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Oxidation of Lipids. IV. Formation and Reaction of Chromanoxyl Radicals as Studied by Electron Spin Resonance

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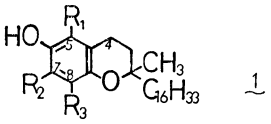
In order to study the inhibition of oxidation by vitamin E, the formation and reaction of chromanoxyl radicals and hindered phenoxyl radicals were examined by ESR. *t*-Butoxyl radical abstracted the phenolic hydrogens of α-, β-, γ-, and δ-tocopherols, 2,4,6-tri-*t*-butylphenol (TBP), and 2,6-di-*t*-butyl-4-methylphenol (BMP) to give corresponding phenoxyl radicals which were stable enough to be observed by ESR. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) also induced the formation of chromanoxyl and phenoxyl radicals. The ESR spectra of chromanoxyl radicals were recorded and their hyperfine coupling constants were determined. The phenoxyl radicals from TBP and BMP disappeared quite rapidly when excess *t*-butyl hydroperoxide was added, whereas α-chromanoxyl radical from α-tocopherol remained considerably. α-Chromanoxyl radical and phenoxyl radicals reacted with *p*-*t*-butylbenzenethiol to regenerate α-tocopherol and corresponding phenols respectively. The implications of these results are discussed.

Vitamin E (tocopherols, **1**) is known to contribute in biological systems as a scavenger of peroxyl and alkoxyl radicals, singlet oxygen and superoxide anion radical.[1-3] Among them, the trapping of peroxyl and



1

α-Tocopherol : 1a, $R_1=CH_3$, $R_2=CH_3$, $R_3=CH_3$,
β-Tocopherol : 1b, $R_1=CH_3$, $R_2=H$, $R_3=CH_3$,
γ-Tocopherol : 1c, $R_1=H$, $R_2=CH_3$, $R_3=CH_3$,
δ-Tocopherol : 1d, $R_1=H$, $R_2=H$, $R_3=CH_3$,

alkoxyl radicals, the chain carrying species in peroxidation of lipids, must be the most important role of vitamin E. Although the mechanism of inhibition of autoxidation by synthetic hindered phenols is now well understood,[4,5] it is not fully established how vitamin E suppresses the lipid peroxidation. The objective of the present work is to study by ESR technique the formation and reaction of chromanoxyl radicals[6] from tocopherols and compare them with those from the hindered phenols such as 2,4,6-tri-*t*-butylphenol (TBP) and 2,6-di-*t*-butyl-4-methylphenol (BMP). α-Tocopherol, the major component of vitamin E, was studied most extensively.

Experimental

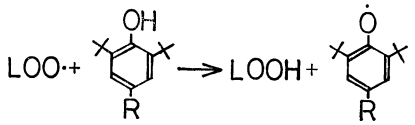
Materials. Natural α-, β-, γ-, and δ-tocopherols were kindly supplied from Eisai Co. Ltd. Commercial TBP and BMP were recrystallized from methanol. *p*-*t*-Butylbenzenethiol was used after distillation. Di-*t*-butyl diperoxyoxalate (DBPO) used as a *t*-butoxyl radical source was prepared by the method described in the literature.[7] Phenylazotriphenylmethane and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Eastman Kodak and used as received. *t*-Butyl hydroperoxide was distilled under reduced pressure prior to use, 42 °C/24 Torr (1 Torr=133.322 Pa). The spin traps, 2-nitroso-2-methylpropane, nitrosobenzene, *N*,*N*'-dioxide-2-methyl-*N*-(4-pyridinylmethylene)-2-propanamine (POBN), and 2,4,6-tri-*t*-butylnitrosobenzene was obtained from Aldrich and used without further purification.

Apparatus and Procedure. ESR spectra were recorded on X-band JEOL FE1X spectrometer at room temperature by the following general procedure. Solid DBPO and TBP or BMP were taken directly into the quartz ESR tube, evacuated and benzene was vacuum transferred into the tube and subjected to ESR analysis. DPPH solution was taken into an ESR tube and the solvent was removed by evacuation. Benzene solution of α-tocopherol and spin trap, when necessary, was taken into a side arm of the ESR tube, frozen, evacuated and then mixed with solution of phenol and DBPO or DPPH. The additives such as *t*-butyl hydroperoxide and *p*-*t*-butylbenzenethiol were introduced into ESR tube by vacuum distillation.

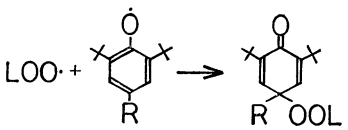
The reaction was carried out at room temperature under vacuum unless otherwise noted. The concentrations of DBPO and DPPH were in the range of 5×10^{-5} to 10^{-2} M (1 M= 1 mol dm^{-3}) and excess amount of α-tocopherol, BMP, or TBP was used.

Results and Discussion

It is now understood that hindered phenols such as TBP and BMP inhibit the autoxidation by trapping the chain carrying peroxyl radicals as shown in Eqs. 1 and 2. The rate determining step is Reaction 1, and Reaction 2 proceeds quite rapidly.[4,5] Each TBP or BMP traps two molecules of peroxyl radicals,[4] and peroxyl radical adduct **2** has been isolated and identified.[8]



(1)

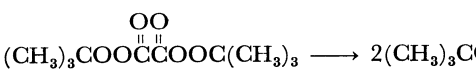


(2)

2

+ : -C(CH$_3$)$_3$

Upon thermal decomposition, DBPO produces two molecules of *t*-butoxyl radicals. When DBPO was decomposed in benzene solution of tocopherols, TBP or



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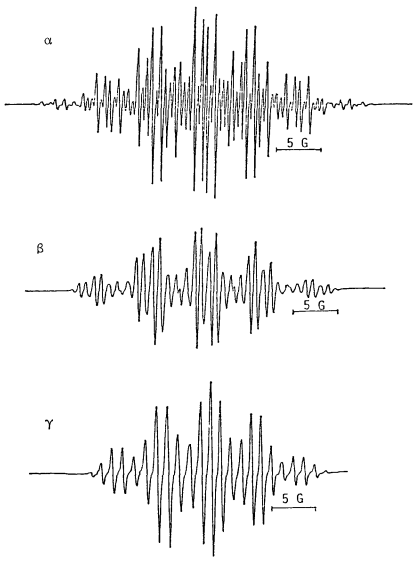


Fig. 1. The ESR spectra of chromanoxyl radicals formed from tocopherols by the reaction with *t*-butoxyl radical.

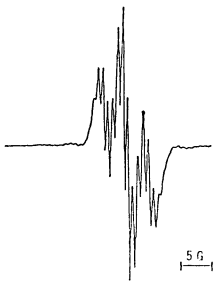


Fig. 2. The ESR spectrum of 2,4,6-tri-*t*-butylphenoxyl radical observed when DBPO was decomposed under vacuum in benzene containing TBP.
[DBPO]$=1\times10^{-4}$ M, [TBP]$=1\times10^{-2}$ M.

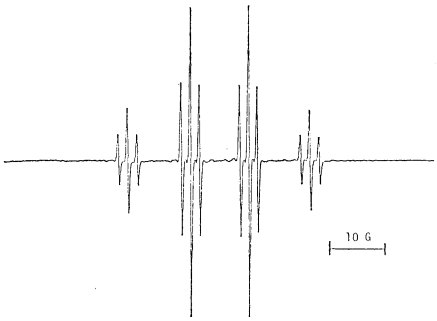


Fig. 3. The ESR spectrum of 2,6-di-*t*-butyl-4-methylphenoxyl radical.
[DBPO]$=1\times10^{-4}$ M, [BMP]$=1\times10^{-2}$ M.

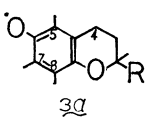
BMP, the corresponding phenoxyl radicals were observed by ESR as shown respectively in Figs. 1, 2, and 3. This shows that *t*-butoxyl radical formed from DBPO abstracts the phenolic hydrogens from tocopherols, TBP, and BMP.

DPPH also gave the same phenoxyl radicals as reported previously,[9-11] that is, when TBP, BMP, or tocopherol was added to the benzene solution of DPPH, the purple color of DPPH, its visible spectrum ($\lambda_{max}=$ 525 nm), and its ESR spectrum disappeared and new ESR spectra of phenoxyl radicals were observed. On the other hand, when phenylazotriphenylmethane was decomposed instead of DBPO, only triphenylmethyl radical was observed and no phenoxyl radical could be observed.

Table 1. Hyperfine coupling constants of chromanoxyl radicals generated from tocopherols[a)]

Radical	5-R_1	7-R_2	8-R_3	4-CH
α-Chromanoxyl	a^{CH_3} 0.602	a^{CH_3} 0.458	a^{CH_3} 0.094	a^{CH_2} 0.148
β-Chromanoxyl	a^{CH_3} 0.625	a^{H} 0.466	a^{CH_3} 0.085	a^{CH_2} 0.168
γ-Chromanoxyl	a^{H} 0.595	a^{CH_3} 0.477	a^{CH_3} 0.116	a^{CH_2} 0.116
δ-Chromanoxyl	a^{H} 0.592	a^{H} 0.460	a^{CH_3} 0.111	a^{CH_2} 0.111

a) In mT, measured in benzene, under vacuum and at room temperature.



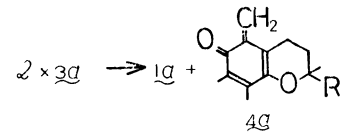
3a

$R=(CH_2CH_2CH_2\underset{\displaystyle CH_3}{\underset{|}{C}}H)_3CH_3$

The ESR spectrum of α-chromanoxyl radical **3a** from α-tocopherol shown in Fig. 1 agrees well with those reported previously.[11-13] As assigned previously,[11-13] the ESR spectrum of **3a** shows seven main lines with additional hyperfine structure. The hyperfine splitting constants measured in this work are summarized in Table 1. It is noteworthy that protons at C-5 and C-7 are not equivalent, the spin density being higher at C-5.

The stability of the phenoxyl radicals is dependent on the substituents on the aromatic ring. α-Chromanoxyl radical and 2,4,6-tri-*t*-butylphenoxyl radical were relatively stable at room temperature in the absence of oxygen. On the other hand, 2,6-di-*t*-butyl-4-methylphenoxyl and 2,4,6-trimethylphenoxyl radicals decayed rapidly even in the absence of oxygen. The phenoxyl radicals from α-tocopherol, TBP, and BMP were stable toward spin traps such as 2-methyl-2-nitrosopropane, nitrosobenzene, 2,4,6-tri-*t*-butylnitrosobenzene, and POBN.

Figure 4 shows the rate of disappearance of α-chromanoxyl radical in the absence of oxygen. The second order plot included in Fig. 4 gives the rate constant for the decay of α-chromanoxyl radical as $k=0.061$ $M^{-1}\,s^{-1}$. It should be noted that the rate of decay depends on the solvent. The self reaction of α-chromanoxyl radicals is reported to give α-tocopherol and *o*-quinone methide **4a** by disproportionation (Reaction 4).[14-16]



4a (4)

The effect of addition of hydroperoxide on the sta-

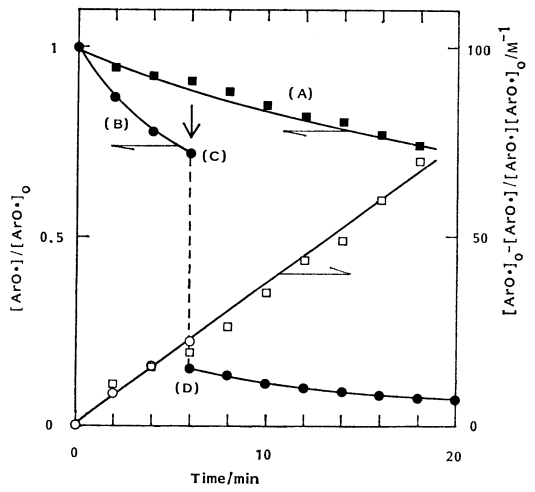
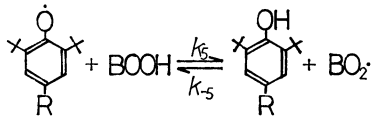


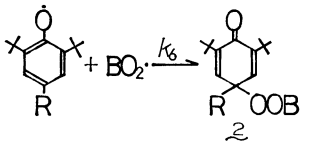
Fig. 4. Decay of α-chromanoxyl radical (ArO·) in benzene at room temperature under vacuum before and after the addition of *t*-butyl hydroperoxide (BOOH).
BOOH was added at the point C indicated by an arrow. There is some time lag (a couple of minutes) between the points C and D due to manipulation.

	ArOH(mM)	DPPH(mM)	BOOH(mM)
(A)	14.9	5.02	0
(B)	24.3	17.7	177

bility of phenoxyl radicals was then studied to know the interaction of α-chromanoxyl radical with peroxyl radical. It was found that, when phenoxyl radical was first generated from TBP or BMP in benzene and then *t*-butyl hydroperoxide was transferred from the side arm of the ESR tube into the above solution, the phenoxyl radicals from TBP and BMP disappeared rapidly and completely. These results suggest that the phenoxyl radicals react with hydroperoxide to give peroxyl radicals, which then react with another phenoxyl radical to give adduct **2** (Reactions 5 and 6).



(5)



(6)

2

Interestingly, however, it was found that, as shown in Fig. 4, α-chromanoxyl radical from α-tocopherol did not disappear completely when excess *t*-butyl hydroperoxide was added. It was confirmed that this variation of ESR spectrum was due neither to dilution effect nor to solvent induced variation in cavity Q.[17]

The hydroperoxidic hydrogen of *t*-butyl hydroperoxide is readily abstracted by oxygen radicals to generate *t*-butylperoxyl radical.[18–22] The rate constant for the hydrogen atom abstraction from α,α-dimethylbenzyl hydroperoxide by 2,4,6-tri-*t*-butylphenoxyl radical has been reported as $\log k$ ($M^{-1}\,s^{-1}$) = 7.1—10900/2.303 RT,[23] which gives k_5 = 0.13 $M^{-1}\,s^{-1}$ at 25 °C.

The rate of disappearance of phenoxyl radical (ArO·) is given by

$$-\frac{d[ArO\cdot]}{dt}=\frac{2k_5k_6[ArO\cdot]^2[BOOH]}{k_{-5}[ArOH]+k_6[ArO\cdot]} \quad (7)$$

and since $k_6[ArO\cdot][BO_2\cdot] \gg k_5[ArOH][BO_2\cdot]$,

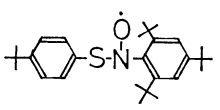
$$-\frac{d[ArO\cdot]}{dt}=2k_5[ArO\cdot][BOOH]. \quad (8)$$

The amount of *t*-butyl hydroperoxide was 0.1 M and if we assume that *t*-butyl hydroperoxide has the same reactivity as α,α-dimethylbenzyl hydroperoxide toward phenoxyl radical, then, $-d[ArO\cdot]/dt = 0.026\ [ArO\cdot]$, which gives the half life of the phenoxyl radical as about 30 s.

The different behavior of α-chromanoxyl radical from the phenoxyl radicals derived from TBP and BMP observed by the addition of *t*-butyl hydroperoxide may be interpreted by an extraordinary stability of α-chromanoxyl radical; that is, a slower interaction of α-chromanoxyl radical with hydroperoxide and peroxyl radical, higher rate constant for the hydrogen atom abstraction from α-tocopherol by peroxyl radical,[24–26] and/or the instability of the adduct of α-chromanoxyl radical and peroxyl radical. Furthermore, α-tocopherol has much higher equilibrium constant than TBP in the reversible reaction with DPPH and galvinoxyl.

We believe the decrease in α-chromanoxyl radical observed by the addition of *t*-butyl hydroperoxide is not due to oxygen which may possibly be formed from the bimolecular interaction of *t*-butylperoxyl radicals. In one experiment, α-chromanoxyl radical was first generated under vacuum by the reaction with DPPH, analyzed by ESR, and then *t*-butyl hydroperoxide was vacuum transferred into the ESR tube. The amount of α-chromanoxyl radical decreased as observed in Fig. 4. Then the ESR tube was evacuated, but the amount and shape of the ESR spectrum did not change, suggesting that the rapid decrease in α-chromanoxyl radical is not due to oxygen formed from *t*-butylperoxyl radical.[27]

We have previously reported that α-chromanoxyl radical reacted with glutathione and ascorbic acid to regenerate α-tocopherol.[28] Similarly it was found that when *p*-*t*-butylbenzenethiol was added to the mixture of DPPH and phenol in benzene, the phenoxyl radicals from α-tocopherol, TBP, and BMP all disappeared rapidly. When both *p*-*t*-butylbenzenethiol and 2,4,6-tri-*t*-butylnitrosobenzene were added, new ESR spectrum was observed in place of the phenoxyl radical. The observed ESR spectrum shown in Fig. 5 is the same as that observed when DBPO was decomposed in benzene solution of *p*-*t*-butylbenzenethiol and 2,4,6-tri-*t*-butylnitrosobenzene and it is ascribed to spin adduct **5**. The coupling constants obtained, a_N = 1.630 mT and a_H = 0.084 mT, agree well with those of phenylthiyl radical spin adduct by 2,4,6-tri-*t*-butylnitrosobenzene reported by Terabe and Konaka, a_N = 1.629 mT and a_H = 0.085 mT.[29]



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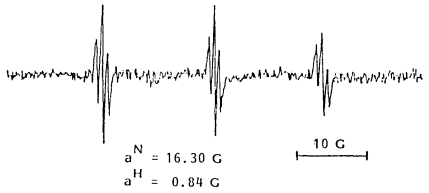
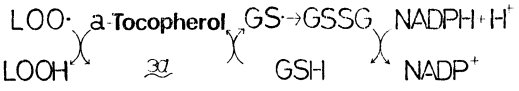


Fig. 5. The ESR spectrum observed when *p*-*t*-butylbenzenethiol was added to the mixture of DPPH, 2,4,6-tri-*t*-butylnitrosobenzene and TBP.

These results suggest that hydrogen atom is quickly transferred from *p*-*t*-butylbenzenethiol to phenoxyl radicals to regenerate the phenols. One of the interesting features of vitamin E in biological systems is the proposed regeneration of vitamin E.[28,30-32] This type of hydrogen transfer from thiol such as glutathione and cysteine to chromanoxyl radicals may contribute in the regeneration and maintenance of vitamin E levels in tissues.



(9)

It must be noted, however, that these observations in vitro in homogeneous solution do not necessarily indicate the contribution of these reactions in heterogeneous systems *in vivo*.

The above results show that *t*-butoxyl radical and DPPH abstract the phenolic hydrogen atom from tocopherols, TBP, and BMP. It has been reported that phenol is more reactive than toluene by a factor of 10^5 to *t*-butylperoxyl radical principally due to a difference in activation energy, even though the heats of these hydrogen atom transfers are comparable.[33] The abstraction of a hydrogen atom by an oxygen radical from oxygen is energetically more favorable than abstraction from carbon. Therefore, the phenolic hydrogen must be abstracted exclusively from α-tocopherol and little benzylic C–H bond should be attacked.

In conclusion, α-tocopherol behaves similarly with TBP and BMP toward alkoxyl radical and DPPH. However, chromanoxyl radical **3** has somewhat different reactivity toward peroxyl radical and/or hydroperoxide.

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