Reduction pathway of *cis*-5 unsaturated fatty acids in intact rat-liver and rat-heart mitochondria: assessment with stable-isotope-labelled substrates

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Besides the conventional isomerase-mediated pathway, unsaturated fatty acids with odd-numbered double bonds are also metabolized by reduction pathways with NADPH as cofactor. The relative contributions of these pathways were measured in intact rat-liver and rat-heart mitochondria with a novel stable isotope tracer technique. A mixture of equal amounts of unlabelled *cis*-5-enoyl-CoA and $^{13}C_4$-labelled acyl-CoA of equal chain lengths was incubated with mitochondria. The isotope distribution of 3-hydroxy fatty acids produced from the first cycle of β-oxidation was analysed with selected ion monitoring by gas chromatograph–mass spectrometer. 3-Hydroxy fatty acids produced from the reduction pathway of unsaturated fatty acids were unlabelled (m+0) whereas those produced from saturated fatty acids were labelled (m+4). The m+0 content serves to indicate the extent of reduction pathway. Rotenone treatment was used to switch the pathway completely to reduction. The extent of m+0 enrichment in untreated mitochondria normalized to the m+0 enrichment of rotenone-treated mitochondria was the percentage of reduction pathway. With this technique, *cis*-4-decenoate was found to be metabolized completely by the reduction pathway in both liver and heart mitochondria. *cis*-5-Dodecenoate was metabolized essentially by the reduction pathway in liver mitochondria, but only to 75% in heart mitochondria. When the chain length was extended to *cis*-5-tetradecenoate, the reduction pathway in liver mitochondria decreased to 86% and that in heart mitochondria to 65%. The effects of carnitine, clofibrate and other conditions on the reduction pathway were also studied. Enrichments of the label on saturated fatty acids and 3-hydroxy fatty acids indicated that the major pathway of reduction was not by the direct reduction of the *cis*-5 double bond. Instead, it is most probably by a pathway that does not involve forming a reduced saturated fatty acid first.

INTRODUCTION

As well as the conventional isomerase-mediated pathway [1], the oxidation of *cis*-5 unsaturated fatty acids also uses reduction pathways that are dependent on NADPH [2]. The major reduction pathway is postulated to mediate through the dehydrogenation of *cis*-5-enoyl-CoA to *trans*-2,*cis*-5-dienoyl-CoA, which is then isomerized to *cis*-3,*cis*-5- and *trans*-3,*cis*-5-dienoyl-CoAs as shown in Scheme 1 [3–8]. This isomerization is catalysed by Δ^3,Δ^2-enoyl-CoA isomerase or peroxisomal tri-functional enzyme [3,5]. $\Delta^{3,5}$-Dienoyl-CoAs are then isomerized to *trans*-2,*trans*-4-dienoyl-CoA under the catalysis of a new enzyme, $\Delta^{3,5}$-*t*2,*t*4-dienoyl-CoA isomerase. This new isomerase has been isolated and purified by us and in another laboratory [4,5]. Deuterium-isotope-labelling experiments indicated that $\Delta^{3,5}$-*t*2,*t*4-dienoyl-CoA isomerase catalysed the conversion by abstracting a proton at C-2 and subsequently shifting the 3,5-double bonds to the 2,4-position [5]. *trans*-2,*trans*-4-Dienoyl-CoA is then reduced to *trans*-3-enoyl-CoA; this reaction is mediated by 2,4-dienoyl-CoA reductase with NADPH as cofactor. Isomerization of *trans*-3-enoyl-CoA to *trans*-2-enoyl-CoA channels the pathway back to the normal β-oxidation sequence.

The existence of the reduction pathway is well established from the study of mitochondrial enzyme extracts [3–7]. In the absence of exogenous NADPH, metabolic intermediates, such as *trans*-2,*trans*-4-dienoate and $\Delta^{3,5}$-dienoates, accumulate [2,3,5–7]. On addition of NADPH, the accumulated metabolic intermediates decrease with time with the appearance of new metabolites, saturated 3-hydroxy fatty acids. With excess NADPH, the metabolic pathway of *cis*-5-enoyl-CoA switches to the reduction pathway [7]. In a previous paper [8] we have demonstrated the existence of the reduction pathway in intact rat-liver mitochondria. The magnitude of this pathway was estimated to be at least 61% on the basis of the ratio of released metabolic intermediates. However, this estimation was based on assumptions that might not be met under the experimental conditions. An underestimation of the reduction pathway by this approach was suggested [8]. Here we report a novel method of determining the degree of pathways in intact mitochondria by using stable-isotope-labelled substrates. The present approach does not rely on the same assumptions as the previous method.

MATERIALS AND METHODS

Reagents and chemicals

cis-4-Decenoic, *cis*-5-dodecenoic and *cis*-5-tetradecenoic acids were synthesized as previously described [9]. Acyl-CoA esters were prepared by a modified mixed-anhydride method with ethyl chloroformate and triethylamine in tetrahydrofuran [10]. Their concentrations were determined with Ellman's procedure [11]. [1,2,3,4-$^{13}C_4$]Dodecanoic acid and [1,2,3,4-$^{13}C_4$]octanoic acid were purchased from MSD Isotopes by custom synthesis. [3,4,5,6-

Abbreviations used: 3OHc5MC12, 3-hydroxy-*cis*-5-dodecenoate; 3OHc5MC14, 3-hydroxy-*cis*-5-tetradecenoate; 3OHMC10, 3-hydroxydecanoate; 3OHMC12, 3-hydroxydodecanoate; 3OHMC14, 3-hydroxytetradecanoate; c4MC10, *cis*-4-decenoyl-CoA; c5MC12, *cis*-5-dodecenoyl-CoA; c5MC14, *cis*-5-tetradecenoyl-CoA; GC, gas chromatograph; TMS, trimethylsilyl.

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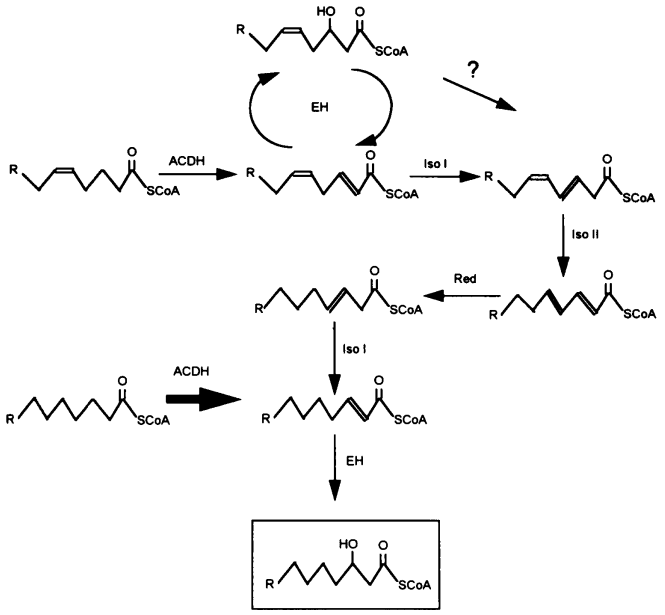

Scheme 1 Metabolic pathways (R = C_5H_{11} or C_7H_{15}) for the production of 3OHMC12 or 3OHMC14 from the incubation of equal amounts of [1,2,3,4-$^{13}C_4$]dodecanoyl-CoA and *cis*-5-dodecenoyl-CoA (or [3,4,5,6-$^{13}C_4$]-tetradecanoyl-CoA and *cis*-5-tetradecenoyl-CoA) in rat-liver or rat-heart mitochondria

The enzymes in each step are: ACDH, acyl-CoA dehydrogenases; EH, enoyl-CoA hydratase; iso I, Δ^3,Δ^2-enoyl-CoA isomerase; iso II, $\Delta^{3,5}$-*t*2,*t*4-dienoyl-CoA isomerase; and Red, 2,4-dienoyl-CoA reductase. The conversion catalysed by enoyl-CoA hydratase between 3-hydroxy-*cis*-5-dodecenoyl-CoA and *trans*-2,*cis*-5-dodecadienoyl-CoA is reversible; however, the conversion between *trans*-2,*cis*-5-dodecadienoyl-CoA and $\Delta^3\Delta^5$-dodecadienoyl-CoAs catalysed by Δ^3,Δ^2-enoyl-CoA isomerase is essentially irreversible. It is not known whether 3-hydroxy-*cis*-5-dodecenoyl-CoA can be converted directly to $\Delta^3\Delta^5$-dienoyl-CoA by one-step dehydration. The dehydrogenation of saturated acyl-CoAs (heavy arrow) is much faster than the corresponding dehydrogenation of *cis*-5-enoyl-CoAs (7).

$^{13}C_4$]decanoic acid was synthesized from [1,2,3,4-$^{13}C_4$]octanoic acid by converting labelled octanoate to octanol with diborane-THF complex in tetrahydrofuran [12]. Labelled octanol was then converted to octyl bromide with 48% HBr in concentrated sulphuric acid [13]. Diethylmalonate was then reacted with labelled octyl bromide under the catalysis of sodium ethoxide in ethanol to yield diethyl octylmalonate [14]. Hydrolysis of this ester, followed by decarboxylation, yielded labelled decanoate. Similarly, [3,4,5,6-$^{13}C_4$]tetradecanoic acid was synthesized from [1,2,3,4-$^{13}C_4$]dodecanoic acid. All labelled compounds contained 99.3 atom% ^{13}C. Mass spectrum (70 eV) for trimethylsilyl (TMS) [3,4,5,6-$^{13}C_4$]decanoic acid: *m*/*z* (percentage relative to base peak) 248 (3, M^+), 233 (91), 205 (4), 189 (4), 146 (18), 133 (8), 132 (59), 131 (7), 130 (40), 117 (100), 75 (79), 73 (80); mass spectrum for [5,6,7,8-$^{13}C_4$]tetradecanoic acid TMS: 304 (3, M^+), 289 (69), 245 (3), 205 (5), 146 (23), 133 (9), 132 (58), 131 (6), 130 (42), 117 (100), 75 (72), 73 (95).

Enzyme measurements

$\Delta^{3,5}$-*t*2,*t*4-dienoyl-CoA isomerase

The formation of *trans*-2,*trans*-4-dodecadienoyl-CoA from $\Delta^{3,5}$-dienoyl-CoA catalysed by this enzyme was measured by the increase in absorption at 300 nm. $\Delta^{3,5}$-Dienoyl-CoA was produced from the incubation of *cis*-5-dodecenoyl-CoA (40 μM) and acyl-CoA oxidase (0.5 unit) in 1 ml of 20 mM phosphate buffer, pH 8 at 37 °C for 10 min. The reaction was started by the addition of the enzyme extract.

Δ^3,Δ^2-Enoyl-CoA isomerase

This enzyme activity was assayed at 37 °C as described in the literature [15] with some modification. The assay mixture contained 17 μmol of EDTA, 2 μmol of NAD, 0.4 unit of β-hydroxyacyl CoA dehydrogenase, 0.4 unit of crotonase and 60 nmol of *trans*-3-hexenoyl-CoA in 1 ml of 0.17 M Tris buffer, pH 9. 2,4-Dienoyl-CoA reductase was measured essentially by the procedure described by Kimura et al. [16] except that *trans*-2,*trans*-4-decadienoyl-CoA was used as substrate. One unit of enzyme activity is defined as the conversion of 1 μmol of substrate/min.

Animal studies

Sprague–Dawley male rats (Charles River Lab., Wilmington, MA, U.S.A.) were divided into three groups. Group 1 was fed with normal Purina rat chow (20% energy from fat-equal mix of lard and corn oil) and were used as controls. Group 2 was fed with high-fat A diet and group 3 was fed with high-fat B diet (40% energy from fat). High-fat diets were custom-formulated by Purina Test Diets (Richmond, IN, U.S.A.). High-fat A diet was derived from lard, and high-fat B diet from corn oil. Rats were allowed food and water *ad lib*. for three weeks before being killed for the studies. Another group of rats was fasted for 24 h. The clofibrate-treated group was fed with Purina rat chow sprayed with 0.5% clofibrate (Aldrich) for 2 weeks. A control group was also studied for comparison.

Isolation of mitochondria

Fresh rat liver was minced and homogenized in a mixture of MSM buffer (220 mM mannitol, 70 mM sucrose, 5 mM Mops, pH 7.4) with 2 mM EDTA. The homogenate was centrifuged at 400 ***g*** (10 min) to remove debris and then centrifuged further at 7000 ***g*** (10 min) to obtain pellets of mitochondria. These mitochondria pellets were washed twice with MSM buffer, then suspended in the same solution. Fresh rat heart, after being rinsed with Chappel–Perry buffer [17], was minced in 10 times the weight of the same buffer, then treated with Nagarse (5 mg/g wet weight) for 30 s. It was homogenized and centrifuged at 12000 ***g*** (10 min). Pellets were further fractionated as described in [17], to obtain mitochondria.

Metabolic studies

These were performed in 25 ml Erlenmeyer flasks in a metabolic shaking incubator (140 cycles/min) at 37 °C. Each flask contained acyl-CoAs (70 μM each of unlabelled unsaturated enoyl-CoA and labelled saturated acyl-CoA), 1 mg of mitochondrial protein and 0.3 mM L-carnitine in 1 ml of incubation mixture (80 mM KCl, 50 mM Mops, 5 mM P_i, 1 mM EGTA and 1 mg of defatted BSA) at pH 7.4. For experiments with mitochondria treated with rotenone, 4 μM rotenone was added and incubated with mitochondria for 1 min before the addition of substrates. Other reagents added in some of the experiments were: dinitrophenol (50 μM) and malonate (5 mM); glutamate (10 mM); and *R*-3-hydroxybutyrate (10 mM). The reaction was stopped by the addition of 1M KOH (120 μl/ml incubation mixture).

Metabolic profiling of incubation mixture

The incubation mixture, pretreated with KOH, was mixed with internal standard (20 μg of pentadecanoic acid) and hydrolysed

at room temperature for 50 min. The mixture was acidified and extracted with ethyl acetate/diethyl ether (1:1). After converting to trimethylsilyl derivatives, the sample was analysed with a dual-capillary-column gas chromatograph (Model 5890, Hewlett-Packard, Avondale, PA), with a bonded dimethylpolysiloxane phase (25 m, SPB-1 from Supelco, Bellefonte, PA) and a bonded 35% diphenyl/65% dimethylpolysiloxane phase (SPB-35, 25 m) fused-silica capillary columns. The initial column temperature was 60 °C and increased at 4 °C/min. to 250 °C with a 50:1 split injection ratio. The quantification was based on the relative peak area to the internal standard. The amount of metabolites was calculated as the mass equivalent to that of internal standard on the basis of area ratio. 3-Hydroxydecanoate (3OHMC10), 3-hydroxydodecanoate (3OHMC12), and 3-hydroxy-*cis*-5-dodecanoate (3OHc5MC12) were quantitatively determined on the SPB-1 column and verified with the SPB-35 column. However, the determination of the amount of 3-hydroxytetradecanoate (3OHMC14) and 3-hydroxy-*cis*-5-tetradecenoate (3OHc5MC14) was based on results from SPB-35 column because 3OHMC14 was co-eluted with palmitate on SPB-1.

A Hewlett-Packard 5985B gas chromatograph–mass spectrometer was used for metabolite identification and isotope enrichment determination. A shorter (12.5 m) fused-silica capillary column (SPB-1) was used with the same temperature program as described for gas chromatography (GC). Electron-impact (70 eV) ionization and repetitive scanning (300 atomic mass units per s) in the range m/z 49–550 was used for obtaining mass spectra. The criteria for identification of compounds were that the retention times on both columns in GC and the mass spectrum were identical with those obtained from authentic samples. 3OHMC10, 3OHMC12, and 3OHMC14 were synthesized from the reaction of ethyl acetoacetate with the respective alkyl halides and the subsequent reduction of the products with sodium borohydride as described [18]. 3OHc5MC12 and 3OHc5MC14 were prepared by the enzymic reaction of *cis*-5-dodecenoyl-CoA (40 μM) and *cis*-5-tetradecenoyl-CoA (40 μM) with acyl-CoA oxidase (0.5 unit/ml; Boehringer-Mannhein, Indianapolis, IN, U.S.A.) and bovine liver crotonase (0.4 unit/ml, Sigma) at 37 °C for 5 min. Subsequent alkaline hydrolysis yielded the authentic samples for metabolite identification by GC and GC/MS.

Determination of enrichments in metabolites by selected-ion monitoring

Derivatized samples were separated through a capillary column of SPB-1 gas chromatograph as described in the previous section. For the analysis of bis-trimethylsilyl 3OHMC10, the column temperature was at 180 °C initially, and was increased at a rate of 4 °C/min after injection. Chromatographic effluent was monitored at m/z 233 (m+0) and 234 (m+1) as well as m/z 317 (m+0) and 321 (m+4) at a dwell time of 100 ms for each ion. For bis-trimethylsilyl 3OHMC12, the initial temperature was 200 °C and was increased at 4 °C/min; the ions monitored were m/z 233 (m+0) and 236 (m+3), and m/z 345 and 349 (100 ms for each ion). The initial temperature was 210 °C and was increased at 4 °C/min for the analysis of bis-trimethylsilyl 3OHMC14. For this compound, m/z 233 (m+0) and 234 (m+1), and m/z 373 (m+0) and 377 (m+4) (100 ms each ion) were monitored. The higher-mass ion pairs for each of these analyses were derived from the loss of a methyl group from the molecular ion, i.e. M^+-15 ions. Peak areas were determined. Metabolites produced from the incubation of unlabelled substrate were also analysed to obtain natural-isotope abundance enrichments. For the calculation of percentage enrichments of m+0 species in 3-hydroxy fatty acids, the relative peak areas of m+1 were corrected for the natural-isotope abundance contribution by the technique described previously [19]. Because there is no contribution of natural-isotope cluster to the m+4 ions, the areas determined from the m+0 and m+4 ions derived from M^+-15 mass fragments did not need any correction before the calculation of m+0 enrichments. The contribution of unlabelled unsaturated enoyl-CoA to the production of 3-hydroxy fatty acids was derived from the m+0 (percentage) enrichment of 3-hydroxy fatty acids. The m+0 percentage enrichments were calculated from the proportion of the m+0 area to the total of m+0 and the area from labelled ions, which could be m+1, m+3 or m+4 depending on the ion pairs. Isotope distribution in dodecanoate and tetradecanoate bis-trimethylsilyl derivatives were determined on m/z 257 and 261 (m+0 and m+4 label for dodecanoate) and m/z 285 and 289 (m+0 and m+4 label for tetradecanoate) with 100 ms for each ion.

Calculation

The percentage of reduction pathway was calculated as the ratio of m+0 enrichment of 3-hydroxy fatty acid in rotenone-untreated mitochondria over the m+0 enrichment in rotenone-treated mitochondria from parallel experiments. This ratio was multiplied by 100%. The m+0 enrichment of 3-hydroxy fatty acids from rotenone-treated mitochondria was regarded as derived from 100% reduction pathway. Data are expressed as means ± S.D.

RESULTS

Gas chromatograph–mass spectrometric determination of the enrichments of 3-hydroxy fatty acids

Bis-trimethylsilyl derivatives of 3-hydroxy fatty acids yielded m/z 233 as the major fragment on 70 eV electron-impact induced fragmentation [20]. This fragment has the composition of CHOTMS-CH_2-CO_2TMS and contains carbons 1, 2, and 3 of the 3-hydroxy fatty acids. For 3OHMC10 produced from [3,4,5,6-$^{13}C_4$]decanoyl-CoA and 3OHMC14 produced from [3,4,5,6-$^{13}C_4$]-tetradecanoyl-CoA, this fragment contained only one ^{13}C label on C-3. Therefore m/z 233 and 234 were monitored for m+0

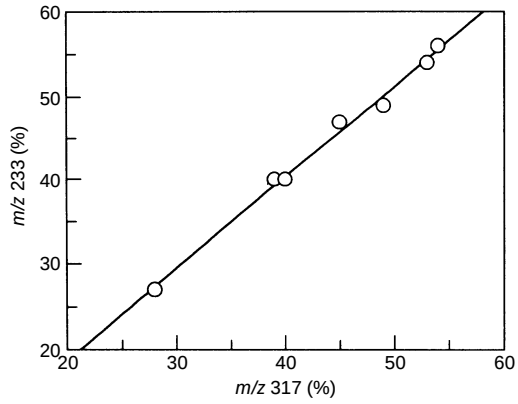

Figure 1 Correlation between m+0 enrichment (%) derived from the monitoring of m/z 233 (m+0) and 234 (m+1), and m/z 317 (m+0) and 321 (m+4) ion pairs for 3OHMC10 produced from the incubation of equal amounts of unlabelled *cis*-4-decenoyl-CoA and [3,4,5,6-$^{13}C_4$]decanoyl-CoA in rat-liver mitochondria

The correlation coefficient (r) was 0.9967. The regression line was $Y = 1.08\ (\pm 0.04)\ X - 2.95\ (\pm 1.77)$. Bis-trimethylsilyl derivative of 3OHMC10 was used for analysis.

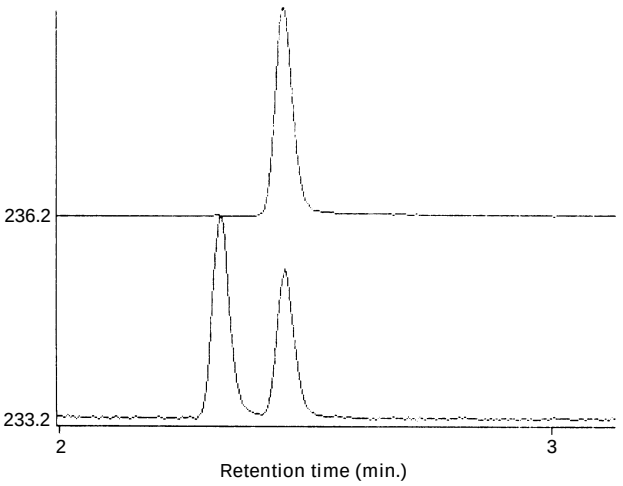

Figure 2 Selected-ion monitoring mass chromatogram of 3OHMC12 bis-trimethylsilyl derivative

The *m/z* ions 233 (m + 0) and 236 (m + 3) were monitored. The ion intensity was maximized for each ion. The earlier peak (retention time 2.3 min.) with *m/z* 233 was derived from 3OHc5MC12. The latter metabolite had a higher *m/z* 233 ion yield than 3OHMC12; the amount of this metabolite therefore seems to be higher in this chromatogram than its actual amount analysed by GC (see Figure 3). The sample was analysed at 200 °C with an increase of 4 °C/min.

and m + 1 of 3-hydroxy fatty acids derived from *cis*-5-enoyl-CoA (unlabelled) and saturated acyl-CoA (labelled), respectively. For 3OHMC12 derived from [1,2,3,4-$^{13}C_4$]dodecanoyl-CoA, this fragment contained three ^{13}C labels, on C-1, C-2, and C-3. Fragments *m/z* 233 and 236 were monitored for m + 0 and m + 3 derived from *cis*-5-dodecenoyl-CoA (unlabelled) and dodecanoyl-CoA (labelled), respectively.

In addition to *m/z* 233 ions, 3-hydroxy fatty acids produced from m + 4 labelled precursors contained m + 4 labels in their $M^+ - 15$ ion (molecular ion minus methyl group). Monitoring of this ion for the determination of isotope enrichment would have the advantage of lower m + 4 background contribution from natural-isotope clusters. Therefore no correction for the enrichments would be needed. However, the abundances of these ions were much lower than the *m/z* 233, which contains only one ^{13}C label on C-3 in fatty acids labelled with 3,4,5,6-$^{13}C_4$. The disadvantage of monitoring the *m/z* 233 ion pair is that correction for natural-isotope contribution has to be performed. Nevertheless, when the enrichments of 3-hydroxydecanoic acid bis-trimethylsilyl ester were analysed for their m + 0 enrichments from the *m/z* 233 and 234 and the *m/z* 317 and 321 pairs, exactly identical results were obtained (Figure 1). Therefore either of these ion pairs can be used for analysis. Because of the higher abundance of the ions, the mass fragments generated from *m/z* 233 were used for the analysis in the present study (Figure 2).

In the incubation period ranging from 1 to 10 minutes, the accumulation of 3-hydroxy fatty acids was not at steady state. The amount of 3-hydroxy fatty acids in the incubation mixture increased with time [21]. However, despite the increase in the total amount and m + 0 enrichments of 3-hydroxy fatty acids with incubation time, the ratio between m + 0 enrichments of rotenone-untreated and rotenone-treated mitochondria stayed relatively constant, as shown in Table 1. The sampling time was therefore not important in the determination of percentage of reduction pathway. Most of the data were obtained from a 3 min incubation.

Table 1 Percentage of m + 0 enrichments in 3-hydroxy fatty acids from non-treated and rotenone-treated mitochondria from liver (*cis*-4-decenoyl-CoA and [3,4,5,6-$^{13}C_4$]decanoyl-CoA as substrate) and heart (*cis*-5-dodecenoyl-CoA and [1,2,3,4-$^{13}C_4$]dodecanoyl-CoA as substrate)

Abbreviations: rot(−), non-treated; rot(+), rotenone-treated; red, percentage of reduction pathway; red = rot(−)/rot(+) × 100. The percentage of m + 0 enrichments of 3-hydroxy fatty acid bis-trimethylsilyl derivative was determined from selected-ion monitoring analysis of *m/z* 233 and 234 for the data from *cis*-4-decenoyl-CoA and decanoyl-CoA. The ions *m/z* 233 and 236 were analysed for studies with *cis*-5-dodecenoyl-CoA and dodecanoyl-CoA as substrates.

	Liver			Heart		
Time (min)	Rot(−) (%)	Rot(+) (%)	Red. (%)	Rot(−) (%)	Rot(+) (%)	Red. (%)
1	17.9	18.5	97	3.1	11.2	27
2	20.0	20.6	97	5.0	16.0	31
5	30.3	29.5	103	6.1	22.7	27

Oxidation of an equal mixture of *cis*-4-decenoyl-CoA and [3,4,5,6-$^{13}C_4$]decanoyl-CoA in rat-liver and rat-heart mitochondria

3OHMC10 was the major metabolite detected in the incubation of an equal mixture of *cis*-4-decenoyl-CoA and [3,4,5,6-$^{13}C_4$]-decanoyl-CoA in rat-liver and rat-heart mitochondria. As shown in Table 2, the concentration of 3OHMC10 was significantly increased after rotenone treatment in both liver and heart mitochondria. However, the increase was much higher in heart than in liver mitochondria. Despite this increase in 3OHMC10 concentration after rotenone treatment, the percentage of m + 0 enrichment (derived from the reduction pathway of unlabelled *cis*-4-decenoyl-CoA) was not changed significantly. The calculated reduction pathway was 96 ± 4 % in liver and 107 ± 6 % in heart mitochondria. Both results indicated that the reduction pathway was the only pathway for the oxidation of *cis*-4-decenoate. This conclusion is consistent with those proposed by other investigators using different approaches [22].

Oxidation of an equal mixture of *cis*-5-dodecenoyl-CoA and [1,2,3,4-$^{13}C_4$]dodecanoyl-CoA in rat-liver and rat-heart mitochondria

The major metabolite detected from the incubation of an equal mixture of *cis*-5-dodecenoyl-CoA and [1,2,3,4-$^{13}C_4$]dodecanoyl-CoA in liver mitochondria was 3OHMC12 (Figure 3A). No measurable amount of 3OHc5MC12 was found. Similar to the studies with *cis*-4-decenoyl-CoA as substrate, the concentration of 3OHMC12 was significantly increased after rotenone treatment and the magnitude of this increase was greater in heart than in liver mitochondria (Table 2). The m + 0 enrichment was not changed significantly after rotenone treatment in liver mitochondria. In heart mitochondria, a significant amount of 3OHc5MC12 was detected, in addition to 3OHMC12 (Figure 3B). The concentration of 3OHc5MC12 in control heart mitochondria was 2.1 ± 0.7 nmol/mg, which increased to 4.2 ± 1.1 nmol/mg after rotenone treatment. The ratio between 3OHMC12/3OHc5MC12 increased significantly ($P = 0.002$) from 3.03 ± 0.48 to 4.40 ± 0.58 after rotenone treatment. Similarly, the percentage of m + 0 in 3OHMC12 was increased significantly after rotenone treatment in heart mitochondria. Therefore in liver mitochondria the reduction pathway was the only pathway for the oxidation of *cis*-5-dodecenoate, but the percentage of the reduction pathway was reduced to 75 % in heart mitochondria.

Table 2 Concentrations and stable isotope enrichments of saturated 3-hydroxy fatty acid metabolites from the incubation of intact rat-liver (L) and rat-heart (H) mitochondria with equal concentrations of unlabelled unsaturated acyl-CoA and ^{13}C-labelled saturated acyl-CoA

Equal concentrations (70 μM of each) of unlabelled unsaturated acyl-CoAs (*cis*-4-decenoyl-CoA, *cis*-5-dodecenoyl-CoA and *cis*-5-tetradecenoyl-CoA) and the corresponding $^{13}C_4$-labelled saturated acyl-CoAs (e.g. decanoyl-CoA, dodecanoyl-CoA and tetradecanoyl-CoA) were incubated with 1 mg of mitochondrial protein in the presence of carnitine (0.3 mM) at pH 7.4 in a shaking incubator for 3 min. Metabolites were extracted, derivatized and analysed with a capillary-column GC for concentration and by selected-ion monitoring GC–MS for isotope enrichments. Data are expressed as means ± S.D. Statistics were performed by paired Student's *t*-test; *P* values less than 0.05 were regarded as not significant (n.s.). The 3-hydroxy fatty acids (3-OH FA) produced in these experiments were 3OHMC10 from *cis*-4-decenoyl-CoA (c4MC10), 3OHMC12 from *cis*-5-dodecenoyl-CoA (c5MC12) and 3OHMC14 from *cis*-5-tetradecenoyl-CoA (c5MC14). Percentage reduction pathway was calculated as the ratio between isotope enrichment of metabolite in the absence of rotenone, rot(−), to that in the presence of rotenone, rot(+), and normalized to 100%.

Substrate	3-OH FA concn. (nmol per mg protein)			Isotope enrichment (%)			Reduction pathway (%)
	Rot(−)	Rot(+)	*P*	Rot(−)	Rot(+)	*P*	
c4MC10							
L ($n = 6$)	10.7 ± 3.7	12.9 ± 3.3	< 0.01	17.2 ± 3.2	17.5 ± 3.8	n.s.	96 ± 4
H ($n = 4$)	9.7 ± 3.0	23.0 ± 6.2	< 0.01	18.0 ± 4.5	16.5 ± 4.5	n.s.	107 ± 6
c5MC12							
L ($n = 4$)	4.7 ± 0.7	5.7 ± 0.8	< 0.03	10.4 ± 1.4	10.6 ± 0.6	n.s.	97 ± 11
H ($n = 5$)	6.5 ± 2.8	17.4 ± 3.2	< 0.001	11.3 ± 2.6	14.9 ± 1.2	< 0.008	75 ± 14
c5MC14							
L ($n = 6$)	2.5 ± 0.8	3.7 ± 0.5	< 0.002	13.9 ± 2.0	15.8 ± 1.1	< 0.009	86 ± 7
H ($n = 5$)	2.8 ± 0.6	12.2 ± 2.0	< 0.001	12.1 ± 2.3	18.6 ± 1.3	< 0.002	65 ± 12

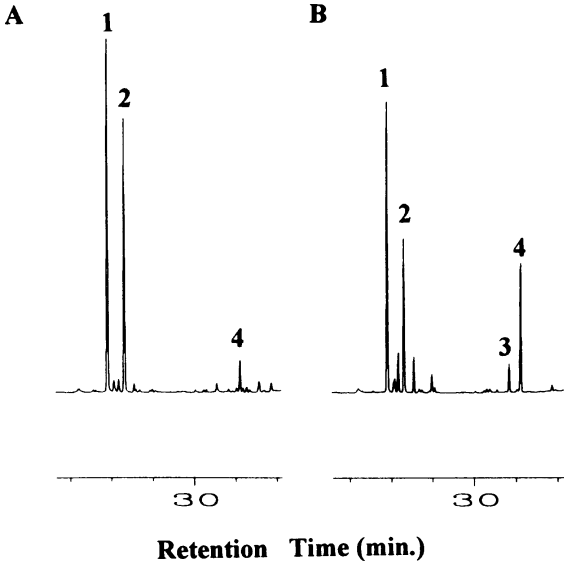

Figure 3 Metabolites produced from the incubation (3 min) of equal amounts of *cis*-5-dodecenoyl-CoA and dodecanoyl-CoA with rat-liver mitochondria (A) and rat-heart mitochondria (B)

The metabolites are: 1, *cis*-5-dodecenoate; 2, dodecanoate; 3, 3OHc5MC12; and 4, 3OHMC12. From equal amounts of substrates, the amount of 3OHc5MC12 produced, relative to 3OHMC12, was greater in heart mitochondria than in liver.

Oxidation of an equal mixture of *cis*-5-tetradecenoyl-CoA and [3,4,5,6-$^{13}C_4$]tetradecanoyl-CoA in rat-liver and rat-heart mitochondria

In liver mitochondria, the incubation of this mixture produced 3OHMC14 as the major metabolite, which was significantly increased after rotenone treatment (Table 2). In contrast with the previous two substrates, the percentage of m+0 in 3OHMC14 was also significantly increased in rotenone-treated samples. The calculated percentage of reduction pathway was 86 ± 7%. In heart mitochondria, in addition to 3OHMC14, a significant amount of 3OHc5MC14 was also produced: the amount of 3OHc5MC14 was 2.1 ± 0.3 nmol/mg in controls, which was increased ($P < 0.001$) to 8.1 ± 1.6 nmol/mg in rotenone-treated samples. The ratios of 3OHMC14/3OHc5MC14 were not significantly changed by rotenone treatment (1.32 ± 0.18 for control compared with 1.52 ± 0.20 for treated). However, the percentage of m+0 in 3OHMC14 was significantly increased in rotenone-treated mitochondria. The calculated percentage of reduction pathway in heart mitochondria was 65 ± 12%. Increasing the chain length from 12 to 14 therefore decreased the percentage of reduction pathway in both liver and heart mitochondria. Nevertheless, the reduction pathway was still the predominant pathway in these organelles.

Percentage of reduction pathways in rat-liver and rat-heart mitochondria from control and high-fat diets

The method for the measuring the percentage of reduction pathway in the oxidation of *cis*-5 unsaturated fatty acids was used to assess whether high-fat diets would induce the enzymes and the extent of the reduction pathway. High-fat diets containing lard (high-fat A) and corn oil (high-fat B) were evaluated. The body weights were 432 ± 53 g for the high-fat A diet group and 412 ± 34 g for the high-fat B diet group. These values were not statistically different from those of controls (383 ± 44 g). The percentage of reduction pathway was studied in liver and heart mitochondria. The results are shown in Table 3. There is no difference ($P > 0.05$) in all three groups in terms of percentage of reduction pathway, either in liver or heart. The enzyme activities for Δ^3,Δ^2-enoyl-CoA isomerase, $\Delta^{3,5}$-*t*2,*t*4-dienoyl-CoA isomerase, and 2,4-dienoyl-CoA reductase related to the metabolism of unsaturated fatty acids were also measured (Table 4). The activities for $\Delta^{3,5}$-*t*2,*t*4-dienoyl-CoA isomerase and Δ^3,Δ^2-enoyl-CoA isomerase were significantly increased by 24–40% in heart mitochondria after both high-fat diets. In addition, high-fat B (corn oil) also significantly increased Δ^3,Δ^2-enoyl-CoA isomerase activity by 36% in liver mitochondria. However, the activity of 2,4-dienoyl-CoA reductase was not changed after the feeding of either high-fat diet. Because the percentage of reduction pathway was not changed after treatment, it seems that the reduction

Table 3 Percentage of reduction pathway of *cis*-5-dodecenoyl-CoA and *cis*-5-tetradecenoyl-CoA in liver and heart mitochondria from rats fed with control and high-fat diets

Abbreviations: L, rat-liver mitochondria; H, rat-heart mitochondria. Control rats were fed with normal Purina rat chow with 20% calories derived from fat (equal mixture of lard and corn oil). High-fat diets derived 40% of calories from fat. Lard was used for high-fat A, whereas corn oil was used for high-fat B. Percentages of reduction pathway in both groups treated with a high-fat diet were not statistically different ($P > 0.05$) from controls. Numbers in parenthesis indicate number of experiments. Data are expressed as means ± S.D.

	Controls		High-fat A		High-fat B	
Substrate	L	H	L	H	L	H
c5MC12	97 ± 11 (4)	75 ± 14 (5)	96 ± 4 (4)	82 ± 9 (4)	108 ± 8 (3)	82 ± 7 (4)
c5MC14	86 ± 7 (6)	65 ± 12 (5)	84 ± 8 (5)	82 ± 12 (4)	89 ± 10 (5)	68 ± 7 (5)

Table 4 Enzyme activities (m-units per mg protein) in mitochondria from rats fed with normal rat chow, high-fat A diet, and high-fat B diet

Abbreviations: isomerase 1, Δ^3,Δ^2-enoyl-CoA isomerase; isomerase 2, $\Delta^{3,5}$-*t*2,*t*4-dienoyl-CoA isomerase; reductase, 2,4-dienoyl-CoA reductase; L, rat-liver mitochondria; H, rat-heart mitochondria. *Significantly different ($P < 0.05$) from controls. Data are expressed as means ± S.D.

	Control ($n = 6$)		High-fat A ($n = 5$)		High-fat B ($n = 5$)	
Enzymes	L	H	L	H	L	H
Isomerase 1	308 ± 20	250 ± 43	350 ± 60	310 ± 35*	420 ± 48*	350 ± 60*
Isomerase 2	89 ± 20	130 ± 11	100 ± 4	180 ± 20*	100 ± 15	168 ± 8*
Reductase	12 ± 2	5 ± 2	13 ± 1	6 ± 1.7	12 ± 1	8 ± 1

Table 5 Percentage of reduction pathway of unsaturated enoyl-CoA in rat-liver (L) and rat-heart (H) mitochondria without the addition of exogenous carnitine

Control rats were fed with normal Purina rat chow; the fasting group was fasted for 24 h; the clofibrate group was fed with rat chow containing 0.5% clofibrate for 2 weeks. $n = 3$ for each group. *Statistically different ($P < 0.05$) from controls. Data are expressed as means ± S.D.

	Controls		Fasting		Clofibrate	
Substrates	L	H	L	H	L	H
c5MC12	41 ± 4	32 ± 3	76 ± 6*	33 ± 3	86 ± 7*	28 ± 6
c5MC14	22 ± 2	34 ± 4	29 ± 4	28 ± 5	41 ± 4*	22 ± 6

pathway might be correlated with the activity of 2,4-dienoyl-CoA reductase instead of the activities of isomerases. However, the increase in the activities of the isomerases after high-fat diet treatment might not be enough for a significant shift in the percentage of reduction pathway.

Effects of carnitine, fasting and clofibrate treatment on the reduction pathway

When the studies were performed under essentially identical conditions as described before but without the addition of exogenous carnitine, the percentage of reduction pathway was significantly reduced. As shown in Table 5, the reduction pathway accounted for less than 50% of the *cis*-5-dodecenoyl-CoA in liver mitochondria. For a longer-chain substrate, e.g. *cis*-5-tetradecenoyl-CoA, the reduction pathway was only 22%. The percentage of reduction pathway was even lower in heart mitochondria for all substrates. In these studies, the amount of 3-hydroxy fatty acids detected was not significantly different from those obtained with the addition of carnitine (results not shown). However, the m+0 enrichments were lower than in studies with carnitine when rotenone was not added. Therefore, in the carnitine-deficient state, the reduction pathway for the oxidation of unsaturated fatty acids was diminished more than the conventional pathways. The decreased reduction pathway in mitochondria without exogenous carnitine was also evident from significantly decreased 3OH-/3OHc5- ratios. In heart mitochondria, where these ratios can be measured, the 3OHMC12/3OHc5MC12 ratio was 1.87 ± 0.07 (compared with 3.03 ± 0.48 from carnitine-treated mitochondria, $P < 0.0001$). The corresponding value for 3OHMC14/3OHc5MC14 was 0.82 ± 0.05 (compared with 1.32 ± 0.18 from carnitine-treated mitochondria, $P < 0.0001$).

Because we have shown that fasting and clofibrate treatment increased the activities of Δ^3,Δ^2-enoyl-CoA isomerase and $\Delta^{3,5}$-*t*2,*t*4-dienoyl-CoA isomerase [5], whether these treatments would stimulate the reduction pathway was tested in the system in the absence of carnitine. As shown in Table 5, fasting increased the reduction pathway slightly in liver mitochondria. A more significant increase was observed in liver mitochondria derived from clofibrate-treated rats. The percentage of reduction pathway in heart mitochondria was not changed either after fasting or clofibrate treatment. This is consistent with our previously reported data that neither fasting nor clofibrate treatment changed the isomerase and reductase activities in heart mitochondria [5].

Effects of dinitrophenol treatment on the reduction pathway

Dinitrophenol uncouples mitochondria and disrupts the production of ATP; this process is expected to decrease the production of NADPH through energy-dependent transhydrogenase and decrease the NADPH/NADP ratio. Together with the addition of malonate to deplete citric acid cycle intermediates and disrupt NADPH production through isocitrate dehydrogenase, this treatment has the potential of diminishing the contribution of the reduction pathway for *cis*-5 fatty acids [23]. As expected, in liver mitochondria with *cis*-5-dodecenoyl-CoA as substrate, the percentage of reduction pathway was decreased ($P = 0.003$, paired *t*-test) from 34 ± 12 % to 26 ± 13 % ($n = 3$) after dinitrophenol uncoupling of mitochondria without the addition of carnitine. In the presence of carnitine, the effect of dinitrophenol on the percentage of reduction pathway was even more pronounced. In liver mitochondria, dinitrophenol plus malonate diminished the reduction pathway from 104% to 35% for *cis*-4-decenoyl-CoA and from 88% to 42% for *cis*-5-dodecenoyl-CoA. In heart mitochondria, dinitrophenol plus

malonate diminished the reduction pathway from 107 % to 10 % for *cis*-4-decenoyl-CoA and from 89 % to 30 % for *cis*-5-dodecenoyl-CoA in paired studies. The addition of glutamate, which has the potential of increasing NADPH production through glutamate dehydrogenase, and 3-hydroxybutyrate, which can change the redox potential of mitochondria, did not change the percentage of reduction pathways significantly for all substrates.

The production of dodecanoate from *cis*-5-dodecenoyl-CoA and tetradecanoate from *cis*-5-tetradecenoyl-CoA

In previous studies with freeze-thawed mitochondria, the results implied that the direct reduction of *cis*-5 fatty acids to their saturated counterparts is possible, but could be only a minor contribution to the overall reduction pathway [7]. The present study examined the m + 0 isotope enrichment of dodecanoate and tetradecanoate from the incubation of *cis*-5-dodecenoyl-CoA and *cis*-5-tetradecenoyl-CoA respectively. After subtracting the background contribution of m + 0 from endogenous substrates, the dodecanoate m + 0 percentage was 5.5 % in liver mitochondria, whereas the m + 0 enrichment of 3OHMC12 was 17.3 %. The significant amount of m + 0 in dodecanoate indicates the production of dodecanoate from *cis*-5-dodecenoyl-CoA. However, the conversion could be through direct reduction of the *cis*-5 double bond or through reduction of *trans*-2-enoyl-CoA after dehydrogenation, isomerization and reduction. Similarly, m + 0 tetradecanoate was detected at a level of 5.4 % in liver mitochondria in studies with *cis*-5-tetradecenoyl-CoA, whereas 3OHMC14 was 23.4 % m + 0. In heart mitochondria, m + 0 tetradecanoate was 6.0 % whereas 3OHMC14 was 14.8 %. Because the m + 0 enrichments of reduced fatty acids were significantly lower than the enrichments in the corresponding 3-hydroxy fatty acids, most of the unlabelled 3-hydroxy fatty acids were not derived from the dehydrogenation and hydration of unlabelled saturated fatty acids. Instead, it was most probably derived from the pathway shown in Scheme 1. For comparison, significant m + 0 enrichment (8.2 %) of decanoate was obtained from the study with *cis*-4-decenoyl-CoA in liver mitochondria, whereas 3OHMC10 was 27.3 % m + 0.

DISCUSSION

Mixtures of an equal amount of unlabelled unsaturated enoyl-CoA and labelled saturated acyl-CoA were used in the present investigation. The rationale behind this approach is based on the production of 3-hydroxy fatty acids from the first cycle of β-oxidation of fatty acids (Scheme 1). For saturated acyl-CoA, this intermediate is produced from the dehydrogenation and hydration in the first cycle. For unsaturated enoyl-CoA, saturated 3-hydroxy fatty acids can be produced only from the reduction pathway. Because m + 0 isotope (5.4–6.0 %) in saturated fatty acids (dodecanoate and tetradecanoate) was significantly less than that (14.8–23.4 %) in 3-hydroxy fatty acids (3OHMC12 and 3OHMC14), it is unlikely that the major reduction mechanism is through the direct reduction of *cis*-5-enoyl-CoA to saturated acyl-CoA, which is then metabolized to 3-hydroxyacyl-CoA, as we proposed originally [2], because if this direct reduction were the major mechanism, the m + 0 enrichment in 3-hydroxy fatty acids would be lower than that of the precursor acyl-CoA from a product–precursor relationship in isotope contents. The actual reduction pathway is most probably through dehydrogenation to *trans*-2,*cis*-5-dienoyl-CoA, which is isomerized to $\Delta^{3,5}$-dienoyl-CoA. Another isomerization converts these compounds to *trans*-2,*trans*-4-dienoyl-CoA, where the reduction occurs. In our study design, the more abundant 3-hydroxy fatty acid produced from saturated acyl-CoA served as carrier and internal standard for the smaller amount of the same intermediate produced from the reduction pathway of unsaturated enoyl-CoAs, yet the two sources are distinguished by their labels. Unlabelled 3-hydroxy fatty acids (m + 0) are produced from unlabelled *cis*-5-enoyl-CoA substrates, whereas [1,2,3,4-$^{13}C_4$]-3-hydroxy fatty acids are produced from [1,2,3,4-$^{13}C_4$]acyl-CoA and [3,4,5,6-$^{13}C_4$]-3-hydroxy fatty acids from [3,4,5,6-$^{13}C_4$]acyl-CoA. Because of the longer pathway, with an additional rate-limiting reduction step (Scheme 1), to the production of 3-hydroxy fatty acids from unsaturated enoyl-CoA in comparison to that produced from saturated acyl-CoA, the accumulation of unlabelled 3-hydroxy fatty acids was much less than the labelled species produced from saturated acyl-CoA.

The production of unlabelled 3-hydroxy fatty acids expected from 100 % reduction pathway was obtained by the incubation of mitochondria with rotenone. Rotenone inhibits complex 1 of the electron transport chain and increases the intra-mitochondrial NADH/NAD ratio. An increase in NADH/NAD ratio is inhibitory to 3-hydroxyacyl-CoA dehydrogenase [24], which then blocks the conventional isomerase-mediated pathway of unsaturated fatty acids. In addition, an increased NADH/NAD ratio enhances the NADPH/NADP ratio through energy-dependent transhydrogenase [25,26]. This contribution further increases the operation of the reduction pathway. It is assumed that, under this condition, 100 % reduction pathway occurred for all unsaturated fatty acids. Two lines of evidence tend to support this assumption. First, *cis*-4-decenoyl-CoA is known to undergo essentially the reduction pathway. No further increase in the ratio of unlabelled 3OHMC10 to labelled 3OHMC10 was observed on the treatment of mitochondria with rotenone. This indicated that essentially 100 % reduction pathway was obtained for *cis*-4-decenoyl-CoA. Secondly, in freeze–thawed mitochondria, the oxidation of *cis*-5-dodecenoyl-CoA was 100 % reduction pathway when exogenous NADPH was added to the incubation [7]. Under this condition, the ratio of unlabelled to labelled 3OHMC12 produced was approx. 20 % when equal amounts of unlabelled *cis*-5-dodecenoyl-CoA and labelled dodecanoyl-CoA were co-incubated. Similar ratios were obtained on the addition of rotenone to intact mitochondria in the present investigation. Even though rotenone treatment of mitochondria inhibits oxygen uptake completely, the first cycle of β-oxidation up to the formation of 3-hydroxyacyl-CoA was not affected [21].

Two isomerases, e.g. Δ^3,Δ^2-enoyl-CoA isomerase and $\Delta^{3,5}$-*t*2,*t*4-dienoyl-CoA isomerase, are needed for the operation of the reduction pathway of *cis*-5 fatty acids [3–8]. In heart mitochondria, the activities of both isomerases are higher than those in liver mitochondria [5]. Despite higher enzyme activities, the smaller contribution from the reduction pathway in heart mitochondria points to a possibility that the activities of these isomerases are probably not rate-limiting in the operation of the reduction pathway. The activity of another enzyme needed in the reduction pathway, e.g. 2,4-dienoyl-CoA reductase, is higher in the liver than in heart [5]. It has been proposed that the activity of 2,4-dienoyl-CoA reductase is rate-limiting in the metabolism of *cis*-4-decenoyl-CoA [24]. It is likely that 2,4-dienoyl-CoA reductase was also rate-limiting in the operation of the reduction pathway of *cis*-5-enoyl-CoA. The diminished reduction pathway with increase in chain length of substrates is consistent with the substrate specificity of 2,4-dienoyl-CoA reductase. This enzyme has an optimal substrate chain length of 10 and the activity is lower towards longer chains [16]. Clofibrate treatment increases 2,4-dienoyl-CoA reductase activity in rat-liver mitochondria [27,28]. The increase in the reduction pathway in liver mito-

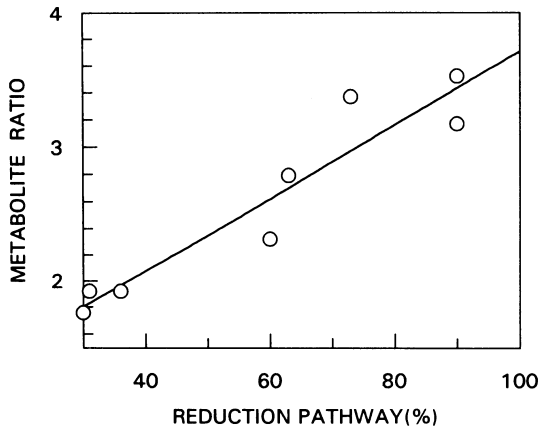

Figure 4 Correlation between the metabolite ratio (3OHMC12 and 3OHc5MC12) and the percentage of reduction pathway calculated from isotope dilution data

The data were obtained from the incubation of equal amounts of unlabelled *cis*-5-dodecenoyl-CoA and [1,2,3,4-$^{13}C_4$]dodecanoyl-CoA in rat-heart mitochondria.

chondria after clofibrate treatment (Table 5) is consistent with the notion that the activity of 2,4-dienoyl-CoA reductase might be the rate-limiting enzyme in the reduction pathway.

If *cis*-5-enoyl-CoAs are metabolized by the conventional β-oxidation pathway, 3-hydroxy-*cis*-5-enoyl-CoA is the metabolic intermediate. On the other hand, saturated 3-hydroxyacyl-CoA is the metabolic intermediate from the reduction pathway. The ratio of 3-hydroxy and 3-hydroxy-*cis*-5 fatty acids produced in the incubation mixture might be a rough estimate of the percentage of reduction pathway even though its magnitude is expected to be underestimated [8]. Nevertheless, a higher percentage of reduction pathway would result in a higher 3OH-/3OHc5-metabolite ratio. The correlation between percentage of reduction pathway calculated from isotope data and that derived from the metabolite ratios is excellent. In studies with *cis*-5-dodecenoyl-CoA as substrate, this correlation is shown in Figure 4. A correlation coefficient (r) of 0.9496 ($P = 0.0002$) was obtained.

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In summary, the reduction pathway has been shown to be the predominant pathway in the oxidation of *cis*-5 fatty acids in both rat-liver and rat-heart mitochondria. In rat-liver mitochondria, the metabolism of *cis*-5 fatty acids was essentially through the reduction pathway. However, in heart mitochondria, the percentage of reduction pathway decreased with increasing chain length.

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