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KINETIC ANALYSIS OF BLOOD-BRAIN BARRIER TRANSPORT OF AMINO ACIDS

WILLIAM M. PARDRIDGE[a,*] and WILLIAM H. OLDENDORF[b,**]

[a] *Department of Neurology, Reed Neurological Research Center, School of Medicine, University of California, Los Angeles, Calif. 90024 and* [b] *Research and Neurology Services, Brentwood Hospital, Veterans Administration, Los Angeles, Calif. 90073 and Department of Neurology, Reed Neurological Research Center, School of Medicine, University of Californa, Los Angeles, Calif. 90024 (U.S.A.)*

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SUMMARY

The Michaelis-Menten kinetics of blood-brain barrier transport of fourteen amino acids was investigated with a tissue-sampling, single-injection technique in the anesthetized rat. Tracer quantities of ^{14}C-labelled amino acids and $^{3}H_2O$, used as a freely diffusible internal reference, were mixed in 0.2 ml of buffered Ringer's solution and injected rapidly into a common carotid artery. Circulation was terminated by decapitation at 15 s following injection. A brain uptake index (I_b) was determined from the ratio of ^{14}C dpm in the brain tissue and the injection mixture divided by the same ratio for the $^{3}H_2O$ reference. Brain clearance of tracer concentration of amino acid was saturable when various concentrations of unlabeled amino acid were added to the injection solution. Double reciprocal plots of the saturation data yielded K_m (mM) values that ranged from a low of 0.09 mM for arginine to a high of 0.75 mM for cycloleucine. Transport V values were determined from the relationship $P = V/K_m$ where P is the blood-brain barrier permeability constant (ml/g per min): P was calculated from the I_b for each amino acid based on a cerebral blood flow of 0.56 ml/g per min and a fractional extraction of 0.75 for the $^{3}H_2O$ reference 15 s following carotid injection. The V values ranged from a low of 6.2 nmol/g per min for lysine to a high of 64 nmol/g per min for L-DOPA. Efflux of the tracer amino acid during the 15-s period after injection was assumed to be slow, since the rate constant of cycloleucine from brain to blood was low, 0.11 min^{-1}.

INTRODUCTION

Since the early studies of Lajtha and Toth [13] on the transport of amino acids across the blood-brain barrier, several investigators have demonstrated the presence

* Present address: University Hospital, Boston University Medical Center, Boston, Mass. 02118.
** To whom correspondance should be addressed at the Department of Neurology, Reed Neurological Research Center.

of carrier-mediated mechanisms responsible for the ready influx of circulating amino acids into brain. Barrier amino acid transport is saturable [3, 14, 16, 17, 30] and stereospecific [14, 16, 17]. Furthermore, neutral and basic amino acids have been shown to enter the brain from blood via two independent transport systems [16, 25]. The significance of amino acid transport systems within the blood-brain barrier is underscored by the recent reports that several pathways of cerebral metabolism, e.g. protein synthesis [26] and neurotransmitter synthesis [9, 11, 12, 27, 28, 31], may be limited by the availability of amino acids in brain.

The role played by blood-brain barrier transport systems in the regulation of amino acid availability in brain may be analysed by quantitating the Michaelis-Menten kinetic parameters (K_m, V) of barrier amino acid transport. The K_m values for barrier transport of three basic amino acids and ten neutral amino acids have recently been determined [19] from saturation uptake data previously reported from this laboratory [17]. These K_m values, however, are preliminary since the values were determined over a range of substrate concentrations, 1 and 4 mM, that greatly exceeded the range of computed K_m values, 0.15–0.63 mM. Furthermore, there are no reported V values for blood-brain barrier amino acid transport. Therefore, the objectives of this report were to determine accurate K_m values over a wide range of substrate concentrations as well as to estimate the V of amino acid transport across the blood-brain barrier. Although K_m and V data for blood-brain barrier sugar transport are well documented [1, 4, 5, 20, 22], there are no such estimates for amino acids.

METHODS

Uptake studies

Uptake by brain of ^{14}C-labeled amino acids was determined with a tissue-sampling, single-injection technique developed by Oldendorf [15] which utilizes ^3H_2O as a freely diffusible internal reference. Wistar rats, 275–350 g, of either sex and fed on routine laboratory diet were anesthetized with intraperitoneal sodium pentobarbital. A common carotid artery was surgically exposed and cannulated with a sharp 27-gauge (0.38 mm outer diameter) needle. The needle did not occlude the artery and free carotid flow persisted throughout the procedure.

An injection mixture was prepared which contained approx. 1 μCi/ml of ^{14}C-labeled amino acid and approx. 5 μCi/ml of ^3H_2O mixed in 0.2 ml of Ringer's solution buffered to pH 7.4 with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, Calbiochem, La Jolla, Calif.). The injection of the 0.2-ml test solution was rapid and was completed within 0.25 s. The anesthetized rat was decapitated 15 s after the injection, a period sufficient to permit a single passage of the injection mixture through the cerebral microcirculation [15]. Any of the test substance not cleared by brain during the single passage was distributed to the general circulation. The cerebral hemisphere ipsilateral to the injection and rostral to the midbrain was immediately removed from the skull, macerated and prepared for liquid scintillation counting by digestion in NCS Solubilizer (Amersham/Searle, Chicago, Ill.). An aliquot of the injection mixture was similarly prepared for liquid scintillation counting. A brain uptake index (I_b) was calculated as follows:

$$I_b = \frac{\frac{^{14}C \text{ DPM (brain)}}{^{14}C \text{ DPM (mix)}}}{\frac{^{3}H \text{ DPM (brain)}}{^{3}H \text{ DPM (mix)}}} \times 100$$

Therefore $I_b = E/E_{H_2O}$, where E and E_{H_2O} represent the extraction fractions of ^{14}C-labeled amino acid and ^3H_2O from blood at 15 s following a single carotid injection. Since the fractional extraction of amino acid was expressed relative to an internal ^3H_2O reference, the need to measure brain weight or injection volume was obviated.

Once the I_b for a tracer concentration of ^{14}C-labeled amino acid was determined, the saturability of brain uptake of labeled amino acid was investigated by adding various concentrations (0.1, 0.5, 1.0 and 4.0 mM) of unlabeled amino acid to the injection solution. A double reciprocal plot of the saturation data yielded the K_m (mM) of amino acid transport, i.e. the concentration of unlabeled amino acid in the injection solution that represented half-maximal saturation of the carrier-mediated uptake. The transport V is not readily calculated from double reciprocal plots of I_b saturation data, since the I_b is a dimensionless index of transport rate, not an absolute measure.

Calculation of transport V

The V of amino acid transport may be measured indirectly from I_b data with the use of Crone's equation [8] derived for single injection techniques, $P = -(F)\ln(1-E)$, where P is the blood-brain barrier permeability constant (ml/g per min) for a given amino acid and F is the rate of cerebral blood flow (ml/g per min). Since $E = I_b E_{H_2O}$, the I_b may be converted to E and P values if E_{H_2O} and F in the barbiturate-anesthetized rat are known. Given the P and K_m values for an amino acid, the transport V may be calculated from the relationship, $P = V/K_m$. The permeability constant is equal to the V/K_m ratio since the Michaelis-Menten equation, $v = V(C)/(K_m+C)$, reduces to a first-order form, $v = (V/K_m)(C)$, when tracer fluxes are measured, i.e. $C \ll K_m$. In this instance, carrier-mediated transport is kinetically equivalent to free diffusion described by the Fick equation, $v = P(C)$. The quantity C (μmol/ml) is the mean capillary concentration.

The determination of V from I_b and K_m values rests on the measurements of of E_{H_2O} and F. The values used in this study are $E_{H_2O} = 0.75$ and $F = 0.56$ ml/g per min and have been reported in previous investigations from this laboratory [22].

Efflux studies

The I_b is an index of the maximal fractional extraction and, therefore, the rate of unidirectional influx, if efflux of the labeled amino acid is shown to be small during the 15-s circulation period subsequent to carotid injection. The rate of exodus of a ^{14}C-labeled amino acid was measured by prolonging the circulation time, i.e. the time interval between carotid injection of the test mixture and decapitation. Labeled cycloleucine, a non-metabolizable amino acid [6], was used, since brain metabolism of the injected amino acid would distort the efflux kinetics. The washout of cyclo[^{14}C]leucine from brain was studied by determining the percent extraction of cycloleucine at 0.25, 1, 2 and 4 min circulation times. The extraction percentage of cyclo[^{14}C]leucine was determined by multiplying the I_b at each respective time interval by the previously reported extraction percentages of the ^3H_2O reference, i.e. 7.73 $\pm$ 1.73% at 0.25 min,

5.16 ± 0.97% at 1 min, 2.84 ± 0.21 % at 2 min and 1.09 ± 0.22 % at 4 min [22]. The rate constant of cyclo[^{14}C] leucine efflux from brain was determined from a logarithmic plot of the percent extraction of the tracer versus time.

RESULTS

Uptake studies

Fig. 1A illustrates the I_b for a tracer concentration of [2-^{14}C]-tryptophan and the saturation of the brain uptake of tryptophan as increasing concentrations of unlabeled tryptophan are added to the injection mixture. The I_b of the extracellular markers (e.g. sucrose or inulin), 2 %, is approximated by high concentrations of amino acid. Fig. 1B represents a double reciprocal plot of the saturation data: a K_m = 0.19 mM for tryptophan transport across the blood-brain barrier is calculated from the slope and intercept of the plot.

Similar saturation data are presented in Table I for ten other neutral amino acids and in Table II for three basic amino acids. Double reciprocal plots of these data similar to that of Fig. 1B yielded theK_m values listed in Table III. Each point in Tables I and II is a mean determination from 3–6 rats. Of the 70 means listed in Tables I and II, 36 have been reported previously [17]. The new data were necessary to make accurate determinations of the K_m values and represent additional experiments on approx. 150 rats.

Calculations of V

As described in Methods, the transport V may be estimated from the I_b and K_m data in Tables I–III from the following relationship.

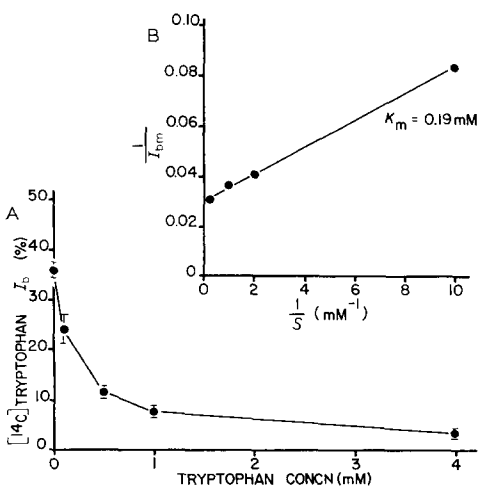

Fig. 1. (A) Saturation of the brain uptake of a tracer concentration (0.025 mM) of L-[^{14}C]tryptophan 15 s after a single carotid injection by increasing concentration of unlabeled L-tryptophan in the injection mixture. Means ±S.D. are based on data from 3 to 6 rats. (B) the saturation data is converted to a form suitable for double reciprocal transformation by plotting the difference (I_{bm}) between the I_b for a tracer concentration and the I_b for the concentration of unlabelled tryptophan in the injection mixture.

TABLE I

SATURATION DATA FOR THE LARGE NEUTRAL L-AMINO ACIDS

I_b values are means ±S.D., n = 3–6 rats.

Amino acids	Trace concentration (uncompeted)*		Brain uptake index (%)			
			0.1 mM	0.5 mM	1 mM*	4 mM*
Phenylalanine	55±5	0.004 mM	31.7±0.4	16.9±2.0	8.7±2.4	5.8±0.2
Leucine	49±4	0.008 mM	28.8±2.0	14.1±1.3	10.6±1.7	6.0±0.5
Tyrosine	53±5	0.002 mM	32.8±0.6	16.6±0.2	11.5±2.3	5.8±0.2
Tryptophan	36±2	0.025 mM	24.3±2.8	10.7±0.4	7.9±1.0	3.3±0.4
Methionine	36±1	0.030 mM	23.7±0.8	16.4±0.4	12.4±0.3	5.6±0.8
Histidine	30±1	0.006 mM	23.1±1.8	13.1±1.8	8.6±0.2	5.8±0.8
Isoleucine	36±2	0.006 mM	27.7±1.3	15.6±0.2	8.5±0.6	5.4±1.0
DOPA	30±3	0.061 mM	27.9±1.0	15.8±1.5	11.8±0.5	6.2±0.4
Valine	17±1	0.010 mM	15.1±0.7	9.8±1.6	7.1±1.3	4.0±0.3
Cycloleucine	16±2	0.094 mM	13.1±1.1	10.6±1.7	7.9±0.7	5.4±0.3
Threonine	10±2	0.009 mM	7.0±1.3	5.6±0.2	4.5±0.2	3.3±0.4

* From ref. 17. This includes all I_b values, trace concentrations (uncompeted) in columns 2 and 3, and all I_b values using 1 and 4 mM concentrations listed in columns 6 and 7, except for all values for cycloleucine which have not been reported previously.

TABLE II

SATURATION DATA FOR THE BASIC AMINO ACIDS

I_b values are means ±S.D., n = 3–6 rats.

Amino acids	Trace concentration (uncompeted)*		Brain uptake index (%)			
			0.1 mM	0.5 mM	1 mM*	4 mM*
Arginine	20±1	0.008 mM	11.2±1.5	5.8±0.9	3.8±0.4	2.6±0.7
Lysine	15±2	0.006 mM	8.4±1.2	4.3±0.6	3.9±0.5	2.3±0.3
Ornithine	11±2	0.023 mM	8.1±0.9	4.2±0.2	3.5±0.5	2.7±0.1

* From ref. 17. As described in footnote to Table I for columns 2, 3, 6, and 7, except for ornithine not previously reported.

$$V = -(K_m)\,(F)\ln(1-E)$$

The resultant V values are listed in Table III. The accuracy of the K_m and V data in Table III may be judged from the S.D. of the data in Tables I and II.

As stated in Methods, reliable kinetic data are obtained only when the tracer concentration of ^{14}C-labeled amino acid in the injection mixture is several-fold less than the transport K_m. The tracer concentrations of the various ^{14}C-labeled amino acids used in these studies ranged from 0.002 to 0.094 mM and the ratio of K_m to tracer concentration ranged from 7 to 120 (mean 40).

Efflux studies

The relationship of the I_b for cyclo [^{14}C]leucine versus circulation times of 0.25, 1, 2 and 4 min is presented in Fig. 2A. The I_b increases with time since the rate of

TABLE III

KINETIC CONSTANTS FOR BLOOD-BRAIN BARRIER AMINO ACID TRANSPORT

Amino acid	K_m (μmol · ml^{-1})	V (nmol · g^{-1} · min^{-1})
Neutral amino acids		
Phenylalanine	0.12	30
Leucine	0.15	33
Tyrosine	0.16	46
Tryptophan	0.19	33
Methionine	0.19	33
Histidine	0.28	38
Isoleucine	0.33	57
DOPA	0.44	64
Valine	0.63	49
Cycloleucine	0.75	55
Threonine	0.73	37
Basic amino acids		
Arginine	0.09	8.6
Lysine	0.10	6.2
Ornithine	0.23	11

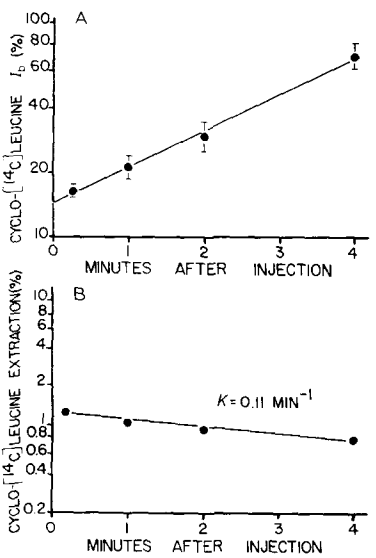

Fig. 2. (A) The I_b of a tracer concentration of cyclo[^{14}C]leucine (0.094 mM) is plotted versus the circulation time. (B) The percent of injected dose of cyclo[^{14}C]leucine, determined from the I_b and the known percent extraction for the ^3H_2O reference for each respective time interval, is plotted against the circulation time.

the ^3H_2O reference exodus is much faster than the rate of efflux of cyclo [^{14}C]-leucine. Fig. 2B illustrates the washout of cyclo[^{14}C]leucine from brain to blood as the circulation time is prolonged up to 4 min. The slope of the curve represents the rate constant of cyclo [^{14}C]leucine efflux, $K = 0.11$ min^{-1}. The accuracy of this value may be judged from the S.D. of the points in Fig. 2B.

DISCUSSION

Methods used in this investigation to obtain kinetic data on the transport of fourteen amino acids across the blood-brain barrier are similar to those recently used to determine the kinetics of barrier sugar transport [22] as well as liver cell membrane amino acid transport [23]. Although kinetic constants for brain cell membrane amino acid transport have been determined [7], K_m and V data for blood-brain barrier amino acid transport have not been reported.

The conversion of I_b data into quantitative transport parameters is dependent on the validity of several important assumptions. The I_b may be converted into fractional extractions if E_{H_2O} is known. The fractional extraction of water 15 s after a single carotid injection is not readily obtained by tissue sampling techniques on small animals. The maximal fractional extraction of water is 0.85 in the barbiturate-anesthetized dog at an unspecified rate of cerebral blood flow [29] and is 0.85 in the barbiturate-anesthetized rhesus monkey at a rate of cerebral blood flow between 0.5 and 0.6 ml/g per min [24]. Since the rate constant of ^3H_2O efflux across the blood-brain barrier is 0.61 min^{-1} in the barbiturate-anesthetized rat [22], approx. 14% of the initial water cleared by brain returns to blood by 15 s. Therefore a corrected E_{H_2O} of 0.75 has been established for experiments with the water reference technique [22].

Extraction fractions may be converted into permeability constants if the rate of cerebral blood flow is known. Based on the rate of efflux of ^3H_2O, cerebral blood flow has been calculated to be 0.56 ml/g per min under the present experimental conditions [22]. It is assumed that the rate of cerebral blood flow is not substantially altered by the rapid injection of 20 μl into the anterior cerebral circulation (only 10% of the 0.2-ml injection bolus enters the internal carotid artery [15, 22]). Raichle and coworkers [10, 24] have shown that a rapid injection of 0.2 ml into the internal carotid artery of the barbiturate-anesthetized rhesus monkey does not alter the rate of cerebral blood flow.

The fractional extractions computed from I_b data are assumed to approximate to the maximal fractional extraction, since only 15 s elapse between the single injection and termination of blood flow. Based on the efflux rate constant of cyclo[^{14}C]leucine, 0.11 min^{-1} (Fig.2), one can conclude that less than 4% of the total amount of cycloleucine cleared by brain has returned to blood by 15 s. Since metabolic sequestration would tend to impede loss of label cleared by brain, the rate of efflux of many of the metabolically active amino acids might be even less than that of cycloleucine.

Pappenheimer and Setchell [20] have argued that the arterial substrate concentration is a poor approximation of the mean capillary concentration. For example, if $E = 0.75$, then the mean capillary concentration is only 54% of the arterial concentration. However, if $E = 0.25$, mean capillary concentration is 87% of arterial concentration based on Pappenheimer and Setchell's equations. Since all the I_b data in Tables I and II are equivalent to extraction fractions of 0.25 or less at the saturating

concentration used in the double reciprocal plots, then the injection solution concentrations of 0.1, 0.5, 1.0 and 4.0 mM are very good approximations of mean capillary concentration.

An important assumption in blood-brain barrier transport studies is that the blood-brain barrier transport site is being saturated by high levels of substrate and not the brain cell membrane transport site or an intracellular enzymatic step. The fact that the brain uptake of cyclo[^{14}C]leucine is readily saturable indicates that an intracellular metabolic step is not being saturated. Furthermore, a comparison of the K_m and V data obtained in this investigation with similar data reported for brain cell membrane transport [7] indicates that the two membranes differ considerably in terms of kinetic constants. The K_m and V values for blood-brain barrier transport are approx. one third and one tenth of the respective values for brain cell membrane transport. Therefore the affinity of blood-brain barrier transport for amino acids is 3-fold that of the brain cell membrane, but the capacity of the brain cell membrane for amino acid transport is approx. 10-fold that of the blood-brain barrier. These relationships infer that in pathologic states of hyperaminoacidemia, i.e. phenylketonuria [18], the inhibition of the influx of circulating amino acids into brain is due to the preferential saturation of blood-brain barrier amino acid transport.

Several investigators [9, 11, 12, 26–28, 31] have recently suggested that many metabolic pathways in brain such as protein or neurotransmitter syntheses are limited by amino acid availability: therefore, changes in plasma levels of amino acids are ultimately referred to changes in brain levels of protein or neurotransmitter. Since the K_m of blood-brain barrier amino acid transport (Table III) approximates to the plasma levels of amino acids [2], changes in blood-brain barrier permeability to amino acids may be just as important as alterations in plasma levels in regulating the cerebral level of key metabolites [21].

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