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PLANT LIPOXYGENASES

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CONTENTS

I. INTRODUCTION

A. *General*

Lipoxygenase (EC. 1.13.11.12) catalyzes the oxidation by molecular oxygen of linoleic acid and of other polyunsaturated fatty acids to hydroperoxides (Fig. 1). It is a prerequisite that substrate fatty acids contain a 1,4-pentadiene system. In general, the product hydroperoxides are optically active. The enzyme is widely distributed in nature and its occurrence in a great variety of plants has been known for a long time.[151] Recently, its presence has also been demonstrated in animal tissue.[96,124]

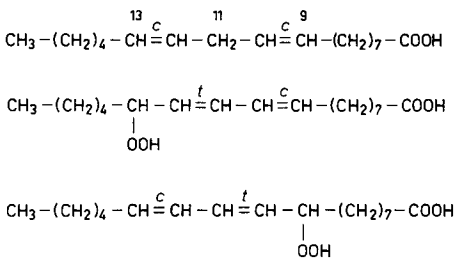


FIG. 1. Substrate and products of lipoxygenase catalysis.

This review deals with the highlights in lipoxygenase biochemistry reported since the excellent review by Tappel.[151] Recently, reviews have been published by Drapron and Uzzan,[36] Axelrod,[12] Galliard[54,55] and Gardner.[62] In this review, special attention has been given to the mechanistic aspects of the reactions catalyzed by lipoxygenases, in particular to the peroxidative pathways recently discovered for the conversion of product hydroperoxides. A critical re-evaluation of some of the earlier results also appeared appropriate since the position of lipoxygenase among the dioxygenases has changed considerably following the discovery of one mole of iron atoms per mole of enzyme.

B. *Assay of Lipoxygenases*

One of the fundamental problems in regard to the investigation of lipoxygenase concerns the reproducibility and reliability of the assay procedure. Linoleic acid or its salts are the most widely used substrates in activity determinations. In principle, the rate of product formation can be derived from a number of parameters:

(a) colorimetric peroxide determination (discontinuous);
(b) monitoring the absorbance change at 234 nm corresponding to the formation of the conjugated diene system in the product;
(c) manometric or polarographic measurement of the oxygen consumption, and
(d) co-oxidation of suitable substrates (e.g. carotene).

Each of the methods has its specific disadvantages mainly depending on the pH optimum and the degree of purity of the enzyme. However, a complication common to all methods is the physical state of the substrate fatty acid during incubation. Even at pH 9.0,

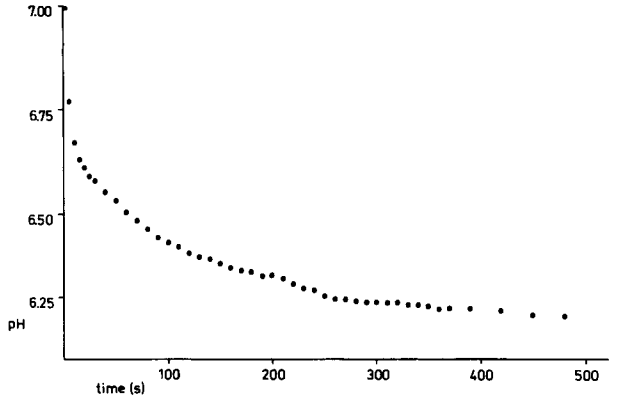


FIG. 2. Effect of enzymic hydroperoxide formation from linoleic acid on the pH. Conditions: 0.5 mM linoleic acid, 0.2 M NaCl, 6 μg lipoxygenase-1, total volume 2 ml.

the substrate forms opaque to slightly turbid solutions. Apparently, the turbidity disappears accordingly as the hydroperoxide formation proceeds.

The risk of artifacts may be illustrated by the following experiment. On incubating linoleic acid (1 μmol) and soybean lipoxygenase-1 at various pH's in a pH-stat, the process of hydroperoxide formation is accompanied by a considerable increase in the amount of titratable protons. Figure 2 shows the gradual drop in pH during lipoxygenase catalysis. In the most extreme case, this corresponds to 40% of the amount of linoleic acid initially present in the reaction vessel. The results of an extensive set of control experiments yielded no indication whatsoever of the formation of a novel reaction product which can be held responsible for the change in apparent acidity of the reaction mixture. This phenomenon is absent when linoleylsulfate instead of linoleate is used as substrate. The solubility of the latter substrate over the pH range 5.5–10.5 is comparable to that of linoleate at pH $\geqslant$ 9. However, serious drawbacks of the use of linoleylsulfate are its limited availability in a pure state and its detergent-like nature. Substrate impurities, particularly those of a peroxidic nature, have adverse effects on the kinetics of product formation. This was already noted by Haining and Axelrod[89] who clearly demonstrated the effect of hydroperoxides on the kinetic lag-phase of the lipoxygenase reaction.

II. ISOLATION AND PURIFICATION OF SOYBEAN LIPOXYGENASE

Crystalline lipoxygenase from soybeans has been prepared by Theorell *et al.*,[156] Mitsuda *et al.*[122,123] and Allen.[1] The crystalline material appeared to be homogeneous according to electrophoresis. Several methods have been described for the isolation of soybean lipoxygenases.[19,25,46,86,141,147,164,175] Starting from a crude commercial lipoxygenase preparation or defatted soybean flour, various lipoxygenase isoenzymes can be found in soybeans.[85,87,90,142,170] On the other hand, Finazzi-Agrò *et al.*[46] used soybeans soaked in water for 52 hr as starting material. A relatively simple procedure involving gel filtration and ion-exchange chromatography gives good yields (1 mg of lipoxygenase-1 from 1 g of dry soybeans) of homogeneous lipoxygenase-1 with a high specific activity (up to 200 μmoles O_2/min/mg protein). Various purity checks have been described including several methods of electrophoresis[19,147,164] and immunoelectrophoresis.[19] In addition to the general protein-staining techniques, procedures for the specific detection of lipoxygenase have been described.[85,87,90,142,164,170]

III. PROTEIN CHARACTERISTICS

A. *Molecular Weight*

In early investigations of the isolation and purification of lipoxygenases, molecular-weight determinations (cf. 156) yielded a value of 102,000 for the crystalline enzyme. More recent studies[25,122,147] invariably mention figures above 100,000 although new computations using both earlier and recent amino-acid analyses and ultracentrifuge data indicate values somewhat below 100,000.[43,70] Stevens *et al.*[147] provided some evidence for the partial dissociation of the protein on treatment with chaotropic agents like guanidine-HCl or sodium dodecylsulfate.

However, recent investigations by Garssen[70] demonstrated that the molecular integrity of pure lipoxygenase-1 is, under various conditions, not affected by these agents, which suggests that the protein consists of a single polypeptide chain. Grosch *et al.*[81] reported the cleavage of the so-called lipoxygenase/guaiacol-linoleic acid hydroperoxide-oxidoreductase into smaller fragments; however, no clear indications were given as to whether this protein is identical with lipoxygenase-1. Both polyacrylamide gel-electrophoresis in the presence of SDS and gel-filtration in the presence of urea[142] did not indicate

that lipoxygenase-1 dissociates under these conditions. In summary, there appears to be no convincing evidence for the existence of regular subunits of lipoxygenase-1.

B. *Amino Acid Composition*

Despite good agreement between the various amino-acid analyses published for lipoxygenase,[105,122,142,145,147] there is a remarkable uncertainty in regard to the exact number of half-cystine residues. Assuming a molecular weight of 100,000, Stevens *et al.*[147] found four cysteines and two disulfide groups, whereas Schroeder[142] found five half-cystines. Garssen[70] detected four cysteines and one disulfide group and Mitsuda *et al.*[122] a total of four half-cystines. Recently, Axelrod reported the presence of twelve half-cystines.[12] Other dissimilarities may be partly due to inherent experimental errors or to varietal differences.

IV. COFACTORS

For a long time, lipoxygenase-1 has been considered as a unique dioxygenase because of the apparent absence of any metal cofactor or a prosthetic group. Although the first metal analyses by Theorell *et al.*[154–156] did indicate the presence of iron, these authors regarded it as an impurity. At that time, spectroscopic and inhibition studies failed to support the view of a direct involvement of a transition metal in the enzymic reaction.[122,147,151]* However, a recent reinvestigation of the metal content of soybean lipoxygenase[21] by atomic absorption spectroscopy revealed the presence of at least one mole of iron in the enzyme. In later reports, it was clearly demonstrated that 1 mole of pure lipoxygenase-1 contains 1 mole of iron atoms which is essential for catalytic activity.[22,31,46,133,140] The possible role of Ca^{2+} ions as an indispensable factor for optimal lipoxygenase activity has been the subject of considerable controversy.[26,104,109,110,164] Little direct evidence seems available for assigning a specific function to Ca^{2+} other than its influence on the physical state of the fatty acid in solution, particularly at lower pH's.[181]

V. SUBSTRATES AND PRODUCTS

A. *Substrates*

Besides linoleic and structurally related fatty acids, several other fatty acid derivatives have been considered as typical lipoxygenase substrates. Koch *et al.*[111] claimed evidence for the existence of a triglyceride (trilinolein) lipoxygenase in soybeans, which is also different from lipoxygenase-1 in its pH-response. Methyl linoleate,[25,88,151,164] unfractionated seed oils,[88] mono- and dilinolein[88] and linoleylsulfate[2,173] have also been used as lipoxygenase substrates. For (kinetic) experiments requiring high substrate concentrations, a system containing Tween-20 as a dispersing agent has been described[149] (cf. 3). In general, there seems to be a relatively large number of lipoxygenases with a pH-optimum $\leqslant 7$ that accept a wider range of substrates than soybean lipoxygenase-1 at pH 9.0.

B. *Products*

The primary products of the aerobic reaction of lipoxygenase and suitable fatty acids are *cis,trans*-conjugated hydroperoxides. Following the first report[94] on the positional specificity of soybean lipoxygenase in the oxygenation of various unsaturated fatty acids, several other aspects related to lipoxygenase specificity have been studied.[12,101,104]

*According to a very recent study[157] dealing with 4-nitrocatechol as an active site-probe for various dioxygenases containing nonheme iron, lipoxygenase appears to be reversibly inhibited by this compound.

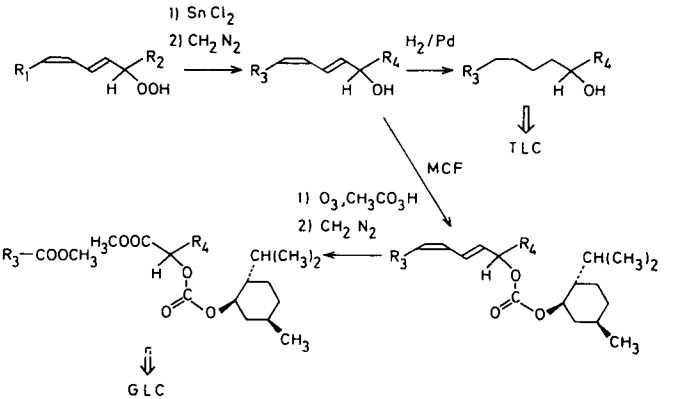


FIG. 3. Reactions used to determine relative amounts of 9D-, 9L-, 13D-, and 13L-hydroperoxyoctadecadienoates formed from linoleic acid. 9-Hydroperoxy-10,12-octadecadienoic acid: $R_1 = (CH_2)_4CH_3$; $R_2 = (CH_2)_7COOH$. 13-Hydroperoxy-9,11-octadecadienoic acid: $R_1 = (CH_2)_7COOH$; $R_2 = (CH_2)_4CH_3$. Methyl 9-hydroxy-10,12-octadecadienoate: $R_3 = (CH_2)_4CH_3$; $R_4 = (CH_2)_7COOCH_3$. Methyl 13-hydroxy-9,11-octadecadienoate: $R_3 = (CH_2)_7COOCH_3$; $R_4 = (CH_2)_4CH_3$. (From: M. Hamberg, *Anal. Biochem.* **43**, 515–526, 1971.)

Stereospecificity in the aerobic reaction was first demonstrated by Privett *et al.* who measured the optical activity of the reaction products.[135] The main product appears to be the (n-6)-L$_S$-hydroperoxy fatty acid.[95] Depending on the conditions and the type of enzyme, also significant amounts of the (n-10)-D$_R$-hydroperoxide are found.[92,139,162] The geometric configuration of unsaturated fatty acid hydroperoxides and some derivatives have been studied by Powell *et al.*,[134] Hall and Roberts,[91] Gardner and Weisleder,[68] and Tallent *et al.*[150] The complete description of the structures of these compounds still requires sophisticated separation procedures[42,92,115] and appropriate derivatization. Hamberg,[92] for instance, described a method to determine the relative amounts of 9D-, 9L-, 13D- and 13L-hydroperoxyoctadecadienoates formed from linoleic acid. Separation of diastereoisomers is accomplished by gas liquid chromatography (GLC) of the menthylchloroformate (MCF)-derivatives (see Fig. 3) of the corresponding hydroxyoctadecadienoates. Possibly, a simpler and more direct approach is the application of chiral nuclear magnetic resonance (NMR) shift reagents for establishing the absolute configuration of the corresponding hydroxy compounds.

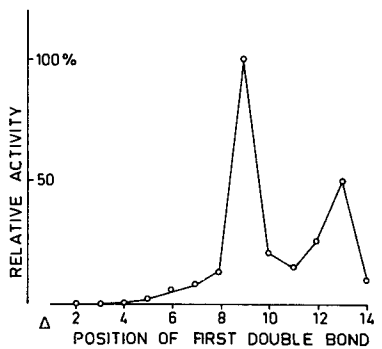


FIG. 4. Substrate specificity of purified soybean lipoxygenase upon positional isomers of octadecadienoic acid in which the methylene-interrupted *cis*, *cis*-diene system occurs from the 2,5- to the 14,17-positions. The abscissa is numbered with reference to the carboxyl group. Rates of reaction are plotted relative to the rate for natural linoleic acid. (Adapted from: R. T. Holman *et al.*, *J. Biol. Chem.* **244**, 1149–1151, 1969.)

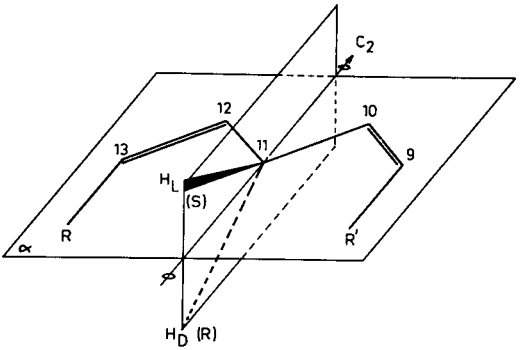


FIG. 5. Head–tail reversion: the 1,4-*cis,cis*-pentadiene system of linoleic acid (in plane α) is reversed around the twofold axis C_2. (From: M. R. Egmond, Thesis, University of Utrecht, The Netherlands, 1973.)

In a study of the substrate specificity of crystalline soybean lipoxygenase, Holman *et al.*[104] assayed a series of octadecadienoic acids with the 1,4-pentadiene system located at various positions in the fatty acid chain (Fig. 4). Apparently, the naturally occurring octadecadienoic acid is the best substrate. Remarkably, the isomer with its double bonds in the ω2,5 position is also a

$$CH_3—CH=CH—CH_2—CH=CH—(CH_2)_{11}—COOH$$

$$CH_3—(CH_2)_4—CH=CH—CH_2—CH=CH—(CH_2)_7—COOH$$

relatively good substrate. The products resulting from incubation of this acid with soybean lipoxygenase-1 were found by Egmond[37] to be 17-L_S-hydroperoxyoctadecadienoic acid (85%) and 13-hydroperoxyoctadecadienoic acid (15%). The formation of mainly the 17-hydroperoxide indicates that the substrate is oriented on the enzyme in the same manner as linoleic acid (cf. 92), i.e. both substrates are oxygenated at the terminal part of the pentadiene system. It also implies that stereospecific H-abstraction can occur from carbon atoms other than (n-8). Furthermore, these findings exclude the possibility of a head-tail reversed orientation of the substrate on the enzyme (Fig. 5).

The aerobic conversion of linoleic acids does not invariably lead to the formation of hydroperoxides. Graveland[74] demonstrated that lipoxygenase—when adsorbed to wheat glutenin—forms a number of epoxy–hydroxy compounds (Fig. 6) from linoleic acid. Also products were found which contained three hydroxy groups derived from the epoxy–hydroxy compounds by hydrolysis. However, the lipoxygenase/glutenin system did not yield the epoxy–hydroxy compounds when incubated with preformed fatty acid hydroperoxides. When this system was kept anaerobic, epoxy–hydroxy fatty acids did accumulate, which may indicate the involvement of a reactive intermediate derived from the hydroperoxide. On incubating methyl linolenate at pH 6 with an aqueous extract of soybean, Roza[138] found, besides monohydroperoxides, an appreciable amount (25% of total product) of substances containing a hydroperoxide plus an endoperoxide

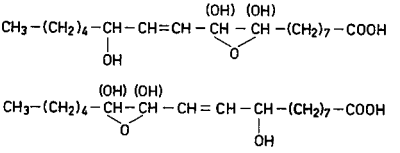


FIG. 6. Structures of isomeric hydroxyepoxy- and trihydroxy acids formed from linoleic acid by the lipoxygenase/wheat glutenin system. (From: A. Graveland, *J. Am. Oil Chem. Soc.* **47**, 352–361, 1970.)

group. The formation of this product was attributed to one of the soybean isoenzymes since pure lipoxygenase-1 did not form this product.

VI. INTERACTION BETWEEN SOYBEAN LIPOXYGENASE-1 AND HYDROPEROXIDES

A. *Kinetic Lag Phase*

In the aerobic reaction, a kinetic lag phase has been observed frequently.[69,89,145] It has been claimed that small amounts of hydroperoxidic compounds are capable of eliminating the lag-period. However, only hydroperoxides, which are formed by the enzyme as main products from substrate fatty acids, are effective in this respect.[89] For example, 13-L-hydroperoxy linoleic [13-ROOH] can shorten the induction period (Fig. 7) whereas the 9-D-isomer has no effect. From this activation of the enzyme by its own product, Smith and Lands[145] and Garssen[69] concluded that the enzyme has a product-binding site. The slow initial reaction rate is mainly due to inhibition by excess fatty acid substrate presumably binding to the product site (Egmond *et al.*[38]). Formation of product by the enzyme or addition of exogenous product leads to an increase in the reaction rate. The interaction between 13-ROOH and enzyme can be monitored by fluorescence measurements,[39,46] spectrophotometry[29,39] and electron paramagnetic resonance (EPR).[29,31,133]

B. *Fluorescence*

Soybean lipoxygenase-1 shows an intrinsic fluorescence at 328 nm on excitation at 280 nm (top curve in Fig. 8). The fluorescence originates only from tryptophan residues and not from tyrosine, since excitation at 295 nm does not significantly alter the emission spectra. Removal of oxygen from the solution causes a decrease in fluorescence without apparent formation of a new absorption band (bottom curve in Fig. 8). Addition of iodide (1 M) to a lipoxygenase-1 solution also causes quenching of its fluorescence.[45] Lowering of the pH enhances this effect (pH 9.4:50% quenching; pH 5.3:65% quenching). Removal of oxygen results in a much higher quenching effect of iodide. This might

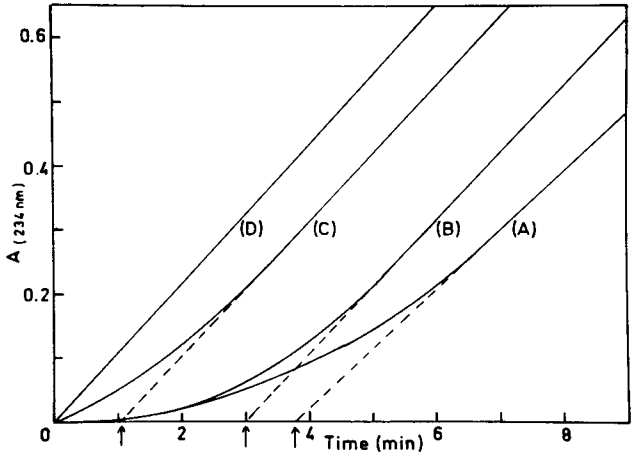


FIG. 7. Effects of 9- and 13-hydroperoxylinoleic acid on the induction period in the lipoxygenase-catalyzed hydroperoxidation of linoleic acid. Conditions: (A) linoleic acid 600 μM; (B) linoleic acid 600 μM + 9-ROOH 2 μM; (C) linoleic acid 600 μM + 13-ROOH 2 μM; (D) linoleic acid 600 μM + 13-ROOH 8 μM. Lipoxygenase: 4 μg; total volume 3 ml. (From: G. J. Garssen, Thesis, University of Utrecht, The Netherlands, 1972.)

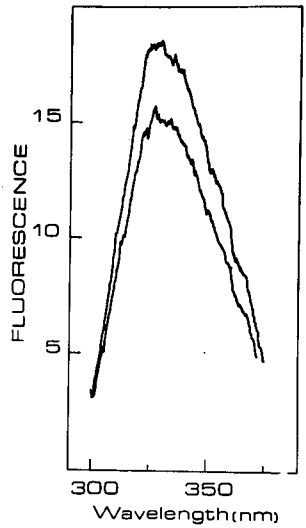


FIG. 8. Effect of oxygen removal on lipoxygenase fluorescence. Top curve: 1.7×10^{-6} M lipoxygenase in 0.1 M sodium acetate. Bottom curve: the same after four cycles of evacuation followed by refilling with argon. Excitation wavelength: 280 nm. (From: A. Finazzi-Agró *et al.*, *Biochim. Biophys. Acta* **326**, 462–470, 1973.)

indicate that oxygen and iodide compete for the same binding site, probably iron. The tryptophan residues in lipoxygenase can be considered to belong to class I.[18] This means that the tryptophan residues are located in a strictly hydrophobic part of the protein, which also contains the iron.

At pH 9.0, 13-ROOH gives a quenching of the fluorescence of the enzyme, which already reaches its maximum after addition of a stoichiometric amount with respect

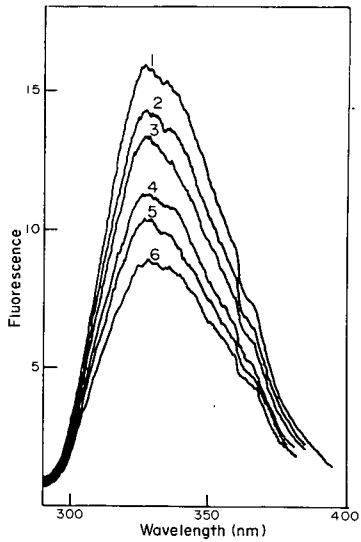


FIG. 9. Additivity of oxygen and linoleate effects on fluorescence. 7×10^{-7} M lipoxygenase dissolved in 0.1 M sodium acetate. From the top: (1) as such; (2) in the presence of 1.6×10^{-7} M linoleate; (3) in the presence of 3.2×10^{-7} M linoleate; (4) in the presence of 6.4×10^{-7} M linoleate; (5) in the presence of 12.8×10^{-7} M linoleate; (6) same as (5) after evacuation. (From: A. Finazzi-Agrò *et al.*, *Biochim. Biophys. Acta* **326**, 462–470, 1973.)

to the enzyme (Fig. 9). However, the shape of the emission curve is unaltered, indicating that all tryptophan residues behave similarly. The changes in fluorescence induced by 13-ROOH are probably directly coupled with changes in the absorbance (see Section VI.C) via a nonradiative energy transfer process. Hydrogen peroxide, which is known to be an irreversible inactivator of lipoxygenase[39,69,123] can also cause fluorescence quenching.[39] However, at an enzyme concentration of 4.6×10^{-5} M, a fourfold molar excess is necessary to obtain the maximum effect. Linoleic acid as such does not alter the fluorescence of absorbance of the native enzyme, provided that it is added under strictly anaerobic conditions (cf. 142). Evidently, linoleic acid is oxidized to hydroperoxide in the presence of oxygen, which gives rise to the above-mentioned fluorescence quenching.

C. *Absorption Spectra*

The optical spectrum of native lipoxygenase-1 has no other characteristics than the protein absorption at 280 nm at pH 9.0. Addition of a stoichiometric amount of 13-ROOH gives rise to an increase in the absorbance of the enzyme in the 300–450 nm region (curve b in Fig. 10). The absorption maximum is located at 330 nm (ϵ 330 $\approx$ 1.5×10^{-3} M^{-1} cm^{-1}). A new absorbance band appears in the presence as well as in the absence of oxygen. In concentrated enzyme solutions, this absorbance change is visible. The colorless solution of the native enzyme turns yellow upon addition of a stoichiometric amount of 13-ROOH. We hold this absorbance change responsible for the fluorescence quenching. The yellow enzyme species represents a well-defined entity. It differs from the native enzyme in the formal charge of iron in the protein. Iron in native lipoxygenase is EPR silent, whereas the EPR spectrum of the yellow form shows the presence of high-spin Fe (III) (see Section VI.D). The fluorescence and absorbance changes during the formation of the yellow enzyme are accompanied by a conversion of 13-ROOH, as could be demonstrated by incubations with 1-^{14}C labelled 13-ROOH under both aerobic and anaerobic conditions. The degree of conversion of

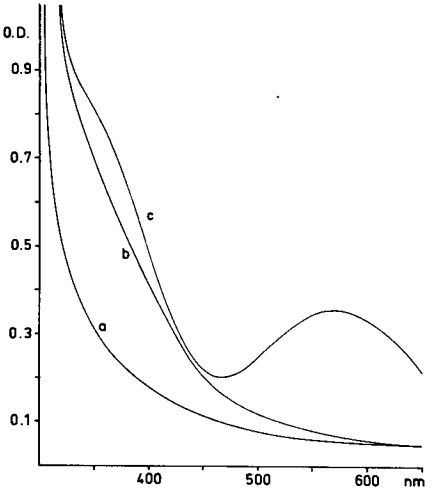


FIG. 10. Effect of 13-ROOH on the ultraviolet and visible absorption spectrum under aerobic conditions at 5°C and pH 9.0. (a) 0.38 mM lipoxygenase solution (38 mg/ml); (b) reaction mixture of 400 μl of a lipoxygenase solution (38 mg/ml) and 84 μl of a hydroperoxide solution (2 mM). Final concentrations: 0.31 mM and 0.34 mM, respectively; (c) reaction mixture of 400 μl of a lipoxygenase solution (38 mg/ml) and 284 μl of a hydroperoxide solution (2 mM). Final concentrations: 0.22 mM and 0.38 mM, respectively. (From: J. J. M. C. de Groot *et al.*, *FEBS Lett.* **56**, 50–54, 1975.)

the hydroperoxide is not dependent on the presence of oxygen. Under anaerobic conditions, only one major reaction product can be detected by thin layer chromatography (TLC) whereas in the presence of oxygen several products appear. The identity of the reaction products cannot be determined easily, because only stoichiometric amounts with respect to the enzyme are involved. It can be concluded that the yellow enzyme is not an enzyme/product complex but a different form of the holo-enzyme. However, upon addition of a 3- to 5-fold molar excess of 13-ROOH to the native or yellow enzyme—under aerobic as well as under anaerobic conditions—another form of the enzyme arises, which has a purple color as can be observed in concentrated enzyme solutions.[29] In the absorption spectrum, a new absorption band with a maximum at 570 nm (ϵ 570 $\approx$ 1 $\times$ 10^3 M^{-1} cm^{-1}) appears whereas the absorbance in the 300–450 nm region is slightly increased (curve c in Fig. 10). The iron occurs in the Fe(III)-state, but the EPR spectrum differs from that of the yellow form (Section VI.D).

The purple species is unstable. Upon standing (21 mg/ml lipoxygenase plus a 3-fold molar excess of 13-ROOH, 5 min at 25°C or 1 hr at 0°C), the color reverts to yellow. The newly formed yellow species is identical to the species derived from the native enzyme by treatment with a stoichiometric amount of 13-ROOH; the absorption and EPR spectra are indistinguishable. The purple form can be obtained by a fresh addition of 13-ROOH. This cycle can be repeated several times. During the purple–yellow transition, the hydroperoxide is concomitantly converted—as is evident from the disappearance of both the hydroperoxy function and the absorbance at 234 nm. These observations suggest that the purple form of the enzyme consists of a ferric form to which 13-ROOH has been liganded. By means of mass spectrometry and NMR, the major conversion product of 13-ROOH could be identified as *threo*-(12,13-*trans* epoxy)-11-hydroxy, 9-*cis*-octadecenoic acid.[29] Experiments with ^{18}O-labelled 13-ROOH indicated a high retention (approx 70%) of both oxygens of the hydroperoxide function in the epoxy-hydroxy-octadecenoic acid (Garssen *et al.*, unpublished). Formally, this conversion can be conceived as an isomerization. Both the yellow and purple forms of lipoxygenase can be evoked by addition of linoleic acid under aerobic conditions, whereas formation of the yellow species on addition of one equivalent of enzymically prepared 9-ROOH is far from complete. Probably, the yellow species formed under these circumstances is caused by a slight contamination of 9-ROOH with 13-ROOH.

D. *EPR Spectra*

The EPR spectrum (curve a in Fig. 11) of native lipoxygenase-1 shows a low-intensity signal at g = 4.3 due to contaminating Fe (III), and a complex signal around g = 2 caused by contaminating Cu (II) and Mn (II). The yellow form, which arises on treatment with a stoichiometric amount of 13-ROOH, has a strikingly different EPR spectrum (curve b). In comparison with the native enzyme, new signals are found at g = 7.5, 6.2 and 5.9. These signals should be ascribed to high-spin Fe (III) as could be concluded from their temperature dependence (De Groot *et al.*, unpublished). The resonances show some similarities to those of an iron–porphyrin system, but no porphyrin system or any prosthetic group could be detected in the enzyme. Therefore, it is highly probable that iron is liganded to amino-acid residues of the polypeptide chain. The spectrum contains also a radical type of signal at g = 2.0. The origin of this resonance is still unclear. The EPR spectrum of the yellow species prepared by anaerobic addition of 13-ROOH is somewhat different (Fig. 12). This indicates that oxygen has an influence on the ligand symmetry of iron. The signal at g = 2 is also affected. The EPR spectrum of the purple enzyme Fe (III)–13-ROOH complex (curve c, Fig. 11) differs significantly from that of the yellow species. The intensities of the signals at g = 7.5, 6.2 and 5.9 are greatly reduced, whereas the signal at g = 4.3 is strongly increased. Also, in this case, oxygen has a similar effect on the spectrum as described for the yellow species.

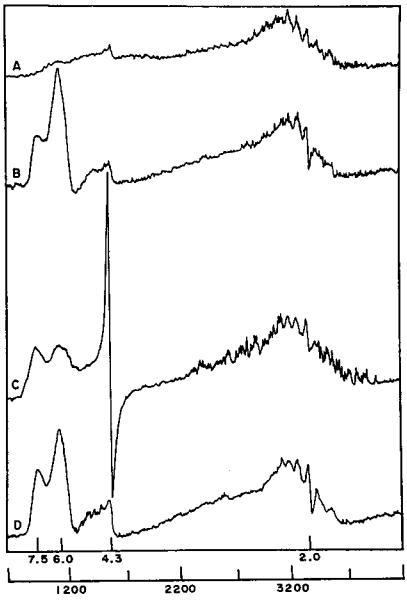


FIG. 11. Effect of 13-ROOH on the E P R spectrum of lipoxygenase-1. (a) 300 μl of a lipoxygenase solution (30 mg/ml); (b) 8 μl of a hydroperoxide solution (11.4 mM) was added to 300 μl of a lipoxygenase solution (30 mg/ml). Final concentrations: 0.29 mM and 0.30 mM; (c) another 16 μl of 11.4 mM hydroperoxide solution was added to the reaction mixture. Final concentrations: 0.28 mM and 0.84 mM, respectively; (d) the same reaction mixture as described under (c) after 1 hr at 0°C. Microwave frequency, 9.307 GHz. (From J. J. M. C. de Groot *et al.*, *FEBS Lett.* **56**, 50–54, 1975.)

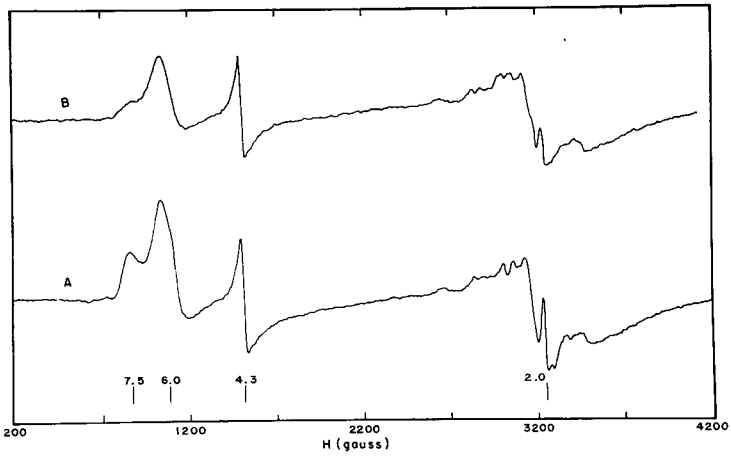


FIG. 12. Effect of 13-L-hydroperoxylinoleic acid on the E P R spectrum of lipoxygenase: (A) under aerobic conditions, 15 μl of a 5.67 mM hydroperoxide solution was added to 300 μl of a lipoxygenase solution (26 mg/ml). Final concentrations: 0.27 mM and 0.25 mM, respectively, in borate buffer pH 9.0. Microwave frequency, 9.079 GHz; (B) under anaerobic conditions, 20 μl of a 5.67 mM hydroperoxide solution was added to 400 μl of a lipoxygenase solution (26 mg/ml). Final concentrations: 0.27 mM and 0.25 mM, respectively, in borate buffer pH 9.0. Microwave frequency, 9.086 GHz. (From: J. J. M. C. de Groot *et al.*, *Biochim. Biophys. Acta* **377**, 71–79, 1975.)

E. *Iron in Soybean Lipoxygenase*

During the 11th congress of the International Society for Fat Research in Gothenburg in 1972, Chan[21] announced that he had found 1–2 mole iron per mole soybean lipoxygenase-1 by means of atomic absorption measurements. The presence of iron was soon confirmed by several other investigators[22,31,46,133,140] who all found an iron content of 1 mole per mole enzyme (mol. wt. 100,000). Axelrod[12] demonstrated that the four soybean isoenzymes (L-1, -2, -3, -4) contain close to 1 mole of iron atoms per mole enzyme. Roza and Francke[140] suggested that iron in the native enzyme occurs in the ferrous form. Garssen *et al.* (unpublished) demonstrated that after treatment of the enzyme with sodium-dodecyl-sulphate or 6 M guanidine under strictly anaerobic conditions, the iron can be trapped by *O*-phenanthroline without addition of a reducing agent indicating that it is liberated in the Fe (II) state. Tiron is an unreliable reagent for deciding on the valence state of iron since it catalyses the autoxidation of ferrous iron. Even under virtually anaerobic conditions, a considerable part of the iron in a solution of Mohr's salt is found as Fe (III) by means of Tiron. This phenomenon may explain Axelrod's[133] tentative conclusion that iron in the native enzyme should be present in the Fe (III) state. So far, no successful attempts to reconstitute the enzyme by treating it with Fe (II) or Fe (III) under various conditions, have been reported.

VII. THE ANAEROBIC REACTION

When an incubation is carried out with soybean lipoxygenase-1 at pH 9.0 and linoleic acid in a closed system using a molar excess of fatty acid with respect to the available amount of oxygen, reaction products other than hydroperoxides are formed.[71,72,166] The products are characterized by an ultraviolet-absorption at 285 nm, indicating the presence of a conjugated dienone chromophore. The reaction can readily be monitored by using a dual-purpose cuvette.[137] This cuvette is provided with a Clark oxygen electrode connected to a Gilson Oxygraph, which allows the simultaneous recording of the oxygen consumption and of the absorbance change at a certain wavelength. The formation of products absorbing at 285 nm starts when the oxygen in the system is

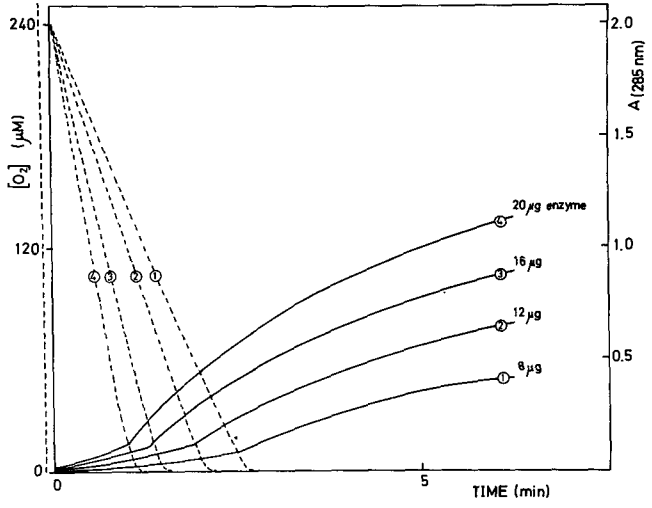


FIG. 13. Progress curves for the lipoxygenase-induced reaction of linoleic acid followed spectrophotometrically at 285 nm (——) and by simultaneous polarographic determination of the O_2 concentration (– – –). To the substrate solution (saturated with air at 25°C and containing 240 μM O_2) was added the indicated amount of enzyme (8–20 μg) dissolved in 10 μl of buffer. (From: Garssen *et al.*, *Biochem. J.* **122**, 327–332, 1971.)

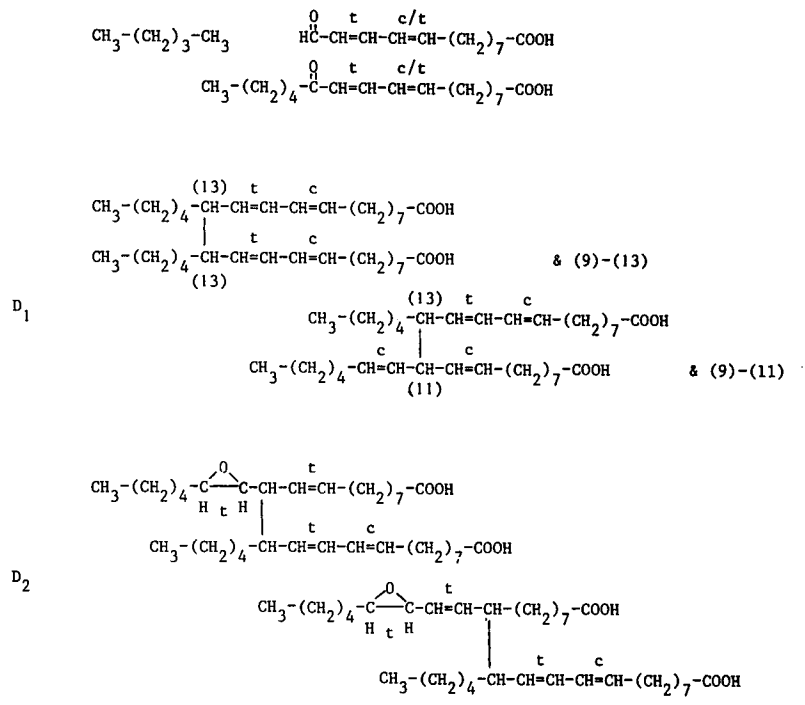


FIG. 14. Products of the anaerobic reaction between lipoxygenase-1, linoleic acid and 13-ROOH.

depleted (Fig. 13). The anaerobic reaction starts immediately when 13-ROOH, linoleic acid and lipoxygenase-1 are incubated in the absence of oxygen. Product hydroperoxide and substrate fatty acid are essential for the reaction. 13-ROOH can be replaced by other hydroperoxides formed as main products by lipoxygenase-1 at pH 9.0 from suitable fatty acid substrates, but not by 9-ROOH or oleic acid hydroperoxides.

Starting from linoleic acid and 13-ROOH, a complex mixture of products is formed in the anaerobic reaction as shown in Fig. 14. The results of experiments with ^{14}C-labelled 13-ROOH or ^{14}C-labelled linoleic acid demonstrated that chain cleavage of 13-ROOH leads to the formation of pentane and 13-oxo-*cis/trans*,9-*trans*,11-tridecadienoic acid. Also 13-oxo-*cis*,9-*trans*,11-octadecadienoic acid is formed from the hydroperoxide. The dimers which contain oxygen originate from one 13-ROOH residue and one linoleic acid moiety, whereas the other dimers are derived from linoleic acid moieties only. The nature of the products formed, especially that of the dimers containing a linkage via C_{11}, suggests that the reaction proceeds via a radical mechanism. This could be proved by applying a radical scavenger to trap the radicals formed during the reaction.[30] To this end, the water-soluble 2-methyl-2-nitroso-propanol was used as a radical trap. Nitroso-alkanes can react with radicals, thereby forming an adduct with considerable stability (Fig. 15). The use of a tertiary nitroso-compound is advantageous to get maximum information on the environment of the unpaired electron in the adduct radical. The absence of a β-hydrogen in the reagent prevents the occurrence of a hyperfine

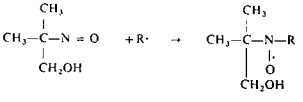


FIG. 15. 2-Methyl-2-nitrosopropanol as a spin-trap in the anaerobic lipoxygenase reaction. (From: J. J. M. C. de Groot *et al.*, *Biochim. Biophys. Acta* **326**, 279–284, 1973.)

splitting due to the structure of the reagent. Since the number of peaks and their hyperfine splitting pattern provide information on the presence of atoms with odd spins near the unpaired electron, the EPR spectrum of the adduct radical gives information on the structure of the trapped radical. [It has to be noted that γ-hydrogen atoms only cause some line broadening.] Other advantages of tertiary nitroso-alkanes are that they do not isomerize to oximes, and that they have only a small tendency to dimerize. In a typical experiment, 0.5 ml of 3.6 mM linoleic acid in an air-saturated 0.2 M borate buffer (pH 9.0) was incubated with 0.2 ml of the lipoxygenase-1 solution (3.75 mg/ml). Owing to the large molar excess of linoleic acid over oxygen, the oxygen is rapidly depleted, followed by the onset of the anaerobic reaction. Immediately after the addition of enzyme, 5 mg of the radical scavenger was added. The corresponding EPR spectrum is depicted in Fig. 16. It shows hyperfine splitting constants of 16.0 and 2.0 Gauss, characteristic of the presence of a β-hydrogen atom in the trapped radical. The signals are centered around g = 2.0056. Upon incubating *cis,cis*-9,12[9,10,11,11,12,13-$^2H_6$] octadecadienoic acid instead of linoleic acid, the EPR spectrum shows only three lines with h.f.s. constants of 16.0 G (Fig. 17). This indicates that the β-hydrogen responsible for the hyperfine splitting has been replaced by deuterium thus excluding the possibility that an enzyme radical had been trapped in the first experiment. Replacing only the hydrogen atoms at C-11 of linoleic acid by deuterium did not result in a loss of hyperfine splitting, which demonstrates that no radical was trapped at C-11. Incubations of *cis,cis*-9,12-[9,10,12,13-$^2H_4$] octadecadienoic acid gave EPR spectra identical to the hexadeuterio-compound. Therefore, the radical scavenger had reacted on the positions 13 and/or 9. The trapped radical stems from linoleic acid and not from the hydroperoxide because incubation of *cis,cis*-9,12-[9,10,11,11,12,13-$^2H_6$] octadecadienoic acid and unlabelled 13-ROOH with lipoxygenase-1 under strictly anaerobic conditions resulted in an EPR spectrum without hyperfine splitting of 2.0 G.

The anaerobic reaction offers an adequate explanation for the formation of carbonyl compounds absorbing at 285 nm as reported frequently in the literature.[17,135,166] Furthermore, this reaction may account for some of the reported discrepancies between oxygen consumption and diene formation. Pattee *et al.*[106,127] observed that ripening peanuts produce pentane; a process paralleled by the actual content of lipoxygenase during ripening (Fig. 18). Curiously enough, an isolated and partly purified peanut lipoxygenase preparation forms pentane *in vitro* also under aerobic conditions.[126] Apparently pentane is produced in the peanut via a chain-fission reaction as we described for soybean lipoxygenase-1 under anaerobic conditions. Axelrod[12] reported that lipoxygenase-3 is capable of forming compounds absorbing at 280 nm under aerobic condi-

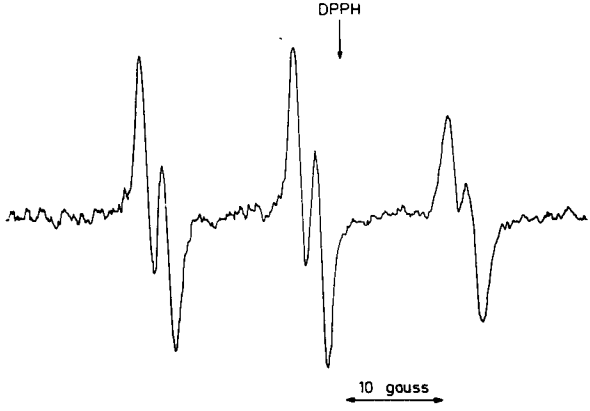


FIG. 16. EPR signal obtained from the incubation of linoleic acid, soybean lipoxygenase-1 and 2-methyl-2-nitrosopropanol. DPPH, diphenylpicrylhydrazyl. (From: J. J. M. C. de Groot *et al.*, *Biochim. Biophys. Acta* **326**, 279–284, 1973.)

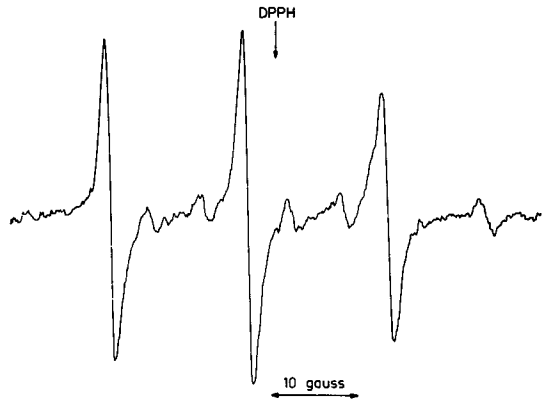


FIG. 17. EPR signal obtained from the incubation of *cis,cis*-9,12-[9,10,11,11,12,13-$^{2}H_{6}$] octadecadienoic acid, soybean lipoxygenase-1 and 2-methyl-2-nitrosopropanol. DPPH, diphenylpicrylhydrazyl. (From: J. J. M. C. de Groot *et al.*, *Biochim. Biophys. Acta* **326**, 279–284, 1973.)

tions. Garssen[69] found that corn germ lipoxygenase with a pH optimum of 6.6 gives no anaerobic reaction. However, the crude enzyme preparation contains also some pH 9.0 activity,[159] leading to 13-ROOH formation and it is at this pH that corn germ lipoxygenase has the capacity to perform the anaerobic reaction.

VIII. STEREOCHEMISTRY OF HYDROGEN ABSTRACTION AND OXYGEN INSERTION

Upon incubating [13-L_s-^{3}H,3-^{14}C] eicosa-8,11,14-*all cis*-trienoic acid with a soybean lipoxygenase preparation, Hamberg and Samuelsson[95] demonstrated that the tritium label was almost completely lost in the main product, the 15-L_s hydroperoxide. In experiments with [13-D_{proR}-^{3}H,3-^{14}C] eicosa-8,11,14-*all cis*-trienoic acid, the tritium label was almost completely retained in the 15-L_s hydroperoxide. Therefore, they concluded that the H-abstraction at C-13 proceeds stereospecifically.

By making use of [11-L_{proS}-^{3}H,1-^{14}C] linoleic acid, Egmond *et al.*[41] demonstrated that at pH 9.0 soybean lipoxygenase-1 abstracts specifically 11-L_{proS}-hydrogen in the formation of 13-L_s-ROOH. For the 9-hydroperoxy linoleic acid, which arises as a minor by-product in the reaction, a preponderance of D_{proR} over L_{proS} hydrogen abstraction

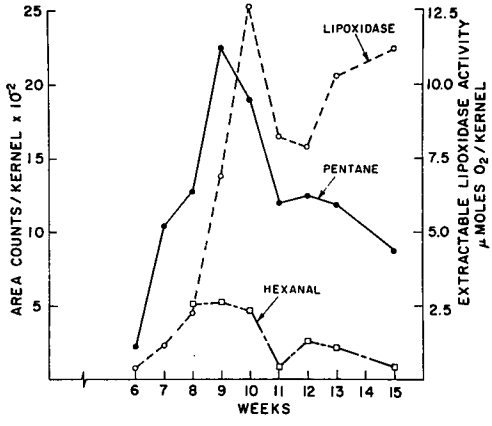


FIG. 18. Lipoxygenase activity and the content of pentane and hexanal in peanut kernels during maturation. (From: H. E. Pattee *et al.*, *Agr. Food Chem.* **18**, 353–356, 1970.)

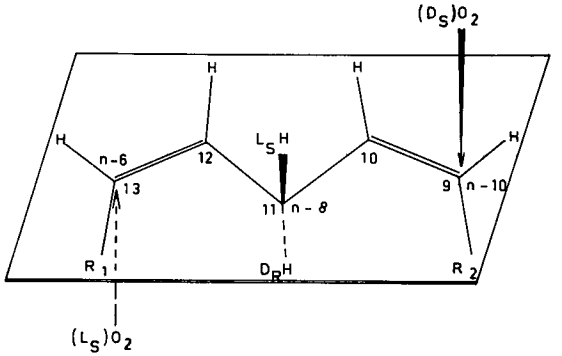


FIG. 19. Stereochemistry of hydrogen abstraction and oxygen insertion in lipoxygenase catalysis. R_1: $CH_3(CH_2)_4$; R_2: $(CH_2)_7COOH$. (From: M. R. Egmond *et al.*, *Biochem. Biophys. Res. Commun.* **48**, 1055–1060, 1972.)

at C-11 exists.[162] A similar experiment with corn germ lipoxygenase at pH 6.6 showed that in the formation of 9-D_R hydroperoxy linoleic acid, the main reaction product, the 11-D_{proR} hydrogen is removed. A comparison of the stereochemistry of the hydrogen abstraction and oxygen insertion for the main reaction products of soybean and corn germ lipoxygenase makes clear that L_{proS} (n-8) hydrogen abstraction gives rise to 13-L_S-hydroperoxylinoleic acid, whereas D_{proR} (n-8) hydrogen abstraction leads to 9-D_R-hydroperoxylinoleic acid. The consequences of these findings are presented in Fig. 19. Since there is ample evidence that a linoleic acid radical is formed upon removal of the hydrogen at C-11, it is reasonable to propose that the pentadiene system of the substrate molecule is oriented on the enzyme as a planar structure.[162] Inspection of Fig. 19 shows that hydrogen abstraction and oxygen insertion take place at opposite sides of the plane. This enzymic oxygenation of unsaturated fatty acids differs strikingly from the oxygenation of olefins by singlet oxygen. In the latter case, the introduction of oxygen and removal of hydrogen occur at the same side of a planar olefinic structure.[49] This excludes the involvement of singlet oxygen in the main course of the reaction.

Hamberg and Samuelsson[95] concluded on the basis of the isotope effect in incubations of L_{proS}(n-8)-^{3}H labelled eicosatrienoic acid with soybean lipoxygenase, that hydrogen abstraction must precede or coincide with irreversible oxygen insertion. They postulated that the reaction is initiated by the removal of a hydrogen atom. In incubations of 11-L_{proS}-^{3}H,1-^{14}C linoleic acid with soybean lipoxygenase-1, we observed a strong enrichment of the ^{3}H label relative to the ^{14}C label[41] in the unreacted substrate, illustrating a large difference in the reaction rates of [11-L_{proS}-^{1}H,1-^{14}C] linoleic acid and the 11-L_{proS}-^{3}H labelled compound. The conversion rates of these acids by corn germ lipoxygenase at pH 6.6 are only slightly different. The large differences in the reaction rates observed between tritiated and nonlabelled substrate with soybean lipoxygenase-1 must be due to the hydrogen abstraction step and/or to the formation of the enzyme/substrate complex. The enrichment of ^{3}H label in the unconverted substrate could point to hydrogen abstraction as the initial and/or rate-determining step in the overall reaction. Kinetic studies on the overall reaction can give further insight into the nature of the isotope effect. However, a quantitative assessment of kinetic parameters using [11-L_S-^{3}H,1-^{14}C] linoleic acid as substrate is inaccurate because, on a molar basis, only a minute fraction of the substrate carries the label. Therefore, we performed experiments[40] with linoleic acid labelled to virtually 100% with deuterium in the pentadiene system {[11,11-$^2H_2$]; [9,10,12,13,-$^2H_4$]-; [9,10,11,11,12,13-$^2H_6$] linoleic acid}. The apparent K_m values (19.6–23·2 μM) are only slightly affected by the type of substrate. Since isotope effects K_{1_H}/K_{2_H} of 8.7 and 9.3 are observed for di- and hexa-deutero linoleic acid, respectively, it can be concluded that hydrogen abstraction from C-11 is the rate-determining step in the overall reaction, and possibly the initial step.

IX. HYDROGEN ABSTRACTION IN THE ANAEROBIC REACTION

To study the stereochemistry of the hydrogen abstraction in the anaerobic reaction, Egmond[37] incubated under strictly anaerobic conditions [11-L$_{proS}$-^{3}H,1-^{14}C] linoleic acid and unlabelled 13-ROOH with soybean lipoxygenase-1 at pH 9.0. Of the various products, only the dimers were labelled. The linoleic acid moieties in the dimers linked through C-13 and C-9 retained only small amounts of ^{3}H-label. This result points to a predominantly enzymic removal of the L$_{proS}$-hydrogen atom from C-11 of linoleic acid. Therefore, it is reasonable to suggest that the enzymic abstraction of hydrogen from the substrate is identical under aerobic and anaerobic conditions. The linoleic acid moieties in dimers containing a C-11 linkage have retained a relatively high ^{3}H-content. This indicates that in the formation of the latter type of dimers, a nonenzymic and a specific hydrogen abstraction occurs. The kinetics of the anaerobic reaction need further investigation in order to establish whether or not the enzymic hydrogen abstraction is the initial and/or rate-limiting step in the overall reaction.

X. KINETICS

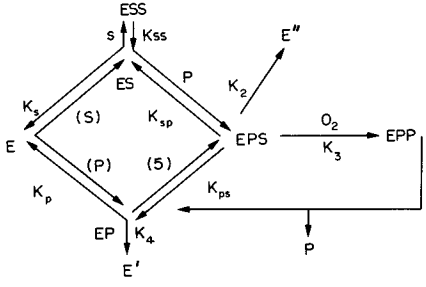
This chapter on the kinetics of the lipoxygenase-catalyzed oxygenation of the various *cis,cis*-methylene-interrupted unsaturated fatty acids is confined to lipoxygenase-1 from soybeans. The rate of oxygenation can be followed by measuring the uptake of O_2 and/or by monitoring the absorbance at 234 nm, which is caused by the formation of the conjugated hydroperoxides ($E_{234} = 25.000\ M^{-1}\ cm^{-1}$,[107]). Recently, the reliability of the continuous spectrophotometric assay of the reaction has been questioned[35] because of the instability of the hydroperoxides formed. However, at 25°C and pH 9.0 the hydroperoxides formed were not found to be degraded during incubation as long as the O_2 concentration in the solution did not drop below approx 5×10^{-6} M.[38]

As early as 1948, Bergström and Holman[15] presented indirect evidence for the involvement of free radical intermediates in the enzymic reaction. Using methyl linoleate as substrate, Kunkel[112] demonstrated that the activity of the enzyme was linear *vs* the square root of the enzyme concentration, which was taken as evidence for an enzyme-catalyzed radical chain process. Thus, the enzymic oxygenation reaction would closely resemble the nonenzymatic autoxidation of unsaturated fatty acids. However, in 1952, Tappel *et al.*[152] showed that a normal linear relation between enzyme activity and concentration is obtained, if a solubilizer is added to the system. They further demonstrated that the enzymatic oxygenation at pH 9 obeys normal Michaelis–Menten kinetics when initial rates are measured at substrate concentrations below 2.50×10^{-4} M. In these experiments, the K_m value found for linoleic acid ($K_m = 2 \times 10^{-5}$ M) in air-saturated solutions at pH 9.0 appeared to differ by more than one order of magnitude from previous determinations of the K_m at high substrate levels (1.4×10^{-3} M[95]). Allen[1] later confirmed the K_m value found by Tappel *et al.*[152] ($K_m = 24.1 \pm 0.04$) 10^{-6} M at 25°C in air-saturated Tris buffer, pH 9.0, ionic strength 0.2. Allen also investigated the effect of the physical condition of linoleic acid on the oxygenation rate and mentioned that measurements of the rates at high linoleic acid concentrations were unreliable because of formation of emulsions. Solubility problems are even more pronounced, when spectroscopic measurements are carried out at low pH. This effect of pH on the solubility of the substrate was overcome by Allen[2] by using linoleylsulfate as substrate for the enzyme. At pH 9, the K_m values for linoleic acid and linoleylsulfate were about equal, indicating that the affinity of the enzyme for the pentadiene system in both substrates remained unaltered.

Tappel *et al.*[152] ascribed lower reaction rates and deviation from simple Michaelis–Menten kinetics at higher substrate levels ($> 2.5 \times 10^{-4}$ M) not only to physical effects but also to substrate inhibition. However, no inhibition constants were reported. Studying the effect of the O_2 concentration on the reaction rate, Tappel *et al.*[152] mentioned that the substrate inhibition caused by linoleic acid is more pronounced at low than at high O_2 concentrations. Thus, molecular oxygen seemed to prevent inhibition by

excess linoleic acid. The apparent K_m values for O_2 at two different (high) concentrations of linoleic acid were found to be 3×10^{-5} M and 2.9×15^{-4} M at 3.6×10^{-4} M and 7.2×10^{-3} M linoleic acid, respectively. Smith and Lands[145] systematically investigated the kinetics of the oxygenation of various unsaturated fatty acids containing a 1,4-*cis,cis*-pentadiene system in air-saturated solutions at pH 9. They observed that the activity of the enzyme decreased during incubation according to a first-order process. This destruction of lipoxygenase was slow if linoleic acid was incubated with the enzyme, but substrates with a higher degree of unsaturation showed much higher inactivation constants. (K_{dest} linoleic acid = 0.05 min^{-1}, K_{dest} linolenic acid = 0.40 min^{-1}.) An important finding by Smith and Lands[145] and Garssen[69] was that the presence of the product (n-6)L_S-hydroperoxy fatty acid is a prerequisite for maximal catalytic activity of the enzyme. These findings explain why contamination of the substrate by small amounts of the product, formed via autoxidation during storage of the substrate, eliminates the induction period.[89,111,152]

Smith and Lands[145] presented the following scheme (Scheme 1) for the kinetic mechanism of the oxygenation reaction:



E: Lipoxygenase ; E', E'' inactive enzyme.

S: Substrate. P: Product.

$K_s = K_p = K_{ps} = K_{sp} = 10^{-6}$ M.

$K_{ss} = 5.10^{-5}$ M.

SCHEME 1.

Assuming that the species E, ES, EP, EPS and ESS are in constant rapid equilibrium during formation of the product, Smith and Lands estimated the equilibrium constants by fitting their experimental results to the rate equation derived from the proposed scheme. Although this scheme comprises substrate inhibition and product activation, it does not account for the effect of varying O_2 concentrations on the inhibition by excess substrate, as previously shown by Tappel *et al.*[152] The same applies to the fact that when linoleic acid is used as substrate, the initial rate is apparently independent of the concentration of the product at linoleic acid concentrations lower than 5×10^{-5} M. This either means that the affinity for the product is extremely high ($K_m \ll 10^{-6}$ M), or that also product-independent conversion of the substrate takes place. On the basis of earlier isotope enrichment studies by Hamberg and Samuelsson,[95] Smith and Lands[145] suggested that the conversion of the substrate in air-saturated solution (step K_3 in the scheme) is the rate-limiting step in the overall oxygenation reaction.

The kinetic isotope effect was studied in more detail by Egmond *et al.*[41] Comparison of the reaction rates for linoleic acid and (11,11-$^2H_2$) linoleic acid as substrates showed that the K_m was not affected by isotope substitution, but that V_{max} of the enzymic reaction was strongly reduced when the dideuterated substrate was incubated ($K_{1H}/K_{2H} \approx 9$). The magnitude of this kinetic isotope effect justifies the suggestion by Smith and Lands that the abstraction of H from the substrate is the rate-limiting step

in the oxygenation reaction in air-saturated solutions. Egmond *et al.*[38] reinvestigated the effect of the O_2 concentration on the aerobic conversion of linoleic acid. They confirmed the previous finding by Tappel *et al.*[152] that substrate inhibition is more pronounced at low O_2 than at high O_2 concentrations. However, substrate inhibition was also found to depend on the presence of the product hydroperoxide (in air-saturated solution at pH 9.0 and 25°C, the inhibition constant K_i for linoleic acid = 5.2×10^{-4} M at 10^{-5} M hydroperoxide), which is in agreement with the previous experiments in O_2-saturated solutions by Smith and Lands.[145] At 2.5×10^{-4} M linoleic acid and 10^{-5} M 13-ROOH, the apparent K_m for O_2 was found to be 3.5×10^{-5} M at 25°C, pH 9.0 (Tappel *et al.*[152] found: K_m (O_2) = 3×10^{-5} M at 3.6×10^{-4} M linoleic acid).

Recent experiments (Egmond *et al.*[38]) have shown that linoleic acid is a hyperbolic competitive inhibitor of the oxygenation reaction catalyzed by lipoxygenase-1 in the presence of 13-ROOH. The inhibition is explained by a kinetic model in which binding sites on the enzyme for linoleic acid and oxygen are proposed, while also the presence of a regulatory site, which can either bind substrate or 13-ROOH, is introduced. At high concentrations of pure linoleic acid substrate, it is thought that both the substrate and the product (regulatory) site are occupied by the substrate, which results in an appreciable kinetic lag-phase. A relatively large amount of 13-ROOH is needed to displace the substrate from the product site resulting in elimination of the lag-phase. On incubating substrate at very low concentrations, the kinetic lag-phase does not show up, which may reflect the higher apparent affinity for O_2. Under these conditions, autoxidation will have a greater impact through subsequent occupation of the product site by 13-ROOH. Furthermore, it was found that prior conversion of the native enzyme into the yellow Fe (III)-form by reacting it with 13-ROOH does not lead to altered steady-state kinetics.[29] The yellow enzyme also shows a kinetic lag-phase which can be eliminated by 13-ROOH.

XI. MECHANISM OF THE AEROBIC REACTION

In the history of lipoxygenase, several schemes for the oxygenation of unsaturated fatty acids have been proposed.[103,151] As early as 1952, Tappel[152] suggested the involvement of fatty acid radicals in the reaction. Although the occurrence of these radicals could not be demonstrated unambiguously,[169] there is now accumulating evidence that they are indeed formed.[30]

Oxygen also serves as a substrate in the aerobic reaction. Some kind of activation of oxygen may play a role in the mechanism. Chan[20] proposed that singlet ($^1\Delta_g$) oxygen is involved. Later on,[13] this suggestion was questioned because the cooxidation of the singlet oxygen scavenger tetraphenyl-cyclopentadienone in the system linoleate–lipoxygenase had been misinterpreted. Also, Smith and Lands[145] presented a mechanism in which activated molecular oxygen (probably $^1\Delta_g$ O_2) participates. They speculated that hydrogen abstraction from the substrate takes place after addition of activated molecular oxygen. The latter species would be generated by the enzyme via a tetroxide intermediate derived from product hydroperoxide and ground-state oxygen. However, besides the stereochemistry of the reaction, a serious objection to this mechanism is the existence of a hydrogen abstraction step in the anaerobic reaction.

Another form of activated oxygen which can be considered is the superoxide anion radical. However, the aerobic reaction cannot be inhibited by superoxide dismutase[12,47] which rules out the possibility that O_2^- as a free species plays a role in the main course of the reaction (see also Section XIII). Accepting that fatty acid radicals are formed from the substrate, the question remains whether the hydrogen atom (proton plus electron) is removed in one step or that proton removal and electron transfer take place separately. So far, no indications in favor of the latter possibility have been found, nor has any clue as to which entity the hydrogen atom would be temporarily attached.

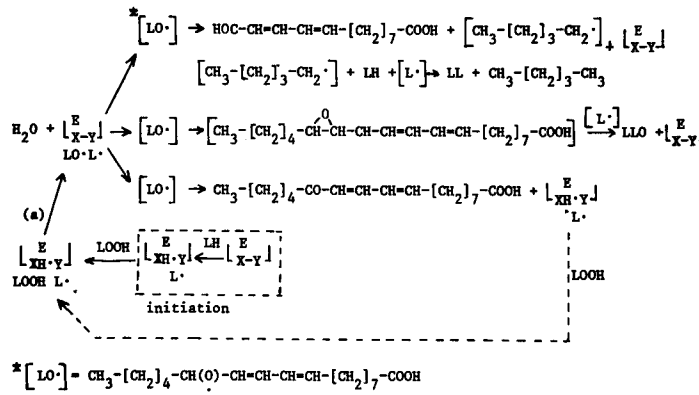


FIG. 20. Mechanistic scheme of lipoxygenase catalysis. Though the nature of [X] and [Y] is unknown, they might represent a disulfide group. (From: G. J. Garssen *et al.*, *Biochem. J.* **130**, 435–442, 1972.)

Before the presence of iron in the enzyme was known, we suggested[71,72] that the protein might temporarily hold the hydrogen atom, resulting in a radical state of the enzyme (Fig. 20). Now we propose that the hydrogen atom disintegrates into a proton and an electron, whereby the electron is transferred to iron. A transient enzyme radical could still occur but this is only a hypothesis. In view of its function as an electron acceptor, iron in the active enzyme must occur in the Fe (III) state. This is in line with the observed reduction by linoleic acid of Fe (III) in the enzyme under anaerobic conditions.[31] The EPR spectra (Section VI) demonstrate this change in valence state. The fluorescence and absorbance characteristics of this reduced lipoxygenase approach those of the native enzyme.[29,39] In the aerobic reaction, the intermediary fatty acid radical is oxygenated. In principle, this can be achieved by molecular oxygen but, as discussed before, activation of oxygen cannot be excluded. This leads to two alternative routes. The radical reacts with $^3\Sigma_g$ O_2 leading to the peroxy radical. Then an electron from Fe (III) is transferred to the peroxy radical, yielding the peroxy anion which is protonated to give the hydroperoxide. Thereby, the enzyme is regenerated and can start again with the conversion of the fatty acid. Alternatively, oxygen is reduced to O_2^- or to $OOH^{\cdot}$, and one of those species reacts with the fatty acid radical. Though it is hard to discriminate, we have some preference for the first route. In regard to the role of 13-ROOH in the mechanism, it could be that the yellow and/or the purple enzyme species are involved. To gain further insight into these possibilities, we prepared the yellow enzyme on a large scale by incubating the native enzyme with an excess of 13-ROOH followed by removal of the low-molecular compounds via filtration over Sephadex G-25. A comparison of the native and yellow enzymes shows that these do not differ in the rates of formation of hydroperoxides from linoleic acid and molecular oxygen (Fig. 21). Both forms have similar lag-periods as is evident from the progress curves. The lag-phase of the yellow form can be eliminated by addition of 13-ROOH, just as for the native enzyme. This result indicates that in order to reach full activity, an occupied product binding site is essential. Since we also suggested that the active enzyme occurs in the Fe (III) state, it is possible that the purple form (Fe (III) complex with 13-ROOH) represents the active form.

A still unanswered question is the initial activation of the enzyme. It is conceivable that upon complex formation of the native enzyme with 13-ROOH in the presence of substrate, an electron transfer from iron to oxygen takes place. The occurrence of trace amounts of $O_2^{-\cdot}$ during the reaction (cf. Section XIII) may support this suggestion. It should be noted that during the reaction dissociation of the enzyme-fatty acid radical complex may take place to a small extent. This leads to fatty acid free radicals in

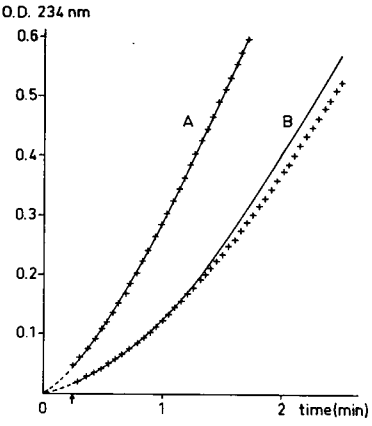


FIG. 21. Progress curves showing the formation of 13-ROOH as a function of time, measured via continuous monitoring of the absorbence at 234 nm in a 1 cm cuvette. The reactions were started at t = 0 by adding 6 μl enzyme solution (1.4×10^{-6} M) to 3.5 ml reaction medium, containing 2.4×10^{-4} M O_2 and either 2.4×10^{-4} M linoleic acid (A) or 4.8×10^{-4} M linoleic acid (B). Measurements started 15–20 seconds after addition of enzyme (mixing time): the arrow indicates the start of the reaction: (——) for the native, EPR-silent form of the enzyme at t = 0; (+ + + +) for the Fe (III) form of the enzyme at t = 0. (From: J. J. M. C. de Groot *et al.*, *FEBS Lett.* **56**, 50–54, 1975.)

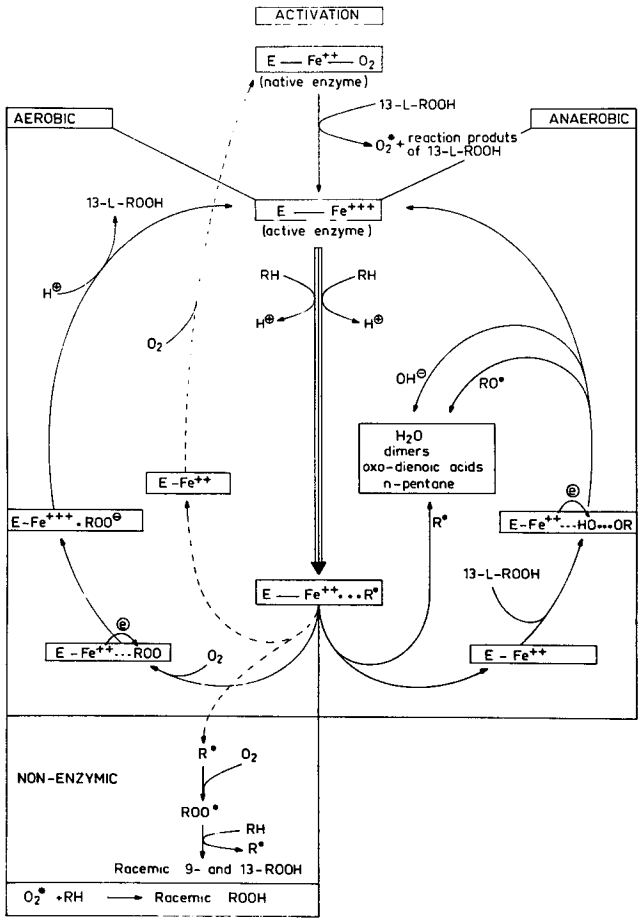
the solution, which would then initiate autoxidation of the fatty acid leading to small amounts of racemic products.

XII. MECHANISM OF THE ANAEROBIC REACTION

The anaerobic reaction is unquestionably a radical reaction. The enzymically formed linoleic acid radicals appear in the solution due to dissociation of the enzyme fatty acid radical complex. As mentioned before, Fe (III) in lipoxygenase is reduced to Fe (II) during the conversion of the fatty acid. To regenerate the enzyme, 13-ROOH is required. Previously, we proposed two possibilities: the formation of alkoxy or peroxy radicals.[72] Now that we know that iron in the ferrous form is involved in this stage, the alkoxy radicals are the most probable species to be formed (cf. 16) together with hydroxyl anions. The alkoxy radicals are the precursors of the oxodienoic acids and of the dimers containing oxygen. The dimers not containing oxygen are formed by combination of enzymically formed linoleic acid radicals or by a chain reaction of such a radical with free linoleic acid[30,72] in the solution. The course of the reactions catalyzed by soybean lipoxygenase-1 under aerobic and anaerobic conditions can be summarized as follows (Scheme 2).[31]

XIII. CHEMILUMINESCENCE DURING LIPOXYGENASE CATALYSIS

The participation of both molecular oxygen and a fatty acid in the lipoxygenase reaction has raised fundamental questions concerning the activation of substrates. The possible involvement of an activated oxygen species was studied by Chan,[20] who observed a conversion of singlet ($^1\Delta_g$) oxygen scavengers during lipoxygenase catalysis. The structures of the oxygenated scavengers were then compared with those obtained in a photochemical oxygenation. A detailed investigation[13] showed that the products obtained from the lipoxygenase-mediated oxygenation and the photochemical oxygenation had different structures. Teng and Smith[153] observed that the cooxidation of cholesterol during lipoxygenase catalysis yielded cholesterol derivatives, which are unlikely to result from a singlet oxygen reaction but can readily be explained by a free radical



SCHEME 2. Proposed reaction scheme for the activation of soybean lipoxygenase-1 and for the catalytic activities at pH 9.0 under aerobic and anaerobic conditions. RH = fatty acid. (From: J. J. M. C. de Groot *et al.*, *Biochim. Biophys. Acta* **377**, 71–79, 1975.)

mechanism. This result excludes the involvement of an activated oxygen species—identical to the one occurring during photochemical oxygenation—in the formation of 13-ROOH by the lipoxygenase reaction. However, the presence of iron at the active site of soybean lipoxygenase and its function during catalysis continues to evoke interest in the process of bonding and possible activation of oxygen by the iron.

The presence of activated reactants may be accompanied by emission of light. In the case of lipoxygenase, a few reports[47,121] have appeared in which chemiluminescence during the oxygenation of unsaturated fatty acids is described. The weak or hardly detectable emission can be amplified considerably by the addition of fluorophores (such as luminol) to the system. The role of luminol in chemiluminescent reactions has been investigated recently.[102] It was found that a variety of free radicals can bring about luminescence. Experiments in the authors' laboratory have confirmed the enhancing effect of luminol provided that oxygen is available in the solution. In a typical experiment (Fig. 22), linoleic acid to which luminol had been added lipoxygenase was allowed to react and the resulting chemiluminescence was measured in a liquid scintillation counter with the coincidence circuitry switched off. In a parallel experiment, the absorbance at 285 nm and the O_2 concentration were measured against time (using the same concentrations of reactants and the same temperature (12°C)). As can be seen from

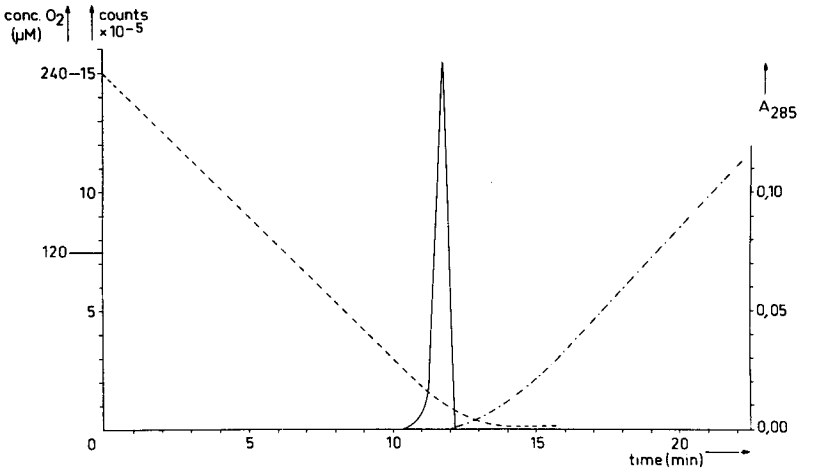


FIG. 22. Relationship between O_2 concentration, chemiluminescence and anaerobic lipoxygenase reaction. Conditions: linoleic acid (1.85 mM), luminol (21 μM), lipoxygenase (5×10^{-3} M), O_2 (240 μM at t = 0), borate buffer, pH 9.0 (– – –; O_2), (——; luminescence) (·–·–; A_{285}). (From: G. A. Veldink *et al.*, unpublished, 1975.)

Fig. 22, a strong and fast-declining emission peak just precedes the onset of absorbance at 285 nm, which is characteristic of the anaerobic reaction. Once the system has become anaerobic, no further luminescence is observed. The luminescence yield is much lower when luminol is omitted from the reaction mixture while also the pattern shows less distinct peaks (Fig. 23). The emission observed in the presence or absence of luminol, is completely quenched by superoxide dismutase and is unaffected by catalase. The slow increase in the luminescence during the steady state of aerobic lipoxygenase catalysis may be due to the increasing amount of hydroperoxide in the solution. This phenomenon may be analogous to the increase in luminescence observed with the xanthine oxidase/hypoxanthine system on addition of extra amounts of hydrogen peroxide.[11] In fact, we observed an enhancing effect of hydroperoxylinoleic acid with xanthine oxidase/hypoxanthine though less pronounced than with H_2O_2. The mechanism through

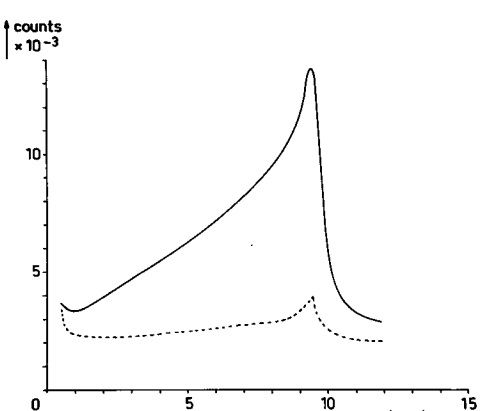


FIG. 23. Effect of catalytic amounts of superoxide dismutase (SOD) on the chemiluminescence during lipoxygenase catalysis. Conditions: linoleic acid (2.8 mM), lipoxygenase (10^{-2} μM), O_2 (240 μM at t = 0), borate buffer, pH 10.0. In the presence of SOD (5×10^{-2} μM), the luminescence is almost completely quenched (lower curve). Addition of 0.1 μM SOD reduces the luminescence to the noise level. (From: G. A. Veldink *et al.*, unpublished, 1975.)

which light is emitted from the lipoxygenase/linoleate/O_2 mixture is not at all clear. The quenching effect of superoxide dismutase points to the involvement of superoxide anions. However, superoxide dismutase does not affect the oxygenation rate of linoleic acid catalyzed by lipoxygenase-1, which may indicate that the release of activated oxygen species is a side-effect of the oxygenation process. The sudden increase in luminescence suggests that, above a certain critical oxygen concentration, the system is forced to continue its aerobic cycles whereas on further oxygen depletion it is caused to shift to the anaerobic pathway. It is conceivable that this low oxygen level causes a significant dissociation of the fatty acid free radical/enzyme complex. In systems containing luminol, the fatty acid radical induces superoxide anion formation via luminol.[102] However, if no luminol is present, activation of oxygen via the reduced enzyme may occur as we proposed for the activation of the native enzyme.

XIV. IRREVERSIBLE INHIBITION OF LIPOXYGENASE

A. *Hydrogen Peroxide*

Hydrogen peroxide is a very potent, irreversible inhibitor of soybean lipoxygenase-1. It is an anologue of the hydroperoxide which attacks iron and its direct coordination sphere.[39,123] At 3.5×10^{-4} M H_2O_2, the activity of 10^{-8} M lipoxygenase-1 at pH 9.0 is completely destroyed. As discussed before, treatment of soybean lipoxygenase-1 with a 4-fold molar excess of H_2O_2 causes changes in the fluorescence and absorbence, which are comparable to the formation of yellow enzyme species from the native one. EPR-spectroscopy shows that the iron is oxidized to the high-spin Fe (III). It is evident that not only the valence state of iron has been altered but also its direct coordination sphere: (1) no purple enzyme species can be formed by addition of 13-ROOH and (2) the greater part of the iron can now be removed from the enzyme by treatment with *O*-phenanthroline + dithionite. The inactivation of lipoxygenase by thiols such as cysteine, 2 mercapto-ethanol and glutathione has to be ascribed to H_2O_2 in such systems. The autoxidation of the thiol compound, particularly in the presence of trace amounts of metals (Cu^{2+}, Mn^{2+}, Fe^{3+}), yields H_2O_2. Since we always found small amounts of these metals as contaminants—even in the purest enzyme preparations—this autoxidation process can easily occur.

B. *Acetylenic Compounds*

Besides hydrogen peroxide and the inhibitors of the free radical chain-breaker type listed by Tappel,[151] another class of inhibitors has been described by Downing *et al.*[32,33] Both soybean lipoxygenase and prostaglandin synthetase from sheep seminal vesicles appear to be irreversibly inhibited by eicosa-5,8,11,14-tetraynoic acid and by octadec-9,12-diynoic acid at very low concentrations. Preincubation of enzyme and acetylenic compound already caused complete inactivation, because on subsequent addition of natural linoleic acid no conversion of the latter is observed. Interestingly, the enynoic acids such as crepenynic acid did not inactivate soybean lipoxygenase. The mechanism of the inhibitory effect has been postulated[32] to involve hydrogen abstraction from the ω 8 carbon atom and a subsequent irreversible reaction of the allene with the enzyme.

XV. HETEROGENEITY OF SOYBEAN LIPOXYGENASE

In recent years, the presence of a large number of enzymes oxygenating unsaturated lipids have been discovered. Only after sufficient purification, could some of these be identified as lipoxygenases. On the basis of differences between carotene bleaching activity, Kies *et al.*[108] concluded that in crude soybean preparations, at least two different

types of lipoxygenase must be present, one of these being responsible for the carotene bleaching activity. The so-called lipoxygenase isoenzymes were subsequently further characterized. One of the pronounced differences between the Theorell-lipoxygenase and other lipoxygenase is their pH-response. So far, soybean lipoxygenase-1 (pH optimum 9.0)—when compared with either the isoenzymes from soybeans or with lipoxygenases from a wide range of other sources—appears to behave exceptionally. The latter type of enzymes shows an optimum activity below pH = 7.0.

Soybean lipoxygenase-1 has been isolated, purified and found homogeneous by several investigators. However, Verhue and Francke[164] found two fractions which were active at pH 9.0 towards linoleic acid as substrate. The methyl esters were oxygenated at significantly lower rates. Furthermore, the low pH activity was found to consist of at least two entities. The functional significance of the heterogeneities within the subfractions is questioned by these authors since no fundamental differences were found in regard to substrate and product specificities. Christopher *et al.*[24] have described four lipoxygenase isoenzymes from soybeans, one active at pH 9.0 (lipoxygenase-1) and three active at pH 6.6 (lipoxygenases-2, -3 and -4). Lipoxygenases-3/-4 appear to have an anomalous pH-response but resemble lipoxygenase-2 in substrate and product specificities. Yamamoto *et al.*[175] also isolated a pH 9.0 enzyme and a pH 6.6 enzyme, which were designated lipoxygenase-a and -b, respectively. In a recent review, Axelrod[12] has summarized the properties of lipoxygenase isoenzymes regarding substrate and product specificities. Despite many efforts, the proteins responsible for the lipoxygenase activity at low pH have not been characterized in sufficient detail to permit a comparison of their amino-acid compositions and active sites.

XVI. COOXIDATION

Unlike soybean lipoxygenase-1, the pH 6.6 enzymes show considerable cooxidizing activity towards various co-substrates, which has found some technical application, e.g. in flour bleaching.[36] The effect may well reflect fundamental differences between the mechanisms through which the lipid substrates are converted by this type of enzymes. Lipoxygenases having this cooxidizing capacity show a less pronounced specificity as to the nature of the primary lipid substrate, i.e. also the methylesters of fatty acids of the linoleic acid type are converted. Recent studies[13,153] have shown that the rather weak cooxidation observed for soybean lipoxygenase-1 does not involve free singlet oxygen as the active chain-carrying species but rather free radicals derived from the fatty acid.

For some time one of the routine assays of lipoxygenase has been based on the rate at which carotene was destroyed during the conversion of unsaturated lipids. However, this method became obsolete when it was recognized that cooxidation of carotene by lipoxygenase preparations is primarily due to lipoxygenase-2 which exerts a strong cooxidation. The cooxidation of carotene by purified lipoxygenases has been studied in some detail with lipoxygenases from peas, soybeans, wheat, flax and alfalfa.[10,172] The pea enzyme (mol. wt. 78,000) was separated into two fractions with both lipoxygenase and carotene oxidase activity at pH 6.3 with a linoleic acid/Tween 20 substrate. In the absence of carotene, the fatty acid was oxidized into hydroperoxides whereas hydroperoxides as such were unable to bleach carotene. From crude soybean homogenates, three lipoxygenases were isolated, two of which showed a carotene oxidase activity at pH 6.5. Also a combination of enzyme and hydroperoxides shows a slow enzymic breakdown of carotene. Lipoxygenases isolated from wheat and alfalfa were separated into various fractions which showed a carotene oxidase activity. Carotene oxidase activity could also be demonstrated in linseed although the maximum activity did not coincide with that of lipoxygenase according to the Sephadex G-200 elution pattern. Besides, a relatively low bleaching activity was found in the fractions showing hydroperoxide isomerase activity. The carotene bleaching activity could always be inhibited by nor-dihydroguaiaretic acid (NDGA). Carotene is converted by soybean lipoxy-

genase-2 and -3 for about 55% and 43%, respectively, when related to the linoleate conversion, whereas lipoxygenase-1 is much less effective (6%).[173] On substituting linoleylsulfate/crocin for the less soluble linoleic acid/Tween or linoleic acid/β-carotene, essentially similar results were obtained regarding the relative amounts of co-substrates oxidized by the soybean enzymes.

These experiments showed that the peroxidation of linoleic acid tends to become more specific in that they show a shift in favor of the 13-ROOH. As cooxidation is assumed to be caused by the release of fatty acid free radicals from the enzyme, a subsequent attack of crocin can to some extent suppress the participation of linoleic acid as the substrate for cooxidation, which results in the formation of a smaller amount of the (racemic) 9-ROOH. The occurrence of small amounts of racemic fatty acid hydroperoxides in normal lipoxygenation reactions[95] should, in our opinion, be attributed to a concomitant cooxidation in which the fatty acid itself is involved. This concept also adequately explains the results of Weber *et al.*[173] who proposed that a fatty acid peroxy radical might be the active species while β-carotene should then be oriented near the active site in such a way that it can be attacked by the free radical. However, it is hardly conceivable that the active site of the enzyme is equally accessible to molecules such as β-carotene, crocin and even chlorophyll.[70,125]

The concept that cooxidation during lipoxygenase catalysis involves free radicals may also be inferred from the observation by Axelrod[12] that lipoxygenase-1 from soybeans efficiently catalyzes dye-destruction under anaerobic conditions. The occurrence of free radicals during the anaerobic reaction is well-established.[30] Some of the products from the anaerobic reaction of linoleic acid, linoleic acid hydroperoxide and lipoxygenase-1 are also formed in an aerobic reaction of the pH 6.6 enzymes from soybeans and linoleic acid. The free radicals released under anaerobic conditions by lipoxygenase-1 most likely originate from the dissociation of the enzyme-fatty acid free radical complex. For lipoxygenase-1, the dissociation of the complex becomes predominant in the absence of oxygen. The difference between the cooxidizing potency of the various lipoxygenase isoenzymes might be due to the varying stability of the respective enzyme-fatty acid radical complexes. However, also the rate of the enzymic oxygenation may vary for the different types of enzyme, which would also result in differences in fatty acid free radical release.

XVII. LIPOXYGENASES FROM OTHER SOURCES

Lipoxygenase activity has been found in a wide variety of organisms. However, relatively few lipoxygenases have been studied in detail. The enzymes from soybeans, especially the type-1, are well-characterized. This Section deals with the enzymes that have been isolated and purified from sources other than soybeans.

A. *Peas*

Lipoxygenase activity in peas was detected several years ago.[143] The enzyme was purified by Eriksson and Svensson.[43] It was found to be homogeneous after ultracentrifugation but it separated into two main fractions and one minor fraction on isoelectric focusing, the pI-values of the main fractions being 5.80–5.82. The molecular weight was calculated to be 72,000 and 67,000, respectively. The presence of lipoxygenase isoenzymes in peas was also demonstrated by several investigators.[4,5,10,90,98] Arens *et al*[10] found three active fractions on DEAE cellulose chromatography, which they designated lipoxygenase-1, -2 and -3, respectively. Fraction 2 was subjected to isoelectric focusing and separated into two fractions showing lipoxygenase activity. A similar phenomenon was observed by Eriksson and Svensson.[43]

1. *Amino Acid Composition*

The amino acid analyses reported by Eriksson and Svensson[43] and by Arens *et al.*[10] are given in Table 1.

TABLE 1.

Residue	PEA lipoxygenase[43] (mol. wt = 72,000)	PEA lipoxygenase[10] (mol. wt = 78,000)
ASP	131	78
THR	35	37
SER	40	48
GLU	58	71
PRO	34	48
GLY	38	49
ALA	29	37
CYS	7	4
VAL	28	34
MET	7	7
ILE	31	34
LEU	61	72
TYR	26	29
PHE	23	29
LYS	36	49
HIS	25	23
ARG	28	34
TRP	—	11

Agents known to affect various metal-containing enzymes have no effect on the activity of pea lipoxygenase.[143]

2. *Reaction Products*

The aerobic incubation of linoleic acid and pea lipoxygenase results in the formation of a mixture of varying amounts of 9- and 13-*cis,trans*-conjugated hydroperoxides. The ratio of the quantities of the 9- and 13-isomers were found to be 55:45[116] and 58:42.[10] The isomer ratio is unaffected by limiting the availability of oxygen. Arens *et al.*[8] found that depending on the enzyme/substrate ratio, besides monohydroperoxides also oxodienoic, epoxyhydroxy, monohydroxy, dihydroxy and trihydroxy fatty acids were formed. At low lipoxygenase concentrations, relatively large amounts of hydroperoxides are formed. The hydroperoxides do not serve as precursors for the monohydroxy and epoxyhydroxy fatty acids. Siddiqi and Tappel[143] noted an increase in absorbance at 280 nm, which indicates the presence of monocarbonyls. The formation of this material was ascribed to secondary reactions of the hydroperoxides with concomitant consumption of additional oxygen.

3. *pH-optimum*

Various pH optima have been reported for the system linoleate/oxygen/pea lipoxygenase. Table 2 summarizes some recent values.

B. *Corn* (Zea mays)

Lipoxygenase activity has been found in corn by several investigators.[50,52,168] The purified enzyme has a maximum activity when linoleic acid is used as substrate at

TABLE 2.

Source	pH optimum	pI
Acetone-defatted peas[143]	6.9	—
Acetone-defatted peas[10]	6.3	6.00–6.15
Acetone-defatted peas[43]	6.5	5.80–5.82
Acetone-defatted pea seedlings[5]	6.2	—
Ground peas[171]	6.5	—

pH values between 6.2 and 7.1.[50,63,64] The reaction products were characterized by Gardner and Weisleder,[67] Hamberg[95] and Veldink *et al.*[159] At optimum pH values, corn germ lipoxygenase forms mainly 9-D-hydroperoxy-10 *trans*,12 *cis*-octadecadienoic acid from linoleic acid[67] whereas at pH 9.0 the production of 13-L-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid prevails.[159] Presumably, this change in specificity is due to the presence of different isoenzymes. A similar influence of pH on product formation was observed by Galliard[57] using soybean lipoxygenase and linoleic acid.

C. *Potato* (Solanum tuberosum)

Lipoxygenase from potatoes has been extensively studied by Galliard and coworkers. Like the corn enzyme, it was found to produce specifically 9-D-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid at pH 5.5.[57] Evidence for the existence of potato lipoxygenase isoenzymes has been found by Pinsky *et al.*[132] These workers also found that the activity of the enzyme was sensitive to agents like hydroxynitrobenzylbromide and N-bromosuccinimide, which may indicate that tryptophan residues are essential for enzymic activity.

D. *Other Species*

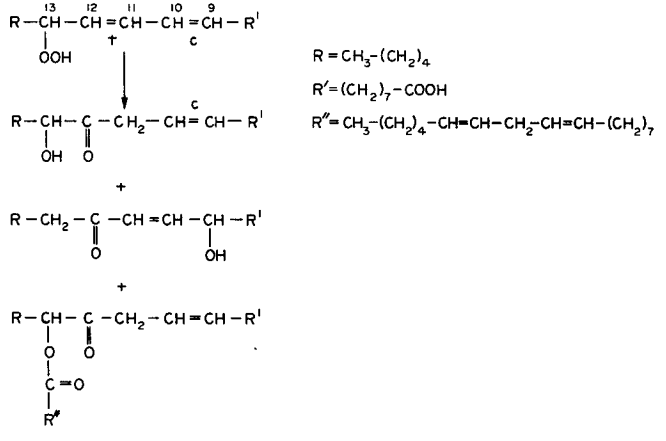
The enzymes from green algae[179] (*Chlorella pyrenoidosa*), horsebean,[14] flaxseed,[177] alfalfa[23] and *Dimorphoteca sinuata*[64] have only partly been characterized. The enzymes from flaxseed and *Dimophoteca sinuata* seed show a product specificity which resembles that of soybean lipoxygenase-1. However, the pH optimum lies at about pH 7.

XVIII. ENZYMIC CONVERSIONS OF FATTY ACID HYDROPEROXIDES

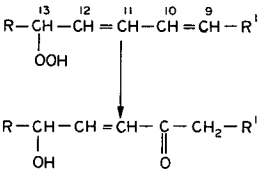
The accumulation of lipid peroxides in living cells can be regarded as harmful as it has been found that a number of enzymic functions can be destroyed.[60,118,119] Therefore, it is reasonable to assume that living cells possess some mechanism which can convert lipid peroxides.

A. In vitro *Studies*

Zimmerman[176] demonstrated that linoleic acid was converted into α-ketols when incubated with a partially purified flaxseed preparation and lipoxygenase. He proposed the intermediate formation of hydroperoxy linoleic acids. Veldink *et al.*[160,161] incubated



Scheme 3



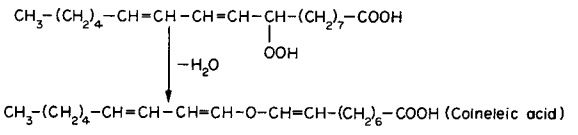
Scheme 4

preformed 13-hydroperoxylinoleic acid with a flaxseed preparation, which indeed yielded 12-oxo,13-hydroxy-9-*cis*-octadecenoic acid. This conversion does not require linoleic acid or oxygen. An analogous conversion of 9-ROOH could not be demonstrated. In contrast to the enzyme preparation from flaxseed used by Veldink *et al.*,[161] a hydroperoxide isomerase preparation from corn germs[61] converted both the 9- and 13-hydroperoxy linoleic acids. In addition to α-ketols, two isomeric γ-ketols and an acylated α-ketol were found (Scheme 3). Recently, Esselman and Clagett[44] observed hydroperoxide isomerase activity in alfalfa which, however, differs from the flax and corn systems in its mode of product formation (Schemes 3 and 4). The only products are γ-ketols in which the hydroxyl function is located at the carbon-atom bearing originally the hydroperoxide group. The different mode of action of this enzyme is also apparent from $^{18}O_2$ studies with the isomerases from flax[163] and alfalfa,[44] respectively.

Veldink *et al.*[167,186] showed that upon incubating ^{18}O-labelled hydroperoxides with an isomerase preparation from either flax or corn germs resulted in the retention of only one ^{18}O atom in the ketols. It appeared that the carbonyl oxygen was the ^{18}O isotope, which suggests that the hydroxyl group stems from the solvent. Esselman *et al.*,[44] using the alfalfa system, found that ^{18}O labelled hydroperoxides were converted into a γ-ketol which had retained both heavy oxygen atoms. Therefore, the corn and flaxseed enzymes may, in a strictly mechanistic sense, not be regarded as true isomerases. The possible involvement of suitable nucleophiles in the replacement of the hydroperoxide group by the corn enzyme has been suggested by Veldink[158] and has gained substantial experimental support by the work of Gardner.[62] It was shown that, besides hydroxy-oxo compounds, also S-ethyl-, methoxy- and oleyl-oxo compounds could be formed enzymically when the isomerase reaction was carried out in the presence of ethylmercaptan, methanol or oleic acid, respectively.

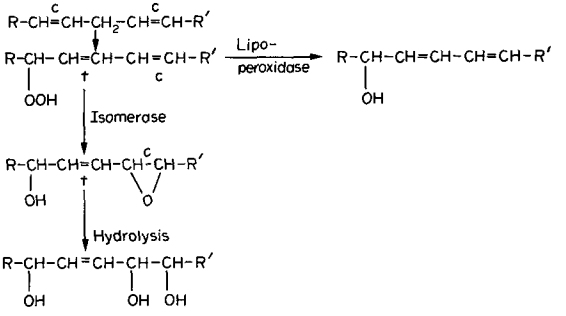
Hydroperoxide isomerase activity has been found and partly characterized in barley,[76,165] wheat[77,78,165,178] and, though less well-defined, in soybeans,[165,178] mung beans,[165] peanuts[146] and potatoes.[55] Following the isolation and characterization of potato lipoxygenase it was found that the hydroperoxides formed by this enzyme were converted enzymically. Lipoxygenase from potato tubers specifically oxygenates the 9-position of linoleic and linolenic acid.[57] Subsequently, the 9-D-hydroperoxide is converted into a fatty acid containing a dienylether function (Scheme 5).[58,59] The 13-hydroperoxide failed to serve as a substrate in this reaction.[55] The colneleic acid is then converted via a chain-fission process into fragments of lower molecular weight, e.g. nona-3,6-dienal. It has been suggested that potato lipoxygenase is involved in all of the chemical steps eventually leading to the production of aldehydes.[54] The degradation of colneleic acid is also catalyzed by small amounts of Fe^{2+} ions and ferredoxin.[54,55]

Enzymic isomerization of hydroperoxylinoleic acids does not always result in the formation of ketols. Heimann *et al.*[100] have described a lipoxygenase from oats which,



Scheme 5

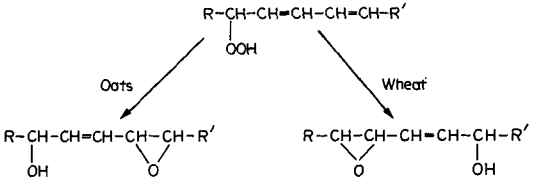
at pH 7.0, forms 88% 9- and 12% 13-hydroperoxylinoleic acid. Incubation of linoleic acid with a crude homogenate from oats does not yield free hydroperoxides but instead trihydroxy fatty acids. Heimann *et al.*[99,100] have proposed the following pathway (Scheme 6) for the metabolism of linoleic acid in cereals. Besides isomerization, the reduction of hydroperoxides seems to proceed independently through the action of a lipoperoxidase.



Scheme 6

According to Heimann *et al.*,[100] the formation of trihydroxy fatty acids by the oats enzyme should be attributed to the subsequent actions of lipoxygenase and an isomerase/hydrolase, whereas Graveland[73] has proposed a modification of the lipoxygenase reaction in the wheat system. An important difference between the systems described by Graveland[75] and Heimann *et al.*,[99] respectively, may be the structure of the precursor of the epoxyhydroxy compound.

Heimann *et al.*[99] have demonstrated that the hydroxy group in the latter compound is attached to the same carbon atom as the hydroperoxide group in the precursor, whereas the mechanism proposed by Graveland implies the participation of this carbon atom in the formation of the epoxy ring (Scheme 7). It should be noted that preformed hydroperoxides are converted by the wheat[75] system only when linoleic acid is also present and the system is kept anaerobic. The so-called lipoperoxidase from plant material which may be responsible for the reduction of fatty acid hydroperoxides to hydroxy fatty acids was shown to be nonseparable from lipoxygenase.[81] When, during lipoxygenase catalysis, suitable hydrogen donors are present (GSH, guaiacol, *p*-phenylenediamine) substantial amounts of hydroxy fatty acids are found.



Scheme 7

In contrast to animal systems (cf. 27, 28), insufficient evidence is available to suggest any physiological role for such systems in plants. It has been proposed earlier[6,131,144,150] that lipoxygenases might be involved in the biosynthesis of, for example, hydroxydienoic fatty acids from *Coriaria nepalensis*, *Monnina emarginata* and *Dimorphoteca sinuata*, which have stored 13-D-hydroxy-9 *cis*,11-*trans*-, 13-L-hydroxy-9 *cis*,11-*trans*- and 9-D-hydroxy-10-*trans*,12-*trans*-octadecadienoic acids, respectively. The lipoxygenase from *Dimorphoteca sinuata* has been characterized by Gardner *et al.*[64] and found to synthesize

predominantly 13-L-hydroperoxy-9 *cis*,11-*trans*-octadecadienoic acid from linoleic acid. The result indicates that no straightforward relationship exists between the mode of action of a lipoxygenase in the resting seed and the structure of major components of the stored lipids. On the other hand, it is conceivable that a different type of lipoxygenase is active during the stage at which the hydroxydienoic acid is biosynthesized or that the 13-L-hydroperoxide is subsequently converted enzymically into the 9-D-hydroxy-10 *trans*,12-*trans*-octadecadienoic acid.

B. *Physiological Effects of Fatty Acid Hydroperoxides*

Peroxidation of unsaturated lipids, particularly in animal systems, has been studied extensively (see, for example, refs. 128–130). The process is thought to be related to aging, membrane permeability, the generation of cancer[53] and atherosclerosis[97] and food deterioration.[36] Lipid peroxidation is often quantified by measuring the amounts of malondialdehyde with the TBA test. As a matter of fact, the peroxidation of unsaturated lipids does not invariably lead to the production of malondialdehyde as is evident from model studies in which purified unsaturated fatty acid hydroperoxides are used.[65,66,93] The adverse effects of fatty acid free radicals in biological systems (cf. ref. 114 and references cited there) and of other radicals, the formation of which is initiated by those derived from the fatty acids, are well documented[48,51,114,120,148,174] though the precise way in which they interfere in cellular processes is still unknown.

C. *Formation of Volatile Compounds*

The origin of volatile compounds in various foodstuffs and plants has been the subject of many investigations because a number of these substances contribute to the flavor properties. Besides, it is not yet clear whether they have any key function in the plant's metabolism. This section only deals with volatiles originating from the metabolism of unsaturated fatty acids in which the involvement of lipoxygenase is likely. Table 3 lists the compounds that have been identified in either intact, mechanically ruptured or homogenized plant parts. The products isolated from model systems containing lipoxygenases are mentioned as well. The mechanisms through which the various volatile compounds are formed have not yet been fully clarified, though several schemes have been proposed.[9,72,84,127] In the suggested mechanisms, key positions are assigned to the fatty acid hydroperoxide and its homolytic and heterolytic scission products which—after rearrangement—may eventually lead to the identified structures.

XIX. ON THE PHYSIOLOGICAL ROLE OF LIPOXYGENASE

It is remarkable that for lipoxygenase, an enzyme which has been known for such a long time and which occurs in such large amounts in various plant seeds, only *ad hoc* reasoning is available with regard to its function in plant lipid metabolism. Recently, Galliard[54,55] and Axelrod[12] reviewed extensively the current theories regarding the biological significance of this dioxygenase. Owing to the presumed toxicity of hydroperoxides for living cells, the production of these compounds cannot be a goal in itself. An efficient conversion of hydroperoxides into less harmful substances is likely to occur. Various plant seeds appear to differ in their routes to metabolize hydroperoxides: e.g. α- and γ-ketols (flax, corn, alfalfa, barley) unsaturated ethers (potato), mono-, di- and trihydroxy acids (wheat, peas), oxodienes, fatty acid dimers (soybean) and alkanes (soybean, peanuts) can be formed. Evidently, no universal principle is operative in the conversion of hydroperoxides, which makes it hard to believe that a comprehensive theory can be given comprising the biological function of the different metabolites. In fact, a large number of possibilities have been put forward, e.g. the possible involvement in wound healing[101,180] and the production of volatile compounds—including ethylene—in the biosynthetic process of unsaturated and oxygenated fatty acids. However,

TABLE 3.

	References	Plant species
ethanal	82, 83, 127	peas, cucumber, peanuts
propanal	82, 83	peas, cucumber
2-*trans*-butenal	82	peas
2-*trans*-pentenal	82	peas, cucumber
2-*trans*-hexenal	82, 117	peas, cucumber, ginkgo*
2-*trans*,4-*cis*-heptadienal	82	peas
2-*trans*,6-*cis*-nonadienal	82, 83	peas
3,5-octadecadiene-2-one	34, 82	peas
hexanal	7, 79, 83, 84	soybeans, cucumber, peas, soybeans, peas, rye, broad beans
pentanol	7, 84	soybeans, peas
hexanol	7, 79	soybeans, peas
heptanol	7	soybeans
2-*trans*-nonenal	83	cucumber
pentanal	83, 84, 127	cucumber, peanuts, soybeans, peas
nonanal	80, 83	cucumber, peas
2-octenal	83, 84	cucumber, soybeans, peas
2-nonenal	83	cucumber
2,4-decadienal	83, 84	cucumber, soybeans, peas
2-heptenal	83, 84	cucumber, soybeans, peas
octanal	80	peas
pentane	127	peanuts
methanol	127	peanuts
ethanol	127	peanuts
acetone	127	peanuts
ethylene, ethane*	56, 136	apple
2,4-nonadienal	84	soybeans, peas

*Doubtful whether lipoxygenase is involved.

common to all reactions is that polyunsaturated fatty acids and oxygen are metabolized. It is, in our opinion, conceivable that conversion of these fatty acids along a pathway which starts with lipoxygenase as the first attacking enzyme, is energetically favorable at certain stages of ripening and/or germination. Here, the bio-energetic aspects would prevail and the metabolites would be of secondary importance. This does not exclude a possible role for some metabolites.

Another point we would like to make concerns the oxygen tension in the seed. When substrate fatty acid is available, lipoxygenase can keep the oxygen tension in the seed very low. The more so, since the presence of hydroperoxide has an activating effect on the enzyme. Interestingly, Leblova *et al.*[113] have shown that in soybean, maize, pea, bean, lentil and broad bean, pyruvate metabolism leads to the formation of lactate and ethanol during the natural anaerobic phase in the early stages of germination. The maximum in ethanol content is reached after 40 hr of germination whereas between 0–30 hr, a concentration peak of lactate appears. Since the above-mentioned species are relatively rich in lipoxygenase, it could well be that lipoxygenase is involved in maintaining the natural anaerobiosis during the germination process.

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NOTE ADDED IN PROOF

Recently, a number of papers have been published dealing with some of the topics discussed in the present review. The kinetics of lipoxygenase catalysis (Section X) have been investigated by Egmond *et al.*,[38] Gibian and Galaway[182] and Cook and Lands.[183] The interaction of the product hydroperoxide with soybean lipoxygenase (Section VI) has now been studied by using ^{18}O-labelled 13-ROOH[184] and 4-nitrocatechol.[185] The process of cooxidation (Section XVI) of carotenoids has been studied in more detail by Weber and Grosch[185] and was found to influence the type of hydroperoxides formed essentially as described in Section XVI.

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