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pH DEPENDENCE OF THE TRANSIENT ABSORPTIONS IN THE FLASH PHOTOLYSIS OF 3-METHYLLUMIFLAVIN

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SUMMARY

1. 3-Methylumiflavin has been studied by flash photolysis in aqueous buffers. Transient absorption spectra have been recorded at pH values varying from 3.1 to 8.8.

2. It is concluded that: (a) The photoflash creates at least two different transient species. (b) One of these transient species, with an absorption maximum at 380–390 nm, has a structure and/or decay that is affected by pH.

INTRODUCTION

Photo-excited flavins can oxidize a great variety of organic substances, among which NADH¹, amino acids² and nucleotides³. Such reactions provide convenient *in vitro* models for the enzyme-catalysed oxidations which flavins perform *in vivo*. Besides, the photo-reactions of flavins might be of interest by themselves, because of their role in photosynthetic electron transport^{4, 5}.

One aspect of the photoreduction of flavins, which is not well understood, is the influence of pH on the reaction rate as reported by PENZER AND RADDA² and PENZER⁶. Because the course and the rate of a photoreaction are often determined by the properties of the intermediate state(s), we undertook a direct study of the excited molecule (or its transient photoproduct) in media of different pH. For this we used the flash photolysis technique.

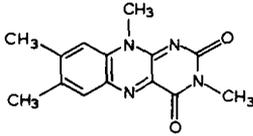
MATERIALS AND METHODS

Materials

3-Methylumiflavin (3,6,7,9-tetramethylisoalloxazine, I), was a generous gift from Prof. P. Hemmerich in Konstanz. It has the same optical properties as FMN or riboflavin, but is much more stable. We used it without further purification.

The following buffers were used: glycine + HCl, pH < 4.0; sodium acetate + acetic acid, pH 4.0–5.5; K₂HPO₄ + KH₂PO₄, pH 5.5–7.5; H₃BO₃ + KOH, pH > 7.5. The buffer concentration was 0.1 M throughout the experiments. All reagents were of *pro analysis* grade. Doubly distilled, deionised water was used throughout.

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Stationary spectra

In order to determine the flavin concn. (ϵ at 440 nm 11500 moles. cm^{-1} . l^{-1} , see ref. 7) and the extent of photodestruction, stationary spectra were recorded on a Cary-14 spectrophotometer before and after each experiment.

Preparation of cells

We used a cylindrical quartz cell with an optical path length of 8.7 cm. A cylindrical quartz cuvette with a path length of 1 cm was attached to the cell by means of a small side-arm. In this cuvette the stationary spectrum could be measured.

O_2 was removed from the cell by the following procedure: The cell was connected by glass tubing to two glass bulbs, and the whole system was attached to a high-vacuum line. The solution was then degassed in one of the bulbs by repeated cycles of freezing, pumping and thawing, and afterwards the solvent water was distilled into the other bulb, frozen, pumped, and distilled back. Finally, the solution was poured into the cell and the cell sealed off.

In this way, an air pressure of less than 10^{-5} mm Hg was obtained, corresponding to an O_2 concentration in the solution of less than 10^{-12} M.

Temperature

All experiments were conducted at room temperature.

Flash apparatus

The cylindrical flashlamp, which had a flash duration of about 5 μsec , produced at a mean energy of about 360 J about $2 \cdot 10^{18}$ light quanta in the region above 380 nm (26000 cm^{-1}). A cylindrical plastic filter around the cell was used to cut off all light below 380 nm.

Two different detection systems were available:

(1) The spectrophotographic method producing at a fixed time delay in one shot a survey of the whole spectrum. This was achieved by means of a spectroflash triggered by the photoflash, and a Hilger quartz-medium spectrograph. The spectra were recorded on Kodak High Speed Infrared 35 mm film, these photographs were traced on a Kipp DD2 densitometer, and the densities were converted to light intensities by means of film-sensitivity curves. As a check several experiments were repeated on Kodak Tri-X Pan film, because High Speed Infrared film has a different sensitivity above and below 370 nm.

(2) The kinetic photorecording method giving the absorption at one wavelength as a function of time. As detection system we used a Zeiss MM 12 double monochromator and the photomultipliers EMI 9558 Q and 9684 A. The electric signal was displayed on an oscilloscope and photographed.

RESULTS

General appearance of the transient spectra

Fig. 1 shows an example of the time dependence of the optical absorption as caused by the photoflash on a pH 5.3 solution. Upon flash photolysis the permanent absorption at 446 nm (22400 cm^{-1}) is bleached (causing a negative change in absorption), while new maxima appear around 295 nm (34000 cm^{-1}), 650 nm (15000 cm^{-1}) and in the region of 480–580 nm ($21000\text{--}18000\text{ cm}^{-1}$).

Between 350 and 400 nm (29000 and 25000 cm^{-1}) the situation is somewhat complicated. After flash photolysis the second absorption band of the flavin at 370 nm should be bleached like the first absorption band at 446 nm, causing likewise a negative change in absorption in the difference spectrum (which compares the situation before and after the flash). At 6 μsec after the flash, however, we observe a temporary increase in absorption, with a maximum around 390 nm (26000 cm^{-1}). This indicates that the photoflash creates a fairly strong transient absorption in this region. At $\Delta t = 50\text{ }\mu\text{sec}$ the difference spectrum shows a negative change in absorption, indicating that the decay of the transient at 390 nm goes faster than the recovery of the ground-state flavin molecules.

Except for a small permanent change, over a long time all absorption changes go to zero. (Photodestruction was less than 1% per flash as determined from the stationary absorption at 440 nm).

We checked if there was any detectable transient absorption in the infrared part of the spectrum at pH 3.1 and 6.9. Because of the lack of film sensitivity at these wavelengths, the observations were made point by point with the kinetic set-up. In the region investigated (800–1100 nm, $12500\text{--}9000\text{ cm}^{-1}$) the results were all negative.

pH effects

Fig. 2 shows the change in absorption of a flavin solution in buffers of different

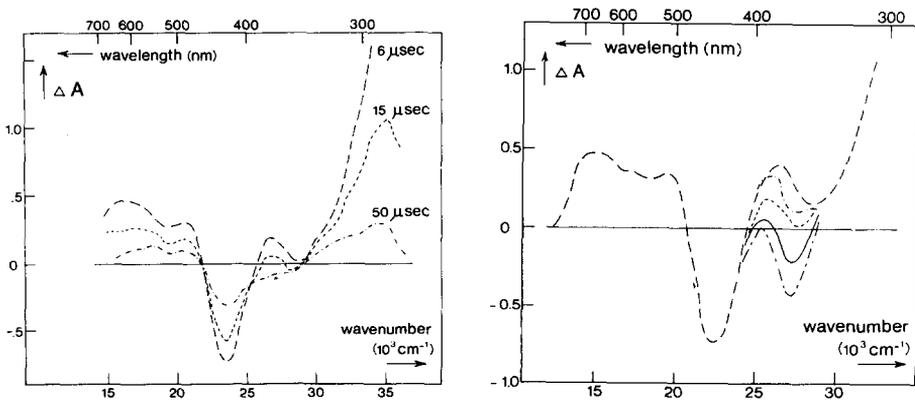


Fig. 1. Transient change in absorption at different time intervals after the photoflash. $25\text{ }\mu\text{M}$ 3-methylumiflavin in 0.1 M acetate buffer, pH 5.3.

Fig. 2. Uncorrected transient change in absorption of $25\text{ }\mu\text{M}$ 3-methylumiflavin in buffers of various pH, at 6 μsec after the photoflash. The spectrum at pH 7.5 is shown completely. The changes in the spectra at the other pH values are shown only in the region where they differ appreciably from that at pH 7.5: —, pH 7.5; - · - · -, pH 8.8; - - - - -, pH 5.3; ———, pH 4.0; - - - - -, pH 3.1.

pH at $\Delta t = 6 \mu\text{sec}$ after the flash. Changing the pH affects the spectrum mainly in the region from 360 to 400 nm (28000 to 25000 cm^{-1}): the absorption of the band at 380–390 nm increases with increasing pH.

Kinetic measurements

In Fig. 3 the transient absorption at different wavelengths is given as a function of time.

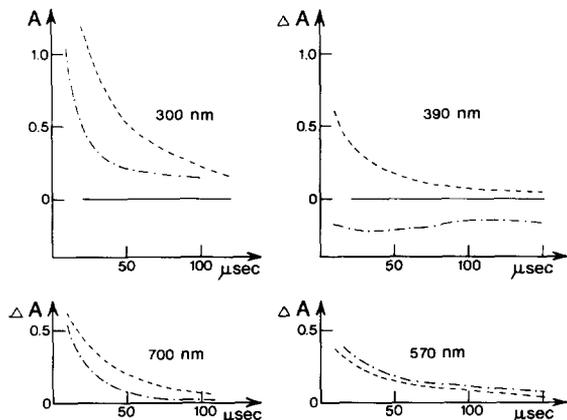


Fig. 3. Transient change in absorption of $25 \mu\text{M}$ 3-methylumiflavin as a function of time, at several wavelengths. -----, pH 8.8; - · - · -, pH 3.1.

At 300 nm (34000 cm^{-1}), 570 nm (18000 cm^{-1}) and 700 nm (14000 cm^{-1}), the decay stays roughly the same throughout the pH range studied.

At every pH, the transient absorption at 570 nm (18000 cm^{-1}) decays several times slower than that at 700 nm (14000 cm^{-1}). We feel that this points to the presence of at least two different transient species.

The behaviour at 390 nm (26000 cm^{-1}) varies with pH. At pH 8.8 there is a transient absorption lasting more than 100 μsec (it decays faster than the transient at 570 nm, but slower than that at 700 nm). When the pH is lowered, the transient absorption in this region becomes smaller and lasts shorter; at about 50 μsec after the flash it even becomes negative. This shows that the decay of this transient goes faster than the total recovery of the ground-state molecules. At pH 3.1 the transient absorption has become so weak that it is overwhelmed by the decrease of the stationary flavin band around 370 nm; the net result is a negative change in absorption.

Analysis of the kinetics showed no clear-cut first- or second-order behaviour at any wavelength.

In acid media the transient absorption at 300 nm had a "tail" that took more than 10 msec to go back to zero. The phenomenon was clearly visible in glycine-HCl buffer at pH 3.1 and in acetate at pH 4.0, and less clearly in acetate at pH 5.3. The addition of $25 \mu\text{M}$ KI to a pH 4.0 solution left this "tail" more or less unchanged. In all these cases the same tail could be recognised in the decrease in absorption at 446 nm and 370 nm; evidently it took more than 200 msec for all flavin molecules to return to their ground state. We did not investigate this very long-lived acid transient any further.

Calculation of the pure transient spectra

The difference spectra compare the situation before the flash, where all flavin molecules are in their ground state, with the situation just after the flash, where some of the flavin molecules have been excited into metastable transient states and are now absorbing at other wavelengths; the background of ground-state absorption has now become proportionally less. If we want to obtain the pure absorption spectrum of the transients, then we must correct the difference spectra for this decrease in ground-state absorption.

In order to get some idea of what the pure transient spectrum should look like, we flashed at different energies a cell with a flavin concentration of 10 μM , (in 0.1 M phosphate buffer, pH 6.9), which was considerably lower than that used in other experiments. When we increased the flash energy from 360 to 600 J or 1000 J, the transient absorptions did not increase much. This indicates, that a 360-J flash produced already enough quanta to excite most of the molecules into some metastable state. We therefore added the ground-state absorption of these molecules to the difference spectrum, and thus obtained an idea of what the pure transient spectrum should look like; it appeared that the pure transient absorption at 440 nm is about equal to the transient absorption around 570 nm, where the ground-state does not absorb.

With this in mind, all other difference spectra can be corrected for the decrease in ground-state absorption, and in this way spectra can be obtained which show absorption of the transient species only.

For this, we used the following method:

(1) The pure transient absorption at 440 nm is about equal to the measured transient absorption at 570 nm. By adding this absorption to the decrease in absorption in the difference spectrum at 440 nm (*cf.* Fig. 2), an estimate could be made of the fraction of flavin molecules that had not yet returned to the ground state at $\Delta t = 6 \mu\text{sec}$.

(2) Before the flash experiment, the initial optical absorption was measured. This spectrum was multiplied by a correction factor so as to make it correspond to the decrease in absorption that the fraction of flavin molecules estimated in (1) would cause in an 8.7 cm cell.

(3) We added this background-correction spectrum to the difference spectrum. In order to relate the experiments at different pH to the same concentration of excited molecules, we divided the resulting spectrum by the true decrease in absorption at 440 nm as estimated in (1).

The method just described may introduce quite a few errors. However, the general appearance of the corrected spectra was not too sensitive to changes in the correction factor, and the effect of pH on the 390 nm-band remained clear whatever the correction.

Fig. 4 shows the result for several pH values. It is to be noted that the result obtained at high pH agrees quite well with the data of KNOWLES AND ROE⁸.

Effect of triplet quenchers

The influence of Cu^{2+} , a typical triplet quencher, was investigated at pH 5.2. At pH 6.9, where Cu^{2+} is insoluble, we investigated the influence of O_2 , which is also paramagnetic. Both 10^{-3} M Cu^{2+} and O_2 at atmospheric pressure (corresponding to a concentration of 250 μM) quenched all transients. At every wavelength the transient

absorption at $t = 6 \mu\text{sec}$ was much lower than without added quenchers, and at $t = 15 \mu\text{sec}$ there was little or no transient absorption left.

The influence of $25 \mu\text{M}$ KI was investigated by the kinetic method at pH 4.0. In this case, both the increase in absorption at 300, 570 and 700 nm and the decrease at 446 and 370 nm decayed much faster than without added I^- .

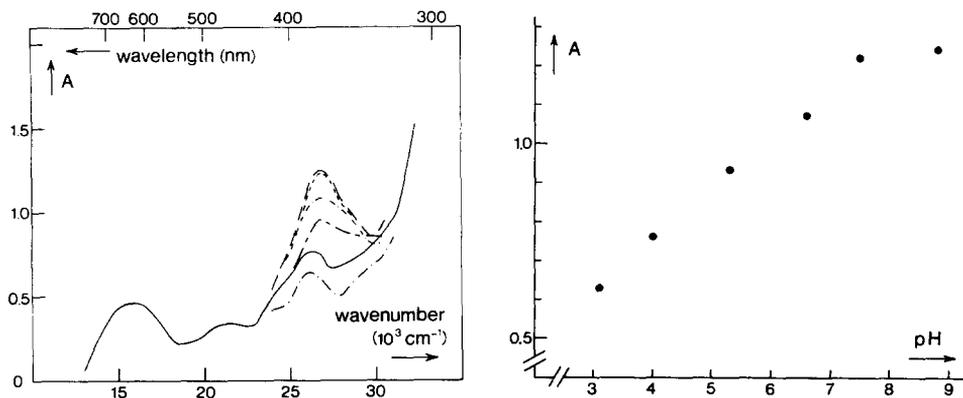


Fig. 4. Absorption spectrum of the pure transient species, at various pH values. Only one spectrum (pH 4.0) is shown completely; the other spectra are only shown in so far as they differ from the spectrum at pH 4.0. —, pH 7.5; - - - - -, pH 8.8; ······, pH 6.6; - · - ·, pH 5.3; ———, pH 4.0; — · —, pH 3.1.

Fig. 5. Pure transient absorption at 380 nm as a function of pH.

DISCUSSION

From the data mentioned above it follows that at least two different transient species are present after the photoflash. Also clear is the fact that the transient absorption at 380–390 nm, as measured at $6 \mu\text{sec}$ after the flash, is the only one that is affected by the pH of the solution.

This dependence, however, can be explained in different ways. Firstly we can assume that the excited state, or some reversibly formed photoproduct, giving rise to the absorption at 380–390 nm, is subject to an equilibrium of the type $\text{M} + \text{H}^+ \rightleftharpoons \text{MH}^+$ which is already established within $6 \mu\text{sec}$ after the photoflash^{8,9}. On the other hand, it could be that the initial decay of the photo-excited state is much faster in acid media than in neutral or slightly basic solutions. To solve this problem additional experiments on a time scale in the nsec range with a pulse laser have to be done.

In Fig. 5 the transient absorption at 380 nm and at $6 \mu\text{sec}$ after the flash is plotted as a function of pH. The result does not seem to point to a specific acidity constant.

As regards the nature of the transient species absorbing at 380–390 nm there are still difficulties in the assignment. KNOWLES AND ROE⁹ obtained similar transient spectra by flashing lumiflavin in aqueous neutral solution and in chloroform. They assigned the absorption maxima at 390 and 650 nm to the absorption from the lowest triplet state of the flavin molecule. The presence of a triplet species was made clear by triplet-triplet energy transfer to acridine.

We do not feel justified to adopt the same assignment for the transient at 380–390 nm because we do not understand why the observed influence of the pH upon intensity and lifetime is not reflected in the absorption at 700 nm.

Whatever the nature of the transient at 380–390 nm may be, our experiments suggest that the influence of the pH on the structure and/or decay of the photo-excited state of the flavin might be responsible for the pH dependence of the rate of the photoreaction between flavin and phenyl acetate² or methionine⁸.

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