40. An ^{1}H-NMR. Spectroscopic Study of Alloxazines and Isoalloxazines

by **Hans J. Grande, Cees G. van Schagen, Tjan Jarbandhan** and **Franz Müller**[1])

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

(17. V. 76)

Summary

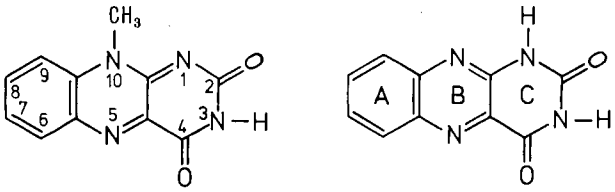
^{1}H-NMR.-spectra of a series of alloxazines and isoalloxazines and certain cationic derivatives were investigated (Tab. 2 and 5). Unequivocal assignment of all resonance signals was achieved in some compounds by selective deuteriation also by double resonance technique. The coupling constants were verified by computer simulation. Considerable enhancement of the signals due to H–C(9) and H–C(6) is found on decoupling of H_3C–C(7), H_3C–C(8) and H_3C–N(10), resp. These results are compared with those obtained with FAD. The methyl resonance signal of the H_3C–C (7) compounds give doublets due to coupling with H–C(6). The difference in chemical shifts observed upon successive formal introduction of methyl groups into the benzene nucleus of (iso)alloxazines indicates that the molecule becomes less planar thereby. The pyrimidine ring of (iso)alloxazines does not contribute to the ring current except by indirect effects *via* the carbonyl groups. The experimental data are compared with published MO calculations and discussed.

1. Introduction. – Higher molecular weight derivatives of isoalloxazine play an important role as cofactors in many flavoenzyme-dependent biological reactions. The great progress achieved in biochemical research of flavoproteins in the past few years has stimulated the investigation of flavocoenzymes and their low molecular weight derivatives in order to obtain better insight into the biological function of flavin-dependent enzymes, see [1]. On the other hand, to be able to give a full account of the observed chemical properties of isoalloxazines and to predict their chemical interaction with various substrates, it is necessary to know their π-electron distribution; a few molecular orbital calculations have been performed [2] but the results do not agree well with published experimental work, by no means surprising considering the chemical complexity of the isoalloxazine molecule. Therefore, a great need still exists for the study of this molecule. For this reason we have undertaken diverse and detailed investigations of a series of isoalloxaines and alloxazzines (*cf. Scheme 1*; for concision the two series will frequently be designated (iso)alloxazines) by different physical methods.

Here we report on the ^{1}H-NMR. spectra of a series of isoalloxazines and their isomeric alloxazines. The aim is threefold, namely to effect a detailed characterization

[1]) To whom correspondence should be addressed.

Scheme 1



10-Methyl-iso-alloxazine Alloxazine

of these molecules by ^{1}H-NMR. spectra, to correlate them with theoretical calculations, and to prepare a basis for an NMR. study of flavoproteins already begun.

2. Spectral analysis. – 2.1 All NMR. spectra were obtained on a *Varian* high resolution XL-NMR. spectrometer operating at 100.1 MHz and the *Fourier* transform technique was used. Usually the following instrumental conditions were observed: pulse width 20 μs (90° pulse equals 28 μs), acquisition time 4 s, without delay. Most spectra were run at 1000 Hz spectral width, peak positions being determined from the computer-generated printout, using TMS as internal standard. The ^{1}H-NMR. probe temperature was 27 $\pm$ 1°. All samples were dissolved in CD_3CN (*Merck* AG, Darmstadt, containing 1% TMS.), the deuterium functioning as an internal lock. The concentration varied from 5 to 15 m*M* depending on the individual solubility; for each at least two spectra were recorded from independent samples; the agreement was within 0.5 Hz. Some spectra were recorded on a *Bruker* HX-360 MHz instrument at the University of Groningen, Holland.

Double resonance experiments were conducted by continuous irradiation of the signal to be decoupled. To separate the nuclear *Overhauser* Effect (NOE) from the decoupling effect, the irradiation frequency was cut off immediately before accumulation and re-established after acquisition for at least 2 s.

2.2. *Simulation of the spectra.* The experimental spectra, where appropriate, have been fitted with the aid of the computer program *LAOCOON* 3 [3] and the DEC-10 computer system of the Agricultural University. The original program was obtained from Dr. *Hollander*, University of Leiden, and was rewritten for the DEC-10 computer. The standard deviations of the calculated parameters were less than 0.05 Hz unless otherwise stated. In fitting the experimental to the calculated spectra, special attention was paid to achieving good agreement with respect to both frequencies and to line intensities.

3. Syntheses (*cf. Scheme 2*). – 3.1. *Generalities.* Analytically pure (iso)alloxazines were obtained by column chromatography. Excellent results were obtained using 'Kieselgel *Mallinckrodt*', 100 mesh (*Serva*, Heidelberg, Germany) as a stationary phase. As mobile phase CH_2Cl_2 was used for the alloxazines, and $CHCl_3$ for the alkylated isoalloxazines. The former were eluted with $CH_2Cl_2/CHCl_3$ and the latter with $CHCl_3/CH_3OH$. Their purity was checked by TLC. employing different solvents ($CHCl_3$, CH_3CN, $CHCl_3/CH_3OH$ 9:1).

For purification precoated thin layer plates, Silica Gel 1B2, *Bakerflex*, were used. The melting points were determined on an electrothermal melting point apparatus and are not corrected. Analytical data and m.p. are given in Tab. 1. For developped formulae of the compounds see Tab. 2 and 5.

3.2. (*Iso*)*alloxazines*[2] (*i.e.* isoalloxazines and alloxazines): alloxazines (compounds IX–XIV, Tab. 2) have been prepared by condensation of the appropriate anilines with 1,3-dimethyl-4-amino-uracil as described by *Goldner* et al. [4].

1,3,6,7-Tetramethyl- (XIII) and 1,3,7-trimethyl-8-trideuteriomethyl-(XII-d_3)alloxazine were obtained from the corresponding riboflavin-5′-monophosphates [5] which were first degraded by periodate to the related 10-formylmethyl-isoalloxazines [6]. Further oxidation of the latter according to *Müller & Dudley* [7] followed by alkylation yielded the desired alloxazines.

[2]) Isoalloxazine = 10-substituted 2,3,4,10-tatrohydro-benzo[g]-pteridine-2,4-dione.

Scheme 2

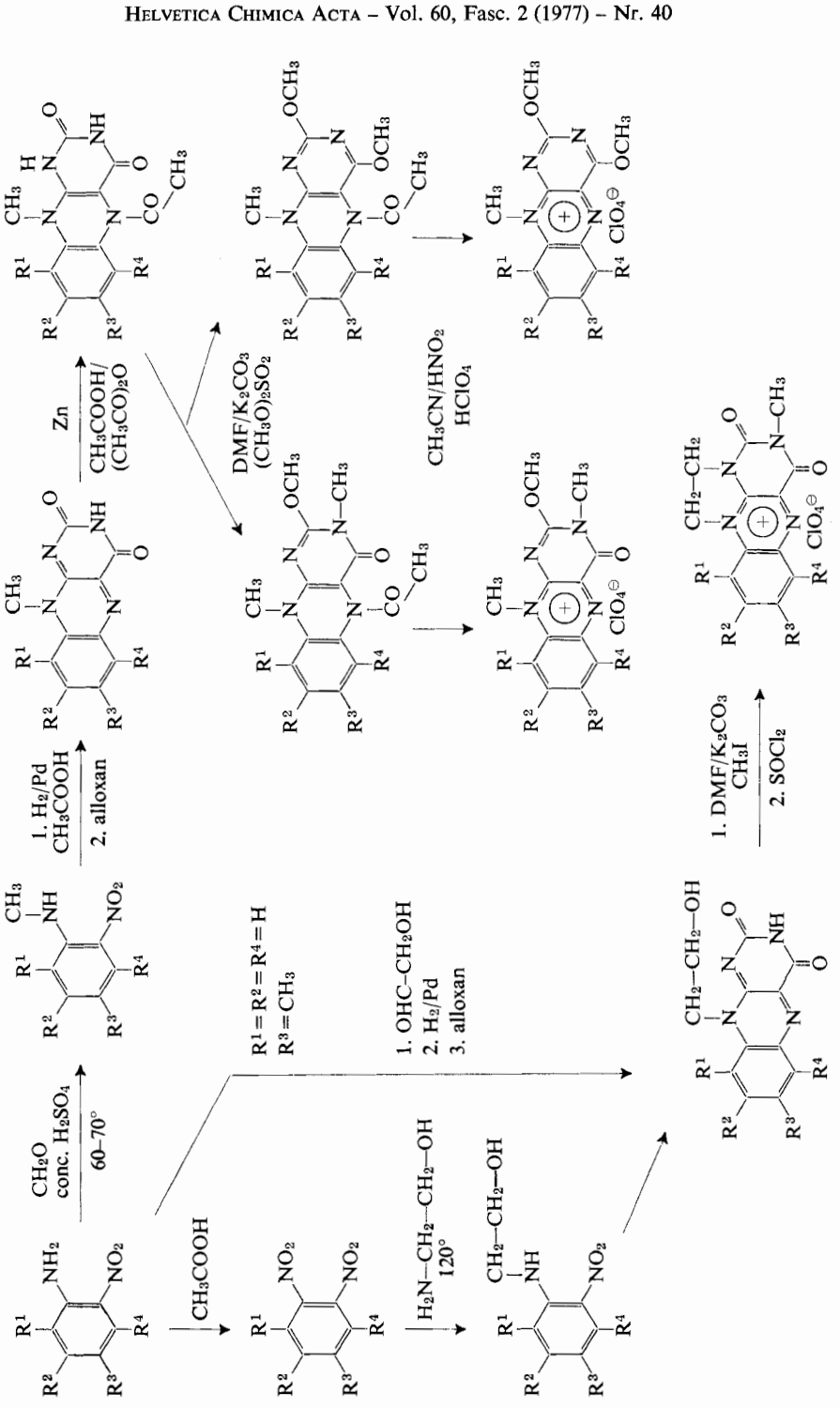
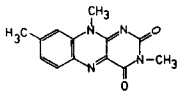
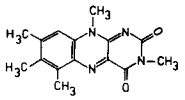
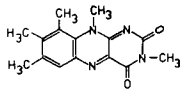
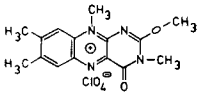
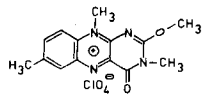
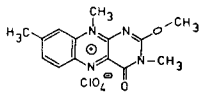
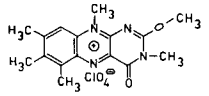
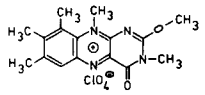
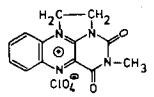
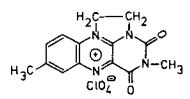
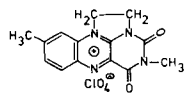
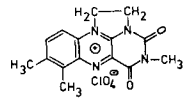
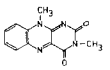
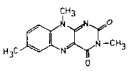
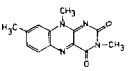
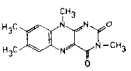
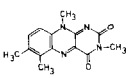
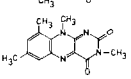
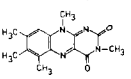
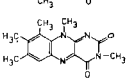
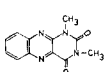
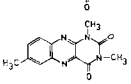
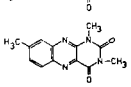
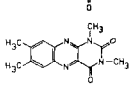
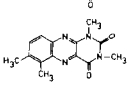
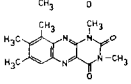

CH2O
conc. H2SO4
60–70°
CH3COOH
H2N–CH2–CH2–OH
120°
R1=R2=R4=H
R3=CH3
1. OHC–CH2OH
2. H2/Pd
3. alloxan
1. H2/Pd
CH3COOH
2. alloxan
Zn
CH3COOH/
(CH3CO)2O
DMF/K2CO3
(CH3O)2SO2
CH3CN/HNO2
HClO4
1. DMF/K2CO3
CH3I
2. SOCl2

Table 1. *Elemental Analyses and Melting Points of the Newly Synthesized Flavin Derivatives*

Compound	Molecular Formula	Mol. Weight		Elemental Analysis %C	%H	%N	%Cl	M.p. (°C)
	$C_{13}H_{12}N_4O_2$	256.26	Calc.	60.93	4.72	21.86	–	281–284
			Found	60.61	4.65	21.89	–	
	$C_{15}H_{16}N_4O_2$ [a])	284.31	Calc.	63.36	5.67	19.71	–	289–290
			Found	63.0	5.6	19.6	–	
	$C_{15}H_{16}N_4O_2$ [a])	284.31	Calc.	63.36	5.67	19.71	–	281–283
			Found	62.9	5.8	19.3	–	
	$C_{15}H_{17}ClN_4O_6$	384.78	Calc.	46.8	4.4	14.56	9.2	221–229 [b])
			Found	46.7	4.5	14.6	9.3	
	$C_{14}H_{15}ClN_4O_6$	370.75	Calc.	45.3	4.1	15.1	9.56	220–221 [b])
			Found	45.2	4.1	15.2	9.7	
	$C_{14}H_{15}ClN_4O_6$	370.75	Calc.	45.3	4.1	15.1	9.56	232–240 [b])
			Found	45.3	4.2	15.2	9.6	
	$C_{16}H_{19}ClN_4O_6$	398.8	Calc.	48.18	4.8	14.05	8.89	>290
			Found	48.0	4.7	14.3	8.7	
	$C_{16}H_{19}ClN_4O_6 \cdot H_2O$	416.8	Calc.	46.20	5.02	13.50	8.55	246–249 [b])
			Found	46.4	4.8	13.5	8.6	
	$C_{13}H_{11}ClN_4O_6$	354.71	Calc.	44.01	3.13	15.80	10.00	300–302
			Found	43.9	3.0	15.8	10.0	
	$C_{14}H_{13}ClN_4O_6$	368.73	Calc.	45.59	3.55	15.2	9.62	301–305
			Found	45.5	3.5	15.3	9.6	
	$C_{14}H_{13}ClN_4O_6$	368.73	Calc.	45.59	3.55	15.2	9.62	313–314
			Found	45.4	3.5	15.4	9.6	
	$C_{15}H_{15}ClN_4O_6$	382.76	Calc.	47.06	3.95	14.64	–	269–271 [b])
			Found	47.0	4.0	14.7	–	

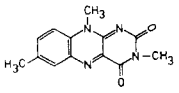
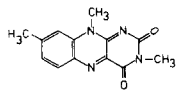
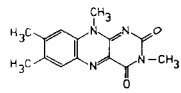
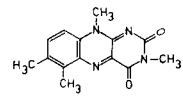
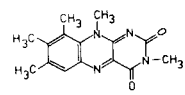
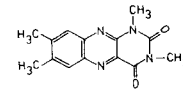
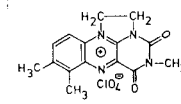
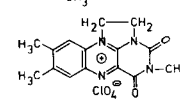
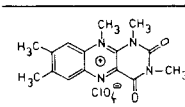
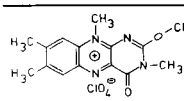
[a]) Contains less than one mole of water.
[b]) Melts under decomposition.

Table 2. *Chemical Shifts and Coupling Constants of Various Neutral Isoalloxazines and Alloxazines*[a])

Compound	No.	Aromatic proton resonances chemical shifts in Hz R.P.[b])				coupling constants in Hz						Methyl resonances chemical shifts in Hz					
		6	7	8	9	J_{6-7}	J_{6-8}	J_{6-9}	J_{7-8}	J_{7-9}	J_{8-9}	6	7	8	9	10	1
	I	811.4	760.3	789.7	777.6	8.3	1.5	0.3	7.2	1.0	8.7	–	–	–	–	402.6	– 336
	II	796.7	–	778.7	772.5	–	2.1	0.4	–	–	8.6	–	252.5 251.7	–	–	400.8	– 335
	III	802.6	748.8	–	764.7	8.5	–	–	–	1.6	–	–	–	260.7	–	400.0	– 335
	IV	791.1	–	–	764.0	–	–	–	–	–	–	–	243.4	253.2	–	400.3	– 335
	V	–	–	776.1	756.6	–	–	–	–	–	8.6	273.4	245.7	–	–	400.8	– 336
	VI	778.8	–	759.6	–	–	2.1	–	–	–	–	–	245.2	–	282.2	415.0	– 335
	VII[c])	–	–	–	748.5	–	–	–	–	–	–	277.4	237.7	255.5	–	402.5	– 337
	VIII	775.3	–	–	–	–	–	–	–	–	–	–	244.2	242.3	259.7	398.1	– 334
	IX	820.1	779.6	794.0	801.4	8.6	1.4	0.6	7.0	1.1	8.6	–	–	–	–	–	371.6 34
	X	798.7	–	780.0	791.8	–	2.1	0.3	–	–	8.8	–	259.3 258.5	–	–	–	370.4 34
	XI	809.0	767.0	–	781.0	8.6	–	0.7	–	2.0	–	–	–	261.9	–	–	370.8 34
	XII	794.4	–	–	777.2	–	–	–	–	–	–	–	249.8	252.7	–	–	368.7 34
	XIII	–	–	776.7	765.2	–	–	–	–	–	8.7	277.8 277.1	252.8 252.0	–	–	–	371.0 34
	XIV	784.8	–	–	–	–	–	–	–	–	–	–	253.1	248.2	273.8	–	374.1 34
	XV[d])	808.3	779.9	779.9	808.3	8.4	1.4	0.5	6.8	1.4	8.4	–	–	–	–	–	–
	XVI[d])	821.9	789.6	789.6	821.9	8.8	1.5	0.3	6.3	1.5	8.8	–	–	–	–	–	–

[a]) Solvent is deuterio-acetonitrile. [b]) R.P. = Ring Position. [c]) Due to low solubility some chloroform was ad to the sample. [d]) For convenience the ring numbering is analogous to that of the quinoxaline residue in isoalloxaz

Table 3. *Percentage Enhancement*[a]) *of Signal Intensities Observed in Double Irradiation Experiments.* The arrows indicate the signal irradiated and the values give the enhancement of the signal due to the indicated position of the (iso)alloxazine ring

Compound	No.	Ring Position				
		6	7	8	9	10
	II	40 ↑	↑ 70	[b])	[b]) 10	 ↑
	III		50	↑	40	
	IV	80 ↑	[c]) ↑ 30	↑ [c]) 30	60	
	V	↑ [c])	[c]) ↑	↑ <10 30 [d])	30 30 <10 ↑ 20	 10 ↑
	VIII	↑	30			
	XII	↑	40	40	↑	
	XXI	↑ [c])	[c]) ↑ 10	50 ↑ [d])	40 [d]) ↑ 20	 [b]) ↑
	XX	↑	40	30	↑ 20	[b]) ↑
	XXIV	↑	30	20	↑ 10	<10 ↑
	XXVIII	↑	40	80	↑ 10	<10 ↑

[a]) The enhancements are due both to decoupling and to NOE. The total enhancement is given as increase of the amplitude of the signal and the stated values have to be considered qualitatively rather than absolutely. The intensities of a spectrum in which a position was irradiated where no absorptions occur served as blanco. Power used, 95 db.

[b]) The enhancement is spread over the multiplets and is thus small.

[c]) Due to the width of the irradiating field the neighbouring signal is affected too.

[d]) The original doublet is transformed into a singlet.

The isoalloxazine derivatives (compounds I–VIII, Tab. 2) were synthesized by virtually following the procedure of *Kuhn & Weygand* [8] except for the preparation of intermediate N-methyl-*o*-nitro-anilines obtained from the corresponding commercially available o-nitro-anilines according to the procedure described by *Halasz* [9].

The general procedure was as follows: *e.g.* to 1.0 g of 4,5-dimethyl-2-nitro-aniline dissolved in 10 ml conc. sulfuric acid 10 ml formaldehyde (37%) were slowly added under stirring during 45 min at 60–70°. This temperature was maintained for an additional 1–5 h depending on the *o*-nitro-aniline derivative employed. The reaction could easily be followed by thin layer chromatography (TLC.) using isopropyl ether. The starting material is yellow, the product is orange coloured and moves ahead of the starting material. After completion of the reaction the mixture was cooled to room temperature (RT.) and poured on 200 ml ice/water, the precipitate filtered off and washed with H_2O. The filtrate was extracted with $CHCl_3$, the organic phase dried with Na_2SO_4, filtered and evaporated. Crystallization of the combined product from ethanol/H_2O yielded 1 g (*ca.* 92%) of red crystals. – The starting materials 3,4,5-trimethyl- and 4,5,6-trimethyl-2-nitro-aniline were prepared according to *Dolinsky et al.* [10]. Clearly this procedure cannot be applied to *o*-nitro-anilines devoid of a methyl substituent *para* to the amino group.

Catalytic reduction of the *N*-methyl-*o*-nitro-anilines derivatives in glacial acetic acid, followed by condensation with alloxan according to *Kuhn & Weygand* [8] gave the desired isoalloxazines in ~82% yield. Methylation at N(3) position of the isoalloxazine ring was carried out as described elsewhere [11].

3,7,10-Trimethyl-8-trideuteriomethyl-isoalloxazine (IV-d_3) was obtained from the corresponding riboflavin-5′-monophosphate analogue [5] by periodate degradation [6] and treatment of the product with 0.1 M NaOH for 1 h at RT. followed by acidification and methylation. 9-Deuterio-3,7,10-trimethyl-(II-d_1) and 9-deuterio-3,7,8,10-tetramethyl-(IV-d_1)isoalloxazines were synthesized from the corresponding aniline derivatives as described elsewhere [5].

3.3. *O(2′), N(3) and O(2′), O(4′) dimethylated quaternary isoalloxazinium perchlorates XXVI–XXX* (Tab. 5) were obtained from the corresponding isoalloxazines following the procedure of *Dudley & Hemmerich* [12] replacing diethyl- by dimethyl sulfate; separation of the two isomers by fractional crystallization analogous to [12] was difficult, but was easily achieved by passing the mixed isomers (1.55 g), dissolved in 10 ml of CH_2Cl_2, through a silicagel column (*cf.* above). The *O,O*-dimethyl isomer was eluted with CH_2Cl_2 whereas *O,N*-dimethyl isomer was eluted with $CHCl_3/CH_3OH$ 99:1.

The 1,3,10-trimethylisoalloxazinium perchlorates (*XXII–XXV*, Tab. 5) were synthesized from the corresponding alloxazines as described by *Dudley & Hemmerich* [13].

3.4. *1,10-Ethano-isoalloxazinium perchlorates XVII–XXI* (*cf.* Tab. 5). Starting materials for 1,10-ethano derivatives were the corresponding 10-(β-hydroxyethyl)-3-methylisoalloxazines [14]. The ethano-bridge closure was achieved according to either *Müller & Massey* [15] or to *Hemmerich et al.* [16]; further details concerning these methods are reported in [17]. *1,10-Ethano-3,7-dimethyl-8-trideuteriomethyl-isoalloxazinium perchlorate* (XX-d_3) was obtained from the corresponding 10-formylmethyl-isoalloxazine [5] [6]. The purity of the isoalloxazinium perchlorates was checked by TLC., solvent CH_3CN. The *analytical data* of the new (iso)alloxazines are presented in Table 1.

3.5. *Structurally related compounds.* 5-Methylphenazinium methylsulfate, purchased from *E. Merck* AG., Darmstadt, Germany, was recrystallized from CH_3CN. The analogous perchlorate was crystallized after adding a small amount of conc. perchloric acid to the aqueous solution of the sulfate. *N*-Methylquinoxalinium methyl sulfate was synthesized according to *Smith et al.* [18] and its perchlorate XXXI prepared as described for *N*-methylphenazinium perchlorate.

4. Results. – The results are divided into three sections, namely those concerning a) isoalloxazines b) alloxazines, and c) the related cationic species. For comparison results from other related heterocycles are included *viz.* quinoxaline (XV), phenazine (XVI) and their cationic species XXXI and XXXII. The structures of *N*(10)-methyl-isoalloxazine and alloxazine are shown in *Scheme 1* together with the (IUPAC) ring numbering [19].

4.1. *The NMR. data for a number of isoalloxazines* are summarized in Tab. 2. The *N–CH₃* resonances of the isoalloxazines appear in the frequency range of about

335 to 400 Hz (Tab. 2, I–VIII), that at lower field being assigned to the N(10)–CH_3 group. Double irradiation experiments show NOE at the 400.3 Hz signal, but not at the 335.1 Hz signal, upon irradiation of the signal at 764.0 Hz of IV or *vice versa*; all isoalloxazines show these effects. The increase in peak amplitude of signals for some compounds observed by double resonance technique, are given in Tab. 3. These experiments were conducted to aid peak assignments, and it is therefore unnecessary to discriminate between NOE and decoupling effects.

Table 4. *Relative Shifts*[a]) (in Hz) *of Aromatic Methyl Resonances due to Methyl Substitution*

Compound	Ring Position							
	Ortho shift due to[b])				*Meta* shift due to[b])			
	6	7	8	9	6	7	8	9
IV	–	−7.5	− 8.7	–	–	–	–	–
V	−6.4	–	–	–	–	–	–	–
VI	–	–	–	–	–	–	–	−6.9
VII	−5.7	–	− 8.0	–	2.3	–	4.0	–
VIII	–	−1.0	−22.5[c])	−10.9	–	–	–	0.8
XII	–	−9.2	− 9.1	–	–	–	–	–
XIII	−6.5	–	–	–	–	–	–	–
XIV	–	–	–	− 4.5	–	–	–	3.3
XX	–	−9.5	−10.0	–	–	–	–	–
XXI	−7.4	–	–	–	–	–	–	–
XXIV	–	−8.6	− 9.2	–	–	–	–	–
XXV	–	–	–	− 9.5	–	–	–	1.1
XXVIII	–	−8.2	− 9.6	–	–	–	–	–
XXIX	−6.3	–	–	–	1.8	–	–	–

[a]) Negative shifts are to high field.
[b]) Shifts observed upon addition of methyl group(s) at the indicated position to methyl group(s) originally present.
[c]) The 8-methyl is thought to be added to compound VI, thus giving ortho shifts at 7 and 9 positions (7/9).

The methyl groups attached to ring A resonate in the frequency range between 235 and 280 Hz. Comparison of the methyl resonances of II and III with those of IV indicates that formal introduction of a methyl group in *ortho* position to that originally present causes an upfield shift of about 8 Hz (*cf.* Tab. 2 and 4). Based on this observation, the resonance at lower field of IV is assigned to H_3C–C(8) which is in agreement with published results [5]. It should be noted that the H_3C–C(7) resonance of II is split into a doublet due to coupling with H–C(6) as verified by decoupling experiments (*cf.* Tab. 3). In fact only the monomethyl analogues II, X (Tab. 2), XVIII, XXII, XXVI (Tab. 5), show this doublet, but the isomeric III, XI (Tab. 2), XIX, XXIII, XXVII (Tab. 5) do not. The methyl resonances of V–VIII have been assigned on the basis of the above mentioned shift induced by additional methyl groups (Tab. 4) combined with double irradiation experiments (Tab. 3).

Signals due to the aromatic protons have been assigned similarey. The chemical shifts induced by an additional methyl group are summarized in Tab. 8 (*cf* chap. 5). The low field resonance of IV at 791.1 Hz has been assigned unequivocally to H–C(6) by selective deuteriation at C(9) [5] and is in agreement with the decoupling data in Tab. 3. For III the low field part of the spectrum consists of an *AB* component with a single line in its centre, the high field *AB* component and the single line are broad as compared to the doublet at lower field. Decoupling the H_3C–C(8) shows that the *AB* system high field component is due to H–C(7). The low field resonances of II are similarly assigned. The resonance positions of H–C(8) and H–C(9) are obtained from the C(9) deuteriated derivative of II. Irradiation of the H_3C–C(7) resonance splits the low field signal into a doublet due to *meta* coupling. These results prove that the signal at low field arises from H–C(6). By fitting the spectrum of II, the starting values for iteration are taken from the deuteriated compound decoupled at 252 Hz (H_3C–C(7')).

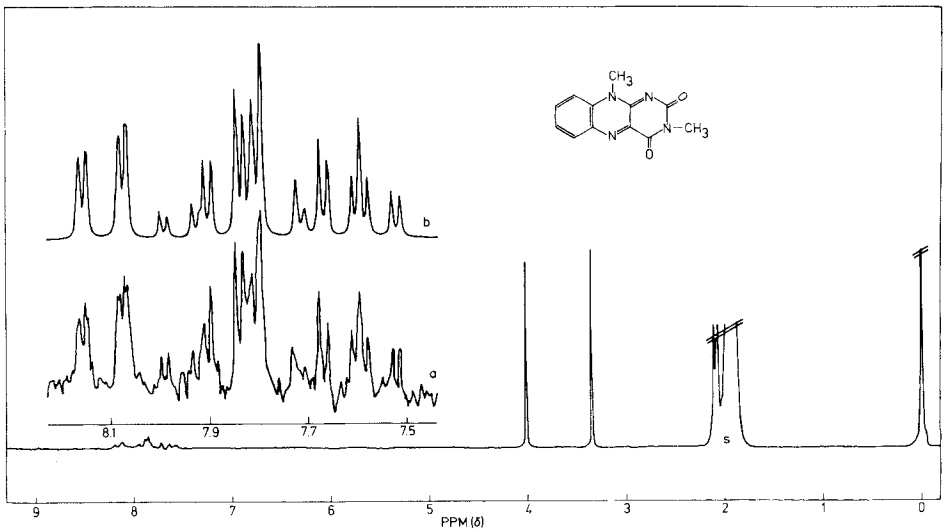


Fig. 1. ^{1}*H-NMR. spectrum of 3,10-dimethylisoalloxazine* (I) *in* CD_3CN (*s* = resonances due to the solvent): a) low field part of the spectrum expanded fivefold in both horizontal and vertical direction; b) calculated spectrum corresponding to a)

The spectrum of I is very complex as shown in Fig. 1; in Fig. 1a its low field component is shown on an expanded scale. It was not possible to obtain derivatives of I selectively deuteriated at one or two atoms[3]), for simplification of the spectrum. In

[3]) Treatment of 3,4-dimethylaniline hydrochloride with D_2O leads to the exchange of the two protons in *ortho*-position to the amino group. From this compound 9-deuterio-7,8,10-trimethylisoalloxazine has been prepared [5]. When the corresponding *N*,4,5-trimethyl-*o*-phenylenediamine dihydrochloride was treated in D_2O under anaerobic conditions the two aromatic protons were exchanged almost quantitatively yielding, after condensation with alloxan, 6,9-dideuterio-7,8,10-trimethylisoalloxazine, the deuterium content of this compound at both C(6) and C(9) is at least 95% as judged by NMR. Therefore, this procedure is much more efficient than that described previously [20] [21]. Similarly, starting from *N*-methyl-*o*-phenylenediamine dihydrochloride 6,7,8,9-tetradeuterio-10-methylisoalloxazine was obtained (D > 95%).

the simulation of the spectrum of Fig. 1a, the only assumption is that the H–C(6) appears at lowest field; this is very reasonable considering results for the other derivatives (Tab. 2). The best fit found by iteration is shown in Fig. 1b; only lines well above the noise level were used for iteration, their frequencies were read off the spectrum computer listing (containing 32 lines from which 20 with a calculated intensity of more than 0.1 were used).

Compound V behaves unexpectedly on application of double resonance technique. Irradiation at 273.4 Hz H_3C–C(6) shows a large signal enhancement at 756.6 Hz (H–C(9)), and a very small one at 776.1 Hz (H–C(8)) (Tab. 3). The mechanism responsable for the relatively stronger enhancement of the *para-* as compared to the *meta*-position is not yet clear but must in part be due to NOE.

4.2. *Alloxazines, and uncharged reference compounds.* The ^{1}H-NMR. resonances of the alloxazines IX–XIV (Tab. 2) are assigned by analogy to those of the isoalloxazines. The influence of further methyl groups on the chemical shifts of those originally present is similar to that described above for the isoalloxazines (Tab. 4); the influence on the chemical shifts of the aromatic protons is given in Tab. 8 (*cf.* chap. 5). Decoupling experiments (Tab. 3) are consistent with the assignments (see also Tab. 2). The

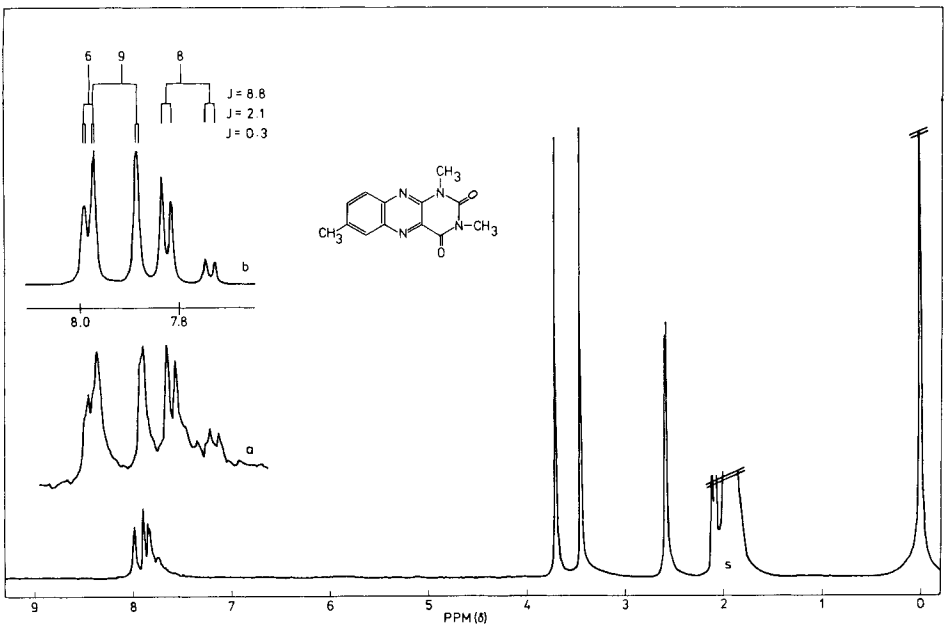


Fig. 2. ^{1}*H-NMR. spectrum of 1,3,7-trimethylallozazine* (X) *in* CD_3CN (*s* = as in Fig. 1); a) expanded low field part of the spectrum (as in Fig. 1), obtained by decoupling the resonance due to the H_3C–C(7); b) calculated spectrum corresponding to a)

spectrum of 1,3,7-trimethylalloxazine (X) is given in Fig. 2; the computer fit of the aromatic region of the spectrum decoupled at H_3C–C(7) (trace a), is represented by trace b. The high field part of trace a represents part of an *AB* system due to H–C(8) and H–C(9), while the signal of the H–C(6) is distorted due to overlap with the low field *AB* pattern.

In contrast to the other studied compounds the aromatic methyl proton resonances of XIII are split into doublets (Tab. 2). Compound XIII shows the same effect as V upon irradiation of H_3C–C(6).

For comparison the spectra of quinoxaline (XV) and phenazine (XVI) in acetonitrile are recorded. These spectra were simulated using the frequencies of our experimental spectra; the coupling constants thus obtained are in agreement with published data [22]. The frequencies of H–C(1), H–C(2) of XV (see footnote c) in Tab. 2) are not reported as they are irrelevant.

4.3. *Cationic derivatives of isoalloxazines and of reference compounds.* Protonation of isoalloxazine or alloxazine eliminates their structural differences likewise effected on replacement of the proton by a methyl (methylene) group; this offers the possibility to study cationic isomeric species, which are otherwise not available in organic solvents. The molecular positive charge anticipates that the chemical shifts of the cations will be located at lower fields than those of the corresponding neutral molecules. The results assembled in Tab. 5 show that the H_3C–N(3) resonances of the cations appear at about 350 Hz and are thus only slightly shifted as compared with those of the neutral alloxazines IX–XIV. The methyl protons of ring A are more sensitive to charge than those of H_3C–N(3) and appear at 260–290 Hz. Methyl substitution has a similar effect on the chemical shift of originally present methyl groups to that found for the neutral molecules (Tab. 4); for the influence on the aromatic protons, see Table 8. To support our assignments compound XX has been selectively deuteriated at H_3C–C (8). The spectrum of XVII is shown in Fig. 3; the complex components (multiplets a and d) are expanded (trace b and e resp.). Double resonance technique (Tab. 3) unequivocally shows that the low field trace e (Fig. 3e) is due to H_2C–N(10). The two methylene groups behave as an *AA'BB'* system and are characterized by their frequencies, and the sum N of their J_{AB} coupling constants as obtained by computer fitting (Tab. 5). These values are derived from the actual spectrum by the method described by *Bovey* [23][4]).

The aromatic protons of the cations exhibit in general the same NMR. properties as their neutral analogues. Again, the calculated spectrum of XVII shown in Fig. 3 (trace c) assumes that the signal at lowest field belongs to H–C(6); the very good fit warrants this assumption. Double resonance experiments with the cations show enhancements similar to their analogues (Tab. 3); compound XXI exhibits the same peculiar behaviour as its neutral analogue V (Tab. 3).

The resonances of XXII–XXV lie at higher field than those of XVII–XXI especially for the aromatic proton signals. The H_3C–N(1) and H_3C–N(3) signals are identified by comparison of the spectrum of 1,7,8-trimethylalloxazine [7] with that of XXIV, both obtained in CD_3CN/CCl_3COOH.

With compounds XXVI–XXIX some uncertainty arises with respect to the assignment of the resonance due to CH_3O–C(2). Assignment of the lower field resonance *e.g.*

[4]) According to [23] the values are calculated by the analytical expression for the first line on the left wing in combination with the second line on the right wing of the low field part multiplet (trace e). Since only a few distinct lines are available extensive iteration would yield very crude values and was therefore not performed. However, in trace f the frequencies of the various lines could be simulated quite well, whereas some of the intensities are not in complete agreement with the actual spectrum.

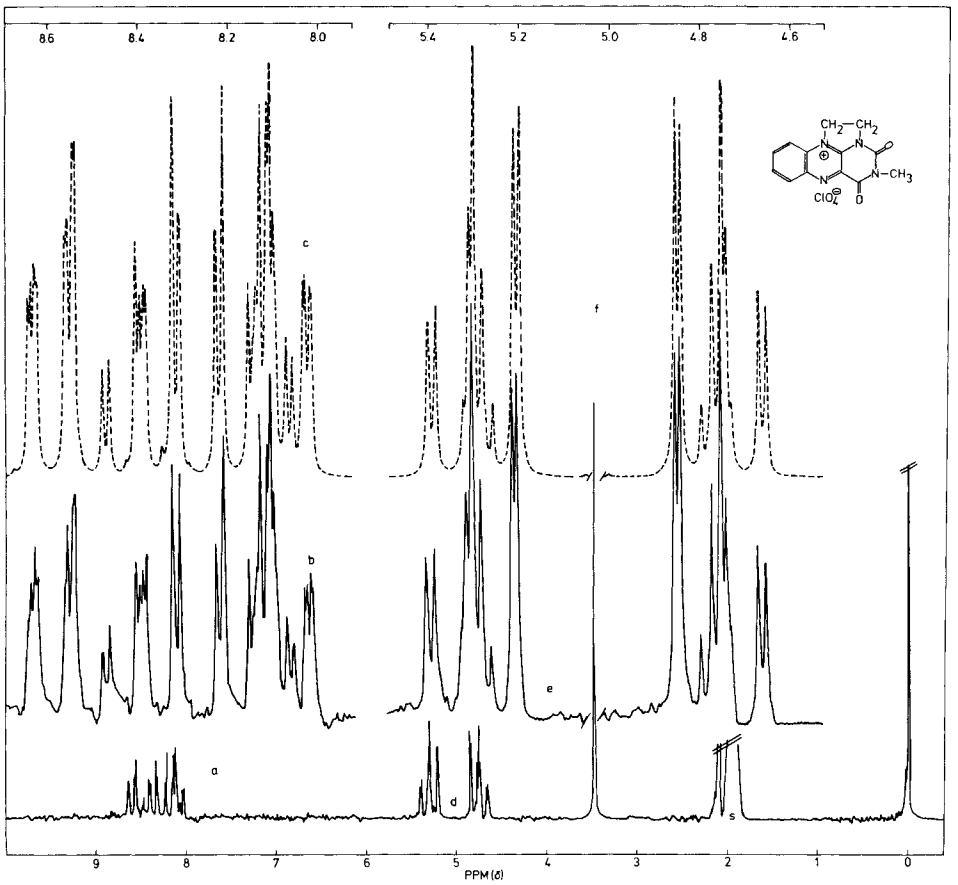
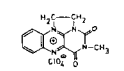
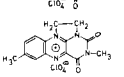
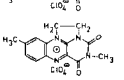
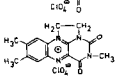
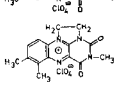
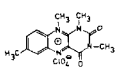
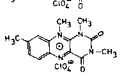
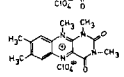
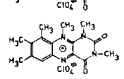
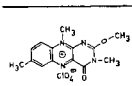
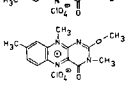
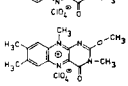
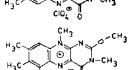
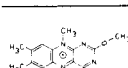
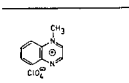
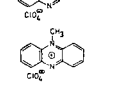


Fig. 3. *^{1}H-NMR. spectrum of 1,10-ethano-3-methylisoalloxazinium perchlorate (XVII) in CD_3CN.* b) and e): expanded parts of the spectrum (as in Fig. 1) corresponding to a) and d); c) and f): computed spectra corresponding to b) and e) resp. (*s* as in Fig. 1)

(450 Hz for XXVIII) to the OCH_3 group is attractive, but irradiation of the signal at 814 Hz (H–C(9)) however enhances the signal at 450 Hz, which proves that the resonance at higher field must be assigned to OCH_3. This is supported by the NMR. data from XXVIII in the presence of methoxide yielding an adduct [24]: they show a slow decrease of the intensity of the signal at 438 Hz due to slow exchange of the CH_3O–C(2) with bulky CD_3O^-. The low field components of the spectrum of XXVI are quite complex, and no accurate values can be determined for the frequencies due to the large overlap of the lines. However, specific deuteriation of C(9) of XXVI allows one to estimate the frequencies of the signals due to H–C(6) and H–C(8). The coupling constants, on the other hand, are somewhat less accurate ($\pm$ 0.1 Hz), than those determined for other compounds. For the computer fit the coupling constants were estimated from the spectrum in CD_3CN/CD_3OD 1:3, which shows a more highly resolution.

Table 5. *Chemical Shifts and Coupling Constants of Various Isoalloxazinium Perchlorates*[a])

Compound No.	Aromatic proton resonances chemical shifts in Hz R.P.[b])				coupling constants in Hz						Methyl(methylene) resonances chemical shifts in Hz							
	6	7	8	9	J_{6-7}	J_{6-8}	J_{6-9}	J_{7-8}	J_{7-9}	J_{8-9}	6	7	8	9	10	1	2α	3
 XVII	858.7	814.9	838.1	806.9	8.6	1.3	0.5	7.3	1.2	8.5	–	–	–	–	N=19.0[c]) 529.0	476.1	–	347.7
 XVIII	838.8	–	823.9	797.6	–	1.8	0.6	–	–	8.7	–	269.5 268.8	–	–	N=19.0 526.3	473.7	–	347.5
 XIX	845.0	799.3	–	790.0	9.0	–	0.7	–	1.4	–	–	–	277.6	–	N=18.9 521.9	473.3	–	346.8
 XX	833.0	–	–	789.5	–	–	–	–	–	–	–	259.1	268.1	–	N=19.0 521.8	471.9	–	346.1
 XXI	–	–	820.6	780.6	–	–	–	–	–	8.7	286.5	261.8	–	–	N=18.5 523.6	473.1	–	347.7
 XXII	827.8	–	816.8	817.3	–	1.6	0.8	–	–	7.6	–	268.2 267.3	–	–	438.8	377.7	–	246
 XXIII	835.6	797.3	–	809.2	8.6	–	0.9	–	1.6	–	–	–	277.6	–	436.4	377.7	–	346
 XXIV	822.8	–	–	807.2	–	–	–	–	–	–	–	258.4	269.0	–	436.6	376.6	–	345
 XXV	809.8	–	–	–	–	–	–	–	–	–	–	259.5	259.5	273.0	423.0	383.5	–	347
 XXVI	832.6	–	822.8	823.4	–	2.0	0.5	–	–	8.0	–	269.5 268.5	–	–	452.2	–	439.1	355
 XXVII	840.6	798.1	–	815.5	8.7	–	0.5	–	1.7	–	–	–	279.2	–	449.8	–	439.0	355
 XXVIII	827.5	–	–	813.7	–	–	–	–	–	–	–	259.4	271.0	–	450.1	–	437.7	354
 XXIX	–	–	–	800.2	–	–	–	–	–	–	290.8	253.1	272.8	–	448.3	–	437.6	355
 XXX	830.7	–	–	822.7	–	–	–	–	–	–	–	262.8	275.7	–	462.2	–	439.0[d])	
 XXXI[e])	852.8	829.1	834.1	845.8	8.4	1.4	0.7	7.0	1.1	9.1	–	–	–	–	468.2	–	–	
 XXXII[e])	864.1	829.3	850.4	865.1	8.8	1.4	0.6	6.8	1.0	9.4	–	–	–	–	492.3	–	–	

a) Solvent is deutero-acetonitrile. b) R.P. = Ring Position. c) See text. d) 4α-methyl resonates at 433.3.
e) For convenience the ring numbering is analogous to that of the quinoxaline residue in isoalloxazine.

The spectra of 1-methylquinoxalinium perchlorate (XXXI) and 5-methylphenazinium perchlorate (XXXII) are recorded (Tab. 5) and simulated for reference. As for quinoxaline the resonances due to HC–N(10) and HC–N(5) of XXXI (see footnote e) in Tab. 5) are not relevant. They form a complex pattern centered at 954.2 Hz (HC–N(10) and 908.5 Hz (HC–N(5)).

As it was very difficult to fit the spectrum of XXXI, a 360 MHz spectrum was also recorded. The calculated parameters for the latter, appropriately corrected for the frequencies, did not yield the 100 MHz spectrum, so it was necessary to fit the coupling constats for the 100 MHz spectrum using the corrected frequencies of the 360 MHz spectrum. Even so it was not completely possible to optimize both the 100 MHz and the 360 MHz spectra with the same optimal parameters; here again it was assumed that 'H–C(6)' (see footnote e) in Tab. 5) is at lowest field.

4.4. *Concentration and solvent effects.* With the data presented in Tab. 2 and 5 it is reasonable to investigate the dependence on concentration and solvent polarity on these values. The choice of solvent in this study is mainly determined by the solubility

Table 6. *The Influence of Solvent Polarity on the Resonances* (in Hz) *of (Iso)alloxazines.* $CDCl_3/CD_3OD$ solvent; no corrections made for dilution

%	3,7,8,10-tetramethyl-isoalloxazine (IV) ring position					
CD_3OD	Aromatic proton resonances		Methyl resonances			
	6	9	7	8	3	10
1.2[a])	806.8	743.8	245.6	255.8	352.8	412.6
11.1	806.5	754.0	248.0	258.7	351.8	414.1
29.8	806.1	762.2	249.7	260.7	351.4	416.0
50.0	803.1	769.2	249.2	260.7	350.0	416.3
68.6	798.7	774.0	248.2	259.8	347.7	414.7
88.0	798.2	772.2	248.0	259.5	346.2	413.8
%	1,3,7,8-tetramethyl-alloxazine (XII) ring position					
CD_3OD	Aromatic proton resonances		Methyl resonances			
	6	9	7	8	3	1
1.2[a])	806.4	779.7	250.8	253.4	359.4	380.8
11.1	803.6	782.0	252.1	254.9	358.6	381.6
29.8	801.3	782.7	253.3	255.3	357.6	380.9
50.0	799.5	783.0	254.0	256.8	357.1	380.7
68.6	793.3	777.7	251.8	254.0	354.2	375.7
88.0	792.7	778.2	252.0	254.3	352.7	375.0

[a]) For stabilisation of the $CDCl_3$ solution a small amount of CD_3OD was added.

of the compounds investigated, thus allowing a direct comparison of the various derivatives of isoalloxazines, alloxazines and their cationic species.

Concentration dependence has been studied in detail employing IV in CH_3CN, the results showing that only the resonances due to H–C(6) and H_3C–N(3) are affected: the signal due to H–C(6) shifts from 805.5 Hz to 807.5 Hz at infinite dilution, whereas that due to H_3C–N(3) shifts from 335.0 Hz to 332.5 Hz, values derived by extrapolation.

The effect of solvent polarity is much more pronounced as illustrated in IV and XII, in $CDCl_3/CD_3OD$, for results see Tab. 6. They show that the resonances of the aromatic protons and the H_3C–N(3) are more influenced than others by solvent polarity. Moreover for the aromatic protons this influence differs for the isoalloxazines and alloxazines: for the former the resonance of the H–C(9) is influenced much more than that of the H–C(6) whereas the reversed holds for the latter.

5. Discussion. – Comparing the NMR. frequencies of aromatic protons of the compounds here described it is seen that the resonance of H–C(6) appears at lowest field. It might be deduced that C(6) of the isoalloxazine ring system possesses the lowest electron density. However, some doubt exists in the literature concerning the NMR. assignment at H–C(6) in flavin-adenine dinucleotide (FAD), and in riboflavin-5'-monophosphate (FMN) [25]; the assignment of a certain resonance to H–C(6) was partly based on published π-electron calculations [26]. Comparing our results with

Table 7. *Comparison of Experimental and Calculated Chemical Shifts* (in Hz) *of Aromatic Protons of 3,10-Dimethylisoalloxazine (I)*

Pisition	Calc.[a])	Observed	Difference
6	795.8	811.4	−15.6
7	784.1	760.3	+23.8
8	781.2	789.7	− 8.5
9	759.4	777.6	−18.2

[a]) Using formula (1) and electron densities as obtained by *Fox et al.*[4]). The theoretical standard deviation as calculated from the standard deviation of the correlation coefficients is 21 Hz.

these calculations we find that hardly any correlation exists between calculated π-electron density and chemical shift. On the other hand, it has been shown by *Sterk & Holzer* [27], that a much better correlation is obtained when the total (π- and σ-)-electron density is taken into account. The results given in Tab. 7 are obtained taking the total electron density as calculated by *Fox et al.*[5]), and the correlation equation (1) [27]:

$$\delta_H = 28.511 - 19.198 q^H - 2.687 q^C \quad (1)$$

where q^H is the electron density at the proton and q^C that at the carbon atom under consideration. The correlation coefficients of equation (1) were derived from MINDO/2 calculations [29]. Although the agreement between experimental and calculated values seems poor, the calculated values are within the theoretical standard deviation of 21 Hz, with the exception of that of H–C(7), and support the assignment of the resonance at lowest field to H–C(6).

It should be noted that the use of slightly different correlation coefficients, which would be obtained from MINDO/3 calculations because of the somewhat different parametrization with respect to MINDO/2, would probably improve the agreement between calculated and experimental values. In addition the consideration of small electric and magnetic field effects due to the *N*-atoms [30], and the consideration of solvent effects (*cf.* Tab. 6 and below) would probably also improve the agreement.

[5]) Personal communication; the data were obtained by an all valence electron molecular orbital calculation within the MINDO/3 formalism [28]. The theoretical values (CNDO calculations) as published by *Grabe* [2] correlate less well than those of *Fox*.

With regards to the dependence of resonances on solvent polarity it should be mentioned that isoalloxazines interact in a specific manner with H_2O [31], so the observed effects, using methanol as solvent (Tab. 6), could be due partially to a specific interaction between solute and solvent.

The different behaviour on solvent change of the H–C(9) resonance of 3,7,8,10-tetramethyl (IV) as compared to that of 1,3,7,8-tetramethylalloxazine (XII) suggests that the electron distributions are affected differently by solvent polarity (Tab. 6).

Theoretical calculations [2] show that a high electron density is located on the carbonyl groups, so that increasing solvent polarity should lead to an even higher electron density. In the case of the isoalloxazines this can lead to a shift of electron density from H–C(9) and H–C(9a) towards the two carbonyl groups and/or withdrawal of electron density from C(6) and C(8). The first possibility leads to some decrease of ring current due to loss of electron density in ring A and B, which is partially compensated by additional delocalization of the π-electron pair of N(10). This would result in a downfield shift for H–C(9), and an upfield shift for H–C(6) of IV, which is actually observed. The second possibility would lead to an upfield shift of the resonances of H_3C–C(7) and H–C(9), and a downfield shift of H–C(6) and H_3C–C(8). This crude evaluation is further supported by the calculations of *Fox*[5]) who found a relatively high excess of electron density at C(9) and C(7), while the C(8) and C(6) atoms are positive. Therefore, withdrawal of electron density from C(9) by a more polarized C(2) carbonyl group is to be expected.

In the case of alloxazines the influence of the carbonyl groups seems to be less important than in isoalloxazines. Electron density withdrawal *via* both carbonyls will affect those at C(6) and C(8) to some degree. From Tab. 6 it may be concluded that with increasing solvent polarity the resonances of isoalloxazines approach those of the alloxazines, as expected from the delocalization of the π-electron pair of N(10) in the isoalloxazines. Comparison of the charged species XXIV, XXVIII and XXX shows that the aromatic proton resonances move gradually to lower field. This illustrates that the carbonyl groups originally present in XXIV withdraw electrons from the *A* and *B* ring on conversion to XXVIII and to XXX, and thus lower the ring current. The latter effect is even more pronounced in the H_3C–N(10) resonances of these compounds (Tab. 5).

In the literature assignment of the two aromatic protons (H–C(6) and H–C(9)) of FAD [25] is contradictory. The results given in Table 6 suggest that in more polar solvents than methanol the resonances of H–C(6) and H–C(9) might cross over and this could be further influenced by the formation of the intramolecular complex [31] [32]. To reconcile the above mentioned contradiction we synthesized 8-trideuteriomethyl-FAD[6]). As expected, the spectrum of this compound (0.05 M in 0.1 M phosphate buffer, pD 7.04[7])) exhibits the H_3C–C(8) resonance (231.5 Hz decreased intensity) at

[6]) 8-Trideuteriomethyl-FAD was prepared in deuterium phosphate buffer as for 8-Trideuteriomethyl-FMN [5]. The exchange reaction for 8-methyl-FAD is much slower than that for CH_3(8) of FMN, 6 h refluxing being necessary to achieve an exchange of about 70%. As could be expected some hydrolysis of the internucleotide pyrophosphate linkage occured during this time; fluormetrical analysis of the reaction mixture according to the method of *Wassink & Mayhew* [32] revealed that 15% of the toal FAD had been hydrolysed.

[7]) pH-Meter reading, thus not corrected for the deuterium activity.

lower field than that of H_3C–C(7). Double irradiation revealed that H–C(9) appears at 749.7 Hz and H–C(6) at 739.7 Hz. An otherwise identical experiment with FAD in pure deuterium oxide (pD 5.60[7]), carried out to check the influence of pH and buffer on the resonance positions showed that H–C(9) again appears at lower field (750.2 Hz), than H–C(6) (742.3 Hz), the difference of the chemical shifts between H–C(9) and H–C(6) being smaller. Upon gradual addition of solid FAD to this sample the signal of H–C(6) gradually moved to lower field and was finally located at lowest field. The position of the resonance of H–C(6) and H–C(9) is therefore determined by concentration, pH of the solution, solvent polarity and intramolecular complexation.

Table 8. *Relative Shifts* (in Hz) *of the Aromatic Protons due to Methyl Substitution*[a])

Compound No	H–C(6)	H–C(7)	H–C(8)	H–C(9)
II	14.7	–	11.0	5.1
III	8.8	11.5	–	12.9
IV	20.3	–	–	13.6
V	–	–	13.6	20.7
VI	32.6	–	30.1	–
VII	–	–	–	29.1
VIII	36.1	–	–	–
X	21.4	–	14.0	9.6
XI	11.1	12.6	–	20.4
XII	25.7	–	–	24.2
XIII	–	–	17.3	36.2
XIV	35.3	–	–	–
XVIII	19.3	–	14.2	9.3
XIX	13.7	15.6	–	16.9
XX	25.7	–	–	17.4
XXI	–	–	17.5	26.3

[a]) The shifts are to high field relative to the resonances of the aromatic protons of 3,10-dimethyl-isoalloxazine (I), 1,3-dimethylalloxazine (IX) and 3-methyl-1,10-ethano-isoalloxazinium perchlorate (XVII), respectively.

The shifts induced on the aromatic proton resonances by further methyl substitution of ring A are summarized in Tab. 8. They show that the effects are much more symmetric for the alloxazines (*e.g.* introduction of CH_3 at C(7) (→ II) produces about the same shift for H–C(6) and H–C(8)) than for the isoalloxazines and their quaternary salts. The substitution effects are not additive, which means that the sum of the *meta* and *ortho* shift (effect) is not identical but smaller than the calculated shift produced on simultaneous introduction of a methyl group in each *meta* and *ortho* position (*e.g.* the sum of the shifts of H–C(9) on introduction of CH_3 at C(7) (→ II) and C(8) (→ III) is smaller than the shift of H–C(9) in IV). The *para* effects are extremely high, *e.g.* in V, VI, VII, VIII, XIII, XIV and XXI. This indicates a less planar conformation of the aromatic ring for these compounds, a conclusion further supported by the enhancement of the signal of H–C(9) upon irradiation of the

H_3C–C(6) in V, XIII and XXI. A bent conformation is expected when ring A, is trimethylated but is already realized in some cases when ring A is dimethylated.

The influence of added methyl groups on those originally present in ring A is, for all compounds studied, almost additive but not symmetric (Tab. 4); exceptions are shown in VI and VIII (Tab. 2) and XXV (Tab. 5), where a larger shift is observed. This effect is caused mainly by the mutual influence of the methyl groups at C(9) and N(10), and is reflected either in the resonance position of H_3C–N(10) or of H_3C–C(9). The relatively low shift values observed for the isoalloxazines (Tab. 4), compared with those for other compounds indicate less aromaticity, apparent from the large resonance differences of H_3C–C(7) and H_3C–C(8) in the two series.

The resonances given in Tab. 2 indicate that the aromaticity of alloxazines is higher than that of isoalloxazines (*e.g. cf.* I and IX). Moreover, comparison of the values for (iso)alloxazines with those of quinoxaline (XV) and phenazine (XVI) clearly shows that (iso)alloxazines resemble more closely the former. It can be concluded that ring C of the (iso)alloxazine system does not directly contribute to the ring current, but exerts indirect effects on ring A and B *via* the carbonyl groups as mentioned above. The same can be said for the cationic species XVII–XXV (Tab. 5) where the H_3C–N(3) group is influenced only little by the positive charge (compare with the resonances of H_3C–N(3) of the (iso)alloxazines). Only when N(1) is unsubstituted XXVI–XXX, allowing conjugation with ring C, a larger shift of the H_3C–N(3) resonance is observed. In addition the comparison of the H_3C–N(1) resonance of IX–XIV with those of the cationic analogues XXII–XXV reveals that the charge at N(1) is not much altered.

On the other hand, in contrast to expectation, the difference between the frequencies of the two methylene groups of the bridged compounds XVII–XXI (Tab. 5) is smaller than that of the H_3C–N(1) and H_3C–N(10) of the cations XXII–XXV (Tab. 5). This indicates that in the bridged compounds the positive charge is distributed between N(1) and (10), while in XXII–XXV the positive charge is mainly localized on N(10). This would explain the relatively high field position of the H–C(9) resonance in the bridged compounds as compared to that in other quaternary salts (Tab. 5). Indeed, in the latter the electron withdrawal from H–C(9) is more effective. This interpretation is supported by other results [24] obtained from isoalloxazines XVII–XXV which form addition products with methoxide at the C(9a). Thus, addition of methoxide to the ethano bridged compounds XVII–XXI leads to a large upfield shift of both methylene groups (about 70 Hz), and addition to the N(1)-methyl compounds XXII–XXV results in an upfield shift of the H_3C–N(10) resonance (to 372 Hz), while the H_3C–N(1) resonance is affected only to a minor extent. Comparison of the resonances of the aromatic protons of XVII (Tab. 5) with those of quinoxalinium (XXXI) or phenazinium XXXII perchlorate shows that XVII resembles the former more closely.

It is evident from these results that ^{1}H-NMR. experiments yield information necessary for elucidation of the molecular structure of isoalloxazines and alloxazines; they also give valuable insight into the conformation of the molecule. More information about the charge distribution in the molecule can be obtained from ^{13}C-NMR. spectra, for which a study has been undertaken, and will be published shortly [34].

We are grateful to Prof. *J. L. Fox*, University of Texas, Austin (U.S.A.), for the communication of the MINDO/3 calculation prior to publication, to Mr. *J. S. Santema* for preparation of some compounds, to Mr. *R. J. Platenkamp* for help in the calculation of the theoretical values and valuable discussion, to Mr. *B. J. Sachteleben* for the preparation of the figures, and to Miss *A. H. W. Trip* for patient preparation of the tables. We are indebted to *Ciba-Geigy A.G.*, Basel (Switzerland), for the elemental analyses.

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] 'Reactivity of Flavins', *K. Yagi*, ed., University of Tokyo Press 1975; 'Flavins and Flavopro teins', *T. P. Singer*, ed., Elsevier Publishing Company, Amsterdam 1976.
[2] *J. L. Fox, K. Nishimoto & L. S. Forster*, Biochim. biophys. Acta *109*, 626 (1965); *B. Grabe*, Acta chem. scand. *26*, 4084 (1972), *ibid A28*, 363 (1974).
[3] *S. Castellano & A. A. Bothner-By*, J. chem. Physics *41*, 3863 (1964).
[4] *H. Goldner, G. Dietz & E. Carstens*, Liebigs Ann. Chem. *694*, 142 (1966).
[5] *F. J. Bullock & O. Jardetzky*, J. org. Chemistry *30*, 2056 (1965).
[6] *H. H. Fall & H. G. Petering*, J. Amer. chem. Soc. *78*, 377 (1956).
[7] *F. Müller & K. H. Dudley*, Helv. *54*, 1487 (1971).
[8] *R. Kuhn & F. Weygand*, Ber. deutsch. chem. Ges. *67*, 1409 (1934).
[9] *A. Halasz*, Chem. and Ind. *1969*, 1701.
[10] *M. Dolinsky, J. H. Jones, C. D. Ritchie, R. L. Yates & M. A. Hall*, J. agric. org. Amer. Chemistry *42*, 709 (1959).
[11] *P. Hemmerich*, Helv. *47*, 464 (1964).
[12] *K. H. Dudley & P. Hemmerich*, Helv. *50*, 355 (1967).
[13] *K. H. Dudley & P. Hemmerich*, J. org. Chemistry *32*, 3049 (1967).
[14] *B. M. Chassy, C. Arsenis & D. B. McCormick*, J. biol. Chemistry *240*, 1338 (1965).
[15] *F. Müller & V. Massey*, J. biol. Chemistry *244*, 4007 (1969).
[16] *P. Hemmerich, A. P. Bhaduri, G. Blankenhorn, M. Brüstlein, W. Haas & W. R. Knappe*, in 'Oxidase and Related Redox Systems', *T. E. King, H. S. Mason & M. Morrison*, eds., University Park Press, Baltimore 1972, p. 2.
[17] *H. J. Grande*, Dissertation, Agricultural University, Wageningen, 1976.
[18] *R. F. Smith, W. I. Rebel & T. N. Beach*, J. org. Chemistry *24*, 205 (1959).
[19] Eur. J. Biochemistry *2*, 5 (1967).
[20] *L. E. G. Eriksson & A. Ehrenberg*, Acta chem. scand. *18*, 1437 (1964).
[21] *J. Fritz, F. Müller & S. G. Mayhew*, Helv. *56*, 2250 (1973).
[22] *D. J. Blears & S. S. Danyluck*, Tetrahedron *23*, 2927 (1967).
[23] *F. A. Bovey*, in 'Nuclear Magnetic Resonance Spectroscopy', Academic Press, New York 1969, p. 119 and references therein.
[24] *F. Müller, H. J. Grande & T. Jarbandhan*, in 'Flavins and Flavoproteins', *T. P. Singer*, ed., Elsevier Publishing Company, Amsterdam 1976, p. 38.
[25] *M. Kainosho & Y. Kyogoku*, Biochemistry *11*, 741 (1972); *G. Kotowycz, N. Teng, M. P. Klein & M. Calvin*, J. biol. Chemistry *244*, 5656 (1969); *R. H. Sarma, P. Dannies & N. O. Kaplan*, Biochemistry 7, 4359 (1968).
[26] *B. Pullman & A. Pullman*, Proc. Nat. Acad. Sci. USA *45*, 136 (1959).
[27] *H. Sterk & H. Holzer*, Org. Mag. Res. *6*, 133 (1974).
[28] *M. J. S. Dewar*, J. Amer. chem. Soc. *97*, 6591 (1975).
[29] *M. J. S. Dewar & E. Haselbach*, J. Amer. chem. Soc. *92*, 590 (1970).
[30] *P. Hamm & W. v. Philipsborn*, Helv. *54*, 2363 (1971).
[31] *G. Weber*, in 'Flavins and Flavoproteins', *E. C. Slater*, ed., B. B. A. Library, Vol. 8, Amsterdam 1966, p. 15.
[32] *Ph. Wahl, J. C. Auchet, A. J. W. G. Visser & F. Müller*, F.E.B.S. Letters *44*, 23 (1974).
[33] *J. H. Wassink & S. G. Mayhew*, Anal. Biochemistry *68*, 609 (1975).
[34] *H. J. Grande, R. Gast, C. G. van Schagen, W. J. H. van Berkel & F. Müller*, Helv. *60*, 367 (1977).