Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection

WILLIAM H. OLDENDORF
Research Service, Wadsworth Hospital, Veterans Administration, Los Angeles, California 90073, and
Department of Neurology, UCLA School of Medicine, Los Angeles, California 90024

Oldendorf, William H. Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. Am. J. Physiol. 221(6): 1629–1639. 1971.—The loss of a 14C-labeled test substance to brain during a single capillary passage following rapid injection into the rat common carotid artery was measured relative to a simultaneously injected highly diffusible reference, 3HOH. Twenty-eight amino acids, thirteen amines, seven hexoses, and 5 relatively nondiffusible substances were studied. Amino acid uptake ranges from unmeasurably low to 55%. Essential nutritional amino acid uptake was greater than nonessential. Two blood-brain barrier (BBB) carrier systems for amino acids were identified. Putative transmitter substances were much less taken up by brain than precursors. Saturability of d-glucose uptake was demonstrated and evidence presented that all of the five hexoses measurably taken up by brain shared a common carrier. Nonmetabolized cycloleucine and 3-O-methylglucose showed saturable uptakes. Amino acid uptake was incompletely stereospecific whereas glucose uptake was stereospecific. Phlorizin inhibits brain uptake of d-glucose. Relative BBB permeabilities to many of the test substances resemble red-cell permeabilities. Carrier systems for amino acids are independent of the glucose carrier.

Blood-brain barrier; tritiated water; amino acid transport; essential amino acids; beta-phenethylamine derivatives; biogenic amines; neurotransmitters; hexose transport; phlorizin

Most blood plasma solutes exchange slowly with brain due to the impermeability of brain capillaries. Certain classes of substances, however, exhibit sufficient blood-brain barrier (BBB) penetration that they come into a measurable partial equilibrium with brain extravascular compartments during a single passage through brain microcirculation. Substances soluble in the lipoprotein of cell membranes, such as ethanol, come into nearly complete equilibrium and largely leave the blood during a single microcirculatory pass (12). A few lipid-insoluble substances, such as glucose (11) and some amino acids (30), also pass the BBB freely when present in blood in low concentrations. It is the characterization of BBB permeability to substances with relatively high uptake rates to which this series of brain uptake studies is directed.

Previous radioisotopic studies of BBB permeability have employed a radiolabeled test substance introduced intra-peritoneally or intravenously. Some minutes to hours later the brain concentration is measured and related either to the terminal blood plasma concentration or the injected dose. A major problem is the lack of a suitable reference with which to compare brain uptake. Ideally, a constant blood concentration is maintained during the equilibration period. Systemic administration with prolonged presence of the test substance in the body commonly results in metabolism of the tracer either before or after entering brain. The novel methodology used in the present investigations (29) circumvents most of the disadvantages of methods requiring systemic administration.

Results presented are of the measurements of brain uptake of a number of radiolabeled substances during their first microcirculatory passage after rapid injection into rat common carotid artery. The test substance, 14C-labeled, is mixed with tritiated water (3HOH) and injected. The rat is decapitated 15 sec later. The amount of test substance remaining in brain at that time is expressed as a percentage of the 3HOH present (29). A large number of substances related to brain metabolism have been examined in this laboratory by this method. These include amino acids, biogenic amines, hexoses, and metabolically inert polar compounds of various molecular weights.

Previous studies from other laboratories have described brain uptake of a great variety of test substances after systemic administration. Various routes of isotope administration and calculation have been used. The present study compares the brain uptake of 28 common amino acids, 13 common biogenic amines, and seven hexoses using a single method, and defines the saturation characteristics of the BBB penetration by some of these substances.

Method

The general methodology has been described elsewhere (29) and is summarized in part here. A mixture is prepared containing 0.1–0.5 μC of a 14C-labeled test substance and 1–2 μC of 3HOH. Isotopes were obtained from New England Nuclear Corp., Billerica, Mass. or Amersham/Searle, Arlington Heights, Ill. A diluent is used to make up a total volume of 0.2-ml solution. This diluent is either Elliot's B electrolyte mixture (14) (Baxter Laboratories, Morton Grove, Ill.) or Ringer solution (Na+ 147, K+ 4, Ca++ 4, Cl− 155 mEq/liter) buffered to pH 7.56 with 4-mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer (Calbiochem, La Jolla, Calif.). Some studies utilize normal rat serum as the diluent. In competition studies the injected solution also contains unlabeled substances which are included to...
measure their ability to inhibit brain uptake of the $^{14}$C-labeled test substance. Wistar rats, 275–350 g, of either sex and on routine laboratory diet are rendered unresponsive with intraperitoneal pentobarbital. The right common carotid artery is surgically exposed and cannulated using a sharp 27-gauge (0.38-mm od) needle. This needle does not occlude the vessel, and free arterial flow past the needle persists throughout the procedure. The animal is positioned in a guillotine (Harvard Apparatus Co., Inc., Dover, Mass.) prior to arterial puncture. The needle is left in the artery after injection since excessive bleeding results if the needle is withdrawn. The temperature of the injected solution is 22–25°C.

The injection of 0.2-ml solution is completed within approximately 0.25 sec. The artery becomes clear during the injection. The rat is decapitated 15 sec after injection. The $^{3}$H in the injected mixture which enters the brain distributes in the course of one capillary passage to a rapidly exchangeable water space which is much larger than the capillary water space. Accordingly, most of the $^{3}$H leaves the brain capillaries (41). The amount of $^{3}$H entering brain tissue is thus flow-dependent and defines the amount of the injected bolus which passed through the piece of tissue examined. Some fraction of the $^{14}$C-labeled test substance also leaves the blood and enters brain tissue. The remaining fraction not taken up by brain during the single passage, is carried on out of the brain blood compartment before decapitation takes place at 15 sec after injection (Fig. 1).

The ratio of $^{14}$C to $^{3}$H remaining in the brain tissue ipsilateral to the injection and rostral to the midbrain is determined by liquid scintillation counting. This tissue is divided into two (approximately 0.25 g) specimens by extruding the brain through a 20-gauge needle into a tissue solubilizer. An aliquot of the injected mixture is similarly counted.

The $^{14}$C-to-$^{3}$H ratio in the tissue is divided by the same ratio in the injected mixture and the result is multiplied by 100 to provide the uptake of the test substance as a percentage of the water uptake under these circumstances of injection. This brain uptake index (BUI) is thus calculated:

$$BUI = \frac{\text{tissue}^{14}\text{C}/\text{tissue}^{3}\text{H}}{\text{mix}^{14}\text{C}/\text{mix}^{3}\text{H}} \times 100$$

RESULTS

Inert Polar Substances

The neutral polar substances urea, mannitol, sucrose, inulin, and 60,000 mol wt dextran were studied. It is assumed these tracers exhibit a negligible penetration of the BBB during a single capillary passage and establish a background level for the method with which other test substances can be compared. The apparent BUI for these inert sub-
BRAIN UPTAKE OF AMINO ACIDS, AMINES, AND HEXOSES

stances is 1.4-2.3 (Table 1). The BUI values for the test substances to be presented have not been corrected for this background level.

Amino Acids

The amino acids studied are indicated in Table 2. The concentration of amino acid injected was only that present in the labeled material obtained from the manufacturer and ranged from 0.002 to 0.76 mM. Ideally the injected concentrations would have been equalized by dilution but this was impractical because of the wide range of specific activities. In addition to the nutritional amino acids, several precursors of central transmitter substances were included as well as thyroxine and nonmetabolites cycloleucine and α-aminoisobutyric acid (AAIBA). Unless otherwise indicated, all labeled and unlabeled amino acids are L-enantiomorphs. Only racemic 5-hydroxytryptophan-14C of relatively low specific activity was commercially available and the injected concentration was accordingly greater than for most of the amino acids studied. Each mean value represents at least three animals. All amino acids were injected mixed with Elliott’s solution with the exception of 3,4-dihydroxyphenylalanine (Dopa), cycloleucine, and AAIBA which were injected in Ringer solution buffered to pH 7.56.

Serum competition. The nutritional amino acids shown in Table 2 were also studied using normal rat serum to make up 90% of the injected solution rather than Elliott’s B solution (Fig. 2). The latter solution has no organic constituent other than 5-mM D-glucose. Serum was obtained from arterial blood collected following decapitation of pentobarbital anesthetized Wistar rats.

The brain uptake of 14C amino acids in the presence of serum should be more representative of the rate of blood-brain exchange in the intact animal than that observed using the inorganic diluent. A reduction in BUI using a test substance in serum would suggest either that the tracer is partially bound in a nondialyzable form to serum proteins or that substances in serum are competing with the tracer for carrier-mediated transport sites in the BBB.

The effects of using serum as a diluent are shown in Fig. 2. All of the amino acids except proline, aspartate, glutamate, and glycine show reductions to 20-50% of the BUI observed with inorganic diluent. The uptakes of proline, aspartate, glutamate, and glycine are below the measurable limits of the method using either diluent.

Amino acid cross-inhibition. The unlabeled amino acids (L-enantiomorphs) in the left column of Table 3 were injected at a 4-mM concentration to measure their ability to inhibit brain uptake of the seven 14C-labeled species indicated at the head of the vertical columns. The values in Table 3 indicate the BUI of the mixture of unlabeled and 14C-labeled amino acid as a percentage of the mean BUI of that labeled amino acid with no unlabeled acid added. Each value represents one animal. Those values with asterisks indicate a competitive BUI more than 5 SD below the mean BUI of the test substance alone. The BUI of cycloleucine-14C was studied only in the presence of 4-mM unlabeled phenylalanine and 4-mM unlabeled arginine.

The unlabeled basic amino acids arginine, lysine, and histidine inhibited arginine-14C uptake, but not the uptake of the other labeled amino acids tested. Conversely all of the other amino acids which exhibited a significant uptake inhibited each other, but not the basic acids.

Methionine self-inhibition. Methionine self-inhibition was studied in detail by adding unlabeled L-methionine to the injected solutions. Concentrations of injected methionine ranged from 0.015 mM (no unlabeled methionine added) to 40 mM (Fig. 3). Inhibition of L-methionine-35S uptake was also studied by adding unlabeled D-methionine in concentrations up to 10 mM (Fig. 3) to assess stereospecificity of brain uptake. Unlabeled D-methionine is about one-half as effective as unlabeled L-methionine as an inhibitor of L-methionine-35S uptake. The L-methionine concentration which reduces the L-methionine-35S BUI by one-half ($K_i$) is approximately 0.5 mM and for D-methionine about 1 mM.

In addition to studies using the Elliott’s B solution a series of self-inhibition studies were conducted using normal rat serum to make up 90% of the volume of the injected solu-

![FIG. 2](http://ajplegacy.physiology.org.by/10.1152/ajplegacy.physiology.org.by) Effect of using rat serum as a diluent (dark bars). All amino acids having a measurable uptake using an inorganic diluent (light bars) show a reduction in presence of rat serum. This reduction could be due to plasma protein binding of amino acids or to competition from serum amino acids. For methionine, this reduction in presence of serum probably is due to serum amino acids (see text).
Using serum as a diluent was, in effect, adding approximately 1 mM unlabeled methionine to the injected mixture.

### Amines

Thirteen amines were studied. Included in this group is β-phenylethylamine because it is structurally related to several of the purative central neural transmitter substances and many sympathomimetic drugs. The choice of the other amines was based, in part, on an attempt at establishing the effect on BBB penetration of substituting various prosthetic groups on the parent molecule, β-phenylethylamine.

Not all amines of potential interest were available from commercial sources. The 14C-test substances were diluted when delivered from the manufacturer with a small amount of Ringer solution and kept frozen at −20°C until a few minutes before use. Immediately after thawing, this solution was diluted to a concentration of approximately 0.5 μC/0.2 ml in buffered Ringer solution.

The BUI of the 13 amines studied are shown in Table 4. Most are near the lower limit of measurability. Acetylcholine is included and also exhibits a barely measurable uptake.

### Hexoses

#### Uptake at low injected concentrations. The hexoses shown in Table 5 were injected with no added unlabeled substance.

### Table 3. Brain uptake index of radiolabeled amino acids studied in presence of twenty 4 mM cold amino acids listed in left column and results expressed as % of mean unoccupied 14C amino acid

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>4-Methyl Cold</th>
<th>Uptake of L-methionine-33S</th>
<th>Uptake of L-methionine-33S</th>
<th>Uptake of L-methionine-33S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>12*</td>
<td>9*</td>
<td>12*</td>
<td>10*</td>
</tr>
</tbody>
</table>
| Leucine | 13* | 10* | 15* | 12* | 26* | 103 | 24* | 59
| Tyrosine | 12* | 15* | 21* | 21* | 49* | 45* | 106 | 122
| Isoleucine | 23* | 17* | 33* | 20* | 29* | 80 | 40* | 58
| Methionine | 16* | 18* | 19* | 18* | 36* | 91 | 63* | 60
| Tryptophane | 13* | 8* | 16* | 12* | 19* | 92 | 38* | 79
| Histidine | 18* | 14* | 18* | 18* | 37* | 55 | 57* | 48
| Dopa | 26* | 14* | 19* | 13* | 21* | 99 | 51* | 59
| Arginine | 94 | 82 | 103 | 100 | 96 | 15* | 110 | 90
| Valine | 35* | 23* | 46* | 32* | 43* | 101 | 52* | 102
| Cysteine | 95 | 85 | 89 | 101 | 129 | 15* | 108 | 94
| Lysine | 115 | 91 | 109 | 83 | 90 | 14* | 97 | 64
| Threonine | 78 | 61* | 69* | 60* | 82* | 51* | 61
| Serine | 110 | 91 | 82 | 107 | 79 | 71 | 53* | 57
| Alanine | 79 | 72 | 92 | 77 | 91 | 81 | 76* | 75
| Glycine | 106 | 96 | 91 | 85 | 98 | 114 | 100 | 73
| Aspartic Acid | 102 | 97 | 119 | 91 | 111 | 89 | 91* | 116

*P > 0.05 as to below unoccupied 14C labeled amino acids. Each percent represents one rat except the two values for cycloleucine-44C, in which n = 3 for each mean value. The 4 mM cold amino acid represents the unlabeled or nonradioactive arm of the study.

### Table 4. Uptake of amines, 14C-labeled

<table>
<thead>
<tr>
<th>Amines</th>
<th>Injected Concentration, μM</th>
<th>Brain Uptake Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-HOH reference</td>
<td>436</td>
<td>67 ± 5</td>
</tr>
<tr>
<td>β-Phenylethylamine</td>
<td>243</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>p-Methoxyphenethylamine</td>
<td>110</td>
<td>12.5 ± 3.2</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>134</td>
<td>11.7 ± 1.3</td>
</tr>
<tr>
<td>3,4-Dimethoxyphenethylamine</td>
<td>030</td>
<td>7.62 ± 0.39</td>
</tr>
<tr>
<td>Glutamine</td>
<td>276</td>
<td>5.6 ± 1.4</td>
</tr>
<tr>
<td>Mescaline</td>
<td>022</td>
<td>4.49 ± 1.3</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>023</td>
<td>4.5 ± 0.9</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>023</td>
<td>3.85 ± 0.38</td>
</tr>
<tr>
<td>Dopamine</td>
<td>023</td>
<td>3.85 ± 0.38</td>
</tr>
<tr>
<td>Tyramine</td>
<td>029</td>
<td>3.07 ± 0.48</td>
</tr>
<tr>
<td>5-Hydroxytryptamine</td>
<td>022</td>
<td>2.63 ± 1.0</td>
</tr>
<tr>
<td>dl-Epinephrine</td>
<td>034</td>
<td>2.38 ± 0.11</td>
</tr>
<tr>
<td>Histamine</td>
<td>023</td>
<td>1.61 ± 0.36</td>
</tr>
</tbody>
</table>

Brain uptake index values are means ± sd. For each mean value n = 3.

### Table 5. Brain uptake of 14C-labeled hexoses

<table>
<thead>
<tr>
<th>Hexoses</th>
<th>Injected Concentration, μM</th>
<th>Brain Uptake Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-HOH reference</td>
<td>42</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>p-Glucose</td>
<td>42</td>
<td>33 ± 3</td>
</tr>
<tr>
<td>3-O-methyl-p-glucose</td>
<td>06</td>
<td>29 ± 2.3</td>
</tr>
<tr>
<td>n-Mannose</td>
<td>05</td>
<td>21 ± 1.3</td>
</tr>
<tr>
<td>N-Butyrolactone</td>
<td>21</td>
<td>14.4 ± 1.6</td>
</tr>
<tr>
<td>N-Acetylcarnitine</td>
<td>43</td>
<td>1.75 ± 0.32</td>
</tr>
<tr>
<td>L-Glucose</td>
<td>42</td>
<td>1.63 ± 0.46</td>
</tr>
</tbody>
</table>

Brain uptake index values are means ± sd. For each mean value n = 3.
BRAIN UPTAKE OF AMINO ACIDS, AMINES, AND HEXOSES

35. I - T 40 i0 mM COLD GLUCOSE

FIG. 4. Self-inhibition of brain D-glucose-14C uptake demonstrated by simultaneous injection of unlabeled (cold) D-glucose in progressively greater concentrations.

TABLE 6. Brain uptake D-glucose-14C versus unlabeled hexoses and amino acids

<table>
<thead>
<tr>
<th>Hexose</th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
<th>40 min</th>
<th>80 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Deoxy-D-glucose</td>
<td>21.9 ±3.5</td>
<td>14.9 ±2.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>22.5 ±1.3</td>
<td>20.4 ±3.7</td>
<td>16.4 ±0.3</td>
<td>11.0 ±1.9</td>
<td>4.4 ±1.2</td>
</tr>
<tr>
<td>3-O-methyl-D-glucose</td>
<td>19.7 ±1.1</td>
<td>17.6 ±1.6</td>
<td>13.9 ±1.1</td>
<td>15.0 ±1.1</td>
<td>11.2 ±1.4</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>23.2 ±2.2</td>
<td>19.4 ±0.9</td>
<td>24.7 ±1.7</td>
<td>19.8 ±1.7</td>
<td>12.6 ±2.4</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>32.0 ±1.7</td>
<td>34.6 ±3.5</td>
<td>22.5 ±3.3</td>
<td>28.7 ±3.3</td>
<td>26.2 ±2.4</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>30.1 ±1.4</td>
<td>32.7 ±3.2</td>
<td>25.7 ±3.2</td>
<td>31.8 ±3.2</td>
<td>26.8 ±2.4</td>
</tr>
<tr>
<td>L-Glucose</td>
<td>37.2 ±1.3</td>
<td>32.7 ±1.3</td>
<td>13.9 ±1.7</td>
<td>13.8 ±1.7</td>
<td>26.8 ±2.8</td>
</tr>
<tr>
<td>Alpha D-glucose</td>
<td>29.4 ±2.4</td>
<td>32.2 ±1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta D-glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Arginine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The uptake of D-glucose-14C observed with the inclusion in the injected solution of various concentrations of unlabeled hexoses, arginine, and phenylalanine. The uptakes of 14C-labeled hexoses at low injected concentrations are shown in Table 5. For each mean value n = 3.

The injected hexose concentration was only that present in the radiolabeled material obtained from the manufacturer. The largest uptake was found with 2-deoxy-D-glucose-14C and no measurable uptake was found with D-fructose-14C or L-glucose-14C. An appreciable BUI (30.2) was found for nonmetabolizable 3-O-methyl-D-glucose-14C.

Self-inhibition of D-glucose-14C uptake. Using D-glucose-14C as a test substance, unlabeled D-glucose was added to the injected solution at concentrations up to 80 mM (Fig. 4). No correction for osmolarity was made except with the 80-mM glucose concentration which was diluted with distilled water so that the total injected osmolarity was 300 mOsm.

When injected without added unlabeled D-glucose the BUI of D-glucose-14C was 32.6. Raising the injected D-glucose concentration to approximately physiological levels of 5 mM reduced the BUI to 22.5. The BUI in the presence of 80-mM cold D-glucose was 9.

The anomeric forms of the unlabeled D-glucose and the D-glucose-14C used in this part of the study were unstated by the manufacturers. Unlabeled D-glucose of known anomeric form (Calbiochem) were studied as competitors of D-glucose-14C uptake. Two groups of animals were injected with D-glucose-14C with 20-mM cold a-D-glucose (three animals) and cold beta-D-glucose (three animals). Both of these cold D-glucose competitive mixtures were injected within 15 min of dissolving to minimize spontaneous mutarotation to the racemic alpha-beta form. This study was undertaken to assess any difference in BBB carrier affinity for these anomers of D-glucose. No significant difference was noted in the ability of these two anomers to inhibit D-glucose uptake (Table 6).
Cross-inhibition of D-glucose-¹⁴C uptake. The hexoses in Table 5 were included in nonradioactive form in the injected solution of D-glucose-¹⁴C at concentrations of 5, 10, 20, or 40 mM (Table 6). There is a good correlation between the degree of suppression of D-glucose-¹⁴C uptake by these unlabeled substances and their uptake when injected radioactively. Those hexoses (such as 2-deoxy-D-glucose) which have a high BUI are effective at relatively low concentrations at inhibiting the uptake of D-glucose-¹⁴C. Figure 5 is a plot of the BUI of each of these radiolabeled test substances, shown in Table 5, vs. 1/Km for that substance when injected as an unlabeled compound. Km in this instance is the millimolar concentration required to reduce the BUI of D-glucose-¹⁴C to one-half its uninhibited BUI of 33. These values for liver were estimated by interpolation of the scattered BUI values at various concentrations shown in Table 2.

Unlabeled L-arginine (10 mM) and 20-mM unlabeled L-phenylalanine were also competed against D-glucose-¹⁴C using three animals for each compound and no inhibition was noted (Table 6).

Phlorizin cross-inhibition. Because phlorizin (4,6-dihydroxy-2-(β-D-glucoside)-β-(p-hydroxyphenyl)propiophenone) competes for glucose carrier-transport sites in intestinal wall (10), renal tubule (13), and red blood cell (26), it was considered of interest to assess its effect on BBB transport of glucose. Injected phlorizin concentrations ranged from 0.3 mM to 10 mM as shown on Fig. 6. The Km of phlorizin is about 0.3 mM. This same degree of inhibition is achieved with 20 mM unlabeled D-glucose.

To establish that phlorizin inhibition of D-glucose-¹⁴C uptake was due to interference with transport and not with subsequent glucose metabolism, in three animals 1-mM cold phlorizin was competed against 3-O-methyl-D-glucose-¹⁴C. The same suppression (BUI 14.2) was found as with D-glucose-¹⁴C (Fig. 6).

Discussion

Methodology

The method employed here to measure blood-brain exchange rates uses 3HOH as a reference with which the ¹⁴C-labeled test substance is compared. Although many highly diffusible reference substances might have been used, 3HOH was chosen because its radiochemical purity cannot deteriorate, simultaneous counting of ³H and ¹⁴C is routine, its rapid passage through BBB is well established (41), and it is inexpensive. The use of 3HOH facilitates the high radioactivity compared to ³H to ¹⁴C desirable in double-isotope counting. Radiolabeled embolic microspheres could have been substituted for the ³HOH but this was abandoned because of the cost, the complexity of simultaneous counting, nonuniformity of suspension, and partial interruption of blood flow. Nevertheless, for some studies, embolic microspheres may be useful since they will not wash out.

Using a ratio of ratios between two easily counted isotopes obviates the need to weigh tissue specimens or injected solutions. The method corrects for regional differences of blood flow.

Direct rapid arterial injection with collection of tissue 15 sec later largely excludes the possibility of chemical alteration of the labeled test substance prior to blood-brain exchange or the loss from brain tissue of products of metabolism of the test substance after exchange. This assures that BBB exchange results are those of the injected substance.

The high rate of injection and consequent minimal mixing of the injected bolus with blood allows the modification of the composition of the injected mixture to include known concentrations of potentially competitive unlabeled substances with assurance that the solution arrives in the brain microcirculation with a composition not greatly differing from that injected.

The minimal mixing with blood prevents removal from free solution by binding of the test substance to plasma proteins. It also prevents competition with the test substance for BBB transport-carrier sites by substances in free solution in blood plasma. Since very little of the injected test substance remains in brain blood, a minimal correction for residual blood content is required.

Carotid-arterial injection delivers a large fraction of the test substance to brain relative to that delivered to brain after intravenous injection. This ability to deliver a large proportion of test substance into brain at a precise time in known chemical form should provide an efficient means of following the subsequent metabolic fate of the radiolabel in brain.

Elliott's B solution was used because its electrolyte composition approximates blood plasma. The pH of this solution varied from 7.5 to 8.5, probably due to escape of CO₂. To obtain a more constant pH, the diluent was changed in later studies to Ringer solution buffered to pH 7.56 with 1-mM HEPES, Good's buffer (17). Duplicate studies of phenylalanine and tyrosine uptake revealed no significant differences in BUI using these two diluents.

The temperature of the injected solution in these studies was 22–25 °C (room temperature). At the microcirculatory site of exchange with tissue the temperature presumably was intermediate between this and brain temperature. To measure the effect of temperature of the injected solution on D-glucose-¹⁴C uptake, three groups of three rats each were studied at 2, 20, and 37 °C. The BUI of these groups were 30.4, 31.9, and 30.7, respectively, indicating independence of BUI and injection temperatures.

The penetration of cell membranes by solutes not having affinity for specific transport-carrier sites is largely a function of the relative affinity of the test substance for water and the protoclipid of the cell membrane. This could be determined by an in vitro oil-water partition coefficient measurement. The in vivo carotid-injection method employed here might be thought of as a form of partition coefficient study. This in vivo method is simpler to perform and is free of many artifacts inherent in in vitro partition coefficient studies where lipids not representative of cell membrane protoclipids and carrier transport systems are used in an artificial environment.

The present method may usefully be applied in the study of any substance which exhibits a BUI more than about 1% above the 1.5–2% background level.

Metabolically Inert Polar Substances

The BUI for these substances, which presumably undergo an unmeasurably small exchange with brain during their
single passage through brain capillaries, probably represents test substance not completely washed out of the brain blood compartment, some recirculation of test substance and uptake of tracer by regions not having a blood-brain barrier, such as in hypothalamus and choroid plexus, but which are included in the brain tissue analysis. Some of this residual may represent test substance adhering to the inner surface of capillary endothelium.

The BUI of these inert substances is 1.4–2.3 and this could be considered the base line or background level with which all test substances might be compared. The BUI values presented here have not been corrected for this residual but such a correction probably would offer a more precise measurement of the test substance which actually passed through the BBB.

Amino Acid Uptake

A considerable range of BUI is observed. When arranged in descending order of uptake (Fig. 7) it was noted that those amino acids nutritionally essential to the rat enter brain more freely than nonessentials (30). Although tyrosine is considered nonessential to the rat (34) it enters brain freely. The high uptake of tyrosine noted in this study is commensurate with previous brain uptake studies of systematically administered tyrosine (9). Since phenylalanine hydroxylase is not present in brain (19), tyrosine presumably must enter through BBB (34) and is thus essential in the sense that it probably cannot be synthesized by brain. The individual amino acid uptakes are unrelated to molecular size or other obvious physical or chemical parameters.

It has been suggested that the low BUI of several of the nutritional amino acids may be correlated with the ability of brain to synthesize them from a glucose substrate (34).

The free entry of many radiolabeled amino acids into brain after systemic administration has been reported (2, 24). Using that route of administration, equilibration half-times between brain and plasma of some minutes have been calculated (23). The present work suggests the rate of equilibration is much faster than this for many amino acids, particularly the essential amino acids, since equilibrations up to about one-half that of $^4$HOH are achieved in the 1–9 sec most of the test substance remains in the brain micro circulation.

The low BUI of $\gamma$-aminobutyric acid (GABA), glutamate, aspartate, and glycine may be related to their putative roles as central transmitter substances since the BBB permeability to all transmitter substances probably is low.

The appreciable uptake of thyroxine is compatible with the observation that cerebrospinal fluid thyroxine levels approximate plasma levels of nonprotein bound thyroxine (20). Although nearly all plasma thyroxine is bound, the small unbound fraction evidently freely equilibrates across BBB. Because the labeled thyroxine in the rapidly injected bolus is largely isolated from plasma protein, the BUI found here probably is representative of the rate of equilibration of the free plasma fraction.

Of the two nonmetabolized amino acids studied, cycloleucine passes the BBB as freely as some of the nutritionally essential amino acids but $\Delta15$IA does not. This suggests that subsequent metabolism of the test substance is not essential to its free BBB penetration.

The unequal uptake of L- and D-enantiomers of tyrosine (Table 2) and the unequal inhibitory effects of unlabeled L- and D-methionine on methionine-35S uptake (Fig. 3) indicate an incomplete stereospecificity of their BBB transport. A similar incomplete stereospecificity of brain uptake of phenylalanine, leucine, and lysine has been demonstrated (25) after intraperitoneal injection. Clearly the L enantiomers have a greater affinity for the BBB amino acid carrier system, but the D enantiomers exhibit an appreciable, although reduced, affinity. The stereospecificity shown for these two amino acids is much less complete than shown for D- and L-glucose using the same technique. There is very efficient uptake of D-glucose, but no measurable uptake of the L-enantiomorph.

The relative uptakes of the various amino acids are quite unlike the relative activity of transport by the inverted hamster small intestine. Proline, threonine, alanine, and glycine are most actively transported by the intestine (40), whereas in the present study they exhibit unmeasurably low brain uptakes.

The relative uptakes found here are also quite different from the uptake by tissue slices in a medium containing radiolabeled amino acids. Glycine, glutamic, alanine, and proline, for example, are very actively concentrated by brain tissue when exposed in this manner (25) but have essentially no uptake by the present method. When relating the present studies of brain uptake of various labeled substances, caution must be exercised to exclude previous studies in which brain tissue has been exposed directly to the test substance either in vivo by ventricular injection or in vitro with introduction into tissue slice supportive media. In these instances the test substance can diffuse directly into the extracellular fluid environment of the nervous system cells and need not traverse the restrictive walls of brain capillaries as in the present studies. The BUI, as determined by the presently employed carotid-injection method, probably represents a product of at least two factors such as freedom of BBB penetration and the distribution space of the test substance in brain once BBB has been penetrated. The latter factor probably corre-
lates with direct tissue uptake by tissue slices or by intact brain after ventricular injection.

The relative rates of uptake observed here approximate those observed for red blood cells in vitro (39).

**Serum competition.** The lower BUI observed for most of the amino acids using a serum vehicle suggests either removal of the test substance from free solution by plasma protein binding or competition for BBB carrier sites by the amino acids normally present in rat serum. Using dialyzed serum, the restoration of the methionine BUI using a serum diluent to level seen with Elliott’s solution suggests plasma protein binding (at least for this amino acid) is not an important factor in the reduced methionine uptake observed using a serum diluent. The observation that dialyzed serum used as a diluent results in nearly the same uptake as with an inorganic diluent suggests that the reduction of methionine BUI in the presence of serum is due to the presence of some dialyzable solute competitive with the methionine-14C for BBB transport; probably amino acids competitive with methionine.

The marked BUI reduction for all of the amino acids when using a serum diluent suggests that the BBB carrier system for many of the amino acids is near saturation in the presence of normal serum levels of amino acids.

The reduction of exchange rates noted in these studies in the presence of serum may explain some of the difference in exchange rates noted when labeled amino acids are administered systemically (23) as against rapid arterial injection of solution containing essentially no unlabeled amino acids reported here. When administered systemically the radiolabeled amino acid species must compete with serum amino acids for BBB carrier sites. When administered rapidly intrarterially there probably is no such competition during the brief period of exchange and the radio labeled species has relatively free access to these carrier sites.

Because of a lack of competition with serum solutes for BBB carrier sites or because of combination with serum proteins, rapid arterial injection probably will result in an exaggerated uptake of many test substances relative to that measured after systemic administration.

**Cross-inhibition studies.** To study the ability of each of the nutritional amino acids to cross-inhibit each of the others would require a prohibitive number of experiments. To restrict this number, the seven labeled amino acids of Table 3 were chosen from various levels down the list of diminishing uptake (Table 2).

The reduction of the radiolabeled amino acid BUI to more than five sd below the uncorrected mean amino acid BUI has a significance of \( P < .001 \). Using this criterion, significant cross-inhibition occurs between all of the high BUI amino acids studied (more than 10% BUI in Table 2) with the exception of arginine, lysine, and histidine which do not cross-inhibit with the others. Unlabeled arginine, lysine, and histidine all inhibit arginine uptake. Unlabeled tyrosine inhibits arginine uptake to a slight but significant degree.

Cycloleucine evidently enters brain via the same carrier mechanism as phenylalanine since 4-methyl phenylalanine inhibits its uptake whereas 4-methyl arginine does not.

Amino acids which have a high BUI, such as phenylalanine, presumably have a greater affinity for their BBB carrier system than those exhibiting a low BUI such as alanine.

This is consistent with the data in Table 3 indicating that, in general, high BUI amino acids are effective cross-inhibitors, but low BUI amino acids are not. Because the uptake of the amino acids toward the lower end of Table 2 is so low and the sd so large, a five sd reduction is unlikely. Thus, the amino acids near the lower end of Table 2 are not accessible to this mode of analysis. Separate carrier systems may facilitate their minimal passage through BBB.

**Methionine self-inhibition.** In the presence of increasing concentrations of unlabeled methionine the methionine BUI falls rapidly until the injected methionine reaches about 2 \( \mu \)M and then falls very gradually at higher concentrations. At 40-\( \mu \)M injected methionine concentration the BUI is 3.26; an elevation about the BUI of urea and mannitol of doubtful significance. These data (Fig. 3) suggest methionine exchange through BBB is, within the limits of the present method, completely saturable.

**Amines**

The putative transmitter substances dopamine, norepinephrine, epinephrine, 5-hydroxytryptamine, and histamine all exhibit very low uptakes. This is in agreement with the very low uptakes by brain after systemic administration described for norepinephrine (37), dopamine (18), 5-hydroxytryptamine (3, 36), and histamine (35). The relatively high uptake (BUI 12.5) of tryptamine is in general agreement with Green and Sawyer (18), although precise comparison with the present study is impossible because the methodologies employed are so different.

Beta-phenethylamine has the greatest uptake of all of the amines studied. Substituting one methoxy group in the para position has a minimal effect on BBB penetration (Fig. 8). Adding methoxy groups in the 3 and 4 positions and 3, 4, 5 positions results in a progressive reduction of brain uptake. Adding hydroxyl groups in 3 or 4 positions reduces the uptake to a level barely measurable by this...
Decarboxylating an amino acid results in a marked reduction of uptake for the resulting amine in the case of tyrosine, Dopas to dopamine, 5-hydroxytryptophane to 5-hydroxytryptamine, histidine to histamine, and tryptophane to tryptamine. Gamma amidation of glutamate results in a greater uptake for glutamine.

To assess whether or not dopamine uptake is significantly above that of neutral polar test substances of similar molecular weight it was compared with mannitol (mol wt 180) as a test substance. The dopamine uptake is greater (3.85) than mannitol (1.94) with \( P < .001 \) indicating that it is above the lower level of measurability.

The putative transmitter precursors 5-hydroxytryptophane, Dopas, and histidine all show substantially higher rates of uptake when studied with the same technique. These findings, together with the observations of low uptakes of GABA, glycine, aspartate, and glutamate are consistent with the generalization that precursors of central nervous system transmitters have high rates of blood-brain exchange whereas as transmitter substances have low rates. If this generalization is correct the search for currently unrecognized transmitters might be assisted by concentrating research efforts on low uptake test substances as measured by the present technique.

The very restricted BBB penetration of all putative transmitters studied here suggests a function of BBB may be to conserve and localize brain extracellular transmitter concentrations. The BBB ordinarily is considered to be a mechanism for excluding plasma solutes from brain extracellular fluid but restriction of passage from brain to plasma may also be a major aspect of BBB teleology.

**Hexoses**

Hexose-uptake at low injected concentrations. The uptakes of the hexoses in Table 5 are in general agreement with previous studies of BBB penetration using a variety of methodologies (1, 3, 7, 22, 27, 31). The particularly high 2-deoxy-D-glucose uptake is in agreement with Fishman's (15) observation that this substance equilibrates more rapidly between blood and CSF than does D-glucose. A very low rate of BBB penetration by D-fructose was also noted by Cron (11) and by Fishman (15).

The complete stereospecificity of the uptake of D- and L-glucose is quite different from the incomplete stereospecificity for L- and D-tyrosine uptake and L- and D-methionine reported above.

There probably is no metabolism of 3-O-methyl-D-glucose in brain or elsewhere (4, 28), yet this substance freely penetrates BBB. This suggests subsequent metabolism by brain is not a requirement of carrier-mediated BBB transport. This observation parallels the observation that cyclulocaine, a nonmetabolized amino acid, also freely penetrates BBB by a saturable mechanism. The 2-deoxy-D-glucose is partially metabolizable since it can be phosphorylated to 2-deoxy-D-glucose-6-phosphate (21), whereas 3-O-methyl-D-glucose is completely nonmetabolizable. (28) Thus, the independence of metabolizability and BBB penetration is more clearly defined with 3-O-methyl-D-glucose.

The number of hexoses studied here is too small to establish precise molecular structure activity relationships for affinity to the BBB glucose carrier. Numerous minor configurational variables found within the pyranose structure correlate importantly with membrane transport characteristics. To establish significant structure-activity relationships would require the study of a much larger number of hexoses.

Despite the small number of hexoses reported here, relevant comparisons can be made with active and passive transport systems in other tissues. Wilson and Landau (38) summarized data on the ability of intestine to actively transport a large number of sugars. n-Mannose and 2-deoxy-D-glucose are not actively transported by intestine, whereas they readily pass the BBB.

The relative permeability of red cell membrane to a large number of sugars (including the hexoses presented here) has been studied (26) and closely resembles those described in the present BBB study. Two important points of similarity are transport of n-mannose and 2-deoxy-D-glucose with the permeability to the latter greatest of all hexoses studied.

These comparisons with active intestinal and passive red-cell membrane transport suggest that BBB transport of glucose is passive. This suggestion of passive transport is supported by evidence of bidirectionality of BBB glucose transport (6, 12, 33).

The similarity of glucose transport by BBB and red cell has been noted by Cron (12) and would be in keeping with the generalization of Krogh (22) that the transfer characteristics of BBB approximate those of plasma membranes.

The similarity of amino acid BBB penetrations of amino acids and red cell permeabilities has been described here earlier. The extension of these selective membrane permeability similarities to hexoses further emphasized the similarity of BBB and red cell membrane selective-transport mechanisms.

Self-inhibition of D-glucose. The brain uptake of D-glucose-\(^{14}C\) in the presence of cold D-glucose confirms earlier observations that the BBB passage of D-glucose is saturable (26). The reduction of D-glucose uptake as a function of glucose concentration noted here parallels the observations of Cron (12) using carotid injection of glucose-\(^{14}C\) relative to a nondiffusible internal standard of reference as described by Chinnard et al. (8).

The similar effectiveness of the alpha and beta anomers of D-glucose as a transport inhibitor parallels the observation of Pratt and Wright (32) concerning the relative ability of these anomers to inhibit D-glucose transport in the posterior choroid plexus of the frog.

Cross-inhibition of D-glucose uptake. Figure 5 indicates that those hexoses in Table 5 which have a high BUI also are more effective inhibitors of D-glucose \(^{14}C\) uptake. This suggests that the BUI of a particular hexose approximately defines the affinity of that substrate for the BBB glucose carrier site. The observed approximately linear correlation of brain uptake of a hexose with its effectiveness as a glucose inhibitor suggests that all of these hexoses enter brain via the same carrier sites as D-glucose.

The failure of high concentrations of L-arginine or L-phenylalanine to alter the BUI of D-glucose suggests that the
carrier systems responsible for BBB passage of amino acids and glucose are independent.

Phlorizin cross-inhibition of D-glucose uptake. The D-glucose uptake inhibitions described here in the presence of phlorizin are compatible with a very high affinity of phlorizin for glucose-transport carrier sites. The relative estimated $K_m$ values for phlorizin and D-glucose as determined by the present methodology suggests that phlorizin has approximately 25 times greater affinity for these transport sites than D-glucose. The similar inhibition of uptake of 3-O-methyl-D-glucose into capillary cells with a thin layer of capillary cell cytoplasm is not metabolized but its BBB transport is still inhibited.

In view of the tight junctions between brain capillary cells, the brain capillary wall probably can be considered to be made up on two uninterrupted layers of plasma membrane with a thin layer of capillary cell cytoplasminterposed between them. Brain capillary transport probably is restricted to transendothelial cell passage through these inner and outer capillary cell walls and the interposed cytoplasm. The BBB thus may be considered to be an extended, continuous plasma membrane. The plasma membrane in Krogh's generalization probably is that of the brain capillary endothelial cell.

The methodology employed here creates an isolated system for conducting transport studies on this membrane in vivo. Its simplicity and rapidity of performance and interpretation exceed methods employed with red cell membrane, many of whose selective transport characteristics the BBB shares. This methodology may make of the BBB an easily accessible, useful model for assessing general membrane transport characteristics in vivo, quite independent of any specific interest in brain.

The author acknowledges valuable discussions with Dr. Arthur Yowiler, Dr. Jared Diamond, and many helpful suggestions from Mrs. Stella Z. Oldendorf. Technical assistance was provided by Mrs. Shigeyo Iyama, Mr. Leon Braun, and Mr. William Partridge.

Received for publication 12 May 1971.

REFERENCES


