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MICROSOMAL OXIDATION OF DODECYLTHIOACETIC ACID (A 3-THIA FATTY ACID) IN RAT LIVER

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Abstract—[1-^{14}C]Dodecylthioacetic acid (DTA), a 3-thia fatty acid, is ω (ω-1)-hydroxylated and sulfur oxygenated at about equal rates in rat liver microsomes. In prolonged incubations DTA is converted to ω-hydroxydodecylsulfoxyacetic acid. ω-Hydroxylation of DTA is catalysed by cytochrome P450IVA1 (or a very closely related isoenzyme in the same gene family), the fatty acid ω-hydroxylating enzyme. It is absolutely dependent on NADPH and inhibited by CO, and lauric acid is a competing substrate. ω-Hydroxylation of DTA is increased by feeding tetradecylthioacetic acid (TTA), a 3-thia fatty acid, for 4 days to rats. ω-Hydroxylation of [1-^{14}C]lauric acid is also induced by TTA and other 3-thia carboxylic acids. A close relationship was observed between induction of microsomal ω-hydroxylation of fatty acid and palmitoyl-CoA hydrolase activity. DTA is ω-hydroxylated at about the same rate as the physiological substrate lauric acid. The sulfur oxygenation of DTA is catalysed by liver microsomal flavin-containing monooxygenase (FMO) (EC 1.14.13.8). It is dependent on either NADH or NADPH. The K_m value for NADH was approx. five times larger than the K_m value for NADPH. It is inhibited by methimazole and not affected by CO. It is not induced by TTA.

Alkylthioacetic acids (3-thia fatty acids) are fatty acid analogues with a sulfur atom in β-position (Table 1). Like several other compounds (e.g. clofibrate, tiadenol§, niadenate) they have been shown to be hypolipidemic and peroxisome proliferating [1]. The inducing effects of alkylthioacetic acids increase with the length of the hydrophobic alkyl end of the analogues and tetradecylthioacetic acid (TTA) has proven to be the most effective inducer [1, 2].

Alkylthioacetic acids are incorporated into triacylglycerols and phospholipids like ordinary fatty acids in isolated rat hepatocytes [3] but are not metabolized by β-oxidation like ordinary fatty acids. *In vivo* they are metabolized to dicarboxylic acids, carboxypropylsulfoxyacetic acid and bis(carboxymethyl)sulfoxide [4]. The proposed metabolic pathway is an initial ω-hydroxylation followed by oxidation to a dicarboxylic acid and β-oxidation from the ω-end. The sulfur atom is also oxygenated to a sulfoxide. Fatty acids are ω or (ω-1)-hydroxylated by a microsomal fatty acid ω-hydroxylase (cytochrome P450IVA1) [5]. Medium-chain lengths (C10–C16) are most easily hydroxylated [6]. The ω-oxidation of these fatty acids produces primary alcohols which are subsequently converted to dicarboxylic acids by alcohol and aldehyde dehydrogenases [7]. Dicarboxylic acids are further metabolized by β-oxidation believed to take place in the peroxisomes [8–11]. The rat liver fatty acid ω-hydroxylation system contains possibly two or three cytochrome P450s, which function in ω and (ω-1)-hydroxylation [12].

Xenobiotic organic sulfides are oxygenated by either of two liver microsomal monooxygenases i.e., flavin-containing monooxygenase (FMO) [13] or cytochrome P450 monooxygenase [14]. Studies have shown that oxygenation of organic sulfides with cytochrome P450 give the corresponding sulfoxide or if the α-atoms of sulfides possess a highly electronegative substituent, such as nitrile, acyl or carbamoyl group, oxidative S-dealkylation might compete with S-oxygenation [14]. Oxygenation of organic sulfides with FMO give only S-oxygenation [13, 14]. Microsomal FMO, bearing a flavin prosthetic group, catalyses oxygenation of nucleophilic organic nitrogen or sulfur compounds [13].

In this paper we report studies on the initial metabolism of dodecylthioacetic acid in rat liver microsomes. We have also studied which effect alkylthioacetic acids in the diet have on their own metabolism and ω-oxidation of fatty acids.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats (170–180 g) were fed a standard pellet diet *ad lib.* until they were killed. Adapted animals were in addition given tetradecylthioacetic acid (TTA), other thio acids and control compounds by a gastric tube once or twice a day for 5 days.

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§ Abbreviations: Tiadenol, 1,10-bis(hydroxyethylthio)decane; DC-Tiadenol, 1,10-bis(carboxymethylthio)decane; DC-6, hexanedioic acid; DC-8, octanedioic acid; DC-14, tetradecanedioic acid; DC-16, hexadecanedioic acid; FMO, flavin-containing monooxygenase.

Materials

[1-^{14}C]dodecylthioacetic acid (DTA) (94 Bq/nmol) was synthesized from dodecanethiol and [1-^{14}C]iodoacetate according to Skrede *et al.* [3]. HPLC of the compound showed that it always contained 1–4% of sulfur oxygenated DTA formed by spontaneous oxidation during the purification procedure. [1-^{14}C]Lauric acid (26 mCi/mmol) was obtained from Amersham International plc (Amersham, U.K.). Palmitic acid was obtained from Sigma Chemical Co. (St Louis, MO). Hexadecanedioic acid (DC-16), tetradecanedioic acid (DC-14), octanedioic acid (suberic acid, DC-8), hexanedioic acid (DC-6) were purchased from Aldrich-Chemie (Stenheim, F.R.G.). 1,10-Bis(carboxymethylthio)-decane (DC-Tiadenol), tetradecylthioacetic acid (TTA) and tetradecylthiopropionic acid (TTP) were synthesized according to Spydevold and Bremer [2]. ω-Hydroxydodecylthioacetic acid was synthesized from ω-hydroxydodecylbromide and mercaptoacetic acid by the same procedure [2]. The corresponding sulfoxide was synthesized by oxidation with an equivalent amount of H_2O_2 in glacial acetic acid. 1,10-Bis(hydroxyethylthio)decane (Tiadenol) was generously supplied by Laboratories Almiral S.A. (Barcelona, Spain). Other chemicals were from the Sigma Chemical Co.

Preparation of liver subcellular fractions

The livers from control and adapted rats were finely minced and homogenized in ice-cold 0.3 M mannitol, 1 mM EDTA and 10 mM Trizma base pH 7.4 by four strokes in a Potter-Elvehjem homogenizer with a loose-fitting teflon pestle. The resulting nuclear plus postnuclear fraction was used as the total homogenate. The homogenate was centrifuged for 10 min at 2200 rpm ($520\,g_{av}$) in a HB 4 rotor in a Sorvall RC-5B centrifuge. The pellet was rehomogenized and the suspension was recentrifuged. The combined postnuclear supernatant was centrifuged at 13,000 rpm ($15{,}700\,g_{av}$) for 20 min in the SS-34 rotor. The supernatant was centrifuged at 37,500 rpm ($105{,}000\,g_{av}$) for 1 hr in the T-865 rotor of a Sorvall OTD65 ultracentrifuge to obtain the microsomal pellet (P-fraction). The remaining supernatant was collected as the cytosolic (S-fraction). All pellets were resuspended in 0.3 M mannitol, 1 mM EDTA and 10 mM Trizma base pH 7.4 and if not used at once, stored at −70°.

Analytical methods

Incubation with DTA as substrate. The ω-oxidation and sulfur oxygenation of DTA was measured in the microsomal (P) fraction from rat liver and determined as relative specific activity. Under standard conditions the incubation mixture contained the following in 0.8 mL of 0.025 M KP_i buffer pH 7.4: 1 mM $MgCl_2$, 1.25 mM NADPH, 1.25 mM NADH, 37.5 μM dodecylthioacetic acid (1.5–2.5 kBq). Normally the amount of protein was 300–400 μg per incubation. In long time experiments a NADPH regenerating system was used instead of NADPH: 5.0 mM glucose 6-phosphate, 1.25 mM NADP and 1.0 units/mL glucose-6-phosphate dehydrogenase and the reaction was flushed with O_2 every 0.5 hr. The incubations normally included a blank where the complete incubation mixture was incubated with heat inactivated microsomes. This enabled us to correct for any spontaneous oxidation of DTA and for the sulfur oxygenated DTA already present in the substrate. No further spontaneous S-oxygenation was detected. The incubation was started with the substrate and the incubation time was 15 min unless stated otherwise. The incubation was stopped with 0.8 mL ice-cold acetonitrile, placed on ice, and centrifuged at 10,000 rpm ($9{,}300\,g_{av}$) for 10 min. The supernatant was carefully removed and stored at −20° before the products were analysed.

Identification of products. The ω-hydroxylation of DTA was determined as the sum of ω and (ω-1)-hydroxylated products. The combined ω and (ω-1)-hydroxydodecylthioacetic acid and dodecylsulfoxyacetic acid were separated by HPLC. The extract was injected directly on a Spherisorb ODS column (4.6 × 250 mm) from Supelco Inc. and a gradient was run with methanol and water in 0.5% formic acid starting at 55% methanol and 45% water and increasing to 80.5% methanol in 17 min, with 80.5% MeOH isocratic for 2 min and again a gradient to 100% methanol in 2.0 min followed by 100% methanol in 3 min and then returning back to the starting point at a flow of 0.8 mL/min. The retention times for the ω and (ω-1)-hydroxylated products of DTA were about 25 min and the sulfur oxygenation product of DTA about 28 min. To separate ω and (ω-1)-hydroxylated DTA, the acetonitrile in the extract of the incubation was evaporated under a stream of nitrogen. Then the extract was dissolved in methanol and injected on a Spherisorb ODS column and eluted with methanol/formic acid/water (66.5:1.0:32.5) isocratic for 35 min and then a gradient to 100% methanol in 1.0 min followed by 100% methanol in 5.0 min at a flow of 0.8 mL/min. The retention times were about 34 min ((ω-1)-hydroxylated DTA), 37 min (ω-hydroxylated DTA) and 44 min (sulfur-oxygenated DTA). The radioactivity was in all cases detected directly on line in a Raytest Ramona 5LS liquid scintillation counter (Isotopenmessgeräte GmbH, Straubenhardt, W. Germany) equipped with a 1.0 mL flow cell. Eluate and liquid scintillator were mixed 1:7. The on line counter automatically calculated the radioactivity as cpm. In a systematic test this calculated radioactivity of the extract of the incubation injected on the HPLC was found to be 100.7 ± 11.5% of that measured in a Packard Scintillation counter.

To identify the metabolites in the chromatograms non-radioactive and radioactive standards, synthesized in our laboratory, were chromatographed, together with extracts from the incubation.

In order to get further proof of the identity of the metabolites, fractions were collected from HPLC and run together with cold standards on a TLC-system consisting of benzene/dioxane/formic acid, 50/50/0.4 in mL. All the samples were run in duplicate tracks. One track was exposed to iodine vapour, localizing the metabolites as brown spots; the other was cut into corresponding zones and immersed in scintillation fluid for counting.

Inhibition studies with competing substrates were done with a standard incubation mixture and 5 mM

methimazole or 0.6 mM lauric acid. To estimate inhibition by CO, incubations were performed in closed test tubes which contained standard incubation mixture and the following mixture of gases: $CO/N_2/O_2$ (40/55/5), N_2/O_2 (95/5), He (100) and one control open to air.

The ω-hydroxylation of laurate was determined in the microsomal (P) fraction from rat liver and separated into two products, ω and ω-1, on HPLC according to Nilsson *et al.* [15].

Palmitoyl-CoA-dependent dehydrogenase (usually termed peroxisomal β-oxidation) and palmitoyl-CoA hydrolase (EC 3.1.2.2) activities were determined in total liver homogenates and in the cytosolic (S) fraction respectively as earlier described [16, 17].

RESULTS

Metabolism of DTA

Two primary metabolites of [1-^{14}C]dodecylthioacetic acid (DTA) from rat liver microsomes have been identified by chromatographing extracts of the incubation in HPLC and TLC-systems together with synthetic products. The least polar of the two products co-chromatographed with dodecylsulfoxyacetic acid (sulfur oxygenated DTA, peak B in Fig. 1a). When peak B in Fig. 1a was isolated by HPLC and applied on TLC plates as described, 93% of the radioactivity co-chromatographed with synthetic dodecylsulfoxyacetic acid with a R_f = 0.66. The most polar of the two products co-chromatographed with ω-hydroxydodecylthioacetic acid (ω-hydroxylated DTA, peak C in Fig. 1a). When this peak was isolated and applied on TLC-plates, 95% of the radioactivity co-chromatographed with synthetic ω-hydroxydodecylthioacetic acid with a R_f = 0.43. Separation of ω and (ω-1)-hydroxylated fatty acids for e.g. lauric acid has been reported [15] and is obtained in a HPLC-system using a gradient with methanol and water. It was also possible to separate ω and (ω-1)-hydroxylated products of DTA. Peak C_1 in Fig. 1a insert co-chromatographed with synthetic ω-hydroxydodecylthioacetic acid and C_2 is assumed to be (ω-1)-hydroxydodecylthioacetic acid.

After prolonged incubation a metabolite with retention time about 13 min appeared (peak D in Fig. 1b). This co-chromatographed with ω-hydroxydodecylsulfoxyacetic acid (ω-hydroxylated and sulfur oxygenated DTA). When this peak was isolated and applied on TLC-plates, 89% of the radioactivity co-chromatographed with synthetic ω-hydroxydodecylsulfoxyacetic acid with a R_f = 0.29. The peak labelled D in Fig. 1b shows a shoulder indicating that there is more than one metabolite in this peak. The shoulder probably represents the (ω-1)-hydroxylated product.

The time curves (Fig. 2) clearly show that both the ω-hydroxylated and the S-oxygenated products of DTA are formed as primary products and that both are further metabolized in rat liver microsomes. The metabolite, ω-hydroxydodecylsulfoxyacetic acid, is thus formed by two pathways. Pre-incubation experiments showed that both the ω-hydroxylation and S-oxygenation enzymes were fully active over the whole 3 hr period (results not shown). Figure 2 also shows that ω-hydroxylation and S-oxygenation are catalysed at approximately the same rates. The K_m value for ω-hydroxylation was estimated to be 0.07 mM and for S-oxygenation to be 0.12 mM (Fig. 3).

Oxygenation of the sulfur atom is dependent on either NADPH or NADH (Table 2). As previously mentioned DTA was contaminated with sulfur oxygenated DTA. The apparent sulfur oxygenation in the absence of NADH/NADPH seen in Table 2, is due to this contamination which was not corrected for in this experiment. No spontaneous S-oxygenation of DTA was detected when incubating with denatured protein, without NADH/NADPH, or without protein. The K_m values for NADH were 5-fold higher than that of NADPH in our system, 0.23 mM and 0.05 mM respectively. ω-Hydroxylation of DTA is dependent on NADPH with a K_m = 0.09 mM. The activity of the ω-hydroxylation enzyme system was barely detectable with NADH as reductant (Table 2). The combined addition of NADH and NADPH as reductants gave no significantly higher rate of ω-hydroxylation than with NADPH alone (Table 2). TTP has also been tested as a substrate. It was converted to the ω-hydroxylated and S-oxygenated products at approximately two thirds the rate of DTA.

Inhibitor studies

Table 3 shows that S-oxygenation of DTA is markedly reduced by methimazole in rat liver microsomes. ω-Hydroxylation of DTA is only slightly reduced by methimazole probably due to unspecific inhibition. Lauric acid markedly reduces ω-hydroxylation activity and CO almost completely turns it off. CO has no effect on the S-oxygenation process (Table 3).

Induction of lauric acid ω-hydroxylation

Table 4 shows the effect on ω-hydroxylation of [1-^{14}C]lauric acid when feeding rats TTA, TTP, Tiadenol, DC-Tiadenol, palmitic acid, and a series of dicarboxylic acids of varying chain lengths at equal molar concentrations. ω-Hydroxylation of lauric acid was preferentially induced over (ω-1)-hydroxylation by TTA, Tiadenol and DC-Tiadenol. These compounds which cannot be β-oxidized have also been shown to induce peroxisomal β-oxidation [1].

In Fig. 4 the increase in palmitoyl-CoA hydrolase and microsomal ω-hydroxylase was compared after feeding with increasing dose of TTA. The calculated correlation value between the two enzymes were 0.906. Induction of peroxisomal β-oxidation was also compared to palmitoyl-CoA hydrolase and microsomal ω-hydroxylase with a correlation values of 0.946 and 0.971 respectively.

Induction of dodecylthioacetic acid ω-hydroxylation

Table 5 shows the effect on ω-hydroxylation and S-oxygenation of DTA when feeding rats TTA. ω-Hydroxylation of DTA in rat liver microsomes was induced by TTA, seen as a rise of specific activity in liver microsomes from TTA-fed rats. The elevated ratio between ω and ω-1 hydroxylation after TTA feeding (Table 5) can be directly compared with the

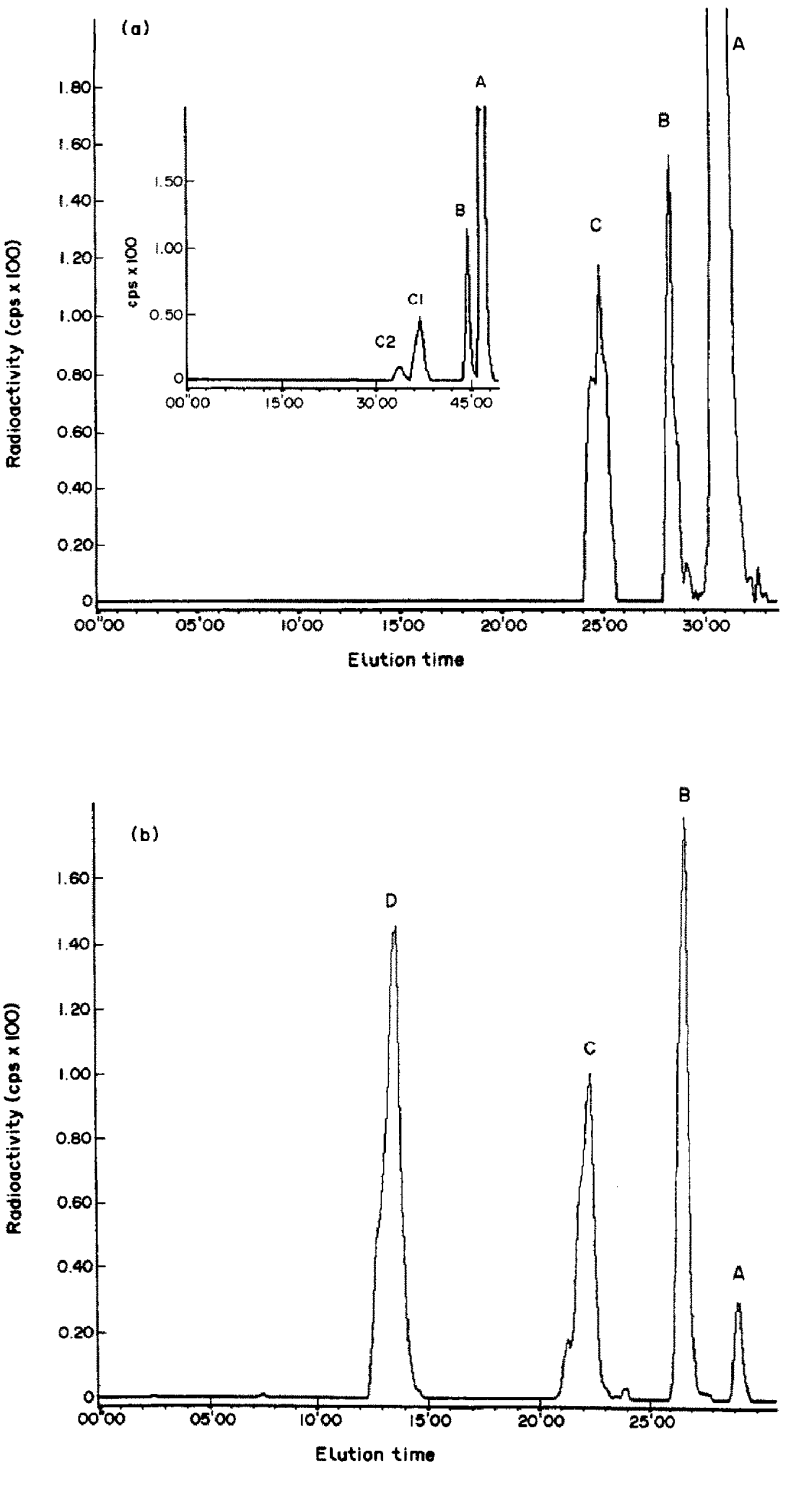

Fig. 1. HPLC analysis of the extract after oxidation of [1-^{14}C]dodecylthioacetic acid by liver microsomes from normal rats. In Fig. 1a liver microsomes from TTA fed rat were incubated for 6.0 min with 9.8 kBq (101 μM) [1-^{14}C]dodecylthioacetic acid as described. Peak labelled B was identified to be dodecylsulfoxyacetic acid (sulfur oxygenated DTA). Peak labelled C was identified to be ω-hydroxydodecylthioacetic acid (ω-hydroxylated DTA). Peak labelled A is dodecylthioacetic acid (DTA). Figure 1a insert shows that peak C (ω-hydroxylated DTA) in Fig. 1a can be separated into two peaks, C_1 (ω-hydroxylated DTA) and C_2 ((ω-1)-hydroxylated DTA). In Fig. 1b liver microsomes from normal rats were incubated for 45 min with 2.3 kBq (31 μM) [1-^{14}C]dodecylthioacetic acid as described. Peak labelled D was identified to be ω-hydroxydodecylsulfoxyacetic acid (ω-hydroxylated and sulfur oxygenated DTA). Peaks labelled A, B and C are identical to those in Fig. 1a.

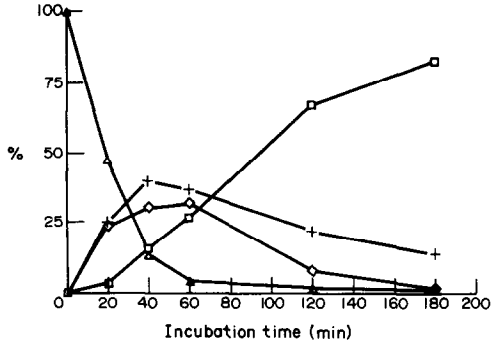

Fig. 2. Time course for the formation of metabolites from dodecylthioacetic acid incubated with liver microsomes from normal rats. Liver microsomes were incubated with [1-^{14}C]dodecylthioacetic acid (117 Bq/nmol) and a NADPH generating system as described. The formation of metabolites were followed for 3 hr. The extract from the incubation was analysed on a Spherisorb ODS column as described. The metabolites were eluted as follows: ω-hydroxydodecylsulfoxyacetic acid (□) 13.0 min, ω-hydroxydodecylthioacetic acid (+) 22 min, dodecylsulfoxyacetic acid (◇) 26 min and the substrate: dodecylthioacetic acid (△) 29 min.

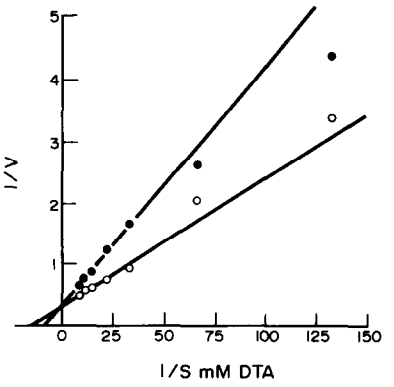

Fig. 3. Double reciprocal plot of initial velocities with variable substrate (DTA) concentration. Rat liver microsomes from normal rats were incubated with increasing concentration of DTA with a specific activity of 48 Bq/nmol as described. Double reciprocal plots of the initial velocities for formation of ω-hydroxylation (○) and S-oxygenation (●) of DTA were drawn. K_m values were estimated using a FORTRAN computer program, published by Cleland [29]. K_m value for ω-hydroxylation was estimated to 0.02 mM and K_m value for S-oxygenation to 0.12 mM.

elevated ratio of ω/ω-1 hydroxylation of lauric acid after TTA feeding (Table 4).

S-Oxygenation of DTA was not induced by TTA. The small decrease in specific activity of S-oxygenation observed after feeding with TTA (Table 5) is not significant.

DTA compares favourably with physiological substrate like lauric acid. Table 6 shows that there is no difference in specific activity of the ω-hydroxylation system when comparing the two substrates DTA and lauric acid in both normal and adapted rats.

Table 1. Structural formulas, names and abbreviations of sulfur substituted fatty acid analogues and their metabolites

Structure of compound	Systematic name and abbreviation
$CH_3—(CH_2)_{11}—S—CH_2—COOH$	Dodecylthioacetic acid (DTA)
$CH_3—(CH_2)_{11}—\overset{\overset{O}{\|}}{S}—CH_2—COOH$	Dodecylsulfoxyacetic acid
$OH—CH_2—(CH_2)_{11}—S—CH_2—COOH$	ω-Hydroxydodecylthioacetic acid
$OH—CH_2—(CH_2)_{11}—\overset{\overset{O}{\|}}{S}—CH_2—COOH$	ω-Hydroxydodecylsulfoxyacetic acid
$CH_3—(CH_2)_{13}—S—CH_2—COOH$	Tetradecylthioacetic acid (TTA)
$CH_3—(CH_2)_{13}—S—CH_2—CH_2—COOH$	Tetradecylthiopropionic acid (TTP)

Table 2. Oxidation of dodecylthioacetic acid in liver microsomes from normal rats with different reductants

Reaction mixture	ω-OH—	—SO—
Complete, − NADH/NADPH	ND	0.22 ± 0.05
Complete, + NADH	0.05 ± 0.01	0.71 ± 0.01
Complete, + NADPH	0.47 ± 0.02	0.59 ± 0.01
Complete, + NADH/NADPH	0.52 ± 0.02	0.58 ± 0.02

Liver microsomes from normal rats were incubated with [1-^{14}C]dodecylthioacetic acid and standard incubation mixture containing: − NADH/NADPH, + NADH, + NADPH and + NADH/NADPH. The extract from the incubation was analysed as described in Materials and Methods. The results are given in nmol/min/mg protein for total $\omega(\omega$-1)-hydroxydodecylthioacetic acid (ω-OH—) and dodecylsulfoxyacetic acid (—SO—) and represents the mean of four measurements ± SED.

ND = not detectable.

Table 3. Inhibition of metabolite formation from dodecylthioacetic acid incubated with liver microsomes from normal rats

Reaction mixture	Activity (% of control) ω-OH—	—SO—
Complete*	100.0 ± 11.6	100.0 ± 9.1
Complete + Methimazole	75.6 ± 6.6‡	10.2 ± 1.4§
Complete + Laurate	32.1 ± 3.7§	105.6 ± 10.2
Complete†	100.0 ± 4.1	100.0 ± 13.3
Complete + CO	0.25 ± 0.05§	112.5 ± 6.4

Rat liver microsomes were incubated for 10 min with 64 μM [1-^{14}C]dodecylthioacetic acid (117 Bq/nmol) with standard incubation mix, competing substrates and CO. The extract from the incubation medium was analysed on a Spherisorb ODS column as described. The results are given in per cent activity of control for total ω (ω-1)-hydroxydodecylthioacetic acid (ω-OH—) and dodecylsulfoxyacetic acid (—SO—) and the tabulated values represent means ± SED for four experiments.

* Values for ω-hydroxylation and sulfur oxygenation of DTA were 2.32 ± 0.20 and 1.43 ± 0.13 nmol/min/mg protein respectively.

† Values for ω-hydroxylation and sulfur oxygenation of DTA were 2.22 ± 0.09 and 1.42 ± 0.20 nmol/min/mg protein respectively.

‡ Significant P = 0.02.

§ Extremely significant P < 0.0001.

Table 4. ω-(ω-1)-Hydroxylation of lauric acid in liver microsomes from rats treated with different compounds

Compounds		ω-(ω-1)-Hydroxylation	ω/ω-1
None	(4)	1.13 ± 0.26	1.1
Palmitate	(2)	3.04 ± 1.55	1.4
DC-6	(2)	0.53 ± 0.28	1.0
DC-8	(2)	1.75 ± 0.32	1.4
DC-14	(2)	0.59 ± 0.41	0.7
DC-16	(2)	1.04 ± 0.64	2.0
Tiadenol	(2)	12.30 ± 1.37*	3.8
DC-Tiadenol	(4)	12.35 ± 2.05*	4.3
TTA	(2)	15.30 ± 2.31*	4.4
TTP	(2)	0.56 ± 0.01	0.6

Liver microsomes from rats treated with different compounds as described in Materials and Methods were incubated with [1-^{14}C]lauric acid for 5 min. The results are given as nmol lauric acid oxidized per min per mg protein and the tabulated values represent means ± SED for the numbers of animals indicated in parenthesis. Statistically significant differences for the controls (none) are indicated by: *0.01 > P.

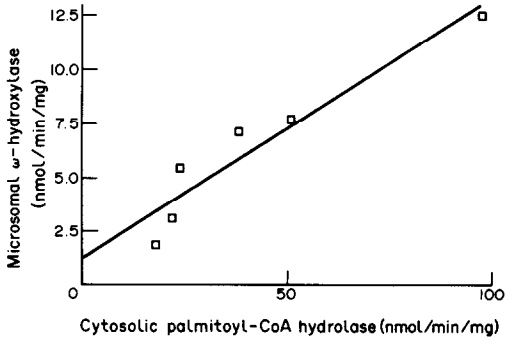

Fig. 4. Dose–response relationship of TTA. Dose–response relationship of TTA on ω-hydroxylation of lauric acid and palmitoyl-CoA hydrolase measured in the microsomal fraction and the cytosolic fraction respectively. Rats were given 0, 75, 150, 250, 500 and 750 mg TTA per day per kg body weight through gastric tube for 5 days. The tabulated values represent the means of three animals in each treatment group and six in the control group and are given as nmol/min/mg protein.

Table 5. Oxidation of dodecylthioacetic acid in liver microsomes from normal and TTA-treated rats

Compounds		ω-OH—	ω/ω-1	—SO—
None	(3)	1.6 ± 0.1	1.5	1.5 ± 0.1
TTA	(4)	8.0 ± 1.1*	4.4	1.3 ± 0.2

Liver microsomes from normal and TTA-treated rats were incubated with 101 μM [1-^{14}C]dodecylthioacetic acid as described. Rats weighing 225–300 g were given 50 mg TTA in 2% carboxymethylcellulose through a gastric tube once a day for 4 days. The results are given in nmol/min/mg protein for total ω (ω-1)-hydroxydodecylthioacetic acid (ω-OH—) and dodecylsulfoxyacetic acid (—SO—) and the tabulated values represent means ± SED for the numbers of animals indicated in parentheses. Statistically significant differences for the controls (none) are indicated by: *0.01 > P.

Table 6. ω-Hydroxylation of lauric acid and DTA in liver microsomes from normal and TTA treated rats

Compounds	Lauric acid ω-hydroxylation	DTA ω-hydroxylation
None (2)	1.1/1.5	1.3/1.7
TTA (2)	10.6/7.6	10.4/9.0

Liver microsomes from normal and TTA-treated rats were incubated with 101 μM (6.5–9.0 kBq) [1-^{14}C]dodecylthioacetic acid and [1-^{14}C] lauric acid. Rats weighing 200–300 g were given 50 mg TTA in 2% carboxymethylcellulose through a gastric tube once a day for 4 days. The results are from two normal and two TTA treated rats and are given in nmol/min/mg protein for the total ω (ω-1)-hydroxylation of lauric acid and DTA.

DISCUSSION

The results in this study indicate that the enzymes catalysing the initial metabolism of DTA in rat liver microsomes are: liver microsomal flavin-containing monooxygenase (FMO), sulfur oxygenation of DTA, and cytochrome P450IVA1 (the lauric acid ω-hydroxylase), ω-hydroxylation of DTA. The conclusions are based on the following observations: the sulfur oxygenation is dependent either on NADH or NADPH as reductants (Table 2). The estimated K_m values for, respectively, NADH and NADPH for sulfur oxygenation of DTA are 0.23 mM and 0.05 mM, giving a K_m(NADH)/K_m(NADPH) ratio of nearly 5. The purified hog liver microsomal FMO enzyme catalysing oxygenation of nucleophilic nitrogen or sulfur containing compounds has been shown to be active with both NADH and NADPH with an estimated K_d value for NADH (0.167 mM) that is much larger than for NADPH (0.008 mM) [18]. Inhibition studies also support the proposal that S-oxygenation is catalysed by a liver microsomal FMO (Table 3). S-Oxygenation activity is not reduced by CO, but markedly reduced by methimazole, a sulfur containing compound that has been shown to be oxidized exclusively by the liver microsomal FMO [19]. Reports on the substrate specificity of the enzyme, however, disagree somewhat with these results. Investigations with purified hog liver FMO have shown that molecules with a carboxylic group one or two carbons removed from the sulfur atom exhibited no substrate activity when measuring O_2 consumption [20]. One explanation for this discrepancy may be our more sensitive analytical method. The existence of isoenzymes with different specificities is another possibility. The native state of the FMO is endoplasmic reticulum bound and a lipid matrix could affect the properties of the enzyme. The results found here agree with recent studies on oxidation of *S*-benzyl-L-cysteine, which has a carboxyl group two carbons removed from the sulfur atom. Its S-oxygenation is inhibited by FMO inhibitors in rat liver microsomes [21].

The oxygenation of the sulfur atoms of Tiadenol, a related hypolipidemic agent, has also been studied and sulfoxide and sulfone formation was detected. The oxygenation of the sulfur atoms was suggested to be catalysed by a cytochrome P450 enzyme system

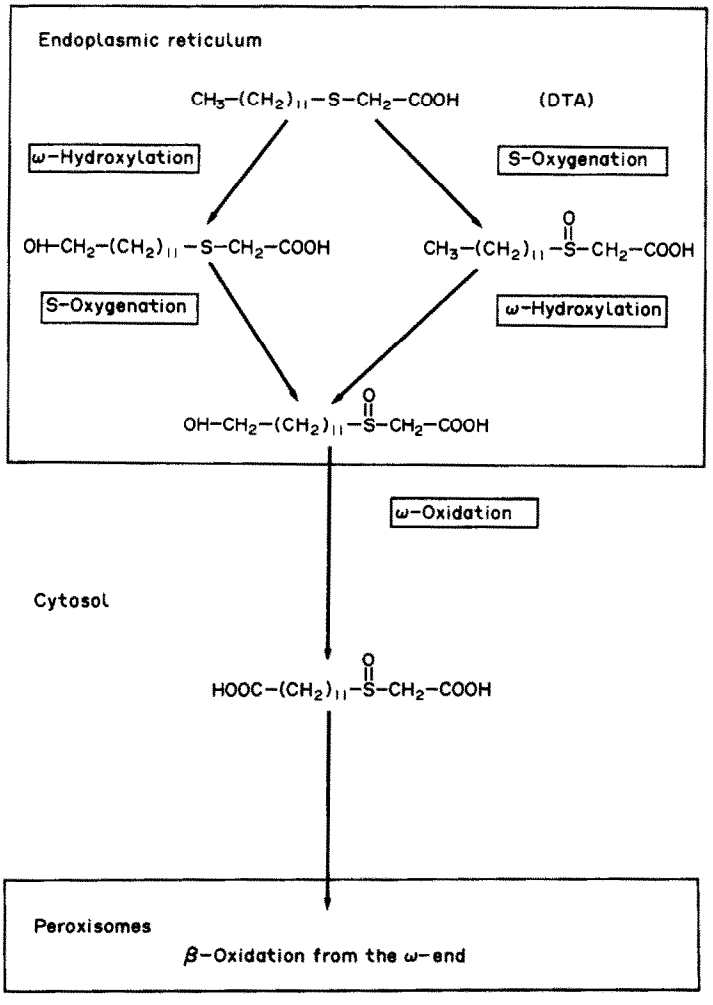

Fig. 5. Scheme to show the proposed first steps in the metabolism of dodecylthioacetic acid (DTA) in rat liver microsomes.

in rat liver microsomes [22]. FMO activity was not observed in that study possibly because the enzyme was inactivated by preincubation above 35° in the absence of NADPH [23]. In this study no evidence was found for the participation of cytochrome P450 in sulfur oxygenation.

ω-Hydroxylation of DTA showed no activity with NADH as reductant. It was absolutely dependent on NADPH (Table 2) and this together with the fact that ω-hydroxylation activity was markedly reduced by CO and by lauric acid and only slightly reduced by methimazole indicate the involvement of a cytochrome P450 isoenzyme, namely cytochrome P450IVA1 and that this isoenzyme is identical with that which ω-hydroxylates laurate and other fatty acids.

Induction of peroxisome proliferation in the liver is associated with proliferation of smooth endoplasmic reticulum [24]. Peroxisome proliferators appear to selectively induce liver cytochrome(s) P450 (cytochrome P450IVA1) that catalyses ω-hydroxylation of medium chain fatty acids [25, 26]. Long-chain alkylthioacetic acids (TTA) also induce both peroxisomal β-oxidation [1] and fatty acid ω-hydroxylation (Table 4). Other studies on the induction of the ω (ω-1)-hydroxylation of fatty acids by different hypolipidemic agents show, as found here, a preferential induction of ω-hydroxylation over (ω-1)-hydroxylation [15, 27]. No substantial increase in fatty acid ω-hydroxylation was found after feeding with different dicarboxylic acids, fatty acid and alkylthiopropionic acid (Table 4). These compounds also have only a marginal effect on peroxisomal β-oxidation [1].

Table 5 shows that feeding rats with TTA also induces ω-hydroxylation of DTA. This is in agreement with our proposal that cytochrome P450IVA1, specific for ω-hydroxylation of lauric acid, catalyses ω-hydroxylation of DTA. Like lauric acid only ω-hydroxylation in contrast to (ω-1)-hydroxylation of DTA is substantially increased after feeding with TTA. The increase in the ratio of ω/ω-1 for DTA after feeding with TTA (Table 5) equals the increase in the ratio for lauric acid (Table 4). A direct comparison of the specific activity of the fatty acid ω-hydroxylation enzyme system between DTA and lauric acid as substrates indicates that DTA is as good a substrate as lauric acid, *in vitro*, both with normal and induced rat liver microsomes (Table 6). A close relationship has been observed between microsomal cytochrome P450IVA1 induction and peroxisome proliferation by different hypolipidemic agents [27, 28]. The results from this study (Fig. 4) also indicate the same. TTA when fed to rats induces peroxisomal β-oxidation, palmitoyl-CoA hydrolase and microsomal ω-hydroxylation of lauric acid and the dose-response relationship of TTA on the three enzymes gives a correlation above 0.91 (Fig. 5 and text). The time–response relationship of TTA on peroxisomal β-oxidation and microsomal ω-hydroxylation of DTA also show a close correlation (results not shown).

In vivo alkylthioacetic acids are excreted in the urine as dicarboxylic sulfoxides [4]. The initial ω-hydroxylation and/or S-oxygenation of DTA in the microsomal fraction is in agreement with the formation of dicarboxylic metabolites *in vivo*. Evidently the ω-alcohol group is further oxidized to a carboxylic group. The long chain dicarboxylic acid thus formed is then shortened, presumably by the peroxisomal β-oxidation enzyme system.

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REFERENCES

1. Berge RK, Aarsland A, Kryvi H, Bremer J and Aarsaether N, Alkylthioacetic acids (3-thia fatty acids)—A new group of non β-oxidizable, peroxisome-inducing fatty acid analogues I. A study on the structural requirements for proliferation of peroxisomes and mitochondria in rat liver. *Biochim Biophys Acta* **1004**: 345–356, 1989.
2. Spydevold Ö and Bremer J, Induction of peroxisomal β-oxidation in 7800 C1 Morris hepatoma cells in steady state by fatty acids and fatty acid analogues. *Biochim Biophys Acta* **1003**: 72–79, 1989.
3. Skrede S, Narce M, Bergseth S and Bremer J, The effects of alkylthioacetic acids (3-thia fatty acids) on fatty acid metabolism in isolated hepatocytes. *Biochim Biophys Acta* **1005**: 296–302, 1989.
4. Bergseth S and Bremer J, *In vivo* metabolism of alkylthioacetic acids (3-thia fatty acids). A new class of hypolipemic and peroxisome proliferating fatty acid analogues. *Biochim Biophys Acta* **1045**: 90–93, 1990.
5. Wada F, Shibata H, Goto M and Sakamoto Y, Participation of the microsomal electron transport system involving cytochrome P-450 in ω-oxidation of fatty acids. *Biochim Biophys Acta* **162**: 518–524, 1968.
6. Kusunose E, Ogita K, Ichihara K and Kusunose M, Effect of cytochrome b5 on fatty acid ω- and (ω-1)-hydroxylation catalysed by partially purified cytochrome P-450 from rabbit kidney cortex microsomes. *J Biochem* **90**: 1069–1076, 1981.
7. Mitz MA and Heinrikson RI, Omega hydroxy fatty acid dehydrogenase. *Biochim Biophys Acta* **46**: 45–50, 1961.
8. Mortensen PB, Kolvraa S, Gregersen N and Rasmussen K, Cyanide insensitive and clofibrate enhanced β-oxidation of dodecanedioic acid in rat liver. An indication of peroxisomal β-oxidation of *N*-dicarboxylic acids. *Biochim Biophys Acta* **713**: 393–397, 1982.
9. Cerdan S, Künnecke B, Dölle A and Seelig J, *In situ* metabolism of 1, omega medium chain dicarboxylic acids in the liver of intact rats as detected by ^{13}C and ^{1}H NMR. *J Biol Chem* **263**: 11664–11674, 1988.
10. Suzuki H, Yamada J, Watanabe T and Suga T, Compartmentation of dicarboxylic acid β-oxidation in rat liver: importance of peroxisomes in the metabolism of dicarboxylic acids. *Biochim Biophys Acta* **990**: 25–30, 1989.
11. Bergseth S, Poisson J-P and Bremer J, Metabolism of dicarboxylic acids in rat hepatocytes. *Biochim Biophys Acta* **1042**: 182–187, 1990.
12. Ortiz de Montellano PR and Reich NO, Specific inactivation of hepatic fatty acid hydroxylases by acetylenic fatty acids. *J Biol Chem* **259**: 4136–4141, 1984.
13. Zeigler DM, Microsomal flavin-containing monooxygenase: Oxygenation of nucleophilic nitrogen and sulfur compounds. In: *Enzyme Basis of Detoxication* (Ed. Jakoby WB), pp. 201–227. Academic Press, New York, 1980.

14. Oae S, Mikami A, Matsuura T, Ogawa-Asada K, Watanabe Y, Fujimori K and Iyanagi T, Comparison of sulfide oxygenation mechanism for liver microsomal FAD-containing monooxygenase with that for cytochrome P-450. *Biochem Biophys Res Commun* **131**: 567–573, 1985.
15. Nilsson A, Arey H, Pedersen JI and Christiansen EN, The effect of high-fat diets on microsomal lauric acid hydroxylation in rat liver. *Biochim Biophys Acta* **879**: 209–214, 1986.
16. Berge RK and Bakke OM, Changes in lipid metabolizing enzymes of hepatic subcellular fractions from rats treated with tiadenol and clofibrate. *Biochem Pharmacol* **30**: 3251–3256, 1981.
17. Berge RK and Dossland B, Differences between microsomal and mitochondrial-matrix palmitoyl-coenzyme A hydrolase, and palmitoyl-L-carnitine hydrolase from rat liver. *Biochem J* **181**: 119–125, 1979.
18. Beaty NB and Ballou DP, The reductive half-reaction of liver microsomal FAD-containing monooxygenase. *J Biol Chem* **256**: 4611–4618, 1981.
19. Ziegler DM and Poulsen LL, Hepatic microsomal mixed-function amine oxidase. *Methods Enzymol* **52**: 142–151, 1978.
20. Taylor KL and Ziegler DM, Studies on substrate specificity of the hog liver flavin-containing monooxygenase. *Biochem Pharmacol* **36**: 141–146, 1987.
21. Sausen PJ and Elfarra AA, Cysteine conjugate *S*-oxidase. *J Biol Chem* **265**: 6139–6145, 1990.
22. Maffei Facino R, Carini M and Tofanetti O, Metabolism of the hypolipidemic agent tiadenol in man and in the rat. *Arzneim-Forsch/Drug Res* **36**: 722–728, 1986.
23. Ziegler DM, Flavin-containing monooxygenases. *Drug Metab Rev* **19**: 33–62, 1988.
24. Reddy JK and Krishnakantha TP, Hepatic peroxisome proliferation: Induction by two novel compounds structurally unrelated to clofibrate. *Science* **190**: 787–789, 1975.
25. Orton TC and Parker GL, The effect of hypolipidemic agents on the hepatic microsomal drug-metabolizing enzyme system of the rat. *Drug Metab Dispos* **10**: 110–115, 1982.
26. Gibson GG, Orton TC and Tamburini PP, Cytochrome P-450 induction by clofibrate. *Biochem J* **203**: 161–168, 1982.
27. Sharma R, Lake BG, Foster J and Gibson GG, Microsomal cytochrome P-452 induction and peroxisomal proliferation by hypolipidaemic agents in rat liver. *Biochem Pharmacol* **37**: 1193–1201, 1988.
28. Lake BG, Gray TJB, Pels Rijcken WR, Beamand JA and Gangolli SD, The effect of hypolipidaemic agents on peroxisomal β-oxidation and MFO activities in primary cultures of rat hepatocytes: Relationship between induction of palmitoyl-CoA oxidation and lauric acid hydroxylation. *Xenobiotica* **14**: 269–276, 1984.
29. Cleland WW, The statistical analysis of enzyme kinetic data. *Adv Enzymol* **29**: 1–32, 1967.