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The Enzymic Conversion of Linoleic Acid into 9-(Nona-1′,3′-dienoxy)non-8-enoic Acid, a Novel Unsaturated Ether Derivative Isolated from Homogenates of *Solanum tuberosum* Tubers

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1. A major component of the lipids in aqueous (pH7.5) homogenates of tuber tissue from *Solanum tuberosum* was isolated and characterized as 9-(nona-1′,3′-dienoxy)non-8-enoic acid. 2. This novel unsaturated ether fatty acid derivative, which contains a butadienyl-vinyl ether function, has the structure:

$$CH_3–[CH_2]_4–CH=CH–CH=CH–O–CH=CH–[CH_2]_6–CO_2H$$

and is formed from linoleic acid by a sequence of enzymic reactions. 3. A precursor of the unsaturated ether derivative is 9-D-hydroperoxyoctadeca-10,12-dienoic acid, formed by the action of *S. tuberosum* lipoxygenase on linoleic acid. 4. An enzyme that converts the fatty acid hydroperoxide into the unsaturated ether derivative was isolated from *S. tuberosum*. The pH optimum of this enzyme is approx. 9, although the overall conversion of linoleic acid into the ether derivative is maximal at pH7.5. 5. An unusual feature of this pathway is the insertion of an oxygen atom into the alkyl chain of a fatty acid. 6. This novel mechanism may play a role in the breakdown of polyunsaturated fatty acids to volatile products in plants.

Although enzymes grouped under the general definition of lipoxygenases (EC 1.13.1.13) occur widely in the plant kingdom and some enzymes of this type (e.g. in legume and cereal seeds) have been known and studied for several years (Holman & Bergström, 1951; Tappel, 1962, 1963), the physiological role of lipoxygenases is not at all understood.

The natural substrates for lipoxygenases are long-chain fatty acids containing $(n-6,n-9)$-diene structures as present in linoleic acid and linolenic acid, major constituents of most lipids from plants. The amounts of free fatty acids in most plants are very low, but disruption of some tissues causes the liberation of free fatty acids through the action of endogenous lipolytic acylhydrolase enzymes; this effect is particularly marked in leaves of some *Phaseolus* species (Sastry & Kates, 1964), carrot root (Dalgarno & Birt, 1963) and potato tubers (Galliard, 1970, 1971*a*,*b*). In the potato tuber, linoleic acid and linolenic acid are further attacked by a very active endogenous lipoxygenase (Galliard & Phillips, 1971; Galliard, 1972).

Oxidative breakdown of polyunsaturated fatty acids has been suggested as the source of some volatile products, responsible for flavour characteristics of plant tissues. Recently, some characteristic components of cucumber flavour (e.g. nona-2,6-dienal, hex-2-enal and non-2-enal) were shown to be formed enzymically from linoleic acid and linolenic acid in homogenates of cucumber (Grosch & Schwarz, 1971). Enzymic reactions are also involved in the conversion of linolenic acid into hex-2-enal in leaf homogenates (Major & Thomas, 1972) and into ethylene in apple fruit extracts (Galliard *et al.*, 1968).

In contrast with the much studied, non-enzymic mechanisms of fatty acid oxidation involving hydroperoxide and free-radical intermediates (Schultz *et al.*, 1962), little is known of the enzymic processes in the breakdown of fatty acid hydroperoxides. An isomerase enzyme that converts fatty acid hydroperoxides into α-ketol derivatives of the same chain length has been studied in seeds of several plants, particularly flax (Zimmerman & Vick, 1970) and corn (Gardner, 1970).

In the present work we describe a novel enzymic pathway by which substrates of lipoxygenase are converted, via hydroperoxide intermediates, into derivatives containing a butadienyl vinyl ether structure in the fatty acid chain.

Materials and Methods

Tubers from potato (*Solanum tuberosum*, vars. Orion and Désirée) were obtained at commercial harvest and stored at 5°C. Lipoxygenase enzyme preparations were obtained from potato tubers; 50%-satd. $(NH_4)_2SO_4$ precipitate fractions (preparation I

of Galliard & Phillips, 1971), stored at 4°C as suspensions in 2M-$(NH_4)_2SO_4$, were dialysed against water to remove salts before use in the preparation of fatty acid hydroperoxides. Linoleic acid (>99% pure by g.l.c.) was purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; stock solutions were stored under an inert atmosphere and checked for the presence of oxidation products by t.l.c. and u.v. analysis. If necessary, traces of oxidation products were removed by chromatography on silicic acid columns. [1-^{14}C]Linoleic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and checked for purity by radio-g.l.c. and radio-t.l.c. of their methyl ester.

The preparation of 9-hydroxy- and 9-hydroperoxyoctadeca-10,12-dienoic acid was described previously (Galliard & Phillips, 1971).

Isolation of 9-(nona-1′,3′-dienoxy)non-8-enoic acid from potato homogenates

Potato tubers were peeled and cut into cubes approx. 1cm^3. After rinsing in ice-cold water the tissue was lightly blotted and 100g portions were homogenized with 0.1M-potassium phosphate buffer, pH7.5 (200ml), at 0°C. The homogenate was incubated with shaking at 25°C for 10–15min. Methanol (600ml) and chloroform (300ml) were added and the resulting monophasic mixture was left for 30min at room temperature with occasional shaking. Chloroform (300ml) and 0.2M-sodium acetate buffer, pH4.0 (300ml), were added with shaking. The lower (chloroform) phase was separated and evaporated to dryness *in vacuo* at 40°C. A chloroform solution of the residual lipid was evaporated *in vacuo*; 5ml of light petroleum (b.p. 60–80°C) was added to the residue and the mixture applied to a column (20cm × 2cm) of silicic acid (CC-4, 200–325 mesh; Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.). The following mixtures were sequentially passed through the column: light petroleum (b.p. 60–80°C), 1 column vol.; light petroleum–diethyl ether (9:1, v/v), 5 column vol.; light petroleum–diethyl ether (3:1, v/v), sufficient to elute the nonadienoxynonenoic acid (approx. 2 column vol.). The E_{250} in ethanol of samples from the eluate was monitored and fractions containing the nonadienoxynonenoic acid were analysed by t.l.c. The peak fractions were pooled and evaporated to dryness, and the residual material was dissolved in light petroleum (b.p. 60–80°C) and stored under N_2 at −20°C. In some cases the nonadienoxynonenoic acid was isolated as its methyl ester; the procedure was similar to that for the free acid except that the lipid mixture was treated with excess of diazomethane before silicic acid-column chromatography and less polar light petroleum–diethyl ether mixtures were used for elution.

Preparation of 9-D-hydroperoxy[1-^{14}C]octadeca-10,12-dienoic acid

Linoleic acid and [1-^{14}C]linoleic acid were mixed to give the required specific radioactivity (0.1–0.4μCi/mol) and converted into the ammonium salt form in aqueous solution. Incubation mixtures contained [^{14}C]linoleic acid (0.25mg/ml) in 0.1M-sodium acetate buffer, pH5.5. Total volumes were 5–25ml depending on the required scale of the preparation. A partially purified preparation of potato lipoxygenase was added, in four portions of 0.02mg of protein/ml of incubation mixture, at 5min intervals during a 20min incubation, with shaking at 25°C. The reaction was stopped by addition of methanol (1.2vol.) and chloroform (1.2vol.). After the two-phase system had been shaken, the lower (chloroform) phase was concentrated *in vacuo* and applied to 0.25 or 0.5mm layers of silica gel G. T.l.c. plates were developed in light petroleum (b.p. 60–80°C)–diethyl ether–acetic acid (60:40:1, by vol.) containing butylated hydroxytoluene (5mg/100ml). The ^{14}C-labelled hydroperoxy acid was located by radio-t.l.c. scanning. The t.l.c. plates were lightly sprayed with water and the area of gel containing the hydroperoxy acid was scraped off and extracted three times with diethyl ether–ethanol (9:1, v/v). After removal of extraction solvent with a N_2 stream, the residual lipid hydroperoxide was dissolved in chloroform and used within 1–2h as substrate for the enzymic preparation of the unsaturated ether acid. (By using successive additions of lipoxygenase in the method described above, 90–95% conversions of linoleic acid into its hydroperoxide were obtained; a single enzyme addition procedure gave conversions of less than 30%.)

Preparation of enzyme extracts from potato tuber

From tuber tissue, diced and washed as described above, 100g was homogenized at 0°C with 200ml of 0.1M-potassium phosphate buffer, pH7.5, containing 0.2mM-mercaptobenzothiazole. The crude homogenate was filtered through Miracloth (Evans, Adlard and Co. Ltd., Cheltenham, Glos., U.K.) and centrifuged for 30min at 15000*g*. The supernatant at 1°C was treated with solid $(NH_4)_2SO_4$ to give fractions precipitating successively at 45, 65 and 85% saturation with $(NH_4)_2SO_4$ at pH7.0. Each precipitate was collected by centrifugation and resuspended in 2M-$(NH_4)_2SO_4$, pH7.0, and stored at 0–4°C. Samples were dialysed against 0.1M-potassium phosphate buffer, pH7.5, before use in enzyme assays.

Incubations with [1-^{14}C]linoleic acid

Linoleic acid and [1-^{14}C]linoleic acid were mixed and converted into the ammonium salt form in aqueous solution (specific radioactivity 0.4μCi/

μmol). Incubation mixtures contained the ^{14}C-labelled linoleic acid (0.5μCi) with enzyme and buffer, as indicated in the text, in a total volume of 2.4ml. Mixtures were incubated for 10min at 25°C with shaking. Reactions were stopped by addition of 9ml of methanol–chloroform (2:1, v/v). Chloroform (3ml) and 0.2M-sodium acetate buffer, pH4.0 (3ml), were added, the mixture was shaken well and the lower (chloroform) phase was separated and evaporated to dryness in a stream of N_2 at room temperature. The lipid residue was dissolved in a small volume of chloroform for t.l.c. separation and subsequent analysis.

Incubations with 9-hydroperoxy[1-^{14}C]octadeca-10,12-dienoic acid

The ^{14}C-labelled hydroperoxy acid, prepared as described above, was dissolved in 0.5M-glycine–NaOH buffer, pH9.6. Routine incubation systems contained the hydroperoxide (0.2–1.0μmol; 0.05μCi) and enzyme, as indicated in the text, in 0.1M-glycine–NaOH buffer, pH9.1; total volume 2.4ml. Incubations at 25°C were stopped after 10min by addition of methanol and chloroform and the products analysed as described above for the [^{14}C]linoleic acid experiments.

General methods

Instrumental methods. U.v. spectra were recorded with a Unicam SP.800 spectrophotometer; i.r. spectra of thin liquid films on NaCl plates were obtained by using a Unicam SP.200G spectrophotometer; n.m.r. spectra were recorded on samples as 1–2% solutions in deuterochloroform by using a Varian XL-100 spectrometer. Liquid-scintillation counting of radioactive materials was performed with a Philips automatic liquid-scintillation analyser; samples were dissolved in toluene–Triton X-100 (2:1, v/v) containing 2,5-diphenyloxazole (4g) and, for aqueous samples, a final concentration of 10% (v/v) water.

Mass spectrometry. An AEI MS 902 spectrometer was operated at 70eV and a source temperature of 180°C. Samples were injected either via the direct-insertion probe or from the effluent of a Pye 104 gas–liquid chromatograph via a silicone-rubber membrane separator. High-resolution (resolving power 8000) data were recorded on magnetic tape and processed by an IBM 1130 computer by using a program developed in this Institute (Johnson *et al.*, 1972).

Gas–liquid chromatography. Methyl esters of fatty acid derivatives were chromatographed on 5ft × 0.25in (1.52m × 6mm) glass columns packed with either 10% polyethylene glycol adipate on 100–120-mesh Diatomite C (Pye–Unicam Ltd., Cambridge, U.K.) at 185°C or 3% SE30 (Applied Science Laboratories Inc., State College, Pa., U.S.A.) on 80–100 mesh Gas-Chrom CL (Johns–Manville, New York, N.Y., U.S.A.) at 190°C. Ozonolysis products were separated on the polyethylene glycol adipate column at 150°C. In all cases argon at 60ml/min was used as carrier gas to the flame-ionization detector of a Pye 104 gas–liquid chromatograph. Fatty acid equivalent-chain-length values were determined from linear plots of carbon number (e.g. methyl stearate = 18.0) versus log(retention time).

Thin-layer chromatography. Methods for standard t.l.c. procedures were described previously (Galliard & Phillips, 1971). For t.l.c. on $AgNO_3$-impregnated plates the silica gel contained 10% (w/w) $AgNO_3$, plates were developed in light petroleum (b.p. 60–80°C)–diethyl ether (7:3, v/v) and spots were located by charring after treatment with aq. 50% (v/v) H_2SO_4. Radioactive materials were located on t.l.c. plates with an automatic scanning instrument (Panax Equipment Ltd., Redhill, Surrey, U.K.). The radioactive contents of individual spots were determined, either by measurement of peak areas recorded by the scanner and calculated from the weight of tracings, or by scraping appropriate areas from the t.l.c. plates into scintillation fluid for liquid-scintillation counting.

Chemical methods. The free carboxyl groups of fatty acids and derivatives were methylated by treatment with excess of diazomethane in ethereal solution at 0°C. Catalytic hydrogenation of unsaturated fatty acid derivatives was achieved by treatment of methanolic solutions with Adams catalyst under H_2 at room temperature and slight positive pressure for 16h. Ozonolysis was performed on solutions of fatty acid derivatives in dichloromethane by passing a 1% mixture of ozone in air through the solution at −70°C for 1min. After 5min at −70°C the ozonides were decomposed with triphenyl phosphine (Stein & Nicolaides, 1962) and samples were injected directly on to the gas–liquid chromatograph. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin [type F, Sigma (London) Chemical Co. Ltd., London, S.W.6, U.K.] as standard.

Results and Discussion

Enzymic conversion of linoleic acid into an unsaturated ether derivative

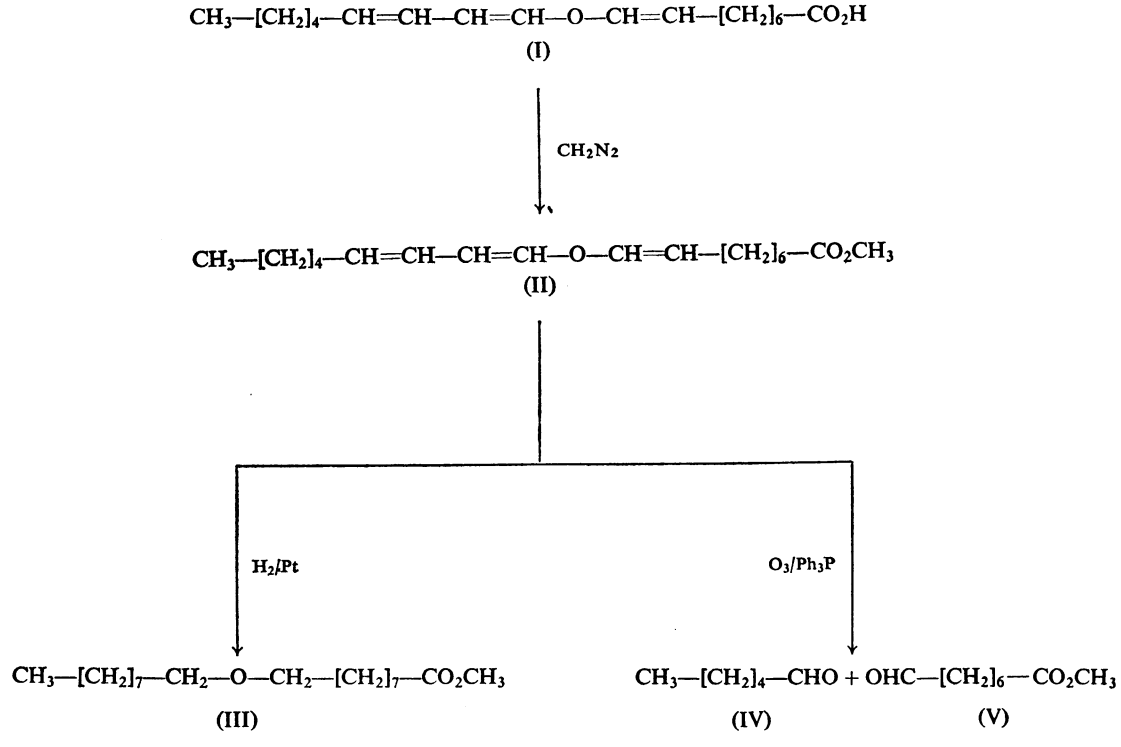
Homogenization of potato tuber tissue in water, or in media buffered at pH5–6, causes a rapid breakdown of endogenous phospholipids and galactolipids to free fatty acids and fatty acid oxidation products, including hydroperoxy and hydroxy derivatives (Galliard, 1970, 1972).

Preliminary studies had indicated that different

Table 1. *Products formed from [1-^{14}C]linoleic acid in crude homogenates of S. tuberosum tubers*

Incubation mixtures contained [1-^{14}C]linoleic acid (0.2μmol; 0.05μCi), 250μmol of buffer (either sodium acetate, pH5.5, or potassium phosphate, pH7.5) and 1ml (equivalent to 0.4g fresh weight) of a water homogenate of potato tuber in a total volume of 2.4ml. After incubation for 10min with shaking at 25°C, reaction products were extracted and analysed (see the Materials and Methods section).

	Radioactivity in ^{14}C-labelled products (% of total)			
	Homogenate		Boiled homogenate	
^{14}C-labelled products	pH7.5	pH5.5	pH7.5	pH5.5
Free fatty acid	5.0	3.5	93.5	89.3
Ether acid derivative (nonadienoxynonenoic acid)	38.0	4.5	3.5	4.1
Hydroperoxyoctadecadienoic acid + hydroxyoctadecadienoic acid	19.0	38.5	3.0	4.1
Unidentified polar lipids	38.0	53.5	0	2.5



Scheme 1. *Proposed structures of the unsaturated ether-acid and derivatives*

products were formed when the homogenizing medium was at pH7–8. Under these conditions a major product was a novel fatty acid derivative containing an unsaturated ether structure (see below), which had a polarity on t.l.c. slightly greater than that of free fatty acids but less than those of hydroxy, hydroperoxy and keto derivatives of fatty acids. In a light petroleum–diethyl ether–acetic acid (70:30:1, by vol.) t.l.c. system on silica gel G, the relative mobilities (linoleic acid = 1.00) were: 9-hydroxyoctadeca-10,12-

dienoic acid 0.52, 9-hydroperoxyoctadeca-10,12-dienoic acid 0.72 and the unsaturated ether derivative 0.92.

When [1-^{14}C]linoleic acid was incubated with crude homogenates of tuber at pH7.5, the ether-acid was a major component in the ^{14}C-labelled reaction products (Table 1). No significant conversion of linoleic acid into ether acid took place in the presence of boiled homogenate. At pH5.5, hydroperoxy and hydroxy derivatives of linoleic acid were major products and little or no labelling of the ether acid was observed. The remaining radioactivity in the products was present in a mixture of unidentified more polar materials which were present at, or near, the origin of the t.l.c. system used in the analysis. These results indicated that the ether acid was formed from a fatty acid precursor by a heat-labile process. Maximal conversion of linoleic acid into the ether acid was obtained at pH7–8.

Characterization of the unsaturated fatty acid ether derivative

The ether acid was isolated from large-scale homogenates of potato tuber tissue and purified chromatographically (see the Materials and Methods section). Yields of approx. 5mg were obtained from 100g fresh weight of tuber. Elucidation of the structure involved derivatives (see Scheme 1) of the unsaturated ether acid (I), namely the unsaturated methyl ester (II) and the saturated methyl ester (III).

Properties of the unsaturated ether acid (I). An ethanolic solution of compound (I) exhibited a strong u.v. absorption with a single peak at $\lambda_{max.}$ 252nm

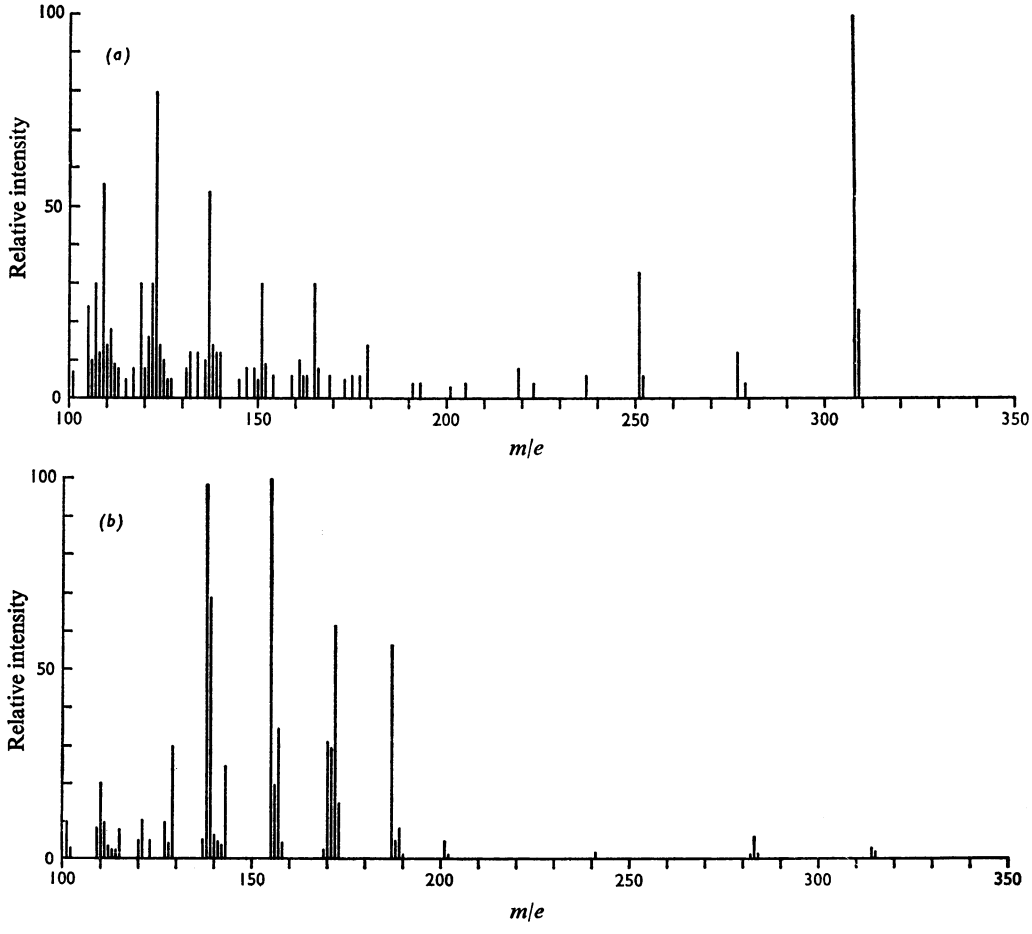


Fig. 1. *Line diagram of mass spectra recorded for compounds (II) (a) and (III) (b) (see Scheme 1)*

For experimental details see the text. Intensities were normalized to the base peak (100%).

($E^{1\%}_{1cm} = 580$). The polarity of compound (I) relative to linoleic acid was indicated above. Diazomethane converted compound (I) into the less-polar derivative (II).

Partial characterization of compound (II). The purity of the preparation was indicated by a single component in several t.l.c. systems, including $AgNO_3$–silica gel layers. [In one preparation of compound (II), a minor impurity was observed by t.l.c. on $AgNO_3$-impregnated plates. The minor component was separated from compound (II) by $AgNO_3$–silicic acid column chromatography and was identified as the methyl ester of 9-(nona-1′,3′,6′-trienoxy)non-8-enoic acid, i.e. an analogue of compound (II) but derived from linolenic acid (T. Galliard & D. Frost, unpublished work).] On silica gel G layers developed in light petroleum–diethyl ether–acetic acid (70:30:1, by vol.) the relative mobility of compound (II) was 0.90 (methyl linoleate = 1.00). No g.l.c. results on compound (II) were obtained because of its decomposition on the columns. The u.v.-absorption characteristics of compound (II) were similar to those of compound (I) with a single peak at 252nm.

The mass spectrum of compound (II) (Fig. 1*a*) showed a base-peak molecular ion at $m/e = 308$. High-resolution mass spectrometry gave an atomic composition for the molecular ion at $m/e = 308.2404$ of $C_{19}H_{32}O_3$. Atomic compositions and assignments for the other high-mass fragments of the following m/e values were: 277.2192 ($C_{18}H_{29}O_2 = M-OCH_3$); 251.1635 ($C_{15}H_{23}O_3 = M-CH_3[CH_2]_3$); 237.1451 ($C_{14}H_{21}O_3 = M-CH_3[CH_2]_4$). These results were consistent with the proposed structure of compound (II) (Scheme 1), giving fragments expected for a fatty acid methyl ester with a double bond in the $n-6$ position. Ion-rearrangement processes in the unsaturated core of the structure precluded information on the location of the other double bonds or of the non-ester oxygen atom.

The i.r. spectrum of compound (II) (Fig. 2*a*) gave characteristic absorptions, suggestive of a conjugated *trans*-substituted vinyl ether (Achmatowicz *et al.*, 1968), at 918cm^{-1} (C–H deformation), 1175cm^{-1}

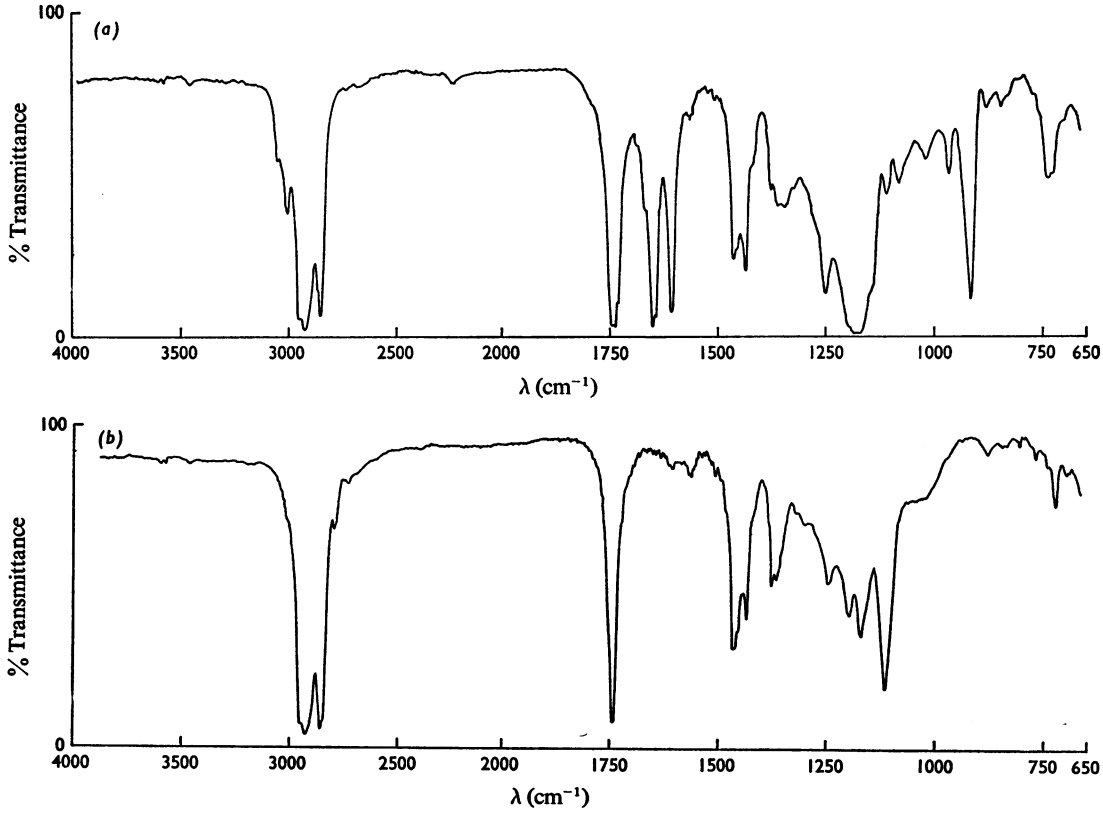


Fig. 2. *I.r. spectra recorded for thin-film specimens of compounds (II) (a) and (III) (b) (see Scheme 1)*

For experimental details see the text.

(C–O–C stretching), 1650, 1645 and 1607cm^{-1} (C=C stretching). The configuration of the non-conjugated vinyl ether linkage (at position 8 in compound II) could not be elucidated from the i.r. spectrum; n.m.r. results (below) indicate a *trans* configuration for this bond.

The 100MHz spectrum of compound (II) showed a methoxyl singlet at τ6.34 (–CO_2CH_3) and a triplet (J = 7Hz at τ7.69 (–CH_2–CO_2CH_3). The four allylic protons appeared as a multiplet centred at τ8.00 and two regions of olefinic signals were present at τ3.12–4.26 (4 protons) and τ4.48–5.00 (2 protons). The higher-field olefinic region was associated with the vinylic protons adjacent to the allylic methylene protons. Olefinic protons on the carbon atoms adjacent to the ether linkage appear in the lower-field olefinic region (Banwell & Sheppard, 1960) together with the two remaining olefinic protons. The n.m.r. spectrum indicated *trans* unsaturation at each side of the ether oxygen atom, but the complexity of the spectrum did not allow any definite conclusions to be made about the stereochemistry of the olefinic linkages.

The position of two of the double bonds in compounds (II) were located by ozonolysis. Reduction of the ozonide and subsequent analysis by g.l.c.–mass spectrometry revealed only two major products, (IV) and (V) (see Scheme 1). The retention volume and mass spectrum of compound (IV) were identical with those we obtained for authentic hexanal. Product (V) was identified as methyl 8-oxo-octanoate by comparison of its mass spectrum with the published spectrum (Withycombe *et al.*, 1971) containing a molecular ion at m/e = 172 and major peaks with m/e values (above 100) of 154, 144, 129, 115, 111 and 101; further evidence was provided by the fact that compound (V) had a fatty acid equivalent-chain-length (see the Materials and Methods section) of 14.25 on the polyethylene glycol adipate column used for the gas-chromatographic separation in the g.l.c.–mass-spectrometry system. The corresponding value for authentic methyl 9-oxononanoate (OHC–$[CH_2]_7$–CO_2CH_3), prepared by ozonolysis of methyl oleate, was exactly one carbon number higher, at 15.25.

Characterization of the hydrogenation product (III). The polarity of compound (III) on t.l.c. analysis was identical with that of compound (II); thus hydrogenation did not change the polarity of the non-ester oxygen atom. Analysis of compound (III) by g.l.c. showed a single component on both polar (polyethylene glycol adipate) and non-polar (SE30) columns with fatty acid equivalent-chain-lengths of 19.8 and 18.6 respectively. The low-resolution mass spectrum of compound (III) (Fig. 1*b*) gave a molecular ion at m/e = 314. High-resolution mass measurements of the major ions are listed in Table 2. The fragmentation pattern was consistent with the proposed structure (Scheme 1), showing particularly intense fragment ions corresponding to cleavage at the ether linkage with charge retention on the carboxyl fragment both with and without additional loss of CH_3OH from the ester group. The presence of an ether group was confirmed by i.r. and n.m.r. spectroscopy. The i.r. spectrum (Fig. 2*b*) was similar to that of methyl stearate (O'Connor *et al.*, 1951) with an additional strong absorption band at 1116cm^{-1} (C–O stretching), characteristic of long-chain aliphatic ethers (Baumann & Ulshoffer, 1968). The n.m.r. spectrum of compound (III) contained typical

Table 2. *High-resolution analysis of ion fragments in the mass spectrum of compound (III) (Scheme 1)*

For experimental details see the text.

Measured mass (mass units)	10^4 × Error (mass units)	Atomic composition	Assignment
(314*)	—	—	$M = CH_3$–$[CH_2]_8$–O–$[CH_2]_8$–CO_2CH_3
283.2637	−1	$C_{18}H_{35}O_2$	$M-OCH_3$
201.1493	+2	$C_{11}H_{21}O_3$	CH_2–O–$[CH_2]_8$–CO_2CH_3
169.1232	+3	$C_{10}H_{17}O_2$	CH_2–O–$[CH_2]_8$–$CO_2CH_3-CH_3OH$
187.1330	−5	$C_{10}H_{19}O_3$	O–$[CH_2]_8$–CO_2CH_3
155.1064	−9	$C_9H_{15}O_2$	O–$[CH_2]_8$–$CO_2CH_3-CH_3OH$
171.1385	−1	$C_{10}H_{19}O_2$	$[CH_2]_8$–CO_2CH_3
172.1457	−7	$C_{10}H_{20}O_2$	$[CH_2]_8$–CO_2CH_3+H
170.1314	+7	$C_{10}H_{18}O_2$	$[CH_2]_8$–CO_2CH_3-H
139.1133	+9	$C_9H_{15}O$	$[CH_2]_8$–$CO_2CH_3-CH_3OH$
138.1048	+3	$C_9H_{14}O$	$[CH_2]_8$–$CO_2CH_3-CH_3OH-H$
157.1183	−2	$C_9H_{17}O_2$	$[CH_2]_7$–CO_2CH_3
143.1082	+9	$C_8H_{15}O_2$	$[CH_2]_6$–CO_2CH_3
129.0915	−1	$C_7H_{13}O_2$	$[CH_2]_5$–CO_2CH_3

* The molecular ion was below the threshold for accurate mass measurement.

peaks of saturated fatty acid methyl esters (Hopkins, 1966) with signals at τ values of 6.3 (CH_3 protons of methyl ester), 7.7 (methylene protons adjacent to ester group), 8.7 (chain methylene protons) and 9.1 (terminal CH_3 protons). In addition a triplet was observed at τ6.6 (aliphatic ether protons, CH_2–O–CH_2).

Summary of evidence for proposed structure of compound (I)

The chain length of 18 carbon atoms and the presence of one non-acidic oxygen atom in compound (I) were shown by mass spectrometry of its unsaturated (II) and saturated (III) methyl esters. The non-acidic oxygen atom was present in an ether linkage (shown by i.r. and n.m.r. of compounds II and III) between C-9 and C-10 (mass spectrometry of compound III). Two double bonds were present at positions 8 and 12 (shown by ozonolysis and mass spectrometry of compound II). The location of the third double bond at C-10 was deduced from the divinyl ether character of compound (II) (by i.r. spectroscopy) and the presence of a conjugated-diene structure in compounds (I) and (II) (by u.v. and n.m.r. spectroscopy). The proposed structure is consistent with the behaviour of compound (I) and its derivatives on t.l.c. and g.l.c. and with the suggestion (see below) that compound (I) is formed enzymically from 9-hydroperoxyoctadeca-10,12-dienoic acid.

Enzymic conversion of 9-D-hydroperoxyoctadeca-10,12-dienoic acid into 9-(nona-1′,3′-dienoxy)non-8-enoic acid

It was known (Galliard & Phillips, 1971) that potato tuber extracts contained a very active lipoxygenase enzyme; to determine whether the fatty acid hydroperoxide produced by the action of this lipoxygenase is an intermediate in the conversion of linoleic acid into nonadienoxynonenoic acid, 9-D-hydroperoxy[1-^{14}C]octadeca-10,12-dienoic acid was prepared and added as a substrate to the potato extracts.

An efficient conversion of the hydroperoxide into the ether acid was obtained with crude homogenates buffered at pH9 (see below). The products formed from the labelled hydroperoxide were monitored; the results obtained with various enzyme preparations

Table 3. *Products formed from 9-hydroperoxy[1-^{14}C]octadeca-10,12-dienoic acid by enzyme preparations from S. tuberosum tubers*

Incubation mixtures contained hydroperoxy[^{14}C]octadecadienoic acid (0.2μmol; 0.05μCi) and enzyme preparations as indicated (equivalent to 0.4g fresh weight of tuber) in 0.1M-glycine–NaOH buffer, pH9.0. Mixtures, total vol. 2.4ml, were incubated for 10min with shaking at 25°C. Products were extracted and analysed as described in the Materials and Methods section.

	Radioactivity in ^{14}C-labelled products (% of total)			
Enzyme preparation	Hydroperoxydienoic acid	Hydroxydienoic acid	Nonadienoxynonenoic acid	Unidentified polar materials
No enzyme	96	0	0	4
Crude homogenate	14	16	35	35
15000*g* supernatant	20	16	37	27
15000*g* supernatant (dialysed)	19	16	36	29
15000*g* supernatant (acetone-dried powder)	32	25	11	32
15000*g* supernatant (boiled)	61	14	0	25
30–45%-satd.-$(NH_4)_2SO_4$ precipitate	57	15	0	28
45–65%-satd.-$(NH_4)_2SO_4$ precipitate	27	14	33	26
45–65%-satd.-$(NH_4)_2SO_4$ precipitate (boiled)	81	17	0	2
65–85%-satd.-$(NH_4)_2SO_4$ precipitate	68	16	0	16
85%-satd.-$(NH_4)_2SO_4$ supernatant	80	12	0	8

are presented in Table 3. The unsaturated ether acid was a major product in crude homogenates and, after partial enzyme purification by centrifugation, dialysis and ammonium sulphate fractionation, the activity responsible for the formation of the ether acid was retained in the 45–65%-satd. $(NH_4)_2SO_4$ fraction (Table 3). The enzymic nature of the reaction was indicated by these purification steps and by the heat-lability of the process (Table 3). The enzyme activity was retained for several weeks at 0–4°C when preparations were stored as suspensions in 2M-$(NH_4)_2SO_4$ at pH7. As was the case with lipoxygenase from potato tubers (Galliard & Phillips, 1971), acetone-dried-powder preparations of the enzyme were less active.

Almost all the radioactivity present in the hydroperoxide substrate was retained in chloroform-soluble materials in the reaction products. Analysis by t.l.c. showed that, in addition to the nonadienoxynonenoic acid and unchanged hydroperoxide, the products contained a hydroxydiene fatty acid derivative and some unidentified, more polar, materials. The hydroxydiene derivative was probably formed, at least in part, by a non-enzymic reduction of the hydroperoxide; some hydroxydiene acid was present in the products formed when only a boiled-enzyme preparation was added (Table 3) and the reduction was proportional to the amount of protein present. It is probable that reducing (e.g. thiol) groups in the enzyme preparations were responsible for some non-enzymic reduction of the hydroperoxide.

The unidentified polar materials were formed in relatively larger amounts when the pH of the incubation system was lowered. It is not known whether these represent further conversions of the ether acid or are alternative products formed from the hydroperoxide; only small amounts of these polar products were formed with boiled-enzyme preparations (Table 3).

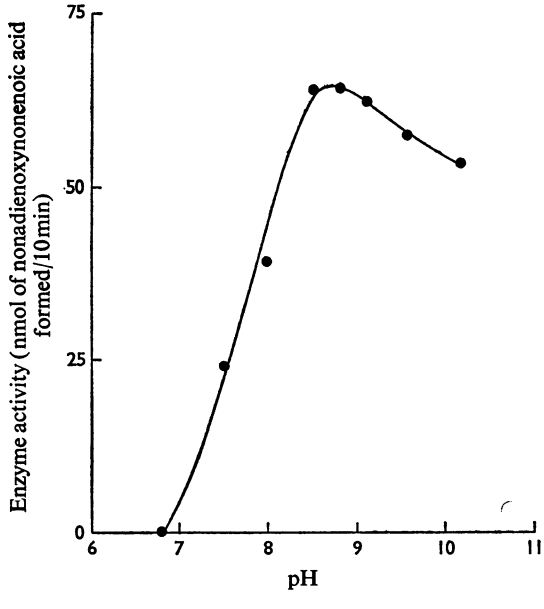


Fig. 3. *pH–response curve for the enzymic conversion of 9-hydroperoxyoctadeca-10,12-dienoic acid into the ether acid derivative*

Incubation mixtures contained [1-^{14}C]hydroperoxyoctadecadienoic acid (1μmol, 0.05μCi), enzyme [0.7mg of protein; 45–65%-satd.-$(NH_4)_2SO_4$ fraction] and 0.1M buffer in a total volume of 2.5ml. Buffers used were potassium phosphate (pH6.5–7.5) or glycine–NaOH (pH8–10.5). Mixtures were incubated for 10min at 25°C.

Properties of the nonadienoxynonenoic acid-forming enzyme

pH optimum. The optimum pH for the formation of the ether acid from 9-D-hydroperoxyoctadeca-10,12-dienoic acid was about pH9 (Fig. 3). This was a higher pH than the optimum (pH7.5) for the conversion of linoleic acid into the ether acid. We propose that the 9-hydroperoxy fatty acid derivative,

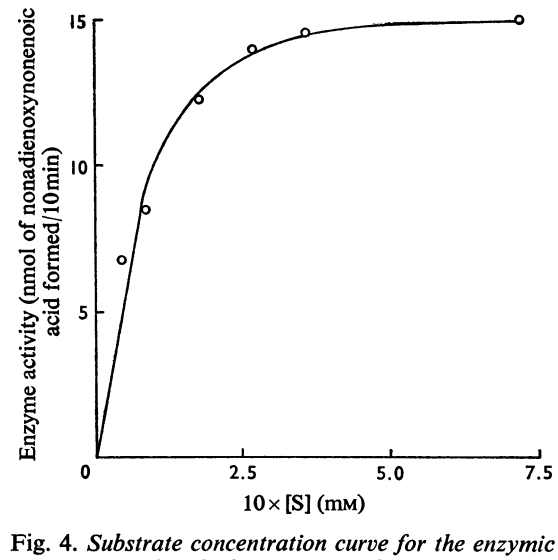


Fig. 4. *Substrate concentration curve for the enzymic conversion of 9-hydroperoxyoctadeca-10,12-dienoic acid into the ether acid derivative*

Incubation conditions were as described in Fig. 3, with 0.1M-glycine–NaOH (pH9.1) as buffer. The range of ^{14}C-labelled hydroperoxide substrate added to the 2.5ml incubation mixture was from 0.11μmol (0.05μCi) to 1.8μmol (0.8μCi).

formed from linoleic acid by lipoxygenase, is an intermediate in the formation of the ether acid. Since the lipoxygenase from potato has a pH optimum at 5.5–6.0 and is inactive at pH9 (Galliard & Phillips, 1971), whereas the enzyme forming the ether acid has an optimum at pH9.0, the value of 7.5 for the pH optimum of the composite reaction appears reasonable.

Kinetics. With the partially purified enzyme preparation [45–65%-satd.-$(NH_4)_2SO_4$ fraction] and 9-D-hydroperoxyoctadeca-10,12-dienoic acid (0.4mM) as substrate, the rate of formation of the ether-acid at pH9.1 was linear with respect to enzyme concentration up to at least 20% conversion of substrate (10nmol of ether acid formed/min per mg of protein). Under the same conditions, the progress curve was linear for up to 10min, and substrate concentration curves (Fig. 4) showed saturation at approx. [hydroperoxide] 0.4mM, with a half-maximal velocity at approx. 60μM substrate. A Lineweaver–Burk reciprocal plot of the results in Fig. 4 was linear and gave values for $V_{max.} = 16$nmol/min per mg of protein and $K_m = 6\times10^{-5}$M.

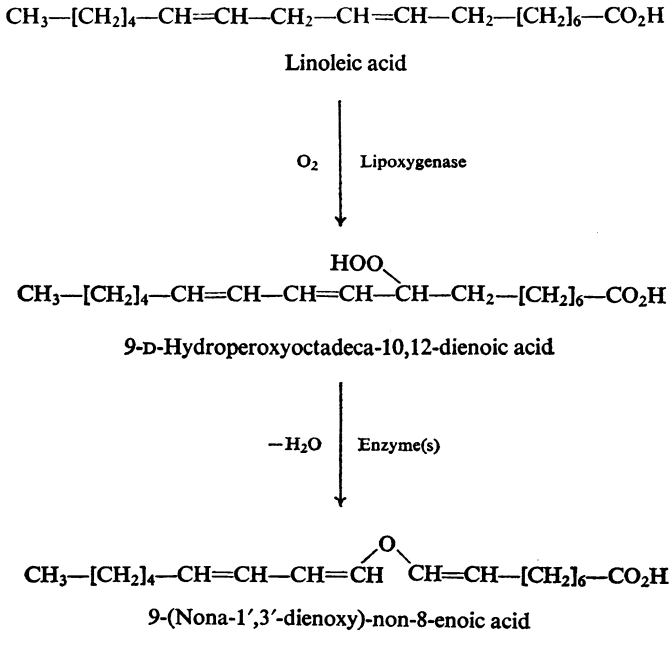
Conclusions

The evidence given in the present paper suggests a novel pathway for the metabolism in plants of polyunsaturated fatty acids via hydroperoxide and unsaturated ether derivatives. The relationship between the compounds involved is presented in Scheme 2, which shows that the conversion of the hydroperoxide into the ether acid formally represents a process involving loss of water and rearrangement involving breakage of a carbon-to-carbon bond and formation of a carbon-to-oxygen bond.

To our knowledge, there is no precedent in the literature for this type of conversion involving fatty acid hydroperoxides. Further, the ether acid produced contains a butadienyl vinyl ether structure; this is a novel derivative in the fatty acid field and we have been unable to find any reference to analogous compounds. A different type of rearrangement of a fatty acid hydroperoxide, leading to an ester rather than an ether, was suggested as a possible reaction in the biosynthesis of stillingic acid and allenic acid in seeds of *Sapium sebiterum* (Gunstone, 1966).

It is possible that the ether-acid derivatives are intermediates in the breakdown of unsaturated fatty acids to some of the volatile compounds produced by plants. Preliminary studies have shown the thermal instability of the ether acid and that acid treatment leads to the production of as yet unidentified volatile fragments containing carbonyl functions.

Very little is known about the mechanisms of fatty acid hydroperoxide metabolism in plants. An isomerase enzyme found in some plants (Zimmerman & Vick, 1970; Gardner, 1970) converts fatty acid



Scheme 2. *Proposed enzymic pathway for formation of nonadienoxynonenoic acid from linoleic acid*

hydroperoxides into α-ketol derivatives. The isomerase from corn forms 9-hydroxy-10-oxo- and 13-hydroxy-12-oxo-octadecenoic acid from 9-hydroperoxy- and 13-hydroperoxy-octadecadienoic acid respectively (Gardner, 1970). The isomerase from flax, however, appears to be specific for the 13-hydroperoxy fatty acid (Veldink *et al.*, 1970). Under anaerobic conditions and in the presence of linoleic acid, soya-bean lipoxygenase catalyses the rearrangement of fatty acid hydroperoxidase to unsaturated carbonyl derivatives (Garssen *et al.*, 1971). The potato lipoxygenase system produces only the (*n*−10)-hydroperoxy derivatives of fatty acids (Galliard & Phillips, 1971); thus, from linoleic acid, only 9-D-hydroperoxyoctadeca-10,12-dienoic acid is formed and subsequently converted into the unsaturated ether derivative with the ether linkage at the 9-position. Work with other hydroperoxy fatty acid derivatives should provide information on the specificity of the enzyme system that produces the unusual ether acid.

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