Oxidation of Docosahexaenoic Acid by Rat Liver Microsomes*

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[1-14C]Docosahexaenoic acid (n-3) was incubated at 37 °C for 30 min in the presence of rat liver microsomes and 1 mM NADPH. The products were isolated using organic solvent extractions, reverse phase, and normal phase high performance liquid chromatography. Isolates were identified using ultraviolet spectroscopy, capillary gas-liquid chromatography, and gas chromatography-mass spectrometry. The major metabolites were: 19,20-, 16,17-, 13,14-, 10,11-, and 7,8-dihydroxydocosapentaenoic acids, 22-hydroxydocosahexaenoic acid, and 21-hydroxydocosahexaenoic acid. The minor metabolites were 17-hydroxy-4,7,10,13,15,19-, 16-hydroxy-4,7,10,17,19-, 14-hydroxy-4,7,10,12,16,19-, 13-hydroxy-4,7,10,14,16,19-, 11-hydroxy-4,7,9,13,16,19-, 10-hydroxy-4,7,11,13,16,19-, 8-hydroxy-4,6,10,13,16,19-, and 7-hydroxy-4,8,10,13,16,19-docosahexaenoic acids. These metabolites of docosahexaenoic acid resulted from four distinct classes of oxidation, ω-hydroxylations, (ω-1)-hydroxylations, epoxidations, and lipoxygenase-like hydroxylations. The similarity of these product profiles to those reported for comparable microsomal incubations with other essential fatty acids suggest that microsome cytochrome P-450 monoxygenases were involved.

Saturated fatty acids are oxygenated by two microsomal pathways. One route, that of ω-oxidation,1 was early recognized when medium chain length dicarboxylic acids were found in human urine (reviewed in Refs. 1-3). Such dicarboxylic acids are produced by the reaction sequence: primary alcohol to aldehyde to carboxylic acid (1, 2, 4, 5). Microsomal cytochrome P-450 monoxygenase from liver (4-6), kidney (6, 7), adrenal glands (8), and lung (6, 9) will catalyze the initial ω-hydroxylations if O2 and NADPH are present (10, 11). The primary alcohol group is oxidized by alcohol and aldehyde dehydrogenases to the carboxylic acid moiety (1, 12, 13).

Saturated fatty acids also undergo monoxygenase catalysis (ω-1)-oxidations (9, 12, 14). The initial product, a secondary alcohol, is converted by microsomal and cytosolic dehydrogenases into (ω-1)-oxo-fatty acids (15-17). A number of factors alter the ω/(ω-1) product ratios; however, because of this and other reasons (18), it is believed that liver contains two distinct monoxygenase systems which are capable of hydroxylating saturated fatty acids. The first catalyzes only ω-1-hydroxylations (19), hydroxylates steroids and xenobiotics (9), is inducible with phenobarbital pretreatment (12), is inhibited by SKF-525A and metyrapone (9), and can utilize hydroperoxides in place of O2 and NADPH (19). A second minor liver monoxygenase which catalyzes both ω- and ω-1-hydroxylations (19) is specific for fatty acids (20), is not inducible by phenobarbital (12, 21-23) but is induced by starvation (22, 24) and by diets high in fatty acids (21), is not inhibited by SKF-525A (6, 9) or metyrapone (9), and specifically requires O2 and NADPH although the latter requirement may be partially satisfied by NADH (3, 20).

Unsaturated fatty acids are converted to epoxides by P-450 monoxygenases in addition to the ω- and (ω-1)-oxidation products (25-29). The epoxide pathway appears to be a major microsomal oxidation route for polyunsaturated fatty acids pretreated with phenobarbital (27-30), which leads to the appearance in animals of a variety of vicinal diols via hydrolysis of epoxides by the microsomal and cytosolic hydrolases (27).

A pathway of microsomal oxidations, unique to polyunsaturated fatty acids, has been studied exclusively with arachidonic acid (27, 30, 31-33). Arachidonic acid is oxidized to compounds containing conjugated double bonds with allylic hydroxyl groups (hydroxyeicosatetraenoics). The isomers formed are 8- (27), 9- (33), 11- (33), 12- (27, 33), and 15-hydroxyeicosatetraenoic acids (27, 33). Generation of these HETEs requires the initial abstraction of bis-allylic hydrogen atoms (30). This lipoxygenase-like reaction is also mediated by a P-450 monoxygenase (31).

Docosahexaenoic acid is the most unsaturated fatty acid normally occurring in mammals and fish (35). It is found in highest concentration in brain, retina, and testis. The concentration ratios of docosahexaenoic acid to arachidonic acid are 1.4 in rat brain (36), 2.4 in rat retina (35), and 0.6 in human testis (37). The concentration of docosahexaenoic acid increases with age, while that of arachidonic acid decreases in gray matter of the adult human brain (38). The source of brain docosahexaenoic acid is either dietary or from docosahexaenoic acid synthesized in liver from dietary linolenic acid (39). Thus liver plays a central role in making docosahexaenoic acid available for peripheral tissues. In mammals the dietary requirement for n-3 essential fatty acids is very low (less than 40 mg/kg of food) in contrast to the relatively large dietary requirements for n-6 essential fatty acids (35). For this reason, it has been postulated that a metabolite of docosahexaenoic acid, required in only low concentrations, may be the basis for docosahexaenoic acid's dietary "essentiality."
Docosahexaenoic acid is a potent inhibitor of mammalian cyclooxygenases, but a poor substrate for cyclooxygenase and lipoxygenase enzymes (40, 41); it is, moreover, a potent inducer of hepatic monoxygenase (54). The present study was designed to determine if liver microsomes could oxidize docosahexaenoic acid and to identify the metabolites produced.

MATERIALS AND METHODS

Preparation of Hepatic Microsomes—Three groups of 3 male Sprague-Dawley rats (240–270 g) were given daily intraperitoneal injections for 4 days of either 2.5 ml of trioctyglycerol, β-naphthoflavone (12.0 mg dissolved in 2.5 ml of trioctyglycerol), or phenobarbital (2.0 mg dissolved in 2.5 ml of trioctyglycerol). After the fourth injection, each animal was deprived of food overnight.

The rats were decapitated 24 h after the last treatment and livers were removed. All subsequent steps were performed at 0–4 °C. The livers were rinsed in isotonic saline, weighed, and minced. Each liver was homogenized (0.25 g/ml) in a buffered salt solution consisting of 150 mM KCl and 50 mM Tris-HCl (pH 7.5) using a loose fitting Teflon pestle. Combined homogenates from livers of the three animals in each treatment group were mixed and centrifuged at 10,000 X g for 10 min. The supernast was then centrifuged at 100,000 X g for 60 min. The microsomal pellets were washed once, re suspended in buffer (the equivalent of 0.5 g of liver/ml), and used rapidly in the incubations.

Substrate Preparation—[1-14C]Docosahexaenoic acid (22:6, 40 Ci/mmol) was made by total organic synthesis (41). Chemical and radiopurity were greater than 99%, as judged by reverse-phase high performance liquid chromatography and by absorbance at 192 nm. The specific radioactivity of 22:6 used for incubations was adjusted to 0.25 Ci/ml using unlabeled 22:6 (>99% purity, Nuchek, Elysian, MN). The substrate was then converted to a water-soluble solution by suspending in 4 ml of 0.1 M Tris-NaOH (pH 8.5).

Incubation Conditions—Microsomes (approximately 50 mg of protein/5 ml of buffer) were added to 45 ml of buffer being shaken at 37 °C. Three 50-μl aliquots containing (a) 500 pmol of glucose 6-phosphate, (b) 165 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and (c) 50 μmol of NADPH were added. After 1 min, 5 μmol of sodium [14C]docosahexaenoate dissolved in 200 μl 0.1 M Tris-NaOH (pH 9.5) were added. After 30 min of shaking at 37 °C in an open 250-ml flask, each 50 ml of incubated microsomes were mixed with 20 ml of ice-cold methanol. The total number of 50-ml incubates was two, six, and six for C, BNF, and PB, respectively. Control incubations using boiled (5 min) microsomal fractions and incubations in which NADPH was excluded were carried out in 5-ml volumes in triplicate.

Extraction Procedures—Ethanol-quenched reaction mixtures were centrifuged at 1000 X g for 10 min at 4 °C. Pellets were suspended in 80% ethanol and recentrifuged. The combined supernatants were then concentrated to near dryness. Further sample concentration was achieved using several methanol and chloroform washes, a procedure also known to improve protein removal (43). Samples were transferred from round bottom flasks using water followed by ethyl acetate. After adjusting the aqueous phase to pH 3 with HCl, an equal volume of ethyl acetate was added. The isolated aqueous phase was reextracted with equal volumes of ethyl acetate. The combined ethyl acetate extracts were then backwashed with water (20% by volume) to remove residual HCl. Samples were concentrated, filtered (MF-1 Centrifugal Microfilter, Cole Scientific, Calabasas, CA), and stored in 1 ml of methanol at −80 °C until RP-HPLC could be performed.

Chromatographic Conditions—RP-HPLC was performed using a guard column containing Apex (Jones Chromatography, Columbus, OH) 5-μm octadecylsilane particles (44), and an analytical column (Ultrasphere-ODS-5, 0.46 (inner diameter) × 25 cm; Beckman, Irvine, CA). The column was maintained at 30 °C using a Model 322 gradient system (Beckman-Alteck, Berkeley, CA) and UV detectors with a cadmium sensor (Beckman Model 160) and deuterium (Shimadzu Model SPD-2A, Kyoto, Japan) light sources. HPLC grade acetoneitrile and phosphoric acid were obtained from Fisher. Solvents were filtered (0.22 μm, Gelman GHP) prior to use.

For quantitative analysis of radioactivity distribution, each sample was injected in 50 μl of methanol and developed (1.6 ml/min) using an initial mobile phase of (43:57) acetoneitrile/aqueous phosphoric acid (pH 2.3). A linear gradient of 1.43%/min of acetoneitrile was applied 85 min after injection. The HPLC effluent was monitored at 239 and 192 nm. Fractions were collected every 0.5 min and radioactivity measured by scintillation counting techniques. The same RP-HPLC conditions described above were used for preparative runs. However, entire peaks containing radioactivity and absorbance at 192 nm were collected. The fractions were neutralized with excess NaHC03 and acetoneitrile was removed in vacuo. The water residue was dissolved to pH 3 with 5% (v/v) acetone and 4 volumes of acetone were added. After centrifugation, the isolated organic extracts were concentrated and methylated using methanol-ethereal diazomethane (19).

Normal Phase HPLC—Methyl esters of isolates from RP-HPLC were injected in 50 μl of isopropyl alcohol:hexane (5:100) and developed (1.6 ml/min) using 5-μm particle, silicic acid guard (0.46 (inner diameter) × 5 cm; Apex), and analytical (0.46 (inner diameter) × 25 cm; Supelco, Bellafina, PA) columns connected directly to each other. The mobile phase was either 0.21:100, 0.5:100, or 1.0:100 isopropyl alcohol:hexane. For quantitation of radioactivity distributions, 1.0-ml fractions were collected and dried prior to scintillation counting.

Desiratization for Gas Chromatography—Methyl esters, isolated following NP-HPLC, were either directly silylated or catalytically hydrogenated with 5% rhodium on alumina and then silylated. Silylation was done by heating the sample 60 °C for 45 min after it was dissolved in 50 μl HCl and anhydride (44) or bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co.).

Capillary GC—The equivalent chain length of each metabolite was determined by measuring its retention time using capillary GC and relating the log of this retention time to the log retention times of 24:0, 26:0, and 28:0 fatty acid methyl esters. A nonpolar (DB-1, J & W, Rancho Cordova, CA) column (0.25 mm (inner diameter) × 30 m with 0.25 μm film thickness) and a “falling needle” all-glass injector (R. A. Allen Co., Boulder, CO) were employed. The carrier gas (helium) was adjusted to a linear velocity of 20 cm/s for the isothermal (240 °C) separations.

GC Mass Spectrometry—Electron impact (70 eV) studies were conducted using a quadrupole mass spectrometer (Model 3200, Finnigan, Sunnyvale, CA) equipped with a Technivend system (St. Louis, MO). The glass column (2.5 mm × 3 ft) used was silanized and then packed with 3% OV-101 on 80/100 Supelcoport (Supelco). The injector temperature was 295 °C, column temperature was increased at a rate of 2 °C/min from 215–255 °C. The flow rate of helium carrier gas was maintained at 30 ml/min.

Spectroscopic Studies—Difference spectra (45) were determined using a Cary 18 spectrophotometer. UV spectra of isolated metabolites were examined using a Cary 14 instrument equipped with a Hennematsu (Type R 466 HA) photomultiplier tube. Protein determinations were performed according to Lowry et al. (46). Cytochrome P-450 was quantitated using the method of Omura and Sato (47).

RESULTS

The average liver weights from C, BNF, and PB-treated rats were 9.30, 9.50, and 12.37 g, respectively. The specific contents of P-450 were 2.2 (C), 2.7 (BNF), and 4.3 (PB) nmol/mg of microsomal protein. Although incubation conditions were not optimized for kinetic analyses, rates of oxygenation were calculated to be 0.24 (C), 0.30 (BNF), and 0.32 (PB) nmol min−1 mg−1 of microsomal protein. Less than 0.5% of 22:6 was oxygenated by boiled microsomes. Maximal absorptions in CO difference spectra were seen at 450 (C), 448 (BNF), and 450 (PB) nm. A Type I binding spectra (53) with 388 nm peak and 420 nm trough values was observed when 100% control of 22:6 was added to 1.0 ml of phosphate buffer (pH 7.4) containing 1.11 mg of microsomal protein isolated from PB-treated rats. The concentration (Kd) of 22:6 that produced half-maximal spectral changes (45) was 130 μM. Together the above findings indicated that the expected drug related effects of the P-450 system had occurred and that 22:6 had a high affinity for the P-450 cytochrome system.

The average recovery of radioactivity from microsomes after ethanol precipitation and ethyl acetate extraction was 55% (C), 47% (BNF), and 46% (PB). The low recoveries of radioactivity presumably resulted from 22:6 incorporation into phospholipids which were not extracted into 80% ethanol.

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Similar 50% recoveries of radioactivity were reported for 20:4 when incubated and extracted in like manner (28). Boiled microsomes in which no phospholipid metabolism would be expected, resulted in over 94% recovery of the radioactivity added to the incubates.

Table I summarizes the group distributions of radioactivity found with RP-HPLC when [14C]22:6 was incubated with microsomes isolated from rats with different treatments. These numbers represent average values from 2–6 duplicate incubations. As expected from the above measurements on 22:6 oxidation rates, the amount of [14C]22:6 remaining was highest in controls. Also, as reported previously for 20:4, Group B metabolites were highest for BNF-induced microsomes (27) and Group A and C metabolites were highest for PB-induced microsomes (27–33). The ratios of compounds I/II + III/IV (see below) in Group A were 4.3:4.1 (C), 5.5:3.1 (BNF), and 5.5:4.1 (PB). Thus minor quantitative differences were suggested when 22:6 was metabolized by microsomes isolated from animals with different drug treatments.

However, drug treatments did not elicit qualitative differences in the way 22:6 was metabolized. The elution profiles from RP-HPLC of the ethyl acetate extracts, as monitored by radioactivity or UV absorption, were identical between all treatment groups. Therefore, ethyl acetate extracts from the three treatment groups were pooled prior to preparative HPLC in order to maximize product yield for identification purposes.

At least 23 radioactive peaks were resolved from 22:6 by RP-HPLC (Fig. 1). The amount of radioactivity in each individual peak was closely paralleled by the magnitude of UV absorption at 192 nm with two exceptions. For radioactivity/absorption at 192 nm, ratios for compounds with retention times of 94 min and 105 min were lower than the observed average. It is interesting that radioactive peaks eluting between 52–56 min and between 118–132 min revealed marked increases in the 229:192 absorption ratio (Fig. 1), suggesting the possible existence of conjugated dienes in these moieties.

Individual fractions separated by RP-HPLC were further resolved using NP-HPLC. Here again changes in radioactivity and UV absorption at 192 nm revealed a close parallelism. However, sensitivity to absorption at 192 nm was dramatically decreased with NP-HPLC, presumably because of the high absorbivity of isopropyl alcohol at 192 nm.

![Graphical representation of Table I](image)

**Table I**

| Drug treatment | A (18–34 min) | B (42–50 min) | C (55–86 min) | D (86–182 min) | 22:6 (140 min) |
|---------------|---------------|---------------|---------------|----------------|---------------|
| Control       | 7.4           | 5.4           | 1.4           | 4.0            | 54.9          |
| β-Naphthoflavone | 7.2           | 8.6           | 1.2           | 4.8            | 42.8          |
| Phenobarbital | 10.3          | 6.2           | 2.2           | 3.9            | 38.3          |

* Retention times for each group are shown in Fig. 1.

Group A Metabolites—NP-HPLC using isopropyl alcohol/hexane (1:100) was performed to further resolve RP-HPLC isolates which eluted between 18–34 min (Fig. 1). The five components in Group A, together representing 8.5% of the radioactivity recovered after RP-HPLC (Table I), have an identical UV spectrum (Fig. 2A). However, the mass spectra of the trimethylsilyl ether methyl esters of these five compounds and their hydrogenated derivatives were distinctive.

The electron impact spectrum of Compound I (Fig. 3A) revealed high mass ions at 520 (M), 489 (M – OCH₃), and 430 (M – Me₅SiO) which suggested a molecular weight of 520. Such a molecular weight was consistent with Compound I containing a 22-carbon aliphatic backbone, five double bonds, and two Me₅SiO groups. The presence of Me₅SiO groups was also supported by the occurrence of the 147 (Me₅SiO=SiMe₃) and 103 (Me₅SiO=CH₃), 74 (Me₅SiOH) and 73 (Me₅Si) ions. Ions at 401 (M – CH₃CH₂CH₂OSiMe₃), 398 (M – CH₃CH₂CH₃SiO), 360 (389 – HCO)², 228 (M – CH₃COCH₂), and 181 (M – CH₂(OSeMe₃)CH₂COCH₂) implied that vicinal Me₅SiO groups occurred at carbons 19 and 20. Furthermore, these ions indicated that all five double bonds were between carbon 18 and the carbomethoxy group.

⁵T means that formation of this ion involved prior transfer of a Me₅Si moiety to the carbomethoxy group (48, 49).

![Graphical representation of Fig. 1](image)

**Fig. 1.** Reverse phase chromatogram of metabolites formed by incubating [14C]docosahexaenoic acid (22:6) with hepatic microsomes. Microsomes and [14C]22:6 were incubated for 30 min at 37°C in the presence of 1 mM NADPH. The ethyl acetate extracts of microsomes from rats treated with vehicle, β-naphthoflavone, or phenobarbital were combined in a 2:5:5 ratio, respectively. An aliquot was applied to an ODS column in 50 μl of methanol. Metabolites, monitored at 229 and 192 nm, were eluted (1.6 ml/min) using 43:57 CH₃CH₂OH (pH 2.3). A linear gradient of 1.43% CH₃CN/min was initiated 85 min after injection. Fractions were collected every 0.5 min and counted.
TABLE II

| Peak* | Radioactivity contenta | Retention time | Equivalent chain lengthb |
|-------|------------------------|----------------|--------------------------|
|       | %                      | min            |                          |
| I     | 2.8                    | 19.4           | 46.7                     | 25.3 (26.3) |
| II    | 1.5                    | 23.2           | 26.9                     | 24.6 (25.3) |
| III   | 1.0                    | 24.3           | 26.9                     | 24.4 (25.0) |
| IV    | 2.6                    | 26.8           | 30.0                     | 24.4 (25.0) |
| V     | 0.6                    | 32.0           | 41.8                     | 24.4 (25.0) |

*Experimental conditions and peak numbering are described in Fig. 1.

†Radioactivity is presented as percentage of total radioactivity recovered from ODS columns. Separations were done isocratically using isopropyl alcohol:hexane (1:100).

‡These were determined on methyl ester trimethylsilyl ether derivatives. Numbers in parentheses represent values for corresponding hydrogenated derivatives.

§Percentage of radioactivity was calculated from data in Fig. 1 and mass ratios obtained by capillary GC.

Fig. 2. Typical UV spectra of metabolites in Groups A, B, and C. Approximately 16, 16, and 8 pg of Compound IV (Group A), VII (Group B), and X-B (Group C), respectively, was dissolved in 1 ml of CH3OH. Each recorded spectrum is labeled by the group letter the compound belongs to.

High mass ions from hydrogenated Compound I (Fig. 3B) indicated a molecular weight of 530, i.e. 515 (M - CH3), 499 (M - OCH3), 457 (M - MeSi), and 440 (M - Me2SiOH). Thus the molecular weight had increased by 10 and verified the presence of five double bonds in the original compound. Ions at 473 (M - CH3CH2CO)7, 472 (M - CH2CH2CHO)7, 411 (M - (CH3)2CH + MeSiOH), 399 (472 - MeSi), 367 (399 - CH2OH), 295 (399 - Me2SiOCH3), 233 (M - (CH3)2COOCH3), and 131 (M - CH(OSiMe3)(CH2)17 COOCH3) confirmed the presence of MeSiO groups at carbons 19 and 20. Assuming the five double bonds retained their original geometries and positions, Compound I was identified as 19,20-dihydroxy-4,7,10,13,16-docosapentaenoic acid.

Mass spectral identification of the other Group A metabolites was essentially carried out as described for Compound I. Details are provided in Miniprint.4 Compound II was identified as 16,17-dihydroxy-4,7,10,13,19-docosahexaenoic acid; Compound III as 13,14-dihydroxy-4,7,10,16,19-docosapentaenoic acid; Compound IV as 10,11-dihydroxy-4,7,10,16,19-docosapentaenoic acid; Compound V as 7,8-dihydroxy-4,10,13,16,19-docosapentaenoic acid.

4 Portions of this paper (including Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-2827, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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In summary, Group A metabolites consisted of five of the six possible diols which would arise if oxygen attacked each of the double bonds in 22:6. The retention times for these vicinal diols (Table II) decreased with RP-HPLC and increased with capillary GC as the pair of hydroxyl groups became located closer to the ω end of the molecule. Surprisingly with NP-HPLC, the retention times for the paired hydroxy moieties increased if the pair of hydroxyl groups were positioned very close to either the ω or the carboxyethoxy end of the molecule.

Group B Metabolites—The Group B metabolites (42–50 min, Fig. 1) consisted of two compounds which together represented 6.4% of the radioactivity recovered after RP-HPLC (Table III). Compounds VI and VII were base-line separated from each other by NP-HPLC using isopropyl alcohol:hexane (0.5:100). After isolation it was found that they had an identical UV spectrum (Fig. 2B) which was different from that of Group A metabolites, which contained a shoulder at 215 nm (Fig. 2A).

The electron impact mass spectrum of Compound VI (Fig. 4) revealed high mass ions at 430 (M), 415 (M - CH3), 399 (M - OCH3), and 309 (M - (Me2SiOH + OCH3)). This confirmed that Compound VI had a molecular weight of 430. The relatively high intensity of the 103 ion also implied that a single Me2SiO group was present at carbon 22. The suggested structure of Compound VI as 22-hydroxy-4,7,10,13,16,19-docosahexaenoic acid was confirmed when a spectrum of the hydrogenated derivative was examined (Miniprint).

The mass spectrum of Compound VII (Fig. 5) revealed high mass ions at 415 (M - CH3) and 340 (M - Me2SiOH). This confirmed that Compound VII had a molecular weight of 430. Ions at 415, 386 (M - CH2CHO)7, 354 (386 - CH2OH), 129 (CH2=CHCHO-OsMe3), 117 (M - C8H6COOCH3), 75 (Me2SiOH), and 73 (Me2Si) also indicated that Compound VII had a single Me2SiO moiety at carbon 21. The suggested structure of Compound VII as 21-hydroxy-4,7,10,13,16,19-docosahexaenoic acid was confirmed when the spectrum of the hydrogenated derivative was examined (Miniprint).

Group C Metabolites—Individual peaks in Group C (58–86 min, Fig. 1) were further resolved by NP-HPLC using isopropyl alcohol:hexane (0.2:100). As with the acetonitrile:water (43:57) mixtures used for RP-HPLC, these conditions were employed because they gave optimal separations for the 10 standards of hydroxylated conjugated dienes derived from 22:6.5 Group C had eight metabolites which together repre-
Fig. 3. Mass spectra of compound I (A) and hydrogenated compound I (B) as methyl ester trimethylsilyl ether derivatives.

Fig. 4. Mass spectrum of compound VI as methyl ester trimethylsilyl ether derivative.

Fig. 5. Mass spectrum of compound VII as methyl ester trimethylsilyl ether derivative.
Oxidation of Docosahexaenoic Acid by Hepatic Microsomes

The essential fatty acid docosahexaenoic acid was oxidized using NADPH-fortified microsomes from rat liver. Over 23 metabolites were found of which the first 13 were identified. The identified monools and diols represent approximately 10% of the docosahexaenoic acid added to microsomes. It is unlikely that these metabolites resulted from autooxidation considering the observation that only 0.5% of the docosahexaenoic acid incubated with boiled microsomes was oxygenated. In fact, less than one-fifth of even these autooxidation products comigrated with identified monools and diols. These metabolites were not due to autooxidation since they were almost absent when boiled microsomes were employed. The major products (Fig. 6) resulted from metabolism along three pathways: 1) \( \omega \)-hydroxylations, 2) \((w-1)\)-hydroxylations, and 3) \((w-3)\), \((w-6)\), \((w-9)\), \((w-12)\), and \((w-15)\)-epoxidations. The minor products, conjugated dienes with allylic hydroxyl groups, could result from lipoxygenase-like activity, but required NADPH addition for their formation, suggesting that docosahexaenoic acid was oxidized via four distinct routes in hepatic microsomes fortified with NADPH.

The microsomal cytochrome P-450 enzyme system was probably responsible for all four pathways since (a) the kinds of metabolites produced have been reported for other polyunsaturated fatty acids exposed to P-450 monoxygenases, (b) docosahexaenoic acid appeared to bind to P-450 with high affinity, and (c) drugs known to induce the P-450 system increased the amount of docosahexaenoic acid oxidized; moreover as reported for arachidonic acid (27), pretreatment of rats with BNF appeared to stimulate the production of \( \omega \) and \((w-1)\)-hydroxylations (Group B), (d) more than that of diols (Group A). It is interesting that pretreatment of rats with PB not only increased the production of vicinal diols but also resulted in doubling the amounts of conjugated dienes produced (Group G, Table I). The number of monoxygenases required for all the observed oxidations is unknown but it is believed that the liver contains at least 10 (50).

Microsomal epoxidases act preferentially toward the \( \omega \) end of unsaturated fatty acids (25, 27–29). This also held true for docosahexaenoic acid as judged by the proportions of diols formed. A 5,6-epoxide was reported to be formed in microsomal incubations with arachidonic acid (28). However in the present study no comparable 4,5-diol from docosahexaenoic acid was isolated. It is possible that the 4,5-diyl of docosahexaenoic acid was converted to a \( \gamma \) or \( \delta \)-lactone, and such lactonization should chromatographically remove the 4,5-diyl from Group A and place it with Group D metabolites which were not analyzed.

Recently human platelets were reported to hydroxylate docosahexaenoic acid at positions 11 and 14 (41). Rat basophils also produce trace amounts of the 4- and 7- hydroxy isomers from docosahexaenoic acid (40). Although the biological actions of these compounds are not yet established, it is known that pathological states which increase free arachidonic acid concentrations also elevate free docosahexaenoic acid concentrations (51, 52). Thus increased enzymatic oxidations of docosahexaenoic acid might be expected to accompany the increased hydroxylation of arachidonic acid typically found with increased levels of free arachidonic acid (53). The biological actions of arachidonic acid HETEs, their precursors, and products, assuming different shapes, are currently the subject of intense investigations (54).

### Table IV

| Peak | Radioactivity content | Retention time |
|------|----------------------|----------------|
|      | %                    | min |
| VIII | A 0.1                | 59.7| 29.2 |
|      | B 0.1                | 59.7| 25.9 |
| IX   | 0.1                  | 64.2| 32.3 |
| X    | A 0.2                | 68.3| 29.9 |
|      | B 0.3                | 68.3| 42.5 |
| XI   | 0.1                  | 72.4| 36.2 |
| XII  | 0.1                  | 76.4| 54.4 |
| XIII | 0.1                  | 80.0| 66.4 |

*Experimental conditions and peak numbering are described in Fig. 1.

Radioactivity is shown as percentage of total radioactivity recovered from ODS columns. For Peaks VIII and X the distribution of radioactivity between the A and B components was determined after NP-HPLC.

### Table V

| Peak | Retention time for Radioactivity content |
|------|----------------------------------------|
|      | min %                                  |
| XIV  | 89 <0.1                                |
| XV   | 88 0.8                                 |
| XVI  | 108 1.0                                |
| XVII | 110                                    |
| XVIII| 113 0.2                                |
| XIX  | 116 0.5                                |
| XX   | 121 0.2                                |
| XXI  | 125 0.2                                |
| XXII | 126                                    |
| XXIII| 131 0.4                                |

*Experimental conditions and peak numbering are described in Fig. 1.

Radioactivity is shown as percentage of total radioactivity recovered from ODS columns.

sent 1.2% of the radioactivity recovered from ODS columns (Table IV). NP- and RP-HPLC retention times of these eight metabolites matched those of standards generated by autoxidation. The UV spectra of the major metabolite in Group C, Compound X-B, is given in Fig. 2C. An absorption maximum at 235 confirmed the presence of a conjugated diene. The mass spectra of the hydrogenated Compound X-B also showed as the major fragments 271 and 273 verifying the presence of a trimethylsilyl group at carbon 10. Thus Compound X-B was identified as 10-hydroxy-4,7,11,13,16,19-docosahexaenoic acid. The other seven metabolites (Table IV) were identified as: Compound VIII-A, 17-hydroxy-4,7,10,13,15,19-docosahexaenoic acid; Compound VIII-B, 16-hydroxy-4,7,10,13,17,19-docosahexaenoic acid; Compound IX, 13-hydroxy-4,7,10,14,16,19-docosahexaenoic acid; Compound X-A, 14-hydroxy-4,7,10,12,16,19-docosahexaenoic acid; Compound XI, 11-hydroxy-4,7,9,13,16,19-docosahexaenoic acid; Compound XII, 7-hydroxy-4,8,10,13,16,19-docosahexaenoic acid; and Compound XIII, 8-hydroxy-4,6,10,13,16,19-docosahexaenoic acid.

Group D Metabolites—Group D metabolites, eluting 86–132 min (Fig. 1), represented 3.5% of the total radioactivity recovered after RP-HPLC (Table V). Radioactive peaks XIV–XVIII (86–114 min) absorbed only at 192 nm. In contrast, peaks XIX–XXIII (114–132 min) also revealed significant absorption at 229 nm. No further analyses were carried out on these metabolites.
mammalian tissues (platelets (41) or ram seminal vesicles (40)).

Arachidonic acid epoxides and diols, the production of which is mediated by microsomal mixed function oxidases, have recently been shown to be potent agonists for the release of luteinizing hormone from anterior pituitary cells (55). Furthermore, factors which enhance P-450 oxidations increase irreversibly binding of arachidonic acid metabolites to proteins with possible carcinogenic implications (26). Thus, examination of the 15 novel metabolites derived from docosahexaenoic acid for biological activity should be of interest, especially in tissues rich in docosahexaenoic acid such as brain, retina, and testis. Perhaps the "essentiality" of the n-3 fatty acids may be due to one or more of these metabolites.

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Oxidation of Docosahexaenoic Acid by Hepatic Microsomes

Table 2. Characteristic Fragments (m/z) in Mass Spectra of Group A Metabolites

| Peak Number | Alpha Cleavage to -OH (m/z) | Other Mass Losses | Hydrogenated | Other Mass Losses |
|-------------|-----------------------------|------------------|--------------|------------------|
|            | Alpha Cleavage to -OH (m/z) | Other Mass Losses | T-2 Migration | Other Mass Losses |
| I           | 369,233,131                  | 520 (M - 489,380, 401,350,299,287, 225       | 369,233,131  | 472               |
|             |                             |                  |              | 515 (M - 490,499, 520,450,411, 401,350,299,287) |
| II          | 452,273,175                  | 520 (M - 489,380, 401,350,299,287, 225       | 452,273,175  | 430               |
|             |                             |                  |              | 499,365,251      |
| III         | 433,323,231                  | 520 (M - 489,380, 401,350,299,287, 225       | 433,323,231  | 430               |
|             |                             |                  |              | 499,365,251      |
| IV          | 371,353,265                  | 520 (M - 489,380, 401,350,299,287, 225       | 371,353,265  | 430               |
|             |                             |                  |              | 499,365,251      |
| V           | 331,229                      | 489,241          | 341 (M - 430,411, 401,350,299,287, 225       | 331,229        |
|             |                             |                  |              | 430               |

1. Electron impact (70 eV) mass spectra were determined on methyl ester, trimethylsilyl ether derivatives. The base (parent) peak for the undetermined derivatives was set at 75.
2. Experimental conditions and peak numbers are described in Figure 1.
3. "T" means that formation of the rearrangement ion involved transfer of a 55(C5H11) group to the carboxylic moiety (48,49).
4. "w" refers to the mass of the molecular ion.

Table 2. Characteristic Fragments (m/z) in Mass Spectra of Group B Metabolites

| Peak Number | Alpha Cleavage to -OH (m/z) | Other Mass Losses | Hydrogenated | Other Mass Losses |
|-------------|-----------------------------|------------------|--------------|------------------|
|             | Alpha Cleavage to -OH (m/z) | Other Mass Losses | T-2 Migration | Other Mass Losses |
| VI          | 103                         | 430 (M - 489,380, 401,350,299,287, 225       | 103           |
|             |                             |                  |              | 427 (M - 449,499, 520,450,411, 401,350,299,287) |
| VII         | 415 (M - 15,117)            | 103              | 415 (M - 15,117) | 103               |

1. Electron impact (70 eV) mass spectra were determined on methyl ester, trimethylsilyl ether derivatives. The base (parent) peak for the unidentified derivatives was set at 75.
2. Experimental conditions and peak numbers are described in Figure 1.
3. "w" refers to the mass of the molecular ion.
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