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Long-chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine*

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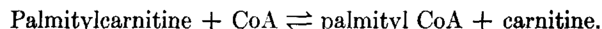
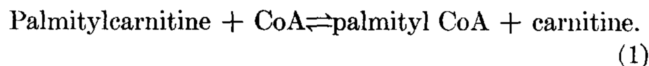
SUMMARY

Carnitine-H^3 or palmitate-C^{14} incubated with heart muscle preparations was incorporated into a compound that had chromatographic behavior in several systems identical to that of palmitylcarnitine chemically synthesized from palmityl chloride and carnitine. Palmitylcarnitine biosynthesis from palmitic acid and carnitine was dependent upon ATP and CoA in addition to substrates and an enzyme preparation. In contrast, palmityl carnitine formation from palmityl CoA and carnitine did not require ATP or CoA. The following reaction, catalyzed by palmitylcarnitine transferase, was shown to be reversible:


Palmitylcarnitine + CoA ⇌ palmityl CoA + carnitine.

General chemical and metabolic properties of palmitylcarnitine were defined. Addition of palmitylcarnitine to heart muscle mitochondria increased respiration more than did addition of palmityl CoA, suggesting that palmitylcarnitine can more readily contribute its acyl group to the fatty acid oxidase system than can exogenous palmityl CoA. Carnitine increased degradation of palmityl-1-C^{14} CoA and stearyl-1-C^{14} CoA to CO_2 and increased total oxygen uptake in the absence of ATP if acyl CoA were present. Carnitine did not appreciably enhance respiration or increase conversion of palmitate-1-C^{14} to CO_2 in the absence of ATP but did augment palmitate oxidation, as previously reported, when ATP and CoA were added to the system. Results are consonant with the hypothesis that the catalytic stimulation by carnitine of long-chain fatty acid oxidation is mediated via acylcarnitine formation, with subsequent transfer of the acyl group to CoA at the site of the fatty acid oxidase system.

In previous publications, we have established that carnitine increased rates of long-chain fatty acid oxidation by a variety of tissues, and that the general site of action is at the level of long-chain acyl CoA formation (1–4). The mechanism of catalysis is not known, but we have recently suggested that carnitine (β-hydroxy, γ-trimethylammonium butyrate) may react with long-chain fatty acids to form acylcarnitine derivatives, which could then be converted to corresponding acyl CoA compounds (5). In a preliminary report (6), we demonstrated that carnitine-H^3 and C^{14}-labeled palmitate were incorporated by heart muscle preparations into a compound having the characteristics of palmitylcarnitine.[1] We also presented evidence suggesting that a transferase exists in heart muscle that catalyzes the reversible reaction:


Palmitylcarnitine + CoA ⇌ palmityl CoA + carnitine. (1)

Palmitylcarnitine added to heart muscle mitochondria increased respiration in the absence of ATP, whereas palmitate and carnitine did not appreciably enhance oxygen uptake unless ATP and CoA were provided. Bremer (7) has also recently demonstrated that palmitylcarnitine and other acylcarnitine deriva-

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[1] Palmitylcarnitine is used to designate the O-palmityl ester of carnitine. Similar terminology has been employed for other acylcarnitine derivatives.

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tives increased respiration by mitochondria prepared from several tissues. From these observations, coupled with information available in the literature concerning the catalytic stimulation of fatty acid oxidation by carnitine (1–5) and concerning carnitine acetyltransferase action (8), it appears possible that carnitine may enhance fatty acid oxidation via acylcarnitine formation (4–7).

This paper presents further studies on carnitine palmityltransferase in various heart muscle fractions and gives additional evidence suggesting that acylcarnitine derivatives are intermediates formed during the catalytic stimulation of long-chain fatty acid oxidation by carnitine.

METHODS

Synthesis and Properties of Palmitylcarnitine HCl. Tritiated DL-carnitine hydrochloride was prepared and purified as previously described (9). It was reacted with equimolar amounts of palmityl chloride under anhydrous conditions, using as a model the method of palmityl choline synthesis of Gomori (10). After petroleum ether (bp 60–70°) extraction, the residue was extracted with boiling anhydrous acetone, and the acetone filtrate was maintained at −15° until crystallization occurred. The granular white crystals were harvested by centrifugation, resuspended in a small volume of warm acetone, and recrystallized twice. The yield was only 1% of theoretical. The material sintered at 137–138° and melted at 141–143°. The compound was water-soluble and yielded equimolar amounts of palmitylhydroxamate when reacted with hydroxylamine in alcohol at pH 12. After hydrolysis at 80° for 15 min in 3 N HCl, carnitine and free fatty acid (FFA) could be identified, using chromatographic procedures. The R_F of DL-palmitylcarnitine was found to be 0.7–0.8 with ascending paper chromatography on Whatman #1 with *n*-propanol–H_2O–NH_4OH 85:10:5, whereas that of carnitine under the same conditions was 0.14. Other chromatographic procedures for separation of palmitylcarnitine from carnitine are described in Results. The R_F values of palmitylcarnitine varied somewhat from day to day between values of 0.7 and 0.9 in solvent systems to be described, requiring standard compounds to be run in the same cylinder with unknowns.

Bremer (7) has recently reported preparation of palmitylcarnitine from an excess of palmityl chloride and carnitine in the presence of concentrated H_2SO_4 and has obtained high yields when calculated on the basis of carnitine added. Since Bremer reports that his sample of palmitylcarnitine HCl melted at 155–175°, and our product melted at 141–143°, it may be useful to give our further characterization of purity:

(1) An infrared absorption spectrum of palmitylcarnitine was identical to that of authentic DL-acetylcarnitine with the exception of strong absorption peaks at 3.42 and 3.54 μ indicative of long-chain alkane groups. Both compounds had absorption peaks at 5.75 μ indicative of an ester link, and little or no peak between 2.8 and 2.9 μ, indicating the absence of a free hydroxyl group.

(2) The elemental composition of the compound was found to be: C, 62.42%; H, 9.87%; and N, 3.38% (Spang's Microanalytical Laboratories, Ann Arbor). Calculated values are: C, 63.32%; H, 10.64%; and N, 3.21%.

Incubation Procedures and Extraction of Palmitylcarnitine. "Intact" heart muscle mitochondria were prepared as previously described (5, 11), while lysed mitochondria were prepared by homogenizing the 8,500 × *g* pellet in a glass homogenizer in distilled water, centrifuging, and homogenizing once again with water. Lysed mitochondria were then resuspended in water at a concentration of approximately 3 mg protein/ml as determined by the biuret reaction (12), while intact washed mitochondria were resuspended in 0.25 M sucrose containing 10^{-3} M sodium ethylenediaminetetraacetate (EDTA) at pH 7.4. In preliminary experiments designed to extend earlier observations on the effects of carnitine on palmitate oxidation by mitochondria (5, 11), we established that lysed mitochondria responded to carnitine addition in accord with earlier results. In experiments in which acylcarnitine formation from carnitine-H^3 was investigated, lysed mitochondria were incubated anaerobically, unless otherwise specified, to favor accumulation of acylcarnitine derivatives. In experiments in which substrate oxidation and respiration were measured, "intact" mitochondria were incubated in a gas phase of air as previously described (5).

Acylcarnitine Formation from Carnitine-H^3 or Palmitate-C^{14}. Components of the system were in separate compartments in Thunberg tubes while anaerobic conditions were established by repeatedly flushing with nitrogen passed through alkaline pyrogallic acid (200 ml 40% KOH combined with 20 ml 25% pyrogallic acid) and evacuating. The tubes were incubated for varying periods of time, usually 30 min, at 37°, time zero being calculated from the time of addition of carnitine from the side arm. At the end of incubation, an equal volume of 15% trichloroacetic acid (TCA) was added, the precipitate was collected by centrifugation and washed with 2.5 ml 7.5% TCA, and TCA fractions were pooled. The washed residue was extracted three times with 2.0 ml of absolute methanol,

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and combined methanol fractions were taken to dryness at room temperature under a stream of nitrogen or air. The dried material was taken up in 0.5 ml methanol and poured into a hexane-washed silicic acid column (0.5 g in a column having 1.0 cm i.d.) with a wash of 4 ml *n*-hexane. Silicic acid (Mallinckrodt, chromatographic grade, 100–200 mesh) had been washed successively with chloroform, methanol, ethanol, water, and finally methanol before activation at 110° for a minimum of 12 hr. When the two-phase system had almost passed through the silicic acid, 5 ml hexane wash solution was poured from the original tube, and the column was developed with 20 to 100 ml additional hexane to free the column of fatty acids. After the hexane fraction had been collected, the column was eluted with 30 ml absolute ethanol adjusted to pH 10.5 with ethanolic sodium hydroxide. In other experiments, it had been shown that this procedure removes only about 1% of added carnitine from the silicic acid column but elutes palmitylcarnitine almost quantitatively. The contaminating carnitine presented no problem since it could be quantitatively separated from acylcarnitine derivatives by paper chromatographic procedures to be described. In addition, over 98% of free carnitine remained in the original TCA supernatant fractions (see Table 5 of reference 5).

The ethanol fraction was taken to dryness at room temperature under a stream of air and then resuspended in 1.0 ml absolute ethanol. Aliquots (0.1 ml) were assayed for radioactivity with a Tri-Carb liquid scintillation spectrometer as previously described (5, 9). Other aliquots were then applied to Whatman #1 filter paper (45 x 10 cm), dried, and analyzed by ascending chromatography in various solvent systems after suitable equilibration. After the solvent front had traveled approximately 30 cm, the papers were removed from cylinders, air-dried, and either stained with iodine vapor or analyzed for localization of radioactivity. In the latter case, thin strips were cut into sequential 1.5-cm segments commencing with the origin and were placed into glass vials for counting. Papers were cut into finer pieces, and 0.1 ml alcoholic sodium hydroxide at pH 10.5 (50% ethanol in water) was added, followed by 1.0 ml absolute ethanol. After thorough mixing, the samples were allowed to stand for 15 min to permit complete elution of material from the paper. Then 5 ml toluene, containing diphenyloxazole and POPOP (1,4-*bis*-2-(5-phenyloxazolyl)-benzene) as previously described (9), was added to vials containing paper strips and ethanol. The counting efficiency for detection of radioactivity in the samples placed in the Tri-Carb spectrometer was approximately 50% for C^{14} and 12% for H^3. Results were corrected for internal quenching.

TABLE 1. R_F VALUES FOR CARNITINE AND ACYLCARNITINE DERIVATIVES*

Compound	Developing Solvent System			
	I	II	III	IV
DL-Carnitine	0.45	0.20	0.14	0.05
DL-Acetylcarnitine	0.50	0.25	0.18	0.14
DL-Palmitylcarnitine	0.80	0.86	0.80	0.74
H^3-Labeled compound obtained after incubation of carnitine-H^3 with mitochondria	0.78	0.84	0.80	0.72

* All values are those obtained with ascending chromatography with the following systems: I. Methanol–H_2O–NH_4OH 93:5:2, developing time 5–6 hr; II. Ethanol–H_2O–NH_4OH 90:5:5, developing time 14–16 hr; III. *n*-Propanol-H_2O-NH_4OH 85:10:5, developing time 15–17 hr; IV. Butanol saturated with water, developing time 15–17 hr. Known carnitine derivatives were localized on paper chromatograms by staining with iodine and by determining H^3 activity in consecutive paper strips. R_F values of the biosynthetic compound were determined by detection of H^3 in strips eluted by procedures given in Methods.

Determination of Carnitine Palmityltransferase Activity. Mitochondrial suspensions were incubated with palmitylcarnitine and hydroxylamine at pH 7.4 in the presence and absence of CoA. After incubation for 30 min at 37°, the reaction was stopped with the ferric-chloride-trichloroacetic acid reagent described by Lipmann and Tuttle (13). The supernatant solution was discarded, the iron-palmitylhydroxamate complex was extracted from the residue with 95% ethanol (2), and absorbancy was determined at 520 mμ, using palmitylhydroxamate as a standard. The use of suitable blanks established that palmitylcarnitine did not react with hydroxylamine at pH 7.4; quantitative conversion of palmitylcarnitine to palmitylhydroxamate occurred nonenzymatically only at pH values above 12.

Materials. Adenosine triphosphate, CoA, and NAD were purchased from Pabst; generous supplies of DL-acetylcarnitine and carnitine HCl were contributed by International Minerals and Chemicals, Skokie, Ill.; tritiation by the Wilzbach procedure was performed by New England Nuclear Corp.; palmitate-1-C^{14} was obtained from U.S. Nuclear Corp. and from Nuclear Chicago Corp.; and all other reagents were of highest purity commercially available. Palmityl CoA was synthesized from palmityl chloride and CoA by the procedure of Seubert (14). Samples of palmityl-1-C^{14} and stearyl-1-C^{14} CoA, synthesized by the same method, were kindly provided by Dr. W. E. Lands.

RESULTS

Palmitylcarnitine Biosynthesis. The R_F values for carnitine, acetylcarnitine, and palmitylcarnitine in

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TABLE 2. INCORPORATION OF CARNITINE-H^3 INTO PALMITYLCARNITINE BY HEART MUSCLE MITOCHONDRIA*

Additions to Basic Medium	cpm Carnitine-H^3 Incorporated into Palmitylcarnitine After Incubation With					
	Palmitic Acid			Palmityl CoA		
	Lysed Mitochondria	600 × g Supernatant Fraction	25,000 × g Supernatant Fraction	Lysed Mitochondria	600 × g Supernatant Fraction	25,000 × g Supernatant Fraction
None	1,400	700	700	28,700	30,800	1,120,000
CoA	2,100	...	...	...	...	...
ATP	2,800	...	...	29,400	...	...
ATP + CoA	26,600	11,900	42,000	...	...	...
ATP + glutathione	2,800	...	...	...	...	...

* Results are expressed as counts per minute H^3 (to the nearest 100) recovered in a compound having the chromatographic properties of palmitylcarnitine. Samples chromatographed were aliquots of the ethanol fraction eluted from silicic acid columns after treatment of the mitochondrial pellet as described in Methods. In a total volume of 2.5 ml, the basic medium contained the following components, neutralized when necessary with $KHCO_3$: 5×10^{-3} M $MgCl_2$, 5×10^{-3} M DL-H^3-carnitine containing 7×10^6 cpm, 3×10^{-2} M KCl, 2.5×10^{-4} M potassium palmitate or palmityl CoA, 3.5×10^{-5} M crystalline bovine serum albumin, and 3×10^{-3} M potassium phosphate at pH 7.4. In addition, the compounds indicated in the first column were present in the following concentrations: 4.25×10^{-5} M CoA (0.068 mg/ml of a Pabst compound assayed at 50% purity), 2×10^{-3} M ATP, and 2.0×10^{-4} M glutathione. After gassing with nitrogen as described in Methods, carnitine was added from a side arm to the main compartment. Tubes were incubated at 37° for 30 min. Experiments on carnitine-H^3 incorporation into lysed mitochondria are representative of eight such experiments. In experiments shown, 3.8 mg protein was present in vessels containing lysed mitochondria, prepared as described in Methods. Other vessels contained either 7.5 mg protein from supernatant fractions spun at 600 × g for 10 min, or 4.6 mg protein from supernatant fractions of heart muscle homogenate spun at 25,000 × g for 1 hr. In these vessels, the final sucrose concentration was 4×10^{-4} M. Results shown with supernatant fractions are representative of at least three separate experiments.

four different systems are summarized in Table 1. Best separation of palmitylcarnitine from carnitine was achieved in systems III and IV; consequently, *n*-propanol and butanol systems were used most often as the developing solvents.

In each of eight separate experiments in which heart muscle mitochondria were incubated with carnitine-H^3, tritium was found in a compound having the chromatographic properties of palmitylcarnitine. Percentage incorporation was low, most probably because the compound was metabolized to palmityl CoA and then deacylated (*vide infra*). Conversion of carnitine into a moiety having the same R_F values as those of palmitylcarnitine was dependent upon added palmitate, ATP, and CoA (Table 2), with glutathione being unable to substitute for the CoA requirement. Incorporation of label into palmitylcarnitine was almost as great after incubation for 5 min as it was after 30 min, indicating either that the enzyme was in excess or that simultaneous addition of a hydrolase in the crude preparation was preventing accumulation of palmitylcarnitine.

Additional proof that the material isolated after incubation of carnitine-H^3 with heart muscle mitochondria was palmitylcarnitine was obtained by identifying compounds after hydrolysis. Chromatograms of aliquots of the ethanol fraction before and after hydrolysis are shown in Fig. 1, demonstrating that carnitine was present in the initial compound. R_F values of the biosynthetic material in different solvents are identical to those of authentic palmitylcarnitine (Table 1). Comparable results were obtained when experiments were performed to measure incorporation of palmitate-C^{14} into palmitylcarnitine by heart muscle mitochondria. In three separate experiments in which palmitate-C^{14}, ATP, and CoA were incubated with unlabeled carnitine and lysed heart muscle mitochondria under conditions identical to those described in Table 2, approximately 0.5% of palmitate-C^{14} was incorporated into the palmitylcarnitine fraction. In contrast, in the absence of ATP or carnitine, only 0.01% appeared in this fraction.

Carnitine Palmityltransferase. When carnitine-H^3 and palmityl CoA were incubated with heart muscle mitochondria, carnitine was incorporated into palmitylcarnitine in the absence of ATP or CoA (Table 2). The lack of requirement for cofactors suggested the presence of an enzyme, carnitine palmityltransferase, which catalyzed the reaction shown in eq. (1). Heart homogenates were fractionated to attempt intracellular localization of the enzyme. All fractions examined were able to convert carnitine and palmityl CoA to palmitylcarnitine when these materials were the only components added to the system (Table 2). Since only inappreciable amounts of palmitylcarnitine were synthesized from free palmitic acid, carnitine, and ATP in the absence of CoA, it appears that CoA is an essential cofactor. The data are consonant with the possibility

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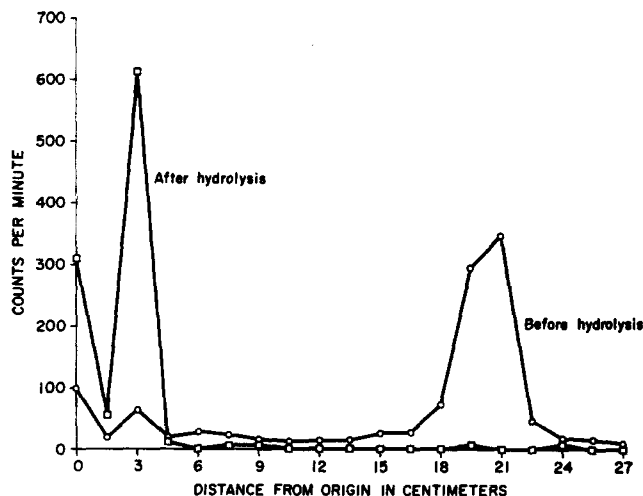

FIG. 1. Carnitine-H^3 incorporation into palmitylcarnitine by heart mitochondria. Details of mitochondrial preparation and incubation were the same as those described for different experiments in the footnote to Table 2. Separate aliquots of the ethanol extract were chromatographed with *n*-propanol–H_2O–NH_4OH 85:10:5 as the developing solvent before and after hydrolysis in 3 N HCl at 80° for 15 min.

that palmityl CoA is an obligatory intermediate in palmitylcarnitine formation.

Although 25,000 × *g* supernatant fractions of rat heart homogenates contained highest apparent enzymatic activity under the conditions of the assay shown in Table 2, we were unable to purify carnitine palmityltransferase from this fraction. A major impediment blocking purification of the enzyme was the persistent association of palmityl CoA hydrolase with all preparations of the transferase obtained. Both enzymes were precipitated with 50% saturated $(NH_4)_2SO_4$, taken up in 0.01 M phosphate buffer at pH 7.7, and adsorbed onto $Ca_3(PO_4)_2$ gel at a final ratio of 3 mg gel to 1 mg protein. A protein solution retaining carnitine palmityltransferase activity was eluted from the gel with 0.5 M potassium phosphate buffer at pH 7.7. We estimated palmityl CoA hydrolase activity in each of the above fractions by adding aliquots to solutions of palmityl CoA and following the reaction at 232 mμ spectrophotometrically (15). Absorbancy at 232 mμ decreased steadily after addition of each fraction, indicating hydrolysis of palmityl CoA (15). Inability to free carnitine palmityltransferase of palmityl CoA hydrolase has prevented the use of more efficient spectrophotometric assay procedures developed during the purification of carnitine acetyltransferase (16). As pointed out above, palmityl CoA hydrolase activity probably is responsible for the relatively small amount of carnitine-H^3 incorporated into palmitylcarnitine by tissue preparations shown in Table 2.

TABLE 3. CONVERSION OF PALMITYLCARNITINE TO PALMITYL CoA*

DL-Palmitylcarnitine Added	Heart Mitochondria	Palmitylhydroxamate Formed −CoA	Palmitylhydroxamate Formed +CoA
μmole		*μmole*	*μmole*
10	−	0	0
0.5	+	0	0.10
5.0	+	0.10	0.47
10.0	+	0.04	0.53

* Each tube contained 500 μmole hydroxylamine at pH 7.4, 5 μmole $MgCl_2$, 125 μmole sucrose, 0.5 μmole sodium EDTA, and 5.0 mg bovine serum albumin in a final volume of 1.5 ml. Aliquots of intact heart mitochondria containing approximately 1.0 mg protein were added to tubes having indicated amounts of DL-palmitylcarnitine and were incubated at 37° for 30 min in the presence and absence of catalytic amounts of CoA (0.17 mg CoA, Pabst). The reaction was stopped by the addition of 2.5 ml ferric chloride reagent (13), and palmitylhydroxamate, in alcohol extracts of the residue, was determined as previously reported (2). Blank tubes consisted of enzyme suspensions incubated in the absence of substrate and enzyme.

Evidence for the conversion of DL-palmitylcarnitine to palmityl CoA is presented in Table 3. At high palmitylcarnitine concentrations, approximately 0.5 μmole palmityl CoA was formed per mg mitochondrial protein in 30 min. In these vessels, lysis of mitochondria occurred, as judged by diminution of turbidity of suspensions, demonstrating that the surface active properties of palmitylcarnitine influence mitochondrial structure.

Carnitine Effects on Respiration and on Acyl CoA Oxidation. Maximal effects of carnitine on oxidation of added palmitate-1-C^{14} and on total respiration were dependent on added ATP, CoA, Mg^{++}, and NAD (Table 4). Oxygen uptake by mitochondria incubated with all cofactors listed was approximately 5 μl/mg protein/30 min in the absence of carnitine and was increased to 20 after carnitine addition under optimal conditions shown in Table 4. These observations confirm and extend earlier reports on optimal conditions required for carnitine to stimulate long-chain fatty acid oxidation (5, 11). Although carnitine had but little effect on oxygen consumption in the absence of ATP or CoA when palmitate was the substrate (5, 11), carnitine *did* increase respiration in the absence of ATP when palmityl CoA or stearyl CoA replaced palmitate as substrate (Table 5 and Fig. 2). Further, carnitine increased the conversion of labeled acyl-1-C^{14} CoA but not palmitate-1-C^{14} to CO_2 by heart muscle mitochondria in the absence of ATP, provided there was a sufficiently high albumin con-

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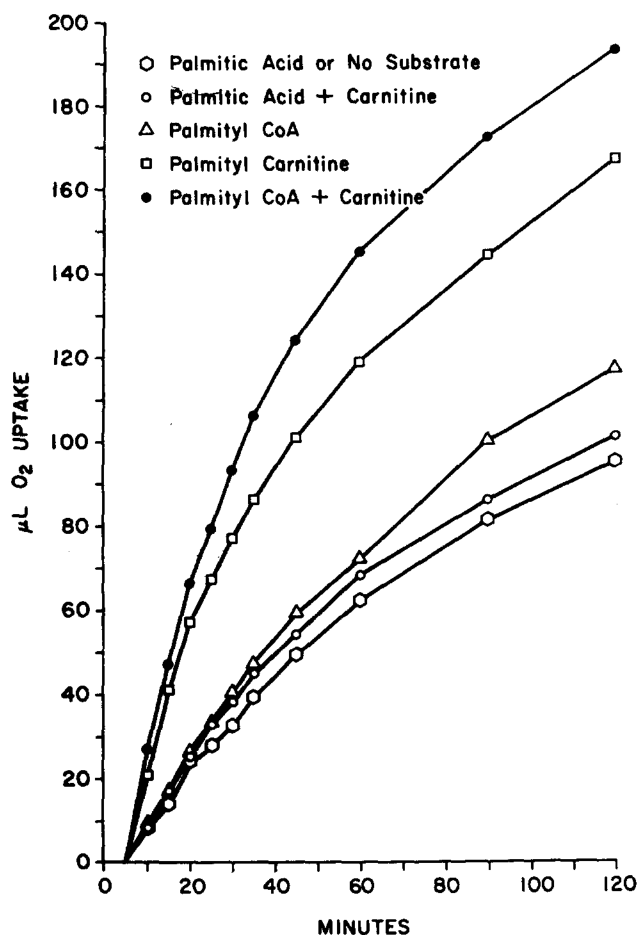

FIG. 2. Effects of various substrates on respiration by heart muscle mitochondria in presence and absence of carnitine. Conditions were identical to those described in the footnote to Table 5, except that substrates were varied as indicated. The final concentration of DL-carnitine when present was 5×10^{-4} M, and substrate concentrations were 1×10^{-4} M. In this experiment, 5.6 mg mitochondrial protein was added to each vessel, and results shown are the average values obtained from duplicate vessels of a single experiment representative of four comparable experiments.

TABLE 4. COFACTOR REQUIREMENTS FOR PALMITATE OXIDATION BY HEART MUSCLE MITOCHONDRIA*

	Percentage Conversion of Palmitate-1-C^{14} to $C^{14}O_2$/mg Protein/30 Min				
	Complete System	Minus ATP	Minus CoA	Minus NAD	Minus $MgCl_2$
Control	1.7	0.88	1.1	1.2	1.9
DL-Carnitine (10^{-3} M)	13.9	2.25	2.75	8.05	1.6

* Intact mitochondria of rat heart muscle, prepared as described in Methods, were incubated for 30 min at 37° in 2.5 ml medium containing 0.5 µc of 1.0×10^{-4} M potassium palmitate-1-C^{14}, 0.14×10^{-4} M bovine serum albumin, 0.1 M sucrose, 4×10^{-4} M sodium EDTA, 1×10^{-5} M L-malate, 8×10^{-2} M KCl, and 8×10^{-3} M sodium phosphate at pH 7.4. The complete medium contained 1×10^{-3} M ATP, 4×10^{-5} M CoA, 2.5×10^{-3} M NAD, and 5×10^{-3} M $MgCl_2$.

TABLE 5. EFFECTS OF CARNITINE ON RESPIRATION OF HEART MUSCLE MITOCHONDRIA INCUBATED WITH DIFFERENT SUBSTRATES IN ABSENCE OF ATP*

DL-Carnitine (5×10^{-4} M)	µl O_2/mg Protein/60 Minutes In Presence of Following Substrate		
	None	Palmitic Acid	Palmityl CoA
−	13.2	13.0	15.3
+	14.5	14.3	31.4

* Heart mitochondrial oxygen consumption, manometrically measured, is reported as µl O_2 consumed during the first 60 min of incubation at 37°. Palmityl CoA, DL-palmitylcarnitine, and palmitic acid were present at a final concentration of 1×10^{-4} M in experiments depicted in appropriate columns. All vessels contained 4.9 mg washed intact heart mitchondrial protein in 2.5 ml of the following composition: 0.25 M sucrose, 1×10^{-3} M sodium EDTA, 1×10^{-3} M $MgCl_2$, 6×10^{-2} M KCl, 3.33×10^{-3} M AMP, 6×10^{-3} M sodium phosphate at pH 7.4, 21 mg bovine serum albumin, and 1×10^{-3} M succinate. Results given are the average values obtained from duplicate vessels of a single experiment representative of at least four experiments for each substrate.

centration in the medium (Table 6). Previous failure to obtain an effect of carnitine on palmityl CoA oxidation by liver particulates may be attributed to the use of fragmented mitochondria from a less responsive tissue incubated in a medium containing low concentrations of albumin (2).

The effects of palmitylcarnitine on oxygen uptake have been reported both by us in a preliminary communication (6) and by Bremer (7). In Table 7 and Fig. 2, we compare the effects of palmitylcarnitine and palmityl CoA on heart muscle mitochondrial respiration. The data indicate that, in the absence of carnitine under conditions of incubation shown on each horizontal line of Table 7, palmitylcarnitine increased oxygen consumption more than palmityl CoA did. High albumin concentrations increased respiration, but the same relative effects of palmitylcarnitine were seen in all cases. The use of AMP as a phosphate acceptor and succinate at 10^{-4} to 10^{-3} M to stimulate oxidation in the tricarboxylic acid cycle (17) proved superior to the use of NAD and malate for obtaining larger effects of palmitylcarnitine on respiration, so we employed the former conditions to examine effects of carnitine on palmityl CoA oxidation. Although palmityl CoA alone slightly stimulated respiration (Table 7 and Fig. 2), the addition of carnitine permitted palmityl CoA to markedly increase oxygen consumption (Tables 5 and 6, Fig. 2). Carnitine alone or with palmitic acid

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TABLE 6. EFFECTS OF CARNITINE ON OXIDATION OF ACYL-1-C^{14} COA AND PALMITIC ACID-1-C^{14} BY HEART MUSCLE MITOCHONDRIA IN ABSENCE OF ATP*

Substrate	Percentage Conversion of C^{14}-Substrate to CO_2/Mg Protein/30 Min		μl O_2/Mg Protein/30 Min	
	Control	DL-Carnitine (1×10^{-4} M)	Control	DL-Carnitine (1×10^{-4} M)
Palmitic acid-1-C^{14}	0.13	0.16	6.0	6.4
Palmityl-1-C^{14} CoA	0.15	1.26	8.7	23.0
Stearyl-1-C^{14} CoA	0.10	2.14	7.8	16.2

* Heart muscle mitochondria (approximately 5 mg protein) were incubated under conditions identical to those cited in the legend to Table 5. Stearyl-1-C^{14} CoA had 100,000 cpm/0.25 μmole, while comparable amounts of palmityl-1-C^{14} CoA and palmitic-1-C^{14} acid had 30,000 cpm and 250,000 cpm, respectively. Vessels were incubated at 37° for 30 min, and results shown on each horizontal line are the average values from duplicate vessels of single experiments. Experiments with palmitate-1-C^{14} and palmityl-1-C^{14} CoA have been repeated three times with comparable results, but the stearyl CoA data are from only one experiment.

TABLE 7. COMPARISON OF EFFECTS OF PALMITYLCARNITINE AND PALMITYL COA ON OXYGEN UPTAKE BY HEART MUSCLE MITOCHONDRIA IN ABSENCE OF ATP*

Components of Medium Varied	μl O_2/Mg Protein/60 Min in Presence of Following Substrate			
	None	Palmityl CoA	DL-Palmityl-carnitine	Palmitic Acid
(21 mg albumin in all)				
NAD + Malate	10.2	11.4	16.6	10.0
AMP + Succinate	11.0	12.6	21.2	11.2
(AMP + Succinate in all)				
Albumin (3 mg)	4.8	9.8	12.1	5.0
Albumin (21 mg)	13.1	17.3	21.4	13.0

* Conditions of incubation in all experiments except those shown on the first line were the same as those reported in the legend to Table 5. Note, however, that albumin concentration was varied as indicated. In experiments shown on the first line, AMP was deleted, NAD concentration was 2.5×10^{-3} M, and L-malate (1×10^{-5} M) was added in place of succinate. Results given are the average values obtained from duplicate vessels of single experiments representative of at least three experiments for each set of conditions. Final concentrations of substrates shown were 1×10^{-4} M.

had negligible effects on oxygen uptake in the absence of ATP. Similarly, carnitine did not increase the conversion of palmitate-1-C^{14} to CO_2 under these conditions. In contrast, label from palmityl-1-C^{14} or stearyl CoA appeared in CO_2 to a much greater extent after carnitine was added (Table 6). It appears, therefore, that carnitine stimulated respiration by increasing oxidation of acyl CoA. Values for the percentage conversion of C^{14}-labeled substrates to CO_2 (Table 6) were lower than those found for palmitate-1-C^{14} conversion to CO_2 by mitochondria incubated under optimal conditions (Table 4) because succinate (10^{-3} M) was present in the experiments shown in Table 6, and this high concentration acted as a trap to dilute labeled acetate generated from acyl CoA as it entered the Krebs cycle. In contrast, malate was present at low concentrations (10^{-5} M) in experiments in which palmitate-1-C^{14} oxidation was estimated (Table 4). In both sets of experiments, the concentration of the long-chain fatty acid or its derivative was 10^{-4} M.

Palmityl-1-C^{14} CoA oxidation to $C^{14}O_2$ by 600 × *g* supernatant fractions of heart homogenates was inhibited by palmitylcarnitine when high albumin concentrations were present in the medium but not at low albumin concentrations (Table 8). It is not known why palmitylcarnitine addition did not suppress the conversion of palmityl-1-C^{14} CoA to $C^{14}O_2$ at low albumin concentrations. This phenomenon may be related to that observed by Bremer (7), who obtained an increase in mitochondrial respiration after palmitylcarnitine addition only at high albumin concentrations. It is also known that the percentage increase of palmitate oxidation induced by carnitine in several tissue preparations is largest at high albumin-to-palmitate molar ratios (4). These phenomena may be correlated in some way with the protective action of albumin against the surface active properties of compounds like palmitylcarnitine on mitochondrial structure.

In experiments reported in Table 8, carnitine increased the conversion of palmityl-1-C^{14} CoA to $C^{14}O_2$ in control vessels, but the effect was not as pronounced as that seen in Table 6 because succinate and AMP were not present in the experiments shown in Table 8. When palmitylcarnitine was added to the system containing palmityl-1-C^{14} CoA and high albumin concentrations, suppression of incorporation of C^{14} into respiratory $C^{14}O_2$ could not be reversed by carnitine. Both in the presence and absence of carnitine, palmitylcarnitine addition decreased the conversion of palmityl-1-C^{14} CoA to $C^{14}O_2$. This probably occurred because labeled palmityl CoA was diluted by unlabeled palmityl CoA generated from palmitylcarnitine, mediated by carnitine palmityltransferase.

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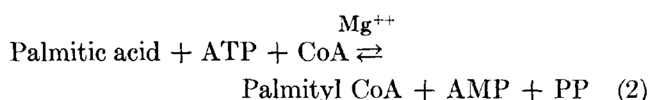
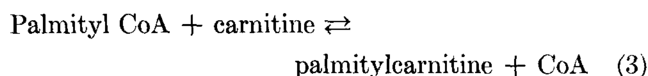
DISCUSSION

Carnitine palmityltransferase, which catalyzes the reversible formation of palmityl CoA from palmitylcarnitine and CoA, may play an important role in making long-chain acyl groups available for oxidation. This enzyme is similar to carnitine acetyltransferase, which was initially described by Friedman and Fraenkel (8) and subsequently purified by Fritz and Schultz (16). Carnitine acetyltransferase is specific for transfer of short-chain acyl groups, since palmityl CoA and palmitylcarnitine are not satisfactory substrates for this enzyme (16). Thus, the enzyme carnitine palmityltransferase reported here is clearly a different transferase. Just as there are separate thiokinases catalyzing the formation of different chain-length acyl CoA derivatives (18), and separate chain-length specific acyl CoA dehydrogenases catalyzing the formation of enolyl CoA derivatives (19), there appear to be at least two carnitine acyltransferases having different chain-length specificities.

We were unable to find a pathway of acylcarnitine synthesis from free fatty acid and carnitine that was not in large part dependent upon added CoA and ATP. Since palmitylcarnitine synthesis readily took place in the absence of ATP and CoA when palmityl CoA and carnitine were the substrates, it seems reasonable to postulate the following pathway for palmitylcarnitine formation:



$$\text{Palmitic acid} + \text{ATP} + \text{CoA} \overset{Mg^{++}}{\rightleftarrows} \text{Palmityl CoA} + \text{AMP} + \text{PP} \quad (2)$$



$$\text{Palmityl CoA} + \text{carnitine} \rightleftarrows \text{palmitylcarnitine} + \text{CoA} \quad (3)$$

Long-chain acylthiokinase, present in largest amounts in the liver cytoplasmic fraction, catalyzes the first reaction, which has been described by Kornberg and Pricer (18). It is known to require ATP, CoA, and Mg^{++}, thereby possibly accounting for the ATP and CoA requirements cited above for palmitylcarnitine formation. After this manuscript was submitted for publication, we received a personal communication from Bremer indicating that he has incubated (—)-carnitine-1-C^{14} with rat liver microsomes. Using different methods of isolation, he has independently shown that C^{14} incorporation into palmitylcarnitine did not proceed in the absence of ATP or CoA, and that the addition of an albumin-palmitate complex increased incorporation 5-fold. Bremer's work was presented in Norway and will appear in abstract form (20). Our findings, a preliminary account of which appeared earlier (6), and Bremer's results are in agreement.

TABLE 8. PALMITYL-1-C^{14} COA OXIDATION BY HEART MUSCLE HOMOGENATES INCUBATED IN THE ABSENCE OF ATP*

Albumin Added Per Vessel (mg)	Percentage Oxidation to CO_2/mg Protein			
	Control		DL-Palmitylcarnitine	
	Minus Carnitine	Carnitine Added	Minus Carnitine	Carnitine Added
3	2.5	3.1	2.1	3.1
21	1.4	3.5	0.34	0.33

* All vessels contained aliquots of 600 × *g* supernatant fractions of heart muscle homogenates (6.5 mg protein/vessel) in 2.5 ml of the following composition: 0.25 M sucrose, 4×10^{-4} M sodium EDTA, 1×10^{-3} M $MgCl_2$, 2.5×10^{-3} M NAD, 2×10^{-5} M CoA, 1×10^{-5} M L-malate, 6×10^{-3} M sodium phosphate at pH 7.4, and 1×10^{-4} M palmityl-1-C^{14} CoA having 30,000 cpm. When present, the final concentrations of DL-palmitylcarnitine or carnitine was 1×10^{-4} M. All vessels were incubated at 37° for 30 min. Results are averages from duplicate vessels of a single experiment representative of at least three experiments performed under each set of conditions.

Addition of palmitylcarnitine to mitochondrial preparations increased respiration to a greater extent than equal or greater amounts of palmityl CoA did (Table 7 and Fig. 2). Palmitylcarnitine may have been metabolized more rapidly than the corresponding acyl CoA compound because the carnitine derivative might penetrate to the site of fatty acid oxidation more readily. If this conjecture proves valid, palmitylcarnitine may function as a carrier to transport the acyl group from acyl CoA past mitochondrial barriers to the fatty acid oxidase system. We wish to offer an hypothesis that the previously observed catalytic stimulation of carnitine on long-chain fatty acid oxidation (1–5) may be mediated via acylcarnitine formation. We base this hypothesis on the following evidence:

(1) Carnitine palmityltransferase has been extracted from various heart muscle fractions.

(2) Carnitine increased acyl-1-C^{14} CoA oxidation in the absence of ATP and also increased total respiration under these conditions, provided acyl CoA was added. In contrast, carnitine had little or no effect on oxygen uptake or on free palmitic acid oxidation when ATP was deleted from the medium containing no acyl CoA.

(3) DL-Acylcarnitine enhanced respiration by heart muscle mitochondria more than equimolar amounts of acyl CoA did under conditions in which free fatty acid and carnitine had slight or no effects on oxygen consumption.

(4) Conditions have been defined in which unlabeled palmitylcarnitine diluted palmityl-1-C^{14} CoA,

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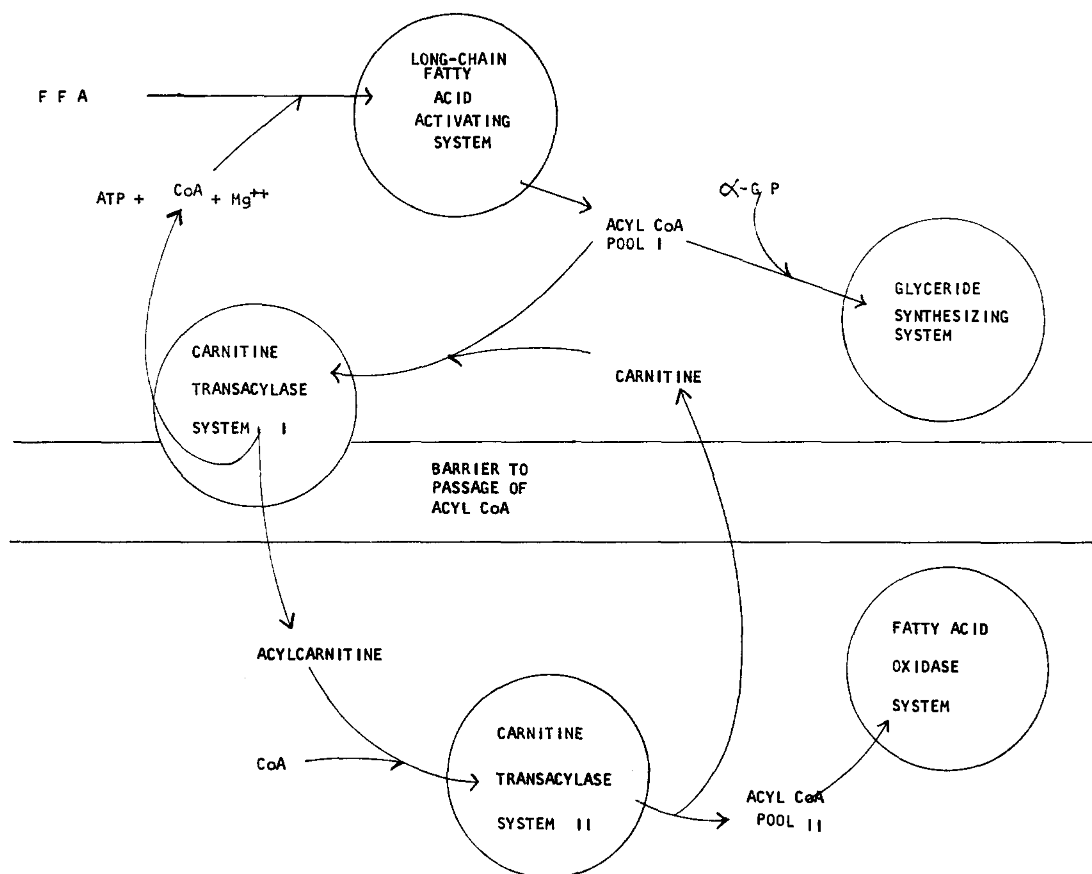

FIG. 3. Hypothetical site of action of carnitine on fatty acid metabolism. This scheme shows functional compartmentation of acyl CoA pools postulated to account for carnitine stimulation of fatty acid oxidation, as discussed in the text.

as evaluated by a decreased appearance of label in respiratory CO_2.

A means by which acylcarnitine derivatives could function as a carrier of acyl groups from extramitochondrial acyl CoA to intramitochondrial acyl CoA, and thereby enhance fatty acid oxidation, is schematically presented in Fig. 3. In this working hypothesis, it is assumed that acyl CoA pools are compartmentalized and that long-chain acylcarnitine derivatives formed from acyl CoA and carnitine have readier access to the fatty acid oxidase system than do fatty acyl CoA compounds. In the absence of carnitine, transport of acyl CoA to the fatty acid oxidase system is postulated to be rate-limiting. The presence of carnitine palmityltransferase in the vicinity of both compartments, which is demanded by the hypothesis, receives experimental support from results shown in Table 2. Other data consonant with this hypothesis are presented in a recent review in which we have examined from a general viewpoint the role of carnitine in fatty acid metabolism (21).

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