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Riboflavin-sensitized photooxidation of ascorbic acid: kinetics and amino acid effects

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The study on the effects of riboflavin contents and light intensity on the riboflavin-sensitized decomposition of ascorbic acid indicated that light and riboflavin accelerated the decomposition of ascorbic acid. The relative photosensitizing activity of riboflavin, methylene blue and protoporphyrin IX was 21:15:1 at 1·2 ppm. The rate constants for reaction of ascorbic acid with singlet oxygen at pH 7·5, 6·0 and 4·5 were $6{\cdot}63 \times 10^8$, $5{\cdot}77 \times 10^8$ and $5{\cdot}27 \times 10^8$ M^{-1} s^{-1}, respectively. Cysteine showed strong antioxidant activity on both riboflavin- and methylene blue-sensitized photooxidation of ascorbic acid and its antioxidant effect was concentration-dependent. Alanine and phenylalanine at 0·1% concentration showed antioxidant effects on the riboflavin-sensitized photooxidation of ascorbic acid but prooxidant effects on the methylene blue-sensitized photooxidation. Tyrosine (0·1%) exhibited strong prooxidant activity on both the riboflavin- and methylene blue-sensitized photooxidation of ascorbic acid. However, tryptophan (0·1%) showed antioxidant or prooxidant activity on the photooxidation of ascorbic acid depending on the storage time.

INTRODUCTION

Ascorbic acid is an important nutrient and very likely to decompose during storage. A number of researches have been reported that temperatures, pH, oxygen concentration, metals and light are important parameters for the destruction of ascorbic acid (Khan & Martell, 1969; Singh *et al.*, 1976; Sahbaz & Somer, 1993). Riboflavin was found to be very photolabile in solution (Sattar *et al.*, 1977*a*; Allen & Parks, 1979; Kearsley & Rodriguez, 1981) and can also photosensitize the destruction of ascorbic acid (Sattar *et al.*, 1977*b*; Sahbaz & Somer, 1993). Riboflavin induced both Type I and Type II photosensitized oxidation (Buettner & Need, 1985; Min, 1991). Type I photosensitized oxidation involves the formation of free radicals or radical ions due to the transfer of hydrogen atoms or electrons by interaction of the triplet sensitizer with other molecules. Type II process involves the generation of singlet oxygen ($^1O_2$) by the energy transfer from an excited triplet sensitizer to a triplet oxygen. Sahbaz and Somer (1993) reported that photodecomposition of ascorbic acid is very efficient in the presence of oxygen, riboflavin and light, resulting in a high yield because of formation of singlet oxygen by an energy transfer from triplet riboflavin to oxygen molecule. The riboflavin-sensitized photooxidation of tryptophan has been studied by Kanner and Fennema (1987). Kanner and Fennema (1987) reported that photooxidation of tryptophan was accelerated in the presence of riboflavin and that the riboflavin-sensitized photooxidation of tryptophan was greatly reduced by ascorbic acid.

Chou and Khan (1983) reported that the rate constant for total quenching of singlet oxygen by ascorbic acid ($k_q + k_r$) was $8{\cdot}3 \times 10^6$ M^{-1} s^{-1} in H_2O at pH 6·8, and $2{\cdot}50 \times 10^6$ M^{-1} s^{-1} in D_2O at pH 7·2. Rooney (1983) reported that the rate constant for total quenching of singlet oxygen by ascorbic acid ($k_q + k_r$) was $8{\cdot}4 \times 10^6$ M^{-1} s^{-1} in pyridine, as determined photochemically, and $1{\cdot}06 \times 10^7$ M^{-1} s^{-1} in pyridine as determined in a dark reaction. Even though the rate constant ($k_q + k_r$) for total quenching of singlet oxygen by ascorbic acid was reported, the reactivity of ascorbic acid toward singlet oxygen (k_r) has not been reported. Bodannes and Chan (1979) estimated that the reactivity of ascorbate toward singlet oxygen was approximately of the same order of magnitude as azide which was $2{\cdot}2 \times 10^8$ M^{-1} s^{-1} in D_2O at pH 7·5. Bodannes and Chan (1979) also reported that methionine reduced riboflavin-sensitized photooxidation of ascorbic acid. However, information on the effects of various amino acids on the riboflavin sensitized photodecomposition of ascorbic acid is not available. Even though the effects of riboflavin on the photooxidation of ascorbic

acid have been extensively studied, the photosensitizing activity of riboflavin on the oxidation of ascorbic acid has not been compared with those of other well known sensitizers such as methylene blue and protoporphyrin IX. The objectives of this study were (1) to investigate the effects of riboflavin content and light intensity on the photodecomposition of ascorbic acid, (2) to compare the photosensitizing activity of riboflavin with those of methylene blue and protoporphyrin IX, (3) to determine the bimolecular rate constant for reaction of ascorbic acid with singlet oxygen at different pHs, and (4) to study the effects of various amino acids on the riboflavin or methylene blue sensitized photodecomposition of ascorbic acid.

MATERIALS AND METHODS

Materials

Ascorbic acid, riboflavin, methylene blue, protoporphyrin IX, alanine, cysteine, histidine, phenylalanine, tryptophan, tyrosine, sodium azide and EDTA were purchased from Sigma Chem. Co. (St Louis, MO). HPLC grade acetonitrile and 85% phosphoric acid were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Serum bottles, teflon-lined rubber septa and aluminum caps were purchased from Supelco Inc. (Bellefonte, PA). Demineralized purified water was produced by using a Milli-Q purification system (Millipore, Bedford, MA). The demineralized purified water was always used for the sample preparation.

Effects of riboflavin contents on the photooxidation of ascorbic acid

To study the effects of riboflavin contents on photooxidation of ascorbic acid, 0, 1·2, 2·4, 3·6 and 6·0 ppm of riboflavin were added to $1·2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 0·1 mM EDTA. The addition of EDTA was to ensure the prevention of the possible metal catalyzed oxidation of ascorbic acid (Bodannes & Chan, 1979). Twenty ml of the prepared sample was transferred into a 30 ml serum bottle. The bottles were sealed with teflon-lined rubber septa and aluminum caps, and then placed, in duplicate, in the light storage box which was described in detail by Fakourelis *et al.* (1987) and Jung *et al.* (1991). The light storage box was a wooden box (70 × 50 × 60 cm) and a transpurent glass plate (thickness, 5 mm) was placed about 20 cm above five cool-white fluorescent lamps within the wooden box. Sample bottles were placed on the glass plate. The light intensity at the sample level was 3300 lux. The light intensity was controlled by adjusting the number of fluorescent lamps on and the distance between the sample level and fluorescent lamps. The temperature within the light box during storage was 25°C. Ascorbic acid content was determined at 265 nm by using a spectrophotometer (UV visible recording spectrophotometer, UV-160A, Shimadzu Corporation, Kyoto, Japan) (Bodannes & Chan, 1979). The accuracy of the data obtained by the direct UV method at 265 nm was confirmed by using a HPLC method (Bradbury & Singh, 1986) on the decrease in ascorbic acid content in $1·2 \times 10^{-4}$M ascorbic acid in 0·01M potassium phosphate buffer (pH 7·5) containing 2·4 ppm riboflavin and 0·1 mM EDTA during 15 min storage. The data obtained by both methods were not significantly different at $P < 0·05$. The relative photosensitizing activity of riboflavin, methylene blue and protoporphyrin IX was studied under the same condition.

Effects of light intensity on the photooxidation of ascorbic acid

To study the effects of light intensity on the photooxidation of ascorbic acid, 1·2 ppm of riboflavin was added to $1·2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 0·1 mM EDTA. Twenty ml of the prepared sample was transferred into a 30 ml serum bottle and sealed with a teflon-lined rubber septum and an aluminum cap. The bottles were placed, in duplicate, in the light storage box. The light intensities at the sample level were 0, 1100, 2200, 3300 or 5500 lux. Ascorbic acid content was determined by using the spectrophotometric analysis (Bodannes & Chan, 1979).

Detection of singlet oxygen by a chemical trapping method

Sodium azide, which is a well known singlet oxygen quencher, was added to detect the singlet oxygen involvement in the riboflavin-sensitized photooxidation of ascorbic acid (Lion *et al.*, 1976; Parkin & Lowum, 1990). In this study, 0·2 mM sodium azide and 2·4 ppm of riboflavin were added to $1·2 \times 10^{-4}$M ascorbic acid in 0·01M potassium phosphate buffer (pH 7·5) containing 0·1 mM EDTA. Twenty ml of the prepared sample was transferred into a 30 ml serum bottle and sealed with a teflon-lined rubber septum and an aluminum cap, and then was placed, in duplicate, in the light storage box. The light intensity at the sample level was 3300 lux. Ascorbic acid content was determined by using the HPLC method (Bradbury & Singh, 1986).

Bimolecular rate constants for reaction of ascorbic acid with singlet oxygen at various pHs

To study the reaction rate constants of singlet oxygen oxidation of ascorbic acid at pH 7·5, 6·0 and 4·5, a steady state kinetic equation was used (Foote, 1979; Jung & Min, 1991; Jung *et al.*, 1991; Lee & Min, 1991). In this study, 6 ppm of methylene blue, which is a well known sensitizer for producing singlet oxygen only, was added to $0·4 \times 10^{-4}$, $0·5 \times 10^{-4}$, $0·6 \times 10^{-4}$, $0·8 \times 10^{-4}$ or $1·0 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5, 6·0 or 4·5) containing 0·1 mM EDTA. Twenty ml of the prepared sample was transferred into a 30 ml serum bottle and sealed with a teflon-lined rubber septum and an aluminum cap. The

bottles were placed, in triplicate, in the light storage box for 3 min at 20°C. The light intensity at the sample level was 5500 lux. Ascorbic acid was determined by measuring the absorbances of the solutions at 265 or 248 nm using a spectrophotometer.

Effects of amino acids on the photooxidation of ascorbic acid

To study the effects of amino acids on the singlet oxygen oxidation of ascorbic acid, 0·1% of alanine, cysteine, histidine, phenylalanine, tryptophan or tyrosine and 2·4 ppm of riboflavin or methylene blue were added to $1·2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 0·1 mM EDTA. Twenty ml of the prepared sample was transferred into a 30 ml serum bottle and sealed with a teflon-lined rubber septum and an aluminum cap. The bottles were placed, in duplicate, in the light storage box. The light intensity at the sample level was 3300 lux. The oxidation of ascorbic acid was monitored by the HPLC method (Bradbury & Singh, 1986) because some amino acids interfere at the absorbance at 265 nm for the determination of ascorbic acid using direct UV method.

Since we found, in the present study, that among the tested amino acids cysteine showed the strongest inhibitory effect on the riboflavin-sensitized photooxidation of ascorbic acid, we studied the quantitative effects of cysteine on the photosensitized oxidation of ascorbic acid. To study the quantitative effects of cysteine on the riboflavin-sensitized photooxidation of ascorbic acid, 0, 0·010, 0·025, 0·050 or 0·100% of cysteine and 2·4 ppm of riboflavin were added to $1·2 \times 10^{-4}$ M ascorbic acid solution in 0·01 M potassium phosphate buffer (pH 7·5) containing 0·1 mM EDTA. Twenty ml of the prepared sample was transferred into a serum bottle and sealed with a teflon-lined rubber septum and an aluminum cap. The bottles were placed in the light storage box. The light intensity at the sample level was 3300 lux. Oxidation of ascorbic acid was monitored by using the HPLC method (Bradbury & Singh, 1986).

HPLC method for ascorbic acid determination

High performance liquid chromatograph (Model 45, Water Associates, Milford, MA) equipped with a UV detector (Waters Associates, Milford, MA) was used (Bradbury & Singh, 1986). The column used was μ-Bondapak C-18 (30 cm × 3·9 mm, Waters Associates, Milford, MA). The mobile phase (flow rate 1·3 ml/min) was aqueous 0·005 M KH_2PO_4 adjusted to pH 4·6 with dilute HCl and acetonitrile (30:70, v/v). Ten μl of sample was injected. The ascorbic acid peak was eluted after 1.75 min. These peaks were monitored at 254 nm by a UV detector. The ascorbic acid contents in samples were calculated by comparing the peak areas of the samples with those of standard ascorbic acid solutions using a standard calibration curve.

Statistical analysis

All the experiments were done in duplicate or in triplicate. Statistical analysis was done by using the Statistical Analysis System (SAS, 1985). Duncan's multiple range test was used to ascertain treatment effects on the photooxidation of ascorbic acid.

RESULTS AND DISCUSSION

Effects of riboflavin contents on the photooxidation of ascorbic acid

The effects of 0, 1·2, 2·4, 3·6 or 6·0 ppm of riboflavin on the photooxidation of $1·2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 0·1 mM EDTA during light storage are shown in Fig. 1. To ensure the accuracy of the data obtained by the direct UV method at 265 nm, the ascorbic acid contents in the system of $1·2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 2·4 ppm riboflavin was measured by both direct UV method and HPLC method during 15 min light storage. The data obtained from both direct UV method and HPLC method were not significantly different at $P < 0·05$. That is, the data obtained by the direct UV method could be used for the determination of ascorbic acid in this system. Riboflavin accelerated the degradation of ascorbic acid during light storage as was expected. As the concentrations of riboflavin increased, the decomposition of ascorbic acid increased considerably. When 6 ppm riboflavin was added, 100% of ascorbic acid was destroyed after 12 min of light storage. Without addition of riboflavin, after 12 min light storage, 98% of ascorbic acid remained unoxidized. These results clearly showed that riboflavin acted as a photosensitizer to accelerate the destruction of ascorbic acid. The oxidation of ascorbic acid might be due to

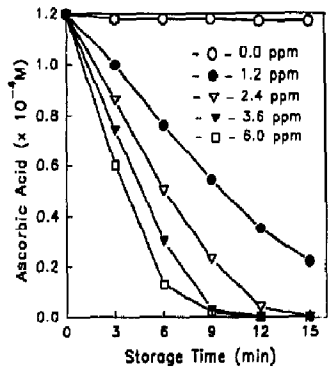


Fig. 1. The effects of 0, 1·2, 2·4, 3·6 or 6·0 ppm of riboflavin on the photooxidation of $1·2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 0·1 mM EDTA during light storage.

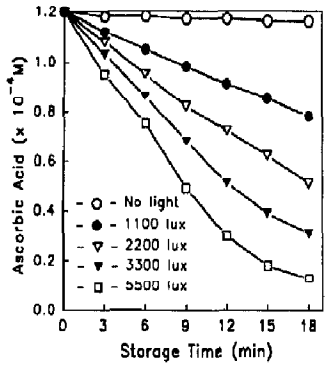


Fig. 2. The effects of light intensity on the photooxidation of $1{\cdot}2 \times 10^{-4}$ M ascorbic acid on 0·01 M potassium phosphate buffer (pH 7·5) containing 1·2 ppm riboflavin and 0·1 mM EDTA during light storage.

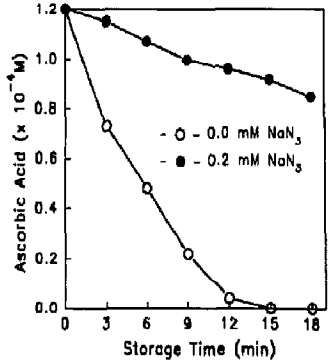


Fig. 3. The effects of 0·2 mM sodium azide on the photooxidation of $1{\cdot}2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 2·4 pmm riboflavin and 0·1 mM EDTA during light storage.

the generation of singlet oxygen, superoxide anion, or excited triplet riboflavin. It has been reported that riboflavin induced both Type I and Type II photosensitized oxidations (Buettner & Need, 1985). The photosensitizing effect of riboflavin on the ascorbic acid oxidation was compared with those of methylene blue and protopopyrine IX (data not shown). The initial destruction rate of ascorbic acid was $1{\cdot}48 \times 10^{-7}$ mol/min for 1·2 ppm riboflavin, $1{\cdot}08 \times 10^{-7}$ mol/min for 1·2 ppm methylene blue, and $0{\cdot}07 \times 10^{-7}$ mol/min for 1·2 ppm protoporphyrin IX. The relative photosensitizing activity of riboflavin, methylene blue and protoporphyrin IX was 21:15:1.

Effects of light intensity on the photooxidation of ascorbic acid

The effects of light intensity on the photooxidation of $1{\cdot}2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 1·2 ppm riboflavin during light storage are shown in Fig. 2. The light intensities at the sample level were 0, 1100, 2200, 3300 and 5500 lux. In the dark, most of ascorbic acid remained unoxidized after 18 min storage. The results indicated that both riboflavin and light are required to accelerate the oxidation of ascorbic acid. As the light intensity increased from 0 to 5500 lux, the destruction of ascorbic acid increased significantly ($P < 0{\cdot}05$). The ascorbic acid contents in samples containing 1·2 ppm riboflavin after 18 min of light storage under 0, 1100, 2200, 3300 and 5500 lux decreased from $1{\cdot}2 \times 10^{-4}$ M to $1{\cdot}12 \times 10^{-4}$, $0{\cdot}83 \times 10^{-4}$, $0{\cdot}67 \times 10^{-4}$ and $0{\cdot}56 \times 10^{-4}$ and $0{\cdot}39 \times 10^{-4}$ M, respectively.

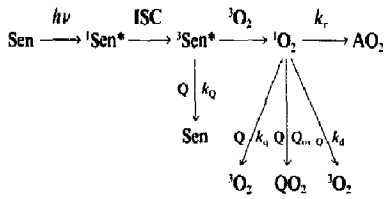
Detection of singlet oxygen in the system

To detect the singlet oxygen involvement in the riboflavin sensitized photooxidation of ascorbic acid, sodium azide, which is a known singlet oxygen quencher, was added to the system. If the addition of sodium azide reduced riboflavin-sensitized photooxidation of ascorbic acid, singlet oxygen was produced in the system. The effect of 0·2 mM sodium azide on the photooxidation of $1{\cdot}2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 2·4 ppm riboflavin during light storage is shown in Fig. 3. Ascorbic acid oxidation accelerated by riboflavin photosensitization was greatly reduced by the addition of 0·2 mM sodium azide. The results in Fig. 3 show that 0·2 mM sodium azide resulted in a 81% inhibition of ascorbic acid photooxidation after 12 min light storage. This result suggested that singlet oxygen was involved in the riboflavin-sensitized photooxidation of ascorbic acid and the oxidation of ascorbic acid was, to at least some extent, due to the reaction between the singlet oxygen and ascorbic acid. The present results are consistent with previous reports (Buettner & Need, 1985; Frenkel, 1983; Chacon *et al.*, 1988) in that riboflavin under light can produce a sufficient level of singlet oxygen to enhance the rate of some photooxidation under some conditions. Min (1991) reported that the singlet oxygen formation in whole and skim milk by riboflavin under light was detected by using an electron spin resonance spectroscopic technique. Kanner and Fennema (1987) also reported that riboflavin-sensitized photooxidation of tryptophan was retarded by sodium azide, and that it seemed likely that the reaction proceeded to some extent, but not entirely, by a type II ($^1O_2$) mechanism. Our results are further experimental evidence indicating that riboflavin produces singlet oxygen in aqueous system under light.

Bimolecular rate constants for reaction of ascorbic acid with singlet oxygen at various pHs

Since riboflavin generated singlet oxygen in the presence of light, the rate constants for reaction of ascorbic acid with singlet oxygen at various pHs were determined.

The schematic diagram for the formation of oxidized ascorbic acid (AO_2) via singlet oxygen oxidation is as follows (Foote, 1979).



When a sensitizer (Sen) absorbs light energy it becomes an exited singlet sensitizer (^{1}Sen*) and then becomes an excited triplet sensitizer (^{3}Sen*) by an intersystem crossing (ISC) mechanism. The energy of ^{3}Sen* is transferred to ordinary triplet state oxygen ($^3O_2$) to produce singlet oxygen ($^1O_2$) by triplet–triplet annihilation. Singlet oxygen reacts with ascorbic acid (A) to produce oxidized ascorbic acid (AO_2). Using the schematic diagram, following steady-state kinetic equation is established (Foote, 1979):

$$\{d[AO_2]/dt\}^{-1} = K^{-1}\{1 + (k_q[Q] + k_{ox\,Q}[Q] + k_d)/k_r[A]\}$$

where K denotes the rate of singlet oxygen formation; AO_2, oxidized ascorbic acid; k_r, bimolecular rate constant for reaction of ascorbic acid with singlet oxygen; k_d, rate of decay of singlet oxygen; A, ascorbic acid; Q, quencher; $k_{ox\,Q}$, rate constant for singlet oxygen chemical quenching by quencher; k_q, rate constant for singlet oxygen physical quenching. In our experimental design, quencher was not added to the system. Thus the equation can be simplified as follows:

$$\{d[AO_2]/dt\}^{-1} = K^{-1}\{k_d/k_r[A]\}$$

If we plot $[AO_2]^{-1}$ vs $[A]^{-1}$, the ratio of slope to intercept of the plot of $[AO_2]^{-1}$ vs $[A]^{-1}$ is k_d/k_r. Thus, the ratio of slope to intercept of the plot of $[AO_2]^{-1}$ vs $[A]^{-1}$ allows the determination of k_d/k_r. If the decay rate of singlet oxygen (k_d) is known, the bimolecular rate constant for reaction of ascorbic acid with singlet oxygen (k_r) can be determined (Foote, 1979).

The effects of different pHs (7·5, 6·0 and 4·5) on singlet oxygen oxidation of 0, $0{\cdot}40 \times 10^{-4}$, $0{\cdot}50 \times 10^{-4}$, $0{\cdot}60 \times 10^{-4}$, $0{\cdot}80 \times 10^{-4}$ and $1{\cdot}00 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer containing 6 ppm methylene blue and 0·1 mM EDTA during 3 min light storage at 20°C is shown in Fig. 4. The linear regression equations of the lines for pH 7·0, 6·0 and 4·5 in Fig. 4 were $Y = 2{\cdot}78X + 7{\cdot}2 \times 10^3$, $Y = 7{\cdot}31X + 1{\cdot}6 \times 10^4$, $Y = 16{\cdot}40X + 3{\cdot}4 \times 10^4$, respectively. The slope/intercept of the regression lines for the ascorbic acid oxidation at the pH 7·5, 6·0 and 4·5 are $3{\cdot}86 \times 10^{-4}$, $4{\cdot}44 \times 10^{-4}$ and $4{\cdot}86 \times 10^{-4}$, respectively. The slope/intercept of the regression lines for the ascorbic acid equals β values (β value = k_d/k_r, where the k_d is the rate of decay of singlet oxygen and k_r is the bimolecular rate constant for reaction of ascorbic acid with singlet oxygen). We calculated the relative reactivity of ascorbic acid to singlet oxygen at different pH using the β values. The calculated relative reactivity of ascorbic acid to singlet oxygen at pH 7·0, 6·0 and, 4·5 were 1:0·86:0·78. Since the k_d for the water is $2{\cdot}56 \times 10^5$ s^{-1} (Rodgers & Snowden, 1982), the bimolecular rate constant (k_r) for reaction of ascorbic acid with singlet oxygen at the pH 7·5, 6·0 and 4·5 equals $2{\cdot}56 \times 10^5/3{\cdot}86 \times 10^{-4} = 6{\cdot}63 \times 10^8$, $2{\cdot}56 \times 10^5/4{\cdot}44 \times 10^{-4} = 5{\cdot}77 \times 10^8$ and $2{\cdot}56 \times 10^5/4{\cdot}86 \times 10^{-4} = 5{\cdot}27 \times 10^8$ M^{-1} s^{-1}, respectively. The rate constant for ascorbic acid with singlet oxygen was very high. That is, ascorbic acid is easily oxidized in the presence of singlet oxygen and ascorbic acid could be also used for the effective singlet oxygen quencher in the singlet oxygen oxidation of other compounds in the aqueous system. The values for ascorbic acid were higher than α-tocopherol (Jung *et al.*, 1991) but lower than the value for β-carotene (Jung & Min, 1991). The present values for ascorbic acid (k_r) are in good agreement with the estimated value of Bodannes and Chan's (1979). Bodannes and Chan (1979) reported that the estimated reactivity of ascorbate toward singlet oxygen (k_r) was approximately of the same order of magnitude as azide which is $2{\cdot}2 \times 10^8$ M^{-1} s^{-1} in D_2O at pH 7·5. All the present values of $6{\cdot}63 \times 10^8$ (pH 7·5) $5{\cdot}77 \times 10^8$ (pH 6·0) and $5{\cdot}27 \times 10^8$ M^{-1} s^{-1} (pH 4·5) were within an order of magnitude of the estimated value of $2{\cdot}28 \times 10^8$ M^{-1} s^{-1}. This result clearly showed that the bimolecular rate constant for reaction of ascorbic acid with singlet oxygen was dependent on the pH of the system. As the pH decreased from 7·5 to 4·5, the reaction rate constants of singlet oxygen oxidation of ascorbic acid decreased. That is, the singlet oxygen oxidation of ascorbic acid occurred more slowly at lower pH.

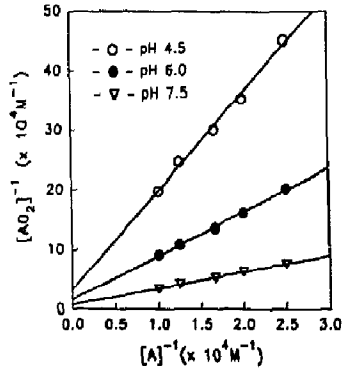


Fig. 4. The effects of different pHs (7·5, 6·0 and 4·5) on singlet oxygen oxidation of 0, $0{\cdot}40 \times 10^{-4}$, $0{\cdot}50 \times 10^{-4}$, $0{\cdot}60 \times 10^{-4}$, $0{\cdot}80 \times 10^{-4}$ and $1{\cdot}00 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer containing 6 ppm methyiene blue and 0·1 mM EDTA during 3 min light storage at 20°C.

Effects of amino acids on the photooxidation of ascorbic acid

The effects of 0·1% amino acids on the photooxidation of $1{\cdot}2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium

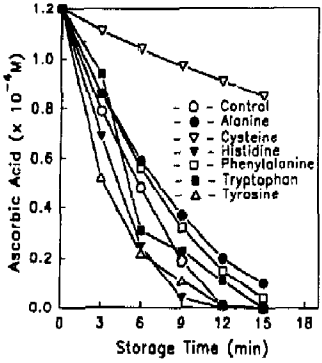


Fig. 5. The effects of 0·1% amino acids on the photooxidation of $1{\cdot}2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 2·4 ppm riboflavin and 0·1 mM EDTA during light storage.

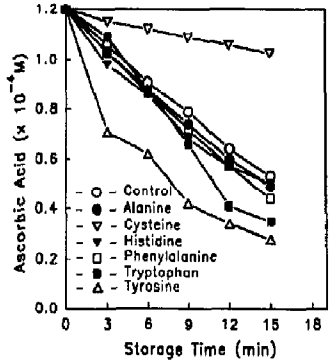


Fig. 6. The effects of 0·1% amino acids on the photooxidation of $1{\cdot}2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 2·4 ppm methylene blue and 0·1 mM EDTA during light storage.

phosphate buffer (pH 7·5) containing 2·4 ppm riboflavin during light storage are shown in Fig. 5. Cysteine showed strong antioxidant effects on the photooxidation of ascorbic acid, and alanine and phenylalanine also showed a protective action in riboflavin sensitized oxidation of ascorbic acid. The ascorbic acid content in the systems treated with alanine, cysteine or phenylalanine was significantly higher than that in control during 12 min storage at $P < 0{\cdot}05$. Cysteine has been known as an effective singlet oxygen quencher. The inhibitory effect of alanine and phenylalanine on the oxidation of ascorbic acid by riboflavin was somewhat unexpected because alanine and phenylalanine have not been known as effective singlet oxygen quenchers. The ascorbic acid contents in the systems treated with histidine and tyrosine were significantly lower than that in control during 9 min storage ($P < 0{\cdot}05$). Histidine and tyrosine greatly accelerated the riboflavin sensitized photooxidation of ascorbic acid. The acceleration of ascorbic acid photooxidation by histidine and tyrosine was also unexpected because both histidine and tyrosine were effective singlet oxygen quenchers (Matheson & Lee, 1979). The only possible explanation that can be offered is that histidine and tyrosine were easily oxidized by reacting with singlet oxygen and produced intermediates which could accelerate the ascorbic acid oxidation. The formation of endoperoxides has been reported for the reaction of singlet oxygen with tyrosine (Seely & Hart, 1976) and with histidine (Tomita *et al.*, 1969) via 1,4-cycloaddition of singlet oxygen. The unstable endoperoxides could produce radical intermediates which are strong reactants. Tryptophan decreased or increased the riboflavin sensitized photooxidation depending on the storage time as shown in Fig. 5. Tryptophan decreased considerably the riboflavin-sensitized photooxidation of ascorbic acid during the initial 3 min but greatly increased the oxidation at 6 min storage. It has been reported that photosensitized oxidation of tryptophan produced *N*-formylkynurenine which can act as a photosensitizer (Walrant *et al.*, 1976; Kanner & Fennema, 1987). Ascorbic acid contents in samples containing cysteine, alanine, phenylalanine, tryptophan, histidine, tyrosine and no amino acid after 9 min illumination were $0{\cdot}97 \times 10^{-4}$, $0{\cdot}37 \times 10^{-4}$, $0{\cdot}32 \times 10^{-4}$, $0{\cdot}23 \times 10^{-4}$, $0{\cdot}04 \times 10^{-4}$, $0{\cdot}11 \times 10^{-4}$ and $0{\cdot}19 \times 10^{-4}$ M, respectively.

To study the possible reasons for the effects of amino acids on the riboflavin-sensitized photooxidation of ascorbic acid, the effects of amino acids on the methylene blue-sensitized photooxidation of ascorbic acid were studied. Figure 6 shows the effects of 0·1% amino acids on the photooxidation of $1{\cdot}2 \times 10^{-4}$ M ascorbic acid in 0·01 M potassium phosphate buffer (pH 7·5) containing 2·4 ppm methylene blue. In the methylene blue-sensitized photooxidation of ascorbic acid, all the amino acids except cysteine increased the oxidation of ascorbic acid. The combined results indicated that the inhibitory effects of alanine and phenylalanine on the riboflavin sensitized photooxidation of ascorbic acid might be due to quenching the excited triplet state riboflavin. The inhibitory effect of cysteine seems to be mainly due to singlet oxygen quenching.

Since cysteine had the most strong antioxidant effects on either riboflavin-sensitized or methylene blue-sensitized photooxidation of ascorbic acid, we studied the quantitative effects of cysteine on the riboflavin-sensitized and methylene blue-sensitized photooxidation of ascorbic acid (data not shown). As the concentration of cysteine increased from 0 to 0·10%, it's protective effect on the photooxidation of ascorbic acid increased significantly ($P < 0{\cdot}05$). Ascorbic acid contents in samples containing 0, 0·010, 0·025, 0·050 and 0·100% cysteine and 2·4 ppm riboflavin after 15 min light storage decreased from $1{\cdot}2 \times 10^{-4}$ M to 0, $0{\cdot}19 \times 10^{-4}$, $0{\cdot}40 \times 10^{-4}$, $0{\cdot}61 \times 10^{-4}$ and $0{\cdot}85 \times 10^{-4}$, respectively (data not shown). Ascorbic acid contents in samples containing 0, 0·010, 0·025, 0·050 and 0·100% cysteine and 2·4 ppm methylene blue after 15 min light storage

decreased from $1{\cdot}2 \times 10^{-4}$ M to $0{\cdot}53 \times 10^{-4}$, $0{\cdot}72 \times 10^{-4}$, $0{\cdot}89 \times 10^{-4}$, $0{\cdot}98 \times 10^{-4}$ and $1{\cdot}03 \times 10^{-4}$, respectively (data not shown).

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