Ascorbic acid and copper in linoleate oxidation. III. Catalysts in combination

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ABSTRACT In promoting oxidation of 0.02 M potassium linoleate in a buffered (pH 7.0) aqueous dispersion at 37°C, ascorbic acid at low concentrations (1.8×10^{-6} and 1.8×10^{-5} M) in combination with copper (1.3×10^{-7} to 1.3×10^{-3} M) had greater catalytic activity than the additive activity of the two catalysts individually. Possible explanations for the enhanced catalysis include reduction of copper by ascorbic acid to the cuprous form, increased concentration of semidehydroascorbic acid radical, and formation of a metal–ascorbic acid–oxygen complex.

Some combinations of ascorbic acid (1.8×10^{-4} and 1.8×10^{-3} M) and copper (1.3×10^{-6} and 1.3×10^{-3} M) inhibited the formation of conjugated dienes but not the oxidation of ascorbic acid, and caused rapid loss of part of the conjugated dienes that were already present. It is suggested that free-radical inhibitors formed by the combination of catalysts inhibit initiation of lipid oxidation but not copper-catalyzed oxidation of ascorbic acid. Effects of the inhibitory combinations on changes in UV absorption by conjugated dienes, and absorbance in the TBA test, indicate the presence of at least two conjugated dienes that differ in stability.

SUPPLEMENTARY KEY WORDS conjugated dienes · lipid oxidation · prooxidants · antioxidant combination · ferric ion

We described previously (1) a study of the catalytic activity of ascorbic acid and of copper in promoting the oxidation of linoleate in a buffered aqueous medium. It was postulated that catalysis by ascorbic acid depends on direct initiation of the oxidation by semidehydroascorbic acid radical. In contrast, the autooxidative nature of catalysis by copper depends, at least in part, on formation of free radicals during decomposition of hydroperoxides.

At all concentrations studied, ascorbic acid was prooxidant. The rates of oxidation in the presence of ascorbic acid were greater than in the control samples that contained neither ascorbic acid nor copper. At high concentrations of ascorbic acid (above 2.0×10^{-2} M) the prooxidant activity was reduced, but inhibition was never observed.

In milk and preparations of fat globule membrane material, ascorbic acid is reported to be prooxidant at low concentrations but antioxidant at high concentrations (2–4). A similar behavior of ascorbic acid has been observed in other materials (5–8). In the studies cited, copper presumably was present in at least trace quantities, and in some experiments it was added. Hence, it is pertinent to consider possible interrelations between ascorbic acid and copper in determining their effect on lipid oxidation.

This paper describes the activity of combinations of prooxidants, principally ascorbic acid and copper.

MATERIALS AND METHODS

Procedures for preparing reagents and materials, conducting the experiments, and making analyses were described previously (9). Briefly, oxidation of an aqueous dispersion (0.02 M) of potassium linoleate in phosphate buffer (pH 7.0, 37°C) was studied by measuring UV absorption of the conjugated dienes produced and by the TBA test. Catalysts were added as appropriate quantities of stock solutions of ascorbic acid, cupric or ferric sulfate, or combinations of them.

RESULTS

A series of experiments was conducted in which the effects of combinations of catalysts were determined, using as-

Abbreviation: TBA, thiobarbituric acid.

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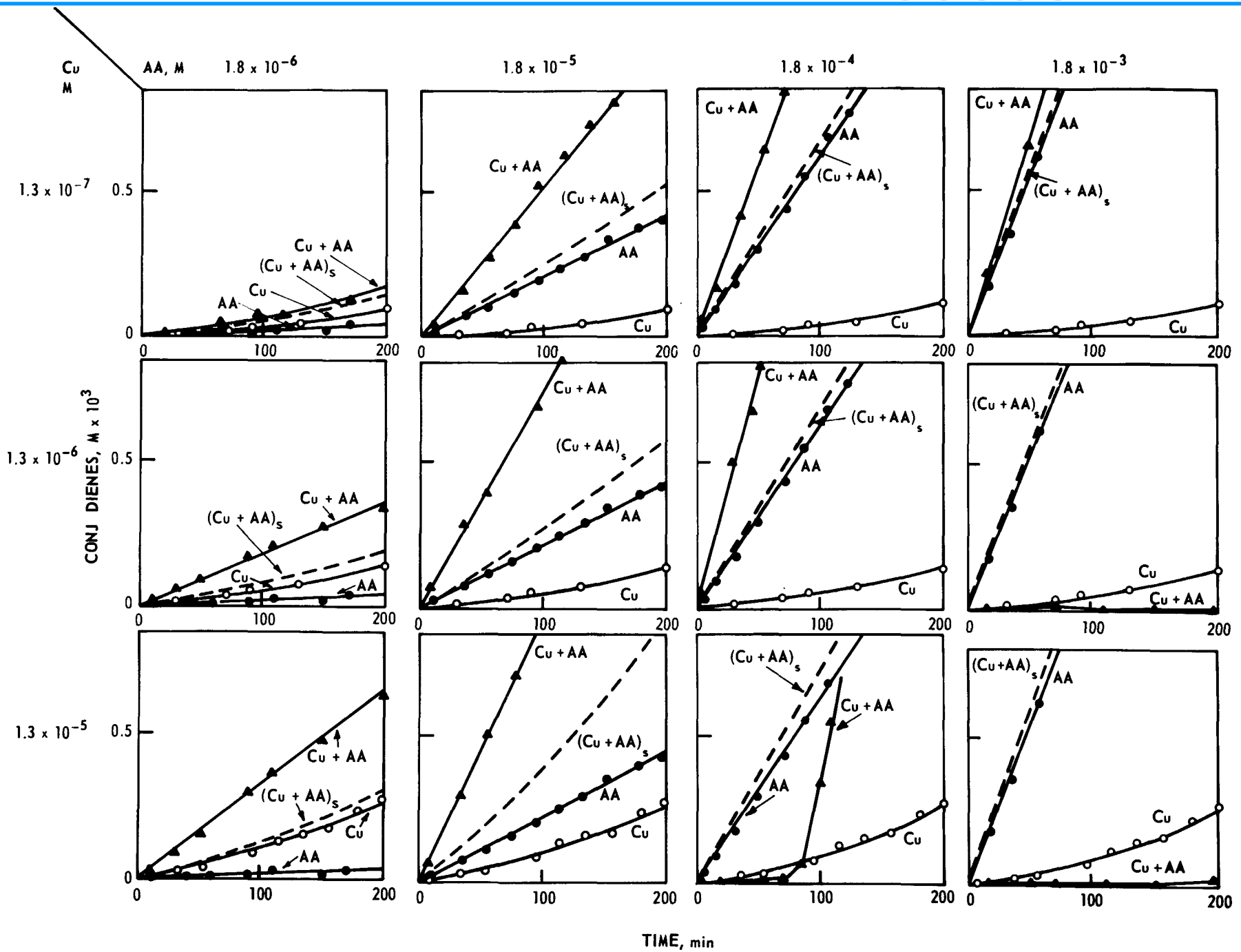

FIG. 1. Catalysis of the formation of conjugated dienes from an aqueous dispersion of 0.02 M potassium linoleate (pH 7.0, 37°C) by ascorbic acid and cupric sulfate. Comparison of the effects of copper (Cu) and ascorbic acid (AA) individually, with the combined effect (Cu + AA), and with the calculated sum of results with the individual catalysts [$(Cu + AA)_s$, broken line].

corbic acid in concentrations ranging from 1.8×10^{-7} to 1.8×10^{-3} M in 10-fold steps, and copper in concentrations from 1.3×10^{-7} to 1.3×10^{-3} M in 10-fold steps. Representative data for ascorbic acid and copper alone over a part of this range, and for both together, are presented in Fig.1. To facilitate comparison of activity of the catalysts in combination with that expected if the effects were additive, we also show the sums of the individual effects in Fig. 1.

Ascorbic acid at 1.8×10^{-7} M did not affect catalysis by copper (not shown). Ascorbic acid at 1.8×10^{-6} or 1.8×10^{-5} M in combination with copper at 1.3×10^{-7}, 1.3×10^{-6}, or 1.3×10^{-5} M had greater prooxidant activity than expected from the sums of the individual effects (Fig. 1). In reaction mixtures containing 1.8×10^{-4} M ascorbic acid initially, with copper at 1.3×10^{-7} or 1.3×10^{-6} M, the combined effects were greater than the sums. With copper at 1.3×10^{-5} M, the oxidation was delayed about 80 min, but then proceeded at a greater rate than expected from the individual effects. When the ascorbic acid was 1.8×10^{-3} M, with copper at 1.3×10^{-7} M, the combined effect was slightly greater than additive. But with copper at 1.3×10^{-6} or 1.3×10^{-5} M, the oxidation was inhibited.

Rate constants for the initial period (i.e. first 100 min) of the various experiments (except with ascorbic acid at 1.8×10^{-7} M) are summarized in Table 1. Whether the

TABLE 1 RATE CONSTANTS FOR THE FORMATION OF CONJUGATED DIENES CATALYZED BY ASCORBIC ACID, COPPER, AND COMBINATIONS OF THEM

Concentration of				
Ascorbic Acid	Copper, 1.3×	$k_i \times 10^6$*	$k_a \times 10^6$†	k_i/k_a
M	M	*moles liter*$^{-1}$*min*$^{-1}$		
1.8×10^{-6}	10^{-7}	1.02	0.79	1.29
	10^{-6}	1.73	1.42	1.22
	10^{-5}	3.09	1.88	1.64
	10^{-4}	4.60	2.13	2.16
	10^{-3}	6.59	2.52	2.62
1.8×10^{-5}	10^{-7}	5.27	2.33	2.26
	10^{-6}	7.37	2.96	2.49
	10^{-5}	8.80	3.42	2.57
	10^{-4}	9.10	3.67	2.48
	10^{-3}	9.78	4.06	2.41
1.8×10^{-4}	10^{-7}	11.4	6.07	1.89
	10^{-6}	12.8‡	6.71	1.92
	10^{-5}	0	7.17	0
	10^{-4}	0	7.42	0
	10^{-3}	0	7.83	0
1.8×10^{-3}	10^{-7}	13.8	11.7	1.18
	10^{-6}	0	12.4	0
	10^{-5}	0	12.8	0
	10^{-4}	0	13.1	0
	10^{-3}	0	13.5	0

* k_i calculated for the first 100 min of the oxidation reaction.
† k_a calculated as sum of the k_i values for each catalyst alone.
‡ k_i for the period 0–70 min.

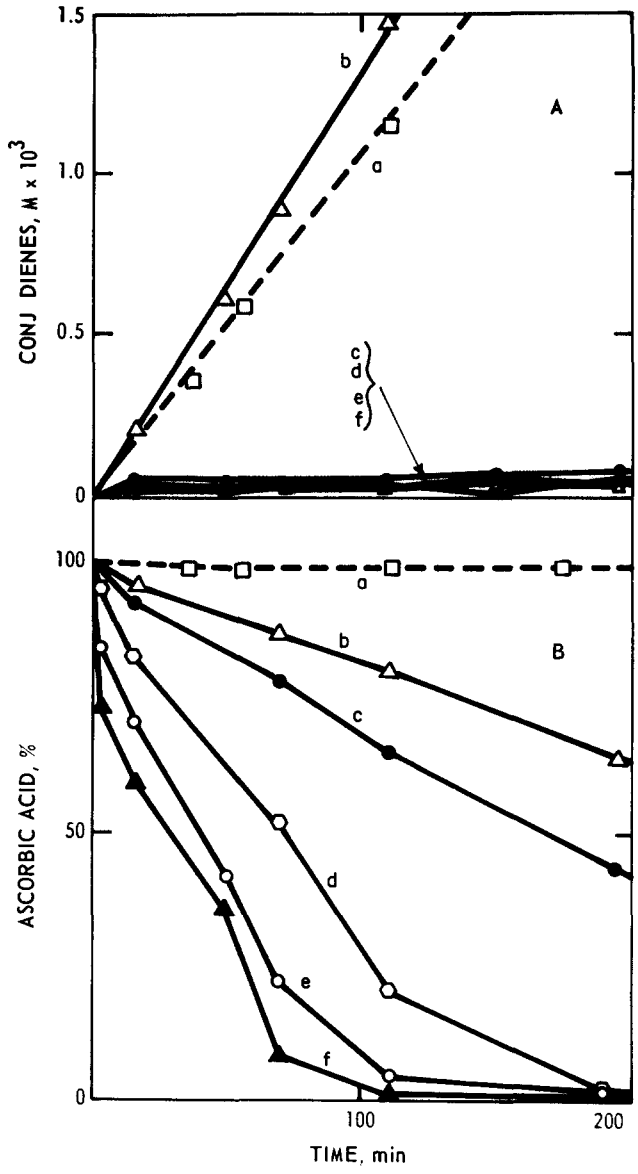

FIG. 2. Concurrent oxidation of (A) linoleate, 0.02 M measured by formation of conjugated dienes, and of (B) ascorbic acid, in a reaction mixture containing ascorbic acid at an initial concentration of 1.8×10^{-3} M, and copper at various concentrations: *a*, no copper; *b*, 1.3×10^{-7}; *c*, 1.3×10^{-6}; *d*, 1.3×10^{-5}; *e*, 1.3×10^{-4}; *f*, 1.3×10^{-3} M.

effect of the combination was to enhance or to inhibit the oxidation is emphasized by the ratio k_i/k_a, which is the ratio of the rate constant for the catalysts in combination to the rate constant assuming an additive effect. The 1.8×10^{-6} and 1.8×10^{-5} M concentrations of ascorbic acid were most effective in enhancing the reaction. The higher concentrations of ascorbic acid when used in conjunction with the higher concentrations of copper, completely inhibited formation of conjugated dienes.

Fig. 2 illustrates the concurrent oxidation of ascorbic acid and linoleate when the reaction mixture initially

contained ascorbic acid at 1.8×10^{-3} M and copper at various concentrations. When no copper was present, conjugated dienes formed rapidly (Fig. 2 A) without oxidation of ascorbic acid (Fig. 2 B). The lowest concentration of copper (1.3×10^{-7} M) enhanced conjugated diene formation and catalyzed oxidation of ascorbic acid. Conjugated diene formation was inhibited at higher concentrations of copper, whereas the rate of oxidation of ascorbic acid increased with increasing copper concentrations. Similar experiments using ascorbic acid at 1.8×10^{-4} M gave similar results.

Table 2 contains results of an experiment in which the degradation of ascorbic acid was measured by determining both ascorbic acid and dehydroascorbic acid. As dehydroascorbic acid was formed, it was further degraded to compounds that were not reducible to ascorbic acid. At the higher copper concentrations, less ascorbic acid was recovered upon reduction. In these experiments, inhibition of conjugated diene formation continued for about 920 and 980 min at respective copper concentrations of 1.3×10^{-3} and 1.3×10^{-4} M. Therefore, the oxidation was inhibited for about 600 min after degradation of the ascorbic and dehydroascorbic acids.

Experiments were conducted in which the oxidation was catalyzed first with ascorbic acid, and then copper was added at various concentrations. The addition of the copper caused inhibition in the same manner as when both copper and ascorbic acid were added initially (Fig. 3 A). At copper concentrations of 1.3×10^{-7} and 1.3×10^{-6} M, the rate of conjugated diene production was increased slightly; but at concentrations of 1.3×10^{-5}, 1.3×10^{-4}, and 1.3×10^{-3} M inhibition occurred immediately (Fig. 3 A). Also, the conjugated diene concentration dropped suddenly, indicating that conjugated dienes were decomposed to substances that did not absorb at 233 nm. After the addition of copper, ascorbic acid was oxidized at rates that increased with copper concentration (Fig. 3 B).

TABLE 2 OXIDATION OF ASCORBIC ACID IN THE MODEL SYSTEM CONTAINING BOTH ASCORBIC ACID AND COPPER

Time	Copper, 1.3×10^{-3} M,* Ascorbic Acid†		Copper, 1.3×10^{-4} M,* Ascorbic Acid†	
	Before	After	Before	After
min	%	%	%	%
137	100	—	100	—
139	78	89	88	95
147	72	79	81	87
172	24	40	56	68
183	17	31	41	58
220	1	9	19	31
240	0	5	13	20
300		0	0	3
360		0	0	1

* Added at 137 min to the model system in which the formation of conjugated dienes was catalyzed by ascorbic acid (1.9×10^{-3} M).

† Determined before (measuring only ascorbic acid) and after measuring both ascorbic acid and dehydroascorbic acid) treatment with homocysteine.

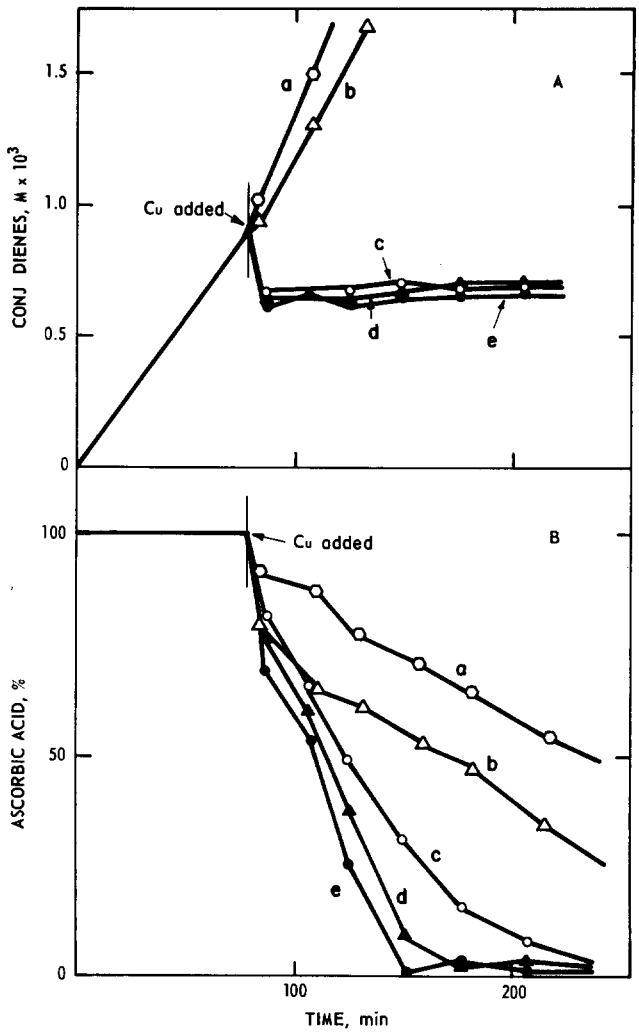

FIG. 3. Effect of addition (at 80 min) of copper at selected concentrations on (A) the formation of conjugated dienes catalyzed by ascorbic acid and on (B) the oxidation of ascorbic acid. Initial concentration of ascorbic acid 1.8×10^{-3} M. Concentrations of copper: *a*, 1.3×10^{-7}; *b*, 1.3×10^{-6}; *c*, 1.3×10^{-5}; *d*, 1.3×10^{-4}; *e*, 1.3×10^{-3} M.

In a similar experiment (Fig. 4) in which further ascorbic acid was added after the reaction had been inhibited by copper, and after the ascorbic acid that was originally present had been oxidized, the addition of further ascorbic acid did not result in formation of conjugated dienes (Fig. 4 A).

Experiments were done to determine whether the transition from enhancement to inhibition of the oxidation with increasing concentrations of copper or ascorbic acid is gradual or sudden. Fig. 5 contains results of an experiment in which effects of concentrations of copper intermediate between 1.3×10^{-6} and 1.3×10^{-5} M were studied. In the presence of ascorbic acid at 1.9×10^{-3} M,

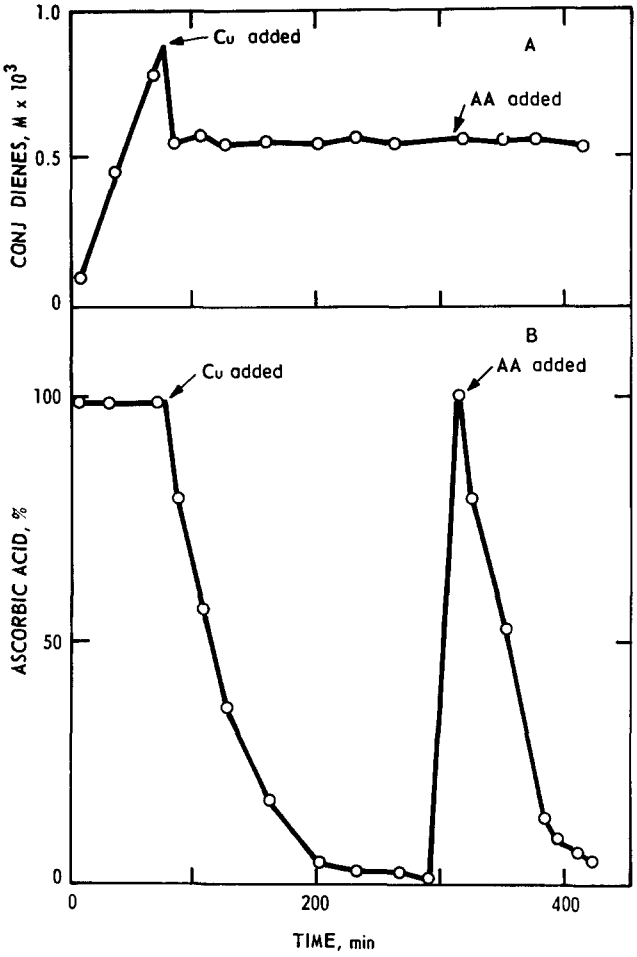

FIG. 4. Effect of additions of copper and ascorbic acid on (A) formation of dienes and on (B) oxidation of ascorbic acid. Ascorbic acid was present initially at 1.8×10^{-3} M; copper at 1.3×10^{-3} M was added after 80 min, and ascorbic acid at 1.8×10^{-3} M was again added after 320 min.

copper added at 4.4×10^{-6} M promoted diene formation; but at 9.9×10^{-6} M it inhibited the reaction. Evidently there are critical combinations of concentrations which cause the effect to change suddenly from one of enhancement to one of inhibition.

To determine whether the sequence of addition influences the effects, the linoleate was oxidized by copper, and ascorbic acid was added later (Fig. 6). In the presence of 1.3×10^{-3} M copper, 2.4×10^{-3} and 2.4×10^{-4} M ascorbic acid caused inhibition, while 2.4×10^{-5} M enhanced the oxidation.

Fig. 7 illustrates how copper affected the TBA test of the model system. As long as ascorbic acid was the only catalyst present, the amount of TBA pigment increased only slightly even though the concentration of dienes indicated considerable oxidation. As soon as copper was added, there was a sharp increase in TBA absorbance, followed by a leveling off.

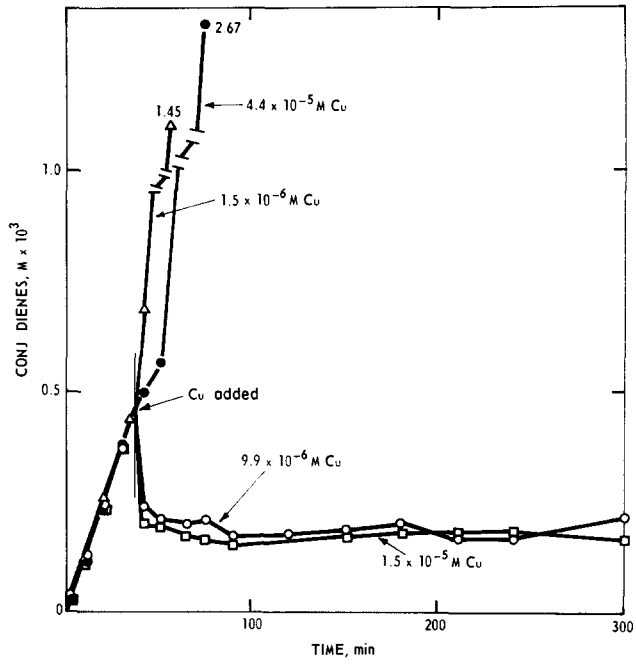

FIG. 5. Effect of addition (at 38 min) of selected concentrations of copper on the formation of conjugated dienes in the presence of ascorbic acid at an initial concentration of 1.9×10^{-3} M.

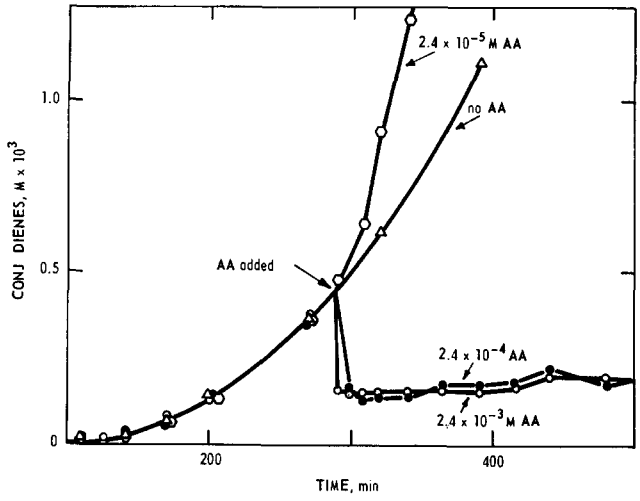

FIG. 6. Effect of addition (at 188 min) of ascorbic acid at selected concentrations on the formation of conjugated dienes catalyzed by 1.3×10^{-3} M copper.

In a similar experiment that was extended for a longer period (Fig. 8), both conjugated diene formation and TBA absorbance increased rapidly after oxidation started again.

In a comparative study, the prooxidant activity of ferric ion, cupric ion, and ascorbic acid increased in that order (Fig. 9). The curvature depicted for iron and copper indicates the autocatalytic nature of the reactions catalyzed by the metals.

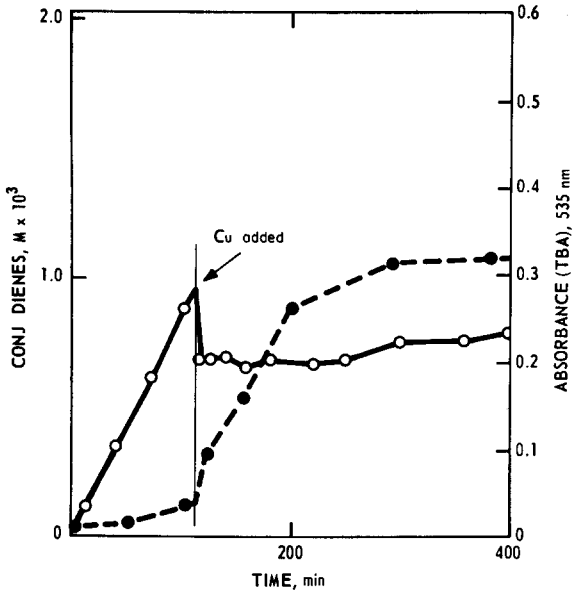

FIG. 7. Formation of conjugated dienes (solid line) and TBA pigment (broken line) during oxidation of linoleate for 400 min. The reaction was catalyzed by 1.8×10^{-3} M ascorbic acid. Copper (1.3×10^{-4} M) was added at 131 min.

The addition of ascorbic acid to a reaction mixture containing iron greatly increased the rate of formation of conjugated dienes (Fig. 10).

DISCUSSION

Enhanced Prooxidant Effect of Ascorbic Acid and Copper Combined

The catalytic activity of ascorbic acid at low concentrations with copper at any of the concentrations tested was greater than the sum of the effects of the catalysts individually. Three possible explanations for this enhanced catalysis are proposed.

1. Ascorbic acid reduces copper to the cuprous form, which, like ferrous ion as compared to ferric, is the more active catalyst (10, 11).

2. The presence of copper increases the concentration of semidehydroascorbic acid radical through catalysis of the oxidation of ascorbic acid. The radical is an intermediate in the oxidation reaction (12–14).

3. The presence of ascorbic acid and copper together favors the formation of a reactive oxygen radical in a metal–ascorbic acid–oxygen complex (13). This complex is formed during oxidation of ascorbic acid in the presence of copper.

The data do not provide information regarding the relative importance of the three factors in contributing to the enhanced catalysis of combinations of ascorbic acid and copper.

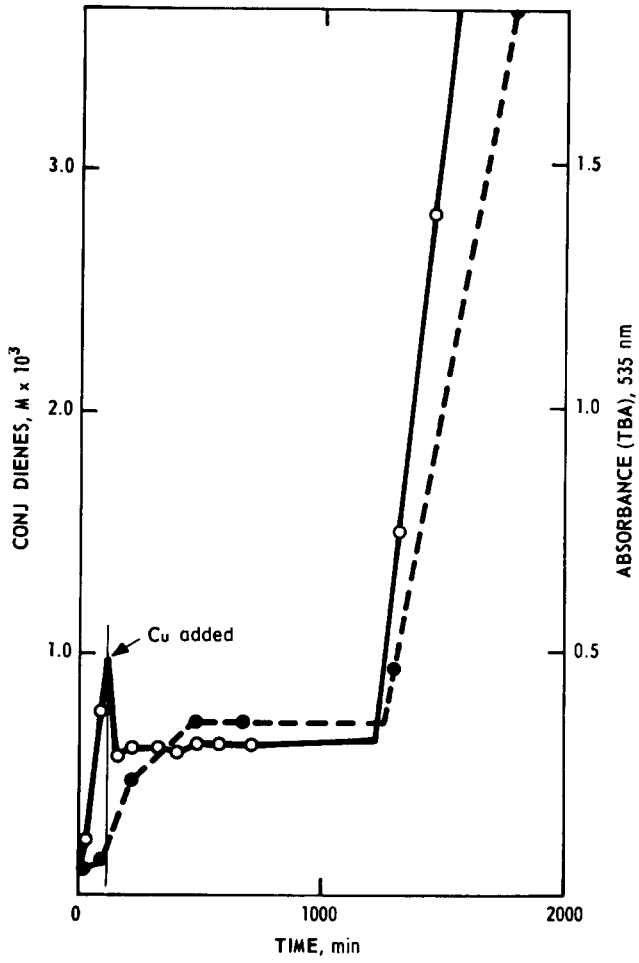

FIG. 8. Formation of conjugated dienes (solid line) and TBA pigment (broken line) during oxidation of linoleate for 2000 min. The reaction was catalyzed by 1.8×10^{-3} M ascorbic acid. Copper (1.3×10^{-4} M) was added at 137 min.

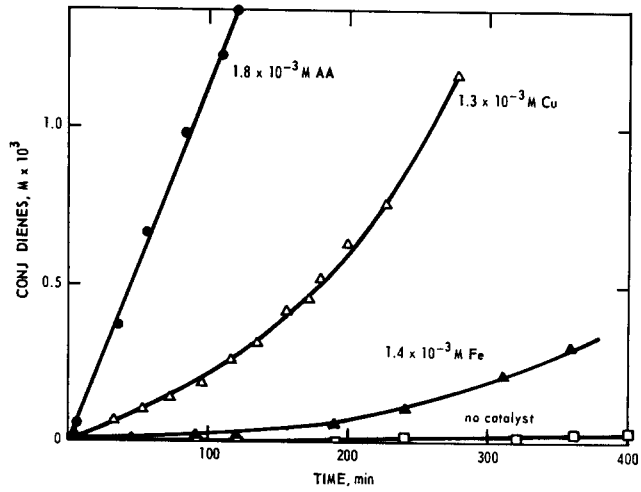

FIG. 9. Comparison of the catalytic effects of ascorbic acid (AA), cupric ion (Cu), and ferric ion (Fe) on the formation of conjugated dienes in linoleate oxidation.

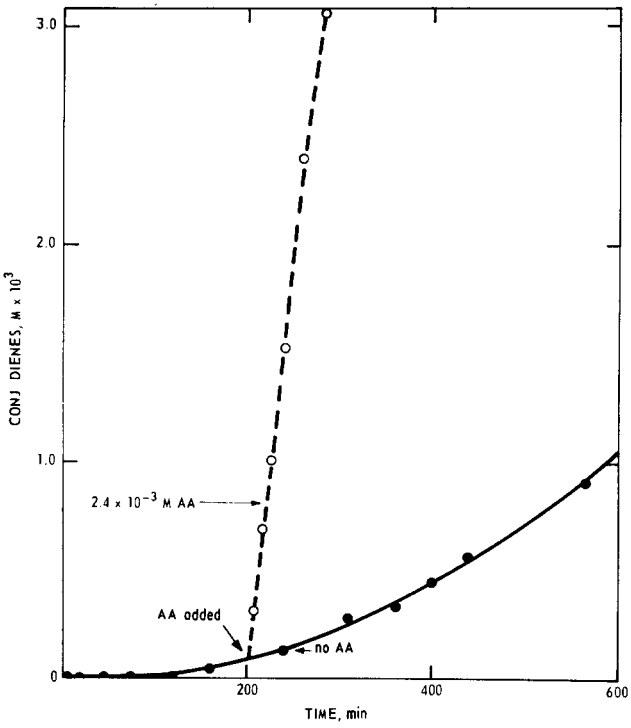

FIG. 10. Effect of addition of ascorbic acid on the formation of conjugated dienes catalyzed by Fe^{+++}. Solid line: 1.4×10^{-3} M iron; broken line: 1.4×10^{-3} M iron with addition of ascorbic acid (2.4×10^{-3} M) at 200 min.

Inhibition by Combinations of Ascorbic Acid and Copper

Some combinations of ascorbic acid and copper inhibited the formation of conjugated dienes but not the oxidation of ascorbic acid, and caused a rapid loss of part, but not all, of the conjugated dienes. The following hypothesis is proposed as a partial explanation for these phenomena. Combinations of ascorbic acid and copper at concentrations that prevent production of conjugated dienes result in the formation of free-radical inhibitors. The inhibitors prevent the initiation step in the oxidation of linoleate, whether the catalyst is ascorbic acid or copper. Formation of the radical inhibitors does not interfere with the copper-catalyzed oxidation of ascorbic acid, or the degradation of lipid oxidation products to TBA-reactive materials. The results are discussed in relation to this hypothesis.

When both ascorbic acid and copper were present in the reaction mixture, and the concentration of either was increased, the activity of the combination suddenly changed from a prooxidant effect that was greater than additive to a termination of conjugated diene formation (Fig. 1). The marked change is emphasized by the ratios of k_i/k_a (Table 1), which are all either greater than 1 or are 0, with no intermediate values. The small difference in concentration of catalyst needed to produce the change is shown in Fig. 5. The inhibition occurred at the higher concentrations of ascorbic acid; and the higher the concentration of ascorbic acid, the lower the copper concentration needed for inhibition.

Inhibition occurred only when both ascorbic acid and copper were added to the reaction mixture, but the order of addition did not influence the result (Figs. 5 and 6). Therefore, the inhibition is attributable to an effect of ascorbic acid and copper in combination.

The rate of oxidation of ascorbic acid increased with the concentration of copper when both ascorbic acid and copper were present (Figs. 2 and 3). Combinations of ascorbic acid and copper that inhibited lipid oxidation did not inhibit oxidation of ascorbic acid.

No relation was observed between either the rate or extent of oxidation of ascorbic acid and the inhibition of conjugated-diene formation. Inhibition continued after the ascorbic acid had been oxidized to a concentration less than that needed to cause inhibition in the fresh reaction mixture at the start of the experiments, and even after all of the dehydroascorbic acid was oxidized. Thus, inhibition of conjugated diene formation continues after the conditions that initially caused the inhibition no longer prevail.

Ascorbic acid inhibited oxidation when added to a reaction mixture in which copper was catalyzing lipid oxidation (Fig. 6). Combinations of ascorbic acid and copper that cause inhibition apparently interfere with the initiation of lipid oxidation by radicals formed during degradation of hydroperoxide. Likewise, the combinations interfere with initiation of the formation of conjugated dienes by ascorbic acid, which we attributed to attack by the semidehydroascorbic acid radical (1).

We postulate that combinations of ascorbic acid and copper that inhibit lipid oxidation do so by the formation of free-radical inhibitors which terminate the free-radical chain reactions, and that the inhibitors are complexes that include the free radicals. The complexes do not include ascorbic or dehydroascorbic acid, because inhibition continues after both are oxidized. The formation of complexes of free radicals with copper has been postulated (15, 16). If the formation of such complexes is influenced by ascorbic or dehydroascorbic acid, the complexes could be of special importance in explaining the inhibition of lipid oxidation by combinations of ascorbic acid and copper. This hypothesis warrants study.

Other explanations of the inhibition of lipid oxidation have been proposed. An explanation based on inhibition by degradation products of ascorbic acid (4) does not conform to the data. Inhibition occurred immediately after addition of the ascorbic acid at concentrations at or above those required for this effect, without a delay for formation of degradation products. Also, at concentrations of either catalyst slightly less than that needed to cause inhibition, degradation products would have accumulated, but more slowly. Hence, in accordance with

this explanation, inhibition would have been expected after enough ascorbic acid had been oxidized to produce inhibiting products. Rapid oxidation that uses up available oxygen (17–19) does not acount for the inhibition, because sufficient oxygen remained to continue oxidation of the ascorbic acid. An explanation for the inhibition of lipid oxidation must account for the continued activity of copper in catalyzing oxidation of ascorbic acid.

Ascorbic acid is considered an antioxidant in many lipid systems. In our experiments, ascorbic acid alone was always prooxidant, a result consistent with previous reports of other investigators (20–22). When inhibition was observed, both copper and ascorbic acid were present. Oxidation was promoted by either catalyst alone, but was inhibited by addition of the other. Therefore, it is probably better to attribute the inhibitory function to a synergistic combination of ascorbic acid and copper, rather than to consider either one as the antioxidant.

In many materials in which lipid oxidation is inhibited by the addition of ascorbic acid (e.g. King's [4] fat globule membrane material), copper, and probably synergists such as polyphenolic compounds, are normally present. Therefore, results of such studies should be interpreted in relation to the combined effects of ascorbic acid and copper rather than as effects of added ascorbic acid alone. Other constituents probably influence the reactions. However, it is not necessary to postulate a specific role for proteins such as that proposed by King (4), because combinations of ascorbic acid and copper can cause either enhanced or inhibited lipid oxidation in simple systems that do not contain protein. But, because of their chelating properties, proteins can influence lipid oxidation catalyzed by copper.

Degradation of Conjugated Dienes

Figs. 3 and 8 illustrate a second effect of catalyst combinations that inhibit formation of conjugated dienes. Addition of either ascorbic acid or copper to a reaction mixture in which diene formation was catalyzed by the other caused a sudden drop in the concentration of conjugated dienes. The catalysts in combination caused a rapid degradation of part, but not all, of the dienes. This indicates the presence of at least two compounds that contain conjugated double bonds, and that differ in stability. In the conditions prevailing in the reaction mixture, one was unstable and the other stable.

The concentration of TBA-reactive compounds continued to increase for some time after the formation of dienes was inhibited, and then leveled off (Fig. 7). This behavior is explainable on the basis that the secondary oxidation products of the linoleic acid continue to be degraded until all are converted to TBA-reactive products, after which the TBA absorbance no longer increases. This explanation also accounts for the observation that after the formation of conjugated dienes is no longer inhibited (Fig. 8), TBA-reactive compounds are formed along with conjugated dienes.

The possibility was considered that the combined catalysts caused such rapid decomposition of hydroperoxides that no increase occurred even though oxidation of the lipid continued. With such a mechanism, it is difficult to imagine why the concentration of conjugated dienes would remain essentially constant at the level to which it decreased immediately after addition of the second catalyst (Figs. 3–7). Also, a continued increase in TBA absorbance would be expected, rather than the changes depicted in Figs. 7 and 8.

Catalytic Activity of Iron

When compared at similar concentrations, iron is less active than copper in catalyzing formation of conjugated dienes (Fig. 9). As with copper, the catalytic activity of iron is enhanced greatly when it is present in combination with ascorbic acid (Fig. 10). The data in Fig. 10, however, illustrate a difference in activity of the metals. At the concentrations used, substitution of copper for iron would have resulted in inhibition, rather than enhancement, of conjugated diene formation.

Interpretation of Resuls of the TBA Test

The red color in the TBA test is believed to result from reaction of TBA with malonaldehyde, or products of lipid oxidation that are degraded to malonaldehyde, during the heating at low pH involved in the test (23–26). Results reported in the first paper in this series (9) support the conclusion that, in the absence of copper, the TBA test measures secondary oxidation products of lipid oxidation, but not hydroperoxides. The presence of copper, and possibly other catalysts, increases the intensity of the red color (27–29). The increase is attributed to catalysis of degradation of the lipid-oxidation products, or of the color-forming reactions, or both.

In accordance with the proposed explanation for the changes in TBA absorbance in Figs. 7 and 8, only some of the conjugated dienes are degraded when the test is made in the presence of copper. The proportion that is converted to TBA-reactive materials influences the relation between results of the TBA test and other measures of lipid oxidation. Although results of TBA tests frequently correlate well with other measures of lipid oxidation, such as absorption by conjugated dienes, further characterization of the reactive compounds is needed to provide a more reliable basis for interpreting the data.

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