Eur. J. Biochem. 57, 35–48 (1975)

Intramolecular Addition of the Riboflavin Side Chain

Anion-Catalyzed Neutral Photochemistry

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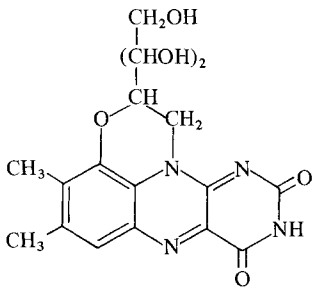
(Received July 4, 1974/February 18, 1975)

The presence of higher (> 0.2 M) concentrations of divalent anions A^{2-} (hydrogenphosphate, sulfate) is found to accelerate as well as to change entirely the course of riboflavin photolysis: instead of 10-dealkylation to yield lumichrome, intramolecular addition of the 2′-hydroxyl group is found to occur at the peri-position C(9). The reaction is analogous to the "photohydration" of the flavin nucleus in the cationic state as described by Schöllnhammer and Hemmerich [*Eur. J. Biochem.* (1974) *44*, 561–577]. The final product of the new addition reaction arises from autoxidation of a dihydroflavin intermediate and exhibits the structure



It is thus representative for a new class of flavins ("cyclo-dehydroflavins"). Earlier reports on "anomalous" flavin photodegradation products absorbing around 410 nm [Holmström (1964) *Ark. Kem. 22*, 281; Massey and Atherton (1962) *J. Biol. Chem. 237*, 2965] are readily explained.

The reaction is found to depend strictly on the presence of a nucleophilic function in the N(10)-side chain, *e.g.* N(10)-CH_2-C(OH)RR′ or even N(10)-$(CH_2)_2$-SO_3^-. Quenching experiments suggest that the new reaction occurs *via* the singlet state $^1Fl^*_{ox}$, while the normal photolysis is mediated by the triplet $^3Fl^*_{ox}$. The new photoaddition is thought to occur *via* a Flavin-A^{2-} complex which creates sterically favorable conditions for C(9)/O(2′α)-interaction.

The neutral photolysis of riboflavin ($\lambda_{max}^{pH\,7}$ = 445, 372 nm) was described as early as 1933 by Kuhn *et al.* [1] to imply complete removal of the ribityl side chain, yielding the parent alloxazine which was named "lumichrome (λ_{max} = 352, 382 sh)" because of its intense blue fluorescence. This N(10)-dealkylation was later on [2] recognised to originate from intramolecular photodehydrogenation of the side chain preferably in position 2′ and subsequent fragmentation. Holmström [3] was first to demonstrate the involvement of the excited flavoquinone triplet ($^3Fl^*_{ox}$) in this reaction. Furthermore, he postulated a flavosemiquinone as the first product of the reaction [4]. This interpretation, however, must be taken with great caution, since recent rapid kinetic studies done on alloxazine photohydration [5] demonstrate a diffusion-controlled secondary formation of semiquinone from comproportionation of a preformed

Abbreviation. NMR, nuclear magnetic resonance.

Nomenclature. The term "flavin" stands exclusively for 7,8-dimethyl isoalloxazine. Cyclodehydroflavin stands for intramolecularly alkoxylated flavin of type Ia (Table 1).

dihydro-intermediate. Hence, the question of flavin photolysis being a $1e^-$ or a $2e^-$ transfer (from the side chain into the heteroaromatic nucleus) still remains open.

More recently, Holmström [6] as well as Massey and Atherton [7] independently observed co-products arising in riboflavin and FMN photolysis, which exhibited a strong absorption maximum at 410 and a long-wave shoulder around 450 nm. Since we assumed that this chromophore might be related to hydroxyflavins described by ourselves in a preceding paper [8], we decided to investigate the whole problem more thoroughly. In the present paper we shall describe conditions which strongly favor formation of the '410-nm species' *via* suppression of normal flavin photolysis, and which therefore permit its isolation and structural elucidation. A preliminary account of these results has been published previously [9].

MATERIALS AND METHODS

Analytical photochemistry of flavins (λ_{max} = 445 nm) was done, unless otherwise stated, in 1-cm quartz cuvettes by irradiation with a specially adapted slide projector with a 24-V/250-W tungsten-halogen lamp. The distance between the light source and the center of the cuvette was 11 cm, the light beam was focussed at the center of the cuvette by two glass lenses. Alloxazines ($\lambda_{max} \approx 350$ nm) were irradiated in a quantum yield photoreactor from Applied Photophysics Ltd (London) equipped with a 250-W medium-pressure mercury lamp. In the case of anaerobic reactions, solutions were purged of oxygen by a stream of (solvent-saturated) argon bubbling through the solutions for 30 min.

Preparative photochemistry was done in open photoreactors of 2-l and 3-l volume equipped with a high-pressure mercury immersion lamp Heraeus TQ 170 (170 W) with glass cooling jacket. The progress of the reaction was monitored by measuring the absorption spectrum of the solution and illumination was stopped when no further increase in absorption at 410 nm was observed.

Light absorption spectra were recorded on a Cary 14 R spectrophotometer. Values for molar absorption coefficients are given in units of $mM^{-1} \cdot cm^{-1}$. Fluorescence spectra were made with a Perkin-Elmer fluorescence spectrophotometer MPF-3.

^{1}H-Nuclear magnetic resonance (NMR) spectra were recorded with a Varian A 60-A or a Bruker HX-90 spectrometer. Chemical shifts (compared to tetramethylsilane) are comparable within 0.05 ppm in both cases. Melting points were determined on a Kofler block and are not corrected. Mass spectral data were obtained with a Varian CH 7 single-focussing mass spectrometer.

Analytical thin-layer chromatography was done on commercially available precoated silica gel glass plates Merck F-254. Preparative chromatography was done on precoated silica gel plates 60 F-254 Merck. The following solvent systems were used.

A = chloroform–methanol (9:2, by vol.), B = chloroform–methanol (9:1), C = chloroform–methanol (95:5), D = butanol–acetic acid–water (4:2:2), E = butanol–acetic acid–water (5:2:3), F = butanol–ethanol–water (4:2:2), DEAE-cellulose was obtained from Whatman.

Riboflavin (VII) was a gift from Hoffmann-La Roche (Basle, Switzerland), 10-(2′-hydroxyethyl)flavin was made according to Fall and Petering [10] and isoriboflavin was prepared as described by Tishler *et al.* [11].

The remaining 10-(2′-hydroxyalkyl)flavin derivatives were prepared with slight modifications according to the general procedures described Karrer *et al.* [12–15] and Moore and Baylor [16].

The experiments were conducted at room temperature when not otherwise stated.

10-(2′-Hydroxypropyl)flavin (I) and Cyclodehydroflavin Ia

1-Chloro-4,5-dimethyl-2-nitrobenzene was prepared according to the procedure of Adams *et al.* [17] from 4,5-dimethyl-2-nitro-aniline in 75% yield, m.p. 59–62 °C.

A solution of 3.3 g (44 mmol) of 1-amino-propan-2-ol and 16.4 g (88 mmol) of 1-chloro-4,5-dimethyl-2-nitrobenzene in 50 ml of dry pyridine was refluxed for 24 h. Excess 1-chloro-4,5-dimethyl-2-nitrobenzene and pyridine were removed by steam distillation, the residue extracted with ether and the ether extract evaporated. The crude product was crystallized from acetic acid/petroleum ether (120–140 °C) to yield 4.2 g (43% as compared to 1-amino-propan-2-ol) of the N-substituted *o*-nitroaniline, m.p. 122–123 °C.

This aniline, 4.2 g (18.7 mmol), was dissolved in 150 ml of absolute ethanol and catalytically hydrogenated over palladium on charcoal. The solution was filtered under nitrogen into a flask containing 3.2 g alloxan monohydrate (20 mmol) and 10 ml of 6.0 M HCl and then refluxed for 15 min. The precipitate, which formed on standing overnight at 0 °C, was filtered, washed with ether and crystallized from acetic acid to yield 2.1 g (37% yield, m.p. 303–305 °C) of 10-(2′-hydroxypropyl)flavin (I) which was pure as judged by thin-layer chromatography (solvent systems A, D).

Found: C 59.4, H 5.4, N 18.1%. $C_{15}H_{16}N_4O_3$ (M_r = 300.3) requires C 59.9, H 5.4, N 18.6%.

NMR(CF_3COOH): δ = 8.47 (6-H), 8.20 (9-H), 5.86–4.66 m (1′-H_2, 2′-H), 2.86 (8-CH_3), 2.74 (7-CH_3), 1.80 d (2′-CH_3, coupled to 2′-H with J_{AB} = 5.6 Hz) ppm.

λ_{max} (ε_{mM}) at pH 7 (neutral species): 445 (12.1), 375 (10.0), at pH 13 (anion): 450 (10.9), 355 (10.9) nm.

The cyclodehydroflavin Ia was prepared from compound I by illuminating a ≈ 0.1 mM solution of 130 mg I in 5 l 2.0 M phosphate buffer pH 7.0. The photoproduct was extracted with chloroform, the chloroform extract was dried over magnesium sulfate and then evaporated. The residue obtained was reprecipitated from 0.1 M NaOH/acetic acid to yield 32 mg (25% yield) of the cyclodehydroflavin Ia. For analysis the product was recrystallized from acetic acid.

Found: C 60.5, H 4.6, N 18.8%. $C_{15}H_{14}N_4O_3$ (M_r = 298.3) requires C 60.4, H 4.7, N 18.8%.

NMR(CF_3COOH): δ = 8.07 (6-H), 5.36–4.20 m (1′-H_2, 2′-H), 2.66 (7,8-CH_3), 1.84 d (2′-CH_3, coupled to 2′-H with J_{AB} = 5.0 Hz) ppm.

λ_{max} (ε_{mM}) in 6.0 M HCl (cation): 408 (21.3), 292 (28.1), 254 (13.4) nm: at pH 7 (neutral species): 412 (16.3), 275 (25.4), 257 (17.0) nm: at pH 13 (anion): 397 (12.6), 280 (32.0) nm.

9-Hydroxy-10-(2′-oxo-propyl)flavin (Cyclic Acetal VIa) by Acid Photolysis of 10-(2′-Hydroxypropyl)flavin (I)

240 mg (0.8 mmol) of compound I were dissolved in 1 l glacial acetic acid which was 0.2 M in CF_3COOH and irradiated for 3 days. After this time the solvent was evaporated, the residue taken up in methanol, filtered and recrystallized from 2.0 M acetic acid. Yield 50 mg (0.15 mmol, 20%) VI a.

m/e 314 [M], 298 [M-OH], 256 [9-hydroxy-lumichrome, formed by complete loss of the side chain].

NMR(CF_3COOH): δ = 8.20 (6-H), 5.72 d, 4.63 d (1′-H_2, AB-system with J_{AB} = 14 Hz), 2.77, 2.70 (7,8-CH_3), 2.20 (2′-CH_3) ppm.

λ_{max} (ε_{mM}) in 6.0 M HCl (cation): 406 (22.2), 290 (32.0), 220 (26.0) nm: at pH 7 (neutral species): 450 sh (5.4), 405 (16.0), 275 (30.0) nm.

10-(2′-Acetoxypropyl)flavin (II)

Compound I, 0.2 g (0.67 mmol), was stirred with 20 ml of pyridine and 20 ml of acetic anhydride for 2.5 h. The solvents were evaporated, the residue was reprecipitated from dimethylformamide/diethylether to yield 0.21 g (88% yield) of compound II. The product was pure as judged by thin-layer chromatography (solvent systems C, E).

Found: C 57.3, H 5.1, N 15.5%. $C_{17}H_{18}N_4O_4 \cdot 1\,H_2O$ (M_r = 342.36) requires C 56.7, H 5.6, N 15.6%.

NMR($C_2{}^2H_6NCHO$): δ = 8.02 (6-H, 9-H), 5.68–4.76 m (1′-H_2, 2′-H), 1.72 (–$COCH_3$), 1.41 d (2′-CH_3 coupled to 2′-H with J_{AB} = 6.0 Hz) ppm. The absorption due to 7,8-CH_3 is masked by the solvent peak.

λ_{max} (ε_{mM}) at pH 7 (neutral species): 446 (11.2), 370 (9.2) nm.

N-3-Methyl-10-(2′-hydroxypropyl)flavin (III)

A suspension containing 0.21 g (0.59 mmol) of compound II, 0.4 g of anhydrous potassium carbonate and 1.1 g (7.8 mmol) methyl iodide was stirred for two days [18]. The reaction mixture was then diluted with 15 ml of water, acidified with acetic acid and extracted with chloroform. The chloroform extract was evaporated and the residue was washed with ether to yield 0.16 g (73% yield) of *N*-3-methyl-10-(2′-acetoxypropyl)flavin. The acetyl group was hydrolyzed by treatment with concentrated HCl for 24 h at room temperature. After neutralisation with 10 M NaOH in an ice-salt bath 0.13 g (96% yield m.p. 296–299 °C) of III was obtained, which was pure in solvent systems A and E.

Found: C 60.6, H 5.9, N 17.5%. $C_{16}H_{18}N_4O_3$ (M_r = 314.35) requires C 61.1, H 5.8, N 17.8%.

NMR(CF_3COOH): δ = 8.47 (6-H), 8.23 (9-H), 5.38–4.67 m (1′-H_2 and 2′-H), 3.76 [N(3)-CH_3], 2.83 (8-CH_3), 2.72 (7-CH_3), 1.77 (2′-CH_3, coupled to 2′-H with J_{AB} = 6.0 Hz) ppm.

λ_{max} (ε_{mM}) at pH 7 (neutral species): 449 (10.3), 368 (8.9) nm.

Small amounts of a compound with the spectral characteristics of a cyclic photoproduct were prepared by illuminating 3 ml of a 0.1 mM solution of compound III in 0.5 M phosphate buffer pH 7. The red non-fluorescent product could be extracted into chloroform and was shown to yield one spot in thin-layer chromatography (systems C and D).

10-(2′-Hydroxy-2′-methyl-propyl)flavin (V) and Cyclodehydroflavin (Va)

1-Amino-2-methyl-propan-2-ol was prepared according to [19] by addition of 414 g concentrated aqueous ammonia to 105 g isobutylene oxide. Yield 36.7 g (29%) 1-amino-2-methyl-propan-2-ol, b.p. 69–71 °C/30 torr (4 kPa).

17 g (200 mmol) 1-amino-2-methyl propan-2-ol and 19 g (100 mmol) 1-chloro-4,5-dimethyl-2-nitro-

benzene [17] were kept overnight in 100 ml boiling pyridine. After this time the pyridine was evaporated, the residue taken up in chloroform and extracted with conc. HCl. Unreacted 1-chloro-4,5-dimethyl-2-nitrobenzene stayed in the chloroform layer and was recovered.

The acid solution was diluted with one part of water, the crude product was then extracted into chloroform, which was then applied on a 2×50-cm column containing 100 g silica gel which retained the impurities quantitatively. After recrystallization from ethanol, 5.8 g (24%) of *N*(2′-methyl-2′-hydroxypropyl)-4,5-dimethyl-2-nitro-aniline were obtained. The substance was pure in thin-layer chromatography (diethylether – petrol ether 1:1) and NMR.

NMR(CF_3COOH): δ = 8.50, 7.86 (3,4-H), 3.95 (1′-H_2), 2.64 (4,5-CH_3), 1.76 (2′-CH_3) ppm.

3.9 g (16 mmol) of this nitroaniline were hydrogenated in 400 ml ethanol with 800 mg 5% palladium on charcoal. After 4 h the hydrogen uptake (theoretical amount 1100 ml) was nearly complete. The solution was acidified with 2 N HCl and filtered under nitrogen into a flask containing 4.57 g of alloxanhydrate. After boiling for 15 min the solution was allowed to cool and the precipate filtered off after standing overnight. After recrystallization from water 1.65 g 10-(2′-hydroxy-2′-methyl-propyl)flavin (V) (5.3 mmol, 33%) were obtained. The substance was pure as checked by thin-layer chromatography in system C.

Found: C 61.2, H 5.9, N 17.3%. $C_{16}H_{18}N_4O_3$ (M_r = 314.35) requires C 61.1, H 5.8, N 17.8%.

NMR(CF_3COOH): δ = 8.56 (6-H), 8.30 (9-H), 5.30 (1′-H_2), 2.88 (8-CH_3), 2.76 (7-CH_3), 1.70 (2′-[CH_3]$_2$) ppm.

λ_{max} (ε_{mM}) at pH 7 (neutral species): 447 (9.9), 374 (8.6) nm.

The cyclodehydroflavin (Va) was prepared by illuminating a solution containing 100 mg (0.32 mmol) of V dissolved in 3 l of 10 mM borate buffer pH 9 which was 0.5 M in ammonium sulfate. The photoproduct was extracted into chloroform, the chloroform layer dried with magnesium sulfate, evaporated and the residue washed with ether to yield 47.6 mg (48%) of the cyclodehydroflavin Va which was pure as judged by thin-layer chromatography in systems E and B.

Found: C 61.3, H 5.4, N 17.5%. $C_{16}H_{16}N_4O_3$ (M_r = 312.3) requires C 61.5, H 5.2, N 17.9%.

NMR(CF_3COOH): δ = 8.37 (6-H), 4.95 (1′-H_2), 2.82 (8-CH_3), 2.77 (7-CH_3), 1.72 (2′-[CH_3]$_2$) ppm.

λ_{max} (ε_{mM}) in 6.0 M HCl (cation): 410 (23.0), 297 (30.1), 253 (14.4) nm: at pH (neutral species): 412 (18.2), 280 (26.8), 257 (19.5) nm: at pH 13 (anion): 400 (14.1), 282 (34.7) nm.

10-(2′-Oxo-propyl)flavin (VI) and Cyclodehydroflavin VIa

3.0 g (10 mmol) 10-(2′-hydroxy-propyl)flavin I were dissolved in 100 ml of a mixture of freshly distilled dimethyl sulfoxide and acetic anhydride [20] (1:1, by vol.) and stirred at room temperature. Samples were taken after appropriate time intervals, diluted with water, extracted into chloroform and monitored by thin-layer chromatography with benzene – diisopropyl ether – ethanol (2:2:1, by vol.). The oxidized product (VI) can be seen as a yellow fluorescent spot travelling slightly slower than the starting material.

After two days the reaction was still not complete but the ratio of starting material to product did not change any more. The precipitate was filtered off, washed with alcohol and ether and dried to yield 2.7 g of a mixture of the starting alcohol and the keto product. Recrystallization from methanol yielded a first fraction of 0.8 g (2.86 mmol, 28%) of pure ketone VI. A second fraction which was obtained on prolonged standing (1.0 g, 3.35 mmol) also showed the presence of some starting material. The filtrate from the second fraction was almost pure starting material. Yield of 10-(2′-oxo-propyl)flavin (VI) 46%, based on consumed I.

Found: C 53.5, H 5.3, N 16.6%, $C_{15}H_{14}N_4O_3 \cdot 2\,H_2O$ (M_r = 298.29) requires C 53.9, H 5.4, N 16.8%.

NMR(CF_3COOH): δ = 8.52 (6-H), 8.00 (9-H), 5.46 (1′-H_2), 2.84 (7-CH_3), 2.74 (8-CH_3), 2.48 (3′-CH_3) ppm.

For oxime formation 100 mg (0.34 mmol) VI, 10 ml 50% acetic acid and 200 mg sodium acetate were warmed on the water bath for 5 min. A precipitate formed which was collected and recrystallized from methanol to yield 57 mg pure oxime of VI as checked by thin-layer chromatography in system C.

Found: C 57.4, H 4.8, N 22.0%. $C_{15}H_{15}N_5O_3$ (M_r = 313.31) requires C 57.5, H 4.8, N 22.3%.

Only small amounts of a cyclodehydroflavin (VIa) were obtained by illuminating 3 ml of a 0.1 mM solution of VI in 2.0 M phosphate buffer at pH 7. Extraction of the solution with chloroform removed lumichrome and starting material. The cyclodehydroflavin VIa was then extracted into butanol and was chromatographically identical (solvent system A) with authentic material obtained from the acid photolysis of I.

Cyclodehydro-riboflavin

A 0.1 mM solution of 320 mg riboflavin (VII) in 8 l of 2.0 M phosphate buffer was illuminated for

8 h. The reacted solution was first extracted with two portions of 1 l chloroform to remove lumichrome and then four times with 500 ml of butanol. The butanol was evaporated, the residue dissolved in a small amount of methanol, insoluble material removed and the material subjected to preparative thin-layer chromatography with solvent system A. After separation of the main red band the product was eluted with methanol and recrystallized from 2 M acetic acid to yield 11 mg (3.5%) of photoproduct VIIa which was pure in thin-layer chromatography in solvent systems A and E.

Found: C 53.1, H 4.9, N 14.6. $C_{17}H_{18}O_6N_4 \cdot {}^1/_2\, H_2O$ (M_r = 374.37) requires C 53.3, H 5.0, N 14.6%.

NMR(CF_3COO^2H): The ^{1}H-NMR was done by computer-averaged Fourier transform analysis (128 runs) with a 20 mM solution of VIIa in CF_3COO^2H.

δ = 7.90 (6-H), 5.8–3.9 m, (7 protons: 1′-CH_2, 2′-CH, 3′-CH, 4′-CH, 5′-CH_2), 2.30, 2.28 (7,8-CH_3) ppm.

λ_{max} (ε_{mM}) in MeOH (neutral species): 450 sh (4.15), 400 (9.8), 278 (19.8) nm; in 6.0 M HCl (cation): 408 (16.1), 290 (20.8) nm; at pH 7 (neutral species): 440 sh (4.15), 410 (12.0), 275 (19.5) nm.

m/e 257 (9-hydroxy-lumichrome, complete loss of side chain).

The cyclodehydroflavin VIIa was silylated [21] with a mixture of *N,O*-bis(trimethylsilyl)acetamide–trimethylchlorosilane–pyridine (10:1:4, by vol.) for 18 h at room temperature. The mass spectrum of the reaction mixture showed *m/e* 734 (M + 5 trimethylsilyl groups).

Degradation of Cyclodehydro-riboflavin (VIIa) by Periodate

2 mg of VIIa were treated overnight with 2 mg periodic acid in 1.0 ml 1.0 M sulfuric acid at room temperature. After neutralisation with sodium acetate chromatography in system B showed only one new spot travelling faster than the starting material VIIa.

The residue from butanol extraction was dissolved in 1 ml 50% methanol and treated with 5 mg $NaBH_4$. After 1 h the solution was acidified with acetic acid and evaporated. After addition of water the product was extracted into chloroform. Thin-layer chromatography in system B showed only one new compound, travelling slower than the starting material VIIa.

The product of the reduction was taken up in 0.5 ml pyridine and 0.5 ml acetic anhydride was added. After stirring overnight the solvent was evaporated, and the material applied on a 5-cm analytical thin-layer chromatography plate to separate the acetylated product from some fluorescent impurities with system C. After elution of the acetate from the plate and purification by distribution between water and chloroform, the product from the chloroform layer was subjected to mass spectrometry. The mass spectrum (200 °C, 70 eV) showed the following pattern, corresponding to (3′-acetoxy-2′-hydroxypropyl)-cyclodehydroflavin:

m/e 356 [M], 313 [M–$COCH_3$], 297 [M–$OCOCH_3$], 283 [M–CH_2OCOCH_3], 240 [M–(CH_2OCOCH_3 + HNCO)].

Isoriboflavin (VIII) and Cyclodehydroflavin VIIIa

A solution ($\approx$ 0.1 mM) of 220 mg (0.58 mmol) of VIII in 6 l of 10 mM phosphate buffer pH 7.0 containing 2.0 M ammonium sulfate, was illuminated for 30 min. Isolumichrome was first removed by exhaustive extraction of the aqueous solution with chloroform, then cyclodehydroflavin VIIIa was extracted with butanol and the butanol evaporated. The residue was dissolved in a minimum amount of trifluoroacetic acid, filtered to remove insoluble material and the filtrate was subjected to preparative thin-layer chromatography, using solvent system A. The main band containing the red product was eluted with methanol, the methanol evaporated and the residue reprecipitated from 0.1 M NaOH–acetic acid. The product (12.4 mg, 5.7% yield), was pure as judged by thin-layer chromatography in solvent systems A and E.

NMR(CF_3COOH): δ = 8.23 (6-H), 5.90–4.22 m (7 protons, side chain), 3.03 (6-CH_3), 2.81 (7-CH_3) ppm.

λ_{max} (ε_{mM}) in 6.0 M HCl; 395 (15.6), 300 (25.0), 247 (10.4) nm: at pH 7 (neutral species): 406 (12.4), 298 (17.4), 276 (18.9), 254 (13.9) nm: at pH 13 (anion): 460 (3.5), 385 (9.9), 285 (24.2), 254 (11.7) nm.

10-(2′-Hydroxy-2′-methylpropyl)isoalloxazine (IX) and Cyclic Photoproduct

Following the procedure described above for compound I, 1-amino-2-methyl-2-propanol was condensed with 2-chloronitrobenzene to obtain a 34% yield of the N-substituted *o*-nitroaniline (m.p. 81–82 °C). A solution of the aniline, 2.3 g (11 mmol) in 92 ml of dimethylformamide was added slowly with stirring to 240 ml of a 50% saturated sodium bicarbonate solution containing 10.6 g (51 mmol) of sodium dithionite. Stirring was continued until the solution was colorless (5–10 min) and the reduced product

was then extracted with chloroform. The chloroform was evaporated and the residue was added to 360 ml of acetic acid containing 2.4 g (15 mmol) of alloxan monohydrate and 6.0 g of boric acid. After stirring for 24 h the acetic acid solution was concentrated and the product was precipitated by adding an equal volume of ether. After reprecipitation from conc. HCl–10.0 M NaOH in an ice-salt bath, 1.72 g (55% yield) of IX was obtained. The product (1.72 g, 55% yield) was pure as judged by thin-layer chromatography (solvent systems A, E).

Found: C 58.4, H 5.0, N 19.3%. $C_{14}H_{14}N_4O_3$ (M_r = 286.29) requires C 58.7, H 4.9, N 19.6%.

NMR(CF_3COOH): δ = 8.64–9.09 m (6,7,8,9-H), 5.38 s (1′-H_2), 1.73 (2′-C[CH_3]$_2$) ppm.

λ_{max} (ε_{mM}) at pH 7 (neutral species): 437 (11.0), 353 (8.8) nm.

The cyclodehydro-derivative (IXa) was prepared by illuminating a solution ($\approx$0.1 mM) of 140 g (0.49 mmol) of IX in 5 l of 2.0 M phosphate buffer pH 7.0. When the reaction was complete (as monitored spectrophotometrically) the solution was extracted with chloroform, the chloroform extract dried with magnesium sulfate and the solvent evaporated. The solid material was reprecipitated from 0.1 M NaOH–acetic acid and the precipitate subjected to preparative thin-layer chromatography (solvent system A). The main band containing the photoproduct was eluted with methanol, the methanol was evaporated and the product reprecipitated from 0.1 M NaOH-acetic acid to yield 38.6 mg (28% yield) of pure IXa as judged by thin-layer chromatography (solvent systems A, E).

NMR(CF_3COOH): δ = 8.20–8.60 m (6,7,8-H), 5.00 (1′-H_2), 1.73 (2′-C[CH_3]$_2$) ppm.

λ_{max} (ε_{mM}) in 6.0 M HCl (cation): 388 (18.4), 292 (28.7), 245 (13.2) nm: pH 7 (neutral species): 390 (13.6), 291 (21.8), 268 (25.6) nm: pH 13 (anion): 452 (6.0), 370 (11.7), 282 (29.0) nm.

When the photoproduct IXa was obtained by photoreaction in ^2H_2O instead of H_2O, no decrease of the 6,8-CH peaks could be detected.

10-(2′-Hydroxypropyl)isoalloxazine (X) and Cyclodehydroproducts Xa and Xb

A solution of 15 g (200 mmol) of 1-amino-propan-2-ol and 63 g (400 mmol) of 2-chloronitrobenzene in 40 ml of dry pyridine was refluxed for 24 h. After evaporation of the solvent the residual oil was dissolved in 160 ml of 6.0 M HCl and extracted with ether. The ether was evaporated, the residual oil taken up in 70 ml of toluene and extracted with 6.0 M HCl. The acid solution was concentrated and neutralized with 10.0 M NaOH in an ice-salt bath to yield 25.5 g (65% yield) of the substituted nitro-aniline, m.p. 65–69 °C.

The nitro-aniline, 6.1 g (31.1 mmol) was dissolved in a solution containing 204 ml of acetic acid and 8 ml of water. Rasped zinc, 33 g (510 mmol) was added and the mixture was refluxed until the solution was pale yellow (approx. 10 min). The hot solution was filtered into a flask containing 500 ml of acetic acid, 6.1 g (38.1 mmol) of alloxan monohydrate and 15.3 g boric acid and stirred overnight. After addition of an equal amount of ether the precipitate was isolated. For purification the product was reprecipitated from 6.0 M HCl with 10.0 M NaOH in an ice-salt bath and from 0.1 M NaOH/HCl. Finally crystallization from acetic acid yielded 1.83 g (22% yield, m.p. 299–302 °C) of X. The product was pure as judged by thin-layer chromatography in systems A and E.

NMR(CF_3COOH): δ = 8.16–8.94 m (6,7,8,9-H), 5.44–4.77 m (1′-H_2, 2′-H), 1.80 d (3′-CH_3, coupled to 2′-H with J_{AB} = 5.5 Hz) ppm.

λ_{max} (ε_{mM}) at pH 7 (neutral species): 437 (9.9), 348 (9.4) nm.

Compound X yielded two red non-fluorescent photoproducts Xa and Xb as detected by thin-layer chromatography (solvent system A). Small amounts were prepared by illuminating 3 ml of a 0.1 mM solution of X in 2.0 M phosphate pH 7.0. Product Xa was extractable with chloroform and product b with butanol. Product Xb (but not Xa) could also be prepared by illuminating a 0.1 mM solution of X in 0.1 N HCl. The reacted solution was evaporated, the residue was dissolved in water and product Xb was extracted with butanol. The major product Xa is formed after short periods of reaction whereas the minor product, Xb, is detected after longer periods of reaction.

Isoalloxazine-10-(2′-ethane Sulfonic Acid) (XI) and Photoproduct XIa

Ethanolamine was condensed with 2-chloronitrobenzene according to the procedure described in the synthesis of X with the modification that the final precipitate was crystallized from 50% ethanol. A 30% yield of the substituted aniline was obtained (m.p. 73–74 °C).

Following the general procedure described by Föry *et al.* [21], *N*-(2-bromoethyl)-2-nitroaniline was obtained in 70% yield (m.p. 78–81 °C) by treatment of *N*-(2′-hydroxyethyl)-2-nitroaniline with triphenylphosphite and bromine.

Found: C 39.1, H 3.5, N 11.2, Br 32.5%. $C_8H_9N_2O_2Br$ (M_r = 245.0) requires C 39.2, H 3.7, N 11.4, Br 32.6%.

The bromoderivative was converted to the thiouronium salt by refluxing 29.6 g (121 mmol) with 10.1 g (133 mmol) of thiourea in 100 ml of 95% ethanol for 6 h. The crude product, which precipitated on cooling (33 g, 85% yield), was hydrolyzed by refluxing under argon for 5 h in 300 ml of 5.0 M NaOH. The hydrolyzed product was precipitated in an ice-salt bath by neutralizing with 6 N HCl. Following air oxidation, 12.9 g (63% yield, m.p. 109–119 °C, decomp.) of the bis[2′-(*N*-2-nitrophenylamino)-ethane]-disulfide was obtained. A solution containing 5.5 g (13.9 mmol) of the disulfide in 270 ml of acetic acid and 7 ml of water was refluxed with 29.3 g (450 mmol) of rasped zinc until the solution was colorless (approx. 10 min). The hot solution was filtered into a flask containing 817 ml of acetic acid, 108 g (680 mmol) of alloxan monohydrate and 272 g of boric acid. After stirring overnight the reaction mixture was filtered and the precipitate washed with 6 l of water. The precipitate was then suspended in 480 ml of 50% saturated sodium bicarbonate, warmed to 60 °C, and the hot solution filtered. The filtrate was discarded and the solid material reprecipitated from concentrated HCl/water 1:1. The non-fluorescent bis[2′-(10-isoalloxazinyl)-ethyl]-disulfide derived from 10-(2′-mercaptoethyl)isoalloxazine was obtained in 43% yield, as based on bis[2′-(*N*-2-nitro-phenylamino)-ethane]-disulfide; m.p. 294–296 °C (decomp.).

Found: C 51.9, H 3.4, N 20.5, S 11.2%; $C_{24}H_{18}O_4S_2$ (M_r = 834.85) requires C 52.5, H 3.7, N 20.4, S 11.7%.

NMR(CF_3COOH): δ = 8.11–8.79 m (6,7,8,9-H), 5.57 broad (1′-H_4), 3.65 broad (2′-H_4) ppm.

The visible absorption spectrum was characteristic of an isoalloxazine with peaks at 435 and 345 nm in aqueous solution.

The bis-isoalloxazinyldisulfide, 0.4 g (0.73 mmol), was dissolved in 80 ml of 0.1 N NaOH and 4 ml of 30% hydrogen peroxide was added. The pH was readjusted by adding sufficient 10 N NaOH to redissolve the isoalloxazine. After 1.5 h the solution was neutralized to pH 5 with acetic acid and the sulfonate XI, 0.19 g (40% yield), was precipitated by adding 200 ml of isopropanol. The fluorescent sulfonic acid derivative was crystallized from water (m.p. 343–346 °C) and was pure as judged by thin-layer chromatography (solvent systems E, F).

The 9-hydroxy derivative of the sulfonate XI (Table 1) was prepared by illuminating a solution containing 90 mg (0.28 mmol) of XI in 2 l of 2.0 M phosphate buffer pH 7.0. Impurities were then extracted from the reacted solution with butanol and the product was then extracted with 100 g of phenol. The phenol extract was diluted with 500 ml of water and the phenol removed by extraction with ether. The aqueous phase was concentrated and the crude product, 18.3 mg (19% yield), precipitated after acidification to pH 1 with HCl. This material was dissolved in 0.1 M borate pH 9.0 and applied to a DEAE-cellulose column (1.7 × 12 cm) equilibrated with the same buffer. The column was washed with the borate buffer until residual starting material XI was completely removed. The product was then eluted in 0.1 M phosphate pH 7.0 and was pure as judged by thin-layer chromatography (solvent systems E, F).

λ_{max} (ε_{mM}) in 6.0 M HCl (cation): 450 sh, 390, 295 nm: in 0.1 M HCl (neutral species): 455 sh, 390, 293, 268 nm: at pH 9 (anion): 550, 428, 413 sh, 328 nm.

The compound has a pK of 7 as was shown by spectral titration. The spectral appearance of the cationic, neutral and anionic forms corresponds with 9-hydroxylation [8]. 6-Hydroxylation was further excluded by the fact, that a chelate is not formed with copper(II) perchlorate [8].

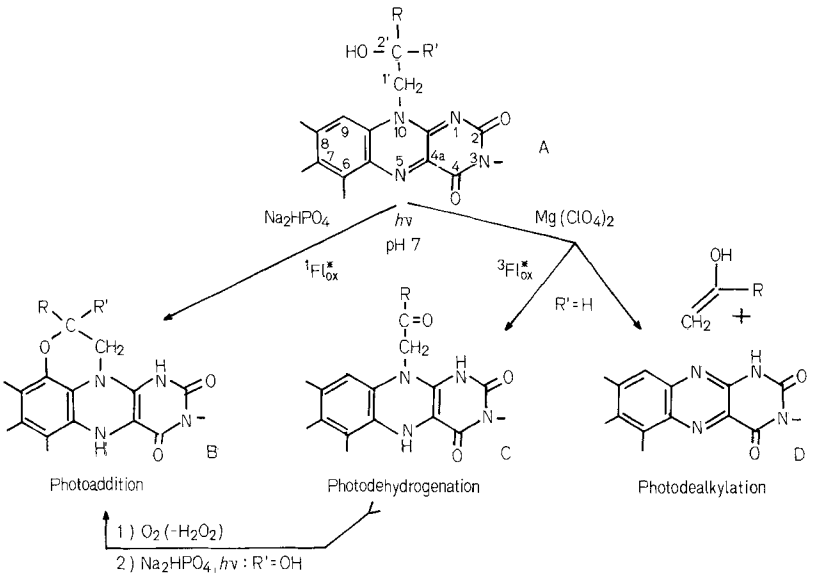
10-(2′-Hydroxypropyl)-[7,9-^2H]isoalloxazin

3.0 g (15.3 mmol) 1-[*N*(2′-hydroxypropyl)]-amino-2-nitrobenzene and 30.00 ml 60% $^2H_2SO_4$ in ^2H_2O (by dilution of 98% $^2H_2SO_4$ of Merck) were heated to 100 °C for 16 h. The dark solution was poured on 100 g of ice and the water phase extracted with chloroform. The organic layer was concentrated and filtered over a column of 60 g silica gel (Merck). Fractions were taken and the orange product which proved to be identical with the starting material was collected to yield 1.53 g (7.8 mmol, 51%) 1-[*N*(2′-hydroxypropyl)]-amino-2-nitro-[4,6-^2H]benzene.

NMR (60% $^2H_2SO_4$): δ = 8.15 (3-H, 5-H), 4.8–3.6 m (1′-H_2, 2′-H), 1.55 d (3′-H_3, coupled to 2′-H with J_{AM} = 6 Hz) ppm.

The aniline was dissolved in 50 ml dry ethanol plus 200 mg palladium (5%) on charcoal and hydrogenated at room temperature and 1 atm (101 kPa). The reaction was stopped after uptake of 620 ml hydrogen and the solution filtered under nitrogen directly into a vessel containing 1 g alloxan monohydrate and 3.5 ml 6.0 M HCl. After boiling for 15 min the solution was allowed to stand overnight. The precipitate was filtered off, washed with water, alcohol and ether to yield 0.92 g of 10-(2′-hydroxypropyl-[7,9-^2H]isoalloxazin which was identical in thin-layer chromatography with non-deuteriated material.

NMR(CF_3COOH): identical with non-deuteriated flavin (X) except for the aromatic region, where δ = 8.50 and 8.65 ppm for the two protons position 6 and 8.



Scheme 1. *Intramolecular photoreaction modes of N(10)-hydroxyalkyl flavins*

Isotope Effect in the Photoaddition of 10-(2'-Hydroxypropyl)-[7,9-^{2}H]isoalloxazin

200 mg samples of the two compounds under investigation, *i.e.* the isoalloxazin X and its deuterated analog, were recrystallized from concentrated acetic acid (20 ml) and the preparations so obtained used for photolysis.

Photolysis was done on 0.01 M, 0.2 M, 0.5 M and 1.0 M phosphate at pH 7 and in 2.0 M ammonium sulfate which was 0.01 M in acetate buffer pH 5. The isoalloxazin concentration was 0.05 mM in all cases. Initial rates were taken from a plot of absorbance at 435 and 390 nm *versus* time.

The isotope effects k_H/k_{2H} in all cases were found to be smaller than the error of our photolytic method ($\pm 10\%$).

RESULTS AND DISCUSSION

Definitions

Flavin photochemistry must be strictly separated into three categories: photoaddition, photoreduction, and photodealkylation (Scheme 1). Photoaddition [8] follows Eqn (1):

$$Fl_{ox} + ROH \xrightarrow{h\nu} RO\text{-}Fl_{red}H \qquad (1)$$

and yields hydroxy or alkoxy dihydroflavins as first intermediates. Flavin reduction is observed spectrally, although the overall reaction involves no net reduction. Dehydrogenation on the other hand, follows Eqn (2) and, therefore, involves a net reduction, yielding free dihydroflavins or alkyl derivatives thereof:

$$Fl_{ox} + RH \xrightarrow{h\nu} R\text{-}Fl_{red}H \xrightarrow{H_2O} H_2Fl_{red} + ROH. \qquad (2)$$

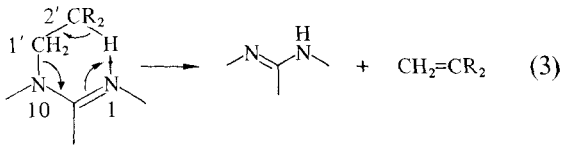
The structure, stability and essentiality of the intermediates R-Fl_{red}H depend very much on the nature of the residue R and have been explicitly dealt with elsewhere [23, 24]. Since H_2Fl_{red} undergoes fast autoxidation [25], photoreductions are in general reversible with respect to the flavin chromophore, while photoadditions (and photodealkylation, see below) are not. Furthermore, photoadditions are necessarily nucleophilic, whereas the character of photoreductions is still much disputed, though the radical course postulated in the earlier literature is now widely abandoned in favor of a "carbanion mechanism" [23], which also seems to apply to reductions of flavoproteins by CH substrates [26]. Third, it appears that photoadditions occur through the excited singlet $^1Fl^*_{ox}$, whereas photoreductions proceed *via* the triplet $^3Fl^*_{ox}$ (see below).

The intramolecular course of photoreduction, involving the N(10)-side chain of 10-hydroxyalkylflavins [27] has previously been designated as photolysis, although mechanistically there may be no difference in the primary steps of photoreduction and photolysis [27]. The ease of intramolecular attack clearly depends on the possibility of "bending back" of the side chain >CHOH group towards the nucleus,

and it is, therefore, certainly true that the hydroxyl next to N(10), *i.e.* C(2′)-OH, does not react faster than C(n')-OH for $n' > 2$ [28].

However, flavin photolysis need not be confined to intramolecular modes of reaction, although these reactions attracted most attention owing to their high velocity and relevance to vitamin B_2 photodegradation. But quite generally, any aliphatic CH center attached to a flavin nucleus is susceptible to intermolecular photodehydrogenation, *i.e.* by a second flavin in the excited triplet state. Schöllnhammer and Haas are presently studying the photolysis of lumiflavin and found C(7)-CH_3 as the preferred center of attack (unpublished results).

The third and strictly intramolecular type of flavin photochemistry, N(10)-photodealkylation, has been studied systematically only very recently [29]. It is much less general as it does not involve a reduction at all, not even a spectrally apparent one under anaerobic conditions, and it requires synchronous breakage (fragmentation) of a N(10)-C(1′) and a C(2′)-H bond [Eqn (3)] in *cis-peri*planar conformation



(3)

with direct proton transfer yielding lumichrome and an olefin.

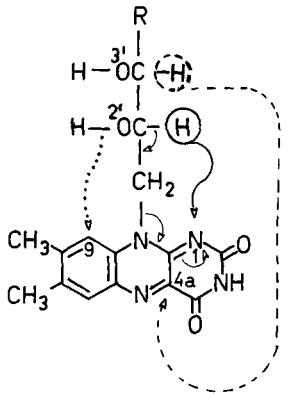
Intermolecular addition and dealkylation involve *peri*-attack at the flavin nucleus, thus favoring reactions at C(2′) of the N(10)-side chain and six membered cyclic transition states, while, as demonstrated in Scheme 2 for the case of riboflavin, 3′-CHOH and 4′-CHOH appears to be preferred in dehydrogenation.

Hence dehydrogenation presumably does not involve *peri*-attack but interaction of the bent-back side chain with the azomethine subgroup C(4a)=N(5) of the flavin nucleus. Obviously N(1) is not an "intake" position for redox equivalents.

In the present paper we are dealing with intramolecular photoaddition, which is the dominating photoreaction of 10-(2′-hydroxyalkyl)flavins, as *e.g.* riboflavin, at > 0.1 M concentrations of divalent anions. The products of this reaction are termed "cyclodehydroflavins".

Reaction Conditions and Products

Since, in general, all three types of flavin photochemistry (Scheme 1) may compete, dehydrogenation being predominant, it was necessary to search for



Scheme 2. *Addition (·····) vs dealkylation (——) and dehydrogenation (– – – –) of flavin side chains.* Addition occurs preferably by the 2′-OH group and involves nucleophilic attack at C(9), dealkylation [29] involves electrophilic attack of C(2′)-H at N(1), while dehydrogenation occurs preferably at C(3′,4′)-H presumably as nucleophilic attack at the C(4a) = N(5) azomethine subgroup

conditions which favor intramolecular photoaddition. It was found that the intramolecular photoaddition exhibits an absolute requirement for divalent anions and occurs at pH values > 6. To facilitate identification of the new "410-nm chromophore" formed during the photoaddition reaction, preliminary studies [9] were performed on flavin derivatives with monofunctional N(10)-substituents (*e.g.* I, Table 1).

The reaction with riboflavin is complicated due to the presence of various reaction sites in the polyhydroxyalkyl side chain, resulting in a more difficult separation of the photoaddition product from the products of the normal photodecomposition reaction. However, an absorption maximum at 400 nm is observed following photoreaction of riboflavin in 0.5 M phosphate, pH 7.0, and corresponds to a ratio of "410-nm product"/lumichrome = 3/1. Lumichrome is the only product formed when the reaction is performed in the absence of phosphate, ionic strength and pH being kept constant (Fig. 1). The purified 410-nm species was examined in the ^{1}H-NMR and showed the absence of C(9)H, while all seven CH protons of the ribityl side chain were retained according to integration of the badly resolved peaks in the 5-ppm region. Direct mass spectroscopy yielded a first main peak corresponding to hydroxylumichrome, while after silylation a molecular peak for penta(trimethylsilyl)riboflavin minus 2 H was obtained. (The heterocyclic nucleus takes up two silyl groups, as was checked by mass spectrometry of riboflavin under the same conditions giving *m/e* 809 which corresponds to a hexa[trimethylsilyl]riboflavin.) Periodate degradation, reduction and acetylation, on the other hand, led to a molecular peak corresponding to

Table 1. *List of cyclodehydroflavins B (Scheme 1) obtained from starting flavin A*
For R and R′ see Scheme 1 B

Starting flavin A						Cyclodehydroflavin B			
Number	Positions					Number	R	R′	
	3	6	7	8	10				
I	H	H	CH_3	CH_3	$CH_2CH(OH)CH_3$	Ia	H	CH_3	
II	H	H	CH_3	CH_3	$CH_2CH(OCOCH_3)CH_3$	no cyclic photoproduct formed			
III	CH_3	H	CH_3	CH_3	$CH_2CH(OH)CH_3$	IIIa	H	CH_3	
IV	H	H	CH_3	CH_3	CH_2CH_2OH	only traces of cyclic photoproduct: not isolated			
V	H	H	CH_3	CH_3	$CH_2CH(OH)(CH_3)_2$	Va	CH_3	CH_3	
VI	H	H	CH_3	CH_3	CH_2COCH_3 (C in scheme 1)	VIa	OH	CH_3	also formed on photolysis of I in acid
VII	riboflavin					VIIa	H	$(CHOH)_2CH_2OH$	
VIII	isoriboflavin					VIIIa	H	$(CHOH)_2CH_2OH$	
IX	H	H	H	H	$CH_2CH(OH)(CH_3)_2$	IXa	CH_3	CH_3	
X	H	H	H	H	$CH_2CH(OH)CH_3$	Xa	H	CH_3	
						Xb	OH	CH_3	
XI	H	H	H	H	$(CH_2)_2SO_3H$	XIa	OH-group in position 9		

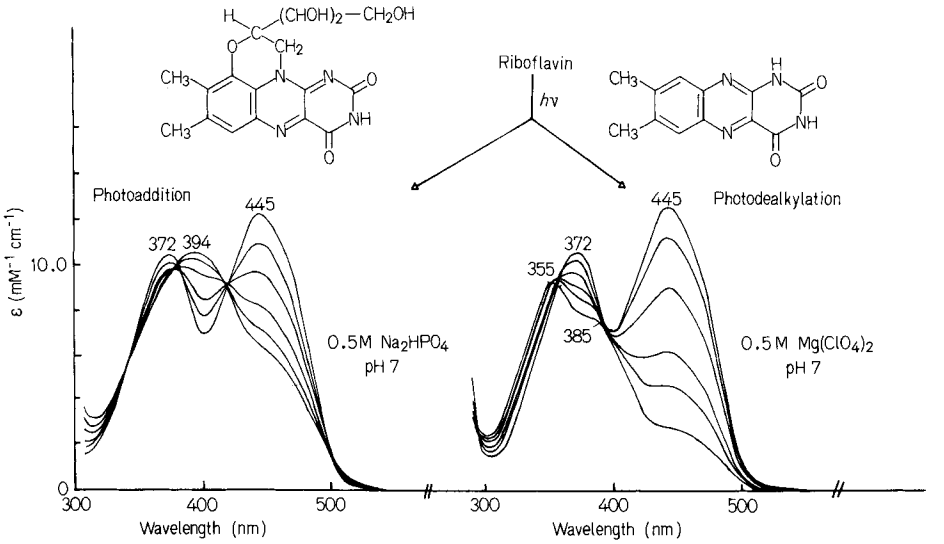


Fig. 1. *Photoaddition* vs *photodealkylation of 0.05 mM riboflavin in the presence of different anions at constant pH and ionic strength.* Spectra were taken after 30, 60, 90, 120 and 150 s illumination

monoacetyl-dihydroxypropylflavin minus 2 H. Elementary analysis was consistent with the formula of "dehydroriboflavin" in correspondence with mass spectrometry after silylation. The chromophore of the new "dehydroflavin" is found to be identical with that of the previously described [8] "cyclic acetal" of 9-hydroxy-lumiflavin-10α-carboxaldehyde (Fig. 2). Spectra are different from those of 9-hydroxyflavins (Fig. 3) only in the anionic state, as expected. The dehydroflavins are non-fluorescent and photochemically rather inert.

The combined results yield strict evidence for the cyclic alkoxyflavin structure shown in Fig. 1, for which we propose the short name "2′,9-cyclo-dehydroriboflavin". The cyclic structure of the riboflavin photoaddition product is completely analogous to the cyclic structures determined for the photoaddition products of flavins bearing monofunctional N(10) substituents, as summarized in Table 1. It should be noted that the extent of photoaddition observed with 10-(β-hydroxyalkyl)flavins decreases as the number of CH groups available for photodehydrogenation

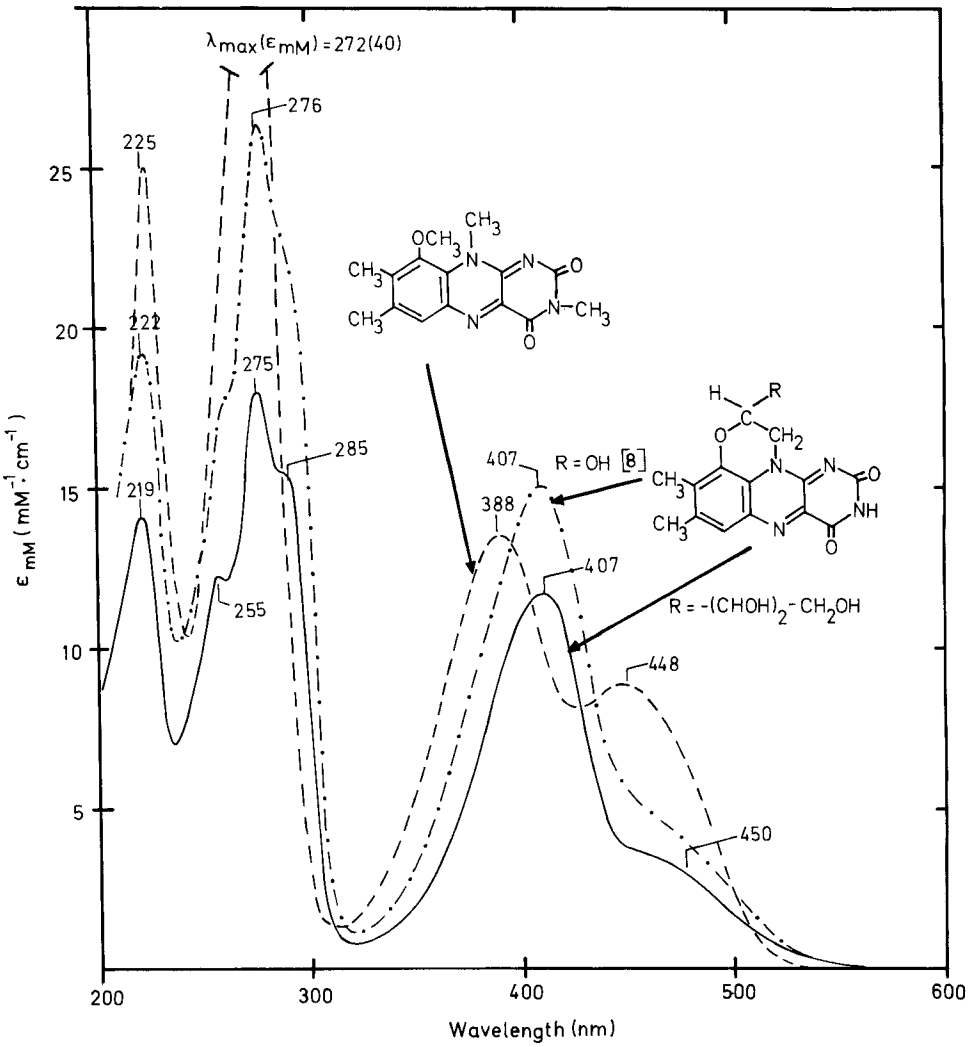


Fig. 2. *Spectral comparison of 9-alkoxylated flavins at pH 7 (neutral species).* (——) Cyclodehydroriboflavin (VIIa); (-·-·-) 9-hydroxy-lumiflavin-10α-carboxaldehyde (cyclic acetal) [8]; (– – –) 9-methoxy-lumiflavin [8]. This compound shows a hypsochromic shift because of 9,10-non-planarity induced by *peri*-overcrowding

increases, *i.e.* tertiary > secondary > primary alcohols. In one case (X) two cyclic products could be isolated from the same reaction, the one (Xa) by the direct photoaddition pathway A → B (Scheme 1), the other (Xb) by an initial dehydrogenation followed by subsequent addition A → C → B. The reaction C → B apparently proceeds *via* the hydrate of the ketone C. The ketone derivatives are quite stable towards dehydrogenation and dealkylation but undergo facile photoaddition as observed directly with C (≡ VI, Table 1).

In contrast to photohydration of flavin cations [8], the anion-catalyzed addition reactions observed under neutral conditions require the presence of a nucleophilic function in the N(10)-side chain. The nucleophilic group may be hydroxyl, as described above, or even sulfonate since a facile photoaddition reaction is observed with isoalloxazine-10-(2′-ethane sulfonic acid) (XI), yielding the 9-hydroxy derivative XIa as the only product. Clearly, the 9-hydroxy group arises from addition of sulfonate at C(9), followed by hydrolysis of the intermediate sultone. The structure and electronic spectra of the product are shown in Fig. 3. Due to the absence of the 7,8-CH_3 groups (as compared to riboflavin), the main absorption of the neutral species is now found at 390 nm. This is clearly attributable to the second π,π^*-transition, while the first one is seen as a shoulder at 455 nm, analogous with the 460-nm shoulder present in cyclodehydroriboflavin (*cf.* Fig. 2). In the anion, this band appears extremely broadened and shifted towards 550 nm.

The catalysis of photoaddition as observed with HPO_4^{2-}, SO_4^{2-} and, to much lesser extent with

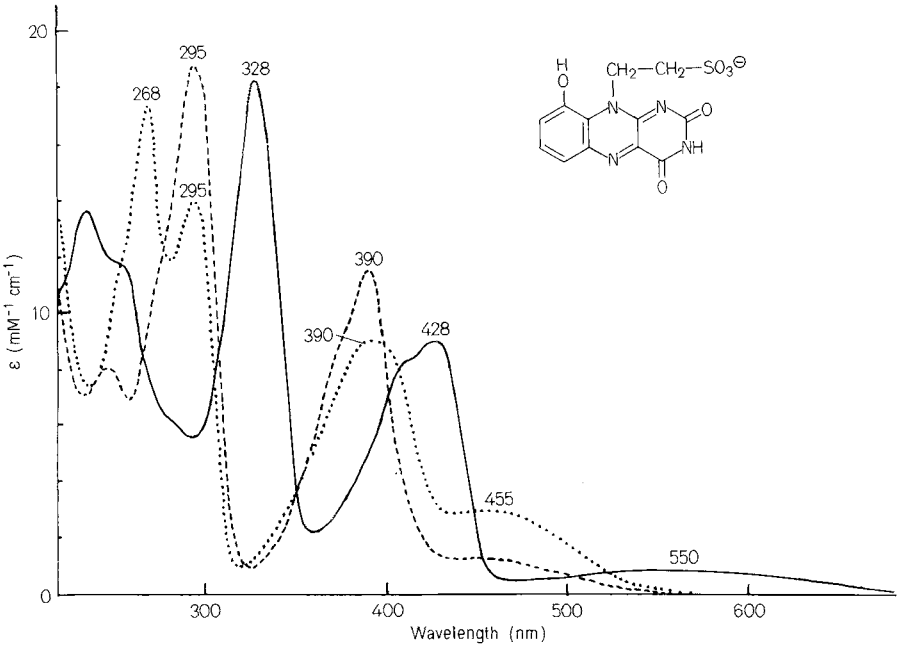


Fig. 3. *Optical spectra and structure of the photoproduct (Xa) of 2'-(10-isoalloxazinyl)-ethanesulfonate (X).* (----) Cation in 6.0 M hydrochloric acid; (·····) neutral species in 0.1 M hydrochloric acid; (——) anion in 0.1 M borate buffer pH 9

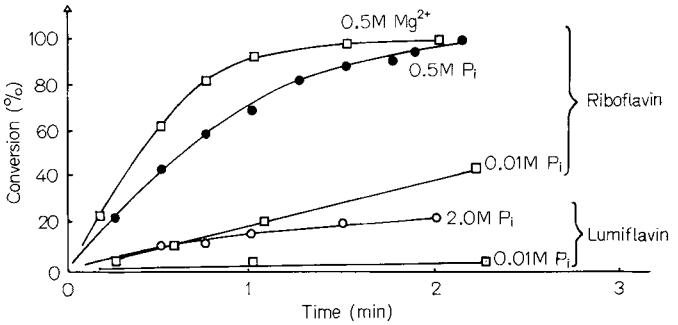


Fig. 4. *Salt effects in the photoreactions of flavins.* 0.05 mM solutions of riboflavin (VII) and lumiflavin were irradiated at pH 7 in the presence of sodium phosphate and magnesium perchlorate (0.01 M in phosphate to maintain pH 7). No precautions were taken to exclude oxygen. The course of the reaction was followed spectrophotometrically at 445 nm taking the end spectrum as 100% conversion to photoproducts. The spectral appearance of the photoreactions of riboflavin in phosphate and perchlorate are shown in Fig. 1. Note that only the closed circles refer to photoaddition, all other curves to photolysis

succinate or phthalate, consists of two effects which can be separated, as shown in Fig. 4.

Firstly, a general salt effect in flavoquinone photochemistry can be demonstrated with lumiflavin in the presence of sodium phosphate as well as with riboflavin in the presence of magnesium perchlorate.

Secondly, a specific switch in favor of photoaddition, however, is only observed with divalent anions A^{2-}, independent of ionic strength, and only in the case of flavins bearing suitable nucleophiles in the N(10)-side chain.

Hence, A^{2-} cannot act as a simple triplet quencher. Experiments with I^- and Mn^{2+} show half-quenching concentrations $[I^-] = 1$ mM and $[Mn^{2+}] = 100$ mM for the addition reaction as compared to $[I^-] = > 10$ mM for 30% and $[Mn^{2+}] = 100$ mM for 10% quenching of the Fl_{ox} fluorescence. Typical flavin triplet reactions, however, afford half quenching concentrations of the order of 1 μM [3]. It could be inferred that, under the present conditions, A^{2-} competes with I^- efficiently for complexation with Fl^*_{ox}, while at the same time it masks Mn^{2+} by complexation. But the photohydration of alloxazine has been shown to involve the excited singlet [5], and no reason can be seen why the photophysics of (iso)-alloxazine additions should differ in intermolecular as compared to intramolecular cases. Furthermore, we found that the photohydration of alloxazine [5], is specifically catalyzed by SO_4^{2-} at pH 3 by a factor of 2 ± 0.2 at constant ionic strength.

The effect of the sulfate concentration on the initial rate of cyclodehydroflavin formation is shown in Fig. 5. Saturation or hyperbolic kinetics are obtained, which yield a linear double-reciprocal plot. These results are consistent with photoaddition *via* formation of a Fl-A^{2-} complex.

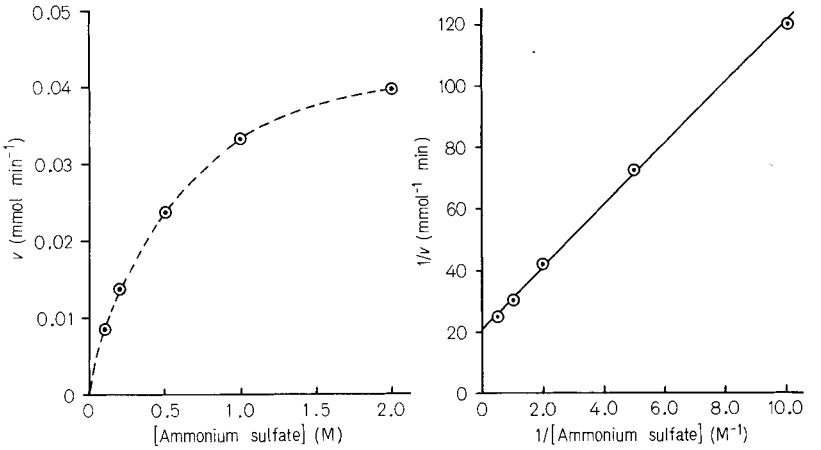


Fig. 5. *Effect of ammonium sulfate concentration on the initial rate of cyclodehydroflavin formation (Ia) from 0.05 mM 10-(2'-hydroxypropyl)-flavin (I) in 10 mM borate buffer pH 9.* The initial rate of product formation was estimated by the increase in absorption at 410 nm using the known absorption coefficient (ε = 16.3 mM^{-1} cm^{-1}) of the product at this wavelength

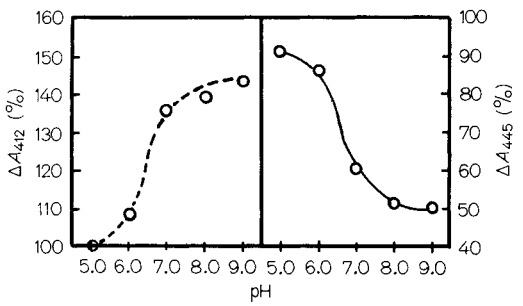


Fig. 6. "*Action pK*" *in the photoreaction of 0.05 mM 10-(2'-hydroxypropyl)flavin (I) in 2.0 M ammonium sulfate.* For maintaining the desired pH the ammonium sulfate solution was ≈ 0.1 M in acetate buffer for pH 5, phosphate buffer for pH 6–8 and free NH_3 for pH 9. Curves show percentage decrease of absorbance at 445 nm (——), representing the disappearance of starting material I and percentage increase of absorbance at 412 nm (– – – –), representing formation of product Ia after 20 s of illumination (*cf.* Methods). Note that at pH < 5 photodealkylation (*cf.* Scheme 1) is predominant resulting in (slow) decrease at 445 nm and no change at 412 nm

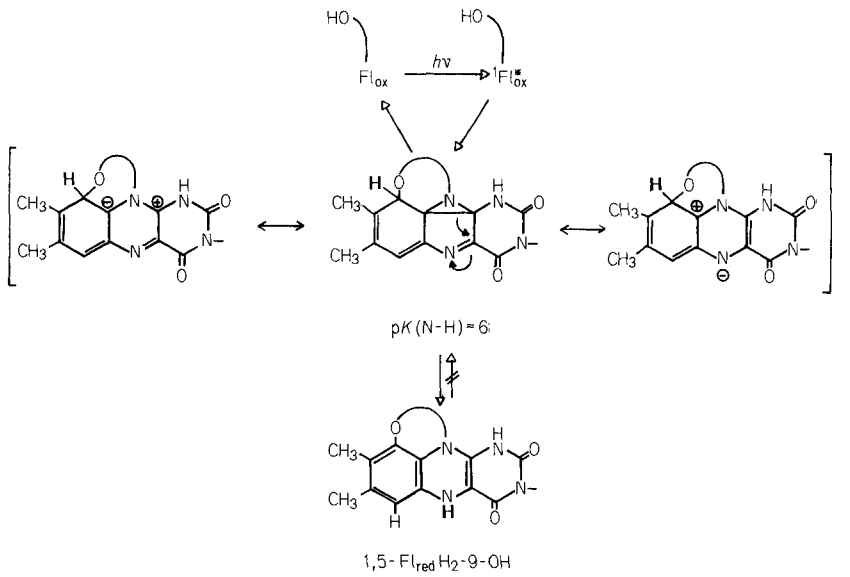
Reaction Mechanism

Photoaddition is prevented by acetylation of the 2'-hydroxyl (*cf.* Table 1). Hence breakage of the C(2')-O bond cannot be involved in the reaction, since this would be facilitated by acetylation. Any primary attack of the A^{2-} catalyst at C(9) with subsequent hydrolysis of an O(9α)-P (or -S) bond and attack of O(9α) at C(2') can thus be eliminated. It would also be difficult to understand why the intramolecular sulfonate reaction would require extraneous sulfate, if covalent attack of S-O^- at C(9) was the primary step of reaction.

If, however, the photoaddition involves a primary nucleophilic attack of 2'-hydroxyl at C(9), the question remains as to the site of proton fixation at the flavin nucleus. The required fast capture of a proton does not occur at C(8), nor at C(6) since photoaddition in ^2H_2O does not lead to hydroxyflavin products deuteriated at C(6) or C(8), as we proved by NMR. Hence, unless intramolecular RO^- attack at C(9) is accompanied by a synchronous sigmatropic shift of the 9α-hydrogen towards N(5), as we postulated earlier [8], we must assume that proton capture takes place at N(1) (according to Scheme 3) and involves subsequent deprotonation at C(9), which becomes the more probable, since we observe a characteristic pH-dependence in the photoaddition with an "action-pK_a" of ≈ 6 (Fig. 6). This pK, measured in the presence of A^{2-}-sulfate, cannot be assigned to any starting product present in solution, while it seems a good guess to connect it with the intermediate shown in Scheme 3.

The only further step required is a 9α,5-proton shift, which leads to 1,5-dihydro-9-alkoxyl-flavin observed as the first stable product in the (anaerobic) photoaddition reaction.

We have tried to further substantiate this proposed scheme starting with 10-(2'-hydroxyethyl)[9-^{2}H]-flavin and we found no isotope effect of C(9)-^{2}H breakage above the error limit of 10% at 5 < pH < 7. Consequently, this proton shift must be fast as compared to the initial addition step. All we have to postulate is reversibility of the photoaddition at pH < 6 with return to the Fl_{ox} ground state.



Scheme 3. *Suggested reaction mechanism of the addition reaction of C(2')-OH at position C(9)*

This investigation was supported by a grant from the American Cancer Society (PF-640 to M.S.J.). We want to thank Miss Jane Rogers and Helga Wilke for their skilful technical assistance and Dr Vince Massey, University of Michigan, Ann Arbor, for helpful discussions. Thanks are due to Prof. Eschenmoser, Zürich, for proposing scheme 3.

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