Sharkey, A. G., Friedel, R. A., and Langer, S. H. (1957), *Anal. Chem. 29*, 770.

Shishiyama, J., Araki, F., and Akai, S. (1970), *Plant Cell Physiol. 11*, 323.

Structure and Biosynthesis of the Hydroxy Fatty Acids of Cutin in *Vicia faba* Leaves†

P. E. Kolattukudy* and T. J. Walton

ABSTRACT: Cutin, the lipid polymer, which is the structural component of cuticle, was isolated from *Vicia faba* leaves by a combination of enzymatic and chemical techniques. Exhaustive hydrogenolysis of powdered cutin followed by thin-layer chromatography and a combination of gas chromatography and mass spectrometry showed that this cutin was composed of 10,16-dihydroxypalmitic acid (77.8%), 9,16-dihydroxypalmitic acid (7.1%), 16-hydroxypalmitic acid (7.1%), palmitic acid (3.6%), stearic acid (2.2%), and oleic acid (0.8%). Disks from young leaves of *V. faba* incorporated sodium [1-^{14}C]acetate (2.4%), [1-^{14}C]palmitic acid (16%), [1-^{14}C]stearic acid (1.5%), and [1-^{14}C]oleic acid (2.7%) into cutin. Only the first two substrates labeled the dihydroxy acids while all four substrates labeled the nonhydroxy and ω-hydroxy acid fractions. The polar acids derived from [1-^{14}C]-palmitic acid were shown to be 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid containing a little 9,16-positional isomer by radiochromatographic techniques in conjunction with analysis of acetylated derivatives and chromic acid oxidation products. The time course of incorporation of [1-^{14}C]palmitic acid into cutin acids showed that at all times the dihydroxypalmitic acid contained most of the ^{14}C and that labeled hydroxy acids did not accumulate in the soluble lipids. Biosynthetically labeled 16-hydroxypalmitic acid was incorporated into cutin directly and after conversion into dihydroxypalmitic acid, when the substrate was fed to disks of young *V. faba* leaves in the presence of oxygen. Exogenous labeled dihydroxypalmitic acid was also directly incorporated into cutin under similar conditions. These results suggest that the following reaction sequence is involved in the biosynthesis of cutin in *V. faba* leaves: palmitic acid → 16-hydroxypalmitic acid → 10,16-dihydroxypalmitic acid → cutin. These reactions occurred much less rapidly in old leaves. Neither [10-^{14}C]palmitoleic acid nor [10-^{14}C]palmitelaidic acid was converted into dihydroxypalmitic acid in *V. faba* leaves, showing that a Δ^9 double bond was not involved in the in-chain hydroxylation. A direct hydroxylation was also indicated by the fact that [9,10-^{3}H,1-^{14}C]palmitic acid fed to *V. faba* leaf disks gave 10,16-dihydroxypalmitic acid with a ^{3}H:^{14}C ratio of 78% of that in the substrate. Phenanthroline and bipyridyl inhibited the hydroxylations of palmitic acid and this inhibition could be partially reversed by Fe^{2+}. These results suggest that a mixed-function oxidase-type enzyme catalyzes the direct hydroxylation at C-10 of ω-hydroxypalmitic acid.

Plant cuticle is made of a polymer, cutin, which is embedded in wax. The chemistry and biosynthesis of cuticular wax has been studied quite extensively in recent years (Eglinton and Hamilton, 1967; Kolattukudy, 1970a; Mazliak, 1968). However, cutin has attracted little attention until recently. This polymer on hydrolysis gives a variety of hydroxy-fatty acids (Matic, 1956; Baker and Holloway, 1970; Crisp, 1965; de Vries, 1969). The hydroxy acids of apple fruit cutin have been analyzed by a combination of gas–liquid chromatography and mass spectrometry (Eglinton and Hunneman, 1968). More recently other plant cutins have been subjected to similar analysis (Holloway and Deas, 1971). The major hydroxy acids thus far observed are ω-hydroxypalmitic acid, ω-hydroxyoleic acid, 10,16-dihydroxypalmitic acid, 18-hydroxy-9,10-epoxystearic acid, and 9,10,18-trihydroxystearic acid (Kolattukudy and Walton, 1972).

Alkaline hydrolysis leaves behind a residue which may consist of up to 45% of the cutin (Crisp, 1965). This residue contains ether bonds and peroxide bridges. For biosynthetic studies a more quantitative method of isolating the cutin monomers from a very small quantity of tissue was needed and such a method has been developed (Kolattukudy, 1970b; Walton and Kolattukudy, 1972b; Kolattukudy *et al.*, 1971).

Autooxidative processes involving the leaf lipids were postulated to be the mechanism for cutin biosynthesis (Priestly, 1943; Huelin, 1959). The only experimental evidence concerning cutin biosynthesis consisted of measurements of lipoxidases and other oxidases that were manifested as a result of wounding of leaves (Heinen and Brand, 1963). In this report we describe the results of the first systematic attempts to study the biosynthesis of cutin with the use of specifically labeled substrates, a novel cleavage technique, and a combination of gas chromatography and mass spectrometry.

On the basis of these studies we propose a biosynthetic pathway for cutin biosynthesis in *Vicia faba* leaves.

Experimental Section

Plants. Broad bean (*V. faba*) plants were grown from seed purchased from Burpee Co., California, in a soil–sand–peat-moss (1:1:1) mixture under wide spectrum Growlux (very

† From the Department of Agricultural Chemistry, Washington State University, Pullman, Washington. *Received November 29, 1971.* Scientific paper 3789, project 3001, College of Agriculture, Washington State University. This work was supported in part by a grant from National Science Foundation GB 23081.

* Author to whom inquiries should be made.

high output) lights (about 1200 ft-candles) supplemented with incandescent lights with 16-hr days. The most rapidly expanding young leaves from several plants were collected. Disks (12 mm) were cut with a stainless steel cork borer and washed thoroughly with distilled water. These discs were used for all the experiments except when old leaves were sampled in which case the fifth leaf from the apex of the plants with seven or eight leaves was used.

Substrates and Reagents. [1-^{14}C]Palmitic acid (specific activity 55 mCi/mmole), sodium [1-^{14}C]acetate (specific activity 57 mCi/mmole), [9,10-^{3}H]palmitic acid (specific activity 40 mCi/mmole), [1-^{14}C]stearic acid (specific activity 48.4 mCi/mmole), and [1-^{14}C]oleic acid (specific activity 57.8 mCi/mmole) were purchased from Amersham–Searle. [10-^{14}C]Palmitelaidic acid was prepared from [10-^{14}C]palmitoleic acid (specific activity 12 mCi/mmole; Schwarz BioResearch Inc., Orangeburg, N. Y.) by isomerization with nitrous acid as catalyst according to the method of Litchfield *et al.* (1965) as modified by Niehaus *et al.* (1970). The methyl esters of the products extracted from the reaction mixture were purified by thin-layer chromatography on silica gel G with benzene as the solvent. The purified methyl ester fraction was then subjected to argentation thin-layer chromatography with hexane–ethyl ether (19:1, v/v) as the solvent system. The major radioactive component had an R_F identical with that of methyl palmitelaidate and the minor component was methyl palmitoleate. The methyl palmitelaidate was isolated and radiopurity established by the same argentation chromatography. Phenanthroline, bipyridyl, purified fungal pectinase, and *Aspergillus niger* cellulase were purchased from Sigma Chemical Co. $LiAlH_4$ was purchased from Pierce Chemical Co. and $LiAlD_4$, minimum isotopic purity 99 atom %D was from Merck, Sharp and Dohme of Canada. Cutin from *V. faba* leaves was isolated as described elsewhere (Walton and Kolattukudy, 1972b).

Preparation of Substrate Dispersions. The labeled lipid material was dissolved in about 10 ml of ethyl ether and small amounts of Tween-20 were added. After mixing well the ether was evaporated off with a stream of N_2. Water was added to the resulting Tween solution of the substrate and shaken to give an almost clear dispersion in cases where the specific activity of the lipid substrates were so high that only submilligram amounts were used. When this technique was not sufficient to give well dispersed substrates, sonication for 1–3 min with the needle probe of Biosonik III at full power was employed.

Incorporation Experiments. Fifteen or twenty well-washed leaf disks were placed in a 125-ml erlenmeyer flask with 0.5–0.75 ml of substrate solution. The disks were individually bathed in the substrate solution so that both sides of the leaf tissue were coated with the substrate. The disks were then spread on the bottom of the flask and incubated in a shaking (100 rpm) water bath at 30° for varying periods of time. At the end of the incubation period the leaf disks were washed twice with 100 ml of water and then ground in a Ten-Broek homogenizer. The homogenate was centrifuged at about 30,000*g* for 15 min and the supernatant was removed. The residue was dispersed in methanol and allowed to stand for 15 min and then centrifuged as described above. The supernatant was removed and the above treatment of the residue repeated with a 1:1 mixture of chloroform and methanol and then four or five times with a 2:1 mixture of chloroform and methanol and then twice with tetrahydrofuran. All the supernatants were pooled for extraction of the soluble lipids, and the final residue was transferred into a 50-ml round-bottom flask with dry tetrahydrofuran (30 ml). Excess $LiAlH_4$ (0.5 g of powder) was added slowly and contents refluxed for 24 hr. The reaction mixture was carefully poured into water (50–100 ml) and concentrated hydrochloric acid was added (3–5 ml). The lipid material from the reaction mixture was recovered by repeated (four times) extraction with ethyl ether (100 ml each time), and the pooled ether extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The product was made up to a suitable volume (0.2–0.5 ml) and aliquots were taken for determination of radioactivity and for thin-layer chromatographic analysis of the products.

Completion of the hydrogenolysis is crucial for the success of this method. The physical state of the residue used for $LiAlH_4$ treatment is very important because it was found that if the finely ground residue was allowed to dry into a clump during the procedure before hydrogenolysis, large quantities of cutin remained unchanged even after 36-hr treatment with $LiAlH_4$. Routine monitoring of the water phase after ether extraction for radioactivity was used to detect possible incomplete hydrogenolysis. The pooled supernatant from the above procedure contained all the soluble lipids which were recovered in the usual manner.

Experiments with Biosynthetically Labeled 16-Hydroxypalmitic Acid and 10,16-Dihydroxypalmitic Acid. In order to prepare labeled hydroxy acids, much larger quantities of substrate were incubated with leaf tissue in incorporation experiments such as those described above. Under such conditions the per cent incorporation obtained was much lower than that in the usual experiments. In a typical preparation 1 mCi of [1-^{14}C]palmitic acid was fed to 60 leaf disks for 6 hr at 30°. At the end of the incubation leaf tissue was processed as described above except that the tetrahydrofuran washes were replaced by an ethanol wash. The final residue was refluxed with 12% ethanolic KOH for 24 hr under nitrogen. The reaction mixture was diluted with water, acidified and extracted four times with ethyl ether. The ether extract was dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure. The residue was applied to 1-mm silica gel G thin-layer plates and developed with ethyl ether–hexane–methanol–formic acid (40:10:1:2, v/v). Three radioactive components corresponding to palmitic acid, 16-hydroxypalmitic acid, and 10,16-dihydroxypalmitic acid were found. The two hydroxy acids were recovered and purified by repeated thin-layer chromatography in the same solvent system. The final radiochemically pure 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid contained 4.5×10^6 and 4.6×10^6 cpm, respectively.

The labeled hydroxy acids obtained as described above were dispersed in water with the aid of Tween-20 and sonication. With these substrate solutions experiments identical with those described in the previous section were done with the following exceptions. At the end of incubation the leaf disks were washed quickly with a 2:1 mixture of chloroform and methanol in an attempt to determine the amount of substrates adhering to the leaf disks. Fully expanded leaves, fifth from the apex, from plants with seven or eight leaves were also used and these were designated old leaves. For experiments under anaerobic conditions the erlenmyer flasks were stoppered with serum caps immediately after adding the substrates, and the air was displaced by passing nitrogen through the flask for 5 min *via* hypodermic needles.

Double-Labeling Experiments. About 300 μCi of [9,10-^{3}H]-palmitic acid (40 mCi/mmole) was mixed with about 100 μCi of [1-^{14}C]palmitic acid (55.5 mCi/mmole) in ethyl ether.

To this solution about 14 mg of Tween-20 was added and substrate solution (5 ml) was prepared as described in a previous section. This solution (1 ml) was incubated with 20 *V. faba* leaf disks for 3.75 hr at 30°, and at the end of the incubation the leaf disks were processed as described in a previous section. The reduction products of 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid were purified by repeated thin-layer chromatography in ethyl ether–hexane–methanol (8:2:1, v/v). The radiochemically pure components were assayed for ^{14}C and ^{3}H as described elsewhere in this paper.

Inhibitor Experiments. In experiment 1, 15 disks (12 mm) from young leaves of *V. faba* were incubated with 5 ml of the inhibitor solution (10^{-2} M) containing 350 μg of Tween-20 in 125-ml erlenmeyer flasks for 1 hr at 30°. The control flasks contained 5 ml of water and 350 μg of Tween. The disks were then washed with water and blotted dry and placed in the flasks with 0.25 ml of [1-^{14}C]palmitic acid (10×10^6 cpm) and 0.5 ml of 10^{-2} M inhibitor solution and incubated for 3 hr at 30°. In expt 2 a similar procedure was used with the following exceptions. The inhibitor in the 1-hr preincubation was 5×10^{-3} M phenanthroline and during the 3-hr incubation with [1-^{14}C]palmitic acid the medium consisted of either 0.75 ml of water or 0.75 ml of 7.5×10^{-3} M $FeSO_4$, but no inhibitor. At the end of the incubation the leaf disks were washed twice with about 150 ml of water, soluble lipids were extracted from the homogenized disks and the insoluble materials were hydrogenolyzed and the ether-soluble material was analyzed by thin-layer chromatography as described below.

Chromatography. Thin-layer chromatography was done with activated 0.5- or 1-mm layers of silica gel G in lined tanks. The noncuticular lipids were analyzed with hexane–ethyl ether–formic acid (40:10:1, v/v) as the solvent system. The hydrogenolysis products of cutin were analyzed with ethyl ether–hexane–methanol (8:2:1, v/v) as the solvent system while hydrolysis products of cutin were fractionated with ethyl ether–hexane–methanol–formic acid (40:10:1:2, v/v). The chromic acid oxidation products of di- and trihydroxyalkanes were separated with hexane–ethyl ether–formic acid (65:35:2) as the solvent system.

Radio gas–liquid chromatography of the diols and triols isolated from the cutin hydrogenolysate and the dimethyl esters of the dioic acids derived from them by chromic acid oxidation was done on 3% OV-1 on 80- to 100-mesh Gas Chrom Q in a coiled stainless steel column (117.6 × 0.6 cm o.d.) with a Perkin-Elmer 800 gas chromatograph attached to a Barber Colman radioactivity monitor as described before (Kolattukudy, 1966). For structure determination of cutin components a combination of a Varian aerograph Model 328 gas chromatograph with a Perkin-Elmer Hitachi mass spectrometer RMU6D was used with a Bieman separator interphase. A coiled glass column (147.0 × 0.31 cm o.d.) packed with 3% OV-1 on Gas Chrom Q was used.

Determination of Radioactivity. Radioactivity in lipid samples and thin-layer chromatograms was determined as described before (Kolattukudy, 1965) except that a Packard liquid scintillation spectrometer was used. Thin-layer plates were also monitored with a Berthold thin-layer scanner. In double-labeling experiments the compounds were first isolated from silica gel and then isotopic ratios were determined. Internal standards were routinely used to determine the counting efficiency which was usually about 70% for ^{14}C. All counting was done with a standard deviation less than 3%.

Preparation of Derivatives. Methyl esters were prepared by refluxing the acid with 14% BF_3 in methanol. Acetates of hydroxyalkanes were prepared by reacting the compounds with 2:1 mixture of acetic anhydride and pyridine at room temperature overnight. Trimethylsilyl ethers of the hydroxyalkanes were prepared by heating these with *N,O*-bis(trimethylsilyl)acetamide (Pierce Chemicals) for 15 min at 80–90°. The reaction mixtures were directly injected into the gas chromatograph. Chromic acid oxidation of hydroxyalkanes was done by heating the sample (1 mg) with Cr_2O_3 (80 mg) in glacial acetic acid (2 ml) at 75° for 90 min. The reaction mixture was diluted with water and products isolated by extraction with ethyl ether. Periodate treatment was performed in pyridine as described by Baumann *et al.* (1969).

Results and Discussion

Structure Determination of Cutin Components of V. faba Leaves. Cutin, being a protective cover, would be expected to be formed during the expanding stages of growth of plants. In order to maximize the probability of finding readily measurable rates of incorporation of precursors into cutin a fast-growing plant, *V. faba* was selected. Since nothing was known about the chemistry of the cutin of *V. faba* leaves, a new technique developed in this laboratory was applied for structure determination. The cutin preparation obtained from these leaves contained lignin from the vascular tissues because the ammonium oxalate–oxalic acid treatment (Walton and Kolattukudy, 1972b) of large amounts of leaves often led to overdigestion of the leaf tissue. Therefore, exhaustive hydrogenolysis of powdered *V. faba* cutin gave only 60–70% yield of the reduced monomers of cutin; the unreacted material consisted of the contaminating lignin.

Thin-layer chromatography of the reduced monomers showed one major component, two minor components, and traces of a more polar material which was not further investigated. We have analyzed the three thin-layer chromatographic fractions by a combination of gas–liquid chromatography and mass spectrometry. A direct analysis of the Me_3-Si ethers without prior thin-layer chromatographic separation gave satisfactory results and therefore only the latter results are discussed here.

The gas–liquid chromatogram of the hydrogenolysis products of *V. faba* cutin is shown in Figure 1. The mass spectrum of peak 1 (Figure 2, top) showed a molecular ion at *m/e* 314 and a major ion at *m/e* 299 indicating methyl loss. The ion at *m/e* 103 and the other ions previously observed in the spectra of Me_3Si ethers of fatty alcohols (Capella and Zorzut, 1968; Esselman and Clagget, 1969; McCloskey *et al.*, 1968) were also found. Thus peak 1 was identified to be cetyl alcohol obviously derived from palmitic acid. Similarly peak 2 was identified to be derived from the C_{18}-acid of the cutin. However in this case, the molecular ions (M^+) and the $M^+ - 15$ ions showed that this peak represented a mixture of saturated and unsaturated alcohol. In contrast to this fraction, no indication of an unsaturated C_{16} acid could be found in peak 1. Cleavage of the cutin with $LiAlD_4$ gave the peaks corresponding to 1 and 2 and their mass spectra showed incorporation of 2 deuterium atoms by the upward shift of M^+ and $M^+ - 15$ ions by 2 amu. The ion at *m/e* 103 was shifted to 105, showing that the 2 deuterium atoms were on the terminal carbon carrying the hydroxyl group. Therefore the alcohols must have originated from C_{16} and C_{18} acids of the cutin.

The mass spectrum of peak 3 showed a weak molecular ion at *m/e* 402 and ions at *m/e* 387 ($M^+ - CH_3$) and *m/e* 371

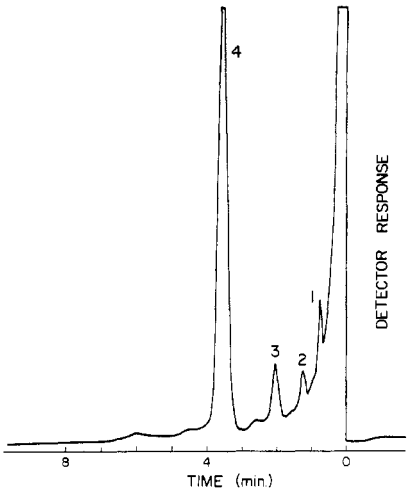

FIGURE 1: Gas–liquid chromatogram of the Me_3Si ethers of the hydrogenolysis products of *V. faba* leaf cutin. Conditions: coiled glass column (147 × 0.30 cm o.d.) packed with 3% OV-1 on 80- to 100-mesh Gas Chrom Q at 235° and 60 cm³/min of carrier gas He.

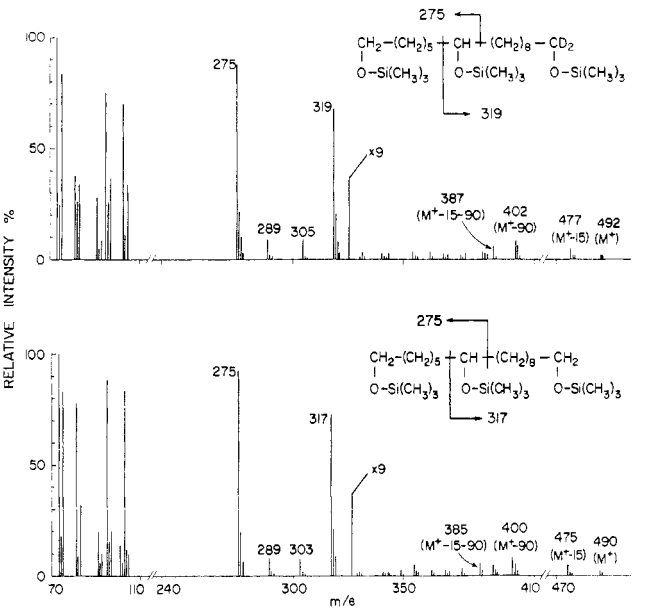

FIGURE 3: Mass spectrum of the component represented by peak 4 shown in Figure 1 (bottom) and that of the deuteriolysis counterpart (top). The spectra were taken at the top of the peaks with 70-eV ionizing voltage.

$(M^+ - CH_3) - CH_4$ (Figure 2, bottom). In this spectrum significant ions were found at *m/e* 312 indicating loss of trimethylsilanol and at *m/e* 297 showing the loss of trimethylsilanol and a methyl. The diagnostic doubly charged ion at *m/e* 186 is characteristic of an α,ω-diol (McCloskey *et al.*, 1968). These ions and the usual low-mass ions showed that peak 3 represented hexadecane-1,16-diol.

The mass spectrum of the corresponding peak from $LiAlD_4$ cleavage of the polymer showed incorporation of two deuterium atoms by the upward shift of M^+, $M^+ - 15$, and $M^+ - 31$ ions by 2 amu. The doubly charged ion was shifted to *m/e* 187 as expected. The two deuterium atoms were shown to be on one of the terminal carbons by the appearance of an ion at *m/e* 105 comparable to the ion at 103 in intensity. Thus the diol originated from 16-hydroxypalmitic acid of cutin. Minor ions in the spectrum showed incorporation of four deuterium atoms indicating the presence of hexadecanedioic acid in the cutin only in trace quantities.

The mass spectrum of the major component (peak 4) of *V. faba* cutin is shown in Figure 3. The molecular ion at *m/e* 490 was relatively weak and the other significant ions at the high mass range were at *m/e* 475 $(M^+ - CH_3)$, 400 $(M^+ - (CH_3)_3SiOH)$, and 385 $(M^+ - CH_3 - (CH_3)_3SiOH)$. Intense ions at *m/e* 275 and 317 represent α cleavage at either side of the Me_3Si ether function in the chain. The corresponding fragments carrying one silyl ether function (at *m/e* 173 and 215) were also observed in the spectrum but they were weak as expected. These α-cleavage ions showed that the in-chain silyl ether function was at C-7. However, a positional isomer with the ether function at C-8 was clearly shown by a set of α-cleavage ions at *m/e* 289 and 303. Thus peak 4 represents mainly hexadecane-1,7,16-triol and a small amount of its positional isomer hexadecane-1,8,16-triol.

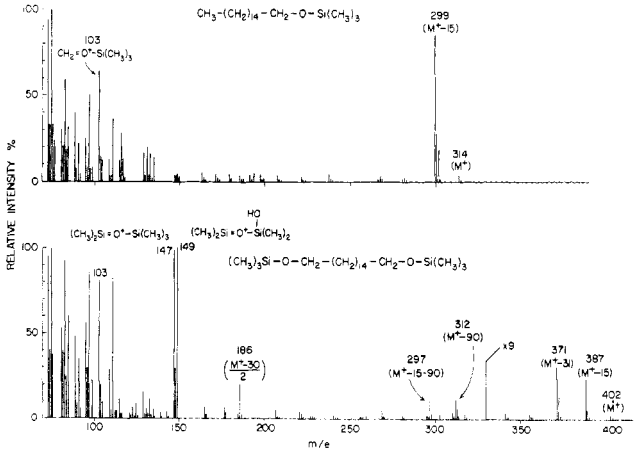

FIGURE 2: Mass spectra of the components represented by peak 1 (top) and peak 3 (bottom) shown in Figure 1. The spectra were taken at the top of the peaks with 70-eV ionizing voltage.

The triols identified in peak 4 obviously originated from a corresponding cutin acid but from the above structure determination it was not possible to determine which end of the triol was derived from the carboxylic ester function in cutin. This problem could be solved with the use of $LiAlD_4$ in place of $LiAlH_4$. On deuteriolysis of cutin the mass spectrum of peak 4 showed incorporation of two deuterium atoms by the upward shift of ions at *m/e* 490, 475, 400, and 385 by 2 amu. Both the deuterium atoms were at one terminal carbon as shown by the ion at *m/e* 105. Of the major α-cleavage ions the one at *m/e* 317 shifted to *m/e* 319 while the other remained unchanged, showing the location of the two deuterium atoms. Similarly the shift of one of the α-cleavage ions of the positional isomer from *m/e* 303 to 305 shows the position of the two deuterium atoms which obviously locates the carboxyl function in the cutin acid. These results clearly show that peak 4 corresponds mainly to 10,16-dihydroxypalmitic acid of *V. faba* cutin together with small amounts of a positional isomer 9,16-dihydroxypalmitic acid.

The gas chromatographic and mass spectrometric analysis discussed above showed that *V. faba* leaf cutin consisted of 10,16-dihydroxypalmitic acid (77.8%), 9,16-dihydroxypalmitic acid (7.1%), 16-hydroxypalmitic acid (7.1%), palmitic acid (3.6%), stearic acid (2.2%), and oleic acid (0.8%).

Incorporation of Labeled Fatty Acids into V. faba Cutin.

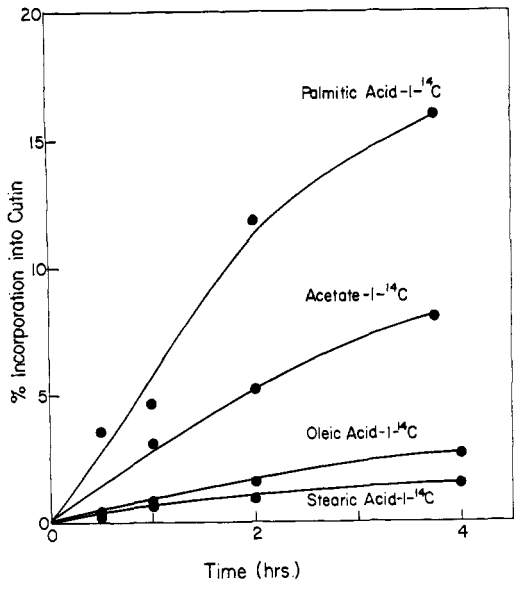

FIGURE 4: Incorporation of [1-^{14}C]acetate, [1-^{14}C]palmitic acid, [1-^{14}C]oleic acid, and [1-^{14}C]stearic acid into cutin in *V. faba* leaf disks. In each expt 20 disks were incubated with 5 μCi of the carboxyl-labeled substrate, and the incorporation into cutin is expressed on the basis of substrate taken up as described under Materials and Methods. In the case of acetate, per cent incorporation is based on the amount of ^{14}C in total lipids.

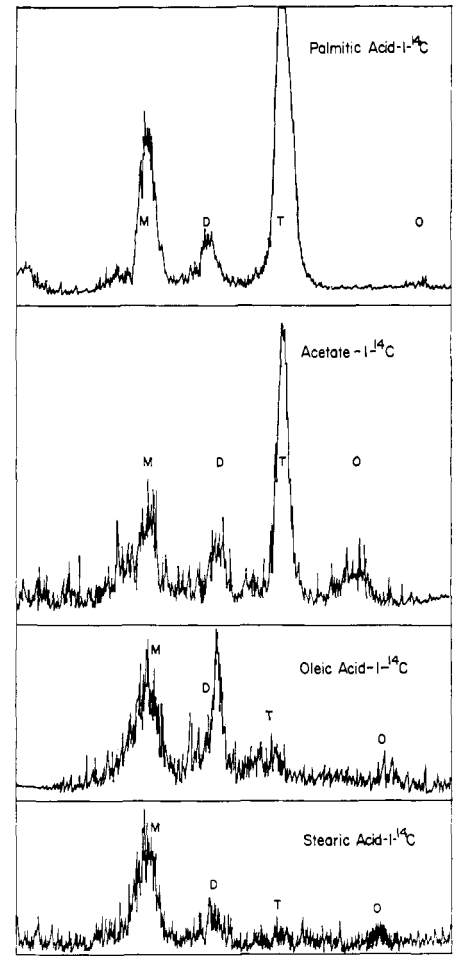

FIGURE 5: Radio thin-layer chromatograms of the hydrogenolysis products of *V. faba* leaf cutin derived from the labeled substrates shown in the figure. Thin-layer chromatography on silica gel G layers with ethyl ether–hexane–methanol (8:2:1, v/v) as the solvent system. The plates were monitored with a scanner. O, origin; T, hexadecanetriol; D, alkanediol; M, alkanol. Identification was done by mixing the labeled products with unlabeled hydrogenolysis products before chromatography and visualizing the components with 2′,7′-dichlorofluorescein.

Incubation of leaf disks with radioactive fatty acids followed by homogenization and thorough extraction of soluble lipids gave an insoluble radioactive residue, which most probably contained cutin, proteins, carbohydrate polymers, and lignin. Exhaustive hydrogenolysis of this residue with $LiAlH_4$ in tetrahydrofuran gave ether-soluble products which contained all the radioactivity of the residue; the water-soluble products were not radioactive. Therefore the radioactivity incorporated into this ether-soluble material may be taken as incorporation into cutin provided that soluble lipids are completely removed prior to hydrogenolysis. When [1-^{14}C]acetate was the substrate rather than a long-chain fatty acid, the water-soluble products obtained by hydrogenolysis contained substantial amounts of radioactivity most probably derived from the noncutin components of the insoluble residue. These water-soluble materials were not further investigated and ether-soluble radioactivity was taken as the incorporation into cutin.

Relative rates of incorporation of [1-^{14}C]acetate, [1-^{14}C]-palmitic acid, [1-^{14}C]oleic acid, and [1-^{14}C]stearic acid into *V. faba* cutin are shown in Figure 4. Palmitic acid was incorporated into cutin most rapidly (15–18%) while oleic (2.7%) and stearic (1.5%) were poorly incorporated. Usually 25–35% of the labeled acetate was converted into lipids in 4 hr, and 6–7% of the radioactivity in the lipid was found in cutin. Each substrate was also incubated with boiled leaf disks and no incorporation was found under these conditions eliminating the possibility of autooxidative formation of insoluble materials. The determination of the composition of *V. faba* cutin discussed above showed that C_{16} acids make up the major components of cutin in this plant and therefore the incorporation data are consistent with the structural information. The incorporation of oleic acid observed is consistent with the identification of this acid in *V. faba* cutin (peak 2, Figure 1). Thus the incorporation of the radioactive fatty acids into the insoluble material is a reliable indication of the synthesis of the natural polymer. In support of this conclusion is the observation that most rapidly expanding leaves incorporated the labeled fatty acids most rapidly (Kolattukudy, 1970c). Such a relationship would be expected if fatty acid incorporation does indeed represent *de novo* synthesis of cutin.

Since hydroxy fatty acids constitute the bulk of the cutin, the labeled fatty acids incorporated into this polymer should be found in hydroxy acids. In order to test this possibility the ether-soluble material containing the polyhydric alcohols, obtained by hydrogenolysis of the radioactive insoluble materials derived from the various labeled substrates, were analyzed by thin-layer chromatography (Figure 5).

[1-^{14}C]Acetate and [1-^{14}C]palmitic acid labeled polar compounds which had R_F values identical to those of hexadecane-1,16-diol and hexadecane-1,7,16-triol. However, the bulk of the radioactivity of the cutin hydrogenolysate derived from [1-^{14}C]oleic acid and [1-^{14}C]stearic acid was found in the fatty alcohol fraction, while a small amount of ^{14}C was found in the alkane-α,ω-diol fraction. These results show that stearic acid and oleic acid are incorporated as such into cutin with very little hydroxylation. The core of the

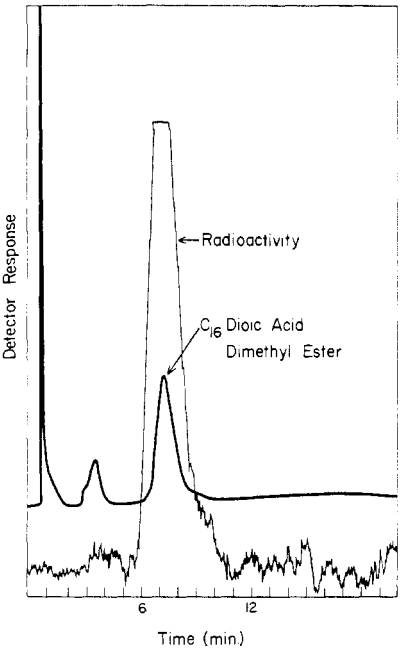

FIGURE 6: Radio gas–liquid chromatogram of the dimethyl esters prepared from the chromic acid oxidation product of component D (Figure 5) obtained from *V. faba* leaves which metabolized [1-^{14}C]palmitic acid. Condition: coiled stainless steel column (117 × 0.61 cm o.d.) packed with 3% OV-1 on 80- to 100-mesh Gas Chrom Q at 190° with 90 cm^3/min of Argon.

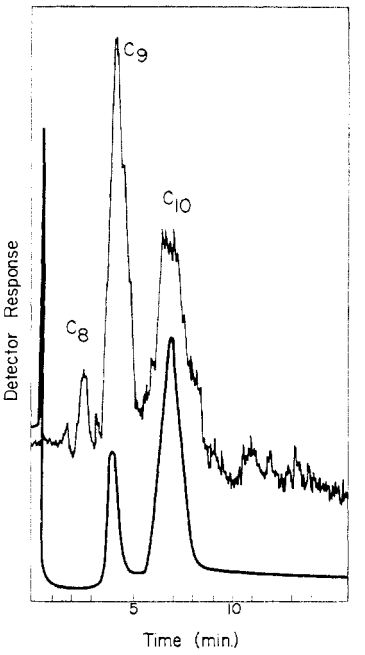

FIGURE 7: Radio gas–liquid chromatogram of the dimethyl esters prepared from the chromic acid oxidation products of component T (Figure 5) obtained from *V. faba* leaves which metabolized [1-^{14}C]palmitic acid. Conditions of chromatography: same as that in Figure 6 except that the temperature of the column was 140°.

polymer in *V. faba* leaves is made of dihydroxy C_{16}-acids and since oleic acid and stearic acid are excluded from the chain hydroxylation reaction, these acids form only minor components of the cutin. The fact that a small part of the radioactivity was found in the ω-hydroxy acid fraction indicates that ω hydroxylation is less specific than the hydroxylation in the middle of the chain.

Identification of the Labeled Polar Cutin Components Derived from [1-^{14}C]Palmitic Acid. Thin-layer chromatography showed that the two polar compounds derived from [1-^{14}C] palmitic acid (D and T, Figure 5), had R_F values identical with those of hexadecane-1,16-diol and hexadecane-1,7,16-triol, respectivelly. Acetylated derivatives of these polar compounds showed R_F values identical with those of authentic hexadecane-1,16-diol diacetate and hexadecane-1,7,16-triol triacetate. Periodate treatment did not affect either of the polar components showing that they did not contain any vicinal diol functions. Radio gas–liquid chromatography of the labeled polar compounds D and T (Figure 5) as their trimethylsilyl ethers showed that the radioactivity coincided with the mass of the trimethylsilyl ethers of hexadecane-1,16-diol and hexadecane-1,7,16-triol, respectively. Thus the component containing the major part of the radioactivity was tentatively identified as hexadecane-1,7,16-triol and the other component as hexadecane-1,16-diol.

In order to confirm these identifications the dioic acids obtained from chromic acid oxidation were analyzed as their dimethyl esters by thin-layer chromatography and radio gas–liquid chromatography. The hexadecane-1,16-diol fraction gave a labeled product which had an R_F identical with that of dimethyl hexadecanedioate and radio gas–liquid chromatography showed that the radioactivity coincided with the mass of the authentic dimethyl ester (Figure 6) proving that the labeled compound was hexadecane-1,16-diol. Chromic acid oxidation of the triol fraction gave labeled dioic acids which when subjected to radio gas–liquid chromatography showed two principal radioactivity peaks which coincided with the mass of dimethyl nonanedioate and dimethyl decanedioate (Figure 7) proving that the radioactive material in the triol fraction was hexadecane-1,7,16-triol.

The small amount of ^{14}C found in octanedioic acid dimethyl ester indicates the formation of a small amount of 9,16-dihydroxypalmitic acid from the labeled palmitic acid fed to *V. faba* leaves. (The expected chromic acid oxidation products of this dihydroxy acid are nonanedioic acid and octanedioic acid.) The occurrence of a small amount of this positional isomer had already been shown by mass spectrometry discussed in a previous section. These results clearly show that palmitic acid was incorporated into 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid in *V. faba* leaves. Since our structural studies showed that these are the major components of the cutin of *V. faba* leaves, these incorporation studies are indeed dealing with the *de novo* synthesis of the biopolymer cutin.

Time-Course of Incorporation of [1-^{14}C]Palmitic Acid into the Cutin Components in V. faba. All the cutin hydroxy acids of *V. faba* contained a hydroxyl group at the ω-carbon atom and therefore it appeared likely that this is the site of the first hydroxylation. The ω-hydroxypalmitic acid would then be hydroxylated at C-10 to give the major component of cutin in this plant. In order to test such a precursor–product relationship, the time-course of incorporation of [1-^{14}C] palmitic acid into the 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid in *V. faba* leaf was studied. The distribution of radioactivity among the three cutin components (palmitic acid, 16-hydroxypalmitic acid, and 10,16-dihydroxypalmitic acid) at various time intervals (Figure 8) showed that at all times the dihydroxy acid contained the major part of the radioactivity while the less hydroxylated acids contained much less label. These results do not show the

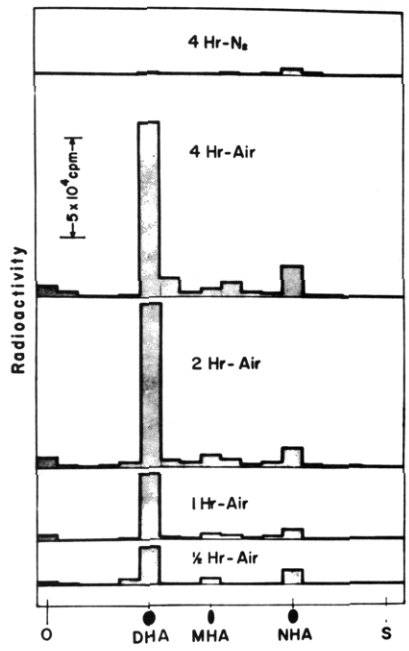

FIGURE 8: Radio thin-layer chromatograms of the hydrogenolysis products of cutin from *V. faba* leaf disks which metabolized palmitic acid for varying periods of time. Twenty disks were incubated with 5 μCi of [1-^{14}C]palmitic acid, and the cutin hydrogenolysates prepared as described under Materials and Methods were thin-layer chromatographed on silica gel G with ethyl ether–hexane–methanol (8:2:1, v/v) as the solvent system. Silica gel from the regions shown were scraped and ^{14}C determined as described before (Kolattukudy, 1965).

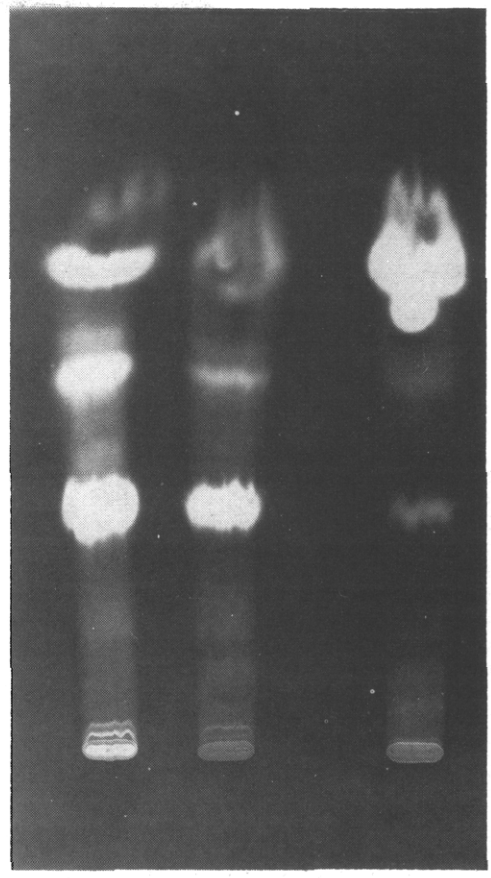

FIGURE 9: Radioautogram of the thin-layer chromatogram of the hydrogenolysis product cutin (far left) and soluble lipid (far right) isolated from *V. faba* leaf disks which metabolized [1-^{14}C]palmitic acid. Cutin hydrogenolysate from disks incubated with sodium [1-^{14}C]acetate is shown in center. The major radioactive components were identified as alkanols (top spot), α,ω-alkanediols (middle spot), and 1,7,16-hexadecanetriol (bottom spot) in increasing order of polarity (origin is visible in all cases). Thin-layer chromatography on silica gel G with ethyl ether–hexane–methanol (8:2:1, v/v) and exposure of X-ray film for 30 days.

expected precursor–product relationship. The times chosen included many points during which incorporation of ^{14}C into cutin was proportional to the time of incubation. Therefore the observation that the dihydroxy acids contained the highest amount of radioactivity is not because of any inappropriate choice of time intervals. However, since only the hydroxy acids already incorporated into the polymer are shown in Figure 8 these data indicate only that the polymer-bound palmitic acid is not subsequently hydroxylated. Apparently the hydroxylation reactions occur on palmitic acid or a soluble derivative of it prior to polymerization. If so the monohydroxy acid and dihydroxy acid in the soluble lipids may show precursor–product relationships. Unfortunately sufficient amounts of the hydroxylated acids did not accumulate in the soluble lipids to make the desired measurements. In order to determine whether the hydroxylated acids can be detected in the soluble lipids, autoradiograms of thin-layer chromatograms of $LiAlH_4$-treated soluble lipids were prepared by a 30-day exposure (Figure 9). By this technique radioactive components with R_F values identical with those of hexadecane-1,16-diol and hexadecane-1,7,16-triol were detected. Here again the dihydroxy acid derivative contained much more radioactivity than the ω-hydroxy acid derivative. The nonhydroxy acids cannot be compared to the others because radioactivity in this fraction came from all the soluble lipids including phospholipids. Thin-layer chromatographic analysis of soluble lipids (data not presented) showed that large amounts of [1-^{14}C]palmitic acid were incorporated into phospholipids. It is therefore clear that only very low levels of 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid were present in leaves which were synthesizing cutin rapidly. These results indicate either that the incorporation of the hydroxylated acids into cutin is so rapid that the monomer concentration in the tissue is extremely low, or that the hydroxylation is on a derivative of the fatty acid (possibly enzyme bound) which merely transfers the hydroxylated acyl moieties on to the growing polymer without involving free hydroxy acids as intermediates. If the latter possibility holds true, the extremely low levels of labeled hydroxy acids detected in the soluble lipids might have been derived from the hydroxylated derivative only as a minor side reaction or by very low levels of cutin hydrolysis. Some recent data we have obtained show that the C-10 hydroxylation involves a CoA ester (Walton and Kolattukudy, 1972a) suggesting that the hypothetical soluble derivative alluded to earlier may be a CoA ester or possibly an acyl carrier protein derivative.

Feeding Experiments with Labeled 16-Hydroxypalmitic Acid and 10,16-Dihydroxypalmitic Acid. In order to determine the precursor–product relationships among the three major cutin components biosynthetically labeled [1-^{14}C]-16-hydroxypalmitic acid was fed to *V. faba* leaf disks. After 4-hr incubation at 30° only 10–20% of the total radioactivity could be removed by washing with water. The remaining 80–90% was either taken into the tissue or adhering to the leaf slices. Of this radioactivity about one-fourth was in cutin. Hydrogenolysis of this labeled cutin followed by thin-

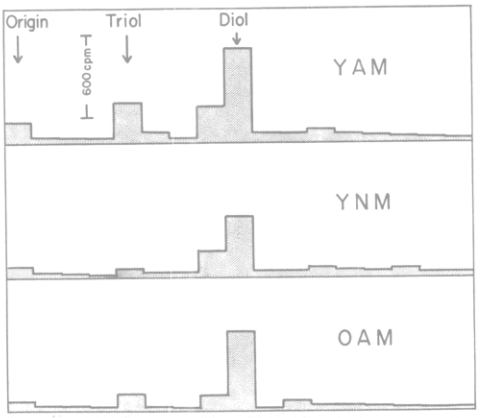

FIGURE 10: Radio thin-layer chromatogram of hydrogenolysis products of cutin of *V. faba* leaf disks which metabolized labeled 16-hydroxypalmitic acid. Twenty-five leaf disks were incubated with 3.12×10^5 cpm of the substrate for 4 hr at 30°. The hydrogenolysates of the insoluble residue was prepared after the residue stopped releasing ^{14}C into chloroform–methanol (2:12, v/v). Thin-layer chromatography procedures as in Figure 8. YAM, young leaves in presence of air; YNM, young leaves under nitrogen; OAM, old leaves (fifth or sixth from the top of plants with six or seven leaves) in the presence of air.

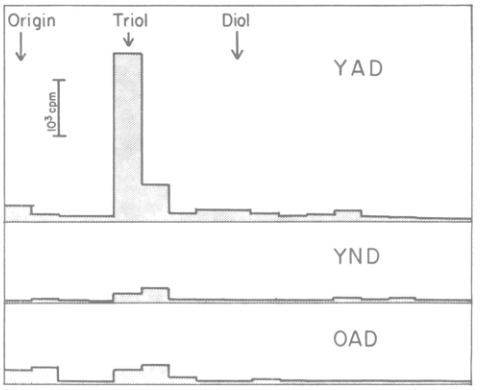

FIGURE 11: Radio thin-layer chromatogram of hydrogenolysis products of cutin of *V. faba* leaf disks which metabolized labeled 10,16-dihydroxypalmitic acid. Experimental procedures are similar to those under Figure 10 except that 1.49×10^6 cpm of the substrate was used. YAD, young leaves in air; YND, young leaves under nitrogen; OAD, old leaves in air.

layer chromatography showed that about 25% of the radioactivity of the cutin was in the 10,16-dihydroxypalmitic acid while essentially all the remaining ^{14}C was in the monohydroxy acid (Figure 10). Similar analysis of the soluble lipids showed that essentially all the radioactivity was in monohydroxypalmitic acid indicating that there was no degradation of the substrate (data not presented). Furthermore, if acetate derived from the degradation of the exogenous acid were converted into the cutin the radioactivity distribution in cutin would have been similar to that in Figure 5; this was not the case. Therefore these results clearly show that exogenous 16-hydroxypalmitic acid is directly converted into 10,16-dihydroxypalmitic acid and incorporated into cutin. Thus the following sequence of reactions gives rise to the major monomers of *V. faba* cutin: palmitic acid → 16-hydroxypalmitic acid → 10,16-dihydroxypalmitic acid. Results in Figure 10 also show that under anaerobic conditions conversion of 16-hydroxypalmitate into dihydroxypalmitate was severely inhibited suggesting that the hydroxylation requires molecular oxygen. It is also clear that exogenous monohydroxy acid can be directly incorporated into the polymer.

In order to determine whether exogenous dihydroxy acid is esterified into cutin, biosynthetically labeled 10,16-dihydroxy acid was fed to *V. faba* leaves. About 35% of the radioactivity could be removed by washing the leaf disks with water after 4-hr incubation with the labeled substrate. Of the radioactivity in the tissue (or adhering to the tissue) about 25–30% was incorporated into cutin. Hydrogenolysis of the labeled cutin and chromatographic analysis of the products showed that virtually all the radioactivity was in hexadecane-1,7,16-triol (Figure 11). Similar analysis of the soluble lipids showed that hexadecane-1,7,16-triol was the only labeled product (data not presented) indicating that the exogenous dihydroxy acid did not undergo much degradation. Therefore these results show that exogenous 10,16-dihydroxypalmitic acid was directly incorporated into cutin in *V. faba* leaf.

Results in Figure 11 also show that under anaerobic conditions incorporation of 10,16-dihydroxypalmitic acid into cutin was inhibited. Uptake of the dihydroxy acid was only slightly inhibited (under 15%) by the anaerobic conditions while incorporation into cutin was severly inhibited (90%). Our present explanation of this observation is that incorporation of the dihydroxy acid into cutin requires the presence of the other cutin components such as ω-hydroxypalmitic acid, the formation of which was severely inhibited by anaerobic conditions. Simultaneous feeding of unlabeled ω-hydroxy acids with the labeled dihydroxy acid would be a method for testing this hypothesis, but such experiments have not yet been done.

It has been shown that the most rapidly expanding leaves of *V. faba* incorporate palmitic acid into cutin components most rapidly (Kolattukudy, 1970c). Fully expanded leaves incorporated very little palmitate into cutin and this small amount only represented incorporation of palmitic acid directly without any hydroxylation. If the incorporation of the labeled 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid into the polymer we observed represent the natural synthesis of cutin, the old fully expanded leaves should not incorporate these hydroxy acids into the polymer. Results shown in Figures 10 and 11 clearly show that incorporation of monohydroxy acid and dihydroxy acid into cutin in the old leaves was less than one-sixth of that in young leaves. Cutin synthesis is thus closely controlled by the physiological state of the plant. This control may either be on the first hydroxlyation step or on the whole complex of hydroxylations and polymerization. The lack of incorporation of exogenous hydroxylated acids into cutin in old leaves may suggest that the polymerization process is also regulated. However, it is possible that the lack of monohydroxy acids in the old leaves would prevent the incorporation of the exogenous dihydroxy acid into cutin.

Studies on the Mechanism of C-10 Hydroxylation. Two hydroxylation steps are involved in the synthesis of the cutin monomers of *V. faba* leaves. The first one is an ω hydroxylation which is a reaction known to occur in animal and microbial systems (Bjorkhem and Danielsson, 1970; Ichihara *et al.*, 1970; Cardini and Jurtshuk, 1970; Heinz *et al.*, 1969; Peterson and Coon, 1970; Lode and Coon, 1971). Although such a reaction has not been demonstrated in higher plants it is probable that a similar reaction gives rise to the ω-hydroxy acids of cutin. The hydroxyl group at C-10 could be introduced *via* a Δ^9 double bond by hydration somewhat similar to the oleic

TABLE I: Incorporation of [1-^{14}C]Palmitic acid, [10-^{14}C]-Palmitoleic acid, and [10-^{14}C]Palmitelaidic Acid into Cutin in *V. faba* Leaves.[a]

Substrate	Radioactivity (10^6 cpm)			
	Administered	Taken Up	Cutin	Cutin % of Uptake
[10-^{14}C]Palmitoleic (cis-Δ^9)	25	18.3	0.84	4.6
[10-^{14}C]Palmitelaidic acid (trans-Δ^9)	4.5	4.2	0.22	5.2
[1-^{14}C]Palmitic acid	8.7	8.4	1.3	15.5

[a] 25 leaf disks (13 mm) were incubated with palmitoleic acid for 6 hr at 30°; 15 disks were incubated for 4 hr with other substrates. The insoluble material obtained after thorough extraction of soluble lipids was hydrogenolized for 48 hr with $LiAlH_4$ in tetrahydrofuran and ether-soluble material obtained is termed cutin. The water-soluble materials did not contain significant amount of radioactivity.

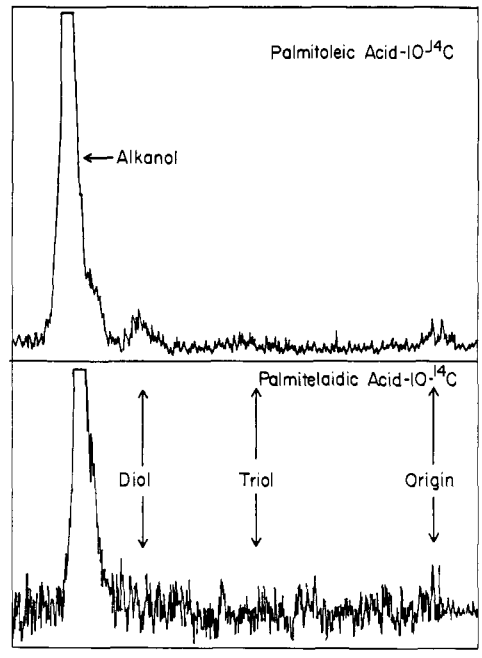

FIGURE 12: Radio thin-layer chromatogram of hydrogenolysis products of cutin of *V. faba* leaf disks which metabolized the substrates shown. Cutin from the experiments shown in Table I was used here. The samples were mixed with unlabeled hydrogenolysis products of cutin before thin-layer chromatography and the components visualized with 2′,7′-dichlorofluorescein are marked in the figure.

acid hydration observed in a pseudomonad (Niehaus *et al.*, 1970; Schroepfer, 1966) or by a direct hydroxylation mechanism. In order to distinguish between these two possibilities [10-^{14}C]palmitoleic acid (cis-Δ^9) was fed to *V. faba* leaf disks. Although this acid was readily taken up and metabolized (about 50% of the ^{14}C fed was incorporated into phospholipids) the amount of radioactivity incorporated into cutin was much less than that from [1-^{14}C]palmitic acid (Table I). Hydrogenolysis followed by thin-layer chromatography showed that the small amount of ^{14}C that was in the cutin was mainly in the non-hydroxylated acids while the ω-hydroxy acid fraction contained a small fraction of this label (Figure 12). No radioactivity could be detected in 10,16-dihydroxypalmitic acid showing that a cis-Δ^9-unsaturated intermediate was not involved in C-10 hydroxylation. Similar experiments with [10-^{14}C]palmitelaidic acid (trans-Δ^9) showed that this acid was also poorly incorporated into cutin, although it was taken up and metabolized (Table I). Less than 25% of the ^{14}C fed remained in the free fatty acids, the rest being in metabolic products, mainly phospholipids. Here the small amount of ^{14}C which was incorporated into cutin was found to be exclusively in non-hydroxy acids (Figure 12). These results ruled out the involvement of a trans-Δ^9-unsaturated intermediate in the introduction of the hydroxy group at C-10. The observation that a small amount of palmitoleic acid was converted into the ω-hydroxy acid fraction together with the fact that stearic acid and oleic acid were also converted into ω-hydroxy acids indicate that the ω-hydroxylase is not as specific as the chain hydroxylating enzyme. However, palmitelaidic acid was not converted into ω-hydroxy acid (Figure 12) indicating that *trans*-Δ^9- unlike *cis*-Δ^9-hexadecenoic acid is not a suitable substrate for the ω-hydroxylase.

The conclusions drawn from the above experiments would be invalid if the exogenous unsaturated acids are excluded from the ω-hydroxylation step and if the C-10 hydroxylation requires 16-hydroxy acids as substrates. Since the *cis*-Δ^9-acid was incorporated at least poorly into the ω-hydroxy acid of cutin, this alternative explanation does not appear to hold for this acid. Nevertheless, another experimental approach which does not involve such an alternate possibility was used to further test whether a double bond is involved in C-10 hydroxylation. If [9,10-^{3}H,1-^{14}C]palmitic acid is converted into 10,16-dihydroxypalmitic acid by a mechanism involving a Δ^9 double bond, ^{3}H from two of the 4 positions at C-9 and C-10 would be lost, giving rise to a product with

TABLE II: Isotopic Ratios (^{3}H:^{14}C) of Cutin Components of *V. faba* Which Metabolized [1-^{14}C-9,10-^{3}H]Palmitic Acid.[a]

Component	Isotopic Ratio ^{3}H:^{14}C
Substrate	2.87
Internal fatty acid	2.70
16-Hydroxypalmitic acid	2.70
	2.52
10,16-Dihydroxypalmitic acid	2.19
	2.29
10,16-Dihydroxy acid expected from direct hydroxylation	2.15
10,16-Dihydroxy acid expected from double-bond hydration	1.44

[a] Twenty leaf disks were incubated with 1.90 μmoles (about 20 μCi) of the doubly labeled fatty acid in 1 ml of water containing 2–3 mg of Tween-20 for 3.75 hr at 30°. The cutin was hydrogenolyzed for 48 hr and components isolated by repetitive thin-layer chromatography. Internal fatty acid was isolated after hydrolysis of the internal lipids.

TABLE III: Inhibition of Incorporation of [1-^{14}C]Palmitic Acid into Cutin Components by Phenanthroline and Bipyridyl and Reversal by Fe^{2+} in *V. faba* Leaves.[a]

	Radioactivity (10^6 cpm)									
			Cutin							
	Taken Up	Phospho-lipids	Total	% Inhib	Non-	% Inhib	Mono-	% Inhib	Di-	% Inhib
Experiment 1										
Control	8.0	1.9	1.65		0.33		0.22		1.1	
Bipyridyl (10^{-2} M)	8.1	2.2	0.54	67	0.21	36	0.12	45	0.21	81
Phenanthroline (10^{-2} M)	7.8	1.3	0.43	74	0.18	45	0.06	73	0.19	83
Experiment 2										
Control	7.6	1.7	1.34		0.18		0.14		1.02	
Phenanthroline (5×10^{-3} M)	7.4	0.9	0.38	72	0.11	39	0.06	57	0.21	80
Phenanthroline (5×10^{-3} M) plus Fe^{2+} (7.5×10^{-3} M)	7.4	0.8	0.64	52	0.11	39	0.05	64	0.48	53

[a] In expt 1, 15 leaf disks were preincubated in 5 ml of inhibitor solution for 1 hr at 30° prior to the incubation with 0.25 ml of [1-^{14}C]palmitic acid (10^7 cpm) and 0.5 ml of 10^{-2} M inhibitor solution for 3 hr at 30°. Control disks were preincubated in water. In expt 2 preincubation was done for 1 hr in 5×10^{-3} M phenanthroline and then the disks were incubated with the radioactive substrate in water or water containing 7.5×10^{-3} M $FeSO_4$ solution for 3 hr. Incorporation into phospholipids was determined by thin-layer chromatography of soluble lipids. Other experimental details are under Materials and Methods.

a ^{3}H:^{14}C isotopic ratio of one-half of that in the substrate. If, on the other hand, a direct hydroxylation at C-10 gives rise to the dihydroxy acid the ^{3}H:^{14}C isotopic ratio of the product would be expected to be 75% of that in the substrate. Experimental results shown in Table II show that the ^{3}H:^{14}C ratio of the 10,16-dihydroxypalmitic acid derived from *V. faba* leaf disks which metabolized [9,10-^{3}H,1-^{14}C]palmitic acid for 3.75 hr was 78% of that in the substrate fed to the disks. This value, within experimental error, is what is expected from a direct hydroxylation mechanism. It appears that significant isotope effects did not influence the observed ^{3}H:^{14}C ratio. Even if the hydroxylation or desaturation process discriminated against tritated molecules such an isotope effect would only lower the ^{3}H:^{14}C ratio. The fact that ω-hydroxypalmitic acid had an isotopic ratio very close to that of the substrate fed and the acid isolated from internal lipids indicates that the ^{3}H loss observed in the formation of the dihydroxy acid is due to the hydroxylation of C-10 rather than by degradation of the substrate followed by resynthesis. The conclusion that a direct hydroxylation is the mechanism for the introduction of the hydroxy group at C-10 is in agreement with our experimental results with labeled palmitoleic acid and palmitelaidic acid.

Such a direct hydroxylation would be expected to be catalyzed by a mixed-function oxidase-type enzyme. Some mixed function oxidases are known to require Fe^{2+}, and therefore are inhibted by chelating agents such as phenanthroline and this inhibition is usually reversed by Fe^{2+} (Kusunose *et al.*, 1964; Kivirikko and Prockop, 1967; Rhoads and Udenfriend, 1970; Galliard and Stumpf, 1966; Hayaishi, 1969). In order to test the possibility that the hydroxylations we propose may involve such enzymes, the effect of several chelating agents on palmitic acid incorporation into cutin acids was studied (Table III). Bipyridyl as well as phenanthroline inhibited the incorporation of [1-^{14}C]palmitic acid into cutin; whereas bipyridyl at this concentration had very little effect on conversion of palmitic acid into phospholipids, phenanthroline did inhibit phospholipid formation. Uptake of the substrate was not significantly affected by these inhibitors. If the primary effect of these chelators is on the hydroxylation reactions, the extent of inhibition may reflect the number of hydroxylation steps involved. In fact the dihydroxypalmitic acid formation was inhibited most severely while the incorporation of [1-^{14}C]palmitic acid into non-hydroxy acid of cutin was inhibited the least. The inhibition of the latter might have been due to the general inhibition of the polymer formation brought about by the inhibition of hydroxy acid synthesis.

If the inhibitory effect of the chelators was due to the removal of Fe^{2+} involved in the hydroxylase reaction, reversal of this inhibition might be expected with the addition of Fe^{2+}. With the rather high concentration of the chelators used in expt 1, addition of 1.5×10^{-2} M Fe^{2+} always gave partial reversal of inhibition of the dihydroxy acid formation. Usually, in the presence of added Fe^{2+}, the amount of label in the dihydroxy acid was 1.5–2.5 times that in the corresponding fraction obtained with phenanthroline. However, quantitatively the extent of reversal was only a few per cent of the incorporation observed in the control. In order to determine whether more substantial reversal can be obtained, leaf disks were preincubated in 5×10^{-3} M phenanthroline for 1 hr and then the disk were washed and transferred to substrate solution containing 7.5×10^{-3} M Fe^{2+}. Under these conditions a substantial reversal of inhibition could be observed (Table III). For example, with phenanthroline the incorporation of palmitic acid into dihydroxypalmitic acid was only 20% of that in the control, whereas with Fe^{2+} and phenanthroline incorporation was 47% of that in the control. Reversal of inhibition of palmitic acid incorporation into the ω-hydroxy acid fraction was so variable that conclusive results could not be obtained. In any case, the results in Table III indicate that mixed-function oxidase-type enzymes, possibly involving metal ions such as Fe^{2+} are involved in the synthesis of the hydroxy acid monomers of

the cutin in *V. faba* leaves. This conclusion fully supports the data discussed in the earlier sections of this paper.

The results presented in this paper show that ω-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid, the major components of *V. faba* cutin, are derived from palmitic acid while oleic acid and stearic acid give rise to only small portions of the non-hydroxylated and ω-hydroxy acid fractions of this cutin. In our experience, rather fast growing plants appear to have similar patterns of cutin monomer distribution, whereas cutin from a slow growing plants such as apple, pear, and peach contain large quantities of polyhydroxy C_{18} acids (Walton and Kolattukudy, 1972b; Kolattukudy *et al.*, 1971). In the fast-growing plants the usual product of fatty acid synthetase is hydroxylated at the ω-position and then at the C-10 position, apparently by mixed-function oxidase-type enzymes (Figure 13). Small amounts of C_{18} chains are hydroxylated at the ω-position but not at the internal carbons of the chain. All these acids are then rapidly transferred to the growing polymer. Our results clearly show that specific hydroxylase enzymes rather than autooxidative-type reactions are involved in the formation of the cutin monomers in *V. faba*, thus disproving the suggestion that cutin is formed by autooxidative reactions on leaf lipids. These reactions appear to occur in the epidermal layer of cells because excised epidermis of *V. faba* incorporated exogenous palmitic acid into ω-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid of cutin (P. E. Kolattukudy, unpublished results). It is not known whether the fatty acid synthetase located in the internal part of the leaf provides fatty acids for cutin formation. However, excised epidermis from leaves of *Pisum sativum* incorporated [1-^{14}C]acetate into ω-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid showing that epidermis does in fact contain the enzymatic machinery for the synthesis of cutin from acetate (J. S. Buckner and P. E. Kolattukudy, unpublished results).

Attempts to further elucidate the enzymatic reactions involved in the biosynthesis of the cutin hydroxy acids as well as the polymerization process, are in progress in this laboratory.

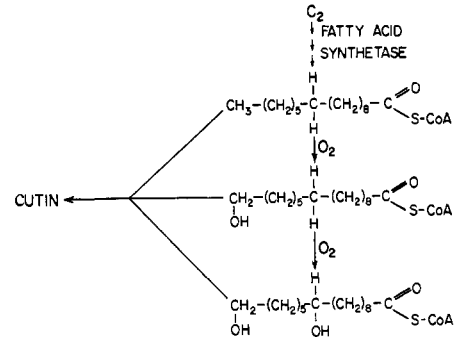

FIGURE 13: Proposed pathway for the biosynthesis of cutin hydroxy acids in *V. faba* leaves.

Acknowledgments

We thank Mrs. Susan Grant, Mrs. Linda Brown, and Mr. Lawrence Ashly for technical assistance, Mr. Charles Oldenberg for raising the plants, and Dr. Milton Zucker for a critical reading of this manuscript.

References

Baker, E. A., and Holloway, P. J. (1970), *Phytochemistry 9*, 1557.

Baumann, W. J., Schmid, H. H. O., and Mangold, H. K. (1969), *J. Lipid Res. 10*, 132.

Bjorkhem, I., and Danielsson, H. (1970), *Eur. J. Biochem. 17*, 450.

Capella, P., and Zorzut, C. M. (1968), *Anal. Chem. 40*, 1458.

Cardini, G., and Jurtshuk, P. (1970), *J. Biol. Chem. 245*, 2789.

Crisp, C. E. (1965), Ph.D. Thesis, University of California, Davis, Calif.

de Vries, H. A. M. A. (1969), *Acta Bot. Neerl. 18*, 589.

Eglinton, G., and Hamilton, R. J. (1967), *Science 156*, 1322.

Eglinton, G., and Hunneman, D. H. (1968), *Phytochemistry 7*, 313.

Esselman, W. J., and Clagget, C. O. (1969), *J. Lipid Res. 10*, 234.

Galliard, T., and Stumpf, P. K. (1966), *J. Biol. Chem. 241*, 5806.

Hayaishi, O. (1969), *Annu. Rev. Biochem. 38*, 21.

Heinen, W., and Brand, I. V. D. (1963), *Z. Naturforsch. B 18*, 67.

Heinz, E., Tulloch, A. P., and Spencer, J. F. T. (1969), *J. Biol. Chem. 244*, 882.

Holloway, P. J., and Deas, A. H. B. (1971), *Phytochemistry 10*, 2781.

Huelin, F. E. (1959), *Aust. J. Biol. Sci. 12*, 175.

Ichihara, K., Kusunose, E. and Kusunose, M. (1970), *Biochim. Biophys. Acta 202*, 560.

Kivirikko, K. I., and Prockop, D. J. (1967), *J. Biol. Chem. 242*, 4007.

Kolattukudy, P. E. (1965), *Biochemistry 4*, 1844.

Kolattukudy, P. E. (1966), *Biochemistry 5*, 2265.

Kolattukudy, P. E. (1970a), *Annu. Rev. Plant Physiol. 21*, 163.

Kolattukudy, P. E. (1970b), *Biochem. Biophys. Res. Commun. 41*, 299.

Kolattukudy, P. E. (1970c), *Plant Physiol. 46*, 759.

Kolattukudy, P. E., and Walton, T. J. (1972), *Progr. Chem. Fats Other Lipids* (in press).

Kolattukudy, P. E., Walton, T. J., and Kushwaha, R. P. S. (1971), *Biochem. Biophys. Res. Commun. 42*, 739.

Kusunose, M., Kusunose, E., and Coon, M. J. (1964), *J. Biol. Chem. 239*, 2135.

Litchfield, C., Harlow, R. D., Isbell, A. F., and Reiser, R. (1965), *J. Amer. Oil Chem. Soc. 42*, 73.

Lode, E. T., and Coon, M. J. (1971), *J. Biol. Chem. 246*, 791.

Matic, M. (1956), *Biochem. J. 63*, 168.

Mazliak, P. (1968), *Progr. Phytochem. 1*, 49.

McCloskey, J. A., Stillwell, R. N., and Lawson, A. M. (1968), *Anal. Chem. 40*, 233.

Niehaus, W. G., Jr., Kisic, A., Torkelson, A., Bednarczyk, D. J., and Schroepfer, G. J., Jr. (1970), *J. Biol. Chem. 245*, 3790.

Peterson, J. A., and Coon, M. J. (1970), *J. Biol. Chem. 245*, 3882.

Priestly, J. H. (1943), *Bot. Rev. London 9*, 593.

Rhoads, R. E., and Udenfriend, S. (1970), *Arch. Biochem. Biophys. 139*, 329.

Schroepfer, G. J., Jr. (1966), *J. Biol. Chem. 241*, 5441.

Walton, T. J., and Kolattukudy, P. E. (1972a), *Biochem. Biophys. Res. Commun. 46*, 16.

Walton, T. J., and Kolattukudy, P. E. (1972b), *Biochemistry 11*, 1885.