November 23, 1973

To the Graduate Council:

I am submitting herewith a thesis written by Susan Goodfellow Polte entitled "A Study of the Quenching of Riboflavin Fluorescence by Metal Ions in Aqueous Methanol". I recommend that it be accepted for eighteen quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor for
Graduate Studies and Research

A STUDY OF THE QUENCHING OF RIBOFLAVIN FLUORESCENCE BY METAL IONS IN AQUEOUS METHANOL

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Susan Goodfellow Polte

March 1974

To Steve and Tom

ACKNOWLEDGMENT

I wish to thank Dr. Wehry for his patience and guidance above and beyond the call of duty.

I was doubly fortunate to share a lab with Dick Leese who was my constant rescuer and advisor.

Fellow graduate students, Norman Canfield and Carol Hammer, were invaluable sources of information and help.

Last, but not least, are my parents and Bob Ralston who were my greatest sources of encouragement for three rather hectic years of graduate school.

1136725

ABSTRACT

The quenching of riboflavin fluorescence by transition-metal ions has been investigated in a solvent medium 80 weight per cent in methanol and 20 weight per cent in water. The quenching data did not exhibit adherence to the classical Stern-Volmer quenching equation. The fluorescence quenching data, reinforced by ultraviolet spectroscopic measurements, indicate that metal ions interact with both the ground and electronically excited states of riboflavin in the alcohol-water mixed solvent. In contrast, riboflavin fluorescence quenching by metal ions in aqueous media appear to involve solely excited-state interactions with the flavin.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	EXPERIMENTAL	10
	A. Materials	10
	B. Solutions for Absorption and Fluorescence Spectra	11
	C. Techniques	13
III.	RESULTS AND DISCUSSION	20
IV.	SUMMARY OF CONCLUSIONS	33
LIST OF REFERENCES		34
VITA		37

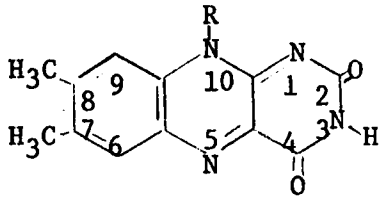
LIST OF FIGURES

FIGURE		PAGE
1.	Variation of Riboflavin Fluorescence Intensity with pH in 80% Aqueous Methanol	14
2.	Fluorescence Intensity of Riboflavin in Water (a) and 80% Methanol (b) -- Absorption Spectra of Metal Ions . .	17
3.	Stern-Volmer Plots for Metal Ion Quenching of Riboflavin Fluorescence	25
4.	Difference Spectrum of Riboflavin	29

CHAPTER I

INTRODUCTION

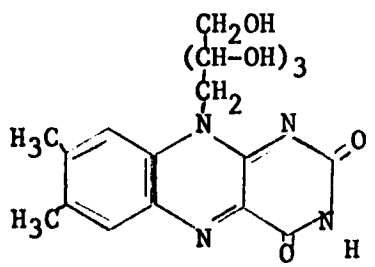
"Flavins" are substituted derivatives of the isoalloxazine heterocyclic ring system:



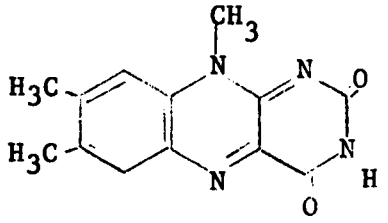
Flavins have a broad range of biological functions, many of which are associated with electron-transfer processes. Flavin can exist in three redox states: (a) fully oxidized ("flavoquinone," depicted in the above structure); (b) half-reduced ("flavosemiquinone"); and (c) fully reduced ("flavohydroquinone"). The properties of these three redox forms of flavin are quite different; the present research is concerned wholly with flavoquinone.

Flavins appear as coenzymes in the oxidation of amino acids, amines, and carboxylic acids.[1] In addition, they are thought to play a part in bacterial dehydrogenation, in nitrate assimilation in fungi and plants, and in thyroid iodinations.[1]

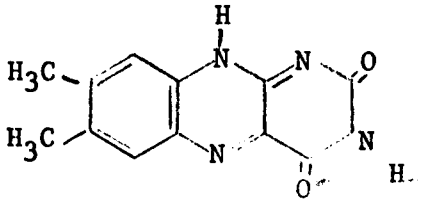
The most common member of the flavin series, riboflavin (vitamin B_2), has a D-ribityl side chain in the 10 position:



Photolysis of riboflavin in basic solution yields, as products, two simpler flavins — lumiflavin and lumichrome:



lumiflavin



lumichrome

The significance of riboflavin in photobiological processes is not yet completely understood, but riboflavin is known to take part in photosynthetic electron transfer[2] and flavins are known to participate in photosynthetic phosphorylation in chloroplasts.[3] Flavins enhance the intensity of bacterial bioluminescence,[4] and a wide variety of other photobiological phenomena exhibit action spectra closely resembling the characteristic visible absorption spectra of flavins.[5]

Because of the possible involvement of electronically excited flavin moieties in photobiological processes, the spectroscopy and photochemistry of "simple" flavins has received considerable attention.[6] Flavins undergo at least two different photochemical decomposition reactions,[7] and they also exhibit both phosphorescence (triplet → singlet emission) and fluorescence (singlet → singlet emission). In aqueous solution, riboflavin exhibits a fluorescence band maximum of 530 nm and a fluorescence quantum efficiency of 0.25.[8] However, in most flavoproteins and flavin enzymes, fluorescence from the flavin entities is undetectably weak; hence, studies of the effects of quenching agents upon flavin fluorescence are of particular interest.

A number of flavoproteins contain metals (especially molybdenum and non-heme iron),[9] and the role of metallic species in flavoprotein-mediated electron-transfer processes and in flavin-dependent photobiology is accordingly of considerable interest.

It has been found that flavins in their ground electronic states are most likely to form complexes with metals when the lowest (normally unoccupied) flavin π^* molecular orbital contains one electron. Occupation of that orbital can be accomplished in three ways. First, the parent flavin can be reduced to a "half-reduced" form. It has been found that this "half-reduced" form (flavosemiquinone) is the only flavin redox state exhibiting general metal affinity in aqueous solution.[9,10] Second, a flavin can form a complex with a strongly reducing metal ion such as Ag(I), Cu(I), or Fe(II), wherein, in effect, electron transfer from metal to flavin occurs.[2] Such metal chelates are regarded as being closely analogous to organic "charge-transfer" molecular complexes. The third process by which the lowest π^* molecular orbital may be occupied is by electronic excitation of the neutral flavin species.[2] It is this third process which is of interest in this research.

The existing data regarding complexation of ground-state flavins with metal ions almost all pertain to aqueous solution; the nature of metal-flavin interactions in solvents which do not coordinate strongly with metals is not presently clear. Hemmerich has reported complexes of flavoquinone with a wide variety of metals in acetone.[11] He determined by NMR that the flavin binding sites were the N(5)-O(4) positions. Recently a report of a stable solid ground-state complex of Cu(II) with

10-methylisoalloxazine was published.[12] A warm, nearly saturated aqueous solution of copper(II) perchlorate was added to a hot saturated solution of 10-methylisoalloxazine in formic acid. Upon slow cooling, orange crystals formed. The resulting compound was identified as bis-(10-methylisoalloxazine) copper(II) perchlorate tetrahydrate. This observation suggests that flavoquinone-metal chelates may exist in a variety of solvent media, but thus far few experimental results in the area have appeared.

The quenching of riboflavin fluorescence by metal ions in aqueous solutions was first studied by Rutter[13] and later by Varnes, Dodson, and Wehry.[14] It has been shown unequivocally that metal ions can quench flavin fluorescence with high efficiency. The order of quenching abilities was determined in aqueous solution to be the following: $Fe^{3+} >> V^{3+} > Cr^{3+} \triangleq Fe^{2+} > Co^{2+} > Rh^{3+} > Ni^{2+} > Ce^{3+}$ with Mn^{2+}, Zn^{2+}, Ba^{2+}, and Pb^{2+} showing no detectable quenching.

Transition-metal ion quenching of riboflavin fluorescence can in principle be rationalized by several mechanisms, including "heavy atom" effects; "paramagnetic" effects; transfer of flavin excitation energy to the metal, producing an excited metal species; ground-state complexation; or formation of a complex of the metal ion with the flavin in its excited singlet state.

It is often observed that the fluorescence of organic solutes is inhibited in the presence of species of high atomic number; "heavy atoms" tend to increase the efficiency of excited singlet-to-triplet intersystem crossing with concomitant decreases in fluorescence

intensity.[15] This effect might be further enhanced by paramagnetism in the quenching species.[16] In aqueous solution, those metal ions which are the most efficient quenchers of riboflavin fluorescence are indeed paramagnetic, but there appears to be no correlation of the quenching ability of metal ions with their paramagnetic moments. Likewise, "heavy atom" effects cannot by themselves rationalize the quenching data; thus, for example, Pb^{2+} (Z = 82) exhibits no quenching action whatsoever.[14]

While electronic energy transfer is a plausible process by which metal ions can quench the first excited singlet state of a flavin, it is very difficult, with most metal ions, to obtain unequivocal experimental evidence for energy-transfer quenching. Energy transfer has in fact been observed in experiments in which the sensitized photodecomposition of a Cr(III) complex was brought about using riboflavin as the sensitizer.[17] Also, there is evidence that quenching of riboflavin fluorescence by Fe^{2+} is accompanied by sensitized photooxidation of Fe^{2+} to Fe^{3+}. There is, however, no evidence that energy transfer is the mechanism by which other metal ions quench riboflavin fluorescence, although the quenching inability of metals with first excited states of high energy is consistent with this mechanism.

If a metal ion were to form a complex with riboflavin in its ground state, the result might be a decrease in the fluorescence efficiency of riboflavin, since it is well known[18] that metal complexes of fluorescent organic ligands usually fluorescence much less strongly than the parent ligand. In aqueous solution, there is no evidence for

complexation of riboflavin with metal ions (other than very strongly reducing ions; see p 3); accordingly, the ground-state complexation ("static") quenching mechanism has not been regarded as relevant in water. In solvents which do not coordinate strongly with metal ions, however, the "static" quenching process may conceivably be of more general significance.

As a final plausible quenching mechanism, one must consider the possibility that, during its short lifetime, the excited riboflavin singlet forms a complex with a metal ion, with the occurrence of this process increasing the efficiency of nonradiative decay of the flavin singlet. Indeed, Hemmerich[9] has speculated that excited states of flavinoquinone should exhibit much greater metal-complexing ability than the ground state (see p 3). While it is an attractive speculation that the order with which different metal ions quench riboflavin fluoresence is in effect a determination of a "stability series" for metal ion-flavin excited singlet complexes, there is no direct evidence for the formation of such transient complexes, and for the present, discussion of the possible metal-complexing ability of flavins in electronically excited states remains wholly speculative.

Despite considerable study, the mechanistic picture for quenching of flavin fluorescence by metal ions is far from clear. All of the studies previously reported have been limited to aqueous solutions. In fact, the photochemistry and photoluminescence of flavins is highly solvent-dependent; we therefore felt that studies of fluorescence quenching phenomena in nonaqueous media might provide additional insight into

the relative validity of the various possible quenching mechanisms.

The fluorescence quantum yields of flavins are strongly solvent dependent, as shown in Table I. The fluorescence quantum yield tends to increase with decreasing solvent polarity and hydrogen-bond-donating ability, while the absorption spectrum is relatively solvent-independent. This fact suggests that either the singlet → triplet intersystem crossing or the singlet → ground state internal conversion efficiencies (or both) are strongly solvent-dependent. The singlet → triplet intersystem crossing efficiency of riboflavin in water has been reported to be 0.70,[6,20] although the validity of this value is questionable. The fluorescence yield of riboflavin in water has been reported to be 0.25.[14] Therefore, internal conversion from the lowest excited singlet to the ground state appears not to be a significant pathway in the absence of quenchers.

There are no reported data on intersystem crossing yields for riboflavin in other solvents, but if the mechanism by which flavin fluorescence is quenched involves enhancement of the singlet → triplet intersystem crossing process, then the quenching of fluorescence in nonaqueous media by metal ions should be greater than in water. Accordingly, in this research the quenching of fluorescence of riboflavin by selected metal ions was studied in 80% aqueous methanol as a solvent medium.

The choice of this solvent medium was dictated by several considerations:

(a) In many nonaqueous solvents, the solubility of metal salts is

TABLE I

DEPENDENCE OF FLAVIN FLUORESCENCE YIELDS
UPON SOLVENT

Solute	Solvent	Φ_F
Riboflavin	H_2O	0.25
	EtOH	0.32
	$(CH_3)_2CO$	0.35
	Dioxane	0.41
Lumiflavin	H_2O	0.28
	EtOH	0.42
	$(CH_3)_2CO$	0.57
	Dioxane	0.64

insufficiently large to generate the metal ion concentrations required to observe significant fluorescence quenching.

(b) The nature of metal-ion solvation phenomena in alcoholic solvents is rather similar to that in water, facilitating comparisons of the data herein reported with those already available for the aqueous solvent system.

(c) The fluorescence spectrum of riboflavin is pH-dependent;[19] thus, it was considered appropriate to choose a solvent in which pH adjustments could be monitored with a glass pH electrode.

These considerations suggest anhydrous methanol as a desirable solvent for these studies. Unfortunately, metal perchlorate salts are notorious for their hygroscopicity (e.g., $Mg(ClO_4)_2$ is a popular desiccant), and it was found infeasible to prepare anhydrous samples of the metal salts employed for these studies. Accordingly, the solvent composition 80% methanol/20% water (by weight) was chosen as the appropriate compromise between the desirability of securing a highly "nonaqueous" medium and the hygroscopic nature of metal perchlorate salts.

CHAPTER II

EXPERIMENTAL

A. Materials

1. Riboflavin

Riboflavin obtained from the Sigma Chemical Corporation was recrystallized three times from an aqueous solution which was 1 M in acetic acid and 10 μ M in EDTA. The solubility of riboflavin in water is very low, making it necessary to use large volumes of solution. The solution was heated to 80° and filtered through a fine fritted glass funnel. The third recrystallization was done in darkness and the riboflavin was finally vacuum dried and stored in a dark vacuum desiccator.

2. Metal Perchlorates

All metal perchlorates were obtained from the G. Frederick Smith Chemical Company. It was found that these salts contained significant amounts of perchloric acid as an impurity, which was largely eliminated by multiple recrystallization of the salts. Since perchlorate salts are extremely soluble in water, 15 to 20 grams of the unpurified crystals generally was soluble in about 2-3 ml of water; ultimately, about 30% of the original material was recovered from each recrystallization. The resulting crystals were stored in a vacuum desiccator.

3. Water

Tap distilled water was redistilled using a Kontes WS-2 still and was stored in polyethylene bottles.

4. Methanol

Reagent-grade methanol from Fisher Scientific Corporation was redistilled over sodium hydroxide and stored in glass containers.

5. Ethylenediaminetetraacetic Acid

Reagent-grade EDTA from Fisher Scientific Corporation was dried in an oven and then stored in a vacuum desiccator.

6. Glassware

All glassware was first cleaned with ethanolic KOH, then with aqua regia, and finally rinsed thoroughly with distilled water.

B. Solutions for Absorption and Fluorescence Spectra

All 80% methanol-20% water (by weight) solutions used in this research were prepared by using calculated volumes of methanol and of water. The following densities were employed to calculate the volumes: 80% MeOH, 0.843 g/ml; methanol, 0.786 g/ml; and water 1.00 g/ml.[22]

Stock riboflavin solutions were prepared by first making a saturated aqueous riboflavin solution, then determining the concentration of the saturated solution by tenfold dilution of an aliquot of the saturated solution to measure its absorbance at 445 nm. (The absorbance of a saturated riboflavin solution was found to fall in the 3.7-4.5 range.) The concentration was determined by using for the molar absorptivity, ε, a value of 1.22×10^4 ℓ $mole^{-1}$ cm^{-1}.[23] The saturated solution was then diluted with water to a concentration which could be

further diluted with methanol to yield an 80% methanolic solution of the proper absorbance for the spectra being obtained. Within experimental error, the molar absorptivities of riboflavin in 80% aqueous methanol and in water were equal at 445 nm. The stock solutions were stored in the dark; new stock solutions were prepared weekly.

Stock metal perchlorate solutions were prepared from recrystallized metal perchlorate by dissolving weighed amounts of the salt in sufficient water and methanol to make an 80% by weight methanolic solution. The water of hydration present in each salt was included in the 20% water content of the solution. Each stock solution was standardized by EDTA titration as described below, and the solutions were stored in the dark.

The concentrations of the nickel(II), manganese(II), zinc(II), and cobalt(II) stock solutions were determined by EDTA titrations using the methods described by Welcher.[24] Murexide was used as the indicator for nickel(II) and cobalt(II), and Eriochrome Black T was used as the indicator for manganese(II) and zinc(II). The copper(II) titration with EDTA proceeded according to the procedure described by Belcher,[25] using Fast Sulphon Black F as indicator.

For each fluorescence quenching study, a series of solutions containing equal amounts of riboflavin but varying amounts of metal ion was prepared. In addition, several blank solutions containing only riboflavin was prepared. First, the stock aqueous riboflavin was quantitatively pipetted into each of a series of 25-milliliter volumetric flasks. Second, enough methanol was transferred into the flasks

to form an 80% by weight methanol solution. Third, the desired amount of stock metal ion solution (in 80% methanol) was added to the flask by buret. Fourth, 80% methanol was added to bring the solution to the mark. Finally, the "pH" was adjusted to the desired value, as described in the next paragraph. This entire process was carried out in the dark using only photographic safelights for illumination.

C. Techniques

Because the fluorescence yield of riboflavin is dependent upon the acidity of the medium (see Figure 1), any study of the quenching of riboflavin fluorescence requires that the pH of all solutions be adjusted to the same value. This requirement poses potentially severe difficulties in nonaqueous media.[26] The measurement of "pH" in non-aqueous solutions, using instruments and electrodes designed to measure hydrogen ion activities in water, poses a number of problems, including dehydration of glass electrodes by nonaqueous solvents and the prolonged period of time needed for equilibration of pH readings.

It has been found that pH measurements in water-alcohol mixtures can be standardized by use of any of a number of standard buffers described by Bates[22] and de Ligny.[27] Use of this approach for measuring the "pH" of solutions in 80% methanol does not necessarily provide accurate values which are easily related to the hydrogen-ion activity in that solvent, but glass-electrode pH measurements in this medium are sufficiently precise that one can employ the measurements to ensure that

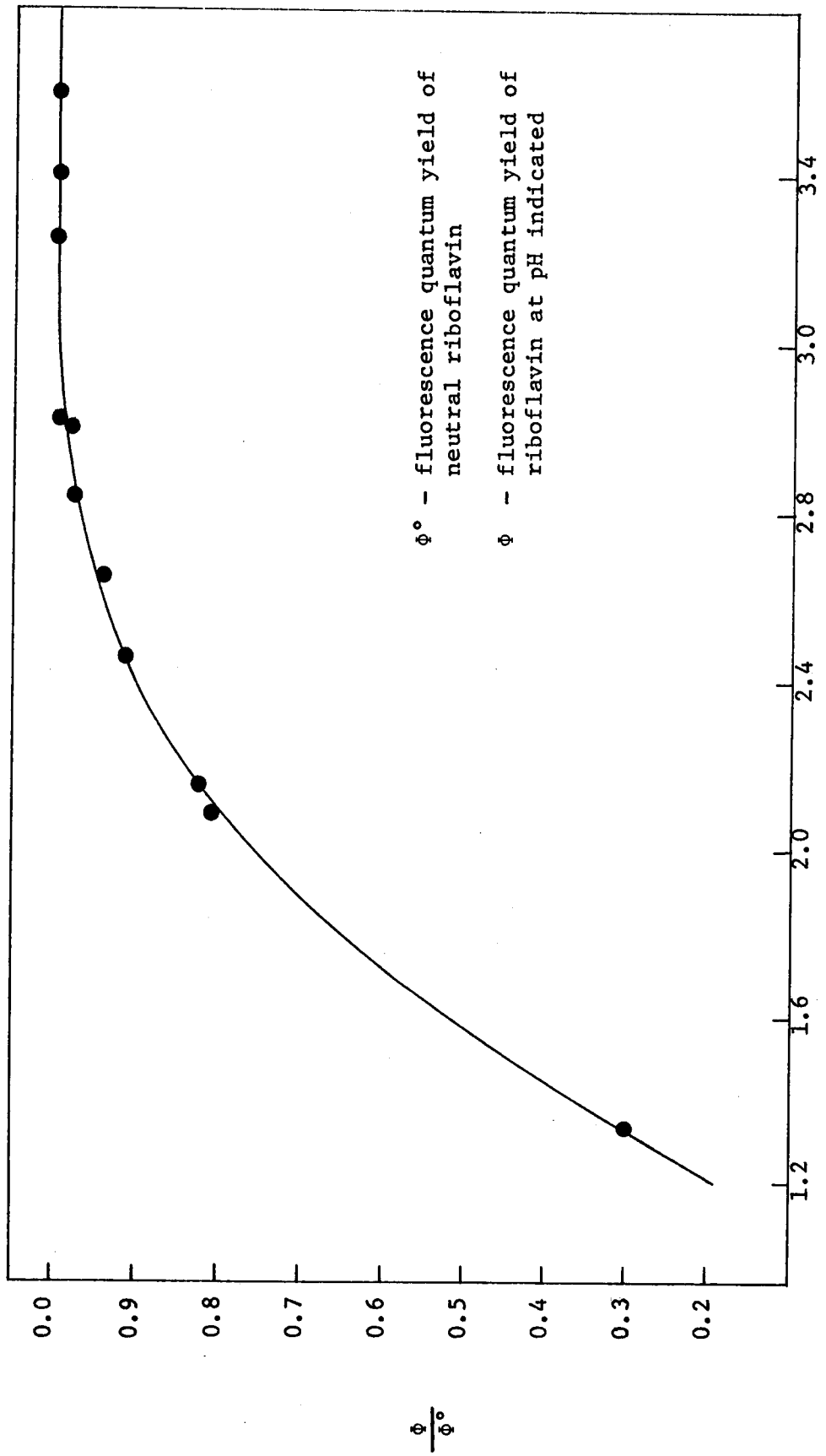


Figure 1. Variation of riboflavin fluorescence intensity with pH in 80% aqueous methanol.

a series of solutions all have effectively the same "pH." Thus, meaningful fluorescence quenching studies can be performed in alcohol-water mixtures.

The standard buffer used in this research was 0.01 M $NH_4HC_2O_4$ in 80% methanol solution, which has a "pH" of 3.12 when measured with a glass electrode (with SCE reference) using a pH meter which has been previously standardized using the customary aqueous buffers. A Beckman "Expandomatic" pH meter was used in this research.

To equalize the "pH" of the solutions prepared, as above, for spectral studies, each solution was transferred from the 25 ml volumetric flask in which it was initially prepared to a 50 ml beaker into which the pH electrodes were inserted. The "pH" was adjusted to the desired value by dropwise addition of concentrated aqueous $HClO_4$. It was desired to use a minimum of aqueous $HClO_4$ since the addition of acid decreased the methanol concentration of the solutions. The number of drops of acid used to adjust the "pH" of the solutions was very small, five drops being the maximum volume of perchloric acid needed for pH adjustment.

In these studies, the "pH" was adjusted to a value lying between 3.0 and 3.5 (the precise value depended upon the metal ion). This choice represented a compromise between the sharp decrease in riboflavin fluorescence intensity observed at pH < 3 (Figure 1) and the formation of metal hydroxide precipitates at higher pH values.

Absorption spectra of all metal ions, riboflavin, and metal-ion-riboflavin solutions were obtained with a Cary Model 17 Spectrophotometer, as were all difference spectra of riboflavin-metal ion solution versus

riboflavin as reference. Absorption spectra of riboflavin and the various metal ions are shown in Figure 2.

Fluorescence intensity measurements were obtained using an Aminco-Bowman Spectrophotofluorometer. All fluorescence measurements were made at a constant temperature of 25° using a water-jacketed "right-angle geometry" cell compartment.

In all quenching studies, the fluorescence intensity of a "blank" (riboflavin solution containing no quencher) was periodically determined. If the intensity of the riboflavin fluorescence "blank" changed between two consecutive measurements (due to changes in the intensity of exciting light), the fluorescence intensity of the riboflavin-metal solutions were redetermined. The fluorescence intensity of a series of riboflavin-metal ion solutions was measured in the order of increasing metal-ion concentration, and the cell was rinsed thoroughly with each subsequent solution before being filled for the next measurement. The transfer of all solutions was carried out in the dark and the fluorescence intensity of each solution was measured at least four times. Errors due to self-absorption of riboflavin fluorescence were minimized by ensuring that the absorbance due to riboflavin in these solutions at 445 nm, the preferred wavelength for fluorescence excitation, was not greater than 0.10.

As shown in Figure 2, there arise problems with respect to overlap of the riboflavin absorption (and/or fluorescence) spectrum with the absorption spectrum of some of the metal ions of interest. For example, Ni^{2+} exhibits an absorption band very near to 445 nm,

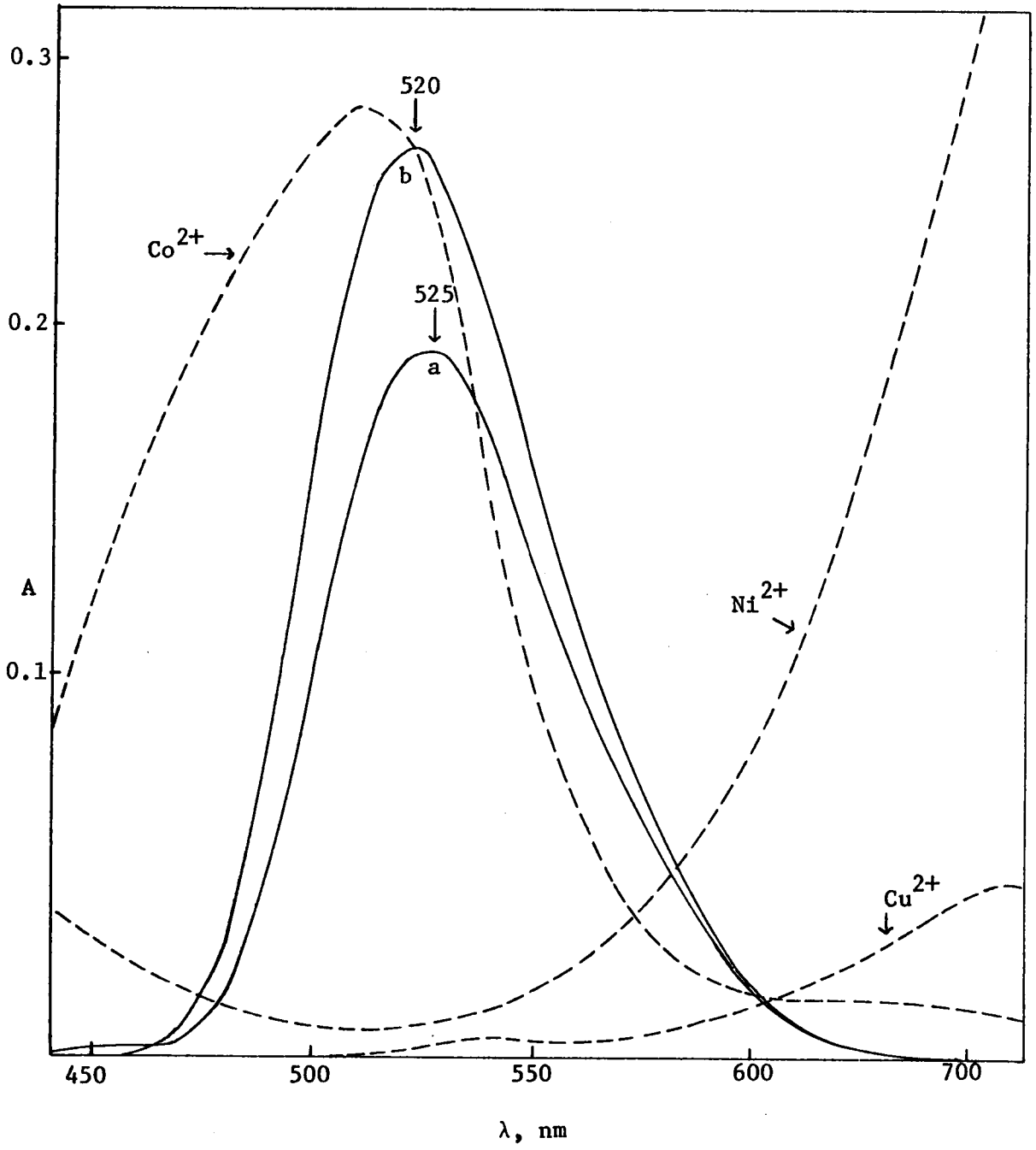


Figure 2. Fluorescence intensity of riboflavin in water (a) and 80% methanol (b) -- absorption spectra of metal ions.

which would normally be the wavelength of choice for excitation of riboflavin fluorescence, and Co^{2+} has an absorption maximum which overlaps both the 445 nm peak in the riboflavin absorption spectrum and the uncorrected riboflavin fluorescence maximum at 520 nm. Such spectral overlap causes "inner filter" errors; that is, a decrease of fluorescence intensity caused by absorption of either the exciting light or of the riboflavin fluorescence, or both, by the quencher.

The inner filter effect of Ni^{2+} was alleviated by employing 480 nm, rather than 445 nm, excitation of riboflavin fluorescence. At 480 nm, riboflavin absorbs rather strongly, but the Ni^{2+} absorbance is negligible. To correct for the Co^{2+} inner filter effect, the excitation wavelength employed was 360 nm, where riboflavin has a second absorption maximum but where Co^{2+} has no measurable absorbance.

Correction for the absorption of riboflavin fluorescence by Co^{2+} was attempted by using two methods. First, the fluorescence intensity was measured at 580 nm, instead of at the uncorrected fluorescence maximum at 520 nm. (The absorbance of 0.05 M Co^{2+} at 520 nm is 0.275, while at 580 nm the Co^{2+} absorbance is only 0.03 absorbance units.) The second correction to overcome the absorption of riboflavin fluorescence by Co^{2+} consisted of the use of front-surface fluorescence measurements, instead of the conventional right-angle geometry used for the other riboflavin-metal-ion solutions. It should be noted that the results for Co^{2+} were obtained by excitation into the second excited singlet state of riboflavin. Thus, if the quantum efficiency of internal conversion to the first excited singlet is less than unity, the quenching

constants obtained for Co^{2+} will not be directly comparable with those for the other metal ions.

CHAPTER III

RESULTS AND DISCUSSION

The uncorrected fluorescence spectra of riboflavin (concentration = 8×10^{-6} M) in water and 80% aqueous methanol are shown in Figure 2 (p 17). Two facts are obvious from these spectra. First, the fluorescence yield of riboflavin in an 80% aqueous methanol system is significantly greater than in water. This is in agreement with the data in Table I (p 8) for other nonaqueous solvents. Second, there is a small shift in the fluorescence peak to shorter wavelength in methanol. This observation is also in agreement with previous work with lumiflavin,[22] in which the position of the fluorescence peak was found to be solvent-dependent.

The intensities of the various maxima in the absorption spectrum of riboflavin appear to be solvent-independent; although exact molar absorptivity values for riboflavin in 80% methanol were not determined, cursory calculations indicate that they are virtually identical with the values observed in aqueous media. Consequently, the greater fluorescence yield for riboflavin in the alcoholic solvent must be indicative of a decrease in the rates of nonradiative decay (either internal conversion or intersystem crossing, or both) of the lowest riboflavin singlet in the nonaqueous medium.

The dependence of the fluorescence intensity for riboflavin in 80% methanol upon "pH" is shown in Figure 1 (p 14). As noted in the figure, the fluorescence intensity is relatively pH-independent at

"pH" $\geq$ 3.0. Whenever possible, therefore, quenching data were obtained in solutions of "pH" greater than 3.0.

It was observed that Co^{2+}, Ni^{2+}, and Cu^{2+} efficiently quenched the fluorescence of riboflavin in 80% methanol, whereas Zn^{2+} and Mn^{2+} effected barely detectable quenching. The quenching data were treated by use of the conventional Stern-Volmer kinetic treatment, which is described briefly in the following paragraphs.

In the usual fluorescence quenching experiment, light of a constant intensity is used to excite the fluorescent species. In that case, a steady state is established in which the rate of excitation of the fluorophore equals the rate of decay of the lowest excited singlet state. The equation describing this condition is

$$I_{(a)} = (k_f + k_{ic} + k_{isc} + k_q [Q])[S_1] \quad . \qquad (1)$$

In Equation 1, Ia denotes the exciting light intensity in quanta per unit time, and $\underline{k}_f$, $\underline{k}_{ic}$, $\underline{k}_{isc}$, and $\underline{k}_q$ denote, respectively, the rate constants for fluorescence, internal conversion, intersystem crossing, and quenching. The quencher concentration is denoted by $[\underline{Q}]$, and $[\underline{S_1}]$ is the concentration of excited singlet molecules.

The fluorescence quantum efficiency (Φ_f) can be represented in terms of the same fundamental parameters:

$$\Phi_f = \frac{k_f}{k_f + k_{ic} + k_{isc} + k_q[Q]} \quad . \qquad (2)$$

The rate of fluorescence emission, in quanta per unit time, is equal to $k_f[S_1]$; consequently, one may write

$$k_f[S_1] = \phi_f I_a = (k_f + k_{ic} + k_{isc} + k_q[Q])[S_1]\ \phi_f \ . \qquad (3)$$

If a solution contains no quenching species, Equation 2 reduces to

$$\Phi_f^\circ = \frac{k_f}{k_f + k_{ic} + k_{isc}} \ , \qquad (4)$$

where Φ_f° denotes the fluorescence yield of the fluorophore in the absence of any quenching agents. To achieve an evaluation of the effect of a quenching species, one can divide Φ_f° by Φ_f, yielding

$$\frac{\Phi_f^\circ}{\Phi_f} = 1 + \frac{k_q[Q]}{k_f + k_{ic} + k_{isc}} = 1 + K[Q] \qquad (5)$$

Equation 5 is commonly known as the "Stern-Volmer equation."

If one plots $\frac{\Phi_f^\circ}{\Phi_f} - 1$ as [Q], one should obtain a straight line, passing through the origin, the slope of which is called the "Stern-Volmer quenching constant." The plot itself is often termed a "Stern-Volmer" plot.

When the exciting light is extinguished, the fluorescence decays exponentially with a lifetime τ_o:

$$1/\tau_o = k_f + k_{ic} + k_{isc} \ . \qquad (6)$$

Then, according to Equation 5,

$$\frac{\Phi_f^\circ}{\Phi_f} - 1 = K[Q] = k_q \tau_o [Q] \ . \qquad (7)$$

Thus, if the fluorescence decay time, τ_o, for the unperturbed fluorophore is known, the quenching rate constant, k_q, can be computed directly from the slope of the Stern-Volmer plot.

In order for a linear Stern-Volmer plot to be obtained in a fluorescence quenching experiment, certain conditions must be satisfied, including the following. First, there must be only one fluorescent excited state of the solute species under consideration. Second, a steady state condition, Equation 1 (p 21) must be attained. Third, the fluorescence must decay exponentially. Finally, the quenching process must be bimolecular and irreversible.

In the case of fluorescence quenching by a metal ion, one must consider the possibility that either the ground state or the excited state (or both) of the fluorophore may interact with the metal ion. The "static" (ground-state) process can be represented by

$$A + Q \rightarrow AQ$$

$$AQ + h\nu \rightarrow (AQ)^* \rightarrow A + Q \ .$$

and the "dynamic" (excited-state) quenching by

$$A + h\nu \rightarrow A^*$$

$$A^* + Q \rightarrow (AQ)^* \rightarrow A + Q$$

in which A is the ground state solute (riboflavin), A* is the flavin in its fluorescent excited state, and Q is the quencher (metal ion). A detailed consideration[21] of both ground- and excited-state complexation by the quenching ion yields the following modified Stern-Volmer equation:

$$\frac{\Phi_f^\circ}{\Phi_f} = (1 + K_0[Q])(1 + k_t^*\tau_o[Q]) \qquad (8)$$

in which K_0 is the equilibrium constant for formation of the ground-state complex and k_t^* is the rate of formation of a complex of the ion with the excited state of the fluorophore.

Equation 8 can be expanded to yield

$$\frac{\Phi_f^\circ}{\Phi_f} = 1 + K_0[Q] + k_t^*\tau_o[Q] + K_0 k_t^*\tau_o[Q]^2 \qquad (9)$$

If <u>either</u> of the two processes, i.e., formation of AQ or formation of A*Q, is relatively inefficient (i.e., if either k_t^* or K_0 is less than unity), the quenching will follow the Stern-Volmer linear dependence upon [Q]. From such a dependence, it is not possible to distinguish between "static" and dynamic quenching mechanisms. However, if <u>both</u> the ground-state <u>and</u> excited-state quenching processes are significant (i.e., $k_t^*\tau_o$ and K_0 both greater than unity), then the term in $[Q]^2$ in Equation 9 will be significant; in that case, a Stern-Volmer plot which is nonlinear in [Q] will result. The Stern-Volmer plot will show increasing positive deviations from linearity with increasing [Q].

In Figure 3, the Stern-Volmer plots for quenching of riboflavin fluorescence by Co^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, and Zn^{2+} in 80% aqueous methanol are shown. As noted, most of the quenching curves are concave upward; accordingly, Stern-Volmer constants, K, can be attained only by using the relatively few points for small values of [Q] and subjecting them to linear least-squares analysis. The resulting quenching constants are compared with those previously reported[14] for water solution in Table II. It can be seen that the Co^{2+} quenching efficiency is only slightly higher in 80% aqueous methanol than it is in water, while the quenching efficiencies of Cu^{2+}, Ni^{2+}, Zn^{2+}, and Mn^{2+} are much higher in 80% methanol than in water. It should be noted that, while all ions studied acted as stronger quenchers in the <u>absolute</u> sense in 80%

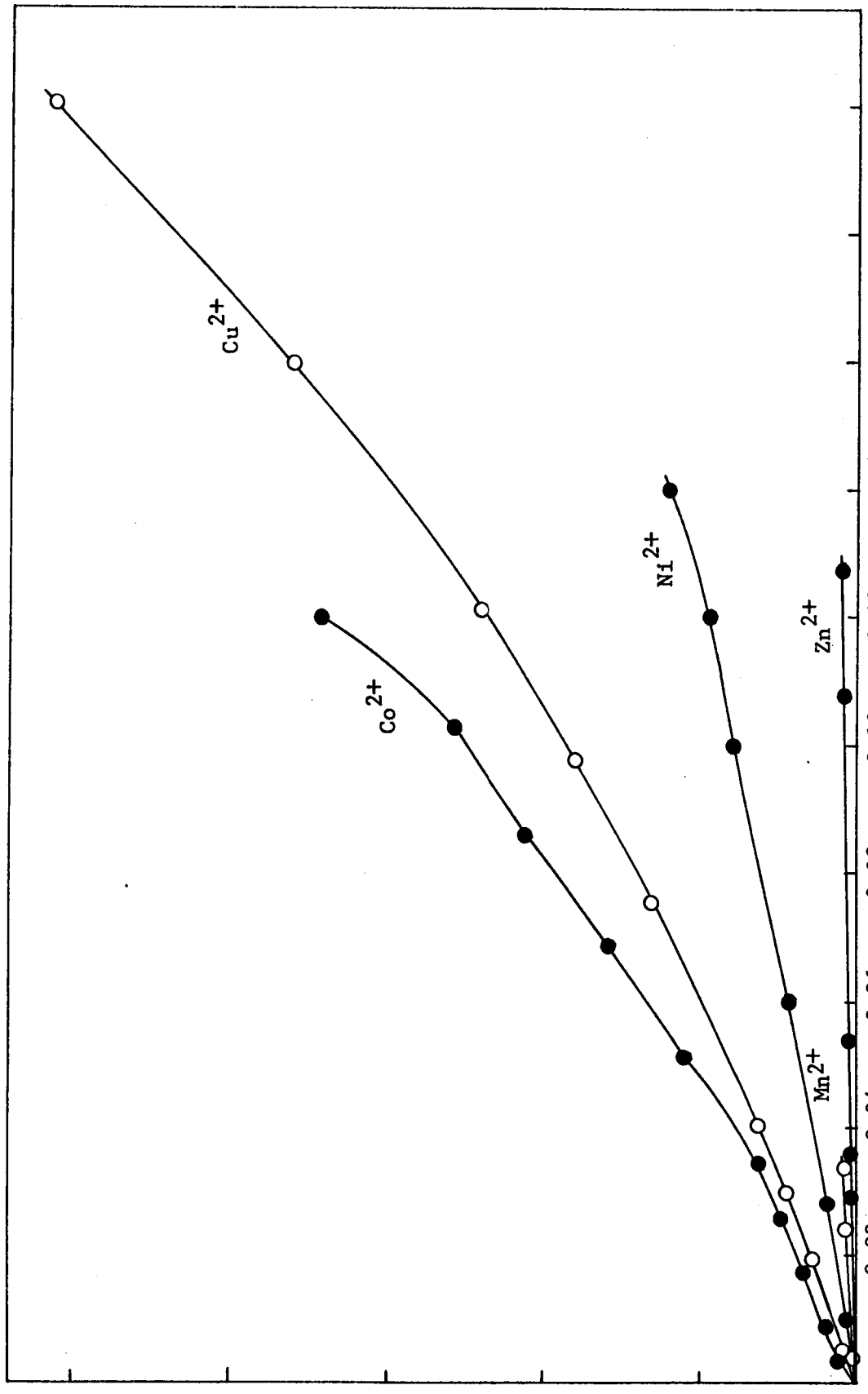


Figure 3. Stern-Volmer plots for metal ion quenching of riboflavin fluorescence.

TABLE II

COMPARISON OF STERN-VOLMER QUENCHING CONSTANTS FOR RIBOFLAVIN FLUORESCENCE BY METAL IONS IN 80% METHANOL AND IN WATER

Ion	80% MeOH (aq.) K (liter $mole^{-1}$)	H_2O K (liter $mole^{-1}$)
Co^{2+}	16.8	15.2
Cu^{2+}	14.3	5.6
Ni^{2+}	7.4	2.6
Mn^{2+}	2.4	< 0.2[a]
Zn^{2+}	0.7	< 0.2[a]

[a]No detectable quenching.

methanol than in water, the relative order of quenching abilities was the same for these ions in both solvents. It is also noteworthy that the most weakly quenching ions (Ni^{2+}, Mn^{2+}) exhibited the greatest increase in quenching efficiency as the solvent composition was changed from aqueous to alcoholic. The differences in quenching ability of the various ions appear to be "levelled" somewhat by use of the alcoholic solvent.

As can be seen in the plot of $(\frac{\Phi_f^\circ}{\Phi_f} - 1)$ vs $[M^{2+}]$, there is a positive deviation from linearity at higher concentrations of strongly quenching ions. Such curvature, as shown in Equation 9 (p 24) may be indicative of formation of complexes between these ions and riboflavin in both ground and excited states. Similar nonlinearity was also observed at high concentrations in the Stern-Volmer plot for Cu^{2+} in the aqueous system,[14] whereas no other ion exhibited a nonlinear Stern-Volmer plot in the aqueous system.

In the previous quenching studies in aqueous media,[14] no spectral (or other) evidence was noted for complexing of ground-state riboflavin with Cu^{2+} (or any other metal ion.) In 80% methanol, however, significant changes in the visible absorption spectrum of riboflavin occur upon introduction of metal ions, especially Cu^{2+}. In the course of the present research using methanolic solutions, a difference spectrum of a solution containing both riboflavin and the ion of interest versus a solution containing the same concentration of riboflavin but no metal ion was obtained for each metal used as a quencher. The difference spectra were then compared with the spectra of each metal alone.

Figure 4 shows the difference spectrum so obtained for a Cu^{2+}-riboflavin solution. If the metal ion exerts no effect on the riboflavin absorption spectrum, then the difference spectrum should be identical with the absorption spectrum of Cu^{2+} itself. Clearly, that is not the case. The difference spectrum for Na^{+}-riboflavin is included on Figure 4 to point out the fact that the change in the riboflavin spectrum is not due to an ionic-strength effect.

The facts that the presence of Cu^{2+} perturbs the riboflavin absorption spectrum, and the Stern-Volmer plot for Cu(II) quenching of riboflavin fluorescence is concave upward may together indicate that, in 80% methanol, <u>both</u> "static" and "dynamic" processes play a role in the interactions of Cu^{2+} with riboflavin excited states in this medium. Interestingly, however, none of the difference spectra for other riboflavin-metal solutions yielded evidence of ground-state interaction, yet the Stern-Volmer plots for Ni^{2+} and (especially) Co^{2+} are also concave upward.

Hemmerich has published a relative order of riboflavin-metal-ion complex stabilities in acetone solutions.[11] This stability series is as follows:

$Cu^{+} > Ni^{2+} > Ag^{+}, Co^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Fe^{2+} >> Mn^{2+}, Mg^{2+}, Fe^{3+}$.

The quenching order found in this research was as follows:

$Co^{2+} > Cu^{2+} > Ni^{2+} >> Mn^{2+} > Zn^{2+}$. Comparison of these two series shows that there is no obvious correlation between ground-state complex stability and quenching ability (though it must be noted that Hemmerich's stability series pertains to <u>acetone</u>, not alcohol, solutions).

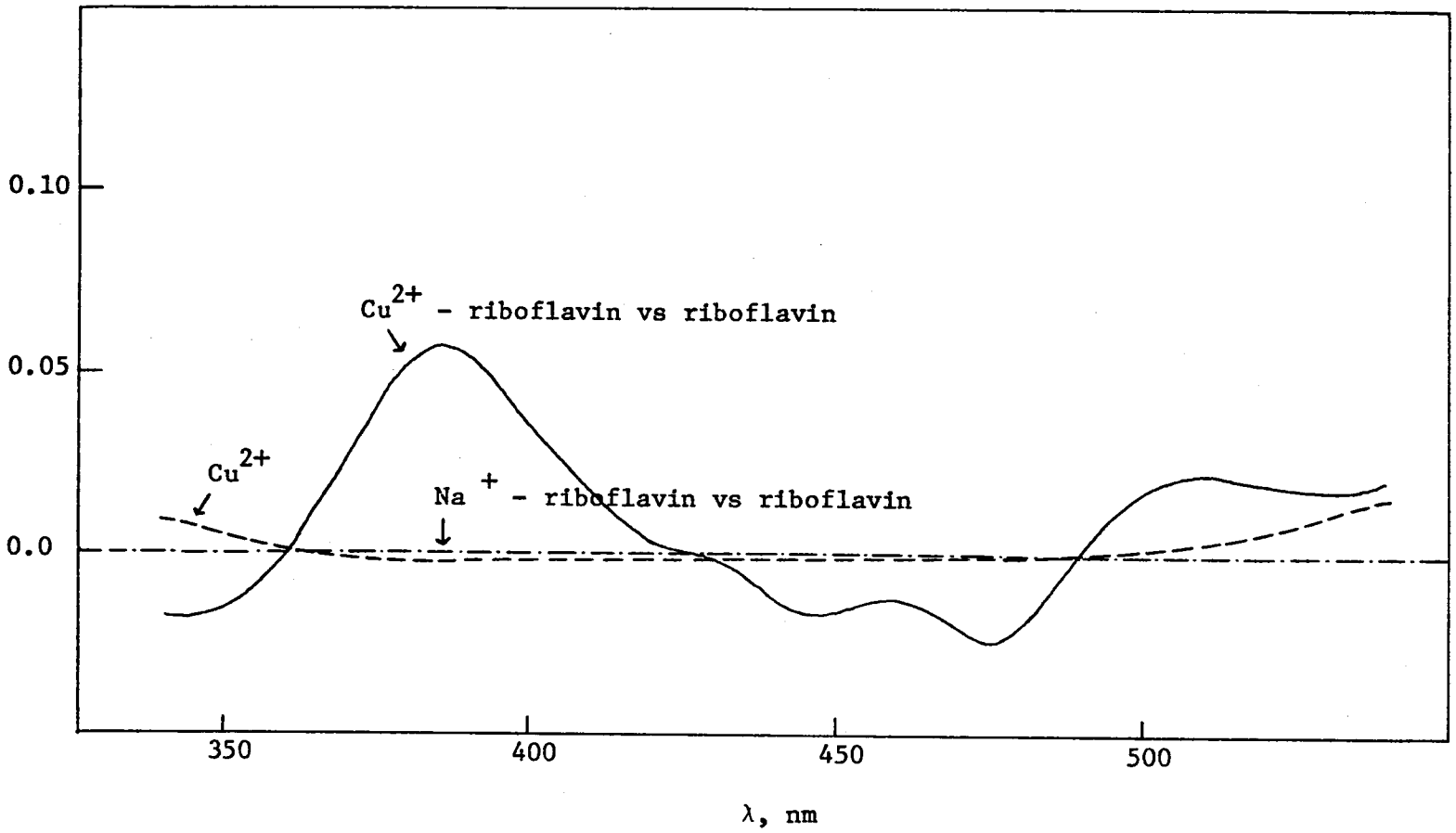


Figure 4. Difference spectrum of riboflavin.

———, 0.1 M $Cu(ClO_4)_2$ - riboflavin vs riboflavin; —· —·, 0.1 M $NaClO_4$ - riboflavin vs riboflavin; ----, 0.1 M $Cu(ClO_4)_2$ vs H_2O.

One must note that, if ground state complexes do form, they must be rather unstable, for the following reasons. First, the Stern-Volmer quenching constant (K_{SV}) for even the most efficient quencher is about 16; thus even if K_{SV} is equal to the formation constant (K_f), the "stability constant" for the metal-flavin complex is of the order of only 10^1. Second, the changes in the riboflavin spectrum effected by Cu^{2+} (Figure 4) are relatively small, even though the concentration of riboflavin is much smaller than the concentration of copper. .

It must be further noticed that the values obtained for the quenching constants are subject to uncertainty because of the inner-filter effect (absorption of exciting light or fluorescence by the metal ions). The inner-filter effect can, in principle, be minimized by proper choice of emission and excitation wavelengths. Particularly in the case of Ni^{2+}, the absorption band of which overlaps the excitation bands of riboflavin, and Co^{2+}, whose absorption band overlaps both the absorption and fluorescence bands of riboflavin, concern regarding possible inner-filter errors must be manifested. As mentioned in the Experimental Section, front-surface fluorescence measurements were employed, especially for studies of quenching by Co^{2+}. This precaution reduces, but does not necessarily eliminate, inner-filter errors. Unfortunately, finite inner-filter errors are virtually incapable of exact correction by mathematical procedures.

Figure 4, which is the difference spectrum of a 0.1 M $Cu(ClO_4)_2$ - 5×10^{-5} M riboflavin solution, shows that the absorbance of the riboflavin-Cu^{2+} solution at 520 nm is increased by .015

absorbance units over the absorbance of the Cu^{2+} and the riboflavin separately at that wavelength. If this difference spectrum is indicative of a ground state complex of riboflavin with Cu^{2+}, then the decrease of fluorescence intensity at the 520 nm maximum might be further decreased by the complex itself acting as an inner filter. This effect is probably of minor significance, but it constitutes an additional precaution which must be considered in studies of these systems in nonaqueous media.

In proposing further studies of this research, determination of Stern-Volmer plots by fluorescence lifetime (τ) data, to supplement the quantum yield data, should be of first priority. When the effect of a quenching agent upon the fluorescence decay time of a species is measured, then one may be certain that the observed effects are due to true excited-state "dynamic" quenching. Any discrepancies in the Stern-Volmer constants obtained for a given quenching ion by τ and Φ measurements can then be utilized to establish quantitatively the extent of "static" (ground state) quenching.

Unfortunately, fluorescence quenching rate constants by τ measurements are exceedingly difficult to obtain in the riboflavin system. The fluorescence decay time for riboflavin in aqueous solution in the absence of quenchers is of the order of 5×10^{-9} sec.[14] In the presence of a quenching agent, τ will be decreased. With conventional pulse-discharge flash-lamp apparatus, fluorescence lifetime measurements are effectively limited to $\tau \geq 2 \times 10^{-9}$ sec. Thus, reliable Stern-Volmer constants from lifetime data are difficult to acquire, because only

relatively small quencher concentrations can be employed (i.e., few data points on the Stern-Volmer plot can be obtained).

In addition to fluorescence lifetime studies, other experiments which may assist in elucidating the relative importance of "dynamic" and "static" quenching mechanisms include:

(1) Study of flavins containing "simpler" substituents at the 10-position. The D-ribityl group of riboflavin may have significant metal affinity, especially in weakly hydrogen-bonding solvents. Use of lumiflavin or lumichrome (p 2) will eliminate this possible complication.

(2) Continued variation of the solvent medium (including the use of nonhydroxylic solvents) is suggested by the large and unanticipated changes in quenching ability effected by changing the flavin environment from aqueous to alcoholic.

(3) The continued study of "ground-state" flavin-metal complexes in nonaqueous media should be undertaken by the use of techniques such as nuclear magnetic resonance, electrochemistry, and infrared spectroscopy.

(4) Finally, the viscosity of the medium should be varied in a systematic way in order to estimate the extent to which flavin fluorescence quenching by metal ions approaches diffusion control.

CHAPTER IV

SUMMARY OF CONCLUSIONS

(1) The abilities of metal ions to quench riboflavin fluorescence are substantially greater in 80% methanol than in aqueous solution, especially among the less strongly quenching ions.

(2) There is definite spectral evidence of ground-state complexation in the case of copper(II), but no apparent evidence for ground state complexes between riboflavin and other metal ions in 80% methanol.

(3) The shapes of Stern-Volmer plots are consistent with the hypothesis that quenching of riboflavin fluorescence by metal ions in 80% methanol involves contributions from both "static" and "dynamic" processes, whereas only the latter are generally operative in aqueous systems.

(4) There is no spectral evidence for "exciplex" formation between excited singlet riboflavin and metal ions in 80% methanol. There is, however, definite kinetic evidence that interactions of some type between excited states of riboflavin and metal ions occur in the mixed solvent system.

LIST OF REFERENCES

LIST OF REFERENCES

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

2. G. Forti and G. Zanetti in "Progress in Photosynthetic Research," Vol. 3, H. Metzner, Ed., Tübuingen, 1969, p 1213 et seq.

3. F. R. Whatley, M. B. Allen, and D. I. Arnon, Biochim. Biophys. Acta, 32, 32 (1959).

4. W. D. McElroy and H. H. Seliger in "Advances in Enzymology," F. F. Nord, Ed., Interscience, New York, N.Y., 1963, p 119.

5. K. Bergman, P. V. Burke, E. Cerda-Olmedo, C. N. David, M. Delbrück, K. W. Foster, E. W. Gooddell, M. Heisenberg, G. Meissner, M. Zalokar, D. S. Dennison, and W. Shropshire, Jr., Bacteriol. Rev., 33, 99 (1969).

6. P. S. Song in "Flavins and Flavoproteins," H. Kamin, Ed., Butterworths, London, 1971, p 37, et seq.

7. W. E. Kurtin, M. A. Latino, and P. S. Song, Photochem. Photobiol., 6, 247 (1967).

8. A. Bard, P. Byrom, J. B. Hudson, and J. H. Turnbull, Photochem. Photobiol., 8, 1 (1969).

9. P. Hemmerich, F. Müller, and A. Ehrenberg in "Oxidases and Related Redox Systems," Vol. 1, T. E. King, H. S. Mason, and M. Morrison, Eds., Wiley, New York, N.Y., 1965, p 128 et seq.

10. A. Ehrenberg, Vitamins and Hormones, 28, 467 (1970).

11. J. Lauterwein, P. Hemmerich, and J. M. Lhoste, Z. Naturforsch, 27b, 1047 (1972).

12. C. J. Fritchie, Chem. Comm., 1220 (1972).

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

14. A. W. Varnes, R. B. Dodson, and E. L. Wehry, J. Amer. Chem. Soc., 94, 946 (1972).

15. R. Sahai, R. H. Hofeldt, and S. H. Lin, Trans. Faraday Soc., 67, 1690 (1971).

16. B. Brocklehurst, Radiat. Res. Rev., 2, 149 (1970).

17. E. L. Wehry, J. Amer. Chem. Soc., 95, 2137 (1973).

18. F. E. Lytle, Appl. Spectrosc., 24, 319 (1970).

19. J. Koziol, Photochem. Photobiol., 9, 45 (1967).

20. B. Nathanson, M. Brody, S. S. Brody, and S. B. Broyde, Photochem. Photobiol., 6, 177 (1967).

21. V. M. Vaugh and G. Weber, Biochemistry, 9, 464 (1970).

22. R. G. Bates, "Determination of pH," Wiley, New York, N.Y., 1969, p 74 et seq.

23. L. G. Whitlby, Biochem. J., 54, 440 (1953).

24. F. A. Welcher in "The Analytical Uses of Ethylenediamine Tetraacetic Acid," Van Nostrand, Princeton, N.J., 1958, pp 149, 217, 230, and 234.

25. R. Belcher, R. A. Close, and T. S. West, Chem. Ind., 1647 (1967).

26. O. Popovych, Crit. Rev. Anal. Chem., 1, 73 (1970).

27. C. L. DeLigny, P. F. M. Luykx, M. Rehback, and A. A. Wienkeke, Rec. Trav. Chim., 79, 713 (1960).

VITA

Susan Goodfellow Polte was born December 7, 1939, in Springfield, Ohio.

She was graduated from Springfield High School in 1957 and from The Ohio State University in 1960. She attended the Graduate School of The University of Tennessee from 1970 through 1973, and held a teaching assistantship in chemistry throughout this period.

She taught at Springfield South High School in Springfield, Ohio, from 1961 through 1967, and is presently a chemistry teacher at Tecumseh High School in Springfield, Ohio.