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KINETIC ESR STUDY ON THE REACTION OF VITAMIN E RADICAL WITH VITAMIN C AND ITS LIPOPHILIC DERIVATIVES IN CETYLTRIMETHYLAMMONIUM BROMIDE MICELLES

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The reaction between α-tocopheroxyl radical (VE˙) and ascorbic acid (VC) and its lipophilic derivatives ascorbyl-6-caprylate (VC-8), 6-laurate (VC-12) and 6-palmitate (VC-16) was studied by stopped-flow ESR spectroscopy in cetyltrimethylammonium bromide (CTAB) micelles. The second-order rate constants for the reaction were found to be 9·0, 3·0, 0·7 and $0{\cdot}03 \times 10^5$ l mol^{-1} s^{-1} for VC, VC-8, VC-12 and VC-16, respectively, indicating a remarkable influence of the aliphatic side-chain on the reactivity. The lifetime of the reaction intermediate, ascorbate radical anion, was greatly enhanced by the lipophilic side-chain, being 0·4, 5 and 110 s for VC-8$^{-\cdot}$, VC-12$^{-\cdot}$ and VC-16$^{-\cdot}$ respectively. Kinetic analysis shows that the inter- and intramicellar diffusion may be the rate-limiting steps for the reaction carried out in micelles.

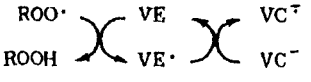
INTRODUCTION

α-Tocopherol (the most active and major component of vitamin E, VE; **1**) is believed to be the principal lipid-soluble chain-breaking antioxidant existing in plasma and erythrocyte membranes against lipid peroxidation,[1] a process implicated in ageing, cancer and a variety of degenerative diseases.[2] The antioxidant efficiency of vitamin E may be greatly enhanced through a synergistic effect of L-ascorbic acid (vitamin C, VC; **2**) because vitamin C can reduce vitamin E radical (VE˙, **1**˙) to regenerate vitamin E, thereby maintaining the vitamin E level in the system (Scheme 1).[3]

This phenomenon is of significance with regard to membrane biochemistry and has attracted much interest in recent years.[3–14] The key step of the antioxidant synergism of the two vitamins is the reaction between vitamin E radical and vitamin C:

$$VE^{\cdot} + VC^{-} \rightarrow VE + VC^{-\cdot} \qquad (1)$$

Packer *et al.*[3] generated vitamin E radical by pulse radiolysis and measured the rate constant for this reaction as $1{\cdot}55 \times 10^6$ l mol^{-1} s^{-1} in homogeneous solutions. Recently, Mukai and co-workers[9–10] reported that introducing a long hydrocarbon chain at the 6- or 5-position in vitamin C did not significantly change its reactivity towards vitamin E radical in homogeneous solutions. On the other hand, Scarpa *et al.*[11] reported that the rate constant for the reaction [equation (1)] decreased to 2×10^5 l mol^{-1} s^{-1} in soybean phosphatidylcholine liposomes, which is about an order of magnitude lower than that in homogeneous solutions. We have found recently[15,16] that the antioxidant activity of vitamin C and its lipophilic derivatives in micelles is remarkably dependent on the length of the side-chain of the antioxidant and the nature of the micelles. These results indicate the critical importance of the microenvironment of reaction media and the lipophilicity of antioxidants on the reactivity. Therefore, it is of interest to examine whether the side-chain of vitamin C derivatives will also influence their reactivity towards vitamin E radical in micelles.



Scheme 1. Antioxidant synergism of vitamin E and vitamin C

In this study we generated α-tocopheroxyl radical (VE˙, **1**˙) by oxidizing α-tocopherol (VE) with Fremy's salt (NO˙, **6**) and investigated the reaction kinetics of VE˙ with ascorbic acid (VC, **2**), ascorbyl-6-caprylate (VC-8, **3**), ascorbyl-6-laurate (VC-12, **4**) and ascorbyl-6-palmitate (VC-16, **5**) in cetyltrimethylammonium

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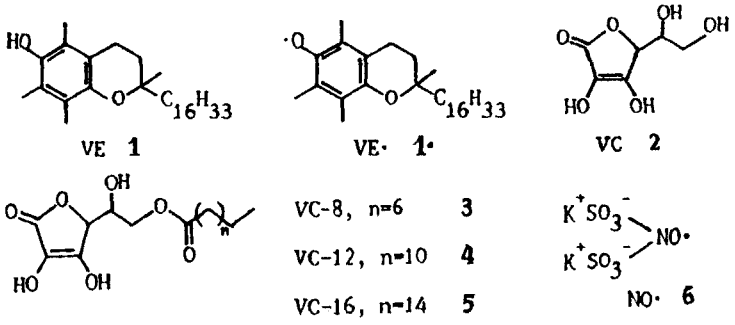

bromide (CTAB) micelles, using a special stopped-flow ESR device which enabled us to detect both VE· and VC^{-}· simultaneously and determine their kinetics.

EXPERIMENTAL

Materials. Fremy's salt (NO·) was synthesized according to the literature.[17] *dl*-α-Tocopherol (VE) and L-ascorbic acid (VC) were obtained from Merck and Beijing Chemicals, respectively, and used as received. Ascorbic acid derivatives (VC-8, VC-12 and VC-16) were prepared according to the literature.[18] CTAB (Sigma) was used as provided.

Sample preparation. Stock solutions of Fremy's salt (0·04–0·16 mM), VE (0·4–1·6 mM) and VC and its derivatives (0·01–0·6 mM) were freshly prepared separately in phosphate buffer (pH 7·2), which was made using doubly distilled water and deaerated by purging with argon. Then CTAB (0·015 M) was added and sonicated for 20 min with a 300 W Shanghai CQ 250 sonicator to facilitate the solubilization. The solutions were protected from air and transferred separately to three syringes in a stopped-flow device for ESR measurement.

Kinetic measurements. Figure 1 shows the device used for kinetic ESR measurements.[19] Aqueous micellar solutions of Fremy's salt, α-tocopherol and ascorbic acid or its derivative were stored separately in syringes L1, L2 and L3, respectively. A small pressure pump was used to perform rapid injections and stopping of the syringes. The solutions containing Fremy's salt in L1 and α-tocopherol in L2 were first mixed in a 1 : 1 ratio and then the mixture, after passing through the tube (L4, diameter 3 mm) was mixed in 2 : 1 ratio with the solution in L3 containing ascorbic acid or its derivative, and then run into a flat quartz cell C (0·4 × 5·5 × 60 mm) which was fixed within the cavity of the ESR spectrometer. The length of L4 was adjusted to ensure that the Fremy's salt was consumed completely before mixing with ascorbic acid or its derivative and the maximum steady-state concentration of tocopheroxyl radical could be obtained. In our system, when the concentration of NO·, VE and CTAB were 0·08 mM, 1·6 mM and 0·015 M, respectively, the length of L4 was *ca* 50 cm with a flow rate of *ca* 60 ml min^{-1}.

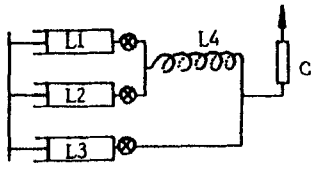

Figure 1. Arrangement for the stopped-flow ESR experiment

ESR measurements were performed with a Bruker ER 200D spectrometer operated in the X-band with 100 kHz modulation, modulation amplitude 0·1–0·25 mT, time constant 0·1 s and microwave power 10 mW. The concentrations of VE· and VC^{-}· were calibrated with Fremy's salt under the same experimental conditions.

RESULTS AND DISCUSSION

α-Tocopheroxyl radical (VE·) was generated from VE by oxidation with Fremy's salt:

$$VE + (SO_3K)_2NO\cdot \rightarrow VE\cdot + (SO_3K)_2NOH \quad (2)$$

As indicated in a previous paper,[15] ESR signals of NO· and VE· could be detected simultaneously under fast flow and, after stopping the flow, NO· decayed very rapidly, whereas VE· decayed relatively slowly in CTAB micelles. Therefore, if VC or its derivative is introduced after all of the Fremy's salt has been consumed, the reaction between VE· and VC or its derivative can be followed kinetically by ESR spectroscopy. Figure 2(a) shows a representative steady-state ESR spectrum obtained during the reaction of VE· and ascorbyl-6-palmitate (VC-16) under fast flow. The strong doublet superimposed on the signal of VE· can be unambiguously assigned to the reaction intermediate, ascorbyl-6-palmitate radical anion ($VC\text{-}16^{-}$·)[16] [Figure 2(b)]. Similar spectra were obtained from reactions of VE· with VC-12 and VC-8.

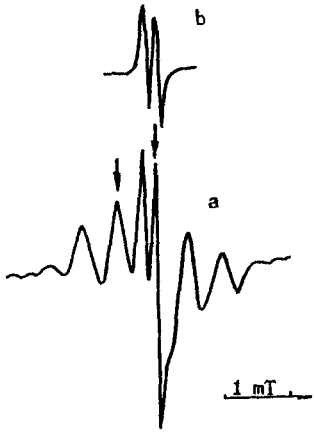

Figure 2. Steady-state ESR spectra of VE· and VC-16^{-}· obtained during the reaction of VE· and VC-16 in 0·015 M CTAB micelles (pH 7·2) at 20 °C. Arrows indicate the positions of locking the magnetic field for kinetic determination. (a) Superposition of VE· and VC-16^{-}· obtained under fast flow, flow rate 60 ml min^{-1}; (b) unique VC-16^{-}· obtained after stopping the flow. $[NO^{\cdot}]_0 = 8 \times 10^{-5}$ M; $[VE]_0 = 1{\cdot}6 \times 10^{-3}$ M; $[VC\text{-}16]_0 = 1 \times 10^{-4}$ M

The kinetic traces of both VE· and the radical anion of ascorbic acid derivatives were obtained by locking the magnetic field at the strongest peak of VE· and the high-field peak of the radical anion as shown in Figure 2(a) and then stopping the flow. Representative kinetic curves are given in Figures 3 and 4.

It can be seen from Figure 3 that the α-tocopheroxyl radical is fairly stable and decays slowly in deaerated

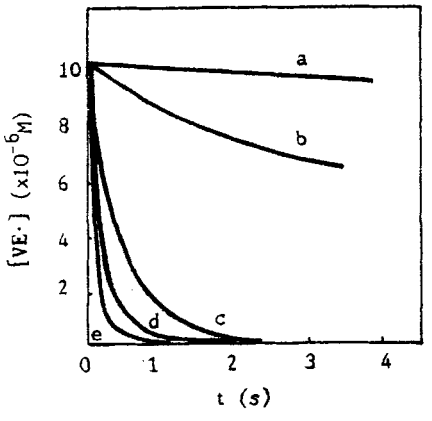

Figure 3. Decay curves of VE· in the absence and presence of VC or its derivatives in 0·015 M CTAB micelles (pH 7·2) at 20 °C. (a) Self-decay; (b) $[VC\text{-}16]_0 = 1{\cdot}0 \times 10^{-4}$ M; (c) $[VC\text{-}12]_0 = 1{\cdot}0 \times 10^{-4}$ M; (d) $[VC\text{-}8]_0 = 1{\cdot}0 \times 10^{-5}$ M; (e) $[VC]_0 = 5 \times 10^{-6}$M

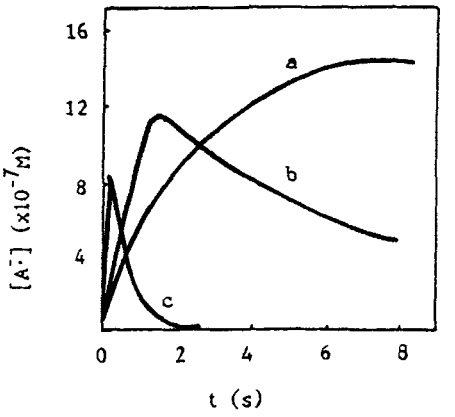

Figure 4. Formation and decay of the radical anion of lipophilic ascorbic acid derivatives during their reactions with VE· in 0·015 M CTAB micelles (pH 7·2) at 20 °C. $[NO^{\cdot}]_0 = 8 \times 10^{-5}$ M; $[VE]_0 = 1{\cdot}6 \times 10^{-3}$M; $[VC\text{-}8]_0 = 2{\cdot}7 \times 10^{-5}$ M; $[VC\text{-}12]_0 = 1{\cdot}0 \times 10^{-4}$ M; $[VC\text{-}16]_0 = 2{\cdot}0 \times 10^{-4}$ M. (a) VC-16·; (b) VC-12·; (c) VC-8·

CTAB micelles. However, the decay rate was greatly enhanced when ascorbic acid or its lipophilic derivative was added. The steady-state ESR spectrum and the formation of the radical anion of the ascorbic acid derivative, as shown in Figure 2, provide good evidence for the synergism of VE with VC and its lipophilic derivatives [equation (1)].

The decay kinetics of VE· was found to be pseudo-first order in the presence of a large excess of VC-16 or other derivatives. Plotting the pseudo-first-order rate constant, k_{obs}, versus the initial concentration of VC-16 etc. gave straight lines from which the second-order rate constant, k_{eff}, was obtained (e.g. Figure 5) and listed in Table 1. The rate constant for the reaction with VC was obtained by dividing k_{obs} by the initial concentration of

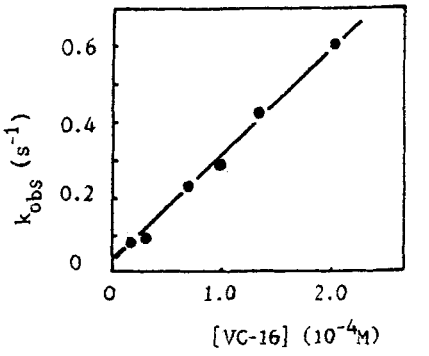

Figure 5. Plot of the pseudo-first-order rate constant k_{obs} versus the initial concentration of VC-16 in 0·015 M CTAB micelles

Table 1. Rate constants for the reaction of VE˙ with VCs in 0·015 M CTAB micelles, the binding constants of the antioxidants and the half-lives of the corresponding radicals

Species	k_{eff} ($l\,mol^{-1}\,s^{-1}$)	k_3 ($l\,mol^{-1}\,s^{-1}$)	K_2[a] ($l\,mol^{-1}$)	k_{-2} (s^{-1})	$t_{1/2}$ (s)
VC	$(9{\cdot}0 \pm 1{\cdot}4) \times 10^5$	$1{\cdot}1 \times 10^6$	$9{\cdot}2 \times 10^2$	$1{\cdot}1 \times 10^6$	
VC-8	$(3{\cdot}0 \pm 0{\cdot}6) \times 10^5$	$9{\cdot}2 \times 10^5$	$9{\cdot}3 \times 10^3$	$1{\cdot}1 \times 10^5$	
VC-12	$(7{\cdot}0 \pm 2.0) \times 10^4$	$7{\cdot}9 \times 10^5$	$4{\cdot}6 \times 10^4$	$2{\cdot}0 \times 10^4$	
VC-16	$(3{\cdot}0 \pm 0{\cdot}8) \times 10^3$	$1{\cdot}2 \times 10^5$	$1{\cdot}5 \times 10^5$	$6{\cdot}7 \times 10^3$	
VE˙					20
VC-8^{-}˙					0·4
VC-12^{-}˙					5
VC-16^{-}˙					110

[a] From Ref. 20.

VC because at higher concentration of VC the rate was too fast to be directly determined.

From comparison of the rates for the reaction of VE˙ with ascorbic acid or its lipophilic derivatives (Figure 3 and Table 1), it is interesting that the effective reactivity in CTAB micelles is remarkably influenced by the length of the side-chain according to the sequence VC > VC-8 > VC-12 ≫ VC-16, in spite of their similar reactivity in homogeneous solutions.[10] The reaction rate for VC is about $10^6\,l\,mol^{-1}\,s^{-1}$, which is close to the result obtained by Packer *et al.*[3] using homogeneous solutions, but the rate dropped to $3 \times 10^3\,l\,mol^{-1}\,s^{-1}$ with VC-16, with as much as a 300-fold decrease. This result reveals the critical importance of the lipophilic side-chain of VC derivatives on the reactivity. Clearly, this must be related to the formation of the micelle–VE˙ and micelle–VC aggregates. Hence the decay mechanism of VE˙ in micelles during its reaction with ascorbic acid or its derivative may be described by the following equations:

$$M + VE^{\cdot} \underset{}{\overset{K_1}{\rightleftharpoons}} M\text{–}VE^{\cdot} \qquad (3)$$

$$M + A \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} M\text{–}A \qquad K_2 = k_2/k_{-2} \qquad (4)$$

$$M\text{–}VE^{\cdot} + A \xrightarrow{k_3} M\text{–}VE + A^{-\cdot} \qquad (5)$$

$$VE^{\cdot} + A \xrightarrow{k_4} VE + A^{-\cdot} \qquad (6)$$

$$M\text{–}VE^{\cdot} + M\text{–}A \xrightarrow{k_5} M\text{–}VE + M\text{–}A^{-\cdot} \qquad (7)$$

where M and A represent the micelle and ascorbic acid or its derivative, respectively. Equations (5), (6) and (7) represent the possible reaction paths. However, the reaction shown in equation (7) must be difficult owing to the electrostatic repulsion between the micelles and can be ignored. Thus we have

$$-d[VE^{\cdot}]_T/dt = k_3[M\text{–}VE^{\cdot}][A] + k_4[VE^{\cdot}][A] \qquad (8)$$

When the equilibria of equations (3) and (4) are considered, we obtain

$$-d[VE^{\cdot}]_T/dt = k_3[M\text{–}VE^{\cdot}][A]_T/(1 + K_2[M]) + k_4[M\text{–}VE^{\cdot}][A]_T/K_1[M](1 + K_2[M]) \qquad (9)$$

where $[VE^{\cdot}]_T$ and $[A]_T$ represent the total concentration of VE˙ and VC or its derivative present in the bulk water and in the micellar phase, respectively.

To a first approximation, the water-phase reaction [equation (6)] can be neglected because vitamin E or its radical is generally believed to reside within micelles,[4] i.e. $[M\text{–}VE^{\cdot}] = [VE^{\cdot}]_T$, hence we have

$$-d[VE^{\cdot}]_T/dt = k_3[VE^{\cdot}]_T[A]_T/(1 + K_2[M]) \qquad (10)$$

The effective rate constant obtained experimentally may be expressed as

$$k_{eff} = k_3/(1 + K_2[M]) \qquad (11)$$

That is, k_{eff} is related to the reaction rate of micelle-aggregated tocopheroxyl radical with the water-phase located ascorbic acid or its derivatives and the aggregation ability of the ascorbic acid derivative with the micelle. Because the equilibrium constant is determined by the entry rate, k_2, and exit rate, k_{-2}, of A, k_{eff} is also related to these rates.

It has been proved that[21] the association rate constant of a substrate with micelles, i.e. the entry rate, is close to diffusion controlled, i.e. *ca* $10^9\,l\,mol^{-1}\,s^{-1}$, and does not change significantly with variation in the length of the side-chain, especially for ionic substrates bearing an opposite charge to the charge of the micellar head group.[22] The exit rate, however, is much slower and strongly dependent on the length of the side-chain. If we assume the entry rates of ascorbic acid and its derivatives are all the same and equal to $1 \times 10^9\,l\,mol^{-1}\,s^{-1}$, the exit rates can be estimated from equation (4). The values obtained are listed in Table 1, where the equilibrium constants K_2 were determined by Griller's method.[20,23] With the K_2 values determined, the rate constants k_3 can be calculated from equation (11) (Table 1).

It can be seen from Table 1 that the exit rate k_{-2} decreases strongly with increase in the side-chain length, which is parallel to the reactivity sequence of k_{eff}. The k_3 values also change in the same direction, but to a much smaller extent. These results enable us to formulate a mechanism for the reaction of tocopheroxyl radical with ascorbic acid derivatives. For a lipophilic ascorbic acid derivative which shows a strong preference for the micellar environment to react with a tocopheroxyl radical which resides within another micelle, it must diffuse out from the micelle in which it is originally located and enter into another micelle, and do so repeatedly until it finds one tocopheroxyl radical. In this case the reaction may follow second-order kinetics with the exit process being the rate-determining step.[24] Therefore, the effective rate is greatly influenced by the side-chain length of the ascorbic acid derivatives, because it is the side-chain length that governs the intermicellar diffusion rate, i.e. the longer the side-chain length, the slower is the exit rate.[21,24]

Moreover, the variation of k_3 with the side-chain length, especially with VC-16, indicates that the entry of a molecule of the VC derivative into a micelle containing VE$^{\cdot}$ does not necessarily bring about a reaction, because the VE$^{\cdot}$ radical may not be located in the same place where the VC molecule enters. Hence the VC molecule must move around the micellar surface to encounter a VE$^{\cdot}$ radical before the reaction can take place. This means that the intramicellar diffusion may also contribute to the reactivity. Indeed, Miyashita *et al.*[25] have recently shown that the intramicellar diffusion rate is also dependent on the side-chain length of the substrate.

It is known that ascorbate radical anion decays by disproportionation at a very high rate in solutions ($k_{disprop.} \approx 10^7\,l\,mol^{-1}\,s^{-1}$ at pH 7.2[26]). Therefore, it is difficult to measure the decay kinetics of ascorbate radical anion in homogeneous solutions by ESR spectroscopy. We have found, however, that the steady-state concentration and the lifetimes of the radical anion of the lipophilic VC derivatives in CTAB micelles are greatly enhanced by the side-chain, as shown in Figure 4. The half-life is about 0·4, 5 and 110 s in 0·015 M CTAB micelles (pH 7·2) for VC-8$^{-\cdot}$, VC-12$^{-\cdot}$ and VC-16$^{-\cdot}$, respectively. This is evidence that intermicellar diffusion may also be the rate-limiting step for the disproportionation of the lipophilic VC$^{-\cdot}$ in micelles. A similar micellar effect on increasing the lifetimes and concentrations of free radicals in sodium dodecyl sulphate (SDS) micelles has been reported by Burkey and Griller.[24]

In conclusion, this work has shown that the reactivity of lipophilic derivatives of ascorbic acid towards tocopheroxyl radical in CTAB micelles is greatly dependent on their side-chain length. Kinetic analysis reveals that the intermicellar diffusion and, to a lesser extent, the intramicellar diffusion play a crucial role in the reactivity. The efficient antioxidant synergism of VC with VE in biomembranes may be attributed to its free migration on the membrane surface. The result may be helpful in understanding the antioxidant mechanism in biological systems.

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