Photo-sensitized Oxidation of Unsaturated Fatty Acid Methyl Esters. The Identification of Different Pathways

HENRY W.-S. CHAN, Agricultural Research Council Food Research Institute, Colney Lane, Norwich, NR4 7UA (UK).

ABSTRACT AND SUMMARY

The photo-sensitized oxidation of methyl linolenate and methyl oleate was studied using erythrosine and riboflavin as sensitizers. The complex mixtures of hydroperoxides obtained were analyzed for the proportion of conjugated products and, after reduction to the corresponding mixtures of hydroxystearates, for the distribution of positional isomers. By comparing the mixtures with that obtained from autoxidation, it was shown that the riboflavin reaction involved the "Type 1" mechanism of photo-sensitized oxidation which proceded via the formation of diene-radicals and yielded the same positional isomers of hydroperoxides as autoxidation. Thus, mixtures of the 8, 9, 10, and 11 positional isomers of allylic hydroperoxides were formed from oleate and the 9, 12, 13, and 16 isomers of conjugated diene-hydroperoxides from linolenate oxidation. The erythrosine reaction, on the other hand, proceded via the "Type 2" mechanism which involved singlet oxygen as the oxygenating species. The mixtures of isomers resulting from oxidation involving singlet oxygen were different from those obtained by autoxidation. Oleate oxidation gave rise to a mixture of only the 9 and 10 positional isomers while the mixture obtained from oxidation of methyl linolenate contained non-conjugated hydroperoxide isomers (with the hydroperoxide group at positions 10 and 15) as well as the conjugated—9, 12, 13, and 16—isomers.

INTRODUCTION

A major pathway for the production of flavor components from fats, oils, or fatty foods is initiated by the oxidation of unsaturated fatty acid moieties. These give rise to hydroperoxides whose decomposition results in the formation of volatile compounds. For this reason, the formation of hydroperoxides from autoxidation and from enzyme-catalyzed oxidation of unsaturated lipids has received considerable attention (1,2). There is, however, a third pathway for the production of hydroperoxides from unsaturated fatty acids, i.e., photo-oxidation. Although the unsaturated fatty acids do not absorb visible light and are not normally subject to direct ultraviolet irradiation, they can undergo photo-sensitized oxidation owing to light absorption by coloring matters present in a foodstuff.

There are two pathways for photo-sensitized oxidations (3). In Type 1, the sensitizer reacts, after light absorption, with the substrate (A) to form intermediates which then, in turn, react with ground state (triplet) oxygen to yield the oxidation products. In Type 2 photo-sensitized oxidation, molecular oxygen rather than the substrate is the species which reacts with the sensitizer after light absorption. In both cases more than one intermediate may be involved. In the case of Type 2 photo-sensitized oxidation, singlet molecular oxygen is generally regarded as the reactive species responsible for oxygenation of the substrate. This excited molecular species is formed by reaction of ground state oxygen with an excited triplet state of the sensitizer formed, via inter-system crossing, from its first excited singlet state.

Type 1.

$$\text{Sens} + \text{A} + h\nu \rightarrow [\text{intermediates I}]$$

$$[\text{intermediates-I}] + O_2 \rightarrow \text{products} + \text{Sens}$$

Type 2.

$$\text{Sens} + O_2 + h\nu \rightarrow [\text{intermediates} - \text{II}]$$

$$[\text{intermediates} - \text{II}] + \text{A} \rightarrow \text{products} + \text{Sens}$$

$$\text{Sens} + h\nu \rightarrow {}^1\text{Sens}$$

$${}^1\text{Sens} \rightarrow {}^3\text{Sens}$$

$${}^3\text{Sens} + {}^3O_2 \rightarrow \text{Sens} + {}^1O_2$$

Both types of photo-sensitized oxidation produce hydroperoxides from olefins (4) and are hence of potential interest to the accelerated deterioration of fatty foodstuffs in the light. This is specially relevant in cases where artificial food colors are added to products that are subjected to illumination. A recent study (5) has shown that of all the synthetic food colors, erythrosine is unique in its ability to sensitize photo-oxidation. A previous study (6) has pointed to the possible role of single oxygen in the initiation of fatty acid autoxidation. We now report the delineation of the two pathways of photo-oxidation of unsaturated fatty acid methyl esters sensitized by food colors.

EXPERIMENTAL PROCEDURES

Materials

Unsaturated fatty acid methyl esters were supplied by Sigma Chemical Co. (St. Louis, MO). Riboflavin was obtained from British Drug Houses Ltd. (Poole, England) and a sample of erythrosine was donated by Williams (Hounslow) Ltd. (Hounslow, U.K.) Kieselgel H and Silicar CC-7 (Mallinkrodt) were supplied by Camlabs (Cambridge, U.K.).

Methods

Peroxide titrations were carried out according to a standard procedure (7). Mass spectra were obtained on an AEI-MS 902 by direct insertion at 200 C. Methyl linolenate was purified before use by chromatography on "Silicar" CC-7 (Mallinkrodt) using 19:1 hexane/ether as the eluting solvent.

Oxygen-Uptake Experiments

Oxygen-uptake, in air, at 25 C was measured in a photochemical Warburg Apparatus fitted with a bank of tungsten light bulbs giving a light intensity of 5000 lux at the bottom of the manometric flasks. An aqueous emulsion (5 ml) of unsaturated fatty acid methyl esters (830 mg) was prepared using Triton X-100 (1 g). An aliquot of this emulsion (0.5 ml) was transferred into a manometric flask together with an aqueous solution (1 ml) of riboflavin (0.4 mg/ml). Butylated Hydroxy Toluene (BHT) where appropriate was added as an aqueous emulsion (2-4% Triton X) to the required concentration and the total volume of each flask was made up to 2 ml.

Experiments in which erythrosine was the sensitizer were conducted in ethanol as previously described (5).

To obtain the ratio of oxygen-uptake to peroxide, aliquots were taken from the flasks at the end of the experiments for peroxide titrations.

Analysis of Mixtures of Hydroperoxides

To prepare quantities of hydroperoxides for analysis, the unsaturated fatty acid methyl esters (332 mg) were illuminated in the presence of erythrosine (0.8 mg) or riboflavin (0.8 mg) in the same respective solvent systems (8 ml) as described above. Photo-oxidation was allowed to proceed until oxygen-uptake corresponded to ca. 5% oxidation (molar baisis, 1-1.5 hr). The contents of the Warburg flasks were diluted with an equal volume of water and extracted with petroleum ether (b.p. 40-60 C, 3 ml x 10 ml). The combined extracts were concentrated to 4 ml and the hydroperoxides were then separated from unreacted starting material by the counter-current method of Zilch et al. (8).

The hydroperoxides were further purified by percolating through a column (4.5 cm) of Kieselgel H in a Pasteur pipette using hexane/ether (90:10). The effluent was monitored for hydroperoxides by the ferrous thiocyanate spot test and fractions containing hydroperoxides were pooled. The solvent volume of the pooled fractions was reduced to ~1 ml by a stream of argon and the volume made up to 2 ml with ethanol.

Ultraviolet (UV) Absorption and Peroxide Value

50 μl of the solution was diluted to 10 ml with ethanol, the UV spectrum measured and absorbance at 234 nm determined. 0.8 ml of the solution was used for peroxide titrations which were carried out in duplicate. The molar ratio of UV absorption to peroxide content of the sample was calculated by assuming a molar absorbance at 234 nm of 26,000 for conjugated diene hydropeoxides.

Reduction to Hydroxystearates and Mass Spectral Studies

An ethanolic solution (2 ml, 1 mg/ml) of hydroperoxides was hydrogenated with Adams Catalyst for 18 hr. The products were evaporated to dryness and purified on a column (4 cm) of Kieselgel H prepared in a Pasteur pipette. Stepwise elution [hexane/ether 95:5 (4 ml), 90:10 (4 ml), 80:20 (4 ml), 50:50 (4 ml)] was used and 1 ml fractions collected. Fractions (8-11) containing the hydroxystearates were pooled, solvent evaporated, and the mass spectrum of the mixture of hydroxystearates determined.

Autoxidation of Unsaturated Fatty Acid Methyl Esters

The methyl esters of fatty acids were oxidized in a Warburg apparatus, in air, at 30 C in the dark until oxidation as measured by oxygen-uptake had reached 5% (molar basis) (60 hr). The hydroperoxides were purified by the counter-current procedure and column chromatography and analyzed as above.

For the autoxidation of methyl oleate, ascorbyl palmitate (1 mg) and ferric palmitate (1 mg) was added to initiate the reaction. (Although ascorbyl palmitate is known for its properties as an antioxidant, a *combination* of ascorbyl palmitate and ferric palmitate was found to promote oxidation in this case.)

RESULTS AND DISCUSSION

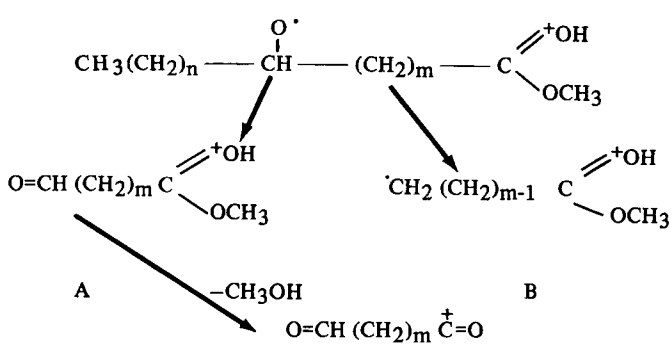
In order to study photo-oxidation with minimal interference from autoxidation, the experiments were conducted under mild conditions and with very pure materials. A measurement of the relative degrees of autoxidation and photo-oxidation in an experiment can be obtained from the rates of oxygen-uptake in successive periods during which the light is switched on and off. In this way, the preponderance of photo-oxidation was demonstrated in erythrosine-(5) and riboflavin-sensitized (Figure 1) photo-oxidation of methyl linolenate.

The rate of oxidation during the "dark" period before illumination is a measure of the rate of autoxidation of the unsaturated fatty acid esters. As this may vary with the purity of the fatty acid methyl esters used, a routine check was made in all photo-oxidation experiments on the rate of autoxidation in a preliminary dark period before the light was switched on. In all cases the rate of autoxidation was <1% of that of photo-oxidation. The rate of the "dark" reaction after one or two periods of illumination is a measure of autoxidation in the presence of hydroperoxides accumulated in photo-oxidation. Although these rates were higher than the rate of autoxidation before illumination they were still small (2%) compared with the rate of photo-oxidation and showed that initiation of autoxidation by hydroperoxide decomposition was not significant in the photo-oxidation experiments.

Under the mild conditions of the experiments and short exposure times (<2 hr) the products of oxidation were almost entirely hydroperoxides. The lack of decomposition products was shown by the oxygen-uptake to hydroperoxide ratio (0.94 and 0.98 for the erythrosine and riboflavin reactions respectively) and by the UV absorption of the reaction mixture which showed only the broad absorption band (234 nm) due to conjugated hydroperoxides.

For the identification of the presence of different positional isomers, the mixtures of hydroperoxides obtained from photo-oxidation and autoxidation were reduced to the corresponding hydroxystearates and the mass spectra of the mixtures determined. The purity of the hydroxystearates obtained from reduction was ca. 90% (as judged by thin layer chromatography and chromic acid charring), the main contaminants being by-products from the hydrogenation procedure. As these may interfere with the mass spectral analysis of isomers, a simple chromatographic procedure was routinely used to purify the hydroxystearates. Although more sophisticated methods of chromatography can separate positional isomers of hydroxystearates (9), no such separation was observed in the simple procedure which ensures that the mass spectra of the mixtures should contain only peaks due to hydroxystearates.

The mass spectrum of a methyl hydroxystearate is characterized by the presence of three major peaks in the high mass-number region of the spectrum (10). These are due to fission of the molecule adjacent to the hydroxyl group (fragments A & B) and the subsequent loss of CH_4O from



C

one of these fragments (A→C)—a process associated with a metastable ion. The calculated m/e values for the ions A, B, and C as well as the metastable ions for the transitions A→C for hydroxystearate isomers with the hydroxygroup in positions from 8 to 16 are shown in Table I. The triplets of fragments derived from individual isomers in the mixtures of hydroxystearates obtained from the sensitized photo-oxidations and autoxidation of methyl linolenate and methyl oleate are indicated in Figures 2 and 3.

In all the spectra, the only major peaks in the high mass-number regions are derived from the different mixtures of

TABLE I

Metastable Ioins in the Mass Spectra of Isomers of Methyl Hydroxystearate

Position of hydroxyl group in isomer	Calculated (m/e) Ions A	B	C	Metastable Ion for A→C	Observed metastable ions (m/e) Sensitized photo-oxidation Riboflavin L[a]	O[b]	Erythrosine L	O	Autoxidation L	O
8	173	144	141	114.92	–	114.9	–	–	–	114.9
9	187	158	155	128.48	128.5	128.4	128.5	128.5	128.5	128.5
10	201	172	169	142.09	–	142.2	142.1	142.1	–	142.1
11	215	186	183	155.76	–	155.8	–	–	–	155.8
12	229	200	197	169.47	169.5	–	169.5	–	169.4	–
13	243	214	211	183.21	183.2	–	183.4	–	183.3	–
14	257	228	225	196.98	–	–	–	–	–	–
15	271	242	239	210.78	–	–	210.6	–	–	–
16	285	256	253	224.59	224.5	–	224.6	–	224.5	–

[a]Linolenate
[b]Oleate

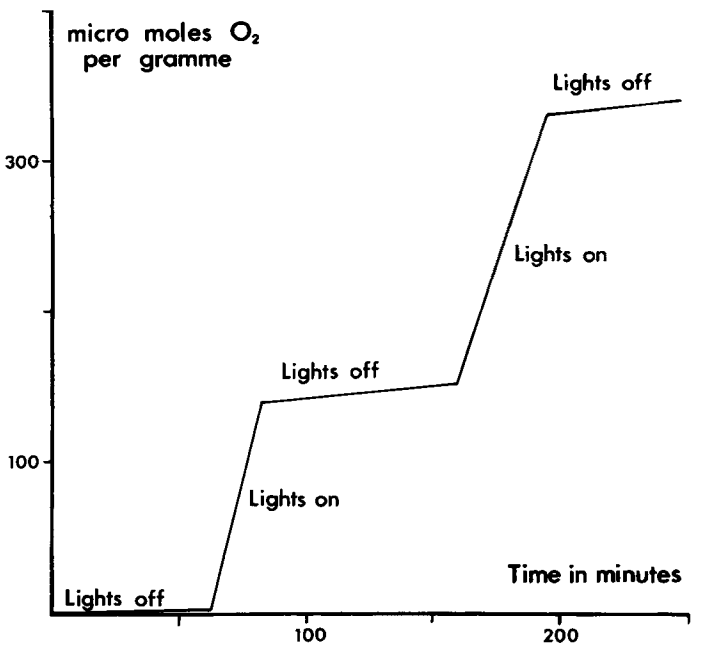


FIG. 1. Riboflavin-sensitized photo-oxidation. Oxygen-uptake per gram of methyl linolenate. Illumination switched ON and OFF as indicated.

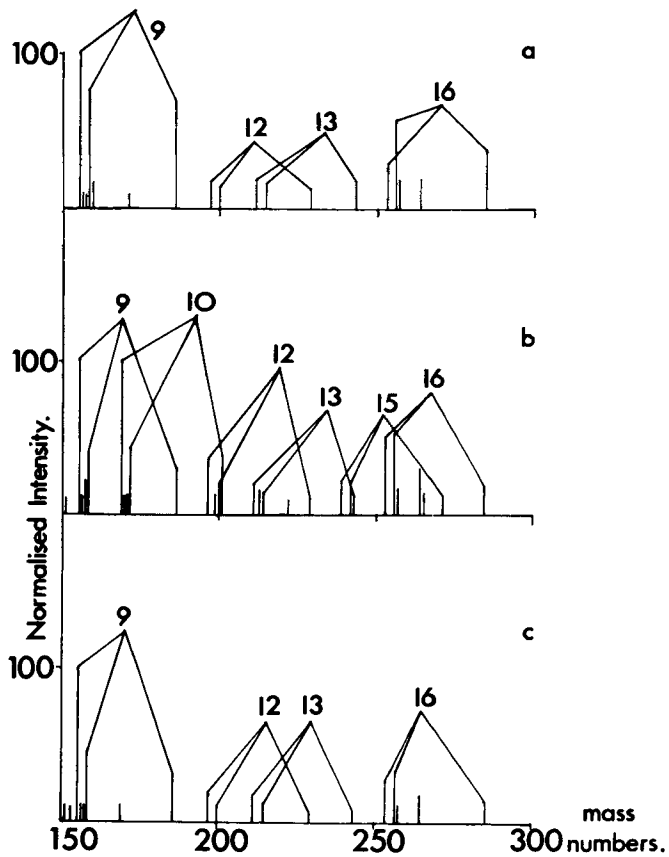


FIG. 2. Mass spectra of methyl hydroxystearate mixtures derived from hydroperoxides obtained by oxidation of methyl linolenate: (a) autoxidation, (b) erythrosine-sensitized photo-oxidation, (c) riboflavin-sensitized photo-oxidation.

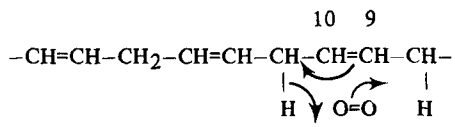
hydroxystearates. The fact that the peaks are due to the fragmentation of the hydroxystearate molecules as outlined above rather than to impurities is supported by the presence of metastable ions for the transitions A→C. These ions appear only when the peaks corresponding to A and C are also present in the individual spectrum and confirm the presence of the mixtures of derived hydroxystearates obtained from different methods of oxidation as indicated in Figures 2 and 3.

The most common mode of attachment of an oxygen molecule to the hydrocarbon chain in the oxidation of unsaturated fatty acids is the reaction of molecular oxygen with a conjugated free radical as, for example, in the propagation step of autoxidation. Preferential attack occurs at the extremities of the longest possible conjugation such as the radicals formed at the ends of a 1,4-diene system. For a complex molecule such as linolenate, two separate 1,4-diene systems are present and autoxidation of linolenate has been shown (11) to give rise to a hydroperoxide mixture containing the 9, 12, 13, and 16 positional isomers. Similarly, methyl oleate which can, by hydrogen abstraction, give rise to allylic radicals between positions 8-10 and 9-11, is autoxidized to a mixture of 8, 9, 10, and 11 hydroperoxide isomers (12).

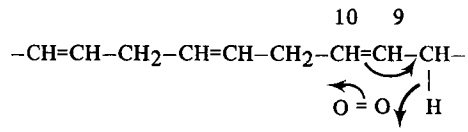
In contrast to this, the reaction between singlet oxygen and olefinic bonds does not involve conjugated free radicals but proceeds via a spin-allowed addition reaction, i.e. the ene-reaction (13). This is a concerted reaction between singlet oxygen and a carbon-carbon double bond in which the oxygen molecule is inserted at either carbon atom of the C=C bond which is shifted to yield an allylic hydroperoxide (see illustrations for methyl linolenate).

It follows that in singlet oxygen reactions with polyunsaturated, methylene-interrupted fatty acids, the reactive moieties in the hydrocarbon chain are the *isolated* olefinic bonds rather than the 1,4-diene system. The reaction of

singlet oxygen with methyl oleate would give rise to two hydroperoxides with oxygenation at positions 9 and 10 and the shift of the double bond to positions Δ-10 and Δ-8 respectively. By applying this to each of the double bonds in methyl linolenate, it is seen that singlet oxygen would give rise to hydroperoxides with the oxygen function at positions 9, 10, 12, 13, 15, and 16. Attack of singlet oxygen at positions 9, 12, 13, and 16 would move an olefinic bond into conjugation with another double bond as illustrated here for the 9 position:



Attack at positions 10 and 15, however, would not result in a conjugated hydroperoxide:



Thus singlet oxygen would react with methyl linolenate to yield six hydroperoxides of which four are conjugated. This is to be contrasted with oxidations involving free radical intermediates which produce four hydroperoxides all of which are conjugated.

The mass spectrum (Figure 2a) of the mixture of hydroxystearates derived from the hydroperoxides obtained by autoxidation of methyl linolenate shows the expected mixture of isomers—9, 12, 13, 16. Although the relative proportions of isomers obtained are in agreement with previous results in the autoxidation of linolenate (1), peak intensities in the mass spectra of hydroxystearates may not be an absolute measure of the proportions of isomers. In particular, isomers with the hydroxyl group towards the ends of the hydrocarbon chain may show higher intensities than those with the OH- functional group in the middle of the chain since this phenomenon is common to all the spectra. The mass spectrum of the mixture of hydroxystearates derived from erythrosine-sensitized photo-oxidation of methyl linolenate (Figure 2b) shows the presence of 9, 10, 12, 13, 15, and 16 isomers—a mixture indicating the participation of singlet oxygen in the photo-oxidation. In addition to the isomers obtained by autoxidation, the presence of two unconjugated hydroperoxides (viz. isomers 10 and 15) is indicated. The oxidation of a synthetic hydrocarbon bearing a 1,4-diene system by singlet oxygen to yield unconjugated oxygenation products has previously been described (14). The presence of unconjugated hydroperoxides in the mixture obtained by erythrosine-sensitized oxidation of methyl linolenate was quantitated in the present study by determining the ratios of UV absorbance to peroxide in the mixtures obtained by different methods of oxidation. The UV/PV ratio (Table II) of the mixture of hydroperoxides obtained by autoxidation is, as expected, close to unity since all the isomers are conjugated. The ratio for products obtained from the erythrosine reaction however is only 0.69. This is close to the expected ratio of 2:3 if the attack of singlet oxygen on the double bonds in methyl linolenate is random so that equal quantities of the unconjugated isomers (10 and 15) and conjugated (isomers 9, 12, 13, and 16) hydroperoxides are formed. Although erythrosine-sensitized photo-oxidation of methyl linolenate produces additional isomers. with methyl oleate as the substrate (Figure 3) it results in fewer isomers (isomers 9 and 10) than autoxidation (isomers 8, 9, 10, 11) as required by the participation of singlet oxygen.

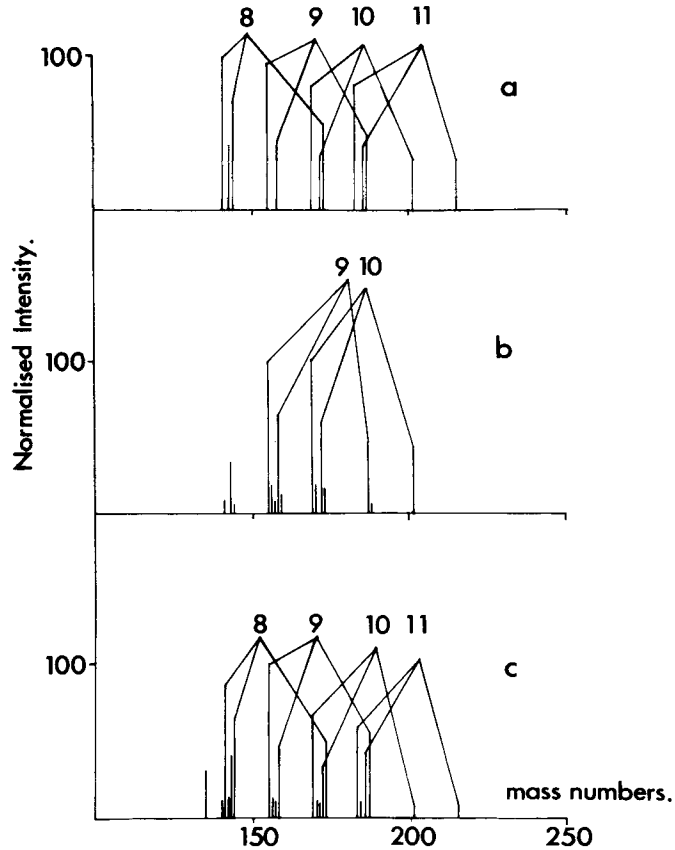


FIG. 3. Mass spectra of methyl hydroxystearate mixtures derived from hydroperoxides obtained by oxidation of methyl oleate: (a) autoxidation, (b) erythrosine-sensitized photo-oxidation, (c) riboflavin-sensitized photo-oxidation.

TABLE II

Molar Ratio of UV Absorbance (234 nm)/Peroxide in Mixtures of Hydroperoxides Obtained from Oxidation of Methyl Linolenate

← Sensitized Photo-Oxidation		
Riboflavin	Erythrosine	Autoxidation
0.97	0.69	1.01

A similar analysis of the products formed by photo-oxidation of unsaturated fatty acid methyl esters sensitized by riboflavin shows that they are, in contrast to products obtained by singlet oxygen, those expected from a reaction involving conjugated free radical intermediates and are identical to those obtained by autoxidation. Thus, the UV absorbance to peroxide ratio (1.01) of the mixture of hydroperoxides obtained from oxidation of methyl linoleante indicates the presence of only conjugated products. The mass spectra of the derived hydroxystearates from the oxidation of linolenate (Figure 2c) and oleate (Figure 3c) show the presence of only those isomers resulting from reaction of oxygen with the two ends of conjugated free radicals. This would be expected if riboflavin-sensitized photo-oxidation of unsaturated fatty acid methyl esters involves activation of the substrate, e.g. by hydrogen abstraction, prior to reaction with oxygen, i.e. if it takes place via Type 1 mechanism. The formation, via photo-induced hydrogen abstraction, of products different from singlet oxygen, in riboflavin-sensitized photo-oxidation has previously been reported (15). Although singlet oxygen may be produced by riboflavin under certain conditions,

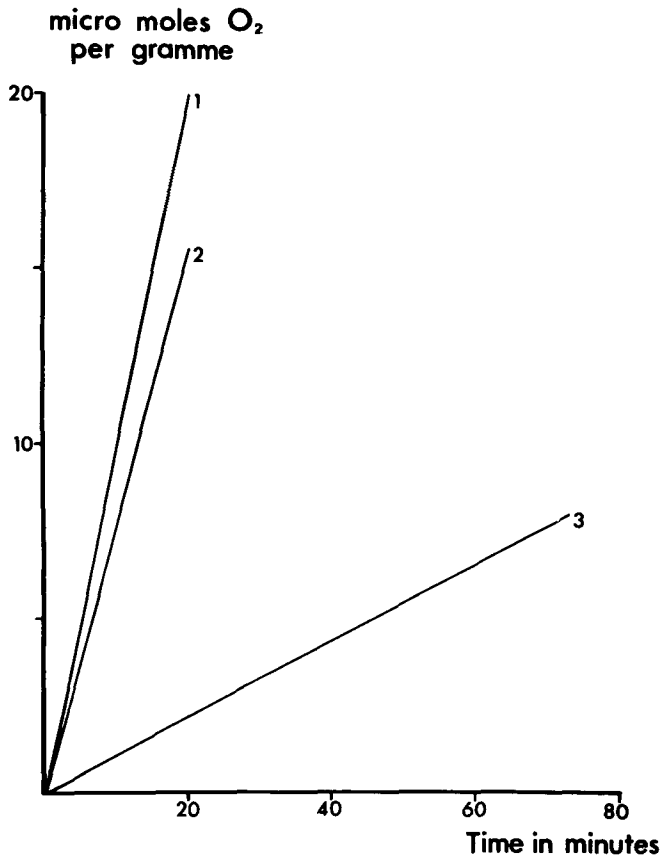


FIG. 4. Effect of BHT on riboflavin-sensitized photo-oxidation of methyl linolenate. Concentration of BHT: (1) 0. (2) 100 ppm. (3) 500 ppm.

e.g., very low (10^{-5}M) substrate concentrations, the ease of formation of conjugated radicals in the case of unsaturated fatty acids results in the exclusive formation of conjugated hydroperoxides.

It should be emphasized, however, that, although the same mixture of hydroperoxides is obtained by autoxidation and riboflavin-sensitized photo-oxidation and possible similar intermediates are involved, the former mode of oxidation is characterized by chain reactions which is not present to any appreciable degree in the latter. This is readily seen by the lack of further reactions when the light is switched off in the photo-oxidation for which there is no induction period (Figure 1) and is further supported by the relatively small inhibitory action of the antioxidant BHT in the riboflavin reaction (Figure 4) as compared with those generally observed for autoxidation where the induction period is lengthened by the addition of antioxidants.

The results reported here show that unsaturated fatty acid moieties are readily subject to photo-sensitized oxidation by both types of pathways which could be distinguished by the analysis of products. The singlet oxygen pathway, exemplified by the action of erythrosine, may lead to the formation of novel hydroperoxides. Thus, the qualitative as well as the quantitative aspects of oxidation may be affected where erythrosine is present in a foodstuff. On the other hand, type 1 sensitized photo-oxidation exemplified here by the action of riboflavin, is also important in the light-induced deterioration of a foodstuff even though the products may be indistinguishable from those of "dark" autoxidation.

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