Characteristics of the Thiobarbituric Acid Reactivity of Human Urine as a Possible Consequence of Lipid Peroxidation

Hiroko Kosugi[a,*], Takashi Kojima[b] and Kiyomi Kikugawa[b]

[a]Ferris University, 4-5-3 Ryokuen, Izumiku, Yokohama 245 and [b]Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

A 532 nm red pigment formed in the thiobarbituric acid (TBA) assay of human urine was characterized after separation of the pigment by high-performance liquid chromatography. The yield of the red pigment was somewhat higher at pH 2 than at pH 5; its development was not inhibited by ethylenediaminetetraacetic acid. The characteristics of the pigment were similar to those of the pigment derived from standard malonaldehyde. The amount of the pigment formed was roughly equal to the content of malonaldehyde derivatives estimated as 1-(2,4-dinitrophenyl)pyrazole. Pigment formation was significantly enhanced by *t*-butyl hydroperoxide (*t*-BuOOH) and ferric ions, which may be due to pigment formed from aldehydes other than malonaldehyde; the presence of these aldehydes was confirmed by the formation of the corresponding 2,4-dinitrophenylhydrazones. The amount of pigment produced from 24-h urine samples of 12 healthy subjects was estimated to be 26–95 nmol/kg, and 65–182 nmol/kg in the presence of *t*-BuOOH. These values are lower than those for urine of rabbit or rat. The TBA reactivity in the absence and presence of *t*-BuOOH of human urine was not related to age or sex. The TBA reactivity of human urine collected in the afternoon and in the evening was higher than that of urine collected in the morning. ***Lipids 28,*** **337–343 (1993).**

Lipid peroxidation has been implicated as being involved in various pathological conditions including aging, carcinogenesis, atherosclerosis, inflammation, ischemia and drug toxicities. Lipid peroxidation products excreted in human urine may thus reflect lipid peroxidation of the whole body. The thiobarbituric acid (TBA) assay (1) has previously been used for the measurement of lipid peroxidation products in rat and in human urine (2–5). Thus, heating of urine with TBA in acidic medium has been shown to give a red malonaldehyde TBA (1:2) adduct with a maximum absorption at 532 nm (6).

Although the red pigment had been assumed to be specific for malonaldehyde, other aldehydes generated during lipid peroxidation have been shown to produce the same pigment (1,7–14). Pigment formation from malonaldehyde is quantitative and is little affected by pH, added organic hydroperoxides or ferric ion. In contrast, pigment formation from alkadienals and alkenals is not quantitative, is highly pH-dependent (12,13) and is greatly enhanced by added organic hydroperoxides (8–14) and ferric ions (13,14). Our previous studies have shown that the pigment formed from oxidized fats and oils is due to alkadienals and/or alkenals (11,13), and that the pigment formed from rat liver and brain homogenate is due to both alkadienals and malonaldehyde (12,14).

In the present study, we characterized the red pigment formed in the TBA assay of human urine and determined which aldehydes generated during lipid peroxidation are responsible for pigment formation. We also determined the amounts of pigment formed from urine of healthy human subjects.

MATERIALS AND METHODS

Materials. TBA, 2,4-dinitrophenylhydrazine (DNPH), propanal, butanal, hexanal and 2,4-heptadienal were obtained from Wako Pure Chemical Industries (Osaka, Japan). 2-Butenal, 2-hexenal, 2,4-hexadienal and tetramethoxypropane (TMP) were obtained from Tokyo Kasei Kogyo Company (Tokyo, Japan). 2-Pentenal and 2-heptenal were obtained from Aldrich Chemical Company (Milwaukee, WI). *t*-Butyl hydroperoxide (*t*-BuOOH) (70% in water) was obtained from Sigma Chemical Company (St. Louis, MO); its concentration was determined to be 7.0 M by iodometric titration. Butylated hydroxytoluene (BHT) was obtained from Nikki Universal Company (Tokyo, Japan). *N*-(2-propenal)aminoacetic acid was prepared as described (15). Glacial acetic acid was a special reagent grade product of Wako Pure Chemical Industries.

Analysis. Absorption spectra were measured on a Hitachi U-2000 (Tokyo, Japan) or a Shimadzu UV-240-Visible (Osaka, Japan) spectrophotometer. High-performance liquid chromatography (HPLC) was carried out on a Hitachi L-600 or L-655-A11 liquid chromatograph equipped with a column (4.6 mm i.d. × 250 mm) of YMC A-303 ODS (Yamamura Chemical Laboratories, Kyoto, Japan). The fractions were detected with a Hitachi L-4200 UV-VIS or a Shimadzu SPD-6A detector.

Urine. Urine was collected from healthy human male and female subjects, Japanese White male rabbits and Wistar male rats and used for analysis within several hours. For determination of one-day amounts of the red pigment, 24-h urine was pooled for the assay.

Major TBA-reactive substances of human urine. The TBA-reactive substances of human urine were partially purified through an anion exchange column and by gel filtration according to the method of Hadley and Draper (16). Human urine (250 mL) was applied to a column (3 cm i.d. × 22 cm) of AG1-X8 (chloride form), the column was eluted with 1 L of linear gradient sodium chloride solution (0–1 M), and 5-mL fractions were collected. Most of the color of urine due to urobilin, and about 30% of the TBA-reactive substances were eluted in fractions (20–60) (200 mL) (shown later in Fig. 2). About 70 mL of the fractions were condensed into 6 mL and passed through a column (3 cm i.d. × 21 cm) of Sephadex G-10 equilibrated with water, and the column was eluted with 100 mL of water. The TBA-reactive substances loaded were completely eluted, and the eluate was condensed to 10 mL.

TBA assay. All TBA assays were performed by the two-step mode to obtain reproducible data for alkadienals (11).

*To whom correspondence should be addressed.

Abbreviations: BHT, butylated hydroxytoluene; DNPH, 2,4-dinitrophenylhydrazine; DNPP, 1-(2,4-dinitrophenyl)pyrazole; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; TBA, thiobarbituric acid; *t*-BuOOH, *tert*-butyl hydroperoxide; TMP, tetramethoxypropane.

The assays were performed with addition of BHT to avoid undesirable peroxidation during the assays (13,14).

Method A. Two mL of a solution containing 20 nmol TMP or urine (human urine 2.0 mL, rabbit and rat urine 0.5 mL) in water, 0.10 mL of 0.5% BHT solution in glacial acetic acid (the final concentration of acetic acid was 2%) and 3.0 mL of 0.5% TBA solution in water were placed in this order into a screw-cap test tube. For studying the effect of *t*-BuOOH, 10 μL of a 0.5–2 M *t*-BuOOH solution in acetic acid was added to the mixture. For studying the effect of ferric ion and ethylenediaminetetraacetic acid (EDTA), 0.5% TBA solution was replaced by 0.5% TBA/0.2–4 mM $FeCl_3$ (or EDTA disodium salt) in water. The mixture was kept at 5°C for 60 min and then was heated at 100°C for 20 min. After cooling, the mixture was extracted with 3 mL of chloroform and then centrifuged at 650 × *g* for 10 min. Absorbance at 532 nm of the aqueous phase was determined, and the amount of red pigment was calculated on the basis of the absorbance and the molecular extinction coefficient (156000) of red pigment (17). The aqueous phase was subjected to HPLC, and the column was eluted with 0.04 M acetate buffer (pH 5.5)/methanol (6:4 vol/vol) at a flow rate of 0.8 mL/min. A peak was detected at 532 nm. Red pigment from a standard TMP solution appeared at a retention time of 8.0 min. The amount of red pigment from urine was determined by comparing the peak area of red pigment with that of the standard TMP solution. The amounts of red pigment from human urine linearly increased with the volume up to 2.0 mL, and those from rabbit and rat urine linearly increased with the volume up to 0.5 mL.

Method B. Method B is a modification of the method of Ohkawa *et al.* (18). Two mL of a solution containing 20 nmol TMP in water or 2.0 mL of human urine, 1.50 mL of 20% acetic acid adjusted at the indicated pH value by addition of 10 N NaOH, 50 μL of 1% BHT solution in glacial acetic acid and 1.50 mL of 0.8% TBA solution in water were placed in this order into a test tube. For studying the effect of *t*-BuOOH, 10 μL of 1 M *t*-BuOOH solution in acetic acid was added. For studying the effect of ferric ions and EDTA, 0.1 mL of 100 mM $FeCl_3$ or EDTA disodium salt solution in water was added. The mixture was kept at 5°C for 60 min and was then heated at 100°C for 60 min. After cooling, the mixture was extracted with 3 mL of chloroform and centrifuged. The aqueous phase was subjected to HPLC as described.

Determination of malonaldehyde as DNPH derivative. Malonaldehyde was determined as 1-(2,4-dinitrophenyl)pyrazole (DNPP) by reaction with DNPH according to the established method (19,20) with slight modifications (14). Reference standard DNPP was prepared as described (14). Thus, a mixture of 1.5 mL of urine or an aqueous solution containing 30 nmol TMP, along with 1.2 mL of 0.25% (wt/vol) DNPH solution in 1 N HCl, was heated at 100°C for 30 min. The reaction mixture was extracted with chloroform as described (14), and the extract was subjected to HPLC. The column was eluted with acetonitrile/0.01 N HCl (45:55, vol/vol) at a flow rate of 1.5 mL/min. The peak due to DNPP appeared at a retention time of 7.0 min and was detected at 300 nm. Malonaldehyde content of urine was determined by comparing the peak height of DNPP from urine with that of DNPP from the TMP standard solution.

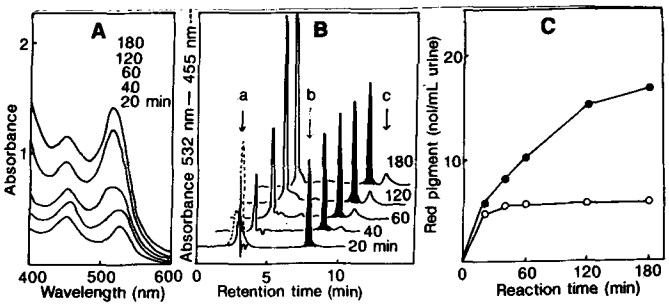

FIG. 1. Profiles of pigment formation in the thiobarbituric acid (TBA) assay of human urine. A: Absorption spectra of the TBA reaction mixture (method A) of human urine (2 mL) heated at 100°C for the indicated period. The reaction mixture of standard tetramethoxypropane (TMP) heated for 20–180 min showed the same absorbance with a single absorption maximum at 532 nm. B: High-performance liquid chromatography (HPLC) pattern of the TBA reaction mixture (method A) of human urine heated at 100°C for the indicated period. Three peaks, a, b and c, were detected. Red pigment (532 nm) from standard TMP eluted at a retention time of 8.0 min (peak b). C: Time course of 532 nm red pigment determined by absorbance (A) (●), and that determined by peak b in HPLC analysis (B) (○). The control reaction mixture without TMP and urine showed no significant coloration and HPLC peaks.

Determination of aldehydes with DNPH. Aldehydes other than malonaldehyde were determined as previously described (11). DNPH (50 mg) was dissolved in 100 mL of 1 N HCl, and the solution was extracted twice with 50 mL of *n*-hexane to remove impurities. Two mL of urine or an aqueous solution containing 5 μmol standard aldehyde was added to 2.0 mL of the solution. The mixture was vigorously shaken at room temperature for 5 min in the dark and extracted six times with 3 mL of benzene. The organic layers were combined and evaporated to dryness, and the residue was redissolved into 0.5 mL of benzene. The solution was applied to a column (1 cm i.d. × 3.5 cm) of silica gel, and the column was eluted with benzene. The hydrazone fractions (3–8 mL) and DNPH fractions (15–32 mL) were separated, the former being evaporated to dryness to be redissolved into 5.0 mL of methanol. Absorption spectra of the solution gave maxima at 360 nm. The amounts of aldehyde were calculated on the basis of the average molar extinction coefficient (26000) of the various hydrazones at 370 nm (21). The methanolic solution was evaporated to dryness and dissolved into 0.2 mL of acetonitrile for HPLC analysis. The column was eluted with acetonitrile/water (3:2, vol/vol) at a flow rate of 1.5 mL/min (14). The peaks were detected at 370 nm (shown later in Fig. 5).

RESULTS

The TBA reaction of the reference standard TMP and human urine was carried out by heating at 100°C for 20 min in 2% acetic acid containing 0.01% BHT (method A). While TMP produced 532 nm red malonaldehyde TBA (1:2) adduct quantitatively, human urine produced pigments with two absorption maxima at 455 and 532 nm (Fig. 1A). The absorbance was gradually increased by prolonged heating up to 180 min, and the maximum at 532 nm was shifted to 518 nm. The 532 nm red pigment could not be accurately determined by direct spectrophotometry

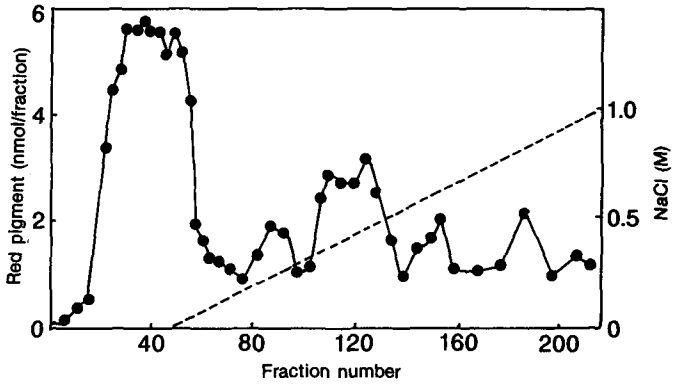

FIG. 2. Anion exchange column chromatography of the thiobarbituric acid (TBA)-reactive substance of human urine. Human urine (250 mL) containing the substances to produce 660 nmol 532 nm red pigment was applied to an AG1-X8 column (3 × 22 cm) with sodium chloride linear gradient elution, and 5-mL fractions were collected. The TBA-reactive substances were detected in the assay by their absorbance at 532 nm (method A).

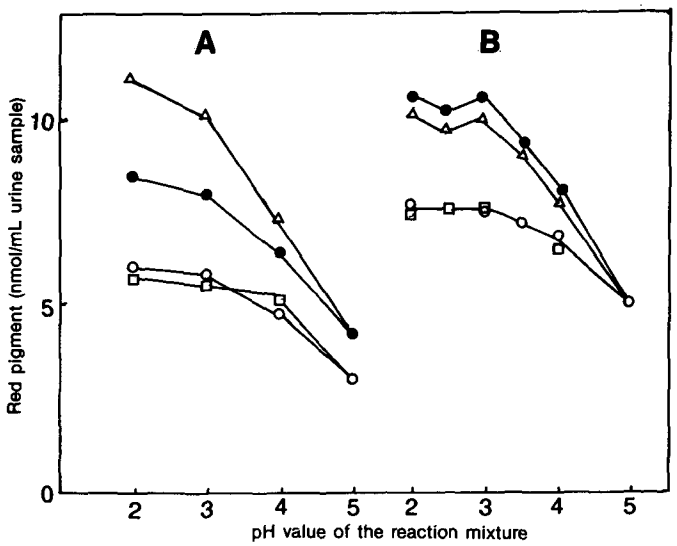

FIG. 3. Effect of pH, *t*-BuOOH, ferric ions and EDTA on the development of the red pigment in the TBA assay of human urine. Human urine (A) and the major TBA-reactive substances obtained by anion exchange column chromatography (see Fig. 2) (B) were subjected to the TBA assay (method B) in the absence (○) or presence of 2 mM *t*-BuOOH (●), 2 mM ferric ions (△) and 2 mM EDTA (□). The reaction mixture heated at 100°C for 20 min was subjected to HPLC analysis. Abbreviations as in Figure 1. *t*-BuOOH, *tert*-butyl hydroperoxide; EDTA, ethylenediaminetetraacetic acid.

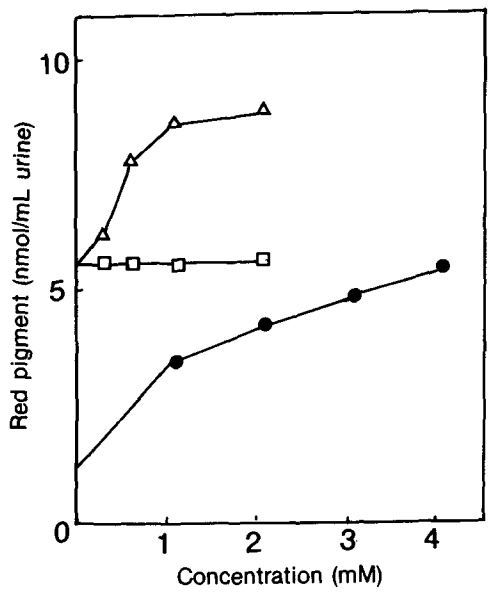

FIG. 4. Concentration-dependent effect of *t*-BuOOH, ferric ions and EDTA on the red pigment development in the TBA assay of human urine. Urine samples were subjected to the TBA assay (method A) in the presence of *t*-BuOOH (●), ferric ion (△) and EDTA (□) at the indicated final concentration. The reaction mixture heated at 100°C for 20 min was subjected to HPLC analysis. Abbreviations as in Figures 1 and 3.

owing to the production of the interfering pigments. In HPLC, the 532 nm red pigment was separated from the interfering pigments (peak a and peak c) and eluted at a retention time of 8.0 min (peak b) (Fig. 1B). The absorption spectrum of peak b showed a single maximum at 532 nm of red malonaldehyde TBA (1:2) adduct. The amount of the red pigment determined in peak b was constant during the heating periods from 20 to 180 min (Fig. 1C). The amount of the red pigment produced during 20 min as estimated by direct spectrophotometry was 20–30% higher than that measured by HPLC. Hence, HPLC separation was necessary for accurate quantitation of the red pigment from urine.

When human urine was dialyzed against water, about 90% of the TBA-reactive substances that produce the red pigment were removed (data not shown), indicating that most of the TBA-reactive substances were low molecular weight compounds. When human urine was passed through an anion exchange column according to the method of Hadley and Draper (16), the TBA reactive-substances were resolved into several peaks (Fig. 2). The chromatographic profile was similar to that of rat urine (16), and the TBA-reactive substances of human urine were not single. Major TBA-reactive substances (about 30%) of human urine were eluted in the first fraction. When the red pigment from human urine was determined by the methods of Ohkawa *et al.* (18), Buege and Aust (22) and Uchiyama and Mihara (23), similar results were obtained as long as the pigment was determined after HPLC separation (data not shown).

The effect of the pH of the TBA reaction mixture (method B) on the red pigment development from human urine (Fig. 3A) and from the major TBA-reactive substances separated by anion exchange column chromatography (Fig. 3B) was further investigated (open circles). Pigment formation was somewhat dependent on the pH value of the reaction mixture; it was maximal at pH 2, and 1.5-fold higher than at pH 5. Addition of 2 mM *t*-BuOOH (Fig. 3; closed circles) or ferric ions (Fig. 3; open triangles) increased pigment development in the pH ranges between 2 and 5, suggesting the presence of alkadienals and/or alkenals whose reactivity is known to be enhanced by these reagents (8–14). Addition of 2 mM EDTA (Fig. 3; open squares) did not affect the pigment development throughout the pH ranges, suggesting that a trace amount of contaminated ferric and ferrous ions had been already chelated and inactivated in the reaction mixtures containing urine samples so as not to enhance the TBA reactivity due to alkadienals and alkenals. Figure 4 shows

TABLE 1

Comparison of the Amount of Red Pigment Produced in the Thiobarbituric Acid (TBA) Assay of Human Urine Containing Malonaldehyde Derivatives and Other Aldehydes

Urine sample	532 nm Red pigment[a] (nmol/mL urine)			Malonaldehyde derivatives[b] (nmol/mL urine)	Other aldehydes[c] (nmol/mL urine)
	+ None	+ *t*-BuOOH	+ $FeCl_3$		
Urine					
1	10.6			15.3	
2	8.0			6.5	
3	11.0			19.5	
4	7.0			5.0	
5	4.3	6.1	7.9		11.5
6	3.9	5.7	8.3		9.1
7	4.7	7.6	8.3		9.0
8	2.2	3.6	5.0		5.4
Major TBA-reactive substances of urine[d]					
9	19.2	20.9	24.1	24.2	24.2

[a]Red pigment (532 nm) was determined by high-performance liquid chromatography of the TBA reaction mixture (method A) heated at 100°C for 20 min in the absence or presence of 2 mM *tert*-butyl hydroperoxide (*t*-BuOOH) or $FeCl_3$.

[b]Malonaldehyde content was determined as 1-(2,4-dinitrophenyl)pyrazole by 2,4-dinitrophenylhydrazine (DNPH) method.

[c]Other aldehyde content was determined as hydrazones of DNPH by silica gel column chromatography.

[d]The major TBA-reactive substances were obtained by anion exchange column chromatography (see Fig. 2).

concentration-dependent enhancement of the red pigment development by *t*-BuOOH and ferric ions in the TBA assay (method A) of human urine samples. The pigment development from malonaldehyde derivatives, TMP and *N*-(2-propenal)aminoacetic acid was not enhanced by 2 mM ferric ions and not inhibited by 2 mM EDTA under the same conditions (data not shown).

The amounts of red pigment formed from human urine (Table 1, samples 1–4) and the major TBA-reactive substances obtained by anion exchange column chromatography (Table 1, sample 9) were compared with the contents of malonaldehyde derivatives determined as DNPP by the DNPH method (19,20). The amounts of red pigment obtained without added *t*-BuOOH and ferric ions were roughly equal to or even lower than the levels of malonaldehyde derivatives measured. Hence, the TBA reactivity of human urine without added *t*-BuOOH and ferric ions was ascribed to malonaldehyde derivatives.

The enhanced amounts of the red pigment that developed in the presence of *t*-BuOOH or ferric ions were compared with the levels of other aldehydes determined as the hydrazones of DNPH (Table 1, samples 5–8). The amounts of other aldehydes were high enough to account for the enhanced pigment formation. When the hydrazones from the major TBA-reactive substances obtained by anion exchange column chromatography (Table 1, sample 9) were analyzed by HPLC (Fig. 5), several peaks due to the hydrazones of aldehydes were detected. One of the hydrazones was identified as the hydrazone of 2,4-hexadienal by cochromatography with an authentic standard (data not shown). Hence, the red pigment development from urine enhanced by *t*-BuOOH and ferric ions may be due to alkadienals and/or alkenals.

One-day amounts of the red pigment from 24-h urine of 12 healthy subjects were determined and compared (Table 2, lane 1). The amounts of the pigment were 26–95 nmol/kg · day in the absence of *t*-BuOOH and 65–182 nmol/kg · day in the presence of *t*-BuOOH. All the subjects excreted both malonaldehyde derivatives and other aldehydes as TBA-reactive substances. However, the amount of pigment formed varied from subject to subject and also varied by the day of collection of urine from a specific subject. The TBA reactivity of urine with and without added *t*-BuOOH was not related to age or sex. One-day amounts of the pigment from rabbit urine (Table 2, lane 2) and rat urine (Table 2, lane 3) were similarly

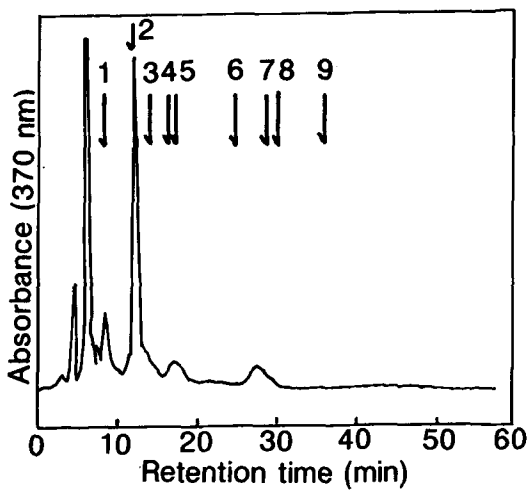

FIG. 5. HPLC chromatogram of the hydrazone fraction of silica gel column chromatography from the major TBA-reactive substances obtained by anion exchange column chromatography (see Fig. 2 and Table 1, lane 9). Hydrazones of standard aldehydes were eluted at the retention times indicated by arrows; 1, propanal; 2, butenal; 3, butanal; 4, pentenal; 5, hexadienal; 6, hexenal; 7, heptadienal; 8, hexanal; and 9, heptenal. The control reaction mixture without sample revealed no hydrazone fraction in silica gel column chromatography and no peaks in HPLC. Abbreviations as in Figure 1.

TABLE 2

One-Day Amounts of Red Pigment Produced in the Thiobarbituric Acid (TBA) Assay of Urine of Human, Rabbit and Rat[a]

Subject			One-day 532 nm red pigment (nmol ± SD/kg•day)		
Sex	Age (year)	Body weight (kg)	Number of determinations	+ None	+ *t*-BuOOH
Human male	22	57	4	44 ± 7	92 ± 13
	23	54	1	54	65
	24	58	1	87	107
	25	58	1	87	107
	34	65	4	30 ± 4	76 ± 13
	71	53	1	92	119
Human female	18	51	1	60	90
	18	55	1	88	106
	46	44	4	84 ± 11	156 ± 26
	49	50	1	69	110
	50	58	5	73 ± 10	117 ± 18
	85	36	1	76	87
Rabbit male	1	3.25	4	616 ± 121	1932 ± 338
	1	3.20	4	445 ± 83	1415 ± 269
	1	3.15	4	524 ± 163	1527 ± 430
	2	4.30	7	363 ± 117	817 ± 261
	3	4.45	5	312 ± 92	733 ± 255
Rat male	0.13	0.18	2	88	578
	0.13	0.23	2	148	860
	0.13	0.21	2	195	354
	0.13	0.22	2	192	703
	0.13	0.20	2	155	849
	1	0.44	4	125 ± 30	1079 ± 308
	1	0.42	4	112 ± 24	868 ± 147
	1	0.38	4	224 ± 16	1017 ± 154
	1	0.44	4	122 ± 30	911 ± 182
	3	0.43	6	63 ± 38	370 ± 139
	3	0.40	2	55	267
	3	0.35	6	57 ± 31	371 ± 230

[a]24-h Urine was collected and subjected to the TBA assay (method A) heated at 100°C for 20 min in the absence or presence of 2 mM *t*-BuOOH. The red pigment was determined by high-performance liquid chromatography. The one-day amount of pigment (nmol ± SD/kg•day) was corrected for the volume of urine and the body weight. *t*-BuOOH, *tert*-butyl hydroperoxide.

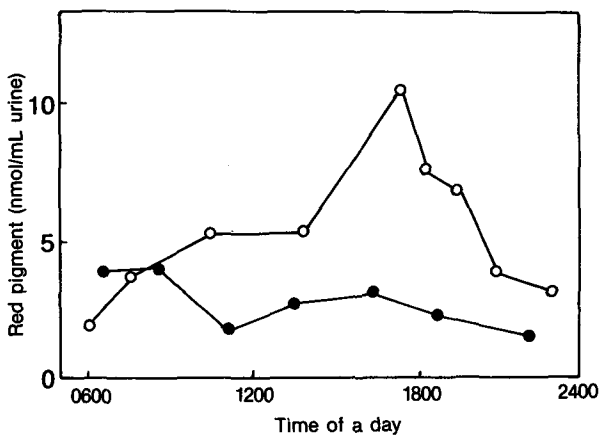

FIG. 6. The TBA reactivity of human urine collected at different times throughout a day. Two human urine samples were subjected to the TBA assay (method A), and the reaction mixture heated at 100°C for 20 min was subjected to HPLC analysis. Abbreviations as in Figure 1.

determined. The TBA reactivity of urine of these animals was much higher; thus humans excreted lower amounts of malonaldehyde derivatives and other aldehydes than did the animals.

Urine was collected from healthy human subjects at different times of the day, and the amounts of the pigment produced without *t*-BuOOH were determined and compared (Fig. 6). It was found that the TBA reactivity of urine collected in the afternoon or in the evening was higher than that collected in the morning.

DISCUSSION

The TBA reaction of human urine produced at least three pigments, namely 455 nm yellow, 532 nm red and 518 nm orange pigments, as shown in Figure 1. The yellow pigment may be derived from alkanals (24,25) and/or sugars (26) and cannot be regarded as an index of lipid peroxidation. The red pigment is only the pigment that appears to reflect lipid peroxidation. The orange pigment was produced when the reaction mixture was heated for prolonged periods. Bidder and Sipka (27) have demonstrated that the absorbance at 532 nm of the TBA reaction mixture of urine progressively increased with heating time, and they suggested that the abnormal increase is due to materials other than malonaldehyde derivatives. As alkadienals and alkenals produce 532 nm red pigment in a time course similar to that of malonaldehyde derivatives (7,8), the abnormal increase may be due to the development of

chromogens other than 532 nm red pigment. Several substances in urine have been shown to produce these different chromogens (1,28–31). HPLC separation of the pigment is necessary for accurate quantitation of the red pigment that appears to reflect lipid peroxidation products in urine.

The TBA reactivity of urine that produces the red pigment is barely dependent on the method used, whereas the reactivity of fats and oils (13) and rat liver and brain homogenate (12,14) have been shown to be greatly dependent on the method used. The yield of the red pigment from urine was somewhat higher at pH 2 than at pH 5, and its development was not inhibited by EDTA. The characteristics of the pigment development were similar to those of standard malonaldehyde derivatives. The amount of red pigment formed was roughly equal to the content of malonaldehyde derivatives estimated as DNPP. The TBA reactivity of urine thus appears to be due to malonaldehyde derivatives. However, the pigment development enhanced by *t*-BuOOH and ferric ions seem to be due to aldehydes other than malonaldehyde derivatives (13,14), whose presence was confirmed by hydrazone formation with DNPH.

The present results are consistent with earlier observations. Draper *et al.* (2–4) had demonstrated that the TBA reactivity of rat urine increased when supplemented with malonaldehyde derivatives, a vitamin E-deficient diet, iron or carbon tetrachloride, and that the TBA reactivity of human morning urine increased by consuming a supplement of n-3 fatty acids (5). They have identified malonaldehyde derivatives, such as *N*α-acetyl-ε-(2-propenal)lysine (32), *N*-(2-propenal)serine (16), *N*-ε-(2-propenal)lysine (33), *N*-(2-propenal)ethanolamine (34) and guanine-malonaldehyde adduct (35) as rat urinary metabolites. Ekstrom *et al.* (19,36) demonstrated that administration of chloroform or hydroquinone to rats resulted in high excretion of malonaldehyde derivatives as estimated by the DNPH method. Our results give additional evidence for the excretion of malonaldehyde derivatives in urine. Lee *et al.* (37) have recently demonstrated that HPLC separation of rat urine gives six TBA-reactive substances, including aldehydes other than malonaldehyde derivatives. Our results on the TBA reactivity of human unseparated urine in the presence of *t*-BuOOH or ferric ions clearly show the presence of aldehydes other than malonaldehyde derivatives.

It has been shown that the TBA reactivity of oxidized fats and oils is due to alkadienals and/or alkenals (11,13,14), and that the reactivity of tissue homogenate is mainly due to alkadienals and, to a lesser extent, to malonaldehyde derivatives (12,14). By contrast, the TBA reactivity of urine was found to be due mainly to malonaldehyde derivatives and, to a lesser extent, to other aldehydes only when the reaction was carried out in the presence of *t*-BuOOH or ferric ions. It is interesting to note that the TBA-reactive substances of urine were different from those of tissues. Nonpolar TBA-reactive substances composed of alkadienals generated in tissues may stay longer in tissues, and the polar TBA-reactive substances composed of malonaldehyde derivatives may be more readily excreted in urine.

The amounts of red pigment produced from 24-h urine of 12 healthy subjects were estimated to be 26–95 nmol/kg, and 65–182 nmol/kg in the presence of *t*-BuOOH, the values being lower than those from urine of rabbit or rat. Excretion of malonaldehyde derivatives and other aldehydes had no relationship to the age or sex of the subjects. TBA reactivity due to malonaldehyde derivatives was higher in the afternoon or in the evening than in the morning, suggesting that the physical activity of a subject during daytime may contribute to increased excretion. It has also been suggested that intake of polyunsaturated fatty acids leads to higher excretion of malonaldehyde derivatives in urine (5).

ACKNOWLEDGMENTS

We thank K. Kiryu, T. Sugawara and H. Nakamura for technical assistance.

REFERENCES

1. Janero, D.R. (1990) *Free Rad. Biol. Med. 9,* 515–540.
2. Draper, H.H., Polensek, L., Hadley, M., and McGirr, L.G. (1984) *Lipids 19,* 836–843.
3. Draper, H.H., McGirr, L.G., and Hadley, M. (1986) *Lipids 21,* 305–307.
4. Dhanakoti, S.N., and Draper, H.H. (1987) *Lipids 22,* 643–646.
5. Piche, L.A., Draper, H.H., and Cole, P.D. (1988) *Lipids 23,* 370–371.
6. Bird, R.P., Hung, S.S.O., Hadley, M., and Draper, H.H. (1983) *Anal. Biochem. 128,* 240–244.
7. Kosugi, H., Kato, T., and Kikugawa, K. (1987) *Anal. Biochem. 165,* 456–464.
8. Kosugi, H., Kato, T., and Kikugawa, K. (1988) *Lipids 23,* 1024–1031.
9. Kosugi, H., and Kikugawa, K. (1989) *J. Jpn. Oil Chem. Soc. 38,* 224–230.
10. Kosugi, H., and Kikugawa, K. (1989) *Free Rad. Biol. Med. 7,* 205–207.
11. Kosugi, H., Kojima, T., and Kikugawa, K. (1989) *Lipids 24,* 873–881.
12. Kikugawa, K., Kojima, T., and Kosugi, H. (1990) *Free Rad. Res. Commun. 8,* 107–113.
13. Kosugi, H., Kojima, T., and Kikugawa, K. (1991) *J. Am. Oil Chem. Soc. 68,* 51–55.
14. Kikugawa, K., Kojima, T., Yamaki, S., and Kosugi, H. (1992) *Anal. Biochem. 202,* 249–255.
15. Crawford, D.L., Yu, T.C., and Sinnhuber, R.O. (1966) *J. Agric. Food Chem. 14,* 182–184.
16. Hadley, M., and Draper, H.H. (1988) *FASEB J. 2,* 138–140.
17. Sinnhuber, R.O., Yu, T.C., and Yu, T.C. (1958) *Food Res. 23,* 626–633.
18. Ohkawa, H., Ohishi, N., and Yagi, K. (1979) *Anal. Biochem. 95,* 351–358.
19. Ekstrom, T., Garberg, P., Egestad, B., and Hogberg, J. (1988) *Chem. Biol. Interact. 66,* 177–187.
20. Tomita, M., and Okuyama, T. (1990) *J. Chromatogr. 136,* 271–277.
21. Esterbauer, H. (1982) in *Free Radicals, Lipid Peroxidation and Cancer* (McBrien, D.C.H., and Slater, T.F., eds.) pp. 101–128, Academic Press, New York.
22. Buege, J.A., and Aust, S.D. (1978) *Methods Enzymol. 52,* 302–310.
23. Uchiyama, M., and Mihara, M. (1978) *Anal. Biochem. 86,* 271–278.
24. Jacobson, G.A., Kirkpatrick, J.A., and Goff, Jr., H.E., (1964) *J. Am. Oil Chem. Soc. 41,* 124–128.
25. Marcuse, R., and Johansson, L. (1973) *J. Am. Oil Chem. Soc. 50,* 387–391.
26. Keeney, M., and Bassette, R. (1959) *J. Dairy Sci. 42,* 945–960.
27. Bidder, G.T., and Sipka, B.M. (1989) *Lipids 24,* 656–658.
28. Gutteridge, J.M.C., and Tickner, T.R. (1978) *Anal. Biochem. 91,* 250–257.
29. Gutteridge, J.M.C., and Tickner, T.R. (1978) *Biochem. Med. 19,* 127–132.

30. Gutteridge, J.M.C. (1981) *FEBS Lett. 128,* 343–346.
31. Baumgartner, W.A., Baker, N., Hill, V.A., and Wright, E.T. (1975) *Lipids 10,* 309–311.
32. McGirr, L.G., Hadley, M., and Draper, H.H. (1985) *J. Biol. Chem. 260,* 15427–15431.
33. Draper, H.H., Hadley, M., Lissemore, L., Laing, N.M., and Cole, P.D. (1988) *Lipids 23,* 626–628.
34. Hadley, M., and Draper, H.H. (1989) *Free Rad. Biol. Med. 6,* 49–52.
35. Hadley, M., and Draper, H.H. (1990) *Lipids 25,* 82–85.
36. Ekstrom, T., Warholm, M., Kronevi, T., and Hogbeerg, J. (1988) *Chem. Biol. Interact. 67,* 25–31.
37. Lee, H.-S., Shoeman, D.W., and Csallany, A.S. (1992) *Lipids 27,* 124–128.

[Received March 31, 1992, and in revised form December 7, 1992; Revision accepted February 6, 1993]