Plant Physiol. (1974) 54, 670–677

Structure and Biosynthesis of Cuticular Lipids

HYDROXYLATION OF PALMITIC ACID AND DECARBOXYLATION OF C_{28}, C_{30}, AND C_{32} ACIDS IN *VICIA FABA* FLOWERS[1, 2]

Received for publication June 3, 1974 and in revised form June 27, 1974

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ABSTRACT

The structure and composition of the cutin monomers from the flower petals of *Vicia faba* were determined by hydrogenolysis ($LiAlH_4$) or deuterolysis ($LiAlD_4$) followed by thin layer chromatography and combined gas-liquid chromatography and mass spectrometry. The major components were 10,16-dihydroxyhexadecanoic acid (79.8%), 9,16-dihydroxyhexadecanoic acid (4.2%), 16-hydroxyhexadecanoic acid (4.2%), 18-hydroxyoctadecanoic acid (1.6%), and hexadecanoic acid (2.4%). These results show that flower petal cutin is very similar to leaf cutin of *V. faba*. Developing petals readily incorporated exogenous [1-^{14}C]palmitic acid into cutin. Direct conversion of the exogeneous acid into 16-hydroxyhexadecanoic acid, 10,16-dihydroxy-, and 9,16-dihydroxyhexadecanoic acid was demonstrated by radio gas-liquid chromatography of their chemical degradation products. About 1% of the exogenous [1-^{14}C]palmitic acid was incorporated into C_{27}, C_{29}, and C_{31} *n*-alkanes, which were identified by combined gas-liquid chromatography and mass spectrometry as the major components of the hydrocarbons of *V. faba* flowers. The radioactivity distribution among these three alkanes (C_{27}, 15%; C_{29}, 48%; C_{31}, 38%) was similar to the per cent composition of the alkanes (C_{27}, 12%; C_{29}, 43%; C_{31}, 44%). [1-^{14}C]Stearic acid was also incorporated into C_{27}, C_{29}, and C_{31} *n*-alkanes in good yield (3%). Trichloroacetate, which has been postulated to be an inhibitor of fatty acid elongation, inhibited the conversion of [1-^{14}C]-stearic acid to alkanes, and the inhibition was greatest for the longer alkanes. Developing flower petals also incorporated exogenous C_{28}, C_{30}, and C_{32} acids into alkanes in 0.5% to 5% yields. [G-^{3}H]*n*-octacosanoic acid (C_{28}) was incorporated into C_{27}, C_{29}, and C_{31} *n*-alkanes. [G-^{3}H]*n*-triacontanoic acid (C_{30}) was incorporated mainly into C_{29} and C_{31} alkanes, whereas [9, 10, 11-^{3}H]*n*-dotriacontanoic acid (C_{32}) was converted mainly to C_{31} alkane. Trichloroacetate inhibited the conversion of the exogenous acids into alkanes with carbon chains longer than the exogenous acid, and at the same time increased the amount of the direct decarboxylation product formed. These results clearly demonstrate direct decarboxylation as well as elongation and decarboxylation of exogenous fatty acids, and thus constitute the most direct evidence thus far obtained for an elongation-decarboxylation mechanism for the biosynthesis of alkanes.

Plant cuticle consists of a cross-esterified hydroxyfatty acid polymer, cutin, which is embedded in a mixture of relatively nonpolar, very long chain compounds collectively called wax (12, 14). In recent years much progress has been made in the determination of the structure of plant cuticular lipids. However, much of the work has been confined to leaves and fruits from which fairly intact cuticles can be isolated. The nature of the protective polymer on other parts of the plant such as flowers and roots still remains unknown. Even though recent work on the biochemistry of the cuticular lipids has enabled us to propose the most probable pathways involved in their biosynthesis, several key enzymatic steps still remain to be elucidated in cell-free preparations (12). If the flower petal synthesizes the same cuticular lipids as those found on the other parts of the plant, it might offer certain advantages as an experimental material for enzyme level studies. The results presented here constitute the first analysis of a flower cutin and show that the cutin on the *V. faba* flower petals is very similar, if not identical, to that found on the leaves of the same plant.

One of the most common components of the soluble lipids of plant cuticle is alkane. On the basis of structural considerations, a head-to-head condensation pathway had been suggested for the biosynthesis of alkanes (5). However, experimental evidence thus far obtained is not consistent with such a hypothesis (12). On the basis of a variety of experimental results summarized elsewhere (12), it was proposed that alkanes are formed by elongation of a fatty acid followed by decarboxylation. The results presented in this paper show that the major alkanes of *V. faba* flowers are *n*-C_{27}, *n*-C_{29}, and *n*-C_{31}, and that they are synthesized by an elongation-decarboxylation mechanism. The direct decarboxylation of labeled *n*-C_{28}, *n*-C_{30}, and *n*-C_{32} acids to the corresponding alkanes, demonstrated by the data contained in this paper, constitute the most direct evidence presented to date for the elongation-decarboxylation mechanism.

MATERIALS AND METHODS

Plants. Broad bean (*V. faba*) plants were grown from seeds purchased from Burpee Co., Calif., in a soil-sand-peatmoss (1:1:1) mixture under wide spectrum Gro-lux (very high out-

[1] This work was supported in part by National Science Foundation Grant GB-23081 and United States Public Health Service Grant GM-18278. Scientific paper No. 4250, Project 2001, Agricultural Research Center, College of Agriculture, Washington State University, Pullman, Washington 99163.

[2] Dedicated to the memory of our friend Milton Zucker.

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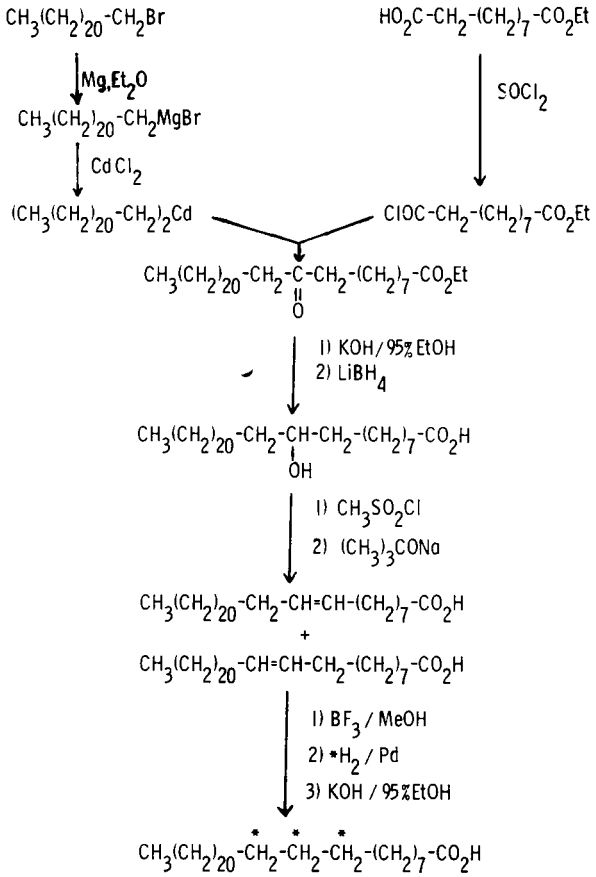
put) lights, with 16-hr days. Only the flowers which were just beginning to open were picked, and the wing petals collected from them were washed and blotted dry.

Isolation of Reduced Monomers from the Cuticular Polymer. Three grams of petals were homogenized in 12 ml of distilled water with a TenBroeck glass homogenizer. The residue was collected by centrifugation at 27,000*g* for 15 min and dispersed in 20 ml of methanol. After 15 min, the residue was recovered by centrifugation and the treatment was repeated once with methanol, three times with a 2:1 mixture of chloroform and methanol and twice with tetrahydrofuran. The final residue (156 mg) was refluxed with $LiAlH_4$ or $LiAlD_4$ (99 atom %D, Merck, Sharp, and Dohme of Canada) in tetrahydrofuran for 24 hr. Excess reagent was carefully decomposed and the chloroform soluble products (56 mg) were recovered after acidification as previously described (18).

Substrates. [1-^{14}C]Palmitic acid (58 Ci/mole) and [1-^{14}C]stearic acid (57 Ci/mole) were obtained from Amersham/Searle Corp., Arlington Heights, Ill. The preparation of [G-^{3}H]*n*-triacontanoic acid (5.86×10^7 cpm/mg) by $^3H_2$ exposure, and purification of this material by TLC have been described previously (10). [G-^{3}H]*n*-Octacosanoic acid (4.62×10^8 cpm/mg) was prepared and purified in a similar manner. [9,10,11-^{3}H]*n*-Dotriacontanoic acid was synthesized by the procedures outlined in Scheme 1. The half-ester of sebacic acid was prepared by standard procedures (3) and was converted to the acid chloride with thionyl chloride. Docosyl cadmium was prepared via the Grignard reagent of docosyl bromide. Coupling of the alkyl cadmium to the acid chloride (16) yielded ethyl-10-ketodotriacontanoate. Hydrolysis of the ester, followed by reduction with $LiBH_4$ afforded 10-hydroxytriacontanoic acid, which was mesylated (2). Elimination of the mesyl group with freshly prepared sodium *t*-butoxide afforded an equal mixture of dotriacont-9-enoic acid and the Δ^{10}-isomer (as shown by conversion of the olefin to the vicinol diol with OsO_4, followed by mass spectrometric analysis of the silylated products (1)). The mixture of unsaturated acids was then methylated and catalytically reduced with $^3H_2$ (New England Nuclear Corp., Boston, Mass.) to yield methyl-[9,10,11-^{3}H]*n*-dotriacontanoate. Rigorous purification of the methyl ester, followed by hydrolysis and repurification of the acid by TLC gave chemically and radiochemically pure [9,10,11-^{3}H]*n*-dotriacontanoic acid (3.75×10^{10} cpm/mg). Substrates were dispersed in water containing Tween 20 by sonication (10).

Incorporation Experiments with Palmitic Acid. A weighed amount of wing petals, excised from the rapidly expanding flower buds, was incubated with 1 ml of a solution containing 172 nmoles (10 μCi) of [1-^{14}C]palmitic acid in a 125-ml Erlenmeyer flask for 4 hr at 30 C. The petals were carefully bathed in the radioactive solution by gentle mixing with a very thin glass rod. At the end of the incubation period, the tissue was homogenized and the residue containing cutin was prepared, hydrogenolyzed, and the products were isolated as described previously (11). This procedure involved removal of soluble lipids by extraction with the solvents indicated above and the soluble lipids were recovered from the pooled extract. These lipids were also treated with $LiAlH_4$ in tetrahydrofuran and the products were isolated in the usual manner.

Incorporation Experiments with C_{18}, C_{28}, C_{30}, and C_{32} Acids. Wing petals, prepared as described above, were incubated with 1 ml of a solution containing 5×10^6 cpm of C_{28}, C_{30}, or C_{32} acid in a 125-ml Erlenmeyer flask for 5 hr at 30 C. For experiments with [1-^{14}C]-stearic acid, identical procedures were employed except that incubation was with 1 ml of a solution containing 2.5 μCi of substrate. In experiments where trichloroacetate was employed, petals were preincubated for 30 min at



Scheme 1. Outline of procedures for the synthesis of [9,10,11-^{3}H]*n*-dotriacontanoic acid. *denotes ^{3}H.

30 C in 0.5 ml of a solution containing the appropriate amount of trichloroacetic acid (neutralized with NaOH). At the end of the pre-incubation, the appropriate amount of substrate was added, and the total liquid volume was brought to 1 ml with water. At the end of the 5-hr incubation period, 50 ml of a mixture of chloroform and methanol (2:1) were added to the flask which was gently shaken for an additional 30 min. The petals were then removed by filtration, and the lipids were extracted from the filtrate after the addition of water and acidification.

Chromatography and Mass Spectrometry. TLC was done on 20×20 cm glass plates coated with 0.5 or 1.0 mm layers of silica gel G activated overnight at 110 C. Procedures used, including detection methods, were essentially the same as before (10, 11, 18). Unsaturated components were fractionated by argentation TLC as described previously (13). Combined gas chromatography-mass spectrometry was done with a Varian gas chromatograph attached to a Perkin Elmer-Hitachi RMU6D mass spectrometer with a Biemann separator interphase. Mass spectra were recorded at the apex of each gas chromatographic peak with an ionizing voltage of 70 ev. A coiled glass column (183×0.31 cm o.d.) packed with 5% OV-101 on 80 to 100 mesh Gas Chrom Q was used. In order to quantitate the saturated and unsaturated ω-hydroxy acids, the diols derived from them were analyzed as their diacetates on a 12% diethylene glycol succinate column. For radio GLC, a Perkin Elmer gas chromatograph attached to a Barber Colman radioactivity monitor was used. The column and experimental conditions used are described under the appropriate figures.

Determination of Radioactivity. Radioactivity in lipid sam-

ples and thin layer chromatograms was determined as described before (11). Internal standards were routinely used to determine the counting efficiency which was usually 70% for ^{14}C and 14% for ^{3}H. All counting was done with a standard deviation of less than 3%. Radioactivity on thin layer chromatograms was routinely monitored with a Berthold thin layer scanner.

Chemical Conversions of Metabolic Products. Acetylation with acetic anhydride and pyridine, CrO_3 oxidation of alkane triol, esterification with 14% BF_3 in methanol, and isolation of products were done as described before (11). Alkane-α, ω-diol was oxidized with 25 mg CrO_3 in 95% acetic acid at room temperature for 16 hr and the products were recovered and esterified as before (11). The trimethylsilyl ether derivatives were prepared by heating the material with N,O-bis(trimethylsilyl)acetamide (Pierce Chemicals) for 15 min at 90 to 100 C. The excess reagent was evaporated off with a stream of N_2, and products were dissolved in a 2:1 mixture of chloroform and methanol for gas chromatography.

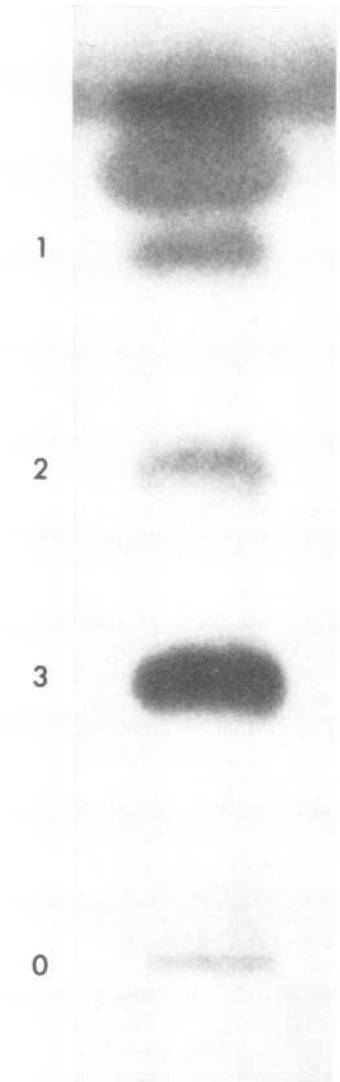


FIG. 1. Thin layer chromatogram of the hydrogenolysate of the insoluble material obtained from the flower petals of *V. faba*. Chromatography was done on Silica Gel G with ethyl ether-hexane-methanol (8:2:1 v/v) as the solvent system. Visualization was done by the sulfuric acid-dichromate charring method. Fatty alcohols (1), alkane-α,ω-diols (2), hexadecane triols (3), and origin (O). The band above fraction 1 did not contain aliphatic components; it may be some phenolic material from the petal residue and it was not investigated further.

RESULTS AND DISCUSSION

In order to determine whether flower petals are coated with a hydroxyfatty acid polymer similar to that on leaves, *V. faba* flowers were chosen for analysis because the composition of the leaf cutin of this plant is known (11). Since the techniques used for isolation of intact cutin layers from leaves could not be readily used on the extremely thin flower petals, a different technique had to be used. Removal of soluble lipids from a petal homogenate resulted in a finely divided residue. The cutin of the petals should be contained in this residue. Therefore, this material was subjected to hydrogenolysis with $LiAlH_4$, a treatment known to reductively depolymerize cutin (18). TLC of the chloroform-soluble materials released by this treatment revealed three components (Fig. 1). This pattern, and the R_F values of the three components, suggested that the flower petals contained cutin which might have a monomer composition similar to that of the leaf cutin.

Fraction 1 had an R_F identical to that of hexadecanol suggesting that this fraction contained fatty alcohols. GLC of this fraction (as trimethylsilyl ethers) revealed three components. The major component showed a retention time identical to that of the trimethylsilyl ether of hexadecanol. Furthermore, its mass spectrum contained a weak parent ion at m/e 314 and an intense ion at m/e 299 (M^+-CH_3) with no other significant ions in the high mass region. In addition, the expected ions characteristic of the trimethylsilyl ethers of primary alcohols were found at m/e 73, 75, 89, and at 103 (17, 18). Thus, the major component of fraction 1 was identified as hexadecanol, obviously derived by the $LiAlH_4$ reduction of hexadecanoic acid of cutin. Similarly, the other two gas chromatographic peaks were identified to be from octadecanol and octadecenol, derived from the corresponding acids. Since a nonpolar liquid phase was used, monounsaturated C_{18} alcohol did not separate from diunsaturated alcohol, but the unsaturated acids constituted only a relatively minor fraction of the cutin acids.

Fraction 2 had an R_F identical to that of hexadecane-1,16-diol suggesting that this fraction constituted alkane-α, ω-diols. GLC of this fraction (as trimethylsilyl ethers) showed one major component and several smaller components. The major component had a retention time identical to that of authentic hexadecane-1,16-diol. The mass spectrum of the major component showed a very weak molecular ion at m/e 402 corresponding to the mol wt of the trimethylsilyl ether of hexadecane diol. Consistent with this structural assignment, significant fragment ions were noted at m/e 387 (M^+-CH_3), 371 (M^+-CH_3-CH_4), 312 M^+-$(CH_3)_3SiOH$, 297 (M^+-CH_3-$(CH_3)_3SiOH$), and at 281 (M^+-CH_3-CH_4-$(CH_3)_3SiOH$). Confirming the proposed structure, a doubly charged ion was found at m/e 186 ((M^+-30)/2)) accompanied by a first isotope ion at m/e 186.5 (15, 18).

Since hexadecane-1,16-diol could have originated either from the corresponding dicarboxylic acid or from the ω-hydroxy acid of cutin by the $LiAlH_4$ treatment, a deuterolysis ($LiAlD_4$) technique was used to distinguish between these two possibilities. The high mass region of the mass spectrum of the trimethylsilyl ether of hexadecanediol obtained by deuterolysis showed that most of the molecules were dideuterated, but a small amount of tetradeuterated species were present. The former would be derived from 16-hydroxyhexadecanoic acid and the latter from hexadecane-1,16-dicarboxylic acid of cutin. From the relative intensities of the doubly charged ions at m/e 187 and 188, it was estimated that only about 10% of this diol originated from the corresponding dicarboxylic acid. Similar examination of the mass spectra of the other gas chromatographic peaks indicated the presence of hexadecene-1,16-diol, heptadecane-1,17-diol, octadecane-1,18-diol, octadecene-1,18-diol, octadecadiene-1,18-diol, and octadecatriene-1,18-diol. Mass spectral analysis of the deuterolysis products showed

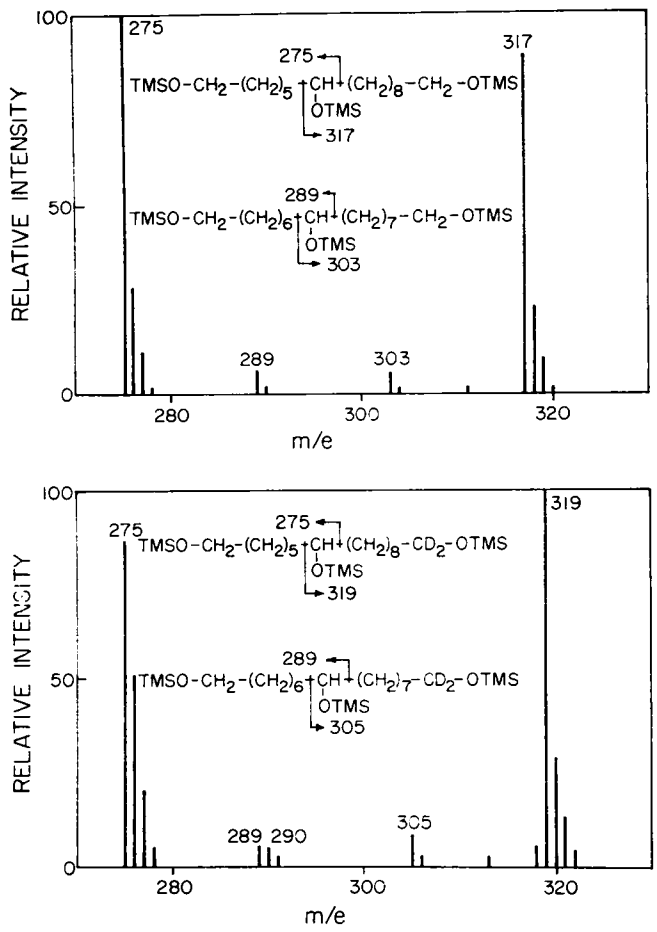


FIG. 2. The α-cleavage region of the mass spectrum of the trimethyl silyl (TMS) derivative of the C_{18} triol derived by hydrogenolysis (top) and deuterolysis (bottom) of the cutin of *V. faba* flower petals. The structures indicated do not include the trideuterated species indicated in the spectrum but they are discussed in the text.

that only minor quantities of these diols were derived from the corresponding dicarboxylic acids, the bulk of each component being derived from the corresponding ω-hydroxy acid of cutin.

Since the mono-, di-, and tri-unsaturated C_{18} diols could not be separated as their trimethylsilyl ethers on the nonpolar (OV-101) column, the diacetates of the diols were subjected to combined GLC-mass spectrometry with a diethylene glycol succinate column. With this technique, the mono-, di-, and tri-unsaturated C_{18} diols were resolved and their mass spectra were consistent with the structures assigned. The above structure assignments were also confirmed by argentation TLC of the diol diacetates followed by combined GLC and mass spectrometry.

Fraction 3, by far the largest fraction, had an R_F identical to that of the C_{16}-triol previously obtained from the cutin of *V. faba* leaves. GLC revealed only one component, with a retention time identical to that of the leaf triol. The high mass region of its mass spectrum was identical to that of the leaf triol. The major ions were at m/e 490 (M^-), 475 (M^+-CH_3), 400 (M^+-$(CH_3)_3SiOH$), and 385 (M^+-CH_3-$(CH_3)_3SiOH$). A fairly intense ion at m/e 103 ($CH_2 = O^+ Si(CH_3)_3$) showed the presence of at least one primary alcohol function. The positions of the other hydroxyl functions were revealed by the α-cleavage pattern (Fig. 2). The two intense ions in this region at m/e 275 and 317 showed that the major component of this triol fraction was hexadecane-1,7,16-triol. The small pair of ions at m/e 289 and 303 indicated that a small amount of hexadecane-1,8,16-triol was also present. Since the hydrogenolysis technique could convert several positional isomers of dihydroxyhexadecanoic acid into indistinguishable C_{16}-triol, a deuterolysis ($LiAlD_4$) technique was also used. The α-cleavage pattern of the trimethylsilyl ether of the hexadecane triol derived from deuterolysis (Fig. 2) showed that the major ions were at m/e 275, 289, 305, and 319. The replacement of the ions at m/e 303 and 317 by the dideuterated species at m/e 305 and 319 showed that these fragments originated from the carboxyl end of the dihydroxy hexadecanoic acid. Thus, the major component was 10,16-dihydroxyhexadecanoic acid while the minor component was 9,16-dihydroxyhexadecanoic acid in the flower cutin. From the relative intensities of the α-cleavage ions, the proportion of the 9-hydroxy isomer was calculated as follows:

$$\% \text{ 9-hydroxy isomer} = \frac{I_{289} + I_{303}}{I_{289} + I_{303} + I_{275} + I_{317}} \quad (1)$$

where I_x represents the intensity of the ion at m/e = x. Thus, only about 5% of the dihydroxy acid was found to be the 9-hydroxy isomer. A close examination of the α-cleavage ion clusters revealed that there was a small amount of monodeuterated fragments with a mass of 276 and 290. These ions indicate the presence of small amounts of 16-oxo-9-hydroxy and 16-oxo-10-hydroxyhexadecanoic acid, two components previously discovered in the cutin of *V. faba* leaves (9). From the relative intensities of the α-cleavage ions, it was estimated that about 20% of the triols originated from the aldehyde components using the formula

$$\% \text{ aldehyde} = \frac{(I_{276} - 0.3\ I_{275}) + (I_{290} - 0.3\ I_{289})}{I_{275} + (I_{276} - 0.3\ I_{275}) + I_{289} + (I_{290} - 0.3\ I_{289})} \quad (2)$$

where I_x = intensity of the ion at m/e = x.

Gas chromatographic analysis of the trimethylsilyl ethers of the reduced monomers on a nonpolar liquid phase, in conjunction with argentation TLC and analysis of diol diacetates on a polar (diethylene glycol succinate) liquid phase, gave the monomer composition summarized in Table I. The monomer composition is very similar to that reported for the cutin of *V. faba* leaves (11). The major components were hexadecanoic acid, 16-hydroxyhexadecanoic acid, 9,16-dihydroxyhexadecanoic acid and 10,16-dihydroxyhexadecanoic acid. Even though small amounts of C_{18} acids were present, significant amounts of in-chain substituted C_{18} acids could not be found, just as in the case of the leaf cutin. The observations that 9,16-dihydroxy acid was only a minor part of the dihydroxy C_{16} acid fraction, and that a substantial part of the 9-hydroxy acid contained a 16-oxo function, also illustrate the extremely close similarity between the leaf cutin and the flower cutin. This report appears to be the first chemical examination of a flower cutin.

Table I. *Monomer Composition of Cutin of Flower Petals of V. faba*

Monomers	%
Hexadecanoic acid	2.4
Octadecenoic acid	0.98
Octadecanoic acid	0.35
16-Hydroxyhexadecanoic acid	4.2
18-Hydroxyoctadecanoic acid	0.5
18-Hydroxyoctadecenoic acid	1.6
18-Hydroxyoctadecadienoic acid	0.9
18-Hydroxyoctadecatrienoic acid	0.4
10,16-Dihydroxyhexadecanoic acid	79.8
9,16-Dihydroxyhexadecanoic acid	4.2

Since the cutin of flower petals contains the same constituents as the leaf cutin, it is reasonable to assume that the biosynthetic reactions involved in their formation are the same. All of our attempts to demonstrate the ω-hydroxylation reaction involved in cutin synthesis with *V. faba* leaf preparations failed, most probably because of competing reactions. If the expanding flower petals synthesize cutin rather rapidly, this might be a suitable system for enzyme level studies on cutin synthesis. In order to investigate this possibility, flower petals from the most rapidly developing flower buds were incubated with [1-^{14}C]palmitic acid. The residue remaining after the removal of all soluble lipids was radioactive. Exhaustive hydrogenolysis with $LiAlH_4$ released all this radioactivity into a chloroform-soluble fraction which contained reduced cutin monomers. About 30% of the ^{14}C administered was recovered in this fraction, indicating rapid cutin synthesis. TLC of this material revealed that all the radioactivity was contained in the three cutin monomer fractions (Fig. 1). The bulk of the radioactivity (87%) was contained in the triols, while the diols and fatty alcohols contained about 4% and 9%, respectively (Table II). This distribution is quite similar to the per cent composition (mass) of the monomers.

Table II. *Incorporation of [1-^{14}C]Palmitic Acid into Cutin Monomers by V. faba Flower Petals*

In experiment 1, 1.27-g petals and in experiment 2, 1.6-g petals were incubated with 1.0 ml of a solution containing 172 nmoles of [1-^{14}C]palmitic acid (10 μCi) for 4 hr at 30 C. The hydrogenolysis products of the labeled cutin were analyzed by TLC with ethyl ether-hexane-methanol (8:2:5 v/v) as the solvent system.

Experiment	Incorporation (cpm $\times 10^{-3}$)		
	Triol	Diol	Alcohol
1	40.6	2.45	5.90
2	50.2	1.74	3.13

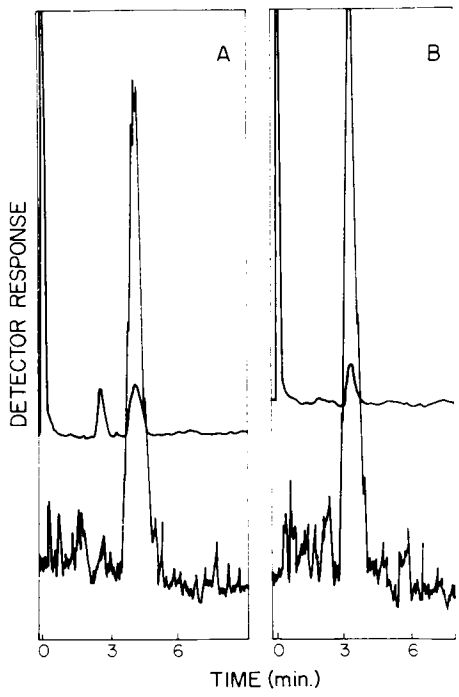


FIG. 3. Radio gas-liquid chromatogram of the labeled diol diacetate (A) and the dicarboxylic acid dimethyl ester derived from the diol by CrO_3 oxidation (B). The diol was isolated from the hydrogenolysate of cutin of the *V. faba* flower petals which were incubated with [1-^{14}C]palmitic acid. In each case, the upper tracing represents the flame ionization detector response and lower tracing radioactivity. The peaks on the upper tracing were due to added authentic standards, hexadecane-1,16-diol diacetate (A) and hexadecane-1,16-dioic acid dimethyl ester (B), respectively. The peak in A with a retention time shorter than that of hexadecane-1,16-diol diacetate was due to hexadexadecane-1,2-diol diacetate which was added to illustrate the separation between the positional isomers of alkane diols. Components were analyzed on 5% OV-1 packed in a coiled stainless steel column (6.5 ft × 0.25 in optical density) held at 247 C with carrier gas (Ar) inlet pressure of 31 p.s.i.

In order to identify the radioactive products, each fraction was isolated by TLC. Since the fatty alcohol fraction represented unhydroxylated palmitic acid, it was not examined further. When the diol fraction was acetylated and rechromatographed, virtually all the radioactivity migrated with authentic hexadecane-1,16-diol diacetate. Radio GLC of the diacetate showed that all of the radioactivity was contained in a component with a retention time identical to that of hexadecane-1,16-diol diacetate (Fig. 3A). In order to confirm the identity of the diol, it was oxidized with CrO_3 at room temperature. The radioactive product co-chromatographed with hexadecane-1,16-dioic acid when subjected to TLC on Silica Gel G with hexane-ethyl ether-formic acid (65:35:2) as the solvent system. The dimethyl ester of the labeled material, when subjected to TLC, co-chromatographed with authentic dimethyl hexadecane-1,16-dioate. Radio GLC of the labeled dimethyl ester fraction showed that all of the radioactivity was contained in a component which had a retention time identical to that of dimethyl hexadecane-1,16-dioate (Fig. 3B). Thus, the radioactivity of the diol fraction was contained in hexadecane-1,16-diol which was obviously derived from ω-hydroxyhexadecanoic acid of cutin.

The labeled triol fraction, which contained the most radioactivity, was acetylated and rechromatographed. All of the radioactivity co-chromatographed with hexadecane-1,7,16-triol triacetate. Radio GLC revealed that all of the radioactivity was contained in a component with a retention time identical to that of hexadecane-1,7,16-triol triacetate. Chromic acid oxidation of the labeled triol gave labeled dicarboxylic acids. The dimethylesters of these acids, when subjected to radio gas-liquid chromatography, revealed three labeled components (Fig. 4). The retention times of the two major components were identical to those of dimethyl esters of C_9 and C_{10} dicarboxylic acids. These are the two products expected to be formed from the CrO_3 oxidation of the $LiAlH_4$ reduction product of [1-^{14}C]10,16-dihydroxyhexadecanoic acid of cutin (Fig. 5). The minor radioactive component had a retention time identical to that of the dimethyl ester of C_8 dicarboxylic acid which obviously was derived from [1-^{14}C]9,16-dihydroxyhexadecanoic acid. These results confirm that [1-^{14}C]hexadecanoic acid was rapidly incorporated into the authentic cutin acids in *V. faba* flower petals. No other tissue is known to incorporate precursors into cutin more rapidly than the *V. faba* flower petals used in the present study. The unusually high incorporation of exogenous precursors into cutin might be brought about by two factors: the relatively large surface area per unit mass of the tissue and the rapid rate of expansion of the petals.

The soluble lipids were also reduced with $LiAlH_4$ and the products were examined by TLC. Small amounts of labeled triol and diol were detected. The triol was isolated and identified to be mainly [1-^{14}C]hexadecane-1,7,16-triol by chromic acid oxidation followed by radio GLC of the resulting dicarboxylic acids as their dimethyl esters. Thus, cutin monomers could be detected in the soluble lipids, but only in much smaller quantities than those found in the polymer. This observation is con-

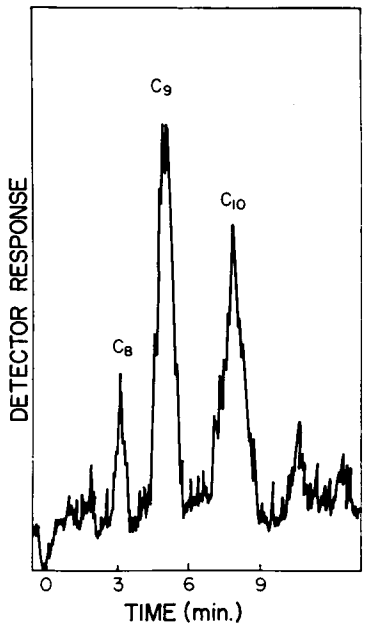


FIG. 4. Radio gas-liquid chromatogram of the dimethyl esters of the dicarboxylic acids obtained by CrO_3 oxidation of the C_{16} triol derived from the cutin of *V. faba* flower petals which were incubated with [1-^{14}C]palmitic acid. The number on each peak represents the chain length determined by comparison of the retention times with those of authentic standards. Components were analyzed on 5% OV-1 packed in a coiled stainless steel column (6.5 ft × 0.25 in optical density) held at 140 C with carrier gas (Ar) inlet pressure of 31 p.s.i.

sistent with the conclusion arrived at earlier (11, 12) that the monomers are transferred to the polymer as soon as they are formed, so that the monomers do not accumulate in the soluble phase.

Another interesting finding was that a radioactive material less polar than fatty alcohol was detected in the thin layer chromatograms of the $LiAlH_4$ reduction products of the soluble lipids. This material, containing about 1% of the total ^{14}C of the soluble lipids, when subjected to TLC co-chromatographed with octacosane in two solvents, benzene and hexane. Radio GLC of this material showed that it contained three labeled components with retention times identical to those of *n*-heptacosane (C_{27}), *n*-nonacosane (C_{29}), and *n*-hentriacontane (C_{31}). The radioactivity distribution among these three alkanes was similar to the per cent composition of the naturally occurring alkanes of the flower petals of *V. faba* (Table III), as determined by combined GLC mass spectrometry. In the $LiAlH_4$ reduction products of the soluble lipids, substantial amounts of ^{14}C were found only in the alkanes, fatty alcohols, and cutin components. Any acyl lipids would have been converted into fatty alcohols by the $LiAlH_4$ treatment. Radio gas-liquid chromatographic analysis of the fatty alcohols showed the presence of measurable amounts of C_{18} and C_{20}, but not longer chains. Thus, alkanes are the major chain elongated products derived from exogenous hexadecanoic acid in the flower petals. Thin layer chromatographic analysis of the surface lipids, extracted by a 20-sec immersion of flower petals in chloroform, showed that alkanes constituted a major component of the surface lipids. Thus, the ^{14}C found in alkanes of the soluble lipids represents conversion of the exogenous [1-^{14}C]hexadecanoic acid into a major soluble cuticular lipid.

A 20-sec immersion of flower petals in chloroform removed specifically *n*-C_{29} and *n*-C_{31} alkanes (70% and 29%, respectively, of the hydrocarbon fraction), while the alkanes obtained by a more complete extraction of the petals showed substantial amounts of *n*-C_{27} alkane. Analysis of the alkanes obtained from the internal lipids of petals did indeed show that internal alkanes contained a higher proportion of shorter chains than did the surface lipids (C_{31}, 26%; C_{29}, 43%; C_{27}, 31%). Compositional differences between hydrocarbons from the surface lipids and the internal lipids of leaves have been noted previously (7, 8).

Experimental evidence thus far obtained with plants indicates that alkanes are synthesized by elongation of fatty acids followed by decarboxylation (10, 12). Trichloroacetate inhibits alkane synthesis, most probably by inhibiting the elongation process (12). In order to determine if fatty acids are incorporated into alkanes of *V. faba* flowers via elongation, the effect

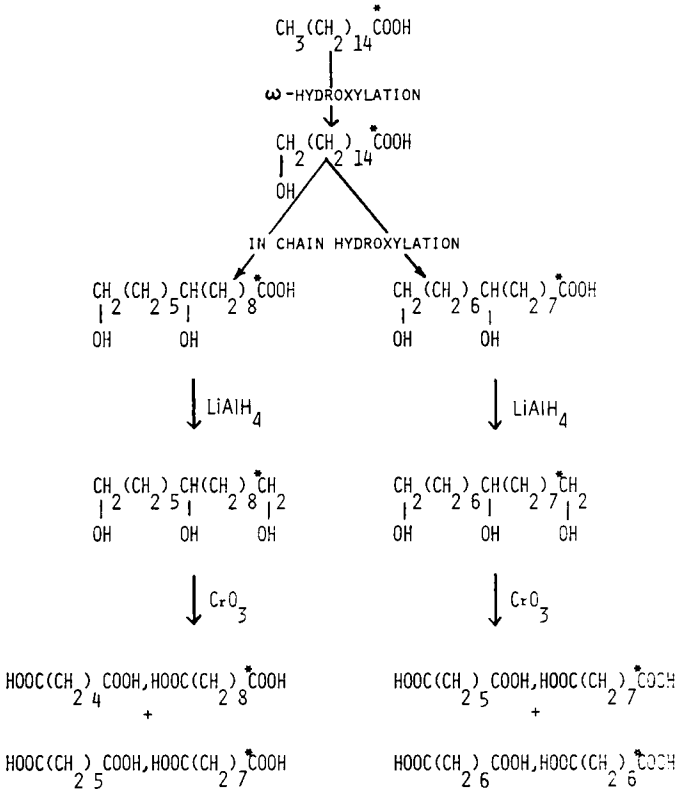


FIG. 5. Hypothetical pathway for the incorporation of [1-^{14}C] palmitic acid into the cutin monomers of *V. faba* flower petals and the chemical reactions used to identify the structure of the labeled C_{16} triol derived from the labeled acid. Although the biochemical reactions probably occur on a thioester of the fatty acids, for illustration of the fate of the carbon chain such details are omitted from this figure. The asterisk (*) indicates the location of ^{14}C.

Table III. *Incorporation of [1-^{14}C]Palmitic Acid into Major Alkanes by Flower Petals of V. faba*

Wing flower petals (4 g) were incubated with 864 nmoles (50 μCi) of [1-^{14}C]palmitic acid for 4 hr at 30 C. Total hydrocarbons isolated by TLC were analyzed by radio GLC on 5% OV-1 packed in a coiled stainless steel column (6.5 ft × 0.25 in o.d.) held at 275 C with carrier gas (Ar) inlet pressure of 30 p.s.i.

Chain Length	Mass	Radioactivity
	%	
C_{27}	12.5	14.5
C_{29}	43.4	47.6
C_{31}	44.1	37.9

Table IV. *Effect of Trichloroacetate on Incorporation of [1-^{14}C]-Stearic Acid into n-Alkanes of V. faba Flower Petals*

In each experiment, 1.7 g of petals were preincubated at 30 C for 30 min with 0.5 ml of a solution containing the appropriate amount of sodium trichloroacetate followed by incubation for 5 hr with 44 nmoles of [1-^{14}C] stearic acid (2.5 μCi) in a total volume of 1 ml of distilled water. Products were analyzed as described in Table III.

Concn of Trichloroacetate	Inhibition of Alkane Synthesis	Radioactivity in *n*-Alkanes			
		C_{27}	C_{29}	C_{31}	C_{33}
μmoles flask	*%*	*% distribution*			
0	0	5	26	54	15
5	46	12	30	47	11
10	83	15	37	38	10
20	95	26	40	34	<1

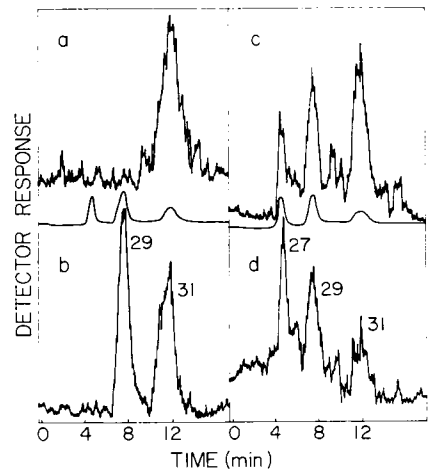


FIG. 6. Radio gas-liquid chromatograms of the alkane fractions isolated by TLC from the lipids of *V. faba* flower petals which were incubated with: (a), [9,10,11-^{3}H]*n*-dotriacontanoic acid (C_{32}); (b), [G-^{3}H]*n*-triacontanoic acid (C_{30}); (c), [G-^{3}H]*n*-octacosanoic acid (C_{28}); and (d), [G-^{3}H]*n*-octacosanoic acid in the presence of trichloroacetate. The middle traces represent the flame ionization detector response. The number on each peak represents the chain length of the *n*-alkane. Chromatographic conditions are described in Table III.

of various concentrations of trichloroacetate on the incorporation of [1-^{14}C]stearic acid into alkanes was examined (Table IV). Moderate levels of trichloroacetate exhibited a marked inhibition of alkane synthesis from stearic acid in the petals, and radio gas-liquid chromatographic analysis of the alkanes showed that the inhibition was greater for the longer alkanes. Thus, fatty acids appear to be incorporated into alkanes by an elongation-decarboxylation mechanism in *V. faba* flowers.

Since developing flower petals synthesize mainly *n*-C_{27}, *n*-C_{29}, and *n*-C_{31} alkanes, attempts were made to test the decarboxylation hypothesis directly by incubating petal tissue with [G-^{3}H]*n*-octacosanoic acid (C_{28}), [G-^{3}H]*n*-triacontanoic acid (C_{30}), and [9,10,11-^{3}H]*n*-dotriacontanoic acid (C_{32}). When each of these long chain fatty acids was administered to *V. faba* flower petals, the hydrocarbon fraction isolated from the lipids was radioactive. Radio gas-liquid chromatographic analysis of the hydrocarbon fraction derived from [^{3}H]C_{32} acid showed one major radioactive component with a retention time identical to that of *n*-C_{31} alkane (Fig. 6a), the expected decarboxylation product of C_{32} acid. Radio GLC of the hydrocarbon fraction derived from [^{3}H]C_{30} acid showed two major radioactive products whose retention times were identical to those of *n*-C_{29} and *n*-C_{31} alkanes (Fig. 6b), suggesting that the C_{30} acid had undergone both direct decarboxylation to C_{29} alkane and further elongation to C_{32} acid, which was subsequently decarboxylated to C_{31} alkane. Consistent with this interpretation was the fact that [^{3}H]C_{28} acid gave rise to radioactive *n*-C_{27}, *n*-C_{29}, and *n*-C_{31} alkanes, as determined by radio GLC (Fig. 6c). Thus, the specific pattern of alkanes formed from each substrate strongly suggests that direct decarboxylation and elongation-decarboxylation occur. *n*-Tritriacontane (C_{33}) is a minor component of the alkane fraction derived from the cuticle lipids of *V. faba* flowers, and small amounts of radioactive *n*-C_{33} alkane were formed from the tritiated long chain acid substrates. Therefore, it appears that little elongation beyond C_{31} acid occurs.

In order to demonstrate that C_{28}, C_{30}, and C_{32} acids undergo elongation and subsequent decarboxylation to alkanes, the effect of trichloroacetate on the incorporation of these long chain acids into alkanes was determined. Since 5 μmoles of trichloroacetate resulted in approximately 50% inhibition of alkane synthesis from [1-^{14}C]stearic acid, and produced a detectable change in the pattern of hydrocarbons formed (Table IV), this level of inhibitor was utilized in experiments with C_{28}, C_{30}, and C_{32} acids. The effect of trichloroacetate on the incorporation of C_{28} acid into alkanes of *V. faba* flower petals is shown in Figure 6, c and d, and in Table V. In the presence of trichloroacetate, less *n*-C_{29} and *n*-C_{31} alkanes were produced than in the control, while the amount of *n*-C_{27} alkane formed increased significantly. Thus, *n*-C_{29} and *n*-C_{31} alkanes must have been derived via elongation of C_{28} acid to C_{30} and C_{32} acids, respectively, followed by decarboxylation. Similar results were observed with C_{30} acid and C_{32} acid as substrates. In each case, the addition of trichloroacetate resulted in a decrease in elongated products and a net increase in the direct decarboxylation product (Table V). These results clearly demonstrate that exogenous C_{28}, C_{30}, and C_{32} fatty acids undergo direct decarboxylation to produce alkanes in *V. faba* flowers.

Thiols have been shown to inhibit alkane formation in pea leaves, presumably by affecting the decarboxylation step (4). Therefore, as a further test for the elongation-decarboxylation mechanism, the effect of dithioerythritol on the conversion of

Table V. *Effect of Trichloroacetate on Incorporation of C_{28}, C_{30}, and C_{32} Acids into n-Alkanes of V. faba Flower Petals*

In each experiment, 1 g of petals was preincubated at 30 C for 30 min with 0.5 ml of distilled water (control) or 0.5 ml of a solution containing 5 μmoles of sodium trichloroacetate, followed by incubation for 5 hr with 5×10^6 cpm of substrate in a total volume of 1 ml of distilled water. Products were analyzed as described in Table III. To permit a meaningful comparison of results between different substrates, data are presented as a per cent of ^{3}H in the alkane fraction of the control. Since the small amount of even chain hydrocarbons formed are excluded from the table, the values for controls do not total 100%.

Substrate and Conditions	Radioactivity in *n*-Alkanes			
	C_{27}	C_{29}	C_{31}	C_{33}
	(% of total alkanes in control)			
C_{28} Acid—control	9	33	52	
C_{28} Acid—with trichloroacetate	23	25	12	
C_{30} Acid—control	5	50	45	
C_{30} Acid—with trichloroacetate	7	62	20	
C_{32} Acid—control	<1	5	82	9
C_{32} Acid—with trichloroacetate	2	7	110	6

[9,10,11-^{3}H]*n*-dotriacontanoic acid (C_{32}) to *n*-hentriacontane (C_{31}) was examined. This dithiol, at 1 μmole and 5 μmoles/flask, inhibited the conversion of exogenous *n*-C_{32} acid to *n*-C_{31} alkane by 10 and 60%, respectively, in experiments identical to those described in Table V.

The results presented in this paper show for the first time that the flower petals are protected by cutin which has a composition extremely similar to that of the leaf cutin. Furthermore, it was demonstrated that the step-wise hydroxylation of palmitic acid at the C-16 and the C-9 or C-10 position gave rise to the cutin monomers in developing flower tissue. Recently, the enzyme that builds the cutin polymer from monomer acids (hydroxylacyl-CoA-cutin transacylase) was demonstrated in cell-free preparations from young *V. faba* flowers (6). Flower tissue was found to be advantageous for biosynthetic studies on soluble cuticular lipids also. Unlike many plant tissues which produce one major hydrocarbon, *V. faba* flowers produce three major alkanes, *n*-C_{27}, *n*-C_{29}, and *n*-C_{31}. Therefore, this tissue provided a suitable system for the demonstration of direct decarboxylation of fatty acids, as well as elongation followed by decarboxylation. Since cuticular lipid synthesis may be a major function of flower petals, this tissue may be more suitable than leaf tissue for enzyme level studies on cuticular lipid biosynthesis.

Acknowledgment—We thank Charles Oldenburg for raising the plants.

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