Blue Fluorescence Generated during Lipid Oxidation of Rat Liver Microsomes Cannot Be Derived from Malonaldehyde but Can Be from Other Aldehyde Species

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Generation of blue fluorescence together with phospholipid hydroperoxides and aldehyde species in rat liver microsomes during oxidation with $FeCl_2$-ADP-ascorbic acid was monitored, and the kind of lipid oxidation products participating in the formation of blue fluorescence was investigated. Contents of phospholipid hydroperoxides were increased in an early stage of oxidation, and were decreased in an advanced stage of oxidation. Contents of components that liberated malonaldehyde, 4-hydroxyalkenals and other unsaturated aldehydes under the acidic assay conditions were increased in the advanced stage of oxidation. Water-soluble blue fluorescence with a maximum at 440—450 nm determined after separation through gel filtration accumulated in the advanced stage of oxidation, and was characterized as resistant to borohydride treatment and to be little dependent on pH values of the solvent. Wavelength of the maximum fluorescence and characteristics of the fluorescence were similar to those of fluorescence with maxima at 440—450 nm formed by reaction of unoxidized microsomes, bovine serum albumin or methylamine with alkenals, and different from those of fluorescence with maxima at above 460 nm obtained by the reaction with a mixture containing malonaldehyde. Hence, blue fluorescence accumulated in oxidized microsomes cannot be derived from free malonaldehyde but can be from other aldehyde species including alkenals.

Key words lipid peroxidation; malonaldehyde; alkenal; microsome; blue fluorescence; 1,4-dihydropyridine 3,5-dicarbaldehyde

Blue fluorescence generated in living cells has been referred to as an age-related lipofuscin-like substance and also an index of aging.[1—3] Blue fluorescence is believed to be derived from the reaction of proteins with lipid oxidation products in living cells *in situ*.[1—5] However, because of the difficulty in isolating blue fluorescence from tissues its characteristic nature remains obscure. In earlier studies, lipid-soluble blue fluorescence showing an excitation maximum at 340—380 nm and a fluorescence maximum at 420—490 nm was extracted with chloroform-methanol,[6,7] and many reports using this organic solvent extraction method have appeared.[3,5] Several other studies have claimed that water-soluble blue fluorescence is a better index than lipid-soluble blue fluorescence.[4,8—12] Furthermore, our recent studies have demonstrated that chloroform-methanol extraction produces an artificial blue fluorescence and that the amount of blue fluorescence generated in tissues may be overestimated by this organic solvent extraction method.[13]

It has been found in several model systems that blue fluorescence is generated by reaction of proteins/amino acids with lipid oxidation products.[1—5,14,15] Malonaldehyde (MA), one of these lipid oxidation products, has received attention because the aldehyde produces blue fluorescent 1,4-dihydropyridine 3,5-dicarbaldehyde derivatives by reaction with proteins under physiological conditions.[1—3,14,15] However, it has been shown that aldehyde species other than MA[3,14,15] and lipid peroxy radicals[16,17] can also contribute to the formation of similar blue fluorescence on proteins.

In the present study, rat liver microsomes were oxidized and the oxidation products were monitored. Phospholipid hydroperoxides were found to be generated in an early stage of oxidation, and components that liberated MA and other aldehyde species under acidic conditions were accumulated in an advanced stage of oxidation. It was further shown that water-soluble blue fluorescence accumulated in the advanced stage of oxidation was originated not from free MA but from other aldehyde species including alkenals.

MATERIALS AND METHODS

Analysis Fluorescence spectra and intensities were measured using a Hitachi 650—60 fluorescence spectrophotometer (Tokyo, Japan) with a xenon-lamp with excitation and fluorescence slit widths set at 10 nm. The instrument was standardized with a solution of 0.1 μM quinine sulfate in 0.1 M sulfuric acid to give a fluorescence intensity of 1.00 at 450 nm when excited at 350 nm. Relative fluorescence intensity (R_{fi}) of the solution against that of the quinine sulfate solution was determined. R_{fi} unit was calculated by multiplying R_{fi} by the volume (ml) of the solution, and the relative molar fluorescence intensity (R_{mi}) of the compound was calculated by multiplying R_{fi} by 0.1/the molar concentration of the compound. Buffers used for fluorescence measurement were 0.1 M HCl–0.1 M KCl (pH 1.0), 0.1 M HCl–0.1 M glycine (pH 2.0), 0.1 M tartaric acid–0.1 M NaOH (pH 3.0), 0.1 M acetate (pH 5.0), 0.1 M phosphate (pH 7.0), 0.1 M Tris–0.1 M HCl (pH 9.0), 0.1 M NaOH–0.1 M NaH_2PO_4 (pH 11.0) and 0.1 M NaOH–0.1 M KCl (pH 13.0).

^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker AM-400 FT-NMR spectrometer (Switzerland) using $CDCl_3$ as a solvent and tetramethylsilane as an internal standard. Characterization of ^{13}C-NMR signals was done by distortionless enhancement by polarization transfer (DEPT) technique. Mass spectrum was obtained with a Hitachi M-80 machine (Tokyo) by electron impact (EI) technique.

Materials Adenosine-5′-diphosphate monopotassium salt (ADP·K) was obtained from Oriental Yeast Company (Tokyo). Phosphatidylcholine (egg yolk) (PC) and phosphatidylethanolamine (egg yolk) (PE) were obtained from Nippon Fats and Oil Liposome Company (Tokyo). PC hy-

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droperoxide (PCOOH) and PE hydroperoxide (PEOOH) were prepared just before use according to the method of Miyazawa *et al.*[18] Briefly, PC and PE were photooxidized at 10 °C for 10 h in the presence of methylene blue, and methylene blue was removed by passing through a column of florisil. Peroxide values of PCOOH and REOOH were estimated to be 1380—1680 and 770—840 neq/mg, respectively. Cytochrome c and luminol were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.) and Wako Pure Chemical Industries (Osaka), respectively. Bovine serum albumin (BSA) was obtained from Sigma. Thiobarbituric acid (TBA), silica gel 60 (70—230 mesh) and silica gel 60 F-254 for thin-layer chromatography (TLC) were obtained from Merck (Darmstadt, Germany). LPO-586 (*N*-methyl-2-phenylindole) was obtained from Bioxytech S.A. (Bonneul sur Marne, France). Sephadex G-10 was obtained from Pharmacia Company (Upsala, Sweden).

cis-3-Hexen-1-ol, *cis*-3-nonen-1-ol, 3-chloroperoxybenzoic acid (50—60%), pyridinium chlorochromate, *trans*-2-hexenal (hexenal) and *trans*-2-nonenal (nonenal) were obtained from Aldrich Chemical Company (Milwaukee, WIS, U.S.A.). Tetramethoxypropane (TMP) was obtained from Tokyo Chemical Company (Tokyo). Free MA was prepared just before use by dissolving TMP in 0.1 M HCl, and the pH value was immediately adjusted with 0.1 M NaOH. 4-Hydroxy-2(E)-nonenal (HNE) and 4-hydroxy-2(E)-hexenal (HHE) were prepared according to the method of Gardner *et al.*[19] For preparation of HNE, a solution of *cis*-3-nonen-1-ol (71 mg) in 2 ml of chloroform, 130 mg of 3-chloroperoxybenzoic acid was added at 0 °C. Resulting 3,4-epoxynonanol (66.7 mg) was obtained as colorless oil, after addition of 2 ml of 10% $NaHCO_3$ to the reaction mixture, extraction with diethyl ether, and finally silica gel column chromatography using a solvent system composed of *n*-hexane : ethyl acetate (3 : 1, v/v). ^{1}H-NMR spectrum (ppm): 3.85 (2H, m, 1-CH_2), 3.09 (1H, m, 3-CH), 2.94 (1H, m, 4-CH), 1.87, 1.65(2H, m, 3-CH_2), 1.53 (4H, m, 5,6-CH_2), 1.32 (4H, m, 7,8-CH_2) and 0.89 (3H, t, CH_3). ^{13}C-NMR (ppm): 60.8 (1-C), 56.7 (4-C), 55.0 (3-C), 31.7 (7-C), 30.7 (2-C), 27.9 (5-C), 26.2 (6-C), 22.6 (8-C) and 14.0 (9-C). To a solution of 3,4-epoxynonanol (60 mg) in 0.7 ml of methylene chloride 87 mg of pyridinium chlorochromate was added and the solution was rigorously stirred at room temperature. Resulting HNE (8.6 mg) was obtained as colorless oil, after silica gel column chromatography of the reaction mixture as described above. ^{1}H-NMR spectrum (ppm): 9.62 (1H, d, CHO, J=7.7 Hz), 6.85 (1H, dd, 3-CH, J=15.7, 4.7 Hz), 6.35 (1H, ddd, 2-CH, J=15.7, 7.7, 1.6 Hz) and 4.46 (1H, dtd, 4-CH, J=4.7, 1.6 Hz). ^{13}C-NMR (ppm): 193.4 (1-C), 158.7 (3-C), 130.8 (2-C), 71.2 (4-C), 36.6 (5-C), 31.6 (6-C), 24.9 (7-C), 22.5 (8-C) and 13.9 (9-C). Similarly, HHE (26 mg) was prepared from *cis*-3-hexen-1-ol as colorless oil. ^{1}H-NMR spectrum (ppm): 9.60 (1H, d, CHO, J=7.9 Hz), 6.80 (1H, dd, 3-CH, J=14.3, 4.7 Hz), 6.32 (1H, ddd, 2-CH, J=14.3, 7.9, 1.6 Hz) and 4.4 (1H, m, 4-CH).

Rat Liver Microsomes Wistar male rats were fed a normal solid diet CE-2 type containing 4.4% soybean oil and 7.3 mg vitamin E (Nippon-Haigo Shiryou, Tokyo) for 10—20 weeks. Rats were sacrificed by bleeding from common carotid arteries after anesthetization with chloroform. The liver was quickly isolated and washed well with cold physiological saline. Microsomes were obtained from 12.0 g wet weight of liver according to the method of Albro *et al.*[20] A microsomal suspension in 30 ml of 25 mM 3-(*N*-morpholino)-propanesulfonic acid buffer (pH 7.4) containing 0.25 M mannitol was obtained.

Oxidation of Rat Liver Microsomes A 30-ml microsomal suspension was mixed with 30 ml of 0.05 M Tris–0.15 M KCl buffer (pH 7.5) containing 2.0 mM ADP·K–2.0 mM sodium ascorbate–24 μM $FeCl_2$, and the mixture was incubated at 37 °C for 6 h. A 2.0-ml suspension containing unoxidized or oxidized microsomes was extracted with an equal volume of chloroform–methanol (2 : 1, v/v), and the mixture was centrifuged at 3000 rpm for 10 min. The organic layer was dehydrated with dried sodium sulfate and evaporated at below 30 °C to dryness to be redissolved into 100 μl of chloroform–methanol (1 : 1, v/v) for PCOOH and PEOOH determinations. The suspension of unoxidized or oxidized microsomes, or its supernatant obtained by centrifugation at 4300 rpm for 10 min was subjected to TBA and LPO-586 assays. For determination of blue fluorescence, the supernatant (60 ml) was lyophilized to subject to a column (0.9×100 cm) of Sephadex G-10 equilibrated with 0.01 M NaCl containing 0.02% NaN_3.

Reaction of Rat Liver Microsomes with Lipid Oxidation Products A 30-ml microsomal suspension was mixed with 30 ml of 0.05 M Tris–0.15 M KCl buffer (pH 7.5) containing 1.0 meq/ml PCOOH, 2.0 mM hexenal, 2.0 mM HNE or 2.0 mM free MA, and the mixture was incubated at 37 °C for 6 h. The supernatant obtained by centrifugation of the suspension at 4300 rpm for 10 min was lyophilized and passed through the column of Sephadex G-10 to obtain blue fluorescent fractions.

Synthesis of 1,4-Dihydropyridine 3,5-Dicarbaldehyde Derivatives Ia—Ic Three 1,4-dihydropyridine 3,5-dicarbaldehyde derivatives **Ia—Ic** substituted with unsaturated or hydroxyunsaturated alkyl chains at the 4-position were prepared.

Ia A mixture of 147 mg (1.5 mmol) of methylammonium chloride, 147 mg (1.47 mmol) of hexenal and 492 mg (2.9 mmol) of free MA in 15 ml of 70% methanol–0.1 M phosphate buffer (pH 7.0) was incubated at 37 °C for 24 h. The reaction mixture was extracted with 50 ml of chloroform. The extract was evaporated to dryness and subjected to silica gel column chromatography using ethyl acetate as a solvent. The product was further purified by TLC using ethyl acetate as a solvent. Greenish yellow crystals of **Ia** (12.8 mg) were obtained in a yield of 4%. ^{1}H-NMR spectrum (ppm): 9.36 (2H, s, 3,5-CHO), 6.72 (2H, s, 2,6-CH), 5.49 (1H, tdd, C$\underline{H}$=CHCH_2, J=6.1, 15.4 Hz), 5.31 (1H, dtd, CH=C$\underline{H}$CH_2, J=14.3, 15.4 Hz), 4.42 (1H, d, 4-CH, J=6.1 Hz), 3.37 (3H, s, NCH_3), 1.90 (2H, q, CH=CHC$\underline{H}_2$, J=14.3 Hz), 1.31 (2H, m, $CH_2C\underline{H}_2CH_3$) and 0.82 (3H, t, $CH_2C\underline{H}_3$).

Ib A mixture of 147 mg (1.5 mmol) of methylammonium chloride, 210 mg (1.48 mmol) of nonenal and 492 mg (2.9 mmol) of free MA in 15 ml of 70% methanol–0.1 M phosphate buffer (pH 7.0) was incubated at 37 °C for 24 h. From the reaction mixture greenish yellow crystals (20.5 mg) of **Ib** were obtained in a yield of 5.5%. EI mass spectrum (*m/z*): 261 [M^+] (4), 232 [M^+-29] (6), 176 (12), 151 (100), 122 (5), 108 (4) and 94 (3). ^{1}H-NMR spectrum (ppm): 9.30 (2H, s, 3,5-CHO), 6.72 (2H, s, 2,6-CH), 5.47 (1H, tdd, C$\underline{H}$=CHCH_2, J=6.0, 15.8 Hz), 5.29 (1H, dtd, CH=C$\underline{H}$CH_2, J=

15.8 Hz), 4.42 (1H, d, 4-CH, J=6.0 Hz), 3.35 (3H, s, NCH_3), 1.90 (2H, q, CH=CHC$\underline{H}_2$), 1.30 (8H, m, $(C\underline{H}_2)_4CH_3$) and 0.81 (3H, t, $CH_2C\underline{H}_3$).

Ic A mixture of 41 mg (0.6 mmol) of methylammonium chloride, 95 mg (0.61 mmol) of HNE and 199 mg (1.2 mmol) of free MA in 15 ml of 70% methanol–0.1 M phosphate buffer (pH 7.0) was incubated at 37 °C for 24 h. From the reaction mixture yellow oil (5 mg) of **Ic** was obtained in a yield of 2.7%. EI mass spectrum (m/z): 277 [M^+] (6), 259 [M^+-18] (6), 242 (8) 230 (12), 176 (16), 151 (100), 135 (4), 112 (8), 86 (16) and 67 (4). ^{1}H-NMR spectrum (ppm): 9.30 (2H, s, 3,5-CHO), 6.75 (2H, s, 2,6-CH), 5.70 (1H, dd, C$\underline{H}$=CHCH$_2$, J=6.0, 15.8 Hz), 5.33 (1H, d, CH=C$\underline{H}$CH$_2$, J=15.8 Hz), 4.47 (1H, d, 4-CH, J=6.0 Hz), 3.25 (3H, s, NCH_3), 1.90 (2H, m, CH=CHCH$_2$), 1.20 (8H, m, $(C\underline{H}_2)_4CH_3$) and 0.86 (3H, t, $CH_2C\underline{H}_3$).

HPLC-Chemiluminescence Assay of PCOOH and PEOOH The contents of PCOOH and PEOOH in microsomal suspensions were determined by HPLC-chemiluminescence method after extraction with chloroform–methanol.[18] A 50-μl portion of the solution of the extract was subjected to HPLC using a Hitachi L-6200 Intelligent Pump equipped with a column (4.6×250 mm) of YMC A-012 S-5 120 A SIL (Yamamura Chemical Laboratories, Kyoto) and a mobile phase composed of chloroform–methanol (1 : 9, v/v) at a flow rate of 1.1 ml/min. A solution composed of 10 μg/ml cytochrome c and 1 μg/ml luminol in 50 mM borate buffer (pH 9.3) was mixed with the eluate at a flow rate of 1.0 ml/min and chemiluminescence generated was detected using a JASCO 825-CL Intelligent CL detector (Tokyo). The chemiluminescent peaks due to PEOOH and PCOOH exhibited retention times of 3.1 min and 5.5 min, respectively. The amounts (neq) of PCOOH and PEOOH were determined by comparing their peak areas with a calibration curve of the peak areas of the standard solutions of PCOOH and PEOOH (0—100 neq).

TBA Reactive-Substances (TBARS) Assay TBA-reactive substances in the microsomal suspension and its supernatant were determined according to the previously described method[21,22] with slight modification. Into a test tube with a screw cap, a 200-μl microsomal suspension or its supernatant, 650 μl of a mixture of 0.20 ml of 5.2% sodium dodecyl sulfate, 50 μl of 0.8% butylated hydroxytoluene solution in glacial acetic acid, 1.50 ml of 0.8% TBA solution in water and 1.70 ml of water with or without EDTA at the final concentration of 2 mM, and finally 150 μl of 20% acetate buffer (pH 3.5) were placed in this order. The mixture was kept at 5 °C for 60 min and then heated at 100 °C for 60 min. The mixture was extracted with 1.0 ml of a mixture of 1-butanol–pyridine (15 : 1, v/v). Absorbance at 532 nm of the extract was measured. The amount of red pigment reflecting the TBA-reactive substances was calculated using an extinction coefficient of the pigment: 156000.

LPO-586 Assay LPO-586 assay of the microsomal supsension and its supernatant was carried out according to the reagent manual (Bioxytech S.A.).[22] To 200 μl of the microsomal suspension or its supernatant, 650 μl of 8.6 mM N-methyl-2-phenylindole in acetonitrile–methanol (3 : 1, v/v), and 150 μl of concentrated hydrochloric acid or 10.4 M methane sulfonic acid were placed in this order. The mixture in hydrochloric acid was heated at 45 °C for 60 min, and the mixture in methane sulfonic acid at 45 °C for 40 min. Absorbance at 586 nm of the mixture was measured. The standard solutions of 0—1 mM TMP in Tris buffer or HNE diethylacetal in acetonitrile were similarly treated. Content of MA-precursors was calculated from the calibration curve of TMP treated in hydrochloric acid, and content of MA- plus 4-hydroxyalkenal-precursors was calculated from the calibration curve of TMP or HNE diethylacetal in methane sulfonic acid.

RESULTS

Lipid Oxidation Products in Oxidized Rat Liver Microsomes Rat liver microsomes suspended in Tris–KCl buffer (pH 7.4) were oxidized with 1.0 mM ADP·K–1.0 mM sodium ascorbate–12 μM $FeCl_2$ at 37 °C for 6 h. It has been shown that the combination of Fe(II) and ascorbate effectively induced lipid peroxidation of microsomes.[23] Fe(II) chelated with ADP may reduce molecular oxygen to produce superoxide and finally hydroxyl radical to cause lipid peroxidation. Primary lipid oxidation products PCOOH and PEOOH in the unoxidized (control) and oxidized microsomal suspensions were determined by HPLC-chemiluminescence method[18] after extraction of the suspensions with chloroform–methanol. HPLC of unoxidized microsomal suspension did not give any significant chemiluminescent peaks due to PEOOH and PCOOH (Fig. 1A). HPLC of 10-min oxidized microsomal suspension gave two chemiluminescent peaks due to PEOOH and PCOOH at retention times of 3.1 and 5.5 min, respectively (Fig. 1B). Contents of PEOOH and PCOOH produced were estimated to be 1.81 and 3.63 neq/ml suspension, respectively. In contrast, HPLC of 60-min (Fig. 1C) or 6-h oxidized (data not shown) microsomal suspension gave no chemiluminescent peaks due to these hydroperoxides. The results indicate that primary lipid oxidation products of phospholipids were produced in the early stage of oxidation, but they were completely destroyed and changed into the secondary lipid oxidation products in the advanced stage

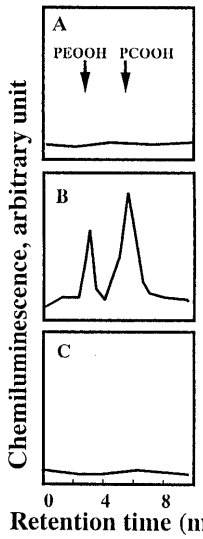


Fig. 1. HPLC-Chemiluminescence Determination of PCOOH and PEOOH of Unoxidized and Oxidized Microsomal Suspensions

Rat liver microsomes suspended in Tris–KCl buffer (pH 7.4) were unoxidized (A) or oxidized at 37 °C for 10 min (B) and for 60 min (C) with 1.0 mM ADP·K–1.0 mM sodium ascorbate-12 μM $FeCl_2$ and extracted with chloroform–methanol.

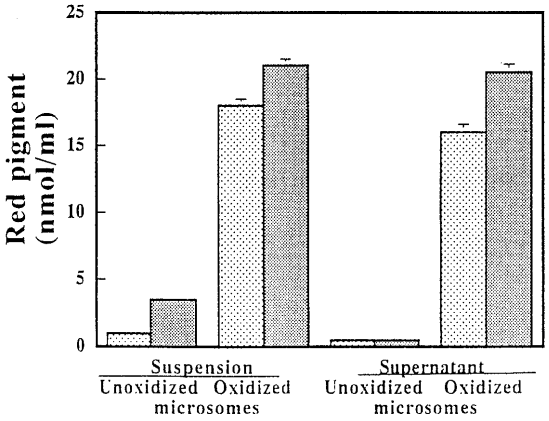


Fig. 2. Red Pigment Formation in TBA Assay of Unoxidized and Oxidized Microsomal Suspensions and Their Supernatants

Rat liver microsomes suspended in Tris-KCl buffer (pH 7.4) were unoxidized or oxidized at 37 °C for 6 h with 1.0 mM ADP·K–1.0 mM sodium ascorbate–12 μM $FeCl_2$. TBA assay with EDTA (▦) and without EDTA (▨) (see Materials and Methods section) of the microsomal suspensions and their supernatants was performed.

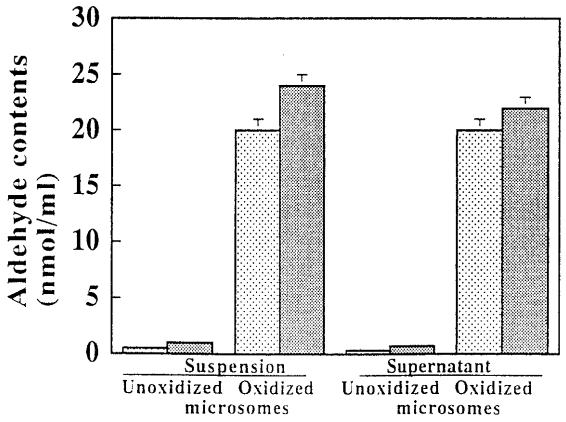


Fig. 3. Contents of MA-Precursors, and MA- plus 4-Hydroxyalkenal-Precursors in Unoxidized and Oxidized Microsomal Suspensions and Their Supernatants Determined by LPO-586 Assay

Rat liver microsomes suspended in Tris–KCl buffer (pH 7.4) were unoxidized or oxidized at 37 °C for 6 h with 1.0 mM ADP·K–1.0 mM sodium ascorbate–12 μM $FeCl_2$. MA-precursors (▦) and MA- plus 4-hydroxyalkenal-precursors (▨) (see Materials and Method section) of the microsomal suspensions and their supernatants were determined.

of oxidation.

Secondary lipid oxidation products, *i.e.*, precursors that liberated MA and other aldehyde species under the acidic assay conditions, were determined by TBA and LPO-586 assays. It is claimed that in the TBA assay with EDTA precursors liberating MA produce red pigment quantitatively, and in the assay without EDTA precursors liberating alkenals and alkadienals produce the same red pigment in a lesser amount.[21,22] Figure 2 shows the amounts of red pigment from the microsomal suspensions and their supernatants. In the assay with EDTA, the amount of red pigment in the microsomal suspension was extensively increased after 6-h oxidation, and similar extent of increase in the amount of the pigment in the supernatant was obtained. The results indicate that MA-precursors were produced during the oxidation and most of them became water-soluble and were released into the supernatant. The levels of MA-precursors were estimated to be about 22 nmol/ml suspension, which were much higher than those of the phospholipid hydroperoxides (PCOOH+PEOOH, 5.4 neq/ml) produced in the early stage of oxidation. Primary phospholipid hydroxperoxides formed in the earyl stage of oxidation were transitory components and readily degraded in the advanced stage of oxidation, while MA-precursors were stable and accumulated in the advanced stage of oxidation. In the assay without EDTA, the amount of red pigment in the microsomal suspension and in the supernatant increased to a slightly higher extent than that in the assay with EDTA. The results indicate that alkenal- plus alkadienal-precursors were also generated during the oxidation to be released into the supernatant.

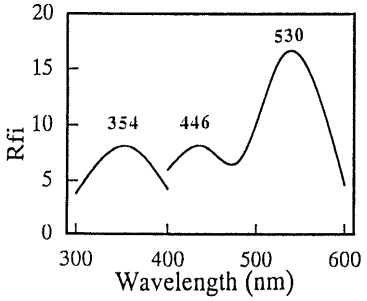


Fig. 4. Fluorescence Spectrum of the Supernatant of Microsomal Suspension Oxidized at 37 °C for 6 h with 1.0 mM ADP·K–1.0 mM Sodium Ascorbate–12 μM $FeCl_2$

Contents of MA-precursors, and MA- plus 4-hydroxyalkenal-precursors were determined by LPO-586 assay in hydrochloric acid and methane sulfonic acid, respectively.[22] Because violet-color development from 4-hydroxyalkenal-precursors may be suppressed in the presence of other aldehydes, their contents may be underestimated under the assay conditions.[22] Figure 3 shows the contents of MA-precursors, and MA- plus 4-hydroxyalkenal-precursors in the microsomal suspensions and their supernatants. Contents of MA-precursors in the microsomal suspension were increased by the oxidation, and a similar amount of the precursors was released into the supernatant. Contents of MA- plus 4-hydroxyalkenal-precursors were increased to a slightly higher extent by the oxidation and were released in the supernatant. MA-precursor contents obtained by LPO-586 assay were similar to those obtained by TBA assay.

The results obtained by TBA and LPO-586 assays indicate that precursors of MA, alkenals, alkadienals and 4-hydroxyalkenals were accumulated during oxidation and most of them became water-soluble to be released into the supernatant. It is not known what kinds of precursors accumulated, but MA-precursors cannot be produced by the reaction of free MA and amino components on a basis of the fluorescence study described below.

Blue Fluorescence in Oxidized Rat Liver Microsomes The supernatant of the microsomal suspensions oxidized for 6 h showed blue fluorescence at 446 nm together with green fluorescence at 530 nm when excited at 354 nm (Fig. 4). Green fluorescence may be due to flavin-containing substances because the fluorescence maximum was similar to that of flavins.[13] Blue fluorescence was separated by passing through a column of Sephadex G-10; blue fluorescence was eluted between the void and the bed volumes of the column and green fluorescence was tightly adsorbed to the gels (Fig. 5). The blue-fluorescent peak fractions composed of multiple components were collected, and the fluorescence spectra and intensities of the fractions were measured (Fig. 6A). Blue flu-

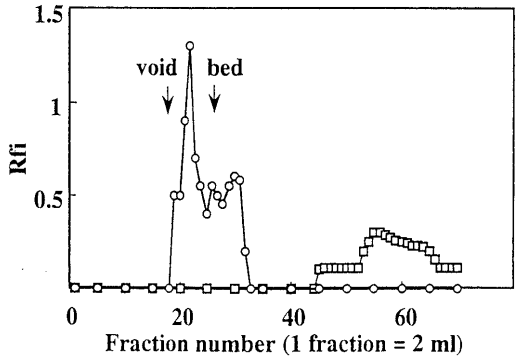


Fig. 5. Gel Filtration of the Supernatant of the Oxidized Microsomal Suspension

The supernatant of the microsomal suspension (30 ml) oxidized at 37 °C for 6 h with 1.0 mM ADP·K–1.0 mM sodium ascorbate–12 μM $FeCl_2$ was lyophilized and subjected to gel filtration through a column (0.9×100 cm) of Sephadex G-10 equilibrated with 0.01 M NaCl containing 0.2% NaN_3. Blue fluorescence at 433—458 nm when excited at 352—360 (○) and green fluorescence at 508—528 nm when excited at 373—380 nm (□) were monitored.

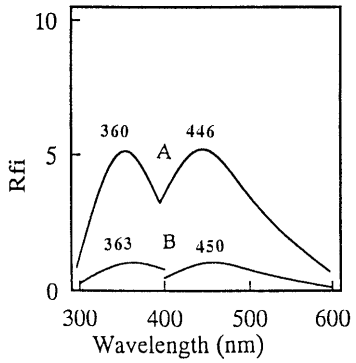


Fig. 6. Spectra and Intensities (R_{fi} Unit) of Blue Fluorescence of the Supernatants of Oxidized (A) and Unoxidized Microsomal Suspensions (B) (30 ml) after Gel Filtration Shown in Fig. 5

orescence showed a single fluorescence maximum at 446 nm when excited at 360 nm. Fluorescence intensity (R_{fi} unit) of the fractions was 5 times as high as that of the same fractions obtained from unoxidized microsomal supernatant (Fig. 6B). Hence, oxidation of microsomes resulted in generation of water-soluble blue fluorescence that was released into the supernatant. Blue fluorescence from oxidized microsomes was quite resistant to treatment with 0.1 M sodium borohydride (data not shown). Fluorescence spectra and intensities were only slightly affected by the pH values of the solution between pH 1 and 13 (Fig. 7).

In order to know participation of primary and secondary lipid oxidation products in the blue fluorescence formation in the supernatant of microsomal suspension, maximum wavelength of blue fluorescence from oxidized microsomes was compared with that from unoxidized microsomes treated with 0.5 meq/ml PCOOH, 1 mM hexenal, 1 mM HNE and 1 mM MA (Table 1). Wavelength of the maximum fluorescence appearing at 440—450 nm when treated with PCOOH or hexenal were similar to that from oxidized microsomes, that appearing at 430 nm on treatment with HNE was somewhat shorter, and that appearing at 460 nm on treatment with MA was somewhat longer, suggesting that phospholipid hydroperoxides and their degradation product alkenals contributed to the blue fluorescence formation in the oxidized microsomes.

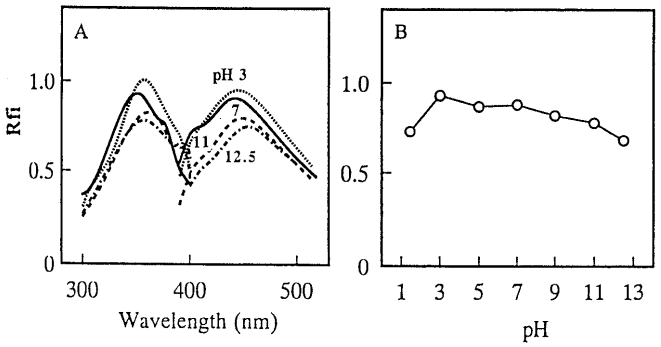


Fig. 7. Spectra (A) and Intensities at the Maximum Fluorescence (B) at the Different pH Values of Blue Fluorescesscence After Gel Filtration Shown in Fig. 5

Table 1. Spectra and Intensities of Blue Fluorescent Fractions Obtained through Gel Filtration of the Supernatants of Microsomal Suspensions Oxidized or Treated with Lipid Oxidation Products

Microsome	Maximum Excitation/fluorescence (nm)	R_{fi} unit
Oxidized microsomal suspension[a]	362/446	5.66
Unoxidized microsomal suspension[b]		
+0.5 meq/ml PCOOH	362/441	13.56
+1.0 mM hexenal	372/443	6.90
+1.0 mM HNE	354/430	9.90
+1.0 mM MA	396/460	6.00

a) Microsomal suspension oxidized at 37 °C for 6 h with 1.0 mM ADP·K–1.0 mM sodium ascorbate–12 μM $FeCl_2$. *b*) Microsomal suspension incubated with the indicated lipid oxidation product at 37 °C for 6 h.

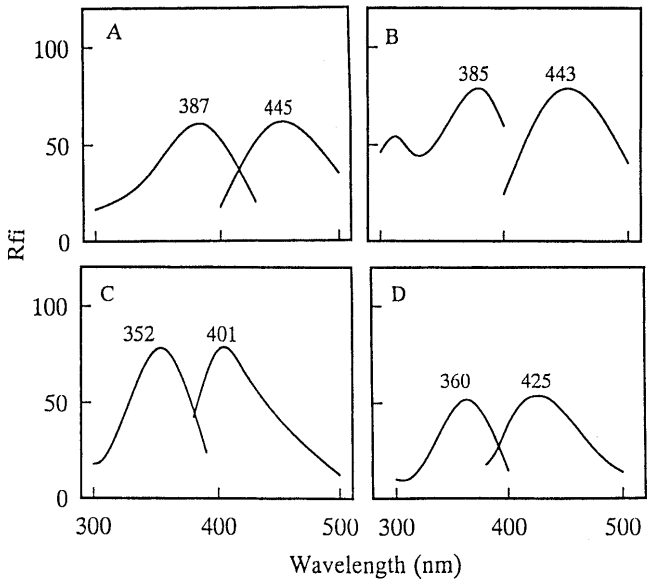
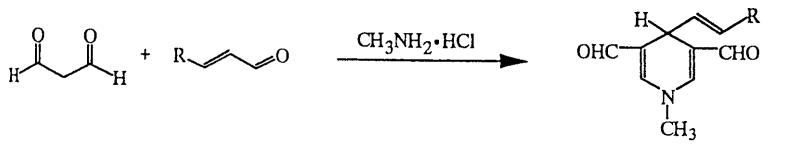


Fig. 8. Spectra of Blue Fluorescence Generated in the Reaction of BSA with Hexenal (A), Nonenal (B), HHE (C) or HNE (D)

A mixture of BSA (10 mg/ml) and 10 mM hexenal, nonenal, HHE or HNE in 0.5% sodium dodecyl sulfate–0.05 M phosphate buffered saline (pH 7.4) was incubated at 37 °C for 24 h. Fluorescence spectra of the mixture were measured.

Fluorescence spectra of the reaction mixture of BSA treated with hexenal, nonenal, HHE and HNE were compared (Fig. 8). BSA treated with hexenal and nonenal showed wavelengths of maximum fluorescence at 440—450 nm whereas those treated with HHE and HNE showed shorter wavelengths of maximum fluorescence at 400—430 nm. Maximum wavelength of blue fluorescence from oxidized



Ia, R = $(CH_2)_2CH_3$
Ib, R = $(CH_2)_5CH_3$
Ic, R = $CH(OH)(CH_2)_4CH_3$

Fig. 9. Structure of Fluorescent 1,4-Dihydropyridine 3,5-dicarbaldehyde Derivatives with Substitution of Unsaturated or Hydroxyunsaturated Groups at the 4-Position

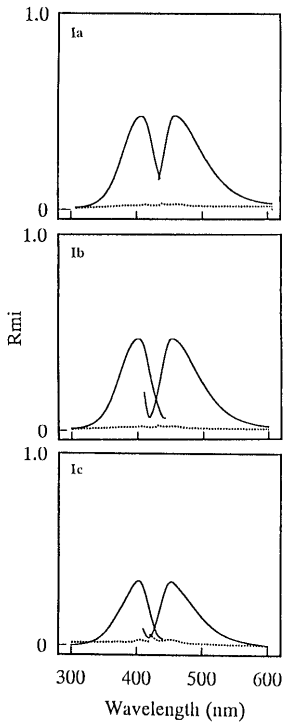


Fig. 10. Fluorescence Spectra of **Ia**—**c** in Methanol before (—) and after (---) Treatment with 0.1 M Sodium Borohydride

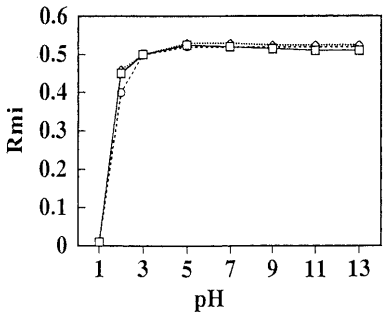


Fig. 11. Fluorescence Intensities of **Ia** (○), **Ib** (□) and **Ic** (△) at the Fluorescence Maximum in the Aqueous Solution at Different pH Values

microsomes was closer to that from the reaction with alkenals than from the reaction with 4-hydroxyalkenals. Fluorescence from these aldehydes was resistant to treatment with 0.1 M sodium borohydride, and fluorescence spectra and intensities of these mixtures were little affected by the pH values of the solution between pH 3 and pH 11 (data not shown). The results suggest that alkenals contributed to the blue fluorescence formation in the oxidized microsomes.

Fluorescence characteristics of highly fluorescent 1,4-dihydropyridine 3,5-dicarbaldehyde derivatives produced in the reaction of protein amino groups and MA alone[24—30] or in combination with alkanals under physiological conditions have been demonstrated.[31—34] All of the known derivatives show fluorescence maxima at above 460 nm.[24—33] Fluorescence of the derivatives is destroyed by borohydride treatment,[26] which may be due to the reduction of one of two 3- or 5-aldehyde groups.[35] Fluorescence intensities of the derivatives are decreased in a strongly acidic medium.[3] Here, alternative 1,4-dihydropyridine 3,5-dicarbaldehyde derivatives produced from MA in combination with alkenals or 4-hydroxyalkenals under physiological conditions were isolated to compare their fluorescence characteristics with those from oxidized microsomes. Highly fluorescent 3,5-dihydropyridine 3,5-dicarbaldehydes with unsaturated or hydroxyunsaturated alkyl chains at the 4-position were obtained (Fig. 9) from the reaction of methylamine and MA in combination with hexenal (**Ia**), nonenal (**Ib**) or HNE (**Ic**). **Ia**, **Ib** and **Ic** showed excitation and fluorescence maxima at 406/464 (R_{mi}: 0.5), 405/464 (R_{mi}: 0.5) and 405/463 nm (R_{mi}: 0.5), respectively, in 0.1 M phosphate buffer (pH 7.0) (Fig. 10). Wavelengths of maximum fluorescence of these compounds were above 460 nm as were those of known 1,4-dihydropyridine 3,5-dicarbaldehydes. Fluorescence of these compounds in methanol was readily destroyed by borohydride treatment (Fig. 10), and fluorescence of the compounds was quenched completely at pH 1 (Fig. 11). Hence, characteristics of blue fluorescence from oxidized microsomes were far different from those of 1,4-dihydropyridine 3,5-dicarbaldehydes produced in the reaction of MA alone or in combination with alkanals, alkenals and 4-hydroxyalkenals.

DISCUSSION

For the assays of secondary lipid oxidation products by TBA and LPO-586 which involve heating in acidic media, it is claimed that in addition to free aldehydes, components liberating free aldehydes under the acidic assay conditions can be determined. Because of high reactivity of aldehyde species to proteins,[14,15] free aldehydes generated in the advanced stage of oxidation of microsomes may have reacted to proteins or other materials. Hence, bound forms of these aldehyde species to proteins, *i.e.*, precursors of these aldehydes, can be exclusively determined in these assays. Water-soluble blue fluorescence generated in the oxidized microsomes showed a fluorescence maximum at 446 nm. Fluorescence spectra and intensities of blue fluorescence were little changed by borohydride treatment and by the pH value of the solution between pH 1 and 13. Generally, blue fluorescence

has been believed to originate from the reaction of proteins with primary lipid hydroperoxides[16,17] or secondary lipid oxidation aldehydic products[14,15] based on observations in model systems. Various fluorescent 1,4-dihydropyridine 3,5-dicarbaldehydes with saturated, unsaturated or hydroxyunsaturated alkyl chains at the 4-position obtained earlier[24—33] and in the present study showed fluorescence maxima at above 460 nm. The fluorescence of all the derivatives was completely destroyed by borohydride treatment, and their fluorescence intensities were greatly decreased in a strongly acidic medium. Because characteristics of blue fluorescence generated in oxidized microsomes are different from those of various 1,4-dihydropyridine 3,5-dicarbaldehydes, free MA cannot participate in the blue fluorescence generation in the oxidized microsomes. Although a high amount of MA-derivatives was produced during microsome oxidation, they may exist in forms which cannot participate in the generation of blue fluorescence.

Aldehyde species other than MA also produce similar blue fluorescence by reaction with ε-amino groups of proteins in model systems.[3,14,15] Alkanals may give blue fluorescence with maxima at 400—430 nm by reaction with primary amines in the presence of oxygen, which is resistant to borohydride treatment.[36] However, studies of the chemistry of alkanals are complicated by their tendency to undergo aldol condensation to give branched chain alkenals in the presence of primary amines.[36] Various alkenals produce fluorescence with maxima at 410—460 nm by reaction with ε-amino groups that is partially resistant to borohydride treatment and whose fluorescence intensity is slightly decreased in an acidic medium and is higher in an alkaline medium.[36—40] Blue fluorescence from HNE shows an emission maximum at 430 nm.[41] In the present model studies, fluorescence spectra of the reaction mixture of BSA treated with alkenals showed wavelengths of maximum fluorescence at 440—450 nm, whereas those treated with 4-hydroxyalkenals showed shorter wavelengths of maximum fluorescence at 400—430 nm; fluorescence from both aldehydes was resistant to borohydride, and fluorescence spectra and intensities were little affected by the pH values of the solution. Maximum wavelength of blue fluorescence from oxidized microsomes was closer to those from the reaction with alkenals than from the reaction with 4-hydroxyalkenals.

Another possible candidate is fluorescent dityrosine formed by reaction of tyrosine residues in proteins by reaction of phospholipid hydroperoxides, but the major blue fluorescence generated in oxidized microsomes may not be due to dityrosine because dityrosine fluoresces with a maximum at shorter wavelength of 410 nm.[16,17]

Hence, the major blue fluorescence generated in oxidized microsomes is not due to free MA but to alkenals, and MA-precursors in oxidized microsomes in high content do not contribute to the formation of blue fluorescence.

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REFERENCES

1) Tappel A. L., "Pathobiology of Cell Membrane," Vol. 1, ed. by Trump B., Arstila A., Academic Press, New York, 1975, pp. 145—170.
2) Tappel A. L., *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **32**, 1870—1874 (1973).
3) Kikugawa K., *Adv. Free Rad. Biol. Med.*, **2**, 389—417 (1986).
4) Tsuchida M., Miura T., Aibara K., *Chem. Phys. Lipids*, **44**, 297—325 (1987).
5) Hammer C., Braum E., *Comp. Biochem. Physiol.*, **90B**, 7—17 (1988).
6) Fletcher B. L., Dillard C. J., Tappel A. L., *Anal. Biochem.*, **52**, 1—9 (1973).
7) Dillard C. J., Tappel A. L., *Methods Enzymol.*, **105**, 337—341 (1984).
8) Desai I. D., Fletcher B. L., Tappel A. L., *Lipids*, **10**, 307—309 (1981).
9) Csallany A. S., Ayaz K. L., Menken B. Z., *Lipids*, **19**, 911—915 (1984).
10) Katz M. L., Robinson W. G., Herrmann R. K., Groome A. B., Bieri J. G., *Mech. Ageing Dev.*, **25**, 149—159 (1984).
11) Csallany A. S., Ayaz K. L., Csallany A. S., *Int. J. Vit. Nutr. Res.*, **56**, 143—147 (1986).
12) Menken B. Z., Su L.-C., Ayaz K. L., Csallany A. S., *J. Nutr.*, **116**, 350—355 (1986).
13) Kikugawa K., Kato T., Yamaki S., Kasai H., *Biol. Pharm. Bull.*, **17**, 9—15 (1994).
14) Kikugawa K., Beppu M., *Chem. Phys. Lipids*, **44**, 277—296 (1987).
15) Kikugawa K., "Membrane Lipid Oxidation," Vol II. ed. by Vigo-Pelfrey C., CRC Press, Boca Raton, 1991, pp. 171—189.
16) Kikugawa K., Kato T., Hayasaka A., *Lipids*, **26**, 922—929 (1991).
17) Kikugawa K., Kato T., Beppu M., Hayasaka A., *Biochim. Biophys. Acta*, **1096**, 108—114 (1991).
18) Miyazawa T., Yasuda K., Fujimoto K., *Anal. Lett.*, **20**, 915—925 (1987).
19) Gardner H. W., Bartelt R. J., Weisleder D., *Lipids*, **27**, 686—689 (1992).
20) Albro P. W., Corbelt J. T., Schroeder L. S., *Lipids*, **22**, 751—756 (1987).
21) Kikugawa K., Kojima T., Yamaki S., Kosugi H., *Anal. Biochem.*, **202**, 249—255 (1992).
22) Inoue T., Ando K., Kikugawa K., *J. Am. Oil Chem. Soc.*, in press.
23) Wills E. D., *Biochem. J.*, **113**, 315—324 (1969).
24) Kikugawa K., Maruyama T., Machida Y., Kurechi T., *Chem. Pharm. Bull.*, **29**, 1423—1432 (1981).
25) Kikugawa K., Machida Y., Kida M., Kurechi T., *Chem. Pharm. Bull.*, **29**, 3003—3011 (1981).
26) Kikugawa K., Ido Y., *Lipids*, **19**, 600—608 (1984).
27) Kikugawa K., Takayanagi K., Watanabe S., *Chem. Pharm. Bull.*, **33**, 5437—5444 (1985).
28) Beppu M., Murakami K., Kikugawa K., *Chem. Pharm. Bull.*, **34**, 781—788 (1986).
29) Beppu M., Fukata Y., Kikugawa K., *Chem. Pharm. Bull.*, **36**, 4519—4526 (1988).
30) Itakura K., Uchida K., Osawa T., *Chem. Phys. Lipids*, **84**, 75—79 (1996).
31) Kikugawa K., Ido Y., Mikami A., *J. Am. Oil Chem. Soc.*, **61**, 1574—1578 (1984).
32) Nair V., Offerman R. J., Turner G. A., *J. Am. Chem. Soc.*, **108**, 8283—8285 (1986).
33) Nair V., Offerman R. J., Turner G. A., Pryor A. N., Baenziger N. C., *Tetrahedron*, **44**, 2793—2803 (1988).
34) Xu D., Thiele G. M., Kearley M. L., Haugen M. D., Klassen L. W., Sorrell M. F., Tuma D. J., *Chem. Res. Toxicol.*, **10**, 978—985 (1997).
35) Kikugawa K., Nakahara T., Sakurai K., *Chem. Pharm. Bull.*, **35**, 4656—4660 (1987).
36) Kikugawa K., Sawamura A., *J. Am. Oil Chem. Soc.*, **64**, 1156—1162 (1987).
37) Kikugawa K., Iwata A., Beppu M., *Chem. Pharm. Bull.*, **36**, 685—692 (1988).
38) Kikugawa K., Kato T., Iwata A., Hayasaka A., *Chem. Pharm. Bull.*, **37**, 3061—3065 (1989).
39) Kikugawa K., Kato T., Iwata A., *Lipids*, **24**, 962—969 (1989).
40) Yamaki S., Kato T., Kikugawa K., *Chem. Pharm. Bull.*, **40**, 2138—2142 (1992).
41) Jurgens G., Lang J., Esterbauer H., *Biochim. Biophys. Acta*, **875**, 103—114 (1986).