Interaction of Galvinoxyl Radical with Ascorbic Acid, Cysteine, and Glutathione in Homogeneous Solution and in Aqueous Dispersions

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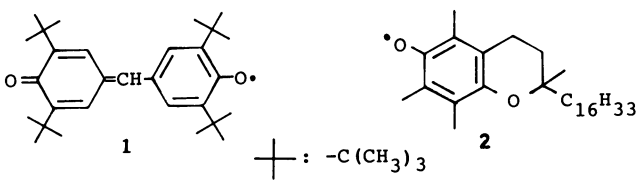
The rates of interaction of galvinoxyl radical with ascorbic acid, cysteine, and glutathione have been studied in homogeneous solution and in water dispersions as a model reaction of vitamin E radical with these reducing agents in biological systems. The rate was measured by following the disappearance of galvinoxyl radical with absorption spectroscopy and electron spin resonance. In every system, galvinoxyl reacted with these reducing substrates and their relative reactivities decreased in the order of ascorbic acid>cysteine>glutathione. For any reducing substrate, the rate was fastest in homogeneous solution and slowest in micelle in Triton X-100 aqueous solution, and the rate in liposome system was in between.

The oxidation of polyunsaturated fatty acids in the lipids and its inhibition has received much attention recently in connection with its pathological effects in biological systems and preservation of foods.[1-8] Vitamin E has been known to act as an effective antioxidant.[9-12] A synergistic inhibition of oxidation by the combination of vitamin E with reducing agents has been suggested[13-19] and it has been observed experimentally that vitamin E radical reacts with ascorbic acid[13,20,21] and glutathione[21,22] to regenerate vitamin E.

In biological systems, most of vitamin E is present in lipophylic membranes, whereas ascorbic acid, glutathione, and cysteine are found in aqueous phase. Therefore, the interaction of vitamin E radical with these reducing agents must be more restricted than in homogeneous system and the effect of reaction medium must be quite important. In order to study the effect of reaction medium, the rates of interaction of galvinoxyl radical (**1**) with ascorbic acid, glutathione, and cysteine were measured both in homogeneous solution and in aqueous dispersions. Galvinoxyl was chosen as a model of vitamin E radical, since both are relatively stable phenoxyl radicals and galvinoxyl is much easier to locate in liposomal membranes and micelles than vitamin E radical (**2**).



Experimental

Materials. Galvinoxyl, L-ascorbic acid, cysteine, and glutathione (reduced form) were of the highest grade available and used as received. *N,N'*-dioxide-2-methyl-*N*-(4-pyridinylmethylene)-2-propanamine (POBN) used as a spin trap was obtained from Aldrich. Dimyristoylphosphatidylcholine (PC) was obtained from Sigma and used without further purification. Ethyl palmitate, ascorbyl-6-stearate, and Triton X-100 were purchased from Tokyo Kasei Kogyo Co. and used as received.

Procedure. The micelle was prepared as follows using Triton X-100 as a nonionic surfactant. Appropriate amount of galvinoxyl was dissolved into ethyl palmitate and then 0.01 M (1 M=1 mol dm^{-3}) Triton X-100 aqueous solution was added. The mixture was shaken vigorously with a Vortex mixer for 1 min to obtain pale brownish micelle solution.

The liposome was prepared by the following method. PC and galvinoxyl (and other oil-soluble additives, when necessary) were dissolved in benzene or in benzene–ethanol (1:1 by volume) and the solution was taken into a small flask. The solvent was removed by evacuation on a water aspirator using a rotary evaporator to obtain a thin film on the flask wall. It was further evacuated under high vacuum. Appropriate amount of 0.1 M NaCl aqueous solution was then added and the film was slowly peeled off by shaking to obtain a milky liposome solution. In several experiments, the solution was sonicated using a Branson Sonifier Model 185.

The rate of disappearance of galvinoxyl was followed by absorption spectroscopy at 429 nm. The reaction was started by mixing the galvinoxyl solution with the reducing agent solution in a Pyrex glass ampoule immersed in a water bath maintained at the desired temperature. The solution was taken out periodically and analyzed.

The rate was also followed by electron spin resonance (ESR) spectroscopic analysis. ESR spectra were recorded on X-band JEOL FE1X spectrometer using a quartz flat cell.

Results

ESR Spectra of Galvinoxyl Radical. Figure 1 shows the ESR spectra of galvinoxyl radical in different media at different temperatures. In homogeneous solution, an isotropic ESR spectrum of galvinoxyl radical with good hyperfine splitting was observed. In micelle, the hyperfine structure showed good split at higher temperature. Similarly, the hyperfine splitting

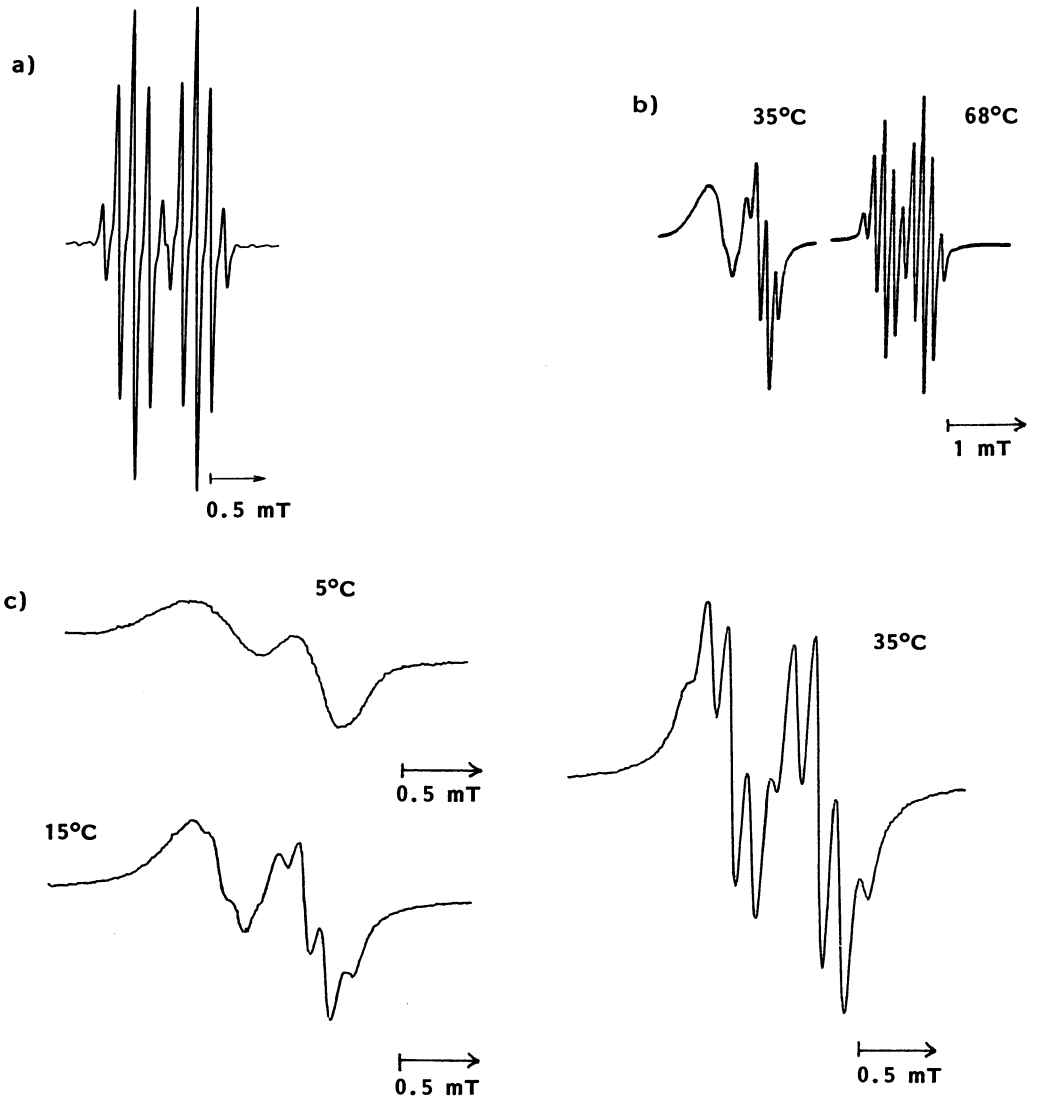


Fig. 1. ESR spectra of galvinoxyl radical in various media.
a) 0.50 mM galvinoxyl in benzene at room temperature under vacuum. b) In 10 mM Triton X-100 aqueous dispersion. c) In dimyristoyl PC liposome dispersed in 0.1 M NaCl aqueous solution.

was not satisfactory below 15 °C for the galvinoxyl incorporated into dimyristoyl PC liposome. The coupling constants for galvinoxyl are summarized in Table 1.

Interaction of Galvinoxyl Radical with Reducing Agents in Homogeneous Solution. When two methanol solutions containing 2.5 mM galvinoxyl and 25 mM ascorbic acid, respectively, were mixed at room temperature, galvinoxyl disappeared instantaneously. On the other hand, when galvinoxyl was mixed with cysteine or glutathione, galvinoxyl disappeared gradually with time and the first order plot gave a good straight line. The pseudo-first order rate constants obtained from this plot are summarized in Table 2.

Interaction in Micelle System. The galvinoxyl incorporated into micelle reacted with ascorbic acid, cysteine, and glutathione. Figure 2 shows an example

Table 1. Coupling constants of galvinoxyl radical in various media[a)]

Medium	Temp °C	a_p^{CH} mT	a_m^H mT
In homogeneous solution of benzene			
	25	0.583	0.140
		0.590[b)]	0.140[b)]
In ethyl palmitate micelle in Triton X-100			
aqueous dispersion	38	0.548	0.137
	48	0.552	0.140
	68	0.554	0.139
In dimyristoyl PC liposome in 0.1 M NaCl			
aqueous dispersion	25	0.594	0.142
	35	0.591	0.143
	46	0.588	0.142

a) Numbers in three decimal places may not be so accurate. b) Ref. 23.

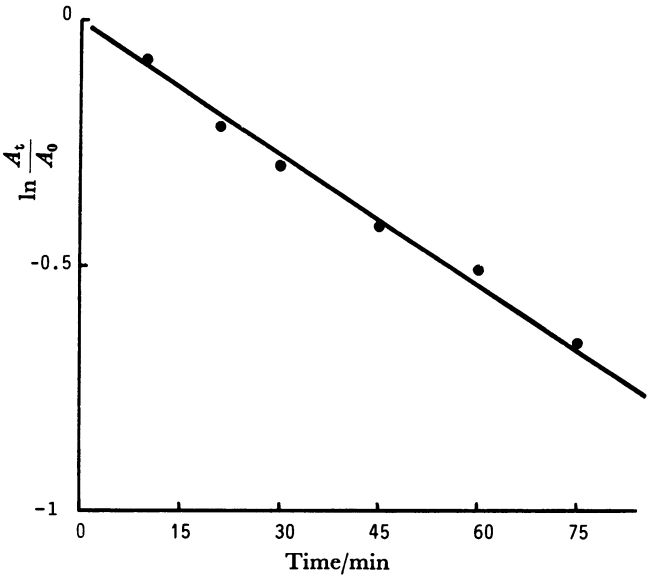


Fig. 2. Pseudo-first order plot of galvinoxyl radical for the reaction with ascorbic acid at 37 °C in Triton X-100 aqueous dispersion: 4 ml ethyl palmitate containing 10 mM galvinoxyl and 4 ml of 50 mM ascorbic acid in 10 mM Triton X-100 aqueous solution were mixed.

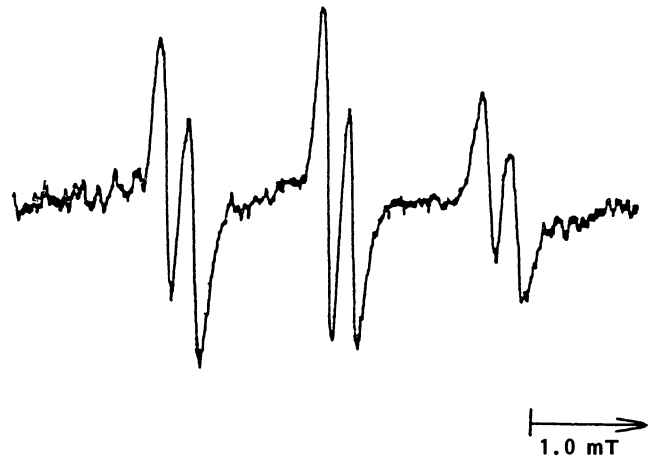


Fig. 3. ESR spectrum observed when POBN was dissolved in an aqueous solution during the interaction of galvinoxyl radical with glutathione in micelle system.

Table 2. Rate of reaction of galvinoxyl radical (G) with ascorbic acid (AsA), Cysteine (CSH), and glutathione (GSH) at 37 °C in homogeneous solution[a] measured by absorption spectroscopy

[G] mM	Reactant	mM	$10^4 k$[d] s^{-1}
2.5[b]	AsA	25	e)
2.5[c]	AsA	25	e)
1.5	CSH	15	10
2.5	GSH	15	1.8
1.5	GSH	7.7	1.1
1.5	GSH	15	1.8
1.5	GSH	30	2.4
1.5	GSH	60	3.3
1.5	GSH	150	3.2
0.75	GSH	15	1.8
0.195[f]	GSH	0.81	2.8

a) Acetone/water=9/4 by v/v. b) In methanol. c) In acetone/methanol=15/8 by v/v. d) Pseudo-first order rate constant. e) The rate was too fast to be measured. f) In acetone/water=5/3 by v/v measured by ESR.

Table 3. Interaction of galvinoxyl radical (G) dissolved in ethyl palmitate (EP) micelle with ascorbic acid (AsA) in 10 mM triton X-100 aqueous dispersion at 37 °C measured by absorption spectroscopy

G in EP [G] mM	ml	AsA in 10 mM Triton X-100 aq [AsA] mM	ml	$10^4 k$ s^{-1}
5	3	0	3	0
5	4	100	4	1.7
5	4	50	4	1.5
5	4	25	4	1.4
5	4	5	4	0.64
10	4	50	4	1.5
2.5	4	50	4	1.7
5	1	50	7	2.8
5	2	50	6	1.8
20	1	50	7	2.4
5	1	50	7[a]	1.7

a) In 100 mM Triton X-100 aqueous solution.

of the first order plot of the galvinoxyl radical in the reaction with ascorbic acid at 37 °C. The galvinoxyl is stable in the absence of ascorbic acid. The results with ascorbic acid are summarized in Table 3. The pseudo-first order rate constant was insensitive to the concentration of ascorbic acid and surfactant and also to the ratio of organic to aqueous fractions.

Interaction in Liposome System. Galvinoxyl incorporated into dimyristoyl PC liposome reacted with ascorbic acid, cysteine, and glutathione faster than in micelle system. Satisfactory first order plot was obtained. The results obtained in different systems are summarized in Table 4. In every case, the results obtained by absorption spectroscopy and ESR were in satisfactory agreement.

Discussion

Galvinoxy radical must interact with the reducing agents by a hydrogen atom abstraction. In fact, when a spin trap, POBN, was also dissolved in an aqueous phase in micelle system, new ESR spectra were observed as galvinoxyl disappeared in the presence of cysteine and glutathione. The example of the ESR spectrum is shown in Fig. 3 and the hyperfine

Table 4. Pseudo-first order rate constant (s^{-1}) for the interaction of galvinoxyl radical with ascorbic acid, cysteine, and glutathione in various reaction media at 37 °C

	Homogeneous solution[a]	Micelle[b]	Liposome[c]
Ascorbic acid	Too fast	1.7×10^{-4}	Too fast
Cysteine	1.0×10^{-3}	4.5×10^{-5}	6.5×10^{-4}
Glutathione	1.8×10^{-4}	1.7×10^{-5}	2.0×10^{-4}

a) In acetone/waser (9/4 by v/v). b) Galvinoxyl dissolved in ethyl palmitate in 10 mM Triton X-100 aqueous disperson. c) Galvinoxyl incorporated into dimyristoyl PC in 0.1 M NaCl aqueous dispersoin.

Table 5. Hyperfine splitting constants for the spin adducts of glutathione and cysteine radicals by POBN[a]

	a^N/mT	a^H_β/mT
Glutathione	1.495	0.230
Glutathione[b]	1.513	0.232
Cysteine	1.504	0.230

a) 1.18 mM galvinoxyl in Triton X-100 aqueous dispersion was reacted with 10 mM glutathione or cysteine. b) In ethanol/water (5/1 by v/v).[21]

splitting constants are given in Table 5. The same ESR spectra were observed when *t*-butoxyl radical was generated from di-*t*-butyl diperoxyoxalate containing POBN and glutathione or cysteine. Therefore, these spectra may well be ascribed to the spin adduct of glutathione radical or cysteine radical by POBN.[21]

Table 4 summarizes the rate constants for the interaction of galvinoxyl with the reducing agents in different systems. It shows that in every system the relative reactivities decrease in the order of ascorbic acid>cysteine>glutathione. It also shows that for any reducing agent the interaction proceeds most rapidly in homogeneous solution and most slowly in micelle system. The rate of interaction in liposome system was in between.

The lower reactivities in liposome and in micelle systems than in homogeneous solution must be ascribed to a lower mobility of galvinoxyl and to a lower accesibility of the reducing agent to galvinoxyl.

It must be important to compare the present results with the antioxidant activities of vitamin E and reducing agents. As reported previously,[13] vitamin E and vitamin C inhibited synergistically the oxidation of methyl linoleate in homogeneous solution. It was suggested that vitamin E scavenges the chain carrying peroxyl radical quickly and the resulting vitamin E radical reacts with vitamin C to regenerate vitamin E. As a consequence, only vitamin C was consumed at first and vitamin E decayed after vitamin C was depleted.

In the oxidation of soybean PC liposome initiated with an oil-soluble azo compound, vitamin C was not as good antioxidant as in the oxidation initiated with a water-soluble azo compound, but vitamin C was still suggested to react with vitamin E radical in the bilayer, regenerate vitamin E and reduce the rate of disappearance of vitamin E.[24]

Interestingly, in contrast to the oxidation in homogeneous solution and in liposome system mentioned above, vitamin C was not an effective antioxidant in the oxidation of methyl linoleate micelle in Triton X-100 aqueous dispersion initiated with an oil-soluble azo compound. However, even in this case, vitamin C could prolong the suppression period when 2,2,5,7,8-pentamethyl-6-chromanol, a vitamin E model, was located in micelle.

It may be also worth noting, that, although galvinoxyl incorporated into liposome reacted with vitamin C in an aqueous phase so rapidly, it reacted only slowly with ascorbyl-6-stearate incorporated into different liposome in the same water dispersion.

The results obtained in the present study and those of oxidations described above are in accordance with each other, although it must be considered that the location and behavior of galvinoxyl and vitamin E radicals in the membrane may not be identical. Furthermore, the location of phenoxyl radicals in micelle and liposome and the accesibility of reducing agents must also depend on the type of surfactant, composition of fatty acids in micelle and liposome, and other experimental variables.

In conclusion, the present results show that stable phenoxyl radicals react with reducing agents such as ascorbic acid, cysteine, and glutathione by a hydrogen atom abstraction not only in homogeneous solution but also in heterogeneous aqueous dispersions. This is another experimental support to the possible contribution of vitamin E and reducing agents in synergistic inhibition of peroxidation in biological systems.

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