The metabolism of tetradecylthiopropionic acid, a 4-thia stearic acid, in the rat

In vivo and *in vitro* studies

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The metabolism of [1-¹⁴C]tetradecylthiopropionic acid (TTP), a 4-thia stearic acid, and its sulphoxide, [1-¹⁴C]tetradecylsulphoxypropionic acid (TTP-SO), has been studied in intact rats, in isolated rat hepatocytes, and in rat liver mitochondria. Two pathways of oxidation (β -oxidation and ω -oxidation) have been demonstrated. TTP is incorporated, *in vivo*, into tissue triacylglycerol and phospholipids, it is oxidized to CO₂, and it is excreted in urine, mainly as carboxypropylsulphoxypropionic acid and a little as carboxymethylsulphoxypropionic acid. TTP-SO is metabolized, *in vivo*, more rapidly to the same two ω -oxidation products. In hepatocytes TTP is incorporated into triacylglycerol and phospholipids even more rapidly than stearic acid. It is recovered mainly in the 1-position of phosphatidylcholine. Some is oxidized to CO₂ and acid-soluble products. TTP-SO is mainly ω -oxidized to the same metabolites as are found in urine. A small fraction is incorporated into phospholipids or oxidized to CO₂. In isolated mitochondria [1-¹⁴C]TTP is converted into ¹⁴CO₂, radioactive malonic semialdehyde, and addition products of malonic semialdehyde. In the presence of phenylhydrazine, malonic semialdehyde phenylhydrazone is the dominating product. In soluble extracts of mitochondria [1-¹⁴C]malonic semialdehyde is oxidized directly to ¹⁴CO₂ in the presence of CoA and NAD⁺, probably by the (methyl)malonic acid semialdehyde dehydrogenase (EC 1.2.1.27).

INTRODUCTION

We have synthesized fatty acid analogues with a sulphur atom substituting for the methylene group in either position 3 or 4 of the carbon chain of the fatty acid. These 3- and 4-thia fatty acids have different metabolic effects when administered to rats.

The 3-thia analogues decrease the serum concentration of triacylglycerol and cholesterol (Aarsland *et al.*, 1989) and induce peroxisomal β -oxidation (Berge *et al.*, 1989a) and microsomal ω -oxidation (Hvattum *et al.*, 1991).

In hepatoma cells and hepatocytes in cell culture the 3-thia fatty acids induce peroxisomal β -oxidation more efficiently than normal fatty acids (Spydevold & Bremer, 1989). Thus, in this respect, the 3-thia acids introduce a fortified fatty acid signal into the cells.

The 4-thia analogues inhibit β -oxidation in peroxisomes and mitochondria (Hovik *et al.*, 1990) and cause hepatic lipidosis when administered to rats (Berge *et al.*, 1989b). They have only a minimal inducing effect on the peroxisomal β -oxidation (Berge *et al.*, 1989a).

4-Thia-fatty-acid analogues have been shown to be substrates for purified acyl-CoA dehydrogenase (EC 1.3.99.3) (Lau *et al.*, 1989). The analogues are metabolized to 4-thia-*trans*-2-enoyl-CoA. Lau and colleagues have further shown that treating this molecule with purified enoyl-CoA hydratase (EC 4.2.1.17) will effect fragmentation of the acyl chain to malonylsemialdehyde-CoA and an alkylthiol, presumably via formation of a thiohemiacetal.

Because of the pronounced differences in metabolic effects of the 3- and 4-thia fatty acids we found it interesting to compare their metabolism. We have previously investigated the metabolism of 3-thia fatty acids in isolated rat liver microsomes (Hvattum *et al.*, 1991), in isolated rat hepatocytes (Skrede *et al.*, 1989) and

in vivo, and have shown that the 3-thia fatty acids are metabolized by sulphur- and ω -oxidation to short dicarboxylic acids which are excreted in the urine (Bergseth & Bremer, 1990). We have now studied the metabolism of tetradecylthiopropionic acid (TTP), a 4-thia fatty acid, *in vivo*, in rat hepatocytes and rat liver mitochondria.

In part, the 4-thia fatty acid was found to be metabolized by the same mechanisms as the 3-thia acids. In addition, malonic semialdehyde was identified as an oxidation product. The metabolism of this compound was therefore also investigated.

Fig. 1. Structure of the various thia-substituted fatty acids and their metabolites

(a) Tetradecylthiopropionic acid (TTP); (b) tetradecylsulphoxypropionic acid (TTP-SO); (c) carboxypropylsulphoxypropionic acid; (d) carboxymethylsulphoxypropionic acid.

Abbreviations used: DMSO, dimethyl sulphoxide; DTA, dodecylthioacetic acid; DTT, DL-dithiothreitol; TTP, tetradecylthiopropionic acid; TTP-SO, tetradecylsulphoxypropionic acid.

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EXPERIMENTAL

Materials

[1-¹⁴C]Palmitic acid and [1-¹⁴C]stearic acid were purchased from Amersham International (Amersham, Bucks, U.K.) and diluted to suitable specific radioactivities with the corresponding unlabelled acids. Ethyl 3,3-diethoxypropionate was purchased from Aldrich-Chemie (Steinheim, Germany).

Synthesis of [1-¹⁴C]TTP

[1-¹⁴C]TTP (Fig. 1) was synthesized as an ethyl ester in the following way.

2-(Tetradecylthio)ethanol synthesis. To a stirred solution of mercaptoethanol (5 ml) and KOH (6 g) in CH₃OH (200 ml) was added tetradecyl bromide (2 ml). After 4 h the tetradecylthioethanol was separated as an oil upon addition of water. The oil crystallized upon cooling. It was recrystallized from ethanol/water (4:1, v/v).

2-Chloroethyltetradecyl thioether synthesis. To a stirred solution of thionyl chloride (0.77 g, 6.5 mmol) in dry chloroform (5 ml) was added a solution of 2-(tetradecylthio)ethanol (1.4 g, 5.0 mmol) in dry chloroform (10 ml). The reaction mixture was stirred at room temperature for 3 h, then concentrated, and the residue was distilled in a Kugelrohr apparatus (oven temp. 130–140 °C/0.05 mmHg). The product was a clear liquid which solidified in the refrigerator. Yield achieved was 1.4 g (96 %).

Data from m.s. analysis: electron impact (% relative abundance): 292/293 (3.1/1.1 *M*⁺), 229 (100, C₁₄H₂₉S).

3-(Tetradecylthio)[1-¹⁴C]propionitrile synthesis. The reaction was run under nitrogen. 2-Chloroethyltetradecyl thioether (41.4 mg, 141 μmol), K¹⁴CN (1.2 mg, 18 μmol) and KCN (6.3 mg, 97 μmol) were dissolved in dry, freshly distilled dimethyl sulphoxide (DMSO) (2.5 ml). The reaction mixture was stirred at 40 °C for 1 day. Solvent was distilled off at reduced pressure. The residue was taken up in hexane/diethyl ether (2:1, v/v) and filtered through a silica column (20 mm × 5 mm). After concentration, 37 mg of a semi-solid material was obtained (corresponding to 91 % recovery of organic material). Unchanged chloride constituted 20 % (g.l.c.-m.s. analysis), in accordance with the excess of starting material used.

Data from m.s. analysis: electron impact (% relative abundance): 285 (1.2, *M*⁺[¹⁴C]), 283 (5.7, *M*⁺), 229 (100, C₁₄H₂₉S).

Ethyl 3-(tetradecylthio)[1-¹⁴C]propionate synthesis. The crude product from the synthesis above was dissolved in ethanol (15 ml). Dry, gaseous hydrochloric acid was let into the reaction mixture at regular intervals during the total reaction time of 3 days. The reaction mixture was stirred at room temperature, and the conversion into product was monitored by capillary g.l.c. The reaction mixture was poured on ice, neutralized with solid NaHCO₃, and the solution was extracted several times with ether. The solution was dried (0.3 nm molecular sieve), and after concentration 35 mg of a semi-solid material was obtained. The product mixture was analysed by g.l.c.-m.s. and consisted of the propionate and 20 % unchanged chloride.

Hydrolysis of ethyl 3-(tetradecylthio)[1-¹⁴C]propionate. Alkaline hydrolysis of the ethyl ester gave decomposition of the thia acid. The ethyl ester of TTP was therefore hydrolysed with 130 units of esterase (Boehringer-Mannheim, Mannheim, Germany) in 90 mM-potassium phosphate, pH 7.4, with 11 % (v/v) acetone to a total volume of 4.6 ml. After 24 h of incubation the solution was extracted with diethyl ether, evaporated to dryness and dissolved in acetonitrile. TTP was isolated by h.p.l.c. by injecting the acetonitrile solution on a Spherisorb ODS column (5 μm; 4.6 mm × 250 mm; Supelco, Bellefonte, PA, U.S.A.). A gradient was run with water and acetonitrile, both containing 0.5 % formic acid. The gradient started with 85 % (v/v) acetonitrile,

isocratic for 18 min, increasing to 100 % (v/v) acetonitrile in 2 min, followed by 100 % (v/v) acetonitrile for 10 min and back again to 85 % (v/v) acetonitrile in 2 min at a flow rate of 0.8 ml/min. The purity of [1-¹⁴C]TTP was established by co-elution of a standard non-radioactive TTP on both the above described h.p.l.c. system and chromatography on silica-gel/aluminium t.l.c. plates eluting with benzene/dioxane/formic acid (50:50:4, by vol.). The isolated radioactive peak was applied with standard TTP in duplicate tracks, one of these was exposed to iodine vapour localizing TTP as a brown spot. The other track was cut into 0.5 cm zones and immersed in scintillation fluid for radioactive counting. Approx. 100 % of the radioactivity co-eluted with standard TTP in both chromatograms.

[1-¹⁴C]Tetradecylsulphoxypropionic acid (TTP-SO) synthesis.

[1-¹⁴C]TTP-SO was prepared by oxidizing [1-¹⁴C]TTP with a 10 % molar excess of H₂O₂ in acetic acid at room temperature.

Carboxypropylsulphoxypropionic and carboxymethylsulphoxypropionic acid were synthesized as described (Bergseth & Bremer, 1990).

Synthesis of malonic semialdehyde

Malonic semialdehyde was prepared from ethyl 3,3-diethoxypropionate (Yamada & Jakoby, 1960). An aliquot (20 μl) of the compound was suspended in 1.0 ml of 0.5 M-KOH and shaken vigorously for 2 h at room temperature. The solution was cooled in an ice bath and 1.0 ml of 2.5 M-sulphuric acid was slowly added. The acid solution was left to incubate for 2 h at room temperature. The solution was neutralized with 6.0 M-KOH and used the same day. The hydrazone of malonic semialdehyde and phenylhydrazine was prepared by addition of phenylhydrazine dissolved in 2.5 M-sulphuric acid.

Metabolism of TTP *in vivo*

Rats were either injected intraperitoneally with 0.06 mg (1.8 μCi) of neutralized TTP dissolved in iso-osmotic NaCl (2.0 ml) or given neutralized TTP-SO, 30 mg (0.5 μCi), by means of a gastric tube. Each rat was placed in an air-tight metabolism cage and urine and CO₂ were collected as previously described (Bergseth *et al.*, 1988). Respiratory ¹⁴CO₂ was measured as described (Woeller, 1961; Bergseth *et al.*, 1988). Urine samples were analysed by h.p.l.c. after filtration through a Millipore Millex-AA (0.8 μm) filter.

Two urine metabolites were purified and analysed by n.m.r. spectroscopy. To get a sufficient quantity of these urine metabolites, rats were given approx. 30 mg of [1-¹⁴C]TTP-SO. Urine was collected for 19 h, and after filtration and evaporation the residue was extracted with ethanol and refrigerated for approx. 2 h. The extract was then centrifuged at 500 *g*_{av} for 5 min and evaporated to half the volume before being applied on an anion-exchange AG 1-X8 column (200–400-mesh formate form) and eluted with a gradient produced by allowing 250 ml of 3 M-formic acid to enter a 250 ml mixing chamber filled with distilled water. The column dimensions were 1.8 cm × 11 cm and the elution rate was 1 ml/min; 5 ml fractions were collected and the peaks were detected by pipetting samples of each fraction into scintillation fluid for counting. Two major radioactive peaks were found and the radioactive column fractions in each peak were combined. After evaporation each peak was dissolved in 6.5 mM-sulphuric acid (1.0 ml) and injected separately on an Aminex HPX-87H column (300 mm × 7.8 mm) (Bio-Rad Laboratories, Richmond, CA, U.S.A.), eluted with 6.5 mM-sulphuric acid at a flow rate of 0.4 ml/min and fractions collected. A Gilson fraction collector (Gilson Medical Electronics, Middleton, WI, U.S.A.) was used and a sample of each fraction was counted for radioactivity. Only one radioactive peak appeared in each case. The radioactive fractions were combined and treated

with 2 molar equivalents of BaCO_3 to remove the sulphuric acid from the solution. After centrifugation ($9300 g_{av}$, 10 min), the supernatant was evaporated to dryness under air and dissolved in distilled water. The purity of each metabolite was assayed by h.p.l.c. using the Aminex column as described above. By monitoring u.v. absorption at 210 nm and counting radioactivity, we obtained only one radioactive and u.v.-absorbing peak for each metabolite. Each peak was then subjected to n.m.r. analysis.

Metabolism of TTP by hepatocytes

Hepatocytes were prepared according to Seglen (1973) and suspended in Krebs–Henseleit bicarbonate buffer containing 1% (w/v) fatty-acid-free BSA as previously described (Bergseth *et al.*, 1986).

The hepatocytes (4–5 mg of protein/ml) were incubated at 37 °C in Krebs–Henseleit bicarbonate buffer with 3.5% (w/v) fatty-acid-free BSA and 0.5 mM-[^{14}C]palmitic acid, [^{14}C]stearic acid, [^{14}C]TTP or [^{14}C]TTP-SO as previously described (Skrede *et al.*, 1989).

The incubation was stopped by adding 280 μl of 2.0 M- HClO_4 /ml, and liberated $^{14}\text{CO}_2$ was measured as described previously (Woeller, 1961; Bergseth *et al.*, 1988).

Incorporation of radioactive substrates into cellular, complex lipids was analysed using t.l.c. after extraction of the cellular lipids with butanol (Skrede *et al.*, 1989).

Before analysis by h.p.l.c., acid-soluble extracts were prepared by adding 70 μl of 2.0 M- HClO_4 to 250 μl of hepatocyte mixture and centrifuging the sample to remove proteins and lipids.

Metabolism of TTP in mitochondria

The rat livers were finely minced and homogenized in ice-cold 0.3 M-mannitol containing 1 mM-EDTA and 10 mM-Trizma base, pH 7.4, and fractionated as described (Myers & Slater, 1957).

Freshly isolated mitochondria (1.4 mg) were incubated with 10–50 μM -[^{14}C]TTP (8.9 $\mu\text{Ci}/\mu\text{mol}$) and an incubation mixture containing 25 mM-Hepes, pH 7.3, 100 mM-KCl, 5 mM- MgCl_2 , 5 mM-ATP, 0.1 mM-(–)carnitine, 0.1% BSA, 25 μM -CoA, 125 μM -DL-dithiothreitol (DTT) and 5 mM-potassium phosphate, pH 7.3. Additions of 5 mM of 2-oxoglutarate, succinate, malate or glutamate and of 0.5 mM malonic semialdehyde and malonate were made as stated in the Table legends. The incubations were performed at 30 °C. Vessels were flushed with O_2 for 30 s and preincubated for 1 min before the incubation was started with [^{14}C]TTP. Incubation time was 10–20 min and the reaction was stopped with 140 μl of 2 M- HClO_4 /ml and centrifuged before h.p.l.c. analysis of acid-soluble products.

When collecting $^{14}\text{CO}_2$ the incubation was performed in closed vessels and the liberated $^{14}\text{CO}_2$ was trapped and measured in hanging wells with $\pm$ (1-phenylethylamine/methanol (1:1, v/v) as described (Woeller, 1961; Bergseth *et al.*, 1988).

To identify malonic semialdehyde as a metabolic product from mitochondrial incubation with [^{14}C]TTP, the products were derivatized with phenylhydrazine to obtain a product more stable than the semialdehyde itself. Phenylhydrazine was added either to the incubation mixture [30 μl of 0.4% (w/v) phenylhydrazine dissolved in water], to the acid extract after proteins had been removed by centrifugation [300 μl of 0.4% (w/v) phenylhydrazine dissolved in 2.0 M-HCl], or to the isolated products [50 μl of 0.4% (w/v) phenylhydrazine in 2.0 M-HCl].

Metabolism of malonic semialdehyde by mitochondrial extract

Mitochondria were isolated as described above and resuspended in 2.0 ml of 10 mM-potassium phosphate buffer, pH 7.4, and sonicated (A 350 G Ultrasonic Ltd., Shipley, Yorks., U.K.) for 3×10 s (on/off) at maximum strength. The solution was then centrifuged at 15000 rev./min (20760 g_{av}) for 30 min

with an SS-34 rotor and Sorvall RC-5B centrifuge and the mitochondrial extract was either used at once or stored at –70 °C.

[^{14}C]Malonic semialdehyde was isolated by h.p.l.c. from a mitochondrial incubation with [^{14}C]TTP and was immediately incubated with mitochondrial extracts in 20 mM-potassium phosphate buffer, pH 7.4, containing 1 mM-CoA, 5 mM-DTT, 4 mM- NAD^+ and 2 mM-ATP. Malonic semialdehyde (0.5 mM) or malonyl-CoA (0.5 mM) was added as stated in the legend to Table 3. The incubation time was normally 10 min and the reaction was stopped with 140 μl of 2.0 M- HClO_4 /ml. $^{14}\text{CO}_2$ was trapped as described earlier.

In addition, isolated and neutralized [^{14}C]malonic semialdehyde was incubated without enzyme and with either 1 mM-CoA, 5 mM-DTT or 1 mM-glutathione in 20 mM-potassium phosphate buffer, pH 7.4.

H.p.l.c. analysis

The metabolites in urine and in extracts from hepatocyte and mitochondria experiments were routinely analysed and isolated on a reverse-phase Polymer column PLRP-S 5 μm 100 Å (Polymer Laboratories Ltd., Church Stretton, Shropshire, U.K.). The column was eluted with a gradient of acetonitrile and water, both in 1.4% (v/v) orthophosphoric acid, starting with 1% acetonitrile, increasing to 100% acetonitrile in 20 min, followed by 100% acetonitrile for 3 min and returning to 1% acetonitrile in 2 min, at a flow rate of 0.4 ml/min. In addition, metabolites were analysed and isolated on the Aminex HPX-87H column eluted with 6.5 mM-sulphuric acid at an isocratic flow rate of 0.4–0.5 ml/min. The radioactivity was in all cases detected directly on-line in a Raytest Ramona 5LS liquid scintillation counter (Isotopenmessgeräte GmbH, Straubenhardt, Germany) equipped with a 1.0 ml flow cell. Eluate and liquid scintillant were mixed in a ratio of 1:7 (v/v).

Malonic semialdehyde was also analysed on the Polymer column. The column was eluted with acetonitrile and water, both in 1.4% (v/v) orthophosphoric acid, starting with 25% acetonitrile and increasing to 60% acetonitrile in 20 min at a flow rate of 0.6 ml/min.

Dicarboxylic sulphoxides were analysed with unlabelled standards on the Polymer column and the Aminex column. The Polymer column was eluted with 2.5% acetonitrile and 97.5% water, both in 1.4% (v/v) orthophosphoric acid, isocratic at a flow rate of 0.6 ml/min. The Aminex column was eluted with 6.5 mM-sulphuric acid at an isocratic flow rate of 0.6 ml/min.

T.l.c.

Silica G plates were used in all the t.l.c. analyses. The samples, together with carrier, were run in duplicate tracks. One track was exposed to iodine vapour and the other was cut into corresponding zones and immersed in scintillation fluid for counting.

Lipids. The butanol-extracted lipids from experiments *in vivo* and with hepatocyte suspensions were applied on t.l.c. and developed with hexane/diethyl ether/acetic acid (70:30:2, by vol.). The subclasses of phospholipids were separated using a mobile phase of chloroform/methanol/acetic acid/water (65:25:4:4, by vol.). The zones corresponding to standards of phosphatidylcholine and phosphatidylethanolamine were scraped from the silica plate and extracted. These purified phospholipids (approx. 200 μg) containing [^{14}C]stearic acid or [^{14}C]TTP were then exposed to hydrolysis with phospholipase A_2 in a Ca^{2+} -containing ethereal solution (Wells & Hanahan, 1969) and rechromatographed in the same system.

Dicarboxylic sulphoxides. The isolated short-chain dicarboxylic sulphoxides from [^{14}C]TTP were chromatographed on Silica

plates using a mobile phase of dioxane/benzene/formic acid (50:50:4, by vol.).

Malonic semialdehyde phenylhydrazone. Before t.l.c. analysis, the radioactive product formed from malonic semialdehyde and phenylhydrazine was isolated by h.p.l.c. and extracted twice from the eluate with ethyl acetate, dried under N_2 and applied as an ethanol solution on silica plates. The mobile phase was benzene/dioxane/formic acid (78:20:2, by vol.) ($R_f = 0.34$).

N.m.r. spectroscopy

N.m.r. spectra were recorded on a Varian (Sao Paulo, CA, U.S.A.) Gemini 200 spectrometer.

Other analytical methods

Free CoA was measured essentially as described (Garland *et al.*, 1965; Baqir & Booth, 1977), measuring formation of NADH at 340 nm using a Beckman Model 26 spectrophotometer.

Acyl-CoA esters were separated by h.p.l.c. essentially as described (Corkey *et al.*, 1981).

Protein was measured by the method of Lowry *et al.* (1951).

Animals

Male Wistar albino rats (200–300 g) were obtained from Veterinær Møllegaards Avlsstasjon, Havdrup, Denmark.

RESULTS

Metabolism *in vivo*

Fig. 2 shows that after intraperitoneal injection of $[1-^{14}C]TPP$, about 20% of the radioactivity was found in the urine and 7% as CO_2 within the first 24 h after the injection. After 54 h about 34% of the radioactivity had been recovered, 24% in the urine and 10% as CO_2 . When TTP-SO was given by stomach intubation, 75% of the radioactivity was recovered in the urine after 19 h (results not shown). The rat which was given TTP was killed after 54 h and liver, kidney and heart were removed. Lipids were extracted by the method of Folch *et al.* (1957). Of the administered activity, 3% was recovered in the lipids from the three organs. The lipids from liver, kidney and heart were analysed using t.l.c.. In liver, TTP was mainly incorporated into phospholipids and a trace amount into triacylglycerol. In kidney and heart TTP was incorporated equally into triacylglycerol and phospholipids (results not shown). The incorporation into complex lipids was further studied in isolated hepatocytes (see below).

The chromatograms in Fig. 3(I,II) show that the urine contained at least three metabolites from TTP. The metabolite labelled A in Fig. 3(I,II) was missing from urine of the rat given TTP-SO [Fig. 3(III)]. Peak C in Fig. 3(I) was isolated and shown to co-chromatograph with synthetic carboxypropylsulphoxypropionic acid in two h.p.l.c. systems (see the Experimental section). It also co-chromatographed with the same compound in t.l.c., $R_f = 0.20$ (see the Experimental section).

The metabolites from TTP-SO were isolated as described and carboxypropylsulphoxypropionic acid [peak C in Fig. 3(III)] was also found in this system, and was identified by n.m.r. The 1H n.m.r. (200 MHz) spectra for authentic carboxypropylsulphoxypropionic acid and peak C were identical, δ (p.p.m.) [2H_2O] 1.96–2.10 (2H, m, $CH_2CH_2CH_2S$), 2.53 (2H, t, $CH_2CH_2CH_2S$), 2.8–3.2 (6H, m). In addition peak B in Fig. 3(III) was identified as carboxymethylsulphoxypropionic acid by co-chromatography and n.m.r. The 1H n.m.r. (200 MHz) spectra for authentic carboxymethylsulphoxypropionic acid and peak B

Fig. 2. Excretion curve for labelled urine metabolites and $^{14}CO_2$.

Each rat was injected with 0.06 mg (1.8 μCi) of $[1-^{14}C]$ tetra-decylthiopropionic acid (TTP). Radioactivity in urine and CO_2 was measured as described. Δ , $^{14}CO_2$; $\square$, urine metabolites.

Fig. 3. Urine metabolites from TTP and TTP-SO separated on h.p.l.c.

Chromatograms were run on a Polymer column for the h.p.l.c. analysis of samples of urines from experiments carried out *in vivo* after either injecting 0.06 mg (1.8 μCi) of TTP or giving 30 mg (0.5 μCi) of TTP-SO by gastric tube, to rats. The column was eluted with acetonitrile and water both with 1.4% (v/v) orthophosphoric acid, starting with 1% acetonitrile and increasing to 100% acetonitrile in 20 min, at a flow rate of 0.4 ml/min. See the Experimental section for more details. (I) 2 h after injection of TTP. (II) 24 h after injection of TTP. (III) 19 h after injection of TTP-SO. Peak B is carboxymethylsulphoxypropionic acid. Peak C is carboxypropylsulphoxypropionic acid. (Peak A not identified.)

Table 1. Metabolism of TTP, palmitic acid and stearic acid in rat hepatocytes

The hepatocytes were incubated as described for 1 h with 0.5 mM- $[1-^{14}C]TTP$, $[1-^{14}C]$ palmitic acid or $[1-^{14}C]$ stearic acid. Results are given as nmol of substrate oxidized or esterified into triacylglycerol or phospholipids per mg of protein ($\pm$ S.D.). Numbers of experiments are given in parentheses.

Substrate	Distribution of metabolites (nmol/mg)				
	Acid-soluble products	CO_2	Diacylglycerol	Triacylglycerol	Phospholipids
Palmitic acid (4)	26.7 $\pm$ 7.0	3.7 $\pm$ 2.1	2.2 $\pm$ 0.8	19.9 $\pm$ 7.8	15.5 $\pm$ 6.0
TTP (3)	7.8 $\pm$ 1.9	0.8 $\pm$ 0.1	0.9 $\pm$ 0.2	11.8 $\pm$ 3.7	22.4 $\pm$ 7.2
Stearic acid (3)	12.8 $\pm$ 5.8	2.6 $\pm$ 0.5	0.8 $\pm$ 0.3	5.0 $\pm$ 1.0	10.3 $\pm$ 3.2

Fig. 4. Incorporation of stearic acid and TTP into total cellular phospholipids

Hepatocytes were incubated with $[1-^{14}\text{C}]$ stearic acid and $[1-^{14}\text{C}]$ TTP as described. Phospholipids were isolated by t.l.c., extracted from the silica, and rechromatographed in chloroform/methanol/acetic acid/water (65:25:4:4, by vol.). (a) Incorporation of stearic acid. (b) Incorporation of TTP. Most of the radioactivity is recovered in the phosphatidylcholine fraction, but some is recovered in phosphatidylethanolamine and phosphatidic acid (with solvent front) in both chromatograms. The phosphatidylcholine fractions from (a) and (b) were isolated separately, extracted from the silica plates, treated with phospholipase A_2 and rechromatographed in the above-mentioned system. Chromatogram (c) shows that stearic acid is incorporated mainly into position 1 in phosphatidylcholine, as most radioactivity is recovered in the lysophosphatidylcholine fraction. Little radioactivity is found in the substrate fraction, while the free fatty acids in the solvent front reflect incorporation into the 2-position of the lipid class. Chromatogram (d) shows a similar pattern of incorporation of TTP to that for stearic acid shown in (c).

were identical δ (p.p.m.) $[^2\text{H}_2\text{O}]$ 2.67–2.74 (2H, m, SCH_2CH_2), 2.82–3.22 (2H, m, SCH_2CH_2), 3.72 and 3.87 (2H, AB-q, J 15 Hz, COCH_2S). When rats received TTP these metabolites decreased steadily with time [Fig. 3(II)]. They were not detectable after 54 h, while peak A was still present (results not shown).

Peak A in Fig. 3(I,II) has not been identified. When purification was attempted, it proved to be volatile.

Metabolism in hepatocytes

Table 1 shows that $[1-^{14}\text{C}]$ TTP is oxidized relatively slowly to CO_2 and acid-soluble products compared with $[1-^{14}\text{C}]$ palmitic acid and $[1-^{14}\text{C}]$ stearic acid in isolated hepatocytes. TTP is taken up by the cells at a rate between that of palmitic acid and stearic acid. Because of the slow oxidation rate, TTP shows a high esterification rate. It shows a similar distribution between phospholipids and triacylglycerol to stearic acid. In phosphatidylcholine both TTP and stearic acid were found mainly in the 1-position (Fig. 4). Similar results were found for the phosphatidylethanolamine fractions (results not shown). Previously, we have shown that a shorter 3-thia fatty acid, dodecylthioacetic acid (DTA), is incorporated more into triacylglycerol, and thus is more similar to palmitic acid in its behaviour (Skrede *et al.*, 1989).

The h.p.l.c. of the acid-soluble extract from TTP showed products that were impossible to identify. The products could not be isolated into distinct peaks.

The metabolism of $[1-^{14}\text{C}]$ TTP-SO was compared with that of $[1-^{14}\text{C}]$ TTP in hepatocytes. TTP-SO is taken up at a slower rate than TTP (results not shown). In contrast with TTP, most of the radioactivity from TTP-SO was oxidized to acid-soluble products (70.4% of the radioactivity taken up by the cell). Some $^{14}\text{CO}_2$ is also released from TTP-SO (1.9%) and t.l.c. of the phospholipids showed some incorporation of radioactivity into products corresponding to phosphatidylcholine and lysophosphatidylcholine (13.8%). No incorporation into triacylglycerol was found. The identity of the phospholipids were not verified by other methods.

Fig. 5. Time curve for mitochondrial acid-soluble products and CO_2 from TTP

Rat liver mitochondria were incubated with $13.2 \mu\text{M}$ $[1-^{14}\text{C}]$ TTP and the release of $^{14}\text{CO}_2$ and labelled acid-soluble products was measured. For details see Experimental section. $\square$, CO_2 ; $\triangle$, acid-soluble products.

Unmodified TTP-SO accounted for 13.8% of the radioactivity in the cells.

The h.p.l.c. of the acid-soluble extract on the Polymer and Aminex columns showed the same two products resulted from $[1-^{14}\text{C}]$ TTP-SO metabolism in hepatocytes as were found in urine (results not shown). The major peak (approx. 88%) was carboxypropylsulphoxypropionic acid and the other (approx. 12%) carboxymethylsulphoxypropionic acid.

Metabolism in mitochondria

When $[1-^{14}\text{C}]$ TTP was incubated with rat liver mitochondria, radioactive CO_2 was released and, in addition, radioactivity was also found in the HClO_4 extract of the incubation mixture. Fig. 5 shows the time course for CO_2 release and production of acid-soluble products. No radioactive CO_2 nor radioactivity in the acid extract were found, without the addition of ATP in the

Fig. 6. Mitochondrial acid-soluble metabolites from TTP

Chromatograms were run on a Polymer column for h.p.l.c. analysis of acid-soluble products after mitochondrial incubation with $34 \mu\text{M}$ $[1\text{-}^{14}\text{C}]\text{TTP}$ and 0.024% phenylhydrazine. The column was eluted with acetonitrile and water containing 1.4% (v/v) orthophosphoric acid, starting with 1% acetonitrile and increasing to 100% acetonitrile in 20 min , at a flow rate of 0.4 ml/min . For more details see the Experimental section. (I) Acid-soluble products after mitochondrial incubation with standard incubation mixture. (II) Acid-soluble products after phenylhydrazine was added to the incubation mixture. Peak A is malonic semialdehyde, peak C is a thiohemiacetal of malonic semialdehyde and CoA, and peak D is malonic semialdehyde-phenylhydrazone.

incubation mixture (results not shown). Evidently activation to CoA ester is necessary.

Three radioactive peaks from $[1\text{-}^{14}\text{C}]\text{TTP}$ metabolism were found when the acid extract of the incubation was analysed by h.p.l.c. [Fig. 6(I)]. Because malonylsemialdehyde-CoA was identified as a product from a 4-thia-fatty-acid CoA ester (Lau *et al.*, 1989) (see also Introduction), phenylhydrazine was included in the incubation mixture to see whether malonic semialdehyde was a product from TTP. Fig. 6(II) shows that a new product appeared in the chromatogram (peak D) when phenylhydrazine was added. It co-eluted with freshly synthesized malonic semialdehyde phenylhydrazone both in h.p.l.c. and on t.l.c. silica plates, where 88% of the radioactivity was recovered from the synthetic malonic semialdehyde phenylhydrazone spot ($R_F = 0.34$) (results not shown).

When peak A in Fig. 6(I) was isolated and derivatized with phenylhydrazine the same product (malonic semialdehyde phenylhydrazone) was formed.

When malonic semialdehyde [A in Fig. 6(I)] was isolated and incubated with CoA, DTT or glutathione (in the absence of mitochondria or enzymes), new products appeared in the chromatograms (results not shown). The product formed after incubation with CoA co-eluted with peak C in Fig. 6(I). The products formed after incubation with DTT and glutathione eluted between peak A and peak C.

These results indicate that malonic semialdehyde reacts readily with free thiol groups and that peaks B and C in Fig. 6(I) are probably thiohemiacetals, formed spontaneously between malonic semialdehyde and either DTT or CoA. When DTT was omitted from the incubation mixture, peak B [Fig. 6(I)] disappeared.

The non-enzymic reaction of malonic semialdehyde with free CoA was further investigated by incubating freshly prepared malonic semialdehyde with CoA. Free CoA disappeared from the reaction mixture at an accelerated rate in the presence of malonic semialdehyde (results not shown). Besides both the product derived from mitochondrial incubation with TTP, peak C in Fig. 6(I) and the product formed spontaneously between CoA and malonic semialdehyde reacted with phenylhydrazine, giving malonic semialdehyde phenylhydrazone.

Table 2. Oxidation of $[1\text{-}^{14}\text{C}]\text{TTP}$ and of $[1\text{-}^{14}\text{C}]\text{palmitic acid}$ in liver mitochondria in the presence of tricarboxylic acid cycle intermediates

Rat liver mitochondria were incubated for 15 min with $6\text{--}7 \mu\text{M}$ $[1\text{-}^{14}\text{C}]\text{TTP}$ or for 6 min with $10 \mu\text{M}$ $[1\text{-}^{14}\text{C}]\text{palmitic acid}$ and standard incubation mixture with 5 mM of 2-oxoglutarate, malate/glutamate or succinate. The tabulated values represent means $\pm$ s.d. Numbers of experiments are shown in parentheses. (For CO_2 measurement with TTP and succinate the values represent only three experiments.)

Reaction mixture	Activity (% of control)	
	CO_2	Acid-soluble products
TTP (4)	100.0 ± 7.3^1	100.0 ± 22.6^2
TTP + 2-oxoglutarate (4)	50.6 ± 14.0	145.9 ± 19.9
TTP + malate/glutamate (4)	57.9 ± 8.5	224.0 ± 19.9
TTP + succinate (4)	50.6 ± 20.7	329.4 ± 41.1
Palmitic acid (3)	100.0 ± 10.9^3	100.0 ± 5.6^4
Palmitic acid + 2-oxoglutarate (3)	18.1 ± 1.1	144.4 ± 8.1
Palmitic acid + malate/glutamate (3)	5.8 ± 1.1	114.5 ± 10.5
Palmitic acid + succinate (3)	19.3 ± 1.8	156.0 ± 4.0

Absolute activity in nmol/min per mg of protein: $^1 0.016$; $^2 0.015$; $^3 0.057$; $^4 0.248$.

Table 3. Inhibition of CO_2 formation from TTP in rat liver mitochondria by malonic semialdehyde and not by malonic acid

Rat liver mitochondria were incubated for 15 min with $14.7 \mu\text{M}$ $[1\text{-}^{14}\text{C}]\text{TTP}$ with standard incubation mix (control) and with either 0.5 mM of freshly synthesized malonic semialdehyde or malonic acid. Results are given as $10^2 \times \text{nmol}$ of $[1\text{-}^{14}\text{C}]\text{TTP}$ oxidized to $^{14}\text{CO}_2$ or radioactive acid-soluble products/min per mg of protein and the tabulated values represent means $\pm$ s.d. for three experiments. Statistical significance: * $P < 0.0001$; † $P = 0.004$.

	$10^2 \times$ Activity (nmol/min per mg of protein)	
	CO_2	Acid-soluble products
Control	1.36 ± 0.02	2.78 ± 0.06
+ Malonic semialdehyde	$0.43 \pm 0.04^*$	$3.58 \pm 0.12^\dagger$
+ Malonic acid	1.26 ± 0.07	2.68 ± 0.10

When peak C in Fig. 6(I) was isolated and re-chromatographed in the h.p.l.c. system for CoA esters (see the Experimental section) no radioactivity was eluted with carriers acetyl-CoA and malonyl-CoA (results not shown).

Table 2 shows that addition of different tricarboxylic acid cycle intermediates to a mitochondrial incubation of $[1\text{-}^{14}\text{C}]\text{TTP}$ only moderately reduced the formation of radioactive CO_2 , while the radioactivity in the HClO_4 extract of the incubation mixture increased correspondingly. This is in marked contrast with palmitic acid, where tricarboxylic-acid-cycle intermediates strongly reduce the release of CO_2 (Table 2).

Table 3 shows that malonic semialdehyde strongly reduced the formation of radioactive CO_2 from $[1\text{-}^{14}\text{C}]\text{TTP}$ metabolism in mitochondria. Malonate had no such effect.

Table 4 shows that in mitochondrial extracts the release of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{malonic semialdehyde}$ is absolutely dependent on NAD^+ , partially dependent on CoA, and not dependent on ATP. Table 5 shows that malonyl-CoA had no effect on the production of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{malonic semialdehyde}$ in mitochondrial extracts, except malonyl-CoA as an intermediate.

Table 4. Co-factor requirement for maximal release of $^{14}\text{CO}_2$ from [1- ^{14}C]malonic semialdehyde

[1- ^{14}C]Malonic semialdehyde was isolated after incubation with [1- ^{14}C]TTP and used as a substrate for further incubation with mitochondrial extract as described in the text. Incubation time was 10 min. The following co-factors were tested: CoA (1 mM), NAD (2 mM) and ATP (2 mM). The results are presented as means $\pm$ S.D. for three experiments in $10^3 \times \text{nmol/min per mg of protein}$.

Additions	$10^3 \times \text{CO}_2$ release (nmol/min per mg of protein)
None	1.7 ± 0.2
CoA	2.4 ± 0.4
NAD ⁺	13.0 ± 0.6
CoA/NAD ⁺	18.8 ± 0.6
CoA/NAD ⁺ /ATP	18.6 ± 0.5

Table 5. Oxidation of [1- ^{14}C]malonic semialdehyde to $^{14}\text{CO}_2$ in the presence of malonyl-CoA

Rat liver mitochondrial extract was incubated for 10 min with $0.4 \mu\text{M}$ -[1- ^{14}C]malonic semialdehyde which was isolated after rat liver mitochondrial incubation with $57 \mu\text{M}$ -[1- ^{14}C]TTP. Either 0.5 mM unlabelled malonic semialdehyde or 0.5 mM -malonyl-CoA was added to the standard incubation mix. The results are given as $10^3 \times \text{nmol}$ of [1- ^{14}C]malonic semialdehyde oxidized to $^{14}\text{CO}_2/\text{min per mg of protein}$ and the tabulated values represent means $\pm$ S.D. of three experiments. Statistical significance: * $P < 0.0001$.

	$10^2 \times \text{Release of } ^{14}\text{CO}_2$ (nmol/min per mg of protein)
Control	2.27 ± 0.006
+ Malonic semialdehyde	$0.18 \pm 0.03^*$
+ Malonyl-CoA	2.30 ± 0.06

DISCUSSION

Metabolism *in vivo*

The results of this study indicate that TTP is metabolized by at least two pathways *in vivo* (β -oxidation and ω -oxidation) (see Scheme 1). A few percent of the injected [1- ^{14}C]TTP were identified as carboxypropylsulphoxypropionic acid in the urine. We have previously shown that 3-thia acids are metabolized by an initial ω -oxidation and/or sulphur-oxygenation (Hvattum *et al.*, 1991). Identification of carboxypropylsulphoxypropionic acid and carboxymethylsulphoxypropionic acid as urine metabolites from a 4-thia acid, TTP, and a 4-thia acid sulphoxide, TTP-SO, indicate that TTP is, in part, metabolized via this pathway. These metabolites correspond to those found in the metabolism of a 3-thia acid (Bergseth & Bremer, 1990).

The experiments with [1- ^{14}C]TTP-SO indicate that this compound is mainly metabolized via ω -oxidation from the methyl end, since it was more rapidly excreted as short dicarboxylic sulphoxides in the urine.

Significant amounts of [1- ^{14}C]TTP (10% after 54 h) were recovered as $^{14}\text{CO}_2$. This indicates that direct β -oxidation is more important in the metabolism of TTP, especially since the dicarboxylic metabolites disappeared from the urine with time.

The unidentified urine metabolite from TTP [peak A in Fig. 3(I,II)] was absent in animals given TTP-SO [Fig. 3(III)] and therefore is most likely a β -oxidation product of TTP. It may be a malonic semialdehyde addition product formed in the kidneys (see below).

Metabolism in hepatocytes

This study shows that TTP, like 3-thia acids (Skrede *et al.*, 1989), is incorporated into lipids as efficiently as ordinary fatty acids. TTP is taken up by the cells even more rapidly than stearic acid, which is the same length. Both are incorporated mainly into phospholipids in the 1-position. This compares well with results found for 14(*R,S*)-[^{18}F]fluoro-6-thiaheptadecanoic acid (DeGrado *et al.*, 1991), where, in mouse, the 6-thia acid was mainly incorporated into phospholipids in heart and liver.

In hepatocytes, no formation of short dicarboxylic sulphoxides from TTP could be detected, but the acid extracts contained several metabolites, which are probably secondary products of malonic semialdehyde.

The results from incubating [1- ^{14}C]TTP-SO with hepatocytes correlate well with results *in vivo*. Dicarboxylic sulphoxides were the main metabolic products. Clearly 4-thia sulphoxide is poorly incorporated into lipids and poorly β -oxidized. This agrees well with a study on the activation of these fatty-acid analogues. No activation to a CoA-ester was found with a 3-thia sulphoxide (Aarsland & Berge, 1991).

The small amount of CO_2 released when incubating with TTP-SO might even be explained by α -oxidation or enzymic reduction of the oxygenated sulphur atom before release of CO_2 . Sulphoxide reductase activity has been shown, e.g. in guinea pig liver (Yoshihara & Tatsumi, 1990), where diphenyl sulphoxide was reduced to diphenyl sulphide.

Metabolism in mitochondria

The main metabolic product from TTP metabolism in mitochondria was identified as malonic semialdehyde by derivatization with phenylhydrazine. This indicates that malonic semialdehyde-CoA, shown to be the product when 4-thia-acyl-CoA is oxidized with purified acyl-CoA dehydrogenase and hydrated with enoyl-CoA hydratase (Lau *et al.*, 1989), is easily hydrolysed to malonic semialdehyde in intact mitochondria.

Aldehydes are reactive compounds, making hemithioacetals with SH-compounds and Schiff's bases with amino groups in spontaneous reactions. Some of these hemithioacetals are relatively stable (Lienhard & Jencks, 1966; Field & Sweetman, 1969). We have, in this study, confirmed that malonic semialdehyde forms addition products with CoA, glutathione and DTT. These observations agree with results found when studying the effects of ethanol in liver and brain of white mice. Acetaldehyde, a metabolic product of ethanol, was found to inactivate CoA (Ammon *et al.*, 1969).

The enzymic metabolism of malonic semialdehyde has been a subject of discussion (Griffith, 1986). Malonic semialdehyde is generated from β -alanine by transamination (Roberts & Bregoff, 1953; Pihl & Fritzson, 1955; Kupiecki & Coon, 1957), probably from 3-methylthiopropionate formed in the transamination pathway of methionine (Steele & Benevenga, 1978), and from propionyl-CoA by β -oxidation. Two different mechanisms have been described for the conversion of malonic semialdehyde into acetyl-CoA and CO_2 , a direct conversion of malonic semialdehyde into acetyl-CoA with the concomitant release of the carboxyl group as CO_2 (Yamada & Jakoby, 1960) or conversion via malonic semialdehyde-CoA ester into malonyl-CoA followed by decarboxylation to acetyl-CoA (Vagelos, 1960). It has been shown that malonyl-CoA is not an intermediate in the metabolism of β -alanine to acetyl-CoA (Scholem & Brown, 1983). Moreover, a methylmalonic semialdehyde dehydrogenase has been purified from rat liver and shown to be active with both methylmalonic semialdehyde and malonic semialdehyde, the products being propionyl-CoA and acetyl-CoA respectively (Goodwin *et al.*, 1989). Our results all agree with a metabolism of malonic

Scheme 1. Metabolic pathway of TTP

Scheme to show the two metabolic pathways of TTP in rat liver. (a) Mitochondrial. (b) Extra-mitochondrial.

semialdehyde via the methylmalonic semialdehyde dehydrogenase to acetyl-CoA and CO_2 . Conversion of $[1\text{-}^{14}\text{C}]$ malonic semialdehyde into labelled malonic acid or such as labelled CoA esters such as acetyl-CoA or malonyl-CoA was not detected. The presence of unlabelled malonic acid and tricarboxylic-acid-cycle intermediates had little or no inhibitory effect on $^{14}\text{CO}_2$ formation, while unlabelled malonic semialdehyde had a diluting

effect. These results suggest that $[1\text{-}^{14}\text{C}]$ malonic semialdehyde is oxidized by the mitochondrial methylmalonic semialdehyde dehydrogenase to $^{14}\text{CO}_2$ and unlabelled acetyl-CoA, i.e. $^{14}\text{CO}_2$ is formed independently of the tricarboxylic acid cycle.

In mitochondrial extracts the release of $^{14}\text{CO}_2$ was absolutely dependent on NAD^+ and CoA, and not dependent on ATP (Table 4).

Altogether our results indicate that TTP is metabolized by Scheme 1.

An interesting question is why the effects of 3-thia and 4-thia fatty acids are so diverse (see Introduction). Most likely the striking differences are connected with their different pathways of metabolism.

The 3-thia acids can only be ω -oxidized. They probably accumulate as free acids and as CoA esters in the cell and thereby trigger induction of lipid-metabolizing enzymes.

TTP shares the ω -oxidation pathway with 3-thia fatty acids. Most likely, therefore, the inhibition of fatty-acid oxidation and induction of hepatic lipidosis by TTP is caused by metabolites formed in the direct β -oxidation pathway. The enoyl-CoA is easily formed and only slowly hydrated (Lau *et al.*, 1989). This intermediate may therefore accumulate and cause inhibition of fatty-acid oxidation. The subsequently formed long-chain thiol and malonic semialdehyde, with a very reactive aldehyde group, may also accumulate and cause metabolic disturbances, e.g. by formation of addition products with CoA.

Very little is known about the metabolism of long-chain alkylthiols. Some of the radioactivity from the earlier mentioned 6-thia fatty acid, 14(*R,S*)-[^{18}F]fluoro-6-thiaheptadecanoic acid, was found bound in the protein fraction (DeGrado *et al.*, 1991). This 6-thia fatty acid is probably metabolized in a similar way to our 4-thia acid (TTP) in the mitochondria, the result being a radioactive long-chain thiol. DeGrado *et al.* speculate whether the long-chain thiol reacts with proteins. Short-chain thiol compounds have been studied (methanethiol, ethanethiol, etc.) and have been shown to inhibit cytochrome *c* and cytochrome *c* oxidase non-competitively (Wilms *et al.*, 1980). Other thiol compounds (e.g. mercaptopropionic acid) were found to be potent inhibitors of fatty-acid oxidation, after activation to its CoA ester (Sabbagh *et al.*, 1985).

The metabolic effects of the thia fatty acids make them interesting 'probes' with which to reveal regulatory mechanisms in the metabolism of fatty acids.

We are grateful to Dr. Siw B. Fredriksen for her help with running the n.m.r. spectra. This work has been supported by the Norwegian Research Council of Science and the Humanities.

REFERENCES

- Aarsland, A. & Berge, R. K. (1991) *Biochem. Pharmacol.* **41**, 53–61
- Aarsland, A., Aarsaether, N., Bremer, J. & Berge, R. K. (1989) *J. Lipid Res.* **30**, 1711–1718
- Ammon, H. P. T., Estler, C.-J. & Heim, F. (1969) *Biochem. Pharmacol.* **18**, 29–33
- Baqir, Y. A. & Booth, R. (1977) *Biochem. J.* **164**, 501–508
- Berge, R. K., Aarsland, A., Kryvi, H., Bremer, J. & Aarsaether, N. (1989a) *Biochim. Biophys. Acta* **1004**, 345–356
- Berge, R. K., Aarsland, A., Kryvi, H., Bremer, J. & Aarsaether, N. (1989b) *Biochem. Pharmacol.* **38**, 3969–3980
- Bergseth, S. & Bremer, J. (1990) *Biochim. Biophys. Acta* **1044**, 237–242
- Bergseth, S., Christiansen, E. N. & Bremer, J. (1986) *Lipids* **21**, 508–514
- Bergseth, S., Hokland, B. M. & Bremer, J. (1988) *Biochim. Biophys. Acta* **961**, 103–109
- Corkey, B. E., Brandt, M., Williams, R. J. & Williamson, J. R. (1981) *Anal. Biochem.* **118**, 30–41
- DeGrado, T. R., Coenen, H. H. & Stöcklin, G. (1991) *J. Nucl. Med.* **32**, 1888–1896
- Field, L. & Sweetman, B. J. (1969) *J. Org. Chem.* **34**, 1799–1803
- Folch, J., Lees, M. & Sloane Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Garland, P. B., Shepherd, D. & Yates, D. W. (1965) *Biochem. J.* **97**, 587–594
- Goodwin, G. W., Rougraff, P. M., Davis, E. J. & Harris, R. A. (1989) *J. Biol. Chem.* **264**, 14965–14971
- Griffith, O. W. (1986) *Annu. Rev. Biochem.* **55**, 855–878
- Hovik, R., Osmundsen, H., Berge, R. K., Aarsland, A., Bergseth, S. & Bremer, J. (1990) *Biochem. J.* **270**, 167–173
- Hvattum, E., Bergseth, S., Pedersen, C. N., Bremer, J., Aarsland, A. & Berge, R. K. (1991) *Biochem. Pharmacol.* **41**, 945–953
- Kupiecki, F. P. & Coon, M. J. (1957) *J. Biol. Chem.* **229**, 743–754
- Lau, S.-M., Brantley, R. K. & Thorpe, C. (1989) *Biochemistry* **28**, 8255–8262
- Lienhard, G. E. & Jencks, W. P. (1966) *J. Am. Chem. Soc.* **88**, 3982–3995
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Myers, D. K. & Slater, E. C. (1957) *Biochemistry* **67**, 558–572
- Pihl, A. & Fritzson, P. (1955) *J. Biol. Chem.* **215**, 345–351
- Roberts, E. & Bregoff, H. M. (1953) *J. Biol. Chem.* **201**, 393–398
- Sabbagh, E., Cuebas, D. & Schulz, H. (1985) *J. Biol. Chem.* **260**, 7337–7342
- Scholem, R. D. & Brown, G. K. (1983) *Biochem. J.* **216**, 81–85
- Seglen, P. O. (1973) *Exp. Cell Res.* **82**, 391–398
- Skrede, S., Narce, M., Bergseth, S. & Bremer, J. (1989) *Biochim. Biophys. Acta* **1005**, 296–302
- Spydevold, Ø. & Bremer, J. (1989) *Biochim. Biophys. Acta* **1003**, 72–79
- Steele, R. D. & Benevenga, N. J. (1978) *J. Biol. Chem.* **253**, 7844–7850
- Vagelos, P. R. (1960) *J. Biol. Chem.* **235**, 346–350
- Wells, M. A. & Hanahan, D. J. (1969) *Biochemistry* **8**, 414–418
- Wilms, J., Lub, J. & Wever, R. (1980) *Biochim. Biophys. Acta* **589**, 324–335
- Woeller, F. H. (1961) *Anal. Biochem.* **2**, 508–511
- Yamada, E. W. & Jakoby, W. B. (1960) *J. Biol. Chem.* **235**, 589–594
- Yoshihara, S. & Tatsumi, K. (1990) *Drug Metab. Dispos.* **876**–881

Received 5 December 1991/2 February 1992; accepted 10 March 1992