Peroxisomal Metabolism of Adrenic Acid; No Δ4 Desaturase Detected in Rat Liver Peroxisomes

T.S. Haugen[a], V. Mimouni[b], J.I. Pedersen[a], H.J. Grav[a], and E.N. Christiansen[a,*]

[a]Institute for Nutrition Research, University of Oslo, Oslo, Norway, and [b]IUT Biologie Appliquée, Université du Maine, Laval, France

ABSTRACT: The existence of a peroxisomal Δ4 desaturation of 22:4n-6 and 22:5n-3 to yield, respectively, 22:5n-6 and 22:6n-3 has been questioned. An alternative pathway has been formulated to include microsomal chain elongation and Δ6 desaturation and peroxisomal chain shortening. We incubated [1-^{14}C]adrenic acid (22:4n-6) in a system for desaturation (i.e., in the presence of NADH) with purified rat liver peroxisomes. The fatty acids were separated as methyl derivatives by high-performance liquid chromatography. Four ultraviolet-absorbing product peaks appeared, three of which contained radioactivity, which we investigated as methyl, trimethylsilyl, and oxazoline derivatives on gas chromatography–mass spectrometry. In addition to adrenic and arachidonic acids, the product peaks were *trans*-enoyl, hydroxy, and keto derivatives of adrenic acid: the three first steps of β-oxidation cycle. This indicated that the NAD-dependent dehydrogenase step in the peroxisomal β-oxidation cycle of adrenic acid was inhibited due to a high concentration of added NADH. Incubation in the presence of NAD instead of NADH reduced radioactivity in the peaks that corresponded to intermediates, while radioactivity in the acid-soluble fraction increased considerably, consistent with a complete β-oxidation cycle of adrenic to arachidonic acid. There were no indications of Δ4 desaturation in purified peroxisomes incubated in a standard desaturation system. Instead, adrenic acid as substrate underwent β-oxidation.
JAOCS 75, 255–259 (1998).

KEY WORDS: Adrenic acid, Δ4 desaturation, fatty acid separation, fatty acids, GC–MS, HPLC, oxazoline fatty acid derivatives, peroxisomal β-oxidation, peroxisomes, trimethylsilylmethyl fatty esters.

The mechanism of introducing a double bond in the polyunsaturated adrenic (22:4n-6) or docosapentaenoic acids (22:5n-3) is under discussion. Martinez (1) has suggested the existence of a new enzyme defect in peroxisomal disorders that involve a missing Δ4-desaturase reaction. She found a drastic decrease in the putative Δ4-desaturation products, docosapentaenoic (22:5n-6) and docosahexaenoic acids (22:6n-3), especially in the kidney and the liver, indicating the absence of a peroxisomal Δ4 desaturase. Mimouni *et al.* (2) investigated the elongation and desaturation of ^{14}C-labelled arachidonic (20:4n-6) and eicosapentaenoic acids (20:5n-3) in normal rat liver and found significant amounts of ^{14}C-labelled products, identified tentatively as 22:5n-6 from arachidonic acid and 22:6n-3 from eicosapentaenoic acid. Also, clofibrate stimulated the synthesis of these products as catalyzed by the light mitochondrial fraction, which could indicate the presence of a peroxisomal Δ4-desaturase reaction in rat liver.

Voss *et al.* (3), however, suggested that, instead of direct Δ4 desaturation, a reaction sequence might take place that involves microsomal elongation of adrenic or docosapentaenoic acids to 24-carbon fatty acids, followed by microsomal Δ6 desaturation, and finally peroxisomal chain shortening to the postulated Δ4-desaturation products. Further evidence that this reaction sequence indeed occurs has been presented and reviewed by Sprecher *et al.* (4).

In this work, we wanted to investigate if a Δ4-desaturase activity could be detected in purified peroxisomes with minimal contamination of microsomes. We attempted to identify the reaction products from adrenic acid by gas chromatography–mass spectrometry (GC–MS). We could not confirm the presence of a Δ4-desaturation product by GC–MS, but instead we found evidence for the presence of intermediates that correspond to partial peroxisomal β-oxidation of adrenic acid.

MATERIALS AND METHODS

Materials. Clofibrate was obtained from Weiders Pharmaceutical Co., A/S (Oslo, Norway). [1-^{14}C] Adrenic acid was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Nycodenz was obtained from Nycomed A/S (Oslo, Norway). Other chemicals were commercially available products of high purity from Sigma Chemical Co. (St. Louis, MO). All solvents were of analytical or high-performance liquid chromatography (HPLC) grade.

Animals and diets. Adult male Wistar Moll rats, weighing 180–200 g were purchased from Møllegaard Breeding Laboratory (Ejby, Denmark) and fed a standard chow diet from B & K Universal Ltd. (Hull, United Kingdom) contained 0.3% (w/w) clofibrate for 10 d.

Preparation of liver peroxisomal fraction. The liver was homogenized in 250 mM sucrose, containing 0.2 mM EDTA and 2 mM Hepes buffer at pH 7.4, and treated by differential and Nycodenz gradient centrifugations essentially as de-

*To whom correspondence should be addressed at Institute for Nutrition Research, University of Oslo, P.O. Box 1046 Blindern, N-0316 Oslo, Norway. E-mail: e.n.christiansen@basalmed.uio.no.

scribed by Prydz *et al.* (5). The light mitochondrial fraction from differential centrifugation was enriched for peroxisomal activity by Nycodenz gradient centrifugation with linear Nycodenz gradients that ranged from 17% (wt/vol) in 250 mM sucrose, 1 mM Hepes buffer pH 7.4, 1 mM EDTA to 45% in 1 mM Hepes buffer, 1 mM EDTA. The peroxisomes were identified with catalase as marker enzyme (6), and the microsomal fraction was identified with esterase as marker enzyme (7). We estimated that the peroxisomal preparation contained 4–5% microsomal protein (not shown). Proteins were estimated by the method of Lowry *et al.* (8) with bovine serum albumin as standard.

Incubation and extraction. The incubation medium used for measuring the Δ4-desaturase activity with adrenic acid as substrate essentially followed Mohammed *et al.* (9) and contained in a total volume of 1.5 mL: 10 µmol ATP, 10 µmol $MgCl_2$, 2 µmol NADH, 0.4 µmol CoA, 150 µmol K-phosphate buffer pH 7.4, 120 nmol [1-^{14}C]22:4n-6, and 0.2 mg peroxisomal protein. The incubation was stopped after 15 min by adding 0.25 mL 4 M NaOH and 3.3 mL methanol; further procedure of hydrolysis, acidification, and final extraction by chloroform/methanol (2:1, vol/vol) was in Reference 9.

Chromatographic conditions. Lipids were methylated as given by Christie (10), and the fatty acids were separated by reversed-phase HPLC as described by Narce *et al.* (11) in a Merck LiChroCart Supersphere 100RP 18 column (Darmstadt, Germany). A Waters model 6000A HPLC chromatograph (Milford, MA) was used. The products were detected by ultraviolet (UV) at 218 nm (Waters Lambda 481 spectrophotometer) and by radioactivity (Wallac 1414 Liquid Scintillation Counter; Wallac Oy, Turku, Finland).

Selected peaks from the UV detection of the HPLC separation were identified in a Shimadzu GC-MS QP2000 (Tokyo, Japan) and postchromatographic analysis by the Shimadzu MSPAC 200 data program as described by Grav *et al.* (12). The fatty acids were also analyzed as oxazoline derivatives (13) by condensation to 2-amino-2-methylpropanol. Hydroxylated fatty acids were derivatized as trimethylsilyl (TMS) derivatives (14).

RESULTS AND DISCUSSION

Incubation of purified liver peroxisomes (with 4–5% microsomal contamination) from clofibrate-treated rats with [1-^{14}C] adrenic acid as substrate in a system for fatty acid desaturation (in the presence of NADH) gave a number of products, which were separated by HPLC. The product peaks were visualized by UV and radio detection as demonstrated in Figure 1. Peak 5 represents the substrate, as identified by GC–MS. The spectrum of oxazoline–adrenic acid (not shown) was identical to the spectrum published by Fay and Richli (15).

The other peaks were further analyzed by GC–MS. The mass spectrum of peak 1 is presented in Figure 2. This product corresponds to the TMS derivative of the methyl ester of 3-hydroxy-22:4n-6, with a molecular ion of *m/z* 434. The hydroxy fatty acid was assumed to be an intermediate in the β-oxidation cycle from adrenic acid. To confirm this result, we modified the incubation system for measurement of desaturation, NADH was replaced with an equimolar amount of NAD^+ or by pyruvate (10 mM) to reoxidize endogenous NADH by endogenous lactate dehydrogenase. The amount of radioactivity from [1-^{14}C] adrenic acid in the acid-soluble fraction increased considerably (16- and 9-fold, respectively)

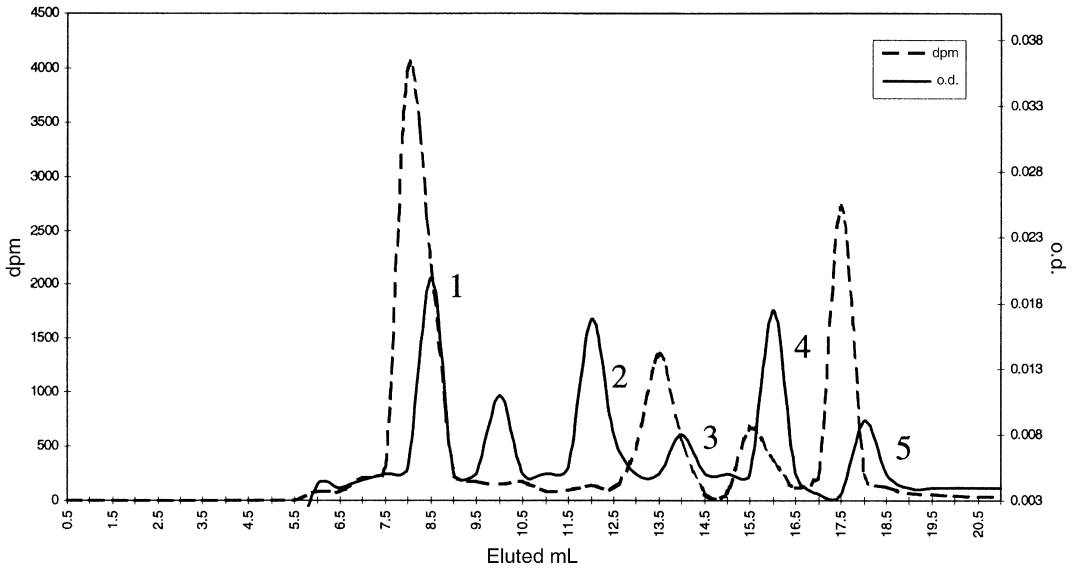

FIG. 1. High-performance liquid chromatography of fatty acid methyl esters from desaturation experiment, run on a Supersphere 100RP 18 (Merck, Darmstadt, Germany) with acetonitrile/water (9:1). The peaks were visualized with ultraviolet (UV) and radio detector, indicated as o.d. (optical density) and dpm, respectively. The UV detection was delayed 0.7 mL in elution volume compared to the radio detection. For further experimental details, see the Materials and Methods section.

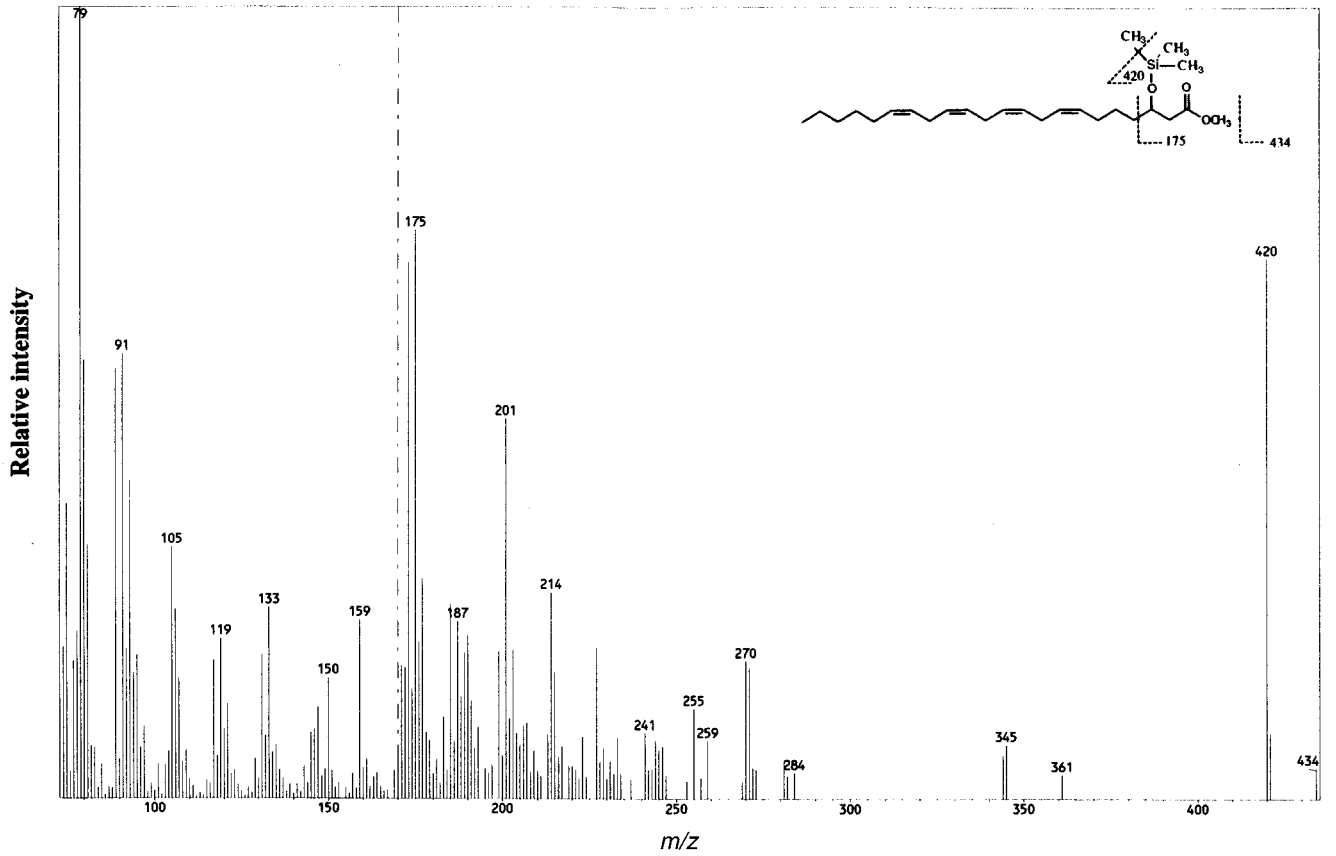

FIG. 2. Mass spectrum of trimethylsilyl-derivatized methyl ester of peak 1 from Figure 1. Insert represents the presumed origin of fragments, in which the ion of *m/z* = 420 probably is the protonated species of M – 15. The vertical broken line indicates lower limit for a range of fragments multiplied by a Factor of 4. For experimental details, see the Materials and Methods section.

with these additions. However, it was further demonstrated that, under desaturation conditions, there was a measurable peroxisomal β-oxidation. A similar accumulation of hydroxy–adrenic acid was identified by Luthria *et al.* (16) when NAD^+ was absent from the incubation mixture. Peak 1 corresponds to a peak that was tentatively identified by HPLC as 22:5n-6 in previous experiments (17). The conclusion drawn there that this implied the existence of a peroxisomal Δ4 desaturase proved untenable due to the use of 22:5n-3 as HPLC standard instead of 22:5n-6; 22:5n-3 was later shown to have the same elution volume as 3-hydroxy-22:4n-6.

Peak 2 was registered only by UV detection (Fig. 1), which indicated that the substance had lost the [1-^{14}C] label. The elution volume corresponded to arachidonic acid; further analysis of the methyl ester by GC–MS gave a molecular ion of *m/z* = 318, which also corresponds to arachidonic acid. Finally, the oxazoline derivative of the fatty acid (not shown) had a mass spectrum identical to that of oxazoline–arachidonic acid with a molecular ion of *m/z* = 357 as demonstrated by Zhang *et al*. (13). These results showed that peak 2 was a chain-shortened product from adrenic acid, with loss of [1-^{14}C] label after one β-oxidation cycle.

Peak 4 possessed a radioactive label, and the mass spectrum of the methyl derivative gave a molecular ion at *m/z* = 344 (not shown), two units less than the *m/z* = 346 for methylated adrenic acid. The oxazoline derivative of peak 4 gave a molecular ion at *m/z* = 383 (Fig. 3), also 2 units less than the expected molecular ion of the oxazoline derivative of adrenic acid. Thus, peak 4 is suggested to represent the first step in the β-oxidation with introduction of a double bond, yielding *trans*-2-enoyl- adrenic acid. The accumulation of a *trans*-fatty acid during peroxisomal β-oxidation of hexadecanoic acid in the absence of NAD^+ has also been demonstrated by Bartlett *et al.* (18).

Finally, peak 3 could not be identified by GC–MS because too little of the compound was available. It was, however, presumed to be 3-ketoacyl-adrenic acid as indicated by a smaller HPLC elution volume than the *trans* adrenic acid (peak 4), in accordance with the analogous results on chain-shortening of 3α,7α,12α-trihydroxy-5β-cholestanoic acid (19). Furthermore, because peak 3 contained radioactivity, it had to consist of at least 22 carbon atoms.

The UV peak with a retention volume between peaks 1 and 2, which contained no radioactivity, was not identified (Fig. 1).

In conclusion, we have not been able to detect Δ4 desaturation or elongation products from adrenic acid after incubation with a purified preparation of liver peroxisomes from rats stimulated by clofibrate. The products found were identified by GC–MS to be the peroxisomal β-oxidation intermediates from adrenic acid; *trans*-2-enoyl-, 3-hydroxy- and probably 3-

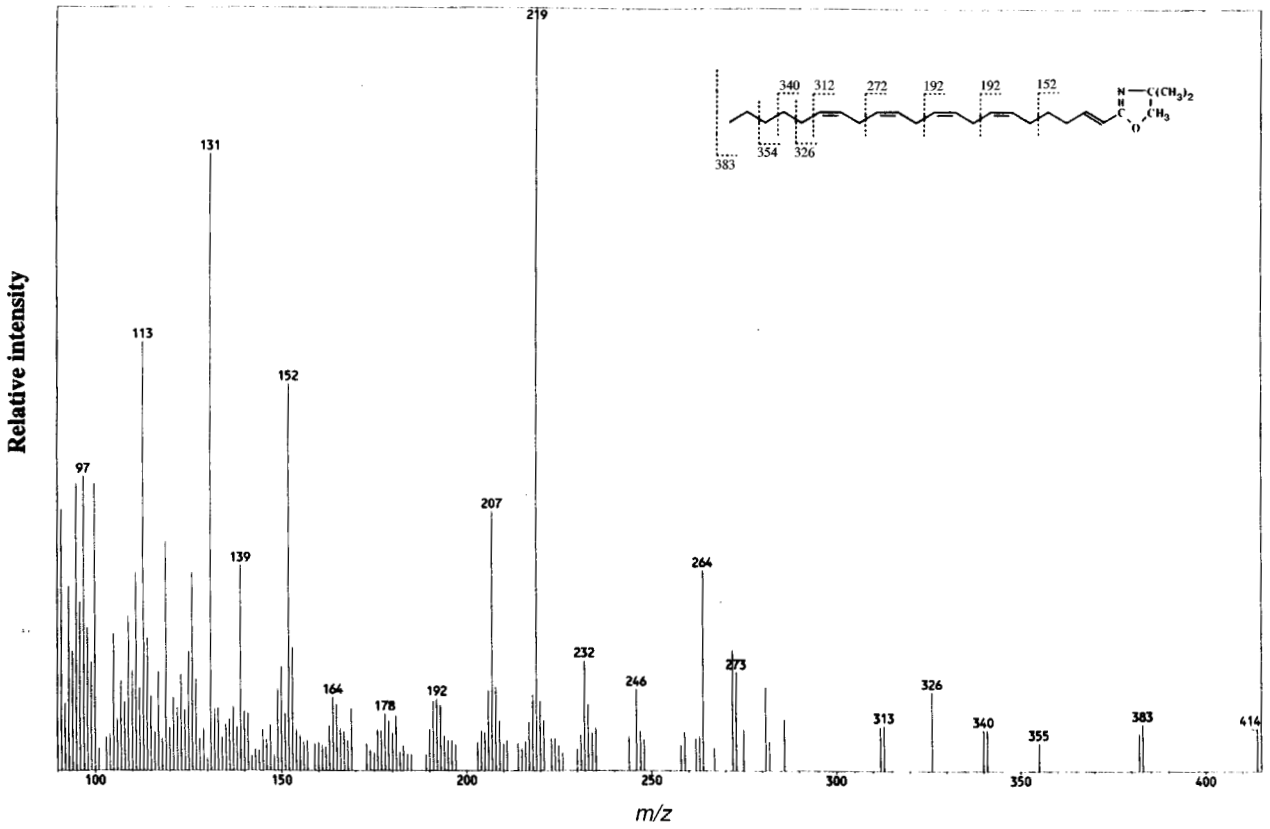

FIG. 3. Mass spectrum of the fatty acid oxazoline derivative of peak 4 from Figure 1. Insert represents the presumed origin of fragments. For experimental details, see the Materials and Methods section.

keto adrenic acid, and finally arachidonic acid to complete one β-oxidation cycle. These products were formed even under the unfavorable conditions for β-oxidation at a high NADH/NAD ratio. Because purified peroxisomes were used in this experiment, it would not be expected that microsomal elongation products of adrenic acid should be found according to the alternative Δ4-desaturation pathway proposed by Sprecher *et al.* (4) because this latter pathway is catalyzed by microsomes.

ACKNOWLEDGMENTS

The authors thank Aase Kopstad and Turid Veggan for technical assistance. This study was supported by The Research Society of the Norwegian Edible Fat Producers (Fredrikstad, Norway); Anders Jahres Research Foundation (Oslo, Norway); The Novo Nordisk Foundation (Bagsvaerd, Denmark); and Freia Chocolate Company Medical Foundation (Oslo, Norway).

REFERENCES

1. Martinez, M., Polyunsaturated Fatty Acid Changes Suggesting a New Enzymatic Defect in Zellweger Syndrome, *Lipids 24*:261–265 (1989).
2. Mimouni, V., E.N. Christiansen, J.P. Blond, L. Ulmann, J.-P. Poisson, and J. Bezard, Elongation and Desaturation of Arachidonic and Eicosapentaenoic Acids in Rat Liver. Effect of Clofibrate Feeding, *Biochim. Biophys. Acta 1086:*349–353 (1991).
3. Voss, A., M. Reinhart, S. Sankarappa, and H. Sprecher, The Metabolism of 7,10,13,16,19-Docosapentaenoic Acid to 4,7,10,13,16,19-Docosahexaenoic Acid in Rat Liver Is Independent of a 4-Desaturase, *J. Biol. Chem. 266*:19995–20000 (1991).
4. Sprecher, H., D.L. Luthria, B.S. Mohammed, and S.P. Baykousheva, Reevaluation of the Pathways for the Biosynthesis of Polyunsaturated Fatty Acids, *J. Lipid Res. 36*:2471–2477 (1995).
5. Prydz, K., B.T. Kase, I. Björkhem, and J.I. Pedersen, Subcellular Localization of 3α,7α-Dihydroxy- and 3α,7α,12α-Trihydroxy-5β-cholestanoyl-coenzyme A Ligase(s) in Rat Liver, *Ibid. 29*:997–1004 (1988).
6. Baudhuin, P., H. Beafay, Y. Rahman-Li, O.Z. Sellinger, R. Wattiaux, P. Jacques, and C. DeDuve, Tissue Fraction Studies, *Biochem. J. 92*:179–184 (1964).
7. Beaufay, H., A. Amar-Costesec, E. Feytmans, D. Thinés-Sempoux, M. Wibo, M. Robbi, and J. Berthet, Analytical Study of Microsomes and Isolated Subcellular Membranes from Rat Liver, *J. Cell Biol. 61*:188–200 (1974).
8. Lowry, O.H., N.J. Rosenbrough, A.L. Farr, and R.J. Randall, Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem. 193*:265–275 (1951).
9. Mohammed, B.S., S. Sankarappa, M. Geiger, and H. Sprecher, Reevaluation of the Pathway for the Metabolism of 7,10,13,16-Docosatetraenoic Acid to 4,7 10,13,16-Docosapentaenoic Acid in Rat Liver, *Arch. Biochem. Biophys. 317*:179–184 (1995).
10. Christie, W.W., Gas Chromatography: Theoretical Aspects and Instrumentation, in *Gas Chromatography and Lipids,* edited by W.W. Christie, The Oily Press, Ayr, 1989, pp. 44–45.
11. Narce, M., J. Gresti, and J. Bezard, Method for Evaluating the Bioconversion of Radioactive Polyunsaturated Fatty Acids by

Use of Reversed-Phase Liquid Chromatography, *J. Chromatogr. 448*:249–264 (1988).
12. Grav, H.J., D.K. Asiedu, and R.K. Berge, Gas Chromatographic Measurement of 3- and 4-Thia Fatty Acids Incorporated into Various Classes of Rat Liver Lipids During Feeding Experiments, *J. Chrom. B 658*:1–10 (1994).
13. Zhang, J.Y., Q.T. Yu, B.N. Liu, and Z.H. Huang, Chemical Modification in Mass Spectrometry. IV. 2-Alkenyl-4,4-Dimethyloxazolines as Derivatives for the Double-Bond Location of Long-Chain Olefinic Acids, *Biomed. Envir. Mass Spec. 15*: 33–44 (1988).
14. Christie, W.W., The Preparation of Derivatives of Lipids, in *Gas Chromatography and Lipids,* edited by W.W. Christie, Pergamon Press, Oxford, 1982, pp. 57–58.
15. Fay, L., and U. Richli, Location of Double Bonds in Polyunsaturated Fatty Acids by Gas Chromatography–Mass Spectrometry After 4, 4-Dimethyloxazoline Derivation, *J. Chrom. 541*:89–98 (1991).
16. Luthria, D.L., S.P. Baykousheva, and H. Sprecher, Double-Bond Removal from Odd-Numbered Carbons During Peroxisomal β-Oxidation of Arachidonic Acid Requires both 2,4-Dienoyl-CoA Reductase and $\Delta^{3,5}$, $\Delta^{2,4}$-Dienoyl-CoA Isomerase, *J. Biol. Chem. 270*:13771–13776 (1995).
17. Mimouni, V., E.N. Christiansen, and J.-P. Poisson. Existence of a Δ4 (n-6) Desaturase Activity in Rat Hepatic Peroxisomes. Effects of Clofibrate, *Biology of the Cell 77*:103 (1993).
18. Bartlett, K., R. Hovik, S. Eaton, N.J. Watmough, and H. Osmundsen, Intermediates of Peroxisomal β-Oxidation. A Study of the Fatty Acyl-CoA Esters Which Accumulate During Peroxisomal β-Oxidation of [U-^{14}C]Hexadecanoate, *Biochem. J. 270*:175–180 (1990).
19. Farrants, A.Ö., I. Björkheim, and J.I. Pedersen, Identification of 3α,7α,12α-Trihydroxy-5β-cholest-24-enoic Acid as Intermediate in the Peroxisomal Conversion of 3α,7α,12α-Trihydroxy-5β-cholestanoic Acid to Cholic Acid, *Biochim. Biophys. Acta 1002*:198–202 (1989).

[Received November 14, 1996; accepted May 3, 1997]