Kinetics of unidirectional leucine transport into brain: effects of isoleucine, valine, and anoxia

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BETZ, A. LORRIS, DAVID D. GILBOE, AND LESTER R. DREWES. Kinetics of unidirectional leucine transport into brain: effects of isoleucine, valine, and anoxia. Am. J. Physiol. 228(3): 995-900. 1975.—The rate of unidirectional uptake, \( u \), of \( L-[^{14}S]H \)leucine was studied in 17 isolated dog brains by means of an indicator-dilution technique using \(^{25}Na\) as the intravascular reference. The arterial leucine concentration was varied in increments by adding unlabeled leucine to the blood and \( u \) was determined after each change. The value and leucine concentrations were varied independently to permit evaluation of their effect on leucine transport. A preliminary analysis indicated that both valine and isoleucine are competitive inhibitors. Therefore, all data were fitted to an equation that describes Michaelis-Menten kinetics in the presence of two competitive inhibitors. These calculations yielded an apparent \( K_m \) for leucine transport of 1.58 mM \( \pm .28 \) SE, a \( V_{max} \) of 0.323 \( \mu \)mol/g per min \( \pm .055 \) SE, and an apparent \( K_i \) for inhibition of leucine transport of 1.76 mM \( \pm .34 \) SE for valine and 0.73 mM \( \pm .14 \) SE for isoleucine. In four isolated brains perfused with blood having a constant leucine level, indicator-dilution injections were made before, and at 1, 5, and 10 min after the start of perfusion with anoxic blood. These findings showed that, unlike glucose transport (Brain Res. 67: 307-316, 1974), the rate of leucine transport is unaffected by 10 min of anoxia.

indicator-dilution technique, unidirectional extraction, blood-brain barrier

IN MOST MAMMALIAN TISSUES the capillary endothelium offers no restriction to the free diffusion of blood constituents into the interstitial space (14). This has been demonstrated for various solutes including large-molecular-weight proteins. However, the capillary endothelium of the brain is thought to function as a selective barrier even for small molecules (13). According to the current concept of this so-called blood-brain barrier the movement of a polar solute from blood to brain requires the presence or absence of transport systems, since only those polar solutes that have affinity for such systems can enter the brain at a significant rate.

During anoxia, the rate-limiting step in cerebral metabolism of glucose appears to be its transport from blood to brain (6). Transport may also be the rate-limiting step in the cerebral metabolism of other solutes. Consequently, a detailed characterization of the control of each transport system will permit a better understanding of the control of cerebral metabolism. Furthermore, knowledge of the structural requirements for solute transport will provide important information for the development of pharmacologic agents designed to act specifically within the central nervous system. Simple kinetic analyses performed under physiological conditions are the basis for the eventual attainment of these goals.

Since the normal function of the blood-brain barrier is dependent on the integrity of the capillary endothelium (8), transport from blood to brain can only be studied on an intact brain. However, the difficulties in controlling important variables with the commonly used in vivo preparations have permitted only semiquantitative estimates of solute uptake and simple kinetic analyses under restricted circumstances. We have recently (7) described a method that utilizes the isolated, perfused dog brain and an indicator dilution-technique to study unidirectional transport kinetics under various conditions (5-7).

The current study utilizes the indicator-dilution method to describe some of the properties of L-leucine transport between blood and brain. Others have shown that L-leucine enters the brain by means of a saturable transport system (3, 24, 28-30, 33, 36) that can be inhibited by L-isoleucine (36) and L-valine (3, 33). Our experiments describe the kinetics of unidirectional leucine transport, and its inhibition by L-isoleucine and L-valine, as well as the effect of anoxia on L-leucine transport. Part of this work was presented in a preliminary communication (21).

MATERIALS AND METHODS

Brain isolation and perfusion. Brains of adult mongrel dogs were isolated during halothane anesthesia by a procedure (22) that involves removal of all extracranial tissues. Arterial blood was supplied through the internal carotid arteries and the anastomotic branch of the internal maxillary arteries. All the venous blood was collected via a threaded Luer connector cemented over a small hole drilled into the bone covering the confluence of venous sinuses. The perfusate consisted of compatible donor blood that had been diluted to a hematocrit of 20-25% and conditioned (7). The plasma glucose concentration, as determined with a Beckman glucose analyzer, was maintained between 4 and 6 mM by the occasional addition of a 5% glucose solution. Electroencephalographic recordings were made intermittently and, with the exception of studies made during anoxia, the experiment was continued only as long as the recording indicated that the preparation was viable. At the
decrease in the $O_2$ concentration.

The perfusion system consisted of two separate pump-oxygenator combinations interconnected through a valve that permitted perfusion from only one oxygenator at a time. The control perfusion system was used to maintain normal brain amino acid concentrations between indicator-dilution injections. The blood in the control oxygenator had a $P_{O_2}$ of 110 mmHg, a $P_{CO_2}$ of 40 mmHg, and a pH of 7.40. The average perfusate concentration of some amino acids other than L-leucine, L-isoleucine, and L-valine is presented in Table 1. In general, these levels vary between 50 and 150% of the corresponding levels in plasma from dogs fasted 24 h (unpublished results). The experimental perfusion system contained identical blood except for increases in the L-leucine, L-valine, or L-isoleucine concentration or a decrease in the $O_2$ concentration.

**Individually dilution injections.** The indicator-dilution technique is described in detail elsewhere. In the present experiment, the 50-$\mu l$ injectate contained 2 $\mu$Ci of $^{22}Na$ (Amersham/Searle Corp.), the intravascular marker, and 10 $\mu$Ci of L-[4,5-$^3$H]leucine (New England Nuclear Corp.), the test molecule. The isotope mixture was injected directly into the blood stream through a rubber injection port located about 4 cm before the bifurcation of the common carotid artery. Except in the anoxic experiments, injections were made over a range of plasma L-leucine concentrations between 0.160 and 6.15 mM and at various fixed levels of L-valine (0.172-3.77 mM) and L-isoleucine (0.052-4.16 mM).

The data treatment for each indicator-dilution injection permitted calculation of the maximal fractional extraction, $E$, for L-leucine. The rate of unidirectional L-leucine transport into the brain, $v$, was calculated from the equation $v = EA_{Leu}F_p/W$, in which $A_{Leu}$ is the arterial plasma L-leucine concentration, $F_p$ is the plasma flow rate, and $W$ is the brain weight. Prior to the end of an experiment, $F_p/W$ could only be estimated since $W$ was unknown. Therefore, the pump speed was adjusted to maintain the estimated $F_p/W$ between 0.40 and 0.50 ml/g per min (7). The average ($\pm$SE) $F_p/W$ calculated from postmortem brain weights was 0.460 $\pm$ 0.006 ($n$ = 108).

**Experimental groups.** Seventeen isolated brains were used in the experiments designed to determine the kinetics of unidirectional L-leucine transport and its inhibition by L-isoleucine and L-valine. Indicator-dilution injections were made over a range of plasma L-leucine concentrations between 0.160 and 6.15 mM and at various fixed levels of L-valine (0.172-3.77 mM) and L-isoleucine (0.052-4.16 mM).

Four isolated brains were used to test the effects of anoxia on L-leucine transport. The experimental procedure was similar to that utilized to study glucose transport during anoxia. The $P_{O_2}$ of the blood in the experimental oxygenator was reduced to less than 10 mmHg. The plasma L-leucine, L-valine, and L-isoleucine concentrations were allowed to remain at control levels (Table 2). Two additional indicator-dilution injections were made 20 s after the switching of perfusion from the control pump to the experimental pump. This procedure minimized the possibility of product inhibition of transport resulting from the elevation of cerebral amino acid levels during perfusion with blood having a high L-leucine, L-isoleucine, or L-valine concentration. Beginning 3 s after the injection, 30 consecutive venous blood samples were collected at 3-s intervals. Immediately after the 30th sample, the blood flow rate was determined by measuring the volume of a 1-min collection of venous blood. A sample of this venous blood was saved for determination of the venous amino acid content while the corresponding arterial sample was taken directly from the oxygenator. Between 6 and 12 separate indicator-dilution injections were made with each isolated brain.

**Table 1.** Average perfusate concentration of some amino acids

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Avg Arterial Conc, mM</th>
<th>Observed Avg $\tau$, min</th>
<th>Adjusted Avg $\tau$, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1.364 ± .046</td>
<td>0.238 ± .011</td>
<td>0.0231 ± .0011</td>
</tr>
<tr>
<td>$\alpha$-Aminobutyric</td>
<td>0.049 ± .002</td>
<td>0.268 ± .028</td>
<td>0.0269 ± .0026</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.013 ± .003</td>
<td>0.278 ± .023</td>
<td>0.0271 ± .0039</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.020 ± .001</td>
<td>0.268 ± .032</td>
<td>0.0270 ± .0043</td>
</tr>
<tr>
<td>Citrulline</td>
<td>0.045 ± .001</td>
<td>0.268 ± .028</td>
<td>0.0269 ± .0026</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.073 ± .002</td>
<td>0.278 ± .023</td>
<td>0.0271 ± .0039</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.141 ± .005</td>
<td>0.278 ± .032</td>
<td>0.0270 ± .0043</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.053 ± .003</td>
<td>0.278 ± .023</td>
<td>0.0271 ± .0039</td>
</tr>
<tr>
<td>Glycerophosphoethanolamine</td>
<td>0.026 ± .001</td>
<td>0.278 ± .032</td>
<td>0.0270 ± .0043</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.390 ± .010</td>
<td>0.287 ± .019</td>
<td>0.0277 ± .0043</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.065 ± .003</td>
<td>0.287 ± .019</td>
<td>0.0277 ± .0043</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.098 ± .004</td>
<td>0.290 ± .016</td>
<td>0.0279 ± .0044</td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>0.050 ± .001</td>
<td>0.290 ± .016</td>
<td>0.0279 ± .0044</td>
</tr>
<tr>
<td>Proline</td>
<td>0.201 ± .006</td>
<td>0.290 ± .016</td>
<td>0.0279 ± .0044</td>
</tr>
<tr>
<td>Serine</td>
<td>0.292 ± .015</td>
<td>0.290 ± .016</td>
<td>0.0279 ± .0044</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.333 ± .013</td>
<td>0.290 ± .016</td>
<td>0.0279 ± .0044</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.040 ± .002</td>
<td>0.333 ± .013</td>
<td>0.0279 ± .0044</td>
</tr>
<tr>
<td>Urea</td>
<td>3.552 ± .092</td>
<td>0.333 ± .013</td>
<td>0.0279 ± .0044</td>
</tr>
</tbody>
</table>

Values, shown with their corresponding SE, are the average arterial amino acid concentrations in the 108 samples used in the study of L-leucine transport kinetics.
tor-dilution injections were made during perfusion of each isolated brain with blood from the control oxygenator. The valve was then switched and the brain was perfused with the anoxic blood. Indicator-dilution injections were made after 1, 5, and 10 min of anoxia, the valve was switched back, and the brain was again perfused with the control blood. The injections were repeated after 10, 20, 30, and 60 min of recovery from 10 min of anoxia.

RESULTS

L-Leucine transport kinetics. Because of the manner of perfusate preparation, it is impossible either to eliminate a constituent or to control its concentration from one preparation to the next. It is well established that L-leucine transport into brain is inhibited by certain other amino acids (3, 28, 30, 33, 36). Therefore, we found it necessary to fit our data to an equation that would take variations in the concentrations of other amino acids into account. To simplify the analysis, we assumed that L-isoleucine and L-valine would be the major inhibiting amino acids and that transport of the branched-chain neutral amino acids could be described by Michaelis-Menten kinetics, i.e.:

\[ v = \frac{V_{\text{max}} \bar{A}}{K_m + \bar{A}} \]  

where \( \bar{A} \), the average of the arterial and venous amino acid levels, is used as an estimate of their concentration in the capillary.

A preliminary analysis of the data was performed in order to determine the type of inhibition that L-isoleucine (Fig. 1) and L-valine (Fig. 2) would exert on L-leucine transport. In both cases the Lineweaver-Burk plots show an effect of inhibition on the slopes, but not the intercepts. Therefore, these data clearly demonstrate that L-isoleucine and L-valine are competitive inhibitors of L-leucine transport. This type of kinetic analysis, however, is not satisfactory for an accurate determination of kinetic constants because of the difficulty in maintaining uniform amino acid levels in the perfusate.

In order to determine the kinetic constants for L-leucine transport at any level of the other two amino acids, we used an equation (12) which describes Michaelis-Menten kinetics in the presence of two competitive inhibitors, equation 2:

\[ v = \frac{V_{\text{max}} \bar{A}}{K_m,\text{Leu} \left(1 + \frac{\bar{A}_{\text{Ile}}}{K_{i,\text{Ile}}} + \frac{\bar{A}_{\text{Val}}}{K_{i,\text{Val}}} \right) + \bar{A}_{\text{Leu}}} \]  

In the equation, \( K_m,\text{Leu} \) is the Michaelis constant for L-leucine transport, while \( K_{i,\text{Ile}} \) and \( K_{i,\text{Val}} \) are the inhibition constants for L-isoleucine and L-valine, respectively. The kinetic constants and their respective standard errors were derived with use of a computer program, similar to the one described by Cleland (11), which fits equation 2 directly by an iterative least-squares method. The apparent \( K_m,\text{Leu} \) was 1.58 mM ± 0.28 SE, the apparent \( K_{i,\text{Ile}} \) was 0.73 mM ± 0.14 SE, the apparent \( K_{i,\text{Val}} \) was 1.76 mM ± 0.34 SE, and \( V_{\text{max}} \) was 0.323 μmol/g per min ± 0.035 SE (n = 108).

L-Leucine transport during anoxia. Although the apparent increase in the rate of transport during and after anoxia is not statistically significant (Table 2), it should be noted that the average arterial L-leucine concentration during control perfusion is lower than during anoxic and postanoxic perfusion. This is due to cerebral L-leucine metabolism during the precontrol maintenance period.

Since the variation in plasma concentrations is small and the levels are about 15% of the \( K_m \), the observed rate of transport can be corrected for the difference in capillary L-leucine concentration by assuming first-order kinetics. The observed rate of transport was adjusted to the average capillary L-leucine concentration of 0.230 mM in the control samples by the equation: adjusted \( v = \) observed \( v \times \frac{0.230}{\bar{A}} \). After correction, it is obvious that the rate of cerebral L-leucine transport is unaffected by 10 min of anoxia.
DISCUSSION

Since maximal extraction of the labeled test molecule is determined before significant backdiffusion of label has occurred, the indicator-dilution technique permits determination of unidirectional transport from blood to brain. Although the location of the transport system observed in indicator-dilution experiments has not been determined, it is probably situated in the capillary endothelium (13, 35, 36). Therefore, this technique is useful for the study of unidirectional transport across the blood-brain barrier.

The technique used in this study is a modification of the method first developed by Chinard et al. (9) and later adapted for use in the canine brain by Crone (13) and Yudilevich and DeRose (35). We have previously presented the details of our modifications of this procedure (7). Briefly, they include use of the maximal observed E as the E for the whole brain (2). 22Na as the intravascular reference (33), and a smaller injectate volume. Furthermore, because of the ability to adjust and measure the perfusate composition and flow rate, use of the isolated perfused brain permits a direct determination of transport kinetics under a wide variety of conditions.

In our previous study of unidirectional glucose transport, we found it necessary to use a factor of 3.6 % to correct for simple diffusion of glucose into the brain (7). This figure was determined from indicator-dilution experiments with a hexose that is not transported into brain (D-fructose). Because of the lack of structural similarity between hexoses and amino acids, it is not reasonable to apply this same correction to the L-leucine data. Initial studies to determine whether the maximal fractional extraction value, E, for transporters would be a suitable diffusion correction showed that D-leucine is itself transported into the brain and that it can inhibit L-leucine transport (unpublished results). Similar results have been obtained by other investigators (25, 26, 30). The arbitrary introduction of even a 1 % diffusion correction to our data resulted in a substantial increase in the SE's for the kinetic constants. In addition, Hoare (23) has shown that L-leucine diffusion into human erythrocytes is insignificant at 37°C. Consequently, we did not use a diffusion correction in this study.

Because the kinetic constants were determined in the presence of amino acids other than L-leucine, L-isoleucine, and L-valine, the values we have calculated are apparent values. Normal dog-to-dog variation in the levels of these other amino acids contributed in part to the standard errors of the K_m and K_i's.

Indicator dilution studies of unidirectional amino acid transport in brain have been reported by Yudilevich et al. (36) and by Murray (28). Analogous studies have been performed by Oldendorf (29, 30) with H_2O as the reference. Yudilevich et al. (36) found that large neutral amino acids were transported across the capillary of the dog brain, while acidic and basic amino acids and short-chain neutral amino acids were not. By contrast, Oldendorf (29), in a study of the rat brain, demonstrated significant uptake of basic amino acids as well as long-chain and some short-chain, neutral amino acids. In the present study the L-leucine (long-chain, neutral amino acid) transport system appears to be similar to the L system for amino acid transport that Christensen (10) considers to be ubiquitous. For example, in the Ehrlich cells, the L system has a K_m for L-leucine of 0.5 mM and is Na independent (32). A similar system in the human erythrocyte has a K_m for L-leucine of 1.8 mM (34).

Christensen (10) states that if one substrate (A) competes with the transport of a second substrate (B), and both are transported by a single "agency," then the K_i,B determined from inhibition of A against B is equal to the K_m,A determined directly. The involvement of a single system in cerebral L-leucine, L-isoleucine, and L-valine transport is suggested by the linearity of the plots in Figs. 1 and 2. Support for this proposition is found in the work of Yudilevich et al. (36) who showed that unidirectional extraction of L-valine could be eliminated by high levels of L-leucine, while L-leucine extraction was abolished by high concentrations of L-phenylalanine and L-tryptophan. The fact that L-phenylalanine extraction was only halved in the presence of high L-leucine levels suggests that L-phenylalanine has affinity for more than one transport system. If there is only one system with affinity for large neutral amino acids, then the K_i's for L-isoleucine and L-valine are identical to their respective K_m's. It must be remembered, however, that the values presented are apparent K_m's, which are valid only when the concentrations of all other inhibitors are similar to those in this study.

Figure 3 shows a comparison of the K_m's for these three amino acids with their respective average concentrations in canine plasma. For all three compounds, the average plasma concentration is about 20 % of the corresponding K_m. This suggests that at average plasma concentrations, unidirectional transport into the brain is nearly a first-order process. In a study of the effects of sodium pentobarbital on the net uptake of amino acids in the isolated dog brain (4), we observed a constant and highly significant uptake of L-leucine and L-isoleucine. This observation was subsequently confirmed in the human brain (17). We suggested that these amino acids play an important role in cerebral metabolism. However, the present study indicates that transport from blood to brain does not function as a control point for their metabolism in the postabsorptive
state since the rate of transport is nearly proportional to the blood concentration. On the other hand, immediately after a meal, a concurrent elevation of all amino acids that use this transport system could result in a limitation in entry of any one of them. This reportedly occurs in rat brain when postprandial elevations of plasma tyrosine, phenylalanine, leucine, isoleucine, and valine effectively limit brain uptake of tryptophan in spite of an elevated plasma tryptophan concentration (19).

Although net uptake of L-leucine was not determined in the present experiments, our study (4) of average net uptake on 53 arteriovenous plasma samples from 11 isolated brains reported net L-leucine uptake of 0.00156 ± 0.00017 SE μmol/g per min at an Ay of 0.0684 mM, aT of 0.0347 mM, and AVal of 0.0878 mM. By substitution of these concentrations and the k’s from the present study into equation 2, we calculate a corresponding rate of unidirectional L-leucine transport of 0.0122 mmol/g per min. This is nearly 8 times the observed net uptake based on the plasma arteriovenous differences. In view of recent reports that erythrocytes contribute substantially to the net interorgan transfer of amino acids both in humans (1, 18) and dogs (16), the actual net uptake of leucine may be closer to this estimated rate of unidirectional transport.

The experiments performed during perfusion with anoxic blood suggest that transport of L-leucine from blood to brain is not an energy-dependent process. Thus, transport must occur by facilitated diffusion. In contrast, a similar study of glucose transport (6), revealed a 60% reduction in the unidirectional flux of glucose (6) after 10 min of anoxia (when whole-brain ATP levels are 23% of normal (15)). However, the possibility remains that L-leucine transport is energy dependent and is more closely coupled to the small amount of energy that is generated after 10 min of anoxia.

Earlier studies (24) suggested that L-leucine was actively transported out of the brain against a concentration gradient. In those experiments, whole-brain L-leucine levels decreased after subarachnoid L-leucine administration, in spite of a higher plasma L-leucine concentration. However, such studies are very difficult to interpret because the actual cerebral L-leucine concentration in the pool in contact with the capillary is unknown.

If L-leucine transport is not energy coupled, then it probably occurs via a mechanism that is distinct from the γ-glutamyl transpeptidase system which has been proposed for amino acid transport in the kidney tubule (27). The latter is a cyclic system that requires three moles of ATP for each mole of amino acid transferred across the membrane. It has been recently reported (31) that there is a high concentration of γ-glutamyl transpeptidase localized in brain capillary endothelium. The possibility remains that the γ-glutamyl cycle is involved in the transport of amino acids other than L-leucine, L-isoleucine, and L-valine from blood into brain.

Finally, this study also demonstrates that use of the appropriate equations permits one to perform in vivo kinetic analyses of transport systems having several naturally occurring substrates. Theoretically, by addition of appropriate A/log K, the system could be simultaneously studied. The competitive effects of additional inhibitors.

REFERENCES


