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On the mechanism of the reactions catalysed by plant lipoxygenases (*)

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Summary

The best investigated species is soybean lipoxygenase-1. It has a molecular weight of approx. 98.500, consists of a simple polypeptide chain. contains 1 gat Fe /mol and has a pH optimum of 9.0. The combination of enzyme with an equimolar amount of 13-L-hydroperoxy-9-cis, 11-trans-octadecadienoic acid (13L-ROOH) gives rise to the formation of a yellow enzymes species. The absorption fluorescence and e.p.r. spectra of this species differ significantly from the native enzyme. Concomitantly with the colour change, the hydroperoxide is converted. This reaction is irreversible. Only under anaerobic conditions the yellow enzyme can be reconverted into a colourless species by substrate fatty acid (linoleic acid). The addition of an excess of 13-L-ROOH to the yellow enzyme yields a purple enzyme species with quite different spectral characteristics. The purple enzyme species represents a complex between the yellow enzyme and 13-L-ROOH. This complex is rather labile at room temperature giving rise to the formation of the yellow enzyme under simultaneous isomerisation of 13-L-ROOH. The kinetics of the formation of the coloured enzyme species have been measured by stopped fow techniques. The results are significant for the role of these species in the reaction mechanism.

It has been found that also the pea lipoxygenase contains iron. Specific interactions between enzyme and hydroperoxide exist for the pea enzyme too.

Key-Words: Soybean-Pea, Lipoxygenase, Mechanism.

Introduction

Lipoxygenase has long been considered as a typical plant enzyme. In animal systems lipoxygenase was postulated as a part of the prostaglandin synthetase but, recently, another lipoxygenase type of enzyme has been isolated from blood platelets which does not seem to be associated with the prostaglandin synthetase (1, 2). From plant sources no new large scale isolations have been reported so most experiments are still carried out with lipoxygenase-1 from soybeans. The latter enzyme is relatively stable and can easily be obtained in large quantities.

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Dioxygenase reactions of lipoxygenase

In figure 1 the aerobic reaction of lipoxygenase with linoleic acid is shown. In general, polyunsaturated fatty acids with a 1,4-cis, cis-pentadiene system (3) can be utilized as a substrate by the enzyme. In the case of linoleic acid the formation of 13-L-hydroperoxylinoleic or 9-D-hydroperoxylinoleic acid occurs through the action of soybean and corn germ lipoxygenase respectively. Formally, the structural features of this reaction are:

i the removal of hydrogen from C-11 which as we could demonstrate (9) occurs stereospecifically and is the rate limiting step.
ii conjugation of the double bond system.
iii introduction of the hydroperoxy group.

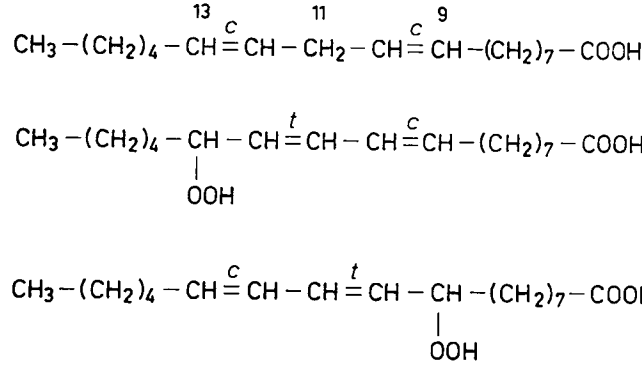


FIG. 1. — *Aerobic conversion of linoleic acid catalyzed by lipoxygenases.*

TABLE I

Metal analysis of soybean lipoxygenase-1

Fe	0.912	mole metal mole enzyme
Cu	0.0018	
Mn	0.086	
Ca	0.21	
Mg	0.18	
Zn	0.03	
Co	—	
V	—	
Mo	—	
Ni	—	

The spin barrier between ground state (triplet) oxygen and linoleic acid has led Chan (8) to reinvestigate the enzyme with respect to the presence of transition metals. It was found that lipoxygenase contains 1-2 moles of iron per mole of enzyme. Subsequent investigations have confirmed the presence of iron in lipoxygenase in a 1:1 ratio (4, 5, 6). The occurrence of iron as a prosthetic group is not limited to soybean lipoxygenase since we could demonstrate its presence also in pea lipoxygenase. We confine ourselves now to soybean lipoxygenase-1 only. A more extensive metal analysis has recently been published by GALPIN et al. (7) (table 1). This enzyme was prepared with EDTA present throughout

the purification procedure in order to reduce the amount of contaminating metal-ions as far as possible. The protein was homogeneous on polyacrylamide gel electrophoresis and had the excellent specific activity of 280 μmol O_2/min/mg. Remarkably enough considerable quantities of divalent ions were detected which all together accounted for almost 1 gramatom. One might wonder whether this represents another metal-binding site or not. Recently, DIXON (15) has pointed out that a wide variety of enzymes which have a transition metal in the active centre, contain a second metal ion. For lipoxygenase this aspect needs further investigation.

The participation of neither free superoxide (O_2^-) nor singlet ($^1\Delta_g$) oxygen

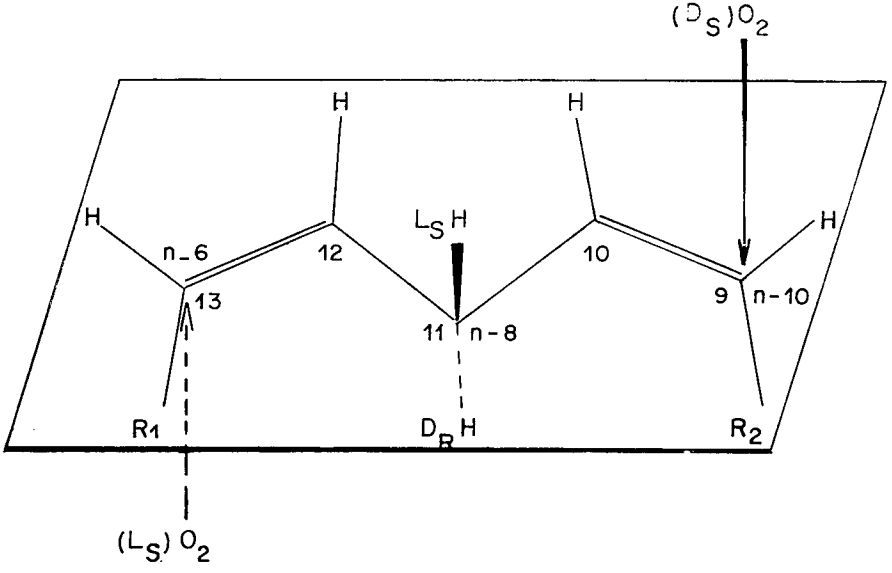


FIG. 2. — *Stereochemistry of hydrogen abstraction and oxygen insertion.*

in the formation of hydroperoxide seems likely. Addition of small amounts of superoxide dismutase to the incubation medium does not affect the rate of hydroperoxide formation. The addition of scavengers for singlet oxygen does not lead to reaction products which might be expected as a result from singlet oxygen reactions. These observations rule out the possibility that one of these free activated oxygen species play a role in the reaction. Moreover, if singlet oxygen were involved the strereochemistry of hydrogen removal and oxygen insertion requires that these processes occur at the same side of the planar pentadiene system (fig. 2). However, the reaction proceeds antarafacially (9). Nevertheless, the possibility that oxygen coordinated to iron has an activating effect cannot be excluded. We have, however, demonstrated that lipoxygenase can activate the fatty acid substrate by generating a fatty acid free radical (10) which would then be able to react directly with ground state oxygen.

Under certain conditions the reaction between linoleic acid and lipoxygenase shows a kinetic lag phase. The lag phase can be eliminated by adding the product 13-L-hydroperoxylinoleic acid. The addition of 9-D-hydroperoxylinoleic acid has no effect. This phenomenon has been interpreted as the result of a specific

interaction between enzyme and product. SMITH and LANDS (11) have proposed on the basis of kinetic measurements that the product hydroperoxide is a compulsory activator of lipoxygenase. However, the kinetic lag phase is a more complicated phenomenon as it is influenced by the concentrations of all reactants. EGMOND et al (17) have shown that at substrate concentrations below 25 μM no sigmoid initial velocity curves are observed. This holds also when the best precautions are taken to avoid contamination with hydroperoxides. The occurrence of a kinetic lag phase is then best explained by substrate inhibition. The

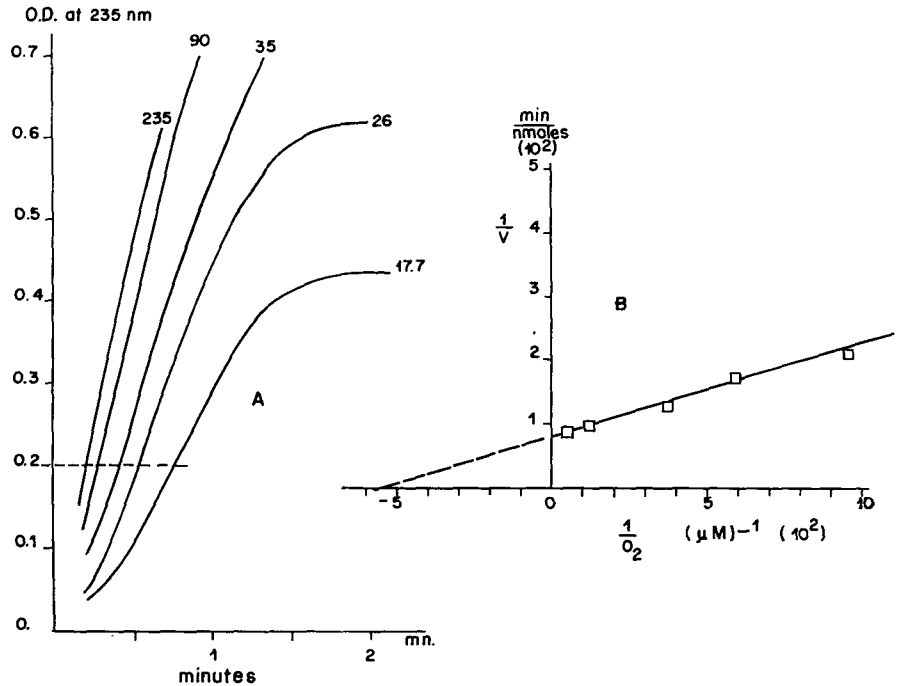


FIG. 3. — A. *Progress curves for the formation of hydroperoxylinoleic acid at various initial O_2 concentrations.*
B. *Double reciprocal plot of the reaction rates obtained from A at 8 μM ROOH and 32 μM linoleic acid vs. the corresponding O_2 concentration.*

concentration of the second substrate—oxygen—also affects the shape of the initial velocity curves as is shown in figure 3. At a fixed concentration of linoleate the curves have a more sigmoid shape at low oxygen concentration. Another factor which is likely to have a significant influence on the kinetics of this reaction is the physical state of the substrate i.e. the occurrence of micelles of linoleate and mixed micelles of linoleate and hydroperoxylinoleate.

Enzyme product interactions

Direct evidence for specific interactions between the enzyme and 13-L-hydroperoxylinoleic acid has been obtained from various spectroscopic studies. Electron paramagnetic resonance (EPR) spectroscopy was applied to investigate the

interactions between the iron of the protein and product hydroperoxide (fig. 4). Spectrum A stems from the native enzyme and shows no significant resonances. Spectrum B was obtained by mixing equimolar amounts of enzyme and product hydroperoxide. The strong absorbance round $g = 6$ must be ascribed to high-

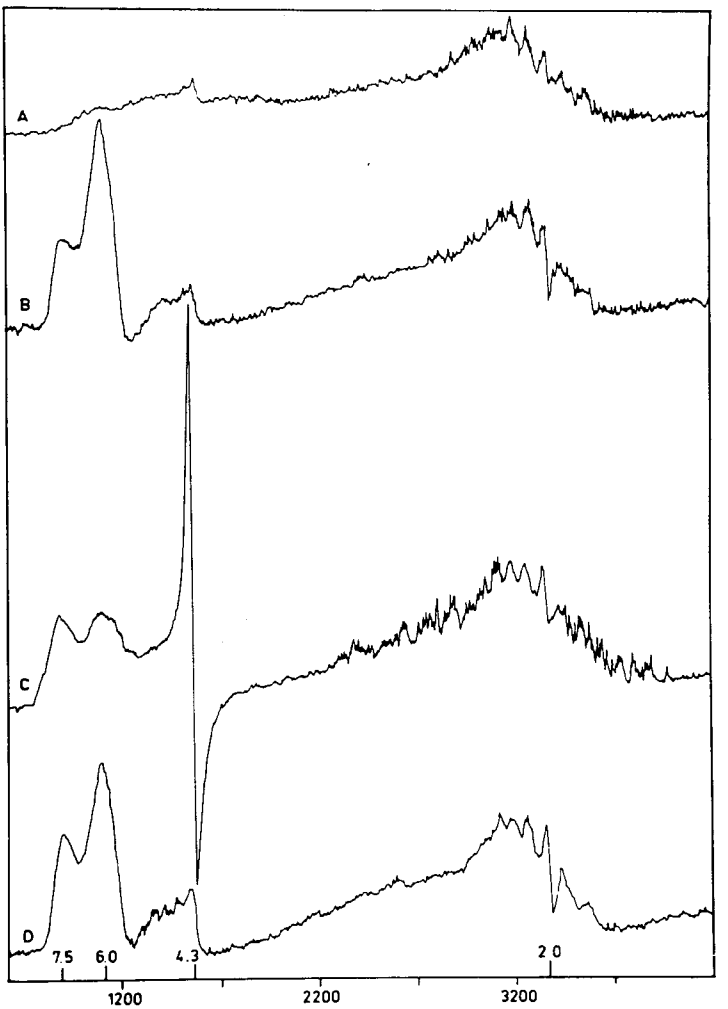


FIG. 4. — *Effect of 13-L-hydroperoxylinoleic acid on the EPR-spectrum of lipoxygenase. A: native enzyme; B: after addition of an equimolar amount of 13-ROOH; C: after addition of an excess of 13-ROOH; D: same as C after 1 h at 0 °C.*

spin Fe (III) in a ligand field of axial symmetry. On the addition of a molar excess of product hydroperoxide the absorbance shifts for the greater part to $g = 4.3$ (fig. 4, C). After 1 h at 0 °C the sample from which spectrum C was obtained gives spectrum D (at 15 °K) which is virtually identical to B. The latter process is reversible. Upon a new addition of product hydroperoxide, again spectrum C arises.

The effect of 13-hydroperoxylinoleic acid on the protein can also be demonstrated with optical spectrophotometry. Figure 5 shows a series of experiments quite similar to those just described with EPR-spectroscopy. The native enzyme (fig. 5, a) shows no specific features. However, upon addition of an equimolar amount of product hydroperoxide the colour of the solution turns into yellow (ε_{330} = 1 600 M^{-1} cm^{-1}, fig. 5, b). A further addition of hydroperoxide causes

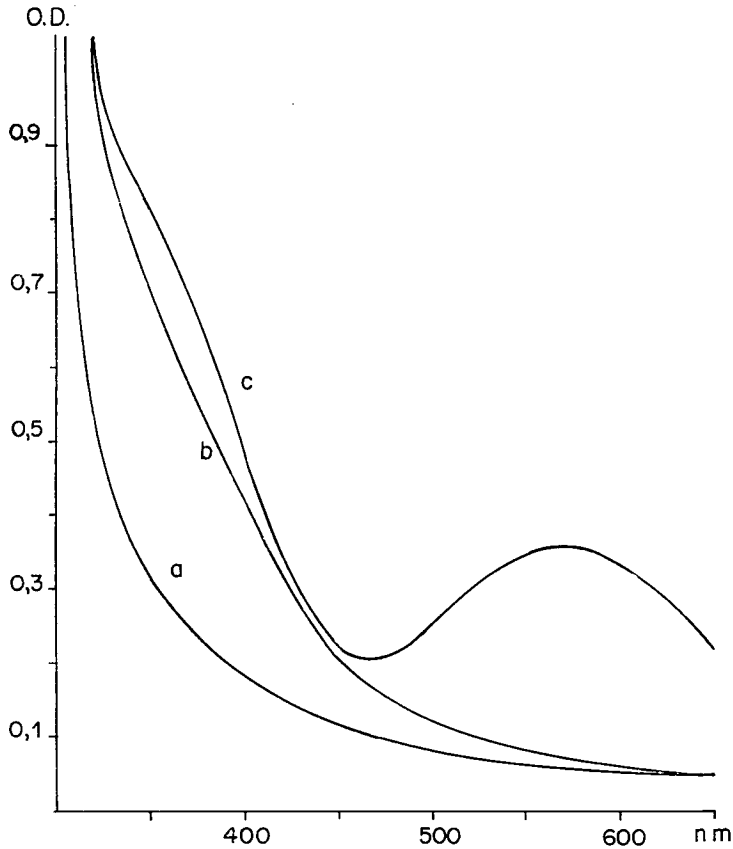


FIG. 5. — *Effect of 13-L-hydroperoxylinoleic acid on the optical spectrum of lipoxygenase.*
a : native enzyme.
b : after addition of an equimolar amount of 13-ROOH.
c : after addition of an excess of 13-ROOH.

the appearance of another chromophore at 580 nm. (ε_{580} = 1 200 $M^{-1}.cm^{-1}$, fig. 5, c) which disappears on standing and the spectrum of the solution becomes indistinguishable from b.

The effect of equimolar amounts of hydroperoxide has also been demonstrated by fluorescence spectrometry (6, 12). An excess of product hydroperoxide does not affect the fluorescence of the protein. The formation of the yellow enzyme species from the native enzyme and product hydroperoxide is a chemical reaction wherein the hydroperoxide is converted into products, which are not yet identified. The purple enzyme species represents a complex between the yellow enzyme and

hydroperoxide. The observed transition of the purple enzyme to the yellow species is accompanied by a conversion of 13-hydroperoxylinoleic acid into mono-unsaturated epoxy-hydroxy fatty acid (fig. 6). When this reaction is carried out with 13-L-hydroperoxy linoleic acid labelled with ^{18}O in the hydroperoxy group, the greater part of label is retained in both new oxygen functions. This suggests that this isomerization proceeds via free radicals in a cage reaction (18).

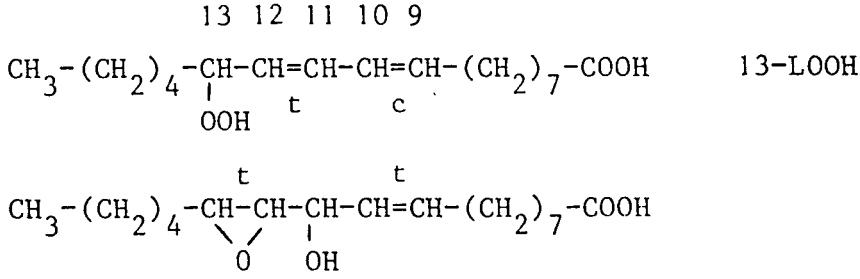


FIG. 6. — *Structure of the mono-unsaturated epoxy-hydroxy fatty acid obtained by enzymic isomerisation of 13-ROOH.*

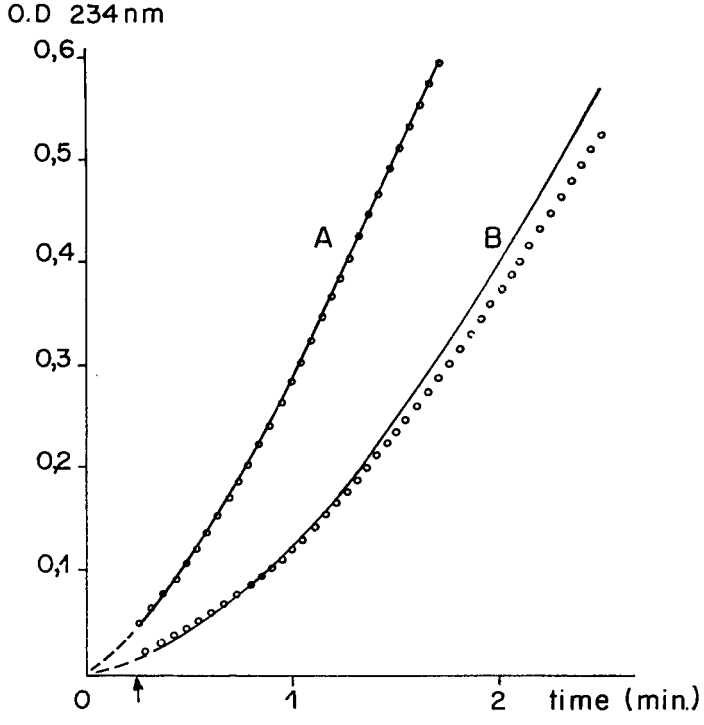
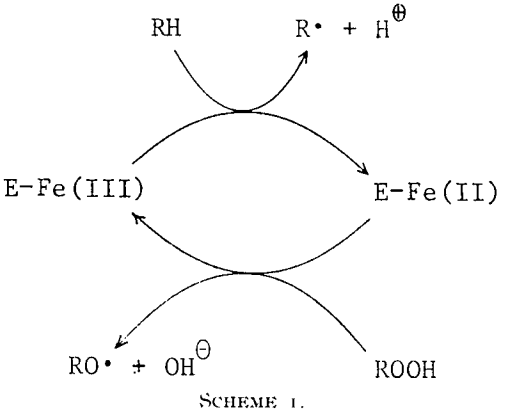


FIG. 7. *Progress curves for the formation of 13-ROOH catalysed by native (——) and by yellow (oo) enzyme at two different substrate concentrations.*
A : 240 µ*M*.
B : 480 µ*M*.

Mechanism of lipoxygenase catalysis

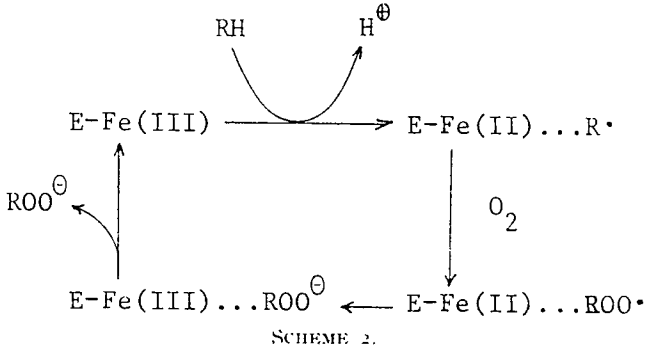
The presence of various enzyme species as detected by spectroscopic techniques raised the question as to which of these species is the active one during the oxygenation reaction. A comparison of the native and the yellow species in a standard assay at two substrate concentrations shows no significant differences (16) (fig. 7). This may be explained by assuming that, regardless of the initial state of the enzyme the same steady state distribution of enzyme species is rapidly attained.

If oxygen is excluded from the reaction medium no reaction is observed with the native enzyme. However, when the yellow enzyme is incubated with a molar excess of linoleic acid under anaerobic conditions a fast bleaching of the yellow species is observed. Concomitantly, the Fe(III) signals in the EPR spectrum have disappeared. Evidently, linoleic acid is an effective reductant for the Fe(III) enzyme. When hydroperoxylinoleic acid is also present the Fe(III) species can be re-oxidised to Fe(III), which can, in turn, be reduced to Fe(II) by linoleic acid (Scheme 1).



SCHEME 1.

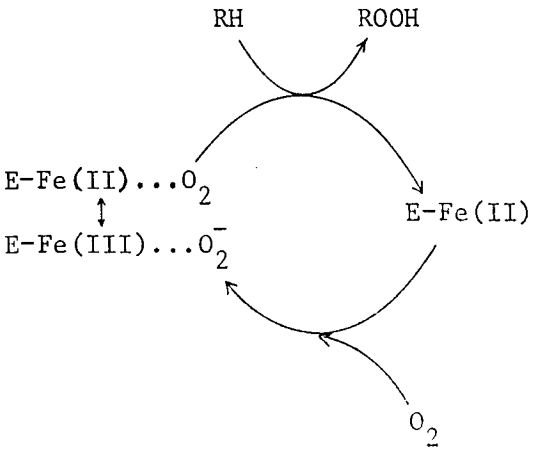
This cycle then represents the anaerobic conversion of hydroperoxylinoleic acid in the presence of linoleic acid (13, 14) and the formation of products resulting from subsequent breakdown and combination of the two types of radicals initially formed by the enzyme.



SCHEME 2.

In the reaction of linoleic acid with molecular oxygen (fig. 1) the role of iron is very similar to the anaerobic case if it is assumed that iron occurs in a Fe(III) state (Scheme 2).

The involvement of a Fe(III)-species is demonstrated by the effect of 4-nitro-



SCHEME 3.

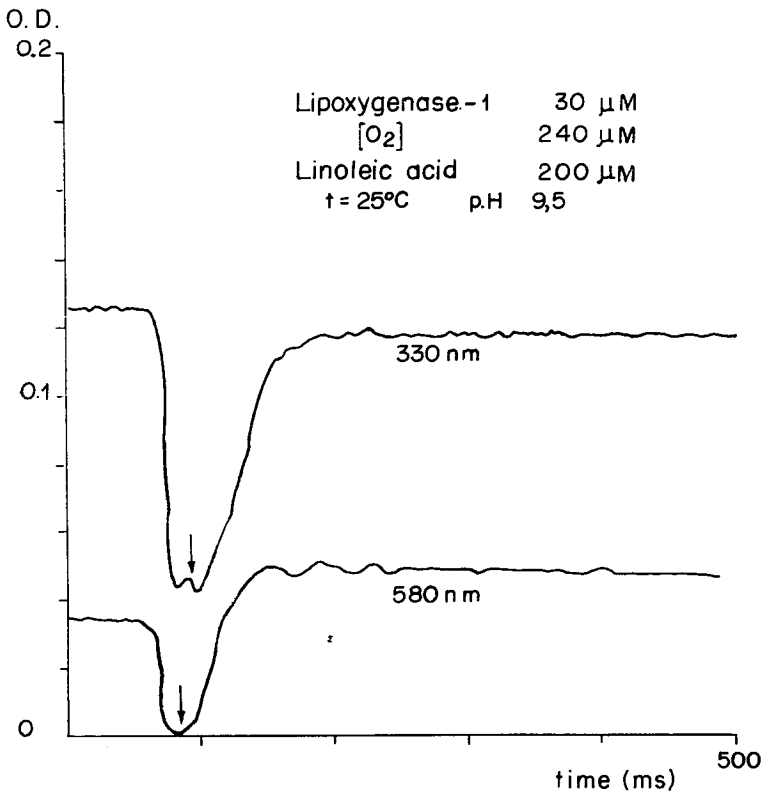


FIG. 8. — *Formation of yellow and purple enzyme species.*

catechol (7) which was found to inhibit the enzyme by binding to the ferric ion. However a preincubation of the native enzyme with this type of inhibitor does not result in a decrease in enzymic activity. This indicates that the native enzyme does not contain a ferric ion accessible for 4-nitrocatechol. Moreover the EPR studies show that the native enzyme is EPR-silent.

Both the diamagnetism and the inaccessibility for 4-nitrocatechol of the native species may be caused by bonded oxygen. Since it has been shown that

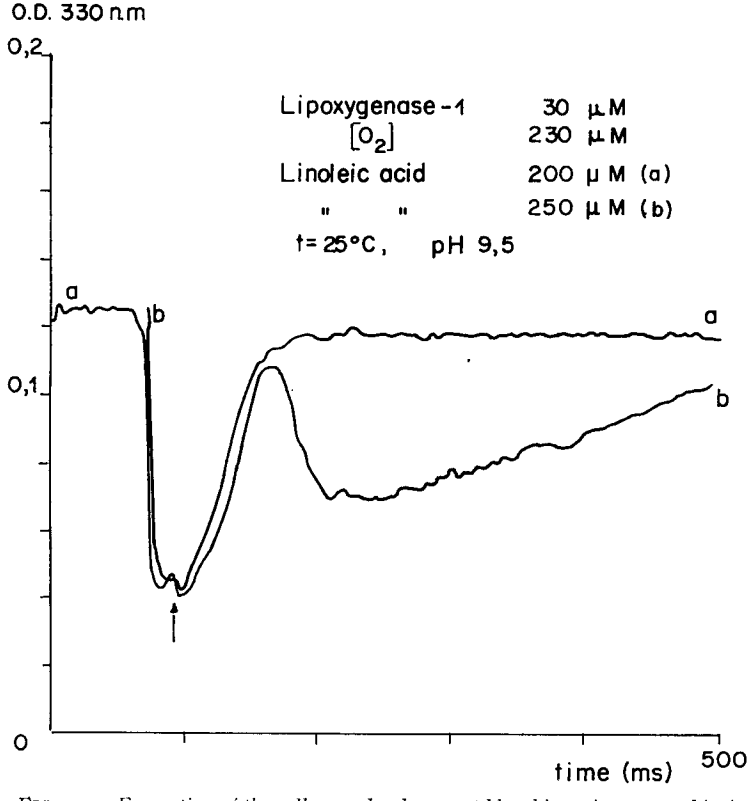


FIG. 9. — *Formation of the yellow and subsequent bleaching after anaerobiosis has been attained.*

the presence of hydroperoxide is not an absolute requirement for the aerobic reaction to start, another enzyme species must contribute to the overall rate of hydroperoxide formation. A ferrous: oxygen complex (or ferric: superoxide) may also be able to produce hydroperoxide from linoleic acid (Scheme 3).

As soon as some hydroperoxide has been produced both the oxygenated and deoxygenated enzyme species (Scheme 3) may become subject to attack by the hydroperoxide which then yields a Fe(III) species capable of contributing to the hydroperoxide formation through the pathway given in Scheme 2. The relative contributions of both pathways to the overall rate are probably dependent on the concentrations of the reactants.

The formation of both the yellow and purple enzyme species can be demonstrated by combining linoleic acid, oxygen and enzyme in a stopped flow

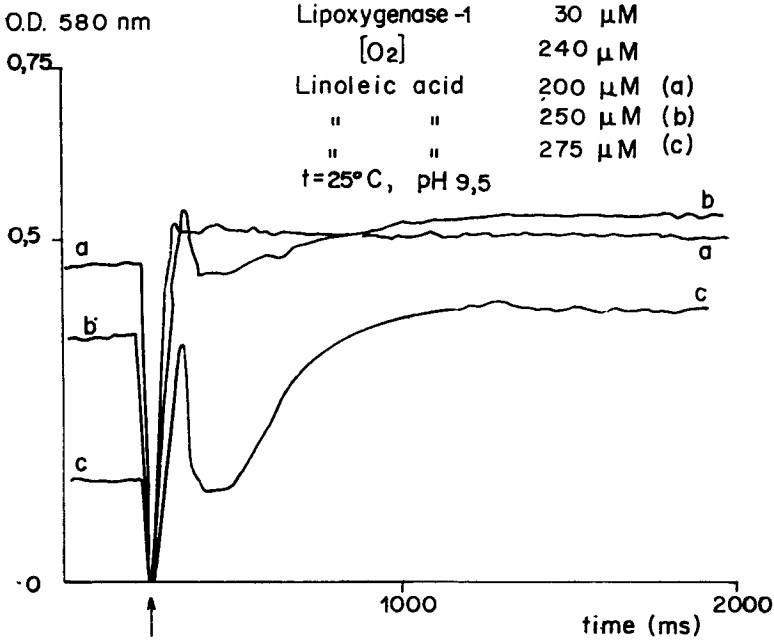


FIG. 10. — *Time dependence of the formation of the purple enzyme at various substrate concentrations.*

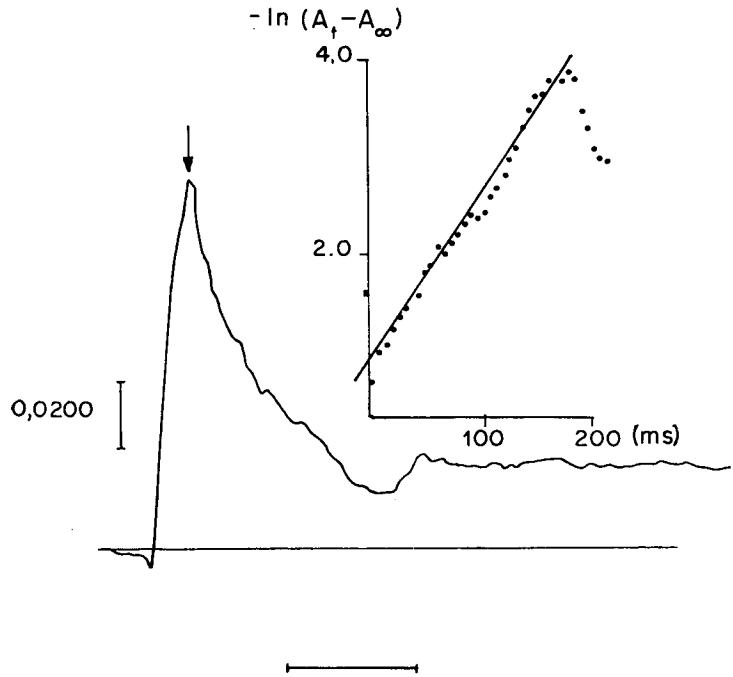


FIG. 11. — *Anaerobic bleaching of the yellow enzyme by deuterated linoleic acid.*

apparatus. Typical experiments are given in figures 8, 9 and 10. Figure 8 shows that the formation of the yellow (330 nm.) and purple (580 nm.) species occurs simultaneously when measured on a millisecond time scale. When the concentration of linoleic acid is higher than the oxygen concentration a partial bleaching of the yellow species is observed (fig. 9, b) due to the anaerobic reduction of the Fe(III)-species by linoleic acid. This phenomenon also be demonstrated by monitoring the system at 580 nm. (fig. 10). After the bleaching step a gradual increase in absorbance occurs, the extent of which depends on the amounts of linoleic acid and hydroperoxylinoleic acid. If an initial concentration of linoleic acid is chosen far above 240 μM all of the hydroperoxylinoleic acid is converted anaerobically and the excess of linoleic acid leaves the enzyme in a ferrous state.

As stated above the mechanism of lipoxygenase catalysis involves the stereospecific removal of one hydrogen from C-11 of linoleic acid. By substituting deuterium for hydrogen at C-11 it has been shown that this step is rate-limiting (9). In Scheme 1 it is proposed that the hydrogen abstraction is accomplished by a Fe(III)-species. Under steady state conditions a rate constant of 32 ± 3 s^{-1} was derived from the maximum rates of hydroxide formation from the deuterated linoleic acid. The rate-limiting nature of the hydrogen abstraction step implies that the rate at which the yellow Fe(III)-species is bleached by the substrate should be close to the overall rate of hydroperoxide formation. In fig. 11 the result is shown from a stopped-flow experiment wherein deuterated linoleic acid is mixed anaerobically with the yellow Fe(III)-species. If the absorbance change at 330 nm is plotted semilogarithmically it can be seen that the reaction follows pseudo first order kinetics between approx. 10 per cent and 75 per cent conversion. The rate constant derived from the first part of the plot is 30 ± 1 s^{-1} which agrees well with the result obtained under steady state conditions.

Despite the considerable progress that has been made in the understanding of the mechanisms of the enzymic conversions of unsaturated fatty acids by lipoxygenase many questions are still unanswered. Undoubtedly one of the most intriguing problems lies in the chemical environment of the iron this enzyme.

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Résumé

Sur le mécanisme des réactions catalysées par la lipoxygénase des plantes

Parmi les lipoxygénases, la plus étudiée est la lipoxygénase 1 du soja. D'un poids moléculaire voisin de 98 500, constituée d'une seule chaîne polypeptidique, elle contient un atome de fer par mole et a un pH optimum de 9. La combinaison de l'enzyme avec une quantité équimolaire d'acide 13-L-hydroperoxy-9-6, 11-trans-octadécadiénoïque (13-L-ROOH) conduit à la production d'une forme de l'enzyme colorée en jaune. Les caractéristiques des spectres d'absorption, de fluorescence et de r.p.e. de cette forme, diffèrent notablement de la forme native. En même temps que le changement de coloration, l'hydroperoxyde est transformé. Cette réaction est irréversible. La forme jaune peut être retransformée en une forme incolore par le substrat (acide linoléique) uniquement en anaérobiose. L'addition d'un excès de 13-L-ROOH à la forme jaune conduit à une forme de couleur pourpre qui possède des caractéristiques spectrales différentes. Cette dernière forme est un complexe entre la forme jaune et le 13-L-ROOH. A température ambiante, ce complexe est relativement labile et conduit à la production de la forme jaune avec une isomérisation simultanée du 13-L-ROOH. Les cinétiques d'apparition des formes colorées ont été mesurées par des techniques d'écoulement interrompu.

La lipoxygénase du pois contient du fer et présente également des interactions spécifiques avec les hydroperoxydes.

Mots clés : Soja, lipoxygénase, mécanisme.

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