Tetrahedron Letters No. 37, pp 3545 - 3548.

0040-4039/79/0908-3545$02.00/0

ACTIVATION AND TRANSFER OF OXYGEN XIV. THE SUCCESSIVE ACCUMULATION OF A 10^A-HYDROXY PSEUDOBASE AND AN ESR-SILENT BLUE ISOMER IN THE N^1-ALKYLFLAVIN MODEL SERIES.

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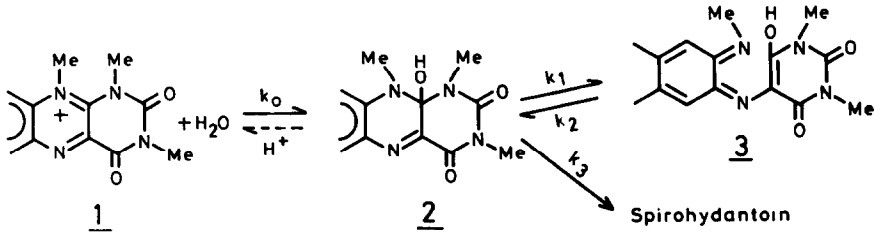
Summary: Kinetic studies have substanciated the idea that the 10^a-hydroxy pseudobase 2 is in equilibrium with a blue transient for which structure 3 has been proposed. The rate-constants and the molar absorbances of the intermediates were estimated.

In 1966 we found that the blue compound, produced in the autoxidation of 1,3,10-trimethyl-5,10-dihydroalloxazine in non-polar solutions, is finally converted into a C^{4a}-spiro-hydantoin.[1]

The nucleophilic trapping reactions in the N^1-alkylflavin series[2-4] put the primary 10^a adduct formation beyond doubt as well as the fact that the primary adducts have absorption maxima at 410-425 nm. This implies that Hemmerich[5,6] has incorrectly assigned the 10^a-adduct structure to ESR-silent flavin transients absorbing at about 610 nm. Alternatively, blue transients have been proposed[3,4] to be 10,10^a-ring opened isomers of 10^a-adducts.

We have now established that blue compounds can also be produced in non-oxidative processes from alloxazinium cations in dependence on the nature of the medium and the nature of the anions present. Good starting materials were found in new salts of the 1,3,10-trimethyl-alloxazinium cation 1, e.g. the benzoate and the trifluoroacetate.

In a fresh solution of the trifluoroacetate in water, the cation 1 is the main species (Fig. 1). The conversion into the spirohydantoin is illustrated by the repititive scan study. The intermediacy of the 10^a-hydroxy pseudobase 2 could not be detected. Fluorescence measurements only showed an emission maximum at about 500 nm with an excitation maximum at 360-370 nm, due to 1. Apparently, the pseudobase formation is the rate limiting step in the disappearance of the cation in aqueous solution. This is in contrast with the behaviour in organic solvents. Due to a retarded spirohydantoin formation ($k_3 < k_1$) a rapid conversion into the pseudobase 2 and a blue transient 3 is observed when 1 is dissolved in organic solvents containing limited amounts



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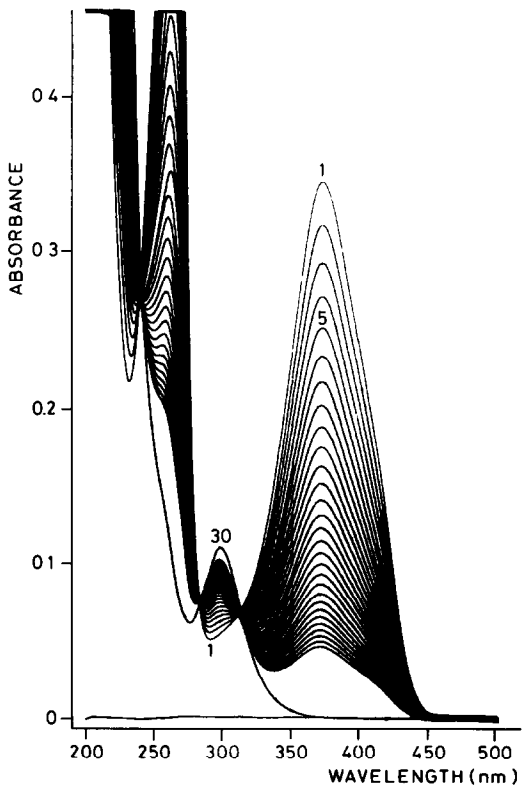

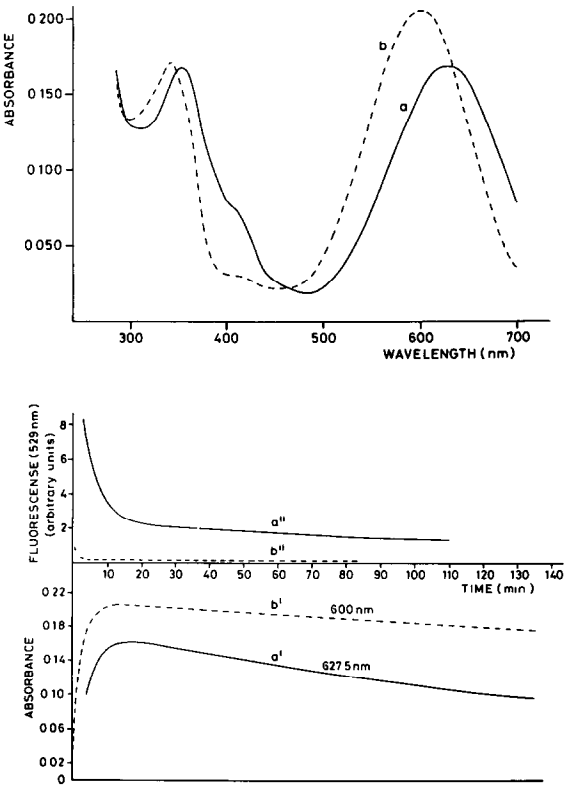

Fig. 1. Repititive scan of 1,3,10-trimethylalloxazinium trifluoroacetate (2.56 x 10^{-5}M) in H_2O, at 22°. λ_{max}= 370 nm; time interval of 230 sec. The maximum of curve 1 was taken 4'29" after dissolving. Isosbestic points at 242, 285 and 314.5 nm.

Fig. 2. Maximal blue colouring of 1,3,10-trimethylalloxazinium trifluoroacetate (2.56 x 10^{-5}M) in H_2O-saturated $CHCl_3$ (—— a) and in H_2O-saturated benzene (---b), at 22°. λ_{max}= 349, 410^{sh} and 627.5 nm (H_2O-saturated $CHCl_3$); λ_{max}= 342, 410^{sh} and 600 nm (H_2O-saturated benzene).

Fig. 3. Absorbance (—— a'; --- b') and fluorescence (—— a"; --- b") versus time of 1,3,10-trimethylalloxazinium trifluoroacetate (2.56 x 10^{-5}M) in H_2O-saturated $CHCl_3$ (—— a', a") and in H_2O-saturated benzene (---- b', b").

of water. It is emphasized that never before experimental conditions have been found to give a detectable presence of the 10^a-hydroxy pseudobase 2. *The blue isomer 3 is an indicator for the 10^a-adduct 2.*

This scheme is supported by quantitative studies:

a) Intensively blue coloured solutions are obtained just by dissolving the trifluoroacetate in water-saturated toluene, benzene, ethyl acetate, diethyl ether and chloroform as illustrated by the curves a and b in Fig. 2. The spectra were taken at the moment that a maximal blue colouring was obtained as shown by the time course curves a' and b' in Fig. 3.

b) The simultaneous presence of the 10^a-hydroxy pseudobase 2 is shown by the absorption shoulder at 410 nm, but in particular by the fluorescence spectrum. In water-saturated chloroform, the pseudobase has an emission maximum at 529 nm (note[7]) with an excitation maximum

at 410 nm (ϕ_f= 0.11). In the same medium (containing 1% TFA), the cation has an emission and excitation maximum at 475 nm and 375 nm (ϕ_f= 6.5 x 10^{-3}), respectively. In 1N sulfuric acid, the cation shows an emission maximum at 500 nm (ϕ_f= 9.0 x 10^{-4}), illustrating the influence of the solvent. In water-saturated chloroform, no fluorescence is given by the blue transient.

c) From the time course fluorescence curve a" it appears that the pseudobase is immediately accumulated prior to the accumulation of the blue isomer. This means that k_o is very large.

The time course curve a', monitored at λ_{max}= 627.5 nm, first shows the increase of the blue intermediate with the time and a subsequent slow decrease after about 18 minutes. This is consistent with the biphasic decrease of the pseudobase concentration as demonstrated by the time course fluorescence curve a" (λ_{exc}= 410 nm).

During the second stage of the process, which is controlled by the irreversible formation of the spirohydantoin, both the absorption at 627.5 nm and the fluorescence decrease according to a first-order rate-constant, for which the consistent values were found of 7.57 x 10^{-5} sec^{-1} and 7.62 x 10^{-5} sec^{-1} (22°), respectively.

The concentration of the pseudobase and the blue transient (BT) in dependence on the time can be represented by Eqs (1) and (2), provided that $k_0 \gg k_1$, k_2, k_3 and $\frac{4k_2k_3}{(k_1+k_2+k_3)^2} \ll 1$

$$\left[RF1OH\right] = \frac{k_1+k_3}{a_1} \cdot \left[RF1^+_{ox}\right]_o \cdot e^{-a_1 t} + \frac{k_2}{a_1}\left[RF1^+_{ox}\right]_o \cdot e^{-\frac{a_2}{a_1}t} \quad \text{--------(1);}$$

$$\left[BT\right] = \frac{k_1+k_3}{a_1} \cdot \left[RF1^+_{ox}\right]_o \cdot (e^{-\frac{a_2}{a_1}t} - e^{-a_1 t}) \quad \text{----------(2)}$$

(a_1= $k_1+k_2+k_3$ and a_2= k_2k_3).

From the time course curve a", the rate-constants in water-saturated chloroform (22°) were calculated to be: k_1= 2.32 x 10^{-3} sec^{-1}; k_2= 5.19 x 10^{-4} sec^{-1}; k_3= 4.88 x 10^{-4} sec^{-1}, giving an equilibrium constant of $K = \frac{k_1}{k_2}$ = 4.47.

From correlating the concentration values calculated from Eq (2) to the extinction values found in the time range of 15-300 minutes (curve a'), the molecular extinction for the blue transient in water-saturated chloroform at λ_{max}= 627.5 nm was calculated to be about 8200.

Both the pseudobase and the blue transient contribute to the decreasing absorbance at 410 nm. From correlating the extinction values of a time course absorption curve taken at 410 nm to the concentrations calculated from Eqs (1) and (2), the molecular extinction for the 10^a-hydroxy pseudobase in water-saturated chloroform was found to be about 6400. For comparison, the 10^a-methoxy adduct derived from the cation 1 has an absorption maximum at 413 nm in methanol[2] with a molecular extinction of 4300.

In water-saturated benzene, the blue compound is even more rapidly formed and less rapidly converted as is illustrated by the absorbance and fluorescence curves b, b' and b" The equilibrium is then shifted more to the side of the blue compound.

In the way as mentioned above, the molecular extinction of the blue compound in benzene

at λ_{max} = 600 nm was calculated to be about 8600.

The optimal blue colouring in water-saturated chloroform (cf. curve a, Fig. 2) corresponds with a maximum accumulation of 81%, while curve b (Fig. 2) shows that in benzene the blue compound has been accumulated up to 93%.

The oxidative formation of the blue intermediate was found[8] to be accelerated by dibenzoylperoxide. The stoichiometric requirement for dibenzoylperoxide was determined by Hemmerich[6] and Müller[9], who arrived at contradicting conclusions. On the basis of the molecular extinction values mentioned above, the maximal blue colouring reported[6] to have arisen in the oxidation of a dihydroalloxazine by dibenzoylperoxide and O_2, is now calculated to have been in the order of 50%.

Since a blue intermediate is an indicator for a 10^a-adduct, it is questioned whether the appearance of a non-radical blue flavin transient in bacterial bioluminescence[10] indicates that a 4^a-hydroperoxy-dihydroflavin is undergoing a HOO^- shift from C^{4a} to the more active 10^a-position.

Evidence for the transient opening of the pyrazine ring will be presented in part XV of these series.

Acknowledgement— We are very much indebted to Mr. T. de Beer for his experimental assistance and to Ir. R.H. Dekker for a helpful suggestion.

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7. Note: All fluorescence excitation and emission data are corrected for the lamp output and the relative spectral sensitivity, respectively. The quantum yields were determined relative to quinine in 1N H_2SO_4 (λ_{exc}= 365 nm; ϕ_f= 0.55).
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(Received in UK 17 May 1979)