Sulfite-Mediated Destruction of β-Carotene

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Sulfite-mediated β-carotene destruction was investigated in two systems. In the first system, which consisted of Mn^{2+}, O_2, glycine, and sulfite, over 90% of the β-carotene was destroyed in 15 s. Destruction occurred concurrently with the aerobic oxidation of sulfite and was effectively inhibited by the free radical scavengers α-tocopherol, 1,2-dihydroxybenzene-3,5-disulfonic acid, and butylated hydroxytoluene. In the second system, which consisted of linoleic acid hydroperoxide and bisulfite, about 80% of the β-carotene was destroyed in 15 s; oxygen was not required. Destruction occurred concurrently with the disappearance of sulfite and hydroperoxide. The free radical scavengers α-tocopherol and butylated hydroxytoluene effectively inhibited the destruction of β-carotene, but the singlet oxygen quencher 2,5-dimethylfuran was only slightly effective. Addition of metal chelators indicated metals were not participating in the reaction. This evidence indicates that in these two systems β-carotene was destroyed by free radicals, though via different mechanisms. A possible mechanism which accounts for such reactions is described.

Sulfur dioxide is a major air pollutant which causes injury to vegetation, animals, and human beings (National Air Pollution Control Administration, 1969). Its ionized species, bisulfite and sulfite, are widely used in foods, beverages, and pharmaceuticals (Schroeter, 1966). Sulfite (sulfite is used to designate the sum of SO_2, SO_3^{2-}, and HSO_3^-) can undergo aerobic oxidation to sulfate via a free radical mechanism forming oxidizing free radicals (Abel, 1951; Fridovich and Handler, 1960; Hayon et al., 1972; Yang, 1970). We have previously shown that chlorophyll was destroyed in vitro by the sulfite–oxygen free radical system (Peiser and Yang, 1977) and by the sulfite–linoleic acid hydroperoxide (LOOH) system (Peiser and Yang, 1978). Since β-carotene is also an important component of the chloroplast pigment system, we have investigated and described in this report the destruction of β-carotene by the sulfite–oxygen and sulfite–LOOH systems.

EXPERIMENTAL SECTION

Materials. Chlorophyll was partially purified, and LOOH and linolenic acid hydroperoxide (LnOOH) were prepared as previously described (Peiser and Yang, 1978). β-Carotene was obtained from Sigma.

Methods. Sulfite-mediated β-carotene destruction was investigated in a Mn^{2+} system and a LOOH system. A standard reaction mixture for the Mn^{2+} system contained in a total volume of 1 mL of 20 μmol of glycine, 1 μmol of $NaHSO_3$, 7.4 nmol of β-carotene, 50 nmol of $MnSO_4$, and 76% ethanol (v/v) at pH 9.2. A standard mixture for the LOOH system contained in a 1-mL volume 20 μmol of sodium acetate, 0.5 μmol of $NaHSO_3$, 7.4 nmol of β-carotene, 0.2 μmol of LOOH, and 76% ethanol (v/v) at pH 6.7. At this ethanol concentration pH values are only empirical and were determined using a glass electrode. For both systems β-carotene was dissolved in $CHCl_3$ and 18 μL of this solution was added to each reaction cuvette. β-Carotene destruction was determined from the loss in absorbance at 454 nm using a Beckman DU spectrophotometer. Absorption spectra were determined using a Beckman DK-2 spectrophotometer. For anaerobic experiments a Thunberg-type cuvette was used with nitrogen replacing air. Sulfite concentrations were determined spectrophotometrically using 5,5′-dithiobis(2-nitrobenzoic acid) (Humphrey et al., 1970).

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Table I. Requirements for the Destruction of β-Carotene in the Mn^{2+} System

components[a]	β-carotene destroyed, %
complete	94
minus HSO_3^-	0.1
minus Mn^{2+}	2.2
minus O_2	2.7
minus glycine	1.0

[a] The compositions of complete reaction were those for the Mn^{2+} system described in the Experimental Section. The reaction time was 15 s.

Table II. Effect of BHT, Tiron and α-Tocopherol upon Inhibition of β-Carotene Destruction in the Mn^{2+} System (Reaction Time Was 4 s)

inhibitor	concn, mM	inhibition, %
BHT	5.5	58
	20	99
tiron	0.009	52
	0.02	97
α-tocopherol	0.006	54
	0.013	97

RESULTS

Destruction of β-carotene occurred in the presence of sulfite in two different systems: Mn^{2+} or with LOOH. When sulfite was present, loss of absorption occurred throughout the visible absorption region with no new peaks or peak shifts occurring for either system (Figure 1).

Characteristics of Mn^{2+} system. Sulfite, Mn^{2+}, oxygen, and glycine were required for β-carotene destruction (Table I). The requirement of glycine for both β-carotene destruction and sulfite oxidation is more clearly shown in Figure 2. In the absence of glycine, but with sulfite and Mn^{2+}, no measurable sulfite oxidation and only a slight amount of β-carotene destruction occurred. When glycine was present, β-carotene destruction occurred concurrently with sulfite oxidation, and rates of both reactions increased as the glycine concentration increased. At 20 mM glycine over 90% of the β-carotene was destroyed in 15 s. The glycine requirement for sulfite oxidation was apparently necessitated by the high ethanol concentration since Mn^{2+} alone readily initiates sulfite oxidation in aqueous solutions (Yang, 1970). If β-carotene destruction was caused by free radicals produced during the aerobic oxidation of sulfite, then free radical scavengers should inhibit destruction.

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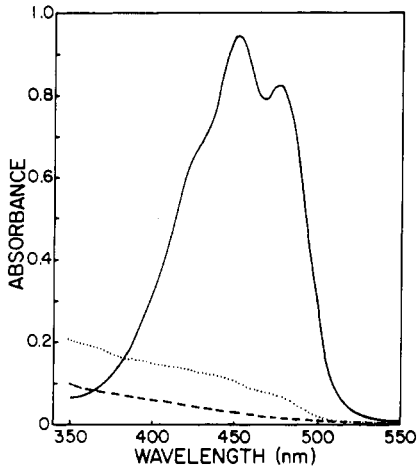

Figure 1. Visible absorption spectrum of β-carotene after incubation for 1 min with bisulfite in the absence (—) or in the presence of Mn^{2+} (- - -) or LOOH (···).

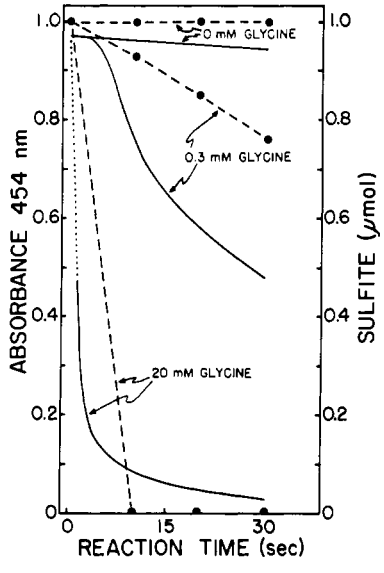

Figure 2. Time courses of β-carotene destruction and sulfite oxidation in the Mn^{2+} system with various concentrations of glycine. The pH of the reaction mixture was maintained between pH 8 and 9 by the addition of HCl or NaOH. The reaction was started by the addition of 1.0 μmol of Na_2SO_3. β-Carotene destruction (—) was followed with a spectrophotometer attached to a recorder and sulfite oxidation (- - -) was determined periodically by the DTNB reagent. β-Carotene destruction and sulfite oxidation in the presence of 20 mM glycine were so rapid that their initial rates could not be followed. The dotted line represents the extrapolation back to zero time.

Table II illustrates the inhibition by the radical scavengers. Butylated hydroxytoluene (BHT), 1,2-dihydroxybenzene-3,5-disulfonic acid (tiron), and α-tocopherol gave approximately 50% inhibition at 5 mM, 9 μM, and 6 μM, respectively, and all three inhibitors gave essentially complete inhibition at higher concentrations.

Characteristics of the LOOH System. β-Carotene destruction occurred very rapidly in the LOOH system with about 80% destruction occurring in 15 s (Figure 3). Both sulfite and LOOH were required, but in contrast to the Mn^{2+} system, neither oxygen nor glycine were necessary in this system. The reaction occurred most rapidly in the acidic pH region with the amount of β-carotene destruction decreasing rapidly as the pH increased (Figure 4). Values below pH 4.5 were not examined because the reaction rate became too rapid to follow. To consider the possible significance of such reaction in biological systems

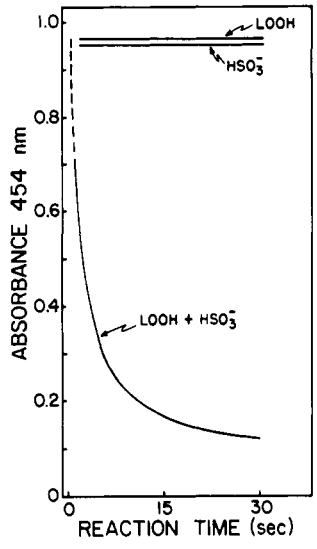

Figure 3. Time course of β-carotene destruction in the presence of LOOH only, bisulfite only, or both LOOH and bisulfite. The absorbance of 454 nm was followed with a spectrophotometer attached to a recorder. When both LOOH and bisulfite were present, the reaction was started by adding 0.5 μmol of $NaHSO_3$. The reaction was so rapid that the initial rate could not be followed and the dotted line represents the extrapolation back to zero time.

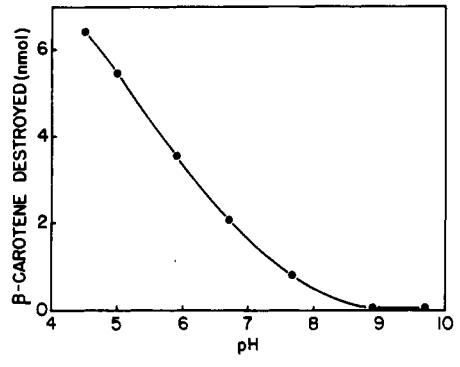

Figure 4. The dependence of β-carotene destruction upon pH in the LOOH system. Incubation time was 1 s.

Table III. Effect of H_2O_2, LnOOH, and LOOH upon β-Carotene Destruction

components	% destruction
control[a]	1
+ H_2O_2 (0.2 mM)	2
+ H_2O_2 (0.2 mM) + HSO_3^-	2
+ H_2O_2 (2 mM)	1
+ H_2O_2 (2 mM) + HSO_3^-	3
+ LnOOH (0.2 mM) + HSO_3^-	77
+ LOOH (0.2 mM) + HSO_3^-	82

[a] The control reaction mixture was composed of 20 mM sodium acetate, 7.4 μmol of β-carotene, and 76% ethanol (v/v) at pH 6.7 in a volume of 1 mL. The concentration of HSO_3^- where indicated was 0.5 mM. Reaction time was 15 s.

most experiments were conducted at pH 6.7 at which destruction was still very rapid (Figure 3). The specificity for LOOH was examined by substituting H_2O_2 or LnOOH for LOOH (Table III). H_2O_2 was not effective in destroying β-carotene, but LnOOH was effective, as expected from their structural similarity.

The effect of radical scavengers upon β-carotene destruction in this system was examined (Table IV). α-Tocopherol was a potent inhibitor and could completely inhibit β-carotene destruction. BHT was not as effective as α-tocopherol while tiron was only slightly effective.

Table IV. Effect of Free Radical Scavengers, Metal Chelators, and Dimethylfuran upon the Inhibition of β-Carotene Destruction in the LOOH System (Reaction Time was 4 s)

inhibitor	inhibitor concn, mM	% inhibition
α-tocopherol	0.032	54
	0.64	97
BHT	15	51
	30	69
tiron	5	16
	10	39
DIECA	0.2	54
	2.0	93
EDTA	5	−24
α,α-dipyridyl	5	−3
benzoin α-oxime	5	−13
dimethylfuran	1	15
	10	33

Table V. Effect of Bisulfite, Chlorophyll, and Light upon β-Carotene Destruction[a]

addition	β-carotene destroyed, %
none, dark	2
none, light	3
HSO_3^-, dark	6
HSO_3^-, light	8
chlorophyll, dark	1
chlorophyll + HSO_3^-, dark	5
chlorophyll + HSO_3^-, light	65

[a] The basic reaction mixture and conditions were those for the Mn^{2+} system listed under Experimental Section, except that Mn^{2+} and HSO_3^- were deleted and the pH was 4.2. Where indicated 14 nmol of chlorophyll or 0.5 μmol of $NaHSO_3$ was added and light was provided with a 15-W daylight fluorescent bulb at 286 μEm^{-2} s^{-1} (400–700 nm).

Since metals can also cause homolytic cleavage of hydroperoxides to form free radicals (Gardner, 1975; Gardner et al., 1976), the possible involvement of metals in this LOOH–sulfite system was examined by employing metal chelators (Table IV). EDTA, α,α-dipyridyl, and benzoin α-oxime did not inhibit β-carotene destruction, but even slightly stimulated its destruction. In contrast, diethyldithiocarbamate (DIECA) was an effective inhibitor. 2,5-Dimethylfuran, a quencher of singlet oxygen, which can be formed during the decomposition of lipid hydroperoxide (King et al., 1975), gave only partial inhibition at 10 mM (Table IV).

Earlier work (Peiser and Yang, 1977) indicated that chlorophyll functioned as a photosensitizer in the light initiating sulfite oxidation with chlorophyll itself subsequently being destroyed. Therefore, the effect of sulfite, light, and chlorophyll upon β-carotene destruction was examined (Table V). Very little destruction occurred in the light or dark with β-carotene alone, and the addition of sulfite caused only 6–8% destruction. However, 65% of the β-carotene was destroyed in the light when both chlorophyll, presumably acting as a photosensitizer, and sulfite were present.

DISCUSSION

The characteristics of β-carotene destruction in the Mn^{2+} system suggests that free radicals produced during the aerobic oxidation of sulfite were responsible for its destruction. It has been demonstrated that glycine was required for the Mn^{2+}-mediated sulfite oxidation in aqueous ethanol medium; other amino acids such as serine, alanine, and methionine could substitute for glycine (Peiser and Yang, 1977). The function of glycine in initiating sulfite oxidation in this system is not known.

The aerobic oxidation of sulfite can be initiated by metals (Abel, 1951; Yang, 1970), photosensitized chlorophyll (Fridovich and Handler, 1960; Peiser and Yang, 1977), illuminated chloroplasts (Asada and Kiso, 1973), as well as by enzymatic reactions (Fridovich and Handler, 1961). A mechanism which explains the Mn^{2+}-initiated oxidation of sulfite (Abel, 1951; Yang, 1970) involves O_2^- and SO_3^- radicals in the initiation steps (eq 1 and 2). Once

$$Mn^{2+} + O_2 \rightarrow Mn^{3+} + O_2^- \quad (1)$$

$$Mn^{3+} + SO_3^{2-} \rightarrow Mn^{2+} + SO_3^- \quad (2)$$

the chain-initiating species are formed, sulfite oxidation is maintained through a series of chain-propagating reactions in which OH and other oxysulfur radicals are generated, and sulfate is the termination product (Abel, 1951; Yang, 1970; Hayon et al., 1972).

O_2^-, OH, and SO_3^- radicals are oxidants and have been implicated to be involved in the destruction of many biological molecules such as methionine (Yang, 1970), tryptophan (Yang, 1973), NADH and NADPH (Klebanoff, 1961; Tuazon and Johnson, 1977), indole-3-acetic acid (Horng and Yang, 1975), and certain nucleosides (Hayatsu et al., 1972). OH radical cannot be involved in the present β-carotene destruction system because ethanol, an effective OH radical scavenger, was employed in the reaction solution. The involvement of O_2^- in Mn^{2+}-initiated sulfite oxidation systems has been demonstrated by using superoxide dismutase (Yang, 1973). In our system the high ethanol concentration excluded the use of this enzyme. However, tiron, a potent O_2^- scavenger (Greenstock and Miller, 1975) was effective in inhibiting β-carotene destruction at low concentrations, suggesting that O_2^- might be involved. A possible mechanism which accounts for the β-carotene destruction is described by eq 3, 4, and 5. The

$$\beta\text{-carotene} + O_2^- + H^+ \rightarrow \beta\text{-carotene}\cdot + H_2O_2 \quad (3)$$

$$\beta\text{-carotene} + SO_3^- \rightarrow \beta\text{-carotene}\cdot + H^+ + SO_3^{2-} \quad (4)$$

$$\beta\text{-carotene}\cdot + O_2 \rightarrow \beta\text{-carotene–OO}\cdot \rightarrow \text{oxidation products} \quad (5)$$

first step consists of a hydrogen abstraction from β-carotene by O_2^- and/or SO_3^- radicals to form β-carotene polyene radical (eq 3 and 4), which then reacts with oxygen to form the oxidation products. The chemical nature of the oxidation products was not characterized.

The characteristics of the reaction between sulfite and LOOH causing β-carotene destruction also imply the involvement of free radicals. Sulfite is known to react with hydroperoxides either through the homolytic mechanism at pH 0–1 (eq 6) or through the heterolytic mechanism at ca. pH 9 (eq 7) (Davies, 1961). At intermediate pH's, both

$$HSO_3^- + ROOH \rightarrow HSO_3\cdot + RO\cdot + OH^- \quad (6)$$

$$SO_3^{2-} + ROOH \rightarrow SO_4^{2-} + ROH \quad (7)$$

mechansism would operate. The pH curve (Figure 4) and inhibitor experiments (Table III) for β-carotene destruction in the LOOH system are consistent with the homolytic free radical mechanism. Although α-tocopherol can quench singlet oxygen (Foote et al., 1974) as well as scavenge free radicals, it is most unlikely that the inhibition was due to its function as a $^1O_2$ quencher because the addition of dimethylfuran, a known $^1O_2$ quencher, gave only slight inhibition.

Since metals can promote homolytic cleavage of LOOH (Gardner, 1975; Gardner et al., 1976), the possible involvement of metal in the present β-carotene destruction

system was investigated. Except for DIECA, none of the chelators employed inhibited the destruction. It is therefore concluded that metals do not participate in the reaction. The effectiveness of DIECA as an inhibitor was probably due to its function as a radical scavenger (Lutz et al., 1973) and not to its chelating properties.

In the LOOH system we suggest that the LO and HSO_3 radicals, produced in the homolytic cleavage of LOOH by bisulfite (eq 6), are the oxidizing agents. As in the Mn^{2+} system, the initial step involves the H· abstraction probably from the C-4 carbon of β-carotene as proposed by Weber and Grosch (1976) and Ikediobi and Snyder (1977) for the lipoxygenase-catalyzed oxidation of β-carotene. The resulting β-carotene polyene radical is subsequently further oxidized by LOOH (eq 10) or oxygen (eq 5), yielding the

$$\beta\text{-carotene} + LO\cdot \rightarrow \beta\text{-carotene}\cdot + LOH \quad (8)$$

$$\beta\text{-carotene} + SO_3^- \rightarrow \beta\text{-carotene}\cdot + H^+ + SO_3^{2-} \quad (9)$$

$$LOOH + \beta\text{-carotene}\cdot \rightarrow \text{oxidation products} \quad (10)$$

colorless oxidation products.

Cooxidation of β-carotene by lipoxygenase while catalyzing the oxidation of linoleic acid (LH) is well known. Much work has been conducted to characterize the relationship between the positional specificities of peroxidation and carotene-bleaching activity of various isolipoxygenases (Grosch et al., 1976; Weber and Grosch, 1976; Ikediobi and Synder, 1977). Early investigators (Tookey et al., 1958) have attributed the cooxidation of β-carotene by lipoxygenase and LH to a free radical intermediate formed during the oxidation of LH to LOOH. Recently Ikediobi and Synder (1977) reported that lipoxygenase and LOOH also caused the cooxidation of β-carotene, but the system required oxygen, and the reaction appeared to occur at a much slower rate than that of the present LOOH–bisulfite system. In these lipoxygenase-catalyzed cooxidations of β-carotene, it was proposed that LOO· was the oxidizing agent either directly oxidizing β-carotene molecule (Weber and Grosch, 1976) or oxidizing the inactive lipoxygenase to an active (oxidized) enzyme which in turn oxidizes β-carotene (Ikediobi and Synder, 1977).

The present and previous studies (Peiser and Yang, 1977, 1978) demonstrate that both β-carotene and chlorophyll can be destroyed by free radicals produced in the Mn^{2+}–sulfite or LOOH–sulfite system. However, it is yet to be demonstrated whether β-carotene may undergo such free radical mediated destruction in plant tissues when they are exposed to sulfur dioxide or treated with sulfite.

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