Tert-BUTYLATED-HYDROXYTOLUENE-SENSITIZED PHOTO-OXIDATION OF FATTY ACIDS: INHIBITION BY 1,4-DIAZABICYCLO-[2.2.2]OCTANE AND ROLE OF SINGLET OXYGEN†

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Summary

The effect of *tert*-butylated hydroxytoluene (BHT) on the photo-oxidation of fatty acids was studied. A marked increase in photo-oxidation was observed in the presence of BHT. The major products of photo-oxidation were identified as epoxides of the fatty acids and only a small amount of hydroperoxides was detected. Formation of epoxides as the major product indicates that enhancement in the photo-oxidation occurs through free radicals, possibly through a type I mechanism. Analyses of the hydroperoxides, however, showed the formation of both conjugated and non-conjugated hydroperoxides, suggesting the involvement of a type II mechanism also in the photo-oxidation. Formation of both free radicals and singlet oxygen products was inhibited by 1,4-diazabicyclo[2.2.2]octane, a widely used singlet oxygen quencher, suggesting that hydroperoxides may be the primary products of photo-oxidation followed by free-radical reactions leading to the formation of the epoxy compounds.

1. Introduction

Environmental UV radiation, primarily of solar origin, is generally conceded to be the primary etiologic agent for common forms of human skin cancer. In recent years, there has been accumulating evidence that antioxidants such as *tert*-butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), glutathione etc. provide considerable protection against photocarcinogenesis [1, 2]. The inhibitory action of antioxidants is believed to act through free-radical quenching, thus preventing lipid peroxidation of cellular membranes. However, photo-oxidation of lipids in biomembranes is believed to occur through photosensitization via type I or type II mechanisms:

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$$\text{sens} + A + h\nu \longrightarrow [\text{intermediates I}] \qquad \text{type I}$$

$$[\text{intermediates I}] + O_2 \longrightarrow \text{products} + \text{sens}$$

$$\text{sens} + O_2 + h\nu \longrightarrow [\text{intermediates II}] \qquad \text{type II}$$

$$[\text{intermediates II}] + A \longrightarrow \text{products} + \text{sens}$$

$$\text{sens} + h\nu \longrightarrow {}^1\text{sens}$$

$${}^1\text{sens} \longrightarrow {}^3\text{sens}$$

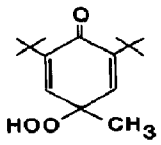
$${}^3\text{sens} + {}^3O_2 \longrightarrow \text{sens} + {}^1O_2$$

which are believed to be unaffected by the common antioxidants used to prevent "dark oxidation". Using two different sensitizers, erythrosine and riboflavine, for oxidation of methyl oleate and methyl linolenate, Chan [3] showed that erythrosine sensitization involves singlet oxygen while riboflavine-sensitized oxidations involve triplet oxygen. It was further shown by Chan that riboflavine-sensitized reactions do not involve an induction period or chain reaction like those seen in dark oxidations. This was supported by the relatively small inhibitory action of the antioxidant BHT in the riboflavine reactions. This observation implies that prevention of photosensitized oxidation involving either singlet or triplet oxygen should not be possible through the antioxidants commonly used to inhibit dark oxidation. A possibility that singlet oxygen initiates peroxidation while propagation still occurs through free radicals resulting from cleavage of hydroperoxides, however, does exist. To determine whether or not the phenolic antioxidants can prevent the photo-oxidation of lipids, we have studied the *in vitro* effect of some commonly used antioxidants on the photo-oxidation of lipids. Irradiation of methyl linoleate with unfiltered UV light ($\lambda > 320$ nm) showed less than 1% consumption. A marked increase in the photo-oxidation of methyl linoleate was observed in the presence of BHT. Similarly, consumption of methyl linolenate and arachidonate was much lower with UV light alone than when BHT was present. Conversely, BHA and tocopherol acetate did not show any significant effect on the photo-oxidation. Enhanced photo-oxidation with BHT was shown to involve free radicals as well as singlet oxygen by characterization of the lipid peroxidation products and by the effect of 1,4-diazabicyclo[2.2.2]octane (DABCO), a singlet oxygen quencher.

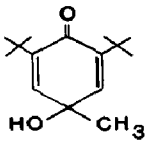
2. Materials and methods

BHA, BHT, D,L-α-tocopherol, tocopheryl acetate, methyl linoleate, methyl linolenate, methyl arachidonate (99.9% pure) and egg phosphatidylcholine (type III E) were purchased from Sigma Chemical Company (St. Louis, MO). DABCO was purchased from the Aldrich Chemical Company. Reference samples of methyl esters of 9-hydroxy-10,12-, 10-hydroxy-12,13-, 12-

hydroxy-9,13- and 13-hydroxy-9,11-octadecadienoate were prepared as described by Thomas and Pryor [4]. Methyl-9,10- and methyl-12,13-epoxy-octadecenoates were prepared by the method of Wu *et al.* [5] and by treating methyl linoleate with 1 molar equivalent of *m*-chloroperbenzoic acid in CH_2Cl_2 at 25 °C. $NaBH_4$ reduction was done in MeOH (Me ≡ methyl), and catalytic hydrogenations were carried out at atmospheric pressure in ethyl acetate with palladium on charcoal (10%). Hydroxy ester derivatives were silylated with 1:3:9 trimethylchlorosilane:hexamethyldisilazane:pyridine. The liposomes were prepared from phosphatidylcholine (5 mg ml^{-1}) by sonication in potassium phosphate buffer (50 mM; pH 7.5). BHT was incorporated into liposomes by dissolving phosphatidylcholine in chloroform with the required amount of BHT. The solvent was removed under nitrogen and the residual lipid was suspended in the buffer and sonicated for 5 min. The lipids from the liposomes were extracted with chloroform:MeOH (2:1) and transesterified with $NaOCH_3$–MeOH [6] to determine the percentage of fatty acids present by gas–liquid chromatography (GLC). For the emulsification of the free fatty acid esters in phosphate buffer, 0.05% sodium deoxycholate was used. 2,6-di-*tert*-butyl-4-hydroperoxy-4-methylcyclohexa-2,5-dienone (I) and the corresponding hydroxydienone (II) were prepared as described by Thomas and Foote [7], and 2,6-di-*tert*-butyl-1,4-benzoquinone (III) was purchased from the Aldrich Chemical Company:



I



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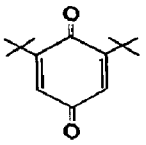


III

2.1. Irradiation

To study the photo-oxidation of fatty acids, a solution of these compounds in hexane or as an emulsion in phosphate buffer (50 mM; pH 7.5) was irradiated in a Pyrex tube with unfiltered UV light using F40 BLB fluorescent lamps (ten fluorescent tubes, 20 W each) which emit a continuous spectrum between 300 and 400 nm with approximately 1% of the fluence below 320 nm. The lamps were arranged in a hemicylindrical well covered with a Plexiglas top. The test-tubes to be irradiated were hung from the top in the middle of the well so that all the samples received equal irradiation. The control and test samples were always put next to each other. The samples were cooled through a constant circulation of air in the well by a fan. The microsomes were irradiated similarly in potassium phosphate buffer (50 mM; pH 7.5). High pressure liquid chromatography (HPLC), GLC, spectroscopic measurements and singlet oxygen quenching studies were carried out as described previously [8].

3. Results and discussion

Figure 1 shows the photo-oxidation of methyl linoleate, methyl linolenate and methyl arachidonate in the presence of BHT. Irradiation of methyl linoleate (3 mg ml^{-1}) in hexane for 4 h with UV light resulted in less than 1% consumption. When this irradiation was carried out in the presence of BHT (5 mg ml^{-1}), a marked increase in the consumption (to approximately 20%) of methyl linoleate was observed. Methyl linolenate and methyl arachidonate showed a similarly enhanced consumption in the presence of BHT as shown in Fig. 1. The increase in photo-oxidation was found to be dependent on the concentration of BHT. At lower concentrations of BHT (up to 1 mg mg^{-1} fatty acid) the enhancement in photo-oxidation was shown to be linear, after which further increases in the concentration of BHT did not show any effect (Fig. 2). Since this effect was observed only in the presence of UV radiation (Fig. 1), it was considered to be photochemical in nature.

The enhancing effect of BHT could be due to a direct or indirect (through photoproducts of BHT) photosensitization by this compound. This possibility was supported by the fact that BHT itself underwent photo-oxidation under our experimental conditions (Fig. 3) and the photo-oxidized mixture of BHT similarly enhanced the photo-oxidation of fatty acids. Irradiation for 4 h consumed more than 70% of the BHT and the solution turned yellow, possibly as a result of the formation of quinones. Two products of BHT photo-oxidation, III and I, were identified by comparing their thin layer chromatograms (silica gel G; hexane:ether, 70:30) and gas–liquid chromatograms (3% OV-101; 170 °C) with those of authentic samples. The latter product has also been reported to form in singlet oxygen oxidation of BHT [7].

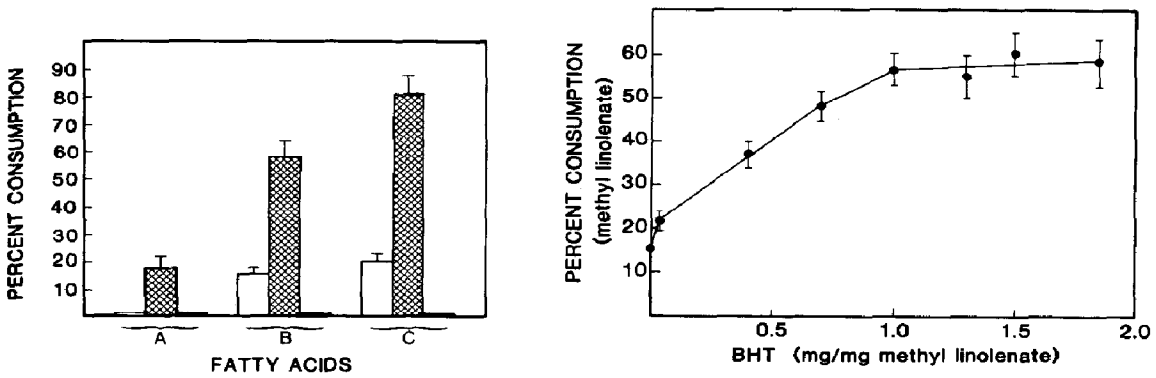


Fig. 1. Photo-oxidation of fatty acids in the presence (▩) or absence (□) of BHT. A solution of these acids in hexane (3 mg ml^{-1}) was irradiated for 4 h in the absence or presence of BHT (5 mg ml^{-1}) in a Pyrex tube with unfiltered UV light: A, methyl linoleate; B, methyl linolenate; C, methyl arachidonate; ■, fatty acids plus BHT in the dark.

Fig. 2. Effect of BHT concentration on the photo-oxidation of methyl linolenate.

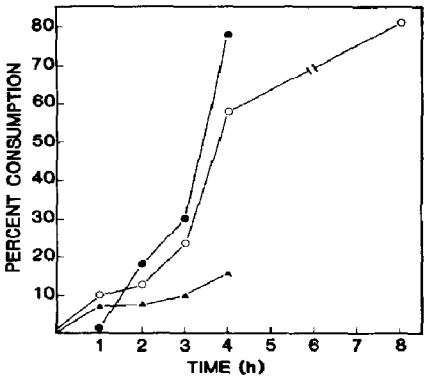


Fig. 3. Photo-oxidation of methyl linolenate in the presence (○) or absence (▲) of BHT. The irradiation was carried out as described in Fig. 1. BHT alone (●) was photo-oxidized under the same conditions.

When methyl linolenate was irradiated in the presence of the photo-oxidized mixture of BHT, a similar enhancing effect in its consumption (as with BHT alone) was observed, indicating that the enhanced photo-oxidation of unsaturated fatty acids was possible because of photosensitized reactions by quinones resulting from the oxidation of BHT. This was further supported by the fact that the time courses of the photo-oxidation of methyl linolenate and of BHT ran parallel to each other (Fig. 3). A marked increase in the consumption was observed after 3 h when considerable accumulation of the oxidized products of BHT had occurred.

Thin layer chromatography (TLC) of the total irradiated mixture showed the formation of several products (Fig. 4). The major oxidation products of methyl linoleate were identified as monoepoxy compounds of methyl linoleate and only a small amount of hydroperoxides was detected. These were isolated by preparative TLC and analyzed by HPLC, GLC and mass spectroscopy. As reported previously [8], HPLC of the hydroperoxides showed the formation of two conjugated (9-hydroperoxy and 13-hydroperoxy isomers) and two non-conjugated diene hydroperoxides (10-hydroperoxy and 12-hydroperoxy isomers), suggesting the participation of singlet oxygen. The formation of these isomers was also confirmed by GLC. For GLC analysis, the hydroperoxides were reduced with $NaBH_4$, and their trimethylsilyl (TMS) ethers were formed (Figs. 5 and 6). It has been shown by Chan [3] and more recently by Thomas and Pryor [4] that oxidation of fatty acids by singlet oxygen can be distinguished from that effected by free radicals by the nature of the hydroperoxides formed. For example, in free-radical oxidation, methyl linoleate gives two conjugated hydroperoxides (9-hydroperoxy-10,12- and 13-hydroperoxy-9,11-octadecadienoate) in equal proportions. Oxidation by singlet oxygen, in contrast, gives a mixture of the two conjugated (9-hydroperoxy and 13-hydroperoxy isomers) and two non-conjugated diene hydroperoxides (10-hydroperoxy-8,12- and 12-hydroperoxy-9,13-octadecadienoate).

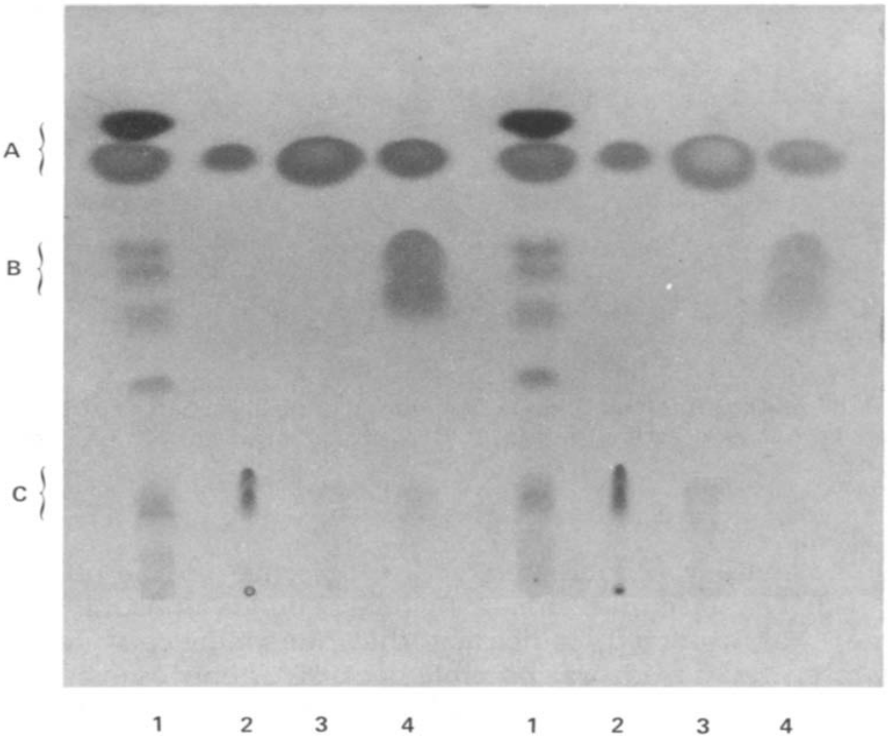


Fig. 4. Thin layer chromatogram of the photo-oxidation products of methyl linoleate: column 1, photo-oxidized in the presence of BHT; column 2, photosensitized oxidation in the presence of methylene blue; column 3, autoxidation at 60 °C for 4 h; column 4, treated with *m*-chloroperbenzoic acid. The samples in columns 1 - 3 were treated with $NaBH_4$ after oxidation: A, unchanged methyl linoleate; B, methyl-12,13- and methyl-9,10-epoxyoctadecenoates; C, methyl linoleate hydroperoxides.

The major fraction separated by TLC was identified as a mixture of methyl-9,10- and methyl-12,13-epoxyoctadecenoates by cochromatography (silica gel G; hexane:ether:acetic acid, 80:20:1) with authentic samples, and by IR and mass spectroscopy as described by Wu *et al.* [5]. The epoxy compounds were also detected in the hydroperoxide fraction and were tentatively identified as monoepoxy hydroperoxy derivatives of methyl linoleate (Fig. 6, peak 4). It should be noted that the epoxy compounds were observed only as a minor product when methyl linoleate was irradiated without BHT. Bulk phase oxidation of methyl linoleate has been shown to produce hydroperoxides predominantly, and the formation of epoxy compounds is not observed [9]. Conversely, monolayer oxidation of methyl linoleate has been shown to give epoxides as the major product of oxidation while hydroperoxides are formed only in minute quantities [5]. In this respect, BHT-induced photo-oxidation mimics oxidation in monolayers. Since singlet oxygen is not known to cause epoxidation of unhindered double bonds, the formation of epoxides should occur through free-radical formation. This expectation was supported by the fact that when photo-oxidation was carried out in polar solvents (phosphate buffer or CH_2Cl_2) the amount of epoxy com-

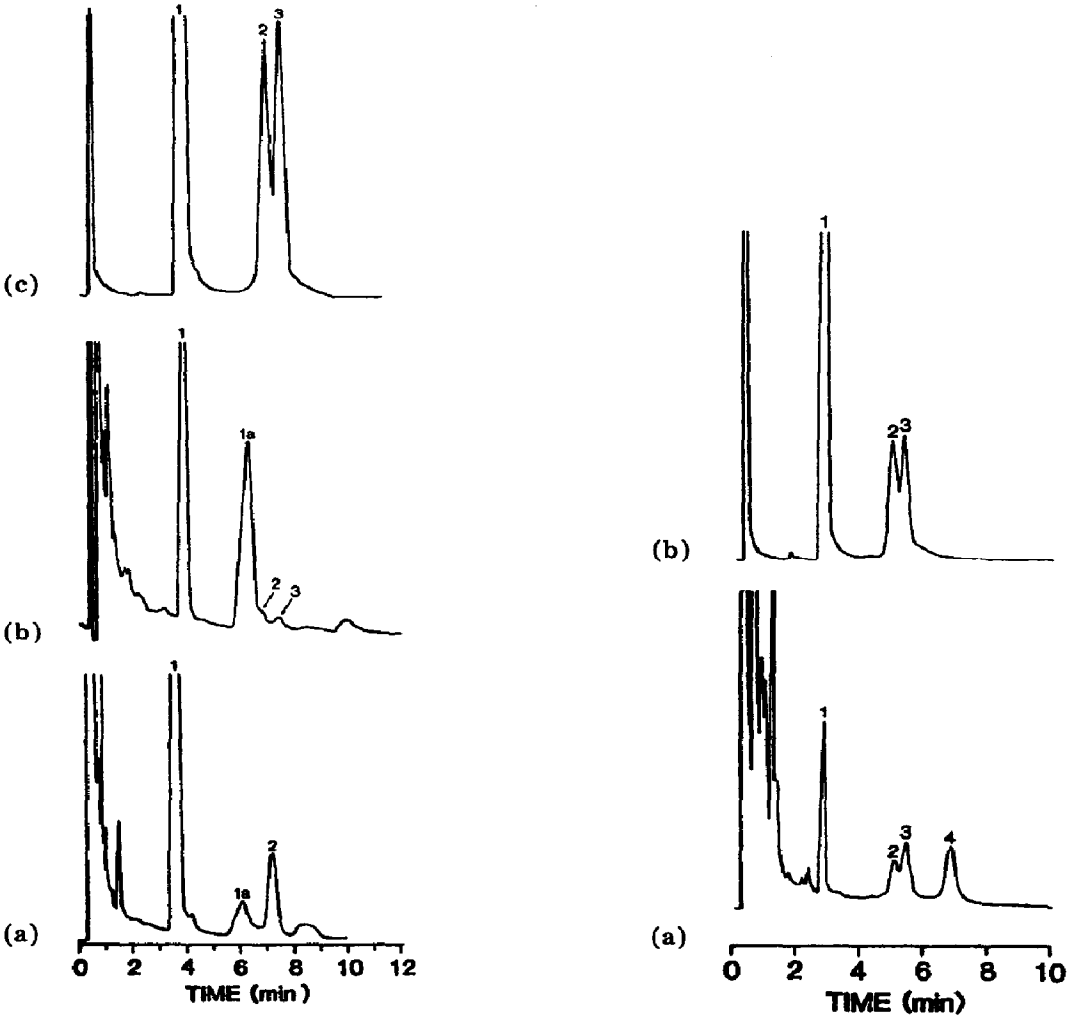
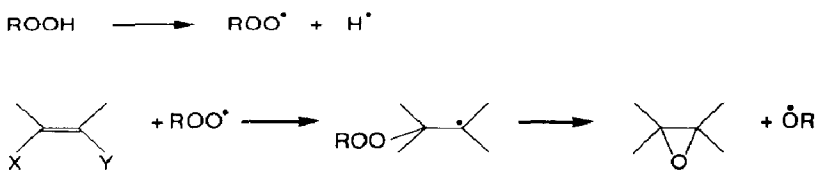


Fig. 5. Gas–liquid chromatogram of silyl ethers of $NaBH_4$-reduced photo-oxidized methyl linoleate (a) without BHT, (b) in the presence of BHT and (c) in the presence of methylene blue: peaks 1, unreacted methyl linoleate; peaks 1a, methyl-9,10- and methyl-12,13-epoxyoctadecenoates; peaks 2, TMS ethers of non-conjugated (10-hydroperoxy and 12-hydroperoxy isomers) hydroperoxides; peaks 3, TMS ethers of conjugated (9-hydroperoxy and 13-hydroperoxy isomers) hydroperoxides.

Fig. 6. Gas–liquid chromatogram of silyl ethers of $NaBH_4$-reduced photo-oxidized methyl linoleate in the presence of (a) BHT (hydroperoxide fraction) and (b) methylene blue: peaks 1, unreacted methyl linoleate; peaks 2, TMS ethers of non-conjugated (10-hydroperoxy and 12-hydroperoxy isomers) hydroperoxides; peaks 3, TMS ethers of conjugated (9-hydroperoxy and 13-hydroperoxy isomers) hydroperoxides; peak 4, TMS ethers of monoepoxy hydroperoxides (tentatively identified).

pounds was greatly reduced (data not shown) as expected for free-radical reactions. Conversely, if singlet oxygen is involved in the formation of epoxy compounds, an increase would be expected in the formation of these compounds in CH_2Cl_2 in which the lifetime of singlet oxygen is known to be

several times higher than in hexane [10]. A plausible mechanism for the formation of epoxides could be through the addition of peroxy radicals on olefinic bonds:



Formation of hydroperoxides, however, involves abstraction of allylic hydrogen from another fatty acid molecule thus propagating the chain reaction. Since the activation energy required for the latter reaction is much lower than that for addition to a double bond, hydroperoxides are presumably formed as the primary product in bulk phase oxidation. To achieve epoxidation of double bonds, the activation energy for the addition reaction must be lowered. It is possible that this occurs through a type I mechanism in which III forms a complex with fatty acids in the presence of UV radiation. When photo-oxidation of fatty acids was carried out in the presence of DABCO, a well-known singlet oxygen quencher [11 - 13], a marked reduction in the consumption of fatty acids was observed (Fig. 7). The presence of DABCO also inhibited the formation of both the hydroperoxides and the epoxy compounds (Table 1), suggesting that hydroperoxides may be the primary products and that the inhibition of photo-oxidation may occur through quenching of singlet oxygen. Alternatively, inhibition of photo-oxidation may occur simply through energy transfer from the excited state of the sensitizer to DABCO molecules.

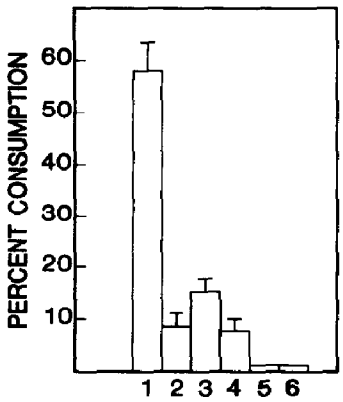


Fig. 7. Effect of DABCO on BHT-induced photo-oxidation of methyl linolenate: bar 1, methyl linolenate + *hν* + BHT; bar 2, methyl linolenate + *hν* + BHT + DABCO; bar 3, methyl linolenate + *hν*; bar 4, methyl linolenate + *hν* + DABCO; bar 5, methyl linolenate + BHT + DABCO; bar 6, methyl linolenate + DABCO. Each bar is the mean plus or minus the standard error of five separate experiments.

TABLE 1

Effect of 1,4-diazabicyclo[2.2.2]octane on the formation of major photo-oxidation products of methyl linoleate

Experimental conditions	*Amount of DABCO* (mM)	*Reduction* (%)	
		Epoxy fatty acids	*Hydroperoxy fatty acids*
Control	None	None	None
Control + DABCO	4.4	58	>90
Control + DABCO	22.0	93	>99

Methyl linoleate was irradiated with BHT for 4 h as described in Section 2 in the presence or absence of DABCO. The percentage reduction in yield of epoxy and hydroperoxy compounds was determined by GLC (3% OV-101; 210 °C) using methyl palmitate as an internal standard.

TABLE 2

The effect of butylated hydroxytoluene on the photo-oxidation of methyl linoleate in liposomes[a]

Experimental conditions	*Methyl linoleate* (%)[b]	*Amount of BHT* ((mg lipid)$^{-1}$)
Control	17.0 ± 3.2	None
Control + $h\nu$[c]	14.5 ± 2.1	None
Control + $h\nu$ + BHT	17.0 ± 3.5	40.0 μg
Control + $h\nu$ + BHT	14.8 ± 3.2	1.5 mg

[a] The liposomes were prepared from egg phosphatidylcholine (5 mg ml^{-1}) in potassium phosphate buffer (50 mM; pH 7.5). BHT was incorporated into liposomes by dissolving phosphatidylcholine in chloroform with the required amount of BHT. The solvent was removed under nitrogen and the residual lipid was suspended in the buffer and sonicated for 5 min.

[b] The lipids from the liposomes were extracted with chloroform:MeOH (2:1) and transesterified with $NaOCH_3$–MeOH. The percentage of methyl linoleate is the weight per cent of the total fatty acids present in liposomes and was determined by GLC (4% OV-1; 200 °C) using methyl palmitate as an internal standard.

[c] Irradiation was carried out for 4 h using unfiltered UV light (ten fluorescent tubes, 20 W each). The output of the source was in the range 320 - 400 nm with less than 1% below 320 nm. Each value represents the mean plus or minus the standard error of three separate experiments.

To determine whether BHT would similarly enhance photo-oxidation in biomembranes, the photo-oxidation of fatty acids in liposomes was studied. As shown in Table 2, no significant effect of BHT was observed in this system. Whether this is due to inadequate distribution of BHT in liposomes or to quenching of type I reactions in polar media is not known at present.

Acknowledgments

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