The oscillopolarographic technique proved to be a valuable tool in characterizing the electrochemical behaviour of this group of substances. A newly developed device[6] enabled the study of the function $dE/dt=f(E)$ separately on individual cycles as well as with a predetermined starting potential. Some applications are shown in Fig. 1. A blank solution and substance I exhibit similar oscillopolarographic patterns (Fig. 1a, b). Under the same conditions, anodic and strong cathodic indentations appear for substance III (Fig. 1c). Besides, these incisions are directly related to the starting potential (Fig. 1 d-f). This is clear evidence that the species being reduced are artefacts, formed by anodic oxidation at more positive potentials of the original substance. Analogous results were obtained with cyclic voltammetry (Fig. 2). The experiments were

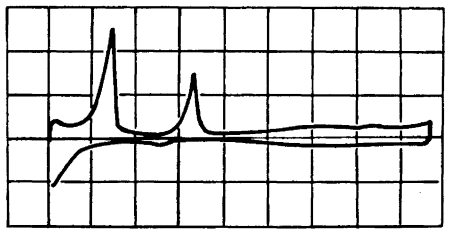

*Fig. 2.* Cyclic voltammogram of substance III under the same experimental conditions as in Fig. 1c. Frequency 30 cycles/s. Potential range −0.1 to −2.0 V *vs.* SCE.

repeated with some compounds related to I, *viz.* its 2,4-dioxa-6,8-dithia, oxatrithia, pentathia, and hexathia analogues (II).[3] These resembled I closely in their electrochemical behaviour, whereas substance V behaved like III.

The following conclusions can be drawn. Compound I and related substances appear as *non-reducible* in the potential range available. Strong oscillopolarographic effects emerge from III and as a result of anodic oxidation at more positive potentials. The appearance of III or analogues as impurities in I may cause the electrochemical effects previously ascribed to I.

*Acknowledgements.* We wish to thank Mr. Rolf Danielsson, Pharmacia AB, for valuable help with the electrochemical work, Professor Nils Hellström for all facilities placed at our disposal and Professor Arne Fredga for his interest in the present work. This was supported by a grant (to K. O.) from the *Swedish Natural Science Research Council.*

*Added in proof.* Professor D. L. Cohen has kindly informed us that he does not dispute our reinterpretation of his results.

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Received December 8, 1970.

# ESR and ENDOR from Neutral Flavin Radicals

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Neutral flavin radicals have been investigated by electron spin resonance (ESR) and isotopic substitution.[1–3] An unambiguous determination of the various hyperfine coupling constants is not straightforward. Electron-nuclear double resonance (ENDOR) has recently proven to be of value for the determination of hyperfine couplings to methyl protons of flavin radicals in liquid and polycrystalline phase.[4] Model studies were performed with anionic and cationic radicals, and also with radical chelates. Samples of

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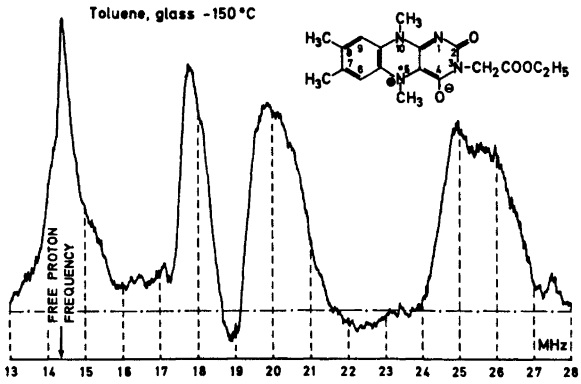


*Fig. 1.* Recording of ENDOR spectrum of the neutral radical of alkylated lumiflavin in vitreous toluene at −150°C. The radical concentration was about 1.5 mM. The microwave power incident on the cylindrical ($TE_{011}$) ENDOR cavity was 15 mW. The broken horizontal line denotes the signal level obtained for zero rf power at 28 MHz.

neutral flavin radicals which should be suitable for ENDOR can now be produced.[2,3]

*Experimental.* Preparation of samples with neutral radicals has been described elsewhere.[2] Toluene was used as solvent. The radical concentration was determined spectrophotometrically and was in the range 1.0−1.5 mM. The radical solutions were transferred anaerobially to quartz sample tubes with about 5 mm i.d., and the tubes were sealed under vacuum. Aqueous ($H_2O$ and $^2H_2O$) samples of lumiflavin-3-$CH_2COO^-$ in 0.1 M phosphate buffer (pH 5.5−6.5) were reduced with $H_2$/Pd. At half-reduction the radical yield is about 6 % of the total flavin concentration, which was 5 or 10 mM.[5] These sample tubes were closed with stop-cocks.

Varian Ass. V-4500 and E-9 ESR spectrometers together with a TMC C-1024 time averaging computer were employed. The ENDOR instrument was a Varian E-700 system. The temperature was regulated with a heater-sensor system using cool nitrogen gas.

*Results and discussion.* A recent ESR study [3] has shown that reductive alkylation of flavin gives a radical with the substituent sitting at N(5); Fig. 1. Instead of chloroform we have here used toluene as solvent.[2] Hence, a larger sample volume could be used. Toluene is preferred in spite of its higher dielectric loss than benzene because of its low melting point (−95°) making it more suitable as an solvent for the ENDOR method. Moreover, toluene can easily be supercooled to form a glass.

The neutral lumiflavin radical with a N(5)−$CH_3$ substituent gives rise to an ESR spectrum with 35 main hyperfine lines at room tenperature. No proton ENDOR signals were detected from samples kept at temperatures around the melting point of toluene. It was necessary to lower the temperature until the sample was highly viscous. It was found that the aggregation of the solvent was extremely important and spectra were only recorded from glassy or polycrystalline samples at about −150° with high spectrometer sensitivity. It has been found that spin exchange is probably not operating in the liquid sample.[2]

Besides the signal around the free proton frequency three other signals are recorded. We have earlier pointed out that methyl protons are especially suitable for ENDOR.[4,6] This is because of the almost isotropic hyperfine tensor for a rotating methyl group. The small inherent anisotropy will contribute to the line width and somewhat skew the signal. The line widths for the signals from the various methyl groups in Fig. 1 are proportional to the estimated isotropic coupling constants. This indicates that the proportion of anisotropy of the hyperfine tensor is fairly constant for the flavin methyl groups.

For a sample with the substituent at N(5) deuterated (methyl-$d_3$) the high frequency signal was absent. The estimated isotropic hyperfine couplings for the signals are 2.4, 3.9, and 7.8 gauss (G). This is in good agreement with couplings for $CH_3(8)$, $CH_3(10)$, and $CH_3(5)$ (2.4, 3.9, and 7.6 G) as found for neutral radicals in chloroform by ESR.[3] A value of 8.5 G has been reported for $CH_3(5)$ in toluene.[2]

The matrix-ENDOR signal [6] around the free proton frequency is narrow and relatively weak in the glassy sample. To the intensity in this area weakly coupled flavin protons also contribute. From the line width we estimate the isotropic coupling of $CH_3(7)$ to be $<0.3$ G. ESR spectra simulations indicated [3] that there must exist a single proton with an isotropic coupling of 1.7 G, which was tentatively assigned to H(6). This α-proton would be expected to give a broad ENDOR signal with a build-up of intensity around the isotropic value. The observed background intensity at 16–17 MHz is therefore in the right region. This intensity is, however, somewhat uncertain because of instabilities of the base line, probably due to rf interference, which even could cause negative signal (Fig. 1). Spectra recorded from polycrystalline and protein samples [7] have shown this intensity more unambiguously.

ESR in combination with isotopic substitution has proven that the unpaired electron of the neutral radical is not delocalized to any appreciable extent to the nitrogens N(1) and N(3).[1,3] Since there is one hyperfine active exchangeable proton it was evident that this must be sitting where a high spin density is located. Reductive alkylation of fully reduced flavin occurs at N(5) as shown by ESR.[3] The neutral alkylated and protonated radicals have very similar light absorption spectra.[2] We have now measured the hyperfine coupling to the exchangeable proton to be about 8 G. This was made possible by an estimation of the change in the total width of the ESR spectrum upon deuterium exchange. The neutral radical of lumiflavin-3-$CH_2COO^-$ in $H_2O$ gives rise to 38 detectable lines, but only 35 in $^2H_2O$-buffer. The spectral resolution of the outermost lines in the latter case was rather poor making the estimation of the total width difficult. The change in total width was found to be 5.5 G. The agreement between the coupling for the exchangeable proton and that for the coupling to $CH_3(5)$ is gratifying. It is well established that a methyl group, as well as a proton, attached to a trigonal nitrogen gives rise to an isotropic coupling close to that for the nitrogen itself. The coupling for N(5) in chloroform has been reported to be 8.0 G.[3] The present measurement of the coupling of the dissociable proton is a direct evidence that it must be sitting at N(5).

From the measured methyl couplings we can estimate the spin densities in the adjacent π-orbitals by the common linear relationships. The spin polarization parameter $Q_{CCH_3}^H$ is assumed to be $+22$ G, as found [8] for substituted naphthalenes. A value of $+27$ G seems to be reasonable for both $Q_{NCH_3}^H$ and $Q_N$.[9,10] The following spin densities are obtained: $\varrho_{N(5)}=0.30$, $\varrho_{N(10)}=0.15$, $\varrho_{C(8)}=0.11$, and $\varrho_{C(7)}<0.01$. Moreover, $\varrho_{N(1)}\sim\varrho_{N(3)}\sim 0$. At least the three first mentioned spin densities are very likely positive. No spin density calculation on the N(5) protonated form of the neutral radical has been reported. However, we notice the reasonable agreement with spin densities calculated on an N(1) protonated radical.[11]

In some flavoproteins "blue" radicals may be formed.[12] The light absorption spectrum indicates that the colour is due to the neutral flavin radical.[2,3] This is consistent with ENDOR results from such flavoproteins. The hyperfine coupling to $CH_3(8)$ is for the *Azotobacter* flavoprotein 2.9 G,[7] glucose oxidase (pH 6) 2.7 G,[13] and about the same for a flavodoxin (*P. elsdenii*).[14] These values should be compared with 2.4 G as measured both by ESR in chloroform [3] and here by ENDOR in toluene. Nothing dramatic seems to happen to the spin density distribution when position N(5) is alkylated. However, some perturbation of the spin density at C(8) upon alkylation is reasonable to assume. Small "solvent" effects, reflecting the difference in environment for a neutral radical in a protein and in our model system may also exist. Unfortunately it has not been possible to detect ENDOR from an aqueous system of neutral radicals. A large (8 mm i.d.) frozen sample at $-170°$ was tried, but we were not able to obtain enough microwave saturation. This was surprising since a liquid sample at $+20°$ started to saturate already at about 3 mW. The poor signal-to-noise ratio with this small (0.9 mm i.d.) sample did not allow an ENDOR study.

*Acknowledgements.* We are indebted to Drs. A. Ehrenberg and J. S. Hyde for valuable discussions. Financial support was received by *Statens Naturvetenskapliga Forskningsråd* (NFR 1187—122, 321—19) and *Statens Medicinska Forskningsråd* (B-70-13X-542-06B). The cost of the ENDOR system was defrayed by *NFR* (321—18) and *Wallenbergsstiftelsen.*

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Received December 5, 1970.