Eur. J. Biochem. 14 (1970) 185–196

The Chemical and Electronic Structure of the Neutral Flavin Radical as Revealed by Electron Spin Resonance Spectroscopy of Chemically and Isotopically Substituted Derivatives

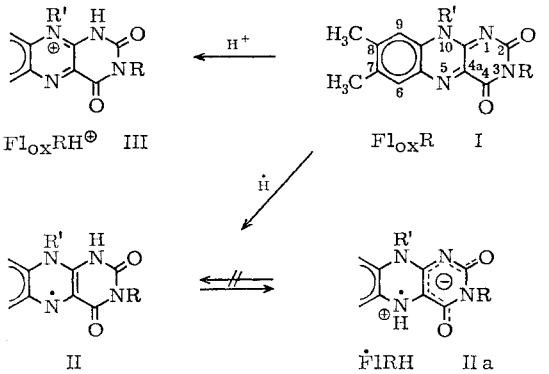
Franz MÜLLER, Peter HEMMERICH, Anders EHRENBERG, Graham PALMER, and Vincent MASSEY

Biofysiska Institutionen, Stockholms Universitet, Medicinska Nobelinstitutet, Karolinska Institutet, Stockholm, Fachbereich Biologie der Universität Konstanz, and Department of Biological Chemistry and Biophysics Research Division, The University of Michigan, Ann Arbor

(Received October 13, 1969/February 7, 1970)

The neutral flavosemiquinone has been studied in detail by electron spin resonance spectroscopy. Isotopic (^{15}N, ^{2}H) and chemical substitutions have been carried out. A hyperfine coupling scheme of the neutral flavosemiquinone is described where N(5), N(10), CH_3(10), CH_3(8) and H(6) are involved. The highest spin density is located at N(5) as has also been found for the anionic and chelated form of the flavosemiquinone. The exchangeable proton in the neutral flavosemiquinone is attached to N(5) having a coupling constant of 7.6 G which is about the same magnitude as the coupling constant to N(5). The hyperfine couplings are interpreted in terms of spin densities and comparison is made with available quantum chemical calculations. Evidence is presented that two tautomeric forms of the neutral flavosemiquinone have to be considered which can be obtained in a pure form by suitable substitution of flavin derivatives. The two types of neutral flavosemiquinones are easily distinguishable by their electron spin resonance and light absorptions.

Since the discovery of flavin radicals by Michaelis and co-workers [1] it has been tacitly assumed that the addition of a hydrogen atom to flavoquinone (I) would occur at N(1) yielding a neutral flavin radical $\dot{F}lRH$ of structure II. This was in analogy to the (in the meantime well established) protonation of flavoquinone, yielding the flavoquinone cation III [2].

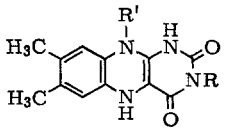


This idea was also supported by the fact that $\dot{F}lRH$ showed an acidity comparable to that of flavohydroquinone (IV) as established by Michaelis and Schwarzenbach potentiometrically [3]. These authors found $pK^{H}_{\dot{F}lRH} \cong 7$ as compared to $pK^{H}_{Fl_{red}RH_2} = 6.5$. Clearly the most acidic proton in IV (R = H) must be assumed to dissociate from the cyclic imide group in either position 1 or 3, and not from the "pyrrole" nitrogen in position 5 of the isoalloxazine ring. The validity of this assumption for 1,5-dihydroflavins was proved by the fact that 5,10-dihydro-1,3-dimethyllumichrome (V) does not exhibit a measurable acidity. (1,3-Dimethyllumichrome was hydrogenated with Pd on SiO_2 as catalyst in 50 % aqueous dimethylformamide and the resulting V titrated under H_2 with OH^- [3a].) On the other hand, alkylation in position 3 (IV, R = alkyl) does not alter the pK value of IV (R = H) [4], proving

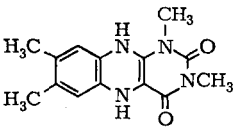
Unusual Abbreviations. Gauss, G; flavoquinone, $Fl_{ox}R$, where R denotes a substituent at N(3) (ring numbering according to IUPAC-IUB nomenclature tentative rules, *Eur. J. Biochem.* 2 (1967) 5, *cf.* formula (1); flavohydroquinone, $Fl_{red}RH_2$; anionic flavosemiquinone, $\dot{F}lR^-$; cationic flavosemiquinone, $\dot{F}lRH_2^+$; neutral flavosemiquinone, $\dot{F}lRH$.

Nomenclature. The term flavin means the 10-alkylated 6,7-benzo-pteridine-2,4-dione, *i. e.* the isoalloxazine nucleus, irrespective of the redox state. Its oxidized form is called "flavoquinone", its fully reduced form "flavohydroquinone", 1,5-dihydroflavin or, for convenience, leucoflavin, though the free leucoflavin is, in fact, not colorless. The term "flavosemiquinone" is assigned to the intermediate radical form. Lumiflavin means 7,8,10-trimethylflavin.

that the measured pK value of about 7 is due to the dissociation of the proton at N(1).



IV



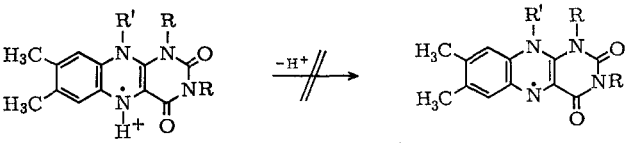
V

Furthermore, on going from $Fl_{ox}H$(I, R = H) to $Fl_{red}H_3$(IV, R = H) the pK for dissociation at N(3) does not change significantly [5].

However, electron spin resonance experiments with half reduced flavins in H_2O and ^2H_2O at neutral pH had shown that one exchangeable proton with a large coupling constant is present in $\dot{F}lRH$ [6,7], whereas $\dot{F}lR^-$ and flavosemiquinone metal chelates do not show any exchangeable, electron spin resonance active protons [6,8,9]. Furthermore, it is well established that there is only a very small spin density located at the nitrogen atoms of the pyrimidine part of the flavin nucleus in $\dot{F}lR^-$ [8,10] and in the flavosemiquinone metal chelates [9,11], and that this spin density is even smaller in $\dot{F}lH_2$ and $\dot{F}lH_3^+$ [12]. From these results it was concluded [7] that the dissociable proton of $\dot{F}lRH$ could not be attached to either of positions N(1) or N(3), but only to N(5) or perhaps O(4). In the case of N(5) there is an obvious and straigthforward coupling mechanism analogous to that of aromatic ring hydrogen [13], whereas in the case of O(4) a mechanism was proposed [7], with the hydroxyl hydrogen swinging out of the molecular plane, which however lacked theoretical testing. From the above it was to be expected that the pK value of $\dot{F}lRH$ would be rather different from that of $Fl_{red}RH_2$ in contrast to the original observation of Michaelis and Schwarzenbach [3]. We therefore reinvestigated the dissociation reaction $\dot{F}lRH \rightleftarrows \dot{F}lR^- + H^+$ by means of electron spin resonance spectroscopy and found pK values of 8.4 for lumiflavin-3-CH_2COO^- and 8.6 for FMN, respectively [10]. Draper and Ingraham [5] have independently reinvestigated this problem by potentiometry and report values of 8.27 for riboflavin and 8.55 for FMN. More recently, Land and Swallow [14] published a value of 8.3 for riboflavin determined spectrophotometrically.

The known results with the flavoproteins, on the other hand, can be outlined as follows: Massey and Palmer [15] investigated a number of flavoproteins and found that there exist two different classes of flavoprotein free radicals, which are readily distinguished on the basis of their optical absorption properties. The purple-blue type of radical, which shows an absorption maximum in the region of 570—600 nm, has also been amply documented with free flavocoenzymes and some of their simple derivatives by optical [2,16] and electron spin resonance spectra as well as by correlation of both methods [17] and must be assigned to the neutral radical species $\dot{F}lRH$. The other class of flavoprotein free radicals exhibits an absorption maximum at about 470 to 490 nm with very little absorption at 600 nm [15]. The solutions of these radicals appear to be red in color. With glucose oxidase, both types of radical were found, the relative concentrations depending on the pH with a pK value of about 7.5 [15]. This suggested that the red type of radical might be due to the anionic radical $\dot{F}lR^-$. This was later confirmed by model studies with lumiflavin derivatives [10].

However, the tautomer and electronic structure of the neutral, blue radical $\dot{F}lRH$ was still controversial. Therefore, we have found it worthwhile to study $\dot{F}lRH$ and elucidate its molecular and submolecular structure. At first, we wanted to find an explanation for the phenomenon observed earlier [2] that 1,3-dialkyl-1,5-dihydrolumiflavins [18] and their corresponding flavoquinonium salts [19] would not comproportionate to give a neutral flavosemiquinone $\dot{F}lR_2$(VI) though the corresponding radical cation $\dot{F}lR_2H^+$(VIa) was perfectly stable [2,20].



VIa VI

This pointed towards an instability of VI which was perfectly in line with the exclusion of structure II for $\dot{F}lRH$ by means of electron spin resonance (large coupling constant of the exchangeable proton [6,7]). If II and VI were unstable, the true structure of $\dot{F}lRH$ and $\dot{F}lR_2$—*i. e.* the neutral, stable blue flavosemiquinone—and with it the site of electron input into $Fl_{ox}R$ had to be reestablished. It will be shown that IIa is the true structure of the neutral blue flavosemiquinone $\dot{F}lH_2$, resp. $\dot{F}lRH$.

MATERIAL AND METHODS

All solvents were reagent grade, except for triethylamine, which was distilled twice over K_2CO_3. Flavin derivatives used in this investigation were the same as reported previously [10]. N(3)-substituted flavin derivatives have been used, since substitution at position 3 has only slight influence on the electron spin resonance spectra [10], while it enhances the solubility of the flavin derivatives in organic solvents. Flavin photoalkylated products were obtained as described elsewhere [21]. Reductive alkylations without N(5)-protection of the flavin derivatives employed were carried out as follows:

A stock solution of 10 mM flavoquinone was prepared in $CHCl_3$ and shaken with 0.1—0.5 M

$Na_2S_2O_4$ solution, (kept neutral with 0.1 N Na_2HPO_4 and half saturated with NaCl) until full reduction of the flavin was achieved. NaCl is needed to keep the water content of the organic phase low and to ensure ready phase separation. 2 ml of the $CHCl_3$ layer, containing the 1,5-dihydroflavin, were transferred anaerobically into an argon flushed tube containing 0.1—0.5 ml alkylating agent. Finally, sufficient triethylamine, 0.1 M in $CHCl_3$, was added in order to ensure excess of base and complete formation of the anion of 1,5-dihydroflavin ($Fl_{red}RH^-$) to be alkylated. After about 5 min at room temperature, the reaction was stopped by shaking with aqueous aerobic 1 M acetic acid (half saturated with NaCl) where upon the N(5)-alkylated blue green radical developed gradually, whereas any residual unsubstituted 1,5-dihydroflavin was oxidized straight to the quinoid state. After a further 5 min, the blue-green $CHCl_3$ layer was flushed with argon and transferred into an argon-flushed electron spin resonance tube. The following alkylating agents have been used: CH_3I, C^2H_3I (*gift from* CIBA AG, Basel, Switzerland), $BrCH_2COOC_2H_5$, $BrCH_2C_6H_5$, C_2H_5I.

The system for the preparation of aqueous $\dot{F}lRH$ consisted of 50 mM veronal buffer, pH 6.5—7.7, 0.1 N in $NaClO_4$. The radical solutions were prepared in a similar manner as described earlier [12], by hydrogenation of the flavin with Pd-asbestos as catalyst, filtration under purified argon and addition to a solution containing an equivalent amount of unreduced flavin. Deuterium oxide (99.8 %) was supplied by Norsk Hydro, Norway.

The electron spin resonance spectra were recorded with a Varian Model V-4500 X-band spectrometer. The 100 kHz field modulation unit Model V-4560 was modified with a filament stabilizer and a Philbrick P2A solid state amplifier. The 12-inch magnet was regulated by a Varian "Fieldial" unit. To avoid saturation, the microwave power in the cavity waveguide was kept below 2.5 mW, as measured with a Hewlett Packard Model 430 C power meter. The cavity was a Varian V-4531 multipurpose cavity. Quartz capillaries of 1.3 mm inner and 4.4 mm outer diameter were used. All experiments were carried out at 20—22°.

The hyperfine coupling constants were evaluated as described elsewhere [8,10] from differences in total width between outermost hyperfine lines of pairs of electron spin resonance spectra differing in a specific isotopic or chemical substitution. Such a procedure can only be employed when the number of lines and the position of the outermost lines of the electron spin resonance spectra are known. In addition, the accuracy of this procedure is dependent on the shape of the lines, *i. e.*, broadened lines lead to less accurate results (*cf.* Fig. 4, Table 2).

It is conventionally assumed that the wave function for the unpaired electron is unchanged when a nucleus is substituted by one of its isotopes. The isotopic hyperfine splitting arising from the Fermi contact term is proportional to the g-factor for that nucleus (g = nuclear magnetic moment (μ)/nuclear spin [I]) [21]. Accordingly when protium ($g = 5.585$) is replaced by deuterium ($g = 0.8570$) the doublet with a spacing of a^H will be transformed into a triplet with the much smaller spacing of $a^{2H} = 0.154\, a^H$. Consequently, the hydrogen hyperfine splitting will mostly collapse upon exchange with deuterium. On the other hand, when ^{14}N ($g = 0.404$) is replaced by ^{15}N ($g = -0.566$) the triplet with a spacing between components of $a^{14}N$ will be transformed into a doublet with a spacing of $a^{15}N = -1.40\, a^{14}N$.

We have also introduced chemical rather than isotopic substituents into a few positions of the isoalloxazine ring in order to facilitate the interpretation of the electron spin resonance hyperfine interactions. Thus, we have replaced protons or methyl groups in the benzene ring of the flavin molecule by chlorine. The hyperfine interaction of the residual protons in benzosemiquinones does not vary appreciably upon substitution of one or more chlorine atoms in the ring [22]. Therefore, we assume that chlorine substitution of the flavin nucleus does not change the charge distribution significantly. Although the nuclear spin of chlorine is 3/2, its interactions are seldom seen in electron spin resonance spectra of solutions [22].

Electron spin resonance spectra were simulated using a simple extension of the approach described by Stone and Maki [23] written in FORTRAN IV for the University of Michigan IBM 360/67 computer. Either Lorentzian or Gaussian lines of constant width could be employed. The computed spectrum was scaled to the amplitude of the largest line in the experimental spectrum and the result plotted automatically on transparent paper. This allowed easy comparison of computed and experimental spectra by using the former as an overlay.

The simulation of the spectra was carried out by using a constant line width and for economic reasons by searching for the best fit of the low field part of the experimental spectrum. However, when a whole spectrum was then simulated, the high field part did not coincide with the experimental spectrum as well as the low field part. The hyperfine lines of the high field part of the experimental spectra always appeared somewhat wider than those of the computed ones. This was also observed for the radical anion, and was taken as an indication that the nitrogen hyperfine splittings are positive [8,26].

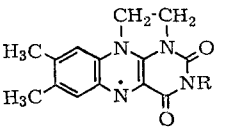
RESULTS AND DISCUSSION

N(1),N(3)-Dialkylflavosemiquinones (VI)

The actual concentrations of free radical in a partially reduced aqueous solution of flavin depend on radical disproportionation [10] and intermolecular

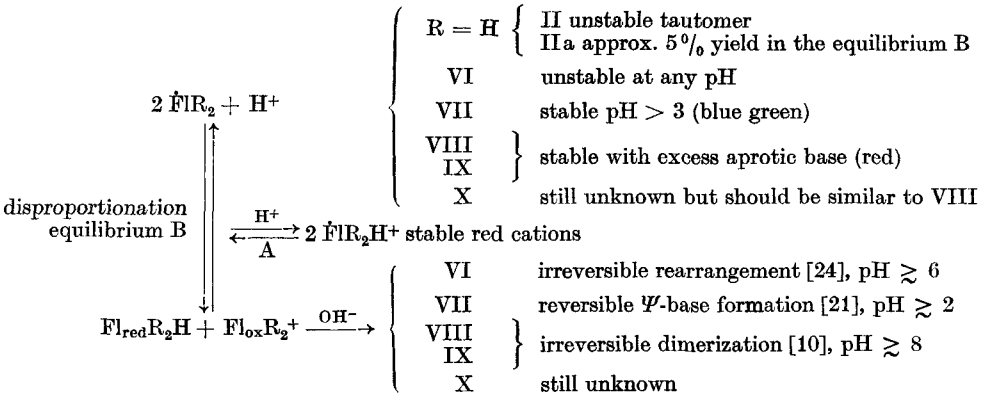
association [24] equilibria which in turn are governed by the acidities of the flavin species involved. Hence, in the neutral pH region, less than 5 % of the total flavin is present as the free radical [10]. Higher radical yields may be obtained by any means which will shift or block flavin deprotonation and suppress intermolecular association, that is aprotic environments and/or alkyl substitution. (The various $\dot{F}lR_2$-isomers dealt with in the present paper and their properties are summarized in Chart I.)

positions 1 and 10, though such steric effects would have to be seriously considered as disturbing the planarity of the radical. The cyclic analog VIb behaves like VI, though steric hindrance is absent.



VIb

Chart I



The first radicals stabilized by alkylation that we tried to synthesize were the 1,3-dialkyl-1-hydroflavins (VI) [19]. We were surprised to find that the blue-to-green colored intermediates obtained upon oxidation of the parent leuco-flavins were extremely unstable even under strictly anaerobic conditions and showed no electron spin resonance signals. Thus we previously assigned an iminol structure to the flavosemiquinone ḞlRH [*e. g.* VIII, IX or X(R = H), Chart I] [2]. Correspondingly we proposed that all stable alkyl flavosemiquinones subsequently obtained were *O*-alkyl isomers [2, 17]. This assignment has now been proven to be wrong (see below). The instability of VI, however, can now be understood as follows.

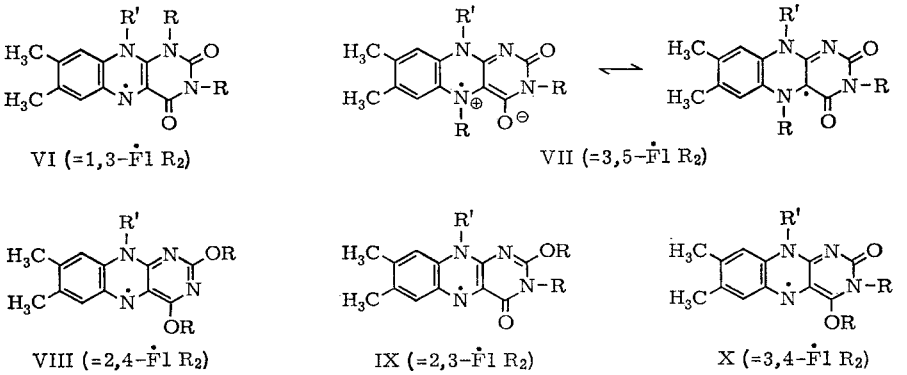
A solution of 50 % reduced 1,3-dialkylflavoquinonium salt comproportionates under acidic conditions to yield 100 % radical cation VIa (equilibrium A, Chart I), while the latter disproportionates quantitatively upon deprotonation. If stronger base is used in order to remove 2 protons, forcing the equilibria A, B towards the upper left, and even if water is excluded by working in dimethylformamide-triethylamine to prevent the known OH^--catalyzed spirohydantoin rearrangement [25] of 1,3-$Fl_{ox}R_2^+$ (*cf.* Chart I), still no trace of VI could be detected by electron spin resonance, though the previously reported transient green color was observed.

We find furthermore that the instability of VI is not due to substituent overcrowding in the *peri*

From this we draw the following conclusions: (a) The proton to be liberated must come from position 5 in 1,3-$R_2Fl_{red}H$ (Chart 1, equilibrium B). This proton has no acidity whatsoever, *cf.* V, which prevents the upper state of B being reached with 1,3-dialkylated flavins. (b) Since the 1,3-isomer of $\dot{F}lR_2$ (VI) is thermodynamically unstable, the non-alkylated stable blue-green $\dot{F}lH_2$ must have its most acidic proton at some position other than 1. This agrees with the electron spin resonance data [6, 7] (see also below). Consequently, the structure of the free neutral radical $\dot{F}lH_2$ must be represented by one of the formulae VII—X, resp. IIa. Among those, VIII—X are excluded by the following evidence.

O-Alkylated Flavosemiquinones VIII—X ($\dot{F}lR_2$)

Solutions of these flavin radicals in chloroform are available by mixing equal parts of oxidized [19] and fully reduced species (*cf.* Methods section). Flavosemiquinones of type VIII, IX are sufficiently stable to permit electron spin resonance spectra to be obtained. The spectra of these $\dot{F}lR_2$-type radicals [9] are practically identical with those of the analogous anions $\dot{F}lR^-$ [10]. This is understandable since in VIII, IX the additional R-substituent of $\dot{F}lR_2$ is located in the pyrimidine part of the flavin nucleus (together with the negative charge of $\dot{F}lR^-$), where very little spin density is observed [8—10]. Hence both electron spin resonance and optical spectra, of



O-alkylated red species VIII, IX on the one hand and of blue F̈lRH on the other hand are totally different. This is direct proof that our previous [2,17] assignment of an iminol [O(2) and/or O(4)-protonated VIII—X, R = H] structure for F̈lH_2 was wrong. At the same time it is indirect evidence, that the acidic proton in F̈lRH must be located at N(5), yielding structure VII.

VIII and IX as iminol esters are much more strongly basic compared with the "amide" VI. VIII and IX need therefore excess base (dimethylformaamide/triethylamine) to be liberated from their cations, while at the same time the oxidized cations again become unstable, because of slow self condensation [10]. The resulting radical solutions exhibit brownish-red color slowly turning into green along with loss of electron spin resonance hyperfine resolution [10]. The green color belonging to the "biflavin" dimer [10], must not be confused with the similar green color of VII (*cf.* below).

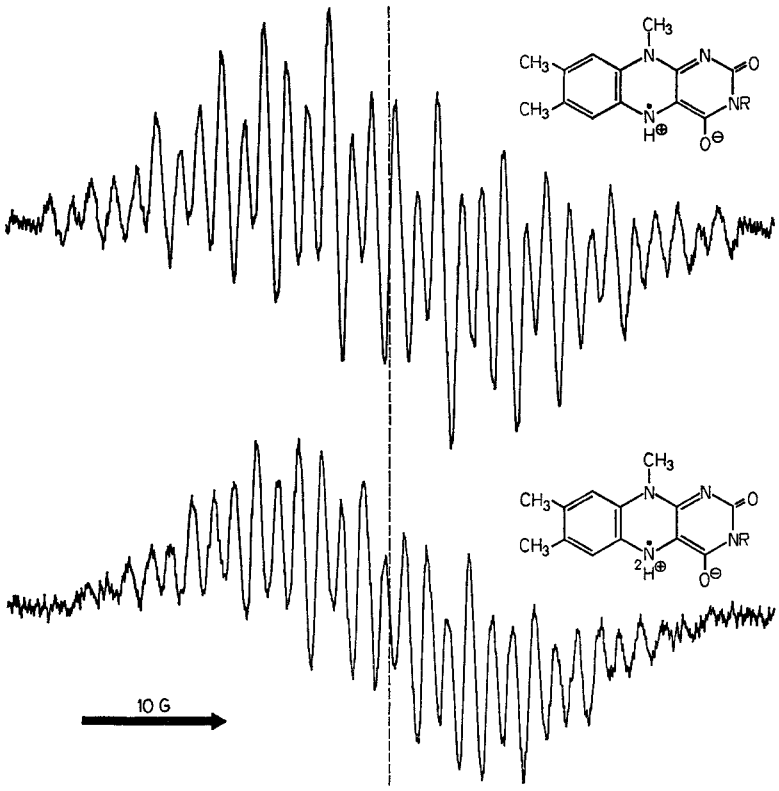


Fig. 1. *Electron spin resonance spectra of 5 mM half-reduced lumiflavin-3-acetic acid in 50 mM veronal and 100 mM $NaClO_4$: at pH 7.5 (upper curve) and at p ^2H 7.6 (lower curve) obtained as described under Methods*

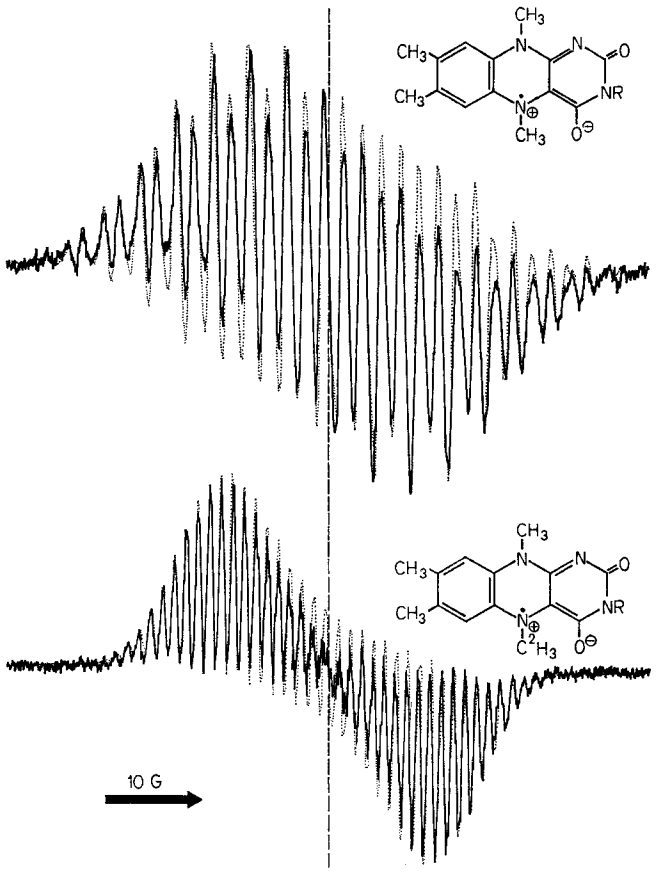


Fig. 2. *Experimental (——) and simulated (·····) electron spin resonance spectra of 10 mM N(5)-CH_3ĖlR (upper curve) and 10 mM N(5)-C^2H_3ĖlR (lower curve) in $CHCl_3$ (for the preparation of the solution see Methods)*

Unsubstituted (IIa, ĖlH$_2$) and N(5)-alkylated Flavosemiquinones (VII, ĖlR$_2$)

We did now confirm our earlier observation [6,7] that there is one exchangeable proton with a large hyperfine coupling in ĖlRH. A half-reduced solution of lumiflavin-3-CH_2COO^- in 50 mM veronal buffer, pH 7.5, produced a well resolved electron spin resonance spectrum consisting of an even number of evenly spaced hyperfine lines with a separation between components of 1.40 G, as shown in Fig. 1. When, in the above experiment, water was replaced by deuterium oxide, the electron spin resonance spectrum thus obtained is composed of an odd number of lines (Fig. 1). The radical yield thus obtained is low (*cf.* Chart I) and accordingly the hyperfine coupling of the active proton could not be evaluated quantitatively from these spectra.

In order to obtain a thermodynamically more stable model we tried reductive alkylation of N(3)-prealkylated flavoquinone ($Fl_{ox}R$) (*cf.* Methods). The resulting blue-green stable radicals were originally mistaken for O(2)- or O(4)-alkylated derivatives, as mentioned above. The electron spin resonance spectra of the radicals alkylated with CH_3I and C^2H_3I showed, however, dramatic differences (Fig. 2). The total widths could easily be determined and they differed with as much as 16.0 G (Table 1, derivatives No. 1 and No. 2). This large difference means that the coupling is so strong that the deuterium splitting can not be neglected in comparison to the line width. The difference thus represents $3\{a_5^{H}(NCH_3) - 2\,a_5^{2H}(NC^2H_3)\} = 3 \times 0.692\, a_5^{H}(NCH_3)$. Hence we obtained $a_5^{H}(NCH_3) = 7.7$ G. The only comparable coupling constant is that obtained for $a_5^{N} = 8.7$ G

Table 1. *Total width between outermost hyperfine lines and mean spacing of main lines for electron spin resonance spectra of various substituted neutral flavosemiquinones in chloroform, obtained as described under Methods*

The total widths are measured between outermost lines of the main hyperfine components with a mean spacing of about 1.85 G (1.3 G for derivative No. 2). These outermost lines are not always seen at the sensitivity used for recording the spectra shown in the Figures. For derivatives No. 13 and No. 14 the position of these lines was estimated under the broader envelope

Derivative No.	Fig. in which spectrum shown	Substituent (position) R = $CH_2COOC_2H_5$	Mean spacing	Total width of spectrum
			G	G
1	2	R(3) CH_3(5,7,8,10)	1.94	65.4
2	2	R(3) CH_3(7,8,10) C^2H_3(5)	1.3	49.4
3	3	R(3) CH_3(5,10) H(7,8)	1.88	60.0
4	3	R(3) CH_3(10) H(7,8) CH_2CH_3(5)	1.75	45.4
5	3	R(3) CH_3(10)H(7,8) $CH_2C_6H_5$(5)	1.90	45.7
6		R(3) CH_3(7,8,10) $CH_2C_6H_5$(5)	1.97	51.0
7		R(3) CH_3(7,8,10) CH_2CH_3(5)	2.02	52.2
8	4	R(3) CH_3(5,7,8,10) ^{15}N(1,3)	1.96	66.3
9	4	R(3) CH_3(5,7,8,10) ^{15}N(1,3,5)	1.91	61.1
10	4	R(3) CH_3(5,8,10) Cl(7) ^{15}N(10)	1.93	61.2
11		R(3) CH_3(5,8,10) Cl(7)	1.86	63.2
12	4	R(3) CH_3(5,7,10) Cl(8)	1.83	56.7
13		R(3) CH_3(5,7,8) C^2H_3(10)	7.60	54.0
14	4	R(3) CH_3(5) Cl(7,8) C^2H_3(10)	6.90	46.5

(Table 2, derivatives No. 8 and No. 9 of Table 1). Since the spin polarization parameters of a trigonal nitrogen and a methyl group bound to this nitrogen are approximately equal [8], the results demonstrate that the methyl group is bound to N(5). This is also the only possible position for the exchangeable proton with high coupling constant in the unalkylated semiquinone. Further support for the structure of the 5-alkylflavosemiquinones is obtained from the synthesis of the same type of radical by photobenzylation of flavoquinone with phenylacetic acid [21].

The coupling constant 2.7 G of CH_3 or H in position 8 is obtained from the difference between the total widths of spectra of derivatives Nos. 1 and 3 (Table 1). Somewhat larger values are obtained when

Table 2. *Isotropic hyperfine coupling constants for N(3,5)-dialkylated lumiflavin neutral radicals in chloroform*

Coupling constant	Hyperfine coupling constants	
	from difference in total width	from simulation fit
	G	G
a_6^N	8.7 ± 0.4 [a]	8.0 ± 0.2 [b]
$a_5^H(NH,NCH_3)$	7.7 ± 0.2	7.6 ± 0.2
a_6^H		1.7 ± 0.2
$a_8^H(CH,CCH_3)$	2.7 ± 0.3	2.4 ± 0.1
a_{10}^N	3.3 ± 0.4	3.6 ± 0.2
$a_{10}^H(NCH_3)$	3.8 ± 1.5	3.9 ± 0.2

[a] Maximal errors estimated from the accuracy of the total width determinations.
[b] Ranges within which the coupling constants could be varied without appreciable decrease of fit between simulated and experimental spectra.

the Cl(8)-compound, derivative No. 12, is compared with derivatives Nos. 1 and 3, which might be due to a perturbation of the spin density distribution caused by the Cl-substituent.

For the coupling of the methyl hydrogens in position 10 the value 3.8 G is obtained from derivatives Nos. 1 and 13, but this figure is somewhat uncertain since the hyperfine lines between which the widths were measured were not resolved. From the spectra of derivatives Nos. 10 and 11 the coupling of N(10) is calculated to be 3.3 G. This is close to the coupling of the N(10)-CH_3 group as would be expected.

The coupling constants obtained from the total width differences as described above are collected in Table 2. Starting from these values simulation was attempted and the constants adjusted in order to obtain good fit to the experimental curves. However, the simulation could only be made to fit by including one proton hyperfine coupling of 1.5—1.9 G, *i. e.* about the magnitude of the mean spacing (*cf.* Table 1). The most likely origins of this coupling is H (6), since this position shows considerable spin density in flavosemiquinone anions and metal chelates [8—10].

The ranges for the values of the coupling constants which gave the best-fitting simulations (Figs. 2, 3 and 4) are also given in Table 2. It should be noted that an increase/decrease of any one coupling constant is coupled with a corresponding decrease/increase of one or several of the other constants. The line width used was 0.70 G. Lorentzian line shape gave better intensity ratios than Gaussian line shape. The simulation experiments showed that the intensity ratios of the alternating lines of N(5)-CH_3-lumiflavin (Fig. 2, top spectrum) were extremely sensitive to the chosen values of $a_8^H(CCH_3)$. Thus the best fits were obtained with values of this coupling constant within the narrow range of 2.3—2.5 G. The other couplings could be varied over a somewhat larger region. From Table 2 it is seen that the center of the region of the improved coupling constants obtained by the simulation technique in most cases fall within the estimated limits of error of the more crude values obtained from the total width differences.

Comparison of the spectral width of derivatives No. 1 and No. 11 (Table 1) suggests a coupling constant of about 0.8 G to the (7)-CH_3 group. This value is too large since the simulations not including this coupling required a line width of 0.7 G, which would not permit the coupling to be larger than 0.2—0.3 G. As in case of Cl-substitution in position 8, the 7-Cl substituent of derivative No. 11 could perturb the spin density distribution somewhat and hence cause an exaggeration of the measured coupling.

When CH_3 at N(5) is replaced by the benzyl group only one of the 5′-methylene protons couples, with approximately the same splitting constant as the hydrogens of the N(5)-CH_3 group, that is 7.5 G (it should be noted that N(5) benzylated flavin derivatives are available only by the photochemical procedure as mentioned [21]). The other 5′-methylene proton has such a small coupling that it can be neglected to the first approximation, *i. e.* the coupling must be considerably smaller than the line width. Since two proton couplings of 7.5 G (4×1.9 G) are eliminated the spectrum should have 8 lines less than the corresponding N(5)-methylated analog (Fig. 3). Indeed, the spectrum of the benzyl derivative consists of 25 evenly spaced lines, whereas the methyl derivative has 33 lines (Fig. 3). This particular behaviour of the 5′-methylene protons is analogous to that of the 10′-methylene protons of the ribityl side chain of riboflavin [8] and indicates that the benzyl residue is not free to rotate. However, when the benzyl group is replaced by an ethyl group, a spectrum showing 27 lines with reduced spacing is obtained (Fig. 3) indicating that in this case the second proton of the 5-CH_2-R group interacts to a certain degree with the

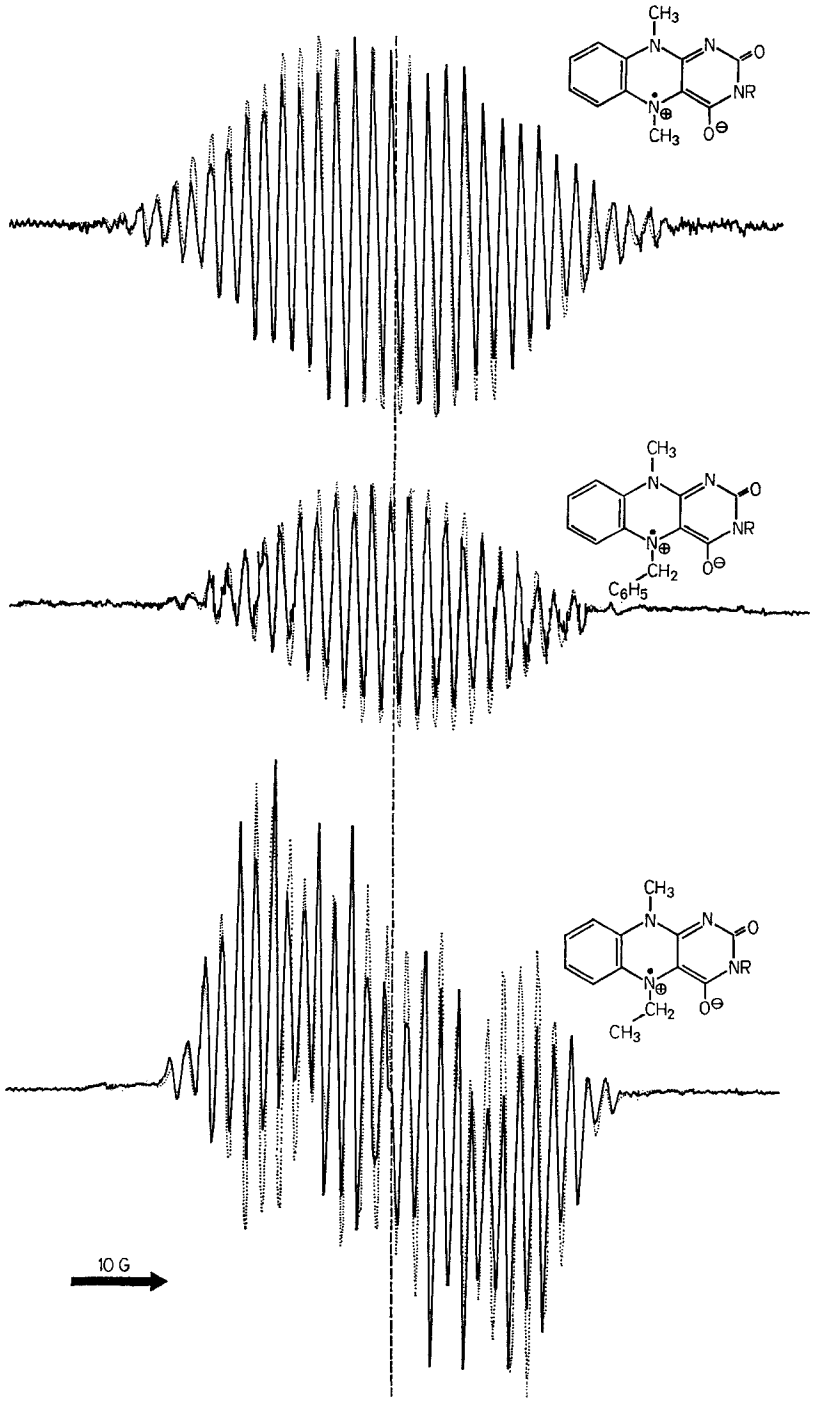


Fig. 3. *Experimental (——) and simulated (·····) electron spin resonance recordings of the neutral radicals of 10 mM various N(5)- substituted 10-methyl-isoalloxazine in $CHCl_3$, obtained as described under Methods*

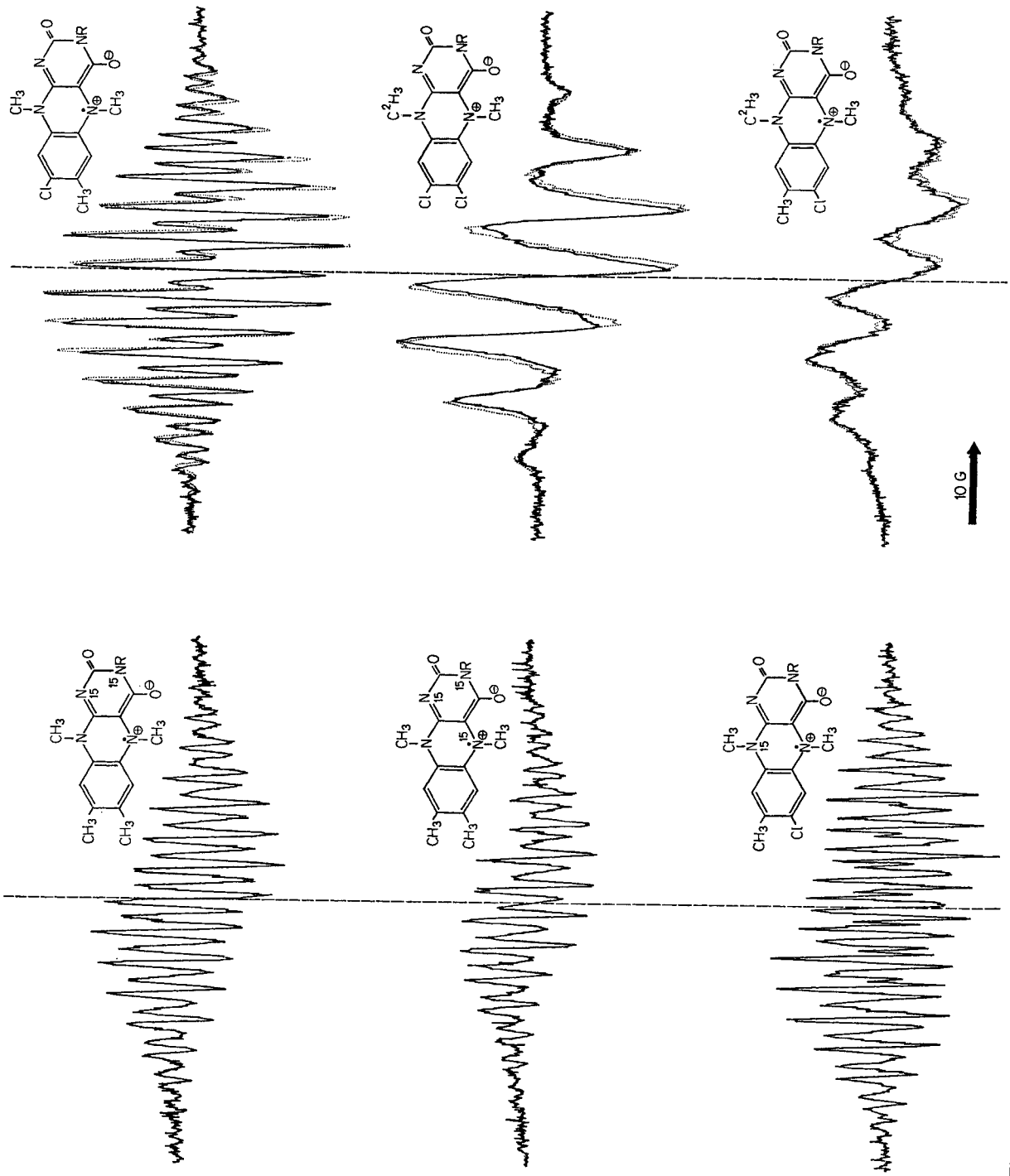


Fig. 4. *Experimental (——) and simulated (·····) electron spin resonance spectra of chemically and isotopically substituted neutral flavin radicals, 10 mM in $CHCl_3$, obtained as described under Methods*

N(5)-p_z-orbital due to its particular steric location. Thus one of the protons couples with a constant of 1.6 G whereas the other proton has a coupling constant of 6.7 G.

Substitutions of ^{14}N with ^{15}N in positions 1 and 3 of *N*(5)-methyl-lumiflavin yields a spectrum consisting of 35 lines (Fig. 4) with a mean spacing of 1.9 G, *i. e.* the same number of lines as obtained with the natural lumiflavin analog (Fig. 2). However, the superhyperfine shape of the two sets of spectra differs somewhat indicating that there is a very small but detectable spin density located at the nitrogen atoms in position 1 and/or 3. In acetate-cellosolve no hyperfine interaction could be observed for the nitrogens in position 1 and 3 [12].

As shown in Fig. 4, replacement of the protons by deuterons in the methyl group in position 10 yields electron spin resonance spectra with fewer lines and a much larger separation between the components than expected. Thus, the 7,8-dichloro-10-C^2H_3 lumiflavin derivative produces an electron spin resonance spectrum of seven lines (Fig. 4) (instead of the predicted 26 lines spectrum) with a mean spacing of about 6.9 G. Similar results were obtained with other 10-deuterated lumiflavin derivatives (Fig. 4). However, the decrease of lines and the increase of the spacing is not due to an increase in the line width, but is caused by the small coupling of the deuterons (about 0.5G). Thus, the deuteromethylated lumiflavin electron spin resonance spectra (Fig. 4) have been simulated by using the line width, as used for the simulation of the other spectra (0.70 G) and including the small deuterium coupling. However, when the deuterium coupling was omitted from the simulation, a spectrum consisting of 26 lines was obtained for the 7,8-dichloro-10-C^2H_3-lumiflavin derivative as to be expected.

Spin Densities and Molecular Orbital Calculations

Some approximate relationships have been established which relate, in a linear fashion, the p_z spin density on a trigonal carbon or nitrogen atom to the hyperfine coupling of the attached proton or methyl group [13,22,27]. For the calculation of the spin density at various atoms of the isoalloxazine ring we used the following simple expression:

$$a^x = Q_x \varrho_x$$

where a^x is the hyperfine coupling constant of the atom x, ϱ_x is the spin density in the p_z-orbitals of the respective C- or N-atoms and Q_x is the spin polarization parameter of the atoms in question. The values for the different Q_x are as follows:

$$Q_N = Q_{NCH_3} = 18.5-28.5 \text{ G}$$

$$Q_C = Q_{CCH_3} = 21-27 \text{ G}.$$

(For a more detailed discussion of the given equation and of the Q_x values see *e. g.* [8,13,27,28].)

From the hyperfine coupling constants, given in Table 1, and by means of the spin polarization parameters, we can now estimate roughly the spin densities at some atoms of ḞlRH.

The results are given in Table 3, where for comparison results from quantum chemical calculations as obtained by Song [29] are also included. The calculated values are, except for the value of the spin density on N(10), in good agreement with our experimental results.

Table 3. *Comparison between experimental spin densities and theoretically calculated distributions of the unpaired electron in the flavin neutral radical*

The range of values stated for electron spin resonance is determined by the limits of the spin polarization parameters (Q) found in the literature. All values are given in units of the negative electronic charge. Only positions relevant for the interpretation of our spectra are included

Position	Atom	Electron spin resonance	Calculated [29]
1	N	approx. 0	0.020
3	N	approx. 0	−0.021
5	N	0.282−0.432	0.382
6	C	0.067−0.085	0.146
7	C	approx. 0	0.085
8	C	0.089−0.115	0.120
9	C	approx. 0	0.077
10	N	0.126−0.194	0.328

CONCLUSIONS

Two flavin radical species are firmly established to exist under physiological conditions. They differ distinctly both in light and electron spin resonance absorption, the blue-green neutral ḞlR(3)H(5) [2,15,16] and the red anionic ḞlR$^-$ [10,15]. In addition, we have observed a tautomer form of the neutral radical species, namely $\dot{F}lR_2$(2,3, or 4) [9]. Both types of neutral radicals can be stabilized as alkyl derivatives. N(5)-alkylated blue radicals are the products of flavin photoalkylation as well as reductive alkylation, whereas O(2)- and/or O(4)-alkylated red radicals ($\dot{F}lR_2$) can be obtained upon alkylation of *N*(5)-acetylleucoflavins followed by removal of the protecting group.

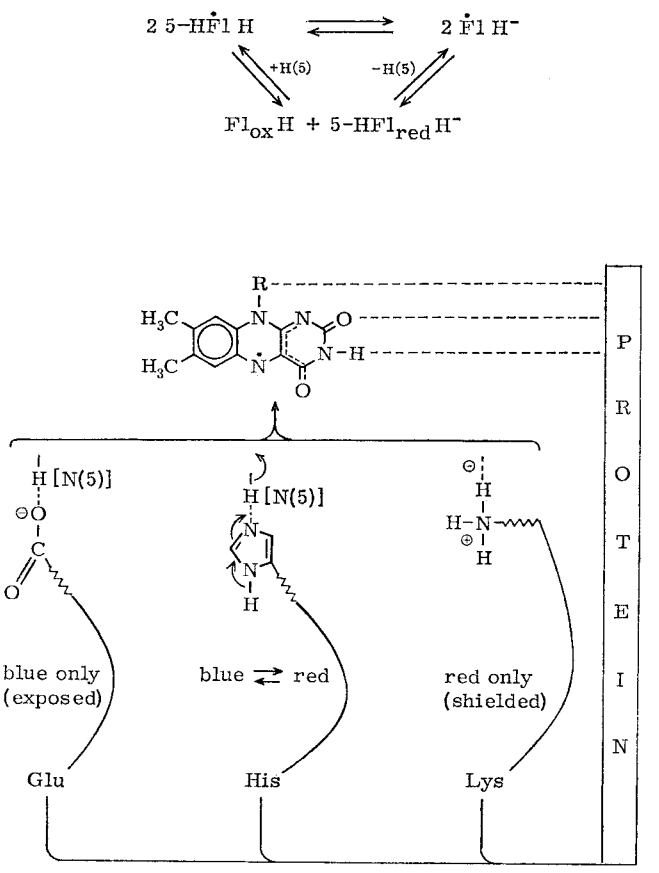
The blue type of flavoprotein radicals is found to be consistent with $\dot{F}lH_2$, the two labile protons occupying positions N(3) and N(5). As with $\dot{F}lH_2$, the proton at N(5) of the flavocoenzyme in blue flavoprotein radicals is electron spin resonance active [30]. However, with the exception of glucose oxidase, the blue flavoprotein radicals can not be transformed into the anionic red form, even at relatively high pH values [15].

The red type of flavoprotein radicals is either due to the corresponding anion (one acidic proton left at

N[3]) or to the tautomeric neutral species, the two protons occupying positions 2, 3 and/or 4, but not position 5. Thus, different apoproteins might favor the formation of different semiquinones, while the free chemical system under neutral conditions exhibits the blue (N[5]-protonated) tautomer only. In some flavoproteins the red radical anion could conceivably be stabilized by chelate formation with a redox-inactive metal ion [9,31].

Furthermore, non-alkylated $\dot{F}lH_2$ exists at neutral pH only in a small equilibrium concentration, whereas protein-bound flavocoenzymes yield the radical almost quantitatively [15]. This raises the question, whether this radical stabilization by the apoprotein is caused by hindered flavin-flavin contact at the enzyme active site (kinetic stabilization of the radical) or by a true shift of equilibrium B (Chart I, R = H). However, while the self reaction of flavocoenzyme is slowed down markedly at the enzyme surface compared to the free chemical system, it is still fast enough to allow adjustment of equilibrium B within minutes. Thus, what is the reason for the apparent thermodynamic stabilization of the flavosemiquinone bound to the protein? Equilibrium B (Chart I) is governed by the *pK* values of the contributing species [4,10]. It has been shown experimentally that the dissociation at N(3), *i.e.* $Fl_{ox}H \rightleftarrows Fl_{ox}$ (*pK* 10.4 in FMN and FAD) can be modified significantly in either direction depending on the protein environment [32]. Hence, for stabilization of $\dot{F}lH^-$ in the bound state it would be sufficient to assume that the *pK* of the flavin radical ($pK^{H}_{FlH_2}$ = 8.4 in the free state [10]) is decreased by the protein, in the same way as shown with chelating metal ions [4]. The stabilization of either form of the flavin radical in flavoproteins could be explained, if the proton at N(5) is stabilized by a strong hydrogen bond to the protein. The stabilization of both, $\dot{F}lH_2$ and $\dot{F}lH^-$ could thus be explained by the presence of either an H^+-donating (*e.g.* COOH from glutamic acid) or an H^+-withdrawing group (*e.g.* NH_2 from lysine) in the neighborhood of flavin-N(5) or O(4) at the active site of the enzyme (Chart II). The exceptional case of glucose oxidase, where blue and red semiquinones are stabilized depending on pH, can be visualized, on the other hand, by a flavin-*N*(5)-imidazole connection (*cf.* Chart II, below) which allows regulation of the charge of the radical without removing the stabilizing group from N(5).

Chart II



ADDENDUM

After this work was finished, and that of a forthcoming paper on flavin cation radicals [20], and while these papers were in preparation, the possibility to make ENDOR measurements on these radicals was discovered [33,34]. Based on the present results and on the cation radicals [20] a search was made for the best solvents for ENDOR experiments. It was found that these conditions also gave better resolved electron spin resonance and optical spectra. These later results have already been published separately [35].

This work was supported in part by a stipend of the CIBA Foundation, Basel, Switzerland and Career Development Award (K4-GM-42,599) (to F. M.), by grants from Deutsche Forschungsgemeinschaft (Projekt: He 5681), and Schweizerischer Nationalfonds (Projekt 4201), (to P. H.), by U. S. Public Health Service (AM-05895), Statens Medicinska Forskningsråd and Statens Naturvetenskapliga Forskningsråd, (to A. E.), by U. S. Public Health Service (GM 11106), (to V. M.), by U. S. Public Health Service (GM 12176) and Career Development Award (GM-K3-31,213), (to G. P.).

REFERENCES

1. Michaelis, L., Schubert, M. P., and Smythe, C. V., *J. Biol. Chem.* 116 (1936) 587.
2. Dudley, K. H., Ehrenberg, A., Hemmerich, P., and Müller, F., *Helv. Chim. Acta*, 47 (1964) 1354.
3. Michaelis, L., and Schwarzenbach, G., *J. Biol. Chem.* 123 (1938) 527.
3a. Hemmerich, P., unpublished result.
4. Hemmerich, P., *Helv. Chim. Acta*, 47 (1964) 464.
5. Draper, R. D., and Ingraham, L. L., *Arch. Biochem. Biophys.* 125 (1968) 802.

6. Ehrenberg, A., *Arkiv Kemi*, 17 (1962) 97.
7. Ehrenberg, A., Eriksson, L. E. G., and Müller, F., In *Flavins and Flavoproteins* (edited by E. C. Slater), Elsevier, Amsterdam 1966, Vol. 8, p. 37.
8. Eriksson, L. E. G., and Ehrenberg, A., *Acta Chem. Scand.* 18 (1964) 1437.
9. Müller, F., Eriksson, L. E. G., and Ehrenberg, A., *Eur. J. Biochem.* 12 (1970) 93.
10. Ehrenberg, A., Müller, F., and Hemmerich, P., *Eur. J. Biochem.* 2 (1967) 286.
11. Müller, F., Ehrenberg, A., and Eriksson, L. E. G., In *Magnetic Resonance in Biological Systems* (edited by A. Ehrenberg, B. G. Malmström and T. Vänngård),, Pergamon, Oxford 1967, p. 281.
12. Ehrenberg, A., and Eriksson, L. E. G., *Arch. Biochem. Biophys.* 105 (1964) 453.
13. McConnell, H. M., and Chesnut, D. B., *J. Chem. Phys.* 28 (1958) 107.
14. Land, E. J., and Swallow, A. J., *Biochemistry*, 8 (1969) 2117.
15. Massey, V., and Palmer, G., *Biochemistry*, 5 (1966) 3181.
16. Beinert, H., *J. Amer. Chem. Soc.* 78 (1956) 5323.
17. Ehrenberg, A., Eriksson, L. E. G., and Hemmerich, P., In *Oxidases and Related Redox System* (edited by T. E. King, H. S. Mason, and M. Morrison), John Wiley and Sons, New York 1965, p. 179.
18. Hemmerich, P., Prijs, B., and Erlenmeyer, H., *Helv. Chim. Acta*, 43 (1960) 373.
19. Dudley, K. H., and Hemmerich, P., *Helv. Chim. Acta*, 50 (1967) 355.
20. Müller, F., Hemmerich, P., and Ehrenberg, A., unpublished results.
21. Walker, W., Hemmerich, P., and Massey, V., *Helv. Chim. Acta*, 50 (1967) 2269.
22. Ingram, D. J. E., *Free Radicals as Studied by Electron Spin Resonance Spectroscopy*, Butterworth, London 1958.
23. Stone, E. W., and Maki, A. H., *J. Chem. Phys.* 38 (1963) 1999.
24. Gibson, Q. H., Massey, V., and Atherton, N., *Biochem. J.* 85 (1962) 369.
25. Dudley, K. H., and Hemmerich, P., *J. Org. Chem.* 32 (1967) 3049.
26. Carrington, A., and Longuet-Higgins, H. C., *Mol. Phys.* 5 (1962) 447.
27. McLachlan, A. D., *Mol. Phys.* 1 (1958) 233.
28. Bernal, J., Rieger, P. H., and Fraenkel, G. K., *J. Chem. Phys.* 37 (1962) 1489.
29. Song, P. S., *Ann. N. Y. Acad. Sci.* 158 (1969) 410.
30. Müller, F., Palmer, G., and Massey, V., unpublished results.
31. Müller, F., Hemmerich, P., and Ehrenberg, A., *Eur. J. Biochem.* 5 (1968) 158.
32. Massey, V., and Ganther, H., *Biochemistry*, 4 (1965) 1161.
33. Ehrenberg, A., Eriksson, L. E. G., and Hyde, J. S., *Biochim. Biophys. Acta*, 167 (1968) 482.
34. Eriksson, L. E. G., Hyde, J. S., and Ehrenberg, A., *Biochim. Biophys. Acta*, 192 (1969) 211.
35. Walker, W., and Ehrenberg, A., *FEBS Letters*, 3 (1969) 315.

F. Müller and V. Massey
Department of Biological Chemistry
The University of Michigan Medical School
Ann Arbor, Michigan 48104, U. S. A.

P. Hemmerich
Fachbereich Biologie der Universität
BRD-7550 Konstanz, Postfach 733, Germany

A. Ehrenberg
Biokemiska Avdelningen, Karolinska Institutet
Solnavägen 1, S-10401 Stockholm 60, Sweden

G. Palmer
Biophysics Research Division
218 Institute of Science and Technology
The University of Michigan
Ann Arbor, Michigan 48105, U.S.A.