BBA 75551

SOME ASPECTS OF UPTAKE OF NON-METABOLITES IN SLICES OF MOUSE BRAIN

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(Received August 17th, 1970)

SUMMARY

1. The uptake of six compounds, sulfate, D-mannitol, urea, cadaverine, creatine, and 2,4-dinitrophenol, was measured in incubated slices of mouse brain. The first three of these were not actively transported; the other three were accumulated against a concentration gradient.

2. Sulfate penetrated a space somewhat smaller than inulin space and thus was restricted to the extracellular space; D-mannitol penetrated, in addition to the extracellular space, a part of the intracellular space; urea penetrated into all tissue water.

3. Incubation in the presence of cyanide resulted in penetration of sulfate into almost all of the extra- but none of the intracellular space, and in penetration of mannitol into all of the intracellular spaces; the penetration of urea was not affected.

4. Cadaverine, creatine, and 2,4-dinitrophenol were accumulated by the slices against a concentration gradient; this uptake was strongly inhibited by cyanide.

5. The exit of 2,4-dinitrophenol was rapid, that of cadaverine was slower, exit of creatine was the slowest. Accordingly, 2,4-dinitrophenol levels reached equilibrium rapidly, but creatine accumulation continued with time. The slow exit of creatine could not be explained by its conversion to creatine phosphate.

6. Transport of 2,4-dinitrophenol is related to that of tyrosine and phenylalanine as shown by the exchange of 2,4-dinitrophenol with these two amino acids and by the resistance of about one-third of 2,4-dinitrophenol, tyrosine and phenylalanine uptake to inhibition by cyanide.

7. Transport of cadaverine is different from that of the basic amino acids: cadaverine uptake was inhibited by putrescine and spermine, whereas lysine had no effect in uptake or in heteroexchange experiments.

8. Arginine has affinity to both lysine and cadaverine carriers, since it inhibited the uptake of lysine and increased the flux of cadaverine. Cadaverine flux increased only if arginine and cadaverine were on the same side of the membrane: extracellularly for an increase in influx and intracellularly for an increase in efflux. Cadaverine uptake and its increase by arginine were abolished by cyanide.

9. The results show that a variety of compounds, some not present under physiological conditions, can be transported in brain by processes with broad

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specificity for metabolites. In addition, a number of specific transport mechanisms may be present in this tissue whose function is not clear at the present time.

INTRODUCTION

The passage of metabolites into and out of the nervous system has been the subject of a number of investigations in recent years[1-5]. In a number of these studies brain slices were used because they can be investigated under easily defined and controlled conditions. Active transport processes were found for a number of metabolites present under physiological circumstances in the nervous system, and properties of transport such as substrate specificity[2,6,7], energy requirements of transport[8-10], and factors influencing transport mechanisms[11] were investigated. The studies with slices showed transport processes with high activity in nervous tissue[3,5]. Studies with the living brain showed that processes with essentially similar properties are present under physiological conditions; *in vivo* transport from the spinal fluid to blood of several compounds was also shown[4,12].

These studies established that the passage of metabolites into and out of the brain occurs mostly through mediated transport, but little is known about the passage of compounds not present in the brain under physiological conditions. The transport from the spinal fluid into the blood against a concentration gradient, of foreign compounds such as Diodrast[13], and the uptake by brain slices of organic compounds[14-19] indicated the possibility that compounds other than the physiological metabolites may act as substrate for cerebral transport processes. In order to study the possible presence of transport mechanisms with specificities different from those for physiological metabolites, the uptake, exit, and exchange of several compounds were measured in incubated brain slices. These compounds included those reported to be restricted to extracellular space, those that penetrate all tissue water, and those that could be metabolic by-products of cerebral metabolism and drugs. Of the six compounds studied, sulfate, mannitol, and urea did not show accumulation against a concentration gradient in brain slices, but their penetration into the various spaces of the slice was not the same; however, the other three compounds, cadaverine, creatine, and 2,4-dinitrophenol, were accumulated by the slices against a concentration gradient, and some characteristics of this accumulation are reported.

EXPERIMENTAL

The preparation of brain slices has been described in detail[6]. Young adult Swiss mice with an average body weight of 28 g and brain weight of 440 mg were used. Brain slices, 0.416 mm thick, where prepared from each cerebral hemisphere, and approx. 150 mg of tissue was placed in a 25-ml erlenmeyer flask containing 4.5 ml of the indicated medium at 0° and oxygenated for 1 min. The flask was stoppered and placed in a shaker bath at 37° for a period of 30 min, unless otherwise indicated. For the uptake experiments, at the end of the first period of incubation 0.5 ml of the labeled substrate was added and the incubation was continued. The final concentration of the substrate in the medium and the time of incubation are given in the legends to the tables. When the effect of inhibitors (cyanide or ouabain)

was measured, they were added to the first 30-min incubation medium. At the end of incubation with labeled substrate, the slices were filtered, frozen, weighed, homogenized in 2 ml of 3 % (w/v)$HClO_4$, and centrifuged; 0.5 ml of the clear supernatant was mixed with 16 ml of modified Prockop–Ebert scintillation fluid[6], and the radioactivity was counted in a liquid scintillation counter. The method of determining the concentration of a compound in tissue on the basis of radioactivity assumed that the ^{14}C label remains in the original compound. The concentrative uptake was calculated according to the following formula: $(T-M \times E/1-E) - M$, where T = concentration of compound in the tissue (μmoles compound per ml tissue water) M = concentration of compound in the medium at the end of incubation (μmoles compound per ml medium), and E = extracellular (inulin) space of the slices. In the exit experiments we assumed that $M = 0$.

The ^{14}C-labeled compounds: uniformly labeled [^{14}C]urea, uniformly labeled D-[^{14}C]mannitol, [1,5-$^{14}C_2$]cadaverine·2HCl, [1-^{14}C]creatine monohydrate and uniformly labeled 2,4-dinitro[^{14}C]phenol were from New England Nuclear Corp., Boston, Mass., $Na_2{}^{35}SO_4$ from Nuclear Equipment Corp., Farmingdale, N. Y.

For the colorimetric determination of creatine the modification of THOMAS[20] of the method of EGGLETON *et al.*[21] was used: creatine phosphate was measured enzymatically[22,23], the enzymes (glucose-6-*P* dehydrogenase, hexokinase and creatine phosphokinase) were purchased from Sigma Chemical Co. (St. Louis).

The standard incubation medium contained 119 mM NaCl, 5.0 mM KCl, 0.75 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 1.0 mM $NaHCO_3$, 5 mM HEPES, and 10 mM glucose. It had a computed tonicity of 300 mosM and pH 7.21 at 37° (ref. 24).

Further experimental details are given in the legends to the tables.

RESULTS

Penetration of sulfate, D-mannitol, and urea into incubated mouse brain slices

Differences in the extent of penetration were found among the compounds tried. The initial rate of penetration of all three compounds into the inulin space was very high. Sulfate penetrated more than two-thirds of the extracellular (inulin) space within 3 min. In spite of such rapid initial penetration, even after 90 min sulfate did not penetrate the inulin space completely (Table I). Mannitol reached complete equilibrium with the inulin space within 3 min and slowly penetrated the intracellular (non-inulin) space[25], but it did not reach diffusion equilibrium with the non-inulin space. The penetration of urea was at the highest rate; it penetrated the total tissue water space (extra- and intracellular space) rapidly, reaching equilibrium within 3 min (Table I). The addition of a metabolic inhibitor, NaCN, to the incubation medium at levels at which it almost completely inhibits the active uptake of amino acids by the slices[9,10] did not significantly affect urea distribution, but it increased the penetration of sulfate into the extracellular space and mannitol penetration into intracellular space. In the presence of cyanide mannitol reached diffusion equilibrium with the total tissue water as opposed to sulfate, which still did not come to complete equilibrium with the extracellular space and was still excluded from the intracellular space (Table I).

TABLE I

PENETRATION OF SULFATE, MANNITOL, AND UREA INTO INULIN AND NON-INULIN SPACE IN INCUBATED SLICES OF MOUSE BRAIN

Mouse brain slices were incubated in 4.5 ml of buffer with or without $1 \cdot 10^{-3}$M NaCN for 30 min; then 0.5 ml of 10 mM $Na_2{}^{35}SO_4$, [^{14}C]mannitol, or [^{14}C]urea was added, and the incubation was continued for 3, 10, 60, or 90 min. The level of the compound in the tissue was calculated from the content of radioactivity of the extract (see EXPERIMENTAL). Results are expressed as % of inulin (extracellular) or non-inulin (intracellular) space penetrated by the compound. Averages of 6–9 experiments ± S.E. are given.

Incubation time (min)	*% of inulin space*		*% of non-inulin space*			
	Sulfate		*Mannitol*		*Urea*	
	Control	*NaCN*	*Control*	*NaCN*	*Control*	*NaCN*
3	69 ± 2.1	80 ± 1.8	10 ± 0.9	33 ± 1.2	98 ± 1.2	100 ± 0.6
10	75 ± 1.9	90 ± 0.9	40 ± 0.8	60 ± 1.4	110 ± 2.6	110 ± 0.9
60	73 ± 2.4	93 ± 2.6	7.8 ± 3.1	101 ± 2.2	105 ± 1.9	103 ± 2.0
90	81 ± 2.2	90 ± 1.7	77 ± 2.3	100 ± 0.3	103 ± 0.4	105 ± 1.3

TABLE II

UPTAKE OF CADAVERINE, CREATINE, AND 2,4-DINITROPHENOL IN SLICES OF MOUSE BRAIN

Mouse brain slices were incubated in 4.5 ml of buffer for 30 min; then 0.5 ml of cadaverine, creatine, or 2,4-dinitrophenol was added, to obtain the final concentration shown in the second column. The incubation was continued for 10, 60, and 90 min. Uptake is expressed as tissue to medium ratio (μmoles of compound per ml of total tissue water over μmoles of compound per ml of medium) at the end of incubation. Averages of 6–9 experiments ± S.E. are given.

Compound	*Concn. (mM)*	*Tissue to medium concentration ratio*		
		10 min	*60 min*	*90 min*
Cadaverine	0.05	1.97 ± 0.06	8.30 ± 0.09	10.10 ± 0.41
	0.10	1.77 ± 0.02	7.32 ± 0.14	8.20 ± 0.21
	1.00	1.65 ± 0.03	5.58 ± 0.11	6.48 ± 0.31
	10.00	1.50 ± 0.08	4.32 ± 0.06	5.41 ± 0.12
Creatine	0.10	1.70 ± 0.07	9.15 ± 0.24	13.15 ± 0.65
	0.50	1.58 ± 0.04	8.90 ± 0.31	13.01 ± 0.24
	1.00	1.50 ± 0.09	8.40 ± 0.44	12.60 ± 0.30
2,4-Dinitrophenol	0.003	3.40 ± 0.12	4.00 ± 0.09	4.08 ± 0.11
	0.006	3.01 ± 0.09	3.88 ± 0.14	3.94 ± 0.23
	0.030	2.55 ± 0.17	3.85 ± 0.21	3.90 ± 0.34

Uptake and exit of cadaverine, creatine, and 2,4-dinitrophenol

The three other compounds tried, cadaverine, creatine, and 2,4-dinitrophenol, were accumulated by brain slices against a concentration gradient, as shown by a tissue to medium concentration ratio greater than one (Table II). The initial rate of uptake (as measured in a 10-min experiment) was the highest with 2,4-dinitrophenol, but the degree of accumulation (as measured in the experiments of longer duration) was higher with the other two compounds tried, cadaverine and creatine. Uptake of cadaverine, but not of creatine, seemed to show saturation under these experimental conditions, in that at higher substrate concentrations the tissue to medium concen-

tration ratio decreased (Table II). Tissue to medium ratios decreased with increasing 2,4-dinitrophenol concentration in the influx (10-min) experiments and did not change when equilibrium was established (in the 60 and 90-min experiments).

The uptake of these compounds by brain slices against a concentration gradient was strongly inhibited in the presence of $1 \cdot 10^{-3}$ M NaCN. The concentrative uptake of creatine was completely inhibited by cyanide; the influx of cadaverine was also

TABLE III

INHIBITION BY CYANIDE OF THE UPTAKE OF CADAVERINE, CREATINE, AND 2,4-DINITROPHENOL

The experimental conditions were the same as reported in the legend to Table II except for the presence of $1 \cdot 10^{-3}$ M NaCN in the medium. Results are expressed as % inhibition of concentrative uptake of control (μmoles/ml of intracellular water *minus* μmoles/ml of medium at the end of incubation). Each value represents the average of 6–12 experiments. The standard error was $\pm$ 5–10%.

Compound	*Concn. (mM)*	*% Inhibition*		
		10 min	*60 min*	*90 min*
Cadaverine	0.05	100	93	85
	0.10	99	86	85
	1.00	95	82	81
	10.00	93	83	81
Creatine	0.10	100	100	100
	0.50	100	100	100
	1.00	100	100	100
2,4-Dinitrophenol	0.003	69	64	65
	0.006	67	65	66
	0.030	66	64	64

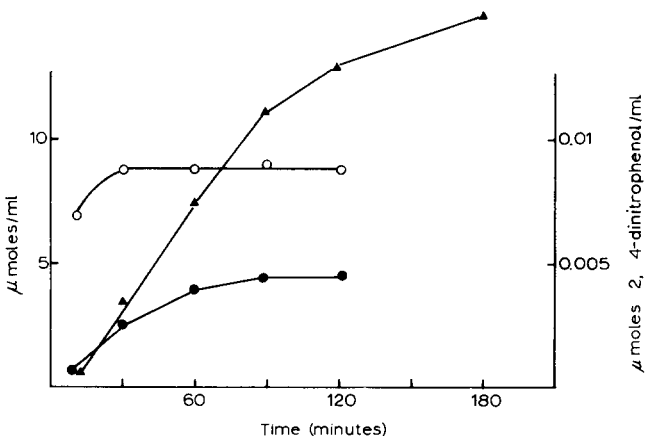

Fig. 1. Time-curve of the uptake of cadaverine, creatine, and 2,4-dinitrophenol. Mouse brain slices were incubated in 4.5 ml of buffer for 30 min; then 0.5 ml of the compound to be measured was added, to obtain a final concentration of 1 mM in the case of cadaverine and creatine and 0.003 mM in the case of 2,4-dinitrophenol. The incubation was continued for 10–180 min. Uptake is expressed as μmoles of compound per ml of intracellular water *minus* level of the medium (uptake above diffusion equilibrium levels). Left ordinate, cadaverine and creatine; right ordinate, 2,4-dinitrophenol. ●, cadaverine; ○, 2,4-dinitrophenol; ▲, creatine.

strongly inhibited, although in the longer-term experiments some uptake of cadaverine remained. About one-third of the concentrative uptake of 2,4-dinitrophenol in the short-term and also in the long-term experiment was insensitive to the inhibition by cyanide (Table III). The pattern of inhibition by cyanide was similar at all concentrations of the substrates studied. The cyanide-resistant uptake of 2,4-dinitrophenol seemed to be resistant also to inhibition by ouabain, since $1 \cdot 10^{-5}$ M ouabain (which itself inhibited uptake, although somewhat less strongly than cyanide) when added to cyanide-containing medium did not increase the inhibition over that of cyanide alone.

There seemed to be differences among the three compounds in the time needed to reach equilibrium levels in the tissue. Equilibrium was reached more rapidly with 2,4-dinitrophenol (30 min) than with cadaverine (90 min), while it was not reached with creatine in 180 min (Fig. 1). Significant differences among the three compounds were found also in the apparent kinetic constants of uptake: apparent K_m cadaverine 7.7, creatine 3.7, 2,4-dinitrophenol 0.6 mM; and v_{max} cadaverine 1.7, creatine 0.6, 2,4-dinitrophenol 0.03 μmole/ml per min. Equilibrium is reached in slices, as has been discussed elsewhere[7,26], when the rate of exit becomes equal to the rate of uptake. A measurement of exit rates showed that in a 30-min period the exit of 2,4-dinitrophenol was complete (all the slice content was lost within this time); there was also a significant (20–50 %) exit of cadaverine, while no creatine exit occurred during this time (Table IV). This rapid exit of 2,4-dinitrophenol and absence of exit of creatine explains why equilibrium in the slice is much more rapidly established with 2,4-dinitrophenol than with creatine (Fig. 1). The presence of cyanide did not significantly affect exit, although the exit of amino acids from the slices was found to be increased in the presence of cyanide[26]. In particular, creatine taken up by the slices was retained even in the presence of cyanide in the exit experiments. Although the rate of uptake of creatine decreased somewhat after 90 min, uptake

TABLE IV

EXIT OF CADAVERINE, CREATINE, AND 2,4-DINITROPHENOL FROM BRAIN SLICES

Mouse brain slices were incubated for 60 min in the presence of cadaverine, 2,4-dinitrophenol, or creatine at the concentrations noted in the second column. The uptake is shown in the third column. To measure exit the slices were transferred into fresh standard medium. After 15 min the medium was replaced to keep the level of the compound negligible in the medium during the exit period. Results are expressed as % decrease during 30 min of exit. Each value represents the average of 6–9 experiments. The standard error was ± 5–10%.

Compound	*Concn. (mM)*	*Control concentrative uptake*	*Exit (%)*	
			Medium	*$1 \cdot 10^{-3}$ M NaCN*
Cadaverine	0.05	0.40	21	24
	0.10	0.71	22	25
	1.00	5.45	51	58
Creatine	0.10	0.89	0	0
	1.00	8.19	0	0
2,4-Dinitrophenol	0.003	0.012	100	100
	0.03	0.115	100	100

increased up to the longest time measured (180 min). Since in 30 min no significant exit of creatine occurred, the exit of this compound was measured also in longer-term experiments, estimating creatine content by a colorimetric method as well as measuring the amount of label in the tissue. Longer incubation time for exit (60 min) resulted in each case in a small but measurable loss of creatine from the slices (Table V). The exit measured with colorimetric methods was somewhat greater than by measuring radioactivity. This indicates a conversion of a portion of labeled creatine taken up to creatine phosphate; this conversion, however, was not responsible for the retention of creatine taken up by the tissue since only a relatively small portion of creatine taken up was converted to the phosphate.

TABLE V

EXIT OF CREATINE FROM BRAIN SLICES

Experimental details were as described in Table IV, except that in the 60-min exit the medium was changed each 15 min. Each value represents the average of 6 experiments.

Incubation uptake	*Time of exit (min)*	*μmoles creatine per ml tissue water (concentrative uptake)*			
				Exit	
		^{14}C *	*Colorimetric* **	^{14}C *	*Colorimetric* **
60	—	7.52	9.79		
	60	5.91	6.62	1.6	3.2
90	—	11.2	12.4		
	60	9.4	9.9	1.8	2.5
180	—	14.9	15.3		
	60	11.9	11.5	3.0	3.8

* Estimating creatine by measuring the label taken up.
** Estimating creatine by the colorimetric procedure[20].

In agreement with THOMAS[20] we found a loss of endogenous creatine upon incubation of slices in a creatine-free medium. Brain slices before incubation contained about 9.5 μmoles of creatine per g tissue and 3.1 μmoles after a 90-min incubation in a creatine-free standard medium. Assuming that all the creatine in the tissue was in the intracellular water and correcting for the swelling of the incubated slices, it appears that about one third of the endogenous creatine was lost during the 90-min incubation in a creatine-free medium. In agreement with THOMAS[20] we found that the creatine phosphate content of slices after 90 min was 1.5 μmoles/g in the absence and 2.2 μmoles/g in the presence of 1 mM creatine in the incubating medium. Less than 20 % of the creatine taken up was converted to creatine phosphate. Increasing creatine in the incubation medium to 5 mM from 1 mM in the 90-min experiment increased creatine uptake about 2.5-fold, but increased creatine phosphate content only 30 %.

Substrate specificity of uptake

It is not clear whether the compounds tested in these experiments are taken up by a transport process with the function of transporting physiologically important metabolites. It is possible, for example, with the not very high substrate specificity

TABLE VI

EXCHANGE OF CADAVERINE, CREATINE, AND 2,4-DINITROPHENOL

In the homoexchange experiments mouse brain slices were incubated for 60 min (preloading time) in the presence of unlabeled compound (cadaverine, 2,4-dinitrophenol or creatine) at the concentrations noted in the second column. At the end of the preloading time the slices were transferred to a medium containing corresponding ^{14}C-labeled compound at the same concentration, and accumulation into the slices was measured after 30 min of incubation. For the heteroexchange experiments the slices were preloaded with L-lysine for 60 min; [^{14}C]cadaverine was then added, and incubation was continued for 30 min. The concentration of lysine in the medium was 10 or 40 times that of cadaverine. The same procedure was used in the case of 2,4-dinitrophenol, where the slices were preloaded with tyrosine, phenylalanine, or a 1 : 1 combination of the two at the indicated concentrations (Column 4). The results are expressed as % increase of influx. Each value represents the average of 3–6 experiments. The standard error was ± 5–10%.

Compound	*Concentration (mM)*	*Homoexchange*	*Heteroexchange Lysine*	
			1 : 10	*1 : 40*
Cadaverine	0.05	8	0	0
	0.10	15	0	0
	1.00	19	0	0
	10.00	22	0	0
Creatine	1.00	0		
			1 : 10 tyrosine	*1 : 10 phenylalanine*	*1 : 10 tyrosine + phenylalanine*
2,4-Dinitrophenol	0.003	0			
	0.030	0	12	9.5	16

of some of the amino acid carriers, that related compounds are taken up by means of an amino acid transport process. In order to study the possible relationship of the uptake of these compounds to amino acid uptake, uptake of cadaverine and 2,4-dinitrophenol was measured in slices preloaded with amino acids. If cadaverine and lysine have affinity to the same carrier, intracellular lysine could be expected to exchange with the cadaverine in the medium, and thus influx of cadaverine would be greater in slices preloaded with lysine. Cadaverine and lysine seem to have different transport specificities, since intracellular cadaverine increased cadaverine influx whereas intracellular lysine had no effect at any of the lysine or cadaverine concentrations tried (Table VI). 2,4-Dinitrophenol, on the other hand, may be taken up by amino acid transport process, since 2,4-dinitrophenol influx was increased by preloading the slices with tyrosine, or phenylalanine, or a mixture of these two amino acids. Homoexchange (preloading with a compound increasing its own influx) was not found with creatine of 2,4-dinitrophenol under our experimental conditions (Table VI). Since 2,4-dinitrophenol influx and the establishment of equilibrium is rapid, the 30-min time period may have been too long to measure an increase in influx of 2,4-dinitrophenol; creatine uptake, however, is linear in the first 30 min.

The possible relationship of 2,4-dinitrophenol transport to that of tyrosine and phenylalanine was studied by a comparison of the effects of a metabolic inhibitor. In the presence of 1 mM NaCN the uptake of most amino acids is completely inhibited[10]. Cyanide did not inhibit 2,4-dinitrophenol uptake completely; about one-third of this uptake seemed to be cyanide-insensitive. A similar percentage of tyrosine

and phenylalanine uptake was resistant to inhibition by cyanide (Table VII), further strengthening the relationship between the transport of these amino acids and that of 2,4-dinitrophenol.

Specificity of cadaverine transport

The specificity of cadaverine transport was studied further by measuring the effect of structurally related compounds on cadaverine uptake. Cadaverine uptake was not influenced by the presence of 10–40-fold higher lysine in the medium, but the presence of putrescine and especially of spermine was strongly inhibitory (Table VIII). The effect of arginine differed from that of lysine; in the presence of 40 mM but not 10 mM arginine in the medium cadaverine uptake greatly increased.

TABLE VII

INHIBITION BY CYANIDE OF THE UPTAKE OF 2,4-DINITROPHENOL, TYROSINE AND PHENYLALANINE

Mouse brain slices were incubated in 4.5 ml of buffer containing $1 \cdot 10^{-3}$ M NaCN for 30 min; then 0.5 ml of 0.3 mM 2,4-dinitro[^{14}C]phenol or, L-[^{14}C]tyrosine or L-[^{14}C]phenylalanine were added, and the incubation was continued for 60 min. For other details see legend to Table III. Each value represents the average of 4 experiments ± S.E.

Compound	% *inhibition*
2,4-Dinitrophenol	64 ± 1.5
L-Tyrosine	70 ± 1.8
L-Phenylalanine	57 ± 0.9

TABLE VIII

CONCENTRATIVE UPTAKE OF CADAVERINE IN THE PRESENCE OF DIFFERENT SUBSTRATES

Mouse brain slices were incubated in 4 ml of buffer with or without $1 \cdot 10^{-3}$ M NaCN for 30 min; then 0.5 ml of 10 mM [^{14}C]cadaverine and 0.5 ml of a substrate were added, to obtain the final concentration shown in the second column. The incubation was continued for 5 and 30 min in the case of arginine and for 30 min in the other experiments.

Substrate	*Concentration (mM)*	*μmoles of cadaverine per ml of intracellular water above medium*		
		5 min	*30 min*	*30 min + NaCN*
None		0.11 ± 0.00	2.71 ± 0.08	0.38 ± 0.02
Arginine	10	0.12 ± 0.01	2.69 ± 0.09	
Arginine	40	3.52 ± 0.12	17.50 ± 0.85	0.54 ± 0.04
Arginine * (preincubation only)	40		2.24 ± 0.09	0.40 ± 0.01
Lysine	10		2.81 ± 0.11	
Lysine	40		2.73 ± 0.09	
Putrescine	10		1.39 ± 0.02	
Putrescine	40		0.50 ± 0.02	
Spermine	10		0.49 ± 0.03	
Spermine	40		0.0	

* In this set of experiments, the slices were incubated in the presence of 40 mM arginine for 30 min. The slices were transferred in a fresh, arginine-free buffer and 0.5 ml of 10 mM [^{14}C]-cadaverine was added. The incubation was continued for 30 min. Results are expressed in μmoles of cadaverine per ml of intracellular water above the medium (see EXPERIMENTAL). Averages of 6 experiments ± S.E. are given.

The increase was greater in short-term experiments (in 5 min, 32-fold) than in longer term experiments (in 30 min, 6.5-fold), and it occurred only when arginine and cadaverine were both present extracellularly. If slices were preloaded with arginine and were transferred in arginine-free medium, cadaverine uptake was slightly inhibited by the intracellular arginine rather than increased. The uptake of cadaverine was strongly inhibited by cyanide, and in the presence of cyanide high extracellular arginine had no significant effect on cadaverine uptake (Table VIII).

The fact that arginine increased cadaverine uptake more in shorter-time than in longer-time experiments is probably explained by its effect on cadaverine exit. The exit of cadaverine was greatly increased by arginine (Table IX). For arginine to have an effect, again it had to be on the same side of the membrane as cadaverine. Exit of cadaverine into arginine-containing media was not greater, but if slices were preloaded with both cadaverine and arginine the intracellular arginine increased the rate of exit of cadaverine from the cells (Table IX).

TABLE IX

THE EFFECT OF ARGININE ON THE EXIT OF CADAVERINE

Slices from mouse brain were incubated in 4.5 ml of buffer (1,2) for 30 min; after 0.5 ml of 10 mM [^{14}C]cadaverine was added, the incubation was continued for 30 min. The slices were then transferred to a fresh cadaverine-free buffer without (1) or with (2) 40 mM arginine, and the incubation was continued for 10 min. In the last set of experiments, after the first 30 min of incubation in 4 ml of buffer, 0.5 ml of 10 mM [^{14}C]cadaverine and 0.5 ml of 400 mM arginine were added and the incubation was continued for 5 min. Then the slices were transferred to a fresh medium containing 40 mM of arginine and the incubation was continued for 10 min. Averages of 4–6 experiments ± S.E. are given.

Substrate	*Experimental conditions*	*μmoles cadaverine per ml intracellular water*		*% exit*
		Beginning of exit	*End of exit*	
None (1)	30 min uptake cadaverine 10 min exit buffer	3.66 ± 0.09	2.55 ± 0.06	30.3
Arginine (2)	30 min uptake cadaverine 10 min exit buffer + arginine	3.66 ± 0.09	2.59 ± 0.02	29.3
Arginine (3)	5 min uptake cadaverine + arginine 10 min exit buffer + arginine	4.43 ± 0.13	0.54 ± 0.03	87.9

DISCUSSION

Penetration of compounds that do not accumulate in the slice above the concentration in the medium

A number of compounds have been used both *in vivo* and *in vitro* to measure extracellular space in tissues: sulfate, mannitol, inulin, sucrose, sorbitol, and others[24–33]. Work with brain slices showed that all compounds do not penetrate to the same degree, and therefore different spaces are delineated. A second inulin space was shown by the finding of a space in slices penetrated by inulin at 37° but not at 0° (refs. 24, 25). Sulfate in our experiments penetrated a space somewhat smaller than the inulin space, which in the presence of cyanide increased to almost

the same size as the inulin space. The mannitol space was larger than the inulin space. The larger mannitol space has been observed previously[24]. The most plausible explanation for these findings seems to be that mannitol, with a lower molecular weight, penetrates a portion of the intracellular space. Similar intracellular penetration was indicated also in *in vivo* experiments[29,30]. The fact that in the presence of cyanide mannitol penetrates all of the extra- and intracellular space, while sulfate seems to be restricted still to the extracellular space, indicates that in spite of alterations in membrane permeability by metabolic inhibitors a portion of selectivity remains. Modifications in the structural elements and membranes in incubated slices have been observed in a histological and electronmicroscopic study[34,35] in which we found that the effects of cyanide were more pronounced on neuronal structures such as mitochondria, and that at least part of the morphological alterations caused by a short-term cyanide treatment were reversible. It is not clear whether inulin or sulfate space is the truer measurement of the extracellular space; the negative charge of sulfate may restrict its diffusion to some spaces by electrostatic repulsion.

Urea in the slices penetrates the total water space, although it does not do so in the living animal. KLEEMAN *et al.*[36] concluded that the major barrier to urea penetration into the nervous tissue is at the capillary level and not at the level of plasma membrane of the glial or neuronal cells. More recently, POLLAY AND KAPLAN[37] showed that urea diffuses into brain tissue approximately according to Fick's law of diffusion. Our results showing a rapid diffusion of urea into the intracellular spaces of brain slices where there is a direct contact of neuronal and glial surfaces with the urea solution are in accord with the above findings. The slightly larger urea space than non-inulin space which occurs after longer incubation time is probably only apparent; it may be the result of the increase of intracellular water water (due to swelling) and may also be related to the level of ATP in the slices during the incubation in the presence of urea. It was found that after 30 min of incubation in the presence of urea the ATP level in the slices decreases to less than one-third of the original value, and does not reach the control values for an additional 40 min of incubation (F. PICCOLI, unpublished data). It is clear from the lack of inhibition by cyanide that the penetration of sulfate, mannitol, or urea into cerebral slices does not involve a process requiring metabolic energy.

Uptake of compounds above medium levels

There is relatively little information available about the uptake of compounds that are not present in the brain under physiological conditions. Some time ago MCILWAIN AND GRINYER[38] investigated the effects of some pyridine and phenozine derivatives on the metabolism of carbohydrates, and showed that brain cortex slices from guinea pig accumulated such derivatives as pheniafromine, diazine green, neutral red, ethyl red, nicotinamide, and nicotine. The concentration in the tissue, for most of them, was 50–300 times that of the substance remaining in solution. The author emphasized that these concentrations were not reached by a process of combining with tissue constituents but were dependent on metabolic activities in the slice. More recently, energy-dependent active uptake was found with the following compounds: carbachol[39], methonium compounds[39,40], and metaraminol[41]. Calcium was found to be actively concentrated by slices of brain cortex from rat[42] and guinea pig[43], but this uptake was not affected by inhibitors such as ouabain

or 2,4-dinitrophenol[42,43]. The level of creatine in cerebral tissue is high, close to 10 μmoles/g, part of which is lost when slices are incubated in a creatine-free medium[20]. Creatine is taken up by such slices from a medium against a concentration gradient; the uptake is inhibited by the absence of O_2 or glucose[20]. The uptake of creatine is accompanied by resynthesis of creatine phosphate; with 0.15 mM creatine in the medium about 20 % (ref. 44), with 1 mM creatine about 10 % (ref. 20), of the compound taken up was converted to the phosphate. Our studies confirmed these findings and showed that in longer-term incubation or with higher levels in the medium slices accumulate creatine above the level in the living brain. Although a part of the creatine taken up is converted to creatine phosphate the metabolic conversion is not mainly responsible for the uptake or retention of creatine in brain slices.

Cadaverine is formed by enzymatic decarboxylation of lysine in microorganisms; there is no evidence that it is formed in the brain. The need for a mechanism to transport cadaverine into the brain is not clear at the present time. The lack of effect of lysine in the exchange or the uptake experiments makes it unlikely that cadaverine has affinity to the lysine transport mechanism, and the distinction of the transport mechanism of diamine from that of lysine is also supported by the finding that cadaverine and putrescine did not inhibit the uptake of lysine by brain slices[7].

The inhibition of cadaverine uptake by diamines with related structure (putrescine and spermine) shows the presence in brain tissue of a transport mechanism with affinity to a number of related amines. The dramatic increase of cadaverine flux by arginine demonstrates the complexities in the substrate specificity of cerebral transport. It seems likely from our results that cadaverine flux and lysine flux are mediated by two separate carriers and neither compound has a significant affinity toward the carrier of the other. Arginine seems to have affinity to both of these carriers, since it inhibits lysine uptake[7] and it also increases cadaverine flux. The effect of arginine on cadaverine flux probably occurs through a process of "competitive stimulation"[4–6] since the effect occurs only if the two compounds are on the same side of the membrane: extracellularly for an increase in uptake, and intracellulary for an increase in exit. The "competitive stimulation" of uptake, like uptake itself, is dependent on the availability of metabolic energy, as shown by the inhibition of both by cyanide.

There was no significant exit of creatine in short-term experiments, as was also indicated in the time-curve of uptake (Fig. 1), where the linear increase of slice levels with time showed a break only after 90 min. Creatine exit was relatively small also in long-term experiments. High uptake of some compounds, coupled with low rate of exit, points out the possibility of considerable accumulation of a substrate in the brain even if its level in the blood is relatively low.

The uptake of 2,4-dinitrophenol seems to be partially energy-independent, since more than one-third of 2,4-dinitrophenol uptake is unaffected by cyanide or by ouabain. This might be related to a certain degree of heterogeneity of the transport system of 2,4-dinitrophenol. 2,4-Dinitrophenol itself inhibits transport in brain slices. We found that near the levels used in the present study (0.02 mM) 2,4-dinitrophenol inhibited the uptake of a number of amino acids 10–20 % and lowered the ATP level in slices by about 30 %. Morphological studies[34,35] indicate that in incubated slices of brain the neurons are more sensitive than glial cells to cyanide, suggesting that in the presence of cyanide it is still possible to measure the residual glial transport

of 2,4-dinitrophenol. In its specificity 2,4-dinitrophenol transport is related to the carrier system of structurally related amino acids, since heteroexchange between phenylalanine or tyrosine and 2,4-dinitrophenol could be demonstrated and the percentage of uptake of these compounds that is resistant to inhibition by cyanide is also similar. 2,4-Dinitrophenol therefore may be taken up because of its affinity to a carrier whose physiological function is the transport of normally occurring metabolites.

The presence in brain tissue of transport mechanisms for compounds not ordinarily present in the tissue and the possibility of physiological transport mechanisms being involved in the transport of foreign substrates emphasize the possible participation of active processes in the movement not only of natural substrates but also of products not normally components of the brain. The different behavior of the three actively accumulated compounds in turn emphasizes the selectivity of the brain barrier system also with foreign compounds. The possibility of a complex interaction between non-metabolites and metabolites is indicated by the finding that one structural analog inhibits cadaverine uptake while another one increases cadaverine uptake or exit depending on its location.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service Research Grant No. NB 04360 from the National Institute for Neurological Diseases and Stroke.

The valuable help of Mrs. A. Grynbaum is gratefully acknowledged.

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