Articles

Solubilization and Reconstitution of Rat Liver Mitochondrial Carnitine Acylcarnitine Translocase†

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ABSTRACT: Carnitine acylcarnitine translocase has been solubilized from inverted inner membrane vesicles of rat liver mitochondria with octyl glucoside and reconstituted into asolectin liposomes. For both processes, optimization of the detergent to phospholipid ratio was found crucial for obtaining reconstitutively active liposomes. Reassembly of the solubilized carrier into asolectin liposomes was achieved either by the octyl glucoside dilution method or by Extracti-Gel D column chromatography. The reconstituted system catalyzed exchange diffusion of carnitine, exhibited the expected inhibitor and temperature sensitivity, and discriminated between stereoisomers of octanoylcarnitine. The activity of unidirectional import of carnitine was low compared to exchange diffusion. It showed high-temperature sensitivity and a loss of activity on prolonged sonication that was regained by an appropriate freeze–thaw step subsequently.

The mitochondrial oxidation of fatty acids requires the transport of activated acyl groups through the inner membrane. This process necessitates the participation of (−)-carnitine, an outer carnitine acyltransferase which converts the external acyl-CoA to (−)-acylcarnitine, carnitine acylcarnitine translocase which catalyzes the import of acylcarnitine in exchange for matrix carnitine, and an inner carnitine acyltransferase which converts the entered acylcarnitine to acyl-CoA, rendering the latter accessible to the enzymes of β-oxidation (Bremer, 1983). Our recent experiments with intact mitochondria have shown that the translocase is unquestionably involved in the import of long-chain acylcarnitines and that the overall mechanism of transport is similar to that of short-chain acylcarnitines or of unesterified carnitine (Murthy & Pande, 1984). It is not known, however, whether the carnitine acylcarnitine translocase and the carnitine acyltransferase activities of mitochondria are different functions of the same or of different proteins (Bremer, 1983). The consensus now is that although the inner and outer carnitine palmitoyltransferases show different properties in their membrane-bound forms, at least these two activities represent expressions of the same protein (Bergstrom & Reitz, 1980; Clark & Bieber, 1981). Indirect evidence based on differences in substrate specificity and inhibitor sensitivity has indicated that carnitine acylcarnitine translocase is an entity distinct from carnitine acyltransferases (Pande & Parvin, 1976; Schulz & Racker, 1979; Parvin et al., 1980). A possible separation of the translocase from the acyltransferase activities has not been attempted so far owing to the unavailability of a method for monitoring the translocase activity during protein fractionation. To enable this and eventually a better understanding of the transport mechanism, we have sought conditions for the active solubilization and reconstitution of carnitine acylcarnitine translocase.

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Materials and Methods

Materials. [*methyl*-^{3}H]Carnitine was synthesized according to Daveluy et al. (1982). Octanoyl[^{3}H]carnitine was synthesized by acylation of [^{3}H]carnitine using octanoyl chloride (Pande, 1981) and then purified by silicic acid chromatography (Murthy & Pande, 1984). After elution of octanoic acid with chloroform–methanol (9:1 v/v), tritiated octanoylcarnitine was eluted with 90% methanol. The esterified carnitine content of the purified product was 99%. *n*-Octyl β-D-glucopyranoside (OG),[1] SB_8, SB_{12}, and CHAPS were obtained from Calbiochem (La Jolla, CA). PMSF was purchased from Boehringer Mannheim (Canada), Extracti-Gel D from Pierce Chemical Co., soybean lecithin (asolectin) from Associated Concentrates (Woodside, NJ), and Sephadex G-50 (fine) from Pharmacia (Canada) Ltd.

Preparation of IMV. Mitochondria were isolated from normal rat livers by the procedure of Bustamante et al. (1977), and mitoplasts were obtained by the digitonin procedure (Greenawalt, 1974). IMV were prepared according to Hackenbrock & Miller-Hammon (1979). The pelleted IMV were washed once with buffer A [100 mM potassium phosphate, pH 7.5, 20% (v/v) glycerol, 2 mM EDTA, 2 mM PMSF, and 25 mM (−)-carnitine]; they were then suspended in the same buffer at 4–6 mg of protein/mL and stored at −80 °C.

Preparation of Liposomes. Asolectin was washed with acetone (Kagawa & Racker, 1971) and stored at −20 °C as a chloroform–methanol (4:1 v/v) solution. Aliquots of this solution were dried under nitrogen in preweighed tubes. The residues were dissolved in diethyl ether, and the solvent was evaporated again. After overnight drying in vacuo, the tubes were weighed, flushed with nitrogen, capped tightly, and stored

[1] Abbreviations: IMV, inner membrane vesicles; DTT, dithiothreitol; OG, *n*-octyl β-D-glucopyranoside; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-[(cholamidopropyl)dimethylammonio]propanesulfonate; SB_8 and SB_{12}, *N*-octyl- and *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate, respectively; NEM, *N*-ethylmaleimide.

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at −20 °C. Before use, the dry lipid was dispersed in water (usually 125 mg/mL) by vigorous vortexing. With the use of a bath sonicator (Heats System W-375, Heats System–Ultrasonic Inc., Farmingdale, NY), the suspension was clarified at room temperature under nitrogen (3–5 min). These liposomes, kept at 4 °C, were used within a week.

Solubilization of Carnitine Acylcarnitine Translocase from IMV. Variations from the standard conditions are described in the legends etc. Optimized conditions were as follows: to a 100-μL suspension of IMV in buffer A were added with mixing 41 μL of water, 2 μL of 0.1 M freshly dissolved DTT, 32 μL of the liposomes (125 mg of asolectin/mL), and 25 μL of OG (10% w/v). The mixture was left on ice for 15 min and then centrifuged for 30 min at 30000*g* at 4 °C. A higher speed centrifugation (140000*g* for 30 min) decreased neither the amount of solubilized proteins nor the "activity" recovered in the supernatant. These OG extracts were used immediately for reconstitution.

Reconstitution of the Translocase Activity. (*a*) *OG Dilution Method.* For most reconstitutions, the OG dilution procedure of Racker et al. (1979) was followed: to 100 μL of the OG-solubilized extract were added with mixing, in the order given, 75 μL of buffer A, 200 μL of water, 25 μL of 0.5 M potassium phosphate (pH 7.5), 50 ± 10 μL (see Results) of the asolectin liposomes (125 mg/mL), and an additional 10% OG (50 μL) to bring the final total OG concentration to 1.25% (w/v). After standing for 20 min on ice, the mixture (0.5 mL) was added, with rapid stirring, to 14.5 mL of buffer B (50 mM potassium phosphate, pH 7.5, 5% glycerol, 0.5 mM EDTA, and 0.5 mM PMSF) containing 0.1 mM DTT and, unless stated otherwise, 1 mM (−)-carnitine to load the intraliposomal space. The proteoliposomes were collected by centrifugation for 60 min at 140000*g*. The pellet was usually suspended in 200 μL of dilution buffer for transport assays.

(*b*) *Extracti-Gel D Method.* For this procedure, the aforementioned reconstitution mixture was applied to a 1-mL column of gel packed in a 1-mL tuberculin syringe and equilibrated at room temperature in buffer B containing 0.1 mM DTT and the desired (−)-carnitine concentration (usually 1 mM). Elution was performed with the same buffer; the turbid fractions were collected and assayed for transport activity.

Assay of Translocase Activity. Transport activity was measured as the NEM-sensitive uptake of radioactive substrate by the proteoliposomes. To 40 μL of the reconstituted proteoliposomes was added with mixing 5 μL of buffer C (buffer B having 0.1 mM DTT and 1 mM carnitine) with (control tubes) or without (experimental tubes) 9 mM NEM. The mixtures were incubated at 30 °C for 3 min. The assay was then started by the addition of 5 μL of an isomolar concentration of the labeled substrate (2 μCi per assay tube for carnitine) in the same buffer mixture. Following incubation at 30 °C, the control tubes received 50 μL of chilled buffer C and the experimental ones 50 μL of the same buffer containing 1 mM NEM. All tubes were then transferred to an ice bath. Thirty seconds later, 90 μL of the cold mixture was pipetted on the top of chilled Sephadex G-50 (fine) columns prepared in 1-mL tuberculin syringes, as described by Penefsky (1977), which had been equilibrated in 50 mM potassium phosphate (pH 7.5) containing 0.5 mM NEM and the same concentration of unlabeled substrate as that present during the assay. The eluent having proteoliposomes was collected into 1.5-mL Eppendorf tubes by centrifugation. After being mixed with 0.8 mL of a scintillation fluid (EP of Beckman), the radioactivity was measured by using 25-mL glass vials as holders of the Eppendorf tubes.

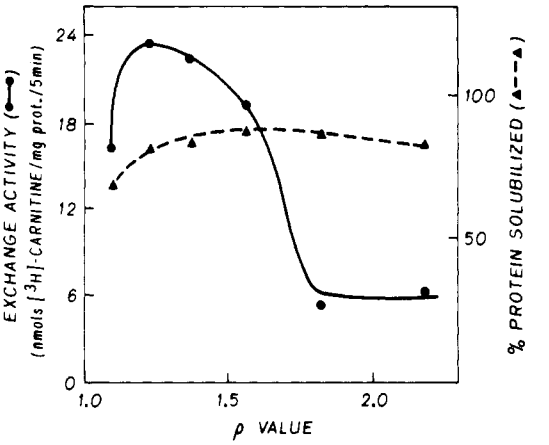

FIGURE 1: Effects of exogenous asolectin liposomes on reconstituted (−)-carnitine–(−)-carnitine exchange activity. To 100-μL aliquots of IMV (5.3 mg/mL) were added increasing quantities of asolectin liposomes, while the OG concentration was kept constant at 1.25% (w/v), in a final volume of 200 μL. After centrifugation, 100 μL of each supernatant was reconstituted as described under Materials and Methods, except that the total asolectin in each sample was adjusted to the same final concentration of 14 mg/mL. The mixtures were diluted 20-fold with buffer B and centrifuged. The proteoliposomes were resuspended in buffer B and assayed for carnitine–carnitine exchange as described in the text (●). The ρ values have been calculated by assuming that 65% (w/w) of asolectin is phospholipid and that the critical micellar concentration of octyl glucoside is 20 mM (Rivnay & Metzger, 1982). Percent of proteins solubilized (▲).

For time course studies, a different procedure was used. IMV were first incubated for 15 min at room temperature either with 1 mM DTT (for experimentals) or with 5 mM NEM (for controls), and then solubilization proceeded as described above, except that control IMV were diluted with buffer containing 1 mM NEM instead of DTT. The pelleted proteoliposomes were concentrated 4-fold [see (a) OG Dilution Method]. The required reaction medium (40 μL per assay) was tempered at 30 °C for 5 min and the reaction started by the addition of 10 μL of proteoliposomes. At appropriate times, the reactions were stopped and the proteoliposomes collected as described above.

Other Procedures. Protein was measured by the method of Peterson (1977) and lipid phosphorus by that of Lanzetta et al. (1979). Carnitine acetyltransferase was assayed according to Parvin & Pande (1977) and carnitine palmitoyltransferase according to Bremer & Norum (1967).

RESULTS

Solubilization of the Translocase Activity. Although Schulz & Racker (1979) mentioned that active carnitine acylcarnitine translocase preparations could be reconstituted from OG-solubilized bovine heart mitochondria, details of those experiments were not described. Initially, we repeatedly failed to obtain active preparations with OG-solubilized IMV of rat liver mitochondria. Because solubilization of certain receptors has shown that active preparations are obtained only when phospholipids are added along with the detergent (Chang & Bock, 1979; Rivnay & Metzger, 1982), we examined this aspect for carnitine acylcarnitine translocase. The results showed that, whereas lipid inclusion did not affect the quantity of protein solubilized (Figure 1), transport-active proteoliposomes were obtained only on incorporation of asolectin in the solubilization medium and that for this only a narrow range of asolectin concentration was acceptable at both the solubilization and the reconstitution steps. The optimum ratios of OG to lipid, expressed as the ρ factor (Rivnay & Metzger,

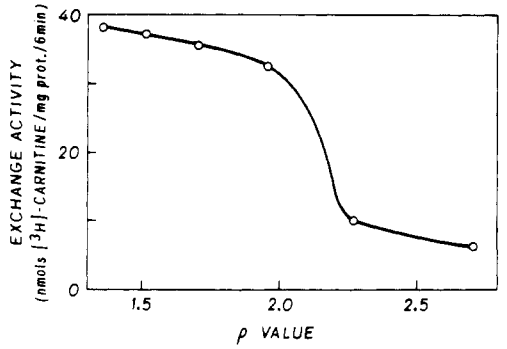

FIGURE 2: Effects of the quantity of asolectin added at the reconstitution step on carnitine–carnitine exchange activity. To 100 μL of OG extract, prepared under optimal conditions of solubilization, were added exogenous asolectin and OG (1.25% w/v) in a final volume of 500 μL to achieve the indicated ρ values. Other details were as specified in the text. Proteoliposomes were prepared and assayed as in Figure 1, using aliquots of 13–20 μg of protein (0.2 mg of lipid phosphorus).

1982), were 1.2–1.5 during solubilization (Figure 1) and 1.4–2.0 during reconstitution (Figure 2). Subsequently, we employed a ρ value of 1.4 during solubilization but found that even then for each batch of OG solution, the optimum quantity of asolectin to add had to be first determined by titration. Whereas addition of cardiolipin has been found essential for obtaining several mitochondrial translocators in the active state (Cheneval et al., 1983; Mende et al., 1983), inclusion of cardiolipin in our experiments did not prove helpful. We found that at lower concentrations cardiolipin had no effect while at concentrations above 1 mg/mL it decreased the recovered activity. Because the presence of substrate and its analogues has been reported to stabilize many carriers, we included 25 mM carnitine in the solubilization medium; however, later experiments showed that omission of carnitine had no noticeable effect on the subsequently recovered activity. Unlike OG, active proteoliposomes were not obtained when the detergent employed was SB_{12} or CHAPS.

Characteristics of the Uptake of Tritiated Carnitine. The proteoliposomes showed a time-dependent uptake of substrate consistent with the reconstitution of active translocase which catalyzed the exchange of medium carnitine for intraliposomal carnitine. In the absence of NEM, the carnitine uptake was hyperbolic with a half-time of about 15 min at 30 °C (Figure 3). The major part of this uptake was inhibited by NEM. Because the activity of the translocase in intact mitochondria (Pande & Parvin, 1980a) and in submitochondrial particles (Schulz & Racker, 1979) is inhibited by NEM, this property was expected in the proteoliposomes. However, with proteoliposomes, a NEM-insensitive uptake was also found to proceed (Figure 3, curve B). It showed a faster rate in the first several minutes followed by a near-linear protracted one, indicating that perhaps a rapid, complete inhibition of the translocase was not being manifested. This could occur, for example, upon the incorporation of some of the carrier molecules within the bilayer in a configuration that rendered certain thiol groups relatively resistant to NEM. Indeed, proteoliposomes prepared from NEM-exposed IMV showed a much lower uptake of medium carnitine (Figure 3 curve C). Incubation of proteoliposomes (3 h, 25 °C) with 0.2 mg of trypsin/mL reduced the carnitine uptake by about 70%; no loss was found in controls lacking trypsin. This showed that the reconstituted transport activity depended on the presence of protein(s).

If the observed NEM-sensitive exchange of carnitine was due to the reconstituted translocase activity, then other known inhibitors of the translocase in intact mitochondria should exert

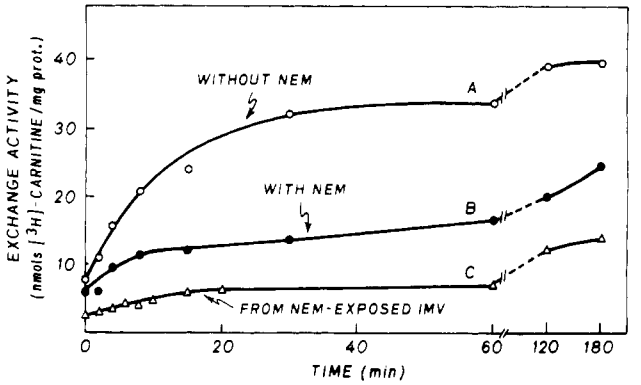

FIGURE 3: Time course of carnitine–carnitine exchange in the absence and in the presence of NEM. Proteoliposomes were prepared as described in the text under optimal conditions of solubilization and reconstitution. The assay medium (40 μL) was tempered at 30 °C for 5 min; the reaction was started by adding 10 μL of proteoliposomes (5–14 μg of protein and 0.2 mg of phospholipids). As indicated, 50 μL of a stop mixture was added to each tube which was then transferred to an ice bath. Proteoliposomes were separated from the medium by rapidly filtering through 1-mL G-50 columns, as described in the text. Curve A, total radioactivity associated with proteoliposomes in the absence of NEM. "Zero-time point" represents the values obtained on adding the stop mixture 30 s after the addition of proteoliposomes. Curve B, carnitine exchange in the presence of 1 mM NEM; the conditions were as for curve A except that the assay medium contained 1 mM NEM. Curve C, exchange in the presence of NEM-treated IMV. IMV were allowed to react with 5 mM NEM for 15 min before being extracted with OG. Then the extract was reconstituted, and the resulting proteoliposomes were assayed as described for curve A.

Table I: Inhibition of Reconstituted Carnitine Exchange Activity by Known Inhibitors of Carnitine Acylcarnitine Translocase[a]

addition	concn (mM)	carnitine exchanged (nmol)
none		0.22[b]
NEM	0.5	0.00
mersalyl	0.125	0.04
mersalyl	0.25	0.01
SB_8	5	0.03

[a] Proteoliposomes were prepared by the detergent dilution procedure and the proteoliposome pellets suspended in buffer B–DTT as described under Materials and Methods. To aliquots containing 6 μg of protein and 0.2 mg of phospholipids, inhibitors were then added, and the reactions were started by the addition of 5 mM tritiated (−)-carnitine. Incubations were for 6 min at 30 °C. Proteoliposomes were separated for determining the uptake of [^{3}H]carnitine as described under Materials and Methods. [b] The values shown have been corrected for the uptake observed in controls containing 1 mM *N*-ethylmaleimide as described under Materials and Methods.

a similar effect on the proteoliposomal system, and such indeed proved to be the case. Table I shows that both mersalyl and SB_8 inhibited the carnitine uptake to an extent approaching that observed with NEM. In intact mitochondria, this translocase is specially sensitive to inhibition by micromolar concentrations of mersalyl (Pande & Parvin, 1980a), and in this regard, it should be noted that the 82% inhibition observed with 0.125 mM mersalyl (Table I) was manifested despite the presence of 0.1 mM dithiothreitol in the medium.

Criteria for Transport. A number of observations demonstrated that the carnitine uptake by liposomes resulted from transport and was not due to protein binding. (a) Freeze–thaw sonication is frequently used to prepare proteoliposomes; this technique requires that the conditions be controlled precisely since the transport activity declines on extended sonication as a result of a decrease in the intraliposomal space (Kasahara & Hinkle, 1977). Figure 4 shows that a similar effect of sonication time was observed on the carnitine translocase and

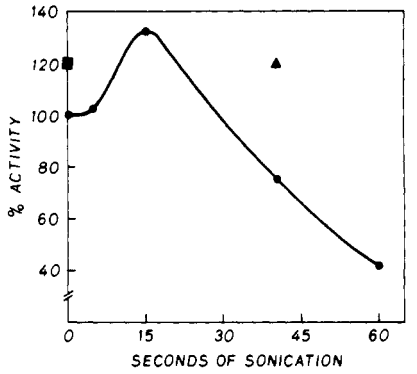

FIGURE 4: Effects of sonication on the exchange activity reconstituted by the OG dilution procedure. Proteoliposomes were prepared under optimal conditions by the OG dilution method. One portion was assayed for carnitine–carnitine exchange in a 10-min incubation; its activity is shown as the 0-s sonication. The remaining portion was frozen, thawed, vortexed for 10 s (Wohlrab, 1984), and then similarly assayed without [(■) point at 0 s] or with sonication for the times shown (●). One sample which had gone through the freeze–thaw 40-s sonciation was subjected to a second freeze–thaw cycle, then vortexed 10 s, and assayed (▲). The results are expressed as the percentage of the activity observed with proteoliposomes not subjected to the freeze–thaw sonication procedure.

Table II: Effects of Carnitine Concentration during the OG Dilution Step on the Maximum Quantity of Substrate Exchanged[a]

[carnitine] (mM)	[^{3}H]carnitine (nmol in proteoliposomes per assay tube)[b]
1.0	0.13
2.5	0.33
5.0	0.70
10.0	1.49

[a] Proteoliposomes were prepared as specified under Materials and Methods, except that the buffers at the dilution step contained 1, 2.5, 5.0, or 10.0 mM (−)-carnitine. For assay of carnitine–carnitine exchange, aliquots of proteoliposomes (9 μg of protein and 0.2 mg of phospholipids) were resuspended in the corresponding buffers containing the same external radioactive (−)-carnitine concentration. Incubations were for 3 h at 30 °C. [b] Corrected for 0-min exchange.

that the activity lost on prolonged sonication was fully recovered on subsequent freeze–thaw vortexing (compare the two 40-s points). (b) The carnitine uptake measured with proteoliposomes lacking internal carnitine was only 16% of that observed with the usual carnitine-loaded vesicles. This showed that the rate of unidirectional net influx of carnitine was low in proteoliposomes, as is known for mitochondria (Pande & Parvin, 1980b). (c) When the concentration of intraliposomal carnitine was changed by varying the carnitine concentration at the OG dilution step, both the initial uptake rates (data not shown) and the quantity of substrate taken up after a 3-h incubation at 30 °C rose with an increase in the intraliposomal carnitine content (Table II), as was expected for a process involving exchange diffusion. (d) The uptake of carnitine was too low to be measured precisely when the incubations were at 0 °C. If the translocase in proteoliposomes had a similar high-temperature dependence as in mitochondria where a Q_{10} of about 6 is observed (Pande & Parvin, 1980b; Murthy & Pande, 1984), then the uptake rate at 0 °C would have been only 0.5% of that at 30 °C.

Substrate Specificity. The carnitine acylcarnitine translocase of intact mitochondria uses octanoylcarnitine and longer chain acylcarnitines with much greater affinity than free carnitine (Tubbs & Ramsay, 1979; Murthy & Pande, 1984), and the same appeared to apply to the reconstituted system. Figure 5 shows that 20 μM (−)-octanoylcarnitine was readily transported into carnitine-containing proteoliposomes with a half-maximal uptake time of about 5 min while the corresponding time for the uptake of 1 mM carnitine was about 15 min (Figure 3). Moreover, Figure 6 shows that the uptake of radioactive 1 mM (−)-carnitine was inhibited by micromolar concentrations of unlabeled octanoylcarnitine esters. Inasmuch as (+)-octanoylcarnitine was more inhibitory than the corresponding (−) isomer, it is evident that the reconstituted translocase discriminated between the two stereoisomers. In intact mitochondria, it is known that (+)-acylcarnitines are transported slower than the (−) isomers and, when present together with (−)-carnitine, the fatty (+)-acylcarnitines inhibit the carnitine transport effectively (Pande, 1975).

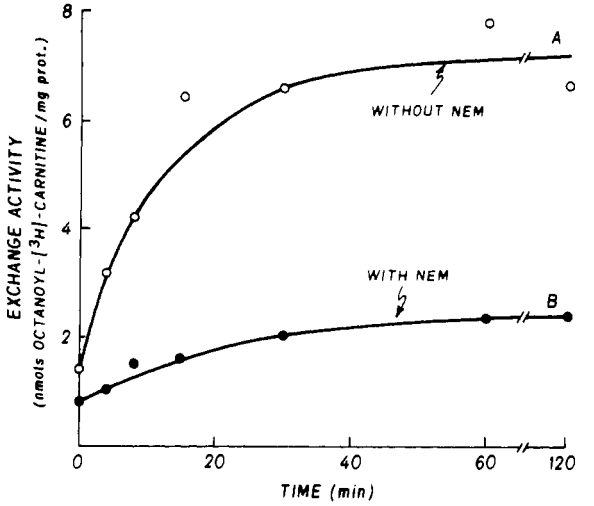

FIGURE 5: Time course of (−)-octanoyl[^{3}H]carnitine–(−)-carnitine exchange. Conditions were as in Figure 3, except that the external medium contained 20 μM (−)-octanoyl[^{3}H]carnitine instead of (−)-[^{3}H]carnitine. Curve A, exchange in the absence of NEM (○) (8 μg of protein and 0.2 mg of phospholipids). Curve B, exchange using proteoliposomes (9 μg of protein and 0.2 mg of phospholipids) prepared from NEM-treated IMV as described for curve C in Figure 3 (●).

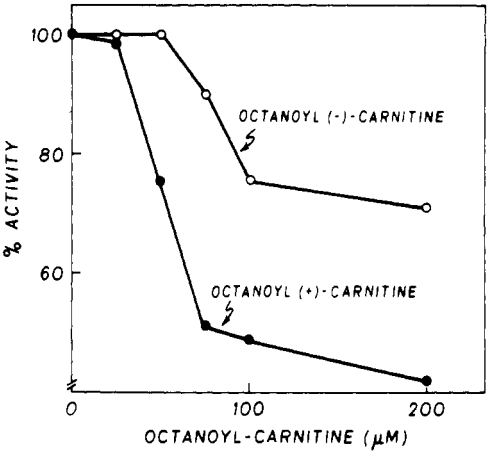

FIGURE 6: Inhibition of (−)-carnitine–(−)-carnitine exchange by octanoylcarnitine isomers. Proteoliposomes were prepared as described under Materials and Methods. To 40 μL of medium containing 1 mM (−)-[^{3}H]carnitine and either (−)-octanoylcarnitine or (+)-octanoylcarnitine at 30 °C was added 10 μL of proteoliposomes to start the reaction. After 10 min at 30 °C, the reactions were stopped, and the filtered proteoliposomes were assayed for radioactivity. The values shown have been corrected for the exchange observed in controls having NEM.

The possibility that the observed carnitine transport in proteoliposomes resulted from the incorporation of some other transporters which fortuitously accommodated carnitine and its esters as substrate is remote. In intact mitochondria, we have shown that substrates of other transporters neither exchange with carnitine nor inhibit the carnitine–carnitine ex-

change (Pande & Parvin, 1976; Parvin & Pande, 1978). The same applied to carnitine transport into proteoliposomes. We found that whereas loading of proteoliposomes with carnitine promoted carnitine uptake this was not the case when, during loading, 1 mM (−)-carnitine was replaced by pyruvate, malate, or glutamate. Moreover, the uptake of 1 mM (−)-[^{3}H]-carnitine by carnitine-loaded proteoliposomes was not noticeably affected by the presence in the medium of either 5 mM pyruvate, malate, citrate, or γ-aminobutyrate. The presence of the known (Ramsay & Tubbs, 1975; Pande, 1975; Pande & Parvin, 1976) substrates of the carnitine acylcarnitine translocase, γ-butyrobetaine, (+)-carnitine, and (−)-acetylcarnitine, on the other hand, inhibited the uptake of [^{3}H]-carnitine by 75%, 43%, and 66%, respectively.

Discussion

The key observations showing that a successful solubilization and reconstitution of carnitine acylcarnitine translocase have been realized in the present work may be summarized as follows: (a) Active proteoliposomes were obtained by keeping the ρ factor within a narrow range at both the solubilization and reconstitution steps. (b) Significant uptake was measured only with carnitine-containing proteoliposomes. (c) The rate of this uptake and the extent of substrate exchanged on prolonged incubation increased with an increase in the intraliposomal carnitine concentration. (d) Carnitine uptake was low at 0 °C compared to that at 30 °C. (e) The uptake process showed a sensitivity to inhibitors and a substrate specificity similar to those of the translocase in intact mitochondria. (f) The reconstituted translocase lost activity on prolonged sonication which was restored on subsequent freeze–thawing and vortexing.

The remote possibility that the observed NEM-sensitive carnitine uptake represented binding to a protein such as carnitine palmitoyltransferase is eliminated from points b, c, and e above; furthermore, like carnitine acetyltransferase (Parvin & Pande, 1977), the carnitine palmitoyltransferase activity is quite resistant to NEM inhibition (unpublished results) in contrast to the carnitine acylcarnitine translocase (Pande & Parvin, 1980a). Moreover, the quantity of (−)-carnitine taken up by proteoliposomes at 30 °C under the standard assay conditions, corresponding to about 30 nmol/mg of protein [Figure 3; up to 167 nmol/mg was taken up by proteoliposomes containing 10 mM (−)-carnitine (Table II)], is too high to be compatible with protein binding.

Although indirect evidence has indicated that carnitine acyltransferases are not likely to possess carnitine acylcarnitine translocase activity, a definitive proof of this has not yet emerged. The successful solubilization and purification of the translocase should allow this aspect to be examined. Meanwhile, we do know that, under conditions that yielded transport-active proteoliposomes using proteins solubilized from liver mitochondrial IMV, active reconstitution was not obtained with a commercially available carnitine acetyltransferase. Moreover, we found that although acetylcarnitine was effectively transported in proteoliposomes, carnitine acetyltransferase activity was not demonstrable in IMV. Carnitine palmitoyltransferase activity was present in the proteoliposome at a specific activity similar to that in intact mitochondria, but this enzyme is known not to accept acetyl esters as substrates (Clarke & Bieber, 1981).

On incubation of mitochondria with radioactive carnitine, the carnitine acylcarnitine translocase catalyzed exchange influx of medium carnitine reaches a steady state as the external carnitine equilibrates with the matrix carnitine; under these conditions, practically all of the mitochondrial carnitine is replaced by medium carnitine (Pande & Parvin, 1980a). This shows that each mitochondrion which contains carnitine has at least one functionally active carnitine acylcarnitine translocase. For the reconstituted system, however, such was not the case. Proteoliposomes whose internal carnitine content was varied 10-fold, when allowed to reach steady state with respect to the NEM-sensitive uptake of radioactive carnitine and then analyzed, showed that the specific radioactivity of the proteoliposome–associated carnitine was only 20% of that of the medium carnitine. On the incorporation of solubilized transporters into liposomes, it is to be expected that not all the transporter molecules would necessarily become incorporated into the lipid layer in a functionally competent configuration nor would all the proteoliposomes necessarily have at least one functionally active translocase per liposome (Gorga & Lienhard, 1984). The above results indicate, therefore, that, under the standard conditions employed presently, only one-fifth of the liposomes had functionally competent translocase(s) incorporated in them.

We have recently shown that measurements of exchange influx of carnitine in mitochondria underestimate the true transport rate, because such an assay measures the rate of replacement of the bulk-phase matrix carnitine by medium carnitine rather than the true translocase-catalyzed exchange that proceeds between medium carnitine and carnitine present in an inner microcompartment near the membrane (Murthy & Pande, 1984). Thus, an exact comparison of the specific activity of the translocase in intact mitochondria with that in the reconstituted system is not possible; however, an assessment indicates that only a small fraction of the total mitochondrial carnitine acylcarnitine translocase activity was being measured in the present reconstitution experiments, as has frequently been the case for other transporters (Kramer & Klingenberg, 1979; Baldwin et al., 1981). Thus, the rate of 2.2 nmol of carnitine min^{-1} (mg of protein)$^{-1}$ at 30 °C, calculated from the first-order kinetic plot of the data of Figure 3, corresponds to only 3% of the expected uptake rate of 1 mM carnitine for liver mitochondria (Parvin & Pande, 1979) at 30 °C, calculated by using a Q_{10} of 6 (Pande & Parvin, 1980b). It is likely, however, that further optimization with respect to the specific lipid requirement (Kramer & Klingenberg, 1977; Wohlrab et al., 1984) and the reconstitution method (Carruthers & Melchior, 1984) would increase the specific activity of the reconstituted translocase.

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Registry No. NEM, 128-53-0; SB_8, 15178-76-4; mersalyl, 492-18-2; carnitine acylcarnitine translocase, 56093-16-4; (*R*)-(−)-octanoylcarnitine, 25243-95-2; (*S*)-(+)-octanoylcarnitine, 96999-03-0; (−)-carnitine, 541-15-1.

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Thyroid Hormone Regulates Transcription of the Gene for Cytosolic Phosphoenolpyruvate Carboxykinase (GTP) in Rat Liver†

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ABSTRACT: Using an in vitro assay with isolated rat nuclei, we have determined that thyroid hormone causes a 4–6-fold increase in the synthesis of mRNA coding for phosphoenolpyruvate carboxykinase. Proportional changes were seen in the steady-state cytosolic mRNA levels for phosphoenolpyruvate carboxykinase. Dibutyryladenosine cyclic 3′,5′-monophosphate, which stimulates transcription of the phosphoenolpyruvate carboxykinase gene in normal rats, remained effective in hypo- or hyperthyroid animals. The effect of epinephrine on transcription of the gene for phosphoenolpyruvate carboxykinase appears to be modulated by thyroid hormone.

Phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is a key regulatory enzyme in gluconeogenesis. The regulation of enzyme levels is complex, but increasing evidence points to gene transcription as the major point of control in governing cellular enzyme activity. Insulin (Granner et al., 1983), cAMP, and glucocorticoids (Lamers et al., 1982) have been shown to regulate the transcription of the gene for phosphoenolpyruvate carboxykinase.

It has recently been shown that altered thyroid status produces marked changes in the activity and synthesis rate of phosphoenolpyruvate carboxykinase (Sibrowski et al., 1982; Muller et al., 1982). In the present paper we deomonstrate that thyroid hormone increases the transcription rate of the phosphoenolpyruvate carboxykinase gene. This leads to elevated steady-state levels of cytosolic mRNA and hence enzyme synthesis.

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Materials and Methods

Male Sprague-Dawley rats, 200–250 g, were obtained from Zvic-Miller (Pittsburgh, PA). Thyroidectomy was performed by the supplier, and rats wer allowed to recover for 2 weeks before treatment.

Isotopes. [^{32}P]UTP (760 Ci/mmol) and [^{32}P]CTP (800 Ci/mmol) were obtained from New England Nuclear, Boston, MA.

In Vitro Transcription. Details of the in vitro transcription assay have been previously described (Lamers et al., 1982). Rats were starved 48 h, refed glucose (5 g/kg of body weight), and after 2 h injected with saline, dibutyryladenosine cyclic

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