The Photochemical Degradation of Flavins as Influenced by the Length and Extent of Hydroxylation of the Side Chain[1]

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Anaerobic photobleaching and aerobic photodecomposition have been examined with flavins which have varying polyhydroxyalkyl or ω-hydroxyalkyl substituents to elucidate the effects of length and extent of hydroxylation of the side chain on its photochemical degradation. Flavins with polyhydroxyalkyl chains of three through six carbons in length are comparably photobleached, whereas those with corresponding ω-hydroxyalkyl chains exhibit generally similar characteristics, but are more slowly bleached. The aerobic photodecomposition of the latter is not only slower over-all than with polyhydroxyalkylflavins, but in an inverse manner, ω-hydroxyalkyl chains are lost more rapidly with longer exposure to light. Lumichrome and several new products result from photolysis of ω-hydroxyalkylflavins. These results substantiate the preferential abstraction of hydrogen from the 2'-carbon during photoreduction, but indicate lack of an absolute dependence on a hydroxyl group at this position. Moreover, free-radical initiated reactions that are more rate limiting during aerobic photolysis may occur with ω-hydroxyalkylflavins which function at least in part through a singlet state.

Introduction

Illumination of riboflavin solutions leads to degradation of the D-ribityl chain in position 9 of the isoalloxazine ring system. Elucidation of the mechanisms for this photolysis is becoming more certain through recent efforts.[2-4] The photobleaching which occurs under anaerobic conditions results in generation of a flavin semiquinone with reduction of the ring and concomitant oxidation of the side chain. The photodecomposition which occurs under aerobic conditions results in the intermediate formation of 6,7-dimethyl-9-formylmethylisoalloxazine and ultimate production of lumiflavin and lumichrome. In the present context, delineation between anaerobic photobleaching and aerobic photodecomposition is based on the experimental conditions and measurements employed rather than referring to exactly distinguishable reactions. The specific degree to which the photochemical process is altered by oxygen has not been clearly established. Moreover, there is evidence that the reduced form of 6,7-dimethyl-9-formylmethylisoalloxazine is formed under anaerobic conditions.[2]

Though the photochemical degradation of other flavins had not been investigated adequately, in a recent review on the work with riboflavin it was sur-

mised that photolysis occurs only with such flavins as have a hydroxyl group in the 2'-position.[5] The present study was undertaken, therefore, to obtain general information on the effects of light on flavins with different side chains and to clarify specifically the presumed necessity for the 2'-hydroxyl group. Both relative rates for anaerobic photobleaching and aerobic photodecomposition have now been examined with flavins which have either polyhydroxyalkyl or ω-hydroxyalkyl chains of three through six carbons in length at position 9.

Experimental Section

Materials. D-Riboflavin (6,7-dimethyl-9-(1'-D-ribityl)isoalloxazine) was obtained from Eastman Organic Chemicals. The following flavins were synthesized by procedures reported previously[6]: 3'-hydroxypropylflavin (6,7-dimethyl-9-(3'-hydroxypropyl)isoalloxazine), DL-glyceroflavin (6,7-dimethyl-9-(1'-DL-glyceryl)isoalloxazine), 4'-hydroxybutylflavin (6,7-dimethyl-9-(4'-hydroxybutyl)isoalloxazine), D-erythroflavin (6,7-dimethyl-9-(1'-D-erythrityl)isoalloxazine), 5'-hydroxypentylflavin (6,7-dimethyl-9-(5'-hydroxypentyl)isoalloxazine), 6'-hydroxyhexylflavin (6,7-dimethyl-9-(6'-hydroxyhexyl)isoalloxazine), and D-alloflavin (6,7-dimethyl-9-(1'-D-allityl)isoalloxazine).

Apparatus. An Aminco-Bowman spectrophotofluorometer (No. 4-8106) which was equipped with a xenon–mercury lamp (200 w.) was used as the source for illumination of flavin solutions. The monochromator was set for an activating wave length near 445 mμ, and slit arrangements were omitted to allow maximum intensity.

A Beckman DU spectrophotometer was used to measure the absorbancy of flavin solutions following anaerobic photobleaching. The wave length for absorption was set at 445 mμ.

A Densicord 542 recording densitometer which was equipped with a low-pressure mercury lamp (253-mμ emission maximum), a primary fluorimetry filter, a secondary fluorescence filter (525-mμ fluorescence maximum), and a type B phototube was used to detect the fluorescent material at the R_f of the original flavin upon paper chromatography following aerobic decomposition. The areas under curves from the chart recordings were measured with a planimeter to calculate the amounts of original flavin which remained.

A Mineralight V 41 ultraviolet lamp was used to scan paper chromatograms of the photodecomposed flavins for identification of primary fluorescent products.

Anaerobic Photobleaching. Flavins were dissolved in sodium phosphate buffer (pH 7) and the solutions were

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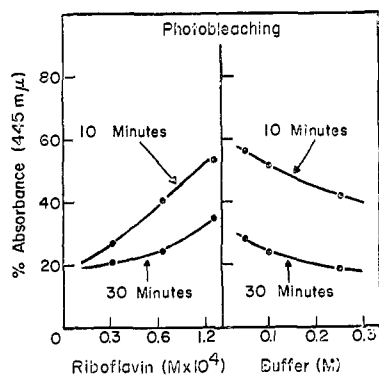
Figure 1. Extent of anaerobic photobleaching of riboflavin solutions as affected by flavin and buffer concentrations. When flavin concentration was varied, 0.1 M buffer was used; when buffer concentration was varied, 1.25×10^{-4} M flavin was used.

Figure 2. Rates of anaerobic photobleaching of flavins with different side chains. The 0.1 M buffer was used with 1.25×10^{-4} M solutions of flavins with the following 9-substituents: 1,4'-hydroxybutyl, 2,6'-hydroxyhexyl, 3,5'-hydroxypentyl, 4,3'-hydroxypropyl, 5,DL-glyceryl, 6,D-allityl, 7,D-ribityl, and 8,D-erythrityl.

pipetted into Thunberg cuvettes which were wrapped in aluminum foil to exclude light. The cuvettes were evacuated with a water aspirator and refilled with nitrogen gas. This process was repeated three times with shaking. The cuvettes were unwrapped, positioned in the spectrophotofluorometer, and illuminated at constant intensity. Decreases in absorbancy of the solutions were measured at intervals against a buffer blank in the spectrophotometer.

Aerobic Photodecomposition. Buffered solutions of the flavins in open cuvettes were illuminated at constant intensity in the spectrophotofluorometer. At intervals, 20-μl. aliquots were withdrawn in micropipets and applied in the dark to Whatman No. 1 paper. The compounds were resolved with *n*-butyl alcohol–acetic acid–water (4:1:5, upper phase), as ascending solvent. The chromatograms were dried and cut into vertical strips, and the amounts of original flavin remaining were measured in the densitometer.

Results

Anaerobic Photobleaching. The extent of photobleaching of riboflavin solutions as affected by varying concentrations of the flavin and phosphate buffer is illustrated by the data in Figure 1. The higher the flavin concentration, the smaller the per cent of total flavin bleached by photoreduction. These results appear to obey an integrated rate law.[7] However, the higher the buffer concentration, the larger the per cent of total flavin bleached. Proportionally greater effects due to varying concentrations are seen at 10 min. than at 30 min.

The various rates of photobleaching of flavins with different side chains are shown in Figure 2. The photobleaching rates for all these flavins decrease in a quasi-exponential manner. Those flavins which bear polyhydroxyalkyl substituents of different lengths seem to have generally similar rates of bleaching, whereas those with ω-hydroxyalkyl substituents are somewhat more slowly bleached and appear to follow an order of 3'-hydroxypropylflavin > 5'-hydroxypentylflavin > 6'-hydroxyhexylflavin > 4'-hydroxybutylflavin.

Aerobic Photodecomposition. The extent of photodecomposition of riboflavin solutions as affected by

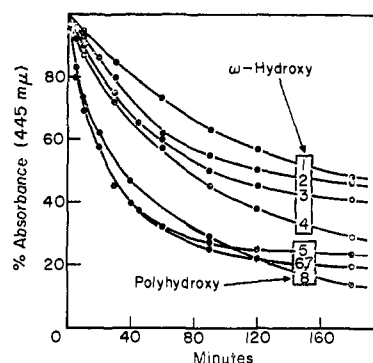(7) G. K. Radda and M. Calvin, *Biochemistry*, 3, 384 (1964).

varying concentrations of the flavin and phosphate buffer is illustrated by the data in Figure 3. The higher the flavin concentration, the smaller the per cent of original flavin decomposed by photolysis. However, the higher the buffer concentration, the larger the per cent of original flavin decomposed. As with photobleaching, the general relationship of photodecomposition to concentration changes is not linear.

The various rates of photodecomposition of flavins with different side chains in position 9 are shown in Figure 4. Only those flavins which bear polyhydroxyalkyl substituents are photodecomposed in a quasi-exponential manner. The 3'-hydroxypropylflavin has an asymmetric decay curve. The other flavins with longer ω-hydroxyalkyl substituents exhibit an over-all inverse behavior, wherein their photolysis becomes more rapid upon prolonged exposure to light. The general sequence for increased photodecomposition of all the flavins appears to follow their approximate order of increased photobleaching.

Upon examination of paper chromatograms, the yellow color or fluorescence in the total of the remaining flavin and its products does not constitute as much as that in the original flavin. After 30 min. of illumination at 445 mμ of an aerobic solution of riboflavin under conditions given for Figure 3, approximately 55% of riboflavin and 30% of additional products with characteristics of fluorescence similar to riboflavin can be accounted for following development of chromatograms with the *n*-butyl alcohol–acetic acid–water solvent. Products with R_f values of 0.11, 0.19, and 0.45 are seen in addition to riboflavin at 0.28 and lumichrome at 0.65. A similar exposure of 5'-hydroxypentylflavin solution to 3 hr. of illumination causes 45% of the original flavin to be decomposed, but only 65% of the total flavin plus products can be detected by scanning the fluorescence at the emission maximum of the original flavin. In addition to 5'-hydroxypentylflavin and lumichrome with similar R_f values near 0.65, new products were found at 0.20 and 0.30. The 3'-hydroxypropylflavin with an R_f value of 0.50 yielded lumichrome and other products at 0.12, 0.15, and 0.36. For all the flavins, different times, intensities, or wave lengths of illumination and changes in concentration of buffer somewhat alter the amounts and partially the nature of products formed.
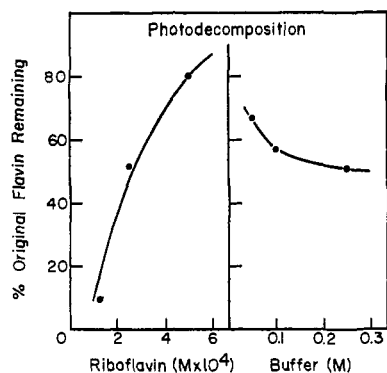
Figure 3. Extent of aerobic photodecomposition of riboflavin solutions as affected by flavin and buffer concentrations. When flavin concentration was varied, 0.1 M buffer was used; when buffer concentration was varied, 2.5 × 10⁻⁴ M flavin was used. Solutions were exposed to light for 30 min.

Figure 4. Rates of aerobic photodecomposition of flavins with different side chains. The 0.1 M buffer was used with 2.5 × 10⁻⁴ M solutions of flavins with the following 9-substituents: 1,4'-hydroxybutyl, 2,6'-hydroxyhexyl, 3,5'-hydroxypentyl, 4,3'-hydroxypropyl, 5,DL-glyceryl, 6,D-allityl, and 7,D-ribityl.

Discussion

The effects of concentration changes demonstrated with riboflavin to establish satisfactory conditions for comparison with other flavins and previous work are in general agreement with recent results.[4] The slight proportional decrease in extent of anaerobic photobleaching with higher concentrations of flavin, especially after brief illumination, may reflect in part the self-quenching due to some dimerization of reduced with oxidized flavin. This is not found under conditions for aerobic decomposition where insufficient reduced flavin can accumulate during the course of photolysis. The nonlinear increase in extent of anaerobic bleaching with increasing phosphate buffer is not strictly proportional to the square of buffer concentration as formerly believed.[8] Similarly the buffer effect is considerable with aerobic photodecomposition, as was reported earlier for the rate of aerobic photobleaching.[9]

The rate of anaerobic photobleaching determined for riboflavin at 445 mμ agrees well with that found by others.[2,8,10] Other flavins with D-polyhydroxyalkyl chains of three through six carbons in length are comparably bleached, whereas those with corresponding ω-hydroxyalkyl chains exhibit generally similar characteristics, but are more slowly bleached. These comparisons are consistent with the evidence that electrons for the photoreduction without additional reducing agents are derived from the side chain of the flavin by an intramolecular reaction.[3,7,8,11] Though hydrogen abstraction appears to occur preferentially from a 2'-carbon with a secondary hydroxyl group as previously suggested,[3,12] there certainly is no such obligatory dependence on the hydroxyl group at this position, as was concluded by some.[5,7,12] In fact, the rate of photo-
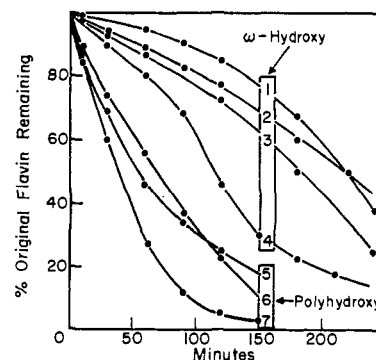
bleaching of DL-glyceroflavin, like the other polyhydroxyalkylflavins, is only moderately faster than that of 3'-hydroxypropylflavin. Molecular models show that each of the aliphatic hydrogens on the side chain of all the flavins studied herein can be placed in proximity to the initial acceptor center at N-1.

The rates of aerobic photodecomposition of flavins indicate similar over-all mechanisms for photolysis of D-polyhydroxyalkyl chains, but a marked difference from ω-hydroxyalkyl chains, especially when longer than three carbons. The relative slowness in cleavage of the latter flavins again supports the early suggestion of secondary hydroxyl group enhancement for rapid photodecomposition.[13] Acetylated riboflavin[14] and derivatives like 9-(3'-hydroxypropyl)isoalloxazine,[15] which lack the 2'-hydroxyl, likewise were found to be photolyzed slowly. The inverse decay patterns of the ω-hydroxyalkylflavins, which begin to lose the side chain more rapidly after longer exposure to light, are suggestive of free-radical-initiated reactions which are much more rate limiting during scission that photoreduction in the total photolytic process.

It has been recently shown that the primary photochemical species from riboflavin leading to lumiflavin is the triplet state, while in the presence of oxygen or a triplet quencher such as dioxane, lumichrome is the dominant product.[16] The occurrence of lumichrome from such flavins as 3'-hydroxypropylflavin and 5'-hydroxypentylflavin points to the probable function of a singlet state during aerobic photolysis of ω-hydroxyalkyl chains as well. More specific interpretations of the individual steps and intermediate species involved in photolysis of these compounds must await identification of the several different products.

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