The cytosolic fraction of human polymorphonuclear leukocytes precipitated with 60% ammonium sulfate produced 5-lipoxygenase products from [14C]arachidonic acid and 13-0H linoleic acid products from both [14C]linoleic acid and, to a lesser extent, [14C]- and [3H]-arachidonic acid. The arachidonyl 5-lipoxygenase products 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) and 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) derived from [14C]arachidonic acid, and the ω-6 lipoxygenase products 13-hydroperoxy-9,11-octadecadienoic acid (13-OOH linoleic acid) and 13-hydroxy-9,11-octadecadienoic acid (13-OOH linoleic acid) derived from [14C]linoleic acid and 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE), and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) derived from [14C]- and [3H]-arachidonic acid were identified by TLC-autoradiography and by reverse-phase high-performance liquid chromatography (RP-HPLC). Products were quantitated by counting samples that had been scraped from replicate TLC plates and by determination of the integrated optical density during RP-HPLC. The arachidonyl 5-lipoxygenase had a pH optimum of 7.5 and was 50% maximally active at a Ca** concentration of 0.05 mM; the Kₘ for production of 5-HPETE/5-HETE from arachidonic acid was 12.2 ± 4.5 μM (mean ± S.D., n = 3), and the Vₘₐₓ was 2.8 ± 0.9 nmol/min·mg protein (mean ± S.D., n = 3). The ω-6 linoleic lipoxygenase had a pH optimum of 6.5 and was 50% maximally active at a Ca** concentration of 0.1 mM in the presence of 5 mM EGTA. When the arachidonyl 5-lipoxygenase and the ω-6 lipoxygenase were separated by DEAE-Sephadex ion exchange chromatography, the ω-6 lipoxygenase exhibited a Kₘ of 77.2 μM and a Vₘₐₓ of 9.5 nmol/min·mg protein (mean = n = 2) for conversion of linoleic acid to 13-OOH/15-OOH linoleic acid and a Kₘ of 63.1 μM and a Vₘₐₓ of 5.3 nmol/min·mg protein (mean = n = 2) for formation of 15-HPETE/15-HETE from arachidonic acid.

The existence of an arachidonyl 5-lipoxygenase that produces 5-HPETE from arachidonic acid was first suggested in 1976 when Borgeat et al. (1) isolated the reduction product of 5-HPETE, 5-HETE, from rabbit peritoneal leukocytes activated with the calcium ionophore A23187. The sequence of products generated from the 5-lipoxygenase pathway, by activation of rabbit peritoneal and human peripheral blood neutrophils with calcium ionophore A23187 was identified by analysis of 0-labeled products of arachidonic acid as 5-HPETE, LTA₄ derived by dehydration of 5-HPETE, and LTß₄ obtained by enzymatic hydrolysis of LTA₄ (2-7). A calcium-activated arachidonyl 5-lipoxygenase that converts arachidonic acid to 5-HPETE has been identified in a cytosolic fraction of guinea pig peritoneal neutrophils (8) and rat basophilic leukemia cells (RBL-1) (9), respectively, but not in a similar fraction of rabbit peritoneal neutrophils, which contained only an arachidonyl 15-lipoxygenase (10). Calcium was not required for the activation of partially purified rabbit neutrophil 15-lipoxygenase (10).

In the presence of exogenous arachidonic acid, human neutrophils activated with calcium ionophore A23187 produced not only arachidonyl 5-lipoxygenase pathway products but also a racemic 15-HETE (5) when incubated for 4 min at 37°C, suggesting the nonenzymatic conversion of arachidonic acid to 15-HETE and possibly the additional action of an arachidonyl 15-lipoxygenase. Subsequently, intact human neutrophils stimulated with ionophore A23187 in the presence of exogenous arachidonic acid and sonicates of human neutrophils were shown to produce 8,15-diHETE and 14,15-diHETE from exogenous arachidonic acid (11) as well as from exogenous 15-HPETE substrate (12, 13). The 17,000 × g postgranule supernatant of human PMN disrupted by nitrogen cavitation has been used to generate 5-, 8-, 9-, and 12-

1 The abbreviations used are: 5-HPETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; LTA₄, leukotriene A₄; LTß₄, leukotriene B₄; (55,6S)-oxido-7,9-trans-11,14-cis-eicosatetraenoic acid; LTB₄, leukotriene B₄; (55,12R)-dihydroxy-6,14-cis,8,10-trans-eicosatetraenoic acid; 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 8,15-diHETE, 8,15-dihydroxy-5,9,11,13-eicosatetraenoic acid; 14,15-diHETE, 14,15-dihydroxy-5,8,10,12-eicosatrienoic acid; 15-HETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; PMN, polymorphonuclear leukocytes; 13-OOH linoleic acid, 13-hydroperoxy-9,11-octadecadienoic acid; 13-OOH linoleic acid, 13-hydroperoxy-9,11-octadecadienoic acid; 13-OOH linoleic acid, 13-hydroxy-9,11-octadecadienoic acid; EGTA, ethylene glycol bis(8-aminoethyl ether)-N,N,N',N'-tetraacetic acid; TLC, thin layer chromatography; EPA, eicosapentaenoic acid; 5-HEPE, 5-hydroxy-6,8,11,14-eicosapentaenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; Kphos buffer, potassium phosphate buffer; RP-HPLC, reverse-phase high-performance liquid chromatography; SP-HPLC, strong-phase high-performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; 9-OOH linoleic acid, 9-hydroxy-10,12-octadecadienoic acid.
monohydroxyeicosatetraenoic acids in very low yield during incubation with \(^{[3]H}\)arachidonic acid for 6 h at 37 °C, conditions suggesting their nonenzymatic formation \((14)\). We now report the initial identification, characterization, and determination of distinctions between two soluble lipoxygenase activities present in the cytosol of human PMN: a calcium ion-dependent arachidonyl 5-lipoxygenase with a pH optimum of 7.5 and a lipoxygenase with a pH optimum of 5.6-6.5 that acts at the \(\omega\)-6 position of \(\omega\)-unsaturated fatty acids to produce 13-OOH linoleic acid/13-OOH linoleic acid from linoleic acid in preference to 15-HPETE/15-HETE from arachidonic acid.

**EXPERIMENTAL PROCEDURES**

**Materials**—Arachidonic acid, docosahexaenoic acid, linolenic acid, linoleic acid, \(\gamma\)-homolinoelic acid (Supelco Inc., Bellefonte, PA); 9,12-trans,trans-octadecadienoic acid (linoleic acid), soybean lipoxygenase type \(I\) (55,000 units/mg protein), ECTA, and EDTA (Sigma); sodium azide, potassium cyanide, calcium chloride, chloroform, methanol (reagent grade), acetic acid, and diethyl ether (Fisher); high-performance liquid chromatography grade methanol (Sigma); sodium azide, potassium cyanide, calcium chloride, chloroform, and methanol (Pharmacia Fine Chemicals); \([\text{14C}]\)arachidonic acid, \([\text{3H}]\)arachidonic acid, \([\text{14C}]\)arachidonic acid, \([\text{14C}]\)linoleic acid (New England Nuclear); and \([\text{14C}]\)9,12,15-octadecatrienoic acid (linolenic acid) (Amersham Corp.) were purchased from the manufacturers. Synthetic 5-HETE, 15-HETE, LTB\(_4\), (5,5,12S)-6-trans-LTB\(_4\), and 5-HEPE were supplied by E. J. Corey (Department of Chemistry, Harvard University, Cambridge, MA) (15-17). 12-HETE standard was prepared by the incubation of sonicates of fresh human platelets (18) with arachidonic acid (10 \(\mu\)g/ml) and resolved by RP-HPLC on a 10-\(\mu\)m C\(_{18}\) Lichrosorb column (Altex-Rainin, Berkley, CA) with an isocratic mobile phase of methanol:water:acetic acid (78:22:0.01) at a flow rate of 1 ml/min.

**Assessment of Arachidonyl Lipoxygenase Activity of Human PMN**—Four to 10 \(\times\) 10\(^6\) PMN were isolated from 500 ml of citrate anticoagulated blood from each of six donors to a purity of \(\geq\)97% by sequential dextran sedimentation, hypotonic lysis, and Ficoll/Hyphae gradient centrifugation (19). The PMN were washed three times in 50 mM Kphos buffer, pH 7.0, 1 mM EDTA, resuspended to a concentration of 10\(^6\) cells/ml in the same buffer, and homogenized on ice for 5-10 s in a Potter-Elvehjem homogenizer (Tri-R Instruments, Rockville Centre, NY). Unbroken cells and debris were sedimented at 400 \(\times\) g for 5 min, and the supernatant was then centrifuged at 18,000 \(\times\) g for 1 h at 4 °C to remove granules. The 18,000 \(\times\) g supernatant was centrifuged at 150,000 \(\times\) g for 1 h, and the 150,000 \(\times\) g supernatant was stored as the cytosolic fraction of the PMN. The preparations from different cell donors. The residue was precipitated by 60% saturation with \((\text{NH}_4)\text{SO}_4\), and the precipitates were resuspended to their initial volume in 10 mM Kphos buffer at pH 7.5. Protein concentration was determined by the method of Lowry et al. (20). The resuspended (NH\(_4\))\text{SO}_4 fraction of 10\(^6\) cells contained 109 \(\pm\) 103 \(\mu\)g (mean \(\pm\) S.D., \(n = 21\)) of protein.

For assessment of 5-lipoxygenase, 80-150-\(\mu\)l portions of cytosol or of the resuspended (NH\(_4\))\text{SO}_4 precipitate (34 to 450 \(\mu\)g) were each incubated with 10 \(\mu\)M \([\text{3H}]\)arachidonic acid and 2 mM Ca\(^{2+}\) for 2 min at 22 °C in a volume of 200 \(\mu\)l in 10 mM Kphos buffer, pH 7.5. All reactions were stopped by acidification with 2 N citric acid to pH \(<\) 3, and lipids were extracted by a modification of the method of Bligh and Dyer (21). Each reaction volume was adjusted to 1 ml with distilled water and then to 3 ml with chloroform:methanol, 2:1 (v/v), and the reaction was separated into two phases by centrifugation at 200 \(\times\) g for 5 min. The lower chloroform phase was removed, evaporated to dryness under nitrogen, resuspended in 60 \(\mu\)l of chloroform:methanol, 9:1 (v/v), and chromatographed on 20 \(\times\) 20-cm TLC plates in a solvent system of ether:petroleum ether:ethanol:acetic acid (65:65:1, v/v). Standards of authentic 5-HETE, 15-HETE, LTB\(_4\), and arachidonic acid were run in separate adjoining lanes and identified by exposure to iodine vapors. The reaction products were identified by autoradiography on XR-5 x-ray film (Eastman Kodak Chemical Co., Rochester, NY) by their positions relative to the standard compounds and were quantitated on replicate plates by scraping the corresponding region into glass counting vials containing 10 ml of Aquasol and counting in a model 43 \(\beta\)-scintillation counter (Tracor Analytic, Elk Grove, IL). The recovery of radioactivity from the TLC plates was \(>95\%\) (mean \(\pm\) S.D., \(n = 7\)) of that originally added to the reaction mixture. For analysis of products by RP-HPLC, the chloroform phase of the lipid extract was evaporated under nitrogen, resuspended in 0.5 ml of methanol:water:acetic acid, 78:22:0.01 (v/v/v), injected into a 10-\(\mu\)m C\(_{18}\) Lichrosorb column and eluted isocratically at a flow rate of 1 ml/min in the same solvent; 1-ml fractions were collected. Each 1-ml fraction was diluted with 10 ml of Aquasol (New England Nuclear) and counted. The radiolabeled reaction products were identified by comparison of their retention times relative to those of the unlabeled standards, which were followed by continuous on-line monitoring with a model 100-40 spectrophotometer (Hitachi, Tokyo) at 234 nm for 5-HETE, 12-HETE, and 15-HETE and at 289 nm for LTB\(_4\). For analysis by SP-HPLC, the evaporated chloroform phase was resuspended in 0.3 ml of hexanes:propanoic acid, 98:3:16 (v/v/v) and eluted isocratically with this solvent at a flow rate of 1 ml/min from an Altex UltraSil-Si 0.46 \(\times\) 25 mm 10-\(\mu\)m column (22). The reaction products were identified and quantitated as for RP-HPLC. In experiments that utilized \([\text{14C}]\)EPA, 5-HEPE was served as a standard and was detected on line at 234 nm. Each final experimental variable was established with two or three enzyme preparations from different cell donors.

**Structural Analysis of 13-OOH Linoleic Acid**—Nine mg of cytosol were incubated for 10 min at 22 °C with 2 mM Ca\(^{2+}\) and 40 \(\mu\)M \([\text{14C}]\)arachidonic acid in 10 mM Kphos buffer, pH 7.0, 1 mM EDTA in a volume of 24 ml. The reaction products were then eluted from a reversed-phase Sep-Pak by sequential washes with 10 ml of petroleum ether, ethyl acetate, and methanol. All the radioactivity eluted in the ethyl acetate fraction, and the ethyl acetate was evaporated under nitrogen. The residue was resuspended in methanol:water:acetic acid (78:22:0.01) and eluted from an Altex ODS-10-\(\mu\)m RP-HPLC column at a flow rate of 1 ml/min, collecting 1-ml fractions. On-line monitoring at 234 nm gave one peak which eluted at 27 min and contained all the radioactive product. The reaction product was subjected to GC-MS analysis as the trimethylsilyl ethyl methyl ester derivative following previously published procedures (23). Gas chromatography was carried out on a 200 \(\times\) 0.2-cm OV–101 column (23).

**RESULTS**

**Product Analysis**—The reaction products of 96 \(\mu\)l (270 \(\mu\)g) of human PMN cytosol and \([\text{14C}]\)arachidonic acid incubated for 2 min at 22 °C in the presence and absence of Ca\(^{2+}\) were resolved by both TLC and RP-HPLC. In the presence of 2 mM Ca\(^{2+}\), four products were detected by TLC analysis (Fig. 1A), with respective \(R_f\) values of 0.31 (identical to that of authentic 5-HETE), 0.51 (distinct from that of 15-HETE, \(R_f\) 0.54), 0.07 (identical to that of LTB\(_4\), and its diHETE isomers), and 0; arachidonic acid migrated with an \(R_f\) of 0.72. In the
absence of Ca\textsuperscript{2+} no products were formed. When products of a parallel reaction were analyzed by RP-HPLC (Fig. 1B), the cpm recovered at the retention time corresponding to 5-HETE accounted for 44% of the radioactivity while 22% eluted with LTB\textsubscript{4}; some label appeared in the 15-HETE region, but none co-eluted with 12-HETE. When a parallel reaction was analyzed by SP-HPLC, only one monohydroxyeicosatetraenoic acid product, co-migrating with synthetic standard 5-HETE at a retention time of 18 min, was identified. This profile of products was representative of enzyme preparations from donors 1, 2, and 3.

TLC analysis of the products of \textsuperscript{[\textsuperscript{14}C]}arachidonic acid formed with 73 \textmu g of the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction of human PMN cytosol from donor 1 incubated for 2 min at 22 °C with 2 mM Ca\textsuperscript{2+} revealed three products (Fig. 2A) with RF values of 0, 0.31, and 0.51. No discrete product representing LTB\textsubscript{4} or 6\textendash-trans-LTB\textsubscript{4} was seen between RF values of 0 and 0.31. When the reaction products of a parallel mixture from this and a separate experiment with 54 \textmu g of the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction from the same donor were incubated with a 50 M excess (500 \textmu M) of NaBH\textsubscript{4} for 10 min on ice and then for 1 h at room temperature, there was a redistribution of products from an RF of 0.51 to 0.31. After reduction, products co-migrating at RF 0.31, which corresponds to 5-HETE, accounted for 90.4% of the radioactivity distributed between these two regions, thereby identifying the product with an RF of 0.51 as 5-HPETE. 80% of the radiolabeled product from formation of arachidonic acid by the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction was identified as arachidonyl 5-lipoxygenase products (Fig. 2). When parallel reactions with 34 \textmu g of the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction were analyzed by RP-HPLC, 68% of the radioactivity eluted with 5-HETE/5-HPETE, 11% with LTB\textsubscript{4}, little or none with 15-HETE, and none with 12-HETE (Fig. 2B). This pattern was representative of the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction of PMN cytosol from donors 1, 2, and 3. Because the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction of cytosol produced no 12-HETE and little or no 15-HETE/5-HPETE (<2% of 5-HETE/5-HPETE) from \textsuperscript{[\textsuperscript{14}C]}arachidonic acid, whereas at least 80% of the products of \textsuperscript{[\textsuperscript{14}C]}arachidonic acid could be identified as 5-HPETE/5-HETE and LTB\textsubscript{4}, as assessed by TLC and RP-HPLC (Fig. 2), analysis of 5-HETE/5-HPETE afforded a quantitative measurement of arachidonyl 5-lipoxygenase in the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction of the cytosol from the PMN of these donors.

The recovery of protein after (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fractionation was 25.3 ± 0.7% (mean ± S.D., n = 4) whereas the average recovery of arachidonyl 5-lipoxygenase activity was 131 ± 85%. The purification obtained by the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fractionation was 4.4 ± 1.7-fold (n = 4). Neither the addition of potassium cyanide (2 mM) nor of sodium azide (2 mM) to the reaction mixture had any effect on the total arachidonyl 5-lipoxygenase activity or on the distribution of products as analyzed by TLC, indicating that heme-containing peroxidases were not affecting the reaction.

**Substrate Utilization and Recognition of an \textalpha\textomega-6 Lipoxygenase**—The relative activity of 412 \mu g of the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction of human PMN cytosol incubated with 10 \mu M \textsuperscript{[\textsuperscript{14}C]}arachidonic acid was compared with its activity when incubated with equal concentrations of seven alternative fatty acid substrates after incubation for 2 min at 22 °C with 2 mM Ca\textsuperscript{2+} (Table I). \textsuperscript{[\textsuperscript{14}C]}EPA yielded products detected by TLC-autoradiography with RF values of 0.31 and 0.51, of which the former co-migrated with authentic 5-HEPE. These products were presumed to be 5-hydroperoxy-6,8,11,14,17-eicosaapentaenoic acid and 5-HEPE as indicated by the elution of a single peak of radioactivity on RP-HPLC in the position of a 5-HEPE standard at 27 min. Quantitation of labeled products after TLC revealed somewhat more activity with \textsuperscript{[\textsuperscript{14}C]}EPA than with \textsuperscript{[\textsuperscript{14}C]}arachidonic acid (Table I). \textsuperscript{[\textsuperscript{14}C]}EPA produced 80% of total product, whereas 30% of activity was a substrate as \textsuperscript{[\textsuperscript{14}C]}arachidonic acid; products were not further identified. The same concentrations of the fatty acid substrates 4,7,10,13,16,19-docosahexaenoic acid, \gamma-linoleic acid, and 9,12-trans,trans-10-octadecadienoic acid (linoleic acid) were less than 4% as active as \textsuperscript{[\textsuperscript{14}C]}arachidonic acid as assessed by integrated absorbance at 234 nm during RP-HPLC with elution to 35 min. A 10 \mu M concentration of \textsuperscript{[\textsuperscript{14}C]}EPA (23.7% of total product) and 0.52 (10.2% of total product) were identified as 13-00H linoleic acid and 13-OH linoleic acid, respectively, by reduction with a 50 M excess of NaBH\textsubscript{4} and parallel TLC analysis to demonstrate quantitative conversion of the product with an RF of 0.52 to one with an RF of 0.52. In these experiments, the unidentified radioactivity remaining at the origins was 39.7% and was unchanged by incubation with NaBH\textsubscript{4}.

The predominant product of the reaction of \textsuperscript{[\textsuperscript{14}C]}linoleic acid with 1 \mu g of soybean lipoygenase type I, which is known to be 13-OOH/13-OH linoleic acid (24), co-chromatographed on RP-HPLC with a major product produced by 154 \mu g of the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fraction (Fig. 3B). Gas chromatography-mass spec-
buffer, pH products produced by soybean lipoxygenase type acid was incubated with the (NH₄)₂SO₄ fraction in TLC autoradiography and by alent chain length of 20.1. Electron impact mass spectrometry cant at m/z 382 (m), 335 (m-47), 311 (loss of CH₃(CH₄)₄-) 292 (m-90), and 225 (loss of (CH₃)₂COOCH₃). Since the 13-Oh and 9-Oh linoleic acid derivatives present similar mass spectra (25) and display similar GC retention times (25), catalytic hydrogenation was carried out using 5% Rh on Al₂O₃ (70 eV) displayed a single major component and was significant at m/z 386 (m), 371 (m-15), 355 (m-31) (TMSO-CH(CH₂)₄COOCH₃), 286 (m-90), and 173 (CH₃(CH₂)₆CHOCH₃) (25). Selected ion chromatograms for the 13-Oh linoleic acid (trimethylsilyl ether methyl ester) with ions at m/z 382, 335, 311, 292, 225, and 173 (CH₃(CH₂)₆CHOCH₃) (25). The configuration of the hydroxyl group and geometry of the diene were not determined.

Because partially purified arachidonyl 5-lipoxygenase of the guinea pig peritoneal neutrophil (8) does not utilize linoleic acid or linolenic acid as substrates, it seemed likely that the linoleic acid lipoxygenase activity of the human neutrophil was distinct from the arachidonyl 5-lipoxygenase. Both [¹⁴C]arachidonic acid and [¹⁴C]linoleic acid were then used to define the pH optimum, cation dependence, kinetics, and elution characteristics from DEAE-Sephadex chromatography of these two enzymatic activities.

**Differential Calcium Ion and pH Requirements for Arachidonyl 5-Lipoxygenase and Linoleic Acid Lipoxygenase**—The effect of Ca⁡⁺⁺ on the arachidonyl 5-lipoxygenase activity was examined with 66–75 μg of the (NH₄)₂SO₄ fraction of PMN from donor 1 and a 3-min incubation at 22 °C followed by TLC. No activity for arachidonyl 5-lipoxygenase was found in the absence of calcium, and 0.05 mM Ca⁡⁺⁺ gave 50% maximal stimulation of 5-lipoxygenase (Fig. 4A). Utilizing 46 μg of the (NH₄)₂SO₄ fraction from donor 4, the conversion of linoleic acid to 13-OOH/13-OH linoleic acid was found in the absence of calcium, and 0.05 mM Ca⁡⁺⁺ gave 50% maximal stimulation of 5-lipoxygenase (Fig. 4A). Utilizing 46 μg of the (NH₄)₂SO₄ fraction from donor 4, the conversion of linoleic acid to 13-OOH/13-OH linoleic acid was found in the presence of calcium, and 0.05 mM Ca⁡⁺⁺ gave 50% maximal stimulation of 5-lipoxygenase (Fig. 4A). Utilizing 46 μg of the (NH₄)₂SO₄ fraction from donor 4, the conversion of linoleic acid to 13-OOH/13-OH linoleic acid was found in the presence of calcium, and 0.05 mM Ca⁡⁺⁺ gave 50% maximal stimulation of 5-lipoxygenase (Fig. 4A).
at 6.5. The activity of the lipoxygenase on linoleic acid was more than 80% maximal at pH 5.6 (Fig. 5B), a pH at which the arachidonyl 5-lipoxygenase was only minimally active, and the linoleic acid lipoxygenase was also 50% maximally active at pH 7.5 in Kphos buffer with Ca$^{2+}$ and EGTA.

**Protein Concentration Dependence and Time Course of the Arachidonyl 5-Lipoxygenase**

The production of 5-HPETE/5-HETE from 20 μM [14C]arachidonic acid by the (NH₄)₂SO₄ fractions from donors 1 (Fig. 6A) and 2 showed a linear dependence with the addition of 12–88 μg of protein (60 to 440 μg/ml) and leveled off at higher concentrations. The conversion of 20 μM [14C]linoleic acid to 13-OOH/13-OH linoleic acid by the (NH₄)₂SO₄ fractions from donors 3 and donor 4 (Fig. 6A) was linear with the additions of 12–110 μg of protein (115 to 550 μg/ml) and plateaued at higher concentrations, whereas in the presence of 1 mM EGTA and 2 mM Ca$^{2+}$ 13-OOH/13-OH linoleic acid production was linear with the addition of 115 to 1000 μg of protein (Fig. 6A).

The conversion of 20 μM [14C]arachidonic acid to 5-HPETE/5-HETE by 63 μg of the (NH₄)₂SO₄ fraction from donor 1 accelerated rapidly for 3 min and was complete by 5 min. The conversion of 20 μM [14C]linoleic acid to 13-OOH/13-OH linoleic acid by 143 μg of the (NH₄)₂SO₄ fraction from donor 4, carried out in 10 mM Kphos buffer, pH 7.5, and in the presence of 1 mM EGTA and 2 mM Ca$^{2+}$ accelerated over 10 min and then leveled off at 20 min (Fig. 6B). When the time course was determined in 10 mM Kphos buffer, pH 7.0, without calcium, the plateau also occurred at 20 min for conversion of [14C]linoleic acid to products.

**Kinetics of the Arachidonyl 5-Lipoxygenase**

The kinetics of the interaction of arachidonyl 5-lipoxygenase (51–78 μg of the (NH₄)₂SO₄ fraction from donors 1 and 2) with its two preferred substrates, arachidonic acid and EPA, were determined with substrate concentrations between 2.5 and 100 μM, and the results of a representative experiment (with PMN from donor 1) are shown in Fig. 7, A and B. In this experiment the $K_m$ for arachidonic acid was 12 μM, and the $V_{max}$ was calculated at 1.72 nmol/min·mg protein. For EPA, the $K_m$ was 23 μM, and the $V_{max}$ was 5.5 nmol/min·mg protein. As shown in Fig. 7B, both substrates manifested substrate inhibition, which is most evident above a concentration of 20 μM. When two additional enzyme preparations were studied, the $K_m$ for arachidonic acid was 12.2 ± 4.5 μM and the $V_{max}$ was 2.8 ± 0.98 nmol/min·mg protein (mean ± S.D., n = 3). When one additional determination of kinetics was made for EPA, the mean $K_m$ was 25 μM and the mean $V_{max}$ was 5.0 nmol/min·mg protein.

**Interaction of Linoleic Acid Lipoxygenase with Arachidonic Acid**

The velocity of 13-OOH/13-OH linoleic acid production by 89 μg of the (NH₄)₂SO₄ fraction from donor 4 was measured at pH 7.5, with 1 mM EGTA and 2 mM Ca$^{2+}$ at concentrations of linoleic acid ranging from 10 to 100 μM in buffer alone and in the presence of 1, 5, or 10 μM unlabeled arachidonic acid and analyzed by Lineweaver-Burk plots (Fig. 8).
8). The secondary plot of the slopes of the reciprocal lines versus the concentration of arachidonic acid was then constructed (Fig. 8, inset), and the \( K_i \) for competitive inhibition, determined as the negative of the \( x \) intercept, was 2.8 \( \mu M \). An analogous experiment carried out with 48 \( \mu g \) of the \((NH_4)_2SO_4\) fraction from donor 5 yielded a \( K_i \) of 1.8 \( \mu M \). When the converse experiment was performed with 86 \( \mu g \) of the \((NH_4)_2SO_4\) fraction from the PMN of donor 1 to determine arachidonyl 5-lipoxygenase kinetics with 5 to 75 \( \mu M \) [\(^{14}C\)] arachidonic acid in the presence of 0, 50, 100, and 200 \( \mu M \) unlabeled linoleic acid, no inhibition was seen at concentrations of linoleic acid below 100 \( \mu M \), and 35\% inhibition was seen at 200 \( \mu M \).

The lipoxygenation of linoleic acid at the \( \omega-6 \) position and the inhibition of this reaction by arachidonic acid suggested that the linoleic acid lipoxygenase would also utilize arachidonic acid as a substrate to produce 15-HPETE/15-HETE. The \((NH_4)_2SO_4\) fraction of cytosol from PMN of donors 4 and 6 was examined for arachidonyl 5- and 15-lipoxygenase activities, as well as linoleic acid lipoxygenase activity. When 86 \( \mu g \) of the \((NH_4)_2SO_4\) fraction from donor 4 was incubated with 10 \( \mu M \) [\(^{14}C\)] arachidonic acid and 2 \( mM \) Ca\(^{2+}\), two products with an \( R_F \) of 0.56 and 0.60 were detected by TLC and autoradiography and accounted for more than 90\% of the metabolites (Fig. 9A). Reduction of the products in a parallel reaction with a 50 \( mM \) excess of NaBH\(_4\) resulted in an almost quantitative redistribution of the product with an \( R_F \) of 0.60 to that of 0.56. In addition, when the products of a parallel reaction with [\(^{3}H\)] arachidonic acid used as substrate were analyzed by RP-HPLC (Fig. 9), radioactivity co-eluted at 21 min with a 15-HETE standard; none eluted at 26 min with a 5-HETE standard. These results indicated that the products of arachidonic acid lipoxygenation by enzyme from PMN of donor 4, as assessed by TLC autoradiography, were 15-HPETE (\( R_F \) 0.60) and 15-HETE (\( R_F \) 0.56). 71 \( \mu g \) of the \((NH_4)_2SO_4\) fraction from this donor produced two products from 10 \( \mu M \) [\(^{14}C\)] linoleic acid with \( R_F \) values of 0.58 and 0.52, which accounted for 0.24 nmol of 13-OH linoleic acid. 71 \( \mu g \) of the \((NH_4)_2SO_4\) fraction converted [\(^{14}C\)] arachidonic acid to 0.09 nmol of 15-HPETE/15-HETE. When the products of the reaction of 144 \( \mu g \) of cytosol from the PMN of donor 6 with 10 \( \mu M \) [\(^{3}H\)] arachidonic acid were analyzed by RP-HPLC, 1.12 nmol of 5-HPETE/5-HETE (retention time 30 min) and 0.8 nmol of 15-HPETE/15-HETE (retention time 22 min) were produced but no 12-HETE was identified. The identity of the products was confirmed by analysis of reaction products by SP-HPLC in hexane/isopropanol-acetic acid, 983:16:1 (v/v/v). Three radioactive peaks eluting at 6 min (arachidonic acid), 12 min (co-eluting with 15-HETE standard), and 19 min (co-eluting with 5-HETE standard) were observed.

**Chromatographic Separation of Two Lipoxygenases**—4 ml of cytosol from the PMN of donor 6, which produced both 5- and 15-lipoxygenase products from 20 \( \mu M \) [\(^{3}H\)] arachidonic acid, were applied directly to a 2 \( \times \) 5-cm DEAE-ion exchange column previously equilibrated in 10 mM Kphos buffer, pH 7.0, 1 mM EDTA. The column was washed with 2.5 bed volumes of equilibration buffer and eluted with a 60-ml gradient of 0.0 to 0.5 \( M \) NaCl in 10 mM Kphos, 1 mM EDTA, pH 7.0 (Fig. 10). The identity of the products of arachidonic acid obtained with the pooled fractions of the separated enzymes was established by analysis by both RP-HPLC and SP-HPLC. The initial peak of lipoxygenase activity eluting from DEAE at 5.7 mS yielded one product from arachidonic acid as assessed after reduction with NaBH\(_4\); this product co-eluted with the 15-HETE standard at 23 min on RP-HPLC and at 12 min on SP-HPLC. The lipoxygenase activity eluting from DEAE at 20 mS also formed one product with arachidonyl 5-lipoxygenase as determined after reduction with NaBH\(_4\); the product co-eluted with the 5-HETE standard at 33 min on RP-HPLC and at 19 min on SP-HPLC. 93 \( \mu g \) of protein from the first peak of lipoxygenase activity generated 0.23 nmol of 13-OH/13-OH linoleic acid from [\(^{14}C\)] linoleic acid and 0.11 nmol of 15-HPETE/15-HETE from [\(^{3}H\)] arachidonic acid, respectively, as quantitated by TLC, for a ratio of 2:1:1. In contrast, 126 \( \mu g \) of protein from the second peak of lipoxygenase activity produced 0.29 nmol of 5-HETE and no 15-HETE from [\(^{3}H\)] arachidonic acid, and 0.06 nmol of product from linoleic acid. In a second experiment with 4 ml of cytosol from the cells of the same donor obtained at another occasion, the 0-6 lipoxygenase eluting at 3.4 mS produced 1.4 nmol of 13-OH/13-OH from [\(^{14}C\)] linoleic acid and no products from [\(^{3}H\)] arachidonic acid, and the lipoxygenase activity eluting at 17.9 mS produced 0.88 nmol of 5-HPETE/5-HETE but no arachidonyl 12- or 15-lipoxygenase products from [\(^{3}H\)] arachidonic acid, and no products from [\(^{14}C\)] linoleic acid.

**Kinetics of the Separated w-6 Lipoxygenase and 5-Lipoxygenase**—Four ml of the \((NH_4)_2SO_4\) fraction from the PMN of donor 6 were dialyzed against 10 mM Kphos buffer, pH 7.0, 1

![Fig. 9. Products of the reaction of the NH_4_2SO_4 fraction of PMN cytosol with arachidonic acid. 10 \mu M [\(^{14}C\)] arachidonic acid (A) or 80 \mu M [\(^{3}H\)] arachidonic acid (B) were incubated with the \((NH_4)_2SO_4\) fraction of donor 4 in pH 7.5 buffer for 2 min at 22 \degree C and analyzed by (A) TLC autoradiography and (B) RP-HPLC.](image-url)
washed with equilibration buffer at a flow rate of 30 ml/h.

with two different preparations the mean kinetic parameters obtained at pH 6.5 with two separate preparations of the \( \omega-6 \) arachidonyl 5-lipoxygenase remained bound to the column until eluted with 0.5 mM EDTA, 2 mM Ca\(^{2+} \), for 7 min at 22 \(^\circ\)C. A, substrate versus velocity; B, Lineweaver-Burk plot.

mm EDTA, 0.1 M NaCl until the conductivity was the same as the buffer. The dialyzed fraction was applied to a 2 x 5-cm DEAE column equilibrated in this buffer. The column was washed with equilibration buffer at a flow rate of 30 ml/h. The \( \omega-6 \) lipoxygenase appeared in the effluent, and the arachidonyl 5-lipoxygenase remained bound to the column until eluted with 0.5 mM Kphos buffer, pH 7.5, 1 mM EDTA, and 2 mM Ca\(^{2+} \). The \( K_m \) for the conversion of linoleic acid to 13-OH/13-10 linoleic acid was 83 \( \mu M \), and the \( V_{max} \) 10 nmol/min·mg protein, and the \( K_m \) for the conversion of arachidonic acid to 15-HPETE/15-HETE was 63.4 \( \mu M \) and the \( V_{max} \) 5.3 nmol/min·mg protein (Fig. 11).

For experiments with two different preparations the mean kinetic parameters were a \( K_m \) of 77.2 \( \mu M \) and a \( V_{max} \) of 9.5 nmol/min·mg protein for linoleic acid, and a \( K_m \) of 63.1 \( \mu M \) and a \( V_{max} \) of 5.3 nmol/min·mg protein for arachidonic acid. Kinetic parameters obtained at pH 6.5 with two separate preparations of the \( \omega-6 \) lipoxygenase were a \( K_m \) of 72.5 \( \mu M \) and a \( V_{max} \) of 30.8 nmol/min·mg protein for linoleic acid, and 57.1 \( \mu M \) and 11.3 nmol/min·mg protein for arachidonic acid. For the separated 5-lipoxygenase at optimal pH, the \( K_m \) was 17.5 \( \mu M \) and the \( V_{max} \) was 5.6 nmol/min·mg protein.

**DISCUSSION**

The cytosol of human PMN has been shown to contain two distinct lipoxygenases, an arachidonyl 5-lipoxygenase and an \( \omega-6 \) lipoxygenase with preferential activity for linoleic acid over arachidonic acid. The appreciation of these two activities followed the recognition of marked donor variability in their expression and the finding that linoleic acid was the preferred substrate for the \( \omega-6 \) lipoxygenase. An (NH\(_4\))\(_2\)SO\(_4\) fraction was utilized originally as a source of both lipoxygenases as it afforded a 4-fold purification of the arachidonyl 5-lipoxygenase, substantially eliminated cytosolic reducing activity, and showed significantly less production of diHETE isomers from \(^{14}C\)arachidonic acid as analyzed by TLC autoradiography and RP-HPLC with synthetic standards used as markers (Figs. 1 and 2). The redistribution of radioactivity from an \( R_f \) of 0.51 to 0.31 confirmed the identity of the two major reaction products as 5-HPETE and 5-HETE, respectively. For donors 1, 2, and 3, 80% of the reaction products of \(^{14}C\)arachidonic acid with the (NH\(_4\))\(_2\)SO\(_4\) fraction could be identified as 5-lipoxygenase products. No 12-HETE and only trivial amounts of 15-HPETE/15-HETE were appreciated by RP-HPLC.

Quantitative analysis by TLC of products detected by autoradiography after metabolism of \(^{14}C\)arachidonic acid and \(^{14}C\)EPA by the (NH\(_4\))\(_2\)SO\(_4\) fraction of donors 1 and 2 revealed EPA to be a slightly preferred substrate (Table 1) as reported for the arachidonyl 5-lipoxygenase of the guinea pig PMN. An additional finding was that the cytosolic fraction of human PMN converted linoleic acid to 13-OH (\( R_f \) 0.58) and 13-OH (\( R_f \) 0.52) linoleic acid (Fig. 3). The monohydroxy product derived from linoleic acid was isolated by RP-HPLC, and its identity as 13-OH-linoleic acid was established by GC-MS.

The arachidonyl 5-lipoxygenase and the linoleic acid lipoxygenase represented separate enzymatic activities was initially supported by defining selected properties of the reactions of \(^{14}C\)arachidonic acid and \(^{14}C\)linoleic acid with the (NH\(_4\))\(_2\)SO\(_4\) fraction and confirmed by separation of the enzymes by chromatography on DEAE-Sephadex (Fig. 10). Both the arachidonyl 5-lipoxygenase and the \( \omega-6 \) lipoxygenase required the addition of Ca\(^{2+} \) for full expression of activity, but the \( \omega-6 \) lipoxygenase retained 22% of its activity in the presence of EGTA (Fig. 4). The pH optimum of the arachidonyl 5-lipoxygenase was 7.5, and this enzyme was only minimally active below a pH of 6.5 (Fig. 5A). The \( \omega-6 \) lipoxygenase showed an optimal range of activity between 5.6 and 7.5, with a peak at 6.5 (Fig. 5B) and was highly active at pH 5.6 where almost no arachidonyl 5-lipoxygenase activity could be detected. Linoleic acid did not competitively inhibit the conversion of \(^{14}C\)arachidonic acid to 5-HPETE/5-HETE by the arachidonyl 5-lipoxygenase, further indicating that these fatty acids were substrates for different enzymes.

The production of 13-OH linoleic acid, an \( \omega-6 \) lipoxygenase product, and the inhibition of this reaction by arachidonic acid (Fig. 8) suggested that the \( \omega-6 \) lipoxygenase would convert arachidonic acid to 15-HPETE/15-HETE. This was confirmed by analysis of the lipoxygenase products formed from arachidonic acid and linoleic acid by the (NH\(_4\))\(_2\)SO\(_4\) fraction (Fig. 9) and by the analysis of the lipoxygenase products of \( \omega-6 \)-lipoxygenase following isolation of the enzyme by DEAE chromatography (Fig. 10). The ratio of 13-OH/13-10 linoleic acid to 15-HPETE/15-HETE, produced from linoleic acid and arachidonic acid, respectively, was 2:2:1, indicating that linoleic acid is the preferred substrate for this human \( \omega-6 \) lipoxygenase, as has been reported for the soybean lipoxygenase and the homogeneous rabbit reticulocyte lipoxygenase (27). The high degree of specificity for the \( \omega-6 \)-position of linoleic acid, as determined by GC-MS, is similar to that described for soybean lipoxygenase (26) and the rabbit reticulocyte lipoxygenase (28). Although we did not determine the configuration of the double bonds of 13-OH linoleic acid, we assume the structure is 13-OH,9,11(cis,trans) as is the case for the product of the soybean lipoxygenase (24).

The \( K_m \) of the arachidonyl 5-lipoxygenase was 12.2 ± 4.5 \( \mu M \) (mean ± S.D.), and the \( V_{max} \) was 2.5 ± 0.9 nmol/min·mg protein (0.30 ± 0.11 nmol/min x 10\(^4\) cells) (mean ± S.D., \( n = 3 \)) with the NH\(_4\)SO\(_4\) fraction (Fig. 7B). The \( K_m \) was 17.5 \( \mu M \), and the \( V_{max} \) was 5.6 nmol/min·mg protein with the 5-lipoxygenase obtained after DEAE chromatography. The arachidonyl 5-lipoxygenase shows substrate inhibition with increasing concentrations of its preferred substrates, arachidonic acid and EPA (Fig. 7, A and B), whereas the \( \omega-6 \) lipoxygenase activity does not at increasing concentrations of linoleic acid or arachidonic acid (Fig. 11). \( \omega-6 \) Lipoxygenase, resolved from 5-lipoxygenase by DEAE chromatography, gave a \( K_m \) for the conversion of linoleic acid to 13-OH/13-10 linoleic acid of 77.2 \( \mu M \) with a \( V_{max} \) of 9.5 nmol/min·mg protein (mean, \( n = 2 \)) and a \( K_m \) for the conversion of arachidonic acid to 15-HPETE/15-HETE of 63.1 \( \mu M \) with a \( V_{max} \) of 5.3 nmol/min·mg protein at pH 7.5; thus, the \( K_m \) values for the two substrates were similar, but there was almost a 2-fold increase in the \( V_{max} \) for linoleic acid (Fig. 11). Kinetic parameters determined at pH 6.5 were again similar in terms of \( K_m \), while the
Vmax favored linoleic acid relative to arachidonic acid by about 3-fold. The $K_v$ values determined for both substrates for the $\omega$-6 lipoxygenase were 5-fold higher than that of arachidonic acid for the arachidonoyl 5-lipoxygenase separated from the same set of human PMN.

Unlike platelets (29), human monocytes (30), or alveolar macrophages (31), the plasma membranes of human PMN contain linoleic acid in equal quantity to arachidonic acid (32). It is also present in the phagolysosome of human PMN where the low pH might allow its utilization as a lipoxygenase substrate were it to be released from membrane phospholipids (32). It is also present in the phagolysosome of human PMN and the generation of these hydroxy-7,9,11,13-eicosatetraenoic acid for the arachidonyl 5-lipoxygenase separated from the same set of human PMN.

14,15-$$\Delta$$-trihydroxy-7,9,11,13-eicosatetraenoic acid (lipoxin A) and 5-D-arachidonyl 5-lipoxygenase separated from the $\omega$-6 lipoxygenase may explain why only 20:4 products are detected by RP-HPLC and gas chromatography-mass spectroscopy when intact PMN are induced to release lipoxygenase products by the Ca$^{2+}$ ionophore A23187 at neutral pH (5). In the absence of exogenous arachidonic acid, intact human neutrophils activated with ionophore have been reported not to produce 15-HPETE/15-HETE (5) and to generate only small amounts of 8,15-diHETE (11-13). Serhan et al. have described the biologically active products 5,6,15-$$\Delta$$-hydroxy-7,9,11,13-eicosatetraenoic acid (lipoxin A) and 5- and 14,15-1-trihydroxy-7,9,11,13-eicosatetraenoic acid (lipoxin B) formed from 15-HPETE by human PMN activated with the calcium ionophore A23187 (33, 34). The generation of these products from membrane-derived arachidonic acid would involve the $\omega$-6 lipoxygenase and the 5-lipoxygenase, but any coherent hypothesis for their sequential action must consider their different pH optima and the availability of alternative substrate for the $\omega$-6 lipoxygenase.

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