THE SEARCH FOR MITOCHONDRIAL CARNITINE OCTANOYL TRANSFERASE--AN INVESTIGATION OF CARNITINE ACYLTRANSFERASE ACTIVITIES IN BEEF HEART MITOCHONDRIA

By

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ABSTRACT

THE SEARCH FOR MITOCHONDRIAL CARNITINE OCTANOYLTRANSFERASE--AN INVESTIGATION OF CARNITINE ACYLTRANSFERASE ACTIVITIES IN BEEF HEART MITOCHONDRIA

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The purpose of this study was to characterize the mitochondrial carnitine octanoyltransferase of beef heart. Carnitine acyltransferase activities were solubilized from isolated beef heart mitochondria using KCl and the non-ionic detergent, Triton X-100, at final concentrations of 1 M and 2%, respectively. Upon fractionation of the solubilized protein on Cibacron Blue Sepharose, two protein peaks with carnitine octanoyltransferase were obtained. These two fractions accounted for all carnitine acyltransferase activity present in the original mitochondrial suspension. The first eluting peak was purified 400-fold by Sephadex G-100 gel filtration, CM-Sepharose ion exchange and hydroxylapatite chromatography to a single protein of greater than 95% purity. This carnitine acetyltransferase (CAT) shows highest activity with acetyl and butyryl carnitine and coenzyme A esters. It has a subunit molecular weight of 62,600 daltons on SDS-polyacrylamide gel electrophoresis, a native molecular weight of 60,500 on Sephadex G-200 gel filtration and an isoelectric pH of 8.20 on sucrose density gradient isoelectric focusing.

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Peter R. H. Clarke

The second peak of carnitine acyltransferase activity from Cibacron Blue Sepharose was purified 1600-fold by fractionation on Sephadex G-100 gel filtration, QAE-Sephadex ion exchange and hydroxylapatite chromatography to a single protein of greater than 95% purity. This enzyme was carnitine palmitoyltransferase (CPT). It is most active with decanoyl and lauryl ester substrates, has a subunit molecular weight of 67,000 daltons on SDS-PAGE, an isoelectric pH of 8.05 on sucrose density gradient isoelectric focusing and migrates as part of a detergent micelle of apparent molecular weight 510,000 on Sephadex G-200 gel filtration.

It is concluded that there are only two carnitine acyltransferase proteins present in beef heart mitochondria, one membrane-bound (CPT) and one membrane-associated (CAT). Each has significant activity toward hexanoyl, octanoyl and decanoyl carnitine and coenzyme A esters. The presence of a separate medium chain length-specific carnitine acyltransferase in beef heart mitochondria is not confirmed by our results.

The roles of micelles of the substrates octanoyl-, lauryl-, decanoyl-, myristoyl- and palmitoylcarnitine and of non-ionic detergents in the determination of the substrate specificity for the reverse reaction of a soluble purified beef heart mitochondrial carnitine palmitoyltransferase (CPT) are investigated. It is shown that the discontinuity in double reciprocal plots of substrate concentration versus reaction rate is attributable to the formation of substrate micelles at the critical micellar concentration (CMC) of the acylcarnitine. A 100-fold increase in K_m and a 6-fold increase in V_{max} are obtained when reactions are carried out with micellar as opposed to monomeric concentrations of the

substrate myristoylcarnitine. With all substrates in the monomeric state, reactions performed in the absence of micelles of non-ionic detergent show that carnitine palmitoyltransferase is most specific for medium chain length substrates, with laurylcarnitine having the lowest K_m (7.8 μM) and decanoylcarnitine the highest V_{max} (6.2 U/mg).

The effect of the addition of micellar concentrations of nonionic detergent is to decrease the sharpness of the discontinuity seen at the substrate CMC in double reciprocal plots. At high detergent concentration, where the discontinuity is negligible, a substrate specificity pattern similar to that seen in detergent absence is observed. In the micellar environment, the enzyme shows up to 3-fold increases in V_{max} for the various substrates and a consistent 5- to 6-fold increase in K_m for octanoyl-, decanoyl-, lauryl- and myristoylcarnitine. Thus the kinetic parameters determined for carnitine palmitoyltransferase are shown to depend on the state of the substrates and on the enzyme's environment.

DEDICATION

To David and Maureen

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Bis Tris-	1,3-bis(tris[hydroxymethyl]-methylamino)-
CAT	Carnitine acetyltransferase
CM-	Carboxymethylethyl-
CMC	Critical micellar concentration
CoA	Coenzyme A
CoASH	Reduced coenzyme A
COT	Carnitine octanoyltransferase
CPT	Carnitine palmitoyltransferase
DEAE-	Diethylaminoethyl-
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	(Ethylenedinitrilo)-tetraacetic acid
HAP	Hydroxylapatite
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Ki	Inhibition constant
Km	Michaelis constant
MOPS	Morpholinopropanesulfonic acid
NAD	Nicotine adenine dinucleotide
pI	Isoelectric pH
POPOP	1,4-bis(2-[4-methyl-5-phenyloxazolyl])benzene
PPO	2,5-diphenyloxazole
QAE-	Diethyl-(2-hydroxypropyl)aminoethyl
QO_2	Respiratory quotient
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis
TNS	6-p-toluidino-2-naphthalenesulfonic acid
Tris	Tris(hydroxymethyl)aminomethane
V_{max}	Maximum velocity

BACKGROUND ON THE CARNITINE ACYLTRANSFERASES

Introduction

Carnitine (γ-trimethylamino-β-hydroxybutyrate) was first isolated in 1905 (1) from mammalian muscle but its significance in muscle and function in metabolism remained unknown for half a century thereafter. Despite its structural resemblance to choline, no role for carnitine as a neurotransmitter appeared possible (2). A compound, later identified as carnitine (3), was discoverdd by Fraenkel et al in 1948 (4) to be an essential nutrient for larva of the beetle Tenebrio molitor and was referred to subsequently in the literature as vitamin B_T. Enzymatic acetylation of p-aminobenzoic acid was found by Friedman and Fraenkel in 1955 (5) to be inhibited by carnitine and was apparently due to an enzyme in pigeon and sheep liver extracts which was able to acetylate carnitine by the reaction: O-acetylcarnitine + coenzyme A $\rightleftharpoons$ acetyl-CoA + carnitine.

Four years later Fritz (6) reported the carnitine-dependent stimulation of the oxidation of long chain fatty acids by particulate liver preparations. He reported little effect of carnitine on medium chain fatty acid degradation or on the oxidation of palmitoyl-CoA. This carnitine independence of medium chain fatty acid oxidation has stood the test of time (7, 8). A role for carnitine in palmityl-CoA oxidation was suggested by the mitochondrial metabolism of palmitoyl-carnitine reported by Bremer (9) in 1962. Separate reports by Bremer (10) and Fritz (11) the

following year noted enzymatic synthesis of palmitoyl-carnitine and the finding of the following reversible reaction: palmitoyl-carnitine + CoA $\rightleftharpoons$ palmitoyl-CoA + carnitine.

Thus carnitine acyltransferases are defined to catalyze the following reactions:

Forward Reaction acyl-CoA + carnitine → acyl-carnitine + CoASH

Reverse Reaction acyl-carnitine + CoASH → acyl-6A + carnitine.

During this period (1962) Bremer (12) reported that mitochondria from several rat tissues showed reversible acetylation of carnitine and Fritz (13) in 1963 reported the partial purification of carnitine acetyltransferase (first called carnitine transacetylase (14)) from pig heart mitochondria. The roles of carnitine proposed by these two independent investigators (15, 16) were the same: a means of transporting activated long chain fatty acids across the CoA-impermeable (17) inner mitochondrial membrane to the site of β-oxidation and a means of transporting activated acetyl groups across the membrane. Thus long chain fatty acyl-CoA esters synthesized outside the matrix of the mitochondrion are transported into the mitochondrion by external transfer of acyl groups to form acylcarnitines, movement of the acylcarnitine across the inner mitochondrial membrane, followed by regeneration of acyl-CoA by internal acyl transfer from carnitine.

These roles for carnitine require at least three types of carnitine acyltransferase activity: a long chain transferase (a carnitine palmitoyltransferase or CPT) accessible to the outer surface of the inner mitochondrial membrane, a CPT accessible to the inner surface, and a carnitine acetyltransferase (or CAT) with access to the inner matrix of the

mitochondrion where acetyl-CoA thioesters are formed from fatty acids, pyruvate and amino acids. The CAT partially purified by Fritz from pig heart did not react with long chain fatty acyl substrates, therefore at least two different proteins were necessary to account for the three activities. The simplest system would require only two enzymes, one for short and one for long chain fatty acyl groups, with each enzyme located in the inner mitochondrial membrane with access to both surfaces, possibly acting as a transport factor as well for the carnitine esters.

It is now believed (18) that the forward and reverse carnitine acyltransferase reactions involved in converting cytosolic acyl-CoA into mitochondrial CoA ester are not performed by one protein molecule; there is CPT available to the outer surface only and CPT accessible to the inner surface only, the two "separated" in function by a "translocase" protein (19, 20) which catalyses the one-for-one exchange of free carnitine or carnitine esters.

The evidence to date (18, 21) is in favor of no extramitochondrial sites of CPT activity in the cell. A proposed role for CAT as an acetyl sink (22, 23), saving activated acetyl groups for fuel and allowing release of mitochondrial CoASH, requires only CAT activity inside the mitochondrion. A more extended role of acetylcarnitine to one like that of citrate, providing net export of acetyl groups for lipid synthesis and acetylation reactions without the ATP expense of the citrate system, would require at least one additional site of CAT activity, either on the external surface of the mitochondrial inner membrane or elsewhere in the cell. Bressler and Brendel in 1966 (24) reported that though their calculations indicated that citrate is the major path for acetyl group movement

out of the pigeon muscle mitochondrion, carnitine showed a stimulation of the production of fatty acids and acetylsulfanilamide from labelled pyruvate without any effect on their production from citrate. In one report (25) comparing the carnitine and citrate transport systems, CAT is concluded to "be the most likely candidate for acetyl group transfer out of yeast mitochondria."

Looking for extramitochondrial carnitine acyltransferase activity in liver and kidney, Markwell et al (26) found, isolated and partially purified CAT proteins from peroxisomes and microsomes. (Recently, in the yeast Torulopsis bovina, Emaus (27) has reported CAT associated with the nuclear fraction as well.) Thus though the role of carnitine in long chain fatty acid metabolism is presently thought to be solely associated with mitochondrial β-oxidation, its role in transfer of acetyl residues must be more diverse.

Assay Methods

Assay of carnitine acyltransferase activity is by no means standardized and has yielded results difficult and occasionally impossible to compare. In the forward direction, the synthesis of (^{14}C)-acylcarnitine (28) and acyl-(^{14}C)-carnitine (29) from labelled substrates or the production of CoASH from acyl-CoA has been measured, the free CoA detected spectrophotometrically by reaction with a sulfhydryl reagent (30) such as DTNB (Ellman's reagent) or DPD (4, 4'-dipyridine disulfide) or measured fluorometrically (31) by a coupled assay with alpha keto glutarate dehydrogenase, producing reduced pyridine nucleotide. The reverse

reaction is assayed as the liberation of (^{14}C)-carnitine (32) from labelled substrate or by the detection of acyl-CoA by its characteristic absorption at 232 nm (33) or by the reaction of acyl-CoA with hydroxamate (11).

Other assays involve monitoring changes in radioactive specific activity of substrate/product of the two reactions--the "isotope exchange" assay (10, 34)--coupling acyltransferase to fatty acid activation to measure acylcarnitine production from free fatty acid, and estimating transferase activities from flavoprotein reduction or oxygen consumption during mitochondrial β-oxidation of fatty acids, acyl-CoA's or acylcarnitine esters. Very few investigators (29, 35) have reported carnitine acyltransferase activities as measured by a variety of techniques for purposes of completeness or comparison; the usual practice is to perform the assay in one direction by one method.

Some of the methods (3) do not allow a simple determination of background acyl-CoA or possible acylcarnitine hydrolase activity. Another, the 232 assay used to measure the reverse reaction, requires few assumptions, is simple and measures the concentration of reaction product directly without further manipulation and is therefore preferred in determining kinetic characterization of purified transferases. The high absorption of extraneous protein in crude preparations of enzyme precludes use of the 232 assay with intact mitochondria, tissue extracts or during the entire course of an enzyme purification procedure. Reduced CoA-trapping assays which measure the forward reaction are unaffected by extraneous protein but contain a high background absorbance in the presence of significant concentrations of sulfhydryl agent-reacting substances seen in crude tissue homogenates. These assays must contend with background

production of CoASH due to acyl-CoA hydrolases which can account for as much as 90% of the total activity, requiring large numbers of replications and making reliable values difficult to obtain.

Reliability of values reported and conclusions drawn about amount and locations of transferases is especially suspect when the method infers amount of acyltransferase activity from amount of product of a subsequent reaction such as the reduction of flavoprotein during β-oxidation of acyl-CoA produced in intact mitochondria from external acylcarnitine or such as the consumption of oxygen linked to carnitine-mediated transport of fatty acyl groups. As an example, in a study by Wood and Chang (36) of carnitine palmitoyltransferase (CPT) activity in rat liver and heart mitochondria, comparison of activity of intact organelles reacting with external sustrates to that seen after detergent disruption gave for liver mitochondria a value of 0.54 for the ratio of CPT on the outer to that on the inner surface of the mitochondrial CoA barrier while the oxygen consumption of palmitoyl-CoA dependent on carnitine versus that of palmitoylcarnitine was 0.56, an excellent agreement confirming the notion of carnitine-dependent palmitoyl-CoA oxidation being a measure of outer CPT while palmitoylcarnitine oxidation measures inner CPT. Under identical conditions, however, rat heart mitochondria showed the same value of 0.56 for QO_2: palmitoyl-CoA + carnitine/QO_2: palmitoylcarnitine, but the ratio of outer CPT/inner CPT from the detergent study was 1.16.

More dramatic are the results of Normann et al (37) and of Bergstrom and Reitz (38). The former group found in guinea pig brown adipose tissue mitochondria that estimation of "outer" and "inner" CPT by monitoring acyl-CoA dehydrogenase flavoprotein redox level revealed that the apparent

initial rate of the inner CPT was 1000 times that of outer CPT. Bergstrom and Reitz found a similar result (a 450 fold difference) using this assay with rat liver mitochondria but also reported that assay of total CPT by the DTNB method before and after treatment of mitochondria with digitonin to remove outer CPT showed that the treatment removed 20-25% of total CPT with the remainder tightly bound and resistant to trypsin digestion (presumably corresponding to inner CPT protected by the intact inner mitochondrial membrance).

Comparison of tissue, animal, and laboratory differences in carnitine acyltransferases reported is made difficult by the various ways in which the same assay is carried out. Various concentrations of albumin, whose ratio to fatty acid or acyl ester has a significant effect on fatty acid activation (39) and transfer (40), are included in the reaction mixture by different investigators. Some values reported are obtained from reactions utilizing dl-carnitine or dl-acylcarnitines. Studies on mitochondrial CPT (41) and on pure CAT (42) have shown the enzymes to be specific for l-carnitine and its esters and inhibited by the d isomer with a Ki within the ranges employed for assay. The forward and reverse reactions have been performed at 25, 30, 35, 37 and 40° C. There is no agreement on the concentrations of substrates to be included in the incubation. Ionic strength is not uniform and has been shown (43) to have a significant effect on long chain acyltransferase activity. Finally, detergent is present in some reactions while absent in others; various different detergents have been used, with the concentration of detergent in the final reaction mixture often not reported.

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Intracellular Localization

Carnitine Acetyltransferase (CAT)

The localization of CAT in the mitochondrion has been studied by histochemistry/electron microscopy, by comparison of the mitochondrial oxidation of acetylcarnitine to that of acetyl-CoA + carnitine, and by membrane disruption using detergents, sonication, osmotic shock and freeze-thaw techniques. The electron microscopic method "observes" the electron dense product of reaction between uranyl acetate, potassium ferricyanide, and free CoASH released from acetyl-CoA in the presence of carnitine. Investigations of rat heart (44) and mouse skeletal muscle (45) have localized CAT by this technique to the space between the inner and outer mitochondrial membranes and to the outer surface of the inner membrane, respectively. None was seen by either group associated with the inner surface of the inner membrane or in the mitochondrial matrix.

An opposite result is reported for isolated intact mitochondria of liver and mammary gland of goat, guinea pig and rat by measuring CAT activity before and after disruption of the mitochondria with detergent or freeze-thaw. In this study (46) on average only 7.5% of total CAT activity was attributable to an outer CAT enzyme. Snoswell (47) has reported similar results for sheep liver, heart, skeletal muscle and kidney cortex where at least 90% of mitochondrial CAT was found to be latent. Comparing intact mitochondria to those treated with digitonin, Solberg (34) reported little or no outer short chain acyltransferase for mitochondria of rat and mouse liver, though the results with calf liver mitochondria were not as clear. She also noted that the 13-fold increase in mitochondrial CAT

induced by clofibrate treatment was an increase in inner CAT only and not reflected in CAT activity on the outer surface of the mitochondria.

Using digitonin to strip away the outer membrane of rat liver mitochondria and to solubilize proteins from the outer surface of the inner membrane not integrally bound, Brdiczka et al (48) noted the major part of CAT in the inner mitochondrial space while at least 25% of the total CAT was assigned to an outer compartment, one containing adenylate kinase, a marker for the space between the inner and outer mitochondrial membranes. After treatment with digitonin, these workers reported that the rate of oxidation of acetyl-CoA + carnitine was significantly decreased, confirming that an outer CAT activity had been removed, one which when present is responsible for carnitine-dependent oxidation of acetyl-CoA. Likewise in blowfly flight muscle mitochondria separate investigators (49, 50) have reported the total absence of outer CAT based on a lack of oxidation of acetyl-CoA + carnitine by these organelles but a very high oxidation of acetylcarnitine.

Warshaw (51) however found that though mitochondria from bovine fetal heart oxidized acetylcarnitine but not acetyl-CoA + carnitine, mitochondria isolated from calf heart were not as impaired, suggesting a deficiency of CAT outside the mitochondria only in early development. Similar results for rat heart mitochondria were reported by Tubbs and Chase (52) who found oxidation of acetyl-CoA + carnitine as well as of acetylcarnitine but of acetylcarnitine only if the mitochondria had been preincubated with bromoacetyl-CoA, a proposed irreversible inhibitor of CAT which is unable to cross the CoA barrier of intact mitochondria. They were able to inhibit both oxidations by using bromoacetylcarnitine.

They concluded that there are two pools of CAT, one inner and one outer, and that some preparations of mitochondria have lost the outer CAT during the isolation procedure.

The association of even the inner CAT with the mitochondrial membrane is not a tight one; it is more "membrane-associated" than "membrane-bound". This was shown by Beenakkers and Klingenberg (53) who, by repeated extraction without membrane solubilization by the use of detergent or extensive sonication, were able to fully solubilize CAT from mitochondria of rat heart and locust flight muscle. Freeze-thawing mitochondria at appropriate ionic strength gave the same result for Barker et al (46) with mitochondrial CAT from liver and mammary gland of goat, guinea pig and rat.

In other organelles, Markwell et al (26) found rat liver peroxisomal CAT to be free in the organelle interior and released by its breakage while the liver microsomal CAT was membrane-associated but solubilized by 0.4 M KCl. These two CAT proteins were shown to have identical chromatographic, physical and kinetic properties; the difference in their solubilization may reflect the difference in the microsomal and peroxisomal membranes, the latter being a unilaminar, fragile structure. Thus the in situ evidence from electron microscopy and that from most studies with isolated mitochondria are clearly in conflict.

More definitive proof of the existence or absence of an outer mitochondrial CAT may be lacking due to a lack of theoretical necessity for its presence. Extra-mitochondrial sites of CAT activity in liver, kidney and heart that have been shown by Markwell and others may account for non-mitochondrial metabolism of acetylcarnitine. The presence of fatty acid

synthetases for short and medium length fatty acids in the mitochondrial matrix and the permeability of the inner mitochondrial membrane for these groups would allow mitochondrial utilization of the shorter fatty acids without the carnitine-mediated system of transport.

Carnitine Palmitoyltransferase (CPT)

Though the inner mitochondrial membrane is permeable to free fatty acids, unlike the short and medium length fatty acids (54), long chain fatty acids are not activated to CoA esters within the inner compartment of the mitochondrion. Rather, long chain acetyl-CoA synthetases are found associated with microsomes (55) and the outer mitochondrial membrane (56). Therefore for carnitine to mediate the conversion of cytosolic long chain fatty acyl-CoA to mitochondrial acyl-CoA a long chain carnitine acyltransferase must be present on the outer surface of the inner mitochondrial membrane or elsewhere in the cell.

Careful intracellular distribution studies by Hoppel (35) and Markwell et al (21) have shown carnitine palmitoyltransferase to be exclusively mitochondrial. The results of some early reports of CPT activity in microsomes as well as in mitochondria were later amended after more careful study to exclude a microsomal location (111, 112). The report of Fogle and Bieber (57) of CPT activity in rat heart microsomes has not been confimed or refuted; research into the properties of heart microsomes is still very new.

In 1965 Norum (58) presented data showing that less than 10% of total cellular CPT was extra-mitochondrial but that the amount of extra-mitochondrial CPT increased with diabetes, fasting or a high fat diet.

More recently, Farrel et al (59) have found that extra-mitochondrial CPT is observed in liver from mice treated with clofibrate and that this cytosolic CPT can constitute as much as 40% of the total cellular CPT. One possibility raised by these investigators to explain this extra-mitochondrial CPT is that it may represent a precursor form of mitochondrial CPT which eventually contributes to the 3-5 fold increase in mitochondrial activity reported by Kahonen, Markwell and others (60, 61, 62) after clofibrate treatment. A precedent for an active precursor form of a mitochondrial enzyme is the model presented recently by Kolattakuddy (63) for mitochondrial malonyl CoA decarboxylase.

If it is agreed that the vast majority, if not all of cellular CPT is associated with the mitochondrion, how it is distributed with respect to the inner mitochondrial membrane is still a matter of dispute. A digitonin fractionation and sonication study of rat liver mitochondria by Hoppel et al (35) showed between 15 and 30% of total CPT to correspond to outer CPT. Bergstrom and Reitz (38) have found 20-25% of rat liver mitochondrial CPT digitonin extractable, the rest tightly bound. Yates and Garland (64) also found 20% of total rat liver mitochondrial CPT to be outer CPT; this value was in agreement with the amount of CPT they observed solubilized from the mitochondria by sonication.

Using the detergent Lubrol, Harano (65) found a 1:2 ratio of outer to inner CPT in rat liver mitochondria. A 1:2 ratio was seen using digitonin fractionation by Layzer et al (66) in mitochondria from normal and CPT deficient human sksletal muscle. Wood and Chang (36) using the detergent Triton X-100 reported a 1:2 ratio also for rat liver mitochondria but a 1:1 ratio for rat heart mitochondria assayed under identical

conditions. Bieber et al (67) reported approximately equal amounts of CPT on the two sides of the mitochondrial CoA barrier in livers of newborn, one-day and five-day piglets. A 1:1 ratio is also reported by Patten et al (68) for mitochondria of normal human skeletal muscle. Based on the proposed selective sensitivity of the outer CPT for the inhibitor malonyl-CoA, McGarry et al (69) concluded that one half of the total CPT is outside the rat liver mitochondrion; with sufficient malonyl-CoA, half of total CPT remained when palmitate oxidation, dependent on outer CPT, was totally inhibited. Finally, Swierczynski et al (70) reported that mitochondria from human term placenta oxidized palmitoyl-CoA + carnitine at half the rate of palmitoylcarnitine, implying a 1:2 ratio of outer to inner CPT.

Digitonin fractionation of mitochondrial compartments yields an underestimate for the proportion of total CPT on the outside of the inner membrane because the inner membrane itself begins to dissolve and leak interior marker enzymes before all of the "outer" CPT is extracted. Thus a concentration of digitonin giving a fraction of total CPT clearly defined by outer markers such as adenylate kinease or monoamine oxidase and containing none of the soluble interior enzymes such as fumarase or glutamic dehydrogenase will not have removed all of the external CPT.

The fraction of CPT outside the inner mitochondrial membrane is concluded to be between 20 and 50% of the total activity with liver mitochondria and heart and skeletal mitochondria, respectively, possibly representing these extremes. As with CAT, which is proposed by Tubbs and Chase (52) to be easily lost during preparation, the lowest values of outer CPT may represent a partial loss of the outer enzyme during

mitochondrial isolation.

Changes in the outer CPT/inner CPT ratio have been observed during development and as a result of infectious or genetically acquired disease. In many animals a rise in mitochondrial fatty acid oxidative capacity is noted during suckling and attributed (71) to the lipid content of milk with lowest levels in late gestation immediately before birth and also a decline to adult levels after weaning. Augenfeld and Fritz (72) have shown that changes in total liver mitochondrial CPT parallel those of oxidative capacity. Increases in liver mitochondrial CPT have also been reported during fasting (73), diabetes (74), and high fat diets (75), conditions associated with an increased concentration of circulating triglycerides and fatty acids.

Wolfe et al (76) has reported that high-fat diet-associated increased mitochondrial CPT activity in neonatal pigs is not specific to the transferase enzyme but reflects a higher mg mitochondrial protein per g wet tissue weight seen with the treatment. Perinatal induction of enzymes necessary for fatty acid utilization has been shown by Aprille (77) to be relatively independent of dietary intake with identical increases in fatty acid oxidation being seen with rabbits nest-reared on mother's milk, formula-fed a diet containing 6% lipid or an equicaloric one containing no fat. In rabbits the rate of oxidation of octanoate and laurate was found to be equal to that of their carnitine esters. There was only a two-fold increase in octanoate and laurate oxidation during the first four days of life while four-fold increases were observed for oxidation of the corresponding carnitine esters and of glutamate-malate. This suggests a lag in the development of either an

intramitochondrial fatty acyl synthetase for these medium chain fatty acids or of external carnitine acyltransferase activity which may exert significant control on their metabolism.

Similarly in the rat, based on carnitine-dependent CoASH release from palmitoyl-CoA in the presence and absence of detergent, Harano (65) reported adult levels of inner CPT but virtually absent outer CPT in the early gestation period. Outer CPT is seen to rise above adult levels after birth during suckling. Changes in palmitate oxidation, which is barely detectable in the fetus, are reported to parallel those of outer CPT activity. Tomec and Hoppel (78) reported palmitoyl-CoA + carnitine oxidation as 2-14% of palmitoylcarnitine oxidation in bovine fetal heart mitochondria but did not attribute the difference to a deficiency of outer CPT at this stage of development, citing instead an abnormal CoA saturation curve of fetal heart mitochodrial transferase activity. Likewise Bieber et al (67) presented evidence that CPT activity is equivalent on each side of the mitochondrial CoA barrier in newborn, one-day and five-day piglets despite the finding that though 24 hour old piglets had total CPT equal to that of adults (as measured by the isotope exchange assay), they oxidized palmitoyl-CoA at half the adult rate. Pace and Wannemacher (79) recently reported a decrease in outer CPT with no change in inner CPT in liver mitochondria of rats infected with Streptococcus pneumoniae.

Decreased utilization of fatty acids caused by systemic carnitine deficiency may trigger the same cellular response as that seen with high lipid levels induced by fasting, fat-feeding or diabetes to give the abnormally high CPT activity reported by Boudin et al (80) in liver,

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muscle, myocardium and kidney epithelium of a woman who died of progressive muscle weakness due to carnitine deficiency. More recently, Scholte et al (81) reported a case of a girl with systemic carnitine deficiency who died in acidosis whose inner CPT and palmitoyl-CoA synthetase levels were increased. A younger sister with decreased muscle and blood carnitine was found to also have increased muscle inner CPT but to show normal outer CPT.

The ratio of CPT outside to inside the mitochondrion may depend on the type of tissue, stage of development, concentration of carnitine, and possibly on the dietary or hormonal state. Hereditary deficiency of CPT activity therefore might be expected to present in various forms, depending on the tissues and membrane locations affected. In the first reported case of carnitine palmitoyltransferase deficiency described in 1973 by DiMauro and DiMauro (82), the now-classic sign of CPT deficiency, recurrent paroxysmal myoglobinuria, led to assay of CPT in muscle only, with the finding of very low (0-20% of normal) muscle CPT as measured by three CPT assay methods. A greater impairment of the isolated mitochondria to utilize palmitate than to oxidize palmitoylcarnitine in the presence of normal palmitoyl-CoA synthetase lead to a conclusion of a more severe defect of outer CPT than of inner CPT. Hostetler et al (83) reported finding a complete lack of outer CPT with normal inner CPT in muscle mitochondria of a patient with recurrent myoglobinuria. He also reported that the fasting plasma concentration of ketone bodies increased normally, an indication of normal liver CPT activity.

Two additional patients (68, 84) studied with decreased muscle CPT but normal ketogenic capacity gave a conflicting pattern of CPT deficit:

normal outer CPT but deficient inner CPT in muscle mitochondria. One of these patients (84) was also tested for CPT in leukocytes with the same finding of normal outer CPT and decreased inner CPT.

Leukocyte, platelet, fibroblast and skeletal muscle CPT were all decreased to 23-39% of normal levels in a patient recently described (66) presenting with recurrent paroxysmal myoglobinuria but normal ketones upon fasting. This patient appears to have a deficit of both inner and outer CPT activities: the same fraction of total CPT is extracted with digitonin from the patient's fibroblast mitochondria as from those of normals; the inner and outer CPT activities separated by detergent were each deficient to the same degree. Other CPT deficient individuals have been described, some with normal ketogenic function (85) and some with inadequate increase in ketones with fasting (86, 85), but with no attempt made to distinguish outer from inner CPT deficiency.

That a liver CPT deficiency is responsible for the low fasting ketone production in the latter patients is shown by the observation (86) of a prompt ketonemia after ingestion of medium chain triglycerides. The prominent symptom with or without presumed liver CPT deficiency inferred from decreased ketogenesis is recurrent myoglobinuria. A distinguishing symptom between the two may be greater muscle pain associated with the ketone insufficiency which was alleviated in one patient (85) by β-hydroxybutyrate.

Liver biopsy for determination of carnitine biosynthetic enzymes in patients with systemic carnitine deficiency has been performed and shown no deficit of the enzymes catalyzing the conversion of trimethyllysine to carnitine (99). Post-mortem findings of CAT deficiency with normal

CPT levels in liver and other tissues of a child who died of apparent neurologic and liver dysfunction led to a suggestion of a functional defect of acetyl-CoA utilization in brain mitochondria as a result of CAT deficiency. To date there have been no reports of liver biopsy to confirm the proposed hepatic CPT deficit in patients with recurrent paroxysmal myoglobinuria who are observed to have a diminished ketogenic response to fasting. One might also expect eventually to find an individual with deficient liver CTP and diminished ketogenic response but with normal muscle CPT activity.

Properties of Carnitine Acyltransferases

Substrate concentrations corresponding to half maximal activity in either reaction direction for purified pigeon breast muscle CAT are reported (87) to be independent of the concentration of the second substrate present, therefore a random order of addition of substrates is concluded. The catalysis by CAT of the formation of S-carboxymethyl-CoA-(-)-carnitine ester from CoASH and bromoacetyl-(-)-carnitine (88) suggests the formation of a ternary complex; it has been proposed (87) that the interconversion of such ternary complexes may be the rate limiting step for the reaction. Other CAT enzymes may not be as simple: acetylcarnitine is reported by one group (114) to inhibit purified rat liver mitochondrial CAT, this inhibition is reversed by carnitine but not by acetyl-CoA; double reciprocal plots of forward reaction activity by yeast CAT (25) indicate an ordered addition of the substrates carnitine and acetyl-CoA.

The reaction mechanism of CPT may also be a simple random addition of substrates as concluded by Edwards (89) for outer CPT, but interpretation of results reported is complicated by the hydrophobicity of the palmitoyl esters of carnitine and CoA and of free CoASH whose critical micellar concentrations have been observed to be 15 μM, 3-4 μM and 30 μM, respectively. Unlike the yeast CAT, calf liver mitochondrial CPT purified by Kopec and Fritz (90) shows no effect of carnitine concentration on the Km for acyl-CoA. The other three substrates, however, palmitoyl-CoA, CoASH, and palmitoylcarnitine, do affect their respective second substrates by raising their Km's.

Similar inhibition by palmitoyl-CoA has been reported by numerous researchers. In 1967 Bremer and Norum (32) proposed that besides being a substrate for the enzyme, palmitoyl-CoA also acted as a competitive inhibitor for carnitine with a Ki (3×10^{-6}) lower than its Km (10^{-5}) as a substrate. They concluded in a separate study on the effect of detergents on CPT (91) that the major effect of detergents was to prevent palmitoyl-CoA from acting as a competitive inhibitor of carnitine and acylcarnitine, with less effect on its function as a substrate in the reaction. Other reactions controlling the rate of fatty acid oxidation and energy production for which palmitoyl-CoA has been proposed to be a significant physiological inhibitor are palmitoyl-CoA synthetase (39), the mitochondrial citrate transporter (92), and adenine nucleotide translocase (93).

Changes in mitochondrial CPT activity with ionic strength--a near linear rise in CPT with increasing ionic strength up to 0.06 M--was correlated by Wood (94) with a parallel linear increased association of

palmitoyl-CoA with mitochondria in this range of ionic strength. She found (43) that CPT released from the mitochondrion by digitonin was not affected by changes in ionic strength, showing that the hydrophobic environment provided by the detergent differs significantly from that provided by the outer surface of the mitochondrial membrane. Similarly she attributed (95) changes in kinetic properties of heart mitochondrial CPT resulting from chronic ischmia as follows: "As a result of ischemia, changes in the lipid components in the membrane containing carnitine CPT were postulated: alterations in the hydrophobic environment of the enzyme produce interference in the binding of palmitoyl-CoA to the second substrate site and may result in a decrease in the Km of the enzyme for carnitine."

The nature of the interaction of another CoA ester, malonyl-CoA, with the outer mitochondrial CPT is also dependent upon the membrane association of the enzyme: McGarry and Foster (69) have reported that treatment of rat liver mitochondria with the detergent Tween-20 releases a malonyl-CoA insensitive CPT activity which they conclude is outer CPT which requires membrane association to be inhibited by malonyl-CoA. They have proposed (96) that malonyl-CoA and acetyl-CoA carboxylase may be largely responsible for control of fatty acid metabolism by the effect of the CoA ester on the outer CPT. Because malonyl-CoA is not a substrate for CAT and because the mitochondrial matrix contains a malonyl-CoA decarboxylase, the concentration of malonyl-CoA inside the mitochondria should not reflect that of the cytoplasm, so that inner CPT is not regulated by the concentration of this intermediate of fatty acid synthesis.

McGarry and Foster report that inner CPT is insensitive to malonyl-CoA when the lipid environment of the membrane is undisturbed by detergent but the membrane's integrity is disrupted by osmotic shock, freeze-thawing, or sonication to allow the otherwise impermeable malonyl-CoA access to the inner CPT. But as with outer CPT, this enzyme also changes with Tween-20 treatment, becoming more sensitive to malonyl-CoA, in contrast to the opposite effect of the detergent on the outer CPT. Fritz (41, 97) has reported that only membrane-bound CPT is inhibited by the d isomer of palmitoylcarnitine. Analogous to the ischemia-induced alteration of palmitoyl-CoA binding and outer CPT kinetics attributed by Wood to changes in the lipid environment of the inner mitochondrial membrane, starvation may influence the lipid composition and membrane environment of outer CPT to affect its interaction with malonyl-CoA. Such an influence would explain the recent finding of Cook et al (98) that starvation increases the apparent Ki of rat mitochondrial CPT for malonyl-CoA.

If changes in the membrane environment can change the kinetic properties of carnitine acyltransferase, perhaps the difference between the hydrophobic environment provided by the outer surface of the inner mitochondrial membrane and that provided by the inner surface could account for the difference in properties reported for outer and inner CPT's. (Perhaps tissue and species differences in mitochondrial CPT kinetics can be explained by differences in the lipid and protein composition of mitochondria from different sources.)

Acyl group specificity of carnitine palmitoyltransferases has been reviewed by Hoppel (18). He includes results received by personal communication from Edwards and Tubbs on the substrate specificity of inner

and outer CPT's. Portions of these data have appeared since in an abstract (89). The findings show that outer beef liver mitochondrial CPT (purified 850-fold to near homogeneity in the abstract) assayed in either direction has greatest activity with palmitoyl or myristoyl CoA or carnitine esters but has significant activity with nearly all even chain acyl substrates with values of 41 and 45% (relative to palmitoyltransferase) found for hexanoyl-CoA and hexanoylcarnitine, respectively. The inner CPT cited by Hoppel from the same data source, presumably the other activity found in the beef liver mitochondria, is much more specific for long chain acyl groups with relative activities of 15, 70 and 100 for octanoyl-, lauryl- and palmitoyl-CoA when assayed in the forward direction.

Three years earlier in 1971, Tubbs with West and Chase (113) reported a similar separation of inner and outer CPT from beef liver mitochrondria but with a different substrate specificity for the inner CPT when assayed for the reverse reaction with the 232 assay. In this direction the inner CPT gave equal V_{max} values for palmitoyl- and oxtanoylcarnitines and activity 50% higher for laurylcarnitine. It is possible that the specificity of inner CPT from beef liver differs for CoA and carnitine esters, however it is more likely that the much greater Km's for medium chain acylcarnitine substrates reported in the earlier study explain this discrepancy. Aside from the differences in substrate specificity, beef liver inner and outer CPT were reported to differ in the measures required for their solubilization, in their interaction with bromopalmitoyl-CoA (it inhibited the isolated outer enzyme but was treated as a substrate by the inner CPT), their chromatographic properties during ion exchange, and by their isoelectric points.

Kopec and Fritz (97) also isolated two CPT enzymes from beef liver mitochondria in 1971 and they purified one of them, designated CPT I, to homogeneity. This enzyme was specific for palmitoyl-and myristoylcarnitine with activity decreasing quickly below substrate carbon length of 14 carbons so that the relative activity of octanoylcarnitine was less than 2%. The protein fraction containing the other enzyme, CPT II, was even more specific for long chain fatty acylcarnitine with the rate for myristoyl transfer less than 20% that for palmitoyl-or stearoylcarnitine. Both CPT I and CPT II were more specific for long chain acyl substrates than the CPT's of West et al (113) though all came from the same tissue source; they differed more in substrate specificity from those of West et al than the latter enzymes differed from each other.

Assignment of CPT I and CPT II to sides of the inner mitochondrial membrane was not defined by their isolation procedure and was achieved by the production of antibodies against pure CPT I and their subsequent use to inhibit activity of soluble CPT I and CPT II and to inhibit CPT activity on the beef liver mitochondrial surfaces. In this way CPT I was shown to be the outer enzyme and CPT II to be on the inside. It was found however that CPT II, defined kinetically and by immunological cross reactivity, could be generated from CPT I by treatment with denaturing agents such as urea and guanidium chloride. CPT II and I were originally obtained by elution from calcium phosphate gel in the absence and presence of the detergent Tween-20, respectively. Incubations for their assay did not contain additional detergent so that differences in the environments of the two enzymes during assay may account for their kinetic differences.

Similarly the outer and inner CPT activities isolated by West et al were obtained from a lead acetate precipitate of the 20,000 g supernatant of a homogenate of frozen liver and from a butanol extract of the 20,000 g pellet, respectively. Again, the environments of the two enzymes may be sufficiently different to yield differing kinetic profiles, or one enzyme may be a partially denatured form of the other.

Differences in chromatographic behavior and isoelectric point of these inner and outer enzymes may also be a function of the method of their isolation due to the presence of contaminating lipids. A relatively hydrophobic protein with adhering phospholipids would be expected to have a lowered measured pI than in their absence, with the value of the pI depending on the relative abundance of different phospholipids. CPT studied by West et al had major peaks of activity with pI's of pH 4.8 and 5.7. There is a tendency for mitochondrial proteins to have pI's near pH 8. Markwell (100) reported that CAT isolated from rat liver peroxisomes and microsomes and that of pigeon breast muscle had pI values of pH 8.3, 8.3, and 7.9 respectively.

Members of the West group published a few years later a study (101) on the question of multiple forms of carnitine acetyltransferase which had been reported in the literature. They found that they were able to observe two forms of CAT in extracts of various animal tissues and attributed these to free and membrane-associated CAT with membrane association responsible for differing apparent molecular weight and isoelectric point. They concluded that the two forms were freely interconvertible with similar kinetic properties and suggested the existence of only a single type of CAT.

Similar kinetic properties of soluble and particle-bound enzyme were reported by Bremer and Norum in 1967 (40) for rat liver mitochondrial CPT. More recently Bergstrom and Reitz (38) have reported the similar nature of inner and outer CPT from rat liver mitochondria. After separation of outer and inner activities by digitonin the two enzyme fractions were subjected to identical purification procedures involving Tween-20 extraction, gel filtration and ammonium sulphate precipitation.

Binding of each protein to detergent micelles is apparently responsible for the 430,000 dalton molecular weight seen for each on gel filtration. Because of the masking effect of the detergent micelle, no difference in molecular weight such as that noted between the outer CPT purified by Edwards (89) (50,000) and CPT I of Kopec and Fritz (75,000) could be evaluated. Molecular weight estimates for CPT I given by the later group do not inspire confidence, however. From the near total exclusion of CPT I from a P-150 gel filtration column in the presence of the detergent Tween-20, they subsequently reported CPT I to be a dimer of molecular weight 150,000, a conclusion with little justification considering the expected association of the protein with detergent micelles and the inate unreliability of estimating molecular size of an excluded molecule.

Though the enzymes isolated by Bergstrom and Reitz were not purified sufficiently to allow monomer molecular weight estimate, their Km values for the substrates of forward and reverse reactions are nearly identical as are the relative rates of the two enzymes in the two directions. From their results and the absence of a more definitive study of the physical and kinetic properties of CPT from inner and outer surfaces

of the inner mitochondrial membrane, the possibility cannot be excluded that the same protein may catalyse the transferase reaction at the two locations.

THE PROBLEM--CARNITINE OCTANOYLTRANSFERASE

It was probably Ephriam Racker who said, "One clean experiment is worth a thousand dirty calculations." Along this line, H. Solberg stated as a dedication/credo to her doctoral thesis on the "Acyl Group Specificity of Carnitine Acyltransferases" (102) the words, "Don't waste clean thoughts on crude enzymes." But she accompanied this thought with a second, "Life is too short for enzyme purification." Among the fruits of her labors in the middle ground between the frustrating extremes of the uncertainty of working with crude enzymes and the tedium of enzyme purification, was evidence for the existence of a third carnitine acyltransferase, carnitine octanoyltransferase (COT).

Carnitine palmitoyltransferase as purified to homogeneity by Kopec and Fritz from calf liver (97) was seen to be quite specific for the transfer of long chain fatty acyl residues with activity dropping dramatically with decreasing substrate carbon length below myristoylcarnitine so that the octanoyltransferase activity of this enzyme was reported as less than 2% of that of the palmitoyltransferase. Carnitine acetyltransferase purified to homogeneity from pigeon breast muscle by Chase et al (103) was reported to be correspondingly specific for short chain acyl residues with a precipitous drop in activity of the pure enzyme with substrates of more than four carbons.

Solberg showed that a commercially available preparation of CAT (104) revealed a second peak of activity with maximal activity for acyl

groups between six and nine carbons, apparently containing a co-purifying contaminant carnitine acyltransferase specific for medium-chain acyl esters. She found evidence for the presence of this COT in substrate specificity profiles of carnitine acyltransferase activity in mitochondria of various rat tissues and reported that the optimum chain length of substrates for the inner pool of carnitine acyltransferase activity of rat, mouse and calf liver mitochondria (34) is seven carbons while that of the outer mitochondrial transferase of rat and mouse liver was nine or ten carbons. Thus the existence of a separate carnitine acyltransferase protein specific for medium chain length fatty acid groups was proposed and its purification left to others.

During the isolation of homogenous CPT from calf liver mitochondria, Kopec and Fritz (97) noted fractions differing in substrate specificity from the published CAT profile and from that of CPT when pure. One of these fractions not pursued further was an extract of calf heart mitochondria with activity centered about a peak of the substrate octanoylcarnitine. Markwell et al (105) reported that rat liver mitochondria contained six times as much COT as CAT activity and that COT activity was present in amounts equivalent to CAT in peroxisomes and microsomes of rat liver. These organelles were devoid of CPT and the CAT subsequently purified from them (26) was clearly separated from COT activity. (Subsequent studies by Valkner and Bieber (106) have shown that in microsomes, the location of the two enzymes is different, with CAT associated with both sides of the microsomal membrane and COT on the cytosolic surface exclusively.) Attempts by Markwell et al (26) to isolate and stabilize COT activity from these organelles were unsuccessful.

Medium chain fatty acids are produced by the mammary glands of some mammals such as the goat (107) but are otherwise not plentiful in the diets of the organisms in which COT has been detected. In fact, the provision of medium chain fatty acids and triglycerides for individuals with genetically impaired transfer of long chain fatty acyl groups is an expensive therapy due to their relatively low natural abundance. It has seemed unlikely until recently that medium length fatty acids or acyl residues have any role in normal metabolism other than as short lived intermediates in fatty acid synthesis or β-oxidation.

The recent possible exception involves β-oxidation discovered in liver peroxisomes (108) during which there is evidence that the breakdown of long chain fatty acyl-CoA to acetyl-CoA may be halted before its completion (109) so that medium chain fatty acyl groups are the end product. If CoASH is to be recovered from the acetyl and medium chain acyl esters and if these groups are to be utilized elsewhere in the cell, a function for a mitochondrial COT enzyme can be inferred as well as those of peroxisomal CAT and COT. This perosizome-associated role for mitochondrial COT had not been proposed when the present investigation was initiated, however, and many workers in the field (110) discounted the significance of COT activity and the existence of a separate medium chain acyltransferase in mitochondria. A reasonable argument could be made for the evidence of the existence of mitochondrial COT being artifactual, considering the uncertainty involved in trying to apply clean thoughts to crude enzyme preparations. It was decided that at the present stage of knowledge and inquiry into the roles of carnitine and the carnitine acyltransferases, life was not too short for the enzyme purification required to

either confirm the existence of mitochondrial COT or to clarity why one appears to exist.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials

Coenzyme A and coenzyme A-esters were from P-L Biochemicals. Carnitine was a generous gift from Otsuka Pharmaceutical Co. and Sigma Tau farmaceutici. Ampholines and hydroxylaptite were from Bio-Rad. Sephadex and Sepharose chromatography media were from Pharmacia. Fluorescamine, Tween-20, Triton X-100 and 5,5'-dithio-bis-(2-nitrobenzoic acid) were from Sigma. DL-(methyl-^{14}C)-carnitine hydrochloride was from Amersham. 2-p-Toluidinylnaphthalene-6-sulfonate (TNS) was from Eastman.

Methods

Mitochondrial Isolation

Beef hearts were obtained from a local abbatoir. Immediately upon removal from the carcass, the heart was sliced and packed in ice for transport to the laboratory. All subsequent procedures were performed at 4°C. The ventricle was cleaned of all fat and connective tissue and cut into thin slices. Batches of 60 g of ventricle were homogenized in 500 ml of Sucrose Isolation Buffer (0.25 M sucrose, 5.0 mM HEPES, 0.25 mM EDTA, pH 7.7) for 45 seconds at high speed in a Waring blender. To insure more complete release of mitochondria, this was followed by a 30 second Polytron homogenization using the large probe in batches of 220 ml. Mitochondria were isolated from the homogenate by differential

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centrifugation employing 15 minute spins at 500,15,000, 500, 11,000 and 7000 x g in a Sorval RC-2 centrifuge using the 55-34 and the GSA rotors. High speed pellets were resuspended in Isotonic Sucrose Buffer (0.25 M reagent grade sucrose, 2.5 mM HEPES, 0.25 mM EDTA, pH 7.5) using two strokes of a loose fitting Potter-Elvejhm Teflon-glass homogenizer. The 7000 x g pellet was resuspended in a minimal volume of isotonic sucrose buffer, assayed for enzyme activities and protein, and stored at -80°C.

Solubilization of Mitochondria

Mitochondrial suspensions from five beef hearts were thawed, pooled and added to one half volume of 3 M KCl in 6% Triton X-100. The suspension was inverted for mixing and then treated batchwise with six passes of the Teflon-class homogenizer at 15°C. After centrifugation for 90 minutes at 89,000 x g (29,000 rpm) in the Type 30 rotor, a clear apricot-colored supernatant fluid containing solubilized mitochondrial protein was pipetted from between floating lipid and the flocculant surface of the mitochondrial pellet.

Carnitine Acyltransferase Assays

The forward reaction was assayed at 412 nm by the DTNB method of Bieber et al.(30). The 200 ul reaction volume contained 115 mM Tris-HCl, 1.1 mM EDTA, 0.1% Triton X-100, 1.25 mM l-(-)-carnitine, 250 μM DTNB and 100 μM acyl CoA at pH 8.0 and 25°C. The reverse reaction was assayed by the method of Srere et al (33) in which CoA thioester bond formation is monitored at 232 nm. The 200 ul reaction volume contained 0.2 M Tris-HCl, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 0.1% Triton X-100, 60 μM CoASH and 500 μM acylcarnitine at pH 7.45 and 35°C.

Sephadex G-200 Chromatography

Solubilized protein from beef heart mitochondria (10 ml) was chromatographed on a 90 cm x 1.8 cm column of Sephadex G-200 equilibrated with Isotonic Sucrose Buffer containing 1.0% Triton X-100 and 1 M KCl; 4.0 ml fractions were collected. Catalase and hemoglobin were similarly chromatographed as standards. Void and bed volumes were determined using Blue Dextran and $K_3Fe(CN)_6$, respectively.

Comparison of Beef Liver and Heart Carnitine Acyltransferase Activities

Heart mitochondria were isolated and solubilized as described above. Beef liver was treated in an identical manner with two exceptions: (1) the ratio of liver tissue to Sucrose Isolation Buffer used for homogenization was 140 g: 420 ml and (2) the polytron homogenization/sonication step was omitted. The solubilized protein solutions from liver and heart mitochondria were chromatographed separately on a 112 cm x 4.8 cm column of Sephadex G-100. Samples of 100 ml containing approximately 15 mg protein/ml were applied to the columns and 24 ml fractions were collected.

Purification of COT Activity-Containing Proteins from Beef Heart Mitochondria

Beef heart mitochondria were solubilized as described above. The mitochondrial protein solution was equilibrated with Blue Buffer (2% Triton X-100, 2.5 mM HEPES, 0.25 mM EDTA, 60 mM KCl, pH 7.5) by exhaustive dialysis and applied at a flow rate of 130 ml/hr to a 75 cm x 4.1 cm column of Cibacron Blue Sepharose 4B equilibrated with Blue Buffer.

Four bed volumes of buffer were then passed through the column to wash off unbound proteins before a 1500 ml linear gradient of 60-860 mM KCl in Blue Buffer was used to elute carnitine octanoyltransferase (COT) activity.

Fractions eluting from the Blue Sepharose column containing both COT and carnitine acetyltransferase (CAT) activities (fractions 31-34) were pooled and passed through a 112 cm x 4.8 cm column of Sephadex G-100 equilibrated with CM Buffer (1% Triton X-100, 5.0 mM HEPES, 0.25 mM EDTA, 60 mM KCl, pH 7.3). Octanoyltransferase-containing fractions from the gel filtration column were pooled and applied at a flow rate of 80 ml/hr to a 29 cm x 4.1 cm column of CM-Sepharose CL-6B equilibrated with CM Buffer. The column was washed with four bed volumes of CM Buffer and then eluted with a 1000 ml linear gradient of CM Buffer containing 60-560 mM KCl. Fractions containing COT activity were pooled (117 ml), diluted with 117 ml of 20 mM KPO_4, pH 6.6, and applied at a flow rate of 24 ml/hr to a 13 cm x 2.2 cm column of hydroxylapatite previously equilibrated with HAP(-) Buffer (0.1% Triton X-100, 10 mM KPO_4, 60 mM KCl, pH 6.8). Four bed volumes of HAP(+) Buffer (identical to HAP(-) Buffer but with the addition of 0.25 mM EDTA) were used to wash the column before elution with a 200 ml linear gradient of HAP(+) Buffer containing 10-150 mM KPO_4, pH 6.8. Fractions having COT of a constant specific activity were pooled.

Fractions eluting from the Blue Sepharose column with both COT and carnitine palmitoyltransferase (CPT) activities (fractions 40-48) were pooled and passed through a 112 cm x 4.8 cm column of Sephadex G-100 equilibrated with QAE Buffer (1.0% Triton X-100, 5.0 mM Bis-Tris Propane Buffer, 0.25 mM EDTA, 20 mM KCl, pH 9.7). Carnitine octanoyltransferase-

containing fractions were pooled and applied at a flow rate of 30 ml/hr to a 48 cm x 4.1 cm column of QAE Sephadex Q-25-120 equilibrated with QAE Buffer. The COT activity which washed through the column was pooled, dialyzed against HAP(+) Buffer, and loaded at a flow rate of 24 ml/hr onto a 11.5 cm x 2.2 cm column of hydroxylapatite which was equilibrated with HAP(+) Buffer and eluted with 400 ml HAP(+) Buffer containing a 10-510 mM linear gradient of KPO_4, pH 6.8. Fractions containing COT of a constant specific activity were pooled. The stock enzyme was stored at 4°C at a concentration of 40 µg protein/ml in the presence of 0.025% (v/v) Triton X-100. (All assays contained enzyme at 2 µg/ml and Triton X-100 at 0.0013%. This is below the CMC of 0.012% determined for Triton X-100.)

CMC Determinations

Critical micellar concentrations of acylcarnitines and non-ionic detergents were measured by the fluorescence method of Horowitz (131) using TNS (2-p-Toluidinylnaphthalene-6-sulfonate). The 2.0 ml reaction volume contained 0.2 M Tris-HCl, 1 mM DDT, 0.5 mM EDTA, 11.0 mM TNS, 60 µM CoASH and varying concentrations of acylcarnitine or non-ionic detergent at 25°C and pH 7.70. Relative fluorescence measurements were made on an Aminco-Bowman Spectrofluorometer.

Interaction of Lauryl-^{14}C-carnitine with Micelles of Tween-20

A 36 x 1.2 cm (I.D.) column of Sephadex G-100 was equilibrated at 25°C with a buffer solution (0.2 M Tris-HCl, 1 mM DTT, 0.5 mM EDTA, 60 µM CoASH, pH 7.7) containing 700 µM ^{14}C-lauryl-l-carnitine at 12,400

cpm/umole. After equilibration, a 2.0 ml aliquot of the equilibration solution, but with the addition of 0.4% (v/v) Tween-20, was applied to the column and 1.0 ml fractions were collected. For each fraction, the entire 1.0 ml volume was added to 10.0 ml of scintillation cocktail (1 liter toluene, 1 liter Triton X-100, 4 g PPO and 100 mg Dimethyl POPOP) for radioactivity determination. An identical gel chromatographic procedure was performed using non-labeled lauryl-l-carnitine and fractions were assayed for the presence of micelles by the method of Horowitz (131) to determine the elution volume of Tween-20. In both procedures, the lauryl carnitine concentration (700 μM) was below its CMC (1050 μM) while the non-ionic detergent Tween-20 concentration of 0.4% was in great excess of its CMC (0.0003%).

Other Methods

Carnitine esters were synthesized as described (120, 121). The synthesis of lauryl-^{14}C-carnitine was identical except that a trace amount of DL-(methyl-^{14}C) carnitine hydrochloride was added to the unlabeled L-carnitine before the synthesis. Blue Sepharose 4B was synthesized by the method of Bohme et al (124). Isoelectric focusing was performed in a sucrose density gradient after the method of Vesterberg (125). SDS-PAGE of purified proteins was performed by the method of Laemmli (122) employing bovine serum albumin, catalase, fumarase, and chicken egg albumin as molecular weight standards. Catalase was assayed by the method of Boudhuin et al (126). Protein was estimated with bovine serum albumin as standard according to the method of Lowry et al (127) and, for solutions containing detergent, by the method of Bohlen et al (128). All chromatographic columns were treated with dimethyldichlorosilane.

Preliminary Investigations

Solberg (104) found different carnitine acyltransferase specificities in mitochondria from various organs of the rat. The investigation by Kopec and Fritz (90) of carnitine acyltransferase activities in calf tissue revealed extracts specific for octanoylcarnitine from the heart mitochondria. In deciding to pursue COT-containing enzymes in beef heart mitochondria we foresaw two significant limitations to the interpretation of the results: (1) Release of mitochondria from muscle, skeletal or cardiac, requires more severe treatment of the tissue than their release from most other organs such as liver, kidney, testis or brain. Longer homogenization is required to effectively break down the cells and a greater dilution of the homogenate is required to overcome the gel formed by broken down myosin in order to allow the mitochondria to sediment. It is possible that the harsh mitochondrial isolation procedure causes a relative enrichment of an inner COT due to a greater loss of COT on the outer surface. (Tubbs and Chase (52) proposed that the outer CAT is more easily lost in mitochondrial isolation. The outer CPT of West et al (113) was obtained in the 20,000 g supernatant of ox liver homogenized after being stored frozen. Yates and Garland (64) concluded that the CPT released by mild sonication of rat liver mitochondria was the outer activity.)

(2) At least two species of mitochondria are recognized in heart muscle, intermyofibrillar and subsarcolemmar (115, 116). The former are more easily released by mechanical disruption of the tissue and the latter best obtained after sonication and/or limited treatment with a protease

such as nagarse. Nagarse treatment is reported (117) to preferentially attack proteins of the outer mitochondrial compartment, effectively sparing CPT but decreasing long chain fatty acyl synthetase activity. Because its effects on a possible outer COT were unknown, nagarse was not used in our mitochondrial isolation. Limited sonication, however, was employed as it allowed a near doubling of the yield of mitochondrial protein and carnitine acyltransferase activities. Thus because of the tissue and methods required, our mitochondrial preparation would most likely be a mixture of interfibrillar and subsarcolemmar mitochondria.

Beenakkers and Klingenberg (53) reported that rat heart CAT was completely solubilized by repeated extraction without the use of detergents or exhaustive sonication to disintegrate the inner mitochondrial membrane. Markwell et al (26) found that CAT associated with the microsomal membrane could be released with 0.4 M KCl. Reitz (118) saw that detergent was required to solubilize CPT from rat liver and heart mitochondria and that 98% of total CPT is released by treatment with a solution of KCl and Triton X-100. Using salts and detergent, therefore, the extraction of COT activity from beef heart mitochondria was compared to that of CAT and CPT. The results of the extraction experiments are shown in Table I. It is seen that as in other tissues, CAT is preferentially extracted with salts and CPT with detergent. COT is extracted by both measures, and in these mitochondria, with an approximate 70:30 CAT-like:CPT-like profile of membrane association.

The most significant findings here are inferred from the ratios of COT to CAT or CPT in the extracts: (1) the ratio of COT:CAT in the salt extract is approximately equal to that value which we have observed in

Table I. Solubilization Experiments

Carnitine Acyltransferase	A. 0.25 M Sucrose Freeze-Thaw total nmoles CoASH released			B. 0.5 M KCL Freeze-Thaw total nmoles CoASH released			C. 0.5% Triton X-100 total nmoles CoASH released		
	Pellet	Super	% Solubilized	Pellet	Super.	% Solubilized	Pellet	Super.	% Solubilized
Acetyl (CAT)	9470	529	5.3	618	1550	71.5	1368	262	16.1
Octanoyl (COT)	8456	452	5.1	824	1275	60.8	1103	344	23.8
Palmitoyl (CPT)	1150	71	5.8	224	40	15.2	50	163	76.5

Beef heart mitochondria isolated in icotonic sucrose buffer at a concentration of 38 mg protein/ml were diluted 1:5 (v/v) in 0.25 M sucrose (A above), 0.624 M KCl (B), or 0.625% Triton X-100 (C). Carnitine acyltransferase activity per minute in pellet and supernatant fluid after centrifugation (15 min. at 25,000 x g) has been corrected for background acyl-CoA hydrolase activity. Differing total volumes were used in A, B, and C.

pure commercial pigeon breast muscle CAT. Commercial CAT no longer contains the medium-chain acyltransferase peak reported by Solberg (104) so that the COT activity in the pigeon enzyme preparation and that in the salt extract of beef heart mitochondria might reasonably be interpreted as that catalyzed by the CAT protein, similar to the transfer with acetyl-CoA and butryl-CoA, but at a lesser relative rate. (2) Detergent extraction yields a solubilized protein fraction with COT activity greater than that of CAT or CPT. This fraction was interpreted as corresponding to those reported from calf heart by Kopec and Fritz (90) and to contain a COT protein and possibly also a heart mitochondrial CPT. It is seen from the extraction results that though detergent treatment gives a protein fraction apparently specific for octanoyl transfer, the COT activity associated with the much more abundant CAT enzyme accounts for the majority of carnitine octanoyltransferase in these mitochondria.

Initial attempts to purify the KCl and detergent solubilized COT-containing proteins by ion-exchange chromatography were largely unsuccessful; i.e., less than half of the activity applied was eventually recovered using cation exchangers phosphocellulose and carboxymethyl-Sephadex. (It had been decided that since the object was to identify all enzymes with COT activity, such losses during any one purification step were unacceptable.) During an initial experiment with DEAE-cellulose at pH 8.5, again more than half of the activity was lost. However a significant fraction of COT activity passed through the column without being retarded. CAT activity was greatly decreased in the non-binding fraction. This fraction was chosen for a substrate chain length specificity profile employing the limited stock of acyl-CoA esters synthesized by a previous

worker in the laboratory; their purity and degree of hydrolysis were unknown and were not defined in this study. The resulting specificity profile is shown in Figure 1. This apparent medium chain acyltransferase is clearly different from the CPT purified by Kopec and Fritz (90) from calf liver mitochondria (see pp. 23-24 above).

The differential extraction of COT into CAT-like and CPT-like proteins using salts and detergent suggested that if carnitine acyltransferase activities were extracted by a combination of these agents, the detergent-extracted enzymes would associate with detergent micelles and migrate separately from salt extracted proteins during gel filtration chromatography. Figure 2 shows this separation on Sephadex G-200 where the micellar transferase has an apparent molecular weight of 530,000 and the free enzyme migrates corresponding to a value of approximately 60,500. Again the ratio of COT:CAT in the free protein peak (1:2.8) is similar to that of commercial pigeon breast muscle CAT.

In the micellar peak there is greatest activity with medium length substrates, though the pattern is not identical to that found for the protein which passed through DEAE-cellulose at pH 8.5 (Figure 1). This difference in substrate specificity is attributed to a difference in substrates; for the G-200 profiles, commercial acyl-CoA esters of uniform purity were obtained. The two peaks of COT activity in Figure 2 represent virtually all of the carnitine acyltransferase activities present in the intact mitochondria. Less than 10% of total CAT, COT and CPT activities was lost during each of the isolation procedures: solubilization and gel filtration.

Figure 1. Substrate specificity of carnitine acyltransferase in void volume of DEAE-cellulose experiment.

A solution of beef heart mitochondrial protein solubilized using 1 M KCl/1% Triton X-100 and a column of DEAE-cellulose were equilibrated with Tris buffer at pH 8.5 containing 60 mM KCl, 1 mM EDTA and 1% Triton X-100. Transferase activity was measured by the DTNB assay.

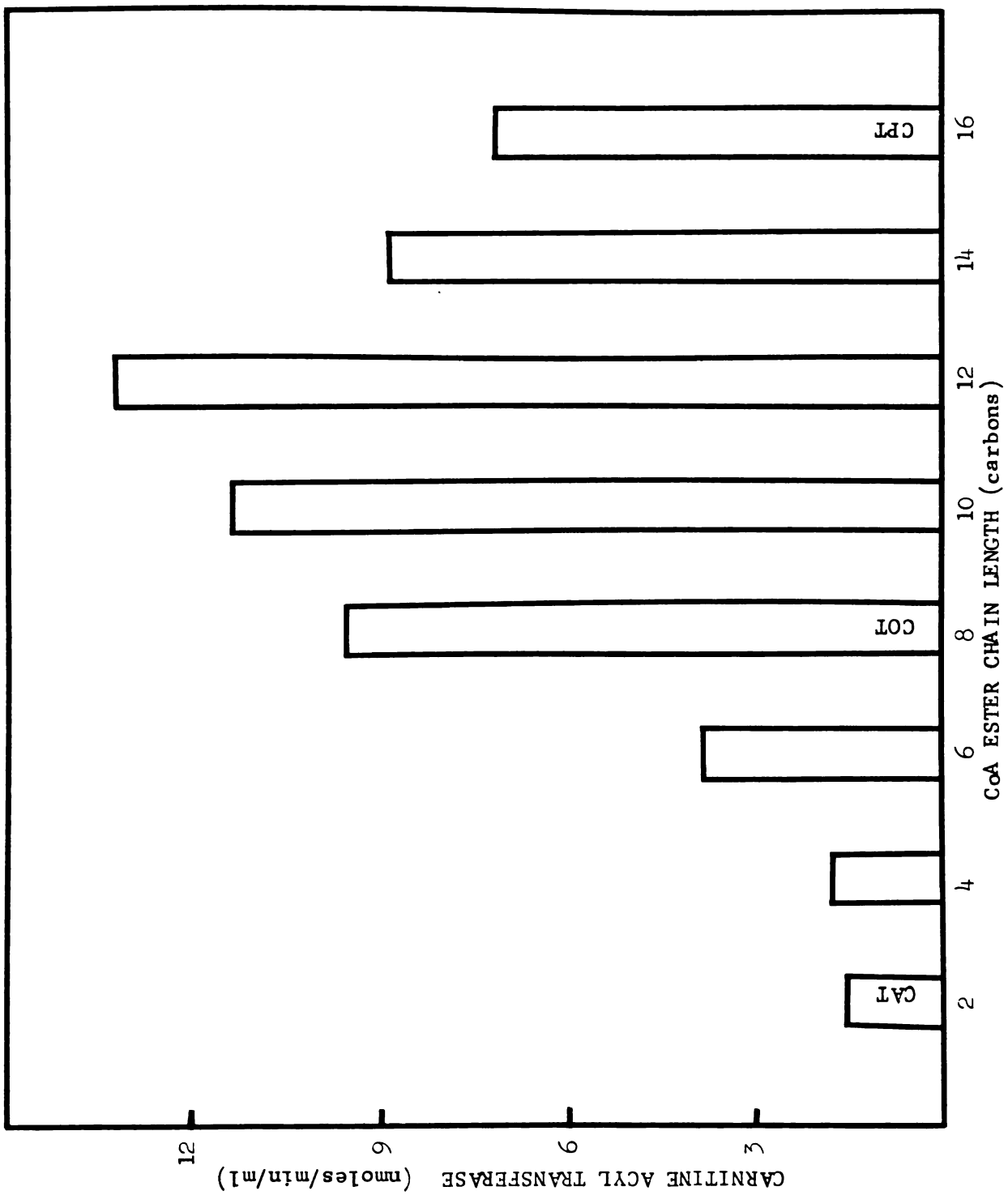
CARNITINE ACYL TRANSFERASE (nmoles/min/ml)
12
9
6
3
CAT
COT
CPT
2
4
6
8
10
12
14
16
CoA ESTER CHAIN LENGTH (carbons)

Figure 2. Sephadex G-200 gel filtration chromatography of beef heart mitochondrial carnitine acyltransferase.

Mitochondrial protein solubilized with 1 M KCl/1% Triton X-100 was chromatographed in sucrose buffer (10 mM HEPES, 1 mM EDTA, 0.25% (w/v) sucrose, pH 7.5) containing 200 mM KCl and 1% Triton X-100. Bed and void volumes were determined using potassium ferricyanate and blue dextran, respectively.

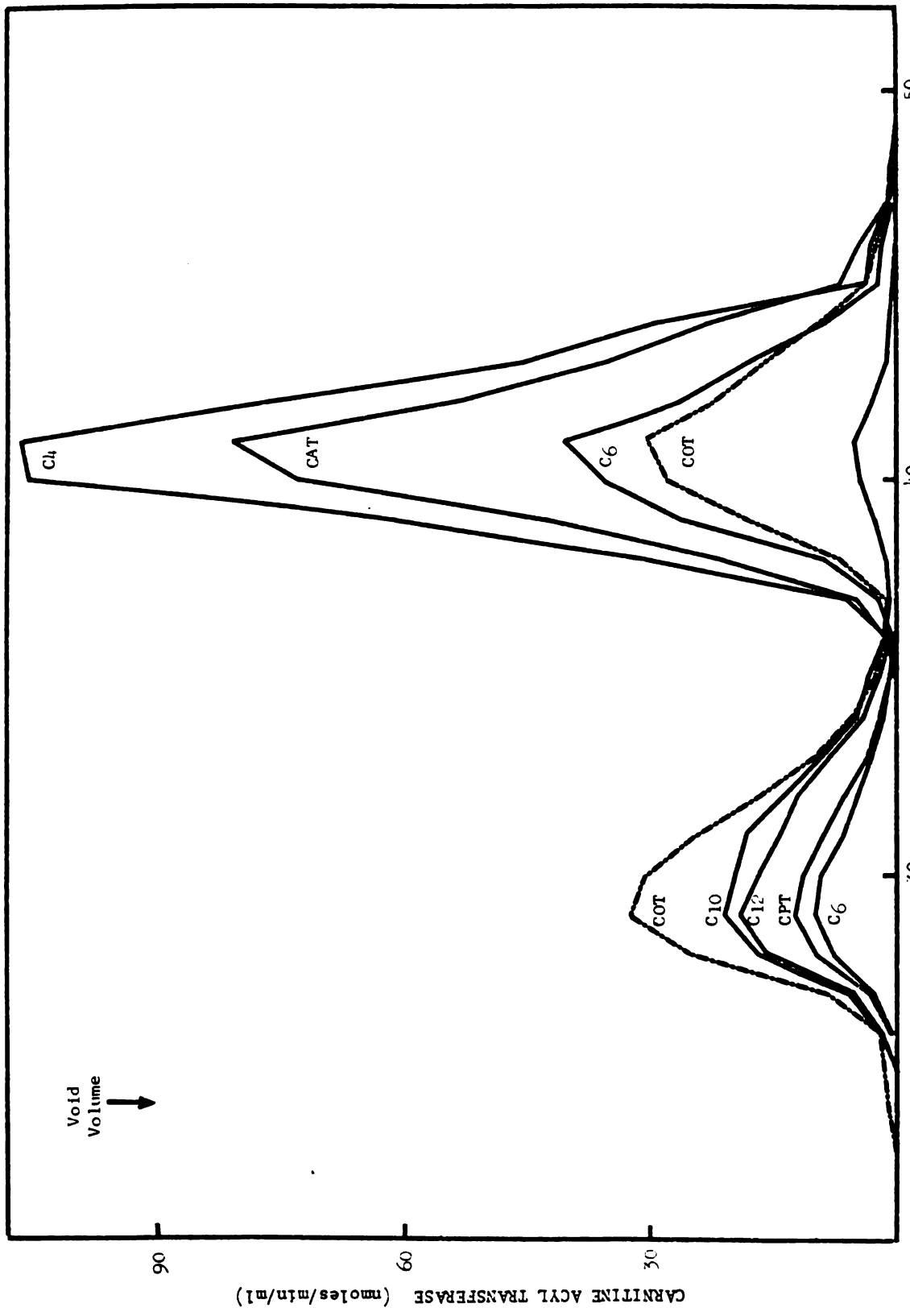
CARNITINE ACYL TRANSFERASE (nmoles/min/ml)
90
60
30
Void Volume
C4
CAT
C6
COT
COT
C10
C12
CPT
C6
30
40
50
FRACTION NUMBER

Figure 2

It was decided to attempt to purify the detergent solubilized COT-containing protein to homogeneity in order to compare its properties and kinetics with those of previously reported carnitine acyltransferases. A number of chromatographic procedures were evaluated for fold purity and percent recovery of COT activity. Commercially available CoA-Sepharoses with the coenzyme attached at the -SH and the ribose positions (PL Biochemicals #s 5504 and 5491, respectively) had no selective affinity for COT under the conditions employed. Nor did carnitine when joined by ether linkage at the β-OH moiety to a spacer arm and thence to Sepharose as synthesized from epoxy-activated Sepharose (Pharmacia) and l-carnitine in the laboratory.

As mentioned above, all attempts at cation exchange were unsatisfactory. Anion exchange was pursued and revealed that as high as pH 10.2 using QAE-Sephadex and the buffer bis-tris propane, COT activity is unretarded with minimal loss of activity while most proteins do bind to the column. Another powerful technique with binding specificity and minimal loss was ersatz-affinity chromatography using Cibacron Blue-Sephadex (Bio-Rad). The enzyme apparently recognizes the dye ligand as a CoA-like nucleotide and is easily extracted by KCl at 300 m$\underline{M}$. Expensive attempts to specifically elute COT from Blue-Sephadex using CoASH, NAD^+ and other adenine nucleotides resulted in no improvement over KCl elution. Elution with a gradient of carnitine as a substitute for KCl also gave no improvement; the COT activity eluted at the same ionic strength with d,l-carnitine-HCl or KCl as the salt used. Finally hydroxylapatite, used by Kopec and Fritz (90) to purify calf liver mitochondrial CPT, bound beef heart mitochondrial COT and released it at

higher phosphate concentration with an acceptable recovery of activity.

Using these methods--KCl/Triton X-100 quantitative solubilization of COT activity, gel filtration chromatography to separate free and micelle-bound acyltransferase proteins, and Blue-Sephadex, QAE-Sephadex and hydroxylapatite chromatography to purify the detergent-extracted COT activity--a COT-containing protein of greater than 90% purity was obtained and some of its properties were examined. Some of these findings are shown in Figures 3-6 and Tables II-IV, on pages 48-58. These data were originally prepared as a poster presentation (119). To summarize these results, a near homogeneous protein of monomer molecular weight approximately 67,000, pI 8.1 and pH optimum between pH 7.4 and 8.4 was isolated from beef heart mitochondria having optimum acyltransferase activity with the substrate nonanoyl CoA, greatest even-chain activity with decanoyl CoA, octanoyl activity greater than palmitoyl, and vanishingly small activity with substrates of less than six carbons in length.

Thus it was shown that beef heart contains a carnitine acyltransferase specific for medium chain length substrates as assayed in one direction. More work was required to synthesize the various acylcarnitine esters in order to assay the enzyme in the opposite direction, the reverse reaction, and thereby compare its specificity with those published for CPT by West *et al* (113) and by Kopec and Fritz (90) using the 232 assay. The even carbon length esters of l-carnitine from acetyl to palmitoylcarnitine were synthetized by the short and long chain methods of Bremer (120, 121); these results are summarized in Table V. Using these substrates, the relative activities of the beef heart enzyme using octanoyl decanoyl-, lauryl-, myristoyl- and palmitoylcarnitine were determined under

Table II. Abstract of COT Studies

ABSTRACT #644

STUDIES ON CARNITINE OCTANOYL TRANSFERASE IN BEEF HEART AND LIVER MITOCHONDRIA P.R.H. Clarke and L.L. Bieber, Biochemistry Dept., Michigan State University, East Lansing, MI 48824

Carnitine octanoyl transferase activity is present in two fractions separable by gel filtration chromatography of extracts from solubilized beef liver and heart mitochondria. The two peaks of activity represent the micellar form of a membrane protein referred to as carnitine octanoyl transferase (COT) and a soluble protein of molecular weight approximately 60,000 daltons emerging from the gel at the same position as the carnitine acetyl transferase (CAT) of the mitochondrial matrix.

In heart mitochondrial extracts, 30 % of the total COT activity is found associated with the membrane protein while the remainder is found in the 60,000 dalton peak. In liver mitochondria, where the highest carnitine acyl transferase activities are for middle length chain fatty acyl CoA esters, fractionation of solubilized mitochondria reveals the bulk of COT activity in the membrine protein fraction.

The membrane COT protein has been purified from beef heart mitochondria and partially characterized. The enzyme has an isoelectric point of 8.3, similar to that reported for the CAT protein, and shows maximal activity with nonanoyl Coenzyme A as acyl donor. The purification and kinetics of the enzyme with respect to middle length chain fatty acyl CoA esters will be presented. (Supported in part by USPHS N.I.H. grant #AM 18427).

Table III. Purification Procedure

PURIFICATION OF CARNITINE OCTANOYL TRANSFERASE FROM BEEF HEART MITOCHONDRIA

Mitochondrial Isolation

Beef hearts were obtained from a local abattoir, where they were sliced in half and packed in ice for transport to the laboratory. All subsequent procedures were performed at 4°C. Connective tissue was cut away from the ventricle which was then sliced into thin pieces, diluted 1 : 8 with Sucrose Buffer (0.25 M sucrose, 5 mM HEPES, 0.25 mM EDTA, pH 7.5), homogenized for 45 seconds in a Waring blender at high speed, and finally treated for 30 seconds with a polytron homogenizer using the large blade/probe. Mitochondria were isolated from the homogenate by differential centrifugation employing spins at 650, 15,000, 650, 11,000 and 7000 x g. The final mitochondrial suspension, yielding approximately 1.2 g of mitochondrial protein per Kg cleaned ventricle, was assayed for carnitine octanoyl transferase activity and frozen at -80°C for later use.

Solubilization

Frozen mitochondrial suspensions from eight beef hearts were thawed, pooled and assayed for protein and enzyme activity. To two volumes of thawed mitochondrial suspension at 19 mg protein/ ml was added one volume of a solubilization solution containing 6% Triton X-100 and 3 M KCl. The mitochondria solution was centrifuged for 90 minutes at 85,000 x g and the supernatant liquid retained as solubilized mitochondrial protein.

Purification

The mitochondrial protein solution was chromatographed in batches over Sephadex G-100 to separate carnitine acyl transferase activities into micellar and free protein populations. The micellar peak fractions of COT activity were pooled and dialyzed against HEPES Buffer (1% Triton X-100, 20 mM KCl, 0.25 mM EDTA, 2.5 mM HEPES, pH 7.5) and centrifuged to remove protein precipitated by the decrease in ionic strength. The supernatant containing COT activity was applied to a Ciba-cron blue Sepharose column to which the activity bound and was eluted by a 20 - 400 mM gradient of KCl in the HEPES Buffer. Peak fractions were pooled, dialyzed against a Bis-Tris Propane Buffer (1% Triton X-100, 20 mM KCl, 0.25 mM EDTA, 5.0 mM Bis-Tris Propane, pH 9.7) and applied to a column of QAE-Sephadex. COT activity did not bind to the column, but was washed through. Finally, the solution containing COT activity was dialyzed against a Phosphate Buffer (1% Triton X-100, 20 mM KCl, 0.25 mM EDTA, 10 mM K^+ Phosphate, pH 6.8) and applied to a hydroxyl apatite column equilibrated with the same buffer sans EDTA. A linear 10 - 400 mM K^+ Phosphate gradient eluted a peak of COT activity which is 700-fold enriched over that assayed in the original mitochondria and is approximately 90% pure by analysis of stained protein banding on SDS polyacrylamide gels.

Table IV. Purification Results

Purification Results

Purification Step	Volume (ml)	Units (umoles/min)	Protein (mg)	Specific Activity	Fold	Percent Recovery
Thawed Mitochondria	628	1079 (335)‡	11,995	0.0899 (0.0279)‡	1.0	100 (31)‡
Solubilized Mitochondria	1084	993	10,623	0.0935	1.04	92
Sephadex G-100	2625	294	6038	0.0487	1.75	88
20 mM KCl Dialysate	2380	281	3165	0.0888	3.19	84
Ciba-cron Blue Sepharose	1195	255	587	0.435	15.6	76
QAE - Sephadex	940	171	52.6	3.25	117	51
Hydroxyl Apatite	92	116	5.93	19.6	702	35

‡ Numbers in parentheses are calculated values for the micellar COT protein correcting for COT activity associated with the free protein peak separated by gel filtration (see Figure 1). The values for fold purification and percent recovery for the Sephadex G-100 and subsequent steps are based on the calculated values.

Figure 3. SDS polyacrylamide gel electrophoresis of purified COT.

Approximately 5 μg of purified beef heart mitochondrial was subjected to SDS-PAGE according to the method of Laemmli (122). Protein was stained with Coumassic Blue and its density in the slab gel was monitored using a Zeiss spectrophotometer. Sharp peaks at the extreme left and right of the tracing represent the top of the gel and the dye front (Bromephenol Blue), respectively.

SCAN OF PURIFIED COT ON SDS-PAGE

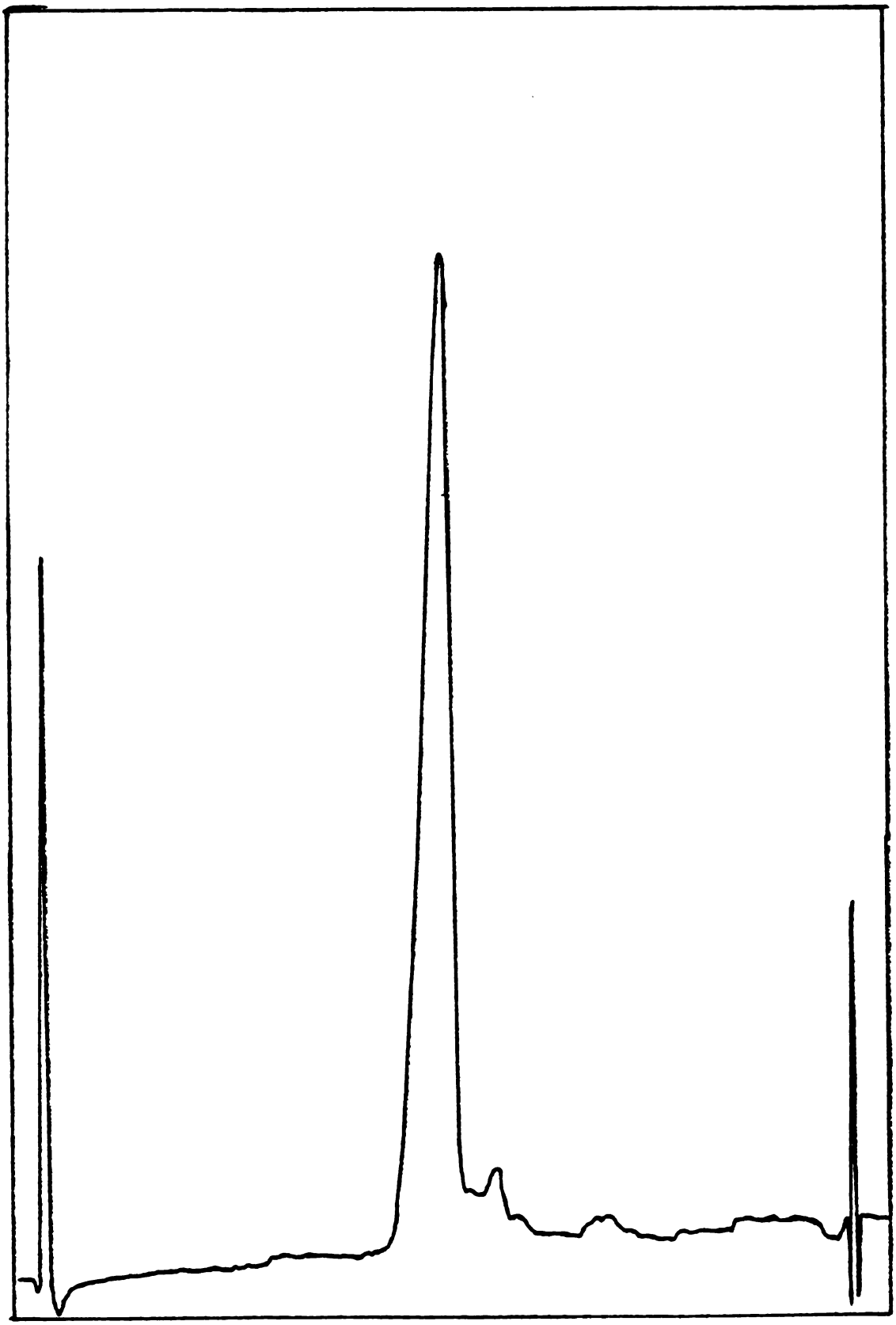

Figure 3

Figure 4. Subunit molecular weight of beef heart mitochondrial COT.

The relative migration of purified COT during SDS polyacrylamide get electrophoresis (see Figure 3) is compared with the migrations of the protein standards bovine serum albumin (BSA), pigeon breast muscle carnitine acetyltransferase (COT), fumarase (FUM) and hen ovalbumin (OVA).

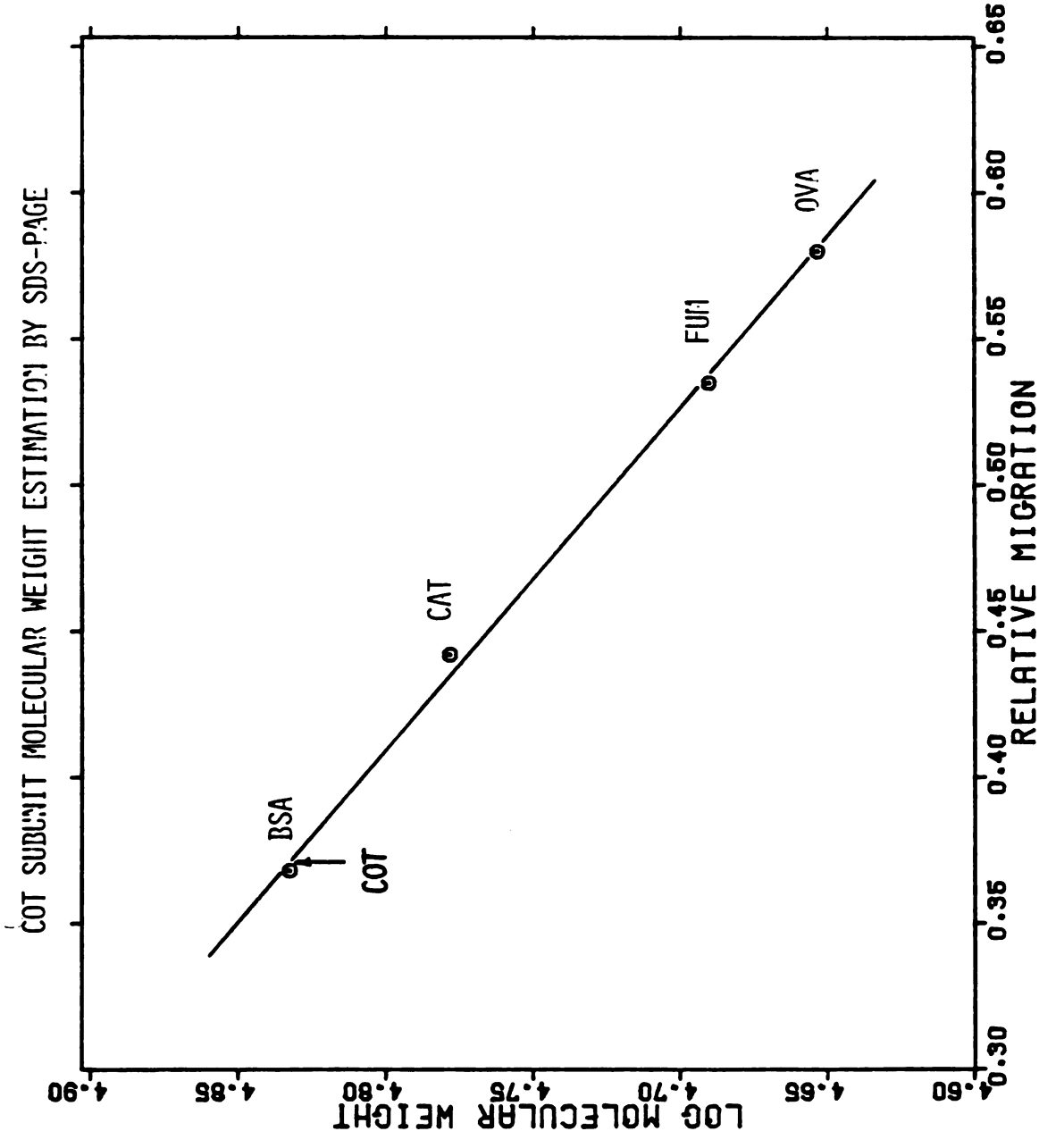
COT SUBUNIT MOLECULAR WEIGHT ESTIMATION BY SDS-PAGE
LOG MOLECULAR WEIGHT
4.90
4.85
4.80
4.75
4.70
4.65
4.60
RELATIVE MIGRATION
0.30
0.35
0.40
0.45
0.50
0.55
0.60
0.65
BSA
COT
CAT
FUM
OVA

Figure 4

Figure 5. Substrate specificity of purified beef heart mitochondrial COT.

The substrate specificity profile of purified COT for the forward reaction (assayed by the DTNB method) shows values of carnitine acyltransferase activity of nmol/min/ml of diluted enzyme. The enzyme concentration is approximately 0.95 mg protein/ml.

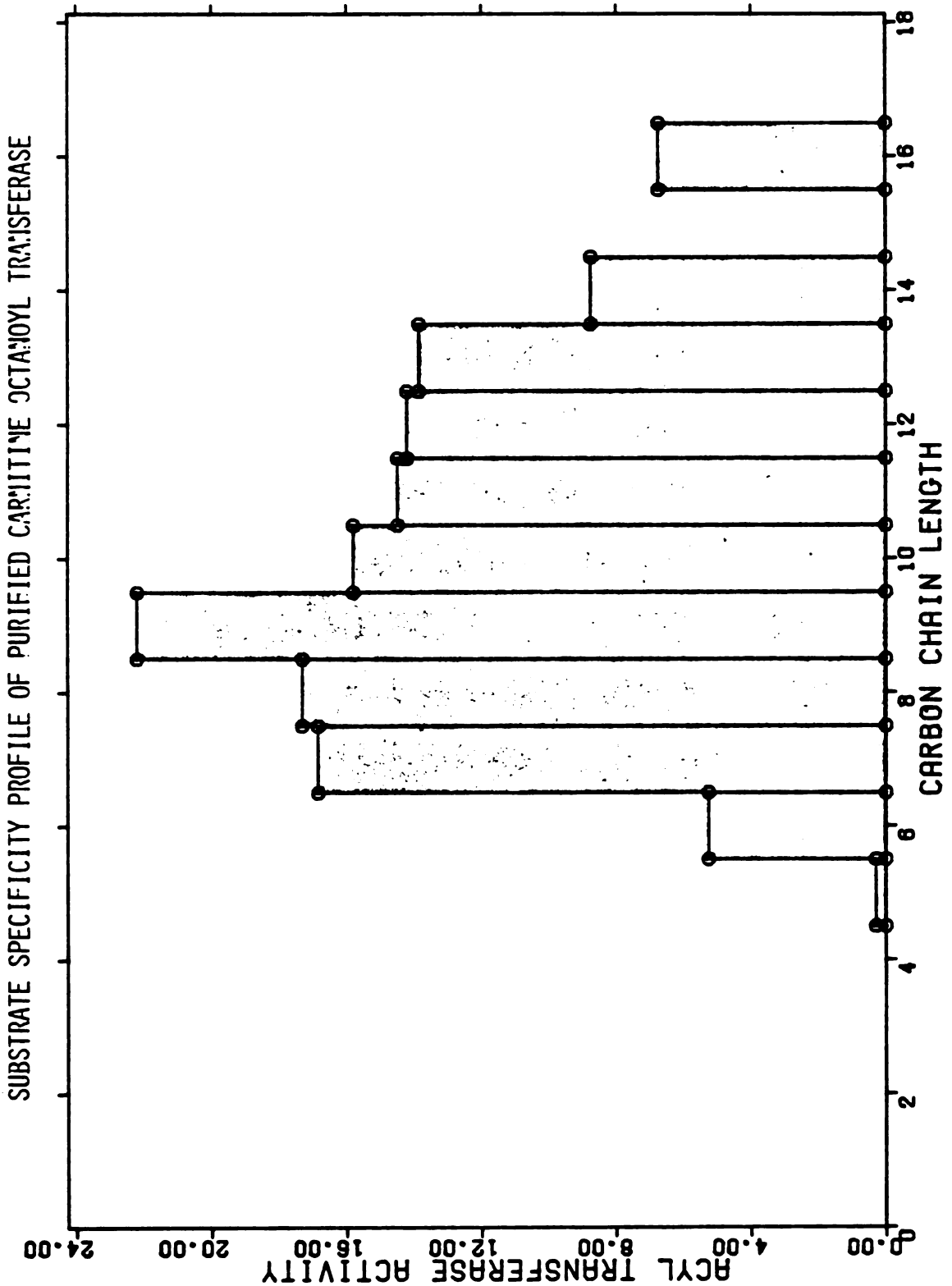
SUBSTRATE SPECIFICITY PROFILE OF PURIFIED CARNITINE OCTANOYL TRANSFERASE
ACYL TRANSFERASE ACTIVITY
0.00
4.00
8.00
12.00
16.00
20.00
24.00
CARBON CHAIN LENGTH
0
2
4
6
8
10
12
14
16
18

Figure 5

Figure 6. pH optimum of beef heart mitochondrial COT.

Carnitine octanoyltransferase activity is expressed in nmol/min/ml. The enzyme concentration is approximately 5.0 mg protein/ml.

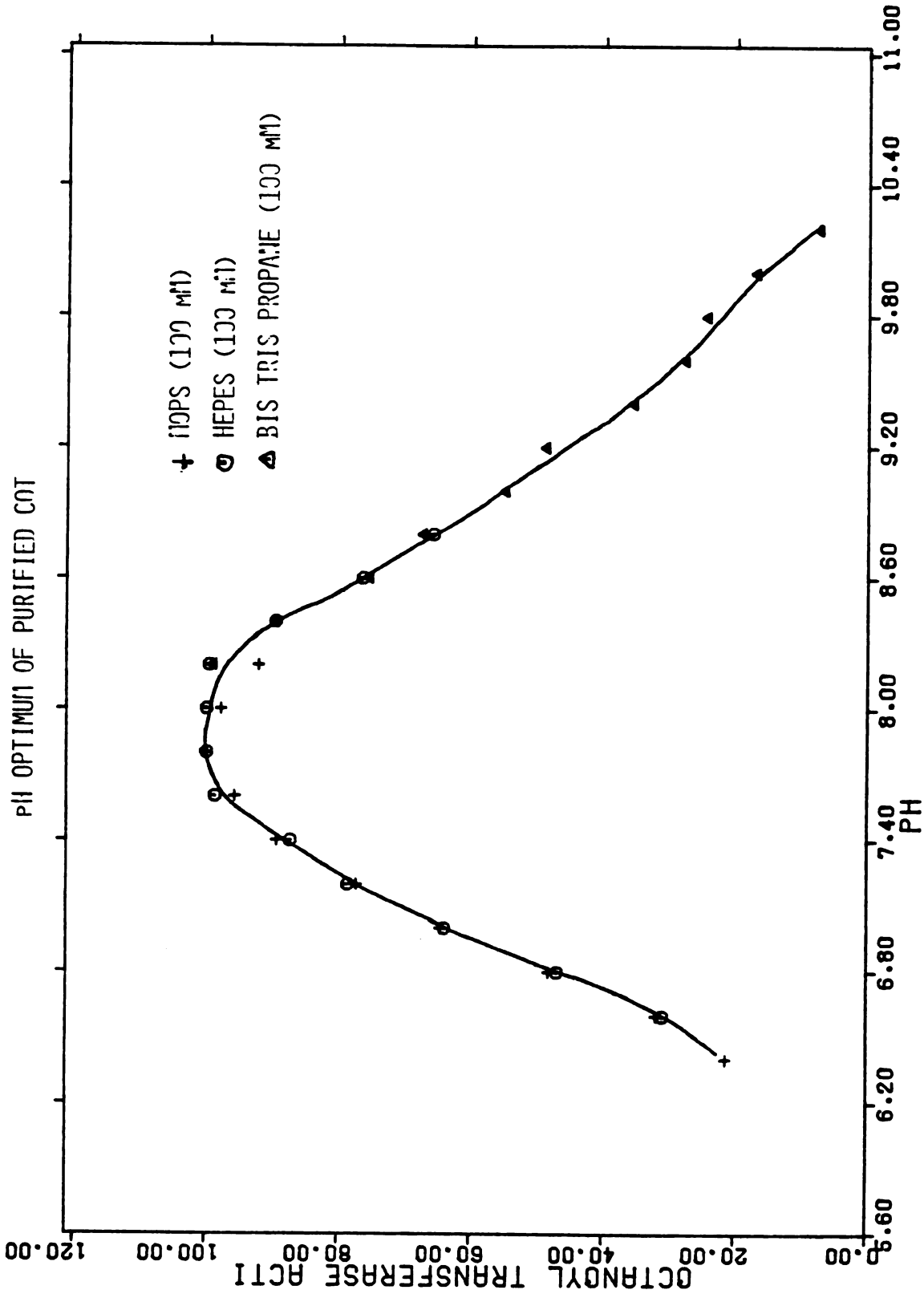
PH OPTIMUM OF PURIFIED COT
+ MOPS (100 mM)
HEPES (100 mM)
BIS TRIS PROPANE (100 mM)
OCTANOYL TRANSFERASE ACTI
0.00
20.00
40.00
60.00
80.00
100.00
120.00
5.60
6.20
6.80
7.40
8.00
8.60
9.20
9.80
10.40
11.00
PH

Figure 6

Table V. Synthesis of Acyl-L-carnitines.

L-Carnitine Ester	% Free L-Carnitine	Specific Rotation
Acetyl	0.95	-7.0
Butyryl	0.97	-7.0
Hexanoyl	0.57	-7.0
Octanoyl	0.49	-7.0
Decanoyl	0.48	-6.8
Lauryl	0.87	-5.0
Myristoyl	0.93	-4.2
Palmitoyl	1.55	-4.0

Acyl-L-carnitines were synthesized by published methods (120, 121) and judged to be approximately 99% pure by TLC analysis (120). Percent contamination by free L-carnitine was determined by radioactive carnitine assay before and after alkaline hydrolysis. Specific rotation measurements were made using a Zeiss polarimeter.

the conditions employed by Kopec and Fritz with their calf liver mitochondrial CPT. The results were nearly identical. The relative rates with these substrates reported by Kopec and Fritz (90) were 2, 26, 31, 99 and 100, respectively; our values were 8, 38, 38, 79 and 100. It was concluded that the beef heart mitochondrial enzyme corresponds to the CPT I isolated from calf liver mitochondria.

At this point it was decided to isolate and identify ALL carnitine acyltransferase proteins from beef heart mitochondria (and to fractionate free and micellar protein from beef liver mitochondria) for the following purposes: (1) to obtain more of the protein previously isolated in order to investigate and explain the apparent differences in substrate specificity of the enzyme for the forward and reverse reactions, (2) to determine whether the free protein fraction seen during gel filtration of solubilized mitochondria indeed contains but one carnitine acyltransferase enzyme, CAT, whose activity with octanoyl CoA totally accounts for COT activity in this fraction, (3) to determine whether a separate CPT enzyme had been overlooked or lost in the previous isolation of COT activity from the micellar protein fraction, and (4) to determine whether, if only two transferase enzymes exist in beef heart mitochondria, is this also true for beef liver mitochondria, based on the substrate specificity of heart and liver mitochondrial free and micelle-bound transferase activities separated by gel filtration. The following section presents the results of this investigation, essentially as submitted for publication (123) under the title of the heading below. Though the free protein fraction was early not considered a source of a separate medium chain

acyltransferase due to its high CAT activity thought to account for the activity with octanoyl-CoA, the procedures described below for the purification of transferase protein from this fraction were developed in parallel with those of the enzyme in the micellar fraction from gel filtration of the solubilized mitochondrial protein.

Isolation and Purification of Mitochondrial Carnitine Octanoyltransferase Activities from Beef Heart

Results

Comparison of Carnitine Acyltransferase Activities in Beef Liver and Heart Mitochondria

Substrate specificity profiles of carnitine acyltransferase activities in liver and heart mitochondrial are presented in Figure 7. Figure 7, A and E show the profiles of intact liver and heart mitochondria. To ascertain whether or not these profiles reflect the activity of one enzyme or more than one enzyme, solubilized liver and heart mitochondria were chromatographed separately on Sephadex G-100. Fractions were assayed for the distribution of carnitine octanoyltransferase activity (B and F). Substrate specificity profiles of the two COT containing peaks separated by gel filtration are pictured in C and D and in G and H for liver and heart, respectively. For each tissue, gel filtration of solubilized mitochondria gave two protein peaks containing transferase activities. Virtually all carnitine acyltransferase activity was released from mitochondria during solubilization (data not shown, but see results from purification of heart mitochondrial transferase activities in Table VI).

Figure 7. Substrate specificity profile of intact and partially fractionated beef heart and liver mitochondrial carnitine acyltransferases.

Carnitine acyltransferase activities were determined by the DTNB assay described in the Methods. Activities are given as nmol x min^{-1} at 25°C.

The substrate specificity profiles of intact liver mitochondria are given in A and intact heart mitochondria in E. Frames B and F show the transferase activities determined with octanoyl CoA of the fractions obtained when liver and heart mitochondria were solubilized in 2% Triton X-100 containing 1 M KCl and chromatographed on a 112 cm x 4.8 cm Sephadex G-100 column as described in Methods. The micellar peak (the first peak) from Figure 7B and 7F were analyzed for substrate specificity; these profiles are shown in frames C and G, respectively. The substrate specificity of the free protein fraction (the second peak) are given in D and H.

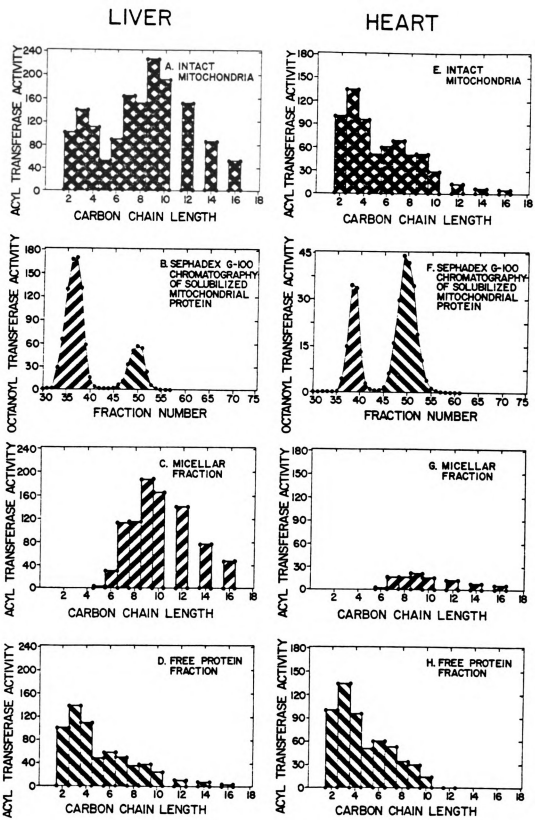
LIVER
HEART
A. INTACT MITOCHONDRIA
E. INTACT MITOCHONDRIA
B. SEPHADEX G-100 CHROMATOGRAPHY OF SOLUBILIZED MITOCHONDRIAL PROTEIN
F. SEPHADEX G-100 CHROMATOGRAPHY OF SOLUBILIZED MITOCHONDRIAL PROTEIN
C. MICELLAR FRACTION
G. MICELLAR FRACTION
D. FREE PROTEIN FRACTION
H. FREE PROTEIN FRACTION
ACYL TRANSFERASE ACTIVITY
OCTANOYL TRANSFERASE ACTIVITY
CARBON CHAIN LENGTH
FRACTION NUMBER

Figure 7

There was no appreciable loss of any of the transferase activities as a result of gel filtration.

The first peak of carnitine octanoyltransferase activity to emerge from each Sephadex column contains enzyme or enzyme aggregates of apparent molecular weight greater than 300,000 daltons. Based on the analysis by other investigators (129) of the gel filtration behavior of detergent-soluble proteins in the presence of non-ionic detergent, we conclude that this peak represents predominately proteins associated with micelles of Triton X-100. For solubilized mitochondrial protein from both tissues, the first peak contains all of the long chain and some of the medium length carnitine acyltransferase activities. All of the short chain transferase activities and the remainder of the medium chain activities eluted with an apparent molecular weight of 60,000 daltons. The substrate specificity profiles of micelle-associated activities solubilized from mitochondria of beef liver and heart are similar (see Figures 7, C and G) as are the profiles of the activities of the 60,000 dalton peak eluted (Figure 7, D and H).

Purification of Carnitine Octanoyltransferase (COT) Activity from Beef Heart Mitochondria

Solubilization. Preliminary studies (data not shown) indicated that beef heart mitochondrial carnitine acetyltransferase (CAT) activity is mostly soluble. More than 90% is released when the mitochondria were disrupted by freeze-thaw or sonication in the presence of 0.5 to 2.0 M KCl. Once the mitochondria were disrupted, 60 mM KCl was required to keep proteins containing carnitine acyltransferase activity in solution. In contrast, carnitine palmitoyltransferase is poorly solubilized by

treatment with KCl requiring instead Triton X-100 (1.0-2.0%) to release greater than 90% of the CPT into the 90.000 x g supernatant fluid. A significant fraction of total COT activity is found in both supernatant fluid and pellet with either the salt or the detergent extraction procedure. Therefore, in order to maximize the release of COT activities by one extraction procedure, thawed mitochondria were solubilized in 2% Triton X-100 in the presence of 1 M KCl. The results in Table VI show that this combined detergent/salt extraction solubilized 79% of the mitochondrial protein and 75, 78 and 90% of CAT, COT and CPT activities, respectively.

Dialysis. Dialysis of the solubilized protein solution preparatory to Blue Sepharose chromatography lowers the effective KCl concentration to 60 mM. At this concentration approximately half of all solubilized protein precipitated out of solution, including a fraction of the carnitine acyltransferase activities. Our preliminary experiments (data not shown) indicated that in dilute (< 1 mg protein/ml) solution, carnitine acyltransferase activity from solubilized beef heart mitochondria is not appreciably precipitated at a KCl concentration of 60 mM. There is measurable precipitation of CAT and to a lesser extent of COT at 50 mM KCl, with near total precipitation of CAT, a lesser fraction of total COT and insignificant loss from the supernatant fluid of CPT at 20 mM KCl.

The precipitation of significant CAT and COT activities during this procedure may be due to a reduction of the actual concentration of KCl inside the dialysis bag because of charge contributions of the protein.

Table VI. Summary of the Purification of Carnitine Acyltransferase Proteins from Beef Heart Mitochondria

Purification Step	Acetyltransferase (CAT)				Octanoyltransferase (COT)				Palmitoyltransferase (CPT)			
	Units	Percent recovery	Specific activity	Fold	Units	Percent recovery	Specific activity	Fold	Units	Percent recovery	Specific activity	Fold
Thawed Mitochondria	1113	100	.182	1	608	100	.0995	1	179	100	.0293	1
Solubilized Mitochondria	833	75	.173	.95	474	78	.0987	.99	161	90	.0335	1.14
Dialysis Supernatant	547	49	.225	1.24	333	55	.1370	1.38	157	79	.0646	2.21
Dialysis Pellet	270	24	.114	.63	130	21	.0549	.55	7	4	.0030	.10
Blue Sepharose (#31-34)*	496	44	4.06	22.3	100	18	.891	8.95				
Blue Sepharose (#40-43)**					181	30	1.95	19.6	128	64	1.38	47.0
Sephadex G-100*					97	16	.819	8.23				
Sephadex G-100**					143	23	4.09	41.2				
CM-Sepharose*					59	10	24.7	248				
QAE-Sephadex**					115	19	37.1	373				
Hydroxylapatite*	165	15	78.5	431	44	7	20.9	219				
Hydroxylapatite**					87	14	53.4	537	77	39	47.5	1620

*Indicates purification procedures performed on Blue Sepharose fractions (#31-34) containing both COT and CAT activities.

*Also corresponds to panels B, C and D of Figure 8.

**Indicates purification procedures performed on Blue Sepharose fractions (#40-48) containing both COT and CPT activities.

**Also corresponds to panels E, F and G of Figure 8.

The protein pelleted after dialysis contains a third of the total CAT activity, a smaller fraction of COT and negligible CPT activity (see Table VI). The ratio of COT to CAT activity assayed in the pellet is less than 0.5.

The dialysis supernatant fluid which was loaded directly onto the Blue Sepharose column contains 67, 72 and 96%, respectively, of the initially solubilized CAT, COT and CPT activities.

Results of Column Chromatography

Greater than 95% of COT activity present in the soluble heart mitochondrial protein fraction after dialysis against 60 mM KCl is retained on Blue Sepharose. This activity elutes as two well-separated peaks after administration of a linear KCl gradient (see Figure 8, A). CAT and CPT activities are completely separated; each co-elutes with one of the COT peaks. Acyl-CoA hydrolase activities are partially removed during the dialysis and are nearly totally absent in the Blue Sepharose peaks of carnitine acyltransferase activity (data not shown). Both CAT and CPT are purified about 20-fold by this step.

Purification of the COT- and CAT-Containing Blue Sepharose Peak

Fractions 31-34. A further 20-fold purification of the CAT/COT activity is effected by CM-Sepharose ion exchange chromatography (Figure 8, B). Chromatography of the peak from CM-Sepharose on hydroxylapatite (HAP) yields nearly superimposable peaks of protein and COT activity (see Figure 8, C). Fractions from the hydroxylapatite column of constant specific activity were pooled and a sample was analyzed for purity by SDS-polyacrylamide gel electrophoresis. This procedure

Figure 8. Purification of carnitine octanoyltransferase from beef heart mitochondria.

In A, transferase was solubilized as described for Figure 7E and the solution was exhaustively dialyzed and applied to a 75 cm x 4.1 cm column Cibacron Blue Sepharose 4B column equilibrated with the Blue Buffer described in the methods. The sample was applied and the column was washed with 4 bed volumes Blue Buffer and then eluted with a 60-860 mM KCl linear gradient in Blue Buffer. The numbers in parentheses represent carnitine acetyltransferase activity (the diamond shaped points in the figure) and the other numbers represent carnitine octanoyltransferase (the open circles) and carnitine palmitoyltransferase activity (the open squares). The solid thin line represents the protein. The KCl gradient is indicated by the dots; the units mS represent millisemens conductivity.

In B, the first transferase peak, fractions 31-34 of Figure 8A, were pooled and passed over a Sephadex G-100 column to equilibrate the protein with the CM Buffer described in the methods. The protein in CM buffer was applied to a 29 cm x 4.1 cm column of CM-Sepharose CL-6B and washed with 4 bed volumes of CM Buffer. The activity was then eluted by 60-560 mM linear gradient of KCl in CM Buffer. The symbols are identical to those described in Figure 8A. The open circles represent carnitine octanoyltransferase activity.

Fractions 37-41 of Figure 8B were pooled and diluted with an equal volume of 20 mM K_2HPO_4, pH 6.6. The solution was applied to a 13 cm x 2.2 cm hydroxylapatite which was equilibrated with the HAP (-) Buffer described in the methods. The column was washed with 4 bed volumes of HAP (+) Buffer and then eluted with a 10-510 mM $KHPO_4$, pH 6.8 linear gradient. The symbols are identical to those described in Figure 8B.

Fraction 17-19 (the peak ones)of Figure 8C were pooled and analyzed for purity by SDS polyacrylamide gel electrophoresis as by the method of Laemmli, see Figure 8D. T represents the top of the gel and B represents the bottom of the gel. The DYE was bromphenol blue.

In E, fractions 40-48 of Figure 8A were pooled and passed through a Sephadex G-100 column equilibrated with the QAE Buffer described in the methods. The protein solution was then passed through a 48 cm x 4.1 cm column of QAE Sephadex Q-25-120 equilibrated with QAE Buffer. The open circles represents carnitine octanyltransferase activity.

Fractions 23-29 of Figure 8E were dialyzed exhaustively against several changes of HAP (+) Buffer and then applied to a 11.5 cm x 2.2 cm column of hydroxylapatite and processes as described in Figure 8C. In this panel, F, the open circles represent carnitine octanyltransferase, but the solid line represents protein. For the peak fractions protein and transferase activity were superimposable. In G, the peak fractions (12-14 of F) were combined and subject to SDS polyacrylamide electrophoresis as described for Figure 8D. The data in Figure 8 are summarized in Table VI where * represents purification described in Panels B, C and D and ** represents the purification shown in panels E, F and G.

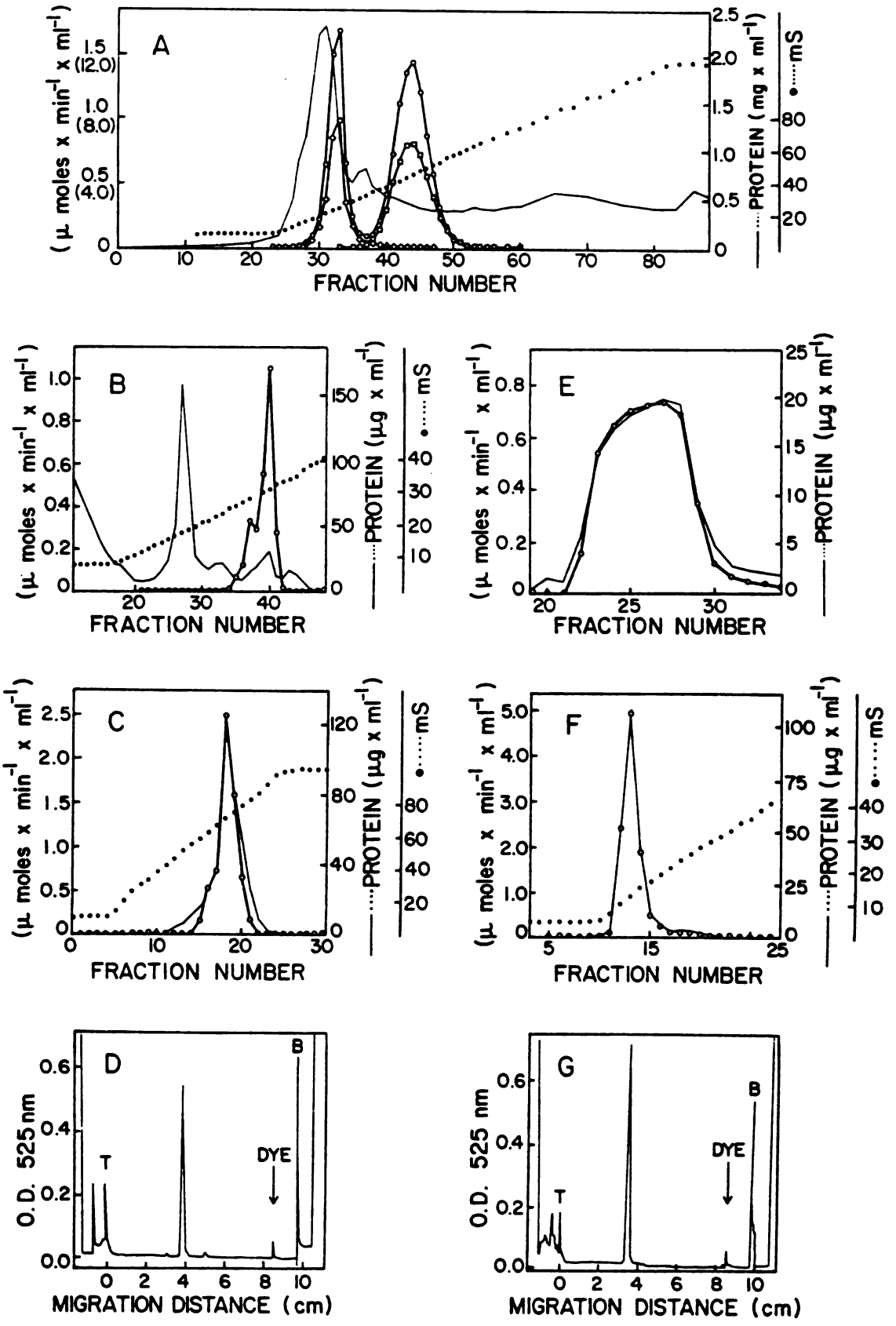
A
B
C
D
E
F
G
FRACTION NUMBER
PROTEIN (mg x ml⁻¹)
PROTEIN (µg x ml⁻¹)
mS
O.D. 525 nm
MIGRATION DISTANCE (cm)
T
DYE
B

Figure 8

revealed the presence of one major protein band and two discernible contaminating proteins of much lesser abundance. The densitometer scan of the gel stained for protein (Figure 8, D) indicates that the purified CAT/COT protein is approximately 95% pure.

Purification of the COT- and CPT-Containing Blue Sepharose Peak

Fractions 40-48. A two-fold purification was achieved during Sephadex G-100 chromatography of the pooled CPT/COT fractions from the Blue Sepharose column. A further nine-fold purification was obtained by passing the protein solution through a column of QAE-Sephadex at pH 9.7 (see Figure 8, E). Hydroxylapatite chromatography of the protein effluent from the QAE-Sephadex column revealed a sharp, symmetrical peak of constant specific activity (Figure 8, F). The CPT/COT protein is estimated to be greater than 95% pure by SDS-polyacrylamide gel electrophoresis (see Figure 8, G).

Molecular Weight Determinations

Comparison of the elution of CAT/COT and CPT/COT activities after Sephadex G-200 chromatography with those of catalase and hemoglobin indicate apparent molecular weights for the two transferases of 60,500 and 510,000 daltons, respectively (see Figure 9). Subunit molecular weight determination by SDS-PAGE using bovine serum albumin, catalase, fumarase and ovalbumin as standards yields values of 62,600 and 67,000, respectively, for beef heart mitochondrial CAT and CPT (Figure 10).

Isoelectric Point

Aliquots of the two purified proteins were combined and subjected to isoelectric focusing in a sucrose density gradient. The results shown

Figure 9. Chromatography of solubilized carnitine octanoyltransferase from beef heart mitochondria.

The solid circles represent carnitine octanoyltransferase activity measured by the DTNB assay. The thin solid line is protein and the arrows indicate the peak positions of molecular weight markers.

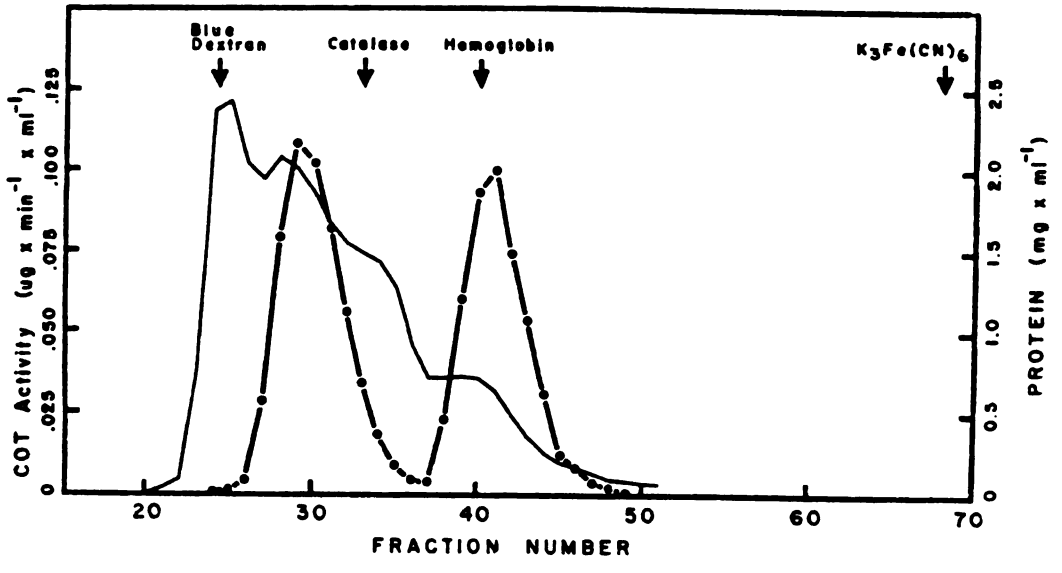
Blue Dextran
Catalase
Hemoglobin
$K_3Fe(CN)_6$
COT Activity (ug x min^{-1} x ml^{-1})
0
.025
.050
.075
.100
.125
PROTEIN (mg x ml^{-1})
0
0.5
1.0
1.5
2.0
2.5
20
30
40
50
60
70
FRACTION NUMBER

Figure 9

Figure 10. Weight determination of carnitine acyltransferases from beef heart mitochondria.

The peak fractions from Figure 8C and Figure 8F were subjected to the polyacrylamide gel (10% gel) electrophoresis as described by Laemmli (122). CPT represents the CPT/COT protein and CAT represents the CAT/COT protein described in the results. BSA is bovine serine albumin.

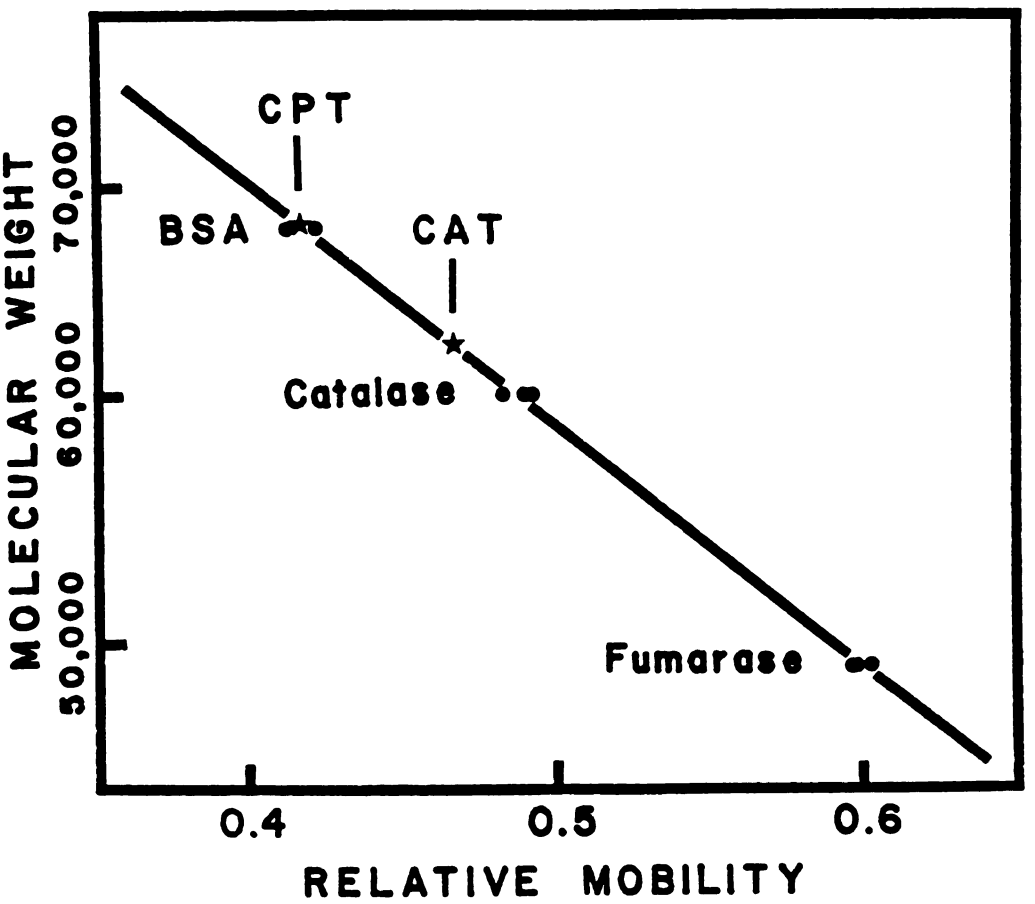
CPT
BSA
CAT
Catalase
Fumarase
MOLECULAR WEIGHT
70,000
60,000
50,000
0.4
0.5
0.6
RELATIVE MOBILITY

Figure 10

in Figure 11 give pI values of 8.20 for the CAT protein and 8.05 for CPT of mitochondria from beef heart.

Isoelectric Focusing--Approach to Equilibrium

In order to investigate the possibility that the micelle-associated COT and CPT activities are associated with two distinct proteins which may have co-purified during our purification procedure, the purified CPT/COT protein was subjected to isoelectric focusing on a pH 3-10 gradient and assayed before the protein(s)' migration had reached equilibrium. Similar but different proteins which have indistinguishable pI's may show different patterns in approaching isoelectric equilibrium. The results presented in Figure 12 show that the two activities are superimposable, suggesting that both activities reside in a single enzyme.

Substrate Specificity

The substrate specificities of the two carnitine acyltransferases purified from beef heart mitochondria are presented in Table VII. Included are results obtained for commercial pigeon breast muscle mitochondrial CAT (Sigma) assayed under identical conditions. For purposes of comparison, literature values for a preparation of purified carnitine palmitoyltransferase (90) are listed, showing relative rates of the reverse reaction. The CPT/COT enzyme has high medium chain transferase activity in the forward direction in contrast to the reverse reaction.

In Figure 13 the substrate specificities of the purified carnitine acyltransferase proteins (lower panel) are compared with that obtained initially for the thawed suspension of beef heart mitochondria. The CAT and CPT enzyme profiles have been normalized to equalize the heights of

Figure 11. Isoelectric point determination of the carnitine acyltransferases from beef heart mitochondria.

Aliquots of the pooled purified proteins described for Figure 10 were subjected to isoelectric focusing, see Methods.

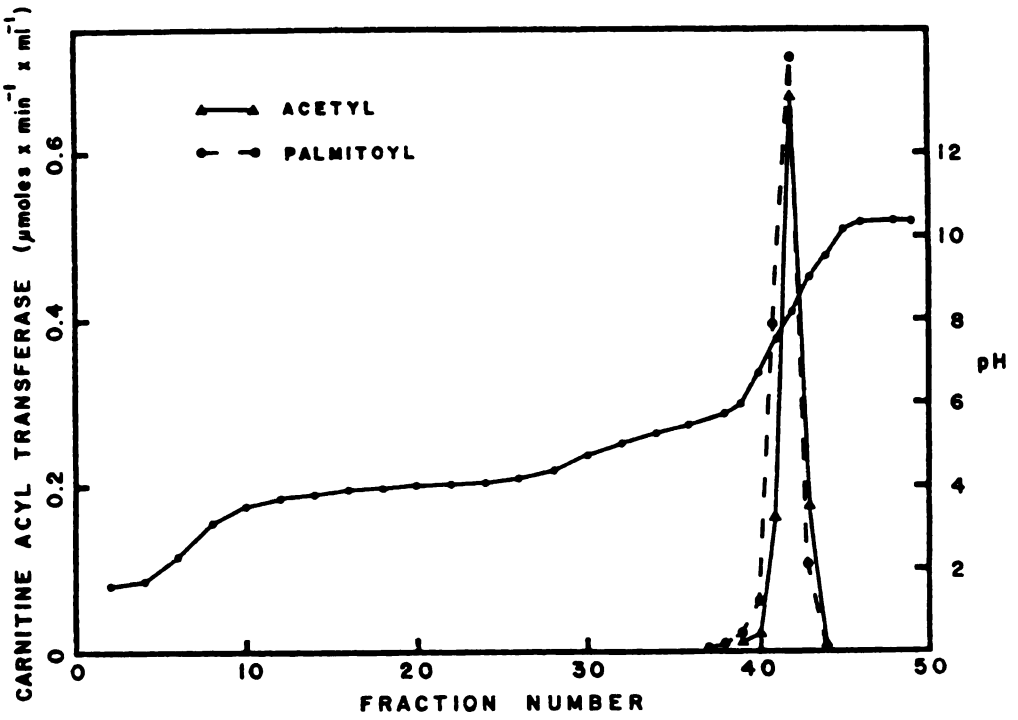
ACETYL
PALMITOYL
CARNITINE ACYL TRANSFERASE (μmoles x min⁻¹ x ml⁻¹)
0
0.2
0.4
0.6
pH
2
4
6
8
10
12
0
10
20
30
40
50
FRACTION NUMBER

Figure 11

Figure 12. Approach to isoelectric equilibrium of the CPT/COT enzyme.

The triangles connected by a solid line represent carnitine octanoyltransferase and the solid squares represent carnitine palmitoyltransferase activity. The pooled peak fractions of Figure 8F were used. The numbers in parentheses represent the palmitoyltransferase.

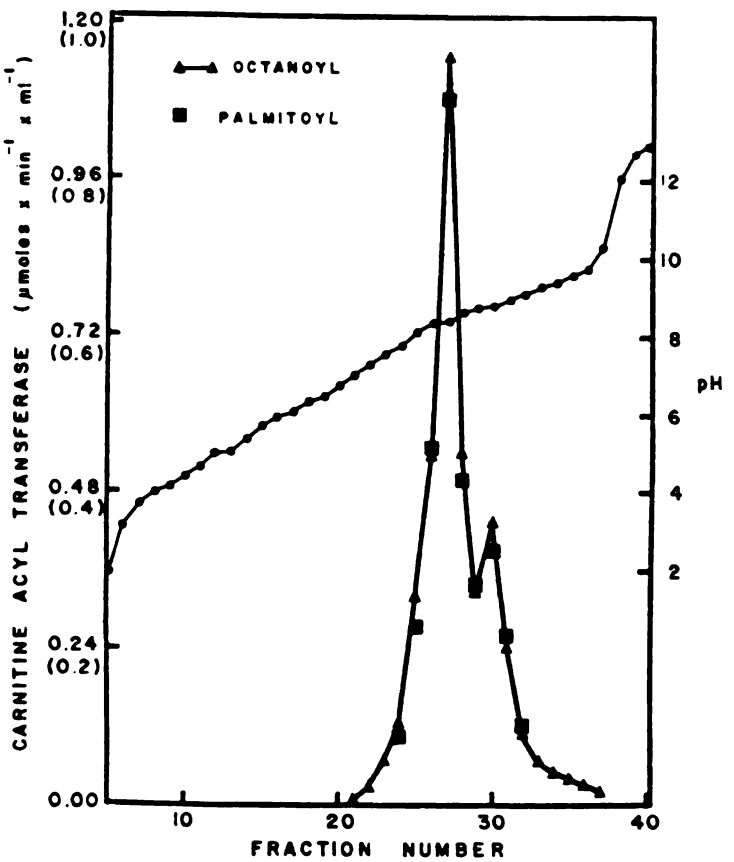
1.20
(1.0)
0.96
(0.8)
0.72
(0.6)
0.48
(0.4)
0.24
(0.2)
0.00
CARNITINE ACYL TRANSFERASE (μmoles x min⁻¹ x ml⁻¹)
OCTANOYL
PALMITOYL
12
10
8
6
4
2
pH
10
20
30
40
FRACTION NUMBER

Figure 12

Table VII. Relative Activities of Beef Heart Mitochondrial Carnitine Acyltransferases.

Acyl Residue	Carnitine Acetyltransferase				Carnitine Palmitoyltransferase		
	Foreard Assay		Reverse Assay		Forward Assay	Reverse Assay	
	CAT/COT	Commercial CAT	CAT/COT	Commercial CAT	CPT/COT	CPT/COT	CPT (ref. 90)
Acetyl	100	100	100	100	0	0	0
Butyrl	109	98	88	86	1	0	0
Hexanoyl	51	41	64	63	43	3	0
Octanoyl	27	20	54	50	113	8	2
Decanoyl	5	3	26	20	226	38	26
Lauryl	1	0	2	2	166	38	31
Myristoyl	0	0	0	---	100	79	99
Palmitoyl	0	0	0	---	100	100	100

The CAT/COT activity represents the peak fractions from Figure 8C and the CPT/COT are the peak fractions from Figure 8F.

The activity of acetyltransferase was arbitrarily set at 100 for CAT/COT and the activity of palmitoyltransferase was set at 100 for the CPT/COT enzymes so relative activities could be expressed.

Figure 13. Comparison of carnitine acyltransferase activity of beef heart mitochondria with the purified transferase enzymes.

See the section on substrate specificity in the Results for details of this experiment.

A. whole mitochondria
B. purified proteins

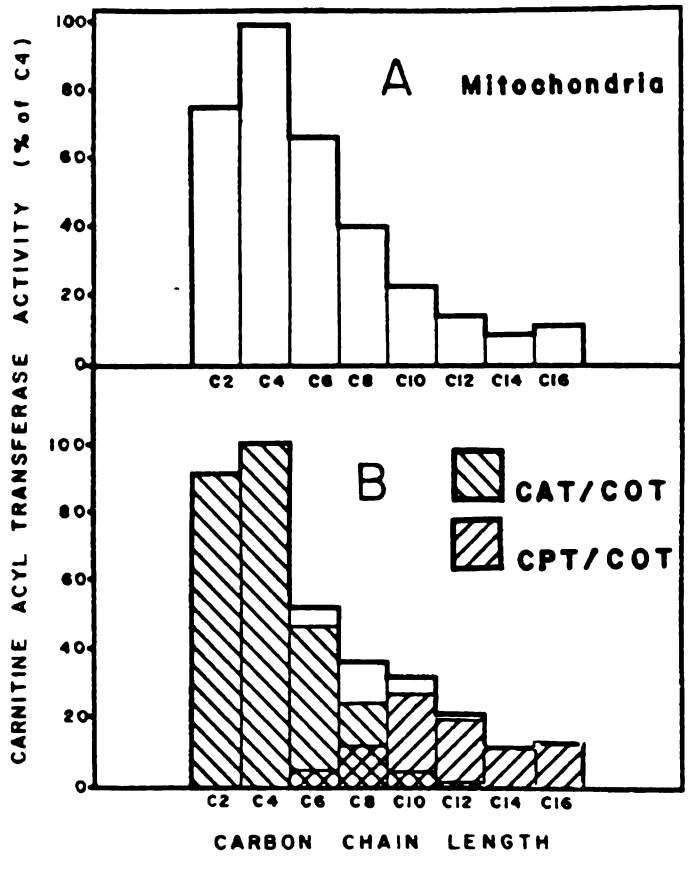
A Mitochondria
B
CAT/COT
CPT/COT
CARNITINE ACYL TRANSFERASE ACTIVITY (% of C4)
CARBON CHAIN LENGTH
C2 C4 C6 C8 C10 C12 C14 C16
0 20 40 60 80 100

Figure 13

C-2 and C-16 in upper and lower portions of the figure. The cross-hatching indicates overlapping of medium chain activities.

Amino Acid Analysis

Beef heart mitochondrial CAT and CPT were subjected to acid hydrolysis for 24 hours and the amino acids were measured using a Beckman amino acid analyzer. The amino acid compositions of the beef heart transferases are shown in Table VIII.

Discussion

Gel filtration of the detergent-solubilized mitochondria separates short and long chain acyltransferase activities, with each fraction contributing COT activity. In beef liver, the majority of COT activity chromatographs with carnitine palmitoyltransferase (CPT) while in heart, in which carnitine acetyltransferase (CAT) is much more abundant relative to long chain transferase activity, the majority of COT is found with the short chain transferase. The subsequent isolation of only two carnitine acyltransferase proteins from beef heart mitochondria, one corresponding to each of the free and micellar peaks seen with gel filtration, together with the similarity of substrate specificity for the corresponding heart and liver free and micellar mitochondrial protein fractions leads to the conclusion that beef liver mitochondria most likely also contain but two carnitine acyltransferase enzymes.

The two proteins obtained from beef heart mitochondria can account for nearly all COT activity initially detected in the intact mitochondria. Carnitine acyltransferase activities which were precipitated during dialysis of solubilized mitochondrial protein preparatory to column

Table VIII. The Amino Acid Compositions of Beef Heart Mitochondrial Carnitine Acyltransferases.*

Amino Acid	CAT/COT	CPT/COT
Asx	8.99	7.72
Thr	4.70	4.49
Ser	6.97	6.33
Glx	11.36	9.46
Pro	6.23	5.95
Gly	5.04	5.26
Ala	8.65	9.76
Val	6.71	5.03
Met	3.13	2.53
Ile	4.40	5.00
Leu	10.77	11.52
Tyr	4.83	4.89
Phe	5.09	8.01
His	2.74	2.40
Lys	5.67	5.70
Arg	4.74	5.94
Cys	nd	nd
Trp	nd	nd

*Values for each amino acid are mole percent of total amino acid detected after 24-hr hydrolysis; nd = not determined.

The CAT/COT is from the pooled peak fractions from Figure 8C and the CPT/COT is from the pooled peak fractions from Figure 8F.

chromatography were not further purified. These precipitated proteins contained 33% of the initially solubilized CAT, 28% of COT and 4% of total CPT activities. It is possible that this fraction contains a COT protein different from those finally purified from the dialysis supernatant fluid. However, based on the COT activities present in the pure CAT/COT and CPT/COT proteins, more than half (17 of the 28%) of total COT found in this fraction can be accounted for by the CAT/COT and CPT/COT proteins which are partially precipitated by this procedure. Though the remaining 11% of COT could represent a separate acyltransferase protein, it should be noted that the precipitate contained all of the acyl-CoA hydrolase activities (data not shown) so that the blanks measured in this fraction are very high and the error for the transferase activities reported in the precipitate is correspondingly large.

The two COT-containing proteins are separated by column chromatography on Cibacron Blue Sepharose and are further purified to near homogeneity by CM-Sepharose and QAE-Sephadex chromatography, respectively, for the acecyl and palmitoyltransferase containing enzymes (see Figure 8, A). The two proteins have similar isoelectric points and amino acid compositions, though the CPT/COT protein is slightly richer in hydrophobic amino acid residues (see Table VIII) as expected from its affinity for the detergent Triton X-100. The native molecular weight (60,600) and pI (8.2) of the CAT/COT protein are similar to those reported by Markwell (26) (MW = 59,000, pI = 8.3) for carnitine acetyltransferases partially purified from rat liver peroxisomes and microsomes. A comparison of the relative activities of the purified CAT/COT protein using acylcarnitine and acyl-CoA substrates with those of commercial purified

pigeon breast muscle CAT (see Table VII) leads us to conclude that this beef heart mitochondrial protein is a carnitine acetyltransferase (EC 2.3.1.7) and not a novel COT enzyme.

Carnitine palmitoyltransferase has been purified to homogeneity from calf liver mitochondria by Kopec and Fritz (90) and the relative rates of the reverse reaction using acylcarnitine substrates are reported therein. A comparison of these data with those obtained for the beef heart mitochondrial CPT/COT protein assayed under identical conditions shows a strikingly similar pattern (see Table VII). For the forward reaction, the purified CPT/COT protein gives a markedly different substrate specificity profile with a preference for medium chain length substrates. Based on the similarity of the substrate specificity of the CPT/COT protein to that of CPT purified by other investigators, it is concluded that the beef heart mitochondrial CPT/COT protein is a carnitine palmitoyltransferase (EC 2.3.1.21), also not a novel COT enzyme.

An investigation of the apparent difference between substrate specificity of purified beef heart mitochondrial CPT for the forward and reverse directions was next undertaken. The following section contains the results of this study as submitted for publication (130) under the title of the heading below.

Effect of Micelles on the Kinetics of Purified Beef Heart Mitochondrial Carnitine Palmitoyltransferase

Results

In the previous paper (123) we reported for purified beef heart mitochondrial carnitine palmitoyltransferase (CPT) rates of the reverse

reaction: acylcarnitine + CoA → carnitine + acyl-CoA using 500 μM acylcarnitines similar to the relative rates described by Kopec and Fritz (90) for CPT purified from calf liver mitochondria. During the investigation of the kinetics of the reaction with various carnitine ester substrates, biphasic kinetic phenomena in the reactions with lauryl- and myristoylcarnitine were observed (these data not shown, but see Figure 15) suggesting that at lower substrate concentrations, a different profile of substrate specificity would be seen. The initial rates of reaction with different substrates at two concentrations were investigated. The data in Table IX give the relative rates of reaction for C_6-C_{16} acylcarnitines at substrate concentrations of 500 and 50 μM acylcarnitine. It is observed that in decreasing the substrate concentration from 500 μM to 50 μM acylcarnitine, the highest activity shifts from palmitoylcarnitine to laurylcarnitine. The greatest decreases in absolute activity are associated with the longer acylcarnitine esters, myristoyl- and palmitoylcarnitine, suggesting a significantly higher K_m for these substrates.

To determine whether the bisphasic kinetics observed with lauryl- and myristoylcarnitine and the apparent shift to higher K_m values for myristoyl- and palmitoylcarnitine were attributable to a shift from monomeric to micellar forms of the substrate, the critical micellar concentrations (CMC's) of the acylcarnitines were determined and these values compared to the substrate concentrations at which points of discontinuity occur in double reciprocal plots for lauryl- and myristoylcarnitine acyltransferase activity. CMC's determined for the various acylcarnitines are shown in Figure 14, indicated by the solid circles. As the acyl chain

Table IX. Rates of Acyl Coenzyme A Formation from Acylcarnitines

Carnitine Ester	500 μM nmol x min^{-1} x $protein^{-1}$	%	50 μM nmol x min^{-1} x $protein^{-1}$	%
Hexanoyl	1800	8	920	23
Octanoyl	2070	12	1110	27
Decanoyl	6810	40	3510	87
Lauryl	6920	41	4050	100
Myristoyl	13210	78	3040	75
Palmitoyl	17000	100	3420	85

Assay conditions are described in the Methods except that the detergent was 0.04% Triton X-100. The enzyme protein concentration was 2.0 μg/ml. The % represents the percent of the greatest rate.

Figure 14. The relationship between the chain length of acylcarnitines to the critical micellar concentration.

The solid circles represent the critical concentrations (CMCs) of the even chained acylcarnitines (CMC determinations for acylcarnitines were performed as described in the Methods). The open circles represent points of discontinuity on Lineweaver-Burk plots of lauryl-and myristoyl-carnitine acyltransferase activities. The enzymatic reactions were performed (as described in the Methods) at a Triton X-100 concentration of 0.0025% and an enzyme protein concentration of 2.0 μg/ml.

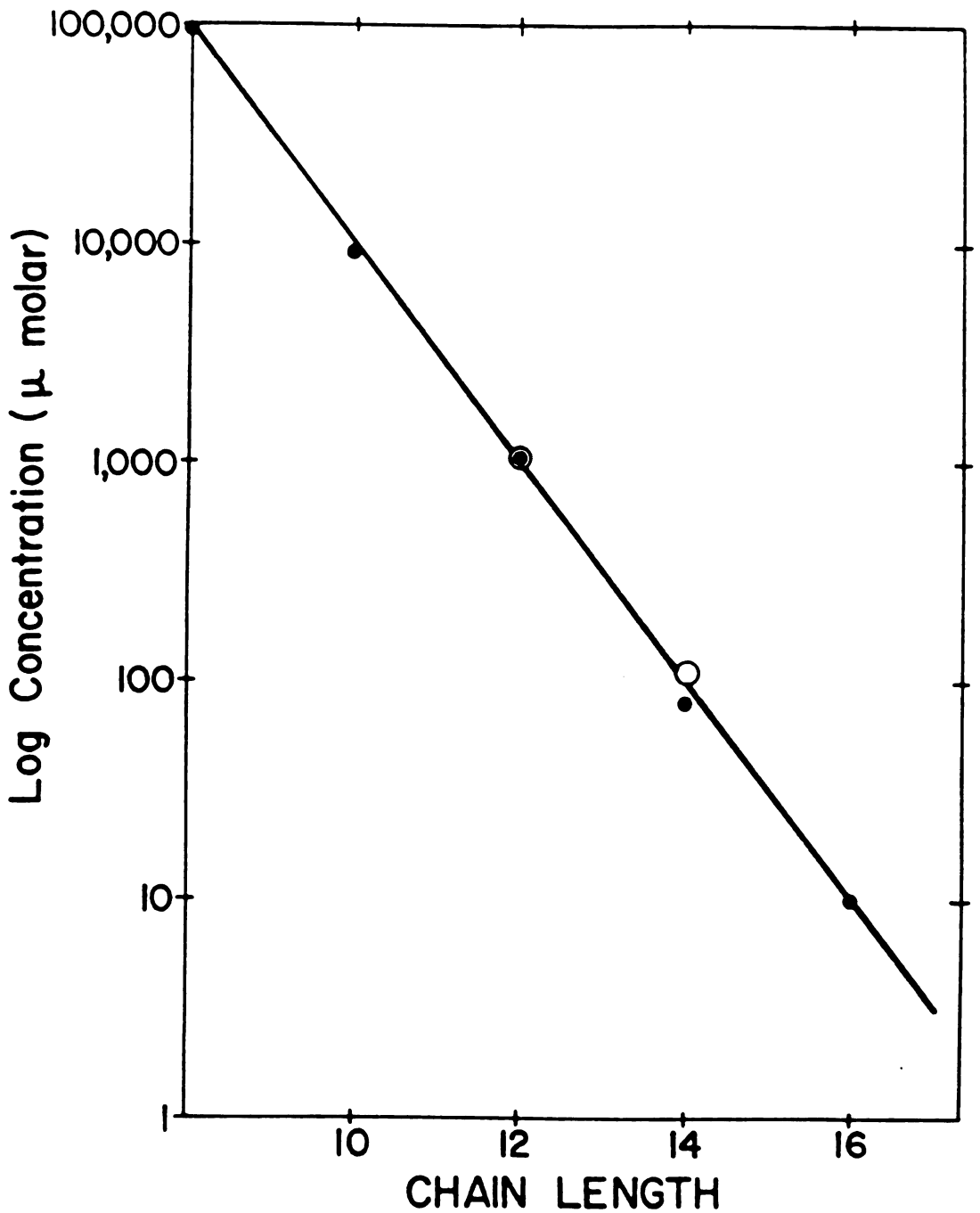
100,000
10,000
1,000
100
10
1
Log Concentration (μ molar)
10
12
14
16
CHAIN LENGTH

Figure 14

length decreases from 16 to 8 carbons by successive two carbon units, the CMC concentration rises correspondingly 10-fold, yielding a linear plot of log [acylcarnitine] versus carbon chain length. As is shown by the open circles in Figure 14, discontinuities in Lineweaver-Burk plots of lauryl-and myristoylcarnitine reactions occur at the same concentrations as their respective CMC's. This indicates that a phase shift of the substrate from monomer to micelle is responsible for the kinetic findings.

To investigate the effect of detergent micelles on this phase shift, kinetics measurements using lauryl-and myristoylcarnitine in the presence of varying concentrations of non-ionic detergents were examined. The data in Table IX are from reactions performed in the presence of 0.04% Triton X-100. Above this concentration the background absorbance at 232 nm of Triton X-100 significantly interferes with activity determinations. Therefore, in order to study the effect of higher detergent concentrations, the non-ionic detergent Tween-20 was used. With either detergent, the effect of increasing the detergent concentration in the assay was to decrease the sharpness of the discontinuity in double reciprocal plots and to lower slightly the substrate concentration at which the break occurs. These data for myristoylcarnitine are summarized in Figure 15, where it is seen that at 0.4% (v/v) Tween-20 (the limiting concentration due its background absorbance) the break in the Lineweaver-Burk plot is virtually absent. The data points indicated by solid circles represent assays done in the absence of detergent micelles while those indicated by X's were performed in the presence of 0.4% Tween-20 (CMC = 0.0003%). Identical data were obtained using laurylcarnitine as

Figure 15. The effect of Tween-20 on the kinetics of myristoyl CoA formation from myristoylcarnitine.

The protein concentration was 2.0 μg/ml and 0.4% Tween-20 was used where indicated. Velocity, V, is expressed as nmol of acyl-CoA formed per minute per μg protein or U x mg^{-1}. The broken line represents the change in the substrate concentration at which the discontinuity occurs with changes in the detergent concentration.

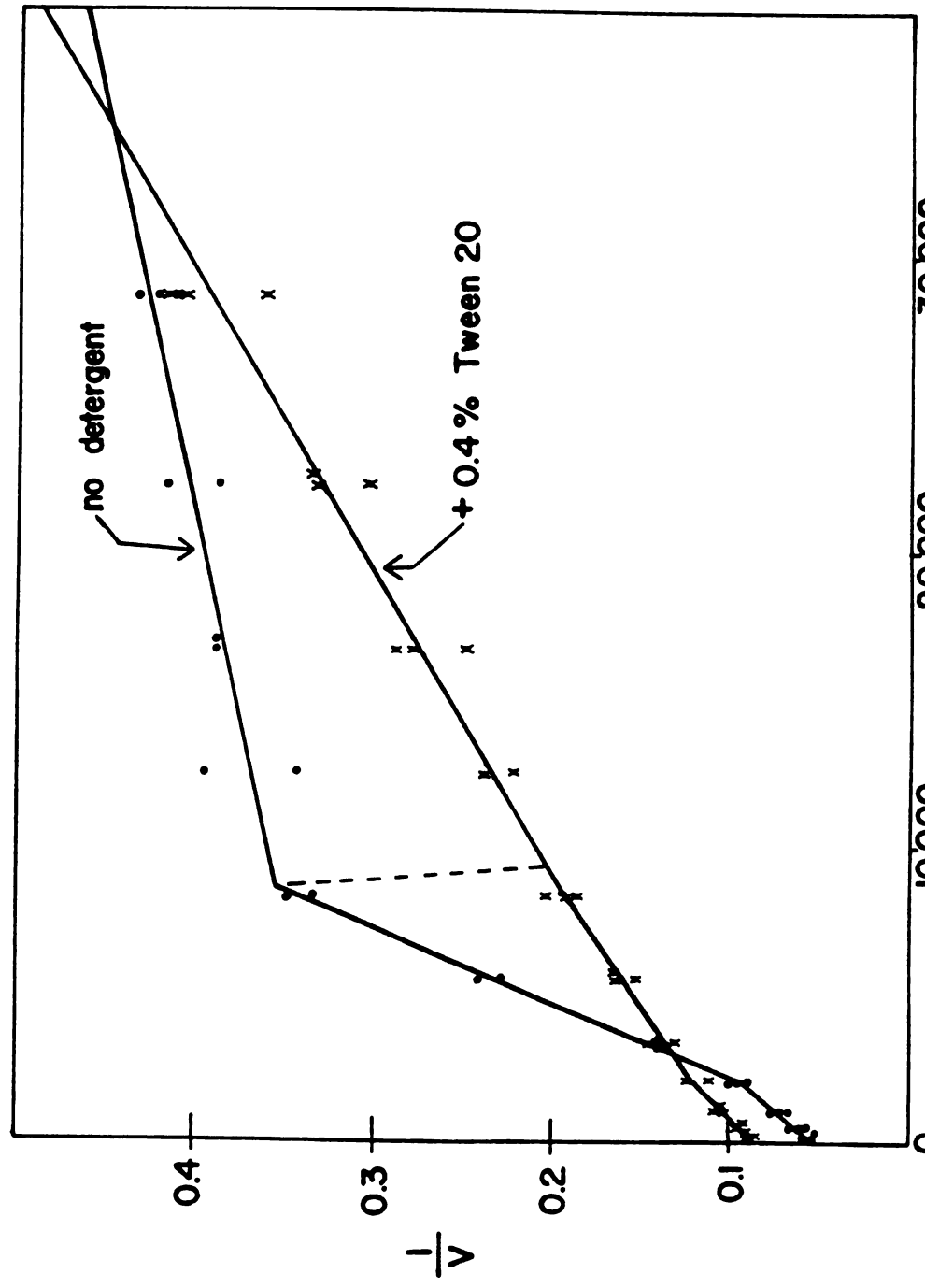
no detergent
+ 0.4 % Tween 20
0.4
0.3
0.2
0.1
1/V
0
10,000
20,000
30,000
C-14 acylcarnitine (M⁻¹)

Figure 15

substrate except that the break occurs around a substrate concentration of 1000 μM.

The smooth transition of enzyme activity with substrate concentrations from below to above the CMC for myristoylcarnitine seen in Figure 15 in the presence of 0.4% Tween-20 implies the presence of mixed micelles in which the acylcarnitine:non-ionic detergent ratio gradually rises as the substrate concentration is increased. That acylcarnitines will form mixed micelles below their CMC in the presence of micelles of Tween-20 is shown in Figure 16 which shows that radioactively labeled laurylcarnitine associates with micelles of Tween-20 at 0.4% (v/v) as observed during gel filtration. As the micelles of Tween-20 pass through the column, monomers of laurylcarnitine preferentially associate with the micelle so that there is an excess of radioactive laurylcarnitine where the micelles elute, leaving a corresponding deficit of labeled laurylcarnitine in its wake.

In order to determine the substrate specificity of the solubilized beef heart mitochondrial CPT with all substrates in the monomeric state and to compare these values to those obtained with the enzyme in a micellular environment, kinetic studies were performed with all substrate concentrations below their micellar concentrations. Activities for palmityocarnitine monomers could not be accurately determined due to the limited sensitivity of the assay at these concentrations (i.e., below 10 μM). Double reciprocal plots of carnitine acyltransferase activity versus concentration of octanoyl-, decanoyl-, lauryl- and myristoylcarnitine are shown in Figure 17 where the solid line denoted $\bar{c}$ indicates reaction in the presence of 0.4% Tween-20 and the broken line denoted $\bar{s}$ indicates

Figure 16. Association of laurylcarnitine monomers in micelles of Tween-20.

Radioactive labeled lauryl (dl) carnitine was synthesized as described (120, 131). Gel filtration chromatography was performed as described in the Methods. The number above the dashed line indicates cpm of ^{14}C-laurylcarnitine bound by micelles of Tween-20. The number below the line indicates the depletion of laurylcarnitine. The lower curve represents the presence of micelles as assayed by the fluorescence method of Horowitz (131) described in the Methods. The scale indicates units of relative fluorescence.

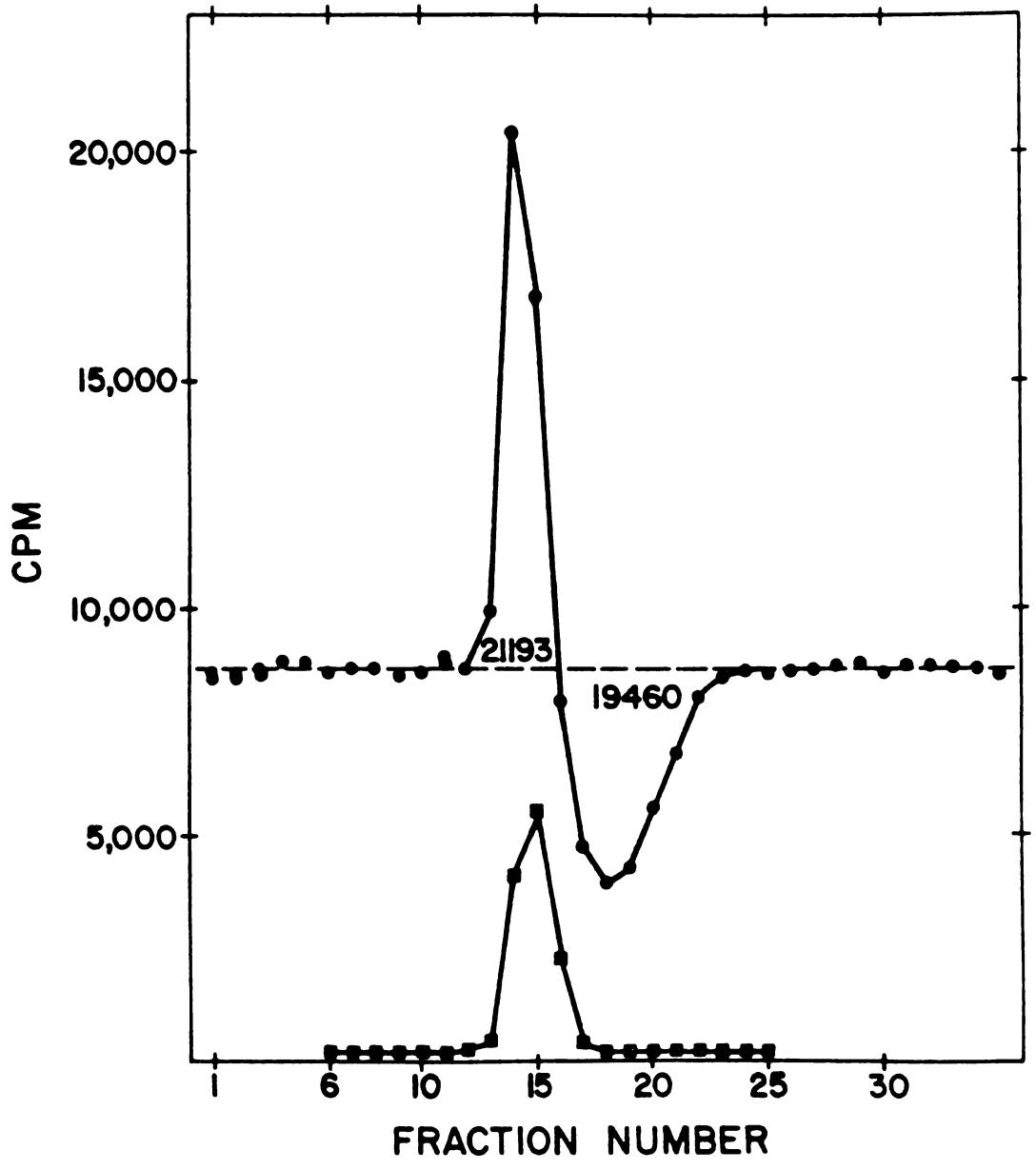
20,000
15,000
10,000
5,000
CPM
21193
19460
1
6
10
15
20
25
30
FRACTION NUMBER

Figure 16

Figure 17. Double reciprocal plots of reaction velocity versus substrate concentration for C_8, C_{10}, C_{12} and C_{14}-carnitine esters.

Enzymes were assayed as described in the Methods. The concentrations of Triton X-100 and enzyme protein were 0.0025% and 2.0 µg/ml, respectively. Solid circles and the line marked $\bar{S}$ represent assays performed in the absence of micelles of non-ionic detergent; Xs indicate the presence of 0.4% (V/V) Tween-20 ($\bar{C}$).

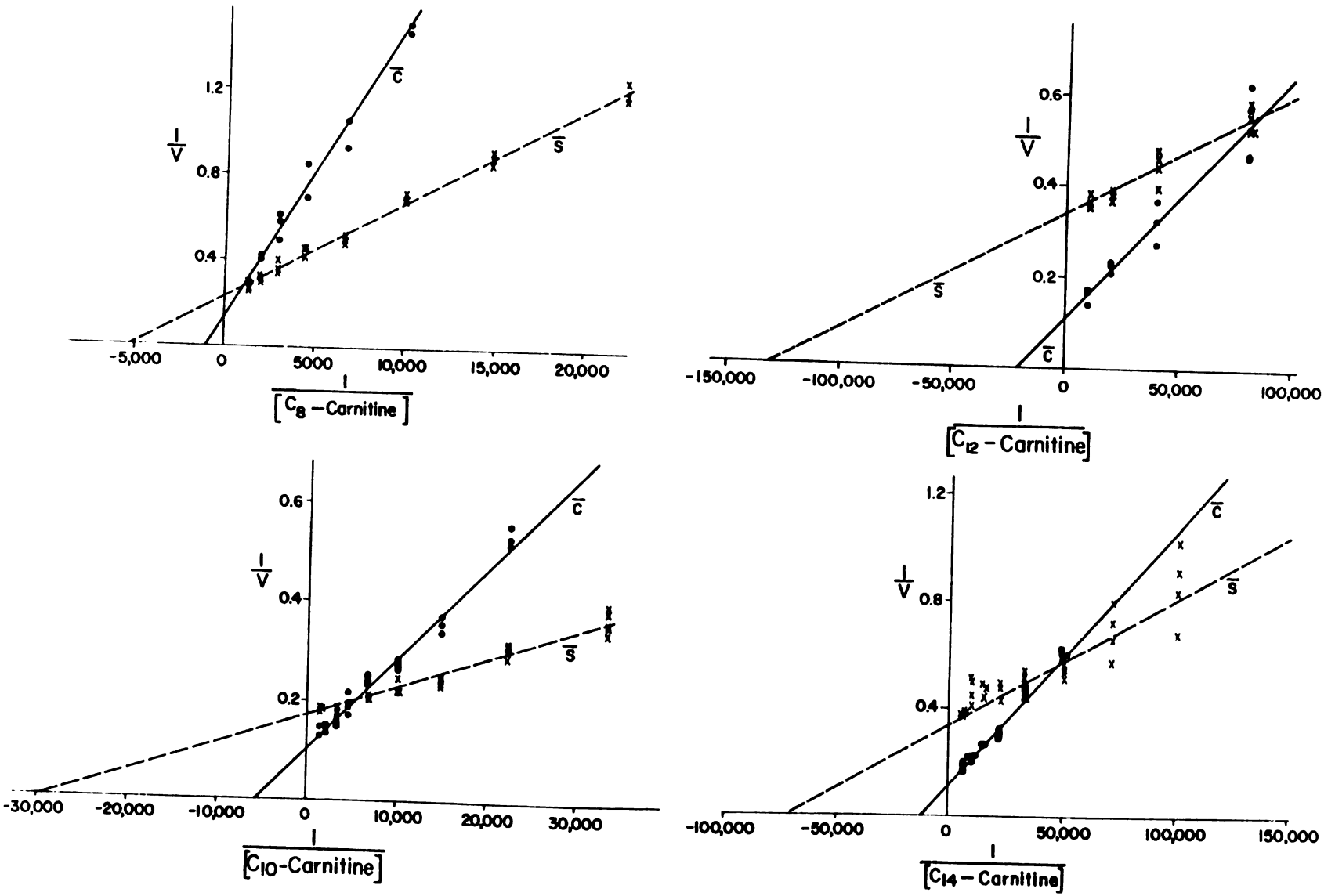
1/V
1.2
0.8
0.4
C̄
S̄
-5,000
0
5000
10,000
15,000
20,000
1/[C8 – Carnitine]
0.6
0.4
0.2
-150,000
-100,000
-50,000
50,000
100,000
1/[C12 – Carnitine]
-30,000
-20,000
-10,000
10,000
20,000
30,000
1/[C10-Carnitine]
-100,000
-50,000
50,000
100,000
150,000
1/[C14 – Carnitine]

Figure 17

its absence. The kinetic parameters V_{max} and K_m obtained from these plots are presented in Table X.

In the absence of detergent, it is seen that the beef heart mitochondrial CPT has its lowest K_m for laurylcarnitine, followed by myristoyl- and octanoylcarnitine. These results showing a specificity for decanoyl and lauryl esters are in accord with those described for the forward reaction with beef heart mitochondrial CPT in the previous paper (123) and to those for ox liver mitochondrial CPT reported by West et al (113) for the reverse reaction. The contributions of acylcarnitine/detergent mixed micelles to the K_m and V_{max} values obtained for reactions performed in the presence of 0.4% Tween-20 may be significant and have not been analyzed further in this study. However, the values given in Table X for reactions done in the presence of detergent are similar to those obtained in its absence: laurylcarnitine shows the lowest K_m, and again the reaction with decanoylcarnitine has the highest V_{max}, though under these conditions, myristoylcarnitine is equally as active as a substrate.

Discussion

Kinetic measurements with purified beef heart mitochondrial carnitine palmitoyltransferase (CPT), revealed biphasic Lineweaver-Burk plots for the reverse carnitine acyltransferase reaction with lauryl- and myristoylcarnitine. These results led us to compare the substrate specificity of beef heart mitochondrial CPT at different substrate concentrations and to investigate the roles of substrate and detergent micelles on the enzymatic activity. We are able to resolve the apparent

Table X. Effect of Tween-20 on K_ms and V_{max} Values for the Formation of Acylcoenzyme A's from Acylcarnitine

	A. No Detergent		B. 0.4% Tween-20			
Acylcarnitine protein	K_m (M)	V_{max} nmol/mg protein	K_m (M)	V_{max} nmol/mg protein	$\frac{K_m\ (B)}{K_m\ (A)}$	$\frac{V_{max}\ (B)}{V_{max}\ (A)}$
Octanoyl	188	4300	1018	7100	5.4	1.65
Decanoyl	34	6200	180	10000	5.3	1.65
Lauryl	7.8	2900	49	8600	6.3	2.96
Myristoyl	14	3000	85	9900	6.1	3.30

discrepancy between significantly different specificity profiles for the reverse carnitine acyltransferase reaction reported for CPT by other investigators by the data presented in Table IX. At a substrate concentration of 500 μM acylcarnitine, our data for beef heart mitochondrial CPT agreed with those of Kopec and Fritz (90) who reported relative rates using calf liver mitochondrial CPT of 2, 31 and 100% for octanoyl-, lauryl- and palmitoyltransferase activities, respectively. At 50 μM acylcarnitine, the profile of substrate specificity shifts to one similar to that described by West et al. (113) for ox liver CPT where they report relative rates of 93, 152 and 100% for the same substrates. Our value for octanoylcarnitine relative to palmitoylcarnitine, 27%, is lower than that reported by the latter investigations (93%). The values for ox liver CPT, however, are listed as relative maximal velocities. The low relative octanoyl activity at 50 μM acylcarnitine is explained by its high K_m relative to the longer chain length acylcarnitines (see data in Table X).

From the data depicted in Figure 15, K_m values of 14, 84 and 2000 μM myristoylcarnitine are observed, depending on the presence or absence of detergent and on the substrate concentration range chosen. In theory, any intermediate value between 14 and 2000 μM can be obtained by choosing the appropriate concentration of detergent, with values between 14 and 55 μM seen for reactions measured below the CMC of myristoylcarnitine and values between 85 and 200 μM possible for the micellar range of substrate concentration. Under these conditions, the V_{max} will correspondingly range from 3.0 to 20 U/mg, these being the respective maximal velocities extrapolated for the monomer and micellar portions of the curve

obtained in the absence of detergent micelles.

To separate the effect of a micellar environment on the enzyme from the micellar effects of substrate, submicellar concentrations of the acylcarnitines were used to determine the kinetic parameters K_m and V_{max} which are presented in Table X. As seen in the column at the right in Table X, V_{max} for octanoyl- and decanoylcarnitine increases by 65% when the enzyme is in a micellar environment while the increase for lauryl- and myristoylcarnitine is approximately 3-fold. The concentrations of substrates employed in this experiment are just below the CMC's of lauryl- and myristoylcarnitine but far below (by one to two orders of magnitude) those for the octanoyl and decanoyl esters so that contributions to V_{max} from the formation of mixed micelles of substrate with detergent would be more significant for the longer chained acylcarnitines and may account for the difference in V_{max} ratios. The ratios in Table X of K_m for acylcarnitine in the presence and absence of detergent micelles, however, reveal a remarkable consistency. That is, the addition of a micellar environment reduces the enzyme's apparent affinity for acylcarnitines by five to six-fold, regardless of the acyl chain length of the substrate investigated. In either the free state in solution or in a micellar environment, the enzyme has its greatest affinity for laurylcarnitine and greatest activity for the reverse reaction with decanoylcarnitine, giving a substrate specificity pattern similar to that reported in the previous paper (123) for the forward reaction using acyl-CoA substrates.

SUMMARY AND CONCLUSIONS

In summary, two carnitine acyltransferase enzymes have been purified from beef heart mitochondria. Comparison of their substrate specificities with those of enzymes purified by other investigators shows them to be carnitine acetyltransferase (CAT) and carnitine palmitoyltransferase (CPT). Partial purification of transferase activities from beef liver mitochondria also reveals two protein fractions whose substrate specificity profiles are very similar if not identical to those of the heart mitochondrial enzymes. The pure beef heart carnitine acyltransferase enzymes have similar amino acid compositions, subunit molecular weights, and isoelectric pH's but differ markedly in their membrane association. The CAT is seen to be membrane-associated and released by salt extraction, while the CPT is found to be membrane-bound, requiring membrane disintegration for its solubilization.

Carnitine octanoyltransferase (COT) activity is present in significant amounts in both of the enzymes purified from beef heart mitochondria and in both of the enzyme fractions separated from beef liver mitochondria. The two proteins purified from beef heart mitochondria satisfactorily account for all of the COT activity detected in the mitochondria initially isolated. In beef heart mitochondria, the greater portion of the total COT activity resides with the CAT enzyme, while in beef liver the majority is associated with the CPT enzyme. This distribution of COT activity between CAT and CPT enzymes parallels the relative abundance of CAT and CPT in beef heart and liver mitochondria.

The substrate specificity of purified beef heart mitochondrial CPT is shown to be dependent upon the presence of micelles of substrate and of non-ionic detergent. By varying the concentration of substrates and detergent, the differing specificity profiles reported by other investigators for preparations of purified CPT can be duplicated. With all substrates in the monomeric state, in the presence or absence of detergent micelles, the preferred substrates for the reverse reaction of beef heart mitochondrial CPT are medium chain length fatty acylcarnitines. Medium chain acyl-CoA esters are likewise the preferred substrates in the forward direction.

It is concluded that no third carnitine acyltransferase enzyme, in addition to CAT and CPT, is present in beef heart mitochondria. Rather beef heart mitochondrial CPT is itself specific for medium chain fatty acyl transfer when assayed in its pure form or in isolated mitochondria. The presence of only two carnitine acyltransferases and a similar specificity of CPT for medium chain fatty acyl transfer is proposed for beef liver mitochondria, based on similar fractionation of transferase activities from beef heart and liver mitochondria.

Because the rate of reaction of beef heart mitochondrial CPT depends upon the form, monomeric or micellar, of the substrate, and because the form itself depends upon the substrate's chain length, concentration and hydrophobic environment, the "physiological substrate specificity" of CPT *in vivo* might be expected to vary with the relative abundance of the various chain length fatty acyl groups.

Finally, as judged by the limited criteria for purity employed in this study--subunit molecular weight, isoelectric pH, approach to

isoelectric equilibrium, and protein/activity superimposition during chromatography--beef heart mitochondria are found to contain one form of CAT and one form of CPT, supporting the conclusion that the inner and outer forms of mitochondrial CPT may not be isoenzymes but are the same protein whose kinetic characteristics may be seen to differ under the appropriate assay conditions due to the different membrane and physiological environments of the two surfaces of the inner mitochondrial membrane.

APPENDIX

COLLABORATIVE STUDIES

APPENDIX

COLLABORATIVE STUDIES

Three investigations performed in collaboration with other workers in Dr. Bieber's laboratory and with members of Dr. Tolbert's group have been prepared for publication. Copies of two of these are contained in the following pages (132, 133). The third (134) is in the press.

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Quantitation of Water-soluble Acylcarnitines and Carnitine Acyltransferases in Rat Tissues*

(Received for publication, April 1, 1977)

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The water-soluble acylcarnitines isolated from rat heart, skeletal muscle, liver, and testis have been characterized. The following acyl residues derived from the acylcarnitine fraction were found: acetyl, propionyl, isobutyryl, butyryl, α-methylbutyryl, isovaleryl, tiglyl, caproyl, β-methylcrotonyl and methacrylyl. The amounts of these acylcarnitines in heart, liver, testis and skeletal muscle from fed rats were determined. Acetylcarnitine was the most abundant acylcarnitine; however, appreciable quantities of propionyl-, isobutyryl-, isovaleryl-, and tiglyl-carnitine were found. The levels of carnitine octanyltransferse, carnitine acetyltransferase and carnitine palmityltransferase activities were determined in several tissues. In addition, carnitine isovaleryltransferase and isobutyryltransferase activities were measured in heart, skeletal muscle, liver, testis and kidney. In all instances the specific activity of isobutyryltransferase was similar to the specific activity of acetyltransferase. The results are consistent with the proposal that carnitine is involved in the catabolism of branched-chain amino acids.

Carnitine acetyltransferase and carnitine octanyltransferase activities are associated with peroxisomes and microsomes as well as with mitochondria in rat liver (1-5). This multiorganelle distribution of the octanyl- and acetyltransferase activities indicates that carnitine has roles other than the well established one of translocating long chain acyl residues across the acyl-CoA barrier of mitochondria (6-9).

As part of an effort to determine whether carnitine has additional roles in intermediary metabolism, we recently reported the occurrence of 4-carbon and 5-carbon acyl esters of carnitine in beef heart (10-12). Herein, we show that these 4-carbon and 5-carbon acyl esters of carnitine occur in rat heart, liver, skeletal muscle, and testis and that these tissues also contain carnitine octanyltransferase as well as carnitine isovaleryl- and isobutyryltransferase activities.

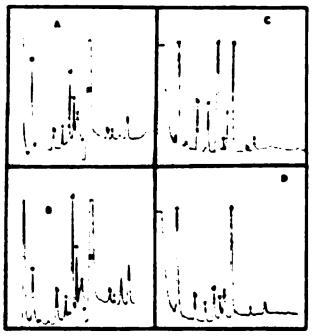
DISCUSSION

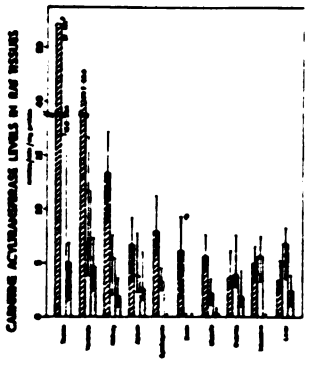
The volatile fatty acids associated with carnitine in rat muscle, liver, heart, and testis are qualitatively similar to those found in beef heart. The presence of branched chain 4-carbon and 5-carbon acyl derivatives of carnitine in rat muscle, liver, heart, and testis is consistent with the previous suggestion that carnitine is involved in branched chain amino acid catabolism (10-12). The presence of large amounts of acetylcarnitine in rat heart, in beef heart (12), in piglet heart (>600 nmol/g of tissue)[1] indicates that acetylcarnitine may function as an immediately available supply of acetyl units that could serve as an energy source during the initial phases of increased energy demands.

The occurrence of carnitine octanyltransferase activity in all of the tissues tested indicates a general role in metabolism for this enzyme activity. The finding that the levels of carnitine isobutyryltransferase activity in heart, muscle, kidney, testis, and liver are similar to the levels of carnitine acetyltransferase activity while the carnitine isovaleryltransferase activity was much lower is surprising. It could mean that the isobutyryltransferase and acetyltransferase activities are due primarily to the same enzyme, namely, carnitine acetyltransferase, while the isovaleryltransferase activity might be attributable to a different enzyme. This is in contrast to the commercial preparation of carnitine acetyltransferase in which the isobutyryl- and isovaleryl transferase activities were much lower than the acetyltransferase activity.

* The methods, Results, and References of this paper (including Figs. 1 and 2 and Tables I and II) are presented in miniprint at the end of this paper. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 77M-469, cite author(s), and include a check or money order for $1.20 per set of photocopies. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Y. R. Choi, P. J. Fogle, P. R. H. Clarke, and L. L. Bieber, unpublished data.





Communication

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Studies on the Oxidation of Isobutyrylcarnitine by Beef and Rat Liver Mitochondria*

(Received for publication, April 2, 1979, and in revised form, April 27, 1979)

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Mitochondria from beef liver oxidize isobutyrylcarnitine at approximately 50% the rate of succinate in the presence of rotenone. However, the oxidation rate of isobutyryl coenzyme A in the presence of *l*(−)-carnitine is very low and can be negligible in both rat and beef liver mitochondria. The limited stimulation of isobutyryl-CoA oxidation by *l*(−)-carnitine appears to be due to inhibition of isobutyrylcarnitine translocation rather than lack of formation of isobutyrylcarnitine. This conclusion is supported by the fact that: 1) isobutyrylcarnitine oxidation is inhibited by *l*(−)-carnitine; 2) some oxidation of isobutyryl-CoA is obtained when a low concentration (50 μM) of *l*(−)-carnitine is used; and 3) under conditions of high isobutyryl-coenzyme A and *l*(−)-carnitine concentrations (1 mM), isobutyrylcarnitine is produced in near theoretical amounts by these rat liver mitochondria. Other studies demonstrated that less than 25% of the carnitine isobutyryltransferase activity of beef liver mitochondria and rat liver mitochondria is located on the cytosol side of the acylcoenzyme A barrier of these mitochondria.

Beef heart (1), as well as testis, skeletal muscle, liver, and heart from rats (2), contain 4-carbon and 5-carbon branched chain acylcarnitines. These tissues also contain considerable quantities of carnitine isobutyryltransferase and carnitine isovaleryltransferase activity (2). The occurrence of 4-carbon and 5-carbon branched chain acylcarnitines and branched chain acyltransferase activity in these tissues indicates that carnitine may be involved in the metabolism of the branched chain amino acids, valine, leucine, and isoleucine. Since the oxidation of the carbon skeletons of these amino acids occurs partly or entirely in mitochondria, a role for carnitine in shuttling the branched chain acyl residues of cytosolic acyl-CoAs across the acyl-CoA barrier of mitochondria was previously proposed (1). In this paper, we show that beef and rat liver mitochondria readily oxidize exogenous isobutyrylcarnitine, but oxidize much less isobutyryl-CoA in the presence of carnitine.

MATERIALS AND METHODS

Mitochondria were isolated from rat liver and bovine liver essentially as described previously (3). Beef livers were obtained from a local abatoir at the time of slaughter; the liver was sliced into thin strips and put in ice-cooled 0.25 M sucrose buffer for transportation to the laboratory. With succinate as substrate, ADP stimulated the respiration with rat liver mitochondria 2- to 5-fold; and with beef liver mitochondria, the increase was 1.5- to 3-fold. Respiration studies were conducted essentially as described in Ref. 3 except that mitochondria (0.1 to 1.3 ml) were added to a buffered KCl solution to make the final concentration in 4.0 ml as follows: 89 mM KCl, 45 mM Tris-HCl, 3.0 mM $MgCl_2$, 6.0 mM P_i, pH 7.5, at a temperature of 33°C. Carnitine acyltransferase assays were performed as described elsewhere (2). The relative distribution of carnitine isobutyryltransferase on the cytosol side and the matrix side of the acyl-CoA barrier of beef liver mitochondria and rat liver mitochondria was determined as previously described for the distribution of carnitine palmityltransferase; see Table II of Ref. 3.

In order to investigate the production of isobutyrylcarnitine by intact mitochondria given a high concentration (1 mM) of *L*-(−)-carnitine and of isobutyryl-CoA, tracer amounts of *dl*-[^{3}H]carnitine were included in incubations identical to those used in the respirations studies. At varying times after the initiation of the reaction by addition of the substrates, aliquots were removed and immediately boiled for 3 min, cooled, and subsequently centrifuged. Tritium-labeled carnitine and isobutyrylcarnitine were then separated from the supernatant liquid by the system of Bohmer and Bremer (4) and quantitated by liquid scintillation counting of the scraped silica areas (scintillation mixture: 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of toluene with 1 liter of Triton X-100). When the *dl*-[^{3}H]carnitine preparation used was chromatographed separately or in the presence of supernatant liquid from a boiled mitochondria solution, a contaminant accounting for 1.0% of the total radioactivity was found to co-migrate with the isobutyrylcarnitine standard. Counts measured in silica scraped from the isobutyrylcarnitine area of the thin layer chromatography plate were corrected for this contaminant. Proteins were determined by a modification of the method of Lowry (described in Ref. 5). Isobutyrylcarnitine, the *l*-(−)-isomer was synthesized as described previously for synthesis of crotonylcarnitine (6). Carnitine was the generous gift of Otsuka Pharmeutical, Tokyo, Japan.

Male rats, 150 to 200 g, were used in this study. During the long term fasting, 8 days, the animals had free access to water.

RESULTS

The purpose of this study was to determine if mitochondria from rat liver or beef liver oxidize isobutyryl-CoA or isobutyrylcarnitine, or both. As shown in Fig. 1*a*, beef liver mitochondria oxidize isobutyrylcarnitine. This oxidation is rotenone-insensitive but cyanide-sensitive. However, these same mitochondria do not oxidize isobutyryl-CoA in the presence of *l*-(−)-carnitine nearly as rapidly; this observation was also made for rat liver mitochondria; see Fig. 1*b*. These experiments were repeated several times with beef liver and rat liver mitochondria and in all instances, the stimulation of oxidation of isobutyryl-CoA by added carnitine was very slight or not detectable, see Fig. 1*b*. The concentrations of isobutyryl-CoA were varied over 2 orders of magnitude with essentially no change. These mitochondria were still capable of β oxidation and the carnitine palmityltransferase was still active under the experimental conditions since addition of palmityl-CoA caused a significant increase in the respiration rate in the presence of *l*-(−)-carnitine, see Fig. 1*b*. When the experiments shown in Fig. 1*b* were repeated, but 50 μM carnitine was added rather than 900 μM carnitine, a small stimulation of oxygen consumption was obtained which increased when isobutyrylcarnitine was added (data not shown). This indicated that the added carnitine might be inhibiting the oxidation of isobutyrylcarnitine.

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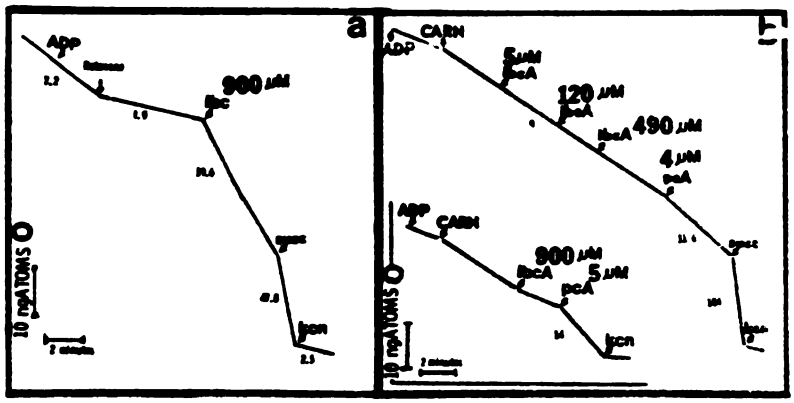

FIG. 1. Oxidation of isobutyrylcarnitine (*A*) and isobutyryl-CoA (*B*) by liver mitochondria. The *numbers below the tracings* indicate the nanogram atoms of oxygen/min/ng atoms of protein. In *A*, 7.1 mg of beef liver mitochondrial protein were used in a final volume of 4.0 ml. In *B*, 5.2 mg of rat liver mitochondrial protein from 5-day-fasted animals were used in a final volume of 4.0 ml. In the *upper curve*, 1.5 mM carnitine and in *lower*, 3.0 mM carnitine. ADP = 0.5 mM; *ibcA* = isobutyryl-CoA; *pcA* = palmityl-CoA; *succ* = succinate, 0.3 mM; *kcn* = K^+ cyanide, 6 mM. The *numbers underneath* are the nanogram atoms of oxygen/min/ng atoms of protein. *ibc*, isobutyrylcarnitine.

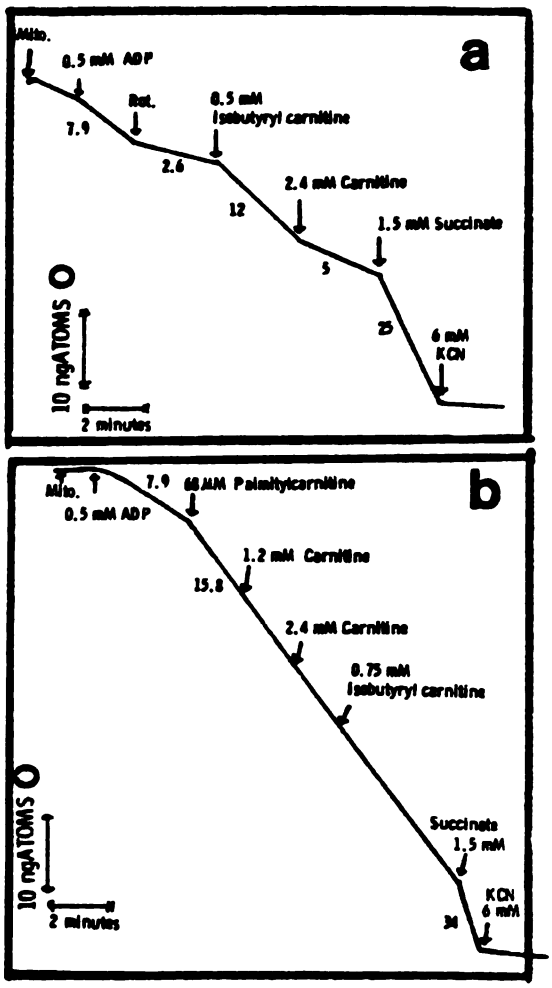

FIG. 2. Effect of carnitine on the oxidation of isobutyrylcarnitine and palmitylcarnitine by beef liver mitochondria. The numbers below the tracings are nanogram atoms of oxygen/min/ng atoms of protein. In *A*, 6.6 mg of mitochondrial protein in a 4.0-ml final volume were used. In *B*, 9.9 mg of mitochondrial protein in 4.0 ml were used. Different preparations of mitochondria were used in *A* and *B*. *Mito* = mitochondria; *rot* = rotenone.

The possible inhibition of isobutyrylcarnitine oxidation by *l*-(−)-carnitine was tested. A series of experiments were performed which showed that *l*-(−)-carnitine, the natural isomer, inhibited the oxidation of isobutyrylcarnitine in both rat liver and beef liver mitochondria. High concentrations of carnitine gave greater inhibition than low concentrations. A typical experiment is shown in Fig. 2*a*. In contrast, palmitylcarnitine oxidation was not inhibited by carnitine; see Fig. 2*b*. The oxidation of isobutyrylcarnitine by rat liver mitochondria from fasted and fed rats was compared. These results are not shown. In some preparations, mitochondria from fasted animals showed greater oxidation rates than mitochondria from fed animals, but the increase was not large.

The very limited oxidation of isobutyryl-CoA in the presence of *l*-(−)-carnitine could also indicate that these mitochondria contain small amounts of isobutyryltransferase on the cytosol side of the acyl coenzyme barrier. This was tested by determining the per cent or relative distribution of carnitine isobutyryltransferase on each side of the acyl-CoA barrier of both beef liver and rat liver mitochondria. As shown in Table I, less than 25% of the total mitochondrial transferase activity was detected on the cytosol side of the coenzyme A barrier. These experiments were repeated several times with essentially the same results. In contrast, the distribution of carnitine palmityltransferase activity on each side of the acyl coenzyme A barrier was about 1:3 for bovine liver mitochondria and near 1:1 for rat liver mitochondria. A similar distribution was found previously for rat liver mitochondria (3). In these experiments, glutamic dehydrogenase was used as a marker for mitochondrial breakage and conditions were adjusted to prevent absorbance changes due to swelling or shrinking of mitochondria. The controls, all components present except 5,5′-dithiobis(2-nitrobenzoic acid) showed negligible absorbance changes.

In the presence of high concentrations (1 mM) of *l*-(−)-carnitine and isobutyryl-CoA, the "outer" mitochondrial transferase is able to produce isobutyrylcarnitine under the conditions used in the respiration studies as demonstrated by the data presented in Fig. 3. The curves containing the solid symbols indicate a near equilibration of the transferase products by 30 min and show further that the transferase activity of intact mitochondria is significantly less than that of mitochondria which have been disrupted with Triton X-100. In an attempt to obtain a more linear initial rate of isobutyrylcarnitine production and to quantitate the relative outer and total transferase activities under these conditions, dilutions of mitochondrial protein of 5-, 10-, and 20-fold were made before initiating the incubation reactions with labeled substrate. The data for a 10-fold dilution are presented by the open symbols

TABLE I

Relative amount of carnitine isobutyryltransferase accessible to exogenous isobutyryl-CoA in beef and rat liver mitochondria

Enzyme	Beef liver			Rat liver					
				Fed			Fasted for 6 days		
	Total activity	Activity in absence of Triton X-100	% of Total	Total activity	Activity in absence of Triton X-100	% of total	Total activity	Activity in absence of Triton X-100	% of total
	nmol/min/mg protein			nmol/min/mg protein			nmol/min/mg protein		
CIBT*	31.3	2.9	9.3	2.1	0.48	20.5	8.2	1.8	15.4
CPT	32.8	8.3	26.3	8.2	4.3	52.4	10.4	3.3	31.7
GDH	5600	None detected	0						

* The abbreviations used are: CIBT, carnitine isobutyryltransferase; CPT, carnitine palmityltransferase; GDH, glutamic dehydrogenase.

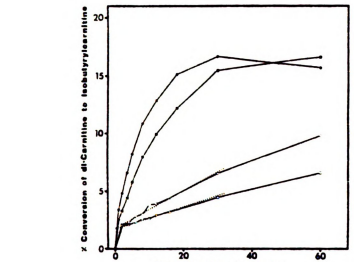

FIG. 3. Production of isobutyrylcarnitine from isobutyryl-CoA and *l*-(−)-carnitine by rat liver mitochondria. Incubations were identical to those performed in the preparation studies with the exception that *l*-(−)-carnitine (1 mM), isobutyryl-CoA (1 mM), and *dl*-[^{3}H]carnitine (4 million dpm/ml) were present from the beginning of the incubation; and aliquots of 150 µl were removed at the times plotted on the graph. Reaction mixtures were 4 ml. The symbols are interpreted as follows: ●—●, mitochondria + 0.1% Triton X-100, 2.10 mg/ml of protein; ■—■, intact mitochondria, 2.10 mg/ml of protein; ○—○, mitochondria + 0.1% Triton X-100, 0.21 mg/ml of protein; □—□, intact mitochondria, 0.21 mg/ml of protein; ·····, least squares regression line for $T = 2$ to 30 min.

in Fig. 3. Analysis of the least squares regression lines for the linear portions of these curves yields an estimate that intact rat liver mitochondria at a high concentration of carnitine and isobutyryl-CoA have 50% of the total transferase activity that is found in the mitochondrial preparations whose CoA barrier has been disrupted by detergent. This value is higher than the amount found with the 5,5′-dithiobis(2-nitrobenzoic acid) assay shown in Table I. However, 4 to 6% of the glutamic dehydrogenase was found in the soluble fraction at the end of the experiments, indicating some breakage of mitochondria. This could contribute to the apparent higher amounts of outer isobutyryltransferase found in Fig. 3.

The equilibrium constant for the reaction: acylcarnitine + CoASH ≈ carnitine + acylCoA has been reported to be 0.6 by Fritz (7) for liver mitochondrial carnitine acetyltransferase. Because *d*-(+)-carnitine is not a substrate for the acetyltransferase (8), one can predict that at equilibrium, 28% of the tritium-labeled *dl*-carnitine will be converted to labeled isobutyrylcarnitine given equal molar initial concentrations of acyl-CoA and *l*-(−)-carnitine. This theoretical maximum is closely approximated in some of the incubations which have been performed. However, most reactions yield maxima between 15 and 20% as in Fig. 3, due probably to the presence of hydrolases, the acyl-CoA hydrolases, the removal of one of the substrates, and the subsequent shifting of the equilibrium toward the initial reactants.

DISCUSSION

The results in Fig. 1 demonstrate that beef liver mitochondria oxidize isobutyrylcarnitine with a rate approximately 50% the rate of succinate oxidation. The rotenone insensitivity of this oxidation is consistent with the flavoprotein-linked dehydrogenase involved in the first step in the oxidation of isobutyryl-CoA. The data given in Table I show that these mitochondria contain much less carnitine isobutyryltransferase activity on the cytosol side of the acyl coenzyme A barrier than on the matrix side, the ratio of activities is about 1:5, respectfully.

The fact that approximately 10% of the isobutyryltransferase activity of bovine liver mitochondria is apparently located on the cytosol side of the acyl coenzyme A barrier is consistent with the limited oxidation of isobutyryl-CoA in the presence of carnitine. However, the finding that *l*-carnitine can inhibit the oxidation of isobutyrylcarnitine indicates that the isobutyryltransferase on the cytosol side of the acyl-CoA barrier may not be the limiting factor in the oxidation of cytosolic isobutyryl-CoA. Appreciable quantities of isobutyrylcarnitine are formed from isobutyryl-CoA and 1 mM *l*-(−)-carnitine by intact rat liver mitochondria as shown in Fig. 3. The inhibition of the oxidation of exogenous isobutyrylcarnitine by free carnitine could be due to the inhibition of isobutyrylcarnitine transport by free carnitine. This type of inhibition is implicated by studies which have shown that carnitine is an inhibitor of acetylcarnitine exchange in rat heart mitochondria (9). A similar exchange system in ox heart mitochondria has been investigated by Ramsay and Tubbs (10). To our knowledge, such an exchange system has not been demonstrated for liver mitochondria.

The lack of stimulation of oxygen consumption by added carnitine in the presence of isobutyryl-CoA and the inhibition of isobutyrylcarnitine oxidation by added carnitine could be caused by inhibition of the formation of isobutyryl-CoA in the matrix. This could occur if the concentration of carnitine in the matrix were elevated to a level whereby the equilibrium of the reaction:

isobutyrylcarnitine + CoASH ⇌ carnitine + isobutyryl-CoA

precluded formation of substrate amounts of isobutyryl-CoA. This seems unlikely because: carnitine did not inhibit palmitylcarnitine oxidation and accumulation of carnitine by liver mitochondria has not been demonstrated. The data imply

that mitochondria would oxidize little isobutyryl-CoA in contact with the cytosolic side of the inner membrane of mitochondria when considerable carnitine is present. In other studies, data to be published elsewhere, we have found that carnitine isobutyryltransferase is also associated with peroxisomes; thus, isobutyrylcarnitine might be formed in other cellular compartments as well as in mitochondria.

The complete subcellular distribution of the enzymes involved in valine metabolism, a source of isobutyryl coenzyme A, is not known. Considerable quantities of the α-keto acid dehydrogenase that oxidizes the keto acid derived from valine are associated with mitochondria (11), but possibly on the cytosol side of the inner membrane (12). The activation of short chain fatty acids can occur in the matrix of beef liver mitochondria (13). Thus, some isobutyryl-CoA could be formed directly from the free acids in the matrix of mitochondria; however, the need for large amounts of isobutyryltransferase in this compartment is not apparent. One possibility is that carnitine acyltransferases such as carnitine acetyltransferase, carnitine isobutyryltransferase, and carnitine octanyltransferase activities are needed to ensure that mitochondria and other cellular compartments always have adequate amounts of free CoASH. This could be accomplished by ensuring that mitochondria and other cellular compartments always have adequate amounts of free CoASH. This could be accomplished by maintaining a relatively constant ratio of free CoASH to acyl-CoA. Since the equilibrium constant for the carnitine acyltransferases is near unity and the amount of carnitine greatly exceeds the total amount of coenzyme A in mammalian tissues, the presence of a broad spectrum of carnitine acyltransferase activity and free carnitine could enable the cell to maintain adequate levels of free reduced coenzyme A. In certain metabolic situations, the short chain acylcarnitines might be exported from the matrix of mitochondria in exchange for free carnitine which could be used to maintain the CoASH/acyl-CoA ratio as recently suggested for skeletal muscle (14). The acylcarnitines could subsequently be utilized as the ratio of CoASH/acyl-CoA increases. Such a general role would still be consistent with the specific functions for carnitine such as translocating long chain acyl residues across the inner membrane of mitochondria and for the roles proposed in branched chain amino acid metabolism.

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