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Synergism between different transport systems stimulates the uptake of neutral amino acids by isolated brain microvessels

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Summary. The polar long-chain amino acids glutamine and methionine can be transported across the endothelial cells of brain microvessels either by an L-system which operates by a facilitated diffusion, exchanging mechanism, or by a concentrating, energy-dependent A-system. The presence of glutamine and/or of methionine can induce a synergism between the two transport systems which results, by a transstimulation mechanism, in a net increased uptake of neutral hydrophobic aminoacids. The methionine analog S-methylthiocysteine, which is the mixed disulfide resulting from the combination of methanethiol with cysteine, behaves similarly to methionine in stimulating the uptake of neutral hydrophobic amino acids. The same transstimulating effect can even be obtained in collagenase-treated, A-system-deprived microvessels by inducing the direct formation of S-methylthiocysteine within the cytoplasmic compartment of the endothelial cells.

Keywords: Amino acid transport – Blood-brain barrier – Glutamine – Methionine – Methanethiol

Abbreviations: MeAIB: α-methylaminoisobutyric acid; BCH: DL-β-aminobicyclo-2,2,1-heptanecarboxylic acid; HEPES: N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid.

Introduction

Previous studies have demonstrated that Gln enters the endothelial cells of isolated brain microvessels *via* the concentrative A-system of amino acid transport and therefore induces, by an L-system-mediated exchange mechanism, a higher rate of uptake of other, more hydrophobic, neutral amino acids [1].

Gln-mediated stimulation of neutral hydrophobic amino acids transport is also involved in the effect exerted on isolated brain microvessels by concentra-

tions of NH_4^+ ions similar to those which are reportedly present in the plasma of patients with hepatic encephalopathy, a neurological syndrome which complicates severe liver failure [2]. Preliminary experiments have shown that effects similar to those exerted by Gln are shared by some other neutral long-chain polar amino acids, such as Asn and Met. Moreover, the stimulation exerted on the subsequent uptake of neutral hydrophobic amino acids upon preloading with Met appeared to be even greater than that exerted by Gln [3, 4].

High plasma and cerebrospinal fluid levels of Met, as usually found in patients with severe hepatic failure and encephalopathy [5], could therefore, by a mechanism similar to those suggested for Gln, be involved in the pathogenesis of this neurological syndrome.

The present study is a characterization of the *in vitro* effects exerted by Met on the uptake of neutral hydrophobic amino acids, leading to the identification of a generalized mechanism by which synergism between the A and L transport systems results in a consistent stimulation of this uptake. It also gives a possible explanation of the pathogenic role of methanethiol, a methionine-derived mercaptan, the levels of which are often elevated in hepatic failure [6, 7] and which has been found to potentiate the ability of ammonia to induce coma in experimental animals [8]: methanethiol, though ineffective *per se*, can lead, upon combination with cystine or cysteine (either of which is *per se* ineffective), to the formation of a mixed disulfide, S-methylthiocysteine, which strongly resembles Met in its chemical structure as well as in its ability to stimulate neutral amino acid transport.

Materials and methods

Chemicals

L-(U^{14}C)Leu (335 mCi/mmol), L-(U^{14}C)Tyr (503 mCi/mmol), L-(methyl-^{3}H)Met (100mCi/mmol), L-(U^{14}C)Lys (300 mCi/mmol), (carboxyl^{14}C) α-MeAIB (35 mCi/mmol), DL-(carboxyl^{14}C)BCH (4.95 mCi/mmol) and Aquasol-2^R were obtained from New England Nuclear (Boston, Mass., U.S.A.). Collagenase from *Clostridium histolyticum* and HEPES were from Sigma (St. Louis, MO., U.S.A.). Carboxyfluorescein diacetate was from Molecular Probes Inc. (Junction City, OR., U.S.A.). Unlabeled amino acids and all other chemicals were from Sigma (St. Louis, MO., U.S.A.), from Merck (Darmstadt, Germany) or from Fluka (Buchs, Switzerland).

Preparation of S-methylthiocysteine and amino acid analysis

10 mM cystine and 20 mM methanethiol were incubated in 20 mM HEPES buffer, pH 7.3, for 45 min at 37°C. The mixture was then acidified to pH 3.0 and the solution, after bubbling with nitrogen for 1 h, was chromatographed on a Dowex 50 column (200–400 mesh, H^+-form). After the absorption step, the column was washed with 200 mM sodium citrate to eliminate the unreacted cystine, and the retained S-methylthiocysteine was eventually eluted with 1 mM HCl. The iodoplatinate- and ninhydrin-positive fractions [9] were collected and liophylized.

Thin-layer chromatography was performed, in the ascending mode, on cellulose aluminium sheets (layer thickness 0.1 mm), using, as solvent, *n*-butanol:acetic acid:water:ethylene glycol monomethylether (1:1:1:1, v/v/v/v). Under these conditions, Met, S-methylthiocysteine, cystine and cysteine had respectively Rf values of 0.77, 0.74, 0.30 and 0.33. The 1 mM HCl eluate from the Dowex 50 column exhibited only the S-methylthiocysteine spot, a very

small amount of cysteine being only occasionally present. In a standard automatic amino acid analysis, performed on a LKB 4400 amino acid analyzer equipped with a Spectraphysics System I computing integrator and operating at 56°C on a Ultropac 8 column with Na^+ citrate buffers (14 min at pH 3.20, then pH 4.25), the retention time of S-methylthiocysteine was 34.9 min, as compared to 35.6 min for Met, 33.7 min for Val, and 32.8 min for Cys. The purity of S-methylthiocysteine in the 1 mM HCl eluate from the Dowex column was constantly above 85%, as confirmed by chemical determination of the SH and S–S content.

Isolation of brain microvessels

Microvessels were isolated from the gray matter of fresh bovine brain essentially as described by Hjelle et al. (10), with minor modifications [1, 2]. Briefly, gray matter was homogenized by hand in a buffer (1:1, w:v) consisting of 122 mM NaCl, 15 mM $NaHCO_3$, 10 mM glucose, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4 and 10 mM HEPES, pH 7.4, equilibrated with 95% O_2 + 5% CO_2. The homogenate was poured on a 86 μm pore size nylon sieve and washed with a spray of ice-cold buffer. The material retained on the sieve was re-homogenized and washed again with a spray of cold buffer. The isolated vessels were then resuspended in buffer and kept, until use, in a plastic tube at 0°C. For some experiments, after the isolation step, the microvessels were resuspended in a sodium-free buffer obtained by substituting NaCl and $NaHCO_3$ respectively with choline-Cl and $KHCO_3$. When indicated, the microvessels were digested with 0.75 mg/ml of crude collagenase for 20 min at 37°C. Collagenase was then removed by extensive washing the microvessels on a 86μm pore size nylon sieve.

Integrity and viability of the microvessels

As previously described [1, 2, 4], scanning electron microscopy and phase contrast light microscopy have shown that the brain microvessels are obtained practically free from nerve or glial cells contamination. The microvessels were also impermeable to trypan blue, and contained approximately 450 ng ATP *per* mg protein, this value decreasing to less than 150 ng/mg protein upon addition of 2, 4-dinitrophenol [2]. Cell permeability was also tested by measuring, at different time intervals, the efflux of carboxyfluorescein from cells previously loaded with carboxyfluorescein diacetate [11]; as shown in Table 1, the release of carboxyfluorescein after 30 min at 37°C was constantly below 20% of its total intracellular concentration. The microvessels suspension was enriched, with respect to the gray matter, of some enzymatic activities such as α-glutamyltranspeptidase and alkaline phosphatase [12, 13]. The 5′-nucleotidase and of Gln-synthetase activities, which were also present in detectable amounts, appeared to be firmly bound to the microvessels themselves, being not removed by extensive collagenase digestion [14, 15].

Met preloading and uptake experiments

In order to increase their internal concentration of Met, isolated microvessels were resuspended in buffer containing 20 mM Met and then incubated for 20 min at 37°C. They were then filtered on a 86 μm pore size nylon sieve, washed with a spray of ice-cold buffer for 2 min, and resuspended, to a final protein concentration of 1.5–2 mg/ml, in warm Met-free buffer which contained the appropriate labeled amino acid. The uptake was then measured, either as a function of the labeled amino acid, or followed cumulatively for 15–30 min [1]. To this purpose, 0.6 ml portions of the microvessels suspension were withdrawn after the desired incubation time, poured on a 44 μm pore nylon sieve on a vacuum manifold, and washed three times with 5 ml of ice-cold buffer. The sieves with the retained microvessels were then placed in plastic tubes containing 1.8 ml of 1 N NaOH, left overnight at room temperature, and then subjected to sonication for 1 min. Portions were withdrawn for protein determination [16] using serum bovine albumin as standard, while 0.5 ml were transferred to liquid scintillation counting vials containing 0.5 ml of 1 N HCl. After addition

of 10 ml of Aquasol 2[R], the vials were counted in a Beckman LCS 9800 liquid scintillation spectrometer.

Similar experiments were also performed by substituting Met, in the preloading step, with cysteine or cystine and/or methanethiol (either methanethiol or, respectively, cysteine or cystine being then, in some experiments, present together with the labeled amino acid in the uptake step).

Non-specific radioactivity due to the binding of the labeled amino acid to the nylon sieve, which was also tested in triplicate by omitting the microvessels from the assay, was found to be constantly below 70 dpm.

Kinetic analysis

When plotted as a function of amino acid concentration, the initial (≤ 2 min) rate of uptake showed the presence of a saturable component superimposed to a non-saturable one [1, 17]. The data were subjected to non-linear regression analysis in order to obtain the optimal estimates of the kinetic parameters (the non-saturable free diffusional component, K_d; the carrier affinity, K_m; and the maximal transport velocity, V_{max}) and to evaluate the standard error impending on them. Alternatively, the non-saturable component could be graphically evaluated and substracted from the overall experimental curve; the values of the kinetic parameters of the saturable component were then plotted in the S/v *vs* S "Hanes plot", which, according to Wilkinson (18), allows straightforward linear statistical analysis.

Results

Influence of Met on the uptake of hydrophobic neutral amino acids

Met appeared to behave similarly to Gln, (which was itself a competitive inhibitor of Met uptake, with a Ki value of 280 ± 7 μM), being transported by both the A and the L systems. Kinetic analysis of Met uptake by the isolated brain microvessels, performed in Na^+-containing buffer, gave, for the saturable component, a V_{max} value of 900 ± 10 pmoles · mg protein^{-1} · min^{-1} and a K_m value of 200 ± 12 μM. Met uptake was competitively inhibited by MeAIB and BCH, which are specific substrates for the A and for the L system respectively, the K_i values being of 200 ± 9 μM for MeAIB and 339 ± 10 μM for BCH.

Incubation of the isolated microvessles with 20 mM Met, followed by removal of this amino acid from the medium, resulted in a considerable increase in the rate of the subsequent Leu uptake (Table 1). This stimulation of hydrophobic amino acid uptake declined to control values within approximately 15 min after removal of the microvessels from the Met-containing buffer, with a time course similar to that of the efflux of Met from the microvessels. When the cumulative uptake of a hydrophobic amino acid was followed as a function of time, a typical transstimulation pattern could be evidenced (Fig. 1). The demonstration of such a transstimulation phenomenon, as well as the reciprocal stimulation of the rate of Met efflux from the intracellular compartment when Leu or Phe were added to the external medium (Fig. 2), confirm that, like Gln, Met acts indeed by allowing a higher rate of exchange through the L system of transport. A similar effect was exerted on the uptake of other hydrophobic neutral amino acids such as Phe, Tyr, BCH and also of Gln, while the uptake of MeAIB, Lys or Glu remained unaffected (Table 2).

Kinetic analysis of the initial rate of uptake of hydrophobic amino acids by Met-preloaded microvessels showed that preloading with Met affected the maxi-

Table 1. Rate of ^{14}C-Leu uptake (expressed as pmoles · mg protein^{-1} · min^{-1}) measured at fixed time intervals following 20 min preloading with methionine, cysteine, methanethiol or cysteine *plus* methanethiol

Addition during preloading	Interval between end of preloading and beginning of uptake		
	0 min	5 min	15 min
Methionine, 20 mM	35.5 ± 8.0	28.0 ± 3.0	40.5 ± 3.5
Cysteine, 20 mM + Methanethiol, 20 mM	32.5 ± 5.0	25.0 ± 4.5	26.0 ± 5.5
Methanethiol, 20 mM	10.0 ± 1.0	—	9.5 ± 2.0
Cysteine, 20 mM	10.5 ± 1.0	—	11.5 ± 2.0
None (buffer alone)	10.0 ± 1.5	—	10.5 ± 1.0

Each result is the mean value (±S.D.) of three different experiments.

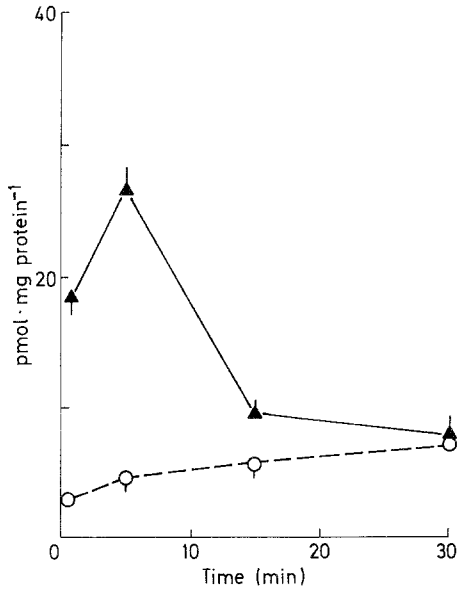

Fig. 1. Overshoot effect in the time course of ^{14}C-Leu uptake by Met-preloaded microvessels (▲) and by microvessels preincubated in Met-free buffer (○). After 20 min of preloading with 20 mM of Met, the microvessels were washed and resuspended in warm (37°C) Met-free buffer. Immediately after the resuspension, labeled Leu was added and the time course uptake followed for 30 min. The data shown are the mean values (±S.D.) of three different determinations

mal influx (V_{max} 646 ± 11 *vs* 284 ± 9 pmol · mg protein^{-1} · min^{-1}) of the saturable component of Leu uptake, while there was little or no modification of either the K_m (148 ± 7 *vs* 144 ± 6 μM) for this amino acid or of the non-saturable diffusional component K_d (0.84 *vs* 0.77 μl · mg protein^{-1} · min^{-1}).

The effect of Met preloading on subsequent hydrophobic neutral amino acid uptake could be counteracted as follows:

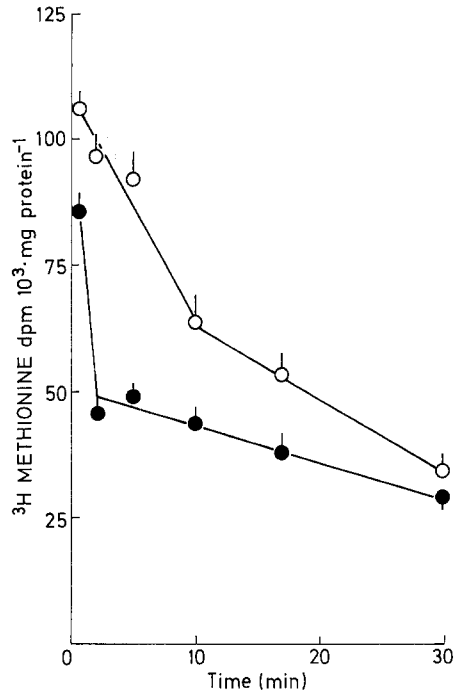

Fig. 2. Effect of the addition of external Leu on Met efflux from brain microvessels. Microvessels were preloaded with ^{3}H-Met (final concentration 20 mM, specific activity 20 mCi/mmol) for 20 min at 37°C, rapidly washed, and then resuspended in warm Met-free buffer (○) or in Met-free buffer containing 0.1 mM (●) cold Leu. At fixed intervals, the isolated microvessels were rapidly washed and the internal radioactivity was measured. The points represent the mean values (±S.D.) of three different determinations

Table 2. Rate of uptake of different ^{14}C-labeled amino acids measured at fixed time intervals following 20 min preloading with either methionine or with 20 mM cysteine + 20 mM methanethiol

Addition during preloading	Labeled amino acid used in the assay	Interval between end of preloading and beginning of uptake		
		0 min	3 min	15 min
Methionine, 20 mM	Gln	68.5 ± 1.0	25.0 ± 4.0	15.0 ± 1.5
	MeAIB	14.5 ± 1.0	14.0 ± 1.0	15.0 ± 5.0
	Lys	6.5 ± 1.5	6.0 ± 1.5	8.5 ± 2.5
	Glu	5.5 ± 1.0	4.0 ± 0.5	6.5 ± 1.5
Cysteine, 20 mM	Gln	70.0 ± 2.0	39.5 ± 0.5	24.5 ± 1.0
+	MeAIB	17.5 ± 5.0	10.5 ± 2.5	12.5 ± 1.0
Methanethiol, 20 mM	Lys	5.0 ± 0.5	4.5 ± 1.0	9.5 ± 2.0
	Glu	9.0 ± 0.5	10.0 ± 3.0	6.0 ± 1.0
None (Buffer alone)	Gln	9.0 ± 0.5	—	9.0 ± 1.0
	MeAIB	11.0 ± 1.5	—	12.5 ± 2.0
	Lys	8.0 ± 0.5	—	8.5 ± 0.5
	Glu	22.0 ± 2.5	—	22.0 ± 1.0

Each result (expressed as pmoles · mg protein^{-1} · min^{-1}) is the mean value (±S.D.) of three different experiments.

Table 3. Rate of ^{14}C-Leu uptake (expressed as pmoles · mg protein^{-1} · min^{-1}) measured at fixed time intervals following 20 min preloading with methionine or with purified S-methylthiocysteine, in the presence or absence of 0.1 mM 2,4 dinitrophenol or of 20 mM MeAIB

Addition during preloading	Interval between end of preloading and beginning of uptake 0 min	3 min
Methionine, 20 mM	35.5 ± 8.0	28.0 ± 3.0
Methionine, 20 mM + DNP, 0.1 mM	11.0 ± 2.0	9.5 ± 1.5
Methionine, 20 mM + MeAIB, 20 mM	16.0 ± 3.0	14.5 ± 2.5
S-methylthiocysteine, 20 mM	36.5 ± 2.0	26.0 ± 5.0
S-methylthiocysteine, 20 mM + DNP, 0.1 mM	11.5 ± 1.5	12.5 ± 2.0
S-methylthiocysteine, 20 mM + MeAIB, 20 mM	12.5 ± 1.5	15.5 ± 2.0
Buffer alone	8.5 ± 1.5	5.0 ± 1.0
Buffer + MeAIB, 20 mM	9.0 ± 1.5	10.0 ± 1.0

Each result is the mean value (±S.D.) of four different experiments.

a) by omitting Na^+ ions from the preincubation medium [4];
b) by including, in the preincubation medium, either the uncoupling agent 2,4-dinitrophenol or substrates (such as MeAIB) which specifically compete with Met for the A-system of active transport (Table 3);
c) by subjecting the microvessels, before preincubation with Met, to an extensive collagenase digestion (Table 4), which inactivates or removes the A-system of transport [19].

Similarly to what had been observed with Gln [2, 3], the Na^+-dependent concentrative A-system of transport appeared therefore to play a crucial role in the uptake of Met by the endothelial cells of the microvessels, this being a pre-requisite for the L-system-mediated stimulation of hydrophobic amino acid exchange.

Methanethiol effect on the uptake of hydrophobic neutral amino acids

Addition of methanethiol to the microvessels had, by itself, no effect on the subsequent uptake of neutral amino acids. A stimulatory effect similar to that

Table 4. Effect of collagenase digestion on the rate of ^{14}C-Leu uptake. Collagenase-treated microvessels, as well as control ones, were preloaded for 20 min with 20 mM S-methylthiocysteine, or with 20 mM cysteine together with 20 mM methanethiol. After the preloading step the microvessels were washed and resuspended in warm 37°C buffer and the rate of uptake of labeled Leu was followed at fixed time intervals

Collagenase treatment	Addition during preloading	Interval between end of preloading and beginning of uptake	
		0 min	5 min
none	S-methylthiocysteine	40.0 ± 2.5	26.0 ± 5.0
none	methanethiol + cysteine	31.5 ± 2.0	24.5 ± 3.0
0.75 mg/ml	S-methylthiocysteine	11.0 ± 2.5	10.0 ± 3.0
0.75 mg/ml	methanethiol + cysteine	37.5 ± 3.0	23.5 ± 2.5
none	buffer	9.0 ± 2.0	6.0 ± 1.5
0.75 mg/ml	buffer	15.0 ± 2.0	14.0 ± 3.0

Each result (expressed as pmoles · mg protein^{-1} · min^{-1}) is the mean value (± S.D.) of three different experiments.

exerted by Met was instead observed upon preincubation of the microvessels with methanethiol in the presence of cysteine or cystine. This behaviour was apparently caused by the formation of a mixed disulfide, S-methylthiocysteine, structurally similar to Met and behaving almost like Met on thin layer chromatography and in standard amino acid analysis [4]. Similarly to what happened with Met, preincubation with purified S-methylthiocysteine could be shown to give a typical transstimulation pattern on the subsequent cumulative uptake of a labeled hydrophobic amino acid (Fig. 3). The effect of S-methylthiocysteine was abolished if the A-system of transport was inhibited by performing the experiment in the absence of Na^+ ions (Fig. 4) or by including either MeAIB or 2, 4-dinitrophenol in the preincubation mixture (Table 3). If the A-system of transport was removed by collagenase digestion, S-methylthiocysteine could no more be concentrated in the endothelial cells and lost therefore its effectiveness, but unpurified mixtures of methanethiol + cysteine were found to be still effective (Table 4); this result indicates that there is, under these conditions, a consistent intracellular formation of S-methylthiocysteine from its "parent compounds" also in the absence of the A-system of transport. It was indeed of interest to observe that, in some experiments, the presence of microvessels appeared to cause a small, but significant, increase in the amount of S-methylthiocysteine formed from methanethiol *plus* cysteine (e.g. 10 nmoles/ml *vs.* 4.2 nmoles/ml).

Discussion

As previously reported, isolated brain microvessels, which can be used as an *in vitro* model of the blood-brain barrier, possess at least two distinct transport systems, named "L" and "A", for neutral amino acids. The characteristics of these systems are the following: the A-system is specific for the small neutral amino

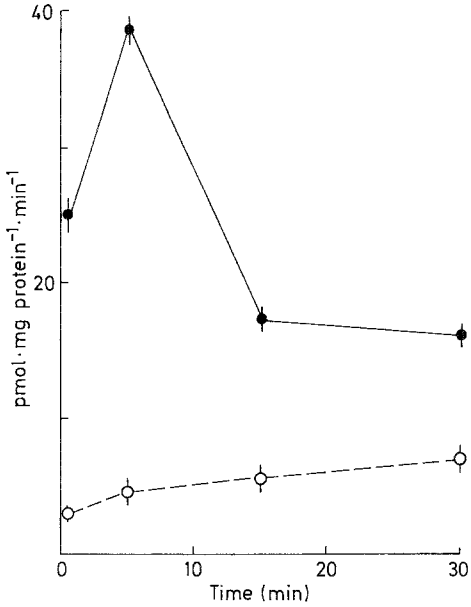

Fig. 3. Progress curves of cumulative uptake of labeled Tyr after preloading with: (●) 20 mM purified S-methylthiocysteine; (○) buffer. Data shown are the mean values (±S.D.) of three different experiments

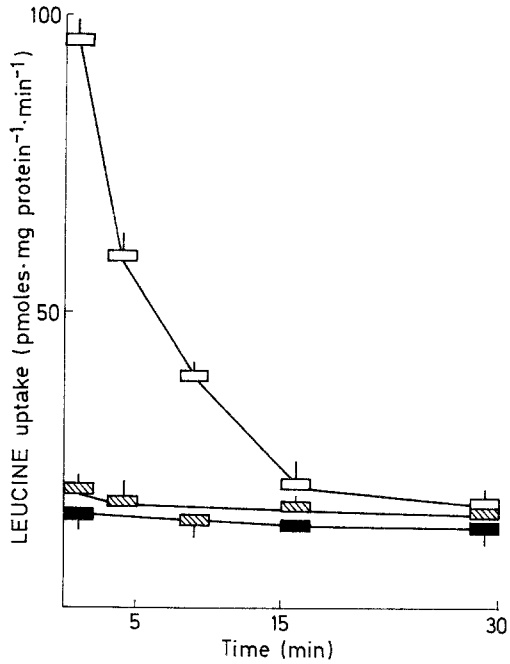

Fig. 4. Rate of Leu uptake measured at fixed intervals after a preloading step performed in the presence (open bars) or absence (shaded bars) of Na^+ ions. The microvessels suspensions were preloaded for 20 min at 37°C, in the presence (▭) or absence (▨) of Na^+ ions, with 20 mM cold S-methylthiocysteine. After the preloading step, the isolated microvessels were rapidly washed and resuspended in warm (37°C) buffer. Labeled Leu (1 μCi/ml) was added at the different time intervals indicated on the abscissa, starting from the resuspension of the microvessels in the warm 37°C buffer after the preloading step, and the uptake in the first minutes was measured. Solid bars indicate the uptake of labeled Leu by microvessels preincubated in buffer alone. The data, expressed as pmoles · mg protein^{-1} · min^{-1}, are the mean values (±S.D.) of three different experiments

acids, is Na^+-dependent, concentrative, has a strong pH sensitivity and is present only on the abluminal side of the brain microvessels; the L-system is specific for the large neutral amino acids, is Na^+-independent, has minimal sensitivity to pH change, possesses very strong exchanging properties, and is active on both the luminal and abluminal sides of the blood-brain barrier. Previous work [1] had shown that the neutral amino acid glutamine can be concentrated inside the endothelial cells *via* the A-system and than exchanged for the large neutral amino acids *via* the L-system of transport. The present work indicates that a similar synergism between the A and the L systems of transport holds also for methionine which, similarly to glutamine, appears to be a dual-affinity amino acid, which can enter the endothelial cells of brain microvessels by using either the Na^+-dependent endoergonic A-system of transport or the Na^+-independent "facilitated" L-system. Under physiological conditions, the bidirectional flux of the neutral amino acids is provided by the close cooperation of the two transport systems, the abluminal A-system being used to concentrate within the endothelial cells long-chain polar aminoacids such as Gln or Met, thus causing an increased rate of exchange through the L-system—and thereby, most probably, a net uptake of hydrophobic aminoacids from the blood to the brain compartment.

A similar effect in enhancing the synergism between the two transport systems can also be attributed to S-methylthiocysteine, an analog of Met which can be formed upon combination of methanethiol with cysteine or half-cystine [4]. As previously reported [4] S-methylthocysteine can indeed utilize both the A and the L systems of amino acid transport. A peculiarity of S-methylthiocysteine, as compared to Met and/or Gln, is the possibility of being directly formed within the intracellular compartment, overcoming the limitations of the A-system of transport. At variance with the NH_4^+-induced synthesis of Gln, which strictly depends on the presence of the glutamino synthetase, the formation of S-methylthiocysteine from its precursors is apparently spontaneous.

Pathological conditions such as severe hepatic failure and portal-systemic shunting are characterized, in patients as well as in experimental animals, both by increased plasma concentrations of NH_4^+ ions and mercaptans and by increased concentrations in plasma, in liquor and in brain tissue of aromatic amino acids and of phenylethyl- and phenylethanolamines with relatively low hydroxylation levels [6, 7, 8, 18]. In previous studies using isolated brain microvessels as an *in vitro* model of the blood-brain barrier, we have checked [2] the validity of the NH_4^+—Gln hypothesis proposed by James et al. (20); this hypothesis can be extended, by the present work, to the methanethiol—S-methylthiocysteine pair: since also S-methylthiocysteine can be exchanged, similarly to Gln, for the neutral hydrophobic amino acids, it seems likely that methanethiol, *via* the S-methylthiocysteine synthesis, can act synergistically to the ammonia—Gln system in causing hepatic encephalopathy.

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References

1. Cangiano C, Cardelli-Cangiano P, James JH, Rossi Fanelli F, Patrizi MA, Brackett KA, Strom R, Fischer JE (1983) J Biol Chem 258: 8989–8954
2. Cardelli-Cangiano P, Cangiano C, James JH, Ceci F, Fischer JE, Strom R (1984) J Biol Chem 259: 5295–5300
3. Strom R, Cardelli-Cangiano P, Fiori A, Ceci F, Rossi Fanelli F, Cangiano C (1984) In: Kleinberger G, Ferenci P, Riederer P, Thaler H (eds) Advances in hepatic encephalopathy and urea cycle diseases. Karger, Basel, pp 273–289
4. Cardelli P, Cangiano C, Fiori A, Cascino A, Ceci F, Rossi Fanelli F, Strom R (1991) Neurochem Int 19: 45–49
5. Cascino A, Cangiano C, Calcaterra V, Rossi Fanelli F, Capocaccia L (1978) Am J Dig Dis 23: 591–598
6. Zieve L, Doizaki WM (1976) Gastroenterology 79: 2–6
7. Al Mandrini H, Bartlett K, Record CO (1984) Gut 25: 284–290
8. Zieve L, Doizaki WM, Zieve FJ (1974) J Lab Clin Med 83: 16–27
9. Awwad AD, Aldstein SS (1966) Anal Biochem 16: 433–437
10. Hjelle TJ, Baird-Lambert J, Cardinale G, Spector S, Udenfriend S (1978) Proc Natl Acad Sci U.S.A. 75: 4544–4548
11. Strom R, Scioscia Santoro A, Crifò C, Bozzi A, Mondovi B, Rossi Fanelli A (1973) Eur J Cancer 9: 103–112
12. Orlowski M, Meister A (1965) J Biol Chem 240: 338–347
13. Torak RM, Barnett RJ (1964) J Neuropathol Exp Neurol 23: 46–59
14. Gerlach U, Hiby W (1974) In: Bergmeyer HV (ed) Methods in enzymatic analysis, vol 2. Academic Press, New York, pp 871–873
15. Wellner VP, Meister A (1966) Biochemistry 5: 872–879
16. Lowry OH, Rosenbrough NH, Farr AL, Randall RJ (1951) J Biol Chem 193: 265–275
17. Cardelli-Cangiano P, Cangiano C, James JH, Jeppsson B, Brenner W, Fischer JE (1981) J Neurochem 36: 627–632
18. Wilkinson GN (1961) Biochem J 80: 324–332
19. Cardelli-Cangiano P, Fiori A, Cangiano C, Barberini F, Allegra P, Peresempio V, Strom R (1987) J Neurochem 49: 1667–1675
20. James JH, Ziparo V, Jeppsson B, Fischer JE (1979) Lancet ii: 772–776

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