An electron spin resonance and time-resolved CIDEP study of the oxidation of ascorbic acid by pyruvic acid, duroquinone, and vitamin K_1

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The photooxidation of vitamin C by pyruvic acid and its derivatives, duroquinone, and vitamin K_1 is systematically studied by the combined esr and time-resolved CIDEP technique. Because of the unique property of the triplet pyruvic acid which is different from that of the duroquinone triplet, the choice of these model systems allowed the CIDEP observations of the initial polarization of the ascorbate radical anion and its counter primary radical in either the enhanced absorptive or the emissive mode. The results demonstrate the efficient photooxidation of vitamin C by the triplet vitamin K_1 and that the latter triplet possesses similar properties as the triplet quinones. In the photooxidation system involving vitamin C and pyruvic acid, the adjustment of the pH of the aqueous solution afforded an opportunity to observe the photochemical reaction between two anions, the pyruvate anion and the ascorbate anion. Arguments were made in favor of an electron transfer mechanism for the oxidation of vitamin C by triplet vitamin K_1 but against the electron transfer mechanism for the oxidation of vitamin C by triplet pyruvate anion. T_1 values of the primary radicals estimated from their CIDEP transient responses at low microwave power are reported and it was suggested that the hydrogen-bonding capacity of the ascorbate radical anion in water does not contribute significantly to the relative difference of T_1's between itself and the counter pyruvic ketyl radical.

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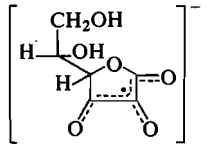
En faisant appel à la rpe combinée à la technique de PEDIC résolue en fonction du temps, on étudie systématiquement la photooxydation de la vitamine C par l'acide pyruvique et ses dérivés, par la duroquinone et par la vitamine K_1. Compte tenu de la propriété unique du triplet de l'acide pyruvique qui est différente de celle du triplet de la duroquinone, le choix de ces modèles permet des observations de PEDIC de la polarisation initiale de l'anion radical ascorbate et de son radical primaire opposé lors de l'absorption ou de l'émission. Les résultats démontrent l'efficacité de la photooxydation de la vitamine C par le triplet de la vitamine K_1 et que le dernier triplet possède des propriétés identiques à celles du triplet des quinones. Dans la photooxydation qui implique la vitamine C et l'acide pyruvique, l'ajustement du pH de la solution aqueuse fournit l'opportunité d'observer la réaction photochimique entre deux anions, l'anion pyruvate et l'anion ascrobate. On suggère un mécanisme par transfert d'électrons pour l'oxydation de la vitamine C par le triplet de la vitamine K_1, mais on n'admet pas ce mécanisme dans le cas de l'oxydation de la vitamine C par le triplet de l'anion pyruvate. On rapporte les valeurs de T_1 des radicaux primaires évalués à partir de leurs réponses intermédiaires en PEDIC en microondes de faible puissance, et on suggère que la capacité en liaison hydrogènes de l'anion radical ascorbate dans l'eau ne contribue pas de façon significative à la différence relative des T_1 entre cet anion et le radical cétylpyruvique opposé.

[Traduit par le journal]

Introduction

Interest in the biological significance of vitamin C and vitamin E continues to be stimulated by reports of their involvement in many biochemical processes including cancer (1) and iron nutrition (2). It has been established that L-ascorbic acid (vitamin C) is readily oxidized in aqueous solution to a relatively stable radical whose controversial structure has been determined by esr spectroscopy (3). The ascorbic acid radicals generated in non-aqueous and aprotic solvents have also been reported (4). A recent theoretical calculation (5) has confirmed the esr assignments of the ascorbate anion free radical (**1**) in aqueous solution with the following structure



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In the non-enzymatic systems, formation of the ascorbate free radical may involve either an electron-transfer process and/or a molecular elimination process. For example, a time-resolved esr study of the oxidation of ascorbic acid by hydroxyl radical produced in pulse radiolysis of water (6) has suggested that addition of ȮH at C-2 followed by rapid elimination of a water molecule resulted in the formation of the ascorbate radical anion. However, a direct electron-transfer process between the ascorbate and the ȮH radical

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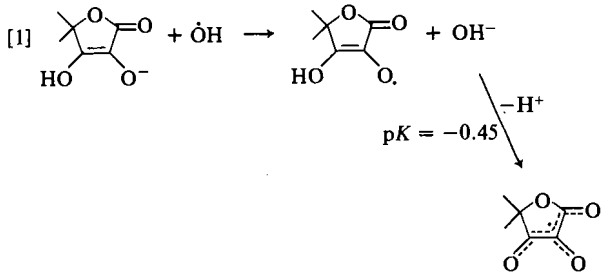


was considered to be also possible.

In the past five years we have extensively used the photoreduction of quinone by phenol as model systems for chemically induced magnetic polarization studies (7). In addition to providing some insights into the mechanistic aspects of free radical reactions, CIDEP could be used to probe the dynamic/structural properties of the transient radicals such as their spin–lattice relaxation times in solution. The interesting ascorbate free radical has a semiquinone-like structure and various probable sites for hydrogen-bonding interaction with the water solvent. It is our hope that a systematic CIDEP study of the photooxidations of vitamin C and vitamin E may yield further valuable information on the mechanism and the dynamic properties of the transient free radicals involved in these processes. In this report we have carefully chosen three model systems: the oxidation of vitamin C by excited pyruvic acid, the oxidation of vitamin C by excited duroquinone, and the oxidation of vitamin C by excited vitamin K_1. The choice of the first two systems is most significant in that the excited triplet states of pyruvic acid and of duroquinone have distinctly different spin dynamic properties (8) which lead to an initial polarization of the ascorbate radical anion being totally enhanced absorptive in the first case and totally emissive in the latter reaction. Spin–lattice relaxation times estimated from both the emissive and the absorptive ascorbate radical anions should be the same and this provides a critical test for the photo-triplet mechanism as well as the reliability of the CIDEP method in estimating T_1 of transient free radicals. Furthermore, by using sodium pyruvate as a control the oxidation of vitamin C by pyruvate affords an interesting opportunity to study a primary photochemical reaction between *two anions*.

Experimental

Pyruvic acid, ethylpyruvate, D-arabascorbic acid, and duroquinone were supplied by Aldrich. L-Ascorbic acid (vitamin C) was purchased from Anachemia and vitamin K_1 was obtained from Sigma. 2,3-Dimethylascorbic acid was custom synthesized and was a gift from Professor W. A. Szarek and Dr. K. S. Kim. The quinones were sublimed before use and laboratory grade benzene and toluene were distilled and dried over $MgSO_4$. A typical organic solution sample was prepared by placing a 0.1 *M* solution of the respective reagents in a Pyrex tubing of 2 mm i.d. and the solution was degassed under high vacuum before being sealed off. For aqueous solution samples, the solution was placed in a quartz flat cell of 0.1 mm thickness and deoxygenated by purging with gaseous nitrogen.

The esr spectra were recorded on a Varian E3 spectrometer equipped with a variable temperature control accessory. The samples were irradiated within the cavity by using a 200 W super pressure mercury lamp. Time-resolved CIDEP experiments were performed on the Varian E3 spectrometer using dc detection. The amplified dc signal was digitized by a Nicolet Explorer III digital scope at 50 ns per point and the digitized data were collected and processed on-line by a Nicolet 1180 computer in a synchronous mode. The design of the dc detection system is similar to that reported by Kim and Weissman (9). The light source used is a Molectron UV24 1-MW nitrogen pulsed laser. A detailed description and evaluation of the present system and the analysis of the data to obtain the spin–lattice relaxation times have been given elsewhere (10).

Results and Discussion

Photooxidation of Vitamin C by Pyruvic Acid in Aqueous Solutions

Photolysis of a saturated aqueous solution of ascorbic acid containing 10% v/v of pyruvic acid at room temperature led to the observation of an esr spectrum which is the superposition of the two primary radicals, the ascorbate radical anion **1** and the pyruvic ketyl radical $CH_3\dot{C}(OH)COOH$. Radical **1** exhibits a hyperfine structure of a doublet (A_H = 1.85 G) which is further split into a triplet (A_{CH_2} = 0.18 G). The pyruvic ketyl radical shows a quartet (A_{CH_3} = 16.9 G) of doublets (A_{OH} = 1.8 G). These assignments are consistent with those reported in the literature (3, 8). Direct inspection of the cw esr spectrum revealed that the low field hyperfine lines of the pyruvic ketyl radical were less intense than the corresponding high field lines. This is typically due to the E/A polarization originating from the radical-pair mechanism of the CIDEP effect (7, 8). Time-resolved CIDEP experiments, however, showed that both primary radicals, **1** and $CH_3\dot{C}(OH)COOH$, exhibit strong *initial* polarization with all hyperfine lines in the enhanced absorption mode. In the time-resolved experiments the effective radical concentrations generated by the 5 ns nitrogen laser pulse were much lower than those achieved by continuous irradiation and thus leading to a predominant *initial* polarization (originated from the photo-triplet mechanism) as observed. The results can be accounted for by the following reactions:

[2] $CH_3COCOOH + h\nu \xrightarrow{ISC} {}^3(CH_3COCOOH)^*$

[3] $^3(CH_3COCOOH)^*$ + Ascorbic acid → $CH_3\dot{C}(OH)COOH^*$ + 1*

The * denotes initial electron polarization.

In a neutral solution ascorbic acid exists mainly in the form of an ascorbate anion and its presence suppresses the ionization of pyruvic acid, as confirmed by nmr (the chemical shifts for the methyl protons are different for pyruvic acid and for pyruvic anion). In the pyruvic/ascorbic acid system most of the excitation light at the laser wavelength of 337 nm would be absorbed by the pyruvic acid only. In a previous study of the photochemical reactions between triplet pyruvic acid and lactic acid, the initial polarization originating from the photo-triplet mechanism led to the polarization of the primary radicals in the enhanced absorption mode (8). To the best of our knowledge, this is the only confirmed photochemical system giving a totally absorptive initial polarization for the primary radical pair. Since the mode of the primary radical initial polarization depends upon the triplet property alone (11), it is expected that in the present pyruvic/ascorbic acid system the reaction of the polarized pyruvic acid triplet should lead to an initial polarization in the totally absorptive mode for both the ascorbate radical anion and the pyruvic ketyl radical. According to the photoexcited triplet model (11) in which the population of each of the spin sublevels can be approximated by

$$P_{\pm 1} \propto (P_x + P_y - 2P_z)(D/B_0)$$

the excited triplet pyruvic acid must either have $D > 0$ and $P_z > P_x, P_y$, or $D < 0$ and $P_z < P_x, P_y$, where D is electron dipolar parameter related to the zero-field splittings of the sublevels. Unfortunately, CIDEP studies in liquid solutions alone can not yield the information of the sign of D. But the observed enhanced absorption for both pyruvic ketyl radical and ascorbate radical anion establishes the oxidation of vitamin C by pyruvic acid via an excited triplet mechanism.

The detailed mechanism of the reaction [3] is of great significance. Although triplet pyruvic acid has been found to abstract hydrogen with a rate similar to benzophenone triplet in various alcohols (12) and thus hydrogen abstraction from ascorbate anion could account for the reaction leading to CIDEP of the primary radicals, it must be recognized that the reaction could also proceed rapidly via an electron-transfer mechanism:

[4] $^3(CH_3COCOOH)^*$ + $ascorbate^-$ → $(CH_3COCOOH)^{\dot{-}*}$ + ascorbic acid radical*

[5] Ascorbic acid radical* → Ascorbate radical anion 1* + H^+

[6] $H^+ + (CH_3COCOOH)^{\dot{-}*} \rightarrow (CH_3\dot{C}(OH)COOH)^*$

In the initial series of experiments the pH of the solutions was at 3.5, so that appreciable concentrations of both the unionized pyruvic acid and the pyruvate anion could be involved in the photooxidation of vitamin C. The triplet property of the unionized pyruvic acid is not expected to be different from that of the triplet pyruvate anion. By adjusting the pH of the solution to 10.2 using KOH, the concentrations of the pyruvate anion and the ascorbate anion would be greatly enhanced so that the actual photooxidation is now represented by the reaction

[7] $^3(CH_3COCOO)^{-*}$ + $ascorbate^-$ → $(CH_3\dot{C}(OH)COO)^{-*}$ + 1*

This presents an interesting opportunity to study the photochemical reaction between two anions and at this time there is no compelling argument that reaction [7] should proceed via an electron-transfer from the ascorbate anion to the excited pyruvate anion as an efficient means to preserve the initial polarization later developed in the observed radicals. The experimental results clearly show that in the basic solution, the CIDEP intensity as well as the mode of polarization were unchanged from those observed in acidic solutions. Similar CIDEP results were obtained in the sodium pyruvate/ascorbic acid system. We thus suggest that the photoredox reaction between triplet pyruvic acid/pyruvate and ascorbate involves mainly a hydrogen abstraction mechanism. The approach of the triplet anion towards another anionic reactant apparently does not slow down the reaction as the triplet anion possesses sufficient excitation energy to overcome any small energy barrier for two approaching anions. We must reiterate that the present results are insufficient to establish the detailed mechanism of the primary process, i.e. whether it involves electron-transfer to be followed by a proton transfer, or it is a direct H atom abstraction. Experimental differentiation of these two mechanisms is extremely difficult at the present time and it is hoped that further technical improvements of the time-resolution to the 10^{-12} s range may help solve the problem.

From the CIDEP observations the T_1's of both the primary radicals/radical ions were estimated and these will be reported and discussed in another section below.

Photooxidation of Ascorbic Acid by Ethyl Pyruvate, 2-Ketobutyric Acid, and Ethylacetoacetate

To further probe the "unique" triplet property of pyruvic acid which leads to enhanced absorptive initial polarization, a systematic study using various available pyruvic derivatives was carried out. In the cw photolysis of ethyl pyruvate and vitamin C in a mixed ethanol/water solvent between 0 and 10°C, both the ascorbate radical anion **1** and the $CH_3\dot{C}(OH)COOC_2H_5$ radical were observed. The latter was identified by its hyperfine pattern with A_{CH_3} = 16.8G and A_{OH} = 1.9G as reported in literature (13). Time-resolved CIDEP experiments again showed that for both primary radicals all their hyperfine lines were in totally enhanced absorption, as expected from the phototriplet mechanism having the ethyl pyruvate triplet similar to the triplet pyruvic acid in the spin sublevel populations. In the cw photolysis of 2-ketobutyric acid and vitamin C in ethanol/water solvent, again both the radical anion **1** and the $CH_3CH_2\dot{C}(OH)COOH$ radical were observed. The latter radical has hyperfine coupling constants A_{CH_2} = 11.6G, A_{OH} = 2.0G, and A_{CH_3} = 0.8G. In the cw spectrum the 2-ketobutyric ketyl radical also exhibits the E/A type polarization originated from the radical-pair mechanism. Time-resolved CIDEP observations in this system also showed a predominant initial polarization with both primary radicals clearly in the enhanced absorptive mode for all their hyperfine components. However, a similar experiment using ethyl acetoacetate as the triplet precursor failed to yield any CIDEP effects. Thus, we are inclined to believe that the similar and "unique" triplet property of pyruvic acid and its derivatives is characteristic of alkyl carbonyl compounds having a functional group —CO—COO—. It is interesting to speculate here that a substitution of the alkyl group next to the carbonyl by an aromatic group might alter the triplet property and thus leading to a different CIDEP pattern.

The photoredox systems were repeated using D-arabascorbic acid instead of vitamin C. In all cases, no significant differences in CIDEP and the T_1's of the ascorbate radical anions were observed.

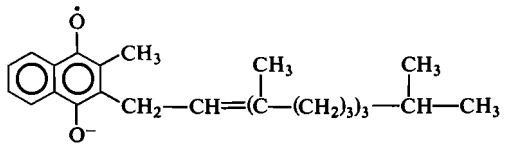
Photooxidation of Vitamin C by Duroquinone

The photoredox reactions between quinones and phenols have been extensively used in this laboratory as model systems for CIDEP study. It is generally recognized that photoexcited triplet duroquinone is spin polarized which in subsequent reactions transfers the initial polarization to the primary radicals in a totally emissive mode. Thus, the triplet property of the duroquinone with respect to spin population of the sublevels is directly opposite to that of pyruvic acid. As a comparison the photooxidation of vitamin C by duroquinone was studied in ethanol solution. Continuous photolysis led to the observation of an esr spectrum which is the superposition of the ascorbate radical anion **1** and the durosemiquinone radical. Time-resolved CIDEP experiments showed again the strong initial polarization of both primary radicals. Because of the triplet duroquinone property, the initial polarizations of both radicals were in the totally emissive mode, as expected by the phototriplet mechanism.

The detailed mechanism of the reaction of duroquinone triplet with the ascorbate anion again deserves some attention. It remains probable that the reaction involves an electron transfer from the ascorbate anion to the triplet duroquinone which is a good electron acceptor. In this regard, we have studied the photooxidation of 2,3-dimethyl ascorbate by duroquinone. Here, no radicals nor CIDEP were observed. Although esr negative results can not be used to exclude the possibility of chemical reactions, it is fairly safe to say that under identical experimental conditions the free radical processes involved in the photooxidation of 2,3-dimethyl ascorbate by quinone are insignificant. This can be attributed to the fact that the "efficient" hydrogen abstraction reaction by the triplet quinone from the —OH group on the vitamin C is no longer operative in the case of the 2,3-dimethyl ascorbate. On the other hand, the 2,3-dimethyl ascorbate is probably unionized in solution but an electron transfer mechanism from the substituted ascorbate to the triplet quinone is still possible. A definitive conclusion of the detailed mechanism of the photooxidation of vitamin C by quinone, however, must await further elucidation.

Photooxidation of Vitamin C by Vitamin K_1

Vitamin K_1 is known to form semiquinone radical with the following structure (14):



The biochemical importance of vitamin K_1 semiquinone suggested that the possible photooxidation of vitamin C by vitamin K_1 would be of some interest.

When vitamin K_1 was photolyzed alone in ethanol, an esr spectrum of the vitamin K_1 semiquinone radical having the same spectral parameters as reported in the literature (14) was observed. Time-

resolved CIDEP study of this system revealed no polarization. However, addition of vitamin C to the solution showed the formation of both the vitamin K_1 semiquinone radical and the ascorbate radical anion **1**, and the CIDEP experiments produced totally emissive polarization for both primary radicals. On the whole, these experiments suggest that the photooxidation of vitamin C involves the spin-polarized vitamin K_1 triplet molecule which possesses property similar to that of other 1,4-quinone triplets. The more salient conclusion to be drawn is the experimental fact that the vitamin K_1 triplet is photoreduced much more efficiently by vitamin C than by ethanol, a suggested by the CIDEP triplet mechanism argument (7). Here the electron transfer mechanism from vitamin C to the triplet vitamin K_1 is particularly attractive as it can account for both the direct formation of the semiquinone radical anion and the efficient reaction rate required by the phototriplet mechanism.

The spin–lattice relaxation times of the vitamin K_1 semiquinone radical and the ascorbate radical anion were measured by CIDEP and reported in the next section.

Spin–Lattice Relaxation Times of Ascorbate Radical Anion, Pyruvic Ketyl Radical, Durosemiquinone Radical, and Vitamin K_1 semiquinone Radical Anion

The choice of the two different triplet precursors, the duroquinone and the pyruvic acid, in the photooxidation of vitamin C affords a unique opportunity to produce the *same* ascorbate radical anion with initial polarization in the emissive mode in the case of pyruvic acid. Figure 1 shows the typical transient enhanced absorptive response from the ascorbate radical anion in the photooxidation by pyruvic acid and Fig. 2 gives the typical transient emissive response from the same radical anion in the photooxidation by duroquinone. In all

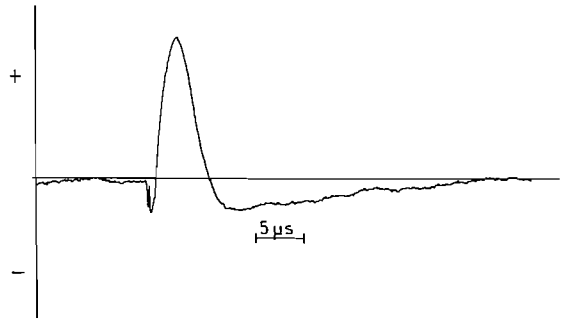


FIG. 1. Transient enhanced absorptive response from the ascorbate radical anion generated in the photooxidation by triplet pyruvic acid in an aqueous solution at 6°C. B_1 is about 40 mG. The curve represents the *difference* between the on-resonance and off-resonance responses.

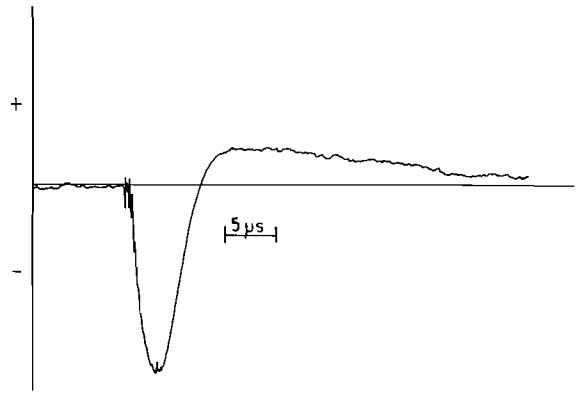


FIG. 2. Transient emissive response from the ascorbate radical anion generated in the photooxidation by triplet duroquinone in an ethanol solvent at −20°C. B_1 is about 40 mG. The curve represents the *difference* between the on-resonance and off-resonance responses.

the CIDEP observations on all systems, the presence of both primary radicals allowed the simultaneous estimates of the T_1's of both radicals under the identical experimental conditions. In all cases, the T_1 measurements used only data obtained from experiments at low microwave power (at or below 1 mW which is equal to B_1 less than 40 mG (10)). Since the Varian E3 is not equipped with a low-power operation configuration, the lowest B_1 usuable is about 15 mG and the extrapolation to $B_1 = 0$ will only provide an estimate of T_1. The estimated values of T_1's for various primary radicals observed in the photooxidation of vitamin C are given in Table 1.

The data show that in general the T_1 of the L-ascorbate radical anion is the same as that of D-arabascorbate radical anion. The apparently shorter spin–lattice relaxation time of the ascorbate radical anion as compared to its counter radicals such as pyruvic ketyl radical under the same experimental conditions is rather surprising. It was expected that in aqueous solutions the ascorbate radical anion may interact with water via substantial hydrogen-bonding and this effect would have increased the value of T_1. In a recent theoretical consideration of T_1's for semiquinone radical in solution (15) it was shown that the T_1 depends upon three major factors which are the orientational correlation time τ_2 for a second rank tensor, the average excitation energy $\Delta\varepsilon$ and some quantities related to **G** tensor deviation. The hydrogen-bonding interaction could conceivably affect the value of τ_2 but Rengan *et al.* (16) have investigated the hydrogen-bonding interaction among semiquinone radicals and obtained a low activation energy, about 4 kJ mol^{-1}. Taking into account that similar hydrogen-bonding interaction is also probable for the counter pyruvic ketyl radical, it is

TABLE 1. Estimated T_1 values from CIDEP measurements of transient radicals in solutions

Radicals in the primary pair	Mode of initial polarization	Temperature (°C)	Solvent	T_1 (μs)
L-Ascorbate radical anion	Absorptive	6	Water	2.1±0.4
Pyruvic Ketyl radical	Absorptive	6	Water	3.4
L-Ascorbate radical anion	Absorptive	29	Water	1.0
Pyruvic Ketyl radical	Absorptive	29	Water	2.3
L-Ascorbic radical anion	Absorptive	27	Water	1.4
Pyruvic Ketyl radical anion	Absorptive	27	(pH = 10.2)	2.5
L-Ascorbate radical anion	Emissive	19	Ethanol	2.0
Vitamin K_1 semiquinone	Emissive	19	Ethanol	4.0
L-Ascorbate radical anion	Emissive	−20	Ethanol	2.4
Durosemiquinone	Emissive	−20	Ethanol	3.0
D-Arabascorbate radical anion	Absorptive	11	Water	2.0
$CH_3CH_2\dot{C}(OH)COO^-$ [a]	Absorptive	11	Water	3.6

[a] T_1 in $CH_3CH_2\dot{C}(OH)COO^-$ radical is slightly dependent on the hyperfine components with T_1 for the center component (0) slightly larger than those associated with the (+1) and (−1) components.

reasonable to argue that the difference in T_1's between the ascorbate radical anion and the pyruvic ketyl radical is not mainly due to hydrogen-bonding. Another factor which comes into the comparison here is the contribution of the hyperfine interaction to the relaxation of these two radicals. Usually the anisotropic contribution is more important because this interaction is readily modulated by the rotational tumbling of the molecule. In the case of the pyruvic ketyl radical with a rotating methyl group, the anisotropic splitting is small and the only contribution to the relaxation will come from the modulation of the isotropic splitting, which is not expected to affect the T_1 significantly. On the other hand, the isotropic hyperfine splitting of the proton at the C-5 position in the ascorbate radical anion may be small but the angular dependence of the anisotropic hyperfine interaction and thus its modulation by the tumbling molecule may contribute to the relaxation in a manner as to reduce the observed T_1. Experimental evidence of such an anisotropic hyperfine interaction contribution to relaxation for the $CH_3CH_2\dot{C}(OH)COO^-$ radical is found in the observed small dependence of T_1's on the hyperfine components with T_1 slightly larger for the central component ($m_I = 0$) than for the outer components having $m_I = \pm 1$. This observation is consistent with those reported for the nitroxide radicals (17). It is hoped that further T_1 studies of vitamin C radical and its derivatives will provide some insight into the dynamics of this class of important radicals.

Another interesting observation is that T_1 of the large vitamin K_1 semiquinone radical appears to be in the same magnitude as the corresponding unsubstituted naphthosemiquinone radical in solution (15). This, however, is not too surprising as the recent theoretical treatments (15) on T_1's of semiquinone radicals in solution showed that a combined argument based upon a modest size effect and upon the extent of the π system provides a better understanding of the T_1 observations than either consideration by itself.

Acknowledgement

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