Formation and Possible Structure of Covalent Hydrates of Alloxazines

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A slow and reversible hydration of N(1)-unsubstituted alloxazines in aqueous alkaline solutions has been studied. Reaction products stable (kinetically) in neutral and acidic solutions were isolated and investigated by IR, NMR, UV and mass spectroscopy. The possible mechanism of the reaction and the structure of the isolated products is discussed.

In the course of a study on spectral behaviour of alloxazine and its methylated derivatives light absorption spectra and apparent pKa values of individual ionic species have been evaluated *. Alloxazine, lumichrome and 3-methylumichrome in aqueous alkaline solutions undergo a slow base-catalysed reaction which manifests by evident changes in the light absorption spectra (Fig. 1), while 1-methylumichrome is stable towards alkaline solutions.

The pKa are 8.1 and 12.6 for alloxazine, 8.23 and 12.82 for lumichrome (7,8-dimethylalloxazine), 8.66 for 1-methylumichrome and 8.71 for 3-methylumichrome. The latter pK values are in good agreement with data obtained independently by MüLLER and DUDLEY.

The reaction does not proceed in methanolic KOH or sodium methoxide. The position of equilibrium between alloxazine or lumichrome and their products depends on the base concentration. However, the equilibrium between 3-methylumichrome and its product does not depend on the base concentration. The reactions are not reversed upon acidification of the reaction mixture.

The reaction products have been isolated by means of preparative TLC on silicagel (they are more polar than the starting compounds) or continuous extraction of the starting materials with chloroform from the neutralised reaction mixtures.

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The isolated crystalline derivatives of lumichrome and 3-methylumichrome decompose without sharp melting points and are sparingly soluble in water and in commonly used organic solvents. Both products are reasonably soluble in acetic acid and its mixtures with organic solvents.

The absorption spectra of the isolated products are shown in Fig. 2. The calculated apparent pKa values are 2.40 and 0.11 for the lumichrome derivative, 2.27 and 0.05 for the 3-methylumichrome derivative. It appears from the pH dependence of the light absorption spectra and fluorescence properties as well as electrophoretic mobility of the product that in neutral pH an anionic, in slightly acidic pH a neutral and in concentrated acids (6 N HCl) a monocationic form is present. In aqueous alkaline solutions both products undergo a base catalysed, slow reverse reaction. The position of the equilibrium depends on base concentration. The reaction is completely reversed in about 2 N NaOH. This is true for the lumichrome derivative only. With 3-methylumichrome always a mixture of starting material and product is found upon acidification of an alkaline solution of the product.

The IR spectra of both products show similar features. These spectra differ from those of the parent compounds as follows: a new band at 3600 cm⁻¹ (OH or NH) is observed, a NH band is extrapolated to zero time (for spectra which changed with time) were obtained according to methods developed by Metzler and Johnson.

* Machine computation of pKa values, spectra of individual ionic species, corrected absorption spectra, absorption spectra extrapolated to zero time (for spectra which changed with time) were obtained according to methods developed by Metzler and Johnson.
Fig. 1. Changes in the absorption spectra of alloxazine, lumichrome (Lc), and 3-methyllumichrome (Lc3M) in 0.1 M aqueous NaOH solution. On each curve is indicated the time interval between the moment when the aqueous solutions of alloxazines were mixed with NaOH solutions and the start of recording at 20 kK (the average recording time from 20 kK to 35 kK was about 3.5 min). The concentration of alloxazines was $2.66 \times 10^{-6}$ M, light path 10 cm. For comparison spectra of each compound in water are shown.

shifted to higher frequencies (from 3150 cm$^{-1}$ to 3250 cm$^{-1}$), the carbonyl band at higher frequencies disappeared or is bathochromically shifted (only one band at about 1695 cm$^{-1}$ is present) and a new band at 1130 cm$^{-1}$ (tertiary cyclic OH) has been found (all spectra were obtained using KBr pellet technique).

In deuterated pyridine, to which a small amount of water was added, a well resolved nuclear magnetic resonance spectrum of the 3-methyllumichrome derivative has been obtained with absorptions at 2.28, 2.32, 3.10 – 3.14 (doublet), 7.46, 8.01, 8.89 (broad multiplet) and at 12.20 ppm, which are assigned to CCH$_3$(7), CCH$_3$(8), NCH$_3$(3), C–H(9), C–H(6), N–H(1) and probably a carboxylic proton, respectively.

Addition of a small volume of $^2$H$_2$O to the sample causes the disappearance of the broad band at 8.89 ppm, conversion of the doublet at 3.10 – 3.14 ppm into a singlet at 3.12 ppm (without change in the integrated area) and disappearance of the absorption at 12.20 ppm. Due to the low solubility of the lumichrome product in organic solvents no spectra could be obtained for it in pyridine and those recorded in deuterated chloroform with small addition of trifluoro-acetic acid did not reveal any useful information concerning the location of the exchangeable protons.

No unambiguous results have been obtained from mass spectrometry, because no parent peaks could be recognized. The decomposition patterns are quite similar for both products and show a sequence of several peak values higher by 2 m/e than those observed for the parent compounds (indicating most probably substitution of a nitrogen atom by oxygen atom in particular steps of decomposition).

Taking into account all the above listed results of preliminary observations and measurements, the lack of reactivity of the products towards carbonyl reagents, the resistance towards oxidizing agents such as H$_2$O$_2$ and KMnO$_4$, the easy reduction with common reducing agents leading to formation of new fluorescent products of unknown structure...
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(derivatives of 6,7-dimethyl-quinoxaline), the difference between the characteristic properties of both isolated products and the comparison with described derivatives resulting from alkaline hydrolysis of isoalloxazines\textsuperscript{3-6} and 1,3-dimethylalloxazine\textsuperscript{7} we propose that monoanions of alloxazines unsubstituted at N-1 undergo covalent hydration most probably across the double bond between N-1 and C-2 according to the following Scheme:

Scheme 1.

The nuclear magnetic resonance spectrum of the 3-methyllumichrome product in pyridine indicates the existence of a structure with open pyrimidine ring (—NH–CH\textsubscript{3} residue) and a carboxylic group. It is possible that such a structure exists only in pyridine (stabilisation of a —NH–COOH residue towards decarboxylation) or that the ring opening leads to the formation of a relatively stable cyclic structure, as suggested recently by P. Hemmerich\textsuperscript{8}.

Additionally we have established that the decomposition of 1,3-dimethylumichrome in aqueous alkaline solutions yields 2-methylamino-6,7-dimethylquinoxaline-3N-methylcarboxamide as described by Brederreck and Pfeiderer\textsuperscript{7}, and proceeds via at least two steps. The first step (slowly reversible after neutralisation) results in the formation of an intermediate with a similar structure as proposed for lumichrome and 3-methylumichrome hydrates (apparently identical absorption spectra).

Work is in progress for further elucidation of the reaction mechanism, structure and properties of covalent hydrates of alloxazines.

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