Enzymic and non enzymic oxidation of polyunsaturated fatty acids

Hilditch lecture

Johannes F G Vliegenthart

In the context of this paper it is appropriate to mention that Professor Hilditch and his coworkers carried out some outstanding investigations into the mechanism of lipid oxidation.[1,2]

Lipid oxidation covers a wide area of enzymic and non-enzymic reactions, many of which have an impact on daily life. For instance, one might think of oxidation of fatty acids as an energy source for the organism, or the conversion of polyunsaturated fatty acids into prostaglandin-like compounds, or the role of lipid oxidation in food deterioration as well as in the ageing process of the organism.

Most of the systems which have been investigated are highly complex. The primary oxidation reactions may be followed by secondary and/or side reactions which ultimately may lead to products far removed from the initially-attacked compounds. The complexity of the systems also makes it difficult to draw reliable mechanistic conclusions. Adequate measurement of lipid oxidation in complex systems remains a problem, in particular when agreement with organoleptic evaluation is required.[3]

In lipid molecules, double bonds are the most accessible structural elements for attack by oxygen. Fortunately, the reaction between double bond systems and molecular ground state oxygen does not take place spontaneously, but needs activation of substrate or oxygen to overcome the spin barrier.

Fatty acid activation

With regard to substrate activation, it is generally accepted that this proceeds *via* radical formation. The scheme:

$$RH + O_2 \rightarrow R\cdot + \cdot OOH \qquad \text{Initiation}$$

$$\left.\begin{aligned} R\cdot + O_2 &\rightarrow ROO\cdot \\ ROO\cdot + RH &\rightarrow ROOH + R\cdot \end{aligned}\right\} \qquad \text{Propagation}$$

shows that hydroperoxides are obtained as the main reaction products,[4,5] and any event which leads to the formation of radicals from RH starts the reaction. To keep the reaction going, decomposition of hydroperoxides to (other) radicals is necessary as given in a modification of Tappel's diagram[6] illustrated in Fig 1. The decomposition of hydroperoxides can be achieved chemically, thermally or photochemically, and depending on the complexity of the system, a wide variety of compounds can arise. Various termination reactions can occur:

$$R\cdot + R\cdot \rightarrow R-R \qquad \text{Termination}$$

$$R\cdot + ROO\cdot \rightarrow ROOR$$

$$ROO\cdot + ROO\cdot \rightarrow \text{Inactive products}$$

Professor Vliegenthart studied chemistry at Utrecht State University, The Netherlands, receiving his doctorate in 1967. In 1975, he was appointed associate professor in bio-organic chemistry at Utrecht. His main research interests are enzymic oxygenation of polyunsaturated fatty acids and carbohydrate chemistry. He presented the Hilditch lecture at the 14th World Congress of the International Society for Fat Research held in Brighton from 17-22 September 1978.

The **Hilditch lecture** was endowed in 1967 by the friends of Professor T. P. Hilditch (1886-1965), the first holder of the Campbell Brown Chair of Industrial Chemistry in the University of Liverpool.

The lecture is sponsored by the Liverpool Section, the Oils and Fats Group, the N.W. Section of the Royal Institute of Chemistry and the N.W. Section of the Oil and Colour Chemists Association.

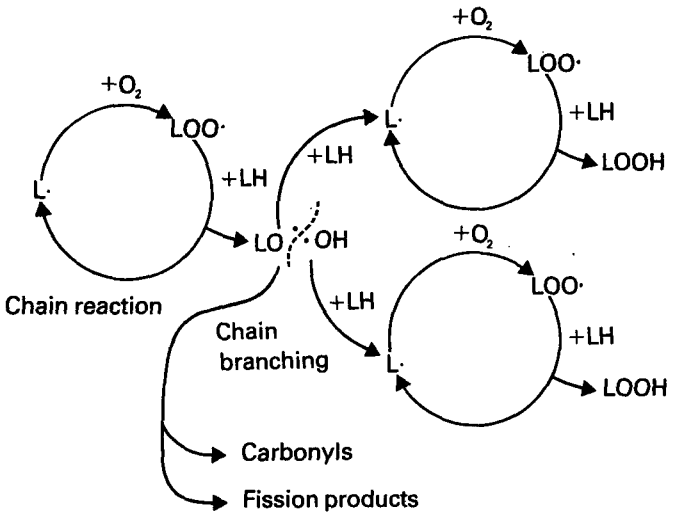
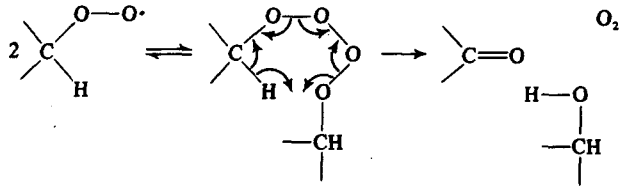


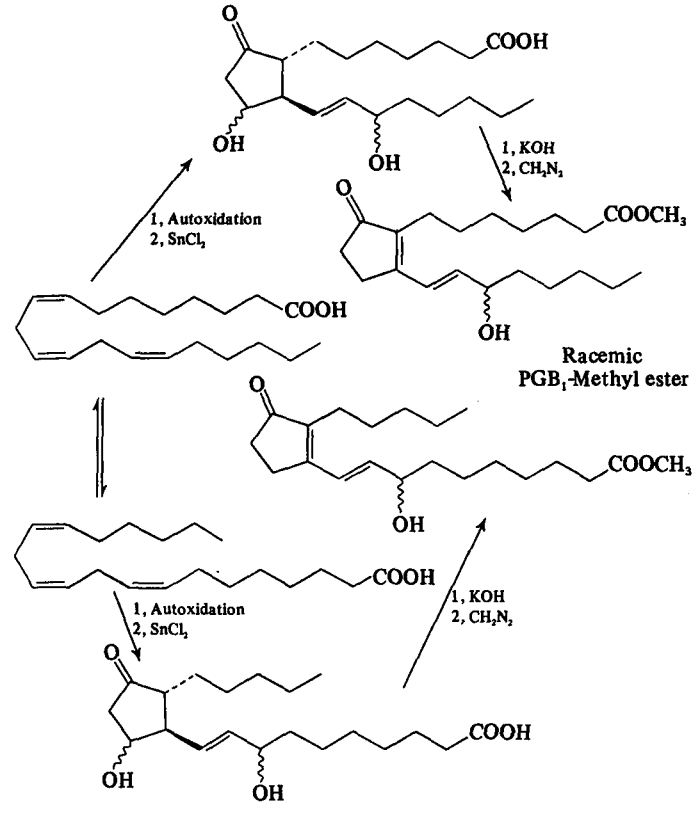
Fig 1 Mechanism of decomposition of hydroperoxides to other radicals

It can be seen that combination of peroxyradicals does not necessarily lead to termination and that decomposition of the intermediary tetroxide may yield reactive species:[7]



The autoxidation of several unsaturated fatty acid esters has recently been analysed by high performance liquid chromatography[8] and by g.c.-m.s.[9-11] It should be noted that Nugteren *et al*[12] showed that prostaglandin E is formed

Fig 2 Formation of PGB_1-methyl ester



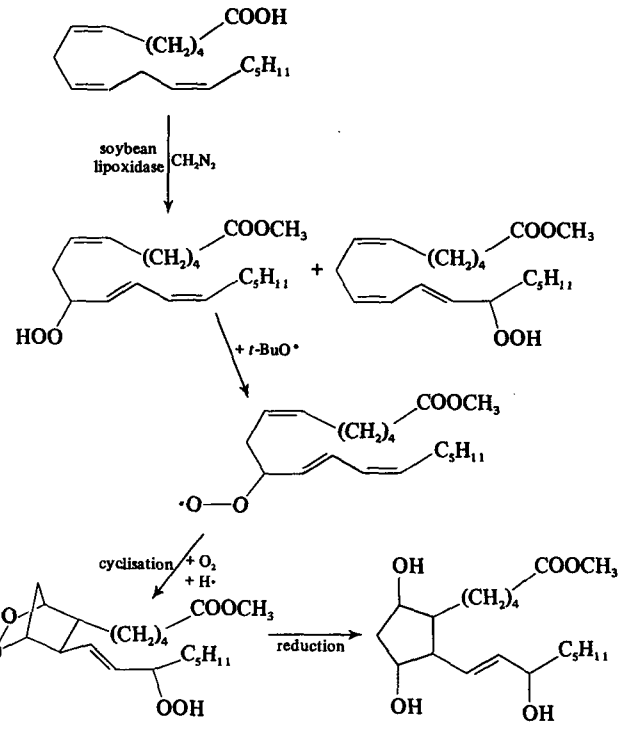


Fig 3 Formation of prostaglandin analogues from γ-linolenic acid

during autoxidation of *all-cis*- 8,11,14-eicosatrienoic acid. They obtained a complex mixture containing 1-2 per cent of prostaglandin-type material and about 0·1 per cent of racemic PGE_1 proper (Fig 2). Porter *et al*[13] tried to get such a process under control by first producing hydroperoxides from γ-linolenic acid in a regio- and stereospecific way by the action of lipoxygenase, followed by the initiation of exclusively peroxy radicals from one of the positional isomers. By means of a di-tert-butyl peroxyoxalate the peroxy radical can produce a radical of the endoperoxide, which in turn could be oxygenated. After reduction and h.p.l.c. of the reaction mixture, it could be demonstrated using g.c.-m.s. that prostaglandin analogues were produced from γ-linolenic acid (Fig 3). It is evident that the system still leaves too many reaction pathways open to be of synthetic importance.

An interesting aspect of Porter's approach is the use of lipoxygenase to obtain hydroperoxides stereospecifically. Recently, Emken[14] discussed the possibilities of applying this type of reaction to the large scale production of compounds which can be derived from those hydroperoxides and from other products formed from unsaturated fatty acids by lipoxygenase action.

The formation of malondialdehyde-like material as detectable by the thiobarbituric acid test is typical of the autoxidation of lipids containing polyunsaturated fatty acids with three or more methylene interrupted double bonds. Some suggestions have been made with regard to the formation of this compound. Dahle *et al*[15] proposed a five membered monocyclic peroxide (Fig 4) as the non volatile precursor of malondialdehyde.

Another proposal emergered from a study of Nugteren *et al*[16] on the enzymic convertion of *all-cis* 8,11,14-eicosatrienoic acid into prostaglandin E_1. Incubations with enzyme preparations in the absence of antioxidants give rise to the formation of relatively

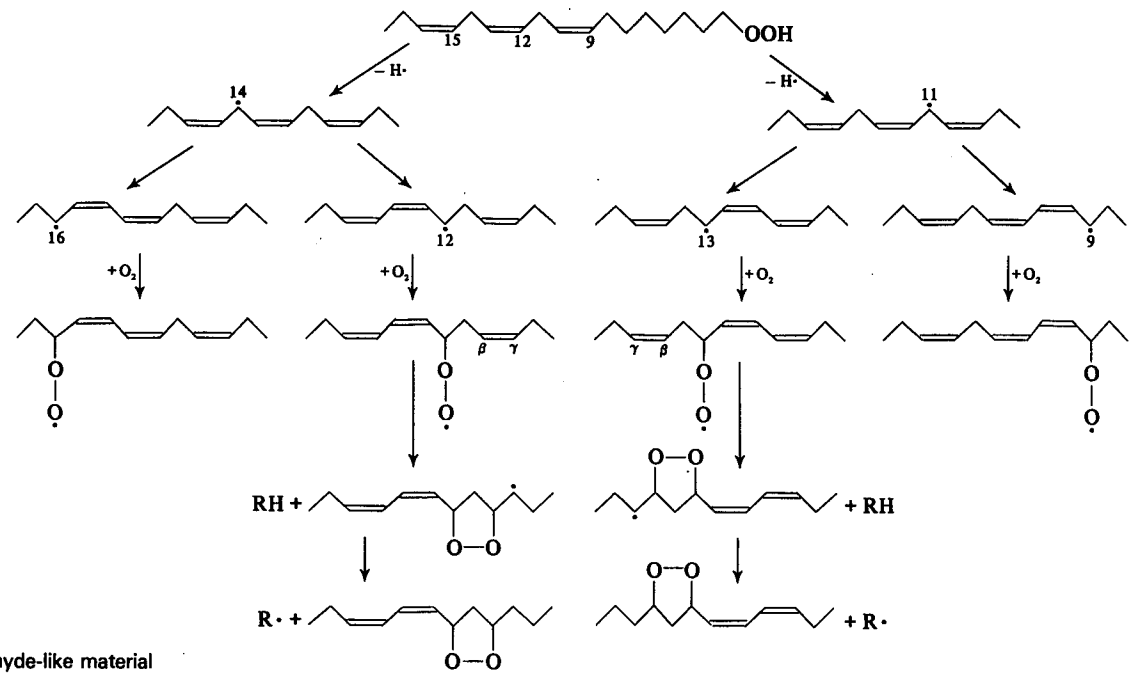


Fig 4 Formation of malondialdehyde-like material

large amounts of 12-hydroxy-8 *trans,* 10 *trans* heptadecadienoic acid in addition to roughly equimolar amounts of malondialdehyde (Fig 5). Apparently, in this system a compound is formed which is a precursor for malondialdehyde. They suggested that the formation of prostaglandin and of the by-products, including malondialdehyde, occurs *via* radical reactions and an endoperoxide intermediate. Pryor and Stanley[17] generalised this suggestion and concluded that autoxidation of polyunsaturated fatty acids containing three or more double bonds yields endoperoxides, which must be considered as the principal non-volatile precursors of malondialdehyde (Fig 6).

Oxygen activation

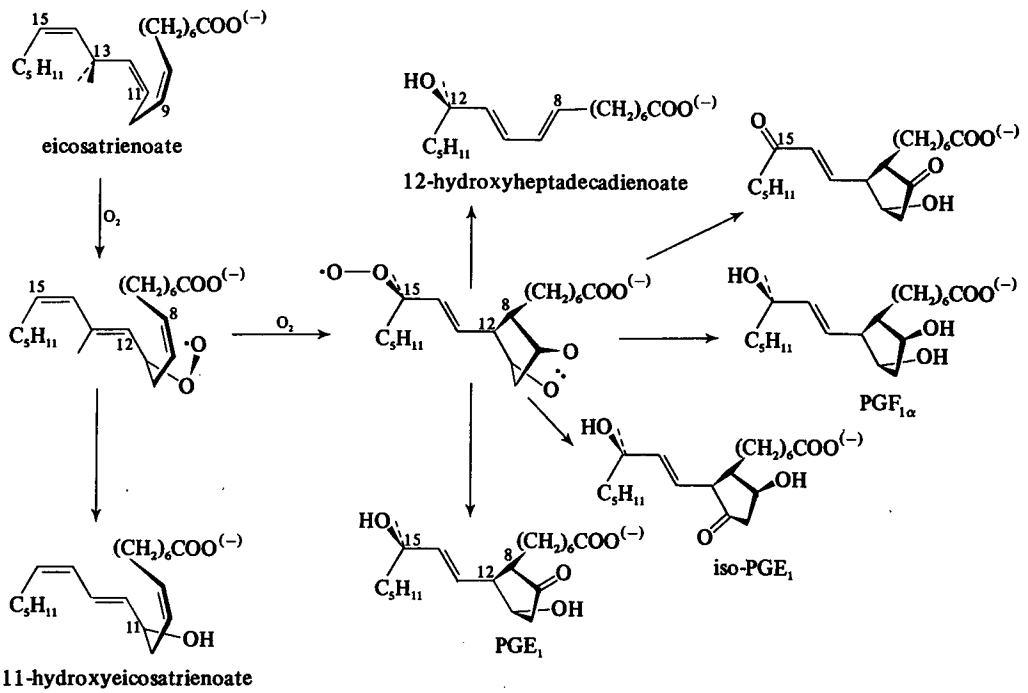
Koppenol[18] has summarised the various oxidation states of oxygen at pH 7 in the energy diagram illustrated in Fig 7. The free energy per oxygen atom $\Delta G_0'$ is plotted against the formal charge, n. The slope of the line joining the species A and B represents the reduction potential E_0' of the couple A/B. All species above the line joining O_2 to H_2O are thermodynamically unstable.

When discussing lipid oxidation it is interesting to consider the possible involvement of $O_2^{-\cdot}$, ·OH and $'\Delta_g O_2$. $O_2^{-\cdot}$ and its conjugate acid, the hydroxyl radical $HO_2^{\cdot}$ gained interest from biochemists when it was demonstrated that some enzymes e.g. xanthine oxidase produce this species. A further stimulus was provided when it was discovered that superoxide dismutases[19] convert $O_2^{-\cdot}$ extremely efficiently according to the reaction:

$$2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$

In particular, Fridovich[20] has stressed the importance of superoxide dismutases for the protection of aerobic cells against attack by $O_2^{-\cdot}$ or by other reactive species derived from $O_2^{-\cdot}$. Relatively little precise information is available on

Fig 5 Proposed conversions of the endo-peroxide of *all-cis* -8,11,14 – eicosatrienoic in the biosynthetic formation of prostaglandins



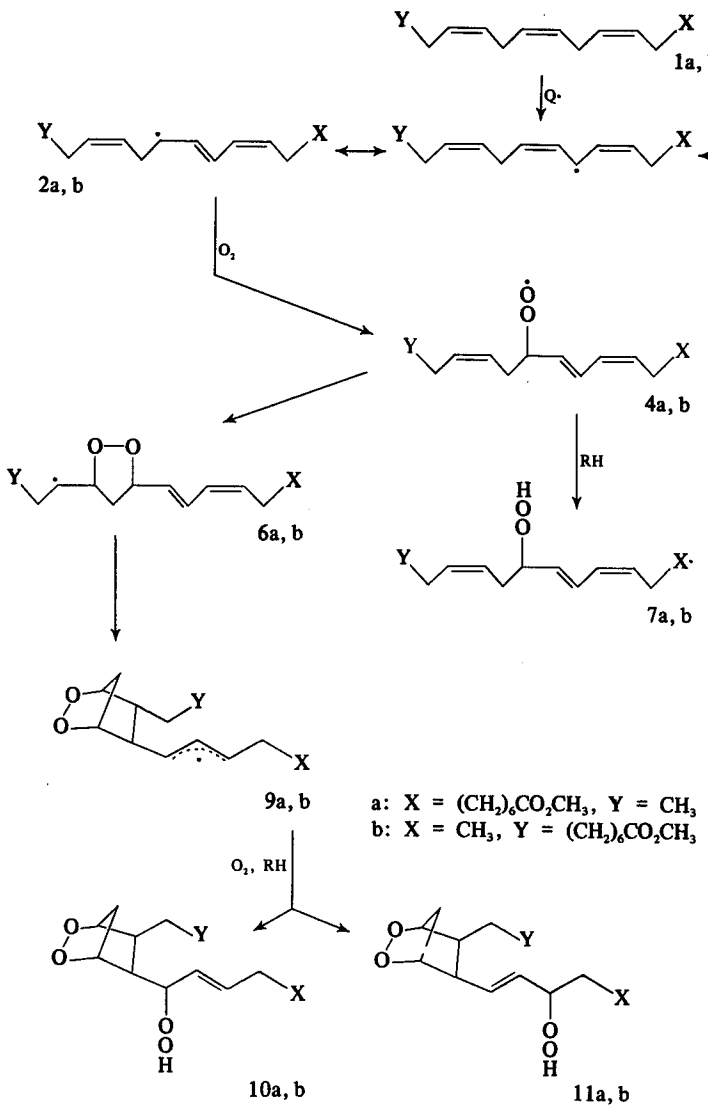


Fig 6 Mechanism for conversion of methyl linolenate to conjugated hydroperoxides and to prostaglandin-like endoperoxides

the chemical reactivity of $O_2^{-\cdot}$ towards various cellular compounds like carbohydrates, proteins, nucleic acids and lipids. It has been proposed that $O_2^{-\cdot}$ or products derived from it *via* the Haber-Weiss cycle:

$$HO_2^{\cdot} + H_2O_2 \rightarrow OH^{\cdot} + O_2 + H_2O$$

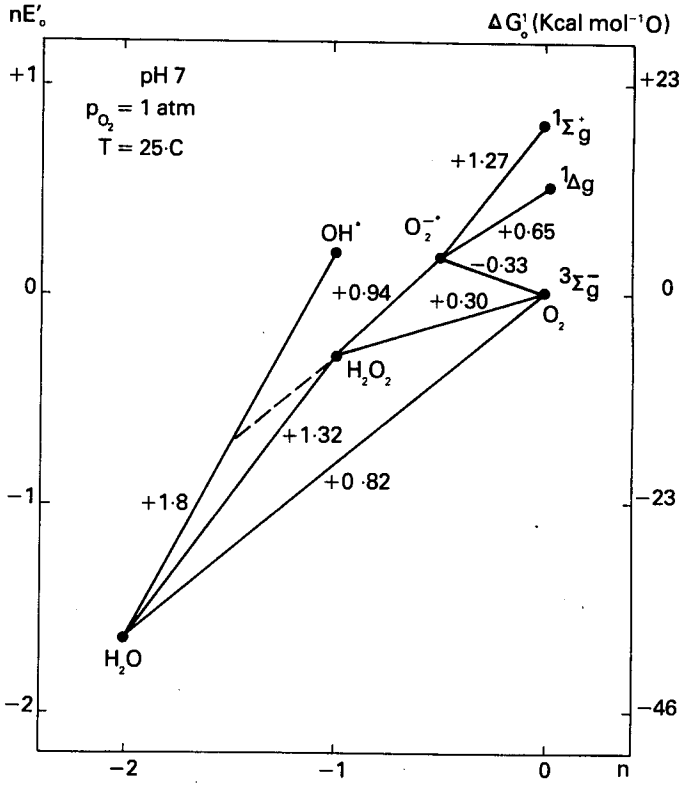
$$OH^{\cdot} + H_2O_2 \rightarrow HO_2^{\cdot} + H_2O$$

are capable of reacting with unsaturated fatty acids.[21]

Complex systems

Most systems which have been used to demonstrate the involvement of $O_2^{-\cdot}$ in lipid peroxidation are highly complex. This makes it difficult to arrive at unambiguous conclusions. Lipid peroxidation, as detected by the formation of malondialdehyde in NADPH (nicotine-amide-adenine-dinucleotide-phosphate [reduced form]) – dependent endogenous lipid oxidation of microsomes has been investigated thoroughly. Hochstein and Ernster[22] showed that the formation of malondialdehyde in isolated microsomes required the presence of NADPH and ADP (Fig 8). This reaction is dependent on Fe^{3+} as shown by Poyer and McCay.[23] Pedersen and Aust[24] demonstrated that a purified preparation of rat liver microsomal NADPH-cytochrome c reductase catalysed the NADPH-dependent peroxidation of isolated microsomal lipid. In addition to ADP and Fe^{3+}, high ionic strength and a critical EDTA concentration are essential. Superoxide dismutase inhibited

Fig 7 Oxidation states of oxygen at pH 7



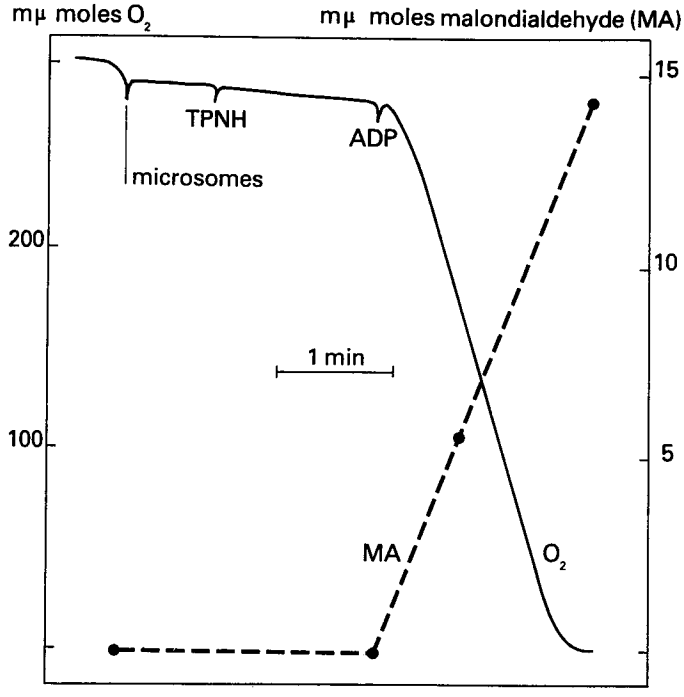


Fig 8 TPNH and ADP-dependent 0_2 uptake and malondialdehyde formation. Additions where indicated were: microsomes containing 6mg protein; TPNH, 0·3mmol; ADP, 1·0mmol

the peroxidation to approximately 65 per cent (Table 1), suggesting that $O_2^{-\cdot}$ produced by the flavoprotein plays a role in this process. When catalase was also included in the system, the inhibition by superoxide dismutase was corroborated. Catalase without superoxide dismutase gives an inhibition of less than 20 per cent. The authors suggest that it could well be that singlet oxygen is involved.

The same authors[25] observed that extracted rat liver microsomal lipids can also be oxidised by the system xanthine oxidase/xanthine, 0·2mmol $FeCl_3$ and 0·1mmol

Table 1 **Effect of superoxide dismutase on lipid peroxidation**

	(nmoles MDA min^{-1} ml^{-1}) Minus catalase	%	Plus 100mg catalase ml^{-1}	
Microsomal lipid liposomes				
NADPH cytochrome reductase				
EDTA, ADP, NADPH	1·46	100	1·28	100
" + 50μg SOD ml^{-1}	0·95	65	0·61	48
" +100μg SOD ml^{-1}	0·75	51	0·45	35
" +200μg SOD ml^{-1}	0·68	47		

Table 2 **Lipid peroxidation promoted by xanthine oxidase**

Reaction system	Malondialdehyde formed (nmol min^{-1} ml^{-1})
Complete, 50μg/ml xanthine oxidase	3·45
" 25μg/ml "	2·80
" 10μg/ml "	1·95
Xanthine, 50μg/ml	0·24
Xanthine oxidase	0·15

Table 3 **The effect of 1,3-diphenylisobenzofuran on lipid peroxidation**

Description	Malondialdehyde formed (nmol min^{-1} ml^{-1}) With xanthine oxidase Activity	% control	With ascorbic acid Activity	% control
Control	2·48		6·40	
+ 1,3-diphenylisobenzufuran:				
0·2 mol/mol lipid P	0·58	23	6·20	97
1·0 mol/mol lipid P	0·15	10	6·15	96

EDTA. The reaction is inhibited by superoxide dismutase as well as by 1,3-diphenylisobenzofuran (Tables 2 and 3). The formation of *o*-dibenzoylbenzene from the latter reagent is interpreted by the authors in terms of the involvement of singlet oxygen. However, this conclusion is not completely justified, as Howard and Mendenhall[26] have shown that the formation of *o*-dibenzoylbenzene is not a sufficient criterion for demonstrating singlet oxygen. Fong *et al*[27] investigated the peroxidation of lysosomal membranes by —

i the oxidation of NADPH by liver microsomes in the presence of ADP and Fe^{3+},

ii oxidation of NADPH by a partially purified NADPH cytochrome P_{450} reductase,

iii oxidation of xanthine by xanthine oxidase in the presence of ADP and Fe^{3+}:

Flavin enzyme + substrate → Reduced flavin enzyme + product

Red. flavin enzyme + O_2 → Flavin enzyme + O_2^-

$2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$

$H_2O_2 + O_2^{-\cdot} \rightarrow O_2 + OH^{\cdot} + OH^-$

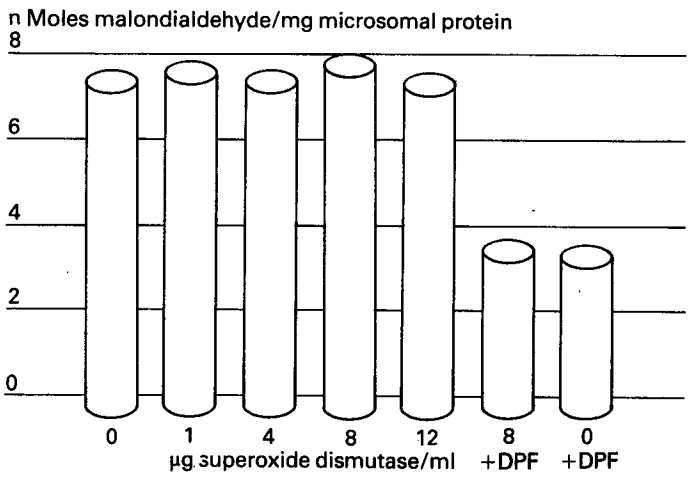
$O_2^{-\cdot}$ + ADP-Fe(III) → O_2 + ADP-Fe(II)

ADP-Fe(II) + H_2O_2 → ADP-Fe(III) + $OH^{\cdot}$ + OH^-

They propose that the lipid oxidation is initiated by ·OH radicals ultimately formed by reaction between $O_2^{-\cdot}$ and H_2O_2. The addition of chelated iron could accelerate the formation of ·OH by reacting with H_2O_2. However, Tyler[28] concluded that ·OH radicals are not involved in the rapid $O_2^{-\cdot}$-dependent lipid peroxidation of liver subcellular fractions.

On the basis of the conversion of 2,5-diphenylfuran into dibenzoylethylene during the NADPH-dependent lipid oxidation by liver microsomes, King *et al*[29] conclude that singlet oxygen is formed. Because the lipid peroxidation and singlet oxygen formation are related to the Fe^{3+} concentration, it is concluded that singlet oxygen probably arises from the breakdown of lipid peroxides (Fig 9). Spectroscopic evidence for the generation of single oxygen in the NADPH-dependent microsomal lipid peroxidation has been given by Horves and Steele[30] and by Nakano *et al.*[31] On the basis of the analysis of a simplified system consisting of NADPH, Fe^{3+}-ADP, EDTA, liposomes, NADPH-cytochrome c reductase and *tris*-HCl buffer (pH 6·8), Sugioka and Nakano[32] suggest that singlet oxygen and a compound in the triplet state, which is probably a carbonyl

Fig 9 Formation of malondialdehyde by NADPH oxidase activity in the presence of superoxide dismutase, with and without diphenylfuran (DPF)



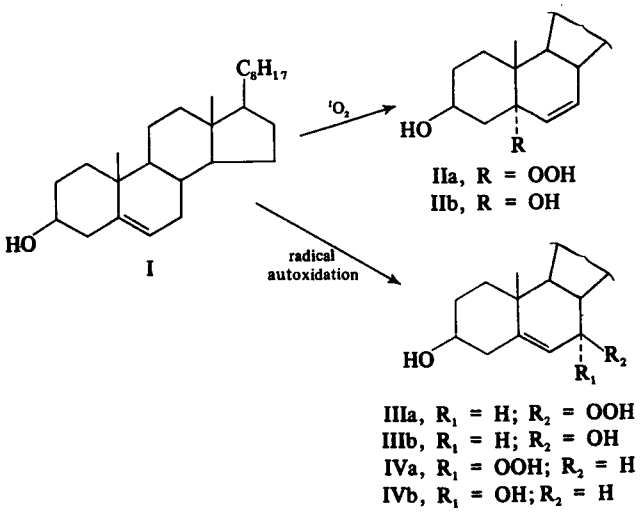


Fig 10 Peroxidation of NADPH-dependent hepatic microsomal lipid in the presence of cholesterol

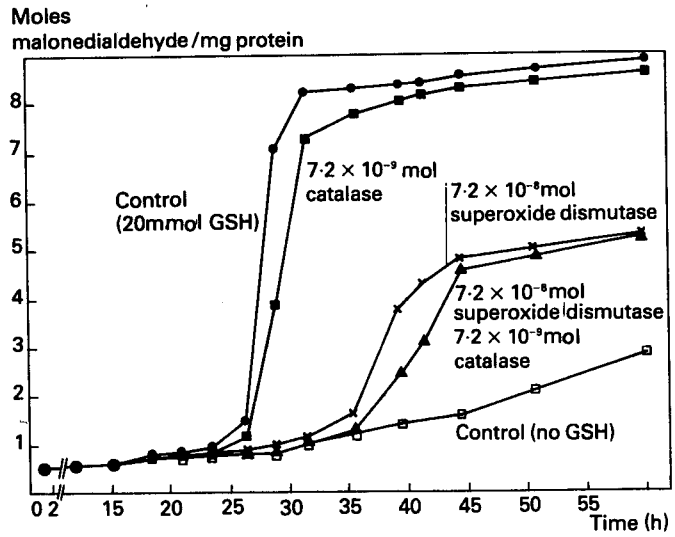


Fig 11 GSH-induced peroxidation of lipids in purified membranes of rat liver mitochondria

compound, are generated by a self reaction of lipid peroxy radicals:

$$2ROOH \rightarrow ROO^{\cdot} + ROH + {}^{\cdot}OH$$

$$2ROO^{\cdot} \rightarrow {}^1\Delta gO_2 + \text{other products}$$

$$2\,{}^1\Delta gO_2 \rightarrow 2\,{}^3O_2 + h\nu$$

The authors maintain that a free radical chain mechanism is responsible for the main process of the lipid oxidation, singlet oxygen being a by-product. Smith and Teng[33] investigated the NADPH dependent hepatic microsomal lipid peroxidation system in the presence of cholesterol. They were unable to detect products (Fig 10),[51] which are characteristic of the oxidation of cholesterol by singlet oxygen. In their opinion, a radical mechanism is involved. Lipid oxidation studies on other systems give a similarly diffuse picture – Fee and Teitelbaum[34] showed that erythrocytes from vitamin E-deficient rats undergo haemolysis in the presence of the rapidly autoxidising dialuric acid. The effect was partially prevented by catalase or superoxide dismutase and more efficiently by a combination of both (Table 4).

Goldberg and Stern[35] investigated the influence on the erythrocyte of $O_2^{-\cdot}$ (autoxidation of dihydroxyfumaric acid as a source of $O_2^{-\cdot}$) generated outside the cell. Cellular damage occurs in the form of haemoglobin breakdown and of hypotonic lysis. No lipid peroxidation nor sulphydryl group oxidation could be observed. The attack of haemoglobin by $O_2^{-\cdot}$ is considered to be the main cause of the observed effects. Kaschnitz and Hatefi[36] studied lipid oxidation in a model system consisting of sonicated phospholipids as substrate and electron transfer proteins found in membranes as possible catalysts. Oxidised haeme compounds were found to be active agents, whereas reduced haeme compounds, flavoproteins and riboflavin were inactive. It could be demonstrated that iron-sulphur proteins were also capable of initiating lipid autoxidation, when they were destabilised by the presence of a suitable iron chelator or chaotropic reagent. Interestingly, superoxide-generating systems did not cause initiation of lipid autoxidation. The presence of superoxide dismutase did not prevent the autoxidation started by haeme compounds or ferredoxin. These observations have led the authors to the conclusion that $O_2^{-\cdot}$ is neither an initiator nor an obligatory intermediate of lipid autoxidation. In studies on the glutathione (reduced) -induced peroxidation of lipids in purified inner membranes of rat liver mitochondria, Zimmermann *et al*[37] found that catalase and superoxide dismutase inhibit this process, whereas a combination of both enzymes was even more effective (Fig 11). Kellogg *et al*[38] found that xanthine oxidase acting aerobically upon acetaldehyde causes the peroxidation of linolenate. Superoxide dismutase and catalase inhibited this reaction which pointed to the involvement of $O_2^{-\cdot}$ and H_2O_2. Singlet oxygen scavengers (2,5-dimethylfuran) but no $\cdot OH$ traps could inhibit the reaction (Table 5). The authors proposed that: singlet oxygen is directly formed from $O_2^{-\cdot}$ and H_2O_2; singlet oxygen is an essential intermediate in the aerobic lipid oxidation in this system.

In a later study, the authors[39] report on liposome oxidation and erythrocyte lysis by $O_2^{-\cdot}$ and H_2O_2 generated by the same system as before. Essentially, comparable results were obtained as with the linolenate system. Singlet oxygen is considered to be responsible for lipid peroxidation as well as for haemolysis.

Table 4 **The effects of catalase and superoxide dismutase on lipid peroxidation in the dialuric acid-vitamin E deficient erythrocyte system**

Solution	A_{535}	nmoles malondialdehyde
No dialuric acid added	0·10	0
System alone without aeration	0·51	33
System alone with aeration	0·48	30
+0·4μmol catalase	0·25	12
+0·4μmol catalase and 5μmol SOD	0·22	9·6

Table 5 **Lipid peroxidation as measured by diene conjugation[38]**

Sample	% control rate at 233nm in presence of 0·1 m mol EDTA	% control rate at 233nm in presence of 0·1 m mol EDTA-Fe^{3+}
Complete	100±10·0	100±3·0
–Acetaldehyde	5·2±0·9	10·3±2·6
–Linolenic acid	10·5±1·6	10·0±3·9
+10μg/ml SOD	17·6±3·0	53·0±13·8
+8·95μg/ml catalase	17·4±1·2	17·7±0·6
+10μg/ml of bovine serum albumin	107·0±0·2	98·5±1·2
0·1 mol mannitol	92·0±2·9	86·0±4·0
0·1 mol +t-butanol	103·0∓3·3	107·0±3·0
10^{-5} mol β carotene	68·0±2·6	96·5±4·6
10^{-5} mol β carotene-linolenic acid	15·1±4·1	

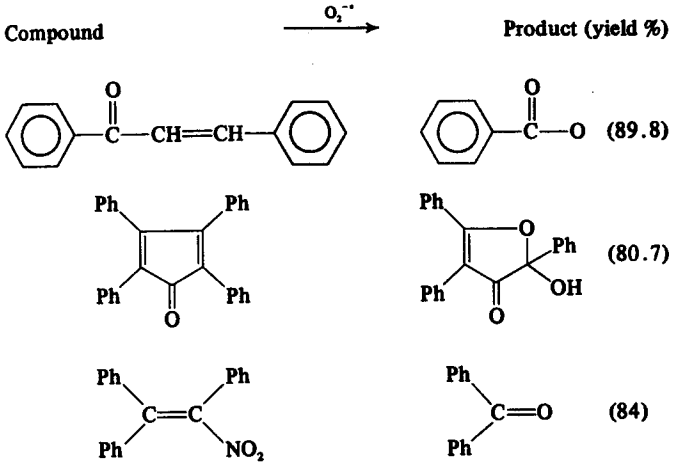


Fig 12 Reaction of $O_2^{-\cdot}$ with electron-poor olefins

This selection of literature data makes clear that from a mechanistic point of view, many contradictions still exist, illustrating the urgent need for simple chemical model systems. All evidence is circumstantial. A few remarks can be made, however. $O_2^{-\cdot}$ as such does not cause lipid peroxidation. Koppenol and Butler[40] concluded, on the basis of theoretical considerations, that $O_2^{-\cdot}$ would not easily react with a simple double bond system because no empty πg orbital is available.

$O_2^{-\cdot}$ has the electron configuration:

$(\sigma_g 1s)^2 (\sigma_u^* 1s)^2 (\sigma_g 2s)^2 (\sigma_u^* 2s)^2 (\sigma_g 2p)^2 (\pi_u 2p)^4 (\pi_g^* 2p)^3 (\sigma_u^* 2p)^0$

Rosenthal and Frimer[41,42] and Frimer *et al*[43] demonstrated that KO, dicyclohexyl-18-crown-6 complex in benzene as source of $O_2^{-\cdot}$ gives no reaction with normal olefins. They have shown, however, that $O_2^{-\cdot}$ reacts readily with electron-poor olefins yielding products corresponding to oxidative cleavage of the double bond (Fig 12). Recently, Niehaus[44] drew attention to another effect of $O_2^{-\cdot}$. He observed that KO_2 in dry dimethylsulphoxide causes rapid de-esterfication of ethyl-hexadecanoate and dilauryl phosphatidyl choline. This has led him to the suggestion that some of the deleterious effects on biological membranes result from de-esterfication of constituting lipids by $O_2^{-\cdot}$ acting as a nucleophile.

The ·OH radical is generally assumed to be an extremely reactive species. An example of the reactivity of complexed hydroxyl radicals towards fatty acids has been given by Hewgill and Proudfoot[45] in a study on the formation of radicals in the $TiCl_3/H_2O_2$ catalysed oxidation of saturated fatty acids (Table 6). In acid solutions of saturated acids hydrogen abstraction mainly takes place at the β and ω-1 positions, whereas in basic solutions the α and ω-1 positions are attacked.

In the shorter unsaturated acids OH· joined at the double bond. For larger unsaturated fatty acids like oleic, linoleic or linolenic acid only α-hydrogen abstraction was observed. This feature is probably due to the inaccessibility of the double bond systems in micelles.

Although these reactions illustrate that OH· is capable of reacting with lipids, there is no convincing evidence of its involvement in lipid oxidation in complex biochemical systems. It has frequently been suggested that OH· can be formed from $O_2^{-\cdot}$ and H_2O_2 via the Haber Weiss cycle under physiological conditions. There is accumulating evidence that this reaction does not occur spontaneously. McClune and Fee,[46] Halliwell,[47] Rigo *et al*[48] and Koppenol *et al*[49] all working with different experimental set ups were unable to demonstrate that the reaction between $O_2^{-\cdot}$ and H_2O_2 proceeds at a significant rate. Rigo *et al* found that the rate constant is less than 10^{-4} mol^{-1} s^{-1}, too low to be competitive with the spontaneous dismqtation of $O_2^{-\cdot}$: $O_2^{-\cdot} + H0_2 \rightarrow 0_2 + H_2O_2$. Koppenol *et al* observed, however, in their radiolysis experiments at low pH, that the cycle:

$$\cdot OH + H_2O_2 \rightarrow HO_2^{\cdot} + H_2O$$
$$HO_2^{\cdot} + H_2O_2 \rightarrow OH^{\cdot} + H_2O + O_2$$

is catalysed by Fe(III) (EDTA):

$$HO_2^{\cdot} + H_2O_2 \rightarrow OH^{\cdot} + O_2 + H_2O$$
$$OH^{\cdot} + H_2O_2 \rightarrow HO_2^{\cdot} + H_2O$$

Reaction with unsaturated fatty acids

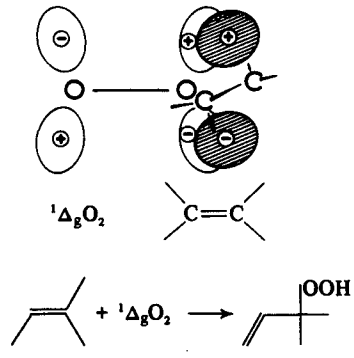
Singlet oxygen is capable of reacting with unsaturated fatty acids. According to Rawls and Van Santen[50] hydroperoxides are formed as primary products. The authors conclude that this reaction can initiate fatty acid autoxidation, which then proceeds along the normal free radical pathway (Fig 13). Suwa *et al*[51] studied the photo-oxidation of cholesterol in liposomes with haematoporphyrin as sensitiser. When haematoporphyrin is incorporated in the liposomes, the yield of the singler oxygen product, 3 β-hydroxycholest-6-ene 5 α-hydroperoxide, was approximately six times greater than in the case when the sensitiser was located outside. These results show that the lifetime of singlet oxygen, when formed in the membrane is long enough to afford cholesterol oxidation (Fig 10). Singlet oxygen formed outside the liposome is mainly quenched by the solvent.

It has been indicated for several systems in which lipid oxidation occurs, that singlet oxygen is present. However, it is conceivable that singlet oxygen should be considered as a

Table 6 **Formulation of radicals in the $TiCl_3/H_2O_2$ catalysed oxidation of saturated fatty acids**

Substrate	Radical	C rel
$CH_3(CH_2)_3COOH \rightarrow$	$CH_3CH_2C^{\cdot}HCH_2COOH$	1
	$CH_3C^{\cdot}H(CH_2)_2$ 1 COOH	
$CH_3(CH_2)_6COO^{\cdot} \rightarrow$	$CH_3C^{\cdot}(CH_2)_5C^{\cdot}HCOO^{-}$	1
	$CH_3C^{\cdot}H(CH_2)_5COO^{-}$	1·2
$CH_3CH_2CH=CH-CH_2COOH-CH_3CH_2CHOHC^{\cdot}HCH_2COO^{-}$		

Fig 13 Overlap between the empty π_g^* orbital of $^1\Delta g0_2$ and the filled π_u orbital of an alkene



secondary product from the oxidising lipid system. For example Hawco *et al*[52] demonstrated that the singlet oxygen trap 2,5-diphenylfuran was rapidly oxidised to *cis*-dibenzoylethylene during the decomposition of linoleic acid hydroperoxide catalysed by ceric ions, methaemoglobin or haematin.

In the literature it has frequently been postulated that activated forms of oxygen are directly involved in lipid oxidation. Upon closer inspection, the arguments favouring such mechanisms are not completely convincing. It is likely that the main process is a more or less controlled (free) radical reaction.

Enzymic oxygenation

The oxygenation of polyunsaturated fatty acids having a 1,4-*cis,cis* pentadiene system to optically active *cis,trans* conjugated hydroperoxides by plant lipoxygenases has been studied in our laboratory for several years. For a recent review see Veldink *et al.*[53] Using linoleic acid as substrate, different positional isomers can be formed depending on the source of the enzyme and the pH of the incubation:

$$CH_3-(CH_2)_4\overset{13}{C}H\overset{c}{=}CH-\overset{11}{C}H_2-CH\overset{c}{=}\overset{9}{C}H-(CH_2)_7-COOH$$
LH

$$CH_3-(CH_2)_4-\underset{\underset{OOH}{|}}{CH}-CH\overset{t}{=}CH-CH\overset{c}{=}CH-(CH_2)_7-C00H$$
13-L-LOOH (soybean)

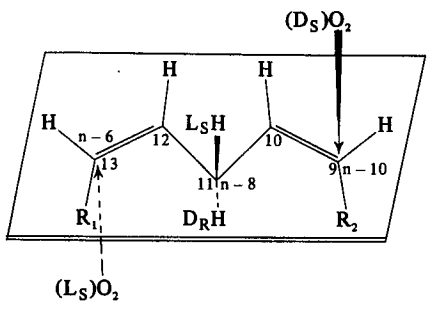
$$CH_3-(CH_2)_4-CH\overset{c}{=}CH-CH\overset{t}{=}CH-\underset{\underset{OOH}{|}}{CH}-(CH_2)_7-C00H$$
9-D-LOOH (maize germs)

Egmond *et al*[54] showed that hydrogen abstraction at C-11 is the rate-determining step in the overall reaction and possibly the initial step. Furthermore, these authors observed that the insertion of oxygen proceeds antarafacially with respect to the hydrogen abstraction. (Fig 14). This stereochemical relationship adequately excludes the involvement of singlet oxygen in the oxygenation process.

From the various plant lipoxygenases, the soybean-1 enzyme is the best investigated species. It consists of a single polypeptide chain and has a molecular weight of approximately 100 Daltons. The enzyme contains 1 gat of non-haeme iron per mole.

It has been shown by De Groot *et al*[55] that iron plays an essential role in the catalytic activity and that its

Fig 14 Stereochemistry of hydrogen abstraction and O_2 insertion in lipoxygenase reactions



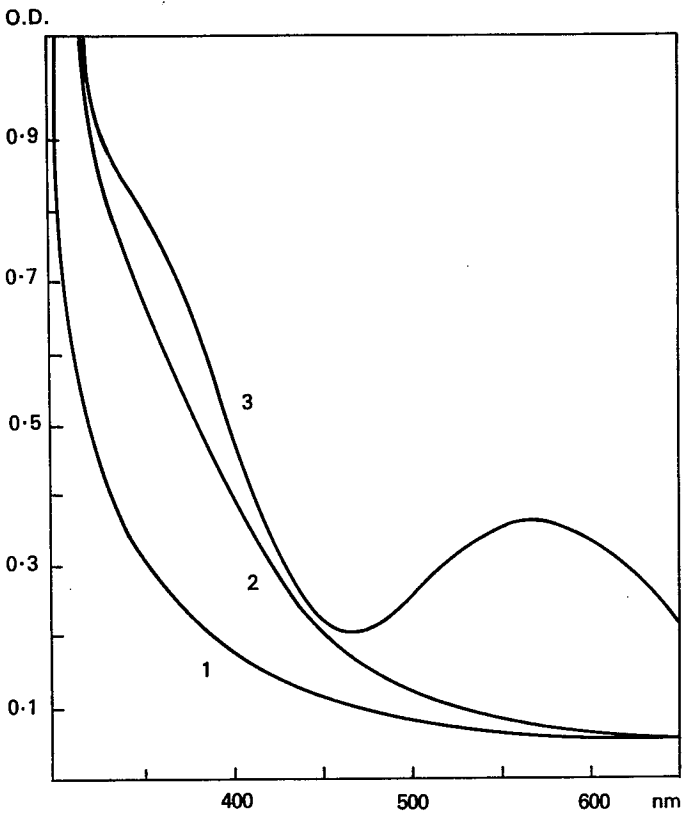
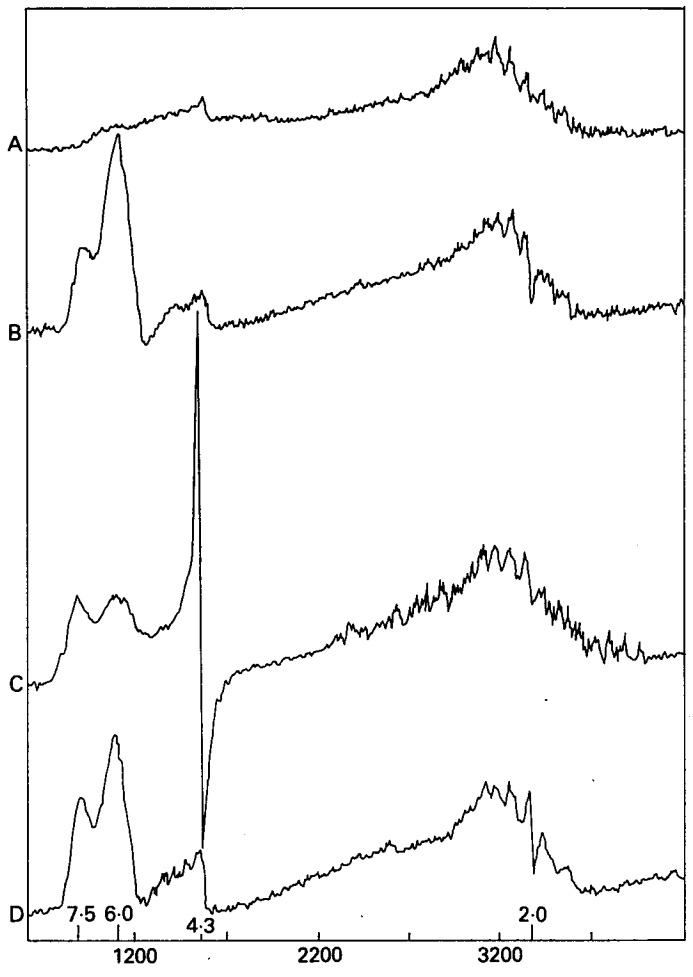


Fig 15 Optical spectra of soybean lipoxygenase-1; 1 native form; 2 yellow form; 3 blue form

Fig 16 Low temperature e.p.r. spectra of the three enzyme forms of lipoxygenase: A native form; B yellow form; C blue form; D yellow form derived from the blue pieces



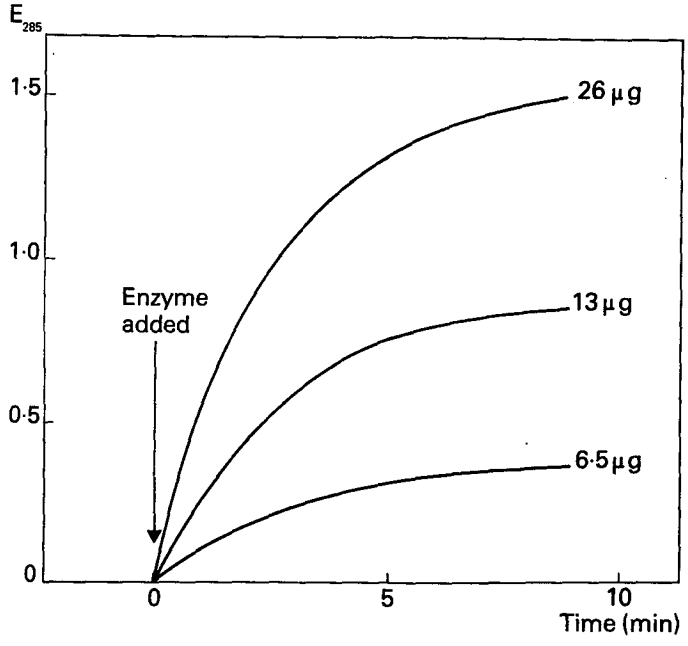


Fig 17 Kinetics of anaerobic conversion of linoleic acid

spectroscopic properties are unique in comparison to other non-haeme iron proteins. Iron is very tightly bound to the polypeptide chain. Removal gives rise to irreversible denaturation.

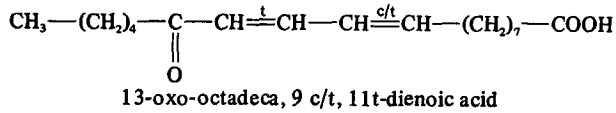
The optical spectrum of native soybean lipoxygenase-1 shows only a protein absorption band with a maximum at 280nm at pH 9·0 (Fig 15). De Groot *et al*[56] demonstrated that addition of stoichiometric amounts of 13-L-hydroperoxy-linoleic acid, results in an increase of the absorbance in the 300-450nm region. The absorption maximum is located at 330 nm (ϵ 330 $\cong$ 1·5 × 10^3 mol^{-1} cm^{-1}). Upon this treatment the colourless solution of the native enzyme turns yellow. This change in absorbance has its counter part in the fluorescence spectrum as demonstrated by Finazzi-Agrò et al.[57] and Egmond et al.[58] The yellow coloured enzyme species can form a 1:1 complex with another hydroperoxide molecule. This labile complex has a new absorption band with a maximum at 570 nm (ϵ 570 $\cong$ 1 × 10^3 mol^{-1} cm^{-1}). The solution of the complex is blue coloured.

The state of iron in the three enzyme forms is different. This can be deduced from the low temperature electron paramagnetic resonance (e.p.r.) spectra (Fig16). Iron in the native enzyme is present as an iron-oxygen complex, which is diamagnetic. Both the yellow and purple forms contain high spin ferric, but the ligand symmetries are different as shown by De Groot *et al*[55] Garssen *et al*[59,60] discovered an important feature of soybean lipoxygenase-1 *viz* its capacity to catalyse the anaerobic conversion of linoleic acid, provided that 13-L-hydroperoxylinoleic acid is present. This reaction proceeds until one of the substrates has been exhausted.

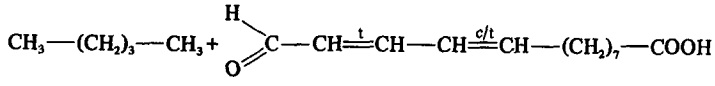
Recently, Verhagen *et al*[61] carried out a study on the kinetics of this reaction, which has provided further insight into the mechanism. The course of the anaerobic reaction can quantitatively be followed on the basis of the absorption at 285nm, which is indicative of the formation of a conjugated dienone chromophore (Fig 17). Apparently, a relatively complex mixture of compounds is formed (Fig 18), although the reaction can easily be understood, when it is known to consist of two steps – first, the ferric enzyme is reduced anaerobically by linoleic acid yielding the ferrous

Fig 18 Compounds formed in anaerobic conversion of linoleic acid

Oxodienoic acids:



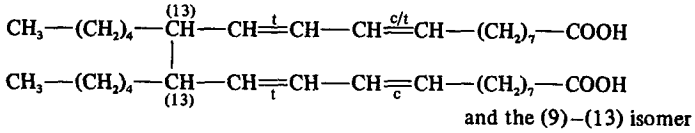
13-oxo-octadeca, 9 c/t, 11t-dienoic acid



n-pentane + 13-oxo-trideca, 9 c/t, 11t-dienoic

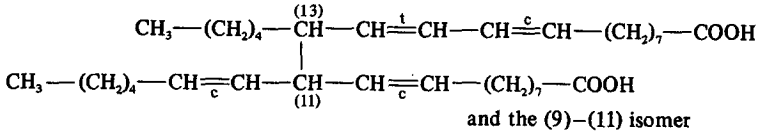
Dimers:

(A)



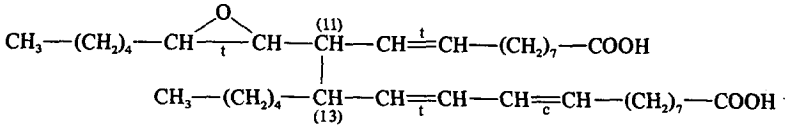
and the (9)–(13) isomer

(B)

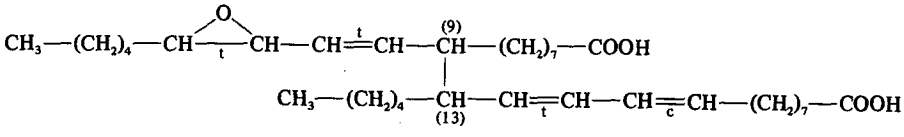


and the (9)–(11) isomer

(C)



(D)



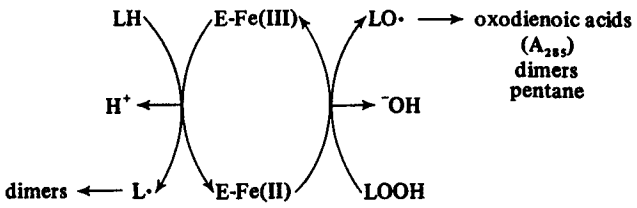


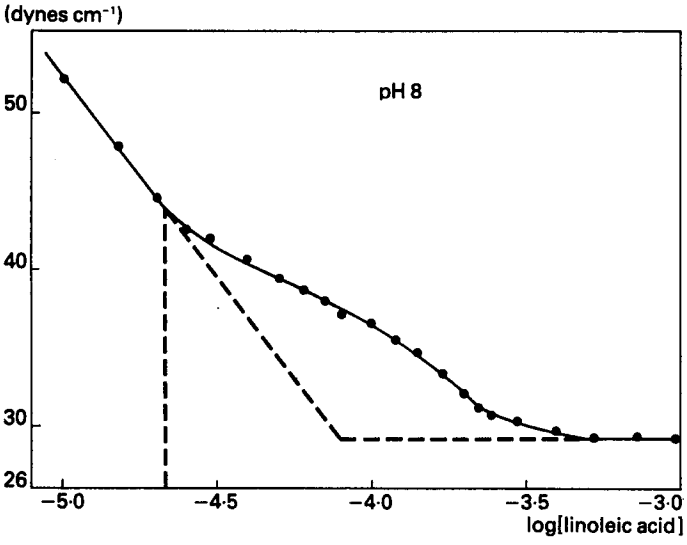
Fig 19 Scheme for the anaerobic reaction of lipoxygenase with linoleic and hydroperoxylinoleic acids

enzyme and linoleic acid radicals, then the ferrous enzyme is oxidised by 13-L-hydroperxylinoleic acid, which leads to the ferric enzyme and alkoxy radicals (Fig 19). The product formation can be explained adequately on the basis of the postulated radicals: combination of linoleic acid radicals with each other or with alkoxy radicals can account for formation of dimers; alkoxy radicals can be considered as precursors of the ketodienes.

Egmond *et al*[62] have demonstrated that the oxidation and reduction steps of the enzyme can be carried out separately. Therefore, a ternary complex of enzyme, linoleic acid and hydroperoxide is not essential for the reaction to proceed. Analysis of the steady state kinetics has shown that the two substrates compete for one binding site. A substituted enzyme mechanism (Ping Pong) with double substrate inhibition is involved.

It should be noted that the physical state of the substrate is an important aspect of kinetic studies on lipoxygenase. Lipoxygenase reacts exclusively with monomers of linoleic acid in the oxygenation reaction, as has been shown by Lagocki *et al.*[63] For linoleic acid a wide variety of critical micelle concentration (c.m.c.) values has been reported, whereas no information is available for hydroperoxides, nor for mixtures of both. During kinetic work on the anaerobic reaction Verhagen *et al*[64] determined the aggregation behaviour of the substrates and mixtures thereof on the basis of surface tension measurements. C.m.c. values were obtained by plotting the measured surface tensions against the logarithm of the concentration of the surface active reagent (Fig 20). Aggregation starts at the concentration at which deviation of linearity occurs in the plot. It is remarkable that in mixtures of linoleic acid and its

Fig 20 Aggregation behaviour of substrates and their mixtures on the basis of surface tension measurements



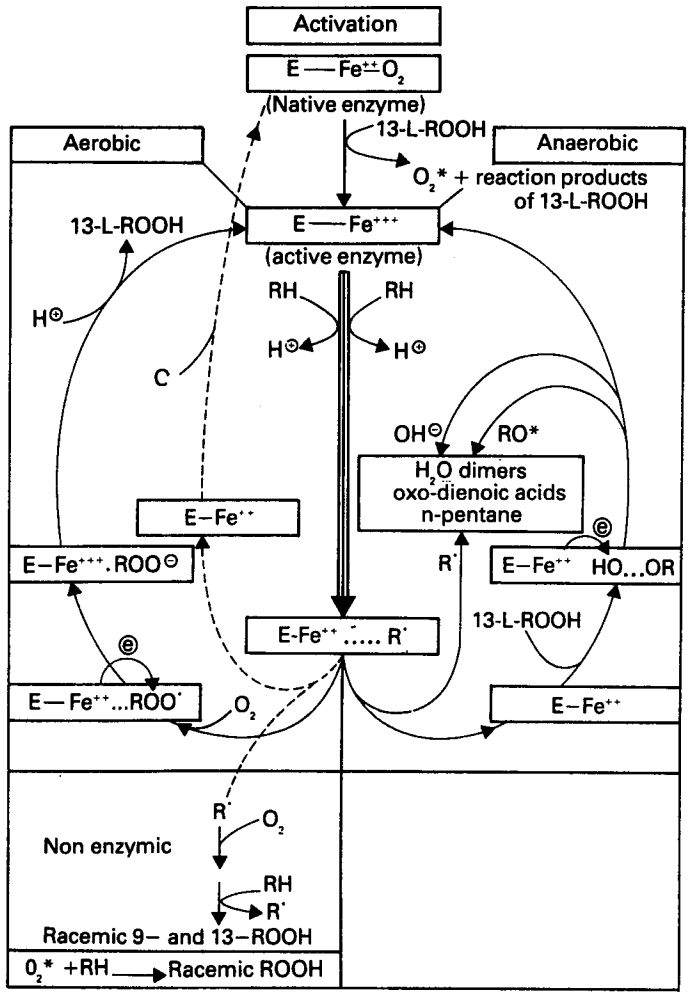


Fig 21 Proposed scheme for the catalytic activities of soybean lipoxygenase-1 at pH 9 under aerobic and anaerobic conditions. RH = fatty acid

hydroperoxide, there is no dramatic increase in c.m.c. value compared with linoleic acid alone.

For the anaerobic reaction, a radical mechanism was postulated. To prove this proposal a radical scavenger was introduced to trap the radicals formed. Although in organic chemistry this technique has already been known for years, the application to biochemical systems presents specific problems. Usually the solubility in aqueous systems of these compounds is rather limited. De Groot *et al*[65] developed a new, simple, water-soluble radical trap, 2-methyl-2-nitroso-propanol, which was the first biochemical application of this technique. The number of peaks and their hyperfine splitting pattern in the e.p.r. spectrum gives information on the presence of atoms with odd spins near the unpaired electron. In this way, it could be demonstrated that linoleic acid radicals were formed. For the identification of the radical, advantage was taken of linoleic acids labelled with deuterium in the pentadiene system.

The anaerobic system has provided much information on soybean lipoxygenase-1. Most important is the observation that this enzyme is capable of generating radicals from the fatty acid substrate. Extrapolating this system to the aerobic situation, it is reasonable to propose that the capacity to produce radicals is retained. For this enzyme a reaction cycle has been proposed[55] (Fig 21) which is a perfectly controlled radical reaction. The iron in the enzyme is alternately in the ferrous and the ferric state.

The steady state kinetics of the aerobic reaction have been investigated by several groups.[66-69] Nevertheless, some very

fundamental questions remain unanswered such as the number of binding sites and the role of the product hydroperoxide in the reaction mechanism.

Several lipoxygenases, having a pH optimum at 6·6 exhibit considerable cooxidising properties towards a variety of structurally unrelated co-substrates like β-carotene, crocin, chlorophyll. This feature has found technical application in flour bleaching. There is ample reason to believe that the cooxidation is a radical reaction, in which the radicals which initiate the reaction can stem from dissociation of an enzyme-radical complex. Two possibilities can be considered in this respect:

- Dissociation of the enzyme-fatty acid radical complex, which leads to a ferrous enzyme and a fatty acid radical. Both can react with oxygen. The peroxy radical can then start the cooxidation process and the enzyme can return to the native state.
- Dissociation of the enzyme-peroxy radical complex, which gives rise to essentially the same products as above, although in this case the introduction of oxygen in the fatty acid radical can proceed under better stereochemical control (compare Van Os *et al.*[70])

In addition, the fatty acid itself can be cooxidised if no co-substrate is present which is more prone to be oxidised. This leads to an apparent loss in specificity in the hydroperoxide formation and to the formation of secondary products, e.g. ketodienes. The interesting observation of Bild *et al*[71] that lipoxygenase-2 is capable of catalysing the conversion of arachidonic acid into prostaglandin-like products fits well to this postulate. Oxygenation of arachidonic acid gives a peroxy radical that can form an endoperoxide fatty acid radical, which can in turn be oxygenated.

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