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Flash Photolysis of Flavins. V. Oxidation and Disproportionation of Flavin Radicals

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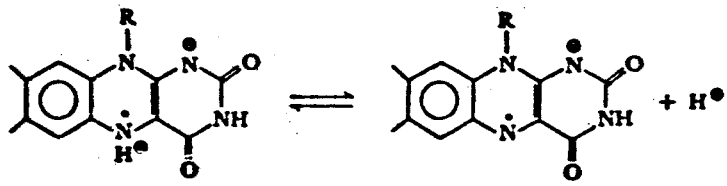
Abstract

Flash photolysis techniques have been utilized to investigate the reactivity patterns of flavin radical species. Rate constants for disproportionation were found to be in the following order: lumiflavin > FMN > FAD and neutral radicals > anionic radicals. Neutral flavin radicals react with oxygen at a rate which is at least 10^4 times slower than the anionic species. No evidence for an intermediate complex or adduct is obtained in this reaction. The pK values for the ionization of the neutral flavin radicals are in the order FAD > FMN > riboflavin = lumiflavin. The rates of reaction of ferricyanide with flavin radicals are essentially independent of pH, whereas benzoquinone reacts slightly more slowly ($\simeq$5 times) with the neutral flavin radical than with the anionic form. Cytochrome c reacts at least ten times more slowly with flavin radicals than does ferricyanide.

Introduction

Radical forms of the flavin coenzymes have been implicated as intermediates in a variety of flavoenzyme catalyzed reactions.[1] These generally function as electron donors to oxidants such as oxygen, cytochromes and quinones. Two such species are known: the neutral radical and the anion radical. These are related by the following equilibrium (only one of the several possible resonance forms is shown):



The pK for the FMN radical has been reported as 8·6.[2] This pK value seems to be quite different for protein-bound flavin radicals, being shifted to considerably higher values in the dehydrogenases and to lower values in the oxidases.[3,4] The flavin radicals are also capable of rapid disproportionation, generating oxidized (F) and fully-reduced (FH_2) species:

$$2FH\cdot \rightleftharpoons F + FH_2$$

This reaction usually does not occur (or occurs only very slowly) in flavoproteins.[4]

Very little information is available concerning the reactivities of the flavin radical forms. Several workers[5–11] have reported values for disproportionation rate constants and have provided evidence for reactions of flavin radicals with oxygen and benzoquinone, but little of a systematic nature has been done and many gaps remain in our knowledge. The present work was undertaken in an attempt to remedy this. Specifically, we have investigated the effect of modification of the flavin ribityl side chain and of pH on disproportionation rates and on rates of reaction of flavin radicals with a variety of oxidants.

Materials and Methods

Lumiflavin was synthesized by the method of Guzzo and Tollin.[12] FMN (B grade), FAD (B grade), riboflavin and cytochrome *c* (equine heart, A grade) were acquired from Calbiochem and used without further purification. *p*-Benzoquinone (Baker grade) was purified by sublimation. Phenol, potassium ferricyanide and EDTA were reagent grade.

Flavin radicals were generated using flash photolysis in the presence of either phenol or EDTA.[10] The flash apparatus, degassing procedures and oxygen measurements were as described earlier.[10] At pH 5–7, where the reactivity of the flavin radical with oxygen is low, solutions were deoxygenated with nitrogen. At higher pH, the solutions were degassed on a vacuum line. Solutions containing cytochrome *c* were also freed from oxygen by purging with nitrogen.

Results and Discussion

When aqueous solutions of flavins are subjected to a high-intensity flash, transients corresponding to the flavin triplet state and the flavin radical are obtained.[5,6,10] If EDTA (or some other hydrogen donor) is also present, the triplet is quenched and a higher yield of radical is produced.[10] It is of interest to compare the yields of these species for various flavin derivatives. In Fig. 1, oscillograms obtained upon flashing solutions of lumiflavin, FMN and FAD in phosphate buffer, pH 7·0, are shown. It is seen that the yields* of radical from lumiflavin and FMN (560 nm traces) are approximately the same, whereas FAD shows essentially no radical formation under these conditions. In the case of the triplet state signals (680 nm traces), lumiflavin gives about twice as much triplet as does FMN, and about thirty times as much as FAD. These triplet yields are rather different from the fluorescence yields,[13,14] which are in the ratio 1:1:0·2. Thus, some quenching at the triplet level must be occurring in FMN and FAD. In our earlier work,[10] we showed that the FMN triplet is quenched intramolecularly, presumably via the side chain. If this process involves hydrogen transfer, it would account for the fact that the radical yield with FMN is approximately the same as with lumiflavin, even though the triplet yields are different. The quenching process in FAD apparently does not involve this type of reaction.

Land and Swallow[7] have studied the kinetics of the disproportionation of the riboflavin radical at different pH values. At pH 5·0, the predominant species is the neutral

* These comparisons are based upon the assumption that the absorptivity of the various species are the same (or closely similar).

radical while at pH 11·4, the anion radical predominates. The second-order rate constants for the disappearance of the riboflavin radical at pH 5·0 and 11·4 were found to be $1{\cdot}14 \times 10^9$ and 7×10^8 $lmole^{-1}$ sec^{-1}, respectively. We have compared the decay of the neutral and anionic forms of the radicals derived from lumiflavin, FMN and FAD by

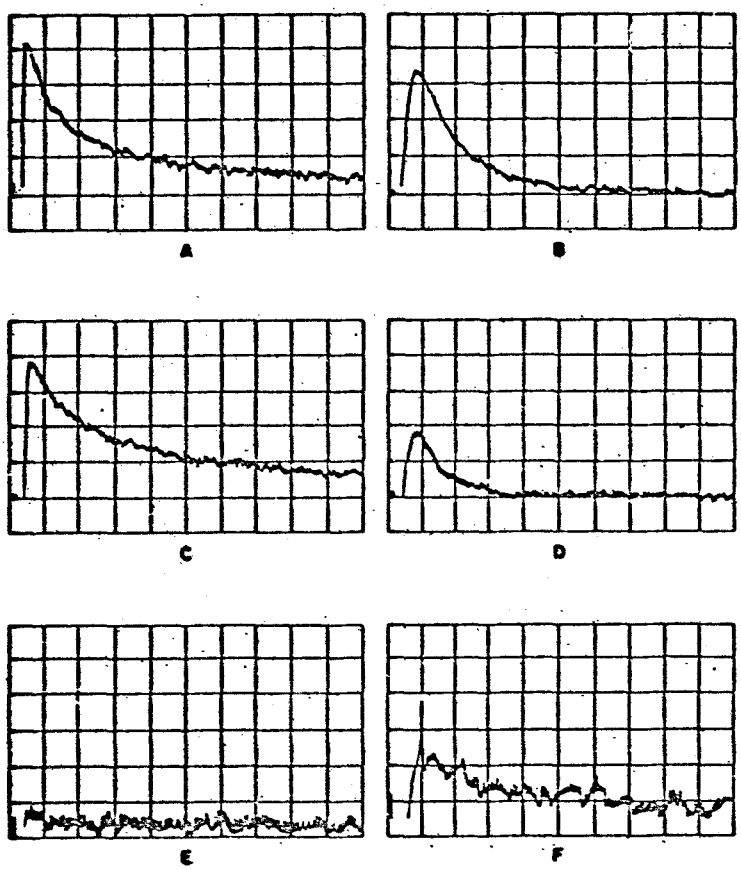


Figure 1. Flash-induced transients obtained from lumiflavin ($1{\cdot}4 \times 10^{-5}$ M), FMN ($1{\cdot}4 \times 10^{-5}$ M) and FAD ($1{\cdot}4 \times 10^{-5}$ M) in 0·025 M phosphate buffer, pH 7·0. A: lumiflavin at 560 nm; ΔA_{max} = 0·10; 0·2 msec per division. B: lumiflavin at 680 nm; ΔA_{max} = 0·20; 0·05 msec per division. C: FMN at 560 nm; ΔA_{max} = 0·11; 0·2 msec division. D: FMN at 680 nm; ΔA_{max} = 0·10; 0·05 msec per division. E: FAD at 560 nm; ΔA_{max} ~ 0; 0·2 msec per division. F: FAD at 680 nm; ΔA_{max} = 0·006; 0·05 msec per division.

flash photolysis in the presence of excess EDTA (Figs. 2, 3 and 4), and have obtained the rate constants shown in Table I.

As is evident from these results, anionic radicals decay more slowly than do the neutral radicals, as was also found for riboflavin.[7] At the same pH, the radicals derived from phosphorylated flavins disproportionate more slowly than that derived from lumiflavin. These differences probably reflect charge effects, i.e., contributions due to electrostatic repulsion. This is further indicated by the larger differences observed at

the higher pH at which an additional negative charge is present in the radical. Furthermore, the differences found between FMN and FAD are probably the result of the intramolecular complexing which is known to exist between the adenine and isoalloxazine

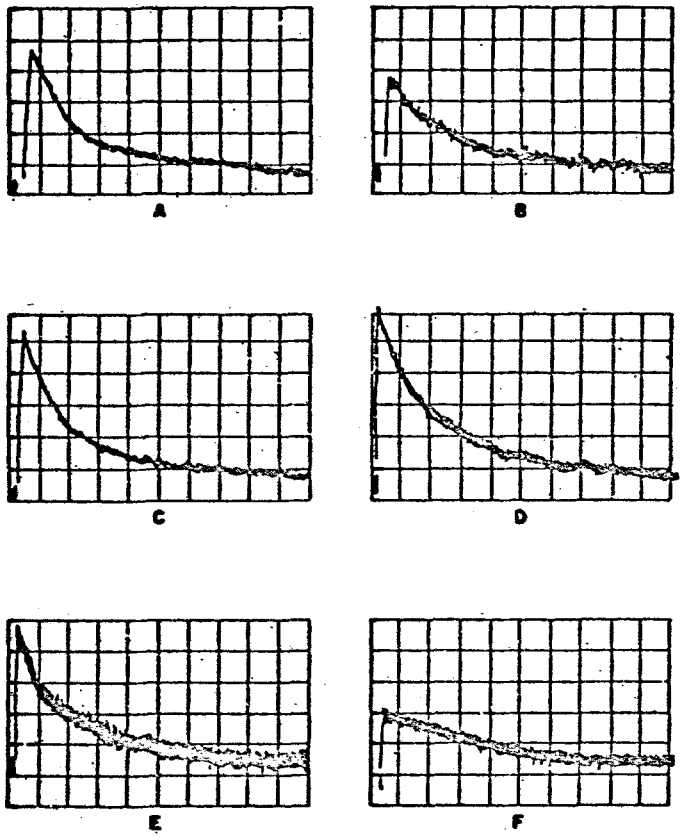


Figure 2. Flash-induced transients obtained from lumiflavin, FMN and FAD in the presence of EDTA. A: lumiflavin ($1{\cdot}2 \times 10^{-5}$ M) + EDTA (1×10^{-3} M) in 0·05 M formate buffer, pH 4·9. 560 nm; $\Delta A_{max.} = 0{\cdot}08$; 0·1 msec per division. B: lumiflavin ($3{\cdot}6 \times 10^{-5}$ M) + EDTA (3×10^{-3} M) in 0·05 M borate buffer, pH 9·6. 510 nm: $\Delta A_{max.} = 0{\cdot}04$; 0·2 msec per division. C: FMN (1×10^{-5} M) + EDTA (1×10^{-3} M) in 0·05 M formate buffer, pH 4·9. 560 nm; $\Delta A_{max.} = 0{\cdot}096$; 0·2 msec per division. D: FMN ($4{\cdot}8 \times 10^{-5}$ M) + EDTA (3×10^{-3} M) in 0·05 M borate buffer, pH 9·6. 510 nm; $\Delta A_{max.} = 0{\cdot}07$; 1 msec per division. E: FAD (5×10^{-5} M) + EDTA (2×10^{-3} M) in 0·05 M formate buffer, pH 4·9. 560 nm; $\Delta A_{max.} = 0{\cdot}03$; 1 msec per division. F: FAD ($4{\cdot}7 \times 10^{-5}$ M) + EDTA (3×10^{-3} M) in 0·05 M borate buffer, pH 9·6. 510 nm; $\Delta A_{max.} = 0{\cdot}03$; 2 msec per division.

rings in the oxidized form of FAD,[13] and which thus probably occurs in the free radical form as well.

In the presence of phenol as a source of hydrogen equivalents,[10] the rate constant (for reverse electron transfer between phenoxy and lumiflavin radicals) at pH = 5·0 is $4{\cdot}7 \times 10^{9}$ lmole^{-1} sec^{-1}. This is not much different than the disproportionation rate constant of $6{\cdot}2 \times 10^{9}$ lmole^{-1} sec^{-1} obtained for lumiflavin radical in the presence of EDTA (Fig. 3).

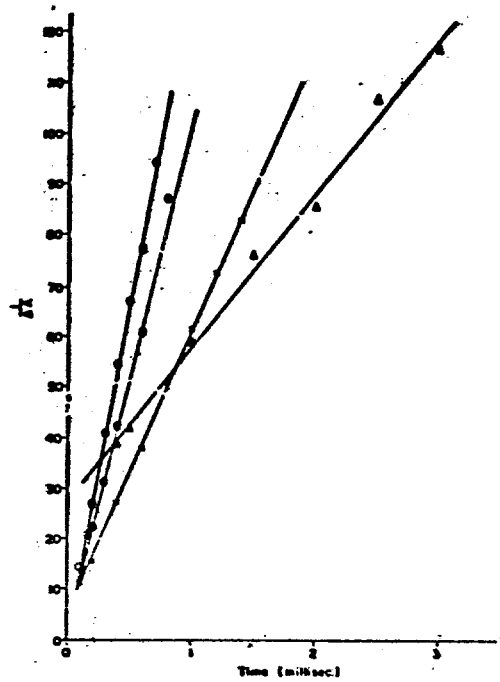


Figure 3. Second-order plots of decay of flavin radicals in 0·05 M formate buffer, pH 5·0. ⊙, lumiflavin ($1{\cdot}2 \times 10^{-5}$ M) + EDTA (1×10^{-3} M); ×, FMN ($1{\cdot}0 \times 10^{-5}$ M) + EDTA (1×10^{-3} M); △, FAD (5×10^{-5} M) + EDTA (2×10^{-3} M); ●, lumiflavin ($1{\cdot}2 \times 10^{-5}$ M) + phenol (1×10^{-3} M). Flash-induced transients were measured at 580 nm. Rate constants were calculated from these data using $\epsilon_{510} = 4700$.

We have also investigated the oxidation of flavin radicals at various pH values using several different oxidizing agents. The apparent second order rate constant (obtained by dividing the pseudo-first-order constant by the concentration of oxidant) for the

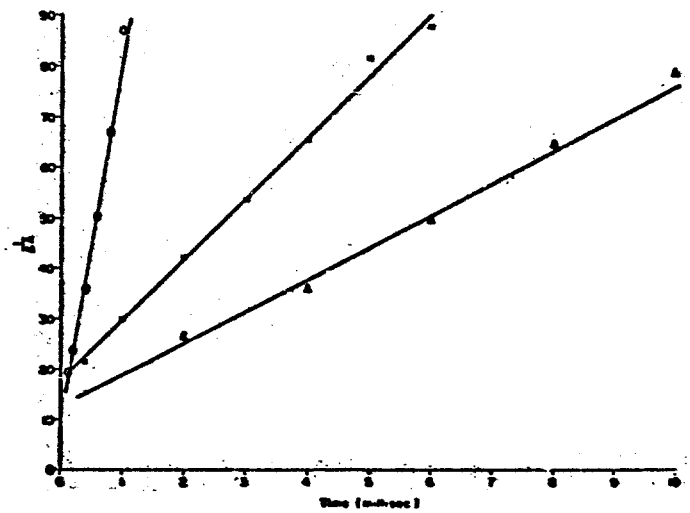


Figure 4. Second-order plots of decay of flavin radicals in 0·05 M borate buffer, pH 9·0. ⊙, lumiflavin ($3{\cdot}3 \times 10^{-5}$ M) + EDTA (3×10^{-3} M); ×, FMN ($2{\cdot}7 \times 10^{-5}$ M) + EDTA (3×10^{-3} M); △, FAD ($3{\cdot}3 \times 10^{-5}$ M) + EDTA (3×10^{-3} M). Flash-induced transients were measured at 510 nm. Rate constants were calculated from these data using $\epsilon_{510} = 1560$.

TABLE I. Second-order rate constants for disproportionation of flavin radicals

	k (lmole^{-1} sec^{-1}) pH 5·0	k (lmole^{-1} sec^{-1}) pH 9·0
Lumiflavin	$6{\cdot}2 \times 10^9$	$1{\cdot}1 \times 10^9$
FMN	$2{\cdot}6 \times 10^9$	$1{\cdot}9 \times 10^8$
FAD	$1{\cdot}9 \times 10^9$	$0{\cdot}95 \times 10^8$

reaction of oxygen with flavin radicals increases with the pH of the solution. This was also observed by Holmström.[9] At pH 5·0, the rate of radical disappearance is not affected by oxygen even when the solution is saturated with this substance.[9] In order to increase the concentration of oxygen still further, a special glass cell was constructed with a glass-to-metal joint which was attached to an oxygen cylinder by Swage-Lok connections. A lumiflavin solution ($1{\cdot}5 \times 10^{-5}$ M) containing 1×10^{-3} M phenol at pH 5·0 was equilibrated with 200 psi of oxygen ($\simeq 1{\cdot}5 \times 10^{-2}$ M) and the resulting solution

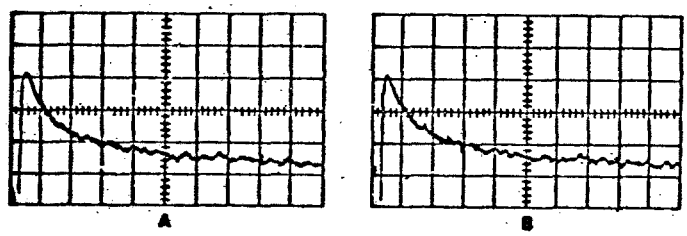


Figure 5. Transients obtained at 580 nm for solutions of lumiflavin ($1{\cdot}5 \times 10^{-5}$ M) and phenol (1×10^{-3} M) in 0·05 M formate buffer, pH 5·0. Each division on the abscissa corresponds to 0·2 msec. A: deoxygenated; B: 200 psi of O_2 pressure.

was flashed. The increased concentration of oxygen caused no observable difference in the radical decay rate (Fig. 5). The transient signal was found to decay by a second-order process with a rate constant of $4{\cdot}4 \times 10^9$ lmole^{-1} sec^{-1}, which is the same as was obtained with oxygen-free solutions.

An upper limit for the rate constant of reaction of the neutral flavin radical at pH 5·0 with oxygen can be calculated from these observations, assuming reverse electron transfer between FH· and phenoxy radical as the major decay path:[10]

$$F_T + \phi OH \rightarrow FH\cdot + \phi O\cdot$$

$$FH\cdot + \phi O\cdot \rightarrow F + \phi OH \qquad (1)$$

$$FH\cdot + O_2 \rightarrow F + O_2^- + H^+ \qquad (2)$$

It is further assumed that a contribution of reaction (2) to an extent of 10% that of reaction (1) could be detected by our methods. Thus:

$$0{\cdot}1k_1[FH\cdot]^2 > k_2[O_2][FH\cdot]$$

reaction (1) reaction (2)

$[FH\cdot] = 1{\cdot}3 \times 10^{-6}$ M (calculated from the peak of the flash-induced transient)
$[O_2] = 1{\cdot}5 \times 10^{-2}$ M
$k_4 = 4{\cdot}4 \times 10^9$ lmole^{-1} sec^{-1}

From these values we can calculate that $k_2 < 3{\cdot}8 \times 10^4$ lmole^{-1} sec^{-1}.

The contribution of the anion radical species to a reaction with oxygen can be analyzed in the following way:

$$FH\cdot \rightleftharpoons F^{\cdot} + H^+ \qquad (3)$$

$$F^{\cdot-} + O_2 \rightarrow F + O_2^{\cdot-} \qquad (4)$$

From these equations, a rate expression can be written:

$$\frac{dx}{dt} = k_4[F^{\cdot-}][O_2] \qquad (5)$$

At a given pH, $[F^{\cdot}_{total}] = [FH^{\cdot}] + [F^{\cdot-}]$. Substituting this into equation (3), we obtain:

$$[F^{\cdot-}] = \frac{[F^{\cdot}_{total}]}{1 + [H^+]/K} \qquad (6)$$

Using this expression for $[F^{\cdot-}]$ and equation (5) we obtain the following rate equation:

$$\frac{dx}{dt} = \frac{k_4[F^{\cdot}_{total}]}{1 + [H^+]/K}[O_2] \qquad (7)$$

From our measurements, a pseudo-first-order rate constant is obtained. If this is divided by the concentration of oxygen, one gets an apparent second-order rate constant (k_{app}). Therefore, from equation (7):

$$k_{app} = \frac{k_4}{1 + [H^+]/K} \qquad (8)$$

This can be arranged to give:

$$\frac{1}{k_{app}} = \frac{1}{k_4} + \frac{[H^+]}{k_4 K} \qquad (9)$$

If the only species which reacts with oxygen is $F^{\cdot-}$, a plot of $1/k_{app}$ versus $[H^+]$ should give a straight line of slope $1/k_4K$ and an intercept of $1/k_4$. At high pH, where $[H^+]$ is very small, k_{app} becomes equal to k_4, which should be the limiting value at a pH sufficiently high for the complete ionization of the neutral radical.

Table II gives the values of k_{app} obtained as a function of the pH of the solution for lumiflavin, riboflavin, FMN and FAD. Figure 6 shows a plot of $1/k_{app}$ versus $[H^+]$ for these compounds. It is seen that straight-line plots are indeed obtained and that all four compounds have approximately the same value for k_4, inasmuch as all of the lines seem to pass through the same intercept. Assuming this, the dissociation constants for the ionization of the neutral semiquinones can be calculated from the slope of the straight lines. These pK values are given in Table III.

The values agree very well with those obtained by other methods. For example, recent potentiometric studies by Draper and Ingraham[3] gave values of 8·3 and 8·6 for the pK's of riboflavin and FMN radicals. According to these workers, the formation

TABLE II. The values of k_{app} versus pH for lumiflavin, riboflavin, FMN and FAD solutions containing 1×10^{-3} M phenol

Lumiflavin								
pH	6·6	6·8	7·0	7·2	7·4	7·83	8·0	
k_{app}	$0{\cdot}82 \times 10^7$	$1{\cdot}39 \times 10^7$	$1{\cdot}82 \times 10^7$	$3{\cdot}49 \times 10^7$	$5{\cdot}16 \times 10^7$	$1{\cdot}21 \times 10^8$	$1{\cdot}6 \times 10^8$	
Riboflavin								
pH	7·0	7·3	7·7	8·01				
k_{app}	$2{\cdot}2 \times 10^7$	$3{\cdot}75 \times 10^7$	$8{\cdot}76 \times 10^7$	$1{\cdot}38 \times 10^8$				
FMN								
pH	6·6	6·8	7·0	7·2	7·3	7·5	7·73	8·0
k_{app}	$0{\cdot}64 \times 10^7$	$1{\cdot}03 \times 10^7$	$1{\cdot}39 \times 10^7$	$2{\cdot}07 \times 10^7$	$3{\cdot}04 \times 10^7$	$4{\cdot}13 \times 10^7$	$5{\cdot}22 \times 10^7$	$8{\cdot}76 \times 10^7$
FAD								
pH	6·7	7·0	7·31	7·71	8·02			
k_{app}	$4{\cdot}4 \times 10^6$	$1{\cdot}13 \times 10^7$	$0{\cdot}85 \times 10^7$	$3{\cdot}4 \times 10^7$	$5{\cdot}06 \times 10^7$			

$k_{app} = k/[O_2]$, where k is the first-order rate constant. The units of k_{app} are $l\,mole^{-1}\,sec^{-1}$

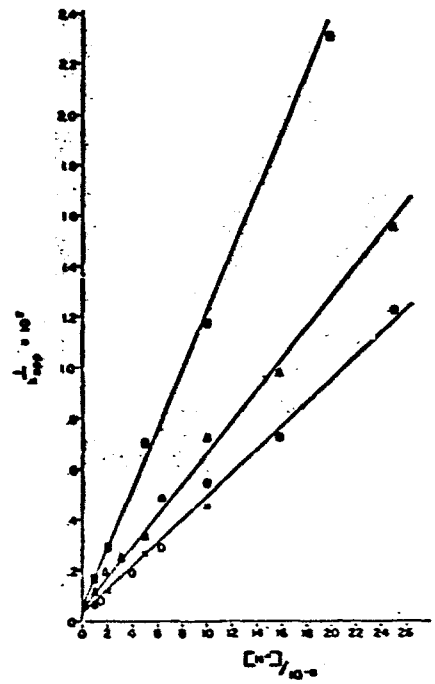


Figure 6. Plot of $1/k_{app}$ versus $[H^+]$ for oxidation of flavin radicals by oxygen in 0·05 M phosphate buffer. $[H^+]$ was varied by adding HCl or NaOH. The oxygen concentration was controlled by bubbling solutions with a known mixture of oxygen and nitrogen. All solutions were $1·4 \times 10^{-5}$ M in flavin and contained 1×10^{-3} M phenol. ⊙, lumiflavin; ×, riboflavin; Δ, FMN; ■, FAD.

of the anionic form of the FMN radical is suppressed due to the electrostatic repulsion effect of the phosphate group. The present work suggests that the adenine ring in FAD provides a further suppression of the proton ionization.

TABLE III. Ionization constants of the neutral radical forms of flavin derivatives

Compound	pK
Lumiflavin and riboflavin	8·36
FMN	8·5
FAD	8·8

The rate constant obtained from the intercept of the plots in Fig. 6 is approximately 3×10^8 $lmole^{-1}$ sec^{-1}. Thus, the neutral flavin radicals react more than 10^4 times less rapidly with oxygen than do the anionic forms. This may be due to the fact that the neutral radicals have a positive charge at the N-5 position which could act to decrease the tendency to lose an electron to an attacking reagent. This would assume that oxygen specifically interacts with flavin radicals at this position. It is interesting that the well-known oxygen stability of neutral flavoprotein radicals[4] is reflected in the properties of the unbound coenzyme. In an evolutionary sense, it would appear that natural selection has utilized this reactivity pattern in generating proteins (dehydrogenases) which specifically stabilize the neutral radical form and others (oxidases) which destabilize it.

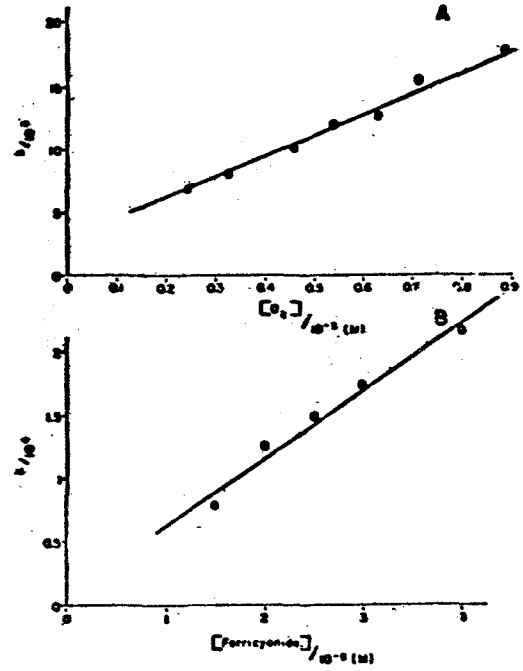


Figure 7. A. Plot of pseudo-first-order rate constants for flavin radical decay versus oxygen concentration, [lumiflavin] = $1{\cdot}4 \times 10^{-5}$ M; [phenol] = $1{\cdot}0 \times 10^{-3}$ M. Reactions run in 0·05 M phosphate buffer, pH 7·0.
B. Plot of pseudo-first-order rate constants for flavin radical decay versus ferricyanide concentration; [lumiflavin] = $1{\cdot}4 \times 10^{-5}$ M, [phenol] = $1{\cdot}0 \times 10^{-3}$ M. Reactions run in 0·05 M formate buffer, pH 5·5.

Gibson and Hastings[16] have observed that the reaction of FH_2 with oxygen proceeds through an intermediate (either an FH_2–O_2 complex or a peroxide). In order to test whether a similar mechanism might occur for the radical reaction, we determined the oxygen concentration dependence of the radical oxidation rate. At a given pH, a plot of the pseudo-first-order rate constants versus the concentration of oxygen gives a straight line (Fig. 7A). Thus, no evidence for an intermediate was obtained. It is, of course, possible that the lifetime of such an intermediate is too short to detect by the present technique (< 50 μsec).

Unlike oxygen, the rate constants for the reaction of ferricyanide with flavin radicals at all pH values are close to 6×10^8 $lmole^{-1}$ sec^{-1} (Table IV). A ferricyanide complex

TABLE IV. Pseudo-first-order rate constants for the reaction of lumiflavin radical with ferricyanide at various pH values

pH	k ($lmole^{-1}$ sec^{-1}) $\times 10^{-8}$
5·0	6·0
6·5	6·3
7·0	5·9
8·0	6·8
9·0	4·6

[Lumiflavin] = $1{\cdot}4 \times 10^{-5}$ M; [Ferricyanide] = 2×10^{-3} M; [Phenol] = 1×10^{-3} M.

apparently does not take part in the reaction, as shown by the straight line obtained when the first-order rate constants are plotted against the ferricyanide concentration (Fig. 7B). It is interesting to speculate on the possible reason for this difference between the reactivity patterns of oxygen and ferricyanide. Electron spin resonance studies[12,17] have shown that an appreciable unpaired spin density occurs in the benzene nucleus of the isoalloxazine ring in flavin radicals (both neutral and anionic). Thus, it is possible that transition metal species such as ferricyanide can abstract an electron from a flavin radical via the pi-electron system of the benzene ring through orbital overlap interaction. This would provide a simple explanation for the lack of dependence of the rate on the state of protonation at N-5.

An attempt was made to determine the rate of reaction of horse heart cytochrome *c* with flavin radicals. However, a solution which is 3×10^{-5} M in this compound (oxidized

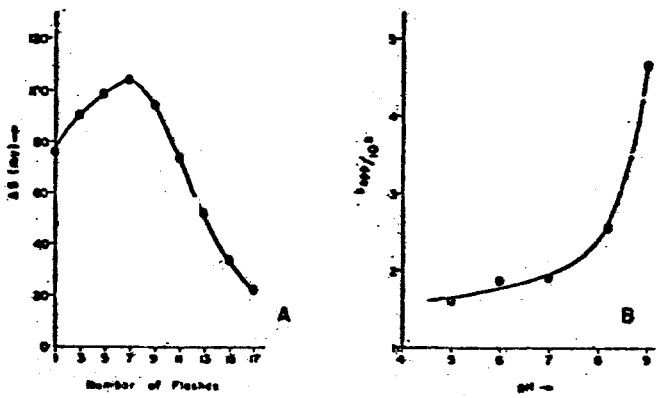


Figure 8. A: Change in ΔS (signal height) with number of flashes sample has received, [lumiflavin] = $1{\cdot}4 \times 10^{-5}$ M, [*p*-benzoquinone] = 5×10^{-3} M, [EDTA] = $1{\cdot}0 \times 10^{-3}$ M. Reaction measured at 560 nm in 0·05 M phosphate buffer, pH 6·0.

B: Rate of radical oxidation versus pH, [lumiflavin] = $1{\cdot}4 \times 10^{-5}$ M, [EDTA] = $1{\cdot}0 \times 10^{-3}$ M, [*p*-benzoquinone] = 5×10^{-3} M at pH 5, 6 and 7, 4×10^{-3} M at pH 8·2, and 3×10^{-3} M at pH 9·0.

form) does not react measurably with the radical at pH 7·0. It is difficult to go to higher cytochrome concentrations in view of its absorption in the 500–600 nm region where the measurements were made. Thus, all that can be said at present is that cytochrome *c* reacts appreciably more slowly (>10 times) with flavin radicals than does ferricyanide.

The rate constants for the oxidation of flavin radical by *p*-benzoquinone increase slowly with the pH of the solution (Fig. 8B). Thus, for this oxidant, the anionic radical reacts somewhat more rapidly ($\simeq 5$ times) than does the neutral radical (k at pH 5·0 is $1{\cdot}6 \times 10^8$ lmole^{-1} sec^{-1} and at pH 9·0 is $4{\cdot}7 \times 10^8$ lmole^{-1} sec^{-1}). It is difficult to obtain a more precise reactivity ratio in this case because of the polymerization of quinone at high pH's.

In Fig. 8A a plot of ΔS (signal height) against the number of flashes at a given pH is shown. ΔS increases at first and then decreases. The decrease in signal is accompanied by a bleaching of the solution, which is due to the formation of fully reduced flavin (in the presence of EDTA). The observed rate of radical decay becomes slower with

an increasing number of flashes until it reaches a constant value (disproportionation). These observations can be interpreted in terms of the following reactions:

$$F_T + AH_2 \rightarrow F^{\cdot}\,(\text{or } FH\cdot) + AH\cdot + H^+$$
$$F^{\cdot}\,(\text{or } FH\cdot) + BQ \rightarrow F + BQ^{\cdot-}(+H^+)$$
$$2H^+ + BQ^{\cdot-} + BQ^{\cdot-} \rightarrow BQ + BQH_2$$
$$2H^+ + 2F^{\cdot}\,(\text{or } 2FH\cdot) \rightarrow F + FH_2$$

Benzoquinone is thus converted to hydroquinone during the course of the reaction and becomes depleted as flashing continues. This causes a temporary increase in the radical yield, which subsequently decreases as F is used up by FH_2 formation.

Acknowledgement

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