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CARNITINE OCTANOYLTRANSFERASE AND CARNITINE ACETYLTRANSFERASE OF MOUSE LIVER PEROXISOMES

by

Shawn O. Farrell

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ABSTRACT

CARNITINE OCTANOYLTRANSFERASE AND CARNITINE ACETYLTRANSFERASE OF MOUSE LIVER PEROXISOMES

by

Shawn O. Farrell

The purpose of this study was to establish the existence of a separate carnitine octanoyltransferase in mouse liver peroxisomes, purify and characterize it, and compare it to the carnitine acetyltransferase purified from the same source. Carnitine octanoyltransferase (COT) and carnitine acetyltransferase (CAT) were solubilized from livers of mice treated with the hypolipidemic drug Wy-14,643 by homogenization and freezing in 8.5% sucrose, 10 mM sodium pyrophosphate, pH 7.5. COT and CAT were separated using Cibacron Blue Sepharose and purified to homogeneity. Both have a molecular weight of 60,000 by Sephadex G-100 chromatography and SDS-polyacrylamide gel electrophoresis. Both have similar pH optima, 8.0 to 8.5, but the pIs are different, 5.2 for COT and 6.8 for CAT. COT and CAT have maximum activities in the forward direction with hexanoyl-CoA and butyryl-CoA, respectively; and in the reverse direction with hexanoylcarnitine and propionylcarnitine, respectively. The K_ms for acyl-CoA are low and suggest that formation of acylcarnitine is favored *in vivo*. With COT, using acyl-CoA with chain-lengths of

4-12, the K_ms for acyl-CoA are between 2 and 4 μM, and are higher for C_2-, C_{16}-, and C_{18}-CoA. With CAT, the acyl-CoA K_ms are between 15-29 μM for C_2-CoA through C_{10}-CoA. For both enzymes, the K_m for L-carnitine varies with the acyl-CoA used. With CAT the K_m for carnitine increases from 86 μM with C_2-CoA to 519 μM with C_{10}-CoA. With COT the K_m for L-carnitine is lower with long-chain acyl-CoAs as cosubstrate. COT retained its maximum activity when preincubated with DTNB at pH 7.0 or 8.5. In contrast, CAT was inactivated at both pH values but could be protected by the substrates. CAT was unaffected by preincubation with trypsin while COT was activated at low trypsin concentrations and inactivated at higher concentrations. Neither enzyme was inhibited by malonyl-CoA.

Antibodies raised against the purified COT did not precipitate with purified CAT, purified beef heart mitochondrial CPT, or solubilized mouse liver mitochondria. The anti-COT serum did react with 10,000g supernatant fluids from homogenates of mouse kidney, mouse intestine, rat liver, dog liver, and beef liver.

It is concluded that carnitine octanoyltransferase is a separate enzyme in mouse liver peroxisomes, with kinetic properties that favor formation of medium-chain acylcarnitines. Peroxisomal COT and CAT probably function in the transport of peroxisomal β-oxidation products out of the peroxisome.

DEDICATION

To my father, George Farrell, who instilled in me a great joy of learning and the will to persevere when the going gets tough.

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CAT	Carnitine acetyltransferase
CoA	Coenzyme A
CoASH	Reduced coenzyme A
COT	Carnitine octanoyltransferase
CPT	Carnitine palmitoyltransferase
DEAE-	Diethylaminoethyl-
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	(Ethylenedinitrilo)-tetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
K_i	Inhibition constant
K_m	Michaelis constant
NADPH	Reduced nicotine adenine dinucleotide phosphate
pI	Isoelectric pH
QAE-	Diethyl-(2-hydroxypropyl)aminoethyl-
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
Tris	tris-(hydroxymethyl)aminomethane
Wy-14,643	[4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]-acetic acid

INTRODUCTION

Background on Carnitine and Carnitine Acyltransferases

Carnitine (gamma-trimethlyamino-ß-hydroxybutyrate) was first isolated in 1905 (1) from mammalian muscle, but its function remained unknown for forty years. Fraenkel *et al.* (2) discovered that carnitine was an essential nutrient for larvae of the beetle *Tenebrio molitor*, and it was subsequently given the trivial name vitamin B_T. In 1955 Friedman and Fraenkel (3) discovered a possible enzymatic role for carnitine when it was found to be acetylated by acetyl-CoA in pigeon and sheep liver. Fritz (4) reported a carnitine dependent stimulation of long-chain fatty acid oxidation in liver preparations, but found little effect of carnitine on medium-chain fatty acid oxidation. Almost simultaneously the laboratories of Fritz and of Bremer provided evidence that carnitine plays a role in mitochondrial ß-oxidation of long-chain fatty acids (5-7).

Carnitine acyltransferases are a class of enzymes that catalyze the reversible reaction:

L-(-)-carnitine + acyl-CoA $\longleftrightarrow$ acyl-L-(-)-carnitine + CoASH,

where the acyl group has a carbon chain-length of 2 to more than 20 and can be straight chained, branched chained, or unsaturated. The reactions are defined as:
Forward reaction:

acyl-CoA + L-carnitine $\longrightarrow$ acyl-L-carnitine + CoASH,

Reverse reaction:

acyl-L-carnitine + CoASH $\longrightarrow$ acyl-CoA + L-carnitine.

The initial investigations dealt with enzymes utilizing predominantly long-chain acyl moieties and acetyl moieties. These enzymes were then given the names carnitine palmitoyltransferases (CPT)(6-8), and carnitine acetyltransferases (CAT)(3,9-11), respectively. Although mammalian systems have a broad acyl specificity of carnitine acyltransferases, in some yeasts, plants, and insects the acyl specificity may be more restricted and depends on the fuel source and metabolism involved (12-18).

Localization and Functions

Studies on the intracellular distribution of CPT have shown that it is a mitochondrial enzyme (19-22). CPT is a membrane associated enzyme that requires the use of

detergents for solubilization and stability (21,23,24), but its distribution in the inner mitochondrial membrane is still the subject of debate.

It has been well established that CPT functions in the mitochondrial ß-oxidation of fatty acids. The inner mitochondrial membrane is permeable to free fatty acids, but only short- and medium-chain fatty acids are activated to acyl-CoAs inside the mitochondria (25), as the long-chain acyl-CoA synthetases are associated with microsomes (26) and the outer mitochondrial membrane (27). CPT I (outer form) converts cytosolic acyl-CoAs to acylcarnitines which pass into the matrix of the mitochondria where they are reconverted to acyl-CoAs by CPT II (inner form) and then undergo ß-oxidation (6,7,19,28-30)(see appendix for further review).

CAT is the predominant acyltransferase in most tissues (31). Early studies indicated a mitochondrial location (32-36), but CAT has also been found in peroxisomes and microsomes from many tissues (20,37,38). The microsomal CAT is tightly membrane associated and labile (37). In contrast, the peroxisomal enzyme is a stable, soluble, matrix enzyme that is easily released by treatments that disrupt the fragile peroxisomal membrane (37,38).

Although the functions of CAT are still being elucidated, several possibilities have been suggested. It is thought that mitochondrial CAT functions to buffer the

CoASH/acetyl-CoA ratio by shuttling acetyl groups out of the mitochondria as acetylcarnitine (18,33,39-41). This would relieve the "acetyl pressure" in the mitochondria that inhibits pyruvate dehydrogenase and the citric acid cycle. A probable function of peroxisomal CAT is the shuttling of acetyl moieties out of the peroxisome after peroxisomal ß-oxidation (20,42-50). The hypolipidemic drugs that increase peroxisomal ß-oxidation also increase CAT in both peroxisomes and mitochondria (46,51,52). It is also thought that CAT plays a role in the development of spermatozoa, where acetylcarnitine has been found in large quantities (53,54).

The Existence of Carnitine Octanoyltransferase

While working with a commercial preparation of CAT, Solberg found a contaminating activity specific for medium-chain (C_6-C_{10}) acyl-CoA, which was subsequently referred to as carnitine octanoyltransferase (COT)(55). This activity was also found in mitochondria of rat liver, heart, and testis. In subsequent studies, COT activity was found in calf heart mitochondria (21) and in an acyltransferase preparation from calf liver (56). In the latter study, four acyltransferase fractions were partially purified that had chain-length optima of 3,6,12, and 16. Three ranges of K_m were found for short-chain, medium-chain,

and long-chain acyl residues, but complete separation of the activities could not be achieved.

Early work in our lab indicated that COT activity exists in mitochondria, peroxisomes, and microsomes of mammalian liver and kidney (20). A microsomal preparation containing COT and CAT activity was purified free of CPT, and when treatment that solublilized the CAT activity completely destroyed the COT activity, a separate COT enzyme was postulated (38). Similarly, with a peroxisomal acyltransferase system free of CPT, purification of CAT with 0.4 M KCl and DEAE-cellulose caused the loss of all COT activity (37). In contrast, COT activity has been purified from beef heart mitochondria, where at least 90% of the activity was shown to be due to a combination of CAT and CPT (23). Clofibrate and other hypolipidemic drugs were found to increase the levels of carnitine acyltransferases (46,51,52,57,58), however all three activities increased to different extents. We interpreted these results as further evidence that a separate COT enzyme must be present.

Peroxisomal ß-Oxidation

Peroxisomes have ß-oxidation capability (44-48), and it has been estimated that as much as 50% of the total ß-oxidation activity of mouse liver may occur in peroxisomes (49). The peroxisomal ß-oxidation process apparently

terminates at medium-chain acyl-CoAs indicating that peroxisomes contain a chain-shortening capacity. This capacity seems particularly important in the metabolism of fatty acids with carbon chain-lengths greater than 20 (59-62). Following peroxisomal ß-oxidation, the products, medium-chain acyl moieties and acetyl moieties, would need to be exported out of the peroxisome (50). Medium-chain and short-chain acylcarnitines formed via medium-chain and short-chain carnitine acyltransferases could serve this purpose (45,59-64).

THESIS STATEMENT

Solberg originally suggested the existence of a separate carnitine octanoyltransferase enzyme after he found an activity specific for medium-chain acyl-coenzyme As in a commerical CAT preparation (55). Other studies then demonstrated this activity in calf heart mitochondria and calf liver (21,56). Markwell found that rat liver peroxisomes and microsomes also contained a medium-chain activity (37,38). However, in none of the above studies were these activities purified. Ironically, the only previous attempt to purify COT showed that beef heart mitochondria did not have a separate COT enzyme and that the observed activity was due to the combination of the broad substrate specificity of CPT and CAT (23).

As enzyme purification and characterization is the only definitive way of demonstrating the existence of an enzyme, the purpose of this thesis was to isolate the carnitine octanoyltransferase enzyme in mouse liver peroxisomes, purify and characterize it, and compare it to its "nearest neighbor", peroxisomal carnitine acetyltransferase.

EXPERIMENTAL PROCEDURES

Materials

Acyl-CoAs were purchased from PL Biochemicals. L-carnitine was a generous gift from the Otsuka Pharmaceutical Company, Naruto, Tokushima, Japan. Nafenopin (2-methyl-2-[p-(1,2,3,4,-tetrahydro-1-napthyl)-phenoxyl]-propionic acid) was a gift from N.E. Tolbert (Michigan State University, East Lansing, MI) and Wy-14,643 ([4-chloro-6-(2,3-xylidino-)-2-pyrimidinylthio]-acetic acid) was a generous gift from J.K. Reddy (Northwestern University Medical School, Chicago, IL.). Ultrathin Serva Precoats pH 3-10, accessories and protein standards were purchased from Serva Feinbiochimica GMBH and Co. QAE-Sephadex was purchased from Pharmacia Fine Chemicals and Sigma Chemical Company. All other reagents were analytical grade.

Methods

Induction of Carnitine Acyltransferases. Five to six week old male Swiss mice or male Sprague Dawley rats were fed diets of ground Purina Chow containing 0.5% w/w clofibrate (p-chlorophenoxy-isobutyrate), 0.125% nafenopin,

or 0.1% Wy-14,643 for two weeks. The animals were sacrificed and the livers rapidly removed and minced, washed, and homogenized in ice cold 10mM sodium pyrophosphate, pH 7.5. Homogenates were frozen at -80° C until used.

Sucrose Gradients. Livers from animals that had been treated with hypolipidemic drugs as above, or from control mice that had been starved for 24 hours were removed and rinsed with ice cold 0.25 M sucrose, 1mM sodium phosphate, pH 7.5. The livers were minced and suspended in 10 volumes w/v of the same buffer, and homogenized by one pass of a loose-fitting Teflon pestle with a glass Potter-Elvejhem homogenizer. After centrifugation at 4° C for 20 minutes at 500g, 6 ml of the supernatant fluid were loaded onto the sucrose step gradients prepared as in (48). These were spun for 3 hours at 25,000 rpm in a Beckman SW 25.2 swinging bucket rotor and 60 drop (2 ml) fractions were collected from the bottom of the gradient.

Purification of COT. Liver homogenates from mice treated with Wy-14,643 as above were thawed and centrifuged at 500g for 15 minutes and the supernatant fluid collected and centrifuged at 10,000g for 15 minutes. The supernatant fluid was made 40% in ammonium sulfate, centrifuged, and the pellet discarded. This supernatant was then made 60% in

ammonium sulfate, centrifuged, and the pellet suspended in 10mM sodium pyrophospahte, 0.25 mM EDTA, 0.02% sodium azide, pH 7.5 (blue buffer). The dissolved pellet was dialyzed overnight in 20 volumes of blue buffer, and applied at a flow rate of 1 ml/min to a column (40 x 2.5 cm) of Cibacron Blue Sepharose CL-6B equilibrated with blue buffer. After washing with 500 ml blue buffer, a 500 ml linear gradient of 0-1M KCl in blue buffer was used to elute the enzyme.

COT fractions (58-68, Fig. 3) were pooled, dialyzed overnight in 20 volumes of blue buffer, and applied at a flow rate of 1 ml/min to a column (35 x 1.5 cm) of QAE Sephadex A-25 (Pharmacia) equilibrated with blue buffer. COT activity washed through without binding. It was pooled, dialyzed overnight in 20 volumes 20 mM sodium phosphate, 0.25 mM EDTA, 0.02% sodium azide, pH 7.5 (HAP buffer), and applied at a flow rate of 1 ml/min to a column (20 x 2.5 cm) of hydroxylapatite equilibrated with 20 mM sodium phosphate, 0.02% sodium azide, pH 7.5. After 400 ml of HAP buffer were passed through, a 500 ml gradient of 20mM sodium phosphate, 60 mM KCl, pH 7.5 to 900 mM sodium phosphate, 60 mM KCl, pH 7.5 was applied. Fractions containing COT were pooled and dialyzed overnight in 20 volumes 20 mM sodium pyrophosphate, 0.02% sodium azide, pH 7.5 (Seph buffer), concentrated to 3 ml using an Amicon PM-10 filter, and applied at a flow rate of 1 ml/min to a column (95 x 2.5 cm) of Sephadex G-100 equilibrated with Seph buffer. COT activity was eluted with

Seph buffer, the fractions pooled, and dialyzed overnight in 20 volumes 2 mM sodium pyrophosphate, 0.002% sodium azide, pH 7.5, and applied at a flow rate of 1 ml/min to a column (2.5 x 8 cm) of QAE-Sephadex A-25 (Sigma) equilibrated with the same buffer. The column was washed with 200 ml of the starting buffer before a 200 ml linear gradient of 2 mM-100 mM sodium pyrophosphate, pH 7.5 was applied to elute the enzyme. Fractions containing COT were pooled.

Purification of CAT. The first carnitine acyltransferase peak to elute from the Cibacron Blue Sepharose column above was used for further purification of CAT (see fig. 3). The fractions were pooled, dialyzed overnight in QAE buffer (25 mM sodium pyrophosphate, 0.25 mM EDTA, 0.02% sodium azide, pH 7.5), and applied at a flow rate of 1 ml/min to a column (35 x 1.5 cm) of QAE Sepahdex A-25 (Pharmacia) that had been equilibrated with QAE buffer. CAT washed through without binding. The effluent was pooled, dialyzed overnight in 20 volumes of CM buffer (5 mM HEPES, 60 mM KCl, 0.25 mM EDTA, 0.02% sodium azide, pH 7.3) and applied at a flow rate of 1 ml/min to a column (35 x 1.5 cm) of CM Sephadex equilibrated in CM buffer. The column was washed with 300 ml CM buffer and the enzyme eluted with a 400 ml linear gradient of 60-560 mM KCl in CM buffer. Fractions containing CAT were pooled and dialyzed overnight in 20 volumes Seph buffer, concentrated to 3 ml using an Amicon PM-10 filter, and applied at a flow rate of 1 ml/min

to a column (95 x 2.5 cm) of Sephadex G-100 equilibrated with Seph buffer. CAT activity was eluted with Seph buffer and the fractions pooled.

Assays. Assays were performed as previously described for catalase (65), fumarase (66), NADPH cytochrome c reductase (67), and glutamate dehydrogenase (68). Carnitine acyltransferases were measured in the forward direction by the DTNB method of (69). The 0.2 ml cuvette volume contained 0.1 mM Acyl-CoA, 1.25 mM L-carnitine, 0.1 mM DTNB, and 115 mM Tris buffer, pH 8.0. All assays were corrected for the carnitine independent release of CoASH (hydrolase). The reverse reaction was assayed as described in (70). Acylcarnitine, 500 μM, and CoASH, 120 μM, were used. For the K_m profiles with various substrates, COT and CAT were assayed in the forward direction by continuously monitoring the release of Coenzyme A with DTNB at 412 nm. The 2.0 ml reaction volume contained 115 mM Tris buffer, 1.1 mM EDTA, 0.1% Triton X-100, 0.1 mM DTNB with varying amounts of L-carnitine and acyl-CoAs at 25° C, pH 8.0. Kinetic data were obtained with a semiautomated system described in (71,72). Raw absorbance-time data were obtained by continuously increasing the substrate concentration of a stirred enzyme assay mixture, with the use of a precision syringe drive during a reaction time of 3.6 minutes, and collected with a Gilford model 2600 spectrophotometer. The

raw data were transformed into velocity-substrate data by a tangent slope procedure and then analyzed as linear plots using the TANKIN program with a Hewlett-Packard 9815 calculator. When C_{12}-CoA was used with the semiautomated system and the TANKIN program, the K_m could not be defined due to strong inhibition by C_{12}-moieties, so a substrate depletion method was used (73).

Preparation of Antibodies and Immunodiffusion. Rabbit antiserum was raised against purified mouse liver COT by Dr. J.K. Reddy. After collecting pre-immune serum, two New Zealand white male rabbits weighting 2-3 kg were immunized with purified COT. Approximately 500-600 µg of COT were emulsified in Freund's adjuvant (Difco, Detroit, MI) and injected subcutaneously at multiple sites at weekly intervals for 4 weeks (74). One week after the last injection a booster dose of COT was administered intravenously and the rabbits bled 5 days later. Double diffusion plates were prepared using 1% agarose in 0.1 M sodium phosphate, 0.02% sodium azide, 0.15% NaCl, pH 7.4, and developed overnight at room temperature.

Trypsin Inactivation. Inactivation by trypsin was accomplished by incubating the enzymes with varying amounts of trypsin (0-2 mg/µg enzyme) for 15 min at 37° C in 10 mM sodium pyrophosphate, pH 7.5. The reaction was stopped by

addition of a 3-fold excess of trypsin inhibitor.

DTNB Inactivation. COT and CAT were preincubated for 15 min at room temperature with 0-1 mM DTNB in 10 mM sodium phosphate buffer pH 7.0 or 8.5. The transferase reaction was then started by addition of a premix containing all of the substrates as described previously (69).

Isoelectric Focusing. Concentrated COT and CAT were focused for 3 hours on ultrathin gels pH 3-10 at 1 1/2 watts per gel on an LKB Multiphor 2117. Bands were fixed with 20% TCA and then stained with 0.1% Coomassie Brilliant Blue.

Other Methods. SDS-polyacrylamide gel electrophoresis was performed as described (75) employing bovine serum albumin, phosphorylase b, ovalbumin, and trypsinogen as molecular weight standards. Native molecular weight was estimated with chromatography on Sepagdex G-100 as in (76). Protein was determined by the fluorescamine method (77) with the exception that 0.2 M borate, pH 9.25 was used. Bovine serum albumin was used as the protein standard.

Amino Acid Analysis. Purified, lyophilized COT and CAT from mouse liver peroxisomes were subjected to acid hydrolysis for 24 hours and the amino acids measured using a Beckman model 121 by Doris Bauer, Michigan State University Biochemistry Dept.

RESULTS

Preliminary Investigations

Effect of Hypolipidemic Drugs on Total Liver Carnitine Acyltransferase Activity. In order to determine if hypolipidemic drugs could be used to increase the level of carnitine octanoyltransferase in mouse liver, mice were fed control diets, or diets containing clofibrate or nafenopin. Table I shows the increase in the specific activities of carnitine acyltransferases in liver 500g supernatant fluids due to these drugs. All three activities increased with treatment of hypolipidemic drugs. COT, which is present at higher levels than CAT and CPT, increased 3.8- and 11.1-fold with clofibrate and nafenopin, respectively. CAT increased 3.1- and 9.6-fold, while CPT increased to a lesser extent.

Subcellular Distribution of Acyltransferases. Previous studies have shown that rat liver has a multiorganelle distribution of short-chain and medium-chain carnitine acyltransferases (20,42,52). To determine the location or locations of carnitine octanoyltransferase in mouse liver, sucrose gradients were prepared to separate the mouse liver organelles. Figure 1 shows the distribution of carnitine

TABLE I

Effect of Clofibrate and Nafenopin on Mouse Liver Carnitine Acyltransferase Activities[a]

	Activity[b] (nmol/min/mg protein)			-Fold increase	
	Control	Clofibrate	Nafenopin	Clofibrate	Nafenopin
COT	7.4 (0.7)	28.2 (3.5)*	82.2 (3.8)*	3.8	11.1
CAT	2.9 (0.8)	8.9 (0.9)*	27.7 (1.4)*	3.1	9.6
CPT	2.9 (0.3)	6.1 (1.0)	12.0 (1.1)*	2.1	4.1

[a] Carnitine acyltransferases were determined in 500g supernatant fluids from mouse livers of control and treated mice as described in Experimental Procedures.

[b] Values given are means (SEM) for six samples.

* indicates that the treated levels were significantly different from control values at a level of $P < 0.01$ as determined by Dunnett's t test for multiple comparisons (78).

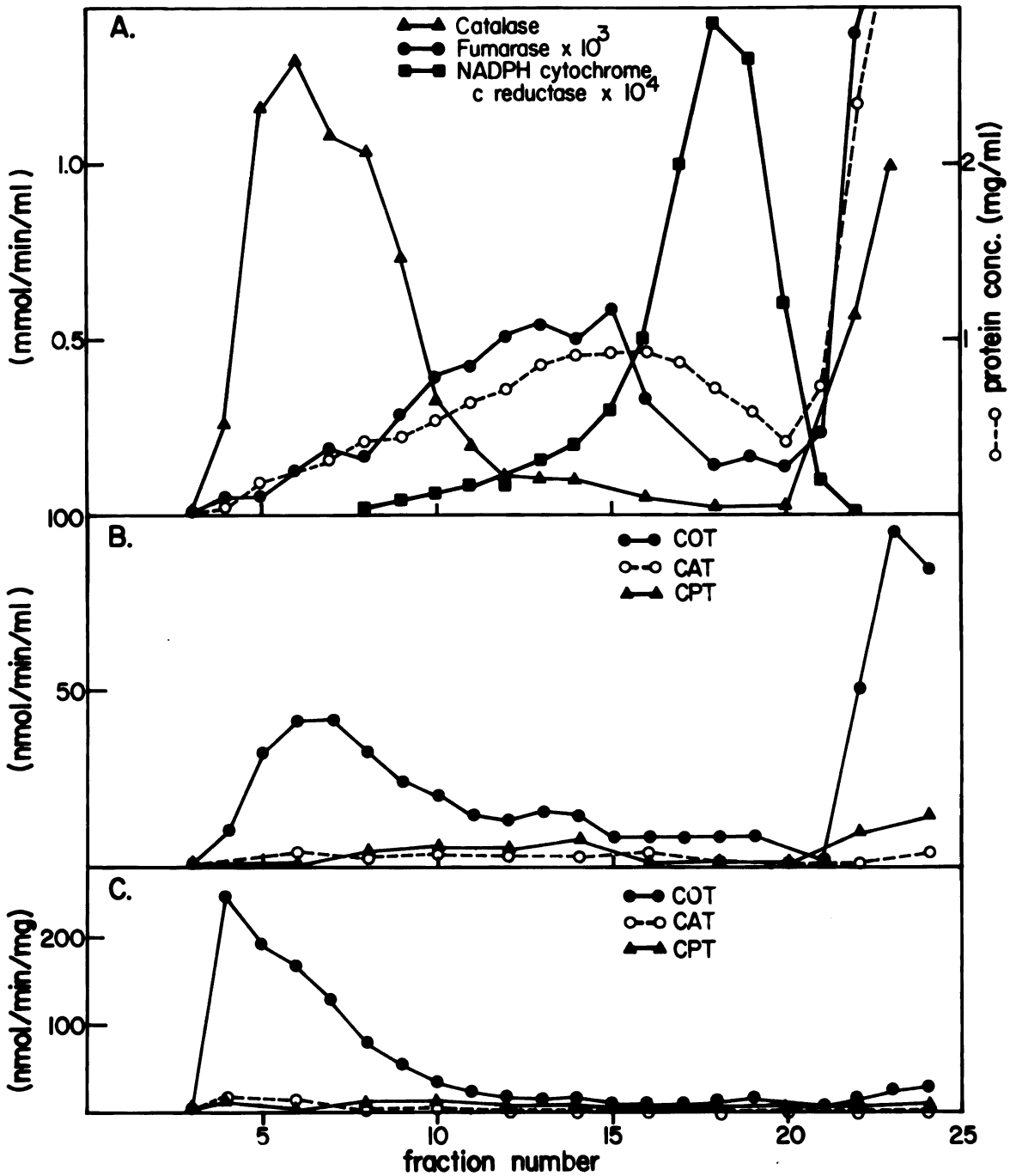
Figure 1. Sucrose gradient separation of mouse liver organelles.

(A) Activity of marker enzymes in mmol/min/ml and protein in mg/ml.

(B) Activity of carnitine acyltransferases in nmol/min/ml.

(C) Specific Activity of carnitine acyltransferases in nmol/min/mg.

Sucrose gradients were prepared and enzymes were assayed as described in Experimental Procedures.


A.
Catalase
Fumarase x 10^3
NADPH cytochrome c reductase x 10^4
(mmol/min/ml)
protein conc. (mg/ml)
1.0
0.5
100
2
1
B.
COT
CAT
CPT
(nmol/min/ml)
50
C.
COT
CAT
CPT
(nmol/min/mg)
200
100
5
10
15
20
25
fraction number

acyltransferase activities from liver homogenates of control mice. The catalase, fumarase, and NADPH cytochrome c reductase distributions shown in Figure 1A represent the distribution of peroxisomes, mitochondria, and microsomes, respectively. A large peak of COT activity coincides with the distribution of catalase, as can be seen in Figure 1B. The COT specific activity in peroxisomes is 10-fold greater than the other two carnitine acyltransferases.

Three different control gradients were assayed and the fractions of the carnitine acyltransferases associated with the peroxisomal, mitochondrial, and soluble fractions of the gradient were determined. Seventy percent of the particulate COT was associated with peroxisomal fraction, and 30% with the mitochondria and microsomes (data not shown). The amount of transferase in the soluble fraction varied with the grinding technique, but the fraction of COT in the soluble fraction coincided with that of catalase.

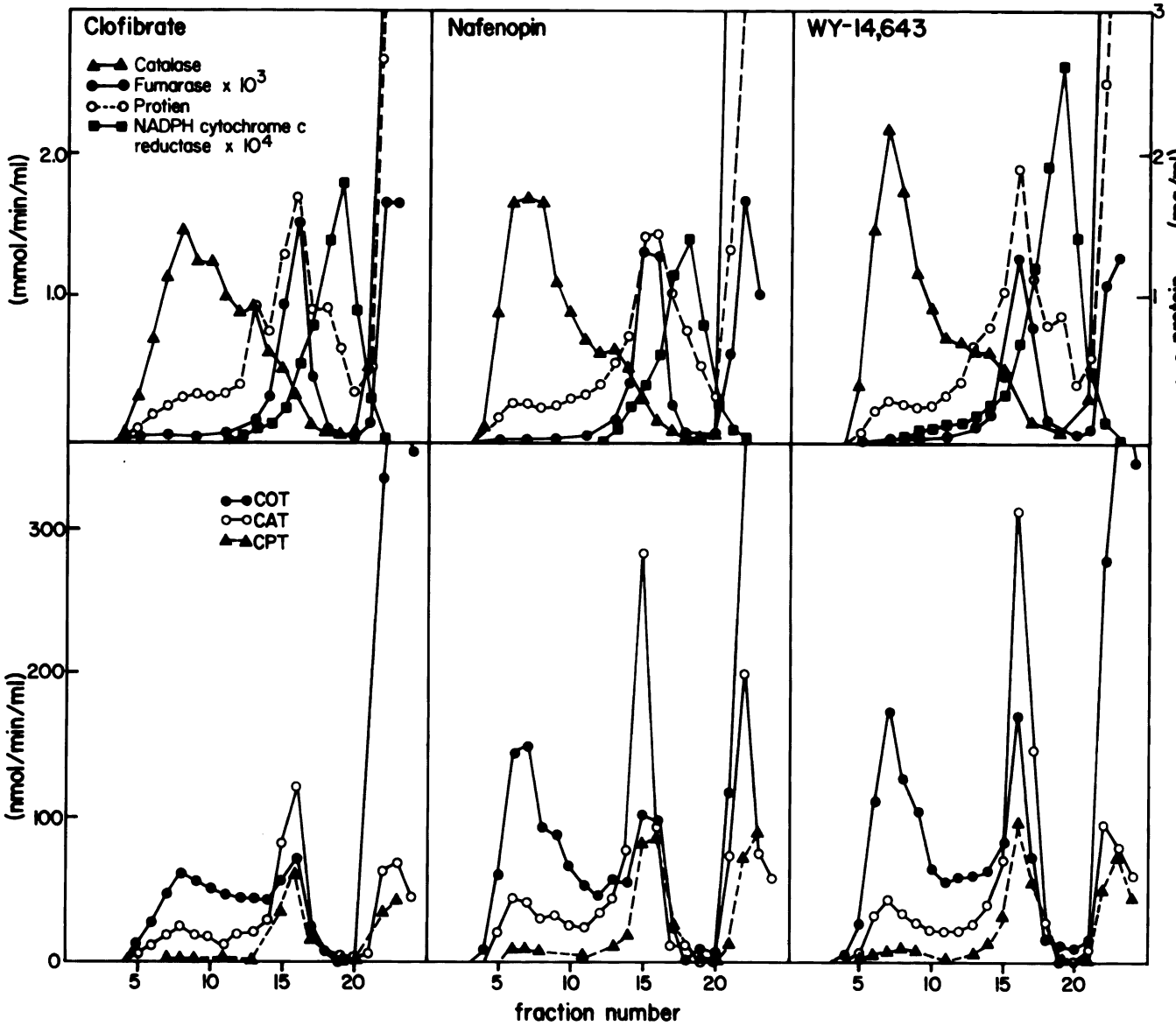
Effect of Hypolipidemic Drugs on the Subcellular Distribution of Carnitine Acyltransferases. In order to determine in which organelles the enzymes are affected by the hypolipidemic drugs, organelles from livers of mice treated with hypolipidemic drugs were separated on sucrose gradients. Figure 2 shows the distribution of carnitine acyltransferases from mice fed clofibrate, nafenopin, or Wy-14,643. COT and CAT increased in the peroxisomes and

Figure 2. Sucrose gradient separation of organelles from livers of mice treated with clofibrate,nafenopin, and Wy-14,643.

Top Panels: Activities of marker enzymes in mmol/min/ml and protein in mg/ml.

Bottom Panels: Activities of carnitine acyltransferases in nmol/min/ml.

Sucrose gradients were prepared and enzymes were assayed as described in Experimental Procedures.


Clofibrate
Nafenopin
WY-14,643
Catalase
Fumarase x 10^3
Protien
NADPH cytochrome c reductase x 10^4
(mmol/min/ml)
protein (mg/ml)
COT
CAT
CPT
(nmol/min/ml)
fraction number

mitochondria with all 3 hypolipidemic drugs. The largest increase, however, was mitochondrial CAT. For all of the drug treatments the peroxisomal breakage was greater, and this was reflected by the catalase and COT activities found in the soluble fractions. Table II gives the specific activities of the carnitine acyltransferases in the peak peroxisomal and mitochondrial fractions. Clofibrate, nafenopin, and Wy-14,643 increased peroxisomal COT 1.1-, 2.9-, and 3.5-fold and mitochondrial COT 4.7-, 8.0-, and 11-fold, respectively. Peroxisomal CAT increased 6-, 12-, and 11-fold, while mitochondrial CAT increased 19-, 54-, and 44-fold, respectively.

Since carnitine octanoyltransferase activity was found in higher levels in mouse liver than could be accounted for by the combination of CAT and CPT, and since hypolipidemic drugs were shown to raise the levels of the 3 carnitine acyltransferases to different extents in different organelles; it was decided that COT must be a separate enzyme, and its purification was undertaken.

Purification of Carnitine Acyltransferases of Mouse Liver Peroxisomes

Solubilization and Purification of Peroxisomal Carnitine Octanoyltransferase. Mice were fed a diet of 0.1% Wy-14,643 for 2 weeks to increase the absolute level of liver

TABLE II

Specific Activities of Carnitine Acyltransferases in Peak Sucrose Gradient Fractions

	Activity(nmol/min/mg protein)							
	Control		Clofibrate		Nafenopin		Wy-14,643	
	P	M	P	M	P	M	P	M
COT	170	8.5	186	40	488	68	600	92
CAT	13.5	3.7	80	72	160	200	148	164
CPT	4.7	7.6	8	40	36	60	32	50

Specific activities were determined for the carnitine acyltransferases in the peak peroxisomal (P) and mitochondrial (M) fractions of the sucrose gradients shown in figures 1 and 2, as determined by catalase and fumarase, respectively. Carnitine acyltransferases were assayed as described in Experimental Procedures.

carnitine acyltransferases. Preliminary studies indicated that peroxisomal but not mitochondrial carnitine acyltransferases are solubilized by the combination of homogenization and freezing in 8.5% sucrose, 10 mM sodium pyrophosphate. Also, previous investigations had indicated that rat liver microsomal COT and CAT were tightly bound and could not be easily released (37,38). Freeze-thawing in the sucrose, sodium pyrophosphate buffer was therefore employed to solubilize peroxisomal COT and CAT, which were then separated from mitochondria by centrifugation at 15,000g (Table III). Eighty percent of the COT was recovered after this step, but only 30% of the CAT and CPT was recovered.

Preliminary results showed that most of the COT in 10,000g supernatant fluids from mouse liver homogenates could be precipitated by ammonium sulfate at concentrations of 40-60% (Table IV). This fractionation range was used to precipitate COT from the 15,000g supernatant fluid above.

Cibacron Blue Sepharose was then used to separate the solubilized COT and CAT. As can be seen in Figure 3, one peak (CAT) contained carnitine acyltransferase with high activity for octanoyl-CoA but a higher activity for acetyl-CoA, while the second peak (COT) contained carnitine acyltransferase with a high activity for octanoyl-CoA and a much lower activity for acetyl-CoA.

This second peak was then purified to homogeneity with the series of chromatographic steps outlined in Table III.

TABLE III

Summary of the Purification of Carnitine Octanoyltransferase from Mouse Liver Peroxisomes

	COT							
	Recovery		Specific Activity		CAT Recovery		CPT Recovery	
Purification step	Units	%	Units/mg	-fold	Units	%	Units	%
Crude Homogenate	382,200	100	136	1	174,000	100	56,800	100
250g Supernatant	368,640	96	154	1.1	115,000	66	43,200	76
15,000g Supernatant	305,226	80	164	1.2	54,684	31	17,112	30
40-60% $(NH_4)_2SO_4$	262,430	69	395	2.9	51,450	30	21,420	38
Cibacron Blue CL-6B	150,716	40	5,690	42	8,364	5	15,622	28
QAE-Sephadex I	180,810	47	11,749	86				
Hydroxylapatite	144,630	38	17,069	126				
Sephadex G-100	122,304	32	39,533	291				
QAE-Sephadex II	74,330	20	72,059	530	1,487	0.9	7,500	13

Carnitine Octanoyltransferase was purified as described in Experimental Procedures. One unit of activity is the amount of enzyme necessary to convert 1 nmol acyl-CoA to acylcarnitine in 1 minute.

TABLE IV

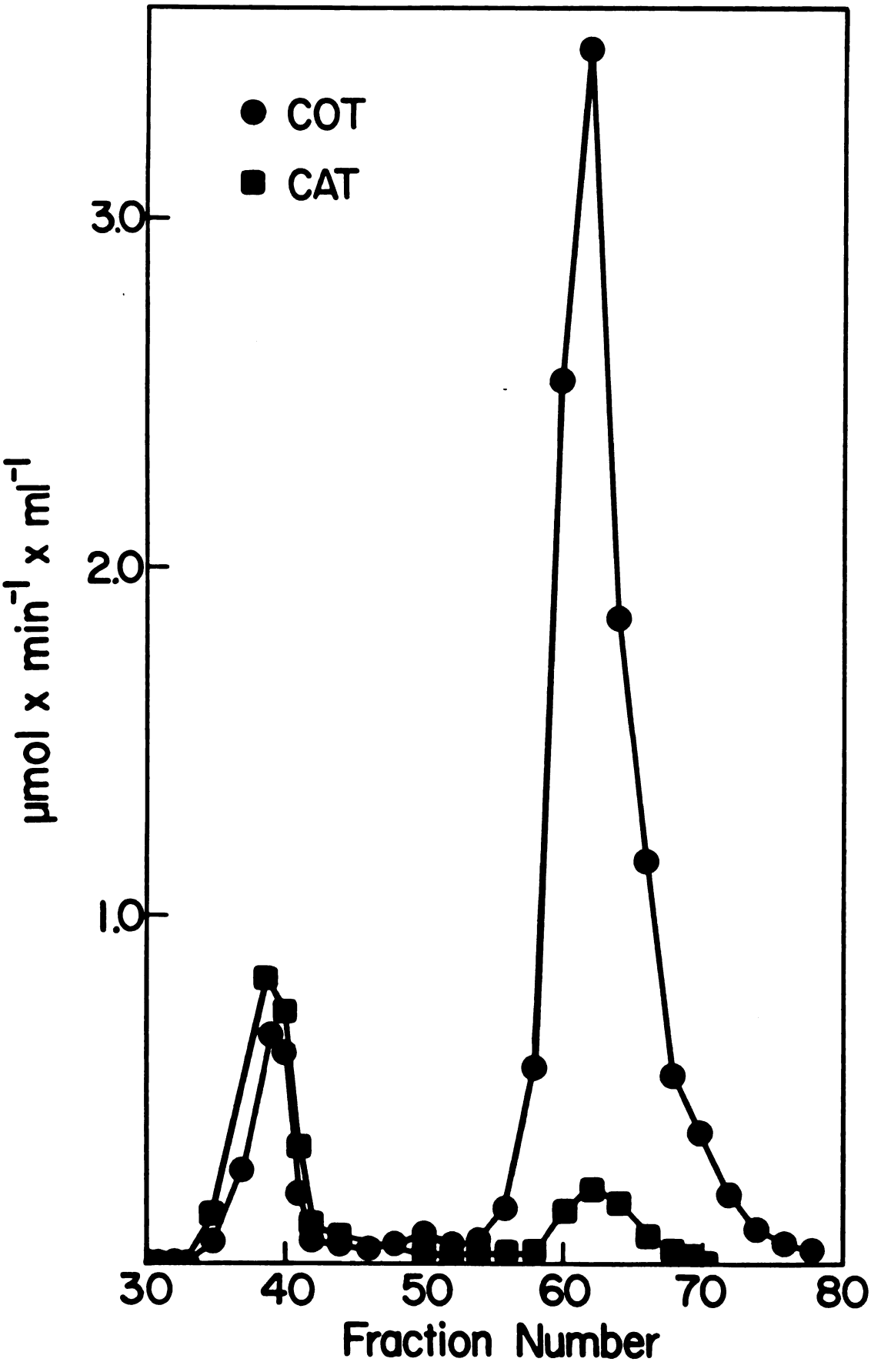
Ammonium Sulfate Precipitation of COT
from 10,000g Supernatant Fluids

Fraction	COT activity (nmol/min/mg protein)
10,000g supernatant	29.7
0-20% Amm. Sulfate	7.0
20-30% "	6.5
30-40% "	5.3
40-50% "	84.3
50-60% "	25.6
60-70% "	5.2

Mouse liver 10,000g supernatant fluids were treated with ammonium sulfate to give varying final concentrations and the COT activity that precipitated measured as described in Experimental Procedures.

Figure 3. Separation of solubilized COT and CAT on Cibacron Blue Sepharose CL-6B.

Activities are in μmol/min/ml. Samples were applied and eluted as described in Experimental Procedures.


COT
CAT
3.0
2.0
1.0
μmol x min⁻¹ x ml⁻¹
30
40
50
60
70
80
Fraction Number

Final recovery of 20% was attained with a 530-fold purification.

Purification of Peroxisomal Carnitine Acetyltransferase. The first peak of carnitine acyltransferase activity from the Cibacron Blue Sepharose column (see Figure 3) was used for further purification of CAT, employing chromatography on QAE-Sephadex, CM-Sephadex, and Sepahdex G-100.

Both COT and CAT were purifed to apparent homogeneity as determined by SDS-polyacrylamide gel electrophoresis, as shown in Figure 4.

Characterization of Carnitine Octanoyltransferase and Carnitine Acetyltransferase

Determination of Molecular Weight. While purifying COT and CAT, Sephadex G-100 was also used to estimate the native molecular weights. Both COT and CAT were determined to have molecular weights of 60,000 by calculating the K_{av}s on Sephadex and comparing them to published values (74). On 10% SDS-gels, both COT and CAT migrated to a distance corresponding to a M_r of 60,000, as can be seen in Figure 4.

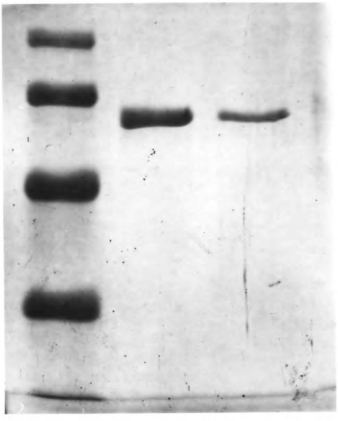
Stability of COT and CAT. Purified COT is stable for months in 10 mM sodium pyrophosphate buffer at pH 7.5 when kept at room temperature or 4° C (Figure 5). Freezing at -20° C or

Figure 4. SDS-polyacrylamide gel electrophoresis of purified carnitine octanoyltransferase and carnitine acetyltransferase.

Top Panel:(Left lane, from top to bottom): 10 µg each of phosphorylase b, BSA, ovalbumin, and trypsinogen. (center lane): 10 µg pure COT. (right lane): 3 µg pure CAT.

Bottom Panel: Relative migration, R_m, is shown versus the log of the molecular weight, MW.

Mouse liver COT and CAT were purified and electrophoresed as described in Experimental Procedures.



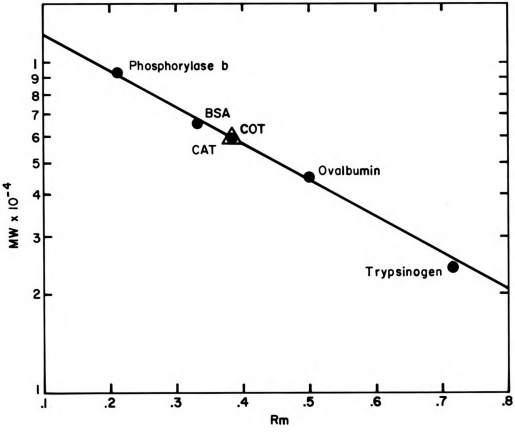
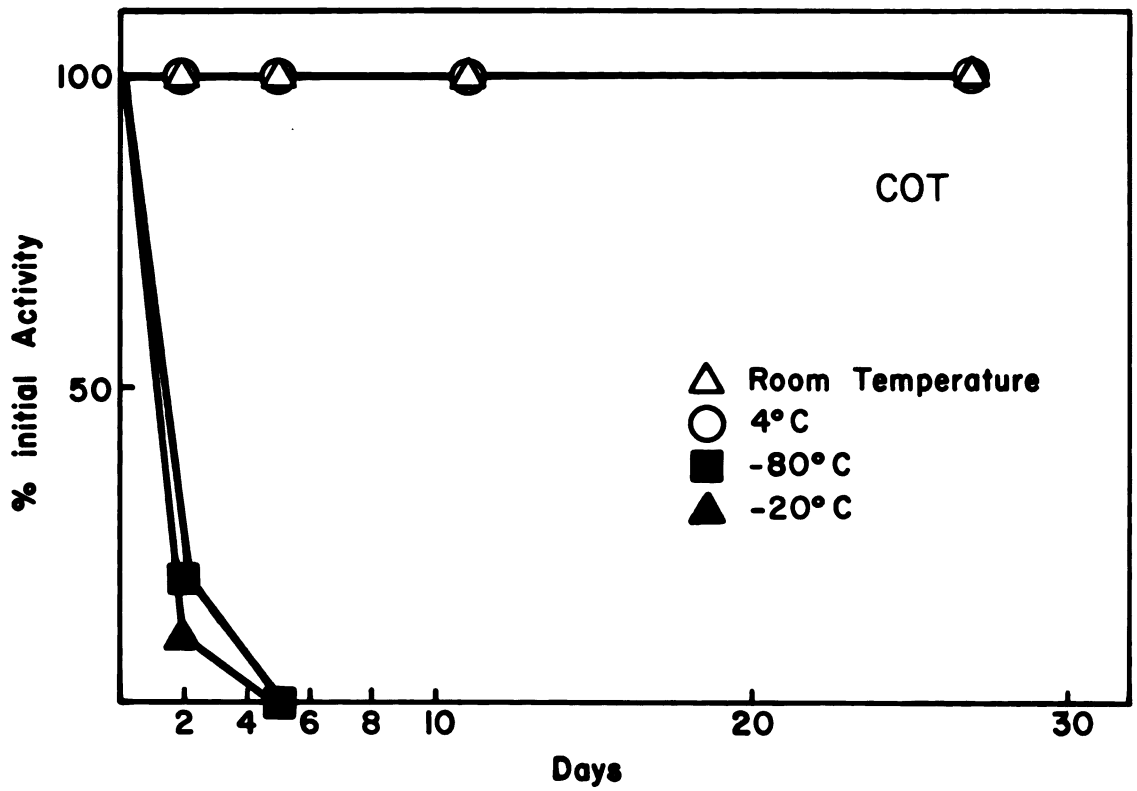

Phosphorylase b
BSA
COT
CAT
Ovalbumin
Trypsinogen
MW x 10⁻⁴
Rm
1
9
8
7
6
5
4
3
2
1
.1
.2
.3
.4
.5
.6
.7
.8

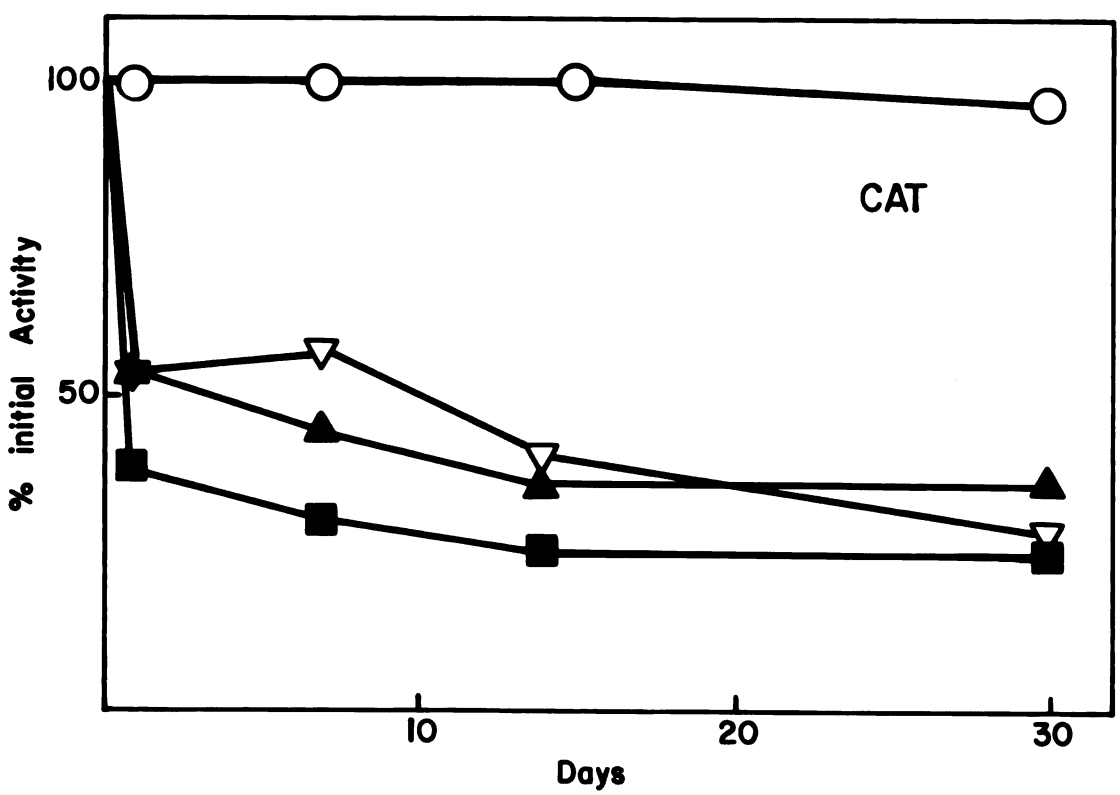
Figure 5. Temperature stability of carnitine octanoyltransferase and carnitine acetyltransferase.

Top Panel: COT

Bottom Panel: CAT

Purified COT and CAT were stored in 20 mM sodium pyrophosphate, pH 7.5 under the conditions indicated.


100
50
% initial Activity
COT
Room Temperature
4°C
-80°C
-20°C
2
4
6
8
10
20
30
Days


100
50
% initial Activity
CAT
10
20
30
Days

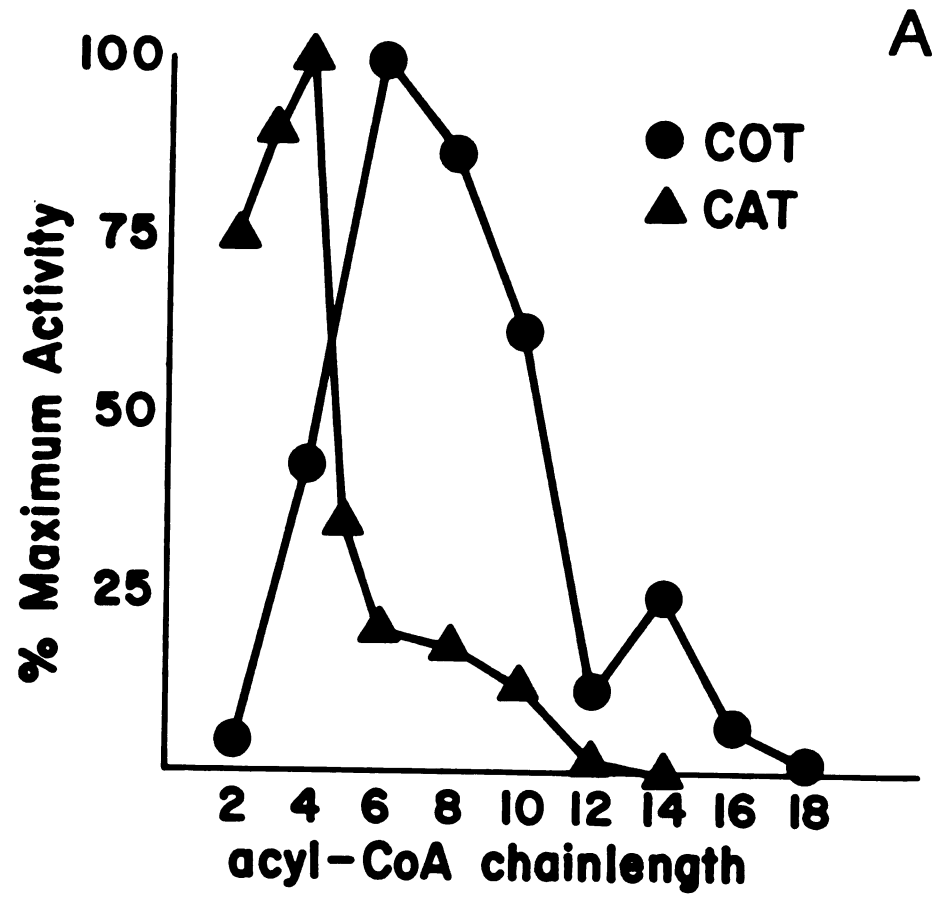
-80° C causes a 90% loss of activity within 2 days, however. CAT is also stable at 4° C, but is less so at room temperature, -20° C, or -80° C. Freezing does not cause as rapid a loss of activity as it does with COT. Neither COT nor CAT require detergents for stability and activity.

Specificity of COT and CAT for Various Substrates. As carnitine acyltransferases are multisubstrate enzymes, a fact which has caused confusion concerning their number and nature, the substrate specificity was determined for the purified COT and CAT. Figure 6A shows the substrate specificity of COT and CAT using acyl-CoAs of different chain-lengths when L-carnitine and acyl-CoA are present at 1.25 mM and 100 μM, respectively. COT had a maximum activity with hexanoyl-CoA, while the maximum for CAT was with butyryl-CoA. Figure 6B shows the substrate specificity for acylcarnitines of different chain-lengths. For COT, maximum activity was obtained with hexanoylcarnitine while CAT had a maximum activity with propionylcarnitine. Table V shows the relationship between acyl-CoA chain-length and the K_ms for acyl-CoAs and L-carnitine for COT and CAT. The K_ms for acyl-CoAs were nearly constant for CAT ranging from 15.3 μM for acetyl-CoA to 28.7 μM for decanoyl-CoA. The corresponding K_ms for L-carnitine increased from 86 μM with acetyl-CoA as cosubstrate to 519 μM with decanoyl-CoA as cosubstrate. With COT the K_ms for acyl-CoA were not

Figure 6. Specificity of COT and CAT for acyl-CoAs and acylcarnitines of varying chain-lengths.

(A) Forward reaction. L-carnitine was 1.25 mM and acyl-CoAs were 100 μM.

(B) Reverse reaction. Acylcarnitines were 500 μM and CoASH was 120 μM.


A
100
75
50
25
% Maximum Activity
COT
CAT
2 4 6 8 10 12 14 16 18
acyl-CoA chainlength

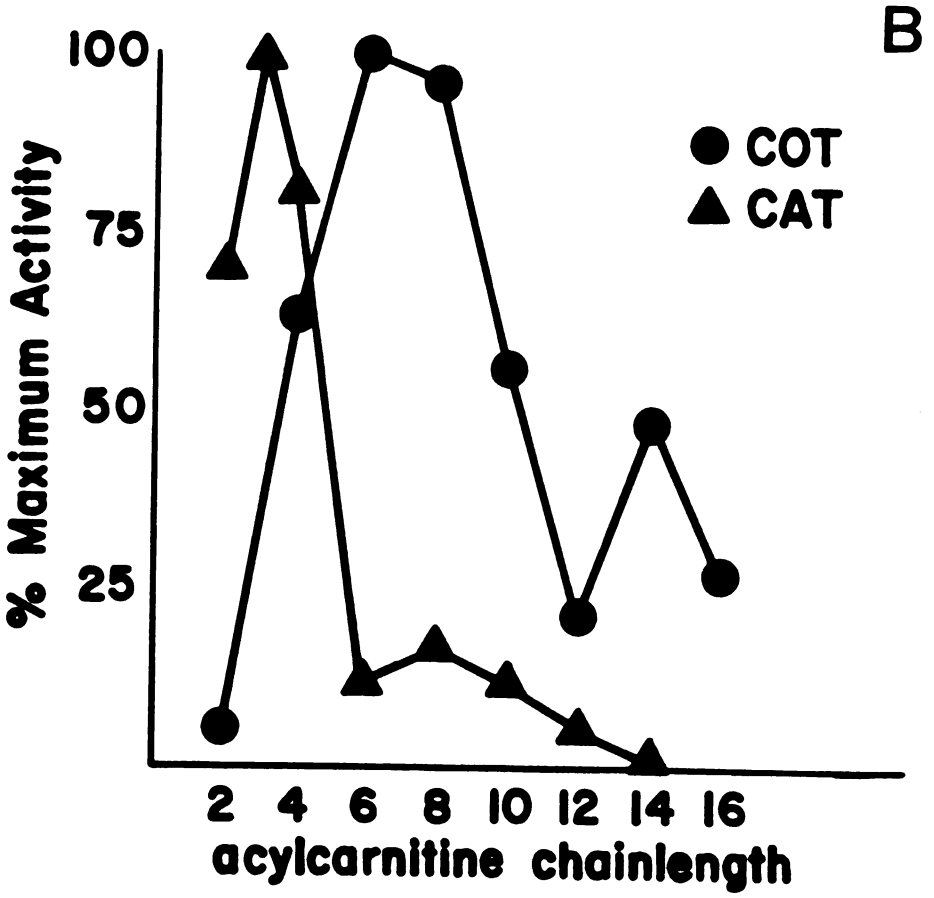

B
100
75
50
25
% Maximum Activity
COT
CAT
2 4 6 8 10 12 14 16
acylcarnitine chainlength

TABLE V

Apparent K_m Values of Substrates for COT and CAT

	COT		CAT	
Substrate	acyl-CoA (μM)	L-carnitine (μM)	acyl-CoA (μM)	L-carnitine (μM)
C_2-CoA	78	83	15.3	86
C_4-CoA	3.2	35	16.6	121
C_6-CoA	2.5	65	20.0	344
C_8-CoA	3.2	97	19.1	395
C_{10}-CoA	2.2	55	28.7	519
C_{12}-CoA	3.7	9	-	-
C_{14}-CoA	10.6	17	-	-
C_{16}-CoA	21	20	-	-
C_{18}-CoA	64	3	-	-

200 μM acyl-CoA was used when determining K_ms for L-carnitine and 1.2 mM L-carnitine was used when determining K_ms of acyl-CoA. Continuous substrate addition assays were performed as described in Experimental Procedures.

constant. They were small for acyl-CoAs with chain-lengths from C_4-C_{12}, but were larger for C_2-, C_{16}-, and C_{18}-CoA. The K_ms for L-carnitine varied with the acyl-CoA cosubstrate used and were smaller for long-chain acyl-CoA than for medium- and short-chain acyl-CoA.

Table VI shows the K_m values for the three traditional substrates of the reverse reaction. All of the K_ms were very high. With COT they were 783 μM, 100 μM, 104 μM and 110 μM for acetylcarnitine, octanoylcarnitine, palmitoylcarnitine, and CoASH, respectively. For CAT, the K_ms were 700 μM, 1250 μM, and 180 μM for acetylcarnitine, octanoylcarnitine, and CoASH, respectively. Palmitoylcarnitine was not a substrate. Hill Coefficients for each acyl-CoA were between 1 and 1.2 for both COT and CAT (data not shown). This is in contrast to the Hill Coefficients of 1.8 to 2.0 for acyl-CoAs with purified beef heart mitochondrial CPT (79).

Isoelectric Focusing of COT and CAT. The pIs of the two enzymes were determined by isoelectric focusing. Figure 7 shows the result of focusing COT and protein markers on ultrathin gels. The pI was determined to be 5.2 by comparing the migration of COT to the migration of the standards (bottom panel). Figure 8 shows the results of a similar experiment with CAT, where the pI was found to be 6.8.

TABLE VI

Apparent K_ms for acylcarnitine substrates of COT and CAT

Substrate	K_m (μM) COT	CAT
Acetylcarnitine	783	700
Octanoylcarnitine	100	1250
Palmitoylcarnitine	104	-
CoASH	110	180

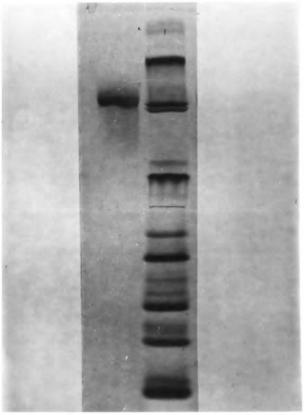
For acylcarnitine K_m determinations, 400 μM CoASH was used as cosubstrate. For the CoASH K_m determinations, 2 mM acetylcarnitine or octanoylcarnitine was used. The 232 reverse assay was performed as described in Experimental Procedures.

Figure 7. Isoelectric focusing of carnitine octanoyl-transferase.

Top Panel:(left lane): pure COT (right lane): Serva Test Mixture #9 (from top) ferritin, BSA, ß-lactoglobulin, conalbumin, horse myoglobin, whale myoglobin, ribonuclease, cytochrome c.

Bottom Panel: Determination of COT pI from isoelectric focusing. Migration is shown versus pI for COT and the Serva Test Mixture #9 markers.

Samples were focused on ultrathin Serva Precoats pH 3-10 as described in Experimental Procedures.



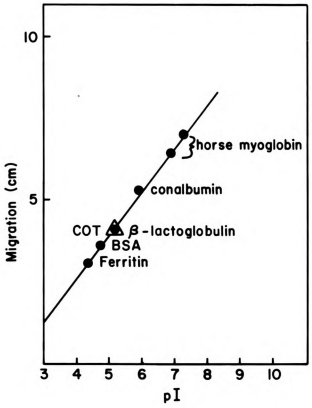
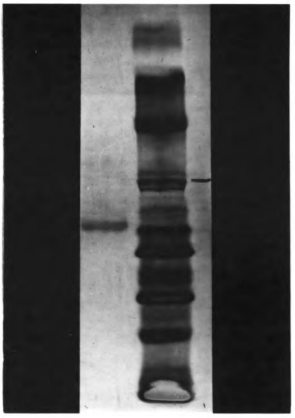
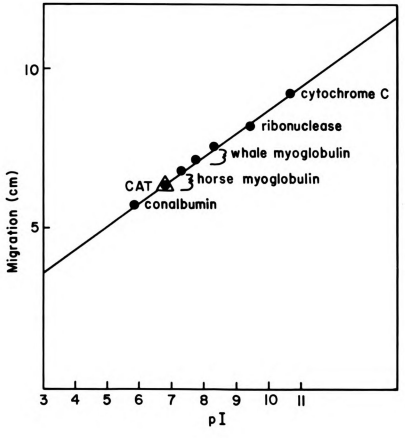

10
5
Migration (cm)
horse myoglobin
conalbumin
COT
β-lactoglobulin
BSA
Ferritin
3
4
5
6
7
8
9
10
pI

Figure 8. Isoelectric focusing of carnitine acetyl-transferase.

Top Panel: (left lane); pure CAT (right lane): Serva Test Mixture #9 (from top) ferritin, BSA, ß-lactoglobulin, conalbumin, horse myoglobin, whale myoglobin, ribonuclease, cytochrome c.

Bottom Panel: Determination of CAT pI from isoelectric focusing. Migration is shown versus pI for CAT and the Serva Test Mixture #9 markers.




10
5
Migration (cm)
cytochrome C
ribonuclease
whale myoglobulin
horse myoglobulin
CAT
conalbumin
3
4
5
6
7
8
9
10
11
pI

PH Optima for COT and CAT. Both COT and CAT were found to have similar pH optima, as shown in Figure 9. Using the reverse reaction with octanoylcarnitine or acetylcarnitine as substrate, the pH optima were found to be 8.0 and 8.5 for COT and CAT, respectively.

Effect of DTNB, Malonyl-CoA, and Divalent Cations. Some investigations have indicated that carnitine acyltransferases may be inactivated by DTNB (80,81). Since this agent is present in our forward assay mixture, its effect was investigated. Figure 10 shows the effect of preincubation with DTNB on COT and CAT. At either pH 7.0 or 8.5, COT was not inactivated. CAT was inactivated at both pH values with greater inactivation at pH 7.0. CAT was completely inactivated by preincubation in 0.95 mM DTNB at pH 7.0. This inactivation was not seen when the substrates were present and there was no difference between CAT activity measured with DTNB and that measured by the direct monitoring of acyl-CoA disappearance at 232 nm in the absence of DTNB (data not shown).

Since recent studies have also indicated that malonyl-CoA and divalent cations affect COT activity in rat liver preparations (80,82-85), we decided to test their effect on purified COT and CAT. Neither COT nor CAT were inhibited by malonyl-CoA at concentrations of 20 μM or 100 μM, and 100 uM malonyl-CoA was not a substrate for CAT or COT,

Figure 9. PH optima for carnitine octanoyltransferase and carnitine acetyltransferase.

500 μM acetylcarnitine or octanoylcarnitine were used to assay CAT and COT in the reverse direction as described in Experimental Procedures, with the exception that the pH of the assay buffer was varied.

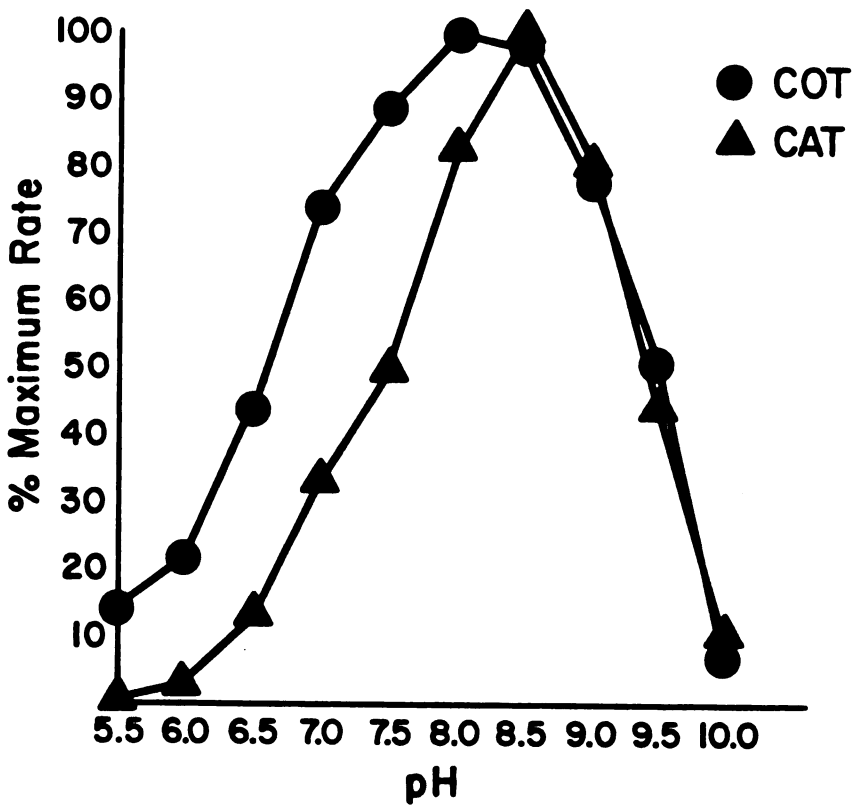
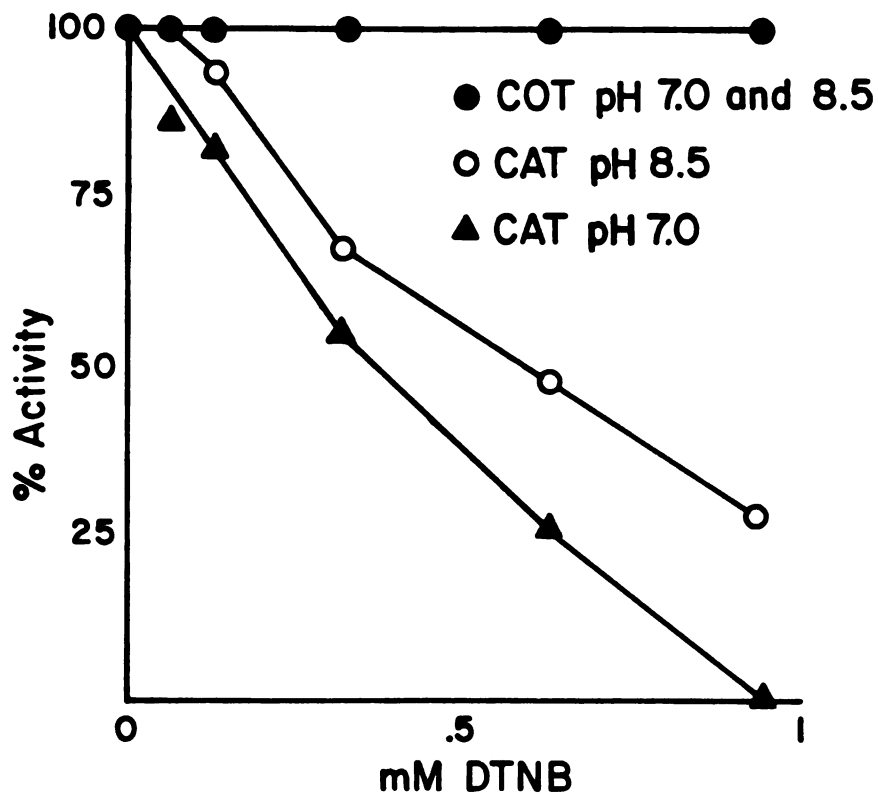

100
90
80
70
60
50
40
30
20
10
% Maximum Rate
COT
CAT
5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0
pH

Figure 10. Effect of DTNB on COT and CAT.

COT and CAT were preincubated for 15 minutes with varying amounts of DTNB at pH 7.0 or 8.5, as described in Experimental Procedures, and then assayed by addition of the substrates (69).


100
75
50
25
0
.5
1
% Activity
mM DTNB
● COT pH 7.0 and 8.5
○ CAT pH 8.5
▲ CAT pH 7.0

indicating that it had not been broken down to acetyl-CoA.

Ten millimolar Ca^{2+},Mg^{2+}, or Mn^{2+} did not inhibit either enzyme. Table VII shows the effect of Zn^{2+} on both enzymes. Only high concentrations of Zn^{2+} were inhibitory, but inhibition was stronger with the reverse assay.

Trypsin Inactivation of COT. Previous investigations in our lab have shown that trypsin affects rat liver microsomal COT and CAT activity. Therefore, the effect of this agent on the mouse liver enzymes was investigated. Figure 11 shows that CAT was unaffected by incubation with as much as 2 mg trypsin/μg CAT. COT, however, was activated at low levels of trypsin (3-5 μg trypsin/μg COT) but was inactivated at higher levels.

Inhibition by D-carnitine. Figure 12 shows the inhibition of COT and CAT by D-carnitine. For both enzymes, D-carnitine altered the slope of the Lineweaver-Burk plot, but not the y-intercept. Figure 13 shows the replots of the slope versus the concentration of D-carnitine, which give K_is of 0.84 mM and 1.0 mM for COT and CAT, respectively.

Amino Acid Analysis. Table VIII shows the partial amino acid analyses of COT and CAT. Although there was much similarity, there were also many differences. Over half of the amino acids analyzed were different by more than 0.5, with the

TABLE VII

Inhibition of COT and CAT by Zn^{2+}

	Forward Assay				Reverse Assay	
	DTNB		232		232	
μM $ZnCl_2$	COT	CAT	COT	CAT	COT	CAT
0	100	100	100	100	100	100
50	"	"	"	"	81	45
100	"	"	"	"	63	43
150	"	"	"	"	49	17
250	"	"	"	69	19	0
450	"	"	"	30	9	0
485	"	"	70	12	6	0

Carnitine acyltransferases were assayed in the forward direction following the release of CoASH with DTNB and by following the disappearance of acyl-CoA at 232. The reverse reaction was followed by monitoring the appearance of acyl-CoA at 232 nm, as described in Experimental Procedures.

Figure 11. Effect of trypsin on COT and CAT.

COT and CAT were incubated at 37° C for 15 min with varying amounts of trypsin and the reaction stopped with trypsin inhibitor. COT and CAT were then assayed in the forward direction as described in Experimental Procedures.

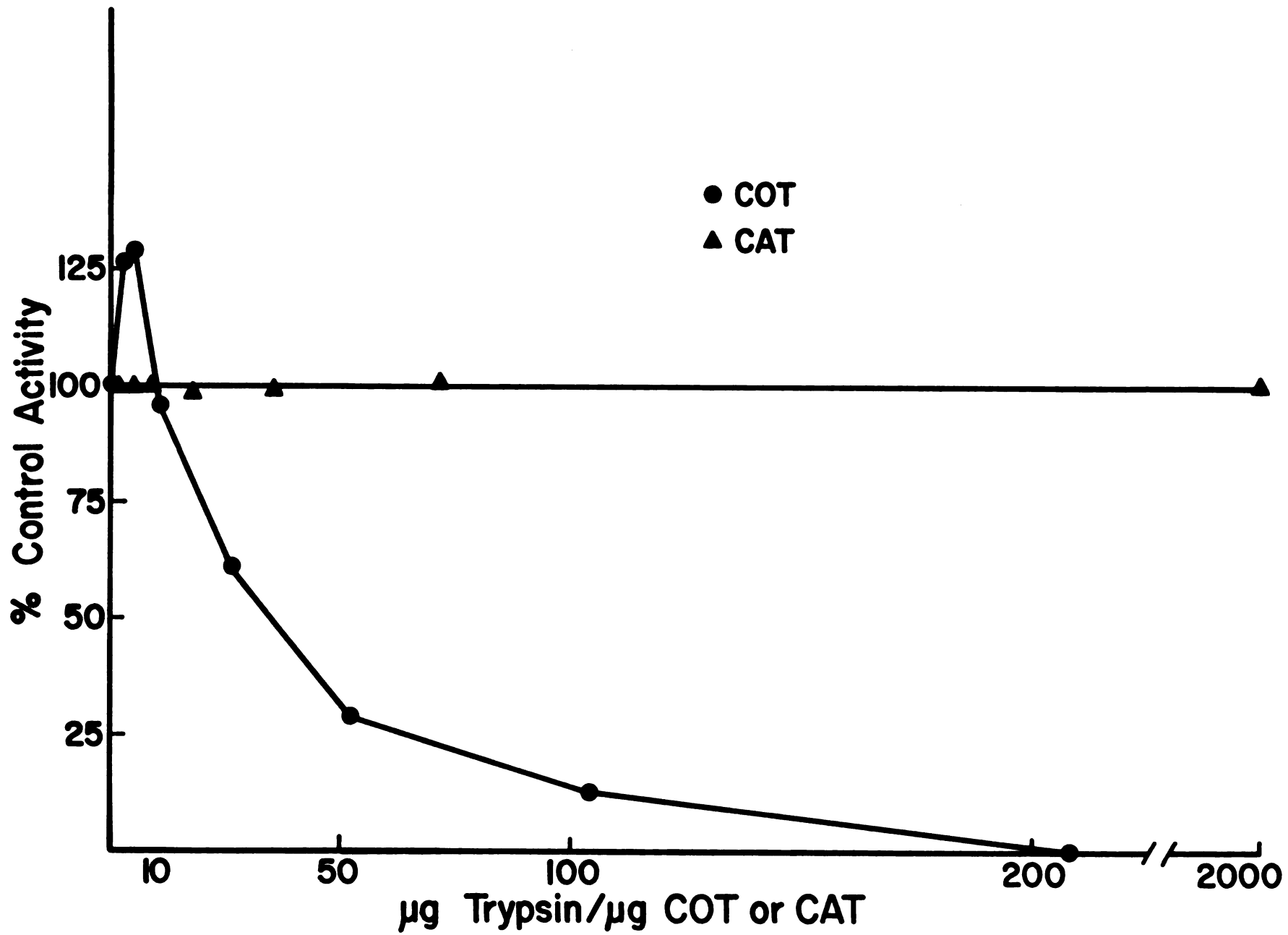

● COT
▲ CAT
% Control Activity
125
100
75
50
25
10
50
100
200
2000
µg Trypsin/µg COT or CAT

Figure 12. Effect of D-carnitine on COT and CAT.

(A) COT

(B) CAT

COT and CAT were assayed with varying L-carnitine concentrations in the presence of 0, 0.62 mM, 1.25 mM, or 2.5 mM D-carnitine with the forward assay described in Experimental Procedures.

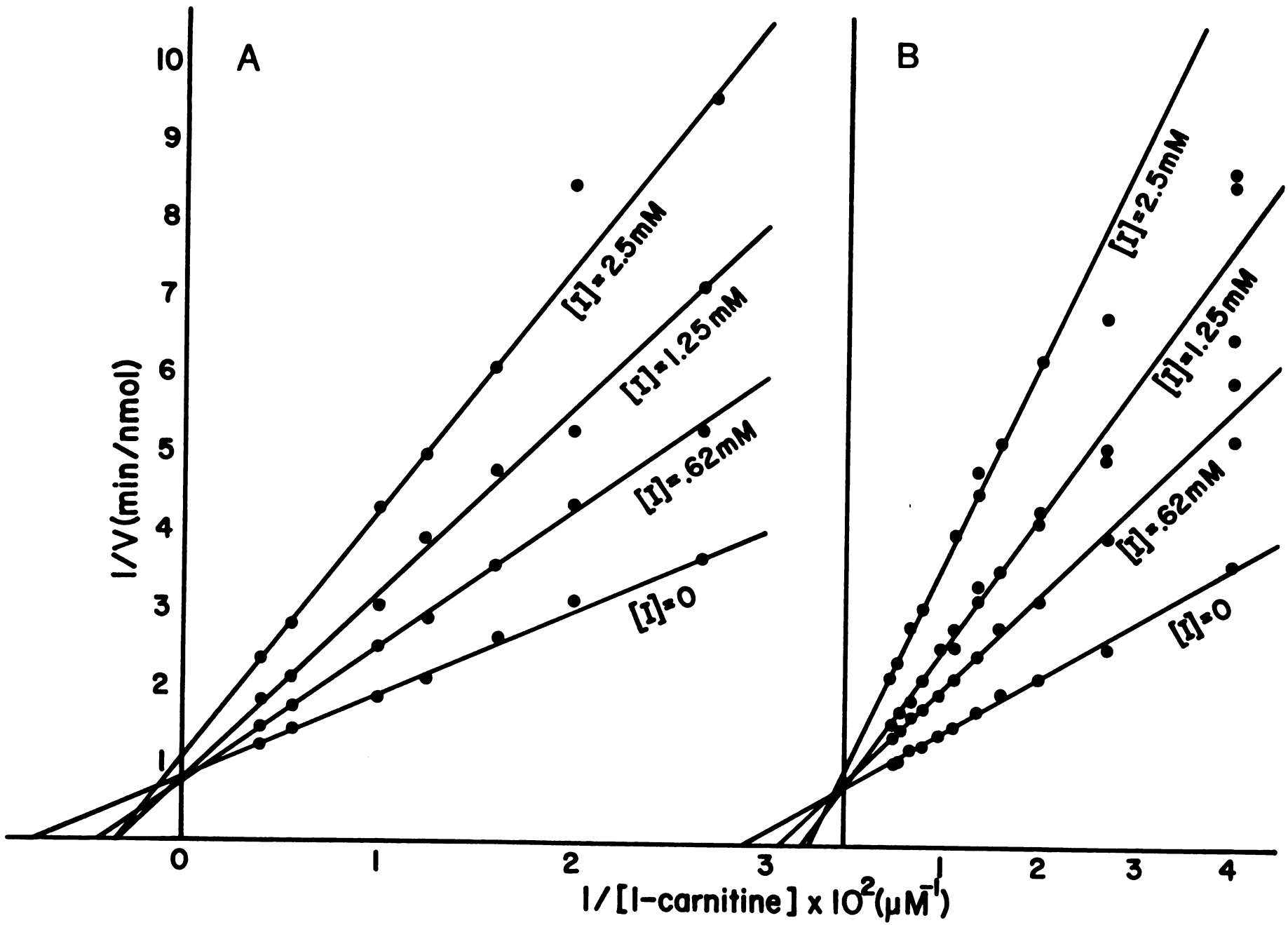
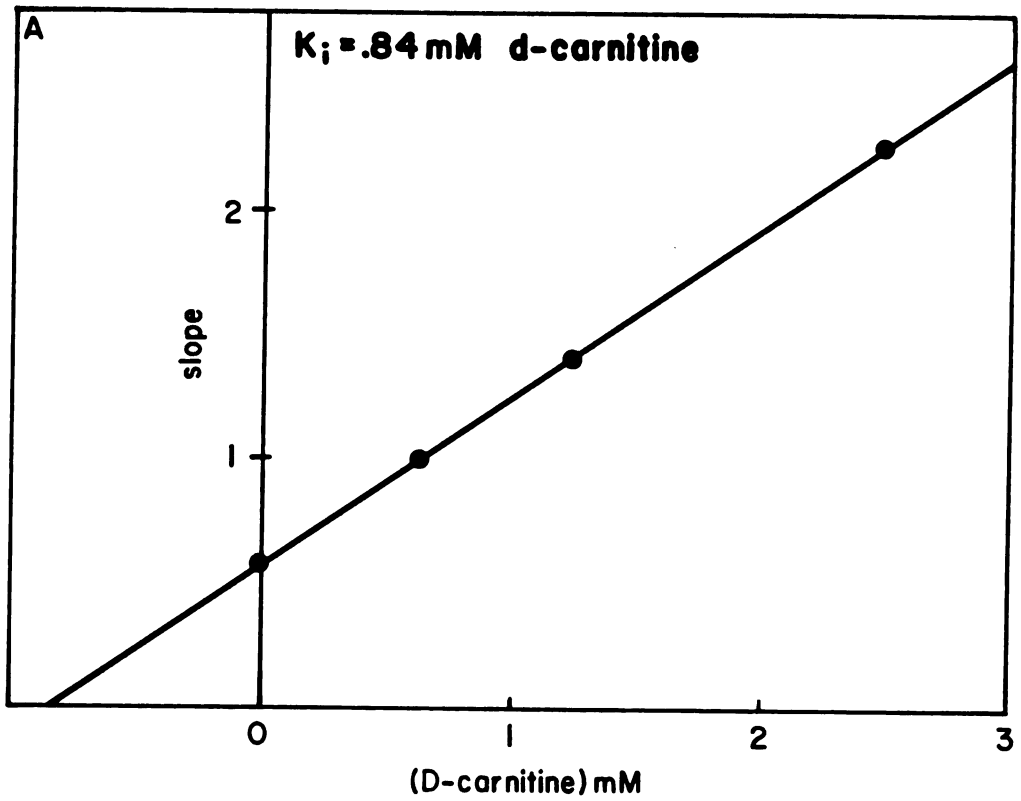

A
B
10
9
8
7
6
5
4
3
2
1
0
1/V(min/nmol)
[I]=2.5mM
[I]=1.25mM
[I]=.62mM
[I]=0
1
2
3
4
1/[I-carnitine] x 10²(μM⁻¹)

Figure 13. Replot of slope versus inhibitor concentration for D-carnitine inhibition of COT and CAT (see figure 12)

(A) COT

(B) CAT


A
K_i = .84 mM d-carnitine
slope
2
1
0
1
2
3
(D-carnitine) mM

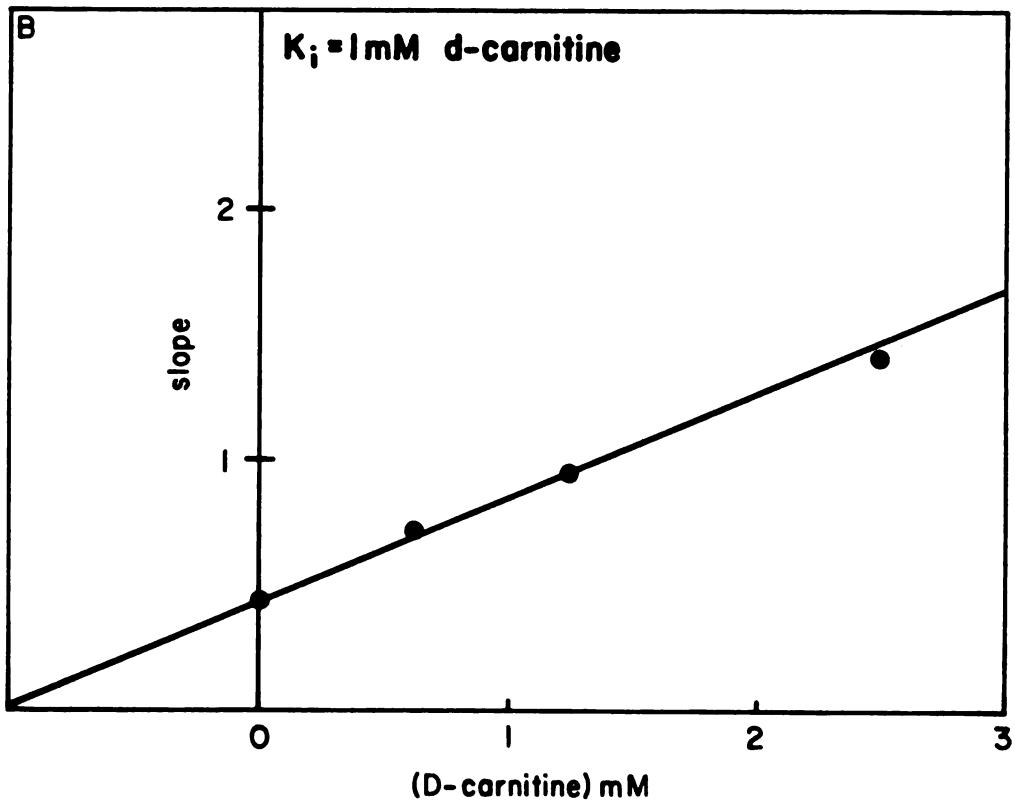

B
K_i = 1 mM d-carnitine
slope
2
1
0
1
2
3
(D-carnitine) mM

TABLE VIII

Amino Acid Compositions of COT and CAT

Amino Acid	COT	CAT
Asx	8.37	10.29
Thr	4.88	4.55
Ser	6.45	6.83
Glx	15.16	14.03
Pro	5.16	5.34
Gly	7.39	6.33
Ala	7.00	7.97
Val	4.37	3.98
Met	2.54	2.58
Ile	3.86	4.70
Leu	10.68	9.98
Tyr	3.50	2.67
Phe	4.87	4.10
His	3.79	3.98
Lys	6.28	7.87
Arg	5.66	4.80

Values for each amino acid are mole percent of detected amino acids. Cysteine and Tryptophan were not determined. Glx and Asx are the combined glutamine/glutamate and asparagine/aspartate values, respectively.

largest differences seen with lysine, glycine, glutamate and glutamine, and aspartate and asparagine; which all varied by more than 1 mole percent of the determined amino acids.

Immunology of Carnitine Octanoyltransferase

Antibodies were prepared against purified mouse liver COT, and their specificity for COT was investigated. Table IX shows the selective immunoprecipitation of COT from 10,000g supernatant fluids of mouse liver homogenates. The original supernatant was high in COT and CAT, but the antibody-antigen complex was low in CAT activity. Figure 14 shows a characteristic immunoprecipitation reaction between rabbit antiserum raised against pure COT and various acyltransferase preparations. Anti-COT serum reacted only with mouse liver COT. There was no reaction with purified CAT from mouse liver nor with mitochondria that had been sonicated, treated with Triton X-100, or both; and no reaction was obtained with untreated mitochondria from mouse liver. Triton X-100 (0.1%) did not interfere with immunoprecipitation of COT by anti-COT serum. No reaction was seen between anti-COT serum and beef heart mitochondrial CPT purified as in (23) (data not shown).

In order to determine if other mouse tissues have proteins immunologically similar to COT, the anti-COT antiserum was diffused towards homogenates of various mouse

TABLE IX

Immunoprecipitation of COT with Rabbit Anti-COT Serum

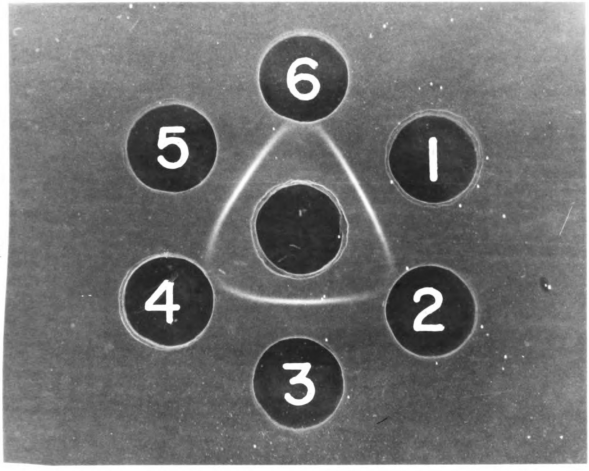
Fraction	CAT/COT ratio
10,000g supernatant	16.4
Immunoprecipitate	4.4

Rabbit anti-COT serum and 10,000g supernatant fluids were mixed to give maximum precipitation, incubated at 37° C. for 15 min., and stored overnight at 4° C. The precipitate was spun down, washed twice with agar buffer, and redissolved in 5mM sodium pyrophosphate, pH 7.5. Activity for octanoyl-CoA and acetyl-CoA were performed as described in Experimental Procedures.

Figure 14. Immunoprecipitation of purified COT with rabbit anti-COT serum.

Center well: 20 µl rabbit antiserum.
Wells 1,3,5: 10 µg pure mouse liver COT
Well 2: 10 µg mouse liver CAT.
Well 4: 20 µl solubilized mitochondria from mouse liver.

Tissues were taken from a mouse treated with Wy-14,643 and the agarose plates developed as described in Experimental Procedures. Well 6 was unused.


6
5
1
4
2
3

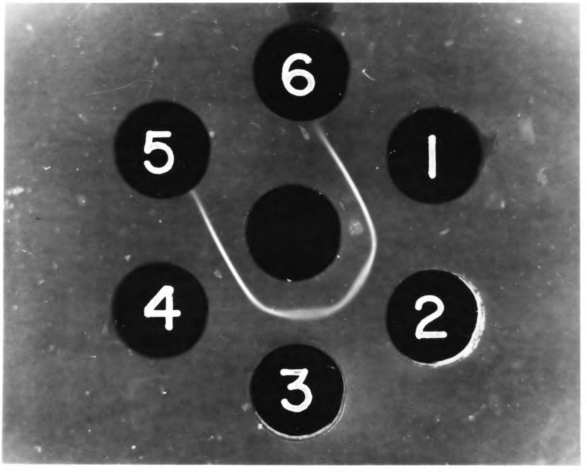
tissues. Figure 15 shows the reaction between anti-COT serum and 10,000g supernatant fluids from kidney, heart, intestine, and skeletal muscle. Kidney and intestine gave precipitin bands of identity with the purified COT. No reaction was detected with heart or muscle.

Livers of other species were then used to see if they contained proteins immunologically similar to mouse liver COT. Figure 16 shows a reaction of partial identity with mouse liver COT and 10,000g supernatant fluids from livers of rats that had been treated with Wy-14,643. The direction of the precipitin spurs indicates that the rat liver preparation is the weaker cross reactant, as expected. Figure 17A compares the reaction between anti-COT serum and mouse liver COT to that with 10,000g supernatant fluids from beef and dog liver. Again the non-mouse tissues gave reactions of partial identity with the pure mouse liver COT. Figure 17B shows the relationship between the preparations from non-mouse livers. The beef and dog liver give reactions of identity with each other, and both give reactions of partial identity with the rat liver supernatant. Although the rat liver titer was much lower than the other two, it also appears to be immunologically more similar to mouse liver COT because the preparations from dog liver and beef liver are weaker cross reactants than the rat liver preparation. For all of the tissues and species tested, mitochondrial pellets were collected.

Figure 15. Immunoprecipitation of purified COT and mouse tissues with rabbit anti-COT serum.

Center well: 20 µl anti-COT serum.
Wells 1,4: 10 µg pure mouse liver COT.
Wells 2,3,5,6: 10,000g supernatant fluids from mouse kidney, intestine, heart, and skeletal muscle, respectively.

Tissues were taken from a mouse treated with Wy-14,643 and the agarose plates developed as described in Experimental Procedures.


6
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1
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2
3

Figure 16. Immunoprecipitation of purified mouse liver COT and 10,000g supernatant fluids from rat Liver.

Center well: 20 µl anti-COT serum.
Wells 2,4,6: 10 µg mouse liver COT.
Well 1: 20 µl 10,000g supernatant fluid from rat liver.

Rat liver was taken from a rat treated with Wy-14,643 and the agarose plates developed as described in Experimental Procedures.

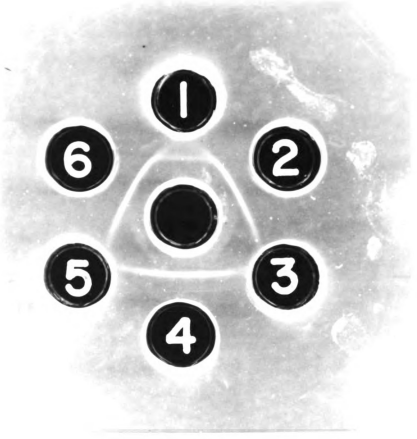
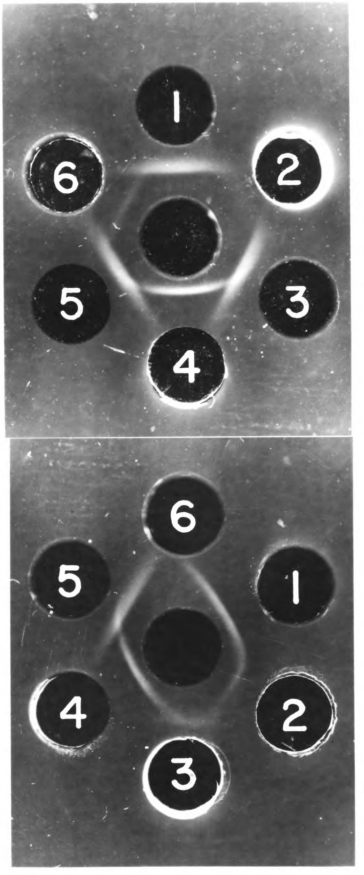

1
2
3
4
5
6

Figure 17. Immunoprecipitation of purified mouse liver COT and 10,000g supernatant fluids from rat, beef, and dog liver.

(A)
Center well: 20 µl anti-COT serum.
Wells 1,3,5: 10 µg mouse liver COT.
Wells 2,4,6: 20 µl 10,000g supernatant fluid from rat, beef, and dog liver, respectively.

(B)
Center well: 20 µl anti-COT serum.
Wells 1,4: 20 µl beef liver 10,000g supernatant fluid.
Well 2: 20 µl dog liver 10,000g supernatant fluid.
Well 3: 20 µl rat liver 10,000g supernatant fluid.
Well 5: 10 µg mouse liver COT.

Rat liver was taken from a rat treated with Wy-14,643 and the agarose plates developed as described in Experimental Procedures.


1
6
2
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3
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6
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1
4
2
3

Samples of each were solublized by sonication, addition of Triton X-100, or both. None of the whole mitochondria or solublized mitochondria reacted with anti-COT serum.

DISCUSSION

Localization and Purification of COT and CAT

Previous studies demonstrated that carnitine octanoyltransferase activity is widely distributed in rat tissues, with the exception of the brain (31), and other investigations with liver indicated a separate COT enzyme in peroxisomes and microsomes (20,37,38). Extensive investigation of beef heart mitochondria, however, attributed this activity to a combination of long-chain and short-chain carnitine acyltransferase instead of a true medium-chain enzyme (23,24).

This work shows that with density gradient separation of mouse liver organelles, most of the COT parallels the distribution of catalase, with 60% in the peroxisomes and 10% in the soluble fractions. The 20-fold greater specific activity of COT in peroxisomes compared to mitochondria in mouse liver is in contrast to the distribution in rat liver where the specific activities of COT and CAT are about equal in peroxisomes (52) and about 60% of the COT activity is associated with mitochondria with a specific activity about 2.5-fold greater than peroxisomal COT.

Clofibrate and nafenopin, two drugs which induce

peroxisome proliferation (86,87), increase ß-oxidation (44,46,88,89), and increase carnitine acyltransferases (52,57,58,89,90) increased the specific activity of COT in mouse liver 500g supernatant fluids 4- and 11-fold, respectively. When mice were fed clofibrate, nafenopin, or Wy-14,643, and the liver organelles separated on sucrose gradients, COT levels were increased in both the peroxisomes and mitochondria with all treatments. The relationship between the increase in carnitine acyltransferases and the lipid-lowering effects of the drugs is not known. Sucrose gradients from mice fed hypolipidemic drugs contained much more catalase, COT and CAT in the soluble fraction than the controls. If all of the soluble catalase came from peroxisomes as has been suggested (91), then 50-60% of the peroxisomes were broken from drug treated animals. This is in contrast to the controls where a maximum of 20% were broken. Thus, it appears that hypolipidemic drugs enhance peroxisomal membrane fragility.

This fragility aided in the separation of the peroxisomal enzymes COT and CAT from the mitochondrial enzymes. Homogenization and freezing released 80% of the COT present in the crude homogenate (Table III), whereas only 30% of the initial CPT and CAT remained in the supernatant fraction after the mitochondria were removed by centrifugation. This remaining activity with palmitoyl-CoA was due to the chain-length specificity of COT rather than

the CPT enzyme on the inner membrane of the mitochondria, as harsher methods are required to liberate and stabilize CPT (21,23,24,92-94). Mitochondria do not appear to rupture during the isolation procedure, as the mitochondrial matrix marker, glutamate dehydrogenase, is not detected in the supernatant after centrifugation at 15,000g. The CAT activity remaining is therefore also due to peroxisomal CAT, and the low percentage recovery after centrifugation is a reflection of the high levels of CAT found in the mitochondria of drug treated mice.

The solubilized peroxisomal COT and CAT were easily separated with the Cibacron Blue Sepharose column, and then both purified to apparent homogeneity by the column chromatographic steps described.

The final purified COT preparation contained 20% of the activity initially present in the crude homogenate. This represents a greater than 20% recovery of the peroxisomal enzyme because the mitochondria have COT activity. The final purification of 530-fold, although low for a liver enzyme, is reasonable considering the 10-fold increase in absolute COT levels in the livers of mice treated with Wy-14,643.

Physical Characterization of COT and CAT

Mouse liver peroxisomal COT and CAT both have molecular weights of 60,000. No aggregation is apparent as Sephadex G-100 and SDS-polyacrylamide gel electrophoresis give the same molecular weight. Recently, carnitine octanoyltransferase was purified from rat liver peroxisomes, and its molecular weight was found to be 66,000 (95). The properties of several carnitine acetyltransferases have been reported. The molecular weight of CAT is 58,000 from pigeon breast muscle (96), 67-69,000 from rat liver mitochondria (97), 62,600 from beef heart mitochondria (23), and 59,000 from rat liver peroxisomes and microsomes (37). Thus, most of the COT and CAT enzymes studied to date are of a similar size. An exception is alkane grown yeast, where both mitochondria and peroxisomes contain a CAT enzyme that has a molecular weight of 420,000 by gel filtration and ultracentrifugation (98). SDS-gel electrophoresis gives subunit weights of 64,000 and 57,000 for the peroxisomal CAT and 64,000 and 52,000 for the mitochondrial CAT, however (15,98).

Peroxisomal COT and CAT have pIs of 5.2 and 6.8, respectively. Other studies have reported pIs for carnitine acyltransferases near 8.3, such as CAT from beef heart mitochondria (23),and from rat liver microsomes and

peroxisomes (37). In (37) a second peak at 5.3 was observed for the microsomal enzyme, and with alkane grown yeast the pIs were 5.11 for peroxisomal CAT and 5.22 for mitochondrial CAT. Proteolytic degradation of carnitine acyltransferases from liver can occur during purification. Rat liver CAT was reported to consist of two non-identical subunits of 34,000 and 25,000 daltons (99). This observation was confirmed in (97), but further analysis showed that CAT is a single polypeptide that can be degraded during purification. When we used mouse liver homogenates stored frozen in the absence of protease inhibitors, COT had 3 major isoelectric peaks at pI 5.9,6.0, and 6.1 (data not shown). It also eluted from Sephadex G-100 as a wide peak spanning a range of 3000 daltons. When fresh liver preparations were used in the presence of 1 mM EDTA, 0.1 mM PMSF, and 5 mg/l pepstatin; COT eluted as a much narrower band from Sephadex G-100 and only 1 band appeared after isoelectric focusing. Therefore, some of the differences between molecular weights and pIs reported in different studies could be due in part to proteolytic degradations during purification, but they could also be due to organelle and species differences, and differences between membrane associated and matrix enzymes. Although apparent proteolytic degradation led to COT eluting as a wide band on Sephadex G-100, fractions taken from the extremes of the peak had the same substrate specificity for acyl-CoAs and similar K_ms for octanoyl-CoA and L-carnitine

(data not shown).

Using a semiautomated kinetic analyzer we determined the K_ms for the even chain-length acyl-CoA substrates and the corresponding K_ms for L-carnitine. For both COT and CAT the K_ms for L-carnitine varied with the acyl-CoA cosubstrate used. With CAT the K_m for L-carnitine increased with increasing acyl-CoA carbon chain-length. This is in contrast to the results with pigeon breast-muscle CAT (100) where no such pattern existed. With COT the K_ms for L-carnitine also varied with the acyl-CoA used but were lower for long-chain acyl-CoAs, as has been reported for rat liver COT (95). With COT, acyl-CoAs from C_4 to C_{12} had very low K_ms, but the K_ms for C_2-CoA, C_{16}-CoA, and C_{18}-CoA were large. This is different from the pattern reported for rat liver COT (95) where the K_ms decreased with increasing acyl-CoA carbon chain-length. Mouse liver CAT had maximum activities with butyryl-CoA and propionylcarnitine. All of the mammalian carnitine acetyltransferases characterized to date have specificities that are similar for the forward and reverse reactions. Maximum activities occur with C_3 or C_4 and the activity drops as the carbon chain-length increases until almost no activity exists at C_8 or C_{10} (23,37,95). Exceptions are with non-mammalian sources, such as alkane grown yeast (15) and T.bovina (17), where virtually no carnitine acyltransferase activity exists with acyl-CoAs of carbon chain-lengths greater than 3.

COT had a maximum activity with hexanoyl-CoA and hexanoylcarnitine similar to the rat liver enzyme (95). We found a biphasic substrate specificity curve, with a local minimum for C_{12} moieties. When this pattern occurred during the reverse reaction, it was noticed that the abnormality occurred at the substrate concentration where acylcarnitines should form micelles, as had been shown in (24). However this transition to micelles should not be present in the mixed micelle environment use for the forward assay containing 0.1% Triton X-100, yet the biphasic specificity pattern existed in the presence and absence of detergents. Also, when C_{12}-CoA was used with the semiautomated system and the TANKIN program the K_m could not be defined due to strong inhibition by C_{12} moieties. In fact, a substrate depletion method (73) was used to determine the K_m for C_{12}-CoA in order to avoid high concentrations of the substrate.

The K_ms for the acyl-CoA substrates of COT and CAT are all very low compared to the K_ms for the corresponding acylcarnitines, which are all over 100 μM. This indicates that acylcarnitine formation would be favored <u>in vivo</u>. The combination of the K_m profiles and the specificity for substrates of varying chain-length under saturating conditions indicates that in the forward direction both the K_m for acyl-CoA and the V_{max} affect the acyl-CoA specificity. With both enzymes there are large differences

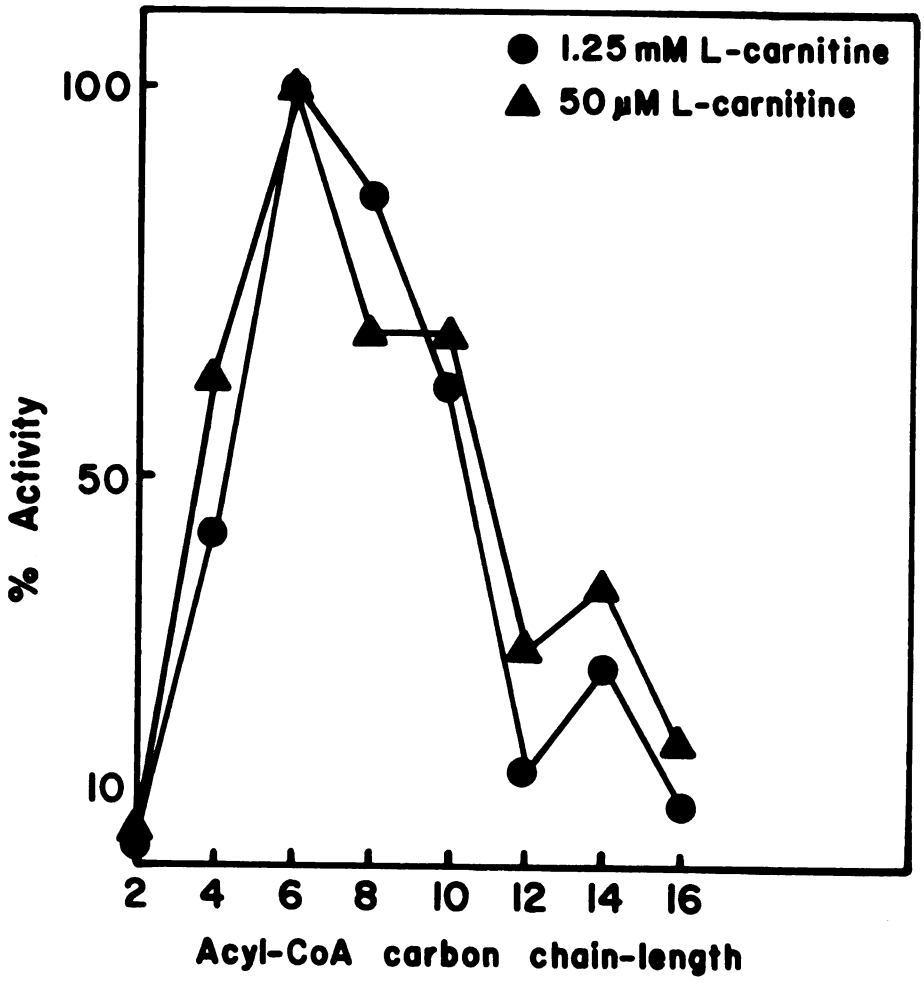
in velocities in the range where the K_m for acyl-CoA does not vary with the carbon chain-length. It is also apparent that the concentration of L-carnitine could be important for the in vivo substrate specificity. With CAT, for example, at concentrations of L-carnitine below 50 μM, acetyl-CoA would become a more favored substrate. This effect can be seen with COT. When subsaturating levels of L-carnitine are used, the acyl-CoA substrate specificity changes so that the relative specificity for long-chain acyl-CoAs increases (see Figure 18).

Inhibitors and Inactivators

It has been suggested that rat liver mitochondria contain a separate carnitine octanoyltransferase because of differences in enzymatic activities with C_8-CoA or C_{16}-CoA in the presence of malonyl-CoA, divalent cations, and DTNB (80,82-85). With purified peroxisomal COT from mouse liver, no effects of malonyl-CoA are seen. Malonyl-CoA was not an inhibitor at concentrations up to 100 μM, which agrees with data reported for the rat liver enzyme (95). This is in contrast to some reported effects of malonyl-CoA on "mitochondrial COT" activity (80,82,83,85) where malonyl-CoA inhibited COT activity and CPT activity to different extents. However, these studies used whole mitochondria that contain at least two carnitine acyltransferases with

Figure 18. **Acyl-CoA specificity for COT at saturating and unsaturating levels of L-carnitine.**

Carnitine octanoyltransferase was measured in the forward direction with 100 μM acyl-CoA and L-carnitine at 1.25mM or 50 μM.


1.25 mM L-carnitine
50 μM L-carnitine
100
50
10
% Activity
2
4
6
8
10
12
14
16
Acyl-CoA carbon chain-length

overlapping specificities for acyl-CoAs of different chain-lengths, along with the possibility of contamination by peroxisomal COT and CAT. It is therefore not suprising that such differences exist between activities with one acyl-CoA versus another, but little information can be attained regarding the actual enzymes involved.

Rat liver mitochondrial CAT shows latent and overt activity, and the overt activity can be inhibited by malonyl-CoA (101). We did not find any malonyl-CoA inhibition, nor inhibition by Ca^{2+} or Mg^{2+} for mouse liver CAT, but both COT and CAT were inhibited by Zn^{2+} at concentrations greater than 50 μM in the reverse direction. In (102) trypsin was shown to inactivate COT and CAT from rat liver microsomes, although the membrane-bound CAT was activated at low trypsin levels. We found that COT from mouse liver peroxisomes is activated by incubation with 3-5 ug trypsin/ug COT, but is inactivated at higher concentrations. CAT was not affected by trypsin under our conditions.

Some sulfhydryl reagents, including DTNB (81), iodoacetimide (99), and p-chloromercuribenzoic acid (15), can inactivate CAT. Mouse liver CAT is inactivated by preincubation with DTNB in the absence of the substrates, but COT was not affected. In the presence of the substrates, no inactivation occurred with either enzyme. Thus DTNB does not affect the initial rates under the

conditions of our standard forward assay.

D-carnitine is a competitive inhibitor of both COT and CAT with respect to L-carnitine. Our data agree with the original investigations of Fritz and coworkers (103), but are in contrast to the data in (104) where parallel Lineweaver-Burk plots were obtained. The K_is were high but might be physiologically important. D-carnitine is virtually absent in biological systems except where it has been introduced. Recently DL-carnitine has become available as a dietary supplement, raising questions about the metabolic consequences of oral ingestion of the D-isomer (105-110). These data show that D-carnitine is inhibitory to both short-chain and medium-chain carnitine acyltransferases, and thus might have adverse effects on systems which involve medium-chain acyl-CoAs and acylcarnitines.

Immunology and COT Localization

Antibodies raised against purified COT from mouse liver peroxisomes do not react with peroxisomal CAT nor with various mitochondrial fractions from mouse liver. In (111) we discussed the possibliity that mitochondria might have a separate COT enzyme. This work, as well as those of (95,97), make it doubtful that a separate COT exists in mouse or rat liver mitochondria. The effects of DTNB,

malonyl-CoA, and divalent cations reported in (80,82-85) are probably not due to a separate enzyme, rather the overlapping specificities of mitochondrial carnitine palmitoyltransferase and acetyltransferase, and possible contamination of the mitochondrial preparations with carnitine acyltransferases from non-mitochondrial sources.

All of the livers tested have proteins that share antigenic determinants with COT from mouse liver. Rat liver has a protein that is more similar to mouse liver COT than the protein from dog or beef liver. However, no mitochondrial fractions reacted with our anti-COT serum. If there is a mitochondrial COT, it would have to be antigenically very different from the COT from liver peroxisomes. It is interesting to note that beef liver has a high titer of a protein antigenically similar to mouse liver COT, but beef heart, which has been studied extensively in the attempt to locate COT (23,24), does not.

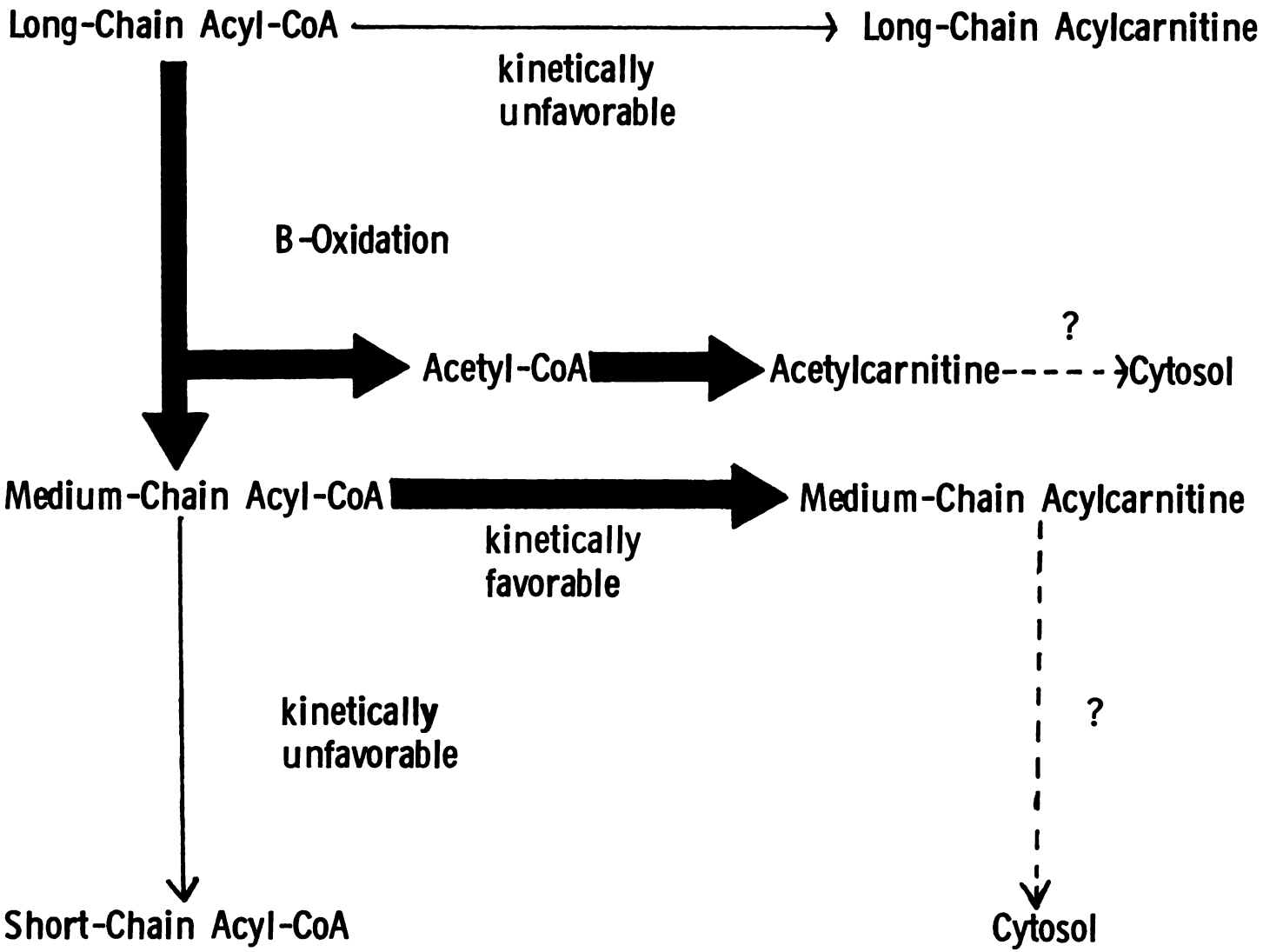
Peroxisomal ß-Oxidation

Peroxisomes have ß-oxidation capability (44-48). Recent studies indicate that the contribution of peroxisomes to the total fatty acid oxidation in mouse liver could be as much as 50% (47-49). If large quantities of fatty acids are undergoing ß-oxidation in the peroxisomes, and the long-chain fatty acyl-CoAs are not oxidized completely to short-chain

acyl-CoAs, as has been reported (44,46,59-63), then medium-chain acyl-CoAs and acetyl-CoA would be formed in the peroxisome and would need to be disposed of (50). Our data are consistent with the proposed pathway of peroxisomal ß-oxidation diagrammed in Figure 19. Long-chain acyl coenzyme As are directed to ß-oxidation rather than acylcarnitine formation due to the K_m and V_{max} effects discussed. Acetyl moieties produced would then be transferred to carnitine by peroxisomal CAT and presumably shuttled out of the peroxisome. Medium-chain acyl coenzyme As are directed to acylcarnitine formation rather than further ß-oxidation, as the enzymes of peroxisomal ß-oxidation are specific for longer chain-length substrates (59,112-115). Medium-chain acylcarnitines should then be shuttled out of the peroxisome and presumably into the mitochondria for further ß-oxidation (30,50,62).

COT activity in rat liver peroxisomes ranges from 6 to 15 nmol/min/mg (37,58). In comparison, COT in mouse liver peroxisomes has a very high specific activity, 250 nmol/min/mg. The finding that mouse liver has more COT than rat liver is consistent with the higher levels of peroxisomal ß-oxidation in mouse liver (49) than in rat liver (46). It is not clear, however, why there is an apparent excess of COT activity compared to CAT activity since much more acetyl-CoA should be produced and shuttled out of peroxisomes than medium-chain acyl-CoA. We therefore cannot

Figure 19. Proposed scheme of relation between peroxisomal COT and CAT and peroxisomal ß-oxidation of fatty acids.


Long-Chain Acyl-CoA
Long-Chain Acylcarnitine
kinetically
unfavorable
B-Oxidation
?
Acetyl-CoA
Acetylcarnitine
Cytosol
Medium-Chain Acyl-CoA
Medium-Chain Acylcarnitine
kinetically
favorable
kinetically
unfavorable
?
Short-Chain Acyl-CoA
Cytosol

discount the possibility that COT has an additional unknown function beyond that proposed for export of fatty acid oxidation intermediates out of peroxisomes.

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APPENDIX

COLLABORATIVE REVIEW OF CARNITINE ACYLTRANSFERASES

18

Carnitine Acyltransferases

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I. Introduction

Carnitine (γ-trimethylamino-β-hydroxybutyrate), $(CH_3)_3N^+-CH_2-CHOH-CH_2-COOH$, was first isolated in 1905 (*1*) from muscle. Forty years later Fraenkel *et al.* (*2*) established that carnitine is an essential nutrient for larvae of the beetle *Tenebrio molitor*, and it was given the trivial name, vitamin B_t. In 1955 Friedman and Fraenkel (*3*) presented evidence for a possible enzymatic role for carnitine, and Fritz (*4*) reported

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2. Fraenkel, G., Blewett, M., and Coles, M. (1948). *Nature (London)* **161**, 981.
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4. Fritz, I. B. (1955). *Acta Physiol. Scand.* **34**, 367.

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a carnitine-dependent stimulation of palmitate oxidation by liver preparations. Almost simultaneously the laboratories of Bremer and of Fritz provided convincing evidence for a role in mitochondrial β oxidation (*5–7*) of long-chain fatty acids.

II. Reactions Catalyzed

Carnitine is a cosubstrate for a family of enzymes that catalyze the reversible reaction

$$\text{acylcarnitine} + \text{CoASH} \longrightarrow \text{acyl-CoA} + \text{carnitine} \quad (1)$$

The enzyme, which has a high transfer capacity for palmityl residues, is named carnitine palmityltransferase (CPT), and the one with a large acyl transfer capacity for acetyl residues is called carnitine acetyltransferase (CAT). The reactions catalyzed by the carnitine acyltransferases are defined:

Forward reaction

$$\text{acyl-CoA} + \text{carnitine} \longrightarrow \text{acylcarnitine} + \text{CoASH} \quad (2)$$

Reverse reaction

$$\text{acylcarnitine} + \text{CoASH} \longrightarrow \text{acyl-CoA} + \text{carnitine} \quad (3)$$

The acyl moieties are aliphatic hydrocarbons that range from 2 carbons to more than 20 carbons in length. They can be straight-chained, branch-chained, or unsaturated. There have been some reports that hydroxyacylcarnitines or CoA are substrates or products (*8–10*), but compounds such as succinyl- and malonyl-CoA are not substrates. The broad acyl specificity of the carnitine acyltransferases in mammalian systems does not occur in all living systems. In some yeasts, plants, and even insects, the acyl specificity may be more restricted and appears to depend on the fuel

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10. Bressler, R., and Katz, R. J. (1965). *JCI* **44**, 840.

source and metabolism involved (*11–14*). The functions of short-chain carnitine acyltransferases require elucidation, especially in non-fatty acid-oxidizing systems such as trypanosomes (*15*) and certain yeasts (*13, 16*), and also in mammals.

III. Number of Carnitine Acyltransferases

A. General Comments

It would be incorrect to state that there are a specific number of carnitine acyltransferases. Rather, the number and nature of the transferases depend on the tissue and animal source. For example, trypanosomes (*15*) and yeast (*13, 16*) contain a short-chain carnitine acyltransferase of very narrow acyl specificity. In contrast, mammalian systems contain more than one enzyme (*17–19*) and the short-chain acyl-specific activity often greatly exceeds that of the long-chain. An exception appears to be honeybee flight muscle mitochondria where short-chain carnitine acyltransferase (*12*) was not detected. Even for mammals the number and nature of transferases vary and are tissue specific. For example, heart may contain as little as two carnitine acyltransferases (*20–22*) and liver clearly has a more complex distribution (*23–27*), whereas sperm has a predominance of carnitine acetyltransferase (*28–31*).

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B. Carnitine Palmityltransferase (CPT)

1. *Background*

Although the intramitochondrial membrane is permeable to long-chain fatty acids, only short- and medium-chain fatty acids (*32*) are activated to CoA esters within the mitochondrial matrix. The long-chain acyl-CoA synthetases are associated with microsomes (*33*), the outer mitochondrial membrane (*34*), and peroxisomes. Some variability exists for the activation of medium-chain fatty acids since rat skeletal muscle mitochondria, in contrast to rat liver mitochondria, oxidize only octanoic acid in the presence of carnitine (*35*). Thus carnitine is required for translocation of the acyl residues across the acyl-CoA barrier of the inner membrane of mitochondria via CPT (*6, 7, 36, 37*), which converts cytosolic long-chain acyl-CoA to long-chain acylcarnitines; they subsequently enter the mitochondrial matrix from the cytosol compartment and are then reconverted to acyl-CoA that can undergo β oxidation:

Cytosol (catalyzed by the outer form of CPT)	*Matrix* (catalyzed by the inner form of CPT)
long-chain acyl-CoA + carnitine → long-chain acylcarnitine + CoASH	long-chain acylcarnitine + CoASH → long-chain acyl-CoA + carnitine

Careful intracellular distribution studies (*20, 23, 24, 36*) have shown that CPT is a mitochondrial enzyme. Reports of CPT in microsomes were amended after more careful studies excluded microsomal location (*38, 39*). An exception is the report that some CPT is associated with rat heart microsomes (*40*). Small amounts of extramitochondrial CPT, which increase with changes in physiological states such as diabetes, fasting, or high-fat diet, have been reported (*41*). However, it seems likely that ex-

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41. Bremer, J. (1981). *BBA* **665**, 628.

tra-mitochondrial CPT activity, especially in liver, is due to the broad-specificity carnitine octanyltransferase associated with peroxisomes. Recent data show that purified, homogenous, medium-chain carnitine acyltransferase from mouse liver peroxisomes has some activity with palmityl-CoA as substrate, but the K_m for this substrate is exceedingly large.

2. *Membrane Distribution of CPT*

Although CPT is associated with mitochondria, how it is distributed with respect to the intramitochondrial membrane has not been unequivocally established. Several approaches have been used to measure the relative proportion of CPT on the matrix and cytosolic face of the inner membrane. Studies using digitonin or low amounts of detergent to remove the easily extractable CPT (presumably the transferase associated with the cytosolic face of the inner membrane) and other more direct assays have yielded distributions between 10 and 35% of the CPT associated with the cytosolic face of the inner membrane (*36*, *41–46*); other investigations using malonyl-CoA inhibition (*47*) and DTNB under nonswelling conditions [(*48*, *49*); see also Ref. (*50*)] give values of approximately 1 : 1 for distribution of the two activities. Thus, the fraction of CPT exposed to the cytosolic face of the inner membrane of mitochondria is between 15 and 50% of the total activity in normal liver, heart, and skeletal muscle mitochondria. This ratio may depend on the type of tissue, the stage of animal development, the concentration of carnitine, and possibly the dietary or hormonal state of the animal. Fasting (*41*, *51–53*), diabetes (*54*), and diet (*55–57*) can all affect CPT levels.

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47. McGarry, J. D., Leatherman, G. F., and Foster, D. W. (1978). *JBC* **253**, 4128.
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52. Norum, K. R. (1965). *BBA* **98**, 652.
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56. Wolfe, R. G., Maxwell, C. V., and Nelson, E. C. (1978). *J. Nutr.* **108**, 1621.
57. Bremer, J., and Norum, K. R. (1982). *J. Lipid Res.* **23**, 243.

As discussed in Section III, the amount of catalytically active outer CPT becomes quite critical when one considers its potential effect on the flux of fatty acids through mitochondrial β oxidation and its regulation by malonyl-CoA. A major factor contributing to the different values obtained for the ratios of the outer and inner forms of CPT is undoubtedly the variable, and sometimes inadequate, methodology. An extreme example of assay variability is a report in which it was estimated that using digitonin approximately 20–25% of rat liver mitochondrial CPT is the outer form, yet using a flavoprotein reduction method the ratio changed from approximately 1 : 5 to 1 : 450 [see Ref. (*42*), Table 3].

C. Carnitine Acetyltransferase (CAT)

1. *Location*

Carnitine acetyltransferase is the predominant acyltransferase in most tissues (*17*). It was initially described by Friedman and Fraenkel (*3*) and was subsequently partially purified from pig heart (*58*). Early studies indicated a mitochondrial location in tissues such as mammary gland, liver, heart, skeletal muscle, and kidney (*59–62*). At least two forms of the enzyme were reported, one outer and the other inner, presumably similar to CPT (*59, 61, 62*). However, Tubbs and co-workers (*63*) questioned the concept of more than one CAT after partially purifying the activity from liver, heart, and muscle. Their data suggested the existence of a single type of CAT. The finding that liver from several species contains extramitochondrial CAT associated with peroxisomes and to a lesser degree with endoplasmic reticulum (*23*) provides a possible explanation for the previous findings. Whereas the microsomal enzyme is tightly membrane associated and very labile (*64*), the peroxisomal enzyme is a stable, soluble enzyme located in the matrix and is readily released by treatments that disrupt the fragile peroxisomal membrane (*64, 65*). Thus in tissues such as liver, CAT is associated with at least three different subcellular structures, while in tissues such as heart and skeletal muscle the distribution of CAT appears to be more limited.

58. Fritz, I. B., Schultz, S. K., and Srere, P. A. (1963). *JBC* **238**, 2509.
59. Barker, P. J., Fincham, N. J., and Hardwick, D. C. (1968). *BJ* **110**, 739.
60. Snoswell, A. M., and Koundakjian, P. P. (1972). *BJ* **127**, 133.
61. Solberg, H. E. (1974). *BBA* **360**, 101.
62. Brdiczka, D., Gerbitz, K., and Pette, D. (1969). *EJB* **11**, 234.
63. Edwards, Y. H., Chase, J. F. A., Edwards, M. R., and Tubbs, P. K. (1974). *EJB* **46**, 209.
64. Markwell, M. A. K., Tolbert, N. E., and Bieber, L. L. (1976). *ABB* **176**, 479.
65. Markwell, M. A. K., and Bieber, L. L. (1976). *ABB* **172**, 502.

2. *Substrate Specificity, Properties, and Mechanism*

Partially purified and purified preparations of CAT from mammalian sources show a broad short-chain acyl-CoA specificity with maximum activities for acetyl- and propionyl-CoA and declining V_{max} values for the longer-chain acyl-CoA derivatives up to about ten carbons in length (*58, 65, 66*). The molecular weights of most of the preparations, regardless of the source, are 58,000 to 61,000 (*5, 66, 67*). Some exceptions are a commercial pigeon breast enzyme, which showed a molecular weight of 51,000 with the identical procedures used for the microsomal and peroxisomal enzyme (*65*), and the report by Mital and Kurup where partially purified rat liver mitochondrial CAT was found to be a dimer of unequal subunits with molecular weights of 25,000 and 34,000 (*68*). However, neither of these studies eliminated proteolytic degradation. Dr. Furuta (Shinshu U.) and his colleagues have submitted for publication extensive studies that show that the enzyme from rat liver mitochondria reported by Mital and Kurup is probably a proteolytic artifact obtained from a 67,500-dalton CAT (recent personal communication).

Some kinetic constants have been determined for purified CAT. The equilibrium constant is 0.6 (reverse direction) for this reversible reaction (*58*) and the apparent K_m values for the substrates, where reported, appear to be in the physiological range (see Table I).

CAT from different sources can be divided into two general categories relative to substrate specificity (see Table I). The mammalian transferases, regardless of organelle source, have a broad acyl specificity from 2 to approximately 10 carbons, with maximum V_{max} values for the shorter acyl chain lengths. In contrast, CAT from yeast has a very narrow acyl-CoA specificity, being optimal for acetyl-CoA and propionyl-CoA with limited butyryl and isobutyryl activity and no acyltransferase activity with acyl carbon lengths greater than 4. As expected for an enzyme that uses a spectrum of acyl-CoA as substrates, the various acyl-CoA derivatives act as competitive inhibitors toward each other (*69, 70*).

Some mechanism studies with the pigeon breast muscle enzyme have been performed (*67, 70, 71*). The enzyme can exist in two or more ternary enzyme complexes in rapid equilibrium with the free substrates. The interconversion of ternary complexes appears to be the rate-limiting step. Evidence has been presented that the enzyme contains a reactive sulfhy-

66. Bremer, J., and Norum, K. R. (1967). *JBC* **242**, 1744.
67. Chase, J. F. A., and Tubbs, P. K. (1969). *BJ* **111**, 225.
68. Mittal, B., and Kurup, C. K. R. (1980). *BBA* **619**, 90.
69. Chase, J. F. A. (1967). *BJ* **104**, 510.
70. Chase, J. F. A., and Tubbs, P. K. (1966). *BJ* **99**, 32.
71. Chase, J. F. A. (1967). *BJ* **104**, 503.

CARNITINE ACYLTRANSFERASE PROPERTIES

Source	Reference	K_m (μM) Acetyl-CoA	L-(−)-Carnitine	CoASH	Acetylcarnitine	M_r	Physical state	Substrate specificity	pH optimum
Rat liver microsomes	*64, 72a*	69	150	—	—	59,000	Tightly membrane-bound (unstable) on both faces of ER	Broad (acetyl to octanyl)	Broad 7–8
Rat liver peroxisomes	*64*	69	143	—	—	59,000	Soluble-state	Broad (acetyl to octanyl)	Broad 7–8
Beef heart mitochondria	*21, 22*	—	—	—	—	61,000 62,000	Membrane associated (stable)	Broad (acetyl to octanyl)	—
Rat liver mitochondria	*68*	30	720 for (±)-carnitine	—	—	56,000 (34,000 + 25,000)	Membrane associated (stable) dimer	—	—
Pig heart	*58, 72*	41	310	—	310	—		Broad (acetyl to octanyl)	
Pigeon breast muscle	*67* *69* *71* *63*	34	120	37	350	58,000	Membrane associated. Stable, 2 PIs on electrofocusing (*63*)	Broad (acetyl to decanoyl)	Broad 7–8
T. bovina	*72b, c*	65	300	—	—	—	Membrane associated, primarily mitochondria	Narrow acyl specificity C_2 to C_4	
C. tropicalis									
Mitochondria	*16*	36	622	257	639	—	—	Very narrow; transfers acetyl and propionyl moieties; alkane-grow yeasts have high CAT, no COT	
Peroxisomes	*16, 72d*	42	719	304	417	—	—		

dryl group at the catalytic site (*72*). It is rapidly inactivated by bromoacetylcarnitine and bromoacetyl-CoA via formation of an *S*-carboxymethyl-CoA (−)-carnitine ester which binds very strongly to the substrate binding sites (*67*).

Numerous studies with isolated mitochondria using acetylcarnitine as substrate show that considerable CAT is located on the matrix side of the inner membrane of mitochondria. The data are ambiguous as to whether mitochondria contain some CAT on the cytosolic face that is loosely associated because when most of these studies were done precautions were not taken to eliminate peroxisomal contamination. However, investigations with insect flight mucle mitochondria (*11*) and heart (*73*) strongly indicate that little, if any, mitochondrial CAT is exposed to the cytosolic surface of the inner membrane. Again, species and organ differences may exist.

D. Carnitine Octanyltransferase (COT)

Although mammalian tissues from several sources contain high amounts of carnitine acetyltransferase activity that has some activity with octanyl-CoA as substrate, unequivocal data demonstrating the existence of a separate (unique) COT had not been published (*17, 18, 20, 23, 26, 74*); that is, COT had not been isolated and purified. However, the liver data from various sources (*18, 20, 23, 26*) strongly indicate the occurrence of a separate COT. The finding that rat liver microsomes and peroxisomes contain COT and CAT activity with little, if any, CPT activity was very suggestive, especially when one considers the acyl-CoA chain-length specificity of peroxisomes. The activity is high for short-chain and medium-chain acyl-CoA derivatives and very low for long-chain derivatives. The acyl-CoA specificity of peroxisomes is much broader than the acyl-CoA specificity of partially purified CAT from peroxisomes (*65*), clearly indicating the existence of a second enzyme. The existence of a separate COT protein has recently been established by Mr. Shawn Farrell of this laboratory, who has purified to homogeneity a medium-chain carnitine acyltransferase from mouse liver; he finds it has high V_{max} and very low K_m values for medium-chain acyl-CoA derivatives, but high K_m and low V_{max}

72. Fritz, I. B., and Schultz, S. K. (1965). *JBC* **240**, 2188.
72a. Valkner, K. J., and Bieber, L. L. (1982). *BBA* **689**, 73.
72b. Emaus, R., and Bieber, L. L. (1983). Submitted for publication.
72c. Emaus, R. K. (1982). Ph.D. Thesis, Michigan State University, East Lansing.
72d. Tanaka, A., Osumi, M., and Fukui, S. (1982). *Ann. N.Y. Acad. Sci.* **386**, 138.
73. Warshaw, J. B. (1970). *BBA* **223**, 409.
74. Saggerson, E. D. (1982). *BJ* **202**, 397.

values for short-chain and long-chain acyl residues (*74a*). The possible occurrence of a unique COT in beef heart mitochondria was extensively investigated (*21*, *22*) and the data indicated the presence of only two carnitine acyltransferases, one a broad-specificity CAT and the other a broad-specificity CPT. More than 90% of the COT activity of beef heart mitochondria was accounted for by the combined activities of CAT and CPT. Thus the data indicate the existence of a separate COT in liver peroxisomes and rat liver microsomes, but its occurrence in tissues such as heart and skeletal muscle remains to be established. The occurrence of a more complex carnitine acyltransferase pattern in liver (a very active catabolic and anabolic tissue) as compared to heart or skeletal muscle, which are more catabolic in nature, seems reasonable.

The finding that liver peroxisomes have considerable β-oxidation capacity (*75*, *76*) that does not go to completion but terminates at medium-chain acyl-CoA derivatives provides a role for the peroxisomal medium-chain carnitine acyltransferase activity, namely, to form acylcarnitines that shuttle acyl residues out of peroxisomes (*57*, *77–79*).

IV. CPT: Purification, Properties, and Regulation

The kinetic and catalytic properties of both membrane-bound and purified CPT have been investigated extensively. As indicated in Section II.B, it is well established that CPT exists in at least two forms on the inner membrane of mitochondria. One, referred to here as the *outer CPT*, is located on the cytosolic face, and the other, referred to as *inner CPT*, is located on the matrix face of the inner membrane of mitochondria. The two forms of the enzyme show different kinetic and catalytic properties consistent with their different functions in the cytosol and matrix compartments.

The data for both the isolated enzyme and the mitochondrial enzyme vary from one laboratory to another. Some data are summarized in Table II in which specific properties of purified or partially purified CPT are

74a. Farrell, S. and Bieber, L. L. (1983). *ABB*, **222**, 123.
75. Lazarow, P. B., and deDuve, C. (1976). *PNAS* **73**, 2043.
76. Lazarow, P. B. (1978). *JBC* **253**, 1522.
77. Leighton, F., Brandan, E., Lazo, O., and Branfman, M. (1982). *Ann. N.Y. Acad. Sci.* **386**, 62.
78. Bieber, L. L., Emaus, R. K., Valkner, K., and Farrell, S. (1982). *FP* **41**, 2858.
79. Osmundsen, H., Christiansen, R. Z., and Bremer, J. (1980). *In* "Carnitine Biosynthesis, Metabolism and Functions" (R. A. Frenkel and J. D. McGary, eds.), p. 127. Academic Press, New York.

given, with selected comments about specific properties of the enzyme preparations used. The bottom portion of Table II summarizes selected investigations with isolated mitochondria that have given specific insights into the nature of this membrane-bound enzyme.

A. Properties of Purified CPT

The kinetic properties of the inner and outer forms of CPT (primarily from liver) have been investigated (*20, 33, 36, 42, 80*), and other studies have been done with purified CPT without designation of the form (*2, 22, 41, 66, 81, 82*). As shown in Table II, the K_m values for palmityl-CoA and CoASH are small, while the K_m values for L-carnitine and palmitylcarnitine are larger. With all of the substrates, the K_m values reported are greater than 10-fold from one laboratory to another. The large differences are at least in part attributable to different assay conditions. Investigations by Bremer, Norum, and colleagues (*66, 81*) demonstrated that palmityl-CoA can be a competitive inhibitor of L-carnitine, and that detergents can alter the palmityl-CoA kinetic parameters. It was recognized by Fritz and co-workers (*20*) that partially purified CPT showed variable K_m values, particularly for carnitine and acylcarnitine, depending on the concentration of the cosubstrate. In a study with highly purified, detergent-bound CPT from beef heart mitochondria (*21, 22*) it was shown that the K_m values for carnitine (particularly the acylcarnitines) can vary greatly depending on the experimental conditions. For example, the K_m for myristylcarnitine varies between 14 and 2000 μM depending on the substrate concentration and detergent concentration. Both the K_m and V_{max} values for specific substrates are dependent on whether the long-chain acyl-substrate, either the acyl-CoA or acylcarnitine, is above or below its critical micelle concentration (cmc), the amount of detergent used, and whether the detergent is above or below its cmc. Curiously, the least-affected kinetic constants were those for the acyl-CoA derivatives. Such data also provide an explanation for the apparent discrepancy between the significantly different specificity profiles reported for CPT. Alternatively, the different chain-length specificities of CPT in the two directions could be explained by an increasing substrate inhibition by acyl-CoA (J. Bremer, personal communication). Almost all data are in agreement that CPT catalyzes reversible reactions involving acyl-CoA and acrylcarnitines as substrates using acyl residues from approximately 6 to 20 carbons in

80. West, D. W., Chase, J. F. A., and Tubbs, P. K. (1971). *BBRC* **42**, 912.
81. Bremer, J., and Norum, K. R. (1967). *JBC* **242**, 1749.
82. Norum, K. R. (1964). *BBA* **89**, 95.

TABLE II

CARNITINE PALMITYLTRANSFERASE (CPT) PROPERTIES

Preparation	Source	M_r	K_m (μM)			
			Palmityl-carnitine	CoASH	Palmityl-CoA	Carnitine
Outer CPT	Ox liver	59,000	12	—	0.59	140
Inner CPT	Ox liver	65,000	60	—	9	2600
CPT	Calf liver mitochondria	—	40	50	10	250
Outer CPT	Calf liver	150,000	136	5.5	17.6	450
Outer CPT	Calf liver	—	170	45	31	210
Inner CPT	Calf liver	150,000	—	—	—	—
Outer CPT	Rat liver mitochondria	430,000	11	35	2.8	280
Inner CPT	Rat liver mitochondria	430,000	11	34	3.5	300
Inner and outer CPT	Beef heart mitochondria	67,000 (Detergent CPT complex = 510,000	—	—	2[a]	—
Intact mitochondria	Bovine heart and rat liver mitochondria	—	—	—	—	—
Heavy mitochondria	Rat liver	—	—	—	1.7	170
Mitochondria	Rat liver and heart	—	—	—	—	—
Mitochondria	Rat liver	—	—	—	—	—
Mitochondria	Rat liver from fed and fasted animals	—	—	—	—	—
Mitochondria	Rat heart and liver	—	—	—	—	—

[a] Unpublished data from Carol Fiol of this laboratory.

length. However, some evidence has been presented indicating the inner form of CPT is not reversible (*83*).

Investigations by Kopec and Fritz (*83*), Bremer and Norum (*81*), Bergström and Reitz (*42*), and Clarke and Bieber (*21*, *22*) have all resulted in data indicating that the outer and inner forms of CPT represent kinetically

83. Kopec, B., and Fritz, J. B. (1973). *JBC* **248**, 4069.

TABLE II

(*Continued*)

References	Acyl group specificity and comments
36, 80	Very broad with moderate C_4 and medium-chain activity.
80, 36	Broad $C_6 \rightarrow C_{16}$ with maximum activity with C_{12}.
66, 81, 82	Broad with moderate medium-chain activity; inhibition by palmityl-CoA.
20, 83	Low medium-chain activity, high long-chain activity.
82	
20, 83	$CPT_{11} \rightarrow CPT_1$ with urea. K_m values for carnitine, CoASH, and acylcarnitine vary.
42	Kinetic data indicate CPT outer and CPT inner are the same enzymes and *in situ* factors alter properties of CPT.
42	
21, 22	Broad acyl specificity with highest activity with C_{10} in forward direction and greatest with C_{16} in reverse direction. CPT outer and inner are the same protein. Great variability in the K_m values for carnitine and acylcarnitines depending on experimental conditions.
37, 83a	Biphasic rates of palmitylcarnitine formation versus palmityl-CoA concentrations. Very high acyl-CoA concentrations required for V_{max}; lag before maximum palmityl-CoA oxidation.
84	Low K_m for palmityl-CoA at low (0.25 m*M*) carnitine and no biphasic saturation curve.
46	Concentration-dependent lag in palmityl-CoA oxidation; possible substrate inhibition.
47, 85, 86	Malonyl-CoA is a potent inhibitor of outer CPT; malonyl-CoA may be the key intermediate for coordinating fatty acid synthesis and degradation in liver.
41, 86–89	The sensitivity of outer CPT to malonyl-CoA may vary depending on the physiological state of the animal.
74	CPT very sensitive to ionic composition of media; CPT sensitive to malonyl-CoA.

different forms of the same protein. Such data provide a possible explanation for the apparent conversion of the inner form to the outer form by treatment with urea (*83*). If CPT is a single protein in mitochondria, then the kinetically different forms must be determined by the membrane and the membrane environment, reminiscent of the concept of allotopy used

83a. Hoppel, C. L., and Tomec, R. J. (1972). *JBC* **247**, 832.

to explain the different catalytic and kinetic properties of other membrane-bound enzymes.

B. Properties of Membrane-Bound CPT

Some of the extensive studies of mitochondrial CPT are summarized in the bottom portion of Table II. A biphasic saturation curve for palmitylcarnitine formation results when the palmityl-CoA concentration is varied (*37*). In some studies extremely high amounts of palmityl-CoA were required for attaining V_{max}, yet in what appear to be essentially identical experimental conditions except the carnitine concentration was approximately 10-fold lower (*84*), no biphasic saturation curve was detected and a K_m of less than 5 μM for palmityl-CoA was obtained. The contribution of lysis or swelling due to detergent effects of palmityl-CoA to the biphasic saturation curves has not been determined. Other studies with intact mitochondria have shown (*37, 46*) concentration-dependent lags in palmityl-CoA oxidation; such lags are not obtained when palmitylcarnitine is the substrate.

1. *Effects of Malonyl-CoA*

The data showing variable kinetics of CPT indicate that other, as yet unidentified factors may affect catalysis by CPT. This has been reinforced by the results of McGarry and Foster (*47, 85, 86*), which show that malonyl-CoA can be a potent inhibitor of the outer form of CPT. The inhibition by malonyl-CoA is lost when the enzyme is solubilized, indicating the importance of membrane factors. Although the physiological significance of the malonyl-CoA inhibition has been questioned by some, because of the amounts of malonyl-CoA *in situ*, others have confirmed and extended the investigations. Very recently, it has been shown that outer CPT sensitivity to malonyl-CoA may vary depending on the physiological state of the animal (*41, 86–89*), including different thyroid states (*90*), and the ionic composition of the assay media (*74*). In all of these studies significant inhibition of CPT by added malonyl-CoA was obtained at concentrations at or below those considered physiological.

84. VanTol, A. (1974). *BBA* **357**, 14.
85. McGarry, J. D., and Foster, D. W. (1979). *JBC* **254**, 8163.
86. McGarry, J. D., and Foster, D. W. (1981). *BJ* **200**, 217.
87. Robinson, I. N., and Zammit, V. A. (1982). *BJ* **206**, 177.
88. Cook, G. A., Otto, D. A., and Cornell, N. W. (1980). *BJ* **192**, 955.
89. Saggerson, E. D., and Carpenter, C. A. (1981). *FEBS Lett.* **129**, 225.
90. Stakkestad, S. A., and Bremer, J. (1982). *BBA* **711**, 90.

a. Speculation. The studies that show that the sensitivity of CPT to malonyl-CoA can vary depending on the physiological state of the animal (*86–90*) have an exceedingly important ramification, namely, that an outer CPT must exist in at least two interconvertible forms on the cytosolic surface of the inner membrane of mitochondria. One is catalytically active both in the presence and absence of malonyl-CoA, and the other is catalytically active but sensitive to malonyl-CoA. Therefore it seems likely that membrane-bound CPT may be a regulated, possibly allosteric, enzyme. If so, modulation of the activity through covalent modification or the existence of a membrane-bound regulator component(s) similar to classical regulator subunits seems plausible. This could provide an explanation for the lack of malonyl-CoA sensitivity when the enzyme is solubilized with detergents, the apparent lack of sensitivity on the matrix side of the inner membrane, and the variability in sensitivity by different physiological states. These speculations are mentioned because most data with intact mitochondria in which latent and overt CPT have been investigated are usually interpreted in terms of two distinct, catalytically different proteins (*91*). Nevertheless, it is evident that mitochondrial CPT can be inhibited by low concentrations of malonyl-CoA, but whether this is the major regulator in coordinating fatty acid synthesis and fatty acid catabolism and ketogenesis remains to be unequivocally determined.

2. *Substrate Analogs and Inhibitors*

Removal of the β-hydroxyl group (deoxycarnitine) from carnitine abolishes its activity (*92*) and produces a competitive inhibitor of the reaction (*93*). Substitution of the β-hydroxyl group with a thiol group does not cause loss of activity (*94*), but removal of a methyl group (conversion of the quaternary ammonium to a tertiary amine) produces an inhibitor norcarnitine (*93*). Palmityl-(+)-carnitine inhibits CPT (*43*, *95*) with a larger K_i for solublized CPT than the membrane-bound enzyme. Fatty acyl-CoA esters of 2-tetradecylglycidic acid (*96*, *97*) and 1-pyrenebutyryl-CoA (*98*) are potent inhibitors of carnitine palmityltransferase; the former affects the outer form of CPT. The 1-pyrenebutyrylcarnitine derivative is

91. Hoppel, C. L. (1982). *FP* **41**, 2853.
92. Fritz, I. B., Kaplan, E., and Yue, K. T. N. (1962). *Am. J. Physiol.* **202**, 117.
93. Norum, K. R. (1965), *BBA* **99**, 511.
94. Tubbs, P. K., and Chase, J. F. A. (1970). *BJ* **116**, 34p.
95. Fritz, I. B., and Marquis, N. R. (1965). *PNAS* **54**, 1226.
96. Tutwiler, G. F., and Ryglak, M. T. (1980). *Life Sci.* **26**, 393.
97. Tutwiler, G. F., Ho, W., and Mahrbrachr, R. J. (1981). "Methods in Enzymology," Vol. 72, p. 533.

a potent inhibitor of the carnitine : acylcarnitine translocase (*98*). The 2-substituted oxiran-2-carbonyl-CoA esters (*99*) and palmityl-CoA analogs in which the carbonyl group is replaced by methylene groups (*100*) are also potent inhibitors of outer CPT.

C. CPT Assays

The large differences in CPT data from one laboratory to another are undoubtedly partly due to the use of different assays and assay conditions. Assay problems include (a) nonlinear rates (i.e., lags in activity, which in our hands are not always reproducible; (b) variable K_m values depending on experimental conditions; (c) latent and overt activity with conversion of one form to the other; and (d) effects of ionic strength, cations, inhibitors, etc. With such an enzyme, rigorously controlled assay conditions must be used that assure linearity during the reaction time. Since there is no ideal assay for CPT, several methods have been used, which are summarized in Table III. Some investigators have used end-point assays and assumed the assay conditions adequate for controls are also adequate for experimental samples. Such assumptions are not always valid and the data obtained may be equivocal.

V. Pathophysiology and Clinical Aspects

It is now well established that low carnitine levels (also low carnitine acyltransferase levels) in human muscle are associated with certain types of myopathies (*101, 102*), and low carnitine levels in liver are associated with serious metabolic problems (*103, 104*). The fact that some muscle myopathies are apparently related to low levels of CPT (*105, 106*) or low levels of carnitine (*102*) is consistent with a lesion in the β oxidation of long-chain fatty acids. However, in systemic carnitine deficiency the decreases in liver carnitine indicate other roles, with different etiologies and

98. Wolkowicz, P. E., Pownall, H. J., and McMillin-Wood, J. B. (1982). *Biochemistry* **21**, 2990.
99. Bartlett, K., and Meredith, P. (1981). *Biochem. Soc. Trans.* **9**, 574.
100. Ciardelli, T., Stewart, C. J., Seeliger, A., and Wieland, T. (1981). *Justus Liebigs Ann. Chem.* p. 828.
101. Karpati, G., *et al.* (1975). *Neurology* **25**, 16.
102. Engel, A. G., and Angelini, C. (1973). *Science* **179**, 899.
103. Ware, A. J., *et al.* (1978). *J. Pediatr.* **93**, 959.
104. Angelini, C., Lucke, S., and Cantarutti, F. (1976). *Neurology* **26**, 633.
105. DiMauro, S., and DiMauro, P. M. M. (1973). *Science* **182**, 929.
106. Bertoni, T., *et al.* (1980). *Neurology* **30**, 263.

TABLE III

CARNITINE PALMITYLTRANSFERASE ASSAYS

Method	Principle	Comments
Exchange	Exchange of radiolabeled carnitine into a pool of acylcarnitine.	Usually performed as an end-point assay. Many do not ensure that the experimental samples respond as controls. Acyl-CoA hydrolase (present in mitochondria, microsomes, and lysosomes) affects final result due to hydrolysis of the acyl-CoA [see Ref. (*90*)]. Particulate preparations can be used.
232 Forward	Rate assay following thioester formation spectrophotometrically at 232 nm.	Difficult to perform with nonpurified systems because of high 232 backgrounds. Preparations should be solubilized. Deviations from linearity and lags occur.
232 Reverse	Same as above but measured in opposite direction by monitoring thioester disappearance.	See comments above.
Radioactive product formation	Determine the formation of radioactive product using either radiolabeled carnitine or radiolabeled acyl groups.	Requires separation and quantitation of radioactive product. Can be used as an initial rate assay, but is usually used as an end-point assay where the limitations of exchange assay can occur.
Hydroxamate formation	Product acyl-CoA or the remaining substrate (acyl-CoA depends on assay direction) converted to hydroxymate that is quantitated.	Same limitations as stated above for end-point assays. Some inhibition by hydroxylamine has been reported. Can be used with nonpurified systems.
CoASH release	Initial rate assay in which the CoASH released is monitored spectrophotometrically with SH reagents such as DTNB and DTBP.	Total CPT can be assayed in crude systems; requires detergent solubilization. Can be used to estimate outer CPT in absence of detergent, under *nonswelling* conditions. Inhibition by DTNB at pH 7.4, but not a problem at pH 8.0.

possibly secondary effects of carnitine. Examples are neurological symptoms due to an apparent inhibition of pyruvate dehydrogenase (*107*), Reyes-like syndrome (*108*), and the effects of carnitine deficiency on propionic acid acidemia (*109*).

Apparently there are no examples of deleterious effects due to tissue carnitine increases. Even when the carnitine levels increase by an order of magnitude such as that in muscle and liver of streptozotocin-induced diabetic sheep (*110*), the carnitine pool appears to function normally. Rather, clinical problems arise due to severe reductions in either mitochondrial carnitine palmityltransferase (the medium- and short-chain activities have not been thoroughly investigated) or tissue carnitine levels. Other possible roles for carnitine unrelated to mitochondrial β oxidation of long-chain fatty acids have been proposed [see Ref. (*78*) for discussion].

107. DiDonato, S. D., Rimoldi, A., Moise, D., Bertagnoglio, B., and Uziel, C. (1979). *Neurology* **29**, 1578.

108. Chapoy, P. R., *et al.* (1980). *N. Engl. J. Med.* **303**, 1389.

109. Roe, C. R., and Bohan, T. P. (1982). *Lancet*, 1411.

110. Fishlock, R. C., Snoswell, A. M., Valkner, K., and Bieber, L. L. (1982). *Int. J. Biochem.* **4**, 451.