62. Absorption and Fluorescence Studies on Neutral and Cationic Isoalloxazines

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(7.III.78)

Summary

The electronic properties of a variety of methylated isoalloxazine derivatives have been investigated with absorption and fluorescence techniques *(Table 1)*. The spectral effects of methyl substitution at different positions in the isoalloxazine ring are interpreted in terms of different bond orders between the C-atoms of the methyl groups and of the aromatic ring. Methyl groups at C(6) or C(9) induce quenching of the fluorescence and shortening of the lifetime whereas a methyl group at C(7) has an opposite effect. The quenching effect might be due to strain in the molecular framework caused by the methyl groups.

The lowest energy levels of the isoalloxazine cations are shifted to higher energy as compared to the neutral isoalloxazines. Apart from these shifts the trends in spectral behaviour upon methylation are similar to those observed for the neutral isoalloxazine *(cf. Table 2)*. The fluorescence of the isoalloxazine cations is strongly quenched in auqeous solution and their lifetime much shortened. These observations indicate that there is substantial interaction between the charged fluorophore and the water dipoles and that this interaction produces efficient radiationless deactivation of the first excited singlet state.

1. Introduction. – Isoalloxazines[2]), *i.e.* flavins, are indispensable constituents of many biological reactions involving both the ground and excited states of these molecules (*e.g.* [1]). The fluorescence and the light absorption properties of protein-bound isoalloxazines vary considerably among the different flavoproteins. In order to understand the underlying physical cause of these differences some model studies were conducted in the past. Thus, the solvent and pH-dependence of the light absorption properties of some isoalloxazines have been investigated by *Dudley et al.* [2]. *Koziol* [3], on the other hand, investigated in detail the solvent dependence of the fluorescence emission spectra and of the quantum yield of

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[2]) Isoalloxazine = 10-substituted 2,3,4,10-tetrahydro-benzo[g]pteridine-2,4-dione; flavin = 7,8-dimethyl-isoalloxazine.

7,8,10-trimethyl-isoalloxazine. Some theoretical as well as further experimental studies were conducted by *Song* (see *e.g.* [4]). The theoretical studies show sometimes poor agreement with published experimental results, mainly for the reason that: a) the luminescence properties of isoalloxazine have not been studied thoroughly experimentally; b) in all theoretical calculations with π-electrons spectral properties are only predicted qualitatively. Therefore, the present study was undertaken to characterize in more detail the fluorescence properties of various neutral isoalloxazines by determining their quantum yields, lifetimes, radiating lifetimes and degrees of polarization under different conditions. Results obtained with cationic isoalloxazines are also reported. Some preliminary results have been reported elsewhere [5] [6].

2. Experimental Part. – 2.1. *Syntheses.* The syntheses of the compounds studied have already been described [7] (and ref. therein), except for the following two new compounds.

3,9,10-Trimethyl-isoalloxazine (cf. Table 1). To 1.5 g of 6-methyl-2-nitro-aniline (*Fluka A.G.*, Buchs) in 50 ml of CH_2Cl_2 4 ml of methyl fluorosulfonate ('Magic Methyl', *Aldrich Co.*) were added and the mixture was stirred first at RT. for 20 min and then heated under reflux overnight until most of the starting material had reacted (TLC. in diisopropyl ether). The cooled mixture was transferred into a separatory funnel, 50 ml of H_2O and 10 ml of 2N NaOH were added and the mixture was shaken vigorously to hydrolyse residual alkylating reagent (all handlings were carried out in a well-ventilating hood). The organic phase was separated, dried with Na_2SO_4 and evaporated to dryness. The residual oily product, contaminated with small amounts of unidentified by-products, was reduced catalytically and the product condensed with alloxan and then alkylated at N(3) as described in [7]. Overall yield 50%, m.p. 298–300° (uncorr.)

$C_{13}H_{12}N_4O_2$ (256.27) Calc. C 60.93 H 4.72 N 21.86% Found C 60.3 H 4.80 N 21.6%

3,6,10-Trimethyl-isoalloxazine (cf. Table 1) was prepared virtually according to the procedure of *Leonard & Lambert* [8] as follows: 6-methyl-2-nitro-aniline was oxidized in peracetic acid to the corresponding dinitro compound which in turn was transformed to 3,*N*-dimethyl-2-nitro-aniline by heating under reflux for 24 h in isopentyl alcohol in the presence of methylamine. Reduction of this product, followed by condensation with alloxan and alkylation, as described above, gave the desired compound in a overall yield of 30%, m.p. 285–290° (uncorr.)

$C_{13}H_{12}N_4O_2$ (256.27) Calc. C 60.93 H 4.72 N 21.86% Found C 59.9 H 4.70 N 21.5%

2.2. *Preparation of samples.* Since isoalloxazines are photolabile all manipulations were performed as far as possible in subdued light. For measurements of the absorption and fluorescence properties stock solutions of isoalloxazines in acetonitrile (*Merck*, 'Uvasol') were prepared. Since many isoalloxazines are poorly soluble in water, their aqueous solutions were prepared by diluting a small amount of a concentrated acetonitrile solution of isoalloxazine with a buffer solution made from doubly distilled water; the final acetonitrile concentration was less than 1%. To prevent decomposition, aqueous solutions of cationic isoalloxazines were kept in 0.1M sodium acetate buffer, pH 5.0. Neutral isoalloxazines were buffered at pH 7.0 with 0.1M sodium phosphate. Absorption spectra were usually measured with a concentration of isoalloxazine in the order of $50 \cdot 10^{-6}$M, fluorescence and polarization spectra as well as the lifetime with concentrations in the range of $1 \cdot 10^{-6}$ to $10 \cdot 10^{-6}$M. Samples for the polarized fluorescence measurements were prepared by diluting concentrated solutions of isoalloxazine in acetonitrile with glycerol (*Merck*, 'doppelt destilliert, reinst').

2.3. *Instrumentation.* Extinction coefficients and absorption spectra were determined with a *Cary* 14 spectrometer. All absorption spectra were converted into the wavenumber scale.

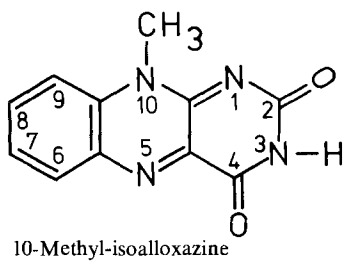
Fluorescence polarization spectra were obtained as described earlier [9]. Depending on the isoalloxazine different sets of cut-off filters were employed: usually *Schott* OG-530 or GG-495 for the neutral isoalloxazines and GG-495 or GG-455 for the isoalloxazine cations. Polarization excitation spectra were run at $-10°$. The excitation band width was 6 nm. The accuracy is ± 0.005 for the higher degrees of polarization (> 0.25) and ± 0.010 for the lower ones (< 0.25).

Fluorescence lifetimes were determined in *Suprasil* cells of standard size with a phasefluorometer described elsewhere [10]. The instrument was equipped with an acousto-optical light modulator operating at frequencies of 15 and 60 MHz. Both frequencies were used in order to check the homogeneity of the lifetimes, *i.e.* small amounts of fluorescent impurities in the investigated solution would yield different values at the two frequencies. The instrument has an accuracy of 2% for lifetimes in the range of 0.2–10 ns.

Fluorescence spectra were recorded on a fluorometer built in our laboratory. The excitation light path contained a 450 W high pressure Xe-arc and a *Zeiss* M4QIII prism monochromator. The emission light path consisted of a *Jarrel-Ash* 82410 monochromator and a *Hamamatsu* R446 photomultiplier. All lenses were from *Suprasil* fused quartz. Part of the beam could be split off with a beam splitter and viewed by a reference photomultiplier (another *Hamamatsu* R446). The emission light was chopped at a frequency of 125 Hz and phase sensitive detection was employed using a *PAR* 128 lock-in amplifier *(Princeton Applied Research Corp.)*. Stray and scattered light were suppressed by using appropriate filters in the exciting and emitting light paths. Whenever necessary solvent blank spectra were recorded and subtracted from the spectra obtained from isoalloxazines. Emission and reference signals could be registered in the ratio mode by a *DANA* 5400 digital voltmeter. In this way fluctuations in the light source were eliminated directly. Another advantage of this set-up was that the digital output of the voltmeter could be used for further data handling. Since the sensitivity of the detection system was dependent on the wavelength, a correction curve was obtained by means of a calibrated tungsten lamp [11]. All emission spectra were taken as the relative photon flux per wavelength interval as a function of wavelength. The emission band width was normally 3 nm. All spectra were corrected with calibration factors and then plotted as photon flux per wavelength interval as a function of wavenumber. Quantum yields of fluorescence were determined relative to that of fluorescein in 0.1N NaOH, which is adopted as 0.92 [12]. The exciting wavelength was 400 or 440 nm. Fully corrected excitation spectra were obtained by the method described by *Chen* [13]. Lifetimes and spectra were obtained at $22 \pm 1°$. No attempts were made to remove oxygen from the samples.

3. Results. – The structure of the isoalloxazine molecule is shown in the *Scheme* together with the internationally accepted ring numbering. The spectral data of neutral (iso)alloxazines and of isoalloxazinium perchlorates are given in *Table 1* and *2*, respectively, the compounds being arranged in successive order of methylation. In *Table 1* two groups may be distinguished: the isoalloxazines I–X and the alloxazines XI and XII, and *Table 2* may be divided into three parts, the N(1), N(10)-ethylene bridged (XIII–XVII), the N(1),N(10)-dialkyl (XVIII–XX) and the N(3),2α-dialkyl cationic compounds XXI–XXIV.

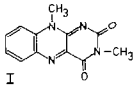
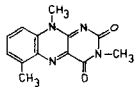
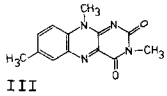
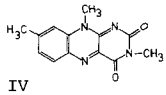
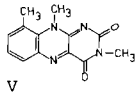
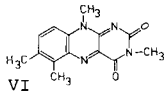
Scheme



10-Methyl-isoalloxazine

3.1. *Neutral isoalloxazines.* The effect of introducing methyl groups into the benzene subnucleus of the isoalloxazine ring becomes apparent when the spectral parameters of the trimethylated derivatives II–V *(Table 1)* are compared with 3,10-dimethyl-isoalloxazine (I), when derivative III is compared with the tetramethylated compounds VI–VIII, or VII with the pentamethylated compounds IX

Table 1. *Spectroscopic data of isoalloxazines*

Compound[a]) No.	$\tilde{\nu}_{max} \cdot 10^{-3}(\varepsilon \cdot 10^{-3})$[b])	f[c])	$F_{max} \cdot 10^{-3}$[d])	$\tilde{\nu}_{cg} \cdot 10^{-3}$[e])	$\Delta\tilde{\nu} \cdot 10^{-3}$[f])	τ[g]) [ns]	τ_0[h]) [ns]	τ/τ_0	Q[i])
 I	22.9 (9.1) 30.5 (7.0) 37.5 (33.6)	0.150	18.9	18.9	4.0	7.0	15.1	0.46	0.38
	23.0 (9.5) 29.2 (7.7) 37.9 (37.5)		19.2	18.8	3.8	4.5			0.15
 II	22.7 (8.6) 29.1 (8.7) 37.0 (35.6)	0.094	20.3	19.1	2.4	1.8	20.6	0.09	0.14
	22.8 (8.5) 27.0 (10.3) 37.6 (37.2)		18.8	18.3	4.0	2.1			0.08
 III	22.4 (9.3) 30.3 (7.2) 37.0 (35.7)	0.097	18.8	18.6	3.6	9.4	23.3	0.40	0.43
	22.4 (9.8) 28.9 (8.2) 37.3 (42.0)		18.1	17.9	4.3	6.9			0.32
 IV	23.0 (11.0) 29.4 (7.8) 37.5 (33.1)	0.128	19.3	19.1	3.7	4.9	16.9	0.29	0.28
	23.1 (11.5) 27.4 (8.8) 37.9 (25.2)		19.3	18.7	3.8	3.0			0.12
 V	22.8 (9.4) 29.1 (9.4) 37.3 (39.4)	0.127	18.8	18.7	4.1	1.8	17.8	0.10	0.07
	22.8 (9.5) 27.0 (12.1) 37.6 (43.6)		18.6	18.4	4.3	2.1			0.06
 VI	22.3 (11.6) 28.6 (12.0) 37.0 (53.7)	0.140	18.4	17.7	3.9	3.9	17.7	0.23	0.17
	22.2 (10.4) 26.3 (15.6) 37.3 (60.6)		17.7	17.7	3.6	4.7			0.03

[a]) The data in the upper row of each individual compound refer to acetonitrile solutions, those in the lower row to aqueous solutions.

[b]) $\tilde{\nu}_{max}$ [cm^{-1}] = wavenumber of the absorption maxima, ε [$l \cdot mol^{-1}cm^{-1}$] = extinction coefficient.

[c]) f is the oxcillator strength of the first electronic transition: $f = 4.32 \cdot 10^{-9} \cdot \int \varepsilon(\tilde{\nu}) d\tilde{\nu}$.

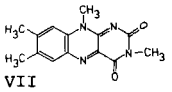
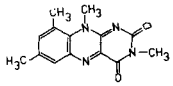
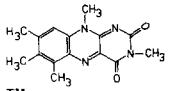
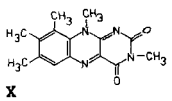
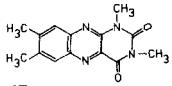
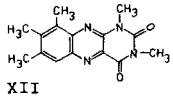
[d]) F_{max} [cm^{-1}] = wavenumber at which the fluorescence maximum is located.

[e]) $\tilde{\nu}_{cg}$ [cm^{-1}] = center of gravity of the fluorescence band ($F(\tilde{\nu})$): $\tilde{\nu}_{cg} = \int \tilde{\nu} F(\tilde{\nu}) d\tilde{\nu} / \int \varepsilon(\tilde{\nu}) d\tilde{\nu}$.

and X. The alloxazines XI and XII form a separate class, not directly comparable with the corresponding isoalloxazines.

There are no dramatic differences in position and extinction coefficient of the first absorption maximum upon formal introduction of one methyl group into I. However, the second absorption band at higher wavenumber is red-shifted, its position and extinction coefficient being strongly influenced by the number and position of the additional methyl substituents. An increase of the solvent polarity has only little influence on the first absorption maximum, whereas the second maximum exhibits a large bathochromic shift. Although the absolute difference between the maxima does not vary much within the methylated compounds, the shape of the spectra changes markedly, especially in the compounds VI and VIII relative to III and IX and X relative to VII. Formal introduction of a methyl group into position 8 (*e.g.* I → IV) leads to a small hypsochromism and an increased extinction coefficient of the first electronic absorption band, and to bathochromism

Table 1 cont.

Compound[a]) No.	$\tilde{\nu}_{max} \cdot 10^{-3}(\varepsilon \cdot 10^{-3})$[b])	f[c])	$F_{max} \cdot 10^{-3}$[d])	$\tilde{\nu}_{cg} \cdot 10^{-3}$[e])	$\Delta\tilde{\nu} \cdot 10^{-3}$[f])	τ[g]) [ns]	τ_0[h]) [ns]	τ/τ_0	Q[i])
 VII	22.5 (12.3) 29.4 (8.8) 37.2 (38.6)	0.147	18.9	18.8	3.6	6.9	15.7	0.44	0.47
	22.5 (12.4) 27.3 (10.0) 37.6 (42.2)		18.9	18.6	4.7				0.25
 VIII	22.3 (6.8) 28.9 (6.8) 36.8 (29.6)	0.084	18.1	17.4	4.2	3.2	33.0	0.10	0.18
	22.1 (6.8) 26.9 (8.4) 37.0 (32.4)		17.5	17.3	4.6	3.2			0.10
 IX	22.4 (9.8) 27.0 (9.3) 36.9 (31.2)	0.119	18.5	18.4	3.9	3.1	19.8	0.15	0.18
	22.3 (8.3) 25.6 (13.0) 37.4 (38.8)		17.5	17.4	4.8	3.9			0.10
 X	22.2 (9.4) 27.0 (10.2) 36.8 (33.8)	0.124	18.1	18.0	4.1	1.7	20.2	0.08	0.06
	22.1 (9.0) 25.3 (14.3) 26.8 (35.9)		17.5	17.4	4.6	1.9			0.03
 XI	26.0 (5.9) 30.0 (5.8) 39.8 (29.2)	0.068	22.4	22.0	3.6	0.72	23.0	0.03	0.05
	25.8 (5.6) 28.3 (7.5) 39.4 (29.9)		21.2	20.6	4.6	2.3			0.07
 XII	25.7 (4.6) 28.4 (7.2) 39.4 (27.6)	0.042	21.3	20.2	4.4	1.5	48.6	0.03	0.04
	25.0 27.5 38.5		19.0	18.1	6.0	5.3			0.04

f) $\Delta\tilde{\nu}$ = difference in wavenumber of the first absorption maximum and the emission maximum.

g) τ = measured lifetime of the fluorescence.

h) τ_0 = radiative lifetime which is obtained from the first resolved absorption and fluorescence bands according to Strickler & Berg [15]: $1/\tau_0 = 2.88 \cdot 10^{-9} \cdot n^2 \cdot <\tilde{\nu}_F^{-3}>^{-1} \cdot \int \varepsilon/\tilde{\nu} d\tilde{\nu}$, with n = refractive index, $<\tilde{\nu}_F^{-3}>$ = third moment of the fluorescence spectrum defined as $\int \tilde{\nu}^{-3} F(\tilde{\nu}) d\tilde{\nu} / \int F(\tilde{\nu}) d\tilde{\nu}$.

i) Q is the quantum yield of fluorescence.

of the second band, while methylation at C(7) (*e.g.* I → III) shifts the first absorption maximum to the red, but has little effect on the location of the second peak. Methyl groups at C(6) or C(9) displace both bands towards the red (the first one to a lesser extent) in comparison to the corresponding reference compounds and cause a distinct hypochromism of the first absorption band in VIII–X (compare with VII).

Some of these spectral effects are illustrated in *Figure 1* where the major absorption bands in the near UV. and VIS. and the fluorescence spectra of four different isoalloxazines in acetonitrile are displayed: all spectra exhibit a partly resolved vibrational fine structure, and some mirror image relationship exists between the first absorption and fluorescence bands. The fine structure is absent in aqueous solution.

For this reason we discuss the data rather qualitatively by comparing peak maxima instead of the more usual 0–0 transitions. In cases where fluorescence peaks are difficult to locate *(e.g. Fig. 1C)* the

average position of the fluorescence band ($\bar{\nu}_{cg}$) is a more meaningful quantity than the peak maximum *(cf. Table 1).*

In order to separate the contribution of the second absorption band from that of the first one in the total spectrum, the absorption bands were resolved into different gaussian components [15], a typical example is given in *Figure 2A* for VII. Only the sum of the components which can be attributed to the first electronic transition was used in calculating the oscillator strength f and the natural lifetime τ_0 *(Table 1).* Resolution into individual components suggests a constant vibrational progression over the first absorption band (about 1220 cm^{-1}).

Generally, the ratio τ/τ_0 should be of the same order of magnitude as the fluorescence quantum yield Q for a given compound. In spite of the inaccuracy of

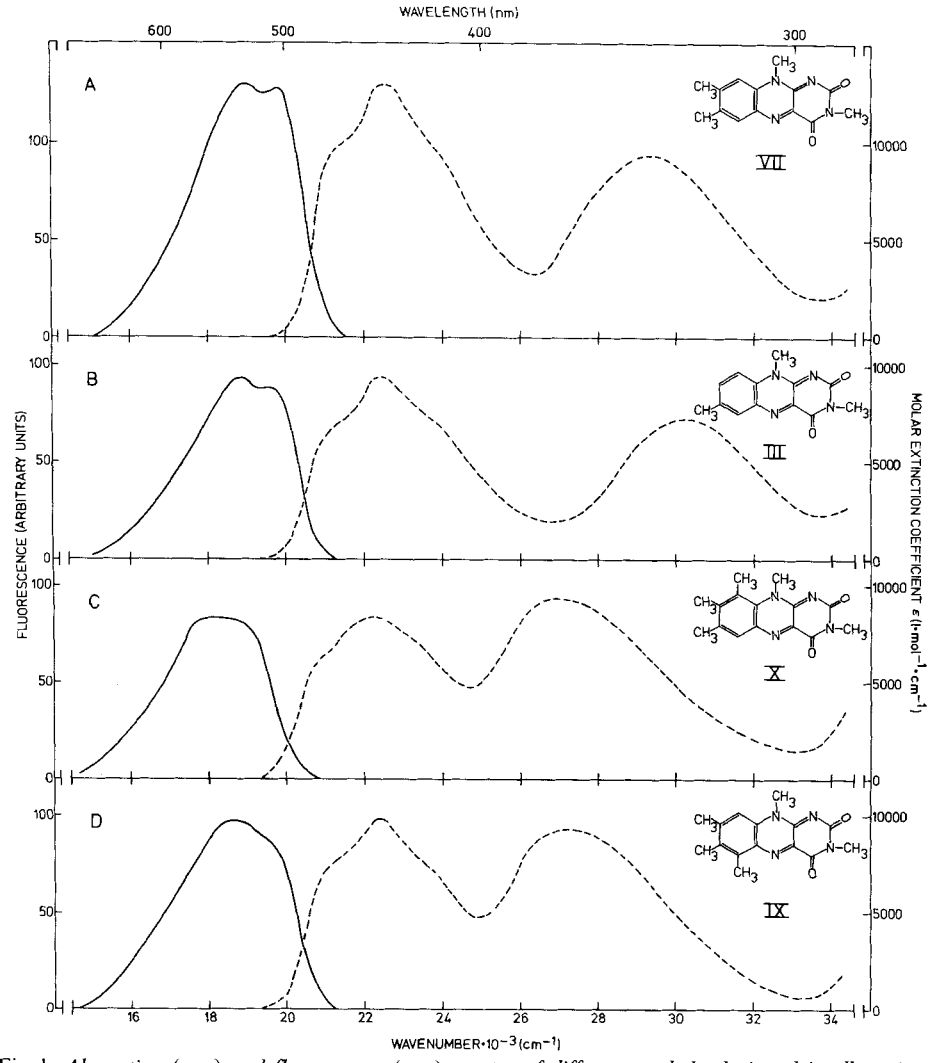


Fig. 1. *Absorption* (---) *and fluorescence* (——) *spectra of different methyl-substituted isoalloxazines in acetonitrile at room temperature* (22°, excitation wavelength 440 nm)

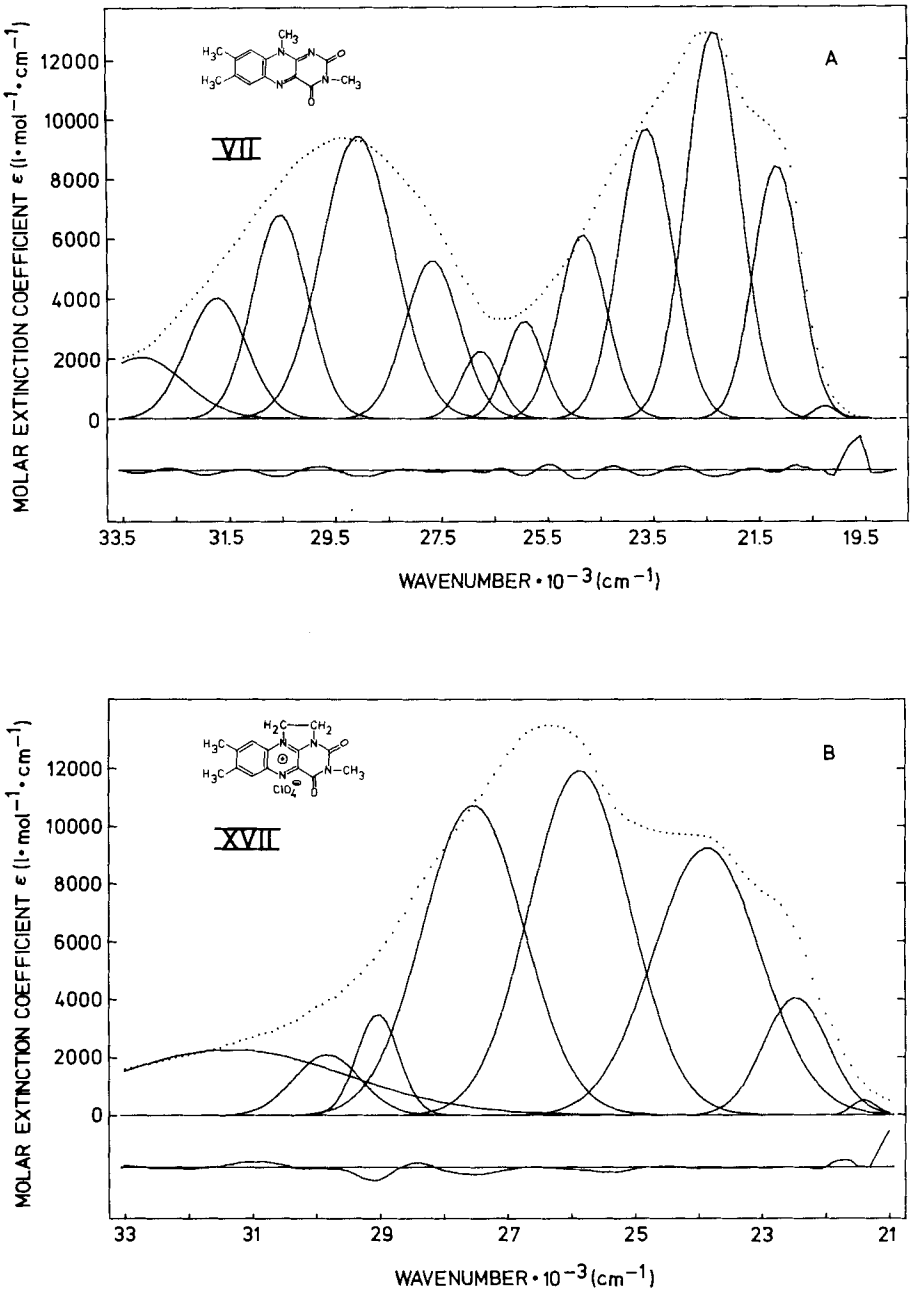


Fig. 2. *Resolution of absorption spectra* (...) *into gaussian components* (——). The lowest curve is the fractional deviation between the experimental curve and the sum of components. For details see text

A. *Compound VII in acetonitrile.* The sum of the first five major components starting at the lowest wavenumber were used in calculating f and τ_0

B. *Compound XVII in acetonitrile.* The sum of the first two components were used

the determination of Q and τ_0 the results obtained for the neutral isoalloxazines point to a fairly good agreement of τ/τ_0 and Q *(Table 1)*.

The values of *Stokes* loss $\Delta\tilde{\nu}$ given in *Table 1* have to be considered merely qualitatively. It can be observed that the *Stokes* shift increases when methyl groups are formally introduced at C(6) and C(9) of III and VII (*cf.* acetonitrile solutions of III, VI and VIII as well as VII, IX and X). The effects are more pronounced in aqueous solution than in organic solvent in the latter case. Note that 6- and 9-

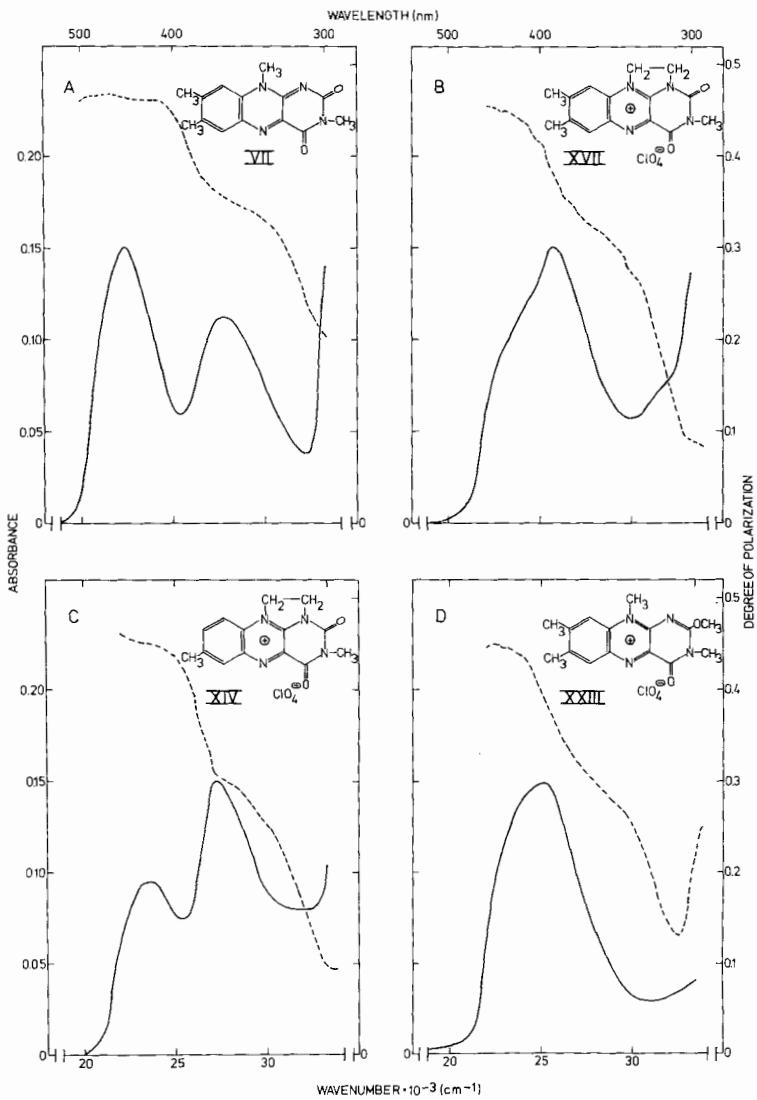


Fig. 3. *Absorption* (——) *and fluorescence polarization excitation spectra* (– – –) *of various neutral and cationic isoalloxazines in glycerol* (absorption spectra at 22°, polarization values at − 10°)

methylated isoalloxazines exhibit a yellow-orange fluorescence in aqueous solution in contrast with the well-known green coloured emission. As expected, generally the *Stokes* loss increases with increasing polarity of the solvent. On the other hand the quantum yield of the fluorescence decreases with increasing polarity of the solvent.

The fluorescence data in *Table 1* lead to the conclusion that a C(7)-methyl group lengthens the lifetime, increases the quantum yield and redshifts the emission spectrum, whereas a methyl group at C(8) has the opposite effect (*cf.* I, III, IV and VII). Both methyl groups at C(6) and C(9) diminish the quantum yields and shorten the lifetime considerably (*cf.* I, II and V; VII, IX and X; III, VI and VIII). Derivatives IX and X show broadened, more diffuse fluorescence bands *(Fig. 1C* and *1D)* and yield equal or longer lifetimes when changing from acetonitrile to water as solvent. This effect on the lifetimes is even more pronounced in the alloxazines XI and XII, whereas the quantum yields are only slightly affected. Noteworthy is the bathochromic effect of the methyl group at C(9) on the lower two absorption maxima in the alloxazines.

Figure 3A shows the polarization excitation spectrum of VII in glycerol together with the absorption spectrum. The polarization measurements were performed in glycerol at $-10°$ to eliminate depolarization by brownian rotations [16]. A region of constant polarization corresponds to the first electronic transition. The point of inflection, indicating the change from the first into the second electronic transition when the excitation wavenumber is varied, coincides approximately with the maximum of the second absorption band. The orientation angle between the first two transition moments can be estimated using a formalism developed by *Jablonski* [17].

In the absence of brownian rotations the theoretical maximal degree of polarization, $p_0 = 0.5$, is not reached in most aromatic molecules. This phenomenon might be due to torsional vibrations in the molecule. This ultra-rapid depolarization effect can be approximated for the first electronic transition by the following equation [17]:

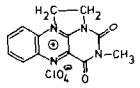
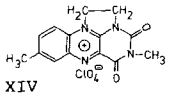
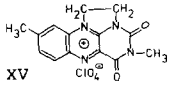
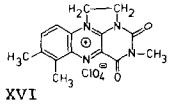
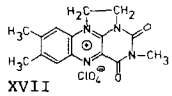
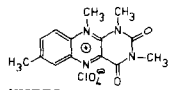
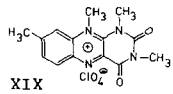
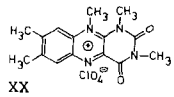
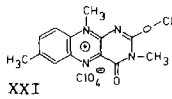
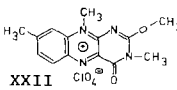
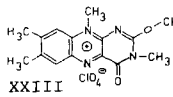
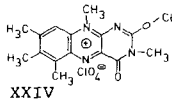
$$r_0(\text{obs.}) = 2p_0/(3 - p_0) = 0.4 - 1.2\,\mu + 0.9\,\mu^2;$$

r_0(obs.) is the limiting observed emission anisotropy, $\mu = <\sin^2\varepsilon>$, where ε is the angle between the mean direction and the instantaneous direction of the transition moment, $r_0 = 0.4$ (for $\mu = 0$) is the fundamental anisotropy for the first electronic transition. We used this expression to estimate μ from the highest observed polarization. We then made the somewhat simplifying assumption that the torsional vibrations give rise to the same depolarization when excited in the second absorption band. The angle between the two transition moments is then calculated from $r_0(\text{obs.}) = r_0(\text{fund.}) = 1.2\,\mu + 0.9\,\mu^2$, with $r_0(\text{fund.}) = 0.4\,(3/2\,\cos^2\theta - 1/2)$. It should be noted that these angles have approximate values. If such corrections are not made the angles are 3–6° larger.

Surprisingly, the angles obtained for the isoalloxazines are all in the same order of magnitude, varying between 24° (X) and 33° (VII).

3.2. *Cationic isoalloxazines.* We have found that, in contrast to published work [18], cationic isoalloxazines are also intrinsically fluorescent. The maxima of the first absorption and fluorescence bands of nearly all the cations exhibit hypsochromic shifts as compared to the corresponding neutral isoalloxazines *(Table 2).* The fluorescence is blue to green and generally weaker than the one of the neutral isoalloxazines. In most cases of the cationic species the first absorption band at lower energy is shifted more to the blue than the second one to the red in comparison

Table 2. *Spectroscopic data of isoalloxazinium salts*[a])

Compound[a]) No.	$\tilde{\nu}_{max} \cdot 10^{-3}(\varepsilon \cdot 10^{-3})$[b])	f[c])	$F_{max} \cdot 10^{-3}$[d])	$\tilde{\nu}_{cg} \cdot 10^{-3}$[e])	$\Delta\tilde{\nu} \cdot 10^{-3}$[f])	τ[g]) [ns]	τ_0[h]) [ns]	τ/τ_0	Q[i])
 XIII	24.6 (8.2) 27.9 (12.0) 39.5 (28.4) 24.5 (6.1) 27.3 (10.4)	0.082	19.8 19.6	19.2	4.8 4.9	4.1 0.83	26.8	0.15	0.22 0.02
 XIV	23.8 (7.1) 27.5 (11.2) 38.2 (31.2) 23.3 (5.0) 27.0 (10.1)	0.067	19.7 18.9	19.3	4.1 4.4	7.2 1.3	31.4	0.23	0.22 0.02
 XV	26.1 (17.0) 39.2 (33.0) 25.6 (20.2)	0.065	20.8	22.1	5.3	4.6 0.61	22.1	0.21	0.13 < 0.01
 XVI	23.2 (4.8) 26.2 (18.0) 37.8 (30.4) 26.0 (18.4)	0.066	17.5	17.5	5.7	7.0 2.6	43.4	0.16	0.08 < 0.01
 XVII	26.4 (13.6) 38.0 (31.3) 25.8 (10.8)	0.081	20.0 19.2	19.4	6.4 6.6	5.5 1.2	25.5	0.22	0.15 0.02
 XVIII	24.7 (6.8) 27.4 (9.2) 37.3 (21.0) 26.9 (6.8)	0.068	19.6	18.9	5.1	2.5 0.57	34.4	0.07	0.07 < 0.01
 XIX	25.5 (20.0) 37.7 (28.8) 25.1 (19.1)	0.065	18.5	18.2	7.0	2.7 0.51	43.1	0.06	0.07 < 0.01
 XX	25.4 (17.0) 25.1 (16.8)	0.043	19.0	18.9	6.4	2.8 0.64	54.8	0.05	0.07 < 0.01
 XXI	23.9 (5.9) 27.1 (9.3) 37.5 (37.2) 23.5 (8.4) 26.8 (10.5)	0.077	20.2 19.3	19.7	3.7 4.2	3.5 2.1	25.6	0.14	0.18 0.05
 XXII	25.0 (15.9) 38.2 (41.5) 25.1 (17.6)	0.122	21.4 20.0	20.8	3.6 5.1	3.2 1.4	14.4	0.23	0.18 0.02
 XXIII	24.2 (12.4) 25.7 (13.0) 38.0 (32.9) 25.4 (16.8)	0.100	20.5 19.4	19.0	3.7 6.0	3.5 2.0	22.3	0.15	0.15 0.03
 XXIV	25.1 (20.1) 36.7 (38.3) 25.0 (20.0)	0.045	17.7	17.9	7.4	10.1 2.0	57.4	0.18	0.05 < 0.01

[a]) See Table 1 for details.

with the neutral species. The result is a merging of the two bands leading to characteristic composite spectra[3]) *(Fig. 4).* The fluorescence spectra are characterized by unresolved bands *(Fig. 4).* In *Figure 4A* and *4B* the corrected excitation spectra are reported indicating that excitation and absorption spectra are identical. Therefore the observed fluorescence emission originates from the cationic isoalloxazines. The general appearance of the absorption spectra depends highly on the number and position of the methyl groups and on the solvent (acetonitrile or water). Like in the neutral isoalloxazines formal methylation at C(7) shifts the first electronic transition bathochromically and the second one hypsochromically, therefore only in the 7-methylated compounds XIV, XVIII and XXI two distinct band maxima are observed in the absorption spectra. Apart from the maximum values other extinction coefficients given in *Table 2* refer either to poorly resolved maxima or to shoulders.

A very clear demonstration that the absorption bands are composed of at least two different electronic transitions is given by the polarization spectra *(Fig. 3B–D).* The absorption spectra in *Figures 3B* and *3D* consist of a single band; however, the great decrease of the degree of polarization (p) within the band definitely points to the presence of a submerged band arising from another electronic transition. The point of inflection is less pronounced than for the neutral isoalloxazines, the inaccuracy in the angle θ is therefore larger, but the values of θ obtained are in the same order as the ones found for the neutral isoalloxazines. The composite nature of the absorption band makes it difficult to estimate the contribution of the first transition, which is needed for the calculation of the oscillator strength f and the natural lifetime τ_0. We estimated it again by resolving the spectrum into different gaussian components *(Fig. 2B).* The contribution of the first transition to the total spectrum was then estimated by a method developed by *Valeur & Weber* [19].

It is assumed that the observed emission anisotropy at each excitation wavelength is composed of 2 different contributions:

$$r_0(\text{obs.},\lambda) = f_a(\lambda)\, r_{0a} + f_b(\lambda)\, r_{0b},$$

with $f_a(\lambda) + f_b(\lambda) = 1$; r_{0a} is the limiting anisotropy of the first transition a, which in all our measurements approaches 0.4, and r_{0b} the anisotropy of the second transition b, estimated at the point of inflection in the polarization spectrum. At each point in the polarization spectrum the fractional contribution f_a of the first absorption band can then be calculated. The corresponding area under the resolved absorption spectrum is estimated from the appropriate sum of gaussian components.

Considering this approximation and the limited accuracy in the quantum yield determination, the agreement between τ/τ_0 and Q *(Table 2)* is reasonable. Methylation at C(6) (XVI and XXIV) leads to a large *Stokes* shift; also the lifetime becomes longer, which is in sharp contrast with the neutral isoalloxazines. Comparing the three different cations of the 7,8-dimethyl derivatives XVII, XX and XXIII the lifetime in acetonitrile of the bridged compound XVII is longest and its quantum yield larger than the one of XX. It should also be noted that water decreases the fluorescence of the isoalloxazinium salts much more efficiently than the one of neutral isoalloxazines.

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[3]) Neutral isoalloxazines dissolved in 6N HCl yield an absorption spectrum composed of only one band in the VIS. and near UV. [2].

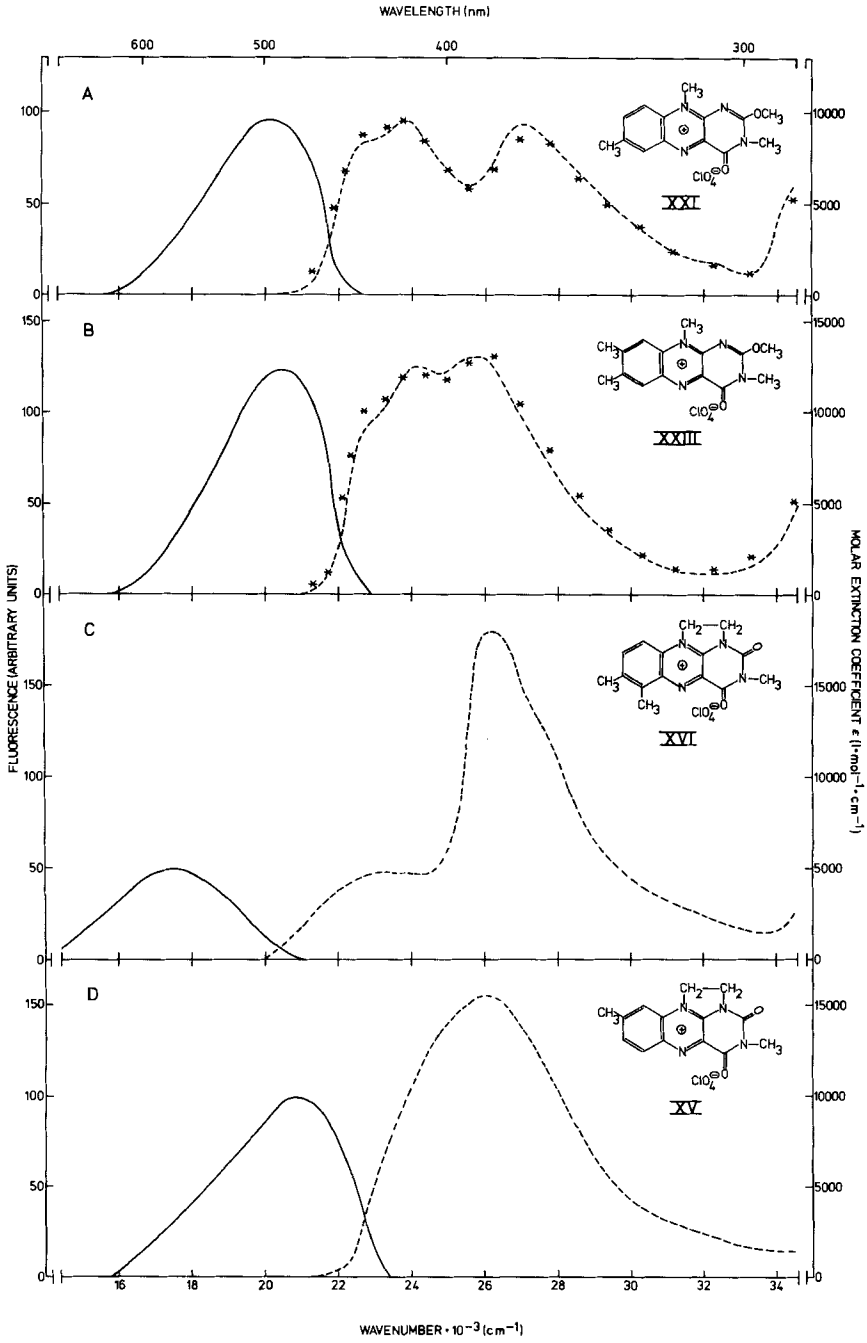

Fig. 4. *Absorption* (---), *fluorescence* (——) *and excitation spectra* (* * *) *of various cationic isoalloxazines in acetonitrile at* $22 \pm 1°$ (excitation wavelength 400 nm)

4. Discussion. – There is a good agreement for nearly all neutral isoalloxazines between data obtained from the first electronic absorption band (*f*, τ_0; *cf. Table 1*) and the fluorescence parameters (τ, Q), implying that the spectroscopic behaviour of the aza-aromatic molecule is normal. The effects of methyl substitution on the absorption spectra of isoalloxazines were predicted by *Song* [20] using quantum chemical calculations with only π-electrons. Some of these predictions are confirmed by our results, *e.g.* the observed bathochromic shift of the second electronic absorption band induced by methylation at C(6) and C(9), and the red shift of the lowest transition of the compounds methylated at C(7). These shifts are definitely due to inductive effects of the methyl groups [21]. Methylation at C(7) gives rise to bathochromism, whereas methylation at C(8) leads to a slight hypsochromism of the energetically lowest absorption and fluorescence bands. This indicates a higher bond order between the carbon atom of the methyl group and C(7) of the isoalloxazine ring in the first excited singlet state. In the second excited state the situation is reversed.

Comparison with NMR. data is not directly relevant since in optical spectroscopy the properties of the excited state are emphasized, leading to a reorganisation of the MO scheme. Nevertheless, *Grande et al.* [7] [22] also concluded from NMR. data that there is a higher electron density in the ground state at C(7) than at C(8).

Introduction of an electron donating group at C(6) and or C(9) decreases the energy of the two lowest excited singlet states. Hyperconjugation appears to be larger at C(6) and C(9), indicating a substantial interaction between σ- and π-electrons. The concomitant hypochromism of the lowest transition in VIII–X might be due to several factors. It is known that the lowest transition is polarized in plane along the long axis C(8)–N(3) of the isoalloxazine molecule [23]. Methylation at C(6) and C(9) might influence the transition in such a way that it becomes more short-axis polarized and has a smaller oscillator strength (more 'forbidden'). A perhaps more plausible explanation is that steric influences cause the hypochromism without having a direct effect on the direction of polarization [24]. Especially the slight crowding of methyl groups in compounds IX and X compared to VII can be responsible for the observed hypochromism, the less resolved vibrational structure of the first absorption band and the broadened emission bands[4]).

The results of the polarization measurements are in agreement with *Song*'s calculations in the sense that methyl groups are small perturbants, the angles being insensitive to the number and position of methyl groups in the aromatic ring of the isoalloxazine [20]. The high degree of polarization was also observed earlier and gives support for two different π–π^* transitions which are polarized non-perpendicularly [25].

The relatively large *Stokes* shift observed in IX and X and the broadening of the emission bands can be partly explained by assuming a strained configuration of the fluorophore. The pentamethylated compound X with its crowded structure exhibits the shortest lifetime and lowest quantum yield of the fluorescence

[4]) ^{13}C- and ^{1}H-NMR. spectra also suggest a similar mutual influence of the methyl groups causing a distortion of the planarity of the molecule [7] [22].

suggesting a very efficient radiationless loss of excitation energy. In IX the mutual influence of the methyl groups at C(6) and the electron lone pair at N(5) might cause some distortion of the planar aromatic ring and thus facilitates radiationless deactivation.

The effect of solvent on the spectral characteristics of 7,8,10-trimethyl-isoalloxazine has already been studied by *Koziol* [3]. We found that the position of the second lowest absorption maximum of all neutral isoalloxazines is very sensitive towards solvent polarity (bathochromic effect in aqueous solutions), while the position of the first maximum is much less affected. According to *Bakhshiev* [26] the *Stokes* shift can be correlated with the dielectric constant and the index of refraction of the solvent and with the dipole moments in ground and excited state of solute. In aqueous solution the interaction between the water dipoles and the changed dipole moment of the excited fluorophore leads to extra energy stabilisation (hydrogen bond terms) and correspondingly to a larger *Stokes* shift. In isoalloxazines there is a substantial interaction between polar solvents and the electron rich carbonyl groups.

The spectral changes occurring in the isoalloxazine upon acidification of the solution have already been described and the absorption in the 450–350 nm region attributed to two π,π^*-transitions [18] [23]. We arrive at the same conclusion for the cationic flavins on the basis of polarized fluorescence. The exceptionally high extinction coefficient observed in some cases also point to merging of two electronic transitions into one band and indicate additivity of extinctions. Thus in isoalloxazinium salts the positive charge seems to shift the lowest electronic energy level more than the second one. In spite of the shift in energy levels the effects induced by 7- and 8-methylation in the absorption spectra indicate the same influences as found in neutral isoalloxazines. Methylation at C(6) (*e.g.* XVI) has an even more profound effect in the cationic than in the neutral isoalloxazines: the fluorescence lifetimes are relatively long, but the quantum yields rather low (*cf.* XVI and XXIV). The fluorescence is shifted towards lower energy *(cf. Fig. 4C).* The low extinction coefficient and the long fluorescence lifetime might suggest that the lowest transition is moderately forbidden.

The bridged cationic derivatives are forced to maintain a planar configuration which should fluoresce more readily. This is demonstrated in the bridged cations XIV, XV and XVII which display much stronger emission than the structurally related compounds XVIII, XIX and XX.

The large solvent dependence of yield and lifetime of the cations is indicative of strong interaction in the first excited singlet state. The dipole moment in the excited state is probably larger than in the ground state because of the strong quenching effect of water. This means that the positive charge might be more localized in the first excited state. The last class of cations (XXI–XXIV) have the largest quantum yields and longest lifetimes in aqueous solution. This can be ascribed to a weaker interaction between the fluorophore and the surrounding water molecules. Since the π-electrons are delocalized over the entire ring system, the positive charge may be distributed over a larger portion of the pyrimidine moiety giving rise to a weaker dipole moment in the excited state as compared to the other cationic isoalloxazines.

5. Conclusions. - Although methyl groups can be considered as weak perturbants, their effect on the light absorption and fluorescence characteristics of isoalloxazine derivatives is appreciable. In several examples of methylation changes of 1000–2000 cm^{-1} in absorption and emission maxima can be observed. These shifts indicate drastic changes in electronic distribution in the first and second excited singlet state. Emphasizing the most important points it is obvious that methyl groups at C(7) and C(8) have counteracting effects while those at C(6) and C(9) lead to diminished fluorescence, shortening of the lifetimes and hypochromism of the visible absorption band. In the latter case a certain distortion of the plane symmetry might be present. Also the bathochromism of the second absorption band at higher energy and the orange-coloured fluorescence induced by higher solvent polarity are points worth noting. Methyl groups at the aromatic part of the isoalloxazine exert their influence especially on the σ-electronic system. More refined theoretical calculations accounting for the contribution of σ-valence electrons, *e.g.* on the spectroscopic CNDO level [27], represent the position of excited energy levels and the electronic distribution more precisely. It is interesting that interactions at specific position in the benzenoid nucleus (C(6) and C(9)) give rise to the largest effects. This might be relevant in the case of some flavoproteins which exhibit not only a distinct hypochromism of the visible absorption band but also a complete absence of isoalloxazine fluorescence.

Preferentially in solvents of low polarity the cationic isoalloxazines exhibit moderately strong fluorescence with blue-shifted maxima as compared with the neutral counterparts. In some cases a single electronic absorption band appears to be present, which on the basis of polarized fluorescence is actually shown to consist of at least two transitions. The bridged cationic compounds show the relatively strongest fluorescence indicating that these cations can be considered as the most planar molecules. Water has a dramatic quenching effect on the fluorescence of the cations. This points to efficient solvent relaxation processes around the fluorophore. The bluish fluorescence of the isoalloxazinium salts in an apolar solvent might also be obtained when such a derivative is bound in a hydrophobic pocket of a (flavo)-protein in analogy to the enhancement and blue shift of the fluorescence observed when a probe like 1-anilino-8-naphthalene sulfonate (ANS) is noncovalently bound to a protein [28]. Because of the similarity of the spectral distribution of fluorescence and bioluminescence, isoalloxazine cations are thought to be possible emitting intermediates in bioluminescence reactions [18]. However, up to now no model has been found to match the bioluminescence spectra. From our data it seems that compounds XVII and XXIII *(Table 2)* mimick the bioluminescence spectra to a much greater extent than any previously investigated isoalloxazine derivatives.

We thank Mr. *B.J. Sachteleben* for preparing the Figures, Mrs. *J.C. Toppenberg-Fang* for preparation of the tables and Mr. *J.S. Santema* and Mr. *T. Jarbardhan* for the help in the preparation of some of the compounds investigated. We also thank Dr. *S.G. Mayhew* for critically reading the manuscript and for linguistic advices.

This investigation was supported in part by the *Netherlands Foundation for Chemical Research* (S.O.N.) and with financial aid from the *Netherlands Organization for the Advancement of Pure Research* (Z.W.O.).

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