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INDEPENDENT BLOOD-BRAIN BARRIER TRANSPORT SYSTEMS FOR NUCLEIC ACID PRECURSORS

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SUMMARY

The blood-brain barrier permeability to certain ^{14}C-labelled purine and pyrimidine compounds was studied by simultaneous injection in conjunction with two reference isotopes into the rat common carotid artery and decapitation 15 s later. The amount of ^{14}C-labelled base or nucleoside remaining in brain was expressed in relation to $^{3}H_2O$ (a highly diffusible internal standard) and ^{113m}In-labelled EDTA (an essentially non-diffusible internal standard).

Of the 17 compounds tested, measurable, saturable uptakes were established for adenine, adenosine, guanosine, inosine and uridine.

Two independent transport systems in the rat blood-brain barrier were defined. One transported adenine ($K_m = 0.027$ mM) and could be inhibited with hypoxanthine. Adenosine ($K_m = 0.018$ mM), guanosine, inosine and uridine all cross-inhibit, defining a second independent nucleoside carrier system. Adenosine inhibited [^{14}C]uridine uptake more effectively than did uridine, suggesting a weaker affinity of uridine for this nucleoside carrier.

INTRODUCTION

The relationship of purines and pyrimidines to nucleic acid metabolism and other metabolic processes accounts for the great interest in membrane transport of these precursors. The brain is one of the most active tissues in nucleotide and nucleic acid synthesis [1] but the role of the blood-brain barrier in regulating transport of these precursors has not, to our knowledge, been completely defined. The apparent absence of the complete apparatus for pyrimidine synthesis in the brain, and the indication of a complete purine pathway [1] further emphasize a possibly important role for the blood-brain barrier. Interest in purine compounds as potential neurotransmitters (purinergic nerves [2–4]) and in association with a sex-linked human neurological disorder (the Lesch-Nyhan syndrome [5–7]) also contributed to the present study.

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METHODS

High specific activity ^{14}C-labelled purine and pyrimidine compounds were injected as a bolus into the surgically exposed right common carotid artery of rats anesthetized with intraperitoneal sodium pentobarbital [8]. In addition to approximately 0.25 μCi of the test substance, the injectate contained $^{3}H_2O$ (about 1 μCi) and ^{113m}In-labelled EDTA (about 50 μCi), prepared as described previously [9, 10]. Millimolar concentrations of unlabelled substances were also added in carrier competition experiments. The rat was decapitated 15 s after injection and the right cerebral hemisphere prepared for liquid scintillation counting for the three isotopes [11]

$$\text{The ratio of } \frac{\frac{[^{113m}In]}{[^{3}H]}_{\text{brain}}}{\frac{[^{113m}In]}{[^{3}H]}_{\text{injectate}}}$$

serves as a plasma space indicator identifying the porportion of nuclides remaining in the brain vasculature. Definition of activity in the vasculature permits calculation of $[^{14}C]$ and $[^{3}H]$ in brain per se and is necessary for accurate measurement of these low uptake compounds. Since $^{3}H_2O$ is highly diffusible [12] and is nearly completely cleared during a single brain passage, the brain uptake index of the $[^{14}C]$ purine and pyrimidine compounds can be expressed [10] as a percentage of the ratio of $[^{14}C]/[^{3}H]$. Exchange occurs within 1–2 s under these conditions, and data obtained are indicative of blood-brain barrier permeability and not a measurement of brain distribution space.

RESULTS AND DISCUSSION

Measurable, saturable uptake was established for adenine, adenosine, guanosine, inosine and uridine (Table I). Our studies of purine and pyrimidine bases suggest that none of the pyrimidines tested (cytosine, uracil, thymine and orotic acid) can measurably cross the blood-brain barrier. In contrast, the purine base adenine is measurably taken up by brain. Self-inhibition studies (Table II) indicate this to be a carrier-mediated process ($K_m = 0.027$ mM) and a double reciprocal plot of hypoxanthine inhibition of adenine uptake indicates that both the slope and intercept are increased (Fig. 1a). The weak affinity of pyrimidine bases for the adenine carrier is also suggested by the similarly low brain uptakes of uracil, thymine and hypoxanthine (Table I). Guanine is virtually insoluble at physiological pH values, and therefore was not included in this study. Uptake of the more soluble guanine sulfate was not measurable. Adenine nucleotides (ADP, AMP and cyclic AMP) also have no demonstrable affinity for this adenine carrier system (Table II). The slight affinity of high concentrations of adenosine, and the absence of effects at 1 mM concentrations of nucleosides, is further discussed below. Blood-brain barrier transport of uric acid, an end product of purine metabolism [1] could not be demonstrated in our study (Table I). Berlin [13] studied this phenomenon in the isolated dog choroid plexus and proposed that, in vivo, the transport system he defined removed uric acid from the cerebrospinal fluid to the blood.

TABLE I

BRAIN UPTAKE OF NUCLEIC ACID PRECURSORS

^{14}C-labelled compound tested	Concentration (mM)	Number of rats tested	I_b* (%)
Adenine	0.0022	3	7.70±0.95
Adenosine	0.0021	8	8.76±3.72
Guanosine	0.0027	7	6.22±1.69
Inosine	0.0025	3	5.40±0.16
Uridine	0.0024	5	4.18±0.78
Cytosine	0.050	3	0.07±0.04
Thymine	0.027	3	2.05±0.24
Uracil	0.019	3	1.13±0.12
Guanine sulfate	0.0112	5	1.77±1.11
Hypoxanthine	0.020	6	1.18±0.22
Orotic acid	0.020	3	0.00
Folic acid	0.023	3	0.01±0.02
Uric acid	0.0104	3	0.06±0.06
Cytidine	0.0024	3	0.33±0.01
[^{3}H]Cytosine arabinoside**	0.0006	3	1.60±0.37
Thymidine	0.0027	3	0.83±0.17
ATP	0.024	5	2.35±0.43

* I_b = brain uptake index. Compounds having an I_b of < 3 % are not considered to be transported to brain, since these values represent the lower (background) limits of the method employed. For example, additional studies indicated that the uptake of thymine was not decreased by the addition of 1 mM thymine, adenine, adenosine or a composite mixture described in Table II.

** I_b for this ^{3}H-labelled compound was not determined by the Indium-corrected method and is therefore probably 1–2 % lower than indicated.

It therefore seems possible that under physiological conditions the blood-brain barrier (presumably located in the cerebral capillary endothelial cells) may make purine compounds available to the brain and the choroid plexus may return purine catabolites to the blood.

The brain apparently cannot synthesize the precursor orotate, but readily forms pyrimidine nucleotides from intraventricularly injected [^{14}C]orotic acid [1]. Because the blood-brain barrier apparently restricts the entry of orotate and other pyrimidine bases (Table I) in spite of a requirement for the pyrimidine nucleus in RNA synthesis, transport studies of pyrimidine and purine nucleosides were initiated.

In addition to the purine carrier system, certain nucleosides also crossed the blood-brain barrier by an independent carrier transport system. Adenosine, guanosine, inosine and uridine all cross-inhibit, indicating a common transport site (Table III). A modified Lineweaver-Burk plot of adenosine uptake (K_m = 0.018 mM) is presented in Fig. 1b. The fact that adenosine more efficiently inhibits [^{14}C]uridine uptake than does uridine (1 mM) suggests that purine nucleosides may have a greater affinity for this carrier system than does uridine. In addition to the purine nucleoside xanthosine, the pyrimidine nucleosides thymidine, cytidine and cytosine arabinoside appear not to be transported across the blood-brain barrier.

Other investigators indicate that uracil [14, 15] and orotate [16] are not readily incorporated into rodent brain tissue but uridine is [16–20]. The present study

suggests that the external source of a pyrimidine nucleus available to the adult rat brain may possibly depend on the availability of circulating uridine and concomitantly low blood levels of nucleosides which share the uridine transport locus. Because cytidine nucleotides are formed by the amination of uridine nucleotides, the rat brain does not require an external source of cytidine nucleotide precursors [21]. An additional report suggests that much of the cytidine nucleotide present in the brain may occur as a polymer, possibly as a storage form [22], and these studies apparently account for the blood-brain barrier exclusion of cytosine and cytidine demonstrated here. These observations seem to be consistent with the proposal of Hogans et al. [16] that a pyrimidine salvage pathway functions in brain RNA biosynthesis.

Cross-inhibitions are seen when [^{14}C]adenine competes with 3–5 mM concentrations of nucleosides but not at 1 mM nucleoside concentrations (Table II). In addition, [^{14}C]adenosine is inhibited by 1 mM adenine but not 5 mM adenine (Table III). These results might be due to metabolic factors rather than competition for a transport locus. For example, Taube and Berlin [23] reported that the differing K_m values for adenosine uptake (0.01 mM) and deamination (0.07 mM) suggest that uptake and metabolism of this nucleoside are separate events in rabbit leukocytes, yet

TABLE II

ADENINE TRANSPORT IN THE PRESENCE OF INHIBITORS

Inhibitor	Concentration of inhibitor (mM)	Number of animals	Brain uptake index	P*
Adenine (uncompeted)	—	3	7.70±0.95	—
Adenine	5	3	1.44±0.06	<0.001
Adenine	1	3	1.69±0.25	<0.001
Adenine	0.1	3	2.81±0.66	<0.01
Hypoxanthine	5	3	2.74±0.07	<0.001
Hypoxanthine	1	3	3.43±0.34	<0.01
Hypoxanthine	0.1	3	5.23±1.06	<0.05
Adenosine	10	3	3.39±0.90	<0.01
Adenosine	5	3	5.37±1.42	<0.1
Adenosine	1	3	7.77±1.40	>0.9
Guanosine	3	3	5.17±1.20	<0.05
Guanosine	1	3	6.97±2.80	<0.7
Inosine	5	3	5.21±1.21	<0.05
Inosine	1	3	7.33±1.55	<0.8
Uridine	1	3	7.75±0.83	>0.9
Cytosine	1	3	9.48±3.53	<0.5
Thymine	5	3	6.38±2.19	<0.4
AMP	5	3	5.74±1.50	<0.2
ADP	5	3	5.33±2.44	<0.2
Cyclic AMP	5	3	6.37±1.22	<0.3
Composite mixture**		3	6.28±0.47	<0.1

* P = probability determined from a 't' test comparing the effect of an inhibitor to uncompeted adenine uptake.

** The composite mixture contained 4 mM phenylalanine, 4 mM aspartic acid, 4 mM arginine, 1 mM pyruvic acid and 10 mM 2-deoxyglucose. Each of these compounds has an affinity for one of the five previously defined transport systems in the rat blood-brain barrier. (L-amino acid and D-hexose forms were used in this mixture.)

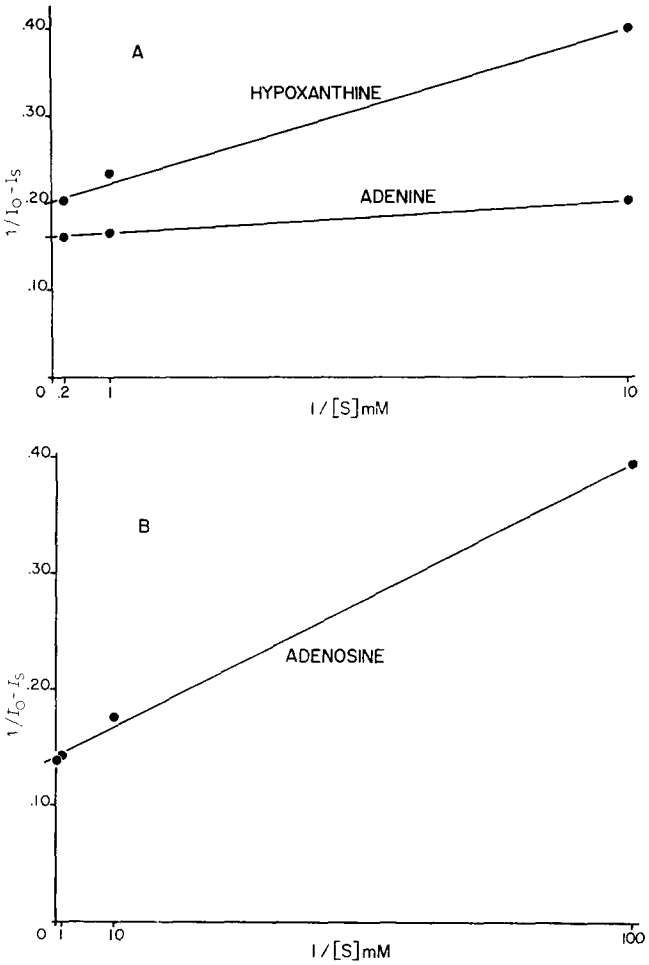

Fig. 1. (A) Lineweaver-Burk plots of adenine self-inhibition and hypoxanthine cross-inhibition were derived from data given in Table II. The inhibition data are converted by the method of Pardridge and Oldendorf [39] to a form suitable for linear transformation by plotting the reciprocal of the difference between the uncompeted brain uptake index (I_0), and the brain uptake index for the given adenine (or hypoxanthine) concentration (I_s), versus the reciprocal of the concentration of unlabelled purine base (S) in the injection mixture. Blood brain barrier adenine K_m = 0.027 mM is within the range reported for two carrier systems in rabbit leukocytes (K_m = 100 mM; K_m = 7 μM) [31]. K_i of hypoxanthine inhibition of adenine uptake = 0.097 mM. (B) A Lineweaver-Burk linear transformation of the adenosine self-inhibition data of Table III prepared in the same manner as (a). Blood brain barrier adenosine K_m = 0.018 mM.

both events may influence membrane transport. In addition, Parks and Brown [24] indicate that if adenosine enters the human erythrocyte in low concentrations (< 0.01 mM) it is converted to AMP, but in high concentrations (> 0.1 mM) adenosine is degraded to hypoxanthine. Similar results from studies of erythrocyte ghosts suggest the same metabolic events occur in the plasma membrane [25] and do not require the participation of intracellular components. Transport systems in the rat blood-brain barrier and erythrocyte membranes share many similar characteristics

TABLE III

EFFECTS OF UNLABELLED INHIBITORY COMPOUNDS ON ^{14}C-LABELLED NUCLEOSIDE TRANSPORT INTO RAT BRAIN

I_b = Brain uptake index (mean ±standard deviation). n = number of animals from which I_b mean is determined. P = statistical probability (determined from a 't' test) of the difference between uncompeted vs inhibited I_b.

Inhibitor	Concentration (mM)	Brain uptake index of ^{14}C-labelled nucleoside								
		Adenosine	n	P	Guanosine	n	P	Uridine	n	P
Uncompeted I_b		8.76±3.72	8	—	6.22±1.69	7	—	4.18±0.78	5	—
Adenosine	5	0.36±0.17	3	<0.001	0.12±0.11	3	<0.001			
	1	0.56±0.32	3	<0.001				0.87±0.20	3	<0.001
	0.1	1.88±0.29	3	<0.001						
	0.01	5.06±0.76	3	<0.05						
Guanosine	1	0.86±0.36	3	<0.001	0.64±0.07	3	<0.001	1.47±0.25	3	<0.001
Inosine	5	0.03±0.06	3	<0.001						
	1		—		0.45±0.08	3	<0.001			
Uridine	1	1.21±0.51	3	<0.001	1.13±0.32	3	<0.001	1.43±0.13	3	<0.001
Adenine	5	9.04±2.22	5	<0.9	3.68±0.30	3	<0.01			
	2	6.10±2.04	3	<0.2						
	1	5.34±0.55	3	<0.05	4.24±0.65	6	<0.02	3.76±0.04	3	<0.3
	0.5	7.98±2.30	3	<0.7						
Hypoxanthine	5	9.78±1.89	3	<0.6						
	1	7.43±0.91	3	<0.4	5.90±2.12	5	<0.8	4.72±0.75	3	<0.4
Xanthosine	1	8.08±2.78	5	<0.8	4.63±0.53	3	<0.1	4.62±1.24	3	<0.7
Thymine	5	9.54±1.12	3	<0.7						
Composite mix*		8.99±2.44	5	<0.9						
UTP	1							4.17±0.71	3	>0.9
ATP	5	9.90±8.01	5	<0.8						
	1	4.00±0.66	3	<0.01						
ADP	5	9.30±5.17	8	<0.9						
	1	5.46±0.46	3	<0.05						
AMP	5	6.41±3.90	8	<0.3						
	1	3.40±0.16	3	<0.01						
Cyclic AMP	5	10.01±1.58	3	<0.5						

* composite mixture, see Table II.

[26]. If similar metabolic events occur in the rat blood-brain barrier any hypoxanthine formed from excess adenosine (or other purine nucleoside) might be expected to saturate or initiate feedback inhibition of transport processes at the adenine locus (Table II). Similarly, 5 mM adenine might be degraded and not expected to compete with adenosine uptake, but 1 mM adenine could possibly become salvaged and appear to compete with adenosine (Table III).

Carrier-mediated transport of adenine and hypoxanthine on the same locus has been demonstrated in yeast [27], tapeworms [28], human erythrocytes [29, 30], rabbit polymorphonuclear leukocytes [31] and human platelets [32]. Sixma et al. [32] in their studies of platelets, reported a competitive effect of high concentrations of adenosine (0.1 mM) on the adenine locus; a finding readily explained by the observations of other workers [24, 25], as discussed above. These reports collectively suggest that purine bases are typically transported at the same site and although nucleoside transport systems have been reported in erythrocytes [33], erythrocyte ghosts [25] and polymorphonuclear leukocytes [23], similarities or differences in the transport of nucleosides and bases have not been fully defined in many biological systems. In the rat blood-brain barrier, however, we believe that our studies indicate two separate transport mechanisms, one an adenine locus, the other for nucleoside uptake. The slight inhibitory effects of 5 and 10 mM adenosine on adenine uptake (Table II) suggest that Lineweaver-Burk analysis of adenosine inhibition would indicate a different intercept and Michaelis-Menten constant($K_i > 10$ mM) than those determined from studies of adenine self-inhibition (Fig. 1). This indicates that the adenyl base and nucleoside do not share the same common transport system and establishes the independence of the adenine carrier from the adenosine (adenosine-guanosine-inosine-uridine) system.

The fact that adenosine inhibits [^{14}C]uridine uptake more efficiently than does uridine (Table III) also suggests that purine nucleosides may have a greater affinity for the nucleoside site than does uridine. Taube and Berlin [23] suggest that conformational changes in the transport carrier protein explain the uptake of both purine and pyrimidine nucleosides by a single carrier system. In the blood-brain barrier nucleoside system this conformational flexibility may be somewhat reduced to an extent that uridine is the only pyrimidine nucleoside with a functional affinity for the transport protein.

In contrast to the results described in the present study, Nakagawa and Guroff [20] reported that the adult rat brain is impermeable to guanine and guanosine, but somewhat more permeable to inosine. We could not solubilize guanine at pH 7.5, but our results indicate that both guanosine and inosine cross the blood-brain barrier. The techniques employed in the present study apparently account for the differing results. Carotid injection and measurement of uptakes within 15 s (employed in our study) effectively minimize any possibility of biotransformation of the labelled precursor. Nakagawa and Guroff [20] injected labelled compounds intraperitoneally and determined brain uptakes 15–60 min later. Paper chromatography additionally indicated to these workers that in many instances the radionuclide present in both plasma and brain was in more than one chemical form [20].

Previous studies in this laboratory using intracarotid injections have defined five separate (hexose [26], organic acid [9] and acidic, neutral and basic amino acid [10, 34]) transport systems in the rat blood-brain barrier. Mixtures containing one

compound with an affinity for each of these defined transport loci (2-deoxyglucose, pyruvate, phenylalanine, arginine and aspartate) hadno significant effect on adenine uptake, or in competition studies for the nucleoside locus, indicating independence of both the purine and nucleoside transport systems from those defined previously. Barbiturate and pyrimidine compounds are chemically similar. All of these studies were performed under pentobarbital (pentabarbitone) anesthesia and therefore subject to the possible effects discussed in a recent paper [35].

Transport studies of isolated cells in culture suggest that nucleotides generally do not cross cell membranes [36]. The minimal uptake of adenine nucleotides measured in this study indicates that these compounds are not transported across the multicellular blood-brain barrier also. This observation may in some way be related to the fact that adenine nucleotide (ATP) may possibly function as a neurotransmitter [2] and this suggests that the blood-brain barrier, like cholinergic neurones [37], is permeable to precursors, but not effectors, of neurotransmission.

Results of the present study are also of interest in relation to the rare disease of pyrimidine nucleotide synthesis, hereditary orotic aciduria. This condition is usually characterized by retarded growth and development, as well as blood dyscrasias and increased urinary orotate and resembles "pyrimidine starvation" of microbial organisms [38]. Strabismus (the only reported neurological symptom) has been observed in less than half of the cases, but the onset of symptoms typically occurs within a few months of birth [38]. This time period coincides approximately with the functional appearance of the blood-brain barrier. The highly beneficial responses to uridine treatment are also well documented. Blood dyscrasias are corrected, normal growth and development restored, but these children are almost always mentally retarded [38]. This suggests to us that if uridine transport sites in the blood-brain barrier of man and rat are similar, this particular locus may play a vital role in early brain development and may be related to the unique effectiveness of uridine in hereditary orotic aciduria. Because orotate would be available to the brain until the functional appearance of the blood-brain barrier, mental retardation might be tentatively attributed to the temporary brain pyrimidine starvation prior to uridine treatment. Oliver and Paterson [33] have defined facilitated uridine transport in human erythrocytes, and reported that counter transport of uridine from pre-loaded erythrocytes can be demonstrated in studies with a variety of nucleosides including adenosine, inosine, guanosine and cytidine. These observations, together with the demonstration that uridine is neither cleaved nor phosphorylated by the red cell [33] suggest that the erythrocyte may function as a transport vehicle, carrying uridine to sites such as the bone marrow and blood-brain barrier. This apparent interaction of transport mechanisms at both unicellular (erythrocytes) and tissue (brain endothelium) organizational levels is but one example of the contribution of transport mechanisms to physiological compartmentation in the organism.

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