Biochemical Pharmacology, Vol. 41, No. 1, pp. 53–61, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

PEROXISOME PROLIFERATING SULPHUR- AND OXY-SUBSTITUTED FATTY ACID ANALOGUES ARE ACTIVATED TO ACYL COENZYME A THIOESTERS

ASLE AARSLAND*† and ROLF K. BERGE
Laboratory of Clinical Biochemistry, University of Bergen, N-5021 Haukeland Sykehus, Norway

(*Received* 25 *May* 1990; *accepted* 10 *August* 1990)

Abstract—In liver homogenates from untreated rats the sulphur-substituted fatty acid analogues tetradecylthioacetic acid (CMTTD) was activated to its acyl-coenzyme A thioester. The activation was found to take place in the mitochondrial, microsomal and peroxisomal fractions. The activity of CMTTD-CoA synthetase was 50% compared to palmitoyl-CoA synthetase in all cellular fractions. When rats were treated with the peroxisome proliferating sulphur-substituted fatty acid analogues CMTTD and 3-dithiahexadecanedioic acid (BCMTD), the CMTTD-CoA synthetase activity was induced in mitochondrial, peroxisomal and microsomal fractions. Palmitoyl-CoA synthetase was induced proportionally. In rats treated with tetradecylthiopropionic acid (CETTD) of low peroxisome proliferating potency, the activities of CMTTD-CoA synthetase and palmitoyl-CoA synthetase were inhibited in mitochondrial and microsomal fractions. In contrast, all three sulphur-substituted acids induced the activity of palmitoyl-CoA synthetase and CMTTD-CoA synthetase in peroxisomes. Both the CMTTD-CoA and palmitoyl-CoA synthetase activities were induced by CMTTD and BCMTD, in close correlation to the induction of peroxisomal β-oxidation. During the three treatment regimes, CMTTD-CoA synthetase activity ran parallel to the palmitoyl-CoA synthetase activity at a rate of 50% in all cellular fractions. Thus, CMTTD is assumed to be activated by the long-chain acyl-CoA synthetase enzyme. Rats were treated for 5 days with sulphur- and oxy-substituted fatty acid analogues, clofibric acid and fenofibric acid. All compounds which induced peroxisomal β-oxidation activity *in vivo* could be activated to their respective CoA thioesters in liver homogenate. CETTD which induced peroxisomal β-oxidation only two-fold, was activated at a rate of 50% compared to palmitate. Fenofibric acid induced peroxisomal β-oxidation 9.6-fold, while it was activated at a rate of only 10% compared to palmitate. Thus, no correlation was found between rate of activation *in vitro* and induction of peroxisomal activity *in vivo*. On the other hand, tetradecylsulfoxyacetic acid (TSOA) and tetradecylsulfonacetic acid (TSA) (sulphuroxygenated metabolites of CMTTD) with no inductive effects, were not activated to their respective CoA derivatives. Altogether the data suggest that the enzymatic activation of the peroxisome proliferating compounds is essential for their proliferating activity, but the rate of activation does not determine the potency of the proliferators. The role of the xenobiotic-CoA pool in relation to the whole coenzyme A profile during peroxisome proliferation is discussed.

It has previously been postulated that the induction of hepatic peroxisome proliferation by various hypolipidemic drugs as well as by different metabolic states is exerted through a common signal. An increased cellular level of long-chain acyl-CoA has been proposed as a common trigger to account for the rather uniform inductive response caused by the different regimes [1, 2]. The postulate was based on the observation that treatment with different peroxisome proliferating compounds caused an increased level of acyl-CoA [1]. Similarly situations characterized by a high hepatic influx of fatty acids, i.e. starvation and uncontrolled diabetes [3–5] and especially high fat diets [6–8], all cause induction of peroxisomal β-oxidation in rodents in conjunction with an enhanced level of acyl-CoA. Proliferation of peroxisomes is encountered in man in situations characterized by a dysfunctional mitochondrial fatty acid β-oxidation and an altered acyl coenzyme A profile [9]. Several peroxisome proliferating compounds are known to be activated to their respective CoA thioesters both *in vivo* and *in vitro* [10–13]. Based on the observation that peroxisome proliferating compounds frequently possess a carboxylic acid functional group, or a group that can be readily oxidized to such, it has been proposed that the proliferators generally are activated to acyl-CoA thioesters and that these derivatives play a causative role in the induction process [12–14].

During treatment with analogues of fatty acids (sulphur-substituted, non β-oxidizable fatty acids) it was confirmed that the induction of peroxisomal β-oxidation correlated well with the enhanced level of long-chain acyl-CoA [15]. The reported acyl-CoA level did not differentiate between long-chain acyl-CoA derived from endogenous fatty acids and coenzyme A thioesters of the proliferators (xenobiotic acyl-CoA). The objective of this investigation was to further penetrate the relationship between endogenous long-chain acyl-CoA, xenobiotic acyl-CoA and peroxisome proliferation. The activation of sulphur- and oxy-substituted fatty acids to acyl-CoA thioesters is investigated. Representatives of another category of proliferators, clofibric and

* To whom correspondence should be addressed.
† Research fellow of Norwegian Research Council for Science and Humanities.

fenofibric acid are also included in the experiment. The rate of activation of the selected compounds *in vitro* is related to their peroxisome proliferating potency *in vivo*. The obtained results substantiate the theory that not merely the long-chain acyl-CoA esters of fatty acids, but the whole acyl-CoA level, including xenobiotic acyl-CoA, may be the stimulus for peroxisome proliferation and induction of associated enzyme activities.

MATERIAL AND METHODS

Chemicals and drugs. The synthesis of the sulphur-substituted fatty acid analogues tetradecylthioacetic acid (CMTTD), tetradecylthiopropionic acid (CETTD), 3-dithiahexadecanedioic acid (BCMTD), tetradecylsulfoxyacetic acid (TSOA) and tetradecylsulfonacetic acid (TSA) was performed as described previously [16]. Similarly the synthesis of oxy-substituted acids, tetradecyloxyacetic acid (CMOTD) and teradecyloxypropionic acid (CEOTD) has been described previously [17]. Palmitic acid and hexadecanedioic acid were purchased from Aldrich-Chemie (Steinheim, F.R.G.) Clofibric acid was provided by ICI Ltd (Macclesfield, U.K.). Fenofibric acid was a gift from Dr A. D. Edgar, Laboratories Fournier s.a. (Dijon, France). Figure 1 gives the structure of the different compounds.

[^{3}H]Coenzyme-A was purchased from New England Nuclear, (Boston, MA, U.S.A.), coenzyme-A was supplied by the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals and diets. Male Wistar rats from Möllegaard Breeding Laboratory (Ejby, Denmark) weighing 170–200 g, were housed individually in metal wire cages in a room maintained at 12 hr light–dark cycles and at a constant temperature of 20 ± 3°. The animals were acclimatized for at least 1 week under these conditions before the start of the experiments. While the animals were administered the selected compounds, they were fed a commercial pelleted food and had free access to water.

In the first series, the animals were given either tetradecylthioacetic acid (CMTTD), tetradecylthiopropionic acid (CETTD) or 3-dithiahexadecanedioic acid (BCMTD), at a dose of 150 mg/kg body weight/day for up to 14 days.

In a separate series of experiments animals were administered all compounds given in Fig. 1 [tetradecylthioacetic acid (CMTTD), tetradecylthiopropionic acid (CETTD), 3-dithiahexadecanedioic acid (BCMTD), tetradecyloxyacetic acid (CMOTD), teradecyloxypropionic acid (CEOTD), palmitic acid (PMA), hexadecanedioic acid (HDDA), clofibric acid and fenofibic acid] at a dose of 250 mg/kg body weight/day. Tetradecylsulfoxyacetic acid (TSOA) and tetradecylsulfonacetic acid (TSA) were administered at a dose of 150 mg/kg body weight/day.

In both series the compounds were suspended in 0.5% (w/v) carboxymethylcellulose and the suspensions were micronized by ultrasonication. The drugs were administered by gastric intubation (gavage) in a volume of 1 mL, once a day. Three animals were used for each treatment and six control animals received the vehicle only. The body weights were measured daily.

At the start of the sixth day, after 12 hr of starvation, the rats were killed and the livers were immediately removed, weighed and chilled on ice.

Preparation of fractions. The livers from individual rats were homogenized in ice-cold sucrose medium (0.25 M sucrose in 10 mM HEPES buffer, pH 7.4, and 1 mM EDTA) using a Potter–Elvehjem homogenizer at 720 rev/min and with two strokes of a loosely fitting Teflon pestle. The resulting nuclear plus post-nuclear fraction was used as the total homogenate. For further analytical differential centrifugation, the post-nuclear fractions from three treated animals were pooled while the homogenate from six control animals were fractionated separately. A mitochondrial-enriched fraction (M), light mitochondrial fraction (L), microsomal fraction (P) and a cytosolic fraction (S) were isolated as previously described [13]. All procedures were performed at 0–4°. The different fractions were stored below −80° until analysed. The variation in the response from animal to animal was estimated separately for selected enzymes in the group of control animals.

Isolation of peroxisomes. Peroxisomes were isolated according to the procedure of Ghosh and Hajra [18] with slight modifications. The light mitochondrial fraction was washed once with the homogenizing buffer and then suspended in the same buffer to approximately 8 mg protein/mL. Two millilitres of the suspended L fraction was layered on top of 15 mL of 30% Nycodenz (10 mM HEPES buffer, pH 7.4, and 1 mM EDTA) (w/v) in a Beckman 338820 tube. The tube was then centrifuged in a Beckman L-8 70 centrifuge at 30,000 rpm (81,043 g_{avg}) for 60 min in a T 30 rotor. After centrifugation a loose pellet was formed (the peroxisomes) which was suspended in Nycodenz medium after the supernatant was removed by gentle suction.

Enzyme assays and other analytical methods. The tetradecylthioacetic-CoA (CMTTD-CoA) synthetase assay was run essentially as the palmitoyl-CoA synthetase [19] using ^{14}C-labeled tetradecylthioacetic acid (CMTTD). Palmitoyl-CoA-dependent dehydrogenase (usually termed peroxisomal β-oxidation) was assayed as described [20]. Protein was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories. Richmond, CA, U.S.A.).

Acyl coenzyme A synthetase activity of unlabeled substrates was measured radiochemically as acid insoluble products of labeled CoA-thioester as previously described [13] with slight modifications. The assay mixture (0.3 mL) contained 100 mM Tris–HCl buffer, pH 7.4, 4 mM $MgCl_2$, 1.5 mM ATP, 0.4 mM [^{3}H]CoA, 0.6 mM DTT and 0.85 mg/mL of protein. Unlabeled substrates to be tested were dissolved in minimum KOH at 37°. The reaction was started with the selected unlabeled substrates (0.33 mM) or an equal volume of water for the blank, run at 37° for various time intervals (0–10 min), and then stopped by adding an equal volume of $HClO_4$ (0.3 mL) giving a concentration of 6% (v/v). The tube was agitated vigorously for 3 min

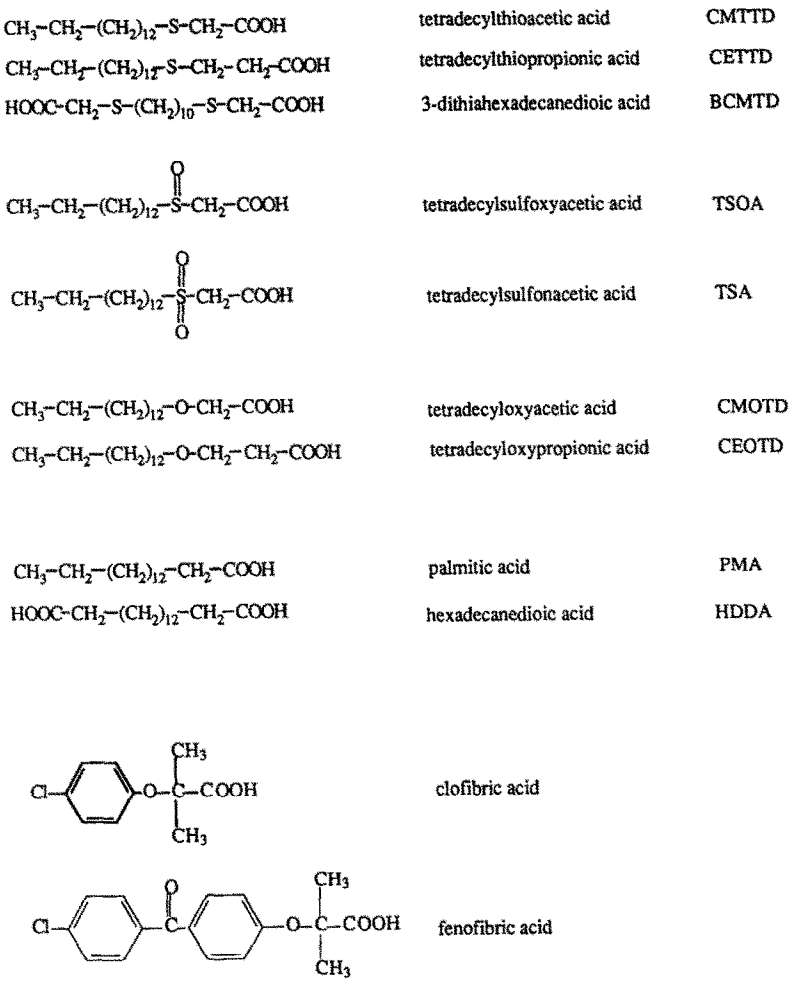

Fig. 1. Structure and abbreviation of the sulphur- and oxy-substituted fatty acid analogues, clofibric acid and fenofibric acid.

and centrifugated at 3000 rpm for 10 min. The pellet was washed in 0.6% $HClO_4$ and centrifuged twice (3000 rpm for 10 min) before finally being resuspended in water (0.75 mL).

With no substrate added to the reaction mixture (only water), an activity of 5% was found, relative to the activity observed with palmitate (100%). This activity was ascribed to endogenous fatty acids being activated to their respective CoA thioesters. No albumin was added to the reaction mixture to entrap fatty acids in liver homogenate. In the following experiments with different substrates for the CoA synthetase, parallels were run with only water added and the calculated activity subtracted from the activities found for selected substrates.

As the method is based on the detection of acid insoluble products, the relative acid to water solubility of the CoA derivative is decisive. To evaluate the method, the reaction was run with ordinary fatty acids with different chain lengths. Acyl-CoA derivatives of fatty acids with chain length of eight carbons or more were detectable, while hydrocarbon-chain lengths of six or less were not detectable (data not shown). This interception corresponds to the water soluble/insoluble schism of ordinary fatty acids. The rate of activation of ordinary fatty acids with chain lengths of eight carbons or more were, relative to palmitate, in accordance with what has been found previously in rat liver [21] (data not shown). The C12-fatty acid (dodecanoic acid), had the highest rate of activation, amounting to 130% relative to palmitate. Thus, the method seemed valid for quantitating the formation of CoA thioesters of water insoluble substrates.

The activities of a number of marker enzymes were measured: for the M fraction, succinate:phenazine methosulfate (PMS) oxidoreductase [22] and glutamate dehydrogenase [23], for the isolated peroxisomes acid phosphatase [24], catalase [25], urate oxidase [26], and for the P fraction, rotenone-insensitive NADPH cytochrome *c* reductase [27].

Statistical analysis. A two-way analysis of variance and the Student's *t*-test was used to evaluate the significance between population means:

Table 1. (A) Palmitoyl-CoA synthetase and (B) tetradecylthioacetic-CoA (CMTTD-CoA) synthetase activity (nmol/min/mg) in mitochondrial (M) and microsomal (P) fractions during 3 days treatment with tetradecylthioacetic acid (CMTTD), dithiahexadecanedioic acid (BCMTD) and tetradecylthiopropionic acid (CETTD) at a daily dose of 150 mg/kg body weight

Days of Treatment	CMTTD		BCMTD		CETTD	
	Subcellular fractions					
	M	P	M	P	M	P
(A) Palmitoyl-CoA synthetase activity						
0	78.7 ± 1.5	161.3 ± 1.9	78.7 ± 1.5	161.3 ± 1.9	78.7 ± 1.5	161.3 ± 1.9
0.5	82.2	181.9	86.5	160.0	71.5	159.1
1.5	65.1	203.6	103.3	226.3	65.9	163.2
3	104.5	261.9	134.0	240.2	59.6	144.7
(B) Tetradecylthioacetic-COA (CMTTD-CoA) synthetase activity						
0	38.0 ± 1.3	80.1 ± 1.7	38.0 ± 1.3	80.1 ± 1.7	38.0 ± 1.3	80.1 ± 1.7
0.5	41.5	87.7	41.9	80.6	32.0	66.6
1.5	34.7	89.9	51.1	111.7	32.9	70.9
3	49.2	121.4	55.5	119.0	30.8	70.7

Data represent the means ± SD for six control animals (0 days of treatment) tested separately and the activity found in the pooled fractions from three animals (for details see Materials and Methods) treated with the individual compounds.

$P > 0.05$ was taken to be statistically non-significant.

RESULTS

Time course of the activities of palmitoyl-CoA and tetradecylthioacetic-CoA (CMTTD-CoA) synthetase during treatment with sulphur-substituted fatty acids

Palmitoyl-CoA synthetase activity. Palmitoyl-CoA synthetase is a multiorganelle localized enzyme involved in formation of long-chain acyl-CoA [19, 28]. During 3 days treatment with tetradecylthioacetic acid (CMTTD) and 3-dithiahexadecanedioic acid (BCMTD) at a dose of 150 mg/kg body weight/day, there was a progressively increasing palmitoyl-CoA synthetase activity in the mitochondrial (M) and microsomal (P) fractions (Table 1). BCMTD as the most potent inducer caused after 3 days treatment nearly a 1.7-fold stimulation of the activity in the M fraction and 1.5-fold in the P fraction (Table 1). The same treatment regime with CMTTD caused a 1.3-fold enhancement of the mitochondrial activity while the microsomal activity was stimulated 1.6-fold compared to control. Tetradecylthiopropionic acid (CETTD) treatment, decreased the palmitoyl-CoA synthetase activity in the two cellular fractions, to 76 and 89% in the M and P fractions, respectively, compared to control (Table 1). On the other hand, in isolated peroxisome (Fig. 2), treatment with all three compounds caused an increased palmitoyl-CoA synthetase activity over the 3 day period. Again BCMTD, which was the most potent inducer, caused an eight-fold increase of activity. With respect to the peroxisomal palmitoyl-CoA synthetase CETTD and CMTTD at their point of maximal stimulation, were found to be almost equipotent both causing an approximate three-fold induction of activity (Fig. 2). No palmitoyl-CoA activity was detected in the cytosolic fraction, neither in control nor treated animals (data not shown).

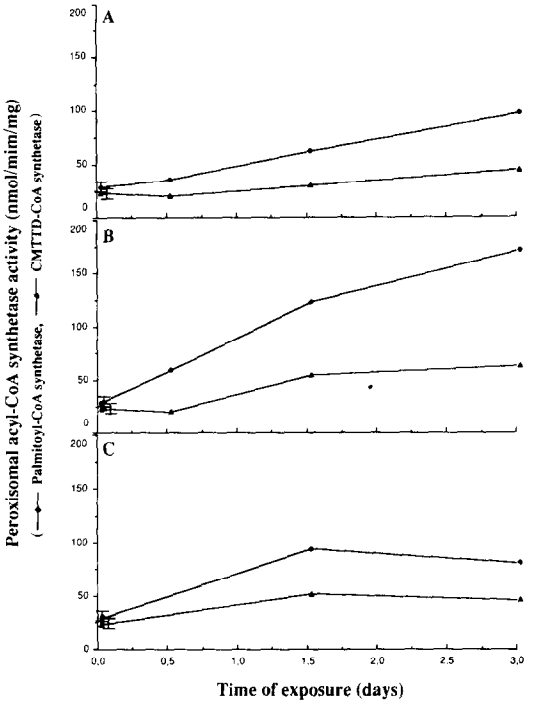

Fig. 2. Palmitoyl-CoA synthetase and tetradecylthioacetic-CoA (CMTTD-CoA) synthetase activity measured in isolated peroxisomes of rat liver during treatment with CMTTD (A), BCMTD (B) and CETTD (C) at a dose of 150 mg/kg body weight/day. For calculations and abbreviations see Table 1 and Fig. 1.

The subcellular fractions were of high purity as the distribution of protein and marker enzymes for mitochondria, microsomes and cytosol were for all groups (data not shown) essentially similar to our previous findings for rat liver homogenates [13]. The

isolated peroxisomes had a high purity. Judged by the distribution of marker enzymes (Materials and Methods) (data not shown) a contamination amounting to 0–1% with mitochondria, 0–2% with microsomes and 0–2% with lysosomes was found. Accordingly, it seems justified to conclude that the enzyme activities found in the M, P and peroxisomal fractions correspond to the mitochondrial, microsomal and peroxisomal palmitoyl-CoA synthetase activities.

CMTTD-CoA synthetase activity. The activation of labeled CMTTD to its coenzyme A thioester derivative was found to take place in mitochondrial, peroxisomal and microsomal fractions (Table 1, Fig. 2). No CMTTD-CoA synthetase activity was found in the cytosolic fraction (data not shown). During treatment with the sulphur-substituted acids, only CMTTD and BCMTD treatment stimulated the activity in the mitochondrial (M) and microsomal (P) fractions, while all three sulphur-substituted fatty acids enhanced the CMTTD-CoA synthetase activity in the isolated peroxisomes. BCMTD stimulated both the mitochondrial and microsomal activities approximately to 1.5-fold. Three days treatment with CMTTD was accompanied by an increased activity amounting to nearly 1.3- and 1.5-fold in the M and P fractions, respectively. CETTD however caused a decrease. Relative to the control the activities were 80% in mitochondrial and 88% in microsomal fraction (Table 1). The peroxisomal synthetase activity was stimulated after 3 days of treatment by all three compounds, five-fold by BCMTD and three-fold by both CMTTD and CETTD (Fig. 2).

Thus, as shown in Table 1 and Fig. 2 the CMTTD-CoA synthetase activity run parallel to the palmitoyl-CoA synthetase activity during treatment with all sulphur-substituted fatty acid analogues.

Time course of peroxisomal β-oxidation in response to treatment with sulphur-substituted fatty acids

In isolated peroxisomes from animals treated at a daily dose of 150 mg/kg body weight of CMTTD, BCMTD and CETTD, the peroxisomal β-oxidation activity was measured. During the feeding period, both CMTTD and BCMTD stimulated the β-oxidation activity (Fig. 3). After 10 days of 3-dithiahexadecanedioic acid (BCMTD) treatment, a maximal stimulatory effect was observed, amounting to approximately a six-fold increase in activity over basal value (control animals). Tetradecylthioacetic acid (CMTTD) treatment at a corresponding dose caused a 4.5-fold increase after 14 days of treatment. These results are all in agreement with what we have found in total liver homogenate after CMTTD and BCMTD feeding previously [29].

On the other hand, tetradecylthiopropionic acid (CETTD) treatment was found to have no effect on the peroxisomal β-oxidation activity in the isolated peroxisomes (Fig. 3). We have previously found that CETTD stimulated the peroxisomal parameter in total liver homogenate [29]. The findings in these experiments could be explained by the isolation procedure which may selectively isolate peroxisomes of a given subpopulation due to a narrow sedimentation window.

Activation of sulphur- and oxy-substituted fatty acid analogues, clofibric acid and fenofibric acid to coenzyme A thioesters in liver homogenate

Rats were treated for 5 days with the tetradecylthioacetic acid (CMTTD) at a dose of 250 mg/kg body weight/day. As reported previously this causes peroxisome proliferation and induction of related enzymes in rat liver [29]. The post-nuclear fraction from these animals was used as the source of protein.

Using unlabeled substrate, the thioacetic acid CMTTD was activated at a rate of approximately 50% compared to palmitate in whole liver homogenate (Table 2). This is in agreement with what was found when labeled CMTTD was used in subcellular fractions (Table 1, Fig. 2). The thiopropionic acid CETTD, was activated at approximately the same rate as CMTTD. Sulphur oxygenation of CMTTD rendered the fatty acid analogue inaccessible for activation. Neither the monooxygenated tetradecylsulfoxyacetic acid (TSOA) nor the dioxygenated tetradecylsulfonacetic acid (TSA) could be activated to their corresponding acyl-CoA derivative (Table 2).

Oxy-substitution also influenced the rate of activation. Tetradecyloxyacetic acid (CMOTD) which is to be compared with CMTTD, was activated to approximately 50% the rate of the sulphur-substituted acid. Tetradecyloxypropionic acid (CEOTD) the oxy-analogue of CETTD, was activated to barely detectable amounts.

The dicarboxylic acids, hexadecanedioic and 3-dithiahexadecanedioic acids (BCMTD), were activated approximately to the same rate, 15 and 10%, respectively, compared to the monocarboxylic acid palmitate.

As well characterized peroxisome proliferators [1, 30], the pharmacological active forms of clofibrate and fenofibrate, were also included in the experiment. Both the CoA thioesters of clofibric acid and fenofibric acid were detectable although to small amounts (Table 2).

Peroxisomal β-oxidation in response to treatment with sulphur- and oxy-substituted fatty acid analogues, clofibric acid and fenofibric acid

Animals were treated for 5 days with the compounds given in Fig. 1 and Table 2 at a daily dose of 250 mg/kg body weight. The activity of peroxisomal β-oxidation was then measured in the post-nuclear fraction. As previously reported, among the sulphur-fatty acids, BCMTD as a non β- and ω-oxidizable dicarboxylic acid, is the most potent inducer of peroxisomal β-oxidation [31]. The non β-oxidizable but ω-oxidizable CMTTD is a somewhat weaker inducer, while CETTD which is ω-oxidizable and accessible for one round of β-oxidation, had only a slight effect on the peroxisomal parameter as measured in the post-nuclear fraction.

Tetradecylsulfoxyacetic acid (TSOA) is a monooxygenase metabolite of CMTTD [32]. At a daily dose of 150 mg/kg for 5 days this metabolite had no stimulatory effect on the peroxisomal β-oxidation, neither had treatment with the corresponding sulfon, tetradecylsulfonacetic acid (TSA), any effect.

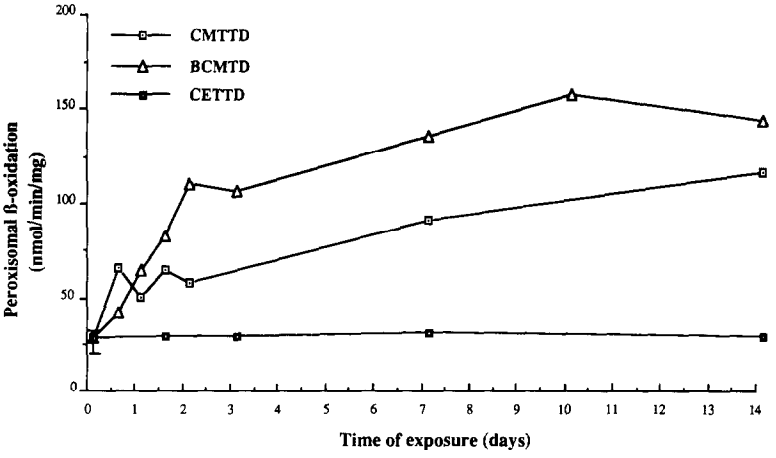

Fig. 3. Peroxisomal β-oxidation activity in isolated peroxisomes of rat liver during treatment with CMTTD, BCMTD and CETTD at a dose of 150 mg/kg body weight/day. For calculations and abbreviations see Table 1 and Fig. 1.

Table 2. The rate of acyl-CoA synthesis of sulphur- and oxy-substituted fatty acid analogues, clofibric and fenofibric acid in post-nuclear fraction from animals treated with 250 mg/day/kg of CMTTD for 5 days, expressed relative to the rate of palmitoyl-CoA synthesis = 0.81 ± 0.05 nmol/min/mg = 100%

Compounds	Acyl-CoA synthetase (%)	Peroxisomal β-oxidation (nmol/min/mg)
Control		3.8 ± 0.3
Palmitate	100	3.9 ± 0.4
CMTTD	53	24.8 ± 7.5*
CETTD	49	7.5 ± 3.8*
BCMTD	10	31.5 ± 3.8*
TSOA	0	3.2 ± 0.3†
TSA	0	3.3 ± 0.2†
CMOTD	25	6.1 ± 0.5*
CEOTD	5	7.7 ± 0.2*
HDDA	15	3.5 ± 0.3
Clofibrate	7	25.4 ± 2.4*
Fenofibrate	10	36.5 ± 3.1*

Peroxisomal β-oxidation activity (nmol/min/mg) measured in the post-nuclear fraction of animals treated with 250 mg/day/kg for 5 days of the respective compounds.

Three animals were tested separately in each treatment group and six animals in the control group. The tabulated values are the means ± SD.

* $P < 0.01$ compared to control.

† Animals treated at doses of 150 mg/kg body weight/day.

Compared to sulphur-substitution, oxy-substitution of the acetic acid had a profound effect on the inductive potency. Tetradecyloxyacetic acid (CMOTD) which is an analogue of the non β-oxidizable thioacetic acid CMTTD, had only a marginal effect on the peroxisomal parameter after 5 days treatment, causing only a two-fold increase of activity (Table 2). The oxypropionic acid tetradecyloxypropionic acid (CEOTD), which is to be compared with the β- and ω-oxidizable CETTD, was of similar potency as the sulphur-acid, causing a two-fold induction of peroxisomal β-oxidation activity (Table 2).

DISCUSSION

Palmitoyl-CoA and tetradecylthioacetic-CoA (CMTTD-CoA) synthetase activity

The palmitoyl-CoA synthetase enzyme also called the long-chain acyl-CoA synthetase, is found both in mitochondria, peroxisomes and microsomes [19, 28]. Induction of palmitoyl-CoA synthetase activity during treatment with peroxisome proliferating compounds is confirmed (Table 1), and the relative highest rate of induction was found in the peroxisomes (Fig. 2) [13]. In accordance with their varying potency as peroxisome proliferators, the

sulphur-substituted fatty acid analogues (CMTTD, BCMTD and CETTD) also have a correspondingly dissimilar effect on palmitoyl-CoA synthetase activity (Tables 1 and 2, Figs 2 and 3).

Results presented in this report (Table 1 and Fig. 2) show that the alkylthioacetic acid CMTTD was activated to its CoA thioester derivative in all three cellular fractions where the palmitoyl-CoA synthetase is known to reside. In untreated animals a CMTTD-CoA synthetase activity running at a rate of 50% compared to palmitate was found in all three cellular fractions. As the three sulphur-substituted acids (CETTD, BCMTD and CETTD) modified the palmitoyl-CoA synthetase during feeding, CMTTD-CoA synthetase activity was found to run in parallel to palmitoyl-CoA synthetase. Thus, the interrelationship of 50% endured either the palmitoyl-CoA synthetase was stimulated (CMTTD and BCMTD in all cellular fractions) or inhibited (CETTD in M and P fractions). These results strongly suggest that the xenobiotic alkylthioacetic acid CMTTD and palmitate are activated by the same enzyme, i.e. mitochondrial, peroxisomal and microsomal long-chain acyl-CoA synthetase. Sulphur-substitution of the fatty acid seems to reduce its availability for activation by 50%.

Activation to acyl-CoA thioesters of substituted fatty acid analogues, clofibric and fenofibric acid, with relation to their peroxisome proliferating capability

Through the application of labeled coenzyme A, the activation of unlabeled xenobiotics to their respective CoA thioesters could be quantitated. Using this method the activation of tetradecylthioacetic acid (CMTTD) in total liver homogenate was found to be approximately 50% compared to palmitate (Table 2). Although the recognized specific activity for CMTTD-CoA synthetase was lower using labeled CoA, the relative activity to palmitate was in agreement with what was observed using ^{14}C-labeled CMTTD (Table 1, Fig. 2).

One of the main metabolites of CMTTD in rats is the product of the liver microsomal flavin-containing monooxygenase enzyme, tetradecylsulfoxyacetic acid (TSOA) [32]. Five days treatment with this sulfoxy-metabolite at a dose of 150 mg/kg/day gave no peroxisome proliferation (Table 2). Neither could the metabolite be activated to its CoA derivative (Table 2). The same findings were made concerning the sulfon-product, tetradecylsulfonacetic acid (TSA). Regarding the peroxisomal activity, similar findings have been reported in cell-free systems [16]. On the other hand Facino *et al.* have compared tiadenol (1,10-bis(hydroxyethylthio)decane with its S-oxidized metabolite with regard to peroxisome proliferating potency [33]. The enzymatic effectors of both tiadenol and the S-oxidized metabolite are thought to be the carboxylic metabolites [13], i.e. the sulphur-substituted fatty acid. Although far less potent than tiadenol, the S-oxidized metabolite was reported to induce peroxisomal palmitoyl-CoA oxidation in rats at high doses (150 mg/kg body weight/day). In the present experiment at a comparable dose, the S-oxidized fatty acid did not induce peroxisomal β-oxidation (Table 2). Thus, the peroxisome proliferating ability of the sulphur-oxidized fatty acids is still controversial. Among the compound tested only the S-oxidized sulphur-substituted acids could not be activated. All the compounds with proliferating activity were activated to their CoA thioesters.

Among the compounds tested, fenofibric acid was the most potent inducer of peroxisomal β-oxidation (almost 10-fold compared to control), while it was only activated to a very low degree (10% compared to palmitate) (Table 2). BCMTD is the most potent peroxisome proliferator of the substituted fatty acids. Compared to CETTD which is a less potent proliferator, BCMTD was only activated at one fifth of the rate of CETTD (Fig. 3, Table 2). The tetradecyloxyacetic acid (CMOTD) which is the oxy-analogue to CMTTD, was activated at a rate of 50% compared to CMTTD. Both the oxy- and thioacetic acids (CMOTD and CMTTD) as non β-oxidizable hydrophobic compounds fulfils what is thought to be the structural requirements for peroxisome proliferating compounds [31]. Their peroxisome proliferating potency was 1 (CMOTD) to 4 (CMTTD) while their rate of activation was 1 (CMOTD) to 2 (CMTTD). Thus, no correlation was found between rate of activation *in vitro* and peroxisome proliferating potency *in vivo*.

Although they do not warrant a generalization, the results obtained suggest that the enzymatic activation of peroxisome proliferating compounds to their CoA thioesters is a prerequisite for the proliferating activity. Their rates of activation to CoA derivatives do not seem to determine their potency as peroxisome proliferators.

The described *in vitro* activation does not necessarily reflect the CoA thioester level of peroxisome proliferating compounds *in vivo*. As the ordinary long-chain acyl-CoA level, the xenobiotic-CoA level may be regarded as a pool of activated intermediates regulated by the flux into different metabolic pathways. Sulphur-substituted fatty acids are known to enter into mitochondrial and peroxisomal β-oxidation and to be incorporated into glycero- and phospholipids [34]. Their CoA derivatives are substrates for hydrolase activity (data to be published). Furthermore, the generally hydrophobic CoA esters are probably compartmentalized at subcellular level by binding to cytosolic fatty acid/acyl-CoA binding proteins or intracellular membranes. This is further complicating speculations on what role quantitatively the xenobiotic-CoA esters play on peroxisome proliferation.

Several authors have dealt with the idea that the CoA esters of peroxisome proliferators could be a common mediator accounting for the uniform inductive response caused by a structurally heterogenous group of compounds. For a review see Ref. 35. An objection raised against this generalization has been the observed proliferating activity of the leukotriene D4 antagonists (tetrazole-substituted acetophenones) [36] with no carboxylic group or groups that can be oxidized to such. At least theoretically, all of these compounds could be

substrates for the hepatic arylamine *N*-acetyltransferase (NAT;EC 2.3.1.5) and in this way attain carboxylic groups [37].

Still, the precise role of the CoA derivative is not elucidated. Both a direct interference with mitochondrial β-oxidation, a sequestration of mitochondrial free CoA which may secondarily inhibit mitochondrial β-oxidation [38] or an inhibition of mitochondrial carnitine palmitoyltransferase I [39] has been proposed to explain the role of the CoA derivatives. All mechanisms could alter the coenzyme A profile. Lately, long-chain dicarboxylic acids stemming from the co-induced microsomal ω-oxidation has been proposed to be the substrate stimulus for proliferation of peroxisomes [40]. As we have failed to observe any peroxisomal proliferation during treatment with dicarboxylic acids of different chain lengths [31] as well as high doses of hexadecanedioic acid [41], dicarboxylic acids alone do not seem to be a sufficient stimuli for proliferation. On the other hand, BCMTD which is a non-metabolizable dicarboxylic acid is both an inducer of cytochrome P452 and peroxisomes [13]. Treatment with this dicarboxylic acid which is activated to its CoA derivative, did not cause any entrapment of free CoA prior to stimulation of peroxisomal activity [15] as proposed for ordinary dicarboxylic acids.

Recently, Bronfman *et al.* [42] have shown that both naturally occurring acyl-CoAs and acyl-CoA thioesters of several peroxisome proliferators, strongly increase the activity of rat liver protein kinase C. Several key enzymes in lipid metabolism are known to be regulated by protein kinases [43]. Although still speculative, it is tempting to propose a common regulating step like the protein kinase C to account for peroxisome proliferation induced by both chemicals and pathophysiological-metabolic states (high fat diets, starvation and diabetes). Thus, the inductive response would not strictly be related to either the quantum of xenobiotic-CoA or endogenous long-chain acyl-CoA alone, but rather to the whole level of acyl coenzyme A.

Acknowledgements—Mr Svein Kryger and Mr Terje Bjørndal have contributed to these experiments with excellent technical assistance. Prof. J. Songstad, University of Bergen, provided the sulphur-substituted fatty acid analogues. The sulphur-oxygenated acetic acids were provided by Prof. J. Bremer and the oxy-substituted fatty acid analogues and the labeled alkylthioacetic acid were provided by Dr Solbakken, both at the University of Oslo. Fenofibric acid was a gift from Dr A. D. Edgar at Laboratories Fournier s.a. Dijon. The authors wish to thank them all. The study was supported by grants from "Nordisk Insulinfond" and "Ragnhild og August Gillums Legat".

REFERENCES

1. Berge RK and Aarsland A, Correlation between the cellular level of long-chain acyl-CoA, peroxisomal β-oxidation, and palmitoyl-CoA hydrolase activity in rat liver. Are the two enzyme systems regulated by a substrate-induced mechanism? *Biochim Biophys Acta* **837**: 141–151, 1985.
2. Berge RK, Hosøy LH, Aarsland A, Bakke OM and Farstad M, Enzymatic changes in rat liver associated with low and high doses of peroxisome proliferator. *Toxicol Appl Pharmacol* **73**: 35–41, 1984.
3. Thomas H, Schladt L, Knehr M and Oesch F, Effect of diabetes and starvation on the activity of rat liver hydrolase, glutatione *S*-transferase and peroxisomal β-oxidation. *Biochem Pharmacol* **38**: 4291–4297, 1989.
4. Suga T and Horie S, Changes in peroxisomal enzymes of rat liver under various physiological and pathological conditions. In: *Annals of the New York Academy of Sciences Vol. 386: Peroxisomes and Glyoxysomes* (Eds. Kindl H and Lazarow PB), pp. 430–432. New York Academy of Sciences, New York, 1982.
5. Horie S, Ishii H and Suga T, Changes in peroxisomal fatty acid oxidation in the diabetic rat liver. *J Biochem* **90**: 1691–1696, 1981.
6. Berge RK, Flatmark T and Christiansen EN, Effect of high-fat diet with partially hydrogenated fish oil on long-chain fatty acid metabolizing enzymes in subcellular fractions of rat liver. *Arch Biochem Biophys* **252**: 269–276, 1987.
7. Osmundsen H, Peroxisomal β-oxidation of long chain fatty acids: effects of high fat diets. In: *Annals of the New York Academy of Sciences Vol. 386: Peroxisomes and Glyoxysomes* (Eds. Kindly H and Lazarow PB), pp. 13–19. New York Academy of Sciences, New York, 1982.
8. Nilsson A, Thomassen MS and Christiansen EN, Long-chain acyl-CoA levels in liver from rats fed high-fat diets: is it of significance for an increased peroxisomal β-oxidation. *Lipids* **19**: 187–194, 1984.
9. Corkey BE, Hale DE, Glennon MC, Kelley RI, Coates PM, Kilpatrick L and Stanley CA, Relationship between unusual hepatic acyl coenzyme A profiles and the pathogenesis of Reye syndrome. *J Clin Invest* **82**: 782–788, 1988.
10. Lygre T, Aarsaether N, Stensland E, Aarsland A and Berge RK, Separation and measurement of clofibroyl coenzyme A and clofibric acid in rat liver after clofibrate administration by reversed-phase high-performance liquid chromatography with photodiode array detection. *J Chromatogr* **381**: 95–105, 1986.
11. Berge RK, Stensland E, Aarsland A, Tsegai G, Osmundsen H, Aarsaether N and Gjellesvik DR, Induction of cytosolic clofibroyl-CoA hydrolase activity in liver of rats treated with clofibrate. *Biochim Biophys Acta* **918**: 60–66, 1987.
12. Bronfman M, Amigo L and Morales MN, Activation of hypolipidaemic drugs to acylcoenzyme A thioesters. *Biochem J* **239**: 781–784, 1986.
13. Aarsland A, Berge RK, Bremer J and Aarsaether N, The hypolipidemic peroxisome-proliferating drug, bis(carboxymethylthio)-1.10. decane, a dicarboxylic metabolite of tiadenol, is activated to an acylcoenzyme A thioester. *Biochim Biophys Acta* **1033**: 176–183, 1990.
14. Hertz R, Arnon J and Bar-Tana J, The effect of bezafibrate and long-chain fatty acids on peroxisomal activities in cultured rat hepatocytes. *Biochim Biophys Acta* **836**: 192–200, 1985.
15. Asiedu D, Aarsland A, Skorve J, Svardal AM and Berge RK, Fatty acid metabolism in liver of rats treated with hypolipidemic sulphur-substituted fatty acid analogues. *Biochim Biophys Acta* **1044**: 211–221, 1990.
16. Spydevold Ø 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.
17. Skorve J, Asiedu D, Solbakken M and Berge RK, Comparative effects of 3- and 4-oxa-, 3- and 4-thia, sulfonyl- and sulfinyl-substituted fatty acids on serum lipids and mitochondrial- and peroxisomal fatty acid oxidation in rats. Submitted, 1990.
18. Ghosh MK and Hajra AK, A rapid method for the

isolation of peroxisomes from rat liver. *Anal Biochem* **159**: 169–174, 1986.
19. Farstad M, Bremer J and Norum KR, Long-chain acyl-CoA synthetase in rat liver. A new assay procedure for the enzyme, and studies on its intracellular localization. *Biochim Biophys Acta* **132**: 492–502, 1967.
20. Mannaerts GP, Debeer LJ, Thomas J and deSchepper PJ, Mitochondrial and peroxisomal fatty acid oxidation in liver homogenates and isolated hepatocytes from control and clofibrate-treated rats. *J Biol Chem* **254**: 4585–4595, 1979.
21. Aas M, Organ and subcellular distribution of fatty acid activating enzymes in the rat. *Biochim Biophys Acta* **231**: 32–47, 1971.
22. Arrigoni O and Singer TP, Limitations of the phenazine methosulfate assay for succinic and related dehydrogenases. *Nature* **193**: 1256–1258, 1962.
23. Schmidt E, Glutamate dehydrogenase. In: *Methods of Enzymatic Analysis, Second English Edn* (Eds. Bergmeyer HU), pp. 650–656. Verlag Chemie, Weinheim, 1974.
24. Bergmeyer HU, Phosphatase, acid. In: *Methods of Enzymatic Analysis, Second English Edn* (Eds. Bergmeyer HU), pp. 495–496, Verlag Chemie, Weinheim, 1974.
25. Aebi H, Catalase. In: *Methods of Enzymatic Analysis, Second English Edn* (Eds. Bergmeyer HU), pp. 673–684. Verlag Chemie, Weinheim, 1974.
26. Bergmeyer HU, Uricase. In: *Methods of Enzymatic Analysis, Second English Edn* (Eds. Bergmeyer HU), p. 518. Verlag Chemie, Weinheim, 1974.
27. Sottocasa GL, Kuylenstierna B, Ernster L and Bergstrand A, An electron-transport system associated with the outer membrane of liver mitochondria. *J Cell Biol* **32**: 415–438, 1967.
28. Berge RK, Flatmark T and Osmundsen H, Enhancement of long-chain acyl-CoA hydrolase activity in peroxisomes and mitochondria of rat liver by peroxisome proliferators. *Eur J Biochem* **141**: 673–644, 1984.
29. Aarsland A, Aarsaether N, Bremer J and Berge RK, Alkylthioacetic acids (3-thia fatty acids) as non-β-oxidizable fatty acid analogues: a new group of hypolipidemic drugs. III. Dissociation of cholesterol- and triglyceride-lowering effects and the induction of peroxisomal β-oxidation. *J Lipid Res* **30**: 1711–1718, 1989.
30. Price SC, Hinton RH, Mitchell FE, Hall DE, Grasso P, Blane GF and Bridges JW, Time and dose study on the response of rats to the hypolipidaemic drug fenofibrate. *Toxicology* **41**: 169–191, 1986.
31. Berge RK, Aarsland A, Kryvi H, Bremer J and Aarsaether N, Alkylthioacetic acid (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.
32. Hvattum E, Bergseth S, Pedersen CN, Bremer J, Aarsland A and Berge RK, Microsomal oxidation of dodecylthioacetic acid (a 3-thia fatty acid) in rat liver. *Biochem Pharmacol*, in press.
33. Facino R Maffei, Carini M and Tofanetti O, Carboxylic metabolites of tiadenol as "proximate" inducers of hepatic peroxisomal β-oxidation activity. *Pharmacol Res Commun* **20**: 265–276, 1988.
34. Bergseth S and Bremer J, Alkylthioacetic acids (3-thia fatty acids) are metabolized and excreated as shortened dicarboxylic acids *in vivo*. *Biochim Biophys Acta* **1044**: 237–242, 1990.
35. Lock EA, Mitchell AM and Elcombe CR, Biochemical mechanisms of induction of hepatic peroxisome proliferation. *Annu Rev Pharmacol Toxicol* **29**: 145–63, 1989.
36. Eacho PI, Foxworthy PS, Dillard RD, Whitesitt CA, Herron DK and Marshall WS, Induction of peroxisomal beta-oxidation in the rat liver *in vivo* and *in vitro* by tetrazole-substituted acetophenones—structure activity relationships. *Toxicol Appl Pharmacol* **100**: 1989.
37. Evans DAP, *N*-Acetyltransferase. *Pharmacol Ther* **42**: 157–234, 1989.
38. Veitch K, Draye J-P and Van Hoof F, Inhibition of mitochondrial β-oxidation and peroxisomal stimulation in rodent liver by valproat. *Biochem Soc Trans* **17**: 1070–1071, 1989.
39. Foxworthy PS and Eacho PI, Inhibition of hepatic fatty acid oxidation at carnitine palmitoyltransferase I by the peroxisome proliferator 2-hydroxy-3-propyl-4(6-(tetrazol-5-yl)hexyloxy)acetophenone. *Biochem J* **252**: 409–414, 1988.
40. Sharma R, Lake BG, Foster J and Gibson GG, Microsomal cytochrome P-452 induction and peroxisome proliferation by hypolipidaemic agents in rat liver. A mechanistic inter-relationship. *Biochem Pharmacol* **37**: 1193–1201, 1988.
41. Berge RK, Aarsland A, Kryvi H, Bremer J and Aarsaether N, Alkylthio acetic acids (3-thia fatty acids)—a new group of non-β-oxidizable peroxisome-inducing fatty acid analogues—II. Dose–response studies on hepatic peroxisomal- and mitochondrial changes and long-chain fatty acid metabolizing enzymes in rat. *Biochem Pharmacol* **38**: 3969–3979, 1989.
42. Bronfman M, Orellana A, Morales MN, Bieri F, Waechter F, Staubli W and Bentley P, Potentiation of diacylglycerol-activated protein kinase-C by acyl-coenzyme A thioesters of hypolipidaemic drugs. *Biochem Biophys Res Commun* **159**: 1026–1031, 1989.
43. Hardie DG, Carling D and Sim ATR, The AMP-activated protein kinase: a multisubstrate regulator of lipid metabolism. *Trends Biochem Sci* 14 January: 20–23, 1989.