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CARNITINE UPTAKE INTO HUMAN HEART CELLS IN CULTURE

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Summary

The uptake of radiolabeled carnitine and butyrobetaine has been studied in human heart cells (CCL 27). The uptake of carnitine is 3—10-fold higher in heart cells than in fibroblasts (pmol · μg DNA^{-1}). The uptake of carnitine increases with temperature coefficient K_T of 1.6 in the interval 10—20°C and with a negligible uptake at 4 and 10°C. The uptake of carnitine follows Michaelis-Menten kinetics with a K_M of 4.8 ± 2.2 μM and V = 8.7 ± 3.2 pmol · μg $DNA^{-1} \cdot h^{-1}$. Carnitine uptake is suppressed 90% by NaF (24 mM). Butyrobetaine is taken up into heart cells to the same extent as carnitine with a K_M of 5.7—17.3 μM and V = 8.7—9.3 pmol · μg $DNA^{-1} \cdot h^{-1}$. Butyrobetaine inhibits competitively the uptake of carnitine and carnitine inhibits the uptake of butyrobetaine to the same extent. No conversion of radiolabeled butyrobetaine to carnitine, or carnitine to methyl choline was observed intra- or extracellulary during incubation. These data are compatible with a selective transport mechanism for carnitine which is also responsible for the uptake of butyrobetaine.

Introduction

Carnitine (L-3-hydroxy-4-*N*-trimethylamino-butyrate) is synthesized from butyrobetaine only in the liver, the conversion of lysine to trimethyllysine and butyrobetaine, however, takes place in most tissues of the rat [1,2]. Thus, butyrobetaine is transported to the liver for conversion to carnitine which is then rapidly transported to a number of tissues [3].

Although different organs are perfused with blood having the same carnitine concentration, the carnitine concentration varies from tissue to tissue. It is specially high in heart and epididymis (60- and 500-fold higher than in plasma [4,5]), indicating that a specific concentrative mechanism for carnitine is present in these tissues.

The presence of such a carnitine concentrative mechanism in heart is also expected both from clinical observations and experimental models in animals: (a) two families with carnitine deficiency have recently been described [6–8], one of these has a reduced level of carnitine in muscle and a normal level in plasma [7]; (b) the lysine deficient rat shows a low level of carnitine in heart and muscle and normal level in the liver [10]; (c) there is a low level of carnitine in the heart of guinea-pigs infected with diptheria [10], there is also a reduced level of carnitine in the failing heart [11] and in mitochondria isolated from dog heart with experimental coronary stenosis [12].

The results of this study show the presence of a concentrative mechanism for carnitine which is most likely an active selective transport.

Materials and Methods

Materials. L-Carnitine was a gift from Otsuka Pharmaceutical Company, Osaka, Japan; butyrobetaine (deoxycarnitine) was obtained from Sigma. St. Louis, U.S.A. [*Me*-^{3}H]Butyrobetaine (spec. act. 143 Ci/mol) was synthesized as previously described [13]. L-[*Me*-^{3}H]Carnitine (spec. act. 80 Ci/mol) was a gift from Professor J. Bremer.

Trypsin was obtained from Difco Laboratories, Detroit, Mich., U.S.A. and Eagle's minimum essential medium with Earle's balanced salt solution from Grand Island Biological Company, New York, U.S.A. The Girardi Human heart cells (CCL 27) and the mouse fibroblast (L 929) were obtained from American Type Culture Collection Cell Repository, Rockville, M.

The tissue culture medium was made from Eagle's minimal essential medium to which the following were added: penicillin 10^5 I.U./l; streptomycin, 100 mg/l; bovine serum (heat inactivated at 56°C for 30 min) 100 ml/l and $NaHCO_3$ 2.2 g/l. The pH of the medium was adjusted to 7.3 by blowing a current of 5% CO_2 in air through the medium for 20 min.

Tissue culture technique. $3 \cdot 10^6$ cells were seeded in Carell flasks and grown for 24 h at 37°C with 5% CO_2 in air as the gaseous phase ($5–6 \cdot 10^6$ cells after 24 h). The growth medium was decanted and the cells were subsequently incubated for 2 h (4 h in experiments depicted in Fig. 1) in 5 ml Eagle's minimal essential medium with/without 10% calf serum. Radioactively labeled carnitine/butyrobetaine were added as indicated in the legends to the figures 370 000–1 500 00 dpm per incubation mixture.

After incubation, the incubation mixture was poured off and the cells were treated with 3 ml 0.05% trypsin at 37°C for 10 min in 1 molar ethylendiamintetraacetic acid, transferred into tubes and centrifuged at $800 \times g$ in cold for 5 min. The cells were twice washed with 1 ml of saline and subsequently extracted with warm trichloroacetic acid which extracted DNA and carnitine.

For separation of methylcholine, butyrobetaine and carnitine an aliquot of the incubation mixture and of trichloroacetic acid extract, after removal of the trichloroacetic acid by three times extraction with 5 volumes of diethylether, was evaporated to dryness under a current of air. The sediment was dissolved in ethanol and chromatographed on thin-layer silicic acid plates [13].

Radiolabeled carnitine and acylcarnitines were separated as previously described [13].

The packed cell volume was determined using a hematocrit centrifuge with cells suspended in a small volume of buffered salt solution after trypsination. Carnitine was determined as previously described [14] and DNA according to Burton [15].

Radioactive counting. Radioactivity was determined by dissolving the samples in Diluene scintillator solution (Packard Chem. Corp.) and counted in a Tri-Carb liquid scintillation spectrometer.

Results and Discussion

Both heart cells and fibroblasts showed a linear uptake of tritiated carnitine with time (Fig. 1). The uptake in heart cells was more than three times higher than in fibroblasts. The heart cells at zero time contained 0.95—1.0 nmol L-carnitine (enzymatically determined) in a volume of 10—12 μl, and the intracellular concentration of carnitine was about 90 μM. The established cell line thus contains less carnitine than heart cells in vivo. Up to 18% of the carnitine initially present (17.5 nmol) in the incubation mixture was taken up into the heart cells (3.6 nmol). The concentration of L-carnitine in the incubation mixture was only 3.5 μM so it was obvious that the heart cells were actually accumulating carnitine against a concentration gradient. During this incubation the heart cells increased their content of L-carnitine three to four times, but the level of carnitine is still lower than in the adult heart cell.

Chromatography of the cell extracts and the incubation mixture revealed no conversion of radiolabeled butyrobetaine to carnitine or conversion of radiola-

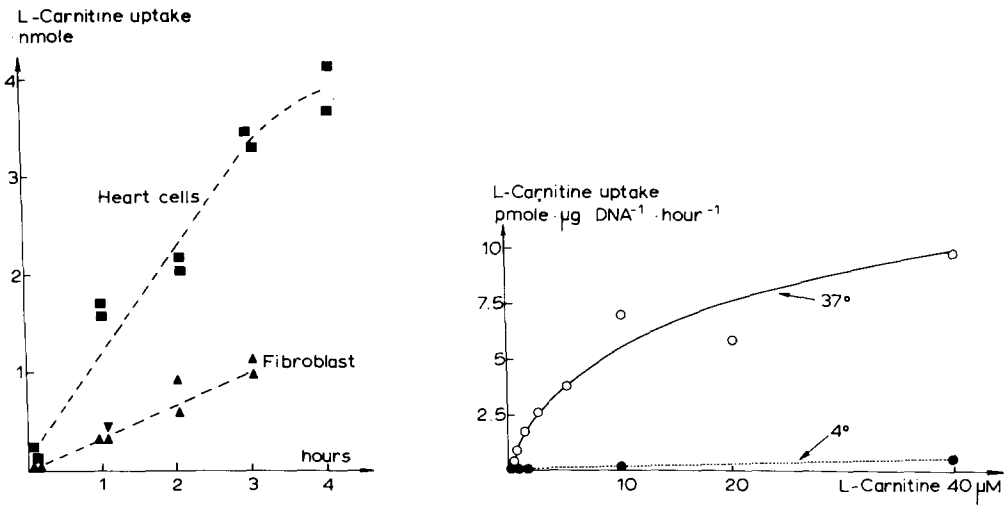

Fig. 1. Carnitine uptake in human heart cells and in fibroblasts. The cells were incubated in 5 ml Eagle's minimal essential medium without calf serum in the presence of L-carnitine 3.5 μM up to 4 h at 37°C. The DNA content was 130—180 μg/dish both in heart cells and fibroblasts. The total uptake is given for heart cells (●) and for fibroblasts (▲).

Fig. 2. Carnitine uptake in human heart cells at 4 and 37°C. The heart cells were incubated for 2 h at 37°C (○) or 4°C (●) with increasing concentrations of L-carnitine as given along the abscissa. The uptake is given as pmol · μg DNA^{-1} · h^{-1}.

beled carnitine to methylcholine. The radioactivity recovered in the trichloroacetic acid extracts therefore was taken as the uptake of L-[*Me*-^{3}H]carnitine and [*Me*-^{3}H]butyrobetaine, respectively in experiments with these substrates.

A hundred percent of the cells were viable (excluded tryphan blue) and the uptake of carnitine was not changed by the absence/presence of serum which was omitted in subsequent experiments.

The fate of the carnitine taken up the cells were also studied. Free carnitine accounted for at least 45% of all radiolabeled carnitine when the cells were incubated in the presence of 5 mM glucose, 2 mM caprylic acid or no addicitional substrate at all (not shown). Thus, sequestration of carnitine or binding as long-chain acylcarnitines (less than 3%) did not account for the ability to taken up carnitine against a gradient.

Very low uptake of carnitine occurs at 4°C (Fig. 2). The total uptake at 37°C was much higher and was saturated with physiological concentrations (10—40 μM) of carnitine in the medium. Similar experiments with tritiated butyrobetaine also showed low proportional uptake of butyrobetaine at 4°C and saturation with increasing concentration of substrate at 37°C (Fig. 3). The absolute uptake of butyrobetaine at 4 and 37°C was of the same magnitude as observed for carnitine, thus both the precursor and carnitine are concentrated to the same extent. The data from the carnitine uptake experiments (total uptake at 37°C minus uptake at 4°C), were also plotted as a Lineweaver-Burk plot, (Fig. 4). Carnitine is taken up according to Michaelis-Menten kinetics with a K_M = 4.3 μM and a V = 8.3 pmol · h^{-1} · μg DNA^{-1} (graphically determined). The absolute uptake of L-carnitine in heart cells, however, showed wide variation in different experiments.

The kinetic constants were therefore calculated by fitting the data to a hyperbolic curve as described by Cleland [16,17]; carnitine K_M = 4.8 ± 2.2 μM (mean ± S.D.), butyrobetaine K_M = 5.7—17.3 μM; carnitine V = 8.7 ± 3.2 and butyrobetaine V = 8.7—9.3 pmol · μg DNA^{-1} · h^{-1}.

Cells prelabeled with 680 pmol [^{3}H]carnitine showed, when incubated in carnitine free medium, an exit of about 45 pmol · h^{-1} of [^{3}H]carnitine. This

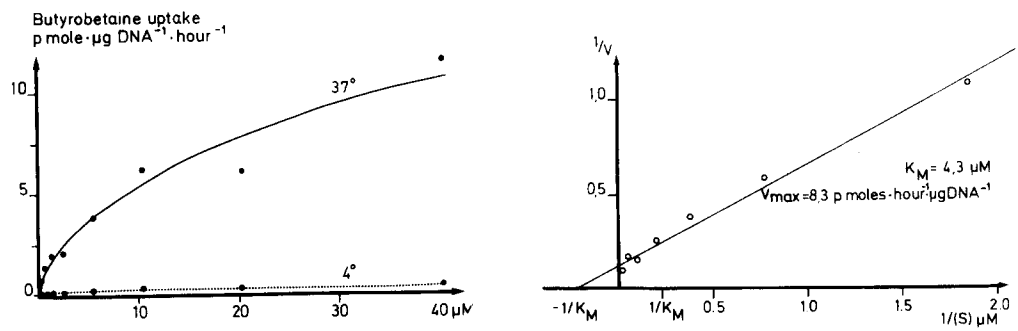

Fig. 3. Butyrobetaine uptake in human heart cells at 4 and 37°C. The heart cells were incubated for 2 h at 37°C (●) or 4°C (●) with increasing concentrations of butyrobetaine as given along the abscissa.

Fig. 4. Lineweaver-Burk plots of carnitine uptake. The data have been plotted inversely 1/[S] (1/substrate concentration) against 1/V (1/net uptake). The net uptake is taken as total uptake at 37°C minus uptake at 4°C. The kinetic parameters have been determined graphically from the plot.

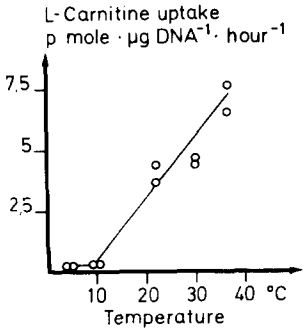

Fig. 5. The effect of temperature on the uptake of carnitine. The incubations were done in temperature-adjusted waterbaths (4, 10, 30, 37°C) and at room temperature (22°C) in the presence of L-carnitine 4.5 µM.

means that about 7% of intracellular carnitine is released per hour. The efflux is small compared to the rate of uptake (0.5 versus 5—10 pmol · h^{-1} · µg DNA^{-1}), and in absolute terms the rate of uptake therefore should be increased with 5—10%. It is rather difficult to achieve saturating concentrations intracellulary of [^{3}H]carnitine to test the maximal efflux. This gives rise to an apparent discrepancy between uptake and efflux. The low efflux means, however, that in our experiments we are mainly observing an unidirectional transport of [^{3}H]-carnitine.

Incubation of the cells at low temperature (Fig. 5) showed only a low uptake of L-carnitine at 4 and 10°C with a rapid rise occurring with increasing temperature up to 37°C. The temperature coefficient K_T was about 1.6 in the interval of 10—20°C. Due to the considerable experimental error we have not tried to fit the data to an Arrhenius plot.

NaF (24 mM) inhibited the carnitine uptake with 90% and 2—4 dinitrophenol (1 mM) reduced the uptake 20% (Table I). There was no effect of NaN_3 (0.5

TABLE I

THE EFFECT OF INHIBITORS AND DENATURATION ON THE CARNITINE UPTAKE

The inhibitors NaN_3 and NaF were added immediately before incubation, in experiments with 2—4 dinitrophenol the cells were also preincubated for 1 h at 37°C with the inhibitor prior to the addition of the substrate. Cells were denatured with 10% trichloroacetic acid for 10 min and subsequently washed (trichloroacetic treated), or heated for 30 min at 60°C (heat denatured) prior to incubation (Expt. 3).

Treatment	Uptake L-carnitine (pmol · µgDN^{-1} · h^{-1}	
Expt. 1 Control	3.3	3.1
NaF, 6 mM	1.1	2.6
NaF, 24 mM	0.36	0.22
Expt. 2 Control	8.7	8.2
2—4 dinitrophenol 0.1 mM	7.9	8.1
2—4 dinitrophenol 0.5 mM	6.2	7.1
NaN_3 0.5 mM	7.5	8.7
Expt. 3 Control	3.3	2.8
Trichloroacetic acid treated	0.10	0.07
Heat-denatured	0.02	0.02

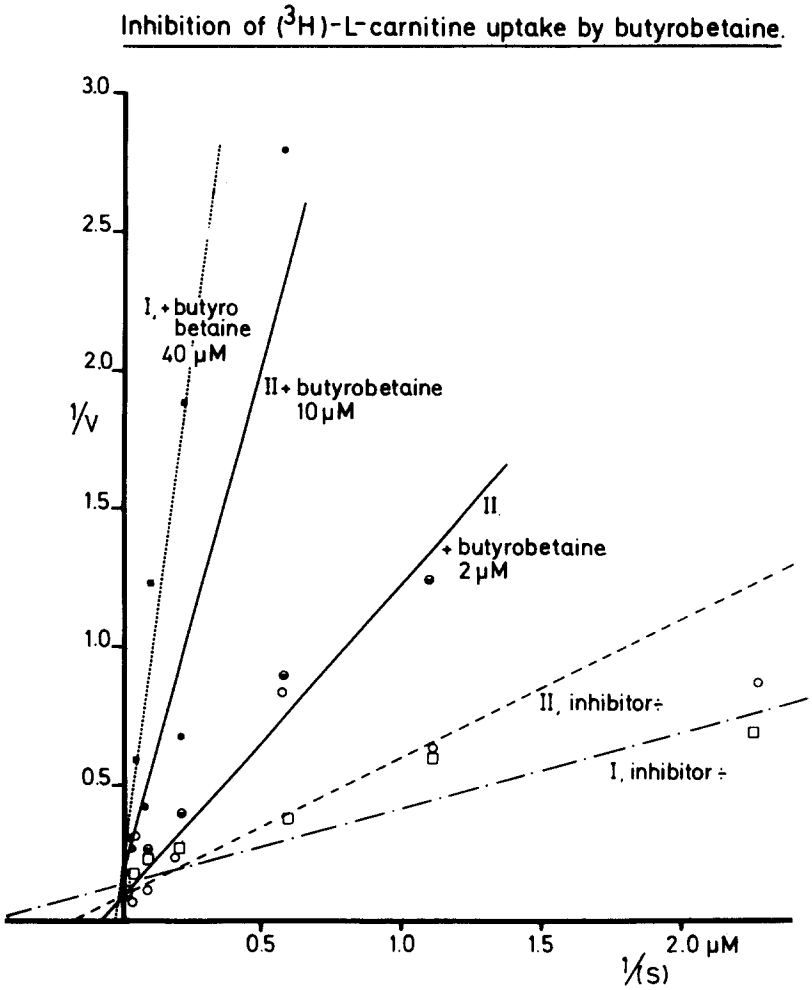

Fig. 6. Competitive inhibition of the carnitine uptake by butyrobetaine. The kinetic parameters were calculated by a computer program [16,17] fitting the data to three different models of inhibition: competitive, non-competitive and uncompetitive. The unexplained variance which remained was lowest when the inhibition was assumed to be of the competitive type. With increasing concentrations of the inhibitor (2, 10, 40 μM) the K_i (inhibitor constant of the slope) was 0.44, 1.5, 3.5 μM, and the $1/V$ (intercept of the y-axis) was 0.07, 0.07, and 0.12, respectively. There is some variation between the two experiments without inhibitor (I and II, inhibitor —). I, inhibitor — (□); II, inhibitor — (○); I, inhibitor + (■); II, inhibitor + (⊖ and ●).

mM) or with higher concentrations (5 mM, not shown). Denaturation of the heart cells with heat or trichloroacetic acid effectively reduced the carnitine uptake.

Thus, the uptake of L-carnitine seems to be dependent upon an active metabolism in the heart cells and is not due to unspecific protein binding.

To reveal a possible competition for the concentrative mechanism the uptake of radiolabeled carnitine was studied in the presence of butyrobetaine (Fig. 6). The slopes of the lines (when plotted $1/V$ versus $1/[S]$ increase with increasing concentrations of the inhibitor. The structural similarity between carnitine and butyrobetaine also fits with an inhibition of the competitive type.

The K_i for the butyrobetaine inhibition of carnitine uptake is 0.45—3.5 μM. This is rather similar to the K_M for the butyrobetaine uptake.

Increasing concentrations of L-carnitine (2—10 μM) inhibited the uptake of radiolabeled butyrobetaine to the same extent as butyrobetaine inhibited the uptake of carnitine. It is likely therefore, that both carnitine and butyrobetaine are taken up at the same site.

A transport mechanism for carnitine has previously been described in bacteria, this was induced by the presence of carnitine in the growth medium [18]. Our results show that such a transport is also present in the eucaryotic cell. It is uncertain, however, whether the transport mechanism of the heart cells is constitutional or inducible as the inactivated calf serum which is added in most tissue cultures contains 15 μM L-carnitine (Böhmer, T., unpublished). However, the uptake mechanism which is hormone dependent in epididymis in the rat can be suppressed by oestrogens and induced by testosterone [19] indicating that at least the carnitine concentrative mechanism in this organ is inducible.

This concentrative mechanisms of carnitine works at physiological level of carnitine in plasma [14], and is probably therefore of importance for normal carnitine uptake in vivo in the heart.

The observed difference in carnitine uptake between heart cells and fibroblasts (Fig. 1) might explain the varying concentrations of carnitine found in different tissues in vivo [4,5].

Acknowledgement

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