Phototautomerism of Lumichromes and Alloxazines[1a,b]

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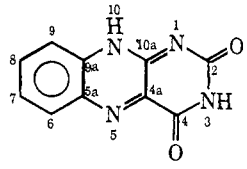
Abstract: Lumichromes and alloxazines, which fluoresce at 420–480 nm (I) and are unsubstituted at N_1, show an additional fluorescence band at 500–560 nm (II) region characteristic of the corresponding isoalloxazines in the mixed solvents dioxane–pyridine and acetic acid–ethanol. Isoemissive points of fluorescence for lumichrome in dioxane–pyridine and acetic acid–ethanol solvents are at 476 and 488 nm, respectively. The increase of the 530-nm emission occurs at the expense of the 440-nm original lumichrome fluorescence. The excitation spectra with respect to the 440- and 530-nm bands are identical. In glacial acetic acid this original lumichrome band (I) disappears completely as the 500–560-nm dimethylisoalloxazine fluorescence (II) reaches the highest intensity. No isoalloxazine fluorescence can be detected when the N_1 position of lumichrome or alloxazine is substituted with a methyl group. Furthermore, no alloxazine and lumichrome emission could be detected when 10-methylisoalloxazine and 7,8-dimethyl-10-methylisoalloxazine (lumiflavine) are excited in dioxane–pyridine, respectively. In the glycerol–pyridine mixture (90:10 and 95:5, v/v) at 298°K lumichrome exhibits a polarization value of 0.3–0.4 across the 530-nm fluorescence band. In 100% glycerol the average polarization is about 0.2. Various isoalloxazines have a fluorescence polarization value of 0.3–0.4 in the same region. Results indicate that in the singlet excited state the N_1 proton of lumichromes as well as alloxazines is shifted to N_{10} and the resulting tautomeric form is similar to or identical with that of isoalloxazines. Thermodynamic data for this phototautomerism are $\Delta H^{\circ *} = 1.06$ kcal/mol, $\Delta S^{\circ *} = -6.4$ eu, and $\Delta G^{\circ *} = 2.97$ kcal/mol.

In a preliminary study[2] we reported a strongly red-shifted fluorescence of alloxazine in solution, when excited in the presence of pyridine. Although the biologically active form (isoalloxazine structure, **1**) of flavines is less stable than the inactive form (alloxazine structure, **2**) in the ground state, it has not been possible to synthesize unsubstituted isoalloxazine. However, it is thought to be feasible to form the former from the excited singlet state of the alloxaxine. This prediction is based on the increased acidity of the N_1 proton in the excited singlet state.[3] Lumichromes carry methyl groups at C_7 and C_8 of compound **2**.



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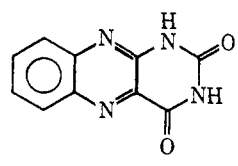

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Some studies[4–7] of thermal tautomerism from alloxazine to isoalloxazine forms under severe conditions (*e.g.*, 15% NaOH[4]) have been reported, but the tautomerism is complicated by several multiequilibria, involving various anionic forms under such conditions. In the present paper, we describe a set of experimental results which are consistent with "clean" phototautomerism between alloxazines and isoalloxazines. Another impetus for our study of the phototautomerism of flavines is the fact that the system to be described can be used as a simple model for a dye laser.

Experimental Section

Materials. The flavines used were the same as those described in our previous paper, unless noted otherwise.[8] 1-Methyl-, 3-methyl-, and 1,3-dimethyllumichromes were gifts from Professor P. Hemmerich, Konstanz. All flavine samples were recrystallized and their purity was checked by either tlc or fluorescence excitation spectroscopy. 2-Hydroxypyridine (Aldrich) was recrystallized, while 2,6-lutidine (Matheson Coleman and Bell) was redistilled. 3,4-Lutidine (Matheson Coleman and Bell) was refluxed over CaH_2. Pyridine, Spectroquality (Matheson Coleman and Bell), and 2,4-lutidine (Aldrich) were used as received. Glycerol and ether, Spectroquality (Matheson Coleman and Bell), and ethanol, Spectroquality (U. S. Industrial), were used without further purification. All other organic solvents were purified and dried as described previously.[9] D_2O (99.8 mol %, Bio-Rad Lab) was used after redistillation.

Methods. Absorption spectra were measured on a Cary 118C recording spectrophotometer or "Specord UV-VIS" spectrophotometer (C. Zeiss, Jena). Corrected fluorescence excitation and emission spectra, electronically compensated for instrumental bias as a function of wavelength, were recorded on a Perkin-Elmer spectrophotofluorometer (Model MPF-3) at an average resolution of 3 nm. Spectral correction for alloxazine (Figure 7) was made according to Lippert, *et al.*,[10] and Argauer and White.[11] The temperature dependence of the fluorescence emission was measured using a high-resolution (0.02 nm) spectrophotometer equipped with a cryocooler (Cryogenic Technology, Model 390400) and SSR photon counter (Model 1120-1105), as described previously.[12] Polarization of fluorescence was measured and corrected according to the Azumi–McGlynn procedure.[13]

Results

Figure 1 shows the fluorescence spectra of lumichrome as a function of pyridine concentration in dioxane. The intensity of red-shifted fluorescence (II) is increased with the pyridine concentration. A clear isoemissive point is obtained (476 nm), indicating two

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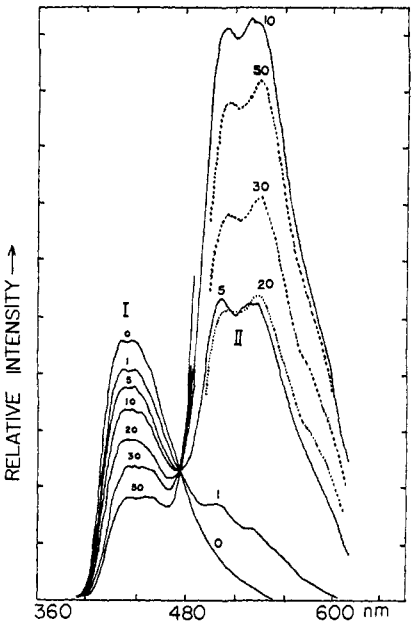

Figure 1. Fluorescence spectra of lumichrome (8.5×10^{-5} *M*) in dioxane at 298°K as a function of per cent pyridine. The excitation wavelength was 385 nm. The dotted line spectra are reduced by $^1/_3$ from their actual intensity.

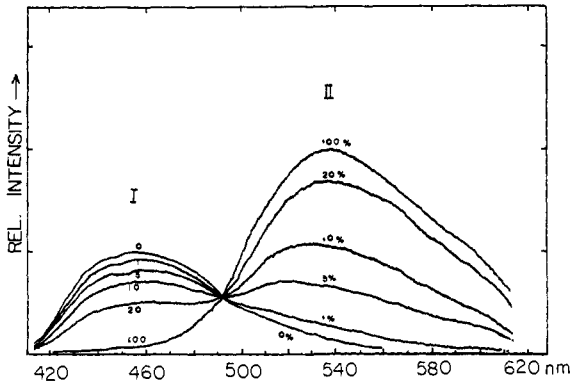

Figure 2. Fluorescence spectra of lumichrome (8.5×10^{-5} *M*) in ethanol at 298°K as a function of per cent acetic acid. The excitation wavelength was 405 nm.

excited species (phototautomers) in equilibrium. Figure 2 shows similar curves as a function of acetic acid concentration in ethanol. Again, an isoemissive point (488 nm) is obtained. It should be noted that the excitation spectra with respect to the two emission bands are identical in both dioxane–pyridine and ethanol–acetic acid mixtures, as illustrated in Figure 3. The excitation spectra in Figure 3 are also identical with those of lumichrome in pure organic solvents where the the two-band system is not observed, except for the usual solvent shift. This indicates that the two emission bands observed are not due to different species such as lumichrome and lumichrome–pyridine or –acetic acid complexes in the ground state.[14]

Figure 4 shows the effect of equimolar concentration

(14) Results in the water–pyridine mixture are more complex. For example, clear isoemissive points are not observed in the fluorescence spectra of lumichrome and alloxazine in the water–pyridine mixture, possibly because of multiple ionic and flavine–pyridine complexation equilibria. However, the dominant fluorescing species are the parent flavines (alloxazine and lumichrome) and their phototautomers even in water–pyridine.

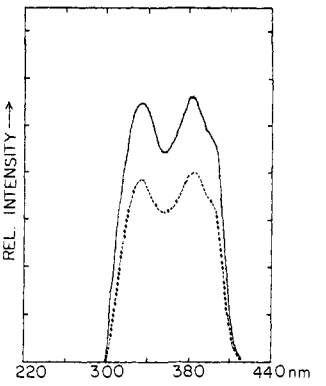

Figure 3. The corrected fluorescence excitation spectra of lumichrome (8.5×10^{-5} *M*) in dioxane–pyridine (50:50, v/v) at 298° with respect to the emission at 540 nm (——) and at 440 nm (------). The excitation intensity below 300 nm is cut off in the presence of pyridine which absorbs uv.

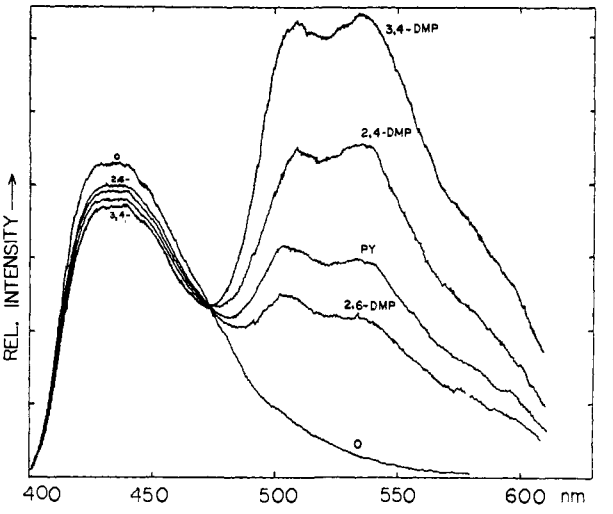

Figure 4. Fluorescence spectra of lumichrome ($\sim 8 \times 10^{-5}$ *M*) in dioxane at 298°K in the presence of 3×10^{-1} *M* pyridine (Py) and lutidines (DMP). The excitation wavelength was 380 nm; O, lumichrome alone.

of methylpyridines (lutidines). It can be seen that 3,4-lutidine is most effective in enhancing band II, apparently reflecting the greater basicity ($pK_a = 6.46$) of the pyridinyl nitrogen relative to pyridine ($pK_a = 5.25$). On the other hand, 2,6-lutidine ($pK_a = 6.6$) is less effective than pyridine, possibly due to the steric requirement around the pyridinyl nitrogen. The effectiveness of 2,4-lutidine ($pK_a = 6.77$) nicely reflects the basicity and steric factor in catalyzing the phototautomerism. Surprisingly, 2-hydroxypyridine was found to be ineffective for catalyzing the excited-state equilibrium of alloxazine, lumichrome, and 3-methyllumichrome.

Band II (*e.g.*, curves in pyridine and in 100% acetic acid in Figures 1 and 2) can be identified by superimposing it with independently measured fluorescence spectra of 10-methylisoalloxazine (equivalent to the alloxazine tautomer) and lumiflavine (equivalent to the lumichrome tautomer). Figure 5 shows the emission spectra of these compounds in dioxane. It also shows that pyridine does not affect the fluorescence spectra of lumiflavine and 10-methylisoalloxazine (not shown). Furthermore, there is no phototautomerism in the presence of 10% or more pyridine in dioxane, when the

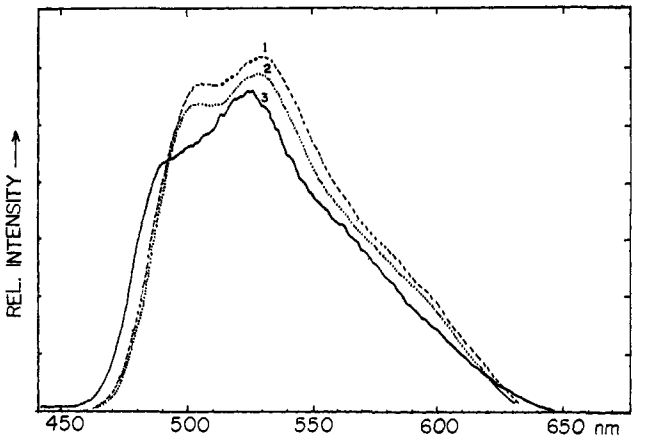

Figure 5. Fluorescence spectra of 10-methylisoalloxazine in dioxane or dioxane–10% pyridine (1) and lumiflavine in dioxane (2) and dioxane–10% pyridine (3) at 298°K. The excitation wavelength was 380 nm (λ_{ex} = 450 nm was also used).

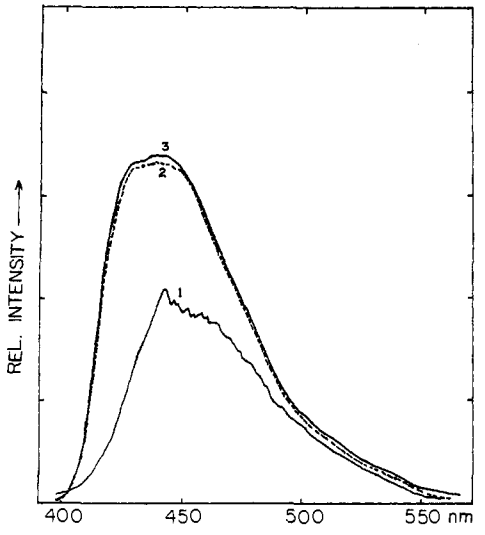

Figure 6. Fluorescence spectra of 1,3-dimethyl-6,9-dibromolumichrome ($>10^{-4}$ *M*) in dioxane or dioxane–10% pyridine (1) and 1,3-dimethyllumichrome (6.2×10^{-5} *M*) in dioxane (2) and dioxane–10% pyridine (3) at 298°K. The excitation wavelength was 380 nm.

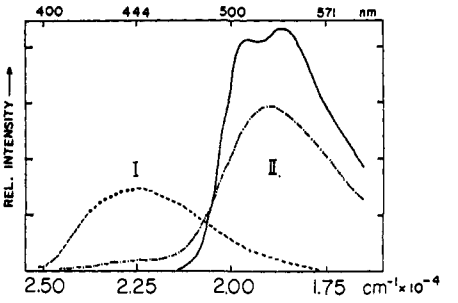

Figure 7. Fluorescence spectra of alloxazine (1×10^{-5} *M*) in ethanol (-----), acetic acid (-·-), and pyridine (——, 5×10^{-6} *M* alloxazine) at room temperature.

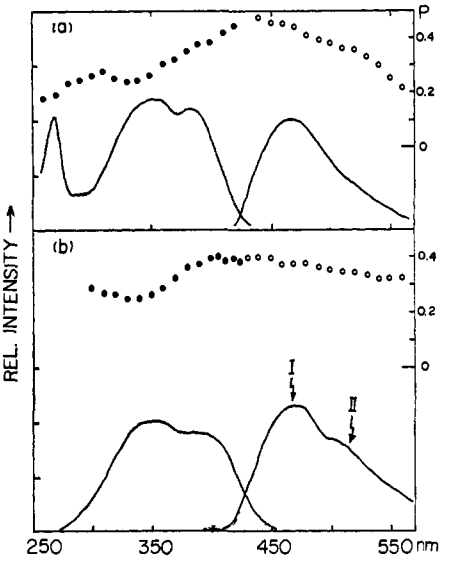

Figure 8. (a) The fluorescence excitation (left) and emission (right) spectra of lumichrome in glycerol–water (95:5, v/v) at 298°K. Relatively weak excitation intensity at 267 nm is due to the front surface imprisonment. (b) Fluorescence excitation and emission spectra of lumichrome in glycerol–pyridine (95:5, v/v) at 298°K. Polarizations (P) of the excitation (●) and emission (○) spectra in (a) and (b) were measured with respect to λ_{em} = 460 nm and λ_{ex} = 395 nm, respectively.

N_1 proton is substituted by methyl group, as illustrated in Figure 6. N_3-Methylation does not negate the phototautomerism. This indicates that N_1-methyl analogs do not equilibrate with the species emitting at the long wavelength(II) in their excited states.

Alloxazine similarly shows all of the characteristics displayed by lumichrome and it appears that 7,8-methyl substitutents have no special effect on the excited-state equilibrium. For example, Figure 7 shows a complete disappearance of band I and a corresponding appearance of band II from alloxazine in pyridine and acetic acid, but only band I appears in ethanol in the absence of either pyridine or acetic acid. Summarizing all of the data mentioned above, band II is definitely attributable to the excited state of the tautomers of the isoalloxazine structure. Further support of this conclusion is provided below.

Figure 8a shows the polarized fluorescence spectrum of lumichrome in glycerol–water (95:5, v/v) with respect to the first absorption band at 395 nm. A sharp decline in polarization degree with wavelength is seen. This is characteristic for alloxazine and lumichrome, as was shown previously in ethanol glass at 77°K.[8] On the other hand, 10-methylisoalloxazine and its derivatives exhibit an essentially constant degree of polarization across the fluorescence band in 95% glycerol (in this work) and in ethanol glass at 77°K.[8] Figure 8b shows the fluorescence spectrum of lumichrome in glycerol–pyridine (95:5, v/v), clearly reflecting the flattened polarization characteristic of lumiflavine. Note that an additional shoulder (band II) appears at 500–600 nm. The band II intensity is not clearly resolved in the presence of 5% pyridine, which is sufficient to show the marked enhancement of its intensity in dioxane (Figure 1). It turns out that the band II intensity was suppressed with gradual increase in the viscosity of the medium. In addition, decrease in temperature also suppresses its intensity, while the band I intensity proportionately increases. Thus, the phototautomerism involves a step which is diffusion controlled.

Figure 9 shows that excitation spectra with respect to bands I and II are different in highly viscous solu-

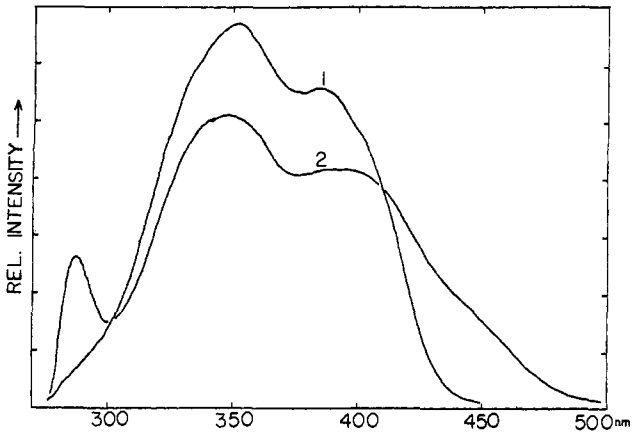

Figure 9. Fluorescence excitation spectra of lumichrome ($\sim 8 \times 10^{-5}$ *M*) in glycerol–pyridine (99:1, v/v) at 298°K with respect to band I at λ_{em} = 460 (1) and band II at 530 nm (2).

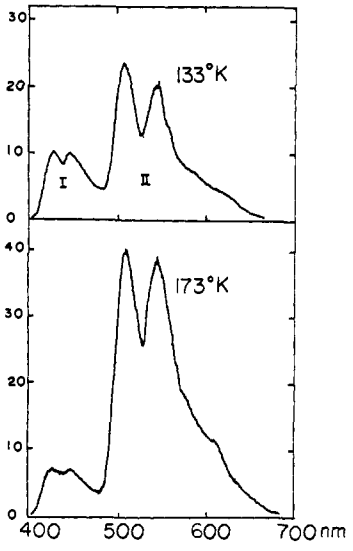

Figure 10. Fluorescence spectra of lumichrome ($\sim 8 \times 10^{-5}$ *M*) in ether–10% pyridine at 133 and 173°K. The excitation wavelength was 380 nm. The uncorrected spectra were recorded at a 0.8-nm resolution.

tion, in contrast with the spectra in other less viscous solvents containing pyridine. This difference is due to the fact that the tautomer (7,8-dimethylisoalloxazine, $\lambda_{abs\ max}$ ~450 nm) formed from its excited state *via* radiative and nonradiative relaxations cannot tautomerize back to the original form (lumichrome) and cannot diffuse out rapidly from the excitation locale within the fluorescence cell. This is further confirmed by the viscosity dependence of the excitation spectrum.

Figure 10 shows typical spectra of lumichrome in ether–pyridine (90:10, v/v) at 133 and 173°K.[15] Figure 11 shows a van't Hoff plot for the temperature-dependence data, after correcting for the quantum yield variation for lumichrome (band I) and lumiflavine (band II). Thermodynamic values for the quasiequilibrium phototautomerism of lumichrome to 7,8-dimethylisoalloxazine in the $^1(\pi,\pi^*)$ state are as follows: $\Delta H^{\circ *}$ = 1.06 kcal/mol; $\Delta S^{\circ *}$ = −6.4 eu; $\Delta G^{\circ *}$ = 2.97 kcal/mol. Assuming that the entropy change is about the same for the ground-state tautomerism and knowing the transition energies of lumichrome (75 kcal/mol) and lumiflavine (64.25 kcal/mol), thermodynamic parameters for the tautomerism between alloxazine and isoalloxazine structures can be estimated, yielding ΔH° = 11.8 kcal/mol, $\Delta S^{\circ} \simeq -6.4$ eu, and ΔG° = 13.7 kcal/mol.

(15) Instead of dioxane, as in Figure 1, ether was used because of its ability to form an optically transparent solution at low temperatures.

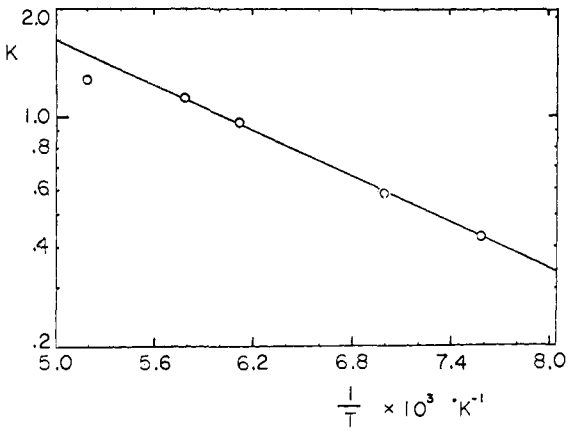

Figure 11. The temperature dependence of quasiequilibrium constant (K) in ether–10% pyridine where $K = (I_F^{II}/I_F^{I})(\Phi_F^{LC}/\Phi_F^{LF})$, and I_F and Φ_F are corrected intensity of bands I and II and quantum yield of lumichrome (LC) and lumiflavine (LF), respectively.

Discussion

All of the fluorescence data described above can be accommodated in terms of tautomerism between alloxazine and isoalloxazine forms in their excited states. It should be emphasized what has been observed is not due to the excited-state ionic equilibria, for example, assigning band II to alloxazine or lumichrome anions. This is because the solvents used do not favor formation of anions.[16] The exciplex formation is not important in the present system. This is apparent, from Figure 1, since the enhancement of band II is about eightfold or more for a given unit of decrease in the band I intensity. This is expected for the phototautomerism since fluorescence quantum yields of isoalloxazines are several times greater than those of alloxazines (for example, $\Phi_{F,\ lumichrome}/\Phi_{F,\ lumiflavine} \simeq 1/6$).[8]

Before illustrating possible mechanisms for the phototautomerism, it is pertinent to examine the electronic structure of the excited singlet states of alloxazines, particularly with respect to the acidity of N_1 proton. Figure 12 indicates that the predicted acidity of the proton at N_1 in lumichrome increases upon excitation to the $^1(\pi,\pi^*)$ state, while the basicity of the N_{10} increases at the same time. Such a charge redistribution pattern is consistent with the acid-base catalyzed phototautomerism, as shown in Figure 13. Pyridine and acetic acid act as general base and bifunctional acid–base catalysts, respectively, for the phototautomerism. The steric effect by 2,6-lutidine can be explained accordingly. The most convincing evidence in support of the phototautomerism is provided by two observations:

(16) In water, lumichrome shows the corrected fluorescence maxima at 478 nm (pH 6.5) and 524 nm (pH 10.5), in agreement with Müller and Dudley.[17] In addition to the 524 maximum, there is a shoulder at about 440 nm indicative of more than one fluorescent species at pH 10.5. The major component of the 524-nm intensity appears to be due to emission from general acid-catalyzed formation of 7,8-dimethylisoalloxazine in the excited state. However, relative contributions of anionic and neutral forms to the long wavelength fluorescence are not determined at the present (unpublished work).

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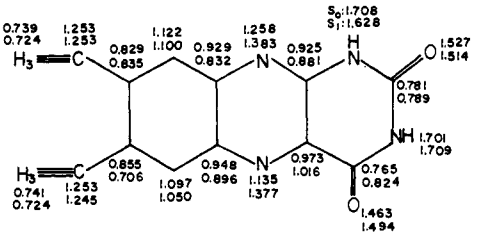


Figure 12. The π-electron density distribution in the ground (S_0) and excited singlet (S_1) states of lumichrome calculated by the SCF MO CI PPP method and the ground orbital model for methyl groups.

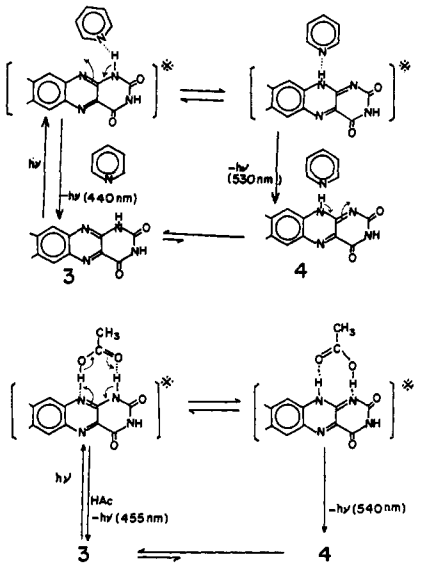
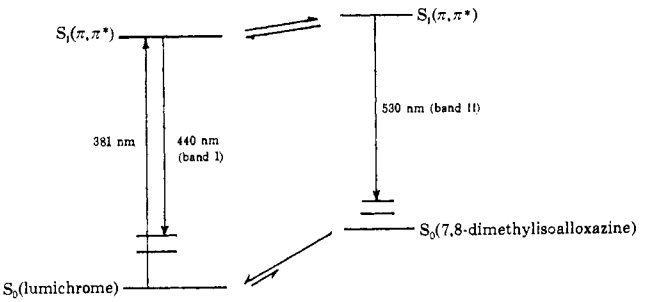


Figure 13. Possible mechanisms for the phototautomerism between lumichrome (3) and 7,8-dimethylisoalloxazine (4) catalyzed by pyridine and acetic acid. The emission maxima are indicated by "$-h\nu$".

(a) N_1-methylalloxazines do not show band II, and (b) the fluorescence spectra of isoalloxazines coincide with the band II spectra. In addition, even when band II is not clearly resolved due to viscosity and water in which resolution of the two-band system is less than that in dioxane, the enhancement in polarization degree of the band II region is characteristic of the emission from the isoalloxazine chromophore.

There was no observable solvent (D_2O) isotope effects on the phototautomeric equilibria in dioxane–pyridine and ethanol–acetic acid mixtures, when the N_1 proton was allowed to exchange with a deuteron in D_2O prior to the fluorescence measurement. Lack of the solvent isotope effect is expected for the proposed phototautomerism, particularly because $\Delta H°*$ is practically zero and the N_1 proton (or deuteron) is merely transferred to N_{10}. The following energy level diagrams summarize the phototautomerism proposed on the basis of the present work. Similar diagrams can be constructed for alloxazine.



In conclusion, the significance of the present work is twofold. First, the system described can serve as a simple model for a dye laser. Second, it is possible to estimate thermodynamic data for the ground states of flavine tautomers, which are not readily obtainable in other ways.

Acknowledgments. We thank Professor Kurt Mislow for suggesting an experiment with lutidines. Support by the Research Institute of Food Science and Nutrition (191-8801) of Texas Tech University is also appreciated.

Communications to the Editor

Thermochemistry of Aliphatic Alcohols Determined by Gas-Phase Ionic Equilibria

Sir:

A number of methods have been used to determine the thermochemical properties of aliphatic alcohols. Gas kinetic methods have been applied to the pyrolysis of compounds of the type ROX where X = OR, NO,

$$ROX = RO\cdot + X\cdot \quad (1)$$

or NO_2.[1-5] Assuming zero activation energy for the recombination reaction between RO· and X·, ΔE for reaction 1 may be set equal to the activation energy for the decomposition reaction. The heat of formation of alkoxy radicals, $\Delta H_f°(RO\cdot)$, can then be calculated if $\Delta H_f°(X\cdot)$ and $\Delta H_f°(ROX)$ are known. Electron impact mass spectrometric methods have been used to measure appearance potentials for processes such as[6,7]

$$ROX + e^- = X^+ + RO\cdot + 2e^- \quad (2)$$

Assuming RO· is an alkoxy radical and not a rearrangement product, it follows that $\Delta H_f°(RO\cdot)$ can be calculated from the measured appearance potential, $\Delta H_f°(ROX)$, and $\Delta H_f°(X^+)$.

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