Brain Research, 192 (1980) 17–28

POLARITY OF THE BLOOD–BRAIN BARRIER: DISTRIBUTION OF ENZYMES BETWEEN THE LUMINAL AND ANTILUMINAL MEMBRANES OF BRAIN CAPILLARY ENDOTHELIAL CELLS

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(Accepted December 6th, 1979)

Key words: blood–brain barrier — capillary endothelial cells — cellular polarity — Na^+, K^+-ATPase — alkaline phosphatase — γ-glutamyl transpeptidase — 5′-nucleotidase — cytochemistry

SUMMARY

The subcellular distribution in brain capillaries of alkaline phosphatase and Na^+, K^+-ATPase was investigated by two methods. Cytochemical studies using whole brain perfusion and electron microscopic examination indicated that alkaline phosphatase activity was located in both the luminal and antiluminal cytoplasmic membranes of the brain capillary endothelial cells. By contrast, the K^+-dependent phosphatase activity associated with Na^+, K^+-ATPase was located in only the antiluminal membrane. Biochemical studies using membranes prepared by homogenization of isolated brain capillaries and density gradient centrifugation resulted in identification of two plasma membrane fractions. The light fraction contained alkaline phosphatase but very little Na^+, K^+-ATPase while the heavier fraction contained both enzyme activities. In addition, γ-glutamyl transpeptidase showed a distribution similar to alkaline phosphatase while 5′-nucleotidase activity was distributed with the Na^+, K^+-ATPase activity. We conclude that the luminal and antiluminal membranes of brain capillaries are biochemically and functionally different. This polarity should permit active solute transport across brain capillary endothelial cells which are the cells responsible for the blood–brain barrier.

INTRODUCTION

For several decades, the term 'blood–brain barrier' (BBB) has been used to

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describe the restricted movement of certain solutes between the blood and the brain. It is now apparent that the brain capillary endothelial cells are responsible for the selective barrier[9,10,13,44,45]. Furthermore, we have begun to recognize that there are at least 4 mechanisms by which the endothelial cells can control solute fluxes across the capillary wall. (A) Initially, the BBB was defined by its impermeability to large molecular weight solutes such as protein-bound dyes[16]. The important ultrastructural work of Reese and Karnovsky[45] and Brightman et al.[9,10] showed that it is the tight junctions between brain endothelial cells and the low rate of pinocytosis across these cells which are responsible for exclusion of proteins by the brain. (B) Subsequently, the BBB permeability to low molecular weight substances was investigated. Two basic principles became apparent. For a solute to easily enter the brain, it must be either highly lipid soluble and able to diffuse through cell membranes[11,39], or it must have affinity for certain specific and selective carrier-mediated transport systems present within the endothelial cell plasma membranes[12,39]. Therefore, a solute which is not lipid soluble and for which there is no transport system across the capillary wall cannot exchange between the blood and the brain. (C) More recent studies have defined a metabolic BBB for certain solutes due to the presence of specific enzymes within brain endothelial cells. For example, L-DOPA cannot readily enter brain from blood because it is metabolized by L-DOPA decarboxylase and monoamine oxidase present in brain capillaries[2]. (D) Finally, it seems likely that the concentration of some solutes in brain interstitial fluid can be kept at a constant low level compared to blood by virtue of specific efflux mechanisms capable of moving such solutes out of the brain against a concentration gradient[5,13,24,49]. However, in vivo studies of efflux at the BBB are difficult to interpret because of the presence of other brain cells. Consequently, the potential importance of active efflux mechanisms located in the endothelial cell has not been generally appreciated. It is this active transcellular transport capability of the BBB which we would like to investigate more fully.

The ability of a single layer of cells to tranport solutes from one side of the cell to the other against a concentration gradient is seen in many epithelia such as those lining kidney tubules and intestinal mucosa. Active transcellular transport in these cells is thought to be due to a selective or polar distribution of transport proteins between the opposite surfaces of the cell[26,36]. As an example, a solute can be greatly accumulated within the cell by active transport across one membrane and subsequently leave the cell by an alternate nonactive process present in the opposite membrane. Therefore, cellular polarity permits vectorial transport against concentration gradients and across cellular barriers.

We recently compared the in vivo and in vitro permeability properties of brain capillaries and proposed that their endothelial cells are polar[5]. In the present study, we use a combination of cytochemical and membrane separation techniques to provide more direct evidence that the luminal and antiluminal membranes of brain capillary endothelial cells are different from each other. A preliminary report of some of this work has been published[21].

METHODS

Cytochemistry

The cytochemical localization of alkaline phosphatase and Na^+, K^+-ATPase in rat brain capillaries was demonstrated as described previously[18,19,21,22]. The brains of adult male rats were perfused for 15 min at 15 °C with a fixative containing 0.25% glutaraldehyde, 2.0% formaldehyde, 5.0% dimethyl sulphoxide and 0.1 M sodium cacodylate, pH 7.4. The brain was then successively perfused with (1) 0.1 M Tris·HCl, pH 9.0, (2) incubation media, (3) 0.1 M Tris·HCl, pH 9.0, (4) 0.25 M sucrose, (5) 1.0% $Pb(NO_3)_2$, (6) 0.25 M sucrose and (7) 0.1 M Tris·HCl, pH 9.0. The incubation medium for alkaline phosphatase contained 0.1 M Tris·HCl, pH 9.0, 10 mM $MgCl_2$, 20 mM $SrCl_2$ and 5 mM disodium p-nitrophenyl phosphate. The incubation medium for Na^+, K^+-ATPase was identical except for the addition of 10 mM KCl and 1 mM L-tetramisole, an inhibitor of alkaline phosphatase. Control incubations contained L-tetramisole and were K^+-free or substrate-free. Pieces of temporal cortex were post-fixed in 1% osmium tetroxide and prepared for electron microscopy.

Isolation of brain capillaries

Because of the large amount of material needed, the rat was not an adequate source of brain capillaries for membrane isolation. Therefore, we isolated capillaries from bovine brains using a modification of the method used for rats[3]. Eight to ten bovine brains were obtained at a local slaughterhouse, packed in ice and transported to the laboratory. The meninges and surface blood vessels were stripped away and the cortex was excised, minced and placed in an iced buffer consisting of oxygen-saturated Ringer solution with 1.2 mM $MgCl_2$, 15 mM N-2-hydroxy-ethylpiperazine-N′-2-ethane sulfonic acid (HEPES), pH 7.4, 5 mM D-glucose and 1% fraction V bovine serum albumin. A 10% (w/v) homogenate was made using a Waring blender turned on for three separate periods of ten seconds each. The homogenate was passed over a 118 μm mesh and the material retained on top of the mesh was saved. This procedure was repeated once with another 118 μm nylon mesh and then with a 70 μm mesh. The material retained by the third mesh was centrifuged at 1000 $\times$ *g* for 10 min. To remove cellular debris and myelin, the pellet was resuspended to a concentration of 16% (w/v) in the same buffer now containing 25% fraction V bovine serum albumin and centrifuged at 1000 $\times$ *g* for 15 min. The new pellet consisted of a mixture of free nuclei and capillary segments of various sizes. To obtain a more uniform suspension of capillaries, the pellet was resuspended in buffer and then passed through a 335 μm nylon mesh under gentle suction. The capillaries were separated from nuclei by passing the suspension through a 1.2 $\times$ 1.5 cm column containing 0.25 mm glass beads. Nuclei were removed by washing the beads with buffer. The capillaries were retained by the beads and could be recovered by gentle agitation in buffer. After the beads settled, the capillaries were collected by centrifugation at 500 $\times$ *g* for 5 min. The quality of each preparation was monitored by phase microscopy.

Preparation of capillary cell membranes

Membranes from brain capillaries were prepared by a modification of the

method that McKeel and Jarret[33] used to make membranes from fat cells. The freshly isolated capillaries were diluted 1 to 10 in the same buffer used for cell isolation but now containing 0.1% (w/v) crude collagenase. After incubation at 37 °C for 30 min, the cells were collected by centrifugation at 500 × *g* for 5 min and washed twice by resuspension and centrifugation in a buffer (TSE) consisting of 10 mM Tris·HCl, pH 7.5, 0.25 M sucrose and 0.1 mM EDTA. A 10% homogenate (v/v) of the collagenase-treated capillaries in TSE was made using 6 up-and-down strokes in a glass and teflon homogenizer (0.15–0.22 mm clearance) at 2300 rpm. After centrifugation at 2000 × *g* for 10 min, the supernatant was removed, made 0.1 mM in $MgSO_4$ by adding the appropriate amount of 10 mM $MgSO_4$ and then saved. The pellet was resuspended in 9 vols of TSE and rehomogenized using 4 up-and-down strokes in the same homogenizer. After centrifugation, the pellet was discarded, the supernatant was adjusted to 0.1 mM $MgSO_4$, combined with the first supernatant and centrifuged at 78,000 × *g* for 1 h. The pellet was resuspended in 10 ml of TSE by 6 up-and-down strokes in a homogenizer at 390 rpm. The microsomal suspension was layered on top of a discontinuous gradient containing layers of 5, 10, 15 and 20% Ficoll in TSE and then centrifuged for 18 h at 34,000 × *g*. The material at each interface was collected and washed once by centrifugation at 78,000 × *g* for 1 h in a buffer containing 10 mM Tris·HCl, pH 7.5, and 0.25 M sucrose.

Enzyme assays

Alkaline phosphatase was determined by the method of Linhardt and Walter[29] and γ-glutamyl transpeptidase by the method of Orlowski and Meister[41]. 5′-Nucleotidase activity was measured as the difference in inorganic phosphate released using 5′-AMP vs 3′-AMP as substrates according to the method of Murer et al.[35].

Na^+, K^+-ATPase was measured as the difference in inorganic phosphate formed from ATP in the presence or absence of 1 mM ouabain. The assay media contained 4 mM Na_2ATP, 4 mM $MgCl_2$, 100 mM NaCl, 30 mM KCl, 0.1 mM EGTA and 50 mM Tris·HCl, pH 7.4. Incubations were carried out for 10 min at 37 °C and stopped by the addition of 30% trichloroacetic acid.

Inorganic phosphate was measured by the method of Fiske and SubbaRow[23] as modified by Bonting[7]. Protein was determined using the method of Lowry et al.[31].

Materials

Disodium ATP was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and ficoll from Pharmacia Chemicals (Uppsala, Sweden). All other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.).

RESULTS

Cytochemistry

The cytochemical localization of alkaline phosphatase in brain capillaries is shown in Fig. 1. Electron-dense reaction product appears as small, granular clumps on the plasma membranes. Both the luminal and antiluminal membranes of the endothe-

Fig. 1. Cytochemical localization of alkaline phosphatase. The incubation mixture contained neither K^+ nor L-tetramisole. Electron-dense reaction product is seen on both the luminal and the antiluminal membranes of the endothelial cells. Bar represents 0.25 μm.

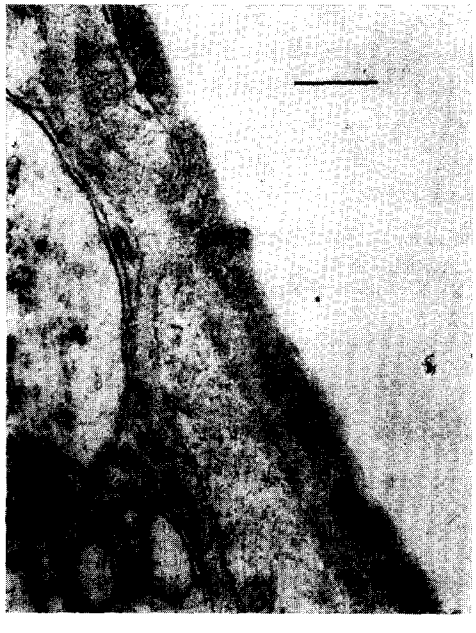

Fig. 2. Incubation in K^+-free media containing L-tetramisole. Reaction product is absent from both endothelial cell membranes. Bar represents 0.25 μm.

Fig. 3. Cytochemical localization of Na^+, K^+-ATPase. The incubation mixture contained L-tetramisole and 10 mM K^+. Electron-dense reaction product is present only on the antiluminal membrane of the endothelial cell. Bar represents 0.25 μm.

lial cell contain reaction product, while there is little or no activity in the other structures of the capillary. Control brains, perfused with media containing the alkaline phosphatase inhibitor L-tetramisole (Fig. 2) or lacking substrate, show no activity.

K^+-dependent p-nitrophenyl phosphatase could be demonstrated by adding K^+ to the incubation media which contained L-tetramisole. In contrast to the distribution

TABLE I

Protein yields and enzyme specific activities in fractions of capillary membranes

Values for protein are in mg and for enzyme activities in nmol of product appearing/mg protein/min. Letters refer to membrane fractions from the Ficoll gradient: A at the 0/5% interface, B at 5/10%, C at 10/15%, D at 15/20% and P was the pellet at the bottom of the gradient. Data shown are the averages of 3 determinations ± S.D.

	Protein	*Specific activity*			
		Alkaline phosphatase	*Na^+,K^+-ATPase*	*γ-Glutamyl transpeptidase*	*5'-Nucleotidase*
Capillary homogenate	104.0 ± 5.0	25.6 ± 1.1	100.0 ± 35.0	647.0 ± 37.0	11.0 ± 0.4
Crude membranes	12.0 ± 1.2	72.4 ± 2.3	151.0 ± 40.0	1100.0 ± 60.0	39.1 ± 0.7
A	1.15 ± 0.05	156.1 ± 9.2	128.0 ± 37.0	2550.0 ± 260.0	55.6 ± 2.3
B	1.11 ± 0.10	101.8 ± 1.9	224.0 ± 35.0	1100.0 ± 170.0	59.3 ± 3.8
C	0.83 ± 0.11	90.1 ± 1.4	204.0 ± 24.0	480.0 ± 110.0	32.2 ± 1.9
D	3.63 ± 0.15	45.9 ± 2.9	222.0 ± 28.0	580.0 ± 220.0	31.5 ± 1.6
P	0.79 ± 0.02	46.5 ± 1.5	67.0 ± 71.0	80.0 ± 220.0	24.2 ± 2.0

of alkaline phosphatase, K^+-stimulated phosphatase activity is absent from the luminal membrane and present in the antiluminal membrane (Fig. 3). This enzyme activity is the result of the phosphatase step of Na^+, K^+-ATPase[17] and has been shown in the renal cortex to coincide with the binding sites both of [^{3}H]ouabain and of ferritin-labeled anti-Na^+,K^+-ATPase antibody[18,19,47]. Na^+,K^+-ATPase activity was not demonstrated in the glial cells surrounding the capillary. These cells are known to contain this enzyme and its activity is well preserved in rat brain under the fixation and incubation conditions employed here[20]. It is possible that presentation of incubation media by perfusion may bias the intensity of reaction so that sites near the vessel lumen appear most active. Unfortunately, Na^+,K^+-ATPase could not be adequately localized in slices of perfusion-fixed brain since under these conditions there was a widely distributed reaction product that was independent of K^+ and insensitive to L-tetramisole.

Our results suggest that Na^+, K^+-ATPase activity is present only in the antiluminal membrane of the brain capillary endothelial cell while alkaline phosphatase is present in both the luminal and antiluminal membrane. However, interpretation of these cytochemical studies may be subject to error and, therefore, we developed a method for membrane isolation in order to further characterize the luminal and antiluminal membranes.

Enzyme activities in isolated membranes

Membranes from homogenized brain capillaries were separated on a Ficoll density gradient and the resultant membrane fractions were assayed for the presence of alkaline phosphatase and Na^+, K^+-ATPase as well as for two other plasma membrane bound enzymes, γ-glutamyl transpeptidase and 5′-nucleotidase. Table I shows the protein yields and the specific activity of these enzymes in the capillary homogenate, the crude membranes and the 5 membrane fractions (A, B, C, D, P) from lightest to heaviest. These data are from a single preparation of capillary membranes but are typical of the results usually obtained.

The relative amounts of different membranes present in each fraction can be better appreciated if the data are analyzed using the total enzyme activity in each fraction rather than the specific activity[15]. Histograms for the distribution of each enzyme in the various capillary membrane fractions are shown in Fig. 4. Most of the alkaline phosphatase activity was evenly distributed between fractions in A and D. In contrast, only 10% of Na^+, K^+-ATPase activity appeared in A while nearly 60% appeared in fraction D. Thus, fraction A contains plasma membranes that are high in alkaline phosphatase activity but low in Na^+, K^+-ATPase activity, while both enzyme activities are present in the membranes of fraction D. Although pure populations of plasma membranes were not obtained, our results show that the activities of two plasma membrane markers can be separated. This suggests that there are two populations of plasma membranes present. In addition to endothelial cells, pericytes are also present in the isolated brain capillaries; however, we have no evidence from the cytochemical studies that either of these enzymes are present in high activity in the pericyte. Therefore, based on our cytochemical data for the distribution of alkaline

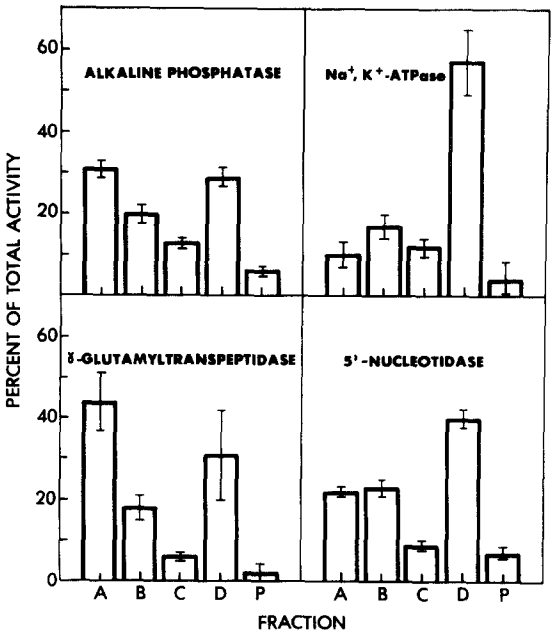

Fig. 4. Distribution of enzyme activities. The activity of each enzyme in the individual fractions is shown as the percent of the total activity in all fractions. Letters refer to fractions as indicated in Table I. The recoveries of activity from the gradient compared to the activity put on the gradient were: alkaline phosphatase, 65 ± 3%; Na^+, K^+-ATPase, 75 ± 20%; γ-glutamyl transpeptidase, 51 ± 7%; 5′-nucleotidase, 61 ± 2%; protein, 62 ± 7%. Data shown are the averages of 3 determinations ± S.D.

phosphatase and Na^+,K^+-ATPase activity in brain capillary endothelial cells, we conclude that fraction A is enriched in the luminal membrane whereas fraction D is enriched in the antiluminal membrane.

The distributions of the other two enzymes shown in Fig. 4 are not as distinct. However, there appears to be a similarity between the distribution of γ-glutamyl transpeptidase and alkaline phosphatase (i.e. both membranes) on the one hand and between 5′-nucleotidase and Na^+, K^+-ATPase (i.e. only antiluminal) on the other.

DISCUSSION

It is well recognized that the BBB plays an important role in regulating the internal milieu of the central nervous system[13,44]. Several different mechanisms are certainly involved; however, promotion of active transport across brain capillaries is potentially the most important as well as perhaps the most susceptible in disease. The existence of active transport out of the brain has been proposed for many years[49] and possible substrates include K^+[8,24,27], amino acids[5,28,30,37], iodide[14], prostaglandins[6], organic anions[1] and proteins[50]. Of necessity, the studies which suggest the existence of these active efflux mechanisms have utilized intact animals. Unfortunately, the presence of other cells such as neurons, glia and choroid plexus which also have active transport capabilities, make the interpretation of these in vivo studies difficult. Likewise,

active transport from blood into the brain is difficult to detect, and to our knowledge, there have been no such active uptake systems demonstrated.

In previous studies, we observed active uptake of amino acids[5] and potassium[24] by isolated brain capillary endothelial cells. Comparison of these results with the well documented low permeability of the luminal aspect of the BBB in vivo to these substances led us to propose a polar model of the brain capillary endothelial cell[5]. Using two different methods, we now show a distinct distribution of plasma membrane markers between the luminal and antiluminal membranes. These data confirm that the brain capillary endothelial cell is polar, and therefore, potentially capable of active transcellular transport.

As in epithelial cells[43,48] the zonula occludens or tight junction of the brain capillary could be responsible for maintaining a polar distribution of membrane enzymes. This is because tight junctions appear to limit lateral diffusion of proteins floating within the plasma membrane. When the tight junctions are disrupted, this restricted diffusion is eliminated and the apical and basal membrane proteins intermix[43]. Tight junctions also contribute to the low protein permeability of brain capillaries[9,10,45]. This structure is present only in capillaries of the brain and retina, and therefore, it is likely that capillary endothelial cells elsewhere in the body are not polar. Thus, brain capillaries are uniquely specialized for providing homeostasis in the central nervous system by means of permeability barriers and active transport processes. The increased energy requirement for active transport by brain capillaries is consistent with the 5-fold greater mitochondrial content of brain endothelial cells compared to systemic vascular endothelia[40].

Our model of the distribution of membrane enzymes in brain capillary endothelial cells is shown in Fig. 5. This schema incorporates the distribution of enzymes demonstrated in the present study as well as the results of previous studies of sugar and amino acid transport at the BBB[4,32,38] and in isolated capillaries[3,5]. Glucose and large neutral (L-system) amino acids readily exchange between the blood and the brain, and therefore, we propose that transport systems for these solutes are located in both the luminal and antiluminal membranes (Fig. 5). This distribution is supported by our finding of γ-glutamyl transpeptidase activity in both membranes since this enzyme may be involved in transport of large neutral amino acids across the BBB[46]. In contrast, the transport activities mediated through the Na^+, K^+-ATPase and the small neutral (A-system, Na^+-dependent) amino acid carrier are restricted to the antiluminal membrane. This proposal is supported by the cytochemical and membrane data presented here as well as the low luminal permeability to these solutes in vivo[8,25,27,38]. The antiluminal distribution of the A-system for amino acid transport would permit active transport of selected neutral amino acids out of the brain. This may contribute to the 5–40-fold lower concentration of most amino acids in the cerebrospinal fluid as compared to plasma[42]. Furthermore, the antiluminal location of Na^+, K^+-ATPase is consistent with its proposed role in maintaining a constant brain K^+ concentration despite large variations in the plasma K^+ level[13,27]. It also would permit formation of cerebrospinal fluid by brain capillaries[24,34].

The BBB is often thought of as a selective but passive sieve and frequently

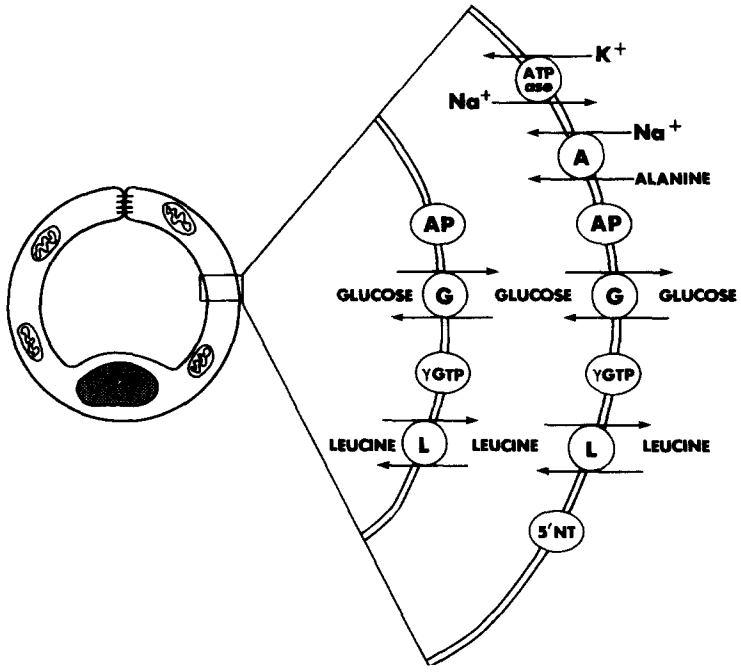

Fig. 5. Polar model of brain capillary endothelial cell. The proposed distribution of enzyme and transport activities between the luminal and antiluminal membranes is shown in the expanded view of the capillary membrane. ATPase, Na^+, K^+-ATPase; A, A-system for neutral amino acids; AP, alkaline phosphatase; G, glucose carrier; GTP, γ-glutamyl transpeptidase; L, L-system for neutral amino acids; 5'-NT, 5'-nucleotidase.

considered important only for the blood-to-brain direction and not the reverse. Our data are consistent with the suggestion that the BBB may mediate active transport of solutes in both directions. Furthermore, these energy requiring processes may be more susceptible to injury than the nonactive aspects of the BBB. Interference with active transport at the BBB could contribute to the neurologic dysfunction observed in a variety of metabolic disorders and in diseases which produce brain edema.

ACKNOWLEDGEMENTS

The authors thank J. Csejtey, B. Evans and A. Ste. Marie for their excellent technical assistance. This work was supported by the National Foundation of the March of Dimes, and by Grants HL-22088, ES-01164, Post-Doctoral Fellowship NS-05807 (A.L.B.) and Research Career Development Award NS-00278 (G.W.G.) from the National Institutes of Health.

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