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THE DETECTION OF LINOLEIC ACID RADICALS IN THE ANAEROBIC REACTION OF LIPOXYGENASE

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SUMMARY

In the anaerobic reaction of soybean lipoxygenase with linoleic acid and enzymically formed hydroperoxy linoleic acid the involvement of linoleic acid radicals is demonstrated.

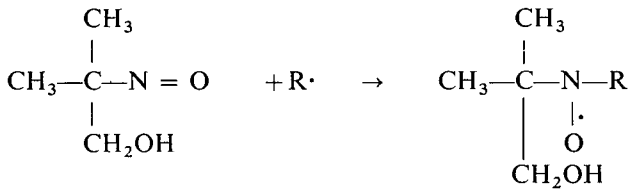
Evidence for this has been obtained by anaerobic incubations of linoleic acid, *cis,cis*-9,12-[11,11-^{2}H]octadecadienoic acid, *cis,cis*-9,12-[9,10,12,13-^{2}H]octadecadienoic acid and *cis,cis*-9,12-[9,10,11,11,12,13-^{2}H]octadecadienoic acid, respectively, together with hydroperoxy linoleic acids in the presence of 2-methyl-2-nitrosopropanol as a water soluble radical scavenger.

The trapped linoleic acid radicals were detected by ESR spectroscopy.

INTRODUCTION

The enzyme lipoxygenase (E.C. 1.13.1.13) occurs in plant tissues and attacks polyunsaturated fatty acids which contain a 1,4-*cis,cis*-pentadiene system. In the presence of oxygen, *cis,trans*-conjugated hydroperoxides are formed. In a system with a limited amount of oxygen and an excess of linoleic acid with respect to the available oxygen, reaction products other than hydroperoxides are also formed[1]. Some reaction products are characterised by ultraviolet absorption at 285 nm indicating the presence of a conjugated oxodiene chromophore. Other reaction products[2] have been isolated and characterised as dimeric fatty acids. Garssen *et al.*[2] have found that the dimeric fatty acids are formed from two molecules of linoleic acid or from one molecule of linoleic acid and one molecule of hydroperoxy linoleic acid. Garssen suggests a radical mechanism for this reaction in which a linoleic acid radical is involved. To prove this a radical trapping technique[3] was used[4].

For this purpose the water soluble 2-methyl-2-nitrosopropanol was used as a radical scavenger. Nitroso alkanes possess the property to react with radicals thereby forming an adduct with considerable stability[5]:



To obtain maximum information about the environment of the unpaired electron in the newly formed radical the use of a tertiary nitroso compound is indicated, because of the absence of a β-hydrogen* in the reagent. The number of peaks and their hyperfine splitting pattern gives information on the presence of atoms with odd spins near the unpaired electron. Every splitting of the pattern that occurs arises therefore from the trapped radical, since the γ-hydrogen atoms only cause some line-broadening. Other advantages are: 1, tertiary nitroso alkanes do not isomerise to oximes; 2, the reactive monomer has only a small tendency to dimerise.

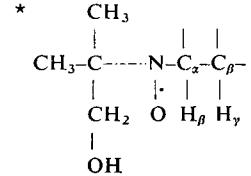
MATERIALS AND METHODS

Linoleic acid (purity > 99%), *cis*, *cis*-9,12-[11,11-^{2}H]octadecadienoic acid (purity > 95%), *cis*,*cis*-9,12-[9,10,12,13-^{2}H]octadecadienoic acid and *cis*,*cis*-9,12-[9,10,11,11,12,13-^{2}H]octadecadienoic acid (purity > 95%) were kindly donated by Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands. The extent of deuterium substitution in the linoleic acids exceeds 95%.

Soya-bean lipoxygenase (spec. act. 106 moles · min^{-1} · mg^{-1}) was isolated according to the method of Finazzi-Agrò *et al* (Finazzi-Agrò, A., Avigliano, L., Veldink, G. A., Vliegenthart, J. F. G. and Boldingh, J., unpublished). 2-Methyl-2-nitropropanol was purchased from Fluka. 2-Methyl-2-nitrosopropanol was synthesized according to Maassen and De Boer[6].

ESR spectra were obtained with a Varian E-4 spectrometer with 100 kcycles/s field modulation. Curves of the first derivative of the microwave absorption were recorded at a microwave power of 5 mW. All experiments were carried out at room temperature in a quartz tube designed for measurement of aqueous solutions and at a modulation amplitude of 0.5 G. g-values were determined with an external standard of solid diphenylpicrylhydrazyl (g = 2.0036).

2-Methyl-2-nitrosopropanol[6,7] was synthesized as follows: 25 g of 2-methyl-2-nitropropanol was dissolved in a solution of 9 g of ammonium chloride in 250 ml of water. During 1 h 45 g of zinc dust was added under vigorous stirring. The temperature was kept at about 60 °C by cooling. After zinc addition the reaction mixture was stirred for another hour. The greyish suspension was filtered and the residue was washed four times with 50 ml of hot water. The combined filtrates were freeze-dried and taken up in about 10 ml of methanol. The non-dissolved part was filtered off and discarded.



The filtrate was concentrated in a rotatory evaporator, yielding the hydroxylamine. This was used for oxidation with silver carbonate without further purification. The silver carbonate reagent was prepared according to Fetizon and Golfier[9], by precipitating silver carbonate on to celite. To a suspension of 17 g of silver carbonate reagent in 150 ml of freshly distilled diethylether, a solution of 2.1 g of the hydroxylamine in 50 ml of water was added. The mixture was shaken vigorously for 2 min. In this reaction the yellow colour turned to black. The celite was removed by filtration, the diethylether layer was separated off and washed with 1 M sulphuric acid to remove unreacted hydroxylamine followed by washing with water. The solution was dried over anhydrous sodium sulphate and the solvent was removed by careful distillation at atmospheric pressure through a Vigreux column. The resulting crystalline 2-methyl-2-nitrosopropanol was used for the radical trapping experiments. The compound was identified on the basis of its ultraviolet-spectrum (λ_{max} at 215, 294 and 675 nm in ethanol) and its infrared spectrum (1545 cm^{-1}, NO-monomer). To prevent the photolytic decomposition of 2-methyl-2-nitrosopropanol and the formation of its nitroxide radical[9] the synthesis and the radical trapping experiments were conducted under exclusion of light as far as possible.

RESULTS AND DISCUSSION

The radical trapping experiments were carried out as follows: 0.5 ml of 3.6 mM linoleic acid in an air-saturated 0.2 M sodium borate buffer (pH 9.0) was incubated at 20 °C with 0.2 ml of the lipoxygenase solution (3.75 mg/ml). Immediately after the addition of the enzyme about 5 mg of the spin trap was added. The reaction mixture was then transferred to the sample cell for ESR measurement. The resulting spectrum is shown in Fig. 1. It shows hfs constants of 16.0 and 2.0 G indicating the presence of a hydrogen atom at the β-position. It proves the involvement of radicals in the reaction. The signals of the ESR spectra were centered around a g-value of 2.0056, which corre-

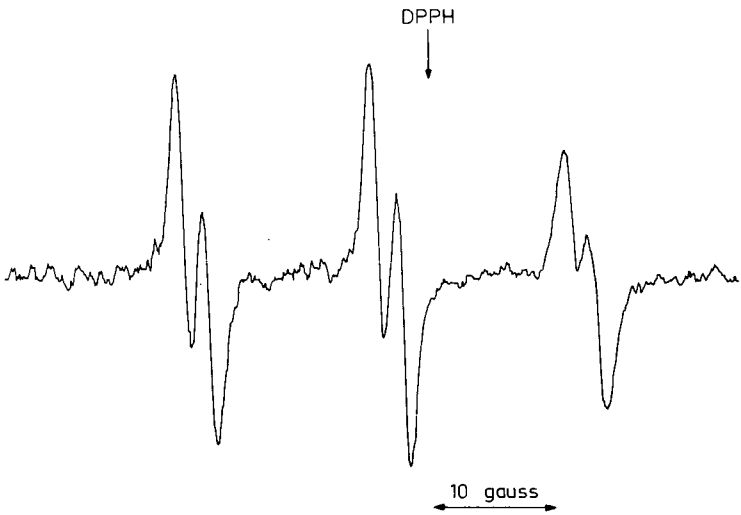


Fig. 1. ESR signal obtained from the incubation of linoleic acid, soybean lipoxygenase and 2-methyl-2-nitrosopropanol. DPPH, diphenylpicrylhydrazyl.

sponds with the *g*-values of di-*t*-butylnitroxides (2.0055–2.0062)[10]. The incubation under these conditions can be regarded to be anaerobic within a few seconds as was demonstrated by absorption at 285 nm which occurred immediately after mixing of the reactants. During the rapid depletion of the dissolved oxygen (about 0.24 μmole/ml) the hydroperoxy linoleic acid, which is required for the reaction, is formed.

To characterise the type of the trapped radicals 0.5 ml of 7.2 mM *cis,cis*-9,12-[11,11-^{2}H]octadecadienoic acid in sodium borate buffer was incubated with 0.3 ml of the lipoxygenase solution. The ESR spectrum of this reaction mixture is shown in Fig. 2. From the spectrum it can be concluded that the same type of radical had been trapped as in the first experiment.

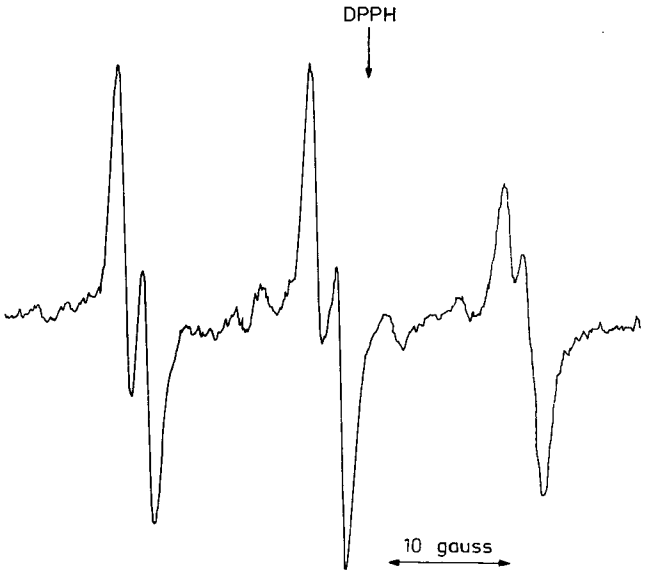


Fig. 2. ESR signal obtained from the incubation of *cis,cis*-9-12-[11,11-^{2}H]octadecadienoic acid, soybean lipoxygenase and 2-methyl-2-nitrosopropanol. DPPH, diphenylpicrylhydrazyl.

Subsequently 7.2 mM *cis,cis*-9,12-[9,10,11,11,12,13-^{2}H]octadecadienoic acid was used as substrate. The resulting ESR spectrum (Fig. 3) now shows only three lines with hfs constants of 16.0 G, indicating that the β-hydrogen that was responsible for the hyperfine splitting in the first two spectra was now replaced by a deuterium atom. This excludes the possibility that an enzyme radical was trapped, because in that case the ESR spectrum should have been identical in all three experiments.

To decide whether the radical stems from linoleic acid or from the hydroperoxide, the spectrum was recorded from a strictly anaerobic incubation of a mixture of hexadeuterolinoleic acid and preformed hydroperoxides, which were prepared from unlabelled linoleic acid. To this end 0.25 ml of 7.2 mM hydroperoxylinoleic acid (mainly consisting of the 13-L-isomer) in sodium borate buffer, which was freed of oxygen and flushed with nitrogen, was mixed with 0.4 ml of the deoxygenated lipoxygenase solution. After a few seconds 0.25 ml of 7.2 mM *cis,cis*-9,12-[9,10,11,11,12,13-^{2}H]octadecadienoic acid in the same deoxygenated buffer was added, followed immediately by the addition of about 5 mg of the spin trap. The resulting spectrum is

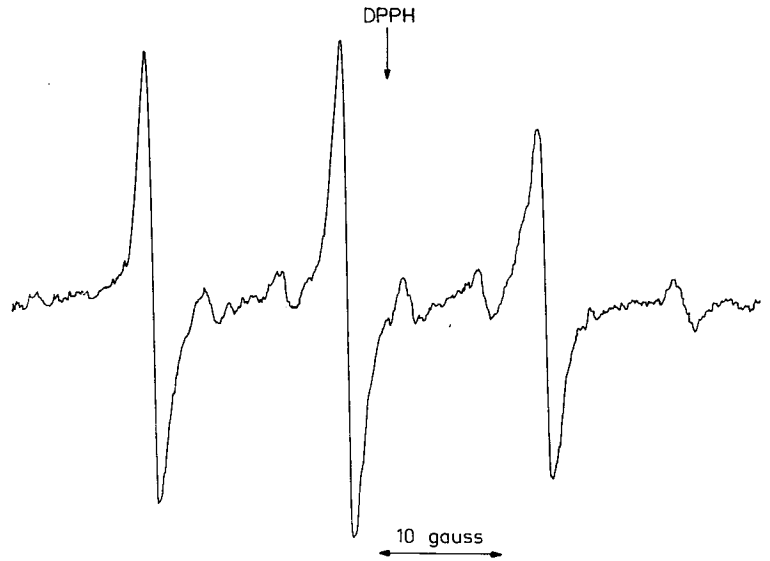


Fig. 3. ESR signal obtained from the incubation of *cis,cis*-9,12-[9,10,11,11,12,13-^{2}H]octadecadienoic acid, soybean lipoxygenase and 2-methyl-2-nitrosopropanol. DPPH, diphenylpicrylhydrazyl.

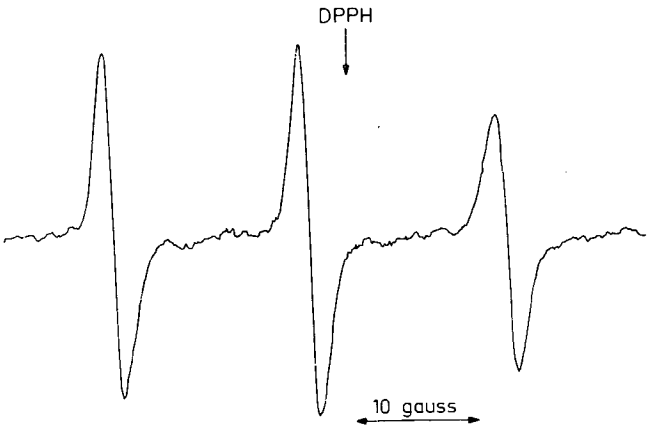


Fig. 4. ESR signal obtained from the incubation of *cis,cis*-9,12-[9,10,11,11,12,13-^{2}H]octadecadienoic acid, hydroperoxy linoleic acid, soybean lipoxygenase and 2-methyl-2-nitrosopropanol. DPPH, diphenylpicrylhydrazyl.

given in Fig. 4. This proves that the trapped fatty acid radical stems from the linoleic acid and not from the hydroperoxide. Moreover it can be concluded that the radical scavenger had mainly reacted at position 13 and/or 9 of the linoleic acid radical.

This conclusion was corroborated by an experiment with 0.5 ml of 3.6 mM *cis,cis*-9,12-[9,10,12,13-^{2}H]octadecadienoic acid. The resulting ESR spectrum is identical to the spectra in Figs 3 and 4, indicating that the nitroso compound has indeed reacted with a linoleic acid radical at C-13 and/or C-9. The additional ESR-signals in Figs 2 and 3 are due to minor impurities in the spin trap and the intensities of these peaks vary in different experiments.

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