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CARNITINE MOVEMENT ACROSS MUSCLE CELL MEMBRANES

STUDIES IN ISOLATED RAT MUSCLE

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Summary

L-Carnitine uptake and exodus was studied in rat extensor digitorum longus muscle in vitro. A saturable transport process was observed, which had an apparent K_m of 60 μM and V of 22 nmol/h per g tissue. Transport was inhibited by 2,4-dinitrophenol, sodium azide, anaerobiosis, ouabain, and sodium ion depletion. Analogs of L-carnitine containing a quarternary ammonium group were found to inhibit uptake (D-carnitine, K_i = 400 μM; γ-butyrobetaine, K_i = 60 μM, choline chloride, K_i = 14 mM), while those not containing this functional group (γ-aminobutyrate, D,L-β-hydroxybutyrate) had no significant effect at concentrations 100 times the apparent K_m of L-carnitine. Carnitine exodus from rat extensor digitorum longus muscle consisted of two phases. The rapid initial phase was attributed to leakage of L-carnitine from damaged muscle fibers, as it proceeded at nearly the same rate at 0° and 37°C, and leveled off to a rate of near zero after 1 h of incubation in vitro. The quantitatively more important phase of exodus showed a latency of 1—2 h and then proceeded at a linear rate of 40—45 nmol/h per g tissue. The results of this study support the contention that L-carnitine is taken up by a carrier-mediated, active transport system in rat extensor digitorum longus muscle. Functionally, the transport system for uptake is distinct from the process by which carnitine is lost from this muscle.

Introduction

The role of L-carnitine in the transfer of long-chain fatty acids across the mitochondrial inner membrane has been well established. Tissues such as heart and skeletal muscle which depend heavily on fatty acids as an energy source must require sufficient carnitine to make these fatty acids available to the enzymes for β-oxidation inside the mitochondrion. Carnitine is synthesized from lysine and methionine in the rat [1]. While most tissues are able to carry

out the early steps in carnitine biosynthesis, the final reaction in the pathway, hydroxylation of γ-butyrobetaine, occurs primarily in the liver, and not at all in the heart and skeletal muscle of the rat [2]. Thus, the carnitine requirement for these tissues must be met entirely by uptake from the serum.

This study attempts to define the mode of uptake of L-carnitine into rat extensor digitorum longus muscle. This muscle was chosen for the ease by which it can be studied intact in vitro, and is considered to be representative of muscles which use fatty acids as an energy source.

Materials and Methods

Materials. L-(−)-Carnitine was obtained from Koch-Light Laboratories, Colnbrook, U.K. D-(+)-Carnitine was purchased from the Biochemical Laboratories, Inc., Redondo Beach, California. [*carboxy*-^{14}C]Carboxy-inulin, [*U*-^{14}C]sucrose, and [1-^{3}H]mannitol were obtained from New England Nuclear Corporation. ε-Trimethylaminocaproic acid and γ-butyrobetaine were synthesized by methylation of the parent primary amines [3]. L-[*Me*-^{3}H]Carnitine was synthesized by the method of Stokke and Bremer [4].

Methods. Carnitine uptake studies were performed on isolated rat extensor digitorum longus muscles. Male rats weighing 90—140 g were anesthetized with ether, whole blood collected by heart puncture, and muscles excised. The muscles were rinsed for 3—5 s in ice-cold Krebs-Ringer bicarbonate solution containing 0.1% glucose, weighed, and pinned at the tendons on either end to paraffin frames. The muscles were incubated at rest length in 12 ml of oxygenated (continuous stream of O_2/CO_2, 95 : 5 v/v) Krebs-Ringer solution containing L[*Me*-^{3}H]carnitine plus appropriate additions at 37°C. In the control experiments designed to measure non-specific uptake, 10 mM unlabelled L-carnitine was added to the assay system. With the sites for carrier-mediated uptake thus saturated, only non-specific, or diffusional uptake was measured [5]. Following a times incubation period muscles were removed from the paraffin frames, rinsed in ice-cold Krebs-Ringer buffer (3—5 s), blotted and weighed. Each muscle was homogenized in 2 ml of water using a "Tissumizer" homogenizer (Tekmar Co., Cincinnati, Ohio) with a micro tip. Following centrifugation of the homogenates, 1-ml aliquots of the supernatants were transferred to vials containing 10 ml of 3a70 Complete Counting Cocktail (Research Products International, Elk Grove Village, Ill.) and counted in a Beckman LS-230 liquid scintillation counter. Total and non-specific uptake calculations were corrected for contribution of labelled carnitine in the extracellular space (determined as described below). Saturable uptake was calculated as the difference of total and non-specific uptake.

Exodus of L-carnitine from rat muscle was measured as follows: Male rats weighing approximately 100 g were given three injections of 0.2 ml of 0.9% NaCl containing 150 μCi (53.2 nmol) of L[*Me*-^{3}H]carnitine at 8 h intervals. Three days after the last injection the extensor digitorum longus muscles were excised, prepared for incubation as described above, and incubated in 15 ml of Krebs-Ringer buffer containing various amounts of unlabeled-L-carnitine. At appropriate time intervals 0.5 or 1.0 ml aliquots of the incubation solution were removed and radioactivity determined by liquid scintillation

counting. At the end of the incubation period the muscle was prepared for radioactivity determination as described above. Exodus of L-carnitine was determined from the radioactivity found in the medium and the specific activity of intracellular L-carnitine.

Total water content was determined by the difference in weight of muscles before and after drying in an oven at 70°C overnight. Extracellular water was determined by incubating intact extensor digitorum longus muscle at 37°C with [*carboxy*-^{14}C]carboxyinulin, [U-^{14}C]sucrose, or [1-^{3}H]mannitol in Krebs-Ringer buffer and determination of radioactivity content as described for L-carnitine uptake experiments. L-Carnitine content of tissue extracts, serum and spent incubation medium was measured by the method of Cederblad and Lindstedt [6]. The specific activity of chemically-synthesized L-[*Me*-^{3}H]carnitine was determined by liquid scintillation counting and enzymatic-spectrophotometric assay according to Pearson et al. [7]. Purity of chemically-synthesized [*Me*-^{3}H]carnitine and metabolism of this compound by rat tissues were determined by chromatography on Whatman No. 3MM paper and silica gel G thin layer plates. Three solvent systems were employed: *n*-butanol/glacial acetic acid/water, 12 : 3 : 5 (v/v) (descending paper chromatography, System I); methanol/conc. ammonium hydroxide, 75 : 25 (v/v) (thin layer, System II) and methanol/acetone/conc. HCl, 90 : 10 : 4 (v/v) (thin layer, System III). The R_F values for carnitine in the three systems were 39, 31 and 53, respectively. Acetylcarnitine had R_F values of 50 and 39 in solvent Systems I and II respectively.

Results

General considerations. Carnitine content of rat serum and extensor digitorum longus muscles was determined. The average concentration of L-carnitine in serum was 44.0 nmol/min (n = 17) with a range of 25.6 to 72.2 nmol/ml. In the muscle the average value obtained was 720 nmol/g wet weight of tissue (n = 17). The average wet weight of muscles used in this determination was 53.4 mg. The range of carnitine content and muscle weights is shown in Fig. 1. The level of carnitine per unit weight of muscle is seen to increase with increas-

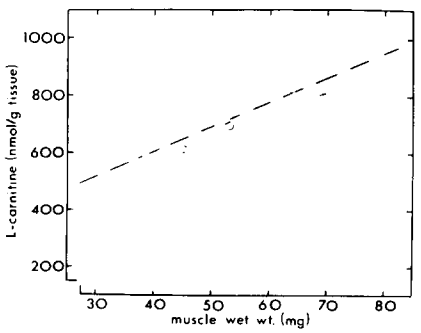

Fig. 1. Carnitine content of rat extensor digitorum longus muscle. Excised muscles were assayed for L-carnitine. Each point represents duplicate determinations from a single muscle. Only one muscle from each rat was assayed. The dashed line represents a linear regression of the experimental points.

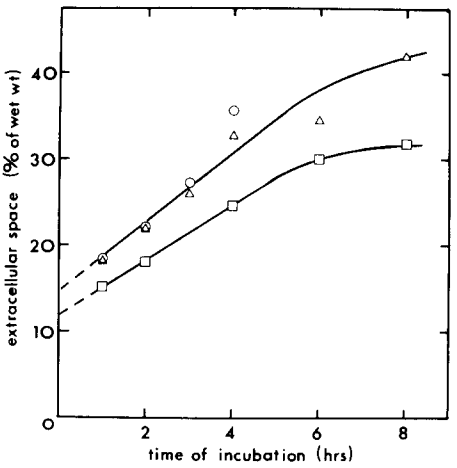

Fig. 2. Extracellular space of rat extensor digitorum longus muscle. Muscles were incubated with [*carboxy*-^{14}C]inulin (□———□), [U-^{14}C]sucrose (△———△) or [1-^{3}H]mannitol (○———○). Following incubation, radioactivity in homogenates was determined by liquid scintillation counting. L-Carnitine uptake results were corrected for extracellular space using the average values for sucrose and mannitol space.

ing weight of muscle. The total water content of fresh rat extensor digitorum longus was 76% of the wet weight.

Extracellular space was measured using three isotopically-labelled compounds unable to penetrate the cell membrane. The extracellular space was seen to increase with increasing incubation time in vitro (Fig. 2). This increase in apparent extracellular space and the range obtained (18—25% of wet weight for 0.5—2 h of incubation) are in agreement with results of other investigators [8,9]. Also in general agreement with reports in the literature is the variability of the measurements from each muscle to another. This variation is thought to be due to differences in muscle size and minor differences in incubation conditions. In the carnitine uptake and efflux experiments reported herein, the results were corrected for the average of measured sucrose and mannitol space. The molecular weights of these compounds more closely approximate the molecular weight of carnitine than does that of inulin. Correction was made in each experiment based on the extracellular space for the same time period of incubation.

Carnitine uptake by rat extensor digitorum longus muscle in vitro. L-Carnitine movement across rat muscle membranes was found to be mediated by a saturable process. Non-specific uptake was negligible in most experiments, however, the values for non-specific uptake when significant were subtracted from total uptake for each experiment as a correction both for diffusion and for small errors in the extracellular space determinations. A time course for uptake by the saturable process at three concentrations of extracellular L-carnitine is shown in Fig. 3. Rates of uptake were essentially linear for the duration of the experiments (4 h). Distribution ratios (ratio of internal to external L-[*Me*-^{3}H]carnitine) greater than one were achieved for muscles incubated in carnitine concentrations as high as 50 μM. Fig. 4 shows the relationship between extracellular L-carnitine concentration and rate of uptake by the

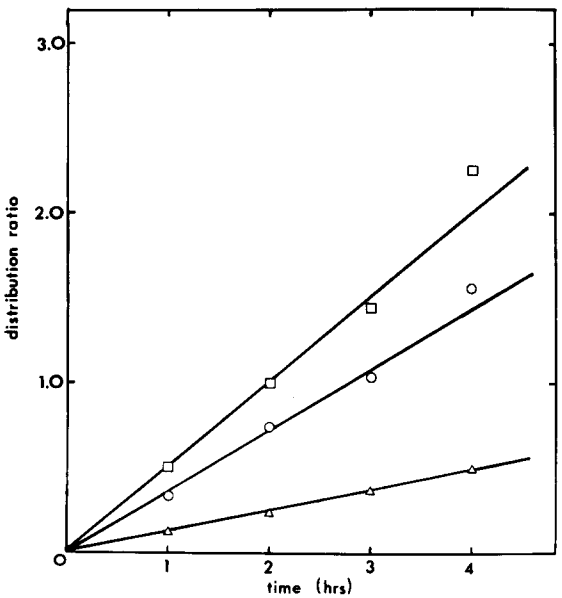

Fig. 3. Carnitine uptake by rat extensor digitorum longus muscle. Muscles were incubatedin 5 (□———□), 50 (○———○) or 250 (△———△) μM L-[*Me*-^{3}H]carnitine for the time periods indicated. "Distribution ratio" is defined as the concentration of intracellular L-[*Me*-^{3}H]carnitine relative to that in the incubation solution (extracellular concentration). Intracellular water was determined from the difference in total water and extracellular water (Fig. 2) at each time period.

saturable process. A Lineweaver-Burk plot (Fig. 4, inset) and an Eadie-Hofstee plot (not shown) of the data yielded values for apparent K_m and V of 60 μM and 22 nmol/h per g wet weight of tissue, respectively.

L-[*Me*-^{3}H]Carnitine taken up by isolated muscles was minimally metabolized. Paper and thin-layer chromatography of deproteinized extracts revealed that acetylcarnitine was the only metabolite, accounting for 20% of the radioactivity, while the remaining 80% migrated with carnitine.

The sites on the muscle cell membrane mediating uptake of L-carnitine by the saturable process are apparently specific for molecules containing quarternary ammonium groups. Two analogs of L-carnitine lacking this function, γ-aminobutyric acid and D,L-β-hydroxybutyrate, failed to significantly reduce the rate of L-carnitine uptake at concentrations 100 times the K_m for L-carnitine (Table I). In contrast, analogs containing the quarternary ammonium group were to varying degrees inhibitory. The most effective inhibitor was γ-butyrobetaine (K_i = 60 μM), which structurally lacks only the β-hydroxy group. The two carbon homolog of γ-butyrobetaine, ε-trimethyl-aminocaproate, was less effective (K_i = 260 μM); and choline, which represents only the quarternary ammonium and hydroxy functions of carnitine, was only slightly inhibitory (K_i = 14 mM). The stereochemistry of the β-hydroxy group is apparently critical in the uptake process, as D-carnitine (K_i = 400 μM) at a concentration five times that of L-carnitine inhibited its transport by only 25%. All of the above mentioned quarternary amines were competitive inhibitors, as deter-

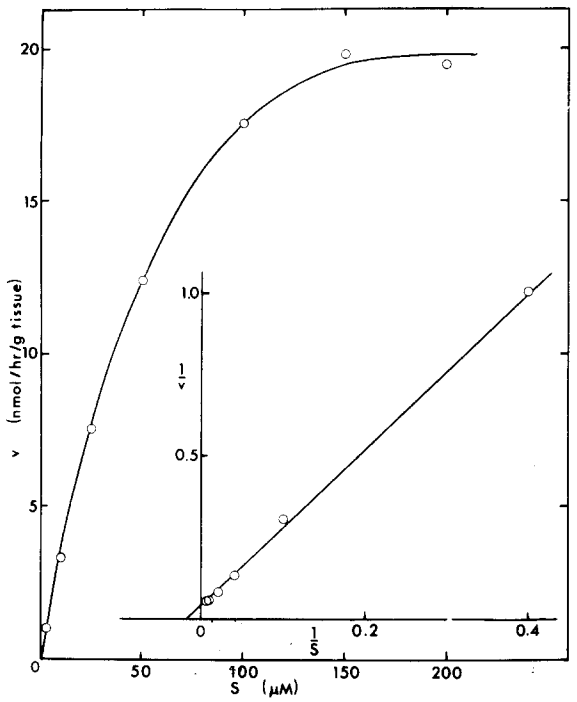

Fig. 4. Kinetics of L-carnitine uptake. The rate of uptake (v) is plotted against extracellular L-carnitine concentration (S). The inset depicts the same data in a Lineweaver-Burk plot. The K_m and V determined from this graph and an Eadie-Hofstee plot of these data were 60 μM and 22 nmol/h per g tissue, respectively.

TABLE I

EFFECT OF STRUCTURAL ANALOGS ON L-CARNITINE UPTAKE BY RAT EXTENSOR DIGITORUM LONGUS MUSCLE

Freshly excised muscles were incubated as described in the text with the indicated analogs and 50 μM L-[*Me*-^{3}H]carnitine for 2 h at 37°C. Rates are expressed as nmol/h per g tissue, with the standard deviation given in parentheses. The reported values are the average of at least three experiments. K_i values were determined from Dixon plots [21] of data for uptake at different concentrations of L-carnitine and analog.

Analog	Rate [a]	% of control	Rate [b]	% of control	K_i
None	9.48 (± 1.66)	(100)	9.48 (± 1.66)	(100)	—
D-Carnitine	7.14 (± 0.36)	75.4			0.40
γ-Aminobutyrate	10.2 (± 2.57)	107	8.68 (± 1.50)	91.6	
γ-Butyrobetaine	0.36 (± 0.24)	3.74			0.06
DL-β-Hydroxy-butyrate	8.80 (± 0.65)	92.8	9.12 (± 0.56)	96.1	
ε-Trimethyamino-caproate	4.52 (± 1.26)	47.7			0.26
Choline chloride	9.41 (± 0.36)	99.3	7.07 (± 1.43)	74.6	14

[a] Analog concentrations are 250 μM.
[b] Analog concentrations are 5 mM.

TABLE II

EFFECT OF INCUBATION CONDITIONS AND METABOLIC INHIBITORS ON UPTAKE OF L-CARNITINE BY RAT EXTENSOR DIGITORUM LONGUS MUSCLE

Muscles were incubated at 37°C for 15 min in the presence of the indicated inhibitor, then transferred to an incubation solution containing the inhibitor plus 50 μM L-[*Me*-^{3}H]carnitine. For the anaerobiosis experiment, 95% N_2: 5% CO_2 replaced 95% O_2: 5% CO_2 as the bubbling gas, while in the sodium depletion experiment, Na^+ in the incubation solution was quantitatively replaced with Li^+. No preincubation period was included in these two experiments. See text for other details of experimental procedures. Rates are expressed as percent of control.

Substance or condition	Rate
Control	(100)
10 mM sodium azide	47.7
0.1 mM 2,4 dinitrophenol	58.2
10 mM KCN	91.3
$-O_2$, $+N_2$	59.0
1 mM ouabain	77.8
$-Na^+$, $+Li^+$	22.8
0°C	0

mined from Dixon plots of uptake at various L-carnitine and analog concentrations (data not shown).

That L-carnitine uptake requires energy was demonstrated by inhibition by the uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, the cytochrome system inhibitor, sodium azide, and by anaerobiosis (Table II). 10 mM potassium cyanide had only a slight inhibitory effect. Ouabain and Na^+ depletion also reduced the rate of uptake. Finally, no uptake of L-carnitine was observed at 0°C.

Carnitine efflux from rat extensor digitorum longus in vitro. Carnitine exodus from rat muscle is shown in Fig. 5. Muscles labelled in vivo with L-[*Me*-^{3}H]carnitine demonstrated a biphasic release of this isotope, with a rapid initial loss followed by a quantitatively more significant efflux characterized by a 1—2 h lag period. The initial loss of carnitine from muscle was greater in 50 μM L-carnitine than when carnitine was absent from the incubation solution. Little difference was seen in this respect in the latent phase of carnitine exodus. 9% of total muscle carnitine was lost at 50 μM external carnitine concentration, while a value of 6% was obtained without external carnitine. The curves for the rapid first phase of carnitine efflux were essentially the same at 0° and 37°C, reaching a plateau after about 30 min. At 37°C and 4 h incubation time muscles lost 18 and 24% of total initial carnitine content at 0 and 50 μM external carnitine concentration, respectively. Paper and thin-layer chromatographic analysis revealed that radioactivity appearing in the medium from 4-h incubations at 0 and 37°C was 90 and 95%, respectively, in the form of free carnitine, and 10 and 5%, respectively, in the form of acetylcarnitine. No other metabolites were detected. Intracellular radioactivity consisted of 74% carnitine, 18% acetylcarnitine, and the remainder in an unidentified metabolite.

Three possibilities were considered concerning the origin of carnitine released from rat muscle during the initial phase (first hour) of incubation in vitro: (a) This material arises from an extracellular pool of carnitine which is somewhat more concentrated than serum carnitine; (2) it arises from damage to

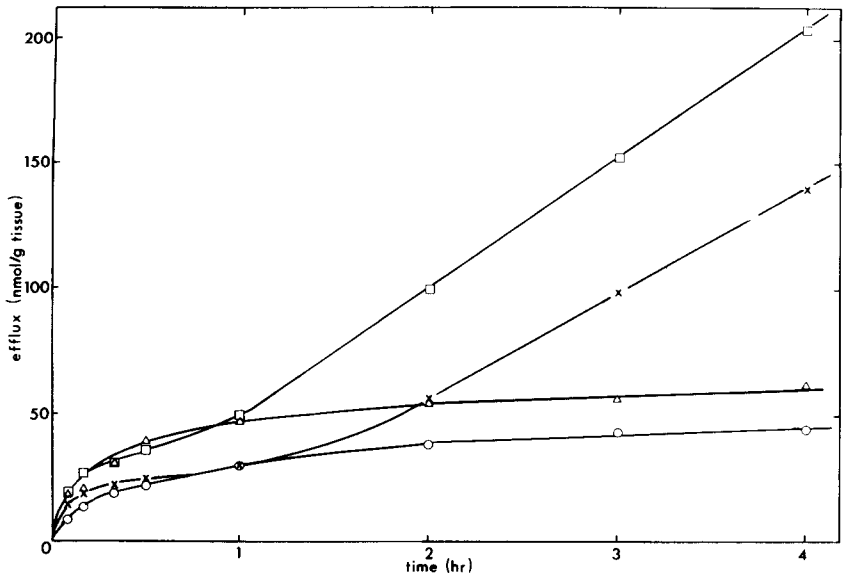

Fig. 5. Carnitine exodus from rat extensor digitorum longus muscle labelled in vivo. Rats were injected with L-[*Me*-^{3}H]carnitine, muscles excised and incubated at 37° or 0°C, as described in the text. (○———○), Incubation at 0°C in the absence of external carnitine; (△———△), incubation at 0°C and 50 μM external L-carnitine; (×———×), incubation at 37°C in the absence of external carnitine; (□———□), incubation at 37°C and 50 μM external L-carnitine. Corrections for contributions of carnitine from the extracellular space were made for each point.

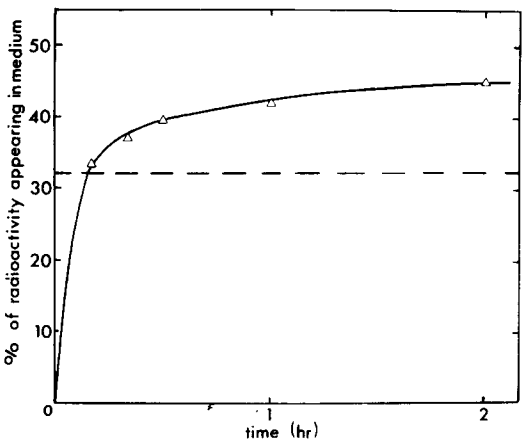

Fig. 6. Carnitine exodus from rat extensor digitorum longus muscle labelled in vitro. Freshly excised muscles were incubated as described in the text with 50 μM L-[*Me*-^{3}H]carnitine (50 μCi/μmol) for 2 h at 37°C. The muscles were then transferred to 15 ml of oxygenated Krebs-Ringer bicarbonate solution containing 0.1% glucose but no carnitine. Muscles were incubated at 0°C for 2 h, with 1-ml aliquots being removed at the indicated times for radioactivity determination, and 0.1 ml aliquots at 10, 30, 60 and 120 min for carnitine assay. Other experimental details are given in the text. The dashed line represents the contribution of radioactivity from the extracellular space (calculated from data of Fig. 1).

a small percentage of the muscle fibers, the contents of which then effectively become part of the extracellular space; or (c) the calculation of extracellular space is lower than the actual extracellular space available to carnitine. All three postulates are supported by the fact that the initial loss of L-carnitine occurs at 0°C as well as 37°C, indicating an extracellular nature for the radioactivity lost to the medium during this period. Alternative (a) above was suggested based on a study by Hider et al. in which they found a pool of amino acids concentrated in the extracellular space of rat muscle [10].

In an attempt to delineate the source of this extracellular carnitine, exodus at 0°C from muscle labelled in vitro was studied. Fig. 6 shows the rate of loss of radioactivity from muscles labelled for 2 h in the presence of 50 μM L-[*Me*-^{3}H]carnitine (50 μCi/μmol) at 37°C. As in the experiments described above, a rapid initial loss at 0°C was observed with the rate falling to near zero after 1 h of incubation. If the contribution of radioactivity from extracellular space (as calculated from Fig. 1) is subtracted, the radioactivity found in the medium at 1 h represents 14.9% of total for muscles pre-labeled in 50 μM L-[*Me*-^{3}H]carnitine. The specific activity of L-carnitine in the medium and cell extracts was 10.7 and 1.90 μCi/μmol, respectively. Further, the specific activity of L-carnitine in the medium remained constant during the 2 h incubation at 0°C. These results suggest that alternative (b) above contributes to the major portion of carnitine efflux observed at 0°C, but the other two considerations cannot be entirely ruled out.

Discussion

Rat skeletal muscle has a concentration of L-carnitine 10—30 times that found in the serum. Muscle, however, cannot synthesize L-carnitine from its immediate precursor, γ-butyrobetaine [11]. Studies by Marquis and Fritz [12] and Ramsay and Tubbs [13] have demonstrated that the carnitine concentration in subcellular organelles is essentially the same as that found in the cytoplasm of heart muscle cells. Although the ratios of acetylcarnitine and long-chain fatty-acyl carnitine to free carnitine vary under different conditions [14], removal of free carnitine by esterification cannot, by means of facilitated diffusion, account for the high concentration of total intracellular carnitine with respect to serum. In addition, studies in this laboratory have ruled out the possibility of "cytoplasmic receptors" for carnitine by binding studies of this molecule to macromolecular components of rat muscle cytosol (Rebouche, C.J., unpublished observations).

L-Carnitine uptake experiments reported herein, however, are consistent with the presence of a carrier-mediated, active transport system associated with the sarcoplasmic membrane. The transport system is saturable and has a degree of specificity for L-carnitine. The apparent K_m value obtained for L-carnitine was 60 μM. Thus, L-carnitine transport would be slightly less than half maximal under normal physiological conditions, as the average serum concentration of the molecule was found to be 44 μM (literature value, 39 μM, ref. 15). Studies with various metabolic inhibitors and different experimental conditions (Fig. 6) clearly demonstrated that metabolic energy is required for function of the transport system. Curious, however, was the relatively small inhibitory effect of

10 mM potassium cyanide. While both azide and cyanide are known to be respiratory chain inhibitors, only azide exhibited a significant inhibitory effect. We are unable to offer an explanation for this anomaly. Involvement of the plasma membrane ($Na^+ + K^+$)-ATPase in saturable L-carnitine uptake was suggested by inhibition of this process by ouabain and the absence of sodium ions in the incubation solution.

Recently a study of carnitine uptake by isolated rat liver cells has appeared [16]. The results revealed the presence of an active transport system with a K_m of 5.6 mM for L-carnitine. In contrast to the high K_m for rat liver cells, and in better agreement with our value for rat extensor digitorum longus, was the K_m of 4.8 μM for rat heart cells in culture [17]. The V for carnitine uptake into isolated liver cells was 2.4 nmol/min per mg protein, which is significantly higher than the value of 22 nmol/h per g tissue obtained for rat extensor digitorum longus. At the physiological serum concentration of 44 μM, the rate of uptake into isolated liver cells (see Fig. 2 of ref. 16) proceeds at a rate approximately 10 times greater than for rat extensor digitorum longus in vitro.

While the initial phase of carnitine efflux from rat muscle (Fig. 5) probably was not physiological, the latent phase may indeed represent a physiological function of the muscle cell. Carnitine efflux from rat quadriceps muscle in vivo has been estimated by Brooks and McIntosh [18]. They found two carnitine pools in this muscle, with fluxes of 14.0 ± 7.7 and 8.0 ± 1.6 nmol/h per g. A sum of 22 ± 8.3 nmol/h per g tissue is obtained, which compares with an efflux of 40—45 nmol/h per g tissue from rat extensor digitorum longus in vitro, after a 1—2 h latency period. The 2-fold difference is probably significant. In a study of creatine loss from rat extensor digitorum longus in vitro a pattern similar to the latent component of L-carnitine loss was obtained [8]. After a lag of about 30 min the rate of creatine loss rapidly accelerated and was approximately linear from 1 to 3 h, with nearly 50% of the total intracellular creatine found in the incubation solution after three hours at 37°C. In contrast, 25% of intracellular L-carnitine was lost from rat muscle after 4 h, with a 1—2 h latency period. Factors such as loss of hormonal control, denervation, etc., may contribute to the rapid loss of these molecules in vitro.

The processes by which carnitine is transported into and lost from the muscle cells are apparently separate and distinct. On the one hand, uptake is linear for the first 2 h while efflux exhibits a lag before reaching linearity between 1 and 2 h of incubation. Secondly, no one-to-one correspondence, or countertransport, can be established for uptake and efflux, as efflux proceeds at nearly the same rate whether or not external carnitine is present. Finally, while uptake increases with external carnitine concentration, little effect on the latent phase of efflux is observed when carnitine is added to the incubation medium.

The role of L-carnitine transport into muscle has recently taken on significance in human disease. Human muscle carnitine deficiency was first reported in 1973 [19]. This case was characterized by an abnormally low level of carnitine in skeletal muscle, with normal liver and serum levels. Extracts of the patient's muscle had an impaired ability to oxidize long-chain fatty acids as compared with controls, but when 1.5 mM L-carnitine was added to the incubation medium, both the patient's and control muscle homogenates oxidized

long-chain fatty acids at the same rate. Numerous cases of this disease have subsequently been reported (cf. ref. 20 for references). While in a few of these cases systemic carnitine deficiency was observed, most had normal liver and serum levels of L-carnitine. These observations suggest that in the patients with low muscle but normal serum and liver carnitine levels, the transport system for L-carnitine across the sarcoplasmic membrane is defective. Efforts are now under way in this laboratory to study human muscle carnitine deficiency in cell culture.

Acknowledgement

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