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Studies on Peroxidized Lipids: Fluorescent Products Derived from the Reaction of Unsaturated Fatty Acids and Methylamine

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Unsaturated fatty acids such as linoleic, linolenic and arachidonic acids produced a complex mixture of fluorescent substances with an excitation maximum at 355—370 nm and an emission maximum at 420—440 nm by reaction with methylamine at pH 7.5 and 37 °C. The excitation and emission maxima of the products shifted to higher wavelength with increasing unsaturation of the fatty acid, and were similar to those of lipofuscin pigments. The fluorescence characteristics of the products indicated that they were different in structure or composition with different fatty acids, and the major fluorescent products were neither 1-substituted-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes [Kikugawa *et al.*, *Chem. Pharm. Bull.*, **29**, 1423 (1981)] nor the conjugated Schiff bases [Chio and Tappel, *Biochemistry*, **8**, 2821 (1969)] which were produced by reaction of malonaldehyde (MDA) with primary amines. Both thin-layer and high performance liquid chromatographies suggested that thiobarbituric acid-reactive substances other than MDA contributed to the formation of the fluorescent substances.

Keywords——linoleic acid; linolenic acid; arachidonic acid; linoleic acid hydroperoxide; malonaldehyde; 1-substituted-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde; conjugated Schiff base; peroxidation; thiobarbituric acid-reactive substance; lipofuscin pigment

A number of studies have demonstrated that aging tissues contain fluorescent pigments, so-called lipofuscin or ceroid pigments.[1,2] Unsaturated fatty acids are readily peroxidized to produce hydroperoxides which are in turn degraded into a complex mixture of secondary products including malonaldehyde (MDA). Tappel and his associates[3–5] suggested that these fluorescent pigments were the conjugated Schiff bases between MDA and the amino groups of proteins or phospholipids. However, our recent studies demonstrated that the reaction of MDA with primary amines under neutral conditions produced strongly fluorescent 1,4-dihydropyridines instead of the conjugated Schiff bases[6,7] (Chart 1).

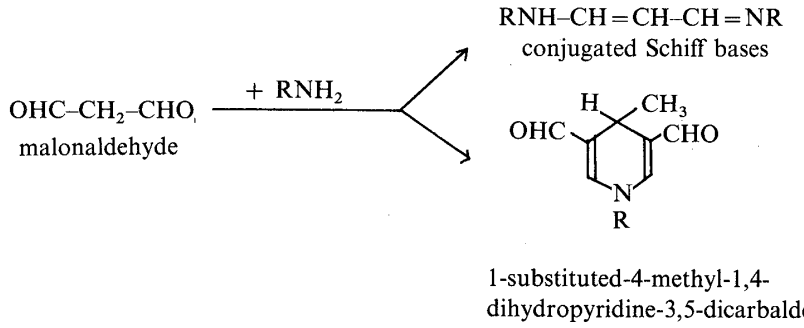

Chart 1

A few chemical studies on the formation of fluorescent pigments from unsaturated fatty acid methyl esters and primary amines have appeared.[8–12] Shimasaki *et al.*[8] reported that the

formation of fluorescent pigments by reaction of methyl linoleate with glycine was due to thiobarbituric acid (TBA)-reactive substances, and the pigments were assumed to be the conjugated Schiff bases between MDA and glycine. Studies of the reaction of methyl linolenate with primary amines suggested that fluorescent pigments were produced from secondary products other than MDA.[10-12] We report here systematic studies on the formation of fluorescent substances by reaction of free unstaturated fatty acids with methylamine, and comparisons of the characteristics of the fluorescent products derived from the fatty acids. The participation of MDA and other TBA-reactive substances in the formation of the fluorescent products is discussed.

Experimental

Materials——Oleic acid (OA), linoleic acid (LA) (Grade III), linolenic acid (LNA) and arachidonic acid (AA) (Grade I) were obtained from Sigma Chemical Company. Linoleic acid hydroperoxide (LOOH) was prepared according to the method previously described[13] and was purified by use of a column of silicic acid.[14] The peroxide value of LOOH thus prepared was 6300 meq/kg (the theoretical value is 6400 meq/kg), and the purity based on a molecular extinction coefficient of ε_{233}: 25300[13] was 85%. TBA and sodium dodecyl sulfate (SDS) were obtained from Wako Pure Chemical Industries, Ltd. Malonaldehyde bis(dimethylacetal) was a product of Aldrich Chemical Company. Butylated hydroxytoluene (BHT) was obtained from Nikki-Universal Company, and was recrystallized from ethanol for use.

Analytical Methods——Absorption spectra were measured with a Hitachi recording spectrophotometer, model 323. Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, model 204, and the relative fluorescence intensity was expressed as a percentage of the intensity of 0.1 μM quinine sulfate.

Thin-layer chromatography was performed on Merck silica gel 5721 with a chloroform–methanol–glacial acetic acid (90 : 30 : 1) solvent system. The spots were extracted with 30 ml of a mixture of chloroform–methanol (1 : 1). The extract was evaporated to a small volume and passed through a column of Sephadex LH 20 (1 × 10 cm) to obtain the fluorescence spectrum. High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-4A liquid chromatograph fitted with a stainless steel column (4 × 250 cm) packed with Lichrosorb RP-18 and a Shimadzu chromatopac C-R2AX. Elution was carried out with methanol–glacial acetic acid–water (50 : 1 : 49) at a flow rate of 1.0 ml/min and at 30 °C. The fluorescent peaks were detected with a Shimadzu fluorescence spectromonitor, model RF-500LC.

The TBA test was performed by the reported method.[15] A 0.6 ml portion of the reaction mixture was mixed with 1.0 ml of 20 mM TBA solution and 1.0 ml of glacial acetic acid, and the mixture was heated at 100 °C for 20 min. After cooling, the reaction mixture was made up to 10 ml with water or methanol to measure the absorbance. A reference standard solution of 1.0 mM malonaldehyde bis(dimethylacetal) was similarly treated.

Reaction of Unsaturated Fatty Acids with Methylamine——A mixture of 0.20 mmol of an unsaturated fatty acid (OA, LA, LNA or AA) and 30 mmol of methylamine hydrochloride in 100 ml of a mixture of 0.1 M phosphate buffer (pH 7.5)–methanol (8 : 2) containing 2 mM SDS was incubated at 37 °C for 120 h with frequent mixing in a Vortex mixer. A mixture of 0.20 mmol of LOOH sodium salt and 40 mmol of methylamine hydrochloride in 100 ml of 0.1 M phosphate buffer (pH 7.5) containing 2 mM SDS was similarly treated. Control experiments without methylamine were carried out simultaneously. Each reaction mixture was almost clear at the start of the reaction, but it gradually produced a small amount of particulate matter which was centrifuged off before spectral estimation. Ultraviolet (UV) and fluorescence spectra were taken after dilution of the reaction mixture with methanol.

Results

A solution containing 2 mM LA was incubated with or without methylamine at pH 7.5 and 37 °C for 120 h. Hydroperoxide produced in the mixtures was estimated by measurement of absorption due to conjugated diene at 233 nm. The hydroperoxide level of the control started to increase at 25 h and reached maximum (0.8 mM) after 90 h of incubation (Fig. 1A). The increase was markedly retarded by a large excess of methylamine; this result can be interpreted in terms of the well-known inhibitory property of amines on lipid peroxidation.[16] TBA-reactive substances in the control exhibited two characteristic maxima at 532 and 455 nm, while standard MDA exhibited an absorption maximum at 532 nm (Fig. 2). TBA-color with a maximum at 532 nm may be due to hydroperoxide[17-19] and secondary products

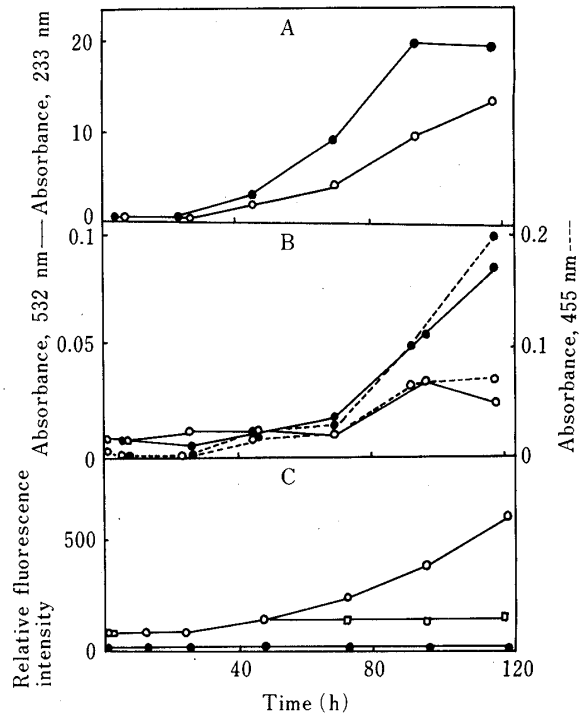

Fig. 1. Time Course of the Derivatization of LA by Incubation with or without Methylamine

A, formation of hydroperoxide; B, formation of TBA-reactive substances; C, formation of fluorescent substances. A solution containing 2 mM LA was incubated: control (●), in the presence of 300 mM methylamine (○) and in the presence of 300 mM methylamine and 0.2 mM BHT (□).

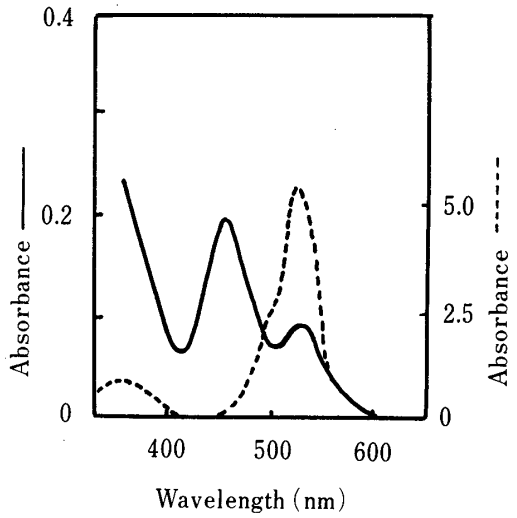

Fig. 2. Absorption Spectrum of a TBA-Treated Mixture of LA Incubated for 120 h

The dotted line indicates the spectrum of TBA-treated 1 mM malonaldehyde bis(dimethylacetal).

TABLE I. Fluorescence Spectra and Intensity of the Fluorescent Substances Produced by Reaction of Unsaturated Fatty Acids with Methylamine

	λ^{ex}_{max} (nm)	λ^{em}_{max} (nm)	Relative fluorescence intensity
Reaction mixture[a]			
LA	360	420	710
LOOH	360	420	16000
OA	—	—	0
LNA	370	420	360
AA	370	440	1530
Standard			
MDD[b]	397	455	

a) A solution containing 2 mM fatty acid was incubated with or without 300 or 400 mM methylamine for 120 h.
b) 1,4-Dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde.

such as 2-alkenals, 2,4-alkadienals,[20] cyclic peroxides[21] or MDA,[22] and the color with a maximum at 455 nm to alkanals[20] or cyclic peroxides.[21] The time course of the increase in the absorbance at 532 and 455 nm of the control (Fig. 1B) showed that TBA-reactive substances were produced after a considerable amount of hydroperoxide had been formed. TBA-values expressed in terms of absorbance were 0.09 at 532 nm and 0.2 at 455 nm after 120 h. The increase in the absorbance was retarded to about 30% by a large amount of methylamine.

This reduction may be due to the inhibition of peroxidation and/or to the derivatization of the substances into non-reactive substances.

While no fluorescence was produced in the control, fluorescence was gradually produced after 70 h in the mixture with methylamine (Fig. 1C). The fluorescence spectrum exhibited an excitation maximum at 360 nm and an emission maximum at 420 nm, and the intensity after 120 h was 710% with respect to quinine sulfate (Table I). The level of fluorescent substances produced may be low since there was no significant increase in absorbance in the region of 260—400 nm. The formation of fluorescence was inhibited by butylated hydroxytoluene (BHT), indicating that it was linked with peroxidation of LA.

In order to obtain further information, a solution containing 2 mM LOOH was similarly incubated with or without methylamine. While the absorbance at 233 nm of the control decreased gradually and the final level of absorbance was higher (Fig. 3A). Absorbance at 280 nm of the mixture increased to a higher level than that of the control, indicating that other substances were formed in the mixture. TBA-coloration of the control revealed two absorption maxima at 532 and 455 nm. The absorbance at 532 nm rapidly increased and then gradually decreased, and the absorbance at 455 nm gradually decreased during the incubation (Fig. 3B). The absorbance remained at values higher than 0.1 and 0.2 at 532 and 455 nm, respectively, throughout the reaction. The mixture of LOOH and methylamine gradually produced fluorescence without any delay period (Fig. 3C). The fluorescence spectrum of the mixture was similar to that of the reaction mixture of LA, and the fluorescence intensity after 120 h was much higher than that of the reaction mixture of LA (Table I). Formation of

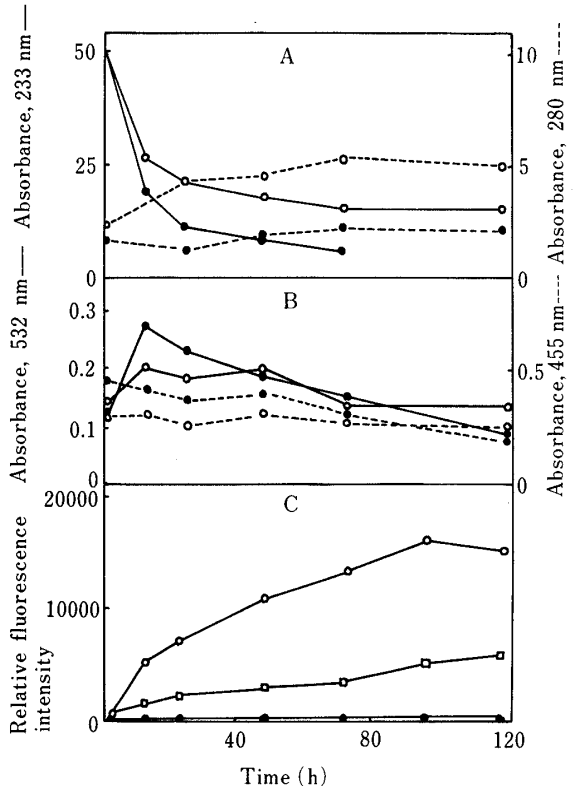

Fig. 3. Time Course of the Degradation of LOOH with or without Methylamine

A, changes in absorbance at 233 and 288 nm; B, changes in TBA-reactive substances; C, formation of fluorescent substances. A solution containing 2 mM LOOH was incubated: control (●), in the presence of 400 mM methylamine (○) and in the presence of 400 mM methylamine and 0.2 mM BHT (□).

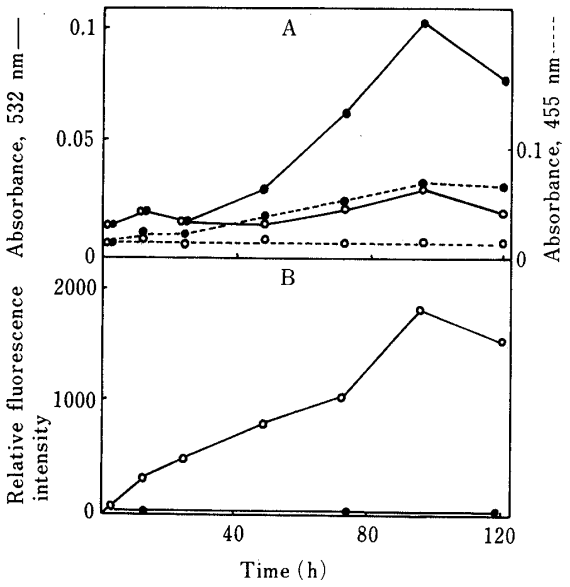

Fig. 4. Time Course of the Derivatization of AA by Incubation with or without Methylamine

A, formation of TBA-reactive substances; B, formation of fluorescent substances. A solution of 2 mM AA was incubated: control (●) and in the presence of 300 mM methylamine (○).

Table II. Effect of Acid, Alkali and Sodium Borohydride on the Fluorescent Intensity of the Reaction Mixtures

	Fluorescence intensity			
	MeOH	MeOH–1 N HCl (9:1)	MeOH–1 N NaOH (9:1)	MeOH–1 M $NaBH_4$ (9:1)
Reaction mixture[a]				
LA	100	90.5	36.2	14.7
LOOH	100	82.9	30.3	20.7
LNA	100	92.8	80.3	78.5
AA	100	87.2	55.4	73.3
Standard				
MDD	100	19.7	100	14.0

a) See footnote to Table I.

fluorescence was inhibited by BHT, indicating that the fluorescent substances were not directly produced from LOOH.

Similar experiments were performed with other unsaturated fatty acids. OA produced no TBA-reactive substances or fluorescent products. LNA produced only a little TBA-reactive substances, and produced fluorescence with much lower intensity than LA (Table I). When AA was incubated, TBA-reactive substances with the absorption maxima at 532 and 455 nm were gradually produced (Fig. 4A), and the absorbance was 0.08 at 532 nm and 0.07 at 455 nm after 120 h. Addition of methylamine markedly retarded TBA-coloration, and produced significant fluorescence without any delay period (Fig. 4B). The fluorescence intensity was higher than that of the reaction mixture of LA (Table I). All these experiments were carried out several times and the results were reproducible. A small amount of particulate matter produced in every incubation mixture was probably due to partial insolubilization of the fatty acid.

The intensity of fluorescence of the reaction mixtures of unsaturated fatty acids with methylamine was in the order of AA > LA > LNA ≫ OA. Excitation and emission maxima of the products shifted slightly to higher wavelength with increasing unsaturation of the fatty acid (Table I), indicating that different kind of fluorescent substances were produced with different fatty acids. The spectrum of each reaction mixture was different from that of 1,4-dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde (MDD) which was obtained by the reaction of MDA and methylamine under similar conditions[6] (Table I). As shown in Table II, the fluorescence intensity of MDD was markedly reduced in the acidic medium and by treatment with sodium borohydride. Though the fluorescence intensity of each reaction mixture was little reduced in the acidic medium, it was decreased in the alkaline medium. The decrease in the alkaline medium was different with different fatty acid reaction mixtures; the intensity of the mixtures of LA and LOOH was decreased to 30—40%, that of LNA to 80% and that of AA to 55%. The effect of treatment with sodium borohydride was also different with different fatty acid reaction mixtures; the intensity of the mixtures of LA and LOOH was decreased to 10—20% and that of LNA and AA to 70—80%. These fluorescence characteristics suggested that the fluorescent substances in mixtures of fatty acids with methylamine were different from those of the other fatty acids, and most of the products were different from MDD.

The reaction mixtures of LA and LOOH showed three major fluorescent spots, and the reaction mixtures of LNA and AA showed two major sports on a thin-layer chromatogram

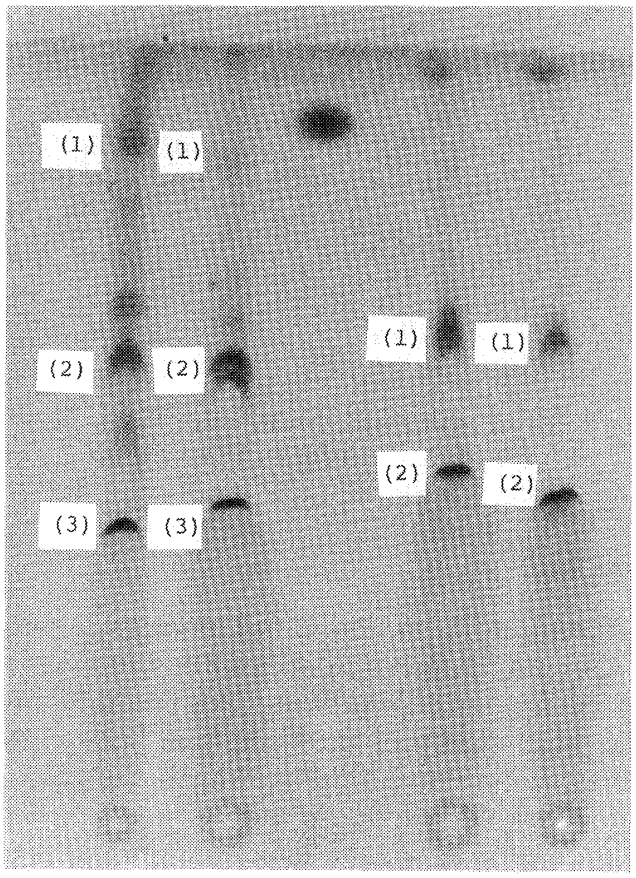

Fig. 5. Thin-Layer Chromatography of the Reaction Mixture of Unsaturated Fatty Acid with Methylamine

Developing solvent: $CHCl_3$–MeOH–AcOH (90:30:1). Fluorescent spots were detected by excitation at 365 nm.

TABLE III. Fluorescence Data for the Extracts of the Thin-Layer Chromatogram Spots

Reaction mixture[a]	Spot number[b]	λ^{ex}_{max} (nm)	λ^{em}_{max} (nm)
LA	1	350	418
	2	363	420
	3	365	413
LOOH	1	353	418
	2	363	425
	3	363	423
LNA	1	365	430
	2	365	430
AA	1	365	433
	2	365	435

a) See footnote to Table I.
b) The spot number is that shown in Fig. 5.

(Fig. 5). The *Rf* values of these major spots were different from that of MDD. Fluorescence spectra of extracts of the major fluorescent spots were similar to that of the reaction mixture (Table III). HPLC of the reaction mixtures of LA and LOOH revealed six fluorescent peaks (Fig. 6). None of these major peaks corresponded to MDD, although a minor fluorescent peak appeared at the same retention time as that of MDD. These results demonstrate that complex mixtures of fluorescent substances were produced in each reaction mixture of fatty acid with methylamine, and that the major fluorescent substances were different from MDD.

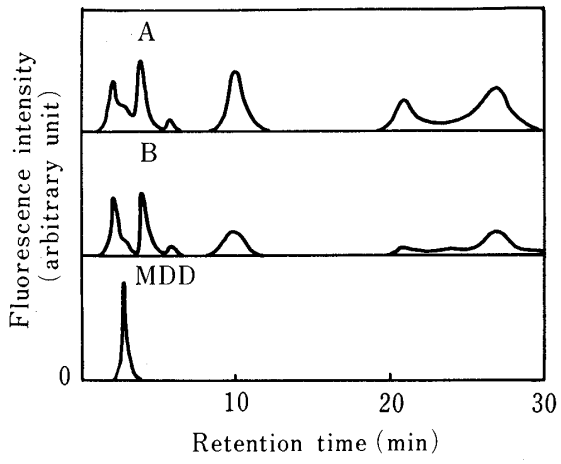

Fig. 6. HPLC of the Reaction Mixture of LOOH (A) or LA (B) with Methylamine

Discussion

The extent of formation of fluorescent substances by reaction of free unsaturated fatty acid with methylamine was in the order of AA > LA > LNA ≫ OA. LOOH produced more fluorescence than LA. While peroxidation of the fatty acid was essential for production of fluorescence, the hydroperoxide was not the direct source of the fluorescent substances. Shimasaki *et al.*[8] reported that increase in fluorescence correlated directly with the decrease in hydroperoxide level and the increase in TBA values at 532 nm during the reaction of methyl linoleate hydroperoxide with glycine in emulsion. They assumed the fluorescent substances to be the conjugated Schiff bases between MDA and glycine. Tabata *et al.*[10,11] suggested that methyl linolenate hydroperoxide was inactive but its secondary products other than MDA were included in the production of fluorescence.

Though TBA-reactive substances are generally regarded as MDA,[22] Marcuse *et al.*[20] expressed some uncertainty as to the origin and character of the pigments absorbing at 532 and 455 nm. Hydroperoxide and secondary products such as alkanals, 2-alkenals, 2,4-alkadienals and cyclic peroxides are converted into MDA during TBA-reaction to produce red pigment absorbing at 532 nm, and certain alkanals and cyclic peroxides react with TBA to produce yellow pigment absorbing at 455 nm.[17-21] The present experiments indicated that the fatty acids with higher levels of TBA-reactive substances showing absorption maxima at 532 and 455 nm produce more fluorescence, and the complex mixture of TBA-reactive substances including MDA contributed to the formation of fluorescence. It is interesting to note that LA produced more TBA-reactive substances and fluorescence than LNA did under the present conditions, although it is generally considered that linolenate produces more MDA than linoleate.[23]

Fluorescence spectra of the products exhibited an excitation maximum at 355—370 nm and an emission maximum at 420—440 nm, which were similar to those of lipofuscin pigments showing a fluorescence maximum at 420—470 nm[2] The fluorescence maximum of the products shifted to slightly higher wavelength with increasing unsaturation of the fatty acids. The fluorescence spectra were different from that of MDD (Table I) and slightly different from those of the conjugated Schiff bases with an excitation maximum at 370 nm and an emission maximum at 450 nm.[4] Both MDD and conjugated Schiff bases are products derived from MDA by reaction with methylamine or primary amines; the former was produced under neutral conditions and the latter under acidic conditions.[4,6] *n*-Hexanal, one of the alkanals produced during lipid peroxidation, was shown to produce fluorescent substances by reaction with primary amines,[12] and the products of *n*-hexanal exhibited a fluorescence spectrum with an excitation maximum at 366 nm and an emission maximum at 428 nm, which is very close to

those of the fluorescent substances produced in the present experiments. Thus, a variety of TBA-reactive substances other than MDA may contribute to the formation of fluorescence.

Shimasaki *et al.*[24] demonstrated that the fluorescence of age-related lipofuscin pigments extracted from rat testicular tissue was quenched at alkaline pH and by metal chelators, and suggested that the pigments contain conjugated Schiff base structures which exhibit the same characteristics.[5] Fluorescence of MDD was, however, little influenced in the alkaline medium and was greatly affected in the acidic medium (Table II). The fluorescence of the substances derived from the fatty acids was little influenced in the acidic medium, and was differently influenced in the alkaline medium with different fatty acids. While the fluorescence of MDD (Table II) and conjugated Schiff bases[5] was markedly decreased by treatment with sodium borohydride, the intensity of the products was influenced differently with different fatty acids. The fluorescence characteristics of the products in the present experiments seem to be similar to those of lipofuscin pigments.

In conclusion, free unsaturated fatty acids such as LA, LNA and AA produced a complex mixture of fluorescent substances by reaction with methyl amine. The substances were different in structure or in composition with the different fatty acids, and most of them may be derived from TBA-reactive substances other than MDA.

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