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ACUTE MODULATION OF RAT HEPATIC LIPID METABOLISM BY SULPHUR-SUBSTITUTED FATTY ACID ANALOGUES

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Abstract—A single oral dose of two 3-thia (3-thiadicarboxylic and tetradecylthioacetic acids) and of 4-thia (tetradecylthiopropionic acid) fatty acids were administered to normolipidemic rats and their effects on lipid metabolism over a 24 hr period were studied. All three thia fatty acids could be detected in plasma 2 hr after treatment. Tetradecylthioacetic and tetradecylthiopropionic acids were detected in different hepatic lipid fractions but were incorporated mainly into hepatic phospholipids. Two hours after administration hepatic mitochrondrial β-oxidation and the total liver level of long-chain fatty acyl-CoA increased with a concomitant decrease in saturated fatty acids, total hepatic malonyl-CoA and plasma triacylglycerol levels in the 3-thia fatty acid groups. Tetradecylthiopropionic acid administration caused a decrease in mitochondrial β-oxidation and an increase in plasma triacylglycerol at 24 hr. The activities of key lipogenic enzymes were unaffected in all treatment groups. Plasma cholesterol level was reduced only at 8 hr in 3-thiadicarboxylic acid treated rats although 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase was suppressed already at 2, 4, 8 and 12 hr. The results show that thia fatty acids are rapidly absorbed and are systemically available after oral administration but the 3-thia fatty acids reached systemic circulation more slowly and less completely than the 4-thia fatty acid. Very low levels of the thia fatty acids are detected in plasma 24 hr after a single administration. They are incorporated into all hepatic lipid classes, especially phospholipids. Rapid incorporation of a non β-oxidizable thia fatty acid into hepatic lipids may cause a diversion of other fatty acids from glycerolipid biosynthesis to mitochondrial β-oxidation. Stimulation of mitochondrial β-oxidation and suppression of HMG-CoA reductase are primary events, occurring within hours, after 3-thia fatty acid administration. The hypotriglyceridemic effect of the 3-thia fatty acids observed at 2–4 hr is independent of the activities of key lipogenic and triacylglycerol synthesising enzymes.

Key words: hepatic lipids; hypolipidemic drugs; 3-thia fatty acids; lipogenic enzymes; cholesterogenic enzymes

Previous reports have shown that repeated administration of the 3-thia fatty acids, 3-thiadicarboxylic [1,10- bis(carboxymethylthio)decane](HOOC- CH_2-S-$(CH_2)_{10}$-S-CH_2-COOH) and tetradecylthioacetic acid (CH_3-$(CH_2)_{13}$-S-CH_2-COOH), cause hypotriglyceridemia and hypocholesterolemia in normolipidemic rats [1, 3]. The 4-thia fatty acid analogue, tetradecylthiopropionic acid (CH_3-$(CH_2)_{13}$-S-CH_2-CH_2-COOH), on the other hand, causes hyperlipidemia and fatty liver, inhibits mitochondrial β-oxidation, and it is a weak peroxisome proliferator [2, 3]. These fatty acid analogues are known to be peroxisome proliferators [1, 4, 5] and can be activated to their CoA-esters [6]. As CoA-esters, they could be incorporated into various lipid classes. Little, however, is known of the possible incorporation of thia fatty acids into lipids.

In a recent study we have shown that after a single administration of 3-thia fatty acids, hepatic mitochondrial β-oxidation and total CPT§ activity were increased within 3–4 hr and this was accompanied by a decrease in plasma triglyceride levels [7]. Since the 3-thia fatty acids are non β-oxidizable, endogenous fatty acids may be used as substrate for the hepatic mitochondrial β-oxidation system. The effect of this event on the total hepatic fatty acid composition has not been studied. Furthermore, the role of lipogenic and triacylglycerol-synthesizing enzymes in the observed rapid hypotriglyceridemia is not known.

In this paper, we studied the incorporation of thia fatty acids into hepatic lipids and the time course of their presence in liver and plasma, their effects on fatty acid composition, key lipogenic and cholesterogenic enzymes and on the flux of acyl-CoA esters after a single oral dose. The results show that the thia fatty acids are systemically available and are rapidly incorporated into hepatic lipid classes, especially phospholipids, after oral administration. Stimulation of mitochondrial β-oxidation and suppression of HMG-CoA reductase activity are primary events after an acute dose of the 3-thia fatty

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§ Abbreviations: CMC, sodium carboxymethylcellulose; CPT, carnitine palmitoyltransferase; DHA, docosahexanoic acid; EPA, eicosapentaenoic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PAP, phosphatidate phosphohydrolase; TG, triacylglycerol.

acids. The results are discussed in relation to their lipid-lowering effects.

MATERIALS AND METHODS

Chemicals. The thia fatty acids were synthesized as previously described [5]. Isotopes used in these experiments were purchased from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were obtained from common commercial sources and were of reagent grade.

Animals. Pathogen-free male Wistar rats weighing approximately 170 g were housed in metal cages and maintained on a 12-hr cycle of light and dark, at a temperature of 20 ± 3°. The rats were acclimatized for 1 week before the start of the experiments and had free access to water and standard commercial chow.

Treatment. The 3-thia fatty acids, 3-thiadicarboxylic and tetradecylthioacetic acid, the 4-thia fatty acid, tetradecylthiopropionic acid and palmitic acid were suspended in 0.5% CMC. A dose of 150 mg/kg body weight was administered by gastric intubation in a volume of 1 mL. Control animals received CMC. There were six rats in each experimental group. At 2, 4, 8, 12 and 24 hr after administration the rats were anaesthetised, cardiac puncture performed and blood was collected in EDTA vacutainers. The livers were removed, weighed and parts were immediately chilled on ice and the other part freeze-clamped in liquid N_2 and stored at −80°.

Preparation of subcellular fractions and determination of enzyme activities. Total homogenates were prepared as described earlier [3, 4, 6]. Microsomal, cytosolic, mitochondrial and peroxisome-enriched fractions were isolated as previously described [1]. The fractions were stored at −80°. Mitochondrial β-oxidation was measured in a mitochondrial-enriched fraction using radiolabelled palmitoyl-carnitine as substrate [8], acetyl-CoA carboxylase [9], fatty acid synthase [10] and ATP-citrate lyase [11] were assayed in the cytosolic fraction while PAP [12] and HMG-CoA reductase [13], assayed in the microsomal fraction were determined as previously described. Furthermore, acetyl-CoA carboxylase activity was determined in the presence of different concentrations (0, 20, 40 and 60 μM) of either palmitoyl-CoA, tetradecylthioacetyl-CoA or tetradecylthiopropionyl-CoA. The activity of HMG-CoA reductase was also determined in an incubation assay containing 20 μM HMG-CoA and in the presence of different concentrations (0, 20, 40 and 60 μM) of either palmitoyl-CoA, tetradecylthioacetyl-CoA or tetradecylthiopropionyl-CoA. All enzyme assays were run in duplicate and performed under conditions where product formation was linear with respect to both the time of incubation and the amount of protein. Protein was determined by Bio-Rad protein kit (Bio-Rad, Richmond, CA, U.S.A.).

Determination of CoA esters. Acetyl-CoA, malonyl-CoA and free CoASH levels were quantitated by a modification of the HPLC method originally described by Corkey *et al.* [14]. Briefly, 0.1 g liver was homogenized in 1 mL 5% sulphosalicylic acid containing 50 mM dithioerythritiol. The sample was centrifuged at 600 *g* for 10 min. Twenty microlitres of the acid-soluble extracts were injected into a Spectra Physics SP 8800 HPLC System. Absorbance measurements were made at 254 nm using a 3 mM Hypersil C18 reverse phase column. The mobile phase was 0.1 M NaH_2PO_4, pH 4.9 (buffer A) and buffer B (a mixture of buffer A and methanol in a ratio 7:3), pH 4.9. The flow rate was 2.0 mL/min. The profile of the elution was as follows: 0–10 min, 10–40% B; 10.1–17.6 min, 40–90% B. The retention times were 9.3, 10.9 and 13.7 min for free CoA, acetyl-CoA and malonyl-CoA, respectively.

Total hepatic long-chain acyl-CoA was estimated by incubating liver tissue precipitates with 1.5 M potassium hydroxide at 55° for 1 hr. The liberated free CoASH found in the supernatant was determined as described above.

Lipid analysis. Plasma was prepared by centrifugation of whole blood at 1000 *g* for 10 min. Triglyceride, cholesterol and phospholipid were measured using the Monotest triglyceride, cholesterol and phospholipid enzymatic kits (Boehringer, Mannheim, Germany). Hepatic lipids were quantified after extraction from total liver homogenates [15].

Quantitation and identification of tetradecylthioacetic and tetradecylthiopropionic acids in plasma and hepatic lipid fractions. Lipids were extracted from liver tissue (1 g) and plasma 400 (μL) by the Folch *et al.* [15] procedure. During the extraction of the liver samples known amounts of heptadecanoic acid, triheptadecanoylglycerol and L-α-phosphatidylcholine-diheptadecanoyl were added as internal standards; the plasma samples were fortified with heptadecanoic acid. Each liver fraction was then subjected to liquid anion exchange chromatography [16] on Superclean (TM)LC-NH_2 SPE columns (Superclo SA, Gland, Switzerland) to effect lipid class separation. The fractions containing triacylglycerol, diacylglycerol, cholesterol esters and phospholipids, as well as the plasma lipid extracts then underwent hydrolysis in 15% methanoic KOH for 45 min at 65°, followed by acidification with HCl and extraction of the liberated fatty acids with hexane. The fatty acids of each fraction were converted to picolinyl esters [17] and separated by capillary gas–liquid chromatography on a 50 m BPI 0.22 mm i.d. column (S.G.E. International, Ringwood, Victoria, Australia) or a 30 m BPX70 0.32 mm i.d. column for samples containing 3- or 4-thia fatty acids, respectively. The columns were fitted in a Carlo Erba Model 4150 gas chromatograph with flame ionization detector connected to an electronic integrator. Injections were made in a splitless mode. For samples containing tetradecylthioacetic acid the columns were initially held for 3 min at 40°, then programmed at 40°/min to 140°, held there for 3 min and finally programmed to 40°/min to 290°. For samples containing tetradecylthiopropionic acid the columns were initially held for 3 min at 40°, then programmed at 40°/min to 160°, held there for 10 min, programmed at 40°/min to 200°, held there for 3 min and finally programmed at 3°/min to 250°. The amounts of 3-

and 4-thia fatty acids were calculated using the internal standard method. The identification of the tetradecylthioacetate and tetradecylthiopropionate peaks were verified by subjecting parallel samples to gas chromatography–mass spectrometry on a Shimadzu GCMS QP-2000 equipped with a 40 m DBI 0.18 mm i.d. capillary column (J&W Scientific, Folsom, CA, U.S.A.).

Total fatty acid determination. Total lipids were extracted from liver as described by Lie *et al.* [18]. The lipid fractions were evaporated, saponified, 19:0 added as internal standard and the fatty acids esterified in 12% BG_3 in methanol. The methyl esters were separated using a Carlo Erba 2900 gas chromatograph ("cold on column" injection, $60^{49°/min}$ $160^{1°/min}$ $190^{4°/min}$ 220°), equipped with a 50 m CP-sil 88 (Chrompack) fused silica capillary column (i.d. 0.32 mm). The fatty acid composition was calculated using a Maxima 820 Chromaography Workstation software, installed in an IBM-AT, connected to the GLC and identification ascertained by standard mixtures of methyl esters (Nu-Chek, Elysian, U.S.A.).

Expression of results. Results are reported as means ± SD. The data were statistically evaluated using ANOVA and Fisher's PLSD used to determine differences between means at 95% confidence interval.

RESULTS

Levels of tetradecylthioacetic and tetradecylthiopropionic acids in hepatic lipids and plasma

The free forms of the fatty acid analogues in plasma and their incorporated residues in hepatic lipids were measured. Already at 2 hr after administration all three thia fatty acids could be detected in plasma (Fig. 1a). At the same point, the level of the 4-thia fatty acid (tetradecylthiopropionic acid) in plasma was 10 times that of the 3-thia fatty acids (3-thiadicarboxylic and tetradecylthioacetic acid). At 4 hr the levels of all the thia fatty acids decreased rapidly. 3-Thiadicarboxylic and tetradecylthioacetic acids were not detectable in plasma at 12 and 24 hr, respectively. Tetradecylthiopropionic acid was, however, detectable in plasma at 24 hr.

Tetradecylthioacetic and tetradecylthiopropionic acids were rapidly incorporated into hepatic lipids (Fig. 1b and c) and this was already observed at 2 hr. Tetradecylthioacetic acid was incorporated mainly into hepatic phospholipids and to a lesser extent into hepatic TG, diacylglycerol, free fatty acids and cholesterol ester. Tetradecylthiopropionic acid, on the other hand, was detectable only in the phospholipid fraction. Notably, at 2 and 4 hr, the concentration of tetradecylthioacetic acid incorporated into hepatic phospholipid was 8 and 2.5 times, respectively, that of incorporated tetradecylthiopropionic acid. The concentration of tetradecylthioacetic acid in hepatic phospholipids reached peak levels at 8 hr and significantly decreased after 12 hr (Fig. 1b). Tetradecylthiopropionic acid was undetectable in hepatic phospholipids after 8 hr (Fig. 1c).

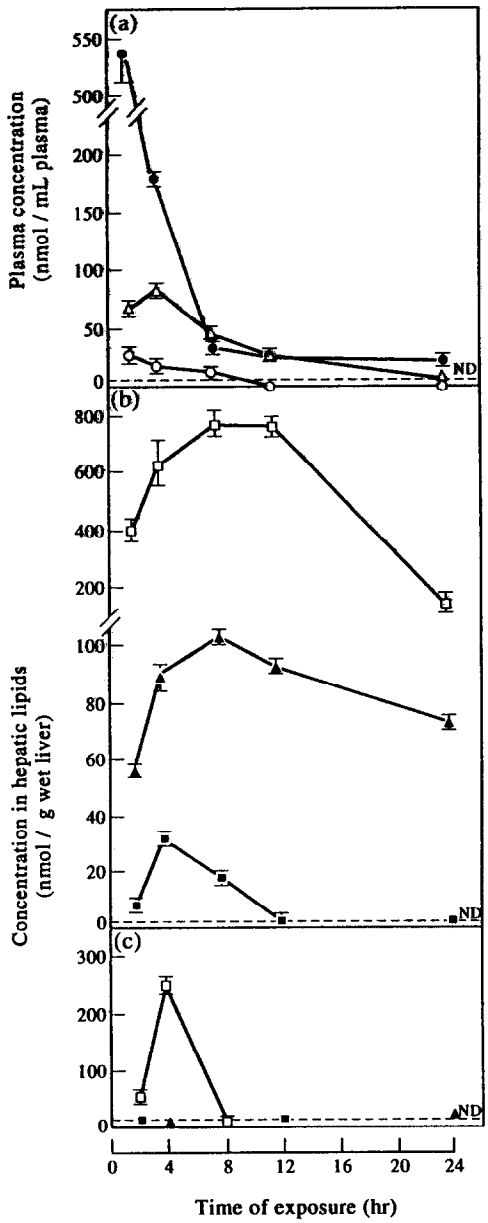

Fig. 1. Plasma concentration (a) of 3-thiadicarboxylic (○), tetradecylthioacetic acid (△) and tetradecylthiopropionic acid (●) as a function of time after a single oral administration of these acids. Concentration of tetradecylthioacetic acid (b) and tetradecylthiopropionic acid (c) in hepatic phospholipids (□), triacylglycerol (▲) and free fatty acid (■) with time. ND, not detectable level denoted by the striped line. Data are expressed as mean ± SEM for six animals in each group.

Effects on hepatic and plasma TG levels

As shown in Fig. 2a, plasma TG levels were decreased at 2 hr after an acute dose of all three thia fatty acids. 3-Thiadicarboxylic acid significantly decreased plasma TG, as compared to controls, by 50% at 2 and 4 hr and 25% at 8 hr. The TG-lowering effect of 3-thia fatty acids, however, disappeared at

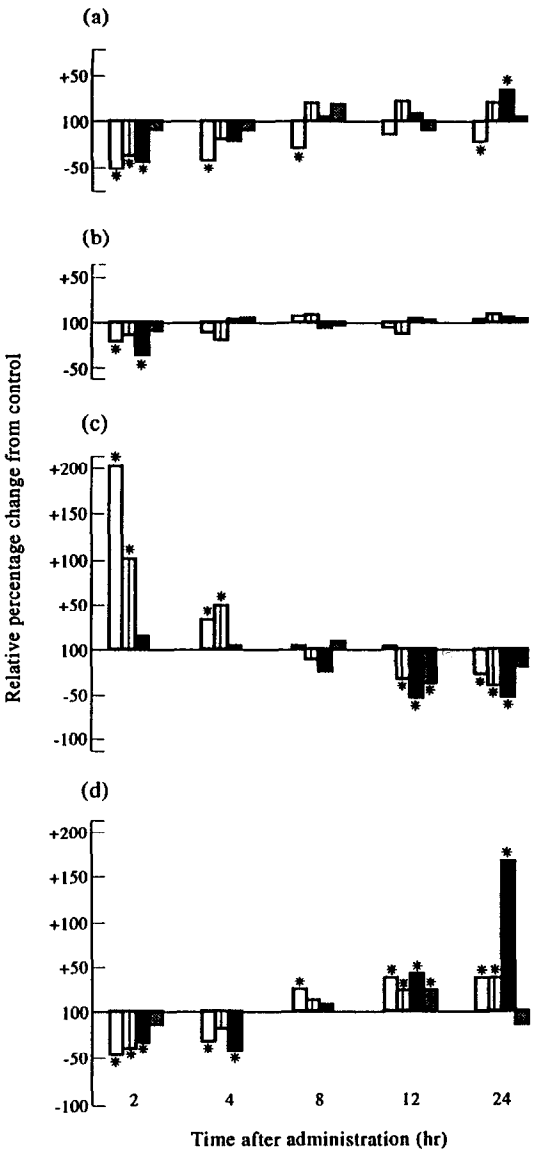

Fig. 2. Time course of plasma (a) and hepatic (b) TG, mitochondrial β-oxidation (using radiolabelled *p*-carnitine as substrate) (c) and total hepatic malonyl-CoA levels (d) after a single oral administration of 3-thiadicarboxylic (□), tetradecylthioacetic (▥), tetradecylthiopropionic (■) and palmitic (▨) acids. The plotted values are relative percentage change of the means from the means of the control values of 1.34 ± 0.14 nmol/L, 3.43 ± 0.09 nmol/g liver, 1.86 ± 0.25 nmol/min/mg protein and 35.76 ± 3.42 nmol/g liver, respectively, plasma TG, hepatic TG, mitochondrial β-oxidation and total hepatic malonyl-CoA levels. The above values represent means ± SEM for six animals in each group. * $P < 0.05$ as compared to controls, represented by the *x*-axis (100%).

12 and 24 hr (Fig. 2a). At 24 hr, plasma TG levels were significantly increased in tetradecylthiopropionic acid treated rats when compared to the control rats.

Hepatic TG levels were significantly decreased at 2 hr by 3-thiadicarboxylic acid treatment (22%) and after administration of tetradecylthiopropionic acid (35%) (Fig. 2b). At 2 and 4 hr, tetradecylthioacetic acid tended to decrease hepatic TG levels but this was not significant. Palmitic acid affected neither plasma nor hepatic TG, as compared to controls, at any time point after acute administration (Fig. 2a and b).

Fatty acid oxidation and lipogenesis

As the availability of substrate (fatty acids) is an important determinant for hepatic TG synthesis, we investigated the effects of a single dose of thia fatty acids on hepatic fatty acid oxidation and lipogenesis. As shown in Fig. 2c, hepatic mitochrondrial β-oxidation was significantly increased in 3-thiadicarboxylate- and tetradecylthioacetate-treated rats by 200 and 100%, respectively, 2 hr after their administration, when compared to control rats. Tetradecylthioacetic acid also significantly increased hepatic mitochondrial β-oxidation at 4 hr. At 8, 12 and 24 hr after acute administration, tetradecylthiopropionic acid significantly decreased mitochondrial β-oxidation.

We have previously reported [7] an increase in total hepatic CPT activity at 2 and 4 hr after a single administration of thia fatty acids. It was therefore of interest to investigate how these fatty acid analogues affected malonyl-CoA levels, since this CoA ester is known to regulate CPT I activity [19] and mitochondrial β-oxidation. At 2 and 4 hr when mitochondrial β-oxidation was high, malonyl-CoA levels, compared to controls, were significantly reduced; 45, 35 and 30%, respectively, after 3-thiadicarboxylic, tetradecylthioacetic and tetradecylthiopropionic acid (Fig. 2d). From 8 to 24 hr after administration, however, malonyl-CoA levels steadily increased, especially in tetradecylthiopropionic acid treated rats at 24 hr, where a significant increase was observed. As shown in Table 1 no significant changes were observed in the activities of ATP-citrate lyase, acetyl-CoA carboxylase and fatty acid synthase at 2 and 4 hr after administration of the thia fatty acids and palmitic acid, as compared to controls. However a significant increase in ATP-citrate lyase activity was observed at 8 hr in the 3-thia acid groups as compared to controls.

Fatty acid composition

The effect of a single dose of tetradecylthioacetic acid on hepatic fatty acid composition is presented in Table 2. Tetradecylthioacetic acid levels were high at 2 hr and this declined steadily until 24 hr. Two hours after administration a significant lowering of saturated fatty acids and a tendency for monounsaturated fatty acids levels to decrease was observed. Both EPA (20:5n − 3) and DHA (22:6n − 3) levels decreased with time and were significantly lower at 24 hr, as compared to control values.

Effect on PAP

Hepatic microsomal PAP activity increased at 2 hr after tetradecylthioacetic, tetradecylthiopropionic and palmitic acid administration and at 4 hr in the thia fatty acid groups, as compared to controls (Table 3). The activity of cytosolic PAP was decreased at

Table 1. Total enzyme activities of ATP-citrate lyase, acetyl-CoA carboxylase and fatty acid synthase after a single dose of 3-thiadicarboxylic (3-TDA), tetradecylthioacetic (TDTAA), tetradecylthiopropionic (TDTPA) and palmitic (PALM) acids

	Time after administration (hr)				
	2	4	8	12	24
ATP-citrate lyase (nmol/min/g liver)					
3-TDA	732 ± 40	727 ± 69	1082 ± 36*	654 ± 11	433 ± 6*
TDTAA	844 ± 59	801 ± 127	1033 ± 70*	632 ± 36	499 ± 10*
TDTPA	607 ± 35	647 ± 38	499 ± 10*	536 ± 4*	620 ± 12
PALM	745 ± 143	551 ± 38	745 ± 36	772 ± 51	630 ± 24
Acetyl-CoA carboxylase (nmol/min/g liver)					
3-TDA	1278 ± 65	1389 ± 13	1701 ± 43*	1481 ± 37	1322 ± 35
TDTAA	1306 ± 64	1295 ± 15	1537 ± 145	1503 ± 65	1445 ± 94
TDTPA	1352 ± 63	1362 ± 68	1309 ± 90	1568 ± 59	1650 ± 170
PALM	1279 ± 58	1399 ± 40	1400 ± 111	1138 ± 22	1280 ± 22
Fatty acid synthase (nmol/min/g liver)					
3-TDA	27 ± 1	41 ± 1	50 ± 7	30 ± 5	31 ± 1
TDTAA	21 ± 1	45 ± 6	36 ± 5	31 ± 4	25 ± 2
TDTPA	23 ± 3	37 ± 3	44 ± 5	35 ± 9	34 ± 1
PALM	32 ± 2	39 ± 4	38 ± 7	32 ± 3	37 ± 1

The results are expressed as mean ± SD for six animals in each group. Significant changes (*$P < 0.05$) are relative to the means of the control values of 859 ± 61, 1400 ± 69 and 37 ± 2 nmol/min/g liver, respectively, for ATP-citrate lyase, acetyl-CoA carboxylase and fatty acid synthase.

Table 2. Fatty acid composition of total liver lipids (% of total lipids) with time after a single administration of tetradecylthioacetic acid (TDTAA) to rats

Fatty acids	Control	Time after administration (hr) 2	4	12	24
TDTAA	—	0.7 ± 0.1	5.0 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
20:5n − 3	1.0 ± 0.0	1.1 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.0*
22:6n − 3	7.7 ± 0.0	8.1 ± 0.2	7.4 ± 1.4	7.6 ± 0.0	6.4 ± 0.1*
Sum sat.	39.4 ± 0.6	36.4 ± 0.3*	38.7 ± 2.0	38.2 ± 0.8	40.3 ± 0.0
Sum monounsat.	13.1 ± 0.3	11.9 ± 1.1	14.9 ± 0.0	14.7 ± 2.1	17.7 ± 2.0
Sum n − 3	10.4 ± 0.1	10.8 ± 0.5	9.7 ± 1.5	9.6 ± 0.3*	8.3 ± 0.2*
Sum n − 6	33.5 ± 1.8	35.9 ± 0.6	33.4 ± 0.3	34.4 ± 2.4	30.7 ± 2.2
Sum polyunsat	45.0 ± 1.7	46.8 ± 0.7	43.2 ± 1.8	44.2 ± 2.7	39.2 ± 1.8
n − 3/n − 6	0.31 ± 0.01	0.30 ± 0.02	0.29 ± 0.04	0.28 ± 0.01	0.27 ± 0.03

Values are means ± SD (N = 4). *$P < 0.05$ as compared to control.

Table 3. Total enzyme activity of microsomal and cytosolic PAP activity with time after a single oral administration of thia fatty acids

	Time after administration (hr)				
	2	4	8	12	24
PAP (microsomal) (nmol/min/g liver)					
3-TDA	308 ± 12*	253 ± 12*	195 ± 11	198 ± 44	182 ± 13
TDTAA	317 ± 36*	254 ± 8*	227 ± 10	191 ± 20	187 ± 9
TDTPA	299 ± 34*	299 ± 29*	240 ± 22	187 ± 5	216 ± 22
PALM	305 ± 8*	241 ± 28	214 ± 7	196 ± 9	237 ± 9
PAP (cytosolic) (nmol/min/g liver)					
3-TDA	137 ± 38	131 ± 11	64 ± 3*	99 ± 3	85 ± 9
TDTAA	120 ± 18	134 ± 15	75 ± 1*	58 ± 11*	79 ± 6*
TDTPA	119 ± 11	83 ± 4	58 ± 7*	75 ± 7*	94 ± 8
PALM	119 ± 10	102 ± 6	93 ± 14	103 ± 7	117 ± 10

The results are means ± SD for six animals in each group. Significant changes (*$P < 0.05$) are relative to the means of the control values of 208 ± 19 and 106 ± 10 nmol/min/g liver, respectively, for microsomal and cytosolic phosphatidate phosphohydrolase. For legend to abbreviations see Table 1.

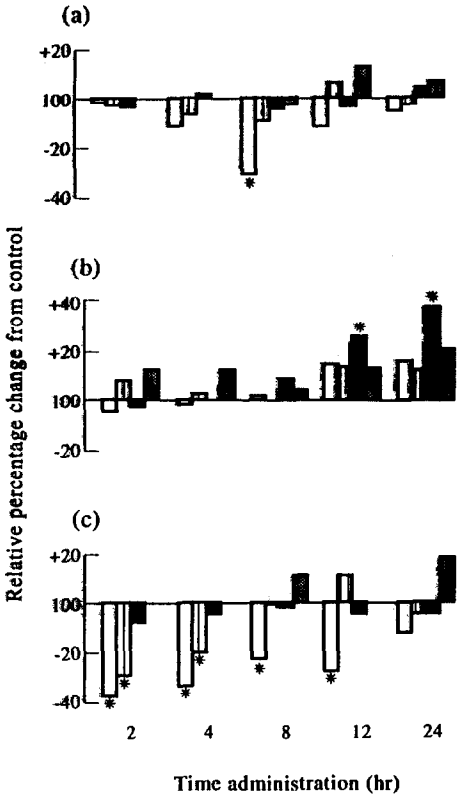

Fig. 3. Effect of a single oral dose of 3-thiadicarboxylic acid (□), tetradecylthioacetic (▨), tetradecylthiopropionic (■) and palmitic (▩) acids as a function of time on plasma cholesterol (a), hepatic cholesterol (b) levels and on HMG-CoA reductase activity (c). The plotted values are relative percentage change of the means from the means of the control values of 1.61 ± 0.26 nmol/L, 2.63 ± 0.25 nmol/g liver, 0.71 ± 0.25 nmol/min/g liver, respectively, for plasma cholesterol, hepatic cholesterol levels and HMG-CoA reductase activity. The above values represent means ± SEM for six animals in each group. *$P < 0.05$ as compared to controls, represented by the x-axis (100%).

8 hr after an acute single oral administration of the thia acids; at 12 hr, after tetradecylthioacetic and tetradecylthiopropionic acid treatment and at 24 hr in tetradecylthioacetate-treated rats (Table 3).

Cholesterol-lowering effect and HMG-CoA reductase activity

Figure 3 (a and b) shows the effect of a single oral dose of thia fatty acids on cholesterol metabolism in normolipidemic rats, as compared to controls. 3-Thiadicarboxylic acid tended to decrease plasma cholesterol levels at all time points, but this cholesterol-lowering effect was only significant (31%) at 8 hr. No significant changes were observed in tetradecylthioacetic, tetradecylthiopropionic and palmitic acid fed rats.

Hepatic cholesterol levels remained generally unchanged in all treatment groups up to 8 hr, after which there was a tendency to increase and this was especially seen after tetradecylthiopropionic administration. As shown in Fig. 3b a significant increase in hepatic cholesterol levels was observed at 12 hr (24%) and 24 hr (35%) after a single treatment with tetradecylthiopropionic acid.

While 3-thiadicarboxylic acid significantly reduced HMG-CoA reductase activity at 2, 4, 8 and 12 hr tetradecylthioacetic acid inhibited this enzyme at 2 and 4 hr, compared to the control value. A non-significant decrease in HMG-CoA reductase activity was observed at all time points after tetradecylthiopropionic acid treatment (Fig. 3c).

The thia fatty acids had no effect on HMG-CoA reductase activity in the peroxisome-enriched fraction (data not shown).

Free CoASH and acyl-CoA ester levels

The effects of a single dose of thia fatty acids on free CoASH and acyl-CoA levels are summarized in Table 4. All the thia fatty acids increased the free CoASH levels up to 8 hr and at 12 hr in the 3-thia fatty acid treatment group. An increase in total hepatic long-chain acyl-CoA levels was observed at 2 and 4 hr in the thia fatty acid groups. Palmitic acid feeding did not change long-chain acyl-CoA levels during the study period, when compared to the controls.

Acetyl-CoA levels remained relatively unchanged in all treatment groups, except at 8 hr in the 3-thiadicarboxylic acid treated rats where a significant increase was observed (Table 4).

Effect of thia acyl-CoA esters on acetyl-CoA carboxylase and HMG-CoA reductase in vitro

The effect of the CoA thioesters of tetradecylthioacetic, tetradecylthiopropionic and palmitic acids on the activities of acetyl-CoA carboxylase and HMG-CoA reductase is shown in Fig. 4a and b. All the three fatty acyl-CoA esters significantly inhibited acetyl-CoA carboxylase, compared to the controls. HMG-CoA reductase activity was significantly suppressed in the presence of tetradecylthioacetyl-CoA (a 3-thia fatty acid) and palmitoyl-CoA. Tetradecylthiopropionyl-CoA (a 4-thia fatty acid) had little effect on HMG-CoA reductase activity.

DISCUSSION

The present study shows that after a single oral administration, all the thia fatty acids were rapidly absorbed and detectable in plasma as early as 2 hr (Fig. 1). At that time, the plasma concentration of tetradecylthiopropionic acid was about 10 times higher than the 3-thia fatty acids (3-thiadicarboxylic and tetradecylthioacetic acids). It is worth noting that the high level of tetradecylthioacetic acid already incorporated into hepatic lipids at 2 hr after administration and its continued decline after 4 hr might indicate that this fatty acid analogue reached its peak in plasma in less than 2 hr. The results, however, suggest that the thia fatty acids can be detected early in plasma after oral administration and that the non-β-oxidizable 3-thia fatty acids appear to reach systemic circulation more slowly and less completely than does the β-oxidizable 4-thia fatty acid (tetradecylthiopropionic acid). Twenty-four hours after administration, however,

Table 4. Total hepatic free CoASH, long-chain acyl-CoA and acetyl-CoA levels in rats after a single oral administration of thia fatty acids

	Time after administration (hr)				
	2	4	8	12	24
Free CoASH (nmol/g liver)					
3-TDA	60 ± 1*	56 ± 8*	70 ± 4*	99 ± 13*	29 ± 7
TDTAA	52 ± 2*	55 ± 1*	40 ± 2*	48 ± 9*	33 ± 3
TDTPA	47 ± 1*	50 ± 9*	45 ± 1*	30 ± 8	32 ± 2
PALM	32 ± 4	45 ± 1*	32 ± 1	38 ± 6	34 ± 6
Long-chain acyl-CoA (nmol/g liver)					
3-TDA	52 ± 2*	55 ± 1*	48 ± 4	51 ± 4	47 ± 1
TDTAA	55 ± 3*	51 ± 1*	48 ± 2	49 ± 3	48 ± 2
TDTPA	55 ± 2*	51 ± 2*	44 ± 2	44 ± 2	45 ± 4
PALM	46 ± 4	49 ± 2	43 ± 1	42 ± 0	49 ± 3
Acetyl-CoA (nmol/g liver)					
3-TDA	18 ± 1	19 ± 0	23 ± 0*	15 ± 2	18 ± 1
TDTAA	20 ± 1	21 ± 1	18 ± 1	16 ± 3	21 ± 2
TDTPA	8 ± 1	18 ± 1	16 ± 1	21 ± 1	18 ± 3
PALM	18 ± 2	21 ± 3	18 ± 2	18 ± 1	20 ± 2

The results are mean ± SD of six animals in each group. Significant changes (*$P < 0.05$) are relative to the mean of the control values of 34 ± 4, 44 ± 2 and 19 ± 2 nmol/g wet liver, respectively, for hepatic free CoASH, long-chain acyl-CoA and acetyl-CoA. For legend to abbreviations see Table 1.

low levels of all the thia fatty acids were detected in plasma.

An important finding was that tetradecylthioacetic and tetradecylthiopropionic acids were rapidly incorporated (at 2–8 hr) into hepatic lipids, especially into the phospholipid fraction (Fig. 1). At 2 hr there were eight times more incorporated tetradecylthioacetic acid residues in phospholipid than tetradecylthiopropionic. This finding may be explained in several ways. Firstly, the thia fatty acids may be taken up by hepatocytes by different mechanisms. Furthermore, since tetradecylthioacetic acid can be activated to acyl-CoA thioester [6], but they (tetradecylthioacetyl-CoA thioesters) are blocked for oxidation, they accumulate in the cells and are subsequently incorporated into phospholipids. In addition, the relatively low levels of tetradecylthiopropionic acid in the phospholipid fraction (Fig. 1) compared to tetradecylthioacetic acid may be due to the fact that the former can undergo one cycle of β-oxidation, and thus are accumulated to a lesser extent. These observations may account for the different effects, i.e. effects on lipid metabolizing enzymes, potency in inducing peroxisome proliferation, etc., observed after repeated administration of tetradecylthioacetic and tetradecylthiopropionic acids [3, 20, 21]. The findings are also significant since a high concentration of 3-thia fatty acids in phospholipids may affect fluidity and signal systems in membranes. It is worth noting that hepatic phospholipid levels were unaffected by treatment with thia fatty acids during the 24 hr study period (data not shown).

Free fatty acids are needed as substrate for the increased mitochondrial oxidation. Two hours after thia fatty acid administration, when mitochondrial fatty acid oxidation was at its peak, a significant decrease in total hepatic saturated fatty acid levels was observed (Table 2). There was also a tendency for total hepatic monosaturated fatty acid levels to decrease, but this was not significant. At this same time point increased incorporation of tetradecylthioacetic acid into hepatic lipids occurred. Tetradecylthioacetic acid is a good substrate for enzymes involved in glycerolipid synthesis (data to be published) and therefore competes with endogenous fatty acids for lipid synthesis. This competition between a non-β-oxidizable and β-oxidizable fatty acids may be an important factor for the rapid hypotriglyceridemia observed after 3-thia fatty acid administration. Thus the non-β-oxidizable fatty acid (tetradecylthioacetic acid) is geared towards incorporation into lipids whereas the easily oxidizable fatty acids are channelled into mitochondria to be oxidized. It is therefore proposed that following acute treatment of rats with a non-β-oxidizable thia fatty acid, they (thia fatty acids) are incorporated into TG and phospholipids while there is diversion of endogenous saturated fatty acids from TG and phospholipid synthesis to mitochondrial β-oxidation.

Prior to incorporation into phospholipids, the thia fatty acids must be converted to their CoA esters. Aarsland and Berge [6] have demonstrated that thia fatty acids can be converted to their CoA esters. The increase in total hepatic long-chain acyl-CoA levels between 2 and 4 hr in the thia acid groups (Table 4), as compared to the palmitic acid and control groups, correspond to the time of maximum incorporation of these fatty acid analogues into hepatic lipids (Fig. 1). Moreover, specific tetradecylthioacetyl-CoA have been detected and their levels are raised at 2 and 4 hr after administration of tetradecylthioacetic acid (data to be published). Free CoASH is required to convert the thia fatty acids to their CoA derivatives. The levels of free CoASH were significantly increased between 2 and 8 hr in the thia acid treated rats (Table 4). It can

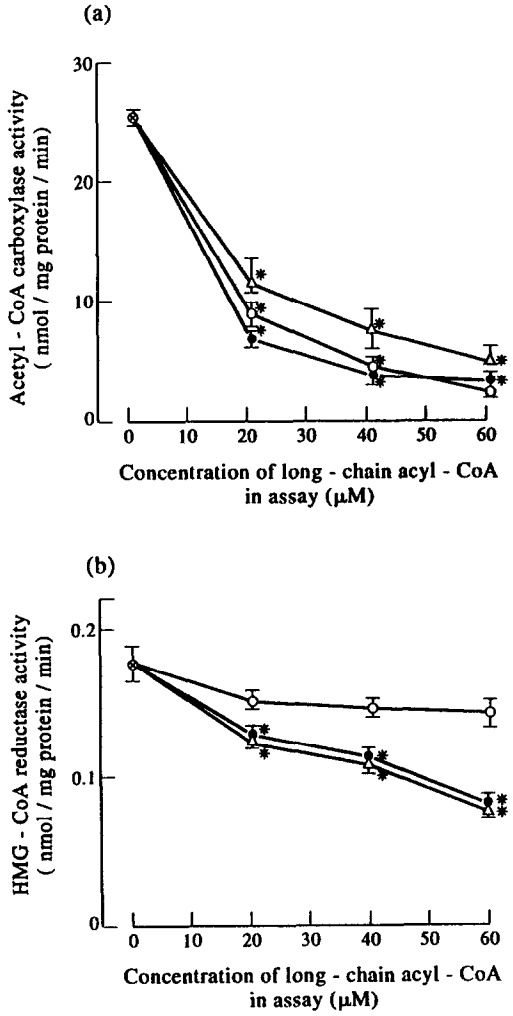

Fig. 4. Effect of tetradecylthioacetyl-CoA (△) and tetradecylthiopropionyl-CoA (○) and palmitoyl-CoA (●) on acetyl-CoA carboxylase (a) and HMG-CoA reductase (b) activity. The incubation assay for HMG-CoA reductase experiment contained 20 μM HMG-CoA. The values are means from two separate experiments. *$P < 0.05$ as compared to controls (⊗).

therefore be inferred that the increased level of long-chain acyl-CoA between 2 and 4 hr can be attributed to acyl-CoA esters of the thia fatty acids. The thia acyl-CoA esters formed at 2 and 4 hr may act as regulators of lipid metabolism (Fig. 5).

Plasma TG levels are determined by a delicate balance between hepatic TG synthesis and secretion on one hand and plasma TG clearance on the other. In liver lipid homeostasis, lipids imported and *de novo* synthesized lipids must equal the sum of consumption of lipid substrates for oxidation and lipids exported to extrahepatic tissues, mainly in the form of VLDL. In the present study, administration of thia fatty acids to normolipidemic rats decreased their plasma. TG levels at 2 hr (Fig. 2a), confirming an earlier report [7]. At this time, mitochondrial β-oxidation increased in the 3-thia fatty acid treatment groups (Fig. 2c), the activities of key enzymes involved in lipogenesis (ATP-citrate lyase, acetyl-CoA carboxylase and fatty acid synthase) were unchanged (Table 1) while the activity of microsomal PAP, the regulatory enzyme in TG synthesis, increased. Prolonged and repeated administration of these fatty acid analogues, however, have been reported to decrease ATP-citrate lyase, fatty acid synthase and PAP activities [3, 22]. Thus, whereas the triglyceride-lowering effect of the 3-thia fatty acids after repeated administration may, in part, be due to decreased lipogenesis and TG synthesis [22], acute hypotriglyceridemia within hours of their administration is independent of this.

An increase in mitochondrial β-oxidation occurs between 2 and 4 hr following administration of 3-thia fatty acids (Fig. 2) and a similar increase has been reported in isolated hepatocytes incubated with tetradecylthioacetic [23] and with tetradecylthiacetic and 3-thiadicarboxylic acids (data to be published). At 2 and 4 hr there was an increase in long-chain acyl-CoA levels (this is attributed to increased thia acyl-CoA) and a decrease in malonyl-CoA levels (Fig. 2d) after 3-thia fatty acid administration. Long-chain acyl-CoA inhibits acetyl-CoA carboxylase allosterically via a protein kinase cascade [24], while high levels of malonyl-CoA inhibit CPT I [17]. Although tetradecylthioacetyl-CoA significantly inhibits acetyl-CoA carboxylase *in vitro* (Fig. 4), this was not observed in the *in vivo* experiment (Table 1). The reason for this is not readily apparent. However, it has recently been shown that in rats given a high carbohydrate diet, administration of tetradecylthioacetic acid results in inhibition of acetyl-CoA carboxylase [25]. Whether acetyl-CoA carboxylase was inhibited between 0 and 2 hr by thia acyl-CoA esters and thereby caused the decrease in malonyl-CoA levels or whether other regulatory mechanisms lead to stimulation of mitochondrial β-oxidation need to be further investigated.

In contrast to the 3-thia fatty acids, tetradecylthiopropionic acid, a 4-thia fatty acid can undergo one cycle of β-oxidation. Metabolites of this fatty acid have been reported to be formed rapidly and to inhibit mitochondrial β-oxidation [26]. This may explain why there was no increase in mitochondrial β-oxidation at 2 and 4 hr after administration of tetradecylthiopropionic acid. This metabolite, tetradecylthioacrylic acid, whose levels may have increased with time, probably inhibited mitochondrial β-oxidation at 12 and 24 hr after a single administration of tetradecylthiopropionic acid.

Hypocholesterolemia was observed in 3-thia-dicarboxylic acid treated rats at 8 hr. An important observation was that this hypocholesterolemic effect was seen at a later time point (8 hr) than the triglyceride-lowering effect (2–4 hr). The observed hypocholesterolemia after administration of 3-thiadicarboxylic acid could be independent of its triacylglycerol-lowering effect. A possible explanation for the hypocholesterolemia is that decreased hepatic TG levels after administration of 3-thiadicarboxylic acid may lead to decreased VLDL (with unchanged composition [22]) secretion, limited conversion of VLDL to LDL, and reduced plasma cholesterol levels. A similar explanation

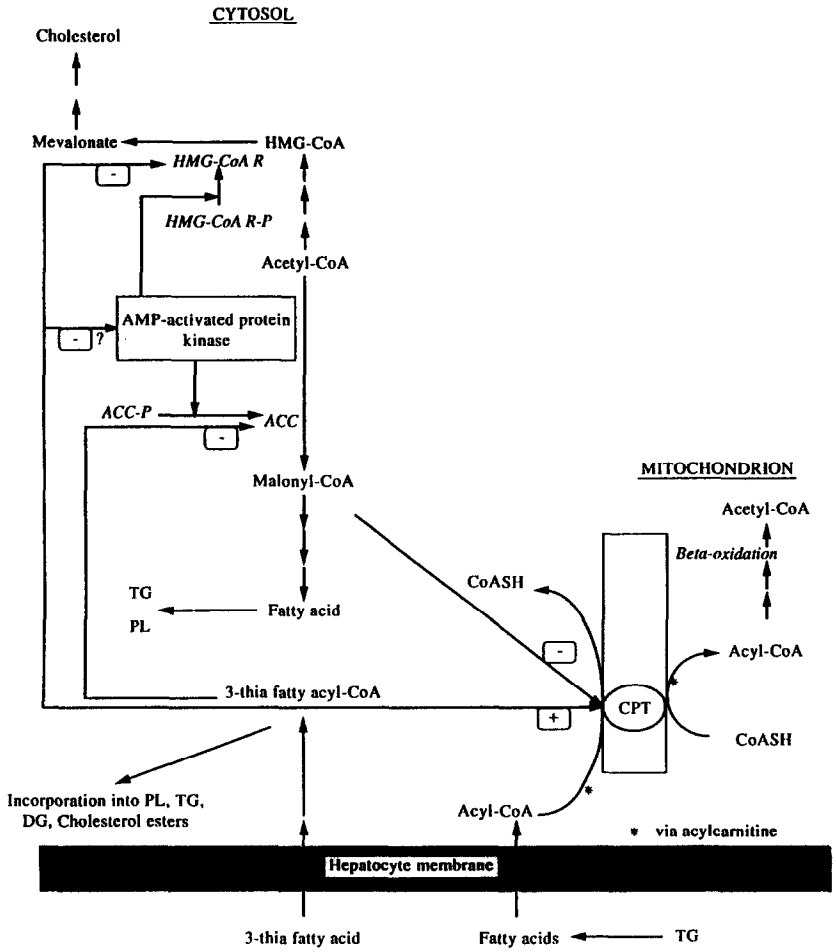

Fig. 5. Schematic presentation of the possible effects of 3-thia fatty acyl-CoA on lipogenesis, mitochondrial β-oxidation and cholesterol synthesis. 3-Thia fatty acyl-CoA stimulates CPT and either directly or via protein kinase cascade inhibits acetyl-CoA carboxylase, leading to decreased synthesis of malonyl-CoA. Stimulated CPT and decreased malonyl-CoA levels will increase mitochondrial β-oxidation. Since the 3-thia fatty acids are non-β-oxidizable, they are incorporated into hepatic lipids instead of endogenous fatty acyl-CoA, which are then β-oxidised. The 3-thia fatty acyl-CoAs inhibit HMG-CoA reductase and decrease cholesterol synthesis. HMG-CoAR, HMG-CoA reductase; HMG-CoAR-P, phosphorylated HMG-CoA reductase; ACC, acetyl-CoA carboxylase; ACC-P, phosphorylated acetyl-CoA carboxylase; (+), stimulation; (−), inhibition.

has previously been proposed for the hypocholesterolemic effect of nicotinic acid and etofibrate [27].

HMG-CoA reductase was significantly inhibited in the 3-thia fatty acid treated groups at 2 and 4 hr and also at 8 and 12 hr in 3-thiadicarboxylic acid group (Fig. 3c). This inhibition may be attributed to the effect of thia fatty acyl-CoA on HMG-CoA reductase (Fig. 4). Inhibition of HMG-CoA reductase is accompanied by a reduction in plasma cholesterol levels. A similar finding has been reported after prolonged and repeated administration of 3-thia fatty acids [22]. Thus, decreased hepatic cholesterol synthesis and possibly reduced hepatic VLDL secretion may all contribute to the hypocholesterolemia caused by the 3-thiadicarboxylic acids.

Tetradecylthiopropionic acid has been reported to cause hypercholesterolemia and increase levels of liver cholesterol [2, 3]. As shown in this short-term study increased levels of liver cholesterol started at 12 hr. Tetradecylthiopropionyl-CoA had no effect on HMG-CoA reductase activity in *in vitro* and *in vivo* studies (Figs 3c and 4). Cholesterol biosynthesis therefore proceeded uninhibited. How tetradecylthiopropionic acid causes hypercholestrolemia needs to be explored further.

In summary, the results of this study show that thia fatty acids are systemically available after oral administration. They suggest that the thia fatty acids can, *in vivo*, be incorporated into hepatic lipids, especially into phospholipids. The data suggest that increased mitochondrial β-oxidation and inhibition of HMG-CoA reductase activity are the primary events after 3-thia fatty acids administration.

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REFERENCES

1. Berge RK, Aarsland A, Kryvi H, Bremer J and Aarsæther N, Alkylthioacetic acids (3-thia fatty acids)—a new group of non-β-oxidizable peroxisome-inducing fatty acid analogues. I. Dose–response studies on hepatic peroxisomal and mitochondrial changes and long-chain fatty acid metabolizing enzymes in rats. *Biochem Pharmacol* **38**: 3969–3979, 1989.
2. Aarsland A, Aarsæther N, Bremer J, 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.
3. Skorve J, Asiedu D, Rustan AC, Drevon, CA, Al-Shurbaji A and Berge RK, Regulation of fatty acid oxidation and triglyceride and phospholipid metabolism by the hypolipidemic sulfur-substituted fatty acid analogues. *J Lipid Res* **31**: 1627–1635, 1990.
4. Spydevold Ø and Bremer J, Induction of peroxisomal β-oxidation in 7800 OO Morris hepatoma cells in steady state by fatty acids and fatty acid analogues. *Biochim Biophys Acta* **1003**: 72–79, 1989.
5. Berge RK, Aarsland A, Kryvi H, Bremer J and Aarsæther 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**: 343–356, 1989.
6. Aarsland A and Berge RK, Peroxisome proliferating sulphur- and oxy-substituted fatty acid analogues are activated to acyl-CoA thioesters. *Biochem Pharmacol* **41**: 53–61, 1991.
7. Asiedu DK, Skorve J, Willumsen N, Demoz A and Berge RK, Early effects on mitochondrial and peroxisomal β-oxidation by the hypolipidemic 3-thia fatty acids in rat livers. *Biochim Biophys Acta* **1166**: 73–76, 1993.
8. 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.
9. Tanabe T, Nakanishi S, Hashimoto T, Nikowa J-I and Numa S, Acetyl-CoA carboxylase from rat liver. *Methods Enzymol* **71**: 5–16, 1981.
10. Roncari DA, Fatty acid synthase from human liver. *Methods Enzymol* **71**: 73–79, 1981.
11. Rose-Khan G and Bar-Tana I, Inhibition of lipid synthesis by $\beta^1.\beta^1$-tetramethyl-substituted C_{14}-C_{22}, a, ω-dicarboxylic acids in cultured rat hepatocytes. *J Biol Chem* **260**: 8411–8415, 1989.
12. Mavis RD, Finkelstein JN and Hall BP, Pulmonary surfactant synthesis. A highly active microsomal phosphatidate phosphohydrolase in the lungs. *J Lipid Res* **19**: 467–477, 1978.
13. Brown MS, Goldstein JL and Diestschy JM, Active and inactive forms of 3-hydroxy-3-methylgluteryl Coenzyme A reductase in the liver of rat: comparison with the rate of cholesterol synthesis in different physiological states. *J Biol Chem* **254**: 5144–5149, 1979.
14. Corkey BE, Brandt M, Williams RJ and Williamson JR, Assay of short-chain acyl-CoA intermediates in tissue extracts in high pressure chromatography. *Anal Biochem* **118**: 30–41, 1981.
15. Folch J, Lees M and Stanley GHS, A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226**: 497–509, 1957.
16. Kaluzny MA, Duncan LA, Merritt MV and Epps DE, Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J Lipid Res* **26**: 135–140, 1985.
17. Christie WW, *Gas Chromatography and Lipids. A Practical Guide*. The Oily Press Air, U.K., p.76, 1989.
18. Lie Ø, Lied E and Lambertsen G, Lipid digestion in cod. *Comp Biochem Physiol* **88B(2)**: 697–700, 1987.
19. McGarry JD and Foster DW, Regulation of hepatic fatty acid oxidation and ketone body production. *Annu Rev Biochem* **49**: 395–420, 1980.
20. Bergseth S and Bremer J, Alkylthioacetic acids (3-thia fatty acids) are metabolised and secreted as shortened dicarboxylic acids *in vivo*. *Biochim Biophys Acta* **1044**: 237–242, 1990.
21. Hvattum E, Skrede S, Bremer J and Solbakken M, The metabolism of tetradecylthiopropionic acid, a 4-thia stearic acid, in the rat. *In vivo* and *in vitro* studies. *Biochem J* **286**: 879–887, 1992.
22. Skorve J, Al-Shurbaji A, Asiedu D, Bjørkhem I, Berglund L and Berge RK, On the mechanism of the hypolipidemic effect of sulfur-substituted hexadecaenoic acid (3-thiadicarboxylic acid). *J Lipid Res* **34**: 1177–1185, 1993.
23. Skrede S and Bremer J, Acylcarnitine formation and fatty acid oxidation in hepatocytes from rats treated with tetradecylthioacetic acid (a 3-thia fatty acid). *Biochim Biophys Acta* **1167**: 189–196, 1993.
24. Hardie DG, Regulation of fatty acid synthesis via phosphorylation of acetyl-CoA carboxylase. *Prog Lipid Res* **28**: 117–146, 1989.
25. Skorve J and Berge RK, The hypocholesterolemic effect of sulfur-substituted fatty acid analogues in rats fed a high carbohydrate diet. *Biochim Biophys Acta* **1167**: 175–181, 1993.
26. Skrede S and Bremer J, Tetradecylthioacrylic acid, a beta-oxidation metabolite of tetradecylthiopropionic acid, inhibits hepatic fatty acid activation and oxidation in rats. In: *Fatty Acids and Lipids from Cell Biology to Human Disease. Abstract Book of ISSFAL*. Lugano, Switzerland, p.61, 1993.
27. Bocos C, Castro M, Quack G and Herrera E, Studies with etofibrate in the rat. Part II. A comparison of the effects of prolonged and acute administration on plasma lipids, liver enzymes and adipose tissue lipolysis. *Biochim Biophys Acta* **1168**: 340–347, 1993.