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Exofacial photoaffinity labelling of the human erythrocyte sugar transporter

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The 4-azidosalicylate derivative of 1,3-bis(D-mannos-4′-yloxy)-2-[2-^{3}H]propylamine (ASA-[2-^{3}H]BMPA) has been tested as a photoaffinity label for the sugar transporter in human erythrocytes. When photolysed in the presence of intact erythrocytes, ASA-[2-^{3}H]BMPA covalently binds to the exofacial surface of the transporter. This labelled protein appears as a broad band in the 4.5 region in sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. The peak of radiolabel incorporation gives an apparent M_r of approx. 50000 on 5–20% acrylamide gels. The binding is 80% inhibitable by 320 mM 4,6-*O*-ethylidene-D-glucose, by 320 mM D-glucose and by 50 μM cytochalasin B. Photoirradiation of a saturating concentration of ASA-BMPA in the presence of erythrocytes results in a 25–30% loss of D-galactose transport activity. From transport inactivation data and estimations of the amount of ASA-[2-^{3}H]BMPA binding to the transporter it is calculated that there are approx. 220000 exofacial hexose-transport binding sites per erythrocyte. The labelling of the transporter has been carried out using freshly drawn blood and 4-weeks-old transfusion blood. No change in the binding profile on SDS-polyacrylamide gel electrophoresis was observed. Proteolytic digestion of the ASA-[2-^{3}H]BMPA-labelled transporter with either trypsin or α-chymotrypsin results in the appearance of a labelled 19 kDa fragment on SDS-polyacrylamide gel electrophoresis.

Introduction

A number of compounds have been reported to be affinity or photoaffinity labels for the hexose transporter in human erythrocytes. These include D-maltosylisothiocyanate [1] and 6-*N*-(4-azido-2-hydroxy-3,5-diiodobenzoyl)-D-glucosamine [2]. However, the most widely used compound is the fungal metabolite, cytochalasin B. This compound is thought to bind to an internal site of the transporter [3–5] and can bind covalently in a light-dependent manner to the hexose transporter of human erythrocytes and rat adipocytes [6–8]. It has been used to investigate the mechanism of insulin action on hexose transport in adipocytes [8,9] and the topographical orientation of the transporter in erythrocytes [10].

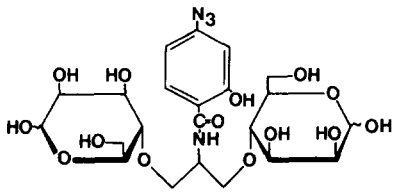
Our aim has been to synthesise a nontransported photoaffinity label which binds specifically to the outside site of the transporter and which cannot enter the cell through the lipid bilayer. A nonpenetrating photoaffinity label would be a useful complement to cytochalasin B in investigations of the relative positions of the internal and external sites on the transport protein. Furthermore, it would be an important tool in the investigation of the insulin stimulation of transport in adipocytes.

We have previously reported the synthesis of a number of bis(D-mannose) compounds [11] which we considered potentially useful as photoaffinity labels for the external site of the transporter. These compounds are synthesised from two D-mannose

Abbreviations: ASA-BMPA, 2-*N*-(4-azidosalicoyl)-1,3-bis(D-mannos-4′-yloxy)propylamine; PMSF, phenylmethylsulphonyl fluoride.

moieties joined at their C-4′ positions by a 2-propylamine bridge. The photolabile substituent is attached through the amino group. We have demonstrated that these compounds have a high affinity for the hexose transporter of both the human erythrocyte and the rat adipocyte [12,13]. We have also shown that these compounds are not significantly taken up into either type of cell over a period of 90 min, neither via the transporter nor through the lipid bilayer.

We now report our results investigating one of these compounds, 2-*N*-(4-azidosalicoyl)-1,3-bis(D-mannos-4′-yloxy)propylamine (ASA-BMPA), as a photoaffinity label for the erythrocyte transporter. The structure of this compound is shown in Scheme I.



Scheme I. The structure of 2-*N*-(4-azidosalicoyl)-1,3-bis(D-mannos-4′-yloxy)propylamine.

Materials and Methods

Reagents. ASA-[2-^{3}H]BMPA (1–2 Ci/mmol) was prepared as previously described [12]. D-[U-^{14}C]glucose and D-[1-^{14}C]galactose were from Amersham International. Cytochalasin B was from Aldrich. Chymotrypsin, trypsin and the proteinase inhibitors PMSF, pepstatin and soybean trypsin inhibitor were obtained from Sigma. Other reagents were from BDH.

Erythrocytes. Erythrocytes were prepared from freshly drawn blood or from 1–4-weeks-old transfusion blood by washing five times in phosphate saline buffer (154 mM NaCl/12.5 mM sodium phosphate (pH 7.2)). Care was taken to remove the 'buffy coat' of white cells and platelets.

Transport experiments. The K_i (the half-maximal inhibition constant) for ASA-BMPA was determined using D-glucose as the substrate. The net uptake of D-glucose in a 10% cytocrit erythrocyte suspension at 20°C was measured at a range of concentrations from 1 to 10 mM with and without the inhibitor (75 μM). The initial rate was taken as the D-glucose uptake in 2 s [2,14,15].

The fractional loss of transport sites following an irradiation of erythrocytes in the presence of ASA-BMPA was determined. The uptake of 100 μM D-galactose was used to assay for residual transport activity. A 10% suspension of cells was irradiated at room temperature for up to 1 min in the presence and in the absence of 900 μM ASA-BMPA. The details of the irradiation procedure are given below. Following irradiation, the cells were washed twice in phosphate saline buffer and then resuspended at 10% cytocrit and at 20°C. The cells were then mixed with 100 μM D-galactose and uptake was measured at zero time, 10 s and 2–3 min (infinite time). Since the substrate concentration is low, the fractional filling at 10 s can be used to calculate the uptake rate constant [12]. The rate constant is equal to $-(\ln(1-f))/t$, where f is the fractional filling and t is the time.

The uptake of sugars was in all cases terminated by adding 3 ml of stopping solution (10 μM $HgCl_2$/0.3 mM phloretin in phosphate saline buffer). Cells were spun briefly in a refrigerated bench centrifuge. The supernatants were removed and then the cell pellets were resuspended in stopping solution and respun. The cell pellets were finally extracted with 10% trichloracetic acid and the radioactivity was estimated by liquid scintillation counting.

Photoaffinity labelling. 5–7 μCi of ASA-[2-^{3}H]BMPA (1–2 Ci/mmol) were mixed with a 400 μl of a 20% cell suspension plus 400 μl of a solution containing transport inhibitors or the nonbinding substitute D-mannitol. Samples were incubated for 10 min at room temperature and then placed in two 400-μl quartz thin film demountable cuvettes (1 mm pathlength). The cuvettes were covered with a large 10 mm pathlength quartz cell containing 1% cumene in 2,2,4-trimethylpentane. The samples were irradiated (usually for 15 s) in a Rayonet RPR-100 photoreactor (Southern New England Ultraviolet Company) using RPR-3000 lamps. The cells were then washed five times in 35 ml of phosphate saline buffer. They were then lysed at 0–4°C in 35 ml of a buffer containing 5 mM sodium phosphate, 1 mM EDTA, 20 μg/ml PMSF, 1 μg/ml pepstatin (pH 7.8). The membranes were collected at 37000

$\times g$ for 20 min. In the experiments in which either trypsin or α-chymotrypsin were used, the membranes were incubated with the enzymes under the indicated conditions. The proteolytic reactions were terminated with either soybean trypsin inhibitor for the trypsin reaction or with PMSF for the α-chymotrypsin reaction and then the membranes were respun at 37000 $\times g$ for 20 min. In all experiments, a small sample of membrane pellet was taken for a protein estimation by the method of Lowry et al. [16]. The remainder was prepared for electrophoresis.

Electrophoresis. Erythrocyte membranes were mixed 7 parts : 1 part with a buffer comprising 40 mM Tris-HCl/8% glycerol/8% 2-mercaptoethanol/8% SDS and were run on 5–20% acrylamide linear-gradient gels using the Neville buffer system [17]. The gel was stained with Coomassie blue, and then destained and sliced (3 slices/cm). The slices (in scintillation vials) were dried at 60°C for 6–8 h and then dissolved in 0.5 ml of alkaline hydrogen peroxide (0.7% ammonium hydroxide in 30% hydrogen peroxide) at 60°C overnight [32]. Samples were counted in 5 ml of scintillation fluid at about 25% counting efficiency.

Results

In a previous publication [12] we showed that the photoaffinity label ASA-BMPA was a good inhibitor of the uptake of 100 μM D-galactose. The half-maximal inhibition constant was 146 ± 4 μM. This value is likely to be more representative of the true K_i than an inhibition constant which is determined in the presence of significant substrate concentrations. However, it was considered to be of interest to measure the inhibition constant in a zero-trans D-glucose influx experiment. The results of this experiment are shown in Fig. 1. The $K_{i,app}$ is 64 ± 12 μM. This may mean that the substrate (D-glucose) can influence the interaction with the inhibitor in some complex kinetic manner to give rise to an apparent slight lowering of the K_i. Alternatively, this effect may be due simply to the difficulties associated with measuring true initial rates for such a rapidly transported sugar as D-glucose. Fig. 1 also shows that ASA-BMPA is a competitive inhibitor of D-glucose net uptake.

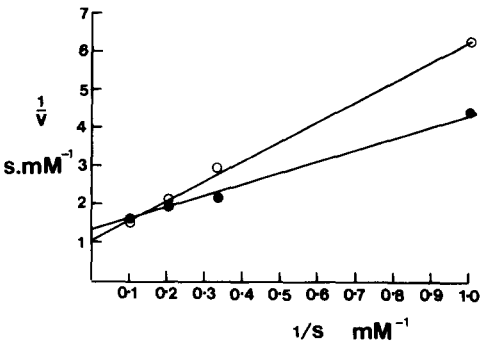


Fig. 1. The net uptake of D-glucose into erythrocytes at 20°C in the absence (●) and in the presence (○) of 75 μM ASA-BMPA. The K_m in the absence of inhibitor is 2.2 ± 0.3 mM ($n = 8$). The $K_{m,app}$ in the presence of inhibitor is 5.0 ± 0.2 mM ($n = 8$). The calculated K_i is 64 ± 12 μM. Estimates are from nonlinear regression.

Before an examination of the binding of radiolabelled ASA-BMPA to erythrocyte membranes was undertaken, it was considered necessary [18] to examine the photolysis of the nonlabelled compound in the presence of cells and to determine whether photolysis in the presence of a saturating concentration of the compound resulted in a significant loss of transport sites. The photolysis of ASA-BMPA is extremely rapid in the absence of erythrocytes (Fig. 2a). The ultraviolet absorption peaks at about 270 nm and at 320 nm are both considerably reduced even after 1 s of irradiation. Within about 4 s the compound is completely photolysed. In the presence of cells at 10% cytocrit and in a cuvette having a pathlength of 1 mm, the photolysis of the compound is considerably slowed. Even after 15 s of irradiation the compound is incompletely photolysed (Fig. 2b). This slowing of the photolysis rate is presumably due to light absorption by haemoglobin. We have found that it is not possible to photolyse the compound using short irradiation times if the cytocrit is above 10%.

Irradiation of erythrocytes for up to 1 min does not result in loss of any D-galactose-transport activity (Fig. 3). However, if cells are irradiated in the presence of 900 μM ASA-BMPA, washed and assayed for residual D-galactose-transport activity, then a significant loss of sites is detectable. There is about a 20% loss of transport activity in 15–30 s, while irradiation for 1 min results in a 25–30% loss of transport activity. Thus, irradiation in the

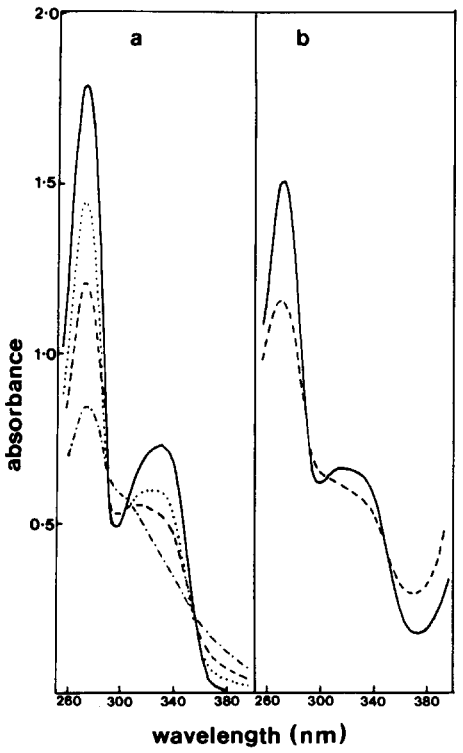


Fig. 2. (a) The photolysis of 150 μM ASA-BMPA in distilled water. The photolysis times are 1 s (· · · · · ·), 2 s (- - - - - -) and 4 s (· – · – ·). (b) The photolysis of 150 μM ASA-BMPA in the presence of a 10% suspension of erythrocytes in a phosphate saline buffer. The photolysis time (- - - - - -) is 15 s. After photolysis, the erythrocytes were sedimented and the supernatants were scanned in a spectrophotometer.

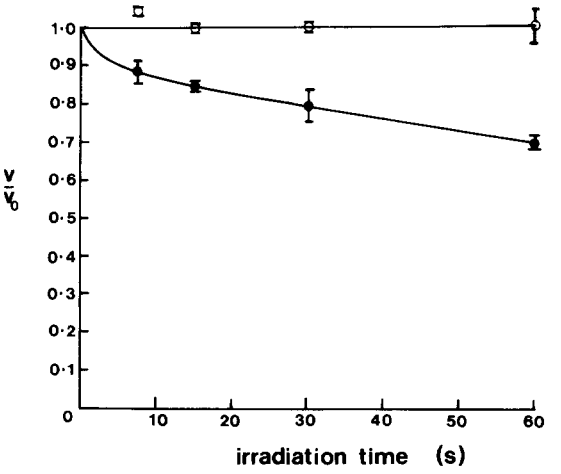


Fig. 3. An estimate of residual D-galactose-transport activity after irradiation in the absence (○) and in the presence (●) of 900 μM ASA-BMPA. Following irradiation, ASA-BMPA was removed by washing and resuspending the erythrocytes twice in phosphate saline buffer. The transport activity is estimated as the ratio of the uptake rate constant following irradiation over the uptake rate constant at zero irradiation time. The fractional loss of sites at 7, 15 and 30 s is 0.14, 0.16 and 0.19, respectively. These values are used to calculate the approximate number of sites as described in the text.

presence of the photoaffinity probe must result in a covalent interaction with the transport system in a region of the transport protein that is responsible for catalysing transport.

Irradiation of erythrocytes in the presence of 7 μCi of ASA-[2-^{3}H]BMPA (1–2 Ci/mmol) for up to 30 s results in a progressive increase in label which is associated with the plasma membrane and which runs in the 4.5 region on SDS-polyacrylamide gels (Fig. 4). The 4.5 region is considered by many investigators to correspond to the D-glucose transporter [2,6,7,19–21] and this is the region to which cytochalasin B has been shown to bind. The apparent molecular weight which can be estimated from the position of the peak of labelling is approx. 50 kDa. There is no incorporation of radiolabel if cells are incubated in the dark with ASA-[2-^{3}H]BMPA.

As an approximation, it can be assumed that pmol bound = (total sites)$\cdot f_o \cdot f_l$, where f_o is the fractional occupancy by radiolabelled ASA-BMPA and f_l is the fractional loss of sites which occurs when the transport system is fully saturated by nonlabelled ASA-BMPA. The fractional occupancy by radiolabelled ASA-BMPA is about 0.05. f_l can be calculated from the data in Fig. 3 where the loss of sites that occurs during the photolysis of 900 μM is estimated. Thus, the ratio of pmol ASA-BMPA bound per cell/$(f_o \cdot f_l)$ gives an estimate of the total number of transport sites. From the data in Fig. 4 we calculate that there are 220000 binding sites for ASA-BMPA at the external surface of red cells. This agrees well with the estimates of the total number of binding sites which can be obtained from cytochalasin B binding data [20]. We have previously shown that erythrocytes are completely impermeable to ASA-BMPA [12]. The apparent fractional filling of erythrocytes by this compound are less than 1% even after 90 min of incubation at 20°C. Thus, our compound provides the first clear demonstration that an external inhibitor binds to the same pro-

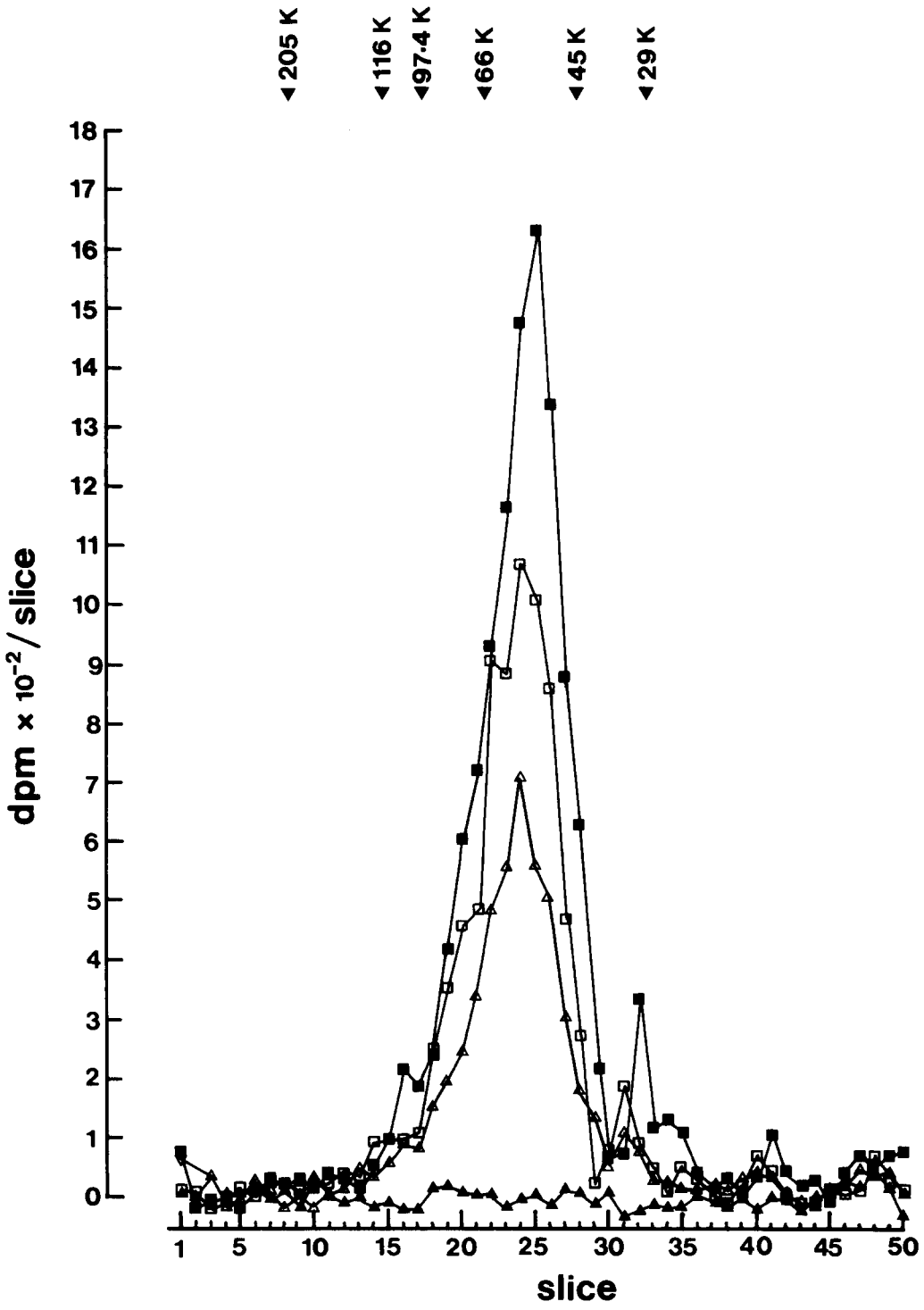


Fig. 4. The effect of increasing irradiation time on the incorporation of radiolabel from 7 μCi of ASA-[2-^{3}H]BMPA in 800 μl of a 10% erythrocyte suspension ($80 \cdot 10^7$ cells). Data from zero irradiation (▲), 7 s irradiation (△) (1.57 pmol bound), 15 s irradiation (□) (2.33 pmol bound) and 30 s irradiation (■) (3.6 pmol bound) were used to estimate the number of sites per cell.

tein as the internal inhibitor cytochalasin B. Weber and Eichholz [2] have also labelled this protein with 6-*N*-(4-azido-2-hydroxy-3,5-diiodobenzoyl)-D-glucosamine. One would suspect that their compound also interacts with the external site of the transporter but this is such a hydrophobic compound that it cannot be confined to the external surface of erythrocytes and thus also labels peptides associated with the internal surface of erythrocyte membranes such as band 6 [2].

The incorporation of label from 5 μCi of ASA-[2-^{3}H]BMPA is more than 80% blocked by 320 mM 4,6-*O*-ethylidene-D-glucose (Fig. 5). 4,6-*O*-Ethylidene-D-glucose is a competitive inhibitor of transport in erythrocytes and interacts only with the outside site of the transporter [22]. Thus, it would be expected to compete with ASA-BMPA for occupancy of the exofacial side of the transporter. A more interesting experiment is to determine whether the binding of ASA-BMPA to the transporter is blocked by an inhibitor which is thought to compete at the internal site. Cytochalasin B is thought to act from the internal surface and it has been shown to be a competitive inhibitor of D-glucose exit [4]. 50 μM cytochalasin B gives more than 80% inhibition of the covalent labelling of the transporter by 7 μCi of ASA-[2-^{3}H]BMPA (Fig. 6). This observation suggests that the transporter cannot be simultaneously occupied by an outside inhibitor and by high concentrations of cytochalasin B.

The transported sugar D-glucose (at 320 mM) is also a very effective inhibitor of ASA-[2-^{3}H]BMPA labelling of the Band 4.5 protein (Fig. 6).

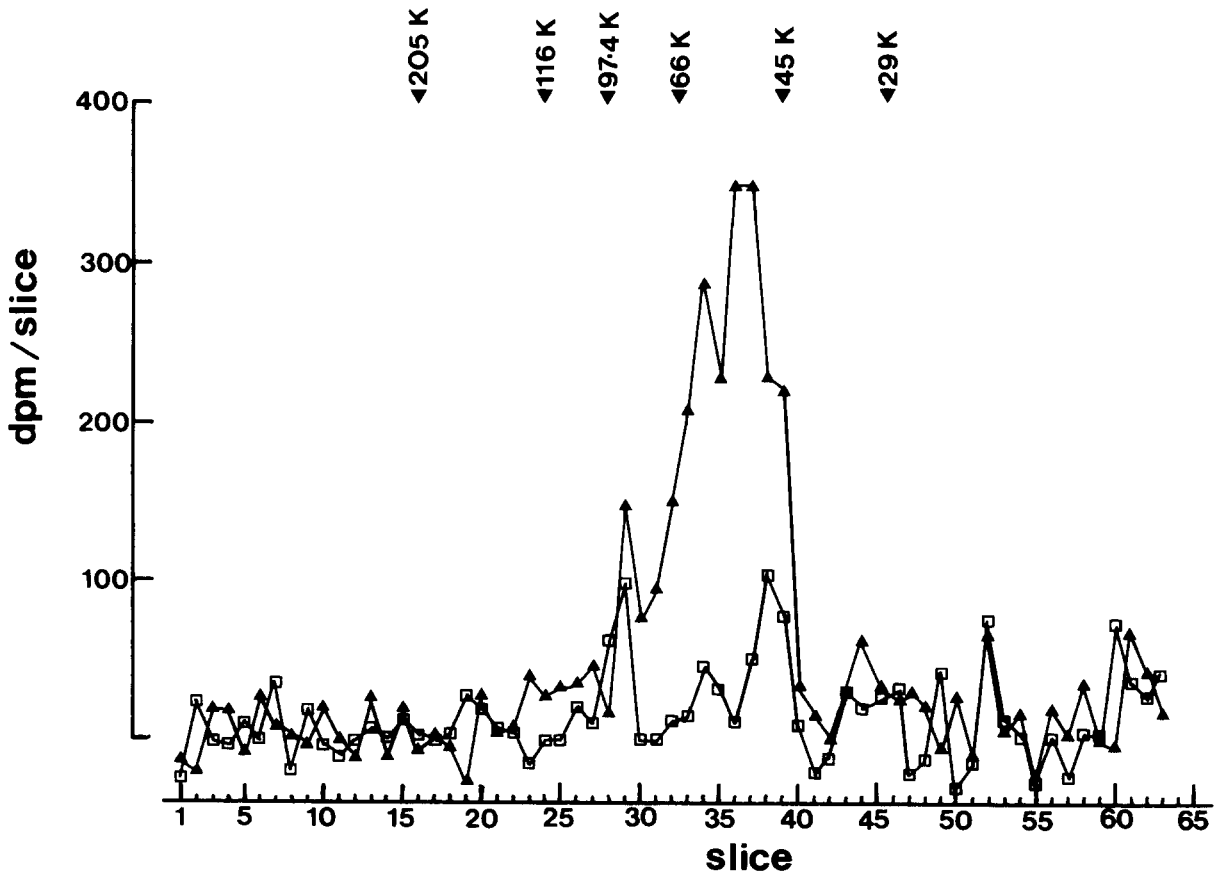


Fig. 5. The labelling of 800 μl of a 10% suspension of erythrocytes which were irradiated for 15 s in the presence of 5 μCi of ASA-[2-^{3}H]BMPA in the presence of 320 mM 4,6-*O*-ethylidene-D-glucose (□) or 320 mM D-mannitol (▲). 310 μg of membrane protein per lane were used.

The experiment shown in Fig. 6 was carried out on freshly drawn blood, while that of Fig. 5 was carried out on 3-weeks-old transfusion blood. A similar experiment using D-glucose instead of 4,6-*O*-ethylidene-D-glucose as an inhibitor of binding to 4-weeks-old transfusion blood also showed

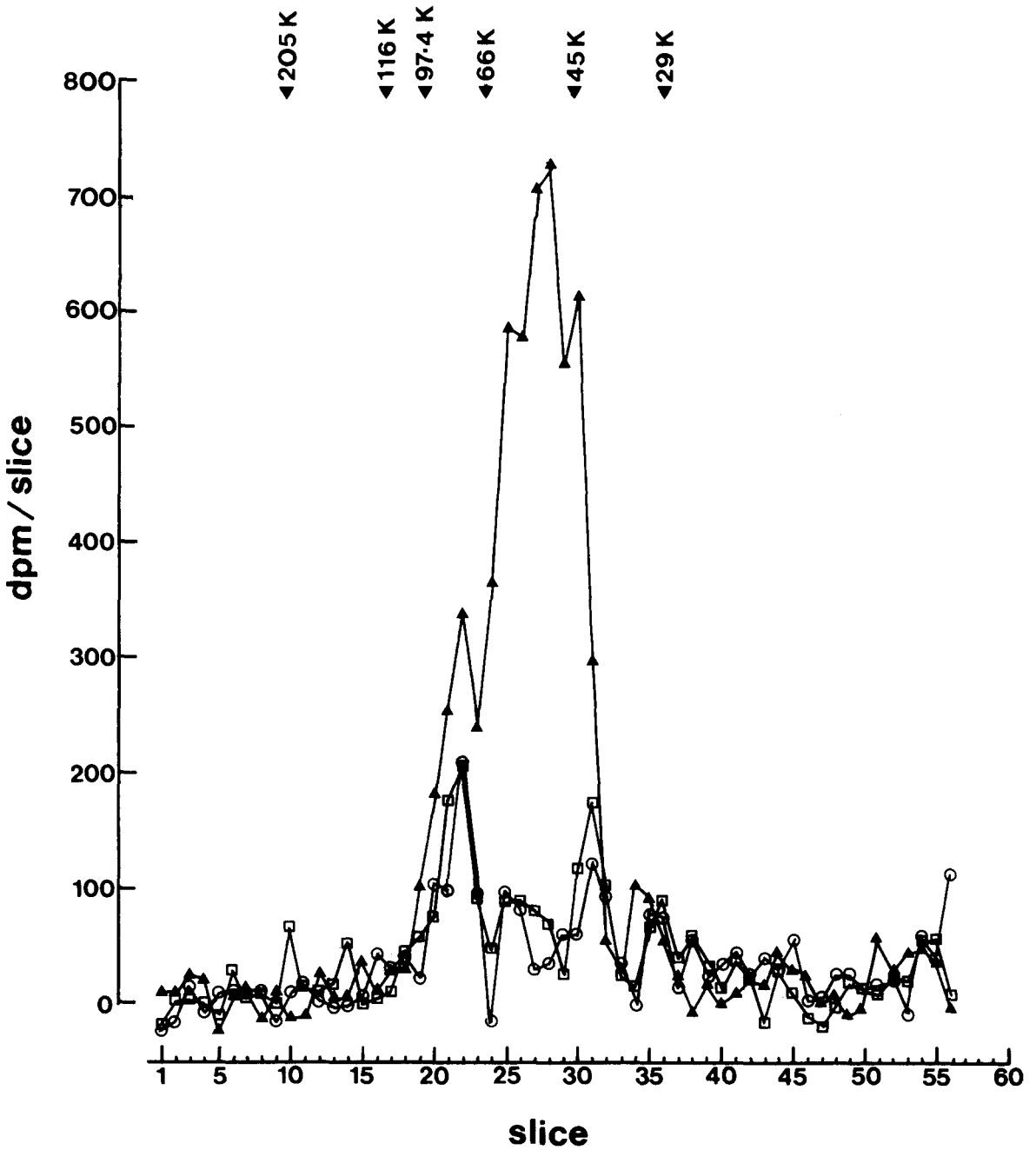


Fig. 6. The labelling of 800 μl of freshly drawn erythrocytes (10% cytocrit) by 7 μCi of ASA-[2-^{3}H]BMPA in the presence of 320 mM D-mannitol (●), 320 mM D-glucose (□) or 50 μM cytochalasin B plus 320 mM D-mannitol (○). 320 μg of membrane protein per lane were used.

greater than 80% inhibition of band 4.5 labelling. In order to more clearly examine any slight differences between freshly drawn and old blood, we have photochemically labelled the transporter in freshly drawn and in 4-weeks-old cells under comparable conditions (Fig. 7). The pattern of labelling is identical and this indicates that the transporter is unaffected by storage of blood for at least 3–4 weeks.

The light-activated covalent labelling of the transporter with cytochalasin B has been very useful in identifying that the internal cytochalasin

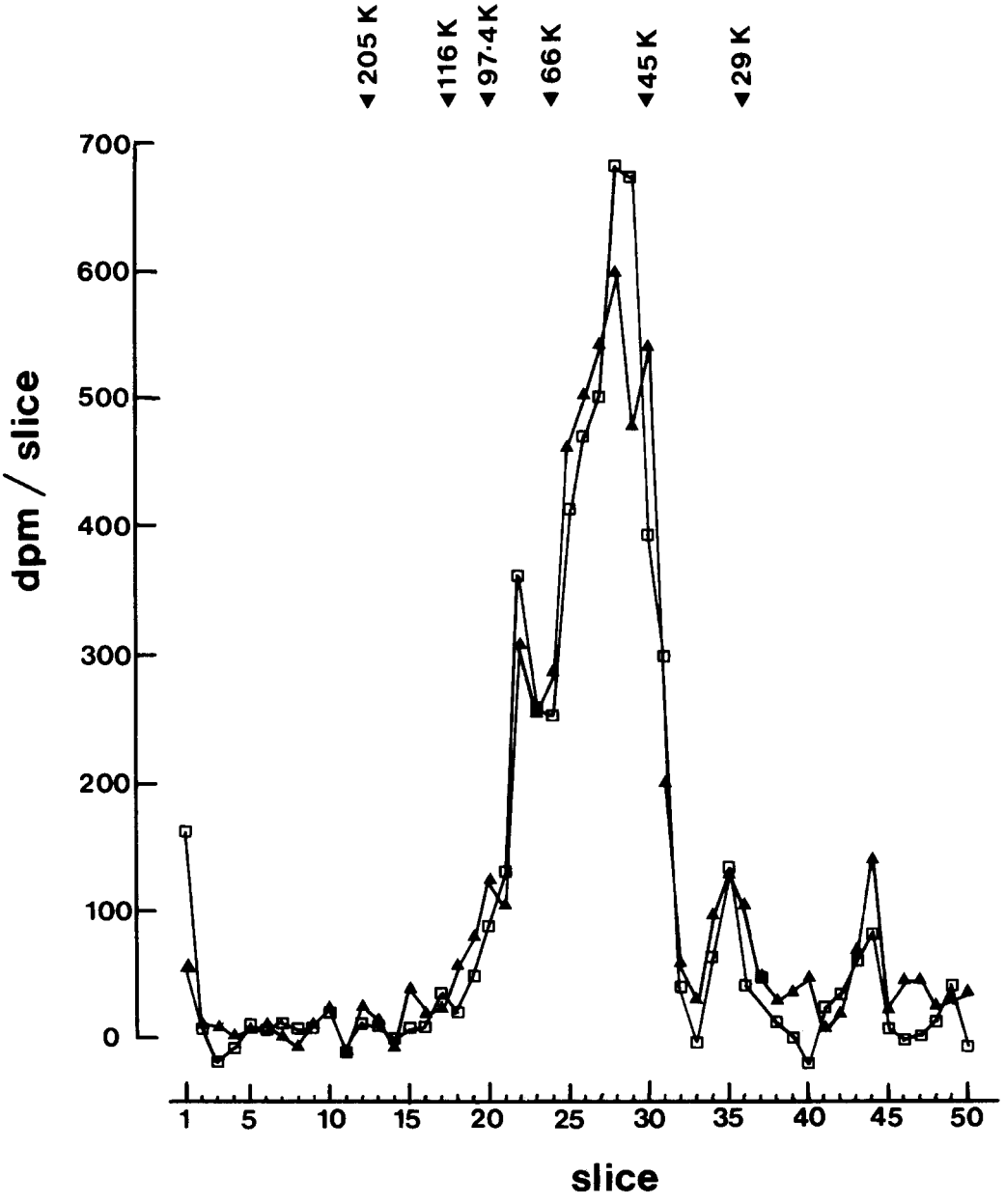


Fig. 7. A direct comparison of the labelling of 800 μl of freshly drawn erythrocytes (10% cytocrit) (▲) or 800 μl of 4-week-old erythrocytes (10% cytocrit) (□) by 7 μCi of ASA-[2-^{3}H]BMPA. Both in the presence of D-mannitol. 304 μg of membrane protein per lane were used.

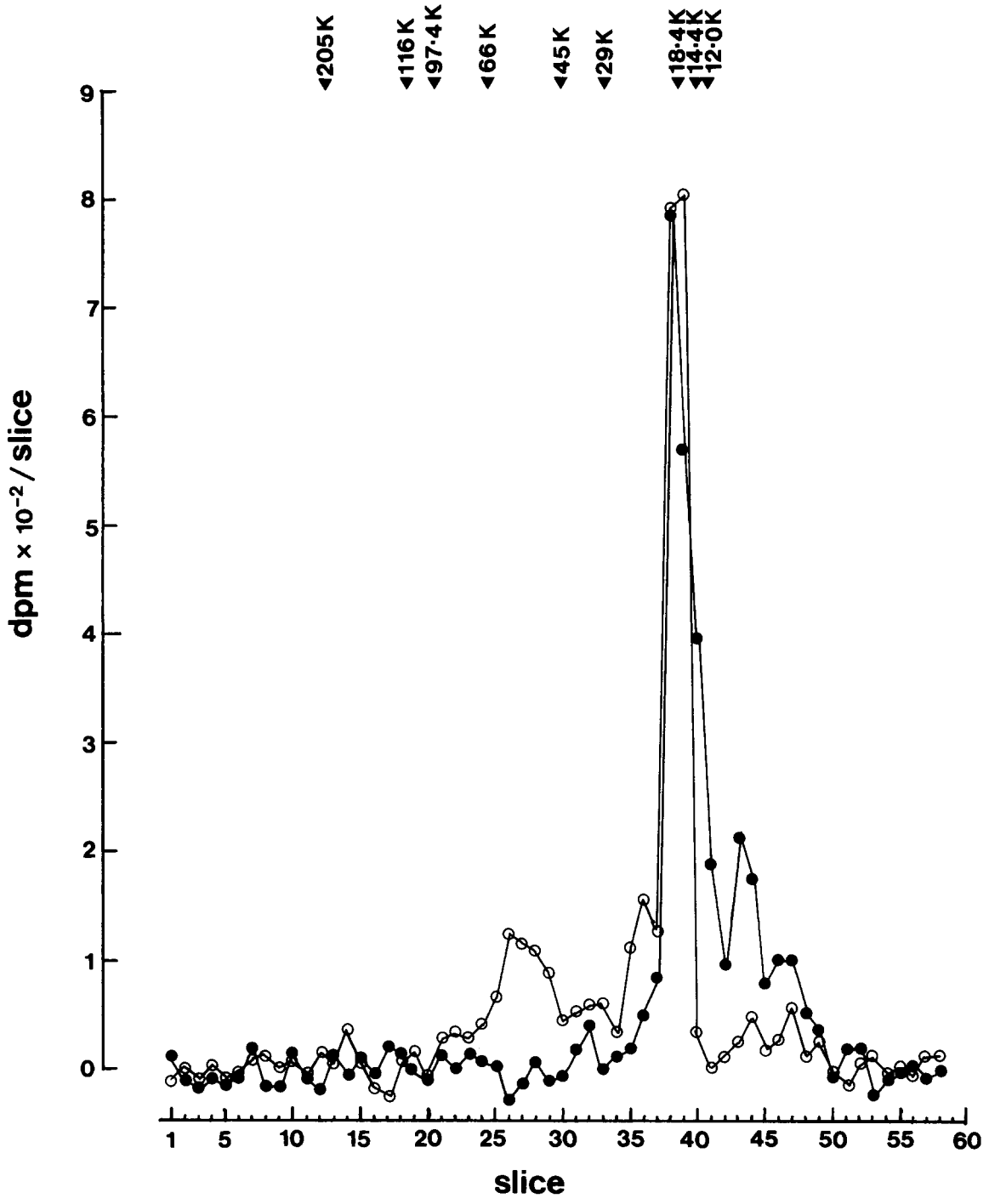


Fig. 8. Proteolytic digestion of ASA-[2-^{3}H]BMPA-labelled transporter. Erythrocyte membranes were isolated from cells which had been photoirradiated in the presence of 7 μCi of ASA-[2-^{3}H]BMPA and 320 mM D-mannitol. Isolated membranes were treated with 550 units/ml of trypsin for 30 min at 25°C (○) or 40 units/ml of α-chymotrypsin for 60 min at 37°C (●). After terminating the reactions, 260 μg of membrane protein were collected and added to the gel.

B-binding site must reside on the 20-kDa trypsin fragment of band 4.5. The results of Cairns et al. [23], of Deziel and Rothstein [24] and of Shanahan and D'Artel-Ellis [10] have all shown that an approx. 20 kDa fragment is formed by trypsin digestion. Deziel and Rothstein have shown that α-chymotrypsin gives a similar fragment to trypsin, while Shanahan and D'Artel-Ellis have suggested that α-chymotrypsin gives a 19 kDa fragment which is somewhat smaller than the trypsin fragment which they estimate to be 21 kDa.

We have proteolysed ASA-BMPA-labelled membranes with trypsin and α-chymotrypsin (Fig. 8). Both enzymes give fragments of 19 kDa, and any differences between the chymotrypsin fragment and the trypsin fragment cannot be resolved in our gel system. The peaks of radiolabelling after proteolytic digestion are much narrower than the broad band of labelling which occurs in the 4.5 region in the membranes of unproteolysed samples. Gorga et al. [25] have shown that the broadness of the band in the 4.5 region is due to heterogeneous protein glycosylation. The sharpening of the labelling profile following proteolysis is thus likely to be due to the removal of a heavily glycosylated unlabelled fragment. The results of our proteolysis experiments using ASA-BMPA are interesting as they show that the ASA-BMPA binding site at the external surface is on the same fragment as the cytochalasin B-binding site. There is some evidence in our data that α-chymotrypsin gives some additional fragments which are smaller than 10 kDa (Fig. 8). Thus, the ASA-BMPA-binding site may be separated from the cytochalasin B-binding site by quite a small span of protein.

Discussion

We have shown that 2-*N*-(4-azidosalicoyl)-1,3-bis(D-mannos-4′-yloxy)propylamine fulfils the requirements for a good photoaffinity label for the erythrocyte sugar transporter. It is impermeable. It is a competitive inhibitor of sugar transport. It is rapidly photolysed even in the presence of a dense cell suspension. The photochemical reaction between the transporter and the compound results in a significant loss of transport activity. The loss of transport activity correllates well with the binding of the radiolabelled compound and provides an estimate of the number of transport sites per cell. Photoirradiation of a red cell suspension in the absence of the compound does not result in a loss of transport activity and this shows that labelling occurs under conditions in which there is no photochemical damage of the transporter. The binding of the radiolabelled photoaffinity probe is more than 80% blocked by D-glucose.

There has been considerable debate concerning the identity of the erythrocyte transporter. Most of the evidence [19–21] indicates that the band 4.5 protein is responsible for transport. The results from our experiments support this. ASA-BMPA labelling shows a broad band in the 4.5 region from which one can estimate that the molecular weight of the transporter is approx. 50000. In addition, our experiments provide evidence that the exofacial binding site of the transporter occurs on the same polypeptide as the internal cytochalasin B-binding site.

Mullins and Langdon [1] have suggested that Band 3 is the transporter. This is based mainly on the use of the low-affinity probe D-maltosylisothiocyanate which labels predominantly band 3. This binding has been shown to be partially blocked by D-glucose [2]. There is also some evidence that fractions of membrane proteins that are enriched in band 3 can catalyse transport in reconstituted phospholipid vesicles just as well as fractions of membrane proteins enriched in band 4.5 [26]. Shelton and Langdon [26] and Kay [27] have suggested that transporter in band 4.5 arises from the action of endogenous proteinases which break down band 3. We have used pepstatin and PMSF as proteinase inhibitors but find no evidence for labelling of band 3 by ASA-BMPA. A small amount of nonspecific labelling occurs as a shoulder infront of the band 4.5 labelling but this corresponds more closely to the periodate-Schiffs staining glycophorin dimer at about 75 kDa rather than to any Coomassie blue staining band. Furthermore, we can show D-maltosylisothiocyanate labelling in the band 3 region in cell preparations which show ASA-BMPA labelling of band 4.5 (Rees, W.D. and Holman, G.D., unpublished results) and we therefore consider it to be unlikely that proteolysis can account for discrepancies in transporter labelling. If band 3 is to be implicated in transport, then we consider that a more likely

possibility for its involvement might be in a band 4.5/band 3 coassociation as an oligomeric assembly. We have compared freshly drawn and 4-weeks-old transfusion blood. The labelling pattern shown by ASA-BMPA is identical in these two preparations and therefore we conclude that the transporter is unaffected by storage of blood for at least 3–4 weeks. Jacquez [28] and Weiser et al. [29] have suggested that there is a change in sugar transport rates due to the storage of blood. It may well be that estimates of transport rates are influenced by factors such as cell shape or volume and that these effects are independent of the number of transporters or their intrinsic rate constants. A change in the intrinsic asymmetry of the transporter during the storage of blood [29] would be expected to influence the binding of an external inhibitor such as ASA-BMPA. The direct comparison of labelling in fresh and 4-weeks-old blood which we have described rules out the possibility that this type of change could be occurring in our experiments.

The binding of ASA-BMPA to the band 4.5 protein is more than 80% blocked by 4,6-*O*-ethylidene-D-glucose, by D-glucose and by cytochalasin B. If one accepts that cytochalasin B binds at just the internal substrate site on the transporter, then our experiments would indicate that external and internal substrate sites cannot be simultaneously occupied by nontransported ligands. Asymmetric conformational states for the transporter with internally and externally bound nontransported ligands were first suggested by Barnett et al. [22]. This possibility has been supported by the experiments of Gorga and Lienhard [5] and by Appleman and Lienhard [30] who further suggested that binding to these sites was mutually exclusive. These experiments on nontransported ligands do not however rule out the possibility of an additional, or allosteric, site for a transported substrate such as D-glucose [31]. The observation that cytochalasin B and the outside inhibitor ASA-BMPA share a single relatively small proteolytic fragment is of great interest and provides evidence that the 20 kDa fragment must span the membrane. Further proteolytic dissection of the system may reveal more details of the arrangement of the transport sites across the membrane.

The demonstration of specific photoaffinity labelling by a bis(hexose) indicates that this class of compound should be a useful addition to the range of reagents that are available for identifying and studying the sugar transporter in various cell types.

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