41. ^{13}C-NMR. Study on Isoalloxazine and Alloxazine Derivatives

by **Hans J. Grande, Robert Gast, Cees G. van Schagen, Willem J. H. van Berkel** and **Franz Müller**[1])

Department of Biochemistry, Agricultural University
De Dreijen 11, Wageningen, The Netherlands

(31. V. 76)

Summary

A series of isoalloxazine and alloxazine derivatives have been investigated by ^{13}C-NMR. The synthesis of selectively ^{13}C-enriched derivatives made it possible to assign unambiguously the signals due to the quaternary carbon atoms at position 4, 4a and 10a of the isoalloxazine ring system. The assignment of the other resonances was ensured by the use of selectively deuteriated and chemically modified compounds as well as by decoupling techniques. The assignments differ in part from those published by *Breitmaier & Voelter* [2] on FMN and FAD. The solvent dependence of the resonances has been studied in dioxan/water mixtures. The experimental data are compared with published MO calculations and discussed.

1. Introduction. – In connection with ^{1}H- and ^{13}C-NMR. studies on flavoproteins it is important to characterize the prosthetic group of these proteins by these techniques. In a previous paper [1] we have described the ^{1}H-NMR. characteristics of alloxazines and isoalloxazines. *Breitmaier & Voelter* [2] studied the ^{13}C-NMR. of flavin-adenine-dinucleotide (FAD) and riboflavin-5'-monophosphate (FMN), the natural constituents of flavoproteins. However, these authors could not assign all resonances unambiguously. Furthermore, preliminary ^{13}C-NMR. results obtained in our laboratory indicated that our assignments did not agree with those published [2]. We have now investigated this problem in detail and found that an unequivocal assignment was only possible employing chemically and isotopically modified flavin (isoalloxazine) derivatives. The results are presented in this paper.

2. Experimental Part. – The ^{13}C-NMR. spectra were taken on a *Varian* XL-100 spectrometer operating at 25.2 MHz. The instrument was equipped with a 16 K *Varian* 620-L computer. All spectra were acquired in the *Fourier* transform mode using 12 mm sample tubes. Porton noise decoupling was used in all experiments, except in those in which single frequency decoupling was employed to aid peak assignments. The following instrumental conditions were used: acquisition time

[1]) To whom correspondence should be addressed.

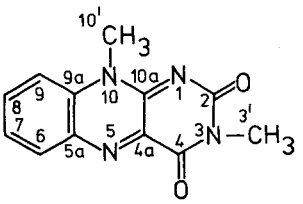
0.8 s, pulse width 10 μs ($\pi/2 = 28$ μs), spectral width 5000 Hz. The peak positions were determined from the computer generated printout, using TMS as internal standard. The chemical shifts given for a particular compound agreed well with values determined independently at different times and are within 3 Hz of each other. The sample temp. was 26°. The compounds were dissolved in deuteriated chloroform (99.8 atom-%, *Merck*, Germany) containing 10% tetradeuterio-^{12}C-methanol (99.5 atom-% D, 99.95 atom-% ^{12}C; *Merck*, Germany). It was necessary to add methanol to the chloroform solution to prevent a slow decomposition of the (iso)alloxazines. The number of transients accumulated varied between 20,000 and 70,000 depending on the solubility of the compound under investigation. Always saturated solutions were used resulting in concentrations varying from 0.1M to 0.01M.

The synthesis of the compounds used in this study has been described elsewhere ([1] and references therein). The selectively ^{13}C-labelled barbituric acids (starting materials for the isoalloxazine synthesis) were obtained by condensation of urea with 2-[^{13}C]- or 1,3-[^{13}C]-diethylmalonate (90 atom-%, *Prochem*, England) in the presence of sodium ethoxide according to *Murray* [3]. *Fieser & Fieser* [4] have suggested that in place of the usually employed equimolar amount of ethoxide with respect to urea and malonate excess of ethoxide might speed up the reaction. We have explored this suggestion and found that a twofold excess of ethoxide does not only shorten the reaction time but also gives higher yields of barbituric acid. Thus a mixture of 1.0 g of ^{13}C-labelled diethylmalonate, 0.336 g of urea, 0.4 g of sodium, and 8 ml of absolute ethanol gave 0.71 g of barbituric acid (91%, [4] 72–78%) with m.p. 254–255° (not corrected, lit. 254°) after 5 h of heating under reflux and work-up.

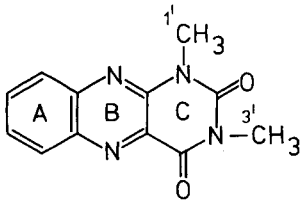
4a-[^{13}C]- and 4,10a-[^{13}C]-7, 8, 10-Trimethylisoalloxazines were obtained by condensation of the appropriate barbituric acids with 2-(*p*-carboxyphenylazo)-*N*,4,5-trimethylanilin in a mixture of glacial acetic acid and butanol as described by *Tishler et al.* [5]. The yield was 92% ([5]: 88%). The compounds were then methylated at N(3) and purified as described in [1].

3. Results. – The structure of 3,10-dimethylisoalloxazine and 1,3-dimethylalloxazine is shown in the *Scheme* together with the recommended ring numbering [6]. The assignment of the ^{13}C-resonances of (iso)alloxazines was based mainly on the comparison of spectra of various derivatives. The correctness of the assignements was then further checked by taking spectra under off-resonance conditions, and also by employing isotopically substituted (deuterium, ^{13}C-enriched) compounds.

Scheme



3,10-Dimethyl-iso-alloxazine



1,3-Dimethyl-alloxazine

Due to the often very weak intensities of resonances of quaternary carbon atoms in noise decoupled spectra it is sometimes a problem to attribute them unambiguously. In the isoalloxazine derivatives at least six quaternary carbon atoms are present, namely at position 2, 4, 4a, 5a, 9a, and 10a. The resonances of C(5a) and C(9a) can be recognized by indirect effects on these resonances, *i.e.* by the influences of methyl substitution in ring *A*. On the other hand, the resonances due to the carbon atoms of

the two carbonyl groups (C(2) and C(4)) can be expected to appear at lowest field. The only two resonances, which will be more difficult to assign, are those due to C(4a) and C(10a). For this reason compounds enriched with ^{13}C at these particular positions were synthesized. In fact derivatives of these compounds were prepared for use in ^{13}C-NMR. experiments on flavoproteins.

Fig. 1 shows the spectra of the selectively ^{13}C-enriched isoalloxazines. In the spectrum of 4a[^{13}C]-3,7,8,10-tetramethylisoalloxazine (Fig. 1A) also some of the resonances due to the natural abundance ^{13}C-atoms are seen. The spectrum of 4,10a-[^{13}C]-3,7,8,10-tetramethylisoalloxazine is shown in Fig. 1B. The through-bond coupling between the two carbon atoms C(4) and C(10a) is 10 Hz. This has been confirmed by ^{13}C decoupling experiments. To reach an unequivocal assignment we took a ^{1}H-NMR. spectrum of this compound. The proton spectrum exhibits two doublets due to coupling of these ^{13}C-atoms with the H_3C–N groups. Irradiating the ^{13}C resonance at lowest field (Fig. 1B) transformed the doublet of the H_3C–N(3) into a singulet. Similarly, irradiating the other ^{13}C resonance gave a singulet of the resonance signal due to H_3C–N(10) (for the ^{1}H-NMR. assignments, see [1]). These results unambiguously show that the resonance signal at lowest field in spectrum B is due to

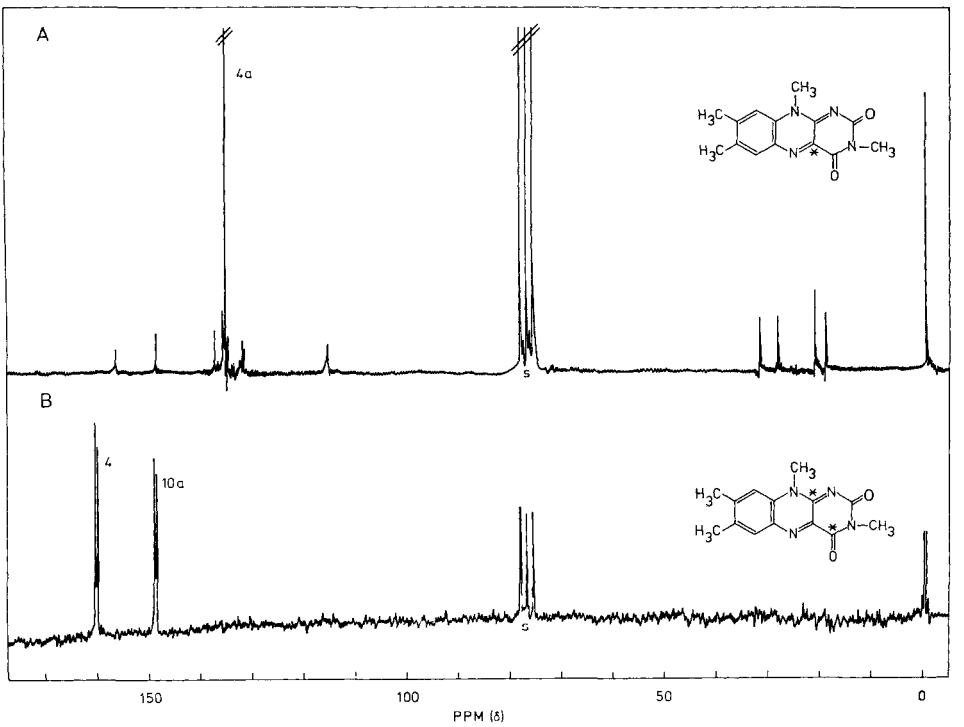
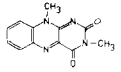
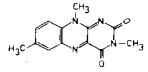
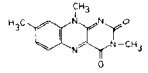
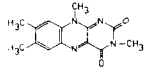
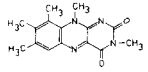
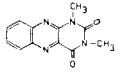
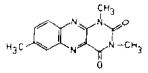
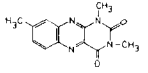
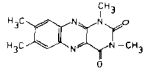
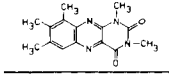


Fig. 1. *Proton noise decoupled ^{13}C-NMR. spectra of 4a-[^{13}C]- and 4,10a-[^{13}C]-3,7,8,10-tetramethylisoalloxazine in $CDCl_3/CD_3OD$.* The asterisk indicates position of ^{13}C-enrichment (90 atom-%) in the isoalloxazine ring system (s = resonances due to the solvent)

Table 1. *^{13}C Chemical Shifts* (in ppm) of Isoalloxazine and Alloxazine Derivatives[a])

Compound	No.	R.P.[b])															
		C(2)	C(4)	C(4a)	C(5a)	C(6)	C(7)	C(8)	C(9)	C(9a)	C(10a)	C(1′)	C(3′)	C(7′)	C(8′)	C(9′)	C(10′)
	I	156.6	160.3	n.o.[c])	136.2	133.5	127.2	136.4	115.8	133.8	149.7	–	28.8	–	–	–	32.3
	II	156.5	160.3	136.7	136.2	132.6	137.9	138.2	115.4	131.7	149.3	–	28.8	20.9	–	–	32.3
	III	156.7	160.5	135.7	134.7	133.0	129.1	149.1[d])	115.4	133.7	149.1[d])	–	28.8	–	23.0	–	32.2
	IV	156.7	160.6	135.5	135.0	132.6	137.5	148.8	115.7	132.0	149.2	–	28.8	19.5	21.6	–	32.2
	V	156.8	160.6	134.9	134.0	130.5	137.5	148.7	124.8	136.0	151.3	–	28.7	19.9	20.7	18.0	41.7
	VI	151.2	160.3	145.7	140.3	129.7	131.0	134.7	128.2	129.8	143.8	29.8	29.4	–	–	–	–
	VII	151.1	160.4	145.2	140.3	129.3	140.4	136.9	127.6	129.2	142.2	29.6	29.2	21.8	–	–	–
	VIII	151.1	160.3	145.6	138.7	130.2	132.1	145.9	126.8	128.7	143.8	29.6	29.2	–	22.4	–	–
	IX	151.2	160.6	145.3	139.3	129.4	140.6	146.2	127.1	128.5	142.8	29.6	29.2	20.3	20.9	–	–
	X	151.8	161.4	n.o.[c])	139.0	127.1	140.3	143.3	133.2	128.0	143.3	29.5	29.1	21.5	17.2	13.4	–

[a]) Spectra were obtained in the solvent mixture $CDCl_3/CD_3OD$. [b]) R.P. = Ring Position. [c]) n.o. = not observed. [d]) Too intense to be only 10a.

C(4), and the resonance signal at higher field due to C(10a). Comparing the spectra B and A it becomes evident that in the latter the natural abundance resonance signal due to the C(4) atom is not observed whereas the resonance signal due to C(10a) is clearly seen.

The other resonances have been assigned studying the influence of methyl substitution of ring *A* which affects the position as well as the intensity of the other resonances in the ring. The results obtained from the isoalloxazines I–V are summarized in Tab. 1. These data show that the resonance at about 156 ppm is independent on substitution of ring *A*., this and the low field position of the resonance makes it feasible to assign it to C(2). The resonances due to C(5) and C(9a) will be influenced by methyl groups placed in *ortho*, *meta* or *para* position to them. The methyl substituent effects are summarized in Fig. 2. Thus, introduction of a methyl group into position 7 of I leading to II influences the resonance position of C(9a) (*para* position) to a greater

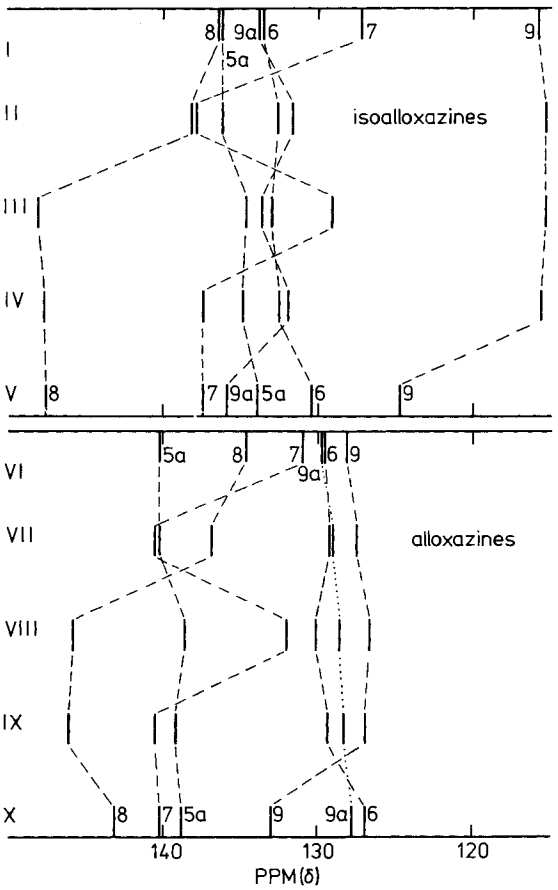


Fig. 2. *Correlation diagram of* ^{13}*C-NMR. chemical shifts of the isoalloxazine and alloxazine derivatives I–X.* Arabic numbers refer to the carbon atoms of the (iso)alloxazine ring (*cf. Scheme* 1)

extent than that of C(5a) (*meta* position). As expected the reverse effect is observed in going from I to III. These assignments to C(5a) and C(9a) are in full agreement with the data obtained for IV and V. In addition the resonances due to C(7), C(8) and C(9) are easily attributed on the basis of the large shifts due to methyl substitution at C(7), C(8), and C(9), respectively. Furthermore, the results obtained from V support the assignment of the resonance signal at about 132 ppm to C(6) (*para* shift; Fig. 2).

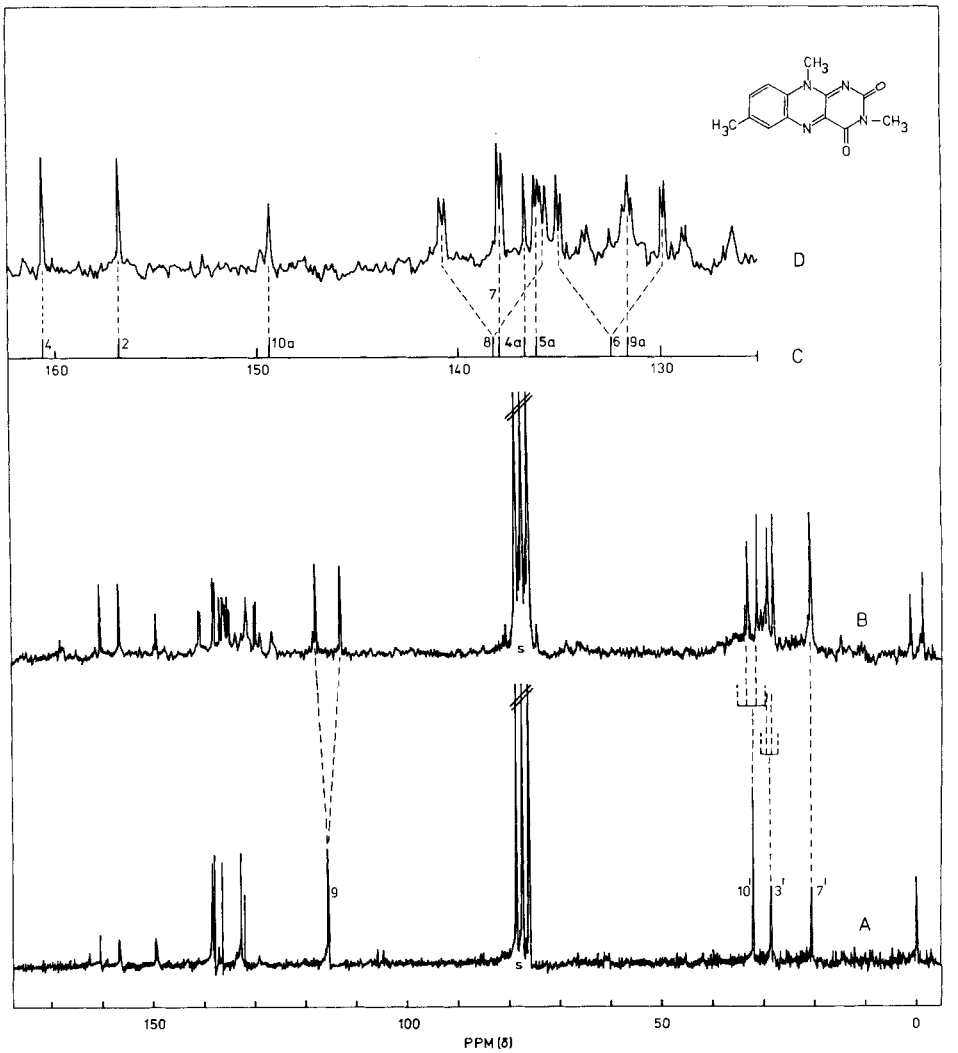


Fig. 3. *Natural abundance ^{13}C-NMR. spectra of 3,7,10-trimethylisoalloxazine (II) in $CDCl_3/CD_3OD$ 9:1.* A: proton noise decoupled; B: spectrum obtained under off-resonance conditions, irradiation frequency at 250 Hz from TMS; D: expanded low field part of the spectrum shown in B; C: stick spectrum corresponding to frequencies observed under noise decoupling conditions; s = as in Fig. 1

The high field resonances, all due to methyl groups, can be divided into two groups (Tab. 1). Comparing the resonances of I with those of II–V it is obvious that the resonances at about 20 ppm are due to methyl groups on C-atoms and those at about 30 ppm due to methyl groups on N-atoms. Mere inspection of the data in Table 1 leads already to the right assignments of the CH_3–C resonances. In order to support further these conclusions and to confirm at the same time the attribution of the other resonances, some off-resonance spectra were recorded and some selectively deuteriated compounds have been employed.

The proton noise decoupled ^{13}C-NMR. spectrum of II is shown in Fig. 3 (trace A). The spectrum B is obtained under single frequency off-resonance conditions [7]. An irradiation frequency of 250 Hz (from TMS) in the proton spectrum is employed. This frequency corresponds almost to the resonance position of H_3C–C(7) [1]. From such an experiment the following is expected: a) the ^{13}C resonances due to quaternary atoms will not be influenced, *i.e.* they will appear as singulets; b) depending on the number of protons, C-atoms bearing protons will split up in multiplets; c) the closer the proton resonance position of a particular group to the irradiating frequency, the more reduced become the H–^{13}C couplings in the ^{13}C spectrum. In the ^{1}H-NMR. spectrum of II the resonance signals due to the following groups appear in the order of decreasing field H_3C–N(3), H_3C–N(10), H–C(9), H–C(8), and H–C(6) [1]. In the high field part of spectrum B two quartets and a broadened singulet are seen which are due to the carbon atoms of the three methyl groups of II. The broadened signal at highest field exhibits in fact a small residual splitting which must be caused by the protons with a resonance closest to the irradiation frequency. Therefore, this signal is assigned to CH_3–C(7). The quartet centered at 28.8 ppm shows a smaller residual splitting than that centered at 32.3 ppm. Taking into account the ^{1}H-NMR. this proves that CH_3–N(3) is located at higher field than CH_3–N(10) (Table 1)[2]). The low field part of spectrum B, except the doublet of C(9), is shown in an (fourfold) expanded scale in trace D together with the corresponding stick spectrum C. The correctness of the assignments to the aromatic C-atoms is again checked by a single frequency off-resonance experiment using an irradiation frequency of 810 Hz from TMS (low field from the H–C(6) resonance) [1]. The residual splittings and NMR. pattern found in this experiment are in full accord with the assignments given in Table 1. It should be noted that in Fig. 3 some small splittings are observed which are due to coupling with protons other than those directly bound to a particular carbon atom.

Furthermore, selective deuteriation of IV at position 9 results in a large decrease of the intensity and a small shift of the signal at 115.7 ppm, as expected for the resonance of C(9). Similarly, selective deuteriation of CH_3–C(8) in IV confirmes the attribution of the resonance at 21.6 ppm to CH_3–C(8).

The signals observed with the alloxazine derivatives VI–X (Table 1) have also been assigned on the basis of the substituent effects (Fig. 2). In addition the results obtained from isoalloxazines were used where possible to achieve an unambiguous

[2]) The same conclusion can be reached comparing the ^{13}C-NMR. data from isoalloxazines with those from alloxazines.

assignment since with alloxazines no enriched compounds were available. The quaternary carbon atoms at positions 2, 4, 4a and 10a are attributed on the basis of the observation that the resonances due to these carbon atoms are not affected upon methylation of ring *A* what has also been observed with isoalloxazines. Furthermore, from a structural point of view with respect to isoalloxazines, the resonance at lowest field (160–161 Hz) is assigned to C(4). This assignment is not really dubious considering that in ^{13}C-enriched *O*(2), 3, 7, 8, 10-pentamethylisoalloxazinium perchlorate [8] the C(4) resonates at about 160 ppm, *i.e.* going from the neutral to the cationic species the resonance of C(4) is affected only to a minor extent. Furthermore, the resonance signal of C(10a) is easily recognized by its much larger amplitude than that of C(4a) due to the methyl group in the neighbourhood of C(10a). The only remarkable difference between the quaternary carbon atoms of isoalloxazines and those of alloxazines is that the intensities of the signals of C(7) or/and C(8) in VII, VIII and IX are much more intense in noise decoupled spectra than those of the corresponding isoalloxazines. The intensities are in fact comparable with those of *C*H resonances.

Similarly as with the isoalloxazines the assigments were confirmed by single frequency (at low and high field) off-resonance experiments. Some of these data are given in Tab. 2.

Table 2. *Off-Resonance Decoupling Experiments on 1,3,7,-Trimethylalloxazine (VII) and 1,3,8-Trimethylalloxazine (VIII)*[a])

	^{13}C chemical shift (in ppm)[b])	^{1}H chemical shift (in ppm)[c])	^{1}H irradiation frequency		
			810 Hz		830 Hz
VII			[d])		[d])
C(6)	129.3	7.98	5		15
C(7)	140.4	–	*s*[e])		*s*[e])
C(8)	136.9	7.79	17.6		32.8
C(9)	127.6	7.91	10		22.7
			820 Hz	850 Hz	371 Hz
VIII			[d])	[d])	[d])
C(6)	130.2	8.08	5	20.2	133.6
C(7)	132.1	7.66	27.7	40.3	118.4
C(8)	145.9	–	*s*[e])	*s*[e])	*s*[e])
C(9)	126.8	7.80	22.7	38	126
C(1′)	29.6	3.70	113.4[f])	118.4[f])	0.0[f])
C(3′)	29.2	3.45	115.9[f])	118.4[f])	10 [f])
C(8′)	22.4	2.62	108.4[f])	110.9[f])	42.8[f])

[a]) The solvent is $CDCl_3/CD_3OD$ 9:1.
[b]) Noise decoupled.
[c]) Taken from ref. [1].
[d]) Residual splittings (in Hz) observed upon irradiation at the indicated frequency.
[e]) *s* = small quartet splitting of about 5 Hz due to CH_3.
[f]) Quartet splittings.

The only assignments not completely sure are those to the carbon atoms C(5a) and C(9a) of the alloxazines since the methyl substituent effects (Fig. 2) are less informative for the alloxines than for the isoalloxazines. Thus, the *para* shift on C(9a) in going from VI to VII is only −0.6 ppm, while the *meta* shift on C(9a) (VI → VIII) is larger (Fig. 2; compare with isoalloxazines). Also the summed effects are small (IX). Furthermore, the resonance signal of C(9a) in VIII is difficult to recognize in the spectrum because of its low intensity compared to the one of C(5a), C(4), C(2) and C(4a)(very narrow and weak signal at 128.7 ppm in the noise decoupled, and very weak signal at 128.7 ppm in the off resonance spectra).

The dependence of the ^{13}C resonances on the polarity of the solvent has been investigated using tetraacetyl-riboflavin which is very soluble in pure deuteriated dioxan and D_2O as well as in mixtures thereof, and allows accumulation of spectra within a reasonable time. The data are collected in Tab. 3. For comparison the data obtained

Table 3. *Dependence of ^{13}C Chemical Shifts* (in ppm) *of Tetraacetyl-riboflavin on Solvent Polarity Using Dioxan/Water*

	D_2O						
	0%	1%	5%	10%	30%	FMN[a]	IV[b]
C(4)	159.8	n.o.[c]	n.o.[c]	n.o.[c]	161.2	160.2	160.6
C(2)	155.7	155.7	–	–	158.0	157.6	156.7
C(10a)	151.7	151.6	151.5	151.6	151.6	149.6	149.2
C(8)	146.4	146.5	147.2	147.5	148.9	150.8	148.8
C(7)	136.4	136.5	136.9	137.3	138.2	139.6	137.5
C(4a)	n.o.[c]	n.o.[c]	n.o.[c]	n.o.[c]	137.1	–	135.5
C(5a)	134.6	134.6	134.7	134.8	135.0	134.2	135.0
C(6)	132.8	132.8	132.7	132.6	132.4	130.0	132.6
C(9a)	132.0	132.0	132.0	132.1	132.2	131.4	132.0
C(9)	116.5	116.5	116.6	116.6	116.8	117.2	115.7

[a] Taken from [2]. Solvent: pure D_2O.
[b] Values given for comparison; solvent $CDCl_3/CD_3OD$ 9:1, see Tab. 1.
[c] Not observed.

from IV in $CDCl_3/CD_3OD$ 9:1 are given as well as those for FMN published by *Breitmaier & Voelter* [2] (the assignments of these resonances are corrected on the basis of the data in Tab. 1). The data of Table 3 show that the resonances of the carbon atoms of ring *B* are not, but the resonances of C(7), C(8), C(2), and C(4) are most influenced by the polarity of the solvent.

4. Discussion. – It is generally agreed that ^{13}C-resonances reflect the electron density distribution in a molecule in a much higher degree than the ^1H-resonances because the ^{13}C-chemical shifts are dominated by paramagnetic effects which in turn are proportional to the π-electron charge densities in the carbon $2p_z$ orbitals and in the neighbouring bonds. Therefore, it can be expected that the large chemical difference between alloxazines and isoalloxazines will be recognized in the ^{13}C-NMR. spectra.

It is interesting to compare our experimental data with data obtained by theoretical calculations. Unfortunately only calculations on isoalloxazines, but not on alloxazines have been published [9] [10]. Comparing our data with *π-electron* density calculations published by *Grabe* [9] and *Fox et al.* [10] little correlation is found. This is in contrast to results where the calculated π-density correlates well with experimental NMR. data on aromatic protons and carbon atoms [11]. However, *Gawer & Daily* [12] observed that such a correlation does not, or to a much lesser degree, exist for protons in heterocycles. A similar trend was observed for the ^{1}H-NMR. data obtained from (iso)alloxazines [1]. It can thus be expected that also ^{13}-NMR. data obtained from heterocycles would show less agreement with calculated data. This would indicate that the σ–π separability conditions [13] do not hold for heterocycles, what is apparent from the high polarization of the σ-core of *e.g.* pyrrole and pyridine as shown by *ab initio* calculations [14]. In calculating the charge distribution with PPP-SCF methods, this polarization is not accounted for, hence they lead to incorrect total electron densities (*cf.* [15]).

Table 4. *Comparison of Calculated Net Charge and ^{13}C Chemical Shifts (in ppm) of Quinoxaline, 3,10-Dimethylisoalloxazine (I) and 1,3-Dimethylalloxazine (VI)*

	Quinoxaline		I		VI
Ring[a]) position	Net charge[b])[c])	Chemical shift[c])	Net charge[b])[d])	Chemical shift	Chemical shift
C(8)	0.009	129.1	0.042	136.4	134.7
C(7)	0.009	129.1	−0.051	127.2	131.0
C(9)	−0.013	129.0	−0.075	115.8	128.2
C(6)	−0.013	129.0	0.021	133.5	129.7
C(9a)	0.087	142.4	0.103	133.8	129.8
C(5a)	0.087	142.4	0.009	136.2	140.3
C(10a)	0.060	144.7	0.264	149.7	143.8
C(4a)	0.060	144.7	−0.099	136.7	145.7
C(2)	–	–	0.705	156.6	151.2
C(4)	–	–	0.624	160.3	160.3

[a]) For comparative purposes the ring numbering assigned to quinoxaline is the same as that attributed to the quinoxaline residue in the (iso)alloxazines.

[b]) The net charge is given as a difference between the total amount of valence electrons (4 for carbon atoms) and the calculated density.

[c]) Taken from [16]: the published chemical shifts are relative to benzene (12 7.7 ppm); those given are calculated relative to TMS.

[d]) See footnote[3]).

In Tab. 4 the net electron densities and the experimental ^{13}C-NMR. data of quinoxaline, 3,10-dimethylisoalloxazine (I) and 1,3-dimethyl alloxazine (VI) are compared. Electron densities, obtained by CNDO/2 calculations, and resonances for quinoxaline, which are given for comparison, are taken from *Pugmire et al.* [16]. The net electron densities for 3,10-dimethylisoalloxazine (I) were calculated by *Fox*[3]).

[3]) Personal communication; the data were obtained by an all valence electron molecular orbital calculation within the MINDO/3 formalism.

To correlate in I the deviation from the (twofold) symmetrical charge distribution with the resonance position the pairs which correspond to equivalent ring positions in quinoxaline are compared in Tab. 4: only a rough correlation is obtained. In quinoxaline the carbon atoms with the lowest electron density are indeed less shielded, but the smaller differences in the calculated electron density are not reflected by the resonances. In accordance with the calculation of *Fox*[3]) in isoalloxazines C(8) is less shielded and thus somewhat positively charged compared to C(7) which is somewhat more shielded. The resonance of C(9) is shifted quite far upfield, the center thus being negative, while that of C(6) is located at relatively low field also as predicted by *Fox*. The resonances of C(9a) and C(5a) are located at high field, but in the opposite order as predicted. On the other hand, the resonance positions of C(4a) (at high field) and C(10a) (at low field) are in agreement with the calculated *relative* charges. Furthermore, the very low field position of the carbonyl carbon atoms is in agreement with the calculated low electron densities on C(2) and C(4) (they miss almost 3/4 electron), the order however is again in disagreement with the prediction. From this it can be concluded that the calculations predict, approximately the chemical shifts, if compared with the data for the symmetric quinoxaline, but the absolute values of the electron densities alone dont correlate[4]). This conclusion is best illustrated by the fact that C(4a) of I resonates at about the same frequency as C(8) and C(5a), but C(4a) possesses the largest calculated negative charge whereas both C(8) and C(5a) carry positive charge.

The results suggest that C(6), C(8), and C(10a) are positive, which means that already in apolar solvents C(8) and C(6) of isoalloxazines are strongly conjugated to C(2) *via* C(10a). It is expected that in polar solvents the carbonyl groups tend to be more electron withdrawing, thus polarizing the molecule to a larger extent. Due to the fact that C(6) and C(8) are already positive, it is reasonable to predict that the withdrawn electrons now have to come from positions 9 and 9a. By this the ring current will not be altered much the electron withdrawal and the π-delocalisation compensating each other. The latter should result in a shift of electrons towards C(10a) causing the molecule to become more alike alloxazine. From Table 3 this solvent effect is not very apparent except from the downfield shift of *both* resonances due to C(7) and C(8). The large solvent induced downfield shift of the resonance of C(7) is quite unexpected. It may indicate that this carbon atom is not as isolated in the isoalloxazine system as usually assumed. The solvent effects are much better reflected in the ^{1}H-NMR. spectra of (iso)alloxazines [1]. The reason for these effects being less apparent in the ^{13}C-NMR. spectra could be ascribed to the higher total valence electron density at the carbon atoms (4 electrons) as compared to that at protons (1 electron), and also to the polarization of the molecule by the carbonyl groups exerting their influence *via* the π-system.

The ^{13}C-NMR. data obtained from alloxazines, when compared with those obtained from quinoxaline and isoalloxazines, yield valuable information for future calculations on these molecules. From Tab. 4 it is apparent that alloxazines resemble

4) We assume that there is a direct correlation between charge density and resonance position. However, there are also indirect effects, such as ring currents, charges on neighbouring atoms *etc.*, which are usually not considered.

quinoxaline more closely than isoalloxazines, a conclusion in agreement with that drawn from data obtained by ^{1}H-NMR. [1]. Thus, the twofold symmetry observed in the data of quinoxaline is decreased to a much lesser degree in alloxazines than in isoalloxazines. In fact the lower symmetry in alloxazines as compared to quinoxaline is reflected most obviously only by the resonances of C(5a) and C(9a). In addition, only the resonance of C(8) of alloxazines appears at significant lower field than that of the corresponding C-atom in quinoxaline, indicating that in the former this atom is involved in conjugation with the carbonyl groups. Moreover, the fact that the chemical shifts of C(4a) and C(10a) of alloxazines are more alike to each other than those corresponding in isoalloxazines suggests that in the alloxazines both carbonyl groups and in the isoalloxazines mainly the C(2)-carbonyl group are involved in the conjugation of the (iso)alloxazine ring system. The relatively high field position of the C(2) resonance of alloxazines, as compared to that of isoalloxazines, suggests delocalization of the lone electron pairs particularly of N(1) and also of N(3) towards C(2). This interpretation is in agreement with acidity constants showing that H–N(1) and H–N(3) of alloxazines are more acidic by about two orders of magnitude than H–N(3) of isoalloxazines [17].

It is noteworthy that the substituent effects observed with V and X differ from those of the other compounds (Fig. 2). The large asymmetry observed in the shifts of the resonances of the carbon atoms in *ortho* position to C(9) in going from IV to V, *i.e.* 4.0 ppm for the resonance of C(9a) and −0.1 ppm for the resonance of C(8) is remarkable; a similar but smaller effect is observed in going from IX to X. This indicates that the mutual influence of the methyl groups causes a distortion from planarity of the isoalloxazine ring as already suggested by the ^{1}H-NMR. spectra [1].

Comparing our resonance assignments with those in [2] it is seen, that five out of the twelve resonances of the isoalloxazine ring were differently assigned in the latter [2]. Since we used an apolar solvent instead of water, the different assignments could be due to this difference, but this is refuted by the results given in Tab. 3 which clearly show that the chemical shifts are not influenced drastically by solvent polarity. In addition, the observed intensities of the lines are much more compatible with our assignments. Thus, the resonance assigned to C(4a) in [2] is a rather intense line, whereas in our spectra this line is weak, as expected for a quaternary carbon atom having no adjacent protons. Only with the aid of the ^{13}C-enriched compounds we were able to assign this weak line unambiguously in most of the spectra. In addition it was observed that in aqueous solution even the resonance of C(4a) of 4a[^{13}C]-FMN was very difficult to detect as compared to the detection of this resonance in organic solvents[5]). This result suggests that probably water interacts quite specifically with the C(4a) atom of the isoalloxazine ring system, which drastically changes the line shape of the C(4a) resonance.

The data here presented show that only a rough correlation exists between calculated electron densities and experimental ^{13}C-resonances. To get a better understanding of the chemical entity of isoalloxazines, the natural constituents of flavoproteins, more accurate theoretical values are required. We believe that better theoretical results could be achieved using the more symmetrical molecule alloxazine for the calculations

[5]) *C. G. van Schagen & F. Müller*, unpublished results.

and employing the methyl substituent effects. The results thus obtained could then form the basis for calculations on isoalloxazines. This approach would require extensive calculations on at least a (low cost) MINDO-level with a different parametrization for each of the derivatives, for which at present our laboratory lacks the necessary means.

We are grateful to Prof. Dr. *J. L. Fox*, University of Texas, Austin (U.S.A), for the communication of the MINDO/3 calculations prior to publication, to Mr. *R. J. Platenkamp* for valuable discussion, to Mr. *B. J. Sachtleben* for the preparation of the Figures, and to Miss *A. H. W. Trip* for preparing the Tables.

This study has been carried out under the auspices of 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.)*.

REFERENCES

[1] *H. J. Grande, C. G. van Schagen, T. Jarbandhan & F. Müller*, Helv. *60*, 348 (1977).
[2] *E. Breitmaier & W. Voelter*, Eur. J. Biochemistry *31*, 234 (1972).
[3] *J. I. Murray*, Org. Synth., Coll. Vol. *4*, 744 (1963).
[4] *L. F. Fieser & M. Fieser*, "Reagents for Organic Synthesis", Vol. I, John Wiley & Sons, Inc., New York 1967, p. 1268.
[5] *M. Tishler, K. Pfister, R. D. Bobson, K. Ladenburg & A. J. Fleming*, J. Amer. chem. Soc. *69*, 1487 (1947).
[6] Eur. J. Biochemistry *2*, (1967).
[7] *B. Birdsall, N. J. M. Birdsall & J. Feeney*, Chem. Commun. *1972*, 316.
[8] *F. Müller, H. J. Grande & T. Jarbandhan*, in "Flavins and Flavoproteins", T. P. Singer, ed., Elsevier Publishing Company, Amsterdam 1976, p. 38.
[9] *B. Grabe*, Acta chem. scand. *A28*, 363 (1974).
[10] *J. L. Fox, K. Nishimoto & L. S. Forster*, Biochem. biophys. Acta *109*, 626 (1965).
[11] *P. C. Lauterbur*, J. Amer. chem. Soc. *83*, 1838 (1961); *H. Spiesecke & W. G. Schneider*, Tetrahedron Letters *1961*, 468.
[12] *A. H. Gawer & B. P. Dailey*, J. chem. Physics *42*, 2658 (1965).
[13] *R. G. Parr*, in "Quantum Theory of Molecular Electronic Structure", W. A. Benjamin, Inc., Publishers, Reading, Massachusetts 1972, p. 42.
[14] *E. Clementi, H. Clementi & R. Davis*, J. chem. Physics *46*, 4725 (1967).
[15] *R. L. Flurry, E. W. Stout & J. J. Bell*, Theor. chim. Acta *8*, 203 (1967).
[16] *R. J. Pugmire, D. M. Grant, M. J. Robins & R. K. Robins*, J. Amer. chem. Soc. *91*, 6381 (1969).
[17] *F. Müller & K. H. Dudley*, Helv. *54*, 1487 (1971).