Is the Thiobarbituric Acid-Reactivity of Blood Plasma Specific to Lipid Peroxidation?

Takashi KOJIMA,[a] Kiyomi KIKUGAWA*,[a] and Hiroko KOSUGI[b]

Tokyo College of Pharmacy,[a] 1432–1 Horinouchi, Hachioji, Tokyo 192–03, Japan and Ferris University,[b] 4–5–3 Ryokuen, Izumiku, Yokohama 245, Japan.
Received June 19, 1990

The fluorometric thiobarbituric acid (TBA) assay of blood plasma was performed under various conditions in order to assess whether the assay reflects lipid peroxidation. The TBA-reactivity of malonaldehyde was not dependent on the pH values of the reaction and was little affected by *tert*-butyl hydroperoxide (*tert*-BuOOH). The reactivity of 2,4-nonadienal and 2-hexenal, which was maximal at pH 3—4 and at above pH 4, respectively, was dramatically enhanced by *tert*-BuOOH and ferric ion, and suppressed by ethylenediaminetetraacetic acid (EDTA). The pigment formation from oxidized low-density-lipoprotein was maximal at about pH 5, markedly enhanced by ferric ion and suppressed by EDTA, indicating that the TBA-reactive substances in the oxidized lipoprotein were composed of malonaldehyde, alkadienals and alkenals originated from lipid peroxidation. The pigment formation from plasma and its phosphotungstic acid precipitate was maximal at pH 4 and at below pH 2, respectively. The effect of *tert*-BuOOH, ferric ion and EDTA was not significant. The major TBA-reactive substances from plasma may be compounds other than malonaldehyde, alkadienals, alkenals and those in the oxidized lipoprotein. The TBA-reactivity of plasma does not appear to be specific to lipid peroxidation.

Keywords thiobarbituric acid assay; plasma; low-density-lipoprotein; lipid peroxidation

Free radical induced lipid peroxidation is a general mechanism involved in cellular injury.[1,2] The level of lipid hydroperoxides and related substances in plasma may be a good indication of the development of a variety of clinical disorders. These substances in plasma have been determined by thiobarbituric acid (TBA) assay after hydrolysis in acidic solvents.[3-17] A red and fluorescent 1:2 malonaldehyde–TBA pigment is produced in the assay, and various means of its sensitive and specific determination have been devised.[3,4,15,17] The origin of the TBA-reactive substances in plasma, however, has not yet been clarified.

Recent chemical studies of the spectrophotometric TBA assay of compounds generated in lipid peroxidation have demonstrated that, in addition to malonaldehyde, alkadienals and alkenals give rise to the formation of the 1:2 malonaldehyde–TBA pigment in substantial yields.[18-24] The formation of the pigment from alkadienals and alkenals is largely dependent on the pH values of the reaction,[24] and is enhanced by *tert*-butyl hydroperoxide (*tert*-BuOOH)[19-21] and ferric ion.[22] The characteristic profiles of the pigment formation from peroxidized edible oils[23] and liver homogenate[24] indicated that the major TBA-reactive substances are alkadienals and alkenals generated during lipid peroxidation.

In the present investigation, we attempted to characterize the TBA-reactive substances in plasma by fluorometry in order to assess whether they were originated from lipid peroxidation. For comparison, the TBA-reactivities of various aldehydes and peroxidized low-density-lipoprotein (LDL) were measured. The 1:2 malonaldehyde–TBA pigment produced from plasma did not appear to be derived from malonaldehyde, alkadienals or alkenals. This assay may thus not be suitable for measurement of the degree of lipid peroxidation of plasma.

Experimental

Materials 2-Thiobarbituric acid (TBA) and 2,4-nonadienal were obtained from Wako Pure Chemical Industries, Ltd., Osaka. Tetramethoxypropane and 2-hexenal were obtained from Tokyo Kasei Kogyo Company, Ltd., Tokyo. *tert*-Butyl hydroperoxide (*tert*-BuOOH, 70% in water) was purchased from Sigma Chemical Company, St. Louis, Mo., and its concentration was determined to be 6.9 M by iodometry. Butylated hydroxytoluene (BHT) was from Nikki Universal Company, Tokyo. Glacial acetic acid was a special reagent grade product of Wako Pure Chemical Industries.

Human Blood Plasma Blood was collected from a healthy donor and added to a citrate–phosphate–dextrose solution. Plasma was obtained by centrifugation and stored at −20 °C before use. Four different preparations were used. The phosphotungstic acid precipitate of plasma was obtained according to the previous method.[4] Thus, to 3 ml of plasma 24 ml of 1/12 N sulfuric acid and 3 ml of 10% phosphotungstic acid solution were added in this order. The mixture was centrifuged at 3000 rpm for 10 min. The sediment was washed similarly and resuspended in 3 ml of water by sonication.

Low-Density-Lipoprotein (LDL) LDL was obtained by preparative ultracentrifugation.[25] Aliquots of 6.5 ml of plasma mixed with ethylenediaminetetraacetic acid (EDTA) at 1 mM were measured into Hitachi 12 polycarbonate ultracentrifuge tubes (1.6 × 7.5 cm), and 3.25 ml of a solution with density of 1.006 solution was layered over the surface. The tubes were centrifuged (Hitachi model 55P-72) in a Hitachi RP65T rotor at 22000 rpm (31000 *g*) and 16 °C for 30 min. The bottom 6.5 ml layer was again overlaid with 3.25 ml of density 1.006 solution and centrifuged at 40000 rpm (115000*g*) for 6 h. Into the bottom 6.5 ml layer was mixed 3.25 ml of density 1.182 solution and the mixture was centrifuged at 40000 rpm for 8 h. The top 2.0 ml layers from 2 tubes were combined and mixed with diisopropylfluorophosphate at 1 mM and sodium azide at 0.01%, and finally with 4.0 ml of density 1.182 solution. Density 1.006 solution (2.0 ml) was layered over the surface. The tube was centrifuged at 40000 rpm for 6 h. The 3—4 ml layer at the top of the higher density solution was collected.

Protein content of the layer was 2—3 mg/ml as determined by Lowry's method using bovine serum albumin as a reference standard.[26] Sodium dodecylsulfate-polyacrylamide gradient gel electrophoresis[27] and sodium dodecylsulfate-glycerol polyacrylamide slab gel electrophoresis[28] of the layer revealed a single protein band corresponding to apolipoprotein B-100 in Coomassie brilliant blue R-250 staining. The layer was stored at 4 °C for later use.

Oxidation of LDL Oxidation of LDL was performed according to the method of Parthasarathy *et al.*[29] The LDL fraction (2.0 ml) was dialyzed against 200 ml of sodium phosphate buffered saline (pH 7.4). The LDL fraction was diluted with the buffer to make a protein concentration at 2.0 mg/ml and mixed with $CuSO_4$ at 5 μM. The mixture was incubated at 37 °C for 4 h, and the reaction was stopped by addition of BHT at 50 μM. The mixture was dialyzed against 100 ml of saline several times, and diluted with saline to make a protein concentration at 0.2—0.3 mg/ml.

TBA Assay by Fluorometry TBA assay was carried out by the following four methods: method A from Kosugi *et al.*,[23] method B from Buege and Aust,[30] method C from Uchiyama and Mihara,[31] and method D from Ohkawa *et al.*[32] Sodium dodecylsulfate used in the original method of Ohkawa *et al.*[32] was eliminated since the introduction of the salt produced

a substantial amount of pigment. In each method, 0.01% BHT was introduced to prevent undesirable autoxidation during the assay.[23,24] *tert*-BuOOH,[19-21,23,24] ferric chloride[22] and EDTA[22] were introduced in order to characterize the TBA-reactivity. Each method was performed by the two-step reaction mode which involved keeping the reaction mixture at 5 °C for 60 min prior to heating at 100 °C.[19-21,23] Major differences of these methods were the solvents employed and thus the pH values of the reaction mixtures.

The reaction mixture was extracted with 4.0 ml of *n*-butanol and centrifuged at 3000 rpm for 10 min. The fluorescence spectra and the intensities at excitation (535 nm) and emission (550 nm)[4] of the *n*-butanol layer were measured on a Hitachi 650-60 spectrophotometer. The amount of pigment (pmol) produced was calculated assuming that tetramethoxypropane was quantitatively converted into the pigment in each method: [RFI (sample) − RFI (blank)/RFI (tetramethoxypropane 800 pmol) − RFI (blank)] × 800, where RFI is a relative fluorescence intensity.

High performance liquid chromatography (HPLC) analysis[23] was carried out using a Hitachi L-6000 pump equipped with a column (4.6 i.d. × 250 mm) of YMC-A303 ODS (Yamamura Chemical Laboratories, Kyoto, Japan). A 50 μl aliquot of the *n*-butanol layer was injected, and 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 fluorescent peak was detected at excitation (515 nm) and emission (550 nm) by a Shimadzu RF-530 fluorescence spectrofluoromonitor. The fluorescent peak due to the 1 : 2 malonaldehyde–TBA pigment appeared at a retention time of 5.8 min. The amount of pigment (pmol) produced in the reaction was calculated assuming that tetramethoxypropane was quantitatively converted to pigment: [peak height (sample) − peak height (blank)/peak height (tetramethoxypropane 800 pmol) − peak height (blank)] × 800.

Method A In a 13-ml test tube with a screw cap, 4.5 ml of 0.44% TBA in water, 0.5 ml of water (or water containing the sample), 0.10 ml of 0.5% BHT solution in glacial acetic acid (or the solution containing the aldehyde), and 10 μl of 30 mM *tert*-BuOOH in acetic acid were placed in this order. The pH value of the reaction mixture was 2.1. The mixture was kept at 5 °C for 60 min and then heated at 100 °C for 20 min.

Method B In a tube, 2.0 ml of 0.375% TBA in 15% trichloroacetic acid/0.25 N hydrochloric acid, 1.0 ml of water (or water containing the sample), 0.05 ml of 0.6% BHT solution in glacial acetic acid (or the solution containing the aldehyde), and 10 μl of 16 mM *tert*-BuOOH in acetic acid were placed in this order. The pH of the reaction mixture was 0.7. The mixture was kept at 5 °C for 60 min and then heated at 100 °C for 15 min.

Method C In a tube, 1.0 ml of 0.6% TBA in water, 0.5 ml of water (or water containing the sample), 3.0 ml of 1.0% phosphoric acid, 0.05 ml of 0.9% BHT solution in acetic acid (or the solution containing the aldehyde), and 10 μl of 28 mM *tert*-BuOOH in acetic acid were placed in this order. The pH value of the mixture was 1.8. The mixture was kept at 5 °C for 60 min and then heated at 100 °C for 45 min.

Method D In a tube, 1.7 ml of 0.71% TBA in water, 0.8 ml of water (or water containing the sample, 5 mM ferric chloride and 5 mM EDTA), 1.5 ml of 20% acetic acid adjusted at pH 3.5 with 10 N NaOH, 0.05 ml of 0.8% BHT solution in acetic acid (or the solution containing the aldehyde), 10 μl of 22 mM *tert*-BuOOH in acetic acid were placed in this order. The mixture was kept at 5 °C for 60 min and then heated at 100 °C for 60 min. For ivestigation of the effect of pH, 20% acetic acid solution was adjusted at the indicated pH value with 10 N NaOH.

Results

Four variations of the TBA assay for liver homogenate were applied to the fluorometric TBA assay of plasma: method A from Kosugi *et al.*,[23] method B from Buege and Aust,[30] method C from Uchiyama and Mihara,[31] and method D from Ohkawa *et al.*[32] Major differences of these methods were the solvents employed and thus the pH values of the reaction mixtures. The blank reaction mixtures of these methods exhibited significant fluorescence with spectra similar to that of the 1 : 2 malonaldehyde–TBA pigment.[4] The intensities were increased by introduction of *tert*-BuOOH and ferric ion, and decreased by the introduction of EDTA. The blank values may have been due to the contaminated aldehydes in the reaction mixtures, and were not reduceable even if the solvents and the reagents were purified. These blank values were taken into account in all the fluorometric TBA assay methods.

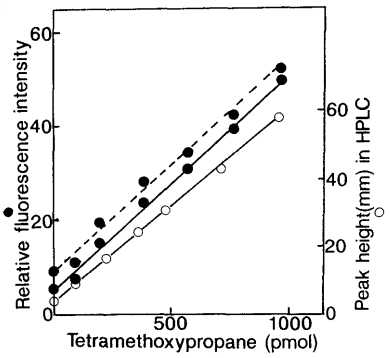

Fig. 1. Standard Calibration Curves of the Pigment Formation from Tetramethoxypropane in Method D (pH 3.5) without (—) and with 50 μM *tert*-BuOOH (– – –) by Direct Fluorometry (●) and Fluorometry after Separation by HPLC (○)

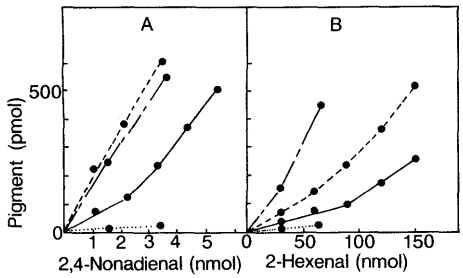

Fig. 2. Dose-dependent Pigment Yield from 2,4-Nonadienal (A) and 2-Hexenal (B) in Method D (pH 3.5) without (—) and with 50 μM *tert*-BuOOH (– – –), 1 mM Ferric Chloride (—·—) and 1 mM EDTA (---) by Direct Fluorometry

Calibration curves of the TBA-reactivities of standard aldehydes that are generated during lipid peroxidation[33] were estimated by the fluorometry of method D (pH 3.5). The reactivity of tetramethoxypropane estimated by direct fluorometry (Fig. 1, closed circles and solid line) and by fluorometry after separation on HPLC (Fig. 1, open circles and solid line) increased linearly up to 1000 pmol of the reagent. Introduction of *tert*-BuOOH did not affect the reactivity throughout the doses except for the blank (Fig. 1, closed circles and dashed line). The reactivities of 2,4-nonadienal and 2-hexenal estimated by direct fluorometry increased with dosage but not linearly (Fig. 2A, B, solid line). The yields of pigment were 5—10% for 2,4-nonadienal (Fig. 2A) and less than 0.2% for 2-hexenal (Fig. 2B). Introduction of *tert*-BuOOH enhanced the reactivities in all doses of the compounds (Fig. 2A, B, dashed lines). Introduction of ferric ion markedly increased the reactivities (Fig. 2A, B, chained lines), and introduction of EDTA completely suppressed the reactivities (Fig. 2A, B, dotted lines). The TBA-reactivities of glucose, galactose, deoxyguanosine, deoxyadenosine and deoxyuridine, which are not due to lipid peroxidation, were lower (data not shown).

The TBA-reactivity of tetramethoxypropane estimated by the direct fluorometry was little affected by the pH values in method D, and *tert*-BuOOH slightly decreased the reactivity throughout the pH ranges (Fig. 3A). In contrast, the reactivity of 2,4-nonadienal was highly pH-dependent and maximal at pH 3—4 in both direct fluorometry and

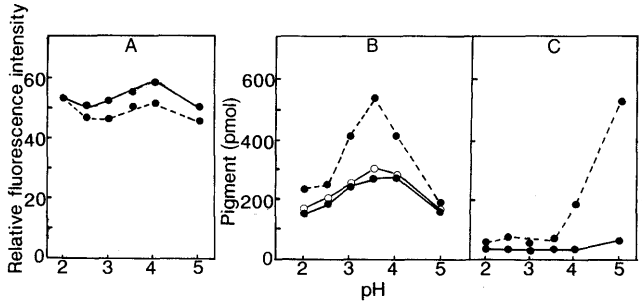

Fig. 3. pH Profiles of the Pigment Formation from Tetramethoxypropane (A), 2,4-Nonadienal (B) and 2-Hexenal (C) in Method D without (—) and with 50 μM *tert*-BuOOH (---) by Direct Fluorometry (●) and Fluorometry after Separation by HPLC (○)

A, relative fluorescence intensity from tetramethoxypropane (760 pmol) after subtracting the blank value was plotted *versus* pH values. B and C, amount of the pigment from 2,4-nonadienal (3.6 nmol) and 2-hexenal (42 nmol) was plotted *versus* respective pH values.

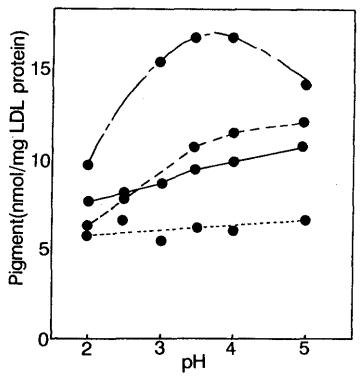

Fig. 4. pH Profiles of the Pigment Formation from Oxidized LDL in Method D without (—) and with 50 μM *tert*-BuOOH (– – –), 1 mM Ferric Chloride (—·—) and 1 mM EDTA (---) by Direct Fluorometry

A 0.1 ml portion of the oxidized LDL solution containing 29 μg protein was used for the assay.

TABLE I. Pigment Yield from 2,4-Nonadienal in Methods A, B, C and D (pH 3.5) by Direct Fluorometry

Method	Reaction mixture		Pigment	
	2,4-Nonadienal (nmol)	*tert*-BuOOH (50 μM)	Amount (pmol)	Yield (%)
A	7.2	–	566	7.9
		+	694	9.6
B	3.6	–	11	0.3
		+	62	1.7
C	3.6	–	0	0
		+	2	0.1
D (pH 3.5)	3.6	–	282	7.9
		+	494	13.7

TABLE II. Pigment Formation from Oxidized LDL in Methods A, B, C and D (pH 3.5) by Direct Fluorometry

Method	*tert*-BuOOH (50 μM)	Pigment (nmol/mg protein)
A	–	7.20
	+	9.20
B	–	3.51
	+	6.84
C	–	3.97
	+	5.75
D (pH 3.5)	–	7.83
	+	8.85

A 0.1 ml LDL solution containing 22 μg protein was used for the assay.

the fluorometry after HPLC separation (Fig. 3B, closed and open circles, solid lines). *tert*-BuOOH enhanced the reactivity throughout the pH ranges (Fig. 3B, dashed line). The reactivity of 2-hexenal was maximal at above pH 4, and enhanced by *tert*-BuOOH (Fig. 3C).

The TBA-reactivity of tetramethoxypropane was similar in methods A, B, C and D (pH 3.5), and it slightly decreased by *tert*-BuOOH in each method (data not shown). In contrast, the pigment yields from 2,4-nonadienal were different depending on the methods applied: more than 7% in methods A and D (pH 3.5) and less than 0.3% in methods B and C (Table I). The differences may be due to the pH values of the reaction mixtures used. The pigment yields were increased by *tert*-BuOOH in all methods. Thus, method D (pH 3.5) may be the most suitable for the fluorometric determination of standard aldehydes that are generated during lipid peroxidation.

LDL isolated from plasma was oxidized by a common procedure.[29] A representative pH-profile of the TBA-reactivity of the oxidized LDL in method D estimated by direct fluorometry is shown in Fig. 4. The pigment was identified as 1 : 2 malonaldehyde–TBA adduct by comparison of its fluorescence spectrum with that of standard pigment from tetramethoxypropane. The reactivity was moderately pH-dependent, and was maximal at above pH 5 (Fig. 4, solid line). *tert*-BuOOH slightly enhanced the reactivity at higher pH values (Fig. 4, dashed line). Ferric ion dramatically enhanced and EDTA significantly suppressed the reactivity throughout all pH ranges (Fig. 4, chained and dotted lines). Similarly, the reactivity of the oxidized LDL was moderately dependent on the methods used, and was highest in method D (pH 3.5) (Table II). *tert*-BuOOH slightly enhanced the reactivity in each method.

The TBA-reactivity of plasma in method D was estimated. The fluorescence spectrum of the reaction mixture exhibited an excitation maximum at 535 nm and an emission maximum at 550 nm. HPLC analysis of the reaction mixture revealed a single fluorescent peak with a retention time at 5.8 min. The pigment was identified as 1 : 2 malonaldehyde–TBA adduct by comparison of its fluorescence spectrum and retention time with those of the standard pigment produced from tetramethoxypropane. The amounts of the pigment estimated by direct fluorometry were identical with those estimated by the fluorometry after HPLC separation. The yields were found to be maximal at pH 4 (Fig. 5A), and those at pH 2 and 5 were one third that at pH 4. Since the lipid fraction of plasma can be purified by precipitation together with proteins using phosphotungstic acid,[4] the TBA-reactivity of the phosphotungstic acid precipitate was similarly estimated. The pigment was also identified as 1 : 2 malonaldehyde–TBA adduct by its spectrum and retention time. The reactivity was maximal at below pH 2 and decreased at higher pH values (Fig. 5B). It is interesting to note that the pH-dependence of the precipitate differed greatly from that of plasma.

Similar pH dependence of the TBA-reactivity was

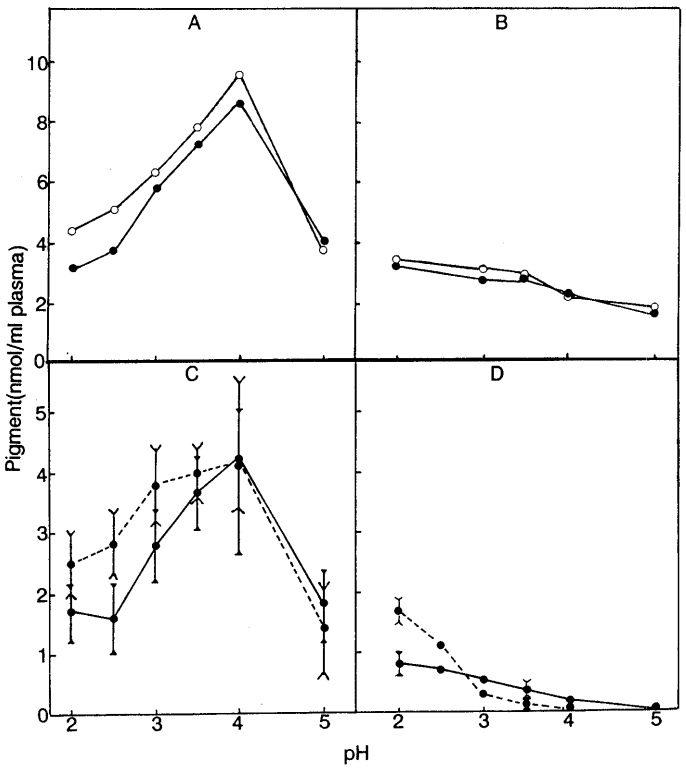

Fig. 5. pH Profiles of the Pigment Formation from Plasma and the Phosphotungstic Acid Precipitate in Method D by Direct Fluorometry (●) and Fluorometry after Separation by HPLC (○)

A, plasma sample 1 (sample size: 0.1 ml); B, the precipitate from plasma sample 1 (sample size: 0.2 ml); C, plasma sample 2 (sample size: 0.1 ml) without (—) and with (– – –) 50 μM *tert*-BuOOH; and D, the precipitate from plasma sample 3 (sample size: 0.2 ml) without (—) and with (– – –) 50 μM *tert*-BuOOH; were measured. The amounts of the pigment in C and D were expressed by the average values ± S.D. (C, $n=5$; and D, $n=3$).

TABLE III. Pigment Formation from Plasma and Phosphotungstic Acid Precipitate in Methods A, B, C and D (pH 3.5) by Direct Fluorometry

Method	Plasma sample 2			Phosphotungstic acid precipitate from plasma sample 4		
	Reaction mixture		Pigment (nmol/ml plasma)	Reaction mixture		Pigment (nmol/ml plasma)
	Plasma (ml)	*tert*-BuOOH (50 μM)		Plasma (ml)	*tert*-BuOOH (50 μM)	
A	0.2	–	0.48	0.4	–	0.19
		+	0.71		+	0.26
B	0.1	–	1.74	0.4	–	0.27
		+	3.17		+	0.38
C	0.1	–	2.65	0.4	–	0.27
		+	3.55		+	0.40
D (pH 3.5)	0.1	–	3.02	0.4	–	0.24
		+	3.39		+	0.13

observed with different preparations of plasma and the precipitate (Fig. 5C, D, solid lines). Introduction of *tert*-BuOOH into plasma increased the pigment formation at pH ranges lower than 3, but not at ranges higher than 4. (Fig. 5C, dashed line). Introduction of the hydroperoxide into the precipitate increased the pigment formation at pH ranges lower than 2, but dramatically suppressed it at ranges higher than 3 (Fig. 5D, dashed line). Introduction of ferric ion and EDTA into plasma and the precipitate did not cause any significant changes in the reactivities (data not shown).

The TBA-reactivities of plasma and the precipitate were dependent on the assay methods used. The reactivity of plasma was highest in method D (pH 3.5) and that of the precipitate was highest in method B or C (Table III); these differences may be due to the pH values of the reaction mixtures. The effects of *tert*-BuOOH were pH-dependent.

Discussion

The spectrophotometric TBA assay has been shown to be useful to measure total degree of lipid peroxidation of edible oils and liver homogenate.[21,23,24] Malonaldehyde, one of the lipid peroxidation products, produces the 1:2 malonaldehyde–TBA pigment quantitatively without dependence on the pH values and the presence of organic hydroperoxides and ferric ion.[18-24] In contrast, other lipid peroxidation products, alkadienals and alkenals, produce the same pigment in lower yields with different characteristics.[18-24] The reactivities of alkadienals are maximal at pH 3—4 and those of alkenals at above pH 5. Both are enhanced by *tert*-BuOOH and ferric ion. The spectrophotometric assay of peroxidized edible oils suggested that the major TBA-reactive substances are aldehydes other than malonaldehyde,[23] and the assay of liver homogenate showed that the substances are alkadienals.[24]

In the present investigation, the fluorometric assay of various aldehydes generated during lipid peroxidation[33] was standardized in order to assess the TBA-reactivity of plasma with low concentration of the TBA-reactive substances. The TBA-reactivities of these standard aldehydes estimated by fluorometry were similar to those estimated by spectrophotometry. Thus, malonaldehyde produced the pigment without dependence on the pH values or the presence of *tert*-BuOOH. The pigment formation from 2,4-nonadienal and 2-hexenal was maximal at pH 3—4 and above pH 4, respectively, was enhanced by *tert*-BuOOH and ferric ion, and was suppressed by EDTA.

The fluorometric assay of the pigment from the oxidized LDL showed that the pigment was produced in a maximum yield at above pH 5. This pH-dependence resembled that of 2-hexenal. *tert*-BuOOH had little effect on the reactivity, but this fact did not imply the lack of effect of the hydroperoxide because the oxidized LDL may contain a substantial amount of endogenous lipid hydroperoxides which may have potentiated the reactivity. The enhancing effect of ferric ion on the reactivity may be reasonably ascribed to the presence of alkadienals and alkenals in the oxidized LDL. The suppression effect of EDTA was significant but not total, which may suggest the presence of malonaldehyde together with alkadienals and alkenals. This result coincided with the previous observation made using different methodology in which oxidized LDL was found to contain malonaldehyde, alkadienals, alkenals and alkanals.[34] Hence, the TBA-reactivity of the oxidized LDL may be the result of a mixture of malonaldehyde, alkadienals and alkenals originated from lipid peroxidation.

The fluorometric assay of the pigment from plasma and its phosphotungstic acid precipitate revealed that the fluorescent pigment was the 1:2 malonaldehyde–TBA pigment. This indicates that the assay reflects the pigment and no other pigments derived from interfering substances such as sialic acid.[3] The pigment formation from plasma and the precipitate was pH dependent, but the pH-

dependences were quite different. The pH-dependence profiles from plasma (Fig. 5A, C, Table III) were similar to that of 2,4-nonadienal (Fig. 3B) but not to those of malonaldehyde (Fig. 3A), 2-hexenal (Fig. 3C) or the oxidized LDL (Fig. 4). The pH-dependence profiles from the precipitate (Fig. 5B, D, Table III) did not resemble any of the profiles from malonaldehyde, 2,4-nonadienal, 2-hexenal or the oxidized LDL. The characteristics of the pigment formation from plasma and the precipitate were greatly different from those of peroxidized edible oils[23] and those of liver homogenate,[24] whose TBA-reactive substances may be alkadienals and/or alkenals generated during lipid peroxidation. Limited enhancing effect of *tert*-BuOOH (Fig. 5C, D, Table III) and no effect of ferric ion or EDTA on the reactivities of plasma and the precipitate indicated that alkadienals and alkenals contributed little to the pigment formation.

It has been shown that plasma or its precipitate contains malonaldehyde at a level between 0.6 and 47 nmol/ml on the basis of the amount of red pigment produced in various TBA assay methods.[3-17] The present investigation demonstrated a similar level of red pigment (0.19—9 nmol/ml) under various conditions (Fig. 5, Table III), but it appeared unlikely that the pigment was derived from malonaldehyde, judging from the pH-dependence profiles. This result was compatible with the observation that no free malonaldehyde was detected in plasma by direct HPLC.[35]

Hence, the TBA assay of plasma may not be specific to the aldehyde species due to lipid peroxidation, and the assay may also measure unidentified components other than those due to lipid peroxidation. It is important to note that although the TBA assay of plasma may reflect lipid peroxidation it may also measure other irrelevant components. The assay may thus not be appropriate for estimating the degree of lipid peroxidation of plasma.

Acknowledgement The authors thank Mr. H. Moriya for his technical assistance.

References

1) A. L. Tappel, *Fed. Proc.*, **32**, 1870 (1973).
2) T. P. Slater, *Biochem. J.*, **222**, 1 (1984).
3) K. Satoh, *Clin. Chim. Acta*, **20**, 37 (1978).
4) K. Yagi, *Biochem. Med.*, **15**, 212 (1976).
5) T. Suematsu, T. Kamada, H. Abe, S. Kikuchi and K. Yagi, *Clin. Chim. Acta*, **79**, 267 (1977).
6) M. T. Santos, J. Valles, J. Aznar and J. Vilches, *J. Clin. Pathol.*, **33**, 973 (1980).
7) M. Maseki, J. Nishigaki, M. Hagihara, Y. Tomoda and K. Yagi, *Clin. Chim. Acta*, **115**, 155 (1981).
8) K. Yasaka, I. Ohya, J. Matsumoto, T. Shiramizu and Y. Sasaguri, *Arch. Intern. Med.*, **141**, 1169 (1981).
9) J. Aznar, M. T. Santos, J. Valles and J. Sala, *J. Clin. Pathol.*, **36**, 712 (1983).
10) L. Viinikka, J. Vuori and O. Ylikorkala, *Med. Sci. Sport Exer.*, **16**, 275 (1984).
11) V. Francesco, A. Cesare, I. Luigi, F. Stefano, G. Andrea and B. Francesco, *Stroke*, **16**, 14 (1985).
12) A. Ledwozyw, J. Michalak, A. Stepien and A. Kadziolka, *Clin. Chim. Acta*, **155**, 275 (1986).
13) D. T. Kirkpatrick, D. J. Guth and R. D. Mavis, *J. Biochem. Toxicol.*, **1**, 93 (1986).
14) I. Schimke and B. Papies, *Z. Med. Lab. Diagn.*, **27**, 71 (1986).
15) S. H. Y. Wong, J. A. Knight, S. M. Hopfer, O. Zaharia, C. N. Leach, Jr. and F. Sunderman, Jr., *Clin. Chem.*, **33**, 214 (1987).
16) J. A. Knight, S. E. Smith, V. E. Kinder and H. B. Anstall, *Clin. Chem.*, **33**, 2289 (1987).
17) V. L. Tatum, C. Changchit and C. K. Chow, *Lipids*, **25**, 226 (1990).
18) H. Kosugi, T. Kato and K. Kikugawa, *Anal. Biochem.*, **165**, 454 (1987).
19) H. Kosugi, T. Kato and K. Kikugawa, *Lipids*, **23**, 1024 (1988).
20) H. Kosugi and K. Kikugawa, *J. Japan Oil Chem. Soc.*, **38**, 224 (1989).
21) H. Kosugi and K. Kikugawa, *Free Rad. Biol. Med.*, **7**, 205 (1989).
22) T. Bigwood and G. Read, *Free Rad. Res. Commun.*, **6**, 387 (1989).
23) H. Kosugi, T. Kojima and K. Kikugawa, *Lipids*, **24**, 873 (1989).
24) K. Kikugawa, T. Kojima and H. Kosugi, *Free Rad. Res. Commun.*, **8**, 107 (1990).
25) F. T. Hatch and D. S. Lee, *Adv. Lipid Res.*, **6**, 1 (1968).
26) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
27) P. Lambin, D. Rochu and J. M. Fine, *Anal. Biochem.*, **74**, 567 (1976).
28) G. F. Maguire, M. Lee and P. W. Connelly, *J. Lipid Res.*, **30**, 757 (1989).
29) S. Parthasarathy, U. P. Steinbrecher, J. Barnett, J. Witztum and D. Steinberg, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 3000 (1985).
30) J. A. Buege and S. D. Aust, *Methods Enzymol.*, **52**, 302 (1978).
31) M. Uchiyama and M. Mihara, *Anal. Biochem.*, **86**, 271 (1978).
32) H. Ohkawa, N. Ohishi and K. Yagi, *Anal. Biochem.*, **95**, 351 (1979).
33) H. Estenbauer, "Free Radicals, Lipid Peroxidation and Cancer," D. C. H. McBrien and T. F. Slater (eds.), Academic Press, New York, 1982, pp. 101—158.
34) H. Esterbauer, G. Jurgens, O. Quehenberger and E. Koller, *J. Lipid Res.*, **28**, 495 (1987).
35) C. Largilliere and B. MeLancon, *Anal. Biochem.*, **170**, 123 (1988).