Ascorbic acid and copper in linoleate oxidation. II. Ascorbic acid and copper as oxidation catalysts

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ABSTRACT Both ascorbic acid and copper were strong prooxidants in the oxidation of linoleate in a buffered (pH 7.0) aqueous dispersion at 37°C. Minimum concentrations at which catalytic activity was detected were 1.3×10^{-7} M for copper and 1.8×10^{-6} M for ascorbic acid. For concentrations up to 10^{-3} M, the increase in rate of oxidation with increase in concentration of catalyst was greater for ascorbic acid than for copper. Ascorbic acid had maximum catalytic activity at 2.0×10^{-3} M, but was still prooxidant at the highest concentration tested (5.0×10^{-2} M). Dehydroascorbic acid was a weaker prooxidant than ascorbic acid. Further degradation products of ascorbic acid were not prooxidant.

In early stages of the oxidation autocatalytic behavior was observed with copper, but not with ascorbic acid. Ascorbic acid functioned as a true catalyst, i.e., it accelerated the reaction but it was not oxidized simultaneously with the linoleate. It is proposed that the dehydroascorbic acid radical initiates the linoleate oxidation reaction.

SUPPLEMENTARY KEY WORDS dehydroascorbic acid · semidehydroascorbic acid radical · autocatalysis · initiation of oxidation

EARLY REPORTS (1, 2) noted that ascorbic acid promotes lipid oxidation in milk products, but Olson and Brown (3) reported the first definitive proof for its role. Since then, many studies have substantiated in other systems the early findings for milk (4–7). The action of ascorbic acid appears to be unique, because other reducing agents and analogous compounds are not able to replace it, or are less effective (8, 9).

Ascorbic acid may also *inhibit* lipid oxidation (7, 10–12). However, the conditions that determine the function are not clear. Thermodynamically, ascorbic acid should be an antioxidant because of its oxidation–reduction potential and the stability of its oxidation products (13).

Cupric copper is an active catalyst of lipid oxidation, but the mechanism of its action is not clear. It is generally agreed that copper acts as a strong prooxidant by catalyzing the decomposition of hydroperoxides (14). However, there is no agreement regarding the importance of copper in the initial step in which hydroperoxides are formed.

The purpose of this study was to clarify the effects, during the early stages of oxidation of linoleate, of ascorbic acid and cupric ion separately. The accompanying paper (14a) deals with the compounds in combination.

METHODS AND MATERIALS

Methods for determining the concentration of conjugated dienes and ascorbic acid, for preparing a buffered dispersion of 0.02 M potassium linoleate, and for preventing metal contamination were as described previously (15). Oxidation of linoleate at 37°C was measured by UV absorption.

Dehydroascorbic acid was prepared by oxidation of L-ascorbic acid with activated charcoal (16). 100 ml of solution containing 1 g of L-ascorbic acid was shaken vigorously for 20 min with 4 g of acid-washed Norit A (Fisher Scientific) and then filtered. The procedure was repeated. Completeness of conversion was determined by

Abbreviations: DHA, dehydroascorbic acid; DKA, diketogulonic acid.

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measurement of the absorption at 265 nm and titration of the solution with 2,6-dichlorophenol–indophenol (17).

Dehydroascorbic acid was estimated by reducing it with homocysteine (18) and measuring the absorption (at 265 nm) of the resultant ascorbic acid in 60% ethanol at pH 7.0.

A crude extract of ascorbic acid oxidase was prepared from cucumber juice (19). Cucumbers were frozen at −20°C and thawed, and the juice was drained off. The juice was transferred to cellophane tubing, concentrated by pervaporation, and filtered.

RESULTS

Catalysis by Ascorbic Acid

Rates of oxidation of linoleate catalyzed by different concentrations of ascorbic acid are depicted in Fig. 1. Catalytic activity was evident at concentrations as low as 1.9×10^{-6} M (0.33 mg/liter). The rate of reaction increased substantially with increases in concentration.

Conjugated dienes started to develop as soon as ascorbic acid was added to the linoleate model system. The initial rate was dependent on the concentration of the catalyst. No time lag or induction period was observed.

During the early stage (first 400 min) of the linoleate oxidation, the increase in conjugated dienes was linearly dependent on time. Rate constants (k_i), equal to the slope of the oxidation curves, could therefore be calculated for the time period 0–100 min. The plot of rate constants against concentration of ascorbic acid (Fig. 2) indicates that catalytic activity increased rapidly between 1.0×10^{-5} and 2.0×10^{-3} M, and then decreased.

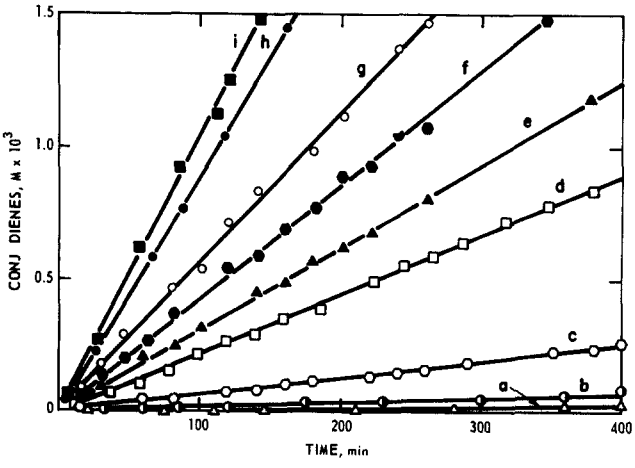

FIG. 1. Effect of various concentrations of ascorbic acid on the formation of conjugated dienes from an aqueous dispersion of 0.02 M potassium linoleate at pH 7.0 and 37°C. The concentrations were: *a*, no ascorbic acid; *b*, 1.9×10^{-6}; *c*, 4.1×10^{-6}; *d*, 1.8×10^{-5}; *e*, 4.1×10^{-5}; *f*, 7.5×10^{-5}; *g*, 1.8×10^{-4}; *h* 1.7×10^{-2}; *i*, 1.8×10^{-3} M.

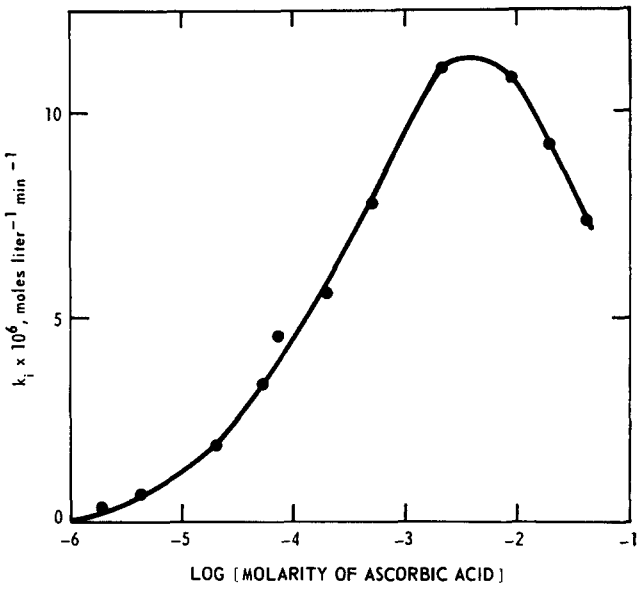

FIG. 2. Depedence of rate constant (k_i) upon the ascorbic acid concentration. k_i is the rate constant for the initial period (0–100 min) of the oxidation of linoleate catalyzed by ascorbic acid.

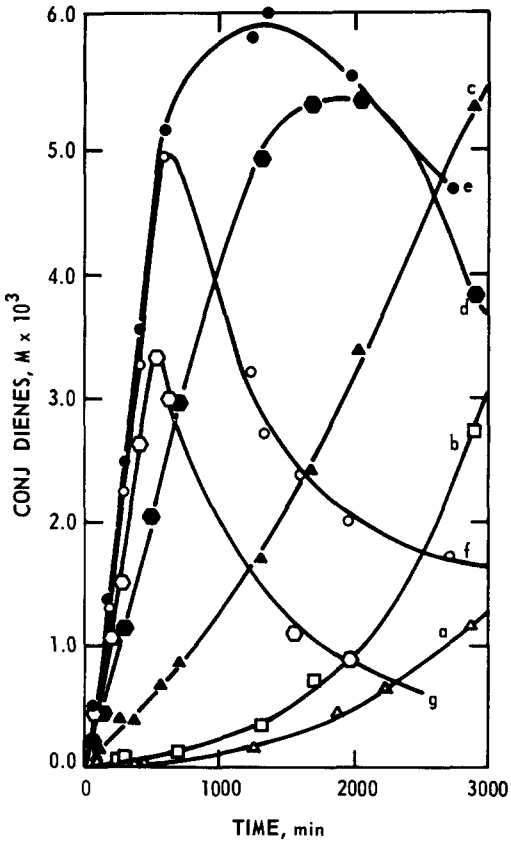

FIG. 3. Change of the concentration of conjugated dienes during linoleate oxidation at selected ascorbic concentrations. The concentrations were: *a*, no ascorbic acid; *b*, 1.9×10^{-6}; *c*, 1.9×10^{-5}; *d*, 1.9×10^{-4}; *e*, 2.0×10^{-3}; *f*, 1.9×10^{-2}; *g*, 5.2×10^{-2} M.

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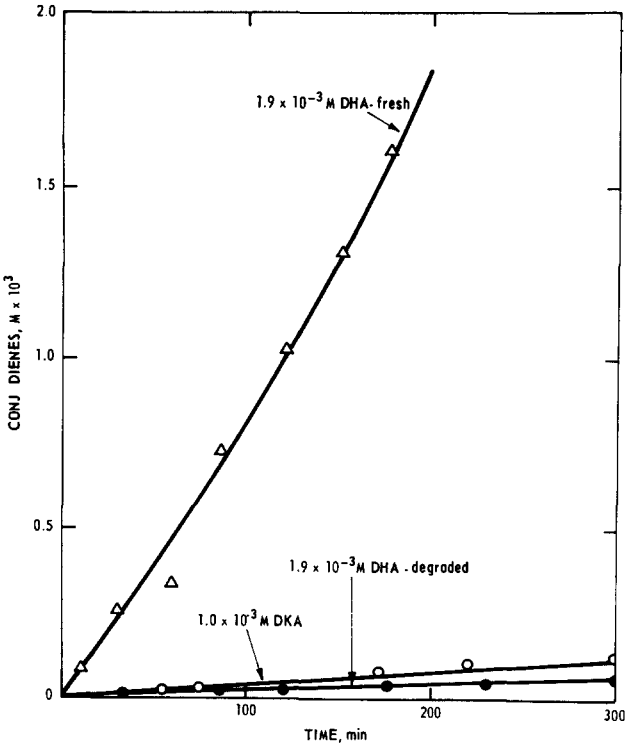

FIG. 4. Effect of fresh and degraded dehydroascorbic acid and 2,3-diketogulonic acid on the formation of conjugated dienes in the linoleate oxidation.

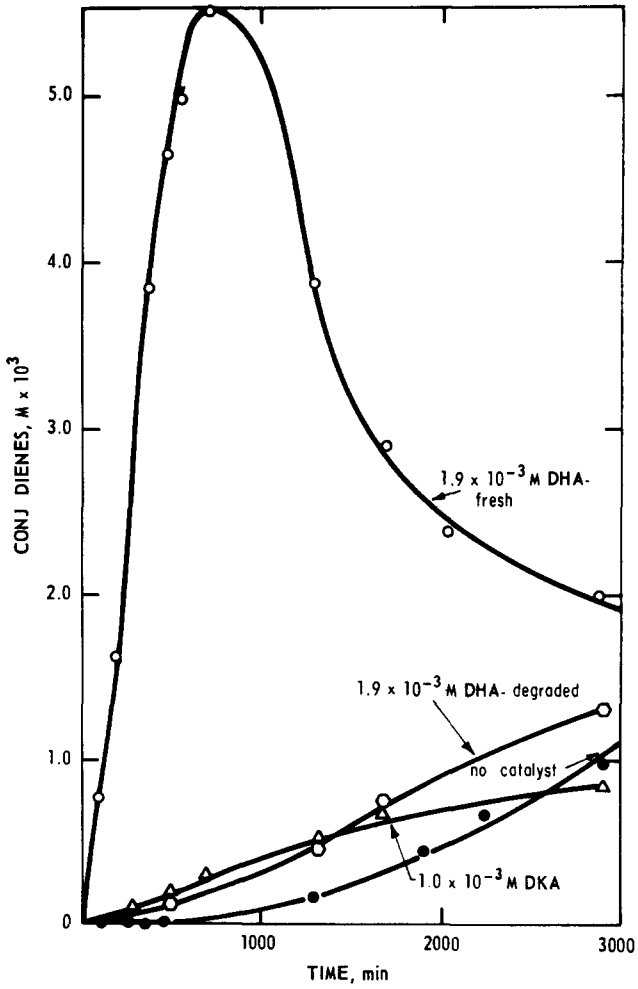

FIG. 5. Effect of fresh and degraded dehydroascorbic acid and 2,3-diketogulonic acid on the concentration of conjugated dienes during linoleate oxidation over an extended period (3000 min).

Although there was a decrease in prooxidant activity at the highest concentration tested (about 5.0×10^{-2} M or 9 g/liter), no inhibition was observed.

The rate of diene production was not linear when the oxidation was studied for longer periods (more than 400 min) (Fig. 3). At the lowest concentrations of ascorbic acid (1.9×10^{-6} and 1.9×10^{-5} M) an autocatalytic behavior was observed. At higher concentrations, the diene concentration increased almost linearly at first, and then dropped sharply. At the higher concentrations of ascorbic acid (1.9 and 5.2×10^{-2} M), the maximum diene concentration appeared earlier. At the lower concentrations (1.9×10^{-5} and 1.9×10^{-6} M) a maximum in diene concentration might have been observed if the experiments had been continued beyond 3000 min.

The concentration of dienes at the maxima varied greatly, but it was highest at an intermediate concentration of ascorbic acid.

In the above experiments the concentration of ascorbic acid was determined in the same sample used for the measurement of diene concentration. No measurable oxidation of ascorbic acid was detected.

We next tested the catalytic activity of solutions of dehydroascorbic acid which were prepared fresh (DHA-fresh) or stored for a week (DHA-degraded) and of 2,3-diketogulonic acid (Fig. 4). Fresh dehydroascorbic acid had high catalytic activity. Absorption measurements at 265 nm and titration with 2,6-dichlorophenol–indophenol indicated that the dehydroascorbic acid was not reduced to ascorbic acid during the oxidation of the linoleate. Neither 2,3-diketogulonic acid nor degraded dehydroascorbic acid catalyzed the linoleate oxidation. None of the degraded dehydroascorbic acid was converted to ascorbic acid upon reduction with homocysteine, which indicates that all of the dehydroascorbic acid had been degraded. Dehydroascorbic acid is unstable (20), and is oxidized upon standing and exposure to atmospheric oxygen.

Extension of the oxidation experiments to 3000 min is shown summarized in Fig. 5. Fresh dehydroascorbic acid gave a maximum similar to that of ascorbic acid. There was some prooxidant activity with degraded dehydroascorbic acid and 2,3-diketoglulonic acid.

Fig. 6 A depicts results of an experiment in which oxidation of ascorbic acid was catalyzed by ascorbic acid

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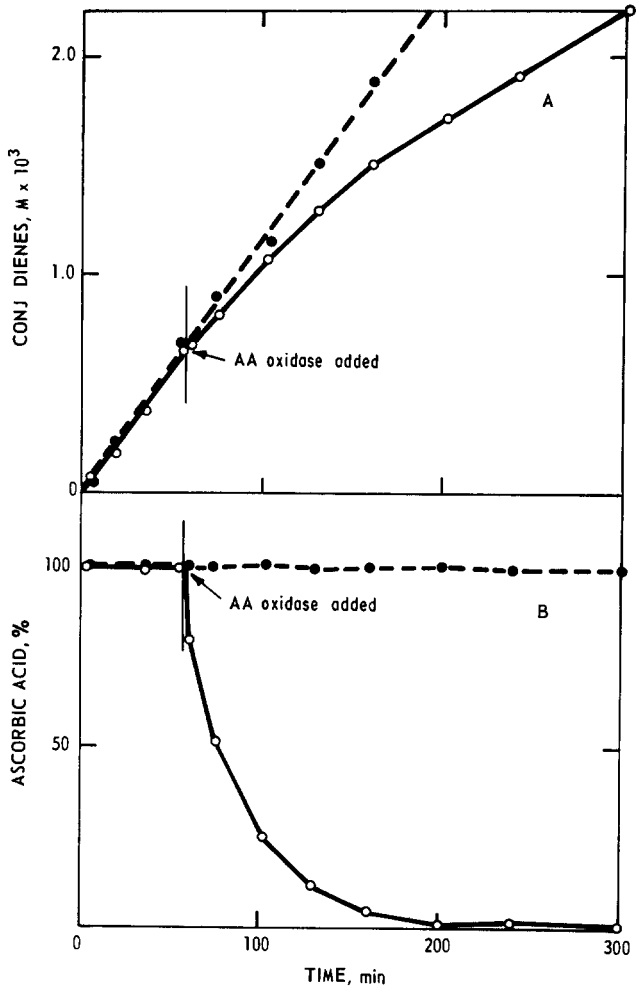

FIG. 6. Influence of addition of ascorbic acid oxidase to a linoleate model system on (A) concentration of conjugated dienes, and (B) of ascorbic acid. Concentration of ascorbic acid, 1.4×10^{-3} M. Solid line, ascorbic acid oxidase added at 58 min; broken line, no oxidase added.

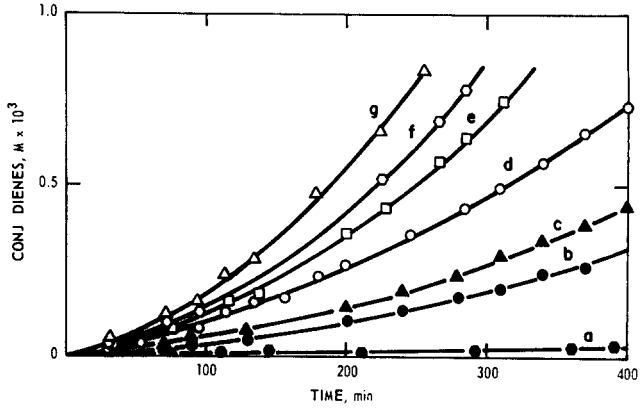

FIG. 7. Effect of various concentrations of copper on the formation of conjugated dienes. Concentrations were: *a*, no copper; *b*, 1.3×10^{-7}; *c*, 1.3×10^{-6}; *d*, 1.3×10^{-5}; *e*, 1.3×10^{-4}; *f*, 2.6×10^{-4}; *g*, 1.3×10^{-3} M.

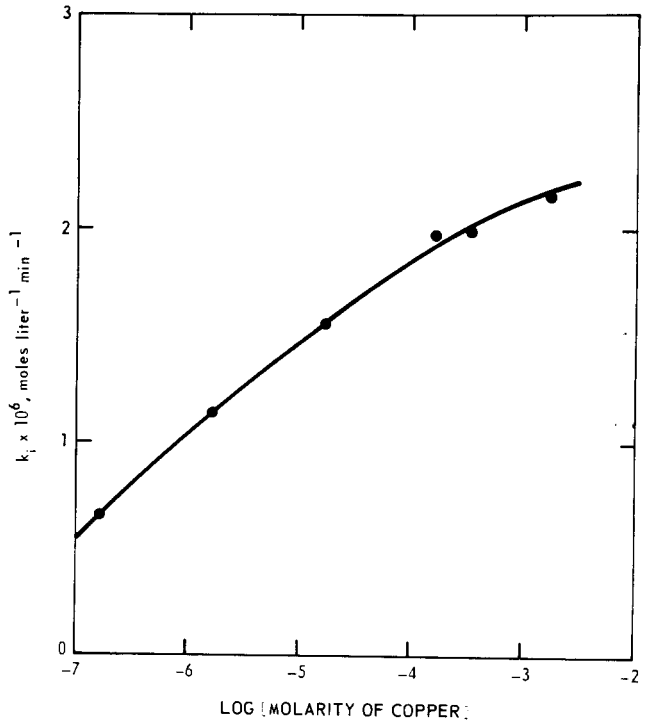

FIG. 8. Dependence of rate constant (k_i) upon the copper concentration. k_i is the rate constant for the initial period (0–100 min) of the oxidation of linoleate catalyzed by copper.

oxidase. The enzyme was added at 58 min to a system containing 1.4×10^{-3} M ascorbic acid as catalyst for the linoleate oxidation. Addition of the oxidase resulted in rapid disappearance of the ascorbic acid (Fig. 6 B). The formation of dienes continued, but at a reduced rate.

Catalysis by Copper

Results of typical experiments showing the catalytic effect of cupric ion on formation of conjugated dienes in linoleate oxidation are presented in Fig. 7. Copper had a prooxidant effect in concentrations as low as 1.3×10^{-7} M (0.008 μg/g or ppm). The rate of reaction increased with the concentration of copper. The highest concentration used was 1.3×10^{-3} M. At concentrations above 1.3×10^{-3} M, heterogeneity of the emulsion increased, as indicated by the formation of blue-green droplets on the surface of the reaction mixture.

The relation between the k_i and copper concentration is shown in Fig. 8. The catalytic activity increased over the entire concentration range from 1.3×10^{-7} to 1.3×10^{-3} M.

Fig. 9 illustrates the change in diene concentration for oxidation experiments extended over longer periods (more than 400 min). After a rapid initial increase, a maximum in the concentration of conjugated dienes was reached. The maximum occurred earlier at higher copper concentrations.

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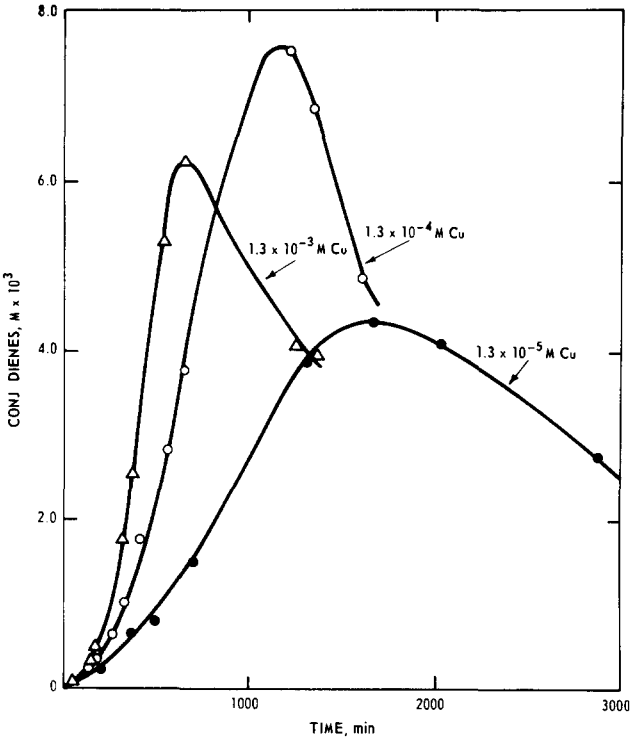

FIG. 9. Influence of concentration of copper on changes in the concentration of conjugated dienes during linoleate oxidation over an extended period (3000 min).

DISCUSSION

Both ascorbic acid and copper catalyze linoleate oxidation. The oxidation is initiated immediately after addition of the catalysts (Figs. 1 and 7), with no induction period. However, differences in the shapes of the curves indicate a different mode of catalysis for copper from that for ascorbic acid.

With copper, the increase in the rate of formation of conjugated dienes with time is typical of an autocatalytic reaction. This increase is attributable to the formation of secondary catalysts from decomposition of hydroperoxides (21).

With ascorbic acid as catalyst, there is a linear relation between concentration of conjugated dienes and time during the early stages of the oxidation. This relation indicates that ascorbic acid initiates the oxidation reaction directly, without the necessity for secondary catalytic agents.

With low concentrations of ascorbic acid, the reaction is autocatalytic after appreciable linoleate oxidation has occurred (Fig. 3). Under these conditions, radicals produced by decomposition of hydroperoxides become more important in initiating the oxidation.

Results of this study support the hypothesis that the prooxidant activity of ascorbic acid in initiating lipid oxidation is attributable to the formation of the semidehydroascorbic acid radical. The ascorbic acid radical was postulated to be an initiator of lipid oxidation in some materials (22), including milk (9). Yamazaki and Piette (23) and others (24–26) have established that an ascorbic acid radical is present in aqueous solution of ascorbic acid, and that it is an intermediate in the oxidation of ascorbic acid. Foerster and coworkers (24, 25) showed by electron-spin resonance measurements that ascorbic acid in aqueous solution is in equilibrium with the semidehydroascorbic acid radical. It can be visualized that this radical abstracts a methylenic hydrogen atom from linoleate, thus causing formation of a linoleate free radical; this is followed by a shift of one double bond, and an attack by oxygen to form a diene-conjugated hydroperoxide. Since the concentration of the semidehydroascorbic acid radical depends on the concentration of ascorbic acid, the increase in catalytic effect with increase in concentration of ascorbic acid can be explained.

Smith and Dunkley (27) considered that their data did not support the theory that the ascorbic acid radical initiates lipid oxidation. They assumed that the concentration of ascorbic acid radical would be proportional to the rate of oxidation of ascorbic acid, and they did not find a relation between the rates of oxidation of lipid and of ascorbic acid. Their studies were done with milk, and in this complex material other constituents probably influenced lipid oxidation.

Ascorbic acid functions as a true catalyst; it enhances the oxidation reaction but is not itself oxidized. Concurrent oxidation of ascorbic acid and lipid is not necessary for the catalytic activity, as has been suggested (8, 28–30). Concurrent oxidation has been observed frequently, though, because some conditions that favor lipid oxidation also promote oxidation of ascorbic acid, as for example the presence of catalytic metals or exposure to light. However simultaneous oxidation is not evidence that the reactions are coupled, or that oxidation of ascorbic acid is necessary to promote lipid oxidation.

A related conclusion is that the rate of oxidation of ascorbic acid does not determine the rate of lipid oxidation. Similar rates of lipid oxidation were obtained under conditions that resulted in marked differences in rates of oxidation of ascorbic acid. For example, when ascorbic acid oxidase was added to the reaction mixture, there was rapid oxidation of ascorbic acid without a corresponding change in rate of linoleate oxidation (Fig. 6). The decrease in rate of formation of conjugated dienes is attributed to the difference in prooxidant activity of ascorbic acid and dehydroascorbic acid.

Our results show that dehydroascorbic acid catalyzes the formation of conjugated dienes. This was demonstrated in experiments with added dehydroascorbic acid (Figs. 4 and 5), and also in experiments in which ascorbic acid oxidase was used to oxidize ascorbic acid to dehydroascorbic acid (Fig. 6). The observed prooxidant

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effect is consistent with results reported previously for milk (3, 31–33), but inconsistent with Smith's (34) findings. Furthermore, the results support the conclusion (10) that the ratio of ascorbic acid to dehydroascorbic acid does not determine the rate of lipid oxidation, as postulated previously (33).

Degradation products of dehydroascorbic acid have little prooxidant activity. In reaction mixtures containing 2,3-diketogulonic acid or degraded dehydroascorbic acid, formation of conjugated dienes was only slightly greater than in control samples without added catalysts. These results are not consistent with a previous report (35) that 2,3-diketogulonic acid is prooxidant, or with the hypothesis (11) that degradation products of ascorbic acid inhibit lipid oxidation.

Rate constants for the early stages of the linoleate oxidation provide a useful criterion for comparing the activity of ascorbic acid and copper in initiating the oxidation. Because of the curvilinear relation depicted with copper as catalyst (Fig. 7), it was necessary to adopt arbitrarily a time interval for the calculation of average rates. The first 100 min were chosen for calculating k_i because the curvilinear relation approximates a straight line over this short interval.

Under our experimental conditions, catalytic activity was evident at a lower concentration with copper (1.3×10^{-7} M) than with ascorbic acid (1.8×10^{-6} M); but the rate of increase with increase in concentration was greater with ascorbic acid (compare Figs. 2 and 8, note difference in scales). As the concentration of the catalyst was increased, a maximum in catalytic activity was obtained with ascorbic acid (Fig. 2) but not with copper (Fig. 8). We hypothesize that the decrease in catalytic activity of ascorbic acid at high concentrations is attributable to a lowering of the oxidation–reduction potential to a level that was less favorable for oxidation of linoleate. However, although ascorbic acid had reduced catalytic activity at high concentrations, it did not act as an antioxidant.

Maximum prooxidant activity was observed in our experiments at an ascorbic acid concentration of about 2.0×10^{-3} M (350 mg/liter). Others have also observed a decrease in rate of lipid oxidation as the concentration of ascorbic acid was increased above a critical level. The critical level, however, varies with the experimental conditions. Examples of such levels are 180 mg/liter for a linoleate model at 2°C (10), 20–40 mg/liter for fat globule membrane material (11, 36), and 15 mg/liter for a mitochondrial suspension (37). Differences in the experimental conditions included pH, presence of tacer amounts of copper, oxidation period, and methods of measuring the lipid oxidation.

It is generally accepted that copper catalyzes the decomposition of hydroperoxides (21). The accumulation of conjugated dienes when the linoleate oxidation was catalyzed by copper (Figs. 7 and 9) is evidence that either the degradation reaction is slow compared with the formation of conjugated dienes, or that only part of the conjugated dienes are degraded by copper. When the oxidation was extended until the concentration of conjugated dienes decreased, similar maximum values in diene concentration and similar rates of increase and decrease were observed whether the oxidation was catalyzed by ascorbic acid (Fig. 3) or by copper (Fig. 9). These data indicate that either the catalysis of degradation of hydroperoxides by copper is slow under the reaction conditions used, or that ascorbic acid has similar effects in promoting the degradation of conjugated dienes.

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