Products and intermediates of the β -oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA by rat liver peroxisomes and mitochondria

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1. The synthesis of [U-¹⁴C]hexadecanedionoyl-mono-CoA is described. 2. The β -oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA by purified rat liver peroxisomes and mitochondria is demonstrated. 3. The products of mitochondrial β -oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA include ketone bodies, citrate and acetylcarnitine. 4. Tetradecanedionoyl-mono-CoA, hexadec-2-enedionoyl-mono-CoA and hexadionoyl-mono-CoA were the only detectable intermediates formed by mitochondrial β -oxidation, whereas acetyl-CoA and all saturated even-numbered intermediates of chain length C₆–C₁₆ were generated by peroxisomal β -oxidation. 5. Hexadecanedionoyl-mono-CoA and hexadecanoyl-CoA were equally effective substrates for peroxisomal β -oxidation, but hexadecanedionoyl-mono-CoA was a relatively poorer substrate for the mitochondrial pathway.

INTRODUCTION

The excretion of dicarboxylic acids of chain length C₆–C₁₀ is observed in a number of metabolic states including diabetic ketoacidosis (Pettersen, 1972), riboflavin deficiency (Goodman, 1981; Gregersen & Kolvraa, 1982; Veitch *et al.*, 1989) and prolonged fasting (Mortensen & Gregersen, 1981). Furthermore, the administration of a high-fat diet (Mortensen, 1981) or of medium-chain triacylglycerols (Shigematsu *et al.*, 1981) also results in dicarboxylicaciduria. In addition, inherited disorders of mitochondrial β -oxidation (Przyrembel *et al.*, 1976; Gregersen *et al.*, 1983; Vianey-Liaud *et al.*, 1987) and acquired disorders such as Jamaican Vomiting Sickness, caused by the ingestion of hypoglycin (Tanaka, 1972), are characterized by the excretion of a constellation of abnormal metabolites which includes medium-chain dicarboxylic acids. The administration of oxirane-carboxylic acids, which, as their corresponding CoA esters, are potent inhibitors of carnitine palmitoyltransferase I, causes a dicarboxylic aciduria (Sherratt & Veitch, 1984). Furthermore, valproate intoxication is accompanied by a dicarboxylicaciduria (Mortensen *et al.*, 1980).

The metabolic origin of dicarboxylic acids remains uncertain. Clearly, ω -oxidation must occur at some stage and, with the exception of the dicarboxylicaciduria due to medium-chain triacylglycerol administration, chain shortening by β -oxidation must also occur, since the majority of long-chain fatty acids have a chain length greater than C₁₆. The question arises as to whether there is first ω -oxidation of monocarboxylic acids followed by chain-shortening of the corresponding dicarboxylates by β -oxidation, or vice versa, or both. If the former pathway is operative, β -oxidation of long-chain dicarboxylates could occur by either the mitochondrial or the peroxisomal pathway. In the present study we demonstrate that both intact purified rat liver peroxisomes and intact purified mitochondria are capable of oxidizing hexadecanedionoyl-mono-CoA efficiently, and we characterize the intermediates and products which are generated.

MATERIALS AND METHODS

Materials

Dicarboxylic acids were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Acetyl-CoA, succinyl-CoA, CoA, ATP, cytochrome *c*, myxathiazole, NAD⁺, NADP⁺, lactate dehydrogenase, acyl-CoA synthetase, acyl-CoA oxidase, crotonase, 3-hydroxyacyl-CoA dehydrogenase and isocitrate dehydrogenase were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). [U-¹⁴C]Palmitic acid (specific radioactivity 828 mCi/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.). Percoll and Dextran T-10 were obtained from Pharmacia, Uppsala, Sweden. Ciprofibrate was a gift from Stirling-Winthrop Research. All other reagents were of the highest available purity unless otherwise specified.

Animals

Male Wistar rats (200–300 g) were maintained on a standard laboratory animal feed (22.7 % protein, 38.5 % carbohydrate, 4.3 % fat, by wt.) supplemented with 0.5 % (w/w) ciprofibrate for 2–3 weeks before they were killed for the preparation of peroxisomal and microsomal fractions. Animals maintained on unsupplemented diets were used for the preparation of mitochondrial fractions.

Synthesis of standard dicarboxyl-mono-CoA esters

The mono-CoA esters of dodecanedioic, tetradecanedioic and hexadecanedioic acids were synthesized enzymically using acyl-CoA synthetase and were purified using Bond Elute cartridges (Analytichem International) as described below for [U-¹⁴C]-hexadecanedionoyl-mono-CoA. The CoA esters of hexanedioic, octanedioic and decanedioic acids were synthesized from the corresponding monochlorides, which were produced by reaction of the free acids with thionyl chloride. Each of the standard CoA esters gave a single peak on analysis by reverse-phase h.p.l.c., as described below, and exhibited u.v. absorption spectra typical of acyl-CoA thioesters. To exclude the possibility

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that di-CoA esters had been generated, each standard compound was analysed by fast-atom-bombardment m.s. Each was shown to have a molecular ion expected of the mono-CoA ester (results not shown).

Preparation of mitochondrial, peroxisomal and microsomal fractions

Mitochondrial fractions were prepared from the livers of male Wistar rats (200–300 g) in 250 mM-sucrose/2 mM-Hepes/0.1 mM-EGTA (pH 7.5) at 0–4 °C and resuspended in isolation medium at a concentration of 25–40 mg/ml (Causey *et al.*, 1986). This crude mitochondrial fraction was further purified using a self-generated Percoll gradient. The fraction of density 1.1–1.108 g/ml, which contained the highest citrate synthase activity (Srere *et al.*, 1965), was collected and resuspended in 3 vol. of medium containing 250 mM-sucrose, 10 mM-Mops, 1% (v/v) ethanol, 10% (w/v) Dextran T-10 and 1 mM-EGTA, pH 7.2, and centrifuged at 2.95×10^8 rad²/s, and the pellet was resuspended in 2 ml of isolation medium. Respiratory control ratios, measured with 10 mM-glutamate plus 1 mM-malate as substrate (Watmough *et al.*, 1988), were routinely 5.5–7.5. Mitochondrial protein was determined by the method of Lowry *et al.* (1951), using BSA as standard.

Rat liver peroxisomal fractions were prepared using self-generated Percoll gradients as described previously (Neat *et al.*, 1981; Osmundsen, 1982). The fraction of density 1.06–1.075 g/ml, which contained the highest activity of acyl-CoA oxidase (Hovik & Osmundsen, 1989) and which was essentially free of citrate synthase activity (Srere *et al.*, 1965), was collected. The specific activity of urate oxidase was measured by the method of de Duve *et al.* (1955) in the mitochondrial and peroxisomal fractions (5.9 ± 1.1 and 185.0 ± 4.3 nmol/min per mg of protein respectively; means $\pm$ S.E.M., $n = 4$). These data suggest that contamination of the mitochondrial fraction by peroxisomes was minimal.

A washed microsomal fraction was prepared from the livers of rats treated with ciprofibrate by high-speed centrifugation of the post-mitochondrial supernatant.

Synthesis and purification of [U-¹⁴C]hexadecanedionoyl-mono-CoA and [U-¹⁴C]hexadecanoyl-CoA

[U-¹⁴C]Palmitate (5.4 μ mol; specific radioactivity 46.5 mCi/mmol) was incubated with washed rat liver microsomes, prepared from ciprofibrate-treated rats, in the presence of 5 mM-MgCl₂, 5 μ M-MnCl₂, 1 mM-NADP⁺, 0.5 mM-NADH, 5 mM-isocitrate, 10 units of isocitrate dehydrogenase, 50 mM-Tris buffer, pH 7.5, and BSA (1 mg/ml) in a total volume of 20 ml for 14 h at 30 °C. The reaction was terminated by the addition of KOH to a final pH of 12, and the reaction mixture was freeze-dried. The residue was resuspended in 2 ml of water and the pH taken to < 1 with HCl. Organic acids were extracted 3 $\times$ 3 vol. of ethyl acetate, the extract dried over anhydrous Na₂SO₄ and the solvent removed by rotary evaporation. Preliminary experiments with unlabelled palmitate and analysis by g.c.-m.s. of the products methylated by the addition of an excess of diazomethane showed that there was about 80% conversion of hexadecanoate into hexadecanedionate (results not shown). However, hexadecanoate and other fatty acids were also present, the latter presumably derived from microsomal lipids. The mass spectra of the biosynthesized hexadecanedionate and the standard compound were identical (results not shown). The radioactive preparation was used without further purification and converted into the corresponding mono-CoA ester by incubation with 1 unit of acyl-CoA synthetase in medium containing 10 mM-MgCl₂, 10 mM-ATP, 3 mM-CoA, 0.1% Triton and 50 mM-KH₂PO₄,

pH 7.4, in a total volume of 5 ml for 4 h at 30 °C. The reaction was terminated by the addition of HCl to bring the pH to 2. The product was purified by preparative h.p.l.c. using a 250 mm $\times$ 10 mm 10 μ m pore size C₁₈ reverse-phase Lichrosorb column developed with a gradient of acetonitrile in 50 mM-potassium phosphate, pH 5.3, as described below. Radioactivity and u.v. absorbance were monitored continuously and the major radioactive peak, which co-chromatographed with standard hexadecanedionoyl-mono-CoA, was collected. This fraction was concentrated and the phosphate was removed using a C₁₈ Bond Elute cartridge which was eluted with 50% (v/v) acetonitrile, freeze-dried and redissolved in water. Re-analysis of the product by radio-h.p.l.c. using an analytical (250 mm $\times$ 4.6 mm) 5 μ m pore size C₁₈ Lichrosorb column revealed a single radioactive peak which co-chromatographed with standard hexadecanedionoyl-mono-CoA and which had a u.v. absorbance spectrum characteristic of that of saturated thioesters (Fig. 1). A portion of the product was saponified and the resultant free acid was derivatized with 4-nitrobenzoyl di-isopropylisourea and analysed by radio-h.p.l.c. as the 4-nitrobenzoyl ester; again a single radioactive peak was observed which co-chromatographed with standard di-(4-nitrobenzoyl)hexadecanedionate (results not shown).

[U-¹⁴C]Hexadecanoyl-CoA was synthesized from [U-¹⁴C]-hexadecanoate as described above for [U-¹⁴C]hexadecanedionoyl-mono-CoA. Analysis of the product by radio-h.p.l.c. revealed a single product which co-chromatographed with standard hexadecanoyl-CoA.

Mitochondria incubation conditions

Mitochondria (2–3 mg of protein) were preincubated at 35 °C for 5 min in a medium containing 110 mM-KCl, 2.5 mM-KH₂PO₄, 5 mM-MgCl₂, 10 mM-Hepes, pH 7.4, 1 mM-EGTA, 1 mM-ADP, 1 mM-ATP, 0.5 mM-L-carnitine, cytochrome *c* (1 mg/ml) and 50 μ M-fluorocitrate. The reaction was started by the addition of the substrate, 22 μ M-[U-¹⁴C]hexadecanedionoyl-mono-CoA (specific radioactivity 1.8–20 mCi/mmol, depending on the nature of the experiment). Reactions were quenched and samples were prepared for the analysis of intermediates of β -oxidation as described previously (Watmough *et al.*, 1989), except that the internal standard used was 20 nmol of undecanedionoyl-mono-CoA. For the analysis of the products of β -oxidation, reactions were quenched by the addition of 50 μ l of 20% (v/v) HClO₄ to 250 μ l aliquots of the reaction mixture, allowed to stand on ice for 30 min and brought to pH 4.5 by the addition of KOH and centrifuged at 12000 *g* for 5 min. The resultant organic acids were analysed directly by radio-h.p.l.c. as described below. For the measurement of acid-soluble radioactivity, 250 μ l aliquots of the reaction mixture were treated with 50 μ l of glacial acetic acid; 300 μ l of 30% (w/v) BSA was then added, followed by 300 μ l of 20% (v/v) HClO₄ and the mixture was allowed to stand for 30 min on ice. After centrifugation (12000 *g* for 5 min), 700 μ l of the supernatant was taken for determination of radioactivity. This procedure was adopted to ensure quantitative precipitation of the substrate.

Peroxisomal incubation conditions

Peroxisomes (0.5–1.0 mg of protein) were incubated in the presence of 130 mM-KCl, 20 mM-Hepes, pH 7.2, 0.1 mM-EGTA, 0.1 mM-NAD⁺, 1 mM-FAD, 0.5 mM-CoA, 5 mM-MgCl₂/ATP, 1 mM-dithiothreitol, 1 mM-NH₄Cl, 1 mM-pyruvate, myxathiazole (3 μ g/ml) and lactate dehydrogenase (1 unit/ml). The reaction was started by the addition of 22 μ M-[U-¹⁴C]hexadecanedionoyl-mono-CoA (specific radioactivity 4.5 mCi/mmol). Reactions were quenched and samples were prepared for the analysis of intermediates of β -oxidation as described previously (Bartlett

Fig. 1. Radio-h.p.l.c. analysis of synthetic $[U-^{14}C]$ hexadecanoyl-mono-CoA

Upper trace, radioactivity; lower trace, A_{260} ; inset in lower trace, u.v. absorption spectrum.

et al., 1990), except that 20 nmol of undecanoyl-mono-CoA was used as internal standard.

Radio-h.p.l.c. analysis of acyl-CoA esters

A Waters 600 solvent delivery system fitted with a Lichrosorb $5\ \mu\text{m}$ pore size C_{18} column (250 mm $\times$ 4.6 mm) (E. Merck, Darmstadt, Germany) was used for h.p.l.c. The column was maintained at 30°C . Samples were introduced with a Waters U6K injector. All solvents were de-aerated with helium. Acyl-CoA esters were resolved by the following binary gradient of acetonitrile in 50 mM- KH_2PO_4 (pH 5.3): isocratic 5% (v/v), 5 min; isocratic 10% (v/v), 0.1 min; linear gradient 10–40% (v/v), 29.9 min; linear gradient 40–50% (v/v), 5 min; linear gradient 50–5% (v/v), 5 min. The flow rate was 1.5 ml/min and the total run time was 45 min. The column was re-equilibrated for at least 10 min under the starting conditions between analyses to maintain reproducibility of retention times.

Analytes were detected with a Waters 990 photodiode array detector (8 μl flow cell; 10 mm path-length). Spectra were acquired at 27 ms intervals over the range 200–300 nm with a band-width of 1.4 nm. Spectral data were acquired on a NEC APC IV microcomputer using dedicated software. Radioactivity associated with eluted compounds was detected on-line with an LKB Betacord radioactivity monitor fitted with a 1 ml flow cell as described previously (Causey *et al.*, 1986; Watmough *et al.*, 1988). Since the flow rate of effluent from the photodiode array detector was 1.5 ml/min, the flow rate of the scintillation fluid (10 g of 2,5-diphenyloxazole, 330 ml of Triton X-100, 150 ml of methanol and 670 ml of xylene) was 6 ml/min. The photodiode array detector and radioactivity monitor were connected in series and analogue signals from each detector were acquired by a Waters chromatography data station (model 840). This allowed the generation of superimposable radiochemical and u.v. traces. The time lag between the detectors was determined using $[U-^{14}C]$ hexadecanoyl-CoA.

Radio-h.p.l.c. analysis of β -oxidation products

The organic acid products of β -oxidation were analysed by radio-h.p.l.c. using a 100 mm $\times$ 4.6 mm $5\ \mu\text{m}$ pore size C_8 Spherisorb cartridge (Technicol Ltd., Stockport, Cheshire, U.K.) connected in series with a 220 mm $\times$ 4.6 mm $10\ \mu\text{m}$ pore size Polypore H cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.), eluted isocratically with 5 mM- H_2SO_4 (0.25 ml/min) at 60°C .

Radio-h.p.l.c. analysis of acylcarnitines

Acylcarnitines were analysed by radio-h.p.l.c. as described previously (Watmough *et al.*, 1988).

RESULTS

Flux of rat liver mitochondrial β -oxidation of $[U-^{14}C]$ hexadecanoyl-mono-CoA

The data presented in Fig. 2 show a comparison of the time course of generation of acid-soluble radioactivity from $[U-^{14}C]$ hexadecanoyl-CoA and $[U-^{14}C]$ hexadecanoyl-mono-CoA by purified intact rat liver mitochondrial fractions. Each substrate was present at a concentration of $22\ \mu\text{M}$. Both substrates were oxidized to completion, the maximal rate of oxidation of

Fig. 2. Time course of the mitochondrial oxidation of $[U-^{14}C]$ hexadecanoyl-CoA and $[U-^{14}C]$ hexadecanoyl-mono-CoA

The oxidations of hexadecanoyl-CoA (■) and hexadecanoyl-mono-CoA (□) were measured as described in the Materials and methods section. The oxidation of hexadecanoyl-mono-CoA was also measured in the absence of carnitine (●) and in the presence of a mixture of rotenone ($2.5\ \mu\text{g/ml}$), myxothiazole ($2.5\ \mu\text{g/ml}$), antimycin A ($10\ \mu\text{g/ml}$) and sodium cyanide ($1\ \text{mM}$) (○).

Fig. 3. Time course of the formation of organic acid products of mitochondrial oxidation of $[U-^{14}C]$ hexadecanoyl-mono-CoA

□, Ketone bodies; ■, acid-soluble radioactivity; ●, citrate. Conditions of incubations and analysis were as described in the Materials and methods section.

Table 1. Time course of [^{14}C]acetylcarnitine production from [$\text{U-}^{14}\text{C}$]hexadecanedionoyl-mono-CoA oxidation by mitochondrial fractions

Conditions of incubation and analysis were as described in the Materials and methods section.

Incubation time (min)	[^{14}C]Acetylcarnitine (d.p.m.)
2	1009
3	1502
5	2650
10	4328
15	7505

the dicarboxylate being about 35% of that of the mono-carboxylate (1.34 and 3.79 nmol/min per mg of protein respectively). Furthermore, the oxidation of [$\text{U-}^{14}\text{C}$]hexadecanedionoyl-mono-CoA was carnitine-dependent and was inhibited by respiratory poisons. It is therefore most unlikely that the oxidation of [$\text{U-}^{14}\text{C}$]hexadecanedionoyl-mono-CoA is the result of contamination by peroxisomes, since peroxisomal β -oxidation is (i) carnitine-independent, and (ii) independent of the respiratory chain. Furthermore, the mitochondrial fractions used in these studies were purified by Percoll gradient isopycnic centrifugation.

Products of rat liver mitochondrial β -oxidation of [$\text{U-}^{14}\text{C}$]hexadecanedionoyl-mono-CoA

Analysis of the organic acid products of the mitochondrial oxidation of [$\text{U-}^{14}\text{C}$]hexadecanedionoyl-mono-CoA revealed the presence of citrate and ketone bodies (Fig. 3). However, only about 50% of the acid-soluble radioactivity could be accounted for by these products. Since the incubations were made in the presence of fluorocitrate, this cannot be attributed to $^{14}\text{CO}_2$ production and, as expected, no other tricarboxylic acid cycle intermediates were detected. However, radio-h.p.l.c. analysis of the acylcarnitines generated revealed a single major peak which co-chromatographed with acetylcarnitine (results not shown). Furthermore, the production of [^{14}C]acetylcarnitine was time-

Fig. 4. Time course of the formation of organic acid products of mitochondrial oxidation of [$\text{U-}^{14}\text{C}$]hexadecanedionoyl-mono-CoA after alkaline hydrolysis

○, Acetate; □, ketone bodies; ■, substrate utilized; ●, citrate. Conditions of incubations and analysis were as described in the Materials and methods section.

Fig. 5. Radio-chromatogram of acyl-CoA intermediates generated by the mitochondrial (a) and peroxisomal (b) oxidation of [$\text{U-}^{14}\text{C}$]hexadecanedionoyl-mono-CoA

The radioactive peaks co-chromatographed with, respectively: 1, succinyl-CoA; 2, acetyl-CoA; 3, hexanedionoyl-mono-CoA; 4, octanedionoyl-mono-CoA; 5, decanedionoyl-mono-CoA; 6, dodecanedionoyl-mono-CoA; 7, tetradecanedionoyl-mono-CoA; 8, hexadec-2-enedionoyl-mono-CoA; 9, hexadecanedionoyl-mono-CoA. Conditions of incubations and analysis were as described in the Materials and methods section.

dependent (Table 1). Thus the acetyl-CoA generated from the oxidation of [$\text{U-}^{14}\text{C}$]hexadecanedionoyl-mono-CoA is used for ketogenesis and the formation of acetylcarnitine. We presume that the citrate observed arose from succinyl-CoA, the terminal

Fig. 6. Time course of formation of the intermediates of mitochondrial oxidation of [$\text{U-}^{14}\text{C}$]hexadecanedionoyl-mono-CoA

●, Hexanedionoyl-mono-CoA; □, tetradecanedionoyl-mono-CoA; ○, hexadec-2-enedionoyl-mono-CoA; ■, hexadecanedionoyl-mono-CoA. Conditions of incubations and analysis were as described in the Materials and methods section.

Fig. 7. Time course of the peroxisomal oxidation of [U-¹⁴C]hexadecanoyl-CoA (■) and [U-¹⁴C]hexadecanedionoyl-mono-CoA (□)

Conditions of incubations and analysis were as described in the Materials and methods section.

intermediate of the β -oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA, since the incubations were made in the absence of malate. This view is supported by the time lag in citrate production (Fig. 3).

In a further experiment (Fig. 4) the products of oxidation were analysed after alkaline hydrolysis. A large peak of acetate is apparent, presumably derived from acetylcarnitine, and in this case in excess of 80 % of the substrate utilized could be accounted for by the sum of acetate, ketone bodies and citrate.

Intermediates of rat liver mitochondrial β -oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA

The acyl-CoA intermediates of the mitochondrial oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA were analysed by radio-h.p.l.c., and a typical chromatogram of a sample in mid-pulse of oxidation is shown in Fig. 5(a). Only the substrate, hexadec-2-enedionoyl-mono-CoA, tetradecanedionoyl-mono-CoA and hexanedionoyl-CoA were detected. The time course of the generation of these intermediates is shown in Fig. 6.

Intermediates of rat liver peroxisomal β -oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA

The time course of rat liver peroxisomal β -oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA, as measured by the production of acid-soluble radioactivity, is shown in Fig. 7. The rate of [U-¹⁴C]hexadecanedionoyl-mono-CoA oxidation was approximately equal to that of the oxidation of [U-¹⁴C]hexadecanoyl-CoA (12.9 and 14.1 nmol/min per mg of protein respectively). Over the time course studied the oxidation of both substrates proceeded to near completion (90.0 % and 91.5 % respectively). The oxidations of both substrates were insensitive to a combination of cyanide, myxathiazole and rotenone (results not shown). The intermediates detected by radio-h.p.l.c. are shown in Fig. 5(b). In contrast with mitochondrial β -oxidation, acetyl-CoA and intermediates of all chain lengths from C₆ to C₁₆ were detected. These data taken as a whole, and the use of Percoll gradient isopycnic centrifugation, indicate that it is most unlikely that the peroxisomal preparations were contaminated with mitochondria.

DISCUSSION

In the present study it is demonstrated unequivocally that the

β -oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA can occur by both the peroxisomal and the mitochondrial pathways. However, the properties of the two systems are, not surprisingly, very different.

The production of acid-soluble radioactivity by mitochondria was used as a measure of the rate of β -oxidation flux. It was clearly of importance to ensure that the substrate, [U-¹⁴C]hexadecanedionoyl-mono-CoA, was pure and not contaminated with oxidizable substrates such as the starting material [U-¹⁴C]hexadecanoate or its CoA ester. This was achieved by purifying the product by preparative h.p.l.c. and demonstrating that all of the radioactivity co-chromatographed with standard hexadecanedionoyl-mono-CoA authenticated by fast-atom bombardment m.s. (Fig. 1).

In Fig. 2 it is shown that the generation of acid-soluble radioactivity by mitochondria is time-dependent and carnitine-dependent, and therefore the substrate traverses the inner mitochondrial membrane by the conventional route, via carnitine palmitoyltransferases I and II and the acylcarnitine/carnitine translocase. Furthermore, β -oxidation was largely inhibited by respiratory poisons, from which it may be concluded that contamination by peroxisomes cannot account for these observations. This possibility is in any event most unlikely, since the mitochondrial fractions used were purified by Percoll gradient isopycnic centrifugation. The mitochondrial oxidation of hexadecanedionoyl-mono-CoA proceeded to near completion and the maximal rate of oxidation was about 35 % of that of hexadecanoyl-CoA. This may be an overestimate, since small amounts of succinyl-CoA and hexanedionoyl-mono-CoA, which are acid-soluble, were detected in some incubations (Fig. 5a). However, as is demonstrated in Figs. 3 and 4, the majority of the acid-soluble radioactivity is associated with products derived from acetyl-CoA: acetoacetate, 3-hydroxybutyrate, citrate and acetylcarnitine. The citrate observed is presumably derived in part from succinyl-CoA, which is the final intermediate of [U-¹⁴C]hexadecanedionoyl-mono-CoA oxidation, since the incubations were carried out in the absence of malate and in the presence of fluorocitrate.

These results apparently contradict those of Cerdan *et al.* (1988) and Poosch & Yamazaki (1989). Both of these groups conclude that the sole site of the β -oxidation of dicarboxylates is the peroxisomal compartment. We believe this view to be in error. The former group show by ¹³C- and ¹H-n.m.r. *in vivo* and *in vitro* that the administration of [1,2,11,12-¹³C₄]dodecanedioate results in the formation of [1-¹³C]adipate and no [1-¹³C]acetyl-CoA-derived metabolites. The latter group demonstrated parallel induction of rat liver peroxisomal acyl-CoA oxidase activity with respect to the C₁₂ substrates dodecanedionoyl-mono-CoA and dodecanoyl-CoA by the administration of di-(2-diethyl-hexyl) phthalate. They further showed parallel loss of activity by treatment of their preparations of rat liver homogenates with antiserum to purified acyl-CoA oxidase. Finally, they reported the kinetic characteristics of acyl-CoA oxidase with respect to chain length (C₆–C₁₂), and demonstrated increasing K_m values with decreasing chain length and marked substrate inhibition. These data and the literature adduced in support of their contention (Kolvaara & Gregersen, 1986; Vamecq & Draye, 1987) are all concerned with the catabolism of medium-chain dicarboxylates, i.e. of chain length < C₁₂. If, as we wish to suggest, a pathway in which ω -oxidation of long-chain monocarboxylic acids precedes chain-shortening by β -oxidation can at least contribute to dicarboxylate metabolism, then it is logical to use substrates of chain length of C₁₆ or greater, since these are the fatty acids of physiological significance. The simplest resolution of this paradox is that medium-chain-length dicarboxylic acids are poor substrates for the mitochondrial acyl-CoA synthetase, or that the

resultant CoA esters are poor substrates for transport across the mitochondrial inner membrane, i.e. for carnitine palmitoyl-CoA transferase I or II or for the translocase. However, as we show here, once a (C₁₆) dicarboxylate is committed to mitochondrial β -oxidation, it proceeds to near completion. Mortensen & Gregersen (1982) have shown that hexadecanedioic acid is toxic to starved rats, whereas the administration of dicarboxylic acids of chain length C₁₀–C₁₄ results in the pronounced excretion of chain-shortened dicarboxylates. This finding argues against β -oxidation of long-chain dicarboxylates *in vivo*. However, from comparisons of the labelling pattern of acetate derived from the oxidation of long-chain fatty acids which are 1-, 2-, 3-, 10- or ω -labelled, it has been suggested that ω -oxidation precedes β -oxidation (Hemmelgarn *et al.*, 1977).

We were unable to detect intermediates of mitochondrial β -oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA other than traces of succinyl-CoA and rather larger amounts of hexanedionoyl-mono-CoA, hexadec-2-enedionoyl-mono-CoA and tetradecanedionoyl-mono-CoA (Fig. 5a). This is in marked contrast with the peroxisomal incubations, where intermediates of all chain lengths were observed (Fig. 5b). This contrast between the peroxisomal and mitochondrial pathways is very similar to our observations of the intermediates of [U-¹⁴C]hexadecanoate oxidation. Thus we reported that tetradecanoyl-CoA was the only intermediate detectable in mitochondrial incubations (Watmough *et al.*, 1989), whereas intermediates of all chain lengths were readily demonstrable in peroxisomal incubations when using a substrate of the same specific radioactivity (Bartlett *et al.*, 1990). These results were consistent with substrate channelling in the mitochondrial oxidation of the monocarboxylate, whereas in the peroxisomal system there was equilibration of intermediates with the bulk phase. The present observations of the oxidation of [U-¹⁴C]-hexadecanedionoyl-mono-CoA can be interpreted similarly.

We conclude that the oxidation of [U-¹⁴C]hexadecanedionoyl-mono-CoA can occur in both the peroxisomal and mitochondrial subcellular compartments. However, the rates of [U-¹⁴C]-hexadecanedionoyl-mono-CoA oxidation relative to the rates of [U-¹⁴C]hexadecanoyl-mono-CoA oxidation are 0.35 and 0.91 in mitochondria and peroxisomes respectively. Furthermore, assuming a ratio of 20:1 for mitochondrial/peroxisomal protein and the observed rates of [U-¹⁴C]hexadecanedionoyl-mono-CoA oxidation (1.34 and 14.1 nmol/min per mg of protein in mitochondria and peroxisomes respectively), it is probable that the oxidation of hexadecanedionoyl-mono-CoA occurs in both compartments *in vivo*. In view of the likely complex competitive interactions with monocarboxylate oxidations *in vivo*, it is not possible to make further extrapolations.

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