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Characteristics of L-Carnitine Transport in Cultured Human Hepatoma HLF Cells

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Abstract

The recently cloned organic cation transporter, OCTN2, isolated as a homologue of OCTN1, has been shown to be of physiological importance in the renal tubular reabsorption of filtered L-carnitine as a high-affinity Na^+ carnitine transporter in man. Although the mutation of the OCTN2 gene has been proved to be directly related to primary carnitine deficiency, there is little information about the L-carnitine transport system in the liver. In this study, the characteristics of L-carnitine transport into hepatocytes were studied by use of cultured human hepatoma HLF cells, which expressed OCTN2 mRNA to a greater extent than OCTN1 mRNA.

The uptake of L-carnitine into HLF cells was saturable and the Eadie–Hofstee plot showed two distinct components. The apparent Michaelis constant and the maximum transport rate were $6{\cdot}59 \pm 1{\cdot}85\,\mu$M (mean ± s.d.) and $78{\cdot}5 \pm 21{\cdot}4$ pmol/5 min/10^6 cells, respectively, for high-affinity uptake, and $590 \pm 134\,\mu$M and 1507 ± 142 pmol/5 min/10^6 cells, respectively, for low-affinity uptake. The high affinity L-carnitine transporter was significantly inhibited by metabolic inhibitors (sodium azide, dinitrophenol, iodoacetic acid) and at low temperature (4°C). Uptake of [^{3}H]L-carnitine also required the presence of Na^+ ions in the external medium. The uptake activity was highest at pH 7·4, and was significantly lower at acidic or basic pH. L-Carnitine analogues (D-carnitine, L-acetylcarnitine and γ-butyrobetaine) strongly inhibited uptake of [^{3}H] L-carnitine, whereas β-alanine, glycine, choline, acetylcholine and an organic anion and cation had little or no inhibitory effect.

In conclusion, L-carnitine is absorbed by hepatocytes from man by an active carrier-mediated transport system which is Na^+-, energy- and pH-dependent and has properties very similar to those of the carnitine transporter OCTN2.

L-Carnitine is as an essential co-factor for transport of long-chain fatty acids across the mitochondrial inner membrane (Fritz 1959). In man it is synthesized only in the liver, kidney and brain, although its precursor, γ-butyrobetaine, is synthesized from L-lysine and L-methionine in all tissues (Siliprandi et al 1990). Despite this, the concentration of L-carnitine in tissues is higher than in plasma (Yokogawa et al 1999), and this concentrative tissue distribution suggests that there is an active cell-membrane transport system for L-carnitine. Indeed, Berardi et al (1995) have shown that size-fractionated rat renal-cortex mRNA induced threefold L-carnitine transport compared with unfractionated mRNA in *Xenopus laevis* oocytes and indicated that the L-carnitine transport is Na^+-dependent. Stieger et al (1995) reported that L-carnitine transport by rat kidney brush-border membranes is Na^+-dependent, and the transport is greater in vesicles with an inside-negative membrane potential, suggesting an electrogenic mechanism. McCloud et al (1996) reported that L-carnitine uptake by intestinal epithelial (Caco-2) cells involves a carrier-mediated system which is temperature-, Na^+-, and ATP-dependent.

In 1988, systemic L-carnitine deficiency mice, denoted juvenile visceral steatosis mice, were

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found in the Laboratory Animal Center of our University (Koizumi et al 1988) and it was suggested they lacked carrier-mediated transport of L-carnitine in the whole body (Hashimoto et al 1998; Yokogawa et al 1999). These mice develop a systemic disorder, including lipid accumulation in the liver, similar to systemic carnitine deficiency in man (Treem et al 1988; Breningstall 1990). Therefore, it is important to clarify the characteristics of the hepatic carnitine transporter. We have recently demonstrated that OCTN2 (organic cation transporter), isolated as a homologue of the organic cation transporter OCTN1 (Tamai et al 1997), is a physiologically important high-affinity Na^+ carnitine transporter in man (Tamai et al 1998). We also showed that a mutation of the OCTN2 gene is directly related to carnitine deficiency both in mice and man (Nezu et al 1999). However, there is little information on the L-carnitine transport system in the liver in man, because it is difficult to use normal hepatocytes for basic studies.

In this study we have investigated the kinetics of the L-carnitine transport system in cultured hepatoma HLF cells from man.

Materials and Methods

Materials

L-Methyl-[^{3}H]carnitine (sp. act. 85 Ci mmol^{-1}) was purchased from Amersham (Buckinghamshire, UK). L-Carnitine, D-carnitine, L-acetylcarnitine, betaine, γ-butyrobetaine, choline chloride, acetylcholine hydrochloride, γ-aminobutyric acid, L-glycine, β-alanine, *p*-aminohippuric acid, tetraethylammonium, sodium azide, dinitrophenol, and iodoacetic acid were purchased from Sigma (St Louis MO). Other chemicals were of analytical grade.

Cell culture

Human hepatoma HLF cells were grown in Dulbecco's modified Eagle's medium containing 20 mM HEPES, 100 μg mL^{-1} kanamycin sulphate, and 10% foetal bovine serum, pH 7·4. Cells were usually seeded into 100-mm plastic culture dishes at a subcultivation ratio of 1 : 3 and grown to confluence at 37°C in a humid atmosphere of 5% CO_2. The culture medium was replaced with fresh medium every 3 days.

Reverse transcriptase-polymerase chain reaction assay

Messenger ribonucleic acid (mRNA) was prepared from HLF cells by means of a QuickPrep micro mRNA purification kit (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions. Reverse transcription reactions were performed in 40 mM KCl, 50 mM Tris–HCl (pH 8·3), 6 mM $MgCl_2$, 1 mM dithiothreitol, 0·1 mg mL^{-1} bovine serum albumin (BSA), 1 mM each dATP, dCTP, dGTP, and dTTP, 10 units RNase inhibitor (Promega, Madison, WI), 100 pmol random hexamer, mRNA and 200 units Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL, Berlin, Germany) in a final volume of 50 μL at 37°C for 60 min. Polymerase chain reaction (PCR) was conducted in a final volume of 20 μL containing 5 μL reverse transcription reaction mixture, 50 mM KCl, 20 mM Tris–HCl (pH 8·3), 2·5 mM $MgCl_2$, 0·1 mg mL^{-1} BSA, 0·2 mM each dATP, dCTP, dGTP, and dTTP, 10 μM of each of the mixed oligonucleotide primers, and 1 unit Taq DNA polymerase (Gibco-BRL). Each cycle consisted of 45 s at 94°C, 60 s at 58°C, and 75 s at 72°C. Reverse transcription reactions performed with 1 μg mRNA and PCR were run for 30 cycles for the reverse transcription-PCR reaction. The sequences of the OCTN1 primers from man were 5′-CAC AGC TGT ACC AGC AGT GAT ATT-3′ (1061–1084) and 5′-TGG ATA AGA AGT AAT AAT CCA CA-3′ (1403–1425) (Tamai et al 1997). The sequences of the OCTN2 primers in man were 5′-TTG AGA TGT TTG TCG TGC TG-3′ (701–720) and 5′-GAA GCA GAT CCA GAA TGT TGT-3′ (1109–1129) (Tamai et al 1998). The sizes of the predicted fragments amplified by PCR are 365 and 429 base pairs, respectively. The sequences of the β-actin primers were given in a previous paper (Waki et al 1995).

Transport study

Cells were peeled from the plastic culture dishes, the culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) solution (2 mL). These cells were adjusted to a concentration of 3×10^6 cells mL^{-1} in Krebs–Ringer phosphate buffer (NaCl 123 mM, KCl 4·93 mM, $MgSO_4$ 1·23 mM, $CaCl_2$ 0·85 mM, glucose 5 mM, glutamine 5 mM, NaH_2PO_4 20 mM, pH 7·4) and pre-incubated for 15 min at 37°C. [^{3}H] L-Carnitine (1·27 nM) was then added to the medium, and samples were taken at designated times from 1 to 180 min. Each sample was centrifuged at 4°C and 1000 *g* for 5 min and the supernatant was removed by aspiration. The pellet was immediately washed twice with ice-cold PBS solution, then digested with NaOH (1 M, 0·5 mL; 1 h at 70°C), and neutralized with HCl (1 M, 0·5 mL). After addition of scintillation fluid (ACS II; Amersham, Arlington Heights IL), the mixture was held at room

temperature for 15 h and the radioactivity was measured by liquid scintillation counting (Aloka LSC-5100, Tokyo, Japan).

The uptake studies were performed at 4°C and 37°C and, unless otherwise stated, intracellular accumulation of L-carnitine was estimated by subtracting the uptake at 4°C from that at 37°C.

Data analysis

The parameters of the Eadie–Hofstee analysis were estimated by the non-linear least-squares method using the Multi program (Yamaoka et al 1981). Student's *t*-test was used to compare the unpaired means of two sets of data. The number of determinations (n) is noted in the tables and figures. A P value of 0·05 or less was taken as indicative of a significant difference between sets of data.

Results

Uptake kinetics of [^{3}H]L-carnitine

We confirmed that HLF cells expressed OCTN2 mRNA more highly than OCTN1 mRNA by reverse transcription-PCR assay (Figure 1).

Figure 2 shows the time courses of 1·27 nM [^{3}H]L-carnitine uptake by HLF cells at 4 and 37°C. The uptake of [^{3}H]L-carnitine at 4°C was significantly lower than that at 37°C, and at 5 min the uptake of [^{3}H]L-carnitine at 4°C was about one-tenth that at 37°C. The net uptake of [^{3}H]L-carnitine from zero to 15 min at 37°C increased linearly and the uptake rate was 41·7 fmol/15 min/10^6 cells. On the basis of these results, a 5-min incubation time was adopted as the standard to determine the initial rate of uptake of [^{3}H]L-carnitine by HLF cells.

As shown in Figure 3, uptake as a function of L-carnitine concentration was saturable and the Eadie-Hofstee plot clearly indicated two distinct components. For the high-affinity component the apparent Michaelis constant (Km) = 6·59 ± 1·85 μM and the maximum transport activity (V_{max}) = 78·5 ± 21·4 pmol/5 min/10^6 cells; for the low-affinity component Km = 590 ± 134 μM and V_{max} = 1507 ± 142 pmol/5 min/10^6 cells.

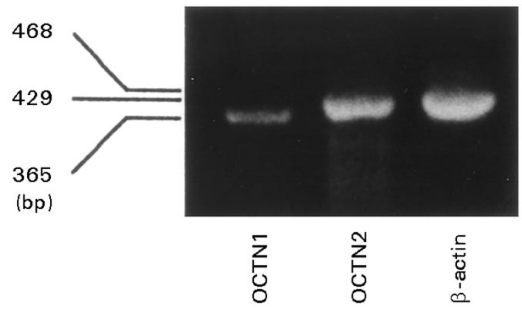

Figure 1. Expression of OCTN1 and OCTN2 mRNAs in HLF cells evaluated by reverse transcription-PCR.

Na^+-dependence of [^{3}H]L-carnitine uptake

To examine the effect of Na^+ ions on the high-affinity transport of [^{3}H]L-carnitine (1·27 nM) by HLF cells, Na^+ was replaced iso-osmotically with chloride salts of other monovalent cations (K^+, Li^+, and choline), or with inert mannitol, at pH 7·4. As shown in Table 1, the uptake of [^{3}H]L-carnitine by HLF cells was significantly reduced by replacing Na^+ ion with other cations or with mannitol.

When the cells were pre-incubated with ouabain (1 mM), an Na^+,K^+-ATPase inhibitor, for 30 min at pH 7·4 at 37°C, the initial uptake rate of [^{3}H]L-carnitine was slightly but significantly reduced ($P < 0·001$); 14·7 ± 0·2 (mean ± s.e.m., n = 4) in the absence of ouabain and 11·4 ± 0·1 (n = 4) fmol/5 min/10^6 cells in its presence.

Energy-dependence of the uptake of [^{3}H]L-carnitine

After treatment with 10 mM sodium azide, dinitrophenol or iodoacetic acid for 30 min at pH 7·4 and 37°C, HLF cells were incubated with 1·27 nM [^{3}H]L-carnitine for 5 min. The initial rate of uptake of [^{3}H]L-carnitine was significantly reduced by ca 25–75% by these compounds (Table 2).

pH-dependence of the uptake of [^{3}H]L-carnitine

Table 3 shows the uptake of 1·27 nM [^{3}H]L-carnitine by HLF cells at external medium pH 5·5, 6·5, 7·4 and 8·5. The uptake activity was highest at pH 7·4, and was significantly lower at lower or higher external medium pH.

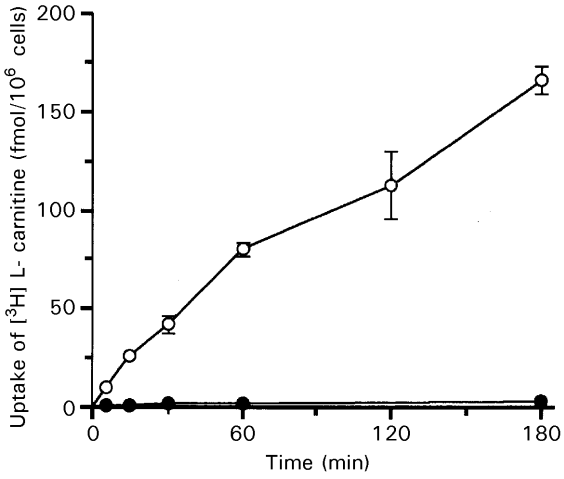

Figure 2. Time-course and temperature-dependency of the uptake of [^{3}H]L-carnitine by HLF cells at pH 7·4. [^{3}H]L-Carnitine was added after 15 min pre-incubation of HLF cells at 4°C (●) or 37°C (○). Each point represents the mean ± s.e.m. of results from four experiments.

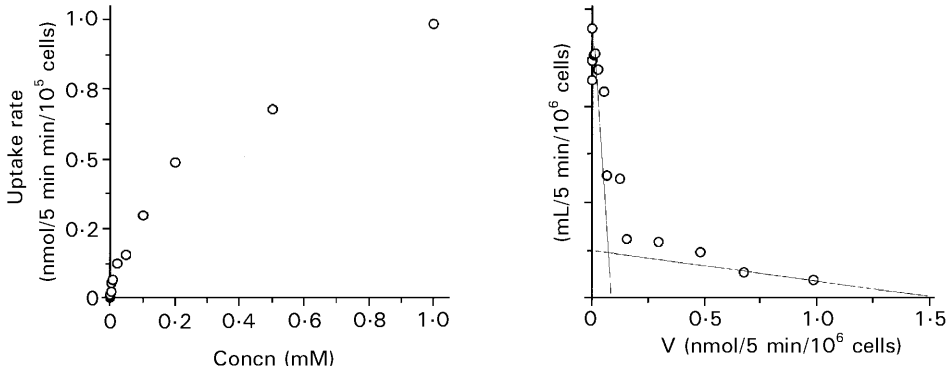

Figure 3. Uptake of L-carnitine by HLF cells as a function of concentration at pH 7·4 and 37°C. HLF cells were pre-incubated for 15 min in Krebs–Ringer buffer in the presence of NaCl, then different concentrations of carnitine were added to the incubation medium and the uptake of carnitine was measured after 5 min. The concentration of [^{3}H]L-carnitine was kept constant at 1·27 nM in this experiment and the concentration of carnitine was varied (range 10 nM–1 mM), as desired, by addition of unlabeled carnitine. A. The carrier-mediated uptake rate of L-[^{3}H]carnitine was estimated by subtracting the uptake at 4°C from that at 37°C. B. An Eadie-Hofstee plot shows the dependence of the uptake rate (V) on uptake rate/carnitine concentration (V/S).

Table 1. Na^+-dependence of the uptake of [^{3}H]L-carnitine by HLF cells at pH 7·4 and 37°C.

Conditions	Uptake rate (fmol/5 min/10^6 cells)
Control (Na^+, 123 mM)	12·4 ± 0·3
K^+ (123 mM)	3·05 ± 0·17*
Li^+ (123 mM)	3·48 ± 0·05*
Choline (123 mM)	3·74 ± 0·07*
Mannitol (246 mM)	3·46 ± 0·31*

HLF cells were pre-incubated for 15 min in Krebs–Ringer buffer containing Na^+, or in buffer in which Na^+ was replaced with K^+, Li^+, choline or mannitol; [^{3}H]L-carnitine (1·27 nM) was then added and the incubation continued for 5 min. Each value is the mean ± s.e.m. of results from three experiments. *$P < 0.001$ compared with control.

Table 2. Energy-dependence of the uptake of [^{3}H]L-carnitine by HLF cells at pH 7·4 and 37°C.

Compound	Uptake (%)
Sodium azide (10 mM)	75·2 ± 1·8*
Dinitrophenol (10 mM)	48·7 ± 2·9**
Iodoacetic acid (10 mM)	25·9 ± 2·4**

HLF cells were pre-incubated for 30 min in Krebs–Ringer buffer containing 10 mM sodium azide, dinitrophenol or iodoacetic acid; [^{3}H]L-carnitine (1·27 nM) was then added and the incubation continued for 5 min. Each value is the percentage uptake of [^{3}H]L-carnitine in medium containing sodium azide, dinitrophenol or iodoacetic acid compared with that in control medium without these compounds. Each value is the mean ± s.e.m. of results from four or five experiments. *$P < 0.01$, **$P < 0.001$ compared with control.

Inhibitory effects of structural analogues, and an organic anion and cation on the uptake of [^{3}H]L-carnitine

To determine the substrate selectivity of the carnitine transport process we examined the inhibition of [^{3}H]L-carnitine uptake by various structural analogues. As shown in Table 4, the L- and D-carnitine isoforms (each 10 μM) both significantly inhibited the uptake of [^{3}H]L-carnitine by HLF cells. However, the inhibitory effect of L-carnitine was stronger than that of D-carnitine (see also Figure 4), and the effect of γ-butyrobetaine, a precursor of carnitine biosynthesis, was even stronger than that of L-carnitine, though that of betaine was weaker. The effect of acetylcarnitine was approximately equal to that of L-carnitine. γ-Aminobutyric acid, β-alanine and glycine had little effect on carnitine transport even at 1 mM. Similarly, choline and acetylcholine had no influence on the carnitine transport process. The inhibitory effects of *p*-aminohippuric acid and tetraethylammonium on the uptake of 1·27 nM [^{3}H] L-carnitine are shown in Table 5. The inhibitory effects of these compounds at 100 μM were very weak, but at 1 mM both significantly inhibited carnitine uptake, although much less potently than did 10 μM L-carnitine (Table 4).

Table 3. Effect of the pH of the medium on the initial rate of uptake of [^{3}H]L-carnitine by HLF cells at 37°C.

pH	Uptake rate (fmol/5 min/10^6 cells)
5·5	3·9 ± 0·3**
6·5	10·6 ± 0·9*
7·4	14·2 ± 0·7
8·5	10·9 ± 0·3*

HLF cells were pre-incubated for 15 min in Krebs–Ringer buffer of different pH values at 37°C; [^{3}H]L-carnitine (1·27 nM) was then added and incubation was continued for 5 min. Each value is the mean ± s.e.m. of results from five experiments. *$P < 0.05$, **$P < 0.001$ compared with medium at pH 7·4.

Discussion

Many reports suggest the existence of a high-affinity carnitine transporter in the plasma membrane

Table 4. Inhibitory effect of structural analogues on the uptake of L-carnitine by HLF cells.

Compound	Concentration (mM)	Uptake (%)
L-Carnitine	10	29·3 ± 2·7**
D-Carnitine	10	60·7 ± 8·2*
γ-Butyrobetaine	10	25·4 ± 0·9**
Betaine	10	62·5 ± 2·1*
L-Acetylcarnitine	10	34·9 ± 0·6**
γ-Aminobutyric acid	1	78·8 ± 2·4
β-Alanine	1	79·4 ± 2·2
Glycine	1	103·2 ± 1·2
Choline	1	80·7 ± 4·9
Acetylcholine	1	97·3 ± 5·4

HLF cells were pre-incubated for 15 min in Krebs–Ringer buffer at 37°C; [^{3}H]L-carnitine (1·27 nM) was then added and incubation was continued for 5 min. Data (uptake of [^{3}H]L-carnitine as a percentage of that in the absence of the analogues) are means ± s.e.m. of results from three experiments. *$P < 0.05$, **$P < 0.01$ compared with control.

Table 5. Inhibitory effect of an organic anion and an organic cation on the uptake of L-carnitine by HLF cells.

Compound	Concentration (mM)	Uptake (%)
p-Aminohippuric acid	0·10	89·7 ± 4·0
	1	63·8 ± 3·7*
Tetraethylammonium	0·1	94·3 ± 2·6
	1	65·7 ± 4·3*

HLF cells were pre-incubated for 15 min in Krebs–Ringer buffer at 37°C; [^{3}H]L-carnitine (1·27 nM) was then added and incubation was continued for 5 min. Data (uptake of L-[^{3}H]carnitine as a percentage of that in the absence of the ions) are means ± s.e.m. of results from three experiments. *$P < 0.05$ compared with control.

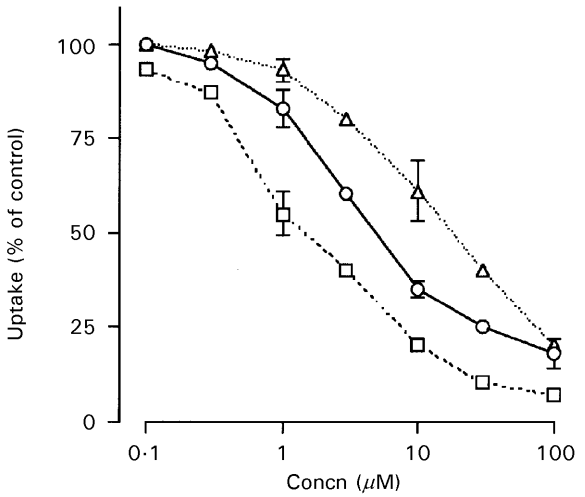

Figure 4. Concentration-dependence of the inhibitory effect of L-carnitine (○),D-carntine (△) and γ-butyrobetaine (□) on the uptake of [^{3}H]L-carnitine by HLF cells at 37°C. HLF cells were pre-incubated for 5 min in Krebs–Ringer buffer at pH 7·4 and 37°C, then 1·27 nM [^{3}H] L-carnitine and a test compound (0·1–100 μM) were added and incubation was continued for 5 min. Each point represents the mean ± s.e.m. of results from three experiments.

of several tissues (Vary & Neely 1982; Shaw et al 1983), but little is known about the physiological functions of the carnitine transporter in the liver. We have suggested, on the basis of a comparison of juvenile visceral steatosis homozygous mutant mice (*jvs/jvs*) with the corresponding wild-type mice (+/+), that a carrier-mediated L-carnitine transporter is present in the plasma membrane of most tissues in wild-type mice (Hashimoto et al 1998; Yokogawa et al 1999) and reported that OCTN2, recently cloned from the kidney of man, is an L-carnitine transporter (Tamai et al 1998). In that paper we showed that the OCTN2 transcript is highly expressed in the kidney, skeletal muscle, heart, and placenta, but only weakly expressed in the liver and other organs. However, we also reported that several cancer-derived cell-lines all strongly expressed OCTN2 mRNA. To examine the characteristics of the L-carnitine transporter in hepatocytes from man, we thus employed the hepatoma cell line HLF from man. As expected, HLF cells strongly expressed OCTN2 mRNA, but only weakly expressed OCTN1, which has high homology with OCTN2 (Tamai et al 1997). The L-carnitine-uptake profile of HLF cells had two components: K_m for high affinity uptake = 6·59 μM and K_m for low affinity uptake = 590 μM. The high-affinity transport of L-carnitine into HLF cells was Na^+ ion- and energy-dependent (Tables 1 and 2), in agreement with our findings using OCTN2-transfected embryonic kidney HEK293 cells from man (Tamai et al 1998). Further, the K_m for high affinity uptake is very similar to the value previously reported for the high-affinity carnitine transporter in other tissues from rodents (Bohmer et al 1977; Martinuzzi et al 1991).

The pH-dependency of the transport process (Table 3) was similar to that of the rat renal carnitine Na^+-dependent transport system expressed in *Xenopus laevis* oocytes (Berardi et al 1995). It is possible that the membrane transport properties are influenced by extracellular pH, because the conformation of the transporter protein in the plasma membrane would be affected by pH changes.

Although carnitine is a zwitterionic substance, it is unlikely that the uptake of carnitine by HLF cells involves the organic-cation or -anion transporter, because the inhibitory effects of *p*-aminohippuric acid and tetraethylammonium were very weak (Table 5). We showed by use of a competition assay that the carnitine transporter can recognize different structural analogues, such as γ-butyrobetaine, L-acetylcarnitine, and betaine, with clear stereoselectivity, but not amino acids or choline compounds (Table 4). These results also agree well with those obtained with OCTN2-transfected HEK

cells (Tamai et al 1998). Thus we conclude that hepatoma HLF cells from man take up L-carnitine through the OCTN2 transporter or a very closely related transporter.

γ-Butyrobetaine was a stronger competitor than L-carnitine and other compounds tested not only in this study using HLF cells (Figure 4), but also in the previous study using OCTN2-transfected HEK cells (Tamai et al 1998). It is well known that γ-butyrobetaine is a precursor in the biosynthesis of L-carnitine, and that in the liver it is converted to L-carnitine by butyrobetaine hydroxylase (Frederic et al 1998). We consider that hepatocytes might actively take up γ-butyrobetaine via the L-carnitine transport system and convert it to L-carnitine. It is possible that the physiological substrate of the carnitine transporter in HLF cells is γ-butyrobetaine rather than L-carnitine, although further experiments are needed to confirm this.

In conclusion, high- and low-affinity L-carnitine transport occurs in human hepatoma HLF cells. The high-affinity transporter is Na^+-, pH- and energy-dependent and has very similar properties to OCTN2. Cancer cells might strongly express the carnitine transporter to supply their energy demand. Loss of the transporter in otherwise normal hepatocytes is likely to result in severe L-carnitine deficiency, because the liver would be unable to take up large amounts of L-carnitine or its precursor γ-butyrobetaine, resulting in severe disorders of liver metabolism.

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