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Effect of L-Ascorbic Acid and its Related Derivatives on the Peroxidation of Linoleic Acid in Neutral Buffer Solution

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Abstract

Catalysis of the peroxidation of linoleic acid (LA) by L-ascorbic acid (AsA) and some of its derivatives was studied by examination of the formation of conjugated dienes in a neutral buffer containing alcohol. The 3, 4-endiol form of 2, 3-diketo-L-gulono-δ-lactone, arising from 2, 3-diketo-L-gulonic acid (DKG), was found in the reaction mixtures when the concentration of DKG was high (4 mM). This product suppressed LA peroxidation, as AsA does. 2-0-Octadecyl-AsA also suppressed LA peroxidation. With 0.1% sodium dodecyl sulphate in the reaction mixture, the degree of LA peroxidation was intermediate to that with 10% EtOH and that with 20%. The addition of Fe^{++} or Cu^{++} accelerated LA peroxidation. Both EDTA and deferoxamine mesylate suppressed the LA peroxidation catalyzed by AsA, but in their presence, dehydro-L-ascorbic acid had no effective suppression.

Introduction

L-Ascorbic acid (AsA) is important in the synthesis of collagen,[1] and also in oxidation and reduction systems *in vivo*,[2] but we do not know much about the effects on linoleic acid (LA) peroxidation caused by degraded products of AsA or by its derivatives.[3] From the chemical structure of AsA, a rather strong reducing capacity can be predicted, so it is used as an antioxidant in foods. In fact, AsA suppresses LA peroxidation in the initial stage of its effects, but then starts to accelerate it.[4] This suggests that AsA might readily be degraded or disappear in a neutral reaction mixture, and also that dehydro-L-ascorbic acid (DHA) or further degraded species may be formed, which might accelerate LA peroxidation. EtOH scavenges active oxygen radicals,[5] so we tried here to use surface-active agents instead of EtOH to disperse LA somewhat in an aqueous medium. We also studied the effects of a 2-0-alkyl derivative of AsA[6] on LA peroxidation, compared with other catalysts. This paper also deals with a substance with reducing power formed from 2, 3-diketo-L-gulonic acid (DKG), namely, the 3, 4-endiol form of 2, 3-diketo-L-gulono-δ-lactone, which suppresses LA peroxidation.

Materials and Methods

Chemicals. LA and methyl linoleate (cis-linoleic acid methyl ester) were purchased from Sigma Chemical Co., and diluted to 100 mM with freshly distilled EtOH under N_2 bubbling, and stored at −20°C until use. DHA and DKG were prepared from AsA as reported previously.[4,7] 6-0-Palmitoyl ascorbic acid (6-0-Pal-AsA) was obtained from Nacalai Tesque. 2-0-Octadecyl-ascorbic acid (2-0-OD-AsA)[6] was the gift of Dr. T. Terao, Central Research Division, Takeda Chemical Industries, Ltd. AsA was purchased from Wako Pure

Chemical Industries, Ltd. Decaglyceryl monolaurate (ML-750) was obtained from Sakamoto Pharmaceutical Co., Ltd. The other reagents used were of analytical grade. For the preparation of the buffer, twice-distilled water was used. The buffer prepared was passed through a column of Chelex as reported previously.[7)]

Analytical methods

LA peroxidation was assayed as reported before.[3,4,7)] To prepare 10 mM LA containing 20% EtOH, we mixed 0.5 ml of 100 mM LA, 0.5 ml of EtOH, 4 ml of 0.1 M phosphate buffer (pH 6.8), and 0.1 ml of an aqueous solution of AsA or one of its oxidized products. The mixture was incubated at 37°C with shaking. A portion (0.3 ml) of the reaction mixture was withdrawn at times, and 3 ml of a 60% aqueous solution of EtOH containing 200 ppm of EDTA was added to the solution. The UV absorption spectrum was recorded over the range of 225–300 nm with a Hitachi Spectrophotometer model 200–10. The responses at 233 and 265 nm were used as the amounts of LA hydroperoxide formed and of AsA or the catalyst remaining, respectively.

Results and Discussion

Suppression by 2-0-OD-AsA of LA peroxidation

The effects on LA peroxidation of two lipophilic derivatives of AsA, 2-0-OD-AsA

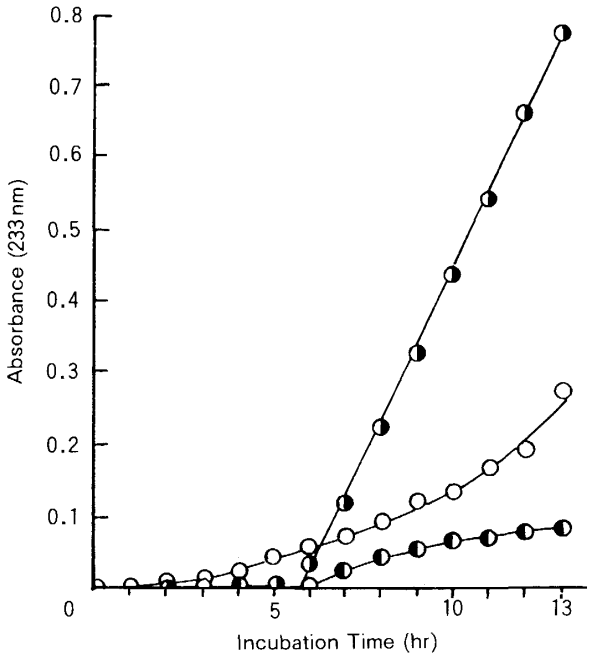

Fig. 1 Peroxidation of Linoleic Acid (LA) Catalyzed by 2-0-Octadecyl-Ascorbic Acid and 6-0-Palmitoyl-Ascorbic Acid
The reaction mixtures contained 10 mM LA. 135 μM of 2-0-octadecyl-ascorbic acid (◐) or 6-0-palmitoyl-ascorbic acid (◑), and 20% EtOH in 80 mM phosphate buffer (pH 6.8), and were incubated in test tubes with shaking. A portion (0.3 ml) of the reaction mixture was withdrawn and added to 3 ml of 60% EtOH solution containing EDTA (200 ppm), and the UV absorption spectrum of the solution was measured. Absorbance at 233 nm is shown. (○), Control values without a catalyst.

and 6-0-Pal-AsA, were tested. Both 2-0-OD-AsA and 6-0-Pal-AsA suppressed the peroxidation for 6 and 5 hr, respectively. 2-0-OD-AsA suppressed more the peroxidation than 6-0-Pal-AsA did (Fig. 1).[8] After the suppression ended, acceleration began. The rate of peroxidation caused by 2-0-OD-AsA was slower than that caused by 6-0-Pal-AsA. These results suggested that the 2-0-alkyl residue of AsA resists hydrolysis, and also that the monodehydro-AsA formed in the reaction mixture was fairly stable.

Effect of DKG at relatively high concentrations on LA peroxidation

A degraded product of AsA, DKG at 1 ~ 4 mM, was studied for its effects on LA peroxidation.[9] At a low concentration of DKG (1 mM), peroxidation was accelerated (Fig. 2A). At higher concentrations of DKG, the slope of autoxidation became less steep,

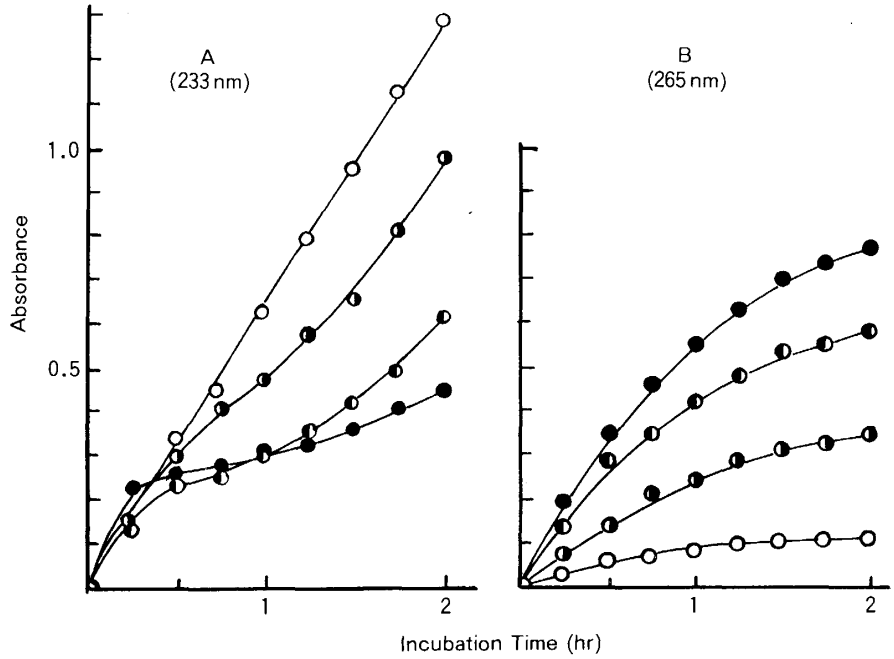

Fig. 2 Effects of 2, 3-Diketo-L-gulonic Acid (DKG) Concentration on LA Peroxidation
Experimental conditions were the same as in the legend of Fig. 1, except for the catalyst. **A** shows the absorbance at 233 nm; **B** shows absorbance at 265 nm. Concn. of DKG: —○—, 1 mM; —◑—, 2 mM; —◑—, 3 mM; —●—, 4 mM.

and a reducing species with absorbance at 265 nm was formed in small amounts. The slope was least steep with 4 mM DKG. These phenomena did not occur with DHA, so the hydrolysis product of DHA, *i.e.* DKG, probably was converted to the 3, 4-endiol form of the δ-lactone ring of 2, 3-diketo-L-gulonic acid under the reaction conditions used, as reported before.[10] The results suggest that during the reaction, the 3, 4-endiol form of 2, 3-DKG arising from DKG suppresses LA peroxidation, but that neither DHA nor DKG itself does so. The suppression of LA peroxidation was not strong, and the peroxidation gradually came to be accelerated. This suggests that the newly formed species was not very stable, and was further degraded.

Effect of pH on LA peroxidation accelerated with DKG

The effect of the pH of the reaction mixture on LA peroxidation was studied with

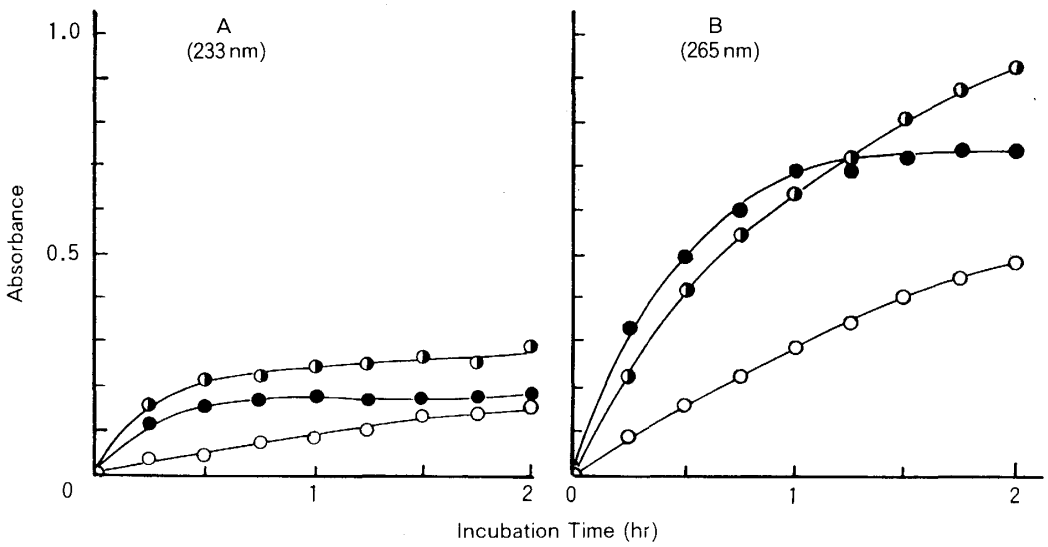

Fig. 3 Effect of pH on LA Peroxidation Catalyzed by DKG
Experimental conditions were the same as in the legend of Fig. 1, except for the pH of the buffer solutions and the catalyst used (5 mM DKG). —○—, pH 6; —◐—, pH 7; —●—, pH 8.

various buffers containing 5 mM DKG, 20% EtOH, and 10 μM LA. With both pH 7 and 8, the patterns of LA peroxidation were similar, but at pH 6, neither conjugated diene nor the reducing species was formed (Fig. 3). At pH 8, the absorbance at 265 nm reached a plateau after 1 hr of reaction. At pH 7, the amount of reducing species continued to

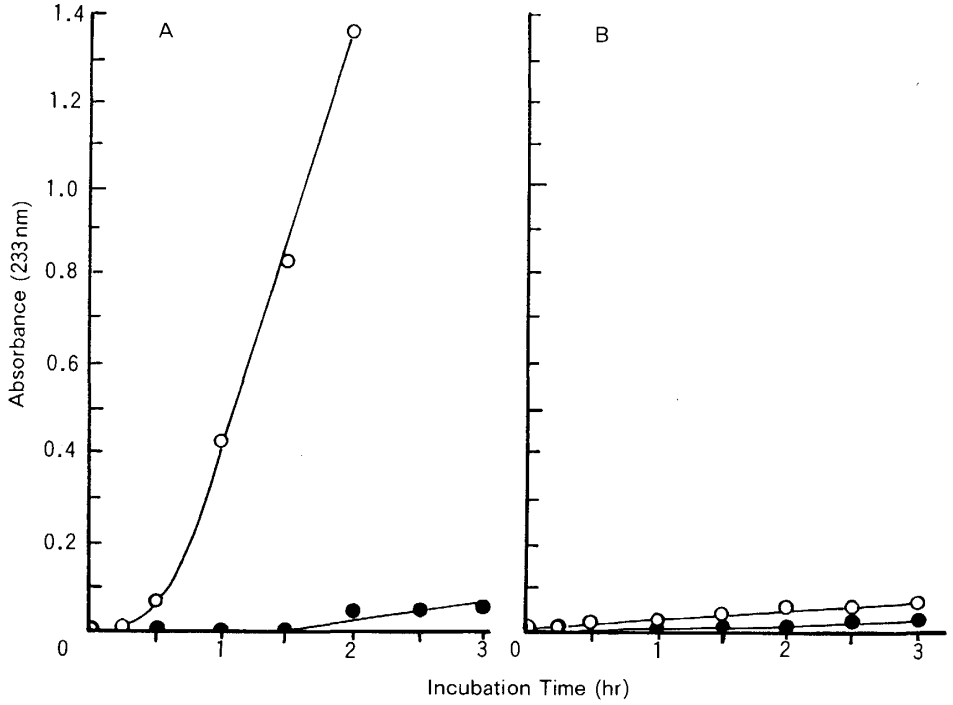

Fig. 4 Peroxidation of LA (A) or Methyl Linoleate (B) Catalyzed by AsA in Phosphate Buffer Containing EtOH
Experimental conditions were the same as in the legend of Fig. 1, except that methyl linoleate (B), and AsA were used here, and a higher concentration of EtOH was also tested. Concn. of EtOH: —○—, 20% EtOH; —●—, 30% EtOH.

increase, probably because at less basic pH, degradation was slight. This result suggested that DKG was stable in relatively acidic reaction mixtures, but unstable in alkaline solutions, and it tended to degrade easily.

Effect of AsA on the peroxidation of methyl linoleate in a neutral buffer containing alcohol

Instead of LA, methyl linoleate was used as the lipophilic material for peroxidation catalyzed by AsA in a reaction mixture containing 20% or 30% EtOH in phosphate buffer. Methyl linoleate was more resistant than LA to peroxidation at both concentrations (Fig. 4). This suggests that because methyl linoleate is more lipophilic than LA, the surface of a microemulsion with methyl linoleate would be less accessible to AsA. The higher EtOH concentration was applied to the LA peroxidation, the more the suppression was observed. This may be due to the scavenging action of EtOH, as reported before.[5)]

Effect of surface-active agents on LA peroxidation catalyzed by AsA or DHA

Two surface-active agents, sodium dodecyl sulphate (SDS) and decaglyceryl monolaurate, were tested at different concentrations for effects on LA peroxidation in reaction mixtures containing 10% or 20% EtOH, and with AsA or DHA as the catalyst. With SDS in the reaction mixture containing AsA as the catalyst, peroxidation was suppressed in the 20% EtOH solution, but accelerated in the 10% EtOH solution (data not shown). In the reaction mixture with DHA, the effects of AsA on peroxidation were the same.

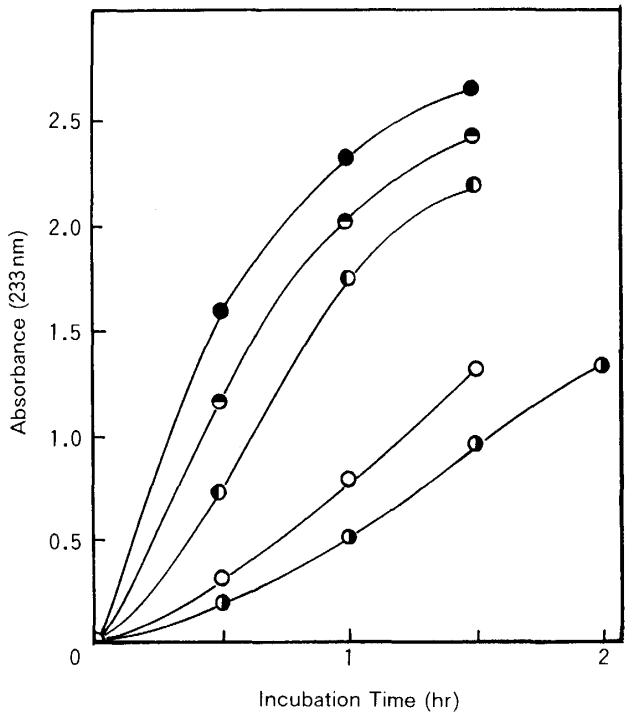

Fig. 5 Effects of Various Concentrations of SDS on LA Peroxidation Catalyzed by DHA.
Experimental conditions were the same as in the legend of Fig. 1, except for the use of DHA at the concentration of 135 μM DHA and the use here of a higher concentration of EtOH as well. The figure shows the absorbance at 233 nm. —◐—, EtOH 10%; —◑—, EtOH 20%; —○—, 0.1% SDS; —◒—, 0.2% SDS; —●—, 0.3% SDS.

Therefore, SDS did not affect LA peroxidation in the presence of alcohol, when AsA was the catalyst.

Figure 5 shows the effects of SDS on LA peroxidation catalyzed by DHA. For the test of the effect of SDS concentrations on LA peroxidation, EtOH was replaced by deionized water. To judge from the appearance of the reaction mixture (which was sometimes turbid), the effects of 0.2% SDS in the reaction mixture without EtOH was similar to that without surface-active agent but with 20% EtOH. SDS at the three concentrations used accelerated LA peroxidation. SDS at 0.3% accelerated the peroxidation more strongly than at 0.2%. This may be caused by an increased area of contact between the emulsion particles and AsA in the smaller particles of LA formed in the 0.3% solution. At 0.1% SDS, the acceleration of LA peroxidation was least of the three concentrations used. The 0.1% SDS may form comparatively large micelles in the reaction mixture with less contact area. The decaglyceryl monolaurate was also tested at concentrations of from 0.02% to 0.2% as described above, but results suggesting the formation of a microemulsion were not obtained. In the same way, the other surface active agents tried, including Tween 80, Span 80, and Triton X-100, did not result in satisfactorily.

Effect of metal ions and chelating agents on LA peroxidation catalyzed by AsA or DHA[11,12]

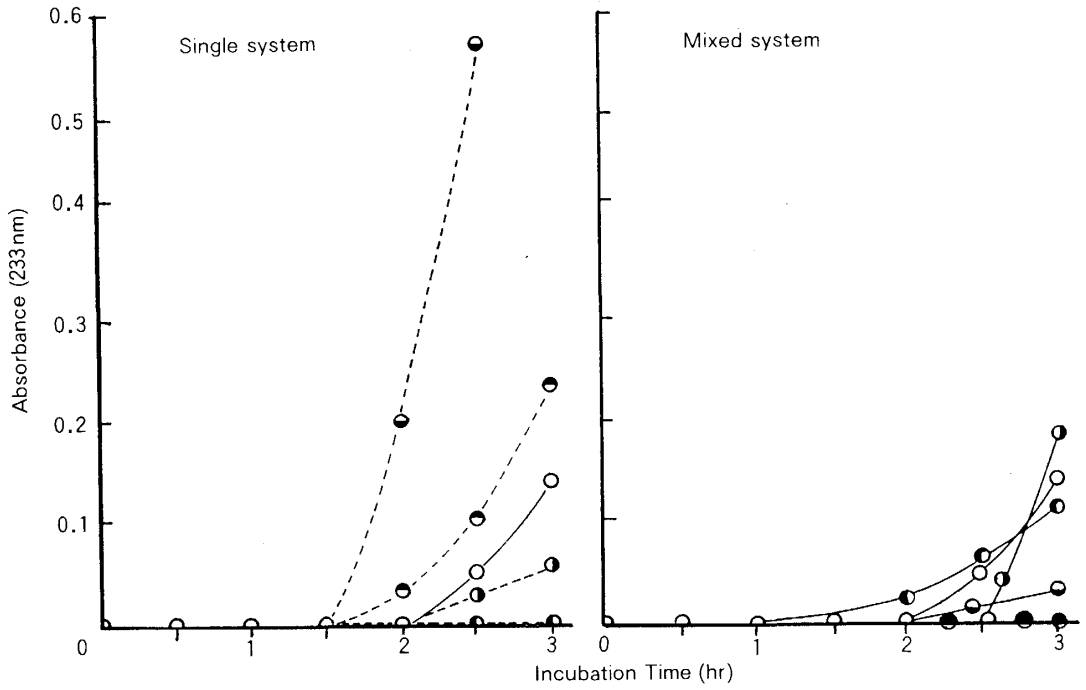

Fig. 6 Effects of Metal Ions and Chelating Agents on LA Peroxidation Catalyzed by AsA
Experimental conditions were the same as in the legend of Fig. 1, except that metal ions (10 μM) or chelating agents (50 μM) or both were used for the reaction and that AsA (135 μM) was the catalyst. —○—, control (135 μM AsA); ——●——, Fe^{++} added; ——◒——, Cu^{++} added; ——◐——, EDTA added; ——◑——, deferoxamine mesylate; —●—, Fe^{++} and EDTA added; —◒—, Cu^{++} and EDTA added; —◐—, Fe^{++} and deferoxamine mesylate added; —◑—, Cu^{++} and deferoxamine mesylate added.

Both metal ions used, Fe^{++} and Cu^{++}, could accelerate LA peroxidation catalyzed by AsA (Fig. 6). Peroxidation was first detected at 90 min after the start of the reaction, and the rate of peroxidation was faster with Cu^{++} than with Fe^{++} in the concentration used. When EDTA was added to the reaction mixture containing AsA, no peroxidation was detected during 3-hr incubation period. When deferoxamine mesylate was added to the reaction mixture instead of EDTA, the degree of peroxidation was between that found when only AsA was added and that found when both AsA and EDTA were added.

Deferoxamine mesylate did not efficiently chelate cupric ions, so the differences in the peroxidation between AsA with deferoxamine mesylate and AsA with EDTA might be due to the Cu^{++} in the reaction mixture.[13)]

In the right half of Fig. 6, EDTA or deferoxamine mesylate was added to the reaction mixture containing AsA and cupric sulphate or ferrous sulphate. The rate of peroxidation was suppressed; with EDTA, the peroxidation was delayed or completely prevented, and even in the case where the peroxidation occurred, the degree of peroxidation was slight. Figure 7 shows the effects of these metal ions and chelating agents on LA peroxidation catalyzed by DHA instead of AsA. Peroxidation started faster and was greater than with AsA. Here also, LA peroxidation seemed to be suppressed most with EDTA. When Fe^{++} or Cu^{++} was added to the mixture with DHA and EDTA, LA peroxidation was accelerated from the start of the reaction (Fig 7A). The rate of peroxidation with DHA, Cu^{++}, and EDTA all present was faster than that with DHA, Fe^{++}, and EDTA present (Fig. 7B); these results with Cu^{++} were close to the control values, with DHA only added. When EDTA was replaced by deferoxamine mesylate, suppression was not found, but only acceleration. The rate of peroxidation with Cu^{++} was faster than that with Fe^{++}. When DHA was the catalyst, no distinct effect of deferoxamine mesylate could be followed. When AsA was the catalyst for the reaction, some positive effect on

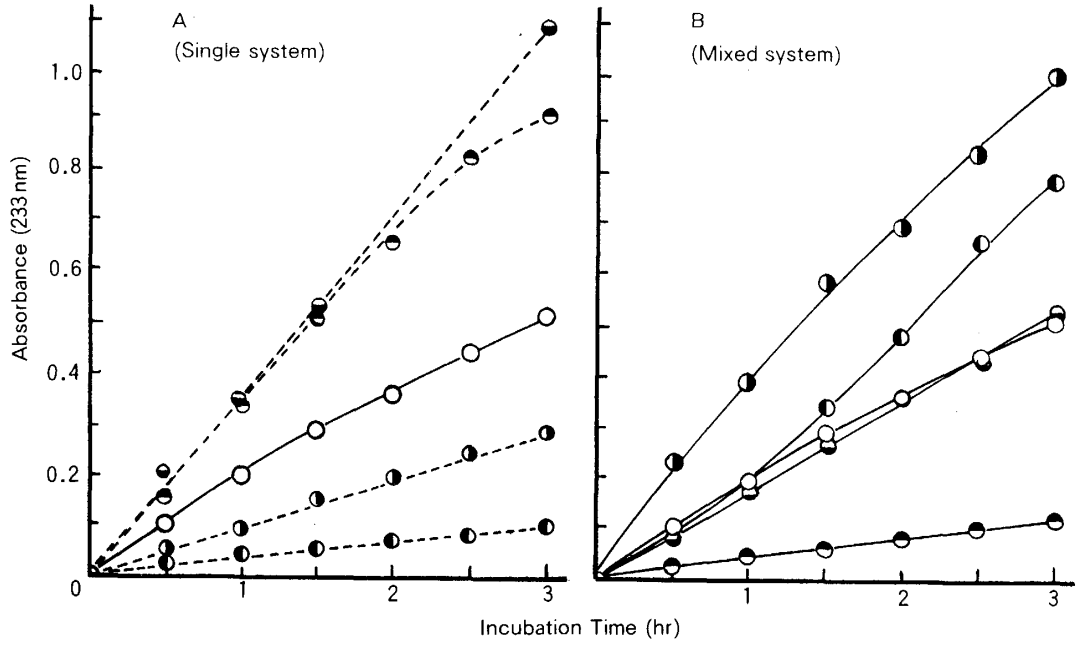

Fig. 7 Effects of Metal Ions and Chelating Agents on LA Peroxidation Catalyzed by DHA
Experimental conditions and symbols are the same as in the legend of Fig. 6, except that 135 μM DHA was used instead of AsA. Peroxidation of separate controls was assayed.

the suppression of LA peroxidation caused by deferoxamine mesylate was observed. When deferoxamine mesylate chelates metal ions, the effect is expected to be the suppression of LA peroxidation, as reported by Buettner.[13] However, in this study, the suppression caused by deferoxamine mesylate seemed to be slight. The reason is not clear.

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