Interactions of Transition-Metal Ions with Photoexcited States of Flavins. Fluorescence Quenching Studies

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Abstract: The influence of 13 metal ions upon fluorescence spectra and quantum yields of riboflavin in acidic aqueous media is described. Those ions which quench riboflavin fluorescence do so without appreciably altering the fluorescence band envelope. The quenching data are interpreted in terms of possible mechanisms. Previous explanations for metal-ion quenching of riboflavin fluorescence are demonstrated to be invalid. Evidence is described which indicates that at least two ions, Fe(II) and Cr(III), act as energy-transfer acceptors in the presence of excited singlet riboflavin, and it is inferred that electronic energy transfer may be an important quenching mechanism in this system. The possibility that other quenching phenomena (heavy-atom or "paramagnetic" perturbations; charge transfer) are important for certain specific metal ions cannot, however, be rejected.

Because metal-containing flavoproteins play important roles in biological oxidation processes, interactions of flavins with metal ions have received considerable study. Hemmerich and coworkers have demonstrated[2] that, although flavosemiquinones complex with a variety of metal ions, the parent flavins form detectable complexes only with strongly reducing ions (*e.g.*, Cu^+). Flavin–metal complexes presumably involve extensive charge transfer from metal d orbitals to flavin π^* orbitals. It has therefore been predicted[2b] that (π,π^*) electronically excited states of flavins should interact much more strongly than the ground state with metal ions. Despite continued interest in flavin–metal interactions and the variety of photoresponsive behavior exhibited by flavins (fluorescence, phosphorescence, photochemical reactions, and chemiluminescence),[3] no recent study of the influence of metal ions upon flavin excited-state processes has appeared.

In 1950, Weber[4] observed quenching of riboflavin fluorescence by Ag^+, which was attributed to ground-state complexation[5a] (Ag^+ is known to complex with ground-state riboflavin[6]). In 1958, Rutter[7] examined several metal ions as quenchers of riboflavin fluorescence in aqueous media and interpreted his findings in terms of ground-state complexation. Since the metal ions studied by Rutter do not complex strongly with ground-state riboflavin,[2] that conclusion requires reexamination; furthermore, several aspects of the experimental techniques introduced significant uncertainty in the results. First, the riboflavin concentrations were sufficiently large (*ca.* 10^{-4} *M*) that errors in fluorescence efficiency determinations could have occurred *via* reabsorption.[5b] Second, no corrections for inner filter absorption of exciting or fluorescent light were reported, though most metal ions absorb in the visible. Third, a uniform anion was not employed; NO_3^-, SO_4^{2-}, Cl^-, and acetate were present in different experiments. Each anion complexes with transition-metal ions in aqueous media and may also quench riboflavin fluorescence. Finally, the solutions were buffered with $NaHCO_3$; bicarbonate quenches riboflavin fluorescence. No studies of metal-ion quenching of riboflavin fluorescence have been reported since 1958. In the present work, we have exercised care to minimize uncertainties in fluorescence measurements from these factors, as well as from thermal reactions of riboflavin or its photodecomposition products with metal ions.

Experimental Section

Materials. Riboflavin was recrystallized three times from aqueous solutions 1 *M* in acetic acid and 10 μM in EDTA. The third recrystallization was performed in the dark; the purified solid was dried at room temperature and stored in the dark. Metal perchlorates were recrystallized from dilute aqueous perchloric acid until supernatants yielded negative tests for Cl^-. Rhodium(III) perchlorate,[8] vanadium(III) perchlorate,[9] potassium trisoxalatoferrate(III),[10] and monothiocyanatopentamminechromium(III) perchlorate[11] were prepared and purified by literature procedures. Fluorescein was purified by the method of Zenk.[12] Water was doubly distilled from alkaline permanganate.

Solutions employed in fluorescence measurements were 1.7×10^{-6} *M* in riboflavin (at 445 nm, the excitation wavelength for most fluorescence measurements, the absorbance was less than 0.02 (1-cm cell) and reabsorption of fluorescence was therefore negligible[6b]) and contained only metal perchlorate salts. The pH was adjusted to 2.6 by addition of concentrated $HClO_4$ or NaOH; no buffer salts were used. Under these conditions, all metal ions (except Fe^{3+} [13]) should have been present predominately as aquo cations.[14] Stock solutions were stored in the dark and were reprepared and restandardized weekly. In the case of reducing ions, such as Fe^{2+}, stock solutions were prepared immediately prior to performance of an experiment.

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(2) (a) P. Hemmerich, C. Veeger, and H. C. S. Wood, *Angew. Chem., Int. Ed. Engl.*, 4, 671 (1965); (b) P. Hemmerich, F. Müller, and A. Ehrenberg in "Oxidases and Related Redox Systems," Vol. 1, T. E. King, H. S. Mason, and M. Morrison, Ed., Wiley, New York, N. Y., 1965, p 157; (c) P. Hemmerich and J. Spence in "Flavins and Flavoproteins," E. C. Slater, Ed., Elsevier, Amsterdam, 1966, pp 82–95.

(3) G. R. Penzer and G. K. Radda, *Quart. Rev., Chem. Soc.*, 21, 43 (1967).

(4) G. Weber, *Biochem. J.*, 47, 114 (1950).

(5) C. A. Parker, "Photoluminescence of Solutions," Elsevier, Amsterdam, 1968: (a) pp 73–74; (b) pp 225–226; (c) pp 226–229; (d) pp 262–263.

(6) I. F. Baarda and D. E. Metzler, *Biochim. Biophys. Acta*, 50, 463 (1961).

(7) W. J. Rutter, *Acta Chem. Scand.*, 12, 438 (1958).

(8) W. Plumb and G. S. Harris, *Inorg. Chem.*, 3, 542 (1964).

(9) W. Kruse and D. Thusius, *ibid.*, 7, 464 (1968).

(10) C. G. Hatchard and C. A. Parker, *Proc. Roy. Soc., Ser. A*, 235, 518 (1956).

(11) E. Zinato, R. D. Lindholm, and A. W. Adamson, *J. Amer. Chem. Soc.*, 91, 1076 (1969).

(12) M. H. Zenk, *Z. Pflanzenp.*, 56, 122 (1967).

(13) L. G. Sillén, *Quart. Rev., Chem. Soc.*, 13, 146 (1959).

(14) As much as 6% of "$V(H_2O)_6^{3+}$" may have been present as $V(H_2O)_5OH^{2+}$ at pH 2.6: L. Pajdowski and B. Jezowska-Trzebiatowska, *J. Inorg. Nucl. Chem.*, 28, 443 (1966).

Methods. Fluorescence spectra were obtained with an Aminco-Bowman spectrophotofluorometer equipped with RCA 1P21 or 7102 photomultipliers. The excitation wavelength was 445 nm unless the metal ion under study absorbed strongly at that wavelength (*e.g.*, Cr^{3+} absorbed at 445 nm and solutions containing that ion were excited at 490 nm). Front-surface excitation was employed to minimize inner filter effects.[5c] For solutions in which inner filter absorption was unavoidable, right-angle illumination was employed and the fluorescence spectra were corrected by the method of Parker and Barnes.[15] Fluorescence spectra were corrected for variations with wavelength in source intensity, photomultiplier response, and monochromator throughput.[16] The sample chamber was thermostated at 30.0 ± 0.1°.

Following measurement of fluorescence, an absorption spectrum of each solution was obtained promptly, using a 10-cm cell. The absorption spectra were generally indistinguishable from superimposed spectra of individual solutions of metal ion and riboflavin at the same concentrations at pH 2.6. For solutions in which this was not true, because of thermal reactions of metal ion with riboflavin or photolysis of either riboflavin or metal ion, the fluorescence data were rejected. When necessary, solutions were degassed[16] by six freeze–thaw cycles at pressures no greater than 2×10^{-4} Torr; luminescence due to impurities distilled from rubber O rings in the Teflon stopcocks[17] was not detected.

Fluorescence quantum yields were determined by the comparative procedure[5d] using fluorescein (ϕ_F = 0.87 ± 0.01[18]) as standard. Fluorescence decay times were measured with a TRW "Nanosecond Spectral Source." Flash photolyses were performed with a commercial apparatus (Xenon Corp., Model 720), using a Spex 1702 analyzing monochromator. The apparatus could be operated in either a decay-kinetics or flash-spectroscopy mode; it was capable of maximum flash energies of 2000 J and a time resolution (using 200-J photolysis flash) of 10 μsec.

In steady-state photochemical sensitization experiments, light from a 2500-W xenon lamp was dispersed by a 500-mm Bausch and Lomb grating monochromator; the band width was 7 nm. The cell compartment temperature was maintained at 5.0 ± 0.5°. In studies of riboflavin-sensitized photoaquation of $Cr(NH_3)_5NCS^{2+}$, free NH_4^+ was determined colorimetrically.[11] Ferrioxalate actinometry[10] was employed.

Results

In the absence of quenchers, solutions of riboflavin in dilute aqueous $HClO_4$ (pH 2.6) fluoresced; the corrected frequency maximum was 18,300 cm^{-1}, the quantum efficiency was 0.25 ± 0.01, and the decay time was 4.7 ± 0.4 nsec. These fluorescence parameters agree well with published data.[4,19] In the presence of Cr^{3+}, Fe^{3+}, Fe^{2+}, V^{3+}, Cu^{2+}, Ce^{3+}, Rh^{3+}, Ni^{2+}, and Co^{2+}, the riboflavin fluorescence was quenched. No discernible changes in the maximum or shape of the fluorescence spectrum accompanied quenching, and none of the ions effected detectable change in the riboflavin absorption spectrum. The quenching behavior generally adhered to the Stern–Volmer equation (1). The

$$\frac{\Phi_F^\circ}{\Phi_F} - 1 = K[Q] = k_Q\tau_0[Q] = \frac{\tau_0}{\tau} - 1 \qquad (1)$$

Stern–Volmer constants (K) for Ni^{2+}, Fe^{3+}, Fe^{2+}, and Cr^{3+} were equal, within experimental error, whether obtained from quantum yields or decay times (*cf.* Figure 1). Because the precision of decay-time measurements was relatively low, all quenching constants were evaluated from quantum-yield data. Degassing of solutions had no effect upon fluorescence yields, either in the absence or presence of quenchers. From the

(15) C. A. Parker and W. J. Barnes, *Analyst*, **82**, 606 (1957).
(16) M. Goldman and E. L. Wehry, *Anal. Chem.*, **42**, 1178 (1970).
(17) J. T. Dubois and F. Wilkinson, *Appl. Spectrosc.*, **18**, 27 (1964).
(18) W. R. Dawson and M. W. Windsor, *J. Phys. Chem.*, 72, 3251 (1968).
(19) R. F. Chen, G. G. Vurek, and N. Alexander, *Science*, **156**, 949 (1967); A. Bowd, P. Byrom, J. B. Hudson, and J. H. Turnbull, *Photochem. Photobiol.*, **8**, 1 (1969).

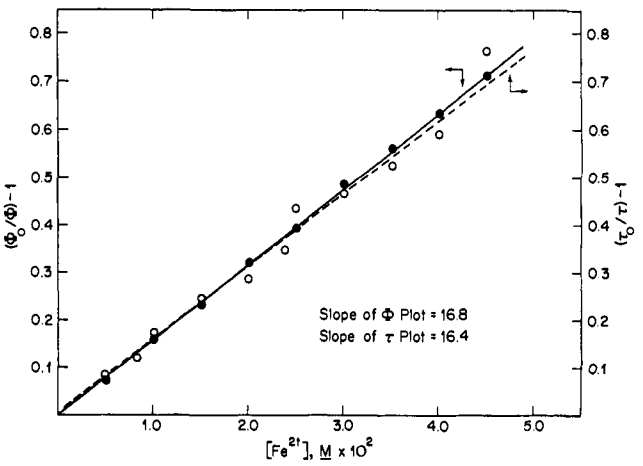

Figure 1. Stern–Volmer plot (——, quantum-yield data; ----, decay-time data) for quenching of riboflavin fluorescence by Fe^{2+}.

experimental quantum yield and decay time for riboflavin fluorescence in the absence of quencher, the bimolecular rate constant, k_Q, for quenching was computed from slopes of the Stern–Volmer plots (Table I).

Table I. Quenching of Riboflavin Fluorescence by Metal Ions[a]

Ion	K, l. mol^{-1}	k_Q, M^{-1}, sec^{-1}, × 10^{-9}	D, cm^2 sec^{-1}, × 10^6 [b]	M[c]	Z	IP, eV[d]
Fe^{3+}	36.0	7.7	*e*	*e*	26	*e*
V^{3+}	21.5	4.6		3	23	48
Cr^{3+}	17.0	3.6	6.0	4	24	49.6
Fe^{2+}	16.8	3.6	7.1	5	26	30.7
Co^{2+}	15.2	3.2	7.3	4	27	33.5
Cu^{2+}	5.6	1.2	7.5	2	29	36.8
Rh^{3+}	3.0	0.64		1	45	
Ni^{2+}	2.6	0.55	7.2	3	28	36.2
Ce^{3+}	0.6	0.13	6.2	2	58	
Mn^{2+}	<0.2	<0.04	7.1	6	25	33.7
Zn^{2+}	<0.2	<0.04	7.0	1	30	39.7
Ba^{2+}	<0.2	<0.04	8.5	1	56	
Pb^{2+}	<0.2	<0.04		1	82	31.9

[a] In aqueous solution; pH = 2.6; temperature = 30.0 ± 0.1°. [b] Diffusion coefficient for aquo ion, calculated from Nernst limiting equation (L. Meites, "Polarographic Techniques," 2nd ed, Wiley-Interscience, New York, N. Y., 1965, pp 144–145). [c] Multiplicity of ground-state aquo ion. [d] Ionization potential; data from C. S. G. Phillips and R. J. P. Williams, "Inorganic Chemistry," Vol. 1, Oxford, London, 1965, pp 58–59. [e] No entries listed for Fe^{3+} due to hydrolysis at pH 2.6.

It was observed that k_Q increased with temperature (Table II); apparent activation energies were in the

Table II. Influence of Temperature upon Quenching Rate Constants for Selected Ions

Ion[a]	Value of k_Q, M^{-1} sec^{-1}, × 10^{-9} at 24°	40°	55°	70°	Apparent activation energy, kcal mol^{-1}
Fe^{2+}	3.6	4.7	6.3	8.1	3.3
Cu^{2+}	1.2	1.6	2.0	2.3	3.0
Cr^{3+}	3.6	4.8	5.9	7.3	1.8
Ni^{2+}	0.55	0.60	0.71	0.78	1.5
Co^{2+}	3.4	4.2	5.0	5.9	1.4

[a] At pH 2.6; concentration of metal ion = 0.05 M; riboflavin concentration = 1.7×10^{-6} M.

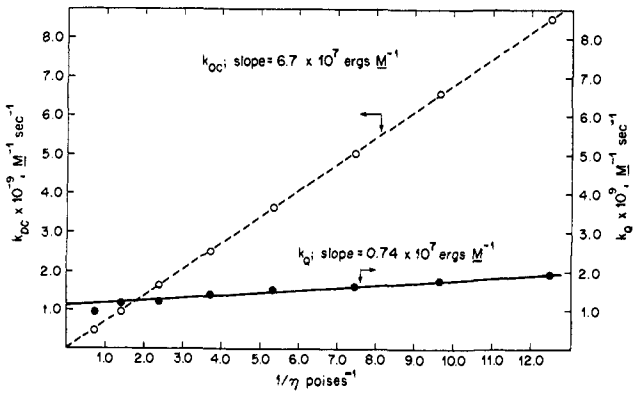

Figure 2. Variation of k_Q for Ni^{2+} (——) and k_{DC} from eq 2 (---) with $1/\eta$ in glycerol–water mixtures.

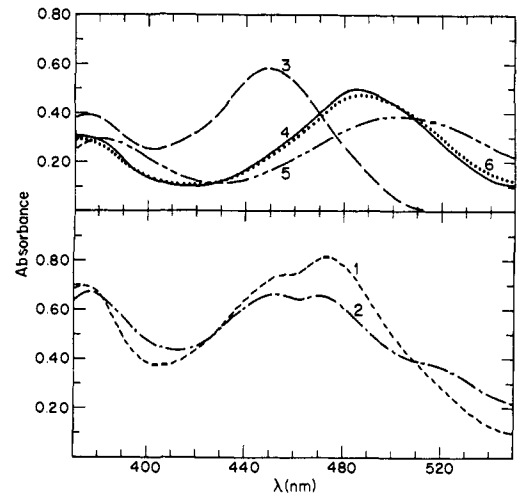

Figure 3. Spectral changes produced by irradiation of oxygen-containing solutions 1.22×10^{-2} M in $Cr(NH_3)_5NCS^{2+}$ and 7.66×10^{-5} M in riboflavin (pH = 4.0): curve 1, absorption spectrum of original (t = 0) solution; curve 2, spectrum after 10-min irradiation; curve 3, spectrum of riboflavin alone (t = 0); curve 4, spectrum of $Cr(NH_3)_5NCS^{2+}$ alone (t = 0); curve 5, subtraction of curve 3 from curve 2 (*i.e.*, spectrum of Cr(III) complex after 10-min irradiation in presence of riboflavin); curve 6, spectrum of solution containing *only* $Cr(NH_3)_5NCS^{2+}$ after 10-min irradiation. For all irradiations, λ = 425 nm; bandwidth = 7 nm; $I_0 \cong 7 \times 10^{16}$ photons sec^{-1}. No detectable changes in riboflavin spectrum (curve 3) were produced by 10-min irradiation unless $Cr(NH_3)_5NCS^{2+}$ was also present.

1.5–3 kcal mol^{-1} range. Stern–Volmer plots remained linear at elevated temperatures; no changes in the shapes of riboflavin absorption or fluorescence spectra were detected. The k_Q values decreased with increasing viscosity in glycerol–water mixtures at 30°; Figure 2 illustrates the data for quenching by Ni^{2+}.

Steady-state irradiation (λ = 480 nm) of degassed solutions 5.0×10^{-2} M in $Fe(ClO_4)_2$ and 8.0×10^{-5} M in riboflavin at pH 2.6 for 15 min resulted in *ca.* 10% conversion of Fe(II) to Fe(III). Blank (unirradiated) solutions decomposed much less rapidly, and the rate of inner filter photooxidation of Fe^{2+} at 480 nm was also slow relative to the rate of generation of Fe(III) in solutions containing riboflavin. Because the "catalysis" occurred at short exposure times, prior to appreciable destruction of riboflavin, it was concluded that photooxidation of Fe^{2+} was sensitized by riboflavin.

It was also observed that, in aqueous solutions (pH 2.6) containing both riboflavin and $Cr(NH_3)_5NCS(ClO_4)_2$ at 5°, sensitized photoaquation of the Cr(III) complex occurred. Spectral changes observed in a typical experiment are depicted in Figure 3. Under the conditions of the experiment, no detectable quantity of riboflavin was destroyed. Spectral changes for the Cr(III) species closely resembled those observed in direct photoaquation,[11] and significant quantities of ammonia were released, demonstrating that photoaquation,[11] rather than primarily thermal aquation, occurred. While inner filter photoaquation of $Cr(NH_3)_5NCS^{2+}$ took place, the aquation rate was enhanced considerably in the presence of riboflavin. The efficiency of sensitized aquation was approximately 15% (*i.e.*, the number of molecules of aquation products formed per unit time was *ca.* 15% as large as the number of excited singlet riboflavin molecules quenched per unit time). In comparison, the quantum yield for direct photoaquation of $Cr(NH_3)_5NCS^{2+}$ is on the order of 0.40.[11]

Discussion

An approximate relationship[20] for the bimolecular rate constant of diffusion-controlled quenching (ignoring transient diffusion phenomena[21]) is

$$k_{DC} = \frac{8RT}{2000\eta} \qquad (2)$$

In aqueous media at 30°, k_{DC} should be on the order of 8×10^9 M^{-1} sec^{-1}. While the most effective quenching ions (Fe^{3+}, V^{3+}, Fe^{2+}) quench at rates comparable to diffusion control, weakly quenching ions (Cu^{2+}, Rh^{3+}, Ni^{2+}) exhibit k_Q values an order of magnitude smaller than k_{DC}. We observe that k_Q (Figure 2) increases linearly with $1/\eta$ (eq 2) in glycerol–water mixtures, but the slope is smaller than that predicted by eq 2 by a factor of ten. For most ions, k_Q is approximately equal to k_{DC} for high viscosities but deviates markedly at lower viscosities. These observations resemble those reported for triplet–triplet energy transfer by Wagner and Kochevar.[22] Increased temperature also enhances the quenching ability of the metal ions, though the apparent activation energies (~1–3 kcal mol^{-1}) are smaller than that for diffusion-controlled quenching in aqueous media (4.3 kcal mol^{-1} [23]). If the data in Table II are plotted as k_Q *vs.* $1/\eta$, the slope is essentially equal to that of a k_Q *vs.* $1/\eta$ plot for the same ion in water–glycerol mixtures, implying that the principal effect of temperature upon k_Q is to decrease the solvent viscosity. Furthermore, in ethanolic glasses at 77°K, metal ions quench riboflavin fluorescence only when present in high concentration (>0.1 M). In aqueous media, quenching of riboflavin fluorescence by metal ions thus appears to require collisional interaction, but not all collisions appear to result in quenching.

Several distinct processes, including ground-state complexation,[7] collisional conversion of electronic to kinetic energy,[3] heavy-atom effects,[24] magnetic perturbations,[25,26] charge-transfer phenomena,[27] and elec-

(20) A. D. Osborne and G. Porter, *Proc. Roy. Soc., Ser. A*, **284**, 9 (1965).
(21) W. R. Ware and J. S. Novros, *J. Phys. Chem.*, **70**, 3246 (1966).
(22) P. J. Wagner and I. Kochevar, *J. Amer. Chem. Soc.*, **90**, 2232 (1968).
(23) J. Feitelson and N. Shaklay, *J. Phys. Chem.*, 71, 2582 (1967).
(24) S. K. Lower and M. A. El-Sayed, *Chem. Rev.*, **66**, 199 (1966).
(25) P. Yuster and S. I. Weissman, *J. Chem. Phys.*, **17**, 1182 (1949).
(26) B. Brocklehurst, *Radiat. Res. Rev.*, 2, 149 (1970).

tronic energy transfer[28] have in the past been invoked to rationalize fluorescence quenching by transition-metal ions. Metal-ion complexation with the ground state of riboflavin[7] cannot be regarded as a general quenching mechanism. Detailed studies[2,6] have failed to reveal formation of soluble complexes between ground-state riboflavin and Fe^{3+}, Fe^{2+}, Cr^{3+}, Co^{2+}, Cu^{2+}, or Ni^{2+} in acidic aqueous media; each of these ions quenches riboflavin fluorescence. Assuming that ground-state complexes were nonfluorescent, their formation constants would equal the measured Stern–Volmer constants (Table I);[5a] complexes of this stability should be detectable. The observations that k_Q increases with temperature, and that k_Q values obtained from decay-time and quantum-yield data are equal, mitigate against ground-state complexation as a quenching process.

Penzer and Radda[3] proposed that "electrolytes, including most metal ions" quench fluorescence of riboflavin by a collisional process wherein electronic energy of the excited flavin is converted to kinetic energy of the colliding species. In this mechanism, specific electronic characteristics of an ion are irrelevant in determining its quenching ability; its mass and diffusion coefficient are the critical parameters. No correlation exists between values of k_Q and diffusion coefficients for the metal ions (Table I); note the wide variations in k_Q for Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}, all of which have essentially equal hydrated radii, diffusion coefficients, and masses. We therefore exclude the Penzer–Radda mechanism from further consideration. Likewise, "external heavy-atom effects"[24] do not appear important in this system, for we observe no correlation between the quenching ability of a metal ion and its atomic number (even Pb^{2+} ($Z = 82$) is an inefficient quencher). Similarly, we note in Table I that, while the efficient quenchers are all paramagnetic, correlations between k_Q and the multiplicities of the ions do not exist, as would be anticipated if a "magnetic" perturbation[25] were responsible for the observed quenching.

A direct test of heavy-atom and paramagnetic quenching mechanisms would consist of measurements of intersystem crossing efficiencies for riboflavin in the absence and presence of quenching ions. The two principal techniques employed for this purpose are, however, inapplicable. Flash photolysis cannot be applied because the riboflavin triplet cannot be detected with microsecond-resolution flash apparatus;[29] triplet–triplet energy transfer[30] fails because the acceptor triplets are susceptible to metal-ion quenching. While we cannot therefore rigorously exclude these mechanisms for specific ions, we conclude that neither satisfactorily rationalizes the body of available data.

The results presented in Table I, pertaining to singlet quenching, resemble those obtained in previous studies of triplet quenching by metal ions.[31] Specifically, Mn^{2+} and Ce^{3+} are weak quenchers, whereas other first-row transition-metal ions effectively quench both singlets and triplets. These observations may be related to the stability of the half-filled shell of Mn^{2+}[32] and the fact that the unpaired electrons in Ce^{3+} are located in f orbitals which may overlap poorly with riboflavin orbitals. It is therefore conceivable that quenching ions engage in charge-transfer interaction[27,33] with the excited riboflavin singlet, increasing the radiationless decay rate. Riboflavin forms complexes in its ground state with electron-donating ions; because the electron affinity of excited riboflavin should be greater than that of the ground state, one might predict that reducing ions should be the most effective quenchers of riboflavin fluorescence by electron transfer from a metal d orbital to a riboflavin π orbital.

In Table I, we note that the quenching abilities of metal ions do not satisfactorily correlate with their ionization potentials. Quenching data for Fe^{3+} are difficult to interpret because of hydrolysis, but none of the hydrolysis products is expected to be strongly reducing. Similarly, Cr^{3+} is not a reducing cation. Many of the other efficient quenchers, in particular V^{3+}, Fe^{2+}, and Co^{2+}, are reducing agents; the inefficient quenchers among the transition-metal ions (Cu^{2+}, Ni^{2+}, Rh^{3+}, and Mn^{2+}) are not reducing species. The quenching inefficiency of Ce^{3+} is not inconsistent with a charge-transfer mechanism because the unpaired electrons in that ion are located in f orbitals.

No direct evidence for charge-transfer interaction of excited riboflavin with metal ions has been detected. Charge-transfer interaction between an excited organic molecule and a quencher can lead to formation of a solvated ion pair, if the electron transfer is essentially complete, or to production of an "exciplex" in which electron transfer is incomplete. In the latter case, the "exciplex" may be fluorescent; if so, its fluorescence spectrum should be different from that of the unperturbed fluorophore.[27,33] We have found no evidence for a fluorescent exciplex between excited riboflavin and metal ions; no alterations in the fluorescence band envelope are introduced by metal ions; no additional fluorescent species are detected in solutions containing riboflavin and quenching ions under either steady-state or flash excitation.

If "complete" electron transfer from a metal ion to excited riboflavin took place, a transient "half-reduced" riboflavin species might be detectable by flash spectroscopy.[23] In flash spectroscopic experiments with degassed riboflavin solutions containing no metal ions, using filtered 500-J photolysis flashes and 30-μsec delays, a transient in the 550-nm region, corresponding to the flavin semiquinone,[29,34] was detected. The intensity of the semiquinone transient decreases in the presence of Co^{2+}, Cu^{2+}, or Fe^{2+}. These observations provide no evidence for half-reduced flavin formation *via* charge

(27) A. Weller in "Fast Reactions and Primary Processes in Chemical Kinetics," S. Claesson, Ed., Almqvist and Wiksell, Stockholm, 1967, pp 413–428.

(28) T. L. Banfield and D. Husain, *Trans. Faraday Soc.*, **65**, 1985 (1969); D. J. Binet, E. L. Goldberg, and L. S. Forster, *J. Phys. Chem.*, **72**, 3017 (1968); A. Vogler and A. W. Adamson, *J. Amer. Chem. Soc.*, **90**, 5943 (1968); G. B. Porter, *ibid.*, **91**, 3980 (1969); M. A. Scandola and F. Scandola, *ibid.*, **92**, 7278 (1970); V. Balzani, R. Ballardini, M. T. Gandolfi, and L. Moggi, *ibid.*, **93**, 339 (1971).

(29) L. Tegner and B. Holmstrom, *Photochem. Photobiol.*, **5**, 223 (1966).

(30) A. A. Lamola and G. S. Hammond, *J. Chem. Phys.*, **43**, 2129 (1965); C. A. Parker and T. A. Joyce, *Photochem. Photobiol.*, **6**, 395 (1967).

(31) H. Linschitz and L. Pekkarinen, *J. Amer. Chem. Soc.*, **82**, 2411 (1960).

(32) In principle, an exactly analogous argument could be applied for Fe^{3+}, which is the strongest quencher encountered in this study! The quenching data for Fe^{3+} must be interpreted cautiously since, at pH 2.6, appreciable concentrations of hydrolysis products, including polynuclear complexes, are present.

(33) T. Okada, H. Oohari, and N. Mataga, *Bull. Chem. Soc. Jap.*, **43**, 2750 (1970).

(34) M. Green and G. Tollin, *Photochem. Photobiol.*, **7**, 129, 143 (1968).

transfer involving the singlet. The present observations contrast with those of Feitelson and Shaklay,[23] who noted an increase in the intensity of the semiquinone transient when benzohydroquinone was flashed in the presence of $Co(CN)_6^{3-}$, an electron acceptor; a charge-transfer quenching mechanism was clearly implicated by that observation.

It is conceivable that a charge-transfer intermediate would be stabilized in rigid media. However, irradiation of ethanolic glasses (77°K) 1.00×10^{-5} *M* in riboflavin, 0.1 *M* in $Co(ClO_4)_2$, $Fe(ClO_4)_2$, or $Cu(ClO_4)_2$, with a 2500-W xenon lamp yielded no evidence for formation of half-reduced flavin species either in absorption or fluorescence spectra; under these conditions, each ion produced significant fluorescence quenching. That no direct evidence for charge-transfer interaction between excited singlet riboflavin and metal ions has been acquired, coupled with the rather poor correlation of k_Q with ionization potential, suggests, but does not prove, that charge-transfer quenching is not the principal mode of fluorescence quenching in this system.

The importance of electronic energy transfer in quenching of organic triplet states by metal ions has recently been recognized.[28] Many of the results in Table I can be rationalized by postulating energy transfer from singlet riboflavin to quenching ions. For example, such ions as Zn^{2+}, Ba^{2+}, and Pb^{2+} exhibit no electronically excited states lower in energy than the first riboflavin singlet and energy transfer would therefore be extremely endothermic. Likewise, Mn^{2+} (d^5 high-spin configuration) possesses no spin-allowed ligand-field excited states; an energy-transfer process such as $^1Rf^* + Mn^{2+}(^6S) \rightarrow {}^1Rf + Mn^{2+}(^4T_1)$ (Rf = riboflavin) is spin forbidden. Indeed, Mn^{2+} is unique among first-row transition-metal ions in not quenching $^1Rf^*$.[32] Because several of the ions employed as quenchers exhibit photochemical reactions (of special interest are Fe^{2+}, which undergoes photooxidation,[35] and Cr^{3+}, which exhibits photochemical water exchange[36]), the ability of riboflavin to sensitize photochemical reactions of metal ions was investigated.

Rutter[7] has referred to "catalysis" of photooxidation of Fe^{2+} by riboflavin, and we have duplicated his observations. An energy-transfer process such as $^1Rf^* + Fe^{2+}(^5T_2) \rightarrow {}^1Rf + Fe^{2+*}(^5E)$ is both spin allowed and energetically feasible. Due to the low extinction coefficients for quintet–quintet transitions in Fe^{2+}, it is highly improbable that such a process could occur by long-range transfer;[37] a collisional transfer mechanism would probably be involved. While the apparent ability of riboflavin to sensitize photooxidation of Fe^{2+} suggests an energy-transfer process, the interpretation is not unequivocal, due to the reducing nature of Fe^{2+} and generation of radicals and/or hydrated electrons in its photooxidation.[38]

A less equivocal test of energy transfer as a quenching mechanism is in principle afforded by Cr^{3+}. The large k_Q for Cr^{3+} (Table I) is noteworthy, as is the reported observation of quenching of triplet excited states of organic molecules by Cr^{3+} complexes.[28] Because the quantum yield for photochemical water exchange in $Cr(H_2O)_6^{3+}$ is small ($\phi = 0.11$ in the 500–700-nm region[36]), we have instead examined the ability of riboflavin to sensitize aquation of $Cr(NH_3)_5NCS^{2+}$, which quenches riboflavin fluorescence with an efficiency ($k_Q = 4.4 \times 10^9\ M^{-1}\ sec^{-1}$) comparable to that for Cr^{3+}. In the visible region, the principal photoaquation product of $Cr(NH_3)_5NCS^{2+}$ is $Cr(NH_3)_4(H_2O)NCS^{2+}$, which is formed with a quantum efficiency of *ca.* 0.40.[11] The principal product of thermal aquation is $Cr(NH_3)_5H_2O^{3+}$ and the spectral changes accompanying photoaquation are marked; it is, therefore, relatively straightforward to distinguish between photo- and thermal aquation. Furthermore, neither the Cr(III) complex nor its photoproducts are strong redox agents and no radicals are generated in the photoaquation. The rate of ammonia release in $Cr(NH_3)_5NCS^{2+}$ (λ = 425 nm) is substantially enhanced (Figure 3) in the presence of riboflavin, suggesting that the Cr(III) complex quenches singlet riboflavin at least partially by intermolecular energy transfer. That the sensitization efficiency (0.15) is substantially smaller than the quantum yield for direct photoaquation (0.40[11]) may indicate either that the presumed energy-transfer process is appreciably less than 100% efficient or that the excited states of the chromium complex populated by sensitization and direct absorption are different.

While it is conceivable that sensitization by riboflavin of photooxidation of Fe^{2+} and aquation of $Cr(NH_3)_5NCS^{2+}$ arises from other processes, the observations are most easily interpreted in terms of electronic energy transfer. No direct evidence implicating energy transfer as a quenching process for other ions has been obtained, but the general pattern of results (especially the inability of Mn^{2+}, Zn^{2+}, Ba^{2+}, and Pb^{2+} to quench) is consistent with an energy-transfer mechanism. As noted above, no other common quenching mechanism satisfactorily rationalizes all the data. It does not, however, appear possible to implicate energy transfer as the sole quenching mechanism, even in the case of quenching by Cr^{3+} species. Quenching of singlet riboflavin by metal ions may thus generally proceed by two or more parallel processes, their relative importance varying with the specific metal ion under consideration.

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(35) J. Jortner and G. Stein, *J. Phys. Chem.*, 66, 1258, 1264 (1962).
(36) R. A. Plane and J. P. Hunt, *J. Amer. Chem. Soc.*, 79, 3343 (1957).
(37) T. Förster, *Discuss. Faraday Soc.*, 27, 7 (1959).
(38) Direct photooxidation of Fe^{2+} proceeds by a mechanism which presumably involves hydrated electron formation.[35] In principle, a useful test of the energy-transfer hypothesis would consist of addition of an $e^-(aq)$ scavenger (such as N_2O) followed by attempted detection of the scavenging product (N_2). For such a test to be positive, it is probable that sufficiently large intensities and/or exposure times would be required that appreciable photobleaching of riboflavin would occur. This would interfere with the scavenging experiment, as would any radicals produced by thermal reactions of riboflavin or its photoproducts with $e^-(aq)$. Because the results of such an experiment would almost certainly be highly equivocal, none has been performed in this work.