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ELECTRON SPIN RESONANCE STUDIES OF FREE RADICALS DERIVED FROM PLASTOQUINONE, α- AND γ-TOCOPHEROL AND THEIR RELATION TO FREE RADICALS OBSERVED IN PHOTOSYNTHETIC MATERIALS

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SUMMARY

Speculation has centered on the semiquinone of plastoquinone as the source of the light induced, broad, structured, slow-decaying free radical (Signal II) generated by O_2-evolving photosynthetic systems. However, neither solution nor low-temperature spectra of plastosemiquinone, which appeared in the presence of KOH, showed substantial asymmetry, while Signal II is characteristically asymmetric. Two types of radicals were observed when α-tocopherol was treated with KOH. One of these radicals was identified as a semiquinone; another free radical was observed at a high power. At 77° K this radical and ones generated from α- and γ-tocopherol by ultraviolet irradiation gave lines which closely mimicked Signal II. We propose that these radicals are of the alkoxy-substituted phenoxy type, *i.e.*, that they are chromanoxyl radicals. The consequences of the presence of these radicals for our present conception of photosynthesis is discussed, and it is proposed that Signal II is predominantly due to plastochromanoxyl free radical.

INTRODUCTION

Free radicals generated by the action of visible light on photosynthetic systems have been observed for many years. Very early work established that two signals can be clearly distinguished[1]. Signal I ($g = 2.0025$) is approx. 7.5 gauss wide without hyperfine structure and decays very quickly in the dark. Signal II ($g = 2.0046$) is about 19 gauss wide, reveals partially resolved hyperfine structure and does not decay rapidly in the dark. Speculation, which has centered on plastoquinone (Fig. 1) as the parent molecule of the radical, usually equates the radical itself with the semiquinone (see, for example ref. 2). It should be noted, however, that the ESR spectrum of Signal II is markedly asymmetric (see Fig. 2) and that the case for plastosemiquinone would be more convincing if plastosemiquinone radicals could be made to show such an asymmetry. Alternatively, if the molecule is in the chromanol configuration, as a result of the intramolecular reaction of the quinol with the double bond in the first isoprenoid unit, then the radical (plastochromanoxyl) produced by the loss of one electron from this plastochromanol would have ESR spectral properties completely different from the semiquinone (for typical chromanol, see tocopherols in Fig. 1). This report characterizes both the semiquinone- and chromanoxyl-type free radicals and compares them with Signal II from photosynthetic preparations.

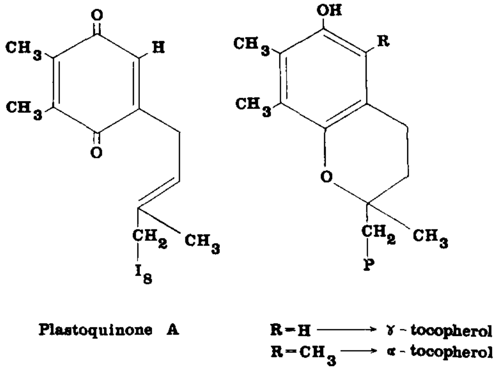
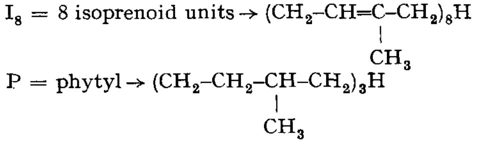


Fig. 1. Plastoquinone, α - and γ-tocopherol.



$$I_8 = 8 \text{ isoprenoid units} \rightarrow (CH_2\text{–}CH\text{=}\underset{\displaystyle CH_3}{\underset{|}{C}}\text{–}CH_2)_8H$$

$$P = \text{phytyl} \rightarrow (CH_2\text{–}CH_2\text{–}\underset{\displaystyle CH_3}{\underset{|}{CH}}\text{–}CH_2)_3H$$

Note that γ-tocopherol is a reduced phytyl analogue of plastoquinone. The chromanol configuration in which the tocopherols are drawn are isoelectronic with a quinol. Tocopherol quinone is formed by the oxidation of tocopherol with the resulting opening of the chromanol ring to form a compound analogous to plastoquinone. However, oxidation of the tocopherol would result in the compound with water added across the double bond to give the γ-hydroxy compound[15].

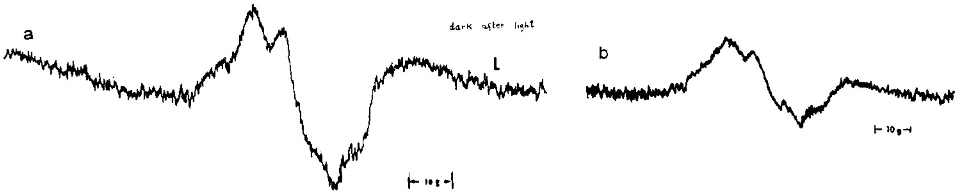


Fig. 2. ESR Signal II is generated by O_2-evolving photosynthetic organisms or appropriate fractions of these organisms. Signal II is most easily observed in the dark seconds after illumination. Signal II spectra from two widely different sources and two laboratories are shown to emphasize that the shape of the line is an intrinsic property of Signal II: a. From tobacco chloroplasts first lyophilized and then resuspended in water. The apparent variable base line is due to a signal from Mn^{2+}. Modulation amplitude = 3.0 gauss. b. From living *Chlamydomonas reinhardti* (after WEAVER[7]).

METHODS AND MATERIALS

Plastoquinone was prepared by extracting lyophilized spinach chloroplasts with a mixture of equal volumes of chloroform, heptane and methanol and by purifying it using ascending thin-layer chromatography on silica gel GHR-uv/254 (Macherey, Nagel and Co.) with 50 % heptane in chloroform as the developing solvent. The silica gel containing the plastoquinone fraction, visualized under ultraviolet light as a dark band migrating to about 2/3–3/4 the distance of the solvent front, was scraped off the chromatogram and was washed with spectral grade ethanol to elute the plastoquinone.

If necessary, this material was subjected to rechromatography. The characteristic ultraviolet spectra of the oxidized and reduced forms[3] and the presence of a single band on the thin-layer plate were taken as criteria for the purity of the compound. For the purposes of this work, no distinction is made between the various plastoquinones (*i.e.*, A, B, C, *etc.*), since this difference would not be expected to influence the ESR spectra.

A chip of KOH was placed at the bottom of a spectrosil quartz tube (2-mm internal diameter), and about 0.1 ml of plastoquinone in ethanol (approx. 0.2 μmole) was then added. A purple band approx. 2 mm deep formed at the bottom of the tube and moved slowly up the tube. In the presence of concentrated KOH, the purple color faded in approx. 2 min. When tocopherols instead of plastoquinone were used, the solvent was acetone and the visible color was yellow. In addition, a band of a slightly different yellow color formed at the air–solvent interface.

Since quinones are known to participate in a complex series of reactions in basic solution, including essentially irreversible changes, radical initiation with base is clearly not the method of choice. However, this method does have the advantage requiring very little material and is reported in the literature[4]. Radicals of α- and γ-tocopherol were also produced by ultraviolet irradiation at 77° K.

α-Tocopherol was obtained from the Sigma Chemical Co. Essentially the same results were achieved in experiments using the commercial preparation compared to experiments in which material was further purified using thin-layer chromatography, except as noted in the text. γ-Tocopherol of high purity was obtained from Distillation Products Industries. Further purification did not influence the experimental results.

Spectra were recorded on a Varian E-3 x-band spectrometer. The effective resolution of spectral lines, imposed by the 100 kcycles/sec modulation frequency, was about 70 mgauss.

RESULTS

Fig. 3 shows a high resolution spectrum generated by plastoquinone in ethanol treated with KOH. A computed spectrum using the coupling constants shown in Fig. 3, together with appropriate adjustments[5], closely fits the experimental spectrum. The

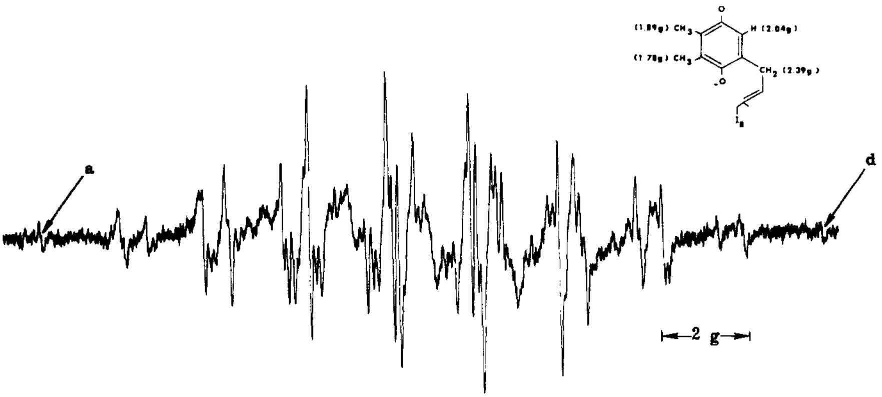


Fig. 3. Plastosemiquinone. Plastoquinone in ethanol treated with KOH. The distance between the extreme lines a–d is 17.7 gauss. Modulation amplitude = 63 mgauss. T = ambient. Microwave power = 1.40 mW. Coupling constants used to reproduce this spectrum by computer[5] are shown.

assignment of the splittings to the specific methyl groups is suggested by both the McLachlan self-consistent field and the Hückel molecular orbital calculations[5]. The details of the interpretation of this and related spectra are the subject of a separate paper. The splittings are all consistent with other data on semiquinones, including particularly those for methyl semiquinones[6]. All of these data tend to support the identification of the radical as plastosemiquinone even though it is a different spectrum from that which WEAVER[7] identified as plastosemiquinone and also from that which CHUMAKOV *et al.*[4] identified as that radical.

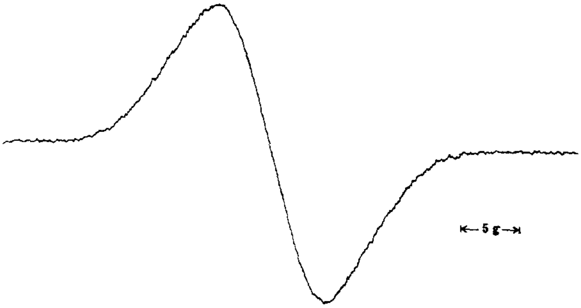


Fig. 4. Plastosemiquinone at 77° K. The same sample used to generate Fig. 3 was placed in liquid N_2 and the above spectrum recorded. Modulation amplitude = 1.0 gauss. Microwave power = 1.40 mW.

This spectrum in no way resembles that of Signal II. This is not surprising since the tumbling in solution, as expected, averages out the anisotropic contributions[8]. The fact that some of the plastoquinone in chloroplasts tenaciously resists extraction with heptane indicates that at least some of it must be immobilized. The parent molecules of Signal II must also be immobilized in order to produce the observed line which we assume to be anisotropic. Plastosemiquinone frozen into a random glass and observed at 77° K would be at least as immobilized as plastoquinone *in vivo*. A spectrum of plastosemiquinone at 77° K is seen in Fig. 4. Although the line is broadened to about twice its original width, as would be excepted from the anistropic contribution of proton hyperfine structure to the line width, it is still essentially symmetric.

Work was undertaken with α-tocopherol because it was readily available and because of the similarity in molecular structure, as compared with plastoquinone. The tocopherols are closely related to plastoquinol (see Fig. 1), ordinarily occurring in the fused ring chromanol configuration rather than as the more symmetrical quinol and having a phytyl side chain in place of isoprenoid units. γ-Tocopherol is the exact phytyl analogue of plastochromanol, while α-tocopherol has a methyl substituent at

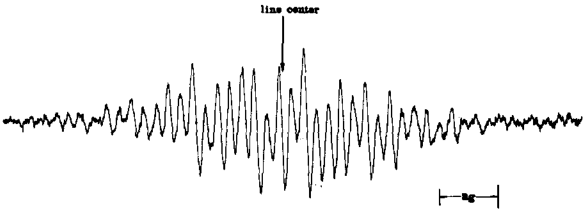


Fig. 5. α-Tocopherylsemiquinone. α-Tocopherol in acetone treated with KOH. T = ambient. Modulation amplitude = 80 mgauss. Microwave power = 1.25 mW.

C-6. γ-Tocopherol is susceptible to nucleophilic attack at C-6, and for this reason α-tocopherol was most often used. A radical generated by treating α-tocopherol in acetone with KOH is seen in Fig. 5. This spectrum is reproduced by a computer-generated spectrum in which the coupling constants are C-2 (methyl) = 2.10 gauss, C-3 (methyl) = 2.10 gauss, C-5 (methylene) = 0.85 gauss, C-6 (methyl) = 1.68 gauss (ref. 5). These coupling constants are very close to those observed by FRITSCH *et al.*[9] who produced the radicals electrolytically. There is no doubt that this spectrum is predominantly that of α-tocopherylsemiquinone. The fact that the same result was obtained when radicals were produced by the more selective electrolytic method makes one confident that the initiation employing KOH often used in this study was appropriate for its purpose. Like the plastosemiquinone, this spectrum broadened in a random glass (Fig. 6) but did not show any of the distinctive asymmetry of Signal II.

α-Tocopherylsemiquinone was saturated at very low microwave power, but as the power was increased another spectrum appeared. The resulting free radical is seen in Fig. 7. We propose that this is the spectrum of a "tocopheroxyl" radical, a member of the class of 4-alkoxyphenoxy radicals. The seven lines observed are from the six approximately equivalent C-2 and C-6 methyl protons, while each of these lines is

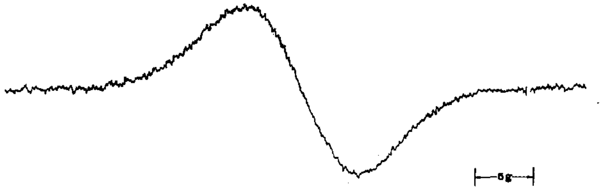


Fig. 6. α-Tocopherylsemiquinone at 77° K. Modulation amplitude = 1.0 gauss. Microwave power = 1.0 mW.

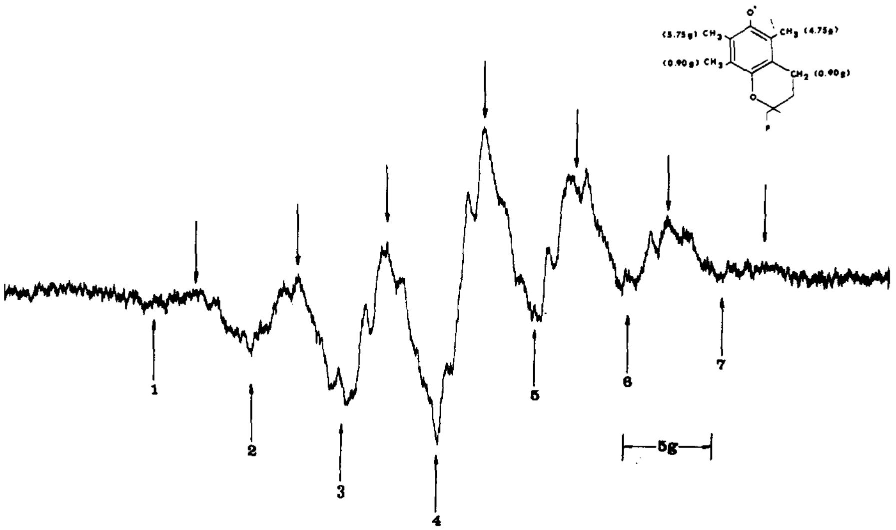


Fig. 7. α-Tocopheroxyl free radical. This spectrum was observed to grow out of the spectrum of α-tocopherylsemiquinone seen in Fig. 5 as the microwave power was increased and the semiquinone was power saturated. Microwave power = 320 mW. Coupling constants used to reproduce this spectrum by computer[5] are shown.

split by the C-3 methyl and C-5 methylene protons. The splittings are of the same order as those observed in substituted phenoxy radicals, namely, approx. 5 gauss for the *o*-substituents and approx. 1 gauss for the *m*-substituents. The spectrum is reproduced by a computer-generated spectrum in which the coupling constants are those indicated in Fig. 7 (ref. 5).

The result seen in Fig. 8 was obtained when this radical was observed at 77° K in a random glass. This spectrum is remarkably like Signal II. The principal difference in the spectra of Fig. 8 *vs.* 2 is the relative height of the peak at field position Z. However, the effect of replacing the methyl at C-6 with a proton—as would be the case if the parent molecule were related to plastochromanol or γ-tocopherol rather than α-tocopherol—would be to reduce the height of the peak at Z.

The identification of this radical as a tocopheroxyl radical is further supported by experiments in which α-tocopherol was frozen into a random glass at 77° K and then briefly subjected to ultraviolet irradiation. The resulting spectra include more than one radical, but when the sample was warmed briefly in acetone–dry ice and then returned to liquid N_2, the spectrum seen in Fig. 9 was observed. This "annealing" procedure worked far better with the crude α-tocopherol than with the highly purified material. We assume that in the latter case only the tocopherol radical was available to accept electrons from the solvent radicals.

This spectrum has the same spacing between hyperfine peaks as that seen in Fig. 8 which we identified as the α-tocopheroxyl radical. The relative amplitudes of the low-field hyperfine components varied somewhat from experiment to experiment. Part of the variation may be the result of using both a crude and a purified preparation of α-tocopherol. However, no systematic investigation of these differences has been undertaken.

γ-Tocopherol treated in the same way produced a spectrum with many of the same characteristics only here the lowest field peak, designated Z in Fig. 10, tended

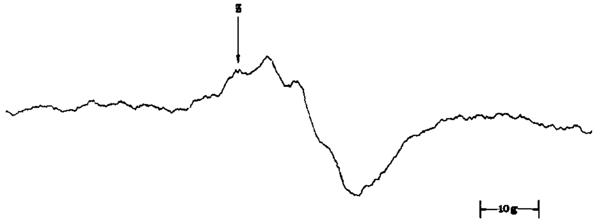


Fig. 8. α-Tocopheroxyl at 77° K. Modulation amplitude = 3.2 gauss. This high modulation amplitude was used since most photosynthetic samples, such as seen in Fig. 2a, were recorded at that value. Microwave power = 2.0 mW.

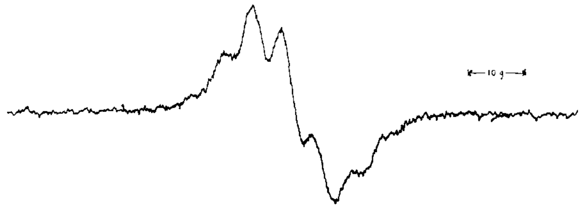


Fig. 9. α-Tocopherol in ethanolic glass (77° K) irradiated with ultraviolet light. This spectrum was recorded at 77° K after the sample was briefly warmed in an acetone–dry ice bath. Modulation amplitude = 1.0 gauss. Microwave power = 1.25 mW.

to be reduced in amplitude relative to the other low-fields peaks. This signal now even more closely mimics Signal II. It is important to note that the same ESR signal would be observed from plastochromanoxyl (that is, from a radical in which the parent compound is in the fused ring plastochromanol rather than in the plastoquinone or plastoquinol configuration), since the only difference between γ-tocopherol and plastochromanol is in the side chain where it would not be reflected in the ESR signal.

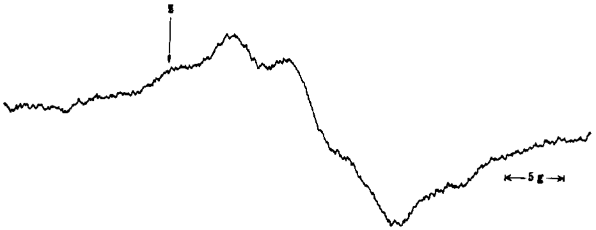


Fig. 10. γ-Tocopherol in ethanolic glass (77° K) irradiated with ultraviolet light. Conditions as in Fig. 9 except modulation amplitude = 3.2 gauss.

The nonspecific nature of the base-catalyzed radical initiation of plastoquinone and the tocopherols makes it necessary to confirm the proposed identifications. A particularly troublesome observation, not dealt with in detail here, was the appearance of a radical in solutions of α-tocopherol in acetone–KOH before the semiquinone developed. Since this radical may reflect events taking place on the surface of the KOH it is particularly important that experiments be done in which the radicals are generated electrolytically and with other oxidizing agents such as PbO_2. The degree to which the peak at field position Z is reduced in γ- as compared to α-tocopheroxyl at 77° K also requires more detailed study.

DISCUSSION

That the slow decaying, light-induced ESR signal seen in O_2-evolving photosynthetic systems (Signal II) is due to plastosemiquinone is a speculation, seen in literature, based almost entirely on correlative evidence. However, the shape of the line produced by immobilized authentic plastosemiquinone radicals is inconsistent with that statement. The evidence presented here suggests that Signal II arises form a substituted phenoxy-type rather than from a semiquinone anion-type radical. We further propose that plastochromanoxyl radicals, rather than α- or γ-tocopheroxyl radicals, are the main contributors to Signal II. The data which favor this choice are the following.

(a) When organisms are cultured over many generations in a totally deuterated medium and their ESR response is observed, both Signals I and II are seen to be markedly narrowed. The half-width of Signal II changes from about 19 to 6 gauss (ref. 10). When lyophilized chloroplasts are extracted with heptane and deuterated plastoquinone is added back, the narrow Signal II generated by fully deuterated organisms is observed, indicating that the unpaired electrons are interacting with deuterons which are present only in plastoquinone or in a molecule directly derived from it[11].

(b) The fact that γ-tocopherol is undetectable in Scenedesmus down to the level of 1 per 2500 chlorophyll (N.I. BISHOP, private communication) and yet gives a normal Signal II is direct evidence that γ-tocopherol radicals cannot be the only contributor to Signal II. Since the signal amplitude is not unduly small in Scenedesmus, it is suggested that γ-tocopherol is not a major contributor to Signal II.

(c) The tentative conclusion that the peak at field position Z in spectra of α-tocopheroxyl free radical at 77° K (Figs. 8 and 9) is elevated compared with Signal II while that of γ-tocopher oxyl (and hence presumably plastochromanoxyl as well) is not, suggests that α-tocopheroxyl is not a dominant contributor to Signal II. Additional experiments are planned in which protonated α-tocopherol will be added back to heptane-extracted chloroplasts prepared from deuterated organisms in order to directly assess the possible contribution of this radical to Signal II.

The participation of plastochromanoxyl free radicals in the photosynthetic electron transport provides a new link between the transport chain and other chemical reactions which are proceeding. The implication of plastochromanoxyl participation in photosynthetic electron transport renews interest in the suggestion that plastoquinone might be involved in photophosphorylation (see, for example, ref. 12). Experiments are in progress to test this hypothesis. This implication raises in addition the prospect of a cycle which must include the plastochromanoxyl free radical as well as plastoquinone and plastoquinol, the latter two being required in order to accommodate the observed increase in plastoquinol and decrease in plastoquinone which accompany the transition of spinach chloroplasts form dark to light[13]. It also provides a possible mechanism for connecting a carrier which is normally thought of as a two-electron carrier to the flow of single electrons[14]. However, again any such proposed mechanism must be consistent with the changes in plastoquinone and plastoquinol concentrations which accompany changes in illumination. In addition, any oxidation–reduction couple in which a plastochromanoxyl free radical participates may have a substantially different standard electrode potential from the quinone–semiquinone–quinol couples which are usually considered.

While we feel that the evidence points most strongly toward the interpretation given above, we cannot rule out the possibility that Signal II contains contributions from a neutral semiquinone, even though the conditions under which the spectrum in Fig. 7 was recorded seem to exclude the radical seen there being a neutral semiquinone. The point is that the difference between the neutral semiquinone and the proposed substituted phenoxy radical would be expected to be the splitting due to the phenolic proton of the former. This splitting would be expected to be of the order of 1–2 gauss, and its presence would have been detected in Fig. 7. However, its contribution might well be obscured when the molecule is immobilized (Fig. 8) or *in vivo*, especially since Signal II is usually observed when using about 3 gauss modulation amplitude. The neutral semiquinone as a contributor to Signal II cannot be ruled out on the basis of the pH of the chloroplast, since the reaction of interest is presumable taking place on a membrane as part of an electron transport system. If under these conditions the local concentration of protons is much higher than considerations of pH would suggest, then the neutral semiquinone may be formed.

NOTE ADDED IN PROOF

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Results of work in progress lend additional weight to the interpretation presented in this paper. (a) KBH_4 reduction of purified plastoquinone permitted higher resolution spectra to be recorded which were identical to the spectrum produced by KOH treatment (Fig. 3). (b) Slightly different coupling constants were recorded for α-tocopherylsemiquinone when the spectrum was produced by KBH_4 reduction of purified α-tocopherolquinone as compared to that produced by KOH treatment of α-tocopherol (Fig. 5). (c) Further experiments comparable to that seen in Fig. 7 support the identification of the free radical as α-tocopheroxyl but make it clear that the spectrum of Fig. 7 is partially power saturated. Thus, the coupling constants indicated in that figure should be taken only as first approximations.

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