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EFFECT OF ETOMOXIRYL-CoA ON DIFFERENT CARNITINE ACYLTRANSFERASES

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Abstract—The effects of etomoxiryl-CoA on purified carnitine acyltransferases and on carnitine acyltransferases of rat heart mitochondria and rat liver microsomes were determined. At nanomolar concentrations, the data agreed with that of other investigators who have shown that etomoxiryl-CoA must be binding to a high affinity site with specific inhibition of mitochondrial carnitine palmitoyltransferase (CPT_o). Micromolar amounts of etomoxiryl-CoA inhibited both short- and long-chain carnitine acyltransferases. The concentrations of etomoxiryl-CoA required for 50% inhibition of the different carnitine acetyltransferases and microsomal and peroxisomal carnitine octanoyltransferase were in the low micromolar range. Mixed-type and uncompetitive inhibition kinetics were obtained, depending on the source of purified enzyme. When purified rat heart CPT was incubated with etomoxiryl-CoA, it increased the $K_{0.5}$ and decreased the Hill coefficient for acyl-CoA. Both proteins and phospholipids of mitochondria and microsomes formed covalent adducts of [^{3}H]etomoxir, with the predominant labeling in phospholipids. None of the purified enzymes formed covalent adducts when incubated with [^{3}H]etomoxiryl-CoA, in contrast to intact mitochondria or microsomes. The major ^{3}H-labeled protein for rat heart mitochondria had a molecular weight of 81,000 ± 4000, and the major proteins from microsomes had a molecular weight of 51,000–57,000. Malonyl-CoA prevented most of the tritium incorporation into the 81,000 Da protein of mitochondria, but it had little effect on incorporation of tritiated etomoxir into the 51,000–57,000 Da proteins of microsomes. When 50 μM etomoxiryl-CoA was added to microsomes and to mitochondria that had been incubated with radioactive etomoxiryl-CoA, much of the radioactive etomoxir disappeared from the major microsomal proteins, but virtually none was displaced from the mitochondrial protein. Thus, at least two different types of covalent etomoxir complexes were formed. This pulse-chase experiment showed that the mitochondrial protein–etomoxir complex was not turned over, consistent with other data showing that etomoxir inhibited carnitine palmitoyltransferase. In contrast, the major protein–etomoxir complex in microsomes was turned over during the pulse-chase experiment.

The carnitine palmitoyltransferase of mammalian mitochondria in contact with the cytosolic compartment (CPT_o)† is a regulated enzyme for the mitochondrial β-oxidation of long-chain fatty acids. Much of the evidence for this conclusion has come from studies of its inhibition by malonyl-CoA [1–3]. A family of epoxy-containing fatty acids (such as etomoxir), whose acyl-CoA derivatives are potent inhibitors of CPT_o in intact mitochondria [4–9], have been used to investigate the mechanism of regulation of CPT_o. It forms a covalent adduct to an approximately 94,000 Da protein in rat liver mitochondria [4, 8] at very low concentrations (<10 nM) of etomoxiryl-CoA. This protein also binds malonyl-CoA.

Lopaschuk and colleagues have studied the effect of etomoxir on the performance of isolated working hearts and have shown that with micromolar concentrations, the amounts of long-chain acylcarnitines are elevated, compared to the levels obtained with 10^{-8} M [10], indicating an inhibition of long-chain acylcarnitine utilization. Their data indicate that at low concentrations *in vivo* etomoxiryl-CoA selectively inhibits CPT_o and, at higher levels, it may also inhibit CPT_i, the form of CPT in contact with the matrix compartment of mitochondria [10, 11]. Thus, their results indicate the etomoxiryl-CoA can inhibit more than one carnitine acyltransferase in a concentration-dependent manner.

As part of ongoing investigations into the properties and functions of carnitine acyltransferases, we have shown recently that the medium-chain/long-chain carnitine acyltransferase activity of rat

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† Abbreviations and definitions: CPT, total carnitine palmitoyltransferase of mitochondria, i.e. CPT_o and CPT_i; CPT_o, mitochondrial CPT in contact with the cytosolic compartment; CPT_i, mitochondrial CPT in contact with the matrix compartment; CAT, carnitine acetyltransferase; the enzymes of mitochondria, microsomes, and peroxisomes that have a kinetic preference for short-chain acyl-CoAs; COT, carnitine octanoyltransferase; the enzymes associated with microsomes and peroxisomes that exhibit both medium-chain and long-chain carnitine acyltransferase activity, but have a kinetic preference for medium-chain acyl-CoAs; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 13-[(3-cholamidopropyl)dimethylammonio]1-propane sulfonate; etomoxiryl-CoA, (B877-38) (*R*)-2-[6-(4-chlorophenoxy)hexyl]-oxirane-2-carboxyl-coenzyme A ester; TDGA-CoA, 2-tetradecylglycidyl-CoA; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; I_{50}, the inhibitor concentration required for 50% inhibition of mitochondrial CPT and microsomal COT; K_i, the inhibitor concentration required for 50% inhibition of the purified enzymes; and $K_{0.5}$, the [*S*] required for attainment of 1/2 V_{max} (equal to the K_m for Michaelis–Menten enzymes).

liver microsomes (COT) can be inhibited completely by both malonyl-CoA and etomoxiryl-CoA [12]. The finding that etomoxiryl-CoA inhibits more than one carnitine acyltransferase raises questions about the specificity of the inhibition of CPT_o by the acyl-CoA derivatives of etomoxir and tetradecylglycidic acid. This study demonstrates that etomoxiryl-CoA inhibits several carnitine acyltransferases. Some of the data have been reported in abstract form [13].

MATERIALS AND METHODS

Materials. Etomoxiryl-CoA (B877-38) and [^{3}H]-etomoxir* with a specific activity of 40 Ci/mmol were a gift from Byk Gulden (D-7750 Konstanz, Federal Republic of Germany). [1-^{14}C]Decanoyl-CoA, with a specific activity of 5053 dpm/nmol, was synthesized as previously described [19]. Pigeon breast muscle carnitine acetyltransferase (acetyl-CoA:carnitine *O*-acetyltransferase, EC 2.3.1.7) was from Boehringer Mannheim Biochemicals.

Isolation of organelles. Rat hearts and livers were obtained from 150–200 g male Sprague–Dawley rats that were stunned lightly in CO_2 and decapitated. Heart mitochondria were isolated by differential centrifugation [14] in 225 mM mannitol, 75 mM sucrose, 1 mM ethyleneglycolbis (aminoethylether) tetra-acetate (EGTA), pH 7.5 (medium A), with minor modifications. No collagenase was used, and the final wash was done with 120 mM KCl, 20 mM sucrose, 10 mM Hepes, 1 mM EGTA, pH 7.5 (medium B). Mitochondria were resuspended in medium B at a protein concentration of 5 mg/mL and either used immediately or subjected to the treatments described in the figure legends. For Fig. 4, bovine heart mitochondrial CPT was isolated as described previously [15] and assayed spectrally at 324 nm in a 2-mL volume containing 50 mM KP_i, pH 7.5, 150 μM dithiopyridine (DTBP), 10 mM L-carnitine, and etomoxiryl-CoA. The concentration of decanoyl-CoA was varied from 0 to 10 μM, and 120 data points were obtained.

Microsomes were isolated from livers which were immediately collected, immersed and coarsely minced in ice-cold 0.25 M sucrose containing 25 μg/mL phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/mL pepstatin A, and 0.05 μg/mL leupeptin (homogenization buffer). Livers were finely minced, rinsed with homogenization buffer, and homogenized at 4° with 4 vol. of buffer using four strokes of a loose-fitting Potter–Elvehjem homogenizer. The liver homogenate was centrifuged at 400 *g* for 10 min, and the supernatant fluids were centrifuged at 10,000 *g* for 15 min in a Beckman JA17 rotor. The supernatant fluids were centrifuged at 100,000 *g* for 60 min in a Beckman Ti70 rotor. The pellet was resuspended in 10 mM KP_i, 1 mM EDTA, pH 7.5, containing 20% glycerol and stored in aliquots at −20°. Detergent solubilization was done by making microsomes 8 mM in 13-[(3-cholamidopropyl) dimethylammonio]1-propane sulfonate (CHAPS), incubating on ice for 1 hr, and storing frozen at −20° for at least 12 hr. They were thawed at room temperature and centrifuged at 100,000 *g* for 30 min at 4° in a Beckman Airfuge.

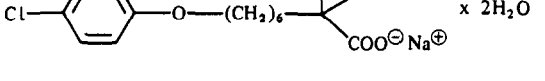
Protein was determined by a modification [16] of the Lowry method.

Carnitine acyltransferase assays. Rat liver microsomal COT, rat liver peroxisomal CAT and rat heart mitochondrial CAT which were purified to homogeneity, and commercial pigeon breast muscle CAT were assayed spectrally at room temperature at 324 nm ($E_{324} = 19{,}600\ M^{-1}\ cm^{-1}$) in 50 mM potassium phosphate, pH 7.5, with 150 μM DTBP. Microsomal COT was assayed using 20 μM decanoyl-CoA, 2 mM L-carnitine, and each assay was corrected for carnitine-independent CoASH release [17]. The ammonium sulfate suspension of pigeon breast muscle CAT was desalted using a Sephadex G-50 column equilibrated in 100 mM KP_i, pH 7.5, 1 mM EDTA. Peroxisomal CAT, heart mitochondrial CAT, and pigeon breast muscle CAT were also assayed in a 2 mL volume containing 1.4 mM L-carnitine; the concentration of acetyl-CoA was varied from 0 to 100 μM, and 120 data points were obtained using a semi-automated kinetic analyzer as described [18]. Every eleventh data point was fitted to a least-squares regression line to calculate kinetic coefficients.

Rat heart mitochondrial CPT activity was assayed using the radiochemical isotope forward method [19]. The assay mixture contained 120 mM KCl, 20 mM sucrose, 10 mM Hepes, 1 mM EGTA, 1 mM dithiothreitol (DTT), pH 7.5, 10 mM L-carnitine, 17 μM [1-^{14}C]decanoyl-CoA in a final volume of 100 μL. When present, malonyl-CoA was 50 μM and etomoxiryl-CoA was as indicated in the figures and tables. The reaction was carried out for 15 or 30 sec at 30°. The reaction was initiated by either adding 5 μg of mitochondrial protein in 5 μL (no preincubation with inhibitor) or by adding 5 μL of a substrate mixture (decanoyl-CoA, L-carnitine) to mitochondria that were preincubated at room temperature with etomoxiryl-CoA (preincubation with inhibitor). The reaction was terminated by adding 0.4 mL methanol and the product was separated from the acyl-CoA substrate on a DE-52 minicolumn (0.5 × 3.0 cm) by washing the column with 1.0 mL of 80% methanol. K_i values were determined by plotting the $1/V$ intercept versus the [I] (see Ref. 28, Chp. 4), and the Hill coefficients (n) were determined by plotting the log $(V/V_{max}-V)$ versus log [S] (see Ref. 28, Chp. 7).

Labeling of heart mitochondria and liver microsomes with [^{3}H]*etomoxir.* Rat heart mitochondria (100 μg) was incubated for 15 min at room temperature with 5 mM ATP, 5 mM $MgCl_2$, 50 μM CoASH, and 1 μM etomoxir (sp. act. 40 Ci/mmol) in a final volume of 100 μL medium B. When 0.5 mM malonyl-CoA was used, it was added 15 min before ATP, Mg^{2+}, etc. The reaction was stopped by dilution with 13 vol. of cold medium B and centrifuged for 15 min in an Eppendorf centrifuge.

*Sodium 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate dihydrate, shown below, was used for these studies. [^{3}H]Etomoxir contained ^{3}H in the chlorophenoxy ring and in the —CH_2-portion of the alkyl chain. The distribution of ^{3}H is not known.



The washing procedure was repeated twice and the mitochondrial pellet was suspended in 100 μL of medium B. For the pulse-chase experiments, after 15 min, etomoxiryl-CoA (unlabeled) was added to a final concentration of 50 μM and the mixture was incubated for 15 min at room temperature.

Rat liver microsomes (540 μg) were incubated for 15 min at room temperature with 5 mM ATP, 5 mM $MgCl_2$, 50 μM CoASH (50 mM KP_i, 50 mM KCl, pH 7.5), and 5 μM [^{3}H]etomoxir (sp. act. 40 Ci/mmol) in a final volume of 100 μL. After 15 min, the reaction mixture was diluted with 1 vol. of cold 50 mM KP_i, 50 mM KCl, pH 7.5, and centrifuged at 100,000 *g* for 30 min in a Beckman Airfuge. The microsomal membrane pellet was rinsed three times with 200 μL of 50 mM KP_i, 50 mM KCl, pH 7.5, and the pellet was resuspended in 100 μL of electrophoresis sample buffer. For the pulse-chase experiments, after the 15-min incubation, etomoxiryl-CoA (unlabeled) was added to a final concentration of 50 μM and the reaction mixture was incubated for an additional 5 min at room temperature.

Mitochondrial (50 μL, 1 mg/mL) and microsomal (50 μL, 5.4 mg/mL) samples were extracted with 5.0 mL of cold hexane:isopropanol (3:2, v/v) by vortexing for 1.0 min, followed by centrifugation for 15 min at 3000 *g*. The pellet was extracted with 200 μL of water plus 5 mL of cold chloroform:methanol (3:2, v/v) and centrifuged, and the protein pellet was then dissolved in 300 μL of 7% sodium dodecyl sulfate (SDS), and aliquots were subjected to SDS–PAGE according to Laemmli [20]. After electrophoresis, the gel was sliced into 2-mm slices, the gel slices were treated with 0.5 mL of distilled water by shaking overnight, and the radioactivity was determined.

Labeling of purified carnitine acyltransferases with [^{3}H]*etomoxiryl-CoA.* [^{3}H]Etomoxiryl-CoA was synthesized enzymatically using rat liver microsomal long-chain acyl-CoA synthetase purified free of acyl-CoA hydrolase activity using Blue Sepharose chromatography as described [21]. The synthesis was done at 35° for 1 hr in 100 μL containing 1 mM [^{3}H]-etomoxir (sp. act. 4.4×10^6 dpm/nmol), 10 mM ATP, 10 mM $MgCl_2$, 5 mM CoASH, 10 mM DTT, 150 mM KCl, 100 mM Tris-Cl, pH 8.0, 0.1% (w/v) Triton X-100 and 60 μg of long-chain acyl-CoA synthetase. [^{3}H]Etomoxiryl-CoA was purified using HPLC with a Water's μBondapak C18 reverse phase column (3.9 mm × 30 cm) with an isocratic buffer system of methanol:water:acetic acid (75:25:0.5) at a flow rate of 1 mL/min. Authentic etomoxiryl-CoA was detected by absorbance at 256 nm, and the fractions containing radioactivity with the retention time of authentic etomoxiryl-CoA were collected and dried under vacuum. The control experiment showed identical labeling of rat liver microsomes with 1 μM [^{3}H]etomoxiryl-CoA, as was seen with 5 μM [^{3}H]etomoxir using the acyl-CoA generating conditions given previously.

Twenty micrograms of purified rat heart mitochondrial CPT [15], rat heart mitochondrial CAT, rat liver peroxisomal CAT, rat liver peroxisomal COT, and rat liver mitochondrial CAT [18, 21, 22] were incubated with 1 μM [^{3}H]etomoxiryl-CoA for 2 min at room temperature in a 35 μL volume. The proteins were subjected to SDS–PAGE according to Laemmli [20]. After electrophoresis, the gel was sliced into 2-mm slices, the gel slices were treated with 0.5 mL of distilled water by shaking overnight, and the radioactivity was determined. Assuming 1 mol of etomoxir interacts with 1 mol of enzyme, sufficient radioactivity was added to detect 0.5% of labeling of a 60,000 mol. wt protein.

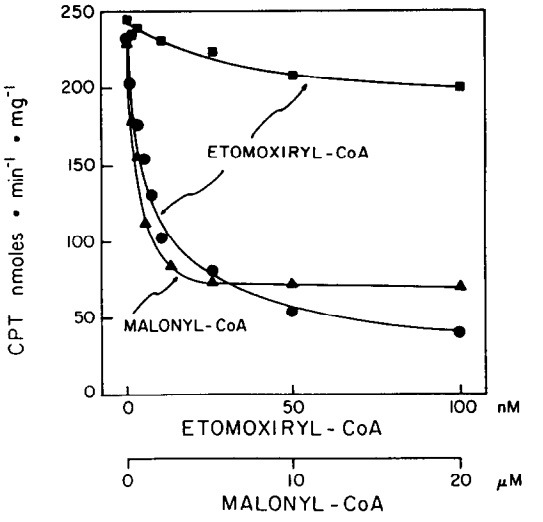


Fig. 1. Effect of etomoxiryl-CoA on the time-dependent and time-independent inhibition of rat heart mitochondrial CPT_o. Key: (■) + etomoxiryl-CoA, no preincubation; (●) 10-min preincubation with etomoxiryl-CoA; and (▲) malonyl-CoA as inhibitor, no preincubation.

RESULTS

In this study, the effects of etomoxiryl-CoA on the kinetic parameters of carnitine acyltransferases of rat liver peroxisomes, rat liver microsomes, and rat heart mitochondria are reported. Rat heart, rather than rat liver, mitochondria were used, as discussed elsewhere [12, 23–25], because it is difficult to isolate rat liver mitochondria that are free of microsomes and peroxisomes using conventional differential centrifugation techniques. Since both of the latter organelles contain carnitine acyltransferases [21], their presence in liver mitochondria could complicate interpretation of data.

Effect of etomoxiryl-CoA on rat heart mitochondria. Inhibition of CPT_o by malonyl-CoA occurred rapidly without preincubation with 50% inhibition by 0.7 μM (see Fig. 1). The studies of others have shown that maximum inhibition of CPT_o of liver mitochondria by nanomolar amounts of 2-tetradecylglycidyl-CoA (TDGA-CoA) is time dependent [4]. With intact rat heart mitochondria, etomoxiryl-CoA inhibited very little CPT_o, even at 100 nM (top curve), but after preincubation for 10 min, CPT_o was inhibited 50% by approximately 9 nM etomoxiryl-CoA. A time course showed that maximum inhibition with 1 μM etomoxiryl-CoA was obtained in less than 3 min (data not shown).

Effect of etomoxiryl-CoA on microsomal COT.

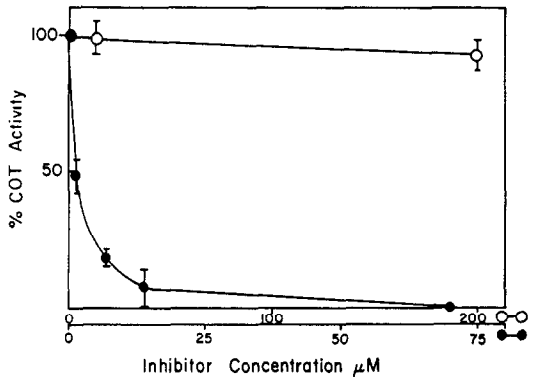


Fig. 2. Effects of etomoxiryl-CoA and malonyl-CoA on CHAPS-solubilized microsomal COT. CHAPS-solubilized microsomes were prepared and COT was assayed spectrally. COT was determined in the presence of the indicated concentrations of etomoxiryl-CoA with a 2-min preincubation (●) or malonyl-CoA (○). COT activity was corrected for carnitine-independent CoASH release at each inhibitor concentration. The concentration of etomoxiryl-CoA required for 50% inhibition of COT activity was 1.5 μM. The initial (100%) COT activity was 4.7 ± 0.6 mUnits/mg. The data are averages ± range (N = 2).

Previous studies demonstrated that both etomoxiryl-CoA and malonyl-CoA inhibit the COT activity of intact rat liver microsomes [12]. Since the inhibition by malonyl-CoA is lost when microsomes are solubilized by the detergent CHAPS, experiments were also done to determine if detergent solubilization effects the inhibition by etomoxiryl-CoA. These experiments are shown in Fig. 2. Complete inhibition of COT activity was obtained by etomoxiryl-CoA, with 50% inhibition at 1.5 μM; this is similar to the I_{50} of 0.6 μM previously reported for intact microsomes [12]. Note that 200 μM malonyl-CoA did not inhibit COT of solubilized microsomes.

Effect of octylglucoside on inhibition by etomoxiryl-CoA. Experiments were also done to determine if the inhibition of microsomal COT by etomoxiryl-CoA is reversible. Since washing experiments are difficult with rat liver microsomes due to inactivation of COT activity by multiple washings, CHAPS-solubilized etomoxiryl-CoA-inhibited microsomes (i.e. the higher concentrations shown in Fig. 2, the closed circles) were passed over a Biogel P6 sieving (desalting) column. Within experimental error, all of the COT activity was restored (two experiments gave an average recovery = 98%). This loss of inhibition of solubilized microsomal COT by etomoxiryl-CoA is very different from the behavior reported for CPT_o of liver mitochondria [8, 9], where detergent solubilization inhibits CPT_o activity [8, 9]. Since previous experiments with heart mitochondria [15] showed that 1% octylglucoside did not destroy malonyl-CoA sensitive CPT_o activity, experiments were done to determine the effect of octylglucoside solubilization on the inhibition of CPT_o by etomoxiryl-CoA. The results are summarized in Table 1. Incubation of heart mitochondria with 1 μM

Table 1. Effect of washing and solubilization with octylglucoside on retention of etomoxiryl-CoA inhibition of mitochondrial CPT

	CPT (nmol/min/mg protein)	
Sample	Without Et-CoA	1 μM Et-CoA
Intact	158	53 (67)
+ malonyl-CoA	68 (57)	
Octylglucoside	382	391

Rat heart mitochondria (1 mg/mL in medium B) were incubated at room temperature with and without 1 μM etomoxiryl-CoA for 10 min, followed by dilution with 10 vol. of medium B and centrifugation for 10 min at 8,000 *g*. This washing was repeated twice and the mitochondria were resuspended in the original volume of medium B. CPT was assayed immediately after resuspension of the mitochondria (intact) and following solubilization of intact mitochondria with 1% octylglucoside. The data are an average of three experiments. The numbers in parentheses represent percent inhibition.

etomoxiryl-CoA decreased carnitine palmitoyltransferase activity by an average of 67%. Treatment with 1% octylglucoside plus 120 mM KCl restored total CPT (CPT_o and CPT_i) activity to an average of 102% of control levels (N = 3); compare 382 to 391 nmol/min/mg protein for controls and 1 μM etomoxiryl-CoA, respectively.

Labeling with [^{3}H]etomoxiryl-CoA. Since inhibition of CPT_o by radioactive TDGA-CoA produces a covalent adduct to an approximately 90,000 Da mitochondrial protein [5, 8], experiments were done in which [^{3}H]etomoxir was incubated with rat heart mitochondria and rat liver microsomes in the presence of an acyl-CoA generating system to determine if similar proteins are labeled by [^{3}H]-etomoxiryl-CoA. Both mitochondria and microsomes showed a large incorporation of radioactivity into detergent-solubilized protein preparations separated by column chromatographic and HPLC techniques. For example, when rat liver microsomes were incubated with [^{3}H]etomoxir in the presence of an acyl-CoA generating system, and then solubilized in detergent, and separated on a G-250 HPLC sieving column, at least five radioactive peaks were obtained, one of which contained free etomoxir and etomoxiryl-CoA; similar, but not identical, results were obtained with rat heart mitochondria (data not shown). When the samples were subjected to SDS–PAGE, < 2% of the total radioactivity was protein-bound, suggesting the presence of [^{3}H]-labeled lipids. Treatment of mitochondria and microsomes by lipid extraction techniques, such as with hexane-isopropanol, showed that > 95% of the radioactivity partitions into the organic solvent. Thin-layer chromatography using solvents that separate phospholipids showed that most of the radioactivity was associated with phospholipids. This was confirmed by passing the chloroform-soluble (Bligh–Dyer) extracts over silicic acid to absorb the phospholipids, washing with chloroform, and then eluting with chloroform:methanol (1:4) to remove

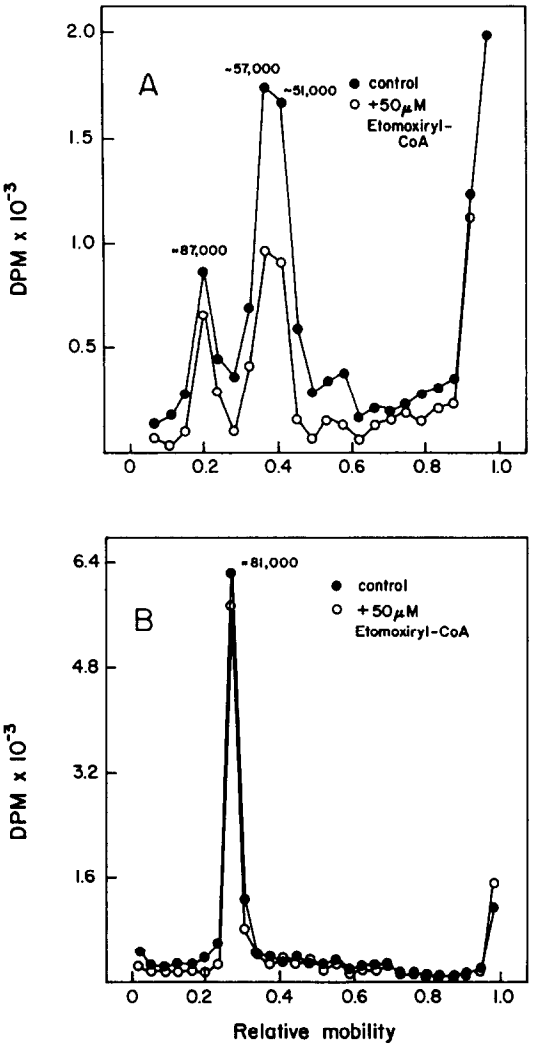


Fig. 3. SDS–PAGE of mitochondrial and microsomal proteins after incubation with [^{3}H]etomoxir. Rat liver microsomes (A) and rat heart mitochondria (B) were incubated for 15 min with [^{3}H]etomoxir, ATP-Mg^{2+}, and CoASH as described in Materials and Methods, and the proteins were separated by SDS–PAGE. The gel was sliced into 0.2-cm pieces, and the radioactivity (●) plotted versus the relative mobility. Molecular weight markers were run on the same gel and silver stained. The open circles (○) show the effect of a 5-min chase incubation with 50 μM unlabeled etomoxiryl-CoA. The molecular weight range of the labeled proteins from four separate gels was 87,500 ± 986, 57,000 ± 577, and 51,600 ± 1250 for panel A and 81,000 ± 4000 for panel B.

most of the phospholipids. The chloroform:methanol 1:4 eluate contained most (> 90%) of the ^{3}H. Thin-layer chromatography demonstrated that most of this radioactivity was associated with phosphatidylethanolamine.

Both mitochondria and microsomes incubated with [^{3}H]etomoxir were subjected to SDS–PAGE. As shown in Fig. 3B, a protein of approximately 81,000 ± 4000 Da was labeled in rat heart mitochondria. Preincubation of mitochondria with malonyl-CoA (0.5 mM) reduced the labeling in this protein about 80% (data not shown). In contrast (see Fig. 3A), the major proteins labeled in liver microsomes had a molecular weight of approximately 51,000–57,000. The labelling was affected only slightly by the addition of large amounts of malonyl-CoA.

The fact that malonyl-CoA reduced the amount of the major labeled protein formed in mitochondria but not in microsomes suggests that the two proteins have different functions. If the ^{3}H-labeled proteins are covalent enzyme substrate intermediates, then addition of unlabeled etomoxiryl-CoA should promote loss of ^{3}H from the protein due to catalytic turnover. If it is a covalent inhibitory complex that is not turned over, then addition of large amounts of etomoxiryl-CoA after preincubation with [^{3}H]-etomoxir should have little effect on the amount of ^{3}H bound to protein. Pulse-chase types of experiments were done with both rat liver microsomes and rat heart mitochondria to determine the effect of a chase with unlabeled etomoxiryl-CoA on the amount of [^{3}H]etomoxir attached to protein. When microsomes were preincubated for 15 min with an acyl-CoA generating system plus radioactive etomoxir, and then 50 μM etomoxiryl-CoA added, the radioactivity in the 51,000–57,000 Da protein fraction was reduced about 60%. In contrast, when rat heart mitochondria were subjected to such treatment, the radioactivity in the ~81,000 Da peak was not reduced, indicating that the covalent adduct was not turned over (see the ○–○ of Fig. 3B).

Each of the purified enzymes used for Table 2 were incubated with [^{3}H]etomoxiryl-CoA as described in Materials and Methods, and then subjected to SDS–PAGE. None of the enzymes contained protein-associated ^{3}H after electrophoresis (data not shown).

Effect of etomoxiryl-CoA on kinetic parameters of purified carnitine acyltransferases. The preceding data show that etomoxiryl-CoA inhibits more than one carnitine acyltransferase, but the concentration of etomoxiryl-CoA required for 50% inhibition varies. Therefore, experiments were performed to determine the effect of etomoxiryl-CoA on the kinetic parameters of purified carnitine acyltransferases. The effect of etomoxiryl-CoA on the velocity versus substrate concentration curve of CPT purified from rat heart mitochondria is shown in Fig. 4A. The purified enzyme did not contain the high affinity etomoxiryl-CoA binding site, but micromolar concentrations of etomoxiryl-CoA shifted the velocity versus substrate concentration curve to the right, similar to the effects of TDGA-CoA on the CPT purified from beef heart mitochondria [26]. Analyses of the curves shown in Fig. 4A show that 0.25 μM etomoxiryl-CoA increased the $K_{0.5}$ for decanoyl-CoA from 1.1 to 3.4 μM and decreased the Hill coefficient for decanoyl-CoA from 2.0 to 1.4. Increasing the etomoxiryl-CoA 10-fold to 2.5 μM increased the $K_{0.5}$ for decanoyl-CoA to > 50 μM and reduced the Hill coefficient to about 1.0.

The effect of etomoxiryl-CoA on commercial CAT purified from pigeon breast muscle was determined. Secondary plots of these data (see Fig. 5) show that etomoxiryl-CoA inhibited CAT in an uncompetitive

Table 2. Summary of the effects of etomoxiryl-CoA inhibition of carnitine acyltransferases

Enzyme source	Comments
CPT_o of intact rat heart mitochondria	I_{50} = 9 nm, time dependent, mol. wt of major protein forming covalent adduct with [^{3}H]-etomoxir = 81,000 ± 4000
CPT purified from beef heart mitochondria	Kinetic constants:* $K_{0.5}$ (μM) / Hill n No etomoxiryl-CoA 2.3 2.2 0.25 μM etomoxiryl-CoA 3.8 1.4 2.5 μM etomoxiryl-CoA >50 No covalent adduct formed with [^{3}H]etomoxiryl-CoA
COT of rat liver microsomes	I_{50} = 0.7 μM, time dependent (intact) I_{50} = 1.5 μM, time dependent, reversible (CHAPS solubilized); mol. wt of major protein forming covalent adduct with [^{3}H]etomoxir = 51,000–57,000; no covalent adduct formed with [^{3}H]-etomoxiryl-CoA
COT purified from liver peroxisomes	K_i = 1 μM, mixed-type inhibition with octanoyl-CoA, time dependent; no covalent adduct formed with [^{3}H]etomoxiryl-CoA
CAT purified from rat liver peroxisomes	K_i = 2 μM, mixed-type inhibition with acetyl-CoA
CAT purified from rat heart mitochondria	K_i = 9 μM, mixed-type inhibition with acetyl-CoA; no covalent adduct formed with [^{3}H]-etomoxiryl-CoA
CAT partially purified from rat liver mitochondria	K_i = 2 μM, mixed-type inhibition with acetyl-CoA; no covalent adduct formed with [^{3}H]-etomoxiryl-CoA
CAT pigeon breast muscle (commercial enzyme)	K_i = 3 μM, uncompetitive inhibition with acetyl-CoA

* Calculated from data given in Fig. 4.

manner, with a K_i equal to 3 μM. The effects of etomoxiryl-CoA on COT and CAT purified from rat liver peroxisomes [22] and CAT purified from rat heart mitochondria were also determined. The data are summarized in Table 2. Etomoxiryl-CoA inhibited peroxisomal CAT with a K_i = 2 μM (mixed-type inhibition), and it inhibited peroxisomal COT with a K_i = 1 μM. Mixed-type inhibition with a K_i = 2 μM was found for CAT purified from rat liver mitochondria and mixed inhibition with a K_i = 9 μM was found for CAT purified from rat heart mitochondria. Surprisingly, the extent of inhibition of peroxisomal COT was time dependent. Little inhibition (20%) was seen when the enzyme was assayed immediately; however, preincubation for 2 min gave > 80% inhibition with 10 μM etomoxiryl-CoA. This phenomenon was not investigated.

DISCUSSION

Etomoxir effects on carnitine acyltransferases. The data presented herein demonstrate that *in vitro* etomoxiryl-CoA inhibited the carnitine acyltransferases of rat heart and liver. At nanomolar concentrations, etomoxiryl-CoA was a specific inhibitor of mitochondrial CPT_o. This agrees with the studies of others showing that nanomolar amounts of etomoxiryl-CoA and TDGA-CoA inhibit CPT_o of intact mitochondria. The I_{50} of 8 nM for etomoxiryl-CoA is similar to reports of others showing an I_{50} of 25 μM for rat skeletal muscle mitochondria [9] and 3 and 9 nM for rat liver mitochondria [7, 9]. At higher concentrations novel data are presented which show that etomoxiryl-CoA inhibited other carnitine acyltransferases, including CPT_i. Although medium-chain acyl-CoA derivatives are very poor substrates for CAT and long-chain acyl-CoAs are not substrates, the fact that CAT was inhibited by etomoxiryl-CoA at low, micromolar concentrations indicates that CAT binds etomoxiryl-CoA. The inhibition of CAT by etomoxiryl-CoA may be similar to the inhibition of CAT by palmitoyl-CoA [27]. The finding that etomoxiryl-CoA inhibits all of the carnitine acyltransferases investigated supports the conclusion of Lopaschuk *et al.* [10, 11] that etomoxiryl-CoA inhibits enzymes such as CPT_i of intact heart mitochondria. One unexpected finding was that [^{3}H]etomoxir enters lipid metabolic pathways; the conversion of [^{3}H]etomoxir to radioactive lipids was quantitatively much greater than the formation of covalent adducts to proteins.

The effect of etomoxiryl-CoA on CPT purified from beef heart mitochondria was almost identical to that reported previously for the effect of TDGA-CoA on this enzyme [19], except that the concentration of etomoxiryl-CoA required for

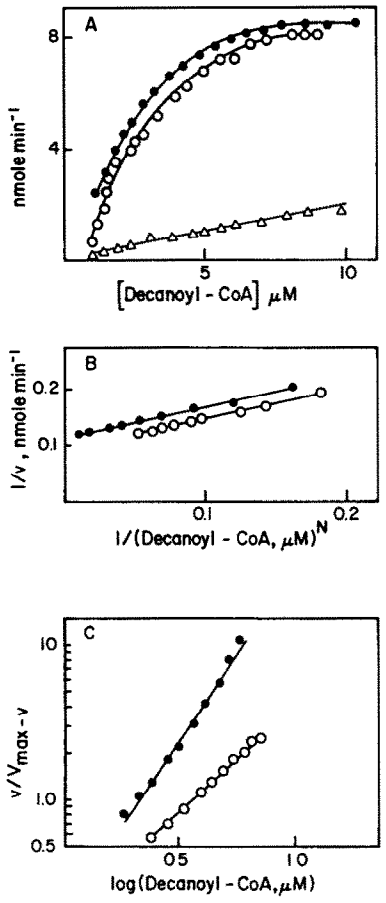


Fig. 4. Inhibition of purified bovine heart mitochondrial CPT by etomoxiryl-CoA. CPT purified from bovine heart mitochondria was assayed spectrally. Key: (●) no etomoxiryl-CoA; (○) 0.25 μM etomoxiryl-CoA; and (△) 2.5 μM etomoxiryl-CoA, for panels A, B, and C. Kinetic constants determined from panel B are given in Table 2. For panel C, lines were fitted by least-squares regression with correlation coefficients (r): (●) $r = 0.995$ and (○) $r = 0.998$. The Hill coefficients (n) from panel C are: (●) $n = 2.2$ and (○) $n = 1.4$.

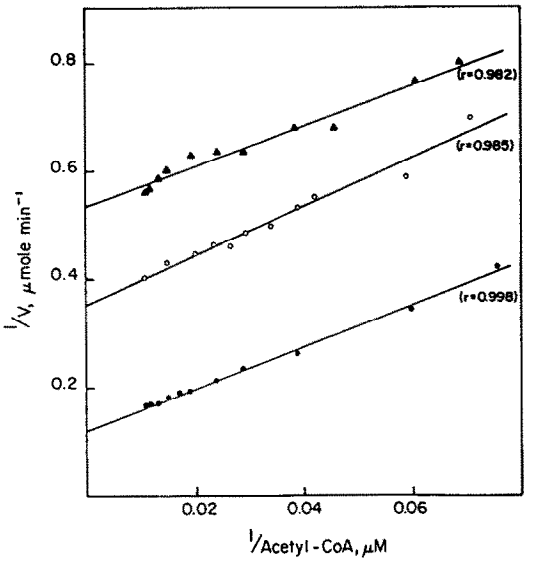


Fig. 5. Effect of etomoxiryl-CoA on purified CAT. Pigeon breast muscle CAT was assayed spectrally in the presence of increasing acetyl-CoA concentrations and plotted as $1/V$ vs 1/acetyl-CoA. Etomoxiryl-CoA was added to the assays as indicated: Key: (●) no etomoxiryl-CoA; (○) 5 μM etomoxiryl-CoA; and 10 μM etomoxiryl-CoA. Lines were fitted by least-squares regression and the correlation coefficient (r) is shown in parentheses. The y-axis intercept ($1/V_{max}$) calculated from the equation for the line and the etomoxiryl-CoA concentration ([I]) were used to replot $1/V_{max}$ versus [I] (plot not shown) which gave a K_i value of 3.3 μM.

decreasing the cooperative behavior of the acyl-CoA substrate was lower. Etomoxiryl-CoA (0.25 μM) shifted the velocity versus decanoyl-CoA curve to the right and apparently decreased the cooperative binding of decanoyl-CoA (Fig. 4); this is indicated by the decrease in the Hill coefficient from 2.2 to 1.4. Thus, etomoxiryl-CoA appears to be binding to the acyl-CoA site that is responsible for the acyl-CoA cooperativity. Substrate cooperativity usually occurs with oligomeric proteins [28]. CPT purified from rat [29] liver is oligomeric, and CPT purified from beef heart mitochondria has a molecular weight of > 600,000 Da [18].

Microsomal and mitochondrial differences. Although the inhibition of both microsomal COT and mitochondrial CPT_o by etomoxiryl-CoA was time dependent and both formed a high molecular weight covalent adduct, the concentration of etomoxiryl-CoA required for 50% inhibition of microsomal COT was ~100-fold higher than that required for 50% inhibition of CPT_o of intact mitochondria. If the mode of inhibition is the same, the concentration of etomoxiryl-CoA required for 50% inhibition is very different. However, the mechanism for etomoxiryl-CoA inhibition of microsomal COT seems different from the mechanism of malonyl-CoA inhibition of CPT_o because CHAPS-solubilized microsomes were inhibited by etomoxiryl-CoA, but not by 200 μM malonyl-CoA (see upper curve of Fig. 2). One possibility is that etomoxiryl-CoA inhibits microsomal COT by more than one mode of action. The experiments in which molecular sieving restored the COT activity of microsomes that were pretreated with etomoxiryl-CoA and then solubilized in CHAPS show that the etomoxiryl-CoA inhibition of liver microsomal COT can be overcome. It is not clear whether this loss of inhibition involves association/dissociation of subunits, i.e. separation of a regulator protein from a catalytic protein, or simple removal of the inhibitor.

Studies with [^{3}H]*etomoxir.* Incorporation of [^{3}H]-etomoxir into two proteins of liver has been reported [30]. Our data agree with this finding. The amount of radioactivity per milligram protein associated with the ~87,000 Da protein of microsomes was much lower than with heart mitochondria; however, the specific activity of heart CPT_o, using decanoyl-CoA as substrate, was about 20-fold greater than the specific activity of liver microsomal COT; thus, the

small amount of 87,000 Da protein might be sufficient to be involved in the inhibition of microsomal COT by malonyl-CoA. The fact that malonyl-CoA reduced the amount of the major labeled protein formed in mitochondria and also in the ~87,000 Da protein of microsomes suggests, but does not prove, that these proteins are involved in the inhibition of carnitine acyltransferase activity by malonyl-CoA. We do not have any evidence that shows that the etomoxiryl-labeled protein (51,000–57,000 Da protein of microsomes) is involved in inhibition of carnitine acyltransferase activity of microsomes. The failure of malonyl-CoA to significantly reduce the labeling of the 51,000–57,000 protein fraction and the dilution of radioactivity by adding a large pool of nonradioactive etomoxiryl-CoA shows that malonyl-CoA is not competing with etomoxiryl-CoA and indicates that it is a covalent protein etomoxir intermediate in lipid biosynthesis.

Relationship of covalent etomoxir adduct to the control of CPT_o. Although the data presented herein agree with the data of others concerning the inhibition of CPT_o by etomoxir, our interpretation differs. It is assumed by many investigators that etomoxiryl-CoA and similar 2-oxirane-CoA compounds inhibit CPT_o by forming a covalent adduct with the "catalytic" species of CPT_o [4, 5, 8, 9]; however, alternative models for 2-oxirane and malonyl-CoA inhibition of CPT_o are compatible with the enzymatic data [23, 31]. The fact that the malonyl-CoA binding protein apparently does not catalyze conversion of acyl-CoAs to acylcarnitines (to date, no *direct* evidence has been published that shows that this protein catalyzes acylcarnitine formation) and the recent reports which show that addition of the malonyl-CoA binding protein containing extracts to malonyl-CoA-insensitive CPT [32–35] can confer malonyl-CoA sensitivity to CPT strongly indicate that the malonyl-CoA binding protein which also forms covalent adducts with etomoxir is a regulatory, rather than a detergent-inactivated, catalytic protein. The restoration of total CPT activity to etomoxiryl-CoA inhibited mitochondria by treatment with octylglucoside plus KCl is consistent with this point of view. If the etomoxirylated protein is a regulatory, rather than a catalytic protein, then KCl plus octylglucoside probably promotes dissociation of the etomoxir-binding protein from CPT_o. It was shown previously that salt plus octylglucoside elutes the 87,000 Da protein, but not CPT, from an anti-CPT affinity column [15], demonstrating that the malonyl-CoA binding protein can be removed from CPT by treatment with salt plus octylglucoside. Our data support some form of model 1 presented in Ref. 31, in which the regulation of CPT by malonyl-CoA involves a catalytic subunit (CPT) and a regulatory subunit, the malonyl-CoA binding protein.

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