Problems in Electronic State Assignment Based on Circular Dichroism. Optical Activity of Flavines and 8-Substituted Lumazines†

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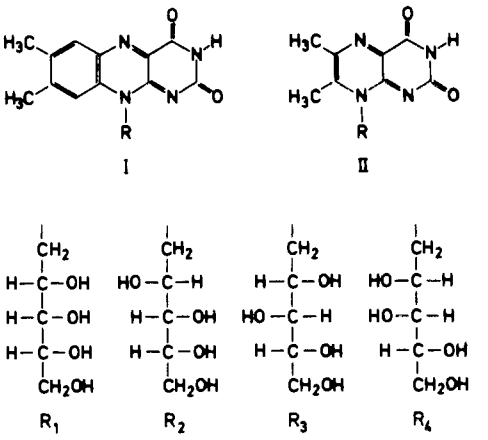
ABSTRACT: Four stereoisomeric flavines and the respective tetraacetyl derivatives were analyzed by means of absorption and circular dichroism (CD) measurements in a variety of solvents. The CD envelope of the lowest energy (π,π^*) state of the flavine chromophore (λ_{max} 445 nm) is sensitive to contributions from each of the centers of chirality in the side chain at position 10. The fine structure of this CD band is also highly dependent on the configuration of the side chain, and in two compounds studied the band is split into parts with opposite sign. This may be explained by contributions from two or more vibronic modes which contribute to the CD envelope with identical or opposite sign depending on the configuration of the side chain, whereas the vibronic contributions to the absorption spectrum always have identical sign. These data suggest that vibronic splitting is not strictly limited to weak Cotton effects. Thus it follows that the observation of two CD bands corresponding to one absorption band provides no compelling evidence for the involvement of more than one electronic state. The shape and sign of the second lowest (π,π^*) state of the flavine chromophore (λ_{max} 370 nm) are mainly determined by the configuration of the 2′-hydroxyl group. The number of electronic states involved in the ultraviolet region cannot be determined with certainty. CD spectra of three 8-substituted derivatives of 6,7-dimethyllumazine provide evidence for a forbidden electronic transition near 320 nm.

Knowledge of the electronic states of the flavine chromophore is essential for the understanding of the optical properties of flavine–protein complexes. A considerable number of experimental studies have dealt with the optical rotatory dispersion, circular dichroism (CD), and magnetic circular dichroism of riboflavine and its derivatives (Gascoigne and Radda, 1965; Simpson and Vallee, 1966; Tollin, 1968; Miles and Urry, 1968; Edmondson and Tollin, 1971; Brady and Beychok, 1971; Scola-Nagelschneider and Hemmerich, 1972). However, the published data show significant discrepancies which seemed to justify a reinvestigation. Furthermore, the electron states of the flavine chromophore are still incompletely understood in spite of considerable theoretical efforts (Fox *et al.*, 1965, 1967; Kurtin and Song, 1968; Song, 1969a,b; Sun *et al.*, 1972). We expected to obtain additional evidence relevant to this problem from detailed CD studies.

Direct evidence concerning the contributions of individual centers of chirality in molecules with more than one such center can be only obtained by comparison of stereoisomeric compounds. Therefore, we recorded the CD spectra of riboflavine and three stereoisomers under a variety of experimental conditions. Since these compounds are poorly soluble in organic solvents, the respective tetraacetyl derivatives were also included in the study. The data show that the envelope of the CD band corresponding to the lowest energy (π,π^*) state of the flavine chromophore is susceptible to all centers of chirality in the side chain at position 10, whereas the second lowest (π,π^*) state is mainly affected by the nearest center of chirality (C-2′). The data further show that vibronic splitting of CD bands into parts with opposite sign is not strictly limited to weak Cotton effects. Hence it follows that band assignments on the basis of CD data are only possible with reservations.



8-Substituted 6,7-dimethyllumazines differ from flavines by the absence of the benzenoid ring. These compounds have met with the interest of organic chemists since the observation that 6,7-dimethyl-8-(D-ribityl)lumazine is the direct biosynthetic precursor of riboflavine (Masuda, 1956; Maley and Plaut, 1959). The CD properties of 8-substituted lumazines have not been studied so far to the best of our knowledge. We investigated three stereoisomeric 6,7-dimethyl-8-pentyllumazines. The CD data demonstrate a weak electronic transition near 320 nm which is consistent with molecular orbital (MO) calculations (Fox *et al.*, 1967; Sun *et al.*, 1972).

Experimental Procedure

Chemicals. Riboflavine was a gift of Hoffmann-La Roche AG, Basel. It was recrystallized repeatedly from water and dilute acetic acid, respectively.

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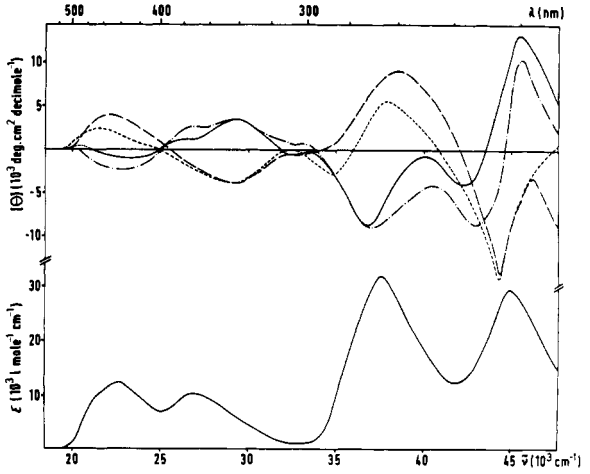


FIGURE 1: Absorption and CD spectra of flavines in 0.1 M phosphate buffer (pH 7): riboflavine (—); D-arabinoflavine (– – –); D-xyloflavine (– · –); D-lyxoflavine (- - -).

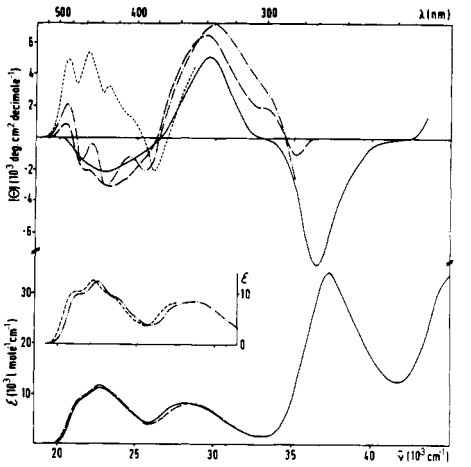


FIGURE 2: Absorption and CD spectra of riboflavine in methanol (—) and of tetraacetylriboflavine in methanol (– –), 1,2-dichloroethane (– · –), and bromoform (- - -).

The following compounds were prepared according to published procedures: D-xyloflavine (Tishler *et al.*, 1947); D-arabinoflavine and D-lyxoflavine (von Euler *et al.*, 1935); tetraacetylflavines (Kuhn and Wagner-Jauregg, 1933); 6,7-dimethyl-8-(D-ribityl)lumazine, 6,7-dimethyl-8-(D-arabityl)lumazine, and 6,7-dimethyl-8-(D-xylityl)lumazine (Winestock and Plaut, 1961). Another sample of 6,7-dimethyl-8-(D-ribityl)lumazine was isolated from the culture fluid of a riboflavine deficient mutant of *Saccharomyces cerevisiae* (A. Bacher, unpublished).

Chromatography. Flavines were purified by chromatography on Dowex 50W-X8 (NH_4^+ form, elution with 0.1 M ammonium phosphate, pH 7). The eluate was adsorbed to magnesium silicate and eluted with acetone–2 N NH_4OH (2:1). The eluate was taken to dryness. The residue was dissolved in 0.1 N HCl and chromatographed on a column of Dowex W-X8 (H^+ form, elution with 0.1 N HCl).

Lumazines were purified by chromatography on Dowex W-X8 (H^+ form, elution with 0.1 N HCl) and subsequent chromatography on Sephadex G-10 (elution with deionized water).

Spectroscopic Measurements. Visible and ultraviolet spectra were run on a photometer DMR 21 from Carl Zeiss Optical Works, Oberkochen, Germany. CD measurements were performed with a spectropolarimeter Cary 60 equipped with a circular dichroism attachment 6002 from Cary Instruments, Monrovia, Calif. The spectral bandwidth was 3 nm throughout. Calibration was performed with an aqueous solution of *d*-10-camphorsulfonic acid. Measurements were performed in fused silica cells with pathlengths from 0.1 to 50 mm. The sample temperature was 23°. The absorbance of samples was less than 2 in the spectral range observed. The validity of the Lambert–Beer law was confirmed by measurements of the same sample solution in cells of different pathlengths. This was routinely performed with all samples. The base line was checked after each measurement. The instrument was checked for absorbance artifacts by measurements of potassium dichromate solutions. The deviation from the base line was negligible below an absorbance of 2 and less than 0.002° at an absorbance of 2.5.

Results

Previous studies in this laboratory have documented that small quantities of impurities can cause significant errors in CD spectra (A. Bacher and H. Rau, unpublished information). For this reason all compounds used in this study were thoroughly purified by repeated liquid chromatography or crystallization as described under Experimental Procedure until the CD spectra were no longer affected by the last respective step of purification.

Figure 1 shows spectra of riboflavine (I, R_1), D-arabinoflavine (R_2), D-xyloflavine (R_3), and D-lyxoflavine (R_4) in phosphate buffer (pH 7). The absorption spectra were found identical within the limits of photometric precision. All compounds under study show CD extrema near 440, 340, 270, and 220 nm which are in reasonable agreement with observed absorption bands. In addition, some compounds show CD extrema near 310 and 230 nm which have no obvious counterpart in the absorption spectra. The CD curves of riboflavine and xyloflavine exhibit shoulders near 380 nm. In the wavelength range above 480 nm (dotted part in Figure 1) the circular dichroism of riboflavine could not be established with full certainty as a consequence of the low ellipticity and high absorbance of the compound.

CD spectra of riboflavine in neutral aqueous solution have been communicated by several authors. Tollin (1968) reported qualitatively different spectra in water and 0.1 M phosphate buffer, respectively, and concluded that the conformation of the ribitol moiety is influenced by the buffer ions. In order to study this interesting detail more closely we recorded spectra in the following solvents: (i) distilled water, (ii) 0.1 M phosphate buffer (pH 5 and 7, respectively), (iii) 0.1 M citrate buffer (pH 5 and 7, respectively), (iv) 0.1 M Tris buffer (pH 7). In order to avoid artifacts caused by fluorescence emission, spectra were also recorded in the same solvents supplemented with 0.1 M KI which effects almost complete quenching of fluorescence. All spectra thus obtained were identical within the limits of instrument precision. The difference between our results and the data of Tollin (1968) cannot be explained. Since the termination of our experimental work, Scola-Nagelschneider and Hemmerich (1972) have communicated similar experiments which are in full agreement with our statements.

Figure 2 shows spectra of riboflavine and tetraacetylriboflavine in organic solvents. The CD spectra of riboflavine and tetraacetylriboflavine in methanol are similar except in the long-wavelength range where the latter compound exhibits fine structure with a weak positive Cotton effect centered at 490 nm. The fine structure of the lowest energy band is more pronounced in the spectra of tetraacetylriboflavine in bromoform and in 1,2-dichloroethane.

CD spectra of riboflavine in methanol and of tetraacetylri-

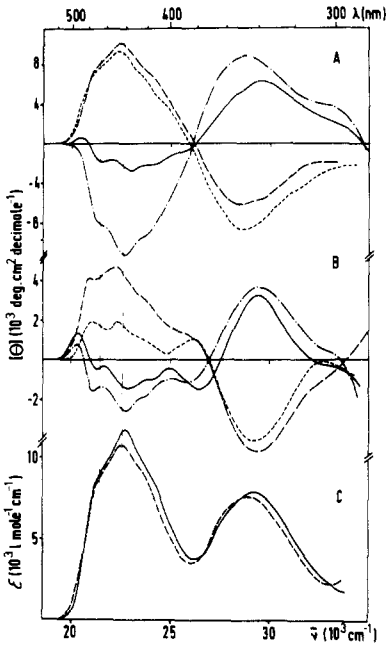


FIGURE 3: Absorption and CD spectra of flavines in dimethylformamide: (A) tetraacetylriboflavine (—), tetraacetyl-D-arabinoflavine (– –), tetraacetyl-D-xyloflavine (– · –), tetraacetyl-D-lyxoflavine (– – –); (B) riboflavine (—), D-arabinoflavine (– –), D-xyloflavine (– · –), D-lyxoflavine (– – –); (C) absorption spectra of riboflavine (—) and tetraacetylriboflavine (– –).

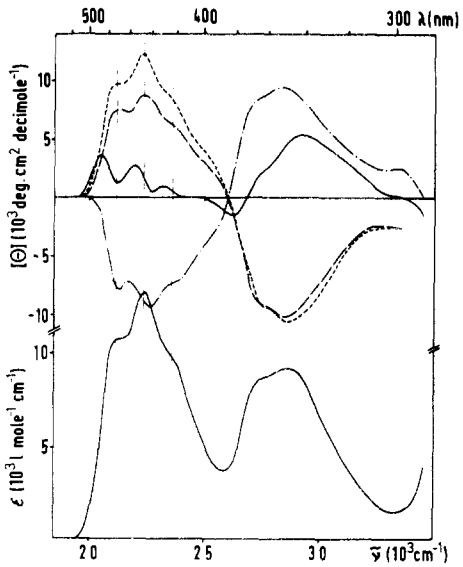


FIGURE 4: Absorption and CD spectra of flavines in chloroform: tetraacetylriboflavine (—); tetraacetyl-D-arabinoflavine (– – –); tetraacetyl-D-xyloflavine (– · –); tetraacetyl-D-lyxoflavine (– –).

boflavine in 1,2-dichloroethane have been reported previously (Tollin, 1968; Edmondson and Tollin, 1971). Again these data are largely incompatible with our results. On the other hand spectra of 3-ethyltetraacetylriboflavine reported by Scola-Nagelschneider and Hemmerich (1972) are in reasonable agreement with our data.

Figure 3 shows spectra of flavines and tetraacetylflavines in dimethylformamide. The shape of the Cotton effect centered near 345 nm is similar in all compounds studied. On the other hand, the shape of the lowest energy Cotton effect is very sensitive to the conformation of the side chain at position 10. Riboflavine and xyloflavine show two CD maxima of opposite sign near 490 and 470 nm, whereas arabinoflavine and lyxoflavine show only one CD maximum in the spectral range above 460 nm. Among the tetraacetyl compounds, the derivative of riboflavine resembles the parent compound, whereas the CD spectra of the other stereoisomers have almost the same shape as the absorption curves.

Figure 4 shows spectra of tetraacetylflavines in chloroform. Again the shape of the Cotton effect near 350 nm is similar for all compounds. With the exception of tetraacetylriboflavine, the shape of the lowest energy Cotton effect imitates the shape of the corresponding absorption band. Tetraacetylriboflavine shows a completely different pattern with rather narrow fine structure bands. Unexpectedly, the fine structure minima rather than the maxima coincide with the fine structure maxima of the other three compounds.

Figure 5 shows spectra of 6,7-dimethyl-8-(D-ribityl)lumazine (II, R_1) and of the corresponding D-arabityl (R_2) and D-xylityl (R_3) compound in phosphate buffer (pH 5). The absorption spectra of these compounds are identical within the limits of experimental error. The shape and amplitude of the CD curves are similar. CD maxima correspond to the absorption bands at 407 and 250 nm and to the shoulder at 280 nm. The Cotton effect near 320 nm has no counterpart in the absorption spectra.

Figure 6 shows spectra of the same compounds in 0.02 M KOH (monoanions). In the long-wavelength range weak Cotton effects are observed at 370, 320, and 280 nm. This pattern is in good agreement with the absorption spectra. In the short-wavelength range all compounds show strong CD bands. However, the compounds behave in a rather different manner with respect to the number and position of CD maxima, and the correlation with the absorption bands is not obvious.

Discussion

In the long-wavelength range, the flavine chromophore shows two absorption bands centered at 445 and 370 nm (in water). It is generally accepted that these bands have (π,π^*) character. This is concluded from absorption and fluorescence measurements and from molecular orbital calculations (Fox *et al.*, 1965, 1967; Kurtin and Song, 1968; Song, 1969a,b; Sun *et al.*, 1972).

Let us consider first the Cotton effects associated with the second lowest $\pi \rightarrow \pi^*$ transition (λ_{max} 370 nm). The sign and shape of this Cotton effect are only affected to a minor degree by the solvent, but the band shows a distinct hypsochromic shift in apolar solvents. Comparison of the stereoisomers shows that the sign of this Cotton effect is determined by the nearest center of chirality (C-2′) only. This confirms the hypothesis of Scola-Nagelschneider and Hemmerich (1972) that the interaction of this electronic transition with the side chain can be understood in terms of "through chain" interaction.

The Cotton effect associated with the lowest energy $\pi \rightarrow \pi^*$ transition is more complex. Shape and sign of this CD band are highly susceptible to solvent influences, and in some spectra the band is split into parts of opposite sign. This raises the question whether more than one electron transition is involved. Kotaki *et al.* (1967) proposed that the long-wavelength shoulder of the absorption band might represent an $n \rightarrow \pi^*$ transition. However, this seems unlikely in the light of more recent fluorescence polarization measurements and molecular orbital calculations (Song, 1969a,b; Sun *et al.*, 1972).

We must next consider the possibility that the CD bands of opposite signs reflect in-phase and out-of-phase components of excitons arising from flavine dimers and aggregates. Formation of flavine mononucleotide dimers in aqueous solution was demonstrated by Gibson *et al.* (1962). Recently Müller *et al.* (1973) observed that the temperature difference spectra of fla-

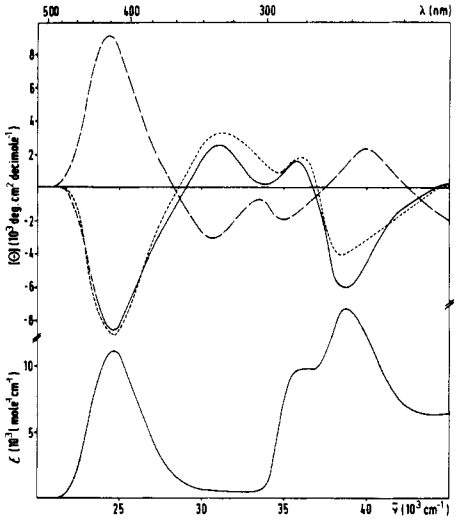


FIGURE 5: Absorption and CD spectra of 8-substituted lumazines in 0.1 M phosphate buffer (pH 5): 6,7-dimethyl-8-(D-ribityl)lumazine (—), 6,7-dimethyl-8-(D-arabityl)lumazine (- -), 6,7-dimethyl-8-(D-xylityl)lumazine (- - -).

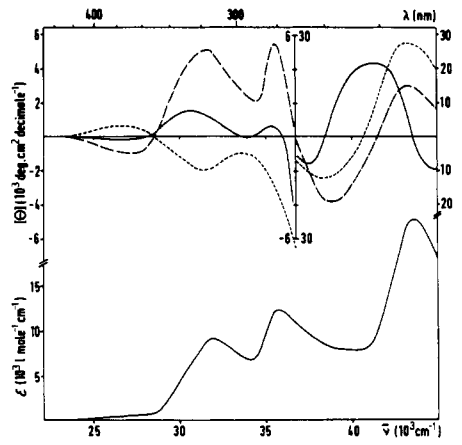


FIGURE 6: Absorption and CD spectra of 8-substituted lumazines in 0.02 M KOH (monoanions): 6,7-dimethyl-8-(D-ribityl)lumazine (—), 6,7-dimethyl-8-(D-arabityl)lumazine (- -), 6,7-dimethyl-8-(D-xylityl)-lumazine (- - -).

vines in aqueous solution are concentration dependent in the range above 10^{-4} M indicating the formation of stacked dimers. On the other hand, no concentration effects on the temperature difference spectra of flavines were observed in organic solvents. Resolution of the 445-nm absorption band into CD components of opposite signs was mainly observed in organic solvents with one exception: 10^{-4} M riboflavine in phosphate buffer shows a very weak Cotton effect near 490 nm which disappears at concentrations above 5×10^{-4} M. Definitive assessment is hampered by experimental difficulties due to the very low ratio of ellipticity and absorbance. CD spectra of riboflavine in dimethylformamide and of tetraacetylriboflavine in 1,2-dichloroethane were determined at various concentrations from 2×10^{-5} to 10^{-2} M. The spectra in dimethylformamide were not concentration dependent. In 1,2-dichloroethane, the only detectable concentration effect was a slight broadening of the fine structure bands at high concentrations near 10^{-2} M. In view of these data it seems unlikely that the two bands with opposite signs are due to dimer formation, although the hypothesis cannot be ruled out definitely in view of the technical impossibility of measurements at concentrations below 2×10^{-5} M. The question then arises whether the observed CD spectra are due to vibronic contributions.

Besides the splitting into parts with opposite signs, the lowest energy Cotton effect presents other peculiarities which deserve special consideration. (1) The degree of fine structure resolution is highly dependent on the configuration of the side chain at position 10. The highest degree of apparent resolution is found in riboflavine which also shows the phenomenon of band splitting. In the absorption spectra which are identical for all compounds under study within the limits of photometric precision, the vibronic structure is poorly resolved. (2) The wavelength position of the lowest energy member of the vibrational sequences is also subject to considerable variation in response to the configuration of the side chain (up to 700 cm^{-1}).

These findings are easily explained if we assume that the observed fine structure results from contributions by two or more vibronic modes contributing to the CD envelope either with identical or with opposite sign depending on the configuration of the side chain at position 10. High resolution of fine structure and splitting of the CD band into parts with opposite signs occur in cases where the different vibronic modes contribute with opposite signs. On the other hand, Cotton effects of high amplitude, but with poorly resolved fine structure, are observed when the different vibronic modes contribute to the CD curve with identical sign. In these cases, the shape of the CD band resembles the absorption band where different vibronic modes always contribute with identical sign.

Vibronic splitting of CD bands into parts with opposite signs was previously observed in cyclic ketones (Weigang, 1965a,b) and in phenylalanine derivatives (Horwitz *et al.*, 1969). The present data suggest that this phenomenon is not strictly confined to weak Cotton effects with ellipticities below 1000 deg cm^2/dmol as suggested by Weigang (1965a). Indeed, Rau *et al.* (1974) observed vibronic splitting of relatively strong CD bands in rigid derivatives of diazines.

This raises the general problem of the possibility of band assignment by means of CD measurements. Generally, separated CD bands corresponding to a single absorption band cannot be accepted as definitive evidence for different electronic transitions.

The comparison of the different stereoisomers further shows that all centers of chirality contribute to the lowest energy Cotton effect. For example, tetraacetylriboflavine and tetraacetylxyloflavine which differ by the configuration of the position 3′-acetyl group show Cotton effects of opposite signs in chloroform. This confirms the hypothesis by Scola-Nagelschneider and Hemmerich (1972) that the interaction of this electron transition with the centers of chirality is of "through space" type.

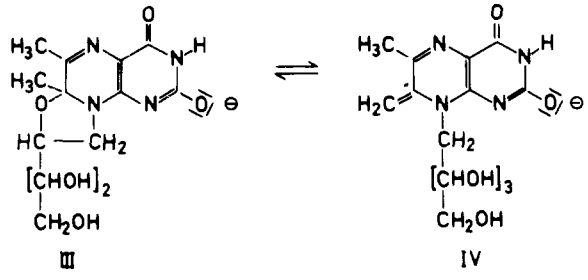
Some of the CD spectra show a Cotton effect near 380 nm. It is not clear whether it is due to a vibronic mode of one of the neighboring $\pi \rightarrow \pi^*$ transitions or to a separate electronic transition which is not observed in the absorption spectrum. Calculations by Sun *et al.* (1972) suggest that the lowest energy $n \rightarrow \pi^*$ transition may be located in this spectral range. A weak Cotton effect near 310 nm was observed previously (Simpson and Vallee, 1966; Miles and Urry, 1968) and was attributed to an $n \rightarrow \pi^*$ transition. Again this assignment is not yet definitively established. A weak $\pi \rightarrow \pi^*$ transition in this spectral range was suggested by molecular orbital calculations (Sun *et al.*, 1972).

In the wavelength range of 300–210 nm, riboflavine and xyloflavine show three CD maxima, whereas arabinoflavine and lyxoflavine show only two maxima. The possibility of vibronically induced band splitting cannot be ruled out. Again the comparison of the different stereoisomers illustrates the problems involved in band assignment based on CD data.

The 8-substituted lumazines studied show CD spectra of similar shape (Figure 5). The ribityl and xylityl type compounds show only minor differences of amplitude indicating that the CD spectrum is mainly determined by the nearest center of chirality (C-2′). All compounds studied show Cotton effects near 320 nm. Since the neighboring absorption bands at 407 and 256 nm are too far removed to account for this Cotton effect, we conclude that it represents an electronic transition which is not observed in the absorption spectrum because of its poor intensity. Calculations predict a weak $\pi \rightarrow \pi^*$ transition near 300 nm (Fox *et al.*, 1967; Sun *et al.*, 1972). However, definitive assignment of the CD band is not possible.

In the short-wavelength region, the ultraviolet spectra show a band at 256 nm with a shoulder at 280 nm, while all CD spectra show two maxima with opposite signs near 280 and 255 nm, respectively. This observation seems to indicate the involvement of two different electronic transitions but the other possibility of vibronically induced band splitting cannot be excluded with certainty.

The CD spectra of the monoanions of the 8-substituted 6,7-dimethyllumazines are strikingly different from the spectra of the neutral molecules and show a discouraging complexity. While our experiments were in progress, detailed nuclear magnetic resonance (nmr) and ultraviolet data of 6,7-dimethyllumazines with a wide variety of substituents in position 8 have been published by Beach and Plaut (1971) and by Pfleiderer *et al.* (1971). These authors have shown that 8-substituted 6,7-dimethyllumazines form an equilibrium mixture in alkaline solutions containing primarily an intramolecular ether formed between the 2′-hydroxyl group of the side chain at position 8 and C-7 of the pyrazine ring (III) and a minor amount of the 7-*exo*-methylene form (IV).



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In the ether form, the proximal part of the side chain in position 8 assumes a rigid conformation which may be responsible for the large Cotton effects observed in the short-wavelength range. Furthermore, the ether form shows a new center of chirality at position 7 of the pyrazine ring, and the equilibrium proportion of the two possible stereoisomers may depend on the configuration of the other centers of chirality of the side chain. This may in part account for the complexity of the spectra and the poor similarity between the different stereoisomers under study.

All compounds show a weak Cotton effect near 370 nm. Beach and Plaut (1971) have shown that the monoanions of 8-substituted 6,7-dimethyllumazines without a hydroxyl group in position 2′ show strong absorption at 366 nm. Since in these compounds the formation of a cyclic ether is not possible, the authors conclude that this absorption band corresponds to the *exo*-methylene form. Possibly the Cotton effects near 370 nm in the present experiments represent the portion of the *exo*-methylene compound present in the equilibrium mixture.

Acknowledgments

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