We want to thank Mrs. M. SappeLT for technical assistance.

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Intermediates in the Neutral Photolysis of Riboflavin

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Photolysis of riboflavin

The formation of a species with an absorption maximum around 410 nm, observed during the neutral photolysis of 10-(2'-hydroxyalkyl) flavins by earlier authors, is shown to depend on the presence of divalent anions. The structure of this new type of compounds is presented and a possible mechanism for the reaction is discussed.

Massey 1 and Holmström 2 have reported that a species with an absorption maximum around 410 nm is formed on illumination of riboflavin and FMN. A similar compound has been isolated as a second chromophore present in preparations of the flavoprotein, glycolic acid oxidase 3. To facilitate identification of this new chromophore we have studied flavin derivatives with monofunctional N(10) substituents.

A non-fluorescent compound with an absorption maximum at 412 nm is formed under both anaerobic and aerobic conditions during the photolysis of 10-(2'-hydroxypropyl) flavin (I, R = CH 3). The 412 nm product is stable towards light. It is formed in the presence of high concentrations of divalent anions A 2- (e.g. 2 M phosphate, sulfate). Only small amounts are formed in the presence of 1 M succinate. It is not formed in the presence of high concentrations of monovalent anions (e.g. 2 M perchlorate, acetate). These results indicate that the formation of the 412 nm product is induced by the presence of divalent anions in which the negative charges are attached to the same central atom (as in phosphate and sulfate) rather than separated as they are in succinate.

In the absence of divalent anions the rate of photolysis is decreased by a factor of 10 and lumichrome is the major reaction product, 10-(2'-keto-propyl) flavin (3, R = CH 3) being formed as a minor product. Analogous products have been obtained from photolysis in water 4. The effect of phosphate concentration on the aerobic photolysis of I (R = CH 3) is shown in Fig. 1.

The amount of 412 nm product formed and the rate of photolysis is also pH dependent. At pH 5 no 412 nm product is formed. The yield of 412 nm product increases as the pH is increased and reaches a maximum around pH 9. Using sulfate as the divalent anion, an apparent pK around 6.5 is obtained by plotting either the rate of disappearance of the starting flavin (as measured by the decrease in absorbance at 445 nm) or the rate of product formation (as measured by the increase in absorbance at 412 nm) as a function of pH. A similar pK has been observed for the photoreduction of flavins by a variety of substrates 5.

Similar to the starting flavin, the 412 nm product exhibits 2 pK's. The spectral properties of the cationic, neutral, and anionic forms are summarized below.

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Fig. 1. Aerobic photolysis of 10-(2'-hydroxypropyl) flavin (1, R = CH₃). A) Illumination in 0.01 M phosphate pH 7.0. Curves 1 - 5 were recorded after 0, 1.5, 3, 6, and 12 min of light, respectively. B) Illumination in 2.0 M phosphate pH 7.0. Curves 1 - 4 were recorded after 0, 5, 10, and 20 sec of light, respectively. (250 W/24 V tungsten-halogen lamp with a filter and lens system transparent from 300 to 800 nm.)

Table I. Absorption Maxima for 2.

<table>
<thead>
<tr>
<th>R</th>
<th>6 N HCl</th>
<th>0.1 M phosphate pH 7</th>
<th>0.1 M NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃</td>
<td>408, 292, 254</td>
<td>412, 275, 247</td>
<td>397, 280</td>
</tr>
<tr>
<td>HOCH₂-CH(OH)-CH(OH)-</td>
<td>408, 290, 252</td>
<td>410, 275 246</td>
<td>395, 281</td>
</tr>
</tbody>
</table>

The NMR spectrum of the photoproduct is compared with the spectrum of the starting flavin in Fig. 2. Integration indicates that the photoproduct contains all the protons present in the original flavin except for the loss of one aromatic proton, indicating substitution at the 6 or 9 position of the flavin ring. Comparison of the chemical shift values for the remaining aromatic proton and the aromatic methyl groups with other flavins substituted at position 6 or 9 with an oxygen function indicate that the remaining aromatic proton in the photoproduct is at position 6. Since the NMR spectrum shows that the side chain is intact, the only possible structure for the product is the cyclic structure shown (2, R = CH₃). This structure is consistent with the elemental analysis (calcd: C 60.4, H 4.7, N 18.8; found: C 60.5, H 4.6, N 18.8) and with the 2 pK’s observed.

Structurally analogous products are formed during the photolysis of 10-(2'-hydroxypropyl) isoisalloxazine and during the photolysis of N-3-methyl-
10-(2'-hydroxypropyl)flavin in the presence of divalent anions. These results indicate that the C-7 and C-8 methyl groups and the N(3)H are not essential for the photoreaction. No 412 nm product is formed during the photolysis of 10-(2'-acetoxypropyl)flavin in the presence of divalent anions. This indicates that the formation of the cyclic product in the case of 10-(2'-hydroxypropyl)flavin involves breakage of the OH bond rather than breakage of the CO bond.

Compound 4 (R = CH₃), which is non-fluorescent and has absorption properties similar to 2 (R = CH₃), is formed during the photolysis of 10-(2'-ketopropyl)flavin (3, R = CH₃) in the presence of divalent anions.

The non-fluorescent compound formed during the photolysis of riboflavin in the presence of divalent anions, under either aerobic or anaerobic conditions, has spectral properties which are very similar to 2 (R = CH₃) (see Table I). Total proof of the structure of the riboflavin product has not yet been obtained. However, the similarity of its spectral properties and the conditions required for its formation suggest a cyclic structure analogous to the 10-(2'-hydroxypropyl)flavin photoproduct, i.e. 2, R = HOCH₂-CH(OH)-CH(OH) - . The ab-
Absorption spectrum of the riboflavin product is compared with the spectrum of the glycolic acid oxidase chromophore in Fig. 3. Preliminary studies indicate that a similar product is formed during the photolysis of FMN in the presence of divalent anions.

It is suggested that the formation of 2 may proceed via the intermediates shown in brackets in the reaction scheme. Intermediate A is formed by the reaction of excited flavin with its side chain in the presence of divalent anions. At pH 5 the decay of this intermediate back to starting flavin predominates. At higher pH values the irreversible elimination of the C-9 proton to yield B is preferred. Rearrangement then yields reduced product C whose reoxidation accounts for the spectral changes observed upon admission of air following anaerobic photolysis. The mechanism by which high concentrations of divalent anions promote 9-substitution remains obscure. However, high concentrations of divalent anions also promote deuterium exchange at the C-8 methyl group. This reaction, initially observed by Bullock and Jardetzky, occurs when flavins are heated in D₂O in 0.2 M Phosphate at pH > 6.5 but does not occur in 0.01 M Phosphate in D₂O.

Photoaddition reactions, involving 6- or 9-substitution of the flavoquinonium cation, are also observed under acid conditions. Under these conditions additions of water and alcohols (both intramolecularly and intermolecularly) are found. Slow addition of...
water to flavin can also occur under the present conditions, as observed with isoxalloxazine-10- \((\beta\)-ethane sulfonic acid). The photodecomposition of this isoxalloxazine is extremely slow, as compared to lumiflavin, which allows photoaddition to become predominant in the presence of divalent anions.

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Circular Dichroism, Self Interaction and Side Chain Conformation of Riboflavin and Riboflavin Analogues

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Circular dichroism, riboflavin

The CD-spectra of riboflavin and riboflavin analogues in aqueous solutions differ very little depending on pH and ionic strength, but are extremely sensitive upon solvent changes. The two bands in the region of 300—500 nm seen in aqueous solutions are split into seven bands in less polar solvents, which can be assigned to seven vibronic transitions. The spectra may be interpreted by “through space” and “through chain” interactions of the sidechain centers of chirality with the flavin chromophore, which influence the two first \(\pi-\pi^*\) transitions in different manner.

Abbreviations used: CD, circular dichroism; FMN, riboflavin-
5'-monophosphate; FAD, flavin-adenine-dinucleotide.

In spite of its scarcity, the literature on CD of riboflavin and its derivatives contains many discrepancies \(^1\) — \(^3\). We have, therefore, reinvestigated the CD spectra of free flavins as a function of flavin concentration, solvent polarity, photolytic “stacking” (out of plane) and “pairing” (in plane) ability, proton activity, ionic strength, and photolytic stability. Under the best conditions (cf. Fig. 1) we could resolve up to seven CD-bands, four in the range of the first \(\pi-\pi^*\) transition \((\lambda_{\text{max}} = 445 \text{ nm})\) and three in the range of the second one \((\lambda_{\text{max}} = 370 \text{ nm})\). In accordance with low temperature absorption spectra (J. M. Lhoste \(^4\)) and calculations by Song \(^5\), but in contrast to interpretations of Tollin \(^2\), we assign these bands to seven vibronic transitions, four belonging to the first Cotton effect, three to the second one.

The dependence of flavin CD on ionic strength was found negligible (cf. Fig. 2) and the large difference in CD-spectra of riboflavin (or FMN) in

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