Thiobarbituric Acid-Reactive Substances from Peroxidized Lipids

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The thiobarbituric acid (TBA) reaction was performed on linoleic acid 13-monohydroperoxide, autoxidized fatty esters, edible fats and oils, rat liver microsomal lipids, and on human erythrocyte ghost lipids in order to determine which substances from peroxidized lipids are TBA-reactive. The reaction was carried out in 2% acetic acid containing butylated hydroxytoluene using two different reaction modes: a one-step mode which involves heating at 100°C, and a two-step mode which involves first treatment at 5°C and subsequent heating at 100°C. Yields of the red 1:2 malonaldehyde/TBA adduct, as estimated by absorbance, fluorescence intensity and high-performance liquid chromatography, were much higher than the malonaldehyde content as determined by direct chemical analysis. Yields of red pigment obtained by the two-step mode were slightly higher than those obtained by the one-step mode. Pigment yields were dramatically increased by addition of *t*-butyl hydroperoxide. Red pigment formation from alkenals and alkadienals was similarly enhanced by the two-step mode or by addition of *t*-butyl hydroperoxide, whereas pigment formation from malonaldehyde was not. It appears likely that a component of the total red pigment formed from the peroxidized lipids was due to aldehyde species other than malonaldehyde.
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The thiobarbituric acid (TBA) test has been commonly used to measure lipid peroxidation ever since it was introduced by Kohn and Liversedge (1-3). Heating of peroxidized lipids with TBA under acidic conditions produces red pigment with an absorption maximum at 532 nm (2). The pigment has been considered to be the 1:2 adduct of malonaldehyde and TBA (4,5). For many years the test was thought to be specific for malonaldehyde. Recently, it has been demonstrated that other lipid oxidation products such as alkanals, alkenals and alkadienals also produce the pigment (6-16). However, it has been shown that malonaldehyde is the aldehyde most reactive with TBA (11,12).

Our recent studies have demonstrated that TBA produces the pigment by reaction with alkanals (13), alkenals (14) and alkadienals (15) via the intermediary 1:1 adducts between TBA and the aldehydes in the presence of water and oxygen. Furthermore, it has been found that mixtures of an alkenal, an alkadienal and an organic hydroperoxide produce unexpectedly large amounts of the pigment (16). The pigment formation from alkenals and alkadienals is markedly enhanced by organic hydroperoxides, and the pigment formation from alkadienals is dependent on the reaction modes: a two-step mode which involves an initial treatment at 5°C and subsequent heating at 100°C produces a much greater amount of the pigment than a one-step mode which involves immediate heating at 100°C (15,16). On the contrary, malonaldehyde produces the pigment without being affected by the presence of other components or hydroperoxides and by the reaction modes.

In the present investigation, the TBA test was performed on various peroxidized lipids in order to elucidate the contributions of various aldehyde species to red pigment formation in the TBA reaction. Yields of the pigment were estimated by the one- and two-step modes, and the effect of an organic hydroperoxide was examined. For comparison, the contents of malonaldehyde, other aldehydes and intrinsic hydroperoxides in the peroxidized lipids were determined by the Hantzsch method (17), the 2,4-dinitrophenyl-hydrazine (11) and dimedone (18) methods, and the sesamol dimer method (19), respectively.

MATERIALS AND METHODS

Materials. TBA, 2,4-dinitrophenylhydrazine (DNPH), 5,5-dimethyl-1,3-cyclohexanedione (dimedone) and 1-hexanal were obtained from Wako Pure Chemical Industries (Osaka, Japan). 1-Propanal, 1-pentanal, 2-hexenal, 2,4-hexadienal, tetramethoxypropane, methyl linoleate and methyl oleate were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 2-Heptenal, 2-octenal, 2,4-heptadienal and 2,4-nonadienal were from Aldrich Chemical Co. (Milwaukee, WI). *t*-Butyl hydroperoxide (*t*-BuOOH) (70% in water) was purchased from Nakarai Chemicals (Kyoto, Japan). Butylated hydroxytoluene (BHT) was from Nikki Universal Co. (Tokyo, Japan). Adenosine-5′-diphosphate monopotassium salt (ADP) was the product of Oriental Yeast Co. (Tokyo, Japan). Glacial acetic acid was a special reagent-grade product of Wako Pure Chemical Industries. Silica gel column chromatography was performed with silica gel for chromatography from Kanto Chemical Co. (Tokyo, Japan).

Soybean oil was a product of Showa-Sangyo Co. (Tokyo, Japan), and sesame oil was Japan Pharmacopoeia grade. Subcutaneous lipids of hog and chicken and whole lipids of sardine meat were extracted and prepared as described by Kosugi and Kikugawa (20). Linoleic acid 13-monohydroperoxide (13-LOOH) (21) was prepared from linoleic acid (Nippon Oil and Fats Co., Tokyo, Japan) and lipoxidase (Type 1: 130,000 units/mg, Sigma Chemical Co., St. Louis, MO). The purity of the hydroperoxide was 96% as estimated on the basis of the molar extinction coefficient (24,500) at 234 nm (22).

Rat liver microsomal suspension was prepared according to the method of Albro *et al.* (23). As detailed therein, livers weighing 120 g from twelve Spraque-Dawley male rats were homogenized, and a microsomal suspension (60 ml) in 25 mM morpholinopropane sulfonic acid buffer (pH 7.4) −0.25 M mannitol was

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Abbreviations: TBA, thiobarbituric acid; *t*-BuOOH, *t*-butyl hydroperoxide; ADP, adenosine-5′-diphosphate; BHT, butylated hydroxytoluene; AOM, active oxygen method, HPLC, high-performance liquid chromatography.

obtained. The suspension was frozen at −20°C until used.

Human erythrocyte ghosts were prepared according to the method of Steck (24) and stored at −20°C in aliquots until used (25).

Analyses. Absorption spectra were measured on a Shimadzu UV-240 UV-visible or a Hitachi U-2000 spectrophotometer. Fluorescence spectra were measured on a Hitachi 650-60 fluorescence spectrophotometer. High-performance liquid chromatography (HPLC) was carried out with a Shimadzu LC-6A liquid chromatograph equipped with a stainless-steel column (4.6 mm i.d. × 25 cm) of YMC A-303 ODS (Yamamura Chemical Laboratories, Kyoto, Japan). For determination of malonaldehyde, the column was eluted with methanol/0.025 M phosphate buffer, pH 7.5 (1:1, v/v) at a flow rate of 0.5 ml/min. The fluorescent peak was detected at 400 nm (excitation) and 465 nm (emission) with a Shimadzu RF-530 fluorescence spectrofluorometer. For determination of red pigment, the column was eluted with methanol/0.04 M acetate buffer (pH 5.5) (4:6 v/v) at a flow rate of 0.8 ml/min. The peak was detected at 532 nm with a Shimadzu SPD-6A spectrophotometer. All analyses were carried out within the linear response ranges of the detectors.

Oxidation of lipid samples. Methyl linoleate, methyl oleate, soybean oil, sesame oil, hog fat and chicken oil were oxidized at 98°C by the active oxygen method (AOM) (26). Sardine oil was oxidized by ultraviolet irradiation as previously described (20).

Microsomal suspension (60 ml) was mixed with an equal volume of 0.05 M Tris-0.15 M KCl (pH 7.5) containing 2.0 mM ADP, 2.0 mM sodium ascorbate and 24 μM $FeCl_2$, and the mixture was incubated at 37°C for 30 min (27). Lipids of the oxidized microsomes were extracted with chloroform/methanol according to the method of Bligh and Dyer (28), and yielded 243 mg of lipid.

Human erythrocyte ghost suspension (40 ml) was mixed with 20 ml of 0.1 M phosphate buffer (pH 7.0) containing 10 μM carbonmonoxy hemoglobin, and the mixture was kept at room temperature for 30 min. A 20-ml solution of 1.0 mM *t*-BuOOH in phosphate buffer was added (29). The mixture was incubated at 37°C for 30 min. Lipids of the oxidized ghosts were extracted and obtained in a yield of 119 mg.

Determination of hydroperoxides. Hydroperoxide content of the peroxidized lipid samples was determined by peroxide value (neq/mg) (30), conjugated diene (31) and the sesamol dimer method (19). For determination by sesamol dimer, 6 mg of the lipid sample was used, and hydroperoxide content was expressed as nmol of 13-LOOH.

Determination of total aldehydes with 2,4-dinitrophenylhydrazine (DNPH) (11). DNPH (50 mg) was dissolved in 100 ml of 1 N hydrochloric acid, and the solution was extracted twice with 50 ml of *n*-hexane to remove impurities. To 2.0 ml of the solution were added 1.8 ml of water and 0.2 ml of glacial acetic acid containing lipid sample (2-4 mg) or standard aldehyde (0-0.2 μmol). The mixture was vigorously shaken at room temperature for five min in the dark and extracted six times with 3.0 ml of benzene. The organic layers were combined and evaporated at 30°C *in vacuo*, and the residue was redissolved in 0.5 ml of benzene. The solution was applied to a column (9 mm i.d. × 3.5 cm) of silica gel, and the column was eluted with benzene. Hydrazone fractions (3-15 ml) and DNPH fractions (19-27 ml) were separated. The hydrazone fractions were combined and evaporated *in vacuo*, and the residue was dissolved in 10.0 ml of methanol in order to measure the absorption spectrum.

Absorption spectra of the hydrazone fractions from the standard aldehydes, 1-hexanal, 2-hexenal and 2,4-hexadienal, showed respective maxima at 358, 373 and 390 nm. Calibration curves of the absorbance at 370 nm of the fractions were linear. Yields of the hydrazones were 55% for 1-hexanal, 103% for 2-hexenal and 118% for 2,4-hexadienal on the basis of the average molar extinction coefficient (26,000) of the various hydrazones at 370 nm (32).

Absorption spectra of the fractions from 13-LOOH, oxidized methyl linoleate, microsomal lipids and ghost lipids showed maxima at 355-380 nm. The amount of total aldehyde in the peroxidized lipid samples was estimated by the absorbance at 370 nm and by comparison with the calibration curve of 2-hexenal. The amount of total aldehyde was expressed as nmol of 2-hexenal.

Determination of total aldehyde with 5,5-dimethyl-1,3-cyclohexanedione (dimedone) (18). 0.5 ml of 2-propanol containing lipid sample (0.5-4.0 mg) or standard aldehyde (0-0.4 μmol) was added to a mixture of 0.5 ml of 0.75 mM dimedone in 2-propanol and 1.0 ml of 1.5 mM ammonium acetate in water. The mixture was heated at 80°C for 60 min after removal of dissolved oxygen. The fluorescence spectrum of each mixture was taken, and fluorescence intensity was determined against blank solution containing no lipid or aldehyde.

Alkanals (1-propanal, 1-pentanal and 1-hexanal) exhibited fluorescence with excitation maxima at 385 nm and emission maxima at 450 nm and displayed similar fluorescence intensities. Alkenals (2-hexenal, 2-heptenal and 2-octenal) showed fluorescence with excitation maxima at 367 nm and emission maxima at 450 nm and displayed similar intensities. Alkadienals (2,4-hexadienal, 2,4-heptadienal and 2,4-nonadienal) showed fluorescence with excitation maxima at 378 nm and emission maxima at 450 nm, and displayed similar intensities. Relative fluorescence intensities measured at 385 nm (excitation) and 450 nm (emission) were 1.0 ± 0.002 for the alkanals, 0.2 ± 0.01 for the alkenals, and 0.34 ± 0.02 for the alkadienals.

Fluorescence spectra of the reaction mixtures of 13-LOOH, oxidized methyl linoleate, microsomal lipids and ghost lipids showed excitation maxima at 380-385 nm and emission maxima at 450-455 nm. The amount of total aldehyde was estimated by the fluorescence intensity relative to that of the reaction of 1-hexanal. The amount of total aldehyde was expressed as nmol of 1-hexanal.

Determination of malonaldehyde. Malonaldehyde content of peroxidized lipid samples was determined by the Hantzsch method after treatment of the samples with acetic acid (17). The lipid sample (5-50 mg) was dissolved in 0.5 ml of 70% acetic acid and heated at 100°C for 30 min, and the mixture was neutralized

by addition of 0.5 ml of 10 N NaOH. Eight ml of 2-propanol, 1.0 ml of 0.1 M methylamine hydrochloride in water and 1.0 ml of 0.1 M acetaldehyde in methanol were added to the above mentioned solution. The mixture was heated at 80°C for two hr after removal of dissolved oxygen. The reaction mixture was subjected to HPLC. The amount of 1,4-dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde was determined by the peak height of the fluorescent peak at a retention time of 6.8 min. The malonaldehyde content of the sample was calculated from the calibration curve of the dihydropyridine versus standard malonaldehyde sodium salt (0–20 nmol).

TBA test. The TBA test was performed according to a previously reported method (16) with slight modification. A TBA solution (0.4%) in water (5.0 ml) was placed into a 13-ml screw-cap tube. For the one-step reaction, the tube was preheated at 100°C, and 0.1 ml of glacial acetic acid containing the lipid sample (0.5–4 mg), 0.5% BHT and 10 μmol *t*-BuOOH were then added. The mixture was vigorously shaken and immediately heated at 100°C for 20 min. For the two-step reaction, the tube containing the TBA solution was precooled to 5°C and 0.1 ml of the sample solution containing BHT and *t*-BuOOH was added. The mixture was kept at 5°C for 60 min and subsequently heated at 100°C for 20 min. Insoluble material in the reaction mixtures was removed by extraction with 1.0 ml of chloroform or 3.0 ml of chloroform/methanol (5:1, v/v). The absorption spectrum of each aqueous phase was recorded, and the amount of red pigment was calculated from the absorbance at 532 nm using the molar extinction coefficient (156,000) of the red 1:2 malonaldehyde/TBA adduct (4).

For measurement of the pigment by fluorometry, fluorescence intensity of each aqueous phase was determined at 515 nm (excitation) and 550 nm (emission) (33) after 50-fold dilution with water. The amount of pigment was calculated from its intensity relative to that of a standard reaction mixture containing tetramethoxypropane.

On HPLC, the peak due to the red pigment with absorption at 532 nm was detected at a retention time of 7.5 min, and the amount of the pigment was calculated by comparing the peak area with that of a standard reaction mixture containing tetramethoxypropane.

RESULTS

Reaction conditions for the TBA test of peroxidized lipids. TBA reaction mixtures in the present experiments contained 0.4% TBA, 2% acetic acid and the large amount of water that is necessary for effective red pigment formation from alkenals and alkadienals (14–16). Although the presence of a large amount of water prevented complete dissolution of lipid samples, the pigment formation was not affected, regardless of whether the reaction was conducted by the one-step or the two-step mode (15,16).

While oxidized methyl linoleate (1350 neq hydroperoxides/mg) produced 5 nmol red pigment/mg at 20-min heating and 8.5 nmol at 90-min heating by the two-step mode, the amount of pigment did not exceed 5 nmol in the presence of 0.01% BHT. The amount of red pigment from unoxidized methyl linoleate at 20-min heating increased from 1.4 to 2.7 nmol by addition of 10 μmol *t*-BuOOH, but the effect of the added hydroperoxide was completely abolished in the presence of 0.01% BHT. The methyl linoleate could be oxidized by the intrinsic hydroperoxides or the exogenously-added hydroperoxide during the TBA reaction, and this oxidation could be prevented by BHT. Since BHT only slightly affected the pigment formation from alkenals and alkadienals (16) and malonaldehyde (34), 0.01% BHT was routinely included in the TBA test as carried out in the present study.

The effect of unoxidized methyl linoleate on red pigment formation from a mixture of tetramethoxypropane, 2-hexenal, 2,4-hexadienal and *t*-BuOOH was examined (Fig. 1). The pigment formation was dependent upon the amount of mixture reacted and was slightly higher by the two-step than by the one-step mode. Most of the red pigment was derived from the combined effect of 2-hexenal, 2,4-hexadienal and *t*-BuOOH (16). The red pigment formation was little affected by methyl linoleate (Fig. 1).

Red pigment formation from linoleic acid 13-monohydroperoxide (13-LOOH). The TBA test of enzymatically-synthesized 13-LOOH (0.5–2.0 mg) in either the one- or the two-step mode produced red pigment. Absorption and fluorescence spectra of the reaction mixtures (Fig. 2B, dotted curves) were similar to those of a standard reaction mixture of tetramethoxypropane (Fig. 2A). The amount of pigment estimated by absorbance and fluorescence intensities increased linearly with increasing amounts of hydroperoxide. The yields of red pigment were slightly higher by the two-step than by the one-step mode, and were estimated to be 12 nmol (one-step) and 13 nmol pigment (two-step)/1.0 mg (or 3.2 μmol) of hydroperoxide (Table 1). When the amount of malonaldehyde produced from the hydroperoxide by acid treatment was determined by the Hantzsch reaction (17), it was less than 1 nmol. A large amount of aldehydes including alkenals and alkadi-

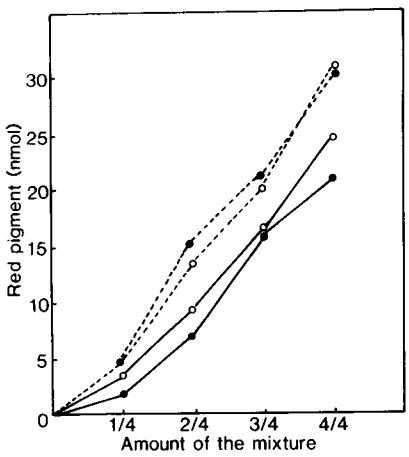

FIG. 1. Effect of unoxidized methyl linoleate on the red pigment formation in the TBA reaction from a mixture of various aldehydes and *t*-BuOOH. The red pigment formation in a mixture of 2.5 nmol tetramethoxypropane, 0.98 μmol 2-hexenal, 0.092 μmol 2,4-hexadienal and 9.4 μmol *t*-BuOOH in the presence (●) and absence (○) of 2.0 mg of unoxidized methyl linoleate is shown. The TBA reaction was performed in the presence of 0.01% BHT by the one-(——) and the two-step (-----) modes.

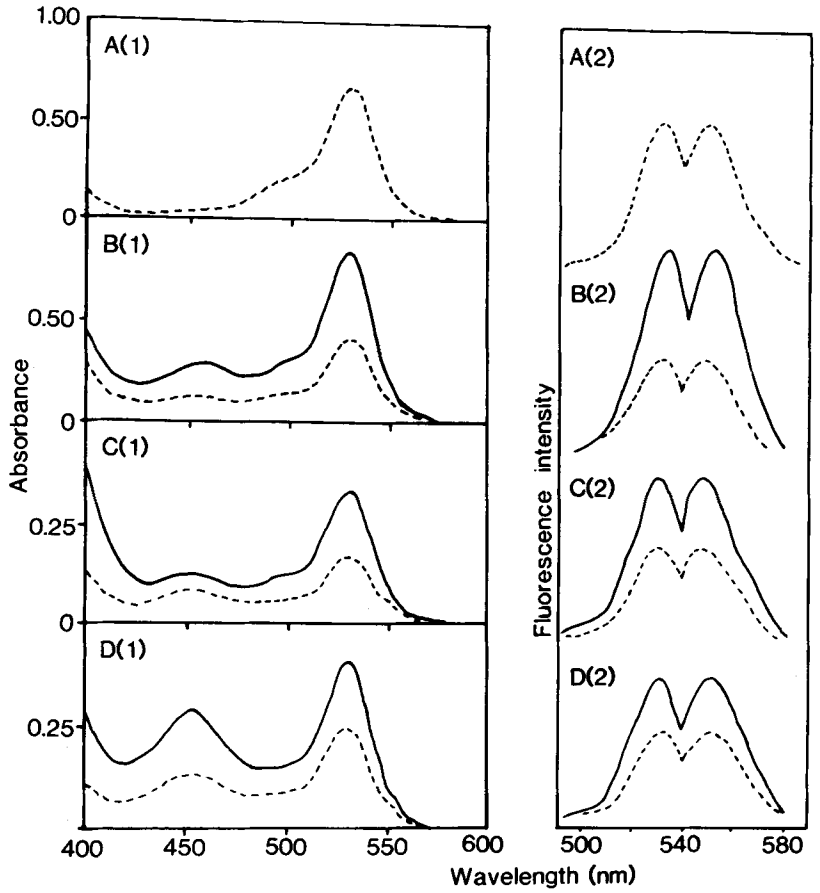

FIG. 2. Absorption (left) and fluorescence (right) spectra of the TBA reaction mixtures. The TBA reaction of tetramethoxypropane (24.4 nmol) (A), 13-LOOH (1.0 mg) (B), oxidized rat liver microsomal lipids (1.8 mg) (C) and human erythrocyte ghost lipids (3.9 mg) (D) with (——) and without (- - - -) 10 μmol *t*-BuOOH was performed in the presence of 0.01% BHT by the two-step mode. Absorption spectra of the reaction mixtures were recorded directly and fluorescence spectra were recorded after dilution into water.

TABLE 1

Amounts of Red Pigment, Total Aldehydes, Malonaldehyde and Hydroperoxides Formed from 13-LOOH and Oxidized Fatty Esters

	Red pigment (nmol/mg lipid) in the TBA test				Total aldehyde (nmol/mg lipid)		Malonaldehyde (nmol/mg lipid)	Hydroperoxide (μmol/mg lipid)
	One-step mode		Two-step mode					
Sample	None	+ *t*-BuOOH (10 μmol)	None	+ *t*-BuOOH (10 μmol)	DNPH	Dimedone	Hantzsch	Peroxide value
13-LOOH	11.4[a]	31.8[a]	13.4[a]	27.5[a]	87 ± 58*	231 ± 119*	<1.0	3.21
	11.8[b]	30.8[b]	13.0[b]	25.9[b]				
Methyl linoleate AOM-12 hr	7.7[a]	20.0[a]	8.9[a]	21.9[a]	67	202	0.7	0.81
	7.3[b]	22.1[b]	11.1[b]	23.2[b]				
	7.0[c]	19.6[c]	8.6[c]	21.2[c]				
Methyl oleate								
AOM-3 hr	1.2[a]	2.9[a]	2.4[a]	3.4[a]				0.11
27 hr	1.5[a]	4.5[a]	3.3[a]	4.3[a]				0.49

The TBA reactivity of each sample was assessed in the presence of 0.01% BHT. The amounts of red pigment are expressed as the mean values ± 1.0 (S.D.) of more than two different experiments. The amounts of total aldehyde liberated from 13-LOOH varied from experiment to experiment, and are expressed as the mean values ± S.D. of four different experiments (*).

[a]The amount of red pigment was determined by absorbance.

[b]The amount of red pigment was determined by fluorescence intensity.

[c]The amount of red pigment was determined by HPLC.

enals were detected by the DNPH method (about 87 nmol) and by the dimedone method (about 231 nmol) (Table 1). Addition of about 3 equivalent amounts (10 μmol) of *t*-BuOOH to the hydroperoxide dramatically increased the yields of the pigment. Absorption and fluorescence spectra of the reaction mixtures with *t*-BuOOH (Fig. 2B, solid curves) were similar to those without *t*-BuOOH (Fig. 2B, dotted curves). The yields of the pigment were 31 nmol (one-step) and 27 nmol (two-step) (Table 1). The enhanced pigment formation by *t*-BuOOH is characteristic to alkenals and alkadienals (16). The red pigment produced in the TBA reaction with 13-LOOH was most likely derived from the combined effect of alkenals, alkadienals and the intrinsic hydroperoxide content of the sample.

Red pigment formation from autoxidized fatty esters. Methyl linoleate was oxidized by the active oxygen method (AOM) for up to 24 hr (Fig. 3). The peroxide values of the ester increased during the first 12 hr and decreased thereafter (Fig. 3A). The amount of total aldehyde, as estimated by the DNPH and dimedone methods, increased during the first 12 hr and then decreased (Fig. 3B). Since volatile aldehydes may be removed during the oxidation, the aldehydes thus estimated appear to be those nonvolatile species liberated from the hydroperoxide compounds. The amount of total aldehyde was lower than that of the amount of hydroperoxide at any stage of autoxidation. The TBA reaction by the one- and the two-step modes showed that amounts of the pigment estimated by absorbance increased during the first 15 hr of oxidation and then decreased (Fig. 3C). Addition of 10 μmol *t*-BuOOH dramatically increased the amount of the pigment at any stage of autoxidation. No further pigment increases were observed by addition of 20 μmol *t*-BuOOH.

The reaction mixtures of the 12 hr-oxidized methyl linoleate with and without added *t*-BuOOH exhibited absorption spectra, fluorescence spectra and HPLC peaks identical with those due to a standard reaction mixture of tetramethoxypropane. The yields of red pigment from the 12 hr-oxidized methyl linoleate, estimated by absorbance, fluorescence intensity and HPLC, were much greater than the amount of malonaldehyde (Table 1). Aldehydes other than malonaldehyde and the hydroperoxide derivatives must have produced the pigment.

Time courses of peroxide values and red pigment formation associated with AOM-oxidized methyl oleate are shown in Figure 4. Red pigment formation was much greater by the two-step mode than by the one-step mode at all stages of autoxidation. The amounts of the pigment from the 3 hr and 23 hr-oxidized methyl oleate were markedly increased by addition of *t*-BuOOH (Table 1). The pigment derived from the oxidized methyl oleate may be due to the combined effect of aldehyde species other than malonaldehyde and the intrinsic hydroperoxide components.

Red pigment formation from autoxidized edible fats and oils. Soybean oil, sesame oil, hog fat and chicken oil were oxidized by AOM, and sardine oil was oxidized by ultraviolet light irradiation. The time courses of peroxide values and red pigment formation are shown in Figure 5. All the fats and oils showed similar parallel increases and decreases in the peroxide value and amount of red pigment. The red pigment formation from soybean, sesame, hog and chicken oils was slightly higher by the two-step TBA-reaction than by the one-step mode at all stages of autoxidation. The amounts of red pigment from soybean, sesame, chicken and sardine oils were significantly increased by addition

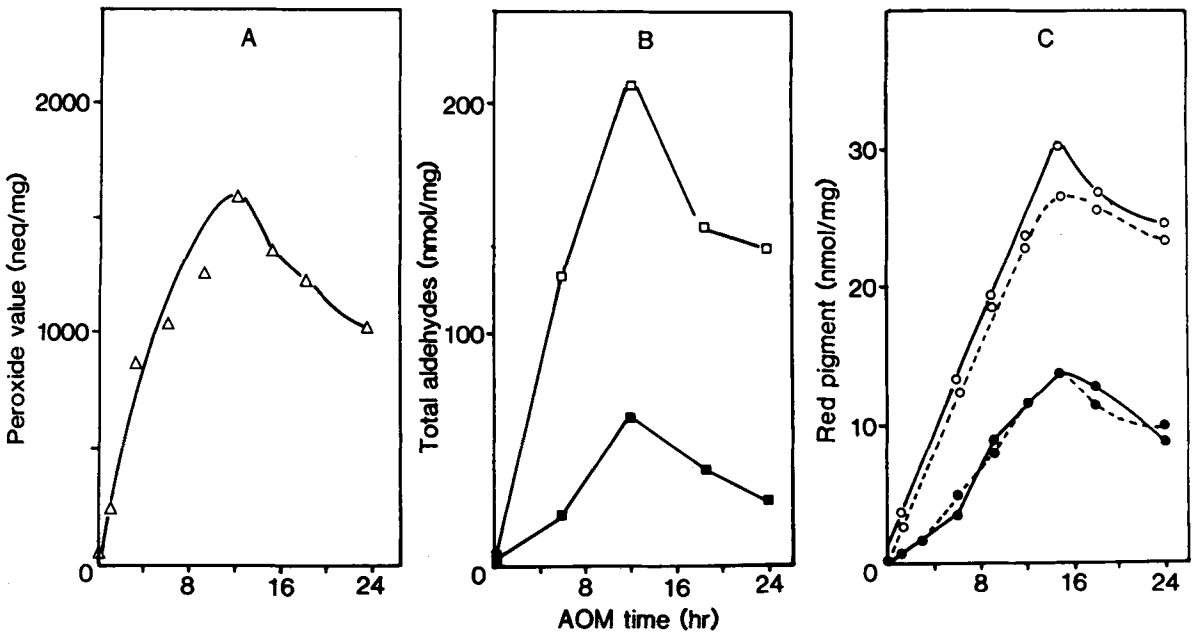

FIG. 3. Time courses of peroxide value (A), total aldehyde formation (B) and red pigment formation (C) of the AOM-oxidized methyl linoleate. Peroxide value (△) and amount of total aldehyde determined by DNPH (■) and dimedone (□) are presented. The TBA test was performed in the presence of 0.01% BHT by the one- (——) and the two-step (- - - -) modes. Absorbance at 532 nm of each sample linearly increased with the sample amount of 0.5–2.5 mg. Red pigment produced in the absence (●) and presence (○) of 10 μmol *t*-BuOOH was estimated by absorbance.

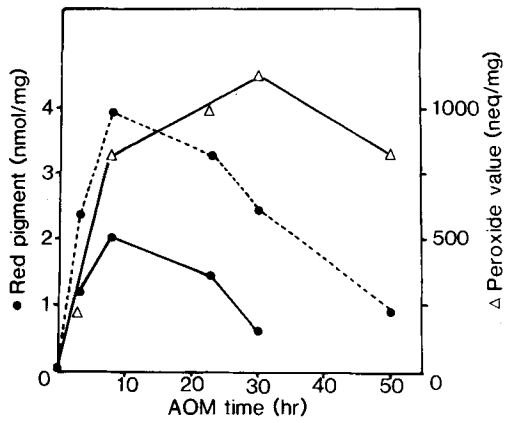

FIG. 4. Time courses of peroxide value and red pigment formation from the AOM-oxidized methyl oleate. The TBA test was performed in the presence of 0.01% BHT by the one- (——) and the two-step (— — —) modes. Absorbance of each sample linearly increased with the sample amount of 0.5–2.5 mg.

of *t*-BuOOH (Table 2). The results suggest that red pigment observed was derived from the combined effect of alkenals, alkadienals and intrinsic hydroperoxides.

Red pigment formation from oxidized rat liver microsomal lipid and oxidized human erythrocyte-ghost lipid. Rat liver microsomes were oxidized with ADP-ascorbate-Fe^{2+}, and human erythrocyte ghosts were oxidized with *t*-BuOOH in the presence of catalytic hemoglobin. The one- and the two-step modes of the TBA-reaction with either the extracted microsomal or ghost lipids yielded absorption spectra, fluorescence spectra (Fig. 2, C and D, dotted curves) and HPLC peaks identical with those of a standard reaction mixture of tetramethoxypropane. The microsomal lipids yielded 3 nmol pigment/mg by the one-step mode, and a little higher amount by the two-step mode (Table 3). The malonaldehyde content was lower than 1 nmol, but a larger amount of other aldehydes (more than 15 nmol) and hydroperoxide derivatives (about 10 nmol) were detected. The ghost lipids yielded 2 nmol pigment/mg by the one-and the two-step modes (Table 3). The malonaldehyde content was about 10% of the pigment content. A large amount of other aldehydes (more than 8 nmol) and hydroperoxide derivatives (about 25 nmol) were detected.

Addition of *t*-BuOOH to the microsomal or the ghost lipids yielded absorption and fluorescence spectra (Fig. 2, C and D, solid curves) and HPLC peaks identical with those of a standard reaction mixture of tetramethoxypropane, but the pigment yield in each case was greatly increased (Table 3). In the TBA reaction of these biological lipids, aldehydes other than malonaldehyde must have contributed to pigment formation.

DISCUSSION

Red pigment produced in the TBA test has been used as a measure of lipid peroxidation (1–3). It has been long held that the red pigment is derived from malonaldehyde generated from peroxidized lipid samples, since Sinnhuber *et al.* (4) and Nair and Turner (5) demon-

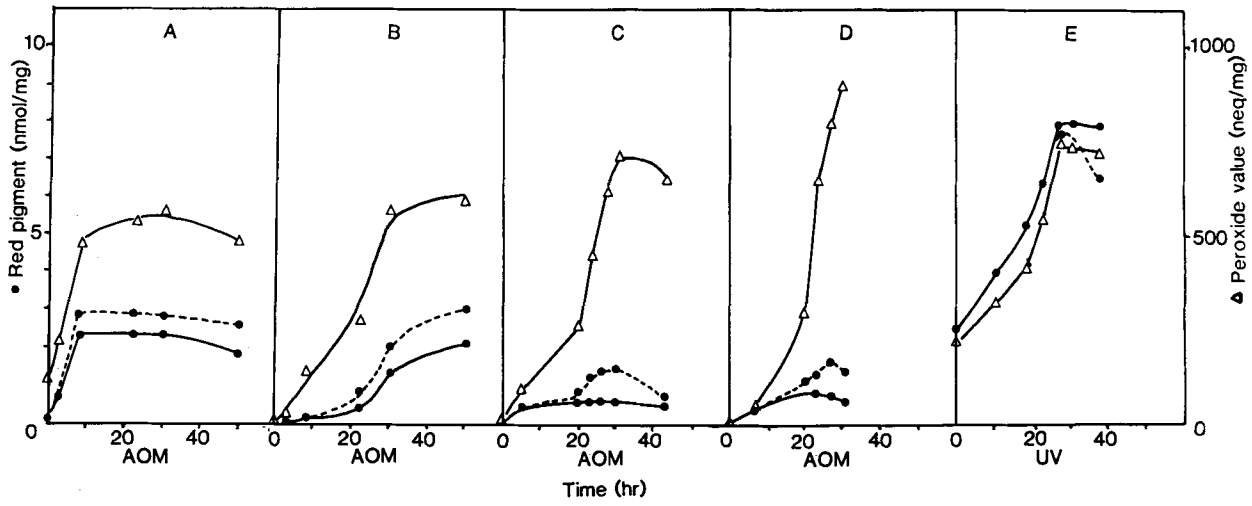

FIG. 5. Time courses of peroxide value and red pigment formation from oxidized soybean oil (A), sesame oil (B), hog fat (C), chicken oil (D) and sardine oil (E). The TBA test was performed in the presence of 0.01% BHT by the one- (——) and the two-step (— — —) modes. Absorbance of each sample linearly increased with the sample amount of 0.5–2.5 mg.

TABLE 2

Red Pigment Formation from Autoxidized Fats and Oils in the TBA Test

Sample	Red pigment (nmol/mg lipid) in the TBA test				Hydroperoxide (μmol/mg lipid)
	One-step mode		Two-step mode		
	None	+ *t*-BuOOH (10 μmol)	None	+ *t*-BuOOH (10 μmol)	Peroxide value
Soybean oil					
AOM-3 hr	0.7	1.3	0.8	1.3	0.11
AOM-30 hr	2.3	4.1	3.0	4.6	0.29
Sesame oil					
AOM-23 hr	0.5	0.9	0.8	1.0	0.14
AOM-50 hr	2.0	3.6	3.1	4.2	0.29
Hog fat					
AOM-5 hr	0.4	0.4	0.5	0.6	0.05
AOM-27 hr	0.6	1.2	1.5	1.5	0.31
Chicken oil					
AOM-27 hr	0.8	1.2	1.7	1.9	0.40
Sardine oil					
UV-37 hr	8.0	9.2	6.5	9.2	0.36

Fats and oils were autoxidized by AOM or ultraviolet (UV) irradiation. The TBA reaction of the sample was performed in the presence of 0.01% BHT, and the amount of red pigment formed was quantified by its absorbance.

TABLE 3

Red Pigment, Total Aldehyde, Malonaldehyde and Hydroperoxide Formation from Peroxidized Microsomal and Erythrocyte-Ghost Lipids

Sample	Red pigment (nmol/mg lipid) in the TBA test				Total aldehyde (nmol/mg lipid)		Malonaldehyde (nmol/mg lipid)	Hydroperoxide (nmol/mg lipid)	
	One-step mode		Two-step mode						
	None	+ *t*-BuOOH (10 μmol)	None	+ *t*-BuOOH (10 μmol)	DNPH	Dimedone	Hantzsch	*Sesamol* dimer	*Conjugated* diene
Peroxidized rat liver microsomal lipid	2.6[a] 2.6[b] 2.5[c]	5.1[a] 6.1[b] 5.0[c]	2.9[a] 2.8[b] 2.7[c]	4.8[a] 6.0[b] 4.4[c]	15.2	16.9	<1.0	9.9	11.8
Peroxidized human erythrocyte-ghost lipid	1.8[a] 1.6[b]	3.6[a] 3.4[b]	1.8[a] 1.7[b]	3.7[a] 3.3[b]	60.3	8.2	0.23	26.7	23.3

Rat liver microsomes and human erythrocyte ghosts were oxidized, and the oxidized membrane lipids were extracted. The TBA reactivity of each oxidized lipid sample was performed in the presence of 0.01% BHT. The amounts of red pigment are expressed as the mean values ± 0.5 (S.D.) of more than two different experiments.

[a]The amount of red pigment was determined by absorbance.

[b]The amount of red pigment was determined by flourescence.

[c]The amount of red pigment was determined by HPLC.

strated that the pigment is the 1:2 adduct of malonaldehyde and TBA. The amount of pigment can be determined by absorbance (4) and fluorescence intensity (33) and, more specifically, by HPLC (35). However, malonaldehyde determination by various methods, i.e., acid decomposition-acetylation (36), dansyl hydrazine (37), direct HPLC (38–40), aromatic amine-fluorometry (41) and Hantzsch method (17), have consistently shown that the malonaldehyde contents of various peroxidized lipid and membrane samples are much lower than those estimated by the TBA test. Hence, the TBA test can now be considered nonspecific for malonaldehyde and nonquantitative as a measure of malonaldehyde.

Standard aldehydes other than malonaldehyde may form red pigment in the TBA test (6–16). However, according to the experiments of Esterbauer *et al.* (11) and Witz *et al.* (12), yields of red pigment from the other aldehydes are much lower than that from malonaldehyde when each of them is reacted with TBA. We have previously reported that there are significant differences in the pigment formation between malonaldehyde and the other aldehydes (14–16). Although malonaldehyde produces the pigment without being affected by the reaction modes and by the presence of other aldehydes and organic hydroperoxides, alkenals and alkadienals produce red pigment depending on the reaction modes, the presence of oxygen and water, and the presence of other aldehydes and organic hydroperoxides (14–16). Pigment formation from alkenals and alkadienals is synergistically enhanced, and it is mark-

edly enhanced by organic hydroperoxides. While the reaction of each alkenal or alkadienal produces 0.2-0.5% (14) and 5-10% (15) pigment, respectively, yields of the pigment are greatly increased (by >30%) when they are combined with each other and with organic hydroperoxides (15,16). The organic hydroperoxides may act as a strong oxidant to promote the red pigment formation (15,16).

The present study was undertaken in order to elucidate which components of peroxidized lipid samples (i.e., peroxidized fatty esters, 13-LOOH, peroxidized edible fats and oils, and peroxidized microsomal and erythrocyte ghost lipids) are TBA-reactive. For this purpose, yields of red pigment formed by two TBA-reaction modes, one-step and two-step, were compared. Yields of the pigment in the absence and presence of *t*-BuOOH were also compared. Yields of the pigment were higher by the two-step mode than by the one-step mode, and much higher in the presence of *t*-BuOOH than in the absence of the agent. These characteristics of red pigment formation from the peroxidized lipid samples were similar to those from alkenals and alkadienals and different from that from malonaldehyde (15,16). Total aldehyde content of the peroxidized lipid samples estimated by the DNPH (11,32) and the dimedone (18) methods was much higher than the malonaldehyde content of the same sample, as estimate by the Hantzsch method (17). Under these TBA-test conditions, red pigment produced in the TBA test from peroxidized lipid samples could largely reflect aldehydes other than malonaldehyde.

Ohkawa *et al.* (42,43) used the TBA test for estimation of hydroperoxides in peroxidized lipid samples, but did not offer mechanistic details. In this regard, the red pigment formation from pure 13-LOOH in the present study is interesting. 13-LOOH by itself may not produce the pigment, since organic hydroperoxides, i.e., *t*-BuOOH and cumene hydroperoxide, do not (16). In the present study, 13-LOOH decomposed into various aldehyde products (3% of the hydroperoxide), including malonaldehyde (less than 0.03% of the hydroperoxide). Yield of red pigment from 13-LOOH (0.4% of the hydroperoxide) greatly exceeded the malonaldehyde content. Thus, aldehyde species other than malonaldehyde must have produced the pigment in concert with the intrinsic hydroperoxide function of 13-LOOH.

The TBA test may be a useful method for estimating a broad range of lipid peroxidation products. The red pigment formed in the reaction should be regarded as reflecting some combination of aldehydes and lipid hydroperoxides. Since the presence of *t*-BuOOH in the TBA reaction mixtures used maximized pigment formation, inclusion of *t*-BuOOH is desirable. The degree of lipid peroxidation estimated by the TBA test should be expressed as red pigment and not as malonaldehyde.

For determination of aldehyde species in peroxidized lipids, the DNPH method (11,32) and the dimedone (18) or cyclohexanedione (44) methods are often used. However, it was found in the present experiments that the total aldehyde content of pure 13-LOOH estimated by these methods was surprisingly high, indicating that aldehydes were liberated during the brief treatment of the hydroperoxide under the strongly acidic or heating conditions required by the methodology. These findings indicate that some of the aldehydes estimated by these methods are those liberated from lipid hydroperoxides as a direct result of the analytical procedures themselves.

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