A Possible Role for Singlet Oxygen in the Initiation of Fatty Acid Autoxidation

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Abstract

A mechanism for the initiation of autoxidation in fatty acids is proposed which involves singlet state oxygen, formed through a photosensitization reaction, as the reactive intermediate. Both singlet oxygen generated in a radio-frequency gas-discharge, and photosensitization by natural pigments, were shown to catalyze the oxidation of methyl linoleate. The involvement of singlet oxygen was shown by the identification of nonconjugated hydroperoxides as products common to both photooxidation and singlet O_2 oxidation. Nonconjugated hydroperoxides could not be detected among the free radical autoxidation products. Further proof for the above mechanism was gained by showing that compounds known to react strongly with singlet oxygen, inhibited the photooxidation. With the exception of chlorophyll, all sensitizers could be completely inhibited. Although singlet oxygen formation can account for approximately 80% of the observed chlorophyll photooxidation, at least one other mechanism must be involved. It is postulated that proton abstraction by the photoactivated carbonyl group of chlorophyll could account for the remaining 20% of the observed photooxidation. The conclusion is drawn that oxygen, excited to its singlet state by a photosensitization process, plays the important role of forming the original hydroperoxides whose presence is necessary before the normal free radical autoxidation process can begin.

Introduction

Although the free radical mechanism which makes up the fatty acid autoxidation process has been thoroughly studied (1–3), one factor remains unsatisfactorily explained: the origin of the initial free radicals necessary to begin the process in an oil completely free of hydroperoxides (1,4–7). The oxidation process in the presence of air is usually assumed to be initiated by the breakdown, in some fashion, of hydroperoxides (8). These hydroperoxides are regenerated during the chain process, thus the process is self-catalyzing and is referred to as autoxidation:

$$\mathrm{ROOH} \rightarrow \mathrm{RO}\cdot + \cdot\mathrm{OH}\ \text{[at very low concentrations (8)]}$$
$$2\mathrm{ROOH} \rightarrow \mathrm{ROO}\cdot + \mathrm{RO}\cdot + \mathrm{H_2O}$$
$$\mathrm{ROO}\cdot + \mathrm{RH} \rightarrow \mathrm{R}\cdot + \mathrm{ROOH}$$
$$\mathrm{R}\cdot + \mathrm{O_2} \rightarrow \mathrm{ROO}\cdot$$
$$\text{etc.}$$

Experiments have indicated that the autoxidation observed in supposedly pure oils cannot be satisfactorily explained by the presence of residual, undetectable amounts of hydroperoxides (7). However, even if this were possible, the origin of these hydroperoxides would also have to be explained.

The obvious explanation would appear to be a direct reaction between RH, the unsaturated fatty acids, and O_2. However, this has also been shown to be unlikely (9). The reaction RH + O_2 → ROOH requires a change in total spin, RH and ROOH being in singlet states while O_2 is in a triplet state; moreover, the reaction is endothermic by about 64 kcal/mole (4). Reactions such as this, in which spin is not conserved, are forbidden according to the well-known Wigner rules (10).

Both the energy and spin barriers could be overcome, however, if instead of ordinary, triplet state O_2, singlet state O_2 was the active species. It has been well established that electrophilic singlet O_2 reacts directly with olefinic molecules (11–15); hence the necessary conditions of spin and energy conservation are satisfied. Thus a mechanism which could supply singlet O_2 could explain the formation of the original hydroperoxides in fatty acids. The photosensitized production of singlet O_2 is believed to be the mechanism of many photooxidation reactions (11–15), and since the plant or animal pigments present in most sources of fatty acids could serve as sensitizers, this is a likely mechanism for the production of fatty acid hydroperoxides, the only other necessary ingredients being visible light and oxygen. Thus, the following mechanism is proposed (16):

$$^1\mathrm{S} + h\nu \rightarrow {}^1\mathrm{S}^* \rightsquigarrow {}^3\mathrm{S}^*$$
$$^3\mathrm{S}^* + {}^3\mathrm{O_2} \longrightarrow {}^1\mathrm{O}^*_2 + {}^1\mathrm{S}$$
$$^1\mathrm{O}^*_2 + \mathrm{RH} \longrightarrow \mathrm{ROOH}$$
$$\mathrm{ROOH} \longrightarrow \text{free radical products}$$

S is the sensitizer, the superscripts refer to the spin multiplicity, and the asterisk indicates electronic excitation. This paper reports the results of an investigation to determine whether singlet O_2 is indeed responsible for the initiation of hydroperoxide formation.

In accomplishing this, singlet O_2, generated externally, was shown to react directly with extremely pure samples of methyl linoleate at a rate at least 1450 times that due to ground state O_2. Further, inhibition experiments as well as product analysis were used to show that singlet O_2 was the reactive intermediate in photooxidation. These reactions were sensitized by pigments representing those commonly found in plant and animal sources of fatty acids.

Experimental Procedures

Purification of Materials

Methyl linoleate is extremely difficult to separate from its own oxidation products. However, it was found that the following method would yield small quantities of greater than 99.99% pure methyl linoleate: 200 μl of methyl linoleate which had been separated from other fatty esters by urea fractionation (17) was applied to a 20 × 20 cm, 0.3 mm thick layer of silica gel G and developed in light petroleum-ethyl ether (70:30 v/v). The portion containing the pure ester was scraped off and the ester extracted (nonexhaustively) with light petroleum. This produced about 100 μl, enough for several experiments. It could be stored for about a week under liquid N_2.

The purity could be checked by UV-absorption, since the greater portion of the oxidation products

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contained conjugated hydroperoxides. As a measure of the purity the ratio of the absorbance at 215 nm where the absorbance is mostly due to linoleate, to that at 235 nm, mostly due to conjugated hydroperoxide, was taken. Only samples in which this ratio was greater than 6 were used. This corresponded to a conjugated hydroperoxide concentration of 0.002% or less. Other oxidation products would be expected to be present in amounts of not more than 100 times less than this.

Chlorophyll-a and pheophytin-a were purified from spinach leaf extracts by chromatographing the extracts on silica TLC plates in the dark. The separated pigments were scraped from the plate, extracted and their purity checked by UV-spectrophotometry. This proved to be a simple method for obtaining small quantities of highly purified pigments. Later, when larger quantities were needed, the chlorophyll and pheophytin were used as mixtures which had been roughly separated from the carotenoids in the spinach leaf extracts.

Generation of Singlet O_2

Following the example of Corey and Taylor (13), a surplus army transmitter capable of producing 300 watts at 6.7 mc was altered so that its energy could be coupled into an evacuated pyrex tube through which O_2 could be passed by means of a needle valve. The RF power was coupled into the gas by means of a water cooled conduction coil. Hg was distilled into the system in order to remove oxygen atoms, while water vapor removed $(^1\Sigma_g)O_2$. Thus only $(^1\Delta_g)O_2$ and $(^3\Sigma_g)O_2$ (normal O_2) emerged (18) and reacted with the samples. Control experiments were run simultaneously by using a reaction chamber located upstream from the discharge.

Photooxidation

A submersion-type pyrex reaction cell designed to accommodate a 100 watt tungsten projection lamp was used. This cell also featured water cooling, a liquid filter and three capillary tubes for bubbling O_2 through the sample solution. The liquid filters which were used allowed only those wavelengths to reach the sample compartment which the sensitizer, but not the sample or the inhibitor, would absorb. The concentrations of the various sensitizers were adjusted for equal number of photons absorbed in the various experiments. Control experiments, in which no sensitizer was added were always run; in no single case was a significant degree of oxidation observed. The irradiation time was 10 min.

IR Analysis

Samples for IR analysis were first purified by TLC, the region corresponding to the monohydroperoxides being scraped from the plate and extracted with diethyl ether. Control experiments showed that an insignificant amount of polymeric material known to contain nonconjugated hydroperoxide groups (19) was formed from the hydroperoxide concentrate during the experiments. Either a Grubb-Parsons Spectromaster or a Perkin Elmer 237 IR spectrometer was used. The samples were thin films of unknown thickness deposited on KCl-windows.

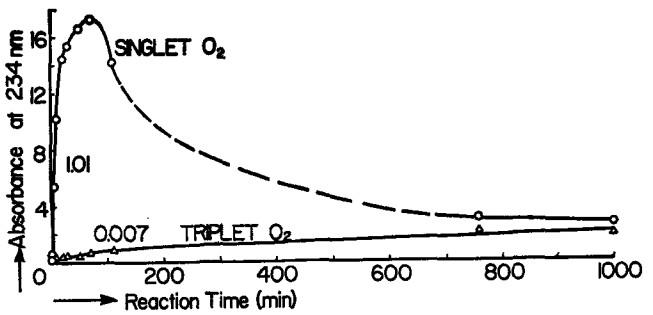
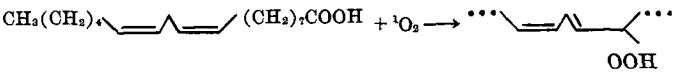


FIG. 1. Variation of absorbance at 234 nm with time of oxidation. The singlet oxygen curve has been corrected for the small increase in absorbance due to the triplet O_2 that was present. The values at the curves indicate the slopes in absorbance units per minute.

Results

The first question is, of course, does singlet O_2 react at a rate sufficiently high, compared to triplet O_2, to bring about the necessary accumulation of hydroperoxides to initiate autoxidation. Singlet O_2, generated externally by a radio-frequency gas discharge, was reacted with methyl linoleate by passing it over a thin film of the linoleate which was deposited on the walls of an evacuated pyrex flask. The progress of the reaction could be followed by observing the increase in UV absorption at about 234 nm due to the formation of the conjugated hydroperoxide:



The results are shown in Figure 1, from which it is obvious that the reaction rate of singlet O_2 with methyl linoleate is several orders of magnitude greater than that of triplet O_2. Thin layer chromatography (TLC) showed that 10% singlet O_2 caused 50% oxidation in 15 min [Gas discharge is supposed (18) to produce a singlet O_2 concentration of about 10%]. After two days in air this sample was nearly 100% oxidized, while oxidation was no more than a few percent in control samples.

The method of purification was such that the freedom of the samples from their own oxidation products varied from sample to sample. It was found that the triplet O_2 oxidation rate was higher in the less pure samples, while there was no significant change in the singlet O_2 oxidation rate. Thus, it would appear that the triplet O_2 oxidation shown in Figure 1 is largely, if not entirely due to residual oxidation products which were not removed in the purification process.

Having established that singlet O_2 indeed reacts very rapidly with methyl linoleate, the role which singlet O_2 plays in photoxidation could now be investigated. The sensitizers were picked for their occurrence in natural sources of fatty acids: chlorophyll-a and pheophytin-a are found in nearly all plant sources and protoporphyrin, the pigment portion of hemo- and myoglobin, was chosen to represent the pigments found in animal sources. Methylene blue, an organic dye, was also used because it was already known to sensitize singlet O_2 production in the photooxidation of various other olefins and dienes (11). As expected, all of these molecules catalyzed methyl linoleate photooxidation. Control samples, having no sensitizer, always showed an insignificant amount of oxidation.

The direct observation of singlet O_2 formed via photosensitization is rather difficult. However, two indirect methods for its detection have recently been used successfully: first, comparison of the products formed in two reactions, common products indicating a common reactive intermediate (12) and secondly, inhibition experiments using molecules known to react

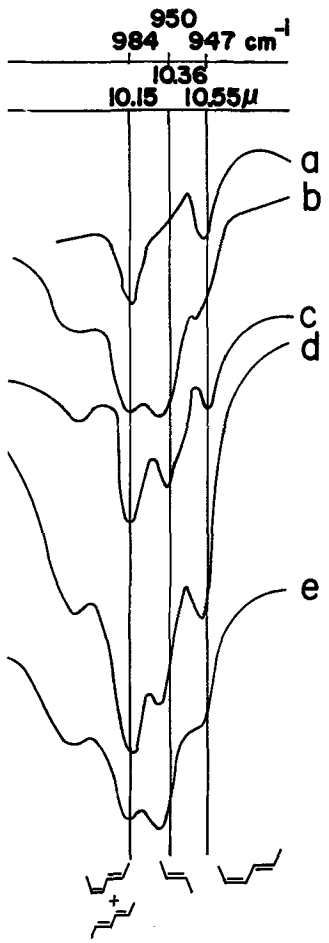


FIG. 2. IR-Analysis of methyl linoleate oxidized in various ways. (a) air-autoxidized; (b) singlet O_2-oxidized; (c) methylene blue photooxidized; (d) chlorophyll photooxidized (upper portion of TLC-spot); (e) Chlorophyll photooxidized (lower portion of TLC-spot).

strongly with singlet O_2, an inhibition indicating competition for the reactive intermediate (14).

Methyl linoleate samples which had been oxidized to approximately the same extent by singlet O_2, by triplet O_2 (air), and by photooxidation with the various sensitizers were chromatographed on thin layers of silica gel (TLC) and the patterns of spots so obtained compared. Although there were some variations among the minor oxidation products, the principal products were the same in all cases. Chlorophyll and pheophytin showed a pattern of spots which differed the most from the others. Chlorophyll-a, pheophytin-a and roughly purified spinach leaf extract all gave the same pattern of TLC spots. Therefore, only the extract was used in subsequent experiments.

Others have shown, by IR analysis, that chlorophyll photooxidation produces nonconjugated as well as conjugated monohydroperoxides as primary oxidation products, while air autoxidation produces only the two conjugated hydroperoxide isomers (20,21). With the solvent system used, conjugated and nonconjugated hydroperoxides are not separated by TLC. Therefore, the spot corresponding to the monohydroperoxides was removed, the hydroperoxides extracted, and their IR spectra were examined.

A band, due to the presence of an isolated *trans* double bond, was observed in the vicinity of 950 cm^{-1} in all but the air autoxidized samples, as shown in Figure 2. This confirms the earlier results (20,21)

TABLE I

Inhibition of Photooxidation by Various Singlet O_2-Reactive Substances, as Estimated by TLC

Inhibitor	Sensitizer	Inhibitor-linoleate molar ratio	Approximate inhibition, %
Tetramethylethylene (TME)	Methylene blue	1 and 100	50 and 100
	Chlorophyll-a	25 and 300	50 and 50
	Protoporphyrin	100	50
Tetraphenylcyclopentadienone (cyclone)	Methylene blue	50	100
	Chl. + pheophytin	50 and 100	80 and 80
	Protoporphyrin	50	100
Diphenylisobenzofuran (DPBF)	Methylene blue	10	100
	Chl. + pheophytin	10 and 20	80 and 80
	Protoporphyrin	10	90
β-Carotene	Methylene blue	25	90 ± 10
	Chl. + pheophytin	25	90 ± 10

concerning the difference between chlorophyll photooxidation and air autoxidation and, in addition, establishes the formation of nonconjugated hydroperoxides among the primary oxidation products of singlet O_2 oxidized and methylene blue photooxidized methyl linoleate. The bands in the vicinity of 984 cm^{-1} and 947 cm^{-1} are characteristic of conjugated diene hydroperoxides and are observed in all cases.

Thus a product common to both photooxidized samples and samples oxidized directly by singlet O_2 was found which could not be detected in samples autoxidized in air.

For the inhibition experiments, the compounds tetramethylethylene (TME), tetraphenylcyclopentadieone (cyclone), and diphenylisobenzofuran (DPBF) were used. They are all known to react strongly with singlet O_2 (12,14) and thus should inhibit, by competition, any reaction involving it. The results are tabulated in Table I.

Inhibition was obtained for all three compounds, their effectiveness increasing in the order TME < cyclone < DPBF, which is the order of their reactivity (11–14,22–24) with singlet O_2.

Oxidation products of chlorophyll, and also DPBF, interfere slightly with the estimation of the amount

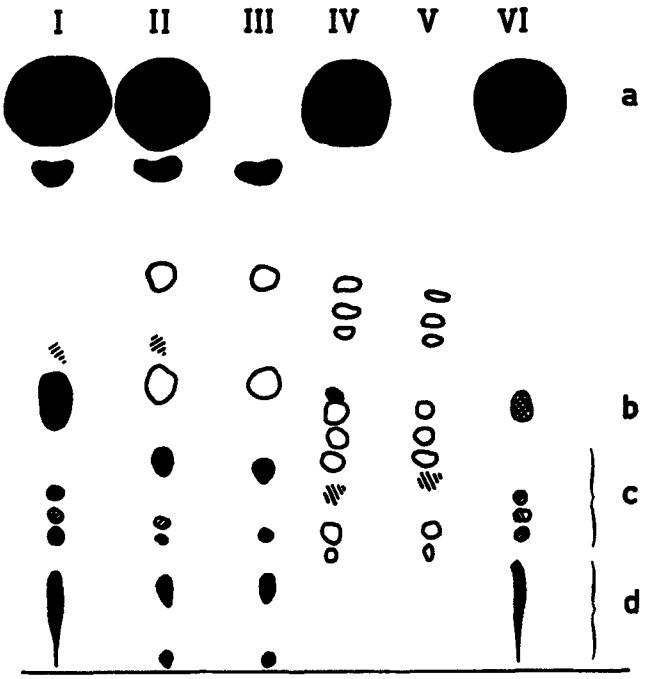


FIG. 3. Thin layer chromatogram of methyl linoleate (ML) after photooxidation with protoporphyrin and inhibition with various substances. I. ML, II. ML inhibited with cylone, III. cyclone, IV. ML inhibited with DPBF, V. DPBF, VI. ML inhibited with TME.

of linoleate oxidation products formed. β-Carotene and its oxidation products interfere seriously with the estimation of linoleate oxidation. Additional products appear to be formed in the combined presence of chlorophyll, linoleate and DPBF which are not found when any two of them are oxidized together.

β-Carotene is a polyolefinic compound which is usually found along with chlorophilic pigments in plants. When subjected to singlet O_2 produced in the gas discharge, β-carotene reacted nearly as rapidly as methyl linoleate. Therefore, it was also used in an inhibition experiment and found to partially inhibit chlorophyll photooxidation.

A thin layer chromatogram of methyl linoleate photooxidized with dimethyl protoporphyrin IX and inhibited with the various inhibiting substances is shown in Figure 3.

Discussion

As was seen in Figure 1, the rate of reaction of singlet O_2 is much higher than that of triplet O_2. If a simple reaction mechanism, first order in singlet O_2, can be assumed, then:

$$RH + O_2 \xrightarrow{k} P \text{ (hydroperoxide)}$$

$$k_1[RH][^1O_2] = \frac{dP_1}{dt}$$

$$k_3[RH][^3O_2] = \frac{dP_3}{dt}$$

$$\frac{k_1}{k_3} = \frac{dP_1/dt}{dP_3/dt}\,\frac{[^3O_2]}{[^1O_2]}$$

Remembering that about 10% singlet O_2 is produced:

$$\frac{k_1}{k_3} = \frac{1.01}{0.007}\,\frac{100}{10} = 1450$$

Thus, singlet O_2 forms hydroperoxides at least 1450 times faster than triplet O_2. However, this figure would probably be at least one order of magnitude higher if a purer sample could be used, since k_3 decreases with increasing sample purity while k_1 remains constant. In addition, as was found in the IR studies, nonconjugated hydroperoxides are formed by the reaction with singlet O_2 but not by triplet O_2 autoxidation. These nonconjugated products do not absorb at 234 nm and therefore go undetected in the method used. Thus, the actual rate is even higher than that shown in Figure 1 and is certainly sufficient to cause a rapid accumulation of enough hydroperoxides to initiate autoxidation.

As would be expected from the extensive photochemistry literature (25), hydroperoxide formation could be photochemically catalyzed using the natural pigments found in both plant and animal sources of fatty acids. Methylene blue also sensitized hydroperoxide formation in fatty acids.

The IR and inhibition studies established the strong possibility that singlet O_2 is the reactive intermediate in these photooxidations. The two most likely mechanisms of photooxidation involve either a biradical "moloxide" (26,27):

$$^1S \xrightarrow{h\nu} {}^1S^* \rightsquigarrow {}^3S^*$$

$$^3S + {}^3O_2 \rightarrow \cdot\, S\text{–}O\text{–}O\,\cdot$$

$$\cdot\, S\text{–}O\text{–}O\,\cdot + RH \longrightarrow ROOH + {}^1S$$

or singlet O_2 as the reactive intermediate.

The biradical intermediate should produce the same oxidation products as the free radical autoxidation mechanism, which is known to proceed via the breakdown of hydroperoxides already present. The singlet O_2 mechanism shown in the introduction section should, on the other hand, produce the same oxidation products as externally produced singlet O_2. A complete product analysis was not made; however, the identification of nonconjugated hydroperoxides in both the direct singlet O_2 oxidation reactions and the photooxidation reactions, while no nonconjugated hydroperoxides could be observed in the autoxidized samples, is strong evidence that singlet O_2 is involved in photooxidation.

If singlet O_2-produced hydroperoxides are to serve as autoxidation initiators, one may wonder why no nonconjugated hydroperoxides are found in autoxidized samples. The reason is that very few hydroperoxides are evidently required for the initiation and any nonconjugated ones initially present would soon be further oxidized and hence, not be found among the primary oxidation products.

The inhibition experiments helped to confirm the IR findings. The fact that the order of effectiveness of the photooxidation inhibitors was the same as the order of their reactivity with singlet O_2, leaves little doubt that singlet O_2 is the reactive intermediate for all three sensitizers used. The measure of this effectiveness is the amount of inhibitor necessary to cause 100% inhibition. Although this quantity was not measured exactly, it is obvious from the Table that it was greatest for TME and smallest for DPBF. Thus, the order of effectiveness is TME < cyclone < DPBF, which is the same as their order of reactivity with singlet O_2.

A closer examination of Table I reveals that none of the compounds used were 100% effective as inhibitors of chlorophyll photooxidation. This is in contrast to the other two sensitizers, whose photooxidizing effect could be completely inhibited. Thus, unless some unexpected mechanism is involved, protoporphyrin and methylene blue photooxidation, but not chlorophyll photooxidation, is due entirely to singlet O_2 formation. From the Table it would appear that about 20% of the chlorophyll photooxidation must be due to one or more entirely different mechanisms.

Chlorophyll has a porphyrin structure very similar to that of protoporphyrin, and it is interesting to speculate as to the reason for the difference in their behavior as photosensitizers. Since chlorophyll and pheophytin give the same results, the Mg portion of chlorophyll can be eliminated as providing an answer. However, the other important structural difference between chlorophyll and protoporphyrin, a five-membered ring condensed to the porphyrin system and containing a carbonyl group, may provide an answer. Electronically-excited carbonyl groups are known to be very effective proton abstractors, and since the visible light which was used for exciting the chlorophyll is sufficient to excite the carbonyl $n \rightarrow \pi^*$ state which lies at 660 nm (28), the following mechanism may account for the nonsinglet O_2 oxidation:

$$Chl + h\nu \rightarrow Chl^* \qquad * = n \rightarrow \pi^* \text{ excited state of the carbonyl group}$$

$$Chl^* + RH \rightarrow R\cdot + \text{(chlorophyll products)}$$

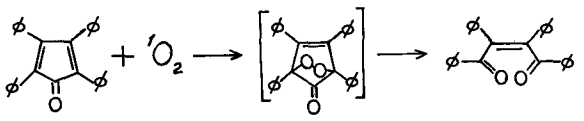
$$R\cdot + O_2 \rightarrow RO_2\cdot$$

$$RO_2\cdot + RH \rightarrow ROOH + R\cdot$$

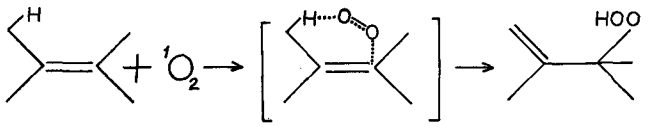
Chlorophyll was slowly destroyed in the photooxidation experiments. This is expected from the above mechanism.

A point which detracts from this interpretation is the fact that TME will not completely inhibit the chlorophyll photooxidation. The other two inhibitors,

cyclone and DPBF, do not have reactive protons available for abstraction, while TME does. The reaction of cyclone with singlet O_2 for example, is believed (12) to be the following:



DPBF behaves similarly, while the TME reaction involves a proton migration (12):



This same proton could be abstracted by an excited chlorophyll and thus TME could compete for the reactive intermediate, Chl* in the one case and singlet O_2 in the other, in both types of photooxidations. The fact that it does not (Table I) is negative but inconclusive evidence against the proton abstraction mechanism. However, the chlorophyll photooxidation could be largely inhibited by the singlet O_2 reactive compounds. Thus, the most important mechanism in chlorophyll photooxidation must be the production of singlet O_2.

In the chlorophyll photooxidation studies, β-carotene was also found to act as an inhibitor. β-Carotene is a conjugated polyene and might thus be expected to react with singlet O_2 in a fashion somewhat similar to that of TME. Photooxidation experiments with compounds structurally similar to the carotenes have been interpreted with a singlet O_2 mechanism (29). Our own studies with singlet O_2 showed that β-carotene was very reactive with singlet O_2, somewhat less than cyclone but more than TME. This helps to explain the inhibiting effect of β-carotene reported in the Table. It is interesting to speculate that perhaps one of the functions of the carotenoids is the protection of lipids or other plant materials from photooxidation. β-Carotene's reactivity with singlet O_2 may well account for its ability to protect the chlorophylls from photooxidation (30). Foote et al. have recently found (31) that β-carotene deactivates singlet O_2 at a rate much higher than the rate at which it reacts with singlet O_2. With this evidence they have postulated a protective role for β-carotene in photosynthesis.

From the above results the conclusion can be drawn that due to its great reactivity with fatty acids, as exemplified by methyl linoleate, plus the great abundance of natural pigments which are able to sensitize its production, singlet O_2 must be a primary source of the original hydroperoxides which initiate fatty acid autoxidation.

ACKNOWLEDGMENTS

Valuable suggestions and discussions by Miss P. Haverkamp-Begemann, urea fractionation of methyl linoleate by J. B. A. Stroink, and technical assistance by Mrs. M. van der Graaf-Wildschut and H. W. Zegstroo.

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[Received June 27, 1969]