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An Anaerobic Reaction between Lipoxygenase, Linoleic Acid and its Hydroperoxides

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In an anaerobic system soya-bean lipoxygenase together with linoleic acid induces a structural rearrangement of 13-hydroperoxyoctadeca-*cis*-9-*trans*-11-dienoic acid leading to the formation of 13-oxotrideca-*cis*(*trans*)-9-*trans*-11-dienoic acid and *n*-pentane as well as 13-oxo-octadeca-9,11-dienoic acid. It is proposed that the 13-peroxyoctadeca-*cis*-9-*trans*-11-dienoic acid radical formed through hydrogen radical abstraction by the linoleic acid radical is the key intermediate for these reactions.

The lipoxygenase (EC 1.13.1.13)-catalysed oxidation *in vitro* of unsaturated fatty acids containing a penta-*cis*–*cis*-1,4-diene system to *cis*–*trans*-conjugated hydroperoxides has been thoroughly investigated with regard to its fatty acid substrate specificity (Holman, Egwim & Christie, 1969).

Dolev, Rohwedder, Mounts & Dutton (1967) demonstrated that both oxygen atoms of the hydroperoxy group stem from molecular oxygen. The specificity of the introduction of molecular oxygen into polyunsaturated fatty acids has been studied by Hamberg & Samuelsson (1967). However, the influence of the concentration of oxygen on the course of the reaction is unknown.

This aspect may be relevant to the situation *in vivo*, as the concentration of oxygen in germinating seed is possibly different from that under the usual conditions *in vitro*. For that reason it seemed attractive to investigate the lipoxygenase reaction in relation to the concentration of oxygen in solution.

We have found that, when an incubation is performed in a closed system with a fixed amount of oxygen and an excess of linoleic acid with respect to the available oxygen, reaction products other than hydroperoxides are also formed. These reaction products are characterized by u.v. absorption at 285nm at pH10, indicating the presence of a conjugated dienone chromophore. The compounds formed under these reaction conditions have been isolated and characterized and the conditions of their formation have been studied in more detail.

MATERIALS AND METHODS

Linoleic acid (purity>99%) was a gift from the Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands. Soya-bean lipoxygenase (activity 8000 units/mg) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

For preparative purposes this enzyme preparation was used; for kinetic experiments, however, soya-bean lipoxygenase purified by the method of Schormüller, Weber, Höxer & Grosch (1969) was used. Two isoenzyme fractions (1 and 2), which were homogeneous in polyacrylamide-gel electrophoresis, were obtained, which differed in their pH optimum (Christopher, Pistorius & Axelrod, 1970). For fraction 1 the specific activity, according to the method of Mitsuda, Yasumoto, Yamamoto & Kusano (1967), was $12\,\text{mM-O}_2\cdot\text{min}^{-1}\cdot E_{280}^{-1}$; the $E_{1\,\text{cm}}^{1\%}$ (280nm) was found to be 12, and this fraction 1 isoenzyme was used for most of the kinetic experiments reported below.

All reagents were of analytical-grade quality and were free of carbonyl compounds.

Linoleic acid hydroperoxides. A 100mg portion of linoleic acid was dissolved in 100ml of 0.04M-NH_3–NH_4Cl buffer, pH9.0, and incubated with 15mg of lipoxygenase (Nutritional Biochemicals Corp.) for 1h at 0°C, while the solution was kept saturated with pure O_2. After acidification to pH3.0 with 2M-HCl, the reaction mixture was extracted with diethyl ether. The hydroperoxides were isolated by preparative t.l.c. on silica gel G (E. Merck A.-G., Darmstadt, Germany) with the solvent system *n*-hexane–diethyl ether–acetic acid (100:100:1, by vol.). The hydroperoxide concentration was determined spectrophotometrically, by using a molar extinction coefficient at 234nm of $25000\,\text{l}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ (Johnston, Zilch, Selke & Dutton, 1961).

Spectroscopy. U.v. spectra were recorded with a Unicam SP.800B spectrophotometer; i.r. spectra were obtained with a Grubb–Parsons Spectromaster instrument; proton-magnetic-resonance spectra were registered with a Varian HA-100 spectrometer; mass spectra were recorded with an AEI-MS9 spectrometer.

EXPERIMENTAL AND RESULTS

Incubations on a preparative scale and identification of reaction products. A 500mg portion of linoleic acid (3.6mM) was incubated at room temperature with 30mg of lipoxygenase (Nutritional Biochemicals Corp.) in 500ml of 0.02M-sodium borate

buffer, pH 8.0, containing 30μM-O_2. Immediately after the mixing of the reactants, the reaction vessel was closed under N_2. During the reaction the O_2 concentration decreased to almost zero. After 2h the reaction mixture was acidified with 2M-hydrochloric acid to pH 2.0 and extracted with *n*-hexane–ether (7:2, v/v).

The extract was washed with water, dried over sodium sulphate and concentrated *in vacuo*. The fatty acids were esterified with diazomethane and the methyl esters were fractionated by preparative t.l.c. on silica gel G in the solvent system *n*-hexane–ether (1:1, v/v).

By spraying the edges of the plates with 0.4% (w/v) DNP-hydrazine in 2M-hydrochloric acid, three carbonyl compounds were located with R_F values 0.56, 0.62 and 0.80. The hydrazones turned violet on spraying with ethanolic alkali, thus indicating their unsaturated character. Spraying with 5% (w/v) phosphomolybdic acid in 96% (v/v) ethanol followed by heating at 110°C for 10min revealed the methyl esters of unchanged linoleic acid (R_F 0.95), the linoleic acid hydroperoxides (R_F 0.74), the carbonyl compounds already mentioned and, additionally, a diffuse zone of material in the region R_F 0.82–0.90.

As the zones with R_F values 0.56 and 0.62 could not be completely separated by the applied t.l.c. system they were collected together from the non-sprayed region of the plate and the carbonyl compounds were converted into their DNP-hydrazones by the method of Haverkamp Begemann & de Jong (1959).

Separation was performed on a preparative scale by chromatography on silica gel G plates with the solvent system carbon tetrachloride–*n*-hexane–ethyl acetate (10:2:1, by vol.). The DNP-hydrazones were thus separated into two zones, both showing u.v.-absorption maxima in chloroform at 390, 305 and 267nm, pointing to the DNP-hydrazones of a 2,4-dienal (Stitt *et al.* 1961).

The mass spectra of the two compounds were identical and in accordance with the structure of the DNP-hydrazone of methyl 13-oxotrideca-9,11-dienoate (Fig. 1). Characteristic is the loss of the fragment containing the conjugated system resulting in a peak at *m/e* 261. The peaks at *m/e* 401 and 383 arise from the parent *m/e* 418 by the loss of a hydroxyl radical and of both a hydroxyl radical and water in accordance with the fragmentation pattern of DNP-hydrazones (Budzikiewicz, Djerassi & Williams, 1967). The peak at *m/e* 231 is formed from the ion at *m/e* 261 by the loss of NO.

The i.r. spectra of both compounds showed absorption peaks corresponding to a DNP-hydrazone (Stitt *et al.* 1961) and an ester group (1733cm^{-1}, 1176cm^{-1}); differences in the spectra could be explained by different configurations of the double bonds.

Proton-magnetic-resonance spectra were obtained in $CDCl_3$ with tetramethylsilane as internal standard. By comparison with the DNP-hydrazones of deca-*trans*-2-*cis*-4-dienal and hepta-*trans*-2-*trans*-4-dienal (Meijboom, 1966), the less-polar compound was identified as the DNP-hydrazone of methyl 13-oxotrideca-*cis*-9-*trans*-11-dienoate.

Absorptions were assigned to the protons of C-9–C-12 of the conjugated diene system as indicated in Table 1. The coupling constant of 10.5Hz observed for $J_{H_{9,10}}$ is typical for a *cis* coupling across a double bond, and the value of 15.0Hz for $J_{H_{11,12}}$ results from coupling across a *trans* bond.

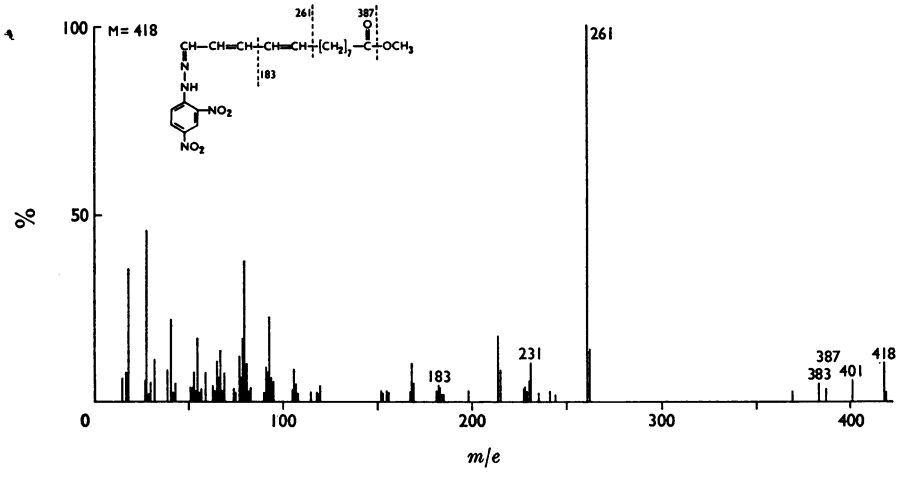


Fig. 1. Mass spectrum of the DNP-hydrazone of methyl 13-oxotrideca-9,11-dienoate.

The other compound was identified as the DNP-hydrazone of methyl 13-oxotrideca-*trans*-9-*trans*-11-dienoate, by comparison with the model compound hepta-*trans*-2-*trans*-4-dienal, and it may be a secondary product resulting from isomerization of the *cis–trans* product. Rechromatography of the isolated DNP-hydrazones showed that no interconversion took place and hence the formation of the *trans–trans* compound occurs either during the hydroperoxide cleavage reaction or by isomerization of the free *cis–trans*-carbonyl compound.

The C_5 fragment of the chain-cleavage reaction could be traced by collecting the volatile reaction products in a cold trap. By gas–solid chromatography (Varian Aerograph 1520 equipped with a flame-ionization detector) at 200°C over silica gel (mesh size 30–60; column 2.5m × 3mm) and also at 80°C over alumina (neutral, Woelm; column 60cm × 3mm) with N_2 as the carrier gas (60ml/min), the main substance of the trapped material was identified as *n*-pentane. Pentan-1-ol and pent-1-ene appeared to be absent.

The carbonyl compound with R_F 0.80 on silica gel G was also isolated and purified as described above. The u.v. spectrum of the DNP-hydrazone in chloroform gave absorption maxima at 388, 304 and 265nm. By mass spectrometry it was found to be identical with the DNP-hydrazone of an oxodiene as described by Vioque & Holman (1962). However, the fragmentation pattern of this compound does not allow differentiation between the DNP-hydrazone of methyl 13-oxo-octadeca-9,11-dienoate and the 9-oxo isomer, and possibly it consists of a mixture of both, which we have not further verified.

It is worthy of mention that in neither of the separations described was any clear indication obtained for the presence of non-ester carbonyl compounds.

From the material in the diffuse zone (R_F 0.82–0.90) on silica gel G, two compounds of R_F 0.63 and 0.50 were isolated by rechromatography in the solvent system *n*-hexane–ether (4:1, v/v). These had u.v.-absorption maxima in ethanol at 237nm and 235nm respectively. Mass spectra of both compounds gave an indication of dimer structures (molecular weights about 600) (cf. Privett, Nickell, Lundberg & Boyer, 1955).

Incubations in a dual-purpose cuvette. Incubations were carried out under various O_2 concentrations in a dual-purpose cuvette of 1cm light-path [made according to the model developed by Ribbons, Smith & Hewitt (1969), and obtained by courtesy of Unilever Research Laboratories], which was provided with a Clark oxygen electrode connected to a GME Oxygraph model KM (GME, Middleton, Wis., U.S.A.) for the polarographic measurement of the O_2 concentration. The O_2 concentration was adjusted to the required value by flushing with O_2-free H_2. Enzyme was then added and the initial O_2 concentration was measured and followed during the incubation reaction. Simultaneously, the reaction was followed by recording the change in the u.v. absorption at either 285 or 234nm with a Unicam SP.800B spectrophotometer.

Fig. 2 gives results obtained from closed-system incubations at room temperature of a fixed amount of linoleic acid and of available O_2 at the start of each experiment over a range of enzyme (fraction 1) concentrations (8–20μg/incubation). The conditions were: 0.02M-sodium borate buffer, pH10.0 (saturated with air at 25°C), linoleic acid concentration 1.9mM, O_2 concentration 240μM and incubation volume 2.20ml. U.v.-absorption measurements at 285nm revealed a lag time that was roughly reciprocally proportional to the enzyme concentration. The lag time corresponds to the time necessary for the depletion of the available O_2.

Table 1. *Relevant chemical shifts and coupling constants of the DNP-hydrazone of* 13-*oxotrideca*-cis-9-trans-11-*dienoate*

Proton	Chemical shifts (δ) (p.p.m.)	Coupling constants (*J*) (Hz)	
$H_{(9)}$	5.77	$J_{H_{8,9}}$	7.5
$H_{(10)}$	6.20	$J_{H_{9,10}}$	10.5
$H_{(11)}$	6.94	$J_{H_{10,11}}$	11.0
$H_{(12)}$	6.43	$J_{H_{11,12}}$	15.0
		$J_{H_{12,13}}$	9.0

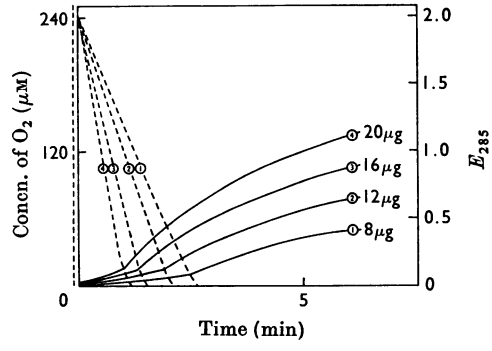


Fig. 2. Progress curves for the lipoxygenase-induced reaction of linoleic acid followed spectrophotometrically at 285nm (——) and by simultaneous polarographic determination of the O_2 concentration (– – – –). To the substrate solution (saturated with air at 25°C and containing 240μM-O_2) was added the indicated amount of enzyme (8–20μg) dissolved in 10μl of buffer.

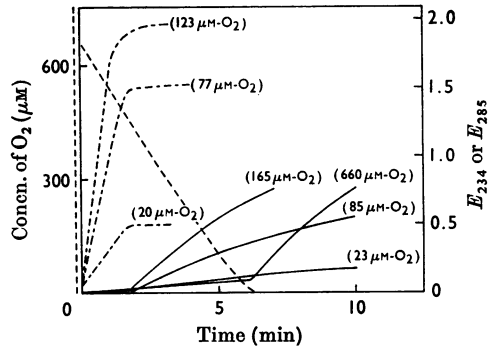


Fig. 3. Progress curves for the diene-forming reaction recorded at 234nm (----) and for the reaction at 285nm (——) over a range of starting concentrations of O_2. The substrate solution was adjusted to the indicated O_2 concentration by flushing with H_2 or O_2, and then enzyme was added. During the reaction the cuvette was stoppered; the temperature was 25°C. ---, Oxygen-electrode tracing for the experiment with initial O_2 concentration 660 μM.

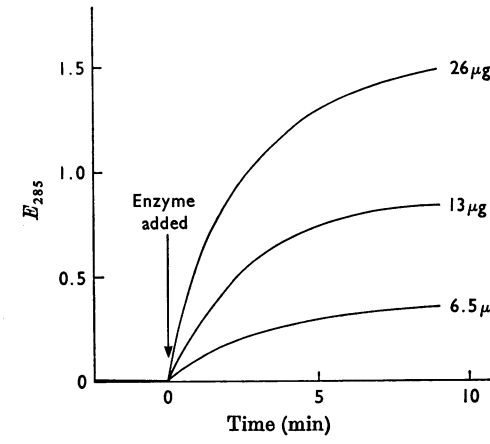


Fig. 4. Progress curves for the anaerobic lipoxygenase-induced reaction of linoleic acid and its hydroperoxides recorded at 285nm. To 1.75ml of O_2-depleted substrate solution (flushed with H_2) was added the indicated amount of enzyme (6.5–26 μg) dissolved in 10 μl of buffer. During the reaction the cuvette was stoppered; the temperature was 25°C.

However, the reaction does not take place when enzymically prepared linoleic acid hydroperoxides are incubated with lipoxygenase in the absence of linoleic acid, with or without O_2. Nor, as expected, did the incubation of linoleic acid with the enzyme give any reaction in the absence of O_2.

Fig. 3 gives results obtained from incubations of a fixed concentration of linoleic acid (1.9mM) and of lipoxygenase (8 μg/incubation) over a range of initial O_2 concentrations (20–660 μM). The data do not permit a quantitative assessment of the relative velocities of hydroperoxide formation versus carbonyl formation. However, the incubation starting with 660 μM-O_2 demonstrates that the onset of the fast formation of carbonyl compounds coincides with the point where O_2 is depleted. The reaction can be set apart from the hydroperoxide formation reaction on incubating enzymically prepared linoleic acid hydroperoxides and linoleic acid (0.23 and 0.54mM respectively) with complete exclusion of O_2; the reaction starts as soon as the lipoxygenase (6–26 μg/incubation) is added (Fig. 4). There is a positive correlation between the velocity of carbonyl compound formation and the amount of enzyme used.

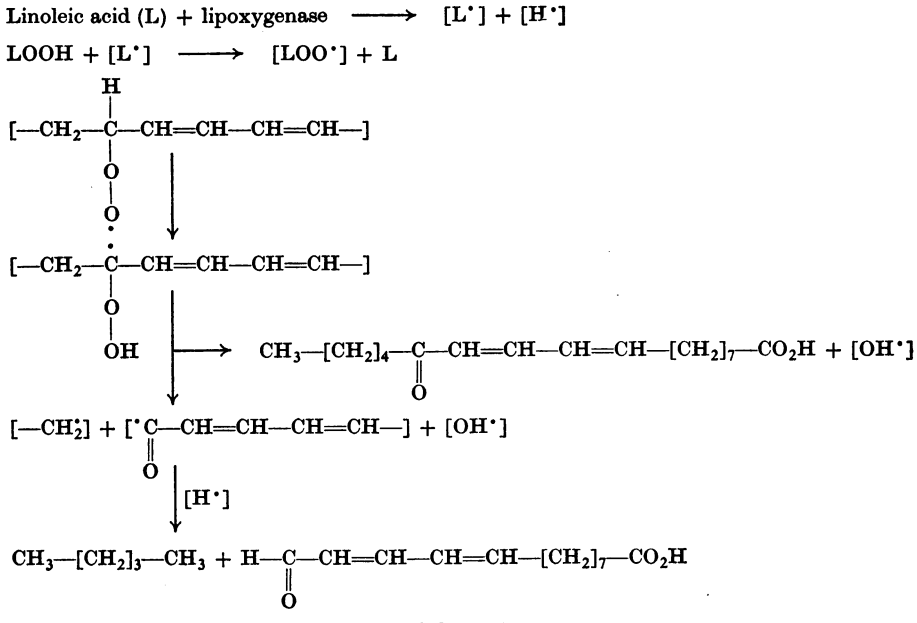
When the enzyme is first inactivated by heating at 100°C for 3min no reaction is observed. Thus the reaction proceeds when both linoleic acid and its hydroperoxides are combined with the native lipoxygenase in the absence of O_2.

Enzyme fraction 2 also gave this reaction, but at lower pH (6–6.5). At this pH fraction 1 is inactive in terms of the conventional lipoxygenase activity.

The reaction is not restricted to linoleic acid and its hydroperoxide. A similar effect has been observed when arachidonic acid, α-linolenic acid and γ-linolenic acid were anaerobically incubated with their respective enzymically prepared hydroperoxides (increase in extinction at 285nm).

Particularly significant is the experiment in which arachidonic acid was anaerobically incubated on a preparative scale with enzymically prepared hydroperoxides of linoleic acid. This yielded exactly the same carbonyl compounds as resulted from the incubation of linoleic acid and its hydroperoxides described above. This unequivocally demonstrates that the carbonyl compounds stem from the hydroperoxides and not from the non-oxidized fatty acid.

The quantitative aspects of the carbonyl formation may become evident from the following typical experiment on a preparative scale. Linoleic acid and linoleic acid hydroperoxides (0.20 and 0.15mmol respectively) were anaerobically incubated with 30mg of lipoxygenase (Nutritional Biochemicals Corp.) in 100ml of 0.02M-sodium borate buffer, pH 9.0, at room temperature. After 1.5h the reaction products were isolated, converted into their methyl esters and fractionated by t.l.c. on silica gel G as described above. After isolation from the plate, the fractions were dissolved in ethanol. Their quantities were spectrophotometrically determined by using molar extinction coefficients at 277nm of 30000 $l \cdot mol^{-1} \cdot cm^{-1}$ for



Scheme 1.

the dienal fraction (Pippen & Nonaka, 1958) and 22000 $l \cdot mol^{-1} \cdot cm^{-1}$ for the C_{18}-oxodiene fraction (Vioque & Holman, 1962).

It appeared that 40% of the hydroperoxides was converted into the carbonyl fraction, 60% of which was in the C_{13}-dienal and 40% in the C_{18}-oxodiene form. Only about 7% unchanged hydroperoxide could be recovered.

DISCUSSION

The function of lipoxygenase in plant lipid metabolism is still unknown but if the enzyme catalyses the formation of hydroperoxides *in vivo*, it is probable that the organism must contain further enzyme systems to metabolize these products. Zimmerman (1966) and Veldink, Vliegenthart & Boldingh (1970) have studied one such enzyme, from flax seed, which catalyses the isomerization of linoleic acid hydroperoxide into a monounsaturated α-ketol, and Gardner (1970) has described a similar isomerization and other secondary reactions of hydroperoxides catalysed by enzymes from corn germ.

We have now demonstrated that under anaerobic conditions the formation of carbonyl compounds from linoleic acid hydroperoxides is dependent on the presence of both native lipoxygenase and of fatty acids that are normal substrates of the enzyme. It is reasonable to assume that the initiation of the reaction is due to the formation of the radicals of these fatty acids, which also occur as intermediates in the aerobic reaction (Dolev, Rohwedder & Dutton, 1967). In the absence of O_2 we believe that this type of radical abstracts a hydrogen radical from the hydroperoxide, yielding a peroxy radical, which is then subject to structural rearrangements (Scheme 1).

From the observation that, in spite of the disappearance of the hydroperoxide, the u.v. extinction at 234nm hardly changes in magnitude it might be concluded that the linoleic acid is partly converted into conjugated material. However, no conjugated monomeric acid could be detected in the incubation mixtures, which leads us to believe that concurrent with the chain-cleavage reaction the linoleic acid radicals form conjugated dienoic dimers in which presumably 50% of the hydroperoxides participate. The dimeric fraction described above shows a u.v. absorption at 234nm. So far no attempts have been made by us to further identify the structure of the compounds.

The chain-cleavage reaction has been proven for the 13-hydroperoxide of linoleic acid, which is the main hydroperoxide formed by soya-bean lipoxygenase. The absence of non-ester carbonyl fractions would indicate that the chain-cleavage reaction does not take place with the 9-hydroperoxide. Final proof can only be obtained when the chain-cleavage reaction conditions are applied to a concentrated 9-hydroperoxide preparation.

It is tempting to assume that this new

lipoxygenase-induced reaction has a distinct function in plant lipid metabolism, e.g. to maintain a very low O_2 concentration in certain stages of seed development and to eliminate the formed hydroperoxides or to provide suitable metabolic substrates during germination.

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