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DETERMINATION OF THE pK VALUES OF THE LUMIFLAVIN TRIPLET STATE BY FLASH PHOTOLYSIS

S. SCHREINER, U. STEINER and H. E. A. KRAMER*
Institut fuer Physikalische Chemie der Universitaet Stuttgart, Germany

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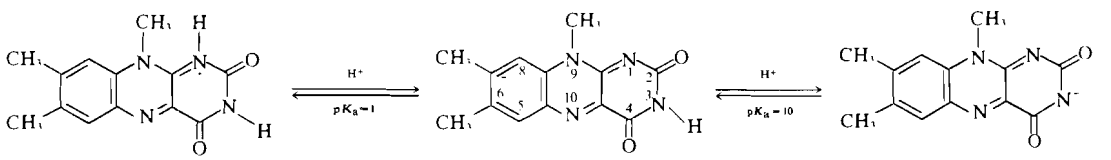
Abstract—The triplet–triplet absorption spectra in aqueous solution of the acid ($^3LfH_2^+$), the neutral (^3LfH) and the basic ($^3Lf^-$) forms of lumiflavin (6,7,9-trimethylisoalloxazine) were measured by flash photolysis. The pK_a values of the corresponding protolytic equilibria of the lumiflavin triplet were found to be $4{\cdot}4_5 \pm 0{\cdot}1$ and $9{\cdot}8 \pm 0{\cdot}15$.

Quantum yield and product distribution of flavin photoreactions are frequently found to be strongly pH dependent (Suelter and Metzler, 1960; Penzer, 1970; Carr and Metzler, 1970; Cairns and Metzler, 1971; Haas and Hemmerich, 1972).

In the case of flavin photoreductions the reaction is known to start from the triplet state (Holmstroem and Oster, 1961; Green and Tollin, 1968; Vaish and Tollin, 1970). So for this type of reaction knowledge of the pK values of the flavin triplets will be of great importance for understanding the pH dependence.

Flavins [in this work lumiflavin (6,7,9-trimethylisoalloxazine)] are amphoteric and, according to Hemmerich (1965), there are three protolytic forms (see also Dudley *et al.*, 1964).



Consequently one has to assume the existence of at least three different protolytic forms for the excited states, too. Kavanagh and Goodwin (1948) found a marked change of fluorescence of lumiflavin and riboflavin in the regions of pH 2–4 and 9–10·5. The pK values of the lumiflavin triplet are still unknown. For flavins, there are only a few investigations of the triplet state with respect to variation of the pH (Shiga and Piette, 1964; Lhoste *et al.*, 1966; Katan *et al.*, 1971).

In this paper we present the results of our investigations on the triplet–triplet absorption spectra of the acid ($^3LfH_2^+$), the neutral (^3LfH) and the basic ($^3Lf^-$) forms of the lumiflavin triplet as detected by flash photolysis.

*Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Lumiflavin was prepared by photolysis of an alkaline aqueous solution of riboflavin (Knowles and Roe, 1968). As a solvent we used triply distilled water, as buffers (0·2 *M*) (Schwabe, 1958):

pH 1–3·7 sodium acetate, hydrochloric acid
pH 3·7–5·6 sodium acetate, acetic acid
pH 6–12 potassium dihydrogen phosphate, sodium hydroxide
pH 13 potassium chloride, sodium hydroxide (E. Merck, Darmstadt, Germany).

The high buffer concentration ensures that the protolytic equilibria in the triplet state are really established.

Oxygen was removed by bubbling nitrogen (>99·9%) through the solutions for 45 min. The results with solutions treated in this way were identical to those with solutions degassed by the 'freeze-pump-and-thaw' procedure.

The flash apparatus was the same as described earlier (Fischer, 1964; Kramer and Maute, 1972). The flash energy could be varied between 300 J and 900 J. The half duration of the flash was about 4·6 μs (300 J) and 6 μs (900 J). The cuvette was surrounded by a Kodak-Wratten filter No. 2A which absorbs light of wavelength shorter than 400 nm.

Using the flash apparatus described above (kinetic spectrophotometry) the spectra were recorded point-by-point at intervals of 10 nm (half-width value 2·5 nm) in the spectral region from 300 to 780 nm; the spectra were corrected for stray light.

RESULTS

Dependence of the lumiflavin triplet–triplet absorption spectrum on pH. Figure 1 shows the transient change in absorption when lumiflavin solutions of pH = 2, pH = 7, and pH = 13 are flashed. From this the triplet–triplet absorption spectra of the acid (pH = 2), neutral (pH = 7),

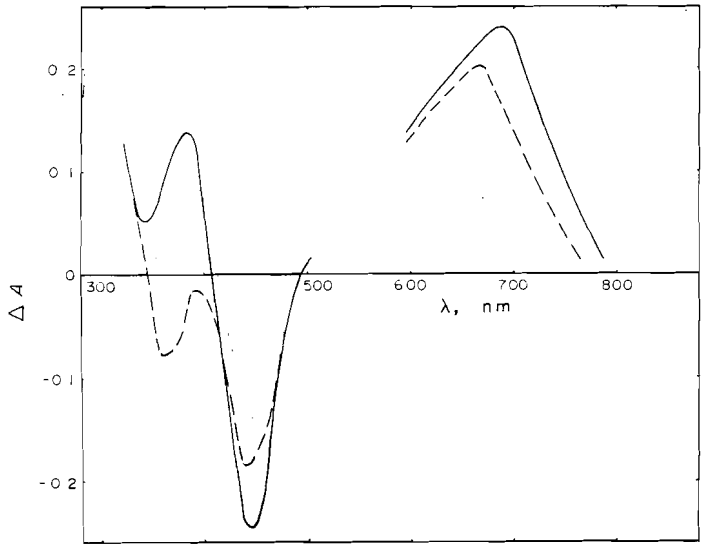


Figure 1. Transient change in absorption observed 8 μs after flashing an aqueous solution of lumiflavin; Oxygen-free. --------- pH = 2 lumiflavin (10^{-5} *M*); ———— pH = 7 lumiflavin (10^{-5} *M*); ..... pH = 13 lumiflavin (2×10^{-5} *M*).

and basic (pH = 13) forms of the lumiflavin triplet were calculated according to the procedure described below (see Fig. 2). The results at pH 7 are in good agreement with those of Knowles and Roe (1968). Correction of the spectra for semiquinone absorption was not necessary, since, using low dye concentrations, almost no semiquinone was formed 8 μs after flashing, the moment when all spectra were taken.

Unlike the case of alloxazine, reported by Dekker *et al.* (1973), in which a considerable amount of long-lived intermediate absorption arises simultaneously with the triplet absorption, there is no long-lived intermediate detectable in our case, when we flash lumiflavin in aerated solutions (pH 2, 7, 13).

In deaerated solutions, only a weak change in optical density is left 300 μs after the flash between 500 and 600 nm, due to a long-lived intermediate. No long-lived absorption could be found at the wavelengths where the pK values were determined.

The absorption of the acid form of the lumiflavin triplet at 370 nm is smaller than that of the neutral form. A similar observation for 3-methyllumiflavin was reported by Katan *et al.* (1971). In the region of longer wavelengths, a small shift of the absorption maximum is found (Fig. 2).

Determination of extinction coefficients. For the calculation of triplet absorption spectra from changes in optical density, there is always the problem that an unknown fraction of ground-state molecules may be present.

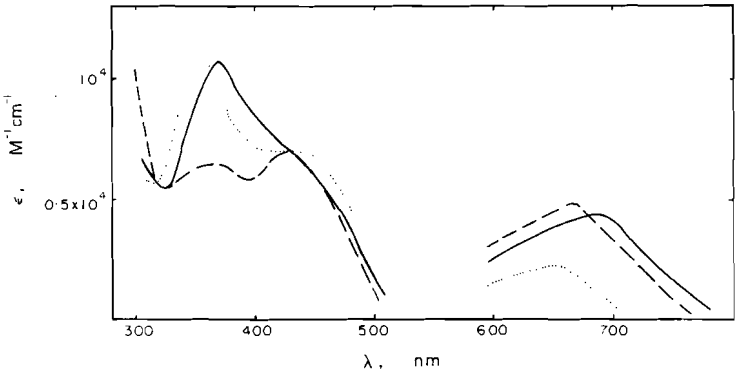


Figure 2. Triplet–triplet absorption spectra (see text) of lumiflavin in aqueous solution 8 μs after flashing: ---- pH = 2 acid triplet form ($^3LfH_2^+$); ——pH = 7 neutral triplet form (^3LfH); ....pH = 13 basic triplet form ($^3Lf^-$); oxygen-free.

As a matter of fact it should be noted that, without knowledge of the molar extinction coefficients, it is not possible to calculate the ratio of concentrations of two absorbing species from a spectrum of their mixture. This is true even if the extinction coefficient of one species is known.

As can be shown mathematically, one cannot gain final information on the starting concentrations by following the spectral changes due to a change of the ratio of concentrations during time. Hence without an additional assumption one cannot determine the absolute values of the triplet extinction coefficients from the spectra (except for the isosbestic points of ground state—and respective triplet absorption spectrum).

Hadley and Keller (1969) suggested an approximate method for the calculation of triplet spectra, based on the assumption that coincidences of sharp peaks of the ground-state spectrum with those of the triplet spectrum should be very unlikely. Therefore one can assume that, among all possible triplet spectra, the correct one will show the least curvature in the ranges of sharp peaks of the ground-state spectrum.

We can show for all three forms of the lumiflavin triplet that, with increasing flash intensity (flash energy up to 900 J), the increase of the optical density, right after the flash, becomes smaller and smaller. From this we conclude that we are close to a triplet population of 100 per cent. The spectra calculated on this assumption (see Fig. 2) are in good agreement with those chosen by means of the Hadley and Keller criteria.

For the extinction coefficients of the long-wavelength maxima we get: acid form $\epsilon_s^{670} = 0{\cdot}48 \times 10^4\,\mathrm{cm}\,M^{-1}$, neutral form $\epsilon_n^{690} = 0{\cdot}44 \times 10^4\,\mathrm{cm}^{-1}\,M^{-1}$, basic form $\epsilon_b^{650} = 0{\cdot}2 \times 10^4\,\mathrm{cm}^{-1}\,M^{-1}$.

*Determination of p*K *values.* The pK_a value in the acid range can be determined from the decrease of absorbance at 390 nm, since the amount of the triplet produced was constant in the pH range $3{\cdot}5 \leq \mathrm{pH} \leq 8$. From Fig. 3a one finds $\mathrm{p}K_a = 4{\cdot}4_5 \pm 0{\cdot}1$. In basic solutions (pH > 9·3) the amount of the triplet produced decreases with increasing pH. The concentration ratio of basic (^{3}Lf$^-$) to neutral

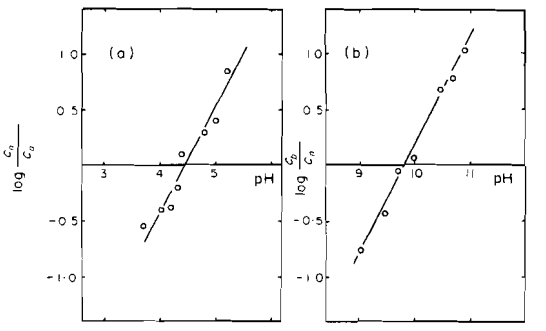


Figure 3. (a) Log plot of the concentration ratio C_n/C_a of the neutral (^{3}LfH) to the acid (^{3}LfH$_2^+$) lumiflavin triplet form vs pH. (b) Log plot of the concentration ratio C_b/C_n of the basic (^{3}Lf$^-$) to the neutral (^{3}LfH) lumiflavin triplet form vs pH. For determination of the concentration ratio, see text.

(^{3}LfH) triplet (C_b/C_n) is obtained from measurements at two different wavelengths ($\lambda_1 = 650$ nm; $\lambda_2 = 700$ nm). One gets:

$$A^{650} = \epsilon_b^{650} \times C_b \times d + \epsilon_n^{650} \times C_n \times d$$

$$A^{700} = \epsilon_b^{700} \times C_b \times d + \epsilon_n^{700} \times C_n \times d.$$

From Fig. 3b one obtains $\mathrm{p}K_a = 9{\cdot}8 \pm 0{\cdot}15$.

Decay of the triplet state. Results of the kinetic measurements at pH = 2, 7, and 13 demonstrate that the first-order decay constant of the acid triplet form is higher than those of the neutral and basic forms, (Fig. 4, Table 1).

DISCUSSION

According to calculations of Song (1968) for the neutral isoalloxazine molecule, the nitrogen atom N_3 carries the highest electron density, the value being nearly the same in all three states [ground state (S_0), triplet state (T_1), first excited singlet state (S_1)]. Likewise, nearly the same value is found for the pK_a in the basic range for the three states (S_0: $\mathrm{p}K_a \approx 10$, Dudley *et al.*, 1964; T_1: $\mathrm{p}K_a = 9{\cdot}8 \pm 0{\cdot}15$, this work. Kavanagh and Goodwin (1948) reported changes of fluorescence in the pH range between 9·0 and

Table 1. Characteristics of the different protolytic forms of the lumiflavin triplet state

	pH	λ_{max} (nm)	$\epsilon \times 10^{-4}$ ($\mathrm{cm}^{-1}\,M^{-1}$)	k* (s^{-1}) apparent first-order decay constant	pK_a
^{3}LfH$_2^+$	2	430; 670	0·7; 0·48	$3{\cdot}4_6 \times 10^4$	$4{\cdot}4_5 \pm 0{\cdot}1$
^{3}LfH	7	370; 690	1·07; 0·44†	$1{\cdot}5_7 \times 10^4$‡	$9{\cdot}8 \pm 0{\cdot}15$
^{3}Lf$^-$	13	350; 650	~1·1; ~0·2	$1{\cdot}6_9 \times 10^4$	

*Lumiflavin concentration $5 \times 10^{-6}\,M$.

†Knowles and Roe (1968): $\epsilon^{680} = 0{\cdot}46 \times 10^4\,\mathrm{cm}^{-1}\,M^{-1}$.

‡Knowles and Roe (1968): $k = 1{\cdot}11 \times 10^4\,\mathrm{s}^{-1}$; for an exact analysis of the triplet decay, see Vaish and Tollin (1970).

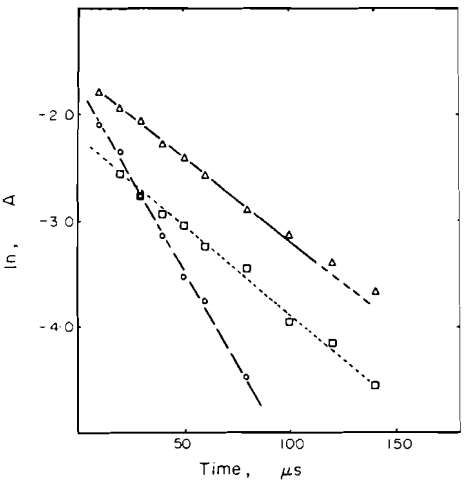


Figure 4. Logarithmic plot of decay of absorbance after flashing 5×10^{-6} *M* lumiflavin in aqueous solution at different pH values: ---- pH = 2, $\lambda_{obs} = 670$ nm; ——— pH = 7, $\lambda_{obs} = 690$ nm; .... pH = 13, $\lambda_{obs} = 650$ nm.

10·5, which leads us to suppose that the pK_a of the S_1 state lies in this range. Therefore, the simplest explanation would be to attribute this pK_a to protonation at N_3. However, our results cannot answer the question of whether or not, in the excited states, protonation at the oxygen atoms could also occur. For the ground state, Dudley *et al.* (1964) excluded such a possibility.

The pK_a in the acid range corresponds to protonation of the neutral lumiflavin triplet, which might occur at N_{10} or perhaps O_4. As J. M. Lhoste has pointed out to us, protonation at N_1 is improbable. The phosphorescence of flavin mononucleotide (Lhoste *et al.*, 1966) in strong acids in low-temperature glasses (where the proton may be considered firmly held at N_1) is blue-shifted relative to the neutral flavin phosphorescence. As follows from the Förster cycle (Förster, 1950; Weller, 1961) the pK_a of the N_1-protonated triplet should therefore be lower than that of the ground state, which is in contradiction to our result. Song (1968) showed that the N_1 atom carries a higher electronic charge in the ground state than N_{10} whereas the opposite holds for the first excited singlet state and triplet state.

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