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Synthesis of Two Hydroxy Fatty Acids from 7,10,13,16,19-Docosapentaenoic Acid by Human Platelets*

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Platelets metabolize 7,10,13,16,19-docosapentaenoic acid (22:5(*n*-3)) into 11-hydroxy-7,9,13,16,19- and 14-hydroxy-7,10,12,16,19-docosapentaenoic acid via an indomethacin-insensitive pathway. Time-dependent studies with 20 μM substrate show a lag in the synthesis of both the 11- and 14-isomers which was not observed for the synthesis of thromboxane B_2 (TXB_2), 5,8,10-heptadecatrienoic acid, and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) from arachidonic acid. When platelets were incubated with increasing concentrations of 22:5(*n*-3), the 11- and 14-isomers were not produced until the substrate concentration exceeded 5 μM unless arachidonic acid was also added to the incubations. The stimulatory effect of arachidonic acid was not blocked by indomethacin thus suggesting that 12-hydroperoxyeicosatetraenoic acid or 12-HETE derived from arachidonic acid may activate the platelet lipoxygenase(s) which metabolize 22:5(*n*-3). Incubations containing 20 μM 22:5(*n*-3) and increasing levels of [1-^{14}C]arachidonic acid show that the (*n*-3) acid inhibits the synthesis of both 5,8,10-heptadecatrienoic acid and TXB_2 from arachidonic acid. At the same time, 12-HETE synthesis increased due to substrate shunting to the lipoxygenase pathway.

Arachidonic acid is metabolized in platelets to TXA_2,[1] HHT, and 12-HETE (1). The low incidence of myocardial infarction and ischemic heart disease observed in Greenland Eskimos has been attributed to their high dietary intake of fish oils which are a rich source of 20:5(*n*-3) (2–4). This acid replaces some of the arachidonic acid in platelet phospholipids. When platelets are stimulated with appropriate agonists, both 20:5(*n*-3) and arachidonic acid are released. 20:5(*n*-3) competes with arachidonic acid for cyclooxygenase to depress the synthesis of TXA_2, which is both a vasoconstrictor and potent stimulator of platelet aggregation (5). Although small amounts of TXA_3 are made from 20:5(*n*-3) (6), this compound is a poor promoter of platelet aggregation (5). Morita *et al.* (7) recently reported that 12-HPETE, produced from arachidonic acid, stimulated TXA_3 synthesis. This interaction between a lipoxygenase metabolite with cyclooxygenase may further help to explain the clinical findings with Greenland Eskimos.

The merits of supplementing the diet with 20:5(*n*-3) imply that this acid must mediate both platelet and endothelial cell metabolism to reduce platelet aggregation. Needleman *et al.* (5) demonstrated that the artery metabolized exogenous PGH_3 to the corresponding prostacyclin and that this compound, like PGI_2, inhibited platelet aggregation. Considerable controversy exists in the literature as to whether endothelial cell cyclooxygenase is able to efficiently metabolize 20:5(*n*-3) to PGH_3 and thus to PGI_3 in sufficient amounts to exert a biological effect (8–12). Fisher and Weber (13) recently identified Δ17-2,3-dinor-6-keto-$PGF_{1\alpha}$ in the urine of human volunteers who had eaten diets elevated in fish oil and thus suggest that PGI_3 can efficiently be made by man.

Any proposal advocating a higher dietary intake of 20:5(*n*-3) fails to recognize that fish oils are a rich source of both 22:5(*n*-3) and 22:6(*n*-3) (14). In addition 22:5(*n*-3) and 22:6(*n*-3) are made from 20:5(*n*-3) and both of these 22-carbon fatty acids are found in platelet phospholipids (3, 9). 22:6(*n*-3) is a competitive inhibitor of both platelet (15) and vesicular gland (16) cyclooxygenase but does not inhibit the synthesis of leukotrienes from arachidonic acid in RBL-1 cells (16). Human platelets metabolize 22:6(*n*-3) via a lipoxygenase pathway into a pair of isomeric acids having their hydroxyl group at carbons 11 and 14 (17). In this study we report that platelets metabolize 22:5(*n*-3) into 11- and 14-hydroxy docosapentaenoic acids via an indomethacin-insensitive pathway and that 22:5(*n*-3) and arachidonic acid interact to regulate TXB_2 production as well as the types and amounts of hydroxy acids produced via the lipoxygenase pathway(s).

MATERIALS AND METHODS

Fatty Acids—7,10,13,16,19-[1-^{14}C]Docosapentaenoic acid (47 Ci/mol) was prepared as previously described (18). [1-^{14}C]Arachidonic acid (56.9 Ci/mol) was obtained from New England Nuclear. Arachidonic acid was purchased from Nu-Chek Preparations, Elsyian, MN while 7,10,13,16,19-docosapentaenoic acid was made by total synthesis.

Platelet Incubations—Blood was drawn from healthy volunteers who had not taken any medication for two weeks. The blood was collected in 7.5% (v/v) 77 mM disodium EDTA and centrifuged for 15 min at 200 × *g*. Platelets were recovered by centrifuging the plasma to 2000 × *g* for 20 min. The platelets were resuspended in 0.15 M NaCl, 0.15 M Tris (pH 7.4), 77 mM disodium EDTA 90:8:2 (v/v) (19) and centrifuged at 2000 × *g* for 15 min. They were then resuspended in the above medium at 3×10^8 cells/ml.

Platelets (0.5 ml) were preincubated for 2 min at 37 °C by stirring in siliconized tubes in a water bath. Reactions were initiated by addition of the potassium salt of the fatty acid (specific activity = 15 Ci/mol). Where noted, indomethacin or 5,8,11,14-heneicosatetraynoic

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[1] The abbreviations used are: TX, thromboxane; HHT, 5,8,10-heptadecatrienoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; HPLC, high-pressure liquid chromatography; PG, prostaglandin; ECL, equivalent chain length; 12-HPETE, 12-hydroperoxyeicosatetraenoic acid;

acid were added in 10 μl of ethanol. This amount of ethanol had no effect on reaction rates. Reactions were terminated by addition of 0.1 ml of 2 N formic acid and the products were recovered by extracting three times with 3 volumes of ethyl acetate. The pooled ethyl acetate extracts were washed with 1 ml of water and the ethyl acetate was removed under a stream of N_2.

High-performance Liquid Chromatography—Reverse-phase HPLC was carried out with a DuPont HPLC consisting of an 870 pump, 8800 series gradient controller, column oven, and a variable wavelength detector. Radioactivity was quantitated with an HP radioactive flow detector (Radiomatic Instruments and Chemical Co., Inc. Tampa, FL). Chromatography was carried out using a Zorbax 10-μm ODS column (0.46 × 25 cm) preceded by a guard column (5 × 0.46 cm) packed with Permaphase ODS (DuPont, Wilmington, DE). Samples were injected in 50 μl of methanol. Chromatography was carried out at 35 °C with a flow rate of 1.5 ml/min while the flow of Flo-Scint II (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL) was 4.5 ml/min through the radioactive detector. Counting efficiency was approximately 70%. Arachidonate metabolites were separated by isocratic elution for 20 min with 30% acetonitrile in water which was adjusted to pH 2.2 with phosphoric acid (Fisher). After this time the concentration of acetonitrile was increased to 42% with a linear gradient over 5 min. At 25 min the concentration of acetonitrile increased to 55% over 30 min using the −2 exponential gradient. Unreacted arachidonic acid was then removed by linearly increasing the concentration of acetonitrile to 100% over 10 min.

Normal phase HPLC was carried out using a Beckman system consisting of two 110 pumps, a 420 controller, and a variable wavelength detector (Bio-Rad). Chromatography was carried out at room temperature using a Zorbax Sil column (0.46 × 25 cm) (DuPont, Wilmington, DE). Hydroxy acids or methyl esters of hydroxy acids were injected in 50 μl of hexane/isopropanol, 99.5:0.5 (v/v). Methyl esters were separated by isocratic elution with 0.6% isopropanol in hexane while the separation of free acids was carried out with hexane/isopropranol/acetic acid 99:1:0.1 (v/v). The flow rate through the column was 1.5 ml/min, while the flow of scintillation fluid was 3 ml/min.

Gas Chromatography-Mass Spectrometry—Hydroxy acids were converted to methyl esters by reaction with ethereal diazomethane. Trimethylsilyl ethers were prepared by reacting the methyl ester with 10 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co.) and an equal volume of pyridine at room temperature for 1 h. Methyl esters were hydrogenated by bubbling hydrogen for 30 s into a solution of the methyl ester in 0.5 ml of methanol which contained about 1 mg of platinum oxide. The reaction mixture was rapidly transferred to a silicic acid column and eluted with 2 ml of methanol. Compounds were dissolved in isooctane for analysis by gas-liquid chromatography.

ECLs were determined using a Varian Vista 6000 gas chromatograph equipped with a glass column (6 foot × 2 mm, inner diameter) packed with 1% SP-2100 on 100/120 mesh Supelcoport (Supelco, Bellefonte, PA). Helium was the carrier gas (30 ml/min) and the temperatures of the injector, oven, and detector were, respectively, 250, 210, and 280 °C. Mass spectrometry was carried out with a Hewlett Packard 5970A mass selective detector and a 5790 gas chromatograph. Separations were carried out on a J and W DB-1 capillary column (15 m × 0.25 mm inner diameter) obtained from Applied Science, State College, PA. Injections were made in the splitless mode with an initial temperature of 70 °C and a valve time of 1 min. The temperature of the injector was 250 °C and the transfer line was 280 °C. One min after injection the oven was programmed at 30 °C/min to 210 or 240 °C, respectively, for analysis of unsaturated and saturated hydroxy acids. The ionizing voltage was 70 eV.

RESULTS

Analysis of metabolites produced from [1-^{14}C]22:5(*n*-3) by reverse-phase HPLC revealed the presence of a single radioactive compound with strong absorption at 234 nm suggesting that it was a hydroxy fatty acid. Fig. 1 shows that [1-^{14}C] 22:5(*n*-3) was metabolized into two metabolites when analysis was carried out by straight-phase HPLC.

The ultraviolet spectrum of compound I had λ_{max} 236 nm with ϵ = 28,000 in methanol. The ECL of the ME-TMS ether was 23.3. The mass spectrum (Fig. 2) had ions at *m/z* 432 (M^+), 417 (M − 15, loss of $\cdot CH_3$), 401 (M − 31, loss of

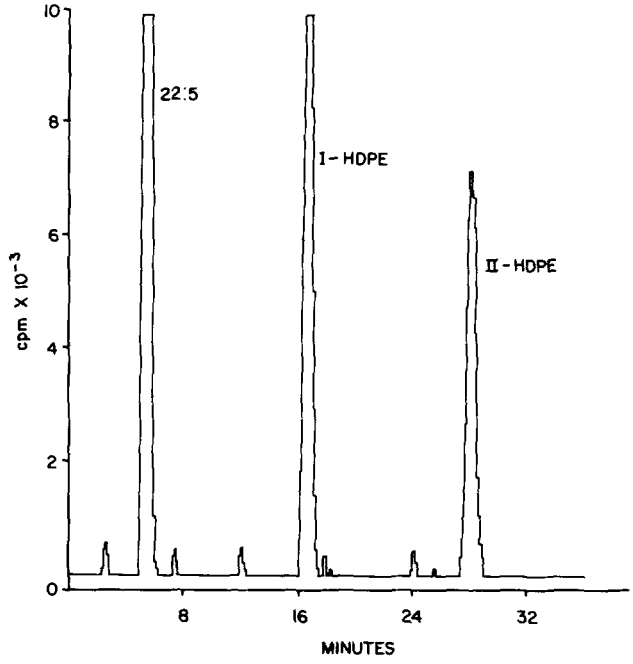

FIG. 1. **Normal phase HPLC radiochromatogram of the metabolites produced from 7,10,13,16,19-[1-^{14}C]docosapentaenoic acid.** Platelets (1.5 × 10^8/0.5 ml) were incubated with 20 μM substrate. After 3 min the metabolites were extracted with ethyl acetate and separated using hexane/isopropanol/acetic acid 99:1:0.1 (v/v).

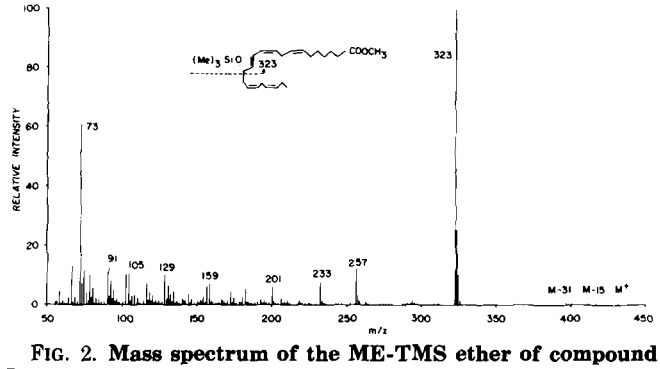

FIG. 2. **Mass spectrum of the ME-TMS ether of compound I.**

$\cdot OCH_3$), 323 (M − 109, loss of $\cdot CH_2$-(CH=CH-CH_2)$_2CH_3$), 257, 233 (323− 90, loss of Me_3SiOH), 201 (323 − (90 + 32)) as well as at 159, 129, 105, 103, 91, and 73. The mass spectrum of the hydrogenated ME-TMS ether of compound I (ECL = 24.0) had ions at *m/z* 427 (M − 15; 0.9%), 411 (M − 31; 2.0%), 395 (M − (15 + 32); 6.0%), 329 ((M − 113, loss of $\cdot CH_2(CH_2)_6CH_3$; 92%), 300 (M − 142, loss of $CH_3(CH_2)_7CHO$ followed by migration of the trimethylsilyl group to the carbomethoxy group 22.3% (20)), 215 (M − 227, loss of $\cdot (CH_2)_{12}COOCH_3$ base peak). Compound I is thus identified as 14-hydroxy-7,10,12,16,19-docosapentaenoic acid and has a pair of conjugated double bonds in the *cis/trans* configuration (21).

Compound II had λ_{max} 234 nm with ϵ = 28,000 in methanol. The mass spectrum (Fig. 3) of the ME-TMS ether (ECL = 23.3) had ions at *m/z* 417 (M − 15), 283 (M − 149, loss of $\cdot CH_2(CH=CH-CH_2)_3CH_3\cdot$); 193 (283 − 90), 161 (283 − (90 + 32)) and ions in the low mass region at 151, 133, 119, 91, and 73. The mass spectrum of the hydrogenated ME-TMS ether of compound II (ECL = 24.0) had ions at *m/z* 427 (M − 15; 0.7%), 411 (M − 31; 1.6%), 395 (M − (15 + 32); 3.6%), 287

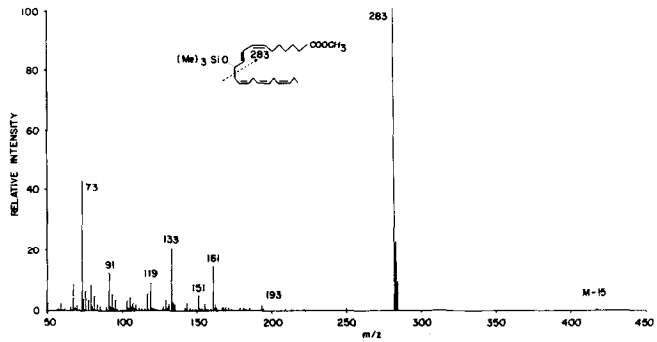

FIG. 3. **Mass spectrum of the ME-TMS ether of compound II.**

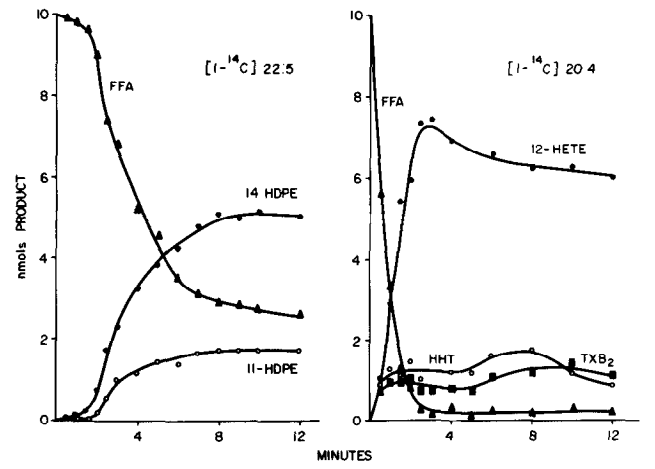

FIG. 4. **Time-dependent metabolism of 7,10,13,16,19-[1-^{14}C]docosapentaenoic acid and [1-^{14}C]arachidonic acid.** Platelets (1.5×10^8/0.5 ml) were incubated with 20 μM substrate. Metabolites from 22:5(n-3) were separated by normal-phase HPLC using hexane/isopropanol/acetic acid, 99:1:0.1 (v/v). Arachidonic acid metabolites were separated by reverse-phase HPLC as described in Fig. 5.

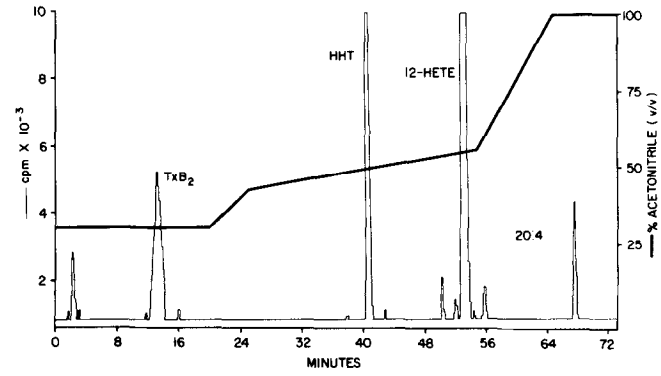

FIG. 5. **Reverse-phase HPLC radiochromatogram showing the separation of metabolites when 20 μM [1-^{14}C]arachidonic acid was incubated with 1.5×10^8 platelets/0.5 ml for 6 min.**

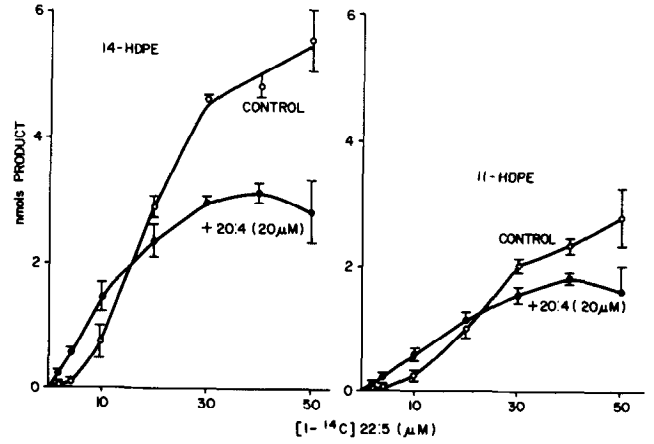

FIG. 6. **Platelets (1.5×10^8/0.5 ml) were incubated with increasing concentrations of 7,10,13,16,19-[1-^{14}C]docosapentaenoic acid (1–25 μM) without (○) and with (●) 20 μM arachidonic acid for 3 min.** Metabolites were separated as free acids by normal-phase HPLC. Results are the average of two experiments ± S.E.

(M − 155, loss of $\cdot CH_2(CH_2)_9CH_3$; base peak), 258 (M − 184, loss of $CH_3(CH_2)_{10}CHO$ followed by rearrangement, 35.8%), and 257 (M − 185, loss of $\cdot CH_2(CH_2)_8COOCH_3$; 82.7%). Compound II is thus 11-hydroxy-7,9,13,16,19-docosapentaenoic acid and probably has a pair of conjugated double bonds in the *cis/trans* configuration.

Fig. 4 compares the time-dependent synthesis of metabolites from arachidonic acid and 22:5(n-3) when 1.5×10^8 platelets/0.5 ml were incubated with 20 μM substrate. A typical HPLC chromatogram of arachidonic acid metabolites is shown in Fig. 5. With arachidonic acid there was rapid metabolism to both HHT and TXB_2 which plateaued after about 1 min. The synthesis of 12-HETE continued until all the substrate was metabolized. Conversely with 22:5(n-3) there was a lag of about 1.5 min before any product was produced and then both hydroxy isomers were produced in an approximately constant ratio.

Fig. 6 shows the effect 20 μM arachidonic acid has on mediating the synthesis of both 11-HDPE and 14-HDPE when platelets were incubated with increasing concentrations of [1-^{14}C]22:5(n-3). In the absence of arachidonic acid and when the concentration of [1-^{14}C]22:5(n-3) was below about 4 μM, it was not possible to detect the synthesis of either 11- or 14-HDPE. Conversely, when incubations contained arachidonic acid, the synthesis of both 11- and 14-HDPE was stimulated at concentrations of [1-^{14}C]22:5(n-3) below 10 μM. The results in Table I show that 10 μM indomethacin completely inhibited the synthesis of both HHT and TXB_2 but did not block the production of either 11- or 14-HDPE. Similar results were obtained when the arachidonic acid concentration was 50 μM. The apparent stimulation of 12-HETE synthesis is probably due to shunting of arachidonate to the lipoxygenase pathway. When incubations, described in Fig. 6, were carried out with 10 μM indomethacin the inhibition in production of both 11- and 14-HDPE at low concentrations of [1-^{14}C]22:5(n-3) was also abolished. These results show that cyclooxygenase-derived metabolites are not involved in regulating 11- and 14-HDPE synthesis but that 12-HPETE or 12-HETE may activate the lipoxygenase(s) acting on 22:5(n-3). As shown in Fig. 6 when the total fatty acid con-

TABLE I

Effect of indomethacin on the production of hydroxy fatty acids by platelets

Platelets (1.5×10^8/0.5 ml) were incubated for 2 min at 37 °C with 10 μM indomethacin after which time the fatty acids were added. After 3 min, the products were isolated and separated by HPLC.

Fatty acid (20 μM)	[1-^{14}C]20:4		[1-^{14}C]22:5	
	HHT	12-HETE	14-HDPE	11-HDPE
	nmol product/3 min		*nmol product/3 min*	
Control	1.5	6.3	4.6	1.7
Indomethacin	0	9.8	4.1	1.5

centration exceeded about 45 μM (20 μM arachidonic plus 25 μM [1-^{14}C]22:5(*n*-3)) there was an apparent inhibition in the synthesis of both 11- and 14-HDPE. Whether this represents true substrate competition for a common enzyme or some nonspecific inhibition due to the detergency effect of high concentrations of fatty acids is not known.

The results in Fig. 7 were obtained when platelets were incubated with and without 20 μM 22:5(*n*-3) in the presence of increasing concentrations of [1-^{14}C]arachidonic acid. These results show that 22:5(*n*-3) inhibited the synthesis of both TXB_2 and HHT thus suggesting that 22:5(*n*-3) inhibits cyclooxygenase even though it is not metabolized by this enzyme. The apparent stimulation in 12-HETE synthesis, at low [1-^{14}C]arachidonic acid concentrations, in the presence of 20 μM 22:5(*n*-3) is probably due to shunting of arachidonic acid to the lipoxygenase pathway. When cyclooxygenase was inhibited by 22:5(*n*-3), the amount of arachidonic acid available for lipoxygenase increased. Again, when the total fatty acid concentration exceeded 50 μM, there was inhibition of 12-HETE synthesis. As noted previously, we do not know if this is a general type of nonspecific inhibition.

Previously we reported that 5,8,11,14-heneicosatetraynoic acid was a selective lipoxygenase inhibitor which did not affect cyclooxygenase activity (22). The results in Fig. 8 confirm our previous results showing that 12-HETE synthesis was inhibited 50% by 0.5 μM levels of this acetylenic acid. Surprisingly, only 0.05 μM levels of this acid were acquired for 50% inhibition of both 11- and 14-HDPE. Virtually identical inhibition curves were obtained when the concentration of fatty acids was 50 μM.

DISCUSSION

Our results show that both 22:5(*n*-3) and 22:6(*n*-3) (17) are metabolized by human platelets into 11- and 14-hydroxy acids

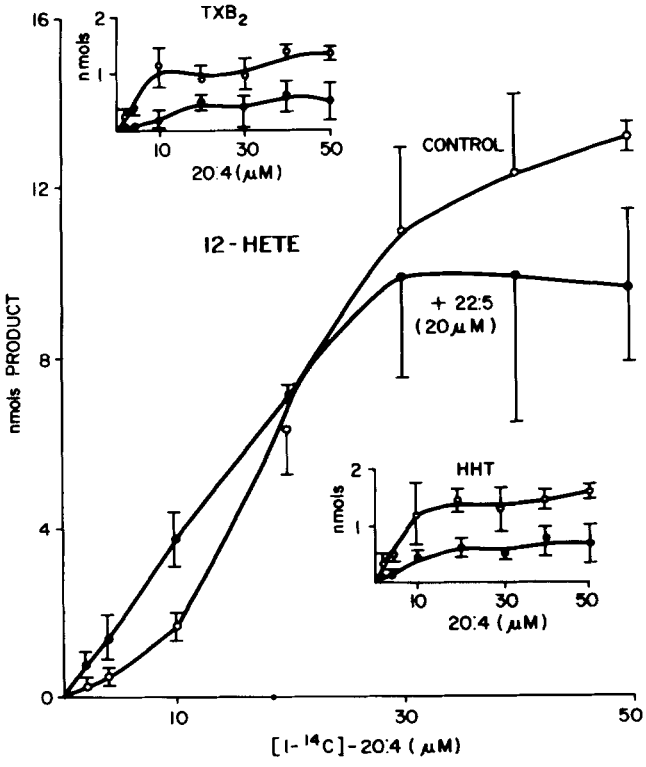

FIG. 7. **Platelets (1.5 × 10^8/0.5 ml) were incubated with increasing concentrations of [1-^{14}C]arachidonic acid without (○) and with (●) 20 μM 7,10,13,16,19-docosapentaenoic acid for 3 min.** Metabolites were separated as shown in Fig. 5. Results are the average of three experiments ± S.E.

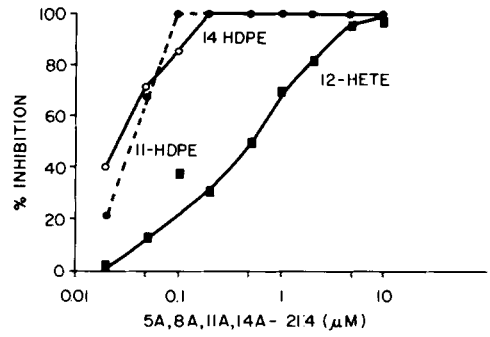

FIG. 8. **Inhibition of hydroxy fatty acid biosynthesis by 5,8,11,14-heneicosatetraynoic acid.** Platelets (1.5 × 10^8/0.5 ml) were preincubated for 2 min at 37 °C with various levels of 5,8,11,14-heneicosatetraynoic acid. Reactions were initiated by addition of 20 μM [1-^{14}C]arachidonic acid or 7,10,13,16,19-[1-^{14}C]docosapentaenoic acid and incubations were continued for 3 min. Metabolites from 22:5(*n*-3) were separated by normal-phase HPLC while those from arachidonic acid were separated by reverse-phase HPLC.

via an indomethacin-insensitive pathway. Platelet lipoxygenase metabolizes arachidonic acid only to 12-HETE (1). The initial step in this reaction involves proton abstraction from carbon 10 (the ω 11 carbon), followed by double-bond shift and allylic attack by the hydroperoxy radical at carbon 12 (23).

If lipoxygenase specificity is dictated by the terminal end of the fatty acid, then both 22:5(*n*-3) and 22:6(*n*-3) would be metabolized to a 14-hydroxy fatty acid since initial proton abstraction would take place at carbon 12, which is the ω 11 carbon. Previously we suggested that the 11-hydroxy isomer might be made by a second lipoxygenase involving initial proton abstraction from position-9 (ω 14) (17). Our present findings are not totally consistent with this hypothesis for the following reasons. There was a lag in the synthesis of both the 11- and 14- isomers in the absence of arachidonate. Secondly, arachidonic acid stimulated the synthesis of both isomers to the same extent. Finally, the synthesis of both isomers was inhibited 50% by the same concentration of 5,8,11,14-heneicosatetraynoic acid and this concentration was 10-fold less than required for 50% inhibition of 12-HETE synthesis. Hamberg (24) recently reported that 6,9,12-18:3 was metabolized by platelets via an indomethacin-insensitive pathway to 10- and 13-hydroxy octadecatrienoic acids. He suggests that platelets may contain a lipoxygenase with dual specificity for proton abstraction. If a single lipoxygenase acts on 22:5(*n*-3) and 22:6(*n*-3) to give the 11- and 14-isomers, presumably it must be different from the lipoxygenase which metabolizes arachidonic acid to 12-HETE. In addition there must be some inherent, as yet unrecognized, structural feature in a fatty acid which will determine whether it is metabolized by platelets into isomeric hydroxy acids. The acids, 22:5(*n*-3) and 22:4(*n*-6), are structurally similar in that their double bonds are located, respectively, at positions 7,10,13,16,19 and 7,10,13,16. Platelets metabolize the latter acid into dihomo-TXB_2, dihomo-HHT, and 14-hydroxy-7,10,12,16-docosatetraenoic acid.[2] This latter acid constituted about 90% of the hydroxy acids made via an indomethacin-insensitive pathway and only 1–2% of an 11-hydroxy isomer was detected.

If 22:5(*n*-3) is released along with arachidonic acid from platelet phospholipids, our results suggest that it will interact with arachidonate in two ways. It inhibits cyclooxygenase as do both 20:5(*n*-3) (4) and 22:6(*n*-3) (16) to depress the syn-

[2] M. VanRollins, L. Horrocks, and H. Sprecher, manuscript in preparation.

thesis of TXB_2 and HHT. In turn, more arachidonic acid is shunted to the lipoxygenase pathway resulting in an increase in 12-HPETE production. The 12-HPETE may then stimulate the synthesis of both the 11- and 14-hydroxy acid isomers from 22:5(*n*-3). This stimulatory effect of 12-HPETE on 11- and 14-HDPE synthesis might then be similar to activation to leukocyte 5-lipoxygenase by 12-HPETE (25). The overall effect would be to increase the amount of hydroxy fatty acids produced in the platelet.

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