Eur. J. Biochem. 116, 17–25 (1981)

On the Molecular and Submolecular Structure of the Semiquinone Cations of Alloxazines and Isoalloxazines as Revealed by Electron-Paramagnetic-Resonance Spectroscopy

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(Received November 7, 1980)

The thermodynamically stable and, therefore, analytically most important alloxazine and isoalloxazine radical cations have been studied in detail by electron paramagnetic resonance (EPR) spectroscopy. Isotopic and chemical substitutions have been made as in earlier studies with the less stable neutral and anionic species. The experimental spectra have been calculated with the aid of a more sophisticated computer-simulation program than previously used. Excellent fits were obtained only when all of the following atoms were taken into account in the hyperfine coupling scheme: N-5 H, N-10 H or CH_3, C-6 H, C-7 H, C-8 H or CH_3 and C-9 H. An additional but small coupling constant was required for the fit. This latter coupling constant is assigned to the nitrogen atom(s) of the pyrimidine subnucleus of (iso)alloxazine radical cations. The EPR-active proton is attached to N-5 as we also found for the neutral flavosemiquinone.

The alloxazine and isoalloxazine radical cations exhibit an identical hyperfine coupling scheme but differ especially in the pyrazine nucleus with respect to the spin density distribution. This suggests that the geometrical structure of the two kinds of radicals is somewhat different. The highest spin density is, however, located at N-5 of (iso)alloxazine as has been found for the other flavosemiquinone species.

The hyperfine coupling constants are interpreted in terms of spin densities and comparison is made with the most recently available quantum chemical calculations. All monomeric flavosemiquinone species are compared with each other and their differences in the submolecular structure are discussed briefly.

Flavin is a heteroaromatic system capable of undergoing two-electron oxidoreduction, i.e. (de)hydrogenation, as well as one-electron transfer. It is ubiquitous in redox metabolism as a means of 'splitting electron pairs' (e.g. [1]) and apparently it is the only available biological system to achieve this goal, which is prerequisite for energy conservation in any biological redox chain. This unique property of flavin implies the thermodynamic stability of an intermediate one-electron redox state (flavosemiquinone) which was recognised as early as 1936 by Michaelis et al. [2]. The first flavosemiquinone was isolated by Kuhn and Ströbele [3]; it was the cationic form and was termed 'rhodoflavin' by these authors.

In previous papers, we have reported on the molecular and submolecular structure of flavosemiquinone radicals in the neutral ($\dot{H}FlR, \dot{F}lR_2$ [4], in the anionic ($\dot{F}lR^-$ [5,6] and the metal-chelated ($[Me\dot{F}lR]^+$ [7]) states. These studies revealed that the unpaired electron in these radicals is mainly localized in the benzene and pyrazine part of the flavin nucleus, whereas the spin density in the pyrimidine subnucleus (position 1–4) is negligible. On the other hand, controversy remains about the flavin radical cation ($H\dot{F}lR_2^+, \dot{F}lRH_2^+$): Guzzo and Tollin [8] have assigned the point of maximum spin density to N-1 in the case of flavin (i.e. isoalloxazine) radical-cation, and to N-5 in the case of alloxazine analogs [9]. It seems very improbable that the flavin and alloxazine radical cations would differ in submolecular structure because the only difference in chemical structure concerns the substituent in position 10, which is an alkyl group for flavins and a proton for alloxazine radical cations.

The special importance of the flavosemiquinone cation resides in its thermodynamic stability. At pH < 0 the half-reduced flavin system is fully comproportionated [Eqn (1)].

$$\begin{array}{ccc} Fl_{ox}R + H_2Fl_{red}R & \overset{2H^+}{\rightleftharpoons} & HFl_{ox}R + H_3Fl_{red}R^+ \\ \updownarrow & & \updownarrow \\ 2\,H\dot{F}lR & \overset{2H^+}{\rightleftharpoons} & 2\,H_2\dot{F}lR^+. \end{array} \quad (1)$$

Since reduction beyond the half-reduced state is thermodynamically disfavored under acid conditions, while autoxidation is slow, any flavin system in acids (6 M HCl, HCOOH,

Abbreviations. Electron paramagnetic resonance, EPR; flavin in oxidized state, $Fl_{ox}R$, where R denotes a substituent at N-3 [ring numbering according to IUPAC-IUB tentative rules (1967) *Eur. J. Biochem.* 2, 5, cf. Scheme]; flavin in fully reduced state, H_2Fl_{red}; neutral flavosemiquinone, $H\dot{F}lR$; anionic flavosemiquinone, $\dot{F}lR^-$; flavosemiquinone metal chelate, $[Me\dot{F}lR]^+$.

Nomenclature. The term flavin means the 10-alkylated 6,7-benzopteridine-2,4-dione, i.e. the isoalloxazine nucleus, irrespective of the redox state. Its oxidized form is called 'flavoquinone' ($Fl_{ox}R$), its fully reduced form 'flavohydroquinone', or 1,5-dihydroflavin ($1,5\text{-}H_2Fl_{red}R$). The term 'flavosemiquinone' is assigned to the intermediate radical form ($H\dot{F}lR$). Lumiflavin means 7,8,10-trimethylisoalloxazine and lumichrome means 7,8-dimethylalloxazine.

CF_3COOH) and in the presence of approximately stoichiometric amounts of reductants ($TiCl_3$, Zn, $SnCl_2$) will appear as 100% radical, even in the presence of small amounts of oxygen. Hence, even a crude biological preparation containing small amounts of flavin ($\approx 10\ \mu M$) can be assayed by EPR after photolysis (see Discussion) and acid hydrolysis. Structural conclusions can be drawn from the hyperfine splitting constants. This procedure has first been utilized successfully in the elucidation of the covalently bound flavocoenzyme from succinate dehydrogenase [10].

The studies presently reported were begun in 1966 and preliminary data of this work have been published elsewhere [11–13] and have been cited on various occasions. In particular Ehrenberg and Eriksson [13] reported preliminary data from isotopically substituted flavin derivatives showing that the radical cations exhibit a spin distribution rather similar to those of the neutral and anionic derivatives as far as the negligible spin density in position 1–4 is concerned.

Meanwhile, Westerling et al. [14] have repeated some of our experimental spectra published by us in a preliminary from [12] and have tried to improve the coupling constants by calculations but were not able to specify the coupling constants due to the benzene subnucleus of the flavin molecule. According to their own statement, only the two coupling constants due to the proton in position 5 and the methyl group in position 10 in 10-methyl-isoalloxazine could be assigned beyond doubt. In this paper we present a complete assignment of the coupling constants. In addition a new computer-simulation program has allowed us to determine the small coupling constants of the positions 7 and 9 of the flavin molecule.

MATERIALS AND METHODS

All solvents used were reagent grade. The synthesis of the alloxazine and isoalloxazine derivatives has been described elsewhere: 1,3-dimethyl-alloxazine [15], lumiflavin, 3,7,10-trimethyl-isoalloxazine [16], 3,8,10-trimethylisoalloxazine, 3,7,8,10-tetramethyl-(6,9-$^2H_2$)isoalloxazine, 3,10-dimethyl-(6,7,8,9-$^2H_4$)isoalloxazine [17], 3,10-dimethylisoalloxazine [5], (9-^{2}H]lumiflavin [18], 1,3-dimethyl-(6,7,8,9-$^2H_4$)-alloxazine was prepared in the same way as 3,10-dimethyl-(6,7,8,9-$^2H_4$)isoalloxazine [17]. N^3-substituted flavin derivatives have been used, since substitution at position 3 has little influence on the EPR spectra [5], while it enhances the solubility of the flavin derivatives in organic apolar solvents.

The flavin radical cation solutions can be obtained in either of the following ways. (a) The flavin derivative to be used can be dissolved in the acid and aliquots of oxidized and reduced flavin solution mixed under anaerobic conditions and transferred to an argon-flushed quartz capillary in a similar manner as described elsewhere [5]. Solutions of reduced flavins in an acid medium can be obtained by catalytic hydrogenation with Pd on asbestos as catalyst as described previously [5]. (b) To an anaerobic solution of oxidized flavin can be added either one equivalent of $SnCl_2 \cdot 2\,H_2O$ dissolved in the acid used or $TiCl_3$ solution. (c) Reduction has been achieved by addition of metallic (granular) Zn. In this study, a mixture of 6 M HCl/CH_3CN (1:1, by vol.) was used as solvent. The reduction was achieved by $TiCl_3$. The degree of reduction of all flavin solutions was kept at about 50%. The concentrations of the red cationic flavosemiquinone solutions thus obtained ranged over 1–5 mM.

Deuterium exchange experiments were carried out in deuterated formic acid (Merck, Darmstadt, FRG) or in 6 M $^2HCl/CH_3CN$ (1:1 by vol.).

The EPR spectra were recorded on a Bruker ER 200 D spectrometer. The instrument was connected to a Data General NOVA 3 computer for storing and handling the experimental spectra. Routinely 4K data points were used for the accumulation of the spectra. Between one and four scans were averaged to obtain smooth spectra. Quartz capillaries of 1.3-mm inner and 4.4-mm outer diameter were used.

All experiments were carried out at 20–22 °C.

The hyperfine coupling constants were obtained by least-squares fitting of the Fourier transform of the EPR data as described by Dunham et al. [19] except for two algorithmic changes. First, the standard (unweighted) least-squares criterion was employed because the rates of convergence of the unweighted and the more expensive weighted criterion were similar. Use of the unweighted criterion has the advantage that minimization in Fourier space of the relative squared error (the sum of the squares of the errors divided by the sum of the squares of the data) is mathematically equivalent to minimization of this same quantity in EPR space because the relative squared error is invariant with respect to the Fourier transform. Second, we incorporated the center correction which appears whenever the center of the spectrum does not occur at a data point. The Fourier transforms of the spectra were found to contain a real component which can be identified as arising from the presence of a small amount of dispersion. In Fourier space, dispersion is a constant times i (the imaginary unit) times absorption. The fitting algorithm, therefore, was applied to the complete Fourier transform of the EPR data with the coupling constants, line width, center correction, and the magnitudes of the dispersion and absorption components as parameters. In the fitting procedure Lorentzian line shape was used.

The usual F-tests for regression and lack-of-fit are not applicable because the theoretical expression for the Fourier transform is nonlinear in the parameters, e.g. the coupling constants. Consequently, a facility for obtaining information on the confidence region was incorporated into the fitting algorithm. Specifically, after an optimal solution was obtained, it was perturbed and, during the subsequent optimization, the minimum and maximum values for all parameters were computed for all points in the parameter space such that the relative squared error was within 10% of its optimal value: a 10% perturbation exceeds the 95% confidence limit for the tests commonly employed in linear least-squares fitting. For the larger hyperfine coupling constants, those greater than 0.1 mT, the limits were never greater than $\pm 5\ \mu T$. During the optimization, 400–2500 points within the confidence region were examined.

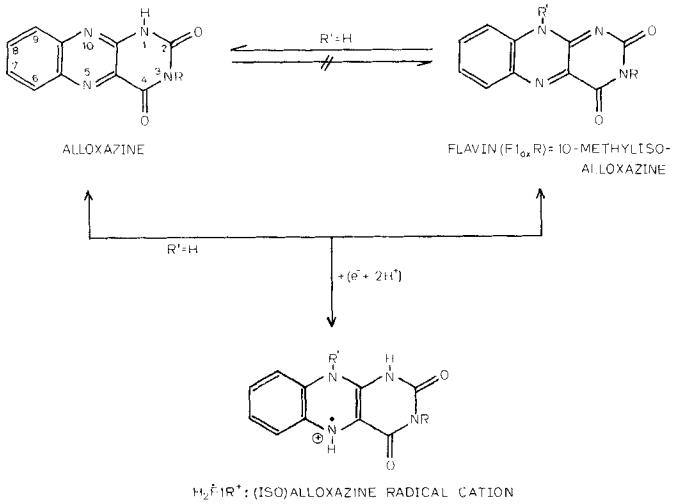
The limits for the smaller coupling constants, 0.02–0.05 mT, were sometimes of the order of 0.02 mT, particularly when there were three or more small singlet couplings of the same type. This lack of precision in the smaller coupling constants is attributed to two factors. First, the algorithm for obtaining the confidence limits is not sophisticated enough to detect when two close coupling constants of the same type were interchanged during the optimization; in this case, the confidence limits for each coupling constant contain both coupling constants. Second, over the relevant portion of Fourier space, the aggregate effect of numerous small coupling constants can be achieved in a number of mathematically equivalent forms; the same conclusion can be reached in EPR space using the continuity of the line shape

function. The basic structure of an EPR spectrum in Fourier space is determined primarily by the larger coupling constants, while the line width and smaller coupling constants serve to eliminate the periodic recurrence of this structure. Consequently, it is the aggregate effect of the small coupling constants rather than their individual effects which decreases the relative squared error (cf. Table 2).

RESULTS AND DISCUSSION

In general, the natural flavocoenzyme radicals yield EPR spectra which are poorly resolved for the following two reasons. First, the free coenzymes can only be handled in highly polar media because of the strictly hydrophilic phosphate and polyhydroxylic side chain. Second, this side chain is not free to rotate because of its strong solvation, which renders the N-10 methylene protons non-equivalent [6]. For these reasons, the side chain should preferably be degraded either to the lumiflavin ($R' = CH_3$) or 7,8-dimethyl-alloxazine ($R' = H$) state before EPR analysis [10] (cf. Scheme). Accordingly, in our present model studies we investigated isoalloxazine and alloxazine derivatives.

The radical cations, in contrast to all other radical (iso)-alloxazine species, are particularly suited for EPR studies for two reasons. First, the radical cations, in contrast to the neutral and anionic radicals, exhibit high thermodynamic stability as already mentioned in the introduction [cf. Eqn (1)]. The radical is more strongly basic ($pK = 2.3$ [20]) than either of the disproportionated constituents ($pK_{ox} \approx 0.5$ and $pK_{red} \ll 0$) of Eqn (1). This was observed for the first time as early as 1937 when Kuhn and Ströbele termed these radicals 'rhodoflavins' [3]. Second, the species $H_2\dot{F}lR^+$ has the same chromophore structure for flavins (isoalloxazines, cf. Scheme and Table 1) and alloxazines (cf. Scheme and Table 1) since all four nitrogen atoms in $H_2\dot{F}lR^+$ are 'three-coordinated', having either H or alkyl as third substituent. Therefore, the difference between the alloxazine and isoalloxazine chromophore is limited to the neutral ($H\dot{F}lR$) and anionic ($\dot{F}lR^-$) states of the radical and the oxidized state ($Fl_{ox}R$). Thus, alloxazine and isoalloxazine derivatives should yield similar EPR patterns for the radical cations, whereas alloxazine neutral and anionic radicals are still unexplored because of their instability, in contrast to the corresponding isoalloxazine (= flavin) radicals $H\dot{F}lR$ and $\dot{F}lR^-$. The overall process of the formation of radical cations (e.g. I, Table 1) involves the addition of one electron and two protons to the stable oxidized forms.



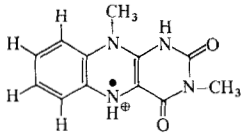
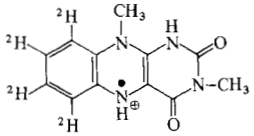
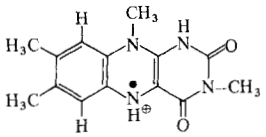
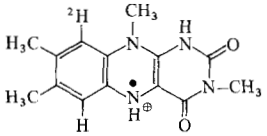
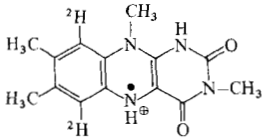
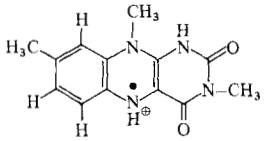
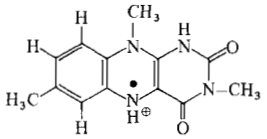
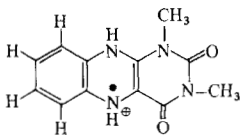
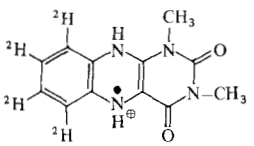
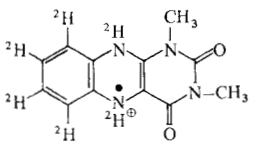
Scheme

Iminol tautomers (H at O-2α or O-4α instead of at N-1) would also be in agreement with the EPR data, but these tautomers are not considered in this study because they are usually present in very small amounts only and the EPR spectra do not yield information in this context. The reason for this is the low spin density in the pyrimidine subnucleus of the isoalloxazine ring as evidenced by replacement of ^{14}N by ^{15}N at positions 1 and 3 of the flavin nucleus which does not affect the electron paramagnetic resonance spectra of $\dot{F}lR^-$ [5] and $H\dot{F}lR$ [4]. For the cationic radical this was already ascertained by the preliminary data of Ehrenberg and Eriksson [13].

Rough coupling constants [12] of the isoalloxazine cation radicals have been estimated from experimental spectra by comparison of the total width of spectra of chemically and isotopically (^2H, ^{15}N) substituted derivatives as described earlier [4]. However, we noticed that this method yielded less reliable results for $H_2\dot{F}lR^+$ than for $\dot{F}lR^-$ and $H\dot{F}lR$ owing to the varying degree of resolution of the spectra from one derivative to another one. For the same reason it was not possible to simulate the experimental spectra by the relatively simple computer program [21] used previously [4]. In addition the fits of the spectra also require some small coupling constants (see below) which could not be estimated from the experimental spectra but which are in fact crucial for a good fit.

Dunham et al. [19] have recently developed a new computer program for the simulation of EPR spectra. With the aid of this program, it was possible to simulate the experimental spectra. The experimental spectrum of 3,10-dimethyl-isoalloxazine radical cation (I) is compared with the simulated one in Fig. 1. The experimental and simulated spectra are given separately because, in an overplot, the very small differences between them cannot be visualized; in fact, the difference shows up only in the intensity of a few of the lines. The Fourier transforms of these spectra are also given in Fig. 1 to demonstrate their features (for details see [19]). The experimental and simulated spectra of 1,3-dimethyl-alloxazine radical cation (VIII) are overplotted in Fig. 2. The error curve included in Fig. 2 demonstrates the excellent agreement between the experimental and simulated spectra. The computed coupling constants of a few compounds are summarized in Table 1. The larger coupling constants agree well with those published by Westerling et al. [14]. However the small coupling constants given in Table 1 are needed for a good fit. In terms of the relative squared error, this is illustrated in Table 2, while Fig. 3 provides visual evidence in EPR space. The results were obtained by varying the coupling constants available to the fitter. The results were accepted as optimal when the addition of a coupling constant or set of related coupling constants failed to improve the relative squared error by 10%, the confidence limit. Small coupling constants known to be present from the structure of the compounds but which did not improve the relative squared error within the 10% limit were not accepted. Although the smaller coupling constants obtained in this manner may be as accurate as the larger coupling constants, these values should be used only to infer the order of magnitude of the coupling constants and to conclude that these and all other coupling constants are strictly limited by their values,

Table 1. *Isotropic hyperfine coupling constants for alloxazine and isoalloxazine semiquinone cations*
The values were obtained by fitting the experimental spectra by computer simulation. The experimental spectra were obtained in 6 M HCl/CH_3CN

Compound	No.	Coupling constant at ring position				
		5		10		
		N	H	N	CH_3	H
		mT				
	I	0.736	0.775	0.484	0.503	–
	II	0.734 0.734	0.784 0.784	0.481 0.481	0.502 0.502	– –
	III	0.732	0.779	0.475	0.497	–
	IV	0.731 0.731	0.779 0.779	0.468 0.468	0.485 0.485	– –
	V	0.734 0.734	0.782 0.782	0.476 0.477	0.501 0.501	– –
	VI	0.746	0.782	0.477	0.481	–
	VII	0.723	0.771	0.489	0.502	–
	VIII	0.762	0.816	0.402	–	0.408
	IX	0.760 0.760	0.807 0.808	0.400 0.400	– –	0.400 0.400
	X	0.751 0.750	0.126[a] 0.125[b]	0.394 0.394	– –	0.062[a] 0.063[b]

[a] Coupling constant due to deuteron.
[b] Spectrum simulated by fixing the deuteron value at the proton value of the parent compound divided by 6.513.

(1:1, by vol.). For the accuracy of the coupling constants, see Materials and Methods. RSE = relative squared error (cf. Materials and Methods)

Coupling constant at ring position						Aggregate coupling (nitrogen)	Line with	Number of main lines	RSE
8		6	9	7					
H	CH_3	H	H	H	CH_3				
mT									μT
0.270	–	0.175	0.049	0.040	–	0.020	0.052	22	0.098
0.040[a]	–	0.035[a]	–	–	–	0.019	0.067	18	0.298
0.041[b]	–	0.027[b]	0.008[b]	0.006[b]	–	0.025	0.066	18	0.300
–	0.322	0.154	0.040	–	0.050	0.021	0.068	22	0.210
–	0.332	0.170	–	–	0.054	0.026	0.078	20	0.237
–	0.332	0.170	0.006[b]	–	0.054	0.026	0.077	20	0.237
–	0.318	0.025[a]	–	–	0.051	0.020	0.074	23	0.413
–	0.318	0.024[b]	0.006[b]	–	0.051	0.025	0.073	23	0.413
–	0.318	0.176	0.060	0.047	–	0.021	0.083	22	0.474
0.287	–	0.156	0.031	–	0.042	0.024	0.057	22	0.256
0.251	–	0.152	0.054	0.040	–	0.018	0.056	21	0.399
0.040[a]	–	0.028[a]	–	–	–	0.038	0.056	10	0.089
0.039[b]	–	0.029[b]	0.008[b]	0.006[b]	–	0.041	0.057	10	0.090
0.040[a]	–	0.029[a]	–	–	–	0.020	0.053	4	1.053
0.039[b]	–	0.023[b]	0.008[b]	0.006[b]	–	0.032	0.052	4	1.168

i.e. the compounds contain no additional coupling constants significantly greater than 0.01 mT. This is particularly true of the small nitrogen coupling constant(s) of the order of 0.020 mT, which should be interpreted as a mathematical representation of the aggregate effect of the coupling constant(s) from the pyrimidine subnucleus of the (iso)alloxazine molecule, i.e. positions 1 and 3.

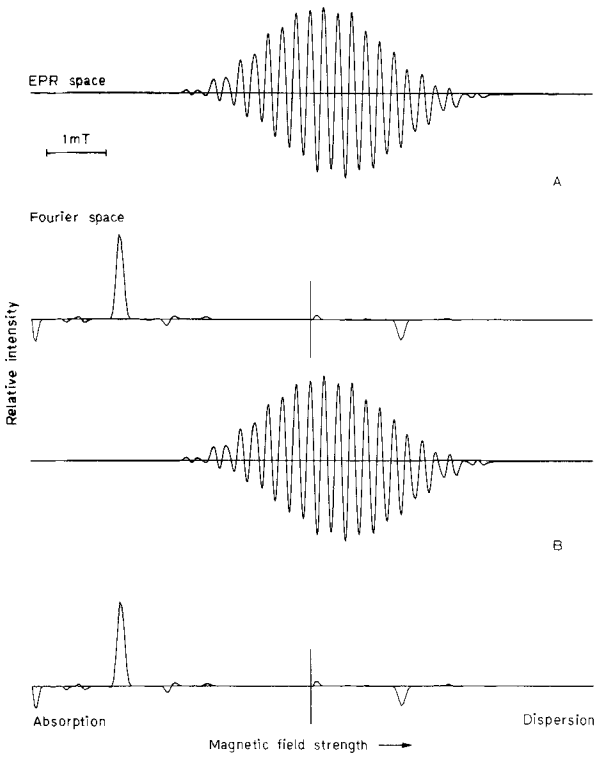


Fig. 1. *Experimental (A) and computer-simulated (B) EPR spectra of 3,10-dimethylisoalloxazine radical cation (I).* The corresponding Fourier transforms of the two spectra are also shown. The experimental spectrum was recorded under the following instrumental settings: centerfield 346.37 mT, sweep width 10 mT, frequency 0.979 mT, power 4.1 mV, modulation frequency 100 kHz, modulation width 0.032 mT

From the foregoing it is evident that using the main coupling constants only, as published by Westerling et al. [14], cannot be expected to produce the quality fit suggested by these authors. A comparison of the simulated spectra of 3,10-dimethylisoalloxazine radical cation (I) using the published coupling constants of Westerling et al. [14] and those given in Table 1 of this paper is given in Fig. 4. This comparison unequivocally shows that the small coupling constants cannot be neglected for the simulation of the experimental spectra.

Guzzo and Tollin assigned the largest coupling constant to N-1 in the case of isoalloxazine cation radicals [8] and to

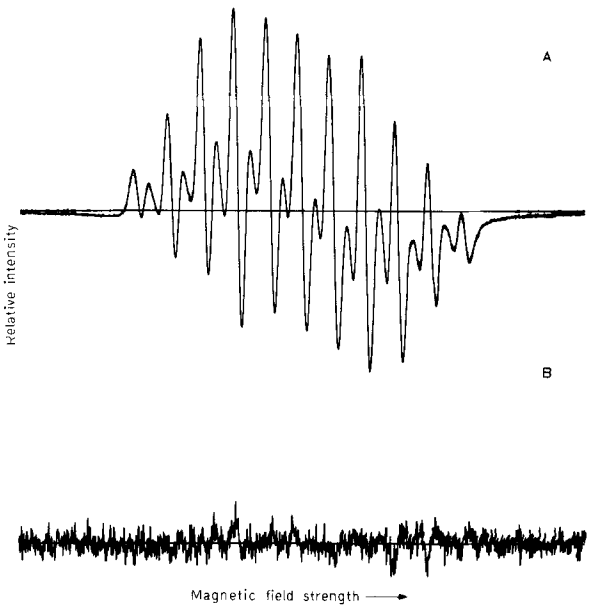


Fig. 2. *Overplot of experimental and computer-fitted EPR spectra of 1,3-dimethylalloxazine radical cation (VIII) (A) and the difference spectrum (B) between the two spectra.* The vertical scale of the error curve is enlarged by a factor of 10 to visualize the small difference. The deviation from the baseline at the beginning and end of the spectra is caused by the presence of dispersion (cf. Materials and Methods). Experimental conditions as in Fig. 1

Table 2. *Variational fits to compounds I, II and VIII obtained by limiting the couplings available to the fitter*
Only couplings assigned to positions 6–9 are given; those in parentheses are for methyl groups, the others for protons; the couplings at positions 10 and 5 showed changes similar to those at positions 8 and 6. Graphs of a portion of the four fits to compound VIII are given in Fig. 3

Number of coumpound	Coupling at ring position				Aggregate coupling (nitrogen)	Line width	Relative squared error
	8	6	9	7			
	mT						μT
I	0.270	0.183	–	–	–	0.073	0.185
	0.270	0.180	0.046	–	–	0.065	0.111
	0.270	0.178	0.048	0.033	–	0.060	0.104
	0.270	0.175	0.049	0.040	0.020	0.052	0.098
III	(0.311)	0.151	–	–	–	0.098	0.897
	(0.317)	0.154	0.069	–	–	0.082	0.311
	(0.322)	0.153	–	(0.053)	–	0.073	0.233
	(0.321)	0.153	0.040	(0.052)	–	0.070	0.213
	(0.322)	0.154	0.040	(0.050)	0.021	0.068	0.210
VIII	0.244	0.145	–	–	–	0.080	2.822
	0.249	0.149	0.055	–	–	0.066	0.909
	0.250	0.150	0.055	0.036	–	0.061	0.529
	0.251	0.152	0.054	0.040	0.018	0.056	0.399

N-5 in the case of alloxazine radical cations [9]. Our results definitely prove that the largest coupling constant must be assigned to N-5 in both classes of compounds. This is further supported by the EPR spectra of alloxazine and isoalloxazine cation radicals, i.e. compounds VIII and I, respectively, where the ^{14}N at positions 1 and 3 were replaced by ^{15}N, yielding spectra which were identical with those of the corresponding parent compounds (see also [13]). As mentioned above, from structural considerations, one would expect that in both classes of compounds the unpaired electron is similarly distributed in the two kinds of molecules, which is indeed found (Table 1). The incorrect assignment by Guzzo and Tollin [8,9] might have been caused by incomplete deuterium exchange experiments due to low deuterium content of the solvent used. The coupling constants as published by various authors are summarized in Table 3. The table shows that the results of Guzzo and Tollin [9] for alloxazine are in better agreement with our results than for isoalloxazine where they assigned the largest coupling constant to N-1.

Although the hyperfine coupling schemes between isoalloxazine and alloxazine cations are very similar, there are some differences which distinguish the two classes from each other (Table 1). Common to both classes of radicals is the number of coupling constants needed to simulate the experimental spectra. Another common feature is that in both classes the proton couplings to N-5 are larger than those of the corresponding nitrogen atom itself. Such effects were also observed with pteridine radical cations [22] and in fact have been observed with many other heterocyclic radical cations [23]. The difference between the two kinds of radicals resides in the coupling constants of the 10 position of the molecules. Whereas in the case of isoalloxazine the proton and nitrogen coupling constants of position 10 behave analogously as those of position 5, the corresponding coupling constants in alloxazine are almost identical. Another difference is a rather drastic decrease of the coupling constants of position 10 and an increase of that of position 5 in alloxazine radical cation as compared to the corresponding coupling constants in isoalloxazine; i.e. the spin density distribution in the two kinds of radicals differs mainly in the pyrazine subnucleus of the molecules. Since the only structural difference between the two kinds of radicals is the substituent at N-10, the substituents in the pyrimidine ring are unimportant as far as the spin distribution is concerned, the results suggest that isoalloxazine cation radicals are probably more planar (probably coplanar) molecules than the alloxazine radical cations. More specifically this could even mean that the configuration of N-10 in the two kinds of radicals is somewhat different.

The relevant hyperfine coupling constants of all flavin radicals are summarized in Table 4. Although some variation of the coupling constants among the different radical species is obvious, the main conclusion which can be drawn from these results is that N-5 always carries the highest spin density irrespective of the species. In addition, for all cases the highest spin density is always located in the pyrazine subnucleus of the flavin molecule whereas the spin density in the pyrimidine subnucleus is very small.

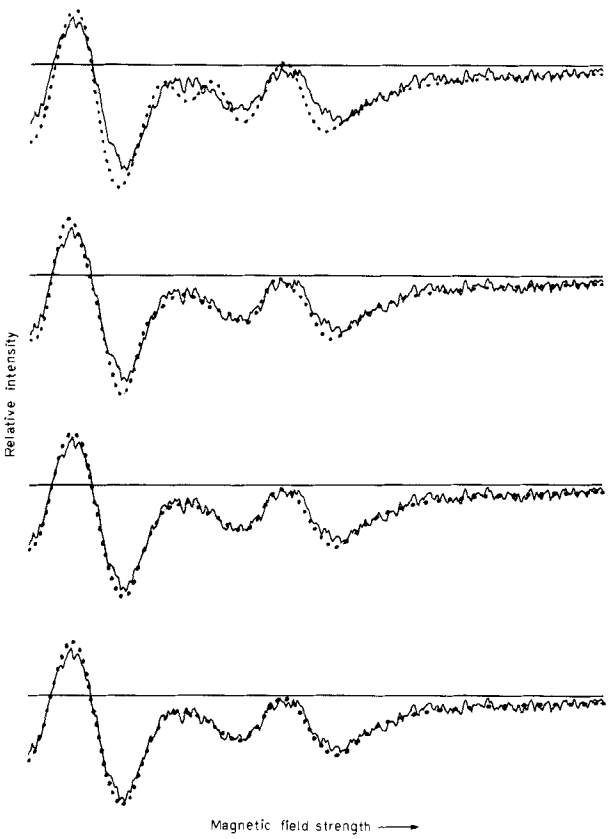


Fig. 3. *Overplots of the data (——) and fits (· · · · ·) for a portion (the three outer most lines on the right hand of the EPR space of compound VIII, cf. Fig. 2) corresponding to the four fits given in Table 2*

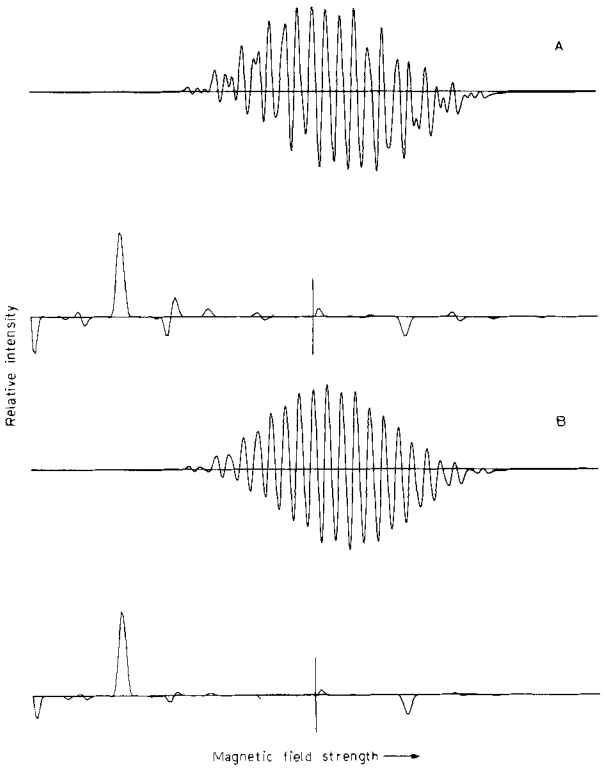


Fig. 4. *Comparison of the computed spectra of 3,10-dimethylisoalloxazine radical cation (I) using the published coupling constants of Westerling et al. [14] (A) and those given in Table 1 of this paper (B).* The corresponding Fourier transforms of the spectra are also given. For the experimental spectrum, see Fig. 1A. Since Westerling et al. [14] did not mention the line width used to simulate their spectrum we optimized the line width (0.08 mT) and center to our experimental spectrum using their published coupling constants

Table 3. *Coupling constants for (iso)alloxazine radical cations as published by various authors*
Values obtained by Guzzo and Tollin [8,9] and by Müller et al. [12] were deduced from experimental spectra; those of Westerling et al. [14] were obtained by fitting of simulated spectra to the experimental traces. The values in this paper were obtained by fitting of simulated spectra to the experimental traces and by isotopic substitution. A question mark is added where assignment was questionable

Ring position	Hyperfine coupling constants by					
	Guzzo and Tollin		Müller et al. [12]	Westerling et al. [14]	this paper	
	isoalloxazine [8]	alloxazine [9]	isoalloxazine	isoalloxazine	isoalloxazine	alloxazine
	mT					
N-5	0.46	0.78	0.85	0.739	0.736	0.762
N-10	0.23	0.39	0.43	0.485	0.484	0.402
H-5	0.46	0.78	1.15	0.775	0.775	0.816
H-10	0.46	0.39	0.47	0.492	0.503	0.408
H-8	0.14	0.23?	0.34	0.291?	0.270	0.251
H-6	–	0.15?	–	0.167?	0.175	0.152
H-7	–	–	–	–	0.040	0.040
H-9	–	–	–	–	0.049	0.054
N-1	0.66	–	–	–	0.020[a]	0.018[a]

[a] See text.

Table 4. *Comparison of the isotropic hyperfine couplings constants for $H_2\dot{F}lR^+$, $H\dot{F}lR$, $\dot{F}lR^-$ and $[Me\dot{F}lR]^+$*
The values represent the individual coupling constants as revealed by EPR spectra simulation techniques ($H\dot{F}lR$, $H_2\dot{F}lR^+$) and as deduced from experimental spectra ($\dot{F}lR^-$, $Me\dot{F}lR^+$)

Ring position	Hyperfine coupling constants of flavin radicals			
	$\dot{F}lR^-$ [5]	$Me\dot{F}lR^+$ [7]	$H\dot{F}lR$ [4]	$H_2\dot{F}lR^+$ (this paper)
	mT			
N-5	0.73 ± 0.03	0.77 ± 0.03	0.80 ± 0.02	0.732
H-5	–	–	–	0.779
N-10	0.32 ± 0.03	0.31 ± 0.05	0.36 ± 0.02	0.475
H-10	0.30 ± 0.02	0.31 ± 0.02	0.39 ± 0.02	0.497
H-8	0.04 ± 0.05	0.39 ± 0.02	0.24 ± 0.05	0.322
H-6	0.35 ± 0.05	0.35 ± 0.05	0.17 ± 0.02	0.134
H-9	0.09 ± 0.01	small	small	0.040
H-7	–	–	–	0.050

Table 5. *Comparison between experimental spin densities and theoretically calculated distribution of the unpaired electron in the cationic (iso)-alloxazine radical*
All values are given in units of the negative electronic charge. The spin polarization parameters of the atoms in question used to calculate the spin densities from the coupling constants are the same as used previously [14]. The dihedral angle is that between the normals of the benzene and the pyrimidine subnucleus of the isoalloxazine molecular [24] (see text)

Position	Atom	Spin densities calculated from the coupling constants of		Theoretically calculated spin densities as depending on the dihedral angle		
		alloxazine	isoalloxazine	32	16	0
1	N			0.023	0.019	0.023
3	N			0.003	0.002	0.003
5	N	0.267 – 0.411	0.258 – 0.398	0.454	0.424	0.338
6	H	0.054 – 0.071	0.065 – 0.084	0.023	0.026	0.019
7	H	0.015 – 0.019	0.015 – 0.019	0.009	0.011	0.008
8	H	0.093 – 0.119	0.110 – 0.129	0.024	0.029	0.024
9	H	0.019 – 0.024	0.019 – 0.024	0.002	0.002	0.002
10	N	0.140 – 0.216	0.169 – 0.261	0.084	0.093	0.100

From the coupling constants given in Table 1 experimental spin densities (ϱ) have been evaluated (Table 5) with the help of the equation

$$a^x = Q_x S_x \qquad (2)$$

with spin polarization parameters (Q) from the literature (for a more detailed discussion of the equation and of the Q_x values see [24–26]). The broad range reported for the experimental spin density is determined by the deviation of these parameters as found in the literature and described earlier [4]. The alloxazine and isoalloxazine radicals are treated separately. In the last two decades quite a few papers have appeared in the literature concerning theoretical calculations on the flavin molecule. Since *ab initio* theoretical studies were carried out on the molecule and described in the most recent paper on this subject by Platenkamp et al. [27], only the values published by these authors are presented in Table 5. In their theoretical study Platenkamp et al. [27] have also investigated the influence of the geometrical structure on the spin density distribution. The calculated values as depending on the dihedral angle between the normals of the benzene and the pyrimidine subnucleus of the isoalloxazine nucleus are also given in Table 5. There is, in general, good agreement between theoretically calculated spin densities and those deduced from experimental coupling constants in so far as the order of spin densities between the different sites is concerned. The calculated spin densities are in general, except for N-5, considerably smaller than the values obtained from the coupling constants. The theoretical calculations also reveal another interesting fact, namely that the spin density at N-5 decreases and that at N-10 increases when the molecule becomes coplanar. Exactly the same trends are observed experimentally, i.e. alloxazine vs isoalloxazine. The theory thus supports our conclusion drawn above with respect to the structural differences between the two kinds of molecules.

Platenkamp et al. [27] concluded from their theoretical data that the flavin radicals are non-planar. It must, however,

be realized that the theoretical data were obtained on a molecule in the gaseous phase carrying a proton at N-10 instead of a methyl group; i.e. alloxazine. From this it can be learned that one must be cautious in deducing conclusions from theoretical calculations where for economical reasons 'innocent' structural simplifications are applied.

In conclusion it can be expected that the results described in this paper will be a useful aid in future structural elucidation of unknown covalently bound prosthetic groups of flavoproteins after degradation to the level of alloxazine or isoalloxazine. Additionally the consistency of the coupling constants within the group of chemically and isotopically substituted (iso)alloxazine cation radicals strongly indicates the possibility of drawing conclusions with respect to the planarity of the molecule from substitutions resulting in a variation of some of the coupling constants.

We are indebted to Mr B. J. Sachteleben for the preparation of the drawings and to Mrs J. C. Toppenberg-Fang for typing the manuscript. This investigation was supported in part by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.), by a U.S. Public Health Service Research Career Development Award (KO4-GM-42,599), and by grants from the Advisory Committee on Computer Activities of The University of Michigan Medical School (to F.M.), by the *Deutsche Forschungsgemeinschaft* (Project: He 5681) (to P.H.), by the U.S. Public Health Service (GM 12176) (to W.R.D.), the Swedish *Statens Medicinska Forskingråd* and *Statens Naturvetenskapliga Forskningsråd* (to A.E.) and a NATO grant (1854) (to H.J.G.).

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