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The Interaction of Riboflavin, FMN, and FAD with Various Metal Ions: The Riboflavin Catalyzed Photochemical Reduction of Fe^{III}, and Photooxidation of Fe^{II}

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(1) The fluorescence of riboflavin, FMN, and FAD solutions is quenched by certain divalent and trivalent metal ions. Of the ions tested, Hg^{II} and Fe^{II}, Fe^{III}, Co^{III}, and Au^{III} were particularly effective.

(2) Riboflavin, FMN, and FAD solutions catalyze a photooxidation of Fe^{II} ions and a photoreduction of Fe^{III} ions.

(3) The riboflavin catalyzed photoreduction of Fe^{III} is inhibited by oxygen, while the photooxidation of Fe^{II} is strongly dependent on oxygen. Catalase stimulates both of these processes.

(4) The mechanism of the photochemical reactions and their possible relationship to the function of riboflavin in biological systems is discussed.

In certain flavoprotein systems, the fluorescence of the free riboflavin coenzyme is lost on combination with the apoprotein [1,2]. This fact together with recent observations concerning possible interactions of metal ions in certain flavoprotein systems [3,4], prompted a study of the effect of various metal ions on the fluorescence of riboflavin, FMN, and FAD **. It was found that Fe^{III} was a remarkably effective quencher of the fluorescence of these compounds. An inquiry concerning the mechanism of the quenching phenomenon resulted in the discovery of a riboflavin-catalyzed photoreduction of Fe^{III}, as well as a riboflavin-catalyzed photooxidation of Fe^{II}. It is the purpose of this paper to report these observations, and to discuss their significance relative to the functions of riboflavin in biological systems.

MATERIALS AND METHODS

FMN and FAD were purified by ionophoresis and kindly supplied by B. Rolander. A sample of riboflavin procured from Hoffman-LaRoche was recrystallized for use in these experiments.

Crystalline catalase was procured from Worthington Biochemicals, Inc. Activity was measured by the method of Beers and Sizer [5]. One unit is defined as that amount of

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** Abbreviations used: FMN — flavin mononucleotide; FAD — flavin adenine dinucleotide; EDTA — ethylene diamine tetraacetate.

enzyme which decomposes 1 mg hydrogen peroxide in 1 min under standard assay conditions.

The concentration of Fe^{II} ions was quantitatively determined with the α,α'-dipyridyl reagent [6], usually from the difference spectra of appropriate experimental samples.

The intensity of fluorescence was measured with the instrument devised and constructed by Theorell and Nygaard [7]. Difference spectra were obtained with the aid of a Cary Recording double beam spectrophotometer.

Photoillumination was accomplished by means of a 250 watt lamp fixed approximately 25 cm from the incubation fluid. The entire system was enclosed in aluminum foil, and partially filled with water so the temperature of the sample remained essentially constant during the photoillumination period.

RESULTS

The fluorescence of riboflavin solutions is quenched by a variety of substances including diphenols, purines, and pyrimidines and electrolytes, particularly [8] I^- and Ag^+. Weber showed that the remarkably strong quenching effect of silver ions is apparently due to the formation of a complex with riboflavin which is non-fluorescent.

The quenching action of electrolytes on riboflavin fluorescence is not a simple function of the ionic strength, but is a function of the particular ionic species present. Theorell and Nygaard [9], for example, have found that halogen ions quench the fluorescence of FMN in the order $I^- > Br^- > Cl^- > F^-$, while $SO_4^=$, $PO_4^=$, and CH_3COO^- ions did not quench at the concentrations tested. $CaCl_2$, KCl, NaCl, and NH_4Cl were equally effective as quenching agents.

Our initial experiments were concerned with the effect of various di- and trivalent metal ions on the fluorescence of riboflavin. Table 1 presents certain of the data obtained. Most divalent ions tested were much less effective quenching agents than silver ions in this regard. Mercury, however, was more effective than Ag^+ in quenching the fluorescence of riboflavin. When $mercury^{II}$ salts were added to aqueous solutions of riboflavin, a light pink color was observed. The color change was similar, but much less pronounced than that produced by silver ions.

Of the trivalent metal ions, Al^{III} was ineffective, but Au^{III}, and especially Co^{III}, and Fe^{III} were remarkably powerful quenching agents. Both Co^{III} and Fe^{III} salts were much more effective than silver salts in quenching riboflavin fluorescence. These results were somewhat unexpected since Albert [10] has shown that divalent metals complex much more strongly with riboflavin than their trivalent counterparts. No binding of Fe^{III} by riboflavin was detected by Albert, for example. The present data nevertheless indicate an Fe^{III}-riboflavin reaction in which either a non-fluorescent complex is involved, or in which the lifetime of the excited state is decreased. A plot of the quenching of fluorescence as a function of Fe^{III} concentration is presented in Fig. 1. The solid points represent experimentally obtained values, whereas the solid curve represents the theoretical curve which would be expected if the reaction involved is simply: (Riboflavin + Fe^{III} → [Riboflavin — Fe^{III}]) and the equilibrium constant

$$K = \frac{[\text{Riboflavin}]\,[Fe^{III}]}{[\text{Riboflavin} - Fe^{III}]} = 4.6 \times 10^{-4}$$

Table 1. Quenching of riboflavin fluorescence by salts. The experimental cuvettes contained 300 mμM riboflavin with ions added as indicated. Volume in all cases 3.0 ml. pH adjusted to 5.5—6.0 with $NaHCO_3$, temperature 30°C. The fluorescence is presented as per cent fluorescence of a control cuvette containing riboflavin alone.

Salt	Concentration (M)	Per cent fluorescence
Monovalent cations		
$NaNO_3$	0.003	87
	0.006	79
	0.01	76
$AgNO_3$	0.003	72
	0.006	58
	0.012	43
	0.018	31
Divalent cations		
$CuSO_4$	0.01	100
	0.10	70 *
$CoSO_4$	0.01	0.77
	0.10	0.29
$FeSO_4$	0.003	0.46
	0.10	0.50
$Hg(CH_3COO)_2$	0.0006	60
$Hg(NO_3)_2$	0.0006	54
$MnSO_4$	0.003	99
	0.01	81
$NiCl_2$	0.01	0.86
$Zn(CH_3COO)_2$	0.003	99
	0.01	90
Trivalent cations		
$AlCl_3$	0.01	100
$AuCl_3$	0.0006	58
$CoNaNO_2$	0.0006	28
$FeCl_3$	0.0001	75
	0.0004	50
	0.0006	28

* visible precipitate.

assuming a non-fluorescent complex [Riboflavin — Fe^{III}] or one in which the lifetime of the excited state is very small compared to that of free riboflavin.

The effectiveness of Fe^{III} as a quenching agent suggested that the quenching observed with Fe^{II} ions (abnormally high for divalent compounds) might in part be due to the formation of Fe^{III} ions. It was observed that with low concentrations (<0.003 M) of Fe^{II}, unlike any of the other divalent ions tested,

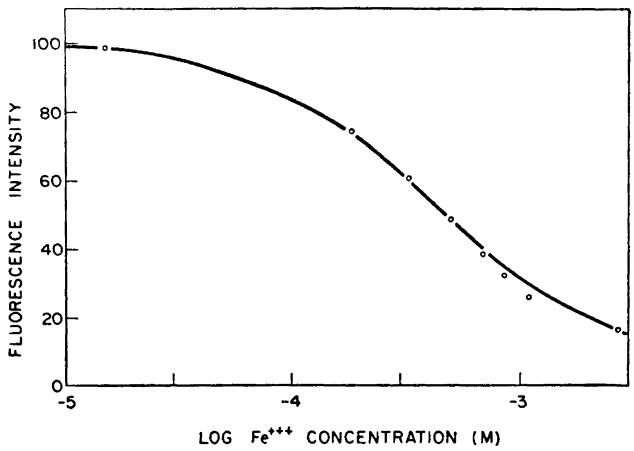

Fig. 1. Quenching of riboflavin fluorescence by Fe^{II} ions. Experimental conditions as described under Table 1.

maximum quenching was obtained only after a considerable time lag (Fig. 2). At higher concentrations of Fe^{II}, the degree of quenching did not change as a function of time; moreover the degree of quenching was approximately the same for 0.003 M and 0.1 M Fe^{II}. Preincubation in the dark did not influence this characteristic behavior. These observations suggest either that Fe^{II} itself is not a particularly effective quenching agent, but that it is converted to Fe^{III} during the course of the measurement of fluorescence, or alternatively that the

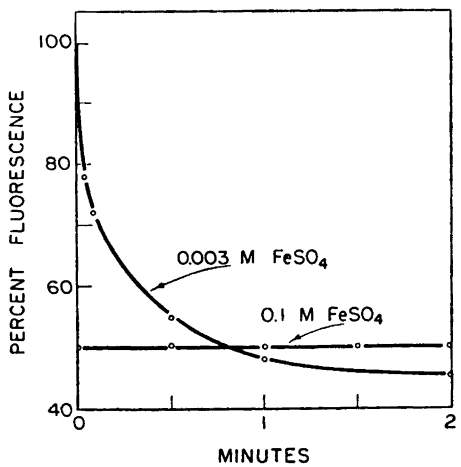

Fig. 2. Quenching of riboflavin fluorescence by Fe^{III} ions. Riboflavin present at a final concentration of 10^{-6} M, $FeCl_3$ added to the concentration indicated, pH adjusted to 5.5 with $NaHCO_3$; temperature 30°C. The per cent of fluorescence of the control riboflavin solution (no $FeCl_3$ added) is plotted as a function of Fe^{III} present.

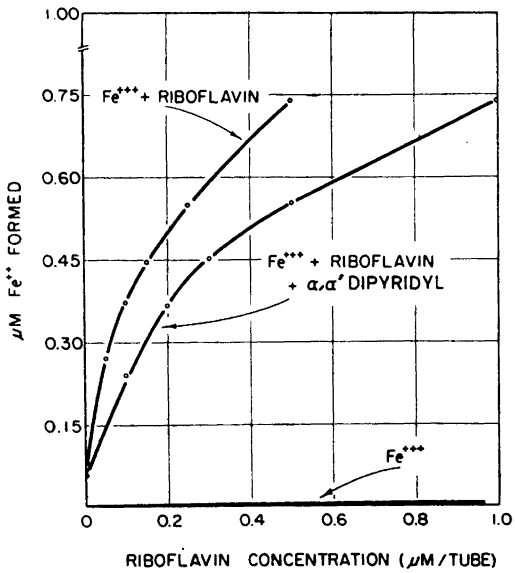

Fig. 3. Riboflavin catalysis of the photoreduction of Fe^{III}. 1 μM $FeCl_3$ was present in all experimental vessels; riboflavin at the concentration indicated; 100 mg α,α'-dipyridyl was added in 0.1 ml ethanol where indicated; pH adjusted to 5.5 in all vessels; period of irradiation, 10 min; temperature 30°C; nitrogen atmosphere. The values for Fe^{II} formation represent increments over Fe^{II} present in similar samples incubated in the dark.

final Fe^{II} riboflavin complex (having about 50 % of the original riboflavin fluorescence) is formed slowly, perhaps through several less stabile Fe^{II} riboflavin complexes.

The other trivalent metal ions which showed inhibition of fluorescence roughly equivalent to Fe^{III}, also showed anomalous quenching behavior. In the experiments with Co^{III} and Au^{III}, the quenching was strongest initially, and decreased with time to a constant level. These observations suggested that a secondary reaction was occurring: one possibility was that these compounds were being partially reduced to the divalent state. Further studies were made with Fe^{III} first, because of the role of Fe in biological systems and secondly, because of the ease of detection of Fe^{II} ions. Appropriate experiments demonstrated that there was indeed a riboflavin-catalyzed photooxidation of Fe^{II}, and a photoreduction of Fe^{III}.

Riboflavin-catalyzed photoreduction of Fe^{III}

After photoillumination of Fe^{III} solutions, an increase in the Fe^{II} concentration was detected in tubes to which riboflavin had been added. As shown in Fig. 3, the rate of formation of Fe^{II} was proportional to the riboflavin present only at comparatively low riboflavin concentrations. The inhibition of Fe^{II} formation by excess α,α'-dipyridyl may be due to complexing with Fe^{III} pre-

Table 2. Effect of oxygen and catalase on riboflavin-catalyzed photoreduction. Experimental cuvettes contained 3 μM $FeCl_3$ and 150 mμM riboflavin and 150 units catalase as indicated; total volume 3.0 ml; pH 5.5; temperature 30°C; illumination period 10 min. The values presented are increments over non-illuminated controls.

Experimental conditions	μM Fe^{II} Formed	
	O_2	N_2
1. a) Fe^{III}	0.04	0.06
b) Fe^{III} + Catalase	0.08	0.085
2. a) Fe^{III} + Riboflavin	0.205	0.500
b) Fe^{III} + Riboflavin + Catalase	0.195	0.595

sent. Fig. 4 shows that the rate of formation of Fe^{II} at a given riboflavin concentration decreases with time. This may be a result either of a conversion of riboflavin to a "less active" form, (*e. g.*, formation of lumiflavin) or perhaps a reflection of approaching the steady state condition.

The data presented in Table 2 illustrate the effect of oxygen and catalase on the photoreduction of Fe^{III} by this reaction. As might be expected, the net formation of Fe^{II} from Fe^{III} is diminished in the presence of oxygen. 10 to

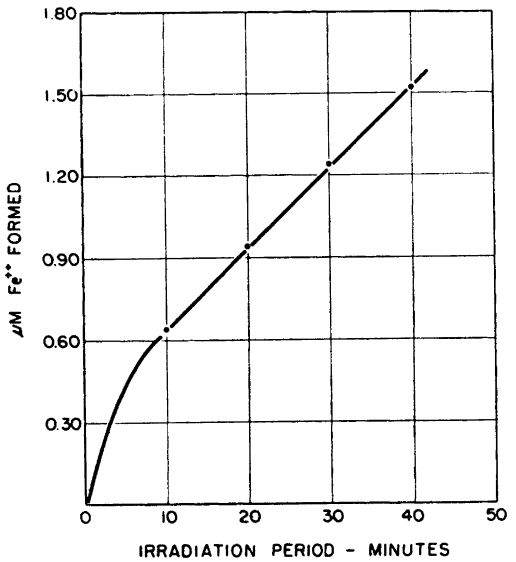

Fig. 4. Riboflavin catalysis of the photoreduction of Fe^{III} as a function of the period of photoillumination. 2 μM $FeCl_3$ and 1 μM riboflavin; 100 mg α,α'-dipyridyl, 0.1 ml 95 % ethanol present in total volume of 3.0 ml; temperature 30.0°C; nitrogen atmosphere. The values of Fe^{II} formed are increments over Fe^{II} present in similar samples incubated in the dark. (Photoillumination of Fe^{III} in the absence of riboflavin for a period of 40 min gives no change over a similar sample incubated in the dark).

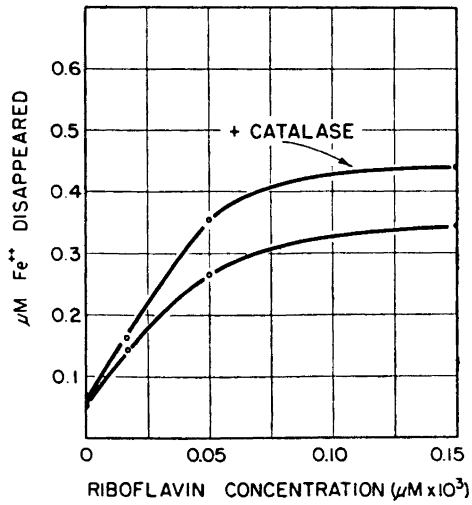

Fig. 5. Effect of catalase on riboflavin catalyzed photooxidation of Fe^{II}. 1 μM $FeSO_4$; riboflavin in the concentration indicated, and sufficient crystalline catalase to decompose 50 mg H_2O_2 per min was added to each sample where indicated; final volume 3.0 ml; pH 5.5; temperature 30°C; oxygen atmosphere; time of photoillumination, 10 min.

20 % increase in Fe^{II} formation were consistently observed in the presence of catalase.

Several attempts to obtain complete conversion of Fe^{III} to Fe^{II} by extended photoillumination periods and by large riboflavin/Fe ratios were unsuccessful. These observations prompted the question of the riboflavin catalysis of the photooxidation of Fe^{II}.

Riboflavin-catalyzed photooxidation of Fe^{II}

The effect of riboflavin on the photooxidation of Fe^{II} salts is shown in Fig. 5. The values represent the difference between illuminated and non-illuminated samples. Catalase consistently stimulated the reaction by 15 to 35 %. The effect of oxygen on the course of the photooxidation is presented in Table 3. It is noteworthy that the absence of oxygen practically eliminates Fe^{II} photooxidation. This observation together with that given in Table 2 for photoreduction of Fe^{III} establishes the conditions under which photoreduction or photooxidation can predominate.

The possibility that riboflavin catalyzed oxidation of Fe^{II} in the dark with oxygen as electron acceptor was tested. Under none of the conditions tested, could any facilitation of this reaction by riboflavin be detected. Even after incubation for 10 h in an oxygen atmosphere there was no difference in the rate of disappearance of Fe^{II} in experimental vessels with riboflavin as compared to controls containing only Fe^{II} ions. Spectral studies of riboflavin in the presence and absence of Fe^{II} ions do not indicate the presence of detectable quantities of reduced riboflavin. During photoillumination there was, of

Table 3. Effect of oxygen on riboflavin-catalyzed photo-oxidation of Fe^{II}. Each experimental cuvette contained 1 μM $FeSO_4$ and 150 mμM riboflavin when present. Total volume 30 ml, pH 5.5, temperature 30°C. The values presented are increments over non-illuminated controls.

Additions	Photoillumination period	Fe^{II} Disappearance (μM)	
		O_2	N_2
1. Fe^{II}	10	0.030	0.045
	30	0.046	0.050
2. Fe^{II} + Riboflavin	10	0.350	0.057
	20	0.437	
	30	0.467	0.067

course, conversion of riboflavin to substances not absorbing in visible region, but this conversion was not markedly influenced by Fe^{II} ions, and bubbling with oxygen did not regenerate the original riboflavin spectra.

FMN and FAD catalyzed photoreduction of Fe^{III} and photooxidation of Fe^{II}

Similar, but not so extensive studies indicate that FMN and FAD also catalyze photooxidation of Fe^{II} and photoreduction of Fe^{III} under similar conditions to those used in the riboflavin experiments.

DISCUSSION

It is known that riboflavin and FMN facilitate photooxidations and photoreductions. Recently Swallow [11] has shown that X-ray irradiation of riboflavin solutions yields a free radical stable in acidic solution, spectrally like the compound produced by chemical reducing agents [12]. Merkel and Nickerson [13] have demonstrated the photochemical reduction of riboflavin salts in aqueous systems containing riboflavin and certain other compounds, *e. g.*, Na_2EDTA, ethylene diamine, cysteine, mercapto succinic, and carboxymethyl succinic acids. Andreae [14] has shown a net formation of Mn^{III} ions upon irradiation of solutions containing Mn^{II}, pyrophosphate, one of a variety of phenols, and riboflavin. Vernon and Ihnen [15] have reported a riboflavin or FMN catalyzed photochemical oxidation of 2,6-dichlorophenol-indophenol and reduced cytochrome c.

The riboflavin catalyzed photochemical oxidation-reduction system reported here is perhaps more easily studied because it is uncomplicated in the sense that a reaction can be observed in aqueous solutions containing only riboflavin and Fe^{II} or Fe^{III}, and because the system is reversible. (This does not necessarily imply that the reduction of Fe^{III} proceeds by a reversal of the same pathway involved in the oxidation of Fe^{II}).

The data obtained are consistent with, but do not prove the postulated series of reactions.

$$R \xrightarrow{h\nu} R^* \quad (1)$$

$$R^* + 2H_2O \xrightarrow{\text{slow}} RH_2 + 2\,{}^{\bullet}OH \quad (2)$$

$$R^* + 2H^+ + 2Fe^{II} \rightarrow RH_2 + 2Fe^{III} \quad (3)$$

$$RH_2 + O_2 \xrightarrow{\text{fast}} R + H_2O_2 \quad (4)$$

$$RH_2 + 2Fe^{III} \xrightarrow{\text{fast}} R + 2Fe^{II} + 2H^+ \quad (5)$$

overall reaction for oxidation of Fe^{II}

$$2H^+ + 2Fe^{II} + O_2 \xrightarrow{h\nu} 2Fe^{III} + H_2O_2 \quad (6)$$

overall reaction for reduction of Fe^{III}

$$2H_2O + 2Fe^{III} \xrightarrow{h\nu} 2Fe^{II} + H_2O_2 + 2H^+ \quad (7)$$

(R = riboflavin; RH_2 = reduced riboflavin; $h\nu$ = photoelectric energy)

The reactions listed should be considered only as one possible formal description of the observations reported.

The interesting observations of Merkel and Nickerson mentioned earlier can perhaps be most easily interpreted in terms of a reaction like (3) above in which the metal chelate acts as an electron donor to riboflavin in the presence of light-energy.

The possible relationship of the present photochemical reactions to the function of riboflavin in biological systems is worthy of consideration. The present observations are most readily explained in terms of a direct electron transference between riboflavin and Fe, and as such, are pertinent to the proposed mechanisms of action of certain of the flavoproteins. The possibility of a role of Fe and riboflavin in photobiologic processes should also not be overlooked.

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