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# EPOXYOCTADECANOIC ACIDS IN PLANT CUTINS AND SUBERINS

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**Key Word Index**—Higher plants; cutin; suberin; alcoholysis; $C_{18}$-epoxy acids; 9,10-epoxy-18-hydroxyoctadecanoic acid; 9,10-epoxy-18-hydroxyoctadec-12-enoic acid; 9,10-epoxyoctadecane-1,18-dioic acid; alkoxyhydrin alkyl esters of epoxy acids.

**Abstract**—Three $C_{18}$ epoxy acids occur in plant cutins and suberins. 9,10-Epoxy-18-hydroxyoctadecanoic acid is a common constituent of both cutins and suberins whilst 9,10-epoxy-18-hydroxyoctadec-12-enoic acid is also present in some cutins. 9,10-Epoxyoctadecane-1,18-dioic acid occurs more commonly in suberins. Epoxy acids may comprise up to 60% of the total monomers obtained from some polymers. The epoxy compounds are readily converted into their corresponding alkoxyhydrin alkyl esters on depolymerization of cutin or suberin by alcoholysis. The chromatographic and MS properties of the alkoxyhydrin derivatives enable them to be readily distinguished from other cutin and suberin hydroxyfatty acids and to be used for the qualitative and quantitative determination of epoxy acids in the polymers.

## INTRODUCTION

EPOXY acids derived from oleic, linoleic or linolenic acids by epoxidation of one of the double bonds are known to occur in several seed oil triglycerides.[1,2] The existence of similar epoxy acids in the lipid polymers cutin and suberin has been the subject of conjecture for a number of years and some workers have suggested that the the compounds may be artefacts.[3,4] Small amounts of epoxy acids, usually comprising less than 10% of the total monomers, have been detected in the alkaline hydrolysis products of some cutins and suberins, e.g. 9,10-epoxy-16-hydroxyhexadecanoic in Agave leaf,[5] 9,10-epoxy-18-hydroxyoctadecanoic in apple fruit,[3] tomato fruit,[6] cranberry fruit[7] and *Betula pendula* cork[8] and 9,10-epoxy-18-hydroxyoctadec-12-enoic in cranberry fruit.[7] Recent work by Kolattukudy and co-workers[9,10] using $LiAlH_4$ and $LiAlD_4$ reduction for depolymerization has shown that apple, pear, peach and grape fruit cutins contain substantial amounts of 9,10-epoxy-18-hydroxyoctadecanoic acid and its $\Delta_{12}$ analogue. The esterified epoxy acids of the polymers were reduced to the corresponding $C_{18}$ triols; MS analysis then showed a deuterium labelling pattern consistent with the presence of an epoxide group. The method does not permit the accurate quantitative determination of the epoxy acids since the $C_{18}$ triol is formed from the reduction of several other naturally occurring $C_{18}$ monobasic and

[1] WOLFF, I. A. (1966) *Science* **154,** 1140.
[2] HITCHCOCK, C. and NICHOLS, B. W. (1971) in *Plant Lipid Biochemistry*, p. 23, Academic Press, London.
[3] BRIESKORN, C. H. and BÖSS, A. J. (1964) *Fette, Seifen, Anstrichm.* **66,** 925.
[4] EGLINTON, G. and HUNNEMAN, D. H. (1968) *Phytochemistry* **7,** 313.
[5] CRISP, C. E. (1965) Ph.D. Thesis, University of California, Davis.
[6] SHISHIYAMA, J., ARAKI, F. and AKAI, S. (1970) *Plant Cell Physiol.* **11,** 323.
[7] CROTEAU, R. and FAGERSON, I. S. (1972) *Phytochemistry* **11,** 353.
[8] HOLLOWAY, P. J. (1972) *Chem. Phys. Lipids* **9,** 158.
[9] KOLATTUKUDY, P. E., WALTON, T. J. and KUSHWAHA, R. P. S. (1971) *Biochem. Biophys. Res. Commun.* **42,** 739.
[10] WALTON, T. J. and KOLATTUKUDY, P. E. (1972) *Biochemistry* **11,** 1885.

dibasic acids and evaluation is based solely on the interpretation of the mixed mass spectrum of the deuterolysis products.

Knowledge of the precise chemical composition of plant cutins is important because the cuticle is the first barrier through which foliar-applied chemicals must pass. Variations in the composition of cutin, or the occurrence of reactive groups such as epoxide in the cutin polymer, may influence the penetration and interaction of applied chemicals. In addition, the presence of epoxy acids may limit the degree of cross-linking of the polyester which in turn could influence the development and structure of the cuticular membrane.

The work described in this paper confirms the presence of epoxy acids in surface lipid polymers and reports a reliable method for the qualitative and quantitative assessment of the acids. The use of alcoholic KOH for depolymerization is shown to be unsuitable and is the main reason why the existence of epoxy acids has not been conclusively shown in earlier work. Epoxy acids are converted into other compounds by the reagents used. An alternative method of depolymerization using alcoholysis demonstrates the widespread occurrence of $C_{18}$ epoxy acids in cutins and suberins. The epoxy acids are readily converted to their corresponding alkoxyhydrin alkyl esters by alcoholysis which provides a convenient means of identification and determination by chromatography and MS.

## RESULTS

### *Experiments with 9,10-Epoxy-18-hydroxyoctadecanoic Acid*

The most widely used method for the degradation of cutin and suberin polymers is hydrolysis with excess of 3% alcoholic KOH, removal of the alcohol, acidification with strong mineral acid and extraction of the monomer acids with ether.[3,4,11–14] Three hours refluxing is sufficient to complete depolymerization.[14] 9,10-Epoxy-18-hydroxyoctadecanoic acid was partially converted (*ca.* 60%) by ethanolic KOH using this method into two products characterized by TLC as methyl esters and by GLC and GLC–MS as methyl ester trimethylsilyl (TMS) ethers. The recovery of unchanged epoxy acid from the reaction mixture was complicated by the occurrence of acid-catalysed reactions and was dependent on the nature of the mineral acid used and the conditions of acidification employed.

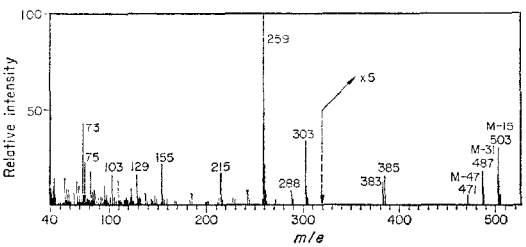



FIG. 1. MS OF GLC PEAK CONTAINING METHYL 9-ETHOXY-10,18-DIHYDROXYOCTADECANOATE AND METHYL 10-ETHOXY-9,18-DIHYDROXYOCTADECANOATE BIS TMS ETHERS.

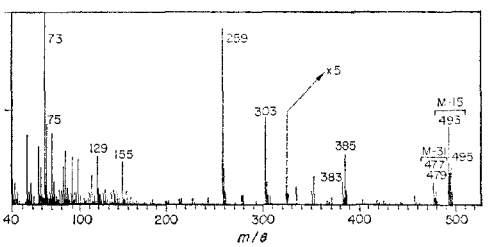
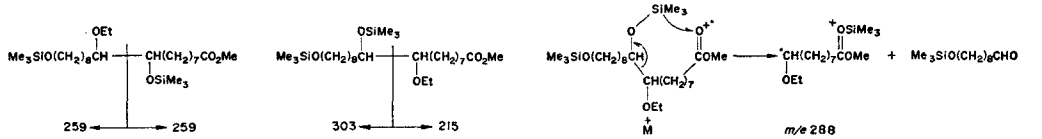



FIG. 2. MS OF GLC PEAK CONTAINING METHYL 9-CHLORO-10,18-DIHYDROXYOCTADECANOATE AND METHYL 10-CHLORO-9,18-DIHYDROXYOCTADECANOATE BIS TMS ETHERS.

[11] ZETZSCHE, F. (1932) in *Handbuch der Pflanzenanalyse* (KLEIN, G., ed.), Vol. 3, part 1, p. 205, Springer Wein.
[12] JENSEN, W. (1950) *Paperi Puu* **32**, 261.
[13] DUHAMEL, L. (1963) *Ann. Chim.* **8**, 315.
[14] BAKER, E. A., BATT, R. F. and MARTIN, J. T. (1964) *Ann. Appl. Biol.* **53**, 59.

The major product of the reaction (40–50%) was 9,10,18-trihydroxyoctadecanoic acid formed by $OH^-$ catalysed hydrolysis of the epoxide group. The acid was identified by TLC and GLC by comparison with authentic material and from the published MS of the methyl ester tris-TMS ether.[4] The second product (*ca.* 10–20%) co-eluted with 10,18-dihydroxyoctadecanoic and 9,10-dihydroxyoctadecane-1,18-dioic acids on TLC and with the latter compound by GLC on SE30 but not on OV210. The MS taken at the apex of the GLC peak (Fig. 1) was typical of long chain aliphatic methyl ester TMS ethers[15] and showed ions at *m/e* 503, 487 and 471 corresponding with M-15, M-31 and M-47 ($M^+$ 518). The most intense fragment ion occurred at *m/e* 259 with smaller ions at 303, 288 and 215; the fragments indicating a mixed MS. The compounds were identified as the methyl esters of the ethanolysis products of the epoxy acid. Because there is an equal probability of attack on the two C–O bonds of the epoxide, a mixture of 9-ethoxy-10,18-dihydroxy- and 10-ethoxy-9,18-dihydroxyoctadecanoic acids is formed. The isomers were not resolved by the chromatographic techniques used. The fragment ions observed in the MS correspond with cleavage between the two carbon atoms bearing the ethoxy and TMS ether groups (Scheme 1). The rearrangement ion *m/e* 288 arises from the isomer with the TMS ether group in the 10 position, the TMS group rearranging on the methyl ester-containing moiety followed by expulsion of the terminal part of the molecule with an ether oxygen (Scheme 1). Analogous rearrangements occur with *vic*-TMS ether methyl esters[16] and certain mono-TMS ether methyl esters[17] but not with alkoxy methyl esters.[18] The identity of the compounds was confirmed by subsequent experiments using ethanolysis.

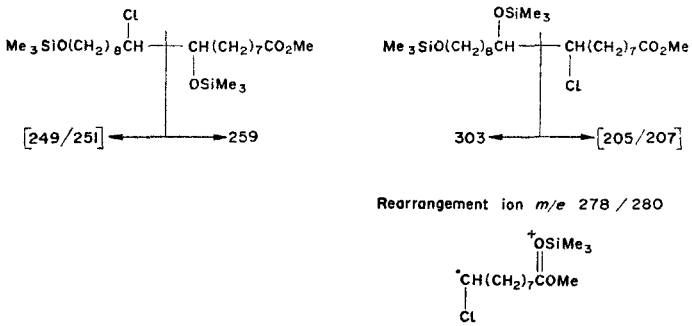



SCHEME 1. MS FRAGMENTATIONS OF METHYL 9-ETHOXY-10,18-DIHYDROXYOCTADECANOATE AND METHYL 10-ETHOXY-9,18-DIHYDROXYOCTADECANOATE BIS TMS ETHERS (see Fig. 1).

The use of hydrochloric acid for acidification after hydrolysis gave a third product which was found in variable amounts depending on the concentration of acid used and the pH of the aqueous suspension of acids prior to recovery with ether. The formation of the product was favoured by the use of conc. HCl or by allowing the pH of the solution to reach 1–3 and resulted in a corresponding reduction in the amount of unchanged epoxy acid recovered from the reaction. The product corresponded with 9,10-dihydroxyoctadecanoic acid by TLC but did not correspond with any of the usual $C_{18}$ hydroxy acids by GLC. The overall appearance of the MS (Fig. 2) was similar to that of the ethoxyhydrin methyl ester TMS ethers but showed ions at *m/e* 493, 495 (M-15) and 477, 479 (M-31) with a 3/1 isotopic ratio of the ions separated by 2 m.u. The abundance ratio is characteristic of compounds containing one chlorine atom. The product was identified as a mixture of the two halohydrins, 9-chloro-10,18-dihydroxyoctadecanoic and 10-chloro-9,18-dihydroxyoctadecanoic acids which were not separated by the chromatographic methods. The major fragment ions *m/e* 259 and 303 correspond with cleavage between the two carbon atoms bearing the chloro and TMS ether groups (Scheme 2) although the chlorine-containing fragments of

[15] EGLINTON, G., HUNNEMAN, D. H. and MCCORMICK, A. (1968) *Org. Mass Spectr.* **1**, 593.
[16] CAPELLA, P. and ZORZUT, C. M. (1968) *Anal. Chem.* **40**, 1458.
[17] RICHTER, W. J. and BURLINGAME, A. L. (1968) *Chem. Commun.* 1158.
[18] NIEHAUS W. G. and RYHAGE, R. (1968) *Anal. Chem.* **40**, 1840.

this cleavage are not visible in the MS. Small rearrangement ions at *m/e* 278 and 280 were also present which result from the rearrangement of the 10-TMS ether group on the methyl ester group. The same compound was produced by reaction of excess concentrated HCl with an aqueous suspension of 9,10-epoxy-18-hydroxyoctadecanoic acid thus providing further proof of identity. Under these conditions complete conversion to halohydrin occurred within 5 min. Acidification experiments using $H_2SO_4$ instead of HCl showed that acid-catalysed hydrolysis of the epoxy acid to 9,10,18-trihydroxyoctadecanoic acid took place. The extent of hydrolysis again was dependent upon the concentration of acid used and the pH of the aqueous solution of acids before recovery with ether. Similar experiments with an authentic sample of 9,10,18-trihydroxyoctadecanoic acid confirmed that neither ethoxy-hydrin, halohydrin nor epoxide formation occurred after treatment with ethanolic KOH or mineral acids.



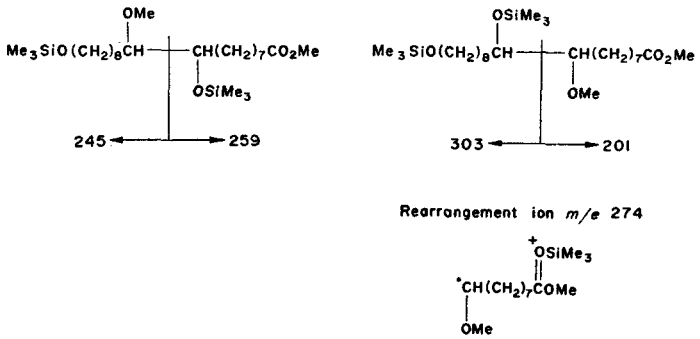

SCHEME 2. MS FRAGMENTATIONS OF METHYL 9-CHLORO-10,18-DIHYDROXYOCTADECANOATE AND METHYL 10-CHLORO-9,18-DIHYDROXYOCTADECANOATE BIS TMS ETHERS (see Fig. 2).

The experiments with the epoxy acid show that although alcoholic alkali may be efficient in hydrolysing the ester bonds of cutin and suberin polymers, if epoxides are present in the polymer or if epoxy acids are liberated during the hydrolysis they are also susceptible to attack by both the alcoholic alkali and the mineral acids used for their recovery. The epoxide group is still reactive when positioned within a long aliphatic chain and undergoes both base- and acid-catalysed reactions. The ethanolysis reaction occurs because in alcoholic solutions of KOH or NaOH the hydroxide/alkoxide equilibrium greatly favours alkoxide formation even in the presence of considerable amounts of water.[19] Also, in the hydrolysis of cutin or suberin with ethanolic KOH, it is likely that the alkali reacts primarily with the ethyl esters of the liberated acids rather than the ester groups of the polymer, i.e. ethanolysis precedes hydrolysis. The experiments led to the identification of two unidentified acids reported in the hydrolysis products of the suberins of *B. pendula* and *Quercus suber*[8] as the ethoxyhydrin and halohydrin derivatives of 9,10-epoxy-18-hydroxyoctadecanoic acid. This provided indirect evidence for the presence of the epoxy acid in both suberins.

The mixed products obtained from the treatment of an epoxy acid with alcoholic alkali also indicate that previous analyses of cutin or suberin based on hydrolysis with alcoholic alkali may not reflect their true qualitative and quantitative composition. The occurrence of unchanged epoxy acids in hydrolysis products does establish the existence of epoxy acids in the original polymer but the amounts found do not represent the total epoxide content.

[19] GLASS, R. L. (1971) *Lipids* **6**, 919.

The amounts of free epoxide recovered depend upon the extent to which epoxide is present in the polymer, the duration of the hydrolysis, the nature and concentration of the mineral acid used for acidification and the derivatization procedure used for GLC analysis. The use of acetic anhydride[5] and trifluoroacetic anhydride[20] for the preparation of acetyl derivatives also converts the epoxide group to the corresponding *vic*-diacetyl derivative. In addition, the estimation of cutin and suberin monomers containing *vic*-diol groups, for example, 9,10,18-trihydroxyoctadecanoic and 9,10-dihydroxyoctadecane-1,18-dioic acids is unreliable using alcoholic alkali because the compounds may be present in the original polymer or be generated from the corresponding epoxide by hydrolysis. The use of aqueous alkali for the depolymerization of cutin[21,22] and suberin[23,24] does not permit any assessment of epoxy acids because they are hydrolysed to the *vic*-diols.




SCHEME 3. MS FRAGMENTATIONS OF METHYL 9,18-DIHYDROXY-10-METHOXYOCTADECANOATE AND METHYL 10,18-DIHYDROXY-9-METHOXYOCTADECANOATE BIS TMS ETHERS (see Fig. 3).

The susceptibility of the epoxide group of an epoxy fatty acid to alcoholysis and the fact that efficient depolymerization of cutin and suberin may also be achieved by this method[25,26] led to further investigations of the use of alcoholysis techniques particularly for the determination of epoxy acids in cutins and suberins. Treatment of 9,10-epoxy-18-hydroxyoctadecanoic acid or its methyl ester at room temperature with an excess of a solution of sodium methoxide in methanol or sodium ethoxide in ethanol gave a rapid conversion to a single product in each case. Alcoholysis gave one spot by TLC and one peak by GLC on both SE30 and OV210. Examination of the MS of the TMS ethers confirmed the formation of two alkoxyhydrin isomers in each case produced by random attack on both carbon atoms of the epoxide group. The most important MS cleavage was between the carbon atoms bearing the alkoxy and TMS ether groups together with characteristic ions produced by rearrangements of the TMS-ether group on the ester group of the molecule. The alkoxy-containing fragment ions in the MS are less intense than the corresponding di-TMS ether and alkyl ester-TMS ether ions.

The mixed MS of methyl 9,18-dihydroxy-10-methoxyoctadecanoate and methyl 10,18-dihydroxy-9-methoxyoctadecanoate bis TMS ethers obtained from a MS scan taken at the

[20] BAKER, E. A. and HOLLOWAY, P. J. (1970) *Phytochemistry* **9**, 1557.
[21] MATIC, M. (1956) *Biochem. J.* **63**, 168.
[22] MEAKINS, G. D. and SWINDELLS, R. (1959) *J. Chem. Soc.* 1044.
[23] GUILLEMONAT, A. and CESAIRE, G. (1949) *Bull. Soc. Chim. Fr.* **16**, 792.
[24] SEOANE, E. and RIBAS, I. (1951) *An. R. Soc. Esp. Fis. Quim.* **47B**, 61.
[25] GUILLEMONAT, A. and STRICH, A. (1953) *Bull. Soc. Chim. Fr.* 378.
[26] BRIESKORN, C. H. and KABELITZ, L. (1971) *Phytochemistry* **10**, 3195.

apex of the single GLC peak is shown in Fig. 3. Fragmentation of the 9-methoxy isomer yields ions *m/e* 303 and 201 and the 10-methoxy isomer, ions *m/e* 259 and 245 with the rearrangement ion *m/e* 274 (Scheme 3). Further structural confirmation of the alkoxyhydrin structure was obtained from the MS of the ethoxyhydrin ethyl ester TMS ethers (Fig. 4) which showed the the corresponding increases in the *m/e* values of the fragment ions and the rearrangement ion (Scheme 4). The high end of the spectrum showed M-15 (-Me), M-45 (-OEt) and M-61 (-Me-EtOH) ions. The ethanolysis product of the epoxy acid after conversion to the methyl ester was identical with the ethoxyhydrin methyl esters produced by ethanolic KOH.

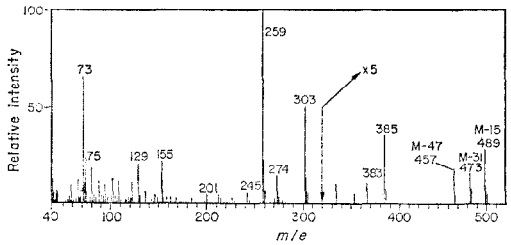



FIG. 3. MS OF GLC PEAK CONTAINING METHYL 9,18-DIHYDROXY-10-METHOXYOCTADECANOATE AND METHYL 10,18-DIHYDROXY-9-METHOXYOCTADECANOATE BIS TMS ETHERS.

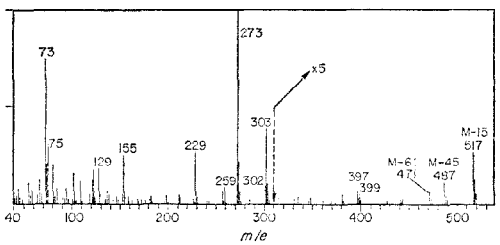
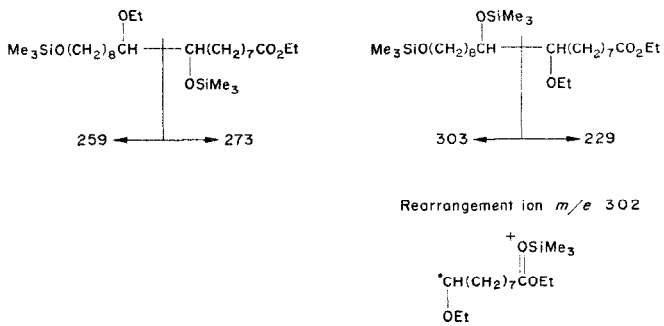



FIG. 4. MS OF GLC PEAK CONTAINING ETHYL 9-ETHOXY-10,18-DIHYDROXYOCTADECANOATE AND ETHYL 10-ETHOXY-9,18-DIHYDROXYOCTADECANOATE BIS TMS ETHERS.

The chromatographic and MS properties of the TMS derivatives of the methoxyhydrin methyl esters and ethoxyhydrin ethyl esters of epoxy acids are sufficiently characteristic to enable them to be distinguished from other cutin and suberin hydroxy-fatty acids.




SCHEME 4. MS FRAGMENTATIONS OF ETHYL 9-ETHOXY-10,18-DIHYDROXYOCTADECANOATE AND ETHYL 10-ETHOXY-9,18-DIHYDROXYOCTADECANOATE BIS TMS ETHERS (See Fig. 4).

*Experiments with Cutins and Suberins*

Alcoholysis experiments with a range of cuticles and corks showed that satisfactory depolymerization of cutin and suberin could be achieved by refluxing with an excess of 3% sodium alkoxide in alcohol for 3 hr. The weights of methyl esters obtained by methanolysis were similar to the weights of $Et_2O$ soluble acids obtained if depolymerization was carried out with ethanolic KOH (Table 1). *Quercus suber* cork and *Lycopersicon esculentum* fruit cutin each gave the same result by the two methods. About 10% more material was obtained by ethanolic KOH from the cuticles of *Agave americana* leaf, *Gasteria planifolia* leaf,

*Malus pumila* fruit and the cork of *B. pendula* but more material was obtained from the methanolysis of *Castanea sativa* cork.

TABLE 1. COMPARISON OF THE WEIGHTS OF MONOMERS OBTAINED FROM CUTICLES AND CORKS BY METHANOLYSIS AND ETHANOLIC KOH

| | Methanolysis | Ethanolic KOH |
|---|---|---|
| Cuticles* | | |
| *Agave americana* leaf | 51 | 60 |
| *Gasteria planifolia* leaf | 44 | 57 |
| *Sansevieria trifasciata* leaf | 47 | 51 |
| *Ilex aquifolium* leaf | 55 | 58 |
| *Lycopersicon esculentum* fruit | 89 | 87 |
| *Malus pumila* cv. "Laxton Superb" fruit | 64 | 74 |
| Corks† | | |
| *Betula pendula* | 51 | 61 |
| *Quercus suber* | 44 | 45 |
| *Castanea sativa* | 46 | 36 |
| *Malus pumila* cv. "Laxton Superb" | 40 | 36 |

* Wt % of dry cellulose-free cuticular membrane.
† Wt % of dry extractive-free cork.

Alkoxyhydrin alkyl esters of $C_{18}$ acids were found in the alcoholysis products obtained from many cutins and suberins, thus showing the widespread occurrence of the esterified epoxy acids in the original polymers. The other constituents of the alcoholysis products were the alkyl esters of fatty and hydroxy-fatty acids. The absence of alkyoxyhydrins in the depolymerization products obtained from cutins and suberins by aqueous KOH confirmed that the compounds did not occur naturally in the polymers. Differences were inevitably found in the qualitative and quantitative compositions of cutins and suberins if determined by both alcoholysis and ethanolic KOH. The occurrence of acids having *vic*-diol groups in the ethanolic KOH products was invariably followed by the identification of both the *vic*-diol acid and the corresponding alkoxyhydrin in the products from alcoholysis. The most marked differences in quantitative composition were found in polymers which yielded large amounts of *vic*-diol acids using ethanolic KOH, alcoholysis giving substantial amounts of the corresponding alkoxyhydrin with reduced quantities of *vic*-diol acids. Ethanolic KOH also produced from these cutins and suberins variable amounts of free epoxide, ethoxyhydrin and also halohydrin if HCl was used for acidification.

The analytical results obtained by alcoholysis were reliable only if precautions were taken to exclude moisture from the reaction. In the presence of water significant amounts of free acids, identified as TMS ether TMS esters by GLC and MS, were formed by hydrolysis of the alkyl esters with hydroxide generated from alkoxide. The problem was minimized by using dried alcohol for preparing alkoxide solutions and using drying tubes on apparatus. For routine analysis methanolysis was preferred because a commercial source of specially dried MeOH was available.

The alkoxyhydrin alkyl esters derived from the cutin and suberin epoxy acids were readily isolated from the total alcoholysis products using preparative-TLC and characterized by GLC and GLC–MS. Three $C_{18}$ epoxy acids were identified and the relative amounts determined in a number of cutins and suberins.

TABLE 2. CHROMATOGRAPHIC DATA OF THE ALKOXYHYDRIN ALKYL ESTER DERIVATIVES OF CUTIN AND SUBERIN EPOXYOCTADECANOIC ACIDS

| Compounds | $R_f$* | Relative $R_t$ SE30† | Relative $R_t$ OV210‡ |
|---|---|---|---|
| Methyl 9,18-dihydroxy-10-methoxyoctadecanoate<br>Methyl 10,18-dihydroxy-9-methoxyoctadecanoate | 0·18 | 1·26 | 1·24 |
| Ethyl 9-ethoxy-10,18-dihydroxyoctadecanoate<br>Ethyl 10-ethoxy-9,18-dihydroxyoctadecanoate | 0·20 | 1·32 | 1·29 |
| Methyl 9,18-dihydroxy-10-methoxyoctadec-12-enoate<br>Methyl 10,18-dihydroxy-9-methoxyoctadec-12-enoate | 0·18 | 1·23 | 1·21 |
| Ethyl 9-ethoxy-10,18-dihydrixyoctadec-12-enoate<br>Ethyl 10-ethoxy-9,18-dihydroxyoctadec-12-enoate | 0·20 | 1·29 | 1·26 |
| Dimethyl 9-hydroxy-10-methoxyoctadecane-1,18-dioate | 0·52 | 1·23 | 1·40 |
| Diethyl 9-ethoxy-10-hydroxyoctadecane-1,18-dioate | 0·62 | 1·33 | 1·52 |

* Silicagel G, $CHCl_3$–EtOAc (7:3).
† TMS ether derivative, column 1·5 m × 2 mm packed 3% SE30, $N_2$ 30 ml/min, 130–300° at 6°/min, internal standard *n*-tetracosane.
‡ TMS ether derivative, column 1·5 m × 2 mm packed 3% OV210, $N_2$ 30 ml/min, 120–250° at 6°/min internal standard *n*-octacosane.

### 9,10-*Epoxy*-18-*hydroxyoctadecanoic Acid*

The alkoxyhydrins of the epoxy acid occurred very commonly in the alcoholysis products of both cutins and suberins. They were obtained as a separate band from preparative-TLC and were identified by comparison of chromatographic data (Table 2) and MS of their TMS ethers with the methoxyhydrin methyl esters (Fig. 3) and ethoxyhydrin ethyl esters (Fig. 4) previously prepared from an authentic sample of 9,10-epoxy-18-hydroxyoctadecanoic acid.

TABLE 3. RELATIVE AMOUNTS (%) OF $C_{18}$ EPOXY AND *vic*-DIOL ACIDS IN THE TOTAL MONOMERS OBTAINED FROM SOME CUTINS AND SUBERINS BY METHANOLYSIS

| | Cutin | | | | Suberin | |
|---|---|---|---|---|---|---|
| Acid | *Agave americana* | *Gasteria planifolia* | *Sansevieria trifasciata* | *Ilex aquifolium* | *Betula pendula* | *Quercus suber* |
| 9,10-Epoxy-18-hydroxyoctadecanoic* | 45·6 | 27·4 | 48·6 | 30·1 | 17·4 | 18·2 |
| 9,10,18-Trihydroxyoctadecanoic† | 17·7 | 9·6 | 27·9 | 13·5 | 30·3 | 6·2 |
| 9,10-Epoxy-18-hydroxyoctadec-12-enoic* | — | 32·9 | — | — | — | — |
| 9,10,18-Trihydroxyoctadec-12-enoic† | — | 5·1 | — | — | — | — |
| 9,10-Epoxy-octadecane-1,18-dioic* | — | — | — | — | 1·1 | 22·6 |
| 9,10-Dihydroxyoctadecane-1,18-dioic† | — | — | — | — | trace | 9·2 |

* Determined by GLC as corresponding methoxyhydrin methyl ester TMS ether.
† Determined by GLC as corresponding methyl ester TMS ether.

The epoxy acid was identified in cutins and suberins from a wide range of species and invariably in those species in which 9,10,18-trihydroxyoctadecanoic acid had previously been identified following alcoholic alkali depolymerization. 9,10-Epoxy-18-hydroxyoctadecanoic acid was found to comprise about 50% of the cutin monomers of *A. americana* and *Sansevieria trifasciata* leaves and was an important constituent of the cutins of *G. planifolia*

and *Ilex aquifolium* leaves and the suberins of *B. pendula* and *Q. suber* (Table 3). The relative amounts of the epoxy acid were determined by GLC as the methoxyhydrin methyl ester TMS ether. The acid was also identified in substantial amounts in the cutins of the fruits of several *M. pumila* cultivars, of the leaves and fruits of *Euonymus europaeus*, of the leaves of several Gramineae including *Lolium perenne*, *Avena sativa*, *Secale cereale*, *Phleum pratense*, *Dactylis glomerata* and *Holcus lanatus* and in the suberins of *M. pumila* cultivars, *Q. robur*, *C. sativa* and *Acer griseum*. Much smaller amounts were identified in cutins where the predominant cutin acid was dihydroxyhexadecanoic, e.g. in those of the leaves of *Coffea arabica*, *M. pumila* cultivars, *Zea mays*, *Citrus aurantifolia* and of the fruits of *L. esculentum* and *Ribes nigrum*.

The presence of 9,10-epoxy-18-hydroxyoctadecanoic acid in a cutin or suberin was usually accompanied by variable amounts of 9,10,18-trihydroxyoctadecanoic acid and 18-hydroxyoctadec-9-enoic acid. The use of alcoholysis permits the reliable assessment of the relative amounts of the epoxy and *vic*-diol acids (Table 3). The amounts of 9,10,18-trihydroxyoctadecanoic acid found in *A. americana* leaf cutin are much less than those reported by previous workers[5,21] for the same species, because their analyses include the acid derived from the hydrolysis of 9,10-epoxy-18-hydroxyoctadecanoic acid. Compounds erroneously reported[8] to be constituents of *B. pendula* and *Q. suber* suberins include the hydrolysis, ethanolysis and HCl products of 9,10-epoxy-18-hydroxyoctadecanoic acid. Analysis by alcoholysis using the same cork samples showed that the major acid of *B. pendula* suberin is 9,10,18-trihydroxyoctadecanoic (30%) but that 18% is present as the corresponding epoxide whereas in *Q. suber* suberin both 9,10,18-trihydroxyoctadecanoic (6%) and 9,10-epoxy-18-hydroxyoctadecanoic (18%) are present.

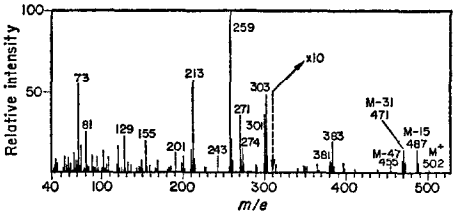



FIG. 5. MS OF GLC PEAK CONTAINING METHYL 9,18-DIHYDROXY-10-METHOXYOCTADEC-12-ENOATE AND METHYL 10,18-DIHYDROXY-9-METHOXYOCTADEC-12-ENOATE BIS TMS ETHERS OBTAINED FROM CUTIN OF *Gasteria planifolia* LEAF.

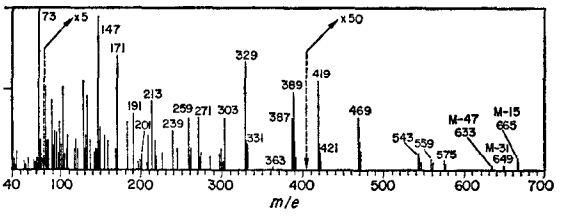
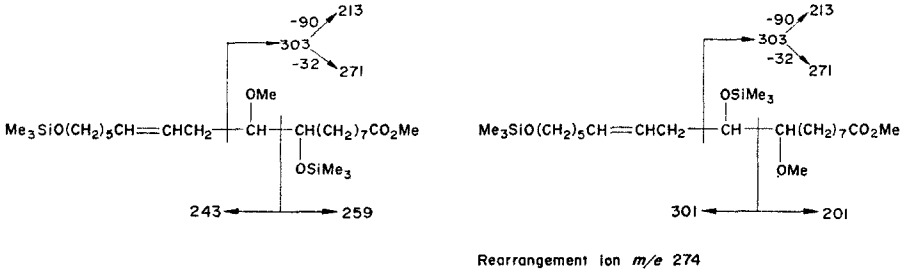



FIG. 6. MS OF GLC PEAK CONTAINING METHYL 9,12,13,18-TETRAHYDROXY-10-METHOXYOCTADECANOATE AND METHYL 10,12,13,18-TETRAHYDROXY-9-METHOXYOCTADECANOATE TETRAKIS TMS ETHERS OBTAINED AFTER OSMYLATION OF COMPOUNDS OF FIG. 5.

### 9,10-*Epoxy*-18-*hydroxyoctadec*-12-*enoic Acid*

The acid was found only in cutin and occurred much less commonly than the corresponding saturated acid. It was the major constituent of *G. planifolia* leaf cutin comprising 33% of the total monomers, and it was also identified but not determined in the fruit cutins of several *M. pumila* cultivars, confirming the results obtained by reduction.[10] The alkoxyhydrins of the epoxy acid were isolated by preparative-TLC as a mixed band containing the saturated analogues and were resolved from the saturated compounds by GLC (Table 2). The unsaturated alkoxyhydrins were identified by MS of both the methyl ester TMS ether and the methyl ester TMS ethers of the osmylation product. The mixed MS of the unsaturated methoxyhydrin methyl ester TMS ethers (Fig. 5) showed a weak $M^+$ 502 and

ions at *m/e* 487 (M-15), 471 (M-31) and 455 (M-47). The fragment ions *m/e* 259, 243 and 301, 201 together with the rearrangement ion *m/e* 274 indicated that the double bond occurred in the fragments containing the terminal TMS ether group (Scheme 5). Additional strong fragment ions at *m/e* 303, 271, and 213 were also present in the spectrum, the ion *m/e* 303 arising from cleavage between carbon atoms 10 and 11 (Scheme 6) and losing MeOH and $Me_3SiOH$ to yield ions *m/e* 271 and 213 respectively. The metastable ions for these transitions were also observed in the MS. This type of fragmentation is characteristic of aliphatic compounds containing in-chain TMS ether groups separated from a double bond by one methylene group and arises from the ease of expulsion of the neutral allylic radical.[10] The presence of the fragment ion *m/e* 303 thus provided strong evidence for the double bond in the 12-position of the unsaturated alkoxyhydrins.



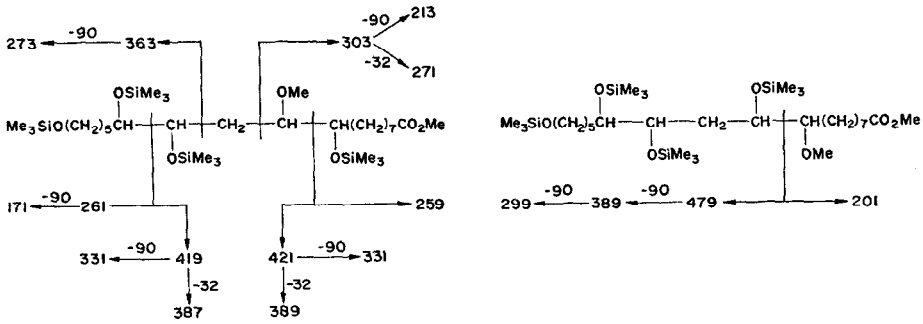

SCHEME 5. MS FRAGMENTATIONS OF METHYL 9,18-DIHYDROXY-10-METHOXYOCTADEC-12-ENOATE AND METHYL 10,18-DIHYDROXY-9-METHOXYOCTADEC-12-ENOATE BIS TMS ETHERS (see Fig. 5).

Confirmation of the 12-position of the double bond was obtained by MS analysis of the product obtained by osmylation of the unsaturated methoxyhydrin methyl esters (Fig. 6). The product was identified as a mixture of methyl 10,12,13,18-tetrahydroxy-9-methoxyoctadecanoate and methyl 9,12,13,18-tetrahydroxy-10-methoxyoctadecanoate tetrakis TMS ethers. The overall appearance of the MS was similar to the published spectra of other aliphatic poly-TMS ether methyl esters[15,26,27] and showed weak ions corresponding with M-15, M-31, and M-47 ($M^+$ 680) and also M-90-15 (575), M-90-31 (559), M-90-47 (543) and M-180-31 (469). The principal fragment ions (Scheme 6) were derived from cleavage between the carbon atoms bearing the *vic*-TMS ether groups of the diol produced from osmylation of the double bond and between the carbon atoms bearing the methoxy and TMS ether groups of the methoxyhydrin, the larger *m/e* ions readily losing MeOH and $Me_3SiOH$. Fragment ions produced by cleavage between the *vic*-TMS ether groups are common to both isomers giving ions *m/e* 261 and *m/e* 419 of low relative intensity which in turn yield ions of greater intensity by loss of MeOH and $Me_3SiOH$ at *m/e* 171 (261–90), 387 (419–32) and 329 (419–90). Common fragments are also formed by a cleavage between $C_{10}$ and $C_{11}$, and $C_{11}$ and $C_{12}$ giving weak ions at *m/e* 363 and 273 (363–90) and stronger ions at *m/e* 303, 271 (303–32) and 213 (303–90). Fragmentation of the 9-methoxy isomer yields ions *m/e* 259 and ions *m/e* 421, 389 (421–32) and 331 (421–90) whilst the 10-methoxy isomer yields ions *m/e* 201 and 389 (479–90).

9,10,18-Trihydroxyoctadec-12-enoic, and 18-hydroxyoctadecadienoic acids were also present in cutins which contained 9,10-epoxy-18-hydroxyoctadec-12-enoic acid (Table 3). The *vic*-diol acid was identified by GLC and from the MS of its methyl ester tris TMS

[27] PERKINS, E. G. and ARGOUDELIS, C. J. (1969) *Lipids* **4**, 619.

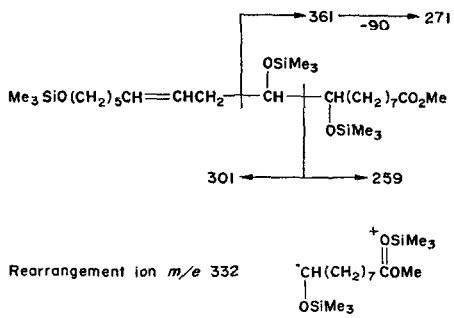
ether. The MS was identical with the published MS of methyl 9,10,18-trihydroxyoctadecenoate tris TMS ether[4] except that ion *m/e* 360 was not present. However, a strong fragment ion *m/e* 361 was present which yielded ion *m/e* 271 by loss of $Me_3SiOH$, the fragmentation establishing the double bond at position 12 on the acid (Scheme 7). The diunsaturated acid was identified by GLC and gave an MS identical with that of methyl 18-hydroxyoctadeca-9,12-dienoate TMS ether.[4]




SCHEME 6. MS FRAGMENTATIONS OF METHYL 9,12,13,18-TETRAHYDROXY-10-METHOXYOCTADECANOATE AND METHYL 10,12,13,18-TETRAHYDROXY-9-METHOXYOCTADECANOATE TETRAKIS TMS ETHERS (see Fig. 6).

### 9,10-*Epoxyoctadecane*-1,18-*dioic Acid*

The alkoxyhydrin derived from the epoxy acid was identified mainly in the alcoholysis products obtained from suberins. The natural occurrence of the epoxy acid has not been previously recorded. The acid is a major constituent of *Q. suber* suberin and a minor one of *B. pendula* suberin (Table 3). It was also identified in significant amounts in the suberins of several *M. pumila* cultivars, *C. sativa*, *Q. robur* and *A. griseum*.




SCHEME 7. MS FRAGMENTATION OF METHYL 9,10,18-TRIHYDROXYOCTADEC-12-ENOATE TRIS TMS ETHER.

The methoxyhydrin methyl ester of the epoxy acid was isolated from the total methanolysis products by preparative-TLC, being present in a band which included ω-hydroxymonobasic acid methyl esters. The compound was resolved from the ω-hydroxymonobasic homologues using GLC. The ethoxyhydrin ethyl ester was resolved from the ω-hydroxymonobasic acid ethyl esters by TLC and could be obtained as a separate band by preparative-TLC of the total ethanolysis products. The chromatographic data of the two alkoxyhydrin alkyl esters are summarised in Table 2.

The alkoxyhydrin alkyl esters were identified from the MS of their TMS ether derivatives.

The MS of the methoxyhydrin methyl ester TMS ether is shown in Fig. 7. The overall appearance of the spectrum was similar to that of the corresponding methoxyhydrin monobasic acids (Fig. 3) except that an ion *m/e* 146 was present which is indicative of a mono-TMS ether.[15] The relative intensities of the M-15, M-31 and M-47 ions were also different from those of the methoxyhydrin monobasic acid, the corresponding dibasic acid showing only a weak M-15 ion. The principal fragment ions occurred at *m/e* 259 and 201 together with a rearrangement ion *m/e* 274 (Scheme 8). A small fragment ion *m/e* 303 arising from a cleavage on both sides of the methoxy-TMS ether groups was also present. The compound was identified as dimethyl 9-hydroxy-10-methoxyoctadecane-1,18-dioate TMS ether. Only one methoxyhydrin isomer is formed because of the symmetrical nature of the original epoxy acid.

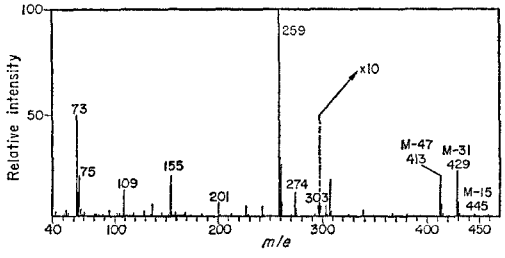



FIG. 7. MS OF DIMETHYL 9-HYDROXY-10-METHOXY-OCTADECANE-1,18-DIOATE TMS ETHER OBTAINED FROM THE METHANOLYSIS PRODUCTS OF *Quercus suber* CORK.

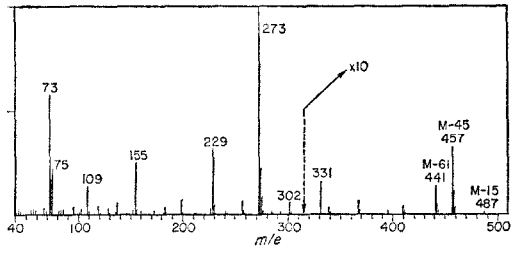
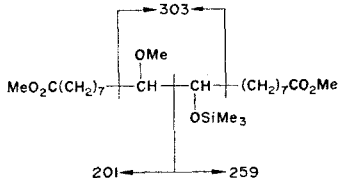


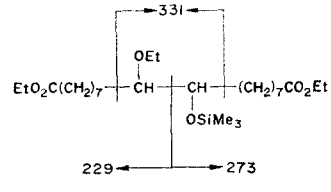

FIG. 8. MS OF DIETHYL 9-ETHOXY-10-HYDROXY-OCTADECANE-1,18-DIOATE TMS ETHER OBTAINED FROM THE ETHANOLYSIS PRODUCTS OF *Quercus suber* CORK.

The identity of the epoxy acid was further confirmed by examination of the MS of the ethoxyhydrin ethyl ester TMS ether (Fig. 8) which showed a corresponding increase of the *m/e* values of the fragment ions to 331, 273 and 229 and the rearrangement ion to 302 (Scheme 9).




Rearrangement ion *m/e* 274

SCHEME 8. MS FRAGMENTATION OF DIMETHYL 9-HYDROXY-10-METHOXYOCTADECANE-1,18-DIOATE TMS ETHER (see Fig. 7.)




Rearrangement ion *m/e* 302

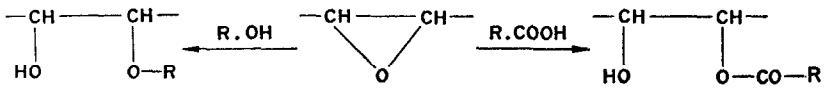
SCHEME 9. MS FRAGMENTATION OF DIETHYL 9-ETHOXY-10-HYDROXYOCTADECANE-1,18-DIOATE TMS ETHER (see Fig. 8).

As was found with the other epoxy acids identified in the present work the corresponding *vic*-diol and unsaturated acids were also present, together with 9,10-epoxyoctadecane-1,18-dioic acid. 9,10-Dihydroxyoctadecane-1,18-dioic and octadec-9-ene-1,18-dioic acids were identified by GLC and by MS of their corresponding methyl ester TMS ether or dimethyl ester derivatives and comparison with published data.[4,8,15] The use of alcoholysis permitted the reliable assessment of the relative amounts of the epoxy acid and *vic*-diol acids in suberins (Table 3). The amounts of 9,10-dihydroxyoctadecane-1,18-dioic acid found in *Q. suber* are less than those reported by workers using hydrolysis methods.[8] These methods also failed to establish the presence of the corresponding epoxy acid.

## DISCUSSION

The epoxy acid contents of the cutins and suberins of different plant species vary from less than 1–60%. The polymers which contain large proportions of epoxide monomers must be essentially linear polyesters because the epoxy acids can be involved in esterification only through their terminal hydroxyl and carboxyl groups. Cross-linking of the polymer chains can be brought about only by other constituent acids which have secondary hydroxyl groups available for esterification, e.g. dihydroxyhexadecanoic and 9,10,18-trihydroxyoctadecanoic. The molecular structure of polymers which contain mainly epoxide monomers must differ from that of polymers made up chiefly of dihydroxyhexadecanoic acid, e.g. the cutins of *C. arabica* leaf,[28] *Vicia faba* leaf[29] and *L. esculentum* fruit.[30] They are likely to be similar, however, to those polymers which contain predominantly ω-hydroxymonobasic and α,ω-diabasic acids, e.g. the suberins of gymnosperms,[31] *Ribes* species[32] and *Solanum tuberosum*.[33]

The epoxide group of the epoxy acid constituents of cutin and suberin may also be involved in the polymerization process and provide an additional mechanism for cross-linking the polymer chains. Reaction of the epoxide groups with the carboxyl groups of other monomers could form ester linkages or with hydroxyl groups may provide ether linkages.[34] Some evidence for the latter in Agave cutin has been presented.[5]

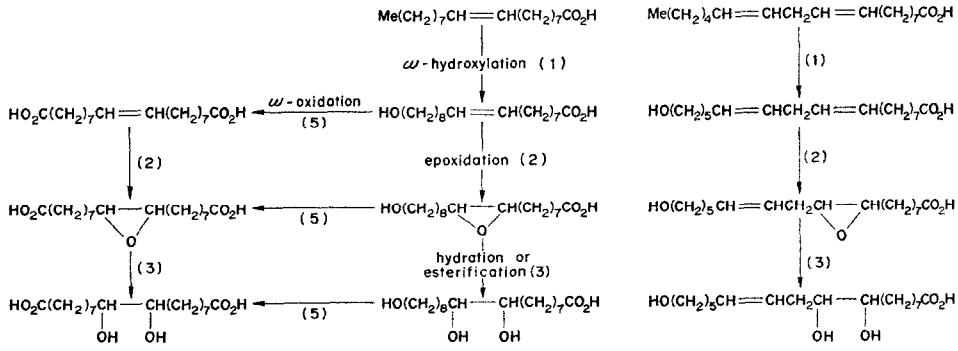



The widespread occurrence of three $C_{18}$ epoxy acid monomers in plant cutins and suberins strongly suggests a specific hydroxylation/epoxidation biosynthetic pathway from oleic and linoleic acids. The biosynthesis of epoxide groups from ethylenic bonds is known in other plant systems[35,36] and involves incorporation of molecular oxygen by a specific enzyme.[37] The pathway also provides a route for the synthesis of several $C_{18}$ hydroxy-fatty acids (Scheme 10) and is supported by the specific incorporation of oleic acid-1-$^{14}$C by apple skin slices into the $C_{18}$ diol, triol and tetraol monomers obtained after reduction of the cutin.[9] Thus, it is unlikely that the formation of $C_{18}$ hydroxy cutin acids occurs by free radical or other reactions of hydroperoxide intermediates generated by autoxidation or lipoxidases as proposed by earlier workers.[5,38]

The epoxidation system of cutin and suberin would appear to be specific for $\Delta_9$ of unsaturated octadecanoic acids. No corresponding 12,13-epoxide was found in the mono-unsaturated epoxyoctadecanoic acid identified in cutin. The formation of 9,10-epoxyoctadecane-1,18-dioic acid presumably takes place by ω-oxidation of 18-hydroxyoctadec-9-enoic acid followed by epoxidation, or by ω-oxidation of 9,10-epoxy-18-hydroxyoctadecanoic acid itself (Scheme 10). The *vic*-diol acids of cutins and suberins may arise from

[28] HOLLOWAY, P. J., DEAS, A. H. B. and KABAARA, A. M. (1972) *Phytochemistry* **11**, 1443.
[29] KOLATTUKUDY, P. E. and WALTON, T. J., (1972) *Biochemistry* **11**, 1897.
[30] BRIESKORN, C. H. and REINARTZ, H. (1967) *Z. Lebensmittelunters. Forsch.* **135**, 55.
[31] SWAN, E. P. and NAYLOR, A. F. S. (1969) *Bi-Mon. Res. Notes Dep. Fish. Forest. Can.* **25**, 32.
[32] HOLLOWAY, P. J. (1972) *Chem. Phys. Lipids* **9**, 171.
[33] RODRIGUEZ-MIGUENS, R. and RIBAS-MARQUES, I. (1972) *An. Quím. R. Soc. Esp. Fis. Quim.* **68**, 303.
[34] LEE, H. and NEVILLE, K. (1967) *Handbook of Epoxy Resins*, McGraw-Hill, New York.
[35] KNOCHE, H. W. (1968) *Lipids* **3**, 163.
[36] MORRIS, L. J. (1970) *Biochem. J.* **118**, 681.
[37] KNOCHE, H. W. (1971) *Lipids* **6**, 581.
[38] HEINEN, W. and VAN DEN BRAND, I. (1963) *Z. Naturforsch* **18b**, 67.

enzymic hydration of the corresponding epoxy acids, a reaction also known in other plant systems.[36,39,40] However, the *vic*-diol acids may not be synthesized as such but may be generated in ester form only during polymerization from the reaction of the epoxide group of the corresponding epoxy acid with the carboxyl groups of other monomer acids. The methods used for depolymerization do not permit any distinction to be drawn between esters formed from the *vic*-diol acids themselves or from the epoxide groups of the epoxy acids. The *vic*-diol acid is obtained in each case. It is unlikely that dihydroxyoctadecanoic acids are formed *via* the epoxidation pathway as suggested by Kolattukudy *et al.*[9] because a number of different positional isomers have been identified in cutin and suberin.[8,41] The occurrence of positional isomers indicates a probable biosynthesis of the acids by direct hydroxylation of stearic acid, a similar mechanism to the biosynthesis of dihydroxyhexadecanoic acids from palmitic acid.[29]




SCHEME 10. BIOSYNTHETIC SCHEME FOR $C_{18}$ CUTIN AND SUBERIN ACIDS.

## EXPERIMENTAL

*Authentication of* 9,10-*epoxy*-18-*hydroxyoctadecanoic acid.* The epoxy acid was initially isolated by E. A. Baker from the cutin acids of *Euonymus japonicus* leaves by fractional recrystallization[42] but was only fully characterized in the course of the present work. IR analysis of the methyl ester (thin film) showed the characteristic epoxide absorption at 850 $cm^{-1}$ [7,43] together with the expected absorptions from the ester, hydrocarbon and hydroxyl functions. The MS of the methyl ester TMS ether was identical to the published MS of methyl 9,10-epoxy-18-hydroxyoctadecanoate TMS ether.[15] The position of the epoxide group was confirmed by hydrolysis with aq. KOH which yielded only 9,10,18-trihydroxyoctadecanoic acid.

*Cutins and suberins.* Cuticular membranes were obtained from mature leaves and fruits using published methods[44–46] and exhaustively extracted with hot $CHCl_3$ and MeOH prior to depolymerization. Cork samples were prepared according to the methods of Holloway.[8]

*Hydrolysis.* Samples (50 mg) of dry cuticles or extractive-free corks were hydrolysed with 100 ml of 3% ethanolic KOH.[20] Samples (5–10 mg) of 9,10-epoxy-18-hydroxyoctadecanoic acid were treated similarly.

*Alcoholysis.* The reagent was always used freshly prepared by dissolving 3 g of freshly cut Na in 100 ml of MeOH (Karl-Fischer grade, specially dried) or freshly distilled Na-treated EtOH. Samples (50 mg) of dried cuticles or extractive-free corks were refluxed for 3 hr with 25 ml of reagent taking precautions to exclude moisture. The reaction mixture was filtered and any residue was refluxed for a further 10 min with 25 ml of alcohol. The combined alcoholic filtrates were acidified by the addition of the calculated amount of

[39] TULLOCH, A. P. (1963) *Can. J. Biochem. Physiol.* **41,** 1115.
[40] MORRIS, L. J. ahd CROUCHMAN, M. L. (1969) *Lipids* **4,** 50.
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[42] BAKER, E. A. and MARTIN, J. T. (1963) *Nature* **199,** 1268.
[43] SHREVE, O. D., HEETHER, M. R., KNOCHT, H. S. and SWERN, D. (1951) *Anal. Chem.* **23,** 277.
[44] HUELIN, F. E. and GALLOP, R. A. (1951) *Australian J. sci. Res.* **4,** 526.
[45] ORGELL, W. H. (1955) *Plant Physiol.* **30,** 78.
[46] HOLLOWAY, P. J. and BAKER, E. A. (1968) *Plant Physiol.* **43,** 1878.

2 M $H_2SO_4$ in MeOH and taken to dryness using a rotary evaporator. The residue was suspended in 50 ml $H_2O$ and the alcoholysis products recovered by $CHCl_3$ extraction (2 × 50 ml), the extract dried over anhyd. $Na_2SO_4$ and evaporated to dryness. Samples (5–10 mg) of 9,10-epoxy-18-hydroxyoctadecanoic acid and its methyl ester derivative were treated with 25 ml of NaOMe or NaOEt reagent for 10 min at room temp. The alcoholysis products from the methyl ester were recovered by $CHCl_3$ extraction as described above. The alcoholysis products from the acid were obtained after extraction with $Et_2O$ and methylated with excess $CH_2N_2$ before analysis.

*Osmylation.* The methyl esters of the unsaturated methoxyhydrins of 9,10-epoxy-18-hydroxyoctadecenoic acid were oxidized with $OsO_4$ using a modification of the method of Capella and Zorzut.[16] The final reaction mixture was diluted with $H_2O$ and the osmylation products recovered by extraction with $Et_2O$. The $Et_2O$ was dried over anhydrous $Na_2SO_4$, evaporated and the residue treated with *N,O*-bis (trimethylsilyl) acetamide[41] before analysis by GLC–MS.

*Chromatographic and MS analyses.* Analytical TLC, preparative-TLC, GLC and GLC–MS were carried out using methods and equipment described previously.[8,41]

*Acknowledgements*—The authors are grateful to Dr. R. L. S. Patterson and the Meat Research Institute, Langford for GLC–MS facilities and to D. Puckey for technical assistance.