Metabolism of 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid by kidney and liver peroxisomes

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Abstract 12(S)-Hydroperoxy-5,8,10,14-eicosatetraenoic acid (12(S)-HPETE) is one of the main products formed from arachidonic acid by animal lipoxygenases. It is usually reduced to the corresponding hydroxy acid, 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE) or it can serve as an intermediate in the biosynthesis of hepoxilins. The functions of 12(S)-HETE are incompletely understood but it has been proposed that it plays a role in the metastatic process of certain cancer cells, including Lewis lung carcinoma cells. 12(S)-HETE is metabolically degraded by β- or ω-oxidation. Indirect evidence has suggested involvement of peroxisomes in the β-oxidative degradation. The purpose of the present investigation was to study 12(S)-HETE metabolism by peroxisomes isolated from rat liver or kidney by sucrose density gradient centrifugation. The purity of subcellular fractions was determined by marker enzyme analyses and electron microscopy. 12(S)-HETE was quantitatively converted by liver or kidney peroxisomes into a single, diethyl ether-extractable metabolite. This was identified by gas–liquid chromatography–mass spectrometry as 8-hydroxyhexadecatrienoic acid. Lewis lung carcinoma cells also metabolized 12(S)-HETE to 8-hydroxyhexadecatrienoic acid.—**Wigren, J., H. Herbertsson, Ö. Tollbom, and S. Hammarström.** Metabolism of 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid by kidney and liver peroxisomes. *J. Lipid Res.* 1993. **34:** 625–631.

Supplementary key words β-oxidation • 12-HETE • 8-hydroxyhexadecatrienoic acid • Lewis lung carcinoma cells • lipoxygenase

12(S)-Hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE) is one of the main products formed from arachidonic acid by animal lipoxygenases (1, 2). It has chemotactic effects on polymorphonuclear leukocytes (PMNLs) (2) and may play a role in the metastatic process of certain cancer cells, including Lewis lung carcinoma cells (LLC) and B16 melanoma cells (3). The corresponding hydroperoxy acid, 12(S)-hydroperoxy-5,8,10,14-eicosatetraenoic acid—an intermediate in the formation of 12(S)-HETE, serves as an intermediate in the biosynthesis of hepoxilins (2). Recent studies have indicated the occurrence of stereo- and regiospecific binding sites for 12(S)-HETE in carcinoma cells (4).

12(S)-HETE is metabolized in several ways. 10,11-Dihydro-12(S)-HETE is formed in porcine PMNLs by a reductase (5). ω-Oxidation of 12(S)-HETE in human PMNLs gives rise to 12,20-dihydroxy-5,8,10,14-eicosatetraenoic acid (6). Human PMNL 5-lipoxygenase converts 12(S)-HETE to 5(S),12(S)-dihydroxy-6,8,10,14-eicosatetraenoic acid, a stereoisomer of leukotriene(LT)B_4 (7). In canine renal tubular epithelial cells (MDCK), 12(S)-HETE was converted to 8-hydroxyhexadecatrienoic acid (8-OH-16:3) (8). Results from experiments using inhibitors have suggested that mitochondrial β-oxidation is not involved in the synthesis of 8-OH-16:3. Based on this indirect evidence, it was proposed that the transformation of 12(S)-HETE to 8-OH-16:3 occurs in peroxisomes.

The purpose of the present investigation was to study 12(S)-HETE metabolism by peroxisomes isolated from liver or kidney by sucrose density gradient centrifugation. The purity of subcellular fractions was assessed by marker enzyme analyses and electron microscopy. 12(S)-HETE was efficiently converted by these peroxisomes into a single, diethyl ether-extractable metabolite. This was identified by gas–liquid chromatography–mass spectrometry as 1,2,3,4-tetranor-5,6-dihydro-12(S)-HETE, i.e., 8-hydroxyhexadecatrienoic acid (8-OH-16:3). The same product was also formed from 12(S)-HETE by Lewis lung carcinoma cells, a cell type in which 12(S)-HETE enhances the metastatic potential.

MATERIALS AND METHODS

Chemicals

Tris-HCl, imidazole, EDTA, di(2-ethylhexyl)-phthalate, HCl, and $MgCl_2$ were purchased from Merck (Darm-

Abbreviations: amu, atomic mass unit; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; E.C.L., equivalent chain length; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; 12(S)-HETE, 12(S)-hydroxy-5,8,10,14-(Z,Z,E,Z)-eicosatetraenoic acid; HPLC, high performance liquid chromatography; 8-OH-16:3, 8-hydroxyhexadecatrienoic acid; LLC, Lewis lung carcinoma; LT, leukotriene; MDCK, Madin-Darby canine kidney; PMNL, polymorphonuclear leukocyte; O-TMS, trimethylsilyl ether.

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stadt, Germany). ATP, CoA, and NAD^+ were Sigma Co. products (St. Louis, MO). 12(S)-Hydroxy-[5,6,8,9,11,12, 14,15-^{3}H(N)]eicosatetraenoic acid (178 Ci/mmol) was obtained from New England Nuclear (Boston, MA). 12(S)-HETE was a product of Cayman Chemicals (Ann Arbor, MI). Sucrose came from BDH Ltd., (Dorset, England). Fetal calf serum, Dulbecco's modified Eagle's medium (DMEM), streptomycin, and penicillin were Gibco products (Renfrewshire, Scotland).

Prepartion of peroxisomes from rat liver and kidney

Male Sprague-Dawley rats, purchased from ALAB (Sollentuna, Sweden), were fed with ordinary rat pellets (Lactamin AB, Stockholm, Sweden) supplemented with 2% (w/w) di(2-ethylhexyl)-phthalate for 7–14 days and fasted overnight prior to experimental use. Peroxisomes were prepared as described previously (9) with a few modifications. A 20% (w/v) homogenate in 0.25 M sucrose containing 3 mM imidazole, pH 7.4, 0.1% ethanol, and 1 mM EDTA was prepared. The homogenate was centrifuged at 3600 *g* for 10 min in a fixed-angle Beckman rotor (70 Ti); the resulting supernatant was centrifuged at 25,300 *g* for 20 min using the same rotor. The pellet from this step, termed the light mitochondrial fraction, was resuspended in 0.25 M sucrose and applied onto a linear sucrose gradient, built from 14 ml each of 34 and 55% sucrose solutions and supported by 9 ml of a 66% sucrose cushion at the bottom. The gradients were centrifuged at 35,800 *g* in a vertical Beckman rotor (JV-20) for 315 min and then divided into 2-ml fractions which were analyzed for marker enzyme distribution. Catalase was used as a marker enzyme for peroxisomes (10), succinate dehydrogenase for mitochondria (11), esterase for microsomes (12), and the Lowry method for protein measurements (13). Pure peroxisome fractions were combined and sedimented by centrifugation at 25,300 *g* for 15 min, suspended in 0.25 M sucrose and stored at −20°C. For electron microscopy (14), peroxisome suspensions were mixed with two parts of 6% glutaraldehyde in 0.2 M sucrose, pH 7.2, and kept at 4°C. They were then sedimented by centrifugation and post-fixed in 1% OsO_4/0.15 M sodium cacodylate-HCl buffer for 90 min at room temperature, dehydrated, and enclosed in Epon 812 for sectioning.

Cell culture

Lewis lung carcinoma cells (LLC), obtained from Dr. Gilbert Vaes or purchased from the American Tissue Culture Collection (ATCC), were grown in DMEM supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) heat-inactivated (30 min at 56°C) fetal calf serum. Cultures maintained at 37°C in a humidified atmosphere containing 10% (v/v) CO_2 were passaged by use of 0.54 mM EDTA in buffered isotonic saline. They were not used for more than 20 consecutive passages.

Incubations with peroxisomes

Peroxisomes, 1–2 mg protein, were suspended in 0.155 M Tris-HCl buffer, pH 8.0, containing 5 mM ATP, 2 mM CoA, 2 mM NAD^+, 10 mM $MgCl_2$, and 1 nM (12-(S)-[^{3}H]HETE, 178 Ci/mmol) in a total volume of 0.5 ml, and incubated at 37°C for 60 min. The reaction mixture was then acidified and extracted with diethyl ether. The extracts were washed with water, dried under argon gas, and redissolved in 100 μl of methanol–water–acetic acid 78:22:0.01 (v/v/v). In order to isolate larger amounts of metabolites, 31 μM 12(S)-[^{3}H]HETE of lower specific activity (2.6 Ci/mol) was incubated with peroxisomes and extracted as described above.

Incubations with Lewis lung carcinoma cells

LLC cells obtained in a tissue culture flask were washed twice with phosphate-buffered saline (PBS) and incubated (4×10^5 cells/ml) with 0.1 nM 12(S)-[^{3}H]HETE (178 Ci/mmol) in DMEM buffered with 10 mM HEPES and supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. The incubation was performed at 37°C for 30 min with gentle shaking. The incubation buffer was removed, acidified to pH 3, and extracted with diethyl ether.

HPLC

Lipid extracts after incubations with liver or kidney peroxisomes or from whole Lewis lung carcinoma cells were analyzed by isocratic reverse-phase HPLC at a flow rate of 1 ml/min, using an M-45 Waters pump and a 4.6 × 250 mm column containing 5 μm C_{18} Nucleosil. The mobile phase consisted of methanol–water–acetic acid 78:22:0.01 (v/v/v). Column effluents were mixed 1:2.5 (v/v) with Quickszint Flow 302 scintillation fluid prior to passing through a Berthold LB 506 radioactivity detector. For the isolation of metabolites for GLC–MS, a Hewlett-Packard model 1040 chromatograph with a built-in model 1090 diode array UV detector was used with the same column and solvent. Fractions were collected and assayed for radioactivity by mixing 10% of each fraction with Quicksafe A scintillation fluid and counting in an LKB model 1214 rack-beta liquid scintillation counter. Fractions containing radioactivity were evaporated to dryness using argon gas and dissolved in 50 μl methanol before derivatization. Isocratic chiral straight-phase HPLC analysis was also carried out on extracts from liver and kidney peroxisome incubations using a dinitrobenzoylphenyl glycine chiral phase HPLC column (250 × 4.6 mm, Supelco Inc., Bellafonte, PA) using a solvent system of hexane–2-propanol–acetic acid 996:3:1 (v/v/v) at a flow rate of 1.5 ml/min.

Gas–liquid chromatography–mass spectrometry (GLC–MS)

A Hewlett-Packard gas chromatograph, 5890A, connected to a mass selective detector (HP 5790B), was used

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to analyze metabolites. A 25 m × 0.2 mm capillary GC column coated with 0.33 μm of a crosslinked 5% phenyl methyl silicone gum phase (Ultra-2, Hewlett Packard) was the stationary phase and helium was used as the carrier gas (flow rate 30 ml/min). Radioactive metabolites were converted to methyl ester, trimethylsilyl ether (O-TMS) derivatives prior to analysis (15). The electron energy was 70 eV, the temperature of the injector, column, and transfer line were 280°C, 220°C, and 280°C, respectively.

RESULTS

Rat liver and kidney peroxisomes were isolated from light mitochondrial fractions on linear sucrose gradients. A typical distribution of marker enzymes on the gradient is shown in **Fig. 1.** Assays were done for catalase (peroxisomes), succinate dehydrogenase (mitochondria), and esterase (microsomes). Protein distribution was determined according to Lowry et al. (13). Fractions 5–7 were combined and used for the incubations of 12(S)-HETE. Electron micrographs of such preparations (**Fig. 2**) showed largely pure peroxisomes some of which appeared normal and well preserved while others were somewhat swollen. Occasional mitochondria, lysosomes, and pieces of endoplasmic reticulum were also detected.

Analysis of the products by reverse-phase HPLC showed that more than 90% of the diethyl ether-extractable radioactivity derived from 12(S)-HETE was converted to a single compound (**I**) (**Fig. 3.A**). The retention time of 12(S)-HETE was 22.4 min, while that of its product was 10.4 min. This compound displayed an absorbance maximum at 235 nm, suggesting the presence of a conjugated diene in its structure.

Analogous incubations were carried out with peroxisomes from rat kidney. Here too, the results showed a conversion of more than 90% of the diethyl ether-extractable radioactivity derived from 12(S)-HETE into a metabolite that co-chromatographed with compound (**I**) on reverse phase HPLC (Fig. 3.B) and on chiral straight-phase HPLC (not shown). When intact LLC cells were incubated with 12(S)-HETE for 30 min, reverse-phase HPLC showed the formation of several products, including one which co-chromatographed with compound (**I**) on reverse-phase HPLC (Fig. 3.C).

For structural analysis, compound (**I**) was isolated from a larger scale experiment and analyzed as the methyl ester, O-TMS derivative. The equivalent chain length (E.C.L. 17.4) was 4.1 units less than the E.C.L. for 12(S)-HETE (same derivative) (16). **Fig. 4** shows an electron impact mass spectrum of compound (**I**). The base peak at *m/z* 241 (M–111), appeared 54 amu lower than the base peak in the corresponding 12(S)-HETE mass spectrum (16), suggesting that this ion has the structure $[(CH_3)_3SiOCH(CH=CH)_2(CH_2)_2COOCH_3]^+$. Compared to the structure of the base peak in the 12(S)-HETE spectrum, this ion is shortened by four carbon atoms and contains one double bond less. High intensity ions also appeared at *m/z* 167 (M–112–73) and 137 (M–111–(73 + 31)). Corresponding ions at *m/z* 191 and 221 in the published 12(S)-HETE spectrum (16) were present, but their relative intensity was considerably lower. Weaker ions were observed at *m/z* 209 (M–111–32), 151 (M–111–90), and 119 (M–111–(90 + 32). Compared to corresponding ions in

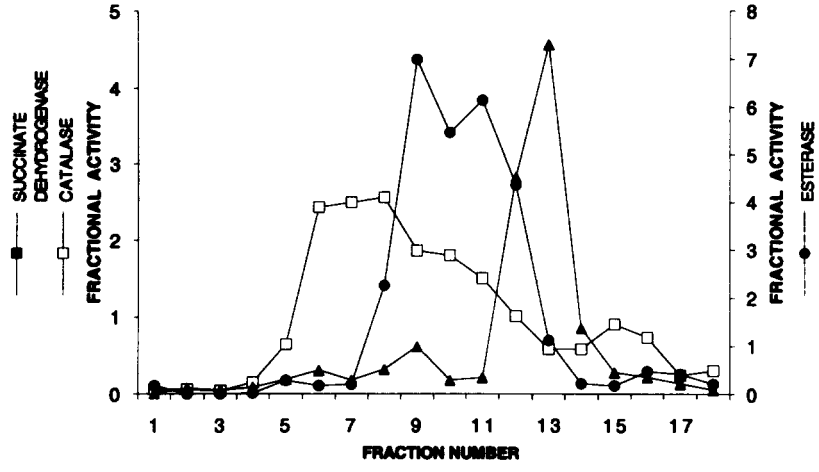

Fig. 1. Marker enzyme distribution of a light mitochondrial fraction centrifuged on a continuous sucrose gradient. Rat liver and kidney homogenates were centrifuged at 3,600 *g* for 10 min to remove large particles. The supernatant, a light mitochondrial fraction, was sedimented by centrifuging at 25,300 *g* for 20 min and applied to a 34–55% sucrose gradient. The gradients were centrifuged at 35,800 *g* for 315 min in a vertical rotor. Fractions numbered from bottom to top were collected and assayed for marker enzymes. Catalase was used as a marker for peroxisomes, succinate dehydrogenase for mitochondria, and esterase for microsomes.

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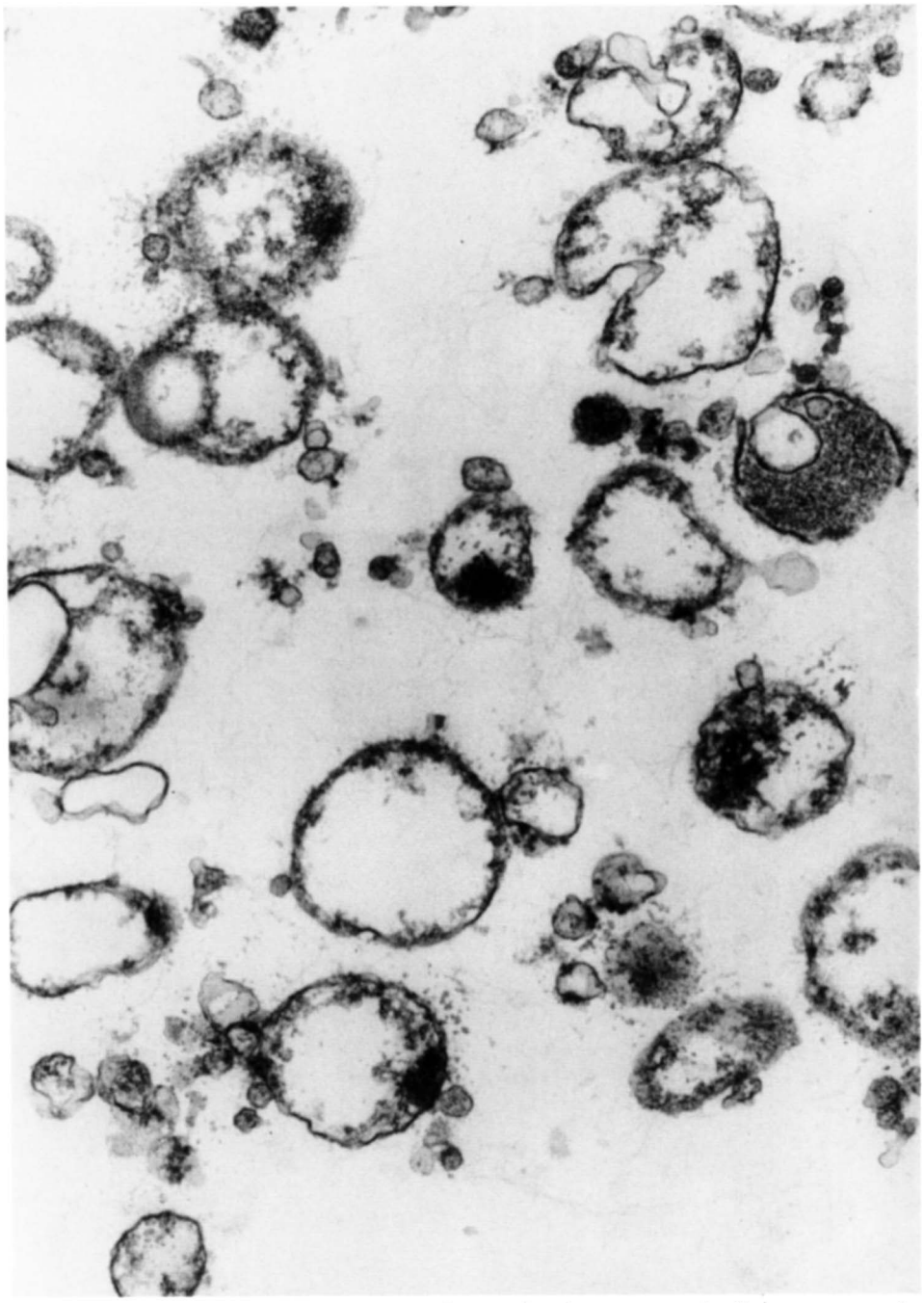

Fig. 2. Electron micrograph of peroxisomes isolated from rat liver homogenates using linear sucrose gradients.

the 12(S)-HETE spectrum, the ions at *m/z* 151 and 119 were of stronger relative intensity. The E.C.L. and mass spectrum demonstrate that compound (**I**) has the structure 1,2,3,4-tetranor-5,6-dihydro-12(S)-HETE, i.e., 8-OH-16:3.

DISCUSSION

Hadjiagapiou et al. (17) and Gordon, Figard, and Spector (8) identified a 12(S)-HETE metabolite in smooth muscle cells from human umbilical vein (17) and the renal cell line (MDCK) (8) as 8-OH-16:3. In later studies, the same group showed that human skin fibroblasts from patients with Zellweger's (cerebrohepatorenal) syndrome (18) and mutant Chinese hamster ovary cells (CHO) (19) with peroxisomal deficiencies failed to transform 12(S)-HETE to 8-OH-16:3. Although these observations suggest a connection between peroxisome activities and the formation of 8-OH-16:3, they are indirect evidence for

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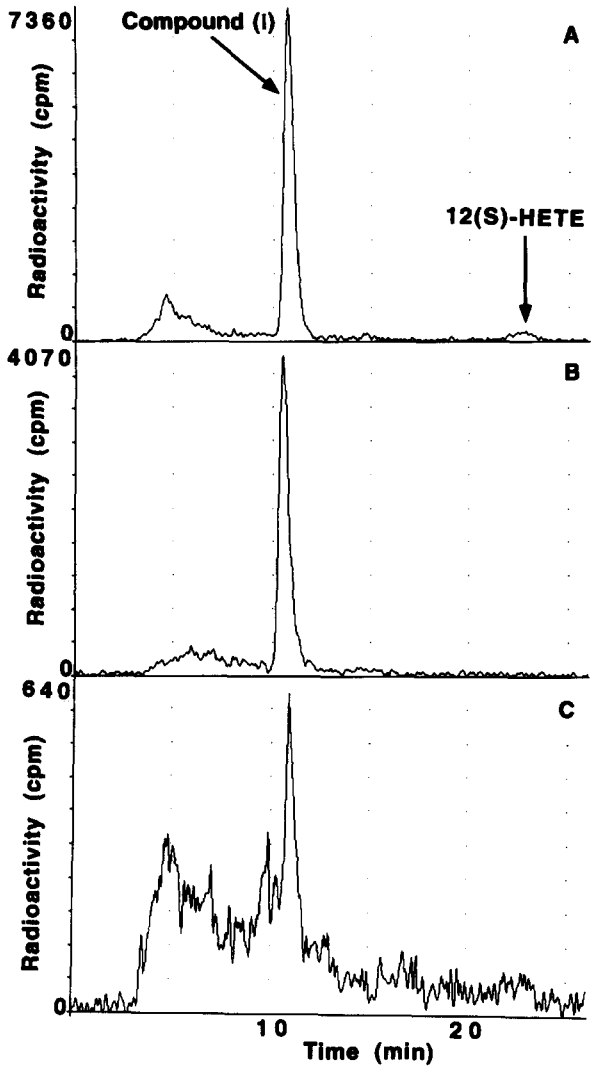

Fig. 3. Reverse-phase HPLC radiochromatogram of the metabolic products formed when 12-(S)-[^{3}H]HETE was incubated with purified rat liver peroxisomes (A), or kidney peroxisomes (B) for 1 h, or Lewis lung carcinoma cells (C). Rat liver and kidney peroxisomes were incubated with 1 nM 12-(S)-[^{3}H]HETE in 0.155 M Tris-HCl buffer, pH 8,0. The medium was acidified and metabolites were extracted with diethyl ether. The extract was dried under argon gas, redissolved in HPLC solvent, methanol-water-acetic acid 78:22:0.01 (v/v/v), and analyzed by isocratic reverse-phase HPLC. Radioactivity was continuously detected using a radioactivity detector with a flow detector. 12(S)-HETE was eluted at 22.4 min and its metabolite at 10.4 min.

peroxisomal metabolism. In this report, we have isolated peroxisomes from rat liver and kidney, and incubated them with 12(S)-[^{3}H]HETE in the presence of ATP, CoA, and NAD$^+$. Both types of peroxisomes quantitatively converted 12(S)-HETE into a single diethyl ether-extractable product. UV spectroscopy demonstrated that the metabolite had retained a conjugated diene structure. The GLC-retention time of the O-TMS derivative suggested that the metabolite had four carbon atoms less than 12(S)-HETE. This was confirmed by mass spectrometry which demonstrated that carbon atoms 1–4 of the substrate had been eliminated. The structure of the metabolite was therefore 8-OH-16:3. The results provide direct evidence for an essential role of peroxisomes in 12(S)-HETE metabolism. Due to their insolubility in diethyl ether (20), CoA derivatives of 12-HETE metabolites remain in the aqueous phase during extraction and therefore are not detected by the present analyses.

The transformation of 12(S)-HETE by peroxisomes (**Fig. 5**) consists of two turns of β-oxidation to give 8-OH-16:3. An NADPH-dependent pathway for the mitochondrial β-oxidation of 5-enoyl-CoAs (21) might be involved. This pathway involves isomerization to a 2,4-dienoyl-CoA intermediate and reduction by an NADPH-dependent 2,4-dienoyl-CoA reductase. Although it has not been demonstrated here, it seems likely that the original stereochemistry of the hydroxyl group, as well as the double bond geometry, have been retained in 8-OH-16:3.

It is of interest that 8-OH-16:3 seems to be an end product of peroxisomal 12(S)-HETE metabolism. Hadjiagapiou et al. (22) found that 6-hydroxy-4,8-tetradecadienoic acid was formed during incubations of 12(S)-HETE with MOLT-4 lymphocytes. These findings provide evidence for a role for 2,4-dienoyl-CoA reductase in the reduction of conjugated trienes, which arise during β-oxidation of 12(S)-HETE. It is, however, unclear from

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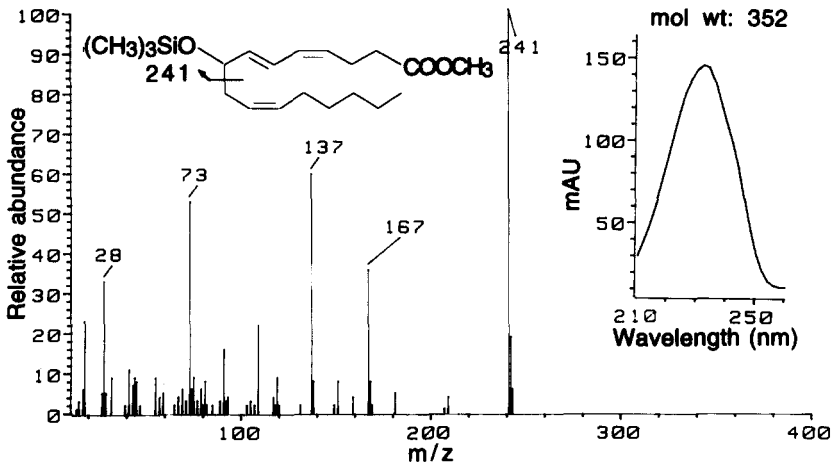

Fig. 4. Mass spectrum of the methyl ester, trimethylsilyl ether derivative of the 12(S)-HETE metabolite (compound **I**) formed during incubations with rat liver peroxisomes. The metabolite was isolated by reverse-phase HPLC, and derivatized prior to GLC–mass spectrometry analyses. Inset: UV spectrum of compound (**I**) recorded in methanol–water–acetic acid 78:22:0.01 (v/v/v).

these studies in which organelle the reduction took place as reductases have been found in both mitochondria and peroxisomes (23).

Peroxisomal metabolism of 12(S)-HETE can be compared to that of arachidonic acid (24). Hiltunen et al. (24) found that arachidonic acid is converted into a major metabolite believed to be either a C16:3 acid or a C14:2 acid. In view of the present results, it seems most likely that the metabolite observed by Hiltunen et al. (24) was hexadecatrienoic acid rather than tetradecadienoic acid. In support of this, Lazarow et al. (25) observed the chain shortening of long chain fatty acids to 16 carbons followed by a subsequent shuttling to mitochondria. If the presence of mitochondrial enzymes is a necessity for the continued β-oxidation of 8-OH-16:3, then the occurrence of 6-OH-14:2 in MOLT-4 lymphocytes after incubations with 12(S)-HETE (22) suggests involvement of mitochondria.

So far the biological roles of 8-OH-16:3 are not known. The dominance of this metabolite in smooth muscle cells (17) and MDCK cells (8) might indicate that 8-OH-16:3 exerts biological effects in these cells and their surroundings.

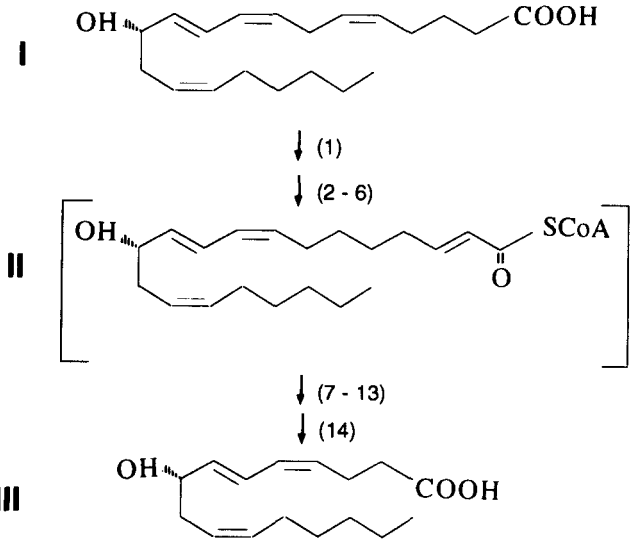

Fig. 5. Proposed scheme of the transformation of 12(S)-HETE to 8-OH-16:3 by peroxisomes. Acyl-CoA synthetase converts 12(S)-hydroxy-5,8,10,14 (Z,Z,E,Z)-eicosatetraenoic acid (**I**) to its CoA derivative (reaction 1). The latter is subsequently converted to 12(S)-hydroxy-2,8,10,14 (E,Z,E,Z)-eicosatetranoyl CoA (**II**) (reactions 2–6, see ref. 21). This product undergoes two rounds of β-oxidation (reactions 7–13), followed by thiolysis (reaction 14) giving 8-hydroxyhexadecatrienoic acid (**III**). The stereochemistry and geometry of the double bonds in 8-hydroxyhexadecatrienoic acid are assumed to be the same as in the substrate. Compound **I** (Fig. 3) = **III**.

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