J. Cell Sci. **85**, 177–185 (1986)
Printed in Great Britain © *The Company of Biologists Limited 1986*

CYTOCHALASIN-B-BINDING PROTEINS RELATED TO GLUCOSE TRANSPORT ACROSS THE BASOLATERAL MEMBRANE OF THE INTESTINAL EPITHELIAL CELL

TADAYOSHI UEZATO[1,*] AND MICHIYA FUJITA[1,2]

[1]*Department of Biochemistry, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431–31, Japan*

[2]*Laboratory of Active Transport, National Institute for Physiological Sciences, Okazaki, Japan*

SUMMARY

Basolateral membrane vesicles were isolated from mouse enterocytes. The vesicles showed Na^+-independent uptake of D-glucose. The uptake was inhibited by cytochalasin B and phloretin with 50% inhibition at 2·0 μM and 25 μM, respectively. 2-Deoxy-D-glucose inhibited D-glucose transport by 50% at 0·5 M. The basolateral membranes bound 13·5 pmol mg protein^{-1} of 0·1 μM-cytochalasin B. The effects of various monosaccharides on cytochalasin B binding were examined; the strongest inhibitor was 2-deoxy-D-glucose (20–30%). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of the basolateral membranes labelled with [^{3}H]cytochalasin B revealed two components with M_r values of 52(±2) and 30($\times 10^3$). Phloretin and 2-deoxy-D-glucose inhibited the photo-incorporation of [^{3}H]cytochalasin B into these components. While phloretin inhibited the photolabelling of the two components to a similar extent, 2-deoxy-D-glucose seemed to inhibit preferentially that into the $52\times10^3 M_r$ component. The similar sensitivity to 2-deoxy-D-glucose of the photolabelling of the $52\times10^3 M_r$ component and of D-glucose transport, together with the fact that dithiothreitol removal increased the incorporation into the $52\times10^3 M_r$ component and decreased that into the $30\times10^3 M_r$ component, seems to suggest that the $52\times10^3 M_r$ component is the major glucose transporter of the basolateral membrane.

INTRODUCTION

It has been reported that there are two types of glucose transporters in the intestinal epithelial cell (for a review, see Kimmich, 1981; Stevens *et al.* 1984). One is located in the microvillous membrane, which is sodium-dependent (Schultz & Zalusky, 1964; Hopfer *et al.* 1973), and the other, located in the basolateral membrane (BLM), is independent of sodium ions (Bihler & Cybulsky, 1973; Murer *et al.* 1974; Ling *et al.* 1981). D-Glucose was actively transported from the intestinal lumen to serosa by these two types of transporters located in opposing membrane domains in the same cell.

Although there have been many studies on the sodium-dependent glucose transporter in the microvillous membrane (for a review, see Kasahara *et al.* 1985), little is known about the glucose transporter located in the BLM of the epithelial cell.

* Author for correspondence.

Key words: intestinal epithelial cells, basolateral membrane, glucose carrier protein, cytochalasin B, photolabelling, 2-deoxy-D-glucose, phloretin.

There is some evidence that cytochalasin B inhibits glucose transport by intestinal BLM (Hopfer *et al.* 1976; Wright *et al.* 1980), an observation similar to that found with membranes of the human red cell (Taverna & Langdon, 1973) and other cells (Mizel & Wilson, 1972; Estensen & Plagemann, 1972). It is also known that glucose transport across BLM is inhibited more effectively by phloretin than by phlorizin (Kimmich & Randles, 1975; Kinne *et al.* 1975).

The techniques of Shanahan (1982) and Carter-Su *et al.* (1982) using photoaffinity labelling of the sugar transport protein in human erythrocytes have been shown to be valid in a number of tissues. Thus, we studied cytochalasin B binding and photolabelling of the BLM of small intestinal epithelial cells and its relationship to D-glucose transport.

MATERIALS AND METHODS

Reagents

[4-^{3}H]cytochalasin B (13·6 Ci mmol^{-1}), [U-^{14}C]sucrose (673 mCi mmol^{-1}), D-[1-^{3}H]glucose (3·5 Ci mmol^{-1}) and L-[1-^{14}C]glucose (58 mCi mmol^{-1}) were purchased from New England Nuclear. Phenylmethylsulphonyl fluoride (PMSF) and leupeptin were obtained from Sigma Chemical Co.

Cell fractionation and assay of marker enzymes

Basolateral plasma membranes were isolated from C3H mouse small intestine by differential and density-gradient centrifugation according to Fujita *et al.* (1972, 1981). Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as substrate in propanediol–HCl buffer, pH 9·4 (Uezato & Fujita, 1983); sucrose activity was measured according to Messer & Dahlqvist (1966). Na^+, K^+-ATPase and glucose-6-phosphatase were assayed as described (Fujita *et al.* 1972).

Assay of D-glucose transport activity

In order to eliminate interference by sucrose during transport and photolabelling, the membrane fractions were washed with 10 mM-imidazole buffer (pH 7·4) and resuspended in 10 mM-phosphate buffer (pH 7·4) or 10 mM-Tris–HEPES (pH 7·4) containing 300 mM-mannitol. Glucose transport was carried out as described (Yamada *et al.* 1983). A sample of the basolateral membrane-rich fraction (1–3 mg protein ml^{-1}) was mixed with 0·5 mM-D-[^{3}H]glucose (5·0 mCi mmol^{-1}), 0·5 mM-L-[^{14}C]glucose in 10 mM-HEPES buffer (pH 7·4) containing 300 mM-mannitol. At an appropriate time the reaction was stopped by adding 1·0 ml of ice-cold stop solution containing 1·0 mM-$HgCl_2$ and 0·5 mM-$MgCl_2$. The mixture was quickly filtered through a Millipore nitrocellulose filter (0·45 μm). The filter was washed with 5 ml of the uptake medium containing 0·5 mM-$HgCl_2$, dissolved in 10 ml of ACS II (Amersham) solution, and the radioactivity counted. Correction for non-specific uptake was made based on the amount of L-[^{14}C]glucose associated with the filters.

Assay of cytochalasin B binding

Binding experiments were carried out as described (Yamada *et al.* 1983; Uezato, 1986). A sample of the membrane fraction suspended in 300 mM-mannitol, 10 mM-HEPES buffer (pH 7·4), 2–3 mg protein ml^{-1}, was incubated with [^{3}H]cytochalasin B (final concn, 0·1 μM) in a total volume of 120 μl at room temperature for at least 20 min. Other additions were 100 μM-cytochalasin E and 2–3 μM-[^{14}C]sucrose. The reaction mixtures were centrifuged at 40 000 rev. min^{-1} for 45 min (Hitachi SPR50 rotor) to recover the pellets and the radioactivity counted as described above. The non-specific binding was estimated by ^{14}C uptake.

Photolabelling with [^{3}H]cytochalasin B

Basolateral membrane was suspended to a final protein concentration of 1–2 mg protein ml^{-1} in 10 mM-phosphate buffer (pH 7·4) containing 300 mM-mannitol, 0·1–1·0 μM-[^{3}H]cytochalasin B and 100 μM-cytochalasin E. Photolysis was carried out using PEN RAY lamp, model SCT-4 (UVP Inc, CA) for 1–5 min at a distance of 10 cm. After photolysis, the sample was diluted with 10 mM-imidazole buffer (pH 7·4) containing 0·5 mM-EDTA and centrifuged at 40 000 rev. min^{-1} for 1 h. The pellet was recovered and applied to a sodium dodecyl sulphate–polyacrylamide gel for electrophoresis (SDS–PAGE).

SDS–PAGE analysis of cytochalasin-B-binding proteins

About 200 μg of photolabelled sample was mixed with the sample buffer (2–4% SDS, 1·0 mM-dithiothreitol), heated at 100°C for 1–3 min, and then applied to a 2 mm thick slab gel (10% gel) according to Laemmli (1970). After electrophoresis the gel was stained with Coomassie Brilliant Blue and destained with 7·5% acetic acid containing 5% methanol. Each strip of the gel was sliced into 2-mm thick segments with a model SL-280 electric gel slicer (Hoefer Scientific Instruments) and then they were dissolved in 0·4 ml of Protosol (New England Nuclear) overnight, added with 3 ml of Econofluor (New England Nuclear), and the radioactivity was counted. Protein M_r standards (BioRad Molecular Weight Standards) contained: myosin (200 000), galactosidase (116 250), phosphorylase *b* (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400).

RESULTS

Cell fractionation

The BLM fraction (Fig. 1) showed about 10- to 20-fold enrichment of Na^+, K^+-ATPase and 5′-AMPase over the homogenate. The specific activities of sucrose and alkaline phosphatase, marker enzymes of microvillous membrane (MVM), were a twentieth to a tenth of those of the original homogenate.

D-*Glucose uptake*

The BLM fraction showed Na^+-independent D-glucose uptake. The K_m for D-glucose was about 35 mM (Fig. 2), confirming results reported earlier (Wright *et al.* 1980). D-Glucose uptake by BLM vesicles was potently inhibited by cytochalasin B and phloretin with K_i values of about 2·0 μM and 25 μM, respectively (Fig. 3). The uptake was also inhibited by 2-deoxy-D-glucose; 50% inhibition being obtained at ≈0·5 M (Fig. 6).

[^{3}H]cytochalasin B binding

The BLM bound 13·5 pmol mg protein^{-1} of 0·1 μM-[^{3}H]cytochalasin B (Table 1). The binding was inhibited by 100 μM-phloretin and 500 mM-2-deoxy-D-glucose, by 30–40% and 20–30%, respectively. The effects of various monosaccharides on cytochalasin B binding were examined (Table 1). Interestingly, however, D-glucose only slightly depressed cytochalasin B binding (5–8% inhibition with 500 mM-D-glucose; Table 1). SDS–PAGE of BLM photolabelled with [^{3}H]cytochalasin B revealed radioactive peaks at M_r values corresponding to 52K (52 000) (±2000) and 30K (Fig. 4). The 50–54K component contained about 20% of the total BLM protein and was apparently heterogeneous (Fig. 4). Phloretin and 2-deoxy-D-glucose

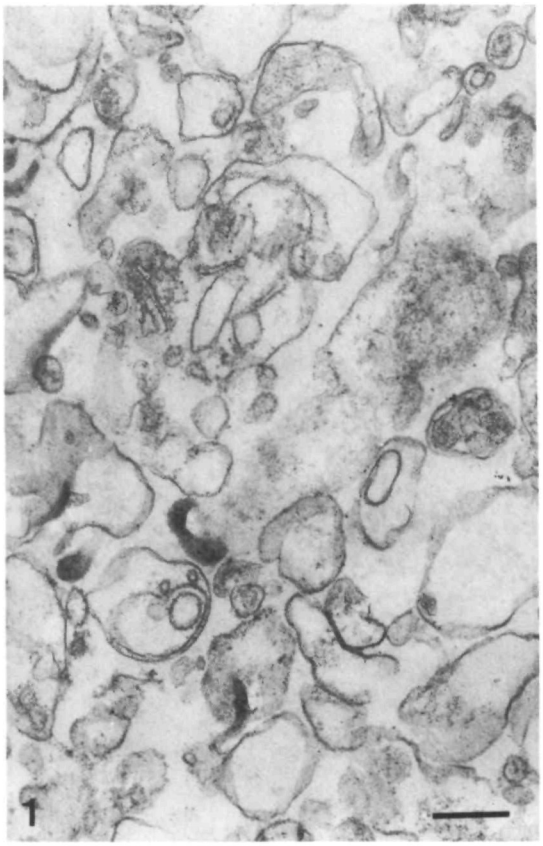

Fig. 1. Electron micrograph of small intestinal basolateral membrane fraction. Bar, 1 μm. ×20 000.

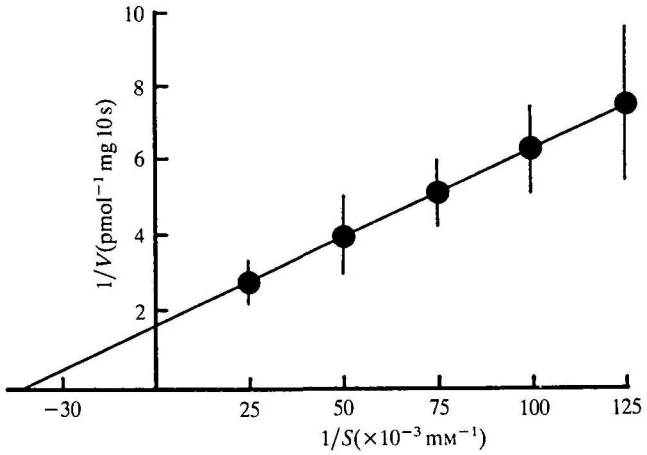

Fig. 2. Kinetics of D-glucose uptake by intestinal BLM vesicles. The rate of uptake at room temperature was determined in the reaction mixture, which contained 2–4 mg protein ml^{-1} and various concentrations (8–40 mM) of D-[^{3}H]glucose. After 10 s the reaction was terminated by addition of 1 ml of ice-cold stopping solution. Other procedures were as described in Materials and Methods. Each point represents the mean ± S.E. ($n = 3$). V, uptake of glucose for 10 s; S, glucose concentration.

inhibited photo-incorporation of [^{3}H]cytochalasin B into the two components. In the presence of increasing concentrations of phloretin, the radioactive peaks at 52K and 30K were progressively reduced in a parallel manner while cytochalasin E did

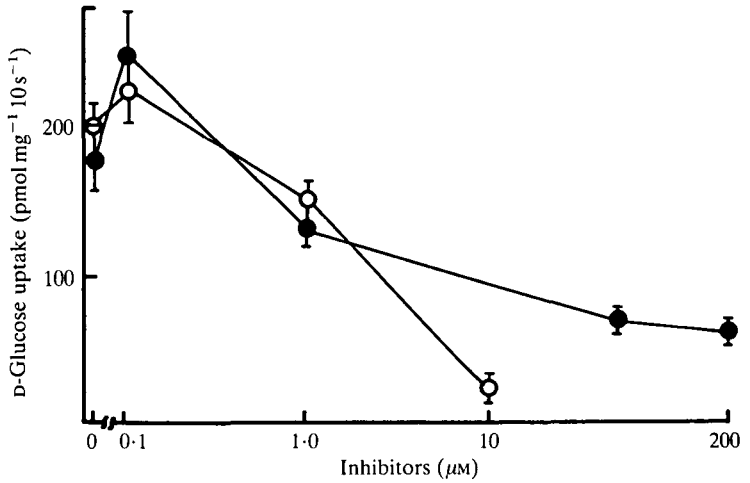

Fig. 3. Inhibition of D-glucose uptake by cytochalasin B and phloretin. Reaction was carried out at room temperature for 10 s. The reaction mixture contained the membranes (2–4 mg protein ml^{-1}), 0·5 mM-D-[^{3}H]glucose, 300 mM-mannitol, 10 mM-Tris–HEPES buffer (pH 7·2) and the indicated amount (final concn) of the reagents; (○) cytochalasin B; (●) phloretin. Each point represents the mean ± S.E. (n = 3).

Table 1. *Effects of various sugars on cytochalasin B binding by mouse intestinal BLM*

Sugars	Concentrations	Binding (pmol mg protein^{-1})
Control		13·48 ± 0·09
D-Galactose	0·5 M	13·62 ± 0·21
L-Glucose	0·5 M	13·84 ± 0·11
D-Sorbitol	0·5 M	13·22 ± 0·46
D-Glucose	0·5 M	12·41 ± 0·07
2-Deoxy-D-glucose	0·5 M	10·00 ± 0·10
3-*O*-methyl-D-glucose	0·5 M	13·31 ± 0·34
D-Fructose	0·5 M	12·57 ± 0·52
D-Mannitol	0·5 M	13·50 ± 0·13
D-mannose	0·5 M	11·89 ± 0·05
D-Ribose	0·5 M	12·50 ± 0·46
D-Xylose	0·5 M	11·13 ± 0·01
N-acetylglucosamine	0·5 M	14·08 ± 0·42
Inositol	0·5 M	13·92 ± 0·39
Phloretin	0·1 mM	8·70 ± 0·09

Membranes (2–3 mg ml^{-1}), one of the sugars (concentrations indicated), 0·1 μM-[^{3}H]cytochalasin B and 100 μM-cytochalasin E were incubated at room temperature for 20 min and centrifuged at 40 000 rev. min^{-1} for 1 h. The pellets were dissolved and the radioactivity was counted. The values indicate the means ± S.E. (n = 3–5).

not affect them (Fig. 5). On the other hand, 2-deoxy-D-glucose appeared to inhibit preferentially the photolabelling of the 52K component. The K_i for the inhibition of photolabelling (data not shown) by 2-deoxy-D-glucose was similar to that for the inhibition of D-glucose transport, i.e. ≈0·5 M (Fig. 6).

The question was asked whether the 30K component was a degradation product of 52K protein resulting from endogenous proteolysis. The inclusion of two protease

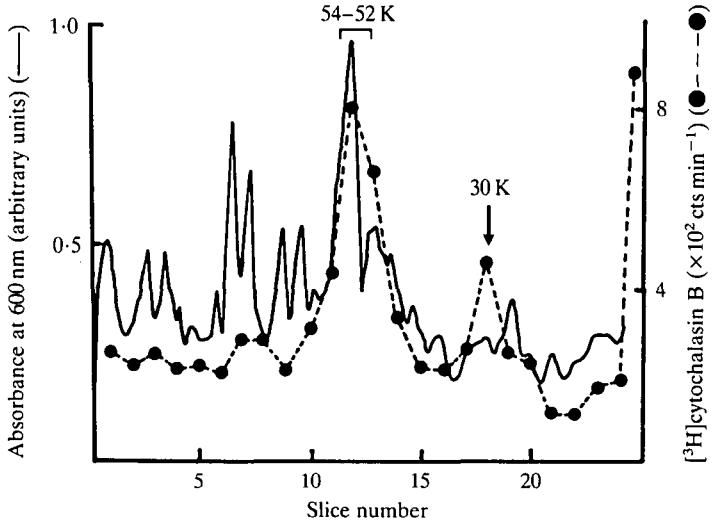

Fig. 4. Distribution of radioactivity and proteins, by SDS–PAGE. A heavy-microsomal fraction (crude BLM) was photolabelled in the presence of 1·0 μM-[^{3}H]cytochalasin B, 100 μM-cytochalasin E and 0·5 M-sorbitol for 3 min, optimal time for the reaction; (●– – –●) radioactivity; (——) densitometric tracing with 600 nm filter.

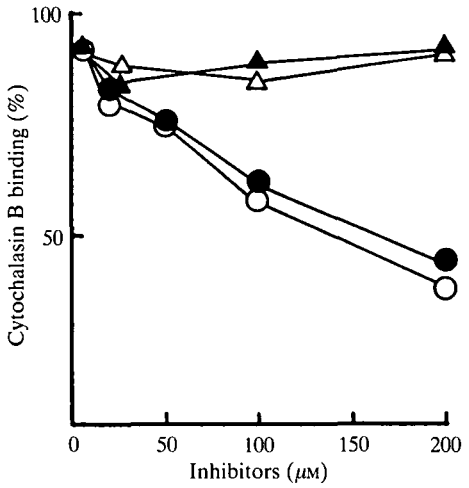

Fig. 5. Effects of phloretin and cytochalasin E on the incorporation of [^{3}H]cytochalasin B into 54K and 30K BLM proteins. The % inhibition was determined from calculation of the peak area on the SDS–PAGE gels. (○) 54K protein (phloretin); (●) 30K protein (phloretin); (△) 54K protein (cytochalasin E); and (▲) 30K protein (cytochalasin E).

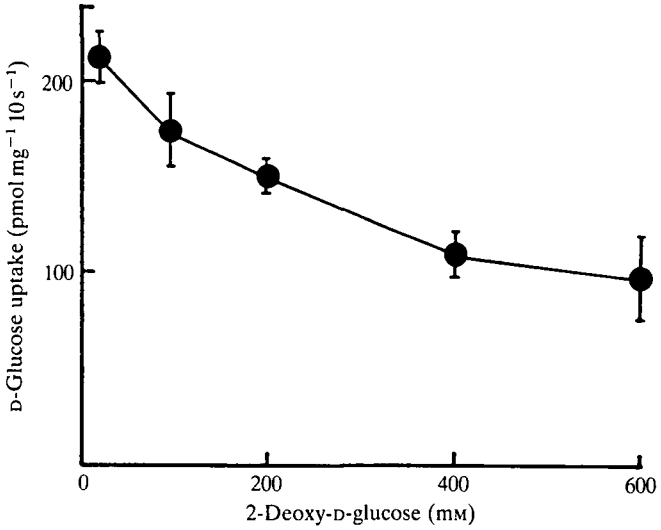

Fig. 6. Effect of 2-deoxy-D-glucose on D-glucose uptake by BLM vesicles. Reaction was carried out at room temperature for 10 s. The condition was the same as for Fig. 3.

inhibitors, PMSF and leupeptin, in the preparation medium did not affect the relative amounts of 52K and 30K proteins (data not shown). When dithiothreitol was omitted from the sample buffer for electrophoresis, the 30K peak was reduced while the 50K peak became more conspicuous.

DISCUSSION

Cytochalasin B inhibited glucose uptake very strongly, whereas glucose and its analogues only weakly inhibited cytochalasin B binding (Table 1). Unexpectedly, 2-deoxy-D-glucose protected the membrane more efficiently from inhibitor binding than D-glucose. Even more puzzling was the finding that D-fructose, D-mannose and D-xylose, sugars with low affinity for glucose transporter in human red cells, were more effective than those with higher affinity, such as D-glucose and D-galactose. These findings suggested that the glucose-binding site and the cytochalasin-B-binding site might be separate. SDS–PAGE analysis of the membrane photolabelled with radioactive cytochalasin B revealed a heterogeneity of the binding site itself. Although the two bands at 52K and 30K were most densely labelled, some incorporation was detected in a higher M_r region (Fig. 4). Therefore, it was likely that the non-specific binding sites were not saturated by cytochalasin E, which was added together with radioactive cytochalasin B to minimize non-specific incorporation (Shanahan, 1982; Carter-Su *et al.* 1982).

The 52K component, one of the two cytochalasin-B-binding proteins, was similar in molecular weight to those found in human red cells (Baldwin *et al.* 1979; Carter-Su *et al.* 1982; Kasahara *et al.* 1985; Kasahara & Hinkle, 1977; Shanahan, 1982), K562 cells (Uezato, 1986) and other types of cells (see Kasahara *et al.* 1985). While phloretin, a specific inhibitor of glucose transport across BLM, inhibited

cytochalasin B incorporation into the two polypeptides with similar K_i values ($\approx$150 μM), 2-deoxy-D-glucose inhibited the incorporation of cytochalasin B more effectively into the 52K polypeptide than into the 30K polypeptide. Thus the 52K polypeptide may be related to D-glucose transport in the BLM of epithelial cells. It was unlikely that the 30K component was a degradative product of the 52K protein because the radioactivity of the 30K component was not affected by the presence of added protease inhibitors such as PMSF and leupeptin during the preparation of BLM. It has been assumed for human heterocarcinoma (Haspel *et al.* 1985) that the glucose carrier was initially synthesized as an $\approx$30K or 38K polypeptide, and then modified to acquire a larger molecular weight, 55K. Thus the possibility cannot be ruled out that the 30K polypeptide might be a deglycosylated product of the 52K protein. More work is necessary to answer this question. Although it is not known why 2-deoxy-D-glucose gave stronger protection than D-glucose against cytochalasin B binding, there is some evidence that 2-deoxy-D-glucose was a much more suitable substrate than D-glucose for transport in the small intestine (Kimmich & Randles, 1975). Thus the specificity of a sugar analogue for protection against cytochalasin B binding seems to be different between the murine intestinal BLM and the human red cell membrane.

We thank Dr H. Ohta, for valuable discussions and suggestions. This work was partly supported by Special Coordination Funds for Promoting Science and Technology for the Science and Technology Agency of Japan.

REFERENCES

BALDWIN, S. A., BALDWIN, J. M., GORGA, F. R. & LIENHARD, G. E. (1979). Purification of the cytochalasin B binding component of the human erythrocyte monosaccharide transport system. *Biochim. biophys. Acta* **552**, 183–188.

BIHLER, I. & CYBULSKY, R. (1973). Sugar transport at the basal and lateral aspect of the small intestinal cell. *Biochim. biophys. Acta* **298**, 429–437.

CARTER-SU, C., PESSIN, J. E., MORA, R., GITOMER, W. & CZECH, M. P. (1982). Photoaffinity labeling of the human erythrocyte D-glucose transporter. *J. biol. Chem.* **257**, 5419–5425.

ESTENSEN, R. D. & PLAGEMANN, P. G. W. (1972). Cytochalasin B: Inhibition of glucose and glucosamine transport. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1430–1434.

FUJITA, M., OHTA, H., KAWAI, K., MATSUI, H. & NAKAO, M. (1972). Differential isolation of microvillous and basolateral plasma membranes from intestinal mucosa: Mutually exclusive distribution of digestive enzymes and ouabain-sensitive ATPase. *Biochim. biophys. Acta* **274**, 316–347.

FUJITA, M., OHTA, H. & UEZATO, T. (1981). Characterization of brushborders purified in iso-osmotic medium and microvillar membranes subfractionated from mouse small intestine. *Biochem. J.* **196**, 669–673.

HASPEL, H. C., BIRNBAUM, M. J., WILK, W. & ROSEN, O. M. (1985). Biosynthetic precursors and *in vitro* translation products of the glucose transporter of human hepatocarcinoma cells, human fibroblasts, and murine preadipocytes. *J. biol. Chem.* **260**, 7219–7225.

HOPFER, U., NELSON, K., PERROTTO, J. & ISSELBACHER, K. J. (1973). Glucose transport in isolated brush border membrane from rat small intestine. *J. biol. Chem.* **248**, 25–32.

HOPFER, U., SIGRIST-NELSON, K., AMMANN, E. & MURER, H. (1976). Differences in neutral amino acid and glucose transport between brush border and basolateral plasma membrane of intestinal epithelial cells. *J. cell. Physiol.* **89**, 805–810.

KASAHARA, M. & HINKLE, P. C. (1977). Reconstitution and purification of the D-glucose transporter from human erythrocytes. *J. biol. Chem.* **252**, 7284–7390.

KASAHARA, M., INUI, K., TAKANO, M. & HORI, R. (1985). Distinction of three types of D-glucose transport systems in animal cells. *Biochem. biophys. Res. Commun.* **132**, 490–496.

KIMMICH, G. A. (1981). Intestinal absorption of sugar. In *The Physiology of the Gastrointestinal Tract* (ed. L. R. Johnson), pp. 1035–1061. New York: Raven Press.

KIMMICH, G. A. & RANDLES, J. (1975). A Na^+-independent phloretin sensitive monosaccharide transport system in isolated intestinal epithelial cells. *J. Membr. Biol.* **23**, 57–76.

KINNE, R., MURER, H., KINNE-SAFFRAN, E., THEES, M. & SACHS, G. (1975). Sugar transport by renal plasma membrane vesicles. Characterization of the systems in the brush-border microvilli and basal-lateral plasma membranes. *J. Membr. Biol.* **21**, 375–395.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, Lond.* **227**, 680–685.

LING, K. Y., IM, W. B. & FAUST, R. G. (1981). Na^+-independent sugar uptake by rat intestinal and renal brush border and basolateral membrane vesicles. *Int. J. Biochem.* **13**, 693–700.

MESSER, M. & DAHLQVIST, A. (1966). A one step ultramicro method for the assay of intestinal disaccharidases. *Analyt. Biochem.* **14**, 376–392.

MIZEL, S. B. & WILSON, L. (1972). Inhibition of the transport of several hexoses in mammalian cells by cytochalasin B. *J. biol. Chem.* **247**, 4102–4104.

MURER, H., HOPFER, U., KINNE-SAFFRAN, E. & KINNE, R. (1974). Glucose transport in isolated brush-border and lateral-basal plasma membrane vesicles from intestinal epithelial cells. *Biochim. biophys. Acta* **345**, 170–179.

SCHULTZ, S. G. & ZALUSKY, R. (1964). Ion transport in isolated rabbit ileum. II. The interaction between active sodium and active sugar transport. *J. gen. Physiol.* **47**, 1043–1059.

SHANAHAN, M. F. (1982). Cytochalasin B. A natural photoaffinity ligand for labeling the human erythrocyte glucose transporter. *J. biol. Chem.* **257**, 7290–7293.

STEVENS, B. R., KAUNITZ, J. D. & WRIGHT, E. M. (1984). Intestinal transport of amino acids and sugars: Advances using membrane vesicles. *A. Rev. Physiol.* **46**, 417–433.

TAVERNA, R. D. & LANGDON, R. G. (1973). Reversible association of cytochalasin B with the human erythrocyte membrane. Inhibition of glucose transport and the stoichiometry of cytochalasin binding. *Biochim. biophys. Acta* **323**, 207–219.

UEZATO, T. (1986). Photoaffinity labeling of the K562 cell membrane D-glucose transporter with cytochalasin B. *Biochem. Int.* **12**, 199–206.

UEZATO, T. & FUJITA, M. (1983). Developmental transition of alkaline phosphatase from suckling to adult type in rat small intestine: Molecular species and effect of injected cortisone and thyroxine. *J. Biochem.* **94**, 1483–1488.

WRIGHT, E. M., VAN OS, C. H. & MIRCHEFF, A. K. (1980). Sugar uptake by intestinal basolateral membrane vesicles. *Biochim. biophys. Acta* **597**, 112–124.

YAMADA, K., TILLOTSON, L. G. & ISSELBACHER, K. J. (1983). Regulation of hexose carriers in chicken embryo fibroblasts. *J. biol. Chem.* **258**, 9786–9792.

(Received 25 March 1986 – Accepted 9 June 1986)