from glycerol and fatty acids by using a lipase preparation without the substrate specificity for 1,2 or 3 position.

Moreover, we found that several aliphatic alcohols, instead of 1,2-diacylglycerol, could be used by the lipase as the fatty acid acceptor in a reverse micellar system. Figure 6 shows the effect of chain length of aliphatic alcohols upon the reactivity of ester formation with oleic acid, which indicates that alcohols with a chain length greater than 4 carbons were effective. However, cholesterol could not be used as the substrate in place of aliphatic alcohols (data not shown).

Triacylglycerol (or ester) synthesis in the reverse micellar system is pictured in Figure 7. The carboxyl group of the fatty acid and hydroxyl group of 1,2-diacylglycerol (or aliphatic alcohols) are in contact with water in reverse micelles in n-hexane and can be esterified by the lipase located inside the reverse micelles. Once products are synthesized, they lose amphipathic properties and are moved from the water phase into the n-hexane phase. In this way the reaction proceeds to triacylglycerol synthesis.

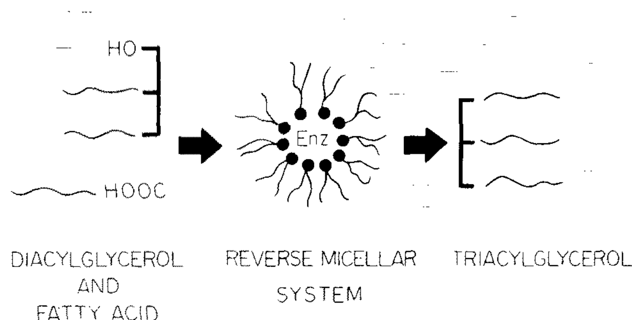

FIG. 7. The reaction scheme of triacylglycerol synthesis in reverse micellar system. See the text.

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[Received Feburary 1984]

☘Studies on Peroxidized Lipids. VI. Fluorescent Products Derived From the Reaction of Primary Amines, Malonaldehyde and Monofunctional Aldehydes

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ABSTRACT

Monofunctional aldehydes such as acetaldehyde, *n*-propylaldehyde, *n*-butylaldehyde, *n*-hexylaldehyde, *n*-heptylaldehyde and benzaldehyde affected the reaction between primary amines and malonaldehyde. While the reaction of primary amines and malonaldehyde at pH 7 produced fluorescent 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes **Ia-f**, the reaction of the primary amines, malonaldehyde and the aldehydes listed above gave fluorescent 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehydes **IIa-j**. The primary amines used for this reaction included alkylamines, amino acids and alkanolamines. The optimal ratio of the amine, malonaldehyde and the aldehyde was 1:2:1-2, at which compounds **II** were produced quantitatively. Peroxidized lipids which may contain malonaldehyde and other aldehydes could react with the primary amines to produce highly fluorescent **II**. Fluorescence spectra of **II** showed excitation maxima at 386-403 nm and emission maxima at 444-465 nm in phosphate similar to those of **I**. The spectra of these 1,4-dihydropyridines **I** and **II** were roughly similar to those of lipofuscin pigment, but they exhibited different characteristics in acid and alkaline media from those of lipofuscin pigment. Compounds **II** may be useful as model compounds to elucidate the chemical structure of lipofuscin pigment.

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INTRODUCTION

The formation of fluorescent lipofuscin pigment has been suggested in the in vivo reaction of proteins and peroxidized lipids (1). The structure of fluorescent lipofuscin pigment has been believed to be the 2:1-conjugated Schiff base (N,N′-disubstituted 1-amino-3-iminopropene) of a primary amine and malonaldehyde, which might be produced by peroxidation of polyunsaturated fatty acids (2-5). In our recent studies of the reaction of primary amines with malonaldehyde, it was shown that the reaction afforded 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes **Ia-e** as major fluorescent products (6-9), which may be formed by the 1:3-reaction of the amines and malonaldehyde.

Peroxidation of polyunsaturated fatty acids has been shown to give various aldehydes besides malonaldehyde (10-12). We found that monofunctional aldehydes stimulated and participated in the formation of fluorescent substances in the reaction of primary amines and malonaldehyde. This paper describes the structures and the properties of the fluorescent products formed from the reaction of primary amines, malonaldehyde and monofunctional

aldehydes. The participation of malonaldehyde and monofunctional aldehydes in the formation of lipofuscin also is discussed.

EXPERIMENTAL PROCEDURES

Materials

Acetaldehyde, *n*-propylaldehyde and *n*-hexylaldehyde were reagent grade products of Kanto Chemical Company, Ltd., Tokyo. Methylamine hydrochloride, *n*-butylaldehyde, *n*-heptylaldehyde, glycine ethyl ester hydrochloride, putrescine, ethanolamine and 3-amino-1-propanol were reagent grade products of Tokyo Kasei Kogyo Company, Ltd., Tokyo. Benzaldehyde was the product of Koso Chemical Company, Ltd., Tokyo.

Tetramethoxypropane (TMP) was the product of Tokyo Kasei Kogyo Company, Ltd., Tokyo. TMP hydrolysate (1 M) was prepared as follows (8). TMP (1.64 g) was shaken with 0.90 ml of 1.0 N HCl at 40 C until miscible, and the mixture was increased to 10 ml with water. The mixture was incubated at 37 C for 1 hr before use. The solution contained no TMP as estimated by gas chromatography. Purified malonaldehyde sodium salt (MDA•Na) was prepared as described elsewhere (8). Authentic compounds, 1,4-dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde **Ia** and 1-methyl-4-dimethoxyethyl-1,4-dihydropyridine-3,5-dicarbaldehyde **IIa**, were prepared by reacting methylamine and TMP hydrolysate as described previously (6). 3,5-Diformyl-4-methyl-1,4-dihydropyridine-1-acetic acid **Id** was similarly prepared (7).

Analytical Methods

Ultraviolet (UV) absorption spectra were determined with a UV-200S Shimadzu double beam spectrophotometer. Solvents for UV analysis were 0.1 M phosphate (pH 7.0), 0.1 M phosphate (pH 7.0)-ethanol (9:1) [N] and ethanol. Fluorescence spectra were determined with a Hitachi MPF-4 fluorescence spectrophotometer, and the relative intensity was expressed against 0.1 μM quinine sulfate in 0.1 N sulfuric acid with excitation at 352 nm and emission at 448 nm. Mass (MS) spectra were taken on a Hitachi double focusing mass spectrometer M-80. Nuclear magnetic resonance (NMR) spectra were taken on a JEOL PS-100 instrument with tetramethylsilane as an internal standard. Thin-layer chromatography (TLC) was performed on a Silica-gel 70F-254 plate-Wako with a solvent system of ethyl acetate-ethanol (4:1). Silica gel column chromatography was performed with silica gel (above 100 mesh) from Kanto Chemical Company, Ltd., Tokyo.

The concentrations of the fluorescent products in the reaction mixtures were determined by high performance liquid chromatography (HPLC) on a Shimadzu LC-2 liquid chromatograph with a stainless steel column (4.6 cm I.D. × 25 cm) of Zorbax ODS. Authentic compounds **Ia** and **IIa** eluted at retention times of 6.8 and 6.0 min, respectively, with methanol-water (1:1) at a flow rate of 0.7 ml/min (8). Compounds **Ia**, **IIa** and **IId** had retention times of 4.0, 4.0 and 8.0 min, respectively, with ethanol-methanol-water (1:2:2). Compound **Id** had a retention time of 2.7 min with methanol-water (3:7). The peak height of each compound showed a linear relationship to the concentration.

4-Substituted 1,4-Dihydropyridine-3,5-Dicarbaldehydes II 1-Methyl-4-*n*-Pentyl-1,4-Dihydropyridine-3,5-Dicarbaldehyde IId

A mixture of 36 ml (0.036 mol) of 1 M methylamine, 18 ml (0.018 mol) of TMP hydrolysate and 18 ml (0.018 mol) of 1 M *n*-hexylaldehyde/methanol in 720 ml of 0.1 M phosphate (pH 7.0) was incubated at 37 C for 67 hr. Crystalline materials separated in the reaction mixture were collected by filtration and recrystallized twice from water. Yellow needles (101 mg) of **IId** were obtained.

3,5-Diformyl-4-*n*-Pentyl-1,4-Dihydropyridine-1-Acetic Acid Ethyl Ester IIg

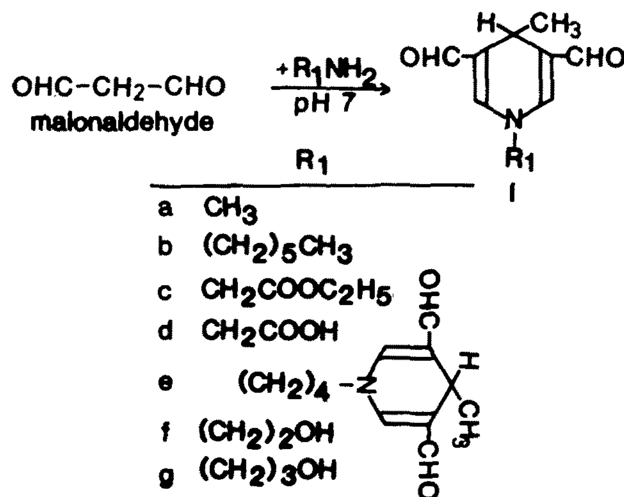
A mixture of 50 ml (0.05 mol) of 1 M glycine ethyl ester, 100 ml (0.1 mol) of TMP hydrolysate and 100 ml (0.1 mol) of 1 M *n*-hexylaldehyde/methanol in 750 ml of 0.1 M phosphate (pH 7.0) was incubated at 37 C for 5 hr. The product was purified by passing through a column of silica gel (3.6 cm I.D. × 36 cm) by use of a solvent system of chloroform-methanol. The product was crystallized and recrystallized from ethyl acetate-*n*-hexane to afford 410 mg of yellow leaflets.

RESULTS

Fluorescent Products of the Reaction of Primary Amines, Malonaldehyde and Monofunctional Aldehydes

Reaction of 10 mM methylamine and 20 mM TMP hydrolysate at pH 7 and 37 C for 24 hr produced two fluorescent stops of Rf: 0.33 (**Ia**) and 0.20 (**IIa**) on TLC. It was shown that compound **Ia** was derived from malonaldehyde, and **IIa** might be produced from malonaldehyde and 3,3-dimethoxypropylaldehyde in the TMP hydrolysate (8). Addition of 10 mM acetaldehyde to the reaction mixture gave a single fluorescent spot corresponding to **Ia** with much higher fluorescence intensity. Addition of 10 mM *n*-hexylaldehyde gave another fluorescent spot (Rf: 0.43) with high fluorescence intensity. Reactions of 10 mM methylamine and 10 mM acetaldehyde or 10 mM *n*-hexylaldehyde produced no fluorescent spots on the chromatogram. Therefore, the presence of other aldehydes affected the formation of fluorescent products in the reaction of methylamine and malonaldehyde.

Compounds **Ia** and **IIb-f** were isolated as single fluorescent products from the reaction mixtures of methylamine, TMP hydrolysate and the monofunctional aldehydes such as acetaldehyde, *n*-propylaldehyde, *n*-butylaldehyde, *n*-hexylaldehyde, *n*-heptylaldehyde and benzaldehyde, respectively. The reaction with acetaldehyde gave a product identical to the authentic **Ia** with respect to mp, NMR and MS spectra. Structures of new compounds **IIb-f** were shown to be 4-substituted 1-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde by elemental analysis, ultraviolet



SCHEME 1

TABLE I

1,4-Dihydropyridine-3,5-dicarbaldehydes I and II

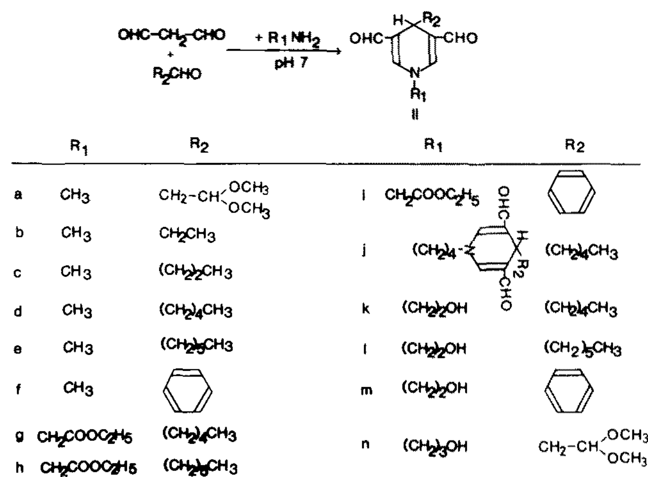
Compound	Reaction conditions[a]			Method of isolation[b]	Purifcn solvent[c]	Isolation yield[d] %	Appearance	Mp, C
	Primary amine R_1NH_2	Monofunctional aldehyde R_2CHO	Time, hr					
If	ethanolamine	–	93	I	A	0.14	yellow plates	102-105
Ig	3-amino-1-propanol	–	49	I	A	0.25	yellow granules	150-153
Ia	methylamine	acetaldehyde	144	II	A	12	yellow leaflets	145-148
IIb	methylamine	*n*-propylaldehyde	64	I	A	7	yellow leaflets	138-139
IIc	methylamine	*n*-butylaldehyde	64	I	A	10	yellow leaflets	130-133
IId	methylamine	*n*-hexylaldehyde	67	III	B	1.4	yellow needles	123-126
IIe	methylamine	*n*-heptylaldehyde	67	III	B	0.9	yellow leaflets	119-123
IIf	methylamine	benzaldehyde	165	II	A	10	yellow plates	162-168
IIg	glycine ethyl	*n*-hexylaldehyde	5	I	C	2.8	yellow leaflets	42-47
IIh	glycine ethyl	*n*-heptylaldehyde	67	I	C	1.6	yellow leaflets	54-57
IIi	glycine ethyl	benzaldehyde	34	I	A	8.4	yellow needles	163-165
IIj	putrescine	*n*-hexylaldehyde	72	I	D	3.8	yellow plates	207-210 (dec)
IIk	ethanolamine	*n*-hexylaldehyde	32	I	A	30	yellow needles	97-100
IIl	ethanolamine	*n*-heptylaldehyde	32	I	A	11	yellow needles	81-84
IIm	ethanolamine	benzaldehyde	67	I	C	3.4	yellow needles	142-147
IIn	3-amino-1-propanol	–	49	I	E	0.36	yellow plates	88-96

TABLE I (Continued)

UV: λmax, nm (mε) [N], pH 7* or ethanol**	NMR[e] d_6-DMSO or $CDCl_3$+ ppm	MS (rel intensity)	Formula
238(23.1)* 265(8.8) 396(11.4)	9.33(1), 7.35(2), 5.05(1H,t,OH), 3.65(5H,m,4-H,N-CH_2CH_2), 0.97(3H,d,4-CH_3)	195(2) 180(100) 136(60)	$C_{10}H_{13}NO_3 \cdot 0.25H_2O$
237(23.6)* 266(8.8) 396(11.6)	9.33(1), 7.32(2), 4.60(1H, bs,OH), 3.6(5H,m,4-H,NCH_2,OCH_2), 1.85 (2H,m,CH_2), 0.97(3H,d,4-CH_3)	209(8) 194(100) 136(56)	$C_{11}H_{15}NO_3$
244(21.5) 267(6.7) 404(10.9)	9.31(1), 7.39(2), 3.75(3), 3.33(4), 1.30(2H,m,CH_2), 0.65(3H,t,CH_3)	179(3) 150(100) 121(3)	$C_{10}H_{13}NO_2$
244(21.9) 270(6.9) 403(11.2)	9.30(1), 7.35(2), 3.74(3), 3.34(4), 1.15(4H,m,$(CH_2)_2$), 0.78(3H,t,CH_3)	193(3) 150(100) 121(2)	$C_{11}H_{15}NO_2 \cdot 0.5H_2O$
246(20.5) 273(6.8) 405(10.6)	9.31(1), 7.41(2), 3.74(3), 3.34(4), 1.15(8H,bs,$(CH_2)_4$), 0.80(3H,t,CH_3)	221(1) 150(100) 121(1)	$C_{13}H_{19}NO_2$
245(20.4) 272(6.8) 406(10.6)	9.27(1), 7.33(2), 3.73(3), 3.30(4), 1.36(10H,bs,$(CH_2)_5$), 0.83(3H,t,CH_3)	235(1) 150(100) 121(1)	$C_{14}H_{21}NO_2$
240(19.2) 265(8.6) 401(10.9)	9.20(1), 7.20(bm,phenyl), 6.80(2), 4.98(1H,s,4-H), 3.23(4)	227(29) 150(100) 122(1)	$C_{14}H_{13}NO_2$
236(22.1) 264(9.1) 386(9.5)	9.31(1), 7.38(2), 4.55(2H,s,NCH_2), 4.22(2H,q,$COOCH_2$), 3.75(3), 1.3(11H, m,$(CH_2)_4$,CH_3), 0.83(3H,t,CH_3)	293(6) 222(100) 194(42),149(8)	$C_{16}H_{23}NO_4$
236(22.9) 262(10.5) 386(9.7)	9.27(1), 7.37(2), 4.55(2H,s,NCH_2), 4.17(2H,q,$COOCH_2$), 3.73(3), 1.2(13H, m,$(CH_2)_5$,CH_3), 0.83(3H,t,CH_3)	307(8) 234(2),222(100) 194(40),149(10)	$C_{17}H_{25}NO_4$
231(20.2) 256(10.9) 381(9.8)	9.27(1), 7.50(2), 7.25(5H,m,phenyl), 4.77(1H,s,4-H), 4.67(2H,s,NCH_2), 4.26(2H,q,$COOCH_2$), 1.27(3H,t,CH_3)	299(35) 270(6),222(100) 194(50),149(4)	$C_{17}H_{17}NO_4$
236(38.9)** 264(17.4) 385(19.1)		468(14) 425(8),397(100) 262(8),220(4)	$C_{28}H_{40}N_2O_4$
238(22.9) 266(8.4) 396(10.9)	9.30(1), 7.37(2), 5.0(1H,s,OH), 3.72(3), 3.63(4H,s,NCH_2CH_2), 1.2 (8H,m,$(CH_2)_4$), 0.92(3H,t,CH_3)	251(10) 220(16),180(100) 136(33)	$C_{14}H_{21}NO_3$
238(22.9) 266(8.4) 396(10.9)	9.30(1), 7.37(2), 5.0(1H,s,OH), 3.72(3), 3.63(4H,s,NCH_2CH_2), 1.2 (13H,m), 0.83(6H,m)	265(6) 180(100) 136(16)	$C_{15}H_{23}NO_3 \cdot 0.33C_6H_{14}$
235(18.2) 262(9.0) 396(9.3)	9.30(1), 7.23(2), 7.2(5H,m,phenyl), 5.1(1H,bs,OH), 4.79(1H,s,4-H), 3.75(4H,s,NCH_2CH_2)	257(26) 180(100) 136(47)	$C_{15}H_{15}NO_3$
237(22.6)* 262(8.1) 396(10.3)	9.33(1), 7.44(2), 4.85(1H,t,OH), 4.20(1H,t,CH), 3.6(5H,m,4-H,NCH_2, OCH_2), 3.10(6H,s,OCH_3), 1.84(2H,t, CH_2), 1.57(2H,t,CH_2)	251(11) 194(100) 136(29)	$C_{14}H_{21}NO_5$

[a]A reaction mixture containing the primary amine (0.025-0.05 M), TMP hydrolysate (0.025-0.05 M) and the monofunctional aldehyde (0.025-0.05 M) in 0.1 M acetate (pH 5) (in case of **If**, **Ig** and **IIn**) or in 0.1 M phosphate (pH 7) was incubated at 37 C.
[b]The fluorescent products were isolated by the following methods: I, extraction with chloroform and successive silica gel column chromatography; II, extraction with chloroform, and III, direct crystallization from the reaction mixture.
[c]Purification solvents were A, chloroform-*n*-hexane; B, water; C, ethyl acetate-*n*-hexane; D, chloroform; and E, ethanol.
[d]Isolation yields were based on the amines.
[e]Signals were assigned as 1: 2H, s, 3,5-CHO; 2: 2H, s, 2,6-H, 3: 1H, t, 4-H; and 4: 3H, s, NCH_3.

absorption, NMR and MS spectra (Table I and Scheme II). Products **Ia** and **IIb-f** produced in the reaction of methylamine, TMP hydrolysate and the aldehydes were isolated in yields of 1-12%, while **Ia** produced in the absence of acetaldehydes was isolated in a yield of less than 1% (6).



SCHEME 2

The reaction of methylamine and TMP hydrolysate was little affected by the presence of the substituted aromatic aldehydes (*p*-hydroxybenzaldehyde, *p*-nitrobenzaldehyde, *o*-nitrobenzaldehyde and *trans*-cinnamic aldehyde), the alkenyl aldehydes (acrolein, crotonaldehyde, *n*-hexenylaldehyde, 2,4-hexadienal and *trans, trans*-decadienal) and the bifunctional aldehydes (glyoxal, methylglyoxal and glutaraldehyde).

While the reaction of glycine ethyl ester with TMP hydrolysate afforded **Ic** (7), addition of *n*-hexylaldehyde, *n*-heptylaldehyde and benzaldehyde to the reaction mixture gave **IIg-i**. The reaction of ethanolamine with TMP hydrolysate produced 4-methyl derivative **If**, and the reaction of 3-amino-1-propanol with TMP hydrolysate gave 4-methyl derivative **Ig** and 4-substituted derivative **IIn**: the latter may be produced from 3,3-dimethoxypropylaldehyde in the hydrolysate. Addition of *n*-hexylaldehyde, *n*-heptylaldehyde and benzaldehyde to the reaction mixture of ethanolamine gave 4-substituted derivatives **IIk-m**. Structures of new compounds **If**, **Ig** and **IIg-n** were rigorously confirmed (Table I, Scheme I and Scheme II).

These experiments demonstrated that several monofunctional aldehydes participated in the formation of fluorescent compounds in the reaction of the primary amines and malonaldehyde. The monofunctional aldehydes were introduced into the 4-position of the dihydropyridine skeleton and produced 4-substituted derivatives **II** instead of 4-methyl derivatives **I**.

Mass Spectrum of II

The most important mass fragmentation process of the 1,4-dihydropyridine derivatives is the formation of the aromatic pyridinium ion, which may take place by loss of the radical of the 4-substituents (13). Mass spectral fragmentations of **IIb-f** with a methyl group at the 1-position indicate that the fragmentation process involved the formation of the aromatic pyridinium ion by loss of an alkyl or aryl radical from the 4-position. An intense peak at m/e 150 corresponded to the stable pyridinium cation. Weak peaks at m/e 121 or 122 indicate successive loss of an aldehyde radical at the 3- or 5-position. The results are the same as those of **Ia** and **IIa** both bearing a methyl group at the 1-position (6). Fragmentations of **IIg-i** with a carboxylmethyl group at the 1-position revealed an intense peak at m/e 222 corresponding to the stable pyridinium cation formed by loss of a radical from the 4-position. A less intense peak at m/e 194 indicates successive loss of an aldehyde radical. Further loss by formation of an ethoxy radical from the 1-substituent may produce a peak at m/e 149. Loss of a pentyl radical from the 4-position of **IIj** produced a peak at m/e 397. Compounds **If** and **IIk-m** with a hydroxyethyl group at the 1-position lost radicals from the 4-position to reveal an intense peak at m/e 180. A less intense peak at m/e 136 indicates successive loss of the 1-substituent. Compounds **Ig** and **IIn** with a hydroxypropyl group at the 1-position revealed an intense peak at m/e 194 and a less intense peak at m/e 136, indicating loss of the 4- and the 1-substituents.

Fluorescence Spectrum of II

Representative fluorescence spectra of 4-substituted derivatives **II** measured in various solvents are shown in Figure 1, and the excitation and emission maxima are listed in Table II. Fluorescence maxima in 0.1 M phosphate (pH 7) were between 386-403 (excitation) and 444-465 (emission) nm. The maxima in organic solvents were shifted to shorter wavelength, and those in chloroform were between 382-398 (excitation) and 430-458 (emission) nm. The relative molar intensity of **IIb-f** with a methyl group at the 1-position was almost equal and comparable to the intensity of quinine sulfate, but the intensity of **IIg-i** with a carboxylmethyl group at the 1-position was lower than that of quinine sulfate. The intensity of **IIj-n** was comparable to that of quinine sulfate. The intensity of every compound in methanol and chloroform was lower than that in 0.1 M phosphate (pH 7).

Fluorescence spectra of **IIb-f** with a methyl group at the 1-position were measured in 0.1 N HCl and 0.1 N NaOH (Table III). While the intensity in 0.1 N HCl was markedly decreased to about 20-30%, that in 0.1 N NaOH did not change significantly. When these compounds were treated with sodium borohydride, fluorescence intensity was reduced to less than 30% of the initial intensity (Table III). These fluorescence properties of **II** were essentially similar to those of 4-methyl derivatives **I** (8).

Quantitative Estimation of the Fluorescent Products in the Reaction Mixtures of the Primary Amines, Malonaldehyde and the Monofunctional Aldehydes

The fluorescent products formed in the reaction mixtures of the primary amines, malonaldehyde and the monofunctional aldehydes were analyzed and determined by HPLC (Table IV). A reaction mixture of 50 mM methylamine,

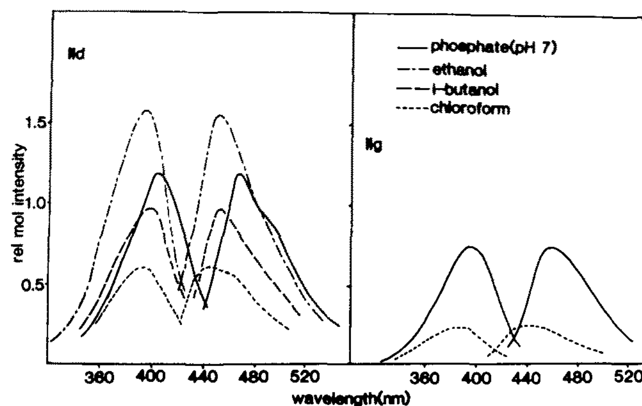


FIG. 1. Fluorescence Spectra of IId and IIg.

TABLE II

Fluorescence Data for I and II

Solvent	If			Ig			IIb			IIc			IId			IIe			IIf		
	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI
0.1 M phosphate (pH 7)	398	460	1.47	401	460	1.59	403	466	1.37	403	466	1.20	403	465	1.21	402	466	1.22	396	453	1.24
methanol	395	450	1.28	395	453	1.35	400	454	1.10	400	453	0.94	401	455	0.96	401	455	0.98	393	443	0.54
ethanol	392	448	1.36	394	450	1.73	396	449	1.56	398	452	1.41	397	451	1.56	398	450	1.49	391	443	0.72
i-butanol	393	448	1.47	395	449	1.51	399	452	1.27	398	452	1.17	398	447	1.11	398	449	1.13	393	441	0.53
chloroform	390	446	1.13	392	446	1.35	395	446	0.70	395	448	0.59	392	446	0.61	392	445	0.64	396	430	0.17

Solvent	IIg			IIh			IIi			IIj		
	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI
0.1 M phosphate (pH 7)	394	458	0.76	395	458	0.76	386	444	0.44	404	463	1.70
chloroform	382	440	0.25	386	440	0.24	398	452	0.05	390	458	0.84

Solvent	IIk			IIl			IIm			IIn		
	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI
0.1 M phosphate (pH 7)	402	464	0.99	403	464	0.96	395	452	0.84	399	461	1.49
chloroform	391	446	0.56	391	446	0.51	393	455	0.26	391	449	0.54

Ex: excitation maximum (nm). Em: emission maximum (nm). Relative molar intensity (RMI) of every compound was determined against quinine sulfate in 0.1 N sulfuric acid.

TABLE III

Alteration of Fluorescence Intensity of II

	Intensity		
	in 0.1 N HCl[a]	in 0.1 N NaOH[a]	treated with $NaBH_4$[b]
IIb	0.22	0.97	0.30
IIc	0.28	1.00	0.19
IId	0.25	1.00	0.16
IIe	0.25	0.93	0.12
IIf	0.21	1.03	0.20

[a]Intensity was expressed against that obtained in 0.1 M phosphate (pH 7).
[b]Sample was treated with 20 mM $NaBH_4$/methanol at room temperature for 5 min and diluted with methanol. Intensity was expressed against that obtained in methanol.

100 mM TMP hydrolysate and 100 mM acetaldehyde produced 23 mM (46%) of **Ia** and 7 mM (14%) of **IIa** (Table IV, lane A). The yield of **Ia** increased 8-fold and the of **IIa** decreased to 42% compared to those of the reaction in the absence of acetaldehyde. The addition of *n*-hexylaldehyde to the reaction mixture of methylamine and TMP hydrolysate produced **IId** in a high yield and inhibited the formation of **Ia** and **IIa** (Table IV, lane B). The addition of acetaldehyde to the reaction mixture of glycine and TMP hydrolysate accelerated the formation of **Id** (Table IV, lane C). The reaction of glycine and acetaldehyde also produced fluorescence, but the fluorescence spectrum was different and the intensity was significantly lower. From the experiments with various ratios of the reactants (Table IV, lane B and C), it was found that the optimal ratio of the primary amine, TMP hydrolysate and the aldehyde for the production of the fluorescent compounds was 1:2:1-2.

In order to clarify the effect of the monofunctional aldehydes, the reaction of methylamine was performed by use of the purified malonaldehyde (Fig. 2). The molar ratio of methylamine, purified malonaldehyde and the aldehyde was 1:2:2. The presence of acetaldehyde and *n*-hexylaldehyde resulted in almost quantitative conversion of methylamine into **Ia** and **IId**, respectively, while the reaction in the absence of the aldehyde converted only 10% of methylamine into **Ia** during the reaction period of 170 hr. Increase in fluroescence intensity of the reaction mixtures with the aldehyde was much higher than that without the aldehyde. While malonaldehyde produced red pigment absorbing at 532 nm by reacting with thiobarbituric acid, 4-methyl-1,4-dihydropyridine derivatives **I** were inert to the reaction (8). The effect of the aldehydes on the loss of malonaldehyde in these reactions was investigated by measuring malonaldehyde with thiobarbituric acid (14). Acetaldehyde and *n*-hexylaldehyde markedly accelerated the loss of malonaldehyde, indicating that they promoted derivatization of malonaldehyde into non-TBA reactive substances.

These results indicate that the monofunctional aldehydes greatly promoted the derivatization of malonaldehyde and the formation of the fluorescent 1,4-dihydropyridine-3,5-dicarbaldehydes.

DISCUSSION

The reaction of the primary amines, malonaldehyde and the monofunctional aldehydes gave fluorescent 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehydes **II**. The primary amines participated in the reaction included alkylamines, amino acids and alkanolamines, and the monofunctional aldehydes were the aliphatic aldehydes and benzaldehyde.

TABLE IV

Reaction of the Primary Amine, TMP Hydrolysate and the Monofunctional Aldehyde

Reaction mixture	Fluorescence of the reaction mixture			Product (mM)[e]			
	Ex(max)	Ex(max)	Intensity[d]	Ia	IIa	IId	Id
A. methylamine (mM)-TMP hydrolysate (mM)-acetaldehyde (mM)[a]							
50-100-0	405	465	15 × 10⁴	2.9	16.8		
50-100-100	405	465	28 × 10⁴	22.9	7.0		
B. methylamine (mM)-TMP hydrolysate (mM)-*n*-hexylaldehyde (mM)[b]							
10-20-0				0.8	1.5		
10-20-10				<0.05	<0.05	4.36	
10-20-20	405	465	17 × 10⁴	<0.05	<0.05	4.90	
10-10-20	405	465	3 × 10⁴	<0.05	<0.05	2.73	
10-5-20				<0.05	<0.05	1.05	
C. glycine (mM)-TMP hydrolysate (mM)-acetaldehyde (mM)[c]							
10-20-0	399	460	2.8 × 10⁴				1.0
10-20-10	398	459	7.5 × 10⁴				5.0
10-20-20	398	460	8.6 × 10⁴				5.3
10-10-20	398	460	5.0 × 10⁴				2.9
10-0-20	395	436	1.05[f]				

[a]Reaction mixture in 0.1 M phosphate (pH 7) was incubated at 37 C for 96 hr.
[b]Reaction mixture in 30% methanol-0.1 M phosphate (pH 7) was incubated at 37 C for 100 hr.
[c]Reaction mixture in 0.1 M phosphate (pH 7) was incubated at 37 C for 52 hr.
[d]Relative intensity against 0.1 μM quinine sulfate in 0.1 N sulfuric acid.
[e]Determined by HPLC.
[f]The mixture produced the different fluorescent compound(s) with low intensity; the retention time of the fluorescent peak was 3.9 min while that of **Id**, 2.7 min.

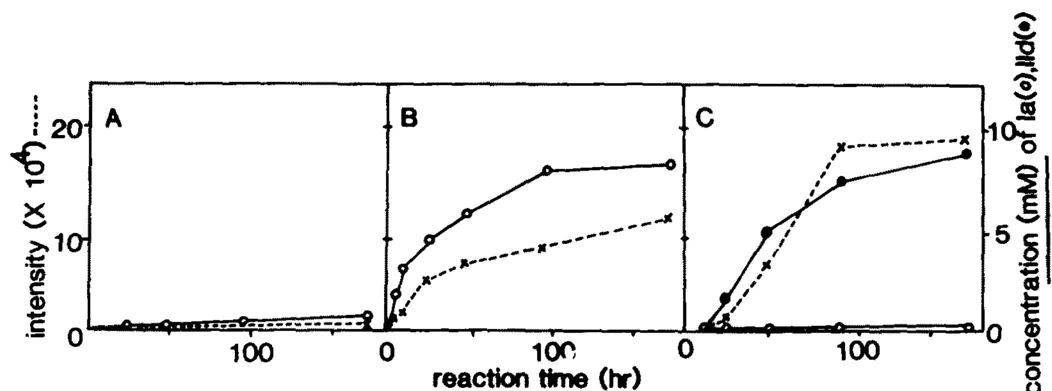
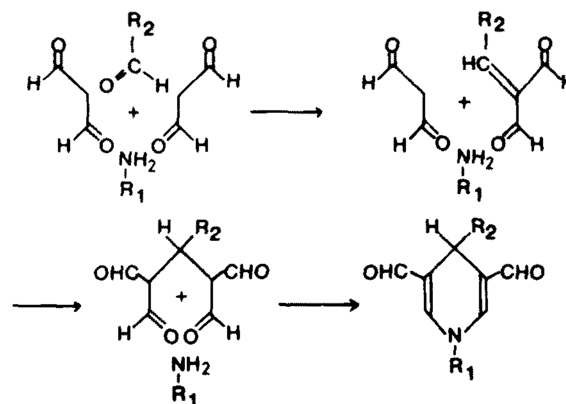


FIG. 2. Formation of Ia and IId by Reaction of Methylamine with Malonaldehyde/Acetaldehyde or Malonaldehyde/n-Hexylaldehyde. Reaction mixtures of 10 mM methylamine and 20 mM MDA·Na in 0.1 M phosphate (pH 7)(A); 10 mM methylamine, 20 mM MDA·Na and 20 mM acetaldehyde in 0.1 M phosphate (pH 7)(B); and 10 mM methylamine, 20 mM MDA·Na and 20 mM n-hexylaaldehyde in 30% methanol-0.1 M phosphate (pH 7) (C) were incubated at 37 C. Concentrations of the products Ia and IId were determined by HPLC. Fluorescence intensity of the reaction mixture was measured at excitation of 405 nm and emission of 465 nm against the intensity of 0.1 μM quinine sulfate.

The optimal ratio of the primary amine, malonaldehyde and the aldehyde was 1:2:1-2, at which the fluorescent compounds **II** were produced almost quantitatively. The mechanisms of the reaction may be explained by the Hantzsch type reaction (13) shown in Scheme III. One mole of the amine, 2 moles of malonaldehyde and one mole of the aldehyde may react to form the stable dihydropyridine skeleton. When the primary amine was treated separately with malonaldehyde or the aldehyde, fluorescent substance(s) also were produced, but their formation was much lower. For instance, reaction of glycine and acetaldehyde produced fluorescence, but the fluorescence intenwity was extremely low with different fluorescence maxima (Table III). Formation of fluorescence was maximized when the three reactants were combined.

The reaction of methylamine and the purified malonal-



SCHEME 3

dehyde gave **Ia**, the same product as that of the reaction in the presence of acetaldehyde. But the rate of formation was much lower than that in the presence of acetaldehyde (Fig. 2A and B). At first it was considered that acetaldehyde was contaminated in the preparation of malonaldehyde. However, this possibility was not supported by measurement of NMR spectrum of the purified malonaldehyde (8). Therefore, the reasons for production of 4-methyl derivatives **I** in the reaction of primary amines and malonaldehyde are still unknown.

Many investigators have suggested that lipid peroxidation produces malonaldehyde (4,5) on the basis of the formation of the red pigment absorbing at 532 nm in the thiobarbituric acid-reaction (15). Various aldehydes including alkanals, alkenals and alkadienals also were produced in peroxidized lipids (10-12). It has been suggested that lipofuscin pigment is produced by the reaction of proteins and peroxidized lipids (1). While the structure of lipofuscin pigment has not yet been elucidated, its structure was supposed to be the 2:1-conjugated Schiff base between the primary amine and malonaldehyde (2). Our recent studies demonstrated that the reaction of the primary amines and malonaldehyde produces 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehydes **I** as major fluorescent compounds (6-9). Present experiments suggest that peroxidized lipids containing both malonaldehyde and other aldehydes could react more readily with proteins to produce highly fluoresent 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehydes **II**.

Fluorescence spectra of 4-substituted derivatives **II** showed excitation maxima at 386-403 nm and emission maxima at 444-465 nm in phosphate, which were quite similar to 4-methyl derivatives **I** (8). The spectra of **I** and **II** were roughly similar to those of lipofuscin pigment (16). However, the fluorescence properties of **I** (8) and **II**, showing diminished intensity in the acidic medium, were different from those of lipofuscin pigment whose fluorescence intensity was quenched in the alkaline medium and not in the acidic medium (17). Several studies on the reaction of primary amines and peroxidized fatty acids (9, 18-21) demonstrated that fluorescence spectra of the reaction mixtures showed excitation and emission maxima with shorter wavelength than those of **I** and **II**. Fluorescence of the products derived from the reactions of methylamine and peroxidized fatty acids showed different characteristics (9).

The mixtures of the primary amines, malonaldehyde and the monofunctional aldehydes readily produced 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehydes **II**. It was found that the reaction of the primary amines in which malonaldehyde participated produced no other fluorescent compounds than 1,4-dihydropyridine-3,5-dicarbaldehydes **I** and **II**. These fluorescent compounds may be useful as model compounds for elucidation of the structure lipofuscin pigment.

ACKNOWLEDGMENT

T. Kurechi provided the opportunity to carry out this research, and T. Suwa provided technical assistance.

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[Received March 26, 1984]