that pathways analogous to mechanism b may only be important in labile complexes for esters composed of alcohols that are good leaving groups. Further experiments are required to resolve this point.

The most important similarity between the zinc ion catalyzed transesterification between *N*-(β-hydroxyethyl)ethylenediamine and *p*-nitrophenyl picolinate and mechanism b is that both reactions involve the nucleophilic attack of one coordinated ligand upon another. Another significant similarity is that in both cases the concentration of the nucleophilic species is increased as a consequence of coordination or proximity to the metal ion. In the present case, the pK_a of the hydroxy group of *N*-(β-hydroxyethyl)-ethylenediamine in the Zn–**1** complex (see eq 3) can be estimated to be about 8.4 by plotting k_c *vs.* $k_c[H^+]$ from the same kinetic data presented in Figure 2. This probably represents a pK_a perturbation of at least 3–4 pK_a units for the hydroxyethyl group in the Zn–**1** complex **6** relative to the hydroxyethyl group of **1** when it is free in solution.

In summary, metal ions which form both labile and inert complexes can catalyze acyl-transfer reactions by mechanisms in which the dual catalytic function of the metal ion is to increase the concentration of an effective nucleophile at neutral pH values and to serve as a template for the reaction between two coordinated ligands. The present study shows that both these catalytic properties are important in reactions of coordinated ligands and suggests that both are likely to be important factors in the catalytic properties of metalloenzymes.

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Molecular Luminescence Studies of Flavins. I. The Excited States of Flavins[1]

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Abstract: The lowest excited singlet and triplet states of flavins have been investigated by means of luminescence polarization (photoselection) and P–P–P SCF–MO CI methods. The fluorescent and phosphorescent states of flavins of varying structures such as isoalloxazines (*e.g.*, riboflavin), alloxazines (*e.g.*, lumichrome), and lumazines (*e.g.*, 6,7,8-trimethyllumazine) have been assigned (π,π^*) symmetry. However, the (n,π^*) states have been found to play a significant role in the luminescence processes of flavins, particularly of alloxazines. Attempts have been made to characterize various electronic transitions of flavins in the visible and near-uv regions of the absorption spectra, and to reconcile diverse spectroscopic data reported in the literature.

Flavins are implicated in a variety of photobiological processes such as photodynamic action, phototropism, phototaxis, and photosynthesis.[3] In order to elucidate the role of flavins in these and related photochemical systems, a considerable amount of work on the photophysics and photochemistry of flavins *in vitro* has been published during the last decade and is summarized in recent review articles.[4,5] The aim of the present paper is to report a comprehensive luminescence study of flavins in an attempt to characterize and reconcile the diverse spectroscopic properties of these molecules. Three aspects of the excited states of flavins are described herein: namely, (a) assignments of various electronic transitions in the near-uv and visible regions, (b) characteristics of the fluorescent state, and (c) characteristics of the phosphorescent state. In the second part of the present work,[6] dimeric and charge-transfer complexes of flavins will be described.

Experimental Section

Materials. Riboflavin (6,7-dimethyl-(1′-D-ribityl)isoalloxazine, RF) was obtained as described elsewhere.[7] Riboflavin tetrabutyrate (RFTB) was purchased from Tokyo Tanabe Co., and was purified by thin-layer chromatography with chloroform–acetone (1,2 v/v) as the developing solvent. Lumiflavin (6,7-dimethyl-9-methylisoalloxazine, LF), 8-methylisoalloxazine (MIS), 3-methyl-6,7-dichlorolumiflavin, ClLF), 1,3-dimethyl-5,8-dibromolumichrome (1,3-dimethyl-5,8-dibromo-6,7-dimethylalloxazine, BrLC), and 1,3-dimethyllumichrome (MLC) were gifts from Dr. J. Koziol, and luminescent impurities were absent as checked on tlc. 3-Methyllumiflavin (MLF) was a gift from Dr. M. J. Gibian and was used without further purification. Alloxazine (benzo[g]pteridine-2,4-(1*H*,3*H*)-dione), pfs grade, was obtained from the Sigma Chemical Co. and was recrystallized or chromatographed on tlc prior to the preparation of samples. When the compound was found to be free of luminescent impurities, as judged from the excitation spectra which were independent of emission wavelengths monitored, no

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(2) (a) Robert A. Welch Foundation Predoctoral Fellow, 1968–1971; (b) National Science Foundation Predoctoral Trainee, 1968–1971.

(3) For a discussion of the photobiological role of flavins, see J. B. Thomas, "Primary Photoprocesses in Biology," Wiley, New York, N. Y., 1965.

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

(5) P. S. Song in "Flavins and Flavoproteins," H. Kamin, Ed., University Park Press, Baltimore, Md., 1971, pp 37–60.

(6) Part II: T. A. Moore and P. S. Song, submitted for publication.

(7) P. S. Song and D. E. Metzler, *Photochem. Photobiol.*, **6**, 691 (1967).

further purification was carried out in subsequent experiments. Several lumazines (2,4(1*H*,3*H*)-pteridinediones) such as 6,7,8-trimethyllumzaine (TL), 6,7-dimethyl-8-(1′-L-threityllumazine) (ThL), 6,7-diethyl-8-methyllumazine (ML), 6,7-dimethyl-8-(1′-D-ribityl)lumazine (RL), 6,7-dimethyl-8-(1′-L-erythrityl)lumazine (EL), 6,7-dimethyl-8-(1′-(3′-hydroxypropyl))lumazine (HL), 6,7-dimethyl-8-(1′-DL-glycerityl)lumazine (GL), 6,7-dimethyl-8-(1′-D-xylityl)lumazine (XL), and 6,7-dimethyl-8-(1′-(2′-deoxy-D-ribityl))-lumazine (DL) were gifts from Dr. R. Beach and were used without further purification as they were free of luminescent impurities as judged from the independence of the excitation spectra upon wavelength of emission over the entire emission bands. 2-Thioriboflavin was a gift from Dr. W. L. Cairns of Dr. D. E. Metzler's laboratory. This flavin was purified on tlc to remove luminescent impurities. Lumichrome (6,7-dimethylalloxazine, LC)[8] was prepared by aerobic photolysis of riboflavin and then separated on tlc, described previously.[7,9]

Absolute ethanol, spectroscopic grade, was purchased from U. S. Industries. Its purity was checked on an emission spectrometer at maximum sensitivity and no detectable luminescence which would interfere with the flavin luminescence was observed. Isopentane, spectroquality, and phosphoric acid (85%) were obtained from Matheson Coleman and Bell.

Preparation of Samples. Unless otherwise indicated, all samples for absorption and luminescence measurements were prepared using a high-purity Amerisil quartz tube (3 mm i.d.; 4 mm o.d.) as the optical cell which was immersed in a liquid nitrogen optical dewar. Flavins were also imbedded in a polymethyl methacrylate plastic by the following procedure. The flavin was dissolved in approximately 10 ml of chromatoquality methyl methacrylate (obtained from Matheson Coleman and Bell) and the mixture in a test tube (*ca.* 1 cm i.d.) was polymerized at about 60° for 4–9 hr until a transparent plastic with imbedded flavin was obtained. Very pure methyl methacrylate could be used without distillation but the best results were obtained by using methyl methacrylate freshly distilled under slightly reduced pressure. It did not seem to be important to exclude oxygen by freeze–thaw cycles prior to polymerization, although the solutions were flushed with nitrogen. It was necessary to add the smallest trace of azoisobutyric dinitrile to initiate the polymerization and careful spectroscopic checks were made to assure the absence of luminescent impurities under the conditions of observation of flavin luminescence. Several typical references describing the methods of preparing solid solutions tried in our laboratory are given by Kellogg and Schwenker,[10] Shaw,[11] and Czarnecki.[12]

Absorption Spectra. The absorption spectra (resolution $\sim$ 0.5 nm) of flavins were recorded on a Bausch & Lomb Spectronic 600 spectrophotometer which was equipped with special cell holders to accommodate the optical dewars.

Luminescence and Polarization Spectra. Fluorescence and phosphorescence emission, excitation, and polarization spectra were recorded and correction of the polarization due to instrumental bias and cylindrical cell geometry was made as described previously.[13–16] A brief description of the polarization measurements is given below.

Unless indicated otherwise, the polarized excitation spectra were obtained by plotting the polarization degrees measured point by point with the fixed emission wavelength at the near 0–0 emission region. Similarly, the polarized emission spectra were obtained with the fixed excitation wavelength at the near 0–0 absorption region. In this way, vibrational depolarization has been minimized in recording polarization spectra. Degree of polarization (p) at each wavelength of excitation or emission was calculated from the following formula[15]

$$p = \frac{I_{EE} - I_{EB}(I_{BE}/I_{BB})}{I_{EE} + I_{EB}(I_{BE}/I_{BB})}$$

where subscripts E and B refer to the vertical and horizontal orientations of the Glan–Thompson prism polarizers, respectively. The first and second letters in the pair of subscripts refer to the exciting and analyzing polarizer orientations, respectively. Polarization measurements were repeated at least five times to ensure reproducibility. For certain flavins, such as riboflavin tetrabutyrate and alloxazine, more than ten measurements were repeated with satisfactory reproducibility over the region longer than 300 nm and with the mean deviation of p value within ± 0.007. The spectral resolution of the polarization across the excitation and emission bands was the same as the fluorescence (2.25 mm) and phosphorescence (5.5 nm) spectra. The theoretical maximum resolution achievable on our modified Aminco-Bowman spectrophotofluorometer equipped with an ellipsoidal condensing system (Xe source, 150 W) and EMI9558QA photomultiplier tube is 2 nm (grating 600 grooves/nm). Phosphorescence lifetimes were measured as described earlier.[14]

Relative Yields of Luminescence. Relative quantum yields were obtained from the corrected emission spectra using a 1P 28 PMT operated at 800 V. The correction factors were obtained following the method of White.[16] The fluorescence quantum yield of riboflavin was calculated based on the fluorescence quantum yield of fluorescein in 0.1 *N* NaOH when excited at either 366 nm, Φ_F = 0.84, or 436 nm, Φ_F = 0.92.[17] Riboflavin was used as the reference compound for the remainder of the flavins investigated. All samples for relative intensity measurements were kept under the same conditions. The relative quantum yields of different flavins were then estimated using the following formula,[18] $\Phi_{sample}/\Phi_{reference} = (F_{area,\ sample}/F_{area,\ reference})(A_{reference}/A_{sample})$, where the ratio on the left is the ratio of the fluorescence quantum yields of sample flavin to that of the reference flavin, riboflavin. The first ratio in the right-hand side is the ratio of the integrated corrected fluorescence spectra in wave-number scale and the last term is the ratio of absorbances at the wavelength of the exciting light. The ratio of absorbances was used as the solutions were dilute (absorbance less than 0.1) and the absorbances of the sample and reference were very nearly equal. Thus, errors from the inner filter effect and from the MacLaurin approximation were negligible.

Fluorescence quantum yields of riboflavin and riboflavin tetrabutyrate evaluated using fluorescein as the reference were found to be 0.32 and 0.33 in ethanol at room temperature, respectively, when excited at 436 nm. These values are in close agreement with previous data.[19] The quartz tube in the optical dewar was used for measuring absorption and emission of samples in the same solvent at both room temperature and 77°K. Since only the relative emission intensities were measured, no attempt was made to correct for the refractive index difference between liquid and frozen solvents and between air and liquid nitrogen in the optical dewar.

Molecular Orbital Computations. The Pariser–Parr–Pople SCF–MO CI calculations[20,21] were carried out in order to calculate the excited states of flavins. The source of semiempirical integrals and details of the calculation have been given previously.[22]

All of the MO calculations reported so far have been based on the assumed geometry of isoalloxazine.[22–24] In the present work, calculations based on the experimental geometry[25,26] for the iso-

(8) In addition to abbreviations used for compounds, the following abbreviations are used in the figures: tlc, thin-layer chromatography; E, uncorrected fluorescence and phosphorescence excitation spectra (excitation spectra of fluorescence and phosphorescence are essentially identical); E_c, corrected excitation spectra; A, absorption spectra; F, uncorrected fluorescence spectra; DF, delayed fluorescence spectra; EP, polarized excitation with respect to the fluorescence emission band; FP, polarized fluorescence emission with respect to the excitation band; P, uncorrected phosphorescence spectra; PEP, polarized excitation with respect to the phosphorescence band; PP, polarized phosphorescence emission with respect to the excitation band; PMA, polymethyl methacrylate; *f*, oscillator strength.

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

(10) R. E. Kellogg and R. P. Schwenker, *J. Chem. Phys.*, **41**, 2860 (1964).

(11) G. Shaw, *Trans. Faraday Soc.*, **63**, 2181 (1967).

(12) S. Czarnecki, *Acta Phys. Polon.*, **32**, 243 (1967).

(13) P. S. Song and W. E. Kurtin, *J. Amer. Chem. Soc.*, **91**, 4892 (1969).

(14) P. S. Song and W. H. Gordon, III, *J. Phys. Chem.*, 74, 4234 (1970).

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(16) C. E. White, M. Ho, and E. Q. Weimer, *Anal. Chem.*, 32, 438 (1960).

(17) W. R. Dawson and M. W. Windsor, *J. Phys. Chem.*, 72, 3251 (1968).

(18) C. A. Parker and W. T. Rees, *Analyst*, **85**, 587 (1960).

(19) (a) J. Koziol, *Photochem. Photobiol.*, **5**, 41 (1966); (b) J. Koziol, *ibid.*, **9**, 45 (1969).

(20) R. Pariser and R. G. Parr, *J. Chem. Phys.*, 21, 466, 767 (1953).

(21) J. A. Pople, *Trans. Faraday Soc.*, **49**, 1375 (1953).

(22) P. S. Song, *Int. J. Quant. Chem.*, **3**, 303 (1969).

(23) B. Grabe, *Biopolymers*, **No. 1**, 283 (1964).

(24) J. L. Fox, S. P. Laberge, K. Nishimoto, and L. S. Forster, *Biochim. Biophys. Acta*, **136**, 544 (1967).

(25) N. Tanaka, T. Ashida, Y. Sasada, and M. Kakudo, *Bull. Chem. Soc. Jap.*, 40, 1739 (1967). This reference was used in the discussion,

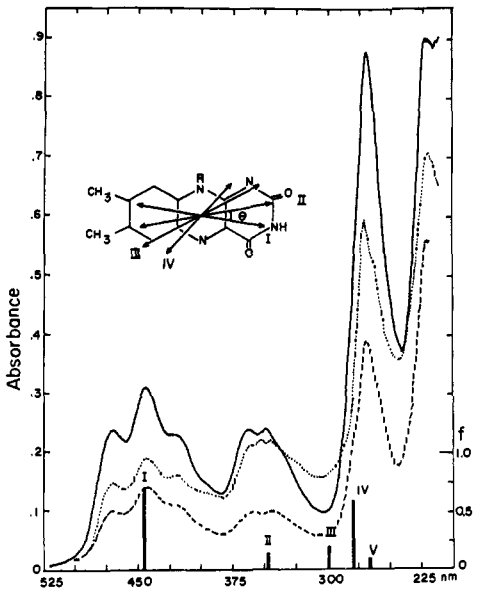


Figure 1. The absorption spectra of riboflavin (———), 3-methyllumiflavin (– – –), and lumiflavin (· · · ·) in ethanol at 77°K. The predicted transition energies and oscillator strengths (f) are indicated by the solid vertical lines. The calculated polarizations of several $\pi \rightarrow \pi^*$ transitions are also included.

alloxazine ring were performed in an attempt to aid the interpretation of observed spectra, and to compare the theoretical electronic spectra of flavins predicted by the MO calculations based on the assumed and observed geometries.

Results and Discussion

The Singlet $^1(\pi,\pi^*)$ States. (a) Isoalloxazines. First, the spectra of riboflavin and riboflavin tetrabutyrate are described in detail as typical isoalloxazines. These two flavins show essentially the same spectral characteristics, and riboflavin tetrabutyrate has a much greater solubility range in organic solvents. Figure 1 shows the absorption spectrum of riboflavin at 77°K, as well as the predicted transition energies, oscillator strengths, and polarizations of the four lowest $\pi \rightarrow \pi^*$ transitions. Agreement between the observed and calculated wavelengths is good for the first two $\pi \rightarrow \pi^*$ transitions, whereas the agreement in the uv region is poor even though the absorption spectrum is expressed in nanometers which makes the comparison artificially more favorable than if reciprocal centimeters are used. This tendency is also noticed in the previous calculations using the assumed geometry.[22,27] We will be mainly concerned with the electronic transitions in the near uv and visible regions. In general, use of the assumed and observed input geometries was found to give very similar spectroscopic data, except for some dependence of the oscillator strengths on the input geometry. Ironically, the predicted intensities in the uv region are better predicted with the assumed geometry than with the observed geometry.

The presence of the two $\pi \rightarrow \pi^*$ transitions in the 450–360-nm region is predicted by MO theory[22,24,27] and supported by circular dichroism[28,29] and polarized

although calculations with the geometry reported in the next reference[26] were also performed yielding similar results.

(26) P. Kierkegaard, R. Norrestam, P.-E. Werner, A. Ehrenberg, L. E. G. Eriksson, and F. Müller, *Chem. Commun.*, 288 (1967).

(27) P. S. Song, *Ann. N. Y. Acad. Sci.*, **158**, 410 (1969).

(28) D. W. Miles and D. W. Urry, *Biochemistry*, 7, 279 (1968).

(29) D. E. Edmondson and G. Tollin, *ibid.*, 10, 113 (1971).

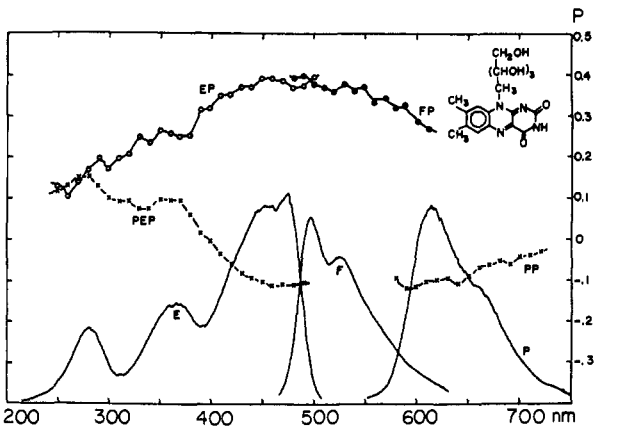


Figure 2. The luminescence and polarization spectra of riboflavin in ethanol at 77°K: E, fluorescence excitation; F, fluorescence; P, phosphorescence; EP, polarized fluorescence exictation; FP, polarized fluorescence; PEP, polarized phosphorescence excitation; and PP, polarized phosphorescence spectra. The polarization spectra are to be referred to the polarization degree (p).

fluorescence excitation[30,31] spectra. However, a third $\pi \rightarrow \pi^*$ transition has been assigned in the region of 340 nm,[29] and the work of Fox, *et al.*,[24] was cited as providing theoretical support. The third $\pi \rightarrow \pi^*$ transition ($f = 0.056$) is actually predicted at 306[24] rather than at 340 nm, in agreement with the transition predicted at 297 nm shown in Figure 1. As far as luminescence polarization data and the present calculations are concerned, the two major absorption bands at 450 and 360 nm can both be attributed to $\pi \rightarrow \pi^*$ transitions. A further comment on this point will follow in the next section (The Singlet $^1(n,\pi^*)$ States).

Figure 2 shows luminescence and polarization spectra of riboflavin. Spectra for riboflavin tetrabutyrate are similar to Figure 2. The polarized fluorescence spectra of riboflavin and riboflavin tetrabutyrate are qualitatively similar to the spectrum of riboflavin in 90% glycerol at 263°K.[31] The 450-nm bands show a maximum degree of excitation polarization with respect to fluorescence, good evidence that the fluorescence arises from the lowest $^1(\pi,\pi^*)$ state. The 360-nm bands show a somewhat lower degree of fluorescence polarization as compared to that of the 450-nm bands. From the value of the polarization in the 450- and 360-nm regions, an angle of approximately 20 ± 5° between the two transition moments can be estimated. This value agrees with the predicted angle shown in Figure 1.[32]

The corrected excitation spectrum of riboflavin tetrabutyrate was found to be similar to that of riboflavin and to its absorption spectrum at 77°K, as expected. Figure 3 shows the delayed fluorescence and

(30) G. Weber in "Flavins and Flavoproteins," E. C. Slater and C. Veeger, Ed., Elsevier, Amsterdam, 1966.

(31) W. E. Kurtin and P. S. Song, *Photochem. Photobiol.*, 7, 263 (1968).

(32) In a previous paper,[31] an angle of 49° was obtained from solution spectra with an invalid assumption. Furthermore, theoretically calculated angles[22,27] without taking into account the configuration interaction are now substantially reduced by virtue of the configuration interaction effect on the direction of polarization. If we assign the polarization between the lowest energy excitation transition and the fluorescent transition a value of 0.5 after a correction for the randomization factor in rigid matrix at 77°K, the estimated angle increases by as much as 10° or more. Thus, angles reported herein are subject to variation depending upon the maximum value of fluorescence polarization taken and should be regarded as only "semiquantitative."

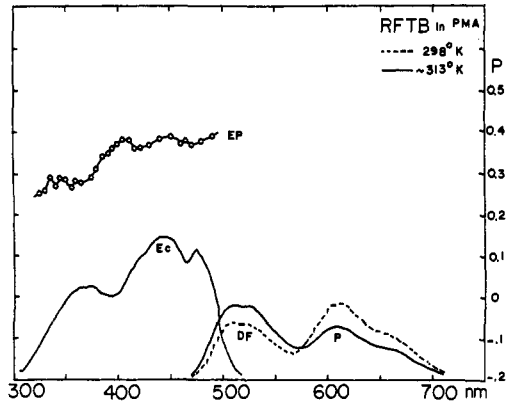


Figure 3. The luminescence and polarization spectra of riboflavin tetrabutyrate in polymethyl methacrylate (see the caption of Figure 2 for an explanation of abbreviations): DF, delayed fluorescence; EC, corrected excitation.

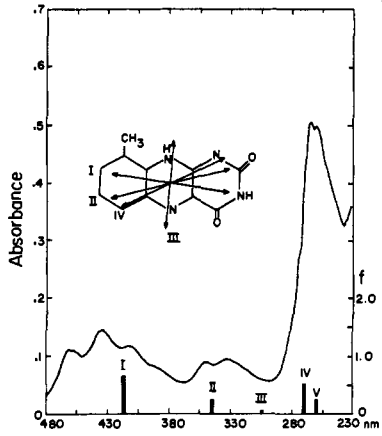


Figure 5. The absorption spectrum of 8-methylisoalloxazine in ethanol at 77°K. The predicted results are also included (see the caption for Figure 1).

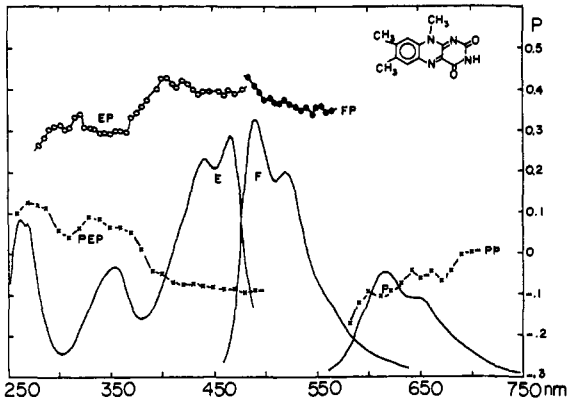


Figure 4. The luminescence and polarization spectra of lumiflavin in ethanol at 77°K (see the caption of Figure 2 for an explanation of abbreviations).

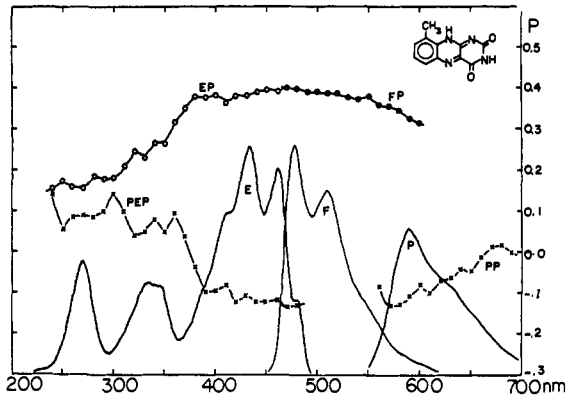


Figure 6. The luminescence and polarization spectra of 8-methylisoalloxazine at 77°K (see the caption of Figure 2 for explanation of abbreviations).

phosphorescence spectra of riboflavin tetrabutyrate in polymethyl methacrylate plastic. The corrected excitation spectrum in the plastic resembles the absorption spectrum in ethanol at 77°K (Figure 1). Results in polymethyl methacrylate are useful in reconciling the phototropic action spectrum in terms of flavin as a photoreceptor pigment.[31] The reciprocal dependence of the delayed fluorescence and phosphorescence intensity in polymethyl methacrylate upon temperature and identity of the excitation spectra for the prompt fluorescence and delayed fluorescence emission further support the assignment of fluorescence and phosphorescence spectra to the emission from the singlet and triplet states, respectively. It can be seen that the polarization spectrum of delayed fluorescence in polymethyl methacrylate at 298°K is essentially identical with that of fluorescence in ethanol at 77°K. This indicates that no new transitions such as $n \rightarrow \pi^*$ are resolved in the nonpolar polymethyl methacrylate matrix.

Figure 4 shows luminescence spectra for lumiflavin. As expected from the similarity of their absorption spectra in Figure 1, the luminescence spectra of these flavins are essentially identical. The angle (θ) between the 450- and 360-nm moments appears to be somewhat less than 20° and the difference may very well be due to experimental errors involved in rather difficult polarization measurements. In all cases of flavins in Figures 2–4, it is nonetheless apparent that the two lowest energy transitions are polarized nearly parallel to each other ($\theta < 30°$).

Figure 5 shows the absorption spectrum of 8-methylisoalloxazine as well as the calculated values of the spectroscopic quantities. A satisfactory account for the blue shift of the first $\pi \rightarrow \pi^*$ in 8-methylisoalloxazine is accomplished by the P–P–P SCF–MO CI calculations. In contrast to the absorption spectra of 6,7-dimethylisoalloxazines (Figure 1), the uv absorption peak splits into two peaks at 265 and 260 nm. Whether this reflects two overlapping electronic transitions, as predicted by the MO calculation, cannot be determined by means of the low-resolution spectrum shown in Figure 6.

From the excitation polarization across the 435- and 330-nm bands shown in Figure 6, the angle θ is estimated to be about 15° in qualitative agreement with the predicted angle of 22° (Figure 5). As in the case of other flavins described so far, the uv band is substantially less polarized than the first $\pi \rightarrow \pi^*$ band at 435 nm. This indicates a larger angle θ between the fluorescence emission oscillator $^1(\pi,\pi^*)$ and the fourth $\pi \rightarrow \pi^*$ band, IV. The theoretical polarizations of these transitions, as shown in Figures 1 and 5, are thus consistent with the polarized fluorescence excitation spectra.

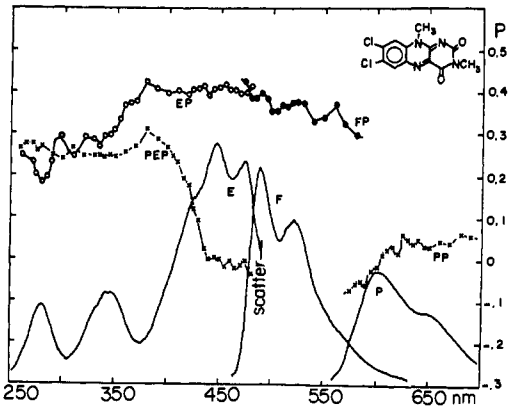


Figure 7. The luminescence and polarization spectra of 3-methyl-6,7-dichlorolumiflavin in ethanol at 77°K (see the caption of Figure 2 for explanation of abbreviations).

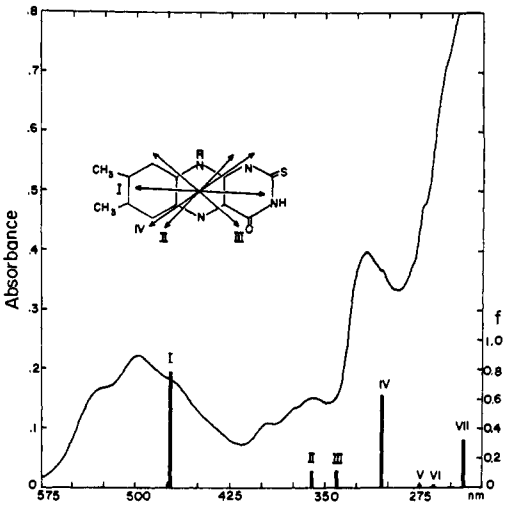


Figure 8. The absorption spectrum of 2-thioriboflavin in ethanol at 77°K. The predicted results are also included (see the caption of Figure 1).

Figure 7 shows luminescence and polarization spectra for 3-methyl-6,7-dichlorolumiflavin. The absorption spectrum of 3-methyl-6,7-dichlorolumiflavin in ethanol was found to be almost identical with that of 3-methyllumiflavin, as was suggested from the similarity of the polarization spectra for these two flavins. Chloro substituents do not appear to affect significantly the polarization of the 450- and 360-nm bands or their mutual transition moment orientation (θ). Thus, the predicted polarization of the two transitions along the long molecular axis is consistent with the above observation. However, the polarization of the uv band at 274 nm is about the same as the band at 340 nm, indicating that polarization of the $\pi \rightarrow \pi^*$ transition at 274 nm is along an axis at a small angle to the axis of the emission moment. The polarized phosphorescence excitation spectrum is also consistent with the above descriptions.

Figure 8 shows the absorption spectrum of 2-thioriboflavin along with the theoretical values of the $\pi \rightarrow \pi^*$ transition energies, intensities, and polarizations. The long-wavelength band I is predicted to be a $\pi \rightarrow \pi^*$ transition which is long-axis polarized. Two relatively weak transitions, II and III, are also predicted in the region of 340–360 nm, and these transitions may correspond to the shoulders of the region of 350–400 nm in the absorption spectrum. A strong uv transition at 305 nm which corresponds most likely to $\pi \rightarrow \pi^*$ transition IV is polarized along an intermediate axis between the long and short axes. Unfortunately, it was not possible to compare these theoretical results with the polarized fluorescence excitation spectrum as 2-thioriboflavin was found to be nonfluorescent. It should be pointed out that dilute tlc-purified samples were found to be fluorescent with a corrected excitation spectrum dissimilar to the absorption spectrum. Namely, no maximum fluorescence excitation at 500 nm was recorded and the excitation and fluorescence spectra resembled the spectra of riboflavin which had been carefully eliminated by tlc. We naively attribute this anomalous spectra to the presence of a flavin impurity.

Summarizing this section, the following conclusions on the spectroscopic properties of flavins arising from $\pi \rightarrow \pi^*$ transitions can be drawn. First, in all cases studied, the fluorescence spectra are attributed to the $^1(\pi,\pi^*) \rightarrow S_0$ (ground state) emission on the basis of a high-polarization degree across the first absorption and emission bands, respectively. The second major absorption band in the region of 360 nm is also assigned to a $\pi \rightarrow \pi^*$ transition, and the direction of its transition moment is only $20 \pm 5°$ displaced from the polarization axis of the first $\pi \rightarrow \pi^*$ band of riboflavin at 77°K. The angles range from 15 to 20° on the average for other isoalloxazines, and are in excellent agreement with the calculated direction of polarizations. On the other hand, an angle of about 30° between the orientation axes of the first and second $\pi \rightarrow \pi^*$ moments can be obtained from the polarized fluorescence excitation spectra[30] in solution as there is a tendency for sharper variations and a higher degree of EP at 450 nm (often approaching the theoretical maximum of 0.5) in solution[31,33] than in rigid organic glasses or polymethyl methacrylate plastics. In any case, it is apparent that the two lowest $\pi \rightarrow \pi^*$ transitions are not mutually perpendicular in their polarization directions. This behavior is understandable in terms of a lack of molecular symmetry and configuration interactions among numerous excited configurations.

The P–P–P SCF–MO CI predicts a weak $\pi \rightarrow \pi^*$ transition to the blue side of the second $\pi \rightarrow \pi^*$ band II, as well as an additional weak band of the $\pi \rightarrow \pi^*$ type V under the uv absorption band, IV. A higher resolution study is desirable to test these predictions. In general, the uv band IV shows a significantly lower polarization degree than the first $\pi \rightarrow \pi^*$ band in the 450-nm region, indicating a large angle between these two transition moments. The P–P–P SCF–MO CI results are also consistent with the above analysis. The magnetic circular dichroic spectrum shows an additional uv band,[34] which may correspond to the transition V.

(b) Alloxazines. Figure 9 presents the absorption spectrum of alloxazine along with theoretical results. Again, agreement between the theoretical and observed transition energies is poor in the uv region, although an excellent correlation between the predicted and observed band locations can be seen in the near-uv region. The polarizations of the weak $\pi \rightarrow \pi^*$ (III) and intense

(33) R. F. Chen, *Science*, 147, 729 (1965).
(34) G. Tollin, *Biochemistry*, 7, 1720 (1968).

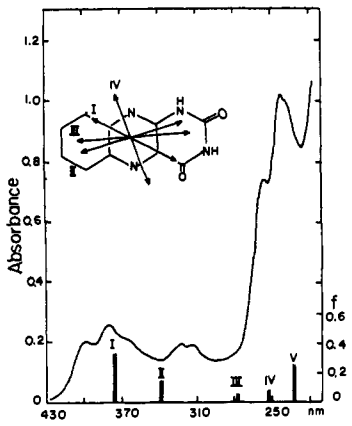


Figure 9. The absorption spectrum of alloxazine in ethanol at 77°K (see the caption of Figure 1 for an explanation of the predicted values).

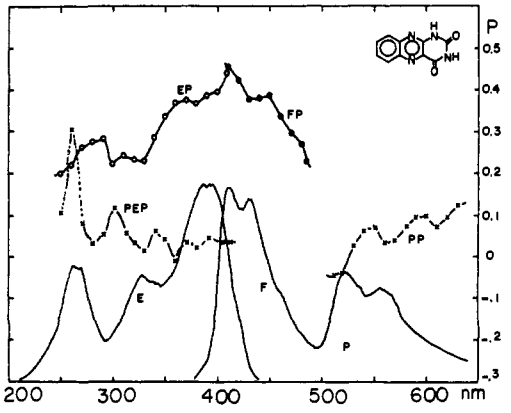


Figure 10. The luminescence and polarization spectra of alloxazine in ethanol at 77°K (see the caption of Figure 2 for an explanation of abbreviations).

$\pi \rightarrow \pi^*$ (IV) bands in isoalloxazines are reversed in the case of alloxazine.. Figure 10 shows the luminescence and polarization spectra of alloxazine. The polarization degree in the red region of the first excitation band is not too far from the theoretical maximum of 0.5. Thus the intense absorption band in this region can be assigned to a $\pi \rightarrow \pi^*$ transition, and the fluorescence is attributable to the emission arising from the $^1(\pi,\pi^*)$ state[31] as in the case of isoalloxazines. The theoretical results shown in Figure 9 are consistent with this assignment. An angle (θ) of about 30° between the transition moments of the two near-uv bands can be estimated from the polarized fluorescence excitation spectrum shown in Figure 10. This angle is somewhat larger than in the isoalloxazines, and has a predicted value of 43° (Figure 9).[32]

Although the shapes of absorption and polarized fluorescence excitation of isoalloxazines and alloxazine are similar except for the blue shift of the bands in the latter, one distinct difference in the polarized fluorescence spectrum was repeatedly found. Frequently it is observed that the maximum polarization degree of 0.5 for the fluorescence of the classical Vavilov–Levshin linear oscillator model is not obtained and that the degree of the fluorescence polarization of complex organic molecules decreases with increasing wavelength of emission.[35]

(35) (a) G. P. Gurinovich, A. M. Sarzhevshii, and A. N. Sevchenko, *Opt. Spectrosc.*, 3, 237 (1957); (b) S. I. Vavilov and V. L. Levshin, *Z. Phys.*, 16, 136 (1923).

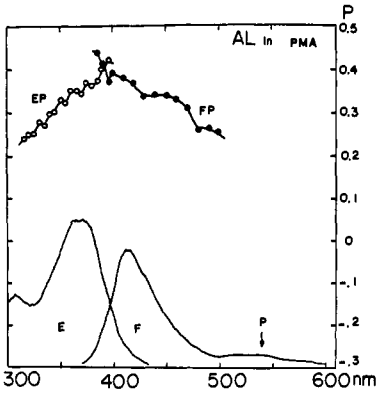


Figure 11. The luminescence and polarization spectra of alloxazine in polymethyl methacrylate at room temperature (see the caption of Figure 2 for an explanation of abbreviations).

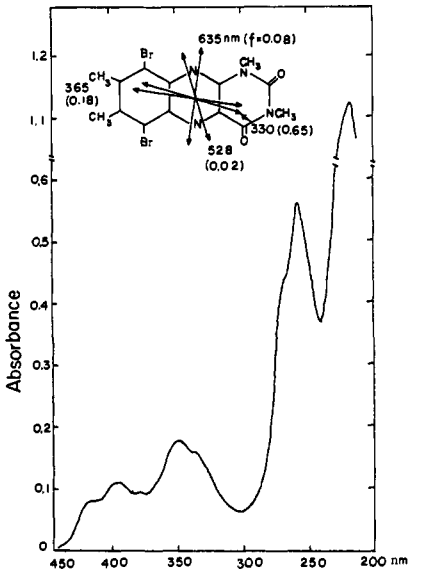


Figure 12. The absorption spectrum of 1,3-dimethyl-5,8-dibromolumichrome in ethanol at 77°K. The theoretical results are also included.

The rate of decrease is significantly sharper in the case of alloxazine than for isoalloxazines even when the emission spectra were expressed in a wave-number scale. A plausible mechanism for the variation of the polarized fluorescence spectra of alloxazine will be offered in a later section (The $^1(n,\pi^*)$ States).

Figure 11 shows the fluorescence and polarization spectra of alloxazine in polymethyl methacrylate at 298°K. Fluorescence polarization of alloxazine in polymethyl methacrylate confirms the spectral characteristics at 77°K described above. Only the prompt fluorescence is recorded in Figure 11, as the phosphorescence and delayed fluorescence intensities of alloxazine appeared to be very weak or absent, as compared to riboflavin tetrabutyrate (Figure 3). Spectra for lumichrome were found to be similar to the alloxazine spectra except for a red shift (*ca.* 10 nm) of the absorption maximum with respect to alloxazine. The first and second $\pi \rightarrow \pi^*$ bands of lumichrome are predicted at 394 (f = 0.513) and 353 nm (f = 0.199), in good agreement with the observed maxima in ethanol at 390 and 340 nm (room temperature), respectively. The predicted angle (θ) is 33° after CI, in qualitative agree-

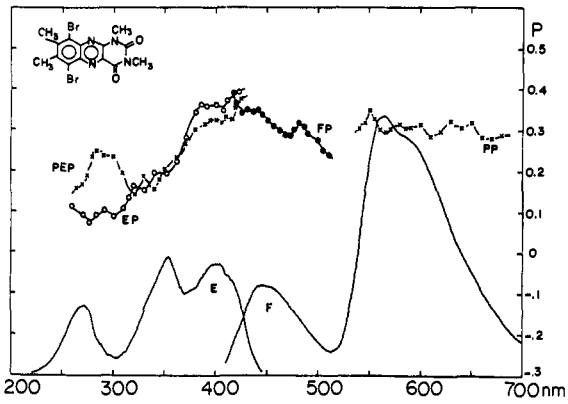


Figure 13. The luminescence and polarization spectra of 1,3-dimethyl-5,8-dibromolumichrome in ethanol at 77°K (see the caption of Figure 2 for an explanation of abbreviations).

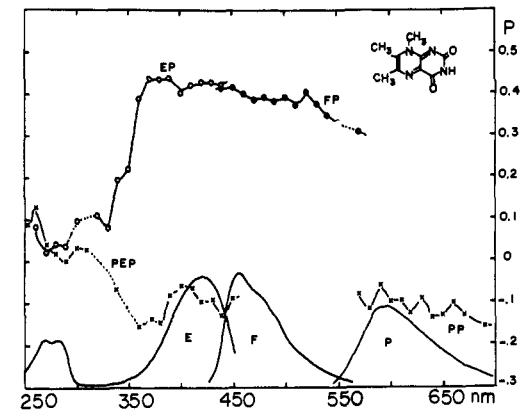


Figure 15. The luminescence and polarization spectra of 6,7,8-trimethyllumazine in ethanol at 77°K (see the caption of Figure 2 for an explanation of abbreviations).

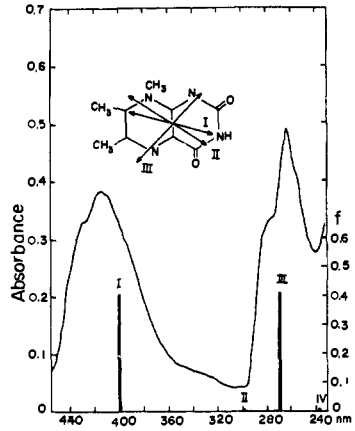


Figure 14. The absorption spectrum of 6,7,8-trimethyllumazine in ethanol at 77°K. The theoretical results are also included.

ment with the polarized fluorescence excitation spectrum.

Figure 12 presents the absorption spectrum of 1,3-dimethyl-5,8-dibromolumichrome at 77°K along with theoretical calculations.[36] Figure 13 shows luminescence and polarization spectra. Inspection of the absorption, polarized excitation spectra, and theoretical transition energies suggests that the lowest $\pi \rightarrow \pi^*$ transition is responsible for the λ_{max} at 398 nm. This corresponds to a red shift of about 10 and 20 nm with respect to the absorption maxima of lumichrome and alloxazine, respectively. The shoulder at 417 nm appears to be due to a vibrational structure associated with the lowest $\pi \rightarrow \pi^*$ band at 77°K. The absorption spectrum of 1,3-dimethyl-5,8-dibromolumichrome in ethanol at room temperature does not reveal any structure in the near-uv region. The second $\pi \rightarrow \pi^*$ at 350 nm is more intense than the first $\pi \rightarrow \pi^*$ at 389 nm, and is polarized along an axis at a substantial angle (θ) to the first $\pi \rightarrow \pi^*$ band. The polarized fluorescence excitations at the red edge of the first excitation band and at the second excitation band (250 nm) show a difference of 0.2 as shown in Figure 13. This corresponds to an angle (θ) of $\sim$20°. The predicted polarizations of the two $\pi \rightarrow \pi^*$ transitions I and II give an angle (θ) of 24° with the lowest energy transition predicted to be polarized along the short axis.

The uv excitation in the 250–290-nm region shows significantly lower polarization than at 398 nm, indicating that the first $\pi \rightarrow \pi^*$ and the uv transition III are far from being parallel. The theory predicts mutually perpendicular polarizations for these transitions, as shown in Figure 12. In general, the 5,8-dibromo substitution has resulted in a red shift of the near-uv absorption bands with respect to the absorption spectra of alloxazine. Furthermore, the intensity of the second $\pi \rightarrow \pi^*$ transition is now greater than the first one. These results are very poorly reproduced by the MO calculations, particularly with respect to the wavelengths of the various transitions. The poor agreement is probably caused by the particular choice of parameters for Br and C–Br, which yielded the highest occupied MO largely made of the AO expansion coefficients on bromine. As a result, the $\pi \rightarrow \pi^*$ transition arising from the configuration (HOMO) $\rightarrow$ (LEMO) contains strong charge-transfer character while the transition monopoles on the bromine atoms are small (0.059 and −0.032).

(c) Lumazines. Figure 14 shows the absorption spectrum of trimethyllumazine at 77°K along with the theoretical transition energies, intensities, and polarizations. The removal of the benzenoid ring from the isoalloxazine chromophore is reflected in the absorption spectrum of the lumazine as evidenced by the absence of the near-uv band.

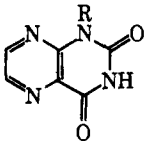
MO calculations predict an extremely weak $\pi \rightarrow \pi^*$ transition II at 298 nm which may correspond to the shoulder at 280 nm of the absorption spectrum shown in Figure 14. The intense uv band at 264 nm can be identified with the third $\pi \rightarrow \pi^*$ band III. This assignment is consistent with the polarized fluorescence excitation spectrum of trimethyllumazine shown in Figure 15. It can be seen that the degree of polarization in the 270-nm region is about zero, while polarization of the 410-nm band approaches the theoretical maximum of 0.5°. From the polarization spectrum, we can estimate an angle (θ) of 45° between the two transition moments. The predicted angle is 62° from Figure 14, in satisfactory agreement with the above assignment and observed polarization data. From the polarization spectrum it is also apparent that there is only one $\pi \rightarrow \pi^*$ transition at wavelengths longer than

(36) Values of semiempirical integrals for Br used in the P–P–P SCF–MO CI calculation were: IP = 19.02 eV, (BrBr|BrBr) = 11.46 eV, and $\beta_{CBr}^{core} = -0.72$ eV.

320 nm in trimethyllumazine. This interpretation is supported by previous theoretical work[24,37] and predictions shown in Figure 14.

On the basis of the polarization characteristics and frequency of the visible and uv bands of lumazines (Figures 14 and 15), the 450-nm band of isoalloxazines can be correlated with the lowest $\pi \rightarrow \pi^*$ transition of the pteridinedione chromophore, whereas one of the uv bands of isoalloxazines and alloxazines may be correlated with the L_b species of the benzenoid ring.

Other lumazines listed in the Experimental Section have also been studied. Results are identical with those described above for trimethyllumazine, as far as the characteristics of absorption, fluorescence excitation, and emission polarization spectra are concerned. Prigge and Lippert[38] have studied lactim (I) derivatives



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in terms of their fluorescence polarization spectra. The absorption and fluorescence spectra of the lactim derivatives are blue shifted in comparison with the spectra of lumazines, as in the case of the alloxazine–isoalloxazine systems. The long-wavelength absorption and fluorescence spectra were attributed to a $\pi \rightarrow \pi$ transition with positive degree of the excitation polarization and the second $\pi \rightarrow \pi^*$ transition at 280–290 nm showed negative polarization indicating that the orientation between these two transition moments is closer to being perpendicular. These observations suggest that the tautomerization of lumazine does not drastically alter the relative polarization of the two lowest $\pi \rightarrow \pi^*$ transitions, although the location of the absorption bands is considerably displaced.

In summary, it is suggested that the lowest energy excitation (absorption) and fluorescence spectra of flavins, namely, isoalloxazines, alloxazines, and lumazines, can be attributed to a $\pi \rightarrow \pi^*$ transition. The lowest $\pi \rightarrow \pi^*$ transition in flavins is predicted to be polarized roughly along the long molecular axis, except for the case of dibromolumichrome where vertically substituted bromines strongly perturb the polarization direction. From the photoselection experiments, the relative polarizations of the low-energy $\pi \rightarrow \pi^*$ transitions have been determined and the results are at least in qualitative agreement with the predicted polarization data. However, absolute polarization directions (experimental) cannot be deduced from the present work.

The Singlet $^1(n,\pi^*)$ States. (a) Isoalloxazines. Information concerning the location and intensity of $n \rightarrow \pi^*$ transitions is useful for elucidating spectroscopic, photochemical, and photobiological properties of flavins. For example, it is well known that $^1(n,\pi^*)$ states play an important role in the spin–orbit interactions which determine the overall luminescence (fluorescence and phosphorescence) behavior of heterocyclic molecules. The $n \rightarrow \pi^*$-like transitions in flavins are potentially intense due to a lack of molecular symmetry, distortion of coplanarity,[26] and the occurrence of two pyridino nitrogens and two carbonyl groups. Several authors have already carried out spectroscopic studies aimed at resolving the $n \rightarrow \pi^*$ band(s).

The absorption spectra of isoalloxazines such as riboflavin, riboflavin tetrabutyrate, and methyllumiflavin in nonpolar solvents at room temperature are characterized by the appearance of a shoulder peak in the 472–480-nm region,[31,39,40] this shoulder being absent in polar solvents at room temperature. Such a solvent dependence of the absorption spectra can be diagnosed as the result of the red-shifted $n \rightarrow \pi^*$ band in nonpolar solvents,[39] thus placing the $^1(n,\pi^*)$ state below the $^1(\pi,\pi^*)$ state. The low-temperature absorption spectrum (Figure 1) shows a similar spectrum with the shoulder at 472 nm which can be described as a resolved vibrational structure of the Franck–Condon envelope belonging to the $\pi \rightarrow \pi^*$ transition rather than to an $n \rightarrow \pi^*$. The following results support this conclusion: (a) polarization of the fluorescence excitation at 420–490 nm approaches the theoretical maximum of 0.5, (b) electron donating substituents such as the methyl group and chlorine do not blue shift the 470–480 = nm shoulder, and (c) flavins show relatively intense fluorescence emission in polar solvents and the fluorescence quantum yield increases considerably in nonpolar solvents.[18,19]

Edmondson and Tollin[29] have found no circular dichroic evidence for $n \rightarrow \pi^*$ transitions in flavins. On the other hand, Miles and Urry[28] suggested the presence of an $n \rightarrow \pi^*$ transition in the 340-nm region. A crude MO estimate of the $n \rightarrow \pi^*$ transition in flavins predicts it at about 370 nm.[22] At 77°K the second major band of riboflavin and other isoalloxazines at 360 nm splits into two peaks at 364 and 348 nm. However, polarization (Figure 2) in this region is constant and thus does not provide information concerning the existence of an $n \rightarrow \pi^*$ if it is hidden under the intense $\pi \rightarrow \pi^*$ band II. It is possible that the sum of the molar extinction coefficients for $n \rightarrow \pi^*$ sources in the isoalloxazine ring may reach a value up to 2000 or more in a given region if all four $n \rightarrow \pi^*$ transitions are nearly degenerate. Slightly positive polarization of the phosphorescence excitation in the 350-nm region and of the 0–0 phosphorescence band[41] are suggestive of the presence of $n \rightarrow \pi^*$ intensities there, provided that $n \rightarrow \pi^*$ transitions and the 0–0 phosphorescence are substantially out-of-plane polarized.[42] Since the 0–0 phosphorescence may have a significant in-plane short-axis component, and the polarization degree of the fluorescence excitation at 77°K (Figure 2) is not as low as the polarization degree in the glycerol solution,[31] the above suggestion cannot be conclusive without additional evidence.

The CNDO/II calculations[43] of isoalloxazine predict the $n \rightarrow \pi^*$ transition to be at a longer wavelength than the second $\pi \rightarrow \pi^*$ transition, consistent with the π–

(37) K. Nishimoto, *Bull. Chem. Soc. Jap.*, **40**, 2493 (1967).
(38) H. Prigge and E. Lippert, *Ber. Bunsenges. Phys. Chem.*, **69**, 458 (1965).
(39) A. Kotaki, M. Naoi, J. Okuda, and K. Yagi, *J. Biochem. (Tokyo)*, **61**, 404 (1967).
(40) H. A. Harbury, K. F. LaNoue, P. A. Loach, and R. M. Amick, *Proc. Nat. Acad. Sci. U. S.*, **45**, 1708 (1959).
(41) P. S. Song and W. E. Kurtin, *Photochem. Photobiol.*, **10**, 211 (1969).
(42) W. E. Kurtin and P. S. Song, *ibid.*, **9**, 127 (1969).
(43) (a) P. S. Song, *J. Phys. Chem.*, **72**, 536, 4724 (1968); (b) unpublished results.

Table I. Relative Fluorescence (Φ_F) and Phosphorescence (Φ_P) Quantum Yields of Flavins in Ethanol

Flavin	$\Phi_{F,298°}$	$\Phi_{F,77°}$	$\Phi_{P,77°}$	Φ_F/Φ_P	τ, sec
RF[a]	0.32 ± 0.02	0.60 ± 0.06	0.004 ± 0.001	150	0.17[c]
RFTB	0.33 ± 0.02	0.77 ± 0.08	0.003 ± 0.001	257	0.16
LF	0.37 ± 0.02	0.78 ± 0.08	0.003 ± 0.001	260	0.15
MLF	0.46 ± 0.02	0.87 ± 0.09	0.003 ± 0.001	290	0.19
MIS	0.40 ± 0.02	0.71 ± 0.07	0.005 ± 0.001	142	0.15
ClLF	0.29 ± 0.03	0.43 ± 0.04	0.003 ± 0.001	143	0.07
2-Thio-RF	?	?	?		?
AL	0.033 ± 0.005	0.068 ± 0.007	0.053 ± 0.005	1.28	0.23[c]
LC	0.076,[b] 0.067 ± 0.01	0.139 ± 0.02	0.066 ± 0.01	2.11	0.24
MLC	0.139,[b] 0.086 ± 0.01	0.148 ± 0.02	0.056 ± 0.01	2.64	0.29
BrLC	0.001 ± 0.0005	0.004 ± 0.001	0.012 ± 0.001	0.33	~0.004
TL		0.98 ± 0.05	0.003 ± 0.001	307	0.32
ThL		0.86 ± 0.05	0.004 ± 0.001	215	0.28
EL		0.90 ± 0.05	0.007 ± 0.002	129	0.30
ML		0.92 ± 0.05	0.005 ± 0.001	184	0.33

[a] See Experimental Section for abbreviations. [b] See ref 14. [c] Previously reported lifetimes in ethylene glycol–water glasss[42] are too large, as they were measured on a filtered microphotometer–recorder unit since an oscilloscope assembly was not available.

MO estimate.[22] Implications of these predictions will be discussed in section b which follows.

(b) Alloxazines. The CNDO/II calculations[43] of alloxazine predict that the second lowest transition is of $n \rightarrow \pi^*$ origin as in the case of isoalloxazine. The crude π–MO estimate yields an $n \rightarrow \pi^*$ transition at 370 nm.[22] The low-temperature absorption spectrum of alloxazine reveals three distinct structures in the 360–400-nm region. Whether one of these shoulders represents an $n \rightarrow \pi^*$ band is not apparent from the polarization spectrum shown in Figures 10 and 11. However, it can be seen from Figures 10 and 11 that polarization of the fluorescence excitation of alloxazine declines more sharply across the first absorption band than the polarization for isoalloxazines such as riboflavin (Figure 2). Furthermore, the fluorescence polarization declines sharply with the wavelength of emission. Such a decline in the fluorescence polarization can be caused by vibrational depolarization and may be explained by the dependence of the transition dipole on the nuclear coordinates.[44] Strong vibronic interactions between the fluorescent (π,π^*) and low-lying (n,π^*) states can effectively result in such a vibrational depolarization of the fluorescence spectrum of alloxazine. Since the energy separation between the fluorescent $^1(\pi,\pi^*)$ and the $^1(n,\pi^*)$ states is expected to be considerably larger for isoalloxazines (see discussion in section a) than for alloxazines, the sharp decline in the fluorescence polarization of alloxazine is suggestive of the presence of the $^1(n,\pi^*)$ state near the lowest $^1(\pi,\pi^*)$ state. To strengthen this point of view, a further discussion follows.

Table I lists the relative luminescence quantum yields of various flavins. It can be seen that the fluorescence emission of alloxazines at 77°K is weaker than that of isoalloxazines, as has been noted previously.[42,45]

The N-methylation at position 3 increases the fluorescence intensity in isoalloxazines and alloxazines, as indicated in Table I. 1,3-Methylation of lumichrome further increases the fluorescence quantum yield by nearly a factor of two (see Table I), however. These results are consistent with the suggestion that the $^1(n,\pi^*)$ state is located near the fluorescent (π,π^*) state which is vibronically coupled with the former. Such an energetic picture is favorable for radiationless transitions, for example, intersystem crossing, thus reducing the fluorescence quantum yield of alloxazines relative to isoalloxazines and also accounting for the Φ_F increase of the former upon methylation as the (n,π^*) states are blue shifted.

If the above interpretation is valid, the fluorescence quantum yield of alloxazines should decrease with a decrease in polarity of the solvent at room temperature, in contrast to the solvent-dependence behavior of isoalloxazines.[46] Indeed, such solvent dependence studies have been carried out for lumichrome and 1,3-dimethyllumichrome by Koziol.[47] Thus, Φ_F values of 1,3-dimethyllumichrome reduce approximately twice and three times, respectively, in going from water to dioxane as the solvent.

It is of interest to examine the effect of acidic solvents on the fluorescence polarization spectra of flavins in order to assess the role of $^1(n,\pi^*)$ states, particularly those in alloxazine. Figure 16 shows the luminescence and polarization spectra of riboflavin in phosphoric acid (85%) at 77°K. The absorption spectrum at 298°K shows only one λ_{max} at 402 nm in the near-uv region, whereas the spectrum in uv region is not altered significantly by the protonation of N and O atoms. The fluorescence polarization degrees of riboflavin are considerably lower in phosphoric acid than in ethanol, except for the red side of the excitation spectrum. This observation can be interpreted in terms of two $\pi \rightarrow \pi^*$ transitions comprising the absorption in the 350–450-nm region with λ_{max} at 402 nm. The effect of eliminating $n \rightarrow \pi^*$ sources by protonation is not obvious from these results. However, the phosphorescence intensity was extremely weak in phosphoric acid.

The P–P–P SCF–MO CI calculation of the protonated riboflavin yields $\pi \rightarrow \pi^*$ transitions at 457 (f = 0.40, polarized 20° clockwise from the long molecular axis) and 410 nm (f = 0.42, polarized along the long axis, 3° counterclockwise). These predictions are qualitatively consistent with the shape of absorption and polarized fluorescence excitation spectra (Figure 16) in the 350–470-nm region where the two overlapping $\pi \rightarrow \pi^*$

(44) S. I. Kubarev, *Opt. Spectrosc.* (English Translation), **10**, 276 (1961).

(45) M. Berezovskii and Zh. I. Aksel'fod, *Dokl. Akad. Nauk SSSR*, **171**, 1101 (1966).

(46) (a) J. Koziol and E. Knobloch, *Biochim. Biophys. Acta*, **102**, 289 (1965); (b) J. Koziol, *Methods Enzymol.*, **18**, 253 (1971).

(47) J. Koziol in "Flavins and Flavoproteins," H. Kamin, Ed., University Park Press, Baltimore, Md., 1971, p 54.

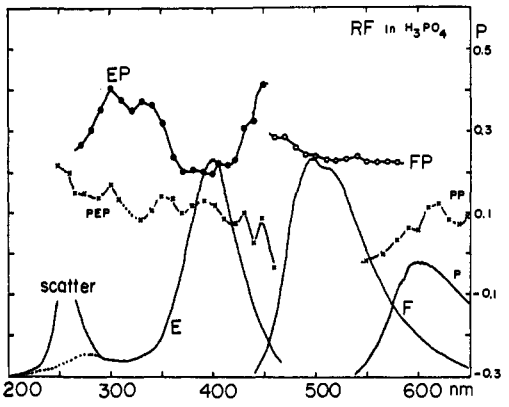

Figure 16. The luminescence and polarization spectra of riboflavin in phosphoric acid (85%) at 77°K (see the caption of Figure 2 for an explanation of abbreviations).

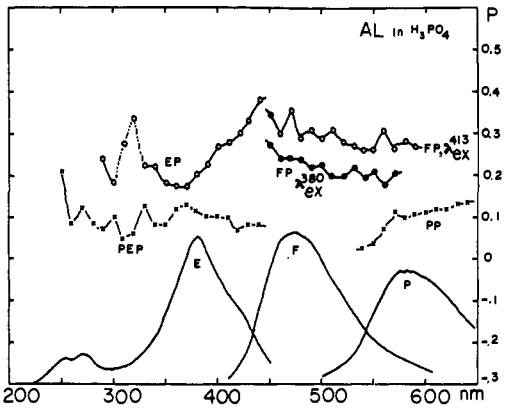

Figure 17. The luminescence and polarization spectra of alloxazine in phosphoric acid (85%) at 77°K (see the caption of Figure 2 for an explanation of abbreviations).

bands may be present. In accordance with this view, the magnetic and electric circular dichroic spectra of protonated isoalloxazines suggest the presence of two transitions in the long-wavelength band.[48]

Figure 17 shows the luminescence and polarization spectra of alloxazine in phosphoric acid. As in the case of riboflavin, only one λ_{max} at 373 nm was observed in the near-uv region, and the absorption spectrum of alloxazine is extended to 460 nm. Since the excitation polarization increases significantly beyond 390 nm, two $\pi \rightarrow \pi^*$ transitions may be involved in the near-uv band centered at 373 nm, as in riboflavin. The P–P–P SCF–MO CI calculation of the protonated alloxazine yields $\pi \rightarrow \pi^*$ transitions at 447 (f = 0.09, polarized 59° clockwise from the long axis) and 387 nm (f = 0.61, polarized along the long axis, 6° clockwise). These predicted results seem to support the two overlapping transitions in the near-uv region where the weak transition extending to 460 nm may correspond to the predicted 447-nm transition. Thus, the theory satisfactorily accounts for the increase in the polarization beyond 410 nm (Figure 17). Furthermore, the emission polarization in phosphoric acid with respect to λ_{ex} = 413 nm does not decline with λ_{em} as sharply as in ethanol and polymethyl methacrylate (Figures 10 and 11). This observation may be taken as indirect evidence for vibrational depolarization of alloxazine fluorescence involving vibronic interactions between the $^1(\pi,\pi^*)$ and $^1(n,\pi^*)$ states. Complications due to ionic and tautomeric equilibria in phosphoric acid prevent the offering of a more conclusive interpretation concerning results shown in Figures 16 and 17.

(c) Lumazine. The locations of the (n,π^*) states in lumazines are also unknown. Lippert and Prigge[49] were unable to observe an $n \rightarrow \pi^*$ band on the longer wavelength side of the absorption spectrum for lactim derivative I, and suggested that a weak $n \rightarrow \pi^*$ transition is hidden under the major absorption band. CNDO calculations[43] predict that the $n \rightarrow \pi^*$ transition is the second lowest energy transition. Strong fluorescence emission from lumazines (Table I) can be taken as evidence for a significant energy gap between the fluorescent (π,π^*) and the $^1(n,\pi^*)$ states.

The Triplet States. (a) Isoalloxazines. The phosphorescent triplet state of isoalloxazines has been assigned $^3(\pi,\pi^*)$ symmetry on the basis of polarized phosphorescence data and theoretical calculations.[41,42,50] Figure 3 shows the delayed fluorescence intensity of riboflavin tetrabutyrate in polymethyl methacrylate at two different temperatures. The excitation spectra for delayed fluorescence and phosphorescence spectra were found to be essentially identical, thus establishing that the emission in the 600-nm region arises from the long-lived triplet state. On the basis of relatively long lifetimes (Table I) and the tendency for negative phosphorescence polarization in the 0–0 region with respect to the lowest $\pi \rightarrow \pi^*$ transitions, the phosphorescent triplet state of various isoallaxazines (Figures 2, 6, and 7) can be assigned as a $^3(\pi,\pi^*)$ type. The PEP spectra in the 350-nm region are generally positive, possibly due to the out-of-plane and in-plane (axis parallel to the polarization direction of the second $\pi \rightarrow \pi^*$ transition) components in the phosphorescence intensity. It should be recalled that the second $\pi \rightarrow \pi^*$ transition is polarized along an axis nearly parallel to the lowest transition (the angle θ smaller than 30°).

The $n \rightarrow \pi^*$ transitions may contribute to the overall oscillator strength in the 350-nm region of the excitation spectra, thus partly accounting for the positive polarization of the phosphorescence excitation. Nonetheless, their exact locations and intensities are still unknown, making it difficult to partition the slightly positive phosphorescence polarization with respect to the 350-nm excitation[41] into the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ polarization directions. Assuming that the out-of-plane component is predominant in the 0–0 phosphorescence band,[42] it can be suggested that spin–orbit coupling between the $^1(n,\pi^*)$ and $^3(\pi,\pi^*)$ states contributes significantly to the singlet–triplet transition probability in flavins.

The 6,7-dichloro substituent exerts its heavy atom effect on the singlet–triplet transition probability possibly by providing $(\sigma,\pi)^*$ and charge-transfer states for the spin orbit coupling scheme. The heavy atom effects are revealed in the shortening of the phosphorescence lifetime (Table I) and positive polarization in the near-uv band at 350 nm.

(b) Alloxazines. The 0–0 phosphorescence band of alloxazine is negative with respect to the excitation at 380 nm (Figure 10) which is predicted to be polarized almost along the long molecular axis (27° clockwise

(48) See ref 34.
(49) E. Lippert and H. Prigge, *Z. Elektrochem.*, 64, 662 (1960).
(50) P. S. Song and W. E. Kurtin, *J. Amer. Chem. Soc.*, 89, 4248 (1967).

from the long axis, Figure 9). Lumichrome showed the same phosphorescence polarization characteristics as alloxazine. Based on the polarization data (see Figure 10) and relatively long phosphorescence lifetime (Table I), the phosphorescent triplet state of alloxazine and of lumichrome is also assigned $^3(\pi,\pi^*)$ symmetry. The positive phosphorescence polarization of alloxazine beyond the 0–0 region may be associated with vibronic coupling between the low-lying $^3(n,\pi^*)$ and phosphorescent $^3(\pi,\pi^*)$ states.[42] This interpretation is consistent with the suggestion that the $^1(n,\pi^*)$ and $^1(\pi,\pi^*)$ states are closely spaced in the case of alloxazines and also provides for the possibility of vibronic coupling in the singlet manifold.

Dibromolumichrome shows exclusively in-plane polarized phosphorescence (Figure 13). If the predicted polarization of the lowest $\pi \rightarrow \pi^*$ transition along the short molecular axis is assumed to be correct (Figure 13), then the phosphorescence of dibromolumichrome is exclusively in-plane and short-axis polarized. The introduction of an in-plane component (subspectra II) into the phosphorescence by halogen substituents is well established in naphthalene in which the halogen also enhances the original naphthalene phosphorescence (subspectra I).[51] The subspectra II type intensity arising from the second-order vibronic spin–orbit coupling involving (σ,σ^*), (σ,π^*), or (π,π^*) states and/or the spin–orbit coupling between $^1(\pi,\pi^*)$–$^3(\pi,\pi^*)$ states where charge-transfer character may be introduced into the $^1(\pi,\pi^*)$ state by the bromine substituents seems to be the predominant mechanism in dibromolumichrome, as compared to the $^{1,3}(,n\pi^*)$–$^{1,3}(\pi,\pi^*)$ spin–orbit interactions (first- and second-order contributions in other flavins).

Figures 16 and 17 show the phosphorescence polarization spectra of riboflavin and alloxazine in phosphoric acid, respectively. The phosphorescence polarization in the acidic glass is higher than in ethanol. The phosphorescence intensities were found to be extremely weak, and the lifetimes were estimated at 0.08 and 0.03 sec for riboflavin and alloxazine, respectively, indicating efficient quenching of the phosphorescence presumably due to the phosphorus-containing solvent (*i.e.*, external heavy atom effects). The positive phosphorescence polarization in the absence of (n,π^*) states (due to protonation) may be accounted for in terms of geometric change (nonplanarity) or in-plane components, particularly along the axis of the second $\pi \rightarrow \pi^*$ transition as the polarization across the second $\pi \rightarrow \pi^*$ band tends to be somewhat more positive than across the first $\pi \rightarrow \pi^*$ band. Thus, the phosphorescence polarization data in phosphoric acid are consistent with the interpretation given for the polarized fluorescence excitation spectra.

(c) Lumazines. The phosphorescence polarization is negative throughout the phosphorescence band of trimethyllumazine (Figure 15) and the polarization across 260–450 nm is negative or zero. Thus, phosphorescence is preferentially out-of-plane polarized and the phosphorescent state can be identified as the $^3(\pi,\pi^*)$ state according to the spin–orbit coupling selection rules.[52] Effects of the external heavy atom perturbation (KI, CH_3CH_2I) on the phosphorescence enhancement were positive in support of the $^3(\pi,\pi^*)$ assignment. The high Φ_F/Φ_P ratios, long phosphorescence lifetimes (Table I), and negative polarization of the phosphorescence excitation are indicative of less vibronic influence of the $^3(n,\pi^*)$ states on the $^3(\pi,\pi^*)$ states in lumazines than in isoalloxazines or alloxazines.

Conclusions

The major absorption bands of flavins in the visible and near-uv regions have been resolved in terms of two $\pi \rightarrow \pi^*$ transitions. The polarizations of these two transitions are not perpendicular. In fact, they are close to being parallel, as revealed in the fluorescence excitation polarization spectra. The polarizations as well as the transition energies for the visible and near-uv bands have been satisfactorily reproduced theoretically by means of the P–P–P SCF–MO CI calculations. The polarization characteristics of the two lowest $\pi \rightarrow \pi^*$ bands of riboflavin described in this paper are generally consistent with recent results from dichroic bleaching[53] and linear dichroic measurements.[54]

The fluorescent and phosphorescent states of isoalloxazines, alloxazines, and lumazines have been characterized as $^1(\pi,\pi^*)$ and $^3(\pi,\pi^*)$ types, respectively, on the basis of polarized fluorescence and phosphorescence spectra in ethanol at 77°K. In general, the phosphorescence intensity of flavins can be described in terms of primarily out-of-plane components with some in-plane contributions. In one particular instance, dibromolumichrome, the in-plane component was found to contribute predominantly to the intensity, thus illustrating the importance of the intramolecular heavy atom effect on the spin–orbit coupling mechansims, apparent even in the heterocyclic carbonyl systems which have permanent dipole moments and $^{1,3}(n,\pi^*)$ states for enhancing the singlet–triplet transition probability.

The $^1(n,\pi^*)$ states seem to play an important role in determining the overall luminescence properties of the flavin systems, particularly alloxazines which appear to possess low-lying (n,π^*) states. In polar solvent at room temperature, the fluorescence quantum yield of some flavins is relatively low and intersystem crossing (ISC) appears to be very efficient ($\Phi_{ISC} > 0.5$).[55] In nonpolar solvents and at low temperature, the Φ_F increases while the Φ_P is extremely low, indicating solvent (microenvironmental) and temperature-dependent barriers to intersystem crossing and also indicating efficient radiationless transitions from the lowest $^3(\pi,\pi^*)$ state to the ground state.

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