Studies of the Fluorescent Products of Lipid Oxidation in Aqueous Emulsion with Glycine and on the Surface of Silica Gel

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ABSTRACT AND SUMMARY

The formation of fluorescent products in the reaction of methyl linoleate hydroperoxide with glycine in aqueous emulsions correlated directly with the decrease in diene conjugation and the increase in thiobarbituric acid (TBA) reactive substances. These correlations also were reflected in the course of the oxidation of methyl linoleate in aqueous emulsions with glycine and indicated that glycine reacted with products of peroxide decomposition as opposed to intermediates of autoxidation in hydroperoxide formation. Thin layer chromatography (TLC) and selective solvent extraction demonstrated that the products of the reaction contained many substances with a fluorescent spectrum similar to those of lipofuscin pigments. When methyl esters of polyunsaturated fatty acids or other polyunsaturated lipids underwent oxidation adsorbed on silica gel particles, products with similar fluorescent spectral properties were formed illustrating that fluorescent substances were formed in a variety of reactions associated with the oxidation of unsaturated lipids.

INTRODUCTION

Lipid peroxidation is generally regarded as the underlying reaction in the formation of aging or lipofuscin pigments that accumulate in the cells of certain organs with age (1-8). These pigments exhibit a characteristic fluorescent spectrum with a maximum wavelength between 430-490 nm (8-10). Compounds with a similar fluorescent spectrum are formed upon the autoxidation of lipids in emulsions with proteins, amino acids, nucleic acids and some phospholipids (11-15). They also are formed by the interaction of malonaldehyde with a variety of model compounds containing amino groups leading to the suggestion that fluorophore of lipofuscin pigments is a conjugated Schiff base (14-16).

The oxidation of unsaturated methyl esters and other lipids adsorbed on the surface of adsorbents have been studied extensively (17-20), but no observations appear to have been made on the fluorescent properties of the products of the reaction. The difficulty of recovering polyunsaturated lipids separated by thin layer chromatography on chromatoplates of silica gel is well known and is generally attributed to irreversible adsorption due to partial oxidation of these compounds during the chromatographic process or subsequent handling of the plates. By chance it was observed that spots of polyunsaturates inadvertently exposed to the atmosphere overnight on chromatoplates exhibited a strong fluorescence when viewed under ultraviolet light.

Reported here are studies of the formation of fluorescent substances upon the incubation of an aqueous emulsion of methyl linoleate hydroperoxide and glycine and upon the autoxidation of methyl linoleate under similar conditions. Also reported are observations of the formation of products exhibiting fluorescent properties upon the atmospheric oxidation of polyunsaturated lipids adsorbed as spots on chromatoplates of silica gel.

EXPERIMENTAL PROCEDURES

Materials and Methods

Highly purified (>99%) methyl linoleate and methyl arachidonate were obtained from the Lipids Preparation Laboratory of The Hormel Institute (Austin, MN). Highly purified methyl linoleate hydroperoxide was isolated from autoxidized methyl linoleate as previously described (21). The peroxide value of the preparation was 6200 me/kg vs. a theoretical value of 6135 for the pure compound.

Lipid emulsions were prepared by a slight modification of the procedure described by Corliss and Dugan (22) as follows. A 0.1 ml aliquot of 0.1 M lipid solution in chloroform was pipetted into a sonication tube. The solvent was evaporated in a stream of nitrogen and 10 ml of 0.1 M borate buffer, pH 7.2, containing 4×10^{-1} moles of glycine, 1×10^{-3} moles of ferric chloride and 2×10^{-3} moles of sodium dodecyl sulfate, was added to the tube. The mixture was emulsified by two 30 sec sonications in an ice-cooled water bath with a sonicator (Sonifier Cell Disruptor Model W140D, Ultrasonics Inc., Long Island, NY).

As controls, solutions without lipid or without glycine were prepared in the same manner. Aliquots of 0.5 ml of the emulsion were incubated at 50 ± 2 C in small test tubes (1.0×10 cm) and extracted with 3.0 ml of ethanol/ether 3:1 v/v by vigorous mixing on a vortex mixer followed by centrifugation for 10 min at 3000 rpm which sedimented particulate material at the bottom of the tube. The fluorescent and ultraviolet spectra of the solutions were measured with an Aminco-Bowman spectrophotofluorometer and a Beckman Model DU spectrophotometer, respectively.

The thiobarbituric acid value (TBA) was determined by a slight modification of the method of Wilbur et al. (23), as follows. An aliquot of 0.5 ml of the emulsion was mixed with 2 ml of 0.67% 2-thiobarbituric acid and 1 ml of 20% trichloroacetic acid and placed in a boiling water bath for 10 min. After the solution cooled to room temperature, it was centrifuged for 20 min at 10,000 rpm and the absorbance of the supernatant phase determined at 535 nm.

For studies of the oxidation of lipids adsorbed on silica gel particles, methyl linoleate and methyl arachidonate, containing methyl palmitate as an internal standard for quantitative analysis, as well as lipid extracts obtained from rat testes, egg yolk, and pig brain were chromatographed on ethyl ether washed layers (0.3 mm) of Silica Gel H 10-40 μ, diameter, (Merck Ag., Darmstadt) spread on glass plates in one-dimension with solvent systems that separated the major components into discrete spots.

In these experiments sufficient sample was applied to the plates to insure at least 20 μg of the major components in each spot. The neutral lipids were chromatographed in a solvent system of petroleum ether/ethyl ether/acetic acid 85:15:1 v/v/v and the polar lipids chloroform/methanol/acetic acid/water 65:25:8:4. Under these conditions it was assumed in accordance with the recent studies of Porter et

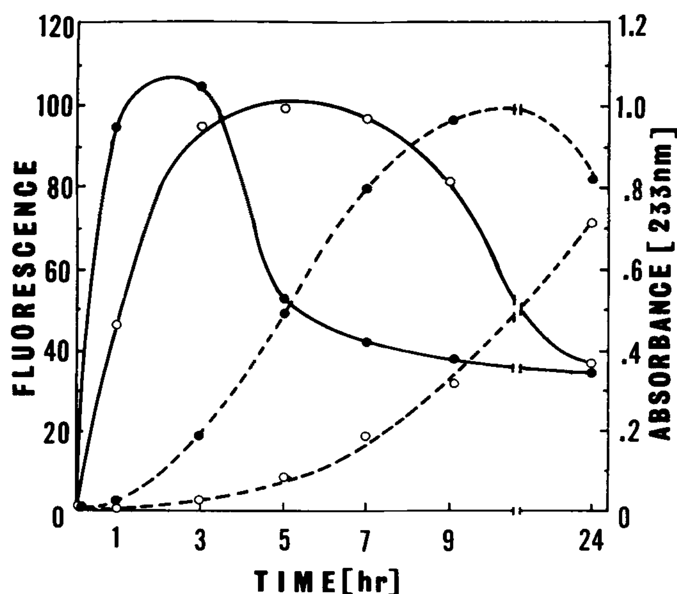

FIG. 1. Formation of fluorescence products (—) and diene conjugation (------) during linoleate oxidation in borate buffer, pH 7.2, emulsified with glycine. Maximum fluorescence intensity (% T) was measured at 435-440 nm with excitation max. 360-365 nm. Solid dots, ferric chloride used as catalyst; open circles, no catalyst.

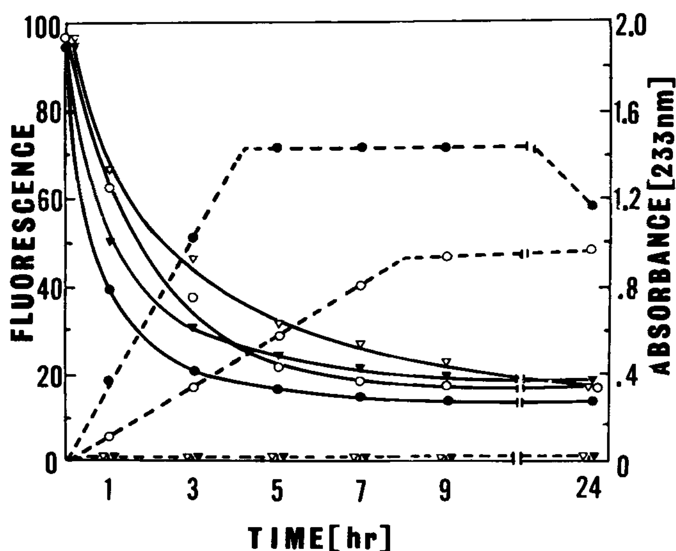

FIG. 2. Production of fluorescence (—) and decomposition of diene conjugation (------) during oxidation of purified linoleate hydroperoxide with glycine (circle) and without glycine (triangle) in emulsions. Maximum fluorescence intensity (% T) 435-440 nm with excitation max. 360-365 nm. Solid circles and triangles, ferric chloride used as a catalyst; open circles and triangles, no catalyst.

al. (18), and earlier work (19,20) that the substances were adsorbed on the surface of the silica gel particles in monolayers inasmuch as the ratio of compound to adsorbent was less than that calculated for a monomolecular film. The plates were exposed to the atmosphere in the dark for periods up to 1 wk. Spots that fluoresced when viewed under ultraviolt (UV) light were circled. At the end of the experiment all of the spots on some plates were made visible by charring them in an oven at 180 C after spraying the plates with chromic-sulfuric acid (24). The spots were identified from their R_f values by comparison with authentic compounds chromatographed on the same or duplicate plates.

For the determination of fatty acid composition of the lipid classes, spots were scraped directly into vials where they were interesterified under an atmosphere of nitrogen with methanol using HCl as a catalyst (25). Gas liquid chromatography (GLC) was carried out with a Barber Colman gas chromatograph equipped with a flame ionization detector using a 6 ft x ¼ in. column of 15% EGSS-X on 100-120 mesh Gas Chrom P (Applied Science Lab., Inc., PA) at 180 C using N_2 as the carrier gas. Fluorescence spectra in this experiment was also determined with an Aminco-Bowman spectrophotofluorometer with 1 μg/ml quinine sulfate in 0.1 N H_2SO_4 solution as a standard.

RESULTS

The relationship between the formation of fluorescent substances and the autoxidation of methyl linoleate in emulsions with glycine (Figure 1) showed that the formation of fluorescent substances was associated with a decrease in diene conjugation. In order to obtain further information on the reaction, experiments were carried out directly on incubations of highly purified preparations of methyl linoleate hydroperoxides under the same conditions (Figure 2). These experiments showed that as the diene conjugation decreased, the formation of fluorescent substances increased. The diene conjugation also decreased at approximately the same rate in incubations that contained no glycine; however, no fluorescent products were formed in the reaction. Decomposition of the hydroperoxides in incubations with glycine (Figure 2) was also indicated by an increase in TBA values which correlated directly with the decrease in diene conjugation, as illustrated in Figure 3A. The increase in fluorescence also correlated directly with the decrease in diene conjugation and the increase in TBA values as shown in Figure 3B and 3C, respectively. These results indicated that the fluorescence substances were formed upon reaction of glycine with products of the decomposition of hydroperoxides.

Extraction of the incubation mixture with different solvents showed that the fluorescent material was almost completely extracted with ethyl ether/alcohol 1:3 v/v, but that only a part of it was extracted with chloroform (Table I). The values obtained with ethyl ether alcohol solutions cannot be compared quantitatively to those in the aqueous buffer solutions because of quenching effects of the organic solvent. However, these results and those of thin layer chromatography (TLC) analysis of the extracts (Figure 4) showed that the fluorescent material consisted of a mixture of substances of different polarities. In the TLC analyses shown in Figure 4, the areas of fluorescence were marked in the silica gel immediately after the plate was developed. In order to identify the major components the plates were sprayed with chromic-sulfuric acid and heated at 180 C to char the spots. There was considerable streaking of the fluorescent material on the plate, but two-dimensional TLC showed that the large number of fluorescent substances was not due to secondary decomposition on the plate during chromatography.

Although the presence of glycine was required for the production of fluorescent substances in the above experiments, it was found that substances with fluorescent properties were produced when polyunsaturated methyl esters or lipid containing polyunsaturated fatty acids were allowed to oxidize on the surface of silica gel separated as spots on chromatoplates as illustrated in Figure 5. In these experiments, after the components were separated the chromatoplates were stored in the dark at room temperature for varying periods of time up to 1 wk. The spots that emitted a fluorescence when viewed under ultraviolet light were marked; for identification some plates (Figure 5) were sprayed with sulfuric acid and heated at 180 C to char all of the spots. Only those spots that contained polyunsaturated fatty acids emitted a fluorescence when viewed under ultraviolet light. Spots that charred but

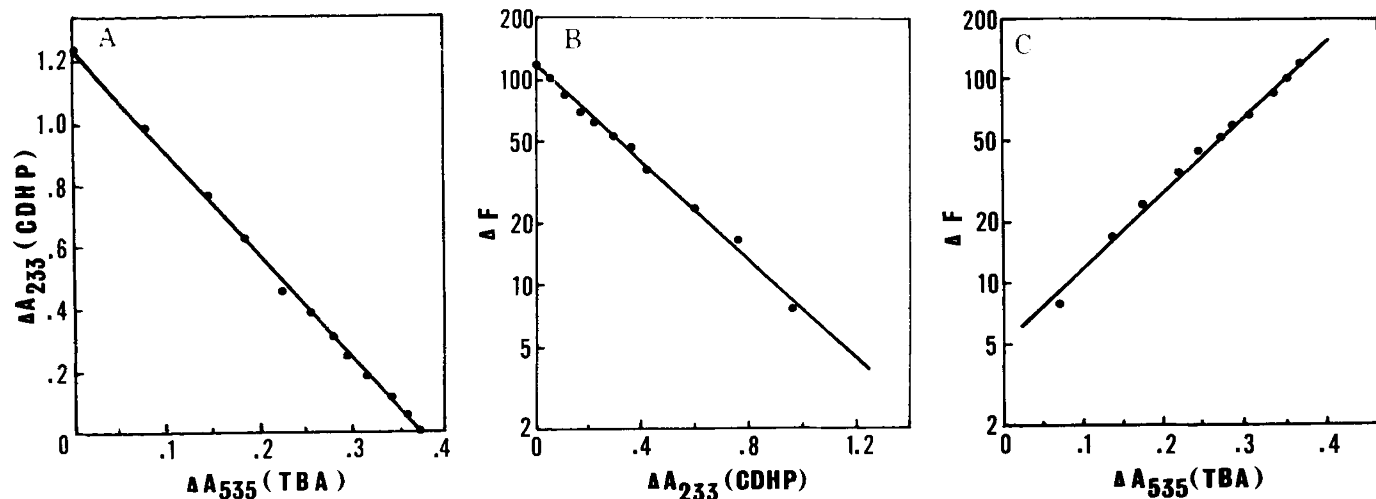

FIG. 3. Relationships between (A) absorbance of conjugated diene hydroperoxide (CDHP) at 233 nm and TBA reactive substances at 535 nm, between (B) fluorescence intensity (% T, max. Em 435-440 nm, Ex. 360-365 nm) and absorbance (CDHP), and between (C) fluorescence intensity and TBA reactive substances during reaction of purified linoleate hydroperoxide with glycine in emulsion incubated at 50 C for period up to 7 hr.

TABLE I

Solvent Extraction of Fluorescent Products of the Reaction of Purified Methyl Linoleate Hydroperoxide with Glycine in Aqueous Emulsions

Incubation at 50 C (hr)	Chloroform		Ethyl ether/ethanol (1:3 v/v)	
	Extract (3 ml)	Extracted[c] buffer	Extract (3 ml)	Extracted[c] buffer
2	0.013[a,b]	6.05	1.90	0.35
5	0.055	9.20	3.08	0.32
10	0.083	8.97	3.12	0.57
22	0.464	4.83	2.76	0.41

[a]Average of duplicate analysis on aliquots of each reactive mixture.

[b]Relative to 1.0 for 1 μg/ml quinine sulfate in 0.1 N H_2SO_4 which gave an intensity (% T) 24 with a meter multiplier setting of 0.3.

[c]Volume, 3 ml of 0.1 M borate buffer pH 7.2 for measurement.

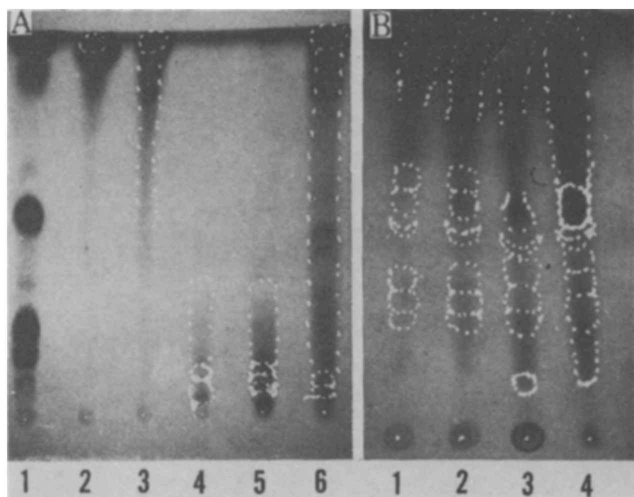

FIG. 4. (A). Thin layer chromatography of (1) reference lipid mixture (diphosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin); (2 and 3) chloroform lipid extracts, of incubation mixture (5 and 22 hr at 50 C) of purified linoleate hydroperoxide with glycine; (4 and 5) nonchloroform soluble fraction of samples (2) and (3); (6) ethyl ether, ethanol extracts of sample (3). Dotted lines show fluorescent substances observed under UV light before charring. Solvent system: chloroform/methanol/water (65/25/4). (B) Thin layer chromatography of fluorescent products during oxidation of linoleate hydroperoxide with glycine in aqueous emulsions. Samples were extracted with ethyl ether/ethanol (1:3 v/v) after incubation of (1) for 2 hr, (2) for 5 hr, (3) for 10 hr, and (4) for 22 hr at 50 C. Solvent system: chloroform/methanol/water (50/45/5).

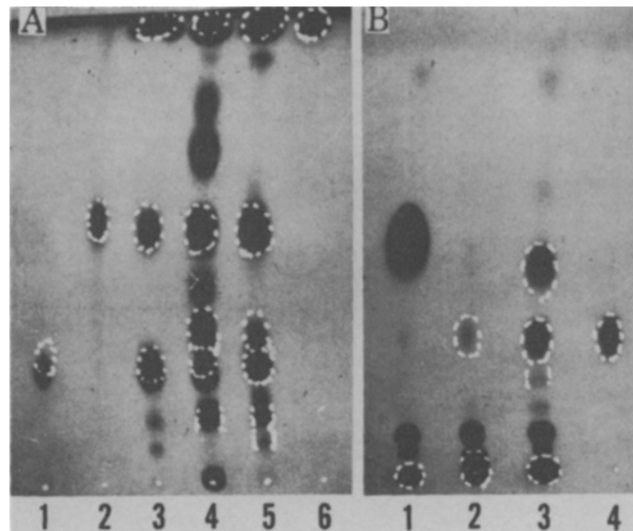

FIG. 5. Fluorescent and nonfluorescent substances of lipids exposed to air for 48 hr on silica gel. A. Solvent system; chloroform:methanol:acetic acid:water (65/25/8/4), (1) 20 μg of phosphatidyl choline, (2) 20 μg of phosphatidyl ethanolamine, (3) 400 μg of egg yolk lipid, (4) 400 μg of pig brain lipids, (5) 400 μg of rat testes lipid, (6) 20 μg of arachidonic acid. B. Solvent system: petroleum ether/ethyl ether/acetic acid (85/15/1), (1), (2), (3) and (4) are the same as (3), (4), (5) and (6) in A, respectively.

which were not circled did not contain any fluorescent material. Only a portion of some spots exhibited fluorescence indicating that fractionation of unsaturated constit-

TABLE II

Fatty Acid Analysis of Testicular Phospholipids after Exposure to Air for 48 hr on Silica Gel (% wt)

Fatty acid	PE[a]		PC[b]	
	Control	Exposed	Control	Exposed
16:0	32.51 ± 0.76[c]	45.52 ± 0.56	43.96 ± 0.44	50.42 ± 0.29
18:0	10.95 ± 0.16	13.10 ± 0.47	8.55 ± 0.11	9.74 ± 0.11
18:1	7.94 ± 0.15	6.46 ± 0.18	15.82 ± 0.19	14.86 ± 0.23
18:2	2.09 ± 0.04	1.51 ± 0.08	4.06 ± 0.03	3.41 ± 0.06
20:4	21.14 ± 0.44	16.49 ± 0.25	13.31 ± 0.31	10.26 ± 0.13
22:5	25.38 ± 0.88	16.94 ± 0.53	14.30 ± 0.40	11.32 ± 0.56

[a]PE = phosphatidyl ethanolamine.
[b]PC = phosphatidyl choline.
[c]M ± SD (4 experiments).

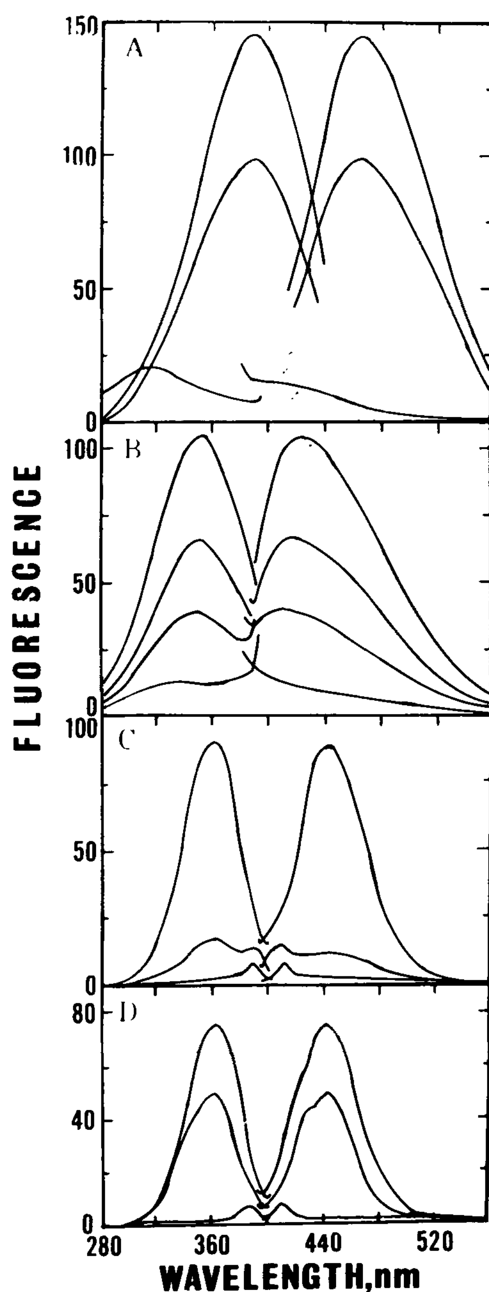

FIG. 6. Excitation and fluorescence spectra of: (A) methyl arachidonate and (B) methyl linoleate exposed to air on silica gel for zero, 48, and 96 hr, and for zero, 48, 96 hr and 1 wk, respectively, (C) linoleate and (D) purified linoleate hydroperoxide incubated with glycine at 50 C in aqueous emulsions for zero, 8, and 24 hr. Meter multiplier, 0.03; sensitivity, 50.

uents frequently occurred within the spot. That the polyunsaturated fatty acids were involved in the reaction that gave substances with fluorescent properties was indicated by determination of the fatty acid composition of phosphatidylethanolamine and phosphatidylcholine before and after oxidation upon exposure to the atmosphere (Table II).

Methyl linoleate and methyl arachidonate oxidized under these conditions exhibited strong fluorescent properties in ca. 48 hr (Figure 5). The fluorescence spectra of recovered spots of these esters are compared to those obtained upon the reaction of methyl linoleate hydroperoxide with glycine in Figure 6. These results showed that the maximum of the spectrum shifted to the longer wavelengths with increasing unsaturation. Determination of the amount of unoxidized material in spots of arachidonate (Table III) showed that the fluorescent substances were produced virtually simultaneously with oxidation. Comparison of the amount of hydroperoxide present in the sample on the basis of diene conjugation with the amount of sample oxidized indicated that the hydroperoxides were rapidly decomposed with the formation of fluorescent compounds.

DISCUSSION

The present study shows that even with simple model systems such as emulsions of methyl esters of polyunsaturated fatty acids or hydroperoxides with glycine, a large number of fluorescent compounds of different polarities (structures) are formed. The experiments with methyl linoleate hydroperoxide indicate that the fluorescent compounds are formed by the interreaction of glycine with products of hydroperoxide decomposition. It has been demonstrated with model systems that malonaldehyde, which is a product of peroxide decomposition, reacts with glycine to form a conjugated Schiff base with spectral characteristics similar to those of lipofuscin pigments (14,16,26). In the present study, the linear correlation of the decrease in diene conjugation with the increase in fluorescence demonstrates a direct relationship between peroxide decomposition and formation of fluorescent substances. The linear correlation of the increase in TBA value with an increase in fluorescence is more difficult to interpret and demonstrates the complexity of the reaction. However, the condensation product of malonaldehyde with glycine as well as malonaldehyde with itself and other aldehydic products of peroxide decomposition give a positive TBA reaction (27,28). Thus, it appears that the TBA value increases because both the products of the decomposition of peroxides and the fluorescent substances give a positive TBA test. The fact that many fluorescent products of different structures are formed also indicates that the reaction is highly complex. Recently it has been suggested (29,30) that in spite of the fact that lipofuscin

TABLE III

Oxidation and Production of Fluorescence of Methyl Arachidonate on Silica Gel[a]

Exposure time (hour)	GLC analysis[b]		Diene conjugation		Fluorescence intensity[d]
	Unoxidized (%)	Oxidized (%) by difference	ΔA233	Hydroperoxide[c] (%)	ΔF
0	100	0	0	0	0
48	99.4	0.6	0.080	0.7	0.013
96	95.8	4.2	0.260	2.3	0.035
168	93.6	6.8	0.360	3.2	0.061

[a]Average value of duplicate experiments.

[b]Determined relative to methyl palmitate used as an internal standard.

[c]Calculated on the basis of a specific coefficient of absorbtivity for a conjugated diene hydroperoxide of 77.

[d]Relative to fluorescence intensity of 1 μg/ml quinine sulfate in 1N H_2SO_4 equal to 1.0.

pigment gives a single strong maxima in the region of 470 nm of the spectrum, it also consists of a complex mixture of compounds.

The formation of fluorescent substances upon the oxidation of polyunsaturated lipids on silica gel has not been observed previously. However, Shanfield et al. (31) has shown that fluorescence can be induced in a variety of organic substances separated as spots on chromatoplates of silica gel by subjecting them to gaseous electrical discharge. Under these conditions, as well as in the method used by Segura and Gotto (32) to produce fluorescence in spots separated on chromatoplates, excited molecules, atoms, and free radicals are produced that react with the adsorbent inasmuch as it is essential for the formation of fluorescent products. Although the conditions in the present study are much milder and accordingly fluorescence is produced only in polyunsaturated lipids, the reactions are analogous inasmuch as free radicals and excited molecules are also produced upon the decomposition of hydroperoxides. No fluorescence substances were produced in the autoxidation of pure polyunsaturated methyl esters in the absence of silica gel as well as glycine. Hence, the adsorbent is undoubtedly involved in the formation of the fluorescence substances under the conditions employed here also. Fluorescence under these conditions may be regarded as an indication of lipid oxidation just as it is in in vivo oxidation in biological systems in which it is believed to be due primarily to the formation of a modified Schiff base (10,33). Obviously, a variety of fluorescent substances may be formed in reactions associated with the oxidation of unsaturated lipids.

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