BBA 46346

CARNITINE: THE CARRIER TRANSPORTING FATTY ACYLS INTO MITOCHONDRIA BY MEANS OF AN ELECTROCHEMICAL GRADIENT OF H^+

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(Received February 29th, 1972)

SUMMARY

The functions of carnitine as a carrier have been studied to verify the hypothesis of the electrophoretic transport of the fatty acyl carnitine cation into mitochondria.

It has been found that both L- and D-palmitoyl carnitines can exist in the form of cations which easily penetrate through phospholipid membranes. Palmitoyl carnitine greatly decreases the electrical resistance of phospholipid membranes, the effect being due to the transmembrane diffusion of the palmitoyl carnitine cation. The concentration gradient of the palmitoyl carnitine cation across the phospholipid membrane, created by transmembrane differences in total palmitoyl carnitine or in pH values, generates a membrane potential with "*plus*" on the side of the lower cation concentration. The magnitudes of this potential correspond closely to those calculated from the Nernst equation for a singly charged ion, *i.e.* 59 mV per 10-fold ion gradient.

Carnitine and acetyl carnitine do not affect the electric characteristics of the phospholipid membrane, which indicates that the cationic forms of these compounds are non-penetrating in the membrane studied. Palmitate induces some decrease in the phospholipid membrane resistance but at concentrations much higher than those of palmitoyl carnitine.

In rat liver mitochondria, it has been found that both L-and D-palmitoyl carnitines, like other penetrating cations, induce the energy-dependent swelling of the mitochondrial matrix, which is shown by the decreased light scattering of the mitochondrial suspension and by characteristic changes in the appearance of the mitochondria in the electron micrographs. The swelling can be supported by respiration or ATP hydrolysis. It is sensitive to the respiratory chain inhibitors in the former, to rutamycin in the latter, and to uncouplers, in both cases.

In polarographic experiments it has been observed that fast oxidation of added L-palmitoyl carnitine requires this compound to be in contact with energized mitochondria for at least several seconds. Energization can be supported by respiration or ATP hydrolysis, rutamycin being inhibitory in the later case. Uncouplers

Abbreviations: $TPMP^+$, triphenyl methyl phosphonium cation; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

prevent L-palmitoyl carnitine oxidation when added before it, and stimulate oxidation when added after preincubation of mitochondria with L-palmitoyl carnitine.

The data obtained are in agreement with the concept according to which the transport of palmitoyl carnitine into mitochondria is a movement of the protonated cationic form of this compound down electrochemical gradient of H^+ which is generated by the redox chain or ATPase.

INTRODUCTION

Carnitine was discovered by Gulewitsch and Krimberg in 1905[1]. In 1963 Fritz and Yue[2] put forward the idea that carnitine is an intermediate of fatty acyl transport into mitochondria. This hypothesis, being confirmed by comprehensive experimental data, is now generally accepted (for review see refs 3–5). However, the function of carnitine in the transport process is still obscure, as well as the forces driving large molecules of fatty acids in the mitochondrial membrane.

Quite independently, Mitchell's pioneering investigation of coupling membranes[6] has resulted in important progress in the understanding of mitochondrial transport of penetrating cations and weak acids. Mitchell postulated the electrochemical potential of H^+ to be a link between oxidation and phosphorylation, and a driving force for the transport of ions against concentration gradients.

Mitchell's idea of membrane potential was confirmed by the discovery of transmembrane electrophoresis of penetrating natural and unnatural ions in mitochondria. Data were obtained indicating that energized mitochondria accumulate some penetrating species down electrical (cations) or chemical (weak acids) gradients of electrochemical potential of H^+. It was found to be the case for such cations as K^+ (+ valinomycin), Na^+ (+ gramicidin), dibenzyl dimethyl ammonium, tetrabutyl ammonium, triphenyl methyl phosphonium, tetraphenyl arsonium, Ca^{2+}, Sr^{2+}, Mn^{2+} and some others (for review see refs 7–9), and for such weak acids as acetic, phosphoric, succinic, malic, α-ketoglutaric, glutamic, citric and some others (see refs 8, 10, 11).

In the same (energized) conditions favourable for the accumulation of cations and weak acids, mitochondria extrude penetrating anions (as was demonstrated with phenyl dicarbaundecaborane[12], tetraphenyl boron[12], thiocyanate[9,11,13] anions) and weak bases (see refs 8, 14).

However, separation of the penetrating molecules by their charge and basicity only seems to be biologically meaningless. And here carriers come into play changing the charge (or basicity) of a compound to be transported across the mitochondrial membrane. This may be exemplified by the K^+ extrusion from energized mitochondria treated with nigericin. K^+ substitutes H^+ in the nigericin molecule, which results in the formation of the electroneutral K^+–nigericin complex transported down the pH gradient from matrix to the extramitochondrial space, *i.e.* in the direction opposite to that of movement of the free cation or of positively charged K^+–valinomycin complex[15].

A problem similar to that of the K^+ extrusion arises when one considers the transport of fatty acids in the respiring mitochondria. Fatty acids are uncouplers of oxidative phosphorylation, as their large and hydrophobic molecules can traverse

mitochondrial membrane in both neutral and anionic forms (see below). To rationalize the mitochondrial fatty acid transport, two questions should be answered: (1) how these compounds, serving as one of the most important substrates whose oxidation starts inside the mitochondrion, get into the mitochondrial matrix in the energized state favourable for extrusion of fatty acid anions, and (2) why fatty acids do not display their uncoupling ability and not discharge a membrane potential generated by their oxidation.

Recently, Severin *et al.*[16] suggested that these problems may be solved by elucidating the role of carnitine. The authors extended the chemiosmotic theory of ion transport to the case of the carnitine-mediated mechanism of fatty acid translocation in mitochondrial membrane. It was postulated that fatty acyl carnitine is transported across the membrane in its protonated (cationic) form, moving down both the electric and H^+ concentration gradients.

In this study, the main postulates of the above hypothesis have been verified. The data obtained are in agreement with the idea of carnitine as a carrier allowing the proton motive force to be used for fatty acid transport in mitochondria.

EXPERIMENTAL

Artificial phospholipid membranes were prepared according to Mueller *et al.*[17]. Phospholipids from beef heart mitochondria or brain were dissolved in heptane or decane up to a concentration of 20 mg phospholipid/ml. Measurements of electric parameters of the membrane were carried out with AgCl electrodes, a VA-j-51 electrometer and a KSP-4 recorder (for details see refs 18–20).

Isolation of rat liver mitochondria. Rats were decapitated and livers were cooled in the cold isolation mixture (0.3 M sucrose, $2 \cdot 10^{-3}$ M Tris–HCl buffer, pH 7.5, 1.10^{-3} M EDTA). Liver was minced by a metallic press with 1-mm holes and homogenized with 10 vol. of isolation mixture in a glass–Teflon homogenizer for 30–40 s. The homogenate was centrifuged at 600 × *g* for 10 min. The supernatant was then centrifuged at 7000 × *g* for 10 min. The precipitate was suspended in the isolation medium, and mitochondria were sedimented at 7000 × *g* for 10 min. The fluffy layer was removed and mitochondria were suspended in a small volume of the isolation mixture and stored in the cold at a concentration of 70–80 mg mitochondrial protein/ml. Protein was determined by the biuret method.

Swelling of mitochondria was measured spectrophotometrically at 520 nm at the right angle.

Electron microscope studies were carried out in a Hitachi HU-11B microscope. Mitochondria were fixed with 5% glutaraldehyde and treated with OsO_4, alcohols, uranyl acetate and epoxide resin 812. The sections of mitochondria were prepared with an LKB-4800 ultramicrotome.

Respiration of mitochondria was recorded polarographically by means of a stationary platinum electrode.

Carnitine and its esters were the kind gift of Professor E. Strack (Institute of Physiological Chemistry, Leipzig). All carnitine derivatives were used as water solutions.

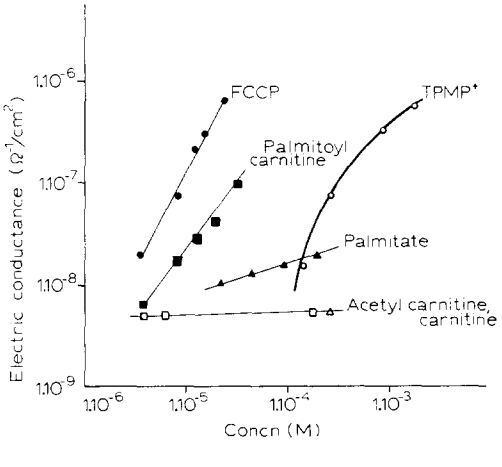
RESULTS

Artificial phospholipid membranes

In this part of the study artificial phospholipid membranes were used as a simple model for analysis of the permeability of hydrophobic barriers to carnitine derivatives.

First of all, the effect of carnitine and its derivatives on electric conductance of the phospholipid membranes has been studied. The data obtained are shown in Fig. 1. It is seen that L-palmitoyl carnitine induced a pronounced increase in the electric conductance of the membrane. In this respect L-palmitoyl carnitine proved much more effective than, for example, the synthetic penetrating cation triphenyl methyl phosphonium ($TPMP^+$), and somewhat less effective than carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), one of the most potent current conductors in the system of phospholipid membranes. Palmitate increased electric conductance only slightly even if added at higher concentrations than L-palmitoyl carnitine. Both acetyl carnitine and unsubstituted carnitine were found to be without measurable effect on the membranes. D-Palmitoyl carnitine (not shown in Fig. 1) caused the same effect as the L-isomer.

In further experiments, the mechanism of the palmitoyl carnitine-induced increase of electric conductance was studied. It was found that a membrane potential arose when concentrations of palmitoyl carnitine in two compartments separated by the membrane were unequal. The "*plus*" sign of the membrane potential was always on the side of the lower palmitoyl carnitine concentration. Varying concentrations of palmitoyl carnitine in one compartment and keeping it steady in the



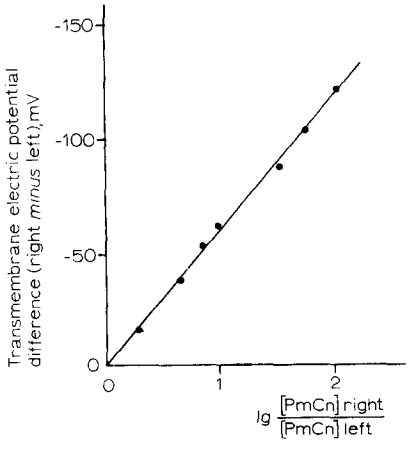


Fig. 1. The effect of carnitine, palmitoyl carnitine, acetyl carnitine and some other compounds on electric conductance of phospholipid membranes. All probes contained 0.25 M sucrose and 0.05 M Tris–HCl buffer, pH 7.5.

Fig. 2. Generation of electric potential across phospholipid membrane by the transmembrane gradient of palmitoyl carnitine concentration ([PmCn] right/[PmCn] left). The experiment was carried out in a solution containing 0.1 M KCl, 0.025 M citric acid, 0.025 M K_2HPO_4, 0.03 M H_3BO_3, pH 3.4. In the left compartment the palmitoyl carnitine concentration was equal to $3.7 \cdot 10^{-7}$ M in all samples; in the right compartment the palmitoyl carnitine concentration was changed from $3.7 \cdot 10^{-7}$ to $3.7 \cdot 10^{-5}$ M.

other one, the membrane potential has been measured as a function of the palmitoyl carnitine concentration gradient. The straight line dependence was revealed. As seen in Fig. 2, the 10-fold gradient of palmitoyl carnitine generated a membrane potential of 59 mV. This value coincides with that calculated from the Nernst equation, assuming the ionized form of palmitoyl carnitine to be a singly charged ion.

A membrane potential could also be generated when not the total concentration of palmitoyl carnitine but the degree of its ionization was changed in one of the compartments. To this end, the pH of the palmitoyl carnitine-containing solution was changed as is demonstrated in Fig. 3 showing the kinetics of the mem-

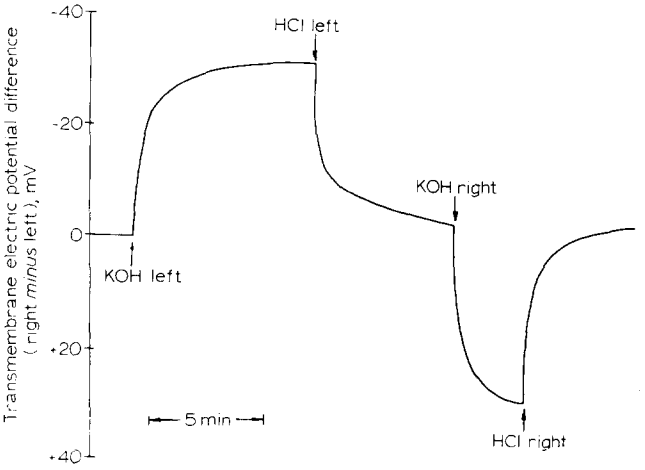


Fig. 3. Generation of electric potential across phospholipid membrane by changing pH of palmitoyl carnitine solution. Initial pH in both compartments was 4.4. Additions of KOH or HCl to one of the compartments induced the pH shift by 1. Concentration of palmitoyl carnitine in both compartments was $3.7 \cdot 10^{-6}$ M. Other conditions as in Fig. 2.

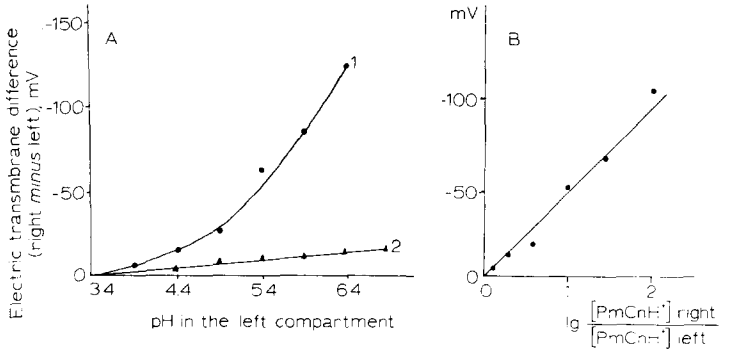


Fig. 4. Electric potential difference across phospholipid membrane as a function of pH and of concentration of the palmitoyl carnitine cation ($PmCnH^+$). pH value in the right compartment was 3.4. In the left compartment pH values were changed by addition of KOH. (A) Curve 1, in the presence of $6.3.10^{-6}$ M palmitoyl carnitine; Curve 2, without palmitoyl carnitine. (B) The palmitoyl carnitine-induced membrane potential, calculated as the difference in the values of Curves 1 and 2 (A), is plotted against the logarithm of the ratios of the $PmCnH^+$ concentrations in the right and in the left compartments. The concentration of $PmCnH^+$ was calculated assuming pK of carboxylic group of carnitine residue as 4.4.

brane potential changes induced by a transmembrane pH gradient. One can see that the addition of KOH to the left compartment resulted in the formation of an electric potential difference with *plus* on the left side of the membrane, as if the concentration of the penetrating cation on the left decreased. Neutralization of the added alkali by HCl reversed the effect. The pH variations in the right compartment induced oppositely directed changes in the membrane potential.

Fig. 4 shows the membrane potential as a function of pH gradient (A) and of concentration of the cationic form of palmitoyl carnitine (B). In this experiment, the pH on the right was kept steady at 3.4 while the pH on the left was changed from 3.4 to 7.0. It is seen (Fig. 4A, Curve 1) that alkalinization in the left compartment resulted in the formation of a membrane potential (*minus* on the right side), whose magnitude was related linearly (Fig. 4B) to the gradient of the palmitoyl carnitine cation calculated, assuming the pK value for palmitoyl carnitine to be 4.4 (this value was obtained by special potentiometric measurements). As is also seen from Fig. 4A, Curve 2, the pH gradient in the samples without palmitoyl carnitine did not form a measurable membrane potential.

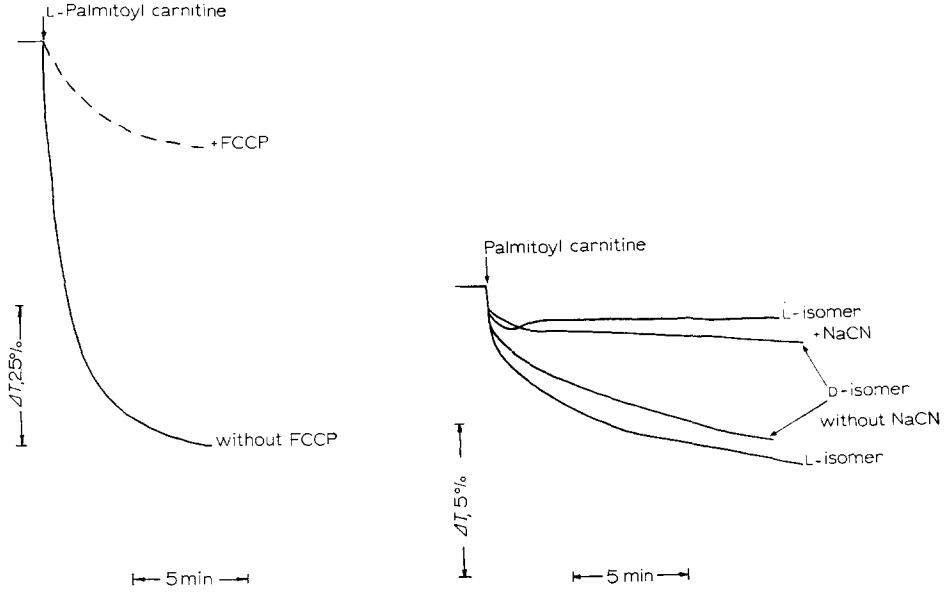


Fig. 5. Energy-dependent swelling of rat liver mitochondria induced by L-isomer of palmitoyl carnitine. Reaction mixture: 0.3 M sucrose, $2.5 \cdot 10^{-3}$ M $MgSO_4$, $5.0 \cdot 10^{-3}$ M Tris–ATP, $2.5 \cdot 10^{-3}$ M sodium phosphate, pH 7.5, $1 \cdot 10^{-6}$ M rotenone and rat liver mitochondria (0.75 mg protein/ml). In the experiment of the upper curve the reaction mixture was supplemented with $2.5 \cdot 10^{-5}$ M FCCP.

Fig. 6. Swelling of rat liver mitochondria in the presence of natural and synthetic isomers of palmitoyl carnitine supported by the succinate oxidation. Reaction mixture: 0.3 M sucrose, $1.25 \cdot 10^{-3}$ M sodium succinate, $2.5 \cdot 10^{-3}$ M sodium phosphate, pH 7.5, $1 \cdot 10^{-6}$ M rotenone and rat liver mitochondria (0.35 mg protein/ml). The process of swelling was initiated by the addition of $2 \cdot 10^{-5}$ M L-palmitoyl carnitine or $2 \cdot 10^{-5}$ M D-palmitoyl carnitine. Concentration of NaCN, $3 \cdot 10^{-3}$ M.

Rat liver mitochondria

Results of the above experiments with artificial phospholipid membranes indicate that palmitoyl carnitine can exist in the form of a penetrating cation. Taking this observation into account, we studied the effects of palmitoyl carnitine on rat liver mitochondria.

It was found that both L- and D-isomers of palmitoyl carnitine, like other penetrating cations, induced energy-dependent swelling of mitochondria, if added together with a penetrating weak acid.

Fig. 5 demonstrates swelling, supported by ATP energy. It is seen that addition of L-palmitoyl carnitine to mitochondria incubated with ATP greatly decreased the light scattering of the suspension, the fact indicating mitochondrial swelling. FCCP strongly inhibited this effect.

Fig. 6 shows similar data for the system where respiration, instead of ATP, was used as an energy source. Swelling was induced by addition of L- or D-palmitoyl carnitine to mitochondria oxidizing succinate. Both isomers induced the swelling in a cyanide-sensitive fashion.

The swelling supported by oxidation of endogeneous substrates is shown in Fig. 7. It is seen, again, that both L- and D-palmitoyl carnitines are effective. FCCP inhibited the swelling.

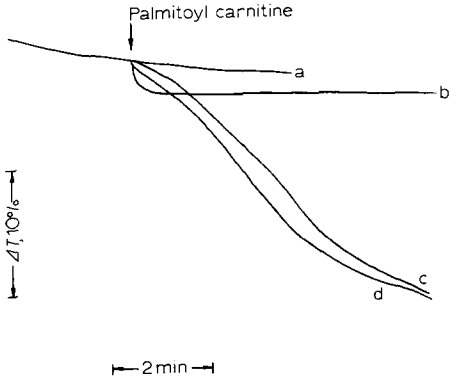


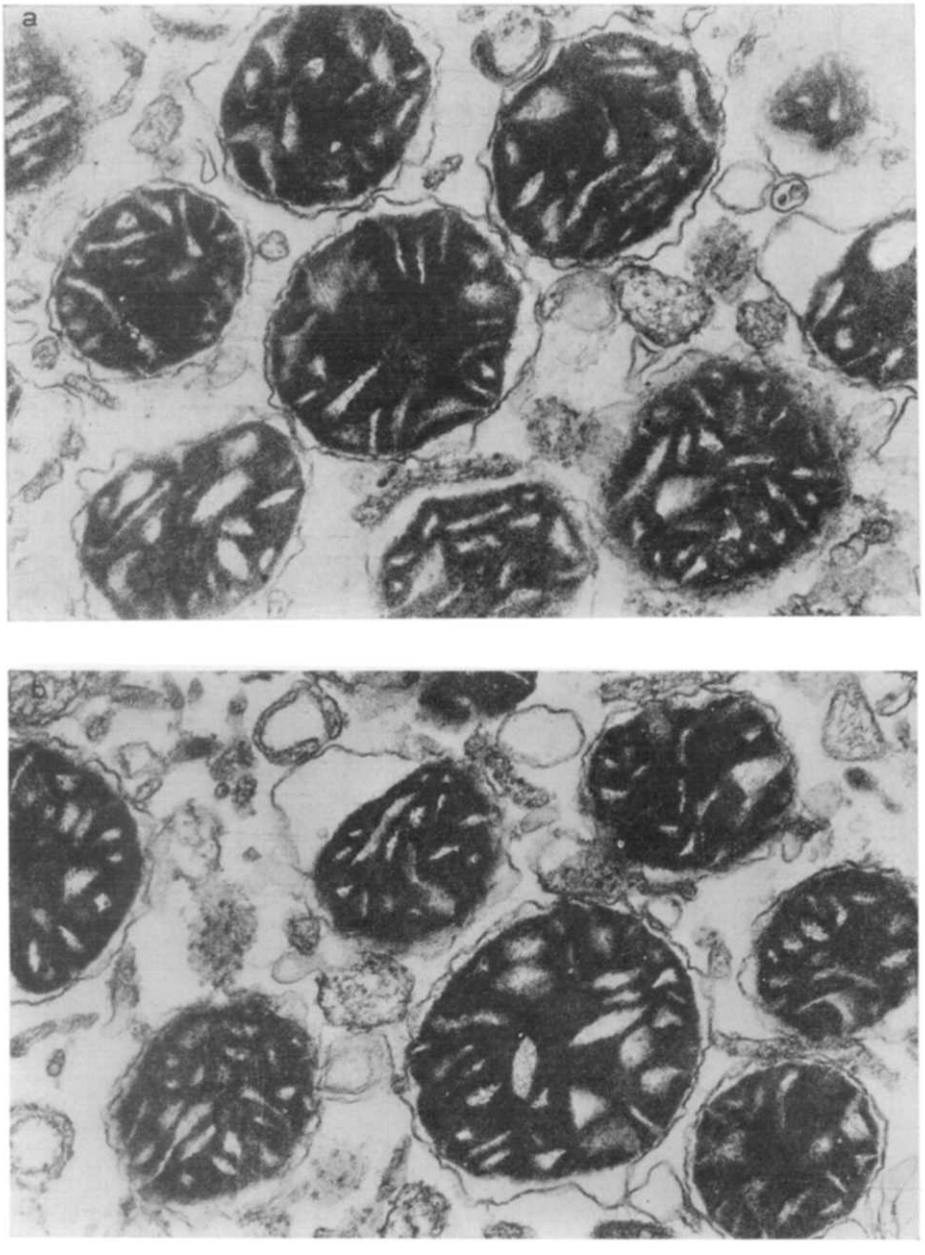
Fig. 7. Palmitoyl carnitine-induced swelling of mitochondria supported by the energy of oxidation of endogenous substrates. Reaction mixture: 0.3 M sucrose, $2.0 \cdot 10^{-2}$ M Tris-HCl buffer, pH 7.5, $1.7 \cdot 10^{-3}$ M $MgSO_4$, $4.2 \cdot 10^{-3}$ M sodium phosphate, rat liver mitochondria (1.6 mg protein/ml). a, without palmitoyl carnitine; b, with $8.5 \cdot 10^{-5}$ M L-palmitoyl carnitine and $4.2 \cdot 10^{-6}$ M FCCP; c, with $4.2 \cdot 10^{-5}$ M D-palmitoyl carnitine; d, with $4.2 \cdot 10^{-5}$ M L-palmitoyl carnitine.

Fig. 8 demonstrates the results of an electron microscope study of mitochondria fixed immediately after light-scattering measurement (see Fig. 7). One can see that it is the matrix space which swells when palmitoyl carnitine is added to the energized mitochondria.

In most of the swelling experiments, rotenone was added to prevent utilization of the palmitoyl carnitine as a substrate of oxidation. In further experiments, the substrate function of palmitoyl carnitine was studied.

It was found that a short-term incubation of L-palmitoyl carnitine with energized mitochondria was necessary to observe the high rate of its oxidation in State

3; uncoupled. As one can see from Fig. 9, Curve a, an uncoupler, FCCP, being added 3 min after palmitoyl carnitine, induced a pronounced and steady increase in the oxidation rate. FCCP, if added 45 s after L-palmitoyl carnitine, stimulates respiration only slightly, the effect changing into the inhibition of oxygen consumption (Curve b). Addition of FCCP before L-palmitoyl carnitine completely prevented the transition of mitochondria to the state of active respiration (Curve c). This effect, as found



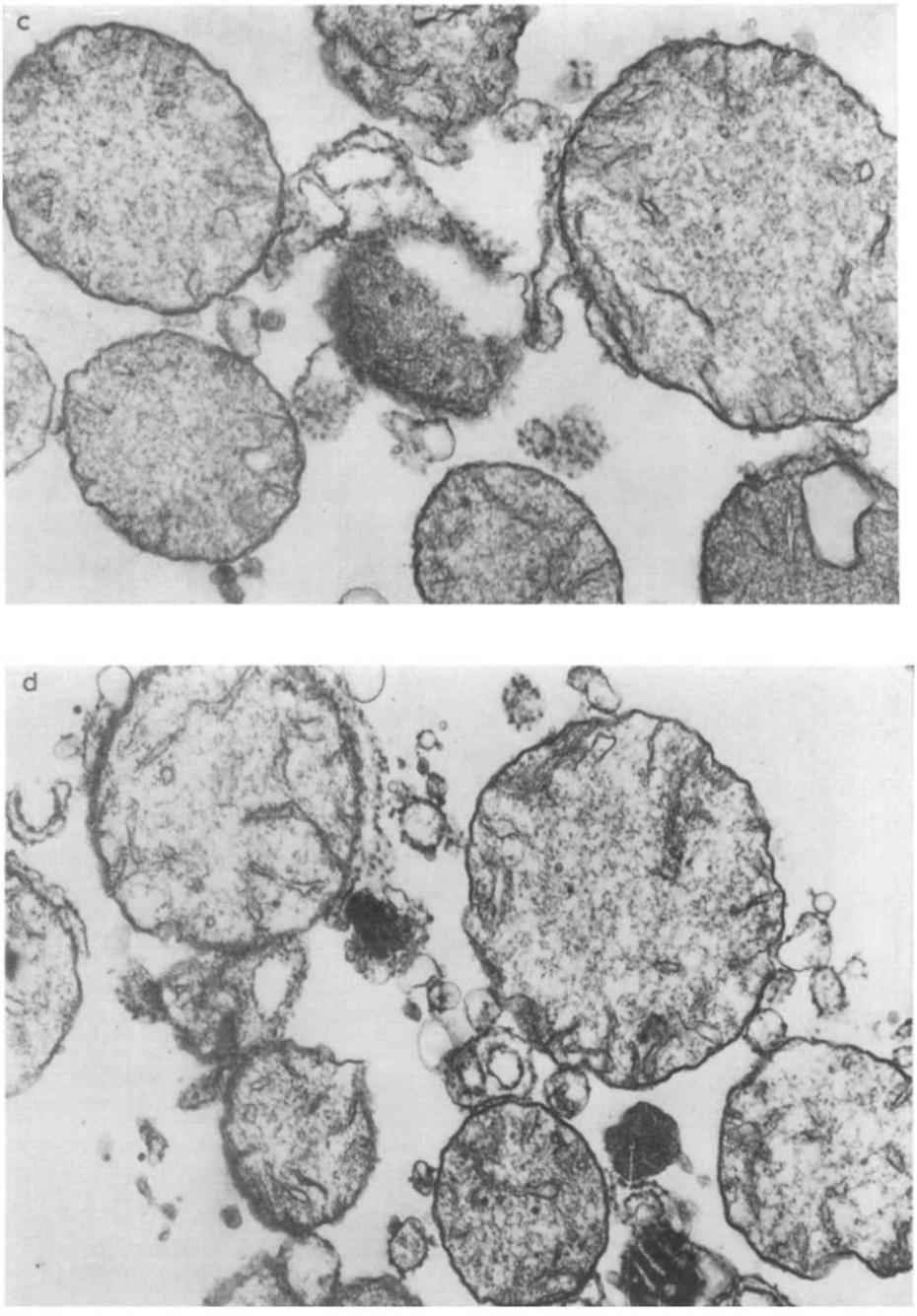


Fig. 8. Electron microscope pictures of slices of mitochondria swollen in the presence of palmitoyl carnitine. Mitochondria were fixed immediately after measurement of light scattering (see Fig. 7). Magnification 44 400 ×. (a) Without palmitoyl carnitine (Fig. 7, Sample a). (b) with L-palmitoyl carnitine and FCCP (Fig. 7, Sample b). (c) With D-palmitoyl carnitine (Fig. 7, Sample c). (d) With L-palmitoyl carnitine (Fig. 7, Sample d).

in further experiments, was due to the lack of oxidizable substrate available for mitochondrial dehydrogenases. Subsequent addition of succinate or glutamate resulted in an increase in the oxidation rate approaching the level of State 3.

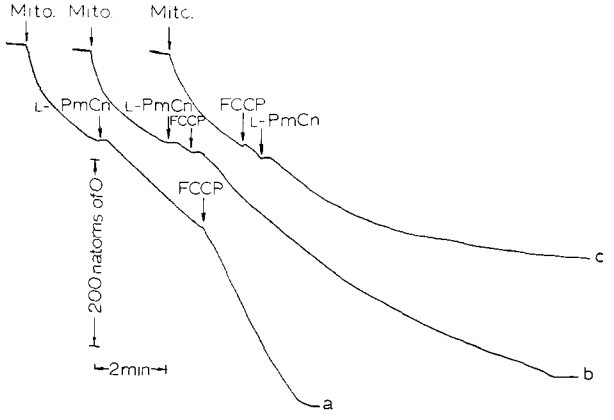


Fig. 9. Requirement of preincubation with palmitoyl carnitine for the FCCP activation of its oxidation in rat liver mitochondria. Reaction mixture: 0.3 M sucrose, $2 \cdot 10^{-2}$ M Tris–HCl buffer, pH 7.5, $2.5 \cdot 10^{-3}$ M $MgSO_4$, $6.3 \cdot 10^{-3}$ M KCl. Additions: 6.5 mg rat liver mitochondria (7.8 mg protein/ml), $7 \cdot 10^{-4}$ M L-palmitoyl carnitine (L-PmCn) and $6.25 \cdot 10^{-7}$ M FCCP.

An effect similar to that of FCCP could be obtained with another uncoupler, 2,4-dinitrophenol (Fig. 10). In the experiment shown in Fig. 10 rutamycin was added to Samples b and d. One can see that the presence of the antibiotic did not abolish the effect of the 2,4-dinitrophenol pre-treatment which prevented active oxidation of palmitoyl carnitine. In the same experiments, it was found that the concentrations of rutamycin used arrested the uncoupler-induced ATPase, so the

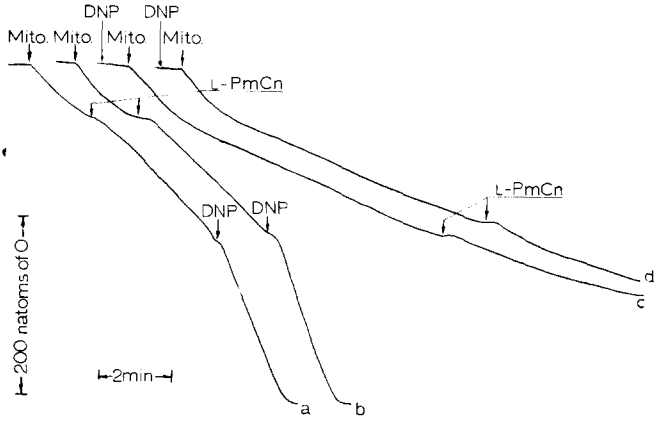


Fig. 10. The effect of the order of additions of L-palmitoyl carnitine and 2,4-dinitrophenol (DNP) on oxidation of L-palmitoyl carnitine by rat liver mitochondria. Reaction mixture (see Fig. 9) was supplemented with rutamycin (6 μg/ml) in Samples b and d. Additions: rat liver mitochondria (4.3 mg protein/ml), $1.2 \cdot 10^{-4}$ M 2,4-dinitrophenol, $1.9 \cdot 10^{-4}$ M L-palmitoyl carnitine (L-PmCn).

effect of the uncouplers on oxidation of palmitoyl carnitine should not be the result of exhaustion of the mitochondrial ATP pool due to uncoupler-activated ATPase.

Neither could it be explained by exhaustion of the pool of endogeneous dicarboxylic acid which might be required for fatty acid oxidation, since, as was

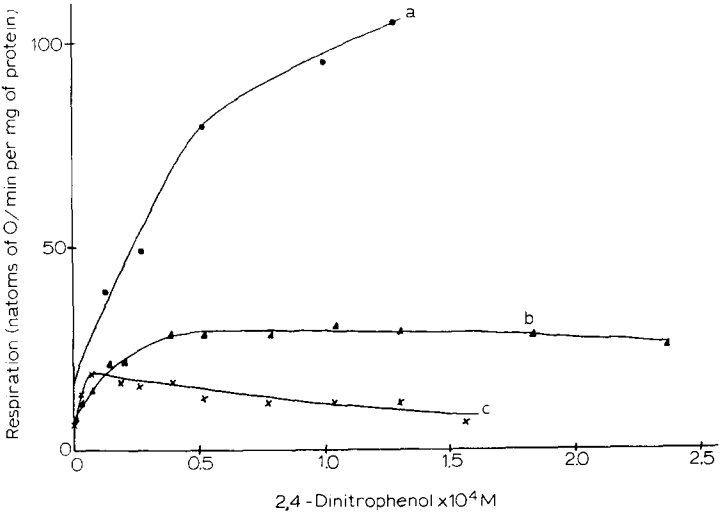


Fig. 11. Oxidation of glutamate + malate and of palmitoyl carnitine as a function of 2,4 dinitrophenol concentration. reaction mixture as in Fig. 9. a, 2,4-dinitrophenol was added to mitochondria after $1.1 \cdot 10^{-2}$ M glutamate + malate; b, 2,4-dinitrophenol was added after 2.5 min incubation of mitochondria with $2.5 \cdot 10^{-4}$ M; c, 2,4-dinitrophenol was added 3.5 min prior to the addition of $2.5 \cdot 10^{-4}$ M L-palmitoyl carnitine. Respiration in the b and c curves was measured 4 min after the addition of L-palmitoyl carnitine.

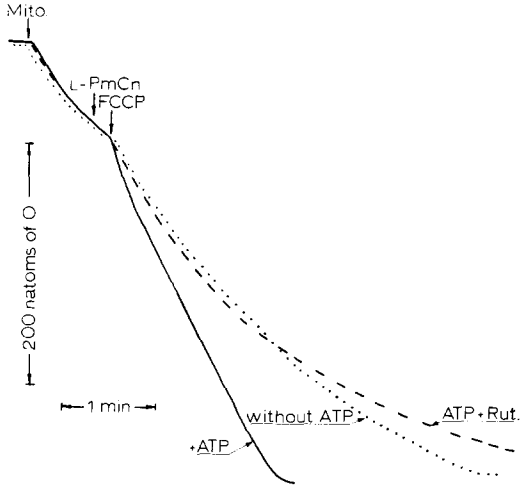


Fig. 12. Stimulation of L-palmitoyl carnitine oxidation in mitochondria by ATP. The incubation mixture as in Fig. 9. Additions: 5 mg of rat liver mitochondria (6 mg protein/ml), $2 \cdot 10^{-4}$ M L-palmitoyl carnitine and $6.25 \cdot 10^{-7}$ M FCCP. The concentration of ATP was $1.25 \cdot 10^{-3}$ M, that of rutamycin 7.2 μg/ml.

shown in other experiments, addition of 0.5 mM malate did not abolish the inhibiting action of the uncouplers.

Fig. 11 demonstrates the respiratory responses of mitochondria as a function of 2,4-dinitrophenol concentration. It is seen that treatment with low concentrations (below $0.25 \cdot 10^{-4}$ M) of 2,4-dinitrophenol added either before or after L-palmitoyl carnitine increased the respiration rate. Higher amounts of 2,4-dinitrophenol inhibited oxidation if added before (but not after) L-palmitoyl carnitine. The concentrations of 2,4-dinitrophenol used were found to produce only a stimulating effect on the oxidation of glutamate and malate.

The following experiments showed that the pre-incubation time between additions of L-palmitoyl carnitine and an uncoupler, required for the steady activation of L-palmitoyl carnitine oxidation could be markedly decreased by the addition of ATP. Fig. 12 shows that the steady activation revealed itself after 10 s of pre-incubation of mitochondria with L-palmitoyl carnitine if ATP was present. In the sample without ATP or with ATP + rutamycin, the oxidation, activated at the first moment after addition of FCCP, strongly decreased during incubation.

DISCUSSION

The results described in the first part of the previous section demonstrate that palmitoyl carnitine exists in the cationic form, easily penetrating across the hydrophobic barrier of the membrane structures. Palmitoyl carnitine greatly increased the electric conductance of artificial phospholipid membranes; the transmembrane gradient of this compound generates a membrane potential whose polarity suggests that diffusion of the cationic form of palmitoyl carnitine is responsible for the electrogenesis; all effects of palmitoyl carnitine on phospholipid membranes prove to be proportional to the concentration of the palmitoyl carnitine cation in the solution.

Apparently, palmitoyl carnitine cation is perfectly adapted to penetrate membraneous barriers. It is much more effective than all the unnatural cationic compounds studied which were found to penetrate phospholipid membranes. For instance, a cation such as triphenyl methyl phosphonium, one of the most effective penetrating synthetic cations, increases the electric conductance of the phospholipid membranes at much higher concentrations than palmitoyl carnitine. The difference in the effectiveness of these two compounds proves to be of several orders of magnitude if one takes into account the concentration of the cationic form of palmitoyl carnitine instead of its total concentration given in Fig. 1.

The fact that the palmitoyl carnitine cation readily penetrates the phospholipid membranes strongly suggests that this compound is also capable of penetrating the mitochondrial membrane.

A study of a wide range of synthetic and natural substances in Dr E. A. Liberman's group and our laboratory resulted in the selection of about fifty compounds which increase the electric conductance of phospholipid membranes. In all cases, without exception, the agents penetrating the artificial membrane affect the mitochondrial functions in a definite, predictable way, as penetrating cations, penetrating anions, or ionophores[7,8,21]. It is most probable that the striking adequacy of the protein-free phospholipid membrane in the study of

mitochondria is due to the fact that some parts of the mitochondrial membrane are actually a phospholipid bilayer, and it is just this area of the mitochondrial structure which is responsible for the effects of the penetrating compounds studied. It is not surprising, therefore, that palmitoyl carnitine, which can penetrate phospholipid membrane as cation, induces the same responses in mitochondrial experiments as other penetrating cations. The results of light-scattering measurements and electron microscope analysis, described in this paper, showed that palmitoyl carnitine being added together with a penetrating weak acid, supported energy-dependent swelling of mitochondrial matrix just as did Ca^{2+}, K^+ (+ valinomycin), dimethyl dibenzyl ammonium, *etc.* These findings confirm some previous observations made by Dargel and Strack[22] concerning an inhibition of the palmitoyl carnitine-induced swelling by 2,4-dinitrophenol.

Subsequent experiments revealed that the transport of palmitoyl carnitine to the compartment of its oxidation in the mitochondrial interior requires energy which can be supplied by respiration or ATP hydrolysis, the transport being sensitive to rutamycin in the latter case. It was found that a high rate of palmitoyl carnitine oxidation can be attained only if this substrate was pre-incubated for a short time with mitochondria in the energized state. De-energization of mitochondria by uncouplers or (in the case of ATP) by rutamycin results in the subsequent addition of palmitoyl carnitine not inducing stimulation of oxygen consumption.

It should be mentioned that all the above relationships can be demonstrated only if the palmitoyl carnitine concentration is not too high. At palmitoyl carnitine concentrations of about 1 mM disruption of the artificial membrane and energy-independent swelling followed by lysis of mitochondria took place. These effects were apparently due to the detergent action of palmitoyl carnitine, whose structure resembles that of lysophosphatides (about the detergent action of palmitoyl carnitine, see refs 23, 24).

Another observation of interest for this discussion is the dependence of the rate of the palmitoyl carnitine oxidation on the uncoupler concentration. It has been found that 2,4-dinitrophenol added before palmitoyl carnitine, completely prevents the stimulation of oxidation by the latter only if the concentration of this uncoupler is as high as $1.5 \cdot 10^{-4}$ M. When lower amounts of 2,4-dinitrophenol were added, subsequent addition of palmitoyl carnitine induced some increase in the oxidation rate. Similar amounts of 2,4-dinitrophenol, as found in this laboratory by I. I. Severina, have been required for the complete inhibition of the accumulation of Ca^{2+}, K^+ (+ valinomycin) and dimethyl dibenzyl ammonium in mitochondria. Under the same conditions, stimulation of the oxidation of palmitoyl carnitine added before 2,4-dinitrophenol requires lower concentrations of the uncoupler. This observation can be compared with the fact that the State 4–State 3 transition of the respiratory chain as well as inhibition of coupled phosphorylation occurs under a relatively small decrease in the respiration-supported membrane potential, whereas the ion transport system, having no threshold membrane potential level, is still operative even when this level is significantly decreased by an uncoupler[8].

Generally, the data obtained are in agreement with the predictions of the hypothesis which considers the electrophoretic movement of the cation of fatty acyl carnitine as a mechanism of the transport of fatty acids in the mitochondrial membrane[16].

Accepting the point that palmitoyl carnitine is a penetrating cation which is prompted by the above experiments, one inevitably comes to the conclusion that palmitoyl carnitine, like other penetrating ions, can be involved in electrophoretic ion transport through the mitochondrial membrane.

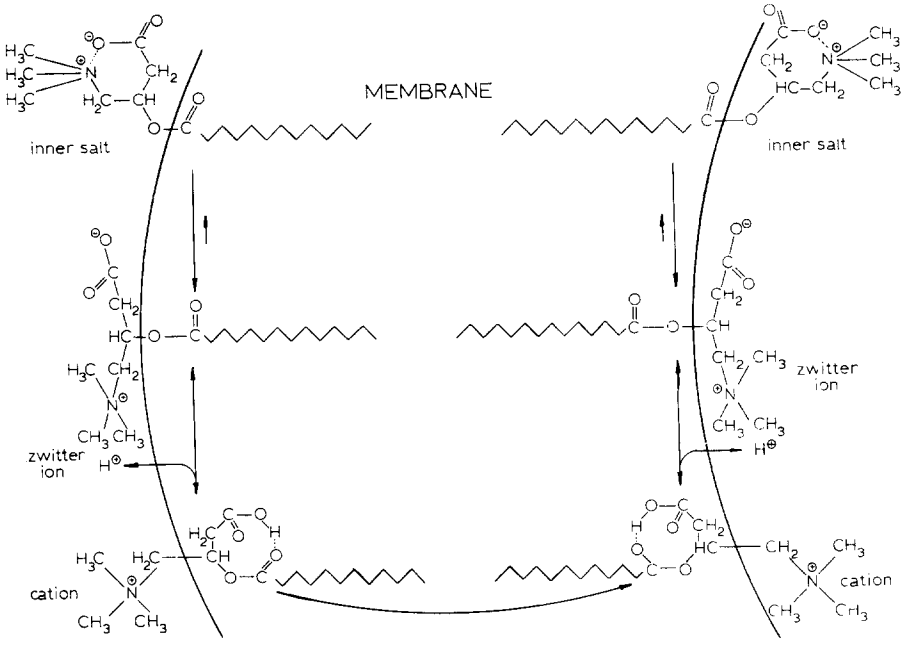


Fig. 13. Mechanism of acyl carnitine transport into mitochondria down electrochemical gradient of H^+.

The scheme illustrating the possible mechanism of palmitoyl carnitine transport is given in Fig. 13. On the scheme, three forms of palmitoyl carnitine are shown: two electroneutral ones (inner salt and opened zwitterion) and the third, cationic, protonated at the carboxylic group. When there is no difference in the electrochemical potentials of the H^+ across the membrane, the palmitoyl carnitine concentrations on both membrane sides should be equal. Formation of a transmembrane electric potential and (or) pH differences must result in the removal of the protonated (cationic) form of palmitoyl carnitine from the positively charged (acidic) compartment and in its transport through the membrane to the negatively charged (alkaline) compartment, *i.e.* in the case of energized mitochondria, from outside to inside. In the outer compartment, decrease in the concentration of the cationic form should shift the equilibrium between the deprotonated palmitoyl carnitine and the palmitoyl carnitine cation, the effect resulting in protonation of a new portion of the zwitterion. Respectively, an increase in the palmitoyl carnitine cation concentration in the inner compartment should result in the opposite shift of the equilibrium, *i.e.* in deprotonation of the cation. So, transport of palmitoyl carnitine into mitochondria proves to be accompanied by H^+ translocation in the same direction (from outside to the mitochondrial interior). This process can be defined as a symport of palmitoyl carnitine and proton down the electrochemical gradient of H^+ ($\Delta\bar{\mu}_H$). It is of impor-

tance that both components of $\Delta\bar{\mu}_H$, *i.e.* electrical ($\Delta\psi$) and chemical (ΔpH), can be utilized for the uphill transport of palmitoyl carnitine, the situation being different from that for penetrating cation and weak acids transport studied previously when $\Delta\psi$ or ΔpH are used[6,8]. Apparently, similar relationships take place in the mechanism of lactose transport across a bacterial cell membrane. As West[25] has shown, the energy-dependent accumulation of lactose by *E. coli*, resulting in the 100–2000-fold increase in inner lactose concentration, represents a symport of one lactose molecule and one H^+. In this case, as in that of palmitoyl carnitine, the transport of an electroneutral compound (lactose) coupled with proton movement seems to be supported by the total $\Delta\bar{\mu}_H$.

Participation of carnitine in fatty acid transport solves not only the problem of the maintenance of a fast and uphill movement of the translocated species but also prevents uncoupling which might arise if a fatty acid had been transported in the unesterified form. At physiological concentrations (below $5 \cdot 10^{-4}$ M), palmitoyl carnitine did not induce measurable proton conductance, apparently due to the low penetrating ability of the deprotonated (zwitterion) form. So, using carnitine, the mitochondrion is able to transport a large, negatively charged molecule of a fatty acid inclined to be localized on the membrane surface and capable of discharging the membrane potential, into the negative charged mitochondrial interior.

When any scheme of a carnitine-mediated fatty acyl transport in mitochondria is considered, there is no getting away from the question of what happens with the carnitine released after transfer of acyl from acyl carnitine to the intramitochondrial CoA. There is some evidence for impermeability of the inner mitochondrial membrane for carnitine[26–28]. So, it is not clear how carnitine, transported into the mitochondrial interior in the acylated form, returns to the outer space to fetch a new acyl residue. The results of our experiments support the opinion

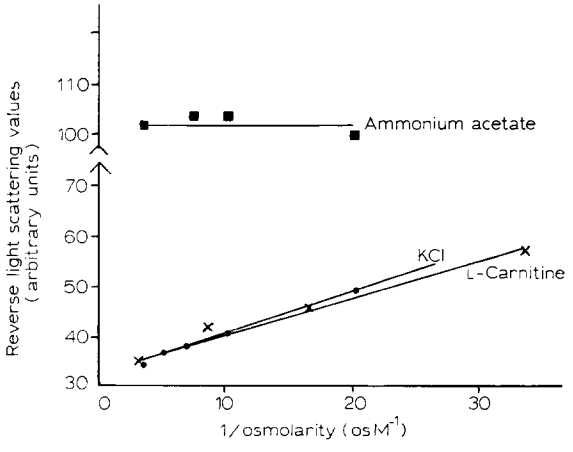


Fig. 14. Swelling of rat liver mitochondria in the solutions of L-carnitine, KCl and ammonium acetate of various tonicity. Reverse values of osmolarity (osM^{-1}) are plotted against reverse values of the light scattering. Samples contained $1 \cdot 10^{-2}$ M Tris–HCl buffer, pH 7.5, and 2.7 mg of rat liver mitochondria (2.7 mg protein/ml). Before the experiment, mitochondria were stored for 30 min in a suspension containing 135 mg protein/ml and $2 \cdot 10^{-4}$ M rotenone dissolved in acetone.

of the low penetrating ability of carnitine. It was found, in particular, that carnitine does not penetrate phospholipid membrane in the cationic form. It was also observed that carnitine can substitute a non-penetrating salt, KCl, when mitochondrial swelling was studied as a function of the medium tonicity after the method of Chappell and Crofts[29]. These data are presented in Fig. 14 where light scattering of mitochondria is plotted against concentrations of carnitine, KCl or a penetrating salt, ammonium acetate.

To bypass the question of the carnitine transport, Garland and co-workers[26,30] proposed that the interaction of extramitochondrial acyl carnitine with intramitochondrial CoA occurs in the middle part of the inner mitochondrial membrane and is organized in such a way that the reaction products, carnitine and acyl CoA, could be released into different compartments: carnitine into outer solution and acyl CoA into the mitochondrial matrix.

Another way to return carnitine to the outer space may involve carnitine translocation between two molecules of acyl carnitine transferase localized on the opposite sides of the inner mitochondrial membrane, a process which might occur without an exchange of the enzyme-bound carnitine with external and (or) internal water solution[21] (Fig. 15). It is postulated that carnitine (Cn) firmly bound to the outer acyl carnitine transferase (E_0) releases in the outer space after acylation by acyl CoA (Stage 1). Acyl carnitine released (acyl Cn) is equilibrated with its protonated cationic form (acyl CnH^+, Stage 2) which traverses the membrane moving down the electrochemical gradient of H^+ (Stage 3). On the inner surface of the membrane, acyl CnH^+ is equilibrated with deprotonated acyl Cn (Stage 4) which interacts with the inner acyl carnitine transferase (E_i) and CoA giving $E_i \cdot Cn$ and acyl CoA (Stage 5). The cycle is completed when Cn is translocated from E_i back to E_0 (a rotation of $E_i \cdot Cn$ and E_0 might be involved in this process, Stage 6). It is essential that the exchange between the free and enzyme-bound pools of carnitine, a process which can be very slow, is not required for the postulated cycle to be

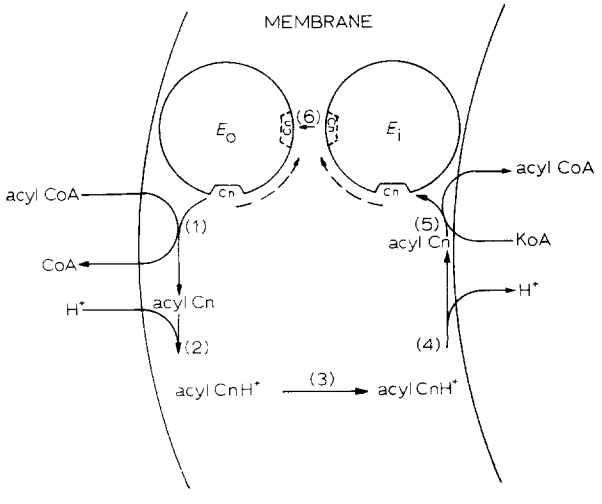


Fig. 15. A scheme illustrating the possible role of carnitine in fatty acid transport through the mitochondrial membrane.

operative. Only the enzyme-bound form of carnitine participates here, whereas acyl carnitine moves in its free form with no acyl carnitine transferase involved. The latter point is confirmed by the fact that both L- and D-palmitoyl carnitine induce an energy-dependent swelling of mitochondria while only the L-isomer can serve as the substrate of acyl carnitine transferase.

One more possibility may be discussed, when considering the fate of carnitine in mitochondria, namely, that carnitine *per se* does not leave the mitochondrial interior until the membrane permeability is kept low. It might be possible for mitochondria oxidizing fatty acids to accumulate free carnitine in the matrix space. If it were the case, carnitine would be returned into cytosol after the mitochondrial swelling which increases the ion permeability of the inner membrane. This event may accompany the swelling–shrinkage cycles which, apparently, always happen to a mitochondrion *in vivo*[21].

One can also think that carnitine accumulating in the matrix of the mitochondria can undergo partial or even complete degradation[31]. It is known that various organisms possess a special enzyme, carnitine decarboxylase, converting carnitine to β-methyl choline[32–35]. There are some indications to the matrix localization of carnitine decarboxylase[30].

Other possible pathways of carnitine degradation in mitochondria have not been studied, being investigated in some microorganisms only. In particular, dehydrogenation of carnitine into its keto-derivative[36] as well as conversion of carnitine into malate[37] were described in bacteria. If the latter pathway were operative in mitochondria, an attractive possibility would arise that acyl carnitine could be considered as a supplier not only of fatty acyl but also of oxaloacetate whose catalytic amounts are necessary for fatty acyl oxidation in mitochondria.

All the above considerations concerning the role of carnitine are relevant to the transport of long-chain fatty acids whose carnitine esters can penetrate through the membrane in the cationic form. The hydrophilic acetyl carnitine cation was found to be non-penetrating for phospholipid membranes. It is hardly possible, therefore, that acetyl carnitine is involved in a transport mechanism similar to that for palmitoyl carnitine. Apparently, some functions, other than transport of acetyl through the membranes, should be discussed for acetyl carnitine, among them the storage of a high-energy acetyl groups[38,39].

ACKNOWLEDGEMENT

We would like to thank professor E. Strack for the carnitine and its derivatives, Miss L. E. Bakeeva and Miss Elena V. Lebedinskaya for participation in experiments and Miss T. I. Kheifets for correcting the English version of the paper.

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