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CHLOROPHYLL DESTRUCTION IN THE PRESENCE OF BISULFITE AND LINOLEIC ACID HYDROPEROXIDE*

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Key Word Index—Bisulfite oxidation; linoleic acid hydroperoxide decomposition; chlorophyll destruction; free radicals.

Abstract—Chlorophyll was rapidly destroyed in the presence of bisulfite and linoleic acid hydroperoxide. Both bisulfite and linoleic acid hydroperoxide were required for chlorophyll destruction and both were consumed in the reaction; however, there was no oxygen requirement. Chlorophyll destruction occurred most readily in the slightly acidic pH region with little destruction occurring above pH 8. The free radical scavengers, hydroquinone and α-tocopherol, were very effective at inhibiting chlorophyll destruction, but the singlet oxygen quenchers, β-carotene, 2,5-dimethylfuran and 1,3-diphenylisobenzofuran, were only slightly effective. The addition of metal chelators indicated that metals were not participating in the reaction. The evidence indicates that chlorophyll was destroyed by a free radical mechanism. Based on the present results and that of others, it is suggested that chlorophyll was destroyed via oxidation by the alkoxy radical which was produced during the decomposition of linoleic acid hydroperoxide by bisulfite.

INTRODUCTION

Sulfur dioxide is one of the major air pollutants which causes damage to plants, and leaf chlorosis is one of the main symptoms. We have demonstrated that chl† was destroyed in aqueous ethanol solution by free radicals produced during the aerobic oxidation of bisulfite, which was initiated either by light or by Mn^{2+}-glycine [1].

The destruction of chl by a lipoxygenase-unsaturated fatty acid system has been studied by several workers [2–4]. Linoleic and linolenic acids were the most effective fatty acids [2], and it has been suggested that chl was destroyed by co-oxidation during the formation of fatty acid hydroperoxides by a specific lipoxygenase [3] or the subsequent enzymatic decomposition of the hydroperoxides [2, 4]. We have therefore examined the effect of bisulfite on the destruction of chl in the presence of LOOH or LnOOH. In this paper we describe the essential role of bisulfite in the presence of LOOH to destroy chl by a free radical mechanism.

RESULTS

Comparison of the absorption spectra of chl plus LOOH and chl plus LOOH and bisulfite revealed that there was a marked loss in absorbance in both the red and blue absorption regions when bisulfite was present, and no new peaks or peak shifts were observed. Chl *a* appeared to be destroyed more readily than chl *b*, and this differential rate of destruction has been observed in other systems for chl destruction with bisulfite [1, 5].

Destruction of chl occurred most readily at a slightly acid pH and the apparent optimum was near pH 4 (Fig. 1). However, the real optimum for the reaction may be at a lower pH, but could not be determined due to the instability of chl at low pH. To investigate the possible physiological importance of this reaction pH 6.3 was used for the experiment. At this pH about 70% of the chl was destroyed in 15 sec (Fig. 2). Fig. 2 also shows that when only LOOH was present, no destruction of chl occurred, and it was very slight when only bisulfite was present. There was no oxygen requirement for the destruction of chl with LOOH plus bisulfite since the same amount was destroyed in N_2 as in air. This was in contrast with the other two systems previously described for chl destruction which were dependent on oxygen for oxidation of bisulfite [1].

The relationship between chl destruction and bisulfite loss, as affected by various LOOH concentrations, is shown in Fig. 3 and the relationship between chl destruction and LOOH decomposition, as affected by various bisulfite concentrations, is illustrated in Fig. 4. It is apparent that the amount of chl lost during the reaction was closely related to both the amount of bisulfite lost and LOOH decomposed. It can be estimated that at low concentrations of LOOH or bisulfite, for each mole of chl destroyed, about 20 moles of bisulfite and 10 moles of LOOH were lost. Thus, for each mole of LOOH decomposed, about 2 moles of bisulfite were lost.

To examine the specificity of the reaction, H_2O_2 or LnOOH was substituted for LOOH (Table 1). Although H_2O_2 readily oxidizes bisulfite to sulfate [6], it caused little chl destruction either in the absence or presence of bisulfite. As could be expected from the structural similarity, LnOOH could substitute for LOOH in the reaction

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† Abbreviations. chl: chlorophyll; DNP-cysteic acid, *N*-2,4-dinitrophenyl-L-cysteic acid; DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid); LOOH, linoleic acid hydroperoxide; LnOOH: linolenic acid hydroperoxide; $^1O_2$: singlet oxygen; bisulfite is used to designate the sum of SO_2, SO_3^{2-} and HSO_3^-.

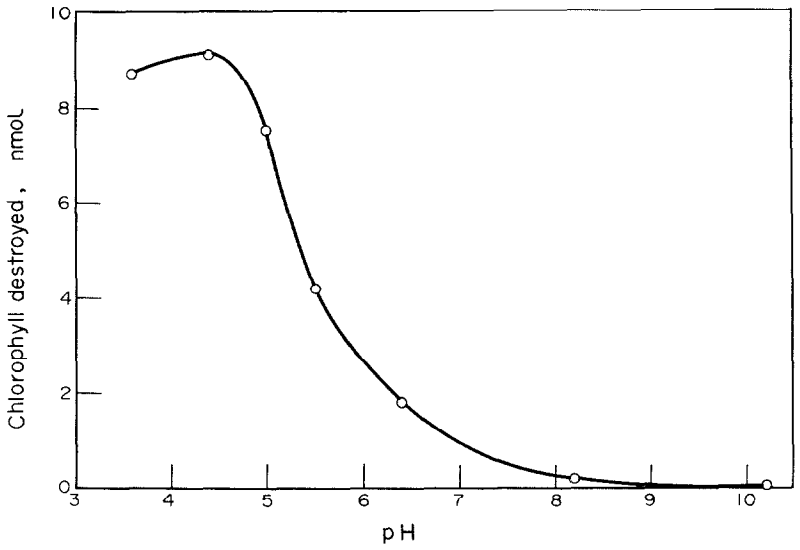

Fig. 1. The dependence of chl destruction upon pH. Concentration of reactants was 0.25 mM HSO_3^-, 0.1 mM LOOH and 14 μM chl. Incubation time was 4 sec.

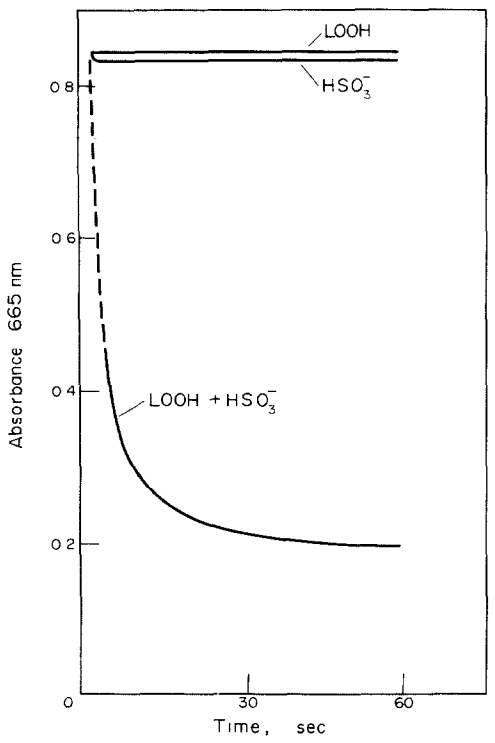

Fig 2. Time course of chl destruction in the presence of LOOH only, HSO_3^- only or both LOOH and HSO_3^-. The absorbance at 665 nm was followed using a spectrophotometer with attached recorder. When both LOOH and HSO_3^- were present, the reaction was started by adding 0.5 μmol $NaHSO_3$. The reaction was so rapid that the initial rate could not be followed and the dotted line represents the extrapolation back to zero time.

with bisulfite. At the same concentrations, both LOOH and LnOOH gave comparable rates of chl destruction. Substitution of LOOH with linoleic acid resulted in only a slight amount of chl destruction over the short reaction times of our experiments, and this probably resulted from LOOH contaminating the linoleic acid sample.

LOOH decomposition is known to proceed via a free radical mechanism [7–11], and this can be mediated by metal ions [7, 8, 10] and may generate $^1O_2$ [12]. In order to gain more information concerning the mechanism of chl destruction in the present system, three classes of inhibitors were used: free radical scavengers, $^1O_2$

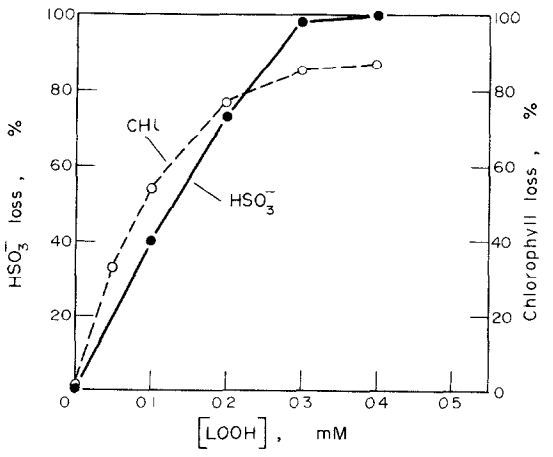

Fig. 3. Relationship between chl destruction and bisulfite loss as affected by the concentration of LOOH Initial concentrations of HSO_3^- and chl were 0.5 mM and 14 μM, respectively and incubation time was 3 min.

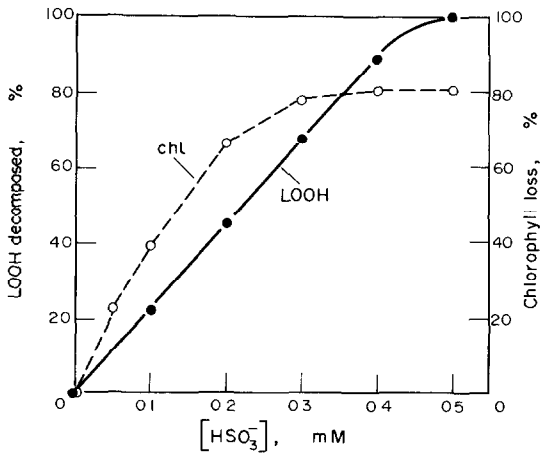

Fig. 4. Relationship between chl destruction and LOOH decomposition as affected by the concentrations of bisulfite. Initial LOOH and chl concentrations were 0.2 mM and 14 μM, respectively, and the incubation time was 3 min.

quenchers and metal chelators. We have previously shown that the radical scavengers hydroquinone and α-tocopherol inhibit chl destruction by the bisulfite-O_2 system which was initiated either by light or Mn^{2+}-glycine [1]. Results presented in Table 2 illustrate that they were also effective in inhibiting chl loss in the presence of LOOH and bisulfite. α-Tocopherol was more effective than hydroquinone, and essentially complete inhibition could be attained with these inhibitors.

To investigate the possibility that $^1O_2$ was involved in the destruction of chl, the effects were examined of the $^1O_2$ quenchers β-carotene, 2,5-dimethylfuran and 1,3-diphenylisobenzofuran [13]. These gave some inhibition of chl destruction, but were not as effective as the free radical scavengers (Table 2). Higher concentrations of 1,3-diphenylisobenzofuran were not used because it became insoluble. At 0.02 mM, β-carotene caused 17% inhibition of chl destruction, but it too was destroyed in this LOOH-bisulfite system. At 0.01 mM, in the absence of chl, 60% of the β-carotene was destroyed in 15 sec as determined by the loss in absorbance at 451 nm.

Several workers have reported that LOOH reacts with metals leading to homolytic cleavage of the hydroperoxide resulting in radical formation [7, 8, 10]. To consider the possibility that metals were involved in the LOOH-bisulfite reaction causing chl destruction, the effects were examined of EDTA, α,α-dipyridyl, benzoin α-oxime

Table 1. The effect of H_2O_2, LOOH and LnOOH upon chl destruction

Components	% Chl destroyed
Control	1
+ H_2O_2 (0.2 mM)	2
+ H_2O_2 (0.2 mM) + HSO_3^-	1
+ H_2O_2 (2 mM)	3
+ H_2O_2 (2 mM) + HSO_3^-	3
+ LOOH (0.2 mM) + HSO_3^-	79
+ LnOOH (0.2 mM) + HSO_3^-	76

Reaction conditions were those given in the Experimental and incubation time was 2 min.

Table 2. Effect of free radical scavengers, metal chelators and $^1O_2$ quenchers upon chl destruction

Compounds tested	concn (mM)	% Inhibition of chl destruction
Free radical scavengers		
Hydroquinone	3	52
Hydroquinone	30	83
α-Tocopherol	0.003	49
α-Tocopherol	0.1	94
Metal chelators		
Diethyldithiocarbamate	0.2	48
Diethyldithiocarbamate	1.5	98
Benzoin α-oxime	5	5
α,α-Dipyridyl	5	7
EDTA	5	0
$^1O_2$ quenchers		
β-Carotene	0.02	17
Dimethylfuran	1	10
Dimethylfuran	10	38
Diphenylisobenzofuran	0.5	33
Diphenylisobenzofuran	1	33

Reaction conditions were those listed under Experimental and incubation time was 4 sec.

(cupron) and diethyldithiocarbamate (Table 2). At 5 mM, α,α-dipyridyl and EDTA gave little or no inhibition. Although both diethyldithiocarbamate and benzoin α-oxime are copper chelators, they gave opposite results in our experiments. Diethyldithiocarbamate at 1.5 mM almost completely inhibited chl destruction while benzoin α-oxime caused little inhibition at 5 mM.

Since homolytic cleavage of LOOH by the Fe^{3+}-*N*-acetylcysteine system has been studied in detail [9, 10], we have compared it with bisulfite for its effect upon chl destruction. In the presence of *N*-acetylcysteine, $FeCl_3$ and LOOH, chl destruction was somewhat slower compared with bisulfite and LOOH (Table 3). By extending the reaction time and increasing the concentrations of *N*-acetylcysteine and LOOH comparable amounts of chl destruction between this system and the LOOH-bisulfite system were obtained.

To study the interaction between bisulfite and LOOH, in the absence of chl, $H^{35}SO_3^-$ and [1-^{14}C]LOOH were employed to facilitate the identification of reaction products. Paper radio-electrophoresis revealed that an adduct was formed between bisulfite and LOOH. It had an electrophoretic mobility of 0.7 relative to DNP-cysteic acid with phosphate buffer at pH 6.8 when either $H^{35}SO_3^-$ and LOOH or bisulfite and [1-^{14}C]LOOH

Table 3. Chl destruction in the *N*-acetylcysteine–$FeCl_3$–LOOH system and in the LOOH–bisulfite system

Components	% chl destroyed	
	30 sec	5 min
LOOH (0.2 mM) + *N*-acetylcysteine (1 mM) + Fe^{3+} (10 μM)	18	61
LOOH (0.6 mM) + *N*-acetylcysteine (3 mM) + Fe^{3+} (10 μM)	42	87
LOOH (0.6 mM) + *N*-acetylcysteine (3 mM)	1	18
LOOH (0.2 mM) + HSO_3^- (0.5 mM)	79	83

Table 4. Ethane and ethylene production from the reaction between bisulfite and LnOOH

Additions	Products (ρmol)	
	ethane	ethylene
pH 3.6		
none	2	2
bisulfite	14	48
bisulfite + α-tocopherol	4	10
pH 6.3		
none	2	2
bisulfite	3	12
bisulfite + α-tocopherol	3	5
pH 10.2		
none	1	2
bisulfite	1	3

Reaction conditions were those given in the Experimental except that LOOH was replaced with LnOOH and glycine–HCl, Na acetate and glycine–NaOH were used to obtain pH 3.6, 6.3 and 10.2, respectively. The concentration of α-topcopherol was 50 μM.

were used. This adduct comprised about 40% of the total [1-^{14}C]LOOH radioactivity. When $H^{35}SO_3^-$ was used, in addition to the adduct of LOOH and bisulfite, the major product was $^{35}SO_4^{2-}$ indicating that oxidation of bisulfite to sulfate was a major reaction in this system. No adduct was found when $H^{35}SO_3^-$ was reacted with linoleic acid.

Decomposition of lipid hydroperoxides via free radical mechanisms yielding pentane, ethane and ethylene [8, 11, 14] have been described. As bisulfite promotes decomposition of LOOH and LnOOH, we examined its effect on the formation of these hydrocarbons from LOOH and LnOOH. No pentane was detected from the reaction between bisulfite and LOOH at pH 6.3 as determined by GLC with a detectability limit of *ca* 50 ρmol. However, very small amounts of ethane and ethylene were detected from the reaction between bisulfite and LnOOH (Table 4). The greatest amounts of ethane and ethylene were produced at pH 3.6, but essentially none at pH 10.2. Both ethane and ethylene production were inhibited by α-tocopherol.

DISCUSSION

The characteristics of the reaction between bisulfite and LOOH causing chl destruction are consistent with the view that free radicals produced during the homolytic cleavage of LOOH by bisulfite were responsible for the destruction of chl.

Davies [15] has reported that the reaction between bisulfite/sulfite and hydroperoxides can occur by either the homolytic or heterolytic mechanism, depending upon pH. At about pH 9 the heterolytic mechanism predominates (equation 1), and at pH 0–1 the homolytic mechanism would predominate (equation 2). At intermediate pH's both mechanisms would operate.

$$SO_3^{2-} + ROOH \rightarrow SO_4^{2-} + ROH \quad (1)$$

$$HSO_3^- + ROOH \rightarrow HSO_3\cdot + RO\cdot + OH^- \quad (2)$$

Chl destruction was effectively inhibited by the free radical scavengers hydroquinone and α-tocopherol. Since α-tocopherol can also quench $^1O_2$ [16, 17], as well as scavenger free radicals, there was the possibility that $^1O_2$ was involved in chl destruction. However, this seems unlikely because other $^1O_2$ quenchers gave only partial inhibition (Table 2). Also β-carotene, which has been reported as being the most effective $^1O_2$ quencher known [17], caused only 17% inhibition at a concentration of 0.02 mM. This concentration is almost 10 times that of α-tocopherol which gave about 50% inhibition. These results indicate that α-tocopherol inhibited chl destruction by functioning as a radical scavenger and not as a $^1O_2$ quencher.

During the homolytic cleavage of LOOH by bisulfite, both alkoxy and bisulfite radicals are formed (equation 2). Assuming that the alkoxy radical was important in the destruction of chl, then other systems that produce it should also destroy chl. We found this to be the case with the *N*-acetylcysteine–$FeCl_3$–LOOH system (Table 3), in which Gardner *et al.* [9, 10] have proposed that the alkoxy radical is formed (equations 3 and 4, where *N*-acetylcysteine is denoted as RSH).

$$Fe^{3+} + RSH \rightarrow Fe^{2+} + RS\cdot \quad (3)$$

$$Fe^{2+} + LOOH \rightarrow Fe^{3+} + LO\cdot + OH^- \quad (4)$$

Equation 4 is very similar to equation 2 but with Fe^{2+} substituted for bisulfite.

Since chl was destroyed in the *N*-acetylcysteine–$FeCl_3$–LOOH system and metals have been reported to promote homolytic cleavage of LOOH in other systems [7, 8, 10], there was a possibility that metals were directly responsible for LOOH cleavage with bisulfite only keeping the metal ions in the reduced state, similar to the effect of *N*-acetylcysteine as depicted in equation 3. However, experiments with metal chelators (Table 2) suggest that this was not so. The effectiveness of diethyldithiocarbamate as an inhibitor was probably due to its radical scavenging ability [18] and not to its chelating properties.

The above discussion strongly suggests that the alkoxy radical which was formed from the reaction between LOOH and bisulfite was responsible for the destruction of chl through a free radical mechanism. We propose the following scheme which explains the reaction between bisulfite and LOOH leading to chl destruction.

$$HSO_3^- + LOOH \rightarrow LO\cdot + HSO_3\cdot + OH^- \quad (5)$$

$$LO\cdot + HSO_3^- + H^+ \rightarrow LOH + HSO_3\cdot \quad (6)$$

$$2HSO_3\cdot + H_2O \rightarrow SO_4^{2-} + HSO_3^- + 3H^+ \quad (7)$$

$$HSO_3\cdot + O_2 + H_2O \rightarrow SO_4^{2-} + O_2^- + 3H^+ \quad (8)$$

$$LO\cdot + chl + H^+ \rightarrow LOH\cdot + chl^+ \quad (9)$$

$$O_2^- + chl + 2H^+ \rightarrow H_2O_2 + chl^+ \quad (10)$$

$$chl^+ \rightarrow \text{oxidation products} \quad (11)$$

$$LOH + HSO_3\cdot \rightarrow \cdot L'(OH)SO_3^- + H^+ \quad (12)$$

$$\cdot L'(OH)SO_3^- + HSO_3^- + H^+ \rightarrow L'H(OH)SO_3^- + HSO_3\cdot \quad (13)$$

$$LO\cdot \rightarrow \text{decomposition products} \quad (14)$$

Equation 5 was proposed by Davies [15] for the homolytic cleavage of LOOH forming alkoxy and bisulfite radicals. The bisulfite radical can undergo dismutation (equation 7) or aerobic oxidation (equation 8) yielding sulfate ion, which was identified as one of the major products in this system. The alkoxy radical or O_2^- formed (equations 5 and 8) would then react with chl

leading to its destruction (equations 9–11). This is in keeping with earlier proposals [1, 19] that the first step is a one-electron oxidation of chl to chl^+ which subsequently forms irreversible oxidation products. Under anaerobic conditions, reactions 8 and 10 would not occur, but chl destruction could proceed through equation 9. This would explain why chl destruction was observed under anaerobic conditions. The above scheme also indicates that chl destruction was closely linked to bisulfite oxidation, and that neither would occur in the absence of LOOH. These predictions are in full agreement with our present observations. The conjugated diene hydroxy acid (LOH) formed by equations 6 and 9 should then readily react with bisulfite forming sulfonic acid adduct, L'H(OH)-SO_3^- (where L'H is the monoenoic analogue of linoleic acid) presumably through a free radical mechanism as depicted by equations 12 and 13. Although its sulfonic acid function was shown by our electrophoresis data, we did not obtain chemical confirmation that the adduct was $L'H(OH)SO_3^-$. However, this would be in line with the results of Gardner *et al.* [10] who studied the reaction between LOOH and *N*-acetylcysteine and found that the latter adds to the LOH which was an intermediate.

The route for the formation of ethylene and ethane from the decomposition of LnOOH can be readily visualized. Some of the 16-hydroperoxide of linolenic acid can be decomposed yielding the corresponding 16-alkoxy radical analogous to the reaction described by equation 5. This alkoxy radical would undergo β-scission [20] with the production of $Me\text{-}CH_2\cdot$ radical which would in turn form ethane or ethylene by the addition or abstraction of hydrogen.

No attempt was made in this study to characterize the breakdown products of chl. As in other systems [1, 2, 5] the absorption decreased greatly throughout the visible range without the formation of new peaks, suggesting that chl was rapidly converted into colorless products.

The co-oxidation of chl coupled to a lipoxygenase–linoleate system has been studied by several workers [2–4]. Chl was bleached by co-oxidation of unsaturated fatty acids catalyzed by extracts from various plants [2, 3]. This bleaching was inactivated by heat and inhibited by antioxidants. Although lipoxygenase appears to be necessary, the purified enzyme alone was ineffective, suggesting that some other unknown factor was essential [2]. Based on the observations that the pH optimum (*ca* 6) for chl bleaching by linoleate-soya extract was different from that for LOOH formation (pH *ca* 8), Holden [2] proposed that chl was bleached during the breakdown of lipid hydroperoxide which was formed by lipoxygenase. Later, Zimmerman and Vick [4] presented evidence indicating that the hydroperoxide isomerase which catalyzes the conversion of hydroperoxide to the corresponding ketohydroxy compound was involved in the chl bleaching reaction. However, Imamura and Shimizu [3] recently questioned the participation of hydroperoxide isomerase in this bleaching reaction. The results reported here show that bisulfite was very effective at decomposing LOOH, and bleaching chl coupled to the decomposition of LOOH. This function of bisulfite may be similar to that of the heat-labile factor of plant extracts that was necessary for the co-oxidation of chl and fatty acids [2]. Recent work from Kaplan *et al.* [21] and from this laboratory (C. Lizada, unpublished results) has indicated that bisulfite can react with linoleic acid *in vitro* to form LOOH. Linoleic and linolenic acids comprise approximately 80% of the fatty acids in the chloroplast [22] but there is as yet no evidence that SO_2 could cause peroxidation of unsaturated fatty acids, and subsequent breakdown of the lipid hydroperoxides with co-oxidation of chl in the chloroplasts *in vivo*. However, investigation of this possibility seems warranted.

EXPERIMENTAL

Chl was extracted and partially purified from market spinach (*Spinacia oleracea*) by the procedure of ref. [23] using MeOH, dioxane and H_2O. Chl concn was estimated spectrophotometrically using the equations of Wintermans and De Mots [24] for chl in EtOH. LOOH was prepared by reacting linoleic acid with soybean lipoxygenase [25]. [1-^{14}C]LOOH was prepared from [1-^{14}C]linoleic acid by the same procedure. LnOOH was prepared using 1 mM linolenic acid and 10000 units/ml of soybean lipoxygenase with a 5 min reaction time at 0° [26]. Purification of LOOH, [1-^{14}C]LOOH and LnOOH was carried out by TLC using Si gel 7GF developed with hexane–Et_2O–HOAc (9.1:1); the spots were detected using $Fe(SCN)_3$ reagent. After elution from the gel, samples were stored in 95% EtOH at −20°. LOOH and LnOOH were estimated spectrophotometrically using the molar A of 25250 at 233 nm [7]. Chl destruction was determined from the loss in A at 665 nm. A standard reaction mixture in a semimicro cuvette contained in 1.0 ml 20 nmol NaOAc, 0.2 μmol LOOH, 0.5 μmol $NaHSO_3$ and 14 nmol chl in 65% EtOH at pH 6.3. In 65% EtOH, NaOAc has a good buffer capacity at pH 6.3. For an anaerobic experiment a Thunberg-type cuvette was used with N_2 to replace air. LOOH decomposition was followed using the thiocyanate method for peroxides [27]. For the assay 200 μl of reaction mixture was added to 2.75 ml 95% EtOH followed by 20 μl conc HCl and 10 μl 0.1 M $FeSO_4$. Exactly 1 min after adding the $FeSO_4$ 20 μl of 2.5 M NaSCN was added and 3 min later, the A at 480 nm was determined. Although $NaHSO_3$ interferes in this assay, no $NaHSO_3$ remained in the reaction mixture when the ratio of $NaHSO_3$ to LOOH was $\leqslant 2$. When $NaHSO_3$ was present, LOOH was extracted into Et_2O and the thiocyanate assay was conducted after evaporation of Et_2O. Bisulfite was determined spectrophotometrically using DTNB reagent [28]. Analysis of pentane, ethane, and ethylene was made on the gas phase of the enclosed reaction flasks using GLC with an Al_2O_3 column and FID.

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