Bioenergetics (1970), **1**, 181–192.

Flash Photolysis of Flavins. IV. Some Properties of the Lumiflavin Triplet State

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Abstract

Analysis of lumiflavin triplet-state decay kinetics in aqueous solution has given the following results: k_1 (first-order decay) = 670 sec^{-1}, k_2 (triplet–triplet quenching) = $8{\cdot}9 \times 10^8$ M^{-1} sec^{-1}, k_3 (triplet–ground-state quenching) = $3{\cdot}7 \times 10^8$ M^{-1} sec^{-1}. The FMN triplet decays mainly via intramolecular quenching by the ribityl side chain and triplet–ground-state quenching. Ferricyanide and phenols are shown to be excellent quenchers of the flavin triplet (comparable to KI and O_2). In the case of phenols, quenching occurs via hydroxyl hydrogen abstraction to generate flavin radical and phenoxy radical. Recombination of these radicals (by reverse hydrogen transfer) competes effectively with flavin radical disproportionation. The lumiflavin triplet is also able to abstract hydrogen from a ground state lumiflavin molecule (probably from the 10-methyl group). The radicals so generated can either recombine or undergo a buffer-catalyzed reaction leading to permanent bleaching. Evidence is presented for rapid oxidation of lumiflavin radical by both oxygen and ferricyanide. In dry non-polar solvents, lumiflavin triplet formation is prevented; addition of small amounts of water restores the ability to produce triplet state molecules. This is probably due to an effect of water on intersystem crossing.

Introduction

The photochemical properties of riboflavin and its analogs have been widely studied,[1] not only because of their intrinsic interest, but also because flavins have been implicated in a variety of photobiological phenomena such as phototropism,[2] *Euglena* phototaxis,[3,4] and chloroplast phototaxis.[5] Although many investigations[6] have shown that the triplet state is an important photochemical intermediate, very little is known concerning its properties.[7,8] The present investigation utilizes flash photolysis techniques to elucidate some of the kinetic and chemical behavior of the lumiflavin triplet. This compound was chosen to avoid complications arising from ribityl side chain photooxidation,[1] although some evidence will be presented which demonstrates that the FMN triplet behaves qualitatively in a similar manner.

Methods

Materials

Lumiflavin was synthesized by the method of Guzzo and Tollin[9] and dried under high vacuum. FMN (riboflavin-5′-phosphate dihydrate, Na salt, B grade) was obtained from Calbiochem and used without further purification.

2,6-Dimethyl phenol and EDTA (disodium salt) were obtained from Eastman

Organic Chemicals. Tyrosine, phenol, *p-tert.*-butyl phenol, 3,4-dimethyl phenol, and 2,3-naphthalenediol were reagent grade. Potassium ferricyanide and potassium iodide were Mallinckrodt A.R. grade. Analyzed reagent grade sucrose was supplied by Allied Chemical.

tert.-Butyl acetate, D_2O (99·5%), and 1,2-dichloroethane were obtained from Matheson, Coleman and Bell. *tert.*-Butyl acetate was washed with 5% Na_2CO_3 solution, then with saturated aqueous $CaCl_2$, dried three times with $CaCl_2$ and distilled. 1,2-Dichloroethane was dried twice with $CaCl_2$ and then fractionally distilled from phosphorous pentoxide.

Deoxygenation of Samples

Lumiflavin solutions were degassed on a high-vacuum line (10^{-6} torr). The solution was placed in a bulb which was attached to a 10 cm cylindrical spectrophotometer cell through a teflon high-vacuum valve. Six cycles of freezing, pumping and thawing removed all dissolved gases, as was shown by reading the pressure with a McCleod gauge. The degassed solution was then transferred into the cell and the teflon valve closed. No air leakage through the valve was detectable even several hours after degassing.

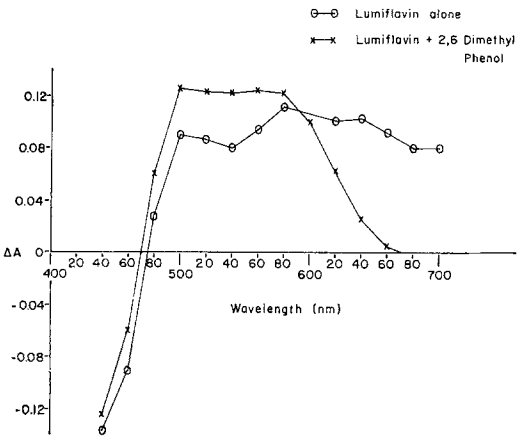

Figure 1. Flash-induced difference spectra for degassed lumiflavin solutions (6×10^{-6} M) in distilled water (pH = 7·0) with and without 2,6-dimethyl phenol (1×10^{-3} M). Points were taken 150 μsec after the flash in order to reduce possible contribution of the triplet state between 500 nm and 600 nm.

Because of their high viscosity, sucrose solutions could not be degassed by the freezing and pumping method. Instead, they were deoxygenated by purging the solution for 45 min with nitrogen gas directly from a tank. No difference was observed in the results when the nitrogen was purified by passing over hot copper turnings.

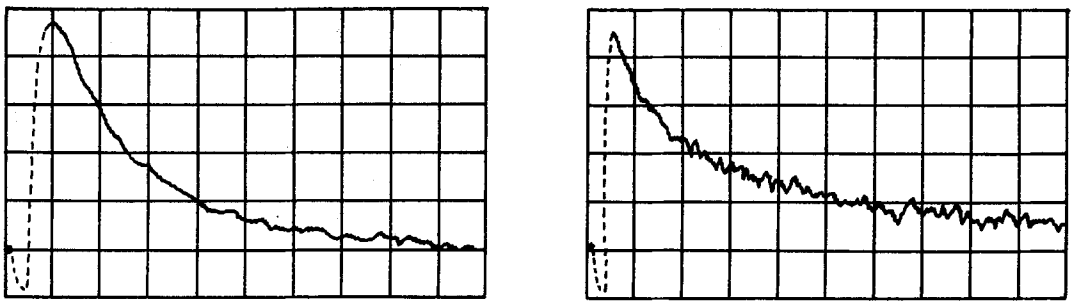

Figure 2. Typical oscillograms observed at 680 nm (A) and 560 nm (B) upon flashing a $6{\cdot}1 \times 10^{-6}$ M lumiflavin solution in distilled water. Each division along the abscissa in (A) represents 50 μsec and in (B) represents 200 μsec. In (A), seven divisions along the ordinate corresponds to a 50% transmission; in (B), nine divisions along the ordinate corresponds to 50% transmission

Oxygen Measurements

The amount of oxygen in solution was measured by using a Yellow Springs Instrument Co. oxygen monitor (Model 55). The oxygen concentration was varied by passing a known mixture of nitrogen and oxygen through the solution in the spectrophotometer cell.

Flash Spectrophotometer

The flash photolysis apparatus was of conventional design. The flash source was a Xenon Corp. lamp (type FP-5). This was charged to 6–7 kV using a 7·5 μF Sangamo low inductance capacitor and fired by triggering an E, G and G model GP-22B ceramic-metal spark gap with an E, G and G model TM-11 trigger module and appropriate pulse circuitry. In order to improve the efficiency and reliability of firing of the flashtube, it was further triggered with an auxiliary Xenon Corp. trigger module (Model C) with a trigger wire wrapped around the tube. The flash duration was 15 μsec (half decay time). In the measurement of decay kinetics, zero time was chosen to be 60–75 μsec (depending on the wavelength of the measurement) to avoid flash artifacts. An infrared heat absorbing filter and a Corning CS-7-59 filter were mounted between the flashtube and the sample cell. The entire sample and flashtube compartment was cooled by forced air.

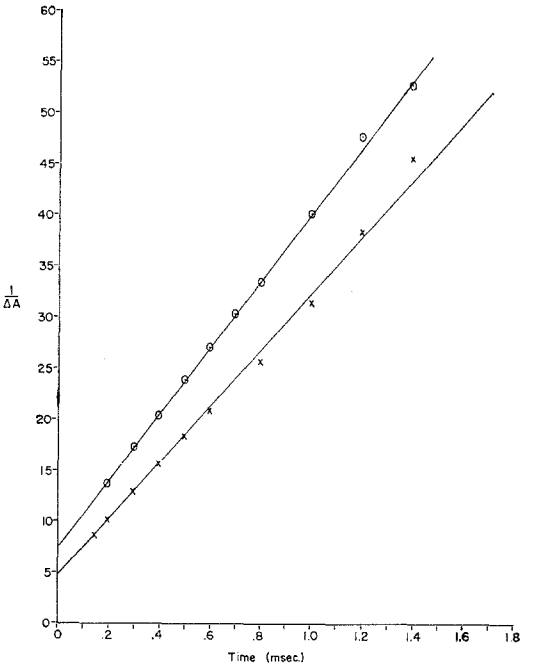

Figure 3. Second-order plots of lumiflavin semiquinone decay obtained with lumiflavin alone (⊙) and with 2,6-dimethyl phenol (×) in distilled water. Data represent approximately 80% of decay curve; [lumiflavin] = 6·1 × 10^{-6} M; [2,6-dimethyl phenol] = 1·0 × 10^{-3} M.

The monitoring beam was a Sylvania 650 W tungsten-halogen lamp (120 V, DVY) which was collimated to pass through the sample cell and filtered using a water-cooled infrared filter and appropriate band pass filters (usually Corning CS 3-70). A d.c. power supply was used for the monitoring lamp. The transmitted light passed through the sample cell into a Jarrel-Ash monochromator (Model 82-410) and onto the photomultiplier detector (RCA 4463, S-20 response). In order to reduce scattered light, a series of baffles was placed between sample and monochromator. The output from the phototube (50 K load resistor) was fed into a Tektronix type 533 oscilloscope.

Results and Discussion

When lumiflavin solutions in phosphate buffer (pH = 7·0) or distilled water are flashed, lumiflavin triplet and semiquinone (free radical) species[7] are formed (Fig. 1). The absorption spectra of these two materials overlap considerably in the visible region.[10]

However, the extinction coefficients of the triplet at 560 nm and that of radical at 680 nm are small and thus the 680 nm absorbance can be assumed to be primarily due to triplet and that at 560 nm due to radical. Typical oscilloscope traces at these two wavelengths are shown in Fig. 2. The transient observed at 560 nm decays by a second-order process (Fig. 3) with a rate constant of $1{\cdot}5 \times 10^9$ lmole^{-1} sec^{-1}. This can be compared with a value of $0{\cdot}75 \times 10^9$ reported by Knowles and Roe.[10]

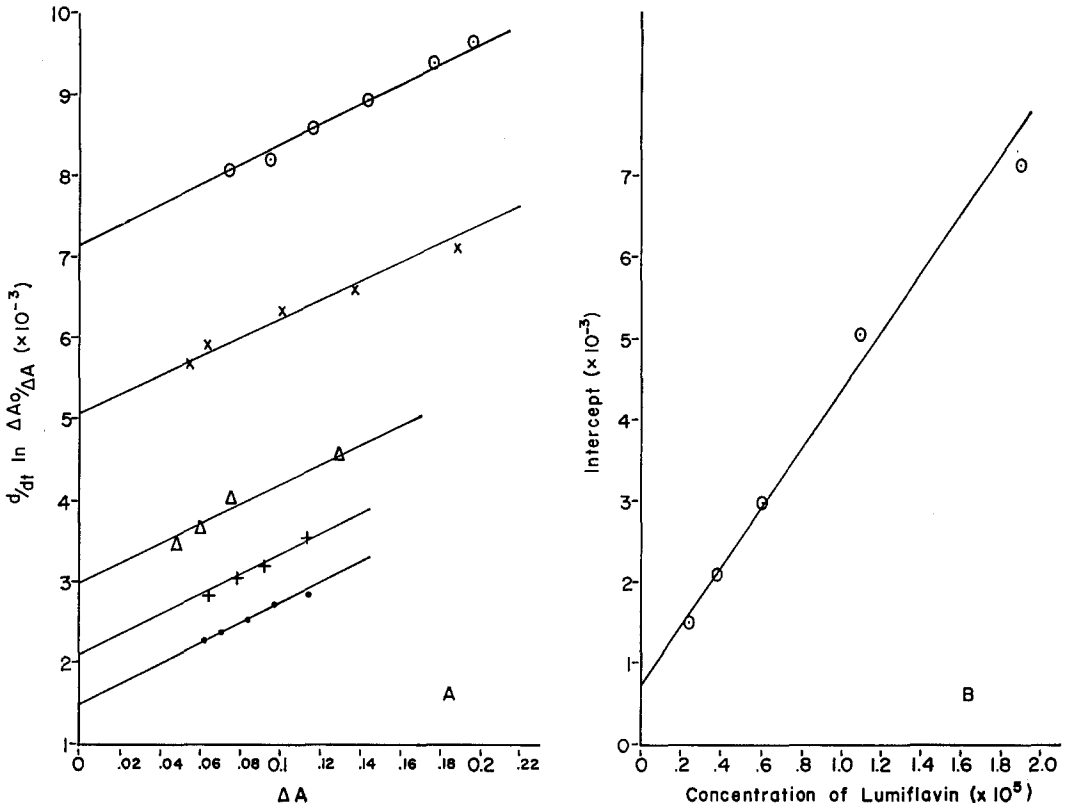

Figure 4. A: Plot of d/dt ln $\Delta A_0/\Delta A$ versus ΔA for various concentrations of lumiflavin in distilled water. ⊙, $1{\cdot}9 \times 10^{-5}$ M; ×, $1{\cdot}1 \times 10^{-5}$ M; △, $0{\cdot}61 \times 10^{-5}$ M; +, $0{\cdot}39 \times 10^{-5}$ M; ●, $0{\cdot}25 \times 10^{-5}$ M.
B: Plot of intercepts from Fig. 4A versus lumiflavin concentration.

The triplet, observed at 680 nm where there is no ground state or radical absorbance,[11] does not decay by a first-order process except at high concentrations of lumiflavin. At lower concentrations of lumiflavin, the kinetics of triplet decay are mixed, although Knowles and Roe[10] report that the lumiflavin triplet decays to the ground state by a first-order process with a rate constant of $1{\cdot}1 \times 10^4$ sec^{-1}. However, this was determined on the basis of an analysis of a decay curve which was partly due to radical and partly to triplet. We have studied the triplet decay at 680 nm in solution as a function of the

concentration of lumiflavin. At low concentrations of flavin, the triplet decay obeys the following rate law reasonably well:

$$-\frac{dc}{dt} = k_1 C_T + k_2 C_T^2 + k_3 C_T C_G \qquad (1)$$

This type of equation was used by Linschitz and Sarkanen[12] to explain chlorophyll triplet decay in pyridine and benzene solutions. In this equation,

k_1 = first-order radiative and radiationless rate constants for the triplet decay;
k_2 = rate constant for triplet-triplet quenching processes;
k_3 = rate constant for triplet-ground state quenching.

Values for these three rate constants were calculated by the method given by Linschitz and Sarkanen.[12] Only the essential terms needed for this analysis are defined here.

Let C_0 = total flavin concentration;
C_T = concentration of flavin triplets;
C_G = concentration of ground-state flavin.

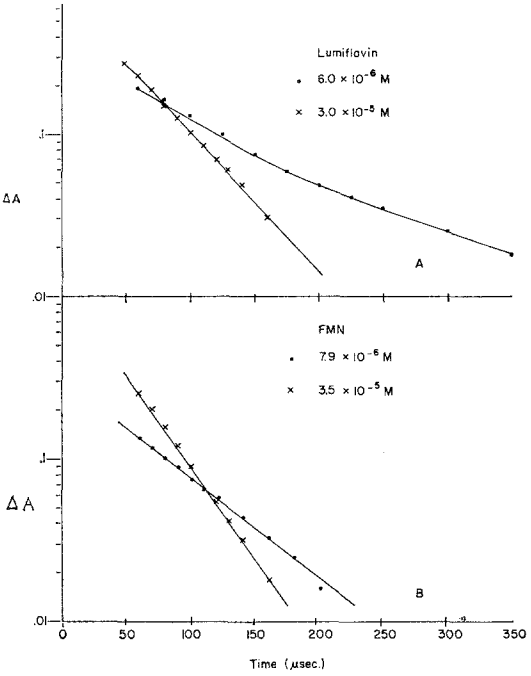

Figure 5. Semilog plots of triplet decay curves for lumiflavin and FMN in distilled water.

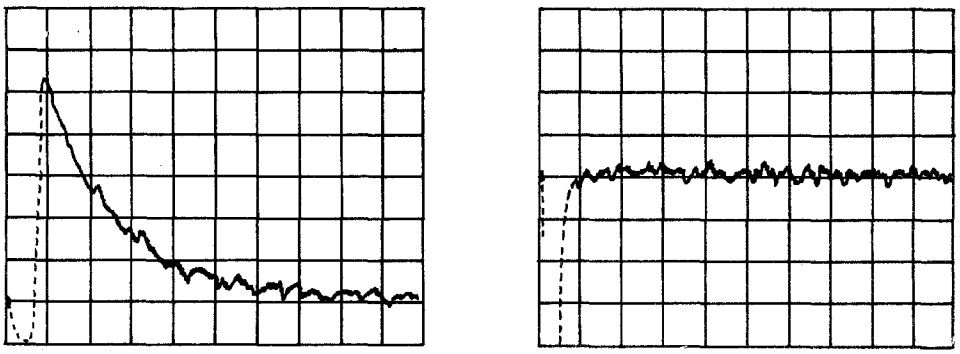

Figure 6. Oscillograms observed at 560 nm for air-saturated solutions of lumiflavin in 0·025 M phosphate buffer (pH = 7·0) with and without 2,6-dimethyl phenol. A. [lumiflavin] = $1{\cdot}9 \times 10^{-5}$ M; [2,6-dimethyl phenol] = 1×10^{-3} M. B. [lumiflavin] = $1{\cdot}9 \times 10^{-5}$ M.
Time scale in A is 100 μsec/division and in B is 200 μsec/division.

Since $\Delta A^{680} = \epsilon_T^{680} C_T l$, where ϵ_T^{680} = molar absorptivity of triplet at 680 nm (= 4600 as given by Knowles and Roe, ref. 10), and l = length of the cell (= 10 cm), we can rewrite equation (1) as follows:

$$\frac{d}{dt} \ln \frac{\Delta A_0}{\Delta A} = a + b\Delta A \qquad (2)$$

where ΔA_0 = change in absorbance measured 60 μsec after the flash;

$$a = k_1 + k_3 C_0 \qquad (3)$$

and

$$b = \frac{k_2 - k_3}{\epsilon_T^{680} . 1} \qquad (4)$$

The time-derivative in equation (2) is obtained by drawing tangents to a plot of $\log (\Delta A_0/\Delta A)$ versus t. The slopes are plotted against the corresponding ΔA values giving a family of lines of constant slope but increasing intercept with increasing C_0 which allow a and b to be determined (Fig. 4A). The variation of a with C_0 gives k_1 and k_3 (Fig. 4B) and k_2 can then be calculated from equation (4). The values of the three rate constants for lumiflavin in distilled water are as follows:

$$k_1 = 670 \text{ sec}^{-1}$$

$$k_2 = 8{\cdot}9 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$$

and

$$k_3 = 3{\cdot}7 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$$

Rate constants of appsoximately equal value were obtained in 0·025 M phosphate buffer, pH 7·0. Note particularly the rather large values for the triplet–ground-state quenching constant k_3. This would suggest that in those flavoproteins in which two flavin molecules are bound in close proximity to one another, triplet quenching would be quite effective.

In Fig. 5, the triplet decay rates at 680 nm for FMN and lumiflavin at low and high concentrations are compared. The FMN triplet decays to the ground state by a first-order process at concentrations at which the lumiflavin decay is of mixed order. This is probably due to intra-

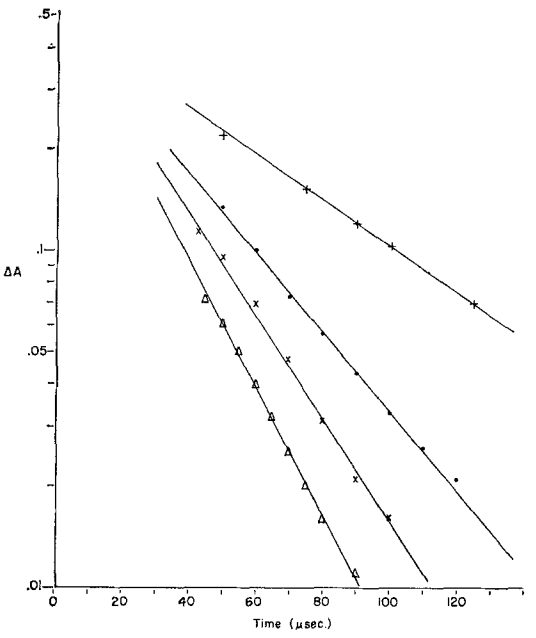

Figure 7. Effect of various quenching agents on lumiflavin triplet decay in 0·025 M phosphate buffer, pH 7·0; [lumiflavin] = $1{\cdot}4 \times 10^{-5}$ M; [quencher] = $1{\cdot}0 \times 10^{-5}$ M. +, lumiflavin alone; ●, lumiflavin plus 2,6-dimethyl phenol; ×, lumiflavin plus ferricyanide; △, lumiflavin plus KI.

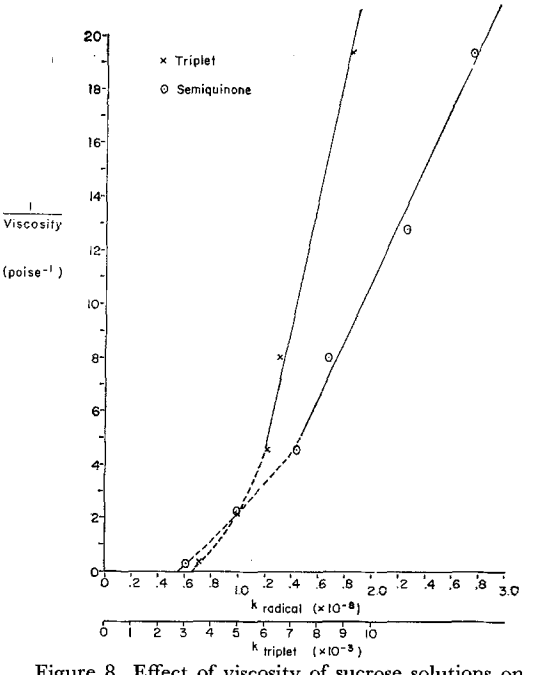

Figure 8. Effect of viscosity of sucrose solutions on decay constants for lumiflavin triplet and semiquinone; [lumiflavin] = $1{\cdot}3 \times 10^{-5}$ M.

molecular self-quenching by the ribityl side chain, although intermolecular quenching is also occurring inasmuch as the rate of decay is concentration dependent. Note also that the lifetime of the FMN triplet at the lower concentrations is shorter than that of the lumiflavin triplet.

Tegner and Holmström[7] have calculated the rate constant for the reaction between triplet lumiflavin and iodide ion to be 7×10^9 M^{-1} sec^{-1}. Inasmuch as ferricyanide ion and phenols are also good triplet quenchers (Fig. 6), we have compared the effectiveness of triplet quenching by these compounds with KI in aqueous solution (Fig. 7). Using the above value for the iodide quenching constant, we obtain values of $5{\cdot}7 \times 10^9$ and $4{\cdot}9 \times 10^9$ M^{-1} sec^{-1} for ferricyanide and 2,6-dimethyl phenol, respectively.

Values of the triplet quenching constants for ferricyanide, dimethyl phenol and oxygen in 70% sucrose solution were directly determined.* Except for altering viscosity, sucrose was found to be photochemically non-reactive. Triplet decay in sucrose solutions follows first-order kinetics. The first-order triplet and second-order radical decay rate constants are viscosity-dependent, but do not follow the expected inverse proportionality to viscosity except at lower viscosities (Fig. 8). The rate of triplet decay is found to increase with increasing concentration of the quencher (Fig. 9A). The quenching constants were calculated from the slope of a straight line obtained by plotting rate constants against quencher concentration (Fig. 9B). The quenching constants in 70% sucrose for ferricyanide, 2,6-dimethyl phenol, and oxygen are $2{\cdot}4 \times 10^8$, $1{\cdot}2 \times 10^8$, and $1{\cdot}3 \times 10^8$ M^{-1} sec^{-1}, respectively. From these data, it is apparent that dimethyl phenol and ferricyanide quench the lumiflavin triplet as effectively as does oxygen. Thus, a tyrosyl side chain, or perhaps nonheme iron, in a flavoprotein would provide an efficient pathway for triplet degradation.

* The high viscosity of the sucrose solutions (321·6 cP) slows the reactions sufficiently to allow this type of measurement to be made over a wide range of quencher concentrations.

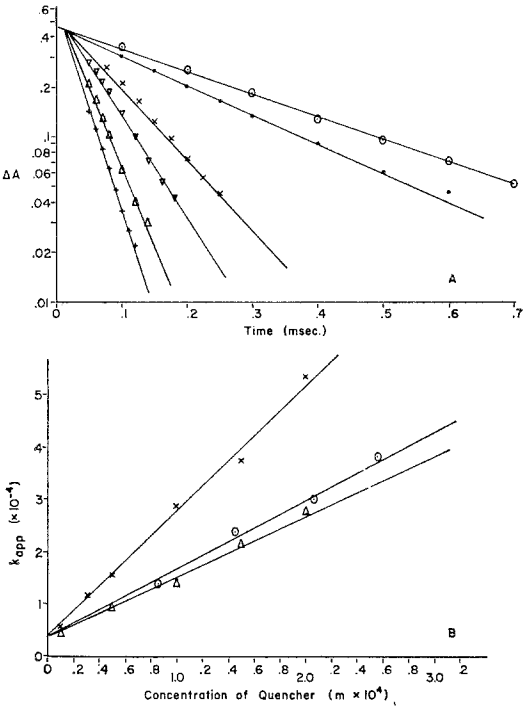

Figure 9. A: Effect of 2,6-dimethyl phenol concentration on lumiflavin triplet decay curves in 70% sucrose solution; [lumiflavin] = $1{\cdot}7 \times 10^{-5}$ M. ⊙, none; ●, $1{\cdot}0 \times 10^{-5}$ M; ×, $5{\cdot}0 \times 10^{-5}$ M; ▽, $1{\cdot}0 \times 10^{-4}$ M; △, $1{\cdot}5 \times 10^{-4}$ M; +, $2{\cdot}0 \times 10^{-4}$ M.

B: Apparent first-order rate constants for lumiflavin triplet decay in 70% sucrose versus concentration of quencher; [lumiflavin] = $1{\cdot}7 \times 10^{-5}$ M. ×, ferricyanide; ⊙, oxygen; △, 2,6-dimethyl phenol.

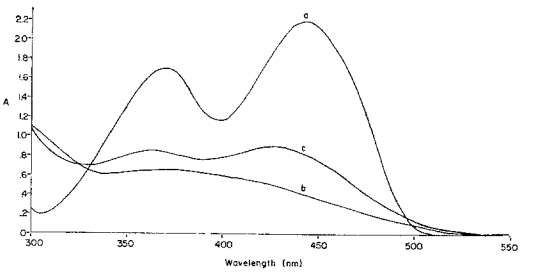

Figure 10. Absorption spectra of lumiflavin solutions in 0·1 M phosphate buffer (pH = 7·0) before and after flashing. (a) before flashing; (b) after twenty flashes; (c) after allowing air to enter cell.

Lumiflavin solutions in 0·1 M phosphate buffer (pH = 7·0) *in vacuo* undergo appreciable photobleaching.[10] The absorption spectrum of such a solution, after about twenty flashes, does not return to its original shape and height when air is allowed into the sample cell (Fig. 10). This is probably due to lumichrome formation. At lower buffer concentrations, less photobleaching occurs.* In distilled water (pH = 7·0), permanent photobleaching after many flashes is minimal (5% or less). When phenols, such as 2,6-dimethyl phenol, tyrosine, *p-tert.*-butyl phenol, 3,4-dimethyl phenol or 2,3-naphthalenediol, are present in 0·1 M buffer, no permanent bleaching occurs. In the presence of these compounds, the triplet is completely quenched and the semiquinone yield is increased (Fig. 1). Radical decay is second order with a rate constant (with 2,6-dimethyl phenol) of 1×10^9 M^{-1} sec^{-1} (Fig. 3).

We have also observed (Fig. 11) that the presence of phenols in a solution of lumiflavin plus EDTA in phosphate buffer markedly reduces the extent of photoreduction to the fully reduced form (which is due to disproportionation of lumiflavin radicals) and increases the radical yield.

If one measures the extent of decrease in absorbance at 445 nm induced by a single flash in lumiflavin solutions in distilled water, one finds the following: lumiflavin alone $\simeq 5\%$, lumiflavin plus 2,6-dimethyl phenol $\simeq 9\%$, and lumiflavin plus EDTA $\simeq 35\%$. The amount of radical generated by the flash in the EDTA and phenol solutions were approximately equal.

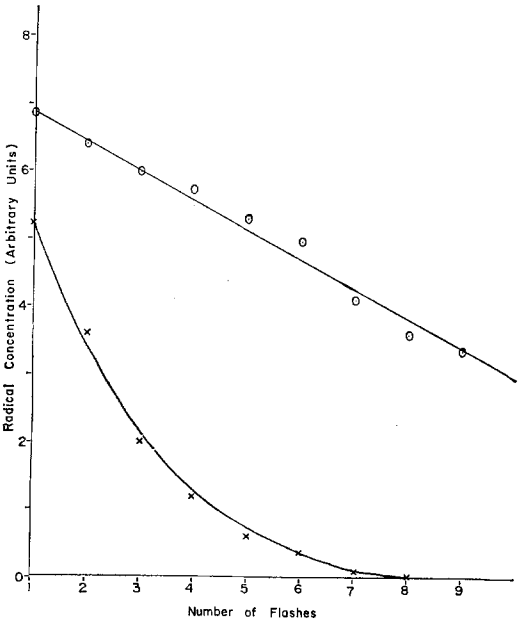

Figure 11. Amount of lumiflavin semiquinone generated by a single flash versus number of flashes which sample has received. 0·1 M phosphate buffer, pH 7·0. ⊙, lumiflavin plus 5×10^{-3} M EDTA and 2×10^{-3} M tyrosine; ×, lumiflavin plus 5×10^{-3} M EDTA.

The above results suggest that phenols can react with the lumiflavin triplet to generate lumiflavin radical and that decay occurs predominantly via a second-order process which competes with disproportionation. Furthermore, the radical decay process in solutions of lumiflavin without an added reductant also proceeds partly via a second-order process which does not involve formation of fully-reduced flavin.

In air-saturated solutions (0·1 M phosphate at pH = 7·0) no transients can be seen in pure lumiflavin solutions (Fig. 6B). This is undoubtedly due to triplet quenching by oxygen. However, the addition of 2,6-dimethyl phenol (or other phenols) causes the appearance of a semiquinone transient at 560 nm (Fig. 6A), which decays more rapidly than in anaerobic solution and by first-order kinetics (Fig. 12). The calculated second-order rate constant ($2{\cdot}2 \times 10^7$ M^{-1} sec^{-1}) is independent of the concentration of phenol, from about 10^{-5} M to 10^{-2} M, and also of phenol structure, thus demonstrating that the phenol is not participating in the decay process. Inasmuch as phenols are effective

* This is suggestive of buffer ion catalysis, particularly since the triplet decay is unaffected by buffer.

quenchers of the flavin triplet, they can compete effectively with oxygen and thus radical formation can occur. The first-order kinetics and more rapid rate of radical decay provide clear evidence for an oxidation of the lumiflavin radical by oxygen. We are presently investigating this in more detail* (see below for further comment).

Potassium iodide quenching provides further support for the concept that radical formation, with and without phenols, proceeds via the lumiflavin triplet state. The results are shown in Fig. 13. Thus, KI reduces both the radical and triplet yields with lumiflavin alone (Fig. 13A–D), at concentrations too low to measurably affect flavin fluorescence. In the presence of 1×10^{-4} M dimethyl phenol one has to go to a higher concentration of iodide ions for a marked reduction in the radical yield (Fig. 13E–H), although one is still below the fluorescence quenching level.

We have also obtained evidence that the FMN triplet can react with phenols. The addition of 5×10^{-4} M dimethyl phenol to $1{\cdot}5 \times 10^{-5}$ M FMN in 0·025 M phosphate buffer, pH 7·0 causes the following changes to occur:

(a) photobleaching of the FMN (due to side chain oxidation) is almost completely prevented;
(b) the FMN radical yield is increased;
(c) the FMN triplet state is completely quenched.

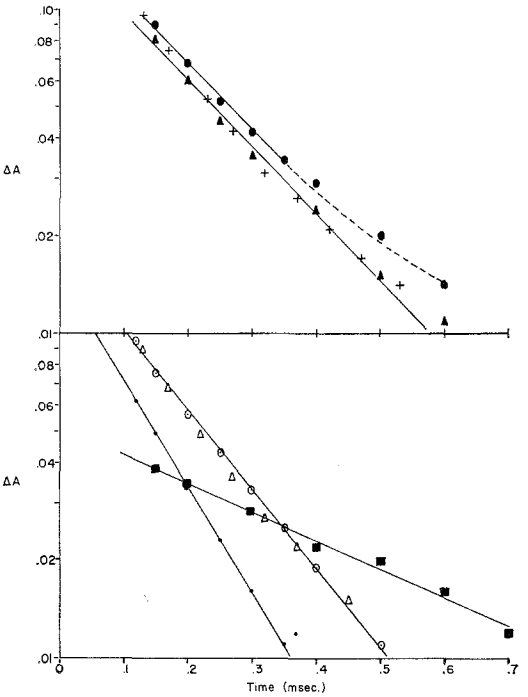

Figure 12. Semilog plots of lumiflavin semiquinone decay curves in air-saturated solutions in distilled water containing various phenols ($1{\cdot}0 \times 10^{-3}$ M) and for lumiflavin alone at low oxygen concentration (1×10^{-4} M); [lumiflavin] = $1{\cdot}9 \times 10^{-5}$ M. ▲, 3,4-dimethyl phenol; +, phenol; ●, tyrosine; •, 2,3-naphthalenediol; ⊙, 2,6-dimethyl phenol; △, *p-tert.*-butyl phenol; ■, lumiflavin alone (×2).

It is necessary now to comment on the source of reducing equivalents for radical production in these systems. The phenol reaction is reasonably simple to explain. The fact that phenol itself is quite reactive suggests hydroxyl hydrogen abstraction generating a phenoxy radical:

$$Lf_T + \phi OH \rightarrow LfH\cdot + \phi O\cdot$$

The low absorptivity of phenoxy radicals and the fact that they absorb in the same region as does lumiflavin[13] precludes a direct observation of these species. The small amount of fully reduced flavin formed suggests that lumiflavin radical decay proceeds mainly by recombination:

$$LfH\cdot + \phi O\cdot \rightarrow Lf + \phi OH$$

rather than by disproportionation:

$$2LfH\cdot \rightarrow Lf + LfH_2$$

* The possibility remains open that the flavin anion radical is actually the reactive species, rather than the neutral radical. Measurement of the pH dependence should resolve this question.

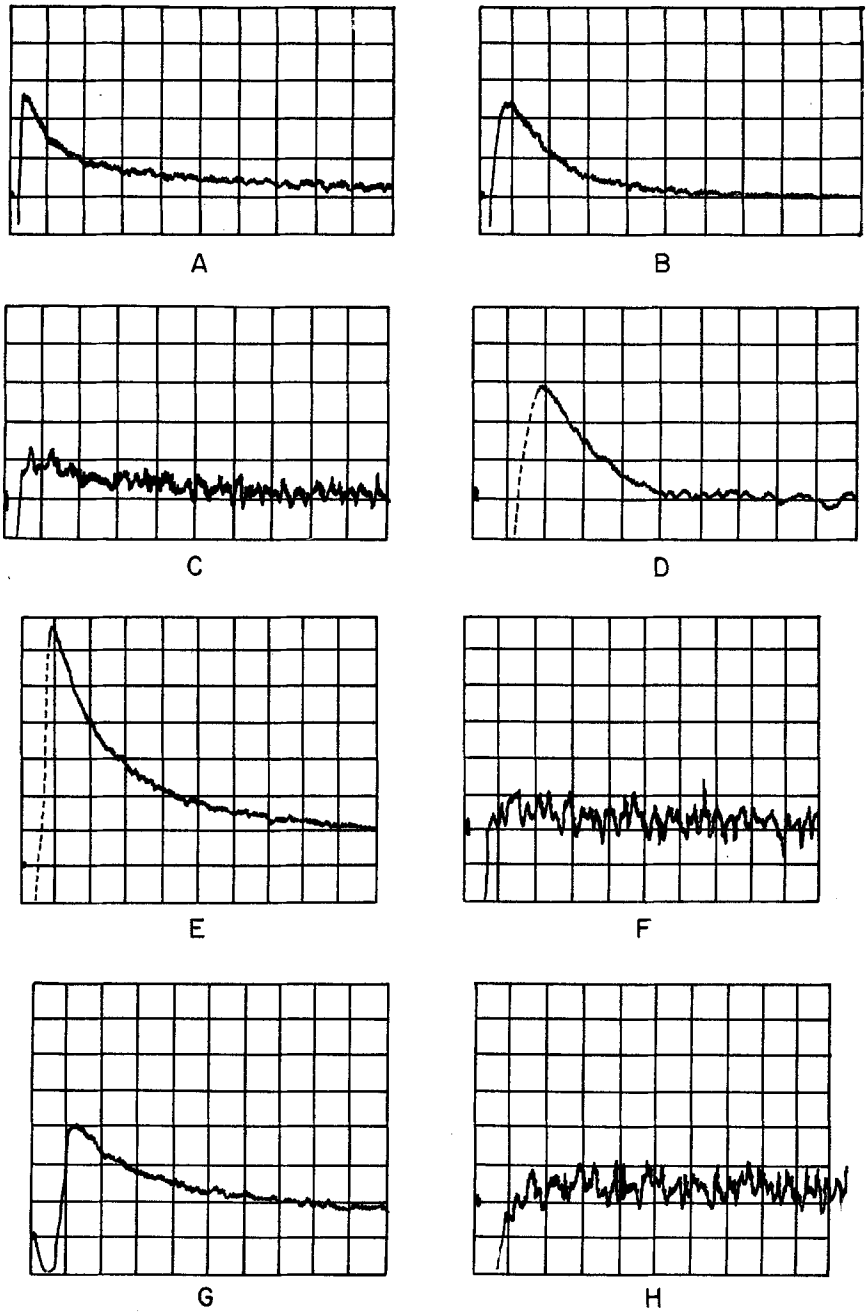

Figure 13. Effect of KI in the presence and absence of 2,6-dimethyl phenol on flash-induced transients at 560 nm and 680 nm in lumiflavin solutions in 0·025 M phosphate buffer, pH = 7·0; [lumiflavin] = $1·5 \times 10^{-5}$ M. A. 560 nm—lumiflavin alone. B. 680 nm—lumiflavin alone. C. 560 nm—lumiflavin plus $1·0 \times 10^{-5}$ M KI. D. 680 nm—lumiflavin plus $1·0 \times 10^{-5}$ M KI. E. 560 nm—lumiflavin plus $1·0 \times 10^{-4}$ M 2,6-dimethyl phenol. F. 680 nm—lumiflavin plus $1·0 \times 10^{-4}$ M 2,6-dimethyl phenol. G. 560 nm—lumiflavin plus $1·0 \times 10^{-4}$ M 2,6-dimethyl phenol and $1·0 \times 10^{-4}$ M KI. H. 680 nm—lumiflavin plus $1·0 \times 10^{-4}$ M 2,6-dimethyl phenol and $1·0 \times 10^{-4}$ M KI.

This lends credence to the suggestion made above that O_2 reacts directly with LfH· (it is in principle possible, although unlikely in view of the kinetics, that the oxygen results are due to reaction with LfH_2).

The large rate constant found for the reaction of lumiflavin triplet with ground-state lumiflavin and the observation of buffer-catalyzed irreversible photobleaching of lumiflavin proceeding via the triplet state, suggest that lumiflavin radical formation in pure water occurs by intermolecular hydrogen abstraction.* This probably occurs from the N-10 methyl group, inasmuch as we have observed that 10-methyl isoalloxazine produces comparable amounts of radical on flash excitation in water. Again, radical decay is partly via recombination, as evidenced by the small amount of photoreduction observed. As a further confirmation of an oxygen–lumiflavin radical reaction, we have made measurements with lumiflavin solutions without phenols containing very low concentrations of oxygen. Although the data are not very accurate because of the small signals, we observe a rapid first-order decay of the lumiflavin radical (Fig. 12). Analysis of the decay kinetics gives a rate constant approximately equal to that obtained in the presence of dimethyl phenol ($2{\cdot}0 \times 10^7\ M^{-1}\ sec^{-1}$).

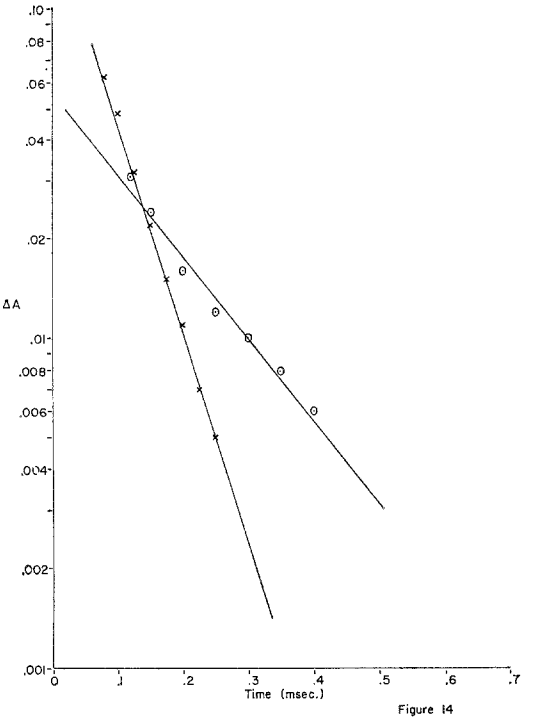

Figure 14. First-order plots of lumiflavin semiquinone decay in the presence of ferricyanide with and without 2,6-dimethyl phenol in 0·025 M phosphate buffer, pH = 7·0; [lumiflavin] = $1{\cdot}5 \times 10^{-5}$ M. ×, lumiflavin plus 5×10^{-4} M 2,6-dimethyl phenol and 4×10^{-5} M ferricyanide; ⊙, lumiflavin plus 1×10^{-5} M ferricyanide.

Additional evidence that the 560 nm absorbing species generated with lumiflavin

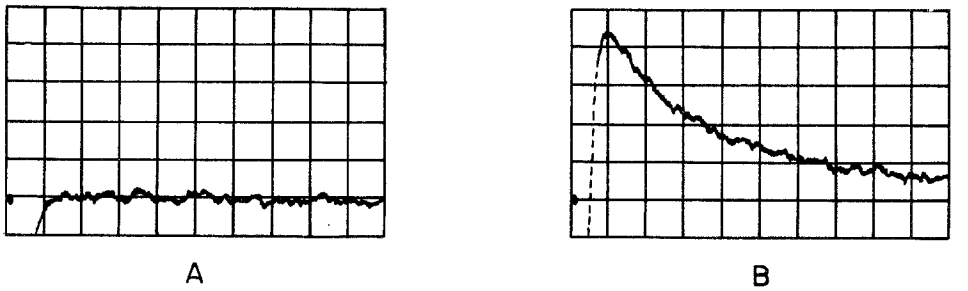

Figure 15. Flash-induced transients at 680 nm for lumiflavin in dry and wet dichloroethane; [lumiflavin] = $1{\cdot}6 \times 10^{-5}$ M. Time scale is 50 μsec per division. A: dry; B: shaken with water.

* The possibility of water oxidation was considered, but experiments using sodium formate (which is a good hydroxyl radical scavenger[14]) showed no effect on radical decay rates.

alone and with phenols is the same compound, namely LfH•, is provided by observations made in the presence of ferricyanide. We find that the decay rate increases and changes from second-order to first-order kinetics (Fig. 14). The calculated second order rate constants are approximately the same for the two systems ($3{\cdot}8 \times 10^8$ M^{-1} sec^{-1} with phenol and $5{\cdot}1 \times 10^8$ M^{-1} sec^{-1} without phenol).

In dry 1,2-dichloroethane and dry *tert.*-butyl acetate, no triplet or radical signal is observed in degassed solutions (Fig. 15A). However, when the solvent is shaken with H_2O or D_2O, a large triplet signal is observed (Fig. 15B). In the latter case, the triplet decay is slightly faster. Similar results are observed with the radical signals, although, because of irreversible photobleaching, the signal at 560 nm in the wet solvent was always associated with a very slowly decaying transient. This did not interfere with the observation of the triplet at 680 nm, where no such slower processes are observed. These results, when considered in relation to observations of increased fluorescence yields for flavins in non-polar solvents,[15] suggest that the presence of water increases the rate constant for intersystem crossing from the singlet to the triplet manifolds of the flavin molecule. Thus, a non-polar environment for flavin in a flavoprotein would provide still another mechanism for preventing triplet state population.

Acknowledgement

This work was supported in part by the U.S. Atomic Energy Commission (Contract AT(11-1)908). The funds for the construction of the flash photolysis spectrometer were obtained from an NIH Biomedical Sciences Support Grant given to the University of Arizona.

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