Formation of Fluorescent Substances in Reaction of Aliphatic Aldehydes and Methylamine

Kiyomi Kikugawa* and **Akiko Sawamura**
Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03 Japan

Treatment of monofunctional aliphatic aldehydes (alkanals, 2-alkenals and 2,4-alkadienals) with methylamine at pH 7 produced fluorescence with excitation maxima at 340–360 nm and emission maxima at 410–470 nm. The reaction of 1-butanal and methylamine gave 2-ethyl-2-hexenal (ii), an aldol condensation product of 1-butanal, and 3,5-diethyl-2-propyl-1-methylpyridinium salt (i). The fluorescence was formed by the reaction of 1-butanal and/or ii with methylamine in the presence of molecular oxygen. Fluorescent products I, II and III formed by the reaction were partially purified. Fluorescence characteristics of I, II and III were close to those of the fluorophores derived from the reaction of oxidized fatty acids and primary amines, with respect to maximum wavelengths in excitation and emission, and the resistance to sodium borohydride reduction.

Aging tissues contain lipofuscin or ceroid pigments composed of characteristic fluorescent components (1–3). The fluorescent components increase with aging of the tissues (4,5). It has been believed that the fluorescent components are derived from oxidized lipids in tissues or cells (6,7), and fluorescence measurement has become an excellent index of lipid oxidation of biological materials (8,9).

Unsaturated fatty acids are oxidized to hydroperoxides and their secondary degradation products, including malonaldehyde and monofunctional aliphatic aldehydes (10). Although amino acid residues in proteins are destroyed or produce fluorescence and cross-links by interaction with the oxidized lipids, the mechanisms of the reactions have not yet been elucidated (11). Tappel and his associates (12–14) suggested that fluorescence and cross-linking of proteins were due to the formation of the conjugated Schiff bases between proteins and malonaldehyde. We have shown that fluorescence derived from the reaction of amino acids and malonaldehyde is due to N-substituted 1,4-dihydropyridine-3,5-dicarbaldehydes (15–21). Other carbonyl compounds in oxidized lipids can react with proteins or primary amines to produce fluorescence (20–24).

It is important to clarify the mode of the reaction between monofunctional aliphatic aldehydes and primary amines, and the characteristics of the fluorescence produced. We will describe here the reaction of monofunctional aliphatic aldehydes with methylamine, and the properties of the fluorescent products.

MATERIALS AND METHODS

1-Propanal, 1-butanal and 1-hexanal were the products of Wako Pure Chemical Industries, Ltd., Osaka, Japan. 1-Pentanal, 1-heptanal, 2-butenal (crotonaldehyde), *trans*-2-hexenal and 2-ethyl-2-hexenal were the products of Tokyo Kasei Kogyo Company, Ltd., Tokyo, Japan. *trans,trans*-2,4-Nonadienal and *trans,trans*-2,4-decadienal were the products of Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

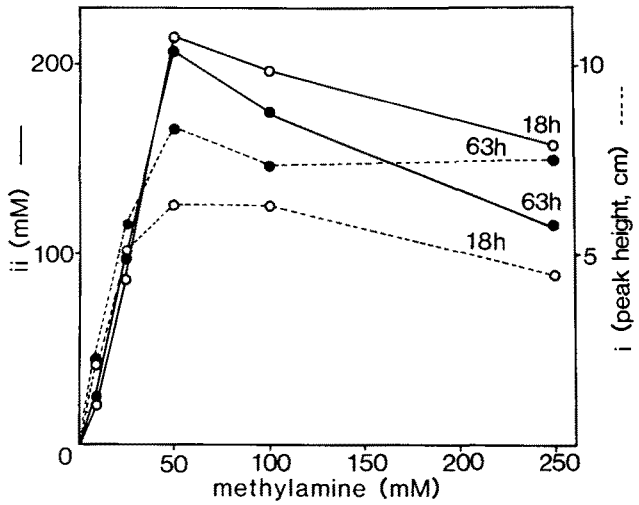


FIG. 1. Effect of methylamine on the formation of 2-ethyl-2-hexenal (ii) from 1-butanal. A solution of 500 mM 1-butanal and methylamine at the indicated concentration in 85% methanol/0.1 M phosphate buffer (pH 7.0) was incubated at 37 C for 18 or 63 hr. Products i and ii were analyzed by HPLC with solvent system A at a flow rate of 0.6 ml/min. The peaks corresponding to i and ii were detected at 280 and 230 nm, respectively. The heights of the peak at the retention time of 7.5 min (i) and the peak at the retention time of 10.0 min (ii) were obtained after injection of 1 μl of the reaction mixture. The concentration of ii in the reaction mixture was calculated from the peak heights and those of the calibration curve of the standard 2-ethyl-2-hexenal solution.

Analysis. Fluorescence spectra were taken with a Hitachi 650-40 fluorescence spectrometer. Ultraviolet absorption spectra were measured with a UV-200S Shimadzu double beam spectrophotometer. Nuclear magnetic resonance spectra were taken in $CDCl_3$ on a Bruker AM-400 NMR spectrometer with tetramethylsilane as an internal standard. Mass spectra were obtained with a Hitachi M-80 double focusing mass spectrometer. Thin layer chromatography was done by use of Wakogel B-5F (Wako Pure Chemical Industries, Ltd.). The chromatogram was developed with chloroform/methanol (9:1). Silica gel column chromatography was performed by use of Silica gel for column chromatography (above 100 mesh) from Kanto Chemical Company, Ltd., Tokyo, Japan. The column was eluted successively with chloroform and chloroform/methanol (95:5). High performance liquid chromatography (HPLC) was carried out by use of a Shimadzu LC-6 liquid chromatograph equipped with a YMC A-303 ODS column (4.6 × 250 mm) (Yamamura Chemical Laboratories, Ltd., Kyoto, Japan). The chromatograph was operated with the following solvent systems: A, methanol/0.025 M phosphate buffer (pH 7.0) (8:2); B, methanol/0.01 M triethylamine bicarbonate buffer (pH 7.6) (5:5), and C, methanol/0.01 M triethylamine bicarbonate buffer (pH 7.6) (8:2). The ultraviolet absorbing peaks were detected by use of a Shimadzu SPD-6A UV

*To whom correspondence should be addressed.

spectrophotometric detector. The fluorescent peaks were detected by use of a Shimadzu RF-530 fluorescence spectrometer. Preparative HPLC was done by use of a YMC S 343 ODS column (20 × 250 mm) (Yamamura Chemical Laboratories, Ltd.) with solvent system B or C.

Identification of ultraviolet absorbing products **i** *and* **ii** *formed by the reaction of 1-butanal and methylamine.* A 750-ml solution of 500 mM 1-butanal and 50 mM methylamine in 85% methanol/0.1 M phosphate buffer (pH 7.0) was incubated at 37 C for 24 hr. Thin layer chromatography of the reaction mixture revealed two ultraviolet absorbing products, **i** (Rf 0.12) and **ii** (Rf 0.69); Rf value of the latter was identical to that of the authentic 2-ethyl-2-hexenal. HPLC of the reaction mixture on a YMC A-303 ODS column with solvent system A at a flow rate of 0.6 ml/min revealed two 260 nm-absorbing peaks at retention times of 7.5 min (**i**) and 10.0 min (**ii**); the latter corresponded to that of the authentic 2-ethyl-2-hexenal. The reaction mixture was extracted twice with 500 ml chloroform. The extracts were evaporated to dryness, and the residue was applied to a silica gel column (2.8 × 13 cm) and the column eluted with chloroform and chloroform/methanol. Products **i** and **ii** were obtained as oils that were homogeneous on a thin layer chromatogram. Ultraviolet absorption spectrum of product **ii** showed a maximum at 230 nm (methanol), which is identical with that of the authentic 2-ethyl-2-hexenal. Product **i** subsequently was purified by preparative HPLC through a YMC S-343 ODS column with solvent system C. A single peak fraction that absorbed at 280 nm was evaporated to dryness to give a brown oil of **i** (30 mg). Ultraviolet absorption spectra of product **i** exhibited maxima at 278 (0.1 M phosphate, pH 7.0), 278 (0.1 N HCl) and 278 nm (0.1 N NaOH). Nuclear magnetic resonance spectrum ($CDCl_3$): ppm; 1.16 (3H, *t*, CH_3), 1.34 (3H, *t*, CH_3), 1.36 (3H, *t*, CH_3), 1.63–1.73 (2H, *m*, CH_2), 2.63–2.91 (4H, *m*, CH_2 × 2), 3.06 (2H, *t*, CH_2), 4.60 (3H, *s*, NCH_3), 7.90 (1H, *s*, pyridinium H), 9.50 (1H, *s*, pyridinium H). Mass spectrum: m/e (relative intensity); 191 [M^+-1] (41), 176 [M^+-1-15] (100), 163 [M^+-1-28] (35). High resolution mass spectrum of the fragment ion peak of mass weight 191.1653 revealed formula of $C_{13}H_{21}N_1$. Products **i** and **ii** were thus identified as 3,5-diethyl-2-propyl-1-methylpyridinium salt and 2-ethyl-2-hexenal, respectively.

Partial purification of fluorescent products I, II and III derived from the reaction of 2-ethyl-2-hexenal (ii) and methylamine. A 250-ml solution of 500 mM 2-ethyl-2-hexenal (**ii**) and 50 mM methylamine in 85% methanol/0.1 M phosphate buffer (pH 7.0) was incubated at 37 C for 112 hr. HPLC of the reaction mixture on a YMC A-303 ODS column with solvent system B revealed three fluorescent peaks (Fig. 2B). The reaction mixture was divided into 10 portions, and each portion was evaporated below 40 C to remove solvents. The treatment of the reaction mixture must be carried out carefully to avoid degradation of the fluorescent products. The extracts were combined and evaporated to leave brown oil (ca. 1 ml). Fluorescent products **I**, **II** and **III** were separated and purified by preparative HPLC on a YMC S-343 ODS column with solvent system B. The fluorescent peak fractions corresponding to **I**, **II** and **III** were collected separately, and the fractions were evaporated to dryness to leave colorless oils, **I** (3 mg), **II** (2 mg) and

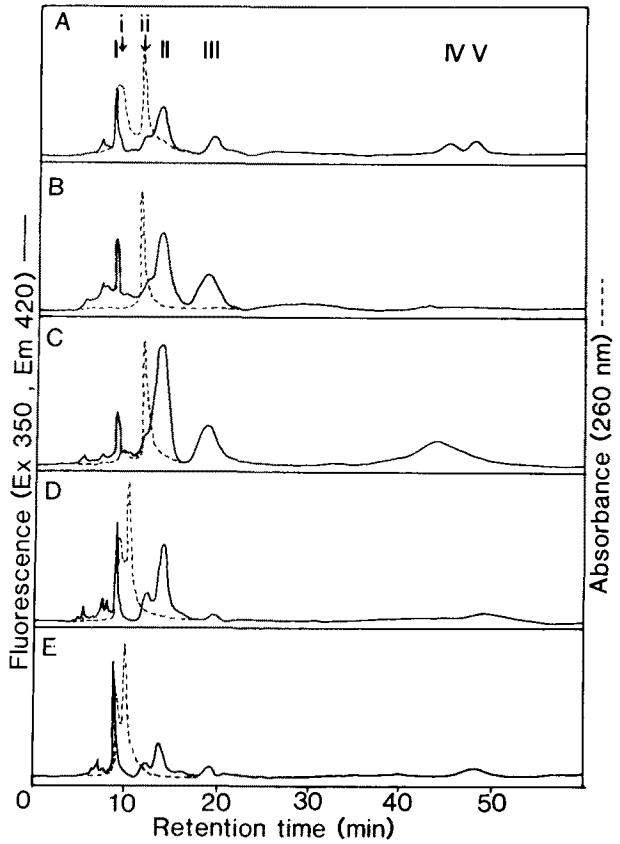


FIG. 2. HPLC of the reaction mixtures of 1-butanal and/or 2-ethyl-2-hexenal (ii) and methylamine. HPLC of reaction mixtures A–E shown in Table 2 (reaction time: 114 or 107 hr) was performed with solvent system B at a flow rate of 0.6 ml/min. Fluorescent peaks were detected at 350 (excitation) and 420 nm (emission). Numerals I–V indicate fluorescent products. Ultraviolet absorbing peaks were detected at 260 nm. Numerals i and ii indicate ultraviolet absorbing products.

TABLE 1

Formation of Fluorescence in the Reaction of Aliphatic Aldehydes and Methylamine

Aliphatic aldehyde	Fluorescence λmax(nm) Excitation	Fluorescence λmax(nm) Emission	Relative intensity
1-Propanal	355	430	220
1-Butanal	353	429	110
1-Pentanal	348	415	260
1-Hexanal	350	430	100
1-Heptanal	347	414	240
2-Butenal(Crotonaldehyde)	354	460	300
trans-2-Hexenal	340	415	230
trans,trans-2,4-Nonadienal	350	465	1200
trans,trans-2,4-Decadienal	350	465	1200

A mixture of 500 mM aliphatic aldehyde and 50 mM methylamine hydrochloride in 85% methanol/0.1 M phosphate buffer (pH 7.0) was incubated 4 days at 37 C. Fluorescence spectra of the reaction mixtures were measured after 50- 1000-fold dilution with methanol, and the relative intensities were expressed against the intensity of 0.1 μM quinine sulfate in 0.1 N sulfuric acid measured at excitation (350 nm) and emission (450 nm). Control experiments without methylamine produced no significant fluorescence.

TABLE 2

Fluorescence Spectra and Intensities of the Reaction Mixtures of 1-Butanal and/or 2-Ethyl-2-hexenal (ii) and Methylamine

Reactants	Solvent	Reaction time (hr)	Fluorescence λmax(nm) Excitation	Fluorescence λmax(nm) Emission	Relative intensity
A. 500 mM 1-Butanal 50 mM methylamine	85% methanol	22	348	412	41
		48	349	418	53
		114	353	429	110
B. 250 mM ii 50 mM methylamine	85% methanol	22	353	423	35
		48	349	419	75
		114	349	420	183
C. 100 mM 1-Butanal 200 mM ii 50 mM methylamine	85% methanol	22	346	409	139
		48	346	407	218
		114	345	410	445
D. 500 mM 1-Butanal 50 mM methylamine	85% ethanol	22	348	414	74
		48	349	419	100
		107	355	425	167
E. 500 mM 1-Butanal 50 mM methylamine	85% dimethylformamide	22	347	421	25
		48	348	419	40
		107	351	426	98

The reaction mixtures in 0.1 M phosphate buffer (pH 7.0) containing organic solvents were incubated at 37 C for indicated periods. Fluorescence spectra were measured after dilution into methanol, and the relative intensities were expressed against the intensity of 0.1 μM quinine sulfate.

III (2.5 mg). These peak fractions were found homogeneous on HPLC with respect to fluorescence and ultraviolet absorption (Fig. 5). They were stable when kept in methanol at 4 C. Fluorescence spectra of **I, II** and **III** are listed in Table 3. Ultraviolet absorption spectra of these products dissolved in methanol (1 mg/10 ml) showed maxima at 276 nm (absorbance: 0.50) for **I**; 235 (0.63) and 268 nm (0.40) for **II**; and 278 (0.24) and 355 nm (0.13) for **III**.

RESULTS

Formation of fluorescence in reaction of monofunctional aliphatic aldehydes and methylamine was investigated. The aliphatic aldehydes were alkanals with 3-7 carbon atoms, 2-alkenals with 4 and 6 carbon atoms and 2,4-alkadienals with 9 and 10 carbon atoms, most of which are secondary products of lipid oxidation (10). Homogeneous mixtures of 500 mM aldehyde and 50 mM methylamine in 85% methanol were incubated at 37 C for four days. Every reaction mixture exhibited blue fluorescence when exposed to ultraviolet light at 360 nm. Fluorescence spectra measured in methanol revealed excitation maxima at 340-360 nm and emission maxima at 410-470 nm (Table 1). Fluorescence intensities of the reaction mixtures of the alkanals and alkenals were roughly the same levels, and those of the alkadienals were slightly higher.

The mixture of 500 mM 1-butanal and 50 mM methylamine was incubated for 24 hr. The ultraviolet absorption spectrum of the reaction mixture showed a strong absorption at 230 nm, while that of the control mixture without methylamine showed no significant absorption. Thin layer chromatography and HPLC of the reaction mixture revealed two ultraviolet absorbing products **i** and **ii**. The Rf values and the retention time of **ii**

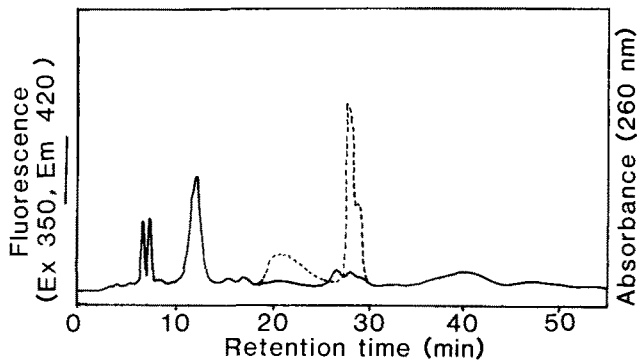


FIG. 3. HPLC of the reaction mixture of 1-hexanal and methylamine. A mixture of 500 mM 1-hexanal and 50 mM methylamine in 85% methanol/0.1 M phosphate buffer (pH 7.0) was incubated at 37 C for 114 hr. HPLC of the reaction mixture was performed with solvent system C at a flow rate of 0.6 ml/min.

coincided with those of the authentic 2-ethyl-2-hexenal, an aldol condensation product of 2 mol 1-butanal. Products **i** and **ii** were isolated by chloroform extraction, silica gel column chromatography and preparative HPLC. Product **ii** showed an absorption maximum at 230 nm characteristic to 2-ethyl-2-hexenal. Product **i** showed an absorption maximum at 278 nm and a mass fragment ion peak at m/e 191. High resolution mass spectrum revealed that the fragment had a formula of $C_{13}H_{21}N_1$. From the earlier observations (25) that condensation of alkanals and primary amines produces 1,2,3,5-substituted pyridinium salts, the structure of **i** could be determined as 3,5-diethyl-2-propyl-1-methylpyridinium salt. Nuclear magnetic resonance spectrum of **i** supported the structure.

Quantitative estimation of **ii** in the reaction mixture was assessed by use of HPLC (Fig. 1). While incubation of 500 mM 1-butanal in the absence of methylamine for

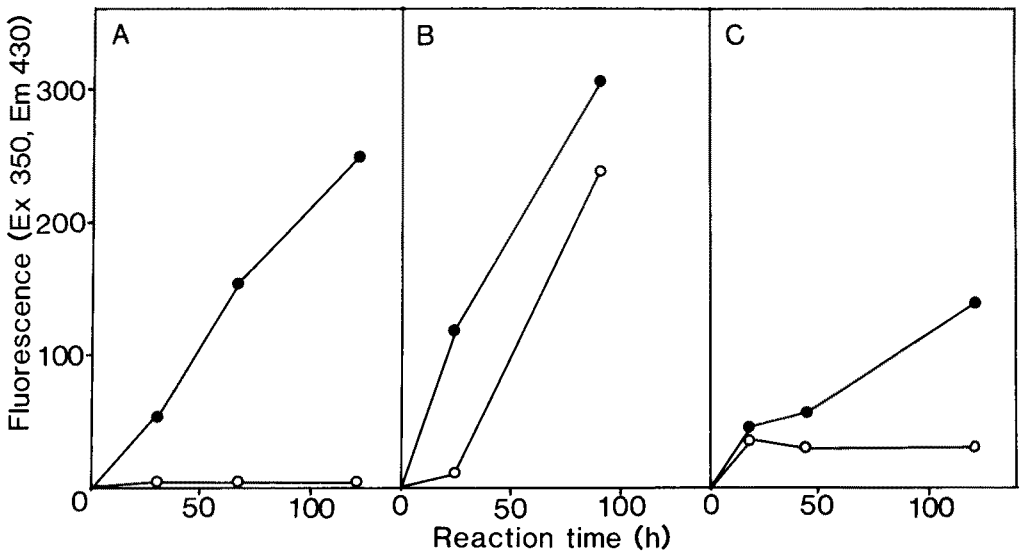


FIG. 4. Effect of molecular oxygen on the formation of fluorescent substances. The reaction mixtures of A, 500 mM 1-butanal; B, 500 mM 2-ethyl-2-hexenal (ii), and C, 500 mM 1-hexanal and 50 mM methylamine in 85% methanol/0.1 M phosphate buffer (pH 7.0) were incubated at 37 C under aerobic (●) and anaerobic (○) conditions. Under anaerobic conditions, molecular oxygen was removed by substitution of the reaction mixtures with nitrogen gas.

18 or 63 hr produced no significant amount of **ii**, the concentration of **ii** increased to 200 mM as the methylamine concentration increased to 50 mM, but it decreased at the methylamine concentrations higher than 50 mM. Rate of conversion of 1-butanal into **ii** in reaction with 50 mM methylamine was 80% after 18 hr. This result indicates that **ii** is the major product of the reaction and is formed by the catalytic action of methylamine. Formation of **i** was also dependent on the methylamine concentration and it reached a maximum level at the methylamine concentrations higher than 50 mM. Removal of molecular oxygen from the reaction mixtures did not affect the formation of **i** and **ii**, indicating that the reaction required no molecular oxygen.

While treatment of **i** with an excess amount of 1-butanal or methylamine at 37 C for 100 hr did not afford any ultraviolet absorbing or fluorescent products, treatment of **ii** with methylamine afforded fluorescent substances. Formation of fluorescence was compared with the following three reaction systems: A, 500 mM 1-butanal and 50 mM methylamine; B, 250 mM **ii** and 50 mM methylamine, and C, 100 mM 1-butanal, 200 mM **ii** and 50 mM methylamine. The reactions were performed in 85% methanol at 37 C for up to 114 hr. Reactions B and C were the models of reaction A in which 100 and 80% of 1-butanal was converted into **ii**, respectively. Three reactions showed similar excitation and emission maxima, and showed increasing fluorescence intensity with the reaction time (Table 2). While the rate of fluorescence formation in reaction B was similar to that in reaction A, the rate in reaction C was much higher.

Reaction A revealed five fluorescent peak fractions (**I–V**) in HPLC, whose retention times were different from those of the ultraviolet absorbing peak fractions (**i** and **ii**) (Fig. 2A). Chromatographic profiles of fluorescent peak fractions in reactions B and C were essentially similar to those of reaction A, and both reactions B and C revealed fluorescent peak fractions **I–III** (Fig. 2B and C). These results demonstrate that fluorescent products **I, II** and **III** were derived from 2-ethyl-2-hexenal (**ii**) as an intermediate.

When methanol in reaction A was replaced by ethanol (reaction D) or dimethylformamide (reaction E), fluorescence spectra and development of fluorescent intensities were unchanged (Table 2). Chromatographic patterns of the fluorescent peak fractions in reactions D and

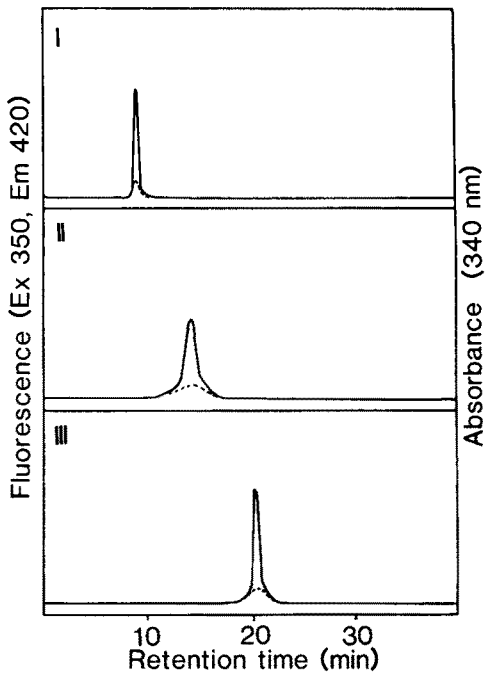


FIG. 5. HPLC of fluorescent products I, II and III. HPLC of the purified products derived from the reaction of 2-ethyl-2-hexenal (ii) and methylamine was performed with solvent system B at a flow rate of 0.6 ml/min.

TABLE 3

Fluorescence Characteristics of I, II and III

Solvent	I			II			III		
	Fluorescence λmax (nm)		Relative intensity	Fluorescence λmax (nm)		Relative intensity	Fluorescence λmax (nm)		Relative intensity
	Ex[a]	Em[b]		Ex[a]	Em[b]		Ex[a]	Em[b]	
Methanol	350	423	3.3	345	407	12.3	362	431	263
Ethanol	349	419	3.7	346	409	11.1	360	430	363
Chloroform	350	418	2.8	348	412	2.4	362	432	168
0.1 M Phosphate (pH 7)	350	417	3.2	348	397	2.9	382	436	21
0.1 N HCl	345	420	1.5	347	390	3.5	377	434	14
0.1 N NaOH	345	414	3.3	337	383	1.6	376	434	21
10 mM $NaBH_4$/methanol	347	421	4.5	347	406	20.3	358	431	313

[a]Ex, excitation.

[b]Em, emission.

Relative intensity of the solution of **I, II** and **III** [absorbance: 1.0 at 276 nm (**I**), 268 nm (**II**) and 355 nm (**III**)] was expressed against the intensity of 0.1 μM quinine sulfate.

E were also similar to those in reaction A (Fig. 2D and 2E). When the content of methanol in reaction A was reduced from 85% to 10%, the reaction mixture became heterogeneous, but the yield of fluorescence increased and the same fluorescent peak fractions were obtained. The formation of the fluorescent products was independent of the organic solvents used.

The reaction of 500 mM 1-hexanal and 50 mM methylamine in 85% methanol at 37 C for 114 hr produced 230 nm-ultraviolet absorbing materials and fluorescent products. HPLC of the reaction mixture revealed two ultraviolet absorbing peak fractions and many fluorescent peak fractions (Fig. 3). The reaction might afford ultraviolet absorbing and fluorescent products similar to the reaction of 1-butanal and methylamine.

Effect of molecular oxygen on the formation of the fluorescent products was investigated. Removal of molecular oxygen by purging with nitrogen gas prior to the reaction prevented the formation of fluorescent products in the reaction of 1-butanal (Fig. 4A), 2-ethyl-2-hexenal (ii) (Fig. 4B) and 1-hexanal (Fig. 4C). It is interesting that the presence of molecular oxygen in the reaction mixtures was essential for efficient production of fluorescent compounds.

Isolation of fluorescent products **I, II** and **III** in reaction B (Fig. 2B) was attempted. Since the fluorescent products in the reaction mixture tended to be degraded in the isolation steps, great care had to be taken. The fluorescent products in the reaction mixture were extracted with chloroform and purified by preparative HPLC. Small amounts of oily products **I, II** and **III** were obtained. They were homogeneous in fluorescence and ultraviolet absorption when analyzed by HPLC (Fig. 5).

Fluorescence spectra and fluorescence characteristics of **I, II** and **III** are listed in Table 3. These products showed excitation maxima at 340–370 nm and emission maxima at 400–440 nm in methanol and ethanol. While relative fluorescence intensities of **I** and **II** as measured against absorbance 1.0 were extremely low, those of **III** were high. **I** and **II** may contain a large amount of ultraviolet absorbing materials other than fluorescent compounds. Mass fragment patterns of **III** were very complex, indicating that **III** was also heterogeneous.

The fluorescence intensities of **I, II** and **III** were lowered when the spectra were taken in aqueous solvents, i.e. 0.1 M phosphate buffer (pH 7), 0.1 N HCl and 0.1 N NaOH. Treatment of these products with sodium borohydride resulted in no significant decrease in fluorescence intensity.

DISCUSSION

Fluorescent components increased with aging of the tissues (4,5), and they have been assumed to be derived from the reaction of oxidized lipids and proteins in tissues or cells (6,7). For instance, Fletcher et al. (4) demonstrated that chloroform-methanol extracts of tissues showed fluorescence with excitation at 340–380 nm and emission at 420–470 nm, and Shimasaki et al. (26) showed that 2,2-dimethoxypropane extract of rat testes had fluorescence with excitation at 355 nm and emission at 490 nm. The fluorescence was quenched in alkaline media (26,27). While fluorescence has become an excellent index of lipid oxidation of tissues (8,9), the properties of the fluorophores and the mechanisms of their formation were still obscure.

Since malonaldehyde, one of the secondary degradation products of lipid oxidation, readily produces fluorescence in reaction with proteins or primary amines, earlier workers suggested that the increased fluorescence in tissues was due to malonaldehyde (13,14). Carbonyl compounds in oxidized lipids other than malonaldehyde can, however, react with proteins to produce fluorescence. Reaction of bovine serum albumin with carbonyl compounds afforded the modified albumin with fluorescence (22). Treatment of polylysine or human erythrocyte ghosts with monofunctional aliphatic aldehydes at pH 7 yielded fluorescence and cross-links (20,21). Treatment of lysozyme in the solid state with vaporized 1-hexanal resulted in the production of fluorescent and cross-linked lysozyme (24).

This investigation was done to clarify reaction profiles for fluorescence production from monofunctional aliphatic aldehydes and primary amines, and the fluorescence

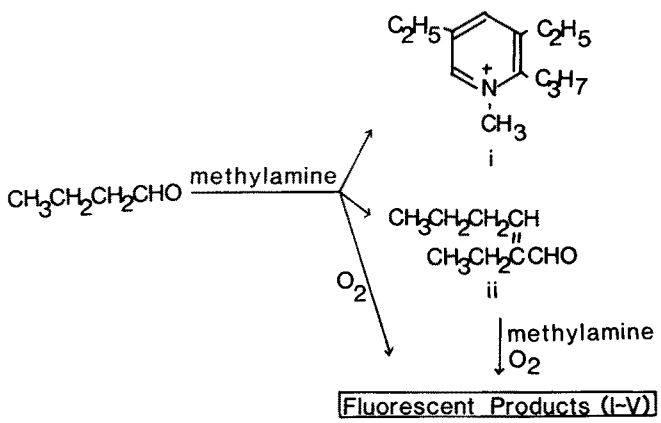
TABLE 4

Fluorescence Characteristics of the Fluorescent Products Derived from the Oxidized Fatty Acids and Their Secondary Degradation Products

Fluorescent products	Fluorescence λmax(nm)		Fluorescence characteristics
	Excitation	Emission	
Oxidized fatty acids modified			
Proteins or polypeptides (20-22,28)	340-360	420-440	Partially lost by $NaBH_4$ (20)
Amino acids or primary amines (29-31)	355-370	420-440	Not lost (30) or partially lost by $NaBH_4$ (31) Quenched in alkaline media (31)
Malonaldehyde modified			
Proteins or polypeptides (13,19-21)	390-400	460-470	Lost by $NaBH_4$ (20)
Amino acids (conjugated Schiff bases) (12)	370	450	Lost by $NaBH_4$ (12) Quenched in alkaline media (32)
Amino acids or primary amines (1,4-dihydropyridine-3,5-dicarbaldehydes) (17,18)	385-405	440-470	Lost by $NaBH_4$ (17,18) Quenched in acidic media (17,18)
Aliphatic aldehydes modified			
Proteins or polypeptides (20-22,24)	340-360	410-440	Only partially lost by $NaBH_4$ (20)
2-Phenylethylamine (23)	366	428	
Methylamine (present paper)	340-370	400-440	Not lost by $NaBH_4$ (present paper) Quenched in aqueous media at any pH ranges (present paper)

characteristics of the fluorescent products. Aliphatic aldehydes produced blue fluorescence by reactions with methylamine at pH 7. The reaction of 1-butanal and methylamine at pH 7 gave 2-ethyl-2-hexenal (**ii**) preferentially. Another product was identified as 3,5-diethyl-2-propyl-1-methylpyridinium salt (**i**), probably as a consequence of the reaction of the Schiff base of **ii** and 1-butanal, as has been suggested (25). The reaction required no molecular oxygen, which is consistent with earlier observations (25). Formation of fluorescence may be due to **ii**, since production of fluorescence in the reaction of **ii** was at a rate similar to that in the reaction of 1-butanal. Combination of 1-butanal and **ii** produced much more fluorescence (Table 2). Chromatographic patterns of the fluorescent products derived from 1-butanal, **ii** and the mixture of 1-butanal and **ii** were similar (Fig. 2). It is interesting to note that the formation of fluorescence required molecular oxygen. Reaction scheme for the formation of fluorescence is shown in Scheme 1.

Fluorescence characteristics of several models for the fluorescent components in lipofuscin are shown in Table 4. The fluorophores derived from the reaction of oxidized fatty acids and amino compounds had fluorescence (20-22,28) that was not or partially lost by treatment with sodium borohydride (20,30,31). The fluorescence was quenched in alkaline media (31). Fluorophores derived from the reaction of malonaldehyde and amino compounds were different from those derived from oxidized fatty acids. Conjugated Schiff bases (12) and 1,4-dihydropyridine-3,5-dicarbaldehydes (17,18) showed fluorescence with rather higher wavelengths that was lost by treatment with sodium borohydride. While the fluorescence of the conjugated Schiff bases was quenched in alkaline media (32), that of the 1,4-dihydropyridines was quenched in acidic media (17,18). Fluorescence spectra of products



SCHEME 1

I, II and **III** obtained in the present experiments exhibited maximum excitation at 340–370 nm and maximum emission at 400–440 nm, which are close to those obtained by reactions of aliphatic aldehydes and proteins (20–22,24) and to those from the reaction of 1-hexanal and 2-phenylethylamine (23). The fluorescence characteristics of the products were close to those of the fluorophores derived from oxidized fatty acids with respect to maximum wavelengths in excitation and emission, and to the resistance to sodium borohydride. However, they were different with respect to the fluorescence intensities in aqueous media. Fluorescence of **I, II** and **III** was quenched in aqueous media at any pH ranges.

We have suggested that malonaldehyde is not a likely molecule for production of fluorophores in lipofuscin (17,18). It is not, however, clear that monofunctional aliphatic aldehydes contributed to the formation of fluorophores in lipofuscin. Further investigation may be necessary for the contribution of the secondary degradation products of oxidized lipids to the fluorescence formation in lipofuscin.

REFERENCES

1. Hyden, H., and B. Lindstrom, *Disc. Faraday Soc. 9:*436 (1950).
2. Hartroft, W.S., *Science 113:*673 (1951).
3. Eldred, G.E., G.V. Miller, W.S. Stark and L. Feeney-Burns, *Science 216:*757 (1982).
4. Fletcher, B.L., C.J. Dillard and A.L. Tappel, *Anal. Biochem. 52:*1 (1973).
5. Tappel, A.L., *Fed. Proc. 32:*1870 (1973).
6. Chio, K.S., R. Reiss, B. Fletcher and A.L. Tappel, *Science 166:*1535 (1969).
7. Dillard, C.J., and A.L. Tappel, *Lipids 6:*715 (1971).
8. Gray, J.I., *J. Am. Oil Chem. Soc. 55:*539 (1978).
9. Logani, J.I., and R.E. Davies, *Lipids 15:*485 (1980).
10. Frankel, E.N., *J. Am. Oil Chem. Soc. 61:*1908 (1984).
11. Gardner, H.W., *J. Agric. Food Chem. 27:*220 (1979).
12. Chio, K.S., and A.L. Tappel, *Biochemistry 8:*2821 (1969).
13. Chio, K.S., and A.L. Tappel, *Ibid. 8:*2827 (1969).
14. Tappel, A.L., in *Free Radicals in Biology,* edited by A.W. Pryor, Academic Press, New York, NY, 1950, Vol. IV, pp. 1–47.
15. Kikugawa, K., T. Maruyama, Y. Machida and T. Kurechi, *Chem. Pharm. Bull. 29:*1423 (1981).
16. Kikugawa, K., Y. Machida, M. Kida and T. Kurechi, *Ibid. 29:*3003 (1981).
17. Kikugawa, K., and Y. Ido, *Lipids 19:*600 (1984).
18. Kikugawa, K., Y. Ido and S. Mikami, *J. Am. Oil Chem. Soc. 61:*1574 (1984).
19. Kikugawa, K., H. Kosugi and T. Asakura, *Arch. Biochem. Biophys. 61:*1574 (1964).
20. Kikugawa, K., K. Takayanagi and S. Watanabe, *Chem. Pharm. Bull. 33:*5437 (1985).
21. Beppu, M., K. Murakami and K. Kikugawa, *Ibid. 34:*781 (1986).
22. Fletcher, B.L., and A.L. Tappel, *Lipids 6:*172 (1971).
23. Yoden, K., R. Matsuzaki, T. Iio and T. Tabata, *Yakugaku Zasshi* (Tokyo) *102:*768 (1982).
24. Tashiro, Y., A. Okitani, N. Utsunomiya, S. Kaneko and H. Kato, *Agric. Biol. Chem. 49:*1739 (1985).
25. Suyama, K., and S. Adachi, *J. Org. Chem. 44:*1417 (1979).
26. Shimasaki, H., N. Ueta and O.S. Privett, *Lipids 15:*236 (1980).
27. Trombly, R., and A.L. Tappel, *Lipids 10:*441 (1975).
28. Shimasaki, H., N. Ueta and O.S. Privett, *Lipids 17:*878 (1982).
29. Shimasaki, H., O.S. Privett and I. Hara, *J. Am. Oil Chem. Soc. 54:*119 (1977).
30. Tabata, T., K. Yoden, H. Takei and T. Iio, *Yakugaku Zasshi (Tokyo) 99:*711 (1979).
31. Kikugawa, K., S. Watanabe and T. Kurechi, *Chem. Pharm. Bull. 32:*638 (1984).
32. Malshet, V.G., A.L. Tappel and U.M. Burns, *Lipids 9:*328 (1974).

[Received April 22, 1986]