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MAJOR THIOBARBITURIC ACID-REACTIVE SUBSTANCES OF LIVER HOMOGENATE ARE ALKADIENALS

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Thiobarbituric acid (TBA) assay of rat liver homogenate was performed by four general variations with 0.01% butylated hydroxytoluene. Development of red pigment was greatly dependent on the methods. The pigment formation by each method was dramatically increased by introduction of 2 mM *t*-butyl hydroperoxide (*t*-BuOOH). The pH values of the reaction mixtures greatly affected the pigment yield both in the absence and presence of *t*-BuOOH, and the maximal pigment yield was obtained at pH 3–4. These characteristic profiles of the pigment formation were similar to those from alkadienals and essentially different from those from malonaldehyde. Alkadienals were likely candidates for the TBA-reactive substances in the homogenate.

KEY WORDS: TBA assay, liver homogenate, malonaldehyde, alkadienal, alkenal, hydroperoxide.

INTRODUCTION

The thiobarbituric acid (TBA) assay has been used for determination of lipid peroxidation of biological samples, since it was introduced by Kohn and Liversedge.[1] Several variations of the TBA assay have been developed for determination of lipid peroxidation of liver homogenate or microsomes[2–4] and of blood plasma.[5] In the TBA assay the red 1:2 malonaldehyde-TBA adduct with an absorption maximum at 532 nm has been used as an index for lipid peroxidation.[6] For many years it has been considered that the pigment is derived from malonaldehyde in the samples.[1–6] However, it has been demonstrated that malonaldehyde contents determined by alternative methods were much lower than those estimated by the TBA assay.[7–13] It remains unclear what substances are detected in the TBA assay.

Recently we have demonstrated that the TBA assay of alkenals[14] and alkadienals,[15] both being possible components in peroxidized lipids,[16] in aqueous acetic acid produce the red 1:2 malonaldehyde-TBA adduct. 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.[15,17] It has been shown that most of the TBA-reactive substances in peroxidized edible fats and oils, and peroxidized microsomal and erythrocyte ghost lipids are not due to malonaldehyde but to the combined effects of alkenals, alkadienals and hydroperoxide components.[18,19] This time, we investigated what substances are detected in the TBA

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assay of rat liver homogenate by use of four general variations of the TBA assay. Method A by the present authors,[18] method B by Buege and Aust,[2] method C by Uchiyama and Mihara,[3] and method D by Ohkawa *et al.*[4] were employed for this purpose, the latter three methods having been applied to the determination of lipid peroxidation of liver homogenate.

MATERIALS AND METHODS

Absorption spectra were measured on a Hitachi U-2000 spectrophotometer. Thiobarbituric acid (TBA) was obtained from Wako Pure Chemical Industries, Osaka. Tetramethoxypropane (malonaldehyde source) and 2,4-hexadienal were obtained from Tokyo Kasei Kogyo Company, Tokyo. *t*-Butyl hydroperoxide (*t*-BuOOH) (70% in water) was obtained from Sigma Chemical Company, St. Louis, and its concentration was determined to be 7.0 M by iodometry. Butylated hydroxytoluene (BHT) was obtained from Nikki Universal Company, Tokyo. Glacial acetic acid was a special reagent grade product of Wako Pure Chemical Industries.

Wistar male rats weighing 180–200 g were sacrificed by bleeding from common carotid arteries, and livers were quickly isolated and homogenized with a cold 1.15% KCl solution to make a 30% homogenate. Two different preparations of the homogenate were used in the experiment. Lipid fraction of the homogenate was extracted according to the method of Bligh and Dyer.[20] Protein content was determined by Lowry method.[21]

The TBA assay was carried out by use of four variations: method A by the present authors,[18] method B by Buege and Aust,[2] method C by Uchiyama and Mihara,[3] and method D by Ohkawa *et al.*[4] The amount of red pigment produced was determined by absorbance at 532 nm and the extinction coefficient of the 1:2 malonaldehyde-TBA adduct: 156000.[6]

Method A

In a test tube was placed 0.5 ml of water containing the homogenate, 4.5 ml of 0.44% TBA solution in water, 0.1 ml of BHT solution (final concentration: 0.01%) in glacial acetic acid, and 10 μl of *t*-BuOOH solution (final concentration: 2.0 mM) in glacial acetic acid, in this order. The pH value of the reaction mixture was about 2.1. For the assay of standard aldehydes, the homogenate was replaced by water and the BHT solution was by the sample solution in glacial acetic acid containing BHT. The mixture was immediately heated at 100°C for 20 min. The mixture was extracted with 4.0 ml of *n*-butanol.

Method B

In a test tube was placed 1.0 ml of water containing the homogenate, 2.0 ml of 0.375% TBA solution in 15% trichloroacetic acid-0.25 N hydrochloric acid, and 50 μl of BHT solution (final concentration: 0.01%) in glacial acetic acid, in this order. The pH value of the reaction mixture was about 0.7. For the assay of standard aldehydes, the homogenate was replaced by water and the BHT solution was by the sample solution in glacial acetic acid containing BHT. Ten microliters of *t*-BuOOH solution (final concentration: 2.0 mM) were introduced in the reaction mixture. The mixture was

heated at 100°C for 15 min. After cooling, the mixture was centrifuged at 3500 rpm for 15 min to remove insoluble materials.

Method C

In a test tube was placed 0.5 ml of water containing the homogenate, 3.0 ml of 1% phosphoric acid, 10 μl of a mixture of BHT (final concentration: 0.01%) and *t*-BuOOH (final concentration: 2.0 mM) in glacial acetic acid, and 1.0 ml of 0.6% TBA solution in water, in this order. The pH value of the reaction mixture was about 1.8. For the assay of standard aldehydes, 0.5 ml of water, 3.0 ml of 1% phosphoric acid, 1.0 ml of the TBA solution, 50 μl of the sample solution in glacial acetic acid containing BHT, and 10 μl of the *t*-BuOOH solution were mixed in this order. The mixture was heated at 100°C for 45 min. After cooling, the mixture was extracted with 4.0 ml of *n*-butanol.

Method D

In a test tube was placed 0.1 ml of water containing the homogenate, 0.7 ml of water, 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid solution adjusted at pH 3.5 with 10 N NaOH, 1.5 ml of 0.8% TBA solution in water, 50 μl of BHT solution (final concentration: 0.01%) in glacial acetic acid, and 10 μl of *t*-BuOOH solution (final concentration: 2.0 mM) in glacial acetic acid, in this order. For the assay of standard aldehydes, the homogenate was replaced by water and the BHT solution was by the sample solution in glacial acetic acid containing BHT. The mixture was heated at 100°C for 60 min. After cooling, the mixture was extracted with 1.0 ml of water and 5.0 ml of *n*-butanol-pyridine (15:1).

RESULTS

The degree of red pigment development from rat liver homogenate by four different variations of the TBA assay, methods A, B, C and D, was compared. Major differences of the reaction conditions of these methods were those of the solvents employed: acetic acid in method A, trichloroacetic acid-hydrochloric acid in method B, phosphoric acid in method C, and acetate adjusted at pH 3.5 and sodium dodecyl sulphate in method D. The reaction periods of these methods were varied from 15 to 60 min. The pH values of the reaction mixtures of these methods were different. In order to prevent possible autoxidation of lipid fraction of the homogenate during the assay, 0.01% BHT was added to each reaction mixture. It was found that the differences of the yields of red pigment produced per ml of the same preparation of the homogenate were unexpectedly large depending on the methods (Table I, left column). Time-course studies of the pigment formation by methods A–D indicated that the pigment formation reached plateaus at the standard reaction times. Differences in the pigment formation by these methods could not be accounted for by the different reaction periods (Data not shown). While the yields of the pigment estimated by methods C and D were little dependent on the sample size, those estimated by methods A and B decreased with increasing sample size (Table I, left column). Low solubilities of the sample in the solvents of methods A and B might partly reduce the yields in the larger sample sizes. The yields by method D were the highest, and they were 15–20 times as

TABLE I
Amounts of red pigment formed by four different methods of TBA assay of rat liver homogenate

Method	Preparation of homogenate[a]	Sample size (ml)[b]	Red pigment (nmol/ml homogenate)	
			− *t*-BuOOH	+ 2 mM *t*-BuOOH
Method A	1	0.5	4.31	10.77
	2	0.4	4.77	9.83
		0.5	4.27 ± 0.44[c]	8.50 ± 0.75[c]
		0.6	3.67	8.33
		1.0	2.70	5.83
Method B	1	0.5	3.92	8.25
		1.0*	2.76	5.63
	2	1.0*	3.90	7.15
Method C	1	0.25	25.8	59.2
		0.5*	21.8	57.5
Method D	1	0.1*	43.8 ± 5.2[d]	92.1 ± 16.3[d]
		0.2	48.3	75.2
	2	0.1*	75.3	127.6

All the methods were performed in the presence of 0.01% BHT. *a*) Two preparations of homogenate (1 and 2) containing 7 mg lipid and 36 mg protein per ml were used for the assay. *b*) Asterisk* indicates sample size in the original methods. *c*) Mean value ± S.D. of six determinations. *d*) Mean value ± S.D. of seven determinations.

high as those by method B. The high yields by method D may be due to not only the solubilities of the samples but also the high ability of the TBA-reactive substances for the red pigment formation under the assay conditions.

When 2 mM *t*-BuOOH was introduced in the reaction mixtures of methods A-D, the yields of the pigment increased about 2-fold with all the methods (Table I, right column). Since this agent alone did not produce any significant amount of the pigment in these methods, the increases could be ascribed to the enhancing effect of the agent on the pigment formation. Time-course studies of the pigment formation from the homogenate by methods A and D indicated that the amounts of the pigment produced in the absence and presence of *t*-BuOOH increased with the reaction time and reached plateaus at the standard reaction time (Data not shown). Thus, autoxidation of lipid fraction of the homogenate did not take place even in the presence of *t*-BuOOH. Enhancing effect effect of the hydroperoxide indicates that the TBA-reactive substances in the homogenate may be alkenals and/or alkadienals, since it has been shown that the pigment formation from these aldehydes in aqueous acetic acid is markedly enhanced by the hydroperoxide.[17]

Standard aldehydes, tetramethoxypropane (malonaldehyde) and 2,4-hexadienal, were subjected to the TBA assay with these methods (Table II). Tetramethoxypropane has been used as a standard in all the variations of the TBA assay.[2-6] 2,4-Hexadienal is a representative of alkadienals that are generated during lipid peroxidation.[16] Tetramethoxypropane produced an almost quantitative amount of the pigment in all the methods as has been reported,[2-4,17] and the pigment formation was not enhanced by introduction of *t*-BuOOH in the reaction mixtures. In contrast, the yields of the pigment from 2,4-hexadienal estimated by the extinction coefficient of the red 1 : 2 malonaldehyde-TBA adduct[6] greatly depended on the methods, and the yield of the pigment was the highest by method D. The yields of the pigment were dramatically increased by introduction of *t*-BuOOH in the reaction of these methods.

It has been shown that the pH values of the reaction mixture greatly influence the

TABLE II
Comparison of the amounts of red pigment formed by four different methods of the TBA assay of standard tetramethoxypropane (malonaldehyde) and 2,4-hexadienal

Method	Red pigment yield (%) from			
	Tetramethoxypropane[a]		2,4-Hexadienal[b]	
	− *t*-BuOOH	+ 2 mM *t*-BuOOH	− *t*-BuOOH	+ 2 mM *t*-BuOOH
Method A	96	96	0.06 ± 0.03	2.02 ± 0.28
Method B	94	96	0.11 ± 0.29	2.19 ± 0.44
Method C	94	96	0.08 ± 0.08	1.46 ± 0.53
Method D	95	98	3.49 ± 0.64	7.66 ± 1.40

All the methods were performed in the absence of BHT. *a*) The amount of the aldehyde was 10 nmol. *b*) The amount of the aldehyde was 0.50 μmol, and the yields were expressed as mean values ± S.D. of three different determinations.

pigment formation from liver homogenate,[4,22] but no reasonable mechanistic consideration has been offered. Red pigment formation from the homogenate by method D was dependent on the pH values of the reaction mixture, and the pigment formation was optimal at pH 3–4 (Figure 1A). Introduction of *t*-BuOOH enhanced the pigment formation throughout the pH ranges, and again the optimal pH was 3.5. Red pigment formation from tetramethoxypropane was little affected at the pH ranges between 2 and 4 as has been reported,[4] and it was unchanged by introduction of *t*-BuOOH throughout the pH ranges (Figure 1B). The pigment formation from alkenals, 2-hexenal (Figure 1C) and 2-octenal (Figure 1D), was much lower than that of malonaldehyde throughout the pH ranges, and the optimal pH was larger than 5. Introduction of *t*-BuOOH enhanced the pigment formation throughout the pH ranges and the optimal pH was also larger than 5. In contrast, the pigment formation from alkadienals, 2,4-hexadienal (Figure 1E) and 2,4-nonadienal (Figure 1F), was higher and pH-dependent; the optimal pH being 3–4. Introduction of *t*-BuOOH enhanced the pigment formation throughout the pH ranges, and again the optimal pH was 3–4.

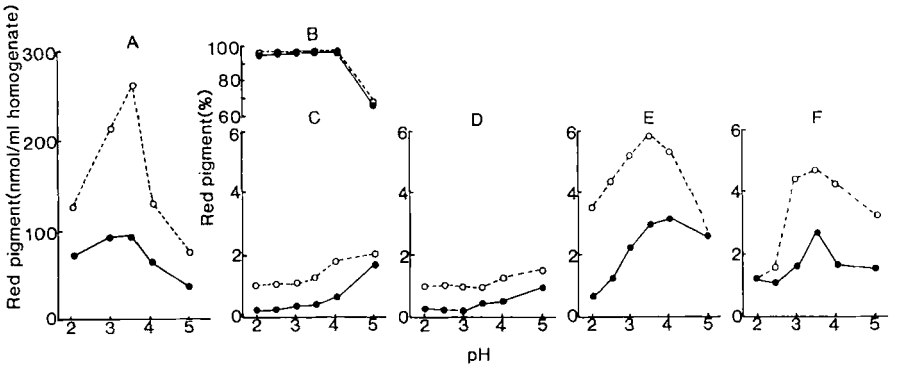

FIGURE 1 pH Dependence of the red pigment formation from rat liver homogenate (A), tetramethoxypropane (B), 2-hexenal (C), 2-octenal (D), 2,4-hexadienal (E) and 2,4-nonadienal (F) in the TBA assay with method D. The assays were performed without (——) and with (– – –) 2 mM *t*-BuOOH in the presence of 0.01% BHT. The homogenate (0.1 ml), tetramethoxypropane (20 nmol), 2-hexenal and 2-octenal (1 μmol), and 2,4-hexadienal and 2,4-nonadienal (0.50 μmol) were used. The yields of red pigment were estimated by the extinction coefficient of the red 1:2 malonaldehyde-TBA adduct: 156000.

The results indicate that the profiles of the red pigment development from rat liver homogenate were similar to those from the alkadienals, and most of the TBA-reactive substances in the homogenate may be a mixture of alkadienals and those liberated from hydroperoxides and cycloendoperoxides by carbon-carbon scission[16] under the assay conditions. The high yields of the red pigment from the homogenate by method D is ascribable to the pH value (pH 3.5) of the reaction mixture that is optimal for the pigment formation from the TBA-reactive substances. The lower yields of the red pigment by other methods may be explained by the lower pH values of the reaction mixtures: pH 2.1 for method A, pH 0.7 for method B and pH 1.8 for method C.

DISCUSSION

Previously we have shown that the TBA assay of alkenals[14] and alkadienals[15] in aqueous acetic acid produces the red 1:2 malonaldehyde-TBA adduct. Although the potencies of alkenals and alkadienals for the pigment formation are very weak,[23,24] they are dramatically enhanced by combination with hydroperoxide components.[15,17] Furthermore, we have investigated the red pigment formation from autoxidized lipid samples such as fatty esters, edible fats and oils, rat liver microsomal lipids and human erythrocyte ghost lipids in the TBA assay in aqueous acetic acid,[18] and found that yields of the pigment were much higher than malonaldehyde contents as determined by direct chemical analysis. Total aldehyde contents including alkenals and alkadienals were high enough to produce the red pigment. Pigment yields were greatly increased by introduction of *t*-BuOOH in the reaction mixture. Thus, it is concluded that the TBA-reactive substances in the lipid samples are likely to be alkenals and/or alkadienals and those liberated under the assay conditions.

Liver homogenate contains variouis components other than lipids. Many variations of the TBA assay for the homogenate have been used, but it is still unclear what components are detected in the assay. In the present investigation, it was found that the characteristics of red pigment formation from rat liver homogenate were similar to those from alkadienals and different from those from malonaldehyde. Pigment formation from the homogenate and an alkadienal was similarly dependent on the methods, similarly enhanced by *t*-BuOOH and similarly dependent on the pH values of the reaction mixture, whereas that from malonaldehyde was little affected by the methods, by addition of *t*-BuOOH and by the pH values of the reaction mixture. It looks more likely that most of the TBA-reactive substances in the homogenate are a mixture of alkadienals and those liberated under the assay conditions.

Present investigation offered other important suggestions for the TBA assay. Method D by Ohkawa *et al.*,[4] whose reaction mixture is adjusted at pH 3.5, was most sensitive to the TBA-reactive substances. The pigment formation by this method was not influenced by the sample size. Introduction of 2 mM *t*-BuOOH in the reaction mixture gave maximal pigment development.

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