A STUDY OF THE KINETICS, SPECIFICITY, AND REGULATION OF HEART MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASE

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ABSTRACT

A STUDY OF THE KINETICS, SPECIFICITY, AND REGULATION OF HEART MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASE

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A study of the kinetic properties of heart mitochondrial carnitine palmitoyltransferase (CPT) was undertaken using both CPT purified to apparent homogeneity from beef heart and membrane bound CPT-I (outer form of CPT) from rat heart. Purified CPT is an aggregate of molecular weight 660,000 by Fractogel TSK chromatography with a subunit molecular weight of 67,000 by SDS PAGE. This aggregate contains 19 moles of phospholipid per mole of enzyme which are primarily cardiolipin, phosphatidylcholine and phosphatidylethanolamine. Purified beef heart CPT has sigmoidal kinetics with both acyl-CoA and L-carnitine. It has higher affinity for L-carnitine in the presence of long-chain acyl-CoA derivatives, but it has the highest absolute catalytic rate with hexanoyl-CoA. Its catalytic activity is strongly pH dependent with a pH optima of 7. The $K_{0.5}$ for palmitoyl-CoA is 1.9 µM and 24.2 µM at pH 8 and 6, respectively. The $K_{0.5}$ for L-carnitine is 0.2 mM and 2.9 mM at pH 8 and 6, respectively. Malonyl-CoA (20-600 µM) has no effect on the kinetics of purified CPT with palmitoyl-CoA. In contrast, TDGA-CoA increased the $K_{0.5}$ for palmitoyl-CoA and reduced the Hill coefficient.

When octylglucoside is substituted for Triton X-100, the specificity of purified beef heart CPT in the forward direction shifts towards the long-chain acyl-CoAs and large changes in the kinetic constants are observed. At pH 8.0 and 200 μM palmitoyl-CoA, the $K_{0.5}$ for L-carnitine is 4.9 mM in 12 mM octylglucoside compared to 0.2 mM in 0.1% Triton X-100. At pH 6.0, the $K_{0.5}$ for palmitoyl-CoA is 24.2 μM in 0.1% Triton X-100, compared to 3.1 μM in 12 mM octylglucoside. Octylglucoside is an apparent competitive inhibitor of CPT reaction with octanoyl-CoA with a K_i of 15 mM.

Membrane bound CPT-I from rat heart mitochondria shows substrate cooperativity of similar magnitude to that exhibited by the purified enzyme from beef heart mitochondria. The $K_{0.5}$ for decanoyl-CoA is 3 μM with mitochondria from both fed and fasted rats. Addition of malonyl-CoA increased the $K_{0.5}$ for decanoyl-CoA with no apparent increase in sigmoidicity or V_{max}. With 20 μM malonyl-CoA, the $K_{0.5}$ for decanoyl-CoA is 185 μM and the Hill coefficient is 2.1. CPT-I from fed rats had an apparent K_i for malonyl-CoA of 0.3 μM while that from fasted rats was 2.5 μM. The kinetics with L-carnitine were variable: the $K_{0.5}$ ranged from 0.2 mM to 0.7 mM and the Hill coefficient varied from 1.2 to 1.8.

These data are consistent with the conclusion that native CPT exhibits different catalytic properties on either side of the inner membrane of mitochondria due to its

non-Michaelis-Menten kinetic behavior, which can be affected by pH differences and differences in membrane environment. The data suggests that the physiological levels of L-carnitine may a be determing factor in the specificity of CPT *in vivo*. It is concluded that malonyl-CoA is not a competitive inhibitor of CPT but acts like a negative allosteric modifier of CPT-I.

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LIST OF ABBREVIATIONS

Bis Tris-	1,3-bis(tris[hydroxymethyl]-methylamino)-
BSA	Bovine serum albumin
CAT	Carnitine acetyltransferase
CMC	Critical micellar concentration
CoA	Coenzyme A
CoASH	Reduced Coenzyme A
COT	Carnitine octanoyltransferase
CPT	Carnitine palmitoyltransferase
CPT-I	Outer form of carnitine palmitoyltransferase
CPT-II	Inner form of carnitine palmitoyltransferase
DTBP	4,4'-dithiobispyridine
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	(Ethylenedinitrilo)-tetra-acetic acid
EGTA	Ethyleneglycol-bis-(-amino-ethyl ether)N,N'-tetra-acetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hill n	Hill coefficient
I_{50}	the inhibitor concentration that causes 50% inhibition under a specific set of assay conditions
K_i	Inhibition constant
K_m	Michaelis constant
$K_{0.5}$	Hill constant

QAE	Diethyl-(2-hydroxypropyl)aminoethyl-
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis
TANKIN	Tangent slope kinetic analysis
TDGA	2-tetradecyl-glycidic acid
Tris	tris-(hydroxymethyl)aminomethane
V_{max}	Maximum velocity

INTRODUCTION

Carnitine (gamma-trimethylamino-beta-hydroxybutyrate), discovered as a component of muscle tissue in 1905 (1), is ubiquitous in the animal kingdom. Most species are capable of its synthesis, with one notable exception, the larvae of Tenebrio molitor (2). Its four carbon chain originates from lysine (3) and its methyl groups from methionine (4). The concentration of carnitine varies between tissues and amongst species (5). Heart and skeletal muscle normally have a concentration of a few millimoles per liter, while adipose tissue and liver have lower concentrations. The initial observations of Fritz (6) on the stimulation of fatty acid oxidation by L-carnitine in liver, and those of Bremer (7) on the mitochondrial metabolism of palmitoylcarnitine led to the proposal of a role for carnitine in the oxidation of palmitoyl-CoA. The discovery of carnitine palmitoyltransferase (CPT) (8,9) established carnitine as the carrier of activated fatty acids through the CoA impermeable barrier of the inner mitochondria. The identification and isolation of other carnitine acyltransferases with specificity for short and medium-chain acyl residues and their multiple organelle distribution demonstrate other roles for carnitine (10).

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

L-(-)-carnitine + acyl-CoA $\rightleftharpoons$ acyl-L-(-)-carnitine + CoASH.

These enzymes are classified according to their acyl substrate specificity into short-, medium- and long-chain acyltransferases. Carnitine acetyltransferase (CAT) is the predominant acyltransferase in most tissues (11). It is found in mitochondria and peroxisomes (12) where it appears to facilitate the shuttle of acetyl units generated from ß-oxidation into the cytosol, thereby maintaining a free CoASH pool for continuing reactions and possibly providing acetyl units for use in synthesis elsewhere in the cell (13). CAT is also associated with the endoplasmic reticulum in mammalian species where it has been proposed to have a role in synthesis (10). Carnitine octanoyltransferase (COT) has been isolated from liver peroxisomes (14,15) where it forms medium-chain acylcarnitine esters from the chain shortened acyl-CoAs produced by peroxisomal ß-oxidation of very long-chain acyl-CoAs. COT also exists in mammalian microsomes (16), whereas CPT is believed to be primarily a mitochondrial enzyme (17).

The current view of the role of CPT in mitochondrial fatty acid oxidation is illustrated in Figure 1. Two forms of CPT (18) are present on the inner mitochondrial membrane.

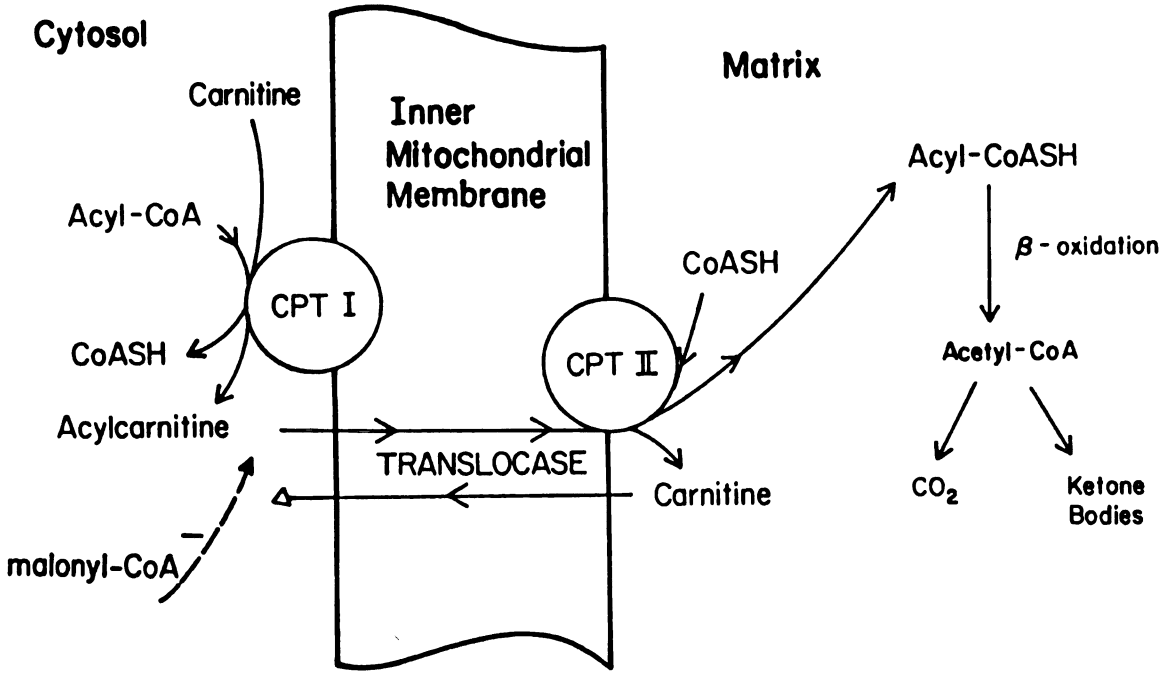

Figure 1. Current view of the role of CPT in mitochondrial fatty acid oxidation.

The outer form of CPT (CPT-I) is exposed to the cytosolic components and catalyzes the formation of acylcarnitine esters from cytosolic acyl-CoAs. The acylcarnitines are transported into the matrix of the mitochondria by a translocase system, in a one to one carnitine/acylcarnitine exchange (19). There they become the substrates of the inner form of CPT (CPT-II) which regenerates the acyl-CoAs in the mitochondrial matrix. Current evidence (20-22) strongly supports the proposal that one of the major mechanisms for control of fatty acid oxidation and ketogenesis is by regulation of the activity of CPT-I through its inhibition by malonyl-CoA. The most significant difference between the inner and outer forms of CPT appears to be the sensitivity of CPT-I to inhibition by malonyl-CoA and the relative insensitivity of CPT-II (23). Though these differences have suggested that the two forms of CPT are distinct enzymes (18,23), when CPT is solubilized from the mitochondrial membrane it loses its capacity to interact with malonyl-CoA (23) and CPT-I and CPT-II become indistinguishable (24). Thus it appears that the membrane environment plays a pivotal role in the regulation of CPT activity (24,5).

CARNITINE PALMITOYLTRANSFERASE

Assay Methods

Two general types of assays have been used to measure the activity of carnitine acyltransferases: one, the continuous assays which measure directly the formation or disappearance of acyl-CoA derivatives at 232 nm (25,26), or indirectly measure the release of CoASH by reaction with sulfhydryl reagents such as DTNB (27) or DTBP (28); and two, discontinuous (end point) assays for which three general approaches have been used, measurement of the formation of a specific radioactive acylcarnitine (29,30), measurement of the formation of acylhydroxamates (31), and measurement of the exchange of radioactive carnitine into the acylcarnitine fraction (25). Assays measuring the formation of acylcarnitines or release of CoASH are known as forward assays and those which measure the formation of acyl-CoA or free carnitine are known as reverse assays. In addition, it is possible to estimate CPT activity in mitochondria with functional assays measuring flavoprotein reduction (32) or oxygen consumption (33) during the oxidation of fatty acids, acyl-CoAs, or acylcarnitines.

None of these methods is ideal and the best choice of

method will depend on the problem and purity of the system studied. When measuring acyltransferase activity in impure preparations or tissue homogenates with assays that measure the formation or disappeareance of acyl-CoA or depend on maintenance of saturating amounts of acyl-CoAs, acyl-CoA hydrolase activity must be quantitated. This is difficult with exchange assays or assays which measure acyl-CoA formation. In addition, tissues such as liver contain a family of carnitine acyltransferases, with overlapping substrate specificity, thus the activity measured in homogenates may represent the contribution of more than one enzyme.

Isotopic exchange methods, which are run at or near equilibrium, are inappropriate for kinetic measurements. The isotope forward assay is very sensitive and is frequently used in kinetic studies (20,34) but special care must be taken to ensure that initial rate conditions are maintained throughout the incubation period. This procedure can be tedious and is often times omitted. In some studies the use of long incubation times at low substrate concentrations suggest that the reaction has gone to near completion (35). Continuous spectrophotometric assays, though less sensitive than the isotope forward assay, are more convenient for kinetic measurements since changes in enzyme activity with time can be monitored to ensure that a true initial rate of reaction is being measured. Due to a

high background absorbance in homogenates, the 232 assay is used only with enzymes of a high degree of purity. With the 232 assay the product formation is measured directly with no further manipulation. However the low extinction coefficient of the acyl-CoAs at 232 nm requires higher product accumulation, therefore there is an increased possibility of product inhibition. In addition, the reversibility of the reaction is not prevented and may affect the initial rates. The DTNB assay can be used to measure CPT activity in homogenates as well as pure preparations, allows for easy determination of hydrolase activity, and the reaction is essentially irreversible by the removal of free CoASH. However, high concentrations of DTNB (greater than 0.25 M) have been reported to abolish both the sensitivity of CPT-I to malonyl-CoA and the apparent sigmoidal kinetics of CPT-I with palmitoyl-CoA (36). From these studies and other studies (37) on the inhibitory effects of disulfides, the presence of reactive thiol groups in the enzyme essential for catalysis has been proposed.

In order to measure the inhibitory action of malonyl-CoA on CPT-I activity, as well as to measure CPT-I independent of CPT-II activity, membrane integrity must be maintained. Albumin is commonly used in the assays to minimize the detergent effects of the long-chain acyl-CoA substrates on the mitochondrial membrane. McCormick and

Notar-Francesco (38) emphasized the need for precise and standard assay conditions by demonstrating the striking dependence of the $K_{0.5}$ for palmitoyl-CoA on albumin concentration. They also demonstrated that varying assay conditions can modify substrate binding to albumin thus changing the effective concentration of the acyl-CoA substrates. This results in an artifactual effect on CPT activity. Zammit (39) has shown that lags in the time course of acylcarnitine formation arise from the time necessary to obtain equilibrium between free, micellar and albumin-bound forms of the substrate.

Recently, Zierz and Engel (40) have reviewed the clinical studies on CPT deficiencies. They have shown that conflicting results on the nature of the disease are due, in great part, to methodological problems in the CPT assay. Their results demonstrate that patients can show severe CPT deficiency with the isotope exchange assay but normal CPT activities with the forward assay.

Intracellular Distribution

The proposed role for L-carnitine as an acylcarrier across the CoA-impermeable inner mitochondrial membrane requires that there be two locations for CPT in the cell. Early studies on the intracellular localization (8,41,42) showed that CPT was primarily associated with mitochondria,

but suggested an extramitochondrial location as well. Later, a more careful investigation by Norum and Bremer (43), using sucrose density fractionation techniques showed that the low residual activity in the microsomal fractions was a result of mitochondrial contamination. Subsequently, evidence in favour of a dual localization of CPT in the mitochondria itself was presented by Yates and Garland (44). They showed that rat liver mitochondria contained an overt CPT activity accessible to inhibition by 2-bromostearoyl-CoA and a latent activity inaccessible to inhibition by 2-bromostearoyl-CoA, unless it was previously exposed by ultrasonic disintegration of the mitochondria. Hoppel and Tomec (45) used a digitonin fractionation technique to separate the outer and inner mitochondrial membranes in sucrose density gradients and showed that CPT was exclusively located on the inner membrane of mitochondria. Using phospholipase C to strip beef liver mitochondria of the outer membrane, Brosnan et al. (46) demonstrated that both the outer and inner surfaces of the mitochondrial inner membrane contain CPT which is inhibited by antibodies to the enzyme (47).

Though it is now generally agreed that CPT is an exclusively mitochondrial enzyme, there have been further reports of a microsomal location. Van Tol (48) measured CPT activity in the rat liver microsomal fraction that could not be attributed to mitochondrial contamination. In fasted

rats, the microsomal activity is increased to about 60% of CPT activity in mitochondria. Fogle and Bieber (49) found CPT activity associated with rat heart microsomes which had been clearly separated from mitochondria by sucrose density gradient centrifugation. However, the finding that rat liver contains extramitochondrial COT associated with peroxisomes and microsomes (50) now suggests that extramitochondrial long-chain acyltransferase activity results from the overlapping specificity of medium-chain acyltransferases. The isolation of microsomal medium-chain acyltransferases has been made difficult by the apparent lability of the enzymatic activity (50), but peroxisomal COT has been purified from both mouse (14) and rat liver (15). Purified COT has low but significant activity with long-chain acyl-CoAs and has been shown to be a distinct enzyme from CPT (51).

Mitochondrial Distribution

Several different methods have been used to estimate the proportion of CPT-I to CPT-II activity in mitochondria. One common procedure is to measure the overt CPT activity under non-swelling conditions, and then measure the total CPT activity by disruption of the mitochondria by sonication or detergent lysis (29,52-55). CPT-II is estimated from the difference between total and overt activity. Most of these

studies estimate that 25-50% of total CPT activity is due to CPT-I. Though both activities are usually measured with the same assay, it is not clear what effect the sonication or detergent have on the measurement of total activity since the properties of the enzyme may have changed. Selective inhibition of CPT-I with malonyl-CoA (20) or bromostearoyl-CoA (44) has also been used to measure the remaining CPT-II. These methods estimate CPT-I activity to be 15%-50% of total CPT.

Another method uses digitonin to selectively solubilize CPT-I. Hoppel and Tomec (45) showed that CPT activity could be released with digitonin from the inner membrane without releasing the matrix enzymes. This resulted in a preparation of mitoplasts that had severely limited capacity to oxidize palmitoyl-CoA + L-carnitine, but retained the ability to oxidize palmitoyl-L-carnitine. From these data, Hoppel and Tomec proposed that CPT released by digitonin was CPT-I that was loosely associated with the external surface of the inner membrane of mitochondria and the remaining CPT activity was tightly membrane bound CPT-II, exposed to the matrix side of mitochondria. They estimated that only 15% of total CPT activity is due to CPT-I. Assuming that only the outer transferase is released, this method is likely to yield an underestimate of CPT-I activity if the membrane begins to disintegrate before all of the outer CPT has been removed. Hoppel and Tomec (45) used sonication after

digitonin treatment to ensure complete release of CPT-I and increased the proportion of outer CPT measured from 15% to 25%. Recently, other investigators (20) have reported that with increasing concentrations of digitonin significant solubilization of CPT coincides closely with that of citrate synthase, a matrix enzyme, and have challenged the validity of this fractionation method.

Others (32,33,53,56) have used indirect methods involving multienzymatic pathways in an attempt to measure the "in situ" activity. Such methods are less reliable since they do not yield a true measurement of the enzymatic activity. Wood and Chang (53) compared the oxidation rate of palmitoyl-CoA + L-carnitine with the rate of oxidation of palmitoylcarnitine by intact mitochondria to estimate the CPT-I/CPT-II ratio. They observed a 1:2 ratio which is in agreement with their estimate obtained using detergent lysis. Bergstrom and Reitz (32) estimated the CPT-I/CPT-II ratio from measurements of flavoprotein reduction rates during oxidation of acyl-CoA/acylcarnitine by mitochondria. Their estimate of CPT-II activity exceeded that of CPT-I by a factor of 450. They proposed that "in situ" factors regulate the activity of CPT-I because of the difference in CPT I/CPT II ratio obtained with the "in situ" versus the direct digitonin extraction. A more direct approach to measure the "in situ" activity of CPT-II using inverted submitochondrial particles estimates that CPT-I is

approximately 30% of total CPT activity (57).

Tissue origin, as well as physiological and developmental factors, seem to affect the CPT-I/CPT-II ratio. The proportion of latent CPT appears to be higher in adipocyte and mammary gland than in liver and kidney, while heart and skeletal muscle may have the largest proportion of overt activity (58). Bovine fetal heart mitochondria oxidize palmitoyl-CoA at 20-30% of the rate of calf heart mitochondria, but can oxidize palmitoyl-L-carnitine at similar rates (59). In addition, the proportion of CPT-I to CPT-II appears to be altered in many cases of CPT deficiency (60).

In summary, between 15 to 50% of the total mitochondrial CPT is believed to be exposed to the cytosolic surface of the inner mitochondrial membrane. Low estimates of CPT-I activity (33,55) may result from the use of low ionic strength assay medium since CPT-I but not CPT-II has been shown to be inactivated in ionic strength below 0.06 M (61).

CPT Deficiency

Numerous cases of muscle CPT deficiency have been reported (40,60,62,63). A benign condition, unlike muscle carnitine deficiency, the disease is manifested only when lipid metabolism is stressed. Clinical symptoms include

episodes of muscle weakness, pain, and myoglobinuria induced by prolonged exercise and aggravated by prior fasting. In most clinical studies, CPT activity measurements are not initial rate measurements, but measurements at or approaching equilibrium. The assumption that optimal conditions for the assay of CPT in normal tissue are optimal for the assay of CPT in a patient's tissue is questionable, in light of the documented changes in the kinetic parameters of CPT as a response to environmental changes (64,65).

Absolute or partial deficiencies have been reported for a given patient depending on the methodology used. In several of these studies (60,62,63) a nearly absolute deficiency is apparent with the hydroxamate assay as well as with the isotope exchange assay under the usual assay conditions. In contrast, significant activity can be measured in the same patient's tissue with the forward assay (60,62) and with the isotope exchange assay using lower concentrations of substrates (62,63). Clearly, the choice of methodology affects the results. The studies of Zierz et al. (40) show that assays measuring initial velocity at low substrate concentrations and minimal product build up can give normal values of CPT activity in patients manifesting the disease. From these data, Zierz and Engel (40) have proposed that CPT deficiency is caused by altered regulatory properties of a mutant enzyme, which is extremely sensitive to inhibition by its substrate and/or products.

This enzyme also appears to be unusually sensitive to inhibition by detergents and malonyl-CoA. They have offered two interpretations for the increased malonyl-CoA sensitivity; the absence of only malonyl-CoA insensitive CPT (CPT-II), or a mutant enzyme with increased sensitivity to malonyl-CoA.

Purification of CPT

The first attempt to purify CPT (25) involved a salt extraction of lyophylized calf liver mitochondria followed by chromatography on DEAE-cellulose. This yielded a 22 fold increase in specific activity. Though the extraction from the membrane was incomplete and the preparation still contained a small amount of carnitine acetyltransferase, these studies helped establish the existance of a separate long-chain acylcarnitine transferase distinct from CAT. Since CPT is an integral membrane protein, more successful purification schemes (24,15) have used non-ionic detergents to solubilize the enzyme and keep it in solution. Attempts have been made to purify CPT-I and CPT-II separately (66,67). With this approach it is uncertain whether a given extraction procedure will yield only CPT-I or CPT-II. It is also uncertain whether differences in the two final preparations are due to differences in the intrinsic

properties of the enzyme or caused by modified environments which result from the purification techniques used for each pool. For these reasons efforts have been made to treat each pool in an identical manner during purification (32) or to extract total CPT and attempt to separate the putative distinct enzymes using chromatographic techniques (15,24). Table I gives a summary of the properties of several purified forms of CPT.

West et al. reported the separation of two forms of CPT (66). Using frozen ox liver they isolated a soluble enzyme with carnitine palmitoyltransferase activity and purified it 850 fold. This preparation was termed "outer" CPT and was found to be inhibited by 2-bromopalmitoyl-CoA. Another fraction of CPT activity was extracted from the membrane sediment by either treatment with Triton X-100 or butanol and purified 10 fold. This fraction was designated as CPT-II and was insensitive to inhibition by 2-bromopalmitoyl-CoA. Other significant differences in the kinetic constants and physical properties of these two enzymes were observed (see Table I) which suggested that inner and outer CPT are distinct enzymes. Since peroxisomes are known to contaminate mitochondrial preparations, it is possible that the soluble enzyme prepared by West et al. is not CPT, but COT that was leaked by the rupturing of the fragile peroxisomal membrane with freeze thawing of the samples. Interestingly, Miyazawa et al. (15) have shown

TABLE I

SUMMARY OF THE PROPERTIES OF PURIFIED CARNITINE PALMITOYLTRANSFERASES

Source	Form	Specific Activity	-fold purified	Molecular Weight	K_m (μM)				Ref.
					C_{16}-carnitine	CoASH	C_{16}-CoASH	Carnitine	
ox liver	CPT-I	13	850	59,000	12	-	0.59	140	66
	CPT-II	0.4	10	65,000	60		9	2600	
calf liver	CPT (I+II)	0.48	22	-	40	50	10	250	25
calf liver	CPT I	10-38	1200	150,000*	40	5.5	18	450	67
	CPT II			75,000 150,000*					
beef heart	CPT (I+II)	47	1620	530,000* 67,000	-	-	2-24	200-10,000	24,64**
rat liver	CPT-I	6.8	15	430,000*	11	35	2.8	280	32
	CPT-II	36.7	23	430,000*	11	34	3.5	300	
rat liver	CPT (I+II)	29.6	75	280,-320,000* 69,200	67	77	6.3	2000	15

* These are the native molecular weights of the detergent-protein complexes.

** Kinetic parameters of this pure enzyme have been shown to vary 50-fold with different assay conditions.

that purified rat liver COT is inhibited by 2-bromopalmitoyl-CoA, while purified rat liver CPT is not. The identity of the soluble fraction isolated by West et al. is questionable.

Kopec and Fritz (67) extracted CPT from calf liver mitochondria using the detergent Tween 20 and purified it by adsorption onto calcium phosphate gels. They obtained several enzyme preparations of specific activity between 10-38 units/mg of protein. The preparations with the highest specific activity were homogenous by SDS-PAGE and were arbitrarily designated as CPT I. Another protein fraction which did not adsorb to the calcium phosphate gel was partially purified and designated as CPT II. This fraction had no activity with acyl-CoAs, required preincubation with CoASH to manifest activity and was unstable at $4^{o}C$. Later, it was shown that CPT I could be transformed into CPT II by treatment with urea and guanidium chloride. In addition antibodies produced against pure CPT I could inhibit CPT II activity. These data suggested that CPT II was a partially denatured form of CPT and not a distinct enzyme.

More convincing evidence that both pools of CPT are the same enzyme has been presented by Bergstrom and Reitz (32) and Clarke and Bieber (24). The former group separated the outer and inner forms of CPT of rat liver mitochondria using the digitonin fractionation procedure. They partially

purified each fraction independently, but using identical procedures. The partially purified enzymes had identical elution volumes on a Sephadex G-200 column and the same kinetic constants (see Table I), although significant kinetic differences in the "in situ" forms of the enzymes had been observed. Clarke and Bieber extracted all of the CPT activity of beef heart mitochondria by treatment with Triton X-100 and KCl and purified it to homogeneity. Using the technique of isoelectric focusing in approach to equilibrium a single protein band was observed, suggesting that inner and outer forms of CPT are the same enzyme. Recently, Miyazawa et al. (15) have purified CPT to near homogeneity from rat liver and have also found a single polypeptide. Using antibodies to the purified enzyme (68), they obtained only a single precipitation line with liver extracts, and a single <u>in vitro</u> translation product from total liver RNA. This translation product appears to be a precursor of CPT with a slightly higher molecular weight of 71,600. In summary, all of the detergent purified forms of CPT are apparent aggregates of molecular weight between 150,000-700,000 daltons and subunit molecular weight between 65,000-75,000.

Kinetics and specificity.

CPT catalyzes reversible reactions involving acyl-CoAs and acyl-carnitines as substrates with a broad specificity for acylgroups from C6 to greater than C20. CPT is highly specific for L-(-)-carnitine, but also reacts with norcarnitine (3-hydroxydimethylaminobutyrate) (69) and thiocarnitine (beta-sulfhydryl-gamma-trimethylaminobutyric acid) (70) with reduced reaction rates. Deoxycarnitine (beta-hydroxyl group removed) (71) and aminocarnitine (3-amino-4-trimethylaminobutyrate) (72) are not substrates for CPT. These carnitine analogs are competitive inhibitors of the reaction (69,72). Acyl-(+)-carnitines inhibit membrane bound CPT and to a lesser extent the soluble enzyme (73).

Table II summarizes the data from several studies on the acyl-group specificity of CPT from various tissue sources and varying degrees of purification. Using intact mitochondria from calf liver and the reverse isotope assay Solberg (74) selectively assayed the "in situ" acylcarnitine specificity of the inner and outer pools of CPT in the absence and presence of external CoASH, respectively. While the inner transferase pool had an optimal V_{max} with C_7- moieties, the outer transferase pool had an optimal V_{max} with C_9- and C_{10}- moieties. He suggested that the two pools

TABLE II

ACYL-GROUP SPECIFICITY OF CARNITINE PALMITOYLTRANSFERASES*

	Source	Form	Substrate Concentration (μM)	Relative Velocities C_2	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	Ref.
	beef liver	CPT-I		1	22	41	57	69	85	98	100	66
		CPT-II					15		70		100	
acyl-CoAs	beef heart	CPT (I+II)	100			43	113	226	166	100	100	64
	rat liver	CPT (I+II)	100 +Tween 20			35	41	88	53	112	100	15
			-Tween 20			25	47	112	112	119	100	
	calf liver	CPT-I	200	45	41	62	69	41	35	65	100	74
		CPT-II		155	150	260	310	160	75	85	100	
	calf liver	CPT (I+II)		15	6		32	50	65	88	100	25
	calf liver	CPT (I+II)	500				2	26	31	99	100	67
acyl-carnitines	ox liver	CPT-II	varied	0	0	19	93		152		100	66
		CPT-I			40	45	45		76		100	18
	beef heart	CPT (I+II)	500			8	12	40	41	78	100	64
			50			27	32	102	118	88	100	
	rat liver	CPT (I+II)	1000 -Tween 20			15	33	63	59	162	100	15
			+Tween 20			19	31	103	103	108	100	

* Partially pure preparations were used in (66,67,25). Homogenous preparations were used in (64,15). Intact mitochondria were used in (74). Preparations in (74,25) are known to contain CAT.

contained different acyltransferases with different relative velocities in different chain regions. However, it is not clear from these data what are the specific optima and relative velocities of CPT-I and CPT-II, since mitochondria contain CAT with overlapping specificity for the medium-chain acyl-groups.

Other investigators have used partially purified preparations of CPT which are assumed to represent "outer" or "inner" pools. Differences in profiles of these preparations have been advanced as further evidence that CPT-I and CPT-II are distinct enzymes. Tubbs et al. (75) characterized the substrate specificity of the soluble acyltransferase activity from ox liver isolated by West et al. (66) which had been postulated to be "outer" CPT from mitochondria. This enzyme has the highest activity with myristoyl- and palmitoyl- esters in either direction. Though is has been purified 850 fold it still retains activity with short-chain acylgroups. Its broad substrate specificity resembles that of peroxisomal COT (51). In contrast, the activity purified by butanol extraction from the mitochondrial membrane by West et al., which is postulated to be "inner" CPT, has relative velocities of 15 and 100 for octanoyl-CoA and palmitoyl-CoA, respectively and no short-chain acyltransferase activity.

Calf liver CPT purified 1200 fold after extraction with detergent by Kopec and Fritz (67), was free of short-chain

acyltransferase activity, but showed a very dissimilar pattern of substrate specificity from that of the membrane bound enzyme isolated by West et al. The relative activity with octanoylcarnitine was only 2% compared to 93% observed by West et al. Clarke and Bieber (65) were able to resolve the apparent discrepancy in these profiles by studying the effect of two different acylcarnitine concentrations on the acylcarnitine specificity of purified beef heart CPT. At 500 µM acylcarnitine, the specificity profile of purified beef heart mitochondrial CPT agrees with that reported by Kopec and Fritz for calf liver mitochondrial CPT, with increased specificity for long-chain acylcarnitines. At 50 µM acylcarnitine the substrate specificity pattern shifts to one similar to that described by West et al. for ox liver CPT with relative rates of 100 and 118 for palmitoylcarnitine and laurylcarnitine, respectively. The acylcarnitine specificity of CPT is also markedly altered by detergents. Table II shows the effect of Tween 20 on the substrate specificity of mouse liver CPT observed by Miyazawa et al. (15).

The kinetic parameters reported for purified CPT from similar sources can differ by an order of magnitude, see Table I. These variations can result, in part, from differences in the state of the enzyme after several purification steps. For example, the dissociation of the membrane bound enzyme with butanol extraction results in an

increase in the K_m from 2.2 μM to 9 μM (18). In addition, assay conditions such as pH and choice of detergent influence the kinetic parameters of CPT (64,65). Bremer and Norum showed that increasing concentrations of palmitoyl-CoA raise the apparent K_m for L-carnitine. Therefore, they proposed that palmitoyl-CoA can behave as a competitive inhibitor to its cosubstrate L-carnitine (30). In a separate study they showed the effects of several detergents, in particular d-palmitoylcarnitine and Tween-80, on their partially pure preparation from calf liver (34). At a high concentration of palmitoyl-CoA, the detergents stimulated the forward reaction and the apparent K_m for L-carnitine decreased. At low palmitoyl-CoA, the detergents were inhibitory and no changes in the K_m for L-carnitine were observed (35). They concluded that the main reason for this effect was that the detergent prevented the substrate inhibition at high concentrations of palmitoyl-CoA. Phospholipids and albumin were later found to have a similar inhibitory and stimulatory effect on the activity of CPT with low and high concentrations of palmitoyl-CoA (76), respectively. However, phospholipids and albumin had these effects without lowering the K_m for L-carnitine. Recently, Bremer has postulated that increasing substrate inhibition by acyl-CoAs as the acyl-chain lengthens explains the profile of maximum relative activity in the forward direction in the medium-chain region (5).

Clarke and Bieber studied the effect of micelles of Tween-80 on the kinetic parameters of purified beef heart CPT in the reverse direction (65). In the micellar environment the enzyme shows a 3-fold increase in V_{max} for the various acylcarnitine substrates and a 5 to 6 fold increase in K_m for C_8-, C_{10}-, C_{12}- and C_{14}-carnitine esters.

Properties of Membrane Bound CPT

The properties of membrane bound CPT differ significantly from the properties of the enzyme in solution. Lags in the time course of the reaction of membrane bound liver and heart CPT-I with palmitoyl-CoA have been observed (77-79). These lags decrease with increasing concentrations of K^+ or Mg^{+2} (77), with preincubation of mitochondria with palmitoyl-CoA (78), and with sonication or detergent solubilization of CPT from the membrane (77). In addition, they are absent when the substrate used is octanoyl-CoA (77). Cook (78) has suggested that these slow changes in reaction rate after the addition of substrates are due to hysteretic behavior of the enzyme and therefore can be prevented by preincubation with the substrates. Similarly, Bremer et al. (79) showed that CPT remaining in membrane residues after extraction with Triton X-100 is subject to an apparent activation by the substrate. However, Zammit (39) has shown that these lags are minimal when the reactions are

started by addition of palmitoyl-CoA/albumin mixtures. He has suggested that the lags are not dependent on intrinsic properties of CPT-I but may represent the time necessary to obtain an equilibrium between free, micellar and albumin-bound forms of the substrate. In albumin containing media, salts may affect the binding of palmitoyl-CoA to albumin (38). The association of palmitoyl-CoA with the mitochondrial membrane appears to increase with rising ionic strength (61). In this context, the non-specific activation of CPT by various metal ions may be related to an increase in the concentration of palmitoyl-CoA in the vicinity of the enzyme's active site.

Due to the diverse assay conditions, particularly the molar ratio of albumin and substrate, reports in the literature on the K_m for palmitoyl-CoA and I_{50} values for malonyl-CoA of membrane bound CPT-I are extremely variable. In a comparative study (38), the K_m reported for palmitoyl-CoA is 47 µM in the absence of albumin, and 450 µM in 2% albumin. Substrate sigmoidicity in the kinetics of membrane bound CPT with palmitoyl-CoA, has been reported in some studies (21,80), while linear hyperbolic kinetics have been reported in others (34,81). In some of these studies hyperbolic kinetics were reported which became sigmoidal after addition of malonyl-CoA to the assay medium (29,34). The relationship between CPT activity and L-carnitine concentration over a limited range of concentrations (20-400

μM) has been reported as hyperbolic (80). The K_m for L-carnitine is apparently not altered by malonyl-CoA or by fasting, though significant tissue variation in the K_m for L-carnitine has been observed. Interestingly, higher values of the K_m for L-carnitine have been obtained for tissues in which CPT has a lower sensitivity to malonyl-CoA inhibition (82). In contrast, the K_m for palmitoyl-CoA does not appear to vary significantly among tissues (82). Mills et al. (83) observed that an increase in pH results in a decrease in the K_m for L-carnitine with no change in the K_m for palmitoyl-CoA.

McGarry and Foster (20,84,85) showed that malonyl-CoA can be a potent inhibitor of membrane bound CPT. They concluded that only CPT-I is inhibited by malonyl-CoA, since the oxidation of palmitoylcarnitine is not affected by malonyl-CoA and in ruptured mitochondria malonyl-CoA inhibits approximately one-half of the total CPT activity. Direct measurements of the effect of malonyl-CoA on CPT-II are not possible because of the impermeability of the inner membrane to the CoASH esters. A moderate inhibition by malonyl-CoA of CPT in inverted mitochondrial vesicles suggests that CPT-II may be sensitive to malonyl-CoA, though these preparations could be contaminated with exposed CPT-I (86). Disruption of the membrane with detergents results in the release of malonyl-CoA insensitive CPT, but the sensitivity of CPT activity left associated to the membrane

fragments is increased (79,84). This observation stresses the importance of membrane factors. Nevertheless, the apparent insensitivity of CPT-II has suggested to some that CPT-I and CPT-II are different proteins. An alternative explanation is that CPT-II does not interact with a membrane component which confers sensitivity to CPT-I. The observation that other acyl derivatives such as succinyl-CoA which are primarily located in the matrix compartment of mitochondria also inhibit CPT (87), provides a possible mechanism by which CPT-II could be regulated.

Several other CoA esters are potent inhibitors of membrane bound CPT. Overt CPT activity of liver mitochondria is sensitive to inhibition by 2-bromopalmitoyl-CoA, but latent CPT is not (88). Latent CPT can generate bromopalmitoyl-CoA within the matrix from external 2-bromopalmitoylcarnitine in a reversible reaction. Palmitoyl-CoA analogs in which the carbonyl group is replaced by methylene groups (89) and the 2-substituted oxiran-2-carbonyl-CoA esters (90) are potent inhibitors of CPT-I. Like malonyl-CoA, 2-tetradecylglycidyl-CoA is an inhibitor of membrane bound CPT-I but it does not appear to inhibit the solubilized enzyme (91,20). Both malonyl-CoA and palmitoyl-CoA protect the enzyme from irreversible inactivation by TDGA-CoA.

Differences in the phospholipid environment of the matrix and cytosolic faces of the inner mitochondrial

membrane (92,93), may result in different properties of the two forms of CPT. The functional activity of CPT appears to be altered in galactosamine hepatotoxicity, a condition in which the phospholipid composition of the mitochondrial membrane is altered (94). Phospholipids can activate partially purified CPT with a low specificity for phosphatidylcholine (76). Brady et al. (86) have demonstrated that small changes in membrane fluidity affect CPT activity. They have also measured increased membrane rigidity upon addition of malonyl-CoA using fluorescent probes. They suggested that malonyl-CoA may affect both CPT-I and CPT-II through changes in the membrane fluidity. However, attempts to modify the lipid environment of solubilized CPT have not restored malonyl-CoA sensitivity (76,92).

At present, the study of CPT is directed towards understanding the biochemical mechanism by which malonyl-CoA is inhibitory. Initially, the removal of malonyl-CoA inhibition by increasing the concentration of palmitoyl-CoA suggested a simple competitive interaction (20,36,96) at the active site of the enzyme. More recently, it has been suggested that malonyl-CoA acts by a mechanism involving cooperative inhibition due to the apparent ability of malonyl-CoA to induce sigmoidal kinetics of CPT-I (34). Since solubilized CPT-I is not inhibited by malonyl-CoA (2,7), the participation of an additional membrane

component(s) has been suggested (64,83,20,97). Kiorpes et al. (30) demonstrated that TDGA-CoA can inhibit membrane bound CPT-I irreversibly, and malonyl-CoA can protect against this inhibition. Recently, it was shown (20) that solubilization of CPT-I "irreversibly" inhibited by TDGA-CoA restored CPT-I activity similar to the effect of solubilization on malonyl-CoA inhibition. Kiorpes et al. detected in SDS gels, the irreversible binding of ^{14}C-TDGA-CoA to a 90,000 molecular weight mitochondrial protein from rat liver (91). This protein could be a regulatory subunit of CPT, but it is not the catalytic subunit since the catalytic subunit of rat liver CPT has a molecular weight 63,000 (15). Though these recent data strongly suggest a separate binding site for malonyl-CoA in the mitochondrial membrane in liver, the nature of a malonyl-CoA binding component remains unknown.

Role of CPT in Control of Fatty Acid Oxidation

Initial studies on the control of fatty acid oxidation by CPT demonstrated enzyme rates far in excess of the capacity of mitochondria for fatty acid oxidation when high concentrations of substrates were used (98-100). It was proposed that substrate availability might control the rate of oxidation of fatty acids (101-103). However, the observation was made that when equal amounts of long-chain

fatty acids are supplied to the perfused liver, starved rats produced ketone bodies at a higher rate than fed rats (104,105). Differences in the rate of ketogenesis from the oxidation of octanoic acid were less pronounced, suggesting that the carnitine acyltransferase system was a primary site for the regulation of hepatic fatty acid oxidation and ketogenesis (104). Subsequently, McGarry and Foster demonstrated the inhibitory action of malonyl-CoA on fatty acid oxidation and CPT-I reaction (23,84). They proposed a key role for this metabolite (the first committed intermediate in the conversion of glucose into fat) in the coordinated control of fatty acid oxidation and synthesis, with an auxiliary control of CPT activity effected through the fluctuation in the levels of fatty acid and L-carnitine (95).

The control of CPT by malonyl-CoA appears to be modified in two ways which may act to amplify each other (106). Hepatic levels of malonyl-CoA vary with the physiological state of the animal (22,106) and hepatic CPT-I is less sensitive to malonyl-CoA inhibition in the fasted and ketotic state (96,29,97,106,108-110). In the fed state where a high rate of fatty acid synthesis occurs, both tissue levels of malonyl-CoA and the sensitivity of CPT-I to malonyl-CoA inhibition are highest. In the starved state, a decrease in malonyl-CoA concentration is amplified by the decreased sensitivity of CPT-I to inhibition by this

metabolite, resulting in maximal rates of fatty acid oxidation. Starvation may affect the properties of CPT through changes in the lipid composition of the membrane. Alternatively, changes in the regulatory properties of liver CPT may be under hormonal control by the insulin/glucagon ratio (94). Changes in the sensitivity of hepatic CPT to malonyl-CoA inhibition apparently result from changes in the K_i values of CPT for malonyl-CoA (34). The K_i value of CPT for malonyl-CoA from liver of diabetic animals is approximately 10-fold greater than in controls (111). Treatment with insulin returns the K_i to control levels. This finding suggests that in addition to facilitating an increase in the levels of fatty acids available for oxidation through enhanced lipolysis (112), a decrease in insulin levels may modulate ketosis through the inhibition of CPT-I by malonyl-CoA. High glucagon appears to decrease the activity of acetyl-CoA carboxylase thus controlling the malonyl-CoA concentration (113). Recent studies suggest a direct effect of glucagon on CPT activity. After incubation of rat hepatocytes with glucagon, SDS electrophoresis of immunoprecipitates obtained using antibodies to purified CPT show a phosphorylated protein band which has the molecular weight of the purified CPT subunit (114).

The regulation of fatty acid oxidation by malonyl-CoA inhibition of CPT-I appears to be a general phenomenon. Inhibition by malonyl-CoA is more pronounced in heart and

skeletal muscle than in liver, kidney, or adipocyte (114). Under identical assay conditions, the I_{50} values for malonyl-CoA of rat liver CPT is 2.7 μM while the I_{50} for dog heart CPT is 20 nM. Though heart and skeletal muscle do not have active fatty acid synthesis they contain considerable amounts of malonyl-CoA in the fed state (82). Other hormones, in particular those associated with stress may have an effect in tissues such as heart. Methylmalonate, an intermediate in propionate metabolism, may also have an important role in the regulation of CPT, especially in tissues with low levels of fatty acid synthesis but high gluconeogenesis, such as sheep liver, where methylmalonate has been shown to be an inhibitor of CPT-I (115).

The therapeutic possibilities of specific inhibitors of fatty acid oxidation in the treatment of hyperglycemia have been proposed (116). Inhibition of CPT-I appears to be an ideal target. Octanoyl-D-carnitine and decanoyl-D-carnitine have antiketogenic and hypoglycemic effects when administered to fasted or diabetic rats (117), but can be toxic at relatively low concentrations presumably due their detergent properties. TDGA is a potent oral hypoglycemic and hypoketonemic agent in animals under conditions in which fatty acids are the main fuel (118). Recently the antiketogenic effects of DL-aminocarnitine (3-amino-4-trimethylaminobutyrate) and its acyl derivatives have been demonstrated (119). These inhibitors of CPT

require concentrations that are 0.1-1% of those required for effective inhibition by acyl-D-carnitines and they show a more pronounced hypoglycemic effect.

EXPERIMENTAL PROCEDURES

Materials

Acyl-CoAs were purchased from PL Biochemicals. L-carnitine was a generous gift from Otsuka Pharmaceutical Co., Japan. Blue Sepharose 4B had been previously synthesized in the laboratory according to (119). Sephacryl S-300, Fractogel TSK HW-55, and QAE-Sephadex were purchased from Pharmacia. Immersible-CX ultrafilters (Polysulfone membrane with a nominal molecular weight limit of 30,000 daltons) were from Millipore. Fluorescamine, collagenase, DTNB, DTBP, and pinacyanol chloride, were from Sigma. Molybdenum Blue spray for phosphorus detection was from Applied Science Laboratory (2051 Waukegan Road, Deerfield, IL). Phospholipid standards and Redi-Coat 2D precoated TLC plates were from Supelco. Triton X-100 was purchased from Research Products International Corporation. Purified mouse liver peroxisomal COT was kindly provided by Dr. Shawn Farrell. Octylglucoside was kindly provided by Dr. Ferguson-Miller (Michigan State University, East Lansing, MI). TDGA-CoA was a generous gift from Drs. Linda and Paul Brady (Washington State University, Pullman, WA). All other reagents were of analytical grade.

Methods

Purification of carnitine palmitoyltransferase from beef heart mitochondria

Several modifications to the procedure in (24) were made in order to obtain a higher yield of pure enzyme.

Mitochondrial Isolation. Beef hearts were obtained from the MSU abbatoir, sliced and packed on ice for transportation. The beef heart ventricles were soaked in ice cold sucrose buffer (0.25 M sucrose, 5.0 mM Hepes, 0.25 mM EDTA, pH 7.7) while the fat and connective tissue were throughly removed. The tissue was homogenized for 45 seconds at high speed in a Waring blender in batches of 240 g in 2 L of buffer, followed by 30 second Polytron homogenization in 250 ml centrifuge tubes. Cellular debris was pelleted with a 15 min spin at 500 x g, and the supernatant was collected by filtering through cheesecloth. The first mitochondrial pellet was obtained by centrifugation at 15,000 x g for 15 min. The pellet was resuspended with two strokes of a loosely fitting Potter-Elvehjem teflon-glass homogenizer in 500 ml of isotonic sucrose buffer (0.25 M reagent grade sucrose, 2.5 mM HEPES, 0.25 mM EDTA, pH 7.5). The

mitochondrial suspension was centrifuged again at 500 x g to remove remaining debris. A second mitochondrial pellet was obtained by centrifuging the supernatant at 11,000 x g and resuspended as previously described. The final mitochondrial preparation was obtained by a third centrifugation at 7000 x g, and resuspension in a minimal volume of isotonic sucrose buffer. The mitochondria were stored at -80^{o}C.

Solubilization of Mitochondrial CPT. Mitochondrial suspensions were thawed, pooled, and mixed with one-half volume of 3 M KCl in 6% Triton X-100. The suspension was homogenized in 15 ml batches with six strokes of a Potter-Elvehjem homogenizer at 15^{o}C and centrifuged for 90 min. at 89,000 x g (29,000 rpm). Lipid in the supernatant was removed by suction and the clear apricot-colored supernatant was collected with care not to disturb the pelleted membranous debris. The solubilized protein was equilibrated with Blue buffer (1.0% Triton X-100, 2.5 mM Hepes, 0.25 mM EDTA, 60 mM KCl, pH 7.5) by exhaustive dialysis. The dialyzed preparation was centrifuged at 15,000 x g for 15 min. to remove protein that was precipitated by the decrease in ionic strength. The ionic strength of the supernatant was then increased to 300 mM KCl by a second dialysis against the Blue buffer containing 0.3 M KCl.

Chromatography of Solubilized CPT. The dialysate was applied in 35 ml batches to a 40 x 5.0 cm column of Sephacryl S-300 equilibrated with Blue buffer containing 300 mM KCl and eluted at a flow rate of 75 ml/hr with the equilibrating Blue buffer. Peak fractions of CPT activity were pooled and dialyzed against QAE buffer (0.5 % Triton X-100, 5.0 mM Bis-Tris Propane, 0.25 mM EDTA, 20 mM KCl, pH 9.7) and applied to a 30 cm x 4.1 cm column of QAE-Sephadex Q-25-120. CPT activity washed through the column, was pooled, and concentrated to a final volume of 1 ml using the immersible ultrafilters describe in Materials. The activity was dialyzed extensively against buffer (0.1 % Triton X-100, 2.5 mM Hepes, 0.25 mM EDTA, 60 mM KCl, pH 7.6) and loaded on a 12 cm x 1.6 cm column of Cibacron Blue Sepharose. Four bed volumes of buffer were used to wash off unbound protein before a 50 ml linear gradient of 60-860 mM KCl in Blue buffer was used to elute CPT. Fractions containing CPT were pooled. Cibacron Blue Sepharose was treated regularly with pronase to maintain a high binding capacity.

Exchange of Triton X-100 for Octylglucoside. The final step in the purification of CPT, Cibacron Blue Sepharose Chromatography, served to exchange Triton X-100 for octylglucoside. The concentration of Triton X-100 was reduced to 0.002% in the equilibrating buffer prior to the

loading of the enzyme to the column. The unbound protein was washed off with Blue buffer in 0.002% Triton X-100 followed by three bed volume washes of Blue buffer in 25 mM octylglucoside. The enzyme was eluted with a similar salt gradient as previously described, in which Triton X-100 was substituted with 25 mM octylglucoside.

Physical Characterization of Purified CPT

SDS-Polyacrylamide Gel Electrophoresis. Purified CPT was electrophoresed on 7% polyacrylamide gels (120) and stained with Coomasie Brilliant Blue. BSA, ovalbumin, myosin, and phosphorylase b were used as molecular weight standards.

Phospholipid Analyses. The total phosphorus content of purified CPT was determined by ashing of the protein and phosphate quantitation (121). Phospholipids were extracted with 20 volumes of chloroform:methanol (2:1) and the total organic solvent soluble phosphate was determined by the Ames procedure (121). The phospholipids were chromatographed in two dimensions (122) on Supelco Redi Coat 2D precoated TLC plates, and localized by spraying with Molybdenum Blue spray. The phospholipids produced blue spots on a white background immediately after the application of the spray. The phospholipids were identified by comparison to

standards.

Native Molecular Weight Determination. A 50 x 1.5 cm Fractogel TSK HW-55 column was equilibrated with buffer containing 2.5 mM Hepes, 0.25 mM EDTA, 300 mM KCl and 12 mM octylglucoside at pH 8.0. The column was calibrated using 10 mg/ml each of ovalbumin, BSA, aldolase, thyroglobulin and 1mg/ml of ferritin. A 1 ml sample of purified CPT containing 200 munits was loaded and 0.3 ml fractions were collected.

Kinetic Characterization of CPT.

Activity Assay. The forward reaction (formation of acylcarnitines) was monitored at 412 nm following the release of CoASH with DTNB. The 2.0 ml reaction volume contained 115 mM Tris Buffer, 1.1 mM EDTA, 0.1% Triton X-100, 150 um DTNB, with varying concentrations of L-carnitine, and acyl-CoA at 25^{o}C. When pH effects were being studied, DTNB was substituted with DTBP and the buffer used was 50 mM Bis Tris Propane.

Kinetic Measurements with Purified CPT. Kinetic data were obtained with a semiautomated kinetic analyzer described in (123,124). A schematic drawing of this system is shown in Figure 2. Absorbance-time data were obtained by continously

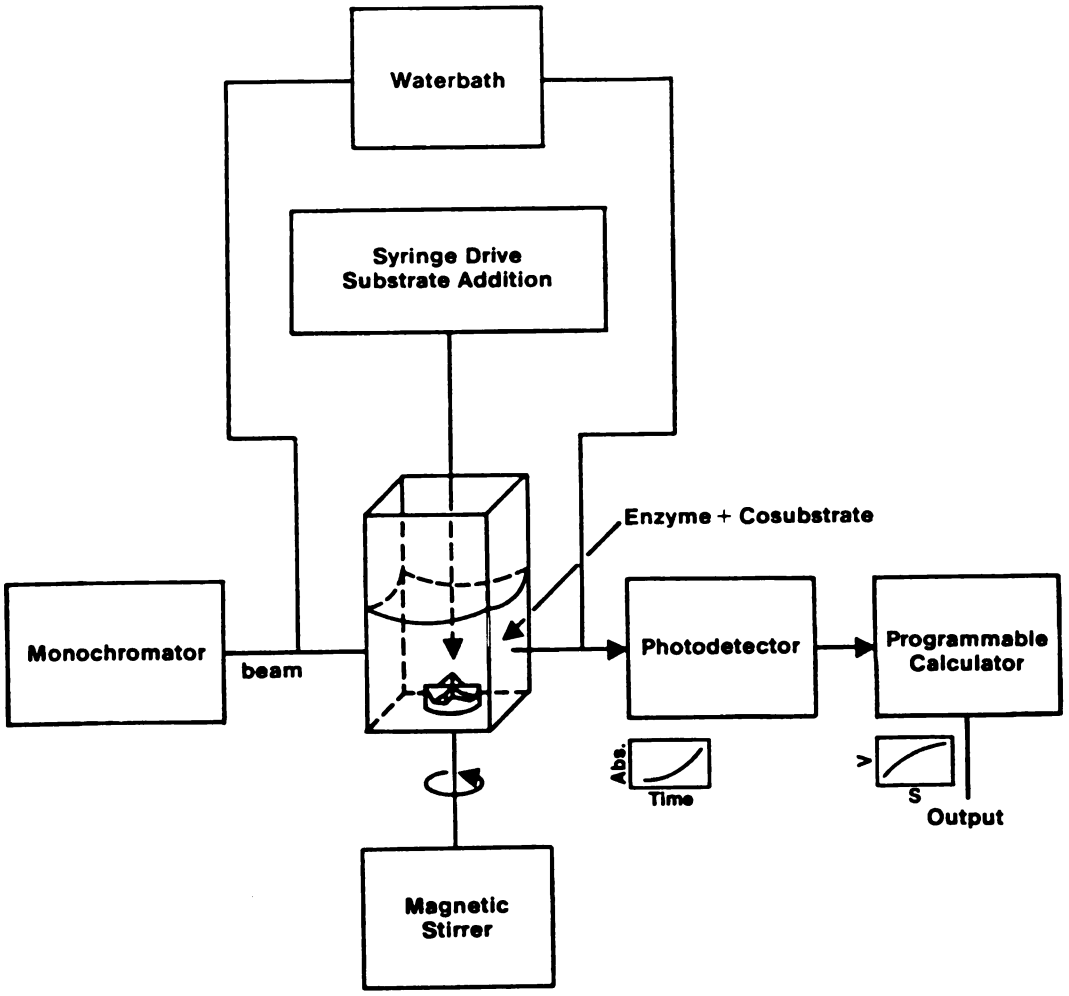

Figure 2. Schematic representation of the semi-automated kinetic analyzer. It consists of a Gilford 2600 spectrophotometer equipped with a microprocessor, a five significant digit absorbance readout, water circulation with temperature control, and magnetic cuvette stirrer. It is interfaced with a programmable desk-top Hewlett Packard calculator which runs the program TANKIN. The reaction is started by addition of substrate with a pump driven syringe to a stirred cuvette containing the enzyme and cosubstrates. The raw time-absorbance data are converted to substrate-velocity data by the method of tangent-slope analysis, and the optimal Hill parameters (V_{max}, $S_{0.5}$, Hill n) are obtained from the best linear fit to the data.

increasing the substrate concentration of a stirred enzyme assay mixture with the use of a precision syringe drive and an automated Gilford model 2600 spectrophotometer. The reaction time was 3.6 min. The concentration of added substrate was adjusted so that the increase in volume during the assay was limited to less than 5%. The final concentration of substrate was optimized for each individual measurement to be within 3-5x the $K_{0.5}$. Absorbance data were obtained to five significant digits. The data were transformed into velocity-substrate data by a tangent slope procedure, analyzed as linear plots using the TANKIN program in a Hewlett-packard 9815 calculator, and plotted with a Hewlett-Packard 9872 A plotter. Time- and product-dependent effects on the reaction rate were determined by systematic analysis of the data (123).

Kinetic Characterization of Membrane Bound Rat Heart CPT-I.

The activity of rat heart mitochondrial CPT was measured at pH 8.0 using the DTNB assay and decanoyl-CoA as substrate. Triton X-100 was omitted and 150 mM KCl was added to the assay mixture to prevent mitochondrial swelling. When used as cosubstrates L-carnitine was 6.0 mM and decanoyl-CoA was 100 uM. For each run a background change in absorbance upon addition of acyl-CoA was measured with a control run which lacked L-carnitine. This background absorbance was subtracted from the absorbance data by an added subroutine

to the TANKIN program. The flow chart for this subroutine is shown in Figure 3.

Other Methods

CMC Determinations. The formation of micelles was monitored by addition of acyl-CoA substrate or octylglucoside to a cuvette containing the activity assay mix and 4 uM pinacyanol chloride. Formation of micelles was observed by a change in the extinction coefficient of the dye at 610 nm (125).

Protein Determination. Protein was determined by the fluorescamine method (126) throughout the purification procedure and the Fractogel Chromatography using BSA as standard. Mitochondrial protein was determined by solubilization with deoxycholate with the Coomasie Blue Method (127).

Isolation of Rat Heart Mitochondria Mitochondria were isolated from rat hearts by the procedure described in (128). Three male Sprague-Dawley rats, 21 days old, were decapitated and the hearts rapidly removed, rinsed, and minced in an ice cold medium containing 0.225 M mannitol, 0.075 M sucrose, and 0.2% (w/v) fatty acid free BSA. The minced hearts were rinsed three times and treated with 200

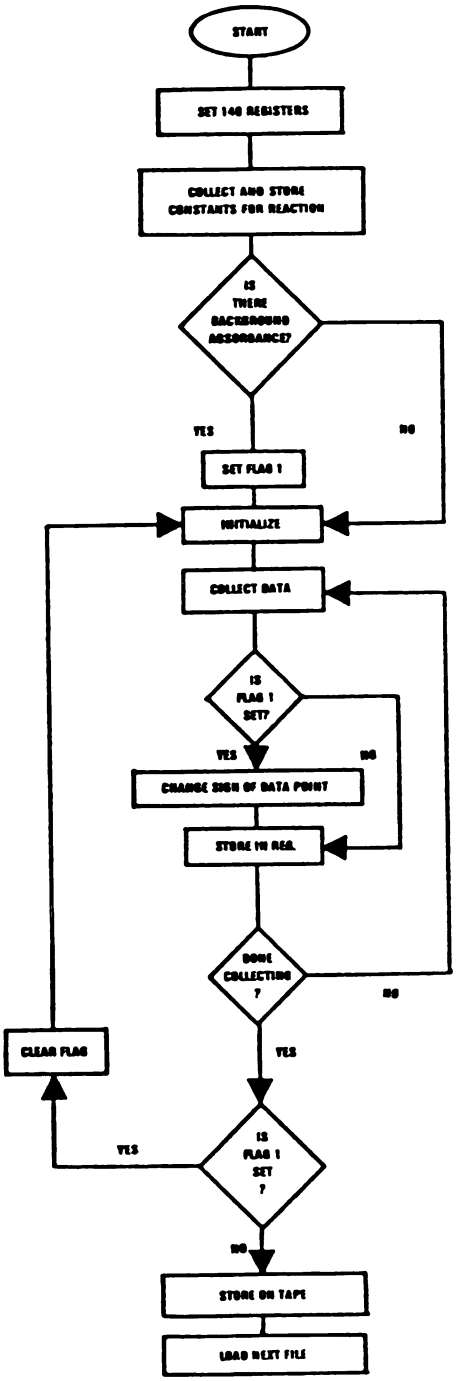

Figure 3. Flowchart for the added subroutine to the computer program TANKIN. This subroutine eliminates absorbance changes due to mitochondrial swelling.

units of collagenase in 15 ml of medium. After 1 min. the preparation was homogenized using a Potter-Elvehjem motor driven pestle. After 3 min. on ice, EGTA was added to a final concentration of 1 mM. The homogenate was centrifuged at 600 x g for 5 min. and the supernatant fluid was collected by filtering through cheesecloth. The mitochondria were pelleted by centrifugation at 8,000 x g for 10 min. The mitochondria were washed twice by resuspension and recentrifugation at 8,000 x g for 10 min and were finally suspended in 0.225 M mannitol/0.075 M sucrose. Heart mitocondria isolated in this manner had respiratory control ratio of 10 or higher with 5 mM pyruvate/2.5 mM malate as substrates. Acyl-CoA hydrolase activity was not detectable. The specific activity of CPT-I was 22-27 (munits/mg of protein) measured with the DTNB assay at 25^{o}C.

RESULTS

Purification of Carnitine Palmitoyltransferase from Beef Heart Mitochondria

Preliminary experiments using the available Cibacron Blue Sepharose resin synthesized in this laboratory revealed that it was no longer effective in the separation of CPT and CAT under the conditions reported earlier (24). A loss of CPT activity in the wash, and an overlap in the elution peaks for CAT and CPT, indicated a reduction in the binding capacity of the resin which could not be restored by treatment with either pronase or denaturing agents. The use of Cibacron Blue Sepharose from Pharmacia also yielded unsatisfactory results. New sets of conditions for binding were tested keeping in mind that non-ionic detergents can interfere with affinity chromatography by encapsulation of the dye in the detergent micelles which results in a reduction of the binding capacity of the resins (129). When a lower concentration of Triton X-100 (0.1% Triton X-100) was used, the enzyme bound tightly at low ionic strength. However, it was deemed necessary to maintain a high concentration of detergent during the early stages of the purification of CPT, an integral membrane protein, to prevent the reaggregation of membrane fragments which could

interfere with the separation of the individual proteins. It was decided that Cibacron Blue Sepharose Chromatography should be used as a later step in the purification procedure. At this time, it was also decided to omit the hydroxyapatite column chromatography used in earlier purifications because of a low enzyme recovery after this step.

A high yield of CPT of high specific activity was obtained following the chromatographic steps outlined in Table III. Sephacryl S-300 was selected as the initial step since its fractionation range is well suited to separate CPT associated with detergent micelles (530,000 daltons) from soluble CAT (60,000 daltons). As shown in Figure 4, CPT and CAT activity elute as two well separated peaks. This step also separates CPT from a bulk of the solubilized mitochondrial protein giving an overall 20-fold purification and a 75% recovery (see Table III). A further 2-fold purification with a 60% overall recovery was obtained with QAE-Sephadex Chromatography at pH 9.7. Cibacron Blue Sepharose at a low concentration of Triton X-100 (0.1%) yielded a final enzyme preparation with specific activity of 42 units/mg of protein which was apparently homogenous as determined by SDS-gel electrophoresis (see Figure 5). The overall recovery was 44%.

TABLE III

SUMMARY OF THE PURIFICATION OF CPT FROM BEEF HEART MITOCHONDRIA

Purification Step	Specific activity units/mg	Percent Recovery	Fold
Thawed Mitochondria	.03	100	1
Solubilized Mitochondria	.04	92	1.3
Dialysis Supernatant	.065	85	2.1
Sephacryl S-300	.60	75	20.0
QAE-Sephadex	1.5	60	50.0
Cibacron Blue Sepharose 4B	40.0	44	1333

One unit of activity is the amount of enzyme necessary to convert 1 umol of acyl-CoA to acylcarnitine in 1 minute.

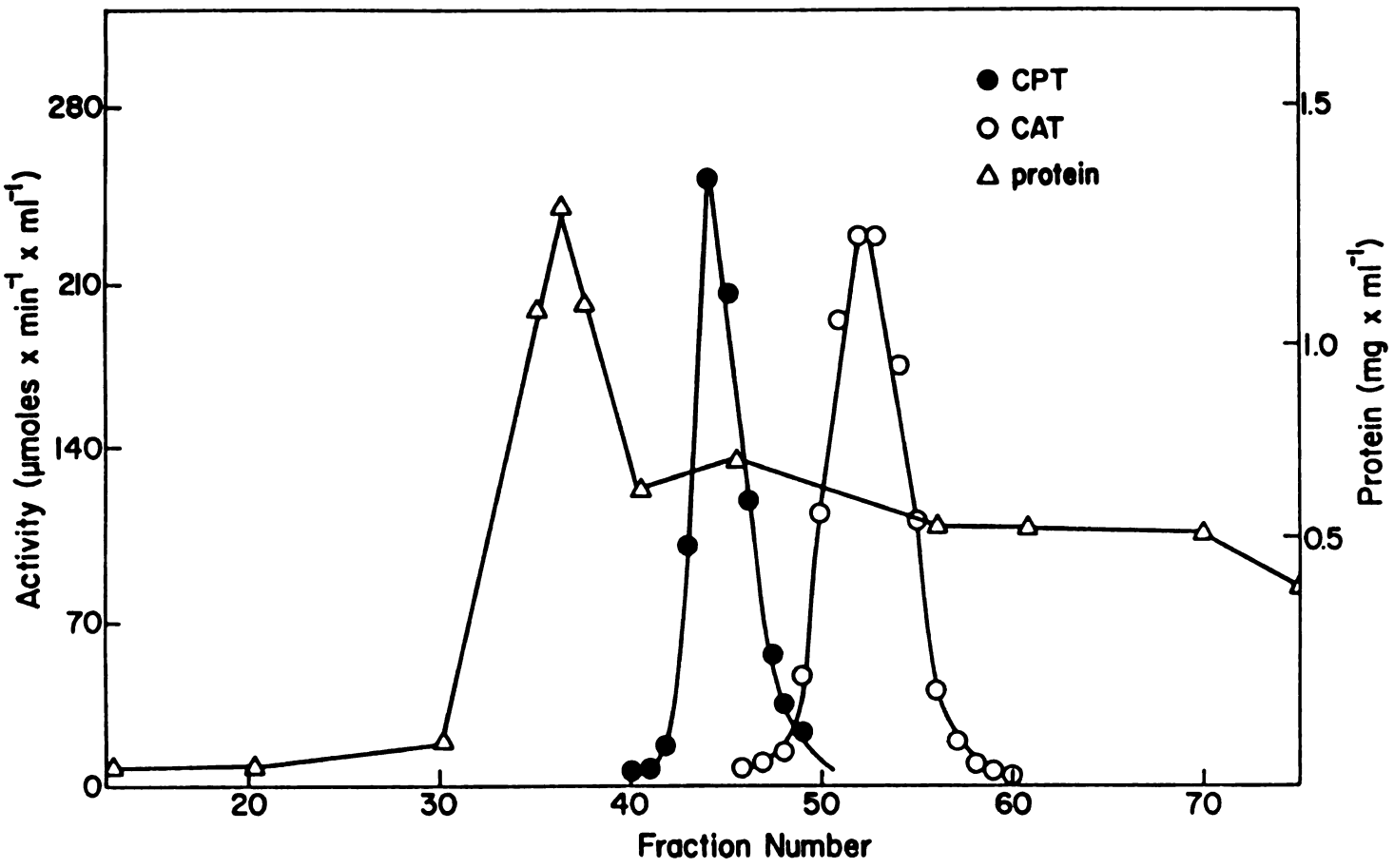

Figure 4. Separation of solubilized CPT and CAT on Sephacryl S-300. Samples were applied in 35 ml batches to a 40 cm x 5.0 cm column at a flow rate of 75 ml/hr as described in Methods.

Figure 5. SDS-polyacrylamide gel electrophoresis of purified beef heart mitochondrial CPT stained with Coomassie Blue.

Left lane: 10 µg pure CPT

Middle lane: (from top to bottom): 10 µg each of myosin, phosphorylase, BSA, ovalbumin.

Right lane: 5 µg pure CPT

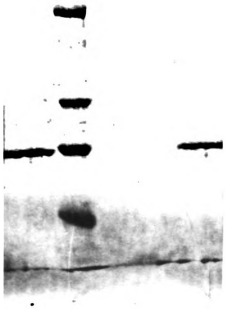

Figure 5.

Physical Characterization of Purified Carnitine Palmitoyltransferase

SDS-polyacrylamide gel electrophoresis of the purified enzyme confirmed the previously reported subunit molecular weight of 67,000 (24). Since CPT is a tightly membrane bound protein, experiments were performed to determine if the purified enzyme contained bound phospholipids. It contained approximately 19 mol of phosphorus/mol of enzyme as determined by protein ashing and phosphate quantitation. All of the phosphorus prior to ashing was soluble in 20 volumes of chloroform:methanol (2:1), indicating that this phosphate was not covalently bound. Two-dimensional thin layer chromatography showed three major phospholipid components identified by comparison to standards as cardiolipin, phosphatidylcholine, and phosphatidylethanolamine. Three other unidentified minor spots were also present. Though direct quantitation was not done, the size and intensity of the spot indicated that cardiolipin was the major component. A tracing of the TLC plate is shown in Figure 6.

Using molecular sieving columns, it had been previously shown (24) that in the presence of Triton X-100 micelles the detergent-protein complex has an apparent molecular weight of 530,000. In this study, Triton X-100 was substituted for

Figure 6. Two dimensional thin layer chromatography of extracted phospholipids from purified CPT. The solvent system used was:
(1) Chloroform:methanol:water:aqueous ammonia (30:70:8:0.5)
(2) Methanol:Acetic acid:water:chloroform:acetone (20:20:10:100:40)
The plates were developed for 35-40 min, dried for 10 min, and sprayed with Molybdenum Blue Spray. A tracing and a photograph of the developed plate are shown.

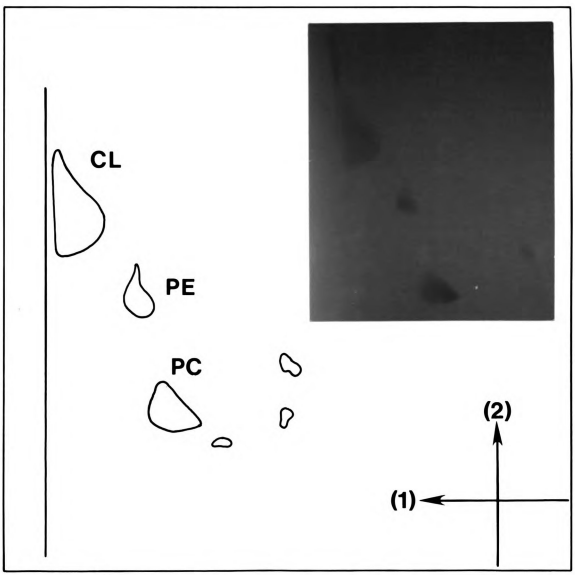

Figure 6.

octylglucoside and the molecular weight of CPT in the absence of detergent micelles was determined by gel filtration in Fractogel TSK-55 at 12 mM octylglucoside. Octylglucoside was chosen since the purified enzyme remains stable at concentrations below the cmc of the detergent. CPT activity migrates as a single peak of constant specific activity with a molecular weight of 660,000 daltons (see Figure 7). The absence of detergent micelles in the eluant was confirmed with the pinacyanol chloride method described in Methods. Figure 8 shows that CPT activity with palmitoyl-CoA is optimal at 12 mM octylglucoside. Though there are no detergent micelles at this concentration (see below), the enzyme remains in suspension after centrifugation at 100 x g. However, the activity of CPT with octanoyl-CoA decreased with increasing concentrations of octylglucoside and was not optimal at 12 mM.

Kinetic Characterization of Purified Carnitine Palmitoyltransferase

Preliminary Tests. For accurate determination of the kinetic parameters of CPT using the TANKIN program, it is necessary to obtain initial velocity measurements that are linear for the duration of the assay, i.e., there should be no hysteretic behavior and no significant product inhibition. Figure 9 shows a time course for the DTNB assay

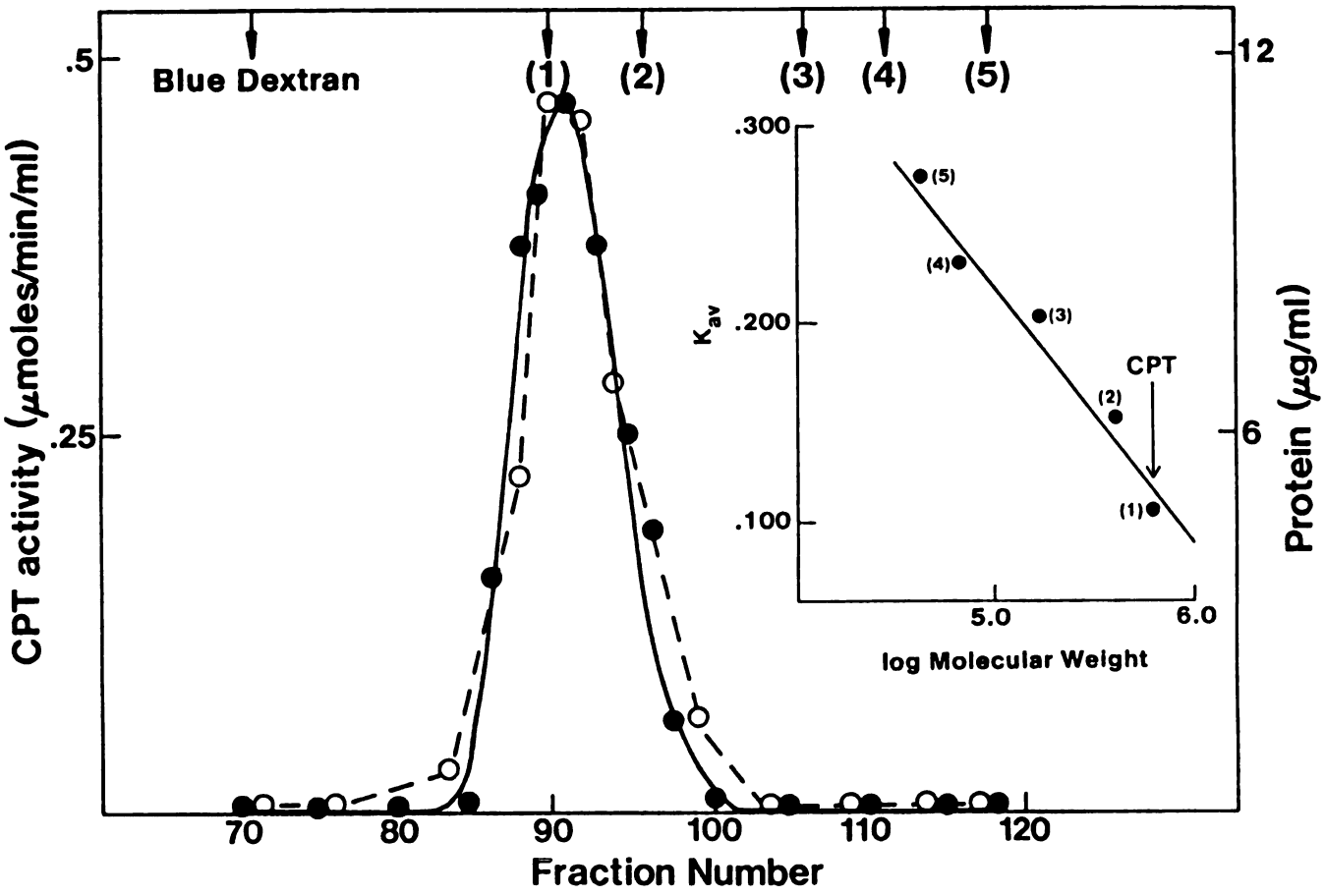

Figure 7. Fractogel TSK HW-55 chromatography of purified CPT. Molecular Weight standards used were: (1) thyroglobulin, (2) ferritin, (3) aldolase, (4) BSA, and (5) ovalbumin; 200 munits of CPT were eluted as described in Methods.

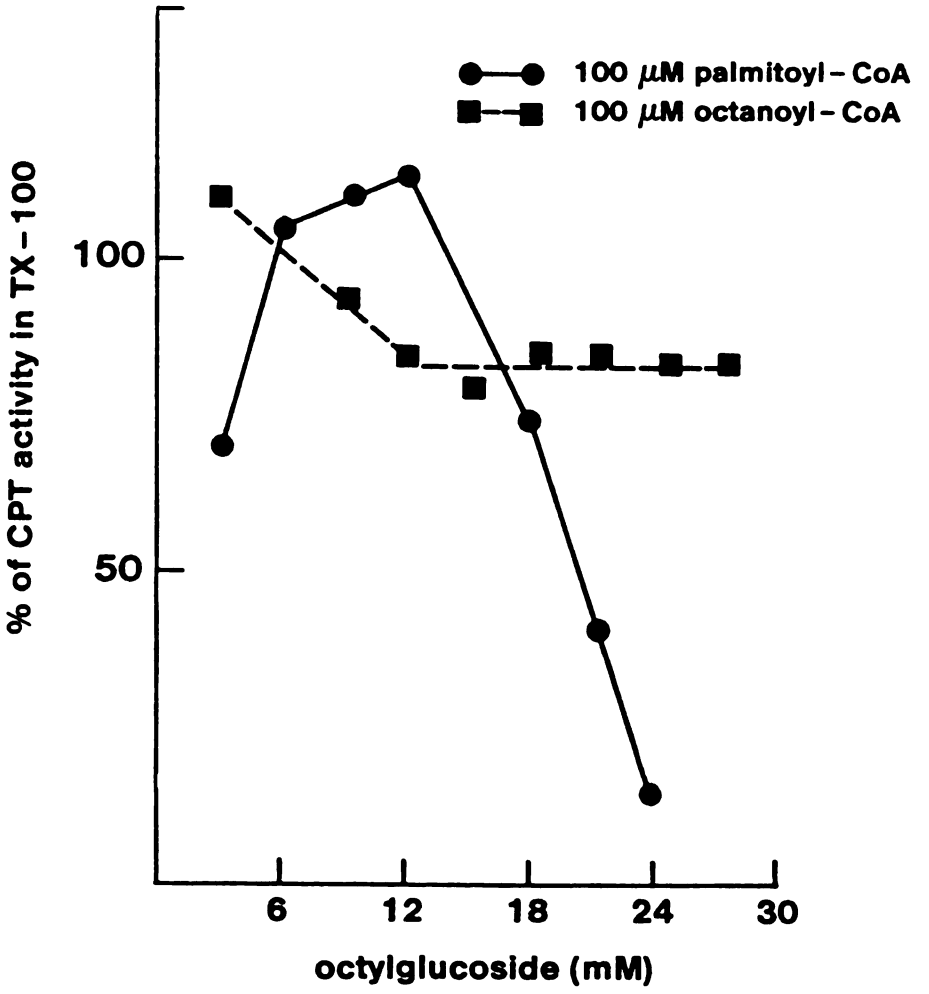

Figure 8. Effect of octylglucoside on CPT activity. Activity was measured with the DTNB assay. L-carnitine was 1.1 mM and the acyl-CoA was 100 μM. Velocities are relative to the V_{max} in 0.1% Triton X-100 for each substrate.

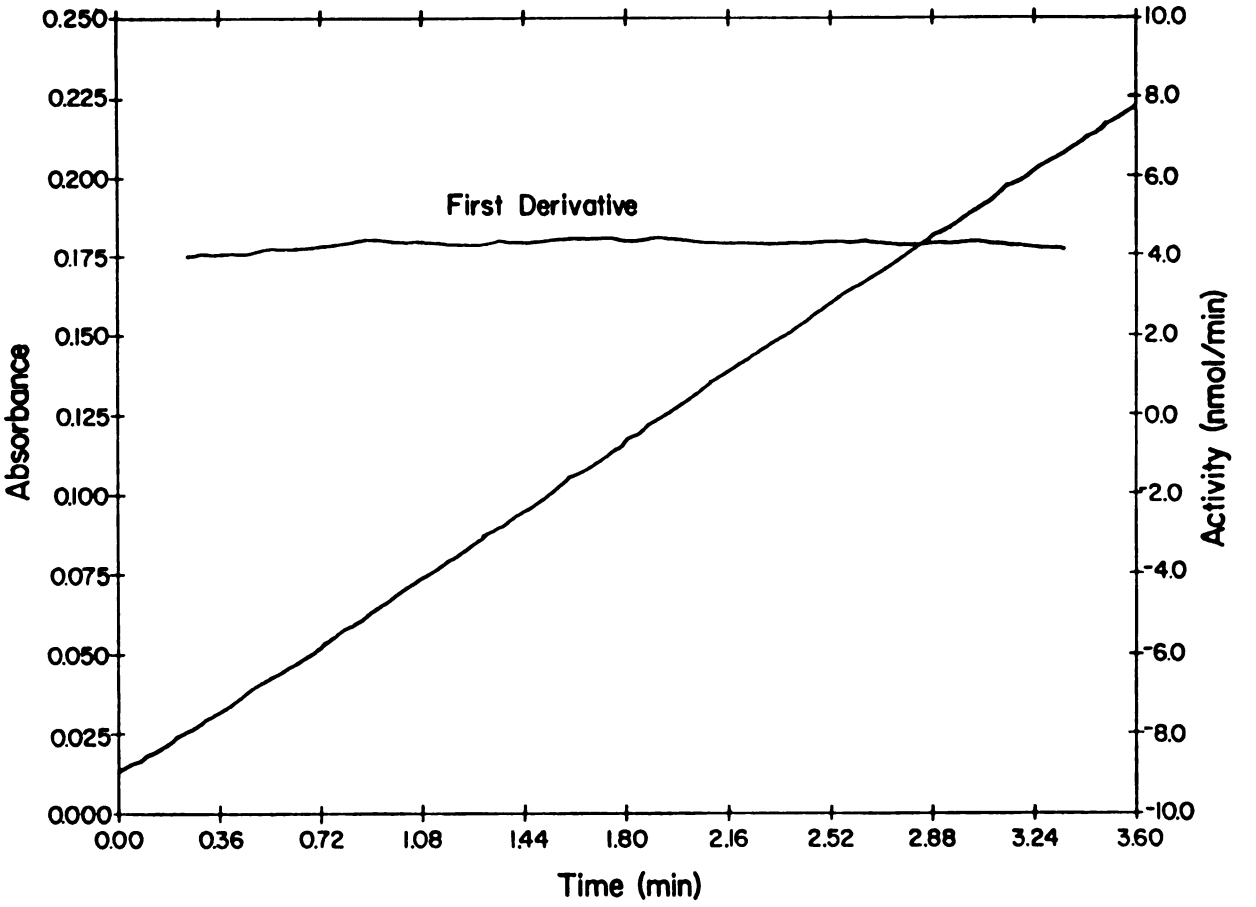

Figure 9. Time course for CPT reaction with DTNB assay. The initial rate was measured in the presence of 0.1% Triton X-100, 1.1 mM L-carnitine, 200 µM palmitoyl-CoA, 200 µM DTNB, 116 mM Tris-HCl, 1.1 mM EDTA at pH 8.0 and 25°C for 3.6 min with an enzyme concentration of 0.1 µg/ml.

used in our studies. The initial rate (first derivative) is constant throughout the duration of the assay, when the substrates are saturating. End point deletions (123) of the velocity-substrate data obtained from a substrate addition experiment will detect enzyme inactivation. The deletions in the data produce variation in the calculated Hill n and the correlation coefficient if there is significant enzyme inactivation. No effect of end point deletion on the Hill coefficient can be detected in the substrate-velocity data obtained for CPT, see Figure 10. In addition, Figure 11 shows that the V_{max} is not affected by different amounts of product accumulated in the reaction mixture. Therefore, it can be concluded that there are no apparent hysteretic effects or significant product inhibition under the conditions of the DTNB assay.

Determination of Nonmicellar Assay Conditions. Suitable conditions in which to investigate the kinetics of CPT in the absence of detergent micelles, substrate micelles, or mixed substrate detergent micelles were selected. The dye pinacyanol chloride was used to detect the formation of micelles in the assay mixture. The cmc of octylglucoside in the DTNB assay mixture is 30 mM. In the absence of detergent, palmitoyl-CoA forms a micelle at 5.4 μM. A logarithmic relationship between the cmc of the acyl-CoAs and their carbon chain length is observed (see Figure 12),

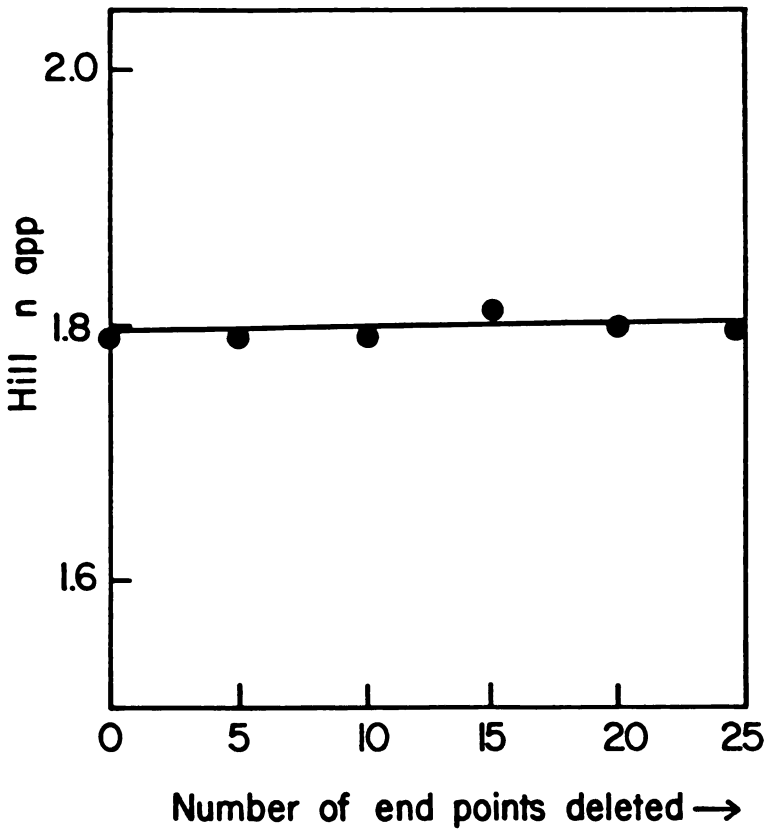

Figure 10. Effect of end point deletion on apparent Hill n. Raw data points from a typical substrate-addition experiment with CPT in which palmitoyl-CoA is the varied substrate were progressively deleted from the end of the data set, i.e., from the highest substrate concentration. The "new" data set was analyzed with the TANKIN program.

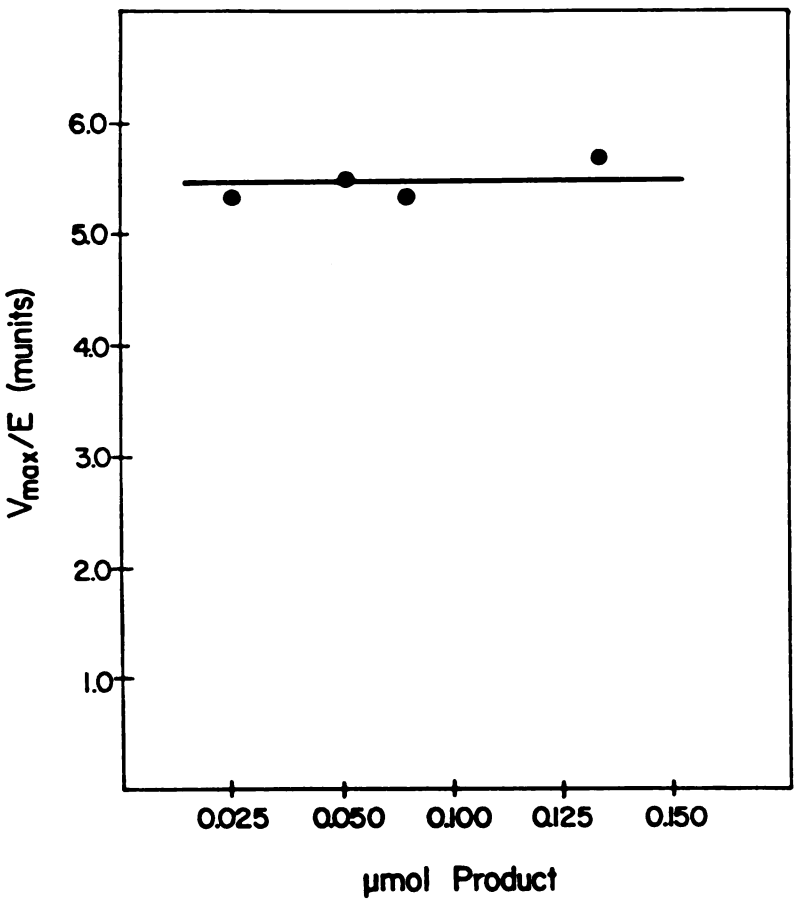

Figure 11. Effect of product accumulation during a substrate addition experiment. The V_{max} of CPT reaction with L-carnitine as the varied substrate and octanoyl-CoA as the cosubstrate was determined for a series of substrate addition experiments in which the amount of enzyme added was varied to obtain different amounts of product accumulation at the end of each run.

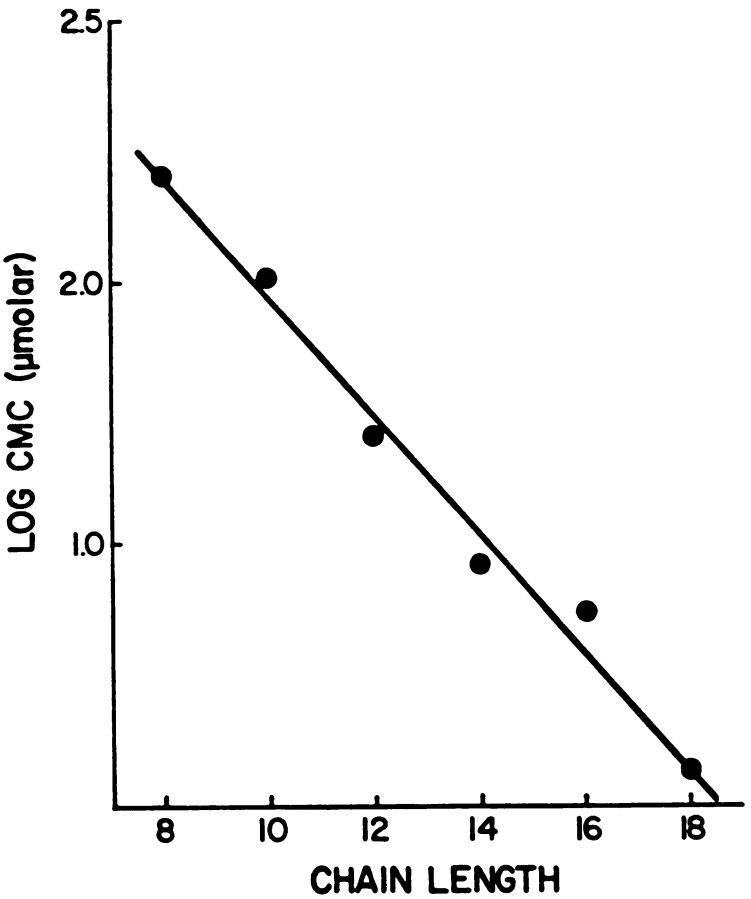

Figure 12. The relationship between the carbon chain-length of the acyl-CoAs and their cmc. The cmc's of the acyl-CoAs were determined using the dye pinacyanol chloride as described in Methods.

thus the cmc of medium-chain acyl-CoAs is much higher than that of long chain acyl-CoAs. As shown in Figure 13, the cmc of palmitoyl-CoA is reduced from 5.4 μM to 1 μM in the assay mixture containing 12 mM octylglucoside. Octanoyl-CoA has a cmc greater than 200 μM in the assay mixture in the absence of detergent. Addition of octanoyl-CoA to the assay mixture containing 12 mM octylglucoside shows no micelle formation up to 150 μM octanoyl-CoA (see Figure 13). Since octanoyl-CoA did not form micelles under these assay conditions, it is used as substrate for kinetic studies in a non-micellar environment.

Nonlinear Kinetics of Purified CPT. The kinetics of purified beef heart mitochondrial CPT were found to be sigmoidal with its acyl-CoA substrates as well as with L-carnitine under all the different assay conditions used. Double reciprocal plots showing non-hyperbolic substrate-enzyme interaction are shown in Figures 14 and 15 for a variety of conditions. When octanoyl-CoA is the varied substrate at pH=8.0 in 0.1% Triton X-100, a double reciprocal plot as in Figure 14A, shows non-linearity. A Hill plot of the same data shown in Figure 14B, gives a linear fit when Hill n=2.3, indicating strong positive cooperativity.

Hill plots also show non-hyperbolic enzyme-substrate

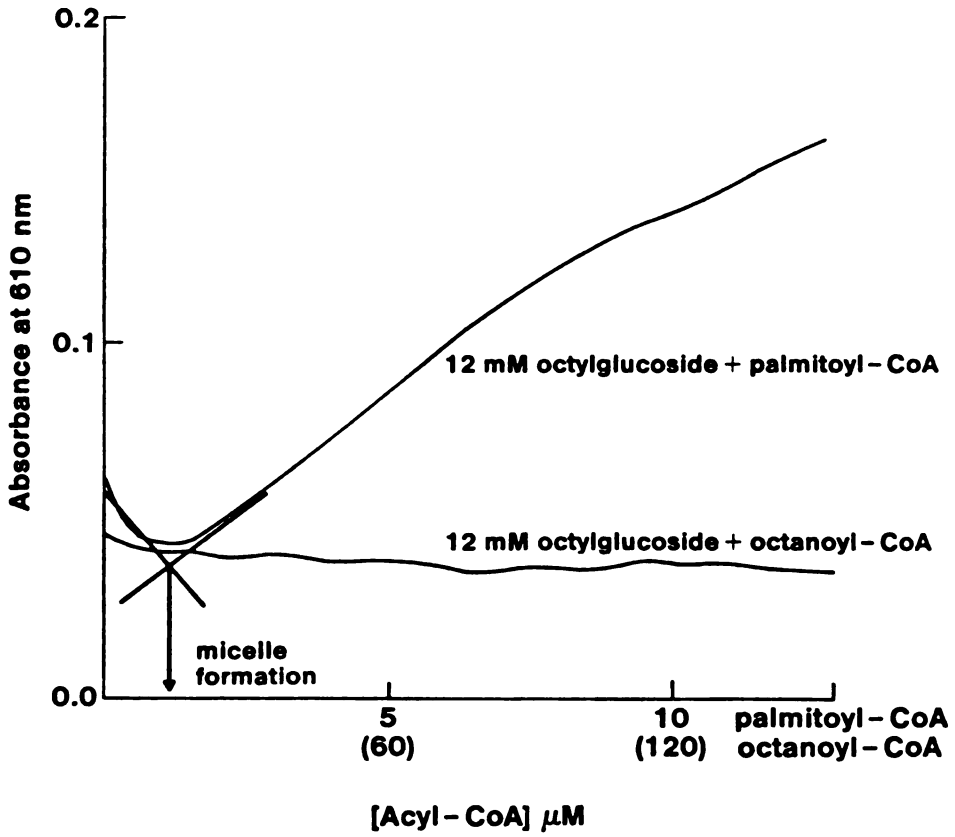

Figure 13. Determination of acyl-CoA micelle formation in octylglucoside. Piracyanol chloride (4 μM) was added to the DTNB assay media containing 12 mM octylglucoside. The absorbance change at 610 nm was recorded upon continuous addition of palmitoyl-CoA or octanoyl-CoA with a pump driven syringe.

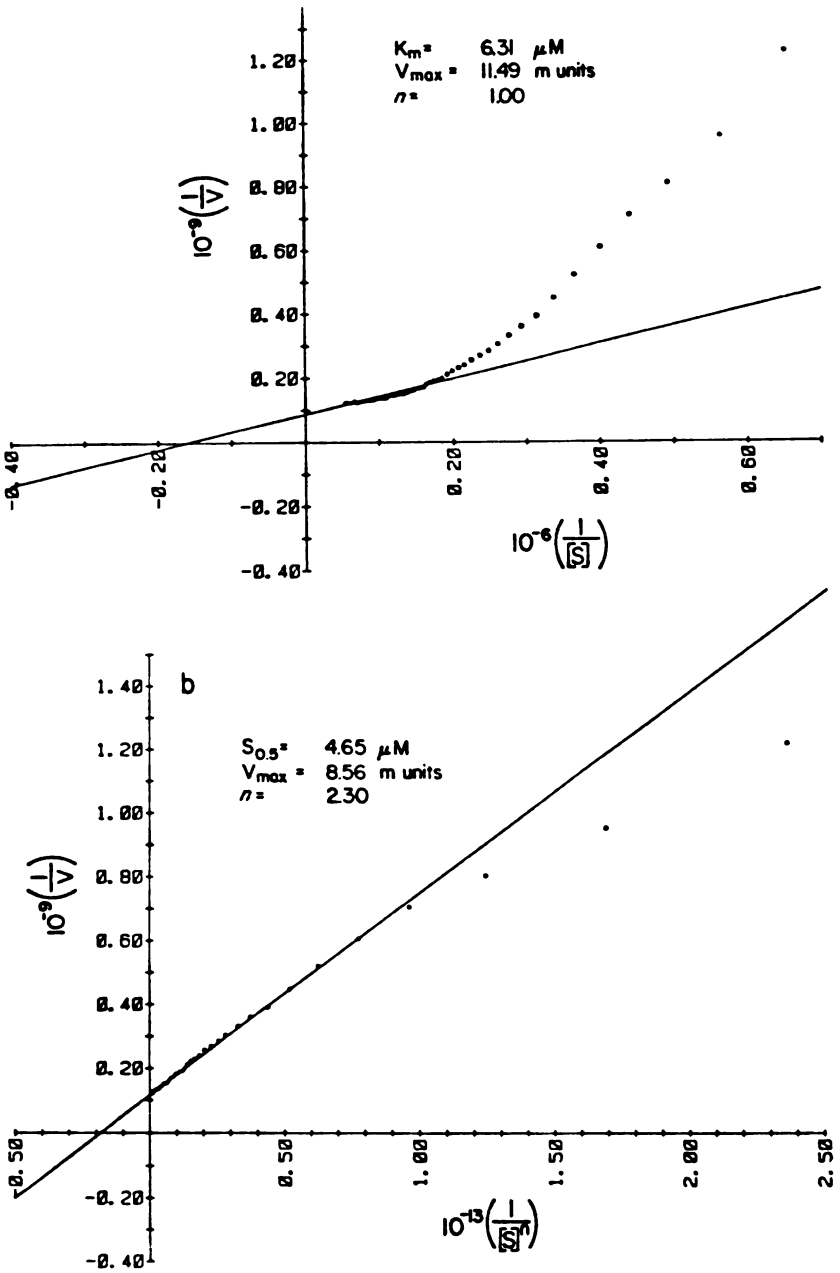

Figure 14. Double reciprocal plots of CPT reaction velocity versus octanoyl-CoA concentration. L-carnitine is fixed at 3.0 mM. Other assay conditions are as described for the DTNB assay in Methods. (a) 1/V versus 1/[S]. (b) 1/V versus $1/[S]^n$, Hill n=2.3. The data were obtained with the kinetic analyzer described in Methods.

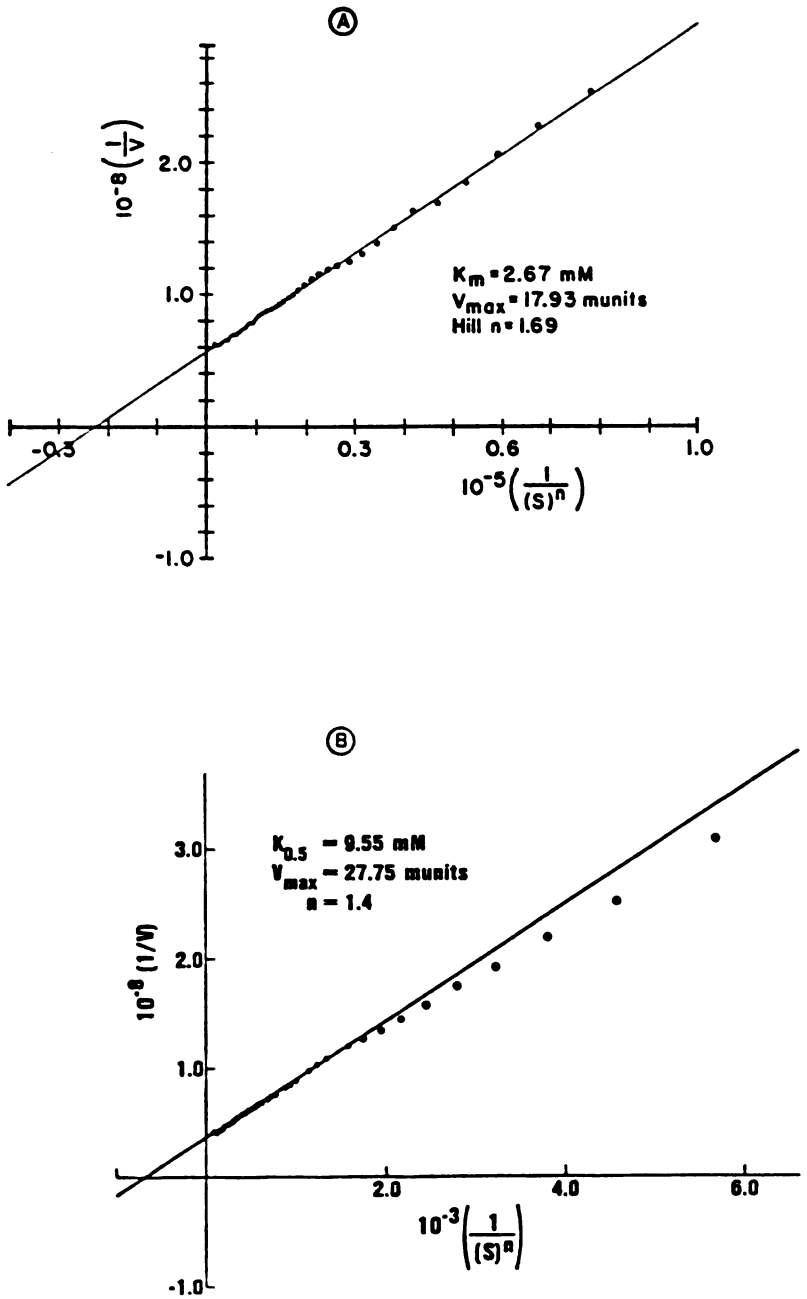

Figure 15. Double reciprocal plot of CPT reaction velocity versus L-carnitine concentration. (a) 1/V versus $1/[S]^n$, Hill n=1.7. Palmitoyl-CoA is 100 µM, Triton X-100 is 0.1 %, pH is 6.0. (b) 1/V versus $1/[S]^n$, Hill n=1.4. Octanoyl-CoA is 100 µM, octylglucoside is 12 mM, pH is 8. Other assay conditions are as described in Methods.

interaction when L-carnitine is the varied substrate. The data from a typical experiment in which palmitoyl-CoA is at saturating levels at pH 6.0 in 0.1% Triton X-100 is shown in Figure 15A. A Hill n=1.7 gives the best linear fit of the experimental data. In 12 mM octylglucoside and 100 µM octanoyl-CoA (non-micellar assay conditions) a Hill plot of the L-carnitine data gives n=1.4 (see Figure 15 B).

In contrast, mouse liver peroxisomal COT exhibits linear kinetics. A double reciprocal plot of data obtained using the same assay, at pH 8.0 with stearoyl-CoA as substrate is shown in Figure 16. A Hill n= 1.0 gives the best linear fit.

Substrate Specificity. CPT substrate specificity profiles in the forward direction in the presence of 0.1% Triton X-100 and 12mM octylglucoside for two fixed concentrations of L-carnitine, are shown in Figure 17. It is clear from the profiles in Triton X-100 that the substrate specificity will depend on the fixed concentration of L-carnitine. The $K_{0.5}$ and V_{max} for L-carnitine were determined in the presence of a fixed saturating concentration of various even-chain acyl-CoAs in both detergents. These data are given in Table IV. In Triton X-100, CPT has higher affinity for L-carnitine in the presence of long-chain acyl-CoA derivatives, but it has the highest absolute catalytic rate with hexanoyl-CoA. A logarithmic relationship exists at

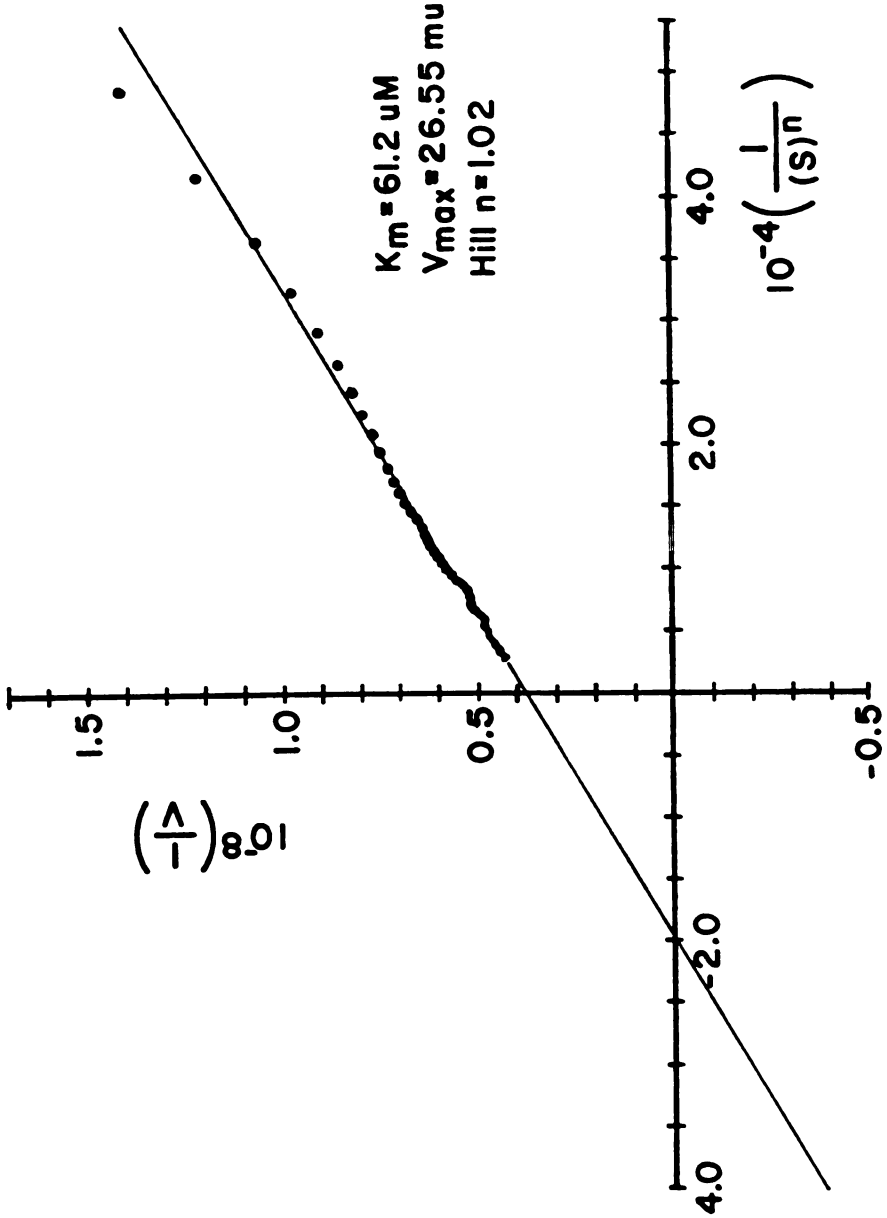

Figure 16. Double reciprocal plot of COT reaction velocity versus acyl-CoA concentration. 1/V versus 1/[S], the substrate, S, is stearoyl-CoA. L-carnitine concentration is 3.0 mM. The assay contains 0.1% Triton X-100 and the pH is 8.0. Other assay conditions are as described for the DTNB assay in Methods.

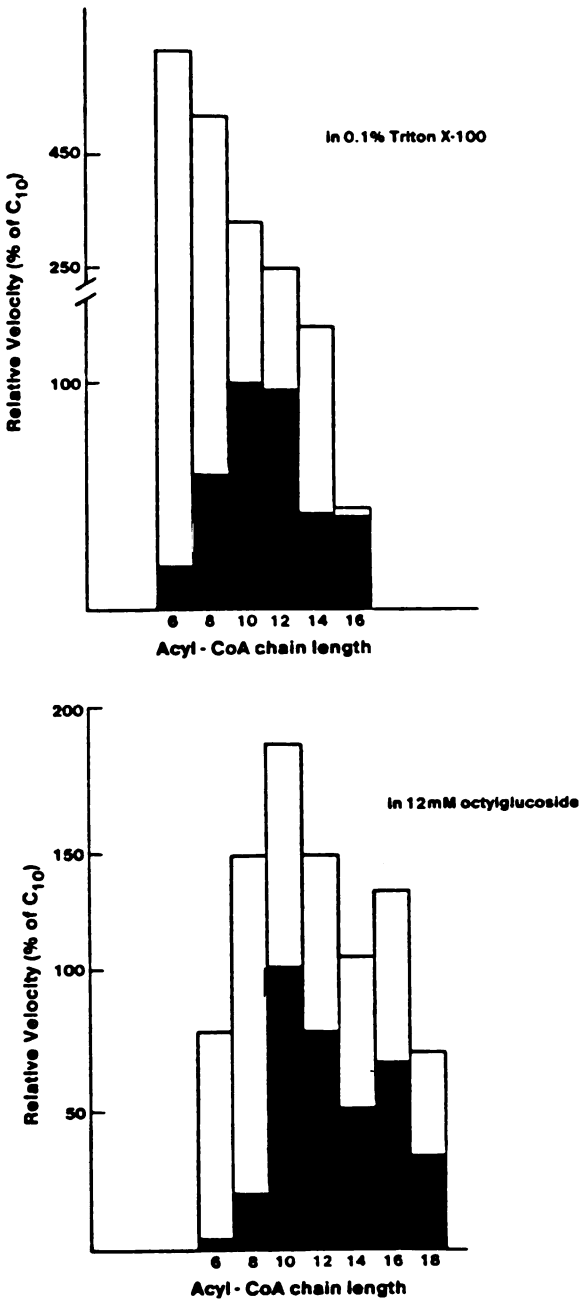

Figure 17. Specificity of CPT for acyl-CoAs of varying chain-length. Measurements were made in 0.1% Triton X-100 and in 12 mM octylglucoside. In shaded areas, the activity was measured with 1.1 mM L-carnitine and 100 µM acyl-CoA using the DTNB assay. In unshaded areas V_{max} was determined using the kinetic analyzer, with L-carnitine as the varied substrate and 200 µM acyl-CoA. The specific activity of CPT with palmitoyl-CoA measured with the standard DTNB assay in Triton X-100 was 40.

TABLE IV

KO.5 AND Vmax VALUES OF CPT FOR L-CARNITINE AT A CONSTANT CONCENTRATION OF ACYL-CoAs OF VARYING CARBON CHAIN-LENGTH

acyl-CoA chain-length	Triton X-100		Octylglucoside	
	KO.5 (mM)	Vmax munits	KO.5 (mM)	Vmax munits
6	2.9	3.0	11.1	15.2
8	1.5	2.5	10.2	27.7
10	0.64	1.6	11.1	35.3
12	0.53	1.2	4.2	27.5
14	0.35	0.6	4.9	25.2
16	0.2	0.2	4.9	25.2
18			3.8	13.5

Acyl-CoAs were 200 uM. Octylglucoside was 12 mM and Triton X-100 was 0.1 %.

saturating acyl-CoA levels between the $K_{0.5}$ for L-carnitine and the acyl- carbon chain-length (see Figure 18). In contrast, mouse liver peroxisomal CAT has higher affinity for L-carnitine in the presence of short chain acyl-CoAs and the K_m for L-carnitine of mouse liver peroxisomal COT, does not show this type of relationship with the acyl-group chain-length, see Table V.

It is apparent from these data that the specificity of CPT for any given acyl-CoA depends both on the binding capacity and on the catalytic rate for each individual acyl-CoA substrate at a specific concentration of L-carnitine. Therefore, a better determination of the enzyme's capacity to use a particular acyl-CoA could be made if one determined the apparent kinetic constants within a limited range of the cosubstrate L-carnitine. With this in mind, the L-carnitine concentration was fixed at 1.0 mM (nonsaturating for medium chain acyl-CoAs) or 5.0 mM (overall saturating) and the kinetic parameters for several acyl-CoAs were determined. The $K_{0.5}$ and V_{max}, with a calculated $V_{max}/S_{0.5}$ ratio, are shown in Table VI, The data show that the beef heart enzyme is selective for long-chain acylcarnitine formation at low concentrations of L-carnitine. Low concentrations of L-carnitine decrease the enzymes catalytic efficiency with hexanoyl-CoA and octanoyl-CoA but do not affect the catalytic efficiency with the long-chain acyl-CoAs.

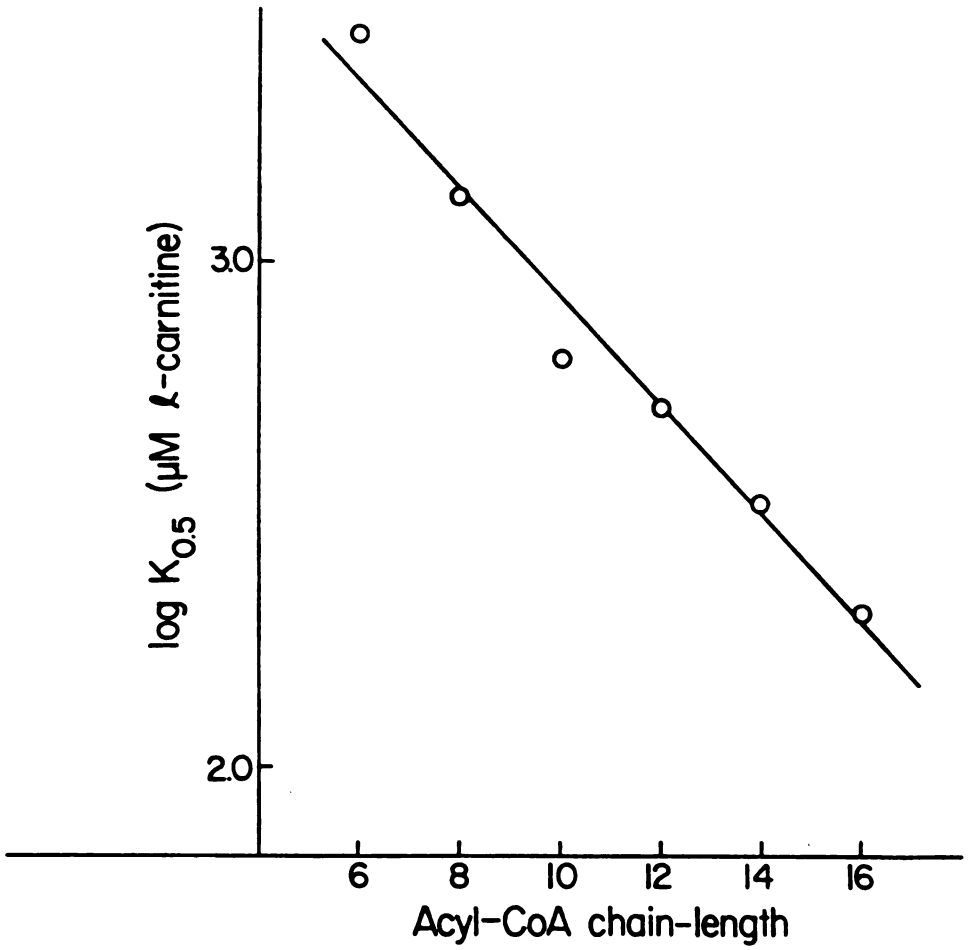

Figure 18. Relation between the chain length of the acyl-CoA and the $K_{0.5}$ of CPT for L-carnitine. $K_{0.5}$ values are determined using the kinetic analyzer described in Methods with the DTNB assay. Acyl-CoAs are 200 μM.

TABLE V

$K_{0.5}$ AND V_{max} VALUES OF COT AND CAT FOR L-CARNITINE AT A CONSTANT CONCENTRATION OF ACYL-CoAs OF VARYING CARBON CHAIN-LENGTH

Acyl-CoA chain-length	$K_{0.5}$	
	COT	CAT
2	78	15
4	3.2	17
6	2.5	20
8	3.2	19
10	2.2	29
12	3.7	-
14	10.6	-
16	21	-
18	64	-

L-carnitine was 1.2 mM. $K_{0.5}$ values were obtained with the kinetic analyzer described under Methods using the DTNB assay at pH 8.0.

TABLE VI

KINETIC PARAMETER OF CPT FOR ACYL-CoAS OF VARYING CHAIN-LENGTH AT TWO FIXED CONCENTRATIONS OF L-CARNITINE

	Concentration of L-carnitine					
	1.0 mM			5.0 mM		
acyl-CoA chain length	$K_{0.5}$ (μM)	V_{max} (munits)	$V_{max}/K_{0.5}$	$K_{0.5}$ (μM)	V_{max} (munits)	$V_{max}/K_{0.5}$
6	69.5	5.9	0.09	43.6	16.3	0.37
8	4.9	4.2	0.86	2.3	11.8	5.11
10	1.0	4.7	4.70	0.6	10.2	17.28
12	0.7	2.8	4.13	0.7	3.2	6.15
14	0.7	0.9	1.20	1.2	1.4	1.12
16	1.2	1.5	1.25	1.5	1.5	1.0
18	1.3	0.6	0.44	1.5	0.6	0.39

Kinetic parameters are determined with the kinetic analyzer using the DTNB assay as described in Methods.

When octylglucoside is substituted for Triton X-100, changes in the substrate specificity pattern of CPT assayed in the forward direction with 1.1 mM L-carnitine and 100 µM acyl-CoA are apparent (compare shaded areas in Figure 17). Although maximum activitity under these conditions is obtained with decanoyl-CoA with both detergents, the relative activity for octanoyl-CoA is much less in octylglucoside and the relative activity with long-chain acyl-CoAs is greater. Also shown in Figure 17 is the acyl-CoA specificity of CPT in 12 mM octylglucoside at saturating levels of L-carnitine (unshaded area). Saturation of the enzyme with L-carnitine increased the V_{max} with medium-chain acyl-CoAs relative to its V_{max} with long-chain acyl-CoA but to a significantly lesser extent than in Triton X-100. Octylglucoside alters the kinetic constants of the enzyme significantly. As shown in Table IV, at pH=8.0 and 200 µM palmitoyl-CoA the $K_{0.5}$ for L-carnitine is 4.9 mM in 12 mM octylglucoside. This is in contrast to assays in Triton X-100 where the $K_{0.5}$ for L-carnitine is only 0.2 mM. A 10-fold difference observed in Triton X-100 between the $K_{0.5}$ for L-carnitine with hexanoyl-CoA as cosubstrate and the $K_{0.5}$ for L-carnitine with palmitoyl-CoA as cosubstrate is reduced to a 2-fold difference in octylglucoside.

The effect of increasing concentrations of octylglucoside on the kinetics with acyl-CoAs is shown in

Table VII and Figure 19. Octylglucoside lowers the $K_{0.5}$ for palmitoyl-CoA. At pH 6.25 the $K_{0.5}$ for palmitoyl-CoA is 24.2 μM in 0.1% Triton X-100 in contrast to 3.1 μM in 12 mM octylglucoside. However, octylglucoside inhibits CPT activity with octanoyl-CoA raising its $K_{0.5}$ for octanoyl-CoA and acting like a competitive inhibitor with an apparent K_i of 15 mM for octylglucoside (see Figure 19).

The Effect of pH on the Kinetics of CPT Since the response of membrane bound CPT to malonyl-CoA is very pH dependent (130), the effect of pH on the kinetics of CPT was determined. Substrate-velocity plots at pH 6.0 and pH 8.0 are shown in Figure 20. In Figure 20A, palmitoyl-CoA is the variable substrate and, in Figure 20B, L-carnitine is the variable substrate. The sigmoidal behavior of CPT with palmitoyl-CoA is more apparent when low substrate concentrations are used at pH 6.0 (see Figure 20A). The insets show the differences in V_{max} at pH 6.0 and 8.0. The $K_{0.5}$ for both L-carnitine and palmitoyl-CoA is larger at pH 6.0 than at pH 8.0, the V_{max} was greater at the lower pH (see Table VIII). Likewise, in octylglucoside the $K_{0.5}$ for L-carnitine increases as the pH decreases from 2.2 mM at pH 8.0 to 9.8 mM at pH 6.25 and the highest V_{max} is at pH 7.0 (see Table IX).

Effect of Malonyl-CoA and TDGA-CoA on CPT Preliminary

TABLE VII

EFFECT OF OCTYLGLUCOSIDE ON THE KINETIC PARAMETERS OF CPT FOR PALMITOYL-CoA

Octylglucoside (mM)	$K_{0.5}$ (μM)	V_{max} munits	Hill n
0	10.8	5.8	1.4
6	2.6	5.6	1.8
12	3.1	7.7	1.8
18	3.1	6.1	1.9

L-carnitine was 40 mM and the palmitoyl-CoA concentration was varied. Assays were done at pH 6.25, with the DTBP assay using the kinetic analyzer described under Methods.

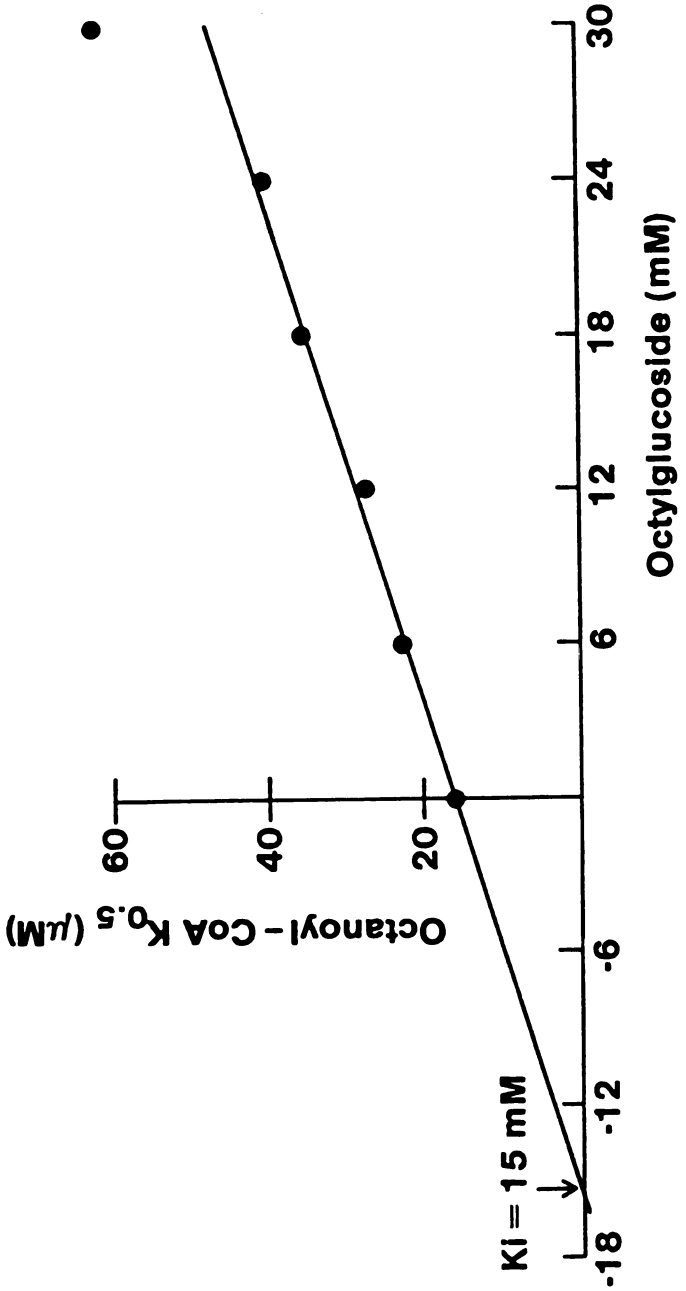

Figure 19. Effect of octylglucoside on the $K_{0.5}$ of CPT for octanoyl-CoA. The $K_{0.5}$ values are determined using the kinetic analyzer described in Methods. L-carnitine is 40 mM and the pH is 8.0.

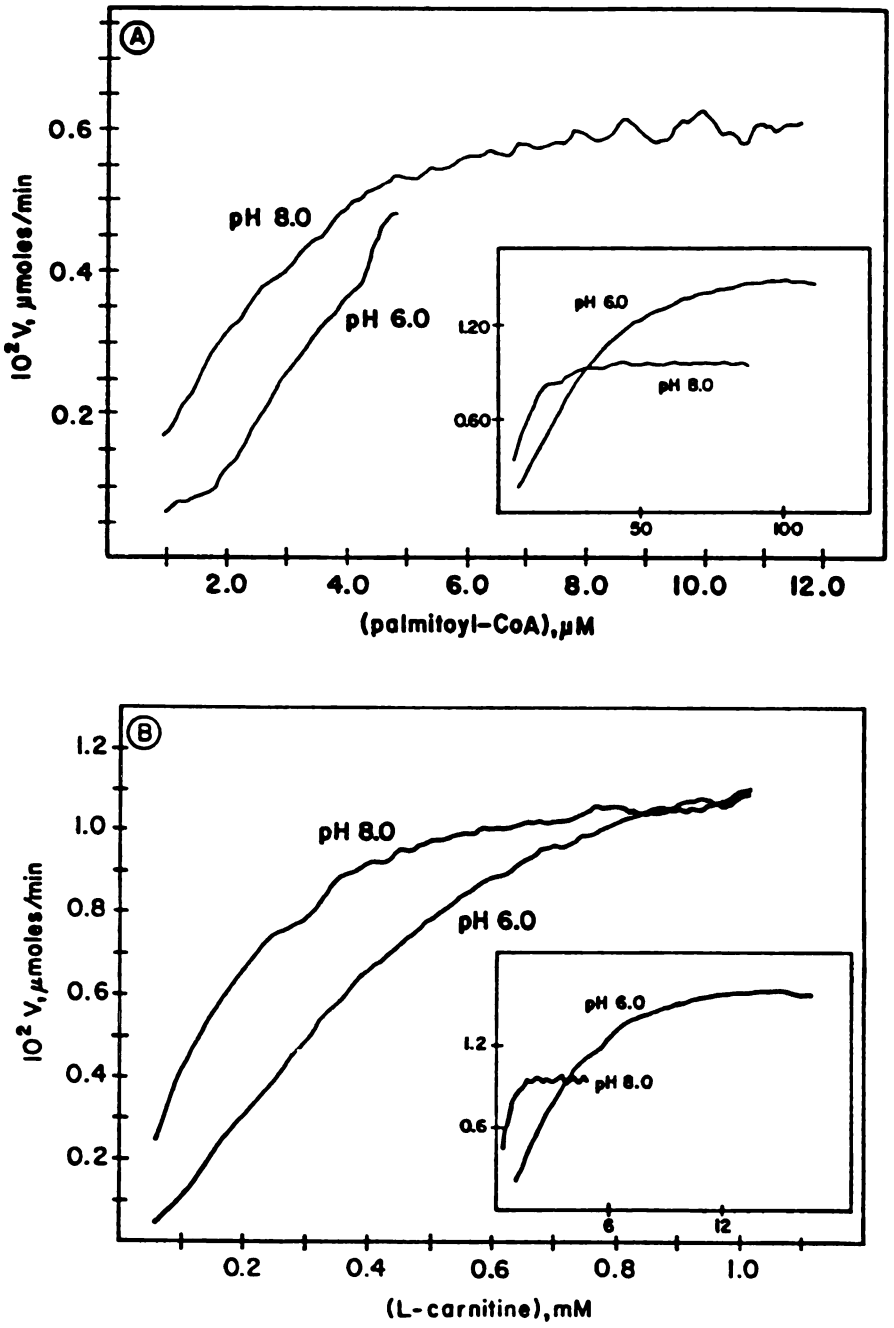

Figure 20. Effect of pH on the velocity versus substrate concentration curves for CPT. In A, palmitoyl-CoA concentration is varied. The concentration of L-carnitine is 6.0 mM. In B, the concentration of L-carnitine is varied. The concentration of palmitoyl-CoA is 200 µM. The insets show the velocity versus substrate profile over a greater range of substrate concentrations. The units for the insets are the same as the units of the figure. Experimental details are described in Methods.

TABLE VIII

EFFECT OF pH ON THE KINETIC PARAMETERS OF CPT IN TRITON X-100

	L-Carnitine			Palmitoyl-CoA		
pH	$K_{0.5}$ (mM)	V_{max} (munits)	Hill n	$K_{0.5}$ (μM)	V_{max} (munits)	Hill n
6.0	2.89(2.5-3.3)	18.0(17.5-19.0)	1.69(1.65-1.75)	24.2(23.6-25.5)	15.5(15.2-16.2)	1.73(1.7-1.8)
7.0	1.10(0.99-1.3)	15.5(15.4-15.8)	1.50(1.45-1.60)	10.4(9.6-11.0)	14.1(13.6-14.6)	1.70(1.65-1.75)
8.0	0.20(0.18-0.25)	9.25(8.0-9.5)	1.33(1.30-1.40)	1.9(1.2-2.6)	8.6(7.0-9.8)	1.7(1.6-1.8)

The data represent the mean and range for three measurements. L-carnitine is 6.0 mM. Palmitoyl-CoA is 100 μM. The data were obtained with the kinetic analyzer using the DTBP assay as described in Methods. One munit of activity is the amount of enzyme necessary to convert one nmol of acyl-CoA to acylcarnitine in one minute.

TABLE IX

EFFECT OF pH ON THE KINETIC OF CPT FOR L-CARNITINE IN OCTYLGLUCOSIDE

pH	$K_{0.5}$ (mM)	V_{max} munits	Hill n
6.25	9.8	14.8	1.5
6.5	7.4	14.7	1.5
6.75	5.0	13.3	1.6
7.0	4.6	17.8	1.5
7.25	3.9	12.5	1.5
7.5	3.0	12.0	1.5
8.0	2.2	10.1	1.5

Palmitoyl-CoA is 100 μM. Octylglucoside is 12 mM. The kinetic parameters for L-carnitine were determined with the kinetic analyzer using the DTBP assay as described in Methods.

initial rate assays at pH 8.0 showed no effect of malonyl-CoA on the V_{max}. The purified enzyme has a low $K_{0.5}$ (1.9 μM) for palmitoyl-CoA at pH 8.0, and since malonyl-CoA inhibition had been reported to be of a competitive type, it was uncertain whether the lack of malonyl-CoA inhibition resulted from oversaturation with the acyl-CoA and malonyl-CoA concentrations used in the initial rate assay, 37.5 μM and 1-5 μM, respectively. Though membrane-bound CPT had been reported to show significant inhibition with similar assay conditions (34) the use of BSA or the presence of a membrane could have reduced the effective concentration of palmitoyl-CoA. Alternatively, the $K_{0.5}$ for palmitoyl-CoA could be higher in the membrane bound state. The kinetic analyzer is very well suited for covering a range of palmitoyl-CoA concentrations below and above the enzyme's $K_{0.5}$ for the substrate, conditions which allow the effects of a "competitive" inhibitor to be best observed. Experiments were performed with palmitoyl-CoA concentrations above and below the $K_{0.5}$ using high and low malonyl-CoA concentrations. No effect of malonyl-CoA on CPT activity was obtained in any of the experiments, even when the malonyl-CoA concentration was 600 μM. The malonyl-CoA effects on the kinetics of CPT at pH 6.0 with saturating levels of L-carnitine are summarized in Table X. When subsaturating levels of L-carnitine are used, again no effect of malonyl-CoA is obtained.

TABLE X

EFFECT OF MALONYL-CoA ON THE KINETICS OF CPT WITH PALMITOYL-CoA

	Malonyl-CoA		
	none	300μM	600μM
K_m	24.2	22.1	25.4
V_{max}	15.5	14.98	15.0
Hill n	1.73	1.8	1.9

Palmitoyl-CoA is the variable substrate and L-carnitine is 6.0 mM. The kinetic determinations were done in 0.1% in Triton X-100 at pH 6.0 as described for the DTBP assay in Methods.

In contrast, 2-tetradecylglycidyl-CoA does have an effect on the kinetic parameters of CPT for palmitoyl-CoA. As shown in Figure 21, TDGA-CoA altered the shape of the V versus [S] curve for palmitoyl-CoA, changing the kinetics from sigmoid to hyperbolic. The effect of TDGA-CoA on the kinetic parameters of CPT with palmitoyl-CoA are summarized in Table XI. At pH 6.0, the Hill coefficient for palmitoyl-CoA is reduced to 1.0 in the presence of 40 μM TDGA-CoA.

Kinetic Characterization of Membrane Bound Rat Heart Mitochondrial Carnitine Palmitoyltransferase

Effect of acyl-CoA on mitochondrial swelling. As shown in Figure 22, continuous addition of low concentrations of palmitoyl-CoA to the assay mixture containing intact heart mitochondria causes a large drop in absorbance which is apparently due to the swelling and rupturing of the mitochondrial membrane caused by the detergent properties of the palmitoyl-CoA. Addition of 1.4% BSA eliminated the swelling but the enzymatic activity can only be measured at much higher concentrations of palmitoyl-CoA (100 μM). With such high concentrations of palmitoyl-CoA, BSA did not completely prevent swelling. In contrast, decanoyl-CoA, which has a higher cmc, does not induce swelling of the mitochondria during the course of the reaction even with no

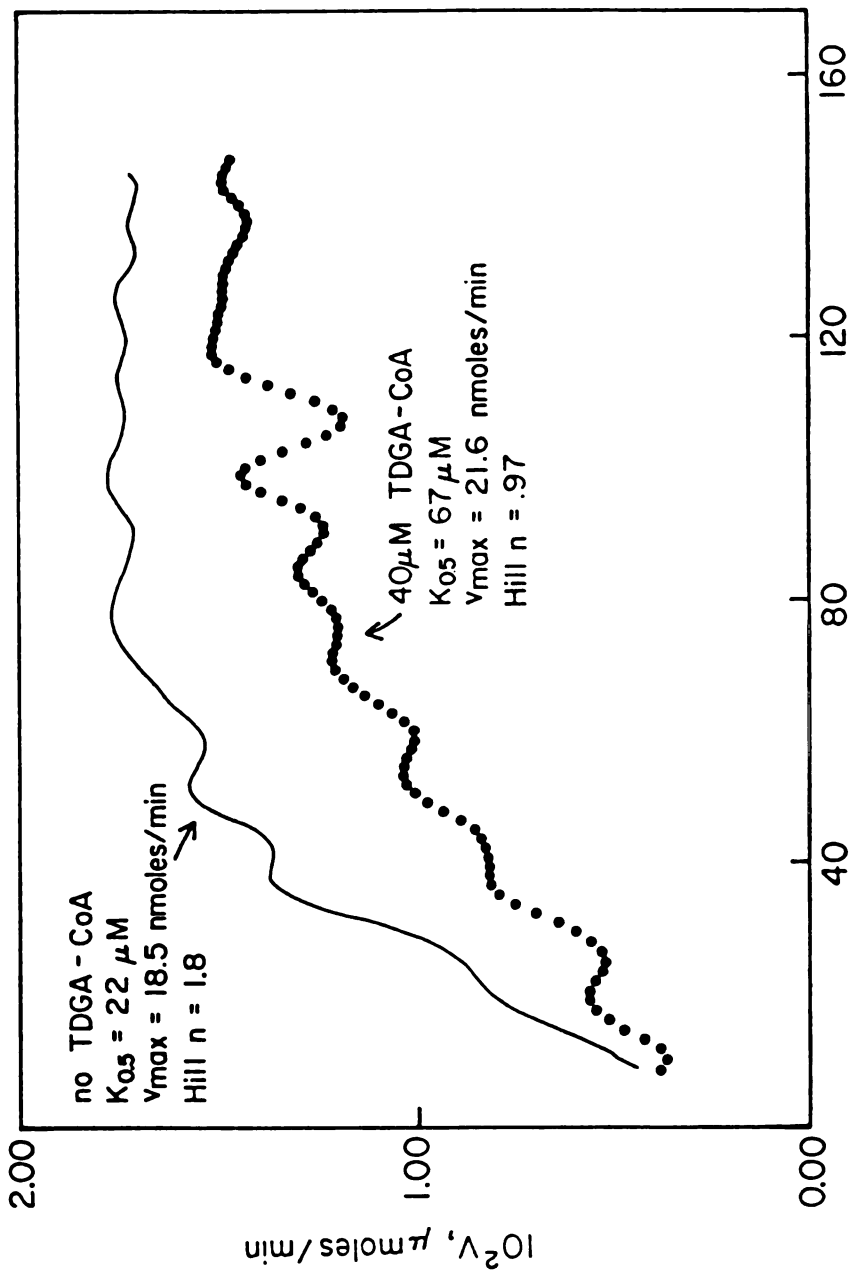

Figure 21. Effect of TDGA-CoA on CPT. Kinetic parameters are determined using the kinetic analyzer with the DTBP assay at pH 6.0. L-carnitine is 6.0 mM. The assay mixture contains 0.1% Triton X-100.

TABLE XI

THE EFFECT OF TDGA-CoA ON THE KINETICS OF PURIFIED CPT WITH PALMITOYL-CoA

	TDGA-CoA		
	none	20 µM	40 µM
$K_{0.5}$ (uM)	22	41	68
V_{max} (munits)	19	18	21
Hill n	1.8	1.5	1.0

Palmitoyl-CoA is the variable substrate and L-carnitine is 6.0 mM. The assays are done in 0.1% Triton X-100 at pH 6.0 as described for the DTBP assay in Methods.

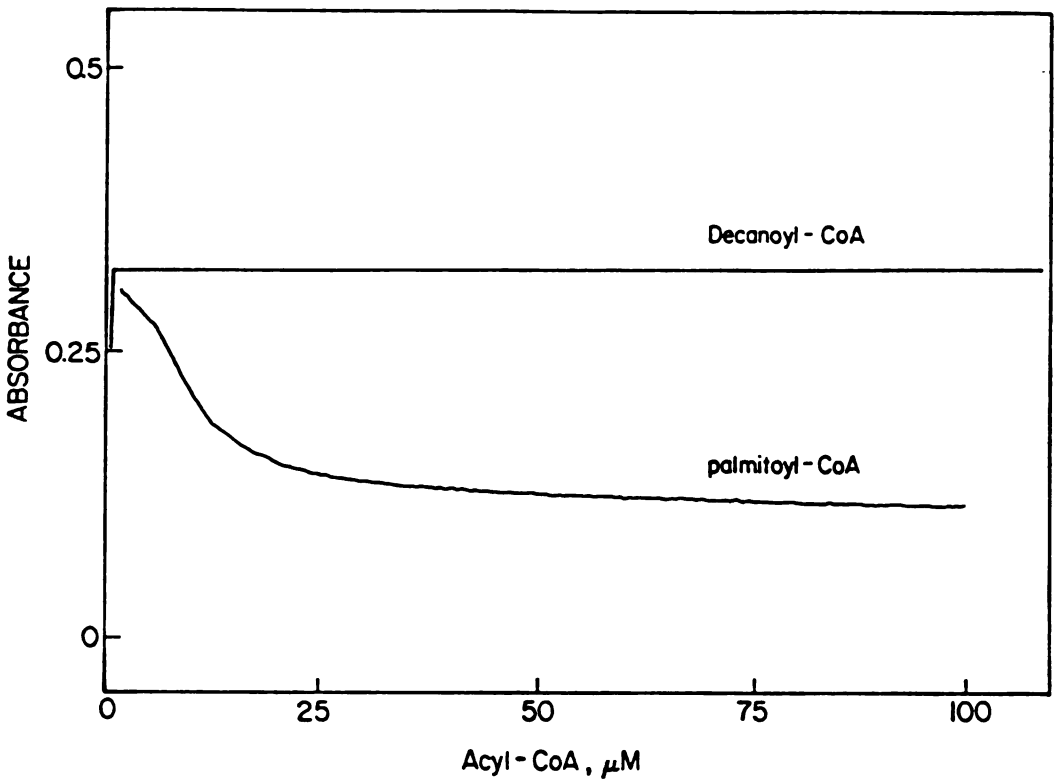

Figure 22. Effect of decanoyl-CoA and palmitoyl-CoA on mitochondrial swelling. Absorbance changes were monitored at 412 nm in a Gilford 2600. The acyl-CoA concentration was continuously increased with a pump driven syringe into a 2 ml cuvette containing 20-50 µg of mitochondrial protein in the DTNB assay buffer with no detergents, 150 mM KCl, and pH 8.

BSA added; see top curve of Figure 22. These data show that decanoyl-CoA can be used in the kinetic study of membrane bound CPT-I similar to the substrate addition experiments with purified CPT.

Kinetics of CPT-I and Effect of Malonyl-CoA. Figure 23 shows that the kinetics of CPT-I with decanoyl-CoA at saturating levels of L-carnitine are sigmoid rather than hyperbolic. The $K_{0.5}$ for decanoyl-CoA is 3 μM and the Hill n is 2.0 in both fed and fasted states. Figure 24A and 24B show the effect of varying concentrations of malonyl-CoA on the V versus [S] curve. The saturation curve is displaced to the right and the $K_{0.5}$ for decanoyl-CoA increases without affecting the V_{max} or the Hill coefficient. At 20 μM malonyl-CoA almost 100% of the activity is inhibited at low decanoyl-CoA concentrations, but as shown in Figure 24B, this inhibition can be reversed by increasing the decanoyl-CoA concentration. Table XII shows the effect of malonyl-CoA on the $K_{0.5}$ for decanoyl-CoA of mitochondrial CPT-I from fed and fasted rats determined under optimized conditions. The $K_{0.5}$ for decanoyl-CoA increases to 185 μM in the presence of 20 μM malonyl-CoA in fed rats.

Effect of Feeding and Fasting on the $K_{i(app)}$ for Malonyl-CoA. Figure 25 shows the effect of feeding and fasting on malonyl-CoA inhibition of heart CPT-I. Fasting

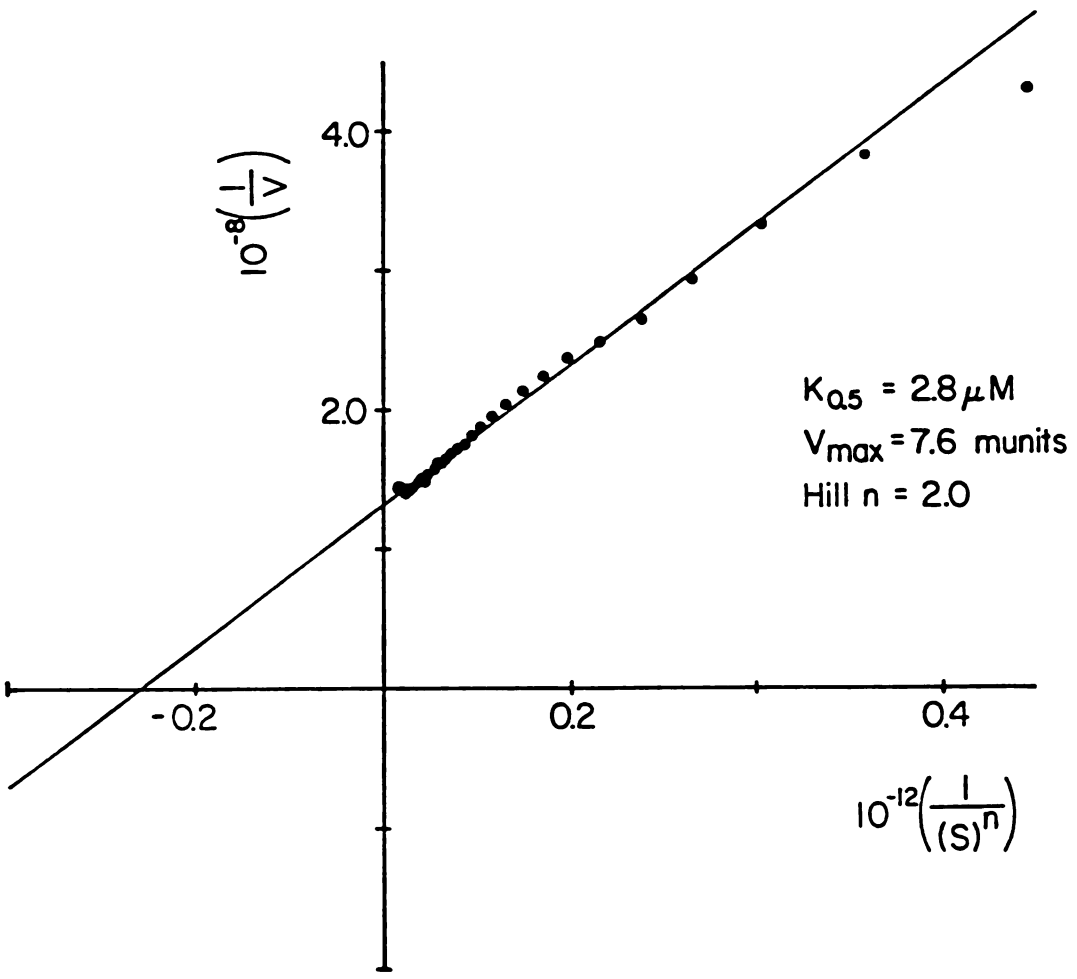

Figure 23. Double reciprocal plot of mitochondrial CPT-I reaction velocity versus decanoyl-CoA concentration. L-carnitine is 6.0 mM. Assay conditions are as described in Figure 22. Mitochondria were isolated from fed rats as described in Methods.

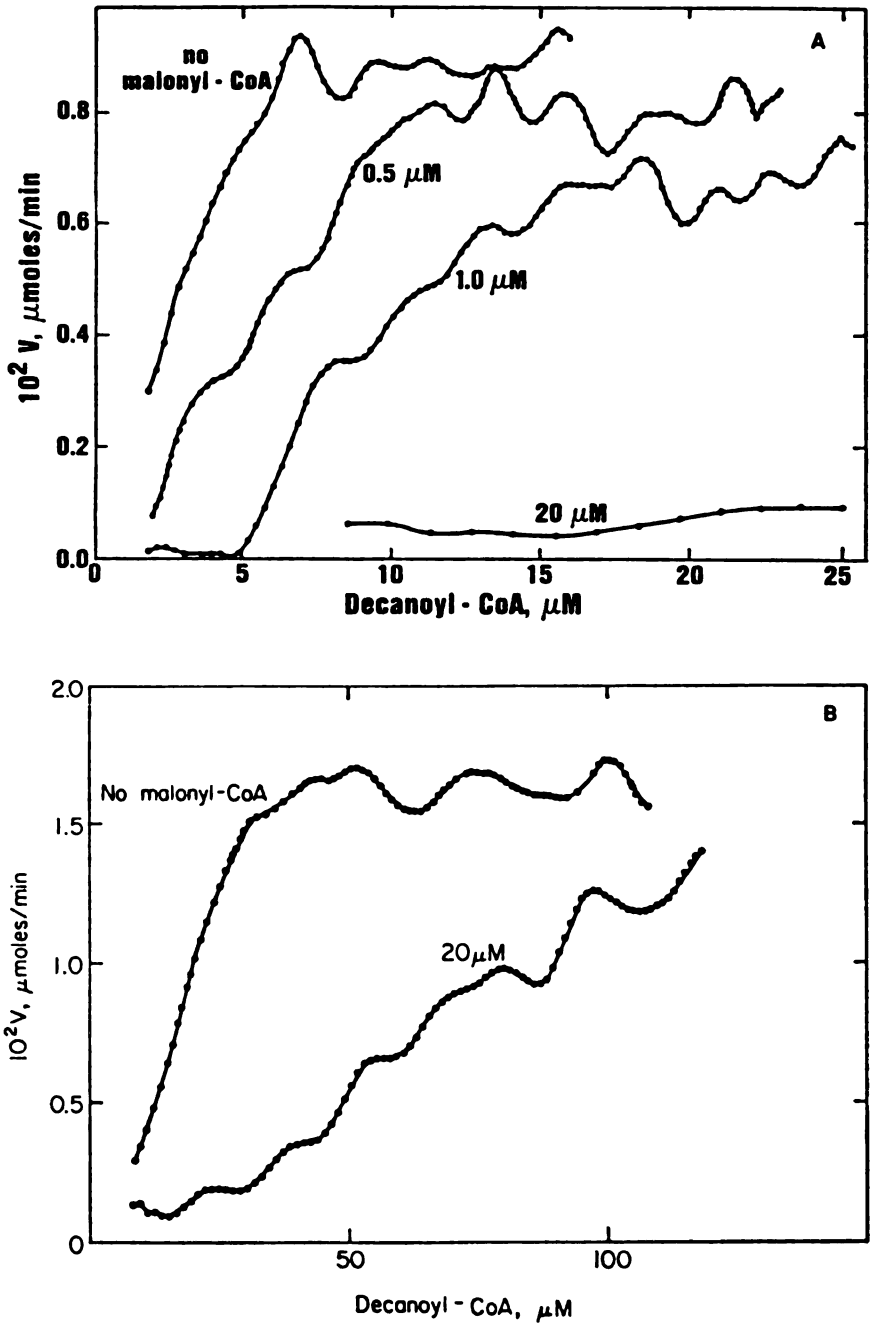

Figure 24. Effect of malonyl-CoA on membrane bound CPT-I. L-carnitine is 6.0 mM. Activity is measured using the DTNB assay at pH 8.0. All other conditions are as described in Figure 22. The assay is started immediately after the addition of malonyl-CoA. Heart mitochondria were isolated from fed rats as described in Methods.

TABLE XII

EFFECT OF FEEDING AND FASTING ON MALONYL-CoA INHIBITION OF MEMBRANE BOUND CPT-I

	$K_{0.5}$ for decanoyl-CoA	
Malonyl-CoA (μM)	Fed	Fasted
none	3	3
0.5	5	-
1.0	9	-
5.0	34	7
10.0	-	14
15.0	-	18
20.0	185	25

The $K_{0.5}$ values are determined from Hill plots similar to the one shown in Figure 23. The assay conditions are as described in Figure 22.

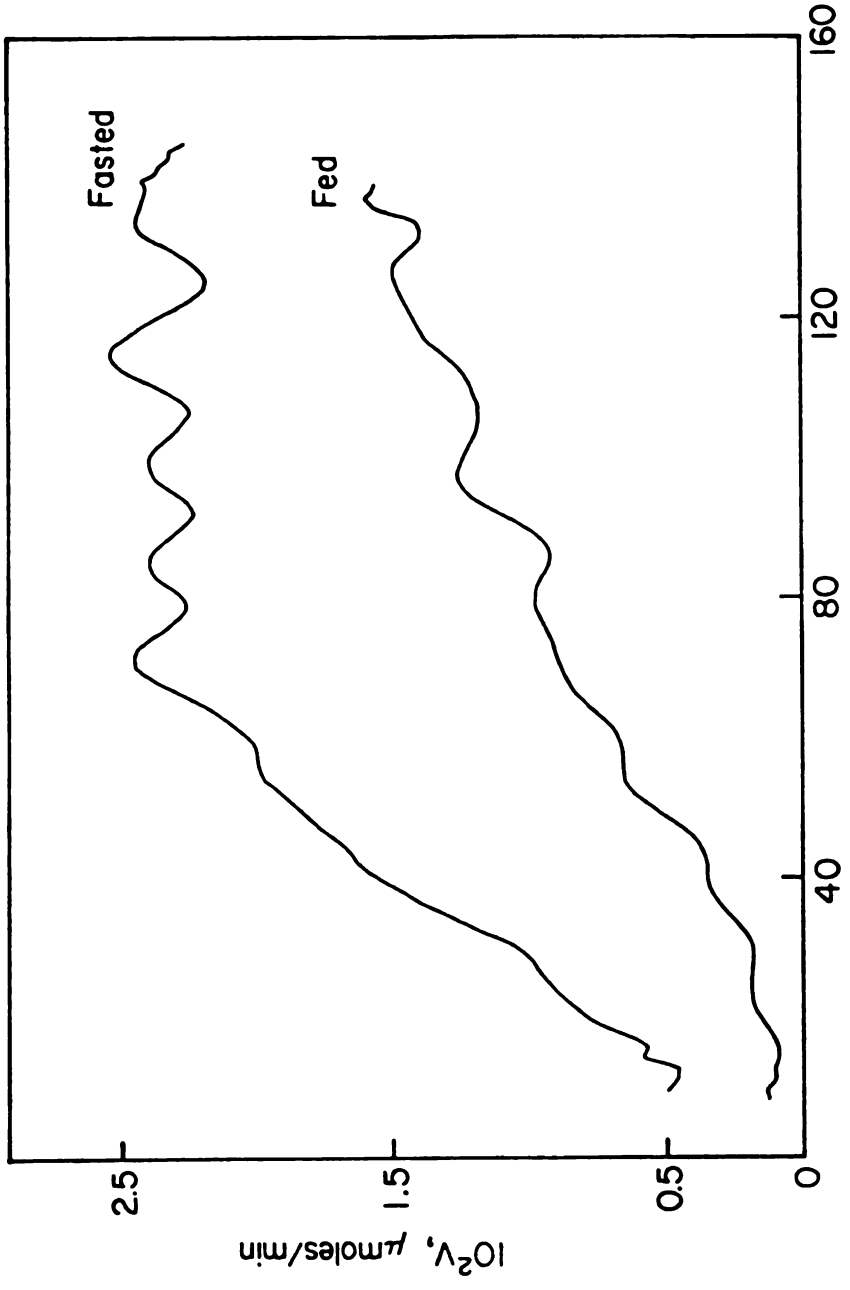

Figure 25. Effect of feeding and fasting on malonyl-CoA inhibition of membrane bound CPT-I. Malonyl-CoA was 20 μM. All other conditions are as described in Figure 22.

significantly reduces the response of CPT-I to a fixed concentration of malonyl-CoA. The secondary plots given in Figure 26 show that there is a ten fold increase in the $K_{i(app)}$ for the inhibitor due to a 48 hr fast. CPT-I of mitochondria from fed rats has a $K_{i(app)}$ for malonyl-CoA of 0.3 µM while those from fasted rats is 2.5 µM. The replots of $K_{0.5}$(app) against malonyl-CoA concentration are linear in the fasted state, but appear to curve upward at high inhibitor concentrations in the fed state. This suggests that there are two inhibitor binding sites in the fed state. However, from the shape of these curves, it cannot be determined if there is any cooperativity between these sites (131).

Kinetics of CPT-I with L-carnitine. Figure 27 shows a substrate-velocity plot with L-carnitine as the varied substrate. The $K_{0.5}$ and Hill coefficient for L-carnitine varied considerably from preparation to preparation of mitochondria. The $K_{0.5}$ varied between 0.2-0.7mM and the Hill coefficient varied between 1.2-1.8. The cause for this variability is unknown. Addition of malonyl-CoA did not alter the $K_{0.5}$ for L-carnitine. For example, for a given preparation of mitochondria which had a $K_{0.5}$ for L-carnitine of 0.6-0.7 mM, the $K_{0.5}$ for L-carnitine in the presence of 20 µM malonyl-CoA was an average of 0.65 mM.

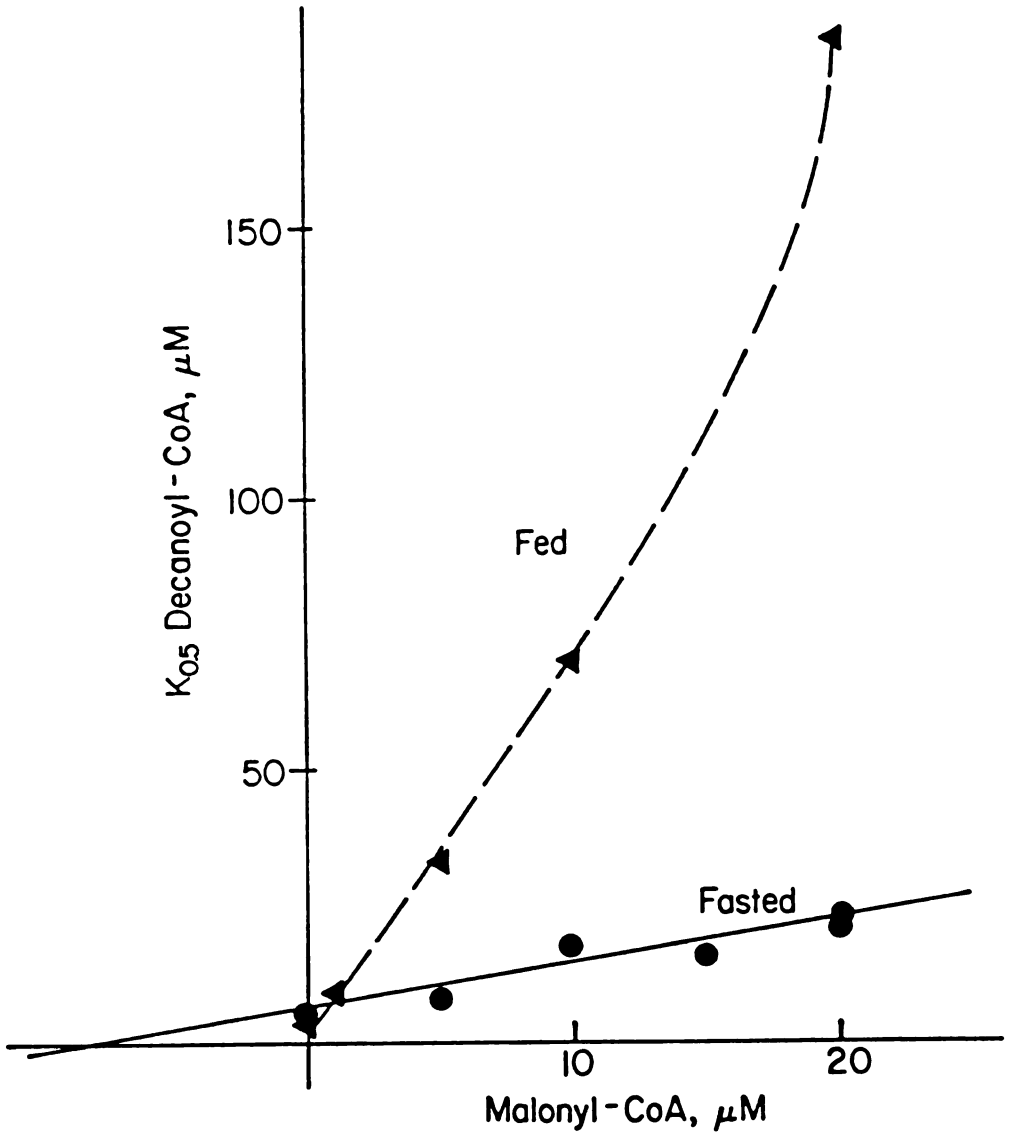

Figure 26. Replot of $K_{0.5}$ versus malonyl-CoA concentration. The $K_{0.5}$ are determined from the best linear fit to the Hill equation of the substrate-velocity data obtained as described in Figure 23. Data collection is started immediately after addition of malonyl-CoA.

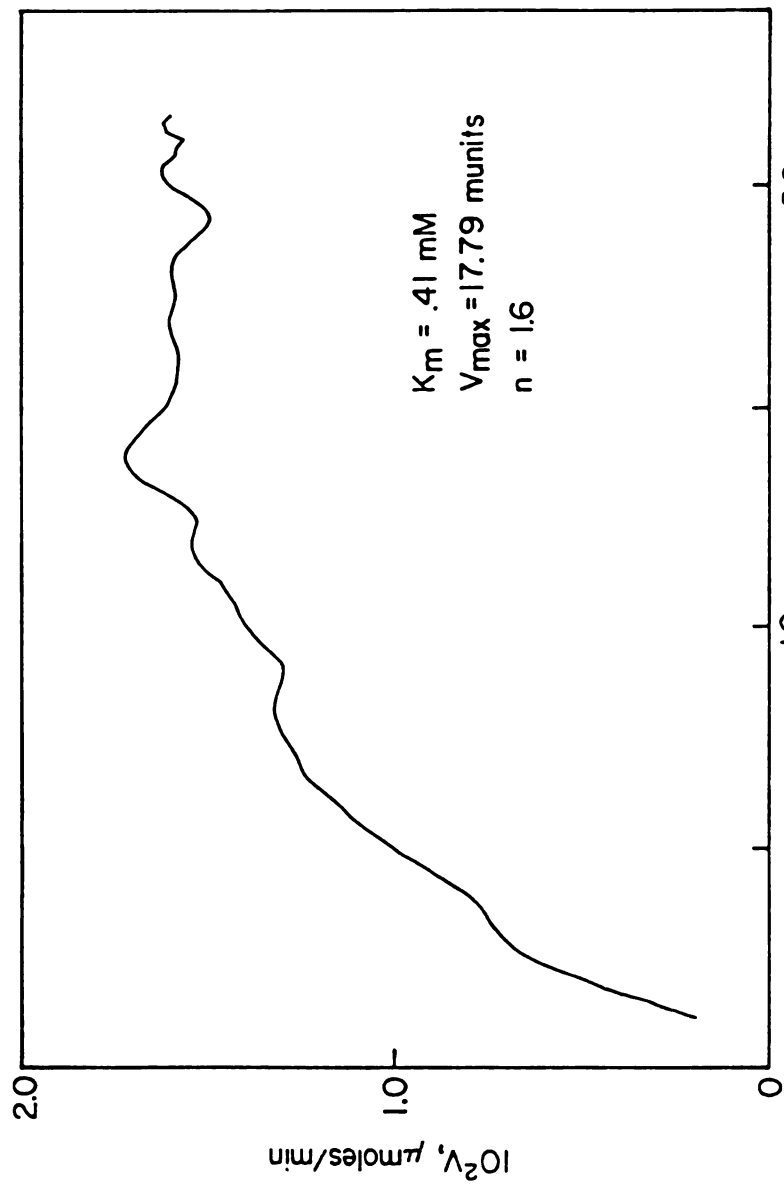

Figure 27. Membrane-bound CPT-I reaction velocity versus L-carnitine concentration curve. Decanoyl-CoA is 100 μM. The assay conditions are as described in Figure 22.

DISCUSSION

The kinetic data show that purified beef heart mitochondrial CPT exhibits non-Michaelis-Menten kinetics with both substrates, the acyl-CoAs and L-carnitine. These data suggest that CPT is allosterically regulated. This suggestion is consistent with the proposed role for CPT in the regulation of fatty acid metabolism. In contrast, when soluble monomeric COT purified from mouse liver peroxisomes (14) is used as a control in kinetic studies, with identical assay conditions, linear kinetics are obtained with Hill coefficients near 1.0.

The sigmoidal kinetics of CPT could result from subunit interaction of varying degrees in purified CPT aggregates. Alternatively, sigmoidal kinetics could arise from allosteric interactions between more than one substrate binding site on the enzyme. The high molecular weight of purified beef heart mitochondrial CPT and of other forms of CPT purified in detergents (see Table I), suggests that CPT is an aggregated enzyme. Taking into account the phospholipid bound to the enzyme and the contribution of a detergent micelle, one can estimate that an associated form of CPT in Triton X-100 might contain at least 4-6 monomers. If several monomers were anchored through a hydrophobic region into a detergent micelle, an apparently associated

form of CPT could result, in which subunit interaction was minimal. However, these studies show that in the absence of detergent micelles, CPT has a molecular weight of 660,000 on Fractogel TSK. These data suggest that CPT is an enzyme aggregate that interacts with detergents. The higher molecular weight of CPT in octylglucoside could be caused by increased aggregation of CPT monomers in the absence of detergent micelles, or perhaps more likely, from a large amount of octylglucoside binding to the aggregates (membrane proteins can bind up to their own weight in detergents (132)). A simple explanation for the oligomerized form of purified CPT is that CPT is an oligomer in the membrane, though, the subunit structure of the native membrane bound CPT might be different from the subunit structure of the protein-detergent complex.

There are several other possible causes for non-hyperbolic dependence of initial velocity upon substrate concentration. Non-linear kinetics can be seen when two enzymes catalyzing the same reaction have significantly different affinities for the substrates. This situation could arise in our system in the unlikely event that the two forms of mitochondrial CPT were indeed separate enzymes with very similar physical properties which had co-purified. However, in this case double reciprocal plots would resemble those of a system exhibiting negative cooperativity with Hill n less than 1.0 (131). The data with purified CPT

shows strong positive cooperativity for CPT with both acyl-CoA and L-carnitine and therefore does not indicate the presence of isozymes.

The continuous addition of substrate and rapid mixing enabled the calculation of initial rates with the TANKIN program at low substrate concentrations (0.1-12 μM palmitoyl-CoA) facilitating the study of the kinetic properties of CPT. This is in contrast to previous studies where the precision and assay sensitivity were limited due to manual addition and mixing of the substrate, the rapid conversion of the substrate to product, and a lower sensitivity of the spectrophotometer used. In order to determine whether the kinetic parameters obtained with the TANKIN program were in any way artifactual several tests for product inhibition and enzyme inactivation were conducted as described by LeBlond et al. (123). In all instances, no evidence was obtained that indicated a bias in the parameters. Evidence has been presented by some investigators that DTNB is inhibitory when preincubated with pigeon breast muscle carnitine acetyltransferase (133) and that DTNB can abolish the apparent cooperativity with respect to palmitoyl-CoA of membrane bound CPT (36). Under the assay conditions used in this study, no evidence of enzyme inactivation was obtained. The rates and progress curves obtained using the DTNB assay were compared to rates and progress curves obtained by following the disappeareance

of the acyl-CoA substrates at 232 nm in the absence of sulfhydryl reagents. Within experimental error, these were identical. Therefore we can conclude that under the conditions of our assay, DTNB does not cause enzyme inactivation nor does it abolish substrate cooperativity.

These data also show that CPT exhibits very different kinetic constants depending on the experimental conditions in which its activity is assayed. As a consequence, the choice of experimental conditions for the determination of substrate specificity profiles can greatly influence the results. This, undoubtedly, is a major factor in the large differences in the data from different laboratories reporting on the same enzyme, see Table II. When the kinetics of purified beef heart mitochondrial CPT are studied in micellar concentration (0.1%) of Triton X-100, the data show that the affinity of the enzyme for L-carnitine depends on the acyl-CoA chain-length, namely a decrease in the chain-length of the acyl-CoA increases the $K_{0.5}$ for L-carnitine. Low concentrations of L-carnitine decrease the catalytic efficiency with hexanoyl-CoA and octanoyl-CoA shifting the specificity of the enzyme towards long-chain acyl-CoAs. As shown in Table VI, at low concentrations of L-carnitine, $V_{max}/K_{0.5}$ is maximized, while that of medium chain acyl-CoAs's is not. This would indicate that the enzyme has evolved to use long-chain acyl-CoAs at low, physiological L-carnitine levels. This

also suggests that fluctuations in the concentration of L-carnitine can affect the "physiological substrate specificity" in the forward direction. The data do not support the suggestions that the enzyme is inhibited by increasing substrate concentrations (30), nor that increasing substrate inhibition (as the acyl- chain lengthens) at the L-carnitine site is a valid explanation (5) for the higher activity of CPT with medium-chain acyl-CoAs.

Major differences in the kinetic parameters of CPT occur when the detergent is changed from Triton X-100 to octylglucoside and when the concentration of detergent is altered thus affecting the substrate specificity pattern of CPT. In 12 mM octylglucoside the $K_{0.5}$ for L-carnitine with medium-chain acyl-CoA's is near 10.5 mM. This large increase in the $K_{0.5}$ for L-carnitine results in very low activity with the assay conditions in Figure 17 where the L-carnitine is 1.1 mM. However saturation with L-carnitine does not shift the specificity of the enzyme towards the medium-chain acyl-CoAs, as it does in Triton X-100. The shift in the substrate specificity of CPT towards higher activity with long-chain acyl-CoAs in octylglucoside appears to result from a combination of effects. Octylglucoside lowers the $K_{0.5}$ of CPT for long-chain acyl-CoAs and this may be related to the formation of mixed micelles with these substrates. The lower activity of CPT with medium-chain acyl-CoAs apparently is due to a moderate competition by

octylglucoside monomers with octanoyl-CoA for the enzyme's active site. In addition, octylglucoside may increase catalysis with the long-chain acyl-CoAs by interacting with the enzyme to give increased rates. In a submicellar concentration of octylglucoside (12 mM) the enzyme remains in solution after centrifugation at 100,000 g for 15 min, while in the absence of detergent significant activity is removed from the supernatant. This suggests that the enzyme can interact with detergent monomers perhaps through hydrophobic regions of the protein. Though direct measurements of detergent binding to CPT were not made, the effects of octylglucoside on the enzyme's kinetic parameters, in particular its apparent competitive inhibition of CPT reaction with octanoyl-CoA and its capacity to increase the $K_{0.5(app)}$ for L-carnitine 20-fold from approximately 0.2 mM to 4.5 mM, indicate a strong interaction at specific sites. One can speculate that hydrophobic site(s) in the enzyme, may have a functional role in the control of enzyme activity through allosteric effects, by binding a membrane component, or acyl-CoA substrate. Such an allosteric site could be involved in the effect of varying hydrophobicity of the acyl-CoA substrates on the enzyme's $K_{0.5}$ for L-carnitine. It is interesting that octylglucoside abolishes in part this effect, see Table IV.

Studies with crude homogenates (134) and with isolated

mitochondria (82) have shown that the K_m for L-carnitine spans a 20 fold range depending on the tissue and species examined. This variation in the K_m for L-carnitine may be a function of the membrane environment. It would be interesting to see if, once solubilized from the membrane, CPT from different tissues still shows significantly different affinities for L-carnitine.

Palmitoyl-CoA and L-carnitine were used to investigate the pH effects on the non-Michaelis-Menten kinetics of CPT. DTBP was used rather than DTNB because it has a higher extinction coefficient that is constant in the range of pHs studied (28), and because it is not inhibitory to carnitine acetyltransferase (133). This makes the assay more sensitive, which allows the use of lower enzyme concentrations with reduced product accumulation. At pH 6, Hill coefficients for both substrates were in the vicinity of 1.8. CPT activity is strongly pH dependent in the range where respiring mitochondria develop a pH gradient during oxidative phosphorylation. Since the matrix pH normally is considerably higher than the inter-membrane pH in mitochondria, this pH difference could affect the catalytic properties of membrane bound carnitine palmitoyltransferase depending on which surface of the inner membrane the enzyme is located. As shown in Figure 20A, a low pH causes low amounts of L-carnitine to greatly limit the enzyme's catalytic capacity. However, at saturating amounts of

L-carnitine, the enzyme attains a V_{max} that is nearly twice that attained at high pH (Table I). Thus low amounts of L-carnitine might affect the activity of the outer form of CPT more, since this form should be exposed to a lower pH. This could be important in certain systemic carnitine deficiencies where low tissue carnitine causes metabolic abnormalities (135). The inner form of carnitine palmitoyltransferase, being exposed to a higher pH, should have a lower maximum capacity to catalyze the formation of acylcarnitines conforming to its ascribed function in the matrix of mitochondria, but it would not be as sensitive to regulation by L-carnitine levels. Besides pH differences, the membrane environment on both faces of the inner membrane contain different protein and phospholipid composition (92). These differences would also be expected to contribute to the catalytic behavior of CPT in vivo.

A rise in pH has been reported to result in a marked decrease in the sensitivity of membrane bound CPT (130) to malonyl-CoA inhibition. Although lowering the pH increased the $K_{0.5}$ for palmitoyl-CoA of purified CPT, (this in contrast to the data reported by Mills et al.(83) where no effect of pH on the K_m for palmitoyl-CoA of membrane-bound CPT was observed) no effect of malonyl-CoA on the kinetics of purified CPT was observed. The lack of malonyl-CoA inhibition of the pure enzyme is not considered as evidence that malonyl-CoA does not affect the membrane bound enzyme.

Rather, it seems likely that the purified enzyme has been removed from some component which can influence its catalytic activity, i.e., a regulator subunit or a special membrane environment. Alternatively, the purified enzyme in the high detergent concentrations may have lost a separate malonyl-CoA binding site. We tested concentrations of malonyl-CoA ranging from 20 μM to 600 μM at saturating and non-saturating levels of L-carnitine over a wide range of palmitoyl-CoA concentrations and neither $K_{0.5}$, V_{max}, or Hill n for palmitoyl-CoA were affected. This indicates that malonyl-CoA is not a competitive inhibitor in contrast to the tentative conclusions by others (29). However, the fact that the enzyme has a high affinity ($K_{0.5}$'s=2-24 μM) for its acyl-CoA substrate and completely lacks a response to malonyl-CoA at concentrations 100-500 times greater than concentrations that are inhibitory with intact mitochondria indicate malonyl-CoA normally must bind at some other site on the native enzyme. Thus, it seems likely that malonyl-CoA is exhibiting some allosteric interaction which is lost upon solubilization of the enzyme. Recent data in the literature strongly suggest a separate binding site for malonyl-CoA. These reports have been discussed previously, see Introduction.

Unlike malonyl-CoA, TDGA-CoA, a substrate analog, did have an effect on the kinetics of CPT. Figure 21 shows that TDGA-CoA altered the shape of the velocity versus

palmitoyl-CoA concentration curve, changing it from sigmoidal to hyperbolic and increasing the $K_{0.5}$ for palmitoyl-CoA. These effects of TDGA-CoA on the kinetics of CPT are those expected of a system with cooperative substrate binding, in which an inhibitor mimics the substrate (127) producing competitive inhibition at two sites. As [I] increases, $K_{0.5}$ increases, and the Hill n approaches 1. Therefore the kinetic data indicate that TDGA-CoA is inhibiting the enzyme by mimicking the acyl-CoA substrate and destroying the substrate cooperativity, but it is not inhibiting CPT irreversibly, since increasing concentrations of palmitoyl-CoA restore V_{max}. This observation is in contrast to the effect of TDGA-CoA on membrane bound CPT, which appears to be irreversible (91). Thus, it appears that TDGA-CoA may have more than a single site of action in vivo. The effect of TDGA-CoA on the purified enzyme, occurs at much higher concentrations of inhibitor than are necessary to produce an irreversible inactivation of membrane bound CPT. The effect of TDGA-CoA on hepatic mitochondria and inverted submitochondrial particles has been recently investigated by Brady et al. (95). Their studies demonstrate that it is possible to almost totally eliminate malonyl-CoA sensitivity in inverted vesicles while retaining a relatively high degree of sensitivity to TDGA-CoA. This result also suggests multiple sites of action for this inhibitor.

Reports in the literature about the kinetics of CPT-I are discrepant. Substrate sigmoidicity has been reported in (21,81) while other studies (29,34) report linear hyperbolic kinetics, which become sigmoidal after addition of malonyl-CoA to the assay medium. Therefore, it has been suggested (34) that malonyl-CoA acts by a mechanism involving cooperative inhibition. CPT of other nonhepatic tissues such as heart and skeletal muscle have greater sensitivity to inhibition by malonyl-CoA and contain significant quantities of malonyl-CoA in the fed state (113,83). Though these tissues do not have coordinated control of fatty acid synthesis and oxidation, their energy demands can vary greatly.

There is increasing interest in the study of the mechanism of malonyl-CoA inhibition of CPT in both hepatic and nonhepatic tissues. Our data for heart mitochondria show that CPT exhibits the same degree of sigmoidicity with decanoyl-CoA in the absence or in the presence of malonyl-CoA. In addition, the kinetic parameters determined for membrane bound CPT-I are similar to those of the purified enzyme from beef heart mitochondria.

One possible explanation for the failure of some other groups to detect sigmoidicity in the absence of malonyl-CoA is that the relative sigmoidicity of two curves is not always apparent when the displacement along the x- axis differs due to different values of $K_{0.5(app)}$. Sigmoidicity

can remain undetected if the substrate range examined is high compared to the $K_{0.5(app)}$. This is common in the progress curves for CPT-I with no malonyl-CoA because of the low $K_{0.5}$ for the acyl-CoAs. Addition of malonyl-CoA increases the $K_{0.5}$ sufficiently to make the substrate range examined optimal for visual detection of sigmoidicity. Analyses of the steepness of an apparently hyperbolic progress curve of data reported by others shows considerable cooperativity. This can be done by determining the $[S](.9V_{max})/[S](.1V_{max})$ ratio (131). This ratio is 81 for a hyperbolic curve and 9 for a curve with a Hill n value of 2. Any value in between 9 and 81 indicates varying degrees of sigmoidicity. For example, if this criteria is applied to the data in Figure 1 of Reference (81), a [S].9/[S].1 ratio of about 9 is obtained for the palmitoyl-CoA saturation curve with no added malonyl-CoA. However, hyperbolic kinetics are described in the paper. Analysis of the data by obtaining the best fit to the Hill equation seems warranted in such situations.

The change in $K_{i(app)}$ for malonyl-CoA as a result of fasting has been shown for liver CPT-I. Our data suggest that a similar mechanism for sensitization-desensitization of mitochondrial CPT to inhibition by malonyl-CoA found in liver exists in heart. Our studies were conducted with freshly prepared heart mitochondria isolated by a new procedure (128) using very young rats. Although the

conditions we used for studying CPT-I interaction with malonyl-CoA, (DTNB assay and pH 8.0) have been avoided by some because of the potential reduced sensitivity of CPT to inhibtion by malonyl-CoA, our data clearly show strong interaction of the same magnitude reported in (34) where a different assay was used. These observations suggest that the mitochondrial preparation procedure and/or the time elapsed from the isolation of the mitochondria to the time analyses are done can affect the interaction of malonyl-CoA with CPT-I. Time dependent changes in the capacity of malonyl-CoA to inhibit CPT-I and to bind to rat liver mitochondria in vitro have been described (136,137). The secondary plots presented in (34) for the rat liver enzyme are very similar to the ones presented here for the heart enzyme. In the fed state there appear to be two inhibitor binding sites. Whether these sites can interact in a cooperative manner cannot be determined from our data. In the starved state, the data shows one inhibitor site of lower affinity for malonyl-CoA.

Higher values of the $K_{0.5}$ for L-carnitine have been measured in tissues with a lower sensitivity to malonyl-CoA inhibition (82). In an attempt to correlate a change in the $K_{0.5}$ for L-carnitine of rat heart CPT-I to changes in the sensitivity to malonyl-CoA inhibition, we encountered considerable variability in the $K_{0.5}$ for L-carnitine in the control (fed state). This variability suggests a mechanism

to regulate the catalytic efficiency of CPT which remains to be elucidated.

The data presented here can be explained by several kinetic models which involve the binding of the inhibitor to a regulatory subunit. For example in the "ligand exclusion" model an inhibitor can bind to a separate regulatory site which may have overlapping functional groups with two or more substrate binding sites. For an enzyme with a high degree of interaction among catalytic subunits, an infinite amount of inhibitor would increase $K_{0.5}$, but would not significantly alter the sigmoidicity of the progress curve. If there are multiple substrate binding sites on the enzyme, not all catalytically active, then dissociation of the enzyme would yield a catalytic subunit insensitive to inhibitor and a regulatory subunit that binds both substrate and inhibitor. An alternate model, would involve a partial competitive inhibition by malonyl-CoA with different substrate and regulatory binding sites. When there is cooperative substrate binding in the absence of inhibitor, addition of the inhibitory regulator makes the enzyme behaves like a new enzyme with the same interaction between subunits but a new $K_{0.5}$. Both these models agree with our data which show strong positive cooperativity for the acyl-CoAs. The increasing amount of indirect evidence in the literature for the interaction of malonyl-CoA with a membrane component, possibly a regulatory subunit of CPT-I,

other than the catalytic subunit itself, strongly support these conclusions.

SUMMARY AND CONCLUSIONS

These data strongly indicate that CPT is a highly regulated, allosteric enzyme which exhibits cooperativity towards its multiple substrates. The molecular weight of CPT by Fractogel TSK chromatography in the absence of detergent micelles indicates that CPT is an aggregate of 4-6 monomers. The similarity in the substrate kinetics of the purified and membrane bound forms of CPT suggests similar subunit aggregation in the membrane bound state.

The data show that the affinity of the enzyme for L-carnitine depends on the acyl-CoA chain-length, namely a decrease in the chain-length of the acyl-CoA increases the $K_{0.5}$ for L-carnitine. This suggests that the concentration of L-carnitine can affect the "physiological substrate specificity". In addition, the data do not support suggestions that the enzyme is inhibited by increasing substrate concentrations. These data indicate that some of the differences observed in the specificity pattern of CPT from various preparations or laboratories reflect the level of saturation of the enzyme with its substrates which result from the variable $K_{0.5}$s with different assay conditions.

It is concluded that malonyl-CoA is not a competitive inhibitor of CPT but acts like a negative allosteric

modifier of membrane bound CPT-I by binding at a site other than the catalytic site. These data indicate that during purification, CPT has been separated from the malonyl-CoA binding component of the mitochondrial membrane since it has lost all sensitivity to the inhibitor. The increased sensitivity to malonyl-CoA inhibition with changes in the physiological state of the enzyme result from changes in the apparent K_i for the inhibitor of the enzyme.

The complex response to changes in substrate concentration, pH, and hydrophobic environment, provides an explanation for the observation that CPT from beef heart mitochondria appears to be a single protein, yet *in vivo* exhibits different kinetic properties on the cytosolic face as compared to the matrix face of the inner membrane of mitochondria. The data support the proposal that CPT is the key regulated step in the control of mitochondrial fatty acid oxidation.

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