METHODS

Thiobarbituric Acid Reaction of Aldehydes and Oxidized Lipids in Glacial Acetic Acid

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ABSTRACT

Thiobarbituric acid (TBA) reaction of several aldehydes and oxidized lipids in glacial acetic acid was performed. All the samples were freely soluble in the solvent used. Saturated aldehydes produced a stable yellow pigment with an absorption maximum at 455 nm, a red pigment derived from malonaldehyde at 532 nm, and an orange pigment due to dienals at 495 nm. The absorbance maximum was 7–9 per μmol for saturated aldehydes, 27.5 per μmol for malonaldehyde and about 2 per μmol for dienals. Autoxidation of unoxidized lipids increased progressively in glacial acetic acid. When the TBA test was performed under nitrogen, autoxidation of unoxidized lipids was inhibited completely. While saturated aldehydes produced no yellow pigment under nitrogen, oxidized lipids produced a considerable amount of stable yellow pigment. The value for absorbance at 455 nm as a function of autoxidation time paralleled those of peroxide values. The absorbance of most oxidized lipids at 455 nm was higher than at 532 nm. Yellow pigment formation in the TBA test under nitrogen could not be ascribed to free saturated aldehydes but rather to unspecified closely related substances. The stable yellow pigment was found to be an excellent indicator of lipid oxidation.

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INTRODUCTION

The TBA test generally is used for determination of lipid oxidation. TBA produces a red pigment with an absorption maximum at 532 nm by reaction with malonaldehyde formed during lipid oxidation (1,2). However, the oxidation of polyunsaturated fatty acids has been shown to give various aldehydes besides malonaldehyde (3,4). Several reports have discussed the reaction of TBA with various saturated and unsaturated monofunctional aldehydes. Jacobson et al. (5) reported that the reaction between TBA and a saturated aldehyde in a single-phase solvent of *iso*-octane, 1-propanol and water produced a yellow pigment with an absorption maximum at 452 nm, while the reaction between TBA and a dienal produced a red pigment with a maximum at 532 nm. Marcuse and Johansson (6) demonstrated that the reaction of a saturated aldehyde in water produced a yellow pigment with a maximum at 450 nm. Measurement of the yellow pigment for determination of lipid rancidity is limited, because the pigment is very unstable in an aqueous medium. Thus, the yellow pigment generally is recognized as an interfering component for measurement of red pigment (7–9).

We investigated the reaction of several aldehydes and oxidized lipids with TBA in glacial acetic acid and found that saturated aldehydes and oxidized lipids produced extensive amounts of the stable yellow pigment. In this paper, we describe the use of this yellow pigment for measurement of lipid oxidation.

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MATERIALS AND METHODS

Materials

TBA, glacial acetic acid (special grade), butylated hydroxytoluene (BHT), 1-propanal, 1-butanal and 1-hexanal were the products of Wako Pure Chemical Industries Ltd. (Osaka, Japan). Malonaldehyde bis(dimethylacetal), 1-heptanal, 1-propanal dimethylacetal, 2-ethyl-2-hexenal, *trans*-2-hexenal, methyl oleate and methyl linoleate were obtained from Tokyo Kasei Kogyo Company Ltd. (Tokyo, Japan). 2,4-Hexadienal and *trans,trans*-2,4-decadienal were from Aldrich Chemical Co. Inc. (Milwaukee, Wisconsin). Purified malonaldehyde sodium salt was prepared as described elsewhere (10). Linoleic acid 13-hydroperoxide (13-LOOH) was prepared enzymatically (11) from pure linoleic acid (Sigma Chemical Co. Ltd., St. Louis, Missouri).

Purified lipids of hog, beef and chicken were prepared by warming and pressing the corresponding subcutaneous fats obtained commercially and washing with hot distilled water several times. Lipids of sardine, beef liver and beef muscle were extracted as follows. The

sample was mixed with an equal volume of 1.15% KCl and homogenized; the homogenate then was mixed with an equal volume of methanol and two vol of chloroform and centrifuged at 3000 rpm for 10 min. The chloroform/methanol layer was collected and evaporated to dryness.

Autoxidation of Lipids

Methyl oleate, methyl linoleate and purified lipids from hog, beef and chicken tissues were oxidized at 98 C by the active oxygen method (AOM) (12) for 70 hr. Chloroform/methanol extracts of the tissue were dissolved in benzene (2.0 g/20 ml) and placed in culture dishes (diameter 10 cm). Samples then were irradiated at a distance of 30 cm from two Toshiba U.V. lamps at 325 nm (Toshiba Company Ltd., Tokyo, Japan) at room temperature for 70 hr. Solvent was supplied intermittently to replace that lost by evaporation. The peroxide value of each oxidized lipid was determined according to the Wheeler method (13).

TBA Reaction

An indicated amount of each lipid sample, which included the standard aldehyde, was dissolved in 5.0 ml of 0.4% TBA/glacial acetic acid in a test tube with a screw cap. The mixture was heated at 100 C either in air or under nitrogen gas for up to five hr. After cooling, the absorption spectrum of the clear reaction mixture was measured with a UV-200S Shimadzu double beam spectrophotometer or a UV-240 UV-visible Shimadzu recording spectrophotometer.

RESULTS

Reaction of Various Aldehydes with TBA in Glacial Acetic Acid

The saturated and unsaturated aldehydes as well as malonaldehyde and their acetals were reacted with TBA in glacial acetic acid at 100 C. 1-Hexanal produced a yellow pigment showing a single absorption maximum at 455 nm. Characteristics of the formation of the yellow pigment are shown in Figure 1. The absorbance at 455 nm increased gradually up to five hr. The relationship between the amount of 1-hexanal and the absorbance at 455 nm was linear after the five hr reaction period (Fig. 1 insert). The presence of 2% BHT suppressed formation of the yellow pigment by about 40%. The reaction in the presence of 20% water yielded only 10% yellow pigment. This lower yield could be due to the lability of the yellow pigment in the aqueous medium. The pigment produced in glacial acetic acid after three hr of reaction was shown to be labile in aqueous media by demonstrating that it was degraded progressively by subsequent addition of water. When the reaction was performed under nitrogen, yellow pigment formation was inhibited completely. A reaction system that had been bubbled with nitrogen gas and subsequently aerated produced yellow pigment to the same extent as that in the same system under air, indicating the aldehyde was not purged by bubbling. The aldehyde pretreated in glacial acetic acid at 100 C for five hr also produced no yellow pigment in subsequent reaction with TBA under nitrogen, indicating that the yellow pigment formation was not due to oxidation of the aldehyde in glacial acetic acid.

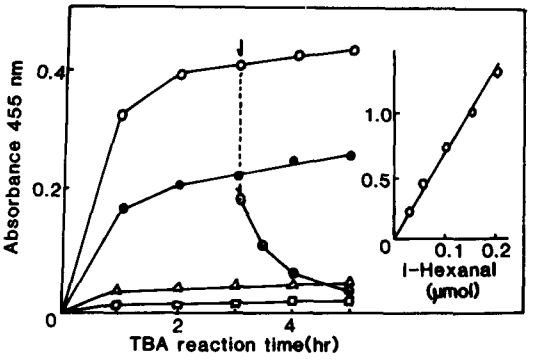

FIG. 1. Time-course of yellow pigment formation in reaction of 1-hexanal with TBA. 1-Hexanal (0.05 μmol) was dissolved in 5.0 ml of TBA/glacial acetic acid (○), TBA/glacial acetic acid containing 2% BHT (●) and TBA/water-glacial acetic acid (1:4, v/v) (△), and the mixtures were heated at 100 C. As indicated by an arrow, 1.0 ml of water was added (⊙). A mixture of 1-hexanal in TBA/glacial acetic acid was gassed with nitrogen and similarly heated (□). The insert indicates a calibration curve of 1-hexanal treated with TBA/glacial acetic acid at 100 C for 5 hr.

Other saturated aldehydes, 1-propanal, 1-butanal, and 1-heptanal, showed the same type of reaction with TBA. The amounts of yellow pigment as estimated by absorbance at 455 nm were between 7–9 per μmol aldehyde (Table 1). When two aldehydes were mixed and reacted with TBA, the absorbance value observed was the sum of that obtained for each aldehyde. 1-Propanal dimethylacetal produced yellow pigment under air but not under nitrogen. The yield of yellow pigment under air was similar to that for 1-propanal (Table 1). The reaction of the free and acetal forms of the aldehyde were quite similar.

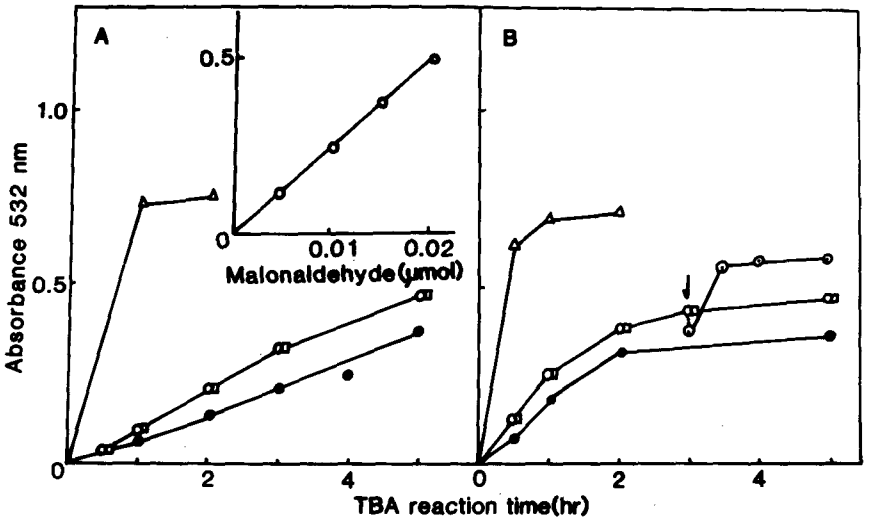

FIG. 2. Time-course of red pigment formation in reaction of free malonaldehyde (A) and malonaldehyde bis(dimethylacetal) (B) with TBA. Malonaldehyde sodium salt or malonaldehyde bis(dimethylacetal) (0.02 μmol) was dissolved in 5.0 ml of TBA/glacial acetic acid (○), TBA/glacial acetic acid containing 2% BHT (●) and TBA/water-glacial acetic (1:4, v/v) (△), and the mixtures were heated at 100 C. As indicated by an arrow, 1.0 ml of water was added (⊙). A mixture of malonaldehyde sodium salt or malonaldehyde bis(dimethylacetal) in TBA/glacial acetic acid was gassed with nitrogen and similarly heated (□). The insert indicates a calibration curve of malonaldehyde treated with TBA/glacial acetic acid at 100 C for 5 hr.

TABLE 1

Absorbance of the Reaction Mixture of the Aldehyde in 5 ml of 0.4% TBA/Glacial Acetic Acid at 100 C for 5 Hr

Aldehyde	Absorbance/μmol aldehyde		
	455 nm	495 nm	532 nm
1-Propanal	7.4		
1-Butanal	8.2		
1-Hexanal	8.2		
1-Heptanal	9.0		
1-Propanal dimethylacetal	7.4		
Malonaldehyde sodium salt			27.5
Malonaldehyde bis(dimethylacetal)			27.5
trans-2-Hexenal	0.4		
2-Ethyl-2-hexenal	0.1		
2,4-Hexadienal	1.2	2.5	1.0
trans,trans-2,4-Decadienal	2.1	2.0	1.0

Malonaldehyde sodium salt and malonaldehyde bis(dimethylacetal) produced a red pigment with a single absorption maximum at 532 nm, both having a similar absorbance intensity (Fig. 2). The relationship between the amount and absorbance at 532 nm was linear (Fig. 2A insert). The absorbance at 532 nm per μmol free malonaldehyde or malonaldehyde bis(dimethylacetal) was 27.5 (Table 1); this value was about four times as large as that at 455 nm obtained from the saturated aldehydes. Addition of water to the reaction mixtures increased the formation of red pigment about two-fold. The presence of BHT suppressed the formation of red pigment from both the free and acetal forms. In contrast to the reaction of the saturated aldehydes, this reaction was not influenced when carried out under nitrogen.

Monoenals, *trans*-2-hexenal and 2-ethyl-2-hexenal, produced a yellow pigment with a single absorption maximum at 455 nm, but the absorbance per μmol of aldehyde was extremely low (Table 1). Dienals, 2,4-hexadienal and *trans,trans*-2,4-decadienal, produced an orange pigment with three absorption maxima at 455, 495 and 532 nm, with the absorbance at 495 nm being the highest (Fig. 3A). The absorbance at 455 and 532 nm was much lower than that of the saturated aldehydes and malonaldehyde, respectively (Table 1). Formation of the orange pigment was greatly suppressed under nitrogen and by water (Fig. 3A). The lower yield of the orange pigment in aqueous acetic acid could not be ascribed to the lability of the pigment, since

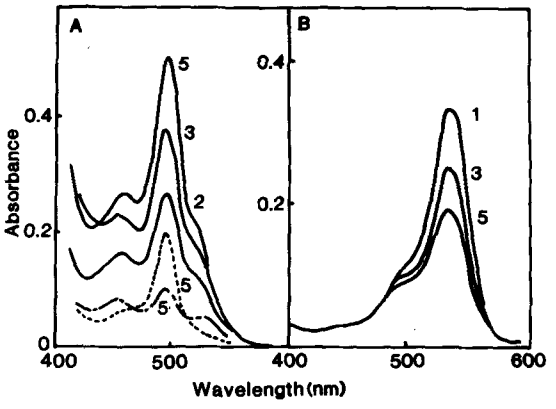

FIG. 3. Absorption spectra of the reaction mixtures of 2,4-hexadienal with TBA. A: 2,4-Hexadienal (0.2 μmol) was heated at 100 C in TBA/glacial acetic acid (———), TBA/glacial acetic acid-water (4:1, v/v) (— - —) and TBA/glacial acetic acid gassed with nitrogen (- - - -). B: 2,4-Hexadienal (1 μmol) was heated at 100 C in TBA/water. Numerals indicate time (hr) of reaction.

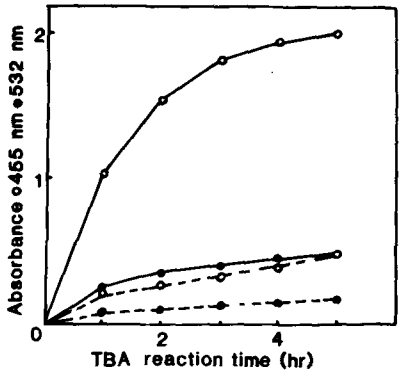

FIG. 4. Time-courses of yellow and red pigment formation during reaction of 13-LOOH with TBA. 13-LOOH (1.0 mg, 3.2 μmol) was dissolved in TBA/glacial acetic acid and the mixture was heated at 100 C (———). The mixture was gassed with nitrogen and heated at 100 C (- - - -).

the pigment produced in glacial acetic acid was not destroyed by subsequent addition of water. It is interesting to note that when the dienals were treated with TBA in water alone, a red pigment having a single absorption maximum at 532 nm was produced (Fig. 3B). Although the absorption spectrum of the red pigment was indistinguishable from that of the pigment derived from malonaldehyde, they were different in stability. Thus, the red pigment from 2,4-hexadienal was destroyed progressively in water (Fig. 3B), while the red pigment from malonaldehyde was stable.

Reaction of Linoleic Acid 13-Hydroperoxide with TBA in Glacial Acetic Acid

Reaction of 13-LOOH with TBA in glacial acetic acid at 100 C showed three absorption maxima at 455, 495 and 532 nm, with the absorbance at 455 nm being the highest. 13-LOOH may be degraded into a complex mixture of compounds (3,4) under the reaction conditions. The time-course of increase in absorbance at 455 and 532 nm is shown in Figure 4. The increase in absorbance at 532 nm probably is due primarily to malonaldehyde, which was estimated to be 0.015 μmol (0.5% of the hydroperoxide) after reaction for five hr. The reaction was suppressed under nitrogen to 40% of the control value, and may reflect retarded degradation of the hydroperoxide. The increase in absorbance at 455 nm might be due to the saturated aldehydes produced by degradation of the hydroperoxide. The amount of saturated aldehydes liberated was estimated to be 0.24 μmol (7.5%) after reaction for five hr. Yellow pigment formation was suppressed to about 25% under nitrogen. Formation of the yellow pigment under nitrogen was high in spite of the retarded degradation of the hydroperoxide and the unreactivity of the saturated aldehydes (Fig. 1). Yellow pigment formation from the hydroperoxide under nitrogen could not be due to the free saturated aldehydes but, rather, to other closely related substances.

Reaction of Oxidized Methyl Oleate and Linoleate with TBA in Glacial Acetic Acid

When unoxidized methyl oleate and linoleate were treated with TBA in glacial acetic acid at 100 C, absorbance at 455 and 532 nm increased progressively, indicating that the esters were oxidized during TBA reaction. Addition of 2% BHT prevented the autoxidation, but not completely. Treatment of unoxidized methyl esters with TBA under nitrogen produced no yellow or red pigments. Thus, oxidation during the TBA reaction was inhibited completely under nitrogen. Therefore, measurement of the extent of oxidation of lipids must be performed under nitrogen.

Methyl oleate and linoleate were oxidized by the AOM, and the oxidized esters were reacted with TBA in glacial acetic acid at 100 C for five hr under nitrogen. As oxidation of the esters proceeded, the reaction mixtures with TBA revealed three absorption maxima at 455, 495 and 532 nm. Relationships between the amount

of the oxidized esters and absorbance at 455 nm (Fig. 5 insert) and at 532 nm were linear. Figure 5 shows the time-course of absorbance at 455 and 532 nm against the AOM time of the esters. Absorbance at 455 nm was much higher than at 532 nm throughout the oxidation time of the esters. Profiles of absorbance at 455 nm were correlated roughly to the peroxide values of both the oxidized esters. The absorbance at 455 nm may be an excellent indicator of peroxidation of these esters. Formation of the pigment could not, however, be ascribed to free saturated aldehydes, but instead to closely related substances. The increase in absorbance at 532 nm was lower than that at 455 nm with both oxidized esters. Absorbance at 532 nm was extremely low with the oxidized methyl oleate throughout the AOM time.

Reaction of Oxidized Fats and Oils with TBA in Glacial Acetic Acid

Hog, beef and chicken fats, which had been oxidized by the AOM, were reacted with TBA in glacial acetic acid at 100 C for five hr under nitrogen. The time-course of the peroxide value and the absorbances at 455 and 532 nm against AOM time are shown in Figure 6. It was found

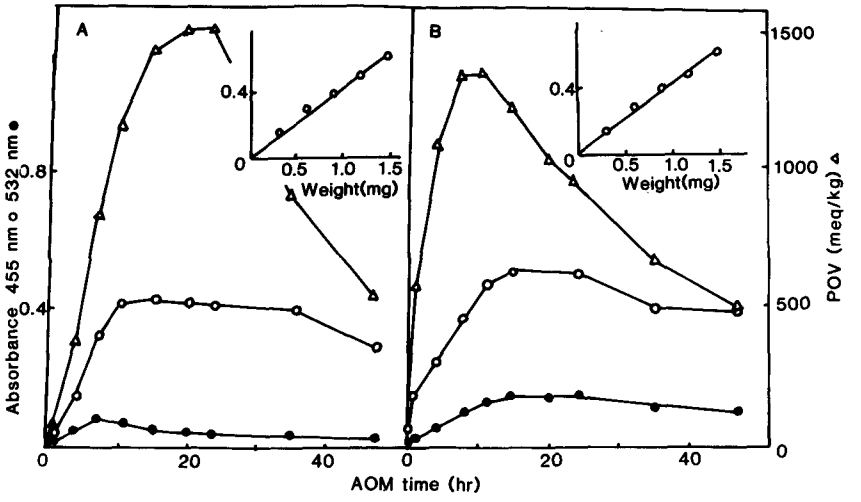

FIG. 5. Time-courses of yellow and red pigment formation in oxidized methyl oleate (A) and methyl linoleate (B). Methyl oleate or methyl linoleate was autoxidized by the AOM, and the oxidized ester (1.0 mg) was reacted in TBA/glacial acetic acid at 100 C for 5 hr under nitrogen. Absorbance at 455 and 532 nm was plotted against AOM time. Peroxide values also were plotted. Inserts indicate calibration curves of absorbance at 455 nm of the oxidized esters (AOM time: 20 hr).

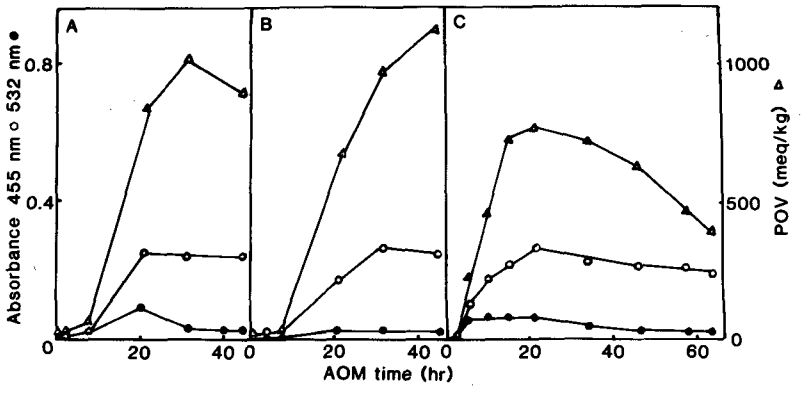

FIG. 6. Time-courses of yellow and red pigment formation in oxidized hog (A), beef (B) and chicken (C) fat. Hog, beef or chicken fat was autoxidized by the AOM, and the oxidized fat (1.0 mg) was reacted with TBA as described in Fig. 5.

that absorbance at 455 nm was more intense than at 532 nm under these conditions, and paralleled the increase and decrease in peroxide values.

Chloroform/methanol extracts of sardine, beef liver and beef muscle, which had been oxidized by ultraviolet irradiation, were reacted with TBA. The time-course of the peroxide value, and the absorbances at 455 and 532 nm as a function of ultraviolet irradiation time of the extract, are shown in Figure 7. The absorbance at 455 nm showed a parallel increase and decrease with the peroxide values. The absorbance at 455 nm was more intense than the absorbance at 532 nm except for sardine extract. The sardine extract showed a higher absorbance at 532 nm, which may be due to its higher polyunsaturated fatty acid content.

DISCUSSION

The TBA test generally is used for measurement of oxidation of lipids. It forms a red pigment with an absorption maximum at 532 nm by reaction with malonaldehyde and its precursors (1,2). Aqueous acidic solutions have been used in the TBA tests reported so far. It was found in the present experiments that deterioration of lipids could be monitored sensitively at 455 nm when the TBA test is carried out in glacial acetic acid.

Performing the TBA reaction in glacial acetic acid had advantages. Glacial acetic acid could solubilize all the lipid samples for the test. Most procedures described in the literature were performed in aqueous acidic media, and lipids are not completely solubilized, possibly interfering with spectrophotometric measurement of the red pigment. For instance, unsolubilized lipid has been removed by extraction (14) or by centrifugation (15) before spectrophotometric measurement. It is doubtful whether a reaction in which not all lipid is solubilized in the reaction mixture accurately reflects the degree of oxidation of the samples. It was found in the present experiment that the formation of red pigment from malonaldehyde and malonaldehyde bis(dimethylacetal) in glacial acetic acid was only about half that obtained in the aqueous acetic acid (Fig. 2).

The yellow pigment produced by the reaction of the saturated aldehydes with TBA was stable in glacial acetic acid under the reaction conditions. Jacobson et al. (3) and Marcuse and Johansson (6) demonstrated that a saturated aldehyde produced a yellow pigment in the reaction with TBA in aqueous media, but the pigment was very unstable. Therefore, the yellow pigment has been considered an interfering product for measurement of the red pigment (7–9). Under the present conditions, the saturated aldehydes produced a stable yellow pigment, which could be measured by its absorbance at 455 nm. The absorbance at 455 nm obtained with saturated aldehydes was about one-fourth of that in 532 nm from malonaldehyde or malonaldehyde bis(dimethylacetal) (Table 1).

The dienals produced an orange pigment with an absorption maximum at 495 nm on reacting with TBA in glacial acetic acid. The dienals produced a different type of pigment in glacial acetic acid, although they produced the red pigment in water (5,6). But the absorbance at 495 nm was much lower than that at 455 nm from the saturated aldehydes, and also lower than that at 532 nm observed for malonaldehyde and malonaldehyde bis(dimethylacetal).

Oxidation of lipids during the TBA reaction in glacial acetic acid increased progressively. 13-LOOH was degraded during the reaction in glacial acetic acid (Fig. 4). Some investigators have used antioxidants such as BHT to prevent autoxidation of lipids during TBA reaction in aqueous acidic media (15,16). It was found in the present experiments that BHT could not inhibit completely autoxidation of unoxidized methyl oleate and linoleate during TBA reaction in glacial acetic acid. Furthermore, BHT influenced formation of the yellow and red pigments from the saturated aldehydes and malonaldehyde (Figs. 1 and 2). Autoxidation of unoxidized lipids during the TBA reaction in glacial acetic acid was inhibited completely under nitrogen. Therefore, it is recommended that measurement of the degree of lipid peroxidation with TBA in glacial acetic acid be performed under nitrogen. It is interesting to note that while formation of the red pigment from malonaldehyde and its acetal was affected little under nitrogen (Fig. 2), formation of the yellow pigment from saturated aldehydes and their acetals was inhibited dramatically under nitrogen (Fig. 1). The reason for this effect is not known. The yellow pigment formed in the reaction of oxidized lipids with TBA (Figs. 5, 6 and 7) cannot be derived from the saturated aldehydes but from other closely related products. Nevertheless, the increase in absorbance at 455 nm always was higher than the increase at 532 nm throughout the oxidation process of lipids except for sardine lipid. Thus, absorbance at 455 nm was a better indicator of oxidized lipids than that at 532 nm.

It has been demonstrated that the yellow pigment also was produced during the reaction of TBA with sugars and other water-soluble impurities (17–20). The specificity of the reaction

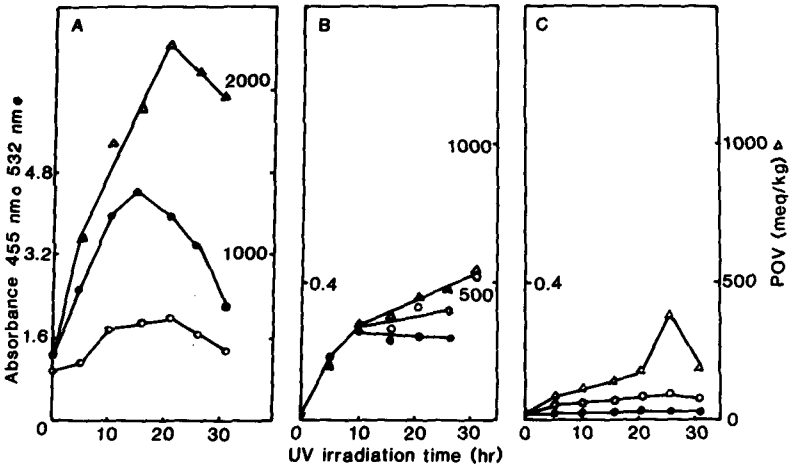

FIG. 7. Time-courses of yellow and red pigment formation in oxidized chloroform/methanol extract of sardine (A), beef liver (B) and beef muscle (C). The chloroform/methanol extract was oxidized by ultraviolet irradiation, and the oxidized extract (1.0 mg) was reacted with TBA as described in Fig. 5.

for lipids can be maintained, however, if the lipid is isolated by extraction with organic solvents prior to the assay. The lipids used in the present experiment were free of these contaminants, and the absorbance at 455 nm can be ascribed to some unspecified species derived from lipid oxidation.

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