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Conversion of 15-Hydroxyeicosatetraenoic Acid to 11-Hydroxyhexadecatrienoic Acid by Endothelial Cells†

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ABSTRACT: Cultured endothelial cells take up 15-hydroxyeicosatetraenoic acid (15-HETE), a lipoxygenase product formed from arachidonic acid, and incorporate it into cellular phospholipids and glycerides. Uptake can occur from either the apical or basolateral surface. A substantial amount of the 15-HETE incorporated into phospholipids is present in the inositol phosphoglycerides. 15-HETE is converted into several metabolic products that accumulate in the extracellular fluid; this conversion does not require stimulation by agonists. The main product has been identified as 11-hydroxyhexadecatrienoic acid [16:3(11-OH)], a metabolite of 15-HETE that has not been described previously. Formation of 16:3(11-OH) decreases when 4-pentenoic acid is present, suggesting that it is produced by β-oxidation. The endothelial cells can take up 16:3(11-OH) only 25% as effectively as 15-HETE, and 16:3(11-OH) is almost entirely excluded from the inositol phosphoglycerides. These results suggest that the endothelial cells can incorporate 15-HETE when it is released into their environment. Through partial oxidation, the endothelium can process 15-HETE to a novel metabolite that is less effectively taken up and, in particular, is excluded from the inositol phosphoglycerides.

15(*S*)-Hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE)[1] is an arachidonic acid derivative formed by cells that contain 15-lipoxygenase activity (Ford-Hutchinson, 1985). 15-HETE inhibits several of the enzymes involved in eicosanoid synthesis, including phospholipase A_2 (Chang et al., 1985), 5-lipoxygenase (Borgeat & Samuelsson, 1979), 12-lipoxygenase (Vanderhoek et al., 1980), and cyclooxygenase (Vanderhoek et al., 1984). In this regard, 15-HETE reduces prostacyclin (PGI_2) formation when it is added to endothelial cultures (Hadjiagapiou & Spector, 1986), suggesting that localized concentrations of this lipoxygenase product might comprise the antithrombotic and vasodilator capacity of the endothelium.

Since the endothelium synthesizes small quantities of 15-HETE (Gorman et al., 1985; Mayer et al., 1986), the amount available to the cells probably is regulated in part through an autocrine mechanism. In addition, several cells that are either adjacent to, or can come in contact with, the endothelium such as smooth muscle (Larrue et al., 1983), eosinophilic leukocytes (Turk et al., 1982), and macrophages (Rabinovitch et al., 1981) release 15-HETE. A number of different cells can take up monohydroxyeicosatetraenoic acids from the extracellular fluid (Bonser et al., 1981; Pawlowski et al., 1982; Stenson et al., 1983), including endothelial cells (Kühn et al., 1985; Schafer et al., 1986; Richards et al., 1986), suggesting that 15-HETE released from adjacent cells also may influence the amount available to the endothelium. Therefore, we have examined the factors that influence the interaction between 15-HETE and endothelial cells to further evaluate the potential role of exogenously derived 15-HETE in endothelial function.

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[1] Abbreviations: 15-HETE, 15-hydroxyeicosatetraenoic acid; PGI_2, prostaglandin I_2 or prostacyclin; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; GLC, gas-liquid chromatography; ECL, equivalent chain length; HHT, 12-hydroxyheptadecatrienoic acid; NDGA, nordihydroguaiaretic acid; 8,15-diHETE, 8,15-dihydroxyeicosatetraenoic acid; 16:3(11-OH), 11-hydroxyhexadecatrienoic acid; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; MEM, minimal essential medium; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; CoA, coenzyme A.

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Experimental Procedures

Cell Culture. Endothelial cells were isolated from the human umbilical vein or bovine aorta by treatment with collagenase (Jaffe et al., 1973). Primary cultures of human umbilical vein endothelial cells were maintained in medium 199 (KC Biological, Lenexa, KS) containing 20% fetal bovine serum, MEM nonessential amino acids, MEM vitamin solution, 2 mM glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma, St. Louis, MO), and 100 μg/mL neomycin at 37 °C in a humidified 5% CO_2 atmosphere (Czervionke et al., 1978). Bovine aortic endothelial cells between passages 4 and 15 were grown in Dulbecco's minimal essential medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum supplemented with MEM nonessential amino acids, MEM vitamin solution, HEPES, glutamine, and 50 μg/mL gentamycin. Serial passage was achieved following treatment of confluent monolayers with a solution containing 0.25% trypsin and 0.5% EDTA. Cells were seeded in six-well tissue culture plates (Costar, Cambridge, MA) and grown to confluence before use. Cell protein was measured by a modification of the Lowry method (Lees & Paxman, 1972).

Bovine aortic endothelial cells were grown as confluent monolayers on micropore filters (Shasby et al., 1982). Polycarbonate filters (0.8-μM pore size, 13-mm diameter, Nucleopore, Pleasanton, CA) were gelatin impregnated and glued to polystyrene cylinders (ADAPS Inc., Dedham, MA) which were suspended in wells of a 24-well tissue culture plate and sterilized with ethylene oxide at 130 °C (Shasby et al., 1982). The gelatin-impregnated filters were exposed to 30 μg/mL fibronectin (Collaborative Research, Cambridge, MA) for 30 min and washed, and 0.5 mL of a suspension containing 4×10^5 bovine aortic endothelial cells was placed on one surface of the filter for 4 h. Excess medium with unattached cells was removed, and fresh medium was added to the cylinder and the well of the tissue culture plate. Endothelial monolayers on micropore filters were used 6–7 days after plating. The integrity of each monolayer was determined prior to the experiment by measuring the transfer of albumin from the apical to basolateral fluid (Hennig et al., 1984). Only monolayers having an albumin permeability <2%/h were used for the experiments.

Incubations. Confluent endothelial cells were washed with Dulbecco's phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 15 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4) and then incubated at 37 °C with 15(*S*)-[5,6,8,9,11,12,14,15-^{3}H]HETE (223 Ci/mM) or [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (91 Ci/mM) obtained from Amersham (Arlington Heights, IL) or with 2 μCi/mL [^{14}C(U)]glycerol (8.7 mCi/mM) obtained from New England Nuclear (Boston, MA). Arachidonic acid was purchased from NuChek Prep (Elysian, MN) and 15(*S*)-HETE was obtained from Cayman Chemical Co. (Ann Arbor, MI). When inhibitors were added, they were incubated with the cells for 30 min and left in the medium after addition of the radioactive substrate. After incubation, the media were collected and the cells were washed with ice-cold buffered saline and harvested with a rubber policeman. In experiments with cell monolayers grown on filters, the media were collected from the apical and basolateral compartments separately, and the filters containing the cells were removed from the cylinders after washing.

Lipid Extraction. The media and cells were extracted with chloroform/methanol containing 1% acetic acid (2:1 v/v) (Folch et al., 1957). The lower phase was collected and dried under nitrogen while being maintained at 35 °C. The radioactivity present in the lipid extracts was measured in a Beckman LS 7000 liquid scintillation system. Quenching was monitored with a ^{226}Ra external standard and by channel ratio measurements.

High-Performance Liquid Chromatography (HPLC). Lipid extracts from the medium were dissolved in methanol and separated by reverse-phase HPLC with a Beckman 332 system (Palo Alto, CA) equipped with a 4.5 × 150 mm column containing Adsorbosphere C_{18} reverse-phase 3-μm spherical packing (Alltech Associates, Deerfield, IL). The elution gradient contained water adjusted to pH 3.4 with phosphoric acid and acetonitrile (Van Rollins et al., 1982). For isolation of the metabolites, the gradient started with 27% acetonitrile and increased over 50 min to 100% acetonitrile. A linear gradient of 50–100% acetonitrile was used for analysis of the metabolite derivatives. After the column effluent was mixed with Budget Solve scintillation fluid (RPI Corp., Mount Prospect, IL), the radioactivity was detected and assayed with a Radiomatic Flow 1B monitor and software provided by Radiomatic Instruments & Chemical Co. (Tampa, FL).

Assay of Cell Lipids. Neutral lipids in the endothelial cell extracts were separated from phospholipids by thin-layer chromatography (TLC) on LK5D silica gel thin-layer plates (Whatman, Clifton, NJ), using a solvent mixture of hexane/diethyl ether/acetic acid (50:50:1 v/v/v). After separation, the phospholipids were extracted with chloroform/methanol/0.9% sodium chloride containing 4 mM HCl (5:5:4 v/v/v). The recovery of phosphatidylcholine (1-stearoyl-2-[^{3}H(N)]arachidonoyl-) (137 Ci/mM, New England Nuclear) from the silica gel with this procedure was 94.3 ± 2.4% (mean ± SEM, 10 determinations). The isolated phospholipids were separated further by TLC with a mixture of chloroform/methanol/methylamine (60:36:5 v/v/v). Distributions of radioactivity on TLC plates were estimated by a radio thin-layer chromatography scanner Model RS (Radiomatic Instruments & Chemical Co.) or by counting the silica gel scraped from the plates into vials containing scintillation fluid. In some experiments a combination of autoradiography using Kodak X-O-mat AR film and lipid staining, by spraying the plates with 1 mM 8-anilino-1-naphthalenesulfonate and viewing under fluorescent light, was employed to locate the lipids.

For phospholipase assays, the phospholipids dissolved in diethyl ether were incubated with phospholipase A_2 (Sigma) in 4 mM $CaCl_2$, 0.1 M Tris-HCl, and 0.1 M sodium borate at 37 °C for 30 min (Yamada et al., 1977). The mixture was adjusted to pH 6.5 and shaken vigorously during the incubation. After the reaction was terminated by addition of 0.1 M EDTA, the lipids were extracted 3 times with 1 mL of *n*-heptane, and the solvent was evaporated under N_2. The residue was resuspended in methanol and analyzed by HPLC. Phosphatidylcholine (1-stearoyl-2-[^{3}H(N)]arachidonoyl-) was used as a standard. For lipase assays, the isolated neutral lipids were dissolved in diethyl ether and mixed with sodium phosphate, pH 5.4. The mixture was sonicated for 3 s and incubated with 800 units/mL *Rhizopus arrhizus* lipase (Sigma) for 15 h at 37 °C with vigorous shaking (Linfield et al., 1984, 1985). The lipids were extracted and assayed by HPLC.

Lactate Dehydrogenase Activity. Lactate dehydrogenase activity contained in the medium of endothelial cultures was assayed by following the reduction of nicotinamide adenine dinucleotide (Amador et al., 1963), using a computer-aided fluorometric rate assay system (Sando & Sando, 1986).

Preparation of Derivatives. The major metabolite of 15-HETE was isolated by reverse-phase HPLC and methylated with diazomethane in ether (Fales & Jaouni, 1973). The methyl ester of the metabolite was converted to the trimethylsilyl ether by incubation with bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane (Sylon BFT, Supelco, Inc., Bellefonte, PA) in pyridine (Maas et al., 1982). Catalytic hydrogenation was performed with 0.1 mg of platinum oxide added to the methyl ester, trimethylsilyl ether derivative of the metabolite in 0.3 mL of ethanol. After H_2 was bubbled through the solution for 5 min, the mixture was diluted with 0.7 mL of water and extracted 3 times with 1 mL of ethyl acetate (Granstrom & Samuelsson, 1971). The methyl esters of the metabolite dissolved in pyridine were acetylated by reaction with acetic anhydride for 2 h. After addition of cold water, the mixture was acidified to pH 3 with 2 N HCl and extracted 3 times with ether. The extracts were neutralized by washing before removal of the solvent (Granstrom & Samuelsson, 1971). To check for the presence of keto groups, the purified metabolites dissolved in 50 μL of cold methanol were treated with 500 μg of sodium borohydride. After the mixture was allowed to warm to room temperature for 1 h, it was diluted with 500 μL of water, acidified to pH 3 with 2 N HCl, extracted with 1-butanol, neutralized, and dried by evaporation (Granstrom & Samuelsson, 1971). To check for epoxides, the metabolites were treated with 1:100 (v/v) methanol/water (Hamberg et al., 1986).

Gas–Liquid Chromatography (GLC) and Mass Spectrometry. Fatty acid methyl esters were separated by GLC using a Hewlett-Packard Model 5700 system containing a 1.8-m glass column (2-mm i.d.) packed with 3% SP-2250 on 100/120 mesh Supelcoport (Supelco Inc.). Equivalent chain length (ECL) was determined by comparing retention times with a saturated series of fatty acid methyl esters.

The hydrogenated and unhydrogenated methyl ester, trimethylsilyl ether derivatives of the main metabolite of 15-HETE were analyzed with a Riber R 10-10 quadropole mass spectrometer containing a 25 m × 0.2 mm GLC column packed with 5% phenylmethylsilicone maintained at 195 °C. The energy of the electron beam was 22.5 eV.

Results

15-HETE Uptake. Human and bovine endothelial cells were found to take up 15-HETE from the extracellular fluid. The uptake of [^{3}H]-15-HETE was time dependent, as shown in Figure 1A. A comparison of the uptakes during a 1-h incubation indicated that from 35% to 70% as much 15-HETE was incorporated as arachidonic acid. The uptake of 15-HETE increased as the concentration was raised, and the total amount taken up was similar in human umbilical vein and bovine aortic endothelial cells (Figure 1B). 15-HETE uptake decreased but was not abolished when fetal bovine serum was added to the medium (Figure 1C). Additional experiments indicated that the uptake of 15-HETE was similarly reduced when bovine serum albumin was added in concentrations comparable to those present in the serum. This suggests that the reductions produced by serum can be accounted for by its albumin content. Even though serum decreases uptake, considerable uptake of 15-HETE still can occur in the presence of serum. As seen in Figure 1D, 1.5 nmol/mg of cell protein was taken up when the human cells were exposed for 2 h to 5 μM 15-HETE bound to 10% fetal bovine serum, and a level of about 2 nmol was maintained in the cells between 16 and 48 h.

In the studies described above, the endothelial cells were grown in plastic dishes so that only the apical surface of the endothelial monolayer was exposed to the medium containing

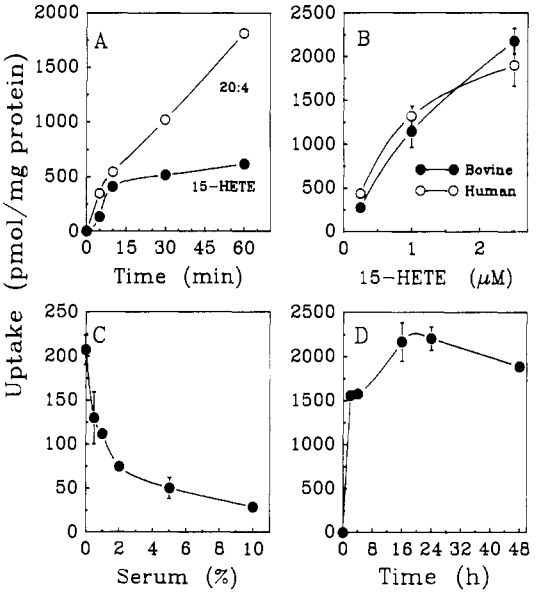

FIGURE 1: Uptake of 15-HETE. All of the studies were done with human umbilical vein endothelial cells except in the concentration dependence study, where bovine aortic endothelial cells also were employed. In the time dependence study comparing [^{3}H]-15-HETE and [^{3}H]arachidonic acid (A), the concentration was 0.25 μM. The time of incubation in the concentration dependence study was 2 h (B). In the fetal bovine serum concentration study (C), the 15-HETE concentration was 0.25 μM and the time of incubation was 2 h. In the long-term uptake study (D), the medium contained 5 μM 15-HETE and 10% fetal bovine serum. Each point is the mean ± SEM of results obtained from four separate cultures, except in (A) where the values are averages obtained from two separate cultures that are within 15% agreement.

15-HETE. It was important to determine whether uptake also might occur at the basolateral surface, which under physiological conditions is likely to be exposed to 15-HETE generated within the tissue. To investigate this, bovine aortic endothelial monolayers grown on micropore filters were incubated with 15-HETE added to either the medium bathing the apical surface or the medium below the filter. Bovine cells were employed because these cultures form monolayers that retard the passage of macromolecules when grown on micropore filters (Henning et al., 1984), whereas the human umbilical vein endothelial cells do not. Separate cultures were exposed to [^{3}H]-15-HETE contained in either the apical or basolateral fluid. As seen in Figure 2, uptake occurred when 15-HETE was present in either compartment, although the amount taken up at the earlier times was somewhat larger from the apical fluid. Almost no radioactivity was recovered in the apical fluid during the first hour when [^{3}H]-15-HETE was added initially to the basolateral compartment. Therefore, it is unlikely that the initial uptake from the basolateral fluid occurred because 15-HETE first transferred across the monolayer and entered the apical fluid.

Formation of Metabolic Products. Several products were formed from [^{3}H]-15-HETE during continuous incubation with the endothelial cells. Figure 3A shows the radioactive compounds detected in the culture medium by reverse-phase HPLC at the end of a 2-h incubation of the human cells with 0.25 μM [^{3}H]-15-HETE. In addition to 15-HETE, which has a retention time of 36.5 min in this gradient elution system, two metabolites with retention times of 27.0 and 29.5 min, designated as products A and B, respectively, were detected. Small amounts of a third metabolite with a retention time of 34.0 min also were observed in some chromatograms. Product B accounted for 75% of the radioactivity converted to metabolites. These products did not form when [^{3}H]-15-HETE

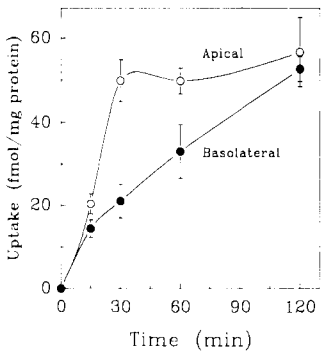

FIGURE 2: Comparison of 15-HETE uptake from the apical and basolateral surfaces. Bovine aortic endothelial cells were grown to confluence on micropore filters impregnated with gelatin and coated with fibronectin. These monolayers were incubated in polystyrene chambers containing 0.1 μCi/mL [^{3}H]-15-HETE in either the fluid above the filter (apical) or the fluid beneath the filter (basolateral). As a control, monolayers were immersed in 3.7% formaldehyde prior to incubation, and the small amount of radioactivity accumulated in these filters was substracted from the uptakes occurring when the filters contained viable cells. Different monolayers were utilized for the apical and basolateral uptake measurements. Each point is the mean ± SEM of values obtained from four separate monolayers.

was incubated in a cell-free medium or with cells killed by 3.7% formaldehyde.

The time dependence of the conversion of [^{3}H]-15-HETE to products A and B by the human endothelial cells is shown in Figure 4A. In this study the concentration of [^{3}H]-15-HETE initially present in the culture medium was 0.25 μM. During the first 4 h, there was a 90% decrease in 15-HETE radioactivity contained in the medium, and the amount of radioactivity present as products A and B increased progressively. At both 2 and 4 h, there was 3–4 times more product B present in the medium than product A.

Figure 4B shows that increasing amounts of product B were formed by the human endothelial cells during a 2-h incubation as the concentration of [^{3}H]-15-HETE in the culture medium was raised. At each concentration, much more product B than product A was formed. Additional studies demonstrated that the bovine aortic endothelial cells also converted [^{3}H]-15-HETE to these metabolites and, as in the case of the human cells, product B was the main radioactive metabolite. As seen in Figure 4C, however, the human cultures formed considerably more product B than the bovine cultures at all concentrations of 15-HETE tested.

Metabolic products were detected in the medium by HPLC when the human umbilical vein endothelial cultures were incubated continuously with [^{3}H]arachidonic acid. About 80% of the radioactivity was contained in prostaglandins, mostly as 6-ketoprostaglandin $F_{1\alpha}$. Radioactive prostaglandin E_2 and $F_{2\alpha}$ also were detected. Although radioactivity eluted between 25 and 40 min, none of these components had the same retention time as any of the metabolites formed from 15-HETE. The main radioactive component formed from [^{3}H]arachidonic acid in this region of the chromatogram eluted at 31.4 min, 1.9 min later than product B. This metabolite had the same retention time as a 12-hydroxyheptadecatrienoic acid (HHT) standard, a product known to be formed by endothelial cells (Mayer et al., 1986).

Incorporation into Endothelial Lipids. The human umbilical vein endothelial cultures incorporated much more radioactivity into neutral lipids than into phospholipids when they were incubated with 0.25–2.5 μM [^{3}H]-15-HETE (Figure 5A). The opposite occurred with the bovine aortic endothelial cultures, the incorporation into phospholipids being 5–10-fold greater than that into neutral lipids (Figure 5B). In both cases,

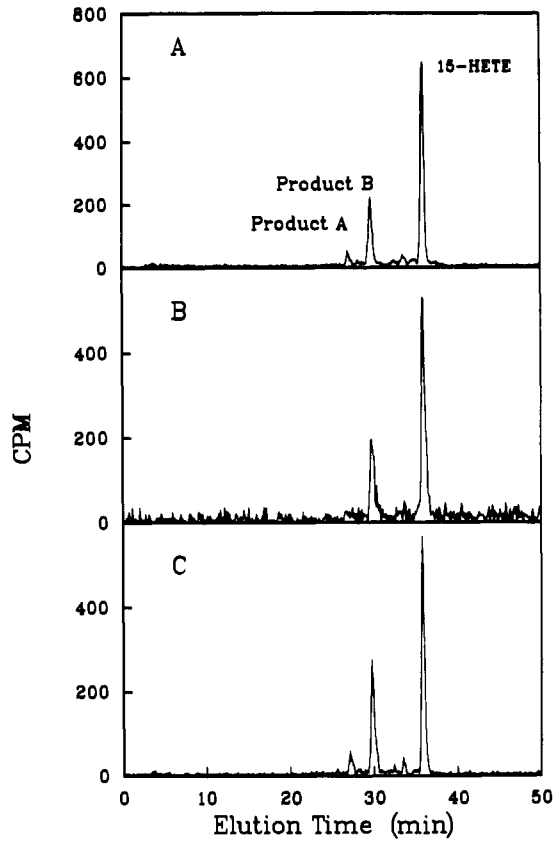

FIGURE 3: Separation of radioactive metabolites by reverse-phase HPLC. Human umbilical vein endothelial cells were incubated with [^{3}H]-15-HETE. Panel A is a chromatogram of the radioactivity contained in the culture medium after a 2-h incubation of the cells with 0.25 μM [^{3}H]-15-HETE. The retention times of product A, product B, and 15-HETE are 27.0, 29.5, and 36.5 min, respectively, when a gradient from 27% to 100% acetonitrile is used. Panel B is a chromatogram of the radioactive products released following hydrolysis of the cell neutral lipids with *R. arrhizus* lipase. The retention times of the two main radioactive compounds correspond to product B and 15-HETE. In panel C, the cells were initially incubated for 2 h with [^{3}H]-15-HETE, washed, and then incubated for an additional 6 h in a medium containing 100 μM albumin. A chromatogram of the radioactive compounds released into the medium during the 6-h incubation is shown. The retention times of the three compounds correspond to product A, product B, and 15-HETE.

the largest amount of the phospholipid radioactivity was present in phosphatidylinositol (Figure 5C,D).

Hydrolysis of the labeled cellular phospholipids with phospholipase A_2 indicated that about 85% of the radioactivity was present in the *sn*-2 position. Analysis of the released radioactivity by HPLC indicated the presence of only one prominant peak, corresponding to the [^{3}H]-15-HETE.

To assess the composition of the large amount of neutral lipid radioactivity in the human umbilical vein cells, this fraction was separated by TLC. As shown in Figure 6, three bands of radioactivity were observed. One component comigrated with [^{3}H]-15-HETE. The other two components, designated NL1 and NL2, migrated between di- and triacylglycerol standards.

In separate experiments, human umbilical vein endothelial cultures were incubated with [^{14}C(U)]glycerol, and the distribution of the radioactivity in the cell lipids was determined. As seen in Table I, most of the radioactivity was contained in triacylglycerol and phospholipids. However, radioactivity also was present in two neutral lipid components having the same mobility on TLC as NL1 and NL2 detected in Figure 6 when corresponding cultures were incubated with [^{3}H]-15-HETE. Following labeling with [^{3}H]-15-HETE, these com-

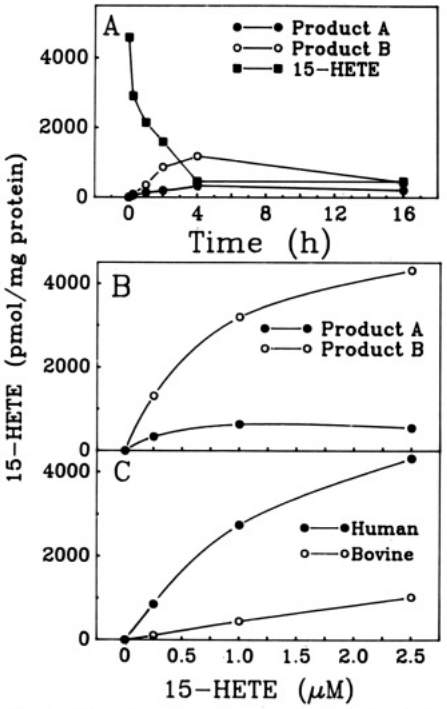

FIGURE 4: Formation of radioactive products. Panel A shows the amounts of product A, product B, and 15-HETE radioactivity present in the medium during a 16-h incubation of 0.25 μM [^{3}H]-15-HETE with human endothelial cultures. These values were obtained from reverse-phase HPLC analysis of the culture medium at each time. Panel B shows the distribution of the radioactive compounds in the medium at the end of a 2-h incubation of the human cells with different amounts of [^{3}H]-15-HETE. Panel C illustrates the results of another experiment in which product B formation by the human and bovine endothelial cells was compared. The time of incubation was 2 h, and the cultures were exposed to different amounts of [^{3}H]-15-HETE. Each point is the mean ± SEM of results obtained from three separate cultures.

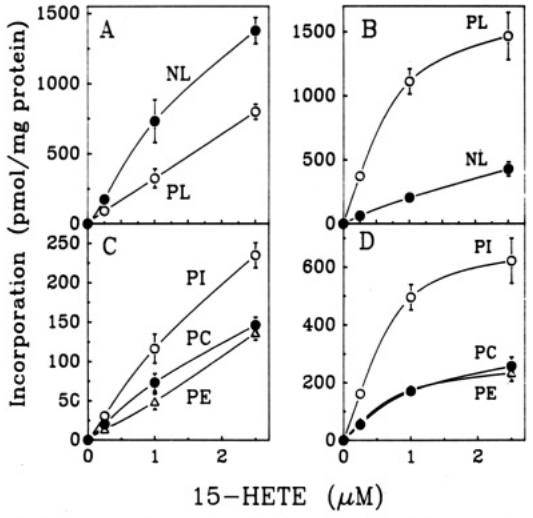

FIGURE 5: Incorporation of 15-HETE into endothelial cell lipids. The cells were incubated for 2 h with varying amounts of [^{3}H]-15-HETE. After removal of the medium and washing, the cell lipids were extracted and separated by TLC. Each value is the mean ± SEM of results obtained from three separate cultures of either human umbilical vein (A and C) or bovine aortic endothelial cells (B and D). The abbreviations are as follows: PL, phospholipids; NL, neutral lipids; PI, phosphatidylinositol; PC, choline phosphoglycerides; PE, ethanolamine phosphoglycerides.

pounds were hydrolyzed with *R Arrhizus* lipase and the radioactive products assayed by HPLC. A typical chromatogram, shown in Figure 3B, indicates the presence of two radioactive compounds. The component eluting at 36.5 min

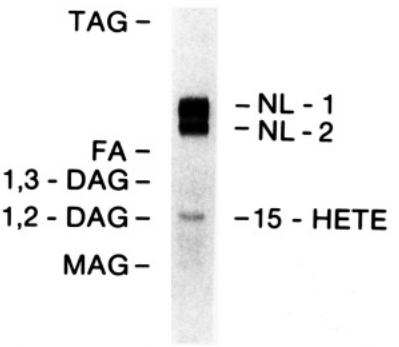

FIGURE 6: Radioactive compounds present in the neutral lipid fraction of human umbilical vein endothelial cells. The neutral lipid extract was separated on a Whatman LK5D silica plate with a solvent system of hexane/diethyl ether/acetic acid (50:50:1 v/v/v), and after the plates were sprayed with En-^{3}Hance, they were subjected to autoradiography at −70 °C. The mixture of nonradioactive lipid standards was visualized by exposure to I_2 vapor. The abbreviations are as follows: TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; FA, fatty acid; NL1 and NL2, unknown neutral lipids containing 15-HETE radioactivity.

Table I: Distribution of [^{14}C(U)]Glycerol Radioactivity in Lipids of Human Umbilical Vein Endothelial Cells[a]

cell lipids	radioactivity (%)	cell lipids	radioactivity (%)
triacylglycerol	51.6	monoacylglycerol	2.6
NL1[b]	5.9	phospholipids	35.5
NL2[b]	2.1		

[a] Endothelial cultures were incubated with [^{14}C(U)]glycerol (8.7 mCi/mmol) for 1 h. After removal of the medium and washing, the lipids were extracted from the cells and separated by TLC. Segments of the silica gel corresponding to appropriate lipid standards were scraped and assayed for radioactivity in a liquid scintillation counter. Each percentage is the average of values obtained from two separate cultures that were within 5% agreement. [b] These components have the same R_f values as the compounds containing [^{3}H]-15-HETE designated in Figure 6 as NL1 and NL2.

corresponds to the [^{3}H]-15-HETE standard and accounts for 75% of the radioactivity. Most of the remaining radioactivity has a retention time of 29.5 min, corresponding to product B. Taken together, these results suggest that NL1 and NL2 are glycerides containing 15-HETE and product B.

Efflux from the Cells. When human endothelial cultures previously labeled with [^{3}H]-15-HETE were incubated in a medium containing serum, radioactive products were released into the extracellular fluid (Figure 7). No stimulus was necessary to elicit this release. After 6 h, 70% of the radioactivity initially present in the cells was released into the medium. Under comparable conditions, monolayers loaded with [^{3}H]arachidonic acid release only 20% of their initial radioactivity content. Assuming that the specific radioactivity of the released material was the same as those of [^{3}H]-15-HETE and [^{3}H]arachidonic acid, respectively, to which the cultures were exposed initially, 2–3 times more material was released from the cells containing [^{3}H]-15-HETE than from those containing arachidonic acid.

The radioactivity released from the cells containing [^{3}H]-15-HETE after 6 h of incubation was assayed by HPLC. As shown in Figure 3C, 50% of the released radioactivity remained as 15-HETE, 30% corresponded to product B, and 10% corresponded to product A. A similar analysis indicated that all of the radioactivity released during the 6-h incubation of the cells containing [^{3}H]arachidonic acid remained as fatty acid.

Bovine monolayers grown on micropore filters were tested to determine whether this release was polarized. Albumin was

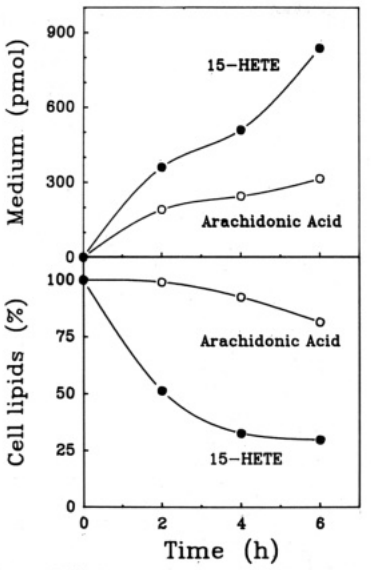

FIGURE 7: Release of [^{3}H]-15-HETE and [^{3}H]arachidonic acid from human umbilical vein endothelial cell lipids during incubation. One set of cultures was incubated for 2 h with 0.25 μM [^{3}H]-15-HETE, the other with 0.25 μM [^{3}H]arachidonic acid. After removal of these media and washing, two cultures from each group were extracted to determine the content of radioactivity in the cell lipids. The remaining cultures were incubated for varying times in a medium containing 100 μM albumin, and the radioactivity in the cell lipids and the medium was assayed. Radioactivity remaining in the cell lipids is expressed as a percentage of that present following the initial 2-h incubation. Each point is the average of results obtained from two separate cultures, which varied by no more than 10%.

Table II: HPLC Retention Times[a]

compound	retention time (min)	compound	retention time (min)
product B	9.9	15-HETE methyl ester	28.3
product B methyl ester	18.6	15-HETE methyl ester acetate	38.4
product B methyl ester acetate	29.8	HHT	12.5
15-HETE	18.8	8,15-diHETE	6.7

[a] Each compound was chromatographed separately on the reverse-phase column with a linear gradient of acetonitrile and H_2O acidified with H_3PO_4, starting with 50% acetonitrile and increasing to 100% over 50 min.

added to the apical and basolateral fluid to bind the released radioactivity and thereby retard redistribution across the cell monolayer. The monolayers were initially exposed for 2 h to [^{3}H]-15-HETE, washed, and then incubated for up to 4 h. Release occurred to both the apical and basolateral fluid in roughly equal amounts. In each case about 60% of the total release occurred during the first hour. Analysis by HPLC indicated that, at the end of the 4-h incubation, more than 90% of the radioactivity present in each compartment was 15-HETE. However, 5% of the radioactivity in the basolateral fluid and 3% in the luminal fluid eluted as product B.

Identification of Product B. Product B, collected following reverse-phase HPLC, exhibited a single absorbance maximum at 234 nm. An identical UV absorbance spectrum was observed with the 15-HETE standard, indicating retention of the conjugated diene structure. No absorbance maxima in the range of 255–285 nm indicative of a conjugated triene derivative of 15-HETE (Hopkins et al., 1984) were detected.

Product B and appropriate standards were assayed by reverse-phase HPLC, as indicated in Table II. Product B eluted after 8,15-dihydroxyeicosatetraenoic acid (8,15-diHETE) and

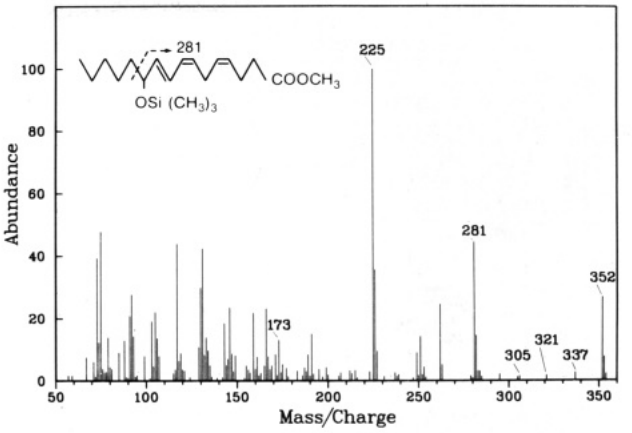

FIGURE 8: Mass spectrum of the trimethylsilyl ether, methyl ester derivative of the main metabolite formed by human umbilical vein endothelial cells from 15-HETE. The compound corresponding to product B in Figure 3 was isolated by HPLC, derivatized, and isolated by GLC.

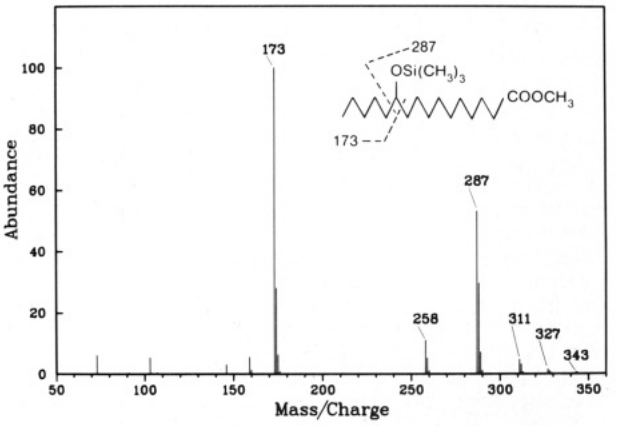

FIGURE 9: Mass spectrum of the trimethylsilyl ether, methyl ester, reduced derivative of product B. The compound was isolated and prepared as described in Figure 8, except that it was also hydrogenated.

before HHT in this gradient system. The retention times of product B and 15-HETE increased to about the same extent following methylation, suggesting that product B retained the carboxyl group of 15-HETE. Likewise, acetylation of these methyl esters increased their retentions times to about the same extent, suggesting that product B also retained the hydroxyl group of 15-HETE. Additional studies with a second gradient elution system indicated that the retention time of product B was essentially unchanged following treatment with sodium borohydride or 1% methanol. GLC of the product B methyl ester indicated an ECL of 17.8. Taken together, these results suggest that product B is a monohydroxy fatty acid derivative of 15-HETE containing 16 carbons.

The methyl ester of product B was silylated and assayed by GLC combined with mass spectrometry. Figure 8 shows that a molecular ion is detected at m/z 352. Additional ions are present at m/z 321 (M − 31, loss of OCH_3), 281 [M − 71, loss of $(CH_2)_4CH_3$], 262 [M − 90, loss of $(CH_3)_3SiOH$], 252 [M − 100, rearrangement followed by loss of OHC-$(CH_2)_4CH_3$], and 173 [$(CH_3)_3SiO^+$=$CH(CH_2)_4CH_3$]. The formation of the large ion at m/z 225 is a somewhat unusual fragmentation that has been detected as a result of a vinylic cleavage in a structurally related compound, 13-hydroxy-*c*-9,*t*-11-octadecadienoic acid (Hubbard et al., 1980; Claeys et al., 1985). This spectrum is consistent with a trienoic 16-carbon derivative containing a hydroxyl group at carbon 11, as indicated by the α cleavage ions at m/z 173 and 281.

Table III: Effect of 4-Pentenoic Acid on Conversion of 15-HETE to 16:3(11-OH) by Human Umbilical Vein Endothelial Cells[a]

4-pentenoic acid (μM)	16:3(11-OH) (dpm/well)	15-HETE (dpm/well)	lactate dehydrogenase (units/well)
0	9219 ± 546	18933 ± 1116	1.6 ± 0.1
50	5069 ± 145	25706 ± 1738	1.2 ± 0.3
100	4462 ± 140	30143 ± 742	1.6 ± 0.1

[a] The cultures were incubated with 4-pentenoic acid for 30 min before addition of [^{3}H]-15-HETE (56000 dpm/well), and the incubation was continued for 2 h. An aliquot of the medium was assayed for lactate dehydrogenase activity, and the remainder was extracted and assayed for radioactivity by HPLC. Each value is the mean ± SEM of results obtained from four separate cultures.

The mass spectrum of the hydrogenated derivative of product B, shown in Figure 9, is consistent with this postulated structure. Although a molecular ion is not detected, ions are present at m/z 287 and 173, formed by cleavage on either side of the trimethylsilyl carbon atom. This suggests that the molecular ion of the saturated derivative is m/z 358. In addition, small ions are present at m/z 343 (M − 15), 327 (M − 31), 311 (M − 47), and 258 (M − 100). Taken together, these spectra are consistent with the interpretation that product B is an 11-hydroxyhexadecatrienoic acid [16:3(11-OH)] in which the three double bonds are located in the segment of the hydrocarbon chain between the carboxyl terminus and the 11-hydroxyl group.

Effects of Metabolic Inhibitors. The effect of various inhibitors on the conversion of [^{3}H]-15-HETE to [^{3}H]-16:3-(11-OH) was examined in the human umbilical vein endothelial cells. Inhibition was produced by 4-pentenoic acid, a β-oxidation inhibitor, as shown in Table III. Incubation with 4-pentenoic acid did not increase lactate dehydrogenase release from the cells, indicating that the decrease in 16:3-(11-OH) formation is not due to nonspecific cell injury. A concentration-dependent reduction in [^{3}H]-16:3(11-OH) formation also occurred when the endothelial cultures were incubated with 5–35 μM nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor. However, exposure to NDGA caused the cells to take on a granular appearance, and the cultures began to lift off the plastic surface at concentrations above 20 μM. These findings suggest that the reduction produced by NDGA probably is due at least in part to generalized cytotoxicity. No inhibition of [^{3}H]-16:3(11-OH) formation occurred when the cultures were incubated with 2 mM acetylsalicylic acid, 100 μM ibuprofen, 500 μM metyrapone, 20 μM butylated hydroxytoluene, 100 μM glutathione, 150 μM allopurinol, or 1 mM 3-amino-1,2,4-triazole. Taken together, these findings suggest that the conversion of 15-HETE to 16:3(11-OH) occurs by β-oxidation, with the removal of four carbons from the carboxyl end of the fatty acid.

Incorporation of 16:3(11-OH). The capacity of the human endothelial cells to incorporate 16:3(11-OH) was investigated (Figure 10). [^{3}H]-16:3(11-OH) was collected by HPLC following a 2-h incubation of endothelial cultures with [^{3}H]-15-HETE. Additional cultures were incubated for 1 h with either the isolated [^{3}H]-16:3(11-OH) or [^{3}H]-15-HETE. In comparing the uptakes, it is assumed that the specific radioactivity of [^{3}H]-16:3(11-OH) is the same as that of the starting material, [^{3}H]-15-HETE. Under similar conditions, the uptake of 16:3(11-OH) was 70% less than the the amount of 15-HETE taken up. Incorporation into total cell phospholipids and the neutral lipids was reduced to about the same extent. Among the phospholipids, equal amounts of 16:3-(11-OH) and 15-HETE were incorporated into the choline phosphoglycerides, whereas there was essentially no incorporation of 16:3(11-OH) into phosphatidylinositol. Furthermore, [^{3}H]-15-HETE was incorporated into both NL1 and NL2, whereas [^{3}H]-16:3(11-OH) was only present in NL2.

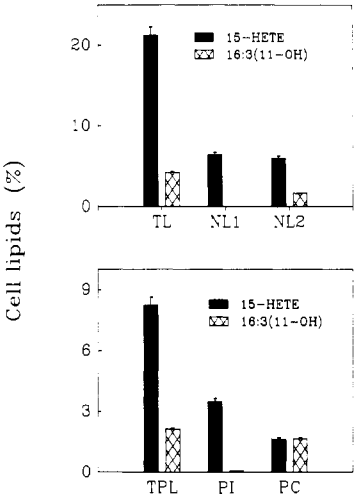

FIGURE 10: Comparison of 15-HETE and 16:3(11-OH) incorporation into endothelial lipids. Human umbilical vein endothelial cells were incubated with [^{3}H]-15-HETE or [^{3}H]16:3(11-OH) prepared biologically from [^{3}H]-15-HETE for 2 h. Each value is the mean ± SEM of results obtained from three separate cultures. The abbreviations are as follows: TL, total cell lipid; NL1 and NL2, unknown neutral lipids; TPL, total cell phospholipid; PI, phosphatidylinositol; PC, choline phosphoglyceride.

Discussion

These findings demonstrate that endothelial cells convert 15-HETE, a lipoxygenase product derived from arachidonic acid, primarily to 16:3(11-OH). On the basis of the structure of the metabolite and the inhibitory effect of 4-pentenoic acid, 16:3(11-OH) appears to be formed through partial β-oxidation of 15-HETE. Although the positions of the three double bonds in 16:3(11-OH) have not been established, the data suggest that their relationship to the hydroxyl group may be the same as in 15-HETE. The four double bonds in 15-HETE are present between the carboxyl and hydroxyl groups, being c-5,c-8,c-11,t-13. An ion with m/z 173 is present in the mass spectrum of 16:3(11-OH) before and after hydrogenation, indicating that as in 15-HETE, no unsaturation is present between the methyl terminus and the hydroxyl group. Prior to hydrogenation (Figure 8), an ion with m/z 225 is prominent. This ion also is detected in the methyl ester, trimethylsilyl ether derivative of c-9,t-11-18:2(13-OH) and represents a fragment from the methyl terminus (carbon 18) to carbon 9 (Hubbard et al., 1980; Claeys et al., 1985). A similar fragmentation pattern probably produced the m/z 225 ion of 16:3(11-OH), making it likely that two of the unsaturated bonds are c-7,t-9; i.e., they retain the same relationship to the hydroxyl group as in 15-HETE. Since 15-HETE can undergo two β-oxidations without affecting the position of the c-8 double bond, this unsaturation probably remains in the c-4 position of 16:3-(11-OH). The maintenance of the single UV absorbance maximum, without any indication of a conjugated triene absorbance in the 255–285-nm range (Hopkins et al., 1984), also is consistent with a lack of double bond rearrangement in the product. On the basis of such a mechanism of formation, we speculate that the structure of the main metabolite is c-4,c-7,t-9-16:3(11-OH), as indicated in Figure 8. The catabolism of 15-HETE through β-oxidation and the formation of this product to our knowledge have not been described previously.

Why the β-oxidation process should terminate after removal of only four carbons from the carboxyl end of 15-HETE is not

apparent. The presence of a *c*-4 unsaturation does not impede the β-oxidation of n-6 polyunsaturated fatty acids (Yang et al., 1986; Dommes & Kunau, 1984) for it is encountered in acyl-CoA intermediates during the oxidation of linoleate (*c*-4-10:1) and arachidonate (*c*-4,*c*-7,*c*-10-16:3). This suggests that either the presence of the hydroxyl group or the conjugated cis–trans unsaturation of the 16:3(11-OH) acyl-CoA intermediate slows β-oxidation sufficiently to allow the accumulation of this metabolite.

Endothelial cells can convert the hydroperoxide precursor of 15-HETE, 15-hydroperoxyeicosatetraenoic acid (15-HPETE), to 8,15-diHETE (Hopkins et al., 1984). While it is possible that product A in Figure 3 may be 8,15-diHETE, it is clear that 8,15-diHETE is not the major product formed from 15-HETE. Thus, it appears that the 15-HPETE, which is derived in the endothelium primarily from the oxidation of endogenous arachidonic acid (Gorman et al., 1985; Mayer et al., 1986), is metabolized differently from the 15-HETE that is taken up from the extracellular fluid. Like endothelial cells, macrophages convert 15-HETE to compounds that have retention times similar to those seen in Figure 3 (Pawlowski et al., 1982). Although these products are thought to be diHETEs or more polar metabolites, the possibility that they may be related to 16:3(11-OH) cannot be excluded. Furthermore, endothelial cells and kidney tubular cells convert 12-hydroxyeicosatetraenoic acid (12-HETE) to a metabolite with related chromatographic properties (Kaduce et al., 1985; Gordon & Spector, 1987), and this product also appears to be a monohydroxy 16-carbon trienoic acid.[2] Therefore, the oxidation pathway that we have observed may not be confined to the endothelium, and it may operate with HETEs other than 15-HETE.

In addition to uptake from the apical surface, the endothelial cells can take up 15-HETE from the basolateral surface (Figure 2). This suggests that any 15-HETE released within the tissue or the vascular wall, such as from smooth muscle, eosinophilic leukocytes, or macrophages (Larrue et al., 1983; Turk et al., 1982; Rabinovitch et al., 1981), is potentially available for direct incorporation into the endothelium without first having to enter the plasma. Likewise, release of 15-HETE and 16:3(11-OH) can occur from both the apical and basolateral surfaces of the endothelial cell. Therefore, at least part of the release also should be directly available to other cells within the vascular wall or the tissue. Cooperative metabolism of 5- and 12-HETEs by two different types of cells already has been established in the case of platelets and granulocytes (Marcus et al., 1984) or endothelium (Schafer et al., 1986). Likewise, leukotriene A_4 derived from an exogenous source can be converted to other leukotriene products by endothelium (Feinmark et al., 1986). On the basis of these precedents, we suggest that the endothelium also may be involved in the further processing of 15-HETE when it is released from adjacent cells.

Although 15-HETE is incorporated more extensively into glycerides than phospholipids by cultured endothelial cells derived from the human maternal–fetal circulation, it is incorporated primarily into phospholipids in the bovine cells that were isolated from systemic arteries (Figure 5). 15-HETE that becomes incorporated in phospholipids probably is contained within membrane structures. Because of the presence of the hydroxyl group, it is possible that the packing of the hydrocarbon chains in certain localized membrane microenvironments may be perturbed when the endothelium begins to accumulate 15-HETE. In both types of endothelial cells, much of the 15-HETE incorporated into the phospholipids is present in phosphatidylinositol (Figures 5 and 10), suggesting that regions of the membrane containing phosphatidylinositol may be particularly susceptible to such perturbations when 15-HETE accumulates. Similarly, a large fraction of the 15-HETE incorporated into macrophage phospholipids is contained in phosphatidylinositol (Stenson et al., 1983; Pawlowski et al., 1982). As compared with 15-HETE, substantially less 16:3(11-OH) accumulates in the cells, and it is almost entirely excluded from phosphatidylinositol (Figure 10). Therefore, conversion of 15-HETE to 16:3(11-OH) may be a protective mechanism, serving to minimize the accumulation of 15-HETE in phospholipids and particularly the phosphatidylinositol fraction of endothelial membranes.

Registry No. 15-HETE, 73180-00-4; 16:3(11-OH), 112088-55-8.

[2] C. Hadjiagapiou, H. Sprecher, T. L. Kaduce, P. H. Figard, and A. A. Spector, unpublished observation.

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Reversible Dissociation of the B873 Light-Harvesting Complex from *Rhodospirillum rubrum* G9+[†,‡]

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ABSTRACT: The light-harvesting complex (B873) from the carotenoid-less mutant *Rhodospirillum rubrum* G9+ was isolated and used for studying the detergent-induced reversible changes in absorption and circular dichroism (CD) spectra. Addition of lauryldimethylamine *N*-oxide (LDAO) to the detergent-free B873 causes a rapid loss of the absorption maximum at 873 nm with the concomitant appearance of an absorption maximum at 775 nm. The absorption change is completely reversible by dilution of the LDAO-solubilized B873 into detergent-free buffer and is partially reversible by the addition of sodium cholate to a final concentration of 1% (w/v). Similar changes are observed by using near-IR CD, although no changes of secondary structure were seen by using UV CD. Detergent-free B873 dissolved in 0.2% octylpentakis-(oxyethylene) (O-POE) shows an absorption maximum principally at 873 nm and was shown by analytical ultracentrifugation measurements to exist as tetramers and hexamers (24-37K). O-POE concentrations above 0.5% cause the 873- to 780-nm transition described above to occur, and this is always associated with the formation of dimers (13K) and monomers (6.5K) containing bound bacteriochlorophyll. ESR studies performed with *N*-ethylmaleimide nitroxide labeled B873 dissolved in various ratios of LDAO and sodium cholate showed that the 873- to 780-nm transition in LDAO and cholate is also due to dissociation of the B873 into smaller units.

The bacteriochlorophyll-containing light-harvesting complexes of the chromatophore membrane of photosynthetic bacteria are responsible for the absorption and transfer of light energy to the photoreaction centers, which catalyze the primary process in energy transduction in photosynthesis [Clayton, 1978; see Cogdell and Thornber (1980)]. Many light-harvesting complexes from purple non-sulfur bacteria have now been isolated [Sauer & Austin, 1978; Cogdell et al., 1982; Picorel et al., 1983; Varga & Staehelin, 1985; see Zuber et al. (1985)]. They have been found to be of three principal types: LHC I, exhibiting an IR absorption maximum between 870 and 890 nm (designated B870/890);[1] LHC II, with ab-

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[‡] Dedicated to Prof. Dr. Hans Wanner on the occasion of his 70th birthday.
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[1] Throughout the text we refer to the B873 and B875 complex from *R. rubrum* G9+. These complexes are essentially identical: in some laboratories (including ours) the complex exhibits an absorption maximum at 873 nm, whereas in other laboratories an absorption maximum at 875 nm is apparently observed. It is generally believed that these small differences arise from a slight alteration of the parent genotype that has occurred in the years following its isolation and/or differences in the growth conditions used by various groups.

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