Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 7233–7237, November 1984
Neurobiology

Identification and characterization of the glucose transporter of the blood–brain barrier by cytochalasin B binding and immunological reactivity

(brain microvessels/capillary endothelium/hexose transport/photoaffinity labeling)

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Communicated by Oscar D. Ratnoff, July 23, 1984

ABSTRACT **[^{3}H]Cytochalasin B was used as a ligand to identify and characterize the glucose transporter in cerebral microvessels of the rat and the pig. Specific cytochalasin B binding, defined as that fraction of the total binding that is stereospecifically displaced by excess (500 mM) D-glucose, is saturable. Kinetic studies of this specific binding to cerebral microvessel preparations showed a dissociation constant (K_d) of 0.65–0.88 μM and a maximal binding (B_{max}) of 60–80 pmol/mg of protein. In comparison, the B_{max} of particulate fractions of the cerebral cortex was about one-tenth that of cerebral microvessels. The ability of various hexoses to displace specific cytochalasin B binding to cerebral microvessels *in vitro* correlated well with the capability of these hexoses to cross the blood–brain barrier *in vivo*. Irreversible photoaffinity labeling of the glucose transporter of cerebral microvessels with cytochalasin B followed by solubilization and polyacrylamide gel electrophoresis labeled a polypeptide(s) with a molecular weight of about 53,000. Antibodies prepared against the glucose transporter of human erythrocytes also reacted with a polypeptide(s) with a molecular weight of about 53,000 on electrophoresed preparations of cerebral microvessels. These results indicate that cerebral microvessels are richly endowed with a glucose transporter moiety of similar molecular weight and antigenic characteristics as the glucose transporter of human erythrocytes and other mammalian tissues.**

Under normal conditions, the central nervous system of mammals relies on a large and uninterrupted supply of D-glucose for its oxidative metabolism. The mammalian nervous system is also isolated from the systemic circulation by a unique capillary endothelium possessing tight cell junctions, which is referred to as the blood–brain barrier (BBB) (1, 2). Because this barrier is poorly permeable to polar molecules, Crone (3) suggested the existence of a carrier-mediated facilitated transport system in brain capillary endothelium that enables D-glucose to cross the BBB. In recent years, a variety of studies using *in vivo* (4–7) and *in vitro* (8, 9) techniques has confirmed Crone's original ideas and has established that the transport of glucose by the endothelial cells of brain capillaries is saturable, stereospecific, nonconcentrative, nonenergy dependent, and not influenced by insulin. This subject has recently been extensively reviewed (10–12).

The glucose transporter in human erythrocytes has been characterized by D-glucose-displaceable specific cytochalasin B binding [see review by Jones and Nickson (13)]. Similar techniques have been used to characterize glucose transporters in a variety of mammalian tissues (14–16). More recently, photoaffinity covalent labeling of cytochalasin B to the glucose transporter (17) has allowed further identification of the transport polypeptide (18, 19). Immunological labeling with antiserum against the erythrocyte glucose transporter has also established the antigenic similarity of the glucose transporters of different cell types (19, 20).

We reasoned that the endothelial cells of brain capillaries, which transport glucose not only for their own use but, more importantly, for the much larger mass of metabolically active neurons and glia, should be particularly rich in glucose transporters. We now report that particulate fractions of cerebral microvessels from the rat and pig are richly endowed with D-glucose-displaceable cytochalasin B binding sites. This binding, which is about 10-fold higher than that of other tissues, is saturable and stereospecific (displaced by D-glucose but not by L-glucose). We also found excellent correlation between the ability of other hexoses to displace specific cytochalasin B binding to cerebral microvessels *in vitro* and their ability to cross the BBB *in vivo* (6). The glucose transporter of cerebral microvessels appears to be a polypeptide (M_r ≈53,000), which crossreacts with antiserum raised against the erythrocyte glucose transporter.

MATERIALS AND METHODS

Cerebral cortical mantles were quickly removed from male Wistar rats (100–150 g) that were killed by decapitation or from adult pigs killed by exsanguination at a local slaughterhouse and cleaned of meninges and choroid plexus. Microvessels were isolated by bulk separation using the method of Betz (21), with minimal modifications. All procedures were carried at 0–4°C unless stated otherwise.

The purity of the microvessel preparations was routinely checked by light microscopy. In several instances, purity of cerebral microvessels and their fractions was assessed by assaying their enrichment with γ-glutamyl transpeptidase, an endothelial cell membrane enzyme marker of brain microvessels (22). γ-Glutamyl transpeptidase (γ-glutamyltransferase; EC 2.3.2.2) activity was assayed by the method of Orlowski and Meister (23).

Particulate fractions of cerebral microvessels were prepared by homogenization in 0.01 M Tris buffer (pH 7.4) with a Brinkmann Polytron for 10 s at a setting of 6. The homogenate was centrifuged at 49,000 × *g* for 15 min and the pellet was washed twice and then resuspended in the same Tris buffer to obtain 0.5–1 mg of tissue protein per ml.

In two experiments, subfractions of pig cerebral microvessels were obtained by the method of Lidinsky and Drewes (24) with modifications. Microvessels were suspended in 0.01 M Tris buffer (pH 7.4) and stirred continuously for 2 hr. The microvessels were then sonicated twice for 2 min. The suspension was centrifuged at 1000 × *g* for 10 min and the pellet was designated fraction A. The supernatant was cen-

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Abbreviation: BBB, blood–brain barrier.

trifuged at 8000 × *g* for 15 min and the resultant pellet was designated fraction B. The supernatant was then centrifuged at 100,000 × *g* for 90 min and the pellet was designated fraction C. Fractions A, B, and C were suspended in Tris buffer to about 1 mg of tissue protein per ml.

Particulate fractions of rat and pig cerebral cortex were prepared by homogenization in 0.01 M Tris buffer (pH 7.4) in a motor-driven glass/Teflon homogenizer. The homogenates were centrifuged at 49,000 × *g* for 15 min and the resulting pellets were resuspended in Tris buffer, disrupted in a Brinkmann Polytron, and washed twice in Tris buffer. The final pellet was suspended in Tris buffer to obtain 0.5–1 mg of tissue protein per ml.

Erythrocyte membranes were prepared from freshly drawn human, rat, or pig blood, collected in heparinized tubes. Erythrocytes were harvested and washed four times with ice-cold, buffered 0.9% NaCl solution before hemolysis in 0.09% NaCl solution. The erythrocyte membranes were washed three times in the hypotonic solution and suspended in 0.01 M Tris buffer (pH 7.4) to a concentration of 0.5–1 mg of protein per ml.

Protein concentrations of tissue suspensions were measured after solubilization in 1 M NaOH by the method of Lowry *et al.* (25) with bovine serum albumin as a standard.

The particulate fractions of tissues were assayed for their D-glucose-displaceable [^{3}H]cytochalasin B binding. [^{3}H]Cytochalasin B (specific activity, 17 Ci/mmol, Amersham; 1 Ci = 37 GBq) was dried under reduced pressure, immediately before use, to remove volatile ^{3}H contaminants. The incubation mixture contained about 60 μg of tissue protein, 5 μM cytochalasin E, varying concentrations of [^{3}H]cytochalasin B and nonradioactive cytochalasin B (total cytochalasin B concentration ranged from 0.05 to 9 μM), 500 mM (either) D-glucose or L-glucose, and 0.01 M Tris buffer (pH 7.4). In some experiments other hexoses replaced D-glucose, as detailed in the legend of Fig. 2. The total incubation volume was 0.1 ml. Cytochalasin E was used to decrease the nonspecific binding of cytochalasin B. Cytochalasin B and cytochalasin E were dissolved in ethanol, but the final concentration of ethanol in the incubation mixture never exceeded 3%. After incubation at 22°C for 15 min, the reaction mixture was rapidly filtered through Whatman GF/B glass fiber filters under reduced pressure and the filters were rinsed twice with 5 ml of ice-cold 0.9% NaCl solution. The filters were assayed for their ^{3}H content in a β liquid scintillation spectrometer at about 45% efficiency.

Specific cytochalasin B binding to tissue particulate fractions was calculated as the difference between the total binding in the presence of 500 mM L-glucose and the nonspecific binding in the presence of 500 mM D-glucose. All determinations were done in triplicate. Binding was expressed as pmol of cytochalasin B bound per mg of tissue protein. The equilibrium constant of binding was estimated according to Scatchard (26). Because of the short half-time reported for the dissociation of cytochalasin B from the erythrocyte glucose transporter (27), each assay sample was filtered and washed individually within 3–4 s to minimize the dissociation of bound cytochalasin B during the washing procedure. Also, in two experiments, equilibrium binding of [^{3}H]cytochalasin B was determined at 4°C followed by centrifugation at 200,000 × *g* for 40 min, as described by Deziel *et al.* (28). The results of specific cytochalasin B binding obtained by the two methods were similar. As an example, the specific binding of cytochalasin B to fraction C of pig brain microvessels, when incubated with about 1.5 μM cytochalasin B, was 50.0 pmol/mg of protein by the centrifugation method and 49.8 pmol/mg of protein by the filtration method.

Photolabeling with [^{3}H]cytochalasin B followed by PAGE was performed as described by Klip *et al.* (19). Microvessels, in the presence of 2 μM [^{3}H]cytochalasin B, 5 μM cytochalasin E, and either D- or L-glucose (180 mM), were exposed to UV light at 280 nm in an Aminco Bowman spectrofluorometer for 2 hr. Tissues were washed twice to remove unbound [^{3}H]cytochalasin B and then solubilized in Na-DodSO$_4$ followed by PAGE. After electrophoresis, the proteins were electroblotted into nitrocellulose sheets and the proteins photolabeled with [^{3}H]cytochalasin B were estimated by cutting the nitrocellulose sheets every 2 mm and assaying the slices for their ^{3}H content.

In other experiments, the nitrocellulose sheets were immunoreacted with antiserum raised against the purified glucose transporter of human erythrocytes according to Lienhard *et al.* (29). Immunoreaction of the antiserum with the nitrocellulose gel was detected by overlaying it with ^{125}I-labeled goat anti-rabbit IgG (New England Nuclear) followed by autoradiography as described by Lienhard *et al.* (29).

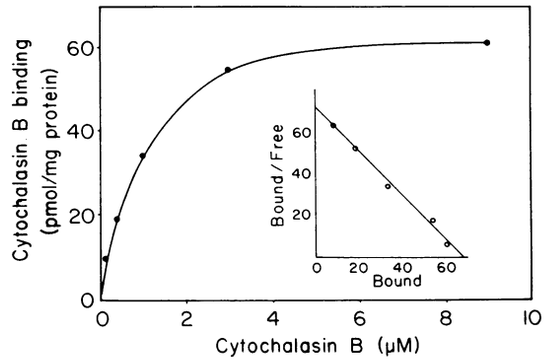

FIG. 1. A typical plot demonstrating saturable D-glucose-displaceable cytochalasin B binding to rat cerebral microvessels. A Scatchard plot (*Inset*) was used to calculate the K_d (1 μM) and B_{max} (66 pmol/mg of protein). The straight line indicates the presence of a single class of binding sites.

RESULTS

The microvessel preparations, assessed by light microscopy, appeared free of identifiable neuronal or glial elements, but small amounts of unidentifiable debris and free nuclei were noted. Consistent with our previous results (30), we estimated that such contaminants constituted <5% of the preparation. Also, γ-glutamyl transpeptidase activity was assayed in cerebral microvessels and in the cerebral cortex to determine the degree of enrichment in microvessels. The enzyme activity of cerebral microvessel preparations was 16-fold higher than that of the cerebral cortex.

Table 1. Specific cytochalasin B binding to particulate fractions of the cerebral cortex, cerebral microvessels, and erythrocytes

Particulate fraction	*n*	K_d, μM	B_{max}, pmol/mg of protein
Cerebral cortex			
Rat	4	0.33 ± 0.17	4.1 ± 0.9
Pig	4	0.99 ± 0.30	8.5 ± 0.5
Cerebral microvessels			
Rat	5	0.88 ± 0.15	62.9 ± 15.3
Pig	4	0.65 ± 0.09	61.7 ± 7.1
Erythrocyte membranes			
Human	2	0.66	285
Rat	2	*	*
Pig	2	*	*

Values denote means ± SEM of the number of observations (*n*). The dissociation constant (K_d) and maximal binding (B_{max}) were calculated by the method of Scatchard (26).
*No detectable binding.

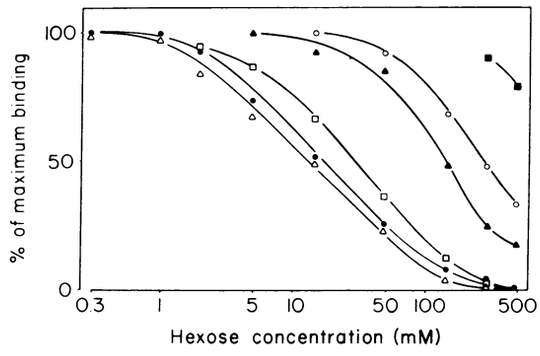

FIG. 2. Displacement of cytochalasin B binding to pig cerebral microvessels as a function of increasing concentrations of 2-deoxy-D-glucose (△), D-glucose (●), 3-*O*-methyl-D-glucose (□), D-(+)-mannose (▲), D-(+)-galactose (○), and β-D-(−)-fructose (■). The concentration of cytochalasin B was 0.5 μM. The specific binding obtained at each concentration of hexose was calculated as the difference between binding in the presence of the hexose and that in the presence of 500 mM L-glucose. The results are expressed as percent of the maximal specific binding. The total solute concentration of the incubation mixture was kept constant by the addition of sucrose. The experiment was done twice and the average IC_{50} values for the different hexoses are given in the results section.

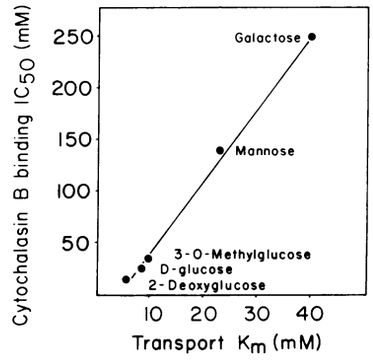

FIG. 3. The IC_{50} values of the various hexoses, derived from the experiments detailed in Fig. 2, are correlated with the transport K_m of the same hexoses in the rat, *in vivo* [taken from Pardridge and Oldendorf (6)].

In preliminary experiments we determined that specific cytochalasin B binding to cerebral microvessels was linear with respect to tissue protein concentrations ranging from 20 to 200 μg per assay. Heating of microvessels to 95°C for 20 min abolished specific binding without altering nonspecific binding.

Specific cytochalasin B binding to particulate fractions of cerebral microvessels was saturable (Fig. 1). Scatchard analysis shows a linear relationship, indicating a single class of binding sites (Fig. 1 *Inset*). The apparent K_d and B_{max} values for particulate fractions of the cerebral cortex and cerebral microvessels of the rat and the pig are presented in Table 1. The results of cytochalasin B binding to human, rat, and pig erythrocyte membranes are also shown in Table 1. Human erythrocyte membranes have a very high density of binding sites (B_{max} of 285 pmol/mg of protein), whereas rat and pig erythrocytes were devoid of detectable specific binding. The absence of specific cytochalasin B binding to rat and pig erythrocytes is consistent with the lack of glucose transporter in these cells (13) and assured us that erythrocytes trapped in microvessels obtained from these two species did not contribute to their cytochalasin B binding. The affinity of specific cytochalasin B binding (K_d) to microvessels from the rat and pig was very similar to that of the human erythrocyte membranes, but the density of binding sites (B_{max}) was approximately one-quarter that of the human erythrocyte. The densities of binding sites in cerebral microvessels of the rat and pig were similar to each other and about 10-fold higher than that of cerebral tissue particulate fractions.

The ability of a variety of hexoses to displace specific cytochalasin B binding to cerebral microvessels was used to assess the biological significance of the specific cytochalasin B binding. The dose–displacement curves for these hexoses are shown in Fig. 2. The concentrations of hexoses that displace 50% of the specific cytochalasin B binding (IC_{50}) to particulate fractions of pig cerebral microvessels are 2-deoxy-D-glucose, 13 mM; D-glucose, 22 mM; 3-*O*-methyl-D-glucose, 33mM; D-(+)-mannose, 145 mM; D-(+)-galactose, 250 mM. β-D-(−)-fructose did not achieve IC_{50} at 500 mM. Both L-glucose and sucrose had no effect on specific cytochalasin B binding at concentrations of 500 mM. These results suggest that the affinity of hexoses to the glucose transporter of cerebral microvessels is in descending order: 2-deoxy-D-glucose > D-glucose > 3-*O*-methyl-D-glucose > D-(+)-mannose > D-(+)-galactose. These IC_{50} values of the hexoses were plotted against apparent K_m values of hexose transport across the BBB previously reported in the rat *in vivo* (6). The excellent correlation (Fig. 3) suggests a high degree of biological specificity of the *in vitro* binding of cytochalasin B to the glucose transporter of cerebral microvessels.

An appreciable portion of the microvessel protein content is derived from the tough basement membrane that surrounds the endothelial cells of microvessels. Since it is most likely that the glucose transporter of the BBB is located on membranes of endothelial cells, we reasoned that removal of the basement membrane should result in a higher density of specific cytochalasin B binding sites in subfractions containing primarily plasma membranes. The results of the crude fractionation of pig cerebral microvessels are shown in Table 2. γ-Glutamyl transpeptidase activity and the density of cytochalasin B binding sites were 3-fold and 2-fold higher in fraction C compared to the original microvessel preparation.

The molecular weight of the glucose transporter of cerebral microvessels was determined by covalent labeling with

Table 2. Specific cytochalasin B binding and γ-glutamyl transpeptidase activity in particulate fractions of the cerebral cortex and cerebral microvessels of the pig

Particulate fraction	Specific cytochalasin B binding			γ-Glutamyl transpeptidase activity, μmol/mg of protein per hr
	pmol/mg of protein*	K_d, μM	B_{max}, pmol/mg of protein	
Cerebral cortex	7	0.58	8	0.6
Cerebral microvessels	38	0.53	44	9.6
Fraction A	25			9.0
Fraction B	68			10.2
Fraction C	77	0.64	90	27.6

The results represent one of two experiments.
*Results in this column were obtained at a cytochalasin B concentration of 3 μM.

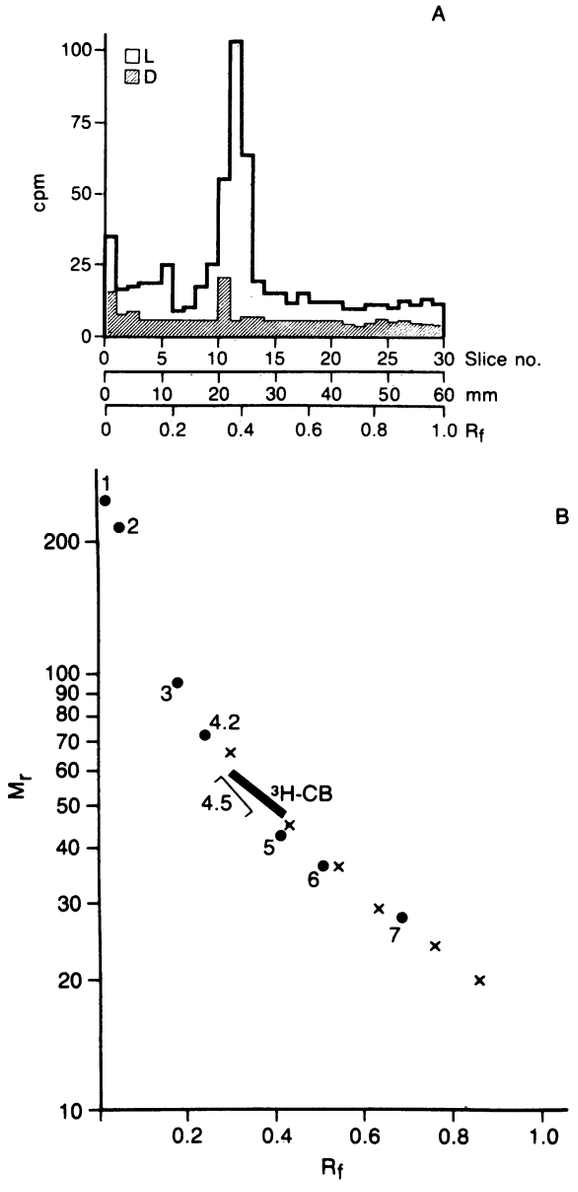

FIG. 4. (*A*) Covalent photolabeling of pig cerebral microvessels with [^{3}H]cytochalasin B. The radioactivities of slices from one representative NaDodSO$_4$/PAGE gel are plotted against the relative positions (R_f) in the gel. The open area is the radioactivity in the sample labeled in the presence of L-glucose and the shaded area represents radioactivity in the parallel sample that was labeled in the presence of D-glucose. The gel was sliced every 2 mm. (*B*) Molecular weight calibration of the [^{3}H]cytochalasin B binding polypeptide(s). Molecular weight standards (×) and human erythrocyte cell membrane proteins, bands 1–7 (●), are plotted against the relative positions (R_f) of the [^{3}H]cytochalasin B binding polypeptide(s) in the protein electrophoresis gel shown in *A*. Molecular weight standards (shown as $M_r \times 10^{-3}$) were trypsin inhibitor, M_r 20,100; trypsinogen, M_r 24,000; carbonic anhydrase, M_r 29,000; glyceraldehyde-3-phosphate dehydrogenase, M_r 36,000; egg albumin, M_r 45,000; and bovine serum albumin, M_r 66,000. The position of the [^{3}H]cytochalasin B-labeled peak of pig cerebral microvessels is indicated by the dark bar and corresponds to band 4.5 of human erythrocyte membranes (bracket) of M_r about 53,000.

[^{3}H]cytochalasin B followed by NaDodSO$_4$/PAGE. Fig. 4*A* shows the results of such an experiment using fraction C of pig brain microvessels. A single peak of radioactivity was found in the sample irradiated in the presence of L-glucose. This peak was extensively (83%) inhibited by the presence of D-glucose. The peak corresponded to an average M_r of 53,000, which matches exactly the molecular weight of the protein band 4.5 of human erythrocyte membranes (Fig. 4*B*). This band has been associated with the glucose transporter of the human erythrocyte. Similar results were obtained with rat cerebral microvessels with the labeled peak corresponding to an average M_r of 55,000 (data not shown).

Antiserum to the glucose transporter of human erythrocytes was used to identify crossreacting polypeptides of pig brain microvessel preparations after NaDodSO$_4$/PAGE. Fig. 5 shows that, of the numerous proteins which constitute these membranes, only one region of M_r 43,000–55,000 reacted with the antiserum. Although direct quantitation of the immunoreactive material by this procedure is not possible (31), confidence in the use of this technique to identify the glucose transporter stems from parallel increases in immunoreactivity and cytochalasin B binding in insulin-treated adipocytes (20, 29) and transformed fibroblasts (31).

DISCUSSION

We have extended methodologies that have previously been used successfully to study the glucose transporter of human erythrocytes and other mammalian tissues to identify, characterize, and quantify the glucose transporter of cerebral microvessels. Our findings indicate the presence of a high density of cytochalasin B binding sites in particulate fractions of cerebral microvessels of the rat and pig. There are several reasons to suggest that these binding sites represent the glucose transporter of the BBB. First, the K_d of binding is <1 μM, which is similar to the concentration of cytochalasin B that inhibits 50% of glucose transport across the BBB *in vivo* (32). Second, there is excellent correlation between the *in vivo* K_m of transport across the BBB for a number of hexoses and their ability to displace the specifically bound cytochalasin B from the microvessel preparations *in vitro* (Fig. 3). Last, the very high density of specific cytochalasin B binding sites suggesting the existence of a high density of the glucose transporter moiety is predictable because brain capillaries that constitute much less than 1% of the total brain weight must transport glucose for the whole brain.

Based on *in vivo* studies of glucose transport across the BBB (33), we estimate that cerebral microvessels can transport an amount of glucose that is about 10% of their wet weight per min. Our results of cytochalasin B binding, which indicate that the density of the glucose transporter in preparations of brain capillaries is 10–20 times higher than the den-

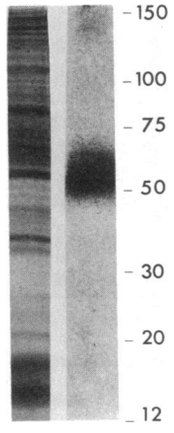

FIG. 5. Immunoreactivity of pig cerebral microvessel proteins with antiserum to the glucose transporter of human erythrocytes. (*Left*) Coomassie blue-stained proteins. (*Right*) Autoradiogram of immunoreactive proteins. The positions of the molecular weight standards (shown as $M_r \times 10^{-3}$) of this gel, performed as in Fig. 4*B*, are indicated.

sity of the transporter in membranes of the cerebral cortex (Tables 1 and 2), adipocytes (34), and muscle cells (16), support this idea. These results also indicate that cerebral microvessels are more richly endowed with the glucose transporter than any other tissue known, with the exception of the human erythrocytes.

The exact cellular and subcellular localization of the cytochalasin B binding sites in microvessel preparations remains to be investigated. From the preliminary experiments described in Table 2, it is apparent that fraction C, which is highly enriched with γ-glutamyl transpeptidase activity (a marker of endothelial cell membranes; ref. 22), has the highest density of cytochalasin B binding sites, thus supporting a plasma membrane location for the cytochalasin B binding sites.

A second objective of our study was to characterize the molecular identity of the glucose transporter. Specific covalent incorporation of [^{3}H]cytochalasin B to the BBB glucose transporter and immunological studies using antiserum raised against the purified glucose transporter of human erythrocytes have both indicated a polypeptide(s) with an M_r of about 53,000. These results point to a similar identity of the BBB glucose transporter to the transporter of other tissues (17–19, 35, 36).

In addition to identification of the glucose transporter, our findings offer practical suggestions for the study of glucose transport across the BBB. *In vivo* studies of the kinetic properties of BBB glucose transport are difficult to perform because of a number of variables that include cerebral blood flow, capillary recruitment, and difficulties in separating glucose transport from glucose metabolism. *In vitro* studies of glucose transport in isolated cerebral microvessels have not been successful in clearly establishing a transport K_m or maximum because such transport is both rapid and bidirectional. *In vitro* studies are also complicated by "leakiness" of the isolated microvessels, as evidenced by their failure to exclude vital dyes and L-glucose (37). Cytochalasin B binding studies allow the direct measurement of the density of the glucose transporter and the affinity of binding without these limitations and complications.

We are indebted to Drs. G. Lienhard and M. Weber for providing us with the antiserum to the human erythrocyte glucose transporter. This research was supported in part by U.S. Public Health Service Grants NS 18150 and NS 05820 and the Diabetes Association of Greater Cleveland to S.I.H., by a grant from the Northeast Ohio Chapter of the American Heart Association to A.P.K.D., and by a grant from the Medical Research Council of Canada to A.K.

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