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Characterization of the unidirectional transport of carnitine catalyzed by the reconstituted carnitine carrier from rat liver mitochondria

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The carnitine carrier from rat liver mitochondria was purified by chromatography on hydroxyapatite and celite and reconstituted in egg yolk phospholipid vesicles by adsorbing the detergent on polystyrene beads. In the reconstituted system, in addition to the carnitine/carnitine exchange, the purified protein catalyzed a uni-directional transport (uniport) of carnitine measured as uptake into unloaded proteoliposomes as well as efflux from prelabelled proteoliposomes. In both cases the reaction followed a first-order kinetics with a rate constant of 0.023–0.026 min^{-1}. Besides carnitine, also acylcarnitines were transported in the uniport mode. *N*-Ethylmaleimide inhibited the uni-directional transport of carnitine completely. The uniport of carnitine is not influenced by the ΔpH and the electric gradient across the membrane. The activation energy for uniport was 115 kJ/mol and the half-saturation constant on the external side of the proteoliposomes was 0.53 mM. The maximal rate of the uniport at 25 °C was 0.2 μmol/min per mg protein, i.e. about 10 times lower than that of the reconstituted carnitine transport in exchange mode.

Introduction

The carnitine carrier of mitochondria catalyzes the entry of acylcarnitines into the mitochondrial matrix in exchange for free carnitine, thus supplying acyl units to the β-oxidation pathway. In studies performed with intact mitochondria it has been shown that this carrier accepts L-carnitine and acylcarnitines of various length as substrates [1] and is specifically inhibited by -SH reagents and substrate analogues [2–3]. Ramsay and Tubbs proposed that the carnitine carrier may catalyze a uni-directional transport besides the more efficient exchange reaction between acylcarnitines and carnitine [3]. This hypothesis was supported by Pande and Parvin [4] who found a uni-directional transport of carnitine in liver and heart mitochondria. It was also proposed that the carrier-mediated uniport of carnitine plays a role in controlling the carnitine level in mitochondria. This in turn would regulate the acylcarnitine import.

Recently, we have isolated the carnitine carrier from rat liver mitochondria by chromatography on hydroxyapatite and celite [5]. In SDS-containing gels, the purified fraction consists of a single band with an apparent molecular mass of 32.5 kDa. After incorporation into liposomes, the purified protein shows substrate and inhibitor specificity similar to that found in intact mitochondria [5]. Furthermore, the reconstituted carnitine/carnitine exchange activity is strongly influenced by cardiolipin and shows well defined kinetics [6].

In this paper we demonstrate that the purified carnitine carrier, reconstituted into liposomes, catalyzes, besides the exchange reaction, a uniport of substrates. The basic properties and kinetic parameters of the uni-directional transport of carnitine in the reconstituted system are described.

Materials and Methods

Materials. Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad, Celite 535 from Roth, Amberlite XAD-2 from Fluka, Sephadex G-50 and G-75 from

Abbreviations: Pipes, 1,4-piperazinediethanesulphonic acid; SDS, sodium dodecyl sulphate.

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Pharmacia, L-[^{3}H]carnitine from Amersham, egg yolk phospholipids (L-α-phosphatidylcholine from fresh turkey egg yolk), cardiolipin, Pipes, Triton X-100, L-carnitine and L-octanoylcarnitine from Sigma, and L-acetylcarnitine from Serva. L-α-Methylbutyrylcarnitine and L-isovalerylcarnitine were a gift of prof. N. Siliprandi and Dr. F. Di Lisa. All other reagents were of analytical grade.

Purification of the carnitine carrier. The carnitine carrier was purified from rat liver mitochondria as described in Ref. 5. The completely purified preparation, i.e. the third celite fraction, was used in all the experiments.

Reconstitution of the purified carnitine carrier into liposomes. The purified protein was reconstituted by removing the detergent with a hydrophobic ion-exchange column [7–8]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through the same Amberlite XAD column. The composition of the initial mixture used for reconstitution was: 380 μl of the purified carnitine carrier (1–2 μg protein in 3% Triton X-100), 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as described in Ref. 9, L-carnitine at the indicated concentrations, 30 mM sodium phosphate (pH 7) in a final volume of 680 μl. After vortexing, this mixture was passed 15 times through the same Amberlite column (0.5 × 3.6 cm) preequilibrated with a buffer containing 30 mM sodium phosphate (pH 7) and the same concentration of L-carnitine present in the starting mixture. All the operations were performed at 4 °C except the passages through Amberlite, which were carried out at room temperature.

Transport measurements. In order to remove the external substrate, 550 μl of proteoliposomes were passed through a Sephadex G-75 column (0.7 × 15 cm) preequilibrated with 10 mM Pipes (pH 7) and 90 mM NaCl. This chromatography was performed in 3 min and at 2 °C to minimize the efflux of carnitine from the proteoliposomes. The transport activity was determined by measuring the flux of labelled carnitine from outside to inside (uptake experiments) or from inside to outside (efflux experiments).

When measuring efflux, the proteoliposomes containing 5 mM internal carnitine were prelabelled by carrier-mediated exchange equilibration. This was achieved by incubating the proteoliposomes with 5 μM [^{3}H]carnitine at high specific radioactivity (50 μCi/nmol) for 20 min at 25 °C. After this incubation period the external radioactivity was removed by passing the liposomes through a Sephadex G-75 column as described above. The concentration of carnitine in the active liposomes, i.e. the carrier-loaded liposomes, at the end of the prelabelling procedure was estimated in parallel samples (incubated with 5 μM or 5 mM unlabelled carnitine for 20 min and passed through Sephadex G-75) by measuring the radioactivity taken up after 90 min incubation at 25 °C in the presence of 2 mM external [^{3}H]carnitine. Under these conditions the uptake of [^{3}H]carnitine is proportional to the amount of substrate present in the active liposomes. Since one can assume that in those samples incubated with 5 mM unlabelled external carnitine the internal concentration is close to 5 mM, we can estimate the concentration of carnitine present in the active liposomes of the samples incubated with 5 μM carnitine as $(dpm_2/dpm_1) \times 5$ mM, where dpm_2 and dpm_1 are the radioactivities taken up by the proteoliposomes incubated with 5 μM and 5 mM carnitine, respectively. With this method we found that the internal concentration of carnitine at the end of the prelabelling procedure ranged between 3.8 and 4.3 mM in different experiments. The same method was used to estimate the internal substrate concentration in the experiments reported in Fig. 3.

Transport was started, in the case of uptake experiments, by adding labelled carnitine to the proteoliposomes or, in the case of efflux experiments, by adding unlabelled carnitine in buffer (90 mM NaCl/10 mM Pipes (pH 7)) or buffer alone to the prelabelled proteoliposomes. In both cases transport was stopped by adding 0.5 mM *N*-ethylmaleimide. In control samples the inhibitor was added at time zero according to the inhibitor stop method [10]. The assay temperature was 25 °C. Finally each sample of proteoliposomes (100 μl) was passed through a Sephadex G-50 column (0.6 × 8 cm) in order to separate the external from the internal radioactivity. Liposomes were eluted with 1.3 ml 50 mM NaCl and collected in 4 ml of scintillation mixture, vortexed and counted.

The isotope equilibration kinetics were followed by stopping the transport reaction after different time intervals. In the case of the uptake experiments, the experimental values were corrected by subtracting the respective control. The *N*-ethylmaleimide-insensitive radioactivity associated to the control samples was always less than 8% and 40% with respect to the *N*-ethylmaleimide-sensitive exchange and uniport of carnitine, respectively. The corrected data were then fitted by a computer program according to a first-order process equation allowing to calculate the initial transport rate, which is expressed as μmol/min per mg protein.

In the case of the efflux determinations, the decrease of radioactivity inside the liposomes was fitted according to a single-exponential decay equation, from which the rate expressed in $-\Delta$dpm/min was derived. The initial transport rate expressed in μmol/min per mg protein was calculated by dividing Δdpm/min by SA × mg protein, where SA is the specific activity of the radioactivity present in the active liposomal space.

The radioactivity present in the active liposomes was determined by measuring the decrease of radioactivity inside the liposomes after incubation with 10 mM external carnitine for 120 min at 25 °C. The amount of substrate present in the active liposomes was calculated by multiplying the internal substrate concentration at the end of the prelabelling procedure (estimated as described above) by the active internal volume.

The active intraliposomal volume was determined by incubating the proteoliposomes (without internal substrate) with 0.1 mM [^{3}H]carnitine for 90 min at 25 °C. Since at equilibrium the concentration of carnitine inside and outside the active liposomes is the same, the ratio between the radioactivity taken up after 90 min (corrected for the *N*-ethylmaleimide-insensitive aliquot) and the total radioactivity added represents the ratio between the active internal volume and the total assay volume. In the experiments reported in this paper the internal active volume of the proteoliposomes was 4 ± 0.3% of the total intraliposomal volume. The total internal volume of the liposomes (i.e., liposomes with and without incorporated carrier protein) was measured as described in Ref. 7.

Other methods. Protein was determined by the Lowry method modified for the presence of Triton [11]. All the samples used for protein determination were subjected to acetone precipitation and redissolved in 1% SDS [5].

Results

In liposomes reconstituted with the purified carnitine carrier we have investigated the uptake of [^{3}H]carnitine as uniport (in the absence of internal carnitine) or as exchange (in the presence of 13 mM carnitine). Fig. 1A compares the time-course of the *N*-ethylmaleimide-sensitive uniport and *N*-ethylmaleimide-sensitive exchange of carnitine at 2.5 mM labelled external substrate. The data of both curves fitted a first-order rate equation. In both cases maximum uptake was reached after about 90 min being 16.7 μmol per mg protein in the case of the exchange and 3.3 μmol per mg protein in the case of the uniport reaction. The ratio between the maximal amount of substrate taken up by the exchange and by the uniport was 5.0, in good agreement with the expected value of 5.2 derived from the intraliposomal concentrations at equilibrium (2.5 mM for the uniport and 13 mM for the exchange). The first-order rate constants resulted to be 0.036 min^{-1} for exchange and 0.026 min^{-1} for uniport. The calculated initial rates of carnitine uptake were 602 and 88 nmol/min per mg protein for exchange and uniport, respectively, with a ratio of 6.8 (exchange/uniport).

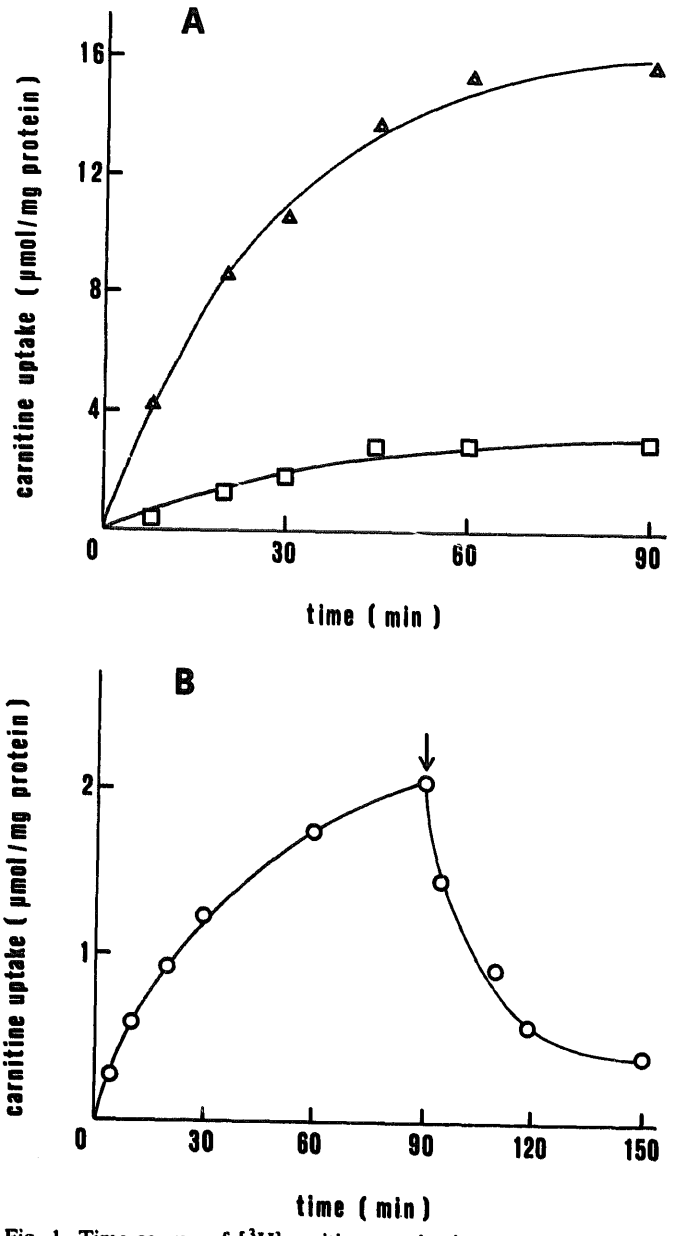

Fig. 1. Time-course of [^{3}H]carnitine uptake in reconstituted liposomes and its efflux after addition of an excess of unlabelled carnitine. In A, 2.5 mM [^{3}H]carnitine was added to unloaded proteoliposomes (uniport, □), or to proteoliposomes loaded with 13 mM carnitine (exchange, △). In B, 1 mM [^{3}H]carnitine was added to unloaded liposomes. Where indicated by the arrow, 20 mM unlabelled carnitine was added.

In other experiments (not shown) the specificity of the uni-directional transport of carnitine was investigated by adding other mitochondrial substrates, instead of carnitine, to unloaded liposomes reconstituted with the purified carnitine carrier. It was found that none of the labelled substrates used (phosphate, malate, malonate, oxoglutarate, citrate, aspartate, glutamate and ADP) was taken up by the proteoliposomes, indicating that the uniport transport of carnitine catalyzed by the carnitine carrier is specific. The same substrate specificity was found for the carnitine exchange catalyzed by the same reconstituted protein [6].

Fig. 1B shows the effect of adding 20 mM unlabelled carnitine to liposomes which had been incubated with 1 mM [^{3}H]carnitine in the absence of internal substrate. The unlabelled substrate was added after 90 min incubation, i.e., when the radioactivity taken up by the proteoliposomes had approached equilibrium. The addition of 20 mM unlabelled substrate caused an extensive efflux of the intraliposomal radioactivity, suggesting that the [^{3}H]carnitine taken up by uniport can be released by exchange for externally added substrate.

In order to obtain further information about this uniport mechanism, the transport of carnitine has been measured as efflux of [^{3}H]carnitine from prelabelled active proteoliposomes. In this technique the ratio between specific and unspecific (inhibitor-insensitive) fluxes of radioactivity is much higher as compared to that in uptake experiments. In Fig. 2 the time-course of the uniport measured as efflux of 5 mM [^{3}H]carnitine is compared to the exchange reaction measured under the same conditions in the presence of 5 mM external carnitine. In both cases the data fitted a single exponential decay equation when the first 60 min were considered. The extrapolated rate constants were 0.17 and 0.023 min^{-1} for the exchange and the uniport, respectively. The transport rates derived as described in Materials and Methods were 2440 and 230 nmol/min per mg protein for the exchange and the uniport, respectively, with a ratio of 10.6. Both uniport and exchange were completely inhibited by *N*-ethylmaleimide (upper curves of Fig. 2). In our previous paper [5]

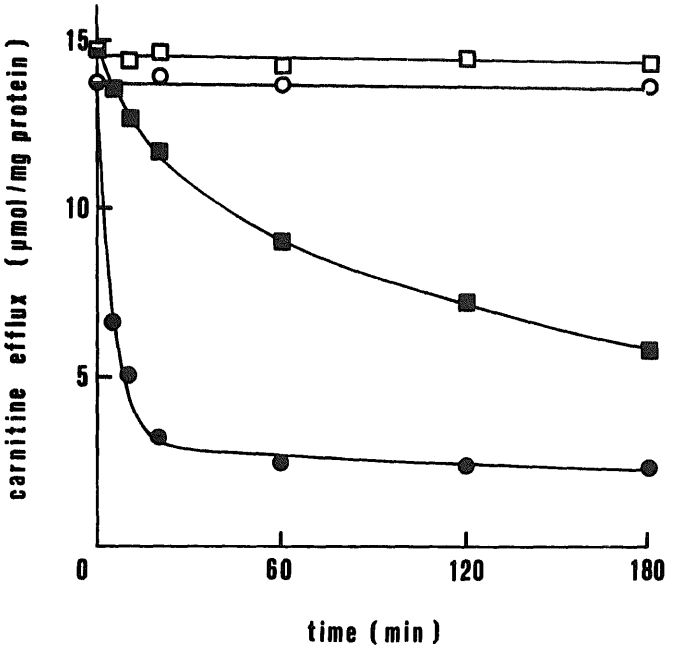

Fig. 2. Time-course of [^{3}H]carnitine efflux from proteoliposomes. The efflux of 5 mM [^{3}H]carnitine from prelabelled proteoliposomes was measured in the presence of buffer (90 mM NaCl/10 mM Pipes, pH 7.0) (uniport, ■ and □) or in the presence of 5 mM unlabelled carnitine in the same buffer (exchange, ● and ○). In □ and ○, *N*-ethylmaleimide was added at time zero.

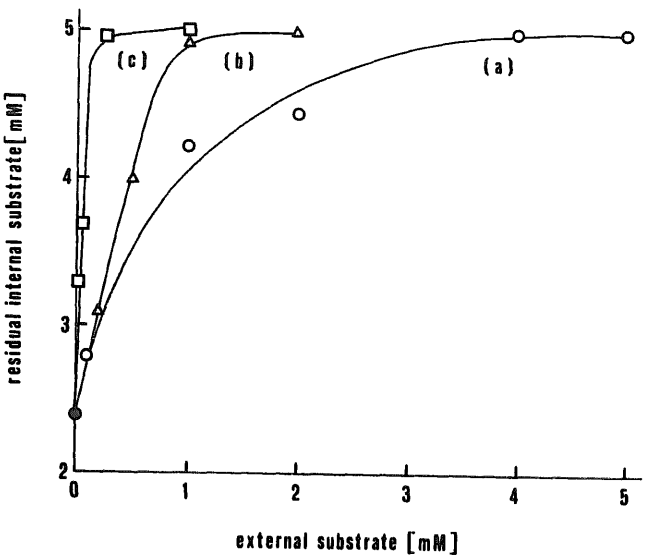

Fig. 3. Dependence of the net efflux of carnitine from proteoliposomes on the external substrate concentration. Proteoliposomes containing 5 mM internal carnitine were incubated in the presence of increasing concentrations of carnitine (○), acetylcarnitine (△) or octanoylcarnitine (□) and in the absence of external substrate (●) for 1 h. After this incubation time, the external substrate was removed and the intraliposomal concentration of substrate was measured as described in Materials and Methods.

the uni-directional transport of carnitine was not observed because the efflux of [^{3}H]carnitine from prelabelled active liposomes was not measured, and because the reconstituted system was not optimized and the uni-directional uptake of [^{3}H]carnitine was not distinguishable from the background.

The experiment in Fig. 3 has been performed to analyze the dependence of the net efflux of carnitine on the concentration gradient of substrate across the membrane (kinetic trans effect). Proteoliposomes containing 5 mM internal carnitine were incubated with increasing concentrations of carnitine for 1 h (curve a). In order to estimate the internal concentration of the substrate at the end of the incubation time, the external substrate was rapidly removed and 2 mM [^{3}H]carnitine was added to proteoliposomes (see Methods). When no substrate was present outside, the residual concentration of carnitine in the active liposomes was 2.4 mM (Fig. 3). This means that about one half of carnitine was lost after 1 h, in agreement with the data of Fig. 2. The net efflux was decreased on increasing the concentration of the external substrate. The concentration of carnitine necessary to stop the efflux completely was about 4 mM. Similar results were obtained when the proteoliposomes were incubated with two other substrates of the carnitine carrier, namely acetylcarnitine and octanoylcarnitine, instead of carnitine (Fig. 3, curves b and c). In these cases a lower concentration of external substrate was sufficient to stop the uni-directional efflux, i.e., 0.2 mM octanoylcarnitine and 1 mM acetylcarnitine. All these concen-

trations necessary to stop efflux are about 10 times higher than the corresponding half-saturation or half-inhibition constants of the same substrates for the carnitine carrier [6], i.e., 15 μM for octanoylcarnitine, 82 μM for acetylcarnitine and 500 μM for carnitine.

In further experiments (not shown) internal carnitine was substituted by acetylcarnitine, isovalerylcarnitine or α-methylbutyrylcarnitine at the same concentration (5 mM). In the absence of external substrate, the concentration of acetylcarnitine, isovalerylcarnitine and α-methylbutyrylcarnitine in the active liposomes was reduced to 2.2, 2.8, and 2.6 mM after 1 h incubation. The uni-directional efflux of the three internal substrates was progressively decreased by increasing concentrations of externally added carnitine or acetylcarnitine, and was completely abolished in the presence of 3–4 mM carnitine or of 0.8–1.3 mM acetylcarnitine.

In order to investigate the dependence of carnitine uniport on ΔpH and the electrical gradient across the proteoliposomal membrane, the experiments reported in Fig. 4 were performed. In these experiments the efflux of 5 mM carnitine was measured as a function of time under different conditions. In A, efflux was initiated in proteoliposomes with an internal pH of 7.0 in the presence of an external unbuffered medium. After 10 min, 20 mM Pipes buffer pH 8.5 or pH 5.5 was added, thus creating a positive or negative ΔpH of 1.5 units each. The data show that the rate of carnitine efflux was not changed by generation of a ΔpH indicating that the uni-directional transport of this substrate does not involve net H^+ movement. In B, efflux was initiated in the presence of transmembrane K^+ gradients of 50 mM KCl external/1 mM KCl internal or vice versa. After 10 min, valinomycin was added, thus creating a transmembrane potential of about 90 mV (in/out and vice versa). No significant change in the efflux rate was observed, indicating that the uniport of carnitine is independent of the electric gradient and the transported species is electroneutral.

Fig. 5 shows the temperature dependence of the uni-directional transport of carnitine. In an Arrhenius plot a straight line was obtained in the range from 10 °C to 30 °C. The activation energy as derived from the slope was 115 kJ/mol, a value very close to that measured for the exchange of carnitine in proteoliposomes [6].

In order to determine K_m and V_{max} of the uni-directional transport of carnitine in the reconstituted system, the dependence on substrate concentration of the rate of carnitine uptake was studied by changing the concentration of externally added [^{3}H]carnitine in the absence of internal substrate. The data from a typical experiment (Fig. 6) show a hyperbolic concentration dependence of the carnitine uniport. The K_m and the V_{max} values for the uniport of carnitine, as derived

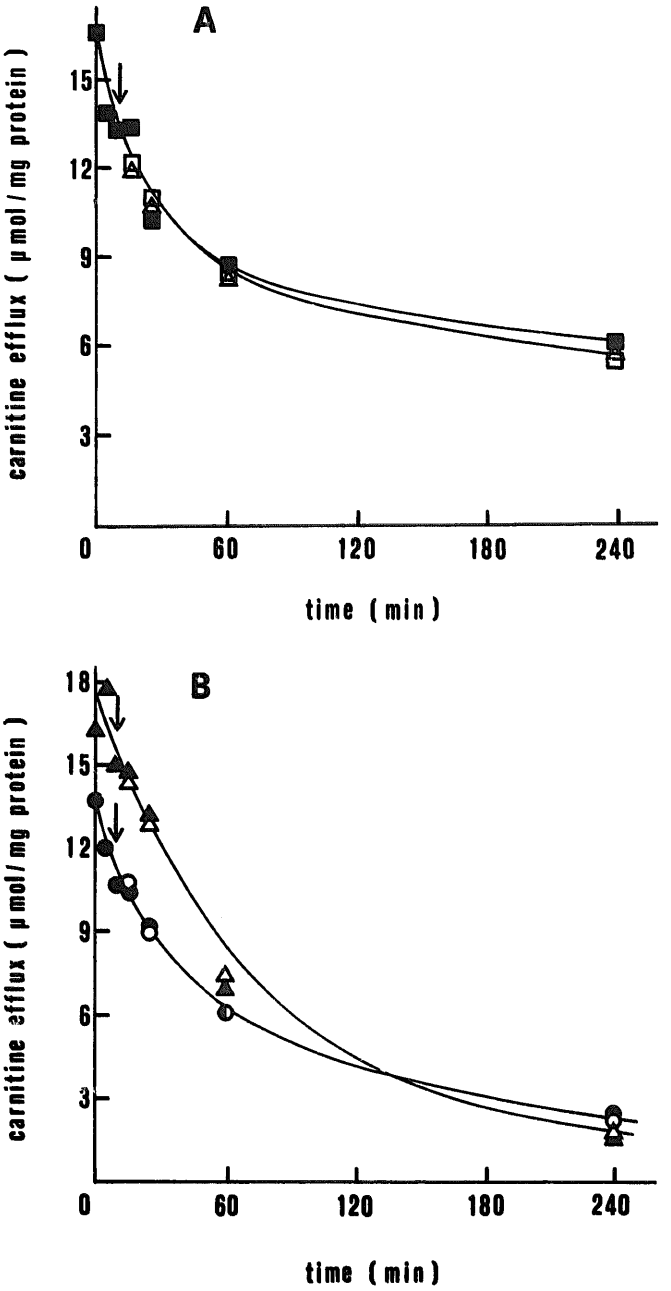

Fig. 4. Dependence of carnitine efflux on the ΔpH and the membrane potential across the liposomal membrane. (A) Efflux of 5 mM [^{3}H]carnitine from prelabelled proteoliposomes (with internal pH 7.0) was measured in the presence of an external unbuffered medium consisting of 90 mM NaCl (■). In □ and △ 20 mM Pipes buffer pH 8.5 (□) or pH 5.5 (△) was added after 10 min from the start of the incubation, as indicated by the arrow. (B) Efflux of 5 mM [^{3}H]carnitine from prelabelled proteoliposomes was measured at different KCl_{in}/KCl_{ex} ratios. (▲ and △) 1 mM internal KCl and 50 mM external KCl; (● and ○) 50 mM internal KCl and 1 mM external KCl. In △ and ○ 40 μg valinomycin was added 10 min after starting the incubation, as indicated by the arrow. In order to balance the osmolarity 49 mM NaCl was present in the same compartment containing 1 mM KCl.

from double-reciprocal plots of six experiments, were 0.53 ± 0.12 mM and 0.2 ± 0.09 μmol/min per mg protein, respectively. As compared to the values previously determined for the reconstituted carnitine/carnitine

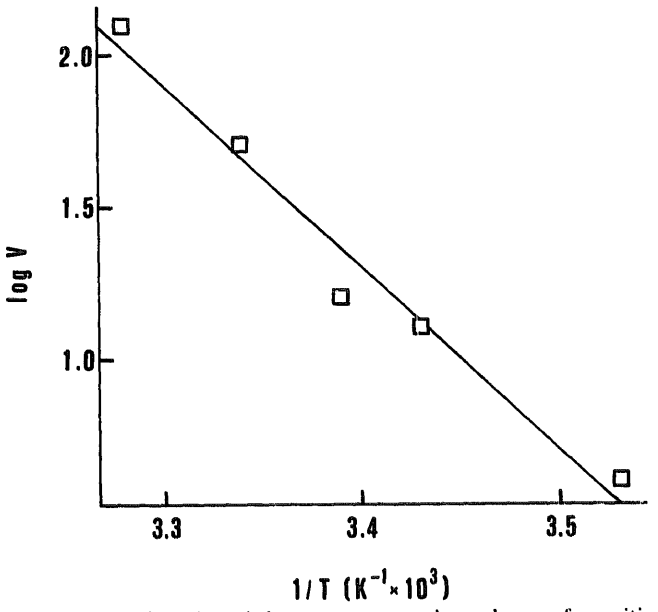

Fig. 5. Arrhenius plot of the temperature dependence of carnitine efflux from proteoliposomes. The efflux of 5 mM [^{3}H]carnitine from prelabelled proteoliposomes in the absence of external substrate (uniport) was measured at the indicated temperatures. The rate of efflux, V, is expressed in nmol/min per mg protein.

exchange [6], we observed a very similar value for the half-saturation constant for uniport whereas the maximal rates of the uniport were about 10 times lower. It should be noted that the standard error of the V_{max} values was rather high when comparing different experiments, presumably due to variations in the amount of active carrier molecules present in each preparation of the purified carrier. We have not been able to determine the affinity of the carrier for carnitine on the internal side of the membrane, since with the available methods it was not possible to evaluate the intraliposomal substrate concentration at the start of the measurements with sufficient accuracy. However, since the rate of the efflux of carnitine in the presence of 5 mM internal substrate (Fig. 2) agrees well with the V_{max} of carnitine uptake in the uniport mode (Fig. 6), it is clear that the K_m on the internal side of the liposomes must be substantially lower than 5 mM.

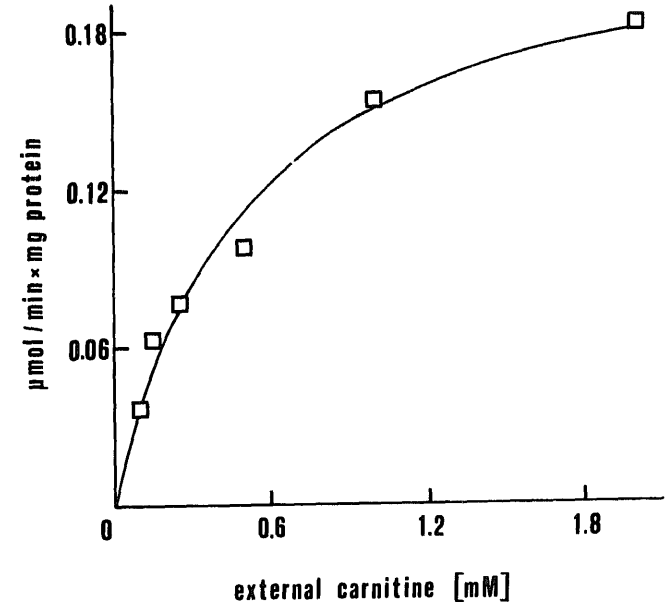

Fig. 6. Dependence of the rate of carnitine uptake in proteoliposomes on external substrate concentration. [^{3}H]Carnitine was added at the concentrations indicated to unloaded proteoliposomes (uniport).

Discussion

The data presented in this paper show that the purified and reconstituted carnitine carrier catalyzes a uni-directional transport of substrates, carnitine and acylcarnitines, besides the well known exchange reaction. The apparent ability of the reconstituted carnitine carrier to catalyze uniport of substrates is in agreement with the data from intact mitochondria as reported by Pande and Parvin [4], although we found a higher activity of the uniport. Our results indicate that the same transport protein is able to catalyze both the exchange and the uniport process. This is demonstrated by the following observations: (i) the ratio between carnitine taken up by exchange and by uniport at equilibrium agrees with the value which would be expected if all the carrier-loaded liposomes catalyze both exchange and uniport of carnitine; (ii) nearly all [^{3}H]carnitine taken up by the uniport mechanism is released after addition of unlabelled carnitine, i.e. by exchange; (iii) both exchange and uniport of carnitine are completely inhibited by *N*-ethylmaleimide; (iv) the activation energy and the K_m for uniport are practically the same as those measured for exchange [6].

Our results also show that the rate of the uni-directional transport of carnitine is regulated by the counter-substrate, since the uniport progressively decreases on increasing the concentration of the counter-substrate (Fig. 3). Physiologically, this means that a net flux of carnitine may occur into carnitine-depleted mitochondria to equilibrate the matrix level of carnitine with that present in the cytosol. On the other hand, uniport-mediated efflux of carnitine from mitochondria is unlikely to occur during β-oxidation, when the concentration of acylcarnitines is high in the extramitochondrial compartment.

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