Water-soluble products of UV-irradiated, autoxidized linoleic and linolenic acids

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ABSTRACT The water-soluble products of the UV-initiated autoxidation of linoleic and linolenic acids emulsified in water were separated into volatile and relatively involatile components, each of which reacted with both thiobarbituric acid (TBA) and peroxidase. The volatile TBA-reactive compound is probably malonaldehyde and the volatile peroxidase-reactive compound is hydrogen peroxide. Additional compounds which absorb UV light were present in the volatile fraction.

After thin-layer chromatography of the involatile fraction, reactivity toward TBA and peroxidase was found in the same spot. Approximate molar yields of hydrogen peroxide, malonaldehyde, "hydroperoxides", and other TBA-reactive compounds were estimated. The ratio of "hydroperoxide" to TBA reactivity was lower for linoleic than for linolenic acid.

The mass of relatively involatile compounds was about 20 times greater than that predicted from either peroxidase or TBA assays of water extracts of oxidized linolenic acid. The properties of the water extract were similar to those shown by others for the products of prolonged autoxidation (without UV-irradiation) of emulsified methyl linoleate.

KEY WORDS polyunsaturated fatty acids · autoxidation · water-soluble products · thiobarbituric acid reactivity · hydrogen peroxide · hydroperoxides · metabolic inhibitors · UV-irradiation

After UV-irradiation of methyl linoleate or linolenate, aqueous extracts of these lipids contain a substance or substances that inhibit oxygen consumption and aerobic and anaerobic glycolysis of tumor cells. Shuster observed (1) that these extracts react with thiobarbituric acid (TBA) and attempted to correlate their TBA reactivity with inhibition of tumor respiration.

Abbreviations: TBA, thiobarbituric acid; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

We have carried out a number of experiments, based upon Shuster's observations, in which we try to establish, first, whether irradiated linoleate and linolenate yield a single TBA-reactive substance in common. Second, we have attempted to learn the chemical nature of the latter compound(s). Third, we have tried to determine whether, among the oxidation products, a TBA-reactive material, or some other substance which does not react with TBA, inhibits tumor respiration. The present paper is concerned with the separation and partial characterization of at least 17 different substances in the water extract of UV-irradiated linoleic and linolenic acids.

METHODS

Sealed vials of linoleic and linolenic acids (A grade, California Corporation for Biochemical Research, Los Angeles) were stored at −16°C. GLC of methylated linoleic acid revealed no contaminating linolenic (or higher unsaturated long-chain) acid.

Aliquots (1 ml) of linoleic or linolenic acid were emulsified in 16 ml of water by mechanical shaking for 4 min in glass-stoppered tubes. The emulsion did not separate during the subsequent 2 hr even though no stabilizer was added. Either 4 or 16 ml of emulsion was then transferred to Petri dishes (5.7 or 8.9 cm in diameter, respectively) under a Hanovia Utility Model mercury arc rated at an intensity of more than 250 $\mu w/cm^2$ at 50.8 cm distance for wavelengths of 3130 A and shorter (1). The distance from the light source was 8.6 cm and time of irradiation 90 min. The temperature of the emulsion was between 24° and 31°C. Control samples of the emulsion were exposed simultaneously to air but in the absence of appreciable UV light.

After the irradiation, 4 ml of emulsion was transferred with water to a glass-stoppered centrifuge tube. The

total volume was made up to 30 ml by the addition of water and the emulsion extracted with petroleum ether (bp 60–110°C; 2 × 20 ml). The phases were separated by centrifugation (900 × *g* for 10 min) and the extracts were discarded. The aqueous emulsion, containing some trapped petroleum ether, was stored at −16°C overnight, thawed the next day, and recentrifuged. A relatively clear water phase separated from the remaining petroleum ether, which was removed by aspiration and discarded. The washed aqueous extracts of four tubes were then combined to give approximately 120 ml (representing 1 ml of UV-irradiated fatty acid) and then divided into two portions: 10 ml for analysis and about 110 ml for flash evaporation (outer bath at approximately 35°C) to complete dryness (or, in earlier experiments, to approximately 20 ml).

The residual dried material was taken up in either 1 ml of 95% ethanol or 5 ml of water and centrifuged to remove insoluble material. When centrifugation did not suffice to remove the insoluble material, ultrafiltration through Millipore filters was used. In numerous instances the previously flash-evaporated, redissolved material was placed in a Thunberg tube, and residual volatile substances were removed by sublimation (2) to dryness at reduced pressure (2.5 mm Hg). The initial extract, which was frozen prior to evacuation of the Thunberg tube, was placed at ambient temperature so that volatile substances would sublime into the other arm of the Thunberg tube (placed in liquid nitrogen). In several instances portions of the unconcentrated aqueous extract of irradiated linolenic or linoleic acid were concentrated directly in the Thunberg tube to determine the percentage of volatile peroxidase-reactive and TBA-reactive compounds.

Peroxidase-reactive material was measured before or after treatment with catalase either by the use of Glucostat (Worthington Biochemical Corporation, Freehold, N.J.) which contains peroxidase, glucose oxidase, and *o*-dianisidine hydrochloride or by horseradish peroxidase and *o*-dianisidine in the concentrations used by Kingsley and Getchell (3). These methods were subsequently modified (4) so that 0.010 μmole (1 ml final volume) of H_2O_2-oxidized dye, when acidified, gave an OD of 0.11 at 400 mμ. Molar concentrations of H_2O_2 and "hydroperoxide" were calculated on this basis. The presence or absence of glucose oxidase did not influence appreciably the assay either of H_2O_2 or of the "hydroperoxide" formed by UV-irradiation of linoleic or linolenic acid. ("Hydroperoxide" is defined here as the material which oxidizes *o*-dianisidine in the presence of peroxidase but which is not removed by catalase.) Beef liver catalase (A grade, C. F. Boehringer & Soehne, GmbH, Mannheim, Germany) was dissolved in Krebs-Ringer phosphate buffer pH 7.4 (5) and added to samples (0.20 μg of catalase per ml, final concentration) which were also dissolved in the same buffer. Catalase-catalyzed reactions proceeded for 5–30 min at ambient temperature and were stopped by placing tubes in a boiling water bath for 5 min. Absence of H_2O_2 or presence of "hydroperoxide" was then measured by addition of dye and peroxidase (15 min, ambient temperature). Earlier experiments established that neither H_2O_2 nor the "hydroperoxide" present in the extract was destroyed during 5 min in a boiling water bath.

TBA-reactive compounds were assayed by adding 1.3 ml of 0.67% 2-thiobarbituric acid and 0.7 ml of 20% trichloroacetic acid to 1 ml of sample, heating 15 min in a boiling water bath, and reading OD at 535 mμ (6). To conserve material and for convenience we frequently performed these assays by adding 2.0 ml of 0.45% TBA in 6.7% trichloroacetic acid to 1.0 ml of the acidified reaction mixture which had been used for assay of hydroperoxides and H_2O_2. The yellow color due to the presence of oxidized dianisidine disappeared during the heating procedure of the TBA assay. Data are expressed in terms of equivalent micromoles of conjugated aldehyde (7) ($\Sigma = 1.4 \times 10^5$ at 535 mμ, with malonaldehyde generated from its tetraethyl acetal as a reference).[1] All colorimetric assays were performed with a Beckman DU spectrophotometer and cuvettes having a 1 cm light path. UV spectra (Beckman Model B) of water extracts of UV-irradiated linoleic and linolenic acid were determined after addition of HCl or NaOH to give the desired pH, with water similarly adjusted with acid or alkali used as a blank.

Involatile water- and ethanol-soluble oxidation products of irradiated linoleic and linolenic acids were chromatographed (after having been dried at reduced pressure and redissolved) by TLC on Silica Gel G. After development in butanol–ethanol–acetic acid (4), the chromatograms were examined under UV light, and then some were sprayed with a colorimetric reagent (anisaldehyde) which has been useful in detecting a variety of aldehydic substances (4, 8).

Peroxidase-reactive and TBA-reactive substances were assayed in water-eluates from various regions of developed chromatoplates which had not been sprayed with anisaldehyde.

RESULTS

After exposure of aqueous emulsions of di- or trienoic fatty acids to UV light and air, significant quantities of water-soluble TBA- and peroxidase-reactive compounds

[1] This assay may be used as a minimum estimate for conjugated aldehydes. Most of these compounds form derivatives with TBA which have lower molar extinction coefficients than that of malonaldehyde (7).

TABLE 1 FORMATION OF WATER-SOLUBLE TBA-REACTIVE AND PEROXIDASE-REACTIVE COMPOUNDS AFTER IRRADIATION OF LINOLEIC AND LINOLENIC ACIDS UNDER ULTRAVIOLET LIGHT IN AIR

Expt.	Acid Irradiated	Amount	Conjugated Aldehyde Formed*	"Hydroperoxide" + H_2O_2 Formed†
		μl	*μmoles/mmole fatty acid*	
1	Linoleic	1200	0.13	0.7
2		1100	0.13	1.6
3		1150	0.10	1.5
4		1200	0.13	1.8
5		1000	0.25	2.8
6		1000	0.18	2.5
7		2100	0.10	1.2
Mean			0.15	1.7
8	Linolenic	250	—	6.7
9		250	—	7.6
10		250	—	6.6
11		250	3.4	7.8
12		250 × 3	2.5	4.5
13		250 × 3	4.5	6.9
Mean			3.5	6.7
14	Linolenic	1000	1.6	2.6
15		1000	3.4	—
16		1000	2.6	2.7
17		1000	2.8	2.6
18		1000	3.7	3.9
19		1750	3.1	3.6
20		1700	2.5	3.3
21		1000	2.2	2.8
22		1000	1.5	2.7
Mean			2.6	3.0

The acids were irradiated for 90 min in aqueous emulsion (250 μl of fatty acid per 4.0 ml of water), 8.6 cm from light source.

* Calculated using malonaldehyde as the standard in the thiobarbituric acid assay (6).

† Represents the total amount of compounds (including H_2O_2) which oxidize *o*-dianisidine in the presence of horseradish peroxidase.

were formed. Analyses of the unconcentrated aqueous extract of irradiated linoleic and linolenic acid are shown in Table 1. The yield of TBA-reactive compounds was about 20 times greater from irradiated linolenic than from linoleic acid. The yield of "hydroperoxide" plus H_2O_2 was also greater from linolenic than from linoleic acid; however, the difference in yields was not as great. In experiments 8–13 (Table 1), in which smaller quantities of linolenic acid were exposed, the highest yields of "hydroperoxides" plus H_2O_2 (average, 6.7 μmoles/mmole of irradiated linolenic acid) were obtained. No TBA-reactive or peroxidase-reactive compounds could be detected in aqueous extracts of unsaturated fatty emulsions which were not UV-irradiated.

Both the volatile and relatively involatile components, separated by sublimation or flash evaporation, contained TBA- and peroxidase-reactive materials.

Properties of the Peroxidase-Reactive Compounds

In the presence of peroxidase the original water extracts of irradiated linoleic and linolenic acids oxidized *o*-dianisidine (9). Oxidation of dye by the extracts did not take place in the absence of peroxidase. The reaction proceeded more slowly than it did when hydrogen peroxide was used as substrate. This difference between hydrogen peroxide and the peroxidase-reactive products of autoxidation was observed in the residue of sublimated or flash-evaporated water extracts, as shown in Fig. 1. However, the peroxidase-reactive material in the volatile fraction of the water extracts oxidized *o*-dianisidine at the same rate as did H_2O_2.

Catalase, in sufficient concentration to remove H_2O_2 within 5 min, was able to remove a large proportion, but not all, of the peroxidase-reactive material in the original aqueous extract of irradiated unsaturated fatty acids. A typical experiment is shown in Fig. 2.

After fractionation of the water extract into volatile and involatile fractions by sublimation, virtually all of the catalase-reactive material was recovered in the sublimate. All of the "hydroperoxide" (not destroyed by catalase), whose reduction by dye and peroxidase proceeded relatively slowly, was found in the involatile residue.

Attempts to remove H_2O_2 from aqueous extracts of UV-irradiated linoleic and linolenic acids by means of flash evaporation were unsuccessful. Invariably, a large proportion of the H_2O_2 remained associated with the dry

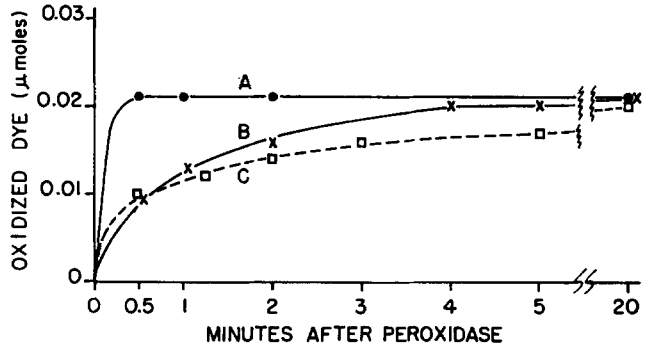


FIG. 1. Peroxidase-catalyzed oxidation of dye by "hydroperoxide" and H_2O_2 formed by UV-initiated autoxidation of linoleic and linolenic acids. Six cuvettes were charged with excess reduced dye (*o*-dianisidine) and duplicate aliquots of (*A*) H_2O_2, (*B*) water extract of irradiated linoleic acid (dried and subjected to TLC) and (*C*) water extract of irradiated linolenic acid (flash evaporated to dryness but not chromatographed). Approximately 60% of the peroxidase-reactive material of the dried linolenic acid water extract was later shown to consist of bound H_2O_2 which could be removed either by sublimation (2.5 mm Hg, from ambient temperature to liquid N_2) or by catalase treatment. One of each pair of duplicates was assayed at ambient temperature with a commercial mixture of buffered peroxidase, dye and glucose oxidase (Glucostat). The duplicate sample was assayed with peroxidase and dye in the absence of glucose oxidase (3). Only the latter results are shown since the time course of development of color was virtually the same with either enzyme preparation. No appreciable oxidation of dye occurred in 20 min in the absence of enzyme.

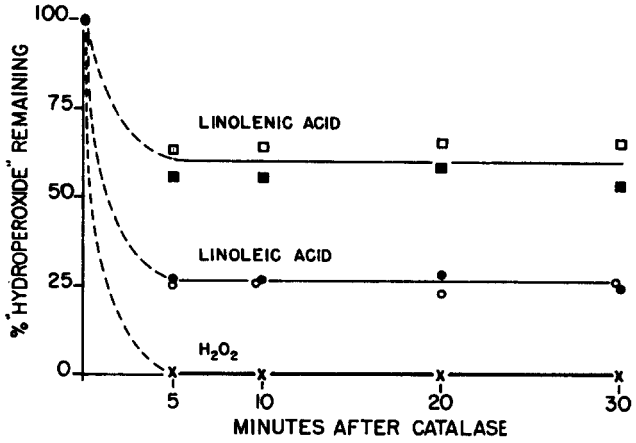


FIG. 2. Removal of hydrogen peroxide by catalase treatment of the water extract of UV-irradiated linolenic and linoleic acids. Aqueous extracts of four separately irradiated preparations and a control sample of hydrogen peroxide were diluted to a final concentration of 3.6×10^{-5} M with respect to total hydroperoxide (based upon separate peroxidase–dye assay). Each preparation was then treated with sufficient catalase to remove all of the H_2O_2 present within 5 min. Approximately 65% and 25% of the original peroxidase-reactivity in the extracts of irradiated linolenic and linoleic acids, respectively, could not be removed by prolonged catalase treatment.

residue. It could be removed either by subsequent treatment with catalase or by sublimation. We have previously reported that hydrogen peroxide is concentrated during removal of water under the present conditions of flash evaporation (10). However, pure solutions of H_2O_2 (in the absence of oxidation products of unsaturated fatty acids) leave no detectable peroxidase-reactive compounds when dried completely by flash evaporation. We believe, therefore, that hydrogen peroxide and one or more involatile "hydroperoxide(s)" are formed during UV-irradiation of aqueous suspensions of linoleic or linolenic acids; a part of the H_2O_2 is bound to some relatively involatile substance(s) from which it is removed not by flash evaporation but by sublimation or catalase treatment.

The results of 14 experiments in which the concentrations of H_2O_2 in the original extracts were determined by combined catalase and peroxidase treatments are shown in Table 2. An average of 75% of the total peroxidase-reactive material (Table 1) was present as H_2O_2 in extracts of irradiated linoleic acid and 50% in those from linolenic acid. Without exception, all of the "hydroperoxide" not destroyed by catalase in 5 min remained for 30 min, as shown in Fig. 2.

Yields of Malonaldehyde and of Involatile TBA-Reactive Compounds

A significant percentage of the TBA-reactive material in each aqueous extract of irradiated unsaturated fatty acid was volatile. Unlike H_2O_2, this volatile material was not concentrated by flash evaporation. Its TBA derivative had an absorption spectrum (visible light) indistinguishable from that of malonaldehyde. Like malonaldehyde, it could be recovered by sublimation in a Thunberg tube. To a large extent it could be made involatile by converting it to a sodium salt. Malonaldehyde is quantitatively converted to its involatile enolate salt in the presence of alkali (11). The acidified volatile fraction of the aqueous extract from irradiated linolenic acid contained a substance(s) which absorbed light in the region 220–250 mμ so intensely that the light absorption of malonaldehyde (λ_{max} 245 mμ) would be undetectable if this substance were present at the concentration indicated by TBA assay. However, λ_{max} of malonaldehyde is shifted from 245 to 265 mμ if the pH is raised from 2 to 10 (9, 12). When the volatile portion of the extract of irradiated linolenic acid was made alkaline, its absorbance of UV light at λ_{max} = 265 mμ was that expected if all TBA-reactive material in the extract were malonaldehyde (Fig. 3). Because of the low yield of TBA-reactive material in the aqueous extract of irradiated linoleic acid and the presence of other intensely absorbing compounds (λ_{max} = 225 mμ), UV spectral data were of little help in confirming other evidence that the volatile TBA-reactive material in the extract of irradiated linoleic acid was primarily malonaldehyde.

TABLE 2 HYDROGEN PEROXIDE AND ORGANIC "HYDROPEROXIDE" IN WATER EXTRACT OF UV-IRRADIATED LINOLEIC AND LINOLENIC ACIDS

Expt.*	Acid Irradiated	Percentage of Total Peroxidase-Reactive Material as: H_2O_2†	Organic Hydroperoxide
5	Linoleic	74	26
6		75	25
7		77	23
Mean		75	25
8	Linolenic	71	29
9		40	60
10		39	61
11		59	41
13		68	32
Mean		55	45
14	Linolenic	43	57
17		48	52
18		34	66
20		56	44
21		17	83
22		23	77
Mean		37	63

* Numbers of experiments correspond to those of Table 1, in which experimental details are given.

† Percentage of enzymatically determined hydroperoxide which could be removed by prior exposure of water extract to catalase.

TABLE 3 APPROXIMATE PERCENTAGE OF THIOBARBITURIC ACID (TBA)-REACTIVE MATERIAL WHICH IS MALONALDEHYDE IN WATER EXTRACTS OF UV-IRRADIATED LINOLEIC AND LINOLENIC ACIDS

Expt.*	Acid Irradiated	Malonaldehyde (Percentage of Total TBA Reactivity)†
3	Linoleic	57
4		46
5		90
6		87
7		44
Mean		65
12	Linolenic	65
13		89
14		85
17		96
18		98
19		92
20		75
21		47
22		40
Mean		76

* Numbers of experiments correspond to those of Table 1, in which experimental details are given.

† Determined by calculating percentage of initial TBA reactivity which could be removed by flash evaporation (or in expt. 12 by lyophilization).

The average percentages of TBA-reactive material present as malonaldehyde equivalent in the initial water extracts of irradiated linoleic and linolenic acids were 65 and 76, respectively (Table 3).

TLC of Aqueous Extracts

After drying the aqueous extracts of irradiated linoleic and linolenic acids by sublimation (thus removing H_2O_2, malonaldehyde, and a volatile substance(s) which absorbed UV light at λ_{max} = 225 mμ), the residue when redissolved in water or in ethanol contained peroxidase- and TBA-reactive substances. The involatile material was subjected to TLC and sprayed with anisaldehyde for detection of aldehydic compounds (4, 8). As shown in Fig. 4, a strongly positive reaction was observed at region B (R_f = 0.75) in the extracts from both linoleic and linolenic acid. When sufficient material was chromatographed, the unsprayed, developed chromatogram showed a narrow yellow band at the position corresponding to the main anisaldehyde-reactive substance(s). The material at this position also strongly absorbed UV light. Virtually all of the "hydroperoxide"-containing and TBA-reactive compounds also were eluted from region B (Fig. 4) at R_f = 0.75. These studies suggested that the relatively involatile fraction contained highly polar compounds of medium chain length which could be purified by TLC. The material is remarkably similar to fraction Vb of Schauenstein and Schatz (13) (obtained from aqueous emulsions of ethyl linoleate and linolenate, autoxidized in the absence of UV light) with respect to the presence of hydroperoxide, acid, and conjugated aldehyde (TBA-reactive) groups, water solubility, and relative involatility. The only difference observed in the chromatographic behavior of extracts of irradiated linoleic and linolenic acid was the ratio of hydroperoxide to TBA-reactive compounds eluted from position B (Fig. 4). This ratio was 2.7 μmoles of hydroperoxide per μmole of conjugated aldehyde for linolenic and 5.3 for linoleic acid.

Other Analyses of Dried, Water-Soluble Extracts of Irradiated Linolenic Acid

Aqueous extracts of 14 separately UV-irradiated, oxidized aliquots of linolenic acid (total fatty acid oxidized, 17 g) were combined, flash evaporated, dissolved in 95% ethanol, and sublimated in Thunberg tubes. The residue was dissolved in water, passed through a Millipore filter, and dried again by sublimation to yield 364 mg of a viscous yellow liquid. It contained acid, 2.4 μeq/mg; "hydroperoxide" (peroxidase), 0.1 μmole/mg; and conjugated aldehyde (TBA), 0.03 μmole/mg.

When the viscous yellow liquid was redissolved in water the solution was colorless; however, upon titration with NaOH the solution became intensely yellow. On a weight basis, the colorless acidic solution was 10 times more reactive in the Schiff test for aldehydes (14) than pyruvaldehyde.

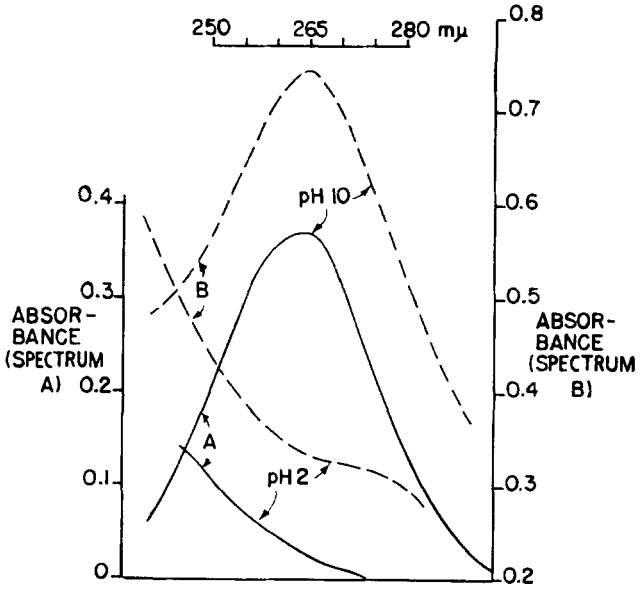
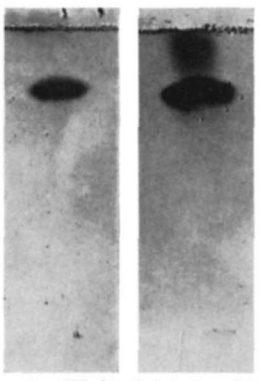


FIG. 3. UV absorption spectra of (*A*) 4.2 × 10^{-5} M malonaldehyde, a solution which gives, with TBA, 1.92 OD units/ml, and (*B*) volatile fraction of UV-irradiated linolenic acid water extract with the same TBA reactivity. The absorbance of malonaldehyde at pH 2 was 0.17 OD at its maximum (245 mμ); that of UV-irradiated linolenic acid (pH 2) was 1.1 at its maximum (225 mμ).



	PER CENT DISTRIBUTION OF REACTIVITY RECOVERED FROM CHROMATOGRAM			
	18:2		18:3	
	ROOH	TBA	ROOH	TBA
A	22	16	0	2
B	67	84	94	87
C	11	0	6	8
D	0	0	0	3

FIG. 4. TLC of dried residues of UV-irradiated linoleic (18:2) and linolenic (18:3) acids. Photographs of two chromatograms, sprayed with anisaldehyde, are shown. Region B corresponds to R_f = 0.75. Distribution of peroxidase- and TBA-reactive compounds eluted from other unsprayed chromatograms is indicated for regions designated *A*, *B*, *C*, and *D*.

The relatively involatile liquid was shown by GLC to be a mixture of at least 14 compounds, none of which had appreciable peroxidase or TBA reactivity after GLC (15).

The UV spectra at acid and alkaline pH of the dried yellow liquid dissolved in 0.1 N HCl or NaOH are shown in Fig. 5. UV absorption was subsequently shown to be the result of absorption by at least eight different components of the mixture, each of which showed minor absorption at 260–280 and major absorption at 200–230 mμ (15).

The IR spectrum in CCl_4 (29 mg/ml) indicated the presence of a carbonyl group, probably as dimeric dicarboxylic acid. There was a complete lack of absorption at the frequencies characteristic of the presence of aldehydes (e.g., at approximately 2720 cm^{-1}). This observation was unexpected in view of the intensely positive Schiff reaction of the material used for IR analysis. Results were the same whether the IR spectra and Schiff tests were performed on freshly prepared material or on extracts which had been stored dry, under N_2, at 0–4°C for over a year.

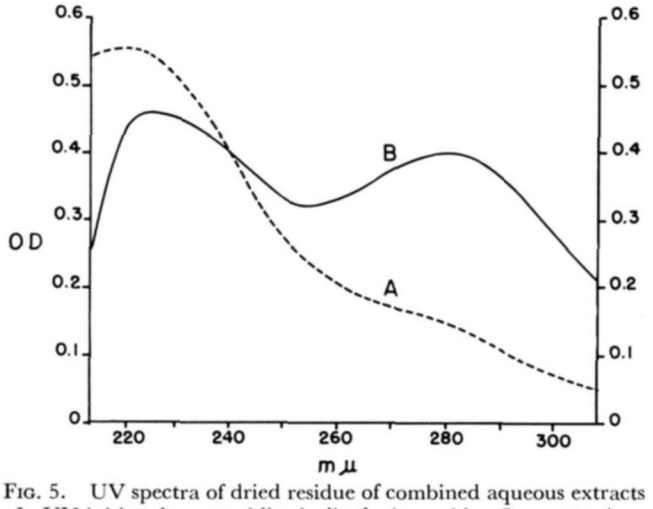


FIG. 5. UV spectra of dried residue of combined aqueous extracts of UV-initiated, autoxidized linolenic acid. Concentration: 0.0955 mg/ml. Solvents: 0.1 N HCl (spectrum A); 0.1 N NaOH (spectrum B).

DISCUSSION

At least 17 water-soluble products were formed after 90 min of UV-initiated autoxidation of aqueous emulsions of linoleic or linolenic acid. One of these was hydrogen peroxide; another was probably malonaldehyde. In one series of experiments, 1.0 and, in another, 3.7 μmoles of H_2O_2 were formed per millimole of linolenic acid irradiated. The yield from linoleic acid was approximately 1.3 μmoles of H_2O_2 per mmole fatty acid. In the case of linolenic acid the molar yield of malonaldehyde was about equal to that of H_2O_2, but for linoleic acid, only about 1 μmole of malonaldehyde was formed per 10 μmoles of H_2O_2. A third, unidentified component sublimed together with H_2O_2, malonaldehyde, and water, and was detected by its intense absorption of light at 225 mμ.

After the more volatile components had been sublimed, the residue of each water extract was found to contain a yellow material, which was isolated by TLC. The solubility properties of the residue, its chromatographic behavior in various solvent systems, and its reactivity with anisaldehyde were suggestive of a medium-

chain, highly polar, aldehydic substance(s). The residue reacted strongly in the TBA reaction for conjugated aldehydes[2], and substituted for H_2O_2 in the peroxidase-catalyzed oxidation of *o*-dianisidine. The relatively involatile material formed from linolenic acid has been shown to be a yellow, viscous, highly acidic liquid which contains at least 14 components in addition to the hydroperoxide and conjugated aldehyde, as demonstrated by GLC (15).

We have proposed earlier (10) that UV-irradiation would be expected to activate oxygen which would then combine with unsaturated fatty acids to form some type of ozonide. In the presence of water, an ozonide would give rise to H_2O_2 and the corresponding aldehydes. The formation of H_2O_2 and malonaldehyde as products of UV-initiated autoxidation of linoleic and linolenic acids was demonstrated. However, other oxidation mechanisms are undoubtedly also operative. Indeed, the similarity between the properties of our involatile fraction with those of the involatile water-soluble product(s) of ethyl linoleate and methyl linolenate which had been autoxidized in water emulsions without UV-irradiation (13) suggests that this fraction is derived from autoxidation processes that do not require activation of oxygen. On the other hand, controlled studies with and without UV light under the present conditions clearly showed that the rate of formation of the involatile fraction was greatly increased by UV-irradiation.[3]

Dahle, Hill, and Holman (17) have reported that no detectable TBA-reactive compounds were formed upon prolonged autoxidation of methyl linoleate and have attributed earlier observations of TBA reactivity in autoxidized linoleate to the presence of contaminants. We were unable to detect (by GLC) any more highly unsaturated fatty acid as a contaminant of the linoleic acid used in the present study. In view of the low TBA reactivities which we observed prior to concentrating our extracts, we feel that it is advisable to concentrate extracts of autoxidized lipid under the mild conditions used here before the presence of TBA-reactive compounds be ruled out. Our data are consistent with those in earlier publications (1, 18) in that linolenic acid formed volatile and involatile TBA-reactive compounds at least 10 times faster than did pure linoleic acid under the present conditions.

Saslaw, Anderson, and Waravdekar have emphasized (19) that malonaldehyde was absent from their aqueous extracts of UV-irradiated linolenate (free acid or methyl ester). Since 96% of their original aqueous extract was evaporated by lyophilization before any chemical analyses were performed, and since malonaldehyde is volatile at pH 5 or less, their data did not actually rule out formation of malonaldehyde nor indicate the percentage of water-soluble TBA-reactive material originally formed which was malonaldehyde. Our data indicate that malonaldehyde is probably responsible for most of the TBA reactivity in the original aqueous extracts of linoleic and linolenic acids irradiated by UV in air; they also provide independent evidence in support of the observation of Saslaw et al. that a viscous, yellow, relatively involatile, water-soluble, TBA-reactive substance that absorbs UV light maximally at about 225 mμ is formed from UV-irradiated linolenic acid. A comparison of our data with those of Shuster (1) and of Saslaw et al. (19) leads us to conclude that this involatile fraction [which we have shown to be responsible for the inhibition of endogenous respiration of tumor cells (15)] is formed upon UV-irradiation of linoleate, linolenate, or arachidonate whether they are in the form of free acids or methyl esters, whether irradiated in the absence of added water or in the form of a water emulsion, and whether irradiated for 90 min or for 18 hr.

Our initial aim was to isolate the microgram quantities of TBA-reactive material which Shuster had calculated was present in an aqueous extract of autoxidized linolenic acid. However, this has proved to be a formidable task, since about 1 g of relatively involatile water-soluble material that is not reactive with TBA is formed for every 3 mg of TBA-reactive material (assuming, as a first approximation, a molecular weight of 100 and a molar extinction at 535 mμ equal to that of malonaldehyde). In addition, we have found a peroxidase-reactive compound which, because of its solubility properties and high oxidation potential, might be expected to be at least partly responsible for the metabolic inhibitory properties described by Shuster. About 10 mg of this material (assuming a molecular weight of 100) was formed per gram of water-soluble extract. In the following paper (15), evidence will be presented that the portion of the extract that reacts with neither TBA nor peroxidase may be isolated by GLC. However, either thin-layer or column chromatography is undoubtedly better suited for isolation of the hydroperoxide(s) and unsaturated aldehydes. Similar conclusions have been reached independently by Schauenstein and his coworkers, who have made outstanding progress towards separation and identification of the water-soluble products that are produced after several days of autoxidation (in the absence of UV-irradiation) of methyl linoleate in aqueous emulsion. Among the 18 compounds separated by column and thin-layer chromatography they have identified *n*-amyl hydroperoxide (20), 2-octenal (21), and 4-hydroxy-2-*trans*-

[2] Although we have assumed that the structure responsible for TBA reactivity is a conjugated aldehyde (7), Schmidt has proposed that the essential structure is HO—|*x*|—CHO (16).

[3] In fact, we were unable to detect any involatile ROOH or TBA-reactive material after allowing emulsions to stand for 90 min at ambient temperature in a room from which sunlight was excluded but which was generally illuminated by fluorescent light.

octenal (22). At least three other hydroperoxides have been separated by these workers (23).

The formation of water-soluble, TBA-, catalase-, and peroxidase-reactive substances is of special significance in view of the recent report by Neubert, Wojtczak, and Lehninger that a combination of catalase and peroxidase is effective in reversing inhibition of the mitochondrial contractile mechanism under conditions which are probably associated with autoxidation of mitochondrial lipids (24).

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