Isolated microvessels: The blood–brain barrier in vitro

(amino acid transport/cerebrospinal microcirculation)

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ABSTRACT Isolated bovine retinal and brain microvessels, exhibiting a patent lumen, were used to study the contribution of the microvasculature to the blood–brain and blood–retina barriers. The diffusion marker, sucrose, was taken up slowly by the isolated microvessels in contrast to leucine, tyrosine, and valine which were taken up at a considerably faster rate. Uptake of leucine was temperature dependent but resistant to inhibition by ouabain and sodium azide. The large neutral amino acids exhibited stereospecificity and cross-competition for uptake by the isolated microvessels. The apparent $K_m$ for uptake for tyrosine, leucine, and valine were 111 μM, 133 μM, and 500 μM, respectively.

The penetration into or removal of a substance from the central nervous system is a function of the interaction between that substance and the various brain barrier systems (1). These systems include the partitions between brain regions, various enzymatic barriers, the clearance and sink actions of the cerebrospinal fluid, choroid plexus and pial vessel transport, and partitioning at the blood–brain interface. From numerous morphological studies using dyes and electron-dense tracers, the barrier to the passage of substances from the blood into the brain has been thought to reside at the level of the intracerebral microvasculature (2). An indication that the microvascular endothelium, with its tight junctions, was the structural entity responsible for this barrier action and not the astrocytic processes adjacent to the microvessels was obtained when it was demonstrated that intraventricularly injected horseradish peroxidase appeared in the pericapillary space (3). Similarly, the impenetrability of the retinal microvasculature to dyes and electron-dense tracers (4) has been attributed to its endothelial tight junctions. In addition, both the retinal and brain microvascular endothelium have low rates of vesicle formation which further decreases the movement of molecules across this cell layer (5). Additional involvement of the cerebral microvasculature in blood–brain partitioning has been demonstrated by in situ carotid artery injection quickly followed by decapitation and determination of brain extraction of the injected test substance (6). Because of the short time interval between injection and decapitation, it has been assumed that the extraction of the test substance was an index of microvascular activity.

To more directly characterize the role of the cerebral microvasculature in the partitioning of small molecules between the blood and the brain and to develop methods for the study of such phenomena in vitro, we examined isolated intracerebral bovine brain microvessels and the anatomically similar retinal microvessels for their abilities to exclude certain polar substances and to transport large neutral amino acids.

Materials and Methods. Materials. L-[2,6-3H]Tyrosine (50 Ci/mmol), [fructose-1-3H]sucrose (15.81 Ci/mmol), and Aquasol were purchased from New England Nuclear, Boston, MA. Ouabain, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), bovine serum albumin fraction V, dopamine, and the unlabeled amino acids were obtained from Sigma Chemical Co., St. Louis, MO. L-[4,5-3H]Leucine (1 Ci/mmol), D-[4,5-3H]leucine (1 Ci/mmol), L-[2,3-3H]valine (2 Ci/mmol), and D-[2,3(n)-3H]valine* (33 Ci/mmol) were purchased from Amersham/Searle Corp., Arlington Heights, IL. Evans blue stain was obtained from the Fisher Scientific Co., Waltham, MA.

Beef brains and enucleated eyes from yearling animals were obtained from a slaughterhouse, packed in ice, and taken to the laboratory within 30 min of death.

Preparation of Microvessels. The microvessels were isolated by a modification of methods of Meezan et al. (7) and Brendel et al. (8). Briefly, after the removal of the pial membrane, pieces of cortical gray matter were collected and homogenized by hand in a 1:1 tissue to buffer volume with five up-and-down strokes in a smooth glass tube equipped with a tapered, serrated Teflon plunger (Arthur Thomas Co., Philadelphia, PA). The buffer used for isolation and incubation contained 122 mM NaCl, 25 mM NaHCO$_3$, 10 mM dextrose, 3 mM KCl, 1.2 mM MgSO$_4$, 0.4 mM K$_2$HPO$_4$, and 1.4 mM CaCl$_2$ at pH 7.3 which was subsequently gassed with 95% O$_2$/5% CO$_2$ to a final pH of ~7.4. The homogenate was poured onto an 86-μm pore size nylon sieve (Tetko Inc., Elmsford, NY) and gently washed with a spray of buffer. The material retained on the sieve after extensive washing was washed into a clean glass tube and rehomogenized with five additional up-and-down strokes. The plunger was immediately removed, allowing the microvessels to float to the surface of the medium and thereby creating a layer of microvessels. The microvessels were taken up in a plastic-tipped dispenser and deposited on an 86-μm pore size sieve for a final wash with a spray of buffer to remove free nonvascular tissue. The collected microvessels were washed into a plastic beaker and placed on ice under a 95% O$_2$/5% CO$_2$ atmosphere until incubation.

The retinas were removed and placed in buffer. After five up-and-down strokes in the hand homogenizer, the retinal homogenate was poured onto an 86-μm pore size sieve and washed with a gentle spray of buffer from a squeeze bottle. Macroscopic retinal blood vessels retained on the sieve were easily removed with forceps. Collection of the microvessels on the 86-μm sieve separated the microvessels from surrounding nonvascular tissue and provided a concentrated source of microvessels for additional fractionation. The material retained on the sieve was washed onto a 210-μm pore size sieve and again washed with a gentle spray of buffer. The material passing through the 210-μm pore size sieve was collected on an 86-μm

* Custom synthesized for us.
pore size sieve and resuspended in buffer. Isolation of the brain or retinal microvessels was routinely accomplished within 1 hr.

To prepare retinal microvessels for light microscopic cinematography, enucleated bovine eyes with attached portions of the optic nerve were cannulated through the ophthalmic artery and perfused with 10 ml of a 1% suspension of a uniform 0.481-μm diameter polystyrene microspheres (Dow Diagnostics, The Dow Chemical Co., Indianapolis, IN) in Earle’s balanced salt solution, buffered with 28 mM Hepes, fortified with 0.1% bovine serum albumin, and containing 0.01% Evans blue dye. It was necessary to mix the microspheres thoroughly with the injection solution before perfusion to dissociate aggregates of the microspheres. The microvessels were then isolated by the procedure described above.

Incubation and Assay of Transport. Aliquots of isolated microvessel suspensions of various volumes were used. For time course experiments 2-ml aliquots of microvessel suspensions containing 1 mg of protein were warmed to 37°C under 95% O2/5% CO2. A 100-μl aliquot of the radiolabeled substance (10 μCi) with or without unlabeled carrier or competitor in buffer was then added to the incubation tube and thoroughly mixed, and the tube was replaced in the 37°C bath. At various times after the addition of the radiolabeled substance, 600-μl aliquots of the incubation suspension were withdrawn with a plastic-tipped dispenser and placed on a 44-μm pore size nylon sieve attached to a suction filtration device. The retained microvessels were washed with four 500-μl aliquots of ice-cold buffer and blotted. The sieves with their collected, washed microvessels were placed in a scintillation vial and then covered with 1.8 ml of 0.1 M NaOH. After standing overnight at room temperature the soaked sieves were sonicated for 1 min. Aliquots were taken for protein determination by the method of Lowry et al. (9) employing bovine serum albumin as standard. Aliquots (0.5 ml) of the sonicate were diluted with an equal volume of 0.1 M HCl in a scintillation vial to which was added 10 ml of Aquasol for scintillation counting.

Kinetic experiments were performed in 700 μl of microvessel suspension containing 0.3–0.5 mg of protein, to which 55 μl of radiolabeled compound, with or without carrier or competitors, was added. Incubation was for 1 min at 37°C followed by determination of the uptake of radiolabel as described above.

Apparent Kmax values were obtained from Lineweaver–Burke plots of amino acid uptake by the isolated microvessels (10) expressed as nmol taken up per mg of protein during the initial minute of incubation. γ-Glutamyl transpeptidase activity was determined by the method of Orlowski and Meister (11). Free amino acids were obtained by sonication of the microvessels in 0.1 M sodium azide and subsequent centrifugation at 10,000 × g for 20 min. The supernatant was analyzed for amino acids by the method of Stein et al. (12).

The metabolism of the radiolabeled amino acids and the microvessel concentration of free amino acids was determined by sonicating the incubated and washed microvessels in distilled water for 1 min. Addition of trichloroacetic acid to make a final concentration of 10% and subsequent centrifugation at 10,000 × g for 5 min produced a supernatant which was then extracted with ether. An amino acid analyzer equipped with split stream was used to isolate individual amino acids for liquid scintillation counting.

RESULTS
The definition of the purity of isolated microvessel preparations has been based largely on light microscopic examination of dilute suspensions of the tissue. The presence of globular shaped tissue either free or attached to the long microvessel segments was considered contamination (7). More recently various enzyme activities have been reported to be concentrated in isolated brain microvessels (13) and may provide another criterion for the purity of microvessel preparations. The microvessels obtained by the procedure described in this communication were found to be 99% free of contaminating nonvascular structures, as determined by light microscopic examination. The isolated bovine microvessels were also found to have 18.1 times more γ-glutamyl transpeptidase activity/mg protein than the cortical grey matter from which they were obtained. This level of enrichment of transpeptidase activity is consistent with the highest relative enrichment reported for isolated microvessels (13, 14).

During microscopic examinations, residual red blood cells and other smaller blood cells within the vessels were observed to exhibit both Brownian movement and migration along the isolated microvessel segments. To document such movement within the vessel segment, we perfused uniform polystyrene microspheres, coated with bovine serum albumin and Evans blue dye, into enucleated eyes via the ophthalmic artery. The perfusions did not interfere with the subsequent isolation of the microvessels. Light microscopic cinematography demonstrated both Brownian and migratory movement of the microspheres within both large- and small-diameter microvessel segments. The microspheres moved rapidly in and out of the plane of focus and from one side of the vessel wall to the other, exhibiting a three-dimensional space for movement which is indicative of a patent lumen. Furthermore, exit of the microspheres from the intravascular space was observed to occur only at the ends of microvessel segments, indicating that the segments were open-ended and that the microvessel walls were intact.

When L-[^3H]leucine was added to suspensions of isolated brain or retinal microvessels, radiolabel was taken up by the microvessels. The two types of microvessels exhibited identical rates of accumulation of [^3H]leucine; a substantially lower rate of radiolabel was accumulated during incubation with [^3H]sucrose (Fig. 1). This indicated that the procedure employed was reproducible and that the capability of transporting large neutral amino acids and excluding sucrose was similar in retinal and brain microvessels. These similarities extended to all the other properties discussed below.

Two polar molecules, 5-hydroxy-3-indoleacetic acid and p-aminobipuric acid, exhibited rates of uptake similar to that of sucrose (data not shown). Probencid, an inhibitor of carrier-mediated organic acid transport (15), had no effect on their rate of uptake indicating that these organic acids entered the brain and retinal microvessel segments by diffusion.

The uptake of L-[^3H]leucine was not affected by a 15-min preincubation of the isolated microvessels with 1 × 10^-4 M sodium azide and was only decreased 10% by preincubation with 1 × 10^-5 M ouabain (data not shown). This suggested that the uptake of L-[^3H]leucine was independent of a high energy and sodium requiring process.

Whereas sucrose uptake was unchanged when the incubation temperature was lowered from 37°C to 4°C, L-leucine uptake was markedly reduced (Fig. 2). The efflux of L-leucine from isolated rat microvessels is also temperature dependent (16). This indicated that the greater uptake of L-leucine was accounted for by a carrier-mediated transport process, whereas that of sucrose occurred by diffusion.

To further examine the specificity of L-leucine uptake, we incubated isolated microvessels with L-[^3H]leucine and various nonradioactive substances. As shown in Fig. 3, L-3,4-dihydroxyphenylalanine and L-valine, both of which are known to be transported in vivo by the neutral amino acid carrier system (17, 18), decreased L-[^3H]leucine uptake. On the other hand
dopamine and sucrose, which do not readily enter the brain from the blood, did not inhibit.

The best example of stereospecificity for the uptake of the large neutral amino acids by the isolated microvessels was shown when radiolabeled L- and D-valine were used as substrates. L-valine was taken up at a several-fold greater rate than D-valine (Fig. 4). The preferential entry of the L isomer of the naturally occurring amino acids into the brain has been shown previously (19–22).

As can be seen in Figs. 1–4 a significant amount of [3H]-labeled amino acid was taken up during the first 20 sec of incubation. This uptake was much greater than that of the diffusion marker sucrose but could be depressed to the same level as sucrose when the incubation temperature was reduced (Fig. 2). The amount of uptake at 20 sec could also be depressed by adding competitive inhibitors (Figs. 3 and 4). In the studies shown in Figs. 1–4 less than 5% of the radiolabel present in the isolated incubated microvessels was precipitable with trichloroacetic acid after 30 min of incubation.

To examine the stereospecificity of various substances in their ability to inhibit the uptake of L-[3H]tyrosine by isolated brain microvessels, 1-min incubations at 37° were employed to rule out any possibility of artifacts due to amino acid metabolism. After 1 min of incubation, 98% of the L-[3H]leucine taken up by the isolated microvessels cochromatographed as leucine. Those substances which are known to enter the brain from the blood via the large neutral amino acid carrier system—leucine, tyrosine, valine, and dihydroxyphenylalanine—were effective in decreasing L-[3H]tyrosine uptake (Table 1). Of these, the L-

FIG. 1. The uptake of L-[3H]leucine and [3H]sucrose by isolated bovine brain and retinal microvessels. The microvessel suspensions in plastic tubes were warmed to 37° before the addition of 10 μCi of [3H]sucrose. Aliquots were removed at 20 sec, 15 min, and 30 min, placed on nylon sieves attached to a suction device, and washed four times with 0.5 ml of ice-cold buffer. [3H]Sucrose points are the average of two retinal microvessel samples (0-0). [3H]Leucine uptake by brain (△) and retinal (○) microvessels represent mean ± SD. n = 4.

FIG. 2. The effect of 37° versus 4° incubation temperature on isolated retinal microvessel sucrose and leucine uptake. Suspensions of microvessels were incubated with 20 μCi of [3H]sucrose at 4° (●—●) and 37° (○—○) or L-[3H]leucine at 4° (●—●) and 37° (○—○) with unlabeled carrier to give incubation concentrations of 1 × 10−4 M.

isomers were more potent than the D isomers with the exception of the isomers of leucine which were equipotent. Glycine, which only slowly enters the brain, did not depress L-[3H]tyrosine uptake. A 15 min preincubation with 1.94 × 10−4 M ouabain depressed L-[3H]tyrosine uptake by only 10% suggesting that sodium was not required for tyrosine transport.

FIG. 3. Inhibition of isolated retinal microvessel L-[3H]leucine uptake by L-system substrates. Suspensions of microvessels were incubated with 10 μCi of L-[3H]leucine (○) or with L-[3H]leucine plus 1.2 × 10−4 M sucrose (△), dopamine (●), L-dihydroxyphenylalanine (□), or L-valine (△).
FIG. 4. The stereospecificity of valine uptake by isolated retinal microvessel suspensions. L-[3H]Valine (O-O) and D-[3H]valine (●-●) with their corresponding unlabeled isomers were incubated at concentrations of 1.1 × 10⁻⁴ M at 37°C.

The kinetics of amino acid uptake were studied using isolated microvessel suspensions and 1 min incubations. The apparent Kₘ values were 500 μM, 133 μM, and 111 μM for L-valine, L-leucine, and L-tyrosine, respectively. These values are similar to those previously obtained by the arterial injection technique for rat brain accumulation; 600 μM for valine (23) and 150 and 160 μM for leucine (23, 24). The amount of free neutral amino acids in the isolated microvessels, 80 nmol/2 mg of wet weight, was too small to interfere with kinetic measurements.

DISCUSSION

The barrier to the penetration of substances into the brain from the blood consists of at least two components: a barrier to the diffusion of molecules and an enzymatic barrier for the inactivation of endogenous neurotransmitters. From morphological studies, the barrier to the diffusion of molecules into the brain and retina from the blood is believed to reside at the level of the microvascular endothelium with its tight junctions (1, 4). Studies into the nature of the selective entry of substances into the brain have been reported using in vitro and in situ techniques (17–28). From such studies it is known that nonpolar substances rapidly enter the brain by diffusion whereas polar molecules do not enter except by selective carrier systems (6). These carrier systems have been defined by the molecules that compete for transport via each system. One such system has been termed the large neutral amino acid system (17, 18). To study the contributions of the microvessel wall to the selective transport of amino acids into the brain and retina, we have examined isolated microvessel preparations from the central nervous system for the presence of such carrier systems. These studies indicate that isolated microvessels from both the retina and brain can take up radiolabeled large neutral amino acids at rates far greater than that of the diffusion marker, sucrose. The large neutral amino acids that were used exhibited cross-competition and (with the exception of leucine) stereospecificity for the microvascular uptake system. In addition, the apparent Kₘ for uptake of leucine and valine were found to be similar to those obtained by the in situ injection technique for rat brain (23, 24). The uptake of amino acids by isolated microvessels in suspension is anatomically equivalent to the passage of substances from the brain into the blood whereas the in situ injection technique is from the blood into the brain. The finding that the apparent Kₘ values for the transport of large neutral amino acids are similar on both sides of the microvascular wall suggests that the transport mechanism is not asymmetric (25–28).

Some preliminary studies were performed to determine whether amino acids taken up by microvessels in vitro, as described in this paper, find their way into the lumen (16). [3H]Inulin, which is excluded from cells, was preincubated with microvessels at 4°C to preload the lumen. When the vessels were subsequently incubated at 37°C, the rate of efflux of inulin was very low, indicating that loss of solutes from the cut ends of the microvessel segments was not rapid. By contrast, the rate of efflux of similarly preloaded [3H]leucine was rapid and, therefore, indicative of a transport mechanism. Such a mechanism would require that the amino acids pass from the lumen through the microvessels into the medium. The apparent symmetry of the amino acid transport mechanism in the microvessels, which was alluded to above, gives us reason to believe that at least part of the amino acids taken up by microvessel segments in vitro passes into the lumen.

The observation that ouabain, an agent that inhibits Na⁺,K⁺-ATPases, decreases by only 10% the uptake of L-[3H]tyrosine and L-[3H]leucine is in agreement with reports that leucine transport in rat brain microvessels is not significantly decreased in sodium-deficient medium (29). The similarities between the sodium and energy independent large neutral amino acid carrier system and the l system proposed by Christensen et al. (30) have been noted (18).

The finding of relatively large amounts of aromatic L-amino acid decarboxylase (31, 32), monoamine oxidase (33, 34) and catechol-O-methyl transferase (33) in isolated intracerebral microvessels from various species indicates the presence of the enzymatic barrier to the infiltration of neurotransmitters from the blood into the brain in such preparations. This is consistent with the numerous reports of such a trapping mechanism for the amine precursors in intracerebral capillaries (35–38). Thus,

<table>
<thead>
<tr>
<th>Test substance</th>
<th>% inhibition</th>
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<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>L-Dopa</td>
<td>65</td>
</tr>
<tr>
<td>D-Dopa</td>
<td>2</td>
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<tr>
<td>L-Tyrosine</td>
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<tr>
<td>D-Tyrosine</td>
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<tr>
<td>L-Valine</td>
<td>67</td>
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<tr>
<td>D-Valine</td>
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<tr>
<td>Glycine</td>
<td>4</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>80</td>
</tr>
<tr>
<td>D-Leucine</td>
<td>86</td>
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<tr>
<td>Ouabain</td>
<td>9</td>
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</table>

Isolated brain microvessel suspensions were incubated for 1 min in a volume of 755 μl containing 10 μCi of L-[3H]tyrosine with the test substance listed. dopa, dihydroxyphenylalanine.
* Final concentration = 8.92 × 10⁻⁵ M.
† Preincubated for 15 min at 37°C at 1.94 × 10⁻⁴ M.
both of these components of the blood–brain barrier have now been shown to reside in the intracerebral microvessel wall. The use of these isolated microvessels provides an opportunity to study the transport of substances across the blood–brain barrier in vitro.

It is becoming more and more evident that brain microvessels are not mere conduits. They are involved in many functions other than the barrier and transport systems alluded to above. Catecholamines and their synthetic and degradative systems are present in isolated brain microvessels (31, 34). The isolated microvessels have also been found to be highly enriched with respect to histamine (39), angiotensin converting enzyme (40–42), and angiotensinase-A (40). Whether these substances or mechanisms have only a local or a more systemic physiological role is not known. Isolated microvessel preparations provide a useful method for the study of such questions.