Neutral Amino Acid Transport by the Blood-brain Barrier

MEMBRANE VESICLE STUDIES

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Endothelial cell membranes, the site of the blood-brain barrier, were obtained from the capillaries of cow brain. The luminal and abluminal membranes were separated by centrifugation on a discontinuous Ficoll gradient. Electron microscopy revealed that the membrane preparations consisted almost entirely of sealed vesicles. The release of latent enzyme activity showed that both membrane preparations were primarily right side out. Radiolabeled L-phenylalanine uptake by luminal vesicles was proportional to membrane protein concentration, with less than 10% binding. Transport was by a high affinity carrier (Kₐ, 11.8 ± 0.1 μM, asymptotic standard error) that showed little or no stereospecificity, and was independent of Na⁺ or H⁺ gradients. Transport was inhibited by L-tryptophan, L-leucine, 2-amino-2-(2,2,1)heptane-2-carboxylate and D-phenylalanine, but not by N-(methylamino)-isobutyrate. Abluminal membranes showed an additional component in which a Na⁺ gradient accelerated the transport of both phenylalanine and N-(methylamino)-isobutyrate. These studies demonstrate the utility of membrane vesicles as a model to characterize the transport properties of the distinct membranes of the polar endothelial cells that form the blood-brain barrier.

Thus, transport across the BBB may be an important mechanism of metabolic regulation. Morphologically brain endothelial cells do not show obvious polarity, but biochemical polarity is well established (10, 11). For instance the abluminal membrane has Na⁺/K⁺-ATPase, Mg⁺-ATPase, and Ca²⁺-ATPase activities, but no detectable activity is present on the luminal membrane (11). On the other hand γ-glutamyl transpeptidase (γ-GT) and alkaline phosphatase are restricted to the luminal membrane (11). Some evidence suggests that polarity also exists concerning the transport of amino acids and glucose. For instance evidence exists for a Na⁺-dependent system located in the abluminal membrane. This A-type amino acid transporter, which transports small non-essential amino acids such as alanine and glycine, may pump amino acids from the extracellular fluid (10, 12-15). There may also be another Na⁺-dependent system, the so-called ASC system, but its location is uncertain (9, 16). Most essential neutral amino acids enter the brain by the L-system (4) or perhaps more specifically the L1-system (9). The current idea is that the L-system is located in both membranes of the BBB and facilitates the entrance and exit of essential large neutral amino acids (LNAA). The methods on which this concept rests are relatively insensitive. The questions of whether the L-system is present in each membrane and what its distribution is remain unanswered. It seems likely that the transport polarity of endothelial cells is important to BBB function. However, transport polarity and its physiological significance are difficult to study by in vivo techniques. This is because the presence of two membranes in series, each with different properties, complicates the study of the BBB by previously used methods. Experiments on the kinetic characteristics of the BBB in vivo rely on the introduction of an artificial medium into circulation. The infusion may be for only a few seconds as with the “brain uptake index” (3, 17), or for longer periods (e.g. 30-60 s), with the “high flow perfusion” technique (8). In either case the only variables that can be measured directly are the concentration in the infusion medium and the accumulation of label in brain tissue. These techniques have been used to demonstrate the existence of several carrier families and to make estimates of their apparent kinetic constants (4, 9). Moreover information about the organization of the BBB is difficult to determine because in vivo techniques measure at best net transependothelial flux across both membranes. The separate contribution of the luminal and abluminal membranes is difficult to deduce. Other limitations include: the presence of transport systems in the choroid plexus (10); discriminating between transependothelial transport and endothelial cell metabolism (7, 18, 19); distinguishing between substrate binding and transependothelial transport (20); and lastly, the composition of the medium at both sides of the...
membranes (both luminal and abluminal) is not accurately known (19). Isolated microvessels offer the advantage that it is possible to control the external substrate concentration (21), but there are also difficulties: substrates may enter endothelial cells through both membranes simultaneously; it is difficult to distinguish whether the substrate has actually entered the endothelial cells or is bound to a membrane or retained in the lumen of the capillary (22); and lastly, the cells in isolated microvessels are leaky. Partridge (4) noted that microvessels are depleted of ATP, do not exclude Trypan blue, and that the sucrose space is indistinguishable from the tritiated water space (23). It would appear that isolated microvessels are more useful for studies of substrate binding than for studies of transport (24).

In the present series of experiments we used isolated, partially purified luminal and abluminal endothelial membrane vesicles to study BBB transport. This methodology provides an opportunity to study the carrier composition and kinetics of the respective membranes separately (25). The advantages include: control over the conditions on both sides of the membrane (intra- and extravascular) (26-28); vesicles have almost no internal metabolism allowing natural substrates to be used instead of analogs; appropriate controls for binding can be included; more studies can be conducted than with intact tissues (29, 30); and, membrane vesicle preparations are a first step toward the identification, purification, and isolation of membrane components (25). Because this approach has not been used before to study transport in brain endothelial cell membranes, the purpose of our experiments was to determine feasibility and to establish the necessary conditions. At this point we thought that it was best to focus on the transport of a single amino acid, and we chose L-phenylalanine as an example of an essential aromatic

**EXPERIMENTAL PROCEDURES**

**Materials—**L-[ring-2,3,4,5,6-H5]Phenylalanine (192 Ci/mmol), N-[1-14C](methylamino)isobutyric acid (48.4 mCi/mmol), and [3H]Ouabain (51.5 mCi/mmol) was obtained from Du Pont-New England Nuclear. L-[L-14C]-Phenylalanine (513 mCi/mmol) was purchased from ICN (Costa Mesa, CA) and used for the laboratory in ice-cold buffer A (101 mM NaCl, 4.6 mM KCl, 2.5 mM CaCl2, 2.5 mM KH2PO4, 1.2 mM MgSO4, and 14.5 mM HEPES, pH 7.4). Either whole brains or cerebral cortices were cut into pieces, after which the pial membranes were removed. Homogenization was carried out in buffer B (buffer A plus 25 mM NaHCO3, 10 mM glucose, 1 mM pyruvate, and 0.5% bovine albumin) using a Waring blender at maximum speed for three periods of 5 s each, separated by 10 s. The final volume of the homogenate was about 350 ml/brain, to which an equal volume of a 26% dextran solution in storage buffer and incubated for 10 min at 37 °C. The pellet was resuspended in storage buffer (290 mM mannitol and 10 mM HEPES-Tris, pH 7.4), and left overnight in an ice bath. Samples were centrifuged at 78,000 × g for 1 h at 4 °C, and the pellet was resuspended in one volume of TSEG with a tissue grinder and layered on top of a discontinuous (5, 10, 15, and 20%) Ficoll gradient in TSEG. After centrifuging at 160,000 × g for 2.5 h at 4 °C in a fixed angle rotor, nine fractions/centrifuge tube were collected. (The fractions corresponded to the interfaces and the segments between them.) All fractions and the pellet were diluted in storage buffer (290 mM mannitol and 10 mM HEPES-Tris, pH 7.4) and left overnight in an ice bath. The final concentration was 10 mM. After 30 min of stirring at 4 °C, the solution was centrifuged at 3,000 × g for 15 min at 4 °C, and the supernatant was removed and centrifuged at 78,000 × g for 1 h at 4 °C. The pellet was resuspended in one volume of TSEG with a tissue grinder and layered on top of a discontinuous (5, 10, 15, and 20%) Ficoll gradient in TSEG. After centrifuging at 160,000 × g for 2.5 h at 4 °C, the solution was centrifuged at 3,000 × g for 15 min at 4 °C, and the supernatant was removed and centrifuged at 78,000 × g for 1 h at 4 °C. The pellet was resuspended in the same buffer. Aliquots (0.2 ml) were stored at -80 °C.

**γ-Glutamyl Transpeptidase Activity—**γ-GT was used as a marker for the luminal plasmalemma (11). A set of dilutions containing 0.5, 1, 2.5, and 5 μg of protein was incubated for 10 min at 37 °C. The reaction was started by adding the indicated reaction media to either the membrane or the abluminal membrane (11). The enzyme was quantified by measuring o-phenylenediamine as described below. Membrane protein (20 μg) was incubated in the presence of binding buffer (2 mM ATP-Tris, 100 mM NaCl, 2 mM MgCl2, 60 mM EGTA, and 10 mM Tris-HCl, pH 7.4) and [3H]Ouabain (100-300 nM) in a final volume of 100 μl. After 30 min of stirring at 4 °C, the reaction was started by the addition of the indicated reaction media to either the membrane or the abluminal membrane. The reaction was stopped by adding 1 ml of 1.5 M acetic acid solution, after which absorbance at 410 nm was measured. p-Nitroanilide was used as the standard, and activity was expressed as mmol p-nitroanilide/mg protein. The orientation of luminal membrane vesicles was determined by measuring latent activity of γ-GT in the presence of 0.1% (w/v) Triton X-100. Since γ-GT is a surface enzyme (32), latent activity would be due to inside-out vesicles.

**Ouabain Binding—**ATPase was used as a marker for the abluminal membrane (11). The enzyme was quantified by measuring o-phenylenediamine, using the rapid filtration technique described below. Membrane protein (20 μg) was incubated in the presence of binding buffer (2 mM ATP-Tris, 100 mM NaCl, 2 mM MgCl2, 60 mM EGTA, and 10 mM Tris-HCl, pH 7.4) and [3H]Ouabain (100-300 nM) in a final volume of 100 μl. As a control for nonspecific binding, incubations were performed in the presence of stopping solution (100 mM NaCl and 10 mM Tris-HCl, pH 7.4). Scatchard analysis was used to quantify binding, expressed as picomoles of ouabain-milligram protein. The orientation of abluminal membrane vesicles was determined by measuring the latency of ouabain binding. The method described above, except that the membranes were pretreated 30 min at room temperature with a mixture of deoxycholate and EDTA, at final concentrations of 0.06% (w/v) and 1 mM, respectively (33).

**Other Markers—**Alkaline phosphatase was used as an additional marker for the luminal plasmalemma (11) and was measured by standard techniques (34). Isolated membranes were assayed for contamination by lysosomes and mitochondria. Thus, the respective relative specific activities of N-acetyl-β-D-glucosaminidase (35) and cytochrome oxidase (36) were determined by quantifying activity in the peak fraction of the isolated membranes and comparing the value to that of the isolated microvesicle homogenate.

**Electron Microscopy—**To confirm that the isolated membranes form vesicles that are free of contaminating organelles, they were prepared for electron microscopy. The peak fractions for the respective marker enzymes were centrifuged at 24,000 × g for 30 min at 4 °C, and the pellet membranes were fixed in modified Tyrode's solution (37), post-fixed in osmium tetroxide, dehydrated in an ethanol series, cleared in propylene oxide, and embedded in Embed 812 (Electron Microscope Sciences, Fort Washington, PA). Thin sections were stained with lead citrate and uranyl acetate and examined under a Zeiss EM 900 electron microscope.

**Uptake Measurements—**Transport of radiolabeled amino acids into membrane vesicles was characterized using the rapid filtration technique (26). Membrane vesicle aliquots were equilibrated overnight in ice-cold storage buffer, after which they were centrifuged at 24,000 × g for 10 min at 4 °C, and the pellet membranes were fixed in modified Tyrode's solution, post-fixed in osmium tetroxide, and any remaining drops were wiped off with filter paper. The reaction was started by adding the indicated reaction media to either the membrane pellet or a membrane suspension. Unless otherwise stated, the final volume was equivalent in weight to microvesicles used (0.1% (w/v) collagenase IV in buffer B and incubated at 37 °C for 30 min. Following incubation, the suspension was centrifuged at 1,000 × g for 5 min at 4 °C, and the pellet was washed twice with TSEG buffer (250 mM sucrose, 0.1 mM EGTA, and 10 mM Tris-HCl, pH 7.4). Ten volumes of TSEG buffer were added to the TSEG buffer to which the membrane suspension was homogenized with a Polytron Tissuemixer (Tefen, Cincinnati, OH) for 90 s in an ice bath before centrifuging at 4,500 × g for 10 min at 4 °C. The supernatant was saved, and the pellet was rehomogenized in nine volumes of TSEG and re-centrifuged as above. Both supernatants were pooled, and solid MgSO4 was added to a final concentration of 10 mM. After 30 min of stirring at 4 °C, the solution was centrifuged at 3,000 × g for 15 min at 4 °C, and the supernatant was removed and centrifuged at 78,000 × g for 1 h at 4 °C. The pellet was resuspended in one volume of TSEG with a tissue grinder and layered on top of a discontinuous (5, 10, 15, and 20%) Ficoll gradient in TSEG. After centrifuging at 160,000 × g for 2.5 h at 4 °C, the solution was centrifuged at 3,000 × g for 15 min at 4 °C, and the supernatant was removed and centrifuged at 78,000 × g for 1 h at 4 °C, and the pellet was resuspended in the same buffer. Aliquots (0.2 ml) were stored at -80 °C.
concentrations of protein and radiolabeled \( \text{L-phenylalanine} \) or MeAIB were 5 mg/ml, 10 and 100 \( \mu \text{M} \), respectively, in a total volume of 10 \( \mu \text{l} \). After incubation at 37 °C for the specified time, the reaction was stopped by diluting with 1 ml of an ice-cold stopping solution (145 mM NaCl and HEPES-Tris, pH 7.4), followed by rapid filtration over a 0.45-\( \mu \text{m} \) Gelman Metricel filter. The filters were washed with 4 aliquots (1 ml) of the ice-cold stopping solution, and dissolved in 1 ml of Soluene 350 for 1 h. The solution was neutralized with 200 \( \mu \text{l} \) of concentrated HCl before adding scintillation fluid. To correct for retention of radiolabeled material by the filter, the background values were determined by stopping the reactions at zero time and subtracting these values from the experimental data.

**Determination of Binding**—To distinguish transmembrane transport from membrane binding, uptake was measured as a function of the reciprocal of medium osmolarity. Luminal vesicles were incubated 45 min in the presence of radiolabeled \( \text{L-phenylalanine} \), 10 mM HEPES-Tris, pH 7.4, and the appropriate concentrations of mannitol sufficient to vary the external osmolarity from 200 to 600 mosm/liter. Under these conditions, the vesicular volume decreases with increasing medium osmolarity, resulting in a reduction of uptake at equilibrium due to the restricted intravesicular space. The origin represents infinite osmolarity, at which measured uptake could only be due to surface binding. Expressing this value as a ratio over uptake measured under normal experimental conditions gives the percent binding. As an additional control, measurements were made in the presence of pronase (5 \( \mu \text{g/ml} \)) to eliminate surface binding.

**Protein Concentration Dependence**—Uptake of radiolabeled \( \text{L-phenylalanine} \) by luminal vesicles was measured over the following membrane protein concentrations: 2, 3, 4, and 5 mg/ml. Incubations were performed in the presence of storage buffer for 1 min.

**Time Dependence**—Transport was studied at the time intervals indicated in the figures. Incubations were performed under three different conditions in the presence of: 1) storage buffer; 2) an inwardly directed sodium gradient (50 mM NaCl, 190 mM mannitol, and 10 mM HEPES-Tris, pH 7.4); and 3) an inwardly directed proton gradient (290 mM mannitol and 10 mM MES-Tris, pH 6.2). For the proton gradient experiments, membranes were equilibrated in 290 mM mannitol and 10 mM MES-Tris, pH 7.4, instead of the usual storage buffer. In some abluminal experiments, 1 mM MeAIB was added to the sodium gradient buffer to specifically inhibit the \( \text{A}^- \)-system amino acid transporter.

**Substrate Concentration Dependence**—Uptake of \( \text{L-phenylalanine} \) into luminal vesicles was measured over a final concentration range of 1 to 100 \( \mu \text{M} \) in storage buffer for 15 s.

**Inhibition Studies**—Transport of radiolabeled \( \text{L-phenylalanine} \) was measured in the presence of potential inhibitors under the conditions specified in the figures.

**Protein Determination**—Protein concentration was measured using the BCA Protein Assay Reagent (Pierce Chemical Co.), with bovine serum albumin as the standard.

**RESULTS**

**Separation of the Membranes**

In order to separate luminal from abluminal membranes, a discontinuous Ficoll gradient was used. Fig. 1 shows that the marker enzymes \( \gamma \text{-GT} \) (luminal) and \( \text{Na}^+/	ext{K}^+\text{-ATPase} \) (abluminal) (11) were separated within the gradient. Usually, 7-20- and 2-4-fold enrichments for \( \gamma \text{-GT} \) and \( \text{Na}^+/	ext{K}^+\text{-ATPase} \) were obtained, respectively. Sometimes, a small luminal contamination was observed in abluminal fractions. However, this contamination was almost completely eliminated by recentrifugation of the abluminal fractions under the same conditions. The data imply that the two membranes are characterized by a marked difference in density, which was also observed by Betz et al. (12). Alkaline phosphatase was used as an additional marker for the luminal membrane (11) and was found to localize in the same peak as \( \gamma \text{-GT} \). The morphology of both luminal and abluminal membranes was examined by transmission electron microscopy. These observations revealed that the membrane preparations consist mainly of sealed vesicles, without significant contamination by other organelles including mitochondria, nuclei, lysosomes, or rough endoplasmic reticulum (Fig. 2). This was confirmed by additional measurements of potential contaminants in a representative luminal membrane preparation. Although the marker for luminal membrane (\( \gamma \text{-GT} \)) was enriched greater than 10-fold, marker enzymes for lysosomes (\( \text{N-acetyl-\( \beta \)-D-glucosaminidase} \)) and mitochondria (cytochrome c oxidase) gave relative specific activities of less than 1 (i.e. 0.94 and 0.22, respectively).

For this study 10 separate luminal membrane preparations were made. On average, 3.01 ± 1.30 mg (± S.E.) of luminal...
membrane protein were recovered from 16.67 ± 6.16 g (wet weight) of microvessels. Of four abluminal membrane isolations, 6.13 ± 3.77 mg of membrane protein were recovered from 17.03 ± 4.19 g (wet weight) of microvessels.

**Orientation of the Vesicles**

The orientation of the vesicles was estimated with latency experiments. The active site of γ-GT (32) and the ouabain-binding site of the Na⁺/K⁺-ATPase (33) are located on the outer face of the respective membranes. Total enzyme activity (right-side-out plus inside-out) was measured in the presence of detergents and compared with the activity measured in the absence of detergent (right-side-out activity). Triton X-100 was used to expose γ-GT inside luminal membranes. Deoxycholate was used for the abluminal membranes (38) because gentler treatment is required to measure ouabain binding by the rapid filtration technique. The rates of activities under both conditions indicated that the percentage of inside-out vesicles was about 37% for luminal vesicles and 13% for abluminal vesicles.

**Uptake Studies into Luminal Vesicles**

In order to characterize the transport of amino acid into the vesicles, luminal membrane vesicles were used initially because of the higher enrichment of the marker enzyme. L-Phenylalanine was chosen as an example of a LNAA. Several studies were done to define the conditions for kinetic measurements.

**Uptake Versus Protein Concentration—[14C]-Phenylalanine uptake was a linear function of membrane protein concentration over a range of 2-5 mg/ml (data not shown). For the remaining experiments, 5 mg of protein/ml of incubation medium were used.**

**Uptake Versus Medium Osmolarity—**To determine the binding component to the membranes, transport studies were done at different medium osmolarities. The γ intercept of a plot of uptake versus the inverse of the osmolality (Fig. 3) depicts the apparent uptake at infinite osmolality. Under this condition there is no intravesicular space available for the substrate, so that the observed uptake is a measurement of binding. The data in Fig. 3 indicate that [14C]-phenylalanine was transported into an osmotically active space, with a binding component of less than 10% of the total uptake measured under the normal conditions of the assay (0.3 osmol/liter). As an additional control for binding, uptake studies were carried out in the presence of Pronase. Such treatment should remove L-phenylalanine bound to membrane proteins and therefore eliminate or decrease the binding. Thus, a plot of uptake measured at different osmolarities in the presence of Pronase would be expected to parallel the original data points, without pronase, but pass through the origin. This was the case as indicated by the broken line in Fig. 3. These experiments confirm that binding represents a minor component of uptake when measured under the usual incubation conditions described and that the data primarily represent transmembrane transport of L-phenylalanine.

**Uptake Over Time—**Radiolabeled [14C]-phenylalanine uptake was measured at 37 °C as a function of time to determine the equilibrium value (influx = efflux) and to define an incubation time that represents initial uptake (linear portion of the uptake plot) for later kinetic measurements. Equilibrium was achieved within 3 min (Fig. 4). The rate of release of [14C]-phenylalanine from preloaded vesicles incubated in a phenylalanine-free medium followed the same time course (not shown). These results suggest that, under our experimental conditions, the carrier present in the luminal membranes works in both directions and that vesicle orientation does not affect the transport measurements. The time chosen for kinetic and inhibition experiments was always on the linear portion of the curve. The average vesicle volume at equilibrium, estimated from these experiments, was about 4 μl/mg of protein, which is similar to that obtained for other membrane vesicle preparations (26, 39).

**Cation Dependence of Uptake—**To analyze the presence of a secondary active transport component, uptake of [14C]-phenylalanine was measured in the presence of inwardly directed gradients of Na⁺ or H⁺. No significant difference in the rate of transport was observed under these conditions (Fig. 4), indicating an apparent energy-independent L-phenylalanine transport across the luminal membranes. The results also indicate that uptake of L-phenylalanine by luminal vesicles was not sensitive to pH changes, showing similar rates of uptake when the pH of the incubation medium was 7.4, 6.2, or 5.5 (not shown).

**Uptake as a Function of Substrate Concentration—**Uptake of [14C]-phenylalanine was measured as a function of substrate concentration over a range of 1 to 100 μM. The data

![Fig. 3. Effect of medium osmolality on [14C]-phenylalanine (10 μM) transport into luminal membrane vesicles (5 mg of protein/ml) at 37 °C for 45 min. The percentage of radio-labeled L-phenylalanine due to surface binding was determined by measuring uptake as a function of the reciprocal of medium osmolality. The γ intercept depicts binding at infinite osmolality, when there is no intravesicular space. Experiments were done in the absence or presence of 5 μg/ml pronase to remove binding material during the experiment. This study was repeated with the same results.](image)

![Fig. 4. Effect of inwardly directed sodium gradient on uptake of [14C]-phenylalanine (10 μM) into luminal membrane vesicles (5 mg of protein/ml). Uptake was measured at the indicated times at 37 °C in the presence of storage buffer or an inwardly directed sodium gradient. This study was repeated with the same results.](image)
were fitted by non-linear regression analysis, using the specific radioactivity of $[^{14}C]$L-phenylalanine as a weighting factor. Fig. 5 shows that L-phenylalanine was transported by both saturable and non-saturable components. The calculated $K_a$ for the saturable component is $11.8 \pm 0.1 \mu M$ (ASE), and the $V_{max}$ is $94.1 \pm 0.6 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein. The $K_D$ value for the non-saturable component is $1.57 \pm 0.01 \mu l \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein.

Inhibition of Uptake—To further characterize the carrier, uptake was determined in the presence of potential inhibitors. Fig. 6A shows that L-tryptophan and L-leucine inhibited, almost completely, $[^{3}H]$L-phenylalanine uptake. This supports the concept that both amino acids enter via the same carrier. Fig. 6B depicts the percentage of $[^{14}C]$L-phenylalanine uptake in the presence of L-tryptophan, BCH, D-phenylalanine, L-phenylalanine, and MeAIB compared to control values in their absence, after subtraction of the non-saturable component. Only MeAIB showed no inhibitory effects.

Uptake Studies in Abluminal Vesicles

Preliminary uptake studies were performed with ab luminal membranes to confirm that the luminal membranes have separate and distinct transport characteristics.

Cation Dependence of L-Phenylalanine Uptake—Transport of $[^{14}C]$L-phenylalanine into abluminal vesicles in the presence of an inwardly directed sodium gradient showed a small but consistent overshoot (Fig. 7). This phenomenon, not observed in luminal vesicles, indicated that L-phenylalanine was cotransported with Na$^+$ ions. Moreover, this overshoot was abolished by the presence of MeAIB in the reaction mixture. An inwardly directed proton gradient had no effect on the uptake of L-phenylalanine.

$N$-(Methylamino)-isobutyrate Uptake—The above results suggested the presence of an A-system for amino acid transport in the abluminal membrane. To further investigate this possibility, uptake of $[^{14}C]$MeAIB, the specific substrate for the A-system (40), was measured in both luminal and abluminal membranes. As shown in Fig. 8, sodium markedly enhanced the initial rate of MeAIB uptake in abluminal membranes, which was significantly greater ($p < 0.05$ than that without sodium. The first order kinetic constants for both curves were determined by non-linear regression analysis fitted to an exponential plot and compared by a $t$ test to the

![Fig. 5](image-url)  
**Fig. 5.** $[^{14}C]$L-phenylalanine transport into luminal membrane vesicles (5 mg of protein/ml) as a function of substrate concentration. L-Phenylalanine transport occurred over 15 s at 37°C. The broken lines are the saturable and non-saturable components fitted to the data (solid line). The calculated kinetic parameters are: $K_a$, $11.8 \pm 0.1 \mu M$ (ASE); $V_{max}$, $94.1 \pm 0.6 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein; and $K_D$, $1.57 \pm 0.01 \mu l \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ of protein. Final concentrations of tritiated L-phenylalanine were achieved by combining 1-9 $\mu M$ $[^{14}C]$L-phenylalanine with unlabeled L-phenylalanine.

![Fig. 6](image-url)  
**Fig. 6.** Uptake of radiolabeled L-phenylalanine into luminal membrane vesicles at 25°C was determined in the presence of potential inhibitors. A, transport, over 30 s, of the indicated concentration of $[^{3}H]$L-phenylalanine was measured in the presence of 1 mM of L-tryptophan or L-leucine, and in their absence. Final concentrations of tritiated L-phenylalanine were achieved by combining 0.5-0.84 $\mu M$ $[^{3}H]$L-phenylalanine with unlabeled L-phenylalanine. B, net uptake of $[^{14}C]$L-phenylalanine (10 $\mu M$) in a 1-min period in the presence of indicated inhibitors at two concentrations (after subtracting the non-saturable component). When L-tryptophan and BCH were used together, the indicated concentration is for each inhibitor.

![Fig. 7](image-url)  
**Fig. 7.** Effect of inwardly directed sodium gradient on uptake of $[^{14}C]$L-phenylalanine (10 $\mu M$) into abluminal membrane vesicles. Uptake was measured at the indicated times at 37°C in the presence of storage buffer or an inwardly directed sodium gradient in the presence or absence of 1 mM MeAIB, the specific substrate of the A-system. These data are representative of three separate experiments.

95% confidence level. A minimal stimulation of transport by sodium was observed in the luminal membrane as well, which most likely was due to minor contamination of the vesicles by abluminal membrane.

DISCUSSION

The results established the following points: enriched membranes can be isolated from the luminal and abluminal sides
of brain endothelial cells; these membranes form vesicles that are primarily right-side-out in orientation that are suitable for the study of blood-brain barrier transport; the L-system for amino acid transport in the luminal membrane is similar to that described in vivo; there is no substantial evidence of a Na+ or H+-dependent transport system for L-phenylalanine on the abluminal membrane; a Na+-dependent system for the transport of amino acids is apparently present in the abluminal membrane.

Isolation of luminal and abluminal membranes from brain endothelial cells was first described by Betz et al. (12). They showed that enriched membrane fractions could be obtained, and they demonstrated that the membranes were biochemically different. We began with the procedure of Betz et al. (12) and made some modifications to get further enrichment of membranes. Na+/K+-ATPase was used as a marker for the abluminal membrane because of its very high activity in cerebral capillaries and the fact that it is clearly limited to the abluminal membrane (11, 41). γ-GT is abundant in brain and is associated with the endothelial cells. For this reason it is often used as a marker of isolated brain capillaries (42). γ-GT was chosen as the most suitable marker for luminal membrane because of its high activity, and the fact that histochemical and immunological studies have shown it to exist almost exclusively on the luminal membrane (11, 43, 44).

The described procedure allowed a separation of luminal membranes in which the activity of γ-GT activity was increased as much as 20 times over the activity in capillary homogenates. Abluminal membranes were enriched to a lesser degree: up to four times the control value. Alkaline phosphatase, an additional marker for the luminal membrane (11), localized in the same peak as γ-GT. Electron microscopy revealed no significant contamination of either membrane preparation by nuclei, lysosomes, rough endoplasmic reticulum, or mitochondria. This was confirmed by additional measurements of the luminal membrane for lysosomal and mitochondrial markers enzymes, both of which gave relative specific activities of less than 1, although the luminal membrane was enriched more than 10 times. It has been suggested that pericytes may contribute γ-GT to cerebral microvessels (45). Pericytes are present at the abluminal aspect of endothelial cells (46) and are only occasionally observed in association with true capillaries (47). Thus, it appears likely that contamination by these cells would be minimal. This interpretation is reinforced by the separation of membranes from endothelial cells in tissue culture, which are uncontaminated by other cell types. Thus, luminal and abluminal membranes were prepared from cultured bovine aortic endothelial cells in a sucrose gradient (48) and gave a pattern of separation similar to that observed for membranes derived from the cerebral microvessels.

The membrane preparations formed sealed vesicles as was expected. Their orientation was primarily right-side-out, with an estimated volume of 4 μl/mg of protein for luminal vesicles. This is similar to membrane vesicles from other types of cells (27, 28). Transport occurred into an osmotically active space (the vesicles behaved as osmometers) providing further evidence that the membranes existed as sealed vesicles under the experimental conditions.

Most studies of amino acid transport have been conducted in vivo, where the movement of a labeled amino acid is from plasma to brain parenchyma. These studies have shown that phenylalanine and a range of other LNAA pass the luminal membrane of brain endothelial cells by an L-system. The L-system, for leucine preferring, carries leucine, isoleucine, methionine, valine, tyrosine, tryptophan, phenylalanine, histidine, and threonine (49, 50). The L-system is facilitative, Na+ independent, and blocked by BCH. It has a preference for L-amino acids but carries D-amino acids as well. Uptake of the D-isomers by the L-system was shown early by Oxender (51) and has been confirmed in the BBB in vivo (17) and in isolated microvessels in vitro (22). The L-system of the BBB has a much higher affinity for LNAA than that described in isolated cells, and this may be due to the presence of the high affinity form L1, as pointed by Smith et al. (52).

The results obtained with luminal membrane vesicles depict an L-system and agree well with the studies in vivo. L-Phenylalanine transport was inhibited almost completely by other LNAA, BCH, and the D-isomer of phenylalanine. Transport was independent of Na+ and H+ gradients and therefore independent of an energy source. The calculated K_m, 11.8 ± 0.1 μM (± ASE) for the saturable component is nearly identical to the most accurate measurement in vivo (52) but higher than the affinity 0.3 μM found in isolated human capillaries (23). Whether the affinity in human capillaries represents the luminal or abluminal membrane or some combination is unclear.

A second, non-saturable component is often seen in vivo (e.g. (51). We saw a similar phenomenon in membrane vesicles (Fig. 5). There are two possibilities. The non-saturable component could be a low affinity, high capacity transport system, or it could be non-facilitated diffusion. The data gathered in the present studies were insufficient to distinguish between the two possibilities.

As mentioned there is no evidence in vivo for the existence of the A-system in the luminal membrane of the BBB, the only membrane directly accessible to material injected into the vascular system (4). However, an A-system has been identified in isolated microvessels (10), and it has been postulated to be on the abluminal side (10, 12, 14, 15). Our results provide direct evidence that there is indeed an A-system in the abluminal membrane. It was possible to demonstrate the transport of MeAIB, the specific substrate for the A-system, in abluminal membranes, as well as a stimulation of transport by a Na+ gradient (Figs. 7 and 8). Although the A-system has a high affinity for small amino acids, there was some Na+ stimulation of L-phenylalanine transport. Thus, it appears that Na+-driven transport systems do exist in the abluminal membrane capable of removing amino acids from the extra-
cellular fluid. Moreover, it seems possible that this transport mechanism may carry a wider range of amino acids than originally thought. The stimulation of L-phenylalanine transport by Na⁺ was completely inhibited by MeAIB indicating that it was due to the A rather than the ASC system, the other Na⁺-dependent neutral amino acid transport system. Thus, no evidence for L-phenylalanine transport by the ASC system was seen in either luminal or abluminal membranes.

The L-system for LNAA is often assumed to be on both membranes. Our data are compatible with the presence of an L-system in the abluminal membranes as well as the luminal membranes, but the degree of activity in the abluminal membranes must be explored by kinetic experiments. On the other hand there was no substantial evidence of Na⁺-dependent transport of L-phenylalanine by luminal membranes, providing further evidence that contamination of luminal vesicles by abluminal vesicles was minimal.

In conclusion, membrane vesicles made from endothelial cells are a good biochemical model to study transport in the BBB. The results reported here show that L-phenylalanine transport into luminal membrane vesicles isolated from brain endothelial cells has the same characteristics as those described in vivo. This supports the view that membrane vesicles retain the carrier composition of the intact cells. The use of isolated membrane vesicles should allow for characterization of both luminal and abluminal membranes of the BBB, thereby building a more comprehensive understanding of the physiological function of the BBB.

REFERENCES