Incomplete Fatty Acid Oxidation
THE PRODUCTION AND EPIMERIZATION OF 3-HYDROXY FATTY ACIDS*

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3-Hydroxydicarboxylic acids are major urinary metabolites derived from fatty acid metabolism. These compounds are produced from the ω-oxidation of 3-hydroxy fatty acids. The production of the precursor 3-hydroxy fatty acids from incomplete β-oxidation of fatty acids in rat liver mitochondria was investigated.

Independent of the chain length or the concentration of fatty acid substrates, the accumulation of 3-hydroxyacyl intermediates was relatively constant at the concentration of 3-5 nmol/mg of mitochondrial protein. The extent of the incomplete oxidation was the same in Percoll gradient-purified mitochondria. Rotenone treatment increased the production of 3-hydroxy fatty acids.

3-Hydroxy fatty acids did not exist as pure l-enantiomer as expected from β-oxidation. Instead, these metabolites were epimerized to a near racemic mixture of D- and L-isomers with a slightly dominant D-isomer (58 ± 3%). By using deuterium-isotope labeling, the mechanism of epimerization was shown to be a rapid dehydration-rehydration through trans-2-enoyl-CoA. In addition, cis-3 and trans-3 fatty acids were produced; these metabolites were derived from the isomerization of trans-2-enoyl-CoA.

Epimerase and isomerase were thought to be enzymes involved in the oxidation of unsaturated fatty acids. Current data have shown that the metabolism of these acids is actually through NADPH-dependent reduction pathways. The activities of epimerase and isomerase detected in rat liver mitochondria possibly function mainly in the metabolism of saturated fatty acids in a reverse role to the conventional concept.

3-Hydroxydicarboxylic aciduria, the increased urinary excretion of 3-hydroxydicarboxylic acids with 6-14 carbons, is of considerable interest, in part, because of the observation of a new defect of fatty acid oxidation, i.e. long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (1-8). We have found that the increased urinary excretion of these metabolites is not limited to patients with long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency; other physiological (fasting) and pathological conditions (dicarboxylic aciduria) are also associated with an increase in these urinary metabolites (8). Based on metabolic studies in rat liver homogenates and the metabolites identified in the urine of patients with 3-hydroxydicarboxylic aciduria, we proposed that the metabolic origin of urinary 3-hydroxydicarboxylic acids is from the ω-oxidation of 3-hydroxy fatty acids (8, 9).

While not elucidated, precursor 3-hydroxy fatty acids are assumed to be released in the course of β-oxidation of fatty acids in mitochondria and/or peroxisomes. Since 3-hydroxydecenoic and 3-hydroxydodecanoic acids are the most significant metabolites in urine, we speculated that the incomplete oxidation of fatty acids, releasing 3-hydroxyacyl intermediates, was greatest at the stage of 10 and 12 carbons. When using rat liver mitochondria, early studies failed to detect intermediates from fatty acid oxidation (10, 11). Because of the efficiency in the multiple step metabolism of fatty acid oxidation, an enzyme complex for fatty acid oxidation has been proposed (11).

However, metabolic intermediates, especially 3-hydroxyacyl metabolites, were found in later studies (12-17). The accumulation of 3-hydroxyacyl metabolites is enhanced under conditions of rotenone treatment and hypoxia. These data indicate that the oxidation of fatty acids could be incomplete and the metabolic intermediates can be isolated. With the discovery that the pathway of peroxisomal fatty acid oxidation is incomplete (18), the "leaky" pathway is generally believed to be due to the contaminating peroxisomes in mitochondrial preparations.

An exclusive peroxisomal origin of 3-hydroxy fatty acids does not explain the low urinary excretion of short chain 3-hydroxydicarboxylic acids in patients with medium-chain acyl-CoA dehydrogenase deficiency (8). Thus, at least, a partial mitochondrial origin is indicated. This report describes the release of 3-hydroxy fatty acids in rat liver mitochondria during the β-oxidation of fatty acid substrates.

MATERIALS AND METHODS

Chemicals—DL-3-Hydroxy fatty acids were synthesized from sodium borohydride reduction of 3-oxo fatty acid ethyl esters, which were prepared by the coupling of ethyl acetoacetate and alkyl halides as described previously (9). Acyl-CoAs were synthesized by the procedure of Goldman and Vagelos (19). Their purity and concentration were determined by the fatty acids released after alkaline hydrolysis. The identity and the amount of fatty acid released were determined by dual capillary column gas chromatograph. In these preparations, no free fatty acid was detectable before alkaline hydrolysis. In addition, the purity was also determined by the absorption spectra and the concentration determined by Eillman's method (20). 3-Hydroxyacyl-CoA dehydrogenase, ATP, and CoA were obtained from Sigma.

Isolation of Rat Liver Mitochondria (21)—Fresh rat liver was minced and then homogenized in a mixture of MSM buffer (220 mM mannitol, 30 mM sucrose, 5 mM Mops, pH 7.4) with EDTA (2 mM). After centrifugation at 400 × g to remove the debris, the homogenate was further centrifuged at 7,000 × g. After twice washing the resultant pellets with MSM solution, the mitochondria were suspended in

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The protein concentration was determined with the Biuret method. Rat liver mitochondria isolated by this method contained less than 5% of peroxisome contamination when determined by protein distribution after Percoll-gradient centrifugation.

Purification of Mitochondria on Percoll Gradient (22)—The mitochondrial preparation from the above procedure was further purified in a self-forming 50% Percoll gradient made isotonic with sucrose. After centrifuging at 79,000 x g for 30 min using a vertical rotor (Beckman VTI 50), the gradient was collected in 3-ml fractions. The mitochondrial fractions (density, 1.08-1.10) were located using citrate synthase activity in the peroxisomal fraction (density, 1.04-1.05) and the lactate dehydrogenase activity located with catalase activity. Protein concentrations were determined by the method of Lowry. The purified mitochondria were obtained by diluting density 1.10 mitochondrial fractions, which contained less than 5% of the original citrate synthetase activity but more than 60% of the original citrate synthetase activity in prefurished mitochondrial preparations, with 9 volumes of MSB buffer and centrifuging at 32,000 x g for 10 min. Therefore, the peroxisome contamination in these purified mitochondria is negligible (less than 0.5%).

Mitochondrial Respiration Study with Oxygen Electrode (21)—The polarographic “oxygen electrode” technique for measuring changes in oxygen utilization by mitochondria was carried out in a 0.5-ml chamber at 30 °C. A mixture of 1 mg of mitochondrial protein, MgCl₂ (1.6 μmol), ATP (1.6 μmol), CoA (0.9 μmol), l-carnitine (0.3 μmol), and malate (2.5 μmol) in 0.5 ml of incubation mixture (80 mM KCl, 50 mM Mops, 5 mM P, 1 mM EGTA, and 1 mg of defatted, dialyzed bovine serum albumin/ml) was preincubated in the chamber. The final concentration of the deproteinized substrates was 1 mm. A mixture of ADP (0.4 μmol) was added and the oxygen consumption rates measured. When acyl-CoAs were used as substrate (40 μmol), cofactors (ATP, CoA, and MgCl₂) were omitted from the mixture and the “state 3” oxygen consumption rate was initiated by the addition of ADP (0.2 mM). In some experiments, rotenone (4 μM) was added and incubated for 1 min before the addition of substrates. Respiratory control ratios were determined by the method of Estabrook (23). Only mitochondrial preparations with respiratory control ratios larger than 6 (glutamate as substrate) were used for the studies.

Metabolic Incubation Studies—The studies were carried out in 25-ml Erlenmeyer flasks in a metabolic shaking incubator (140 cycles/min) at 37 °C with the same composition of incubation mixture, cofactors, and substrates; however, the mitochondrial protein was 1 mg/ml and the total volume was 3 ml. After 2, 5, 10, and 30 min of incubation, the metabolic reaction was terminated by quick freezing in an ice-acetone mixture.

Metabolic Profiling of Incubation Mixture (24)—An aliquot (1 ml) of the incubation mixture was mixed with internal standard (20 μg of pentadecanoic acid) and hydrolyzed at 90 °C for 30 min with the addition of 100 μl of 3 N KOH. The mixture was acidified and extracted with isooctane (dideethyl ether (1:1)). After addition of 1 μl of 15% tris(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane, the sample was analyzed with a dual capillary column gas chromatograph, using a bonded dimethylpolysiloxane phase (SPB-1 from Supelco) and bonded 35% diphenyl, 65% dimethylpolysiloxane phase (SPB-35) fused silica capillary columns. The column temperature was programmed to increase at 4 °C/min from 60 to 250 °C and a 50 to 1 split injection ratio. The quantitation was based on the relative peak area to internal standard area ratio. The amount of metabolites was calculated as the weight equivalent to that of internal standard on the basis of area ratio.

A Hewlett-Packard 5890B gas chromatograph/mass spectrometer was used for metabolite identification. A shorter (15 m) fused silica capillary column was used with the same temperature program as described for GC. Electron-impact (70 eV) ionization and repetitive scanning (300 atomic mass units) from m/z 49-550 was used for metabolite identification. The criteria for identification of a compound were that retention times on both columns in GC and the mass spectrum identical to those obtained from authentic standards.

Synthesis of 3-Hydroxydecanoic-3-d and 3-Hydroxydecanoic-2-3-H-Acids—Ethyl 3-oxodecanoate was reduced with sodium borohydride (28) in methanol to yield ethyl 3-hydroxydecanoate-3-d. Alkaline hydrolysis produced 3-hydroxydecanoic-3-d acid. The product contained 96% d, and 4% d as determined from the mass spectrometric analysis of trimethylsilyl derivative.

3-Hydroxydecanoic-2,2-d₂ acid was prepared by deuteration isotope exchange. Ethyl 3-hydroxydecanoate (10 mmol) was refluxed for 15 min in a solution of sodium methoxide (0.7 M) in 3.5 ml of CH₃OD. After evaporation to dryness under dry nitrogen, the mixture was treated twice with fresh portions of CH₃OD. The mixture was acidified with 10 N DCI (2 ml) and the product was extracted into ether. Alkaline hydrolysis of the dried extract yielded 3-hydroxydecanoic acid, which was analyzed by gas chromatography-mass spectrometry and the product contained: 29.9% d, 16.2% d₂, and 53.9% d₃. All the deuterium labels were accounted for at the 2,2'-position. No label was found in other positions.

Metabolic Studies with Deuterium-labeled 3-Hydroxydecanoic Acids—Deuterium-labeled 3-hydroxydecanoic acids were studied under the same conditions as described for unlabeled substrates. The samples were extracted and derivatized as in previous section. For the determination of isotopic enrichments of the metabolites (cis-3-, trans-3-, and trans-2-dodecanoic acids), the samples were obtained using selected ion monitoring analysis using mass spectrometers. The chromatographic column temperature was increased from 185 °C at a rate of 4 °C/min until 3-hydroxydecanoic acid bis(trimethylsilyl) derivative was eluted. The ions monitored were m/z 255, 256, and 257 (from 3 to 4.5 min) for cis-3-, trans-3-, and trans-2-dodecanoic acids; it was switched to m/z 233, 234, and 235 (from 5.5 to 6.5 min) for 3-hydroxydecanoic acids.

The dwell time of each ion was 100 ns.

For unsaturated metabolites, the ions monitored were those derived from M-15 fragment, while ions containing carbons one, two, and three were used for 3-hydroxy compound. In the latter case, the enrichments in M-15 ions (m/z 345, 346, and 347) were also determined. The difference in enrichments was observed as the integration of selected ions. The calculation of the enrichments was as described before (25). This method can determine an enrichment of at least 1% above unlabeled blank values.

Determination of d- and L-Isomers of 3-Hydroxy Fatty Acids—The metabolites were extracted from the incubation mixture as described in previous section. After converting to methyl esters with 100 μl of methanolic hydrogen chloride (20 μl of acetyl chloride in 1 ml of methanol) at 90 °C for 1 h, the samples were dried and purified by a silica gel column chromatograph (in a disposable Pasteur pipet packed to a height of 7 cm and equilibrated with isooctane). The column was eluted stepwise with 5 ml each of 10 and 50% of ethyl acetate in isooctane. The former fraction contained methyl esters of d-3-hydroxy fatty acids and the latter fraction contained methyl esters of 3-hydroxy fatty acids. After evaporating to dryness, the methyl 3-hydroxy fatty acid fraction was derivatized with (S)-(+)2-phenylpropionyl chloride in benzene (40 μl) and pyridine (20 μl) at 60 °C for 1 h (26). The solvent was removed and the derivatives were purified on another silica gel column to remove the unreacted derivatizing reagent. The desired derivatives were eluted with 5 ml of isooctane/ethyl acetate (7:1) while the reagent was retained on the column. After evaporation to dryness, the sample was dissolved in isooctane (40 μl) and analyzed with dual capillary column gas chromatography, repetitive scanning mass spectrometry, or selected ion monitoring mass spectrometry.

In dual capillary column gas chromatographic analysis, 3-hydroxydecanoic acid, 3-hydroxyhexadecanoic acid, and 3-hydroxytetradecanoic acid were monitored from 60 °C at a 4 °C/min increase in temperature; 3-hydroxytetradecanoic and 3-hydroxyhexadecanoic acid derivatives were analyzed from 160 °C at the same rate. Base-line separation was achieved for all the isomeric pairs. For repetitive scanning mass spectrometry, the derivatized sample was introduced through the capillary column GC and the effluent was repetitively scanned from m/z 49 to 550. In the case of selected ion monitoring analysis using mass spectrometer, the ions m/z 105, 155, 151, 209, and 237 were used. The ion m/z 105 was for all 2-phenylpropyl derivatives and the rest of the ions were for 3-hydroxydecanoic, 3-hydroxydecanoic, 3-hydroxytetradecanoic, and 3-hydroxyhexadecanoic acid derivatives, respectively.

For the determination of absolute configuration of the isomeric 3-hydroxy fatty acid pairs, the accumulation of 3-hydroxydecanoic acids were prepared from the enzymatic reaction of racemic 3-hydroxyacyl-CoAs with 3-hydroxyacyl-CoA dehydrogenase. A mixture of 3-hydroxyacyl-CoA (140 mmol), NAD (10 mmol), and bovine serum 3-hydroxyacyl-CoA dehydrogenase (4 units, from Sigma) in 3 ml of Tris buffer (0.2 M, pH 9.0) was stirred at 37 °C for 30 min. After sequential alkaline hydrolysis, acidification, extraction, and derivatization, the samples were analyzed with GC and GC/MS.

1 The abbreviations used are: GC, gas chromatography; GC/MS, gas chromatography-mass spectrometry; 3OHMC10, 3-hydroxydecanoic acid; 3OHMC12, 3-hydroxydecanoic acid; 3OHMC14, 3-hydroxytetradecanoic acid; 3OHMC16, 3-hydroxypalmitic acid; c3, cis-3; t3, trans-3; c2, cis-2; t2, trans-2; 15, 15-dodecanoic acid; 2-phenylpropionic acid; 3-deuterium; EGTA, ethylenebis(oxyethylenenitritilo)ethylenediacetic acid; Mops, 4-morpholinopropanesulfonic acid.
RESULTS

β-Oxidation of Dodecanoic Acid in Rat Liver Mitochondria—When dodecanoic acid and cofactors (ATP, CoA, MgCl2, l-carnitine) were incubated with intact rat liver mitochondria, intermediates resulting from the incomplete oxidation of substrate were detected (Fig. 1). These metabolites consisted of 3-hydroxydodecanoic acid (3OHMC12) as the major component together with smaller amounts of 3-hydroxydodecanoic (3OHMC10), trans-2-dodecanoic (t2MC12), and dodecanoic acids. The concentrations of these metabolites with incubation time are shown in Fig. 2. When the precursor dodecanoic acid was completely metabolized, the metabolic intermediates were no longer detectable in the incubation medium. These data indicate that the release of metabolic intermediates was in a steady state as long as there was unmetabolized precursor. When the precursor was completely metabolized, the intermediates reentered the β-oxidation pathway and continued to be metabolized to completion.

The accumulation of metabolic intermediates in control, rotenone-inhibited, frozen-thawed, and Percoll gradient-purified mitochondria after 10 min of incubation are shown in Table I. Prolonging the incubation up to 30 min did not change significantly the composition of the metabolites. Omitting carnitine did not change the accumulation of 3-hydroxydodecanoic acid in paired experiments, i.e. 3.20 ± 0.6 nmol/mg of protein comparing to 4.20 ± 0.9 with carnitine, n = 5. Mitochondria treated with rotenone as well as by freezing-thawing increased the accumulation of 3-hydroxy fatty acids from the first β-oxidation cycle. In addition, two more metabolites, i.e. cis-3-dodecenoic and trans-3-dodecenoic acids, became detectable with a slightly higher trans-3 than the cis-3 isomers. The metabolic profile from Percoll gradient-purified mitochondria was not different from the control mitochondria; therefore, the possibility that the accumulation of intermediates was due to contaminating peroxisomes can be ruled out.

The disappearance rates of dodecanoic acid were comparable among control, rotenone inhibited, and frozen-thawed mitochondria, i.e. 3.6 ± 0.7 (n = 7), 3.4 ± 0.8 (n = 3), and 2.7 ± 0.9 (n = 4) nmol/min/mg of protein, respectively. However, the oxygen consumption rates showed considerable difference among these mitochondria. The oxygen consumption rates of dodecanoic acid in control mitochondria were 64 ± 3 nanomols of O2/min/mg of protein (n = 7). In contrast, the rates were completely inhibited in rotenone treated as well as frozen-thawed mitochondria. These data indicate that the metabolic rates of fatty acids in the first cycle of β-oxidation up to the formation of 3-hydroxyacyl-CoA were not affected by rotenone treatment as well as by freezing-thawing. The metabolic step affected by these treatments is the oxidation

![Figure 1](image1.jpg)

**Fig. 1.** Metabolites produced from the incomplete oxidation of dodecanoic acid in rat liver mitochondria. A, control mitochondria; B, mitochondria plus rotenone. Metabolites are identified as: 1, dodecanoic; 2, 3-hydroxyoctanoic; 3, cis-3-dodecenoic; 4, trans-3-dodecenoic; 5, dodecanoic (starting substrate); 6, 3-hydroxydodecanoic; 7, trans-2-dodecenoic; 8, 3-hydroxydodecanoic acids, and 9, internal standard. Rotenone was added at a concentration of 4 μM. Rotenone increased the accumulation of 3-hydroxy fatty acids, but did not change the metabolic rate of dodecanoic acid. These samples were extracted and analyzed as trimethylsilyl derivatives on SPB-1 fused acid capillary column (0.25 mm ID × 25 m) gas chromatograph. The column temperature was from 60 to 250 °C at an increase of 4 °C/min.

![Figure 2](image2.jpg)

**Table I**

Incomplete oxidized metabolites accumulated during the oxidation of dodecanoic acid in rat liver mitochondria

Other metabolites that were detectable, but of smaller quantity were cis-3-dodecenoic and trans-3-dodecenoic acids. Dodecanoic acid was incubated with rat liver mitochondria (1 mg/ml) together with cofactors (1.6 μmol ATP, 1.6 μmol MgCl2, 0.9 μmol CoA, and 0.3 μmol l-carnitine). Control mitochondria were those isolated by the usual procedure without further purification to remove trace of peroxisomes; the rotenone-treated mitochondria were preincubated with 4 μM of rotenone for 1 min before the addition of substrate; frozen-thawed mitochondria were those obtained from control mitochondria by freezing and thawing once; Percoll-purified mitochondria were those obtained from Percoll-gradient centrifugation. Data are means ± S.D. Initial substrate concentrations were 160 μM (150 nmol/mg of mitochondrial protein) and the substrate was incubated for 10 min.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Concentration of metabolites</th>
<th>nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 5)</td>
<td>6.0 ± 2.8; 3.7 ± 2.7; 3.8 ± 2.5; 6.4 ± 2.9</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Rotenone (n = 4)</td>
<td>13.4 ± 6.0; 6.4 ± 3.2; 2.5 ± 1.3; 1.7 ± 1.5</td>
<td>1.7 ± 1.5</td>
</tr>
<tr>
<td>Freeze-thaw (n = 4)</td>
<td>22.7 ± 2.8; 2.7 ± 0.5; 2.5 ± 3.1; 3.5 ± 1.2</td>
<td>3.0 ± 1.5</td>
</tr>
<tr>
<td>Percoll (n = 3)</td>
<td>6.5 ± 2.3; 2.7 ± 2.7; 1.9 ± 1.9</td>
<td>8.7 ± 9.9; 0.5 ± 0.5</td>
</tr>
</tbody>
</table>

These data indicate that the accumulation of intermediates was shown in right-hand expanded scale (0-25 nmol/ml) while the substrate (dodecanoic acid) uses the left-hand scale (0-130 nmol/ml).
mediated by 3-hydroxyacyl-CoA dehydrogenase, which oxidizes 3-hydroxyacyl-CoA to 3-oxoacyl-CoA.

In contrast to the measurement of oxygen consumption rates, the metabolic disappearance rates of the substrates were not dependent on the presence of malate or malonate, where the products would be citrate or acetoacetate (27). The accumulation of the intermediates from the incomplete oxidation of substrates was also independent of these factors. ADP-induced oxidation produced lower amount of 3-hydroxydodecanoic acid, i.e. 1.3 ± 1.0 nmol/mg of protein (n = 4), than when ATP was present. However, the difference in the extent of accumulation of 3-hydroxydodecanoic acid was not due to the energy state of the incubation conditions (12). The oxygen consumption rates of dodecanoic acid plus cofactors (ATP, CoA, carnitine, and MgCl₂ but without ADP) were the same as the state 3 rates (68 ± 11 nanomols of O₂/min/mg of protein, n = 4) of dodecanoyl-CoA plus carnitine and ADP.

**3-Oxidation of Myristic, Palmitic, and Oleic Acids—**These substrates were studied as acyl-CoA plus carnitine. The 3-hydroxy fatty acids in the incubation milieu were from 1.2 to 4.7 nmol/mg of protein (n = 5). In rotenone-treated mitochondria, these values increased to 3.7–14.3 nmol/mg of protein (n = 5), while in frozen-thawed mitochondria, these values were 5.1–13.4 nmol/mg of protein (n = 7). Compared to the data obtained from dodecanoic acid (Table I), the incomplete oxidation of longer chain fatty acids was of the same degree as dodecanoic acid. There is no preferential leakage of 3-hydroxy fatty acids at dodecanoic acid (9). Other metabolic intermediates accumulated in these studies were similar to those observed in dodecanoic acid studies. These metabolites included fatty acids with two and four less carbons, 3-hydroxy fatty acids with 2 and 4 less carbons, as well as trans-2, cis-3, and trans-3 fatty acids.

**Absolute Configuration of the 3-Hydroxy Group in 3-Hydroxy Fatty Acyl Intermediates—**The 3-hydroxy fatty acid accumulated from the peroxisomal metabolism of linoleic acid has been reported to be a racemic D- and L-isomer mixture (28). This occurrence is explained by the dehydration and rehydration by enoyl-CoA hydratases in peroxisomes. Since mitochondria are thought to be lacking epimerase activity (29, 30), the racemization of 3-hydroxyacyl-CoA was not expected. The absolute configuration of the 3-hydroxy group in 3-hydroxyacyl intermediates were studied by derivatization with chiral reagent. Successful separation of the D- and L-isomers of 3-hydroxy fatty acids was achieved using methyl ester (S)-(−)-2-phenylpropyl ester derivatives (25, 26).

The synthetic 3-hydroxydecanoic and 3-hydroxydodecanoic acids were racemic mixtures; their chromatographic separations are shown in Fig. 3. After enzymatic treatment with 3-hydroxyacyl-CoA dehydrogenase, the original racemic 3-hydroxy fatty acids were converted to one predominant peak, which had the same retentions as the earlier eluted peak in the racemic mixture. Therefore, the earlier eluting peak was assigned to D-enantiomer, while the later eluting peak was the L-enantiomer. This assignment is in the same sequence as those indicated for longer chain homologues (26, 28).

For the analysis of 3-hydroxy fatty acids in biological matrix, the extract was derivatized and purified through silica gel columns. After these treatments, the chromatogram contained essentially only 3-hydroxy fatty acids (Fig 4). The enantiomeric composition of 3-hydroxy fatty acids can be determined directly from the gas chromatogram. However, for confirmation, these samples were usually analyzed with gas chromatograph-mass spectrometry using either repetitive scanning or selected ion monitoring (Fig. 4). The enantiomer composition determined from all three techniques were identical.

The electron impact (70 eV) induced mass spectra were identical for D- and L-enantiomers. Molecular ions were detectable, but the abundance was less than 1% of the base ion (m/z 105). For 3OHMC10 and 3OHMC12 derivatives, the mass fragments of significant abundance are: m/z (percentage of base ion): 3OHMC10, m/z 216(5), 185(8), 153(21), 135(18), 132(19), 111(8), 105(100), 97(8), 69(18); 3OHMC12, m/z 244(2), 213(9), 181(11), 163(11), 132(23), 105(100), 97(10), 83(9), 69(7). The fragments, m/z 132 and 105 are derived from the 2-phenylpropionyl group. The fragments of importance for compound identification are the series derived from the successive loss of 2-phenylpropionyl (M-149) and methanol (M-149-32). These fragments occur at m/z 185 and 153 for 3OHMC10; while they are at m/z 215 and 181 for 3OHMC12.
3-Hydroxydodecanoic acid accumulated from the metabolism of dodecanoic acid consisted of 58 ± 3% D-enantiomer and 42 ± 3 L-enantiomer (n = 6). Identical results were obtained in Percoll gradient-purified mitochondria: D-enantiomer (57 ± 2%) and L-enantiomer (43 ± 2%), n = 3. When longer chain fatty acids (MC14 and MC16) were used as the substrate, the 3-hydroxy fatty acids (30HMC14 and 30HMC16) showed similar epimerization (D-isomer: 54 ± 2%, n = 5). In contrast, chemically synthesized racemic 3-hydroxy fatty acids (30HMC10, 30HMC12, 30HMC14, and 30HMC16) showed 49 ± 1% of D-isomer and 51 ± 1% of L-isomer. In repetitively freezing and thawing mitochondria in phosphate buffer (31), the metabolism terminates at 3-hydroxy fatty acids obtained from these experiments showed a racemic composition with 49 ± 1% of D-isomer (n = 6). The difference between the isomer composition in freshly prepared versus frozen-thawed mitochondria must be further metabolism. 3-Hydroxyacyl-CoA dehydrogenase is specific only for the oxidation of L-3-hydroxyacyl-CoAs. The slightly higher D-isomer in the 3-hydroxy fatty acids isolated from intact mitochondria was the result of selective further metabolism of L-3-hydroxyacyl-CoA. This speculation was confirmed from the experiments with synthetic racemic 3-hydroxydodecanoic acid as substrate (Fig. 4). The unmetabolized substrate, after 10-60 min of incubation in mitochondria, showed the same predominance of D-isomer, 58 ± 4% (n = 3), a change from the initial composition of 49% D-isomer.

**Mechanism of the Epimerization of 3-Hydroxy Fatty Acids—**
Since enoyl-CoA hydratase (crotonase) is specific only for producing L-3-hydroxyacyl-CoA, the metabolite produced initially must be L-form. The subsequent epimerization then converted L-isomer to D-isomer. A rapid interconversion between L- and D-isomers must have occurred since the 3-hydroxy fatty acids isolated were always a racemic or near racemic mixture. Racemization of 3-hydroxy group can be mediated through either dehydration-rehydration or oxidation-reduction. To distinguish between these mechanisms, deuterium labeled 3-hydroxydodecanoic acids were synthesized with deuterium at 2,2'-position or 3-position. The disappearance and appearance of the D- and L-isomers stayed relatively constant with a slightly predominant D-isomer. As shown in Table I, the 2,2'-d2-labeled 3-hydroxydodecanoic acid lost its double d2 label at the rate of 5%/min. This tracer lost its label by dehydration to trans-2-dodecanoic acid (Fig. 5A). No nonspecific chemical dehydrogenation or isotope exchange occurred since the deuterium label was stable in buffer incubated under the same condition for as long as 120 min. A mitochondrial enzyme extract is needed to effect the loss of deuterium label.

In the first cycle of dehydration, the product t2MC12 retains one deuterium; however, both deuterium labels can be lost from more than one cycle of dehydration and rehydration by enoyl-CoA hydratases. The back conversion from t2MC12 to 30HMC12 is not expected to yield any significant doubly labeled 30HMC12, since the rehydration used water that was relatively low in deuterium content. Therefore, the rate of loss of deuterium can be an estimate to the rate of epimerization. Apparently, the rate of epimerization (5%/min) was faster than the further β-oxidation (2%/min); therefore, a near racemization occurred in 30HMC12. Since the regular enoyl-CoA hydratase catalyzes only the reversible conversion between t2MC12 and L-30HMC12, another novel enoyl-CoA hydratase which is specific for catalyzing the reversible conversion between t2MC12 and D-30HMC12 is probably present in mitochondria. It is likely that the catalytic rate of this novel enoyl-CoA hydratase is the same as the regular enoyl-CoA hydratase in order to reach a final racemic product. The reversible interconversion between L-3-hydroxydodecanoic-CoA and cis-2-dodecenoic-CoA, allegedly mediated by the regular enoyl-CoA hydratase (32), apparently did not occur in mitochondria since no cis-2-dodecanoic acid could be detected as metabolic intermediates under all conditions.

That the mechanism of epimerization was through the dehydration and rehydration (Fig. 5A) is further supported by the data from 3-d1-labeled 30HMC12 shown in Table III. The loss of deuterium through oxidation-reduction (Fig. 5B) was only about 0.04%/min, too small to account for the epimerization observed.

Besides t2MC12, cis-3-dodecenoic (c3MC12) and trans-3-dodecenoic (t3MC12) acids were also formed from the metabolism of dodecanoic acid (31). These metabolites can be formed from either the direct dehydration to 3-enoyl-CoA or the isomerization from trans-2-enoyl-CoA. The labeling pattern determined in c3MC12 and t3MC12 (Table II) indicates that these metabolites were formed from the isomerization of t2MC12 since the direct dehydration to 3-enoyl compound would retain doubly labeled deuterium as the precursor 30HMC12. Instead, c3MC12 and t3MC12 did not have significant d2 label, while the extent of d1 labeling was consistent with t2MC12 as the metabolic precursor.

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**Table II**

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>30HMC12</th>
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<td>7.1</td>
</tr>
</tbody>
</table>

**Table III**

<table>
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<tr>
<th>Time</th>
<th>30HMC12-d1</th>
<th>t2MC12-d1</th>
<th>c3- and t3MC12-d1</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>mol % excess</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>96.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>96.4</td>
<td>94.9</td>
<td>94.7</td>
</tr>
<tr>
<td>30</td>
<td>95.5</td>
<td>93.9</td>
<td>93.4</td>
</tr>
</tbody>
</table>

---

**Fig. 5.** The loss of deuterium label through dehydration-rehydration mechanism using 3-hydroxydodecanoyl-2,2-d2-CoA as substrate (A) and through oxidation-reduction mechanism using 3-hydroxydodecanoyl-3d1-CoA as substrate (B).
DISCUSSION

Dodecanoic acid was chosen as a model substrate based on: (a) 3-hydroxydodecanedioic acid is one of the major urinary organic acids in dicarboxylic aciduria and (b) there is no significant amount of endogenous dodecanoic acid to complicate the analysis of metabolites or the interpretation of data. Independent of the chain length or concentration of the substrates, the accumulation of 3-hydroxyacyl intermediates was relatively constant at the concentration of 3–5 nmol/mg of protein. Since the mitochondrial CoA content is 2 nmol/mg of protein (11), the accumulated intermediates probably existed as a mixture of carnitine and CoA esters as well as free acids (17). This incomplete oxidation is consistent with the “leaky hosepipe” model proposed by Stanley and Tubbs (13) that fatty acid metabolism consists of two pools. The major pool is the fast turnover pool of main pathway of β-oxidation; another smaller and slower pool is the leakage of metabolites from the main β-oxidation pathway. The intermediates in the smaller stagnant pool can reenter the β-oxidation pathway. Under the in vitro study conditions, the leakage and the reentry of the intermediates appears to be in equilibrium. Since peroxisomes are known to undergo incomplete oxidation of fatty acids and intermediates are released (18), this “leaky hosepipe” model was regarded by some as a possible peroxisomal metabolism contaminated in mitochondrial preparations. In this investigation, Percoll gradient-purified mitochondria, which contained negligible peroxisomes, also show the same accumulation of incompletely oxidized intermediates; therefore, the incomplete oxidation of fatty acids also occurs in mitochondria.

Bremer and Wojtczak (12) observed 3-hydroxybutyramide ranging from 0.1 to 0.2 nmol/mg of protein (recalculated from the original percentage of substrate data) when 20% of the palmitoylcarnitine substrate was metabolized under ADP stimulated or DNP uncoupled conditions. In the presence of ATP, the oxygen consumption rates were significantly lower and the accumulation of 3-hydroxybutyramide increased to 3.4–4.5 nmol/mg of protein. Whether in the presence of ADP or ATP, the treatment with rotenone resulted in the accumulation of 3-hydroxyacyl intermediates in the range of 3.4 to 6.3 nmol/mg of protein. These results are comparable with the data obtained from the present investigation.

The 3-hydroxy fatty acids released were epimerized to a near racemic mixture of D- and L-isomers instead of a pure L-enantiomer as would be predicted from the β-oxidation of fatty acids. This epimerization is through a rapid dehydration and rehydration via trans-2-acyl intermediates. For decades, the epimerase activity was thought to be an integral part in the metabolism of saturated fatty acids. The net epimerization from D-3-hydroxy to L-3-hydroxy, the β-oxidation activity actually serves the function of a net conversion of 3-hydroxyacyl-CoA to 3-hydroxyacyl-CoA in the metabolism of saturated fatty acids. The biological function of this epimerization is not known.

Fig. 6. The postulated pathways for the racemization of 3-hydroxy fatty acids and the production of cis-3 and trans-3 fatty acids. β-Oxidation of dodecanoic acid (MC12-CoA) releases L-3-hydroxydicarboxylic acid (L-3OHMC12-CoA), which is converted to trans-2-dodecenoyl-CoA through the action of crotonase (I). A novel soybean hydroxylase (II) converts dodec-2-enoyl-CoA to dodecanoic acid. cis-3-Dodecenoic acid (c3MC12-CoA) and trans-3-dodecenoic acid (t3MC12-CoA) are produced from the isomerization of MC12-CoA through enoyl-CoA isomerase (III). Therefore, epimerase and isomerase, the alleged enzymes in the metabolism of unsaturated fatty acids, are actually involved in the metabolism of saturated fatty acids. The net production from these enzymes is D-3-hydroxy, cis-3, and trans-3 fatty acids, a reversal from the classical concept. The function of this epimerase is to convert D-3-hydroxyacyl-CoA to L-3-hydroxyacyl-CoA for the further β-oxidation catalyzed by 3-hydroxyacyl-CoA dehydrogenase. D-3-Hydroxyacyl-CoAs are formed from the hydration of cis-2-enoyl-CoA by enoyl-CoA hydratase. Recently, the metabolism of even-numbered unsaturated fatty acids has been shown to be exclusively or predominantly by the NADPH-dependent 2,4-dienoyl-CoA reductase pathway instead of the epimerase pathway (33). cis-2-Enoyl-CoA is not formed from the further metabolism of cis-4-trans-2-dienoyl-CoA since the latter is a poor substrate for enoyl-CoA hydratase. Furthermore, a single epimerase activity was not detected; instead, the epimerization is mediated by the concerted action of two enzymes, the regular enoyl-CoA hydratase (cetonase) and a novel enoyl-CoA hydratase II which uses D-3-hydroxyacyl-CoA as substrate and converts it to trans-2-enoyl-CoA (32, 34). Our data are consistent with this mechanism of epimerization. Using an enzymatic method, Schulz and colleagues (29, 30) have indicated that rat liver mitochondria has a low activity of epimerization. This apparent discrepancy can not be explained at the present time. In the enzymatic assay, the epimerization is assumed to be a rate limiting step; however, the direct measurement of epimerization in the present study indicates that the epimerization is faster than the subsequent β-oxidation reactions. Therefore, a near racemic mixture of 3-hydroxy fatty acids was maintained at all times. Contrary to the conventional concept of net epimerization from D-3-hydroxy to L-3-hydroxy, the epimerase activity actually serves the function of a net conversion from L-3-hydroxyacyl-CoA to D-3-hydroxyacyl-CoA in the metabolism of saturated fatty acids. The biological function of this epimerization is not known.

Besides trans-2 fatty acids, which are intermediates in the epimerization of 3-hydroxyacyl metabolites, the metabolism of saturated fatty acids also produced cis-3 and trans-3 fatty acids. Through deuterium isotope labeling, the metabolic origin of these cis-3 and trans-3 fatty acids is shown to be through the isomerization of trans-2 fatty acids possibly through its CoA ester (Fig. 6). The isomerization may be effected by enoyl-CoA isomerase thought to be involved in the metabolism of odd-numbered double bond unsaturated fatty acids (33). In the classical hypothesis, the β-oxidation of odd-numbered unsaturated fatty acids produces cis-3-enoyl-CoA, which is then isomerized to trans-2-enoyl-CoA by the isomerase. The isomerases have been isolated and purified from both mitochondria and peroxisomes (35, 36). Recently, we have shown that the metabolism of odd-numbered unsaturated fatty acids does not go through the isomerase pathway (31). The reason is that cis-5-enoyl-CoA cannot be metabolized further to cis-3-enoyl-CoA. Instead, these unsaturated fatty acids are metabolized by a NADPH-dependent reduction of 5-enoyl-CoA. The function of the isomerase appears to be in the net isomerization of trans-2-enoyl-CoA to cis-3- and trans-3-enoyl-CoA, a reversal of the classical concept. Overall, both the activities of epimerase and isomerase are actually involved in the metabolism of saturated fatty acids in the reverse function to what was originally proposed.

Do these pathways, i.e., the incomplete oxidation, epimerization of 3-hydroxy fatty acids, and the isomerization, occur under physiological conditions in vivo? The metabolites found in the urine support the occurrence of these pathways. Urine from rats and humans contains 3-hydroxydicarboxylic acids, which are derived from the ω-oxidation of 3-hydroxy fatty acids (8, 9). Preliminary data of urinary 3-hydroxydicarboxylic acid from rats show the same degree of racemization as the present data. In addition, cis-3- and trans-3-dicarboxylic acids
Incomplete Fatty Acid Oxidation

exist in the urine of humans and rats (24). Originally, we thought that these metabolites were derived from the β-oxidation of cis-5 and trans-5 precursors. Since a cis-5-monocarboxylic and -dicarboxylic acids are not converted to a cis-3 product, the likely metabolic origins of these compounds are from the incomplete oxidation of saturated fatty acids.

REFERENCES