

Chemical Synthesis and Some Properties of 6-Substituted Flavins[†]

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ABSTRACT: A number of derivatives of riboflavin and of 3-methyllumiflavin substituted in the 6 position have been synthesized starting with 6-nitro flavins, reduction to the 6-amino flavin, and diazotization, followed by reaction with the appropriate nucleophile. The absorption spectra, oxidation-reduction potentials, and the electron spin resonance spectra of the radical cationic forms of several of these synthetic compounds have been determined, including 6-*S*-cysteinyl-3-methyllumiflavin and 6-*S*-cysteinylriboflavin. The latter has been shown to be identical with the dephosphorylated form

of the aminoacyl flavin isolated from trimethylamine dehydrogenase [Steenkamp, D. J., Kenney, W. C., & Singer, T. P. (1978) *J. Biol. Chem.* 253, 2812-2817; Steenkamp, D. J., McIntire, W., & Kenney, W. C. (1978) *J. Biol. Chem.* 253, 2818-2824] in regard to absorption spectrum, photochemical properties, and mobility in high-voltage electrophoresis and in thin-layer chromatography. An unusually pronounced interaction between the amino group and the isoalloxazine ring system was deduced from the absorption spectra of 6-amino-3-methyllumiflavin and 6-aminoriboflavin.

Until about 1976 all covalently bound flavins were thought to be attached to proteins via the 8 α position (Edmondson & Singer, 1976). Recently, however, evidence was presented to show that in bacterial trimethylamine dehydrogenase the flavin is linked to the protein by a thioether bridge between a cysteine residue and C(6) of the isoalloxazine nucleus (Steenkamp et al., 1978a,b). The natural occurrence of flavin nucleotides substituted in the 6 position has also been reported by Mayhew et al. (1974). These authors reported that pig liver glycolate oxidase contains 6-OH-FMN and that the electron-transferring flavoprotein from *Peptostreptococcus elsdenii* contains, besides FAD and 8-OH-FAD, 6-OH-FAD, although the flavins in these instances are noncovalently attached to the protein. Collectively, these data point to the importance of 6-substituted flavins in biological systems and suggest the need to elaborate methods for their chemical synthesis and characterization.

The present paper describes the synthesis and properties of several derivatives of riboflavin and of 3-methyllumiflavin

substituted at C(6), including 6-*S*-cysteinylriboflavin thioether. The latter compound is shown to be identical with the dephosphorylation product of the aminoacyl flavin isolated from trimethylamine dehydrogenase, providing further, conclusive evidence for the previously proposed structure of the covalently bound flavin prosthetic group of that enzyme.

Experimental Section

Materials

Trimethylamine dehydrogenase (EC 1.5.99.7) was purified to homogeneity as previously described (Steenkamp & Malinsson, 1976). Preparation of the aminoacyl flavin of this enzyme and derivatives thereof has been documented (Steenkamp et al., 1978a,b). Riboflavin-binding protein from chicken egg white was obtained by the method of Blankenhorn et al. (1975). Thin-layer chromatography was performed with Merck precoated plates.

Methods

6-Amino-3-methyllumiflavin (1). To an aqueous suspension of 2.0 g (6.35 mmol) of 3-methyl-6-nitrolumiflavin (Knappe, 1979), 12.0 g of sodium dithionite in 40 mL of 10% ammonia was added, resulting in the formation of a black precipitate. After this solid was heated to 90 °C, it dissolved, the solution turned yellow, and light yellow crystals began to form. After 5 min at 100 °C, the solution was cooled to and kept at 0 °C for 1 h. The solid was filtered off and washed with water, dilute acetic acid, and methanol. The crystals of 6-amino-1,5-dihydro-3-methyllumiflavin turned dark brown on exposure to oxygen. The yield of **1** was 400 mg (88%). Anal. Calcd for C₁₄H₁₅N₅O₂·H₂O: C, 55.43; H, 5.65; N, 23.09. Found: C, 55.87; H, 5.81; N, 23.23. MS¹ (70 eV, 225 °C) *m/z* (rel

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intensity) 285 (M^+ , 21), 284 ($M^+ - H$, 100), 227 ($M^+ - H - CH_3NCO$, 53).

6-Aminoriboflavin (2). This compound was obtained by the reduction of 2',3',4',5'-tetraacetyl-6-nitroriboflavin (Litvak & Berezovskii, 1978; Knappe, 1979) with dithionite in the presence of ammonia analogously to the reduction of 3-methyl-6-nitrolumiflavin. Under these conditions the acetyl groups are hydrolyzed. The yield of **2** was 74%. 1H NMR (in CF_3CO_2H) δ 2.49 (s, 3 H, 7- CH_3), 2.80 (s, 3 H, 8- CH_3), 4.06–5.35 (m, 8 H, side chain protons and NH_2), 7.68 (s, 1 H, 9-H).

2',3',4',5'-Tetraacetyl-6-aminoriboflavin. This was synthesized the same way as **2** but at pH 10.5, in sodium carbonate buffer, in the absence of ammonia. The yield was 78%. 1H NMR (CF_3CO_2H) δ 1.96, 2.20, 2.31, 2.57, and 2.80 (s, 6 CH_3 groups), 4.25–6.10 (m, 8 H, corresponding to ribityl side-chain protons and NH_2), 7.82 (s, 9-H).

6-Bromo-3-methylumiflavin (3). Five hundred milligrams of **1** was dissolved in 5 mL of concentrated H_2SO_4 and poured on 25 g of ice. Sodium nitrite (175 mg; 50% excess) was added slowly at 0 °C, resulting in a color change from red brown to golden orange. The solution was stirred for 15 min; then 180 mg of urea was added slowly to destroy excess nitrite and, after further 15-min stirring, saturated with potassium bromide. After the solution was stirred at room temperature overnight, the pH was adjusted to between 5 and 8; the orange precipitate was filtered off and washed with water and ethanol: yield, 270 mg (52%). MS (70 eV, 300 °C) m/z (rel intensity) 350 ($M^+ + 2$, 100), 348 (M^+ , 95), 293 ($M^+ + 2 - CH_3NCO$, 69), 291 ($M^+ - CH_3NCO$, 66).

6-Bromoriboflavin (4). This was obtained by the same procedure as **3** from compound **2** in 40% yield. Anal. Calcd for $C_{17}H_{19}BrN_4O_6$: C, 44.9; H, 4.21; N, 12.3; Br, 17.5. Found: C, 45.1; H, 4.15; N, 12.3; Br, 16.5. Absorption spectrum: λ_{max} = 445 ($\epsilon \sim 9700$) and 397 nm ($\epsilon \sim 12500$). 1H NMR (CF_3CO_2H) δ 2.80 (s, 3 H, 7- CH_3), 2.84 (s, 3 H, 8- CH_3), 4.05–5.85 (m, 7 H, ribityl side-chain protons), 8.12 (s, 1 H, 9-H).

6-(Thiocyanato)-3-methylumiflavin (5). Compound **1** (300 mg, 1.05 mmol) was dissolved in 1.5 mL of concentrated H_2SO_4 ; 4.5 g of ice was added and diazotization was carried out as described above for **3**. Saturated KSCN solution (0.5 mL) was then added slowly and the solution was kept at 0 °C until nitrogen evolution ceased. The pH of the solution was then adjusted to 2 with concentrated ammonium hydroxide, and the crystalline product was filtered, thoroughly washed with water and a little methanol, and dried in vacuo: yield, 250 mg. The product contained only a trace of 3-methylumiflavin by thin-layer chromatography (silica plates; 1-butanol–acetic acid–water, 6:2:2 v/v/v). MS (70 eV, 250 °C) m/z (rel intensity) 327 (M^+ , 26), 313 (30), 270 ($M^+ - CH_3NCO$, 100); IR (KBr) 2160 ($C\equiv N$), 1700 [$C(4)=O$], 1660 cm^{-1} [$C(2)=O$].

The corresponding 6-(thiocyanato)riboflavin was synthesized analogously, but the product could not be crystallized and was thus used without further purification.

6-(Ethylxanthyl)-3-methylumiflavin. This compound was prepared from 6-amino-3-methylumiflavin (110 mg, ~ 0.4 mmol) by reaction of the diazotate (cf. above) with potassium ethylxanthate (96 mg, 0.6 mmol) dissolved in 1 mL of water at 0 °C. The reaction mixture was brought to ambient tem-

perature for 20 min and then warmed at 60 °C for 5 min. The precipitate was filtered, washed with water, and recrystallized from ethanol to yield 60 mg of the product. This compound showed on thin-layer chromatography (silica plates; 1-butanol–acetic acid–water, 6:2:2 v/v/v) a major nonfluorescent spot corresponding to the 6-ethylxanthate derivative and a minor fluorescent spot corresponding to 3-methylumiflavin.

Hydrolysis of this product yields as a main product 6-thio-3-methylumiflavin. For hydrolysis, 20 mg of the 6-xanthate derivative was refluxed in 2 mL of 6 N HCl for 6 h, the solvent was evaporated in vacuo, and the residue was dissolved in a minimum volume of warm glacial acetic acid. Upon addition of water and cooling, a precipitate was obtained which was filtered and washed with water. This material was free of 3-methylumiflavin, consisted of a mixture of 3-methyl-6-thiolumiflavin and its oxidation product, the 6-SS-dimer, and, according to thin-layer chromatography (silica plates; 1-butanol–acetic acid–water, 6:2:2 v/v/v), was identical with the products obtained as described below.

6-Thio-3-methylumiflavin. Fifty milligrams of **5** was suspended in 2 mL of water under a stream of nitrogen, solid sodium dithionite was then added until the color of the suspension turned yellow, and a precipitate of reduced 6-thio-3-methylumiflavin was formed. During the procedure the pH of the solution was held at 8–9 with dilute ammonia. The precipitate was then filtered and washed with water and a little methanol. The reduced compound was rapidly reoxidized by air. When a suspension is stirred in aqueous solution at pH at 6–7, the green color of the 6- S^- compound is rapidly formed, which then turns to yellow orange due to formation of the dimer (6-SS-).

6-S-Cysteinyl-3-methylumiflavin. Ten micromoles of **3** was dissolved in 16 mL of dimethylformamide and 144 mL of 0.1 M Na_2CO_3 , pH 10.9. To this 16 mL of freshly prepared 0.5 M cysteine hydrochloride, adjusted to pH 9.0, was added. Following incubation for 22 h at ambient temperature, the pH of the reaction mixture was adjusted to 7.0 and the solution was extracted 3 times with an equal volume of $CHCl_3$ to remove any unreacted starting material. To the aqueous phase 0.1 volume of 55% (w/v) trichloroacetic acid was added, and reduced pressure was applied to remove CO_2 . The solution was then applied to a column of Florisil (0.9 \times 6 cm) equilibrated with 5% (v/v) acetic acid. The column was washed with 5% acetic acid and water, and then the flavin was eluted with 20% (v/v) pyridine. The eluate was directly applied to a column (0.9 \times 5 cm) of DEAE-cellulose (acetate form), equilibrated with 20% pyridine. 6-S-Cysteinyl-3-methylumiflavin came off the column in 20% pyridine, while some green-colored material (presumably 6-N-cysteinyl-3-methylumiflavin) was retained on the column. The yield of 6-S-cysteinyl-3-methylumiflavin was 55%.

6-S-Cysteinylriboflavin. Five micromoles (2.3 mg) of 6-bromoriboflavin was dissolved in 6 mL of dimethylformamide, and this was diluted with 50 mL of 0.1 M sodium carbonate, pH 11. A freshly prepared solution of 486 mg of cysteine (4 mmol) in 8 mL of water (pH adjusted to ~ 9) was added (pH of mixture ~ 10) and then stirred at 22 °C for 22 h in the dark. The pH of the solution was then adjusted to 7 with acetic acid, the precipitate which formed was filtered off, and the filtrate was evaporated in vacuo to a volume of ~ 20 mL. A 10-mL aliquot was applied to a Florisil column (1 \times 10 cm), equilibrated with 5% acetic acid. The column was washed with 5% acetic acid and then water, and the total flavin was eluted with 20% pyridine. The eluate was evaporated to dryness in vacuo; the residue was dissolved in 0.1 M potassium phosphate

¹ Abbreviations used: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; MS, mass spectrum; IR, infrared spectrum.

buffer, pH 7.0, and then applied to an affinity column consisting of riboflavin-binding protein immobilized to Sepharose B [riboflavin-binding capacity was 1.5 μmol [prepared by the method of Mayhew & Strating (1975)]]. Riboflavin derivatives which do not carry a charged function are bound by this column, while 6-S-cysteinylriboflavin is not retained. The compound was obtained with a yield of 35–40% and is pure by chromatographic (silica plates; 1-butanol–acetic acid–water, 6:2:2 and 4:3:2 v/v/v) and spectroscopic criteria. Upon aging, small quantities of the rearrangement product, 6-N-cysteinylriboflavin, may form, which can be separated by DEAE chromatography as described above for 6-S-cysteinyl-3-methyllumiflavin.

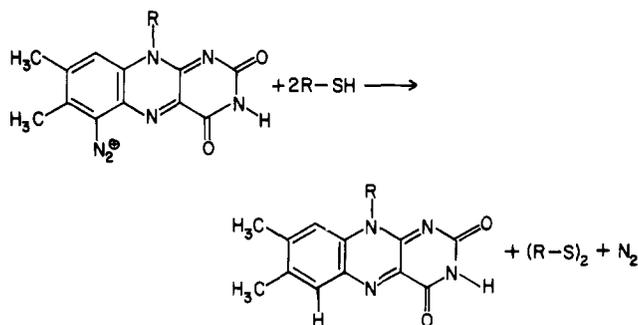
6-(N-Acetylamino)-3-methyllumiflavin. Acetylation of 6-amino-3-methyllumiflavin (30 mg) was achieved by gently heating in 1 mL of acetic anhydride–acetic acid (2:1 v/v) and 1 drop of trifluoroacetic acid until the dark brown solid was dissolved and a clear yellow solution was formed. The solvent was evaporated to dryness in vacuo, and the yellow residue was recrystallized from the minimal amount of acetic acid–water to yield 25 mg of the pure acetylated compound. ^1H NMR (D_2O) δ 2.30 (6 H, 7- and 8- CH_3), 2.68 (s, 3 H, 6-NH-COCH₃), 3.40 (s, 3 H, 3- CH_3), 4.13 (s, 3 H, 10- CH_3), 8.03 (s, 1 H, 9-H).

Measurement of Oxidation–Reduction Potentials. These were determined by anaerobic titrations with dithionite of a solution of the particular flavin in the presence of a suitable dye in 0.1 M potassium phosphate, pH 7.0, at 22 °C, essentially as previously described (Edmondson & Singer, 1973; Müller & Massey, 1969). Indigo disulfonate ($E_{m,7} = -116$ mV; Clark, 1960) and safranin T ($E_{m,7} = -289$ mV; Clark, 1960) were the mediator dyes used in the titration of 6-bromoriboflavin and of 6-amino flavins, respectively. Because of their low solubility at pH 7.0, 6-aminoriboflavin and 6-amino-3-methyllumiflavin were first dissolved at high concentration (~ 7 mM) in 6 N HCl, followed by dilution into phosphate buffer and adjustment of the pH to 7.0. The maximal concentration of 6-amino-3-methyllumiflavin achieved even in this manner is 11 μM . The relative concentration of the oxidized and reduced forms of flavin was determined by the procedure of Moore et al. (1978), and the potential of the system was calculated from the Nernst equation after each addition of dithionite. The oxidation–reduction potential was also determined coulometrically (Wilson, 1978) under anaerobic conditions, with 40–50 μM flavin, 0.5 mM methylviologen, and 0.5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ present, in order to avoid interference by the sulfite generated in dithionite titrations (Müller & Massey, 1969).

EPR Spectra. Since the reduction of 6-S-cysteinyl flavins with TiCl_3 in acid media produces flavin hydroquinones, not flavin cation radicals, the latter were generated by mixing equivalent amounts of oxidized and reduced flavins under Ar. The former was dissolved in 12 N HCl and the latter, prepared by reduction with a stoichiometric amount of dithionite, was dissolved in 0.1 M potassium phosphate, pH 7.2.

Other Methods. Absorption spectra were recorded with either a Cary 14 spectrophotometer interfaced with a Nova 2/4 computer (Data General Corp.) or a Cary 219, a Cary 118C, or a Varian 635 Superscan spectrophotometer. Mass spectra were obtained with a Varian CH-7 instrument. NMR spectra were obtained with a Varian EM-390 instrument at 35 °C by using tetramethylsilane as the internal standard. Infrared spectra in KBr pellets were measured with a Perkin-Elmer 621 IR instrument. The photochemical experiments were carried out with the instrumentation described elsewhere

Scheme I



by Knappe (1975). EPR spectra were recorded with a Varian E-4 spectrometer.

Results and Discussion

Chemical Synthesis of 6-Substituted Flavins. In principle there are two approaches to the synthesis of flavins substituted in the 6 position. They may be prepared by condensing smaller ring systems, such as appropriately substituted *o*-phenylenediamines and alloxane [for a review, see Lambooy (1967)], or the desired group may be inserted into the intact isoalloxazine ring system (Berezovskii et al., 1972), the approach we have used in the present study. Since complications may occur during these syntheses, it seems appropriate to comment briefly on the procedures.

Although it has been reported that nitration of riboflavin or lumiflavin yields 9-nitroriboflavin or 6,9-dinitrolumiflavin, respectively (Berezovskii & Aksel'rod, 1968; Litvak & Berezovskii, 1978), more recently it has been established that the product believed to be 9-nitroriboflavin is the 6 isomer and that 6-nitrolumiflavin is the main product obtained on nitration of lumiflavin (Knappe, 1979). Moreover, the nitro group of these flavins can be reduced to the amino function either photochemically or with dithionite (Knappe, 1979). Such 6-amino flavins then become suitable starting compounds for the derivatization of the 6 position via the Sandmeyer reaction with (pseudo) halogens (Weygand & Hilgetag, 1970). This reaction has been successfully applied to the synthesis of various 8-substituted flavins from the parent 8-amino flavins (Ghisla & Mayhew, 1980). With the 6-amino flavins, the formation of the 6-diazotized compounds proceeded readily, but a high acid concentration (5 M H_2SO_4) was required to prevent the formation of unidentified dark decomposition products.

These diazonium salts are characterized by an orange color with absorption maxima at 462, 343, and 275 nm in 2 N HCl. The 6-diazonium flavins may be converted to the corresponding halide substituents under Sandmeyer conditions (Weygand & Hilgetag, 1970). The order of reactivity observed was $\text{I}^- > \text{Br}^- > \text{Cl}^-$, the 6-chloro derivative being formed in 5–10% yield.

Attempts to convert 6-diazonium flavins to 6-alkylthio or to 6-thio flavins by reaction with either alkylthiols or HS^- , as had been successfully carried out with 8-diazotized flavins (Ghisla & Mayhew, 1980), invariably failed. Although the expected products were formed in minor amounts, as shown by thin-layer chromatography, the major products obtained were the 6-unsubstituted flavins [C(6)-H], which are probably formed via reductive elimination of the diazonium groups (Scheme I).

6-Iodo flavins were found to be essentially unreactive toward displacement with thiols in the pH range 9–11. The 6-bromo analogues, on the other hand, reacted albeit very slowly. For example, after 24–48 h of incubation under aerobic conditions at pH 9.5 and 25 °C, over 90% of the 6-bromo flavin had

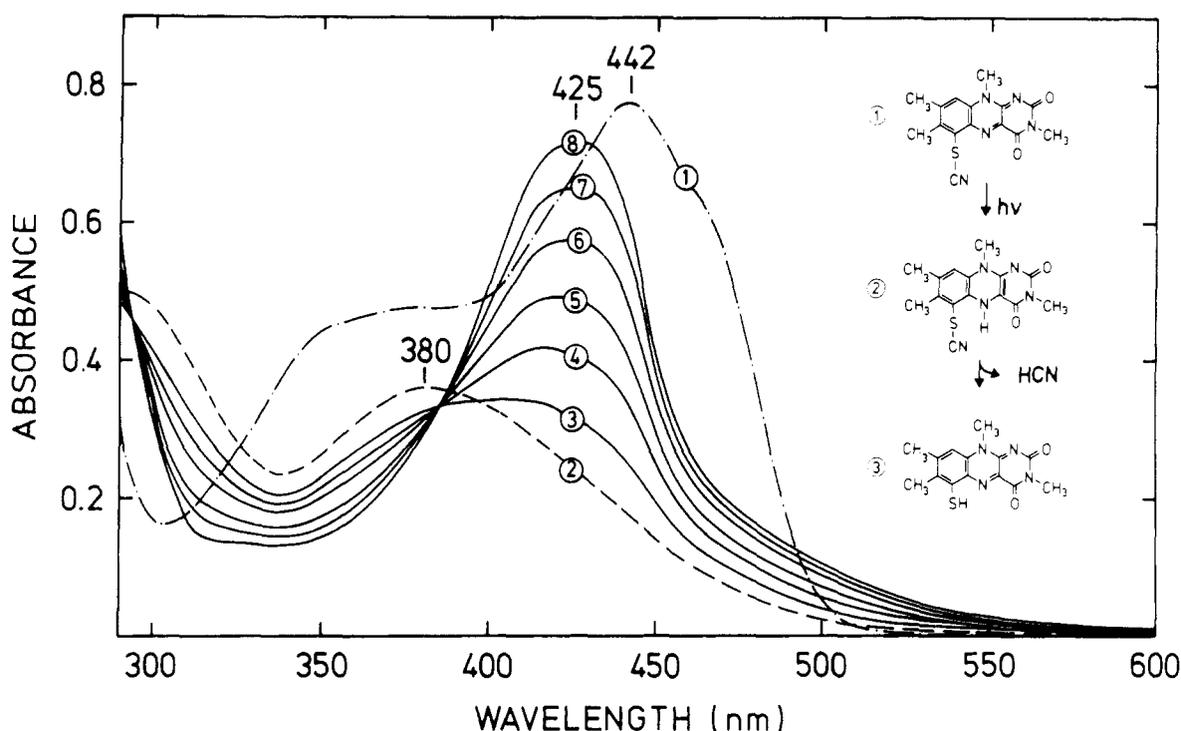
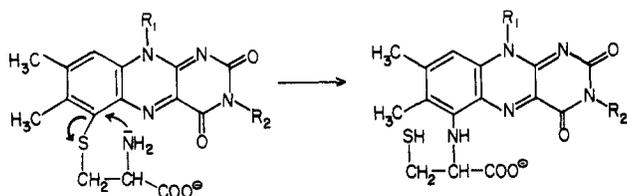


FIGURE 1: Spectral course of the intramolecular decomposition of 1,5-dihydro-6-(thiocyanato)-3-methylflavin yielding 6-thio-3-methylflavin and hydrogen cyanide. Curve 1: spectrum of 6-(thiocyanato)-3-methylflavin, 6 μ M, in 20% aqueous methanol containing 4 mM EDTA and 0.05 M sodium sulfate, pH 2.5. Curve 2: 1,5-dihydro-6-(thiocyanato)-3-methylflavin obtained by illumination of 1 for 10 s with blue light (420–480 nm) under strictly anaerobic conditions. The following traces were obtained after 5, 10, 15, 25, 40, and 80 min, respectively, of standing in the dark. Curve 8 represents the final product which is identical with 6-thio-3-methylflavin.

Scheme II



reacted in the presence of a 50-fold molar excess of cysteine. The reaction must be carried out under aerobic conditions, since the rate of reduction of the flavin via the thiolate is comparable to the rate of substitution. Since reduced 6-bromo flavins are unreactive toward nucleophilic displacement with thiolates, the oxygen serves to reoxidize the flavin fraction reduced via the mercaptides. At higher pH values (pH > 10), position N(3) of the flavin ionizes and this prevents nucleophilic attack at C(6). The N(3) position may be alkylated, as in 3-methylflavin, but decomposition occurs at pH > 11 (Smith & Bruce, 1975).

A further complication observed in this approach to the syntheses of 6-S-cysteinyll flavins was the formation of a rearrangement product, particularly at elevated temperatures. This can be separated from the desired product (6-S-cysteinyll flavin) on a column of Dowex 50 or DEAE-cellulose. The major contaminant has spectral properties very similar to those of 6-amino flavins (cf. Figure 3). This compound differs, however, from unsubstituted 6-amino flavin, as verified by thin-layer chromatography and its behavior on cation-exchange resins. This derivative is most probably a compound in which a Smiles S \rightarrow N rearrangement (Truce et al., 1970) has occurred and in which the cysteinyl moiety still remains attached to the flavin (Scheme II). A similar rearrangement has been observed with 8-S-cysteinyll flavins. Indeed, the reaction is sufficiently fast in this case that the amino group of the cysteine must be protected by acylation during synthesis

(Moore et al., 1979; S. Ghisla, V. Massey, and P. Hemmerich, unpublished experiments).

A thio group can also be introduced into the 6 position by incubation of the 6-diazonium flavin with thiocyanate or ethylxanthate. For preparative purposes this is the preferred method. The 6-thiocyanate and 6-ethylxanthate derivatives of 3-methylflavin were obtained in crystalline form and in good yield. These reactions proceed equally well with the riboflavin analogues, although the products failed to crystallize readily. The conversion of these compounds to the 6-SH compound by classical methods (Weygand & Hilgetag, 1970) is feasible, but a much simpler procedure was developed, based on the peculiar reactivity of 6-thiosulfato flavin, which eliminates sulfite upon reduction (Steenkamp et al., 1978b). Thus, reduction of 6-thiocyanato flavins at pH > 4 yields immediately the green color of 6-thio flavin anion. That this reaction proceeds through formation of a reduced flavin intermediate is shown in Figure 1. When 6-(thiocyanato)-3-methylflavin was reduced with EDTA and light (Massey et al., 1978) under anaerobic conditions, a species rapidly formed which had the characteristic absorption spectrum of reduced flavins carrying electron-withdrawing substituents and which have the 1,5-dihydro flavin structure (Knappe, 1980). An isosbestic reaction with a first-order rate constant of 0.046 min^{-1} then follows, leading to the formation of 6-thio-3-methylflavin (Figure 1). 6-Thio-FMN, derived from the covalently bound flavin component of trimethylamine dehydrogenase, is known to react rapidly with oxygen to form the dimeric flavin disulfide (Steenkamp et al., 1978b). Analogous behavior has been observed with synthetic 6-thio flavins in the present study. As shown in Figure 2, the oxidation of 6-thio-3-methylflavin by oxygen proceeds isosbastically to form the sulfide dimer. This species can be reconverted to the monomeric anion by reduction with dithionite, sodium borohydride, or EDTA and light.

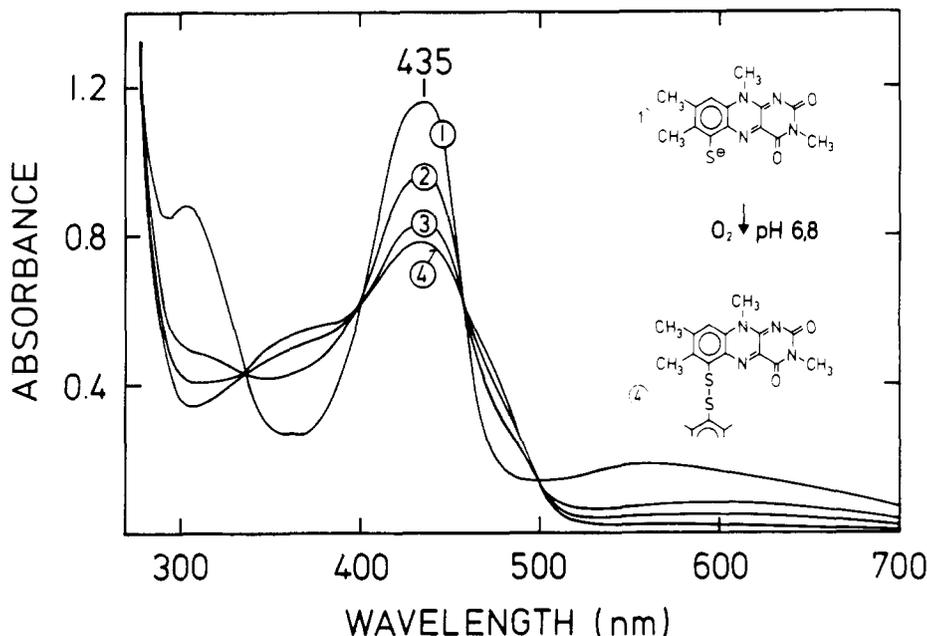


FIGURE 2: Spectral course of the oxidation of 6-thio-3-methylflavin (anion) by air, yielding the corresponding disulfide. Curve 1: 6-thio-3-methylflavin anion in 20% aqueous methanol, 0.05 M in sodium phosphate, pH 6.8, was formed in situ via photoreduction of 6-(thiocyanato)-3-methylflavin in the presence of EDTA (cf. legend of Figure 1). Upon admission of air, spectra 2, 3, and 4 were recorded after 5, 15, and 30 min. Note the disappearance of the long-wavelength absorbance at 560 nm. Curve 4 represents the spectrum of 6,6'-bis(3-methylflavin) disulfide.

Qualitatively, the reactivity of 6-bromo, 6-iodo, and 6-diazonium flavins toward nucleophilic displacement is similar to that of the vinyl analogue 8-substituted flavins. However, while the latter shows a reactivity which is comparable to that of dinitrobenzene derivatives (Moore et al., 1979), with 6-substituted flavins this reactivity is lowered by several orders of magnitude. Worth noting also is the rapid oxidation of 6-thio flavins to the corresponding disulfide by oxygen, as compared to 8-thio flavins (Moore et al., 1979). These properties probably reflect the difference in electron density at the 6 and 8 positions, respectively, a feature which is also shown by the differences in pK_a values of 6-hydroxy [$pK_a = 7.1$ (Mayhew et al., 1974)] and 8-hydroxy [$pK_a = 4.8$ (Ghisla & Mayhew, 1976)] flavins.

Spectral Properties of 6-Amino Flavins. Flavin coenzymes carrying a nitrogen substituent in the C(6) position of the isoalloxazine ring system have not as yet been described as occurring in nature. In view of the number of different types of modification which have been uncovered so far (Edmondson & Singer, 1976; Kenney, 1980), it seems probable that 6-amino flavins might also occur as natural cofactors. One possibility is covalent linkage of the flavin via the ϵ -amino group of a lysyl residue in a protein. The spectral properties of 6-amino flavins, the putative parent compound of such coenzymes, are, therefore, of potential interest and were investigated in some detail. The 6-amino flavin chromophore is characterized by relatively small absorbance changes with pH at wavelengths greater than 500 nm and by a single, relatively narrow band in the 390–430-nm region, the position of which is pH dependent (Figure 3). Their spectral properties are similar to those of 6-hydroxy flavin anions (Mayhew et al., 1974; Schöllnhammer & Hemmerich, 1974) and to 6-keto flavins, in which an "orthoquinoid structure" is obtained by alkylation at the N(1) position of the flavin (Schöllnhammer & Hemmerich, 1974). By analogy, it appears that the neutral and the monoanionic forms of 6-amino flavins, which have very similar spectra, should have the orthoquinoid structure shown in Figure 3. Consequently, the deprotonation step with a $pK_a \cong 10.3$ would be expected to occur at N(1)-H. That the

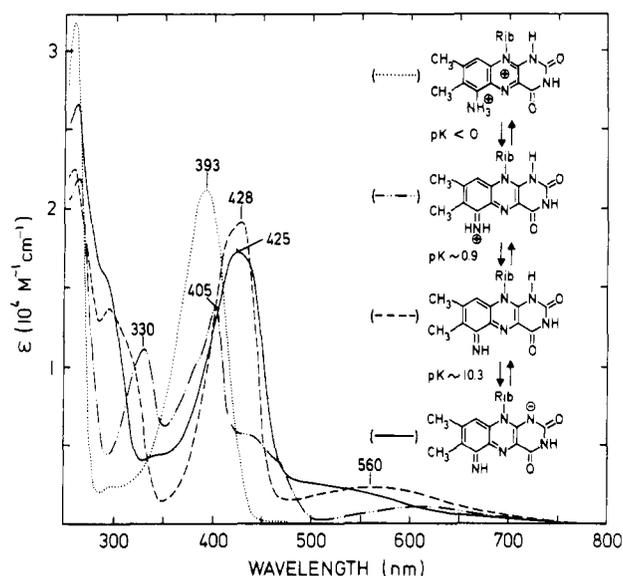


FIGURE 3: Absorption spectra of 6-aminoriboflavin as function of the ionization state. Curve \cdots , in 70% HClO_4 ; curve $-\cdot-, in 2 N HCl ; curve $---$, in 0.1 M sodium phosphate, pH 7.0; curve $---$, in 0.01 N NaOH solution.$

neutral orthoquinoid form shown in Figure 3 is present in the pH range 2–8, and not the possible mesoionic structure [carrying a positive charge on the 6-amino function and a negative one on the N(1)–C(2)=O function], is indicated by the lack of solvatochromy, when the spectra are recorded in solvents of different polarity (water to benzene). The protonation with a pK_a of 0.9 occurs predominantly at the 6-amino function (Figure 3), but the complexity of the spectrum indicates that other tautomers possibly exist as well. In strong acid, the dicationic form must be present, as shown by the single absorption band at 393 nm, which is characteristic of isoalloxazines carrying a positive charge on the nucleus and in which an exocyclic conjugation is absent (Dudley et al., 1964).

Acylation of the amino group of 6-amino flavins gives rise

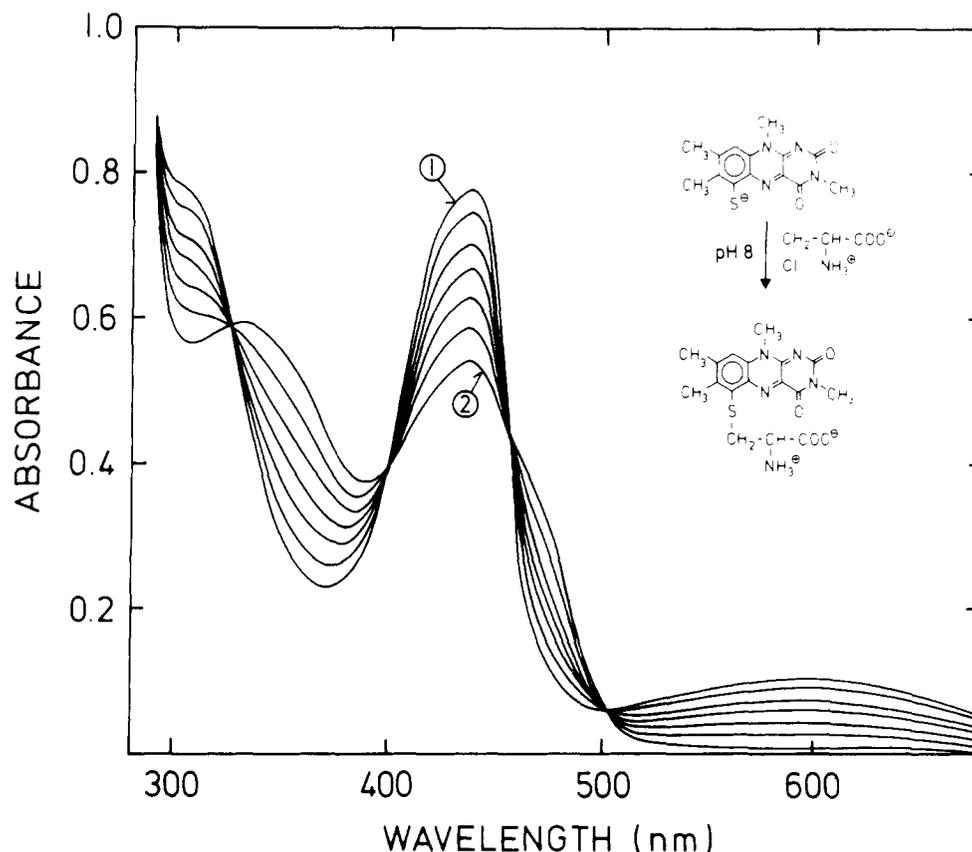


FIGURE 4: Spectral course of the alkylation of 6-thio-3-methylflavin anion with β -chloroalanine (0.4 M) at pH 8 and 25 °C. Curve 1: spectrum of 6-thio-3-methylflavin anion produced in situ by photoreduction of 6-(thiocyanato)-3-methylflavin (cf. Figure 1). The following curves, which are corrected for dilution, were recorded after anaerobic addition of β -chloroalanine at 3, 5, 7, 10, 14, and 30 h. Curve 2 represents the spectrum of 6-S-cysteinyl-3-methylflavin. Note the decrease of the long-wavelength absorbance at 600 nm and the formation of a new second absorbance band at 340 nm.

to a typical flavin-type absorption spectrum with maxima at 450 and 350 nm. In this compound, conjugation of the nitrogen with the aromatic ring system is blocked. Similar effects have been obtained upon oxidation of the sulfur moieties in 6-thio flavins (Steenkamp et al., 1978a) and in 8-thio flavins (Moore et al., 1979), as well as acetylation of 6-hydroxy and 8-hydroxy flavins (Schöllnhammer & Hemmerich, 1974; Ghisla & Mayhew, 1976).

Identity of Native and Synthetic 6-S-Cysteinyl Flavins. Alkylation of synthetic 6-thio flavin anion with an excess of β -chloroalanine yields a compound with spectral properties indistinguishable from those of the aminoacyl coenzyme of trimethylamine dehydrogenase (Steenkamp et al., 1978a,b) (Figure 4). The properties of this synthetic compound (6-S-cysteinylriboflavin) are identical with those of the dephosphorylated form of the aminoacyl coenzyme of trimethylamine dehydrogenase in every respect examined. Both compounds are converted to the thiolate on illumination at pH 8.0. This is accompanied by absorbance changes which are just the reverse of those in Figure 4. Synthetic 6-S-cysteinylriboflavin and the natural compound (Steenkamp et al., 1978a) are both converted by peroxy acids to the same chromophore. The synthetic and natural compounds show the same absorption spectra in 6 N HCl, at neutral pH, and in 0.1 N NaOH, indicating the presence of the same ionizing groups. Their mobilities at the riboflavin level (-0.6 , as compared to mobility of FMN = 1.0) are identical in high-voltage electrophoresis at pH 1.9 [5% (v/v) formic acid] and on silica gel thin-layer chromatography ($R_f \approx 0.36$ in 1-butanol-acetic acid-water, 4:3:2 v/v/v). Further, illumination of the spots following such chromatographic separation at pH 2.5 results in the formation

Table I: Oxidation-Reduction Potentials of 6-Substituted Flavins

flavin	method of redn ^a	E_0' (mV)	n value
riboflavin	C	-199	2.1
6-bromoriboflavin	C	-144	2.1
6-bromoriboflavin	D	-149	2.4
6-aminoriboflavin	D	-293	1.7
6-amino-3-methylflavin	D	-320	2.0
6-(N-acetylamino)-3-methylflavin	C	-185	2.0
6-S-cysteinyl-FMN	C	-154	2.0

^a C is coulometric titration; D is dithionite reduction in the presence of dyes.

of a product with a whitish blue fluorescence ($\lambda_{max} \approx 500$ nm), a property previously noted with the naturally occurring compound when illumination was performed at acid pH values (Steenkamp & Singer, 1976).

These data confirm, beyond doubt, that the coenzyme of trimethylamine dehydrogenase is the monophosphate of 6-S-cysteinyl-7,8-dimethyl-10-D-ribitylisoalloxazine, as was previously postulated (Steenkamp et al., 1978a,b).

Oxidation-Reduction Potentials of 6-Substituted Flavins. The results of the determination of the oxidation-reduction potentials of 6-substituted flavins are summarized in Table I. From the data presented, the profound influence of 6-substitution on the oxidation-reduction potential of the flavin is apparent. Whereas 6-bromoriboflavin has a redox potential of -144 mV at pH 7, 6-aminoriboflavin has a potential of -293 mV. These may be compared with a value of -199 mV for the parent compound, riboflavin.

Scheme III

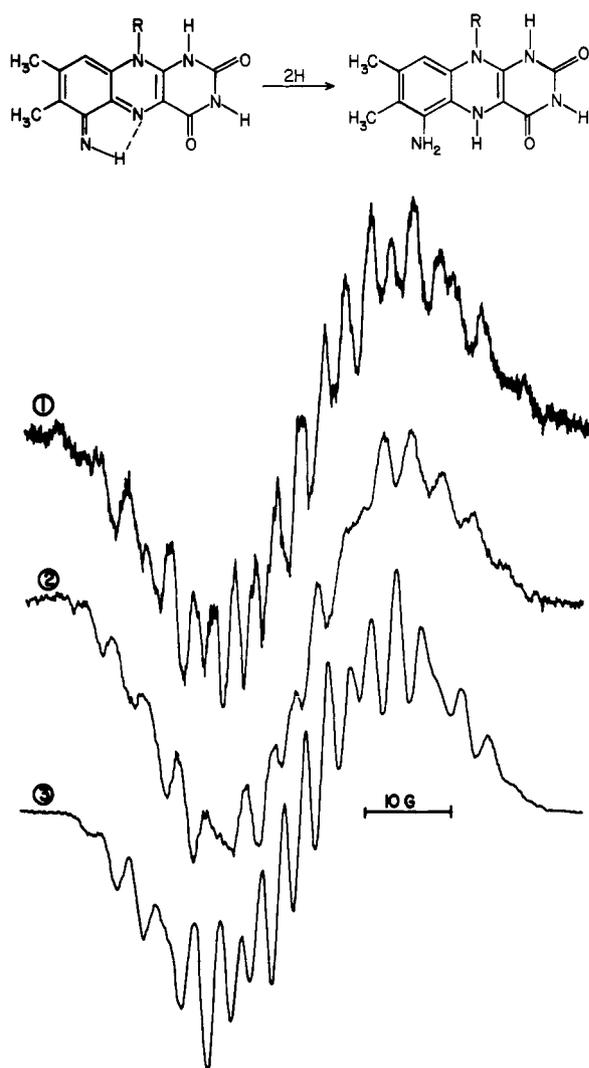


FIGURE 5: EPR spectra of cationic semiquinone forms of 6-S-cysteinyll flavins. Flavin concentrations were 3–4 mM in 6 N HCl. Upper: 6-S-cysteinyll-3-methylumiflavin (microwave frequency, 9.062 GHz; microwave power, 3.5 mW; modulation frequency, 100 kHz; modulation amplitude, 0.8 G). Middle: 6-S-cysteinyll-FMN from trimethylamine dehydrogenase (microwave power, 1.0 mW; modulation amplitude, 1.6 G). Lower: 6-bromoriboflavin (microwave power, 2 mW; modulation amplitude, 1.0 G).

The reason for the unexpected behavior of 6-amino flavins may be due to the formal reduction of the N(5)–C(6)–N system rather than to the “usual” reduction of the N(1)–N(5) system of other flavins (Scheme III). When the amino group is acetylated, the potential is reconverted to nearly that of normal flavin.

The oxidation–reduction potential of 6-S-cysteinyll-FMN, the covalently bound cofactor of trimethylamine dehydrogenase, is –154 mV, considerably more positive than that of FMN (–216 mV; Clark, 1960). Estimation of the oxidation–reduction potential of this aminoacyl coenzyme via flavin–sulfite complex formation according to the procedure of Müller & Massey (1969) gave comparable results.

It may be mentioned that the potentials obtained by the two procedures in Table I were always in good agreement. Examples of this are the values for 6-bromoriboflavin (Table I). The coulometric procedure is particularly useful when no indicator dyes are available with suitable potential and spectral properties as is the case for the naturally occurring 6-S-cysteinyll-FMN.

EPR Spectra of 6-S-Cysteinyll Flavins. Figure 5 shows the EPR spectra of the cationic radicals of 6-S-cysteinyll-3-methylumiflavin, 6-S-cysteinyll-FMN isolated from trimethylamine dehydrogenase, and 6-bromoriboflavin. The first of these has a line width of 60 G with 20 to 21 peaks and a g value of 2.0070; the second compound has a line width of 57 G with 16 to 17 peaks and a g value of 2.0073; the third one has a 57-G line width, 18 peaks, and a g value of 2.0085. The difference in the line width and the number of peaks is due to the different substituents at the C(6) and N(10) positions of these three compounds. The total width is comparable to that of the parent flavin in accordance with the rather small effect expected from the removing of the small coupling constant of the 6-H. A sulfur or bromine should not give major hyperfine couplings. These results may be contrasted with those obtained for 8 α -substituted flavins in which the total width of the spectrum is reduced relative to that of the parent compound (Salach et al., 1972).

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