notes on methodology

Reaction of linoleic acid hydroperoxide with thiobarbituric acid

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Summary The linoleic acid hydroperoxide obtained by enzymatic peroxidation of linoleic acid was found to react with thiobarbituric acid to yield a red pigment. The optimum pH for the reaction was found to be 4.0. In the early stages of peroxidation of linoleic acid, thiobarbituric acid value, the amount of conjugated diene, oxygen consumption, and peroxide value were in parallel with one another. The data were compared with those on peroxidation of linolenic acid and arachidonic acid.

Supplementary key words lipid peroxide · linolenic acid hydroperoxide · arachidonic acid hydroperoxide

Linoleic acid is an essential fatty acid and can be converted in the animal to arachidonic acid which is the precursor of prostaglandins. It has been claimed by Holman and Greenberg (1, 2) and Anderson et al. (3) that linoleic acid peroxide has pathological consequences. Lipid peroxidation in living cells is associated with serious damage to essential structural proteins and enzymes (4–6). Accordingly, metabolic pathways can be altered significantly by lipid peroxidation or by the products resulting from it (6). It has been shown that in the process of lipid peroxidation, the amount of linoleic acid as well as other polyunsaturated fatty acids existing in certain phospholipids decreases concomitantly in vivo (7) and in vitro (8, 9).

Since Bernheim, Bernheim, and Wilbur (10) and Wilbur, Bernheim, and Shapiro (11) found that the oxidation products of unsaturated fatty acids reacted with TBA to form a red-colored compound, the reaction with TBA has been widely adopted as a sensitive assay method for lipid peroxidation in animal tissues. Later, Kenaston et al. (12) reported that this method is sensitive and precise in determining peroxides of linoleic acid, linolenic acid, and arachidonic acid. Tarladgis and Watts (13) also reported the reactivity of linoleic acid peroxide with TBA. On the other hand, Dahle, Hill, and Holman (14) reported the failure of reaction of linoleic acid peroxide with TBA in relation to the mechanism of TBA reaction. Pryor, Stanley and Blair (15) reported a similar result. These discrepant results led us to examine the conditions for the reaction of linoleic acid hydroperoxide with TBA. The present report describes clear evidence that linoleic acid hydroperoxide obtained by a lipoxygenase system reacts with TBA. The stoichiometry of this reaction is described in comparison with reactions of linolenic acid and arachidonic acid hydroperoxides.

Experimental procedures

Materials. Linoleic acid and linolenic acid were purchased from P-L Biochemicals Inc., Milwaukee, WI, and arachidonic acid from Sigma Chemical Co., St. Louis, MO. Samples of fatty acids were analyzed by gas-liquid chromatography to check their purity. The purities of linoleic acid and linolenic acid were approximately 99%, and that of arachidonic acid was about 92% (contaminated with approximately 8% of linolenic acid). TBA was obtained from BDH Chemicals Ltd., Poole, England; TMP from Tokyo Kasei Kogyo Ltd., Tokyo; lipoxygenase from Miles Laboratories Ltd., Stoke Poges, England; and cumene hydroperoxide from Nakarai Chemicals, Kyoto. All other chemicals were of reagent grade and were used without further purification. Thin-layer chromatography plates (Kieselgel 60) were purchased from E. Merck, Darmstadt.

Enzymatic peroxidation of unsaturated fatty acids. Each acid (0.1 g) was dissolved in 30 ml of ethanol with shaking and diluted with distilled water to 50 ml (stock solution). Immediately before use, 20 ml of the stock solution was diluted with a mixture of 20 ml of 0.2 M borate buffer (pH 9.0) and 40 ml of distilled water; 1.5 ml of the diluted solution, which contained 0.75 mg of the acid, was then mixed with 3.75 ml of distilled water and 15–40 μl of lipoxygenase solution (1 mg/ml of 0.2 M borate buffer, pH 9.0; 117,000 U/mg) and incubated at 23°C. Peroxidation was followed by measuring oxygen consumption in a Beckman oxygen sensor.

Assay for conjugated diene. After a definite period of oxygen consumption, the reaction mixture (0.3 ml) was diluted with 5.0 ml of 80% ethanol, and absorbance at 233 nm was determined. Concentration of conjugated diene was calculated on the basis of a

Abbreviations: TBA, thiobarbituric acid; TMP, 1,1,3,3-tetramethoxypropane; POV, peroxide value.

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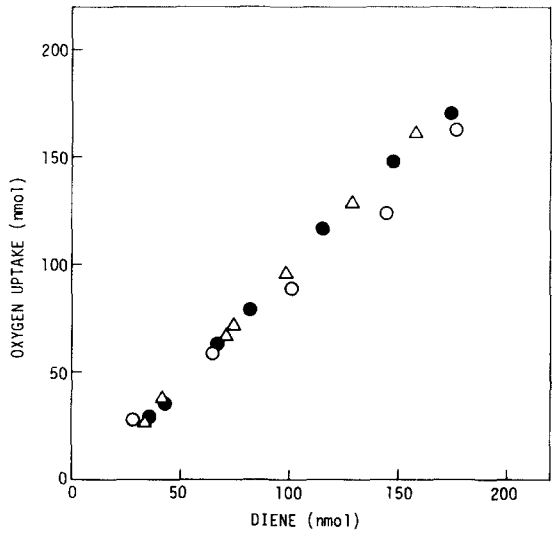

Fig. 1. Relationship between conjugated diene and oxygen uptake during enzymatic peroxidation of unsaturated fatty acids. The peroxidation of unsaturated fatty acids was performed enzymatically with lipoxygenase as described in Experimental Procedures. Open circle, linoleic acid; closed circle, linolenic acid; open triangle, arachidonic acid.

molar absorptivity ϵ of 27.4×10^3 (ref. 16), and the value was corrected for the absorbance at zero time of the oxygen consumption.

Iodometric determination of hydroperoxides. Unsaturated fatty acid hydroperoxides were also determined by a method based on that of Swoboda and Lea (17). Aliquots (2 ml) of the enzymatic reaction mixture were acidified with glacial acetic acid and mixed with 5 ml of the mixture of chloroform and methanol 2:1 (v/v). The mixture was shaken and centrifuged. To the chloroform layer (3 ml), 0.5 ml of glacial acetic acid was added, and N_2 gas was bubbled through the mixture for 1 min. KI solution (0.2 ml) (1.2 g/ml distilled water, freshly prepared) was added. The mixture was left in the dark at room temperature for 30 min. Then 5 ml of 0.5% cadmium acetate was added, and the mixture was shaken and centrifuged. Absorbance at 350 nm of the aqueous layer was measured. POV was calculated using cumene hydroperoxide as standard and expressed as POV, nmol equivalents to the standard. This method can be adopted to determine the amounts of hydroperoxide in the range of 30 nmol to 400 nmol equivalents to cumene hydroperoxide.

Thin-layer chromatography of hydroperoxides. To check the reactivity of pure lipid peroxide with TBA, unsaturated fatty acid hydroperoxide obtained by lipoxygenase reaction was subjected to thin-layer chromatography. The pH of an aliquot (5 ml) containing about 1 μmol of conjugated diene was adjusted to 4.0 with glacial acetic acid and the aliquot was shaken with chloroform–methanol 2:1 (v/v). After centrifugation, the organic layer was evaporated to dryness and the residue was treated with diazomethane in an ice bath for 30 min. The ester of hydroperoxide was dried, dissolved into a minimal amount of hexane, and subjected to thin-layer chromatography at 7°C. The solvent system was hexane–diethyl ether 8:2 (v/v). After development, the plate was divided into four parts and treated with the following four procedures: *1*) charring with 50% sulfuric acid; *2*) spraying with KI solution (1 ml of KI saturated aqueous solution + 30 ml of ethanol–glacial acetic acid–chloroform 5:3:2 (v/v)) for the detection of hydroperoxide; *3*) spraying with 0.4% 2,4-dinitrophenyl hydrazine in 2 N HCl for detection of keto compounds; and *4*) the zones detected by procedure 1 were scraped off and eluted with diethyl ether. The residues after evaporation were dissolved in 3 ml of ethanol and were subjected to the assay of conjugated diene and to TBA reaction.

Results and discussion

Unsaturated fatty acid hydroperoxide was prepared by using the lipoxygenase system as described in Experimental Procedures. **Fig. 1** shows the relationship between the degree of peroxidation of unsaturated fatty acid and oxygen consumption. The extent of peroxidation was expressed as the amount of conjugated diene measured at 233 nm. As can be seen from the figure, the ratio of conjugated diene to oxygen uptake was 1 for linoleic acid as well as for linolenic acid and arachidonic acid. The linear correlation indicates the stoichiometric peroxidation of these fatty acids by lipoxygenase, which is in accord with those of many previous reports claiming the currently accepted mechanism of this enzymatic reaction. Accordingly, the following experiments were performed by using these preparations which contain the desired amounts of hydroperoxides.

Linoleic acid hydroperoxide prepared as described was subjected to TBA reaction under our previously reported conditions (18, 19): a 4.0-ml aliquot of the diluted sample, which contained ca. 150 nmol of conjugated diene, was mixed with 1.0 ml of TBA reagent (mixture of equal volumes of 0.67% TBA and glacial acetic acid), and heated at 95°C for 60 min. The reaction mixture turned red, and the absorption spectrum of the mixture (**Fig. 2**) was the same as those of the other higher unsaturated fatty acid hydroperoxides.

As described above, discrepant results have been reported on the reactivity of linoleic acid hydroperoxide with TBA. Dahle et al. (14) claimed that the low TBA color observed by Wilbur et al. (11) and Kenaston et al. (12) for oxidized linoleic acid could

be attributed to the contamination of linolenic acid in their samples. To check this point, the present sample of linoleic acid hydroperoxide prepared enzymatically was subjected to TBA reaction under the conditions adopted by Dahle et al. (14). The reaction of our preparation with their reagent containing trichloroacetic acid gave no color (pH of the reaction mixture, 1.1).

From the facts mentioned above, it was suspected that the pH of the reaction mixture is a factor that affects the reactivity of linoleic acid hydroperoxide with TBA to a great extent. To check this point, the effect of pH on the reaction rate was examined. Reaction mixtures containing 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution of various pH values adjusted with NaOH (in the range of pH 1.0–2.5, 20% acetic acid solution contained 0.27 M HCl), 1.5 ml of 0.8% aqueous solution of TBA, and an appropriate amount of lipid peroxide were examined. Each mixture was finally made up to 4.0 ml with distilled water and was heated at 95°C for 60 min. The results are shown in **Fig. 3.** In the case of linoleic acid hydroperoxide, the optimal pH of the reaction was found to be 4.0, and the reactivity decreased sharply with a decrease in pH. The pH profiles of the reactions of linolenic acid and arachidonic acid hydroperoxide were different from that of linoleic acid hydroperoxide and their optimal pH values were around 3.0–3.5. The reactivity of the standard, TMP, scarcely changed between pH 1.0 and 4.5 (Fig. 3, insert).

From these results, the disagreement on the reactivity of linoleic acid hydroperoxide with TBA among

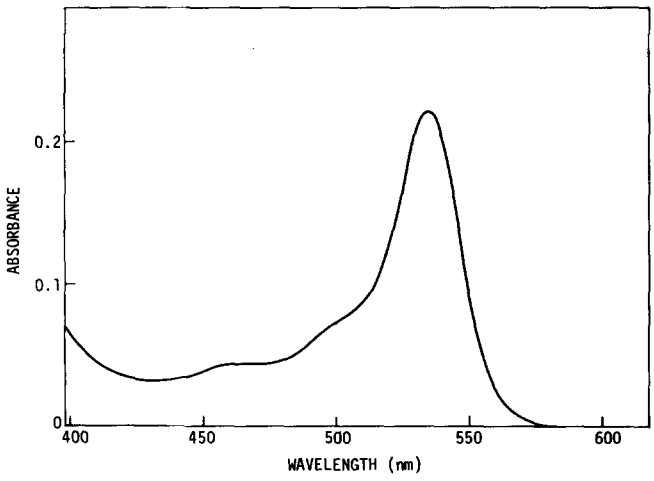

Fig. 2. Absorption spectrum of the red pigment produced by the reaction of linoleic acid hydroperoxide with TBA. The hydroperoxide prepared enzymatically was used. TBA reaction was carried out in a mixture consisting of 1.0 ml of TBA reagent (mixture of equal volumes of 0.67% TBA and glacial acetic acid) and 4.0 ml of the diluted sample (ca. 150 nmol hydroperoxide) at 95°C for 60 min.

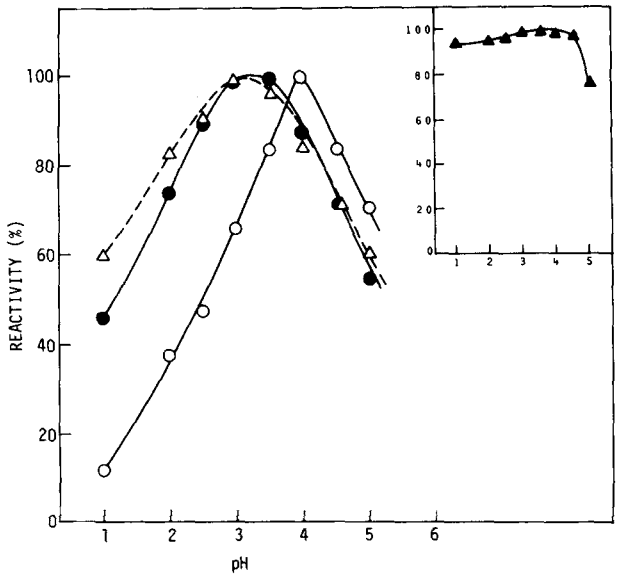

Fig. 3. pH profile of the reaction of unsaturated fatty acid hydroperoxides with TBA. See text for details. Open circle, linoleic acid hydroperoxide; closed circle, linolenic acid hydroperoxide; open triangle, arachidonic acid hydroperoxide; closed triangle, TMP.

the data of different authors was considered to be ascribed to the differences in pH of the reaction mixtures. In the following reaction, therefore, the pH value of the reaction mixture was adjusted to 4.0 by using acetic acid (in final, 7.5%)–NaOH solution.

Fig. 4 shows the time course of the reaction. To reach the maximum, heating at 95°C in an oil bath for 60 min was required in all cases examined.

Taking these results into account, the reaction conditions were finally set up as follows. To the sample containing hydroperoxides (0.3 ml) were added 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution adjusted to pH 4.0 with NaOH, and 1.5 ml of 0.8% aqueous solution of TBA. The mixture was made up to 4.0 ml with distilled water, and was heated in an oil bath at 95°C for 60 min. After cooling with tap water, absorbance at 532 nm was measured. TMP was used as an external standard and the TBA values were expressed as nmol of malondialdehyde.

Under these conditions, the reaction products obtained from linoleic acid, linolenic acid, and arachidonic acid hydroperoxide have the same absorption spectrum. The absorption spectrum of the product obtained from TMP was in good agreement with that reported by Sinnhuber, Yu, and Yu (20), who used 1,1,3,3-tetraethoxypropane. The spectrum of the product obtained from these lipid peroxides is identical to that obtained from TMP except for one slight difference; the former had a slight absorption band around 450 nm. The molecular extinction coefficient

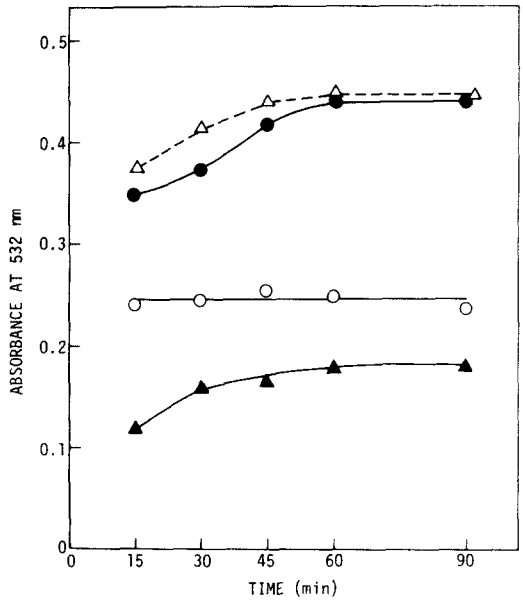

Fig. 4. Time course of the reaction of unsaturated fatty acid hydroperoxides with TBA. Reaction conditions were the same as those for Fig. 3, except that the reaction pH was fixed at 4.0 and that the reaction time was changed as shown in the figure. The reaction was followed by the value of absorbance at 532 nm. Open circle, linoleic acid hydroperoxide; closed circle, linolenic acid hydroperoxide; open triangle, arachidonic acid hydroperoxide; closed triangle, TMP.

at 532 nm of the chromogen from TMP in this system was calculated to be 1.48×10^5.

The enzymatic peroxidation mixture of linoleic acid was subjected to thin-layer chromatography. Nonreacted linoleic acid (R_f 0.71), linoleic acid hydroperoxide (R_f 0.22), and a minor compound (R_f 0.13) were detected upon charring with 50% sulfuric acid. Among the compounds detected, only linoleic acid hydroperoxide reacted with TBA to yield red pigment, absorbed at 233 nm, indicating the occurrence of conjugated diene, and was not positive to the reaction with 2,4-dinitrophenyl hydrazine. The minor product did not react with 2,4-dinitrophenyl hydrazine and TBA and seems to be the polymeric oxidation product described by Vioque and Holman (21). The same results were also obtained in the cases of linolenic acid and arachidonic acid hydroperoxide. These data show that the substances reacted with TBA in our system are unsaturated fatty acid hydroperoxides.

The relationship between the amount of conjugated diene and TBA value expressed in terms of nmol of malondialdehyde is shown in **Fig. 5.** The linear correlation between them is clearly observed in these three fatty acid hydroperoxides. However, the relation was not equimolar, which coincides with the data reported by Dahle et al. (14). The molar ratio of TBA value to conjugated diene was 5/100 for linoleic acid hydroperoxide. For linolenic acid and arachidonic acid hydroperoxide, the molar ratio was 10/100. The linear relationship was also observed between TBA value and oxygen uptake for the three fatty acids.

The concentration of hydroperoxide formed during the peroxidation by the enzymatic reaction system was also determined by an iodometric method. The linear relationship between POV and the amount of conjugated diene and that between POV and TBA value were observed in the process of peroxidation of linoleic acid as well as linolenic acid and arachidonic acid.

From the present data, it is clear that linoleic acid hydroperoxide reacts with TBA to give a red pigment. The pigment formed is identical with those formed from linolenic acid and arachidonic acid hydroperoxide. As to the reaction of linolenic acid and arachidonic acid hydroperoxide with TBA, the reaction mechanism involving a bicyclic endoperoxide as an intermediate was proposed (15). However, this mechanism does not seem to be the case for linoleic acid hydroperoxide for the following reasons. Upon heating linoleic acid hydroperoxide under the above experimental condition in the absence of TBA reagent, POV decreased with the decrease in TBA value, while in the cases of linolenic acid hydroperoxide and arachidonic acid hydroperoxide, their POV decreased but their TBA values were essentially unchanged. Accordingly, a mechanism different from that of linolenic

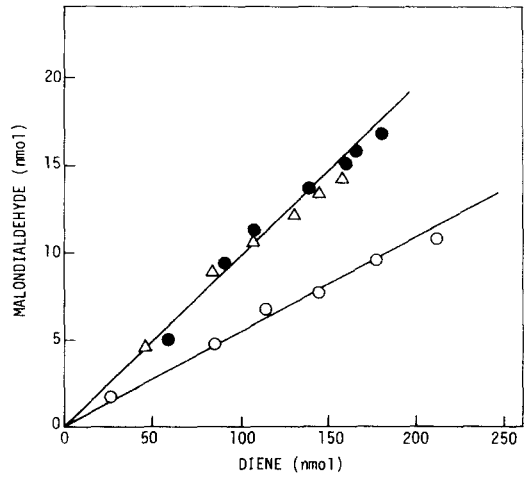

Fig. 5. Relationship between conjugated diene and TBA value during enzymatic peroxidation of unsaturated fatty acids. Reaction conditions were the same as those for Fig. 3, except that the reaction pH was fixed at 4.0. The amount of conjugated diene was calculated from the absorbance at 233 nm. TBA value was calculated from the absorbance at 532 nm of the product of TBA reaction using TMP as an external standard, and expressed in terms of nmol of malondialdehyde. Open circle, linoleic acid hydroperoxide; closed circle, linolenic acid hydroperoxide; open triangle, arachidonic acid hydroperoxide.

acid and arachidonic acid hydroperoxide should be considered for the reaction of linoleic acid hydroperoxide. Linoleic acid hydroperoxide seems to react directly with TBA to produce a red pigment.

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