PHOTOCHEMISTRY OF N$_5$- AND N$_{10}$-SUBSTITUTED ALLOXAZINIUM CATIONS: PHOTOHYDRATION IN NEUTRAL AND ACIDIC SOLUTIONS

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(Received November 20, 1979)

Summary

The photohydration of N$_5$- and N$_{10}$-substituted alloxazinium cations is described. In dilute buffer the photoreaction of 1,3,5,7,8-pentamethyl-alloxazinium (5-PMA$^+$) perchlorate appears to be much faster than the competing dark reaction, *i.e.* the N$_5$ demethylation of 5-PMA$^+$. Photohydration of 5-PMA$^+$ in 1.0 mM phosphate buffer at pH 7 occurs at C$_9$, whereas the yield of the C$_6$—OH isomer increases upon decreasing the pH. Irradiation of the N$_5$-demethylated product of 5-PMA$^+$, *i.e.* 1,3-dimethyllumichrome, in acidic aqueous solution yields both isomers in almost equal amounts. These findings are in contrast with the exclusive character of the photoaddition site observed in most of the (iso)alloxazines which have been investigated. The results of a flash photolysis study of 1,3,10-trimethylalloxazinium perchlorate are compared with those of previous work on lumichrome.

1. Introduction

The photohydration* of alloxazines [1] and flavins [4] has been studied by irradiation of these compounds in acidic solution. Recently evidence has been found that protonation of the excited singlet (iso)alloxazine is the first step of the reaction [2]. If the function of the proton is restricted to the formation of an excited singlet cation, then photohydration of alloxazinium cations, obtained by substitution of N$_5$ or N$_{10}$, should be possible in neutral solution. In this work we report on the irradiation of the 1,3,5,7,8-penta-methylalloxazinium (5-PMA$^+$) cation under these conditions.

An interesting aspect of the reaction is the specificity of the addition site which, as shown by the compounds studied so far, is either the C$_6$ or the C$_9$ position. Based on this observation and the requirement of the excited

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*Photohydration is a more appropriate description of the process, *i.e.* an attack of water on the excited molecule. The term photohydroxylation, which we have used in previous publications [1 - 3], implies a reaction with hydroxylating species.

singlet cation, a mechanism has been proposed which relates the probable position of protonation with the photoaddition site [2]. An investigation of model compounds such as N_5- and N_{10}-substituted alloxazinium cations can contribute to an examination of the validity of this scheme.
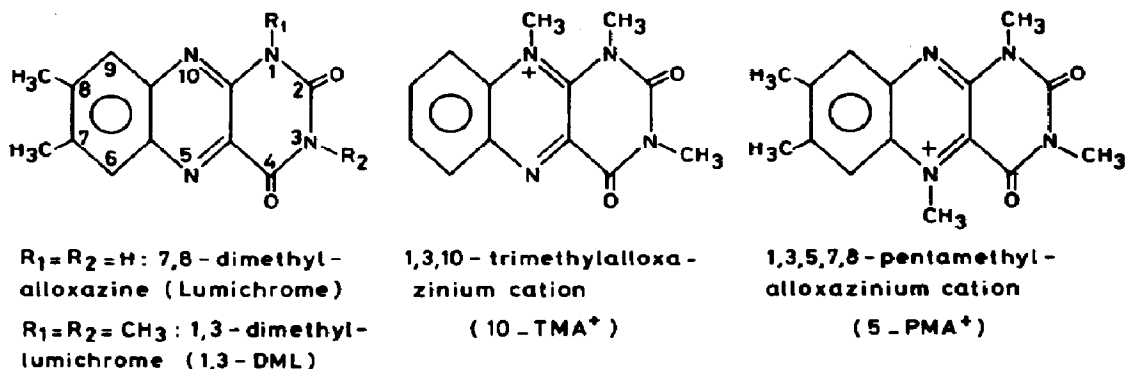

$R_1 = R_2 = H$: 7,8 - dimethyl - alloxazine (Lumichrome)

$R_1 = R_2 = CH_3$: 1,3 - dimethyl - lumichrome (1,3 - DML)

1,3,10 - trimethylalloxa - zinium cation

(10 - TMA⁺)

1,3,5,7,8 - pentamethyl - alloxazinium cation

(5 - PMA⁺)

Fig. 1. The structures of the compounds investigated.

2. Experimental

The syntheses of 5-PMA⁺ perchlorate, 10-TMA⁺ perchlorate and 1,3-DML have been described by Mager and Berends [5, 6]. Methylation of the photoproduct of lumichrome [3] was carried out according to the procedure of Mager and Berends [6].

2.1. Irradiation experiments

The light source for the analytical photochemistry was a 150 W xenon arc. For the irradiation of 10-TMA⁺ and 1,3-DML a Pyrex glass filter was inserted between the light source and the 1 cm quartz cuvette (Hellma 220-QS) containing the sample. Additional filtering (aqueous 0.4 M $CuSO_4$, an optical path length of 6 cm and a type GG 455 Schott filter) was applied for the experiments with 5-PMA⁺. The samples were either saturated with oxygen or deoxygenated by flushing with Cr^{2+}-scrubbed nitrogen.

Preparative photochemistry was carried out with a 1600 W xenon arc. A cooled water filter (a Pyrex glass cylinder 15 cm long) was placed close to the light source. For the experiments with 5-PMA⁺ an internally cooled Pyrex glass cylinder 15 cm long, containing an aqueous 0.4 M $CuSO_4$ solution, and a 5 mm GG 455 Schott filter were also used. All irradiation experiments were carried out at 20 - 22 °C and the samples were stirred.

2.2. Spectral measurements

UV absorption spectra were recorded with a Perkin−Elmer 356 or a Beckman UV 5260 spectrophotometer and the IR spectra were recorded with a Hilger Watts Infrascan. ^1H nuclear magnetic resonance (NMR) spectra were obtained with a Varian A-60 using tetramethylsilane as an internal

reference. Mass spectral analyses were carried out with a Varian Mat 311A. Electron spin resonance (ESR) spectra were recorded with a Varian X-band E4 apparatus. The samples were prepared by adding 20 μl of 1 M $TiCl_3$ to a solution of 0.02 mmol of the alloxazinium cation in 1 ml of 6 M HCl, which had been previously bubbled with argon. The mixture was transferred under anaerobic conditions into the sample cell. An aqueous solution of Fremy's salt ($K_2NO(SO_3)_2$) saturated with sodium carbonate was used for the calibration of the field scale of the spectrometer. Flash photolysis experiments were performed with a conventional single-beam flash spectrometer which has been described previously [2]. The data given in Fig. 7 were determined with a fixed flash energy of 676 J (8 μF, 13 kV). Sample cells of various optical path lengths were used: a 2 cm cell for the two more concentrated solutions, a 4 cm cell for the standard sample (18 μM) and a 17 cm cuvette for the three less concentrated solutions. The conversion percentages calculated upon flashing these samples were 5.7, 5.8, 5.7, 2.9, 1.5 and 0.6% respectively.

2.3. Irradiation of 5-PMA$^+$

Nine portions of 50 mg each (0.13 mmol) were successively dissolved in 100 ml of 1.0 mM phosphate buffer of pH 6; each sample was irradiated for 1 h. The samples were pooled, the buffer concentration was increased tenfold and the pH was maintained at 7. The solution was kept in the dark overnight. During this time the N_5 demethylation of the alloxazinium species took place and a precipitate of the hydrolysed products was formed. The suspension was shaken with three 50 ml portions of $CHCl_3$, the extract was concentrated and was applied to preparative precoated silica gel plates (Merck). The compounds which migrated from the start upon elution with $CHCl_3$ were pooled and extracted with chloroform. After concentration the extract was applied to a prepacked silica gel column (31 cm × 2.5 cm, Merck). The separation of the mixture was performed under a pressure of 2 atm with chloroform as eluent. The unreacted hydrolysed starting compound, i.e. 1,3-DML (first fraction), and two main products were collected, crystallized from $CHCl_3$, filtered off and washed with diethyl ether. Minor fractions, with smaller R_f values than these three, and the tarry material remaining at the start were not analysed further. The overall yield was 38% and consisted of a yellow compound I (9-hydroxy-1,3-dimethyllumichrome) (100 mg, 30%) and a bright-yellow product II (6-hydroxy-1,3-dimethyllumichrome) (30 mg, 8%). Analysis data for products I and II are given in Table 1.

2.4. Irradiation of 1,3-DML

The solubility of 1,3-DML in aqueous solution (28 μM at 20 °C) was increased twentyfold without affecting the reaction by dissolving the compound in acetonitrile and diluting the solution with aqueous HCl until it contained 20% CH_3CN and 10^{-1} M HCl. This solution (1 l) was irradiated for 48 h. The reaction mixture was extracted with three 50 ml portions of $CHCl_3$ and the products were purified according to the procedure described earlier.

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TABLE 1

Analysis data for products I and II

	Product I	Product II
Elemental analysis[a] (%)	C, 58.4; H, 4.9; N, 19.5	C, 58.3; H, 4.9; N, 19.6
m/e	286 (M^+, 100%)	286 (M^+, 100%)
	201 (M^+-85, 46%)	243 (M^+-43, 45%)
	174 (M^+-112, 59%)	174 (M^+-112, 39%)
IR (KBr) (cm^{-1})	3400	3320
UV (H_2O), λ_{max} ($\epsilon \times 10^{-3}$)	213 (21.3)	210 (24.0)
	246 (17.7) shoulder	248 (19.7) shoulder
	284 (35.3)	271 (29.6)
	374 (14.0)	281 (30.0)
		372 (17.3)
NMR (CF_3COOD)[b], δ	2.62 (3H, s, C—CH_3)	2.49 (3H, s, C—CH_3)
	2.73 (3H, s, C—CH_3)	2.67 (3H, s, C—CH_3)
	3.74 (3H, s, N_3—CH_3)	3.77 (3H, s, N_3—CH_3)
	4.02 (3H, s, N_1—CH_3)	4.01 (3H, s, N_1—CH_3)
	7.87 (1H, s, Ar—H)	7.62 (1H, s, Ar—H)
m.p. (°C)	295 - 296	268 - 268.5

[a]Calculated for $C_{14}H_{14}N_4O_3$ (286.28): C, 58.72%; H, 4.93%; N, 19.57%.
[b]For 1,3-DML, δ = 2.72 (6H, s, C_7—CH_3 and C_8—CH_3); 3.72 (3H, s, N_3—CH_3); 4.02 (3H, s, N_1—CH_3); 8.27 (2H, s, Ar—H). s ≡ singlet.

Two main photoproducts were collected and identified as 9-hydroxy-1,3-dimethyllumichrome and 6-hydroxy-1,3-dimethyllumichrome, based on m.p., elemental analysis, IR, mass spectrometric, UV and NMR data. The overall yield was 47%, consisting of 150 mg (28%) of the C_9—OH product and 100 mg (19%) of the C_6—OH isomer.

2.5. Methylation of 9-hydroxylumichrome

400 mg (1.56 mmol) of the photoproduct of lumichrome [3] was methylated according to the procedure described by Mager and Berends [6]. When the reaction was complete, which was apparent from a change of colour of the suspension from brownish to bright yellow, the solvent was evaporated under reduced pressure and the residue was washed with diethyl ether and extracted with chloroform. The extract was concentrated and applied to a prepacked silica gel column. The elution was carried out with chloroform under a pressure of 2 atm. The yield of 9-hydroxy-1,3-dimethyl-lumichrome was 330 mg (74%). The analysis data (m.p., elemental, mass spectrometric, IR, UV and NMR) of this compound were similar to those of the C_9—OH product obtained from the irradiation of 1,3-DML and 5-PMA$^+$.

2.6. Irradiation of 10-TMA$^+$

285 mg (0.8 mmol) of 10-TMA$^+$ in 200 ml of 1 M HCl was irradiated for 20 h and resulted in a conversion of approximately 90%, as estimated from the optical density at 295 nm. The solvent was evaporated under

reduced pressure and the residue was dissolved in 25 ml of 0.1 M HCl. The precipitate obtained upon addition of 0.5 ml of 1 M $HClO_4$ was filtered off and successively washed with cold water, ethanol and diethyl ether. The red crystals were dried *in vacuo* over P_2O_5. The yield of 6-hydroxy-1,3,10-trimethylalloxazinium (10-TMAO$^+$) perchlorate was 150 mg (50%); analysis data are given in Table 2.

3. Results

Experiments with alloxazinium cations in neutral or weakly acidic aqueous solution are complicated by the instability of the compounds under these conditions. The N_5 demethylation of 5-PMA$^+$ has been studied by Mager and Berends [5] in a 0.5 M phosphate buffer of pH 6.50. The reaction, as formulated by these authors, is initiated by the attack of N_5—CH$_3$ by HO$^-$, leading to an N_5-hydroxymethyl intermediate which decomposes to afford formaldehyde and the N_5-demethylated dihydroalloxazine compound. In the presence of oxygen the latter reacts further yielding H_2O_2 and 1,3,7,8-tetramethylalloxazine (1,3-dimethyllumichrome). The UV spectra of 5-PMA$^+$ (Fig. 2) and the final product 1,3-DML (Fig. 3) show the large hypsochromic shift involved. The stability of 5-PMA$^+$ could be enhanced considerably by decreasing the buffer concentration. Furthermore it was found that, under the experimental conditions with 1.0 mM phosphate buffer of pH $\leqslant$ 7, the photoreaction was much faster than the decomposition of the cation, the rate of which was approximately 10% h^{-1} as measured from the optical

TABLE 2

Analysis data for 10-TMAO$^+$

Elemental analysis[a] (%)	C, 39.9; H, 3.9; N, 14.2
m/e (field desorption method)	273 (M$^+$-99, 52%)
	272 (M$^+$-100, 100%)
	258 (M$^+$-114, 30%)
IR (KBr) (cm^{-1})	3340
UV (1 M HCl), λ_{max} ($\epsilon \times 10^{-3}$)	210 (16.3)
	255 (9.35)
	295 (23.5)
	395 (15.40)
NMR (CF$_3$COOD)[b], δ	3.69 (3H, s, N$_3$—CH$_3$)
	4.03 (3H, s, N$_1$—CH$_3$)
	4.57 (3H, s, N$_{10}$—CH$_3$)
	7.48 - 8.31 (3H, m, Ar—H)
m.p. ($^\circ$C)	>300

[a]Calculated for $C_{13}H_{13}ClN_4O_7 \cdot 1H_2O$ (390.74): C, 39.96%; H, 3.87%; N, 14.36%.
[b]For 10-TMA$^+$, δ = 3.66 (3H, s, N$_3$—CH$_3$); 4.01 (3H, s, N$_1$—CH$_3$); 4.60 (3H, s, N$_{10}$—CH$_3$); 8.15 - 8.58 (4H, m, Ar—H). s $\equiv$ singlet; m $\equiv$ multiplet.

density decrease at 450 nm. The alloxazinium cation was stable in the dark at pH $\leqslant$ 2.

3.1. Irradiation of 5-PMA$^+$

The UV absorption spectrum of an oxygen-saturated solution of 5-PMA$^+$ in a 1.0 mM phosphate buffer of pH 7 changed upon irradiation with $\lambda_{exc} \geqslant$ 450 nm from spectrum 0 into spectrum 1 (Fig. 2). After standing in the dark for several hours spectrum 1 disappeared and new maxima appeared at 374 and 283 nm. The general appearance of this spectrum strongly resembles the spectrum of the photohydrated product of lumichrome [1 - 3]. Thus the initially formed alloxazinium photoproduct is also apparently subject to N_5 demethylation, as has been observed for 5-PMA$^+$. Photochemical N_5 demethylation was found upon irradiation with $\lambda_{exc} >$ 310 nm.

Spectrum 1 changed into spectrum 2 when the pH of the solution was adjusted to a pH of 1. This spectral change is caused by an acid–base equilibrium of the photoproducts because the greater part of the initial spectrum 1 was restored upon readjustment of the pH to 7. The spectrum was distorted at 374 nm owing to N_5 demethylation of the photoproducts during this latter procedure.

Curve 3 represents the absorption spectrum of a solution of 5-PMA$^+$ which was irradiated at pH 1 (0.1 M HCl or 0.1 M HClO$_4$). Isosbestic points were observed at 512, 437, 405, 325 and 277 nm. The fact that spectra 2 and 3 are not identical indicates that there is a difference in product forma-
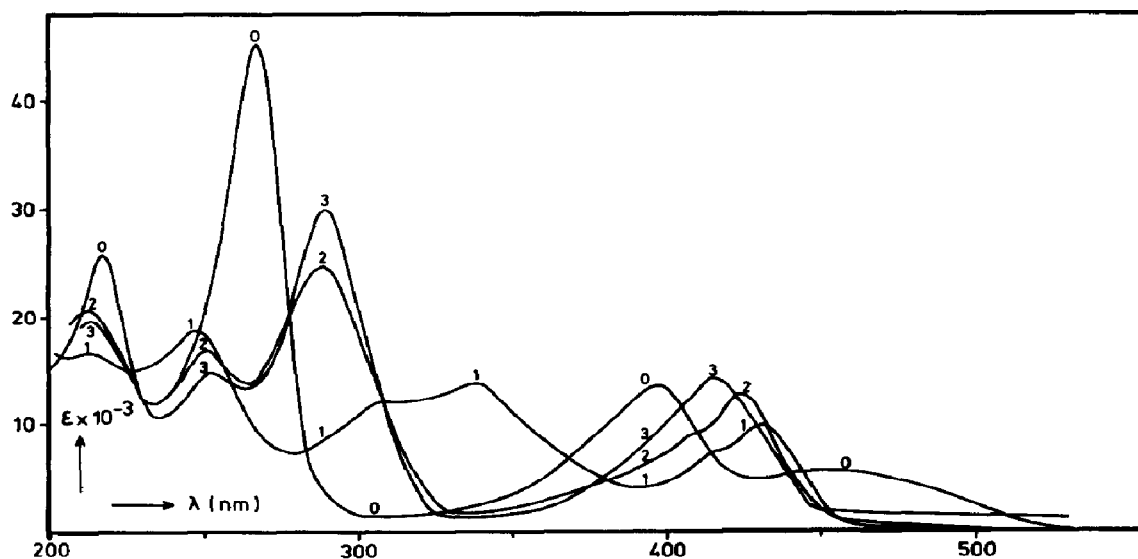

Fig. 2. The absorption spectrum of 5-PMA$^+$ in a 1.0 mM phosphate buffer of pH 7 (0), the spectrum after 30 min irradiation with $\lambda >$ 450 nm (1), the spectrum after changing the pH of the irradiation sample from 7 to 1 (2) and the spectrum of a solution of 5-PMA$^+$ irradiated at pH 1 (3).

tion at pH 7 and pH 1. However, the characteristics of the two spectra suggest a structural relation between the photoproducts formed at these pH values. These results can be explained if it is assumed that irradiation of 5-PMA$^+$ yields two isomeric hydroxyalloxazinium cations, the relative amounts being determined by the pH. The following experiments are in agreement with this interpretation. Irradiation of 5-PMA$^+$ in phosphate buffers of pH values between 7 and 5, in pure water or in aqueous $HClO_4$ at lower pH values afforded reaction mixtures showing UV spectra which were intermediate between spectra 1 and 3. Analysis of these samples by thin layer chromatography on silica gel, subsequent to N_5 demethylation at pH 7, showed two main photoproducts moving right behind the unreacted N_5-demethylated starting compound. The sample irradiated at pH 7 contained almost exclusively the fastest moving product (this appeared to be the 9-hydroxy product), whereas the product ratio of the samples irradiated at lower pH values continuously changed in favour of the slower moving compound (the 6-hydroxy product) in such a way that the latter became predominantly present in the reaction mixture of pH 1.

A significant change of the rate of photolysis, determined from the optical density decrease at 460 nm, was observed during these experiments. Taking the rate at pH 7 as a reference of 100%, the relative rates measured at the other pH values were as follows: 90% at pH 6; 80% at pH 5; 30% at pH 3 and 20% at both pH 2 and pH 1. Photochemical demethylation upon excitation with $\lambda \geqslant 450$ nm does not occur, or at least is insignificant since spectra 2 and 3 (Fig. 2) show no distortions at 355 nm (demethylated starting material) or at 375 nm (demethylated photoproducts).

Attempts to isolate the products as cationic species, by liquid chromatography on a silica gel column with a mixture of isopropanol and HCl (9:1) as eluent, failed because of excessive N_5 demethylation. Therefore we decided to separate the products in the reaction mixture only after complete N_5 demethylation in the dark at pH 7. Two main photoproducts obtained in this way were identified as 6-hydroxy-1,3,7,8-tetramethylalloxazine and the 9-hydroxy isomer. The assignment of the position of the OH group was made by comparing these isomers with the compound obtained after methylation of the photoproduct of lumichrome, which has been identified as 9-hydroxylumichrome [7]. Furthermore, the assignment is in agreement with the results of the NMR study of Grande *et al.* [8]. They have attributed the lower field signal of the aromatic proton in the NMR spectrum of (iso)-alloxazines to the C_6 proton, which is consistent with the earlier findings of Bullock and Jardetzky [9]. Consequently we concluded that the spectrum showing the aromatic proton resonance at lowest field corresponds to the C_9—OH photoproduct.

3.2. Irradiation of 1,3-DML

As a check we compared the photoproducts of 5-PMA$^+$ with those isolated after irradiation of 1,3-DML in 0.1 M HCl. Acetonitrile, which was added to increase the solubility, did not affect the photoreaction. In view of

the photohydration of lumichrome [7] and the proposed reaction mechanism [2], 1,3-DML was expected to afford the C_9—OH photoproduct exclusively. However, 6-hydroxy-1,3-DML was also formed, and in quantities comparable with those of the C_9—OH isomer. In contrast with the results of the irradiation of 5-PMA$^+$, the isomer ratio was not affected upon increasing the acidity of the solution even up to 5 M HClO$_4$. The photoproducts of 1,3-DML and those obtained from 5-PMA$^+$ after N$_5$ demethylation appeared to be identical. The UV absorption spectra of 1,3-DML and both its photoproducts are shown in Fig. 3. The spectra of the two isomers are very similar, showing the characteristic maxima for photohydrated alloxazines at 373 and 283 nm.

3.3. Methylation of 9-hydroxylumichrome

The photoproduct of lumichrome was methylated at both the N$_1$ and N$_3$ positions according to the method of Mager and Berends [6]. The methylated product proved to be identical with the C_9—OH photoproduct of 1,3-DML. Traces of the C_6—OH isomer were detected by thin layer chromatography on silica gel. The quantity of the latter product was estimated to be less than 5% of the total yield.

3.4. Irradiation of 10-TMA$^+$

The study of the dark reaction of this compound by Mager and Berends [6] has shown that neutral or weakly acidic solutions of 10-TMA$^+$ are not stable. On dissolving 10-TMA$^+$ in aqueous solutions of pH exceeding 1 a rearrangement took place, leading to a spirohydantoin. This reaction could
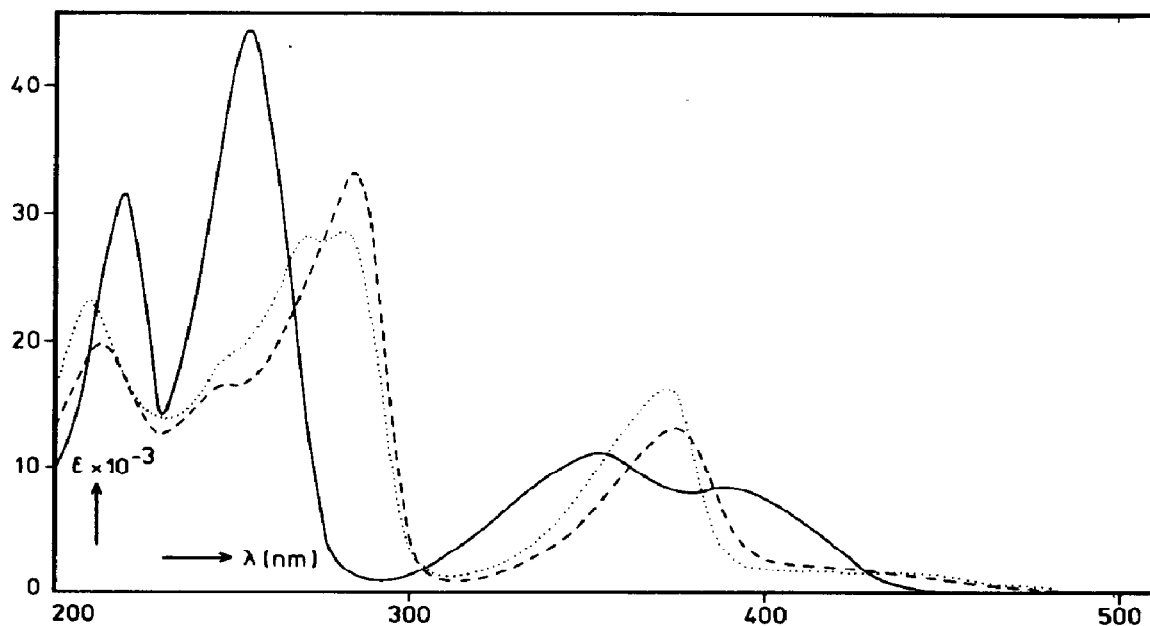

Fig. 3. Absorption spectra of 1,3-DML (——), the 9-hydroxy photoproduct (- - -) and the 6-hydroxy isomer (· · ·) in H$_2$O.

be inhibited by increasing the ionic strength of the solution, e.g. by addition of $NaClO_4$. However, the extent of inhibition appeared to be insufficient to permit irradiation experiments under neutral conditions. We irradiated $10\text{-}TMA^+$ in various acidic aqueous solutions (0.1 M HCl, 1 M HCl and 5 M $HClO_4$). Irradiation of oxygen-saturated samples of $10\text{-}TMA^+$ showed similar changes of the UV absorption spectrum in these three cases. The conversion of $10\text{-}TMA^+$ was detected by the optical density decreases at 212, 285 and 380 nm, whereas the product formation was followed at 295 and 395 nm (Fig. 4).

The spectrum obtained after complete anaerobic irradiation of $10\text{-}TMA^+$ (Fig. 4) shows that, as well as the characteristic dihydroalloxazinium absorption at approximately 300 nm [10], there is also a remarkable increase of the optical density at about 480 nm, indicating the presence of a significant quantity of cationic free radicals. The concentration of these species increased with the acidity of the solution. The reduced products were slowly oxidized upon admission of oxygen; the absorption spectrum changed and became similar to that obtained after aerobic irradiation of $10\text{-}TMA^+$. It has been reported [4] that irradiation of a similar compound, i.e. 1,3,7,8,10-penta-methylalloxazinium perchlorate, results in hydration at the C_6 position. Furthermore, it has been shown that the photoaddition site of isoalloxazines is not affected by the CH_3 substituents at the C_7 and C_8 positions. Based on these findings and the analysis of the product we assume that photohydration of $10\text{-}TMA^+$ also occurs at the C_6 position. Evidence to support this assumption is supplied by the ESR spectra of $10\text{-}TMAO^+$ (Fig. 5(a)) and $10\text{-}TMA^+$ (Fig. 5(b)). A comparison of these spectra shows that the width measured between the outer lines of the spectrum of $10\text{-}TMAO^+$ is smaller than that determined from the spectrum of $10\text{-}TMA^+$. Since this decrease of the spectral width is caused by the loss of contribution of the C_6 proton and

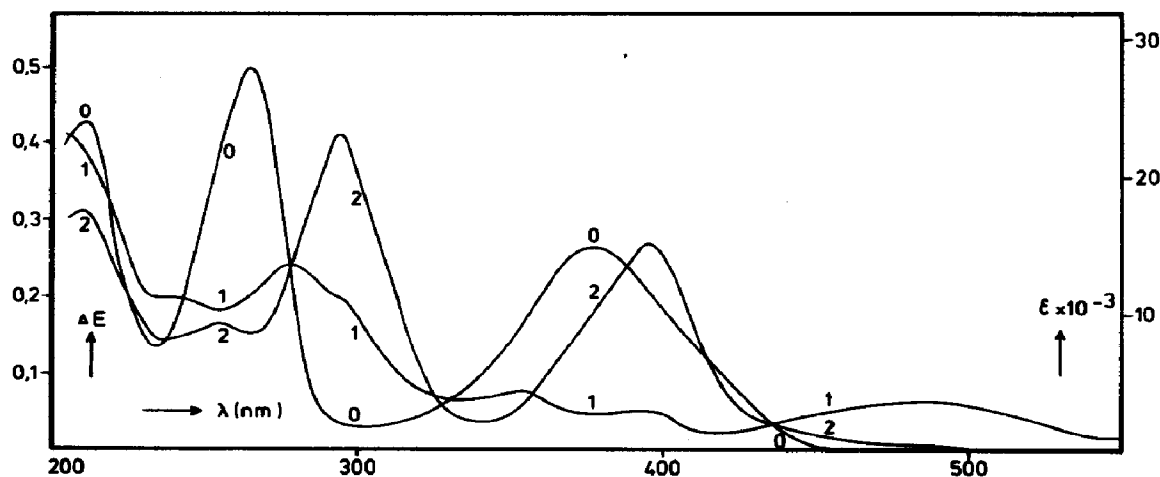Fig. 4. Absorption spectrum of $10\text{-}TMA^+$ in 5 M $HClO_4$ (0), the spectrum after 6 h anaerobic irradiation in 5 M $HClO_4$ (1) and the spectrum after 1 h aerobic irradiation or after oxidation of the anaerobic irradiated solution (2).

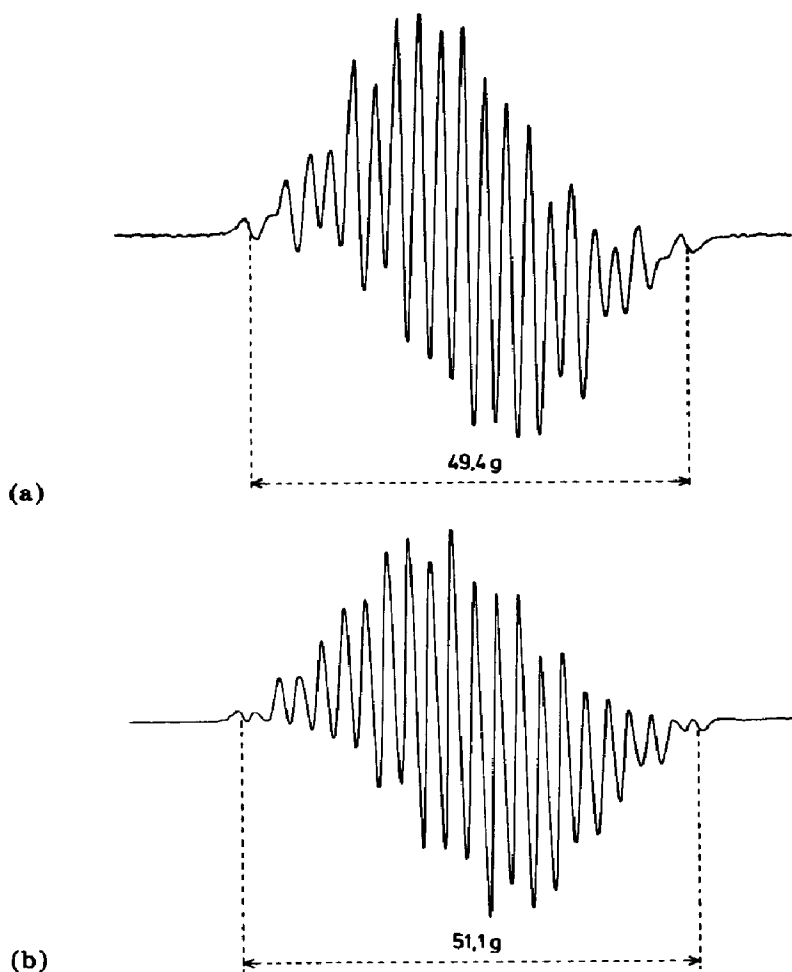Fig. 5. ESR spectra of (a) 10-TMAO$^+$ and (b) 10-TMA$^+$.

since no contribution has been found of the C_9 proton in any (iso)alloxazine radical [4], the ESR spectrum of 10-TMAO$^+$ is consistent with that expected for the C_6—OH isomer. The value of the difference is 1.7g, which is close to the data reported for C_6-photohydrated alloxazinium cations [4]. In view of these findings the coupling constant of 1.60g, which has been computed from the ESR spectrum of 10-TMA$^+$ by Westerling *et al.* [11], can probably be ascribed to the C_6 proton.

3.5. Flash photolysis of 10-TMA$^+$

The relatively high concentration of radicals present during the anaerobic irradiation of 10-TMA$^+$, estimated from the optical density at 480 nm, and the slow oxidation of these species by oxygen are indicative of their remarkable stability. This picture is distinctly different from that found for lumichrome, in which case the amount of radical species was negligible whereas the dihydro products and the oxidized photoproduct were predominantly

present during the early stage of the anaerobic irradiation [3]. This alternative reaction pattern, which does not originate from the photochemical step but rather from the subsequent dark reaction, prompted the flash photolysis study of 10-TMA$^+$.

Transient spectra generated upon flashing an oxygen-saturated solution of 10-TMA$^+$ (18 μM) are shown in Fig. 6. Analogous to the spectra observed upon flashing lumichrome [2], the first transient spectrum which appeared 100 μs after the flash is ascribed to the conversion of 10-TMA$^+$ into 10-TMAOH$_2^+$, i.e. the 6-hydroxy-1,3,10-trimethyl-1,5-dihydroalloxazinium cation. Between 440 and 370 nm this spectrum is a mirror image of the absorption spectrum of 10-TMA$^+$, which enabled us to calculate the conversion percentage after each flash. Using the calculated value of 5.7% ± 0.2% the molar absorption spectrum of 10-TMAOH$_2^+$ was determined (Fig. 6).

The shape of this spectrum, showing the characteristic maximum at 300 nm ($\epsilon = 1.3 \times 10^4$ M^{-1} cm^{-1}), is in agreement with previous reports [10, 12]. The transient reacts further with another molecule of 10-TMA$^+$, according to a pseudo first order process, giving rise to two different radical species 10-TMAH$\cdot^+$ and 10-TMAOH$\cdot^+$. The kinetics of this reaction were studied by the formation of the radicals at 480 nm. The apparent first order rate constant was measured at various concentrations of 10-TMA$^+$ and, from the slope of the plot in Fig. 7, a bimolecular rate constant of 1.6 × 10^7 M^{-1} s^{-1} was obtained. The conditions for the determination of the apparent first order rate constant are described in Section 2.
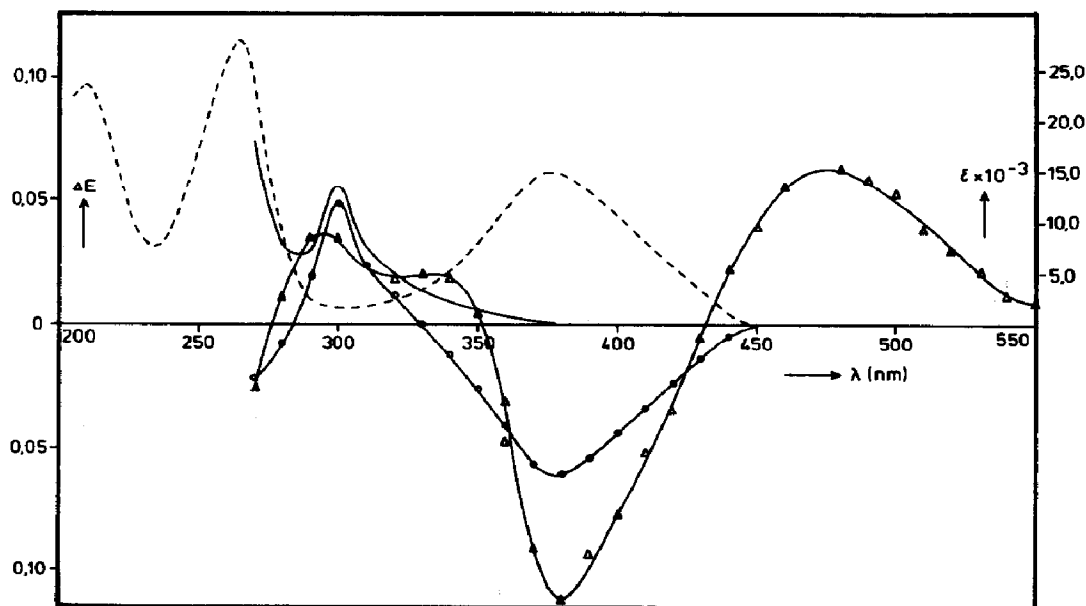

Fig. 6. The absorption spectrum of 10-TMA$^+$ in 5 M HClO$_4$ (- - -), the transient spectrum at 100 μs (O O O), the absorption spectrum of 10-TMAOH$_2^+$ (——) and the transient spectrum at 25 ms (△ △ △).
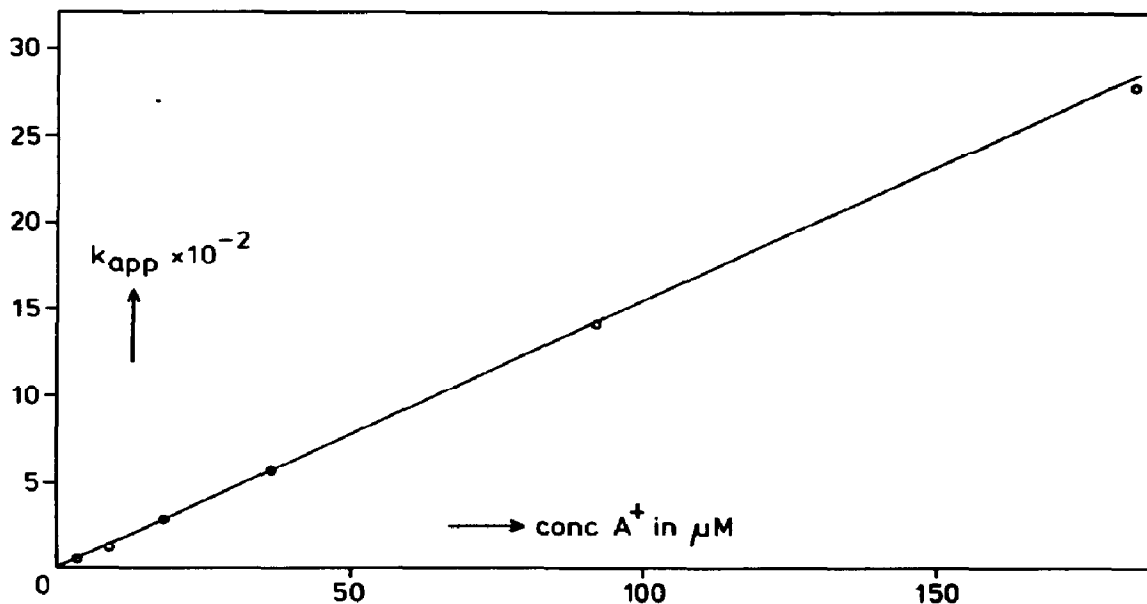
Fig. 7. A plot of the apparent first order rate constant $vs.$ the initial concentration of 10-TMA^+ for the reaction $\text{AOH}_2^+ + \text{A}^+ \rightarrow \text{AH}^{\cdot+} + \text{AOH}^{\cdot+}$ ($k_{app} = C_0 k_{bimol}$).

Thus, the long wavelength part of the transient spectrum measured 25 ms after the flash represents equimolar amounts of the two aforementioned radicals (Fig. 6). From the optical density at 480 nm and the conversion percentage of $2 \times 5.7\%$, a molar extinction coefficient of 0.75×10^4 M^{-1} cm^{-1} was estimated, assuming similar optical densities for both radical species in this area. This figure is very close to the data of Dudley et al. [12] for similar compounds and to that found for the lumichrome radical cation [3].

With this value the concentration of the radicals in the anaerobic sample after the continuous photolysis experiment (Fig. 4) was calculated to be 50% of the initial concentration of 10-TMA^+. The spectrum derived after admission of oxygen appeared to be similar to that obtained after complete irradiation of 10-TMA^+ under aerobic conditions. Thus reduced species of 10-TMA^+ are absent in the fully irradiated anaerobic sample, probably the result of small amounts of residual oxygen. Therefore the reaction mixture prior to oxidation is represented by the equilibrium

$$10\text{-TMAOH}_2^+ + 10\text{-TMAO}^+ \rightleftharpoons 2 \ 10\text{-TMAOH}^{\cdot+}$$

The concentration of 10-TMAO^+ estimated from the optical density at 395 nm is 20% of the initial concentration of 10-TMA^+; this leaves 30% of the initial concentration of 10-TMA^+ for 10-TMAOH_2^+. The value of the disproportionation constant K ($= [10\text{-TMAOH}^{\cdot+}]^2/[10\text{-TMAOH}_2^+][10\text{-TMAO}^+]$) of 4 is consistent with the observations of the flash photolysis study.

4. Discussion

The photohydration of 5-PMA$^+$ in neutral aqueous solution is in agreement with the idea that this reaction, which so far has been found to take place only in acidic solutions, proceeds via an excited singlet cation. This reactive species can be formed either by excitation of N_5- or N_{10}-substituted alloxazinium cations or by excitation and subsequent protonation of neutral (iso)alloxazines. Evidence for this concept has been obtained from a comparison of the quantum yield of product formation with that of fluorescence, both as functions of the pH [2]. Moreover, Lasser and Feitelson [13, 14] have shown that, because of the fluorescence lifetime of several nanoseconds, interaction between excited singlet (iso)alloxazines and H$^+$ ions proceeds at pH values of less than 2. Similar lifetime data have been reported by Fugate and Song [15] and by Grodowski *et al.* [16]. Such data for N_{10}-substituted alloxazinium cations have more recently been reported by Visser and Müller [17]. It appears from their work that, in aqueous solution, the fluorescence and the fluorescence lifetime of these cations both decrease considerably relative to those of the neutral species. These significant differences have been ascribed to strong interactions with the solvating water dipoles, implying an enhancement of the localized character of the positive charge upon excitation. We believe that the formation of the photohydrated products can be considered to be a result of these processes. It is estimated from the product that a substantial amount of the charge in the excited state seems to be located at the C_6 position and/or at the C_9 position.

Based on the proposed mechanism of photohydration [2], it was anticipated that photohydration of 5-PMA$^+$ in neutral aqueous solution not only would be possible but also would take place at the C_9 position as well. Indeed this was found when the experiment was carried out at pH 7. However, irradiation of 5-PMA$^+$ at slightly lower pH values also afforded the C_6—OH isomer. The procedure we used to isolate the photoproducts did not permit a quantitative determination of the yields of both products because of interfering side reactions leading to tarry material. Apart from this, the observed tendency for photohydration at C_6 is significant since the N_5 demethylations of the various samples were carried out under identical conditions.

From the relative rates for the photochemical disappearance of 5-PMA$^+$ as a function of the pH a pK value of about 4 can be estimated, although the relevance of this figure with respect to the photoreactive species involved is unknown. As has been mentioned previously, direct interaction of H$^+$ ions with the excited singlet cation is not likely in view of the fluorescence lifetime of the latter. Therefore we assume that the protons possibly induce certain modifications in the structure of the solvating water molecules, which may cause a change of the site and the rate of photohydration. A still more curious effect of the acidity on the photoaddition site has been reported for 1:10-ethanoflavinium perchlorate [4].

The complexity of the photohydration with respect to the parameters which are determinant for the addition site is further demonstrated by com-

paring the results of lumichrome and 1,3-DML. Lumichrome was found to be quantitatively photohydrated at the C_9 position [7], whereas irradiation of 1,3-DML afforded both isomeric products. Apparently this is caused by the CH_3 substitutent at N_1, since methylation of 10-methyl-isoalloxazine at N_3 has no effect on the photoaddition site [4]. Furthermore, it seems that photohydration of 3-methyllumiflavin occurs at the C_9 position, whereas the C_6—OH photoproduct is obtained from the N_1-methylated derivative of 3-methyllumiflavin. It has been suggested that photoaddition at the 6 position would be favoured with *peri*-overcrowding at N_1/N_{10} [4]. The result of the irradiation of 1,3-DML (unsubstituted at N_{10}) does not support this suggestion; it indicates rather that the photoaddition site may depend on the substituent at N_1. From these results it is clear that the actual mechanism of photohydration remains unsolved. The reason for this lies in the lack of knowledge of the properties of the excited singlet (iso)alloxazines. However, this study emphasizes the importance of the excited singlet cation in this process, irrespective of the way it is formed.

Similar to the results found for lumichrome [2], the first stable product upon flashing 10-TMA$^+$ is the hydroxydihydroalloxazinium cation 10-TMAOH$_2{}^+$. The oxidation of this species by the parent compound is a one-electron process. This is different from the corresponding reduction of lumichrome which is a diffusion-controlled two-electron reaction. This discrepancy arises from the stability of the radicals, *i.e.* the two species formed after transfer of one electron from 10-TMAOH$_2{}^+$ to 10-TMA$^+$ do not react further but instead diffuse away from each other. In the case of lumichrome, the kinetic data [2] were consistent with transfer of two electrons between the reactants prior to escape of the products from the solvent cage. This scheme is in agreement with the previously reported diffusion-controlled rate constant for the disproportionation of lumichrome radicals [3].

Acknowledgments

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