Decomposition of Unsaturated Fatty Acid Hydroperoxides by Hemoglobin: Structures of Major Products of 13L-Hydroperoxy-9,11-octadecadienoic Acid

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ABSTRACT

13L-Hydroperoxy-9,11-octadecadienoic acid was decomposed rapidly in the presence of hemoglobin. The product consisted of five major compounds, i.e. 13-keto-9,11-octadecadienoic acid, 13L-hydroxy-9,11-octadecadienoic acid, *erythro*-11-hydroxy-12,13-epoxy-9-octadecenoic acid, *threo*-11-hydroxy-12,13-epoxy-9-octadecenoic acid, and 9DL-hydroxy-12,13-epoxy-10-octadecenoic acid.

INTRODUCTION

Heme compounds catalyze decomposition of fatty acid hydroperoxides, and free radical intermediates generated during this process initiate oxygenation of unsaturated fatty acids (1). Since heme compounds frequently occur in proximity to oxygen and unsaturated lipids in biological systems, it is probable that heme catalysis is of wide importance. Some aspects of this process, e.g. kinetics, relative effectiveness of different heme compounds, and the effect of inhibitors have been studied in detail. On the other hand, little is known about the structures of the oxygenated fatty acid derivatives that are formed during heme catalysis. In the present work, the product formed on decomposition of 13L-hydroperoxy-9,11-octadecadienoic acid by hemoglobin was studied. The structures of five compounds have been determined. After submission of this paper for publication there appeared a paper by Gardner, et al. (2) in which nine oxygenated fatty acids were identified following treatment of an isomeric mixture of linoleic acid hydroperoxides with an Fe(III)-cysteine couple.

MATERIALS AND METHODS

[1-^{14}C]13L-Hydroperoxy-9,11-octadecadienoic acid (70 μCi/mmole) was prepared as previously described (3). The preparation contained 4-5% of the isomeric 9-hydroperoxide.

erythro- And *threo*-11,12-dihydroxy-1-octadecanols were obtained by *trans*-hydroxylation of *trans*- and *cis*-vaccenic acids, respectively, with performic acid followed by reduction with $LiAlH_4$ (3). Hemoglobin (bovine, mixture of methemoglobin, and oxyhemoglobin) was purchased from Sigma Chemical Co., St. Louis, Mo.

Oxidative ozonolysis of (-)-menthoxycarbonyl derivatives and analysis of the esterified product by gas liquid chromatography (GLC) was performed as previously described (4).

Thin layer chromatography (TLC) of fatty acid hydroperoxides and their reaction products was carried out with plates coated with Silica Gel G. The organic layer of an equilibrated mixture of ethyl acetate-2,2,4-trimethylpentane-water (50:100:100, v/v/v) was used as solvent. Separation of *erythro*- and *threo*-11,12-dihydroxy-1-octadecanols was achieved by TLC on sodium arsenite-impregnated plates and methanol-chloroform (3:97, v/v) as solvent (3). Spots and bands were located by spraying with 2',7'-dichlorofluorescein and viewing by UV. Radioactivity on TLC plates was determined with a Berthold Dünnschichtscanner II.

GLC was carried out with an F&M biomedical gas chromatograph model 402 and columns of 1% SE 30 on Gas Chrom Q (long chain compounds) and 5% QF-1 on Gas Chrom Q (ozonolysis products). Mass spectra were recorded with an LKB 9000 instrument equipped with a column of 1% OV-1 on Supelcoport (Supelco, Bellefonte, Pa.).

RESULTS

[1-^{14}C]13L-Hydroperoxy-9,11-octadecadienoic acid (0.5 mg) in 6 μliter ethanol was added to 1 ml solutions of hemoglobin (0.15%, 0.5%, 1.5%, and 5%, w/v) in 0.1 M potassium phosphate buffer at 37 C. The mixtures were incubated at 37 C for 5 min and subsequently diluted with water, acidified, and extracted twice with diethyl ether. The ether phase was washed until neutral and dried over $MgSO_4$. Evaporation of the ether gave a residue (ca. 60% of the added radioactivity) that was treated with diazomethane and subjected to TLC. Radioactivity assay showed that the fatty acid hydroperoxide had disappeared in all incubations and that five major compounds were present and together constituted ca. 90% of the recovered radioactivity (Fig. 1). Use of high concentrations of hemoglobin (1.5 and 5%)

appeared to favor formation of compound V. To obtain sufficient amounts of compounds I-V for structural work, batches of 8 mg [1-^{14}C] 13L-hydroperoxy-9,11-octadecadienoic acid were incubated with 500 mg hemoglobin in 10 ml buffer and the esterified product subjected to preparative TLC (Fig. 1). The product contained the following percentages of the 5 major compounds: compound I, 11% (R_f = 0.75); compound II, 25% (R_f = 0.60); compound III, 10% (R_f = 0.48); compound IV, 14% (R_f = 0.43); compound V, 31% (R_f = 0.38).

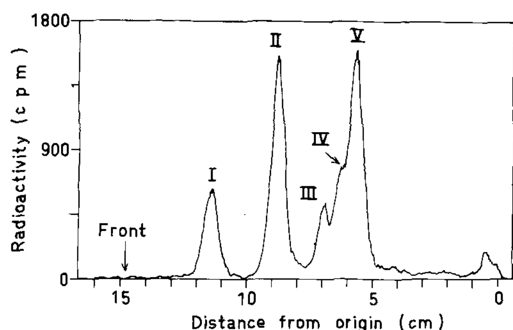


FIG. 1. Thin layer radiochromatogram of esterified material isolated after incubation of 8 mg [1-^{14}C] 13L-hydroperoxy-9,11-octadecadienoic acid with hemoglobin. Solvent system: organic layer of ethyl acetate-2,2,4-trimethylpentane-water (50:100:100, v/v/v). I, II, III, IV, and V = major compounds.

STRUCTURE OF COMPOUND I

The UV spectrum of compound I showed an absorption band with λ_{max}^{EtOH} = 278 nm suggesting a conjugated dienone chromophore. GLC analysis showed a single peak with equivalent chain length corresponding to C-20.1 (column, 1% OV-1, column temperature, 190 C). The mass spectrum recorded on this peak (Fig. 2) showed ions of high intensity at m/e 308 (M), 277 (M-31; loss of $\cdot OCH_3$), 252 (M-56; β-cleavage with loss of CH_2=CH-CH_2-CH_3), 237 (M-71; α-cleavage with loss of $\cdot[CH_2]_4CH_3$), 209 (M-99; α-cleavage with loss of $\cdot\overset{O}{C}$-$[CH_2]_4CH_3$), 177 (209-32), and 151 ($[(CH=CH)_2$-$\underset{O}{C}$-$(CH_2)_4CH_3]^+$).

Catalytic hydrogenation of compound I (0.2 mg; 5% palladium-on-carbon) afforded a tetrahydro derivative, the mass spectrum of which was identical with that of authentic methyl 13-ketooctadecanoate (5,6). Finally, oxidative ozonolysis performed on compound I (0.2 mg) followed by esterification afforded a major compound identified by GLC as dimethyl azelate. Compound I, thus, possessed a keto group at C-13 and a double bond at Δ^9. The second double bond was placed at Δ^{11} by the UV data which had shown the presence of a conjugated dienone structure.

The structure of compound I was, therefore, methyl 13-keto-9,11-octadecadienoate.

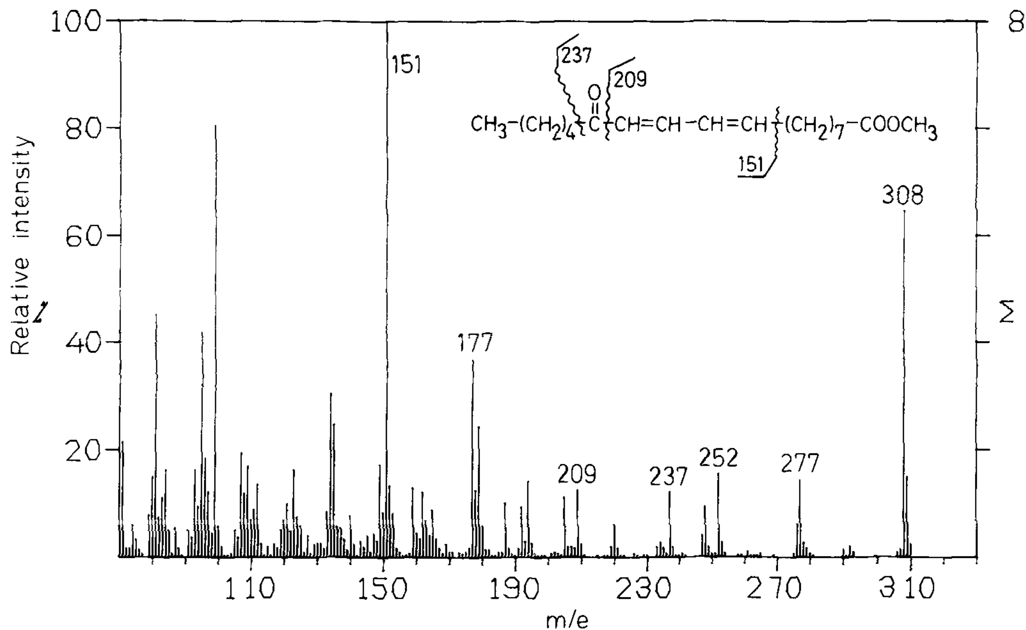


FIG. 2. Mass spectrum of compound I.

TABLE I

Thin Layer Chromatography of Dihydroxyoctadecanols Formed on Reduction of Hydrogenated Derivatives of Compounds III and IV

Compound	R_f	Position of hydroxyls[a]
threo-11,12-Dihydroxy-1-octadecanol	0.59	1,11,12
erythro-11,12-Dihydroxy-1-octadecanol	0.47	1,11,12
Compound III hydrogenated and reduced	0.47	1,11,12
	0.29	1,11,13
Compound IV hydrogenated and reduced	0.59	1,11,12
	0.47	1,11,13 (Major isomer) 1,11,12 (Minor isomer)

[a]Determined by mass spectrometric analysis of the trimethylsilyl derivatives.

STRUCTURE OF COMPOUND II

UV spectrometry showed an absorption band with $\lambda_{max}^{EtOH} = 234$ nm indicating the presence of one pair of conjugated double bonds. The mass spectrum of the trimethylsilyl (TMSi) derivative of compound II (C-19.9) showed ions of high intensity at m/e 382 (M), 311 (M-71; α-cleavage with loss of $\cdot[CH_2]_4CH_3$), 225 ($[(CH{=}CH)_2-CH(OTMSi)-(CH_2)_4CH_3]^+$), 186, 143, and 130, indicating a C_{18} ester carrying a TMSiO group at C-13. Catalytic hydrogenation afforded methyl 13-hydroxyoctadecanoate as shown by GLC-mass spectrometry and comparison with authentic material (5,6). Oxidative ozonolysis performed on the menthoxycarbonyl derivative of compound II yielded dimethyl azelate and the menthoxycarbonyl derivative of methyl 2L-hydroxyheptanoate.

The structure of compound II was, thus, methyl 13L-hydroxy-9,11-octadecadienoate.

STRUCTURE OF COMPOUND III

No absorption band in the range 220-320 nm was present in the UV sprectrum of compound III, showing that the conjugated double bonds of the starting hydroperoxide were not retained. The mass spectrum of the TMSi derivative (C-20.7) showed ions at 398 (M), 327 (M-71; loss of $\cdot[CH_2]_4CH_3$) and 285 (M-113; loss of $\cdot CH-CH-[CH_2]_4CH_3$ with O bridging the two CH) (3). Catalytic hydrogenation shifted the molecular ion to m/e 400, showing the presence of one double bond. The dihydro derivative was not affected by treatment with sodium borohydride. This was in agreement with the presence of an epoxy group at C-12,13 but not with a keto group at any of these positions. The double bond was placed at Δ^9 by oxidative ozonolysis which afforded *inter alia* dimethyl azelate. Finally, the dihydro derivative of compound III (ca. 0.5 mg) was refluxed with 50 mg of $LiAlH_4$ in 5 ml tetrahydrofurane for 18 hr. The product contained comparable amounts of 11,12-dihydroxy-1-octadecanol and 11,13-dihydroxy-1-octadecanol as shown by GLC-mass spectrometry (3), demonstrating conclusively the presence of a hydroxyl group at C-11 and an epoxy group at C-12,13 in compound III.

To obtain information about the configuration at carbons 11 and 12, the above mixture of dihydroxyoctadecanols was subjected to TLC with sodium arsenite-impregnated plates. Two peaks of labeled material appeared (R_f = 0.47 and 0.29; references, *threo*-11,12-dihydroxy-1-octadecanol, R_f = 0.59, and *erythro*-11,12-dihydroxy-1-octadecanol, R_f = 0.47). The more polar material (R_f = 0.29) was due to 11,13-dihydroxy-1-octadecanol as shown by mass spectrometric analysis of the TMSi derivative (ions of high intensity were present at m/e 503 [M-15; loss of $\cdot CH_3$]), 428 (M-90; loss of TMSiOH), 357 (M-[90+71]; loss of TMSiOH plus $\cdot[CH_2]_4CH_3$), 331 (M-187; loss of $\cdot CH_2-CH[OTMSi]-[CH_2]_4CH_3$), and 173 ($TMSiO^+{=}CH-[CH_2]_4CH_3$) (3). The less polar material (R_f = 0.47) similarly was analyzed and found to be 11,12-dihydroxy-1-octadecanol (the mass spectrum showed ions of high intensity at m/e 503 (M-15), 433 (M-85; loss of $\cdot[CH_2]_5CH_3$), 331 (M-187; loss of $\cdot CH[OTMSi]-[CH_2]_5CH_3$), and 187 ($TMSiO^+{=}CH-[CH_2]_5CH_3$) (3). This must be the *erythro*- isomer according to the TLC data. No radioactivity was detected at the position of the *threo*- isomer of 11,12-dihydroxy-1-octadecanol (Table I).

On the basis of these experiments, Compound III was assigned the structure methyl *erythro*-11-hydroxy-12,13-epoxy-9-octadecenoate.

STRUCTURE OF COMPOUND IV

The UV spectrum of compound IV did not

FIG. 3. Mass spectrum of the trimethylsilyl derivative of compound V.

show any absorption band in the range 220-320 nm demonstrating that the conjugated double bonds of the precursor were not retained. The mass spectrum of the TMSi derivative (C-20.7) was almost identical with that of the corresponding derivative of compound III and of methyl 11-hydroxy-12,13-epoxy-9-octadecenoate (3). One double bond was present in compound IV, since catalytic hydrogenation shifted the molecular ion from m/e 398 to m/e 400. Oxidative ozonolysis afforded *inter alia* dimethyl azelate, demonstrating that the double bond was present at Δ^9. These experiments suggested that compound IV was a stereoisomer of compound III. The relative configuration at carbons 11 and 12 was determined by TLC analysis of the derived dihydroxyoctadecanols as described above. Two peaks of radioactivity appeared (R_f = 0.59 and 0.47; coinciding with the references, *threo-* and *erythro-*11,12-dihydroxy-1-octadecanols, respectively [Table I]). The less polar material derived from compound IV was due to 11,12-dihydroxy-1-octadecanol as shown by GLC-mass spectrometry. This must be the *threo-*isomer according to the TLC data. The more polar material (R_f = 0.47) consisted mainly of 11,13-dihydroxy-1-octadecanol but also 11,12-dihydroxy-1-octadecanol. From the relative intensities of the ions at m/e 173 and m/e 187 in several mass spectra recorded on the common peak of the TMSi derivatives and from the relative amounts of the two reduction products isolated by sodium arsenite TLC (Table I), it was calculated that compound IV was ca. 80% *threo-* and 20% *erythro-*isomers of methyl 11-hydroxy-12,13-epoxy-9-octadecenoate.

STRUCTURE OF COMPOUND V

The conjugated double bonds of 13L-hydroperoxy-9,11-octadecadienoic acid were not retained in compound V, since the UV spectrum showed no absorption band in the range 220-320 nm. The mass spectrum of the TMSi derivative (C-21.5) (Fig. 3) showed ions of high intensity at m/e 327 (M-71; loss of $\cdot[CH_2]_4CH_3$), 285 (M-113; loss of $\cdot CH\text{-}O\text{-}CH\text{-}[CH_2]_4CH_3$), 259 (M-139; loss of $\cdot CH{=}CH\text{-}CH\text{-}O\text{-}CH\text{-}[CH_2]_4CH_3$), and 241 ($TMSiO^+{=}CH\text{-}CH{=}CH\text{-}CH\text{-}O\text{-}CH\text{-}[CH_2]_4CH_3$).

Treatment of compound V (0.5 mg) with glacial acetic acid at 70 C for 2 hr yielded a more polar compound (R_f = 0.10-0.15) that was converted into the TMSi derivative and analyzed by mass spectrometry. The mass spectrum showed an ion at m/e 470 (M-60; elimination of CH_3COOH) as well as intense ions at m/e 387 (M-143; loss of $\cdot CH[OCOCH_3]\text{-}[CH_2]_4CH_3$), 259 ($TMSiO^+{=}CH\text{-}[CH_2]_7\text{-}COOCH_3$), and 173 ($TMSiO^+{=}CH\text{-}[CH_2]_4CH_3$), indicating a mixture of monounsaturated C_{18} esters carrying one acetoxy group and two TMSiO groups. Although the positions of the oxygen functions were not analyzed further, it was clear that compound V readily incorporated one molecule of acetic acid in agreement with the presence of an epoxy group.

Oxidative ozonolysis performed on the menthoxycarbonyl derivative of Compound V afforded *inter alia* the menthoxycarbonyl derivatives of dimethyl 2L-hydroxy-(52%) and 2D-hydroxy-(48%) sebacates. This demonstrated the presence of double bond at Δ^{10}, as well as the presence of a hydroxyl group at C-9 (52% 9D, 48% 9L).

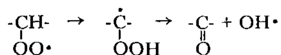
The structure of compound V was, therefore, methyl 9DL-hydroxy-12,13-epoxy-10-octadecenoate.

DISCUSSION

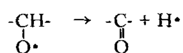
The mechanism proposed (1) for heme catalysis consists of initial formation of an activated coordination compound from the fatty acid hydroperoxide and the heme compound. Subsequent homolytic scission of the O-O bond affords oxy-fatty acid and oxy-heme radicals that may initiate autoxidation of unsaturated fatty acids. The fact that phenolic antioxidants inhibit heme catalyzed autoxidation supports the view that free radicals are involved.

The product formed on decomposition of linoleic acid hydroperoxides by heme compounds has earlier been characterized by UV spectrometry (7). This showed a decreased absorption at 233 nm and increased absorption at 285 nm, indicating partial disappearance of the conjugated diene structure and formation of a conjugated dienone. TLC revealed a "complex range of products" (7). Notably, the same complex mixture apparently was formed when linoleic acid hydroperoxides were treated with transition metals salts or exposed to heat.

The present work describes isolation and structure determination of five major compounds present in the product formed on incubation of 13L-hydroperoxy-9,11-octadecadienoic acid with hemoglobin (Fig. 4). The least polar compound (compound I) was shown to be 13-keto-9,11-octadecadienoic acid (methyl ester). Its strong absorption at 278 nm explained the increased absorption around this wavelength earlier observed in products of heme catalysis. Formation of 13-keto-9,11-octadecadienoic acid from the starting hydroperoxide probably occurs by expulsion of OH• from the 13-peroxy radical:



The same mechanism was postulated for formation of 13-keto-9,11-octadecadienoic acid from 13-hydroperoxy-9,11-octadecadienoic acid incubated anaerobically with linoleic acid and soybean lipoxygenase (8). An alternate mechanism consists of elimination of H• from the 13-oxy radical:



For the formation of 13L-hydroxy-9,11-octadecadienoic (parent acid of compound II), two mechanisms seem possible, i.e. addition of a hydrogen atom to the 13-oxy radical (9):

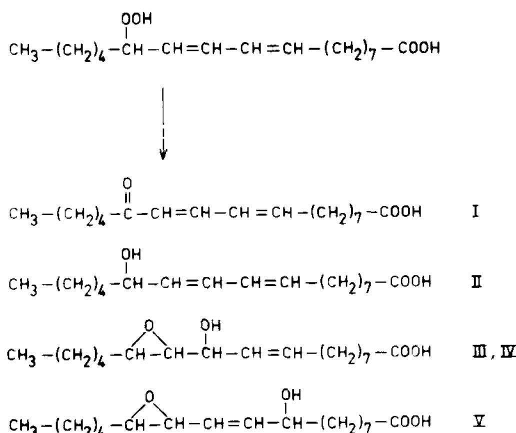
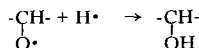
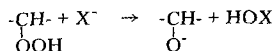


FIG. 4. Structures of the five major oxygenated fatty acids formed from 13L-hydroperoxy-9,11-octadecadienoic acid on incubation with hemoglobin.



and reduction of the hydroperoxide group as a result of attack by nucleophilic groups present in the hemoglobin molecule:

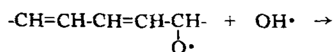


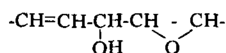
Compounds III and IV were shown to be stereoisomers of 11-hydroxy-12,13-epoxy-9-octadecenoic acid (methyl esters). Compound III was found to be the pure *erythro*-isomer, whereas compound IV was mainly (ca. 80%) the *threo*-isomer. The reason for the presence of *erythro*-isomer (ca. 20%) in compound IV is not known. Possibly, this isomer differed from compound III with respect to the configurations at the epoxy group and the Δ^9 double bond. The 11,12-dihydroxy-1-octadecanols derived from compounds III and IV were accompanied by comparable amounts of two isomeric 11,13-dihydroxy-1-octadecanols of unknown configurations (Table I). It is planned to correlate the latter compounds with the diastereoisomeric pair of 11,13-dihydroxy-1-octadecanol (11D,13D+11L,13L and 11D,13L+11L,13D). Knowledge of the configuration at C-11,13 of compounds III and IV coupled with the configuration at C-11,12 (Table I) will allow assignment of the configuration of the epoxy group (*cis* or *trans*) of compounds III and IV.

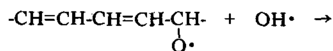
threo-11-Hydroxy-12,13-epoxy-9-octadecenoic acid recently was isolated from the product formed on heat treatment of 13L-hydroperoxy-9,11-octadecadienoic acid (3). 11-Hydroxy-12,13-epoxy-9-octadecenoic acid and its isomer, 11-hydroxy-9,10-epoxy-12-octa-

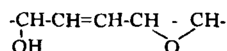
decenoic acid, also were formed during autoxidation of linoleic acid (3).

Compound V was identified as 9DL-hydroxy-12,13-epoxy-10-octadecenoic acid (methyl ester). This acid has been isolated previously after incubation of linoleic acid with flour doughs (10). The mechanism in the formation of compounds III-V probably consists of addition of OH• to the oxy-fatty acid radical without (compounds III and IV) or with (compound V) shift of the Δ^9 double bond:









In the case of *threo*-11-hydroxy-12,13-epoxy-9-octadecenoic acid formed by heat treatment of 13L-hydroperoxy-9,11-octadecadienoic acid (3), isotopic studies showed that the major part of the epoxide was formed by a reaction involving elimination of the distal oxygen of the hydroperoxide group and incorporation of OH (ion or radical) from the aqueous solvent. Similar studies on the formation of the compounds described in the present paper are in progress.

ACKNOWLEDGMENT

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