Action *in vivo* and *in vitro* of 2-tetradecylglycidic acid, 2-tetradecylglycidyl-CoA and 2-tetradecylglycidylcarnitine on hepatic carnitine palmitoyltransferase

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The effects of 2-tetradecylglycidic acid (TDGA), TDGA-CoA and TDGA-carnitine were examined in purified hepatic CPT (carnitine palmitoyltransferase) and in hepatic mitochondria and inverted submitochondrial vesicles derived from Sprague–Dawley rats. Since TDGA has been reported as a specific inhibitor of carnitine palmitoyltransferase-A (CPT-A), the focus was on kinetics and inhibition of CPT-A, and the relationship of this key enzyme to β -oxidation. After administration of TDGA *in vivo* to overnight-starved rats, the $V_{\max}$ of CPT in intact mitochondria and in inverted vesicles (CPT-B) was depressed by 66%. The $s_{0.5}$ for palmitoyl-CoA and K_m for carnitine were unchanged. The I_{50} (concn. giving 50% inhibition) for malonyl-CoA was significantly increased from 20 to 141 μM in intact mitochondria, but unchanged (199 versus 268 μM) in inverted vesicles. The addition *in vitro* of TDGA-CoA (0–1.0 μM) gave I_{50} values of 0.29 and 0.27 μM (S.E.M. = 0.19) in intact mitochondria from fed and 48 h-starved rats, and 0.81 and 1.57 μM (S.E.M. = 0.29) for inverted vesicles derived from fed and starved rats. Addition *in vitro* of TDGA-carnitine to mitochondria from starved rats yielded an I_{50} value of 27.7 mM (S.E.M. = 12.2) for L-[methyl- ^{14}C]carnitine release from palmitoyl-L-[methyl- ^{14}C]carnitine and 0.64 mM (S.E.M. = 0.07) for palmitoyl-L-[methyl- ^{14}C]carnitine formation from L-[methyl- ^{14}C]carnitine in intact mitochondria. Inverted vesicles were not measurably sensitive to TDGA-carnitine up to 500 μM for the assay of L-[methyl- ^{14}C]carnitine release, but were as sensitive as intact mitochondria when inhibition was determined in the direction of palmitoyl-L-[methyl- ^{14}C]carnitine formation ($I_{50} = 0.54 \pm 0.07 \mu\text{M}$). When TDGA-CoA was added to intact mitochondria, then incubated for 5 min at room temperature and subsequently washed out, $V_{\max}$ of CPT decreased from 5.8 to 3.5 (S.E.M. = 0.6) in intact mitochondria, and from 17.2 to 6.3 (S.E.M. = 4.8) in inverted vesicles. The K_m for L-carnitine and the $s_{0.5}$ for palmitoyl-CoA increased 2-fold with TDGA-CoA pretreatment in both intact mitochondria and inverted vesicles. Detergent solubilization (0.05% Triton X-100) resulted in a complete loss of TDGA-CoA sensitivity (up to 1.0 μM measured). Sonicated mitochondria exhibited an I_{50} of $0.72 \pm 0.03 \mu\text{M}$. When sonicated mitochondria were partitioned into the sonicated supernatant and pellet after centrifugation at 100 000 g for 1 h, the supernatant had an I_{50} of $8.8 \pm 3.2 \mu\text{M}$ and the pellet had an I_{50} of $0.84 \pm 0.05 \mu\text{M}$. Purified rat liver CPT had an I_{50} of 60–80 μM , which declined to 9.5 μM in the presence of 20% dimethyl sulphoxide and to 15 μM in phosphatidylcholine or cardiolipin liposomes. The data suggest that the membrane environment may be important to TDGA sensitivity.

INTRODUCTION

2-Tetradecylglycidic acid (methylpalmoxirate; TDGA) has been used *in vivo* as a hypoglycaemic and hypoketonaemic agent (Tutwiler *et al.*, 1985). The ketone-decreasing effect has been attributed to the inhibition of hepatic mitochondrial carnitine palmitoyltransferase-A (CPT-A, 'outer enzyme'), the apparent rate-limiting enzyme for long-chain fatty acid oxidation (McGarry & Foster, 1980; Tutwiler & Ryzlak, 1980; Tutwiler *et al.*, 1981, 1985). More detailed experiments *in vitro* have characterized TDGA-CoA as the active form, binding tightly to the active site for CPT-A with a K_i of 270 nM (Kiorpes *et al.*, 1984). Malonyl-CoA, palmitoyl-CoA and free CoA protected CPT-A from TDGA-CoA inhibition, but palmitoylcarnitine and L-carnitine were without effect. Although it was originally thought that

TDGA-CoA was an irreversible inhibitor, Bieber & Fiol (1986) have shown that the inhibition is reversible with high concentrations of palmitoyl-CoA.

A question which arose during these studies was whether CPT-A alone, or both CPT-A and -B (inner enzyme), were inhibited (Tutwiler & Ryzlak, 1980; Tutwiler *et al.*, 1981), and whether the accessibility of the CoA derivative to the inner enzyme actually limited its effect on CPT-B. Kiorpes *et al.* (1984) have stated that TDGA-carnitine is not formed and have shown that synthetic TDGA-carnitine does not inhibit the enzyme. They postulated that TDGA-carnitine should have permeated the mitochondrial inner membrane and have had access to CPT-B.

Malonyl-CoA has been proposed as a physiologically important inhibitor of CPT-A. Since TDGA-CoA binding to CPT-A is restricted by malonyl-CoA, it can

Abbreviations used: TDGA, 2-tetradecylglycidic acid; CPT, carnitine palmitoyltransferase (EC 2.3.1.21); CPT-A and CPT-B, CPT activity of the outer and the inner surface respectively of the mitochondrial inner membrane; Me₂SO, dimethyl sulphoxide; DPH, 1,6-diphenylhexa-1,3,5-triene; I_{50} , the concentration giving 50% inhibition.

be postulated that both bind to the same site and that this is the enzyme's active site. More recent studies suggest that malonyl-CoA may bind to a protein distinct from CPT-A (Mills *et al.*, 1984) and that TDGA-CoA may also bind to the same site (Declercq *et al.*, 1985). However, sensitivity of purified ox heart CPT to TDGA-CoA, where malonyl-CoA sensitivity is undetectable, has been reported (Fiol & Bieber, 1984; Bieber & Fiol, 1986). We have been using inverted submitochondrial vesicles as a means to probe kinetics and malonyl-CoA inhibition of CPT-B *in situ* (Brady *et al.*, 1985, 1986a). Our purposes in the present studies were (1) to examine effects *in vivo* of TDGA on hepatic CPT-A and CPT-B, β -oxidation and hepatic mitochondrial membrane properties by using intact mitochondria and inverted sub-mitochondrial vesicles, (2) to determine TDGA-CoA inhibition *in vitro* of CPT-A, -B and purified CPT and associated effects, and (3) to examine the effects of TDGA-carnitine on intact and inverted mitochondria.

EXPERIMENTAL

Adult Sprague-Dawley rats of both sexes weighing 250–500 g were fed on stock rodent diet (Purina Rat Chow) and water *ad libitum*. For studies *in vitro*, rats were fed or starved for 48 h. For studies *in vivo*, rats (350–500 g) were starved overnight before administration of 2 mg of TDGA/kg body wt. orally in ethanol at 05:00 h. The rats were killed 3 h later for preparation of mitochondria. Control animals received only the ethanol carrier.

Mitochondria were prepared by differential centrifugation by the technique of Hoppel *et al.* (1979). Inverted vesicles were prepared by nitrogen compression/decompression (Fleischer *et al.*, 1974). After final pelleting and resuspension, to remove the right-side-out vesicles, vesicles were suspended, batch-wise, with cytochrome *c*-Sephacrose-4B, prepared as described by Godinot & Gautheron (1979). In some experiments, intact mitochondria were incubated with 10 μ M-TDGA for 5 min at 25 °C in the presence of either 100 μ M-malonyl-CoA or 10 μ M-palmitoyl-CoA (to provide an alternative substrate for the acyl-CoA hydrolase activity, as described by Kiorpes *et al.*, 1984). The mitochondria were then washed with 5 vol. of the isolation buffer containing 0.05% bovine serum albumin, followed by buffer without albumin. Intact mitochondria and inverted vesicles were handled in the standard manner from this point. No TDGA-CoA inhibition was observed if malonyl-CoA or palmitoyl-CoA was omitted. We had previously determined that 100 μ M-malonyl-CoA and 10 μ M-palmitoyl-CoA gave comparable results: that is, they produced no alteration in CPT kinetics when incubated alone and subsequently washed out. In other experiments the mitochondria were sonicated in 20 mM- K_2HPO_4/KH_2PO_4 /1 mM-EDTA, pH 7.4, for 3×15 s separated by intervals of 20 s. The mitochondria were then centrifuged at 100 000 g_{av} for 1 h. The supernatant and pellet were separated, and protein was determined before the CPT assay. This procedure has been stated to yield a supernatant enriched in CPT-A and a pellet enriched in CPT-B (Hoppel, 1982).

CPT activity was measured in the direction of palmitoylcarnitine formation with L-[methyl- ^{14}C]carnitine as substrate [Hoppel & Tomec (1972), as modified by

Brady *et al.* (1985, 1986a)]. The CPT assay, pH 7.0, contained, in a final volume of 0.25 ml: 80 mM-KCl, 50 mM-Mops, 1 mM-EGTA, 1 mg of dialysed defatted bovine serum albumin/ml, 4 mg of sodium tetrathionate/ml, 10 μ M- or 40 μ M-palmitoyl-CoA, 0.01–2.0 mM total L-carnitine (unlabelled plus ^{14}C -labelled carnitine). After a 5 min preincubation, the reaction was initiated by the addition of 50 μ g of mitochondrial protein, and the reaction was allowed to proceed for 2 min at 30 °C for the kinetic determinations. The specific radioactivity of the L-[methyl- ^{14}C]carnitine was adjusted as necessary to provide adequate sensitivity. Sodium tetrathionate was added to prevent the back reaction (to serve as a CoA-SH sink). The assay was linear for up to 4 min with 100 μ g of protein. Kinetic parameters were measured by varying both palmitoyl-CoA (10 and 40 μ M) and carnitine (0.01, 0.025, 0.05, 0.1, 0.5 and 2 mM at each of the palmitoyl-CoA concentrations). Initial plots of reciprocal velocity versus reciprocal carnitine concentration were replotted to give slope and intercept as a function of reciprocal palmitoyl-CoA concentration. The kinetic parameters were derived from the secondary plots as detailed by Segel (1975). Dixon plots were generated with 10 μ M- and 40 μ M-palmitoyl-CoA, 0.5 mM-L-carnitine, and 0, 5, 10, 25 and 50 μ M-malonyl-CoA, or 0, 0.05, 0.1, 0.5 and 1.0 μ M-TDGA-CoA unless otherwise noted (Segel, 1975). CPT activity measured in the presence of malonyl-CoA or TDGA-CoA was linear during the period measured and exhibited correlation coefficients of 0.99 or better. When TDGA-carnitine inhibition was determined, TDGA-carnitine was added to give a final concentration of 0, 0.05, 0.1, 0.25 or 0.5 mM. The resulting Dixon plots were also linear. L-Carnitine was present at 0.05 or 0.5 μ M and palmitoyl-CoA was used at 40 μ M. The CPT activity in the reverse direction was assayed by a kinetic modification of the assay II of Hoppel & Tomec (1972). The final assay volume of 0.2 ml contained: 80 mM-KCl, 50 mM-Mops, 1 mM-EGTA, 1 mg of defatted and dialysed albumin/ml, 5 mM-CoA, and 60 or 30 μ M-palmitoyl-L-[methyl- ^{14}C]carnitine. TDGA-carnitine was added to give a final concentration of 0, 0.05, 0.1, 0.25, or 0.5 mM. After a 5 min preincubation at 37 °C, the reaction was initiated by the addition of 5 μ g of enzyme protein. The reaction was terminated after 3 min by addition of 0.2 ml of 6% (v/v) $HClO_4$. The reaction was linear up to 10 μ g of protein and for 4 min. After addition of $HClO_4$, samples were cooled on ice for 30 min, centrifuged at 12 000 g_{av} for 5 min in an Eppendorf Microfuge, and a sample of the supernatant, containing the liberated L-[methyl- ^{14}C]carnitine, was counted for radioactivity.

Hepatic CPT was purified from mitochondria isolated from rats fed on stock diet containing 2% diethylhexyl phthalate for 2 weeks. The mitochondrial suspension was added to 0.5 vol. of 3 M-KCl/6% Triton X-100/0.3 mM-phenylmethanesulphonyl fluoride, homogenized with a glass/Teflon homogenizer and centrifuged at 89 000 g for 90 min. The supernatant was collected and dialysed against 1% Triton/2.5 mM-Hepes/0.25 mM-EDTA/20 mM-KCl/0.1 mM-phenylmethanesulphonyl fluoride, pH 7.2. After removal by centrifugation of protein precipitated at the low ionic strength, the supernatant KCl concentration was increased to 300 mM and it was applied in 25 ml batches to a 40 cm $\times$ 5 cm Sephacryl S-300 (Pharmacia) column equilibrated with this buffer. Active fractions were collected (23 munits/mg of protein;

approx. 2.3-fold purification) and dialysed/concentrated with a 30000-Da nominal cut-off membrane (Micro-ProdiCon; BioMolecular Dynamics, Beaverton, OR, U.S.A.) against 0.5% Triton/5 mM-Bis-Tris Propane {1,3-bis[tris(hydroxymethyl)methylamino]propane}/0.25 mM-EDTA/20 mM-KCl/0.1 mM pH 9.7. The dialysis residue was applied to a 40 cm $\times$ 5 cm QAE (quaternary aminoethyl)-Sephadex Q-25-120 column equilibrated with this buffer and eluted with the same buffer. This yielded an approx. 2-fold increase in specific activity (50 munits/mg of protein). These first two steps are essentially as described by Fiol & Bieber (1984) for purification of ox heart CPT. The Cibacron Blue-Sephadex step used by those investigators did not retard the rat CPT, although it did work well for pig hepatic CPT (P. S. Brady & L. J. Brady, unpublished work). The active fractions of the rat liver CPT preparation from the QAE-Sephadex column, which were eluted near the solvent front, were pooled, dialysed/concentrated against 0.5% Tween 20/0.5 mM-dithiothreitol/5 mM-K₂HPO₄/KH₂PO₄/0.1 mM-phenylmethanesulphonyl fluoride, pH 7.5, and applied to a 12 cm $\times$ 1.6 cm Fractogel TSK DEAE-650M (EM Science) column equilibrated with this buffer. The column was eluted with approx. 2 vol. of the start buffer, followed by a 200 ml linear gradient of 5–20 mM-K₂HPO₄/KH₂PO₄ (see Miyazawa *et al.*, 1983). CPT was eluted in the final half or third of the gradient. Clean-up to this point is substantial (40-fold purification, with a specific activity of 8 units/mg of protein), but several contaminants at approximately the same *M_r* are observed on SDS/polyacrylamide-gel electrophoresis. To effect final purification, the CPT active fractions were dialysed and concentrated to less than 5 ml against the 5 mM-K₂HPO₄/KH₂PO₄ buffer used in the previous step. This was applied to a 20 cm $\times$ 1.6 cm PBE 94 column (Pharmacia), previously equilibrated with 0.025 M-acetate, pH 8.3. The CPT was eluted with Polybuffer (Pharmacia) adjusted to pH 5.0 as directed by the manufacturer for this system and containing 25 mM-octyl glucoside. The effect is to produce a pH gradient of 8–5 on the column; CPT was eluted between pH 6 and 5.5. SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) yielded a single band of *M_r* 68000. The specific activity at 25 °C was 22–30 μ mol/min per mg of protein measured by the method of Bieber *et al.* (1972) for CPT activity (total purification was approx. 2400-fold). Ten rat livers yielded 0.75–1 mg of CPT. Subsequent assay of CPT activity was by the spectrophotometric assay of Bieber *et al.* (1972) for the forward reaction. The assay did not contain albumin, but did include 0.1% Triton; palmitoyl-CoA was present at 10, 80 or 160 μ M; L-carnitine was used at 6 mM; TDGA-CoA was present at 0, 5, 10, 50 and 100 μ M. Rates determined by this method were equivalent to rates determined by the radioactive assay. For some studies, dimethyl sulphoxide (Me₂SO) was added at 10–50% of assay volume. Phosphatidylcholine and cardiolipin liposomes were prepared as described by Noel *et al.* (1985), by sonicating 0.25 mg of phosphatidylcholine or cardiolipin/ml of buffer (20 mM-KH₂HPO₄/KH₂PO₄/0.5 mM-dithiothreitol/0.5% Tween 20, pH 7.5) to clarity. Purified CPT (7.5 μ g/ml) was added and mixed. Detergent was removed with Extracti-gel D (Pierce Chemical Co.). The supernatant was used after centrifugation for 5 min at 12000 g. CPT activity in liposomes was assayed without Triton or Me₂SO.

Mitochondrial O₂ consumption with palmitoyl-CoA plus carnitine plus malate as substrates was determined as described by Brady & Hoppel (1983). Palmitoyl-CoA was added at 40 and 10 μ M, and L-carnitine at 2 mM. TDGA-CoA was added in a range of concentrations from 0 to 1.0 μ M. Acid-soluble products of mitochondrial palmitoyl-CoA oxidation were also determined in separate experiments at 30 °C with [1-¹⁴C]palmitoyl-CoA in the presence of 5 mM-malate, 80 mM-KCl, 50 mM-Mops, 5 mM-K₂HPO₄/KH₂PO₄, 1 mM-EGTA, 1 mg of bovine serum albumin (defatted and dialysed) ml, 2 mM-ADP, 0.01–2.0 mM-L-carnitine, and 10 or 40 μ M-palmitoyl-CoA, pH 7.0. Under these conditions, acetoacetate should be the major product (Garland, 1968). Mitochondrial protein was 0.5–1.0 mg/ml. Under these conditions, the rate of accumulation of acid-soluble product was linear for up to 3 min. The reaction was terminated after 1 min with 10% HClO₄. Acid-soluble products were counted for radioactivity in the supernatant after centrifugation for 5 min at 12000 g.

Fluorescence polarization was determined at 30 °C or from 6 to 40 °C with 1,6-diphenylhexa-1,3,5-triene (DPH) as probe as described by Shinitzky & Barenholz (1978). Intact-mitochondrial or inverted-vesicle protein was adjusted to 0.5 mg/ml and incubated with DPH for 1 h before introduction of TDGA-CoA. Data were expressed as the polarization ratio.

Data were analysed by analysis of variance for a factorial design with the potential to accommodate missing data (Federer & Zelen, 1966) or for a completely randomized design (Steel & Torrie, 1960). S.E.M. or S.D. values were derived from the mean-square error of the analysis of variance. Linear-regression analysis was done by standard least-squares techniques (Steel & Torrie, 1960).

Materials

TDGA was given by McNeil Pharmaceuticals, Spring House, PA, U.S.A. The CoA derivative was synthesized as described by Kiorpes *et al.* (1984). Carnitine perchlorate (Chase & Tubbs, 1970) and TDGA chloride (Kiorpes *et al.*, 1984) were made to react and purified as described by Brendel & Bressler (1967) to yield TDGA-carnitine. Palmitoyl-CoA was synthesized as described by Seubert (1960). L-Carnitine was given by Sigma-Tau, Rome, Italy. L-[methyl-¹⁴C]Carnitine was synthesized by using [¹⁴C]methyl iodide (Dupont–New England Nuclear) as described by Ingalls *et al.* (1982). Palmitoyl-L-[methyl-¹⁴C]carnitine was synthesized from L-[methyl-¹⁴C]carnitine perchlorate (prepared as described by Chase & Tubbs, 1970) and palmitoyl chloride as described by Brendel & Bressler (1967). Malonyl-CoA was purchased from P-L Biochemicals. [1-¹⁴C]Palmitoyl-CoA was purchased from DuPont–New England Nuclear. Bovine serum albumin (type V) was defatted (Chen, 1967) and dialysed (Hanson & Ballard, 1968). Sephacryl S-300, QAE-Sephadex, PBE and Polybuffers were products of Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Sephacryl and QAE-Sephadex were purchased from Sigma, and the Chromatofocusing resin and buffers were purchased directly from Pharmacia. Fractogel TSK (Toyopearl) DEAE-650M was purchased from American Scientific Products. All other chemicals were reagent grade or better.

Table 1. Kinetics and malonyl-CoA inhibition of hepatic mitochondrial CPT after administration of TDGA *in vivo*

After starvation overnight, rats were given 2 mg of TDGA (dissolved in ethanol)/kg body wt. orally via stomach tube at 05:00 h ('Treated'). Control rats received ethanol only. At 08:00 h rats were killed. Hepatic mitochondria ('Intact') and inverted submitochondrial vesicles ('Inverted') were prepared and CPT kinetics and malonyl-CoA inhibition determined as described in the Experimental section. Data were analysed as a 2×2 factorial arrangement of treatments. Significant effects at the 5% level (F) are designated 'S' for effect of sidedness, 'T' for effect of TDGA treatment and 'S $\times$ T' for the interaction effect; NS indicates no significant effect. The percentage change with treatment is reported for $V_{\max}$, where a significant effect of treatment was found, and for the malonyl-CoA I_{50} , where a significant interaction was found. The S.E.M. was determined from the error mean square in the analysis of variance. Numbers of replicates are indicated in parentheses.

	Intact		Inverted		S.E.M.	F
	Control	Treated	Control	Treated		
$V_{\max}$ (nmol/min per mg)	9.2 (7)	3.2 (6)	39.4 (6)	10.0 (6)	6.0	S, T
(% change)	-65		-75			
$s_{0.5}$ for palmitoyl-CoA (μM)	11.1 (7)	24.6 (6)	50.9 (6)	20.4 (6)	9.0	NS
K_m for carnitine (mM)	0.31 (7)	0.90 (6)	0.58 (6)	0.37 (6)	0.15	NS
I_{50} for malonyl-CoA (μM)	20 (3)	141 (3)	199 (6)	268 (6)	51	S, S $\times$ T
(% change)	+705		+135			

Table 2. TDGA-CoA sensitivity of intact mitochondria and inverted submitochondrial vesicles

Mitochondria and inverted submitochondrial vesicles were isolated as described in the Experimental section. TDGA-CoA was added to the forward CPT assay at 0, 0.05, 0.1, 0.25, 0.5 and 1.0 μM . TDGA-carnitine was added to the forward and reverse CPT assay at 0, 0.05, 0.1, 0.25 and 0.5 μM . Data were analysed as described in Table 1; means are for four to five rats; S, significant effect ($P < 0.05$) of sidedness. Since no I_{50} could be determined for TDGA-carnitine in inverted vesicles using the reverse reaction, the S.E.M. represents the value for the intact mitochondria. In this case, a significant effect of sidedness is indicated without further statistical analysis.

I_{50}	Rats . . .	Intact		Inverted		S.E.M.	F
		Fed	Starved	Fed	Starved		
Forward reaction							
TDGA-CoA (μM)		0.29	0.27	0.81	1.57	0.29	S
TDGA-carnitine (mM)		—	0.64	—	0.54	0.07	—
Reverse reaction							
TDGA-carnitine (mM)		—	27.7	—	∞	12.2	S

RESULTS

Effects of TDGA administration *in vivo*

Table 1 presents the data for hepatic CPT characteristics after administration *in vivo* of 2 mg of TDGA/kg body wt. The $V_{\max}$ for intact mitochondria was depressed by about 65% by TDGA treatment. The K_m for L-carnitine and the $s_{0.5}$ for palmitoyl-CoA in intact mitochondria were increased 2-fold over the control (not statistically significant), whereas the I_{50} for malonyl-CoA was increased 7-fold over the control.

In the inverted submitochondrial vesicles, the $V_{\max}$ for the control group was 4 times (39.4 nmol/min per mg) that observed in the corresponding intact mitochondria (9.2 nmol/min per mg). This is in agreement with our previous observations using this preparation without the cytochrome *c* affinity clean-up step (Brady *et al.*, 1985, 1986a). The $V_{\max}$ for inverted vesicles from rats

treated with TDGA was decreased to 10.0 nmol/min per mg, or about 25% of the control group. There was not a statistically significant effect of TDGA on the L-carnitine K_m or the palmitoyl-CoA $s_{0.5}$ of inverted submitochondrial vesicles from control and TDGA-treated rats. There was also no effect of TDGA treatment on the malonyl-CoA I_{50} observed in the inverted vesicles.

Effects of TDGA-CoA and carnitine derivatives on intact mitochondria and inverted vesicles *in vitro*

Table 2 presents data for the effects of TDGA-CoA on hepatic mitochondria and inverted submitochondrial vesicles from fed and starved rats, and for the effects of TDGA-carnitine on the same preparations isolated from starved rats. The TDGA-CoA I_{50} was 0.29 μM for fed rats and 0.27 μM for starved rats in the intact mitochondria ($n = 4$), and 0.81 and 1.57 μM in the inverted vesicles ($n = 5$) from fed and starved rats respectively.

Table 3. Effect of addition of TDGA-CoA on intact mitochondria and on inverted vesicles

TDGA-CoA was added to intact mitochondria (10 μM) in the presence of acyl-CoA (100 μM -malonyl-CoA or 10 μM -palmitoyl-CoA) to provide an alternative substrate for the acyl-CoA hydrolase activity and incubated for 5 min at 25 °C. The mitochondria were washed with isolation buffer containing 0.05% albumin, followed by buffer without albumin. Intact mitochondria and inverted vesicles were handled then as described from this point (see the Experimental section). Each value is the mean of three rats. Data were analysed as before, by analysis of variance for intact mitochondria and inverted vesicles separately. The values in parentheses are the standard errors generated from this analysis: *significant effect ($P < 0.05$) of TDGA. Where a significant effect is observed, the percentage change with treatment is noted.

	Intact			Inverted		
	Control	Treated	% change	Control	Treated	% change
V_{max} . (nmol/min per mg)	5.8 (0.65)*	3.5	-40	17.2 (4.8)*	6.3	-63
$s_{0.5}$ for palmitoyl-CoA (μM)	8.9 (7.3)	17.8		9.1 (8.2)	20.2	
K_m for carnitine (mM)	0.11 (0.02)*	0.18	+64	0.09 (0.03)*	0.18	+100

There was no significant effect of starvation on CPT sensitivity to TDGA-CoA, as measured thus. Also of note is that the inverted vesicles possess sensitivity to TDGA-CoA, albeit 2–3-fold less than that of intact mitochondria. It could be argued that this sensitivity is due to contamination of the inverted vesicles with right-side-out vesicles. However, since the vesicles were additionally treated with cytochrome *c*-Sepharose, as a means to remove right-side-out vesicles, this is unlikely as the sole explanation. As a consequence of this additional purification step, malonyl-CoA sensitivity was decreased somewhat compared with what we have previously reported (Brady *et al.*, 1985, 1986a). Intact mitochondria were much less sensitive to TDGA-carnitine (as measured by [*methyl*- ^{14}C]carnitine release in the reverse reaction, TDGA-carnitine competing with carnitine) than to TDGA-CoA, with an I_{50} of 27.7 mM. The inverted vesicles were not measurably sensitive to TDGA-carnitine.

In a separate series of experiments, we presented TDGA-CoA to intact mitochondria, washed the mitochondria, and subsequently produced inverted vesicles from the mitochondria (Table 3). In the intact mitochondria, TDGA-CoA resulted in a 35% decrease in V_{max} , with doubling of the carnitine K_m and palmitoyl-CoA $s_{0.5}$. In the inverted vesicles, the TDGA-CoA pretreatment resulted in a greater than 50% decrease in V_{max} for CPT-B. The specific kinetic values obtained differ somewhat from those presented in Table 1. It should be noted that the control animals, as well as the TDGA-treated animals in the studies *in vivo*, received ethanol orally before the isolation of hepatic mitochondria. We have determined in preliminary studies that ethanol treatment of mitochondria *in vitro* alters membrane characteristics and CPT activity. The isolation of the mitochondria after TDGA treatment *in vitro* also involves some additional steps (two extra washes, one with albumin) which may contribute to the difference from the studies *in vivo*.

Detergent solubilization with 0.05% Triton X-100 resulted in complete loss of TDGA-CoA sensitivity over the range 0–1.0 μM ($n = 3$). Sonicated mitochondria exhibited a TDGA-CoA I_{50} of $0.72 \pm 0.03 \mu\text{M}$ ($n = 3$).

That is, sensitivity was decreased to a point resembling that of inverted vesicles. Sonication does produce inverted submitochondrial vesicles of undetermined purity. However, right-side-out vesicles and membrane fragments are also produced. Thus the lower sensitivity may be due to predominance of inverted vesicles, to membrane disruption or to both factors. The supernatant derived from centrifugation of the sonicated mitochondria had a TDGA-CoA I_{50} of $8.8 \pm 3.2 \mu\text{M}$, and the pellet had an I_{50} of $0.87 \pm 0.05 \mu\text{M}$.

Purified CPT

Purified rat liver CPT was inhibited by TDGA-CoA with an I_{50} of 60–80 μM , whether or not 0.1% Triton was present. In the presence of 20% Me_2SO , this value decreased to 9.5 μM . The inhibition was rapid; a rate

Table 4. Effect of TDGA-CoA on hepatic mitochondrial DPH fluorescence polarization

Mitochondria from fed and 48 h-starved rat liver were prepared as described in the Experimental section. Mitochondria (0.5 mg of protein/ml) were incubated with 5.5 μg of DPH for 30 min at 25 °C and then exposed to various concentrations of TDGA-CoA. Values are means of two to five observations and are expressed as the polarization ratio (p). The s.d. derived from the analysis of variance was 0.004. A significant effect ($P < 0.01$) of starvation was found.

[TDGA-CoA] (μM)	Polarization, p	
	Rats . . .	
	Fed	Starved
0	0.134	0.108
0.05	0.171	0.118
0.10	0.147	0.128
0.25	0.133	0.110
0.50	0.129	0.111
1.0	0.128	0.094
5.0	0.112	0.081
10.0	0.082	0.067

Fig. 1. Relationship between CPT-A activity and the formation of acid-soluble products of palmitoyl-CoA oxidation after TDGA administration *in vivo*

Rats were untreated (a) or treated with TDGA (b) by oral administration of an ethanol solution 3 h before being killed. Hepatic mitochondria were isolated and CPT-A activity (nmol/min per mg of protein) was measured as described in the Experimental section. The formation of acid-soluble products (nmol/min per mg of protein) was measured by using intact mitochondria (0.5–1.0 mg of protein/ml) at 30 °C for 1 min in an assay containing 80 mM-KCl, 50 mM-Mops, 1 mM-EGTA, 1 mg of bovine serum albumin/ml, 5 mM-malate; 0.01–2 mM-L-carnitine, and 5 mM-KH₂PO₄/K₂HPO₄, pH 7.0. Palmitoyl-CoA was present at a total of 40 or 10 μ M containing L-[¹⁴C]-palmitoyl-CoA. The assay was linear with time and protein. For the relationship of acid-soluble product formation to CPT-A activity, untreated rats gave a slope of 0.53 and intercept of 1.50 ($r = 0.7916$; $n = 36$), and treated rats gave a slope of 0.97 and intercept of 0.03 ($r = 0.9331$; $n = 27$).

constant could not be calculated. Essentially all inhibition occurred within 5 s. A difference spectrum (10–100 μ M-TDGA-CoA with 10–100 μ M-palmitoyl-CoA as reference) had an absorbance maximum at 255 nm. The V_{max} increased from 22 to 38 units/mg of protein with 20% Me₂SO; the $s_{0.5}$ for palmitoyl-CoA decreased from 45 to 30 μ M; and the L-carnitine K_m was unchanged. Also, velocity increased in the presence of 0–30% Me₂SO and decreased with higher concentrations (40 and 50%). At 30% Me₂SO, velocity was 2.4 times that in the absence of Me₂SO. CPT in phosphatidylcholine or

cardiolipin liposomes also exhibited increased sensitivity to TDGA-CoA ($I_{50} = 10$ –20 μ M).

Effects of TDGA on the membrane

The dose relationship of DPH fluorescence polarization and TDGA-CoA in fed and starved rats was examined (Table 4). Mitochondria from starved rats exhibited lower polarization (increased fluidity) compared with fed rats. At the same time, TDGA-CoA had no effect on polarization until concentrations of 5–10 μ M were reached. At these values, TDGA produced decreased polarization. Because Mills *et al.* (1984) had reported effects of pH on malonyl-CoA sensitivity, and because Declercq *et al.* (1985) reported that TDGA-CoA and malonyl-CoA may share the same target site, we examined the effect of pH on sensitivity to TDGA-CoA (results not shown); there was no significant effect.

Relationship of CPT-A to β -oxidation

In the present study, we isolated hepatic mitochondria from rats after oral treatment with TDGA and measured CPT activity over a range of palmitoyl-CoA and carnitine concentrations, concurrently measuring the generation of acid-soluble products from palmitoyl-CoA. Fig. 1 shows the relationship between CPT-A activity and acid-soluble-product formation. In mitochondria from both control and TDGA-treated rats, the relationship was linear. The TDGA-treated rats showed a more limited range of response to substrate concentration, but, within this range, acid-soluble product formation was almost twice as sensitive to a given change in CPT-A activity. Inclusion of TDGA-CoA in the incubations *in vitro* also yielded a linear relationship of acid-soluble product formation and CPT-A activity (Fig. 2a). Starved rats exhibited a rightward shift in this relationship regardless of substrate concentration used, indicating a lower rate of acid-soluble product formation at equivalent CPT-A activity. At 10 μ M-palmitoyl-CoA there was a decrease in the slope of the relationship for both fed and starved rats as compared with that at 40 μ M-palmitoyl-CoA (2.14 and 2.15 respectively for fed and starved at 40 μ M, and 0.95 and 0.75 for fed and starved rats at 10 μ M). A linear relationship between mitochondrial State-3 respiration rates and CPT-A activity was also observed (Fig. 2b). The relationship of State-3 oxidation rates for palmitoyl-CoA + carnitine + malate to CPT-A activity also showed a rightward shift with starvation.

DISCUSSION

The carnitine palmitoyltransferases have proved to be a difficult system to study. In part, this results from an inability to distinguish, unequivocally, the activity of the outer surface of the inner membrane (CPT-A) from the activity of the inner surface (CPT-B). Indeed, Miyazawa *et al.* (1983) were able to detect only a single rat liver mRNA translation product with an M_r somewhat greater than that of the purified CPT. Thus CPT-A and -B activities may be the product of a single protein (see also Bergstrom & Reitz, 1980; Clarke & Bieber, 1981; Fiol & Bieber, 1984). The CPT-A activity has been proposed as the rate-limiting enzyme in hepatic mitochondrial oxidation of long-chain fatty acids, with malonyl-CoA acting as a key physiological inhibitor (McGarry & Foster, 1980). Mills *et al.* (1984) have suggested that malonyl-CoA exerts its effect on CPT-A

Fig. 2. Relationship between CPT-A activity and acid-soluble product formation (a) and O_2 consumption (b) with TDGA-CoA addition *in vitro*

Mitochondria derived from fed ($\triangle$, $\blacktriangle$) and 48 h-starved ($\circ$, $\bullet$) rats were isolated, and CPT-A activity (nmol/min per mg of protein) and acid-soluble product formation (nmol/min per mg of protein) were determined as described in the Experimental section. Palmitoyl-CoA containing L-[^{14}C]palmitoyl-CoA was present at a total of 40 μM ($\blacktriangle$, $\bullet$) or 10 μM ($\triangle$, $\circ$). O_2 consumption (ng-atoms/min per mg of protein) was determined as described by Brady & Hoppel (1983), with 2 mM-ADP, 40 μM - and 10 μM -palmitoyl-CoA, 2.5 mM-malate and 2 mM-L-carnitine. TDGA-CoA was added at 0, 0.05, 0.1, 0.25, 0.5 and 1.0 μM to alter CPT activity, rate of acid-soluble product formation, and O_2 consumption.

through a receptor independent of the CPT catalytic site, and Declercq *et al.* (1985) have suggested that TDGA-CoA and malonyl-CoA interact with a common site that is not the CPT active site. Kiorpes *et al.* (1984) had previously proposed TDGA-CoA as an irreversible active-site inhibitor of CPT-A. Our data and those of Bieber & Fiol (1986) may reconcile these differences. We would agree with Declercq *et al.* (1985) that TDGA-CoA resulted in a depression in CPT-A activity and in sensitivity to malonyl-CoA inhibition and that detergent solubilization eliminated sensitivity to TDGA-CoA, over the range of concentrations used (0–1 μM). Kiorpes *et al.* (1984) and Tutwiler *et al.* (1985) have also reported loss of TDGA-CoA sensitivity in the pellet after digitonin extraction of mitochondria. Hoppel & Tomec (1972) and

Hoppel (1982) have proposed that this digitonin solubilization scheme releases CPT-A into the supernatant, leaving CPT-B in the pellet. However, our own direct measurements indicate some sensitivity of the inverted submitochondrial vesicles to TDGA-CoA inhibition. We believe that this underlines the importance of the membrane to the kinetic and inhibitory characteristics of CPT. Bieber & Fiol (1986) reported sensitivity of purified ox heart CPT to TDGA-CoA inhibition, a system in which no measurable sensitivity to malonyl-CoA is retained. These investigators also found the inhibition to be reversible, requiring concentrations of palmitoyl-CoA beyond what could be tolerated by mitochondria without lysis. We observed that the purified rat liver CPT is also sensitive to TDGA-CoA, but with an I_{50} considerably higher than that of any of the other preparations that we used, but the I_{50} was decreased in the presence of Me_2SO or by insertion into phosphatidylcholine or cardiolipin liposomes. Declercq *et al.* (1985) may be correct that TDGA-CoA interacts with the malonyl-CoA site; and this interaction may be responsible for the very high sensitivity of CPT of the intact mitochondria to TDGA-CoA. However, some sensitivity is retained in sonicated mitochondria, in the sonicated supernatant and the pellet after high-speed centrifugation, and in the purified enzyme. In view of the sensitivity of purified CPT to TDGA-CoA, it is likely that even the detergent-solubilized mitochondria retain a greatly decreased sensitivity. This sensitivity may be directed toward the CPT active site or to a regulatory site, but remains when malonyl-CoA sensitivity is not measurable. Thus TDGA-CoA may exert an effect on two sites, the malonyl-CoA receptor and a site on the CPT. In view of the pattern of sensitivity of the various CPT preparations, another possibility remains. Both malonyl-CoA and TDGA-CoA may share a regulatory site on the CPT. The configuration of this site and resulting inhibitor sensitivity may be quite dependent on the membrane environment. Declercq *et al.* (1985) solubilized mitochondria with detergent; then reconstituted CPT in the membrane by dialysing out the detergent. However, the native mitochondrial inner membrane is highly asymmetrical (Daum, 1985). It is not clear to what degree the reconstituted membrane reflects the native state. Also, the proper insertion of CPT into the membrane may require the leader sequence suggested by the data of Miyazawa *et al.* (1983). Finally, the CPT-A activity measured in the intact mitochondria under our conditions is much less sensitive to malonyl-CoA (5–25 μM) (Brady *et al.*, 1985) than to TDGA-CoA (0.05–1 μM). Extrapolating to the purified CPT, it may not be possible to detect malonyl-CoA sensitivity because of the high concentrations that would be required. However, in the presence of Me_2SO and in phosphatidylcholine and cardiolipin liposomes, sensitivity of purified CPT to TDGA-CoA is sharply enhanced. In none of these preparations is malonyl-CoA sensitivity observed. The effects of Me_2SO and of insertion of the enzyme into lipid are similar; that is, Me_2SO decreases water activity, and lipid may also be expected to exclude water from certain regions of the protein. It is not clear why the reconstitution procedure employed by Declercq *et al.* (1985) failed to increase CPT sensitivity to TDGA-CoA. The choice of phosphatidylcholine and cardiolipin in the present study may have been fortunate; sensitivities in other liposome compositions should be examined. Regardless,

it seems clear that a membrane environment is important in expression of sensitivity to TDGA-CoA. It would further appear that the expressed kinetic characteristics and inhibitor sensitivity of purified CPT are potentially modulated by water activity.

Kiorpes *et al.* (1984) further found that TDGA-carnitine did not inhibit palmitoylcarnitine oxidation, and hence did not inhibit the CPT-B. From the observed TDGA-carnitine I_{50} for both intact mitochondria and inverted vesicles, it is obvious that this derivative is not a significant inhibitor.

An important observation in the present study is the indication that CPT-A 'communicates' with CPT-B activity. As stated above, several investigators have suggested that CPT is a single protein. At the same time, the mitochondrial inner membrane, which provides the environment of the CPT, has an asymmetric phospholipid distribution (Daum, 1985). In order to explain the concept of a single membrane protein with activities in two widely different membrane environments, the most reasonable model is a membrane-spanning unit with two active sites, one in each environment. Such a model is in agreement with the observations by Fiol & Bieber (1984) and Bieber & Fiol (1986) of Hill coefficients (h) of 2 for purified ox heart CPT. Such a model would also easily explain the observed communication. The effect of such communication is less clear. CPT-A has been proposed as the rate-limiting enzyme for mitochondrial β -oxidation of fatty acid oxidation. Regardless of sensitivity of CPT-B to TDGA *in vivo* or *in vitro*, at no point do we observe a CPT-B activity less than the corresponding CPT-A activity. Neither have we observed CPT-B activity less than the corresponding CPT-A in our previous use of the inserted vesicle (Brady *et al.*, 1985, 1986a). It is not clear if CPT-B would ever be rate-limiting. Kiorpes *et al.* (1984) and Turnbull *et al.* (1984) did not observe a TDGA-CoA inhibition of palmitoylcarnitine oxidation by intact hepatic mitochondria. Under physiological conditions, the direction of the CPT-B reaction is the reverse of the direction in which we measure kinetics. In addition, the actual concentrations of substrates and inhibitors presented to either CPT-A or CPT-B *in vivo* are not known. The apparent linkage of CPT-A and CPT-B may have important regulatory consequences and certainly warrants further study, but it is impossible to make such a claim at present.

We have noted previously that increased mitochondrial membrane rigidity is associated with decreased CPT activities (Brady *et al.*, 1985). Bulk membrane fluidity changes were not involved in the TDGA-CoA effects on CPT. Neither did altering pH result in changes in CPT-A sensitivity to TDGA-CoA or in changes in membrane fluidity. Mills *et al.* (1984) have previously reported changes in mitochondrial sensitivity to malonyl-CoA with pH. Again, TDGA-CoA may not share all the characteristics of malonyl-CoA.

A good relationship of acid-soluble product formation and CPT-A activity would be expected if CPT-A were controlling the rate of this process. Usually, a good linear relationship of CPT to some measure of the fatty acid oxidative process has been found (Tutwiler *et al.*, 1985; Brady *et al.*, 1985, 1986a), although there are instances where the correlation is lost (Cederbaum *et al.*, 1975; Hoppel *et al.*, 1979; Tutwiler *et al.*, 1985; Brady *et al.*, 1986b). The reason for the different responses of the

process (O_2 consumption) to CPT activity is not clear, but may imply that factors in addition to CPT-A are involved in mitochondrial β -oxidation. In the present studies, starvation also produced a change in the relationship of CPT-A to acid-soluble product formation and to State-3 oxidation when compared with the fed state, where TDGA-CoA was used to perturb the enzyme activity. Starvation resulted in increased membrane fluidity and increased CPT activity *in situ* (Brady *et al.*, 1985, 1986a); however, at the same CPT-A activity, less product appears to be produced. In addition, Turnbull *et al.* (1984) observed a TDGA-CoA I_{50} for palmitoyl-CoA oxidation of 10 μ M, which is much higher than is observed for the CPT-A activity in the present studies (0.29 μ M). Kiorpes *et al.* (1984) found that 1 μ M-TDGA inhibited 80% of palmitoyl-CoA oxidation. Thus the enzyme may be more sensitive to inhibition than the process as measured, and the relationship of CPT and β -oxidation may be more complex. Differences in I_{50} for enzyme and process may reflect the differences in the conditions of measurement, or they may indicate that factors in addition to CPT-A activity exert control over the rate of the fatty acid oxidative process.

Conclusions

In summary, CPT sensitivity to TDGA was altered by membrane integrity. Sensitivity was higher in the CPT-A activity of intact mitochondria and lowest in purified CPT. Sensitivity of purified CPT to TDGA inhibition can be enhanced by insertion into phosphatidylcholine liposomes or inclusion of Me_2SO in the assay. Presentation of TDGA *in vivo* or *in vitro* to the outer surface of the mitochondrial membrane resulted in inhibition of the CPT-B activity. The TDGA effect did not appear to be mediated by changes in bulk membrane fluidity. Finally, inhibition of CPT-A activity *in vitro* by TDGA correlated with inhibition of oxidation of palmitoyl-CoA. However, factors other than CPT-A activity may also be involved in regulating this relationship.

Note added in proof (received 29 July 1986)

Since the studies described here, we have observed that standard commercial Triton X-100 may be inhibitory to CPT. Although this inhibition appears reversible, purification can be accomplished in Tween 20 at somewhat higher concentration without introducing this potential problem.

This work was supported by a grant from the American Diabetes Association, and by the Washington Agricultural Experiment Station. This is Washington Agricultural Experiment Station paper no. 7261.

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Received 7 October 1985/20 May 1986; accepted 6 June 1986