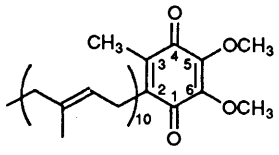
ENDOR and EPR Studies of Highly Isotopically ^{13}C-Enriched Ubiquinone Radicals

Rimma I. Samoilova,[a] Willem van Liemt,[b] Winand F. Steggerda,[b] Johan Lugtenburg,[b] Arnold J. Hoff,[c] Andrei P. Spoyalov,[a] Alexei M. Tyryshkin,[a] Nina P. Gritzan[a] and Yuri D. Tsvetkov[a]

[a] *Institute of Chemical Kinetics and Combustion, Academy of Sciences, Novosibirsk, 630090, Russia*
[b] *Department of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands*
[c] *Department of Biophysics, Leiden University, PO Box, 9504, 2300 RA Leiden, The Netherlands*

ENDOR and EPR measurements of anionic, neutral and diprotonated radical cations of ^{13}C-1-labelled ubiquinone 0† in solution are presented. The ^{13}C-carbonyl hyperfine coupling constant in protic solvents is roughly half of that in aprotic solvents. Preferred conformers of diprotonated ubiquinone and neutral radicals exist as a result of intramolecular H-bonding interactions. The variation of spin densities on the ubiquinone ring carbon atoms correlates with the calculated out-of-plane twist angles of the methoxy groups.

Quinones and dihydroquinones are interconvertible by two one-electron oxidation–reduction steps. They are very often involved in oxidation–reduction processes in the living cell in respiration and in photosynthesis. The reaction centres (RCs) of the photosynthetic bacteria *Rhodopseudomonas* (*Rps.*) *viridis* and *Rhodobacter* (*Rb.*) *sphaeroides* R-26 and 2.4.1 contain two quinones, Q_A and Q_B, which function as secondary and tertiary electron acceptors, respectively. *Rb sphaeroides* contains solely ubiquinone 10 with the structure shown below. In *Rps. viridis*



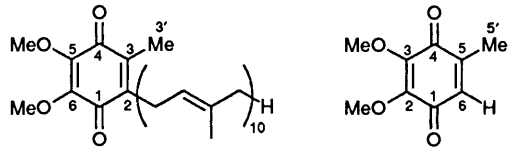
Q_A is a menaquinone and Q_B is ubiquinone 10. Since the radical anions of ubiquinone 10 play such a central role in photosynthesis, it is important to obtain *in-situ* structural information about its radical forms. With EPR and ENDOR spectroscopy information can be obtained on the spin density distribution of the unpaired electron by measuring the hyperfine coupling constants (hfc) of ^1H and natural-abundance ^{13}C. The hfc for ^{13}C especially is very sensitive even to small changes of the radical structure and/or its surroundings.[1]

Unfortunately, the natural abundance of ^{13}C (1.1%) is usually too low for its hfc interaction to be observed. Therefore, in this work we have investigated the radical species produced in some ubiquinone molecules that have been selectively enriched (99%) in ^{13}C. Our approach was, first, to investigate the isolated species in an organic solvent, as an appropriate model system, and then to compare the hfc data with those obtained from the same molecule in RCs with the natural, specific protein environment. We restricted our study to ubiquinone 0, the quinone core of ubiquinone 10 with no isoprenic side chain at C-6, on the assumption that the side chain plays a minor role in the reactivity of radical species.[2]

Experimental

Synthesis

[1-^{13}C]*Ubiquinone* 0.—[1-^{13}C]Ubiquinone 0 was prepared starting from 5 g commercial ethyl [1-^{13}C]bromoacetate (**8a**, Cambridge Isotope Laboratories, Woburn, Mass., USA), that was treated with the anion of diethyl methylmalonate in THF



1 **2**

Ubiquinone 10 (**1**) and ubiquinone 0 (**2**), structure and IUPAC numbering

(Scheme 1). Treatment of the product with aqueous HCl gave [4-^{13}C]methylsuccinic acid, which was subsequently converted into [4-^{13}C]methylsuccinic anhydride, **6a**.

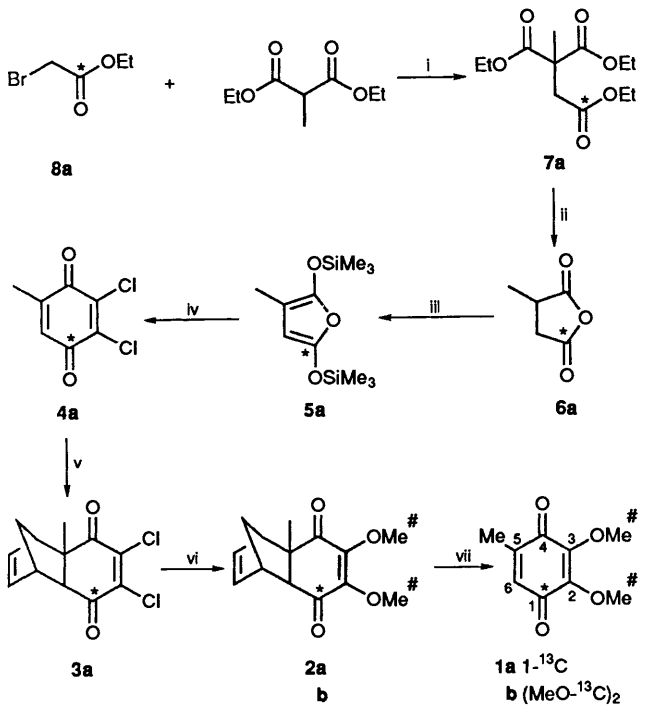
Treatment of **6a** with trimethylsilyl chloride gave [5-^{13}C]-2,5-bis(trimethylsilyloxy)-3-methylfuran, **5a**, that reacted with 1,1,2-trichloroethene to give [1-^{13}C]-2,3-dichloro-5-methylbenzoquinone, **4a**, which in turn reacted with cyclopentadiene to give the ^{13}C-labelled Diels–Alder adduct, **3a**.[3] Treatment of **3a** with sodium methoxide in methanol–toluene yielded the Diels–Alder adduct of [4-^{13}C]ubiquinone 0 and cyclopentadiene (**2a**). A retro Diels–Alder reaction of **2a**, in refluxing toluene, gave [1-^{13}C]ubiquinone 0, **1a** (17%, based on the ethyl [1-^{13}C]bromoacetate).

The 200 MHz ^1H NMR spectrum of **1a** was identical with that obtained for unlabelled ubiquinone 0,[3] apart from a small ^{13}C–^1H coupling of the labelled C-1 to H-6. The 50 MHz ^{13}C NMR spectrum of **1a** had an intense peak at 184 ppm for the 99% enriched C-1. The C-6 signal was a doublet, centred at 131 ppm, due to coupling with the ^{13}C-enriched C-1. These NMR spectra clearly establish C-1 as the only 99% ^{13}C-enriched position.

(MeO-$^{13}C)_2$-*Ubiquinone* 0.— Treatment of the Diels–Alder adduct of the 2,3-dichloro-5-methylbenzoquinone with 99% ^{13}C-enriched methanolate in 99% ^{13}C-enriched methanol gave the Diels–Alder adduct of 2,3-(di[^{13}C]methoxy)-ubiquinone 0 (**2b**). Retro Diels–Alder gave 2,3-(di[^{13}C]methoxy)ubiquinone 0 (**1b**) in 65% yield, based on **3**.

The 200 MHz ^1H NMR spectrum of **1b** showed a large splitting of the methoxy signals due to the large ^{13}C–^1H coupling (147 Hz). The 50 MHz ^{13}C NMR spectrum of **1b** showed an intense peak at 61 ppm for the 99% enriched di([^{13}C]methoxy) product, establishing clearly the position of the labelled carbons.

† 2,3-Dimethoxy-5-methylbenzoquinone.



Scheme 1 Synthesis of [1-^{13}C]ubiquinone and di([^{13}C]methoxy)-ubiquinone. *Reagents:* i, NaH; ii, 8% HCl–$(COCl)_2$; iii, NEt_3–Me_3SiCl; iv, Cl_2=CCl_2=MeOH; v, cyclopentadiene–MeOH; vi, MeONa–MeOH; vii, 120 °C.

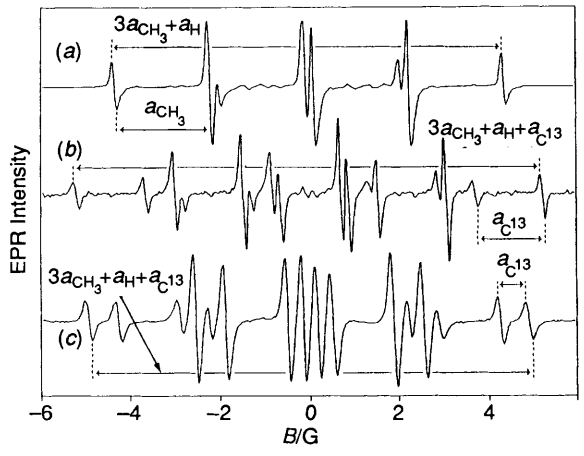


Fig. 1 EPR spectra of radical anions at room temperature: (*a*) ubiquinone in EtOH with KO_2; (*b*) [1-^{13}C]ubiquinone in HMPT with KO_2; (*c*) [1-^{13}C]ubiquinone in EtOH with KBH_4

Preparation of Radical Species.—A ubiquinone can accept one electron to form radical species that can occur in three states which differ in their degree of protonation. The structures of these radical species are shown in Scheme 2 (an asterisk indicates the labelled position).

The radical anion is stable in the absence of a proton. The diprotonated radical cation is stable in acidic solution. The third species (neutral radical) is very labile and can be obtained either by the fast-reaction technique or by light irradiation.

Ubiquinone 0 was converted into its anion by reduction in various solvents (protic, aprotic, aprotic dipolar) and with various reducing agents, in order to obtain reliable information about the hyperfine coupling constant of the ^{13}C probe. The radicals were generated in the usual way.[3–5] The reducing agents were potassium superoxide (KO_2), potassium borohydride (KBH_4), sodium dithionite ($Na_2S_2O_4$ = DTNa), all of the 'Chemically pure' grade, and the metallic liquid alloy K–Na

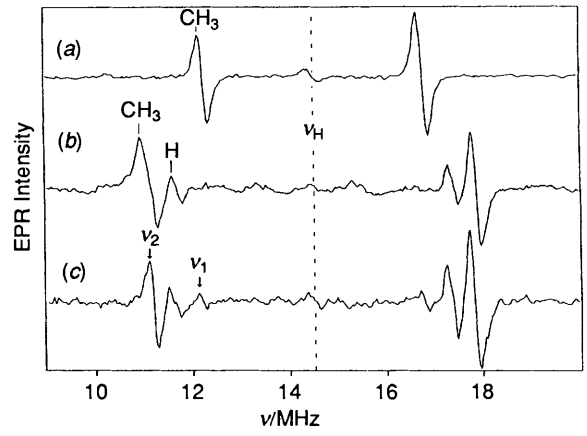


Fig. 2 ENDOR spectra of ubiquinone radical anions at room temperature in IPA with KO_2 taken with different field settings, indicated by arrows in the EPR spectra of Fig. 3(*c*): field setting H_3 (*a*), H_1 (*b*) and H_2 (*c*)

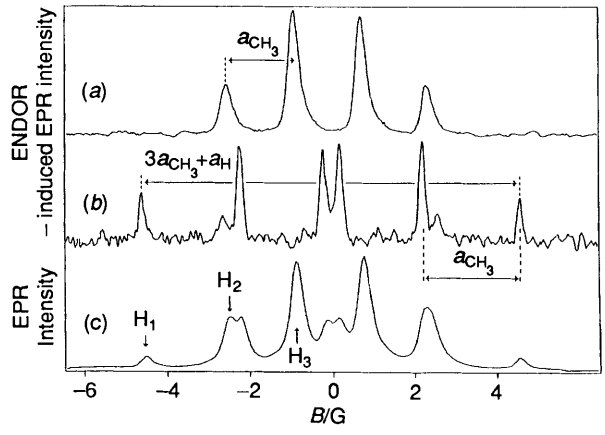
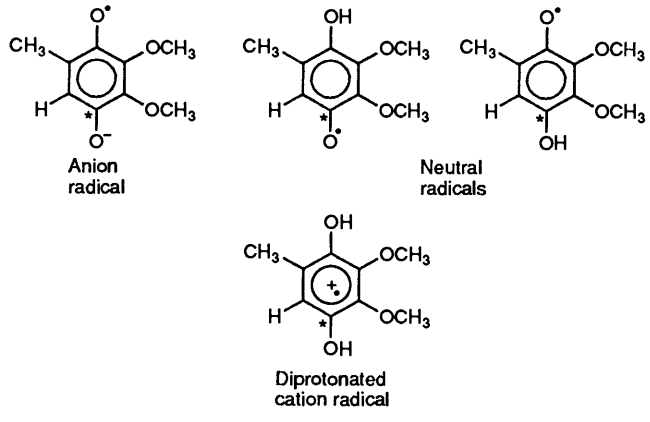


Fig. 3 EI EPR [(*a*), (*b*)] and integrated EPR (*c*) spectra of ubiquinone radical anions radical in IPA with KO_2. (*a*), (*b*) Correspond to the frequencies ν_1 and ν_2 in the ENDOR spectra [Fig. 2(*c*)].



Scheme 2

(30:70) obtained as described in ref. 6. The solvents were commercial materials: dimethyl sulfoxide (DMSO), dimethoxyethane (DME), hexamethylphosphoric triamide (HMPT), ethanol (EtOH), isopropyl alcohol (IPA), and were purified and dried by usual methods.

The diprotonated quinone radical cations were obtained by dissolving the quinones in degassed trifluoromethanesulfonic (triflic) acid at *ca.* 20 °C. Triflic acid was used without purification. This solvent has a high acidity and low nucleophilicity, which contributes to the stabilization of cationic species.[7] Samples were prepared using high-vacuum techniques for the degassing procedure.

Table 1 Isotropic hyperfine constants for the radical of [1-^{13}C]ubiquinone (∓0.05 G)

Splitting constant/G							Reducing agent	Solvent
Radical anion								
a_H^5		a_H^6	a_{C13}^1					
2.42		2.02	0.68				KBH_4	EtOH
2.40		2.05	0.63				DTNa	EtOH
2.42		2.02	0.68				KO_2	EtOH
2.25		2.25	1.33				KO_2	DMSO
2.20		2.20	1.26				Na/K	DME
2.18		2.31	1.53				KO_2	HMPT
2.16		2.28	1.68				NaBuO	HMPT
2.37		2.03[a]	0.68				KO_2	IPS
1.61		<0.05[b]	0.68					
Neutral radical								
a_H^5		a_H^6	a_{C13}^1	a_{OH}^7	a_{OH}^8		UV	EtOH
4.6		<0.15	<0.15	1.35	—			
<0.15		4.6	4.6	—	1.35			
Radical cation							—	Triflic acid
a_H^2	a_H^3	a_H^5	a_H^6	a_{C13}^1	a_{OH}	a_{OH}		
0.75	0.34	4.64	0.75	4.70	2.92	2.61		

[a] Primary anion. [b] Substituted anion.

The neutral, short-lived radicals were studied during photolysis of ubiquinone in alcoholic solutions, in the presence of acetic acid, as described earlier.[8] UV illumination of a quartz flat cell filled with degassed solution was provided by a high-pressure mercury lamp with $\lambda < 300$ nm, using a glass cut-off filter.

The EPR spectra were measured on a Bruker ESP-300 or a Varian E3 spectrometer, ENDOR and ENDOR-induced EPR (EI EPR) spectra were measured on a Bruker ESP-300.

Results and Discussion

Radical Anions.—The reaction of ubiquinone with KO_2 in EtOH, IPS, DME, DMSO and HMPT gave the ubiquinone anions. In addition, in alcohol and DME solutions the radical product of the alkoxylation reaction in position 6 was detected.[9] The yield of this reaction depended on the amount of water in solution, the temperature and the concentration of KO_2. The reaction did not proceed at low temperature ($T \lesssim -40$ °C), if water was present in the solution or at KO_2 concentrations $> 10^{-2}$ mol cm^{-3}.

Figs. 1–3 show some of the experimental EPR, ENDOR and EI EPR spectra of [1-^{13}C]- labelled and unlabelled ubiquinone. The EPR spectrum [Fig. 1(*a*)] of the unlabelled ubiquinone radical anion in EtOH shows a quartet of doublet splittings: the quartet is due to methyl protons with hfc ≈2.4 G and the doublet splitting to a single proton with an hfc of *ca.* 2 G. The hfc of the methoxy proton is small and does not manifest itself as a separate splitting.

In the EPR spectrum of the [1-^{13}C]-labelled ubiquinone anion [Fig. 1(*b*) in EtOH and Fig. 1(*c*) in HMPT], the additional doublet splitting from the ^{13}C-1 carbon atom was observed. As was first shown in ref. 10, the sign of the ^{13}C splitting can frequently be determined by observing the difference in the linewidths of the low and high-field lines of the ^{13}C doublet. Fig. 1(*c*) shows that in alcoholic solution, the high-field side of the ^{13}C doublet is broader than in that of the low-field side. In HMPT [Fig. 1(*b*)] and DMSO, however, the picture is different. These data indicate that the ^{13}C splitting changes from negative to positive upon changing the solvent.

ENDOR spectra of these samples exhibit pairs of lines centred about the free proton frequency (ν_H = 14.52 MHz), which belong to the different groups of quinone protons. The spectra shown in Fig. 2 belong to radical mixtures, and were obtained at room temperature in alcoholic solution with the magnetic field set on different components of the EPR spectrum, as shown by arrows in Fig. 3(*c*). For separating the mixed radical EPR spectra we made use of EI EPR spectroscopy.[11] This analytical method is extremely effective for analysing such mixed spectra, and has been applied by several authors.[12,13] For the measurement of the EI EPR spectrum proton ENDOR lines of different frequency were saturated. Setting ν_i in the position shown in Fig. 2(*c*), we recorded the EI EPR spectra of the individual radicals [Fig. 3(*a*), (*b*)].

The hyperfine coupling constants obtained from all experimental results are collected in Table 1. Since the anion often exhibits pronounced interactions with the counter ion, we also investigated the ubiquinone anion prepared with different reducing agents: KBH_4, $NaBH_4$, $Na_2S_2O_4$ and K–Na alloy, in the same solvent. These results are also summarized in Table 1.

Table 1 shows that $A(^{13}C)$ in protic solvents is about half that in aprotic solvents, and the effect of solvent variation on the spin-density distribution is of the same magnitude as the substituent effects. A detailed investigation of semiquinones in a series of solvents with decreasing solvation capability demonstrated that aprotic solvents (like HMPT) solvate the ions only weakly, whereas protic solvents can form hydrogen bonds to O=C groups.[14] Thus, in alcohols, the formation of hydrogen bonds to the solvent results in a lowering of $A(^{13}C\text{-}1)$ (Table 1). When HMPT is used as solvent, the ^{13}C-carbonyl splitting is the highest splitting observed, thus it is the most weakly solvated ion of those studied.

An attempt to observe the ENDOR response of the ^{13}C nucleus at position-1 was undertaken. As is well known, ^{13}C ENDOR studies, as a rule, involve even greater experimental difficulties than proton ENDOR. We hoped that by using ^{13}C-

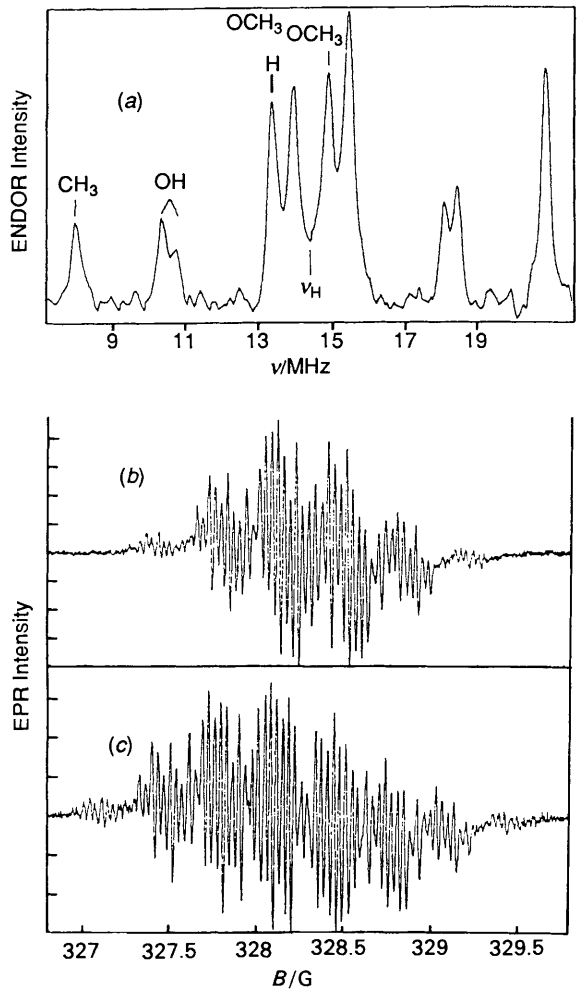


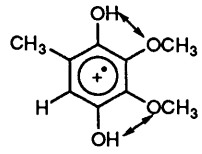
Fig. 4 ENDOR (*a*) and EPR [(*b*), (*c*)] spectra of the ubiquinone radical cation in triflic acid at room temperature: (*b*) unlabelled, (*c*) [1-^{13}C]ubiquinone

enriched samples we would overcome the difficulties associated with ENDOR studies of this nucleus. However, our attempts with [1-^{13}C]ubiquinone failed in all the systems studied. The likely reason for the lack of success of the ENDOR studies is the high anisotropic hfi value ($T_{21}^2 \approx 600$ MHz^2, as estimated from the spin-density distribution in the anion radical), resulting in a considerable shortening of the spin–lattice relaxation time of this particular carbon nucleus. To achieve optimal ENDOR absorption, the condition $T_{1e} \approx T_{1n}$ (where T_{1e} and T_{1n} are the electron and nuclear relaxation times, respectively), must be obeyed, *e.g.*, by changing the temperature or the solvent viscosity. We have tried several solvents and different temperatures, from 160 to 300 K, without success. Taking into account the results of ref. 11, one would expect to obtain an observable ENDOR signal at still higher temperatures (around 350–400 K). We could not work at these temperatures, however, since the ubiquinone radicals are unstable in this region.

When we prepared the radical anions from the ubiquinone with ^{13}C-labelled 5,6-methoxy groups we did not observe the splitting from ^{13}C. The linewidth in our EPR spectra was *ca.* 0.1 G, so we conclude that the splitting from ^{13}C in the 5,6 positions is < 0.1 G.

Radical Cations.—The ENDOR and EPR spectra of the quinone cations are shown in Fig. 4. The spectra were recorded immediately after preparation, because at room temperature the ubiquinone cations are stable for a few hours only. The primary radical cations undergo a slow chemical reaction with triflic acid, producing secondary radical cations with known structures.[15] The ENDOR lines in the region 9–19 MHz indicate the interaction of the unpaired electron with all the quinone proton groups. The proton couplings from the ENDOR spectra are assigned to different ring positions and to the proton nuclei of the hydroxy groups of ubiquinone (Table 1).

In principle, several conformers are possible for the ubiquinone cation.[16,17] However, according to our previous results,[18] for the ubiquinone radical cation the *cis* conformation of hydroxy groups is preferred. In addition to steric factors relating to the methyl group, this is probably due to the fact that the ubiquinone cation has two electron-donating methoxy groups and two proton-donating hydroxy groups. AM1 calculations showed (see below) that intramolecular H-bonding between the phenolic hydrogen and the methoxy oxygen leads to the preferred *cis* conformer of the ubiquinone cation shown below.



Usually, a correlation between the hfc in the radical cations and radical anions is observed. For many oxygen-containing aromatic radicals. the splittings of the cation are *ca.* 20% larger than the corresponding splittings in the anion.[19] Our results (Table 1), however, do not show this correlation. Most likely, this lack of correlation is due to differences in the geometry of the anion and cation substituents and, consequently, in their influence on the unpaired spin distribution within the quinone ring (see also the AM1 calculation below).

Neutral Radicals.—It is known[8] that neutral radicals can be detected during photolysis of quinones in alcoholic solution, in the presence of a small amount of acid. Fig. 5 shows the EPR spectra of radicals generated in this way. We assign the small doublet splitting in these spectra to the protons of the OH groups of the radicals since for radicals generated in deuteriated alcohol, this splitting is absent from the spectrum.

The assignment of the quartet splitting is more complicated. This quartet [Fig. 5 (*a*)] has an anomalous intensity distribution, which may change from experiment to experiment. We interpret this quartet as follows. Protonation of quinone oxygens attached to either C-1 or C-4 can lead to the formation of two or more radical conformers (see Table 2). The experimental data can be explained assuming that only two conformers, **IIa**$_1$ and **IIb**$_1$, contribute to the spectrum in Fig. 5(*a*). The absolute hfc value of the ring proton in **IIa**$_1$ [Fig. 5(*c*)] must be almost equal to the hfcs of the methyl protons in **IIb**$_1$ [Fig. 5(*d*)]. Thus the doublet splitting from the ring proton in **IIa**$_1$ overlaps the quartet splitting corresponding to the methyl protons of **IIb**$_1$. The overlap of the two signals perturbs the intensity correlation in the quartet spectrum [Fig. 5(*b*)]. The hfcs of the ring proton in **IIb**$_1$, the methyl protons in **IIa**$_1$, and the methoxy protons, are so small that they are not resolved and contribute only to the EPR linewidth. A computer simulation taking into account this spectral overlap has been performed [Fig. 5(*c*), (*d*)]; the resulting values of the hfcs of both conformers are presented in Table 1.

Additional confirmation for our interpretation of the anomalous intensity distribution of the quartet can be found in the spectra of ^{13}C-labelled ubiquinones. They show a more complicated hfc structure than the unlabelled case, but the full spectral width is the same. This behaviour can be understood if we assume that two different radicals contribute to the spectra. Then one of these radicals must have three equivalent protons

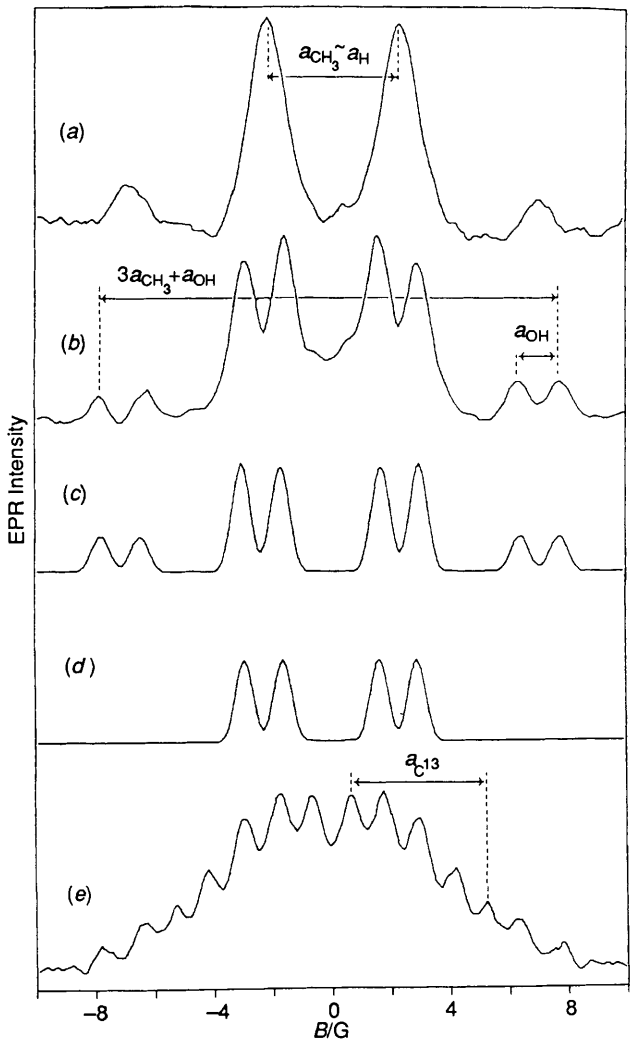


Fig. 5 Integrated EPR spectra observed during photolysis of ubiquinone solutions: (*a*) [$^2H_6$]EtOH with [$^2H_5$]acetic acid; (*b*) EtOH with acetic acid; (*e*) labelled [1-^{13}C]ubiquinone in EtOH with acetic acid. Computer simulations of spectra with the data from Table 1 and a linewidth of 0.05 mT for (*c*) conformer **11a**$_1$, (*d*) conformer **11b**$_1$.

with hfc ≈4.6 G, and a ^{13}C hfc of *ca.* 0 G, whereas the other has one proton with the same hfc of *ca.* 4.6 G, and a considerable ^{13}C hfc, *ca.* 4 G.

Calculations.—MO calculations of the radical anions and the neutral radicals are reported in refs. 19 and 20, but they are not accurate enough for an explanation of our experimental results. We have therefore carried out additional MO calculations, employing the AM1 technqiue[21,22] with the modified MNDO-85 program.[23] In the calculation, the standard Davidon–Fletcher–Powell procedure[24] has been used for the optimization of the geometry. The properties of open-shell systems were calculated by using a method similar to the 'half-electron' method.[25] The conventional AM1 (RHF) procedure was used to calculate the enthalpies of formation, geometries and spin density distributions for the anion, neutral and cation species. The structures obtained are shown in Fig. 6; the parameters are collected in Table 2. The bond lengths and angles in the anion, neutral and diprotonated cation radicals are almost identical, but the out-of-plane twist angles of the methoxy groups are different.

First, the calculations confirmed our experimental result that only conformers with an intramolecular hydrogen bond are present in the observed spectra. The enthalpies of formation for cation *cis* conformers and for conformers **11a**$_1$ and **IIb**$_1$ of the neutral radical are *ca.* 4 kcal mol^{-1} lower than the corresponding enthalpies of formation of cation *trans* conformers and of conformers **IIa**$_2$ and **IIb**$_2$. In the gas phase conformers with an intramolecular hydrogen bond were always the most stable.[26]

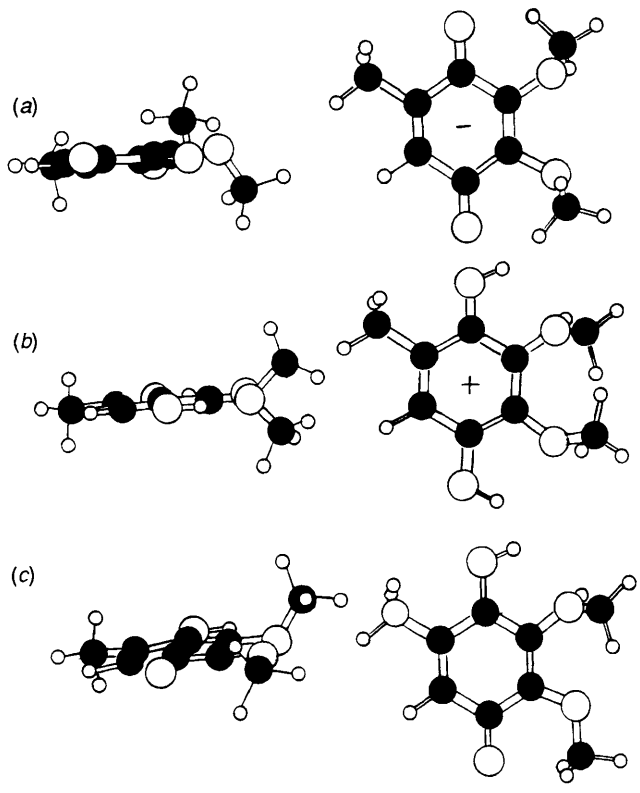


Fig. 6 Optimized geometries for the the ubiquinone radical anion (*a*), radical cation (*b*) and neutral radical (*c*) (conformer **IIa**$_1$)

Although the AM1 method is not particularly suitable for accurate calculation of spin-density distributions, some of the calculated results help us, nevertheless, to explain the experimental observations. In the radical anion, the calculated spin density on the ^{13}C-5 and ^{13}C-6 carbon atoms is almost equal. This agrees with the experimentally observed equality of hfcs for the proton in position 6 and the methyl protons in position 5. In contrast, for the radical cations these values are rather different, in full agreement with the calculations. We have also performed calculations for a quinone with a symmetrical structure (**IV** in Table 2), in which the methyl group is replaced by a ring proton. In this case, the spin densities in positions 5 and 6 are equal, and this change is accompanied by a change in the twist angle of one of the methoxy groups. Therefore, the difference in the hfcs for positions 5 and 6 can be explained by the influence of the methyl group. Finally, our calculations show that the spin densities on C-5 of conformer **IIa**$_1$ and on C-6 of conformer **IIb**$_1$ are almost equal, confirming our assumption about the equality of the hfc constants for the ring proton in **IIa**$_1$ and the methyl protons in **IIb**$_1$. The carbonyl ^{13}C-splitting in the two conformers of the neutral radicals correlates with the value of the π-electron spin density of the carbonyl carbon.

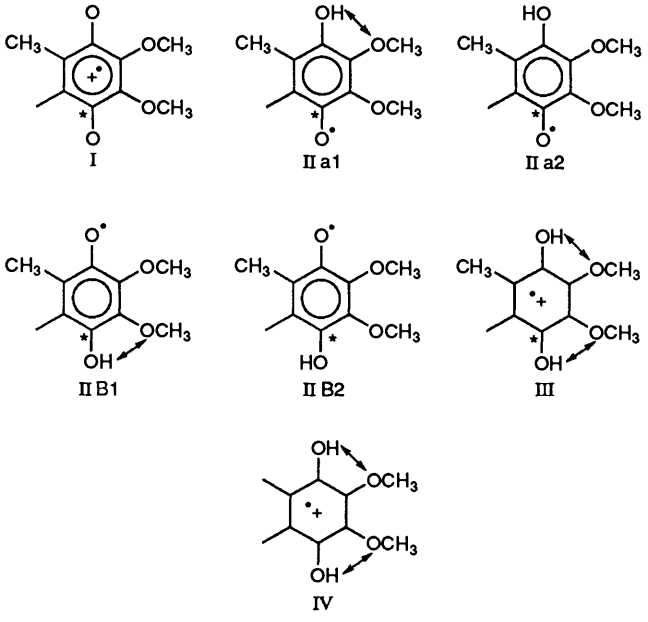
Conclusions

We have demonstrated that EPR and ENDOR studies of isotopically enriched [1-^{13}C]ubiquinone make it possible to distinguish between different types of radical. Hyperfine coupling constants of the carbonyl ^{13}C-1 atom have been determined for the ubiquinone radical anion, neutral radical and radical cation. For the radical anions, the value of the ^{13}C-1 hfc in protic solvents is about half that in aprotic solvents.

Table 2 Calculated enthalpies of formation (ΔH), out-of-plane twist angles of the methoxy group (θ) and spin distribution for the reduced ubiquinone

Radical[a]	ΔH/ kcal mol^{-1}	Twist angles/degrees	Spin density									
			C-4	C-3	C-2	C*-1	C-6	C-5	O-7	O-8	O-9	O-10
I	−154.8	$\theta_2 = 63$ $\theta_3 = 64$	0.108	0.112	0.115	0.107	0.114	0.119	0.142	0.144	0.002	0.002
IIa$_1$	−113.9	$\theta_2 = 63$ $\theta_3 = 5$	0.258	6.10^{-4}	0.220	0.06	0.134	0.033	0.064	0.156	4.10^{-6}	0.064
IIa$_2$	−109.8											
IIB$_1$	−114.5	$\theta_2 = 7$ $\theta_3 = 67$	0.065	0.200	0.002	0.250	0.025	0.16	0.157	0.063	0.057	1.10^{-4}
IIb$_2$	−110.2					0.213						
III	37.46	$\theta_2 = 67$ $\theta_3 = 55$	0.225	0.043	0.101	0.221	0.04	0.102	0.106	0.105	0.004	0.017
IV	46.54	$\theta_2 = 55$ $\theta_3 = 55$	0.221	0.069	0.069		0.070	0.070	0.111	0.111	0.010	0.010

[a] Structures of radicals analysed:



Counterions barely influence the ^{13}C hfc. The preferred *cis* conformer of the ubiquinone cation, and two conformers of the neutral radical, result from intramolecular H-bonding interactions between the phenolic hydrogens and the methoxy oxygens. MO calculations support our conclusions based on the experimental data. Analysis of the calculated data shows that the out-of-plane twist angles of the methoxy groups are quite different in the radical anion, neutral radical and radical cation. These changes result in variations of the spin density on the ubiquinone ring carbon atoms. The hfc for ^{13}C are more sensitive toward small structural changes in the radicals and its surroundings then couplings of peripheral protons. We hope to compare the unique heterogeneous natural protein environment of ubiquinone in photosynthetic reaction centres with the results which we obtained in different solvents. ENDOR spectroscopy of [1-^{13}C]-labelled ubiquinone samples was not successful. We have confidence, however, that ubiquinone enriched in [^{13}C]methyl will be more suitable for ENDOR studies.

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