ACTA CHEMICA SCANDINAVICA 18 (1964) 1437–1453

Electron Spin Resonance Study on Anionic Flavin Free Radicals *

L. E. GÖRAN ERIKSSON and ANDERS EHRENBERG

Medicinska Nobelinstitutet, Biokemiska Avdelningen, Karolinska Institutet, Stockholm 60, Sweden

The anionic lumiflavin radical was studied in detail by ESR and nitrogen-15 as well as deuterium substitutions have been carried out. The free radicals of the flavin coenzymes and a number of isoalloxazine derivatives have also been investigated. A hyperfine coupling scheme of the lumiflavin radical is derived where N(9), N(10), CH_3 (7), CH_3(9), and H(5 or 8) are involved. The hyperfine couplings are interpreted in terms of spin densities and comparison is made with available quantum chemical calculations. The effect of the N(9)-substitutent upon the resolution of the spectra is examined and discussed.

The presence of flavin free radicals as an intermediary red-ox stage in certain flavo-enzymes is rather well established today, see *e.g.* Ref.[1] The method of electron spin resonance (ESR)** has been applied in some studies on flavin containing enzymes and their prosthetic groups. However, our knowledge about the precise function and the detailed electronic structure of the flavin radical is still poor. When the flavin coenzyme is bound to the apoenzyme the resolution of the ESR spectrum in aqueous solution almost completely vanishes since the slow rotational relaxation of the large protein molecule is not effective in averaging out the anisotropic effects.[2] Thus, in order to obtain more detailed information about the properties of the flavin radical it is at present necessary to study the coenzymes or any relevant model compound in dilute solution.

Due to the asymmetry of the isoalloxazine structure (Fig. 1) and the occurrence of proton dissociations of the radical, the interpretations of the

* Some preliminary results were presented by one of us (A. E.) at the Symposium on »Electronic Aspects of Biochemistry», Ravello, Septemper 1963, Academic Press, New York 1964, p. 379.

** Abbreviations used: ESR — electron spin resonance: NMR — nuclear magnetic resonance; LF — lumiflavin; RF — riboflavin; FMN — flavin mononucleotide; FAD — flavin adenine dinucleotide; SCF — self-consistent field calculation.

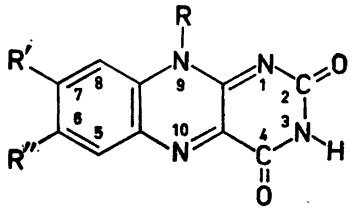

Fig. 1. Structure of the oxidized, neutral form of an isoalloxazine derivative.

hyperfine structures are not straightforward. Nevertheless, some attempts to explain the hyperfine structures of isoalloxazine free radicals in alkaline [2] and acid [3–6] solutions have been reported. These interpretations are based on fitting estimated line intensities and spacings to the various possible coupling schemes by trial and error. However, since there are several nuclei with magnetic moment in the flavin molecule, a number of possible coupling schemes could be *a priori* set up to satisfy each particular experimental spectrum. Our recent results show [7] that the reported interpretations of the spectra of flavin radicals in acid solution are incorrect. Because of the great biological importance of the flavin coenzymes we have undertaken to investigate systematically flavin free radicals by means of ESR in order to interprete hyperfine structures and, if possible, make a comparison with results predicted theoretically from quantum chemical calculations.[8–11] In the first report of this work [2] it was stated that definite conclusions concerning the hyperfine couplings could be deduced only from experiments with flavin free radicals having isotopic substitutions at suitable positions. In the present study experiments of this type have been done using lumiflavin (LF), 6, 7, 9-trimethylisoalloxazine (Fig. 1: R = R′ = R″ = CH_3), as a model compound. Nitrogen-15 has been specifically introduced in positions (1) and (3) (LF-1,3-$^{15}N_2$), in position (10) (LF-10-^{15}N) and in all three positions (LF-1,3,10-$^{15}N_3$). Protons have been exchanged for deuterons in the benzenoid ring and in the 9-(N-methyl)-group. Certain significant isoalloxazine derivatives have been studied when isotope labeling was not possible for some reasons.

From the appearance of the hyperfine spectra it was concluded [2] that there were at least three forms of the FMN radical: a cationic, a neutral, and an anionic form with transitions in the pH-regions 0—2 and 7—9, respectively. The occurrence of dianions should perhaps be considered at very high pH-values. The ESR spectra of the anionic free radicals of LF at pH about 12 consist of 14 evenly spaced hyperfine lines. Also FMN, FAD, and RF give ESR spectra with an even number of lines under the same conditions. There are at least 12 lines but it has not been possible to show unequivocally by direct experiment whether there are further lines. The resolution of the spectra is dependent on the nature of the substituent on nitrogen (9) and decreases in the order LF>FMN> FAD>RF but the spacing remains essentially constant.[2] The simplicity and the rather good resolution of the ESR spectrum of the LF radical in alkaline solution makes this species most convenient for a detailed study. Moreover, in the alkaline solution there should be only a comparatively small tendency for dimer formation. In the neutral pH-region the solubility of LF is low and there are several flavin species

with dissociable hydrogens to account for and thus the ESR spectrum is more complicated.

The oxidized coenzyme is probably linked to the protein in the neutral form as inferred from light absorption and fluorescence spectra. A one electron reduction will transform the neutral oxidized flavin into a radical anion. Therefore it is possible that this entity is biologically active in some way, *e.g.* as a transient intermediary.

EXPERIMENTAL

Methods and materials

The ESR spectra were recorded with a Varian X-band spectrometer Model V-4500 operating at 9.51 kMc/sec and equipped with a Model V-4560 100 kc/sec field modulation unit. Some of the spectra were measured with 400 c/sec field modulation and a Model V-K3525 superheterodyne adapter. A 12 inch magnet was used. When the superheterodyne equipment was employed the magnetic field was regulated with a Varian "Fieldial" accessory kit. Calibration of the field scale and determination of *g*-values were carried out by means of a simple proton resonance magnetic field meter of the marginal oscillator type and an electronic frequency counter as well as a transfer oscillator, Hewlet Packard Model 524D and 540B, respectively. A standard flat quartz aqueous sample cell was used.

The flavin solutions were reduced to the desired degree with the methods and apparatus described before.[2] As reducing reagents we have used $Na_2S_2O_4$ and H_2 in which case a Pd-asbestos catalyst was used. Anaerobic conditions were obtained by repeatedly evacuating the apparatus and flushing with commercial grade argon freed from oxygen by bubbling through two wash bottles containing amalgamated zinc and vanadyl sulfate in dilute sulfuric acid.[12] Hydrogen was purified in the same way. Prior to reduction the samples were freed from oxygen by bubbling with argon.

RF and FMN were gifts from Sigma Co. and were not purified further. Barbituric acid-$^{15}N_2$ (95.3 atom %) was furnished by Isomet Corp., USA. Sodium nitrite-^{15}N (95.7 %) and methyl-d_3-amine HCl (98 %) was purchased from Merck Sharp & Dohme,Canada. Deuterium oxide (99.8 %) was supplied by Norsk Hydro, Norway. No check of the isotopic content of these compounds has been carried out.

Lumiflavin and isotopically substituted lumiflavin

We have used two different ways to synthesize the isoalloxazine namely, condensation of aromatic *o*-diamines with alloxan[13] and condensation of *o*-sec. amino-azo compounds with barbituric acid.[14]

Lumiflavin (LF), 6,7,9-trimethylisoalloxazine, was synthesized according to Hemmerich[14] but in a version modified for semimicro quantities. Starting with 3,4-dimethylaniline (*I*) (L. Light & Co. Ltd, England) N,3,4-trimethylaniline (*II*) was prepared with the N-*p*-toluenesulfonyl derivative as an intermediate. The next step was to couple *II* with diazotized *p*-aminobenzoic acid to produce the 6-*p*-carboxyphenylazo derivative (*III*). The low yield was due to the occurrence of unidentified by-products.* Finally the azo-compound was condensed with barbituric acid. The crude LF was purified by repeated extractions between chloroform and water at controlled pH.[15] (Found: C 60.59; H 5.11; N 21.99. $C_{13}H_{12}N_4O_2$ requires: C 60.91; H 4.72; N 21.86).

LF-10-^{15}N was synthesized by diazotation of *p*-aminobenzoic acid with sodium nitrite-^{15}N and coupling with *II*. The azo-compound (*III*) was then reacted with barbituric acid. *LF-1,3-*$^{15}N_2$ was produced by condensation of *III* with the doubly ^{15}N substituted barbituric acid. *LF-1,3,10-*$^{15}N_3$ was obtained by condensation of isotopically substituted azo-compound (*III*) with barbituric acid-$^{15}N_2$.

* Dr. P. Hemmerich, (University of Basel, personal communication) has pointed out to us that, by using $HClO_4$ instead of HCl in the diazotation reaction, it would be possible to improve the yield.

LF-5,8-d_2. *II* was treated three times for at least two days each time with concentrated D_2SO_4 at about 100°C. D_2SO_4 was prepared by absorption of SO_3 from fuming sulfuric acid into D_2O. The amine was isolated after each treatment and examined in a Varian A-60 NMR spectrometer. At the end the deuterium substitution was found to be better than 95 %. It was observed that during these treatments increasing amounts of impurities were formed, which were difficult to remove. The deuterated amine was then used in the flavin synthesis in the way described above for LF. The NMR spectrum of the purified lumiflavin, dissolved in 0.1 N NaOD (internal standard: sodium β-(trimethylsilyl)propionate), showed absorption at the very field where one of the benzenoid ring protons of normal lumiflavin has absorption. Hence, a partial back exchange of hydrogen had taken place during the final steps of the synthesis. From NMR spectra we estimate the amount of protons in one of the two positions (5) or (8) to be 90 (±10) % (δ = 6.77 ppm) and in the other position 50 (±10) % (δ = 6.55 ppm).

LF-9-methyl-d_3. 1-Amino-3,4-dimethyl-6-nitrobenzene was obtained from *I* by nitration [16] and 1-chloro-3,4-dimethyl-6-nitrobenzene was then produced.[17] The latter compound was reacted with an equimolar amount of methyl-d_3-amine HCl in 50 % ethanol. An equivalent amount of $(C_2H_5)_3N$ was present and Cu powder was used as a catalyst. The reaction was carried out in a pressure vessel at 180°C for 24 h. 1-(Methyl-d_3)amino-3,4-dimethyl-6-nitrobenzene was obtained after extraction and chromatography on Al_2O_3 with petroleum ether (5% chloroform). Reduction with hydrogen and Pd(C)-catalyst in glacial acetic acid, followed by condensation of the diamine with alloxan in the presence of boric acid produced lumiflavin. After purification the compound was checked in the NMR spectrometer and no N(9)-methyl group (δ = 3.21 ppm) was observed.

The particular reactivity of the 7-methyl [18,19] group suggested that an exchange of these methyl protons might be possible. We have therefore dissolved lumiflavin in conc. D_2SO_4 but no tendency of exchange was noticed with NMR even after 2 weeks' treatment at room temperature.

6,7-Dimethyl-9-ethylisoalloxazine was synthesized in a similar fashion as lumiflavin. The N-*p*-toluenesulfonyl derivative of *I* was treated with diethylsulfate. The N-ethyl-N-(*p*-toluenesulfonyl)-3,4-dimethylaniline (m.p. 72–73°C) was then hydrolysed with concentrated HCl. The isolated N-ethyl-3,4-dimethylaniline (b.p.$_{20}$ 129–130°C) was coupled with diazotized *p*-aminobenzoic acid. Condensation of the azo-compound (m.p. 195–198°C) with barbituric acid gave the flavin, but the yield was lower than in case of LF. An alternative route of flavin synthesis was also tried. 1-Amino-3,4-dimethyl-6-nitrobenzene was reacted with an aqueous solution of ethylamine (33 %) at 160°C for 36 h. After chromatography and recrystallisation from methanol, orange coulered needles (m.p. 75–76°C) were obtained. Catalytic reduction to the corresponding diamine and condensation with alloxan yielded a flavin identical with that obtained in the other way in about the same yield.

6,7-Dimethyl-9-(2'-hydroxyethyl)isoalloxazine was prepared from riboflavin by periodate oxidation followed by sodium borohydride reduction.[20]

9-Methylisoalloxazine. N-Methyl-*o*-nitroaniline was produced from *o*-nitroaniline.[21] The catalytic reduction was carried out with hydrogen plus Pd(C)-catalyst in glacial acetic acid following addition of alloxan-boric acid to the diamine solution.[22] (Found: C 57.90; H 3.52; N 24.52. $C_{11}H_8N_4O_2$ requires: C 57.89; H 3.53; N 24.55).

9-Ethylisoalloxazine was prepared in an analogous way. N-Ethyl-*o*-nitroaniline was produced by alkylation with diethylsulfate and was obtained as a red oil after chromatography. After reduction and condensation with alloxan the product was recrystallized from ethanol in the form of yellow needles. (Found: C 59.41; H 4.46; N 23.00. $C_{12}H_{10}H_4O_2$ requires: C 59.50; H 4.16; N 23.13).

The following riboflavin analogues were obtained through the generosity of Dr. J. P. Lambooy, University of Nebraska: *6-chloro-7-methyl-*, *6-methyl-7-chloro-*, *6,7-dichloro-*, *6-ethyl-7-methyl-*, *6-methyl-7-ethyl-* and *6,7-diethyl-9-(1'-D-ribityl)isoalloxazine*.[23,24] *7-Chloro-6,9-dimethylisoalloxazine* [25] and *6,7-dimethyl-9-(1'-D-ribityltetraacetate)isoalloxazine* [26] were prepared by Dr. P. Hemmerich, University of Basel, and investigated in cooperation with him.

RESULTS AND DISCUSSION

The oxidized flavin molecule is converted into the semiquinoid, free radical state by the addition of one electron or one hydrogen atom. The yield of free radicals depends strongly on the pH and the degree of reduction.[2,27] The actual concentration of free radicals in a partially reduced aqueous flavin solution is a result of the equilibrium between: the radical disproportionation, the radical dimerization and, at least in non-alkaline media, the formation of other complexes between flavin molecules of the same or different states of oxidation. In alkaline medium the radical yield curve is a symmetrical function of the degree of reduction. The formation of bimolecular complexes between the free radical and the oxidized or the reduced form is likely to be negligible. Also, the tendency of the radicals to dimerize is comparatively small in this pH-region and might be neglected for most purposes when dealing with ESR absorption.[2,27] At present we have no indication of the existence of a dianion radical at high pH-values and we therefore expect only one radical species to be present in the alkaline medium used for this investigation.

Table 1. g-Values for flavin free radicals in various media.

Free radical	Conc. (mM)	Medium	g-value
Flavin mononucleotide (FMN)	3	6 N HCl	2.0032
	10	0.1 M acetate buffer pH 4.9	2.0033
	10	0.1 M phosphate buffer pH 7.0	2.0032
	10	0.05 N NaOH pH 12	2.0034
Lumiflavin (LF)	1	3 N HCl	2.0032
	3	3 N HCl-cellosolve (1:1)	2.0030
	4	0.1 N acetate buffer-cellosolve (1:1) +65°C	2.0030
(isotopically substituted LF included)	10	0.05 N NaOH pH 12	2.0034

In Table 1 it is seen that the g-values were all in the region 2.0030—2.0034. At present we are not able to state to what extent the observed variations should be considered as significant. As a control the g-value of $K_2(SO_3)NO$ in anaerobic glycine buffer of pH 9.1 was determined to be 2.0055, in agreement with what has been reported.[28]

The LF free radical in alkaline medium (0.05 N NaOH, pH about 12) produces a rather well resolved ESR spectrum consisting of at least 14 evenly spaced hyperfine lines as shown by the derivative representation in Fig. 2.

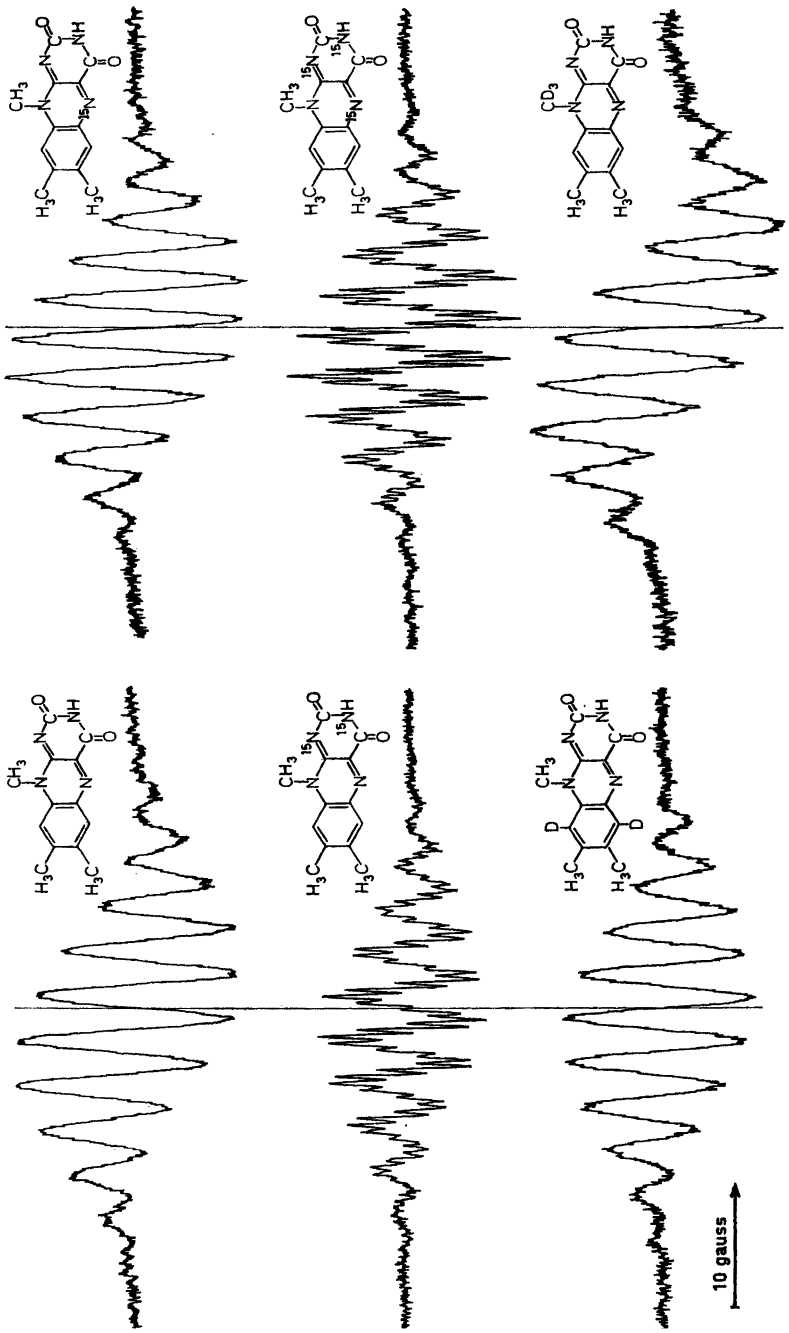

Fig. 2. ESR recordings of the free radicals of lumiflavin and isotopically substituted lumiflavin in aqueous solution at pH 12. Flavin concentration 10 mM; degree of reduction about 50 %.

The spacing between the lines was measured to be 3.51 G. The intensity ratios were estimated after integration of the recorded spectrum (Table 2). The outermost lines are only detectable by means of optimum instrumental sensitivity and thus a detailed study of these is difficult. In an earlier report some likely coupling schemes for this radical were discussed.[2] In order to achieve a more unambiguious interpretation of the hyperfine spectrum we have now used the isotopic substitution technique.

The wave-function for the unpaired electron may be considered unchanged when a nucleus is substituted with its isotope. The isotropic hyperfine splitting arising from the Fermi contact term is proportional to the ratio μ/I (nuclear magnetic moment/nuclear spin). Natural nitrogen consists to more than 99.6 % of ^{14}N which has $I = 1$ and $\mu = 0.404$ nuclear magnetons, whereas these constants for ^{15}N are $I = 1/2$ and $\mu = -0.283$. Hence, if ^{14}N gives rise to three lines with a spacing between neighbouring lines of $a^{^{14}N}$ replacement by ^{15}N in the same position will produce two lines with a spacing of $a^{^{15}N} = -1.40\ a^{^{14}N}$. Similarily, when protium ($I = 1/2$; $\mu = 2.793$) is replaced by deuterium ($I = 1$; $\mu = 0.857$) the doublet with a spacing of a^{H} will transform into a triplet with the much smaller spacing $a^{D} = 0.154\ a^{H}$. The practical consequence of this is the well-known effect that the hydrogen hyperfine splitting will collapse when deuterium is introduced.

The spectrum of the LF radical with ^{15}N in positions (1) and (3) is also shown in Fig. 2. Again we observe 14 main lines as in normal LF and with the same spacing and intensity ratios. In case of LF-1,3-$^{15}N_2$ however, each main line is partially resolved into about 6 sublines. A change in resolution within the main hyperfine lines is not unexpected as the change of ^{14}N into ^{15}N will alter the hyperfine splitting and intensity ratio among the almost coincident sublines that form the main lines. ^{15}N gives two lines with relative intensity 1/2 and spacing $1.4\ a^{^{14}N}$ as compared to the three lines with relative intensity 1/3 and spacing $a^{^{14}N}$ caused by ^{14}N. The resolution of the sublines seems to vary somewhat with the degree of reduction but the mechanism of this effect is not yet known. The ESR spectrum of LF-1,3-$^{15}N_2$ clearly reveals that N (1) and N (3) do not contribute to the main hyperfine splitting at pH 12. The hyperfine interaction of the nitrogens of the reduced pyrimidine ring is only a second order effect, giving rise to a splitting considerably smaller than 3.5 G.

The LF-10-^{15}N radical gives rise to an ESR spectrum with an odd number of hyperfine lines (Fig. 2). Examination of the spectrum reveals that the number of lines is at least 13, with an even spacing equal to or slightly less than that of LF. The intensities are given in Table 2. This demonstrates that N (10) is indeed involved in the main hyperfine interaction.

As a control we have examined LF with ^{15}N simultaneously in positions (1), (3), and (10). The spectrum of this radical is also shown in Fig. 2. As expected, it consists of 13 main lines each of which is further split into about 6 sublines.

The simplest isotope experiment would be to exchange dissociable protons for deuterons. The anionic radical has one such proton, which has been shown to be attached to N (3).[29] In experiments earlier made on LF[2] and now extended

Table 2. Observed and calculated ESR line intensities of flavin radical anions. In each case the strongest line has arbitrarily been given the intensity 100. When there is an open space in the first row, the number of line is even.

Lumiflavin (LF)		LF-1,3-$^{15}N_2$		LF-10-^{15}N		LF-5,8-d_2		LF-9-methyl-d_3		9-methyliso-alloxazine		7-chloro-6,9-dimethyliso-alloxazine	
obs.	calc.	obs.	calc.	obs.	calc.	obs.	calc.	obs.	calc.	obs.	calc.	obs.	calc.
—	—	—	—	100	100	100	100	100	100	—	—	100	100
100	100	100	100	91.2	95.2	92.0	94.7	94.3	95.8	100	100	94.2	95.8
86.6	88.5	85.3	88.5	69.7	78.6	80.3	77.7	75.7	79.2	89.1	89.4	75.8	79.2
64.9	66.1	60.5	66.1	42.1	50.8	52.5	51.1	47.4	50.0	65.7	66.0	48.1	50.0
37.8	38.8	34.9	38.8	18.0	23.0	27.1	24.5	20.0	20.8	35.2	36.2	22.0	20.8
17.1	16.4	14.8	16.4	4.1	6.3	8.0	7.5	4.0	4.2	12.7	12.8	6.8	4.2
5.2	4.4	4.1	4.4	0.4	0.8	2.1	1.1			1.9	2.1		
1.1	0.6	0.7	0.6										

to LF-1,3-$^{15}N_2$ no effect on the ESR spectrum was observed when the radical was dissolved in alkaline D_2O. This may be due to a low spin density on N(3) or else the rate of exchange is too high.

LF-5,8-d_2, in which the two benzenoid ring hydrogens had been substituted with deuterium to ~ 90 % and ~ 50 %, respectively, afforded a spectrum with an odd number of lines (Fig. 2). There are 13 lines with very nearly the same spacing as in normal LF and with the intensities given in Table 2. This proves that the ring hydrogen, which nearly completely had been replaced with deuterium, takes part in the main hyperfine interaction. The measured intensity ratios of the hyperfine lines fit well with this conclusion. For this reason and since the other ring proton was partly replaced by deuterium, we may conclude that this other proton has only negligible hyperfine coupling. Whether it is the proton in position (5) or (8) that has the strong hyperfine coupling remains unsettled at present, since we have not yet been able to give assignments of the NMR peaks to the two ring protons.

In order to investigate the influence from the (1′)-protons of the N(9) substituent we have measured the radical of LF-9-methyl-d_3. In this case 11 lines were observed with the same spacing as for ordinary LF (Fig. 2 and Table 2). Hence, the 9-methyl protons of LF must each have a coupling constant of 3.5 G. Also the line intensity ratios of the methyl-d_3 radical are compatible with this conclusion. Because of the conservation of an even number of lines in the spectra of the RF and coenzyme radicals it is evident that the two (1)′-hydrogens in these cases are not equivalent and can not both have about the same coupling constant as the N(9)-methyl hydrogens of LF. The situation must be such that the ribityl chain, the free rotation of which is sterically hindered, by some means obtains a certain average position so that one of its (1′)-protons has just about the same coupling constant as the three 9-methyl protons of LF, whereas the other (1′)-proton has a much smaller coupling. The probability of this coincidence, that the two different mechanisms would transfer about identical spin density to one of the (1′)-

protons of ribityl as to the methyl-protons of LF, was previously considered rather unlikely.[2] However, our present results show unequivocally that this coincidence is just what happens to be true. Hence, there are in the hyperfine spectrum of RF, FMN and FAD radicals two lines less than in case of LF, even if this is not possible to show by direct measurement at present, because of the poor resolution and the low signal to noise ratio in the outer regions of the spectra.

An obvious possibility is that the ribityl substituent could be locked in a certain rotational orientation, which might be caused by a hydrogen bond from one of the ribityl hydroxyls to some part of the pyrimidine ring. This hypothesis is corroborated by the poorly defined resolution of the ESR spectrum obtained from the radical of RF-tetraacetate. In this radical an intramolecular hydrogen bond of the type discussed can not be formed and hence, the distribution of the hyperfine coupling of the two (1')-hydrogens should become more even.

The same arguments hold for the radicals of the 9-ethyl substituted compounds 6,7-dimethyl-9-ethylisoalloxazine and 9-ethylisoalloxazine, which both give spectra with poor resolution as compared with the corresponding 9-methyl compounds (Fig. 4).

The building of molecular models shows that for steric reasons a hydrogen bond from any of the ribityl hydroxyls to N(1) is quite feasible. We have found that the radical of 6,7-dimethyl-9-(2'-hydroxyethyl)isoalloxazine gives a poorly resolved ESR spectrum. This would mean either that no intramolecular hydrogen bond can be formed from a (2')-hydroxyl or that the strength of such a bond is considerably weaker in case of the hydroxyethyl compound than for the ribityl compounds.

Deuterium substitution in the methyl groups (6) and (7) of LF would be a rather tedious task. Therefore, we have at present restricted ourselves to a study of some ethyl and chloro analogues of RF and LF. When methyl is replaced by ethyl in the benzenoid ring we may expect the change of the spin density distribution within the isoalloxazine framework to be negligible. The effect of the chlorine substitution is less certain. The method of halogen substitution in aromatic radicals has, however, been reported in several ESR studies. Usually no hyperfine splitting from the halogen itself is observed and the influence on the spin density distribution seems to be small, only affecting the resolution of the spectrum.

6-Ethyl-7-methyl-9-(1'-D-ribityl)isoalloxazine afforded a radical ESR spectrum (Fig. 3) indistinguishable from that of RF, *i.e.* 12 lines. The same number of lines were also obtained for 6-chloro-7-methyl-9-(1'-D-ribityl)-isoalloxazine but the spacing was decreased somewhat to about 3.4 G and the resolution was enhanced. These results indicate that the spin density on C(6) is small.

The radicals of 6-methyl-7-chloro- and 6,7-dichloro-9-(1'-D-ribityl)isoalloxazine both exhibit an odd number of lines, probably 9 lines, but the resolution varies (Fig. 3). 7-Chloro-6,9-dimethyl-isoalloxazine gives rise to a spectrum with 11 lines and a spacing of about 3.34 G (Fig. 3; Table 2). 6-Methyl-7-ethyl- and 6,7-diethyl-9-(1'-D-ribityl)isoalloxazine have identical spectra with an odd number of lines, the total number being 9 or 11. This information

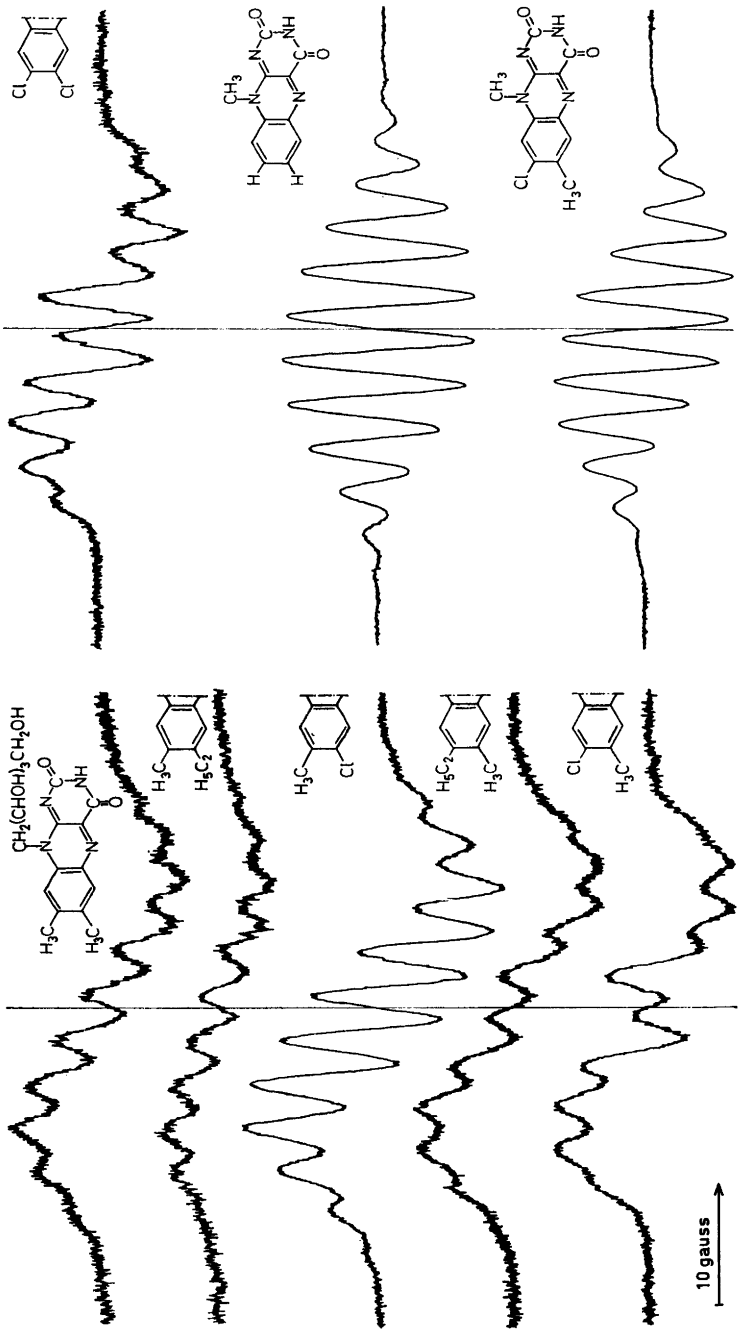

Fig. 3. ESR spectra of flavin free radicals with various substituents in positions (6) and (7). 10 mM aqueous solution of pH 12; degree of reduction about 50 %. Compare spectrum of normal lumiflavin in Fig. 2.

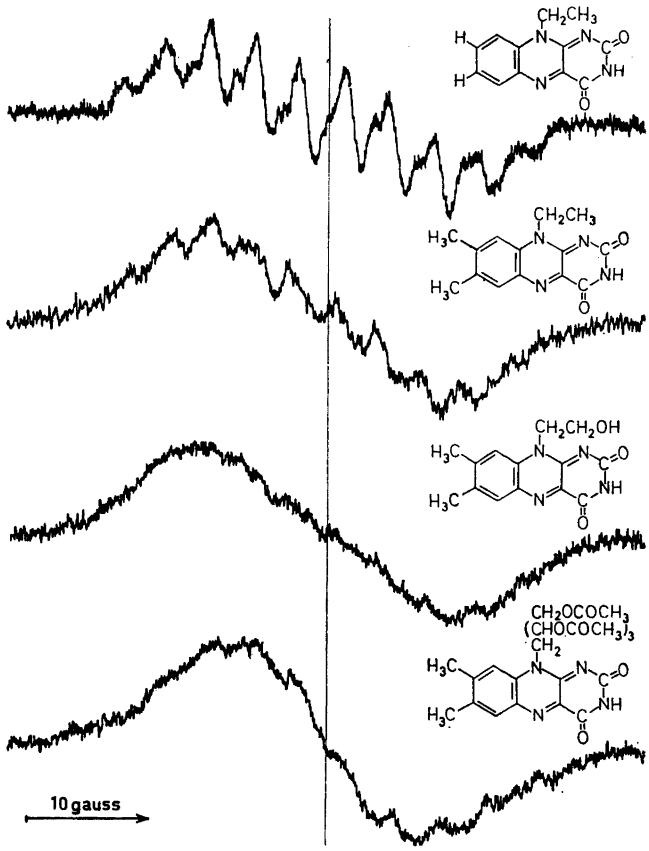

Fig. 4. ESR recordings of flavin free radicals with various N(9)-substituents. 10 mM aqueous solution of pH 12; degree of reduction about 50 %. For comparison see Figs. 2 and 3.

together with the previous results for RF (12 lines) and LF (14 lines) is consistent with a high spin density in the $2p_z$-orbital of C(7). For the three chloro-derivatives no chlorine hyperfine splitting has been observed.

The anion radical of 9-methylisoalloxazine gives an ESR spectrum consisting of 12 rather well resolved lines (Fig. 3; Table 2). The spacing is 3.52 G which is identical with that of the LF radical within the limits of experimental error. It is well-known that the spin polarization parameters for a ring proton and for the protons of a ring methyl group have about the same magnitude. Hence, the observed spectra show that replacement of ring protons by methyl groups in position (6) and (7) perturbs the spin density distribution only to a minor extent.

Because of the unusually good resolution of the ESR spectrum of the 9-methylisoalloxazine radical we have in this case more closely analyzed the

outermost lines which are the least complicated components of the main spectrum. This revealed that their shape does not exactly conform to that of a Gaussian function.

Assignment of hyperfine splittings and reconstruction of spectra

An ESR absorption spectrum was obtained by numerical integration of the recorded derivative spectrum without any further correction than for eventual slope of the base line. To any given absorption spectrum Gaussian hyperfine lines were fitted by trial and error. Since several overlapping sublines contribute to each main hyperfine line it is, however, not likely that the line-shape is simple like that. Nevertheless, the idealized approximation was used for practical reasons. A constant line width was used but it was found that the lines of the low-field part probably are somewhat narrower than those of high-field part. This would indicate that the nitrogen hyperfine splitting a^N is positive.[30] Average line intensities for the two halves of each spectrum were calculated and are shown in Table 2.

The spectra of the various flavin free radicals in alkaline solution may only be explained by assuming that the hyperfine splittings of the nuclei participating in the interaction are multiples, or fairly close to multiples, of the measured spacing. This is confirmed by our substitution experiments. We conclude that the following nuclei should primarily be accounted for when reconstructing the spectra of the anionic radicals: N(10), the ring proton or the methyl protons in position (7), the (1′)-protons in position (9) which are sterically available for interaction, and either of ring protons H(5) ar H(8). Moreover our analysis of the possibilities to reconstruct the spectra shows that still another nitrogen is needed. Since the interactions from N(1) and N(3) are small this nitrogen must be N(9). This seems to be compatible with observed hyperfine interaction of the (1′)-protons.

Table 3. Main isotropic hyperfine coupling constants for the anionic lumiflavin free radical in units of gauss (G). Figures within brackets denote alternative possibilities.

$a_9^N = 3.5$ (7.0)	$a_5^H = 3.5$ (small)
$a_{10}^N = 7.0$ (3.5)	$a_7^H(CCH_3) = 3.5$
	$a_8^H =$ small (3.5)
	$a_9^H(NCH_3) = 3.5$

Only schemes with precisely 14 lines for the LF radical have been considered, since schemes giving more lines, the weakest of which would not have been detected with the present instrumental sensitivity, do not have intensity ratios in the centre in agreement with experiments. The set of hyperfine splittings for the LF radical which fits the available experimental information is given in Table 3. It is not yet settled whether it is N(10) or N(9) that gives the larger splitting. Certain theoretical relationships to be discussed below

make us, however, inclined to assign the larger hyperfine splitting to N(10). As mentioned before, ambiguity also remains whether it is H(5) or H(8) that takes part in the main hyperfine interaction. The hyperfine splittings of N(1), N(3) and CH_3(6) are estimated to be of the order of 0.5 G or less. By means of the values of Table 3, and after adjustment of all splittings to multiples of 3.5 G, we have calculated the line intensities of the radical spectra given in Table 2.

Comparison with calculations. The isoalloxazines retain properties of several groups of compounds. When comparing our experimental results with available quantum chemical calculations [8-11] we have to rely on relationships between hyperfine splittings and spin densities established theoretically and found empirically to be valid more or less rigorously for a number of simpler compounds. However, one must be cautious when applying these relationships to the more complicated chemical structure of isoalloxazine.

The linear relation [31]

$$a_i^H = Q_{CH}^H \varrho_{C_i}^\pi \tag{1}$$

between the proton hyperfine splitting a_i^H and the spin density $\varrho_{C_i}^\pi$ in the $2p_z$-orbital of the carbon atom (i) to which the H-atom is bonded has proved to apply to many aromatic radicals. The absolute value of the spin polarization parameter, $|Q_{CH}^H|$, is usually considered to be within the range 21—27 G and is 22.5 G for the benzene anion. In some cases larger values of $|Q|$ have been found, since the simple molecular orbital theory used fails to predict negative spin densities, which are, however, indicated by more sophisticated theories.

A similiar expression

$$a_i^H(CCH_3) = Q_{CCH_3}^H \varrho_{C_i}^\pi \tag{2}$$

has been deduced [32] for the protons of a methyl group attached to a trigonal carbon atom. Experimental results in agreement with the theoretical value $|Q_{CCH_3}| = 28$ G have been reported.[33] However, for some dimethylnaphthalene anions a value of 19 G was found to be valid.[34] Nevertheless, from our substitution experiments we may conclude that it is reasonable to assume that $|Q_{CH}^H| \approx |Q_{CCH_3}^H|$ for the isoalloxazine free radicals.

Several heterocyclic aromatic radical ions have been studied by means of ESR and the observed hyperfine splittings compared with calculated unpaired electron distributions.[35-41] In analogy with the relationship developed for the splittings of ^{13}C with sp^2 sigma bonds [42] the expression

$$a_i^N = Q_N^N \varrho_{N_i}^\pi + \sum_{j=1}^{3} Q_{X_jN}^N \varrho_{X_j}^\pi \tag{3}$$

has been applied to trigonal nitrogen atoms.[41] Contributions from the spin density $\varrho_{N_i}^\pi$ in the p_z-orbital of the nitrogen itself and from the spin densities $\varrho_{X_j}^\pi$ in the p_z-orbitals of nearest neighbour atoms X_j are included. The spin polarization contributed by the nitrogen lone-pair electrons, when present,

is implicitely accounted for in the first term. The values calculated for Q_N^N and Q_{XjN}^N appear to be quite sensitive to the choice of other spin polarizing parameters of the molecule. The dominating contribution to the nitrogen splitting emerges, however, from the spin density on that very atom. The empirical values for $|Q_N^N|$ range from 18.5 to 28.5 G. The influence from the nearest neighbour C-atoms is small. In the cases when higher values of Q_{CN}^N have been reported the corresponding Q_N^N values are found to be comparatively small. This means that if Q_{CN}^N is neglected also in these cases, the apparent Q_N^N would increase somewhat. Hence it is reasonable to use as a first approximation the simplified linear relationship for trigonal nitrogen

$$a_i^N = Q_N^N \varrho_{N_i}^\pi \tag{4}$$

with Q_N^N within the mentioned range. This can readily be applied in the case of flavin radicals, where the lack of information about the spin densities on the bridge C-atoms prohibits the use of the more complicated expression (3).

A relationship analogous to that derived for the protons of a methyl group bonded to a trigonal carbon atom, eqn. (2), should also be valid in case the bonding atom is a nitrogen with sp^2 sigma bonds and has unpaired spin density in the p_z orbital:[32]

$$a_i^H(NCH_3) = Q_{NCH_3}^H \varrho_{N_i}^\pi \tag{5}$$

ESR studies on the Würster's blue ion [43,44] have shown that the splitting $a_i^H(NCH_3)$ of the methyl protons is practically identical with a_i^N. This means that we have

$$|Q_N^N| \approx |Q_{NCH_3}^H| \tag{6}$$

McLachlan's rough Hückel calculations [32] of the radical indicate the range 21—31 G for $|Q_{NCH_3}^H|$, which is inagreement with (6) and the assumed range of value for $|Q_N^N|$. The recent results by Johnson and Gutowsky on the viologens [40] indicate that $|Q_N^N| \approx 25$ G and that (6) is valid for these N-substituted heterocycles. Hence it is reasonable to expect the equality to hold also in case of LF. This is compatible with our first choice of assignments of the nitrogen splittings of Table 3, with $a_9^N = a_9^H(NCH_3)$. The alternative assignment would require $|Q_N^N| \approx 2|Q_{NCH_3}|$.

In the derivatives with a methylene group bonded to N(9) $Q_{NCH_3}^H$ is, at least in some cases, different for the two methylene hydrogens and depends on the equilibrium conformation of the N(9)-CH_2(1′) grouping. Asymmetric methylene groups in solids are known from ESR studies on radiation induced free radicals. It has been shown theoretically [45] that an asymmetry of this type could be caused either by a rotational distortion of the bond from the methylene carbon to the atom carrying the unpaired spin or a distortion of the bond angle at the methylene carbon. When the temperature of the solid is increased the thermal agitation sometimes restores the symmetry reversibly. The steric requirements and forces for the distortion are implied by the crystalline lattice and field. In the case of isoalloxazine derivatives our results indicate that a hydrogen bond is involved. This could lock the methylene group in a

certain rotational orientation and/or distort the bond angle at the methylene carbon. However, the possibility should also be considered that the asymmetry and its change with different substituents simply could be due to different time averages of the hyperfine interactions of the two methylene hydrogens. This could be caused by the unequal steric hindrance at C(8) and N(1) influencing the rotation of the N(9) substituent.

Table 4. Comparison between experimental spin densities and theoretically calculated distributions of the unpaired electron in the flavin anion radical. The range of values stated for ESR is determined by the limits of the spin polarization parameters (Q) found in the literature. All values in units of the negative electronic charge. Only positions relevant for the interpretation or our ESR spectra are included.

Position	Atom	ESR (ϱ^{π})	Karreman[10] ketoform	Baudet *et al*[11]. ketoform	Grabe* ketoform	Grabe* enolform
1	N	$\leqslant 0.025$	0.037	0.013	0.074	0.057
3	N	$\leqslant 0.025$	0.001	−0.007	0.000	0.094
5	C	0.130−0.167	0.097	0.158	0.044	0.037
6	C	$\leqslant 0.025$	0.014	−0.031	0.000	0.000
7	C	0.125−0.184	0.080	0.098	0.028	0.023
8	C	$\leqslant 0.025$	0.037	0.015	0.011	0.008
9	N	0.123−0.189	0.096	0.107	0.036	0.027
10	N	0.246−0.378	0.282	0.427	0.343	0.321

* Grabe, B., (1964), personal communication

From our experimental hyperfine splittings, given in Table 3, and by means of the spin polarization parameters, we can now estimate roughly the spin density at some atoms of the anionic LF radical. If we use both limiting values of Q in each case and assume all spin densities to be positive we obtain the experimental figures shown in Table 4.

In the table are also included results from quantum chemical calculations. Karreman [10] has applied the Hückel method considering all mobile electrons of the flavin radical in ketoform. The Hückel approximation as well as the self-consistent field (SCF) method were used in calculations on the heterocyclic parts of the enolic anion radical by Grabe.[8] She has [9] extended her calculations to the whole isoalloxazine structure but not included the influence of the methyl groups in positions (6) and (7). Both the keto- and the enolform were investigated. The distribution of the unpaired electron was in all the mentioned calculations obtained by the addition of a single electron to the lowest empty orbital of the oxidized neutral isoalloxazine, without any further SCF calculations. With due permission of Dr. B. Grabe, University of Stockholm, her unpublished recent SCF calculations* on the radical, explicitly considering the unpaired electron, are shown in Table 4. Baudet *et al.*[11] have used a SCF method, calculating with separate orbitals for spin α and spin β. In this way negative spin densities may also be obtained. However, the type of wave

* These calculations were made on UNIVAC 1105 at Illinois Institute of Technology, Chicago.

function used in this calculation has been criticized [46] since it is not an eigenfunction of the total spin operator S^2. In spite of this, as seen from Table 4, the calculations by Baudet *et al.* are in many details in fairly good agreement with our experimental results. According to the calculations ϱ_{10}/ϱ_9 comes out higher than would be expected from the ESR experiments, if the two values of Q_N^N are identical. A somewhat smaller Q_N^N for position (10) could arise from differences in the spin polarizibility of an N-C bond and a nitrogen lonepair, respectively. Also the results by Stone and Maki [41] indicate that Q_N^N is smaller for a nitrogen with a lone-pair than if there is a third N-C bond. There is at present no reason to believe that either Q_{CCH_3} or Q_{CH}^H should vary in the benzenoid ring. Therefore the calculations should give spin density ratios ϱ_6/ϱ_7 and ϱ_8/ϱ_5 in agreement with experiments. The ratio ϱ_8/ϱ_5 may be larger or smaller than unity due to the alternative assignments of H(5) and H(8) shown in Table 3. Because all calculations give the highest spin density in position (5) we believe that H (5) causes the strongest hyperfine splitting of the two.

Acknowledgement. This is part of a program supported by PHS Research Grant AM-5895 from the *National Institute of Arthritis and Metabolic Diseases*, U.S. Public Health Service. The inspiring and valuable discussions with Professor I. Fischer-Hjalmars and Dr. B. Grabe, University of Stockholm, are gratefully acknowledged. We are indebted to Dr. P. Hemmerich University of Basel, for having his advise in the synthetic part of this work, and for collaboration in the work with the radicals of 7-chloro-6,9-dimethylisoalloxazine and 6,7-dimethyl-9-(1′-D-ribityltetraacetate)isoalloxazine. Our thanks are also due to Dr. J. P. Lambooy, University of Nebraska, for the gift of several riboflavin analogues. We wish to thank Miss M. Ahlén and Mr. T. Lauvrud for able technical assistance. A grant from *Statens Naturvetenskapliga Forskningsråd* made it possible for one of us (A.E.) to visit Basel and for P.H. to visit Stockholm.

REFERENCES

1. Beinert, H. and Sands, R. H. in Blois, M. S. *et al. Free Radicals in Biological Systems*, Academic Press, New York 1961, p. 17.
2. Ehrenberg, A. *Arkiv Kemi* **19** (1962) 97.
3. Shiga, T. *J. Physiol. Soc. Japan* **24** (1962) 77.
4. Kubo, H., Watari, H., Shiga, T., Isomato, A., Uozumi, M. and Kadota, K. *Koso Kagaku Shimpoziumu* **17** (1962) 24.
5. Kubo, H., Shiga, T., Uozumi, M. and Isomato, A. *Bull. Soc. Chim. Biol.* **45** (1963) 219.
6. Guzzo, A. V. and Tollin, G. *Arch. Biochem. Biophys.* **103** (1963) 231.
7. Ehrenberg, A. and Eriksson, L. E. G. *Arch. Biochem. Biophys.* **105** (1964) 453.
8. Grabe, B. *Arkiv Fysik* **17** (1960) 97.
9. Grabe, B. *Biopolymers* Symposia No. **1** (1964) 283.
10. Karreman, G. *Bull. Math. Biophys.* **23** (1961) 55.
11. Baudet, J., Berthier, G. and Pullman, B. *Compt. Rend.* **254** (1962) 762.
12. Meites, L. and Meites, T. *Anal. Chem.* **20** (1948) 984.
13. Kuhn, R. and Weygand, F. *Ber.* **68** (1935) 1282.
14. Hemmerich, P. *Helv. Chim. Acta.* **39** (1956) 1242.
15. Warburg, O. and Christian, W. *Biochem. Z.* **266** (1933) 377.
16. Noelting, E., Braun, A. and Thesmar, G. *Ber.* **34** (1901) 2242.
17. Adams, R. R., Weisel, C. A. and Mosher, H. S. *J. Am. Chem. Soc.* **68** (1946) 883.
18. Hemmerich, P., Prijs, B. and Erlenmeyer, H. *Helv. Chim. Acta* **42** (1959) 2164.
19. Hemmerich, P. *Helv. Chim. Acta* **43** (1960) 1942.
20. Fall, H. H. and Petering, H. G. *J. Am. Chem. Soc.* **78** (1956) 377.
21. Usherwood, E. H. and Whiteley, M. A. *J. Chem. Soc.* **123** (1923) 1084.
22. Kuhn, R. and Weygand, F. *Ber.* **67** (1934) 1409.
23. Haley, E. E. and Lambooy, J. P. *J. Am. Chem. Soc.* **76** (1954) 5093.

24. Lambooy, J. P. *J. Am. Chem. Soc.* **80** (1958) 110.
25. Hemmerich, P., Prijs, B. and Erlenmeyer, H. *Helv. Chim. Acta* **42** (1959) 1604.
26. Kuhn, R. and Wagner-Jauregg, T. *Ber.* **66** (1933) 1577.
27. Ehrenberg, A. *In preparation.*
28. Wertz, J. E., Reitz, P. C. and Pravnieks, F. in Blois, M. S. *et al. Free Radicals in Biological Systems*, Academic Press, New York 1961, p. 183.
29. Hemmerich, P. *Helv. Chim. Acta* **47** (1964) 464.
30. Carrington, A. and Longuet-Higgins, H. C. *Mol. Phys.* **5** (1962) 447.
31. McConnell, H. M. and Chesnut, D. B. *J. Chem. Phys.* **28** (1958) 107.
32. McLachlan, A. D. *Mol. Phys.* **1** (1958) 233.
33. Bernal, I., Rieger, P. H. and Fraenkel, G. K. *J. Chem. Phys.* **37** (1962) 1489.
34. de Waard, C. and Henning, J. C. M. *Phys. Letters* **4** (1963) 31.
35. Ward, R. L. *J. Am. Chem. Soc.* **83** (1961) 3623; **84** (1962) 332.
36. Carrington, A. and dos Santos-Veiga, J. *Mol. Phys.* **5** (1962) 21.
37. Bolton, J. R., Carrington, A. and dos Santos-Veiga, J. *Mol. Phys.* **5** (1962) 465.
38. Atherton, N. M., Gerson, F. and Murrell, J. N. *Mol. Phys.* **5** (1962) 509; **6** (1963) 265.
39. Henning, J. C. M. and de Waard, C. *Phys. Letters* **3** (1962) 139.
40. Johnson, Jr., C. S. and Gutowsky, H. S. *J. Chem. Phys.* **39** (1963) 58.
41. Stone, E. W. and Maki, A. H. *J. Chem. Phys.* **39** (1963) 1635.
42. Karplus, M. and Fraenkel, G. K. *J. Chem. Phys.* **35** (1961) 1312.
43. Hausser, K. G. *Bull. Ampère*, **11**e année, fasc.spéc. (1962) 420.
44. Bolton, J. R., Carrington, A. and dos Santos-Veiga, J. *Mol. Phys.* **5** (1962) 615.
45. Morokuma, K. and Fukui, K. *Bull. Chem. Soc. Japan.* **36** (1963) 534.
46. Longuet-Higgins, H.C. and Pople, J.A. *Proc. Phys. Soc. (London)* **A 68** (1955) 591.

Received March 20, 1964.