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^{1}H, ^{2}H, ^{19}F, ^{14}N ENDOR and TRIPLE Resonance Investigations of Substituted Flavin Radicals in Their Different Protonation States

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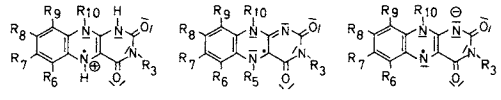
A variety of isotopically labelled and/or substituted flavins have been converted into their corresponding radical states. Cation and neutral radicals were generated chemically and anion radicals were obtained electrochemically. By performing ENDOR and TRIPLE resonance experiments, complete sets of hyperfine coupling constants including their signs were accessible. The hyperfine data allowed (a) identification of the radical state present, (b) information to be obtained about the preferred conformational arrangements of the substituents and (c) conclusions to be drawn about the influences of the substituents on the spin density distributions of the different radical states.

KEY WORDS ENDOR TRIPLE resonance spectroscopy Flavin radicals Electrochemical generation of radicals

INTRODUCTION

Flavins can occur in three different redox states analogous to the quinone redox system, viz. the quinoid, the semiquinoid, and the hydroquinoid form:



Moreover, the three species can exist in different protonation states depending on the pH of the solution. A general rule states that flavoprotein oxidases form the anionic radical state while flavoprotein dehydrogenases and flavodoxins form the neutral radical state in high yields. Flavoprotein monooxygenases form few radicals or do not give semiquinones. Investigations of the flavin redox system and its ubiquitous biological function have been summarized in a variety of reviews; more recent reviews are those by Müller,[1] Edmondson and Tollin,[2] Walsh,[3] Möbius and Lubitz[4] and Kurreck *et al.*[5] Michaelis and Schwarzenbach[6] were the first to detect flavin radical species. This observation was confirmed by Beinert,[7] who published the first EPR spectra of flavin radicals. In the last two decades the radical states of flavoproteins and flavin model compounds have attracted increasing attention. Using frozen solutions of flavoproteins, the structure of the paramagnetic coenzyme in the free and protein-bound state and its protonation state could be evaluated from the application of the ENDOR technique.[8,9] In order to obtain more detailed information about the spin density distribution of the unpaired electron in flavin radicals, it was essential to perform high-resolution ENDOR experiments on flavin radicals in liquid solution. Kurreck and co-workers[9,10] were the first to show that this technique with its significantly increased spectral resolution can, in fact, be successfully applied in this field and that valuable information about hyperfine data could be obtained.

In this paper we report on an ENDOR investigation of a variety of differently substituted and isotopically labelled flavin radicals (cf. Table 1). Since the principles of ENDOR and TRIPLE resonance spectroscopy have been reviewed,[5,11] we focus on a comparison of the different radical protonation states, i.e. cationic, neutral and anionic, by ENDOR techniques.

EXPERIMENTAL

Compounds

In their final steps most of the diamagnetic precursors of the radicals were synthesized according to known procedures;[12,13] hence, in this paper, only basic synthetic routes are described (see Fig. 1 and Table 1); more detailed preparative procedures will be presented elsewhere.[14] Except for **1c** and **1n–q** the flavins were prepared via nitrosative cyclization of anilinouracils **3**, which in turn are accessible through condensation reactions of secondary arylamines **4** with 6-chloro-3-methyluracil (**5**) in refluxing tripropylamine. The

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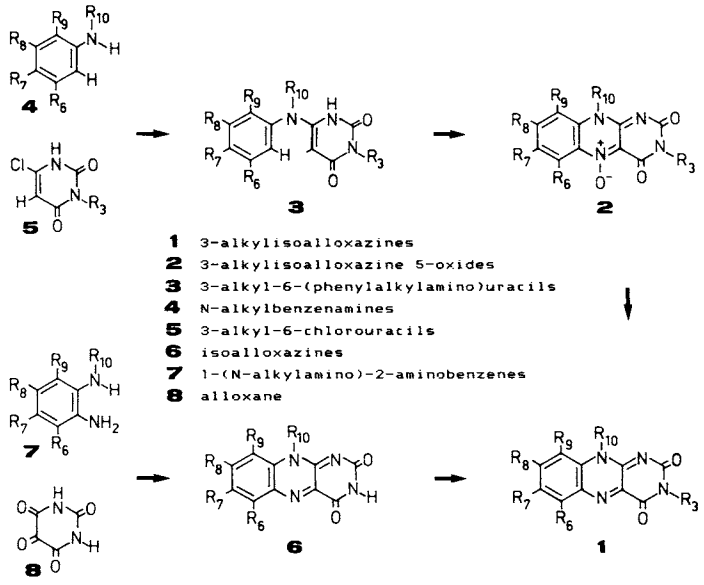


Figure 1. Synthetic pathways, structural formulae and numbering scheme of compounds.

resulting 5-*N*-oxides **2** were reduced and reoxidized to the desired lumiflavins in excellent yields.[14]

N-Alkylanilines **4** were prepared in good yields from the corresponding primary phenylamines by alkylation of the *N*-acetylated amines (sodium hydride, alkyl iodide) and subsequent saponification of the resulting *N*-alkylacetanilides. The (trideuteriomethyl)anilines **4d** and **4e** and the ethylanilines **4h** and **4j** were obtained via lithiation and alkylation of the corresponding trimethylsilyl protected bromoanilines **4r** and **4s** with iodotrideuteriomethane or iodoethane, respectively.

Butylaniline **4k** for the synthesis of 8-normethyl-8-butyllumiflavin (**1k**) was prepared by reacting the appropriately protected aniline **4s** with butyllithium.

The synthesis of *N*,4-dimethyl-3-(trifluoromethyl)-benzenamine (**4t**) was achieved in very poor yield by dilithiating, dimethylating and deprotecting 4-bromo-3-(trifluoromethyl)-*N*-(trimethylsilyl)benzenamine.

Table 1. Flavin compounds used and some of the corresponding peak potentials (E_{pc} ± 0.02 in V vs SCE) in methylene chloride[a]

Compound	R_3	R_5[b]	R_6	R_7	R_8	R_9	R_{10}	E_{pc}
1a	CH_3		H	CH_3	CH_3	H	CH_3	−0.81
1b	CD_3		H	CH_3	CH_3	H	CH_3	
1c	CH_3		D	CH_3	CH_3	D	CH_3	
1d	CH_3		H	CD_3	CH_3	H	CH_3	
1e	CH_3		H	CH_3	CD_3	H	CH_3	
1f	CH_3		H	CH_3	CH_3	H	CD_3	
1g	CH_3	C_2H_5	H	CH_3	CH_3	H	CH_3	
1h	CH_3		H	C_2H_5	CH_3	H	CH_3	−0.82
1i	CH_3		H	CH_3	H	H	CH_3	−0.75
1j	CH_3		H	CH_3	C_2H_5	H	CH_3	−0.82
1k	CH_3		H	CH_3	But[c]	H	CH_3	−0.82
1l	CH_3		H	CH_3	H	CH_3	CH_3	−0.76
1m	CH_3		H	CH_3	CH_3	H	C_2H_5	−0.84
1n	CH_3		H	CH_3	CH_3	H	Octa[d]	−0.83
1o	H		H	CH_3	CH_3	H	HE[e]	
1p	H		H	CH_3	CH_3	H	TAR[f]	−0.71
1q	CH_3		H	F	H	H	CH_3	−0.64
1r	CH_3		H	Br	CH_3	H	CH_3	−0.67
1s	CH_3		H	CH_3	Br	H	CH_3	−0.69
1t	CH_3		H	CH_3	CF_3	H	CH_3	−0.56
1u	CH_3		H	H	CF_3	H	CH_3	−0.54

[a] The values in HMPT are, within the experimental accuracy, the same as those in methylene chloride.
[b] R_5: CH_3 in all neutral radicals except **1g**.
[c] Butyl.
[d] Octadecyl.
[e] 2-Hydroxyethyl.
[f] Tetraacetylribityl.

[6,9-$^2H_2$]lumiflavin **1c** was obtained according to the procedure of Grande *et al.*[13] by treating the intermediate *N*,3,4-trimethyl-*o*-phenylenediamine **4c** several times with dilute deuterium chloride solution (35% in deuterium oxide) and reacting the disalt of the diamine with alloxane which, in turn, had been pre-treated with dilute sodium deuteriooxide (NaOD) solution. The lumiflavin **6c** thus obtained was contaminated with 10% of its demethylation product lumichrome. The mixture was separated by preparative HPLC using 60% methanol, yielding 75% of pure lumiflavin **6c** that was *N*(3)-methylated according to the procedure of Hemmerich *et al.*[15] to yield 96% of lumiflavin **1c** (isotopic purity 98% as determined by mass spectrometry).

Generation of the radicals

The *radical cations* and the *neutral radicals* were obtained by chemical reduction of the oxidized flavins. The radical cations were generated by addition of trifluoroacetic acid to a *ca* 10^{-3} M solution of the flavin in toluene previously reduced with dithionite. To obtain optimum samples the freeze–pump–thaw technique was applied on a high-vacuum line; this proved to be superior to simply flushing with purified nitrogen. Since the neutral radicals are unstable, *N*(5)-alkylation is required in order to obtain a satisfactory radical concentration.[16] To a *ca* 10^{-3} M solution of the flavin in methylene chloride a layer of aqueous buffer (pH 7.6–8.0) and some solid dithionite were added. This mixture was stirred until the fluorescence disappeared. Subsequent alkylation was achieved by addition of alkyl iodide and triethylamine. The *N*(5)-alkyl-1,5-dihydroflavins thus obtained were re-oxidized by air or addition of an appropriate amount of lead tetraacetate. After removal of the buffer layer the radical solution was dried and flushed with purified nitrogen. *N*(5)-Ethylation required heating of the reduced flavin with ethyl iodide.

The *radical anions* were generated electrochemically. One- and two-electron reduction processes with flavins can be observed separately in organic solvents by cyclic voltammetry, the first step being quasi-reversible and the second irreversible.[17,18] This permits electrochemical generation of the paramagnetic reduction step. (It should be noted that in contrast with the interpretation given in the literature cited, some experimental evidence indicates that the first step might already be a two-electron reduction process. Further work on this subject is in progress.) Figure 2 shows, as an example, the cyclic voltamogramm of lumiflavin. In order to obtain the appropriate redox potential prior to electrolysis, the cathodic peak potential, E_{pc}, of the flavin was determined by cyclic voltammetry. It must be pointed out that simultaneous electron spin resonance, i.e. internal electrochemical generation of radicals, is a well established technique that can advantageously be used, for example, for the generation of short-lived radicals.[19,20] On the other hand, electrochemistry and ENDOR cannot be performed simultaneously because of radiation effects on the electronics and heating of the electrolyte solution by the r.f. field. Hence, an appropriate experimental arrangement is needed that allows electrochemical generation of the radicals externally and subsequent transfer of the sample into the ENDOR cavity. The cell design reproduced in Fig. 3 was proposed by Allendoerfer.[21] Electrolysis was carried out at a mercury working electrode and a platinum counter electrode. A silver wire dipping into the solution served as a pseudo reference electrode. The potential of this electrode was 0.04 ± 0.01 V more negative than that of the saturated calomel electrode (SCE). Methylene chloride or hexamethylphosphoric triamide (HMPT) was used as a solvent and tetrabutylammonium tetrafluoroborate as the supporting electrolyte. The concentration of the supporting electrolyte was 100 mM for cyclic voltammetry and 10 mM for electrolysis. The concentration of the flavin was 1 mM in both cases. Electrolysis of the solution was performed with stirring for 30 min under argon using a potential of $E_{pc} - 0.1$ V.

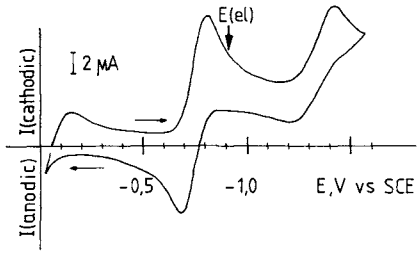


Figure 2. Cyclic voltamogramm of lumiflavin (**1a**) in HMPT. Supporting electrolyte, tetra-butylammonium tetrafluoroborate. $E_{(el)}$ indicates the voltage for electrolysis (cf. Fig. 5).

ENDOR Instrumentation

EPR, ENDOR and TRIPLE resonance spectra were recorded on a Bruker EPR spectrometer (ER 220D) equipped with a Bruker cavity (ER 200ENB) and laboratory-built NMR facilities described elsewhere.[5,22] The spectrometer was interfaced to a minicomputer

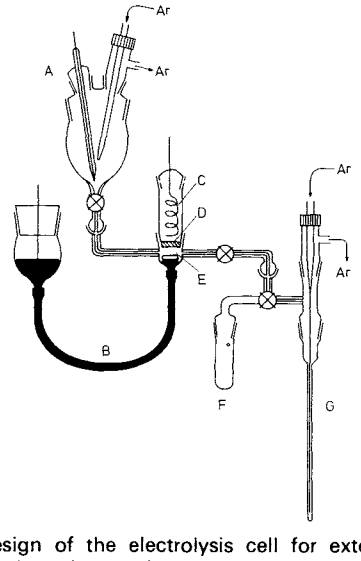


Figure 3. Design of the electrolysis cell for external reductive radical generation. A, solution storage vessel with silver wire pseudo reference electrode; B, mercury working electrode; C, platinum auxiliary electrode; D, glas frit; E, electrochemical cell with magnetic stirring bar; F, waste tube; G, sample tube.

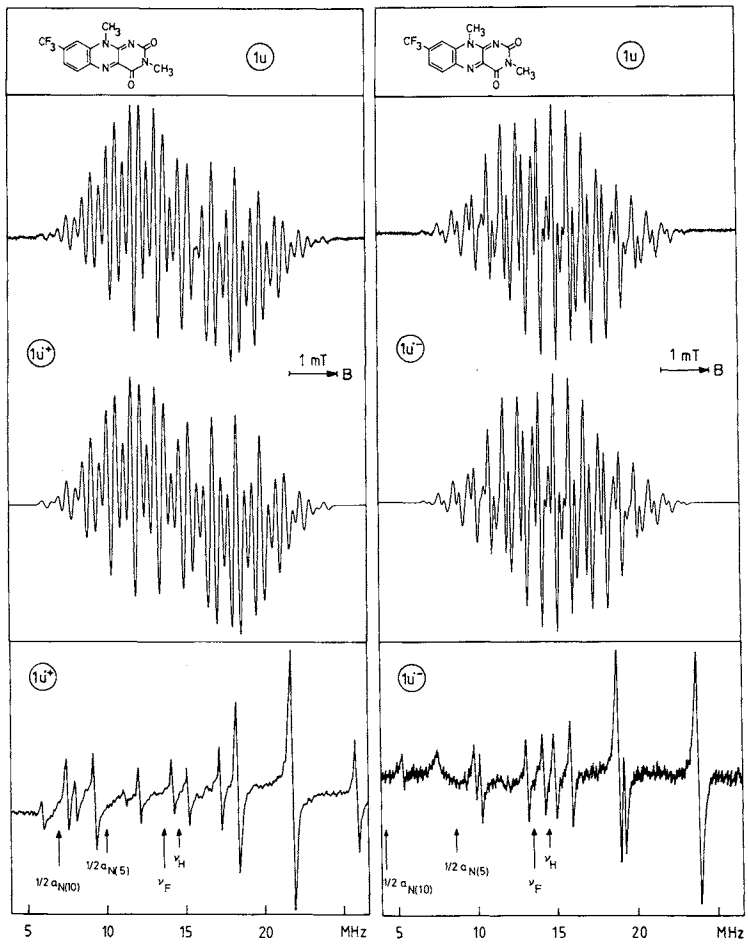


Figure 4. Experimental (top) and simulated (centre) EPR and ENDOR (bottom) spectra of the radical cation (left) and anion (right) of 3,10-dimethyl-8-(trifluoromethyl)isoalloxazine (**1u**) in toluene–trifluoroacetic acid and methylene chloride, respectively.

(HP 1000/A600) used for controlling the scan oscillator and the radiofrequency power (which was kept constant over the frequency range) in addition to data acquisition, handling and storage of the spectra. Microwave and radiofrequency power levels of 130–160 mW and about 250 W (field strength *ca* 0.6 mT in the rotating frame), respectively, were applied. Standard temperatures for ENDOR were 250–280 K for the radical cations, 220 K for the neutral radicals (except **1p**$^{\cdot}$: 290 K) and 220 K (methylene chloride) or 290 K (HMPT) for the radical anions. The accuracy of the hyperfine coupling (HFC) constants was $a(^{14}N) = \pm 0.1$ MHz and $a(^{1}H, ^{2}H, ^{19}F) = \pm 0.04$ MHz. All EPR spectra were recorded at ambient temperature.

RESULTS

The ENDOR resonance condition reads

$$\nu_{ENDOR} = |\nu_n \pm aM_S| \qquad (1)$$

where ν_{ENDOR} is the resonance frequency of the signals, ν_n the free nuclear frequency at the external magnetic field selected, a the HFC constant and M_S, the magnetic spin quantum number. In the case of a doublet radical, i.e. $M_S = \pm 1/2$, each set of equivalent nuclei gives rise to one ENDOR line pair. If $|a/2| < \nu_n$, the lines are centered around the free nuclear frequency ν_n and spaced by the HFC constant a. Given the condition $|a/2| > \nu_n$, the ENDOR lines are centred around $|a/2|$ and spaced by $2\nu_n$. Looking at the non-proton ENDOR experiments, specifically ^{19}F in the α-position and ^{14}N, higher microwave and radio frequency power levels are usually required compared with ^{1}H ENDOR. A detailed outline of the experimental non-proton ENDOR conditions is given elsewhere.[5,23]

Figure 4 shows the EPR and ENDOR spectra of the radical cation and anion of the modified lumiflavin **1u**. Owing to the resolution enhancement of ENDOR spectroscopy, all HFC constants can be unambiguously extracted (see Tables 2–4). Moreover, the resonance signals can be assigned to the respective nuclei, i.e. ^{1}H, ^{2}H, ^{19}F and ^{14}N. On the other hand, the multiplicities of different sets of equivalent nuclei are usually not reflected in the signal intensities[5,23] and hence cannot be deduced from the spectra. In the case of sufficiently resolved EPR spectra, multiplicities can be determined by simulation of these spectra using the experimental HFC constants obtained from ENDOR experiments. As is obvious from Fig. 4, excellent agreement could be achieved between experimental and simulated spectra. This facilitates assignments of the HFC constants to molecular positions. By performing general TRIPLE

Table 2. Hyperfine coupling constants (MHz) of flavin radical cations as deduced from ENDOR spectra

Compound	a(N-5)	a(N-10)	a(H-3)	a(H-5)	a(H-6)	a(H-7)	a(H-8)	a(H-9)	a(H-10)
1a$^{\cdot+}$	20.65	13.20	0.90	−22.70	−3.96	1.64	+9.81	1.64	+13.98
1b$^{\cdot+}$	20.60	13.20	—	22.70	3.96	1.60	9.88	1.60	14.04
1d$^{\cdot+}$	20.60	13.20	1.08	22.74	3.96	—	9.76	1.68	14.02
1e$^{\cdot+}$	20.54	13.18	0.89	22.75	3.95	1.64	—	1.64	13.99
1h$^{\cdot+}$	20.39	13.13	0.88	22.69	3.97	1.42	9.72	1.99	13.96
1i$^{\cdot+}$ [a]	20.32	13.87	0.98	22.71	4.13	1.46	8.64	1.46	14.18
1j$^{\cdot+}$	20.46	13.51	0.80	22.33	4.01	1.52	7.96	1.52	13.95
1k$^{\cdot+}$	20.41	13.28	0.80	22.63	4.08	1.52	6.66	1.52	13.99
1l$^{\cdot+}$	20.15	13.49	0.91	22.34	4.28	2.24	8.54	2.24	14.11
1m$^{\cdot+}$	20.70[b]	13.44	0.86	22.76	3.92	1.64	9.80	1.64	6.88 6.88
1n$^{\cdot+}$	20.70[b]	13.50	0.78	22.74	3.92	1.50	9.64	1.50	7.43 6.71
1o$^{\cdot+}$	20.47	12.83	0.87	22.90	4.14	1.54	10.19	1.54	8.09 6.26
1p$^{\cdot+}$	21.52	12.84	0.90	22.95	4.12	1.51	10.30	1.51	9.46 4.93
1q$^{\cdot+}$	19.67	13.90	1.02	22.04	3.85	1.86[c]	8.33	1.60[b]	14.51
1r$^{\cdot+}$	20.26	13.48	0.99	22.55	4.31	—	8.68	1.20[b]	14.12
1s$^{\cdot+}$	20.40	13.45	0.99	22.59	4.57	1.40	—	1.40	14.10
1t$^{\cdot+}$	19.56	13.96	0.94	22.18	4.43	0.84[b]	9.88[c]	1.15[b]	14.62
1u$^{\cdot+}$	19.98	13.68	0.94	22.39	5.20	0.94	9.13[c]	0.94	14.34

[a] Taken from Ref. 9.
[b] From simulation.
[c] Fluorine HFC constants.

resonance experiments (see, for example, Fig. 5, Table 4; cf. Ref. 9) the determination of the relative signs of HFC constants is possible. Knowledge of these signs is particularly useful in the investigation of spin density transfer mechanisms such as spin polarization or hyperconjugation (see below); it can also be helpful for assignments. However, complete and unambiguous assignment of all HFC constants to molecular positions can only be achieved by elaborate specific deuteriation.

It is well known in flavin chemistry that the flavosemiquinone *cation* is the thermodynamically most stable protonation state.[1] Generation of the *neutral* semiquinone requires stabilization by *N*(5)-alkylation (see above) hence here these species have been investigated

Table 3. Hyperfine coupling constants (MHz) of flavin neutral radicals as deduced from ENDOR spectra

Compound	a(N-5)	a(N-10)	a(H-3)	a(H-5)	a(H-6)	a(H-7)	a(H-8)	a(H-9)	a(H-10)
1a$^{\cdot}$	22.18	9.88	1.46	21.60	3.98	0.76	6.57	0.76	11.00
1b$^{\cdot}$	22.01	9.90	—	21.60	3.96	0.82	6.56	0.82	10.97
1c$^{\cdot}$	22.10	9.88	1.43	21.62	—	0.88	6.59	—	11.06
1d$^{\cdot}$	22.13	9.90	1.44	21.62	3.99	—	6.61	0.60	11.06
1f$^{\cdot}$	22.15	9.98	1.45	21.64	3.94	0.81	6.57	0.81	1.66[c]
1g$^{\cdot}$	23.70	10.04	1.44	13.33	4.09	0.58	6.77	0.58	11.12
1h$^{\cdot}$	22.10	9.80	1.41	21.76	3.98	0.90	6.49	0.90	11.00
1i$^{\cdot}$	22.20[a]	10.05	1.50	21.26	4.03	0.70	6.00	0.70	10.90
1j$^{\cdot}$	22.20[a]	9.85	1.43	21.66	3.98	0.86	5.10	0.86	10.98
1k$^{\cdot}$	22.10[a]	9.99	1.33	21.72	3.97	0.94	4.42	0.94	11.04
1l$^{\cdot}$	22.00	9.60	1.28	21.14	4.00	1.28	5.66	1.28	10.75
1m$^{\cdot}$	22.30[a]	10.22	1.24	21.82	3.82	1.24	6.51	1.24	5.86 4.93
1n$^{\cdot}$	22.30[a]	10.05	1.46	21.44	3.86	1.00	6.41	1.00	6.41 4.57
1o$^{\cdot}$	22.50	9.20[a]	1.10	22.26	3.82	1.10	6.70	1.10	6.70 4.86
1p$^{\cdot}$	22.71	9.05	0.65	21.91	4.01	0.65	6.70	0.65	6.70 4.01
1q$^{\cdot}$	22.36	10.08	1.56	20.48	3.90	0.84[b]	6.10	1.56	10.98
1r$^{\cdot}$	21.56	9.71	1.38	21.06	4.43	—	5.78	1.38	10.51

[a] From simulation.
[b] Fluorine HFC constant.
[c] Deuterium HFC constant.

Table 4. Hyperfine coupling constants (MHz) of flavin radical anions as deduced from ENDOR spectra (in methylene chloride)

Compound	a(N-5)	a(N-10)	a(H-3)	a(H-6)	a(H-7)	a(H-8)	a(H-9)	a(H-10)
1a$^{\cdot-}$	19.7[a]	8.76	0.62	8.38	1.86	11.11	1.86	9.11
1a$^{\cdot-}$[b]	18.9[a]	8.22	0.48	−9.18	−2.33	+11.35	+1.58	+8.37
1b$^{\cdot-}$	19.7[a]	8.78	—	8.32	1.89	11.11	1.89	9.13
1c$^{\cdot-}$	19.7[a]	8.70	0.63	—	−1.91	+11.12	—	+9.13
1c$^{\cdot-}$[b]	18.9[a]	8.16	0.44	—	2.35	11.37	—	8.32
1d$^{\cdot-}$	19.7[a]	8.78	0.62	−8.34	—	+11.14	+1.64	+9.06
1e$^{\cdot-}$	19.7[a]	8.69	0.65	8.31	1.85	—	1.85	9.13
1f$^{\cdot-}$	19.7[a]	8.69	0.59	8.31	1.88	11.08	1.88	—
1f$^{\cdot-}$[b]	18.9[a]	8.09	0.49	9.02	2.28	11.28	1.53	—
1h$^{\cdot-}$	19.7[a]	8.74	0.55	8.38	1.48	11.03	1.48	9.08
1i$^{\cdot-}$	19.6[a]	8.60	0.64	8.56	1.92	10.92	1.24	9.04
1j$^{\cdot-}$	19.8[a]	8.62	0.62	8.40	1.96	7.70	1.58	9.10
1k$^{\cdot-}$	19.7[a]	8.75	0.51	8.45	1.82	6.82	1.82	9.04
1l$^{\cdot-}$	19.6[a]	8.94	0.44	−7.90	−2.24	−10.48	−2.24	+8.72
1m$^{\cdot-}$	19.8[a]	8.90	0.64	8.18	1.76	10.92	1.76	5.41 3.70
1n$^{\cdot-}$	19.8[a]	8.82	0.58	8.26	1.82	10.93	1.82	5.83 3.50
1q$^{\cdot-}$	18.2	8.78	0.72	−8.48	−6.28[c]	−11.34	0.72	+8.98
1r$^{\cdot-}$	19.2	8.38	0.60	−9.30	—	+10.66	+1.08	+8.60
1s$^{\cdot-}$	19.1	8.36		9.35	2.42	—		8.75
1t$^{\cdot-}$	17.4	8.90	0.70	8.77	2.83	22.58[c]	0.00[a]	8.77
1u$^{\cdot-}$	17.5	8.62	0.64	9.38	2.72	20.34[c]	0.00[a]	8.54

[a] From simulation.
[b] In HMPT.
[c] Fluorine HFC constants.

in the *N*(5)-methylated or -ethylated form. The *anionic* semiquinone state can be obtained in fairly stable form in water or dimethylformamide by comproportionation of fully reduced with oxidized flavin.[24] However, these solvents are very unsuitable for solution ENDOR experiments. (Recently, flavin radical anions were stabilized in *reversed micelles* using a hydrophobic bulk solvent.[25] It emerged that stable radical anions can be generated under these conditions.) In this work we used electrochemical reduction for the generation of the radical anions. The appropriate redox potentials were determined by cyclic voltammetry. As is obvious from the peak potentials (E_{pc}) in Table 1, the redox properties of the flavins are affected by substitution. For example, the peak potentials of the trifluoromethyl-substituted flavins are less negative, i.e. they can more easily be reduced, than their parent compound. It is noteworthy that additional proof for the presence of flavin radical anions was obtained by the finding that these species generated electrochemically can be readily protonated to the corresponding radical cations by addition of trifluoracetic acid.

Figure 5 demonstrates that satisfactory ENDOR spectra can be obtained in HMPT or methylene chloride. It is clearly seen by inspection of Fig. 5 and Table 4 that different HFC constants are obtained in the two solvents. This may either be due to different solvation effects (it is known that in HMPT solvation of anions is very weak[26]) or to temperature dependence of the HFC constants since, in order to achieve an optimum ENDOR response, different temperatures (HMPT 290 K, methylene chloride 220 K) had to be used for the two media. Figure 6 shows the ENDOR spectra of two flavin radicals, namely the 8-normethyl-8-butyl and 10-normethyl-10-octadecyl derivatives, in different protonation states, i.e. cationic (**1k**$^{\cdot+}$, **1n**$^{\cdot+}$), neutral (**1k**$^{\cdot}$, **1n**$^{\cdot}$) and anionic states (**1k**$^{\cdot-}$, **1n**$^{\cdot-}$).

Interestingly, the β-protons in the 10-position and, in the case of the *N*(5)-alkylated neutral radicals, additionally those in the 5-position, are non-equivalent, whereas those of the substituent in the 8-position do not show a splitting in their ENDOR spectrum but the line positions differ from those of the corresponding methyl protons (see Discussion).

DISCUSSION

Different protonation states

As already mentioned, the use of selectively deuteriated compounds permitted the unambiguous assignment of the HFC constants to molecular positions. It is obvious from our measurements that a number of previous assignments[9,16,24,27] have to be revised, particularly the couplings in positions 3, 7 and 9. As can be seen by inspection of Tables 2–4, positions 7 and 9 of the lumiflavin radicals have almost identical HFC constants, regardless of their protonation state. This has recently also been deduced from EPR spectra of flavin radical cations using a sophisticated simulation program.[27] Moreover, in all states a small coupling is also found for the 3-position. This assignment was achieved by inspection of a 3-trideuteriomethylated flavin; the ^{1}H ENDOR line pair in question was absent from the spec-

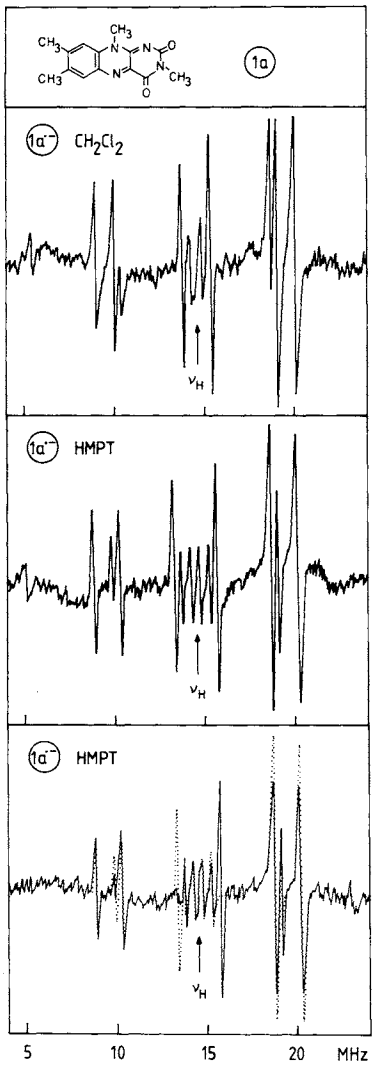
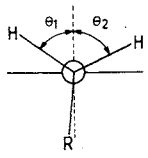


Figure 5. ENDOR spectra of the electrochemically generated 3-methyllumiflavin radical anion (**1a**$^{\cdot -}$) in HMPT and methylene chloride. The general TRIPLE spectrum (HMPT) is also given (bottom spectrum).

trum of the respective radicals (see **1b**, Tables 2–4). In accordance with previous results,[9,16,24,27] the large nitrogen and proton HFC constants of the different protonation states **1a**$^{\cdot +}$, **1a**$^{\cdot}$ and **1a**$^{\cdot -}$ exhibit considerable differences, thus allowing identification of the type of radical present. This proved of high diagnostic value in solid-state ENDOR studies of native flavoproteins.[8,9]

Alkyl-substituted flavin radicals

Whereas α-protons experience spin density through spin polarization effects, in the case of β-protons the dominant spin density transfer mechanism is hyperconjugation. This causes a strong angular dependence (referred to the dihedral angle Θ between the plane containing the C—C—H bonds and that containing the C—C bond and the axis of the $2p_z$ orbital on the carbon) of the β ^{1}H HFC constants as can be expressed quantitatively:[28]



$$\alpha_{\beta(H)} = (B_0 + B\cos^2\Theta)\rho_{C(\pi)} \qquad (2)$$

where B_0 includes contributions from spin density which arise from conformation-independent mechanisms, in particular spin polarization, while B accounts for the hyperconjugative contribution. B_0 is usually assumed to be negligible in comparison with B. If the fragment attached to a π system is free to rotate, which usually holds for methyl groups, one obtains

$$\alpha_{\beta(H)} = \tfrac{1}{2}B\rho_{C(\pi)} \qquad (3)$$

The β ^{1}H HFC constants are then usually close in magnitude to those of the corresponding protons of the unsubstituted analogue (cf., the data of the radicals derived from **1a** with those from **1i** and **1l**, Tables 2–4). In this situation it is, however, indicative of the dominant hyperconjugation effect that the signs of these HFC constants are opposite (see, e.g. **1l**$^{\cdot -}$).

If a methyl group is monosubstituted, the fragment is no longer free to rotate. Actually, in the case of the 5-, 8- and 10-substituted flavin radicals the HFC constant of the β-protons is smaller than that found for the unsubstituted flavin radical, i.e. Eqn (3) is no longer valid. In the case of the 8-substituted flavin radicals the two β-protons did not show a splitting in the ENDOR spectrum (see the results obtained for the radicals from **1j** and **1k**, Tables 2–4). In this case the alkyl groups are not rigidly constrained and Θ varies as the group rotates.[29] In contrast, the ENDOR lines of the β-protons of (non-methyl) substituents in 5- and 10-positions are significantly split, i.e. these protons are non-equivalent (see **1g**, **m**–**p**, Tables 2–4). Assuming that temperature dependence of the HFC constants and the inductive effect of the alkyl groups on the spin density distribution are negligibly small, the above Heller–McConnell relationship [Eqns (2) and (3)] allows the dihedral angles Θ_1 and Θ_2 to be calculated from the HFC constants. If the 'tight locking' model of the CH_2 fragment applies, the sum of the two dihedral angles should be 120°. Table 5 demonstrates that this is actually found for all radicals, regardless of their protonation state.

Table 5. Dihedral angles of 5- and 10-substituents, calculated from β HFC constants

Compound	a[β-H(1)]	a[β-H(2)]	θ(1)	θ(2)	θ(1) + θ(2)
1m$^{\cdot +}$	6.88	6.88	60.3	60.3	120.6
1n$^{\cdot +}$	7.43	6.71	59.0	60.7	119.7
1o$^{\cdot +}$	8.09	6.26	57.5	61.8	119.3
1p$^{\cdot +}$	9.46	4.93	55.0	65.5	120.5
1m$^{\cdot}$	5.86	4.93	59.1	62.9	121.0
1n$^{\cdot}$	6.41	4.57	57.5	63.0	120.5
1o$^{\cdot}$	6.70	4.86	56.7	62.1	118.8
1p$^{\cdot}$	6.70	4.01	56.7	64.9	121.6
1g$^{\cdot}$	13.33	9.05	56.3	62.8	119.1
1m$^{\cdot -}$	5.41	3.70	57.0	63.2	120.2
1n$^{\cdot -}$	5.83	3.50	55.6	64.0	119.6

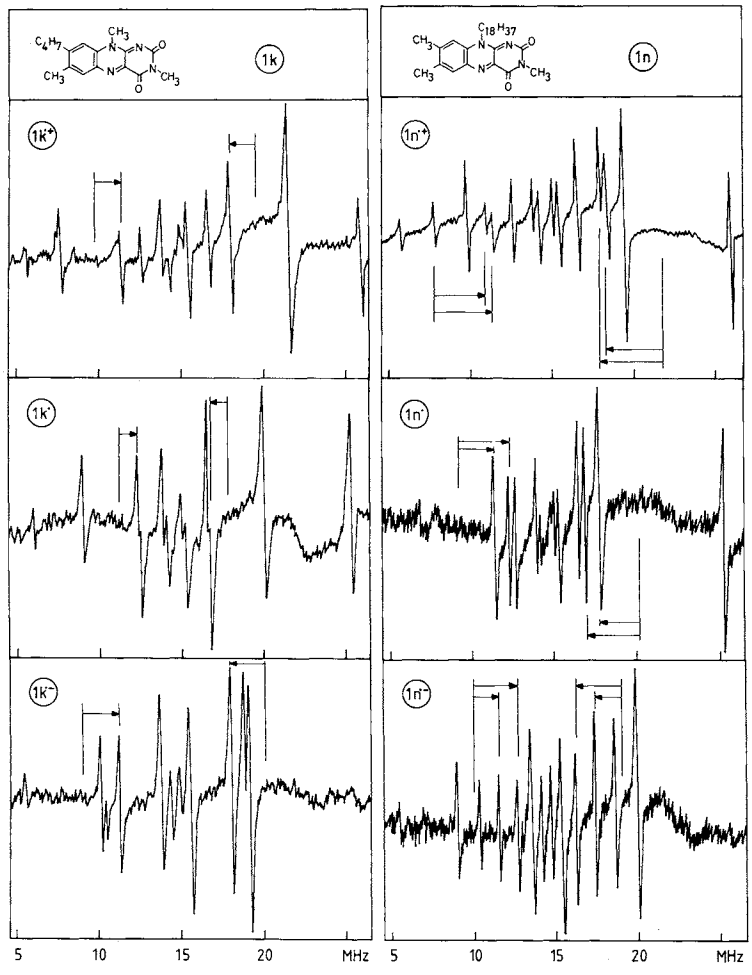


Figure 6. ENDOR spectra of the radicals from 8-normethyl-8-butyllumiflavin (**1k**, left) and 10-normethyl-10-octadecyllumiflavin (**1n**, right) in their different protonation states. It should be noted that the β-proton resonances of the octadecyl substituent in the 10-position are significantly split, whereas those of the substituent in the 8-position are not.

Previously it was postulated that in (diamagnetic) 10-(hydroxyalkyl)isoalloxazines the substituent in the 10-position forms a hydrogen bridge to N-1.[30] In order to look at such effects in radical states of flavins, we studied an appropriate model compound, namely the 10-(hydroxyethyl) derivative **1o**. However, comparison of the hyperfine data of the radicals from **1o** with those from the 10-octadecyl derivative **1n** did not reveal significant differences in the dihedral angles of the substituents in the 10-position. These data indicate that such a hydrogen bond formation is probably confined to oxidized flavin.

Halogen-substituted radicals

In order to study the influence of electron-withdrawing substituents we performed ENDOR measurements on a variety of halogenated flavins. Fluorine is the most convenient halogen substituent since ^{19}F ENDOR experiments can easily be performed, whereas ENDOR signals of the other halogen atoms cannot be obtained.[5] In the case of a freely rotating trifluoromethyl group the fluorines are also equivalent, but the sign of the ^{19}F HFC constant is not changed compared with that of an α-fluorine in the corresponding position since for the latter direct conjugation is the dominant spin transfer mechanism[5,31] (see Table 4). As can be seen by inspection of Tables 2–4, the spin density of the radicals is redistributed by the substituents; this is particularly pronounced in the case of the 8-CF_3 group. The most salient feature is the different influence of this group in the radical cations compared with that of the anions. Whereas the ^{19}F and the ^{1}H HFC constant of the *radical cations* of the CF_3 and the CH_3 groups are almost identical (**1a**$^{\cdot+}$ 9.81, **1t**$^{\cdot+}$ 9.88, **1u**$^{\cdot+}$ 9.13 MHz), in the case of the *radical anions* the former HFC constant is twice as large as that of the latter [**1t**$^{\cdot-}$ 22.58, **1u**$^{\cdot-}$ 20.34 MHz compared with 11.11 MHz (**1a**$^{\cdot-}$)]. This was also found for radicals **1q**$^{\cdot+}$/**1q**$^{\cdot-}$ where the fluorine substituent is in the α-position. The inductive effect of the trifluoromethyl substituent acts differently on the spin density distributions of the different protonation states of the flavin radicals. In the case of the *radical cations* the electron-withdrawing effect is not very pronounced, hence the HFC constant of *N*-5 is only slightly reduced and those of the *N*(10)-CH_3 are larger. In contrast, the *radical anions* show considerable reduction of the HFC constant of *N*-5 and slight reduction of those of the *N*(10)-CH_3 fragment. Similar effects were found for all halogen-substituted radicals from **1q–u**.

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