Breakdown of 2-Hydroxylated Straight Chain Fatty Acids via Peroxisomal 2-Hydroxyphytanoyl-CoA Lyase

A REVISED PATHWAY FOR THE α-OXIDATION OF STRAIGHT CHAIN FATTY ACIDS*

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2-Hydroxyfatty acids, constituents of brain cerebroside and sulfatides, were previously reported to be degraded by an α-oxidation system, generating fatty acids shortened by one carbon atom. In the current study we used labeled and unlabeled 2-hydroxyoctadecanoic acid to reinvestigate the degradation of this class of lipids. Both in intact and broken cell systems formate was identified as a main reaction product. Furthermore, the generation of an n – 1 aldehyde was demonstrated. In permeabilized rat hepatocytes and liver homogenates, studies on cofactor requirements revealed a dependence on ATP, CoA, Mg2+, thiamine pyrophosphate, and NAD+. Together with subcellular fractionation data and studies on recombinant enzymes, this led to the following picture. In a first step, the 2-hydroxyfatty acid is activated to an acyl-CoA; subsequently, the 2-hydroxy fatty acyl-CoA is cleaved by 2-hydroxyphytanoyl-CoA lyase, to formyl-CoA and an n – 1 aldehyde. The severe inhibition of formate generation by oxythiamin treatment of intact fibroblasts indicates that cleavage was apparently independent of the preceding formation of an α-keto fatty acid, found only in small amounts, was formed as an intermediate (5). α-Oxidation of straight chain fatty acids has also been studied in plants, and the formation of an n – 1 aldehyde was reported by several authors (6–8). α-Oxidation has also been described in yeast (9) and in protozoa (10).

In 1964 Levis and Mead (4) reported for the first time the existence of an α-oxidation system for the degradation of the C20 to C26 straight chain fatty acids of rat brain sphingolipids. It was postulated that this pathway would consist of two steps, generating first 2-hydroxy even-numbered fatty acids, and subsequently odd-numbered fatty acids one carbon atom shorter. Later, it was reported that in rat brain the decarboxylation reaction was performed by a microsomal enzyme and that a 2-keto fatty acid, found only in small amounts, was formed as a primary product. α-Oxidation of straight chain fatty acids was also studied in plants, and the formation of α-keto fatty acids was associated with a high increase in myelination, whereas the percentage of odd-numbered fatty acids continues to increase up to the age of 10–15 years (3).

More recently, the involvement of peroxisomes in the α-oxidation of cerebronic acid (2-hydroxytetracosanoic acid) was described. The decarboxylation of 2-hydroxytetracosanoic acid was apparently independent of the preceding formation of an acyl-CoA and was supposed to be distinct from the α-oxidation of 3-methyl-branched fatty acids such as phytanic acid (11). The latter pathway is currently thought to proceed as follows; 1) activation to a CoA ester, 2) hydroxylation of carbon 2 by phytanoyl-CoA hydroxylase (PAHX), and 3) cleavage of the hydroxylated CoA-ester by 2-hydroxyphytanoyl-CoA lyase (2-HPCL) to formyl-CoA (12) and a 2-methyl-branched fatty aldehyde (13) in a TPP-dependent manner (14). Both PAHX and 2-HPCL are peroxisomal enzymes.

According to our data PAHX does not act on straight chain fatty acids or their CoA esters (15, 16) and, hence, cannot be involved in the formation of 2-hydroxyfatty acids; others claim, however, that PAHX can hydroxylate straight chain acyl-CoAs.
α-Oxidation of Straight Chain Fatty Acids

(17). Regardless of this discrepancy, a recently described fatty acid 2-hydroxylase, highly abundant in brain and encoded by the FA2H gene (18), is likely responsible for the formation of 2-hydroxyfatty acids in man.

The current study was undertaken to elucidate the degradation of 2-hydroxyfatty acids and to highlight a possible role of 2-HPCL in this process. Hereby, we made use of 2-hydroxyoctadecanoic acid, labeled and unlabeled, and its unlabeled CoA ester. These substrates are easier to synthesize and manipulate than the more abundant very long chain 2-hydroxyfatty acids. Although 2-hydroxyoctadecanoic acid is a less abundant 2-hydroxyfatty acid, it forms a more than negligible fraction of the 2-hydroxyfatty acids in brain cerebrospines (4% in newborn, 1% in adult brain) and sulfatides (15 and 1%, respectively) (3).

EXPERIMENTAL PROCEDURES

Fatty Acids and Derivatives—[1-14C]Hexadecanoic acid was from PerkinElmer Life Sciences, 2-Hydroxyhexadecanoic and 2-hydroxyeicosanoic acid were from Larodon. 2-Reto-octanoic acid was obtained from Sisco Research Laboratories. Most fatty acid derivatives have been described before: 2-hydroxy-3-methyl[1-14C]hexadecanoic acid, labeled and unlabeled 3-methylhexadecanoic acid, 3-methylhexadecanoyl-CoA, and 2-hydroxy-3-methylhexadecanoyl-CoA (19); 2-methylhexadecanoic acid and 2-methylhexadecanoyl-CoA (20); 3-hydroxy-3-methylhexadecanoyl-CoA (14); 3-hydroxy-2-methylhexadecanoyl-CoA (21); tetradecanoyl, 2-methylpentadecanoyl, and heptadecanoyl (13).

2-Hydroxyoctadecanoic acid was purchased from Larodon or synthesized as follows (adapted from Sandhir et al. (22)). Stearic acid treated with PBr3 was brominated in dry dichloromethane in a closed screw-capped thick-wall vial for 72 h at 70 °C (Hell-Volhard-Zelinski reaction; molar ratio acid/PBr3/Br2, 1/12/36). After removal of the solvent, water was carefully added to hydrolyze the 2-bromo-octadecanoyl bromide, and the 2-bromooctadecanoic acid (Rf 0.58; Silica gel 60 F254 plates; ethylene/acetone/H2O; Rf 0.59) was extracted into diethyl ether. The bromo group was converted to a hydroxyl group in two steps. The dried diethyl ether extract was dissolved in acetic acid containing potassium acetate and placed under reflux at 120 °C for 2 h. After dilution with water, the 2-acetoxyoctadecanoic acid was extracted into diethyl ether, dried, and hydrolyzed in 1.5 M NaOH/methanol (15/85, v/v) for 2 h under reflux. The mixture was acidified, and the formed 2-hydroxyoctadecanoic acid was extracted into diethyl ether and further purified by preparative TLC (Rf 0.14). Overall yield was 35%. 2-Hydroxy[1-14C]octadecanoyl acid and 2-hydroxyoctadecanoic acid were synthesized in a similar manner, starting from [1-14C]octadecanoyl acid (Moravek Biochemicals, Inc.) and nonadecanoic acid (Fluka), respectively. The CoA esters of 2-OH-FA were prepared by transesterification of their thiopeol ester, prepared from the corresponding acid (3-methylhexadecanoyl acid, 0.5 M NaHCO3/hydroxyethanethiol/0.26 M substrate (2-hydroxy[1-14C]octadecanoyl acid, 2-hydroxy-3-methyl[1-14C]hexadecanoyl-CoA, 2-hydroxy-3-methyl[1-14C]hexadecanoic acid, [1-14C]hexadecanoyl-CoA, or [1-14C]hexadecanoic acid) for the competition experiments a substrate concentration of 10 μM was used, and a 50 μM concentration of a related compound was added. When using unlabeled substrates (2-hydroxyoctadecanoyl-CoA, 2-hydroxy-3-methylhexadecanoyl-CoA), 2-HPCL activity was quantified by measuring [14C]formate and 14C-labeled acid-soluble material as determined above.)

Enzyme Activity Measurements—The production of acyl-CoA esters was measured in 20 mM Hepes-NaOH, pH 7.2, 12.5 μM BSA, 4 mM ATP, 0.5 mM CoA, and 50 μM 14C-labeled substrate (2-hydroxy[1-14C]octadecanoic acid). Reactions were terminated by the addition of 2 ml of isopropanol/0.1 N HCl (1:1, v/v); fatty acids were extracted with 4 ml of heptane, and an aliquot of the water layer containing the CoA-esters was counted in a liquid scintillation counter (PerkinElmer Life Sciences).

When using 1-14C-labeled substrates 2-hydroxyhexanoyl-CoA lyase activity was quantified by measuring [14C]formylglycine (23) since the primary 2-HPCL product, [14C]formylglycine, is quickly hydrolyzed to formate (12). Incubations (37 °C) were performed in a final volume of 250 ml containing 50 mM Tris-HCl, pH 7.5, 6.6 mM BSA, 0.8 mM MgCl2, and 20 μM TPP (referred to as standard conditions) with 40 μM substrate (2-hydroxy[1-14C]octadecanoyl acid, 2-hydroxy-3-methyl[1-14C]hexadecanoyl-CoA, 2-hydroxy-3-methyl[1-14C]hexadecanoic acid, [1-14C]hexadecanoyl-CoA, or [1-14C]hexadecanoic acid). The competition experiments a substrate concentration of 10 μM was used, and a 50 μM concentration of a related compound was added. When using unlabeled substrates (2-hydroxyoctadecanoyl-CoA, 2-hydroxy-3-methylhexadecanoyl-CoA), 2-HPCL activity was quantified by measuring the formation of the n + 1 aldehyde.

Lipid Analysis—The identification of the CoA esters was based on the formation of fluorescent acyl etheno-CoA derivatives by bromoacetaldelyde (prepared by refluxing bromodimethylacetal under acidic conditions and brought to pH 4.6 with sodium acetate, up to a final concentration of 100 mM acetate) (adapted from Larson and Graham (33)). Briefly, standard incubations (250 μl final volume) containing 50 mM substrate were stopped by the addition of 25 μl of 1 N H2SO4. After the addition of 25 nmol of the appropriate internal standard, the samples were extracted with 1.2 ml of isopropanol/heptane (4/1, v/v), and the upper phase was evaporated under N2. The residue was reconstituted in 100 μl of water and transferred to a derivatization vial. Bromoacetaldelyde reagent (200 μl; ~250 mM, pH 4.6) was added, the samples were kept in the dark, placed at 80 °C for 15 min, and immediately afterward put on ice. An aliquot of the derivatization mixture was injected onto a C18 column (Symmetry; 150 × 4.6 mm; 5 μm; 100 Å, Waters) on a Waters 1525 HPLC. The acyl-CoA esters were eluted with a gradient of acetonitrile in 0.25 mM ammonium acetate buffer, pH 5.0: linear gradient 10—66%, 15 min; linear gradient 66—80%, 2 min; isocratic 80%, 2 min; linear gradient 80—10%, 2 min; isocratic 10%, 6 min. Detection was performed on a Waters 2475 Fluorescence detector (excitation 230 nm; emission 420 nm).
TABLE I

Oxidation of 2-hydroxyoctadecanoic acid in intact cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Activity CO₂</th>
<th>CO₃₂ Formate</th>
<th>ASM (CO₂ + formate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact mouse hepatocytes (n = 2)</td>
<td>nmol/10⁸ cells/min</td>
<td>6.36</td>
<td>0.67</td>
</tr>
<tr>
<td>Confident mouse hepatocytes (n = 3)</td>
<td>nmol/mg protein/6 h</td>
<td>2.68 ± 0.48</td>
<td>0.11 ± 0.10</td>
</tr>
<tr>
<td>Confident fibroblasts (n = 3)</td>
<td>nmol/mg protein/24 h</td>
<td>2.60 ± 0.56</td>
<td>17.43 ± 4.09</td>
</tr>
<tr>
<td>Confident C6 glial cells (n = 2)</td>
<td>nmol/mg protein/24 h</td>
<td>0.01</td>
<td>5.49</td>
</tr>
</tbody>
</table>

FIG. 1. Breakdown products of 2-hydroxy[1-¹⁴C]octadecanoic acid, 3-methyl[1-¹⁴C]hexadecanoic acid, and ¹⁴C]hexadecanoic acid in isolated intact rat hepatocytes. Isolated rat hepatocytes were incubated with the indicated fatty acids (200 μM for 2-hydroxy[1-¹⁴C]octadecanoic acid and 3-methyl[1-¹⁴C]hexadecanoic acid, 50 μM for ¹⁴C]hexadecanoic acid) in the presence of increasing concentrations of unlabeled formate. Incubations were stopped after 10 min with HClO₄, and the amount of ¹⁴CO₂ (white bars), [¹⁴C]formate (black bars), and [¹⁴C] acid-soluble material (gray bars) was measured.

TABLE II

Oxidation of 2-hydroxyoctadecanoic acid in human and murine fibroblasts

<table>
<thead>
<tr>
<th>CO₂ (nmol/mg protein/24 h)</th>
<th>Formate (nmol/mg protein/24 h)</th>
<th>CO₂ + Formate (nmol/mg protein/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control fibroblasts (n = 3)</td>
<td>2.60 ± 0.56</td>
<td>17.43 ± 4.09</td>
</tr>
<tr>
<td>MADD fibroblasts (n = 1)</td>
<td>2.60</td>
<td>17.54</td>
</tr>
<tr>
<td>MCAD fibroblasts (n = 1)</td>
<td>2.70</td>
<td>13.98</td>
</tr>
<tr>
<td>X-ALD fibroblasts (n = 3)</td>
<td>3.13 ± 1.30</td>
<td>19.90 ± 2.91</td>
</tr>
<tr>
<td>RCDP fibroblasts (n = 1)</td>
<td>0.67</td>
<td>16.10</td>
</tr>
<tr>
<td>Zellweger fibroblasts (n = 1)</td>
<td>1.05</td>
<td>10.34</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pex5⁻/⁻ fibroblasts (n = 1)</td>
<td>2.35</td>
<td>9.61</td>
</tr>
<tr>
<td>Pex5⁻/⁻ fibroblasts (n = 1)</td>
<td>1.36</td>
<td>4.11</td>
</tr>
</tbody>
</table>

For the identification and analysis of aldehydes, reactions were stopped with 125 μl of 2 N HCl. After the addition of 2.5 nmol of the appropriate internal standard and 125 μl of a solution of 2,4-dinitrophenylhydrazine (3.75 mg in 5 ml of 2 N HCl), the samples were incubated for 30 min at 50 °C (in this and subsequent steps samples were protected from light). After adding 0.5 ml of methanol, the hydrazones were extracted into 2 ml of hexane. 1.75 ml of the upper phase was evaporated, the residue was dissolved in 88 μl of acetonitrile, and 50 μl (1.25 nmol of internal standard) was injected on a C18 column (Symmetry 150 × 4.6 mm; 5 μm, 100 Å Waters; Waters 1525 HPLC system) eluted with acetonitrile under isocratic conditions. Detection was done at 360 nm (Waters 484 tunable absorbance detector).

Generation and Purification of Recombinant Hs 2-HPCL—Human 2-HPCL cDNA was amplified from a human liver cDNA library with primers Hs 2-HPCL-F (5'-CCGGGATCCGGATCCTACAGTCATTAGCGG- GTC) and Hs 2-HPCL-R (5'-AAACGATGATGGGCTATCTCACTCATCATCACTGAGG, and Hs 2-HPCL-H2, 5'-ATCCGGATGATGGGATGTGATGATGATGATGATGATGCCCATCCTGGTAC) were allowed to hybridize to generate a small linker. The resulting adaptor sequence was cloned between the KpnI-BamHI sites of yVF3 (yVF7), and S. cerevisiae CBS80 cells, transformed with yVF7, were grown for 18 h in 400 ml of Sc-ura containing 0.5% (w/v) glucose. The fusion protein was purified from cell lysate (prepared as described before in 8 ml of lysis buffer) on nickel nitrilotriacetic acid-agarose essentially as described for the purification of phytanoyl-CoA hydroxylase (16). Analysis of the purified fraction by SDS-PAGE (12% polyacrylamide, w/v) and subsequent immuno blotting with anti-His antibody (Clontech) revealed one single polyhistidine-tagged protein with a molecular mass of ~63 kDa. The yield was 77–400 μg of protein with a specific activity of about 26 milliunits/mg of protein (substrate, 40 μM 2-hydroxy-3-methyl[1-¹⁴C]hexadecanoic acid).
RESULTS

Degradation of 2-OH-FA in Intact and Permeabilized Cells; Indication for a Role of Peroxisomes and 2-HPCL

Analysis of the medium of control human fibroblasts and rat C6 glial cells incubated with 2-hydroxy[1-14C]octadecanoic acid for different possible labeled degradation products revealed formate as the major acid-soluble oxidation product (Table I). In intact isolated rat and cultured mouse hepatocytes, on the other hand, the amount of acid-soluble material was markedly higher than the amount of formate generated (Table I), suggesting that in these cells, apart from formate, another metabolite was generated as the primary reaction product or that formate/CO2 was subsequently converted into another acid-soluble metabolite. When intact rat hepatocytes were incubated in the presence of increasing concentrations of unlabeled formate, the production of 14CO2 from 2-hydroxy[1-14C]octadecanoic acid decreased dramatically (Fig. 1). This decrease was accompanied by a compensatory increase in the generation of [14C]formate (Fig. 1), as was shown earlier for 3-methylhexadecanoic acid (Ref. 35; see also Fig. 1), whereas the \( \alpha \)-oxidation of straight chain fatty acids was unaffected throughout the whole formate concentration range (Fig. 1). Hence, these results support the contention that 2-hydroxyfatty acids are shortened by a process resembling the \( \alpha \)-oxidation of phytanic acid whereby not CO2, but formyl-CoA/formate is the primary oxidation product that is subsequently converted into CO2 and possibly other metabolites.

To obtain a first insight in the organelle(s)/enzymes involved, the oxidation of 2-hydroxy[1-14C]octadecanoic acid was analyzed in fibroblasts from patients affected with various fatty acid oxidation disorders. In patients with the mitochondrial fatty acid oxidation disorders medium chain acyl-CoA dehydrogenase deficiency or multiple acyl-CoA dehydrogenase deficiency, characterized by a deficiency in the medium chain or

### Table III

Cofactor requirements for oxidation of 2-hydroxyoctadecanoic acid in permeabilized hepatocytes

<table>
<thead>
<tr>
<th>Additions</th>
<th>CO2</th>
<th>Formate</th>
<th>CO2 + formate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>13.26</td>
<td>11.83</td>
<td>11.83</td>
</tr>
<tr>
<td>ATP, Mg^{2+}, TPP</td>
<td>100.08</td>
<td>16.49</td>
<td>30.22</td>
</tr>
<tr>
<td>CoA, Mg^{2+}, TPP</td>
<td>7.63</td>
<td>6.24</td>
<td>6.64</td>
</tr>
<tr>
<td>ATP, CoA, Mg^{2+}</td>
<td>101.71</td>
<td>101.71</td>
<td>71.56</td>
</tr>
<tr>
<td>ATP, CoA, Mg^{2+}, TPP</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ATP, CoA, Mg^{2+}, TPP, NAD^+</td>
<td>141.78</td>
<td>118.23</td>
<td>123.64</td>
</tr>
<tr>
<td>ATP, CoA, Mg^{2+}, NAD^+</td>
<td>151.11</td>
<td>93.08</td>
<td></td>
</tr>
<tr>
<td>NAD^+</td>
<td>70.28</td>
<td>78.45</td>
<td>39.35</td>
</tr>
</tbody>
</table>

FIG. 2. Identification of 2-hydroxyoctadecanoyl-CoA (A–C) and heptadecanal (D–F) in incubations of rat liver homogenate with 2-hydroxyoctadecanoic acid and 2-hydroxyoctadecanoyl-CoA, respectively. HPLC analysis of acyl etheno-CoA esters (left panel) and aldehydes (right panel) formed during incubations of rat liver homogenate with 2-hydroxyoctadecanoic acid (100 \( \mu \)M) and 2-hydroxyoctadecanoyl-CoA (40 \( \mu \)M), respectively. Homogenates were incubated for 0 min (A and D) and 10 min (B and E), and reaction products were derivatized after the addition of the appropriate internal standard (IS) as described under “Experimental Procedures.” Elution of standards is shown in C and F.
multiple acyl-CoA dehydrogenases, respectively, no decrease in oxidation rates was observed (Table II). Hence, these data exclude a significant role of these mitochondrial dehydrogenases in the degradation of 2-hydroxylated straight chain fatty acids. Furthermore the oxidation rates of the 2-hydroxyfatty acids were not reduced either in cells from X-linked adrenoleukodystrophy patients, characterized by a deficiency in the peroxisomal activation/import of very long chain fatty acids, or in those from a rhizomelic chondrodysplasia punctata type 1 patient with a proven deficiency of Pex7p, the import receptor for peroxisomal matrix proteins containing a peroxisomal targeting signal-2 (Table II). Only in fibroblasts from a patient with Zellweger syndrome, with a demonstrated defect in the peroxisomal import of proteins containing a peroxisomal targeting signal-1, was a decrease (43%) in oxidation rate noticed. A similar decrease was seen in fibroblasts from Pex5/H11002/H11002 mice, a mouse model for Zellweger syndrome (Table II). These data suggest that peroxisomes are important and that peroxisomal enzymes with a peroxisomal targeting signal are probably involved in the degradation of 2-OH fatty acids. The addition to the culture medium of etomoxir or tetradecylglycidic acid, inhibitors of carnitine palmitoyl-CoA transferase and, hence, of fatty acyl-CoA entry into the mitochondria, did not significantly affect the oxidation of 2-hydroxy[1-14C]octadecanoic acid (results not shown), another indication that mitochondria are likely not involved.

To study the necessary cofactors, rat hepatocytes, permeabilized with α-toxin to deplete the cytosolic cofactors (25), were incubated with different sets of cofactors. In these hepatocytes the oxidation of 2-hydroxy[1-14C]octadecanoic acid was strictly dependent on ATP, Mg2+, and CoA and was enhanced by TPP (Table III). Extra addition of NAD+ resulted in a further 20% increase of total oxidation.

The dependence on TPP was further investigated by culturing fibroblasts and C6 glial cells in the presence of the thiamine antimitabolite oxythiamin. The addition of this compound (1 mM) to the culture medium for several cell divisions reduced the overall oxidation rates of 2-hydroxyoctadecanoic acid by 44, 39, and 60% in control fibroblasts, rhizomelic chondrodysplasia punctata fibroblasts, and C6 glial cells, respectively. These data confirm the involvement of a TPP-dependent enzyme and are similar to the effect of oxythiamin on α-oxidation of 3-methylhexadecanoic acid. The addition of oxythiamin had no effect on the β-oxidation of long chain fatty acids.2

The finding that ATP and CoA are essential suggests that the oxidation of 2-hydroxyfatty acids involves an activation reaction. The dependence of the pathway on TPP points to a reaction similar to that of the TPP-dependent cleavage of 2-hydroxy-3-methylacyl-CoA esters during the α-oxidation of 3-methyl-branched fatty acids.

Degradation of 2-OH-FA in Homogenates; Identification of Intermediates and End Products, Subcellular Localization, and Enzymes Involved

In rat liver homogenates similar results as those in permeabilized hepatocytes were obtained (results not shown): breakdown of 2-hydroxy[1-14C]octadecanoic acid in whole rat liver homogenates was dependent on the presence of CoA, ATP, Mg2+, and TPP. Although the oxidation rates in the presence of ADP were about 65% of those obtained with ATP, no oxidation was detected upon the addition of GTP or AMP. Furthermore, CoA could not be replaced by desulfo-CoA or dephospho-CoA.

In homogenates the main product was formate (90% of total products). The addition of KCN did not affect the oxidation.

Identification of Intermediates and End Products of 2-Hydroxyoctadecanoic Acid Oxidation

When rat liver homogenates were incubated with 2-hydroxy[1-14C]octadecanoic acid in the presence of ATP, CoA, and Mg2+, labeled acyl-CoA esters were generated. HPLC analysis of the etheno-CoA esters revealed a peak with significant fluorescence at 18.7 min coeluting with the 2-hydroxyoctadecanoyl-CoA standard (Fig. 2, A–C). The amount of 2-hydroxyoctadecanoyl-CoA (0.630 nmol/mg of protein/min) agreed well with the total amount of labeled CoA ester formed, based on extraction procedures (0.746 nmol/mg of protein/min) (mean of two measurements). Additionally, the generation of a 2-hydroxyoctadecanoyl-CoA intermediate was also demonstrated in rat brain homogenates and in lysates from rat C6 glial cells.

**Fig. 4.** Subcellular distribution of acyl-CoA synthetase activity (Fractionation A) and the production of heptadecanal from 2-hydroxyoctadecanoyl-CoA (Fractionation B) in rat liver. In two separate experiments fresh liver homogenates were fractionated by differential centrifugation into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and cytosolic (S) fractions. Fractionation A, fractions were incubated with 50 μM 2-hydroxy[1-14C]octadecanoic acid, and the production of the CoA ester (synthetase) was measured as described under “Experimental Procedures.” Fractionation B, the production of heptadecanal from 2-hydroxyoctadecanoyl-CoA (lyase) was measured in each fraction as described under “Experimental Procedures.” Marker enzymes were determined in each fraction: carboxylesterase (endoplasmic reticulum), glutamate dehydrogenase (GDH; mitochondria), catalase (peroxisomal matrix), and lactate dehydrogenase (LDH; cytosol). Results are expressed as relative specific activities versus percentage of total protein. Relative specific activity is defined as the percentage of total recovered activity present in a particular fraction divided by the corresponding percentage of protein. Recovery for synthetase activity was 89%; recovery for heptadecanal production was 109%; recoveries for marker enzymes were between 86 and 119%.
In the presence of Mg$^{2+}$ and TPP, heptadecanal was formed by rat liver homogenates (Fig. 2, D–F) incubated with 2-hydroxyoctadecanoyl-CoA at rates of 0.014 nmol/mg of protein/min ($n = 2$). Overall, these data indicate that 2-hydroxyacyl-CoA esters are intermediates in the degradation of 2-hydroxy straight chain fatty acids. Unfortunately, due to the presence of interfering peaks, the formation of the $n - 1$ aldehyde could not be demonstrated in incubations with brain homogenate.

Characterization of the acyl-CoA Synthetase, Catalyzing the Formation of 2-Hydroxyacyl-CoA Esters—In rat liver homogenates the activation of 2-hydroxyoctadecanoic acid was shown to be linear for up to 3 min (Fig. 3A). The formation of 2-hydroxyoctadecanoyl-CoA reached an optimum at a substrate/albumin ratio ($v$) of 4 (Fig. 3B). Measuring the generation of 2-hydroxyoctadecanoyl-CoA at increasing substrate concentrations (at $v = 4$) resulted in a plateau from 100 μM onward; transformation of the data according to Lineweaver-Burk allowed the calculation of an apparent $K_m$ of 19.5 μM (Fig. 3, C–D). Subsequent experiments in rat liver homogenates and subcellular fractions were, therefore, performed at a substrate concentration of 50 μM and a $v$ ratio of 4 and were terminated after 3 min. For measurement of synthetase activity in lysates of rat C6 glial cells, optimum conditions were 100 μM substrate, a $v$ ratio of 2, and an incubation time of 10 min.

The activation of 2-hydroxyoctadecanoic acid in subcellular fractions of rat liver showed a bimodal distribution coinciding with the light mitochondrial fraction, enriched in peroxisomes, and the microsomal fraction (Fig. 4, Fractionation A). In the light mitochondrial fraction the activity appeared to be predominantly membrane-associated. Further separation of the light mitochondrial fraction on a Nycodenz gradient revealed an association with peroxisomes (data not shown). In rat brain a bimodal distribution was observed as well, the microsomal localization being most prominent (results not shown).

To further characterize the acyl-CoA synthetase responsible for the activation of 2-hydroxy straight chain fatty acids, incubations of lysates from C6 glial cells with 2-hydroxy[1-14C]octadecanoic acid were performed with and without the addition of a range of unlabeled fatty acids. Whereas 3-methylhexadecanoic acid, 2-methylhexadecanoic acid, eicosatetraenoic, and tetracosanoic acid had no major inhibitory effect on the activation of 2-hydroxyoctadecanoic acid, hexadecanoic acid and octadecanoic acid markedly reduced the activation rates (results not shown), indicating that the substrate spectrum of the activating enzyme involved certainly covers long chain fatty acids.

Identification of 2-HPCL as the Enzyme Catalyzing the Cleavage Reaction—Investigation of the formation of heptadecanal from 2-hydroxyoctadecanoyl-CoA in subcellular fractions of rat liver indicated that the responsible enzyme has a peroxisomal localization (Fig. 4, Fractionation B). Both this subcellular distribution and the marked thiamine dependence point toward 2-HPCL as the enzyme catalyzing the cleavage reaction.

In the presence of ATP, CoA, Mg$^{2+}$, and TPP, incubations of the purified recombinant polyhistidine-fused human 2-HPCL with 2-hydroxyoctadecanoyl-CoA yielded heptadecanal, in analogy with the formation of 2-methylpentadecanal from 2-hydroxy-3-methylhexadecanoyl-CoA. The reaction kinetics of recombinant 2-HPCL toward these substrates were compared by measuring the generation of 2-methylpentadecanal and heptadecanal. The cleavage rate of the branched substrate was linear for up to 10 min, whereas for the straight chain substrate it was linear for up to at least 30 min (results not shown). At increasing substrate concentrations a plateau from 40 μM onward was reached for 2-hydroxy-3-methylhexadecanoyl-CoA, whereas for 2-hydroxyoctadecanoyl-CoA a plateau was reached from 20 μM onward (Fig. 5, A and B); apparent $K_m$ values of 15.8 and 6.3 μM, respectively, could be calculated. Furthermore, the amount of aldehyde generated with both substrates increased linearly with the amount of recombinant protein up to at least 10 μg.

The substrate spectrum of recombinant, polyhistidine-fused human 2-HPCL was further investigated using labeled substrates. Incubation of the recombinant 2-HPCL with 2-hydroxy-3-methyl[1-14C]hexadecanoyl-CoA under standard conditions resulted in production of [14C]formyl-CoA/[14C]formate at rates of 26.06 ± 1.17 milliunits/mg of protein (mean ± S.E.; $n = 4$), but the lyase showed no activity toward [1-14C]-labeled 3-methylhexadecanoyl-CoA, hexadecanoyl-CoA, 3-hydroxyhexadecanoyl-CoA, octadecanoyl-CoA, or toward 2-hydroxy-3-methylhexadecanoic acid, 3-methylhexadecanoic acid, hexadecanoic acid, and 2-hydroxyhexadecanoic acid. Furthermore, the production of [14C]formyl-CoA/[14C]formate from 2-hydroxy-3-methyl[1-14C]hexadecanoyl-CoA was not reduced in the presence of 2-keto-octanoate, 2-hydroxyhexadecanoic acid, 2-methylhexadecanoyl-CoA, 2-methylhexadecanoic acid, 3-methylhexadecanoyl-CoA, 3-hydroxy-3-methylhexadecanoyl-CoA, or 3-hydroxy-2-methylhexadecanoyl-CoA (Fig. 6). The addition of 50 μM 2-hydroxyhexadecanoyl-CoA or 2-hydroxyoctadecanoyl-CoA to incubations with 10 μM 2-hydroxy-3-methyl[1-14C]hexadecanoyl-CoA, however, reduced the cleavage rates to a major extent (Fig. 6), indicating that 2-hydroxy straight chain acyl-CoA esters compete with 2-hydroxy-3-methyl-branched acyl-CoAs for cleavage by 2-HPCL.

**DISCUSSION**

In the current study 2-hydroxyoctadecanoic acid was used as a substrate to document the degradation of 2-hydroxy straight
Chain fatty acids. This compound is less abundant than 2-hydroxytetraocsoanoic acid (cerebronic acid) in brain cerebrosides and sulfatides (3), but it offers advantages over the very long chain substrates both in synthesis and solubility. The fact that 2-hydroxy[1,14C]octadecanoic acid gives rise to the generation of a 2-keto fatty acid, formed as an intermediate via the inhibition by oxythiamin suggests that the main pathway, that 2-hydroxyfatty acids can be degraded by other pathways, not all details are yet available. The naturally occurring 2-hydroxyfatty acids possess a D-configuration, but so far racemic 2-hydroxyfatty acids are activated by a very long chain acyl-CoA synthetase. However, because it is well known that the substrate spectra of long chain and very long chain acyl-CoA synthetases overlap (39, 40), no definitive conclusions can be drawn as to the specific enzyme involved. Moreover, it might well be that the chain length of the substrate determines the involved isozyme. Hence, long chain 2-hydroxyfatty acids (e.g. 2-hydroxyoctadecanoic acid) might be activated by a long chain acyl-CoA synthetase and very long chain 2-hydroxyfatty acids might be activated by a very long chain acyl-CoA synthetase.

The contention of 2-HPCL being involved in the degradation of 2-hydroxyfatty acids was strengthened by the increased relative specific activities of heptadecanal formation from 2-hydroxyoctadecanoyl-CoA in subcellular fractions from rat liver, where the activity is associated both with the peroxisomal membrane and with the endoplasmic reticulum. This distribution together with the fact that, among the fatty acids tested, only hexadecanoic acid and octadecanoic acid competitively inhibited the activation of 2-hydroxyoctadecanoic acid, point toward the long chain acyl-CoA synthetase as the activating enzyme. However, because it is well known that the substrate spectra of long chain and very long chain acyl-CoA synthetases overlap (39, 40), no definitive conclusions can be drawn as to the specific enzyme involved. Moreover, it might well be that the chain length of the substrate determines the involved isozyme. Hence, long chain 2-hydroxyfatty acids (e.g. 2-hydroxyoctadecanoic acid) might be activated by a long chain acyl-CoA synthetase and very long chain 2-hydroxyfatty acids might be activated by a very long chain acyl-CoA synthetase.

The fact that 2-hydroxyfatty acid degradation is decreased by only ~50% in fibroblasts from Zellweger patients and Pex5−/− mice, an animal model for Zellweger syndrome (23), might suggest that 2-HPCL, a peroxisomal matrix enzyme (14), remains partially active in the cytosol under conditions where peroxisomal protein import is deficient. This is in contrast with most other peroxisomal matrix enzymes, which are labile in the

![Diagram](http://www.jbc.org/figs/fig6.png)
cytosol when their import is impaired, but in agreement with our previous measurements of 2-HPCL activity in liver homogenates of Zellweger patients and Pex5−/− mice using 2-hydroxy-3-methylhexadecanoyl-CoA as substrate (41). Whether the partial impairment of 2-hydroxyfatty acid degradation leads to an accumulation of 2-hydroxyfatty acids in tissues from Zellweger patients is unknown. Moderate increases in cerebrosides and/or gangliosides have been described in fibroblasts or CHO cells lacking peroxisomes, but their 2-hydroxyfatty acid content was not documented (42, 43). When considering the identification of this second substrate for 2-hydroxyphytanoyl-CoA lyase, one could expect that an isolated deficiency of 2-HPCL, although hitherto not identified, might lead not only to an impaired ω-oxidation of phytanic acid but also to an accumulation of 2-hydroxyfatty acids.

The identification of 2-HPCL as the cleavage enzyme in the degradation of 2-hydroxyfatty acids is also of interest for its reaction mechanism. It demonstrates that the methyl branch at position 3 is not necessary but that both the hydroxy group at position 2 and the CoA moiety are important. A 2-hydroxy carboxyl compound (instead of a 2-keto compound) is an unusual substrate for TPP-dependent enzymes. Only one other enzyme, N²-(2-carboxyethyl)-L-arginine synthase, catalyzing the condensation of L-arginine and D-glyceraldehyde in the presence of TPP (the first step in the biosynthesis of clavulanic acid), has been reported to show activity toward compounds with a hydroxy group at position 2 (44). In all TPP-dependent cleavage reactions described so far decarboxylation involves the activation of the C2-H of the thiazole ring of TPP to an intermediate carbanion. This is followed by a nucleophilic attack at the carbonyl atom of the substrate (carbon 2) (45). Most likely, the formation of a carbanion is also required for the cleavage of 2-hydroxy-(3-methyl)acyl-CoA esters by 2-HPCL. However, this carbanion will then attack carbon 1 of the substrate, which is highly reactive due to the presence of the thioester bond. Ultimately, this will lead to the formation of formyl-CoA and an n − 1 fatty aldehyde (Fig. 8).

The observation that extra addition of NAD⁺ to the incuba-
tion mixture resulted in a further 20% increase of total oxidation (Table III) might be explained by a diminished product inhibition through NADH-dependent dehydrogenation of the generated aldehyde. Fatty aldehyde dehydrogenase activity is found in peroxisomes and endoplasmic reticula (13, 46). It is currently unknown whether fatty aldehydes generated in the peroxisome are dehydrogenated exclusively by the peroxisomal dehydrogenase or also partially by the endoplasmic reticulum enzyme.

In summary, our present work shows that (the bulk of) 2-hydroxyfatty acids undergo an initial degradation that apparently shares three reactions (activation, cleavage of the C1-C2 bond, aldehyde dehydrogenation) with the \( \beta \)-oxidation sequence of 3-methyl-branched fatty acids, giving rise to an odd-numbered fatty acids, which can subsequently be degraded via \( \beta \)-oxidation. It is of interest to note that the second enzyme of the \( \alpha \)-oxidation sequence of 3-methyl-branched fatty acids (the peroxisomal PAHX, which hydroxylates 3-methyl-acyl-CoAs to 2-hydroxy-3-methyl-acyl-CoAs) is likely not involved in the synthesis of 2-hydroxy straight chain fatty acids (see the Introduction). One of the underlying reasons might be that an (imaginary) hydroxylation of straight chain acyl-CoAs by PAHX would lead to the immediate further breakdown of the 2-hydroxylated acyl-CoAs by 2-HPCL within the peroxisome.

To control the levels of 2-hydroxyfatty acids in brain cerebrosides and sulfatides, supposed to play a role in myelinization, a strategy relying on different sets of enzymes for their synthesis and degradation, located at different subcellular sites, might be more beneficial. Moreover, as was reported in older literature and recently discussed by Alderson et al. (18), hydroxylation of straight chain fatty acids might occur only after incorporation in sphingolipids, which would further rule out peroxisomes as a key player in this hydroxylation process. In this context it is of interest to note that 2-hydroxyoctadecanoic acid was hardly incorporated into complex lipids when given to intact cells.\(^3\) Overall, the \( \alpha \)-oxidation of straight chain fatty acids, as has been described especially for brain, appears to proceed as follows: 1) hydroxylation of the fatty acid by a fatty acid 2-hydroxylase (see the Introduction), 2) activation of the 2-hydroxyfatty acid to a 2-hydroxyacyl-CoA, 3) cleavage of

\(^3\) M. Sniekers, V. Foulon, P. P. Van Veldhoven, and M. Casteels, unpublished data.
the CoA ester into formyl-CoA and an \( n - 1 \) fatty aldehyde, and 4) dehydrogenation of the aldehyde to the corresponding odd-numbered fatty acid.

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Breakdown of 2-Hydroxylated Straight Chain Fatty Acids via Peroxisomal 2-Hydroxyphytanoyl-CoA Lyase: A REVISED PATHWAY FOR THE $\alpha$-OXIDATION OF STRAIGHT CHAIN FATTY ACIDS

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