Ascorbic acid and copper in linoleate oxidation. I. Measurement of oxidation by ultraviolet spectrophotometry and the thiobarbituric acid test

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ABSTRACT The UV absorption method and the thiobarbituric acid (TBA) test for oxidation of an aqueous suspension of linoleate were compared. The absorption method depends on the formation of hydroperoxides having conjugated double bonds that absorb strongly at 233 nm. The absorption at 233 nm increased markedly during oxidation of linoleate catalyzed by either ascorbic acid or cupric ions. The concentration of ascorbic acid in the reaction mixture was also measured by UV absorption at 265 nm and pH 7.0.

Color development in the TBA test also increased markedly with the extent of oxidation of linoleate. When ascorbic acid was the catalyst, UV absorption detected early stages of oxidation with greater sensitivity than the TBA test. The reverse was true when Cu^{++} was the catalyst. In general, the relation between the two procedures will depend on whether copper is present when the TBA test is made.

SUPPLEMENTARY KEY WORDS buffered linoleate model system · hydroperoxides · conjugated dienes · secondary products · metal contamination

BOTH ASCORBIC ACID and cupric ions function as catalysts in lipid oxidation processes (1). Much of the literature regarding the mode of action of these oxidation catalysts in foods and biological materials is difficult to interpret because of the large number of variables in the system. Even in simple systems the formation of oxidation products complicates interpretation of results. For example, when copper and ascorbic acid are present together, ascorbic acid is oxidized simultaneously with the lipids; and degradation products of ascorbic acid are reported to influence lipid oxidation (2). Hence, many peculiarities related to the roles of ascorbic acid and copper in lipid oxidation remain unexplained.

Spectrophotometry in the ultraviolet range has become extremely valuable as a means to measure lipid oxidation. For example, as linoleic acid oxidizes, absorption by conjugated double bonds in hydroperoxides increases, with a maximum in the region of 231–234 nm (3). The rate of development of conjugated dienes correlates closely with oxygen uptake by the linoleic acid (4–6) and with iodometric determination of peroxide value (7). Johnston, Zilch, Selke, and Dutton (8) concluded that UV absorption provides a more sensitive insight into the oxidation process than does peroxide determination. However, in linoleate oxidation, only during the first part of the reaction is there correspondence between diene conjugation, absorption of oxygen, and concentration of hydroperoxides as determined by peroxide value and chromatography (3, 8, 9). Despite this limitation, UV spectroscopy has proved an excellent tool in the study of the oxidation of fatty materials, linoleic acid in particular.

The TBA test (2, 10–12) has also attracted considerable attention during recent years. The pigment produced in this sensitive color reaction is a condensation product between TBA and malonaldehyde, a product of fatty acid oxidation (13, 14). A feature of the TBA test

Abbreviations: TBA, thiobarbituric acid; ppm, parts per million.

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is its moderate specificity for oxidation products of polyunsaturated fatty acids and its apparent correlation with flavor deterioration. The latter makes it particularly useful for investigations of food lipids (11).

In this study, a simple system was used as a model to represent the more complicated systems in many biological materials. The model consisted of buffered linoleate as substrate, to which oxidation catalysts were added. UV absorption was used to measure oxidation of both linoleate and ascorbic acid. Comparisons with measurements by the TBA test also are presented.

MATERIALS AND METHODS

Preventing Contamination

Extreme care was taken to avoid contamination by copper from reagents or laboratory apparatus. Glassware and equipment were immersed for 24 hr in a solution (200 ppm) of EDTA and rinsed three or four times with deionized water (11). KOH was freed of metals by extraction of a concentrated solution with a solution of dithizone (Eastman) in CCl_4 (15). HCl was purified by redistillation of a constant-boiling mixture (15). We prepared the potassium phosphate buffer by stirring 1 M KH_2PO_4 with Dowex A-1 (Dow Chemical Co.) for 24 hr, decanting the solution, diluting with deionized water, and adjusting to pH 7 with decontaminated KOH. Decontaminated H_3PO_4 was prepared by passage of a solution of KH_2PO_4 through a Chelex (Bio-Rad Laboratories, Richmond, Calif.) column to remove copper ions, and then through an AG50W-X8 (Bio-Rad Laboratories) column in H^+ form to exchange K^+ for H^+.

Buffered Linoleate

We made a solution of linoleate (16) by neutralizing linoleic acid (Hormel Institute, Austin, Minn.) with KOH and diluting to 0.1 M linoleic acid with deionized water. Portions (12 ml) were pipetted into nitrogen-gassed 125-ml flasks, the flasks were gassed again, corked, waxed, and stored at −20°C. For use, portions were thawed, adjusted to pH about 6.6 with H_3PO_4, and diluted to 0.02 M potassium linoleate with 0.1 M potassium phosphate buffer (pH 7.0). The resulting buffered linoleate had a pH of 7.0 ±0.1 at 37°C.

Reaction Conditions

Oxidations were carried out in 25- or 50-ml flasks containing 15, 20, or 40 ml of buffered linoleate shaken continuously in a constant-temperature water bath at 37°C. L-ascorbic acid (Baker Analyzed) was added to the reaction flask in 0.03- to 0.7-ml quantities of appropriate stock solutions, prepared just before the start of the experiment. When high concentrations of ascorbic acid were used, additional KOH was added to adjust the reaction mixture to pH 7.0. Copper was added in 0.02- to 0.20-ml quantities of appropriate stock solutions of cupric sulfate.

Measuring Oxidation of Linoleate

To measure oxidation of the linoleate, we mixed 0.5 ml of the reaction mixture with 5 ml of 60% ethanol containing 200 ppm EDTA. Absorbance was then determined at 233 nm with a Gilford model 2000 spectrophotometer in 10-mm or 1-mm quartz cells. Calculation of the concentration of conjugated dienes was based on a molar absorptivity ϵ of 26,000 (17). The unoxidized model system always had some absorbance (usually between 0.200 and 0.300) at zero reaction time, and appropriate correction was made.

A reaction mixture containing 1.8×10^{-3} M ascorbic acid was included in most experiments for reference purposes, to provide a basis for comparing results of different experiments, and for detecting contamination by metals. Frequently, buffered linoleate without added catalyst also was included. The results obtained with these reference samples usually were reproducible. Erratic data were thought to result mainly from accidental contamination by traces of metals, despite the care taken to eliminate this source of error. When erratic data were obtained, results of the entire experiment were discarded.

A Cary 15 spectrophotometer was used for observations of ultraviolet absorption spectra within the range of 225–300 nm.

Measuring Ascorbic Acid Oxidation

Absorbance at 265 nm was used as a measure of ascorbic acid concentration (18, 19). The readings were made on the same sample that was used for the measurements at 233 nm. To avoid the necessity for eliminating metal contamination after the sample was taken, EDTA (200 ppm) was included in the 60% ethanol solvent.

TBA Test

For the TBA test the procedure of Dunkley and Franke (11) was used with minor modifications (principally related to reducing the size of the sample to 4 ml).

RESULTS

The Model System

The buffered linoleate was a milky-white suspension. Examination with a phase microscope showed small droplets which did not change in appearance when catalysts were added or during oxidation of the linoleate. The suspension did not separate into layers on standing or when centrifuged at 3000 rpm for 15 min. In most experiments there was no change in macroscopic appearance of

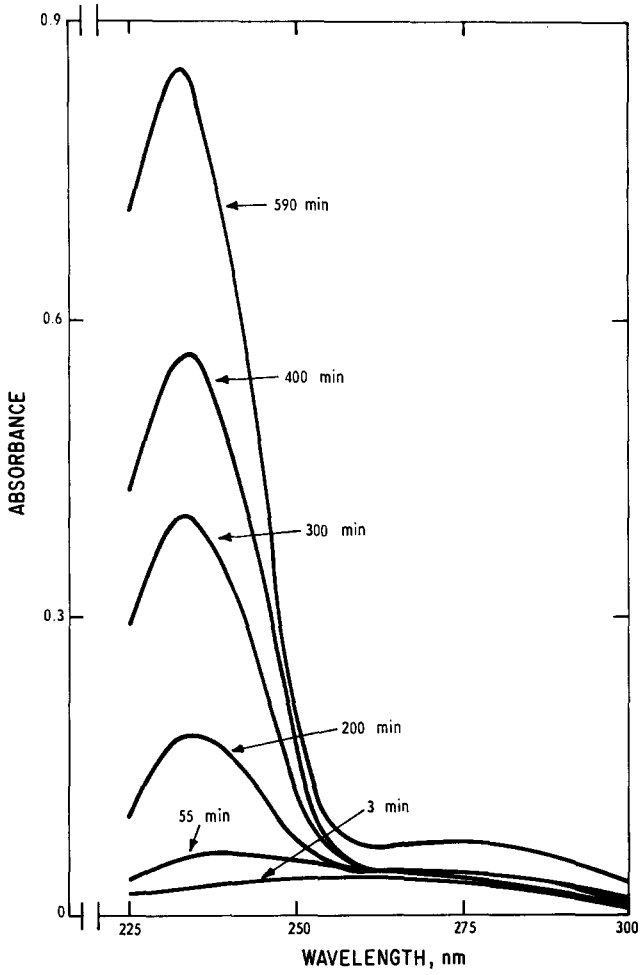

FIG. 1. Changes in UV absorption in a reaction mixture during oxidation of aqueous dispersion of potassium linoleate (0.02 M) at pH 7.0 with Cu^{++} (1.3×10^{-3} M) as catalyst. 1-mm quartz cuvette and 60% ethanol as diluting solvent (1:11 dilution). Cary 15 spectrophotometer.

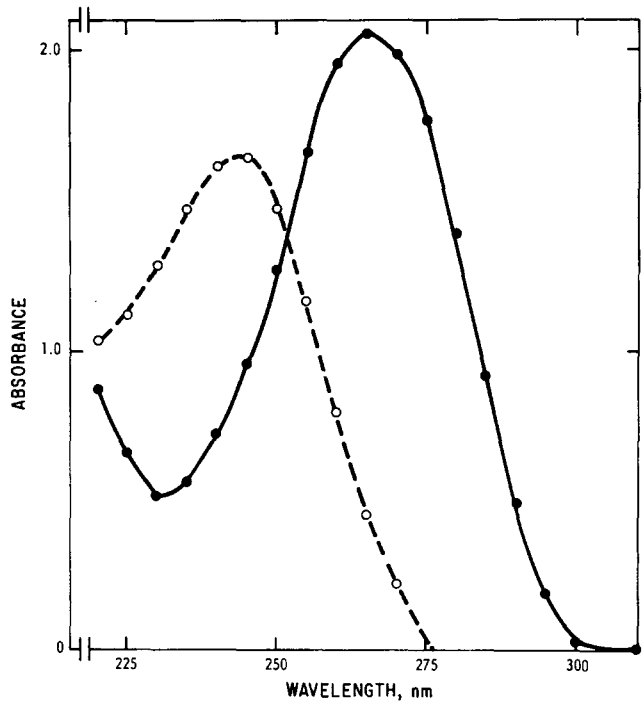

FIG. 2. Absorption spectra of ascorbic acid (concentration, 1.8×10^{-3} M, in 0.1 M phosphate buffer). Solid line, pH 7.0; broken line, pH 1.0. 10-mm quartz cuvette and 60% ethanol containing 200 ppm EDTA as diluting solvent (1:11 dilution). Gilford model 2000 spectrophotometer.

the model system during the oxidation period. In a few, a yellowish-brown hue developed toward the end of long oxidation periods in the presence of ascorbic acid. This was attributed to extensive oxidation accompanied by polymerization of reaction products. Agreement of results of replicate experiments provided evidence that the suspensions were reproducible.

The rate of oxidation of the linoleate was dependent on the buffer concentration. As the concentration was increased, a higher rate of reaction was observed. However, at concentrations of 0.2 M phosphate and above, the system became unstable, and flocculation and separation of the fatty acid occurred. Hence, all experiments were done with a reaction mixture containing 0.1 M phosphate buffer.

UV Absorption of the Linoleate Model System

The buffered linoleate was too turbid for direct measurement of absorbance. Therefore, portions of the reaction mixture were removed at selected intervals and diluted with a solvent that yielded a clear solution. Among the solvents tried (95% ethanol, 60% ethanol, diethyl ether, methylene chloride, hexane, *p*-dioxane), 60% ethanol proved to be the most suitable. An advantage of removing samples for the absorbance measurements was that possible catalysis by the UV irradiation of the reaction mixture was avoided.

Changes between 225 and 300 nm in the absorption spectra of the model system during oxidation of the linoleate are depicted in Fig. 1. Maximum absorption was observed at 233 nm, and its intensity increased with time. If the conjugated hydroperoxides had decomposed during the reaction period, with the formation of aldehydes and ketones, an incease in absorption around 280 nm (20) would have been expected. Such an increase was not observed up to 400 min. At 590 min a slight absorption increase at 280 nm became apparent. On the basis of these results, absorbance at 233 nm was adopted as a measure of the extent of the oxidation during early stages of the reactions.

Ascorbic acid also was determined by absorption measurements. Fig. 2 depicts absorption spectra at pH 1.0 and 7.0. An advantage of using absorption at pH 1.0 and 245 nm to determine ascorbic acid is that the low pH stabilizes the ascorbic acid (21–24). Adjusting the pH to 1.0, however, leads to interference between absorption by

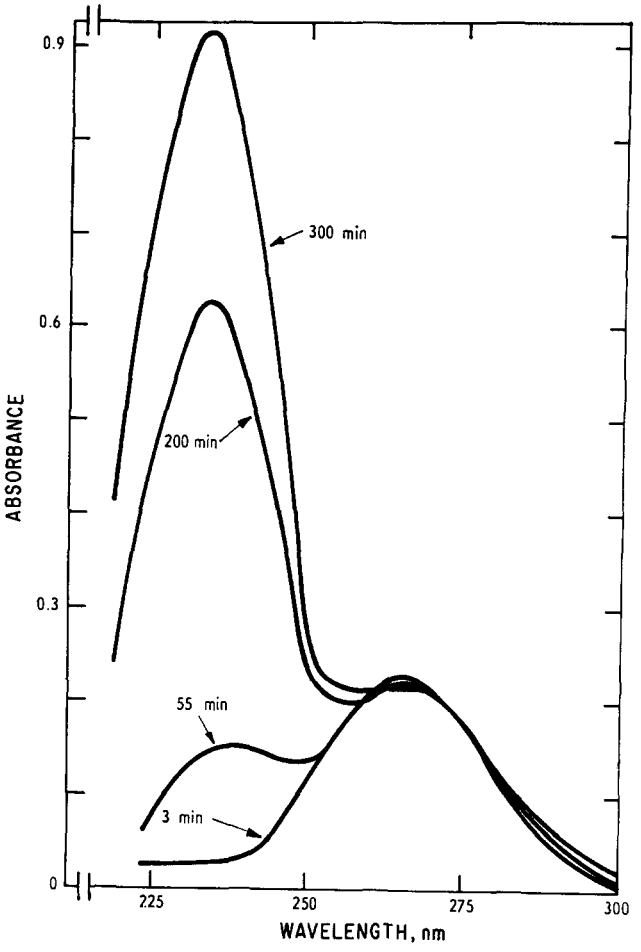

FIG. 3. Spectral changes in a linoleate reaction mixture as a function of time, with ascorbic acid (1.8×10^{-3} M) as catalyst. 1-mm quartz cuvette and 60% ethanol as diluting solvent (1:11 dilution). Cary 15 spectrophotometer.

ascorbic acid at 245 nm and conjugated dienes at 233 nm. Therefore, the absorption at 265 nm at pH 7.0 was used to determine the concentration of ascorbic acid. The ascorbic acid was stabilized by including EDTA (200 ppm) in the solvent. The molar extinction coefficient, ϵ, for ascorbic acid under these conditions was 14,200.

Fig. 3 presents absorption spectra for conjugated dienes and ascorbic acid during an experiment in which the oxidation of the linoleate was catalyzed by ascorbic acid. The absorption of ascorbic acid at 233 nm caused little interference with the absorption of the conjugated dienes at this wavelength. In calculating the concentration of the conjugated dienes, subtraction of the absorption at zero time provided a correction for interference by the ascorbic acid.

Interrelation between UV Absorption and TBA Results

Rates of oxidation of linoleate catalyzed by ascorbic acid or cupric ions and measured by development of conjugated dienes, are illustrated in Fig. 4. With both catalysts, oxidation was detected quickly. Initially, the rate of oxidation appeared to be faster when catalyzed by ascorbic acid than when catalyzed by cupric ions.

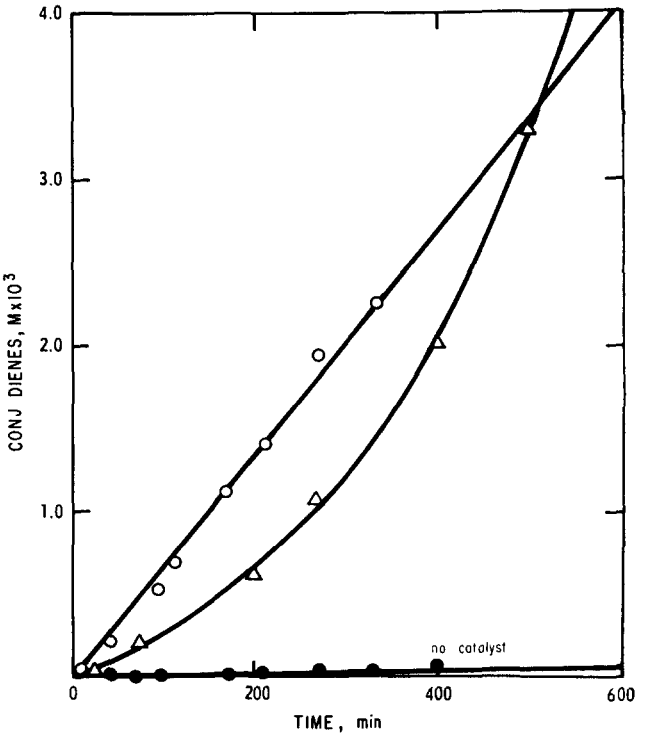

FIG. 4. Formation of conjugated dienes during linoleate oxidation with 1.3×10^{-3} M Cu^{++} (△) or 1.8×10^{-3} M ascorbic acid (○) as catalyst. Molar absorbance of dienes taken as 26,000 (17). ●, no catalyst.

A distinctly different relationship between results with the two catalysts was obtained when the linoleate oxidation was measured by the TBA test (Fig. 5). Absorbance in the TBA test increased immediately with copper as catalyst, but with ascorbic acid, the color increased slowly for the first 150 min, rapidly thereafter. With both tests, little oxidation was detected when no catalyst was added.

The relation between results of the two measures of linoleate oxidation is shown in Fig. 6. When the reaction was catalyzed by ascorbic acid, there was an appreciable formation of conjugated dienes before color development occurred in the TBA test. In contrast, with copper as catalyst, in the early stages of the oxidation, values obtained by the TBA test increased more rapidly than absorption by conjugated dienes.

DISCUSSION

The Model System

The model system that was adopted simulated selected conditions that influence lipid oxidation in foods and

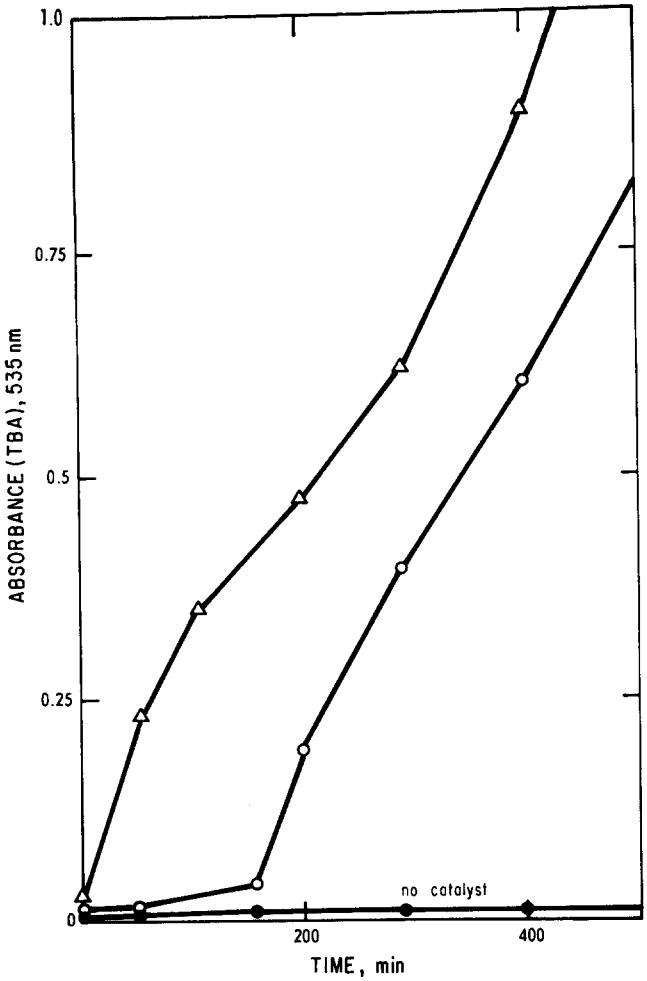

FIG. 5. TBA absorbance at 535 nm, during linoleate oxidation with copper or ascorbic acid as catalyst. △, 1.3×10^{-3} M Cu; ○, 1.8×10^{-3} M ascorbic acid; ●, no catalyst.

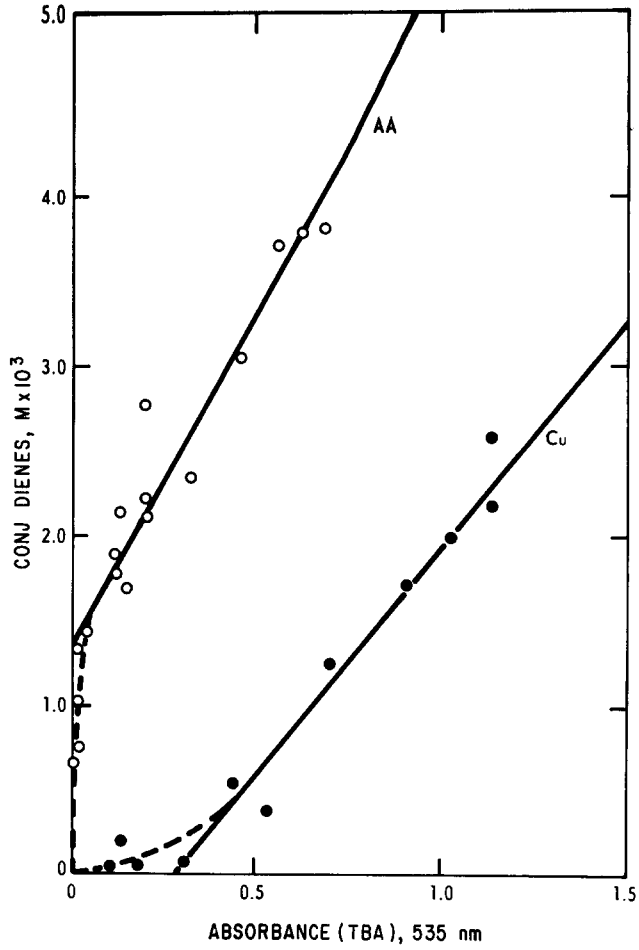

FIG. 6. Relation between concentration of conjugated dienes and TBA absorbance at 535 nm during the early stage of the oxidation of lionleate. *AA*, with ascorbic acid as catalyst, $C_t \times 10^3 = 3.89T + 1.38$; *Cu*, with copper as catalyst, $C_t \times 10^3 = 2.78T - 0.783$ (C_t = molar concentration of dienes at time t; T, absorbance [TBA] at 535 nm). The curves represent a compilation of data for various concentrations of ascorbic acid and copper. For both curves, values below a TBA absorbance of 0.100 were not used in the calculation of the equations.

biological materials. To minimize variables and to provide reproducible conditions, a number of restrictions were arbitrarily imposed. These included standardizing both the lipid substrate and its concentration, and the pH, composition, and concentration of the buffer. The scope of the study was further limited by emphasizing early stages of the oxidation during which secondary reactions did not unduly complicate the interpretation of the results.

Diene Conjugation as a Measure of the Rate of Oxidation

The primary oxidation products of lipids are hydroperoxides (1, 25–27). In the case of linoleic acid, a conjugated system is formed first by detachment of hydrogen from an α-carbon and migration of the double bond (17, 28, 29). Subsequently, reaction with oxygen results in formation of conjugated hydroperoxides. The conjugation gives rise to UV absorption. Since most of the hydroperoxides are conjugated, determination of absorbance gives a measure of the hydroperoxides present (4, 6, 30).

Data obtained by absorption measurements at 233 nm during oxidation of linoleic acid correlate closely with results obtained by other measures of lipid oxidation, such as the Warburg technique and peroxide value determination (4, 30). Moreover, since absorption by conjugated dienes gives an indication of the extent of the first step of the oxidation sequence, the absorption method is especially suited for study of the initiation and early stages of the oxidation. Another advantage of the spectrophotometric procedure is that oxidation of ascorbic acid can also be followed by absorption measurements. Hence, the spectrophotometric procedure was particularly useful in this study.

The presence of the ascorbic acid and its simultaneous oxidation made the use of a manometric technique nonadvisable. Results obtained in the present study (not shown), as well as literature reports (31, 32), indicate that

the oxidation of ascorbic acid goes beyond the dehydroascorbic acid stage. Determining the portion of the oxygen that is consumed by oxidation of ascorbic acid would be difficult.

From the available values for the molar absorbance of conjugated hydroperoxides, the value reported by Sephton and Sutton (17) ($\epsilon = 26{,}000$), which is considered to represent the absorbance of the mixture of hydroperoxides obtained by oxidation of linoleic acid, was chosen for the calculations. We recognize the limitations of this calculation: accurate estimation of the concentration of hydroperoxides is possible only if the relative proportions of the geometric isomers are known. Although any inaccuracy in molar absorbance would influence the absolute values reported, it would not influence relative values.

The TBA Test as a Measure of Lipid Oxidation

The formation of conjugated hydroperoxides is initiated immediately after the addition of ascorbic acid as a catalyst (Fig. 4). However, absorbance in the TBA test increases little until some time has elapsed (Fig. 5) and conjugated dienes have formed (Fig. 6). This delay in response is interpreted as evidence that the TBA test measures secondary oxidation products, not hydroperoxides. Furthermore, these results indicate that conditions prevailing during the TBA test do not degrade the conjugated hyroperoxides to TBA-reactive material, and that the hydroperoxides formed during oxidation catalyzed by ascorbic acid are rather stable.

In the presence of Cu^{++}, the TBA test detects early stages of oxidation more sensitively than does UV absorption by dienes (Fig. 6). Possible explanations are that copper catalyzes the degradation of hydroperoxides to TBA-reactive products (33, 34), or that it catalyzes the TBA reaction itself (35, 36), or both. Thus, the relation between the two measures of oxidation depends on whether copper, or possibly other catalysts, are present when the TBA test is being done. This factor must be considered when results of the TBA test are interpreted.

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