Characteristics of Fluorescence Formed by the Reaction of Proteins with Unsaturated Aldehydes, Possible Degradation Products of Lipid Radicals

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Blue fluorescence with maxima at 350—370/420—440 nm produced by the non-radical reaction of amino groups of proteins with lipid peroxy (or alkoxy) radicals has been suggested to be derived from aldehydes other than malonaldehyde. Characteristics of fluorescence produced in the reaction of amino acids with aldehydes depended on the α- or ε-amino groups and on the unsaturation of the aldehydes. Unsaturated aldehydes, 2-hexenal and 2-octenal, produced fluorescence on ε-amino groups whose maxima at 360—380/430—460 nm were close to those produced by lipid radicals. Intensities of the fluorescence from lipid radicals and unsaturated aldehydes were similarly decreased in an alkaline solution and on borohydride treatment. The fluorescence formation from unsaturated aldehydes was enhanced by organic hydroperoxides without affecting the chromatographic profiles of the fluorescence. In the reaction of lipid radicals, it is suggested that lipid hydroperoxides from the radicals enhance the fluorescence formation from their degradation products, unsaturated aldehydes. Lipid radicals and 2-octenal formed cross-links in protein accompanying a similar decrease in lysyl and histidyl residues. Unsaturated aldehydes may be major causative molecules for protein damage by lipid radicals.

Keywords blue fluorescence; cross-linking; unsaturated aldehyde; lipid peroxy radical; lipid alkoxy radical; amino acid; protein

Lipid radicals generated during lipid peroxidation induce various damages to proteins and amino acids. Degradation of amino acids,[1,2] generation of carbon-centered radicals,[2] formation of blue fluorescence[3,4] and protein cross-linking[3,4] have been demonstrated. Attention has been paid to the formation of blue fluorescence, since *in vitro* lipid peroxidation of biological samples gives rise to the accumulation of blue fluorescence,[5,6] which has become an index for the lipid peroxidation of tissue.[7-9]

Peroxidation of lipids generates lipid peroxy (or alkoxy) radicals as unstable species, which are converted into long-lived aldehydes.[10] We have shown previously that the radical species produce blue fluorescence and cross-link in proteins in two ways: one is a radical reaction with tyrosyl residues leading to the formation of fluorescent and cross-linkable dityrosine, and another is a non-radical reaction with amino groups.[11-13] Aldehydes are possible candidates for the non-radical reactions. Malonaldehyde once received attention as a causative molecule in the non-radical reaction,[14] but its contribution has been found to be small.[3,4] Fragmentary studies of aldehydes other than malonaldehyde reacting with amino-containing compounds giving blue fluorescence[15-25] have suggested the contribution of the aldehydes to the non-radical fluorescence formation induced by lipid radicals.

This paper deals with the characteristics of fluorescence formed in the reaction of amino acids and proteins with unsaturated aldehydes. It was found that unsaturated aldehydes may contribute to the non-radical fluorescence formation in proteins induced by peroxidized lipids.

Experimental

Materials 2-*trans*-Hexenal (hexenal) and 2,4-*trans*-hexadienal (hexadienal) were obtained from Tokyo Kasei Kogyo Company (Tokyo, Japan). Hexanal, glycine and glycine ethyl ester hydrochloride were obtained from Wako Pure Chemical Industries (Osaka, Japan). 2-*trans*-Heptenal (heptenal) and 2-*trans*-octenal (octenal) were obtained from Aldrich Chemical Company (Milwaukee, WI). Bovine serum albumin (BSA), α-crystallin from bovine eye lens, poly-DL-lysine (polylysine), methemoglobin (MetHb), *tert*-butyl hydroperoxide (*tert*-BuOOH) and cumene hydroperoxide (CuOOH) were obtained from Sigma Chemical Company (St. Louis, MO). Nα-carbobenzoxylysine (Nα-Z-lysine) was a product of Fluka Chemie AG (Switzerland).

Linoleic acid 13-monohydroperoxide (LOOH) was prepared by use of linoleic acid (Nippon Oil and Fats Company, Tokyo, Japan) and lipoxygenase type I from soybeans [EC 1.13.11.12] (Sigma Chemical Company) according to the method of Gardner.[26] The purity of the hydroperoxide was 95—105% when estimated on the basis of its molecular extinction coefficient at 234 nm: 24500[27] and 97—106% on the basis of its calculated peroxide value: 6400 meq/kg. Methyl linoleate 13-monohydroperoxide (MLOOH) was prepared from LOOH by methylation using diazomethane.[12,13] The purity of the product was 94% based on its theoretical peroxide value: 6140 meq/kg.

Phosphatidylcholine hydroperoxide (PCOOH) was prepared from egg yolk phosphatidylcholine (PC) (Coatsome NC-10, Nippon Oil and Fats Company) according to the method of Miyazawa *et al.*[28] with slight modifications.[12,13] The product showed an absorption maximum at 233 nm in ethanol, and a peroxide value of 2600—3200 meq/kg.

Analysis Fluorescence spectra were measured on a Hitachi 650-60 or 650-40 fluorescence spectrophotometer (Tokyo, Japan). The instrument was standardized with 0.1 μM quinine sulfate in 0.1 N sulfuric acid to give a fluorescence intensity of 1.00 at 450 nm when excited at 350 nm. Fluorescence intensities relative to that of 0.1 μM quinine sulfate (RFI) were shown. High-performance liquid chromatography (HPLC) was carried out using a Hitachi 655 liquid chromatograph (Tokyo, Japan) equipped with a YMC A-303 ODS column (4.6 mm i.d. × 250 mm; Yamamura Chemical Laboratories, Kyoto, Japan). The column was equilibrated and eluted with methanol/0.1 M ammonium formate (7:3 or 6:4, v/v) at a flow rate of 0.6 ml/min. The fluorescent peak was detected at 430 nm when excited at 360 nm by use of a Shimadzu fluorescence spectromonitor RF-530 (Tokyo, Japan). Amino acid analysis was carried out on a Hitachi L-8500 high-performance amino acid analyzer (Tokyo, Japan) equipped with a No. 2620 ion exchanger column (4.6 mm i.d. × 80 mm). The sample was hydrolyzed in 6 M HCl containing 4% thioglycolic acid at 110 °C for 24 h under vacuum, and the mixture was evaporated to dryness for analysis. Common amino acids were detected by ninhydrin coloration. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under reducing conditions using Laemmli's discontinuous buffer systems[29] composed of 4% stacking and 15% separating gel.

Light Irradiation A quartz cuvette containing the sample was placed in a Hitachi 650-60 fluorescence spectrophotometer. Light with all wavelengths between 300 and 600 nm emitted from the xenon lamp attached to the apparatus was irradiated to the cuvette at room temperature for up to 90 min.

Results

When Nα-Z-lysine, in which the α-amino group was

TABLE I. Maximum Wavelength and Intensity of Fluorescence of the Reaction Mixtures of Amino Acids and Proteins with Aldehydes

Aldehyde or lipid radical	Maximum wavelength (nm) of excitation (Ex) and emission (Em), and relative fluorescence intensity (RFI) of the reaction mixture																	
	Glycine[a]			Glycine ethyl[a]			Nα-Z-Lysine[b]			BSA[c]			Polylysine[c]			α-Crystallin[d]		
	Ex	Em	RFI	Ex	Em	RFI	Ex	Em	RFI	Ex	Em	RFI	Ex	Em	RFI	Ex	Em	RFI
LOOH							362	433	300							356	430	3.9
MLOOH							361	433	72									
PCOOH							362	432	47									
Hexanal	348	416	101	376	428	104	348	416	40							348	401	1.6
Hexenal	453	494	553	392	455	1235	375	453	230	374	442	0.75	360	438	1.9			
Heptenal	468	514	437	390	454	794 (1)												
				470	510	686 (2)												
Octenal	468	516	371	391	454	832 (1)	372	453	440	375	434	1.00				376	441	2.9
				469	508	728 (2)												
Hexadienal	397	470	443 (1)	426	505	335 (1)	380	434	600									
	469	514	523 (2)	472	526	394 (2)												

a) A mixture of 100 mM amino acid and 200 mM aldehyde in 70% methanol/0.1 M phosphate buffer (pH 7.0), *b*) a mixture of 100 mM Nα-Z-lysine and 200 mM aldehyde in 80% methanol/0.1 M phosphate buffer (pH 7.0) or 125 mM Nα-Z-lysine and 6.25 mM lipid hydroperoxide/11 μM MetHb in 66% acetonitrile/0.1 M phosphate buffer (pH 7.0), *c*) a mixture of 1 mg/ml BSA or polylysine and 100 mM aldehyde in 5% methanol/0.1 M phosphate buffer (pH 7.0), and *d*) a mixture of 5 mg/ml α-crystallin and 10 mM aldehyde or 10 mM LOOH/1.3 μM MetHb in 5% methanol/0.1 M phosphate buffer (pH 7.5), were incubated at 37 °C for 24 h. Fluorescence spectra of the reaction mixtures were recorded after dilution into the solvent used for the reaction.

blocked and the ε-amino group was free, and bovine eye lens α-crystallin were incubated at pH 7 or 7.5 at 37 °C for 24 h with lipid peroxy (or alkoxy) radicals generated from LOOH, MLOOH and PCOOH by interaction with MetHb,[30] blue fluorescence with maxima at 350—370 (excitation)/420—440 (emission) nm was produced (Table I). The maximum wavelengths of the fluorescence were similar to those already reported for the reaction of amino acids and proteins with lipid hydroperoxides or radicals.[11-13,15-17,31-39] The reaction of glycine, glycine ethyl ester, Nα-Z-lysine and α-crystallin with saturated aldehyde hexanal gave fluorescence, but the maximum wavelengths of 340—380/400—430 nm were rather shorter. The reaction of glycine and glycine ethyl ester with unsaturated aldehydes hexenal, heptenal, octenal and hexadienal produced fluorescence with maxima at the longer wavelengths of 390—470/470—530 nm. Some of these reactions produced fluorescence with two excitation and emission maxima in these ranges. The reaction of Nα-Z-lysine with unsaturated aldehydes hexenal, octenal and hexadienal produced fluorescence with maxima at 360—380/430—460 nm, which were close to those of the fluorescence from the reaction with the lipid radicals. The reaction of BSA, polylysine and α-crystallin with the unsaturated aldehydes hexenal and octenal gave fluorescence with maxima at 360—380/430—450 nm, which were similar to those produced by the reaction with the lipid radicals. The results indicate that the maximum wavelengths of fluorescence produced by the reaction of amino groups with aldehydes greatly depends on the α- and ε-amino groups and on the unsaturation of the aldehydes. Maximum wavelengths of the fluorescence produced by the reaction of ε-amino groups with unsaturated aldehydes were close to those produced by the reaction with the lipid radicals.

Fluorescence intensities of the reaction mixtures of Nα-Z-lysine with the lipid radicals from LOOH, MLOOH and PCOOH were extensively decreased in an alkaline solution and on borohydride treatment (Table II), which is consistent with earlier observations.[16,38,39] The decreased intensity in the alkaline solution was recovered at pH 7.

TABLE II. Characteristics of the Fluorescence Formed in the Reaction of Nα-Z-Lysine with Aldehydes

Aldehyde or lipid radical	% fluorescence intensity at the maximum emission of the reaction mixture			
	0.1 M phosphate (pH 7.0)	0.1 N HCl	0.1 N NaOH	0.1 M phosphate (pH 7.0) after treatment with 0.2 M sodium borohydride
LOOH[a]	100	94	12[c]	11
MLOOH[a]	100			17
PCOOH[a]	100			19
Hexanal[b]	100	100	50[d]	75
Octenal[b]	100	78	47[c]	34
Octenal + *tert*-BuOOH[b]	100	86	32[c]	56

a) A mixture of 125 mM Nα-Z-lysine and 6.25 mM lipid hydroperoxide/11 μM MetHb in 66% acetonitrile/0.1 M phosphate buffer (pH 7.0) was incubated at 37 °C for 20 h. *b*) A mixture of 20 mM Nα-Z-lysine and 40 mM aldehyde with or without 40 mM *tert*-BuOOH in 80% methanol/0.1 M phosphate buffer (pH 7.0) was incubated at 37 °C for 48 h. *c*) Fluorescence intensity was recovered at pH 7. *d*) Fluorescence intensity was not recovered at pH 7.

Fluorescence intensity of the reaction mixture of Nα-Z-lysine with hexanal was decreased to one half in the alkaline solution and only slightly on borohydride treatment, and the decreased intensity in the alkaline solution was not recovered at pH 7. Fluorescence intensities of the reaction mixtures of Nα-Z-lysine with octenal were similarly decreased in the alkaline solution and even more decreased on borohydride treatment, and the decrease by alkali was recovered at pH 7. The characteristics of fluorescence from octenal were similar to those of the fluorescence from the lipid radicals.

It was found that organic hydroperoxides affected the fluorescence formation in the reaction of amino acids and proteins with aldehydes. When various amounts of *tert*-BuOOH were added to the reaction of Nα-Z-lysine with octenal (Fig. 1A), the formation of fluorescence was enhanced at every reaction period up to 96 h. About a two-

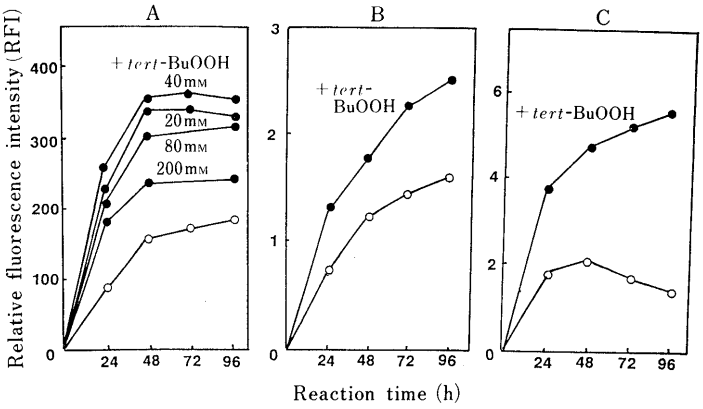

Fig. 1. Time Course of Blue Fluorescence Formation in the Reaction of Nα-Z-Lysine with Octenal (A), BSA with Hexenal (B) and Polylysine with Hexenal (C) in the Presence of *tert*-BuOOH

A: A mixture of 20 mM Nα-Z-lysine, 40 mM octenal and *tert*-BuOOH at the indicated concentration in 80% methanol/0.1 M phosphate buffer (pH 7.0); B: a mixture of 1.0 mg/ml BSA, 100 mM hexenal and 100 mM *tert*-BuOOH in 5% methanol/0.1 M phosphate buffer (pH 7.0); and C: a mixture of 1.0 mg/ml polylysine, 100 mM hexenal and 100 mM *tert*-BuOOH in 5% methanol/0.1 M phosphate buffer (pH 7.0) was incubated at 37 °C. After dilution into the solvent employed for the reaction, RFI at each maximum was measured. Control mixtures with octenal, hexenal or *tert*-BuOOH alone did not show any significant fluorescence during the incubation periods.

TABLE III. Effect of *tert*-BuOOH and CuOOH on the Fluorescence Formation in the Reaction of Amino Acids, BSA and Polylysine with Aldehydes

Reaction mixture	% fluorescence intensity of the reaction mixture		
	None	+*tert*-BuOOH	+CuOOH
Hexanal+ glycine	100	77	65
Hexenal+ glycine	100	241	268
Nα-Z-lysine	100	208	283
BSA	100	173	
polylysine	100	200	
Heptenal+ glycine	100	330	341
Octenal+ glycine	100	328	319
Nα-Z-lysine	100	162	
Hexadienal+ glycine	100 (1)	129	136
	100 (2)	133	139

The reaction conditions were the same as those in Table I: *a*) for glycine, *b*) for Nα-Z-lysine and *c*) for BSA and polylysine. 200 mM *tert*-BuOOH or CuOOH was introduced in the reaction of glycine and Nα-Z-lysine, and 100 mM *tert*-BuOOH was introduced in the reaction of BSA and polylysine. Fluorescence spectra of the reaction mixtures with *tert*-BuOOH and CuOOH were similar to those without the organic hydroperoxides.

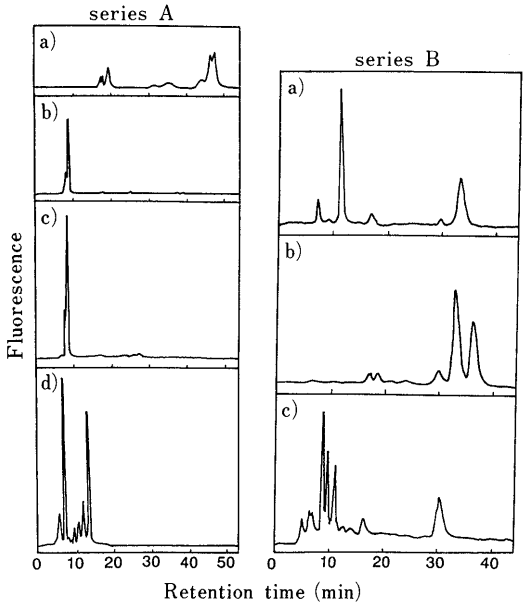

Fig. 2. HPLC of the Fluorescence Formed in the Reaction of Nα-Z-Lysine with Aldehydes or Lipid Radicals

The reaction mixtures shown in Table III were subjected to HPLC with a YMC A-303 ODS column. Elution was carried out using methanol/0.1 M ammonium formate (7 : 3, v/v) (series A) and methanol/0.1 M ammonium formate (6 : 4, v/v) (series B) at a flow rate of 0.6 min. The peaks were detected at 360/430 nm. Series A: a) hexanal; b) octenal; c) octenal+*tert*-BuOOH; d) LOOH. Series B: a) LOOH; b) MLOOH; c) PCOOH.

fold increase was obtained at the reactant ratio of 1 : 2 : 2 (Nα-Z-lysine–octenal–*tert*-BuOOH), the increase being lowered by the addition of higher amounts of *tert*-BuOOH. Fluorescence produced in the presence of *tert*-BuOOH was decreased in alkali and on borohydride treatment, similar to that produced in the absence of the hydroperoxide (Table II). The fluorescence formation in the reaction of BSA (Fig. 1B) and polylysine (Fig. 1C) with hexenal was similarly enhanced by *tert*-BuOOH. The fluorescence formation in the reaction with unsaturated aldehydes hexenal, heptenal, octenal and hexadienal was similarly enhanced by organic hydroperoxides *tert*-BuOOH and CuOOH, whereas fluorescence formation in the reaction with saturated aldehyde hexanal was decreased (Table III). The reasons for the enhancing effect of the organic hydroperoxides were not known.

HPLC profiles of the fluorescent compounds produced in the reaction of Nα-Z-lysine with hexanal, octenal and the lipid radicals from LOOH were different, indicating the formation of fluorescent compounds with different structures (Fig. 2, series A). HPLC profiles of the reaction with octenal and octenal/*tert*-BuOOH were similar, indicating that *tert*-BuOOH enhanced the formation of fluorescent compounds from the aldehyde. HPLC profiles of the fluorescent compounds from the lipid radicals due to LOOH, MLOOH and PCOOH were different, indicating the formation of fluorescent compounds with different structures (Fig. 2, series B). The results indicate that the fluorescent compounds from aldehydes and lipid radicals were not uniform but diverse.

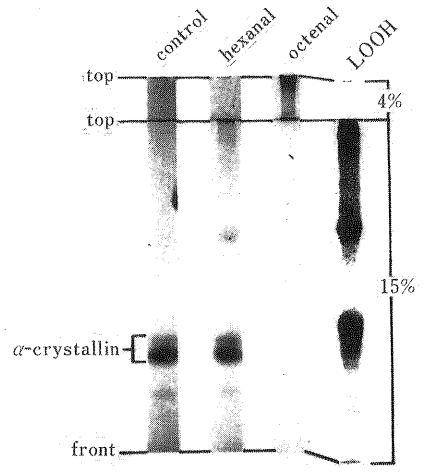

Fig. 3. SDS-PAGE of the α-Crystallin Modified with Hexanal, Octenal and LOOH/MetHb

A mixture of 5 mg/ml α-crystallin and 10 mM hexanal, 10 mM octenal or 10 mM LOOH/1.3 μM MetHb in 5% methanol/0.1 M phosphate buffer (pH 7.5) was incubated at 37 °C for 24 h. The reaction mixture was treated with 50 mM sodium borohydride, dialyzed against water and lyophilized. The protein was redissolved into 1% SDS solution for analysis on SDS-PAGE with 4% stacking and 15% separating gel under reducing conditions.

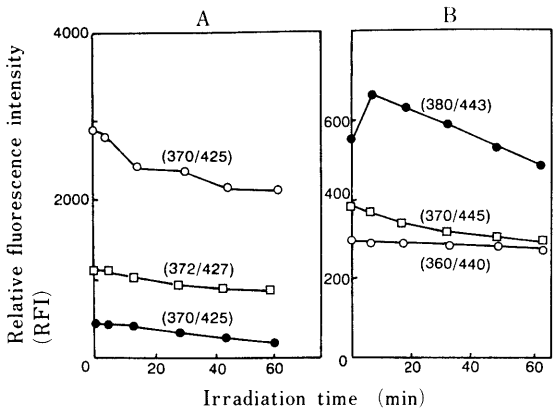

Fig. 4. Time Course of the Light-Induced Change of Blue Fluorescence Intensities of the Reaction Mixtures and Their Chloroform/Methanol Extracts

A mixture of 125 mM Nα-Z-lysine with 6.6 mM LOOH/12 μM MetHb (A) and a mixture of 20 mM Nα-Z-lysine with 40 mM octenal (B) in 70% methanol/0.1 M phosphate buffer (pH 7.0) were incubated at 37 °C for 48 h (○). A 5.0 ml aliquot of the mixture was extracted with 7.0 ml chloroform to give 9.1 ml chloroform/methanol extract (●), the extract being evaporated at 40 °C *in vacuo* to dryness and redissolved into 5.0 ml of a 70% methanol/0.1 M phosphate buffer (pH 7.0) (□). The reaction mixture and the redissolved solution were diluted into a 70% methanol/0.1 M phosphate buffer (pH 7.0), and chloroform/methanol extract was diluted into chloroform. They were irradiated during the indicated periods. RFI at maximum fluorescence wavelength in parenthesis (excitation/emission nm) was calculated on the basis of the initial volume of the reaction mixture. The control reaction mixture with each reactant alone produced fluorescence at only less than 2 RFI throughout the experiments.

Bovine eye lens α-crystallin modified with the lipid radicals from LOOH, hexanal and octenal was treated with borohydride to analyze cross-linking and amino acid damage. Non-disulfide cross-linked high-molecular-weight polymers appeared in each modified protein in SDS-PAGE analysis under reducing conditions (Fig. 3). The amounts of non-cross-linked protein remaining in the modification with the lipid radical and octenal were much smaller than that in the modification with hexanal. The lipid radical-modified protein showed a decrease in lysyl (15%) and histidyl (35%) residues. The octenal-modified protein showed a decrease in lysyl (45%) and histidyl (60%) residues. The hexanal-modified protein showed decrease only in lysyl (45%) residue. The lipid radicals and octenal similarly cross-linked protein accompanying the damage of common amino acids lysine and histidine. No apparent differences between the modifications with the lipid radicals and octenal were obtained.

Fletcher *et al.*[7,8] have proposed a method to measure lipid peroxidation of tissues by blue fluorescence, in which fluorescence is extracted with chloroform/methanol (2 : 1) and the extract is irradiated to eliminate disturbing retinol. Extractability and stability against irradiation of the fluorescent compounds obtained in the present study were investigated (Fig. 4). When the reaction mixture of Nα-Z-lysine with the lipid radicals from LOOH (Fig. 4A), and the mixture of Nα-Z-lysine with octenal (Fig. 4B) were diluted into the solvents employed for the reaction and irradiated for 60 min, the fluorescence decreased slightly during the irradiation (open circles). When the mixture of Nα-Z-lysine with the lipid radical was extracted with chloroform/methanol, the fluorescence intensity of the extract was reduced to 17% that of the reaction mixture, indicating that not all of the fluorescent compounds were extracted or the fluorescence was quenched by chloroform (Fig. 4A). Irradiation of the extract slightly decreased the intensity (closed circles). When the mixtures of Nα-Z-lysine with octenal were extracted with the solvent, the fluorescence intensity increased, but the increase was cancelled after evaporation of the extract and redissolution into the initial solvent (Fig. 4B). In this case, chloroform enhanced the fluorescence intensity of the fluorescent compounds. The fluorescence intensity of the extract was markedly increased by the short time irradiation, but it was gradually decreased by the longer time irradiation (closed circles). The fluorescence from BSA and α-crystallin treated with octenal was not extracted with the solvent (data not shown). The results demonstrated that chloroform/methanol extraction and subsequent irradiation may mislead the estimation of fluorescence induced by lipid peroxidation.

Discussion

Attention has been paid to the formation of blue fluorescence in the reaction of proteins with peroxidized lipids because blue fluorescence is regarded as an index for lipid peroxidation of biological samples.[7-9] The contribution of malonaldehyde in peroxidized lipids to fluorescence formation, a long-held belief,[14] has been brushed aside.[3,4] The radical reaction of peroxidized lipids may lead to a conversion of tyrosyl residue into fluorescent and cross-linkable dityrosine, and a part of the fluorescence formed may be accounted for by the formation of dityrosine.[11-13] However, the content of dityrosine showing maxima at 320/410 nm in proteins treated with lipid radicals is small. It was found in the present study that unsaturated aldehyde species[10] are likely degradation products of peroxidized lipids to participate in non-radical fluorescence formation on amino groups of proteins. Unsaturated aldehydes produced fluorescence on ε-amino groups, whose maxima 360—380/430—460 nm were close to those 350—370/420—440 nm formed by lipid radicals.

In addition to fluorescence formed by lipid radicals, fluorescence formed by unsaturated aldehydes was markedly reduced in alkaline solutions and on borohydride treatment.

It is interesting to note that fluorescence formation in the reaction of unsaturated aldehydes with amino groups was dramatically enhanced by organic hydroperoxides. This enhancement may not be due to the formation of new fluorescent compounds, but to the stimulation of the formation of the same fluorescent compounds. This result suggests that the fluorescence formation from lipid radicals depends on their degradation products, unsaturated aldehydes, and also on the lipid hydroperoxides formed from the lipid radicals.

In the modification of α-crystallin with lipid radicals, extensive cross-linking was observed. This cross-linking was suggested to be due partly to the radical reaction leading to the formation of dityrosine[11-13] and mainly to the non-radical reaction of amino groups.[16] Saturated aldehyde hexanal produced cross-linking but gave damage to lysyl residue alone, and the cross-linking may be produced by the reaction of ε-amino groups of lysyl residue and a dialdehyde formed by self-condensation of hexanal as has been shown.[24] Unsaturated aldehyde octenal produced extensive cross-linking and damaged both lysyl and histidyl residues. The cross-linking may be due to the reaction of ε-amino groups of lysyl residue and a dialdehyde formed by self-condensation of the aldehyde. The characteristics of cross-linking and amino acid damage by octenal were similar to those by lipid radicals. It is likely that the cross-linking and amino acid damage by lipid radicals and octenal are due to a common mechanism.

The fluorometric assay of *in vivo* lipid peroxidation devised by Fletcher *et al.*[7,8] is based on the chloroform/methanol extraction of fluorescence to eliminate water-soluble fluorescent flavin proteins and irradiation of the extract to eliminate organic solvent-soluble fluorescent retinol. Tsuchida *et al.*,[9] however, pointed out that blue fluorescence is not always obtained from tissues by the solvent extraction, and they recommended the assay of water-soluble blue fluorescence as an index of lipid peroxidation. Shimasaki *et al.*[35] showed that blue fluorescence produced in the reaction of proteins with peroxidized lipids is water-soluble and not extracted in the organic solvent system. It was found in the present study that blue fluorescence formed by lipid radicals and unsaturated aldehydes was not always extracted by the organic solvent system. Furthermore, irradiation of the extract greatly affected the intensity of the fluorescence. Chloroform/methanol extraction and subsequent irradiation may not be suitable for the fluorometric assay of lipid peroxidation.

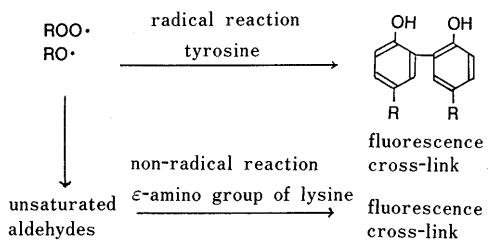

Chart 1. Possible Pathway of the Formation of Fluorescence and Cross-Link in Proteins Treated with Lipid Peroxy (or Alkoxy) Radicals

In conclusion, unsaturated aldehydes may be major causative molecules for protein damage in reaction with peroxidized lipids. The formation of fluorescence and cross-linking by lipid radicals may be due partly to radical-induced dityrosine formation and mainly to the non-radical reaction of unsaturated aldehyde species (Chart 1).

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