BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **241,** 127–131 (1997)
ARTICLE NO. RC977779

Evidence for an Asymmetrical Uptake of L-Carnitine in the Blood-Brain Barrier *in Vitro*

Joanna E. Mroczkowska,* Hans-Joachim Galla,† Maciej J. Nałęcz,* and Katarzyna A. Nałęcz*,[1]
**The Nencki Institute of Experimental Biology, 3 Pasteur St., 02-093 Warsaw, Poland; and †Institut für Biochemie, Westfälische Wilhelms-Universität, Wilhelm-Klemm-Str. 2, D-48149 Münster, Germany*

Received October 27, 1997

The transport of L-carnitine (4-N-trimethylammonium-3-hydroxybutyric acid) was studied with a primary culture of porcine brain capillary endothelial cells (BCEC) as an *in vitro* model of the blood-brain barrier. The measurements with suspended cells and cell monolayers allowed to distinguish a polarized transport phenomena. The part of the BCEC cells exposed to the medium (apical membrane) accumulated carnitine by a sodium-independent, saturable (K_m=28 μM) system, with k=0.018 min^{-1}. Exposure of the basolateral part revealed a presence of a facilitated diffusion process. Carnitine uptake through the saturable system was inhibited by butyrobetaine. Acylcarnitines and choline have no effect on the carnitine accumulation in suspended cells, a process diminished by phenylalanine, leucine, and L system inhibitor. This points to the possibility that carnitine enters through the basolateral membrane using amino acid transporting systems. A different, novel system is postulated to operate in the apical part of the plasma membrane of BCEC.

Carnitine (4-N-trimethylammonium-3-hydroxybutyric acid) is known to be involved in peripheral tissues, such as the liver, kidney and muscles, in a process of long-chain fatty acids transfer from the cytosol to the mitochondrial matrix where these acids are further metabolized. The pathway for carnitine-dependent transport of fatty acids through the inner mitochondrial membrane, the so called "carnitine shuttle", consists of several enzymes synthesizing acylcarnitine derivatives on the cytosolic side of the outer mitochondrial membrane and of enzymes transferring the acyl moieties to CoASH inside mitochondria. There is also a central translocation step occurring through the carnitine carrier transporting carnitine and its acyl derivatives through the inner mitochondrial membrane (1).

In adult brain, where glucose is the main energetic substrate (2), the isolated carnitine carrier (3, 4) was postulated to deliver mitochondrial acetyl moiety to cytoplasm (5), supplying a substrate for acetylcholine synthesis (6, 7), what could explain the beneficial efects of carnitine administration to patients with neurodegenerative diseases (8). Carnitine, although *in vivo* is mainly synthesized in the liver (9), accumulates in nervous tissue (10, 11). The content of carnitine in brain was observed to be lower than in peripheral tissues, with the exception of hypothalamus (10), which is known to have an easy access to many substances through fenestrations. This observation may point to the existence of some limitation in carnitine crossing the blood-brain barrier.

The accumulation of any compound in the brain depends on the selectivity of the brain capillary endothelial cells connected by tight junctions and providing a permeability barrier between blood and brain fluids (12). The uptake of choline into brain of nonanaesthesized rats was reported to be diminished by 20% in the presence of 500 μM carnitine (13). The accumulation of carnitine in immortalized rat brain capillary endothelial cells RBE4 (14) was, however, found to be insensitive to choline or hemicholinium-3, a specific inhibitor of choline transport (15). Due to the fact, that adenovirus transfection and several passages could be responsible for some phenotype changes, the present study was focused on the characteristics of carnitine accumulation in the primary culture of brain endothelial cells, in order to clarify a possible involvement of choline transporter in carnitine accumulation.

MATERIALS AND METHODS

Materials. L-[methyl-^{3}H]carnitine, inulin[^{14}C]carboxylic acid and [methyl-^{14}C]thymidine were purchased from Amersham. Medium M199, antibiotics and antimycotics were from Gibco, ox serum was

[1] To whom correspondence should be addressed. Fax: 48-22-822-53-42. E-mail: knal@nencki.gov.pl.

Abbreviations: BCH, 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid; MeAIB, N-(methylamino)-isobutyric acid.

0006-291X/97 $25.00

obtained from PAA Laboratories. Collagen G was from Seromed. Acylcarnitine derivatives were delivered by Serva, L-carnitine was from Fluka. Butyrobetaine (3-carboxypropyl)trimethyl-ammonium) was synthesized by methylation of GABA by Dr. J. Boksa in the Institute of Pharmacology, Cracow. All other reagents were purchased from Sigma.

Accumulation of carnitine. Brain endothelial capillary cells (BCEC) were obtained from porcine brains according to the method of Mischeck et al. (16). The cells were grown to confluence on a plastic surface covered with collagen G (0.2 mg/ml) in 10% ox serum, 90% medium M199, containing [methyl-^{14}C]thymidine (1 Ci/mol), 25 mM bicarbonate, 100 U penicillin/ml, 100 μg streptomycin/ml, 0.25 μg fungizone/ml, pH 7.4 in a humid atmosphere of 5% CO_2, at 37°C. The cells that reached full confluence were used in experiments (usually on the 7th or 8th day after isolation). Experiments to determine carnitine accumulation were performed either with cells in suspension or in a monolayer, as described in (14). The unspecific binding was subtracted from the content of inulin, administered to the same samples.

Calculation of kinetic data. When the accumulation of carnitine was measured as a function of time, the obtained graphs were fitted to the first order rate equation, y=limit*(1-Exp(-k*t)), where y and limit denominate the amount of product at a given and infinite time t, respectively. The results obtained in two iterations, with $p<0.05$ were taken into account.

RESULTS

Any compound capable of crossing the blood-brain barrier should be transported into the capillary endothelial cells, in addition having the possibility of being further transferred into the intracerebral fluid. In order to cross the plasma membrane, an ionized, low molecular weight compound can be transported either by a facilitated diffusion, or in an uphill transport, coupled to a process in which other molecules (mainly Na^+) move down their free energy gradient (18). Therefore, when studying the transport of carnitine in BCEC cells, it seemed crucial to clarify whether carnitine accumulation, if any, is coupled with a [Na^+] gradient and whether some of the known transporters could be involved in this process.

As shown in Fig.1A, the BCEC cells accumulate carnitine. The amount of this compound taken up by the cells in suspension, when measured as a function of time, resulted in a curved graph, that could be fitted to the first-order rate kinetics with the velocity constant of $k = 0.018 \pm 0.0004$ min^{-1}. The accumulation was inhibited by 36% after preincubation with ouabain, a well established inhibitor of Na,K-ATPase (19). A decrease of carnitine accumulation was observed to be more pronounced (70% inhibition) in the presence of amiloride and furosemide, the compounds known to block the Na-channel and Na/K/2Cl transporter, respectively (20). On the contrary, when the uptake of carnitine was followed in cells grown in monolayer, no [Na^+] gradient dependence was detected - neither of the sodium transport inhibitors revealed any effect (Fig.1B).

When looking for a system that might be responsible

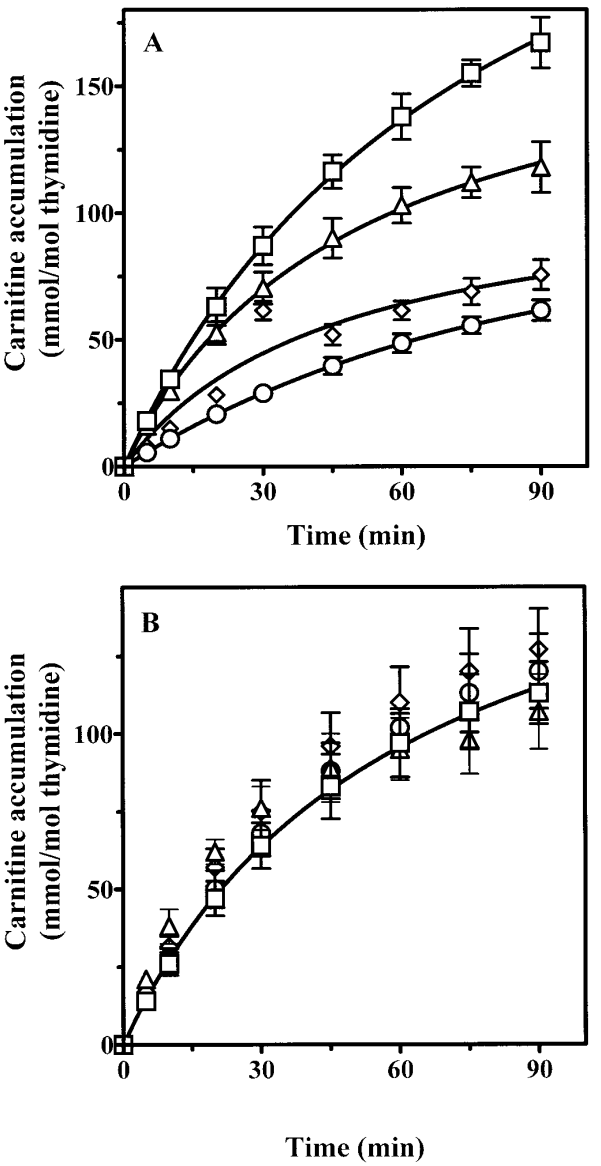

FIG. 1. Time dependence of carnitine accumulation in NB-2a cells. BCEC cells were incubated under conditions described in the Materials and Methods section. The accumulation of carnitine was measured either in suspension (A) or in a cell monolayer (B) in the absence of any additions (squares) or after 30 min preincubation with 0.5 mM ouabain (triangles), 100 μM amiloride and 1 mM furosemide (diamonds), or all inhibitors (circles). Curve fitting was performed as given in the Materials and Methods section.

for carnitine binding and transport in BCEC cells, one has to take into consideration the transporting systems that have been described to be present in the blood-brain barrier. Choline is structuraly related to carnitine, since, in comparison with this compound, is lacking a carboxyl group. The overall uptake of choline by brain (13) was reported to be diminished by concentration of carnitine exceeding 10 times those found in plasma under physiological conditions, i.e. being 10-80

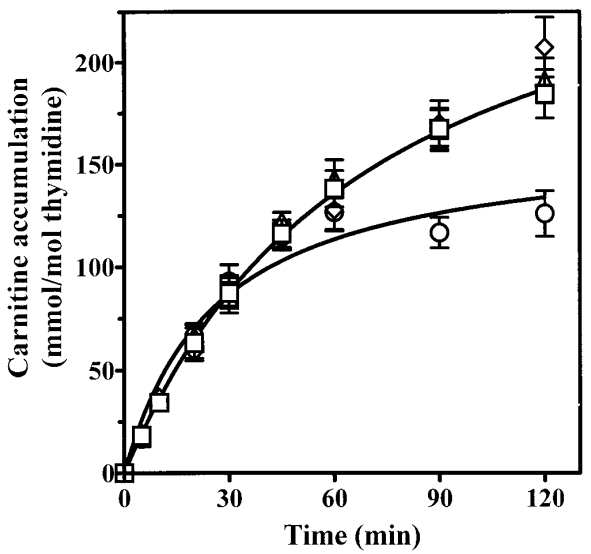

FIG. 2. Effect of choline on accumulation of carnitine in BCEC cells. Cells were incubated in the presence of L-[methyl-^{3}H]carnitine for the indicated times either without any additions (squares) or with choline at 10 μM (triangles) and 1 mM (circles) concentrations. The samples treated with 1 μM hemicholinium-3 (diamonds) were preincubated for 1 h, according to (15). The results represent means±SD from 9 independent experiments.

μM in most mammals (21). As presented in Figure 2, choline was observed to have no effect on carnitine accumulation when applied at a saturating concentration of the high affinity system (22, 23). Carnitine uptake was not changed in the presence of hemicholinium-3, an inhibitor of this system (15). The higher concentration of choline, exceeding saturation of the low affinity system (22), decreased carnitine accumulation measured after longer times, but did not, however, affect the initial velocity of carnitine uptake.

Taking into account the chemical structure of carnitine, a possible involvement of systems transporting the other, structurally related compounds, as amino acids and betaines has been studied in more detail. As presented in Table 1, butyrobetaine, leucine, BCH, and phenylalanine decreased carnitine accumulation by 40-50%, as measured in suspended cells. Betaine, itself, although structurally similar to butyrobetaine does not inhibit carnitine uptake, neither did carnitine acyl derivatives of different chain lengths. MeAIB - an analog of alanine and a known substrate of an A system transporting amino acids did not reveal any effect either. The effect of substances influencing carnitine accumulation was studied further in a monolayer system. Only butyrobetaine was observed to decrease carnitine accumulation through the part of cellular membrane exposed to the incubation medium.

Accumulation of carnitine, measured as a function of its concentration, did not reach saturation even at such high concentrations as 5 mM (Fig. 3A, *inset*). The straight line illustrating accumulation of carnitine as a function of its concentration might be interpreted as a diffusion process, with a diffusion constant of 44 ± 3 mmol/min/mM/mol thymidine. Taking into account this value, the diffusion component was subtracted from the total uptake measured at the lower concentration ranges. The differences obtained in such a way could be fitted to the Michaelis-Menten equation with K_m of 33 ± 4 μM (Fig. 3A). A further kinetics analysis was performed with the monolayer of BCEC cells. As visualized in Fig. 3B, this uptake was also observed to increase with carnitine concentration and the values of carnitine accumulation can be fitted to the Michaelis-Menten equation with K_m of 28 ± 6 μM. This result, as well as the unsuccessful attempts to fit the calculated function from Fig. 3A to the equations describing two systems, could suggest that the saturable component of carnitine accumulation is responsible for uptake of this compound through the apical membrane, whilst the process of carnitine accumulation through the basolateral part of plasma membrane occurs due to a facilitated diffusion.

DISCUSSION

There are reports indicating that the localization of transport systems was different on the brain and blood sides of the blood-brain barrier (24). Our experiments on carnitine transport in the primary culture of BCEC cells demonstrate that additional phenomena were detected when cells were detached from the surface, they were cultured on. There were substantial differences

TABLE 1

Effect of Various Compounds on the Initial Velocity of Carnitine Accumulation in BCEC Cells

	Carnitine accumulation (% of control)	
Addition	Cell suspension	Cell monolayer
None	100	100
Betaine	93 ± 8 (21)	93 ± 10 (16)
Butyrobetaine	64 ± 4 (19)	69 ± 13 (12)
Acetylcarnitine	102 ± 3 (13)	nd
Octanoylcarnitine	114 ± 15 (6)	nd
Palmitoylcarnitine	105 ± 7 (8)	nd
MeAIB	113 ± 10 (5)	102 ± 9 (4)
Leucine	59 ± 6 (7)	110 ± 12 (3)
BCH	55 ± 6 (12)	103 ± 15 (15)
Phenylalanine	44 ± 6 (15)	134 ± 15 (15)

Note. The uptake measurements were performed either in suspension or with a monolayer, as described in the Materials and Methods section. All listed compounds were added at a 1 mM concentration. The number of measurements performed is indicated in parentheses. nd, not determined.

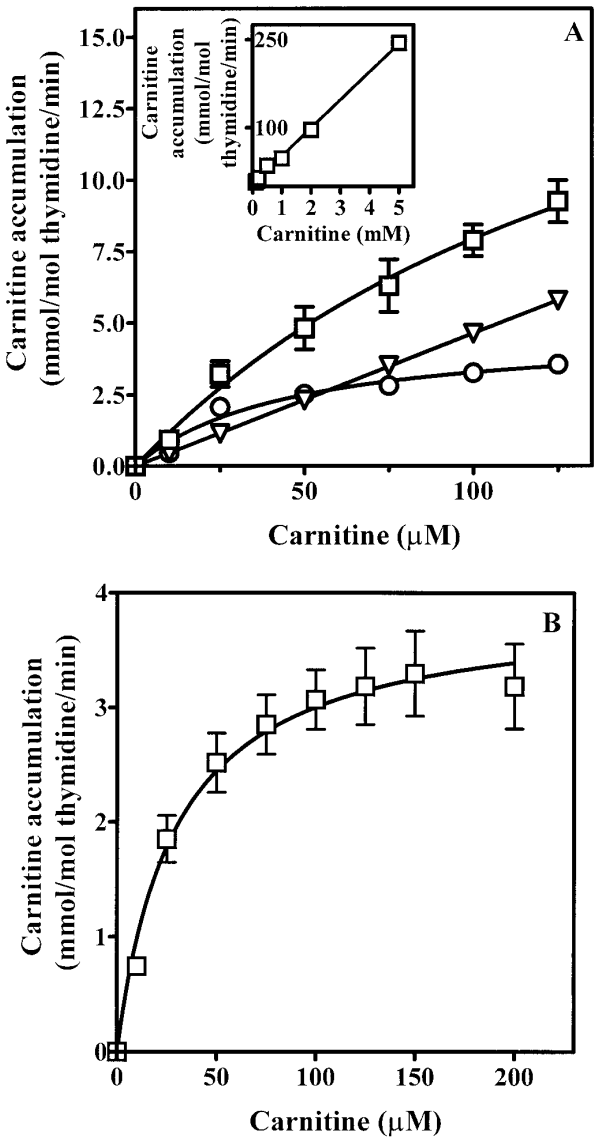

FIG. 3. Concentration dependence of carnitine accumulation in BCEC cells. The cells were incubated in the presence of various concentrations of L-[methyl-^{3}H]carnitine either in suspension (A) or in monolayer (B). The velocities were estimated from the initial rates, measured over 10 min. (A) Analysis of total carnitine accumulation (squares) into diffusion (triangles) and carrier-mediated transport (circles). The diffusion constant was estimated from the velocities measured at high substrate concentrations (*inset*). (B) Accumulation of carnitine in BCEC monolayers. The results of A and B represent means ± SD from three separate experiments.

observed in the sodium gradient dependence, kinetics mechanism and parameters, as well as an influence of different substances affecting carnitine transport followed in both experimental systems. Carnitine accumulation was demonstrated to be Na-independent in the monolayers. On the contrary, when the other side of the cell monolayer was exposed after suspension of BCEC cells, the sodium transport inhibitors partially diminished accumulation of carnitine, pointing to the existence of a different transporting system, dependent on a [Na^+] concentration gradient. It should be emphasized, that the contribution of ouabain-sensitive carnitine accumulation did not exceed 30% of the total uptake, indicating the fact that the main accumulation process is Na-independent, a situation different from those reported for the carnitine carriers in neurones (17), fibroblasts (25), and kidney (26). It is worth mentioning that a similar asymmetry was reported for the amino acids transporting systems, in case of which the sodium-dependent systems are restricted to the abluminal side of brain capillary endothelium (27).

Carnitine, with its ionized trimethylamino- (positively charged) and carboxyl- (negatively charged) groups can be treated as a neutral γ-amino acid and, due to the fact that some amino acids inhibited its uptake by 40%, an involvement of amino acid transporting system seems to be quite probable. Leucine, BCH and also phenylalanine (27, 29) are the known substrates of system L, a system assumed to be present in both membranes of endothelial cells, however, known to be Na-independent. Functioning of systems A and $B^{o,+}$, that transport several amino acids in a Na-dependent way, was ascribed to the abluminal membrane (29). These two systems were distinguished due to their different sensitivity towards MeAIB (29). Since the carnitine accumulation was observed to be MeAIB insensitive, the involvement of the $B^{o,+}$ can be postulated in the mechanism of carnitine accumulation in BCEC cells through the basolateral membrane.

In the monolayer, the only compound affecting carnitine accumulation was butyrobetaine, pointing to the necessity of trimethylammonium- and carboxyl functional groups for transport. The hydroxyl group of carnitine molecule appeared to be less important, since choline was without any effect. Such an observation was also reported for the endothelial cells from rat brain (14), indicating the similarity of observed phenomena in the cells in primary culture and the immortalized cell line. Contrary to observations on the carnitine transporter from kidney (26), various acylcarnitines did not affect carnitine accumulation in BCEC cells. Therefore, it can be concluded that a novel transporting system, Na-independent and sensitive to butyrobetaine, is responsible for carnitine accumulation in BCEC cells through the apical membrane. The novel system described herewith would be responsible for an increase of carnitine content in the brain, especially that its affinity toward carnitine is comparable with physiological carnitine concentration in blood.

ACKNOWLEDGMENTS

This work was supported by the Volkswagen Foundation (Grant I/70 192) and the Foundation for Polish-German Cooperation (Grant 1935/95/LN). The authors thank Professor E. Przegaliński from the Institute of Pharmacology in Cracow, Poland, for the arrangement

of butyrobetaine synthesis, for which Dr. J. Boksa is gratefully acknowledged. We thank Mr. Tomasz Calikowski, Mrs. Magdalena Bachowska-Mac, and Mr. Dirk Grieschie for their help in the preliminary experiments performed for this study.

REFERENCES

1. Bremer, J. (1983) *Physiol. Rev.* **63,** 1421–1480.
2. Nehlig, A., and Pereira De Vasconcelos, A. P. (1993) *Prog. Neurobiol.* **40,** 163–221.
3. Kamińska, J., Nałęcz, K. A., Azzi, A., and Nałęcz, M. J. (1993) *Biochem. Mol. Biol. Int.* **29,** 999–1007.
4. Kamińska, J., Nałęcz, K. A., and Nałęcz, M. J. (1995) *Acta Neurobiol. Exp.* **55,** 1–9.
5. Nałęcz, K. A., and Nałęcz, M. J. (1996) *Acta Neurobiol. Exp.* **56,** 597–609.
6. Wawrzeńczyk, A., Nałęcz, K. A., and Nałęcz, M. J. (1994) *Biochem. Biophys. Res. Commun.* **202,** 354–359.
7. Wawrzeńczyk, A., Nałęcz, K. A., and Nałęcz, M. J. (1995) *Neurochem. Int.* **26,** 635–641.
8. Rebouche, C. J. (1992) *FASEB J.* **6,** 3379–3386.
9. Bøhmer, T. (1974) *Biochim. Biophys. Acta* **343,** 551–557.
10. Bresolin, N., Freddo, L., Vergani, L., and Angelini, C. (1982) *Expl. Neurol.* **78,** 285–292.
11. Shug, A. L., Schmidt, M. J., Golden, G. T., and Fariello, R. T. (1982) *Life Sci.* **31,** 2869–2874.
12. Pardridge, W. M. (1983) *Physiol. Rev.* **63,** 1481–1535.
13. Cornford, E. M., Braun, L. D., and Oldendorf, W. H. (1978) *J. Neurochem.* **30,** 299–308.
14. Mroczkowska, J. E., Roux, F., Galla, H.-J., Nałęcz, M. J., and Nałęcz, K. A. (1996) *Neurosci. Res. Commun.* **19,** 153–160.
15. Happe, H. K., and Murrin, L. C. (1993) *J.Neurochem.* **60,** 1191–1201.
16. Mischeck, U., Meyer, J., and Galla, H.-J. (1989) *Cell Tiss. Res.* **256,** 221–226.
17. Nałęcz, K. A., Korzon, D., Wawrzeńczyk, A., and Nałęcz, M. J. (1995) *Archiv. Biochem. Biophys.* **322,** 214–220.
18. West, I. C. (1980) *Biochim. Biophys. Acta* **604,** 91–126.
19. Uesugi, S., Dulak, N. C., Dixon, J. F., Hexum, T. D., Dahl, J. L., Perdue, J. F., and Hokin, L. E. (1971) *J. Biol. Chem.* **246,** 531–543.
20. Betz, A. L. (1983) *J. Neurochem.* **41,** 1158–1164.
21. Rebouche, C. J. (1989) *Biochim. Biophys. Acta* **1033,** 111–113.
22. Yamamura, H. I., and Snyder, S. H. (1973) *J. Neurochem.* **21,** 1355–1374.
23. Estrada, C., Bready, J., Berliner, J., and Cancilla, P. A. (1990) *J. Neurochem.* **54,** 1467–1473.
24. Betz, A. L., and Goldstein, G. W. (1978) *Science* **202,** 225–226.
25. Tein, I., Bukovac, S., and Xie, Z-W. (1996) *Archiv. Biochem. Biophys.* **329,** 145–155.
26. Berardi, S., Hagenbuch, B., Carafoli, E., and Krähenbühl, S. (1995) *Biochem. J.* **309,** 389–393.
27. Brendel, K., Meezan, E., and Carlson, E. C. (1974) *Science* **185,** 953–955.
28. Sánchez del Pino, M. M., Hawkins, R. A., and Peterson, D. R. (1992) *J. Neurochem.* **267,** 25951–25957.
29. Sánchez del Pino, M. M., Peterson, D. R., and Hawkins, R. A. (1995) *J. Biol. Chem.* **270,** 14913–14918.