The Chemistry of Plant Cuticles: a Study of Cutin from *Agave americana* L.

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The cuticle is a layer covering the epidermis of the exposed parts of plants (e.g. stems, leaves). In spite of very wide occurrence, the chemical composition of plant cuticular layer is practically unknown (cf. Zetsche, 1932). Lee (1925) found that the cuticles of chrysanthemum and rose petals and the petioles of rhubarb leaves consisted of fatty acids, both free and esterified, soaps of fatty acids and some free higher alcohols, resinous substances and tannins. Triterpenoids, such as ursolic acid, were also reported in the cuticular layer of some 300 Japanese plants (Kariyine & Hashimoto, 1953; cf. Huelin & Gallop, 1951). On extraction of the cuticle of *Agave americana* and *Agave rigida* (Legg & Wheeler, 1925, 1929) successively with different solvents, several classes of compounds were obtained, namely glycosides of the saponin type, free acids, saponifiable material and cellulose. The residue remaining after these extractions was defined as cutin and was considered to be the characteristic part of the cuticular layer. The cutin was further resolved, after saponification with ethanolic potassium hydroxide, by a method based on the different solubilities of individual metal salts in various solvents. Four acids were isolated: a semi-liquid acid, $C_{26}H_{50}O_8$ (65% of cutin); a semi-liquid acid, $C_{13}H_{22}O_3$ (10%); an acid melting at 107–108° (10%), thought to be identical with phloionic acid found in suberin, and an acid melting at 88–90° (very small amount). By similar methods, the isolation of only two acids from cutin of an agave had been reported previously (Freymy, 1859, 1881, 1885): a solid acid, named stearo-cutic, m.p. 76°, and a semi-liquid acid, named oleo-cutic. Their relative proportion in the cutin was 1:5 respectively. No attempts have been made to elucidate the structures of any of these acids.

This paper is concerned with the separation, isolation and structure of the acids constituting the ether-soluble fraction obtained on saponification of cutin from *A. americana* L. This fraction represents some 60% of the weight of cutin.

METHODS

Countercurrent distribution. Countercurrent-distribution experiments were performed in a 24-tube stainless-steel apparatus of 6.8 ml./phase capacity or in an all-glass model of 100 ml./phase capacity (Craig & Craig, 1950).

Sixty transfers were applied in the all-glass apparatus, with a single-withdrawal technique. The thirty emerging upper phases were then recycled over thirty fresh lower phases. The solvent system consisted of mutually saturated chloroform-methanol-isohexane-water (10:10:5:4, by vol.).

The distribution in the stainless-steel apparatus was followed by titration to phenolphthalein of the contents of each tube with ethanolic 0.05N-NaOH. Portions (5 ml.) from each phase in each tube were collected from the all-glass apparatus; after drying in a vacuum oven the residues were weighed.

The volume of NaOH used or the weight of the residue was plotted against the tube number.

Analytical-scale reversed-phase partition chromatography. For these chromatographic experiments a water-jacketed column (0.8 cm. $\times$ 80 cm.) was used. The temperature was that of running tap water (18–20°), and small variations did not affect the separations. The eluate was collected in a siphon delivering 1 ml. portions and titrated in a glass vessel as described by Howard & Martin (1950) with 0.01N methanolic NaOH, bromothymol blue being used as indicator (0.2%, w/v, in 70% aqueous acetone). N_2 from a cylinder was used to agitate the eluate, and it was found unnecessary to protect it against atmospheric CO_2 . On plotting the volume of alkali used against the aliquot number, chromatographic curves were obtained.

Three different stationary phases were used: (i) purified medicinal paraffin (Howard & Martin, 1950); (ii) purified castor oil (Savary & Desnuelle, 1953) and (iii) a paraffin-castor oil mixture (50%, w/w). The stationary phases were adsorbed on non-wetting kieselguhr and packed as described by Silk & Hahn (1954a).

Different water-acetone (v/v) mixtures equilibrated with the appropriate stationary phase and clarified by filtration were used as developing solvents.

The material (5–30 mg.) was dissolved in ether and 1 g. of non-wetting kieselguhr containing the stationary phase was added. The ether was slowly evaporated with constant stirring. The powdery mull was suspended in 10 ml. of the mixture having the lowest acetone content that was used in the experiment and bedded by means of a plunger with a perforated disk.

Preparative reversed-phase partition chromatography. It became necessary to extend the reversed-phase partition chromatography in such a way that it could be used as a preparative tool. A column (3.9 cm. $\times$ 1.2 m.) was used in conjunction with a siphon of special construction. The siphon (cf. Silk & Hahn, 1954b) divided each fraction into two portions: (i) the bulk (30 ml.), which was collected; (ii) a small sample (2 ml.), which was titrated and discarded. It was thus possible to follow the development of the chromatogram without contaminating the material

recovered. When 280 g. of non-wetting kieselguhr with 225 g. of castor oil was used, it was found that as much as 18 g. of material could be processed at once without adversely affecting the resolution. The mull was prepared in the usual way and packed in the column as a slurry with the aid of a plunger.

Determination of the position of the secondary hydroxyl group. The following micro method was developed for the detection of the position of the secondary hydroxyl group in molecules of the type

The methyl ester of the acid, dissolved in acetic acid, was shaken in a stoppered flask with the calculated amount of CrO_3 for 18 hr. at 30° . Under these conditions very little or no hydrolysis occurred. The reaction mixture was poured into water and worked up in ether. The product obtained on distillation of the solvent was extracted with pentane to remove the half-ester of dicarboxylic acids formed and to leave free dicarboxylic acids as a residue. Part of the half-ester extract was then saponified. In this way three fractions were obtained: (a) pentane-insoluble (dicarboxylic acids); (b) pentane-soluble (half-esters) and (c) pentane-soluble saponified fraction (saponified half esters) (these will be referred to as fractions *a*, *b* and *c* in the experimental part). All three fractions and a synthetic mixture of adipic, pimelic, suberic, azelaic and sebacic acids were spotted on a sheet of Whatman no. 1 paper and developed with a mixture of *n*-propanol-conc. aqueous NH_3 (60:40, v/v) (Isherwood & Hanes, 1953) by descending chromatography at approx. 20° . When the solvent front had moved about 25 cm. the paper was removed from the tank, dried for 1 hr. at room temperature and sprayed with bromocresol-green indicator prepared according to Lindquist & Störgards (1953).

The longest-chain acid detected from the saponified half-ester fraction gave the distance of the hydroxyl group from the original carboxyl side and the longest-chain acid detected in the dicarboxylic acid fraction gave the position of the hydroxyl relative to the opposite side of the chain, as shown below:

The dicarboxylic acids detected must give the original chain length when the appropriate oxidation fragments are added (e.g. 1 + 2 or 3 + 4), thus providing a check of the results. The half-esters all have R_f values of about 0.9, which is much higher than those of the dicarboxylic acids.

EXPERIMENTAL AND RESULTS

Melting points were determined in capillary tubes immersed in an electrically heated and stirred paraffin bath and are corrected. Infrared absorption spectra were recorded with a Perkin-Elmer infrared spectrometer Model 21.

Isolation of cutin

Leaves of *A. americana* L. were collected near Pretoria and, after washing, the cuticle was stripped off. A method based on that of Legg & Wheeler (1925) was used for the isolation of cutin. Air-dried cuticle (329 g.) was extracted

twice with boiling hexane (2×2 l.) for 3 hr. The hexane solutions were combined and gave 24 g. of yellow wax. The cuticle was refluxed with 2 l. of ethanol for 3 hr. and the solvent decanted while hot. This treatment was repeated eight times. The combined ethanolic solutions gave a further 54 g. of waxy material. The total wax fraction was thus 78 g. (24.1%).

The cuticle was extracted with 6 l. of boiling water for 2 hr. and the water decanted while hot. After four treatments the water was no longer coloured. The extraction was continued in an autoclave under a pressure of 17 lb./sq. in. until a 100 ml. portion left no residue on evaporation. The combined water extracts were worked up to give 27 g. (8.2%) of dark-brown, crisp material.

Cellulose was removed by treatment with Schweizer reagent. The cuticle, suspended in 2 l. of cuprammonium hydroxide solution, was shaken for 8 hr. and filtered. After three extractions all cellulose was removed, since the test with 'chlor-zinc-iodide' reagent (Abderhalden, 1914) on sections of the residual material showed no blue coloration.

The residue, which was defined as cutin, was washed with 4 l. of 10% hydrochloric acid and hot water until neutral. After air-drying, 168 g. (51%) of light-brown elastic tissue was obtained. (Found: ash, 4.2; C, 63.9; H, 9.6%, ash-free basis.)

Saponification of cutin and preliminary fractionation of the derived acids

Saponification of cutin with ethanolic KOH (5%, w/v), sodium ethoxide or boiling Cellosolve-KOH (5%, w/v) was found unsatisfactory, and the method described below was used in the present work.

Finely ground cutin (30 g.) and 5% aqueous KOH (200 ml.) were placed in a rotating 500 ml. autoclave and heated for 17 hr. at 130°. The reaction mixture was filtered hot through a Gooch crucible; the residue was boiled twice with water and filtered off. After drying at 100°, 3.59 g. of a brown powder was obtained. (Found: ash, 10.5; C, 52.6; H, 7.8%, ash-free basis.)

The combined filtrates were extracted with ether but no unsaponifiable material was found.

After acidification with 5% HCl, the precipitated acids were filtered off and repeatedly extracted with ether. The ether-insoluble residue gave, after drying, 4-14 g. of brown powder. (Found: C, 70.3; H, 11.9%; equiv. wt., 470.) The aqueous filtrate was extracted several times with ether and the ethereal solutions were combined with those obtained previously. After distillation of the solvent, 17.23 g. of brown greasy material remained. (Found: equiv. wt., 332.) The fractionation is shown schematically in Fig. 1.

Fig. 1

*Reduction of the ether-soluble fraction
to the parent acids*

The ether-soluble fraction (2 g.), HI (sp.gr. 1.7; 30 ml.) and red P (0.3 g.) were refluxed for 8 hr. with continuous stirring. The reaction mixture was poured into water and extracted with ether. The ethereal solution was washed with water, 5% NaHSO_3 and water, and dried and distilled, leaving a brown oil (3.21 g.). The oil was dissolved in methanol (30 ml.) and refluxed for 4 hr. in the presence of Zn (2 g.) and conc. HCl (15 ml.). The reaction mixture was diluted with water and worked up in ether to give a yellow solid (1.94 g.), which was chromatographed on a column of neutral alumina (40 g.). Three fractions were collected from the chromatogram: (i) pentane-eluted material (0.67 g.); (ii) pentane-ether (1:1, v/v)-eluted material (0.66 g.); and (iii) ether-eluted material (0.13 g.).

Fraction (i) was saponified and some of the acids obtained were subjected to reversed-phase partition chromatography with paraffin as a stationary phase. Two well-resolved peaks were obtained and identified as palmitic and stearic acids by a mixed chromatogram with authentic samples.

Fraction (ii) contained, in addition to a small amount of palmitic and stearic acids, some incompletely reduced material which appeared with the solvent front. This fraction (0.6 g.) was therefore treated again with HI as described above. After purification on an alumina column, the pentane eluate (0.3 g.) consisted again only of palmitic and stearic acids.

The ratio of palmitic to stearic acid in the total ether-soluble fraction was approximately 1:3.

*Separation of the acids constituting
the ether-soluble fraction*

Countercurrent-distribution experiments. The ether-soluble fraction was only partially resolved after thirty transfers in the all-glass apparatus, as can be seen from Fig. 2. Two fractions designated as Z and Y in Fig. 2 were collected, and fraction Z was worked up in ether and then subjected to sixty transfers in the manner described previously. Some further separation was evident, but sixty transfers were still insufficient to resolve this mixture into well-defined peaks. This method was not used for further resolution of the mixture. However, from fraction Y a high concentrate of a relatively pure compound was obtained. This material was designated as concentrate A.

The preparation of the concentrate A was simplified further by using the multiple-batch extraction technique (cf. Zilch, Dutton & Cowan, 1952). The ether-soluble fraction (10 g.) was subjected to four transfers with four separating funnels. The same solvent system was used as in the countercurrent-distribution experiments (150 ml. of each phase). All four upper phases were combined and worked up to give fraction 1 (2.4 g.). The four lower phases were combined and worked up; the material obtained was subjected again to the process described above. The combined upper phases gave fraction 2 (2.5 g.) and the combined lower phases fraction 3 (4.8 g.). This fractionation is shown in Fig. 3.

All three fractions obtained by this procedure were subjected to countercurrent distribution and the curves are reproduced in Fig. 4. Fraction 1 was practically the same as concentrate A. Fractions 2 and 3 were very similar to one another and were therefore combined.

Fig. 2. Countercurrent distribution of the ether-soluble fraction of saponified cutin. Thirty transfers in chloroform-methanol-*iso*hexane-water (10:10:5:4, by vol.).

Fig. 3

Fig. 4. Countercurrent distribution of fractions 1-3, obtained by the batch-extraction technique (see text). Twenty-four transfers in chloroform-methanol-*iso*hexane-water (10:10:5:4, by vol.).

Reversed-phase partition chromatography. Some of the total ether-soluble fraction was subjected to reversed-phase partition chromatography, with castor oil as a stationary phase. Five peaks, designated *A*, *B*, *C*, *D* and *E* respectively, were obtained as recorded in Fig. 5. In this procedure the separation of *A* and *B* was far from complete. When, however, the combined fractions 2 and 3, obtained by the batch-extraction technique as described above, were chromatographed on the same column, the curve reproduced in Fig. 6 was obtained. As was expected, the largest part of *A* was already removed, and therefore the degree of separation which could be achieved between the remaining *A* and *B* was increased.

These experiments showed that excellent separation of cutin acids on a castor-oil column was possible. The bulk of the material was therefore processed in the preparative column described. Four fractions were collected from each chromatogram as indicated in Fig. 6. From each fraction most of the organic solvent was removed by distillation at

reduced pressure. The precipitated acids were extracted with ether, and after removal of the solvent four concentrates, designated *A*, *B*, *C* and *D* respectively, were obtained.

9:10:18-Trihydroxyoctadecanoic acid*
(*phloionolic acid*)

Isolation. Fraction 1 obtained by the batch-extraction technique was recrystallized from ethyl acetate to give the acid (approx. 25% of cutin; approx. 43% of the ether-soluble fraction) (needles, m.p. 102–103°, unchanged on admixture with phloionolic acid isolated from suberin (kindly supplied by Professor Ribas). (Found: C, 65.0; H, 10.9%; equiv. wt., 330. Calc. for $C_{18}H_{34}O_6$: C, 65.0; H, 10.9%; equiv. wt., 332.)

The methyl ester was prepared by refluxing the acid in methanol- H_2SO_4 ; it was crystallized from benzene to m.p. 80.0–80.4°. (Found: C, 66.1; H, 11.0; active H, 0.9 (Zerewitinoff method in anisole). Calc. for $C_{18}H_{34}O_6$: C, 65.9; H, 11.0; active H, 0.87%.)

Reduction to the parent acid. The acid (460 mg.) was treated with HI and Zn as described above and worked up in ether to give a solid (369 mg.) which was purified on a neutral alumina column.

The pentane-eluted material (256 mg.), after one crystallization from acetone, had m.p. 38.2–38.6°, undepressed on admixture with authentic methyl stearate. (Found: C, 76.6; H, 12.9. Calc. for $C_{18}H_{36}O_2$: C, 76.45; H, 12.8%.)

Oxidation with lead tetraacetate. The acid (5 g.) dissolved in benzene (150 ml.) containing acetic acid (15 ml.) was oxidized with lead tetraacetate (20 g.) in benzene (50 ml.) at 55° in the presence of air (cf. Mendel & Coops, 1939). The reaction mixture was poured into 5% aqueous H_2SO_4 (100 ml.) and the aqueous layer extracted three times with benzene. The combined benzene solutions were treated with 50% aqueous KOH and the alkaline extracts acidified and worked up in ether. The residue obtained was esterified with methanol to give a mixture (4.2 g.) which was adsorbed on a neutral alumina (50 g.) column and eluted with (i) isohexane (350 ml.) and (ii) methanol (150 ml.).

(i) The isohexane-eluted material (2.47 g.) was an oil which distilled at 160°/24 mm.; η_D^{20} 1.4365 (dimethyl azelate: η_D^{20} 1.43669). (Found: C, 61.2; H, 9.3. Calc. for $C_{11}H_{20}O_4$: C, 61.1; H, 9.3%.) The distillate was saponified to give azelaic acid, m.p. and mixed m.p. 107°. (Found: C, 57.4; H, 8.45. Calc. for $C_9H_{16}O_4$: C, 57.4; H, 8.6%.) The dianilide had m.p. 186°. (Found: N, 8.1. Calc. for $C_{21}H_{28}O_2N_2$: N, 8.3%.)

(ii) The methanolic eluate was concentrated *in vacuo* to 25 ml., diluted with water and extracted with ether. Distillation of the solvent left an oil (1.4 g.). (Found: active H, 0.57. ($LiAlH_4$ method.) Calc. for $C_{10}H_{20}O_3$: active H, 0.53%.) This was saponified to give the acid, recrystallized from ethyl acetate and water to m.p. 50.0–50.5° (9-hydroxynonanoic acid has m.p. 51–51.5°; Chuit & Hausser, 1929). (Found: C, 62.2; H, 10.5%; equiv. wt., 174. Calc. for $C_9H_{18}O_3$: C, 62.0; H, 10.4%; equiv. wt., 174.)

Reduction by $LiAlH_4$ of the products of oxidation by lead tetraacetate. The mixture of acids (2.59 g.), obtained by oxidation with lead tetraacetate as described above, was dissolved in dry ether (100 ml.) and added dropwise to $LiAlH_4$ (1.1 g.) in ether (50 ml.). It was then refluxed for 1 hr., cooled and decomposed with 5% aqueous H_2SO_4 .

* Geneva numbering, $CO_2H=1$.

Fig. 5. Reversed-phase partition chromatography of the ether-soluble fraction of saponified cutin. The column (0.8 cm. $\times$ 80 cm.) contained non-wetting kieselguhr (12 g.) and castor oil (10 g.). Changes of solvent (% v/v, aqueous acetone) are indicated by the arrows.

Fig. 6. Reversed-phase partition chromatography of the combined fractions 2 and 3, obtained by the batch-extraction technique (see text). Column and solvent as in Fig. 5.

(Nylstrom & Brown, 1947). The reaction product was worked up in ether to give a solid (1.49 g.) which distilled at 123°/0.5 mm. without leaving any residue. After recrystallization from benzene the alcohol was identified as nonane-1:9-diol, m.p. and mixed m.p. 45.2–45.6°. (Found: C, 67.7; H, 12.5. Calc. for $C_9H_{20}O_2$: C, 67.45; H, 12.6%.)

Degradative oxidation with CrO_3 . The methyl ester (100 mg.), acetic acid (1 ml.) and CrO_3 (90 mg.), were shaken for 18 hr. at 30° and worked up in ether to give a solid (120 mg.), which was treated as described under Methods in order to obtain the three fractions. These were chromatographed on paper together with a mixture of authentic dicarboxylic acids. Fraction *b* gave a spot with R_F 0.86. Fractions *a* and *c* both gave a spot (R_F 0.58) identical with that given by azelaic acid. Two more very faint spots, due to suberic acid (R_F 0.48) and azelaic half-ester (R_F 0.86), were also detected in these fractions.

10:16-Dihydroxyhexadecanoic acid

Isolation. The concentrate *B* (obtained from the preparative reversed-phase partition chromatography, see Fig. 6) was rechromatographed on a castor-oil column. From the shape of the curve it was obvious that in addition to a small amount of *C* a certain amount of *A* was also present.

The concentrate *B* (4.7 g.), dissolved in 96% ethanol (150 ml.), was treated with a solution of sodium periodate (2 g.) in 2N- H_2SO_4 (50 ml.) (Karnovsky & Rapson, 1946; cf. Jackson, 1944) in order to oxidize phloionolic acid. After standing at room temperature for 1 hr. the reaction mixture was poured into water and worked up in ether. The solid obtained (3.9 g.) was rechromatographed on a castor-oil column and the fractions were collected in such a way that the right-hand impurity (*C*) was discarded. The acetone was distilled off *in vacuo* and the precipitated solid filtered off and dried over P_2O_5 . The acid (approx. 9.7% of cutin; approx. 17% of the ether-soluble fraction) was crystallized from aqueous methanol to m.p. 75–76° (needles). (Found: C, 66.5; H, 10.9%; equiv. wt., 275. $C_{16}H_{32}O_4$ requires C, 66.6; H, 11.2%; equiv. wt., 288.) The methyl ester, prepared in the usual way, was crystallized from benzene to m.p. 67.5–68.3°. (Found: C, 67.35; H, 11.5; active H, 0.67 (Zerewitinoff method in anisole). $C_{17}H_{34}O_4$ requires C, 67.5; H, 11.3; active H, 0.66%.)

Reduction to the parent acid. The acid (300 mg.) was treated with HI and Zn as described above and worked up in ether to give a slightly yellow oil (268 mg.) which was purified on a neutral alumina column. The pentane (150 ml.)-eluted material (218 mg.) was saponified to give palmitic acid, m.p. and mixed m.p. 62.4–62.8°. (Found: C, 74.9; H, 12.6. Calc. for $C_{16}H_{32}O_2$: C, 74.9; H, 12.6%.) A mixed chromatogram of the acid obtained with authentic palmitic acid gave only one peak.

Oxidation to the ketodicarboxylic acid. To the methyl ester (49 mg.) dissolved in acetone (4 ml.) was added dropwise a solution of CrO_3 (40 mg.), water (2.5 ml.) and conc. H_2SO_4 (0.4 ml.) (cf. Anet, Lythgoe, Silk & Trippett, 1953). After addition of the reagent, the mixture was stirred for 30 min. at room temperature. It was then poured into water, extracted with ether and the ethereal solution washed with water. After removal of the solvent the derived solid (43 mg.) was saponified to give the acid (42 mg.), which was crystallized from benzene to m.p.

112.5–113.5° (7-oxohexadecanedioic acid (Collaud, 1943) has m.p. 113–113.5°). (Found: C, 64.2; H, 9.8%; equiv. wt., 304. Calc. for $C_{16}H_{30}O_5$: C, 64.0; H, 9.4%; equiv. wt., 300.)

The position of the secondary hydroxyl group. The methyl ester (34 mg.) in acetic acid (1 ml.) was oxidized with CrO_3 (45 mg.) as described previously and worked up in ether to give a yellow solid (32 mg.). The three fractions obtained as described under Methods were chromatographed on paper. Fraction *b* gave a spot with R_F 0.89. In the fraction *a* two spots identical in R_F value with those given by adipic (R_F 0.41) and pimelic (R_F 0.47) acids were detected. Fraction *c* gave two spots with R_F 0.58 and 0.69 respectively, identical with those given by azelaic and sebacic acids. In this last fraction another spot (R_F 0.76) identical with that given by the C_{16} ketodicarboxylic acid was also detected.

10:18-Dihydroxyoctadecanoic acid

Isolation. The concentrate *C*, obtained from the preparative reversed-phase partition chromatography (see Fig. 6), was rechromatographed on a castor-oil column and the fractions were collected in such a way that the right-hand impurity (*D*) was discarded. The acetone was distilled off *in vacuo* and the precipitated solid extracted with ether. The ethereal solution was decolorized with charcoal and the solvent distilled off. The acid obtained (approx. 10.3% of cutin; approx. 18% of the ether-soluble fraction) was crystallized from methanol to m.p. 84–85°. (Found: C, 68.5; H, 11.6%; equiv. wt., 298. $C_{18}H_{36}O_4$ requires C, 68.3; H, 11.5%; equiv. wt., 316.) The methyl ester, prepared in the usual way, was crystallized from hexane-benzene to m.p. 62.6–63.4°. (Found: C, 69.2; H, 11.7; active H, 0.57 (Zerewitinoff method in anisole). $C_{19}H_{38}O_4$ requires C, 69.0; H, 11.6; active H, 0.61%.)

Reduction to the parent acid. The acid (206 mg.) was treated with HI and Zn as described above and worked up in ether to give a slightly yellow solid (161 mg.), which was purified on a neutral alumina column. The pentane (150 ml.)-eluted material (132 mg.) had m.p. 37.0–37.7°, which was raised to 37.5–38.5° on addition of methyl stearate of m.p. 38.3–38.6°. The ester was saponified to give the acid, m.p. 69.0–69.4°, undepressed on admixture with authentic stearic acid. (Found: C, 75.8; H, 12.9. Calc. for $C_{18}H_{36}O_2$: C, 76.0; H, 12.8%.) A mixed chromatogram of the obtained parent acid with authentic stearic acid gave only one peak.

Oxidation to the ketodicarboxylic acid. The acid (39 mg.) dissolved in acetone (4 ml.) was oxidized with chromic acid in the way described above. The reaction mixture was worked up in ether to give 10-oxo-octadecanedioic acid, which was crystallized from benzene to m.p. 108–109°. (Found: C, 65.6; H, 10.1%; equiv. wt., 334. $C_{18}H_{32}O_5$ requires C, 65.8; H, 9.8%; equiv. wt., 328.)

The position of the secondary hydroxyl group. The methyl ester (41 mg.) in acetic acid (1 ml.) was oxidized with CrO_3 (51 mg.) as described above and worked up in ether to give a solid (42 mg.). The three fractions obtained as described under Methods were chromatographed on paper. Fraction *b* gave a spot with R_F 0.89. In fraction *a* two spots, identical in R_F with those given by azelaic (R_F 0.62) and suberic (R_F 0.55) acids, were detected. Fraction *c* gave two spots with R_F 0.62 and 0.70 identical with those given by azelaic and sebacic acids respectively. In this last fraction a spot (R_F 0.78), due to a C_{18} ketodicarboxylic acid, was also detected.

18-Hydroxyoctadec-cis-9-enoic acid

Isolation (as 9:10:18-trihydroxyoctadecanoic acid). When the concentrate *D* (obtained from the preparative reversed-phase partition chromatography, see Fig. 6) was worked up in the usual way it was found that it contained, in addition to the acids derived from cutin, a large amount of castor oil washed from the column. The cutin acids were separated from castor oil by extraction with 5% K_2CO_3 and, after acidification, they were extracted with ether. The ethereal solution was passed through a kieselguhr-silica gel (50%, w/w) column in order to remove brown-coloured impurities. The eluate was worked up to give a slightly coloured semi-solid which was partly unsaturated (24.4 mg. in ethanol took up 0.7 ml. of H_2 in presence of Adams's catalyst) and was obviously a mixture of several acids. The infrared spectrum of the mixture (CS_2 solution) was recorded and examined in the 10.35 μ . region for *trans* double bonds. No absorption peaks occurred in this region and therefore the double bond(s) had a *cis* configuration.

When the mixture was subjected to reversed-phase partition chromatography, with paraffin-castor oil (50%, w/w) as stationary phase, only slight resolution was achieved. As it was not possible to obtain pure acid in this way, the mixture was dissolved in formic acid (98–100%) and hydroxylated with performic acid at 50° (Knight, Koos & Swern, 1954; Bergström & Pääbo, 1954). The reaction product was saponified, worked up in ether and chromatographed on a paraffin-castor oil column. Three well-resolved peaks were obtained and three fractions, designated *D1*, *D2* and *D3* respectively, were collected as indicated in Fig. 7.

Although fraction *D2* was made up for the greater part of the unchanged unsaturated acid, a solid acid whose methyl ester had m.p. 42–45° was also isolated in an impure state from this fraction. The small amount of material obtained precluded further purification and identification of this acid.

Fraction *D1* was worked up in the usual way and re-crystallized from aqueous methanol to give phloionolic acid, m.p. 102°, undepressed on admixture with an authentic sample. (Found: C, 64.9; H, 10.7%; equiv. wt., 328. Calc. for $C_{18}H_{36}O_5$: C, 65.0; H, 10.9%; equiv. wt., 332.) The methyl ester had m.p. 79.9–80.2° (from benzene), undepressed on admixture with methyl phloionolate. (Found: C, 65.9; H, 11.0. Calc. for $C_{18}H_{38}O_5$: C, 65.9; H, 11.0%.)

The original unsaturated acid represents approx. 2% of cutin (approx. 3.4% of the ether-soluble fraction).

Degradative oxidation with CrO_3 . The acid obtained from the fraction *D1* (52 mg.) in acetic acid (1 ml.) was oxidized with CrO_3 (20 mg.) as described before. The reaction product was worked up in ether and chromatographed on paper, together with a synthetic mixture of dicarboxylic acids (cf. Methods). In addition to a very strong spot (R_F 0.61) corresponding to that given by azelaic acid, a very faint spot (R_F 0.82), probably due to incompletely oxidized original material, was also detected.

18-Hydroxyoctadecanoic acid

Isolation. Fraction *D3* was still a mixture, as can be seen from the shape of the chromatographic curve in Fig. 7. This fraction was therefore rechromatographed on a paraffin-castor oil (50%, w/w) column, and the curve

obtained is reproduced in Fig. 8. Fraction *D31* (Fig. 8) was collected, worked up in the usual way and the yellow solid obtained was esterified with methanol. The methyl ester was adsorbed on a neutral alumina column and eluted with ether. It was then crystallized from hexane to m.p. 61.5–62.0° (18-hydroxyoctadecanoic acid methyl ester has m.p. 61.5–62.0°, Chuit & Hausser, 1929; 62.4–62.6°, Bergström, Aulin-Erdtman, Rolander, Stenhagen & Östling, 1952). (Found: C, 72.8; H, 12.0. Calc. for $C_{19}H_{38}O_2$: C, 72.6; H, 12.2%.) This acid represents approx. 0.3% of cutin (approx. 0.7% of the ether-soluble fraction).

Oxidation to the dicarboxylic acid. The methyl ester (16.6 mg.), dissolved in acetone (3 ml.), was treated with chromic acid as described above. The oxidation product was saponified with methanolic KOH to give the acid (12 mg.), which was crystallized from benzene to m.p. 122.5–123.5°, undepressed on admixture with authentic octadecanedioic acid. (Found: C, 68.9; H, 11.0. Calc. for $C_{18}H_{34}O_4$: C, 68.75; H, 10.9%.)

Fig. 7. Reversed-phase partition chromatography of concentrate *D* (Fig. 6) after treatment with performic acid. The column (0.8 cm. $\times$ 80 cm.) contained non-wetting kieselguhr (12 g.), paraffin (5 g.) and castor oil (5 g.). Solvent as in Fig. 5.

Fig. 8. Reversed-phase partition chromatography of *D3* (Fig. 7). Column as in Fig. 7, solvent as in Fig. 5.

DISCUSSION

Cutin from *A. americana* was obtained by a slightly modified method of Legg & Wheeler (1925), and then saponified in order to break down the polymer into its constituent acids. Preliminary fractionation of the products obtained was achieved by ether extraction. The percentage recovery of the ether-soluble fraction varied somewhat in different experiments, according to the efficiency of the extraction. When small samples were used, more than 60 % of this material, based on the weight of cutin, was obtained, but only a 50 % yield resulted from large samples. The ether-soluble fraction therefore represented the bulk of cutin. By reduction to palmitic and stearic acids it was shown that this fraction consisted almost entirely of C_{16} and C_{18} normal-chain hydroxylated acids. The ether-soluble fraction was only partly resolved in a 30-tube Craig apparatus. There is no apparent reason, however, why complete separation of acids could not be possible in an automatic apparatus where a sufficient number of transfers could be applied. Further resolution of the ether-soluble fraction was achieved by means of reversed-phase partition chromatography with castor oil or a mixture of paraffin-castor oil (50 %, w/w) as stationary phase. The efficiency of the castor-oil column appeared to be at least 700 transfers, and a very good separation of the constituent acids was obtained. In order to prepare sufficient amounts of pure acids for structural work, the reversed-phase partition chromatography was extended so that it became a preparative tool. It was found that 18 g. of material could be processed without adversely affecting the resolution, but this by no means represents the upper limit of the column's loading. The useful application of the methods described above in the investigation of chemically related natural products (e.g. suberin, shellac) is an obvious possibility.

Five acids—9:10:18-trihydroxyoctadecanoic, 10:18-dihydroxyoctadecanoic, 18-hydroxyoctadecanoic, 18-hydroxyoctadec-*cis*-9-enoic and 10:16-dihydroxyhexadecanoic acids—representing more than 80 % of the ether-soluble fraction, were found to be present. The structures of the first four acids are suggestive of the biological path of their formation from a common precursor. It is also quite possible that their C_{16} homologues are present in cutin in small amounts, as indicated by the isolation of 10:16-dihydroxyhexadecanoic acid. It has not been possible, however, to isolate other C_{16} homologues yet.

The percentage yield of the isolated acids was calculated from Figs. 5, 7 and 8 and it should be considered as approximate only. The major component (about 25 % of cutin) was phloionolic acid

(the name commonly applied to 9:10:18-trihydroxy-octadecanoic acid, without regard to its isomeric form) found also in small amounts in suberin (Zetsche & Bähler, 1931; Seoane & Ribas, 1951; cf. Jensen & Pekka, 1954). As the mixed m.p. of the acids isolated from cutin and suberin showed no depression, it is probable that they are identical. The structure of phloionolic acid was confirmed by the following series of experiments. The acid was reduced to stearic acid to establish its carbon skeleton. Oxidation with lead tetraacetate produced azelaic and 9-hydroxynonanoic acids. In a parallel experiment the mixture obtained on lead tetraacetate oxidation was reduced with lithium aluminium hydride to give nonane-1:9-diol as a sole product. Oxidation with chromium trioxide under mild conditions gave only azelaic acid. By these experiments the position of the hydroxyl groups at C_9 , C_{10} and C_{18} was confirmed. In addition, it was possible partly to elucidate the configuration of phloionolic acid. It seems well established that during the reaction of performic acid with the double bond inversion occurs, so that a *cis*-ethylene produces a diol with a *threo* configuration (e.g. Crombie, 1952; cf. Myers, 1952). It has also been demonstrated (Swern, Witnauer & Knight, 1952) that the low-melting, *threo*-9:10-dihydroxyoctadecanoic acid, obtained on oxidation of oleic (*cis*) acid with performic acid, forms a urea complex, whereas the high-melting *erythro*-9:10-dihydroxyoctadecanoic acid, obtained on oxidation of oleic acid with potassium permanganate, does not. On treatment of 18-hydroxyoctadec-*cis*-9-enoic acid found in cutin, with performic acid, a trihydroxy acid having *threo* configuration, should, therefore, be obtained. This acid was found to be identical with authentic phloionolic acid isolated from cutin. Further, phloionolic acid, from both cutin and suberin, formed a urea complex (cf. Ribas & Gil, 1951), and was the low-melting isomer (a high-melting form, m.p. 133°, was obtained by Ribas & Seoane (1954) by treating 18-hydroxyoctadec-*cis*-9-enoic acid with potassium permanganate; cf. Dupont, Dulou & Cohen, 1955). On the strength of this evidence a *threo* configuration is now assigned to phloionolic acid isolated from cutin.

The structure of 10:16-dihydroxyhexadecanoic acid was proved in the following way. Its carbon skeleton was established by reduction to palmitic acid. The infrared spectrum of the acid (KBr technique) did not show any distinct CH_3 band at 7.28μ , and the presence of an ω -hydroxyl group was suspected. Oxidation with chromic acid in acetone gave an oxothapsic acid, and therefore one of the hydroxyl groups must be attached at C_{16} . For the detection of the position of the remaining hydroxyl group a micro-method was developed, as described under Methods. Adipic, pimelic, azelaic

and sebacic acids were identified as oxidation products, thus establishing the position of the second hydroxyl group at C₁₀:

Similarly, 10:18-dihydroxyoctadecanoic acid was reduced to stearic acid. The infrared spectrum (KBr technique) failed again to show conclusively the absence of a CH₃ band at 7.28 μ., but on oxidation with chromic acid in acetone an oxo-octadecanedioic acid was obtained to fix the position of one hydroxyl group at C₁₈. The position of the second hydroxyl group was established at C₁₀ by oxidation to suberic, azelaic and sebacic acids:

No record of the isolation of 10:16-dihydroxyhexadecanoic and 10:18-dihydroxyoctadecanoic acids has been found in the literature.

It was not possible to isolate the unsaturated acid present in cutin in a pure state, but after treatment with performic acid the trihydroxy acid obtained was isolated and identified by mixed m.p.'s of the acid and its ester as phloionolic acid. Oxidation of the reaction product with chromium trioxide gave only azelaic acid, thus confirming the structure. The position of the glycol grouping established the position of the original double bond, and the absence in the infrared spectrum of a *trans* band in the 10.35 μ. region (determined on the concentrate of the unsaturated acid), established its *cis* configuration. The original acid must therefore have been 18-hydroxyoctadec-*cis*-9-enoic acid. This acid has been recently found in suberin (Ribas & Seoane, 1954).

The structure of 18-hydroxyoctadecanoic acid, the last acid isolated from the ether-soluble fraction, was established by its oxidation to octadecanedioic acid. This acid has been now isolated for the first time from a natural product.

In the light of the evidence presented, it is quite clear that the cutin acids reported on previously

(Freymy, 1859, 1881, 1885; Legg & Wheeler, 1925, 1929) were not pure compounds but mixtures, with the possible exception of Legg & Wheeler's

'phloionic' acid, which was probably impure phloionolic acid (cf. Zetsche & Böhler, 1931).

The nature of cutin itself before hydrolysis is of considerable interest, and some relevant information has become available as a result of the present work. No unsaponifiable material was detected in cutin, and all acids isolated so far from this substance after saponification have functionalities greater than 1. A large proportion (nearly 50 % of

cutin) has one carboxyl and more than one hydroxyl group per molecule. By inter-esterification of such molecules cross-linked materials can be built up, and during the reaction the carboxyl and hydroxyl groups become linked in a random fashion. These polymers are usually insoluble and elastic; they swell in certain solvents and are fairly stable thermally. Cutin possesses all these attributes and is very probably a natural polyester, but there is as yet no information about the randomness of attachment of functional groups. The fairly strong hydroxyl band at 2.92 μ. in the infrared spectrum of cutin (in medicinal paraffin) indicates that some free hydroxyl groups are present in cutin itself. This was also apparent from the examination of the isolated acids, in which a large excess of hydroxyl over carboxyl functions was found.

It was suggested long ago (Freymy, 1882) that suberin, the characteristic constituent of work, is chemically very similar to cutin. This view was supported by Legg & Wheeler (1925) and seems to be generally accepted to-day (e.g. Frey-Wissling, 1948). On considering the acids isolated from suberin so far in a pure state, namely 22-hydroxydocosanoic (phellonic), docosanedioic (phello-genic), 9:10-dihydroxyoctadecanedioic (phloionic),

9:10:18-trihydroxyoctadecanoic (phloionolic), *cis*- and *trans*-octadec-9-enedioic and 18-hydroxy-octadec-*cis*-9-enoic acids, it can indeed be seen that they are closely related to the cutin acids, and that two of them are identical (phloionolic and 18-hydroxyoctadec-*cis*-9-enoic acids). However, no dicarboxylic acids or acids of C₂₂ chain length were found in the cutin preparations here described. Another natural product, this time of insect origin (shellac), appears to be chemically similar to cutin. The major component (about 30–40 %) isolated from this material (Harries & Nagel, 1922) is aleuritic acid (trihydroxyhexadecanoic acid), a homologue of phloionolic acid. Other as yet unidentified hydroxy fatty acids of similar chain length are also present (cf. Wright, 1949).

SUMMARY

1. The ether-soluble fraction, obtained on saponification of cutin from *Agave americana*, was resolved by means of countercurrent distribution and reversed-phase partition chromatography, the latter method being adapted to a preparative scale.

2. Four acids, namely *threo*-9:10:18-trihydroxy-octadecanoic, 10:18-dihydroxyoctadecanoic, 18-hydroxyoctadecanoic and 10:16-dihydroxyhexadecanoic acid, were isolated in a pure state. The fifth, 18-hydroxyoctadec-*cis*-9-enoic acid, was obtained as its dihydroxy derivative.

3. The nature of cutin from *A. americana* and its similarity to certain other natural products is discussed.

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