Linoleic Acid Oxidation Catalyzed by Various Amino Acids and Cupric Ions in Aqueous Media

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ABSTRACT

Model systems were designed to study linoleic acid oxidation in the presence and absence of various amino acids with or without cupric ions. The tested amino acids exhibited a potential prooxidant effect in linoleic acid dispersed in aqueous media. The effectiveness of various amino acids on linoleic acid oxidation decreased in the following order: cysteine > serine > tryptophan > phenylalanine > histidine > alanine. The addition of alanine, serine, phenylalanine, histidine, or tryptophan to linoleic acid showed an autocatalytic chain reaction. With cysteine, there was a linear relation between concentration of hydroperoxides and time during the early stages of oxidation. The prooxidative activity of the tested amino acids in general could be attributed to the presence of the α-amino group in the form $H_3\overset{+}{N}$-R. The apparent difference in the prooxidative activity is mainly due to the functional groups attached in the β-carbon atom in the amino acid molecules. The addition of cupric ions at a concentration of 10^{-5}M to linoleic acid catalyzed with various α-amino acids showed that these amino acids had no significant effect. Increasing the copper concentration from 10^{-5}M to 10^{-3}M had the following effects: a shortening of the induction period of linoleic acid catalyzed by amino acids having an aromatic side chain, no effect on the induction period but an increase in the oxidation rate during the propagation step in the model systems catalyzed by alanine and serine, and in the model system containing cysteine a linear increase in the linoleic acid oxidation from the start of the reaction.

INTRODUCTION

Polyunsaturated fatty acids, especially those with methylene-interrupted diene systems, are extremely susceptible to peroxidation and react readily with atmospheric oxygen. Today there is an increasing interest in antioxidant substances occurring in biological materials with regard to the protection of lipids against oxidation rather than the use of synthetic substances. In emulsified lipid systems with such a large number of parameters, it is not surprising that contradictory results were reported. For instance, Marcuse (1) and Sieihouiski (2) found that some amino acids are effective antioxidants in emulsion. On the other hand, Saunders et al. (3) and Jurewicz and Solmonuioz (4) reported the reverse action.

The present study was undertaken to determine in well-defined model systems the anti- or prooxidative behavior of some individual α-amino acids added to linoleic acid. Also, it seems that no attempt has been made to study the relation between the rate of linoleic acid oxidation in aqueous emulsion and the chemical structure of α-amino acids.

MATERIALS AND METHODS

Linoleic Acid

Linoleic acid, puriss grade, was obtained from Koch-Light Laboratories Ltd., Colnbrook, England. The purity of linoleic acid was checked, and the following characteristics were determined: it gave one spot by thin layer chromatography (TLC), nearly free from peroxides (peroxide no. 0.1) as estimated by the method outlined by the AOAC (5), and the digested linoleic acid solution contained Cu, Mg, Fe, Co, Ni, and Mn not more than 0.007 ppm. Small amounts of linoleic acid were pipetted into 5 ml ampoules which had been gassed with nitrogen, the ampoules were gassed again, and stored in the dark at -60 C until use. Before the beginning of any experiment, some ampoules were left in the dark at room temperature for 15 min. In this way only freshly opened ampoules were used in every experiment.

Amino Acids

Pure amino acids used in the present work were "PROLABO" grade (alanine, serine, and tryptophan) and "BDH" grade (cysteine, histidine, and phenylalanine). The amino acids which gave one spot by TLC and were practically free from heavy metals (0.006-0.008 ppm) after they had been acid digested were applied.

Other Reagents

Cupric sulfate, sulfuric and nitric acids were all "ANALAR" grade. Tween 20 and ethylene diamine tetraacetic acid disodium salt (EDTA) were "BDH" grade.

Deionized Water

Deionized water with conductivity less than 1 megohm-cm^{-1}, was used for the preparation of all aqueous solutions

TABLE I

pH Values of the Model Systems and the Ratio between the Protonated and Unprotonated Amino Nitrogen of Amino Acids

Model system Linoleic acid (10^{-2}M) + amino acid (10^{-5}M)	pH values	$\overset{+}{N}H_3$ pK	$\overset{+}{N}/\overset{..}{N}$ ratio
Linoleic acid	8.5	---	---
Linoleic acid + alanine	8.9	9.69	6:1
Linoleic acid + cysteine	6.8	8.18	24:1
Linoleic acid + histidine	8.8	9.17	2:1
Linoleic acid + serine	8.8	9.15	2:1
Linoleic acid + phenylalanine	8.7	9.13	2:1
Linoleic acid + tryptophan	9.1	9.39	2:1

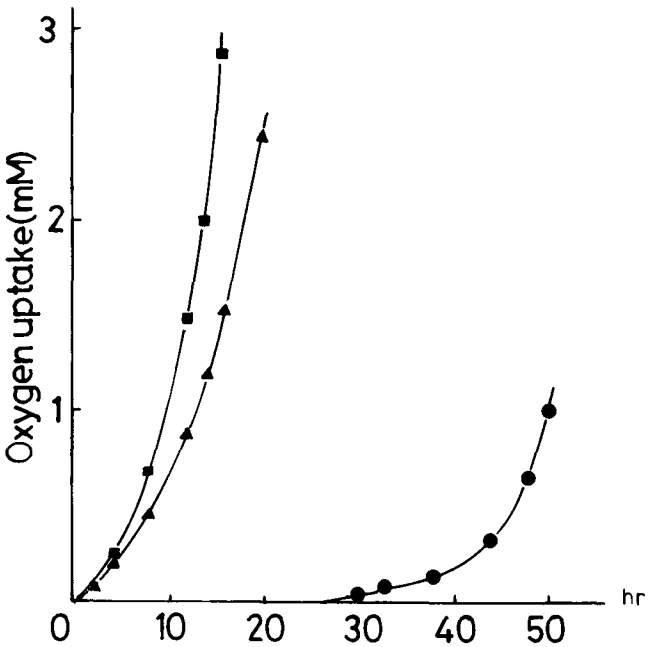


FIG. 1. The effect of cupric ions on the oxygen absorbed by aqueous emulsion of linoleic acid. ● ○ = Linoleic acid (10^{-2}M); ▲ △ = linoleic acid (10^{-2}M) + Cu (10^{-5}M); ■ □ = linoleic acid (10^{-2}M) + Cu (10^{-3}M).

and for the cleaning of all glassware.

Apparatus

Trace metal analyses were performed using a Pye Unicam model Sp 1900 atomic absorption spectrophotometer, and pH values of the linoleic acid emulsion catalyzed by various amino acids were measured using a Beckman (Fullerton, CA) apparatus. Warburg manometric apparatus was used for measurement of oxygen uptake.

Prevention of Contamination by Heavy Metals

Scrupulous care was taken to avoid contamination by heavy metals. All experimental work was carried out in all-glass equipment to minimize metal contamination. All glassware was immersed for 24 hr in a 0.5% solution of EDTA, rinsed several times with deionized water, and dried at 120 C before use.

Preparation of Linoleic Acid Emulsion

A mixture consisting of linoleic acid (0.706 g) and Tween 20 (0.5 ml) was made up to 25 ml with deionized water. Emulsification was achieved by agitation using a Vortex shaker for 15 min. Aliquots (0.2 ml) of this emulsion were quantitatively transferred into Warburg flasks, diluted to 2 ml with deionized water, and amino acid was added. Amino acids were added to emulsified linoleic acid in 0.2 ml quantitites of appropriate stock solution to give a final amino acid concentration of 10^{-3}M. Cupric ions were also added in some experiments to the emulsified linoleic acid in 0.2 ml quantities of appropriate stock solutions of cupric sulfate to give a final metal concentration of 10^{-3}M and 10^{-5}M.

Measurement of Oxidation

Warburg apparatus was used to measure the oxygen absorbed by the linoleic acid emulsion. Experiments were conducted at 40 C with a constant shaking rate of 80 oscillations per minute. The average value of three replicates of each experiment was taken provided that the three figures did not differ by not more than 5%. Also, controls with the individual amino acids were run simultaneously with the corresponding experiment. No appreciable oxygen uptake was recorded for the last controls. The central well of the vessels contained 0.2 ml of 20% (w/v) potassium hydroxide solution to absorb gases such as carbon dioxide or formic acid vapor formed during the oxidation.

RESULTS AND DISCUSSION

Oxidation of lipids in aqueous emulsion is of considerable relevance to the deterioration of many foods and in particular milk and dairy products. It was intended to propose a model system as simple as possible in order to minimize variables and to obtain reproducible results. A number of restrictions were imposed, including the concentration of linoleic acid, the nature of the metal, the type and concentration of emulsifier, as well as the temperature and shaking rate of the reaction vessel. These, together with scrupulous avoidance of contamination by extraneous metal ions and careful adherence to the routine preparation of the emulsions, have produced extremely reproducible and consistent results.

The variables in the model systems were restricted only to the type of amino acids, and no buffers were used for the preparation of the model systems since the results of Haase and Dunkely (6), Allen and Farag (7), and Wills (8) have shown that phosphate, tris and borate buffers increased and decreased the rate of lipid oxidation. It should be emphasized that pH has an important role in lipid oxidation. The variations in pH of the systems employed in the present study as a result of adding six different amino acids to linoleic acid at the beginning of the experiments, however, were small for alanine, histidine, serine, and phenylalanine, but significant differences were found for cysteine and tryptophan as shown in Table I.

Stability of Linoleic Acid in Aqueous Media

The model system consisting of linoleic acid (10^{-2}M) and Tween 20 (0.02%) had an induction period of 46 hr. The autoxidation of linoleic acid could possibly be due to its interaction with other components of the emulsion, such as trace metals which are extremely difficult to eliminate completely. Uri (9) reported that linoleic acid twice-distilled in all-glass apparatus still contained traces of Cu, Fe, and Co. Linoleic acid used in the present investigation contained some trace metals which can certainly promote linoleic acid oxidation. The deliberate addition of cupric ions at concentrations of 10^{-3}M and 10^{-5}M caused an apparent decrease in linoleic acid stability, and the induction periods were 9 hr and 11 hr as shown in Figure 1. Stability and relative stability of linoleic acid when mixed with cupric ions at 40 C are presented in Table II. The results indicate that the rate of linoleic acid oxidation was dependent on copper concentration.

TABLE II

Stability and Relative Stability of Linoleic Acid Catalyzed by Cupric Ions

Parameters	Linoleic acid	Linoleic acid + 10^{-5}M Cu^{2+}	Linoleic acid + 10^{-3}M Cu^{2+}
Stability (hr)	46	11	9
Relative stability[a]	1	0.23	0.19

[a]Relative stability for linoleic acid is given as 1.00.

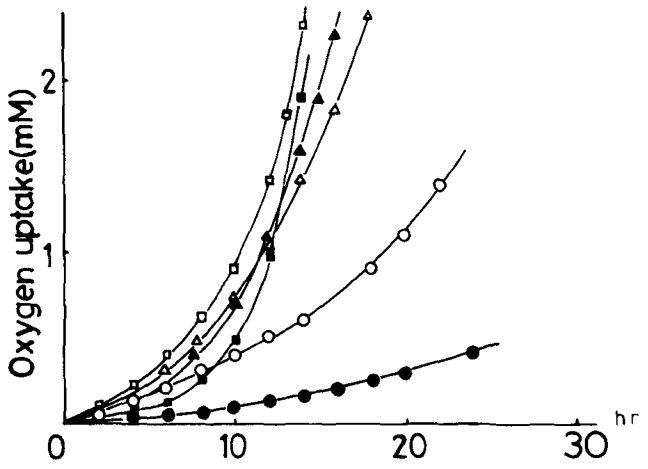


FIG. 2. Oxygen absorbed by alanine or serine and with or without cupric ions. ● ○ = Linoleic acid (10^{-2}M); ▲ △ = linoleic acid (10^{-2}M) + Cu (10^{-5}M); ■ □ = linoleic acid (10^{-2}M) + Cu (10^{-3}M); ● ▲ ■ alanine; ○ △ □ serine.

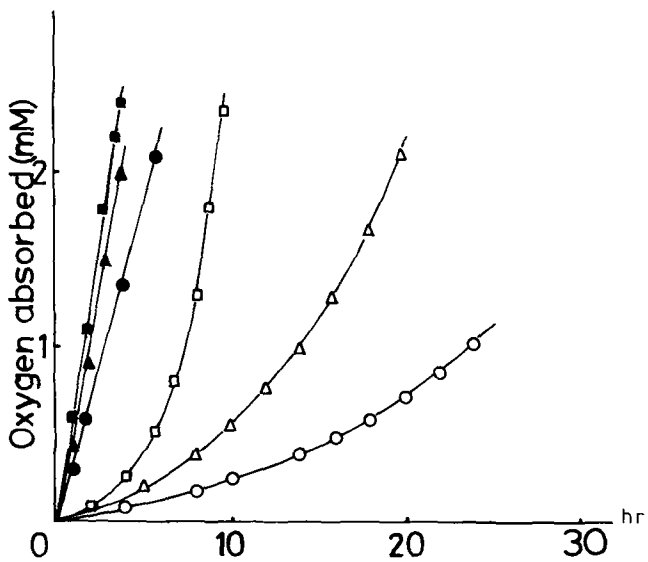


FIG. 3. Oxygen absorbed by cysteine or phenylalanine and with or without cupric ions. ● ○ = Linoleic acid (10^{-2}M); ▲ △ = linoleic acid (10^{-2}M) + Cu (10^{-5}M); ■ □ = linoleic acid (10^{-2}M) + Cu (10^{-3}M); ● ■ ■ = cysteine; ○ △ □ = phenylalanine.

Catalysis of Linoleic Acid by Amino Acids in Aqueous Media

The object of these experiments was to evaluate the specific anti- or prooxidative behavior of the various α-amino acids. The results of individual experiments using

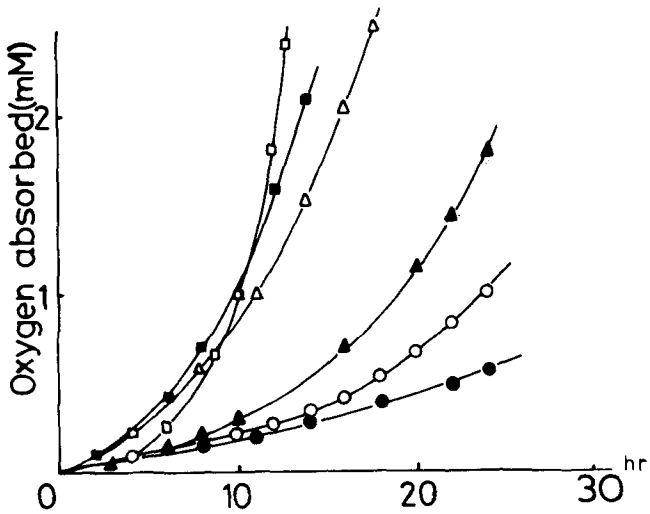


FIG. 4. Oxygen absorbed by histidine or typtophan and with or without cupric ions. ● ○ = Linoleic acid (10^{-2}M); ▲ △ = linoleic acid (10^{-2}M) + Cu (10^{-5}M); ■ □ = linoleic acid (10^{-2}M) + Cu (10^{-3}M); ● ▲ ■ = histidine; ○ △ □ = tryptophan.

model systems containing the amino acids in concentrations of one mole of the amino acid per ten moles of linoleic acid showed considerable variations in the rates of oxidation in comparison with the control experiment (containing no amino acid). The stability and relative stability (the latter means the stability in hours of the lipid materials relative to that of linoleic acid) of pure linoleic acid when mixed with amino acids calculated from Figures 2, 3, and 4 and the obtained results are shown in Table III. A relative induction period value of < 1 indicates a prooxidative effect, whereas a value of >1 indicates an antioxidative effect. The results indicate that the tested amino acids possessed potential prooxidative capacity. The effectiveness of amino acids on linoleic acid oxidation decreased in the following descending order: cysteine > serine > tryptophan > phenylalanine > histidine > alanine. These findings coincide with the results of Marcuse (10) who studied the oxidation of linoleic acid in water catalyzed by glycine, histidine, and tryptophan. On the other hand, Sieihouiski (2) reported that the antioxidant activities of the amino acids for olive oil oxidation were in the following order: cysteine > methionine > cystine. It seems that the discrepancies in the effect of amino acids on lipid oxidation stem from the differences in temperature, metal content, lipid concentration, type of emulsifier, media, pH, etc., in different experimental systems.

The addition of alanine to the linoleic acid model system decreased its induction period to about one-half its value. However, phenylalanine, tryptophan, and histidine shortened the induction period for linoleic acid oxidation

TABLE III

Catalytic Effect of Various Amino Acids and Cupric Ions on the Stability of Linoleic Acid (18:2) in Aqueous Media

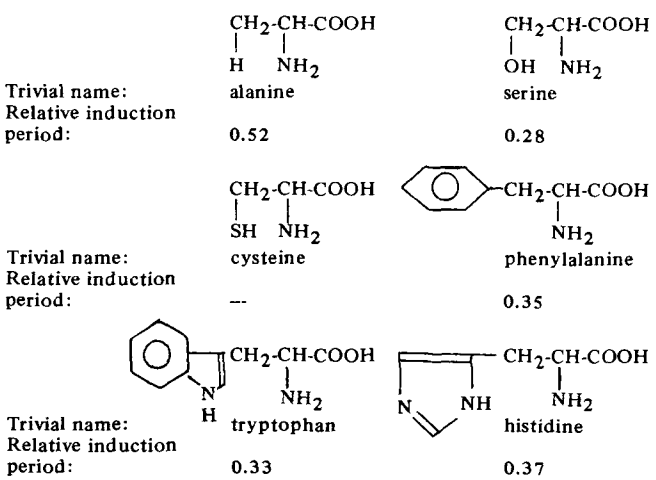
Model system	Induction period (hr)			Relative induction period[a]		
	Without added metal	Cu^{2+} 10^{-5}M	Cu^{2+} 10^{-3}M	Without added metal	Cu^{2+} 10^{-5}M	Cu^{2+} 10^{-3}M
18:2 + alanine	24	11.9	11.5	0.52	0.26	0.25
18:2 + serine	13.3	13.0	11.5	0.28	0.28	0.28
18:2 + cysteine	—[b]	—[b]	—[b]	—	—	—
18:2 + phenylalanine	16.3	14.9	11.0	0.35	0.32	0.23
18:2 + tryptophan	15.3	12.0	7.0	0.33	0.26	0.15
18:2 + histidine	17.3	14.4	10	0.37	0.31	0.22

[a]Relative induction period for linoleic acid is given as 1.00.
[b]Indicates that no induction period was observed.

to nearly one-third of its original value. In other words, experiments with tryptophan and histidine showed an effect similar to phenylalanine. The effect of amino acids used in this work (except for cysteine which had a specific oxidation effect) on linoleic acid oxidation showed features of an autocatalytic chain reaction. This means that the increase in the rate of formation of hydroperoxides with time is typical of an autocatalytic reaction. This increase is attributable to the formation of secondary catalysts from decomposition of hydroperoxides as suggested by Uri (11). With cysteine as a catalyst, there was a linear relation between the concentration of hydroperoxides and time during the early stages of oxidation. In other words, conjugated diene hydroperoxides started to develop as soon as cysteine was added to linoleic acid. Therefore, no time lag or induction period was observed. This linear relation indicates that cysteine initiates the oxidation reaction directly without the need of secondary catalytic agents.

The prooxidant activity of cysteine as a catalyst for linoleic acid oxidation was the highest when compared with all tested amino acids. This lends weight to the results obtained by Lewis and Wills (12) who found a higher rate of HS-group destruction by oxidized linoleic acid emulsion than by fresh emulsions. Cysteine was also shown to be converted to a mixture of cystine, cysteic, and cystine disulfoxide.

The prooxidative activity of the tested amino acids in general could be attributed to the presence of the α-amino groups in the form $H_3\overset{+}{N}$-R. The ratio between the protonated ($H_3\overset{+}{N}$-R) and the unprotonated ($H_2\ddot{N}$-R) amino groups of the amino acids used in the present work were calculated and found to be in favor of the protonated amino groups as shown in Table I. In this respect, Tsai and Smith (13) studied the role of bases and phosphoryl bases of phospholipids in the autoxidation of methyl linoleate emulsion. They reported that the amino group ($-\overset{+}{N}H_3$) of the primary amine accelerated the autoxidation, but the $H_2\ddot{N}$-group had the reverse effect. In this work, six amino acids have been chosen, and their structures are as follows:



The above formulas showed that these amino acids have in common three carbon atoms and α-amino groups. The difference is mainly in the functional groups attached to the β-carbon atom in the amino acid molecules. The catalytic activities of these amino acids are attributed to their functional groups. Serine had more prooxidant activity than alanine and that may be due to the presence of a hydroxyl group in serine moiety. An association (i.e., hydrogen bonds) between the hydroxyl groups of serine and hydroperoxide groups initially present in very small amounts might exist. This assumed that association will extent the lipid micelle and pave the way for oxygen to come into action. Alternatively, or concomitantly, the close adherence of analytically active protonated amino groups in the surface regions of the micelle might be responsible for the rapid linoleic acid oxidation.

With regard to cysteine, the substitution of a hydrogen atom on the β-carbon of alanine by the HS-group caused a remarkable change in linoleic acid oxidation which stems from the rapid destruction of the HS-group as previously mentioned. The linear relation between the rate of linoleic acid oxidation and the oxygen uptake suggested that the HS-group had a more powerful prooxidant effect than did the protonated nitrogen of the amino acids.

Alpha amino acids containing an aromatic ring decreased much more stability of the linoleic acid emulsion relative to linoleic acid catalyzed by alanine. The replacement of the hydrogen atom of the β-carbon in alanine with an aromatic ring increased the linoleic acid oxidation. This effect may be due to the hydrophobic action of the aromatic ring. Hence, the interaction between the α-amino acids containing aromatic rings and the linoleic acid micelle is increased, and the protonated amino groups will be much closer to the lipid micelle which will indeed increase the rate of linoleic acid oxidation. However, this prooxidant effect is less than the effect of hydroxyl group on peroxide decomposition, and hence the prooxidant effect of these amino acids will be less than that of serine.

The results of these experiments support the theory that the coupled oxidation of amino acids with linoleic acid requires a micellar environment and that this process may take place either on the surface or in the interior of the micelles. Results reported by Lohmar and Tookey (14) and Haurowitz et al. (15) also support this theory. Further work in this field necessitates the use of an electron microscope for determination of the changes in micelle structure during oxidation.

Catalysis of Linoleic Acid by Amino Acids and Cupric Ions in Aqueous Media

The effect of amino acids together with cupric ions on linoleic acid stability was investigated, and the results are given in Table III. The comparison between the relative induction period of linoleic acid catalyzed with amino acids and with or without added cupric ions revealed the following points.

Alanine combined with Cu^{2+} (10^{-5}M or 10^{-3}M) shortened the induction period to about one-half that of linoleic acid catalyzed by alanine. The higher copper concentration had a similar effect on shortening the induction period to that of lower copper concentration, whereas, the higher copper concentration had an increasingly greater influence on the propagation step (Fig. 2). Generally speaking, the cupric ions in this model system had a more pronounced effect than those of any other model system.

The model system consisting of serine, Cu^{2+}, (10^{-5}M or 10^{-3}M), and linoleic acid had a relative induction period similar to that containing no added metal. Therefore, cupric ions seemed to have no extra effect on linoleic acid oxidation during the induction period. An increased copper concentration accelerated the formation of hydroperoxides during the propagation process as shown in Figure 2.

In the model system containing cysteine and Cu^{2+} (10^{-5}M or 10^{-3}M) as catalysts for linoleic acid oxidation, there was a linear relation between concentration of hydroperoxides and time during the course of oxidation. The other model systems have shown features of an autocatalytic chain process. Figure 3 shows the combined addition of Cu^{2+} and cysteine to linoleic acid which caused an increase in the rate of oxidation, and the ratio between the catalyzed reaction with cysteine and copper 10^{-5}M or 10^{-3}M was 1:1.6:1.7. In the case of α-amino acids having an aromatic side chain, the presence of Cu^{2+} (10^{-5}M) had

very little effect when compared with the model systems catalyzed only with aromatic amino acids. Copper at a concentration of 10^{-3}M markedly decreased the induction period, particularly in the model system catalyzed with tryptophan (Fig. 4).

One could explain the effect of the addition of Cu^{2+} (10^{-5}M) together with amino acids in the present work (except for cysteine which had a particular oxidation mechanism) as follows. The mean relative induction period for the tested amino acids was 0.28, and with Cu^{2+} all the systems had a relative induction period of ca. 0.28. This indicates that Cu^{2+} at a concentration of 10^{-5}M presents a high metal concentration at which the effect of various amino acids would be nonsignificant. Perhaps at lower Cu^{2+} concentrations than those used in this work the amino acids would have variable significant effects on linoleic acid oxidation. Similar results for nearly similar systems were obtained by Allen and Wood (16), who studied the copper-catalyzed oxidation of linoleic acid dispersed in buffered aqueous solutions and found that ascorbic acid at 10^{-3}M was a more effective prooxidant in the presence of low copper concentrations than at high concentrations especially in the initial stages of oxidation. Increasing the copper concentration from 10^{-5}M to 10^{-3}M had the following effects: a shortening of the induction period of linoleic acid catalyzed by amino acids having an aromatic side chain, no effect on the induction period by an increase in the oxidation rate during the propagation step in the model systems catalyzed by alanine and serine, and in the model system containing cysteine, a linear increase in the linoleic acid oxidation from the start of the reaction.

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