in their work on acylation reactions (Scheme II):

$$R{-}CH_2{-}CO{-}NH{-}Ph \xrightarrow[Ac_2O]{reflux} Ac{-}NH{-}Ph$$

Scheme II

The application of the gas chromatographic procedure was applied to plasma samples. A more detailed discussion (data substantiating the selection of the various parameters described under *Procedure*) of this application for pharmacokinetic studies will be presented at a later date.

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pH Dependence of Fluorescence of Riboflavin and Related Isoalloxazine Derivatives

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Abstract □ pKa values were determined for several flavins by absorptiometry and by fluorimetry. The poor agreement between absorptiometrically and fluorimetrically determined pKa values is attributed to the fluorimetrically measured quantities being due to equilibria in the fluorescent state (lowest excited singlet state). The excited-state pKa's and the ground-state pKa's do not correspond to protonation at the same sites in the isoalloxazine ring. The significance of excited-state equilibria for fluorimetric analysis is considered.

Keyphrases □ Fluorescence, pH dependence—riboflavin, related isoalloxazine derivatives □ Riboflavin, related isoalloxazine derivatives—fluorescence pH dependence □ pH dependence—fluorescence of riboflavin, related isoalloxazine derivatives

Fluorimetric assay is probably the most widely employed method for the determination of riboflavin (1). The intense green fluorescence of riboflavin (2) and that of its photodecomposition product lumiflavin (3–5) have been used in quantitative analysis.

Riboflavin fluorescence is constant in intensity between pH 3 and 8 but is quenched at pH <3 and >8. As a result, it is necessary to maintain pH 3–8 in solutions upon which riboflavin assay is performed. Early studies of the fluorescence intensity of riboflavin as a function of pH yielded two pKa values of 2 and 10 (6). However, absorptiometric determination of the pKa's in the same acidity regions gave values of -0.2 and 9.8 (7). Subsequent measurements of the second pKa of riboflavin by electrochemical methods yielded values of 9.9 (8) and 10.0 (9). In light of the good agreement between the fluorimetrically, absorptiometrically, and electrometrically determined values of the second pKa, it seems reasonable to conclude that they all correspond to the same dissociation. However, the substantial difference between the value of the first pKa as determined by fluorimetric titration and that determined by absorptiometry suggests that the two pKa values do not correspond to identical protolytic processes.

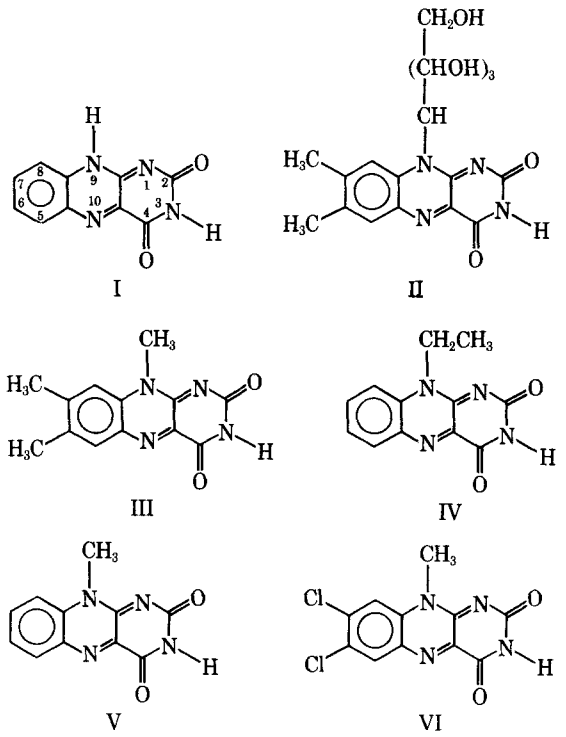
The most obvious explanation for the latter dilemma is that the absorptiometric measurements yield a conventional pKa value corresponding to a protolytic dissociation in the ground state of the molecule, while the fluorescence measurements yield a pKa corresponding to a protolytic reaction of riboflavin in the electronically excited state from which fluorescence originates: the lowest excited singlet state (10). Hereafter, pKa values corresponding to electronically excited states will be referred to as pKa*. Prototropism in the lowest excited singlet state derives from the fact that rates of protonation and dissociation are often fast enough to compete with fluorescence for deactivation of the excited state of a fluorescent molecule. Consequently, the fluorescence intensity may reflect the relative concentrations of acid and conjugate base in the electronically excited state rather than the corresponding quantities in the ground state (as determined by absorptiometry or electrometry). Because the electronic distribution is generally different in an electronically excited molecule from that in the ground state of the same molecule, the binding energy of a proton and hence the pKa is different from that in the ground-state molecule. Obviously, it is as important to know pKa* values in fluorimetric analysis as it is to know pKa values in absorption spectrophotometric analysis in order to establish the pH range of optimum analytical utility.

To investigate more closely the differences between the pH dependencies of the fluorescence spectra and

Table I—Features of the Absorption and Fluorescence Spectra of Riboflavin and Some Related Isoalloxazine Derivatives

	Long Wavelength Absorption Maxima				Fluorescence Maxima, pH 7.00, λ, nm.
	4.65 M $HClO_4$ (H_o = 2.02)		pH 7.00		
	λ, nm.	ε	λ, nm.	ε	
Riboflavin	395	1.18×10^4	447	1.19×10^4	517
Lumiflavin	368	3.43×10^4	433	3.22×10^4	509
9-Ethylisoalloxazine	370	3.36×10^4	433	3.33×10^4	507
9-Methylisoalloxazine	368	3.35×10^4	435	3.33×10^4	507
6,7-Dichloro-9-methyl-isoalloxazine		—		—	514

absorption spectra of riboflavin and some related isoalloxazine derivatives (flavins), the present study was undertaken. The structures of isoalloxazine (I), riboflavin (II), lumiflavin (III), 9-ethylisoalloxazine (IV), 9-methylisoalloxazine (V), and 6,7-dichloro-9-methylisoalloxazine (VI) are relevant to this study.



EXPERIMENTAL

Analytical reagent grade riboflavin was purchased[1] and was further purified by several recrystallizations from distilled deionized water. Pure samples of lumiflavin, 9-ethylisoalloxazine, 9-methylisoalloxazine, and 6,7-dichloro-9-methylisoalloxazine were used without further purification (11).[2] Absorption and fluorescence spectra were taken on 4.00×10^{-5} M solutions of all compounds studied in 1-cm. square quartz cells. Wherever possible, the same solutions were employed in luminesence and absorption studies. All solutions were deoxygenated by purging with dry nitrogen for 10 min. prior to recording of spectra. Mallinckrodt reagent grade perchloric acid and Beckman standard buffers were used to make solutions of known pH and Hammett acidity (12).

Fluorescence spectra were taken on a Farrand Mark I spectrofluorometer and recorded on a Heath model EU-20B strip chart recorder. The emission and excitation monochromators of the spectrofluorometer were calibrated against the mercury lines from a Pen-Ray low pressure mercury lamp. Emission and excitation spectra were not corrected for the wavelength dependencies of monochromator and phototube responses. However, this correction is not critical for the present work.

Absorption spectra were taken on a Beckman DB-G spectrophotometer. The pKa values were calculated from the pH (or Hammett acidity) dependences of the long wavelength absorption bands of the conjugate acid species according to

$$\text{pKa} = \text{pH (or } H_o) - \log \frac{\epsilon_A - \epsilon}{\epsilon - \epsilon_B} \qquad \text{(Eq. 1)}$$

where ϵ_A and ϵ_B are the ratios of absorbance to concentration at the acidic and basic limits, respectively, of the inflection region; ϵ represents the value of absorbance/concentration for any point in the inflection region of the titration; and H_o is the Hammett acidity. The pKa* values were estimated graphically from the value of pH at 50% fluorescence quenching in the plot of relative fluorescence intensity *versus* pH.

RESULTS AND DISCUSSION

The principal features of the absorption and fluorescence spectra of the flavins are listed in Table I. The absence of absorption spectral data for 6,7-dichloro-9-methylisoalloxazine in Table I is a result of the extremely low solubility of that compound in water. Consequently, no pKa was determined for it. However, due to the greater sensitivity of fluorescence compared with absorption, a value of pKa* was estimable even at the low concentration level of the 6,7-dichloro derivative at saturation in water. The absorptiometric titration curves are shown in Fig. 1, while the fluorimetric titration curves are presented in Fig. 2. The pKa and pKa* values estimated from the data of these curves are given in Table II.

The excellent agreement between all of the pKa values of Table II indicates that protonation occurs at the same site in the ground state for all of the compounds studied here. Similarly, the closeness of the pKa* values indicates that in the lowest excited singlet states of the flavins, the same site in the isoalloxazine ring is being protonated for all compounds studied. The slightly lower pKa* value of the 6,7-dichloro compound probably is due to the inductive effects of the chlorine atoms.

That the fluorescences of the flavins arise from electronically excited states having the same structures as the corresponding ground states in neutral solution is strongly suggested by the mirror-image relationships (13) extant between the fluorescence bands and the long wavelength absorption bands of the corresponding flavins.

Clearly, in all cases, the value of pKa* relative to pKa indicates that the protonated form of the fluorescent species is a significantly weaker acid in the excited state than in the ground state. The problem of the discrepancy between the fluorimetrically and absorptio-

Table II—pKa and pKa* Values of the Flavins[a]

	pKa	pKa*
Riboflavin	−0.1	1.7
Lumiflavin	0.0	1.7
9-Ethylisoalloxazine	0.0	1.7
9-Methylisoalloxazine	0.1	1.7
6,7-Dichloro-9-methyl-isoalloxazine	—	1.3

[a] Estimated from the data represented in Figs. 1 and 2, respectively.

[1] Mallinckrodt Chemical Works, Inc., St. Louis, Mo.
[2] Obtained from Dr. Gordon Tollin of the University of Arizona.

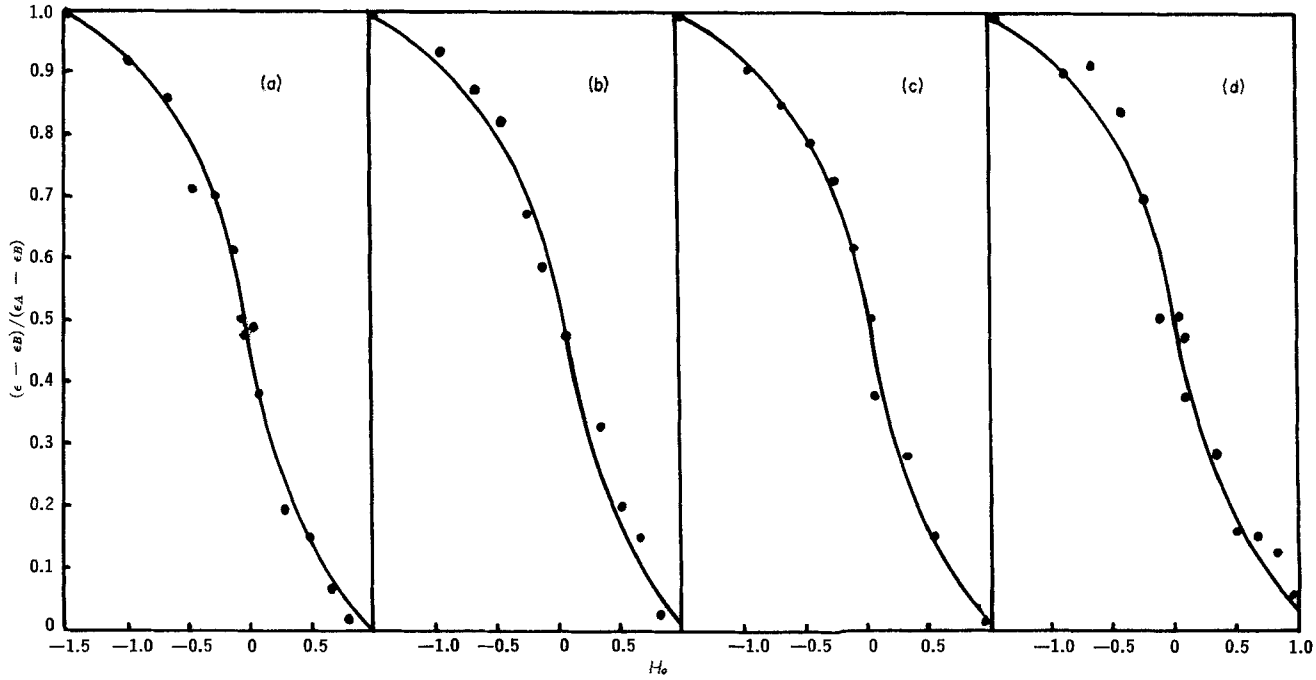


Figure 1—*Absorptiometric titrations of the flavins;* $(\epsilon - \epsilon_B)/(\epsilon_A - \epsilon_B)$ *is the fraction of flavin in the protonated form. Key: (a), riboflavin; (b), lumiflavin; (c), 9-ethylisoalloxazine; and (d), 9-methylisoalloxazine.*

metrically determined values of pKa in acid solution was considered by Weber (14). He concluded, from the shortening of the mean lifetime of the excited state as the riboflavin fluorescence was quenched by hydrogen ion, that the quenching occurred in the excited state (dynamic quenching). This is in agreement with the basic premise here. If quenching of the riboflavin fluorescence occurred as a result of a ground state acid–base reaction (static quenching), it would not be accompanied by an alteration of excited-state properties (lifetime). However, it is implicit in Weber's treatment that the excited-state protonated form and the ground-state protonated form of riboflavin are identical. This is not necessarily the case. In a polyfunctional molecule like a flavin, the redistribution of electronic charge accompanying electronic excitation often results in the site of protonation in the excited state being different from that in the ground state. For example, the quinolinols exist as neutral phenols in the ground state and are protonated at the ring nitrogen in acidic solutions. However, in the lowest excited singlet state, the quinolinols are zwitterions in neutral solution and are protonated at the phenolic oxygen in strongly acidic media (15). A rough estimate of the pKa* value of the excited-state equilibrium corresponding to the species involved in the absorptiometric titration (pKa*′) is to be had from the shift in the long wavelength absorption maxima, upon protonation, according to a thermodynamic treatment by Forster (16). In this treatment, pKa*′ is calculated from

$$\text{pKa}^{*\prime} = \text{pKa} - 2.10 \times 10^{-3}(\bar{\nu}_a - \bar{\nu}_b) \qquad \text{(Eq. 2)}$$

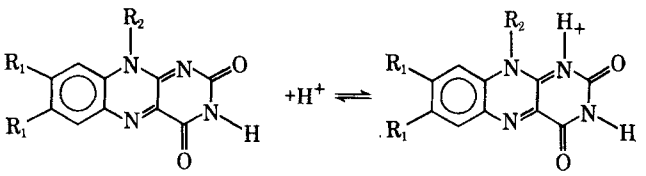
where pKa is the ground-state equilibrium involving the identical conjugate acid–base pair to which pKa*′ corresponds, and $\bar{\nu}_a$ and $\bar{\nu}_b$ are the wavenumbers of the long wavelength maxima in the absorption spectra of acid and conjugate base, respectively. It must be borne in mind that pKa*′ values calculated from absorption spectral shifts frequently are different from those determined by fluorimetric titration, by as much as 3 pH units. This is a consequence of the differences between the vibrational and solvent structures of ground- and excited-state species (15). However, if these calculations are carefully interpreted, they may yield useful information. Application of Eq. 2 to the absorbance data of Table I yields the results presented in Table III.

Table III—pKa*′ Values and Absorption Maxima of Conjugate Acid ($\bar{\nu}_a$) and Conjugate Base ($\bar{\nu}_b$) Species Derived from the Flavins

	$\bar{\nu}_a$ (cm.$^{-1}$)	$\bar{\nu}_b$ (cm.$^{-1}$)	pKa*′
Riboflavin	2.53×10^4	2.24×10^4	−6.2
Lumiflavin	2.72×10^4	2.31×10^4	−8.5
9-Ethylisoalloxazine	2.70×10^4	2.30×10^4	−8.2
9-Methylisoalloxazine	2.72×10^4	2.30×10^4	−8.9

The pKa*′ values of Table III are substantially different from the pKa* values of Table II, even if the approximate nature of the calculation is taken into account. Consequently, it must be concluded that the excited-state prototropic equilibrium responsible for the quenching of the flavin fluorescence does not correspond to the ground-state equilibrium studied absorptiometrically. Since the conjugate base species are probably the same in both equilibria, the excited- and ground-state equilibria appear to differ in the conjugate acid species; *i.e.*, the site of protonation of the isoalloxazine ring is different in the excited state than in the ground state.

Self-consistent field molecular orbital calculations on isoalloxazine (17) have indicated that, in the ground state, the nitrogen atom in the 1-position has the highest π-electron charge density and should therefore be the most basic site on the isoalloxazine ring (18) (Scheme I). Some experimental work supports this conclusion (19). The molecular orbital calculations also predict that, in the lowest excited singlet state, the nitrogen atom in the 1-position becomes considerably less basic, in agreement with the values of pKa*′ calculated here from absorption spectra, and that the nitrogen atom in the 10-position becomes the most basic (Scheme II). In light of the supporting evidence, it is concluded that the ground-state equilibria of the flavins studied by absorptiometric titration are of the type shown in Scheme I, whereas the equilibria



Scheme I

studied in the lowest excited singlet state by fluorimetric titration are of the type shown in Scheme II.

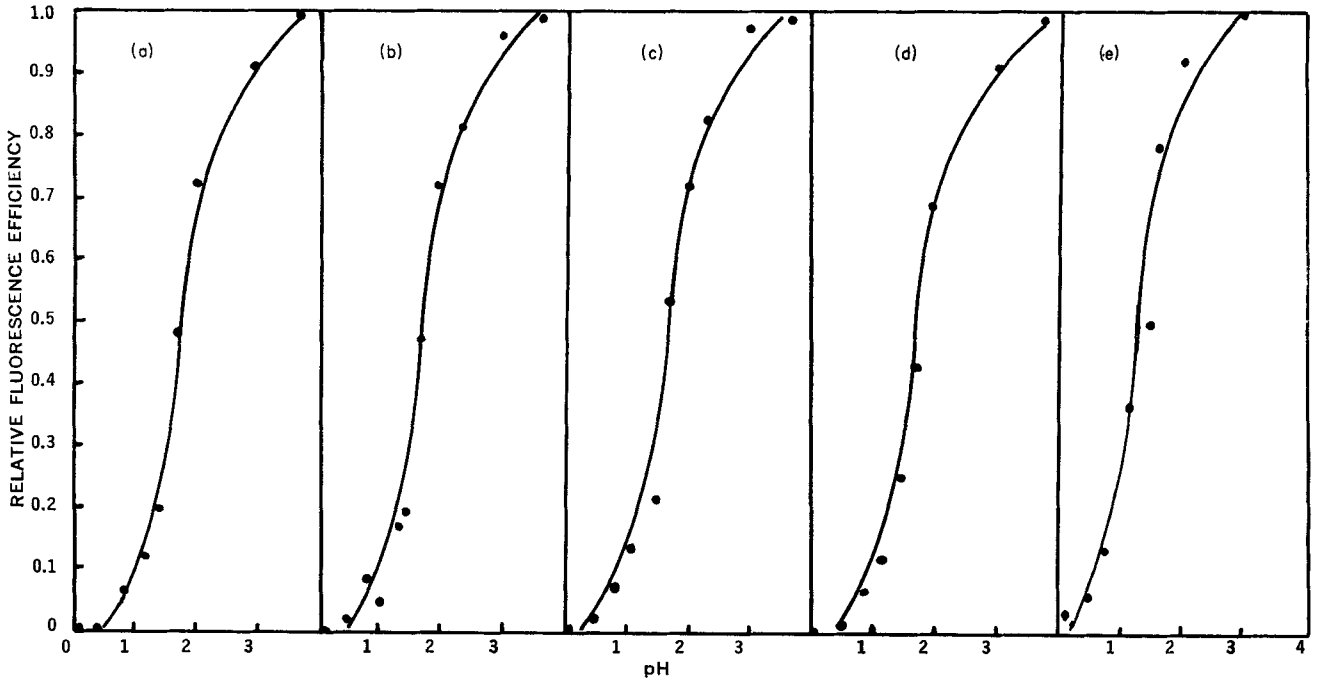
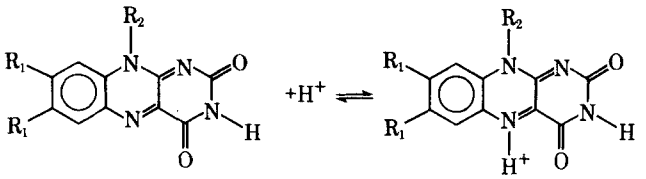


Figure 2—*Fluorimetric titrations of the flavins. Key: (a), riboflavin; (b), lumiflavin; (c), 9-ethylisoalloxazine; (d), 9-methylisoalloxazine; and (e), 6,7-dichloro-9-methylisoalloxazine.*



Scheme II

The excellent agreement between the pKa values of the flavins in the basic region (pKa ~ 10), whether determined by absorptiometry, electrometric measurement, or fluorimetry, is no doubt due to the fact that all three methods are determining the same ground-state pKa (probably of the abstraction of the amidic proton on the nitrogen in the 3-position by OH^-). The failure of fluorimetric titration in weakly basic solution to represent the excited-state dissociation is likely due to the diffusion-limited nature of the hydrogen-ion abstraction by OH^-. Consequently, in weakly basic solution where OH^- concentration is low, the reaction is not fast enough to compete with fluorescence for deactivation of the excited state. Therefore, the concentration of the absorbing species has prime importance in determining the intensity of fluorescence, so that the fluorescence intensity *versus* pH characteristics of the titration are much the same as a plot of absorbance *versus* pH. In support of this conclusion, Weber (14) found the mean lifetime of the excited state of the fluorescent species to vary only slightly as the fluorescence is quenched by OH^-, implying that the quenching in this case is static rather than dynamic.

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