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Skeletal muscle mitochondrial β-oxidation of dicarboxylates

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(1) The oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA by rat skeletal muscle mitochondrial fractions is carnitine dependent and is inhibited by cyanide. (2) [U-^{14}C]hexadecanedionoyl-*mono*-CoA was oxidised at a rate 8% of that of [U-^{14}C]hexadecanoyl-CoA. (3) Oxidations were saturable and no substrate inhibition was observed. (4) We demonstrate the formation of dicarboxylyl-*mono*-CoA esters and the corresponding carnitine derivatives. (5) We conclude that, although skeletal muscle mitochondria are capable of the β-oxidation of dicarboxylic acids, this is unlikely to be of great physiological significance.

Introduction

Dicarboxylic acids administered to humans or to experimental animals can be chain-shortened by units of two carbon atoms, presumably by β-oxidation, and excreted as short-chain dicarboxylic acids. Thus, following the ingestion of long-chain dicarboxylic acids with even-number carbon chains, even-numbered short-chain dicarboxylic acids are excreted and odd-numbered acids give rise to odd-numbered chain-shortened products [50,5,12]. Bergström et al. [4] showed that 2,2-dimethylstearic acid, which is not oxidized by normal β-oxidation, can be omega-oxidized and more than 90% of the ingested fatty acid is excreted in the urine as 2,2-dimethyladipate formed by β-oxidation of the resultant dicarboxylic acid. Medium-chain dicarboxylic acids can also be β-oxidized in vivo [24,28,3,8,11]. Bergseth et al. [3] demonstrated that [1-^{14}C]dodecanedioic acid is relatively efficiently metabolized in the rat, none is excreted but depending on the dose, 10–50% is excreted as chain-shortened dicarboxylic acids, mainly adipate (DC_6) and suberate (DC_8). However, suberic acid is relatively poorly metabolized and is efficiently excreted. There is evidence that short-chain dicarboxylic acids can be further oxidized to succinate. Rusoff et al. [37] showed that administration of [^{14}C]adipic acid to rats treated concurrently with malonate resulted in the excretion of [^{14}C]succinate. 3-Oxoadipic acid was also detected, which strongly suggests that adipate undergoes β-oxidation. Similarly, Pettersen [30] reported that [1,6-^{14}C]adipic acid administered intravenously to dogs is partially oxidised to $^{14}CO_2$.

Interest in the biogenesis of dicarboxylic acid metabolism has been stimulated by the observation of elevated tissue and increased urinary excretion of medium- and short-chain dicarboxylic acid in situations of impaired or stimulated fatty acid oxidation such as inherited disorders of mitochondrial β-oxidation [47,1] or prolonged fasting [31,32,44,18], respectively. In vitro investigation of dicarboxylic acid β-oxidation has focused on hepatic metabolism (see for example Ref. 33 and references therein). Little or no attention has been paid to other tissues. Muscle comprises about 40% of lean body mass of adult humans and makes a major contribution to whole body oxygen uptake and metabolism [39]. Since about 70% of the energy requirements of resting muscle is met by fatty acid oxidation [14], it is important to establish whether muscle mitochondria are capable of the β-oxidation of dicarboxylic acids and if so to what extent.

We have developed methods for the measurement of the intact [^{14}C]acyl-CoA ester intermediates and the corresponding [^{14}C]acylcarnitines of [U-^{14}C]hexadecanedioate oxidation [33,34]. In the present study we apply these methods to investigate the β-oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA by rat skeletal muscle mitochondrial fractions. We compare the products and intermediates of β-oxidation of [U-^{14}C]hexadecanedioate with those arising from the β-oxidation of [U-^{14}C]hexadecanoate.

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Materials and Methods

Materials

Dicarboxylic acids were obtained from Aldrich, Gillingham, Dorset, UK. Acetyl-CoA, succinyl-CoA, CoA, ATP, NAD, acyl-CoA synthetase, acyl-CoA oxidase and crotonase were purchased from Sigma (Poole, Dorset, UK). Acetonitrile (S grade) was purchased from Rathburn Chemicals, Walkerburn, UK. HPLC grade water, AnalaR grade $CHCl_3$, CH_3OH, Scintran grade xylene, Triton X-100 and 2,5-diphenyloxazole were supplied by BDH, Poole, Dorset, UK. All other reagents were of the highest available purity unless otherwise specified. [U-^{14}C]Hexadecanedionoyl-*mono*-CoA and [U-^{14}C]hexadecanoyl-CoA were prepared as described previously [33].

Preparation of rat skeletal muscle mitochondrial fractions

Mitochondrial fractions were prepared from hind-leg muscle of male Wistar rats by a modification of the method of Makinen and Lee [21]. Hind-leg muscle was dissected out from rats killed by cervical dislocation, excess fat and connective tissue were trimmed, weighed and placed in medium A (120 mM KCl/20 mM Hepes/5 mM $MgCl_2$/1 mM EGTA/5 mg/ml bovine serum albumin (BSA), pH 7.4). The muscle (about 10 g) was chopped finely with scissors, rinsed twice with about 30 ml of medium A, passed through a hand mincer in 5 g batches, homogenised (Ystral Y/20, setting 9 for 3 s) and made up to 20 volumes with respect to the original wet weight of tissue with medium A. Cell debris was removed by centrifugation (Sorvall RC5B centrifuge fitted with a w^2dt integrator; rotor SS34) at $600 \times g_{av}$ for 10 min ($4.08 \cdot 10^7$ rad^2/s). The pellet was resuspended in 8 vol. of medium A and recentrifuged. The supernatants were combined, filtered through four layers of cheesecloth to remove fat and centrifuged at $17000 \times g_{av}$ for 10 min ($1.09 \cdot 10^9$ rad^2/s). The pellet was resuspended in 10 volumes of medium A and centrifuged at $7000 \times g_{av}$ for 10 min ($5.03 \cdot 10^8$ rad^2/s). The pellet was then resuspended in 5 vol. of medium B (300 mM sucrose, 2 mM Hepes, 0.1 mM EGTA, pH 7.4) and centrifuged at $3500 \times g_{av}$ for 10 min ($2.51 \cdot 10^8$ rad^2/s). This final pellet was resuspended in 0.5–1.5 ml of medium B to give a final protein concentration of 20–30 mg/ml. Oxygen uptake by mitochondrial fractions was measured polarographically at 30°C and pH 7.2 in a final volume of 0.74 ml containing 1–3 mg of protein, 120 mM KCl/10 mM Hepes/10 mM KH_2PO_4/1 mM EDTA (pH 7.4) supplemented with 2 mg/ml BSA and 0.2 mg/ml cytochrome *c* [39]. Coupling conditions were obtained by adding 0.5 mM ADP and uncoupling conditions by adding 20 μM 2,4-dinitrophenol. Respiratory control ratios were determined as described by Chance and Williams [9] using 10 mM pyruvate plus 1 mM malate and ADP/O ratios as described by Nicholls [25]. Protein was measured by the method of Lowry et al. [20].

Incubation conditions

Incubations were made in a medium containing, unless otherwise stated, 110 mM KCl/2.5 mM KH_2PO_4/10 mM Hepes/1 mM EGTA/1 mM malate/5 mM $MgCl_2$/1 mM ADP/1 mM ATP/0.5 mM L-carnitine/0.3 mg/ml cytochrome *c* (pH 7.4); and mitochondrial suspension (1–3 mg protein/ml) at 35°C in a shaking water bath (130 strokes/min). After 5 min preincubation the reaction was started by the addition of substrate, 21 μM [U-^{14}C]hexadecanedionoyl-*mono*-CoA or [U-^{14}C]hexadecanoyl-CoA (specific radioactivity 1.8–10 mCi/mmol depending on the nature of the experiment). Throughout the study the *mono*-CoA ester of dicarboxylate was used as substrate. In the experiments where the rates of oxidation of monocarboxylate and dicarboxylate were compared the former substrate was also present as its CoA ester. However, in the experiments where the accumulation of β-oxidation intermediates was compared the monocarboxylate was used as free acid (bound to albumin, 5:1 molar ratio). Reactions were terminated at the appropriate times by the addition of 100 μl of either 20% (v/v) $HClO_4$ or 0.1 M H_2SO_4 to 1 ml aliquots of the reaction mixture depending on the type of analysis required.

Measurement of $^{14}CO_2$ production

Incubations were made in a final volume of 1 ml in 20 ml glass scintillation vials fitted with silicone rubber seals. Each vial also contained a 0.5 ml microcentrifuge tube placed inside a 1.5 ml microcentrifuge tube. Reactions were quenched by the addition of 100 μl of 20% (v/v) $HClO_4$, followed by 100 μl of 0.5 M $KHCO_3$. Hyamine hydroxide (300 μl of 11% (w/v) in methanol) was added to the central microcentrifuge tube, and the vials were left at room temperature for 2 h to allow complete trapping of $^{14}CO_2$. Radioactivity was measured in the hyamine hydroxide solution using the external standard channels ratio method.

Determination of acid-soluble radioactivity

Aliquots of 250 μl of the reaction mixture were treated with 50 μl of glacial acetic acid, 300 μl of 30% (w/v) BSA was then added followed by 300 μl of 20% (w/v) perchloric acid and the mixture was allowed to stand on ice for 30 min. Acid-insoluble material was removed by centrifugation ($12000 \times g_{av}$ for 5 min). Total acid-soluble radioactivity was determined in 700 μl of the supernatant. This procedure was adopted to ensure quantitative precipitation of the substrate.

Sample preparation and radio-HPLC analysis of acyl-CoA esters

Incubations were terminated by the addition of 100 μl of 0.1 M H_2SO_4 to 1 ml of the reaction mixture. Internal standard (20 nmol undecanedionoyl-*mono*-CoA) was added followed by 100 μl of saturated $(NH_4)_2SO_4$. Each sample was extracted twice with 10 vol. of ethyl acetate/diethyl ether (1:1, v/v) to remove free carboxylic acids and then extracted with 10 ml $CH_3OH/CHCl_3$ (2:1, v/v) for 1 h with continuous agitation. After centrifugation ($5000 \times g_{av}$ for 5 min) the supernatant was retained and the pellet was re-extracted with 5 ml of $CH_3OH/CHCl_3$ (2:1) and recentrifuged. The combined supernatant was evaporated to dryness under a stream of nitrogen at 30°C. The residue was dissolved in 3 ml of CH_3OH and 1 ml of HPLC grade water was added. This was centrifuged ($5000 \times g_{av}$ for 5 min) and the supernatant was applied to a DEAE-Sephacel column (acetate form; 60 × 6 mm). The eluate was retained and reapplied to the column to promote maximum binding. The column was washed with 4 ml of CH_3OH/H_2O (4:1 v/v). The acyl-CoA esters were eluted with 4 ml of CH_3OH/H_2O (4:1 v/v) containing 0.5 M ammonium acetate and 10 mM acetic acid. Solvents were removed with nitrogen at room temperature, 2 ml of HPLC grade water was added and the sample was freeze-dried. The residue was dissolved in 500 μl of 5% (v/v) CH_3CN/KH_2PO_4 (50 mM pH 5.3) and 200 μl was analysed by HPLC as previously described [34].

Radioactivity associated with eluted peaks was detected on-line with an LKB Betacord radioactivity monitor as previously described [51]. The detector was equipped with a 500 μl flow cell and 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 theory of operation and calculation and interpretation of data arising from radio-HPLC analysis of acyl-CoA and acylcarnitines has been described in detail elsewhere [7].

Sample preparation and radio-HPLC analysis of acylcarnitines

Reaction mixtures were prepared as described above except that 30 nmol of undecanoylcarnitine was used as internal standard. The $CH_3OH/CHCl_3$ extract was evaporated to dryness and the residue dissolved in 1.5 ml of water. After centrifugation ($5000 \times g_{av}$ for 5 min) the supernatent was applied to a Dowex 50X-8W (200–400 mesh) column (pyridinium form; 50 × 6 mm). The eluate was retained and reapplied to the column which was then washed with 2 ml 10 mM HCl and 2 ml of water. The acylcarnitines were eluted with 4 × 1 ml 1 M pyridinium acetate (pH 4.5)/ethanol (50:50, v/v) and the eluate freeze-dried. The resultant acylcarnitines were derivatized and analysed by radio-HPLC as previously described [33,34].

Analysis of organic acids

The organic acids products of β-oxidation were analysed by radio-HPLC as previously described [33].

Results

Counting efficiency and precision of radio-HPLC

The efficiency of counting and precision of estimates of cpm by radio-HPLC were examined by analyses of standard [1-^{14}C]acetyl-CoA, [U-^{14}C]hexadecanedionoyl-*mono*-CoA and [1-^{14}C]acetylcarnitine. Counting efficiencies were about 74% for CoA esters

TABLE I

Counting efficiency and recovery of radioactivity obtained from radio-HPLC analyses of standard compounds

The values shown are the means ± S.D. ($n = 5$). The values in parentheses are coefficients of variation. Counting efficiency is expressed as cpm calculated from the peak area divided by the dpm counted manually in the fraction corresponding to the eluted peak. Recovery is expressed as the dpm measured in the column effluent by manual counting divided by the dpm loaded onto the column.

Analyte	dpm injected	cpm from peak area	Efficiency (%)	dpm in HPLC eluate fraction	Recovery (%)
[1-^{14}C]Acetyl-CoA	2047	1578 ± 87 (5.5)	73.4 ± 3.7	2149 ± 102 (4.7)	105 ± 5
	9872	7430 ± 253 (3.4)	74.0 ± 2.5	10068 ± 285 (2.8)	102 ± 3
[U-^{14}C]DC_{16}-CoA	2010	1465 ± 68 (4.6)	74.0 ± 3.0	1978 ± 115 (5.6)	99 ± 6
	10545	7631 ± 282 (3.7)	74.5 ± 2.8	10229 ± 206 (2.9)	97 ± 2
[1-^{14}C]Acetylcarnitine	3114	2510 ± 114 (4.7)	77.3 ± 3.5	3248 ± 124 (3.8)	104 ± 4
	9251	7293 ± 298 (4.2)	78.0 ± 3.2	9632 ± 277 (2.9)	101 ± 3

(acetyl and DC_{16}) which were analysed together in a gradient run and 77.5% for acetylcarnitine (Table I). The latter was analysed isocratically at two different concentrations of acetonitrile (55% and 85%). These results indicate that the efficiency of counting is independent of chain length of the analytes in a single chromatogram and is also independent of the acetonitrile concentration in the mobile phase.

Flux of mitochondrial β-oxidation of [U-^{14}C]hexadecanedionoyl-mono-CoA

The data presented in Fig. 1 show a comparison of the time course of generation of $^{14}CO_2$ and acid-soluble radioactivity from [U-^{14}C]hexadecanedionoyl-*mono*-CoA and [U-^{14}C]hexadecanoyl-CoA by a skeletal muscle mitochondrial fraction. Each substrate was present at a concentration of 21 μM. Under identical conditions the maximal rate of the production of $^{14}CO_2$ from dicarboxylate was about 8% of that of the monocarboxylate (0.92 and 13.1 nmol/min per mg protein respectively). Similarly, the maximal rate of oxidation of dicarboxylate was about 8.5% of that of the monocarboxylate measured as acid soluble radioactivity (0.11 and 1.25 nmol/min per mg protein, respectively). In contrast to [U-^{14}C]hexadecanoyl-CoA, the oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA did not proceed

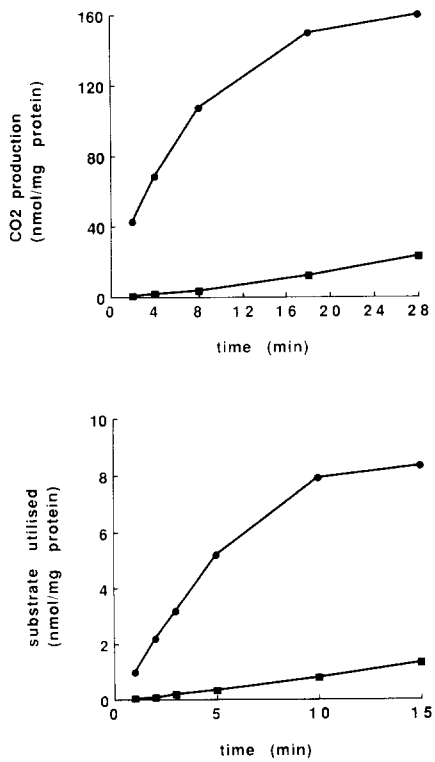

Fig. 1. Time course of skeletal muscle mitochondrial oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA (■) and [U-^{14}C]hexadecanoyl-CoA (●). Upper panel, $^{14}CO_2$; lower panel, substrate utilisation. Conditions of incubation and analysis were as described in Materials and Methods.

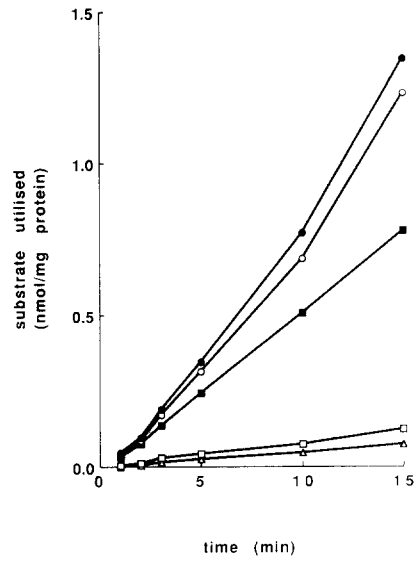

Fig. 2. Effects of carnitine, malate, malonate and cyanide on the β-oxidation by skeletal muscle mitochondria of [U-^{14}C]hexadecanedionoyl-*mono*-CoA. The oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA was measured as described in Materials and Methods (●), in the absence of carnitine (△), in the absence of malate (■), in the presence of cyanide (□) and in the presence of malonate (○).

to completion over the time course studied. However, the oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA was carnitine-dependent and was inhibited by cyanide (Fig. 2) and therefore it is most unlikely to be the result of contamination by peroxisomes or microbodies. The addition of malate (1 mM) to the incubation stimulated the oxidation rate by about 70% whereas the addition of malonate (5 mM) to inhibit the citrate cycle did not have any significant effect on the utilisation of substrate (Fig. 2). The effect of varying carnitine concentration over the range 0.1–2 mM was investigated. Maximal rates were observed in incubations made in the presence of 0.5 mM carnitine.

The effect of substrate concentration on the rate of β-oxidation was examined using concentrations of [U-^{14}C]hexadecanedionoyl-*mono*-CoA ranging from 10 to 100 μM. The results show that the rate of β-oxidation increases with the concentration of substrate and there is no substrate inhibition over the range studied (Fig. 3). There was an initial lag period in the formation of acid-soluble radioactivity resulting in non-linear progress curves which precluded formal kinetic treatment. The rate of oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA was maximal 10 min after the addition of substrate in comparison to [U-^{14}C]hexadecanoyl-CoA which had a maximal rate of oxidation between 1 and 2 min (Fig. 1). These observations suggest that the mitochondrial β-oxidation system in muscle has a low affinity toward dicarboxylates and thus high concentrations of CoA ester intermediates in

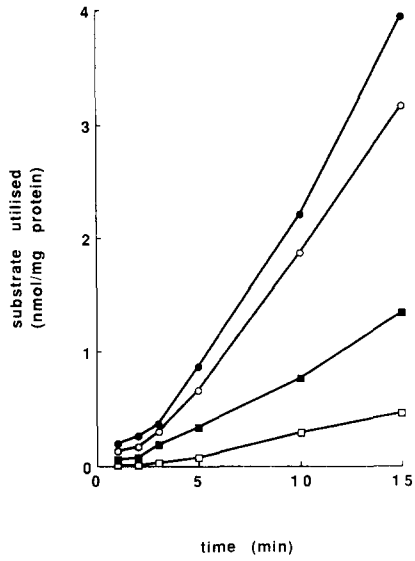

Fig. 3. Effect of substrate concentration on the oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA. Substrate concentration: 10 μM (□), 20 μM (■), 50 μM (○) and 100 μM (●).

the mitochondrial matrix are required to achieve maximal rates of oxidation.

A comparison of the oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA and [U-^{14}C]hexadecanoyl-CoA by isolated rat heart mitochondria is shown in Fig. 4. The results were similar to those obtained using skeletal muscle mitochondrial fractions. The oxidation of both substrates was inhibited by respiratory poisons and the oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA was almost completely inhibited by the absence of carnitine. The maximal rate of [U-^{14}C]hexadecanedionoyl-*mono*-CoA was 18% of the maximal rate of [U-^{14}C]hexadecanoyl-CoA oxidation.

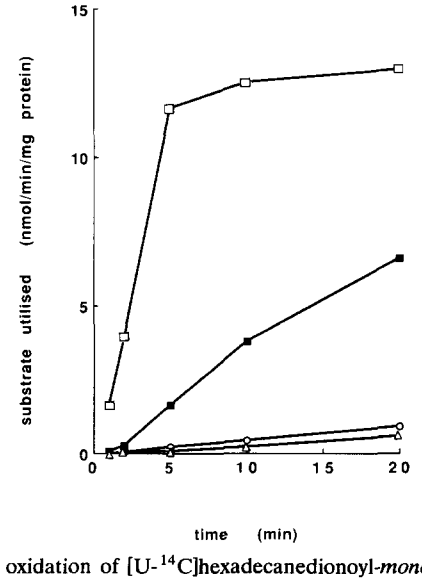

Fig. 4. The oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA (■), in the absence of carnitine and in the presence of respiratory poisons (▲) and of [U-^{14}C]hexadecanoyl-CoA (□), in the presence of respiratory poisons (○) by isolated rat heart mitochondria. Rat heart mitochondria were isolated as described in Materials and Methods for skeletal muscle mitochondria and incubations were made under identical conditions.

TABLE II

Radio-labelled products and intermediates arising from the β-oxidation of [U-^{14}C]hexadecanedionoyl-mono-CoA by isolated rat skeletal muscle mitochondrial fractions

Rat skeletal muscle mitochondrial fractions (3 mg protein/ml) were incubated with [U-^{14}C]hexadecanedionoyl-*mono*-CoA (50 μM) as described in the experimental section. Reactions were terminated after 17 min when about 18% of the substrate was utilized and samples were analysed by radio-HPLC for the ^{14}C-labelled organic acids, acylcarnitine and acyl-CoA esters as described in the experimental section. In calculation of concentrations it is assumed that succinate and citrate contains only two ^{14}C atoms (since the citrate cycle is inhibited by 5 mM malonate) and other compounds are universally labelled. Data from two independent experiments are shown.

Compound	Concentration (nmol/mg protein)
Succinate	32.3, 33.7
Citrate	2.73, 2.60
Hexanedionoyl-*mono*-carnitine	0.07, 0.05
Decanedionoyl-*mono*-carnitine	0.31, 0.45
Dodecanedionoyl-*mono*-carnitine	0.28, 0.41
Tetradecanedionoyl-*mono*-carnitine	0.21, 0.32
Hexadecanedionoyl-*mono*-carnitine	1.45, 1.21
Hexanedionoyl-*mono*-CoA	0.32, 0.26
Decanedionoyl-*mono*-CoA	0.11, 0.20
Dodecanedionoyl-*mono*-CoA	0.28, 0.41
Tetradec-2-enedionoyl-*mono*-CoA and 3-hydroxyhexadecanedionoyl-*mono*-CoA	0.91, 1.35
Tetradecanedionoyl-*mono*-CoA	0.1, 0.21
Hexadec-2-enedionoyl-*mono*-CoA	0.2, 0.15
Hexadecanedionoyl-*mono*-CoA	1.2, 1.51

Identification of the products and intermediates of β-oxidation

The products and intermediates of the β-oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA by skeletal muscle mitochondria were identified using incubation conditions described in the experimental section except that the substrate was present at the concentration of 50 μM and that 5 mM malonate was added to the incubation medium to inhibit the citrate cycle [26]. Preliminary experiments showed that the addition of malonate did not alter the flux through β-oxidation under these conditions. Incubations were quenched after 17 min when about 18% of the substrate was utilized and samples were analysed by radio-HPLC for ^{14}C-labelled organic acids, acyl-CoA and acylcarnitine esters. Analysis of the organic acid products of the oxidation showed the presence of succinate and small amounts of citrate (Table II). Accumulation of citrate was small, presumably because muscle mitochondria lack a citrate-transporting system [10,13]. No acetate or

other citrate cycle intermediates were detected. In some experiments organic acids were analysed before and after alkaline hydrolysis and identical results were obtained. The ^{14}C-labelled acyl-CoA esters detected by radio-HPLC were the substrate, hexadec-2-enedionoyl-*mono*-CoA, 3-hydroxyhexadecanedionoyl-*mono*-CoA, tetradecanedionoyl-*mono*-CoA, tetradec-2-enedionoyl-*mono*-CoA, dodecanedionoyl-*mono*-CoA, hexanedionoyl-*mono*-CoA and traces of decanedionoyl-*mono*-CoA (Fig. 5a; Table II). Acetyl-CoA and succinyl-CoA were not detected. Since the reactions were made in the presence of malonate, it is not possible to conclude whether or not the β-oxidation of hexadecanedionoyl-*mono*-CoA by muscle mitochondria proceeds as far as succinyl-CoA. The absence of acetyl units (acetate, acetyl-CoA and acetylcarnitine) indicates that the capacity for citrate formation exceeds the rate of production of acetyl-CoA under these conditions. The radio-HPLC analysis of ^{14}C-labelled acylcarnitine esters revealed the presence of hexadecanedionoyl-*mono*-carnitine, tetradecanedionoyl-*mono*-carnitine, dodecanedionoyl-*mono*-carnitine, decanedionoyl-*mono*-carnitine and traces of hexanedionoyl-*mono*-carnitine (Fig. 6a; Table II). Very little acetylcarnitine was detected. A number of other peaks were also present which had chromatographic properties consistent with 2-enedionoylcarnitine esters.

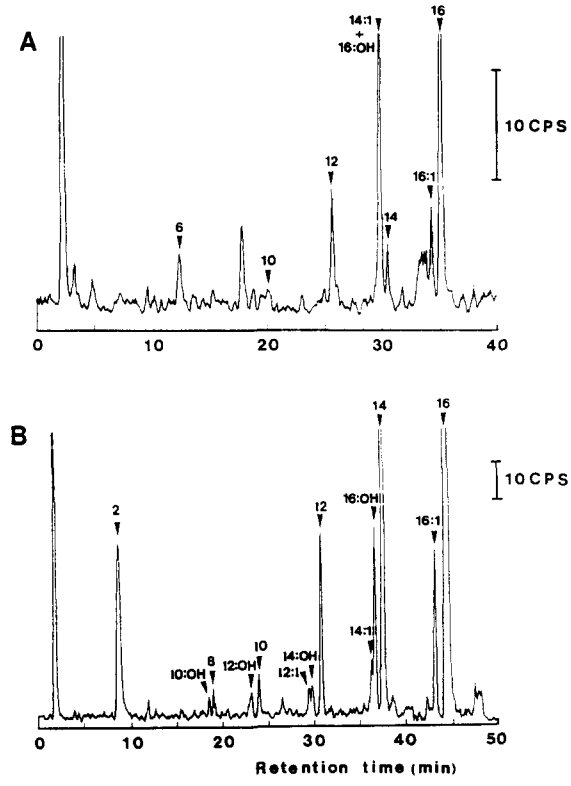

Fig. 5. Radio chromatograms of acyl-CoA intermediates generated by the skeletal muscle mitochondrial oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA (A) and [U-^{14}C]hexadecanoyl-CoA (B). Upper trace dicarboxylyl-*mono*-CoA esters, lower trace monocarboxyl-CoA esters. The carbon numbers are as indicated; 2,3-enoyl esters are indicated by: 1 and 3-hydroxyacyl esters by: OH. The conditions of incubation and analysis are as described in Materials and Methods.

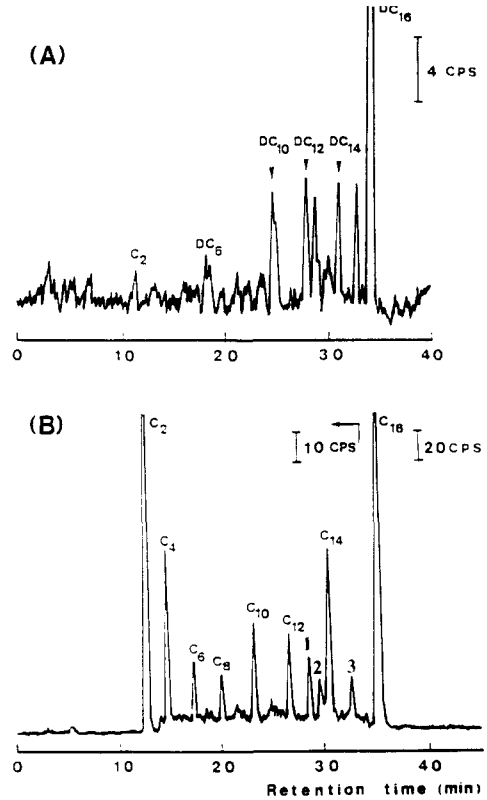

Fig. 6. Radio chromatograms of acylcarnitine esters generated by the skeletal muscle mitochondrial oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA (A) and [U-^{14}C]hexadecanoyl-CoA (B). Upper trace dicarboxylyl-carnitine esters are indicated by (DC), lower trace monocarboxylcarnitine esters are indicated by (C), the carbon numbers are as indicated. The conditions of incubation and analysis are as described in the experimental sectiom. The peaks labelled 1, 2 and 3 correspond to 3-hydroxyhexadecanoylcarnitine plus tetradec-2-enoylcarnitine, 3-oxohexadecanoylcarnitine and hexadec-2-enoylcarnitine, respectively.

For comparison the accumulation of ^{14}C-labelled acyl-CoA intermediates and their carnitine esters during the β-oxidation of [U-^{14}C]hexadecanoate was investigated. The incubation conditions were as described above except that the substrate was 60 μM free acid complexed to albumin (5 : 1 molar ratio) and ATP and CoA were added at concentrations of 5 mM and 0.2 mM, respectively. Incubations were quenched after 5 min when about 30% of the substrate was utilized. Analysis of [^{14}C]acyl-CoA esters by radio-HPLC showed that all the saturated, 2,3-enoyl- and 3-hydroxyacyl-CoA intermediates down to [^{14}C]octanoyl-CoA were present. [^{14}C]hexanoyl-CoA and [^{14}C]butyryl-CoA were also present in small amounts (Fig. 5b, Table III).

Radio-HPLC analysis of ^{14}C-labelled acylcarnitines also showed the accumulation of carnitine esters of

TABLE III

Accumulation of ^{14}C-labelled acylcarnitines and acyl-CoA intermediates arising from the β-oxidation of [U-^{14}C]hexadecanoate by isolated rat skeletal muscle mitochondrial fractions

Rat skeletal muscle mitochondrial fraction (1.84 mg protein/ml) was incubated with 60 mM [U-^{14}C]hexadecanoate complexed to albumin (5:1 molar ratio) as described in Materials and Methods. The reaction was terminated after 5 min when about 30% of the added substrate was utilized and ^{14}C-labelled acylcarnitine and acyl-CoA esters were extracted and analysed by radio-HPLC.

Compound	Concentration (nmol per mg protein)
Acetylcarnitine	25.8
Butyrylcarnitine	0.61
Hexanoylcarnitine	0.23
Octanoylcarnitine	0.12
Decanoylcarnitine	0.18
Dodecanoylcarnitine	0.16
Tetradecanoylcarnitine	0.27
Hexadecanoylcarnitine	1.24
Acetyl-CoA	1.39
Butyryl-CoA	0.33
Decanoyl-CoA	0.04
Dec-2-enoyl-CoA	0.01
Dodecanoyl-CoA	0.15
Dodec-2-enoyl-CoA	0.02
3-Hydroxydodecanoyl-CoA	0.02
Tetradecanoyl-CoA	0.4
Tetradec-2-enoyl-CoA	0.04
3-Hydroxytetradecanoyl-CoA	0.03
Hexadecanoyl-CoA	1.56
Hexadec-2-enoyl-CoA	0.26
3-Hydroxyhexadecanoyl-CoA	0.10

β-oxidation intermediates (Fig. 6b, Table III). These included the saturated [^{14}C]acylcarnitines down to [^{14}C]butyrylcarnitine and some other peaks which were presumed to be 2,3-unsaturated and 3-hydroxyacylcarnitines. Although the chromatographic behaviour of the latter groups of esters are known, their recoveries under our analytical procedure are not, and therefore these compounds can not be precisely quantified.

Discussion

The excretion of dicarboxylic acids of chain length C_6–C_{14} is associated with a number of pathological and physiological states. Dicarboxylic aciduria occurs in states characterised by high rates of hepatic β-oxidation such as diabetic ketoacidosis [29], the administration of a high fat diet [22] or medium-chain triacylglycerols [40] and prolonged fasting [23]. Paradoxically, impaired hepatic β-oxidation such as riboflavin deficiency [15,16,48], inherited disorders of mitochondrial β-oxidation [36,17,47,1] and acquired disorders such as Jamaican vomiting sickness caused by the ingestion of hypoglycin [43] are also characterised by dicarboxylic aciduria. However, in all these cases substrate supply exceeds oxidative capacity and thus long-chain fatty acids are delivered to the liver at rates which exceed the capacity for mitochondrial β-oxidation. Under these conditions fatty acids are diverted to either triacylglycerol synthesis with resultant development of fatty liver or metabolised by alternative pathways such as the formation of dicarboxylic acids. The biogenesis of these compounds is thought to involve Ω- and β-oxidation pathways, although the precise sequence and subcellular localisation of events remains uncertain.

It is well known that rat liver peroxisomes are capable of β-oxidation, although the properties of the peroxisomal system are very different from its mitochondrial counterpart (for review see Ref. 6). The characterisation of peroxisomal β-oxidation with respect to the accumulation of acyl-CoA intermediates of hexadecanoate oxidation, have shown that chain-shortening to the level of butyrate occurs under conditions of low substrate concentrations (50 μM). At higher substrate concentrations (> 100 μM) intermediates consistent with partial chain-shortening occurred suggestive of 2–3 cycles of β-oxidation [2]. Furthermore, the pattern of intermediates observed was dependent on the presence of an NAD^+-regenerating system – its absence caused the accumulation of 3-hydroxyacyl-CoA and 2-enoyl-CoA esters. We have demonstrated that β-oxidation of dicarboxylates occurs by both the mitochondrial and peroxisomal pathways [33]. We have extended these studies to a detailed examination of the characterstics of the peroxisomal system by analysis of the acyl-CoA esters produced by the oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA. We show that, in general, peroxisomal β-oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA is similar to the monocarboxylate, although under most experimental conditions we were unable to demonstrate chain-shortening beyond the C_6 level [35].

The present study shows that [U-^{14}C]hexadecanedionoyl-*mono*-CoA can be oxidized at least as far as hexanedionoyl-*mono*-CoA in skeletal muscle mitochondria. The rate of oxidation, however, is only about 8% of that of the [U-^{14}C]hexadecanoyl-CoA. Muscle contains a small number of microperoxisomes which make a detectable contribution to fatty acid oxidation [38,46]. Since the oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA was carnitine dependent and was inhibited by cyanide it is most unlikely that this oxidation is the result of the contamination of mitochondrial fraction by the microperoxisomes.

A comparison of the data presented here with our previous results [33] shows that hexadecanedionoyl-*mono*-CoA is handled differently by rat liver and skeletal muscle mitochondria, i.e., the rate of skeletal muscle mitochondrial oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA is low relative to that of [U-

[^{14}C]hexadecanoyl-CoA compared to the situation in liver. The reason for this difference is not clear. To our knowledge, there is no evidence suggesting the presence of isoforms of β-oxidation enzymes in different tissues with the exeption of CPT I which is shown to have immunologically distinct isoforms in rat liver and skeletal muscle mitochondria [19]. However, from the data presented in Table II and Fig. 6a it appears that hexadecanedionoyl-*mono*-CoA is efficiently converted to its carnitine ester. Therefore, it seems unlikely that CPT I is restricting β-oxidation in skeletal muscle mitochondria. One likely explanation is that in skeletal muscle the intramitochondrial β-oxidation enzymes have high K_m values towards dicarboxylyl-CoA substrates. This is supported by the finding of a positive correlation between substrate concentration and the rate of β-oxidation (Fig. 3). However, it remains possible that the acylcarnitine/carnitine translocase and/or CPT II are important.

These results also demonstrate the accumulation of β-oxidation intermediates during state-3 oxidation of [U-^{14}C]hexadecanedionoyl-*mono*-CoA. The pattern of intermediates is analogous to that observed during the β-oxidation of [U-^{14}C]hexadecanoate under similar conditions where the whole spectrum of the intermediates accumulate (Figs. 5b and 6b, Table III). This is in marked contrast with the liver mitochondria, in which few, if any intermediates accumulate during β-oxidation [42,51,33]. The reason for this difference between two tissues remains unclear. Van Hinsbergh et al. [45] also reported the accumulation of intermediates during the state-3 oxidation of [^{14}C]hexadecanoate by muscle mitochondria at high substrate concentration (i.e., 120 μM) although the intermediates were not identified. It is of interest to know whether the carnitine esters of β-oxidation intermediates are only formed to release part of the intramitochondrial CoA required for β-oxidation to continue or whether they also play some regulatory role.

Long-chain acyl-CoA esters are known to inhibit mitochondrial adenylate nucleotide translocase and therefore β-oxidation inducing state-4 conditions [27,41,45]. No such inhibition by hexadecanedionoyl-*mono*-CoA was observed in our experimental conditions within the substrate range studied (10–100 μM). Since the mitochondria were coupled as judged by respiratory control ratios, one explanation is that the translocator is not inhibited by hexadecanedionoyl-*mono*-CoA. However, a more likely explanation is that the oxidation of hexadecanedionoyl-*mono*-CoA is too low to be limited by the electron transport at high ATP/ADP ratios.

To our knowledge, there is no previous report demonstrating whether or not long-chain dicarboxylic acids can be β-oxidized by skeletal muscle mitochondria. Bergseth et al. [3] however, found that dodecanedioic and octanedioic acids are metabolized extremely slowly in the perfused hindquarter of the rat, which, in general is in agreement with our finding that hexadecanedionoyl-*mono*-CoA is a poor substrate for β-oxidation in skeletal muscle mitochondria. The higher rate which was measured here, however, may be due to higher activities toward the longer chain substrate used in the present study or that cellular uptake and/or activation to the CoA ester may be limiting. In contrast, Vamecq and Draye [49] reported that dodecanedioate was activated in rat skeletal muscle homogenate in the presence of CoA, ATP/Mg^{2+} and added detergent and measured a carnitine-dependent oxidation of dodecanedionoyl-*mono*-CoA by muscle mitochondria, at a rate of 25% of that of dodecanoyl-CoA, detected by a ferricyanide reduction assay. Even if under these experimental conditions mitochondrial β-oxidation enzymes are active on the medium-chain dicarboxylyl-CoA esters, the apparent poor uptake into and/or activation to the CoA ester may prevent their β-oxidation. Nevertheless, in the case of hexadecanedioic acid, even if uptake into the cell and activation to the CoA ester are not limiting, the mitochondrial oxidation of its CoA ester is very slow (about 8% of that of hexadecanoyl-CoA). Furthermore, there is no report in the literature on the existence of an omega-oxidation system, active on fatty acids in skeletal muscle, thus, in situ formation of dicarboxylic acids in this tissue is highly unlikely. We therefore conclude that β-oxidation of dicarboxylic acids by skeletal muscle mitochondria is of little physiological importance.

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