Biosynthesis of the C_{18} Family of Cutin Acids: ω-Hydroxyoleic Acid, ω-Hydroxy-9,10-epoxystearic Acid, 9,10,18-Trihydroxystearic Acid, and Their Δ^{12}-Unsaturated Analogs†

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ABSTRACT: Biosynthesis of the hydroxy-C_{18} acids which constitute the major components of the polymer, cutin, was studied with specifically labeled fatty acids. Skin slices (but not internal tissue) of rapidly growing apple fruits incorporated exogenous fatty acids into cutin. [1-^{14}C]Acetate was incorporated into all classes of hydroxy acids, whereas [1-^{14}C]-palmitic acid specifically labeled ω-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid. [1-^{14}C]Stearic acid, on the other hand, was not incorporated into polyhydroxy C_{18} acids. Chemical degradation, followed by product analysis, of the labeled components derived from [1-^{14}C]oleic acid showed that this acid was incorporated specifically into 18-hydroxy-9,-10-epoxystearic acid and 9,10,18-trihydroxystearic acid. Conversion of oleic acid into 18-hydroxy-9,10-epoxystearic acid was also shown to occur in grape berry skin and apple leaves by chromatographic identification of the labeled derivatives obtained from the labeled epoxy acid derived from [1-^{14}C]-oleic acid. Biosynthetically labeled 18-hydroxyoleic acid was incorporated into the corresponding 9,10-epoxy acid and 9,10,18-trihydroxy acid in apple skin slices. From these results it is concluded that oleic acid undergoes ω-hydroxylation, epoxidation of the double bond and finally hydration of the epoxide to give rise to the three major components of the C_{18} family of cutin acids. With [1-^{14}C]linoleic acid and [1-^{14}C]-linolenic acid the following evidence was obtained that the Δ^9 double bond of these acids also undergoes the same sequence of reactions as those suggested for oleic acid: (i) [1-^{14}C]-linoleic acid was incorporated specifically into 18-hydroxy-linoleic acid, monounsaturated 18-hydroxy-9,10-epoxy-C_{18} acid and monounsaturated 9,10,18-trihydroxy-C_{18} acid; (ii) [1-^{14}C]linolenic acid was incorporated specifically into triunsaturated 18-hydroxy-C_{18} acid, diunsaturated 18-hydroxy-9,-10-epoxy-C_{18} acid and diunsaturated 9,10,18-trihydroxy-C_{18} acid. The labeled polyunsaturated acids were also incorporated into the ether-linked core of the polymer, suggesting that the *cis*-4-pentadiene system is involved in the formation of ether bonds. With argentimetric thin-layer chromatography and combined gas–liquid chromatography–mass spectrometry the following novel compounds were identified in the cutin of young apple fruits: (i) 18-hydroxylinolenic acid; (ii) 18-hydroxy-9,10-epoxyoctadecadienoic acid; and (iii) 9,10,-18-trihydroxyoctadecadienoic acid. These compounds were either minor components or not detectable in the mature fruits.

Cutin, which constitutes the structural component of plant cuticles, is a polymer of hydroxy fatty acids (Mazliak, 1968; Martin and Juniper, 1971; Kolattukudy and Walton, 1972a). In recent years great progress has been made in the determination of the structures of the cutin acids primarily because of the availability of the modern analytical techniques, especially the combination of gas–liquid chromatography and mass spectrometry (Eglinton and Hunneman, 1968; Kolattukudy *et al.*, 1971; Holloway and Deas, 1971; Croteau and Fagerson, 1972; Brieskorn and Kabelitz, 1972; Walton and Kolattukudy, 1972a; Hunneman and Eglinton, 1972). Two distinct categories of cutin acids are present in the phytopolymer, a C_{16} family and a C_{18} family. Palmitic acid, ω-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid, together with its positional isomers, make up the bulk of the C_{16} family. Oleic acid, ω-hydroxyoleic acid, 9,10-epoxy-18-hydroxystearic acid, and 9,10,18-trihydroxystearic acid together with their Δ^{12}-monoenoic analogs appear to be the most common major components of the C_{18} family of cutin acids (Walton and Kolattukudy, 1972a).

The biosynthesis of cutin was originally proposed to involve excretion of internal lipids onto the surface of the plant where they underwent oxidative polymerization to give cutin (Priestley, 1943). Recently biosynthesis of cutin has been approached with modern biochemical techniques (Kolattukudy, 1970a; Kolattukudy *et al.*, 1971; Kolattukudy and Walton, 1972b). On the basis of such studies with young *Vicia faba* leaves it was proposed that palmitic acid was hydroxylated by mixed-function oxidase-type enzymes first at the C-16 and then at the C-10 position to give ω-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid (Kolattukudy, 1970a; Kolattukudy and Walton, 1972b). The enzyme that catalyzes the second hydroxylation has been demonstrated with epidermal extracts of *V. faba* leaves (Walton and Kolattukudy, 1972b). However, *V. faba* cutin does not contain significant amounts of the C_{18} family of acids and, therefore, this system could not be used for biosynthetic studies on these acids.

In the present paper we describe biosynthetic studies on the C_{18} family of acids with leaves and/or fruits of apple, grape, and *Senecio odoris*. Our results show that the most likely pathway for the biosynthesis of the C_{18} cutin acids involves ω-hydroxylation of oleic acid followed by epoxidation of the double bond at C-9 followed by hydration of the epoxide. We also demonstrate that a similar sequence of reactions on linoleic acid gives rise to the Δ^{12}-unsaturated analogs.

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Experimental Section

Plant Materials. Young McIntosh apple fruits and leaves were picked just prior to the experiment from the orchard of Washington State University, Pullman, or from a commercial orchard of Mr. Leroy Carlson, Troy, Idaho. The young apple fruits were 5–7 cm in diameter. Grape berries were from experimental vineyards of the Irrigated Agriculture Research and Extension Center of Washington State University at Prosser, Washington. *S. odoris* plants were grown in the greenhouse.

Substrates. [1-^{14}C]Acetate (specific activity 60 Ci/mol), [1-^{14}C]palmitic acid (specific activity 55 Ci/mol), [1-^{14}C]oleic acid (specific activity 62 Ci/mol), [1-^{14}C]linoleic acid (specific activity 57 Ci/mol), and [1-^{14}C]linolenic acid (specific activity 58 Ci/mol) were purchased from Amersham/Searle Corp. [10-^{14}C]Palmitoleic acid (specific activity 12 Ci/mol) was purchased from Schwarz BioResearch Inc., Orangeburg, N. Y. Dispersions of the substrates were made in water with the aid of Tween 20 as described before (Kolattukudy, 1967).

Metabolic Experiments. To determine which portion of the fruit synthesizes cutin, cylinders (12 mm in diameter) were cut from freshly picked young apple fruits with a stainless steel cork borer. The skin disks were excised from these cylinders with a razor blade. Then 1-mm thick disks were cut from the cylinders from the outer end toward the center of the fruit. Thus, five internal tissue disks and one skin disk were collected from each cylinder. The disks were washed three times with water and then gently blotted with filter paper. Fifteen disks were placed in 125-ml erlenmeyer flasks and 0.5 ml of a solution containing 14×10^6 cpm of [1-^{14}C]palmitic acid was added. Each disk was carefully bathed in the substrate solution and then spread at the bottom of the flask. The flasks were incubated for 4 hr at 30° in a gyratory water bath shaker. At the end of the incubation period, the tissue disks were washed with water and then processed as described elsewhere in this paper. In the other metabolic experiments with apple fruit skin the same procedure as described above was used with the appropriate substrates.

In the experiments with leaf disks a stainless steel cork borer (12 mm in diameter) was used to cut the disks from young (not fully expanded) leaves of apple or *S. odoris*. The leaf disks were thoroughly washed with water and incubated with substrates in a manner similar to that described for the fruit skin slices.

In experiments with young grape berries (1.2–1.5-cm diameter) a segment of the berry 11 mm in diameter was excised with a stainless steel cork borer. Invariably, internal tissue was attached to the skin and this tissue was carefully scraped off the disk with the curved back side of a pair of forceps. The disks were thoroughly washed and incubated with the labeled substrates at 30° as described above. In this case, at the end of the incubation period the samples were frozen and kept in Dry Ice for transportation and they were processed the next day.

Isolation of Metabolic Products. At the end of the incubation period the tissue slices were transferred to fritted disk funnels and washed twice with a total volume of 100 ml of water. The radioactivity thus removed, together with the small amount of ^{14}C remaining in the incubation flask, was considered to be the amount not taken up, although with the lipid substrates a portion of the material fed probably adhered to the disks. The washed disks were ground in a Ten-Broeck homogenizer with about 10 ml of water. In the case of apple skin and other tough tissues, addition of a few drops of concentrated HCl to the tissue prior to homogenization helped to soften the tissue. However, such drastic changes in pH were avoided when functional groups that are highly susceptible to acid, such as epoxides, were being examined. The homogenate was centrifuged at 27,000*g* for 15 min and the residue was repeatedly treated with methanol and chloroform–methanol mixtures as described before in order to remove all the soluble lipids (Kolattukudy and Walton, 1972b). The final residue, containing the labeled cutin, was refluxed with either $LiAlH_4$ for 24–48 hr in tetrahydrofuran (Kolattukudy and Walton, 1972b) or with 22 mM sodium methoxide in absolute methanol, under nitrogen, for 24–48 hr. The products were isolated in the usual manner and subjected to thin-layer chromatographic analysis.

Cleavage of Ether-Bonded Cutin Core. The dry residue recovered from repeated $LiAlH_4$ treatment of labeled cutin (50 mg) was suspended in a mixture of glacial acetic acid (10 ml) and 47% hydrogen iodide solution (5 ml) and the mixture was heated in a sealed tube under vacuum at 100° for 16 hr.

Chromatography. Thin layers of silica gel G (0.5 or 1 mm) were coated on glass plates (20 × 20 cm) and activated for a minimum of 2 hr at 110°. The solvent systems used are indicated elsewhere in this paper. For separation of unsaturated compounds from the saturated, 13% $AgNO_3$ was used in 0.25-mm thick silica gel G layer. The components were visualized by spraying with a 0.1% alcoholic solution of 2′,7′-dichlorofluorescein, and were eluted with ethyl ether, a 2:1 mixture of chloroform and methanol, or methanol as required. The $AgNO_3$ contained in the recovered fractions was removed by rechromatography on silica gel G plates. A Perkin-Elmer 800 gas chromatograph equipped with a flame ionization detector and an effluent splitter, which sent about 70–80% of the effluent through a Barber-Coleman radioactivity monitor, was used for radio gas–liquid chromatographic analysis. Liquid phase OV-1 was 4% on 80–100 mesh Gas Chrom Q packed in a coiled stainless steel column (0.25 in. × 6 ft). Further details are shown under the appropriate figures.

Analysis of Cutin Composition. Cutin from young McIntosh apples at the same stage of development as those used for the biosynthetic experiments was isolated and hydrogenolyzed as described earlier (Walton and Kolattukudy, 1972a). After preliminary silica gel G chromatography, the isolated alcohol fractions were further fractionated by argentimetric chromatography and finally purified by silica gel G thin-layer chromatography prior to gas–liquid chromatography–mass spectrometry (glc–ms). The solvent systems for thin-layer chromatography were those described previously (Walton and Kolattukudy, 1972a) and the fractions were eluted with methanol. A coiled glass column (147.0 × 0.31 cm o.d.) packed with 3% OV-1 on 80–100 mesh Gas Chrom Q was used in conjunction with a Perkin-Elmer Hitachi RMU 6D mass spectrometer for the glc–ms analysis of the trimethylsilyl (Me_3Si) ethers of the alcohols derived from cutin (Walton and Kolattukudy, 1972a).

Determination of Radioactivity. Radioactivity in liquid samples was determined as described before (Kolattukudy, 1965). Internal standards were always used to determine the efficiency of counting, which was about 74% for ^{14}C. All counting was done with a standard deviation less than 3%. The radioactive components on thin-layer plates were detected with a Berthold thin-layer scanner. Radioactivity on thin-layer fractions was determined by assaying the scraped silica gel directly with a Packard liquid scintillation spectrometer as described before (Kolattukudy, 1965). Radio-

TABLE I: Incorporation of [1-^{14}C]Palmitic Acid into an Insoluble Polymer in Tissue Slices of Young Apple Fruits.[a]

Tissue	[1-^{14}C]Palmitic Acid Taken up (cpm × 10^{-6})	Incorp into Reduced "Cutin" Monomer Fraction (cpm × 10^{-5})
Skin	11.3	1.45
1st mm	11.4	0.37
2nd mm	9.8	0.22
3rd mm	9.4	0.20
4th mm	10.8	0.19
5th mm	12.0	0.30

[a] Fifteen skin slices and internal tissue slices, each 12 mm in diameter, were incubated with 13.9 × 10^6 cpm of [1-^{14}C]-palmitic acid in 0.55 ml of water for 4 hr at 30°. They were processed as described in the Experimental Section. Ether-soluble hydrogenolysis products obtained from the insoluble residue is designated reduced cutin monomer fraction.

activity in the effluent of the gas chromatograph was determined with a Barber-Coleman radioactivity monitor. Determination of ^{14}C in solid samples (insoluble) was done after combustion and absorption of the resulting $^{14}CO_2$ in Hyamine hydroxide solution.

Chemical Conversions of the Metabolic Products. Me_3Si ethers of the reduced monomers derived from cutin were prepared by heating the sample with *N,O*-bis(trimethylsilyl)-acetamide at 100° for 15 min.

The methyl ester of the suspected epoxy acid, isolated by thin-layer chromatography, was acetylated by treatment with a 2:1 mixture of acetic anhydride and pyridine for 12 hr at room temperature. The product, isolated in the usual manner, was purified by thin-layer chromatography and then refluxed with glacial acetic acid for 6 hr. The reaction mixture was diluted with water and the products were isolated by extraction (three times) with ethyl ether. The ether extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. This product, after purification by thin-layer chromatography, was refluxed with excess $LiAlH_4$ in tetrahydrofuran overnight. The reduction product was isolated in the usual manner and subjected to thin-layer chromatography.

Periodate Oxidation. Periodate–permanganate oxidation of the vicinal diol, 1,9,10,18-octadecanetetraol, was carried out essentially by the method of Baumann *et al.* (1969) as modified by Sawaya and Kolattukudy (1972). The tetraol (233,000 cpm) was dissolved in pyridine (7 ml) and vigorously stirred with sodium metaperiodate (75 mg) under a nitrogen atmosphere at room temperature. After 6 hr, 7 ml of a solution containing potassium permanganate (5 mM) and sodium carbonate (2 mM) was added and stirring was continued for 1 hr. The reaction mixture was decolorized with sodium bisulfite, acidified with HCl, diluted with water, and then extracted with $CHCl_3$. The solvent was evaporated and the oxidation products (203,000 cpm) were dissolved in tetrahydrofuran (25 ml) and refluxed with $LiAlH_4$ (250 mg) for 12 hr. The reduction products were isolated as described above and subjected to preparative thin-layer chromatography (ether–hexane–methanol, 8:2:1, v/v) and the diol region was isolated. A portion of the diol (130,000 cpm) was acetylated as described above and chromatographed on a thin layer of silica gel G (ether–hexane, 1:1, v/v) and the diol diacetate region was isolated and eluted (108,000 cpm).

Chromic Acid Oxidation. Radioactive C_{18}-triol (220,000 cpm) was dissolved in 95% acetic acid (3 ml), chromium trioxide (30 mg) was added, and the solution was heated at 70° for 90 min. The reaction mixture was diluted with water (10 ml) and extracted with chloroform (3 × 30 ml). The products (158,000 cpm) were refluxed with 14% BF_3 in MeOH for 14 hr and the methyl esters (156,000 cpm) were extracted into chloroform and purified by thin-layer chromatography ether–hexane, 1:1, v/v) on silica gel G prior to radio gas chromatography.

Results and Discussion

Site of Cutin Synthesis. Young apple fruits were chosen for biosynthetic studies on the C_{18} family of acids because apple fruit cutin contains large proportions of the C_{18} family of acids (Eglinton and Hunneman, 1968; Walton and Kolattukudy, 1972a) and most rapidly expanding tissues are likely to synthesize cutin most rapidly (Kolattukudy, 1970b). In order to test whether this tissue was suitable for biosynthetic studies, [1-^{14}C]palmitic acid, which is known to be incorporated into the cutin acids in *V. faba*, was used. Skin tissue was suspected to be the major site of cutin synthesis. In order to test this possibility, skin tissue slices and 1-mm thick slices from the internal tissue, up to 5 mm in depth into the fruit, were incubated with [1-^{14}C]palmitic acid for 4 hr. Thorough extraction of the soluble lipids left behind a radioactive residue which, when hydrogenolyzed, released all of the ^{14}C into ether-soluble materials. This material represents reduced cutin monomers (Kolattukudy, 1970a; Kolattukudy and Walton, 1972a). The incorporation of the exogenous [1-^{14}C]-palmitic acid into this lipid fraction by the tissues from various depths into the apple fruit is shown in Table I. It is obvious that the skin tissue incorporated [1-^{14}C]palmitic acid into the reduced cutin monomers, whereas the corresponding fraction from the internal tissue contained much less radioactivity. Thin-layer chromatographic analysis showed that the small amount of ^{14}C contained in the hydrogenolysis products of the residue of the internal tissue was exclusively located in the fatty alcohol fraction. On the other hand, hexadecanetriol and alkane-α,ω-diol fractions contained the bulk (>90%) of the ^{14}C found in the hydrogenolysis products of the residue from the skin. Furthermore, visualization of the thin-layer chromatograms showed that the polyhydroxyalkanes typical of apple cutin were found only in the hydrogenolysate of the skin tissue. Therefore, it is clear that the skin tissue is the site of cutin synthesis in apple fruits.

Incorporation of [1-^{14}C]Acetate, [1-^{14}C]Palmitate, [1-^{14}C]-Stearate, and [1-^{14}C]Oleate into Cutin in Apple Fruits. The labeling pattern obtained with [1-^{14}C]palmitic acid (Figure 1) and further analysis of each thin-layer fraction by radio gas liquid chromatography showed that palmitic acid was incorporated specifically into ω-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid (containing a little 9,16-dihydroxypalmitic acid) of the apple cutin. In order to test whether a double bond at Δ^9 is involved in the introduction of the in-chain hydroxyl group of the dihydroxypalmitic acid, apple skin slices were incubated with [10-^{14}C]palmitoleic acid and the hydrogenolysis products of cutin, which contained about 2% of the administered ^{14}C, were analyzed as before. The only fractions which contained ^{14}C were the fatty alcohols (>95%) and alkane-α,ω-diols. Thin-layer chromatographic (tlc) analysis of soluble lipids from the tissue showed that

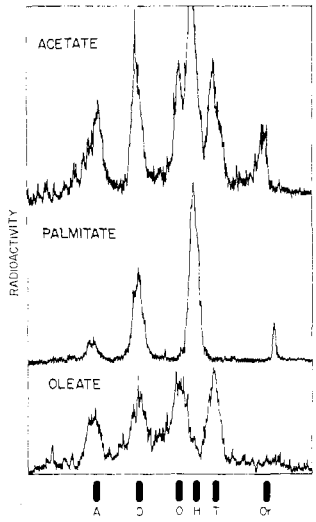


FIGURE 1: Radio thin-layer chromatogram of the hydrogenolysates of cutin derived from [1-^{14}C]acetate, [1-^{14}C]palmitate, and [1-^{14}C]-oleate by skin slices of young apple fruits; Or = origin, T = octadecane-1,9,10-18-tetraol, H = hexadecane-1,7,16-triol, O = octadecane-1,9,18-triol, D = alkane-α,ω-diols, A = fatty alcohols.

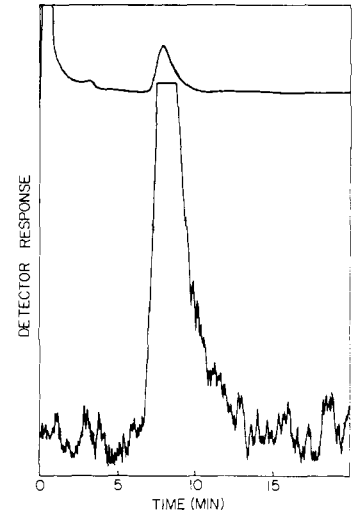


FIGURE 2: Radio gas–liquid chromatography pattern of the radioactive diol diacetate derived from the trihydroxy acid in young apple cutin labeled from [1-^{14}C]oleic acid. Column conditions: 5 ft $\times$ 0.25 in. stainless steel column; liquid phase was 5% OV-1 on 80–100 mesh Gas Chrom Q, argon flow rate 120 cm^3/min, temperature 149°; top, mass; bottom, radioactivity.

palmitoleic acid was incorporated into virtually all the acyl lipids especially phospholipids (data not shown). Similar results were obtained with [10-^{14}C]palmitelaidic acid. The observation that no label could be found in the C_{16}-triol fraction derived from the unsaturated acids strongly suggests that a double bond is not involved in the introduction of the hydroxyl group at C-10. This conclusion is similar to that reached previously with *V. faba* (Kolattukudy and Walton, 1972b) and the biosynthetic pathway for the C_{16} family of acids is probably the same as that in *V. faba*.

In order to determine the biosynthetic pathway for the C_{18} family of cutin acids the apple skin slices were incubated with [1-^{14}C]stearic acid. Only about 1.5% of the label was recovered in the hydrogenolysis products of cutin. Thin-layer chromatographic analysis showed that the bulk (>95%) of this radioactivity was in the fatty alcohol, while the remaining portion was in the α,ω-diol fraction. The in-chain hydroxylated C_{18} fractions contained no detectable radioactivity, strongly suggesting that the saturated hydrocarbon chain of stearic acid does not undergo hydroxylation reaction similar to that observed in the formation of the in-chain hydroxylated C_{16}-cutin acids. The fact that stearic acid does undergo ω-hydroxylation, a somewhat nonspecific step in cutin biosynthesis (Kolattukudy and Walton, 1972b), indicates that stearic acid is accessible to the site of synthesis of the polymer, and is not excluded from synthesis of cutin by a permeability barrier.

Since stearic acid was not incorporated into in-chain hydroxylated C_{18} acids, [1-^{14}C]acetate was used as a precursor. About 29% of the acetate incubated with the apple skin slices was incorporated into soluble lipids in 4 hr while about 2% was converted into cutin. Thin-layer chromatographic analysis of the hydrogenolysis products showed that all cutin components were labeled (Figure 1). The identity of the labeled hydrogenolysis products was confirmed by radio gas–liquid chromatography of the Me_3Si ethers of each fraction. Since acetate was incorporated into the in-chain hydroxylated C_{18} components, for which stearic acid was not an efficient precursor, oleic acid was suspected to be the precursor of the C_{18} family of cutin acids. In 4 hr about 9% of the [1-^{14}C]oleic acid fed to skin slices of young apple fruits was incorporated into cutin. Thin-layer chromatographic analysis of the hydrogenolysis products showed that all the radioactivity was contained in the α,ω-diol, C_{18}-triol, and C_{18}-tetraol components, with no significant label in the C_{16}-triol fraction (Figure 1) demonstrating specific incorporation of the Δ^9-unsaturated C_{18} acid into the in-chain hydroxylated C_{18} acids of cutin. Radio gas–liquid chromatography of the diol fraction showed that the radioactivity was exclusively in the C_{18} components, while the labeled triol and tetraol components corresponded to octadecane-1,9,18-triol and octadecane-1,9,10,18-tetraol. The structure of the radioactive triol was established as octadecane-1,9,18-triol by chromic acid degradation followed by radio gas–liquid chromatography as described later in this paper. The identity of the labeled tetraol was also established by degradation. The radioactive tetraol fraction obtained by $LiAlH_4$ treatment of apple fruit cutin derived from [1-^{14}C]-oleic acid was treated with sodium metaperiodate, and the oxidation products were reduced with $LiAlH_4$ and subsequently acetylated. Analysis of the acetylated product by radio gas–liquid chromatography revealed a single radioactive component, corresponding to C_9-α,ω-diol diacetate (Figure 2). This result confirmed the structure of the labeled tetraol as octadecane-1,9,10,18-tetraol, which obviously was derived from 9,10,18-trihydroxystearic acid of the cutin.

A similar qualitative picture of cutin labeling was observed when [1-^{14}C]oleic acid was incubated with leaves of apple and *S. odoris*, although the incorporations did show significant quantitative differences (Table II). Again oleic acid specifically labeled the C_{18}-cutin components, but in the very young leaves from the seedlings, the amount of radioactivity in the C_{18}-tetraol was low compared with that in the C_{18}-triol fraction. We regard this difference in incorporation as a developmental effect reflecting the age of the tissue. Similar observations were also made in metabolic experiments with grape berries (P. E. Kolattukudy and T. J. Walton, unpublished results) in which the labeling of the polar components of cutin was weak at very early stages of growth.

The labeling results obtained with young apple fruit and apple and *S. odoris* leaves demonstrated that oleic acid was the specific precursor of in-chain hydroxylated C_{18}-cutin

TABLE II: Incorporation of [1-^{14}C]Oleic Acid into Cutin in the Leaves of *S. odoris* and Apple.[a]

Tissue	[1-^{14}C]Oleic Acid Administered (cpm $\times 10^{-6}$)	Radioactivity Taken up (cpm $\times 10^{-6}$)	Radioactivity in Reduced Cutin Monomers (cpm $\times 10^{-5}$)	Primary Alcohol	Diol	C_{18}-Triol	C_{18}-Tetraol
Disks from young leaves of orchard grown apple tree	11.5	8.1	1.34	2.87	1.85	3.62	3.47
Chopped leaves from apple seedlings grown in greenhouse	11.0		4.45	6.55	20.8	12.80	3.20
Disks from leaves of *S. odoris* grown in greenhouse	6.6	6.1	1.74	7.34	3.34	3.52	2.22

[a] Where leaf disks were used 15 disks (12 mm), were incubated with [1-^{14}C]oleic acid for 4 hr at 30°. In the case of the chopped leaf experiment 600 mg of tissue slices was incubated for 5 hr at 30°. The products were obtained and analyzed as described in the Experimental Section.

acids. The absence of label in the α,ω-C_{16}-diol and hexadecanetriol fractions show that there was no significant random labeling of the cutin acids from acetate derived by degradation of the substrate. Thus, the Δ^9 double bond of oleic acid appears to be essential for the formation of in-chain oxygenated C_{18}-cutin acids.

Evidence for Incorporation of [1-^{14}C]Oleic Acid into 18-Hydroxy-9,10-epoxystearic Acid. Structural analyses (Brieskorn and Boss, 1964; Walton and Kolattukudy, 1972a; Croteau and Fagerson, 1972) have established that 9,10-epoxy-18-hydroxystearic acid is an apparently ubiquitous member of the C_{18} family of cutin acids, and indeed in the cutin of grape berry, the ω-hydroxy-9,10-epoxy-C_{18} acid is the major component (Walton and Kolattukudy, 1972a). Both 9,18-dihydroxystearic acid and 10,18-dihydroxystearic acid have also been reported, usually as minor cutin components (Matic, 1956; Croteau and Fagerson, 1972; Brieskorn and Kabelitz, 1971). Since all three of these compounds would give rise to isomeric C_{18}-triols upon treatment with $LiAlH_4$, it was essential to establish the structure of the cutin component derived from [1-^{14}C]oleic acid which gives rise to the C_{18}-triol during reductive depolymerization. If the epoxy acid is, in fact, an intermediate in the synthesis of the 9,10,18-trihydroxy-C_{18} acid, as was suggested earlier (Kolattukudy *et al.*, 1971), the epoxy acid should be expected to be labeled by [1-^{14}C]oleic acid. In order to test this possibility we initially used skin slices of young grape berries because the major component of grape cutin had been shown to be the epoxy acid (Walton and Kolattukudy, 1972a). Preliminary experiments with skin disks of young grape berries showed that the Concord, Nyabell, Kendaia, Buffalo, and 4912-96 varieties incorporated 5–9% of the administered [1-^{14}C]oleic acid into cutin in 4 hr. Alkaline hydrolysis of the labeled cutin followed by thin-layer chromatographic analysis of the products showed that 15–30% of the label in the hydrolysate was contained in a product which was more polar than ω-hydroxy acid, presumably 18-hydroxy-9,10-epoxystearic acid. Since Concord grapes appeared to give the highest incorporation into this product and oleic acid incorporation into this product increased from 7 to 14% as this grape began to expand to about 1.5 cm, further experiments were done with this variety. The distribution of radioactivity among the products obtained by $NaOCH_3$ depolymerization of Concord grape cutin derived from [1-^{14}C]oleic acid is shown in Figure 3. The R_F values suggested that the labeled components are methyl esters of fatty acids (N), ω-hydroxy acid (M), dihydroxy acid (D), trihydroxy acid (T), and an unknown (E), which was the major radioactive product (44.5% of the total). This major labeled component had the expected mobility of 18-hydroxy-9,10-epoxymethyl stearate, and upon acetylation it cochromatographed with authentic 18-acetoxy-9,10-epoxymethyl stearate (R_F 0.5 in ether–hexane–methanol, 20:5:1, v/v). Treatment of this material with glacial acetic acid yielded a more polar compound (R_F 0.23 in the above solvent) which, when reduced with $LiAlH_4$ yielded C_{18}-1,9,10,18-tetraol as the major product, while direct $LiAlH_4$ reduction of the unknown E yielded only C_{18}-triol (Figure 4). These results show (Scheme I) that the major component of the grape cutin derived from [1-^{14}C]oleic acid was 9,10-epoxy-18-hydroxystearic acid, suggesting that this is an important biosynthetic product.

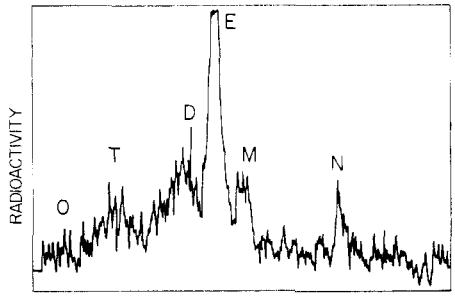
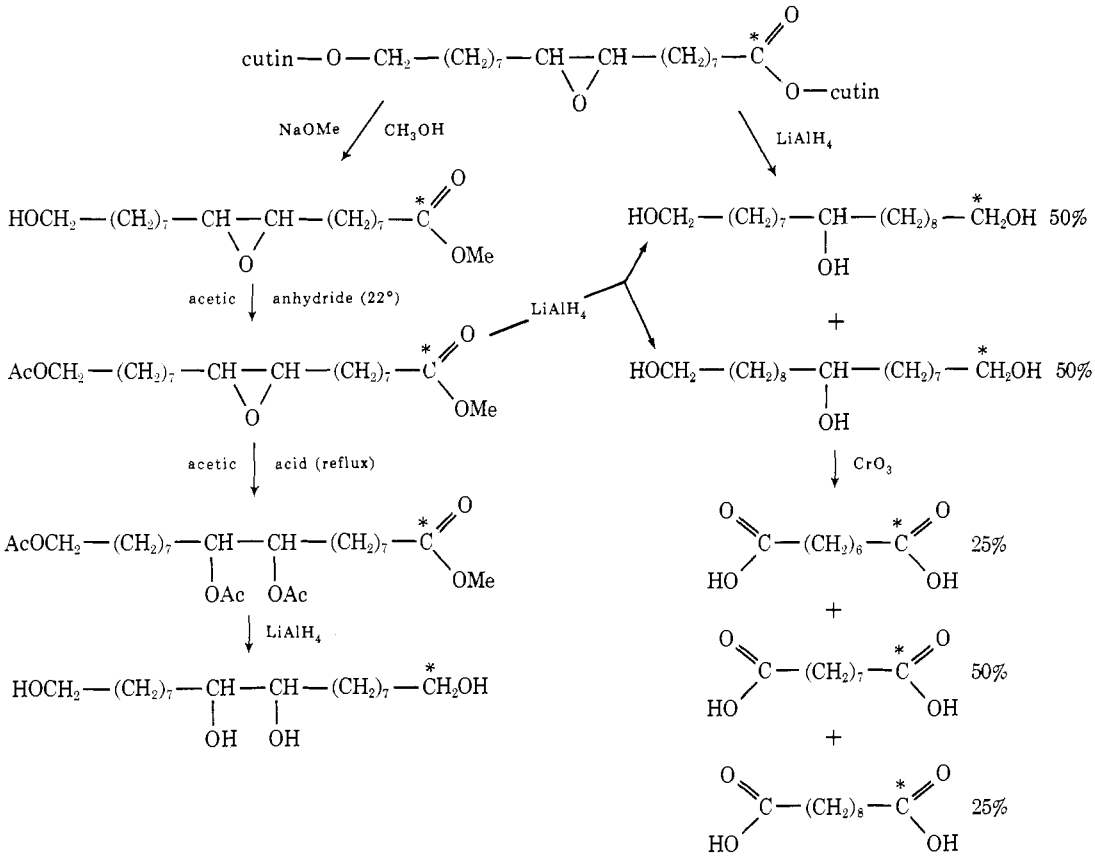


FIGURE 3: Radio thin-layer chromatogram of the ether soluble products derived from grape berry cutin, labeled from [1-^{14}C]oleic acid, by treatment with NaOMe–MeOH. Solvent system ether–HCl (17:3). O = origin, T = trihydroxy-C_{18} acid methyl ester, D = dihydroxy-C_{18} acid methyl ester, E = suspected 9,10-epoxy-18-hydroxymethyl stearate, M = ω-hydroxy acid methyl ester, N = fatty acid methyl ester.

A second approach was used to confirm the presence of labeled epoxy acid in apple fruit cutin derived from [1-^{14}C]-oleic acid. If the labeled C_{18}-triol obtained by hydrogenolysis of cutin derived from [1-^{14}C]oleic acid is the reduction product of the epoxy acid, chromic acid oxidation of the labeled triol should give radioactive C_8-, C_9-, and C_{10}-dicarboxylic acids in a ratio of 1:2:1 (Scheme I). Radio gas–liquid chromatography of the dimethyl esters of the dicarboxylic acids obtained showed the expected pattern (Figure 5). The experimentally determined distribution of label among C_8-dioic

SCHEME I



acid (25%), C_9-dioic acid (51.8%), and C_{10}-dioic acid (23.2%) was in excellent agreement with the expected results. Therefore, the C_{18}-triol was derived from 18-hydroxy-9,10-epoxystearic acid of apple cutin (Scheme I). We have also obtained similar experimental evidence that oleic acid was converted into 18-hydroxy-9,10-epoxystearic acid in apple leaves. Thus, experimental results obtained with several plant tissues and several chemical techniques show that 18-hydroxy-9,10-epoxystearic acid is derived from oleic acid.

Incorporation of Exogenously Labeled ω-Hydroxyoleic Acid into Cutin Components. The results discussed thus far show that oleic acid is converted into ω-hydroxyoleic acid, 9,10-epoxy-18-hydroxystearic acid, and 9,10,18-trihydroxystearic acid. While the order of hydroxylation and epoxidation is not clear, all C_{18} components so far isolated from higher plant cutin contain an oxygen function at the ω-carbon atom, suggesting that ω-hydroxylation is the first step in cutin acid biosynthesis. In order to test this possibility we isolated labeled ω-hydroxyoleic acid from grape berry skin slices which metabolized [1-^{14}C]oleic acid and incubated this material for 6 hr with skin slices from young apple fruits. Under these conditions, 8–10% of the exogenous ω-hydroxyoleic acid was incorporated into cutin. Analysis of the hydrogenolysate of the labeled cutin by thin-layer chromatography showed that C_{18}-tetraol and C_{18}-triol contained the major portion (54%) of the label incorporated into cutin (Table III). These hydroxy alkanes are probably derived from 9,10,18-

TABLE III: Incorporation of [^{14}C]-ω-Hydroxyoleic Acid into Cutin Components in Apple Fruit Skin Slices.[a]

Fraction from Hydrogenolysis of Cutin	Incorp (cpm × 10^{-3})
Total	10.0
Alkane-α,ω-diol	2.5
C_{18}-triol	3.0
C_{18}-tetraol	2.4

[a] Fifteen disks were incubated for 6 hr at 30° with 2 mg of biosynthetically labeled ω-hydroxyoleic acid (1.2 × 10^5 cpm). The disks were then washed and processed as described under the Experimental Section. About 10% of the ^{14}C contained in the hydrogenolysate was spread above the diol region and another 10% between triol and diol regions of the plate.

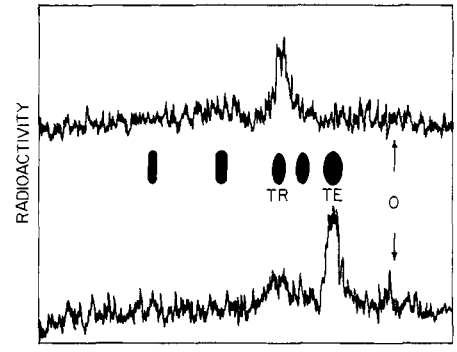
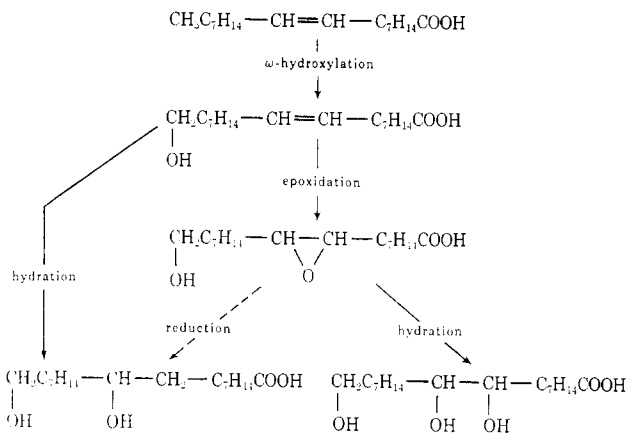


FIGURE 4: Radio thin-layer chromatogram of the $LiAlH_4$ reduction products of 9,10-epoxy-18-*O*-acetoxymethyl stearate (top) and suspected 9,10,18-tri-*O*-acetoxymethyl stearate (bottom). Solvent system ether–hexane–methanol (8:2:1), identification of TE as octadecanetetraol and TR as octadecanetriol by cochromatography with authentic standards.

SCHEME II



trihydroxystearic acid and 18-hydroxy-9,10-epoxystearic acid of the cutin, respectively (Walton and Kolattukudy, 1972a). Therefore, the present experimental evidence strongly suggests that ω-hydroxyoleic acid is the precursor of the epoxy acid and 9,10,18-trihydroxystearic acid. Since the epoxy acid is likely to be the precursor of the trihydroxy acid we attempted to demonstrate conversions of exogenously labeled epoxy acid into the trihydroxy acid. Incubation of labeled 18-hydroxy-9,10-epoxystearic acid (derived from [1-^{14}C]oleic acid in grape) with apple skin slices showed that the epoxy acid was highly toxic to the apple tissue. Presumably for this reason, we observed only a very small amount of incorporation of the epoxy acid into cutin.

Proposed Pathway for the Biosynthesis of the C_{18} Family of Cutin Acids. Based on the structural and labeling studies we propose the following pathway for the biosynthesis of C_{18} cutin acids (Scheme II). While the mechanism of ω-hydroxylation in plants has not been established, it seems likely that it is a direct hydroxylation catalyzed by a mixed-function oxygenase similar to those studied in animals and microorganisms (Hayaishi, 1969; Tyson *et al.*, 1972; Ueda and Coon, 1972; Ellin *et al.*, 1972; Ichihara *et al.*, 1971). As expected from such a reaction incorporation of oleic acid into cutin components by skin slices does indeed require molecular oxygen (data not shown). The epoxidation and hydration steps, besides being suggested by the experimental results presented in this paper, are also supported by the occurrence of chemically analogous reactions in other systems (Knoche, 1968, 1971; Corey *et al.*, 1966; van Tamelen *et al.*, 1966; May and Abbott, 1973; Jerina *et al.*, 1970; Kaubisch *et al.*, 1972; Niehaus *et al.*, 1970; Hartmann and Frear, 1963; Watabe *et al.*, 1971; Watabe and Akamatsu, 1972; Oesch *et al.*, 1971; Sims, 1971).

The minor cutin components, 9,18- and 10,18-dihydroxystearic acids, could arise either by hydration of ω-hydroxyoleic acid or reduction of the corresponding epoxycutin acid. Since enzymes which catalyze hydration of olefins also catalyze hydration of epoxides in other biochemical systems (Niehaus *et al.*, 1970; Albright and Schroepfer, 1971), it is possible that hydration of the 18-hydroxy-9,10-epoxystearic acid and 18-hydroxyoleic acid is catalyzed by the same (or similar) enzyme, giving rise to 9,10,18-trihydroxystearic acid and 9,18- and/or 10,18-dihydroxystearic acids, respectively.

Incorporation of [1-^{14}C]Linoleic Acid and [1-^{14}C]Linolenic Acid Specifically into Unsaturated Cutin Acids. The Δ^{12}-monoenoic analogs of the in-chain substituted saturated C_{18} acids which are significant components of the polymer (Eglinton and Hunneman, 1968; Croteau and Fagerson,

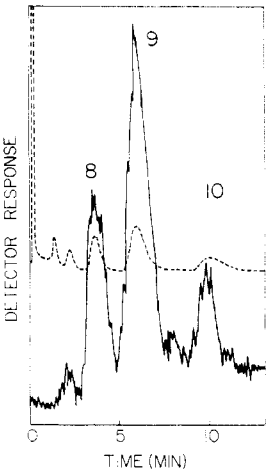


FIGURE 5: Radio gas–liquid chromatogram of the dicarboxylic acids (as dimethyl esters) derived from chromic acid oxidation of the radioactive C_{18}-triol isolated from cutin of young apple skin disks which had metabolized [1-^{14}C]oleic acid. Chromatography conditions: 5 ft × 0.25 in. stainless steel column, liquid phase was 5% OV-I on 80–100 mesh Gas Chrom Q, argon flow rate 120 cm^3/min, temperature 138°. Identification of C_8-, C_9-, and C_{10}-dicarboxylic acid dimethyl esters was based on cochromatography with authentic standards: dotted line, mass of coinjected standards; solid line, radioactivity.

1972; Walton and Kolattukudy, 1972a), may arise from linoleic acid by a series of reactions analogous to those proposed for the formation of the saturated cutin acids. Linolenic acid may also undergo this series of reactions to yield Δ^{12},Δ^{15}-dienoic hydroxycutin acids although such acids have not been reported to be significant components of cutins from any source so far examined. In order to test these possibilities, [1-^{14}C]linoleic acid and [1-^{14}C]linolenic acid were incubated with skin disks from young apple fruits and the ether-soluble hydrogenolysis products of cutin were examined by tlc (Figure 6). In the case of linoleate, the radioactivity (4.6% of administered substrate) in the diol, triol, and tetraol fractions indicated that while this substrate was incorporated into the ω-hydroxy acid and 9,10-epoxy-18-hydroxy-C_{18} acid fractions

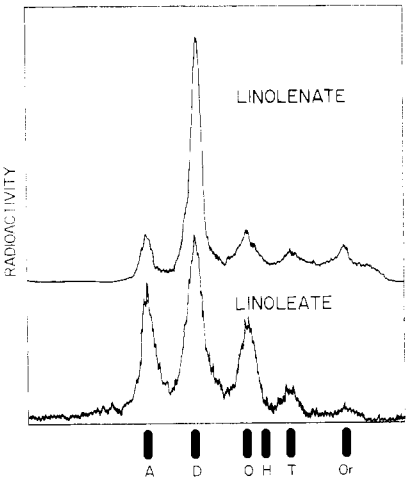


FIGURE 6: Radio thin-layer chromatogram of the cutin hydrogenolysis products isolated from young apple skin disks which had metabolized [1-^{14}C]linoleic and [1-^{14}C]linolenic acids. Solvent system ether–hexane–methanol (8:2:1, v/v), identification was by cochromatography with authentic standards. Or = origin, T = C_{18}-tetraol, H = C_{16}-triol, O = C_{18}-triol, D = α,ω-diol, A = fatty alcohol.

TABLE IV: Distribution of Radioactivity among the Diols Derived from [1-^{14}C]Acetate, [1-^{14}C]Oleate, [1-^{14}C]Linoleate, [1-^{14}C]Linolenate.[a]

Substrate	Distribution of Radioactivity (%)			
	Saturated	Monounsaturated	Diunsaturated	Triunsaturated
[1-^{14}C]Acetate	57.3	24.3	18.5	
[1-^{14}C]Oleate	5.8	76.2	18.0	
[1-^{14}C]Linoleate	8.1	4.7	87.3	
[1-^{14}C]Linolenate	2.3	1.4	1.3	95.0

[a] Radioactivity distribution was determined by argentimetric thin-layer chromatography of the diol fraction isolated from the samples shown in Figures 1 and 7 as described under the Experimental Section.

of cutin, the trihydroxy-C_{18} acid of cutin was not as strongly labeled as in the case of experiments with [1-^{14}C]oleic acid (Figure 1). Linolenic acid, however, labeled principally the diol fraction of the cutin hydrogenolysis products, while the fractions derived from 9,10-epoxy-18-hydroxy-C_{18} acid and 9,10-18-trihydroxy-C_{18} acid contained much less radioactivity. These results suggested that both linoleic acid and linolenic acid can be alternative substrates for the enzymes involved in C_{18}-cutin acid biosynthesis. The presence of the additional unsaturation distal to Δ^9 appeared to make these precursors less suitable substrates for in-chain substitution, this effect being most pronounced in the case of linolenic acid.

The incorporation of the di- and triunsaturated substrates could indicate either specific direct incorporation or an indirect route *via* degradation followed by resynthesis. In the former case, the ω-hydroxylated products derived from [1-^{14}C]linoleic acid and [1-^{14}C]linolenic acid should contain two and three double bonds, respectively, while those components with in-chain functional groups should contain one and two double bonds, respectively. On the other hand, indirect incorporation *via* degradation products (acetyl-CoA) should give a product distribution similar to that obtained with [1-^{14}C]acetate. In order to distinguish between these two possibilities, the diol, C_{18}-triol and C_{18}-tetraol fractions isolated from hydrogenolysis products of cutin derived from each substrate were subjected to argentimetric chromatography. Data in Table IV clearly show that [1-^{14}C]oleic acid and [1-^{14}C]linoleic acid gave rise to mainly mono- and diunsaturated diols, respectively. Similar analysis also showed that most of the label in the diol derived from [1-^{14}C]linolenic acid was in the triunsaturated diol. A substantial portion of the label in the diol derived from [1-^{14}C]oleic acid was in the diunsaturated diol fraction indicating that exogenous oleic acid did undergo some desaturation. The major portion of the label in the diol derived from [1-^{14}C]acetate was in the saturated fraction while monounsaturated and diunsaturated diol fractions did contain substantial amounts of ^{14}C. The triunsaturated diol fraction contained very little ^{14}C when labeled acetate, oleic acid, or linoleic acid was used as the substrate. These results show that ω-hydroxylation of oleic, linoleic and linolenic acids may occur in the apple skin giving rise to the corresponding ω-hydroxycutin acid. Since the ω-hydroxylation step of cutin biosynthesis has been shown to exhibit a low degree of specificity for the nature of the hydrocarbon chain of the administered fatty acid, this observation in itself is not surprising. However, ω-hydroxylinolenic acid has not been heretofore reported in any cutin.

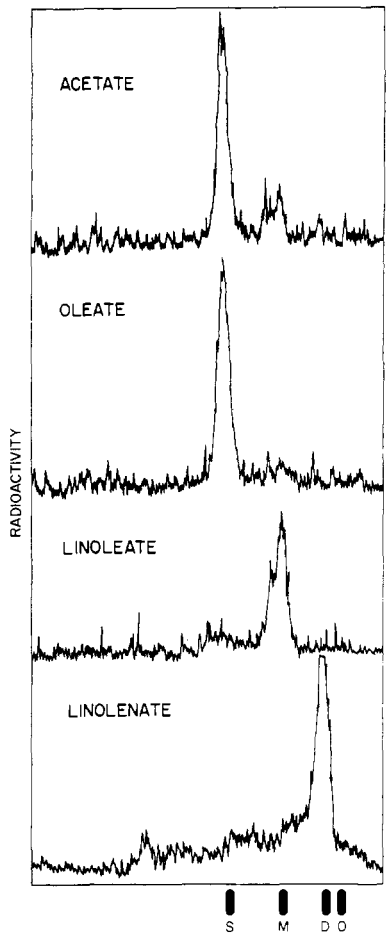


FIGURE 7: Argentimetric radio thin-layer chromatogram of the C_{18}-triol fraction derived from cutin of young apple skin disks which had metabolized [1-^{14}C]acetate, [1-^{14}C]oleate, [1-^{14}C]linoleate, and [1-^{14}C]linolenate. Chromatography was on silver nitrate impregnated silica gel G (solvent system ether–hexane–methanol (16:4:3, v/v)). Identification of components was by glc–ms: O = origin, D = dienoic C_{18}-triol, M = monoenoic C_{18}-triol, S = saturated C_{18}-triol.

Argentimetric thin-layer chromatogram of the triols showed that [1-^{14}C]linolenic acid, [1-^{14}C]linoleic acid, and [1-^{14}C]oleic acid gave rise to mainly diunsaturated, monounsaturated and saturated triols, respectively, most probably derived from the corresponding ω-hydroxy-9,10-epoxy-C_{18} acid (Figure 7). These results clearly show specific incorporation of the unsaturated acids into the products expected from the pathway proposed in Scheme II. [1-^{14}C]Acetate also gave rise to small amounts of monounsaturated triol, probably *via* linoleic acid.

Further unambiguous evidence for the epoxidation of linoleic acid was obtained by the previously described procedure used to establish the labeling of 9,10-epoxy-18-hydroxystearic acid from [1-^{14}C]oleic acid in grape skin. The cutin from grape berry skin which had metabolized [1-^{14}C]linoleic acid was refluxed with sodium methoxide–methanol and the product, with tlc mobility of a 9,10-epoxy-18-hydroxymethyl octadecanoate was acetylated and then subjected to acetoxylation. The acetoxylated product was reduced with $LiAlH_4$ and subjected to argentimetric chromatography. The labeled product cochromatographed with the C_{18}-tetraol derived from 9,10,18-trihydroxyoctadec-12-enoic acid, and was, therefore, clearly derived from 9,10-epoxy-18-hydroxyoctadec-12-enoic

TABLE V: Distribution of Radioactivity among the Tetraols Derived from [1-^{14}C]Acetate, [1-^{14}C]Oleate, [1-^{14}C]Linoleate, and [1-^{14}C]Linolenate.[a]

Substrate	Distribution of Radioactivity		
	Saturated	Monounsaturated	Diunsaturated
[1-^{14}C]Acetate	93.6	6.4	
[1-^{14}C]Oleate	94.6	5.1	
[1-^{14}C]Linoleate	15.5	84.5	
[1-^{14}C]Linolenate	NM	NM	48.0

[a] Radioactivity distribution was determined by argentimetric thin-layer chromatography of the tetraol fraction isolated from the samples shown in Figures 1 and 7 as described under the Experimental Section. NM, measurement of the radioactivity by a scanner showed that some of the ^{14}C was spread over a large area and therefore this activity cannot be attributed to saturated or monounsaturated components.

acid in grape cutin. We have, therefore, established that, in addition to oleic acid, linoleic, and linolenic acids may also undergo epoxidation at the Δ^9 position. The epoxy acids which have been examined so far all contained the oxirane function at the 9,10 position, suggesting that the epoxidation enzyme is specific for Δ^9 of the immediate precursor.

Fractionation of the tetraols derived from specifically labeled substrates showed that 93–95% of the ^{14}C incorporated into this fraction from [1-^{14}C]acetate and [1-^{14}C]oleic acid was in the saturated fraction, whereas the bulk of the radioactivity in the tetraol derived from [1-^{14}C]linoleic acid was in the monounsaturated fraction (Table V). Our previous glc–ms analysis (Walton and Kolattukudy, 1972a) showed that this fraction consisted of 9,10,18-trihydroxyoctadec-12-enoic acid, the product expected to be formed from linoleic acid (Scheme II). Argentimetric thin-layer chromatography of the tetraol derived from [1-^{14}C]linolenate showed that the major labeled component was diunsaturated as expected from Scheme II. However, substantial amounts of ^{14}C were found spread out in the saturated and monounsaturated regions. We did not examine these fractions further. In any case, the results presented above show that oleic acid, linoleic acid, and linolenic acid are converted into the corresponding ω-hydroxy acids, ω-hydroxy-9,10-epoxy acids, and 9,10-18-trihydroxy acids.

In a recent glc–ms analysis 9,10,12,13,18-pentahydroxystearic acid was reported as a significant component of cutin from the leaves of *Rosmarinus officianalis* (Brieskorn and Kabelitz, 1971). Both Δ^9 and Δ^{12} double bonds of linoleic acid presumably undergo epoxidation and hydration to give this acid.

Identification of Polyunsaturated Monomers in the Cutin of Young Apple Fruits. Earlier analyses of cutin from many mature plant tissues (Eglinton and Hunneman, 1968; Croteau and Fagerson, 1972; Walton and Kolattukudy, 1972a) failed to detect the presence of ω-hydroxylinolenic acid, 18-hydroxy-9,10-epoxyoctadecadienoic acid, and 9,10-18-trihydroxyoctadecadienoic acid, all of which appeared to be formed from [1-^{14}C]linolenic acid in the skin slices of young apple fruits. It is possible that these polyunsaturated cutin acids are present in significant quantities only in young tissues, or the observed incorporation of exogenous linolenic acid represents merely unnatural reactions. In order to distinguish between these possibilities, the argentimetric thin-layer chromatograms from the biosynthetic studies discussed above were sprayed with 2′,7′-dichlorofluorescein. Examination of the chromatogram of the diols under uv light revealed the presence of substantial amounts of a component suspected to be trienoic diol. This material was eluted and the Me_3Si ethers examined by glc–ms. The spectrum showed a prominent molecular ion (M^+) at *m/e* 424, and the usual fragment ions at *m/e* 409 ($M^+ - CH_3$) and at *m/e* 334 ($M^+ - (CH_3)_3SiOH$); the latter was 38.5% of base peak. The doubly charged ion, characteristic of Me_2Si ethers of α,ω-diols, at *m/e* 197 ($(M^+ - 30)/2$), accompanied by a first isotope peak at *m/e* 197.5 (Walton and Kolattukudy, 1972a), confirmed the structure as an α,ω-octadecatrienediol which was probably derived from ω-hydroxylinolenic acid in cutin.

Argentimetric tlc of the triol fraction obtained from young apples showed a component with an R_F expected from a diunsaturated triol. When the Me_2Si ether of this component was subjected to glc–ms analysis, a weak parent ion at *m/e* 514 (M^+) and characteristic fragment ions at *m/e* 499 ($M^+ - CH_3$) and *m/e* 424 ($M^+ - (CH_3)_3SiOH$), together with diagnostic α-cleavage ions at *m/e* 303, 313, and 317 (13.5, 1.0, and 31.8% of the base peak, respectively) were observed in the spectrum. As had been observed in the monoenoic analog (Walton and Kolattukudy, 1972a), the α-cleavage ion at *m/e* 317 predominated, arising by cleavage of the bond α to both the substituted carbon atom and allylic methylene group. In the case of the dienoic triol fraction, the predicted α-cleavage ion at *m/e* 313 was present but quite weak, while there was no significant signal at *m/e* 299, the other predicted α-cleavage ion. The fragmentation pattern observed indicated that the dienoic C_{18}-triol was derived from 9,10-epoxy-18-hydroxyoctadec-12,15-dienoic acid in cutin.

The discovery of the novel cutin components, ω-hydroxylinolenic acid and 9,10-epoxy-18-hydroxyoctadec-12,15-dienoic acid, together with the tentative identification of 9,10,18-trihydroxyoctadec-12,15-dienoic acid in the young apple cutin led to a reexamination of the composition of cutin from young and mature apple fruit. Large differences in composition were found. The most striking difference was that all the cutin components which would be derived from linolenic acid (Scheme II) were significant components in the cutin from the young tissue while such components were very minor components or barely detectable in old fruits. (A detailed analysis will be published later.) For example, in young cutin 9,10-epoxy-18-hydroxyoctadec-12,15-dienoic acid represented 27.7% of the epoxy acid fraction, while in old cutin it represented only 2.8% (8.2 and 0.9% of total cutin acids, respectively). These results indicated that cutin composition is highly dependent upon the developmental stage of the tissue. Recent observations in this laboratory have established that not only does cutin composition in other plant tissues vary quite widely with the age of the tissue, but also novel cutin components may be present in significant quantities in the young tissues (Kolattukudy, 1972, 1973). Thus, the failure of earlier analyses to identify these trienoic and dienoic components of cutin (Eglinton and Hunneman, 1968; Walton and Kolattukudy, 1972a) is probably due to the fact that these analyses were conducted on cutin from mature apple fruits. In such apples these polyunsaturated components are only minor components and they would be more susceptible than their saturated analogs to loss through autooxidation during the lengthy procedures generally used to isolate cutin. It is also possible that as the tissue ages the dienoic and trienoic components found in the young tissue might undergo secon-

dary cross-linking reactions such as those indicated in a section below. Thus, such polyunsaturated components might end up in the ether-bonded core, resistant to hydrolysis as well as hydrogenolysis. Therefore, they could not be detected in previous analyses which utilized these depolymerization techniques.

Biogenesis of the Ether-Linked Portion of Cutin. Very little radioactivity was present in the solid material recovered after hydrogenolysis of young apple cutin labeled with [1-^{14}C]oleic acid. This residue is thought to be the cutin core held together by ether bonds (Crisp, 1965) which are not susceptible to $LiAlH_4$ cleavage. However, in the case of both [1-^{14}C]linoleic and [1-^{14}C]linolenic acids, the $LiAlH_4$-resistant cutin core contained appreciable radioactivity. Specific radioactivity measurements of this residue indicated that the core from [1-^{14}C]linoleic acid had over 17 times the specific radioactivity when compared to that derived from [1-^{14}C]oleic acid (2.8×10^4 and 1.6×10^3 cpm mg^{-1}, respectively). When the core derived from [1-^{14}C]linoleic acid was treated with HI–AcOH, a reagent which cleaves ether bonds, ether-soluble products containing nearly 40% of the radioactivity of the core were obtained. When this material was subjected to silica gel G tlc (ether–hexane–methanol, 8:2:1, v/v) at le three radioactive components were detected, but they were not further identified. The final residue after HI treatment had approximately the same specific activity as the untreated material. Thus, there appears to be a specific incorporation of the methylene-interrupted dienoic and trienoic acids into the ether-linked core of cutin, suggesting that the *cis*-1,4-pentadiene structure may be involved in the formation of ether bonds in cutin. One possible route of synthesis could involve displacement of an acyl function from in-chain esterified α-ketol, produced by the action of lipoxygenase and hydroperoxide isomerase on linoleic acid (Veldink *et al.*, 1970, 1972; Zimmerman, 1966) in a manner similar to that observed in glyceryl ether biosynthesis (Hajra, 1970; Snyder *et al.*, 1970; Friedberg and Heifetz, 1973), although a more nonspecific radical mechanism cannot be ruled out.

Acknowledgments

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Organization of the Lipid Phase in Viral Membranes. Effects of Independent Variation of the Lipid and the Protein Composition†

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ABSTRACT: Spin-label electron spin resonance (esr) methods have been used to investigate the effects of independent variation of the lipid and protein composition on the organization of the lipid in viral membranes. Influenza and parainfluenza SV5 virions were grown in BHK21-F and MDBK cells and labeled with stearic acid derivative spin labels. Since the lipid composition of the virus reflects that of the plasma membrane of the host cell but the proteins are virus specified, two different viruses grown in the same cell line contain membranes with similar lipids and different proteins. The esr spectral splittings of such virions were found to be indistinguishable. Growing the same virus in different cells permitted a comparison of membranes with similar proteins but different lipids. The esr spectra of these virions showed significant differences. These results indicate that the rigidity of the viral membrane depends largely on the lipid composition, and is not affected by the differences in the protein composition of the two viruses. Evidence is presented that the lipids of parainfluenza virions are arranged in a bilayer structure.

Enveloped viruses, such as influenza and parainfluenza viruses, possess lipid-containing membranes which are acquired during the process of assembly by budding at the cell surface. The viral lipid composition reflects the composition of the plasma membrane of the host cell, while the proteins are virus specific (Klenk and Choppin, 1969, 1970a,b; Choppin *et al.*, 1971, 1972). The influenza virion contains seven polypeptides (Compans *et al.*, 1970; Schulze, 1970). Four of these are glycoproteins and form projections or spikes on the outer surface of the virion. A major nonglycosylated protein appears to be associated with the inner surface of the viral lipid membrane. The SV5 virion, often used as a model for the parainfluenza group, contains five major proteins, two of which are the glycoproteins forming the surface spikes, and one is a carbohydrate free protein thought to be associated with the viral membrane (Caliguiri *et al.*, 1969; Klenk *et al.*, 1970; Chen *et al.*, 1971; Mountcastle *et al.*, 1971). As discussed previously (Choppin *et al.*, 1972), viral membranes provide useful systems for the study of lipid–protein interactions for several reasons. (1) They are easily obtained in a high degree of purity. (2) They are composed of a limited number of protein species, and there is considerable information about the arrangement of the polypeptides associated with the viral lipid layer. (3) It is possible to alter the lipid composition of the membrane, without altering the proteins, by growing the same strain of viruses in cell types with differing plasma membrane lipids. (4) Since the proteins associated with viral membranes are coded for entirely by the viral genome, one can obtain viruses with a similar lipid composition but a completely different set of membrane-associated proteins by growing two different viruses in the same host cell.

Recently we have studied the organization of the lipid phase in influenza and Rauscher murine leukemia viruses by electron spin resonance (esr) using spin-label methods (Landsberger *et al.*, 1971a, 1972). The results indicate that in each virus the lipids are organized in a bilayer structure. Complete removal of the spikes from influenza virus particles by protease treatment (Compans *et al.*, 1970) had no detectable effect on the organization of the viral lipid (Landsberger *et al.*, 1971a), suggesting that the spikes do not penetrate through the lipid bilayer.

The present communication presents evidence for a lipid bilayer in parainfluenza virus and describes the results of esr studies on the lipid-containing membrane of influenza and parainfluenza viruses grown in different host cells. The effects of independently varying the lipid and the protein composition on the organization of the lipid phase have been determined.

Materials and Methods

Virus and Cells. The WSN strain of influenza virus A_0 and the W3 strain of the parainfluenza virus SV5 were used. Viruses were grown in the MDBK line of bovine kidney cells or the BHK21-F line of baby hamster kidney cells, as described previously (Choppin, 1964, 1969; Compans *et al.*, 1970; Holmes and Choppin, 1966).

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