protein are central to the molecular mechanism of transport, and evidence for the involvement of such conformational changes is strong (Section 1.3.2). However, the identity of those regions of the protein affected by the changes remains largely unknown. A recent report on the inactivation of transport by alkylating agents suggested that substrate-induced conformational changes occur primarily within the hydrophobic membrane-spanning regions of the protein, while the hydrophilic segments outside the bilayer are largely unaffected (Rampal & Jung, 1987). In this study I have used tryptic digestion to investigate more fully the possible involvement of the hydrophilic, cytoplasmic regions of the protein in conformational changes.

3.1.1 Cleavage of the glucose transporter by trypsin.

As discussed previously (Section 1.6.1.1) trypsin cleaves the glucose transporter, in the absence of substrates or inhibitors of transport, in a characteristic manner solely at the cytoplasmic surface of the membrane as summarised in Figure 3.1. This study
Figure 3.1 Tryptic cleavage of the human erythrocyte glucose transporter in the absence of substrates or inhibitors of transport.

Cleavage occurs at a number of sites in the central extramembranous loop between Arg212 and Arg369, and in the C-terminal region of the sequence following Lys456. The order in which cleavages occur is not obligatory, the ultimate products being a glycosylated fragment of Mr 23000 - 42000 and a non-glycosylated fragment of Mr 18000. All potential sites of tryptic cleavage on the cytoplasmic side of the membrane are shown (▲). (For details see text, Section 1.6.1.1.)
describes the effects of D-glucose and of reversible inhibitors of transport on the rate and pattern of these tryptic cleavages of the transporter. The inhibitors used were phenyl β-D-glucopyranoside (phenyl glucoside) and cytochalasin B, which bind preferentially at the cytoplasmic side of the membrane (Barnett et al., 1975; Devés & Krupka, 1978), and 4,6-O-ethylidene-D-glucose (ethylidene glucose) and phloretin, which bind preferentially at the extracellular side of the membrane (Baker & Widdas, 1973; Krupka, 1985). These inhibitors would be predicted by the alternating conformation model for transport to bind preferentially to the inward-facing and outward-facing conformations of the protein respectively, and so cause it to accumulate in one of these forms.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Tryptic digestion

Proteolysis of the transporter using trypsin (from bovine pancreas, diphenylcarbamyl chloride-treated) at a concentration of 1% (w/w) that of the transporter, resulted in a relatively slow rate of cleavage suitable for investigation of the effect of ligands on the rate of cleavage and pattern of fragmentation of the transporter. Digestions were carried out in 50 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, pH 7.4 at a range of temperatures using purified glucose transporter and trypsin at concentrations of 216 μg/ml and 2.5 μg/ml respectively (unless stated otherwise). Such digestions were performed in the presence or absence of sugars or transport inhibitors, which were present at concentrations of about 10-fold their K_m or K_i respectively. Each incubation usually lasted for 2 hours during which time samples were taken for SDS/polyacrylamide gel electrophoresis, the trypsin in them being inactivated by the addition of bovine lung
aprotinin (Boehringer Corp. Ltd., Lewes, East Sussex, U.K.) to a concentration of twice that of the trypsin. Gel samples were prepared directly from these as described in section 2.6.1.

Control experiments were carried out using alkali-stripped human erythrocyte membranes (Baldwin et al., 1982) to ensure that any effects of substrates and inhibitors of glucose transport on the rate of transporter cleavage resulted from their binding to the transporter rather than to trypsin. None of the compounds tested had any effect on the rate of tryptic cleavage of the anion transporter in these membranes under conditions identical to those used for digestion of the purified glucose transporter (results not shown). However, their effects on the rate of production of fragments of the glucose transporter in these membranes were similar to those seen using the purified protein.

The apparent rate of cleavage of the glucose transporter by trypsin varied slightly between transporter preparations, probably because of their variable content of sealed vesicular material in which the cytoplasmic region of the transporter is not accessible for cleavage. Therefore, comparisons of the effects of different sugars or inhibitors on the cleavage rate were always made by performing parallel digestion experiments simultaneously, using identical trypsin and transporter preparations. The proportion of transporters inaccessible to trypsin was estimated from the amount of transporter remaining uncleaved after extended periods of digestion. Typically, about 25% of the transporter remained intact even after treatment with 5% (w/w) trypsin for 26 hours at 25°C, fresh protease being added after 0,3 and 6 hours.
3.2.2 SDS/polyacrylamide gel electrophoresis and subsequent spectrophotometric estimation of intact glucose transporter.

SDS/polyacrylamide-gel electrophoresis was carried out as described (section 2.6.1) using 12% polyacrylamide gels. After staining with Coomassie blue the gels were scanned spectrophotometrically at 530nm using a Joyce-Loebl Chromoscan 3. Previous studies (S.A. Baldwin, unpublished work) have shown a linear relationship between peak area and the amount of transporter on a gel over the concentration range used here. Routinely, the peak area data were corrected for the presence of transporter inaccessible to trypsin by subtracting the value for intact transporter remaining after extended trypsin treatment as described above. After correction in this fashion the data indicated the percentage of the 'accessible' transporters remaining after various periods of tryptic digestion. They were then fitted to straight line, first order plots by linear regression.

3.3 RESULTS

The rate of cleavage of the membrane-bound glucose transporter by trypsin at 25ºC, in the presence of the non-transported substrate analogue 4,6-O-ethyldene-D-glucose (ethyldene glucose) at a concentration of 200 mM was found to be markedly lower than in the presence of 200 mM L-glucose, which does not bind to the protein (Figures 3.2 and 3.3). The extents of cleavage were measured by spectrophotometric scanning of the gels shown in Figure 3.2. This procedure slightly underestimates the true extent of cleavage because the Mr 45000 tryptic fragment (arrowed in Figure 3.2) is not fully resolved from the broad Mr 55000 band of the intact glucose transporter. However, after correction for the presence of 28% of the glucose transporters that were inaccessible to trypsin, the cleavage
Figure 3.2 The effect of ethylidene glucose on tryptic cleavage of the glucose transporter.

Glucose transporter (216 μg/ml) was digested at 25°C with 1.2% trypsin for the times indicated, in the presence of either (A) 200 mM-ethylidene glucose or (B) 200 mM-L-glucose. Samples were electrophoresed on a 12% SDS/polyacrylamide gel and then stained with Coomassie Blue. The positions of marker proteins of known Mr, and the Mr 45000 fragment of the transporter (arrowed), are indicated. DF = tracking dye front.
Figure 3.3 The effect of ethylidene glucose on the rate of tryptic cleavage of the glucose transporter.

The relative amounts of intact transporter remaining after various times of tryptic digestion in the presence of 200 mM-ethylidene glucose (●) or 200 mM-L-glucose (○) were determined from spectrophotometric scans of the gels shown in Figure 3.2. The data are shown after correction for the presence of transporters inaccessible to trypsin.
data appeared to exhibit first order kinetics:

Tryptic cleavage of the transporter can be represented as follows:-

\[
\text{glucose transporter} \rightarrow \text{fragments}
\]

\[
(T) \quad \text{(F)}
\]

If this process obeys first order kinetics, the following relationships exist:

\[
- \frac{d[T]}{dt} = k[T]
\]

\[
- \frac{d[T]}{[T]} = k \ dt
\]

Let \([T]\) at time zero = \([T]_0\) and let \([T]\) at time \(t = [T]_t\). Integrating between these limits we get:

\[
\int_{[T]_0}^{[T]_t} \frac{d[T]}{[T]} = k \int_{0}^{t} dt
\]

Thus:

\[
- \left[ \ln[T] \right]_0^{t} [T]_t = k \left[ t \right]_0^{t}
\]

and therefore:

\[
- (\ln[T]_0 - \ln[T]_t) = -kt
\]

\[
\ln[T]_t - \ln[T]_0 = -kt
\]
Rearranging and converting to log to the base 10 we get:

$$\log[T]_t = -\frac{kt}{2.303} + \log[T]_0$$

Therefore, a plot of $\log[T]_t$ against $t$ would be expected to give a straight line with the gradient equal to (the first order rate constant for cleavage / 2.303). This was indeed seen to be the case (Figure 3.4).

The finding that tryptic cleavage of the transporter approximated to a first order process enabled the effects of transport inhibitors and substrates upon the process to be quantified. Ethylidene glucose was found to decrease the apparent first order rate constant for cleavage by approximately 4-fold relative to L-glucose (Table 3.1). Inspection of the gels showed that the patterns of fragments produced in the presence of the two sugars were the same, but that the rates of production of both the Mr 45000 fragment (arrowed in Figure 3.2) and the Mr 18000 fragment and its precursors were reduced in the presence of ethylidene glucose. This finding was confirmed by spectrophotometric scanning to quantify the Mr 18000 fragment, as described below.

The mechanism of the different effects of L-glucose and ethylidene glucose was further investigated by examining the effects of different concentrations of the two substances on tryptic cleavage. In this experiment the transporter (160 µg/ml) was incubated for a fixed period (4 hours) with trypsin (1% w/w) at 25°C, and the effects of the sugars were monitored by quantifying the amounts of both the uncleaved transporter and its Mr 18000 tryptic fragment on a gel. The extent of cleavage was little affected by L-glucose at concentrations up to 400 mM but decreased as the concentration of ethylidene glucose
Figure 3.4 The effect of ethylidene glucose on the rate of tryptic cleavage of the glucose transporter.

The relative amounts of intact transporter remaining after various times of tryptic digestion in the presence of 200 mM-ethylidene glucose (●) or 200 mM-L-glucose (○) were determined from spectrophotometric scans of the gels shown in Figure 3.2. This semi-logarithmic plot of the data in Figure 3.3 is indicative that the data displays first order kinetics. The straight lines have been fitted by linear regression and the gradient of each line is equal to (the first order rate constant for cleavage / 2.303).
Figure 3.5 The concentration dependence of the effect of ethyldene glucose on tryptic cleavage of the glucose transporter.

Glucose transporter (160 µg/ml) was digested at 25°C for 4 hours with 1% (w/w) trypsin in the presence of various concentrations of ethyldene glucose (●, ▲) or L-glucose (○, △). Samples were electrophoresed on a 12% SDS/polyacrylamide gel and then stained with Coomassie Blue. The relative amounts of the intact transporter (●, ○) and of the tryptic fragment of apparent Mₚ 18000 (▲, △) were estimated by spectrophotometric scanning.
increased up to 400 mM (Figure 3.5). The half-maximal effect of the sugar analogue on the amount of the Mr 18000 fragment produced was seen at approximately 25 mM ethylidene glucose. The concentration required for half-maximal effect on the amount of the intact transporter remaining after digestion appeared to be higher, at approximately 75 mM. However, less confidence can be placed upon this estimate because the presence of the Mr 45000 tryptic fragment precludes accurate quantification of the intact transporter, as mentioned above. Accurate estimation of the Mr 18000 fragment is more easily achieved because it is well resolved from the other fragments on the gel.

The effect of phloretin upon the rate of tryptic cleavage of the transporter was also investigated. This compound is a potent inhibitor of transport and resembles ethylidene glucose in that it binds at the extracellular surface of the transporter (Krupka, 1985). The effect of phloretin (100 μM) was similar to that of ethylidene glucose; the rate of cleavage of the transporter by trypsin was slowed and this effect was manifested as a decrease in the rate of production of both the Mr 45000 and the Mr 18000 tryptic fragments. After correction for the presence of 36% of the transporter which was inaccessible to trypsin, the cleavage data again exhibited apparent first-order kinetics (Figure 3.6). Phloretin decreased the apparent first-order rate constant for cleavage by approximately 3-fold (Table 3.1).

Ethylidene glucose and phloretin bind preferentially at the extracellular surface of the membrane (Baker & Widdas, 1973; Krupka, 1985). For comparison the effects of cytochalasin B and phenylglucoside were investigated. These inhibitors bind preferentially at the cytoplasmic surface of the membrane (Barnett et al., 1975; Devés & Krupka, 1978). Both cytochalasin B (20 μM) and phenyl glucoside (50 mM) slowed the rate of cleavage of the glucose
Figure 3.6 The effect of phloretin on the rate of tryptic cleavage of the glucose transporter.

Glucose transporter (216 μg/ml) was digested at 25°C with 1.2% (w/w) trypsin for the times indicated, in the presence of either 100 μM-phloretin and 1% (v/v) ethanol (●), or 1% (v/v) ethanol alone (○). Samples were electrophoresed on a 12% SDS/polyacrylamide gel, stained with Coomassie Blue, and the relative amounts of intact transporter remaining determined by spectrophotometric scanning. The data are shown as a semi-logarithmic plot after correction for the presence of transporters inaccessible to trypsin. The straight lines have been fitted by linear regression.
transporter at 25°C, relative to a control (Figures 3.7 and 3.8, Table 3.1). However this effect was much smaller than that seen in the presence of either ethylidene glucose or phloretin.

A possible explanation for the protection against tryptic cleavage afforded by ethylidene glucose and phloretin might be that these agents stabilise the transporter in a trypsin-resistant, outward-facing conformation. Conversely, if the alternating conformation model for transport is correct, cytochalasin B and phenyl glucoside might be expected to increase the rate of cleavage by stabilising an inward-facing conformation of the transporter. No such effect was seen at 25°C. However, analyses of the kinetics of glucose transport by Lowe & Walmsley (1986), using such an alternating conformation model, led to the prediction that the proportion of the transporter in each conformation is temperature-dependent. In the human erythrocyte, at 25°C, 75% of the transporters are predicted to be in the inward-facing conformation. Therefore at this temperature any effects of cytochalasin B and phenyl glucoside mediated by conformational changes might be expected to be smaller than those produced by stabilisers of the outward-facing conformation, such as phloretin and ethylidene glucose. To investigate this possibility, digestion experiments were performed at 45°C, at which temperature approximately 50% of the glucose transporters in the erythrocyte are predicted to be in the inward-facing conformation and approximately 50% are predicted to be in the outward-facing conformation. Experiments were also performed at 50°C where the effects, if any, of cytochalasin B and phenyl glucoside could be expected to be even more pronounced. The protective effects of phloretin and ethylidene glucose at 45°C closely resembled those seen at 25°C (Table 3.1). Phenyl glucoside (50 mM) had no significant effect on the rate of tryptic cleavage of the transporter either at 45°C or 50°C (Table 3.1). However, unexpectedly, cytochalasin B
Figure 3.7 The effect of cytochalasin B on the rate of tryptic cleavage of the glucose transporter.

Glucose transporter (216 μg/ml) was digested at 25°C with 1.15% (w/w) trypsin for the times indicated, in the presence of either 20 μM-cytochalasin B and 0.5% (v/v) ethanol (●), or 0.5% (v/v) ethanol alone (○). Samples were electrophoresed on a 12% SDS/polyacrylamide gel, stained with Coomassie Blue, and the relative amounts of intact transporter remaining determined by spectrophotometric scanning. The data are shown as a semi-logarithmic plot after correction for the presence of transporters inaccessible to trypsin. The straight lines have been fitted by linear regression.
Figure 3.8 The effect of phenyl glucoside on the rate of tryptic cleavage of the glucose transporter.

Glucose transporter (162 μg/ml) was digested at 25°C with 1.54% (w/w) trypsin for the times indicated, in the presence of either 50 mM-phenyl glucoside (●), or with no additives (O). Samples were electrophoresed on a 12% SDS/polyacrylamide gel, stained with Coomassie Blue, and the relative amounts of intact transporter remaining determined by spectrophotometric scanning. The data are shown as a semi-logarithmic plot after correction for the presence of transporters inaccessible to trypsin. The straight lines have been fitted by linear regression.
exerted protective effects at both temperatures; the rate constants for tryptic cleavage were decreased by approximately 1.4-fold and 4-fold in the presence of cytochalasin B (20 μM) at 45°C and 50°C respectively (Table 3.1).

The effect of the physiological substrate D-glucose on tryptic cleavage of the transporter was examined at both 25°C and 45°C using trypsin at 1% (w/w). At 25°C, D-glucose (200 mM) increased the first-order rate constant for cleavage by trypsin by approximately 1.5-fold, relative to that seen in the presence of L-glucose (200 mM) (Figure 3.9). Although the effect was small it was reproducible as can be seen in Table 3.1. However, no significant effects of D-glucose were seen at 45°C (Table 3.1).

3.4 DISCUSSION

Both phloretin and ethylidene glucose were found to decrease the rate of tryptic cleavage of the glucose transporter at 25°C and 45°C. Phloretin has been reported to bind exclusively to a site on the transporter that is exposed to the extracellular environment (Helgerson & Carruthers, 1987; Krupka, 1985), whereas trypsin cleaves the protein solely at sites on the cytoplasmic surface of the membrane (Deziel & Rothstein, 1984; Lienhard et al., 1984). The effect of phloretin cannot, therefore, be the result of simple steric hindrance following its occupation of the binding site. Instead it must involve a conformational change to a form of the protein in which the sites of cleavage are less accessible to trypsin.

The same explanation probably also accounts for the effect of ethylidene glucose, although this sugar analogue does not exhibit absolute specificity for the extracellular substrate-binding site (Helgerson & Carruthers, 1987; Baker & Widdas, 1973). The
Figure 3.9 The effect of D-glucose on the rate of tryptic cleavage of the glucose transporter.

Glucose transporter (116 µg/ml) was digested at 25°C with 1% (w/w) trypsin for the times indicated, in the presence of either 200 mM-D-glucose (●) or 200 mM-L-glucose (○). Samples were electrophoresed on a 12% SDS/polyacrylamide gel, stained with Coomassie Blue, and the relative amounts of intact transporter remaining determined by spectrophotometric scanning. The data are shown as a semi-logarithmic plot after correction for the presence of transporters inaccessible to trypsin. The straight lines have been fitted by linear regression.
<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Inhibitor/substrate</th>
<th>10^3 x rate constant (min⁻¹)</th>
<th>Control</th>
<th>10^3 x rate constant (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>Ethylidene glucose (200mM)</td>
<td>4.5 ± 0.6</td>
<td>L-glucose (200mM)</td>
<td>17.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Phloretin (100μM)</td>
<td>5.5 ± 0.4</td>
<td>Ethanol (1% v/v)</td>
<td>15.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Cytochalasin B (20μM)</td>
<td>25.6 ± 1.5</td>
<td>Ethanol (0.5% v/v)</td>
<td>30.9 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Phenyl glucoside (50mM)</td>
<td>17.2 ± 1.5</td>
<td>No additives*</td>
<td>33.6 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>D-glucose (200mM)</td>
<td>21.6 ± 1.6</td>
<td>L-glucose (200mM)</td>
<td>13.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>D-glucose (200mM)</td>
<td>19.8 ± 1.4</td>
<td>L-glucose (200mM)</td>
<td>12.8 ± 0.7</td>
</tr>
<tr>
<td>45</td>
<td>Ethylidene glucose (200mM)</td>
<td>9.6 ± 2.1</td>
<td>L-glucose (200mM)</td>
<td>37.0 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>Phloretin (100μM)</td>
<td>16.3 ± 1.5</td>
<td>Ethanol (1% v/v)</td>
<td>36.2 ± 2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>-------</td>
<td>-----------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>Cytochalasin B (20μM)</td>
<td>26.6 + 1.7</td>
<td>36.2 + 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyl glucoside (50mM)</td>
<td>9.7 + 1.0</td>
<td>L-glucose (200mM)</td>
<td>7.0 + 3.4</td>
<td></td>
</tr>
<tr>
<td>D-glucose (200mM)</td>
<td>10.4 + 2.5</td>
<td>L-glucose</td>
<td>7.0 + 3.4</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 The effects of substrate and inhibitors on the rate of tryptic cleavage of the glucose transporter.

Rate constants were calculated from the tryptic cleavage data after correction for the presence of inaccessible transporters, as described (Section 3.2.2). Each value, and its associated S.E.M., was calculated from the results of a single experiment using linear regression analysis.

* Phenyl glucoside was dissolved directly in the incubation mixture. Therefore its control was an identical incubation without additives.

** The incubation in the presence of L-glucose was used as a control for phenyl glucoside and D-glucose in a single experiment, since identical results had been obtained previously for incubations in the presence and absence of L-glucose.
concentration dependence of the effect revealed in Figure 3.5 indicates that it does not merely reflect a non-specific perturbation of the lipid environment of the protein. The inevitable presence of a proportion of inactive transporters in the preparations used in this study (Baldwin et al., 1982) complicates the kinetic analyses of the cleavage process. However, the concentration of ethylidene glucose required to afford half-maximal protection against cleavage, 25 mM, corresponds closely to the value of 26 mM measured as the $K_i$ for inhibition of cytochalasin B binding to protein-depleted membranes (Gorga & Lienhard, 1981). It has been suggested that such a high $K_i$ value reflects binding to a low-affinity site at the cytoplasmic surface of the membrane (Helgerson & Carruthers, 1987). However, even if a portion of the ethylidene glucose is binding to such a site, it is unlikely for two reasons that the resultant steric hindrance is responsible for the effect on cleavage. Firstly, at the concentration where the half-maximal effect is seen (25 mM), the two-site model predicts that only 17% of the intracellular sites would be occupied (calculated from the data of Helgerson & Carruthers, 1987). Secondly, occupation of presumably the same intracellular sites by the bulky molecules phenyl glucoside and cytochalasin B causes little or no steric hindrance respectively to the action of trypsin at 25°C.

The observation that neither cytochalasin B nor phenyl glucoside increased the rate of tryptic cleavage of the transporter even at 50°C is at first sight difficult to reconcile with a single-site, alternating conformation model for transport in which the outward-facing conformation of the protein is resistant to tryptic cleavage. This model has been supported by a recent series of steady-state kinetic studies (Lowe & Walmsley, 1986; Wheeler, 1986; Wheeler & Whelan, 1988). A possible explanation for the results would be that the purified transporter adopts largely an inward-facing conformation.
even at high temperatures. The lipid environment of the purified protein is known to be different from that which it experiences in the intact erythrocyte membrane, and so the temperature dependence of its conformational states may differ from that which occurs in the intact cell (Baldwin et al., 1981; Wheeler & Hinkle, 1981). Alternatively, the single-site model may not adequately describe the mechanism of transport, and a more complex model, such as that of Helgerson & Carruthers (1987), may be required.

Conformational changes in the protein resulting from the binding of cytochalasin B have been detected by other techniques. For example, the binding of this inhibitor to the cytoplasmic side of the membrane protects an exofacial thiol group from reaction with maleimides (Batt, et al., 1976; Roberts et al., 1982; Deziel et al., 1985). Karim et al. (1987) have reported that photolabelling the transporter with cytochalasin B greatly increases its susceptibility to cleavage by thermolysin. However, it was not reported whether non-covalently bound cytochalasin B also had this effect. 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 on ion exchange columns. Similarly, transporter photolabelled at its extracellular surface with an azidosalicoyl derivative of bis-mannose (ASA-BMPA) has been reported to be resistant to thermolytic cleavage, whereas protein labelled with cytochalasin B is readily cleaved (Holman & Rees, 1987). However, it is not clear whether the non-covalent association of these two ligands with the protein has any effect on the rate of thermolytic cleavage.

The protection against tryptic cleavage of the transporter afforded by cytochalasin B at 45°C and 50°C may result from its ability to prevent or slow the denaturation of the protein at this elevated
temperature. At 50°C in the absence of cytochalasin B the transporter loses approximately 30% of its cytochalasin B binding activity per hour (results not shown). Such stabilization of the transporter structure by cytochalasin B has been reported in a hydrogen exchange study (Jung et al., 1986).

The physiological substrate of the transporter, D-glucose, was found to enhance the rate of cleavage of the transporter by trypsin at 25°C. This finding is reminiscent of the enhanced reactivity of the transporter towards a variety of alkylating agents seen in the presence of D-glucose, and must reflect the presence of a conformation of the transporter in which the trypsin-sensitive peptide bonds are more accessible to the proteinase.

The regions of the transporter that become resistant to thermolytic cleavage upon occupancy of the outward-facing substrate-binding site by bis-mannose derivatives have been suggested to lie in the hydrophobic sequences at the cytosolic ends of membrane-spanning helices 7 and 8 (Holman & Rees, 1987). However, the sites at which this proteinase cleaves the transporter have not yet been definitively established. Similarly the effects of D-glucose on the rates of alkylation of the transporter by a range of reagents have led to the suggestion that it is the hydrophobic membrane-spanning portions of the transporter that experience a conformational change (Rampal & Jung, 1987). These regions probably include the exofacial cysteine residue(s) that is (are) protected from reaction with maleimides by cytochalasin B. The protected residues are known to lie in the C-terminal half of the transporter, and probably correspond to one residue, Cys-429, in the hydrophobic sequence at the extracellular ends of membrane-spanning helices 11 and 12 (Mueckler et al., 1985; Deziel et al., 1985; May et al., 1990).

In this study, ethylidene glucose and phloretin were found to
reduce the rate of production of both the Mr 45000- and Mr 18000-
tryptic fragments of the transporter. This finding implies that both
the central hydrophilic region of the sequence and the C-terminal
region become more resistant to tryptic cleavage upon binding of the
inhibitors to the extracellular surface of the transporter. One or
both must, therefore, undergo a conformational change, although it is
possible that a change at one site sterically hinders the access of
trypsin to the other. These hydrophilic regions of the transporter
have not previously been identified as undergoing translocation-
related conformational changes. These present findings imply that they
have an important role in the mechanism of transport.
CHAPTER 4 INVESTIGATION OF THE SUITABILITY OF A RANGE OF DETERGENTS FOR SOLUBILIZATION OF THE NATIVE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER.

4.1 INTRODUCTION

The investigations carried out in Chapter 3 resulted in the identification of two cytoplasmically-exposed hydrophilic regions of the glucose transporter polypeptide which were involved in a transport-related conformational change. In order to pursue these conformational changes by another technique, that of X-ray solution scattering (Chapter 5), conditions were required which would allow the maintenance of the protein in its native structure whilst solubilized in monodisperse solution (Section 5.2). When solubilized in octyl glucoside - the detergent used in the routine purification of the glucose transporter (Section 2.1.3) - the purified transporter was known to be unstable at 8°C having a half life of approximately 3.8 - 7.2 hours (Baldwin et al., 1982). Before solution scattering measurements could be made it was therefore necessary to find better detergents and/or solubilization conditions which could maintain the solubilized transporter in its native conformation during the extended periods (up to 12 hours) required for sample preparation and data collection.

4.1.2 Choice of detergents

No single detergent has been found which is suitable for solubilizing all membrane proteins whilst retaining their native, active conformation. Normally a detergent is selected empirically following pilot-scale investigations for a particular protein. However there are a number of important considerations. The detergent must
solubilize but not denature the protein; it should be relatively easy to remove for reconstitution purposes; finally it should not interfere with assays which are to be performed on the solubilized material (for example the Triton series of detergents have a strong U.V. absorbance at 280 nm, thus interfering with spectrophotometric assays for protein). The non-ionic acyl polyoxyethylene ethers such as Triton X-100 are usually polydisperse (containing molecules of varying structure) so their properties may vary from batch to batch. Consequently for purification of proteins which are very sensitive to their hydrophobic environment use of a chemically defined homogeneous detergent such as octyl glucoside is often preferable, so that optimal solubilization conditions can be reproducibly obtained.

4.1.2.1 Detergent structure and charge

Detergents contain spatially distinct hydrophobic and hydrophilic regions and can be classified as ionic or non-ionic. The ionic class is sub-divided into anionic, cationic and zwitterionic detergents. Non-ionic detergents (e.g. octyl glucoside) tend to be less denaturing than ionic detergents (e.g. sodium dodecyl sulphate) since they do not generally affect protein-protein interactions.

4.1.2.2 Critical micellar concentration and critical micellar temperature

All amphiphiles possess the capacity to form micelles. These are thermodynamically stable aggregates which form spontaneously if the following two criteria are satisfied: the temperature must be above the critical micellar temperature (CMT) and the detergent concentration must be greater than the critical micellar concentration (CMC) for the detergent in question. Below the CMC the detergent exists in its monomeric form. There appears to be some correlation
between the CMC and the concentration of the detergent required for solubilization, in that detergents with low CMCs are effective in membrane-solubilization at lower concentrations than their high-CMC counterparts (Rivnay & Metzger, 1982). An indirect consequence of the CMC is that detergents with a high CMC are more easily removed from a solution by dialysis against detergent-free buffer to facilitate reconstitution.

A number of factors affect the CMC. In general the CMC is lowered as the size of the non-polar region of the detergent molecule is increased. Counteracting this tendency are structural features which interfere with the packing properties of the detergents. For example double bonds and chain branches act to increase the CMC. The electrostatic repulsion between the head groups of the charged detergents raises their CMC by 100-fold compared with their non-ionic analogues (Rosen, 1984). Increased temperature, above the CMT, has little effect on ionic detergents but markedly reduces the CMC of non-ionic detergents (Helenius & Simons, 1975; Terland et al., 1977).

At temperatures higher than the CMT and detergent concentrations significantly higher than the CMC the micellar size increases until the micelles form superaggregates and come out of solution, forming a detergent-rich phase. The temperature at which this occurs is called the 'cloud point'. This behaviour can be exploited in the purification of membrane-proteins. For example, Triton X-114 has a cloud-point at 20°C. If this detergent is used to solubilize membrane proteins at 0°C and the temperature is subsequently raised to 20°C the protein/detergent micelles will precipitate. Thus the protein can be solubilized and purified in one step (Bordier, 1981).
4.1.3 Stabilization of solubilized membrane proteins

Levels of solubilization are dependent on both the lipid concentration and the micellar detergent concentration. Therefore a useful parameter to quantify the effective concentration of detergent is the $\rho$ parameter introduced by Rivnay & Metzger (1982), which attempts to describe the molar ratio of micellar detergent to phospholipid:

$$\rho = \frac{[\text{detergent}] - CMC_{\text{effective}}}{[\text{phospholipid}]}$$

The $CMC_{\text{effective}}$ describes the CMC under the experimental conditions since this is often decreased in the presence of lipids and proteins. However in practice the literature values of CMCs are often used.

Unfortunately due to the large number of variables involved in reconstitution protocols it is not possible to compare $\rho$ values in the literature except to say that in general as the $\rho$ value increases the degree of solubilization increases. The situation is far more complex than is implied by $\rho$ values alone since account must also be taken of varying lipid-detergent interactions.

It is well established that complete removal of lipids inactivates many membrane proteins even in the presence of detergents (Warren et al., 1974; Robinson et al., 1980). This can occur to such an extent that the result is precipitation of the protein. Partial removal of lipids causes denaturation to varying extents dependent on the protein in question and the detergent used. Such denaturation can be decreased by lowering the concentration of detergent used so as to strip away fewer lipids from around the protein, but this may compromise the extent of solubilization and consequently the yield obtained during
puriﬁcation procedures. Alternatively the temperature, pH or ionic strength can be altered to decrease the activity of the detergent or the time for which the protein is in the solubilized state can be kept as short as possible.

The presence of lipids seems to stabilize the protein in its native form. For example, the addition of exogenous lipids during the puriﬁcation of the glucose transporter has been reported signiﬁcantly to improve the yield of functionally active protein (Baldwin et al., 1982). Delipidation of proteins during puriﬁcation of solubilized material on afﬁnity columns can result in loss of activity in the ﬁnal material. This can be overcome if the buffers used throughout are supplemented with lipid (Chang & Bock, 1977; Talvenheimo et al., 1982). In the light of such ﬁndings, detergents have been sought which by the nature of their structure mimic the endogenous lipid and so might stabilize the solubilized protein. Such detergents include cholate, digitonin and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). These detergents are steroid derivatives and may be able to substitute for cholesterol, a lipid which may stabilize the structures of certain membrane proteins (Rooney et al., 1985).

Addition of speciﬁc ligands has also been employed in an attempt to increase the stability of solubilized membrane proteins. For example, during solubilization of the \( \beta \) -adrenergic receptor by deoxycholate, the addition of agonists protects the protein from inactivation, presumably by locking the protein into a stable conformational state (Nedivi & Schramm, 1984).
4.1.4 Stability of the solubilized glucose transporter

Purification of the glucose transporter as described (Section 2.1.3) utilizes the non-ionic detergent octyl-β-D-glucopyranoside for solubilization of the protein from erythrocyte membranes. This detergent has been used since the discovery that it led to the production of a higher activity preparation than that obtained using Triton X-100 (Baldwin et al., 1982). However, the protein is still very unstable whilst solubilized with octyl glucoside even at cold room temperatures (8°C) (Baldwin et al., 1982). Consequently the preparation is not suitable for physical measurements such as those of X-ray solution scattering which require the protein to be maintained in the solubilized state for up to twelve hours (Chapter 5). Nor is it suitable for attempts at crystallization from solubilized material which requires maintenance of the material in a solubilized state for periods of weeks (for example crystallization of the photosynthetic reaction centre from Rhodopseudomonas viridis, Michel, 1982). In this study I have investigated the suitability of a number of detergents for solubilizing the glucose transporter in a more stable form. The detergents investigated were selected on the basis of their reported potentially useful properties and/or because of their successful use in the solubilization of other membrane proteins.

4.2 EXPERIMENTAL PROCEDURES

Eight detergents were investigated including the non-ionic detergent octyl glucoside used routinely for preparation of the glucose transporter. Details of the eight detergents are given in Table 4.1 and their structures are shown in Figure 4.1. Each was investigated for its ability to solubilize both the glucose transporter and the kinetically- and functionally-similar nucleoside
<table>
<thead>
<tr>
<th>Detergent</th>
<th>Type</th>
<th>Mr</th>
<th>CMC</th>
<th>Reported uses</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octyl-(\beta)-D-glucopyranoside</td>
<td>NI</td>
<td>292.4</td>
<td>25 mM</td>
<td>Solubilization of functional human erythrocyte glucose transporter</td>
<td>Baldwin et al., 1982</td>
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<td></td>
<td></td>
<td></td>
<td>Cairns et al., 1984</td>
</tr>
<tr>
<td>Octyl-(\beta)-D-thioglycopyranoside</td>
<td>NI</td>
<td>308.4</td>
<td>9 mM</td>
<td>Solubilization of the melibiose transporter from <em>E.coli</em></td>
<td>Saito &amp; Tsuchiya, 1984</td>
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<td>Decanoyl-N-methylglucomide (MEGA-10)</td>
<td>NI</td>
<td>349</td>
<td>6-7 mM</td>
<td>Solubilization of plasma-membranes from Epstein-Barr virus-transformed B-lymphoblastoid cells</td>
<td>Hildreth, 1982</td>
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<tr>
<td>Dodecyl-(\beta)-D-maltoside</td>
<td>NI</td>
<td>511</td>
<td>0.6 mM</td>
<td>Solubilization of cytochrome oxidase from beef heart and <em>Neurospora</em></td>
<td>Rosevear et al., 1980</td>
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<tr>
<td>Brij 58 ((C_{16}E_{20}))</td>
<td>NI</td>
<td>1122</td>
<td>77 (\mu)M</td>
<td>Investigation into stabilization of the solubilized (Ca^{2+})-ATPase from Sarcoplasmic Reticulum</td>
<td>Lund et al., 1989</td>
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<td>CHAPS*</td>
<td>Z</td>
<td>615</td>
<td>6-10 mM</td>
<td>Solubilization of non-denatured functional opiate receptor</td>
<td>Simonds et al., 1980</td>
</tr>
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<td>Classification</td>
<td>CMC (mM)</td>
<td>Description</td>
<td>Reference</td>
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<td>----------------</td>
<td>----------</td>
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<td></td>
</tr>
<tr>
<td>CHAPSO*</td>
<td>Z</td>
<td>631</td>
<td>8 mM Further improved solubilization of non-denatured functional opiate receptor</td>
<td>Simonds et al., 1980</td>
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<tr>
<td>Deoxy Big CHAP*</td>
<td>NI</td>
<td>862</td>
<td>1.4 mM Solubilization of functional opiate receptor with the benefit that this detergent does not interfere with anion exchange chromatography</td>
<td>Hjelmeland et al., 1983</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1 Details of the detergents used throughout this study, showing their classification as either non-ionic (NI) or zwitterionic (Z), critical micellar concentration (CMC), relative molecular mass (M_r) and details of their previous use.

* CHAPS = 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate
* CHAPSO = 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulphonate
* Deoxy Big CHAP = [N,N-bis-(-3-D-glucanomido propyl)-deoxycholamide]
Figure 4.1a Structures of the detergents investigated for their suitability for preparation of the human erythrocyte glucose transporter.

The structures shown are those of (A) octyl-\(\beta\)-D-glucopyranoside, (B) octyl-\(\beta\)-D-thioglucopyranoside, (C) decanoyl-N-methylglucamide and (D) dodecyl-\(\beta\)-D-maltoside. Details of each is given in Table 4.1.
Figure 4.1b Structures of the detergents investigated for their suitability for preparation of the human erythrocyte glucose transporter.

The structures shown are those of (A) Brij 58, (B) CHAPS, (C) CHAPSO and (D) Deoxy Big CHAP. Details of each is given in Table 4.1.
transporter in an active form from erythrocyte membranes. Any correlation between lipid:protein and lipid:detergent ratios with the resultant activity of the solubilized proteins was also studied. Finally the effects of temperature on the stability of the glucose transporter whilst in its solubilized state were investigated.

4.2.1 Pilot-scale solubilization of the glucose transporter

Solubilization of the human erythrocyte glucose transporter from alkali-stripped erythrocyte membranes (Section 2.1.2) was carried out for each detergent using a range of detergent concentrations centred around its CMC (Table 4.1). Each incubation contained 6 mg of membrane protein such that final concentrations were 2 mg/ml protein, 46.5 mM Tris-HCl, 2 mM DTT, pH 7.4. Following solubilization of the membranes by incubation on ice for 20 minutes, half of each resulting solution (1.5 ml) was stored on ice. The remaining 1.5 ml was subjected to centrifugation at 45000 rpm (approximately 80000g) for 1 hour at 4°C using a Kontron T-70 ultracentrifuge. The supernatant, containing solubilized material, and the remaining non-centrifuged material (1.5 ml kept on ice) were made 1 mM in EDTA, 100 mM in NaCl. They were then reconstituted, either by dialysis against four 2 litre volumes of 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4 at 4°C over a period of 24 hours, or by removal of the detergent by incubation with Amberlite resin XAD-2 (Sigma,U.K.) (Cheetham, 1979). The method of detergent removal was dependent on the CMC of the detergent as discussed in Section 4.1.2.2. Following reconstitution the resulting material was assayed for protein and lipid concentration; for its content of functional glucose transporters (measured by its ability reversibly to bind cytochalasin B); and for its content of functional nucleoside transporters (measured by its ability to bind NBMPR) (sections 2.2, 2.3, and 2.5 respectively).
4.3 RESULTS

4.3.1 Assessment of the suitability of each detergent for solubilization of the human erythrocyte glucose transporter and nucleoside transporter from alkali-stripped erythrocyte membranes

4.3.1.1 Octyl-\(\beta\)-D-glucopyranoside

The use of octyl-\(\beta\)-D-glucopyranoside (octyl glucoside) for solubilization of the human erythrocyte glucose transporter was investigated over the range of 0-90 mM, the CMC of this detergent being 25 mM. In the routine preparation of the glucose transporter described in Chapter 2 the concentration of this detergent used in membrane solubilization is 46 mM. Investigation of the total protein solubilized, and therefore retrieved in the supernatant after centrifugation, showed that a maximum of about 45% of the membrane protein was solubilized by 50 mM detergent. Paradoxically, higher detergent concentrations decreased the yield of soluble protein, as shown in Figure 4.2. The maximum yield of cytochalasin B binding activity in the supernatant was also obtained using 50 mM detergent. Use of higher concentrations greatly decreased the yield of active protein, no activity being recovered when 90 mM detergent was used (Figure 4.2). A similar loss in activity was observed for the unfractionated detergent-treated membranes at octyl glucoside concentrations greater than 50 mM. The maximum yield of NBMPR-binding activity in the supernatant was also obtained using 50 mM octyl glucoside, and following the same trend as that seen for cytochalasin B-binding activity, greatly decreased above this detergent concentration (Figure 4.2). No obvious correlation was seen between the ratio of lipid:detergent in the supernatant and the specific activity of the solubilized transporters; this ratio
Figure 4.2 Solubilization of the glucose transporter and the nucleoside transporter from alkali-stripped erythrocyte membranes using the non-ionic detergent octyl glucoside.

Alkali-stripped erythrocyte membranes were treated with octyl glucoside over the concentration range indicated. Both the non-centrifuged samples (---) and the supernatants (----) were assayed for protein (□) and for phospholipid (△), and for cytochalasin B- (○) and NBMPR- (●) binding activity following reconstitution by detergent removal. The protein and phospholipid concentrations of the non-centrifuged samples have been omitted from this graph for clarity; these values were fairly constant across the concentration range, being 1.88 ± 0.16 mg/ml and 1.64 ± 0.24 mg/ml respectively. (For details see text.)
increased from 0.06 (w/w) following solubilization in the presence of 50 mM octyl glucoside to 0.07 (w/w) following solubilization in the presence of 70 mM octyl glucoside (Table 4.2).

4.3.1.2 Octyl-β-D-thioglucopyranoside

As shown in Figure 4.2 the optimal concentration of octyl glucoside for solubilization of active glucose transporters and nucleoside transporters was 50 mM. Concentrations of 30 mM or less failed to solubilize these membrane proteins and those of 70 mM or above seemed to have detrimental affects on the activity of the transporters. Octyl-β-D-thioglucopyranoside (octyl thioglucoside) has been reported to solubilize the melibiose transporter from *Escherichia coli* over a wide range of concentrations without loss of activity of the transporter. In addition octyl thioglucoside is dialysable with characteristics identical to those of octyl glucoside and is inherently more stable due to the nature of its thioether bond as opposed to the ether bond in octyl glucoside (Saito & Tsuchiya, 1984).

The use of octyl thioglucoside for solubilization of the human erythrocyte glucose transporter was investigated over the range of 0-50 mM, the CMC of this detergent being 9 mM. The amount of protein solubilized did not reach a plateau value even at the highest concentration of detergent used. However the ligand-binding data indicated that maximal yields of active glucose- and nucleoside-transporters were solubilized by octyl thioglucoside concentrations within this range (Figure 4.3). Maximal cytochalasin B-binding activity (96% of that in the non-solubilized material) was recovered in the supernatant using a detergent concentration of 30 mM. The recovery at detergent concentrations of 20 mM and 50 mM was 85% and 66% respectively (Table 4.2). Recovery of NMPR-binding activity in the supernatant reached a plateau at a detergent concentration of
Figure 4.3 Solubilization of the glucose transporter and the nucleoside transporter from alkali-stripped erythrocyte membranes using the non-ionic detergent octyl thiogluicoside.

Alkali-stripped erythrocyte membranes were treated with octyl thiogluicoside over the concentration range indicated. For details see legend to Figure 4.2. The protein and phospholipid concentrations of the non-centrifuged samples have been omitted from this graph for clarity; these values were fairly constant across the concentration range, being $2.44 \pm 0.33$ mg/ml and $1.17 \pm 0.36$ mg/ml respectively. (For details see text.)
30 mM where approximately 100% of the available NBMPR-binding sites were recovered. However, at detergent concentrations less than 30 mM the recovered NBMPR binding activity was significantly lower. For example, at 20 mM detergent only 23% of the NBMPR-binding activity was solubilized. This value can be contrasted with the 85% recovery of cytochalasin B-binding activity in the reconstituted supernatant obtained using 20 mM detergent. Thus, at this low detergent concentration the glucose transporter was selectively solubilized relative to the nucleoside transporter.

4.3.1.3 Dodecyl-β-D-maltoside

The suitability of dodecyl-β-D-maltoside (dodecyl maltoside) for solubilization of active glucose- and nucleoside- transporters was investigated over the concentration range of 0-20 mM, the CMC of this detergent being 0.6 mM. Due to its low CMC detergent removal was by absorption with Amberlite XAD-2 resin. Following solubilization, and centrifugation where applicable, each 1.5 ml sample was mixed gently on ice with 60 mg of resin for 16 hours. The sample buffer was then exchanged for 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4 by dialysis against four 2 litre volumes of buffer over a period of 24 hours. This was done to render the results directly comparable to those obtained from experiments where detergents were removed by dialysis. The recovery of protein in the supernatant was high with this detergent, 78% of the membrane protein being recovered using 10 mM dodecyl maltoside increasing to 83% at 20 mM. However the cytochalasin B binding activity recovered in the supernatant was extremely low, rising to a maximum of only 19% that of the activity in the non-solubilized material. NBMPR-binding activity was higher, a maximum value of 53% being obtained following solubilization with 15 mM detergent. However the most striking result was that the
Figure 4.4 Solubilization of the glucose transporter and the nucleoside transporter from alkali-stripped erythrocyte membranes using the non-ionic detergent dodecyl maltoside.

Alkali-stripped erythrocyte membranes were treated with dodecyl maltoside over the concentration range indicated. For details see legend to Figure 4.2. The protein and phospholipid concentrations of the non-centrifuged samples have been omitted from this graph for clarity; these values were fairly constant across the concentration range, being $2.08 \pm 0.10$ mg/ml and $2.29 \pm 0.05$ mg/ml respectively. (For details see text.)
cytochalasin B and NBMPR binding activities were decreased to 5% and 50% respectively in the non-centrifuged samples following solubilization with 10 mM detergent. This may have been due to denaturation of the protein whilst solubilized in the presence of this detergent or due to poor detergent removal by the XAD-2 resin which could interfere with the binding assays (Figure 4.4 and Table 4.2).

4.3.1.4 Decanoyl-N-methylglucamide

Investigation of the suitability of decanoyl-N-methylglucamide (MEGA-10) was carried out over the concentration range of 0-90 mM, this detergent having a CMC of 6-7 mM. Due to the poor solubility of this detergent below 10°C, solubilization was performed at room temperature and centrifugation was carried out at 10°C. Reconstitution was by dialysis which was performed at room temperature for 10 hours (one 2 litre volume of buffer) and continued at 4°C for a further 14 hours (three 2 litre volumes of buffer). The protein yield in the supernatants did not plateau but peaked, at 30 mM, falling again in the samples solubilized with 50, 70 and 90 mM MEGA-10. A precipitate formed during dialysis of these samples, even though this procedure was begun at room temperature. This precipitation could account for the loss of protein recovered for assay. Recovery of cytochalasin B-binding activity in the supernatants was maximal at 30 mM, with a value 44% that of the non-solubilized material. Recovery of NBMPR-binding activity in the supernatants was maximal at 30 and 50 mM detergent (being 100% that of the non-solubilized material) and then fell in those treated with MEGA-10 at 70 and 90 mM (Figure 4.5 and Table 4.2).
Figure 4.5 Solubilization of the glucose transporter and the nucleoside transporter from alkali-stripped erythrocyte membranes using the non-ionic detergent decanoyl-N-methylglucamide.

Alkali-stripped erythrocyte membranes were treated with decanoyl-N-methylglucamide over the concentration range indicated. For details see legend to Figure 4.2. The protein and phospholipid concentrations of the non-centrifuged samples have been omitted from this graph for clarity; these values were fairly constant across the concentration range, being 1.74 ± 0.15 mg/ml and 1.56 ± 0.14 mg/ml respectively. (For details see text.)
4.3.1.5 Brij 58

Brij 58 \((C_{16}E_{20})\) was investigated over the range 0-20 mM, the CMC of this detergent being 77 \(\mu\)M. Reconstitution was achieved by absorption of the detergent with XAD-2 resin (Section 4.2.1). Solubilization of the transport proteins with this detergent was very poor over the concentration range used, the maximum protein yield in the supernatants being 7% that of the non-solubilized material. The recovered cytochalasin B- and NBMPR-binding activities of the supernatants were also very low being a maximum of 3% and 5% that of the non-solubilized material at 10 mM and 20 mM detergent respectively. The ligand-binding activities of the non-centrifuged and reconstituted samples also showed a decrease in activity of the transporters, indicating a detrimental affect of this detergent on the structures of the proteins. Cytochalasin B-binding activity seemed to be more sensitive to this effect than NBMPR-binding activity (Figure 4.6). The phospholipid concentrations of the supernatants were also low being 4% that of the non-solubilized material when treated with 20 mM Brij 58 (Figure 4.6 and Table 4.2).

4.3.1.6 CHAPS and CHAPSO

CHAPS and CHAPSO were used over the range of 0-50 mM, the CMCs of both detergents being 8 mM. Detergent removal was by dialysis against 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4. The maximum recovery of protein in the supernatant with both of these detergents was 5%. The maximum recoveries of cytochalasin B- and NBMPR-binding activity in the supernatants were 2% and 12% that of the non-solubilized material respectively, when treated with CHAPS or CHAPSO. The maximum loss of cytochalasin B-and NBMPR-binding activity in the non-centrifuged material was 32% and 8% respectively with either detergent at a concentration of 50 mM, probably due to destabilizing
Figure 4.6 Solubilization of the glucose transporter and the nucleoside transporter from alkali-stripped erythrocyte membranes using the non-ionic detergent Brij 58.

Alkali-stripped erythrocyte membranes were treated with Brij 58 over the concentration range indicated. For details see legend to Figure 4.2. The protein and phospholipid concentrations of the non-centrifuged samples have been omitted from this graph for clarity; these values were fairly constant across the concentration range, being $1.80 \pm 0.11$ mg/ml and $2.28 \pm 0.21$ mg/ml respectively. (For details see text.)
Figure 4.7 Solubilization of the glucose transporter and the nucleoside transporter from alkali-stripped erythrocyte membranes using the zwitterionic detergent CHAPS.

Alkali-stripped erythrocyte membranes were treated with CHAPS over the concentration range indicated. For details see legend to Figure 4.2. The protein and phospholipid concentrations of the non-centrifuged samples have been omitted from this graph for clarity; these values were fairly constant across the concentration range, being $1.60 \pm 0.13$ mg/ml and $1.29 \pm 0.04$ mg/ml respectively. (For details see text.)
Figure 4.8 Solubilization of the glucose transporter and the nucleoside transporter from alkali-stripped erythrocyte membranes using the zwitterionic detergent CHAPSO.

Alkali-stripped erythrocyte membranes were treated with CHAPSO over the concentration range indicated. For details see legend to Figure 4.2. The protein and phospholipid concentrations of the non-centrifuged samples have been omitted from this graph for clarity; these values were fairly constant across the concentration range, being $1.95 \pm 0.18$ mg.ml and $1.78 \pm 0.14$ mg/ml respectively. (For details see text.)
effects of the detergent on the protein structure (Figures 4.7 and 4.8, and Table 4.2). Phospholipid solubilization was very effective by both of these detergents, the yield with 50 mM CHAPS and 50 mM CHAPSO being 72% and 66% that of the non-solubilized material respectively.

4.3.1.7 Deoxy Big CHAP

The suitability of Deoxy Big CHAP for solubilization of active glucose- and nucleoside-transporter was investigated over a concentration range 0-20 mM, the CMC of this detergent being 1.4 mM. Protein recovery in the supernatants did not reach a plateau over the concentration range used, the maximum recovery being 30% at a detergent concentration of 20 mM. Recovery of cytochalasin B- and NBMPR-binding activity in the supernatants was a maximum of 8% and 4% that of the non-solubilized material respectively, over the concentration range used. The maximum loss of NBMPR-binding activity in the non-centrifuged, reconstituted material was 15%, seen at 20 mM detergent. However cytochalasin B binding activity in these samples fell dramatically, falling to 32% that of the non-solubilized material at 3 mM detergent and to 18% at 20 mM detergent (Figure 4.9). The maximal phospholipid recovery in the supernatants was 45% at 20 mM Deoxy Big CHAP. At this detergent concentration the recovery of phospholipid appears to have reached a plateau (Figure 4.9 and Table 4.2).

4.3.2 Stability of the glucose transporter when solubilized with octyl thioglucoside or octyl glucoside

Of all the detergents studied only octyl thioglucoside, besides octyl glucoside, showed any potentially useful properties for solubilization of the glucose transporter: the use of octyl thioglucoside at 20 mM resulted in selective solubilization of the
Figure 4.9 Solubilization of the glucose transporter and the nucleoside transporter from alkali-stripped erythrocyte membranes using the non-ionic detergent Deoxy Big CHAP.

Alkali-stripped erythrocyte membranes were treated with Deoxy Big CHAP over the concentration range indicated. For details see legend to Figure 4.2. The protein and phospholipid concentrations of the non-centrifuged samples have been omitted from this graph for clarity; these values were fairly constant across the concentration range, being 1.95 ± 0.11 mg/ml and 2.60 ± 0.39 mg/ml respectively. (For details see text.)
glucose transporter as opposed to the nucleoside transporter. (Figure 4.3). In order to compare the stability of the glucose transporter purified using octyl glucoside with that prepared using octyl thiogluco-side, two preparations of the glucose transporter were performed in parallel. These were carried out as described in Section 2.1.3, with the exception that 48 mg of alkali-stripped erythrocyte membranes were solubilized and the supernatants were applied to a 10 ml anion exchange column. Octyl glucoside was used at concentrations of 46 mM for solubilization and at 34 mM for anion exchange chromatography, and octyl thiogluco-side was used at concentrations of 30 mM for solubilization and at 20 mM for anion exchange chromatography. Following chromatography, the purified material was incubated at 0°C for a period of 49.5 hours during which time samples were taken and reconstituted by dialysis against 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4. The recoveries of protein using octyl glucoside and octyl thiogluco-side were 3.4 mg and 2.3 mg respectively. The cytochalasin B-binding activities of the two preparations, measured in units of (B/F per mg/ml) (see Section 2.5.2), were 83.7 and 66.1 for that prepared using octyl glucoside and octyl thiogluco-side respectively. Therefore the yield of protein with octyl thiogluco-side was 78% of that using octyl glucoside, and the specific activity of the transporter prepared with octyl thiogluco-side was 62% of that prepared using octyl glucoside. The stability of the preparations obtained was high using both detergents. The specific activity remaining after 49 hours (as a percentage of the specific activity of an aliquot which was dialysed without any incubation at 0°C) was 80% and 77% for transporter prepared using octyl glucoside and octyl thiogluco-side respectively (Figure 4.10). In a previous report by Baldwin et al. (1982) in which human erythrocyte glucose transporter was solubilized and purified using octyl glucoside
Figure 4.10 Stability of the human erythrocyte glucose transporter when solubilized with octyl glucoside or octyl thioglucoside.

Alkali-stripped erythrocyte membranes (48 mg of protein) were solubilized with 46 mM-octyl glucoside (○) or 30 mM-octyl thioglucoside (●) and the resulting supernatants purified by anion exchange chromatography, in buffer containing these detergents at 34 mM or 20 mM respectively. Each extract was then incubated at 0°C for 50 hours, during which time samples were taken and reconstituted by dialysis against detergent-free buffer (for details see text). The resulting samples were assayed for their ability to bind cytochalasin B, and this figure corrected for protein concentration to give the specific binding activity.
Figure 4.11 The effect of temperature on the stability of the human erythrocyte glucose transporter when solubilized with octyl glucoside.

Alkali-stripped erythrocyte membranes (144 mg of protein) were solubilized with 46 mM-octyl glucoside and the resulting supernatants purified by anion exchange chromatography, in buffer containing detergent at 34 mM. Samples of the extract were then incubated at 0°C (O) or 8°C (●) for 18 hours and during this time samples were taken and reconstituted by dialysis against detergent-free buffer (for details see text). The resulting samples were assayed for their ability to bind cytochalasin B, and this figure corrected for protein concentration to give the specific binding activity.
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<th>Recovered NBMPR-binding activity</th>
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Table 4.2 Solubilization of the glucose transporter and the nucleoside transporter from alkali-stripped erythrocyte membranes using the detergents shown.

Where results for a particular detergent are tabulated at more than one concentration this is in order to show interesting trends across the detergent concentration range. The recovery of glucose- and nucleoside-transporter activities in the supernatants are calculated from their cytochalasin B- and NBMPR- binding activities respectively. These are expressed as a percentage of the activities of the non-solubilized material and are calculated from results in triplicate. The figures of phospholipid/protein and phospholipid/detergent indicate the ratios of these constituents (w/w) in the supernatants following centrifugation. The second of these two figures has been calculated assuming all of the detergent to be present in the supernatant; however some may in fact be present in the pellet.
as described here, the half life of the solubilized protein whilst kept at 8°C was reported to be approximately 8 hours. To investigate whether this difference in stability could be explained by the difference in temperatures, the stability of material solubilized in octyl glucoside was investigated again, this time to compare the loss of cytochalasin B-binding activity at 0°C and 8°C. The cytochalasin B binding activity remaining after 18 hours at 0°C was 95% whereas that remaining after the same period of incubation at 8°C was 3% (Figure 4.11).

4.4 Discussion

Of the seven detergents investigated and compared with octyl glucoside, only octyl thioglucoside displayed any potentially useful properties. It was found selectively to solubilize the glucose transporter, as opposed to the nucleoside transporter, when used at 20 mM, therefore decreasing the extent of contamination of the glucose transporter preparation by the nucleoside transporter which is usually present at approximately 3% (w/w) when using octyl glucoside (Section 2.2.1.3). The percentage recovery of the glucose transporter, when using octyl thioglucoside at 20 mM, was 85% and therefore higher than the recovery using octyl glucoside at 50 mM (Table 4.2). Therefore the use of octyl thioglucoside at 20 mM resulted in an improved recovery of the glucose transporter in a more pure form than that obtained using octyl glucoside.

When an anion exchange chromatography step was included in the procedure the specific activity of the material resulting from the use of octyl thioglucoside was only 62% of that prepared using octyl glucoside. However the stability at 0°C of this solubilized material, after chromatography, following preparation using both octyl glucoside
and octyl thioglucoside was high, more than 75% of the cytochalasin B-binding activity remaining after incubation at 0°C for 50 hours.

Increasing the temperature of the incubation to 8°C (the temperature used by Baldwin et al., 1982) dramatically reduced the stability of the octyl glucoside-solubilized glucose transporter, only 3% of the cytochalasin B-binding activity remaining after 3 hours.

Therefore as a result of these investigations, conditions have been found which are suitable for maintaining the activity of the solubilized glucose transporter during X-ray scattering experiments (Chapter 5). If the material, solubilized with either octyl glucoside or octyl thioglucoside, is kept at a constant temperature of 0°C throughout the experimental procedures, only small losses of cytochalasin B-binding activity should be incurred.
CHAPTER 5 A PRELIMINARY INVESTIGATION OF POSSIBLE CONFORMATIONAL CHANGES IN THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER BY X-RAY SOLUTION SCATTERING TECHNIQUES

5.1 INTRODUCTION

A knowledge of the 3-dimensional structure of the human erythrocyte glucose transporter at atomic resolution will ultimately be required for a complete understanding of its mechanism. Ideally, structural information would be obtained by X-ray diffraction analysis of suitable crystals. Unfortunately both the isolation of membrane proteins in undenatured form and the crystallization of these amphipathic proteins have proven very difficult. Successful crystallization and X-ray diffraction analysis has been achieved in very few cases, namely the photosynthetic reaction centre from *Rhodopseudomonas viridis* (Michel, 1982) and porin from *Rhodobacter capsulatus* (Weiss et al., 1990). A degree of success has now been achieved with respect to the solubilization and purification of the glucose transporter in an active form, as described in Chapter 4. However the stability of the solubilized protein (solubilized with the non-ionic detergent octyl glucoside) is temperature-dependent, the half-life being greater than 18 hours at 0°C but less than 2 hours at 8°C. Crystallization often requires higher temperatures for periods of days to weeks. It may be feasible to crystallize the glucose transporter from a detergent solution whilst bound to a stabilizing ligand, this having the added advantage that the transporter molecules would all be present in the same conformation (according to the model of Mueckler et al. (1985)).

Therefore because of the difficulties involved in crystallization of the glucose transporter, a lower resolution technique, that of X-
ray solution scattering, has been employed here. This technique is based on the diffraction properties of the protein when orientated randomly in solution, and as such, if the transporter is eventually crystallized, will act as a control: if the crystallized material is indeed in its native form, the $R_G$ calculated from solution scattering should agree with the $R_G$ of the crystallographic structure.

In X-ray solution scattering a solution of the protein is irradiated by a highly collimated, monochromatic beam of X-rays. The intensity of the scattering curve $I(Q)$ is measured as a function of the scattering angle $Q$ or $2\theta$ (Figure 5.1). Data obtained at low $Q$ values can be used in Guinier plots to determine the radius of gyration, $R_G$, which is a measure of the degree of elongation of the molecule, and also to determine the intensity of forward scattering at zero angle, $I(0)$, which can be used to calculate its molecular mass. At larger scattering angles further macromolecular parameters can be determined, ultimately capable of describing the structure of the protein to a resolution of 2-4 nm.

5.1.1 The theory behind X-ray solution scattering

Scattering occurs wherever electromagnetic radiation interacts with matter. X-rays are scattered by electrons, the resultant scattering effects being divided into two types; coherent and incoherent scattering. The former type leads to interference phenomena and solution scattering.

5.1.1.1 Bragg's Law and the Debye Equation

Diffraction is described by Bragg's Law:

$$\lambda = 2d \sin \theta$$

Equation 5.1
Figure 5.1 General features of a solution scattering curve.

This is based on the neutron solution scattering curve of Clq of complement measured in 100% $^2$H$_2$O buffers (Perkins et al., 1984) to show the Guinier region at low $Q$ (out to $Q$ of $0.16 \, \text{nm}^{-1}$) and its continuation to a $Q$ of $0.7 \, \text{nm}^{-1}$. The experimental data are indicated as points, while the continuous line shows the simulated curve for a Debye sphere model of Clq (Perkins, 1985). (Taken from Perkins, 1988a.)
where $\lambda$ is the wavelength, $d$ is the diffraction spacing, and $2\theta$ is the angle of diffraction. In solution scattering, $d$ corresponds to dimensions of 0-100 nm, and the scattering angle is given in terms of the scattering vector $Q$.

$$Q = \frac{4\pi \sin \theta}{\lambda}$$  \hspace{1cm} \text{Equation 5.2}$$

where $2\theta$ is the scattering angle from the direction of the main beam.

The two chief elements of solution scattering theory are (a) the scattering properties of biological macromolecules, and (b) the geometrical relationship between individual scatterers within the macromolecule. In the case of the scattering of X-rays, for any pair of scattering electrons, when $2\theta$ is zero, the scattered waves are in phase and reinforce each other such that the scattered intensity is at a maximum. At zero scattering angle, the intensity of scattering $I(0)$ is therefore a direct measure of the molecular mass. When $Q$ and $2\theta$ are non-zero, the intensity of scattering is given by the Debye Equation (Debye, 1915 a and b; Guinier & Fournet, 1955):

$$I(Q) = \sum \sum f_p f_q \sin \left(\frac{rQ}{rQ}\right)$$  \hspace{1cm} \text{Equation 5.3}$$

where $f_p$ and $f_q$ are the scattering lengths of the electrons at points $P$ and $Q$ in the molecule, and $r$ corresponds to the distance between $P$ and $Q$. The value of $f$ for an atom is proportional to the atomic number.

In biological systems distinct variations in scattering density can occur within one macromolecule due to the variation between, for example, protein and RNA in the case of an RNA virus, or protein and carbohydrate in the case of a glycoprotein such as the glucose
transporter. Distinction between all four macromolecular classes (lipids, carbohydrates, proteins and nucleic acids) is limited when using X-ray scattering techniques but is possible by neutron scattering due to the wider range of solvent contrasts in the latter case. In neutron contrast variation, solution scattering is capable of distinguishing between every macromolecular class and is consequently a preferable technique in many cases.

5.1.1.2 Guinier Analyses

At sufficiently low Q values, the Debye Equation can be approximated to the Guinier Equation:

\[ \ln I(Q) = \ln I(0) - R_G^2 Q^2 / 3 \]  \hspace{1cm} \text{Equation 5.4}

The radius of gyration, \( R_G \), characterizes the degree of elongation of the structure. \( I(0) \) is the intensity at zero Q and is proportional to \( M_r^2 \). It is important to note that although this approximation only holds at low Q values the lowest Q values cannot be measured for reason of the main beam and the beamstop. The smallest Q required to measure the radius of gyration is dependent on the maximum particle dimension (Glatter & Kratky, 1982) and the largest Q permitted in Guinier plots is dependent on the particle shape. For example, for spheres \( Q R_G \) should be less than 1.3.

Further Guinier-type analyses can be carried out specifically for rod-like and plate-like shapes, yielding a cross-sectional radius of gyration \( R_{XS} \). Combination of this value and the \( R_G \) will give the particle length.
5.1.1.3 Analyses of I(0) values

Molecular mass calculations are based on the I(0) parameter of the Guinier plots. I(0) values normalized by the sample concentration will yield relative molecular masses. Absolute molecular mass data can be obtained by relating $M_r$ to the total number of electrons in the molecule (Kratky, 1963):

$$I(0) = \frac{K M_r (\bar{\rho}_1^2 - \rho_2 \bar{V}_1)^2}{c}$$  \hspace{1cm} \text{Equation 5.5}

where $\rho_2$ is the electron density of the solvent, $\bar{V}_1$ is the partial volume of the macromolecule under investigation, $\bar{\rho}_1$ is the number of electrons per mole, $c$ is the concentration of the macromolecule and $K$ is a constant.

5.1.1.4 Structural analyses of $R_q$ values

The $R_G$ corresponds to the mean square distance of scattering elements from their centre of gravity, the degree of elongation being quantified by the ratio of $R_G/R_o$, where $R_o$ is the $R_G$ of a sphere, the most compact shape (Kratky, 1963). Due to the non-uniformity of the scattering density of most macromolecules, the $R_G$ of a biological macromolecule normally depends on the difference between the mean scattering density of the macromolecule and that of the buffer. This difference in scattering densities is called the contrast $\Delta \rho$. By use of the Stuhrmann Equation (Ibel & Stuhrmann, 1975) it is possible to calculate the absolute external shape of the macromolecule free from fluctuations in density, that is $R_C$, the radius of gyration at infinite contrast.

$$R_G^2 = R_C^2 + \alpha/\Delta \rho - \beta/\Delta \rho^2$$  \hspace{1cm} \text{Equation 5.6}
However these calculation require measurement of the $R_G$ over a range of $\Delta\rho$ values and as explained in section 5.1.1.1 contrast variation is not necessarily an option available with X-ray scattering. Neutron scattering is the method of choice for this measurement. Using contrast variation it is possible to match out regions of one scattering density, for example to match out the protein coat or the nucleic acid core in the case of viruses. It is then possible to calculate $\alpha$ and $\beta$ above. $\alpha$ describes the radial distribution of scattering density fluctuations, and is positive if the outermost scattering density is higher than those near the centre and negative if it is lower. $\beta$ corresponds to the distance between the centres of two distinct components of two largely different scattering densities. To accurately measure $\beta$ one of the components needs to be heavily deuterated (Koch et al., 1978; Perkins & Weiss, 1983; Ramakrishnan, 1986). However these effects are negligible when studying glycoproteins due to the similarity between the scattering densities of proteins and carbohydrates.

5.1.1.5 Scattering curve simulations

Both X-ray and neutron scattering curves measured beyond the range of the Guinier region lead to further information concerning the shape of the molecule. These data are most easily analysed by comparison with simulated scattering curves from models, either simple spherical or elliptical models or more sophisticated models composed of a number of Debye spheres. These models can be visualised by computer graphics, and adjustments made to the model until a good fit is obtained. However the final model only offers one explanation of the scattering curve; unique structural determinations are not possible by this method.
5.1.2 Practical aspects of X-ray scattering

This study was performed using the synchrotron X-ray source, station 8.2, at the SERC Daresbury Laboratory. Synchrotron radiation sources are $10^{-7}$ times more intense than the conventional anode-source of X-rays used in the laboratory and the facility at Daresbury was the world's first high energy electron accelerator dedicated to the production and utilisation of synchrotron radiation.

At the synchrotron radiation source (SRS) electrons are emitted from the hot cathode of a linear accelerator and then accelerated to 12 MeV prior to injection into a storage ring. There, they are further accelerated to a peak energy of 2 GeV. Due to the high speed of the electrons and their constraint in a circular trajectory by the electromagnets of the ring, energy is radiated including X-rays. These X-rays travel tangentially away from the storage ring. Situated around the ring are 10 ports leading to a number of work-stations dedicated to specific technical approaches.

When utilized for X-ray solution scattering, the X-ray beam is passed through a monochromator, a focussing mirror and two pairs of vertical and horizontal slits in order to produce a beam of defined size which then passes through the sample to the X-ray detector. The use of a high flux source such as the SRS, as opposed to a laboratory anode-source, enables the detector to be placed at greater distances from the sample. Consequently this allows the study of smaller $Q$ ranges and larger $R_q$ values. The data is then collected and stored on a VAX computer-system on site for later analysis.

Samples are used at a total constituent concentration of approximately 10 mg/ml and only 50 $\mu$L of sample per run is required. The sample is injected into a Teflon sample-holder with a path-length of 1 mm and composed at two opposite faces of 10-15 $\mu$m thick mica windows. Each sample is normally irradiated for 10 minutes and samples
are monitored for activity both before and after irradiation as a control for structural change. The data collected can be subdivided into time-frames, each of which can be interpreted as an individual entity.

5.2 EXPERIMENTAL PROCEDURES

Initial experiments at the SERC Daresbury Laboratory were carried out using purified human erythrocyte glucose transporter reconstituted into large unilamellar vesicles of average diameter 250 nm, composed of dioleoyl phosphatidylcholine (DOPC). However the X-ray scattering intensity due to the phospholipid vesicle was so great that any effects due to the protein were masked making analysis extremely difficult. These data, though analysed, are therefore not discussed here. Following the discoveries described in Chapter 4 as regards suitable detergents and temperatures to allow investigation of the solubilized glucose transporter in an active form, further investigations were made by solubilizing the transporter in octyl glucoside and keeping the samples at 0°C throughout by means of a cooled sample holder.

5.2.1 Sample preparation

Each sample was required as a dilution series, in order to calculate the $M_r$ of the structure present (Section 5.3.2.3). The purpose of each sample type (Sections 5.2.1.1, 5.2.1.2 and 5.2.1.3) is explained in Section 5.2.3.

73 ml of purified reconstituted glucose transporter (0.152 mg protein/ml) was concentrated by centrifugation at 45000 rpm in a Kontron T70 ultracentrifuge, to a final concentration of 2.02 mg
protein/ml (5.5 ml). A sample (2 ml) of this concentrated preparation was then used to prepare a phospholipid extract of the purified glucose transporter by the method of Folch, Lees and Stanley (1953) (Section 2.3). This extract was resuspended in 50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4 and both the phospholipid extract and the purified glucose transporter were then assayed for phospholipid and protein (Sections 2.2 and 2.3).

These samples of the reconstituted glucose transporter and its phospholipid extract were transported to the SERC Daresbury Laboratory on ice and then kept on ice until approximately 90 minutes before being required for X-ray scattering measurements. Material was then prepared for use in batches:-

5.2.1.1 Glucose transporter/octyl glucoside/endogenous erythrocyte phospholipid mixed micelles (GTS/OG/PL micelles)

576 µl of the concentrated glucose transporter (2.02 mg protein/ml) was solubilized using octyl glucoside at a final concentration of 5% (171 mM). After gentle mixing on ice by passing up and down in a pipette, the resulting material was centrifuged at 55000 rpm for 1 hour in a Beckman TL-100 micro-ultracentrifuge to remove any non-solubilized material, although this was a precautionary measure and none was seen. The resulting material was 1.50 mg protein/ml, 8.47 mg phospholipid/ml, 50 mg octyl glucoside/ml. Part of this was used to prepare two other dilutions of the material by the addition of 50 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, pH 7.4. The resulting three dilutions were of total concentration (protein + phospholipid + octyl glucoside) 60, 52 and 43 mg/ml, and are described in Table 5.1.
<table>
<thead>
<tr>
<th>Dilution series</th>
<th>[GTS] (mg/ml)</th>
<th>[PL] (mg/ml)</th>
<th>[OG] (mg/ml)</th>
<th>[Total] (mg/ml)</th>
<th>( b_{PL} ) (( \mu \text{M} ))</th>
<th>( b_{OG} ) (( \mu \text{M} ))</th>
<th>([\text{micelle}] ) (mM)</th>
<th>([\text{OG}_{\text{free}}] ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTS/OG/PL</td>
<td>1.5 28</td>
<td>8.5 10.8</td>
<td>50 171</td>
<td>60</td>
<td>21</td>
<td>294</td>
<td>515</td>
<td>17</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.3 24</td>
<td>7.2 9.2</td>
<td>43 146</td>
<td>52</td>
<td>21</td>
<td>286</td>
<td>440</td>
<td>17</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.1 20</td>
<td>6.1 7.8</td>
<td>36 123</td>
<td>43</td>
<td>23</td>
<td>317</td>
<td>338</td>
<td>17</td>
</tr>
<tr>
<td>OG/PL</td>
<td>- -</td>
<td>11.6 14.7</td>
<td>68 234</td>
<td>80</td>
<td>20</td>
<td>290</td>
<td>737</td>
<td>17</td>
</tr>
<tr>
<td>&quot;</td>
<td>- -</td>
<td>10.1 12.9</td>
<td>60 205</td>
<td>70</td>
<td>20</td>
<td>292</td>
<td>645</td>
<td>17</td>
</tr>
<tr>
<td>&quot;</td>
<td>- -</td>
<td>8.7 11.1</td>
<td>51 176</td>
<td>60</td>
<td>20</td>
<td>290</td>
<td>550</td>
<td>17</td>
</tr>
<tr>
<td>&quot;</td>
<td>- -</td>
<td>7.2 9.2</td>
<td>43 146</td>
<td>50</td>
<td>22</td>
<td>306</td>
<td>419</td>
<td>17</td>
</tr>
<tr>
<td>&quot;</td>
<td>- -</td>
<td>5.8 7.4</td>
<td>34 117</td>
<td>40</td>
<td>22</td>
<td>306</td>
<td>335</td>
<td>17</td>
</tr>
<tr>
<td>&quot;</td>
<td>- -</td>
<td>4.3 5.5</td>
<td>26 88</td>
<td>30</td>
<td>26</td>
<td>322</td>
<td>212</td>
<td>17</td>
</tr>
</tbody>
</table>

Table 5.1 Characteristics of samples prepared for analysis by X-ray solution scattering.

GTS = glucose transporter, PL = phospholipid, OG = octyl glucoside, \( b_{PL} \) = number of phospholipid molecules per mixed micelle, \( b_{OG} \) = number of octyl glucoside molecules per mixed micelle, \([\text{OG}_{\text{free}}]\) = octyl glucoside not associated with micelles and therefore free in solution. The data for \( b_{PL} \), \( b_{OG} \), \([\text{micelle}]\) and \([\text{OG}_{\text{free}}]\) were calculated using the data of Eidelman et al. (1988).
5.2.1.2 Octyl glucoside/endogenous erythrocyte phospholipid mixed micelles (OG/PL micelles)

In a similar manner, the concentrated phospholipid extract was used to produce a dilution series of 80, 70, 60, 50, 40, and 30 mg/ml total concentration. The 60, 50 and 40 mg/ml samples were approximately equivalent to the three glucose transporter samples (Table 5.1).

5.2.1.3 GTS/OG/PL micelles in the presence of inhibitors of glucose transport

Both types of mixed micelle were prepared twice more, as in Sections 5.2.1.1. and 5.2.1.2. One series was then made 100 μM in phloretin and the other 40 μM in cytochalasin B by the addition of stock solutions of each in ethanol.

5.2.2 Preparation of apparatus and data collection

The distance from sample to linear detector was set at 2.608 metres and throughout data collection beam currents ranged from 273 to 190 mA. The brass sample-holder was cooled by a circulating ethylene-glycol bath set at 0°C and nitrogen gas was continually blown over the mica windows of the sample holder in order to prevent atmospheric condensation. After alignment of the beam, using X-ray-sensitive "green paper" in the sample holder, the Q-range on the linear detector was calibrated using wet, slightly stretched collagen. The wavelength and Q-range were determined to be 1.585 Å and 0.009 Å - 0.141 Å respectively.

The samples were prepared in batches as described in section 5.2.1, then used for data collection. Each sample of each dilution series was run twice using fresh aliquots of the sample. Between each pair of runs a sample of buffer (50 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, pH 7.4) was run, containing transport inhibitors
where appropriate (Section 5.2.3). Immediately after each run the aliquot of sample was retrieved from the sample-holder and dialysed against 50 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, pH 7.4 in order to reconstitute the material in readiness for subsequent binding assays. The remains of the samples which had not been subjected to X-rays were also dialysed for future assay.

5.2.3 Data analysis

Data reduction and subsequent analysis of the spectra obtained at the SERC Daresbury Laboratory were performed on the Vax 11-750 computer system at Daresbury, and on the CYBER 855 mainframe at Imperial College, London. The OTOKO program of the Vax 11-750 was used to inspect, reduce and analyse the data. These reduced data were then transferred to the Cyber 855 via the Transfer routine of the Vax 11-750. Guinier analysis and modelling were performed using the SCTPL3 and the SCT2 and SCT3 programs of the CYBER 855 respectively.

Briefly, the X-ray scattering curves were extracted by simple subtraction of the sample and buffer curves. The buffer data used for this subtraction were those collected in the presence of transport inhibitors where appropriate. The data were then normalized for the detector response:

\[
I(Q) = \frac{I_{\text{sample}}(Q) - I_{\text{buffer}}(Q)}{I_{\text{response}}(Q)} \quad \text{Equation 5.7}
\]

The samples containing glucose transporter (Sections 5.2.1.1 and 5.2.1.3) were comprised of both GTS/OG/PL and OG/PL mixed micelles. Therefore after buffer-subtraction and normalization the data were due to both populations of mixed micelles. In an independent attempt to obtain scattering data due solely to the GTS/OG/PL micelles, the data
obtained for OG/PL micelles (Section 5.2.1.2) were subtracted from the data obtained using samples containing two populations. The subtraction of OG/PL data was performed before subsequent buffer subtraction and normalization.

For modelling, a series of spherical and cylindrical models were constructed, composed of overlapping Debye spheres (Equation 5.3). Each sphere volume was set to be that of a cube of side 8 Å. Layers within the models were assigned to structural groups of varying electron densities; that is to hydrophilic lipid head groups (534 e nm$^{-2}$), hydrophobic fatty acyl chains of the lipids (296 e nm$^{-2}$) and to protein (417 e nm$^{-2}$) (electron densities from Perkins, 1988b). The SCT2 and SCT3 programs were then used to construct scattering curves for the models, which were compared with the experimental data.

5.3 RESULTS

5.3.1 The effect of X-ray analysis on the activity of the glucose transporter

Before solubilization and X-ray irradiation the glucose transporter used throughout these experiments had a cytochalasin B-binding activity of 92 B/F per mg/ml (Section 2.5.1). Glucose transporter which was solubilized using 5% octyl glucoside and kept on ice for 2.5 hours before reconstitution by dialysis, retained 100% of its cytochalasin B-binding activity. However material treated in an identical manner but subjected to 10 minutes X-ray irradiation before reconstitution retained only 53% of its cytochalasin B-binding activity. These results and those of transporter which was solubilized in the presence and absence of cytochalasin B or phloretin
<table>
<thead>
<tr>
<th>Sample</th>
<th>Description *</th>
<th>Time kept in solubilized state on ice before irradiation</th>
<th>Cytochalasin B binding activity (B/F per mg/ml)</th>
<th>Cytochalasin B binding activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTS</td>
<td>GTS preparation at 2 mg protein/ml</td>
<td>Not solubilized or irradiated. On ice for 60 hrs.</td>
<td>69.8</td>
<td>100</td>
</tr>
<tr>
<td>GTS/OG/PL</td>
<td>60 mg/ml total</td>
<td>Solubilized for 2.5 hrs. Not irradiated.</td>
<td>69.9</td>
<td>100</td>
</tr>
<tr>
<td>GTS/OG/PL</td>
<td>60 mg/ml total</td>
<td>2.5 hrs</td>
<td>37.0</td>
<td>53</td>
</tr>
<tr>
<td>&quot;</td>
<td>51 mg/ml total</td>
<td>3.5 hrs</td>
<td>35.9</td>
<td>51</td>
</tr>
<tr>
<td>&quot;</td>
<td>51 mg/ml total</td>
<td>10 hrs</td>
<td>8.5</td>
<td>12</td>
</tr>
<tr>
<td>&quot;</td>
<td>43 mg/ml total</td>
<td>4.5 hrs</td>
<td>37.2</td>
<td>53</td>
</tr>
<tr>
<td>&quot;</td>
<td>43 mg/ml total</td>
<td>9.8 hrs</td>
<td>11.1</td>
<td>16</td>
</tr>
<tr>
<td>GTS/OG/PL</td>
<td>60 mg/ml total + phloretin</td>
<td>2.3 hrs</td>
<td>28.2</td>
<td>40</td>
</tr>
</tbody>
</table>
Table 5.2 The time-dependent loss of cytochalasin B-binding activity of the glucose transporter when solubilized with 5% octyl glucoside and kept on ice at 0°C for the times shown prior to 10 minutes X-ray irradiation.

The degree of cytochalasin B binding activity is expressed as a percentage of the material which was kept in a non-solubilized state on ice for 60 hours. GTS/OG/PL represents mixed micelles of the glucose transport protein, octyl glucoside and endogenous erythrocyte phospholipids.

* mg/ml total refers to the total concentration of constituents (protein + lipid + detergent)
Figure 5.2 The effect of X-ray irradiation on the activity of the solubilized glucose transporter.

The glucose transporter was solubilized using octyl glucoside at 5% (w/w) that of the glucose transport protein, in the absence of ligand (O) or in the presence of 100 μM-phloretin (■) or 40 μM-cytochalasin B (●). The material was then stored on ice at 0°C until required. After irradiation for 10 minutes the sample was retrieved and reconstituted by extensive dialysis against detergent-free buffer. The activity of each sample was determined by its ability to bind cytochalasin B. (For details see text.) The activity is shown as a percentage (100% being that of the non-solubilized material kept on ice and not subjected to X-rays) and is plotted against the time for which the material was solubilized prior to irradiation. The curve joins the data points in the absence of ligand (O).
and kept on ice for various times before X-ray-irradiation are shown in Table 5.2. The binding activities had decreased to values as low as 27% that of the non-solubilized material. A graphic representation of the loss of cytochalasin B-binding activity of all of these samples is shown in Figure 5.2 from which it appears that there was a general trend of exponential loss of activity with time. In addition to this it appears that the presence of the two transport inhibitors had effects on the rate of loss of activity: in the presence of cytochalasin B the rate of loss of activity seemed to be decreased, and in the presence of phloretin this seemed to be increased, when compared to that in the absence of ligand. When the data was examined as 1 minute time-frames it was not possible to see any gross changes in the scattering data throughout the period of irradiation (data not shown).

5.3.2 Guinier Analysis

5.3.2.1 Analysis of the glucose transporter in a solution of GTS/OG/FL and OG/FL micelles

The majority of the data gathered gave reasonable linear fits to the Guinier approximation (Section 5.1.1.2) (Figure 5.3). For each sample at each concentration the data for I(0) (intensity at zero scattering angle) were normalised by dividing through by the total concentration (c) and then plotted against this concentration (Figure 5.4 A). There was a large difference between the values of I(0)/c for OG/FL micelles and GTS/OG/FL micelles in both the absence and presence of ligands. In all dilution series except that in the presence of cytochalasin B, there was a general trend in which the I(0)/c value increased as the total concentration decreased. The values for I(0)/c across the concentration range were very similar for the GTS/OG/FL micelles in both the presence and absence of phloretin, showing a
Figure 5.3 Representative Guinier plots of GTS/OG/PL and OG/PL mixed micelles measured by synchrotron X-ray scattering.

Data were collected using GTS/OG/PL micelles or OG/PL micelles at a total constituent concentration of 60 mg/ml (A) and 50 mg/ml (B). All samples were in 50 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, pH 7.4 at 0°C (for details see text). The Guinier fits were made in the Q range of 0.02 - 0.04 nm⁻¹. This range was used throughout to obtain the data shown in Figure 5.4.
Figure 5.4  Guinier analysis of the glucose transporter in GTS/OG/PL micelles.

OG/PL micelles (▲,–), GTS/OG/PL micelles (○,–) and GTS/OG/PL micelles in the presence of phloretin (●,–) or cytochalasin B (□,–) were subjected to X-ray solution scattering techniques and the data analysed by Guinier analysis. Both the I(0)/c and R_G values were plotted against total constituent concentration (A and B respectively). The values of the standard deviation of I(0)/c in part A fell within the region of the symbols and were omitted for clarity. Straight lines were fitted by linear regression. (See text for details.)
slight divergence as the concentration increased. However there was a large difference in I(0)/c of the GTS/OG/PL micelles in the presence and absence of cytochalasin B. This difference was apparent across the entire concentration range.

The R_g (radius of gyration) data were plotted directly against total concentration (Figure 5.4 B). As with the I(0)/c values, the R_g values of all the dilution series except for that in the presence of cytochalasin B decreased with increasing total concentration. In the case of the GTS/OG/PL micelles in the presence of cytochalasin B the R_g values decreased as the total concentration decreased. The R_g values across the concentration range were similar for the GTS/OG/PL micelles in both the presence and absence of phloretin, showing a slight convergence towards lower concentrations as was seen for the I(0)/c values.

5.3.2.2 Guinier analysis of the glucose transporter in GTS/OG/PL micelles after subtraction of scattering due to OG/PL micelles

The data plotted and discussed in the previous section were due to the scattering effects both of the GTS/OG/PL and OG/PL micelles. Therefore to obtain scattering data due solely to the GTS/OG/PL micelles the data for the OG/PL micelles were subtracted from the data for both micelle-types and the data re-analysed (Section 5.2.3). Although the OG/PL data used here for the subtraction contained neither inhibitor, experiments have been performed which show that both cytochalasin B and phloretin have no effect on either the R_g or I(0)/c values of OG/PL micelles (data not shown).

The resulting plots of (I(0)/c v c) and (R_g v c) are shown in Figure 5.5. From the lines drawn by linear regression it is apparent that the value for I(0)/c across the dilution series is again slightly affected by the concentration. However there is little effect of the
Figure 5.5  Guinier analysis of the glucose transporter in GTS/OG/PL micelles following subtraction of scattering due to OG/PL micelles

OG/PL micelles, GTS/OG/PL micelles (O,—) and GTS/OG/PL micelles in the presence of phloretin (●,—) or cytochalasin B (□) were subjected to X-ray solution scattering techniques. Following subtraction of scattering data due to the OG/PL micelles the data were analysed by Guinier analysis. Both the I(0)/c and R_g values were plotted against protein concentration (A and B respectively). Straight lines were fitted by linear regression. (See text for details.)
addition of the inhibitor phloretin on the \( I(0)/c \) value and at a protein concentration of 1.5 mg/ml the value for \( I(0)/c \) is the same for the glucose transporter in the presence and absence of phloretin. In contrast the value of \( R_G \) is affected by the presence of phloretin and is constant across the concentration series. (The data for glucose transporter in the presence of cytochalasin B were unfortunately very poor after subtraction of the data due to octyl glucoside and phospholipid. Consequently the data points are shown but not subjected to linear regression.)

5.3.2.3 Calculation of the molecular mass of the glucose transporter

This was calculated using the formula of Kratky (1963) (Section 5.1.1.3):

\[
\frac{I(0)}{c} = K M_T (3_1 - \sqrt[2]{2 \gamma_1})^2 \tag{5.5}
\]

The value for the constant \( K \) was calculated using data which were obtained using the plasma protein \( \alpha_1 \text{antitrypsin} \) as a \( M_T \) standard. These data were collected during the same period of data collection as those of the glucose transporter and using \( \alpha_1 \text{antitrypsin} \) suspended in the same buffer (50 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.4):-

\[
3_1 = \text{number of electrons per mole}
\]

\[
= \frac{\text{electrons}}{M_T} \tag{5.8}
\]
\[ \bar{\nu}_1 = \text{partial specific volume of the molecule} \]
\[ = 0.732 \text{ ml/g} \]
\[ \rho_2 = \text{electron density of the solvent (H}_2\text{O)} \]
\[ = \text{density of H}_2\text{O} \times \text{electrons} \quad \frac{\text{e}}{M_r} \]
\[ = 0.99987 \times 10^{-18} \]
\[ = 0.55548 \text{ mole. e per cm}^3 \]

Since the value of I(0)/c for \( \alpha_1 \)-antitrypsin was 7330, and the \( M_r \) of \( \alpha_1 \)-antitrypsin is 51500 daltons, from Equation 5.5:

\[ 7330 = K \times 51500 \left( \frac{0.53650 - 0.55548 \times 0.732}{0.732} \right)^2 \]

\[ K = 8.4362 \]

This value for \( K \) was then used in the calculation of the \( M_r \) of the (GTS/OG/PL) mixed micelle:

The number of phospholipid and octyl glucoside molecules per micelle
were calculated for each of the three (GTS/OG/PL) concentrations used
(\( \delta_{\text{PL}} \) and \( \delta_{\text{OG}} \), Table 5.1). At a total constituent concentration of 60 mg/ml, each micelle was calculated to contain 21 molecules of phospholipid and 294 molecules of octyl glucoside. Assuming the average empirical formula of an erythrocyte membrane phospholipid to be \( \text{C}_{38}\text{H}_{73}\text{NO}_7\text{P} \) (Baldwin & Lienhard, 1989) this is very similar to the empirical formula for dimyristoylphosphatidylcholine (DMPC) which has 374 electrons per molecule. Octyl glucoside has 160 electrons per molecule and the glucose transporter has 33926 electrons per molecule. Therefore:

Total electrons = \( \text{electrons}_{\text{GTS}} + \text{electrons}_{\text{OG}} + \text{electrons}_{\text{PL}} \)

\[
= 33926 + (294 \times 160) + (21 \times 374)
\]

\[
= 88820
\]

Total \( M_T \) = \( 63301 + (294 \times 292) + (21 \times 677) \)

\[
= 163366
\]

Therefore: \[
\frac{\text{Total electrons}}{\text{Total M}_T} = \frac{88820}{163366}
\]

\[
= 0.5437 \text{ electrons/mole}
\]

The total volume of a micelle was calculated from those of its separate components (Perkins 1988b):

Total volume \( = \text{volume}_{\text{GTS}} + (21 \times \text{volume}_{\text{DMPC}}) + (294 \times \text{volume}_{\text{OG}}) \)
\[ = 81339 + 22869 + 114072 \]
\[ = 218280 \times 10^{-3} \text{ nm}^3 \]

Therefore:
\[ \bar{V}_1 = \frac{218280 \times 0.6023}{163366} \]
\[ \bar{V}_1 = 0.8048 \text{ ml/g} \]

From Equation 5.5:

\[ \frac{I(0)}{c} = K M_r \left( \gamma_1 - \rho^2 \bar{V}_1 \right)^2 \]

The value of \( I(0)/c \) extrapolated to \( c = 0 \), for the data due to GTS/OG/PL micelles (Section 5.3.2.2 and Figure 5.5) was 10029.

Therefore:
\[ 10029 = 8.4362 M_r (0.5437 - 0.4471)^2 \]
\[ M_r = 127396 \]

Following similar calculations for GTS/OG/PL micelles at a total concentration of 52 and 43 mg/ml, the \( M_r \) was calculated to be 127529 and 128355 respectively. Therefore:

\[ M_r \text{ of the GTS/OG/PL micelles} = 127760 \pm 13000 \]
An initial attempt at modelling the glucose transporter in GTS/OG/PL micelles

The curves for the (OG/PL) micelles were characteristic of a spherical structure (Figure 5.6). Other spherical structures investigated by solution scattering and seen to produce such curves, displaying peaks and troughs, include DOPC-large unilamellar vesicles, investigated by myself (Section 5.2) and low density lipoproteins (Bellamy et al., 1989). Assuming 21 phospholipid molecules and 294 octyl glucoside molecules per micelle, based on the calculations of Eidelman et al. (1988), the volume of one (OG/PL) micelle was calculated to be 136941 Å³. A modelling system of a sphere composed of an inner and outer shell was chosen, these shells representing the hydrophobic aliphatic side chains (total volume 78246 Å³) and the hydrophilic head-groups (total volume 57918 Å³) respectively. Small variations were made to the electron-densities and radii of the shells in order to improve the fit of the model curve to the experimental data. The best fit was obtained between experimental data for a micelle of 60 mg/ml total constituent concentration and a sphere model of total volume 159232 Å³ with inner and outer shell volumes of 91648 and 67584 Å³ respectively (Figure 5.6). The electron densities used in the model were 534 and 296 e./nm³ for the outer and the inner shell respectively (Perkins, 1988b). The \( R_g \) value for the model was 35.7 Å, compared to a value of 33.6 Å for the experimental data. The main discrepancy between the model and the experimental data was the deep trough seen in the model curve at a \( Q \) value of approximately 0.08 Å⁻¹ (Figure 5.6). The depth of this trough could not be decreased by altering any of the parameters mentioned above. This was attributed to the polydisperse nature of the sample: The modelling program (SCT2) produces a scattering curve for a sample containing one population of micelles. However the sample contained a heterogeneous population
Figure 5.6 Computer-modelling of the OG/PL micelle.

The scattering data obtained for OG/PL micelles is represented above by the wide-angle scattering curve for OG/PL micelles at a total concentration of 60 mg/ml ($\triangle$). This curve is characteristic of a sphere. Models based on a sphere were investigated, incorporating an outer and inner shell, as explained in the text. Using the data of Eidelmann et al. (1988) to calculate the volumes shown above, the best curve-fit (---) was obtained using electron-densities of 534 and 296 e.nm$^3$ for the outer hydrophilic and inner hydrophobic shells respectively.
differing in the number of octyl glucoside and phospholipid molecules per micelle. Therefore the overall effect of a range of scattering curves with their troughs falling over a range of Q values would be smoothing of the trough in the experimental data as seen in Figure 5.6.

In contrast to the scattering data for the (OG/PL) micelles, the scattering data for the (GTS/OG/PL) micelles were not characteristic of a spherical structure. Since the glucose transport protein is exposed at both the extracellular and cytoplasmic surfaces of the erythrocyte membrane in its native state, it was assumed that a modelling system composed of three concentric cylinders would be an adequate representation of the data, in which the transmembrane protein was represented by the central core, surrounded by the octyl glucoside and phospholipid molecules with their hydrophobic regions in the middle layer and their hydrophilic head-groups in the outer layer (Figure 5.7). Trial calculations were carried out assuming 1 glucose transporter molecule, 21 phospholipid molecules and 294 octyl glucoside molecules per micelle (as was calculated from the data of Eidelman et al., 1988). The volume of one GTS/OG/PL micelle was calculated to be 218280 Å$^3$. The volume of the inner layer, that of the protein, was required to be 81339 Å$^3$; the volume of the hydrophobic middle layer was required to be equal to the total volume of the hydrophobic regions of the octyl glucoside and the phospholipid, that is 79023 Å$^3$; and similarly the volume of the outer hydrophilic layer was required to be equal to 57918 Å$^3$ (volumes calculated from Perkins, 1988b). The values for the electron densities of the three layers were 534, 296 and 417 e.Å$^{-3}$ for the outer, middle and inner layer respectively (Perkins, 1988b). During the modelling procedure, small variations were made to the length of the cylinder, and to the electron densities and radii of the layers in order to improve the
Figure 5.7 Computer-modelling of the GTS/OG/PL micelle.

The scattering data obtained for GTS/OG/PL micelles is represented above by the wide-angle scattering curve for GTS/OG/PL micelles at a protein concentration of 1.3 mg/ml (Δ). Models based on three concentric cylinders were investigated, incorporating an outer, inner and middle layer, as explained in the text. Using the data of Eidelmann et al. (1988) to calculate the volumes shown above, the best curve-fit (—) was obtained using electron-densities of 554, 296, and 417 e.nm\(^{-3}\) for the outer hydrophilic layer, the middle hydrophobic layer and the middle protein layer respectively.
The best curve fit was made between data for (GTS/OG/PL) mixed micelles at a total concentration of 50 mg/ml and a model of total volume 212992 Å³ and cylinder length 32Å. The volumes of the layers were 65536, 90112 and 57344 Å³ and the electron densities were 554, 296 and 417 e.nm⁻², for the inner, middle and outer layers respectively (Figure 5.7). The Rₚ values for the experimental data and the model were 38 and 39.5 Å respectively. However, the inaccuracy in this model falls in the dimension which would govern the thickness of the erythrocyte membrane; this could be no more than 32 Å whereas the bilayer is actually approximately 50 Å thick (Figure 5.7).

5.4 DISCUSSION

From the study of the cytochalasin B binding activity of the glucose transporter it is apparent that a substantial loss of binding activity occurred during the time that the solubilized protein was stored on ice and subsequently subjected to 10 minutes of X-ray irradiation. Consequently the X-ray scattering data obtained throughout this study cannot be assumed to be that for the native protein. Since all samples were irradiated for the same length of time and at the same temperature (0°C), the length of time for which the material was solubilized prior to irradiation may have been the important factor. Either the solubilized transporter lost binding activity during its storage time on ice, or during that time it was becoming increasing susceptible to subsequent damage by X-ray irradiation. If the latter were correct one may have expected to have seen a gradual change in the characteristics of the scattering curves when examined in ten 1 minute time-frames. However no such evidence was seen, and it was assumed that the scattering data were unaffected by inactivation of the glucose transporter. The rate of loss of
cytochalasin B binding activity seemed to be enhanced in the presence of phloretin, and decreased in the presence of cytochalasin B. Such an effect of cytochalasin B to stabilize the glucose transporter has been reported previously following a hydrogen exchange study (Jung et al., 1986). Since this inhibitor binds to the inward-facing conformation of the glucose transporter (according to the model of Mueckler et al., 1985) it may be that the inward-facing conformation of the protein is less susceptible to inactivation due to X-ray irradiation than the outward-facing conformation which is prevalent in the presence of phloretin.

Analysis of the Guinier plots showed a decrease in the value of \( I(0)/c \) for the OG/PL micelles as the sample concentration increased. From the calculations summarised in Table 5.1, based on studies of the composition of octyl glucoside/phosphatidylcholine mixed micelles (Eidelman et al., 1988), the \( M_r \) of the the micelles was expected to increase from 101000 to 115000 as the total concentration of constituents decreased from 80 to 30 mg/ml. Since the value of \( I(0)/c \) is proportional to the \( M_r \) of the macromolecule, this effect on the OG/PL micelles was to be expected. The difference in \( I(0)/c \) values between OG/PL micelles and GTS/OG/PL micelles was due to the addition of the glucose transporter, whose \( M_r \) was calculated to be 63300 assuming the carbohydrate moiety to be of the composition described by Sogin & Hinkle (1978). However the effect of the presence of the inhibitor cytochalasin B on the \( M_r \), and therefore the \( I(0)/c \) value of the GTS/OG/PL micelles should have ideally been negligible since only one inhibitor molecule would have been expected to have bound per glucose transporter and therefore per micelle. Therefore the apparent large increase in \( M_r \) when adding the inhibitor cytochalasin B to the GTS/OG/PL preparation may have indicated non-specific aggregation of the glucose transporter, or an altered concentration dependence as
shown in Figure 5.5.

After subtraction of the scattering data obtained for OG/PL micelles from those for the mixture of GTS/OG/PL and OG/PL micelles the Guinier analyses showed little effect of the inhibitor phloretin on the I(0)/c value (and therefore the $M_r$) of the glucose transporter. However this inhibitor did affect the $R_G$ value which was $45.0 \pm 2.0\ \text{Å}$ in the absence of phloretin, but $40.9 \pm 2.1\ \text{Å}$ in the presence of phloretin. Since the $R_G$ is a measure of the degree of elongation of the structure, this indicates a change in shape of the micellar complex in the absence of an increase in $M_r$. This is an indication of a difference in the conformation of the glucose transporter in the presence and absence of phloretin, and could be attributed to an increase in the proportion of the molecules in the outward-facing conformation. Similar substrate-related decreases in $R_G$ have been reported for globular proteins by X-ray solution scattering. The $R_G$ of the L-arabinose-binding protein of *Escherichia coli* has been reported to decrease by $0.94 \pm 0.33\ \text{Å}$ due to the binding of L-arabinose (Newcomer et al., 1981). Similarly the $R_G$ of yeast hexokinase was reported to decrease by $0.94 \pm 0.24\ \text{Å}$ upon binding glucose and $1.25 \pm 0.28\ \text{Å}$ upon binding glucose 6-phosphate (McDonald et al., 1979). Both of these observations are attributed to the closing of a cleft on binding of substrate.

The estimated value for the $M_r$ of the GTS/OG/PL mixed micelle obtained from scattering experiments was $127760 \pm 13000$. Assuming the composition of the non-protein moiety of the GTS/OG/PL micelle to be the same as that described for protein-free micelles by Eidelman et al. (1988), the presence of a protein monomer within a micelle would result in the micelle having a $M_r$ of $163366$, whereas if the micelle contained a protein dimer its $M_r$ would be $226667$. The estimated value of $127760$ obtained from scattering experiments therefore strongly
suggests that each micelle contains a monomer of the glucose transporter.

The basic modelling produced reasonable curve fits for both the OG/PL micelles and the GTS/OG/PL micelles, the former approximating to a sphere and the latter to a cylindrical structure as shown in Figures 5.6 and 5.7 respectively. In future experiments, if samples of a greater stability can be obtained, it may prove possible to detect differences in the dimensions and possibly the overall shape of the GTS/OG/PL mixed micelle in the presence of transport inhibitors such as those investigated here and the physiological substrate for the glucose transporter, D-glucose. A further improvement in strategy would be to employ the technique of neutron scattering which would allow matching out of scattering due to the phospholipid and the detergent components of the micelle by contrast variation, and may allow a more detailed insight into the conformational changes of the glucose transporter. Another advantage of neutron scattering is that no radiation damage effects are encountered with this technique.
6.1 INTRODUCTION

Identification of the regions of the glucose-transporter amino-acid sequence involved directly in the cytoplasmic- and extracellular-sugar-binding sites, and the transmembrane channel through which sugars pass during their transport across the erythrocyte membrane, would be an important advance in our understanding of the transport mechanism. The acquisition of such data would aid in the construction of a tentative 3-dimensional model for the arrangement of the proposed 12 α-helices (Mueckler et al., 1985) in the erythrocyte membrane.

In this study the glucose transporter was covalently radiolabelled at either the cytoplasmic or extracellular sugar-binding sites with [3H]cytochalasin B or [3H]2-N-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)-2-propylamine ([3H]ATB-BMPA) respectively. Cytochalasin B is one of the most potent inhibitors of the glucose transporter, binding exclusively at the cytoplasmic surface of the membrane (Devés & Krupka, 1978 a,b) (Section 1.4) and the tritiated form can be used to radiolabel covalently the transporter in the presence of ultraviolet light (Carter-Su et al., 1982; Shanahan, 1982). This covalent labelling is inhibitable by D-glucose, and therefore may involve covalent interactions in the vicinity of the sugar binding-site at the cytoplasmic surface of the membrane, although one must not dismiss the second possibility that the D-glucose- and cytochalasin B- binding sites are linked allosterically. Conversely, the bis(D-mannose) derivative ATB-BMPA (Clark & Holman, 1990) binds reversibly to the exofacial surface of
Figure 6.1 The synthetic peptides against which the bank of antipeptide antibodies used during this study were previously raised (Davies et al., 1987; Davies et al., 1990).

The nine regions of the peptide sequence indicated above were synthesised and these peptides used as antigens to produce polyclonal antibodies in rabbits. The nomenclature of the antibodies used in this study is as follows, the first number being the residue number of the N-terminal amino-acid and the last being that of the C-terminal amino acid. 'A' is an abbreviation for Anti.

the erythrocyte membrane in a manner inhibitable by D-glucose and
cytochalasin B, and the tritiated form can be used to radiolabel the
sugar binding-site using ultraviolet irradiation. Therefore it can be
assumed that ATB-BMPA binds covalently in the vicinity of the
extracellular sugar-binding site of the glucose transporter, although
allostERIC affects are again a possibility (Clark & Holman, 1990).
The protein was then cleaved by a number of enzymatic and chemical
means in order to produce a range of fragments, some of which were
radiolabelled. All of the fragments were identified by the use of a
bank of polyclonal antibodies previously raised against whole glucose
transporter and against synthetic peptides corresponding to regions
of the transporter primary structure (Davies et al., 1987; Davies et
al., 1990). The synthetic peptides against which these antibodies were
raised are shown in Figure 6.1.

6.2 EXPERIMENTAL PROCEDURES

The purified human erythrocyte glucose transporter was
photolabelled at either its cytoplasmic surface, with \([^3H]\)-
cytochalasin B, or its extracellular surface, with \([^3H]\)-ATB-BMPA, as
described in Section 2.8. This material was then subjected to
proteolytic and chemical cleavage as described below. The products
resulting from the various means of cleavage were identified by
Tricine/SDS/polyacrylamide gel electrophoresis (Section 2.6.2),
followed by electrotransfer and immunoblotting (Section 2.6.4) using
the bank of antipeptide antibodies described in Figure 6.1, and a
polyclonal antibody raised against the whole transporter. The
association of each fragment with the cytoplasmic and extracellular
sugar-binding sites was investigated by the use of radioactivity
profiles of the gels (Section 2.8.3).
6.2.1 Extensive proteolytic cleavage of the photolabelled glucose transporter at lysine and arginine residues with trypsin and endoproteinase Lys-C

Endoproteinase Lys-C cleaves peptides solely at the carboxyl-terminus of lysine residues, whereas trypsin cleaves peptides specifically at the carboxyl-terminus of both lysine and arginine residues. Cleavage of the photolabelled transporter was carried out using trypsin (from bovine pancreas, diphenylcarbamyl chloride-treated) or endoproteinase Lys-C (purified from Lysobacter enzymogenes, Boehringer Corp. Ltd.) at a concentration 5% (w/w) that of the transport protein. These digestions were carried out in 50 mM sodium phosphate, 100 mM sodium chloride, 1 mM EDTA, pH 7.4 at 25°C over a period of 24 hours and were performed on the transporter in its native or solubilized state, in the absence or presence of 0.1% SDS respectively. In case of inactivation of the enzymes over this long incubation period, additions of enzyme were made at 0, 3 and 6 hours by adding the respective enzyme to 5% (w/w) each time. Thus the final concentration of enzyme was 15%. Both enzymes were inactivated after 24 hours by the addition of bovine lung aprotinin to 30% (w/w) that of the transporter. Gel samples were prepared directly from this material (section 2.6.1).

6.2.2 Cleavage of the photolabelled glucose transporter at tryptophan residues with N-bromo-succinimide

Cleavage of the glucose transporter at the carboxyl terminus of tryptophan residues was carried out by cleavage with N-bromosuccinimide (NBS) in the presence of urea. Purified photolabelled glucose transporter was suspended to 0.15 mg/ml in 50 mM sodium citrate (pH 3), 1% SDS, 8 M urea. To this was added NBS (10 mg/ml in 10 M urea) to a final concentration of 75 μg/ml and the
mixture was incubated for 20 minutes at room temperature. Mercaptoethanol was then added to 4% (v/v) followed by sodium borate, pH 9.3 to a final concentration of 0.7 M and the resulting solution was incubated at 37°C for 40 minutes. Finally the cleaved material was de-salted by repeatedly passing through Centriprep-10 concentrators (Amicon Division, Danvers, M.A., U.S.A.). The resulting solutions were used directly to prepare gel samples (Section 2.6.1).

6.2.3 Purification of the M₉ 18000 tryptic fragment of the glucose transporter followed by cleavage with endoproteinase Lys-C

Following cleavage of the photolabelled, native glucose transporter with trypsin (Section 6.2.1) the M₉ 18000 fragment was separated from other fragments and remaining uncleaved transporter by SDS/polyacrylamide gel electrophoresis (Section 2.6.1). The M₉ 18000 fragment was then harvested by electroelution and treated by electrodialysis to reduce the SDS concentration to 1% (w/v) (Section 2.6.3). In readiness for digestion with endoproteinase Lys-C the sample was exchanged into (50 mM sodium phosphate, 100 mM sodium chloride, 0.1% SDS, pH 7.4) by dialysis against 2 changes of 2 litres of this buffer over a period of 20 hours at 4°C. The dialysed sample was further diluted with this buffer to bring the SDS to a final concentration of 0.1% (w/v), this concentration being quantified by SDS assay (Section 2.4). Endoproteinase Lys-C digestion was performed as in Section 6.2.1.
6.3 RESULTS

6.3.1 Extensive cleavage with endoproteinase Lys-C

Glucose transporter photolabelled with either $[^3H]$cytochalasin B or $[^3H]$ATB-BMPA (Sections 2.8.1 and 2.8.3) was digested extensively with endoproteinase Lys-C in the absence of SDS. This resulted in the production of a major fragment of apparent $M_r$ 22000-23000 and a minor fragment of apparent $M_r$ 16000-17000 as seen by SDS/polyacrylamide gel electrophoresis (Figure 6.2, fragments 'a' and 'b' respectively). The fragment of apparent $M_r$ 22000-23000 was radiolabelled by both $[^3H]$cytochalasin B and $[^3H]$ATB-BMPA (Figure 6.3) and when subjected to electrotransfer and immunoblotting was seen to be labelled with antibodies against the whole transporter ($A_{GTS}$) and with antibodies against peptides 293-306, 326-340, 389-403 and 450-467 (Figure 6.2). From its pattern of antibody-labelling and with knowledge of the possible cleavage sites of endoproteinase Lys-C, this fragment was deduced to consist of residues Val$_{257}$-Lys$_{456}$ (theoretical $M_r$ 22027) (Figure 6.2). The fragment of apparent $M_r$ 16000-17000 was produced in greater abundance when cleavage was performed in the presence of SDS and will be discussed below. A broad band of apparent $M_r$ 23000-42000 was also visible on the Coomassie blue-stained gel, which was not radiolabelled and was immunolabelled by $A_{GTS}$. Cleavage of the native transporter with trypsin is known to result in the production of a stable glycosylated fragment of apparent $M_r$ 23000-42000 (Section 1.6.1.1) which has been deduced from amino acid analysis and immunolabelling to consist of residues Met$_1$-Arg$_{212}$ (Cairns et al., 1987; Davies et al., 1990). The fragment of apparent $M_r$ 23000-42000 seen here is possibly a similar fragment, probably of a slightly higher molecular weight, due to cleavage of the protein at a lysine residue rather than at an arginine residue, in the large cytoplasmic
Figure 6.2 Cleavage of the glucose transporter by endoproteinase Lys-C.

Following cleavage of the glucose transporter by endoproteinase Lys-C in the absence (−) or presence (+) of 0.1% SDS, the fragments produced were identified by SDS/polyacrylamide gel electrophoresis and immunoblotting, using the antibodies shown (for details see text). Fragments a and b were both labelled with [3H]cytochalasin B and [3H]ATB-BMPA and by their pattern of antibody-labelling were deduced to be composed of residues Val_{257}–Lys_{456} and Ala_{301}–Lys_{456}, respectively. Fragment 'c' was not radiolabelled and was deduced to be composed of residues Val_{257}–Lys_{300}.
Figure 6.3 Radioactivity profiles of the products of cleavage of the glucose transporter with endoproteinase Lys-C.

Following cleavage of the radiolabelled glucose transporter by endoproteinase Lys-C in the absence (A) or presence (B) of 0.1% SDS, the resulting fragments were visualized by SDS/polyacrylamide gel electrophoresis. The gel was then sliced and dissolved in readiness for liquid scintillation counting to obtain a radioactive profile of the gel (Section 2.8.3). Regions a and b represent locations of the bands indicated in Figure 6.2. (The region of radioactivity from 25-35 mm represents un-digested transporter.) Arrows indicate the positions of the $M_r$ markers shown in Figure 6.2. TD = position of tracking dye.
loop between helices 6 and 7.

When this cleavage with endoproteinase Lys-C was performed in the presence of 0.1% SDS one major fragment was produced of apparent Mr 16000-17000 (Figure 6.2, fragment 'b') which was radiolabelled by both $[^{3}\text{H}]$cytochalasin B and $[^{3}\text{H}]$ATB-BMPA. The immunoblotting showed this fragment to be labelled by $A_{\text{TGS}}$ and with antibodies against peptides 326-340, 389-403 and 450-467, but it was not labelled with antibodies against peptide 293-306. From its pattern of antibody-labelling and with knowledge of the possible cleavage sites of endoproteinase Lys-C, this fragment was deduced to be composed of residues Ala$^{301}$-Lys$^{456}$ (theoretical Mr 17027). A second smaller fragment of apparent Mr 4900 (Figure 6.2, fragment 'c') was not labelled with either radiolabel but was immunolabelled by $A_{\text{TGS}}$ and (weakly) by antibodies against residues 293-306. This fragment was probably composed of residues Val$^{257}$-Lys$^{300}$ (theoretical Mr 5057).

These results suggested that at least one site of covalent labelling by each of the radiolabels $[^{3}\text{H}]$cytochalasin B and $[^{3}\text{H}]$ATB-BMPA was within the region of residues 301-456 of the amino-acid sequence.

6.3.2 Extensive cleavage with trypsin

Extensive tryptic cleavage of the native glucose transporter, previously photolabelled with either $[^{3}\text{H}]$cytochalasin B or $[^{3}\text{H}]$ATB-BMPA, in the absence of SDS resulted in the production of a major fragment of apparent Mr 19000 as seen by SDS/polyacrylamide gel electrophoresis (Figure 6.4, fragment 'a'). This fragment was radiolabelled by both $[^{3}\text{H}]$cytochalasin B and $[^{3}\text{H}]$ATB-BMPA (Figure 6.5), and could be immunolabelled with $A_{\text{TGS}}$ and with antibodies raised against peptides 293-306, 326-340, 389-403, and 450-467 (Figure 6.4). This fragment was immuno-labelled in a manner characteristic of the
well documented $M_t$ 18000/19000 fragment composed of amino-acid residues 270-456 (Davies et al., 1987; Davies et al., 1990) which contains both the cytochalasin B and ASA-BMPA binding sites (Cairns et al., 1984; Holman et al., 1986).

When this extensive tryptic cleavage was performed in the presence of 0.1% SDS, a major fragment of apparent $M_t$ 11400 was produced (Figure 6.4, fragment 'b'). This was labelled with $A_GTS$ and with antibodies raised against peptides 326-340 and 389-403, but not antibodies raised against peptides 293-306 or 450-467, and was radiolabelled by $[^3]H$cytochalasin B but not by $[^3]H$-ATB-BMPA (Figure 6.5). This fragment was apparently derived from the $M_t$ 19000 fragment described above, having lost all or part of the epitopes for the antibodies raised against residues 450-467 and residues 293-306. By examining the immunolabelling of this fragment and the potential cleavage sites for trypsin it was concluded that the fragment of apparent $M_t$ 11400 consisted of residues Ala$^{331}$-Lys$^{451}$ (theoretical $M_t$ 12927) (Figure 6.4).

Both the $M_t$ 19000 fragment (Gln$^{270}$-Lys$^{456}$) and the $M_t$ 11400 fragment (Ala$^{331}$-Lys$^{451}$) were labelled covalently by $[^3]H$-cytochalasin B. Therefore, one site of labelling by this ligand must be within the $M_t$11400 fragment. However, it is not possible to say from these results whether or not there are further sites of labelling within the region Gln$^{270}$-Arg$^{330}$. Because the $M_t$ 19000 fragment was labelled covalently by $[^3]H$-ATB-BMPA, but the $M_t$ 11400 was not, a site of labelling by this ligand must lie within the region of Gln$^{270}$-Arg$^{330}$. 
Figure 6.4 Cleavage of the glucose transporter by trypsin.

Following extensive cleavage of the glucose transporter by trypsin in the absence (-) or presence (+) of 0.1% SDS, the fragments produced were identified by SDS/polyacrylamide gel electrophoresis and immunoblotting, using the antibodies shown (for details see text). Fragments a was radiolabelled by both \(^{3}H\)-ATP-BMPA and \(^{3}H\) cytochalasin B and fragment b was labelled by \(^{3}H\) cytochalasin B only. By their pattern of antibody-labelling fragments a and b were deduced to be composed of residues Gln\(_{270}\)-Lys\(_{456}\), and Ala\(_{331}\)-Lys\(_{451}\) respectively.
Figure 6.5 Radioactivity profiles of the products of cleavage of the glucose transporter with trypsin.

Following cleavage of the radiolabelled glucose transporter by trypsin in the absence (A) or presence (B) of 0.1% SDS, the resulting fragments were visualized by SDS/polyacrylamide gel electrophoresis. The gel was then sliced and dissolved in readiness for liquid scintillation counting to obtain a radioactive profile of the gel (Section 2.8.3). Regions a and b represent locations of the bands indicated in Figure 6.4. (The region of radioactivity from 30-45 mm represents un-digested transporter.) Arrows indicate the positions of the $M_r$ markers shown in Figure 6.4. TD = position of tracking dye.
6.3.3 Cleavage with N-bromosuccinimide

Cleavage of the glucose transporter with N-bromosuccinimide (NBS) appeared to be very inefficient, as can be clearly seen from the Coomassie blue-stained SDS/polyacrylamide gel illustrated in Figure 6.6 and from the distribution of radioactivity within this gel (Figure 6.7). Cleavage of the \[^{3}H\]-ATB-BMPA-labelled transporter with NBS yielded only small amounts of radiolabelled fragments on SDS/polyacrylamide gels but cleavage of the \[^{3}H\]-cytochalasin B-labelled transporter yielded at least two distinct radiolabelled peptides of apparent Mr 14200 and 11500 (fragments 'a' and 'b' respectively in Figures 6.6 and 6.7). Each of these peptides migrated at the same position as a band labelled on blots by antibodies raised against residues 450-467 of the transporter. In contrast a third peptide band of apparent Mr 9000, also recognised by this antibody on blots, was not radiolabelled (fragment 'c', Figures 6.6 and 6.7). Fragment 'a' was also labelled on blots by antibodies against residues 389-403 but fragments 'b' and 'c' were not. Since all three fragments were recognised by A\_450-467, and the C-terminal region of the transporter beyond residue Phe\_450 contains no tryptophan residues, all three fragments must terminate at Val\_492 of the transporter sequence. Comparison of their immunoreactivities and sizes with those predicted from the location of the tryptophan residues in the C-terminal half of the transporter suggests that fragment 'a' corresponds to residues Met\_364-Val\_492 (theoretical Mr 13911), fragment 'b' corresponds to residues Phe\_389-Val\_492 (theoretical Mr 11378) and fragment 'c' corresponds to residues Thr\_413-Val\_492 (theoretical Mr 8829). Because fragment 'b' was labelled covalently with \[^{3}H\]-cytochalasin B but fragment 'c' was not, this site of labelling probably lies within the region Phe\_389-Trp\_412.

A possible source of error which had to be investigated was that of
Figure 6.6 Chemical cleavage of the glucose transporter by N-bromosuccinimide.

Following cleavage of the glucose transporter by N-bromosuccinimide in the presence of urea, the fragments produced were identified by SDS/polyacrylamide gel electrophoresis and immunoblotting, using the antibodies shown (for details see text). Lanes 'N' are non-digested transporter. Fragments a and b were radiolabelled by [³H] cytochalasin B and fragment c was not radiolabelled. By their pattern of antibody-labelling a, b, and c were deduced to be composed of residues Met'⁶⁴-Val'⁴⁹₂, Phe'⁸⁹-Val'⁴⁹₂, and Thr'⁴¹³-Val'⁴⁹₂ respectively.
Figure 6.7 Radioactivity profiles of the \[^{3}\text{H}]-\text{ATB-BMPA}- \text{and } [^{3}\text{H}]-\text{cytochalasin B}- \text{labelled glucose transporter and of the products of its cleavage with N-bromosuccinimide.}

Radiolabelled glucose transporter and the products of its cleavage with N-bromosuccinimide were visualized by SDS/polyacrylamide gel electrophoresis. The gel was then sliced and dissolved in readiness for liquid scintillation counting to obtain a radioactive profile of the gel (Section 2.8.3). The profiles of the radiolabelled glucose transporter and the cleaved material are shown in A and B respectively. Regions a, b and c represent locations of the bands indicated in Figure 6.6. (The region of radioactive labelling from 25-39 mm represents un-cleaved transporter.) Arrows indicate the positions of the M\textsubscript{T} markers shown in Figure 6.6. TD = position of tracking dye.
NBS-cleavage products from other regions of the transporter coincidentally being of the same apparent $M_T$ as the radiolabelled fragments identified by the radioactivity profiles. However, of the 27 possible theoretical products of NBS-cleavage of the glucose transporter, only one was of a $M_T$ which could co-migrate with one of the three fragments mentioned above: a theoretical fragment composed of residues Val$_{49}$-Trp$_{186}$ would have a $M_T$ of 14977 and could therefore coincide with the $[^3]$H-cytochalasin B-labelled fragment ('a') of apparent $M_T$ 14200. However the fragment composed of residues Val$_{49}$-Trp$_{186}$ would not be immunolabelled by antibodies raised against either residues 450-467 or 389-403 and, being a product of the $N$-terminal half of the protein would not be radiolabelled by $[^3]$Hcytochalasin B (Davies et al., 1987). Therefore this fragment can be dismissed as a possible cause of error.

6.3.4 Purification of the $M_T$ 18000 tryptic fragment followed by cleavage with endoproteinase Lys-C

Purification of the $[^3]$H-ATB-BMPA-labelled $M_T$ 18000 tryptic fragment of the glucose transporter by electroelution from an SDS/polyacrylamide gel (Sections 2.6.3 and 6.2.3) resulted in the successful isolation of a radiolabelled fragment which ran as a single band of apparent $M_T$ 18000 on SDS/polyacrylamide gels (Figure 6.8).

Cleavage of the purified $[^3]$H-ATB-BMPA-labelled $M_T$ 18000 tryptic fragment by endoproteinase Lys-C (5% w/w) in the presence of 0.1% SDS resulted in the production of a fragment of apparent $M_T$ 16000 - 17000 as seen previously (Section 6.2.1) and a fragment of apparent $M_T$ 2000 - 3000. The fragment of apparent $M_T$ 16000 - 17000 was radiolabelled, and was immunolabelled by $A_{GTS}$ and by antibodies against residues 326-340, 389-403, and 450-467. The fragment of apparent $M_T$ 2000 - 3000 was not radiolabelled and was immunolabelled by antibodies against
Figure 6.8 Cleavage of the purified $[^3\text{H}]$-ATB-BMPA-M$_r$ 18000 tryptic fragment with endoproteinase Lys-C.

Following cleavage of the $[^3\text{H}]$ATB-BMPA-labelled glucose transporter by trypsin, the radiolabelled M$_r$ 18000 fragment was purified by electroelution from an SDS/polyacrylamide gel. This purified fragment was then subjected to cleavage by endoproteinase Lys-C. The resulting fragments were identified by SDS/polyacrylamide gel electrophoresis and immunoblotting, using the antibodies shown (for details see text). Only fragment a was radiolabelled by $[^3\text{H}]$ATB-BMPA. By their pattern of antibody-labelling a and b were deduced to be composed of residues Ala$_{301}$-Lys$_{456}$ and Gln$_{270}$-Lys$_{300}$ respectively.
Figure 6.9 Radioactivity profiles of the products of cleavage of the purified $[^3H]$ATB-BMPA-$M_T$ 18000 tryptic fragment by endoproteinase Lys-C in the presence of 0.1% SDS.

Following cleavage of the purified $[^3H]$ATB-BMPA-labelled $M_T$ 18000 fragment by endoproteinase Lys-C, the resulting fragments were visualized by SDS/polyacrylamide gel electrophoresis. The gel was then sliced and dissolved in readiness for liquid scintillation counting to obtain a radioactive profile of the gel (Section 2.8.3). Regions $M_T$ 18000 and a represent the locations of the bands indicated in Figure 6.8. Arrows indicate the positions of the $M_T$ markers shown in Figure 6.8. TD = position of tracking dye.
residues 256-272 only (Figures 6.8 and 6.9).

By comparison of the potential cleavage sites for endoproteinase Lys-C in the \( M_r \) 18000 fragment with the patterns of immuno-labelling and the \( M_r \)'s of the fragments produced, it was deduced that the fragment of apparent \( M_r \) 16000 - 17000 was composed of residues Ala_{301}^{456} (the same fragment as that produced by the action of endoproteinase Lys-C on the intact transporter in the presence of 0.1% SDS (Section 6.3.1)). The failure of the antibody raised against residues 293-306 to label this fragment was due to the cleavage of the epitope. The fragment of apparent \( M_r \) 2000 - 3000 was deduced to be composed of residues 270-300 (theoretical \( M_r \) 3453). Both this fragment and the \( M_r \) 18000 tryptic fragment were labelled by the antibody raised against residues 256-272. However both these fragments contain only the C-terminal three residues of the peptide against which the antibody was raised; such a short sequence is likely to be too small to account for the observed immunolabelling. It is likely, therefore, that the \( M_r \) 18000 tryptic fragment contains a small population of fragments whose sequence begins at Ser_{265}.

6.4 DISCUSSION

Digestion of the native photolabelled glucose transporter with trypsin resulted in the production of a fragment of apparent \( M_r \) 18000 which was labelled by both \(^{3}H\)cytochalasin B and \(^{3}H\)ATB-EMPA and due to its pattern of labelling by antipeptide antibodies was identified as consisting of residues Gln_{270}^{456}, plus a small population of Ser_{265}^{456}. This was in agreement with results obtained previously (Cairns et al., 1984, 1987; Deziel & Rothstein, 1984; Holman et al., 1986; Clark & Holman, 1990, Davies et al., 1990). Digestion of the SDS-solubilized glucose transporter with endoproteinase Lys-C resulted
in the production of two major fragments which migrated on gels as sharp bands; one of apparent \( M_r \) 16000 - 17000 which was labelled by both photolabels and from its labelling by antipeptide antibodies was deduced to be composed of residues Ala\(_{301}\)-Lys\(_{456}\), and one of apparent \( M_r \) 4900 which was not labelled by either radiolabel and probably corresponded to residues Gln\(_{270}\)-Lys\(_{300}\). Therefore the sites of labelling by both \(^3\)Hcytochalasin B and \(^3\)HATB-BMPA lie within the region of residues Ala\(_{301}\)-Lys\(_{456}\).

Digestion of the SDS-solubilized photolabelled transporter with trypsin resulted in the production of a fragment of apparent \( M_r \) 11400 which was radiolabelled solely by \(^3\)Hcytochalasin B. By its labelling with antipeptide antibodies this fragment was identified as residues Ala\(_{331}\)-Lys\(_{451}\) (theoretical \( M_r \) 12927). Since the fragment composed of residues Ala\(_{301}\)-Lys\(_{456}\) (produced by the action of endoproteinase Lys-C on the SDS-solubilized transporter) was labelled by both radiolabels, and the tryptic fragment composed of residues Ala\(_{331}\)-Lys\(_{451}\) was labelled only by \(^3\)Hcytochalasin B, \(^3\)HATB-BMPA appears to label the transporter primarily within the region(s) of residues Ala\(_{301}\)-Arg\(_{330}\) or (and) Val\(_{452}\)-Lys\(_{456}\). The latter of these two regions is situated internally (according to the model of Mueckler et al., 1985) whereas this radiolabel binds to the external sugar-binding site. Therefore this region is unlikely to be the site of \(^3\)HATB-BMPA labelling. However the region of residues Ala\(_{301}\)-Arg\(_{330}\), containing helix 8, is more likely as it is exposed extracellularly and is consistent with the model in which helices 3,5,7,8 and 11 are amphipathic and involved directly in the sugar-binding site and the aqueous pore. Therefore the region of labelling by \(^3\)HATB-BMPA has been localized to residues Ala\(_{301}\)-Arg\(_{330}\) and that of labelling by \(^3\)Hcytochalasin B has been localized to residues Ala\(_{331}\)-Lys\(_{451}\).

Chemical cleavage of the photolabelled glucose transporter at
tryptophan residues by N-bromosuccinimide resulted in the production of a $[^{3}H]$cytochalasin B-labelled fragment of apparent $M_r$ 11500, assigned by immunolabelling and possible cleavage sites to residues 389-492 (theoretical $M_r$ 11379), and an unlabelled fragment of apparent $M_r$ 9000, which was assigned to residues 413-492 (theoretical $M_r$ 8829). Therefore it appears that the site of labelling by $[^{3}H]$cytochalasin B is within the region of residues 389-412. This agrees with the model proposed by Holman & Rees (1987) in which the cytochalasin B-binding site lies within the region of the cytoplasmic loop connecting helices 10 and 11 (residues 390-401).

Therefore the region of the protein involved in the binding of $[^{3}H]$ATB-BMPA to the extracellular sugar-binding site probably includes amino acid residues lying between residues 301-330 of the sequence, and the region involved in the binding of $[^{3}H]$cytochalasin B to the cytoplasmic sugar-binding site probably includes amino acid residues lying between residues 389-412 of this sequence (Figure 6.10). Both of these findings are in agreement with the model of Mueckler et al. (1985) in which $\alpha$-helices 3, 5, 7, 8 and 11 are amphipathic and possibly involved in forming the sugar-binding site(s) and an aqueous pore. Although ATB-BMPA is known to bind to the transporter at its extracellular surface (Clark & Holman, 1990), it need not be assumed that the site of labelling within the region of residues 301-330 is actually in the small loop linking helices 7 and 8 (Val$_{328}$-His$_{337}$). It is likely that the binding of ATB-BMPA to the extracellular site involves a residue or residues within helix 8 which is available to the extracellular environment when the transporter is in its outward-facing conformation. Similarly although cytochalasin B is known to bind to the transporter at its cytoplasmic surface (Devés & Krupka, 1978a) it should not be assumed that the residues involved in the binding of cytochalasin B to the cytoplasmic sugar-binding site are
Figure 6.10 Regions of the glucose transporter polypeptide involved in the binding of ATB-BMPA and cytochalasin B to the extracellular and cytoplasmic sugar-binding sites respectively.

Cytochalasin B and ATB-BMPA bind covalently to the glucose transporter polypeptide within the regions Phe\(^\text{389}\)-Trp\(^\text{412}\) (•) and Ala\(^\text{301}\)-Arg\(^\text{330}\) (○) respectively. Since this labelling is specifically inhibited by D-glucose it is postulated to involve binding to the cytoplasmic and extracellular sugar-binding sites respectively. Therefore the regions indicated are postulated to be involved in the sugar-binding site in agreement with the model of Mueckler et al. (1985) in which the amphipathic \(\alpha\)-helices 3,5,7,8 and 11 are involved in a hydrophilic pore.
within the small loop linking helices 10 and 11 (Ile_{390}-Phe_{401}). Binding of the ligand could occur via residues within helix 11 which are exposed to the cytoplasmic environment when the transporter is in its inward-facing conformation.
7.1 CONFORMATIONAL CHANGES OF THE GLUCOSE TRANSPORTER

At the beginning of this study, much evidence existed for the occurrence of conformational changes in the human erythrocyte glucose transporter (Section 1.3.2), but the identity of those regions of the protein affected by these changes was largely unknown. An investigation into the inactivation of the transporter by alkylating agents (Rampal & Jung, 1987) suggested that substrate-induced conformational changes occurred primarily within the membrane-spanning regions of the protein, while the hydrophilic segments outside the bilayer remained largely unaffected. The investigations described in Chapter 3, which followed the effects of transport-inhibitors and the substrate D-glucose on the rate of cleavage of the transport protein by trypsin, resulted in the identification of two cytoplasmically-exposed hydrophilic regions of the polypeptide whose accessibility to trypsin were affected by the conformation of the transporter. These regions were the central hydrophilic loop between helices 6 and 7, and the C-terminal region following helix 12 (according to the model of Mueckler et al., 1985, Figure 1.4). When the transporters were present largely in the outward-facing conformation, due to binding of the transport inhibitor phloretin or ethylidene glucose at the extracellular surface, the accessibility of these regions to trypsin at the cytoplasmic surface was decreased. This may have resulted from conformational changes involving both regions of the polypeptide, or from those involving one region, whose altered conformation then sterically affected the other. In the presence of D-glucose the accessibility of these two regions to trypsin was increased. Because such an increase in accessibility occurred only in the presence of
D-glucose, and not in the presence of one of the non-transported inhibitors, this may have been due to the transporter being in a conformation unique to the transport process; a population of the transporters may have been in a transient state associated with the transport of D-glucose, and in which conformation the two regions were most accessible to the enzyme. This transient state may tentatively be suggested to be the stage when, according to the one-site alternating conformation model (Barnett et al., 1975; Baldwin & Lienhard, 1981; Vidaver, 1966) D-glucose has bound to the glucose transporter at either the cytoplasmic- or extracellular- sugar-binding site, and the transporter is changing conformation in order to release the sugar at the opposite side of the membrane.

Further evidence for the occurrence of a conformational change of the glucose transporter in the presence of the inhibitor phloretin was obtained from the results of the X-ray solution scattering experiments described in Chapter 5. Incubation of the transporter, in GTS/OG/PL mixed micelles, in the presence of phloretin, resulted in a change in the \( R_q \) of the structure without a change in the \( M_r \) compared to those measurements made in the absence of inhibitor; that is a change in the degree of elongation of the structure without a change in its \( M_r \). Such an effect could only be explained by a change in the conformation of the mixed micelle. Since the inhibitor had no effect on the \( R_G \) or \( M_r \) of OG/PL mixed micelles this conformational change was due to the effect of the inhibitor on the protein component of the GTS/OG/PL micelles.

7.2 THE SUBSTRATE-BINDING SITES OF THE GLUCOSE TRANSPORTER

Previous investigations into the regions of the glucose transporter polypeptide involved in both the cytoplasmic- and extracellular- sugar
binding sites have largely involved the use of the transport-inhibitors cytochalasin B and ASA-BMPA (Cairns et al., 1984, 1987; Deziel & Rothstein, 1984; Holman et al., 1986; Davies et al., 1987, 1990). These inhibitors, in a tritiated form, can be used covalently to radiolabel the transporter, in the presence of ultraviolet light, at the cytoplasmic- and extracellular- surfaces of the membrane respectively (Carter-Su et al., 1982; Holman et al., 1986; Holman & Rees, 1987; Shanahan, 1982). This covalent labelling by both inhibitors is itself inhibitable by D-glucose and therefore may involve covalent interactions in the vicinity of the sugar binding-sites at the cytoplasmic- and extracellular- surfaces of the membrane respectively. However one must not dismiss the second possibility that the D-glucose- and inhibitor- binding sites are linked allosterically. Cleavage of the radiolabelled transporter by a variety of enzymatic and chemical means resulted in the tentative identification of one region involved in the covalent binding of each of the inhibitors. $[^3H]$cytochalasin B was postulated to bind to the cytoplasmic surface of the transporter at the extramembranous loop between helices 10 and 11, and $[^3H]$ASA-BMPA was postulated to bind to the extracellular surface at the loop between helices 9 and 10 (according to the model of Mueckler et al., 1985) (Cairns et al., 1984, 1987; Deziel & Rothstein, 1984; Holman et al., 1986; Davies et al., 1987, 1990; Holman & Rees, 1987) (Section 1.7.1 and Figure 1.5). These regions were therefore postulated to be involved in the cytoplasmic- and extracellular- sugar-binding sites respectively.

The study described in Chapter 6 involved the chemical and enzymatic cleavage of the glucose transporter, radiolabelled at its cytoplasmic- and extracellular- surfaces with $[^3H]$cytochalasin B and $[^3H]$ATB-BMPA (Clark & Holman, 1990) respectively. The fragments thus produced were identified by the use of a bank of polyclonal
antibodies previously raised against whole glucose transporter and against synthetic peptides corresponding to regions of the transporter sequence (Davies et al., 1987, 1990). As a result of this study two regions of the polypeptide sequence were identified as being involved in the covalent binding of the two inhibitors to the glucose transporter. The region of the protein involved in the binding of $[^3H]$ATB-EMPA to the extracellular sugar-binding site probably includes amino acid residues lying between residues 301-330 of the sequence, and the region involved in the binding of $[^3H]$cytochalasin B to the cytoplasmic sugar-binding site probably includes amino acid residues lying between residues 389-412 of this sequence (Fig 6.10). These regions are postulated to be involved in the sugar binding sites at the extracellular and cytoplasmic surfaces of the erythrocyte membrane respectively. This would be in agreement with the model of Mueckler et al. (1985) which predicts that $\alpha$-helices 3,5,7,8 and 11 are amphipathic in nature and involved in the formation of a hydrophilic pore through which the transported sugars pass.

7.3 Solubilization and Stability of the Glucose Transporter

Techniques such as X-ray solution scattering (Chapter 5) and protein crystallization require maintenance of the protein in an active form whilst solubilized for periods of days to weeks (Michel, 1982). However, when solubilized in 46 mM-octyl glucoside the glucose transporter was known to be very unstable, having a half-life of 3.8 - 7.2 hours at $8^\circ C$ (Baldwin et al., 1982). The studies described in Chapter 4 involved the investigation of the suitability of a number of detergents (including octyl glucoside) for their use in solubilizing the glucose transporter. Of the eight detergents studied, only octyl glucoside and octyl thiogluconoside were efficient at solubilizing the
glucose transporter from alkali-stripped erythrocyte membranes in an active form. Octyl thioglucoside was more efficient than octylglucoside and was also selective in its solubilization of the glucose transporter as opposed to the nucleoside transporter. However, when the solubilized preparation was purified by anion exchange chromatography, as in the standard purification of the glucose transporter, the glucose transporter solubilized with octyl thioglucoside was less active than that solubilized with octyl glucoside. After this chromatography step the solubilized purified material was stable at 0°C, more than 75% of the cytochalasin B-binding activity remaining after incubation at 0°C for 50 hours, whether solubilized in octyl glucoside or octyl thioglucoside. Increasing the incubation temperature of the octyl glucoside-solubilized purified material to 8°C resulted in a dramatic decrease in stability, only 3% of the cytochalasin B-binding activity remaining after 18 hours. Therefore the instability of the solubilized purified material reported by Baldwin et al. (1982) was due in part to the temperature at which the material was maintained.

As a result of the studies described in Chapter 4, conditions were found which were suitable for maintaining the activity of the solubilized glucose transporter during the X-ray scattering experiments described in Chapter 5. The material was solubilized with octyl glucoside and maintained at a constant temperature of 0°C throughout the experimental procedures by means of a cooled sample holder. Such stabilizing conditions may also be suitable for the production of two-dimensional crystalline arrays of the glucose transporter, since several membrane proteins have been crystallized in this manner at low temperatures; two-dimensional crystalline arrays of Ca\textsuperscript{2+}-ATPase from sarcoplasmic reticulum were produced following incubation in specified conditions at 2°C for 16 hours (Dux et al.,
and two-dimensional arrays of lactose permease from *Escherichia coli* were produced following incubation at 4°C for 4-12 hours (Li & Tooth, 1987). However, the production of three-dimensional crystals suitable for X-ray crystallography may require incubation of the transporter at higher temperatures for similar periods, as was the case for crystallization of matrix protein from *Escherichia coli* (Garavito & Rosenbusch, 1980). This protocol required storage of the solubilized material at room temperature for one week, or 36-45°C for 36-48 hours. It may be feasible to stabilize the glucose transporter during procedures such as crystallization by promoting its interaction with a ligand, such as cytochalasin B, or one of the monoclonal antibodies raised against the native protein (Davies et al., 1990). Cytochalasin B has been seen to have possible stabilizing effects on the transporter before (Jung et al., 1986 and Section 3.4) and its use during the crystallization procedure would have the added advantage that the transporters should be mainly in the inward-facing conformation.

Although substantial progress has been made towards the understanding of the structure and function of the glucose transporter, much work remains to be done. The successful crystallization of the protein in its native form and subsequent X-ray crystallographic analyses should allow its structure to be determined to atomic resolution. Subsequent studies of the transporter crystallized in the presence of transport-inhibitors or D-glucose may allow the characterization of the various conformations of the sugar-binding site(s). Another powerful technique is that of site-directed mutagenesis which can be used to investigate the role of single amino-acid residues or specific sequences in the function and
regulation of proteins; this technique has been used with some success in investigations of the function of the $\beta$-galactoside transport system from *Escherichia coli* (Kaback, 1988). A pre-requisite for site-directed mutagenesis is the expression of the functional protein in a suitable expression system. This has recently been achieved for the GLUT 1 transport protein, which has been expressed in *Xenopus laevis* oocytes (Gould & Lienhard, 1989) and Chinese hamster ovary (CHO) cells (Oka, 1990). A significant discovery has already been made in the latter of these cases which is consistent with the conclusions drawn in Chapter 3; in the absence of its C-terminal region the transporter appears to be 'locked' into the inward-facing conformation and as such is incapable of glucose transport (Oka et al., 1990). With so many potentially useful techniques now available, a complete understanding of the glucose transporter seems to be nearly within our grasp.
REFERENCES


McDonald, R.C., Steitz, T.A. & Engelman, D.M. (1979) Biochemistry 18, 338-342


Miller, D.M. (1968) Biophys. J. 8, 1339-1352


Ramakrishnan, V. (1986) Science 231, 1562-1564


Proteolytic dissection as a probe of conformational changes in the human erythrocyte glucose transport protein

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INTRODUCTION

The amino acid sequence of the human erythrocyte membrane glucose transporter has recently been deduced from the nucleotide sequence of a cDNA clone encoding an apparently identical protein from HepG2 hepatoma cells [1]. The protein is known to be predominantly α-helical [2–6] and hydrophobic analysis of the sequence suggests that it contains 12 hydrophobic, membrane-spanning α-helical segments [1]. Several of these putative membrane-spanning helices are predicted to be amphipathic and may be clustered around an aqueous channel through which the glucose moves [1,6,7]. The major hydrophilic regions of the sequence are proposed to be exposed on the cytoplasmic surface of the membrane. These include the N-terminal region (residues 1–12), the C-terminal region (residues 451–492) and a large extramembranous loop near the centre of the sequence (residues 207–271) [1]. The cytoplasmic locations of the latter two regions have in fact recently been confirmed both by vectorial tryptic-digestion experiments [1,8–10], and by the use of peptide-specific antibodies [11].

Despite these considerable recent advances in our understanding of the transporter structure, the mechanism of transport remains unclear. Two main types of kinetic model have been proposed to account for the observed features of the transport process. In the alternating conformational model, a single substrate-binding site is alternately exposed at the two surfaces of the membrane by means of a conformational change [12–14]. In contrast, a recent model proposed by Carruthers and colleagues on the basis of fluorescence quenching [15,16] and ligand binding [17] studies, suggests that two substrate-binding sites are simultaneously present, one exposed at each face of the membrane. Although the two models are fundamentally different in many respects, both imply that conformational changes in the transport protein are central to the molecular mechanism of transport. Such changes have in fact been detected by a large variety of techniques [5,6,18–23].

Although evidence for the involvement of conformational changes in the mechanism of transport is strong, the identity of those regions of the protein that are affected by the changes remains largely unknown. A recent report on the inactivation of transport by alkylating agents suggested that substrate-induced conformational changes occur primarily within the hydrophobic, membrane-spanning regions of the protein, while the hydrophilic segments outside the bilayer are largely unaffected [24]. In the present study, we have used tryptic digestion to investigate more fully the possible involvement of the hydrophilic, cytoplasmic domain of the protein in transport-related conformational changes. Trypsin cleaves the transporter, in the absence of substrates or inhibitors of transport, solely at the cytoplasmic surface of the membrane [8,9]. Cleavage occurs at several sites in the central extramembranous loop of the sequence between Arg-212 and Arg-269, and in the C-terminal region of the sequence, following Lys-456 [10]. After short periods of digestion, cleavage within the central loop yields a glycosylated fragment, derived from the N-terminal half of the protein, which runs as a broad band of apparent Mr 23000–42000 on SDS/polyacrylamide gels [8,11,25]. The C-terminal half of the protein initially yields a tryptic fragment which migrates as a sharp band of apparent Mr 25500. The latter is subsequently converted via a fragment of apparent Mr 23000 to a stable product of apparent Mr 18000, which lacks the C-terminus of the intact protein [8,11,25]. However, the order in which the cleavages occur appears not to be obligatory; cleavage of the intact transporter can occur first near its C-terminus, yielding substantial amounts of a glycosylated fragment of apparent Mr 45000 after short periods of digestion [11].

We describe in this report the effects of D-glucose and of reversible inhibitors of transport on the rate and...
pattern of these tryptic cleavages of the transporter. The inhibitors used were phenyl β-D-glucoside and cytochalasin B, which bind preferentially at the cytoplasmic side of the membrane [13,26], and 4,6-O-ethylidene-a-D-glucose and phloretin, which bind preferentially at the extracellular side of the membrane [27,28]. These inhibitors would be predicted by the alternating conformation model for transport to bind preferentially to the inward-facing and outward-facing conformations of the protein respectively, and so cause it to accumulate in one of these forms. The resultant effects on the cleavage of the transporter by trypsin have, for the first time, identified specific regions of the protein sequence that are involved in conformational changes.

A preliminary account of part of this work was presented at the 623rd meeting of The Biochemical Society in 1987 [28a].

EXPERIMENTAL

Materials
Cytochalasin B and 4,6-O-ethylidene-a-D-glucose were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Bovine lung aprotinin was from the Boehringer Corporation (Lewes, East Sussex, U.K.). Phloretin, phenyl β-D-glucopyranoside, L-glucose, β-D-glucose, diphenylcarbamyl chloride-treated trypsin and molecular-mass markers for SDS/polyacrylamide-gel electrophoresis were supplied by Sigma (Poole, Dorset, U.K.). New England Nuclear (Southampton, Hants., U.K.) supplied [4-14C]cytochalasin B. Outdated human blood was provided by the blood bank of the Royal Free Hospital.

Preparation of the human erythrocyte glucose transporter
The human erythrocyte glucose transporter was prepared from outdated blood by the method of Baldwin et al. [29], with the modifications described by Cairns et al. [25]. The cytochalasin B-binding activity of each preparation was measured by equilibrium dialysis as previously described [30]. Only those preparations that contained more than 11 nmol of cytochalasin B-binding sites per mg of protein were used in this study. All showed a single broad band of Coomassie Blue-staining material of average apparent Mr 55000 on SDS/polyacrylamide-gel electrophoresis.

Tryptic digestion
Tryptic digestion was performed in 50 mm-sodium phosphate/100 mm-NaCl/1 mm-EDTA, pH 7.4, in the presence or absence of sugars or transport inhibitors. The respective concentrations of purified transporter and of trypsin were 216 μg/ml and 2.5 μg/ml unless stated otherwise. At various times during the digestions, samples were taken for analysis by SDS/polyacrylamide-gel electrophoresis. The trypsin present in these samples was immediately inactivated by the addition of a 2-fold excess (by weight) or bovine lung aprotinin.

Control experiments were carried out using alkaline-stripped human erythrocyte membranes [29] to ensure that any effects of substrates and inhibitors of glucose transport on the rate of transporter cleavage resulted from their binding to the transporter rather than to trypsin. None of the compounds tested had any effect on the rate of tryptic cleavage of the anion transporter in these membranes under conditions identical with those used for digestion of the purified glucose transporter (results not shown). However, their effects on the rate of production of tryptic fragments of the glucose transporter in these membranes were similar to those seen using the purified protein (results not shown).

The apparent rate of cleavage of the protein by trypsin varied slightly between transporter preparations, probably because of their variable content of sealed, vesicular material in which the cytoplasmic domain of the transporter is not accessible for cleavage. Therefore, comparisons of the effects of different sugars or inhibitors on the cleavage rate were always made by performing parallel digestion experiments simultaneously, using identical trypsin and transporter preparations. The proportion of transporters inaccessible to trypsin was estimated from the amount of transporter remaining uncleaved after extended periods of digestion. Typically, about 25% of the transporter remained intact even after treatment with protease for 26 h at 25 °C, fresh trypsin (5%, w/w) being added after 0, 3 and 6 h.

SDS/polyacrylamide-gel electrophoresis
SDS/polyacrylamide-gel electrophoresis was carried out by the procedure of Laemmli [31] using 12% polyacrylamide gels. Proteins of known Mr, used as markers were as previously described [25]. After fixation and staining with Coomassie Blue the gels were scanned spectrophotometrically at 530 nm. Previous studies (D. A. Elliot & S. A. Baldwin, unpublished work) have shown a linear relationship between peak area and the amount of transporter on a gel over the concentration range used here. Routinely, the peak area data were corrected for the presence of transporter inaccessible to trypsin by subtracting the value for intact transporter remaining intact after extended trypsin treatment, as described above. After correction in this fashion the data indicated the percentage of the 'accessible' transporters remaining intact after various periods of tryptic digestion. They were then fitted to straight-line, first-order plots by linear regression.

RESULTS
The rate of cleavage of the membrane-bound glucose transporter by trypsin at 25 °C, in the presence of the non-transported substrate analogue 4,6-O-ethylidene-a-D-glucose (ethylidene glucose) at a concentration of 200 mM, was found to be markedly lower than in the presence of 200 mM-L-glucose, which does not bind to the protein (Figs. 1 and 2). The extents of cleavage were measured by spectrophotometric scanning of the gels shown in Fig. 1. This procedure slightly underestimates the true extent of cleavage because the Mr 45000 tryptic fragment (arrowed in Fig. 1) is not fully resolved from the broad Mr 55000 band of the intact transporter. However, after correction for the presence of 28% of the transporters that were inaccessible to trypsin, the cleavage data showed apparent first-order kinetics (Fig. 2). Ethylidene glucose was found to decrease the first-order rate constant for cleavage by about 4-fold relative to L-glucose (Table 1). Inspection of the gels showed that the patterns of fragments produced in the presence of the
Conformational changes of the glucose transporter

Glucose transporter (216 µg/ml) was digested at 25 °C with 1.2%, (w/w) trypsin for the times indicated, in the presence of either (a) 200 mM-ethylidene glucose or (b) 200 mM-L-glucose. Samples were electrophoresed on a 12%, SDS/polyacrylamide gel and then stained with Coomassie Blue. The positions of marker proteins of known Mr, and of the glycosylated Mr 45000 fragment of the transporter (arrowed), are indicated. DF = tracking dye front.

The relative amounts of intact transporter remaining after various times of trypsin digestion in the presence of 200 mM-ethylidene glucose (○) or 200 mM-L-glucose (●) were determined from spectrophotometric scans of the gels shown in Fig. 1. The data are shown as a semi-logarithmic plot after correction for the presence of transporters inaccessible to trypsin as described in the Experimental section. The straight lines have been fitted by linear regression.

two sugars were the same, but that the rates of production both of the Mr 45000 fragment (arrowed in Fig. 1) and of the Mr 18000 fragment and its precursors were reduced in the presence of ethylidene glucose. This finding was confirmed by spectrophotometric scanning to quantify the Mr 18000 fragment (results not shown).

The mechanism of the different effects of L-glucose and ethylidene glucose was further investigated by examining the effects of different concentrations of the two sugars on trypsin cleavage. In this experiment the transporter (160 µg/ml) was incubated for a fixed period (4 h) with trypsin (1%, w/w) at 25 °C, and the effects of the sugars were monitored by quantifying the amounts of both the uncleaved transporter and its Mr 18000 trypsin fragment on a gel. The extent of cleavage was little affected by L-glucose at concentrations up to 400 mM, but decreased as the concentration of ethylidene glucose increased up to 400 mM (Fig. 3). The half-maximal effect of the sugar on the amount of the Mr 18000 fragment produced was seen at about 25 mM-ethylidene glucose. The concentration required for half-maximal effect on the amount of the intact transporter remaining after digestion appeared to be higher, at approximately 75 mM. However, less confidence can be placed upon this estimate because the presence of the Mr 45000 trypsin fragment precludes accurate quantification of the intact transporter, as mentioned above. Accurate estimation of the Mr 18000 fragment is more easily achieved because it is well resolved from other fragments on the gel.

The effects of phloretin upon the rate of trypsin cleavage of the transporter were also investigated. This compound is a potent inhibitor of transport and resembles ethylidene glucose in that it binds at the extracellular surface of the transporter [28]. The effect of phloretin (100 µM) was similar to that of ethylidene glucose; the rate of cleavage of the transporter by trypsin was slowed and this effect was manifested as a decrease in the rate of production of both the Mr 45000 and the Mr 18000 trypsin fragments.
Table 1. The effects of substrate and inhibitors on the rate of tryptic cleavage of the glucose transporter

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Inhibitor/substrate</th>
<th>$10^3 \times$ Rate constant (min$^{-1}$)</th>
<th>Control</th>
<th>$10^3 \times$ Rate constant (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>Ethylidene glucose</td>
<td>4.5 ± 0.6</td>
<td>L-Glucose</td>
<td>17.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>(200 mM)</td>
<td></td>
<td>(200 mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phloretin</td>
<td>5.5 ± 0.4</td>
<td>Ethanol</td>
<td>15.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>(100 μM)</td>
<td></td>
<td>(1 %, v/v)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D-Glucose</td>
<td>21.6 ± 1.6</td>
<td>L-Glucose</td>
<td>13.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>(200 mM)</td>
<td></td>
<td>(200 mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D-Glucose</td>
<td>19.8 ± 1.4</td>
<td>L-Glucose</td>
<td>12.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>(200 mM)</td>
<td></td>
<td>(200 mM)</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>Ethylidene glucose</td>
<td>9.6 ± 2.1</td>
<td>L-Glucose</td>
<td>37.0 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>(200 mM)</td>
<td></td>
<td>(200 mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phloretin</td>
<td>16.3 ± 1.5</td>
<td>Ethanol</td>
<td>36.2 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>(100 μM)</td>
<td></td>
<td>(1 %, v/v)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochalasin B</td>
<td>26.6 ± 1.7</td>
<td>Ethanol</td>
<td>36.2 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>(20 μM)</td>
<td></td>
<td>(0.5 %, v/v)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>Cytochalasin B</td>
<td>50.0 ± 7.0</td>
<td>Ethanol</td>
<td>201.0 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>(20 μM)</td>
<td></td>
<td>(0.5 %, v/v)</td>
<td></td>
</tr>
</tbody>
</table>

Glucose transporter (160 μg/ml) was digested at 25 °C for 4 h with 1 % (w/w) trypsin in the presence of various concentrations of L-glucose (●, □) or ethylidene glucose (○, △). Samples were electrophoresed on a 12 % SDS/polyacrylamide gel and then stained with Coomassie Blue. The relative amounts of the intact transporter (●, ○) and of the tryptic fragment of apparent $M_r$ 18000 (□, △) were estimated by spectrophotometric scanning.

(results not shown). After correction for the presence of 36 % of the transporter which was inaccessible to trypsin, the cleavage data exhibited first-order kinetics (Fig. 4). Phloretin decreased the first-order rate constant for cleavage about 3-fold (Table 1).

Ethylidene glucose and phloretin bind preferentially to the transporter at the extracellular surface of the membrane [27,28]. For comparison, the effects of cytochalasin B and phenyl β-D-glucoside (phenyl glucoside) on the rate of cleavage of the transporter by trypsin were investigated. These inhibitors bind preferentially at the cytoplasmic side of the membrane [13,26]. Cytochalasin B (20 μM) had no effect on the rate of tryptic cleavage of the transporter at 25 °C (results not shown). Similarly, the presence of 50 mM-phenyl glucoside slowed the rate of cleavage of the transporter, relative to a control, to a much smaller extent than either ethylidene glucose or phloretin (results not shown).

A possible explanation for the protection against tryptic cleavage afforded by ethylidene glucose and phloretin might be that these agents stabilize the transporter in a trypsin-resistant, outward-facing conformation. Conversely, if the alternating conformation model for transport is correct, cytochalasin B and phenyl glucoside might be expected to increase the rate of cleavage by stabilizing an inward-facing conformation of the transporter. No such effect was seen at 25 °C. However, analysis of the kinetics of transport by Lowe & Walmsley [32], using such an alternating conformation model, led to the prediction that the proportion of the transporter in each conformation is temperature dependent. In the human erythrocyte, at 25 °C, 75 % of the transporters are predicted to be in the inward-facing conformation. Therefore, at this temperature any effects of cytochalasin B and phenyl glucoside mediated by conformational changes might be expected to be smaller than those produced by stabilizers of the outward-facing
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**DISCUSSION**

Both phloretin and ethylidene glucose were found to decrease the rate of tryptic cleavage of the purified glucose transporter at 25 °C and at 45 °C. Phloretin has been reported to bind exclusively to a site on the transporter that is exposed to the extracellular environment [17,28], whereas trypsin cleaves the protein solely at sites on the cytoplasmic surface of the membrane [8,9]. The effect of phloretin cannot, therefore, be the result of simple steric hindrance following its occupation of the binding site. Instead, it must involve a conformational change to a form of the protein in which the sites of cleavage are less accessible to trypsin.

The same explanation probably also accounts for the effect of ethylidene glucose, although this sugar analogue does not exhibit absolute specificity for the extracellular substrate-binding site [17,27]. The concentration dependence of the effect revealed in Fig. 3 indicates that it does not merely reflect a non-specific perturbation of the lipid environment of the protein. The inevitable presence of a proportion of inactive transporters in the preparations used in this study [29] complicates the kinetic analysis of the cleavage process. However, the concentration of ethylidene glucose required to afford half-maximal protection against cleavage, 25 mM, corresponds closely to the value of 26 mM measured as the $K_v$ for inhibition of cytochalasin B binding to protein-depleted erythrocyte membranes [33]. It has been suggested that such a high $K_v$ value reflects binding to a low-affinity site at the cytoplasmic surface of the membrane [17]. However, even if a portion of the ethylidene glucose is binding to such a site, it is unlikely for two reasons that the resultant steric hindrance is responsible for the effect on cleavage. Firstly, at the concentration where the half-maximal effect is seen (25 mM), the two-sites model predicts that only 17% of the extracellular sites would be occupied (calculated from the data in ref. [17]). Secondly, occupation of presumably the same intracellular sites by the bulky molecules phenyl glucoside and cytochalasin B causes little or no steric hindrance respectively to the action of trypsin at 25 °C.

The observation that neither cytochalasin B nor phenyl glucoside increased the rate of tryptic cleavage of the transporter even at 50 °C is at first sight difficult to reconcile with a single-site, alternating conformation model for transport in which the outward-facing conformation of the protein is more resistant to tryptic cleavage. This model has been supported by a recent series of steady-state kinetic studies [32,34,35]. A possible explanation for the results would be that the purified transporter adopts largely an inward-facing conformation even at high temperatures. The lipid environment of the purified protein is known to be different from that which it experiences in the intact erythrocyte membrane, and so the temperature dependence of its conformational states may differ from that which occurs in the cell. This altered environment may also be responsible for the finding that the transport activity of the purified transporter is much lower than that seen in the intact cell [36,37]. Alternatively, the single-site model may not adequately describe the mechanism of transport, and a more complex model, such as that of Helgerson & Carruthers [17], may be required.

Conformational changes in the protein resulting from the binding of cytochalasin B have been detected by...
change at one site sterically hinders the access of trypsin to the other. These hydrophobic regions of the transporter have not previously been identified as undergoing translocation-related conformational changes. Our present findings imply that they have an important role in the mechanism of transport.

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REFERENCES


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Conformational changes of the glucose transporter


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