Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 903–907, February 1984
Medical Sciences

12*S*,20-Dihydroxyicosatetraenoic acid: A new icosanoid synthesized by neutrophils from 12*S*-hydroxyicosatetraenoic acid produced by thrombin- or collagen-stimulated platelets

(aspirin/lipoxygenase/cell–cell interactions/thin-layer and high-performance liquid chromatography/gas chromatography–mass spectrometry)

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Communicated by Josef Fried, October 3, 1983

ABSTRACT **A new metabolite of arachidonic acid, formed during interaction between thrombin- or collagen-stimulated platelets and unstimulated neutrophils, has been demonstrated by both thin-layer radiochromatography and high-performance liquid chromatography. Production of the ^{3}H-labeled metabolite in combined suspensions containing [^{3}H]arachidonate-labeled platelets and unlabeled neutrophils from aspirin-treated donors suggested that platelet ^{3}H-labeled 12*S*-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-icosatetraenoic acid (12-HETE) was the precursor. This was confirmed by identification of the same product when purified 12-[^{3}H]HETE was added directly to unstimulated neutrophils. Hydrogenation and oxidation of the isolated product, followed by gas chromatography–mass spectrometry showed the structure to be 12*S*,20-dihydroxyicosatetraenoic acid. These experiments further show that platelet stimuli known to occur *in vivo* may initiate metabolic interactions between different cell types via the arachidonic acid pathway.**

Blood vessel injury with resultant exposure of subendothelial structures such as collagen initiates several hemostatic responses, including platelet aggregation and activation of coagulation, which culminate in thrombin formation (1). Collagen and thrombin also induce release of platelet arachidonic acid and its transformation into icosanoids (1). Cellular components of the hemostatic process such as platelets, neutrophils, and endothelial cells are consequently brought into close apposition—a situation that affords the opportunity for metabolic interchange of biochemical products generated by these cells (2).

Using [^{3}H]arachidonate-labeled platelets, we previously showed direct cell–cell interactions between platelets and endothelial cells via the cyclooxygenase pathway in which platelet-derived precursors (endoperoxides) were used by endothelial cells in the formation of prostacyclin (2). More recently, we reported that stimulated radiolabeled platelets interact with stimulated neutrophils via the lipoxygenase pathway of arachidonate metabolism. Thus, released platelet [^{3}H]arachidonate served as precursor for stimulated neutrophil-derived leukotriene B_4 (LTB_4; 5*S*,12*R*-dihydroxy-6-*cis*,8,10-*trans*,14-*cis*-icosatetraenoic acid) and 5*S*-hydroxy-6-*trans*,8,11,14-*cis*-icosatetraenoic acid (5-HETE), whereas ^{3}H-labeled 12*S*-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-icosatetraenoic acid (12-HETE) from the platelet served as substrate for 5*S*,12*S*-dihydroxy-6-*trans*,8-*cis*,10-trans,14-*cis*-icosatetraenoic acid (5*S*,12*S*-DiHETE) formation (3). The present investigation documents additional platelet–neutrophil interactions involving the lipoxygenase pathway in which a new metabolite of arachidonic acid, 12*S*,20-dihydroxyicosatetraenoic acid is formed by the unstimulated neutrophil in the presence of thrombin- or collagen-stimulated platelets.

MATERIALS AND METHODS

Preparation of Platelet Suspensions. Blood was collected and processed (2, 4) from donors who had not ingested aspirin or who had taken 650 mg of aspirin 12 and 2 hr prior to venipuncture. Platelets were labeled (2) with 50 μCi (1 Ci = 37 GBq) of [^{3}H]arachidonate added during the first wash (4).

Preparation of Neutrophil Suspensions. Suspensions containing 95% neutrophils (5% eosinophils) were prepared from 80–240 ml of whole blood (2) initially centrifuged at 200 $\times$ *g*. After removal of platelet-rich plasma, the remaining leukocytes and erythrocytes were suspended in Dextran T500 (1.5%). Leukocyte-rich plasma was centrifuged at 280 $\times$ *g* (10 min, 4°C), and the resulting pellets were suspended in cold saline and layered on Ficoll-Hypaque (density, 1.077). After centrifugation at 350 $\times$ *g* (30 min, 4°C), the interface was removed and erythrocytes in the neutrophil-containing pellet were lysed with distilled water (25 sec). On return to isotonicity with NaCl, phosphate-buffered saline (pH 7.4) was added, the suspension was centrifuged at 280 $\times$ *g* for 5 min (4°C), and the neutrophils were suspended in Hepes buffer containing calcium and magnesium (3). Trypan blue exclusion by the preparation averaged 94%, and on stained smears no platelets were seen.

Experimental Procedure. For TLC studies, 3×10^8 labeled platelets were incubated at 37°C for 5 min followed by addition of 3×10^7 unlabeled neutrophils (total vol, 1 ml). One minute later thrombin (5 units/ml), collagen (30 μg/ml), or ionophore A23187 (1–2 μM) was added. The same protocol was used in HPLC experiments with unlabeled cells. Reactions were stopped at 5 min and lipids were extracted (4). In experiments with unlabeled neutrophils (3×10^7 per ml), 12-[^{3}H]HETE (0.45 μCi; 9.4 pmol) was added as sodium salt.

Thin-Layer Radiochromatography. Lipid extracts were chromatographed on activated (1 hr, 110°C) silica gel G plates (Analtech, Newark, DE) along with standards using chloroform/methanol/acetic acid/water (90:8:1:0.8) (3) as

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Abbreviations: 12-HETE,12*S*-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-icosatetraenoic acid; 5-HETE, 5*S*-hydroxy-6-*trans*,8,11,14-*cis*-icosatetraenoic acid; LTB_4 (leukotriene B_4), 5*S*,12R-dihydroxy-6-*cis*,8,10-*trans*,14-*cis*-icosatetraenoic acid; THETE, trihydroxy-icosatetraenoic acid; ETYA, 5,8,11,14-icosatetraynoic acid; 5*S*,12*S*-DiHETE, 5*S*,12*S*-dihydroxy-6-*trans*,8-*cis*,10-*trans*,14-*cis*-icosatetraenoic acid; 12,20-DiHETE, (12*S*)-12,20-dihydroxy-5,8,10,14-icosatetraenoic acid; RP-HPLC, reversed-phase HPLC; SP-HPLC, straight-phase HPLC.

solvent. Other TLC-related procedures have been described (5).

HPLC Analysis of Icosanoids. In preparation for reversed-phase HPLC (RP-HPLC), cell suspensions were extracted as follows: reactions were stopped with 1.5 vol of acetone, precipitated protein was removed by centrifugation, and acetone was evaporated under nitrogen. After acidification to pH 4.0 with 1 M H_3PO_4, extraction three times with 1 ml aliquots of ethyl acetate, and evaporation to dryness, residues were dissolved in 50 μl of methanol/water (3:1) and 5 μl was injected into the HPLC column (6). RP-HPLC was carried out on an 8-mm i.d. Radial-PAK C18 (10 μm) column (Waters) with methanol/water/acetic acid (75:25:0.01) as eluting solvent. Flow rate was 0.7 ml/min.

In preparation for mass spectrometry procedures, the product derived from RP-HPLC was esterified using diazomethane and subjected to straight-phase HPLC (SP-HPLC) on a 25-cm Whatman PAC column (10 μm) using a solvent system of hexane/isopropanol (94:6) with a flow rate of 1.5 ml/min. Under these conditions the new metabolite eluted with a retention time of 14 min. In both HPLC procedures, absorbance was monitored at 237 nm.

Preparative Procedures. For larger scale preparations of the new compound, cell ratios were maintained at 3×10^8 platelets per ml to 3×10^7 neutrophils per ml. Total neutrophil numbers ranged from 28 to 67×10^7. The stimulus was thrombin (5 units/ml) and incubations were for 5 or 10 min. Aspirin-treated platelet donors were used to increase yields of 12-HETE and, consequently, the new product. Alternatively, the new metabolite could be prepared from purified 12-HETE added to neutrophils. 12-HETE was synthesized from ionophore-stimulated platelets and purified by HPLC.

Catalytic Hydrogenation and Jones Oxidation. After esterification and purification by SP-HPLC, the new metabolite (11.2 μg) was dissolved in 1.0 ml of methanol. One milligram of platinum oxide catalyst was added, and hydrogen gas was bubbled through the solution for 10 min at 22°C. The solution was then filtered through Celite under constant N_2 pressure to remove the catalyst. The Celite was immediately washed with 30 ml of methanol, and the hydrogenated product was subjected to gas chromatography–mass spectrometry as described (7).

A portion of the hydrogenated product (6.8 μg) was dissolved in 10 ml of acetone and incubated for 20 min at 22°C with 150 μl of Jones reagent (8). The reaction was quenched with 500 μl of isopropanol and the acetone was removed under N_2. Ten milliliters of water was added, and the solution was extracted three times with 5 vol of hexane/ethyl acetate (60:40). Finally, the sample was washed twice with equal volumes of water, rotary evaporated to dryness, esterified, and analyzed by gas chromatography–mass spectrometry (7). Sources of all reagents and standards were identical to those previously published (3, 7).

RESULTS AND DISCUSSION

Thin-Layer Radiochromatographic Studies. Stimulation of cell suspensions containing [^{3}H]arachidonate-labeled platelets and unlabeled neutrophils with thrombin or collagen resulted in production of a labeled compound subsequently identified as (12*S*)-12,20-dihydroxy-5,8,10,14-icosatetraenoic acid (12,20-DiHETE), which was not synthesized by platelets alone. Thin-layer radiochromatography indicated that this new metabolite had a R_f value intermediate between that of 5*S*,12*S*-DiHETE ($R_f = 0.41$) and 5-HETE ($R_f = 0.53$) (3). The R_f value observed (0.47) was incompatible with any previously identified neutrophil or platelet icosanoid (Fig. 1).

Thrombin or collagen did not initiate arachidonic acid metabolism in neutrophils, as evidenced by lack of production of 5-[^{3}H]HETE or [^{3}H]LTB_4 in these experiments. Therefore, the new icosanoid was synthesized by neutrophils (unstimulated with respect to arachidonate oxygenation) from an icosanoid produced by stimulated platelets. This concept was further supported by the observation that when 5.7 μM [^{14}C]arachidonate, a concentration that was nonstimulatory for neutrophils but that did activate platelets, was added to unlabeled platelet–neutrophil mixtures, the new metabolite was produced. In contrast, when platelet–neutrophil suspensions were exposed to ionophore A23187, an activator of neutrophil 5-lipoxygenase, formation of 12,20-DiHETE was markedly inhibited, and 5*S*,12*S*-DiHETE was produced (3, 9).

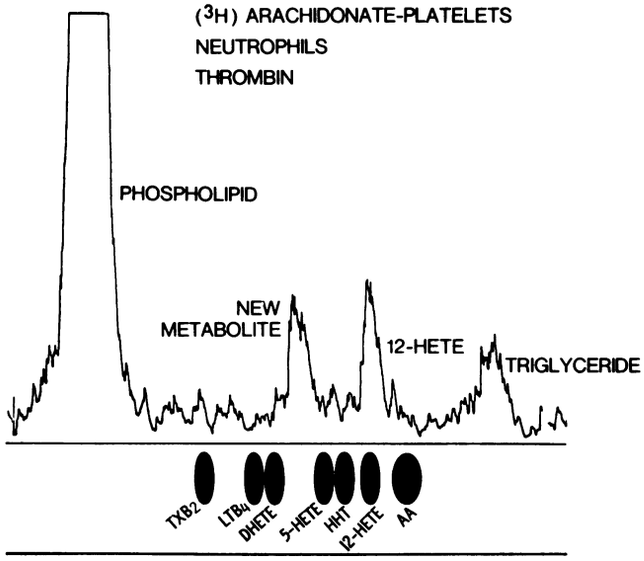


FIG. 1. Radioactivity scan of TLC separation of lipids extracted from combined suspensions of [^{3}H]arachidonate-labeled platelets and unlabeled neutrophils after addition of thrombin. Shown below the tracing is a diagram indicating positions of unlabeled standards added to the extract prior to chromatography. The platelet donor had ingested aspirin, thus eliminating thromboxane B_2 (TXB_2) and 12*S*-hydroxy-5,8,10-heptadecatrienoic acid (HHT) production. The R_f of the new metabolite (12,20-DiHETE) did not correspond to that of known icosanoids. The peak representing 12-HETE is platelet-derived, and production of the new metabolite requires the additional presence of neutrophils. Because platelets were the sole source of radioactivity in these experiments, precursors of all compounds detected were platelet derived. Solvent system: chloroform/methanol/acetic acid/water (90:8:1:0.8). AA, arachidonic acid.

Pretreatment of platelet donors with aspirin did not inhibit synthesis of the new product. Thus, involvement of 12-HETE from the platelet lipoxygenase pathway appeared likely. Table 1 summarizes results of experiments carried out with labeled platelets obtained from such a donor. In the platelet control stimulated by thrombin, 12-HETE was the

Table 1. Metabolism of platelet 12-HETE by neutrophils

Incubation mixture	New metabolite	5*S*,12*S*-DiHETE	12-HETE
Platelets/buffer	40	34	147
Platelets/thrombin	81	54	13,897
Platelets/neutrophils/thrombin	4,645	86	4,129
Platelets/neutrophils/ionophore	312	3,034	5,876

Numbers indicate dpm, corrected to 100% recovery from TLC plates and to 1×10^5 dpm spotted. [^{3}H]Arachidonate-labeled platelets (3×10^8) were incubated with neutrophils (3×10^7) and then with either thrombin (5 units/ml) or 1 μM ionophore. Five minutes later, lipids were extracted, separated by TLC along with authentic standards, stained with iodine, and scanned. Identified areas were scraped and quantified by scintillation counting (4, 5).

only arachidonate metabolite formed. In contrast, when the labeled platelet–unlabeled neutrophil suspension was exposed to thrombin, 12-HETE decreased by 70% and the new metabolite appeared. Also shown is the relative lack of production of the new compound in the presence of ionophore and corresponding synthesis of 5*S*,12*S*-DiHETE, a known metabolite of 12-HETE (3, 9).

To directly ascertain that 12-HETE was the precursor of the new metabolite, 12-[^{3}H]HETE (9.4 pmol/ml) was added to unlabeled neutrophil suspensions. A radioactive product with the identical R_f value as that previously obtained with thrombin- or collagen-stimulated combined cell suspensions was observed (20% of recovered nonphospholipid counts). Addition of thrombin to 12-HETE and neutrophils did not enhance production of the new metabolite. As previously reported, stimulation of neutrophils with ionophore A23187 in the presence of 12-HETE resulted in formation of 5*S*,12*S*-DiHETE (3).

HPLC Experiments. Studies of endogenous platelet–neutrophil arachidonate metabolism by using HPLC confirmed and extended the TLC observations. Results of a group of experiments analyzed by RP-HPLC are summarized in Fig. 2. Thrombin-stimulated unlabeled platelet–neutrophil suspensions generated the new metabolite, which appeared as a distinct peak (retention time, 10.75 min) as shown in curve C. The peak was absent when platelets alone (curve B) or neutrophils alone (curve A) were exposed to thrombin. Furthermore, 5-HETE or LTB_4 (monitored at 269 nm) did not form in thrombin-treated neutrophils. Addition of purified 12-HETE to neutrophils in the absence of platelets resulted in the appearance of a RP-HPLC peak with the identical retention time to that generated by thrombin-stimulated platelets in the presence of neutrophils (curve D).

Production of the new metabolite by neutrophils from purified platelet 12-HETE increased with both time and cell number. Thus, when 3×10^7 neutrophils per ml and 8.8 μM 12-HETE were incubated for 10, rather than 5 min, the quantity of new product increased 1.5-fold. Similarly, increasing the neutrophil concentration from 1×10^7 to 3×10^7 cells per ml, resulted in a 2.9-fold increase in amount of metabolite formed. The inhibitory effect of ionophore was also demonstrable in RP-HPLC experiments. Thus, addition of 5 μM ionophore to 1×10^7 neutrophils per ml in the presence of 8.8 μM 12-HETE (5 min) decreased production of the new product by 95%. Synthesis of the metabolite during 5-min incubations of 3×10^7 neutrophils per ml and 8.8 μM 12-HETE was also inhibited by 5,8,11,14-icosatetraynoic acid (ETYA) (16% and 65% with 10 μM and 30 μM ETYA, respectively). ETYA is regarded as an inhibitor of the cyclooxygenase and lipoxygenase pathways. Thus, interference with formation of 12,20-DiHETE from 12-HETE suggests an additional inhibitory property of ETYA.

To ascertain whether the new product was recoverable in the supernatant or was cell associated, incubation of neutrophils and 12-HETE was stopped by cooling to 4°C and by centrifuging at $280 \times g$ for 5 min (4°C). The supernatant was then extracted and quantified by RP-HPLC. Recovery of the product was 100% when compared to a control in which the entire incubation mixture was processed. Thus, after its generation from platelet 12-HETE, this neutrophil metabolite is present in the extracellular milieu. Stenson and Parker, in a study of 12-[^{14}C]HETE incorporation into neutrophil phospholipids and triglycerides, noted a polar metabolite in the supernatant fluid. The product was not further characterized in detail, but may be similar or identical to the one described here (10).

Thin-Layer and Ultraviolet Spectrometry Studies. After elution from the RP-HPLC column, fractions containing the new metabolite were rechromatographed on TLC. The R_f value was identical to that obtained in the thin-layer radiochromatographic studies shown in Fig. 1. A UV absorption spectrum of the isolated material after esterification and SP-HPLC showed an absorption maximum at 235 nm. This spectrum did not differ significantly from that of the parent compound (12-HETE).

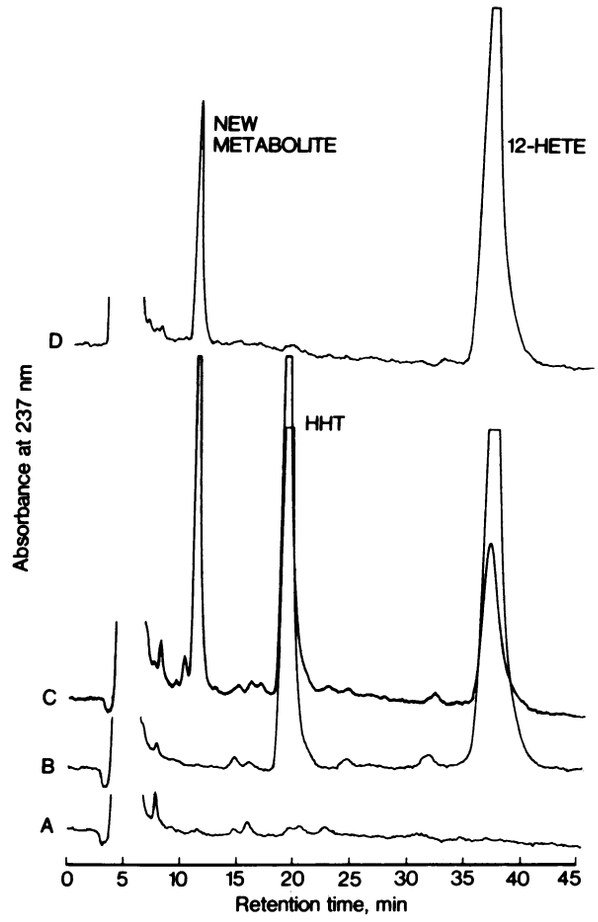
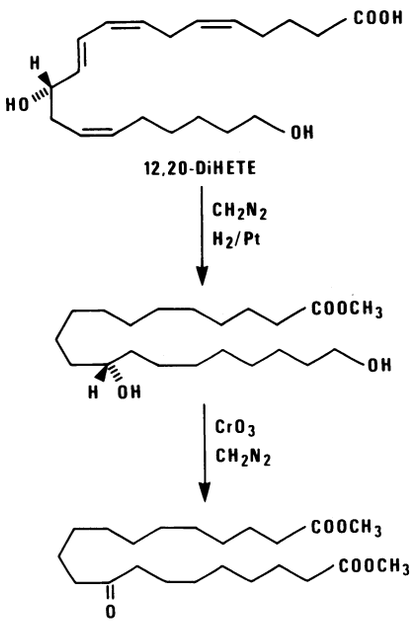


FIG. 2. RP-HPLC elution profiles of hydroxy acids produced under various experimental conditions and monitored at 237 nm. Curve A, neutrophils (3×10^7) plus thrombin at 5 units/ml (5 min). At this wavelength or at 269 nm (leukotrienes), there is no evidence of hydroxy acid production. TLC results also indicated that thrombin does not initiate arachidonic acid metabolism in neutrophils. Curve B, platelets (3×10^8) plus thrombin in the absence of aspirin. The two platelet hydroxy acids 12*S*-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12-HETE are apparent. Curve C, neutrophils (3×10^7) and platelets (3×10^8) plus thrombin at 5 units/ml. The new metabolite, 12,20-DiHETE, is synthesized only when both cells are incubated together in the presence of a platelet stimulus. Curve D, neutrophils (1×10^7) plus 8.8 μM purified 12-HETE. 12,20-DiHETE is also synthesized when the platelet product, 12-HETE, is directly added to unstimulated neutrophils. Known standards were chromatographed prior to injection of test samples. Under the HPLC conditions used, 15(*S*)-hydroxy-9-oxo-5-*cis*-8(12),13-*trans*-prostatrienoic acid (prostaglandin B_2; PGB_2).

Gas Chromatography–Mass Spectrometry Analyses. The purified esterified product obtained by SP-HPLC was converted to the trimethylsilyl ether and analyzed by gas chromatography–mass spectrometry (7). The derivatized product eluted from an SE-30 column with an equivalent chain length value of C-24.6. The mass spectrometry showed prominent ions at m/z 479 ($M^{+\cdot} - 15$), 404 ($M^{+\cdot} - 90$; loss of Me_3SiOH), 353 [$M^{+\cdot} - 141$; loss of $\cdot CH_2CH = CH(CH_2)_3COOCH_3$], 314 [$M^{+\cdot} - (2 \times 90)$; loss of $2 \times Me_3SiOH$], base peak 295 [$M^{+\cdot} - 199$; loss of $\cdot CH_2CH = CH(CH_2)_4CH_2OSiMe_3$], 205 (295 − 90), 179, and 173 (205 −

32). This suggested that the new compound was a dihydroxy derivative of arachidonic acid. The base peak of 295 strongly suggested that the additional hydroxylation was present between C-12 and C-20.



The unknown product was then hydrogenated. The mass spectrum of this material is shown in Fig. 3. Consistent with the assumption that the compound retained a hydroxyl group at C-12 and had an additional hydroxyl between C-12 and C-20, we observed two prominent ions with m/z 301 [α cleavage at C-12 with loss of $\cdot(CH_2)_7CH_2OSiMe_3$] and 303 [α cleavage with loss of $\cdot(CH_2)_{10}COOCH_3$]. Additional ions were observed at m/z 502 ($M^{+\cdot}$), 487 ($M^{+\cdot} - 15$), and 471 ($M^{+\cdot} - 31$). The spectrum also suggested that the additional hydroxyl group was located at C-20, because no other prominent α cleavage products were observed.

Additional procedures were carried out to confirm the C-20 position of the ω hydroxyl. After catalytic hydrogenation, Jones oxidation and methylation, the unknown compound was analyzed by gas chromatography–mass spectrometry on a Finnigan 4401 instrument. The major component detected gave a retention time on SE-30 compatible with an equivalent chain length value of C-24.7. The mass spectrum is shown in Fig. 4, and it conclusively proves that (12*S*)-12,20-dihydroxy-5,8,10,14-icosatetraenoic acid is the structure of the new metabolite. The following ions are interpreted to originate from a dimethyl 12-keto-icosane-1,20-dioate: m/z 384 ($M^{+\cdot}$), 369 ($M^{+\cdot} - 15$), 353 ($M^{+\cdot} - 31$), 321 [$M^{+\cdot} - (31 + 32)$], 279 [$M^{+\cdot} - (32 + 73)$], 227 [$M^{+\cdot} - 157$; α cleavage with loss of $\cdot(CH_2)_7COOCH_3$], 185 [$M^{+\cdot} - 199$; α cleavage with loss of $\cdot(CH_2)_{10}COOCH_3$], and 311 ($M^{+\cdot} - 73$; loss of $\cdot CH_2COOCH_3$). Ions with m/z 200 and 242 arise from McLafferty rearrangement involving the 12-keto functionality.

The mass spectrum obtained from platelet-derived 12,20-DiHETE was identical to that observed when this metabolite was produced by addition of exogenous 12-HETE to neutrophils in the absence of platelets.

12,20-DiHETE Formation and Platelet-Neutrophil Interactions. 5,12,20-Trihydroxy derivatives (THETEs) of both 5*S*,12*S*-DiHETE and LTB_4 have been observed in ionophore-stimulated human leukocyte suspensions (11, 12). We previously reported that despite formation of 5*S*,12*S*-DiHETE by platelets from 5-[^{3}H]HETE, no detectable

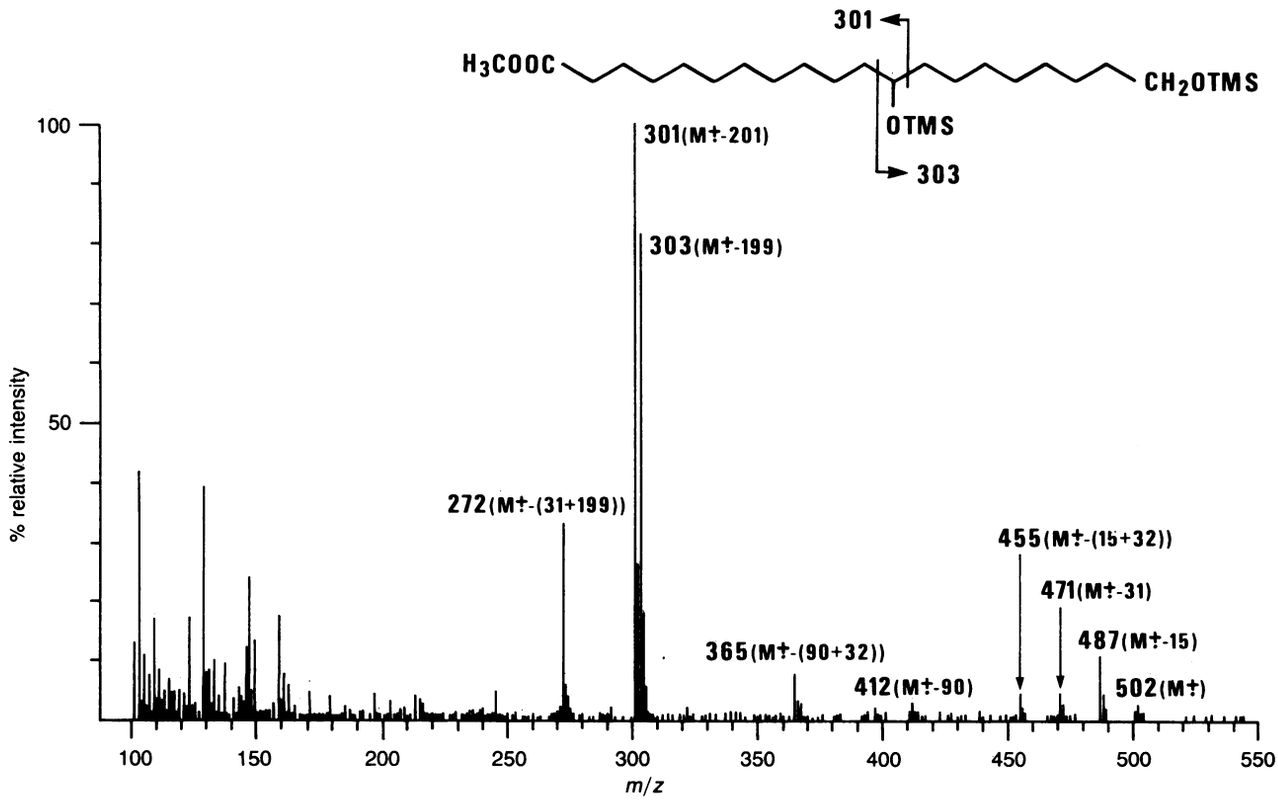


FIG. 3. Mass spectrum of hydrogenated unknown compound. The unknown compound was purified by sequential RP-HPLC and SP-HPLC. The sample (11.2 μg) was dissolved in 1.0 ml of methanol that contained 1.0 mg of platinum oxide catalyst and was exposed to H_2 gas for 10 min. The hydrogenated molecule was then derivatized, and analyzed by gas chromatography–mass spectrometry. Two major ions were observed at m/z 301 and 303 indicating the presence of hydroxyl groups at C-12 and at either C-19 or C-20. TMS, trimethylsilyl.

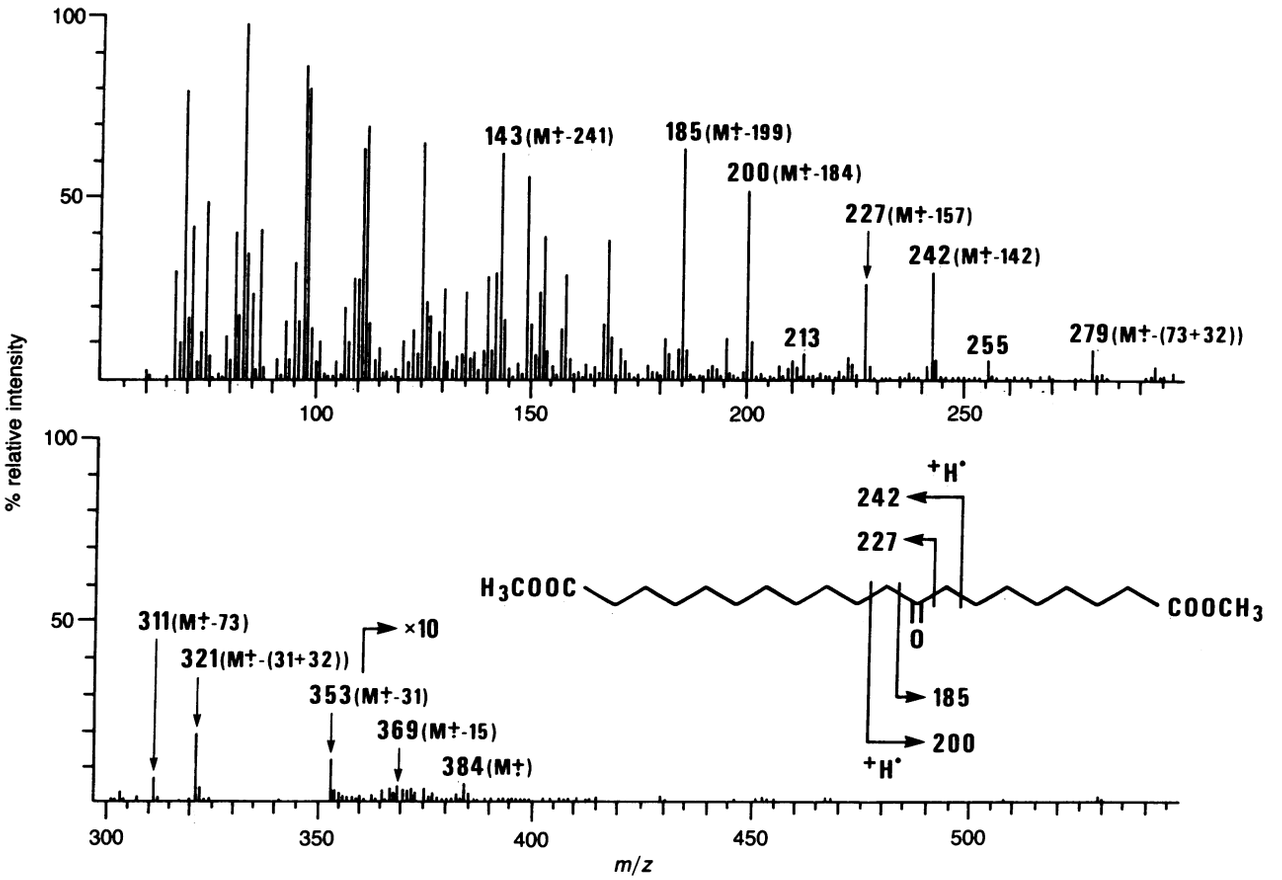


FIG. 4. Mass spectrum of hydrogenated and oxidized unknown compound. The unknown compound (6.8 μg) was hydrogenated as described in Fig. 3, and subsequently exposed to Jones reagent. The resultant oxidized molecule (dimethyl 12-keto-icosane-1,20-dioate) was then esterified and analyzed by gas chromatography–mass spectrometry. Prominent ions were observed at 185 ($M^{+\cdot}$ − 199), 200 ($M^{+\cdot}$ − 184), 227 ($M^{+\cdot}$ − 157), and 242 ($M^{+\cdot}$ − 142) indicating without equivocation that the unknown molecule has hydroxyl groups at C-12 and C-20, and that the unknown molecule is (12*S*)-12,20-dihydroxy-5,8,10,14-icosatetraenoic acid.

THETEs were produced in the absence of neutrophils (3). In contrast, THETEs were formed when 5*S*,12*S*-DiHETE was produced by addition of 12-[^{3}H]HETE to ionophore-stimulated neutrophils (3). Those results indicated that the mechanism for hydroxylation of C-20 in icosanoids was present in neutrophils but not in platelets. The experiments reported here, in which 12,20-DiHETE formation required neutrophils, support this contention.

It was of interest that 12,20-DiHETE production in platelet-neutrophil suspensions (Table 1) or on 12-HETE addition to neutrophils was markedly decreased when ionophore was the stimulus. A possible explanation for this decreased 12-HETE conversion to 12,20-DiHETE is as follows. Ionophore, a strong activator of neutrophil 5-lipoxygenase (13) can divert 12-HETE to 5,12-DiHETE (3). In addition, the 5,12-DiHETE and LTB_4 produced by ionophore stimulation may compete with 12-HETE for a putative C-20 hydroxylating enzyme.

Finally, it should be mentioned that the platelet–neutrophil interaction described herein was always accompanied by a mixed agglutination phenomenon as monitored by phase contrast microscopy.

This work was supported by grants from the Veterans Administration, National Institutes of Health HL 18828-08 SCOR (A.J.M. and M.J.B.), HL 29034,RR05396 (M.J.B.), the New York Heart Association (M.J.B.), and the Edward Gruenstein Fund (A.J.M.). M.J.B. is an Established Investigator of the American Heart Association.

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