Photochemical Degradation of Flavins. VI. A New Photoproduct and Its Use in Studying the Photolytic Mechanism[1]

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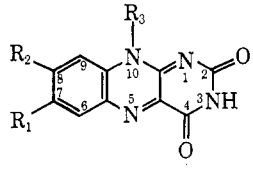
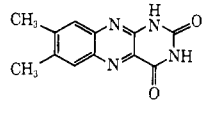
Abstract: Two new products have been isolated from riboflavin (RF) photolysates. The first is the 2′-ketoflavin 7,8-dimethyl-10-(1′-deoxy-D-*erythro*-2′-pentulosyl)isoalloxazine. It is formed over the pH range 4–10 and its production, like that of formylmethylflavin (FMF), is very sensitive to quenching by KI. The other product, believed to be the 4′-ketoflavin, is not produced under alkaline conditions and its formation under neutral conditions (like that of lumichrome, LC) is not easily quenched by KI. The ketones are suggested to result from abstraction of the 2′- and 4′-α hydrogens by the excited ring. Reduction of the 2′-ketoflavin with NaB^3H_4 yielded 2′-^3H-RF, which upon photolysis generated ^3H-FMF. This proves that the 2′-α hydrogen is not abstracted during FMF production. Possible mechanisms for production of FMF are discussed; the observation of an isotope effect when RF is photolyzed in D_2O under alkaline conditions suggests abstraction of the 2′-hydroxyl hydrogen. The rate of photolysis increases 30-fold at pH 8 and 2.5-fold at pH 3 relative to the rate at pH 5. The existence of bent triplet and a planar excited singlet differing in the center for hydrogen abstraction and protonation could explain the dependence of rate and product distribution on pH.

Riboflavin (RF, vitamin B_2) is particularly sensitive to light, the isoalloxazine ring system undergoing intramolecular photoreduction in which the ribityl side chain serves as the electron donor (in the absence of an external reductant). During oxidation of the side chain, fragmentation may occur to produce several photoproducts as summarized in Table I. A large number of other unidentified photoproducts have also been detected chromatographically.[2-8]

Table I. Structure of Riboflavin and Its Photoproducts



isoalloxazine ring system

Compound	R_1, R_2	R_3	Ref
Riboflavin (RF)	$-CH_3$	$-CH_2(CH_2OH)_3-CH_2OH$	
Formylmethylflavin (FMF)	$-CH_3$	$-CH_2CHO$	*a*
Carboxymethylflavin (CMF)	$-CH_3$	$-CH_2COOH$	*b–d*
Lumiflavin (LF)	$-CH_3$	$-CH_3$	*e, f*
Lumichrome (LC)			*g, h*

[a] E. C. Smith and D. E. Metzler, *J. Amer. Chem. Soc.*, **85**, 3285 (1963). [b] G. E. Treadwell, M.S. Thesis, Iowa State University, Ames, Iowa, 1967. [c] C. Fukamachi and Y. Sakurai, *Bitamin*, **7**, 939 (1954). [d] R. Kuhn and H. Rudy, *Ber.*, **68**, 300 (1935). [e] R. Kuhn and K. Reinemund, *ibid.*, **67**, 1932 (1934). [f] R. Kuhn, H. Rudy, and K. Beinemund, *ibid.*, **68**, 170 (1935). [g] P. Karrer, H. Salomon, K. Schöpp, E. Schlittler, and H. Fritzsche, *Helv. Chim. Acta*, **17**, 1010 (1934). [h] R. Kuhn and H. Rudy, *Ber.*, **67**, 1936 (1934).

An earlier publication[6] from this laboratory reported preliminary studies on the structure of two of the other flavin photoproducts, designated A and C, and the conditions under which they were obtained. Both appeared to contain a 5-carbon side chain with one of the positions oxidized to the carbonyl level. The present report establishes the structure of C as the 2′-ketoflavin and that of A as a mixture containing predominately the 4′-ketoflavin. Properties of the two substances are described and they are used together with ^{14}C-labeled riboflavin to study the mechanism of photodestruction.

Experimental Section

Materials and Methods. The sources of standard flavins, the photolytic techniques, and the chromatographic procedures have been described.[6] The degassing procedure for preparing anaerobic solutions consisted of at least 4–5 cycles of evacuation of the Thunberg tubes with an oil pump followed by filling with helium. The Thunberg tubes were shaken vigorously during the entire procedure. A procedure for carrying out characterization reactions directly on thin-layer chromatograms[6] was used extensively in determining the structures of products A and C. The chromatographic localization of the various photoproducts discussed is also described in ref 6.

Radioactive 2-^{14}C-RF and NaB^3H_4 were obtained from Amersham/Searle. The ^{14}C-RF was purified by tlc before use. All chemicals were reagent grade.

Products A and C were isolated on standard tlc plates in small batches and were eluted from the silica gel with methanol or water. Substance A was obtained from the partial anaerobic photolysis of 0.1–0.5 l. of 5×10^{-5} *M* RF in methanol bleached 50% at 445 nm. In all detectable ways, substance A prepared in this manner was found identical with A prepared by photolysis in water. Compound C was obtained from anaerobic photolysis of a 10^{-4} *M* aqueous alkaline solution of RF bleached 50% at 445 nm.

The semicarbazones of A and C were prepared by heating, just to boiling, a 5×10^{-5} *M* solution of the flavin in methanol to which

(1) Supported by Grant No. GB-7889 from the National Science Foundation. Taken largely from the Ph.D. Dissertation of William L. Cairns.

(2) I. M. Hais and L. Pecáková, *Nature (London)*, **163**, 768 (1949).
(3) S. Svobodová, I. M. Hais, and J. V. Kostír, *Chem. Listy*, **47**, 205 (1953).
(4) S. Svobodová-Leblová, J. V. Koštíř, and I. M. Hais, *J. Chromatogr.*, **14**, 451 (1964).
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(6) G. E. Treadwell, W. L. Cairns, and D. E. Metzler, *J. Chromatogr.*, **35**, 376 (1968).
(7) J. Koziol, *Photochem. Photobiol.*, **5**, 55 (1966).
(8) T. Wada, C. Fukai, and Y. Sakurai, *Eiyo to Shokuryo*, **5**, 97 (1952).

had been added 1 drop of a solution of 0.24 g of semicarbazide–HCl and 0.15 g of sodium acetate in 2.5 ml of water. RF did not react with semicarbazide under these conditions.

Tritiated RF was prepared by reduction of aqueous solutions of A and C immediately after they had been isolated. A saturated solution of NaB^3H_4 in pyridine was used for reduction, and the extent of the reaction was followed chromatographically. The reduced products were bound to R-15 resorcinol–formaldehyde resin.[9] The resin was then washed with 100 ml of 1 *N* HCl, followed by two three times the amount of redistilled water necessary to bring the pH to neutrality. The washed flavins were eluted with 50% acetone–water (v/v) and evaporated to dryness at 30–40° in a rotary evaporator. The residue was chromatographed in the ketone solvent system to separate the products of reduction from each other and from nonreduced flavin. The reduced products were scraped and eluted from the silica gel with redistilled water, and the eluate was then filtered through a millipore filter (pore size 0.45 μ, Millipore Filter Corp.) to remove small particles of silica gel before the final vacuum concentration.

Photolysates of tritiated RF were washed with 500–600 ml of redistilled H_2O while bound to the R-15 resin to remove any labile tritium generated during the photolysis.

As an internal standard, 2-^{14}C-RF was always added to the tritiated RF in amounts to give a relative $^3H/^{14}C$ ratio of 0.5–5.0. The absolute ratio was not determined. The $^3H/^{14}C$ ratio of each photoproduct was divided by the ratio in the unphotolyzed RF to give the fraction of tritium remaining in the photoproduct.

After separation of the flavin photoproducts by two-dimensional chromatography using the ketone and BAW solvents,[6] each spot was scraped into a vial containing 15 ml of Bray's scintillation mixture.[10] This mixture readily eluted the flavins from the gel. A linear counting rate for concentrations of 2-^{14}C-RF between 0.0009 μg/15 ml and 4.5 μg/15 ml was obtained without significant quenching problems. An Ansitron II liquid scintillation counter (Picker Nuclear) was used, and all counting was for times long enough that the counting statistics would be at the 95% confidence level or better.

The microbiological assay procedures were modifications of those described by Difco Laboratories[11] using *Lactobacillus casei* ATCC 7469. Samples to be assayed were dissolved in sterile water and pipetted in measured amounts into tubes containing previously sterilized assay medium. The total concentration of flavin in the samples was estimated spectrophotometrically by assuming identical extinction coefficients at 445 nm for the unknown and RF. Turbidimetric readings of the assay samples were made at 640 nm after approximately 18-hr incubation at 37°. The microorganisms and the medium for the assay were purchased from Difco Laboratories.

To determine the effect of pH on the photolysis rate, a bleaching curve (absorption at 445 nm *vs.* time) was plotted for each solution photolyzed, and from these curves the times required to obtain 10 and 20% bleaching were read. After photolysis the Thunberg tubes were opened, the unbuffered solutions were aerated with 100% oxygen (to avoid introduction of carbon dioxide), and a combination electrode was lowered into the tube for the pH measurement.

Results

Structure of Compound C. Compound C can be reduced with sodium borohydride to two products, of which one behaves like RF during chromatography in three different solvents (ketone, BAW, AAW; see ref 6 for solvent compositions). The other migrates in the ketone solvent like the diastereomer, araboflavin (R_f 0.12 compared with 0.18 for RF). Periodate oxidation of C produces carboxymethylflavin (CMF), but if C is reduced with NaB^3H_4 and the reduction products are oxidized with periodate, 95% of the tritium is retained in the FMF produced. Reaction of C with semicarbazide–HCl leads to a new product with an R_f of 0.35 in BAW. Compound C shows no ability to support growth of *L. casei*, but the reduced product corresponding to RF shows complete recovery of biological activity as compared with standard RF. These results clearly indicate that C is the 2′-ketoflavin–7,8-dimethyl-10-(1′-deoxy-D-*erythro*-2′-pentulosyl)isoalloxazine.

Structure of Substance A. Substance A can also be reduced by borohydride to two products, one with the chromatographic behavior of RF, and the other with a slightly lower R_f (0.12) in the ketone solvent system. When reduction is carried out with NaB^3H_4 and the products are cleaved with periodate, less than 5% of the tritium is found in FMF. Like compound C, substance A does not support growth of *L. casei*, but the reduction product of A corresponding to RF had 60% of the biological activity of standard RF.

With semicarbazide–HCl, substance A gives a product of R_f 0.30 in BAW. Periodate cleavage of A yields FMF and formaldehyde, and the periodate oxidation of the semicarbazone of A also produced FMF. Abercrombie and Jones[12] have found that the C_α–C_β bond of sugar phenylhydrazones is resistant to periodate cleavage. Under our conditions, the semicarbazone of C also showed no susceptibility to periodate oxidation.

All of the above observations are consistent with A being the 4′-ketoflavin, but, because only small amounts of A could be prepared, not all of the tests were quantitative. Two observations suggest that substance A is in fact a mixture of predominately the 4′-ketoflavin and up to 30–40% of 3′-ketoflavin and/or 5′-aldoflavin. (1) The borohydride-reduced substance A, after separation from araboflavin, was only 60% as active in supporting microbial growth as RF. This suggests the presence of another, unresolved, biologically inactive diastereomer. (2) When the RF spot from borotritide-reduced A was photolyzed (see below) 29% of the tritium still remained in the compound A which was produced (regenerated) during the photolysis. Attempts to separate A into components have been unsuccessful.

Chemical Properties of A and C. When solutions of A or C were held at pH 10 for 1 hr, then chromatographed, both products were found to have been completely destroyed. Compound C (the 2′-ketoflavin) produced LF as the major degradation product. Substance A was converted to three products, one (but not the most abundant) of which was LF. The other two products of A, which corresponded to compounds 4 and 5 reported previously,[6] were not identified.

It is noteworthy that like compound C the other 2′-carbonyl photoproduct, FMF, is also hydrolyzed to LF in alkali.[13] The possible participation of the flavin ring in these surprising reactions poses an interesting mechanistic question.[14]

Spectra and Protonation of A and C. Spectra of the compounds in various concentrations of HCl and in 0.05 *M* acetate buffers were recorded with a Cary 1501 spectrophotometer. The spectra of the neutral and cationic species of both A and C were found similar to those of the corresponding species of RF.[15] The long-

(9) A. Koziolowa and J. Kzoiol, *J. Chromatogr.*, **34**, 216 (1968).
(10) G. A. Bray, *Anal. Biochem.*, **1**, 279 (1960).
(11) "Media for the Microbiological Assay of Vitamins and Amino Acids," Difco Laboratories, Detroit, Mich., 1964.
(12) M. J. Abercrombie and J. K. N. Jones, *Can. J. Chem.*, **38**, 308 (1960).
(13) P-S. Song, E. C. Smith, and D. E. Metzler, *J. Amer. Chem. Soc.*, **87**, 4181 (1965).
(14) W. L. Cairns, Ph.D. Dissertation, Iowa State University, 1970.
(15) C. H. Suelter and D. E. Metzler, *Biochim. Biophys. Acta*, **44**, 23 (1960).

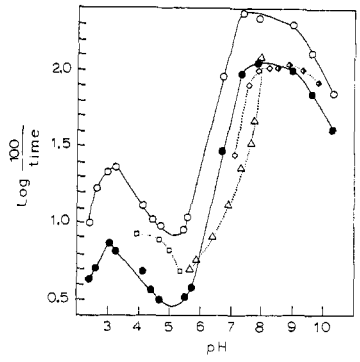


Figure 1. Effect of pH on the time required to reach 10 and 20% bleaching of an 8×10^{-5} *M* riboflavin solution; all buffers were 10^{-2} *M*: ○, unbuffered, 10% bleaching; ●, unbuffered, 20% bleaching; □, acetate buffer, 20% bleaching; △, phosphate buffer, 20% bleaching; ◇, Tris buffer, 20% bleaching; ◊, borate buffer, 20% bleaching.

wavelength peak of C was centered at 437 nm and was broader than that of RF or A.

The p*K* for protonation was estimated from changes in the absorption at 445 nm as approximately −0.1 for A and +0.6 for C. The p*K*'s of RF[16] and FMF[15] are −0.2 and 3.5, respectively.

The presence of a carbonyl group in the side chain of A and C enables these compounds, like FMF,[15] to form cyclized intermediates when protonated, raising the p*K*'s from that of RF. The greater number of possible conformations of the side chain of A and C and the more remote location of the 4′ position in the case of A may explain the lower p*K* of these two flavins relative to that of FMF.

Distribution of Products. Table II shows relative yields of the major fluorescent photoproducts of RF obtained when a 10^{-4} *M* solution of 2-^{14}*C*-RF was photolyzed anaerobically with light of wavelength greater than 400 nm until 50% of the flavin had been photoreduced as judged by the absorption at 445 nm. The products were separated chromatographically and the radioactivity (counts per minute) of each photoproduct was measured and divided by the total counts per minute for all spots on the chromatogram to give the per cent yield.

Table II. Percentage Yields of Photoproducts after 50% Bleaching

Photoproduct[a]	Percentage yields[b] pH 7	pH 10
% destruction of RF[c]	42.9 (44.8)	60.9 (60.4)
LC	16.8 (16.5)	31.7 (34.4)
26	0.7 (0.4)	1.8 (0.7)
FMF	57.4 (59.4)	39.6 (40.5)
LF	0.2 (0.2)	4.6 (4.5)
A + C	17.2 (16.3)	
C		12.8 (10.9)
16	5.6 (5.1)	3.0 (3.0)
B	2.1 (1.6)	6.2 (5.0)
4 + 5		1.1 (1.0)

[a] Unidentified photoproducts are numbered as in ref 6. [b] Figures give the percentage of total riboflavin destroyed which is represented by each product. Results of a duplicate experiment are given in parentheses. [c] The per cent destruction of riboflavin is the sum of the counts of all fluorescent products × 100 divided by the total counts on the plate, assuming that all radioactivity was present in the fluorescent spots. Note that per cent destruction does not exactly equal the per cent of bleaching at 445 nm, especially in alkaline solutions and at extended degrees of bleaching.

The ratio of A/C varied between 0.4 and 4 in a series of experiments in unbuffered solution at pH values between 6.5 and 7.5, probably as a result of the transition in mechanisms discussed below.

Hotta[17] and his coworker Terao[18] reported that they had isolated the 2′-ketoflavin, and that it was produced under all conditions including aerobic photolysis, and in yields as high as 50%. We have not been able to obtain such high yields under any circumstances. FMF, LF, and LC always make up greater than 60% of photoproducts.

Quenching by Potassium Iodide. When 2-^{14}*C*-RF (8×10^{-5} *M* neutral, anaerobic) was photolyzed for 30 min in the presence of 10^{-3} *M* KI, the yields of some photoproducts, *e.g.*, FMF and C, were more drastically reduced than those of others such as A and LC (Table III). Using a Stern–Volmer quenching constant of 50 for KI,[19] a fractional reduction in fluorescence of only 5% would be expected.

Table III. Ratio of Photoproduct Yields in the Presence and Absence of KI[a]

Photoproduct	% yield in absence of KI / % yield in presence of KI[b]
LC	1.5 (1.4)
FMF	26.0 (32.2)
A	1.3 (1.6)
C	∞[c] (∞)[c]

[a] 8×10^{-5} *M* RF was photolyzed in the presence and absence of 10^{-3} *M* KI for 30 min. [b] Results of a duplicate experiment are shown in parentheses. [c] No C detected in the presence of KI.

Note that Song and Metzler failed to resolve A and C and incorrectly reported a strong quenching of the production of A by KI.

Effect of pH on the Rate of Photobleaching. The influence of pH on the time required for the absorption at 445 nm of an 8×10^{-5} *M* solution to fall by 10 and 20% of the original value is shown in Figure 1. The rate at pH 5 is slow, but increases about 2.5-fold at lower pH to a maximum at approximately pH 3, then falls again. Above pH 5 the rate increases about 30-fold, then falls off again at pH values above 9.

Several features of the pH profile can be explained simply. The fall-off below pH 3 is doubtlessly related to the similar decrease in fluorescence of riboflavin[20,21] which results partly from formation of a nonfluorescent cation (p*K* for protonation of ground state ~0), but more from proton quenching of the singlet excited state.[21] The decline in rate above pH 8 resembles that observed by Weatherby and Carr[22] and by Carr and Metzler[23] for RF-catalyzed photooxidations. Flavins

(16) L. Michaelis, M. P. Schubert, and C. V. Smythe, *J. Biol. Chem.*, **116**, 587 (1936).

(17) K. Hotta, *Bitamin*, **8**, 248 (1955).
(18) M. Terao, *Tohoku Igaku Zassi*, **59**, 441 (1959).
(19) P-S. Song and D. E. Metzler, *Photochem. Photobiol.*, **6**, 691 (1967).
(20) O. A. Bessey, O. H. Lowry, and R. H. Love, *J. Biol. Chem.*, **180**, 755 (1949).
(21) G. Weber, *Biochem. J.*, **47**, 114 (1950).
(22) G. D. Weatherby and D. O. Carr, *Biochemistry*, **9**, 344 (1970).
(23) D. O. Carr and D. E. Metzler, *Biochim. Biophys. Acta*, **205**, 63 (1970).

having a nonionizable substituent at position N-3 did not show a decline in rate at high pH suggesting that the anion formed by deprotonation of N-3 is less efficient than the neutral flavin in photooxidation of either external electron donors or of the ribityl side chain.

The other features of the profile are not easily understood and are considered further in the Discussion.

Photolysis of 2′-^3H-RF and 4′-^3H-RF. Preparation of 2′-^3H-RF is described in the Experimental Section. The purity of the compound was checked by oxidation with periodate. Greater than 95% of the tritium was recovered in FMF meaning that less than 5% of the preparation was flavins containing tritium in other than the 2′ position of the side chain.

The fraction of tritium remaining in each of the photoproducts of 2′-^3H-RF is shown in Table IV. The

Table IV. Fraction of Tritium Remaining in Each of the Major Photoproducts of 2′-^3H-RF[a]

Product	Fraction of residual tritium pH 6.2	pH 9.8
RF (unphotolyzed)	1.00	1.00
RF (after photolysis)	1.12	0.95
LC	0.00	0.00
FMF	1.06	0.90
A	0.98	
C	0.29	0.00

[a] Riboflavin (10^{-4} *M*) was anaerobically bleached 47% at pH 6.2 and 52% at pH 9.8.

residual tritium (29%) in C under neutral conditions may be a result of contamination by substance A which migrates slightly ahead of C and may not be completely resolved from A. Certainly under alkaline conditions where no A is formed, C contains no tritium.

Photolysis of 4′-^3H-RF (prepared by reduction of A) gave results shown in Table V. The residual 29%

Table V. Fraction of Tritium in each of the Major Photoproducts of 4′-^3H-RF[a]

Product	Fraction of residual tritium pH 6.3	pH 10.3
RF (unphotolyzed)	1.00	1.00
RF (after photolysis)	1.04	0.99
LC	0.00	0.00
FMF	0.00	0.00
A	0.29	
C	0.93	0.98

[a] Riboflavin (10^{-4} *M*) was anaerobically bleached 46% at pH 6.3 and 48% at pH 10.3.

tritium remaining in A after neutral photolysis indicates that A is probably not pure and contains some 3′-ketoflavin and/or 5′-aldoflavin. The lack of any tritium in FMF suggests that the A was not contaminated by 2′-ketoflavin before reduction.

Discussion

Mechanisms for three distinct types of reactions must be considered: (1) loss of the complete side chain to form LC; (2) oxidation of the side chain to ketones A and C; (3) chain cleavage to form FMF and, in secondary reactions, LF and CMF.

(1) Formation of Lumichrome. The mechanism of side-chain loss from 10-(2′-hydroxyethyl)isoalloxazine has been investigated by Moore and coworkers.[24] When this compound was photolyzed in D_2O, to alloxazine and acetaldehyde, no isotope rate effect was observed, but 10-(2′-hydroxyethyl-2′,2′-$^2H_2$)isoalloxazine was photolyzed 2.5 times more slowly than the undeuterated compound. Thus the primary reaction is apparently abstraction of a hydrogen from the 2′ carbon.

McBride and Metzler[25] and Song and Metzler[19] suggested that for RF a similar reaction might lead to LC. This mechanism (a Norrish type II or photoelimination reaction) could also account for LC production from other flavins containing a hydrogen on the 2′ carbon of the side chain, regardless of whether the hydrogen is α to a hydroxyl group or not.

(2) Formation of Ketones. The formation of products C and A could occur by direct abstraction of a hydrogen atom, either from a carbon atom (α hydrogen) or from a hydroxyl oxygen. Abstraction of an α hydrogen is favored on the basis of a kinetic isotope effect observed by Moore and Baylor[26] for photoreduction of a flavin with a 3′-hydroxypropyl side chain. Similarly, abstraction of the 4′ hydrogen and to a lesser extent of the 3′ and 5′ hydrogens could lead to compound A and to smaller amounts of 3′- and 5′-carbonyl compounds.

(3) Formation of Formylmethylflavin. Moore and Baylor observed that both the 2′- and 3′-hydroxyl groups must be present in the side chain for formation of significant quantities of photoproducts with a formylmethyl side chain.[26] The cleavage presumably follows an intramolecular hydrogen abstraction similar to that yielding A and C, but the position of this oxidation is unknown. The 2′-α hydrogen can be excluded immediately, for, as recorded in Table IV, tritium is retained in FMF after photolysis of 2′-^3H-RF. Moore and Baylor have suggested abstraction of the 3′-α hydrogen and subsequent formation of the 3′-ketone.[26] They suggest that due to the presence of a 2′-hydroxyl-group adjacent to the 3′-carbonyl, the side chain of this 3′-ketoflavin is unstable in the presence of the isoalloxazine ring system and decomposes to FMF. However, the side chains of compounds A and C are in a similar environment and are reasonably stable.

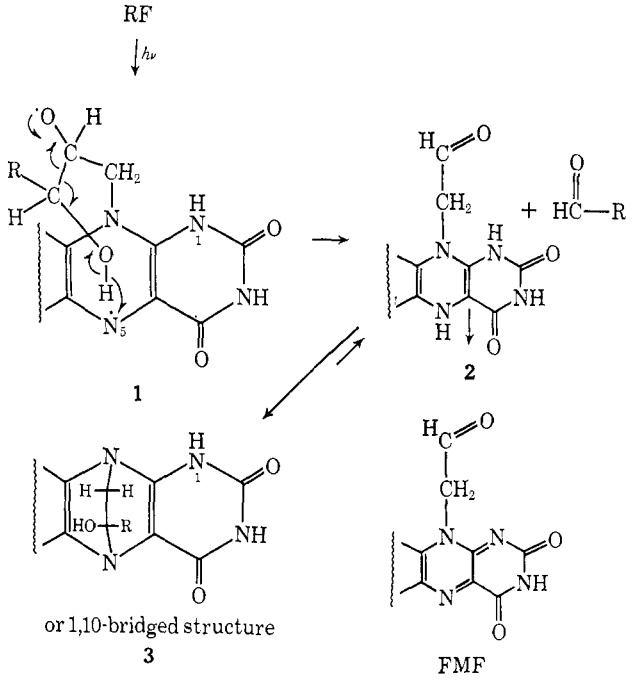
Other possible sites for the initial hydrogen removal leading to FMF are: (1) a 1′-methylene hydrogen; (2) the 4′-α hydrogen; (3) the 2′-hydroxyl hydrogen. In each case, a subsequent β cleavage of the radical formed could lead to FMF.

Examination of a space-filling model of RF shows that abstraction of a 1′-methylene hydrogen by N-1 of the isoalloxazine ring is unlikely, but that abstraction of the 4′ hydrogen by N-1 of the isoalloxazine ring is possible. However, the latter could not explain the formation of a formylmethyl side chain when 10-(2′,3′-dihydroxypropyl)isoalloxazine is photolyzed.[26] Nevertheless, 4′-hydrogen abstraction in RF cannot be ruled out conclusively because 7,8-dimethyl-10-(2′,3′-

(24) W. M. Moore, J. T. Spence, F. A. Raymond, and S. D. Colson, *J. Amer. Chem. Soc.*, **85**, 3367 (1963).
(25) M. M. McBride and D. E. Metzler, *Photochem. Photobiol.*, **6**, 113 (1967).
(26) W. M. Moore and C. Baylor, Jr., *J. Amer. Chem. Soc.*, **91**, 7170 (1969).

dihydroxypropyl)isoalloxazine is photolyzed 7.5 times less efficiently than RF, and different mechanisms may be operating in the two cases. A second objection is that the same radical on C-4′ would lead to both A and FMF. Thus, one might expect a constant ratio of A to FMF independent of pH, but a drastic change in the ratio of these two products is actually observed.

The third possible mechanism for FMF production depends on the abstraction of a hydrogen atom from the 2′-hydroxyl group to give a diradical **1** which could



then yield the reduced FMF (**2**) in a simple chain cleavage as indicated. It is probable that **2** exists predominately as a cyclic structure **3**, either the 5,10-bridged carbinolamine shown or a similar 1,10-bridged structure. Such a structure has been suggested from polarographic studies[24,27] as responsible for the anodic wave which disappears upon aeration of anaerobically photolyzed RF solution.

Photolysis of riboflavin in D_2O shows no isotope effect according to Moore and Baylor,[26] which was interpreted to mean that no hydroxyl hydrogens were abstracted during photobleaching. On the other hand, preliminary studies in our laboratory indicate a distinct and reproducible isotope effect for photobleaching in alkaline solutions.

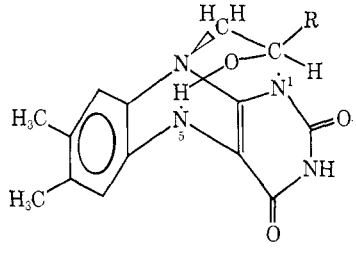
Our most surprising experimental findings are the changes in rate,[28] especially the 30-fold increase centered around pH 6.5, and in product distribution with pH (no substance A at high pH) and in the presence of KI (decrease in yield of FMF and compound C). These results are most easily rationalized by assuming that both singlet and triplet excited states yield photochemical products.[19] The singlet state reacts with lower quantum yields to produce principally LC and substance A. This reaction predominates at low pH and is not effectively quenched by KI in low concentrations. The triplet state yields C and FMF as well as LC and is effectively quenched by KI.[19,29]

An objection to this proposal is that the quantum yields for intersystem crossing (0.72,[30] 0.71[31]) and for fluorescence (0.25[32]) are high. Thus, the rate constant for abstraction of hydrogens by the excited singlet would have to be larger than the rate constants for fluorescence, intersystem crossing, and nonradiative decay to compete with these processes. This might be the case if the hydrogens abstracted by the excited singlet are in close proximity to the abstracting nitrogen (as for example due to side chain–ring interaction through hydrogen bonding).

Whether or not some products arise through a singlet mechanism, the data suggest that the rate of product formation from the triplet state undergoes a large increase with increasing pH centered around a pH of about 6.5 (in unbuffered solution). A simple explanation would be that the triplet possesses a pK_a of about 6.5[33] and that the protonated form is less reactive than the unprotonated form.

There is no experimental evidence indicating a pK for protonation of the flavin triplet as high as 6.5. Indeed, it has been suggested that the pK is close to that of the ground state[34] (*i.e.*, ~0), which would be consistent with the general trend observed by Jackson and Porter[35] for various naphthalene derivatives and heterocyclic amines. Furthermore, Shiga and Piette have reported only an insignificant change in the lifetime of the FMN triplet with pH.[36] (Later studies[37] reported a lifetime at pH 7 ten times greater than that reported in the earlier paper, suggesting some uncertainty in the experiment.) Since the experiments of Lhoste, *et al.*, and of Shiga and Piette were all done at low temperature in the solid phase, they cannot rule out our suggestion.

A possible explanation for both the proposed high pK for the triplet and the increased reactivity at high pH is that the triplet exists in a bent, diradical form



It is apparent that N-5 can be approached by the side-chain hydrogens (the 2′-α hydrogen for formation of LC and compound C and the 2′-hydroxyl hydrogen

(27) M. M. McBride and W. M. Moore, *Photochem. Photobiol.*, **6**, 103 (1967).

(28) Changes in the effects of buffers in different pH regions (Figure 1) also suggest a change in mechanism. Below pH 6 both acetate and phosphate buffers increase the rate of photolysis relative to unbuffered solutions. M. Halwar (*J. Amer. Chem. Soc.*, **73**, 4870 (1951)) has also reported general acid–base catalysis of the aerobic photolysis of riboflavin in the pH range 4–5. In contrast, between pH 6 and 8.0–8.5 marked inhibition is caused by phosphate and Tris buffers.

(29) J. Posthuma and W. Berends, *Biochim. Biophys. Acta*, **112**, 422 (1966).

(30) P-S. Song in "Flavins and Flavoproteins," H. Kamin, Ed., University Park Press, Baltimore, Md., in press.

(31) B. Nathanson, M. Brody, S. Brody, and S. B. Broyde, *Photochem. Photobiol.*, **6**, 177 (1967).

(32) G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, **53**, 646 (1957).

(33) P. Hemmerich, University of Konstanz, Konstanz, West Germany, has suggested the possibility in a personal communication.

(34) J. M. Lhoste, A. Haug, and P. Hemmerich, *Biochemistry*, **5**, 3290 (1966).

(35) G. Jackson and G. Porter, *Proc. Roy. Soc., Ser. A*, **260**, 13 (1961).

(36) T. Shiga and L. H. Piette, *Photochem. Photobiol.*, **3**, 213 (1964).

(37) T. Shiga and L. H. Piette, *ibid.*, **4**, 769 (1965).

for formation of FMF and possibly also compound C) only in such a bent structure and not in a planar conformation. If it is assumed that the N-5 of the triplet is more reactive than N-1 in hydrogen abstraction and that it is also the site of protonation,[38] the pH profile is understandable. Thus, protonation at N-5 could block the most reactive center of the molecule.

The changes in product distribution are harder to explain. In the case of the triplet, although all hydrogens of the side chain can approach the now sp^3-hydridized N-5, the numerous conformational possibilities of the side chain may restrict abstraction to only the nearest hydrogens (1′ or 2′).

If the excited singlet has a planar conformation, N-1 is most likely involved in the abstraction. Products originating from the singlet may reflect preexcitation associations of N-1 and the side chain as, for example, through hydrogen bonding.

A final point concerns the increase in rate below pH 5. Perhaps a second site in the triplet is protonated to give a more reactive species, as has been suggested by Penzer[39] to explain the acceleration of rate of flavin mononucleotide (FMN) catalyzed photooxidation of methionine under acidic conditions in the pH region 5–6.

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(38) P. S. Song (*Ann. N. Y. Acad. Sci.*, **158**, 410 (1969)) has calculated the π-electron density as higher at N-5 than at N-1 in the first excited triplet of 7,8-dimethylisoalloxazine.

(39) G. R. Penzer, *Biochem. J.*, **116**, 733 (1970).

Communications to the Editor

Strategies in the Application of Partially Relaxed Fourier Transform Nuclear Magnetic Resonance Spectroscopy in Assignments of Carbon-13 Resonances of Complex Molecules. Stachyose[1,2]

Sir:

There are at present only two well-known procedures of general applicability for the assignment of resonances in proton-decoupled carbon-13 spectra: comparisons within a series of compounds with similar structures, and the use of splitting patterns arising from incomplete proton decoupling.[3] With the advent of Fourier transform nmr,[4] it has become possible to use partially relaxed[5] Fourier transform (PRFT) spectra[2,6,7] as an additional aid in assignments. We will discuss some strategies and limitations in the use of PRFT spectra for assigning ^{13}C resonances of complex molecules. As an illustration, we will show that the PRFT method is useful in assigning the carbon-13 spectrum of the nonreducing tetrasaccharide stachyose (**1**, Figure 1).

If the ^{13}C nuclei are proton decoupled, then the ^{13}C relaxation is exponential.[8] In this case, intensities in a PRFT spectrum are given by[9]

$$A = A_0[1 - 2\exp(-\tau/T_1)] \quad (1)$$

where A and A_0 are the observed and equilibrium intensities, respectively, τ is the interval between the 180 and 90° pulses,[5] and T_1 is the ^{13}C spin–lattice relaxation time. A resonance will appear inverted, nulled, or positive (with respect to the normal spectrum) depending on whether τ is smaller, equal to, or larger than T_1 ln 2, respectively. Thus, if two carbons have different T_1 values, the intensity of their resonances will have a different dependence on τ. Successful application of PRFT spectra in assignment of ^{13}C resonances requires an appreciation of how ^{13}C T_1 values vary with molecular site.

Theoretical considerations[8,10–12] and experimental work[2,6,7,13] indicate that the following three principles should be considered when using PRFT spectra for assigning ^{13}C resonances of large and asymmetric molecules. **Principle A**: relaxation of protonated carbons is overwhelmingly dominated by dipolar interactions with the attached protons, with T_1 given by[12]

$$1/T_1 = N\hbar^2\gamma_C^2\gamma_H^2 r_{CH}^{-6}\tau_{eff} \quad (2)$$

Here γ_C and γ_H are the gyromagnetic ratios of ^{13}C and ^1H, N is the number of directly attached hydrogens, r_{CH} is the CH distance, and τ_{eff} is the effective correlation time for rotational reorientation. Equation 2 is valid under conditions of complete proton decoupling a ıd only when $1/\tau_{eff}$ is much larger than the ^1H resonance frequency. Principle A may not apply to small or very symmetric molecules, which may have an appreciable contribution to $1/T_1$ from the spin–rotation interaction [14] In addition, contributions to $1/T_1$ from chemical shift anisotropy may be important in some

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