Radicals Formed from α-Tocopherol under Oxidizing and Reducing Conditions. An EPR and ENDOR Study

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Oxidation of α-tocopherol with linoleic acid as hydrogen acceptor gave the neutral radical of the corresponding semiquinone in acetic acid. The hyperfine structure was well resolved. The neutral radical was also formed in methanol in the presence of sodium hydroxide and linoleic acid. The anion radical of the semiquinone appeared when the concentration of the sodium hydroxide was sufficiently high.

EPR and ENDOR spectra showed α-tocopherol to yield a single radical with two different proton couplings under the reducing conditions of a sodium−ammonia system at room temperature. At 193 K the EPR spectrum showed the spectral lines of 12 equivalent protons and the ENDOR spectrum only one proton coupling. The effect of temperature was reversible.

The splitting pattern of ^{13}C in natural abundance was also seen in the EPR spectrum. The 6 coupling constants of the 10 carbon atoms appeared in the satellite spectrum.

The oxidation of α-tocopherol has been studied earlier by EPR but resolution of the spectrum was poor.[1,10] Alkali metal reduction of the quinone of α-tocopherol has given the corresponding anion radical of the semiquinone.[2] The spin relaxation processes have been investigated by EPR and ENDOR spectra.[3] A comprehensive treatise on vitamin E has been edited by Machlin.[4]

α-Tocopherol (=RH₂)

$R_1 = CH_2 - (CH_2 - CH_2 - CH - CH_2)_3H$
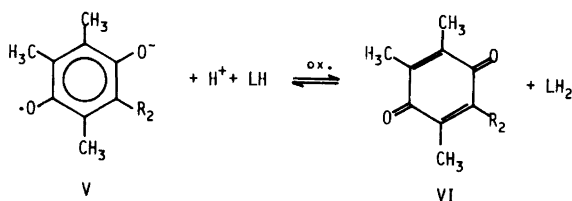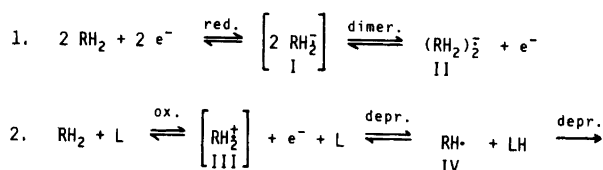$\qquad\qquad\qquad\qquad |$
$\qquad\qquad\qquad\quad CH_3$
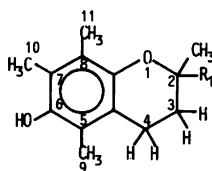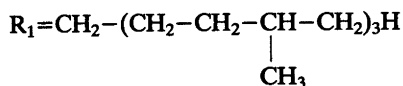
α-Tocopherol produces radicals under oxidizing conditions[4] and we have now extended the studies to cover also the reducing conditions. Two proton and two electron transfers take place in the oxidizing reactions as in the system hydroquinone→quinone. When a hydroquinone loses only one electron it becomes a semiquinone. In this study we used linoleic or oleic acid as the unsaturated compound needed as electron and proton acceptor.

$R_2 = CH_2 - CH = C(CH_3) - R_1$ and L is the hydrogen acceptor. I is the anion radical of the α-tocopherol, which dimerizes into II in liquid ammonia. III is the cation radical of the corresponding semiquinone, IV the neutral radical, V the anion radical of the semiquinone, and VI α-tocopherolquinone. I and III were not seen in this study. IV can polymerize in many different ways.[4]

EXPERIMENTAL

We employed Varian E-12 and E-9 EPR spectrometers equipped with a Varian gaussmeter and a high frequency counter TR 5211 from Takeda Riken Industry Co. The equipment was connected to a computer programmed to

1. $2 RH_2 + 2 e^- \overset{red.}{\rightleftharpoons} \left[2 RH_2^{\bar{\cdot}} \atop I \right] \overset{dimer.}{\rightleftharpoons} (RH_2)_2^{\bar{\cdot}} + e^-$
    $\phantom{1.\quad 2 RH_2 + 2 e^- \overset{red.}{\rightleftharpoons} \left[2 RH_2 \right]} II$

2. $RH_2 + L \overset{ox.}{\rightleftharpoons} \left[RH_2^{\dagger} \atop III \right] + e^- + L \overset{depr.}{\rightleftharpoons} RH\cdot + LH \overset{depr.}{\longrightarrow}$
    $\phantom{2.\quad RH_2 + L \overset{ox.}{\rightleftharpoons} \left[RH_2 \right] + e^- + L \overset{depr.}{\rightleftharpoons} } IV$

$$V \qquad\qquad + H^+ + LH \overset{ox.}{\rightleftharpoons} \qquad\qquad VI \qquad + LH_2$$

calibrate the changes in high frequency during the measurements. The computer prints out the *g*-values and intensities, and lists the values of the coupling constants in order of magnitude. The coupling constant measured in the different parts of the spectrum differed by less than ±0.01 G. The position of the sample relative to the probe of the gaussmeter proved critical and the sample was carefully adjusted for each measurement. As standards in the *g*-value measurements we used the anion radicals of pyrene, naphtacene and perylene.[5,6] The reproducibility was good to six decimals. Second order corrections were not used. The ENDOR spectra were measured with a Varian E-12 spectrometer equipped with a Bruker Physik ENDOR system B-EN200S. The variable temperature regulators from Varian were controlled with a digital thermometer. The INDO calculations were carried out on a UNIVAC 1100/60 computer.

DL-*a*-Tocopherol for biochemical use was a product of Merck. IR, NMR and UV spectra were identical with spectra in the literature.[4,7,8] The linoleic acid was from Fluka AG (*puriss*) and the oleic acid from Merck. The samples were prepared in nitrogen atmosphere and the high vacuum samples were prepared as before.[9] The ammonia was a Merck product of 99.9 % purity. It was distilled into the ampoule under nitrogen atmosphere.

RESULTS AND DISCUSSION

In ethanol with dissolved oxygen, *a*-tocopherol gave a poorly resolved EPR-spectrum. When the sample was illuminated with a UV-lamp, the radical concentration increased slightly. At room temperature the radical disappeared within half an hour. The lifetime of the radical in the corresponding high vacuum sample was clearly longer, but the radical formation was more difficult.

When linoleic acid was added to a sample of *a*-tocopherol in ethanol with dissolved oxygen, the resolution of the EPR spectrum clearly improved and the hyperfine structure appeared. The radical concentration at room temperature as a function of time seemed to decrease in two steps and the signal disappeared entirely in five hours.

In an attempt to increase the lifetime of IV, methanol, carbon tetrachloride and acetic acid were used instead of ethanol in the above system. In all these solvents the hyperfine structure of the EPR spectra was well resolved. Signal intensity was highest in methanol but the signal disappeared in four hours at room temperature. The resolution was poor when the temperature de-

Table 1. The coupling constants (G) of the neutral radical of *a*-tocopherol (IV), linoleic acid as hydrogen acceptor, 295 K, in different solvents.
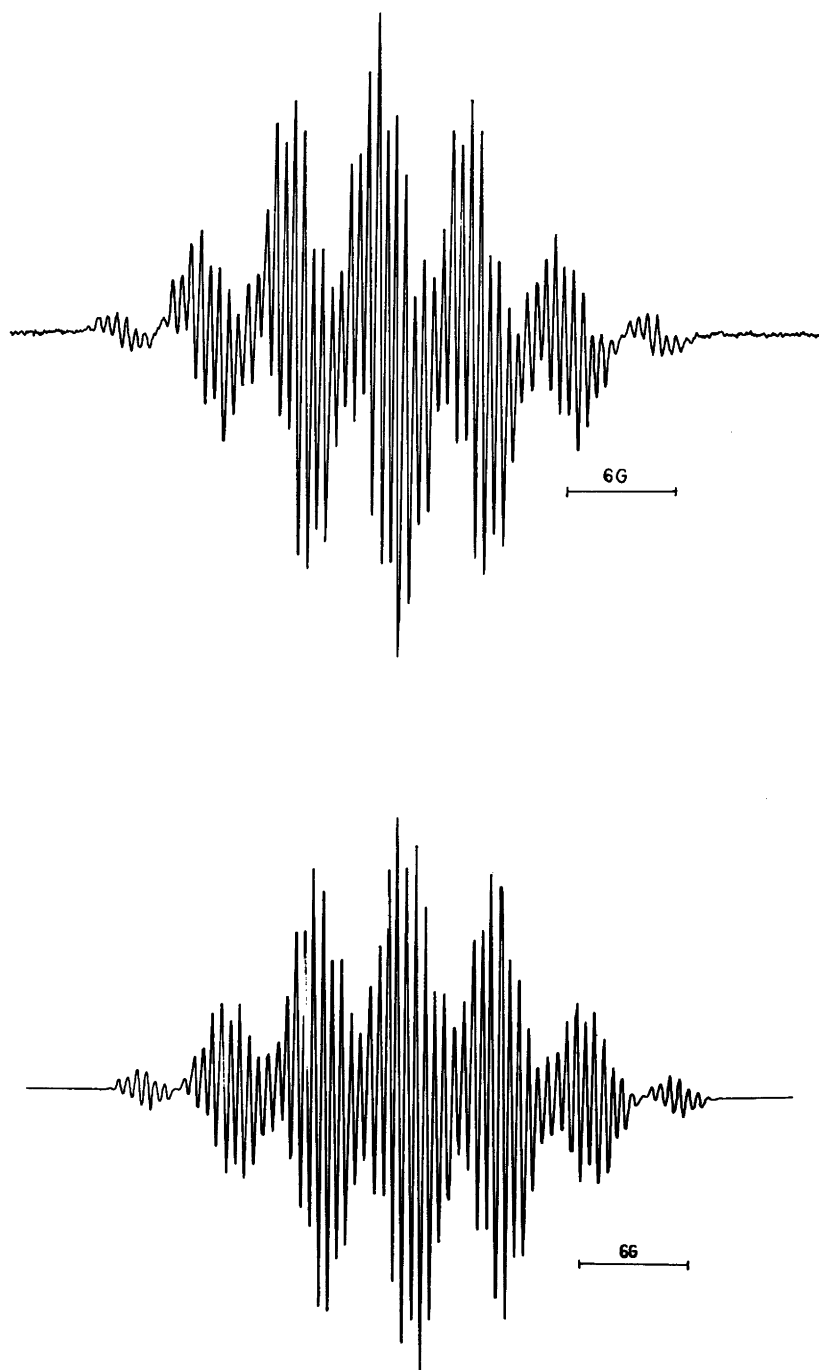
	CH_3COOH	CCl_4	CH_3OH	CH_3CH_2OH	CH_3COCH_3[10]	Indo A	Indo B
$-CH_3(11)$	0.54	0.75	0.57	0.65	0.90	−2.65	−2.69
$-CH_3(9)$	4.13	4.41	4.20	4.23	4.75	4.20	4.18
$-CH_3(10)$	5.63	5.94	5.72	5.79	5.75	4.34	4.38
$-CH_2(4)$	0.98	1.39	1.13	1.09	0.90	−3.48	−3.75

Fig. 1. The EPR spectrum of the neutral radical of α-tocopherol semiquinone (IV) in acetic acid, with linoleic acid as the proton acceptor, at room temperature. (a) Measured spectrum, (b) simulated spectrum.
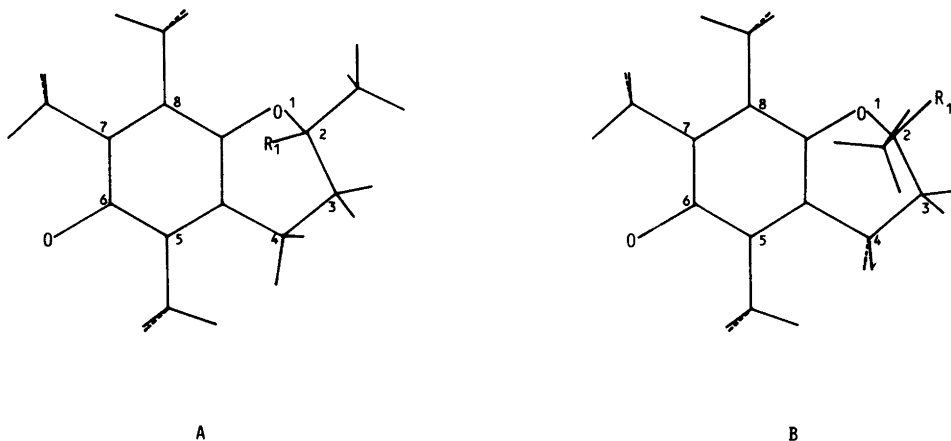
A   B

Fig. 2. The two projected conformations of the α-tocopherol structure.

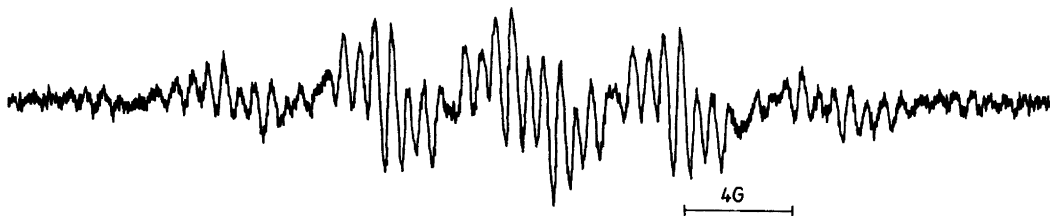*Fig. 3a.* The EPR spectrum of the neutral radical of α-tocopherol (IV) in weak alkaline methanol solution at room temperature with linoleic acid as proton acceptor.
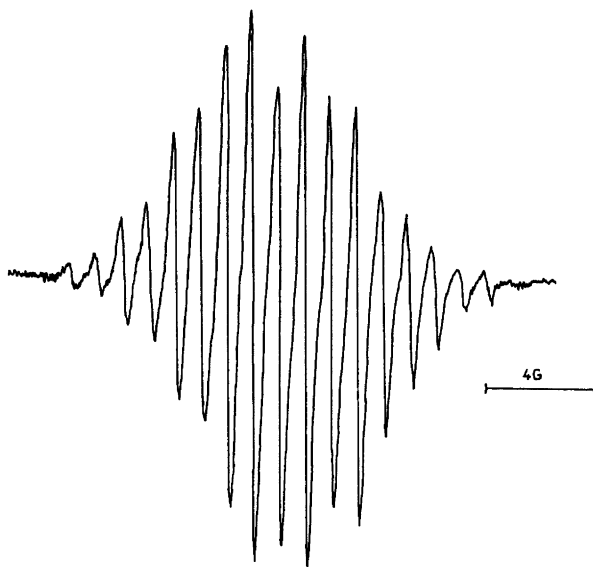

Fig. 3b. The EPR spectrum of the anion radical of the semiquinone (V) in strong alkaline methanol solution at room temperature with linoleic acid as proton acceptor.
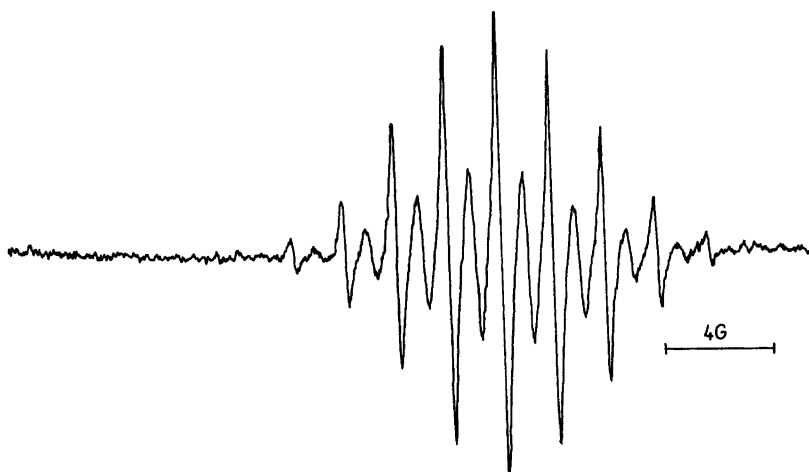
Fig. 4. The EPR spectrum of the anion radical of the α-tocopherol dimer (II) in the sodium−ammonia at room temperature.

creased to 268 K, and increase of the temperature above room temperature accelerated the disappearance of the signal. The best resolved spectrum of IV was obtained in acetic acid (Fig. 1).

When the hydrogen acceptor was oleic acid instead of linoleic acid, the resolution of the EPR spectra was poorer, but the coupling constants were similar. When the solvent was trifluoroacetic acid the EPR spectra showed 7 broad peaks without hyperfine structure.

The coupling constants (Table 1) were somewhat solvent dependent and differed slightly from the values given in the literature.[10] The coupling constants were assigned with INDO calculations. Bond lengths and bond angles were given standard values.[11] Projections of the two conformations of α-tocopherol in the plane of the

aromatic ring are seen in Fig. 2. According to INDO calculations the conformation A is energetically a little more favourable than the conformation B. The spin densities of both conformations differ only slightly and in the side chains the spin densities are very small.

α-Tocopherol and linoleic acid in methanol with sodium hydroxide initially gave a poorly resolved spectrum of the neutral radical of the semiquinone (Fig. 3a). When the solution was saturated with sodium hydroxide and the sample was kept for several hours at room temperature, the spectrum of the anion radical of the semiquinone appeared (V, Fig. 3b). The coupling constants were $a(CH_2)=0.95$ G and $a(3CH_3)=1.90$ G, which agree with the values measured by other methods.[2] The semiquinone anion radical was stable for several days at room temperature

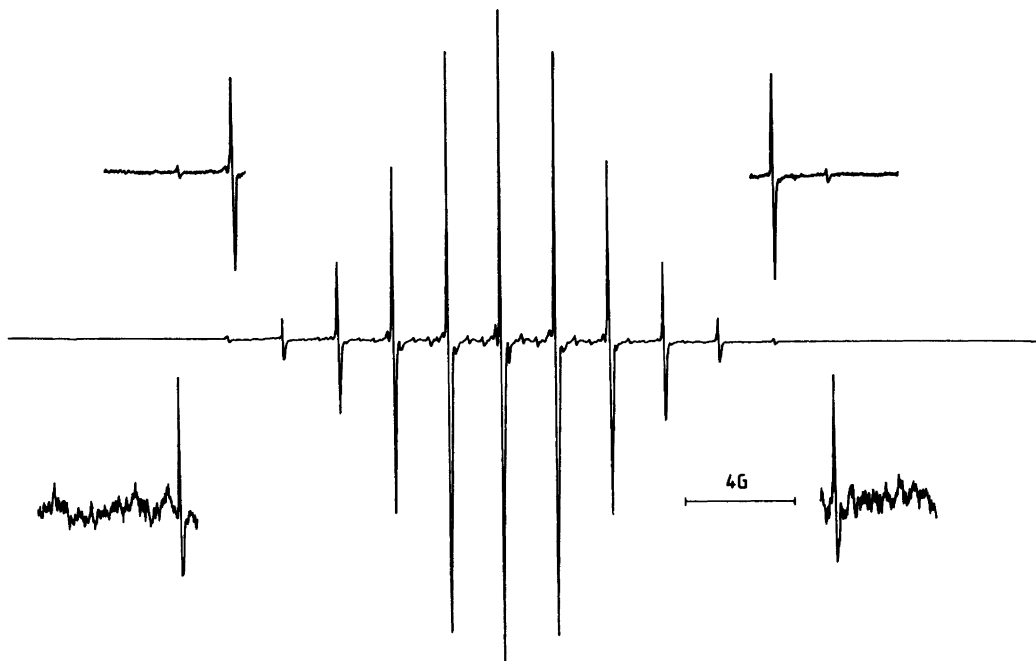*Fig. 5.* The ENDOR spectrum of the same sample as in Fig. 4.

Fig. 6. The EPR spectrum of the same sample as in Fig. 4, at 193 K.

and when the temperature was decreased the hyperfine structure of the EPR spectrum disappeared.

If the concentration of the natrium hydroxide was inadequate the neutral radical disappeared and the spectrum of the anion was not seen. Apparently the neutral radical also may polymerize without anion formation.[4]

Under the reducing conditions in high vacuum in sodium−ammonia without linoleic acid, at room temperature a-tocopherol gave the EPR spectrum seen in Fig. 4 and the ENDOR spectrum seen in Fig. 5. The ENDOR spectrum shows two proton couplings $a(CH_3)=1.94$ G and $a(CH_2)=0.95$ G.

The EPR and ENDOR spectra of the same sample but at 193 K are reproduced in Figs. 6 and 7. There is only one proton coupling $a=1.91$ G in the ENDOR spectrum. In the EPR spectrum in Fig. 6 there are 12 equivalent protons (13 lines). Table 2 shows the effect of temperature on the intensities of the lines. The intensities were
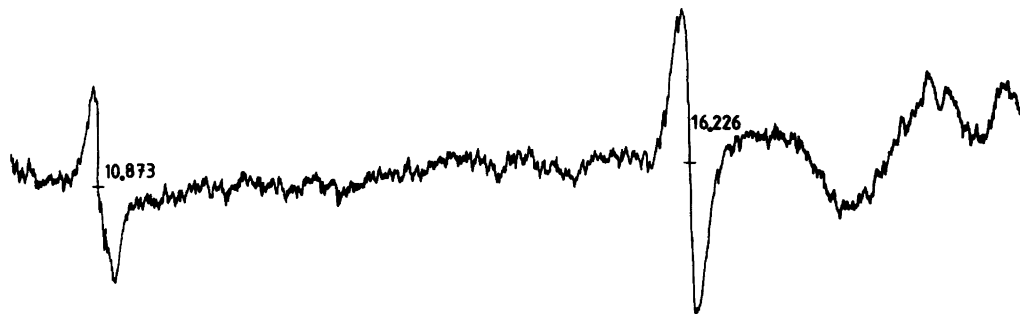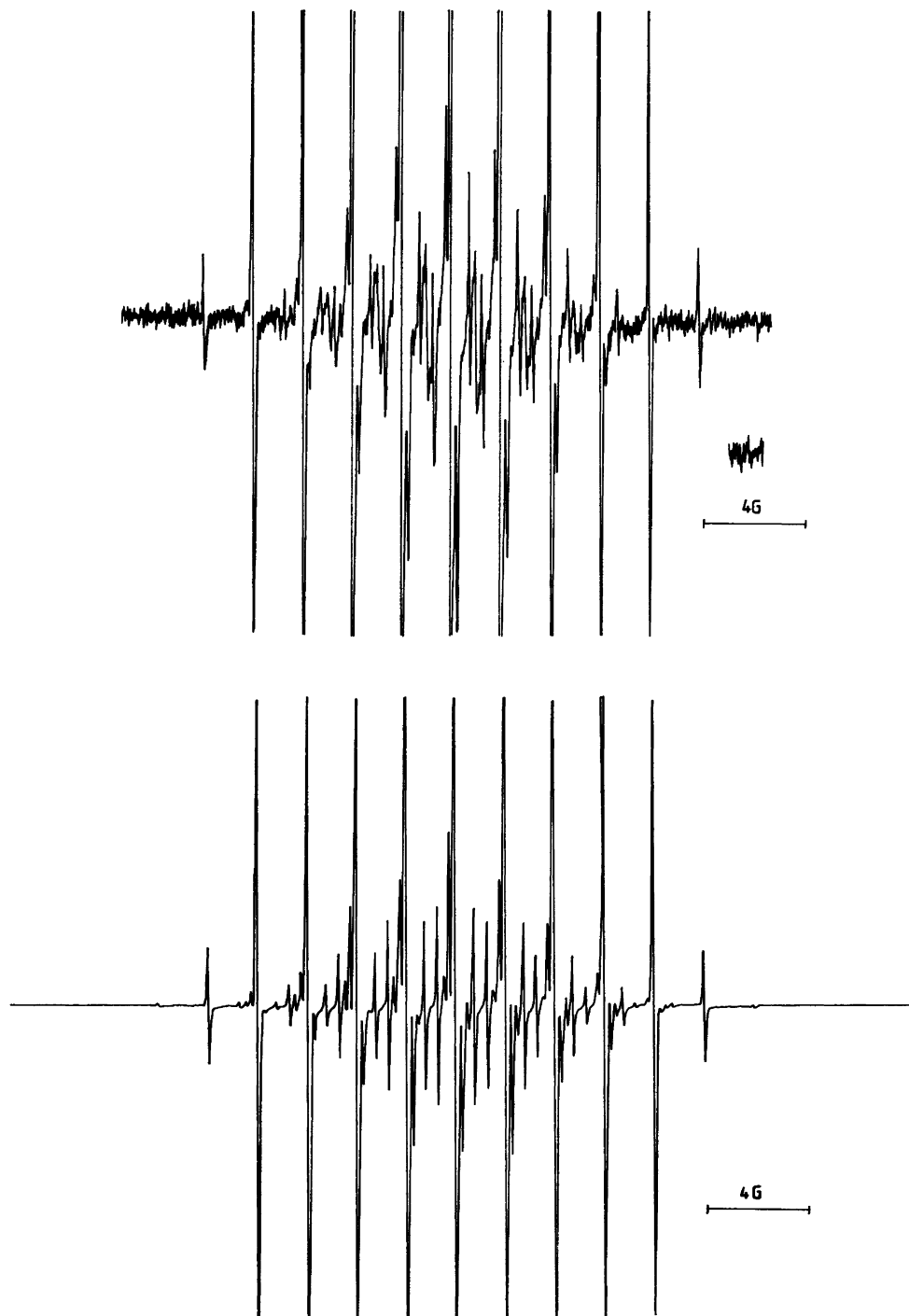

Fig. 7. The ENDOR spectrum of the same sample as in Fig. 4, at 193 K.

Fig. 8. The EPR ¹³C satellite spectrum of the anion radical of the α-tocopherol dimer (II) recorded at 193 K. (a) Recorded spectrum, (b) simulated spectrum.

Table 2. The effect of temperature on the relative intensities of EPR spectral lines of α-tocopherol in sodium−ammonia (II).

12 prot. calc. *I*/K		0.0011	0.013	0.071	0.238	0.536	0.857	1.00
295	g=2.00482 a=1.91 G a=0.95 G	–		0.069	0.226	0.531	0.845	1.00
253	g=2.00482 a=1.92 G	–		0.060	0.240	0.521	0.862	1.00
243	g=2.00482 a=1.92 G	–		0.070	0.243	0.530	0.860	1.00
233	g=2.00482 a=1.92 G	–		0.069	0.239	0.531	0.887	1.00
213	g=2.00481 a=1.92 G	0.009		0.065	0.236	0.531	0.856	1.00
193	g=2.00482 a=1.92 G	0.013		0.060	0.237	0.536	0.858	1.00

measured both using a computer and manually from spectra. The measured intensities agree well with the calculated intensities of 12 equivalent protons even at room temperature. Fig. 4 shows that between the main lines there appear new lines. According to the computer analysis the smaller coupling constant at room temperature is 0.95 G, the same as the value measured by ENDOR. The effect of temperature was reversible. The ENDOR measurements did not show the existence of two radicals, even when the magnetic field was locked to different lines.

The coupling constants (1.94 and 0.95 G) are similar in value to those of the anion radical of the semiquinone (V) (1.90 and 0.95 G). However, intensity analysis of the EPR spectrum and the presence of the 12 equivalent protons clearly prove that they are due to different radicals. Indeed it would be hard to believe that the reduction and oxidation reactions followed by ring opening could lead to the same results.

From the structure of the α-tocopherol it can be deduced that the anion radical of α-tocopherol (I) dimerizes to radical II. Otherwise it would be difficult to explain the 12 equivalent protons. Apparently the dimerization takes place at the hydroxyl groups. The 12 equivalent protons are then the methyl protons of the dimer in the positions 9, 10, 9', 10'. As the temperature increases toward room temperature, the dimer remains stable, but the protons in position 4 start to appear in the spectrum.

Fig. 8a shows the EPR satellite spectrum of the ^{13}C isotope in natural abundance of the anion radical of the α-tocopherol dimer and Fig. 8b the simulated spectrum. Coupling constants of at least ten carbon atoms can be observed. Such a high receiver gain was used in recording the spectrum that between the main lines one can also see the proton coupling of 0.95 G.

The peaks between the main lines become clearly visible near room temperature. The assignment of the methyl carbons (9, 10, 9', 10') and (5, 7, 5', 7') has been made by INDO calculations on I (Table 3). The INDO calcula-

Table 3. The ^{13}C coupling constants (G) of the satellite EPR spectrum of the anion radical dimer of α-tocopherol (II).

	Positions	INDO
a(4 carbons)=0.44 G	9,10,9',10'	−4.1
a(4 carbons)=1.40 G	5,7,5',7'	9.0
a(1 carbon) =4.82 G	6 or 6'	–
a(1 carbon) =4.40 G	6 or 6'	–

tions on the monomer cannot be valid for the largest coupling constant if the dimer is formed through hydroxyl groups, as proposed. The symmetry of the dimer in positions 6,6' is not complete.

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