Biochimica et Biophysica Acta, 751 (1983) 189–200
Elsevier Biomedical Press

BBA 51361

IODINATION OF ARACHIDONIC ACID MEDIATED BY EOSINOPHIL PEROXIDASE, MYELOPEROXIDASE AND LACTOPEROXIDASE

IDENTIFICATION AND COMPARISON OF PRODUCTS

JOHN TURK [a,*], WILLIAM R. HENDERSON [b], SEYMOUR J. KLEBANOFF [b] and WALTER C. HUBBARD [a,**]

[a] *Departments of Medicine and Pharmacology, Vanderbilt University, Nashville, TN 37232 and* [b] *Department of Medicine, University of Washington, Seattle, WA 98195 (U.S.A.)*

(Received November 29th, 1982)

Key words: Arachidonic acid; Iodination; Peroxidase; Myeloperoxidase; Lactoperoxidase

Arachidonic acid undergoes iodination in the presence of hydrogen peroxide, iodide, and either eosinophil peroxidase, myeloperoxidase or lactoperoxidase. The profile of products generated by each of the three peroxidases is similar as determined by reversed-phase high-performance liquid chromatography. Structural analysis of the products indicate that: 1, each of the four double bonds in arachidonic acid is susceptible to iodination; 2, arachidonic acid can be multiply iodinated; and 3, the carboxylate moiety does not participate in the formation of all products. The isomeric composition of the isolated products indicates that peroxidase-mediated iodination of arachidonate is not stereoselective.

Introduction

Eosinophils contain a peroxidase which in the presence of hydrogen peroxide and iodide exerts a cytotoxic effects on tumor cells [1] fungi [2], bacteria [3–5] and parasites [6]. The eosinophil peroxidase/H_2O_2/iodide system is known to catalyze the iodination of proteins [7], as do comparable systems containing the enzymes thyroid peroxidase, lacteroperoxidase or myeloperoxidase [8]. Halogenation of target cell-associated protein may thus be one of the biochemical mechanisms underlying the cytotoxic effects of peroxidase/H_2O_2/halide systems. It is possible that covalent modification of molecules other than proteins also occurs and contributes to the cytotoxic effects of peroxidase-containing systems.

It has recently been demonstrated that unsaturated lipids undergo covalent modification in the presence of peroxidase/H_2O_2/halide systems. Arachidonic acid is transformed to a variety of iodinated compounds, including at least three distinct iodolactones by systems containing lactoperoxidase [9,10] or thyroid peroxidase [9]. Leukotrienes B_4 [11], C_4 [11,12] and D_4 [11] are converted to biologically inactive compounds in the presence of eosinophil peroxidase, H_2O_2 and iodide. Further, intact eosinophils [11,12] and neutrophils [13,14], when appropriately stimulated, can transform prostaglandins [13] and leukotriene C_4 [11,12,14] to inactive products by a mechanism which involves peroxidase, H_2O_2 and halide ions. The transformation of lipid mediators by eosino-

* Present address: Departments of Medicine and Pharmacology, Box 8118,Washington University School of Medicine, 660 S. Euclid, St. Louis, MO 63110, U.S.A.

** To whom correspondence should be addressed.

Abbreviations: BSTFA, *N,O*-bistrimethylsilyltrifluoroacetamide; 6I-5-HET lactone, 6-iodo-5-hydroxyeicosatrienoic acid δ-lactone; 15I-14-HET lactone, 15-iodo-14-hydroxyeicosatrienoic acid ω-lactone; 14I-15-HET lactone, 14-iodo-15-hydroxyeicosatrienoic acid ω-lactone.

phils and neutrophils through the action of the peroxidase/H_2O_2/halide systems may be a mechanism by which these cells modulate hypersensitivity reactions.

We have identified some of the products formed during the incubation of arachidonic acid, eosinophil peroxidase, H_2O_2 and iodide to gain further insight into the nature of the biochemical transformations of unsaturated lipids mediated by eosinophil peroxidase. These products have been compared to those from similar incubations with lactoperoxidase and myeloperoxidase.

Materials and Methods

Materials

Eosinophil peroxidase was purified from horse eosinophils as described by Jörg et al. [5] and myeloperoxidase was purified from canine pyometral pus by the method of Agner [15] to the end of step 6. Lactoperoxidase was obtained from Sigma Chemical Company, St. Louis, MO. Peroxidase activity was determined by guaiacol oxidation [16]. 1 unit of enzyme is the amount which oxidizes 1 μmol of guaiacol/min at 25°C, using a molar absorbancy for the product, tetraguaiacol, of $2.66 \cdot 10^4$ cm^{-1} at 470 nm [17]. [5,6,8,9,11,12,14,15-$^2H_8$]Arachidonate was prepared from eicosatetraynoic acid and deuterium gas as described [18]. Arachidonic acid was obtained from NuChek Prep, Elysian, MN; NaI and H_2O_2 from Fischer Chemical, St. Louis MO; [5,6,8,9,11,12,14,15-$^3H_8$]arachidonic acid and Na^{125}I from New England Nuclear (Boston, MA); guaiacol (anhydrous) from Sigma Chemical Co. and catalase (beef liver; 64700 units/mg) from Worthington Biochemical Corp. Freehold, NJ. The catalase was dialyzed against water before use. All organic solvents were obtained from Burdick and Jackson, Muskegon, MI, and all other reagents were of the highest commercial grade available.

Methods

Peroxidase incubations. Eosinophil peroxidase incubations were performed in 0.02 M sodium phosphate buffer (pH 5.0), with 10^{-4} M NaI (0.5 μCi ^{125}I), 10^{-4} M H_2O_2, 10^{-3} M arachidonic acid (10 μCi [$^3H_8$]arachidonate), and 122 mU eosinophil peroxidase in a total volume of 0.5 ml. Incubations were continued for 30 min at 37°C and the reaction terminated by the addition of 0.10 ml of 0.01 M sodium thiosulfate. Myeloperoxidase incubations were performed under similar conditions except that 0.1 M NaCl was included in the incubation medium. Lactoperoxidase incubations with arachidonic acid at pH 5 and 7 were performed as described elsewhere [9,10].

Product recovery and analysis. After the addition of sodium thiosulfate the incubation medium was diluted with 1 vol. of H_2O and extracted twice with 2 vol. of CH_2Cl_2. The combined extracts were concentrated to dryness under nitrogen, reconstituted in methanol, and chromatographed on a Waters (Milford, MA) μBondapak C_{18} HPLC column (3.9 mm × 30 cm) at a flow rate of 1 ml per min with the solvent methanol/water/acetic acid (80 : 20 : 0.01, v/v), and sequential 1-ml fractions collected. Aliquots of each fraction were employed for detection of ^{3}H activity in a liquid scintillation counter and ^{125}I activity in a gamma scintillation counter. Peaks containing radioactivity were extracted from the HPLC solvent with CH_2Cl_2 and either rechromatographed or derivatized for gas-liquid chromatographic (GLC) and mass spectrometric (MS) analysis.

Product derivatization. Methyl esters were prepared with excess diazomethane in diethyl ether. Catalytic hydrogenation was performed in ethanol with hydrogen gas and platinum oxide as described [9]. Hydrolysis of hydrogenated lactones was performed by dissolving approximately 10 μg of these materials in 1.0 ml of dimethoxyethane, adding 0.2 ml of 3 N LiOH, and heating the sealed reaction vial in a nitrogen atmosphere for 90 min at 60°C. The reaction mixture was then concentrated to dryness under N_2, reconstituted in 3 ml H_2O, acidified to pH 5.0 (1 M HCl), and extracted twice with CH_2Cl_2. Iodohydrins were converted to the corresponding epoxides with sodium hydroxide in tetrahydrofuran, and reduction of epoxides with $LiAlH_4$ was performed as described [9]. Silylation was performed with excess *N,O*-bistrimethylsilyltrifluoroacetamide (BSTFA, Pierce Chemical Co., Rockford, IL) in pyridine at 60°C for 15 min.

Product identification. Gas chromatography was performed at 220 or 230°C with a glass GC column (1 m × 2 mm internal diameter) packed with

3% OV-1 (gcq 100/120) interfaced with a Riber-Mag 10-10 quadrapole mass spectrometer (Nermag, Inc., Santa Clara, CA). Carbon values for materials amenable to GLC analysis were calculated from the retention times of a series of saturated fatty acid methyl esters. Materials not amenable to GC analysis were introduced into the mass spectrometer by a desorption probe and were subjected to chemical ionization with ammonia or methane as reagent gas.

Results

A reversed-phase high-performance liquid chromatogram (RP-HPLC) of the products formed by incubation of [^{3}H]arachidonic acid with H_2O_2, [^{125}I]iodide, and eosinophil peroxidase is shown in Fig. 1. The association of ^{3}H and ^{125}I activities in a number of peaks indicated that several iodinated arachidonate derivatives were formed. The ^{125}I activity eluted slightly after the ^{3}H activity in peaks with larger retention volumes due to the separation of the tritium and protium forms of arachidonate in this RP-HPLC system. Since the protium form was much more abundant than the tritium form of arachidonic acid in the incubation mixture, most of the ^{125}I activity was incorporated into molecules lacking ^{3}H activity. Incubations performed at either pH 5.0 or 7.0 resulted in a similar profile of products, but conversion of arachidonate was approximately 10 times greater at pH 5.0. Substitutions of lactoperoxidase or myeloperoxidase for eosinophil peroxidase resulted in the formation of products with the same retention volumes as shown in Fig. 1 with only minor variations in their relative abundance. Iodinated products were not formed when either eosinophil peroxidase or H_2O_2 was omitted from the eosinophil peroxidase/ iodide/ H_2O_2/ arachidonate system or when azide (which inhibits hemeproteins) or catalase (which degrades H_2O_2) was added (Table I). Heated catalase was ineffective in inhibiting peroxidase-catalyzed iodination of arachidonic acid. These findings indicate that the generation of iodinated arachidonate derivatives was dependent on the peroxidatic activity of the eosinophil peroxidase system.

The identity of compounds present in peaks

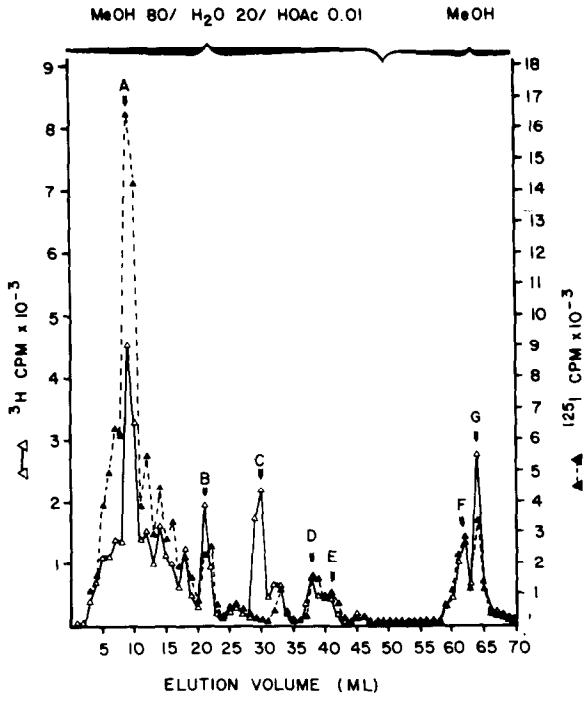

Fig. 1. Reversed-phase high-performance liquid chromatogram of the products formed by incubation of arachidonic acid with eosinophil peroxidase, hydrogen peroxide and iodide. The reaction mixture, incubation, product isolation and separation by RP-HPLC were performed as described in Methods.

TABLE I

IODINATION OF ARACHIDONIC ACID BY THE EOSINOPHIL PEROXIDASE/H_2O_2/IODIDE SYSTEM

The reaction mixture was as described in Methods except that eosinophil peroxidase or H_2O_2 was omitted and 10^{-3} M azide or 25 μg catalase (or catalase heated at 100°C for 15 min) was added where indicated. Incubations werre terminated and the products extracted and analyzed by RP-HPLC as described in Methods. The ^{125}I activity associated with the ^{3}H peaks were summed and the total indicated as a percentage of that observed with the complete eosinophil peroxidase + H_2O_2 + iodide + arachidonic acid system. The results are the mean of four experiments.

Supplements	Iodination
Eosinophil peroxidase + H_2O_2 + iodide + arachidonic acid	100
Eosinophil peroxidase omitted	3
H_2O_2 omitted	3
Azide added	1
Catalase added	1
Heated catalase added	101

A–G in Fig. 1 was investigated. Peak C contained unconsumed arachidonate, as indicated by the lack of ^{125}I activity associated with the ^{3}H activity, by an RP-HPLC retention volume identical to that of standard [^{3}H]arachidonate, and by gas liquid chromatographic and mass spectrometric characterization (data not shown).

Peak B contained material with an RP-HPLC retention volume identical to that of standard 6-iodo-5-hydroxyeicosatrienoic acid δ-lactone (6I-5-HET lactone). Because only limited amounts of eosinophil peroxidase were available, the mass of arachidonate derivatives generated by the enzyme was insufficient for complete GLC-MS characterization. To circumvent this difficulty, octadeutero-(6I-5-HET lactone) was prepared by the action of lactoperoxidase on [5,6,8,9,11,12,14,15-$^{2}H_{8}$]arachidonate [9]. The mass spectrum of this material contains fragment ions from the loss of the lactone ring plus a deuterium atom at *m/z* 338 (*M* − 100), from the loss of iodine at *m/z* 311 (*M* − 127) and from the loss of iodine plus water at *m/z* 292 (*M* − (127 + 19)). The water eliminated during fragmention contains a deuterium atom accounting for the loss of 19 mass units instead of the 18 mass units of H_2O. The corresponding ions in the protium form of the molecule are *m/z* 332, 303, and 285. To demonstrate that this molecule was formed in the eosinophil peroxidase system, 1 μg of octadeutero-(6I-5-HET lactone) was added as an internal standard to the reaction mixture after completion of the incubation. Peak B was then isolated by RP-HPLC, and analyzed by GC-MS with selected ion monitoring. The internal standard is indicated by a peak of ion current at *m/z* 311. The elution time of this peak corresponds to carbon value of 23.9 [9]. Co-eluting peaks at *m/z* 332, 303 and 285 indicated that 6I-5-HET lactone had been formed during the incubation of arachidonic acid with eosinophil peroxidase, H_2O_2 and iodide. The relative abundances of the ions at *m/z* 332, 303 and 285 in the eosinophil peroxidase-derived material were identical to those of the ions at 338, 311 and 292 in the deuterated internal standard.

Peaks D and E were found to contain 15-iodo-14-hydroxyeicosatrienoic acid ω-lactone (15I-14-HET lactone) and 14-iodo-15-hydroxyeicosatrienoic acid ω-lactone (14I-15-HET lactone), respectively [10], by methods similar to those described above.

The predominant peak obtained from incubation of arachidonic acid with the eosinophil peroxidase (EPO) system was peak A, designated A_{EPO}. When A_{EPO} was rechromatographed with a solvent system of methanol/water/acetic acid (75 : 25 : 0.01, v/v), a single, symmetrical peak with an elution volume of 14–19 ml was seen. A quotient of 1.87 was obtained when the ratio of ^{125}I to ^{3}H activities in this peak was divided by the corresponding ratio in the 15I-14-HET lactone peak from the same incubation, suggesting that the material and A_{EPO} was doubly iodinated. The short retention volume of A_{EPO} on RP-HPLC indicated that A_{EPO} was more polar than the iodolactones in peaks B, D and E. These data suggested that A_{EPO} contained materials with free hydroxyl groups and/or a free carboxyl moiety.

Large scale incubations were performed with lactoperoxidase (LPO)/H_2O_2/iodide, and arachidonate (10 mg) to obtain sufficient material (designated A_{LPO}) for structural analysis. The RP-HPLC retention volume of A_{LPO} was identical to that of A_{EPO}. Although neither the underivatized form nor the methyl ester of A_{LPO} could by analyzed by GLC, trimethylsilylation of the methyl ester of A_{LPO} resulted in a derivative which was amenable to GLC analysis (carbon value, 26.0). These observations suggested that A_{LPO} contained compounds with both a free carboxyl moiety and free hydroxyl groups.

A partial electron-impact mass spectrum of the methyl ester/trimethylsilyl ether derivative of A_{LPO} is shown in Fig. 2, panel A. The molecule was postulated to be a double iodohydrin at the 8,9 and 14,15 positions on the basis of prominent ions at *m/z* 383 $(((CH_3)_3SiO)CHCHICH_2(CH)_2(CH_2)_3CO_2CH_3)^+$, *m/z* 313 $(((CH_3)_3SiO)CHCHI(CH_2)_4CH_3)^+$ and a less abundant ion at *m/z* 437 (*M* − 313). These assignments were supported by the mass spectrum of the methyl ester/trimethylsilyl ether derivative of the octadeutero form of A_{LPO} which contained prominent ions at *m/z* 387 $(((CH_3)_3SiO)C^2HC^2HICH_2(C^2H)_2(CH_2)_3CO_2CH_3)^+$, *m/z* 315 $(((CH_3)_3SiO)^2HC^2HI(CH_2)_4CH_3)^+$ and a less abundant ion at *m/z* 443 (*M* − 315) (data not shown).

The postulated molecular ion (*m/z* 750) could

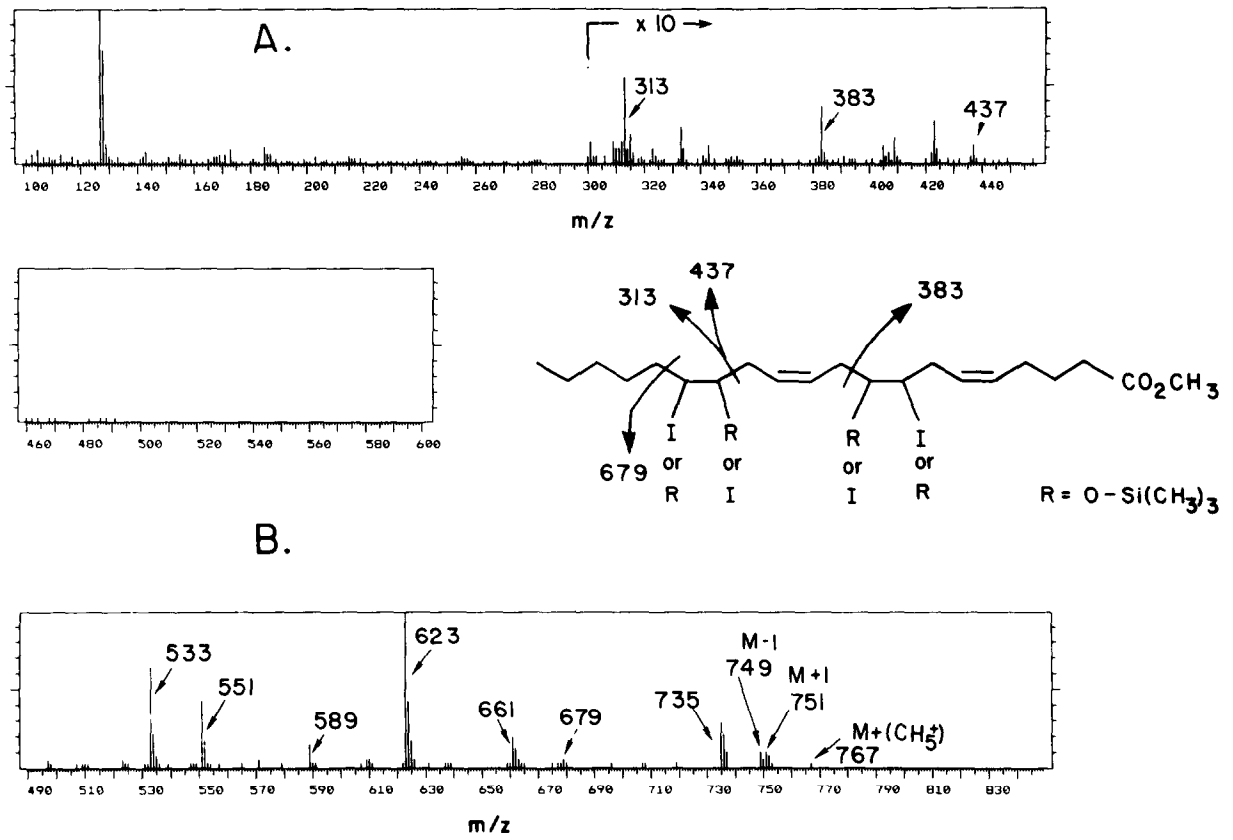

Fig. 2. Panel A, partial electron-impact mass spectrum of the methyl ester/trimethylsilyl ether derivative of the material in peak A_{LPO}. Panel B, partial methane chemical ionization mass spectrum of the methyl ester/trimethylsilyl ether derivative of the material in RP-HPLC peak A_{LPO}.

not be demonstrated by electron-impact mass spectrometry. The methyl ester/trimethylsilyl ether derivative of A_{LPO} was therefore subjected to chemical ionization mass spectrometry with methane (CH_4) as reagent gas (Fig. 2, panel B). The presence of ions at m/z 751 ($M + H^+$) 749 ($M - H^+$) and 767 ($M + (CH_5)^+$) is consistent with a molecular weight of 750 for the material and A_{LPO}. Chemical ionization mass spectrometry with NH_3 as reagent gas gave a prominent ion at m/z 768 ($M + (NH_4)^+$) which further substantiates a molecular weight of 750 for this material. Other ions in the CH_4 chemical ionization mass spectrum of the methyl ester/trimethyl derivative of A_{LPO} include m/z 735 ($M - 15$, loss of CH_3), 679 ($M - 71$, loss of C16–C20), 661 (($M + 1$) − 90, loss of trimethylsilanol), 623 ($M - 127$, loss of I), 589 ($M - (90 + 71)$, loss of trimethylsilanol + C16–C20), 551 (($M - 1$) − (127 + 71), loss of I and C16–C20) and 533 ($M - (127 + 90)$, loss of I and trimethylsilanol). The presence of these fragment ions is consistent with a molecular weight for A_{LPO} of 750, but does not indicate the positions of iodohydrins in the fatty acid chain.

To establish firmly the locations of the hydroxyl groups of the iodohydrins in A_{LPO}, the material was subjected to catalytic hydrogenation. This resulted in saturation of the double bonds and loss of iodine via exchange with hydrogen, leaving the hydroxyl groups as the major fragment-orienting moieties [9,10,19]. The methyl ester/trimethylsilyl ether derivative of the hydrogenation product of A_{LPO} exhibited a carbon value of 24.0. Its electron-impact mass spectrum is shown in Fig. 3. Ions were observed at m/z 502 (M), 501 ($M - 1$), 487 ($M - 15$, loss of CH_3) and 471 (M

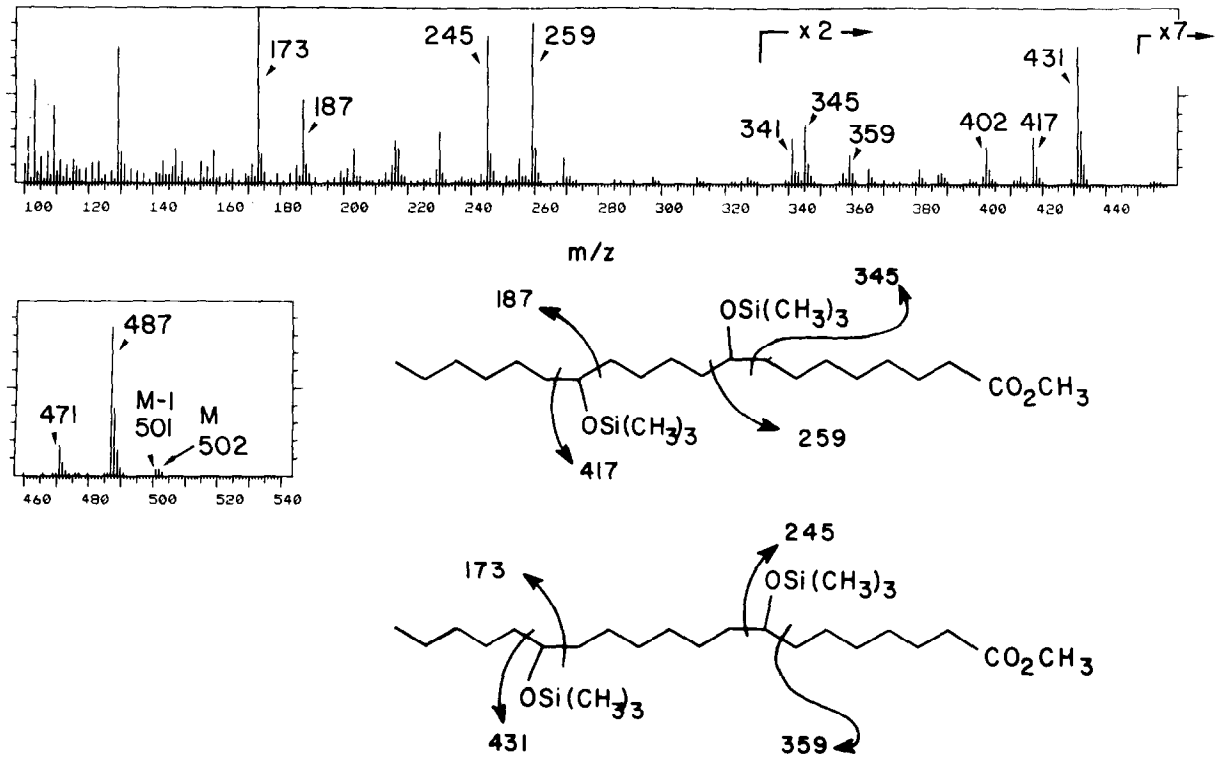

Fig. 3. Electron-impact mass spectrum of the methyl ester/trimethylsilyl ether derivative of the hydrogenation product of the material in RP-HPLC peak A_{LPO}.

-31, loss of OCH_3). These ions are consistent with the presence of one derivatized carboxyl group and two derivatized hydroxyl groups on a saturated 20-carbon chain. The presence of derivatized hydroxyl groups at carbons 8 or 9 and at carbons 14 or 15 is indicated by the fragment ions at m/z: 173 $(((CH_3)_3SiO)CH(CH_2)_3CH_3)^+$, 187 (173 + CH_2), 245 $(((CH_3)_3SiO)CH(CH_2)_6CO_2CH_3)^+$, 259 (245 + CH_2), 345 ($M-157$, loss of $(CH_2)_7CO_2CH_3$), 359 (345 + CH_2), 417 ($M-85$, loss of $(CH_2)_5CH_3$), and 431 (417 + CH_2).

It was anticipated that the iodine atoms in A_{LPO} would be substituents of carbon atoms in arachidonic acid that had participated in iodohydrin formation and that these carbon atoms would be adjacent to those bearing hydroxyl groups [9,10,19]. To evaluate this possibility, A_{LPO} was subjected to base-catalyzed dehydrohalogenation which results in the replacement of each iodohydrin with an epoxy group [9]. The methyl ester of this material was subjected to catalytic hydrogenation to saturate the double bonds. The mass spectrum of the resultant compound (carbon value, 22.8) contained ions at m/z 354 (M), 339 ($M-15$, loss of CH_3), 336 ($M-18$, loss of H_2O), 323 ($M-31$, loss of OCH_3) and 305 ($M-49$, loss of OCH_3 and H_2O). These fragment ions indicate the presence of one derivatized carboxyl group and two epoxy groups on an otherwise saturated 20-carbon chain. The ions of lower mass range in the mass spectrum did not indicate the locations of the epoxy groups. The presence of ether linkages rather than epoxy groups could not be excluded.

The methyl ester of the hydrogenated double epoxide was therefore treated with lithium aluminum hydrided. Such treatment converts the epoxides to a mixture of isomers bearing hydroxyl groups at one or the other of the carbon atoms that had originally participated in the formation of the epoxides [9]. In addition, the carboxylate ester is converted to an alcohol [9]. Ethers are inert to this treatment. The electron-impact mass spectrum

of the trimethylsilyl ether derivative (data not shown) of the resultant material (carbon value 24.4) contained several fragment ions. Ions were observed at *m/z*: 546 (*M*), 545 (*M* − 1) and 531 (*M* − 15). These ions were consistent with the presence of two derivatized secondary hydroxyl groups and one derivatized primary hydroxyl group on a saturated 20-carbon chain. The mass spectrum obtained was felt to reflect a mixture of isomers bearing derivatized secondary hydroxyl groups at carbon atoms 8 or 9 and at carbon atoms 14 or 15 on the basis of the following ions, *m/z*: 173 $(((CH_3)_3SiO)CH(CH_2)_4CH_3)^+$, 187 (173 + CH_2), 289 $(((CH_3)_3SiO)CH(CH_2)_6CH_2O\text{-}Si(CH_3)_3)^+$, 303 (289 + CH_2), 345 (*M* − 201, loss of $(CH_2)_7CH_2OSi(CH_3)_3$); 359 (*M* − 187, loss of 201 minus CH_2), 461 (*M* − 85, loss of $(CH_2)_5CH_3$) and 475 (*M* − 71, loss of 85 minus CH_2).

These mass spectral data indicate that A_{LPO} contains a mixture of isomeric compounds with: 1, a 20-carbon linear chain; 2, two hydroxyl groups with one at either carbon 8 or 9 and the other at either carbon 14 or 15; 3, two iodine atoms with one at either carbon 8 or 9 and the other at either carbon 14 or 15; 4, two double bonds; and 5, one carboxyl group at carbon 1. Assuming that the double bonds remain at C5,6 and at C11,12 and retain the configuration of the precursor fatty acid [9,10,19], the mixture of compounds in peak A can be assigned the following structures: (1) 8,15-diiodo-9,14-dihydroxyeicosa-5,11-*cis*-dienoic acid; (2) 9,14-diiodo-8,15-dihydroxyeicosa-5,11-*cis*-dienoic acid; (3) 8,14-diiodo-9,15-dihydroxyeicosa-5,11-*cis*-dienoic acid and (4) 9,15-diiodo-8,14-dihydroxyeicosa-5,11-*cis*-dienoic acid.

The materials contained in A_{LPO} and A_{EPO} appeared to be identical, based on experiments utilizing A_{LPO} generated from octadeuteroarachidonate ($^2H_8A_{LPO}$). Octadeuterated A_{LPO} was added as an internal standard to the products of an incubation of eosinophil peroxidase, arachidonate, H_2O_2 and iodide. A_{EPO} was then isolated by RP-HPLC converted to the methyl ester/trimethyl derivative, and subjected to GC-MS analysis with selected ion monitoring. Co-eluting peaks in ion current (carbon value, 26.0) were observed at *m/z* 387 (internal standard) and at *m/z* 313,383 and 437 from the double iodohydrin formed during the incubation with eosinophil peroxidase.

Peaks F and G (Fig. 1) contained singly iodinated compounds as judged from the ratio of ^{125}I to ^3H activities, which was identical to that of 15I-14-HET lactone. The extremely non-polar behavior of these materials on RP-HPLC suggested that they contained neither a free hydroxyl nor a carboxyl group. Structural analyses of material in peaks F and G were performed with material derived from incubation with lactoperoxidase, designated, respectively, F_{LPO} and G_{LPO}. These materials were not amenable to GLC analysis before or after treatment with diazomethane and excess silylating reagent.

The scheme depicted in Fig. 4 was employed for structural analysis of the monoiodinated material in peaks F_{LPO} and G_{LPO}. Because the materials contained in these peaks were not amenable to vapor-phase analysis, they were introduced into the mass spectrometer operating in the chemical ionization mode (reagent gas, methane) with a desorption probe. Desorption-chemical ionization mass spectroscopic analysis of the material in peaks F_{LPO} and G_{LPO} in this manner gave a mass spectrum (spectrum not shown) typical of those obtained during similar analysis of iodolactones previously identified [9,10]. Ions in the desorption-chemical ionization mass spectrum typical of iodolactones and their intensities relative to the base peak (in parenthesis) include: *m/z* 431 (*M* + 1; (24%)), 429 (*M* − 1; (6%)), 303 (*M* − 127, loss of I; (base peak)) and 285 (*M* − 145, loss of I + H_2O; (16%)). These data are consistent with the presence of an iodolactone or a mixture of iodolactones in peaks F_{LPO} and G_{LPO}.

Catalytic hydrogenation of unsaturated iodolactones, in addition to saturating the double bonds in the fatty acid chain, results in the substitution of hydrogen for the atom of iodine [9,10,19]. Reduction of the monoiodinated material in peaks F_{LPO} and G_{LPO} with H_2 in the presence of platinum oxide followed by desorption-chemical ionization mass spectrometry indicated the presence of one or more saturated 20 carbon alkyl lactones (Fig. 4). Ions in the mass spectrum and their relative abundancies (in parenthesis) include: *m/z* 311 (*M* + 1; (base peak)), 309 (*M* − 1; (95%)), 327 ((*M* + (CH_5^+); (11%)) and 293((*M* + 1) − 18,

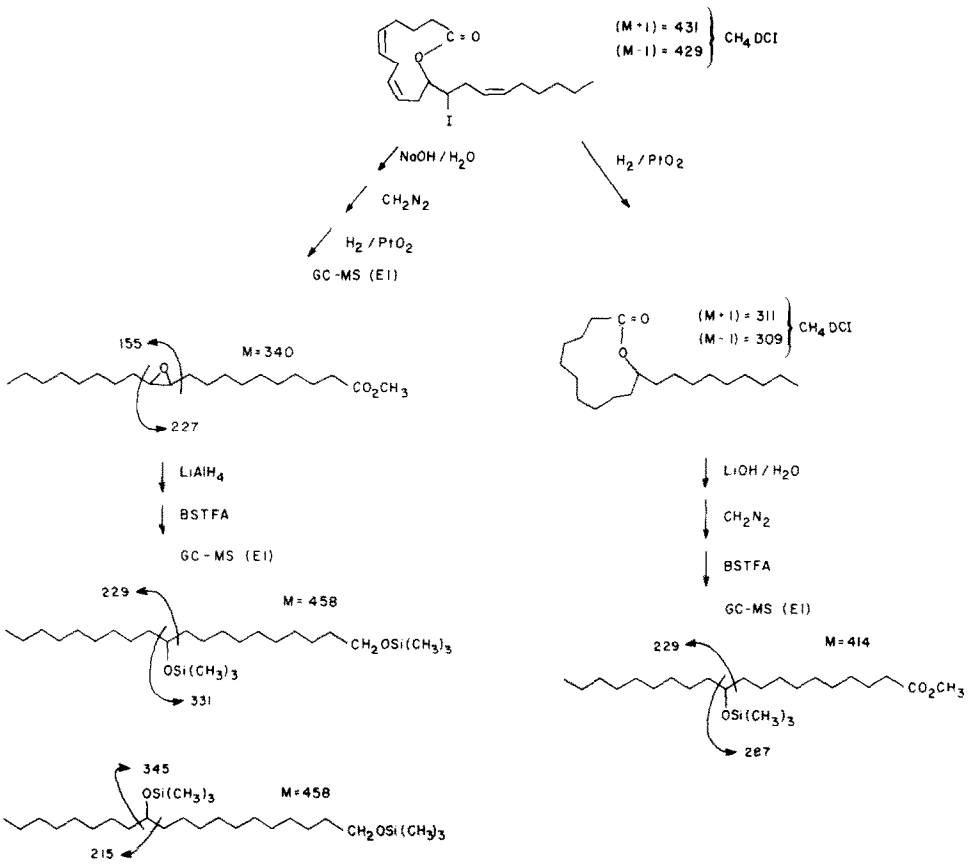

Fig. 4. Scheme for structural analysis of materials contained in RP-HPLC peaks F_{LPO} and G_{LPO}. The sequence of analysis is illustrated with a single compound for clarity. Reaction conditions are detailed in Methods. The modes of mass spectroscopic analysis employed are abbreviated as follows: CH_4DCl, desorption-chemical ionization with methane as reagent gas and GC-MS(EI), electron-impact gas-liquid chromatography-mass spectrometry. *M* denotes the mass of the molecular ion. Chemical modification and derivatization procedures are abbreviated as follows: H_2/PtO_2, catalytic hydrogenation; $NaOH/H_2O$, base-catalyzed dehydroiodination; CH_2N_2, esterification with excess diazomethane; $LiAlH_4$, reduction with lithium aluminum hydride; $LiOH/H_2O$, hydrolysis with lithium hydroxide and BSTFA = silylation. The values of *M* depicted in the figure are those determined during mass spectroscopic analysis of each product or mixture of products.

loss of water; (40%)). These data indicate the presence of one or more saturated 20-carbon alkyl lactones ($M = 310$). Typically, a fragment ion arising from the loss of water is observed in mass spectral analysis of hydrogenated products of monoiodinated iodolactones formed from unsaturated fatty acids [9,10,19]. These data further substantiate the presence of one or more iodolactones in peaks F_{LPO} and G_{LPO}, but does not indicate which of the carbon atoms participated in iodohydrin formation.

Alkaline (lithium hydroxide) hydrolysis of saturated alkyl lactones derived from iodohydrins converts the intramolecular ester to the monohydroxy carboxylic acid (Fig. 4). The position of the hydroxyl moiety of the original iodohydrin can be determined via GLC-MS analysis of the methyl ester/trimethylsilyl ether derivative of the monohydroxy carboxylic acid ($M = 414$, carbon value = 22.0). Typically, fragmentation during electron impact of saturated monohydroxy fatty acids so derivatized occurs between the silylated hydroxyl moiety and α-carbon atoms [20]. The fragment ions that are formed from such compounds derivatized in this manner and the position of the carbon atom bearing the derivatized hydroxyl

TABLE II

FRAGMENT IONS FORMED DURING ELECTRON IMPACT VIA α-CLEAVAGE OF ESTERIFIED, SILYLATED SATURATED 20-CARBON MONOHYDROXY FATTY ACIDS

Fragment ions, m/z	Position of OH moiety
173,343	C-15
187,329	C-14
215,301	C-12
229,287	C-11
257,259	C-9
271,245	C-8
299,217	C-6
313,203	C-5

group giving rise to those fragment ions are listed in Table II. The presence of fragment ions at each of the m/z values listed in Table II from the hydrogenated-alkaline hydrolysis product from peaks F_{LPO} and G_{LPO} indicated that the material present was a mixture of iodolactones arising from iodohydrin formation at each of the four double bonds of arachidonic acid. These data further suggest that the OH moieties of the iodohydrins were introduced at each terminus of the double bonds of arachidonic acid.

In order to determine the presence of iodohydrins, the material in peaks F_{LPO} and G_{LPO} was subjected to base-catalyzed (NaOH) dehydroiodination followed by esterification (diazomethane) and catalytic hydrogenation (Fig. 4). Such treatment converts lactonized monoiodohydrins to the corresponding saturated, esterified monoepoxides [9,10.19]. The fragmentation pattern of the methyl ester of different saturated 20-carbon monoepoxides during electron impact have been determined [21]. Typically, ions in the mass spectra of such compounds include m/z 340 (M), 322 ($M-18$, loss of H_2O) and 309 ($M-31$, loss of OCH_3). Additional fragmentation occurs between the epoxide and α-carbon atoms. Table III lists the characteristic fragment ions arising from α-cleavage of the saturated fatty acid chain defining the position of the different monoepoxides. The presence of these fragment ions in the mass spectrum of material from peaks F_{LPO} and G_{LPO} after base-catalyzed dehydroiodoination, esterification

TABLE III

FRAGMENT IONS FORMED DURING ELECTRON IMPACT VIA α-CLEAVAGE OF ESTERIFIED SATURATED 20-CARBON MONOEPOXIDES

Fragment ions, m/z	Position of epoxide
143,239	C-5,6
197,185	C-8,9
227,155	C-11,12
269,113	C-14,15

and catalytic hydrogenation indicates that the material in these peaks was a mixture of lactonized monoiodohydrins.

Additional evidence for the presence of a mixture of saturated 20-carbon monoepoxides (and not ethers) derived from the material in peaks F_{LPO} and G_{LPO} was obtained via reduction of the products generated by base-catalyzed dehydroiodination and catalytic hydrogenation with lithium aluminum hydride ($LiAlH_4$) as shown in Fig. 4. Reduction of these epoxides with $LiAlH_4$ converts the epoxides to a mixture of isomeric secondary alcohols and the carboxylate ester to a primary alcohol [9,19]. After silylation, the $LiAlH_4$-reduced material was analyzed via GLC-MS analysis. A mass spectrum of the $LiAlH_4$-reduced product (carbon value, 22.5) is shown in Fig. 5. The fragment ions at m/z 457 ($M-1$) and 443 ($M-15$) indicate the presence of a compound or a mixture of isomeric compounds having a molecular weight of 458. The remainder of the fragment ions in the mass spectrum and substituents comprising those fragment ions (Fig. 5) indicate that after $LiAlH_4$ reduction a mixture of silylated diols are present. Specifically, the diols present and fragment ions indicating their presence include: 1,5(m/z 247, 313); 1,6 (m/z 261, 299); 1,8 (m/z 271, 289); 1,9 (m/z 257, 303); 1,11 (m/z 229, 331); 1,12 (m/z 215, 345); 1,14 (m/z 187, 373) and 1,15 (m/z 173, 387).

The mixture of compounds contained in peaks F_{LPO} and G_{LPO} peaks thus may be assigned the following structures: (1) 6-iodo-5-hydroxyeicosa-8,11,14-trienoic acid 1,5-lactone; (2) 5-iodo-6-hydroxyeicosa-8,11,14-trienoic acid 1,6-lactone; (3) 8-iodo-9-hydroxyeicosa-5,11,14-trienoic acid

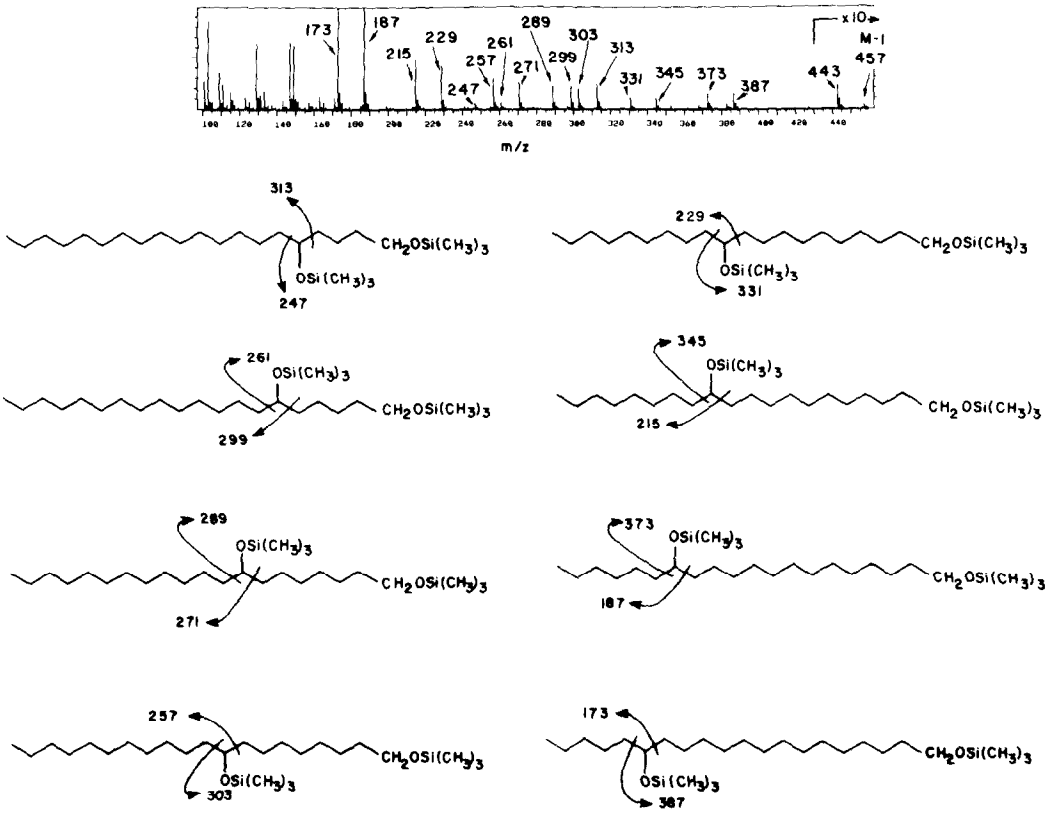

Fig. 5. Electron-impact mass spectrum of material from RP-HPLC peak F_{LPO} after base-catalyzed dehydroiodination, hydrogenation, reduction with $LiAlH_4$, and trimethylsilylation.

1,9-lactone; (4) 9-iodo-8-hydroxyeicosa-5,11-14-*cis*-trienoic acid 1,8-lactone; (5) 11-iodo-12-hydroxyeicosa-5,8,14-trienoic acid 1,12-lactone; (6) 12-iodo-11-hydroxyeicosa-5,8,14-trienoic acid 1,11-lactone; (7) 15-iodo-14-hydroxyeicosa-5,8,11-trienoic acid 1,14-lactone and (8) 14-iodo-15-hydroxyeicosa-5,8,11-trienoic acid 1,15-lactone.

The structural basis for the RP-HPLC separation of compounds in peaks F and G has not been formally established but is likely related to stereochemical configurations of the two populations of compounds. Iodohydrin formation generates two optical centers with four possible diastereoisomers, some of which may be separable by RP-HPLC. The presence of geometric isomers about the double bonds cannot be excluded, but it is not obvious how the geometry of double bonds not participating in iodohydrin formation could be altered by the peroxidase reaction.

It has not been directly established that the materials in peaks F and G derived from incubations with eosinophil peroxidase and myeloperoxidase are identical to those described above, but this is considered likely on the basis of their retention volume on RP-HPLC and the overall similarity of products generated by eosinophil peroxidase, myeloperoxidase and lactoperoxidase.

Discussion

The data presented above constitute the first demonstration of the following features of peroxidase-mediated iodination of arachidonate: 1, eosinophil peroxidase and myeloperoxidase, like lactoperoxidase, can catalyze the iodination of arachidonate, and the profile of products generated by each of the three enzymes is similar; 2, each of the four double bonds in arachidonate is susceptible to iodination in the presence of peroxidase/H_2O_2/iodide systems; 3, multiple iodina-

tion of arachidonate can occur; 4, iodohydrin formation can occur without participation of the carboxyl group. The lack of regional specificity and apparent lack of stereospecificity in the iodination of arachidonate suggests that this process does not necessarily involve direct interaction of arachidonate and the peroxidase enzyme. Participation of the enzyme in this process may be limited to the generation of an oxidized iodine species (e.g., HOI or iodinium ion) which then reacts non-specifically with arachidonate. The similar profile of iodinated arachidonate derivatives produced by three different peroxidases is compatible with this hypothesis. Brominated and chlorinated derivatives of arachidonic acid also may be formed by peroxidase systems containing these halides. The possibility that halogenated derivatives of arachidonic acid may have biological activity remains to be explored.

Formation of compounds analogous to those described here could be among the mechanisms by which myeloperoxidase and eosinophil peroxidase inactivate prostaglandins and leukotrienes [11, 12,14]. Leukotrienes derived from initial dioxygenation of arachidonate at carbon 5 retain a non-conjugated *cis* double bond between carbons 14 and 15 which could undergo transformation to an iodohydrin, or possibly an iodolactone. Similarly, bisenoic prostaglandins and the recently described leukotrienes derived from initial dioxygenation at carbon 15 [22] retain a non-conjugated *cis* double bond between carbons 5 and 6 which could also undergo transformations analogous to those described here. These processes may not represent the sole mechanism of inactivation of prostaglandins or leukotrienes by peroxidase-containing phagocytes. Covalent modification of the conjugated double bond system or the sulfur-containing substituent of leukotriene C_4 could alsoo occur [12,14]. Hydroxyl radicals ($OH^{\cdot}$) generated either by the iron-catalyzed interaction of superoxide and H_2O_2 (Haber-Weiss reaction) or a direct interaction between iron and H_2O_2 (Fenton-type reaction) inactivate the chemotactic activity of leukotriene B_4 [23] and the slow-reacting substance bioactivity of leukotrienes C_4, D_4, and E_4 (Henderson, W.R. and Klebanoff, S.J., unpublished data). Since $OH^{\cdot}$ are generated by phagocytes during the respiratory burst, their interaction with arachidonic acid metabolites may be an additional mechanism by which phagocytes modulate the activity of these potent mediators. The products formed by the interaction of leukotrienes and ($OH^{\cdot}$) have not been defined.

All previously identified iodinated arachidonate derivatives formed by peroxidase-dependent mechanisms have been lactones [9,10]. Formation of such compounds requires the presence of a free carboxyl group in the unsaturated fatty acid in which iodohydrin formation occurs. The isolation and identification of double iodohydrins retaining a free carboxyl group demonstrates that the carboxyl group need not participate in the formation of all iodonated products of arachidonic acid. This suggests that esters of arachidonic acid and perhaps other polyunsaturated fatty acids may also be iodinated by the peroxidase systems. We have recently demonstrated the iodination of methyl arachidonate by eosinophil peroxidase, H_2O_2 and iodide (Hubbard, W.C., unpublished data). It is therefore possible that arachidonate and other polyunsaturated fatty acids esterified to glycerol in membrane phospholipid and other cellular lipids could undergo peroxidase-mediated halogenation. Such a process could contribute to the cytotoxicity of peroxidase/H_2O_2/halide systems.

Acknowledgements

This work was supported by grants GM 15431, AM 28511, AI 17758 and AI 07763 from the National Institutes of Health and by a grant from the Rockefeller Foundation. J.T. was supported by grant GM 07569 and W.R. H. is the recipient of an Allergic Diseases Academic Award AI 00487 from the National Institute of Allergy and Infectious Diseases. The invaluable technical assistance of Gertrude Chiang, John Lawson, Mark Phillips, Ella Stitt and Ann Waltersdorph and the secretarial assistance of Janice Neely are gratefully acknowledged. The advice and interest of Dr. John Oates is much appreciated.

References

1 Jong, E.C. and Klebanoff, S.J. (1980) J. Immunol. 124, 1949–1953

2 Lehrer, R.I. (1969) J. Bacteriol. 99, 361–365

3 Migler, R., DeChatelet, L.R. and Bass, D.A. (1978) Blood 51, 445–456
4 Jong, E.C., Henderson, W.R. and Klebanoff, S.J. (1980) J. Immunol. 124, 1378–1382
5 Jörg, A., Pasquier, J. and Klebanoff, S.J. (1982) Biochim. Biophys. Acta 701, 185–191
6 Jong, E.C., Mahmoud, A.A.F. and Klebanoff, S.J. (1981) J. Immunol. 126, 468–471
7 Henderson, W.R., Jong, E.J. and Klebanoff, S.J. (1980) J. Immunol. 124, 1383–1388
8 Klebanoff, S.J., Yip, C. and Kessler, D. (1962) Biochim. Biophys. Acta 58, 563–574
9 Boeynaems, J.M. and Hubbard, W.C. (1980) J. Biol. Chem. 225, 9001–9004
10 Boeynaems, J.M., Regan, D. and Hubbard, W.C. (1981) Lipids 16, 246–249
11 Henderson, W.R., Jörg, A. and Klebanoff, S.J. (1982) J. Immunol. 128, 2609–2613
12 Goetzl, E.J. (1982) Biochem. Biophys. Res. Comm. 106, 270–275
13 Paredes, J. and Weiss, S.J. (1982) J. Biol. Chem. 257–2738–2740
14 Lee, C.W., Lewis, R.A., Corey, E.J., Barton, A., Oh, H., Tauber, A.I. and Austen, K.F. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 4166–4170
15 Agner, K. (1958) Acta Chem. Scand. 12, 89–94
16 Himmelhoch, S.R., Evans, W.H., Mage, M.G. and Peterson, E.A., (1969) Biochemistry 8, 914
17 Maehly, A.C. and Chance, B. (1954) in Methods of Biochemical Analysis, Vol. 1 (Glick, D., ed.), pp. 357–408, Interscience Publishers, New York
18 Taber, D., Phillips, M. and Hubbard, W.C. (1981) Prostaglandins 22, 349–352
19 Boeynaems, J.M., Watson, J.T., Oates, J.A. and Hubbard, W.C. (1981) Lipids 16, 323–327
20 Boeynaems, J.M., Brash, A.R., Oates, J.A. and Hubbard, W.C. (1980) Analyt. Biochem. 104, 259–267
21 Oliw, E.H., Guengerich, F.P. and Oates, J.A. (1982) J. Biol. Chem. 257, 3771–3781
22 Maas, R.L., Brash, A.R. and Oates, J.A. (1981) Proc. Natl. Acad. Sci, U.S.A. 78, 5523–5527
23 Henderson, W.R. and Klebanoff, S.J. (1982) Biochem. Biophys. Res. Commun. 110, 266–272