A Sensitive Spectrophotometric Method for Lipid Hydroperoxide Determination

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A spectrophotometric method for the quantitation of lipid hydroperoxides is described. The chromophore produced in the reaction is the strongly absorbing I_3^- ion, which has been used previously by many researchers. The present method deviates from earlier methods in three respects—first, the assay is done at low acid concentrations; second, the iodide concentration is reduced; and third, Fe^{2+} is used as a catalyst. As a result of these modifications the interference from oxygen is minimized. The assay is fast, sensitive and simple to perform.

KEY WORDS: Iodometry, lipid peroxidation, method, spectrophotometry.

Over the past few years a number of new methods have been generated for the detection of lipid peroxidation. These methods are based on recent technological developments, as well as on improved insights into the complexity of oxidation reactions. The major initial reaction products for lipid peroxidation are hydroperoxides, which are labile species that can undergo both enzymatic and nonenzymatic degradation to produce a complex array of secondary products, including volatile hydrocarbons, malondialdehyde precursors, malondialdehyde itself, olefins and carbonyl compounds. The assay for these secondary products forms the basis for several tests of peroxidation.

Evidently, the assay for hydroperoxides offers the most direct measure of lipid peroxidation. The methods available to evaluate the hydroperoxide content in a biological sample are based: i) On the spectrophotometric determination of the oxidation of iodine (1,2), leuco-dyes (3), thiocyanate (4) or diene conjugation (5); ii) on oxidation of dichlorofluorescein catalyzed by hematin (6); iii) on oxidation of glutathione S-transferase (GSH) by glutathione peroxidase (7,8); iv) on Fe^{3+}-catalyzed oxidation of thiobarbituric acid (9); v) on chemiluminescence (10,11); vi) on high-performance liquid chromatography (HPLC) measurements (12–16); vii) on gas chromatography-mass spectra (GC-MS) methods (17); and viii) on ethane exhalation (18). Some of the methods are discussed by van Kuijk and Dratz (19) and by Smith and Anderson (20).

The classical method for the determination of liberated iodine, titration with thiosulfate, has been superseded by its estimation as the triiodide anion, Eqs. [1] and [2].

$$ROOH + 2H^+ + 2I^- \rightarrow ROH + H_2O + I_2 \quad [1]$$

$$I^- + I_2 \rightarrow I_3^- \quad [2]$$

A major disadvantage of the iodometric assay is its susceptibility toward background reactions; iodine is easily oxidized to triiodide by molecular oxygen, and unsaturated fatty acids also are oxidized to the parent lipid hydroperoxides.

The purpose of the present investigation was to optimize the iodometric assay. It is demonstrated that this is accomplished by reduction of the acid and the iodide concentrations. It is also shown that Fe^{2+} can be used to accelerate the formation of the I_3^- chromophore from lipid hydroperoxides without promoting oxidation of unsaturated fatty acids.

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MATERIALS AND METHODS

The lipid sample used for peroxidation studies was a triglyceride (capelin oil) with a iodine number of 121.7 and 13.6% polyunsaturated fatty acids (18:4 + 20:5 + 22:6). Peroxides could not be detected by the conventional iodometric titration (AOAC method 28.025, ref. 21). Vitamin E could not be detected. Cumene hydroperoxide (art. no. 1300-23) was of 80% technical quality from Koch-Light (Colnbrook, England), and contained 4.46 M peroxides by titration (AOAC method 28.025). Docosahexaenoic acid (DHA) was from Sigma (cat. no. D 2659; St. Louis, MO).

Chemicals. All solvents were of Pro Analysis quality from Merck (Darmstadt, Germany). I_2 (art. no. 4761), KI (art. no. 5043), and $(Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O)$ (art. no. 3793) were also from Merck. 3N Methanolic HCl (cat. no. 3-3050) was from Supelco (Bellefonte, PA). A saturated solution of KI was made and tested as described in reference 21.

For spectrophotometric measurements a 2:1 mixture of methanol/butanol (MeOH/BuOH) containing 3% of saturated KI-solution was used. The solution is stable when stored in the dark and is sufficiently polar to dissolve both KI, iron salts and lipids.

A 12.5 mM stock solution of ferrous salt was prepared in high-quality distilled water (conductance less than 10^{-9} mho). The pure ferrous salt is stable. Stock ferrous salt should never be prepared in buffered media at neutral pH, where it can rapidly autoxidize. The solution may not be prepared in the presence of chelating agents [*e.g.*, ethylene diaminetetraacetic acid (EDTA) or diethylene triaminepentaacetic acid (DTPA)] because the chelate may be unstable.

25 mM HCl was made by diluting 3M methanolic HCl in methanol. The standard assay was performed as follows: Add 2.2 mL solvent (MeOH/BuOH/KI) to a 3.0-mL cuvette, then add 100 μL of lipid sample (diluted 1:100 in butanol). Read the absorbance for 15 min in a recording spectrophotometer at 360 nm. Precipitates may form at higher ion or lipid concentrations. Peroxide value (PV, in mEq/kg lipid) is calculated by:

$$PV = A_{15} / 18.3 \times D \times V_t / V_s \quad [3]$$

A_{15} is the absorbance value at 360 nm after 15 min, D is the dilution factor for the lipid sample in butanol, V_t is the total volume added to the cuvette and V_s is the sample volume added. The calculations are based on a mM extinction coefficient for the I_3^- ion of 18.3 (360 nm).

Instruments. Spectrophotometric recordings were done in a Hewlett-Packard diode array spectrophotometer (8451A, Hewlett-Packard, Fullerton, CA) with stirring and temperature control (25°C).

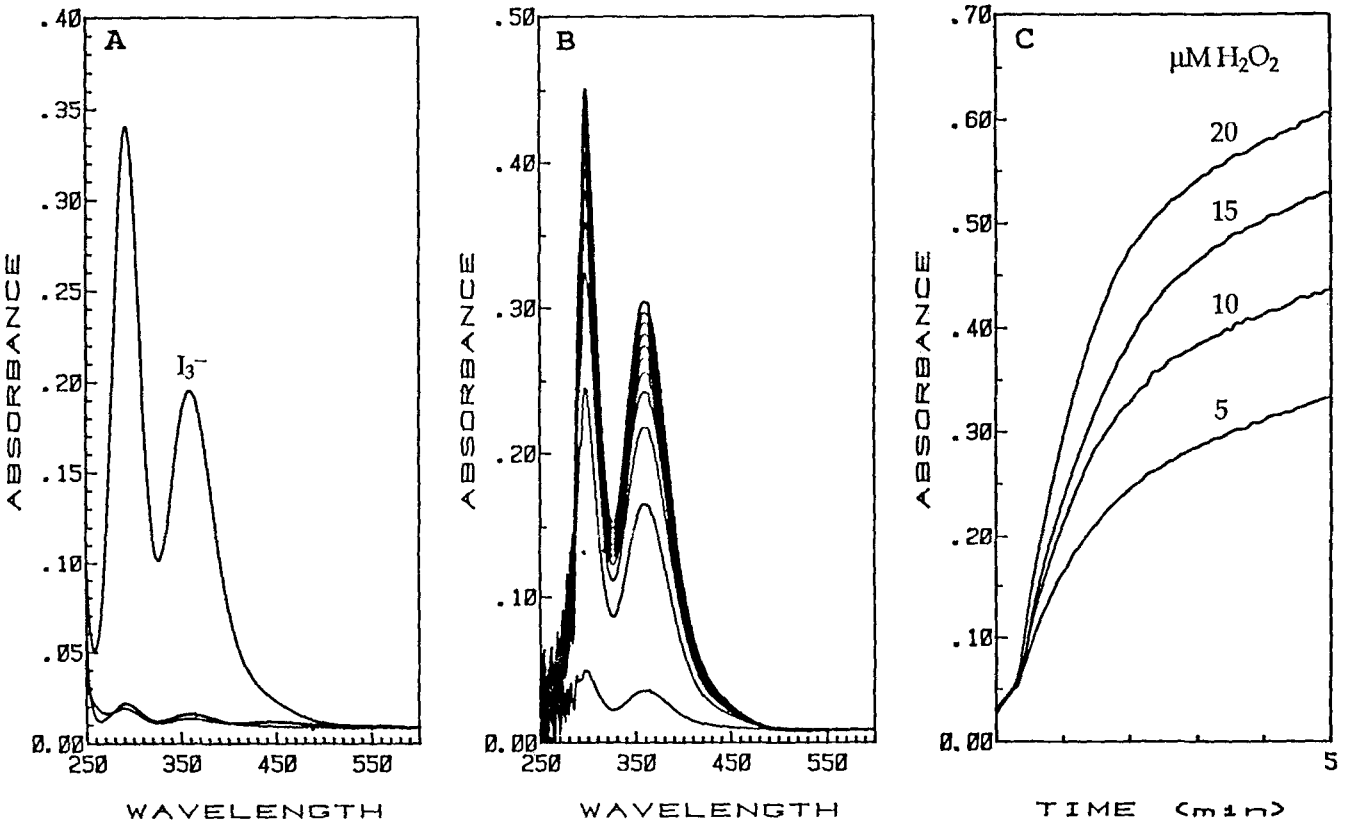

FIG. 1. Spectra of iodine compounds and kinetics for formation of I_3^-. A: Spectra of I_2 (10 μM), I^- (200 μM), and $I_2 + I^-$ (10 μM + 200 μM, upper trace). B: Time-based spectra of 20 μM H_2O_2 in MeOH/BuOH (2:1) containing 3% of saturated KI and 50 mM HCl. The spectra were taken every 30 s, and were corrected for baseline shift due to KCl precipitation. C: I_3^- formation in the presence of 20, 15, 10 and 5 μM H_2O_2 added 20 s after the trace started. Other conditions as in B.

RESULTS AND DISCUSSION

Spectrophotometric determination of I_3^-. The color evolution, which results when a peroxide reacts with iodide, may be used as a convenient vehicle for the determination of peroxides by a spectrophotometric method. The color is due to a reaction product between I_2 and I^-, namely the I_3^- ion, and the molar extinction coefficient is high (Fig. 1A and Table 1). When H_2O_2 is mixed with a solution containing I^- there is a time-dependent formation of the chromophore (Fig. 1B), which is determined by the peroxide concentration (Fig. 1C). After the initial rapid rise in absorbance the reaction levels off to a constant rate, reflecting the background oxidation of I^-. To use this ion as a chromophore for the hydroperoxide determination, its formation must be both stoichiometric and quantitative.

The equilibrium constants for I_3^- in different solvents were calculated from spectrophotometric measurements. Different amounts of iodine were mixed with excess iodide, and the equilibrium constant K could then be derived from Eqs. [4] and [5]:

$$K = \frac{[I_3^-]}{([I_2]_0 - [I_3^-]) \cdot [I^-]} \quad [4]$$

which was rearranged and solved with respect to I_3^-, giving:

$$\frac{1}{A} = \frac{1}{E[I_2]_0} + \frac{1}{EK[I_2]_0} \cdot \frac{1}{[I^-]} \quad [5]$$

A is the absorbance of I_3^- at 360 or 290 nm, and E is the corresponding extinction coefficient; $[I_2]_0$ is the initial concentration of I_2 and $[I^-]$ is the fixed concentration of iodine.

The relationships between 1/A and $1/I^-$ in water, methanol and ethanol are shown in Figure 2, and the calculated equilibrium constants are given in Table 1. The reaction of iodine and iodide is strongly in favor of I_3^- formation, and the iodine formed by redox reactions with peroxides will mainly end up as the strongly absorbing I_3^- ion. The equilibrium constant is inversely related to the dielectric constant of the solvent. In the following studies MeOH/BuOH (2:1) was used as solvent, as this mixture

TABLE 1

Spectral Data and Equilibrium Constants for I_3^- Formation in Different Media

Solvent	Molar extinction 290 nm	Molar extinction 360 nm	K(M^{-1})
H_2O	11600	6200	1.26×10^3
MeOH	32100	18300	9.01×10^3
EtOH	34500	19500	33.30×10^3

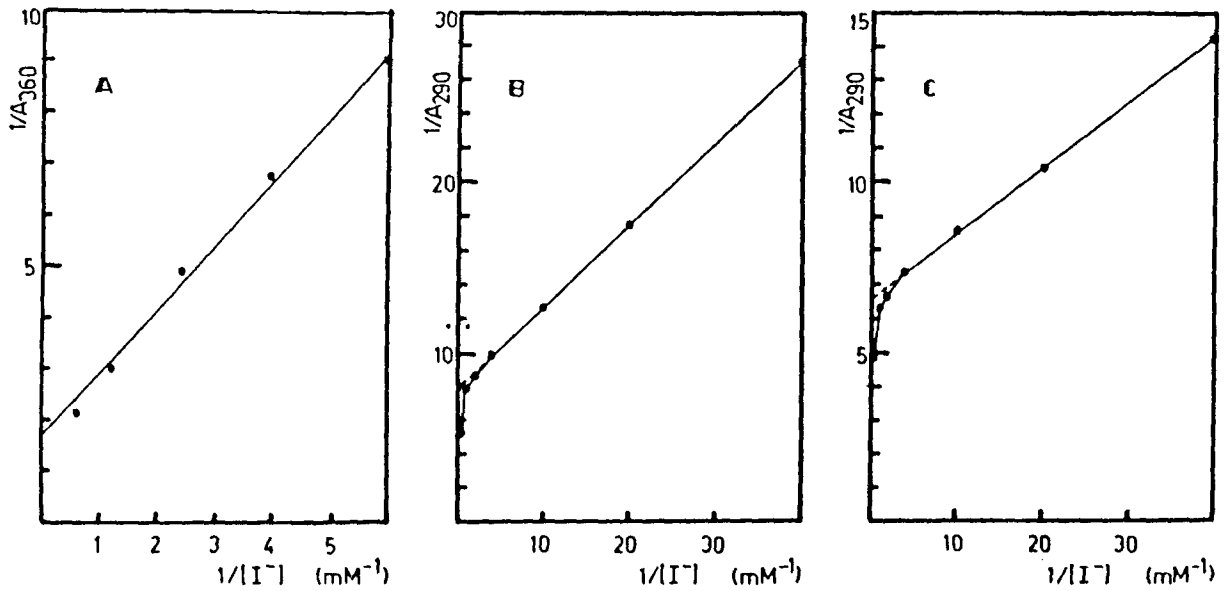

FIG. 2. Determination of equilibrium constants for I_3^- in: A, water; B, methanol; and C, ethanol. 33 μM of I_2 (A) and 5 μM I_2 were mixed with KI at the indicated concentrations.

enabled solution of 3% KI, iron salts and lipid. The equilibrium constant for this mixture was not measured, but it is believed to be higher than that for pure methanol and strongly in favor of I_3^- formation.

Iodide oxidation and lipid peroxidation. The effect of acid on the formation of I_3^- was investigated in a solvent containing MeOH/BuOH (2:1), containing 3% of saturated KI and equilibrated with oxygen (0.2 atm). The investigation was done in the presence of lipid (capelin oil essentially free of peroxides) (Fig. 3A), and in the absence of lipid (Fig. 3B). The traces in Figure 3A represent the sum oxidation of iodide and of polyunsaturated fatty acids, whereas Figure 3B shows the oxidation of iodide alone. The oxidation of lipid was determined by difference (Fig. 3C). The results clearly demonstrate that both iodide oxidation and lipid peroxidation are accelerated by acid, and that iodometric methods for hydroperoxide determination, performed under strongly acid conditions, has an inherent weakness due to background oxidations. This problem has been addressed previously by performing the assay under anaerobic conditions (1,2,22). The problem cannot be solved by omitting acid from the reaction mixture because acid is needed for the reaction between peroxides and I^- (Fig. 4). Furthermore, the rate of I_3^- formation depends on the peroxide, as exemplified by the slow-reacting cumene hydroperoxide and the fast-reacting hydrogen peroxide. These differences in peroxide reactivities prohibit quantitation of peroxides by kinetic measurements.

It was possible that the inherent problems of the assay could be corrected by the addition of a catalyst, as iron compounds previously have been used as catalysts for peroxide determinations (3–5,23). For example, Cathcart *et al.* (6) have used hematin as catalyst in the reaction between lipid hydroperoxides and dichlorofluorescein, while Wade *et al.* (9) and Gutteridge and Quinlan (24) have used Fe^{3+} in their determination of lipid peroxides by a thiobarbituric assay (TBA) method. Iron was thus tested as a catalyst for the formation of I_3^- formation from peroxides.

Fe(II) Catalysis of I_3^- formation from peroxides. In the following experiments 1 mM HCl was included in the solvent. This acid concentration was sufficiently high to neutralize the hydroxyl ions generated by the reaction of peroxides but, at the same time, sufficiently low to prevent a significant contribution from the above-mentioned background oxidations (oxidation of iodide and unsaturated fatty acids).

Under these conditions there was a 1:1 relationship between the added amount of H_2O_2 and formation of I_3^-, and the equilibrium absorbance was consistent with a molar extinction coefficient of 18,300 for the I_3^- ion at 360 nm. However, the reaction was slow, and equilibrium was obtained only after long reaction time (45–60 min). Hicks and Gebicki (2) have reported extinction coefficients for the I_3^- ion that are higher than in this study (44, 100 at 290 nm and 28,000 at 360 nm). These workers used a three-fold excess of I^-, and they may not have corrected for the absorbance of the ion. Inclusion of Fe^{2+} in the reaction mixture gave a 30–80-fold increase in the specific rate of I_3^- formation when hydrogen peroxide or cumene hydroperoxide was used as sample (Table 2).

The rate and equilibrium of I_3^- formation was investigated for variable Fe^{2+} concentrations (Fig. 5A), for variable peroxide concentrations (Fig. 5B) and for variable lipid/peroxide ratios (Fig. 5C), made by mixing capelin oil and cumene hydroperoxide in fixed or variable ratios. The reaction mixture was MeOH/BuOH (2:1) containing 3% saturated KI, 1 mM HCl and Fe^{2+} as indicated. At low Fe^{2+} concentrations there was a background reaction (Fig. 5A). However, at 500 μM Fe^{2+} this reaction(s) vanished, and there was also a strict 1:1 relationship between the amount of peroxide added and the I_3^- formation (Fig. 5B). The equilibrium was not affected by variations of the lipid/peroxide ratio when 500 μM Fe^{2+} was included in the reaction medium (Fig. 5C), suggesting that

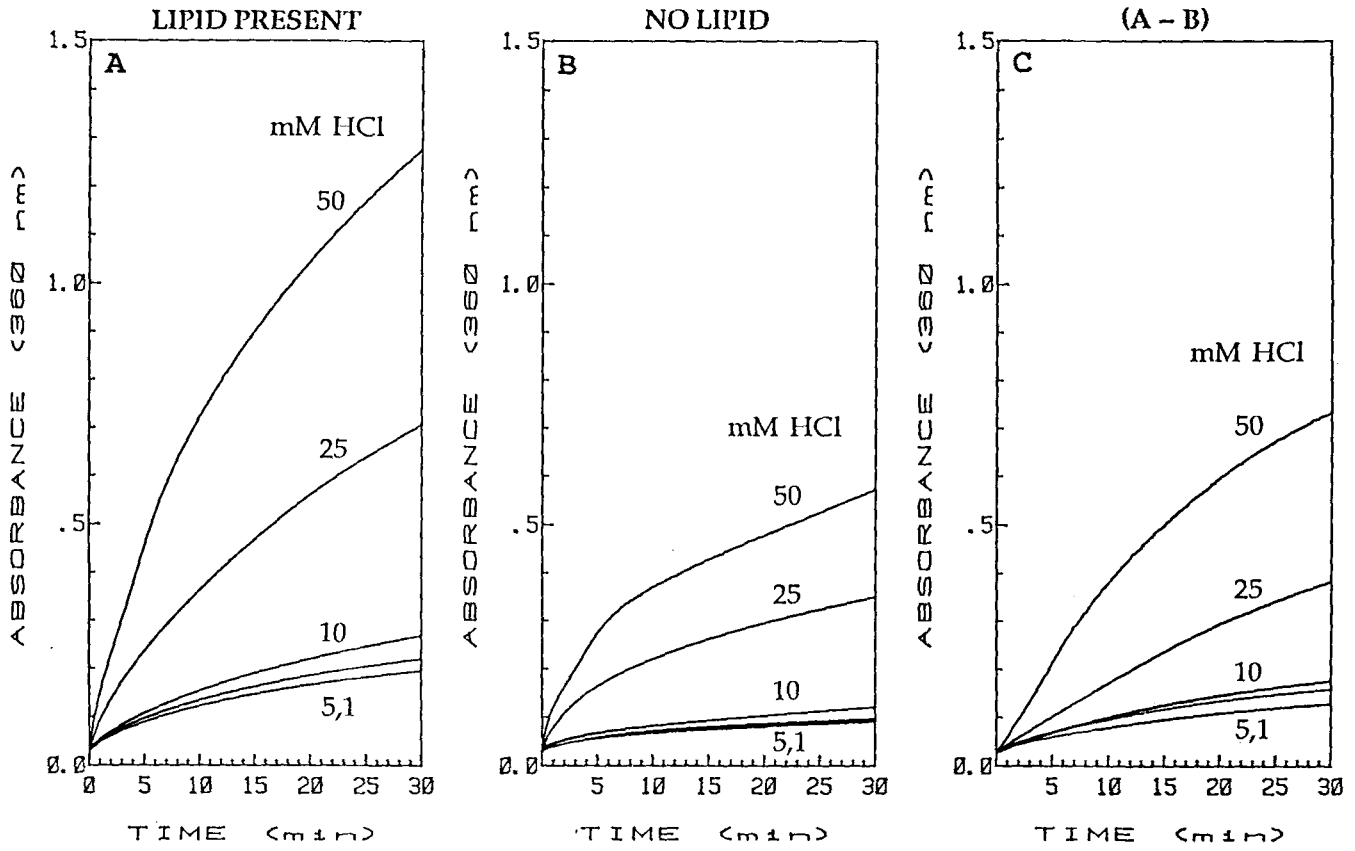

FIG. 3. I_3^- Formation in the presence of 50, 25, 10, 5 and 1 mM HCl. The upper traces had the highest concentration of HCl. The reaction was started by addition of methanolic HCl to the spectrophotometric cuvette. I_3^- formation was monitored at 360 nm. A, 4 mg of lipid/mL solvent; B, no lipid; and C, difference (A–B).

Fe^{2+} does not initiate lipid peroxidation. It is assumed that iron is kept in its reduced state by excess of iodine, and that lipid peroxidation by Fe^{3+} thus is prevented. At lower Fe^{2+} concentrations the reaction with cumene hydroperoxide was slow, and there seemed to be an oxidation of unsaturated lipids.

Peroxide determinations also were determined with lipid samples (DHA) ranging in concentration from 0.1 to 0.6 mg/mL (Table 3). The absorbance readings varied from 0.29 to 1.72. If oxidation of the lipid sample had taken place during the assay, absorbance readings from 5.8 to 35.7 would have been expected (provided formation of 1 peroxide per molecule of DHA). It could thus be inferred that no (or little) peroxidation took place under these assay conditions. The observed formation of I_3^- could be assigned to the presence of peroxides. In the absence of catalysts peroxides are chemically inert (25). The results suggest the following reaction scheme:

$$ROOH + Fe^{2+} \rightarrow RO^{\cdot} + OH^- + Fe^{3+} \quad [6]$$

$$2RO^{\cdot} + 2H^+ + 3I^- \rightarrow 2ROH + I^{3-} \quad [7]$$

$$2Fe^{3+} + 3I^- \rightarrow 2Fe^{2+} + I_3^- \quad [8]$$

$$ROOH + 2H^+ + 3I^- \rightarrow ROH + H_2O + I_3^- \quad [9]$$

The reaction scheme presented in Eqs. [6–9] is similar to a suggestion by Bachowski *et al.* (26) concerning ascorbate-enhanced lipid peroxidation. In their model the peroxyl radical ($RO^{\cdot}$) initiated further lipid peroxidation. Such reactions were not seen in their present work, presumably because I^- efficiently quenches $RO^{\cdot}$. This also suggests that I^- is an efficient antioxidant.

Interactions. In control experiments with 500 μM Fe^{2+}, 3% I^- and 1 mM H^+, no background evolution, *i.e.*, evolution of I_3^-, took place. Under these conditions there was no or little interaction between Fe^{2+} and oxygen. However, when EDTA was added, the solution immediately turned yellow, indicating that Fe^{2+}-EDTA had a much enhanced reactivity towards oxygen. Fe^{2+}-EDTA is known to generate superoxide radicals (27) and to induce lipid peroxidation in model systems (28). Numerous studies with EDTA have demonstrated the complexity it imparts upon the reactivity of iron [references in (28)]. Chelating agents, particularly EDTA, may thus profoundly disturb the assay.

From the work of other researchers (30), it is known that the assay will not tolerate substances that reduce hydroperoxides to the parent alcohol (such as mercaptanes), or compounds that react with iodine (such as acetone). Solvents that are known to peroxidize also should be avoided. These include diethyl ether and tetrahydrofuran. Choice of solvent should be carefully considered (31). Colored material absorbing in the region of 290–360 nm will increase the background absorbance, but this is easily corrected for by modern spectrophotometers. It is recommended that samples are spiked with standard organic hydroperoxides (like cumene hydroperoxide) to

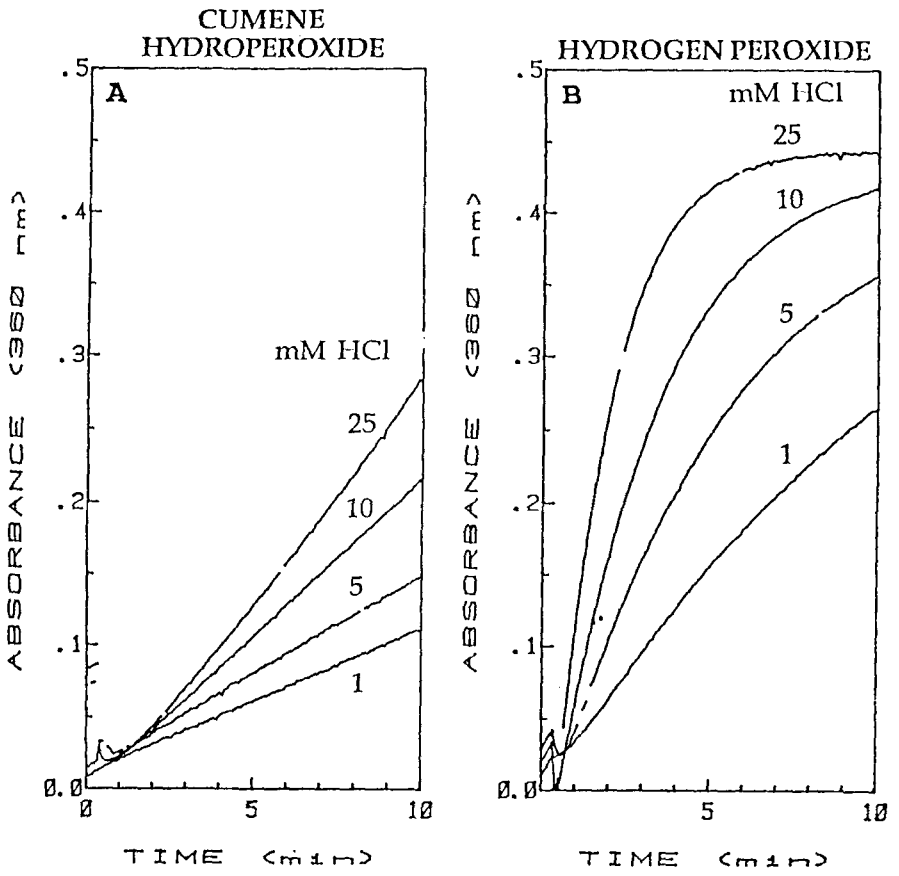

FIG. 4. I_3^- Formation from 20 μM cumene hydroperoxide (A) and 20 μM hydrogenperoxide (B) at 25, 10, 5 and 1 mM HCl. The traces are corrected for background oxidation of iodide. Solvent: MeOH/BuOH (2:1) containing 3% KI.

TABLE 2

Rate Constants for H^+ and Fe^{2+} Catalysis for I_3^- Formation with H_2O_2 and Cumene Hydroperoxide (values are expressed as $M^{-1}s^{-1}$)

Peroxide	H^+ Catalysis	Fe^{2+} Catalysis
H_2O_2	2.25	72
Cumene hydroperoxide	0.17	13

ascertain the performance of the assay. The present investigation demonstrates that the iodometric determination of lipid hydroperoxides can be simplified by reducing the acid and iodide concentrations and by using Fe^{2+} as a catalyst.

A major disadvantage of triiodide assay has been related to the background reactions of molecular oxygen with iodine and unsaturated fatty acids. Chain propagation reactions are known to proceed at extremely low oxygen concentration (32,33). The interference of oxygen on the assay is thus of the greatest importance and has to be dealt with. Either lipid peroxidation must be nullified by performing the assays under strictly anaerobic conditions, or the reactivity of oxygen must be reduced. Without this precaution, high blanks and poor sensitivity result. Recently it has been shown that lipid peroxidation is strongly accelerated by acid (34). This offers an explanation for the high background reactions seen in conventional iodometric assays. These assays are invariantly performed in the presence of high concentrations of acetic acid and will thus give high blank reading in the presence of oxygen. The earlier observed relationship (34) between high acid and high peroxidation is confirmed and extended by the present communication. This relationship also suggests that the iodometric assay should be performed at a low acid concentration.

The strategy of the present investigation was to minimize the interference of oxygen by reducing its reactivity. This has been attained simply by changing the solvent from the conventional acetic acid/methanol to methanol/butanol (2:1). One mM of HCl was added to supply the protons necessary for the conversion of lipid hydroperoxides to the parent alcohol. Under these conditions the autoxidation of lipids was essentially eliminated, and there was only low background oxidation of iodide.

Second, the level of iodide was reduced. It is demonstrated that the formation of triiodide increases when the polarity of the medium decreases. The concentration of iodide thus can be compromised, and the result is prevention of the direct reaction between molecular oxygen and iodide. Third, Fe^{2+} was introduced as a catalyst for the reaction between lipid hydroperoxides and iodide. It is well known that iron is a pro-oxidant, and it was therefore essential to investigate the effects of iron on the oxidation of unsaturated lipids.

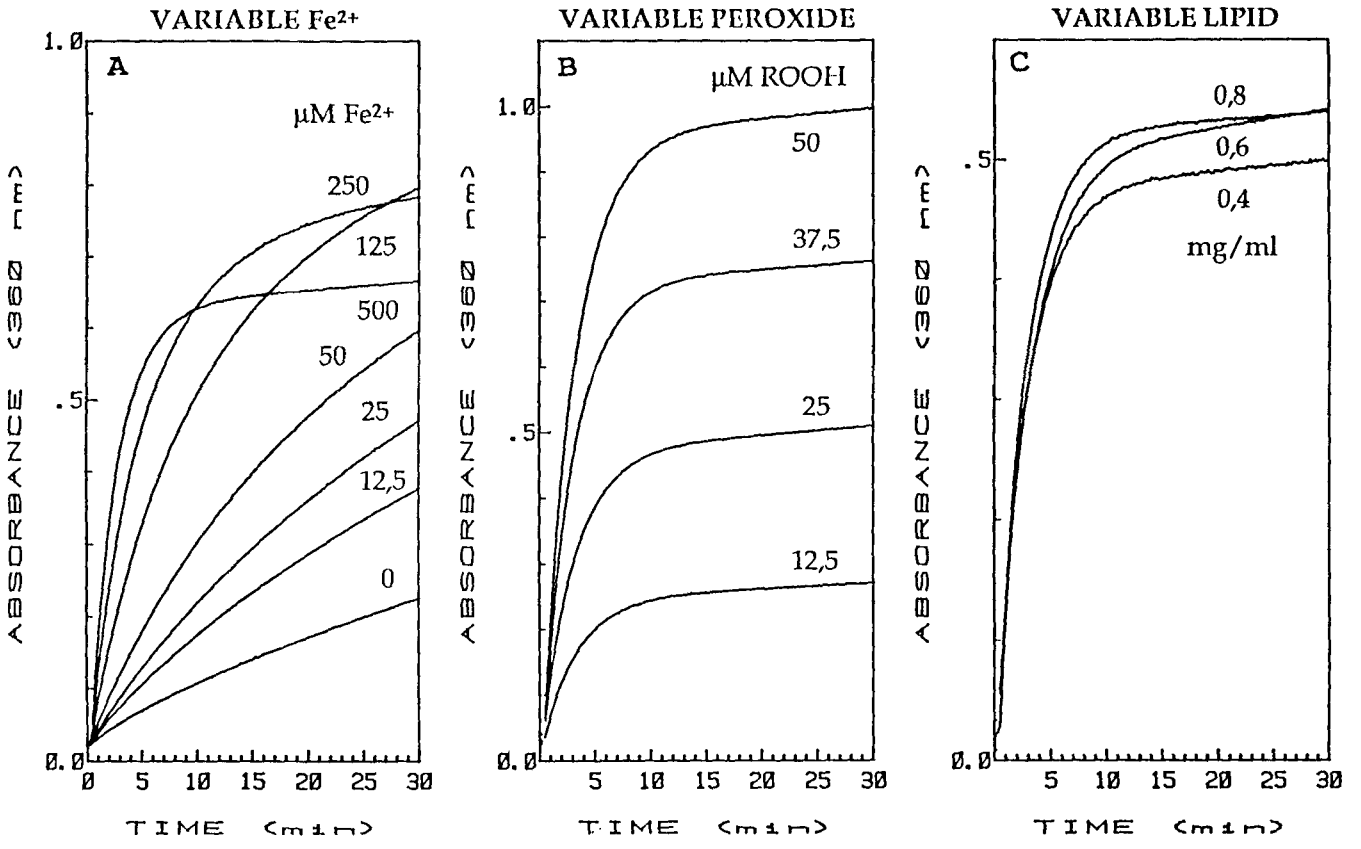

FIG. 5. I_3^- Formation as a function of Fe^{2+}-concentration, peroxide concentration and peroxide/lipid ratio. A, Variable Fe^{2+}-concentration. The lipid concentration was 0.4 mg lipid/mL and cumene hydroperoxide concentration 30 μM. B, Variable peroxide concentration. The Fe^{2+}-concentration was 500 μM. C, Variable lipid/peroxide ratio. The peroxide concentration was 25 μM, and the lipid concentration 0.4, 0.6 or 0.8 mg/mL. The Fe^{2+}-concentration was 500 μM.

TABLE 3

Peroxide Measurements in a Sample of DHA[a] [The sample was diluted in butanol as indicated. 100 μL of the diluted sample was then added to 2.4 mL of MeOH:BuOH (2:1)-3% KI with 1 mM HCl and 500 μM Fe(II)][b]

Dilution factor	mg/mL	A(360)[c]	PV (mEq/kg)[d]
67	0.6	1.7235	87.6
100	0.4	1.1808	90.0
133	0.3	0.8767	89.1
200	0.2	0.6114	93.2
400	0.1	0.2994	91.3

[a]DHA, docosohexaenoic acid (n-3,6,9,12,15,18 C22:6, all *cis*).
[b]Peroxide values were calculated according to Eq. [4].
[c]Absorbance at 360 nm.
[d]Peroxide value.

The extinction coefficient at the I_3^- ion at 360 nm limits the sensitivity to about 500 ng of lipid hydroperoxides, which is less than the sensitivity levels of peroxidized phospholipids on GC-MS (10 ng − 10 pg) (17). The Fe^{2+}-iodide method has, however, the merit of a simple procedure and may have sufficient sensitivity for a number of applications. Apparently, the simplicity of the Fe^{2+}-iodide method enables us to replace more cumbersome methods as a means of estimating lipid peroxides. We should be aware of the limitations of this method when it is applied to biological materials. In particular, we must bear in mind that lipid hydroperoxides in biological membranes are transient species that are rapidly metabolized.

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