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THE LOCALIZATION OF PALMITOYL-CoA:CARNITINE PALMITOYLTRANSFERASE IN RAT LIVER

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SUMMARY

1. The distribution of palmitoyl-CoA:carnitine palmitoyltransferase has been studied in subcellular fractions of rat liver. By using two different estimations for the enzyme activity and by differential centrifugation and linear sucrose density gradient centrifugation, the enzyme is shown to be localized both in mitochondria and microsomes.

2. The mitochondrial palmitoyl-CoA:carnitine palmitoyltransferase is localized in the inner membrane *plus* matrix fraction.

3. During palmitate oxidation by isolated mitochondria, in the presence of a physiological concentration of carnitine, palmitoylcarnitine accumulates. From this and experiments with sonicated mitochondria, it is concluded that the capacities of long-chain fatty acid activation and of palmitoyl-CoA:carnitine palmitoyltransferase *in vitro* by far exceed the capacity of fatty acid oxidation.

INTRODUCTION

The long-chain fatty acyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) was first demonstrated by KORNBERG AND PRICER[1] in rat-liver microsomes. In more recent investigations, palmitoyl-CoA synthetase has been demonstrated by different methods in both mitochondria and microsomes. A thorough study on the intracellular distribution of the enzyme, with the use of marker enzymes for the different subfractions, was made by FARSTAD *et al.*[2]. They reported that the microsomes have 70 % and the mitochondria 30 % of the activity present in rat-liver cells.

On the other hand, the localization of the palmitoyl-CoA:carnitine palmitoyltransferase (trivial name, carnitine palmitoyltransferase) is reported to be 100 % mitochondrial[3] and probably solely in the inner mitochondrial membrane[4].

The concept that long-chain fatty acids are partly oxidized in a carnitine-dependent fashion is generally accepted. It is assumed that acyl-CoA cannot penetrate the inner mitochondrial membrane[5]. Therefore acyl-CoA formed at the site of the endoplasmic reticulum or the mitochondrial outer membrane[2,4] is transported as acylcarnitine through the mitochondrial inner membrane, prior to oxidation of the acyl moiety[6,7]. In this scheme the transport of activated fatty acids is thought to function from the outside to the inside of mitochondria. Rephrasing this scheme,

acylcarnitine is a storage form of activated long-chain fatty acid, which can pass through the mitochondrial inner membrane. In principle the possibility exists that acylcarnitine is not only used for intramitochondrial but also for extramitochondrial reactions. Support for this idea came from the observation that the capacity of mitochondrial fatty acid activation exceeds by far that for fatty acid oxidation[8]. If this excess of activated fatty acid partially exists as acylcarnitine, it must be converted back to the CoA-derivative before utilization. As mentioned already, mitochondria contain carnitine palmitoyltransferase. The literature on the existence of carnitine palmitoyltransferase in the microsomes is rather confusing[3,9,10]. We therefore reinvestigated the distribution of carnitine palmitoyltransferase with the use of marker enzymes. The present paper demonstrates that the carnitine palmitoyltransferase is localized not only in the mitochondria but also in the microsomes.

MATERIALS AND METHODS

Oligomycin and kynuramine were purchased from Sigma Chemical Co. (St. Louis, Mo.), crystalline bovine serum albumin (Fraction V) from Sigma or from Pentex (Kankakee, Ill.), and palmitic acid, sucrose and ascorbic acid from the British Drug Houses.

DL-[*Me*-^{3}H]Carnitine, with a specific activity of 75 μC/μmole, was a gift of Dr. J. Bremer from the Oslo University and L-carnitine was a gift from Otsuka Pharmaceutical Factory (Osaka, Japan). The radioactive carnitine was diluted with L-carnitine to a specific activity of 0.95 or 0.032 μC/μmole L-carnitine. The radioactive fatty acids were purchased from the Radiochemical Centre (Amersham, England) and nucleotides, phosphoenolpyruvate, cytochrome *c*, pyruvate kinase (EC 2.7.1.40) and adenylate kinase (EC 2.7.4.3) from C. F. Boehringer and Sons (Mannheim, Germany).

Carnitine palmitoyltransferase was purified from calf-liver mitochondria according to FARSTAD *et al.*[2]. Palmitoyl-L-carnitine was synthesized according to BRENDEL AND BRESSLER[11] and was found to be chromatographically pure, as tested in the system described by BØHMER *et al.*[12].

Albumin was used, after dialysis and millipore filtration, as a clear neutral solution. Palmitate–albumin solutions were prepared by mixing potassium palmitate with albumin in a molar ratio of 7:1.

Animals

Male rats of the Wistar strain were used for the experiments (weight approx. 250 g). The animals had free access to food and water (except in the fasting experiments where only water was supplied *ad libitum*). The animals were bled after cervical fracture.

Homogenization and centrifugation procedures

Differential centrifugation. 4 g of liver were homogenized in 9 vol. of 0.25 M sucrose in a Potter–Elvehjem homogenizer with a Teflon pestle. The differential centrifugation was performed according to DE DUVE *et al.*[13], with slight modifications. The crude homogenate was centrifuged for 5 min at 900 $\times$ *g* in a Sorvall refrigerated centrifuge (Rotor SS 34). The pellet was rehomogenized in 20 ml of 0.25 M sucrose

and centrifuged again for 5 min at 900 × *g*. The last step was repeated once; and the final pellet, which consists of cellular debris, unbroken cells and nuclei, was suspended and designated nuclear fraction (N). The combined supernatants of fraction N were centrifuged for 10 min at 5100 × *g*. The pellet was resuspended in 30 ml of 0.25 M sucrose and centrifuged for the same integrated field. The last step was repeated once and the final pellet, after washing off the fluffy layer with some sucrose, was designated heavy mitochondrial fraction (M). The combined supernatants of the previous centrifugations were centrifuged for 10 min at 27000 × *g*. The pellet was resuspended in 30 ml of 0.25 M sucrose and centrifuged for the same integrated field. This was repeated once. The final pellet after washing off the fluffy layer was designated light mitochondrial fraction (L). The combined supernatants were centrifuged for 60 min at 131000 × *g* in the Beckman ultracentrifuge (Rotor SW 27); the resulting pellet was designated microsomal fraction (P) and the final supernatant soluble fraction (S).

Gradient centrifugation of 900 × g supernatant. 10 g of liver were homogenized in 4 vol. of 0.25 M sucrose. This homogenate was centrifuged for 5 min at 900 × *g*. 10 ml of the supernatant were layered on 28 ml of a continuous sucrose density gradient, with specific gravity varying from 1.025 to 1.275. The gradient was centrifuged for 2.5 h at 131000 × *g* in a Beckman ultracentrifuge (Rotor SW 27). After centrifugation, the content of the tube was collected in ten fractions. Beginning from the bottom of the tube there were nine fractions of 3 ml and one fraction (No. 10) of 11 ml, which included the soluble proteins from the layered homogenate. Fraction 10 was included to calculate the recoveries but was not considered in the data where percentage of total activity means percentage of activity in the Fractions 1–9. The activity in Fraction 10 was low for all the enzymes tested (see below).

Gradient centrifugation of mitochondria. The heavy mitochondrial fraction was isolated from 10 g of liver as described before, except that the mitochondria were washed twice by resuspending in 0.25 M sucrose and centrifugation at 12000 × *g* (10 min). The fluffy layer was carefully washed off. The final pellet was resuspended in 15 ml of 0.25 M sucrose of which 10 ml were layered on the continuous sucrose density gradient and centrifuged as described before. In the swelling experiments the final pellet was resuspended in 50 ml of 20 mM potassium phosphate buffer (pH 7.2) containing 0.02 % of bovine serum albumin and stirred for 20 min at 0–4°, as described by PARSONS *et al.*[14]. The swollen mitochondria were collected by centrifugation for 20 min at 48200 × *g* and resuspended in 15 ml of 0.25 M sucrose of which 10 ml were layered on the gradient and centrifuged as described before.

Enzyme assays

All the enzymes tested were assayed at 37°. Glucose-6-phosphatase (EC 3.1.3.9) was assayed according to BEAUFAY *et al.*[15]; monoamine oxidase (EC 1.4.3.4) according to WEISSBACH *et al.*[16] with kynuramine as substrate; cytochrome *c* oxidase (EC 1.9.3.1) was measured in an oxygraph, equipped with a "Clark" oxygen electrode; ascorbic acid was used as the electron donor as described by SOTTOCASA *et al.*[17]. Palmitoyl-CoA synthetase was assayed according to FARSTAD *et al.*[2] with several modifications. The incubation medium contained 20 mM KCl, 2 mM $MgCl_2$, 80 mM Tris–HCl, 0.5 mM EDTA, 0.5 mM potassium palmitate (complexed with 0.07 mM bovine serum albumin), 3 μg oligomycin, 5 mM L-[^{3}H]carnitine (specific activity, 0.032 μC/μmole), 0.2 ml of purified carnitine palmitoyltransferase (0.04 unit), 0.2 mM CoA, 2 mM ATP,

5 mM phosphoenolpyruvate and approx. 2 units of pyruvate kinase and adenylate kinase each. The reaction volume was 1 ml, and the pH was 7.4. The reaction was started with 0.1 mg of either mitochondrial or microsomal protein and stopped after 6 min of incubation with 0.1 ml of conc. HCl. After addition of 1.9 ml of water, the palmitoylcarnitine was extracted with 1.5 ml of *n*-butanol. After washing the butanol phase with 2 ml of butanol-saturated water, 0.5 ml of the butanol phase was transferred to a counting vial together with 10 ml of toluene (containing 5 g of 2,5-diphenyloxazole and 0.3 g of dimethyl-1,4-bis-(5-phenyloxazolyl-2)benzene per l) and sufficient Nuclear-Chicago solubilizer (Amersham Searle Corp.) or abs. ethanol to keep the solution clear. The counting efficiency was 29 % as determined by the channels ratio method. Carnitine palmitoyltransferase was assayed by two different methods: firstly, as described by NORUM[18] as the CoA-dependent incorporation of labeled carnitine into palmitoylcarnitine (the exchange reaction, Method I); secondly, as the formation of palmitoylcarnitine from palmitate, ATP, CoA and carnitine in the presence of an excess palmitoyl-CoA synthetase (forward reaction, Method II). The latter enzyme does not have to be added to the reaction medium, since it was found that both in mitochondria and in microsomes palmitoyl-CoA synthetase is present in excess of carnitine palmitoyltransferase. The incubation medium (Method II) is the same as that used for palmitoyl-CoA synthetase, except that the purified carnitine palmitoyltransferase from calf liver is omitted. The rate of the exchange reaction (Method I) was converted into the rate of the forward reaction (palmitoyl-CoA + carnitine $\rightarrow$ palmitoylcarnitine + CoA) by the use of the exchange rate expression as described in ref. 19.

The fractions prepared by differential centrifugation were treated with ultrasonic vibration before incubation (Branson S-75 sonifier; 1 min at 20 kHz). The temperature was maintained between 0 and 4° by cooling in an ice–salt bath.

Protein was determined with the biuret reaction after solubilization of particle-bound protein with deoxycholate according to JACOBS *et al.*[20].

RESULTS

Differential centrifugation studies on the localization of carnitine palmitoyltransferase and palmitoyl-CoA synthetase

Table I shows the distribution of carnitine palmitoyltransferase, palmitoyl-CoA synthetase and some marker enzymes in subcellular fractions from rat liver. The fractions were prepared by differential centrifugation, as described in MATERIALS AND METHODS. The results of this typical experiment are shown graphically in Fig. 1. The relative specific activities (for definition see legend to Fig. 1) are plotted against the percentage of the total protein as proposed by DE DUVE *et al.*[13]. The distribution of the marker enzymes, *i.e.* cytochrome *c* oxidase (mitochondrial) and glucose-6-phosphatase (microsomal), and protein correspond to those observed by other workers[13], except that our Fraction N is richer in protein and in enzyme content. This suggests a gentle homogenization of the liver, leaving a relatively large amount of unbroken cells in Fraction N. From the distribution of the marker enzymes it can be concluded that the mutual contamination of the different fractions is low. The distribution of monoamine oxidase resembles that of cytochrome *c* oxidase, except that a somewhat higher percentage is found in the microsomal fraction. As monoamine oxidase is

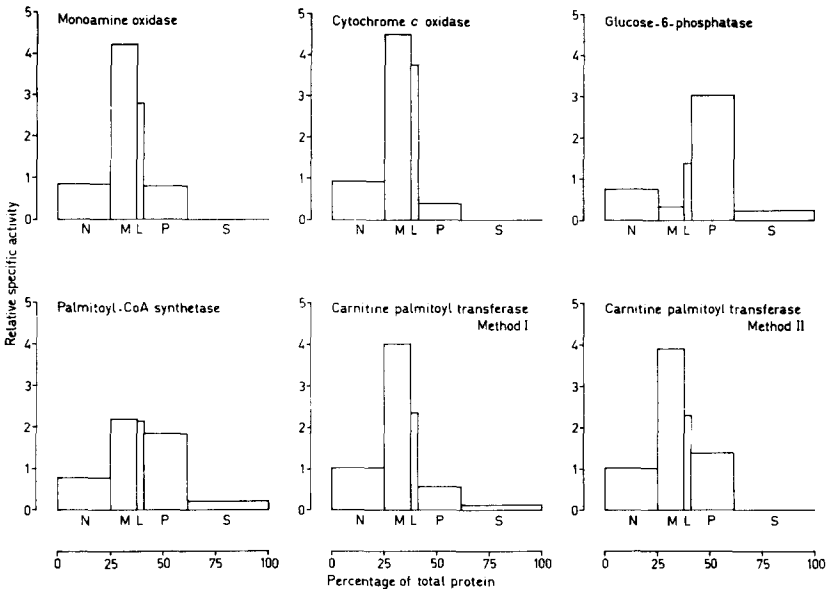

Fig. 1. The distribution patterns of carnitine palmitoyltransferase, palmitoyl-CoA synthetase and and some marker enzymes in fractions from rat liver. The relative specific activities (percentage of total activity divided by the percentage of total protein content) of the fractions are plotted against the percentage of total protein in each fraction. For recoveries and the meaning of N, M, L, P and S see Table I.

TABLE I

INTRACELLULAR DISTRIBUTION OF CARNITINE PALMITOYLTRANSFERASE, PALMITOYL-CoA SYNTHETASE AND SOME MARKER ENZYMES IN RAT LIVER

The enzymes and protein were assayed as described under MATERIALS AND METHODS. Protein is expressed in mg/g of liver (wet wt.). The enzyme activities in the total homogenate are given in μmoles of substrate metabolized per g of liver (wet wt.) per min. Fraction N is the nuclear fraction; M, the heavy mitochondrial fraction; L, the light mitochondrial fraction; P, the microsomal fraction; and S, the final supernatant.

Enzyme	*Total homogenate*	*N* (%)	*M* (%)	*L* (%)	*P* (%)	*S* (%)	*Recovery* (%)
Carnitine palmitoyltransferase (activity determined by Method I)	4.4	26.0	49.9	8.2	11.6	4.3	101
Carnitine palmitoyltransferase (activity determined by Method II)	2.2	26.1	48.5	8.1	28.4	0	111
Palmitoyl-CoA synthetase	12.4	19.1	27.1	7.5	37.7	8.5	91
Monoamine oxidase	0.17	21.7	52.6	9.8	16.0	0	92
Cytochrome *c* oxidase	88.0	23.4	55.5	13.2	7.9	0	92
Glucose-6-phosphatase	11.0	19.3	4.1	4.8	62.7	9.1	108
Protein	200.0	25.2	12.4	3.5	20.5	38.3	93

localized in the mitochondrial outer membrane[21,22], this suggests that a part of the mitochondrial outer membrane is separated from the mitochondria by the fractionation procedure. The distribution of palmitoyl-CoA synthetase supports the dual localization of the enzyme as reported by FARSTAD *et al.*[2]. In our hands a greater percentage is found in the mitochondria. From Table I it can be calculated that the

specific activities measured by us are higher than reported before, especially in the mitochondrial fraction[2,23].

The distribution of carnitine palmitoyltransferase is strongly dependent on the estimation used. When measured with the exchange reaction (Method I), the percentage of the total activity in the microsomal fraction is only slightly higher when compared to cytochrome *c* oxidase. However, when the forward reaction (Method II) is used, the distribution pattern suggests a dual localization of the enzyme with activity both in mitochondria and microsomes. It is suggested by several workers that the ratio of long-chain acylcarnitine to free carnitine is higher in livers from fasted rats than from fed rats (see for instance ref. 12). Microsomal enzymes are in general more sensitive to variations in the diet than mitochondrial enzymes. Therefore, we tested whether fasting activated the microsomal carnitine palmitoyltransferase more than the mitochondrial enzyme.

TABLE II

EFFECT OF FASTING (48 h) ON THE ACTIVITY OF CARNITINE PALMITOYLTRANSFERASE IN FRACTIONS FROM RAT LIVER

The enzyme activity was assayed using the CoA-dependent isotope-exchange reaction (Method I). The enzyme fractions were prepared by centrifugation of a 10% rat-liver homogenate for 5 min at 900 × *g*. The 900 × *g* supernatant is centrifuged for 10 min at 12000 × *g* to obtain the 12000 × *g* supernatant. Incubation was carried out with 2 mg (900 × *g* supernatant) or 6 mg (12000 × *g* supernatant) of protein. The enzyme activity is given as μmoles of substrate metabolized per amount of supernatant corresponding to 1 g of liver per min. The mean and the standard error of the mean of enzyme activity of 4 fasted and 4 normal rats are given. For further details see MATERIALS AND METHODS.

Treatment	*Number of rats*	*900 × g supernatant*	*12000 × g supernatant*
None	4	3.25 ± 0.56	0.83 ± 0.16
Fasted	4	4.00 ± 0.13*	1.19 ± 0.02**

* Statistically significant difference as compared with the values for untreated rats: $0.1 > P > 0.05$.

** $0.02 > P > 0.01$.

It can be seen from Table II, where the activities of carnitine palmitoyltransferase are compared in 900 × *g* and 12000 × *g* supernatants of livers from fed rats and rats fasted for 48 h, that the activity per g of liver is significantly increased by fasting only in the 12000 × *g* supernatant. This suggests that the increment induced by fasting is mainly due to activation of the extramitochondrial enzyme. Moreover, NORUM[9] found that after fasting, fat feeding and in diabetes the total activity increased and that the specific activity in the extramitochondrial compartment increased most. For these reasons and the smaller variation in enzyme activities in fasted rats (compare the standard errors in Table II), we decided to use fasted animals in further localization studies.

The distribution of carnitine palmitoyltransferase as studied with the use of continuous sucrose density gradients

It can be seen from Fig. 2, which shows a representative experiment of a series of 3 experiments, that also in continuous gradient centrifugation studies the enzyme

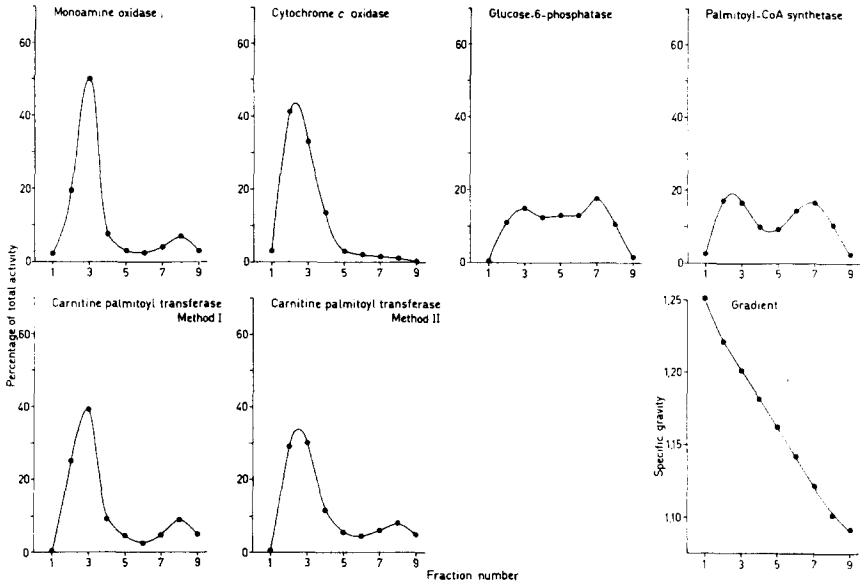

Fig. 2. The distribution of carnitine palmitoyltransferase, palmitoyl-CoA synthetase and some marker enzymes in fractions from rat liver, isolated by continuous sucrose density gradient centrifugation. A 48-h-fasted rat was used. For details see MATERIALS AND METHODS. The percentage of the total activity in Fractions 1–9 is plotted against the fraction number. The recovery of the activities of the different enzymes in the gradient fractions varied between 87 and 107%.

has a dual localization. Fractions 5–9 are very little contaminated with mitochondrial inner membranes (see the distribution of cytochrome *c* oxidase; compare ref. 13).

Fig. 2 shows that some monoamine oxidase is present in fractions not containing cytochrome *c* oxidase. Does the activity of carnitine palmitoyltransferase in gradient Fractions 5–9 correspond to particles containing monoamine oxidase (mitochondrial outer membranes) or particles containing glucose-6-phosphatase (microsomes)? In order to investigate this problem, we reinvestigated the intramitochondrial distribution of carnitine palmitoyltransferase. For these experiments, twice-washed mitochondria were used. The inner and outer membranes were separated by swelling in phosphate buffer as suggested by PARSONS *et al.*[14] (for details see MATERIALS AND METHODS) followed by centrifugation in a continuous sucrose density gradient, prepared exactly as that used in the fasting experiments. Monoamine oxidase (outer membranes) and cytochrome *c* oxidase (inner membranes) were used as marker enzymes for the mitochondrial membranes.

As can been seen from Fig. 3, this method yields a good separation of outer and inner membranes. Whereas after phosphate treatment the activity of cytochrome *c* oxidase is shifted to a region of higher density, about two thirds of the monoamine oxidase activity is shifted to a region of lower density. Carnitine palmitoyltransferase in these experiments behaves quantitatively as cytochrome *c* oxidase (95% of the activity shifts to regions of higher density). These experiments then strongly suggest that the mitochondrial carnitine palmitoyltransferase is localized in the inner membrane, confirming the observations of NORUM *et al.*[4]. Another enzyme also localized in the mitochondrial outer membrane, the rotenone-insensitive NADH–cytochrome

c reductase[17] behaves in these swelling experiments essentially like monoamine oxidase (not shown). These experiments then suggest that in the experiments of Fig. 2 the activity of carnitine palmitoyltransferase in Fractions 5–9 is not due to contamination with mitochondrial outer membranes (compare also the data of Table I) but is to be ascribed to the presence of microsomes. Rather unexpected is the distribution of glucose-6-phosphatase in the gradient experiment of Fig. 2. From differential centrifugation experiments (Table I) it can be seen that glucose-6-phosphatase is localized almost exclusively in the microsomes. However, when a 900 × *g* supernatant is centrifuged in a continuous sucrose density gradient apparently a considerable portion of the enzyme activity is found in the mitochondrial fractions (1–4). The same observation was made by FARSTAD *et al.*[2], when a mixture of isolated mitochondria and microsomes was centrifuged in about the same way. Probably part of the endoplasmic reticulum is rather tightly attached to the mitochondria (compare ref. 24). It is evident then that the percentage of the microsomal carnitine palmitoyltransferase is underestimated in these gradient experiments. This explains the apparent difference

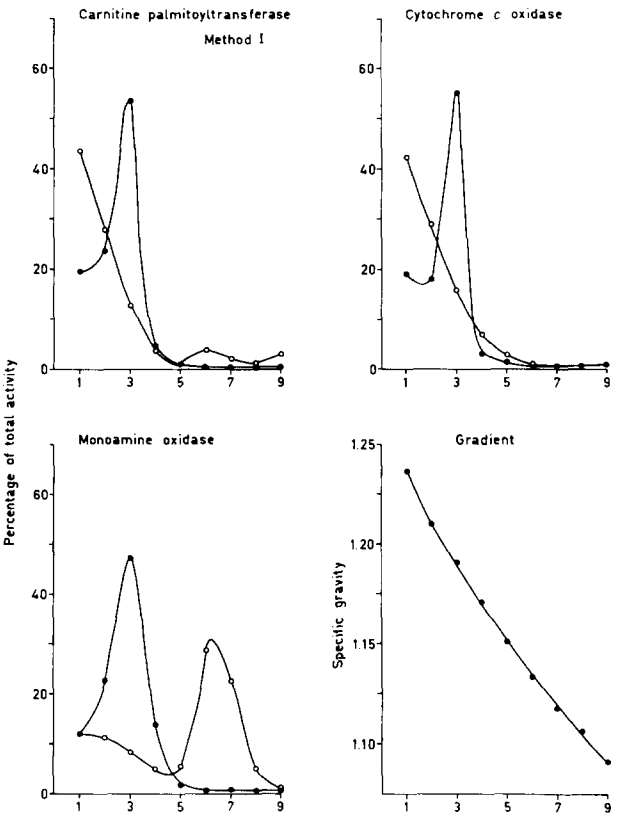

Fig. 3. Intramitochondrial localization of carnitine palmitoyltransferase. After swelling of the mitochondria in 20 mM phosphate buffer, the outer and inner membranes were separated in a continuous sucrose density gradient. For further details see MATERIALS AND METHODS. The percentage of the total activity in Fractions 1–9 is plotted against the fraction number. The recovery of the activities of the different enzymes in the gradient fractions varied between 89 and 103%. ●—●, intact mitochondria; ○—○, swollen mitochondria.

in percentage of microsomal carnitine palmitoyltransferase in the gradient studies (Fig. 2) and the differential centrifugation studies (Fig. 1). The gradient centrifugation studies thus confirm the differential centrifugation studies that carnitine palmitoyltransferase has a dual localization. The activity resides both in mitochondria and in microsomes. In mitochondria at least the bulk of the enzyme activity is localized in the inner membranes. Whether the outer membrane contains no carnitine palmitoyltransferase activity at all is difficult to decide. In the gradient centrifugation studies, the significant difference in transferase activity, measured by methods I and II as observed in the differential centrifugation studies, has disappeared. This requires further investigation.

Palmitoylcarnitine formation during palmitate oxidation in isolated mitochondria

The activities for the palmitoyl-CoA synthetase in sonicated mitochondria, as measured in the present studies, are much bigger than reported before[2,8,23]. From Table I a specific activity of 8.1 μmoles/mg protein per h can be calculated. From earlier experiments, carried out under different conditions, we concluded already that the capacity for long-chain fatty acid activation exceeds by far that for fatty acid oxidation[8]. If the rates of palmitate activation in sonicated and in intact mitochondria were the same, the oxidation of palmitate to acetyl-CoA (acetoacetate) would maximally correlate with an O_2 uptake of 1250 μl O_2 per mg protein per h (Q_{O_2}) at 37°. However, even when palmitoylcarnitine, a substrate which is oxidized more rapidly than palmitate by rat-liver mitochondria, is used, the Q_{O_2}, even for complete oxidation, never exceeds 250 (compare also ref. 25). The activity of mitochondrial carnitine palmitoyltransferase is also relatively high (2.6 μmoles/mg protein per h), so that it is also rather unlikely that this enzyme is rate limiting for long-chain fatty acid oxidation. In fact, as shown in Fig. 4, when palmitate is oxidized by rat-liver mitochondria in the presence of a physiological concentration of carnitine[26], the formation of palmitoylcarnitine can be demonstrated. That more palmitate (the spots marked with an asterisk) disappeared when the incubation was carried out with mitochondria (M) instead of mitochondrial supernatant (P + S) can be concluded from the autoradiogram. This extra amount was not recovered in the other spots, indicating a loss of material not extractable by chloroform–methanol, indicating oxidation of palmitate. Fig. 4 also shows that incubation with the mitochondrial supernatant results in the formation of palmitoylcarnitine (indicated by the arrow). This enzyme fraction is so little contaminated with mitochondria (compare the fractions from Table I), that the palmitoylcarnitine formation cannot be explained by mitochondrial contamination. The spot indicated by the arrow was further identified by two-dimensional thin-layer chromatography. The material was scraped from the thin-layer plate and, after elution from the silica gel with methanol, unlabeled palmitoylcarnitine was added. This mixture was chromatographed in two directions. The chromatogram was developed in chloroform–methanol–water (77:35:7, by vol.) and subsequently in chloroform–methanol–glacial acetic acid–water (50:15:6:2.5, by vol.). 80–96 % of the radioactivity was recovered in the palmitoylcarnitine spot. When in similar experiments [^{3}H]carnitine was used, ^{3}H was present in the palmitoylcarnitine spot.

These experiments then indicate that, even during palmitate oxidation in the presence of carnitine, palmitoylcarnitine accumulates.

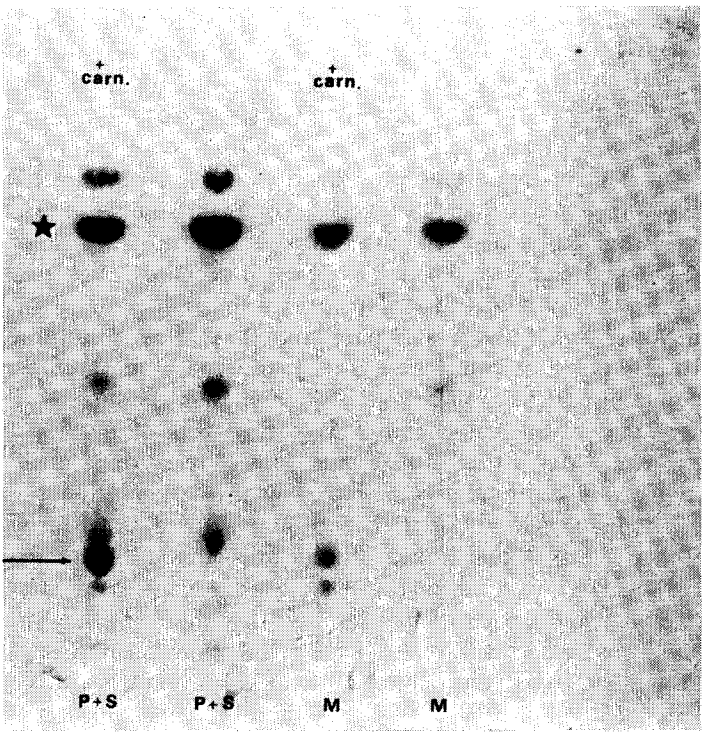

Fig. 4. Autoradiographic demonstration of palmitoylcarnitine synthesis in mitochondria (M) and in 27000 × *g* supernatant (P + S) from rat liver under aerobic condition. The reaction medium contained 80 mM Tris–HCl, 0.25 mM [1-^{14}C]palmitic acid (specific activity, 0.2 μC/μmole) complexed with 0.035 mM bovine serum albumin, 0.5 mM CoA, 2 mM $MgCl_2$, 20 mM KCl, 0.5 mM EDTA, 10 mM P_i, 2 mM ATP, 5 mM phosphoenolpyruvate, 2 units pyruvate kinase and 30 mM sucrose. Where indicated, 2 mM DL-carnitine (carn.) was added. Reaction volume, 1 ml; temp., 37°; pH 7.4. The enzyme fractions were prepared by centrifugation of a rat-liver homogenate in 0.25 M sucrose for 5 min at 900 × *g*. The pellet was discarded and the supernatant was centrifuged for 10 min at 27000 × *g*. The 27000 × *g* supernatant was carefully pipetted off. The 27000 × *g* pellet was washed twice by resuspending in 0.25 M sucrose and centrifugation for 10 min at 12000 × *g*. The fluffy layer was discarded and the mitochondrial pellet suspended in 0.25 M sucrose. After 20 min of incubation with 1.1 or 4.5 mg of protein (mitochondrial or 27000 × *g* supernatant fraction, respectively), the reactions were stopped by extraction with 3 ml chloroform–methanol (1:2, by vol.). The precipitate formed was reextracted with a mixture of 1.6 ml water, 4.0 ml methanol and 2.0 ml chloroform. After centrifugation the supernatants were combined and 7.7 ml of chloroform and 7.7 ml of water added. The resulting lower phase was quantitatively removed and evaporated to dryness. The residue was dissolved in 0.2 ml chloroform–methanol (1:2, by vol.) and applied to a thin-layer plate (silica gel). The chromatogram was developed with chloroform–methanol–water (77:35:7, by vol.). The references used (not shown) were palmitic acid, phosphatidylethanolamine, phosphatidylcholine, palmitoylcarnitine and a mixture of these compounds. Agfa–Gevaert Osray film was used (exposure time, 80 h).

DISCUSSION

The findings of other workers[2,23] that ATP-dependent palmitoyl-CoA synthetase has a dual localization is confirmed by us. The specific activities found by us are (at 37°) about 8 μmoles/mg protein per h in both mitochondria and microsomes. PANDE AND MEAD[23], who measured the activity of the enzyme based on the formation of hydroxamate in the presence of hydroxylamine, reported for microsomes a similar

figure. In mitochondria they found lower activities. FARSTAD *et al.*[2], on the other hand, measured much lower activities. Both FARSTAD *et al.* and we used in principle the same method: formation of palmitoylcarnitine in the presence of excess carnitine and carnitine palmitoyltransferase. Here, the discrepancy between the low activities described by FARSTAD *et al.* and the high activities described by us are probably to be ascribed to differences in incubation conditions. FARSTAD *et al.* report that fatty acid activation in isolated mitochondria and microsomes requires a soluble protein factor for optimal activity. In our experiments, however, such a soluble protein fraction was not required, as is demonstrated by the good recoveries of palmitoyl-CoA synthetase in the experiments on the subcellular distribution of the enzyme.

In agreement with NORUM *et al.*[4], the present paper reports that the mitochondrial localization of carnitine palmitoyltransferase is virtually 100% in the inner membrane *plus* matrix fraction. In fact, these authors found that the enzyme is localized in the inner membrane of the mitochondria. In earlier work of BREMER[10], in which no marker enzymes for the various cellular fractions were used, carnitine palmitoyltransferase activity was also found in the microsomal fraction. Moreover, NORUM[9] also found, especially in livers from fasted, diabetic and fat-fed rats, carnitine palmitoyltransferase activity in the extramitochondrial compartment. In a later publication, NORUM AND BREMER[3] now using marker enzymes state that "carnitine acyltransferases in rat liver are mitochondrial enzymes, and that these enzymes are not associated with the lysosomes, the microsomes or the cellular sap". The present paper, on the contrary, provides strong evidence for a dual localization of carnitine palmitoyltransferase in rat liver. As measured by our method II, which consists of the CoA- and ATP-dependent (endogenous) palmitoyl-CoA synthetase, followed by Reaction 1 from left to right, we find activities (at 37°) of 2.6 and 0.9 μmoles/mg protein per h in mitochondria and microsomes, respectively.

$$\text{Palmitoyl-CoA} + \text{carnitine} \rightleftharpoons \text{palmitoylcarnitine} + \text{CoA} \qquad (1)$$

With the very high activities reported here, it is unlikely that the palmitoyl-CoA synthetase or the carnitine palmitoyltransferase are rate-limiting enzymes in palmitate oxidation by rat-liver mitochondria. The mitochondrion, at least under certain conditions, has the apparatus to synthesize more activated fatty acids (acyl-CoA and acylcarnitine) than it can use for oxidation (compare also the experiment of Fig. 4). With the data presented in this paper we want to stress that acylcarnitine is a storage form of activated fatty acid, which can be formed and used both inside and outside the mitochondria.

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