Acetylation and Methylation of Flavins and the Effects on Isoalloxazine Ring Protonation (1)

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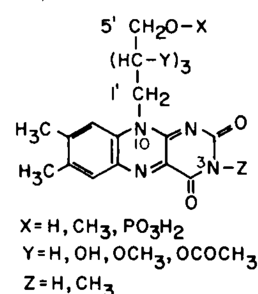
The acetylation of N^{10}-(poly)hydroxy side chains of 7,8-dimethylisoalloxazines has been accomplished in conventional ways using perchloric acid as catalyst with excess acetic anhydride in acetic acid for flavins or in pyridine for the coenzyme, D-riboflavin-5′-phosphate (FMN). Although complete acetylation of the primary and secondary alcoholic functions of flavins with various hydroxyalkyl chains occurs, the primary product purified by DEAE-cellulose chromatotraphy of the reaction with FMN, followed by neutralization, is the 2′,3′-diacetyl derivative. Diacetyl FMN and other acetylated flavins exhibit the usual absorption spectra of protonated isoalloxazinium compounds, with λ max values of 390, 264, and 222 nm, only upon greater acidification than necessary for their hydroxy or especially deoxy counterparts. A similar though less marked effect is found with flavins methylated by treatment with methyl iodide and silver oxide in dimethylformamide. Protonation of the isoalloxazine ring, which occurs on the most basic nitrogen 1, is most effectively impaired by acetylation or methylation of the proximal 2′-hydroxyl group.

Acetylation of the 2′,3′, and 4′-hydroxyls of the 10-(1′-D-ribityl) side chain of 5′-trityl-D-riboflavin in pyridine was claimed for the specific synthesis of the coenzyme, FMN, though the triacetyl FMN was not obtained as a pure compound following detritylation (2). The 2′,3′,4′-triacetyl FMN was isolated after treatment of FMN with acetic anhydride, both with perchloric acid as catalyst and in pyridine (3). A pertrifluoroacetyl cyclic FMN (probably 3,2′,3′-tristrifluoroacetyl-D-riboflavin-4′,5′-diphosphate) was reported as a potentially useful FMN analog (4). More recent syntheses involving flavin modifications, *e.g.*, halogenation at the 9-aryl (5) or 8α-methyl function (6) prior to the conversion to coenzyme forms, have included the complete acetylation of 2′,3′,4′, and 5′-hydroxyls of riboflavin with acetic anhydride in acetic acid containing perchloric acid. A more complete study of the acetylation of other flavins, though, was still needed.

Alkylations of lumiflavin (7,8,10-trimethylisoalloxazine) and 2′,3′,4′,5′-tetra-*O*-acetylriboflavin in the 3-imino position of the isoalloxazine nucleus had been accomplished by treatment of the flavin with an appropriate alkyl iodide in dimethylformamide with potassium carbonate as catalyst (7); however, alkylation of hydroxyalkyl substituents at the 10-position of flavins had not been reported.

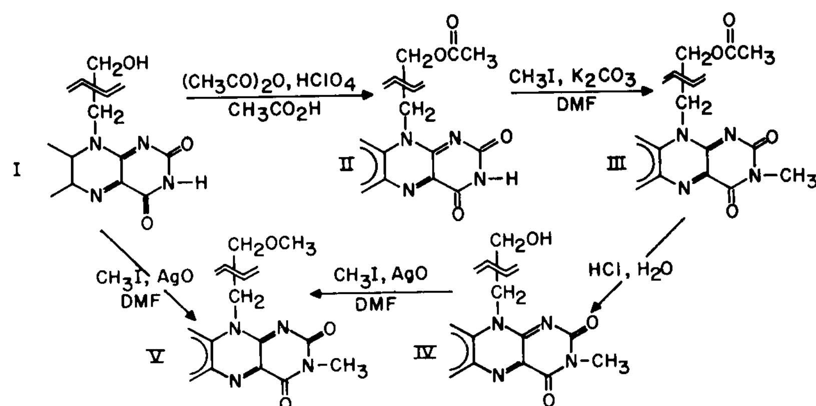
Although the spectral properties of numerous flavins have been published, some with a systematic attempt to identify the chromophoric species responsible (8), the influence that side-chain functions can exert on the absorption spectra of isoalloxazine compounds in acid apparently has been overlooked, and no quantitation of the shifts in pK_a values resulting from such modifications has heretofore been documented.

The present paper describes the synthesis and properties of flavins acetylated or methylated in the side chain at position 10 of the 7,8-dimethyl- and 3,7,8-trimethylisoalloxazine nucleus, as shown in Formula I. Furthermore,

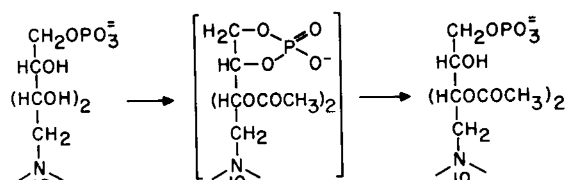


the significant effect of impeding flavin ring protonation, which groups nearby nitrogen 1 can exert, is characterized.

Preferred reactions for acetylation and methylation of flavins are shown in the following scheme. It should be pointed out that acetylation with acetic anhydride and the subsequent chloroform extraction of acetylated flavin from aqueous acetic acid affords a simple, high-yield procedure that is more satisfactory than one obtains with acetyl chlor-



ide, where hydrolysis of product is a greater problem. Interestingly, though some triacetyl FMN can be obtained by the use of acetic anhydride with a suitable basic solvent, such as pyridine (3), the diacetyl compound is a major product that results from the intermediate formation of the 4′,5′-cyclic monophosphate, as shown in the following equation. The similar 2′,3′-di-*O*-butyryl, isobutyryl, and



nicotinoyl 4′,5′-cyclic phosphates of riboflavin have recently been isolated from treatment of FMN in pyridine with anhydrides of the corresponding aliphatic acids (9). Hydrolysis of the diacyl cyclic phosphates with formic acid in ethanol yielded the 2′,3′-diacyl 4′- or 5′-phosphates. Methylations of flavins with stronger base catalysts or higher temperatures than those presently used result in the formation of biflavins *via* bimolecular condensations at the 9-methyl function (10). Aqueous media, *e.g.* sodium hydroxide with dimethylsulfate, cannot be used since hydrolysis of the isoalloxazine system occurs, especially with 3-alkylflavins (11).

Elemental analyses for acetylated and methylated flavin derivatives are given in Table I. Tetraacetyl-3-methylriboflavin now has been made both by acetylation of 3-methylriboflavin, as well as by methylation of tetraacetylriboflavin as reported earlier (7). The 10-(2′-acetoxyethyl)flavin was prepared in the present instance from acetylation of the hydroxyethylflavin in acetic acid but has also been made using pyridine as solvent (12).

Table I

Elemental Analyses of Flavin Derivatives

Compound	Elemental composition	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
7,8-Dimethylisoalloxazine							
2′,3′-Diacetylriboflavin-5′-phosphate (a)	$LiC_{21}H_{24}N_4O_{11}P$	46.14	4.43	10.26	45.85	4.29	10.21
3,2′,3′,4′,5′-Pentamethylriboflavin	$C_{22}H_{30}N_4O_6$	59.18	6.77	12.59	59.04	6.42	12.72
2′,3′,4′,5′-Tetraacetyl-3-methylriboflavin	$C_{26}H_{30}N_4O_{10}$	55.91	5.41	10.03	55.78	5.42	9.97
10-Formylmethyl-3-methylflavin	$C_{15}H_{14}N_4O_3{\cdot}H_2O$	56.96	5.10	17.71	57.41	5.23	17.69
10-(2′-Acetoxyethyl)flavin	$C_{16}H_{16}N_4O_4$	58.53	4.91	17.07	58.72	5.02	16.86
10-(2′-Acetoxyethyl)-3-methylflavin	$C_{17}H_{18}N_4O_4$	59.64	5.29	16.36	59.49	5.25	16.29
10-(2′-Hydroxyethyl)-3-methylflavin	$C_{15}H_{16}N_4O_3$	59.99	5.37	18.65	59.88	5.38	18.71
10-(2′-Methoxyethyl)-3-methylflavin	$C_{16}H_{18}N_4O_3$	61.14	5.77	17.82	61.04	5.80	17.79
10-(5′-Acetoxypentyl)flavin	$C_{19}H_{22}N_4O_4$	61.61	5.98	15.13	61.50	6.14	14.96
10-(5′-Acetoxypentyl)-3-methylflavin	$C_{20}H_{24}N_4O_4$	62.48	6.39	14.58	62.35	6.32	14.62
10-(5′-Hydroxypentyl)-3-methylflavin	$C_{18}H_{22}N_4O_3$	63.14	6.48	16.36	63.24	6.47	16.39
10-(5′-Methoxypentyl)-3-methylflavin	$C_{19}H_{24}N_4O_3$	64.03	6.79	15.72	64.08	6.83	15.90
7,8-Dimethylalloxazine							
3-Methyllumichrome	$C_{13}H_{12}N_4O_2$	60.93	4.72	21.86	60.51	4.55	21.62

(a) Phosphorus was calculated for the lithium salt to be 5.67 and found to be 5.66%.

Table II

Mobilities of Flavin Derivatives on Thin-layer Chromatograms (a)

Compound	R_f value (b)	(c)
Riboflavin-5′-phosphate	0.64	0.01
Diacetylriboflavin-5′-phosphate	0.76	0.03
Riboflavin	0.79	0.05
Tetraacetylriboflavin	0.89	0.75
Pentamethylriboflavin	0.88	0.75
3-Methylriboflavin	0.82	0.10
Tetraacetyl-3-methylriboflavin	0.92	0.84
10-Formylmethylflavin	0.88	0.54
10-Formylmethyl-3-methylflavin	0.91	0.75
10-(2′-Hydroxyethyl)flavin	0.80	0.40
10-(2′-Acetoxyethyl)flavin	0.82	0.73
10-(2′-Acetoxyethyl)-3-methylflavin	0.86	0.81
10-(2′-Hydroxyethyl)-3-methylflavin	0.82	0.73
10-(2′-Methoxyethyl)-3-methylflavin	0.85	0.79
10-(5′-Hydroxypentyl)flavin	0.81	0.51
10-(5′-Acetoxypentyl)-3-methylflavin	0.90	0.87
10-(5′-Hydroxypentyl)-3-methylflavin	0.84	0.75
10-(5′-Methoxypentyl)-3-methylflavin	0.87	0.86
Lumiflavin	0.86	0.55
Lumichrome	0.84	0.68
3-Methyllumichrome	0.88	0.86

(a) Quanta/Gram sheets were developed by ascending solvents of (b) *n*-butyl alcohol:acetic acid:water (2:1:1, v/v/v) or (c) chloroform:methanol:acetic acid (18:1:1, v/v/v).

Mobilities of flavin derivatives upon thin-layer chromatography are presented in Table II. In all instances, as expected, the acetylated flavins migrate faster in the acetic organic solvents used. The most polar phosphate esters, *viz*. FMN and diacetyl FMN, have R_f values lower than the corresponding nonphosphorylated forms. This separation is even greater in the ammoniacal butanolic solvent, but partial deacetylation and formation of the 4′,5′-cyclic phosphates occur in this system. Intermediate values are found for flavins with hydroxyalkyl side chains, and the greatest mobilities occur with methylated and especially acetylated derivatives.

The structure of 2′,3′-diacetyl FMN was established by an extensive examination of chemical and spectral properties in comparison with known flavins. In addition to faster migration than FMN on thin-layer chromatograms developed with *n*-butyl alcohol:acetic acid:water, the diacetyl compound has an R_f value of 0.41, compared to 0.15 for FMN on paper using the same developing solvent. Like tetraacetylriboflavin, the acetylated FMN is not sensitive to photolysis in neutral solution, whereas riboflavin and FMN are photodecomposed to lumichrome and lumiflavin. Moreover, treatment with periodic acid does not degrade the acetylated side chain, unlike riboflavin and FMN, which are converted to the 10-formylmethylflavin (12). However, after hydrolysis of the 5′-phosphate, catalyzed by acid phosphatase at pH 5 (13), periodate treatment results in a new flavin detected on paper chromatograms. Diacetyl FMN is easily deacetylated by heating in strong acid and especially base (2). The pyrimidine portion of the isoalloxazine ring system is further hydrolyzed in strong base to the 2-keto-quinoxaline carboxylate derivative. Small amounts of the 4′,5′-cyclic FMN are also formed in weakly basic solutions. The flavin can be readily reduced by sodium dithionite to the colorless 1,5-dihydro form, which is reoxidized to the yellow flavoquinone upon aeration. The infrared spectrum of diacetyl FMN exhibits the additional carbonyl stretchings near 5.7 μ, indicative of the acetyl functions, and the absorbance due to such acetate esters is also seen near 8.2 μ, as similarly found in tetraacetylriboflavin and other acetylated flavins. Absorbances caused by the phosphate ester in the diacetyl compound are prominent, near 9 μ, as also seen with FMN. In addition to confirming the nature of other hydrogens present in diacetyl FMN, the nmr spectrum further delineates the two methyl groups found at chemical shift positions of 1.8 and 2.3 δ. The more upfield position can be attributed to the methyl of the 2′-acetyl, as the shift position is similar to that observed for the monoacetyl derivative of 2′-hydroxyethylflavin, whereas the resonance of the methyl of the 5′-acetoxypentylflavin in more downfield.

Ultraviolet-visible spectra typical for flavins are exhibited by their acetylated and methylated derivatives in weakly basic, neutral, and weakly acidic media. There are only moderate shifts in the positions of primary absorption bands centered near 450, 370, and 265 nm and but minor changes in amplitudes, even above the pK_a near 10 for ionization of the flavin 3-imino hydrogen (14). There are essentially no changes in the absorption spectra from

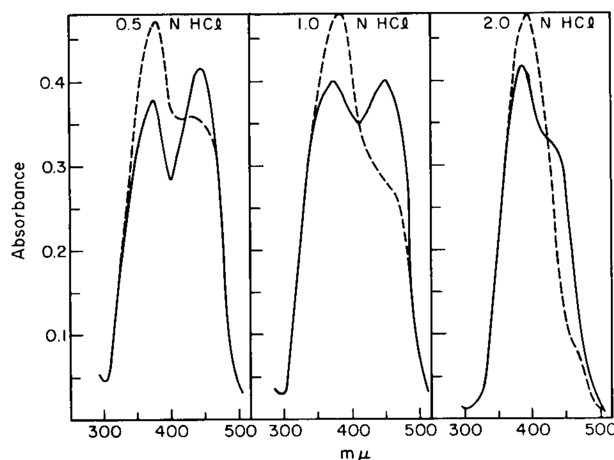
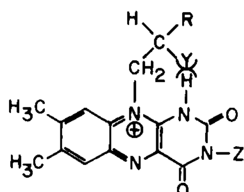


Figure 1. Ultraviolet-visible spectra of diacetyl FMN (——) and FMN (----) in hydrochloric acid solutions. Compounds were approximately 3.5 x 10^{-5} *M*.

neutral to slightly acid pH, as is also characteristic of other 7,8-dimethyl-10-alkylisoalloxazines. In stronger acid, however, peracetylated or permethylated flavins are unlike the parent compounds in that the protonated isoalloxazinium species are less in evidence. As seen in Figure 1, the decrease at 446 nm and increase at 385 to 390 nm, attributed to protonation at the N-1 position of isoalloxazines (8,14) as illustrated in Formula 2, is typical for FMN (and other



flavins with hydroxyalkyl and alkyl side chains) but partially prevented in the diacetyl analog.

Fluorescence emissions of acetylated and methylated flavins at 520 nm also reflect their shift to lower pK_a values. As generally true for 7,8-dimethyl-10-alkylisoalloxazines (15), the anionic quinoid forms, which exist in alkaline solution (0.1 *N* sodium hydroxide), are essentially nonfluoresent, as is also true for the protonated quinoid species. The large decrease in fluoroescence, even in dilute acid (0.1 *N* hydrochloric acid), is mainly attributable to collisional quenching (16). Again, though, the residual fluorescences, for example of FMN and its diacetyl derivative, in stronger aicd (1 *N* hydrochloric acid) reflect the more extensive protonation of the flavin without acetylated or methylated side chain, since the fluorescence ratio of 0.1 : 1.1 for FMN:diacetyl FMN is proportional to the ratio of their absorbancies at 450 nm.

As shown in Figure 2, the spectrum of tetraacetylriboflavin in 1 *N* hydrochloric acid still closely resembles that obtained for acetylated or nonacetylated flavins in neutral solutions, whereas riboflavin is largely protonated in 1 *N* hydrochloric acid. The same behavior was found for other tetraacetyl derivatives, *e.g.* those of D- and L-lyxoflavin and 3-methylriboflavin. With the monoacetyl derivatives of 2′-hydroxyethyl- and 5′-hydroxypentylflavins, an intermediate and only slight prevention of ring protonation is seen, respectively. In all cases, hydrolysis of the acetyl functions, such as by heating to 100° in the 1 *N* hydrochloric acid for 10 to 15 minutes, abolishes the masking effect of the acetyl function, as is seen by loss of the maxima near 450 and 370 nm and minimum at 400 nm with increase at 390 nm. An isosbestic point at 420-425 nm is observed in all such interconversions of neutral and monoprotonated flavins.

From these results, it is clear that an acetyl group proximal to nitrogen 1 is more effective in decreasing its protonation than one further away. Also, two acetyl functions are more effective than one, and four are better than two. Methylations at corresponding positions of 10-substituted flavins result in similar but less marked effects. Methylation at position 3 has no significant influence on the protonation of flavins. Absence of even hydroxyl groups in the side chain allows a shift to higher pK_a values. Hence, it is probable that steric hindrance of protonation is the predominent factor involved.

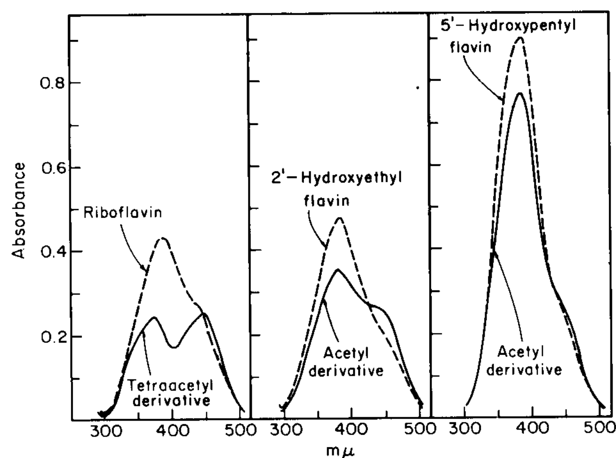


Figure 2. Ultraviolet-visible spectra of flavins and their acetyl derivatives. Compounds in 1 *N* hydrochloric acid were approximately 2.2 x 10^{-5} *M* for riboflavin, 2′-hydroxyethylflavin, and their acetyl derivatives, and 4.2 x 10^{-5} *M* for 5′-hydroxypentylflavin and its acetyl derivative.

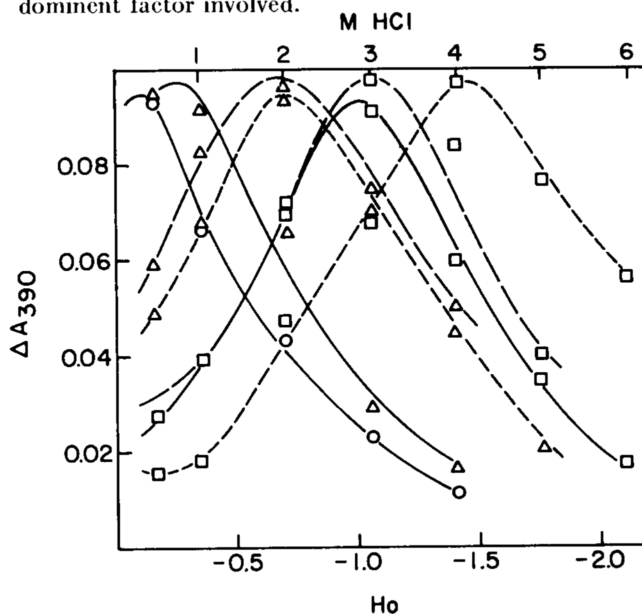


Figure 3. The effect of acidity (*M* hydrochloric acid, H_0) on the protonation (ΔA_{390}) of flavins with different side chains in position 10. Compounds were approximately 3 x 10^{-5} *M*: 10-(5′-hydroxypentyl)flavin (–o–), 10-(2′-hydroxyethyl)flavin (–△–), 10-(2′-methoxyethyl)-3-methylflavin (--△--), 10-(2′-acetoxyethyl)flavin (--△--), riboflavin (–□–), pentamethylriboflavin (--□--), and tetraacetylriboflavin (--□--).

Quantitation of the effects of substituent changes on protonation of flavins is easily followed by measuring the change in absorbance at 390 nm, which increases to a maximum as a 7,8-di- or 3,7,8-trimethylisoalloxazine becomes fully monoprotonated. The difference in this abosrbance (ΔA_{390}) from neutral to acidic species goes through a maximum corresponding to the midpoint or pK_a for the flavin. The data for such spectrophotometric titrations of representative flavins in varying concentrations of hydrochloric acid are shown in Figure 3. The known acidities for such acid solutions (17) allow one to express accurately the pK_{FH^+} values for the equilibrium of $F + H^+ \rightleftharpoons FH^+$, where F is neutral flavin and FH^+ is the protonated form. These values are listed in Table III. It can be seen

Table III

pK_{FH^+} Values for Flavin Derivatives

10-Substituent	pK_{FH^+} (a)
5′-Hydroxypentyl	0.5
5′-Methoxypentyl	0.5
5′-Acetoxypentyl	0.4
2′-Hydroxyethyl	0.0
2′-Methoxyethyl	-0.5
2′-Acetoxyethyl	-0.7
D-Ribityl	-1.0
2′,3′,4′,5′-Tetramethyl-D-ribityl	-1.2
2′,3′,4′,5′-Tetraacetyl-D-ribityl	-1.5

(a) The Hammett acidity function, $H_o = pK_{FH^+} - \log (C_{FH^+}/C_F)$, and at the titration midpoint, $\log (C_{FH^+}/C_F) = 0$ and $H_o = pK_{FH^+}$.

that an increase in size of a group that is distant from the base center that becomes protonated (N-1) has little or no influence, whereas a close and sizable substituent can shift the pK_{FH^+} value by more than a log unit in the negative direction.

The sensitivity of N-1 in flavin coenzymes to steric restriction in the addition of hydrogen necessary in their biological oxidation-reduction reactions must be markedly altered in many instances by the enveloping protein milieu of the enzyme to which they are bound.

EXPERIMENTAL

Analytical Methods.

Light absorption spectra were determined with a Cary Model 14 recording spectrophotometer. Measurements of absorbance at 390 nm were also made with a Bausch and Lomb Spectronic 20. Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorimeter, using a xenon lamp, photomultiplier tube 1P21, and slit arrangement No. 3. Excitation was at 450 nm and emission at 520 nm. Infrared spectra were obtained with potassium bromide press pellets (1 mg./300 mg.) using a Perkin-Elmer Infracord spectrometer. The nmr spectra, in deuterium oxide or deuteriochloroform, were measured in a Varian A-60A spectrometer. Thin-layer chromatograms were run on sheets of Brinkmann silica gel N-HR and of Q5W Quanta/gram (Quantum Industries). These were developed by ascending solvents of chloroform:methanol: acetic acid (18:1:1, v/v/v), *n*-butyl alcohol:acetic acid:water (2:1:1, v/v/v), and *n*-butyl alcohol:2 *N* ammonium hydroxide:ethanol (3:1:1, v/v/v). Compounds were visualized both directly and, more sensitively, by their fluorescence under a Mineralight uv lamp.

Whatman No. 1 paper chromatograms were developed with the ascending butanolic solvents. Melting (decomposition) points were determined with a Fisher-Johns melting point apparatus. Microanalysis was by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

Flavins.

D-Riboflavin-5′-phosphate was the hydrated sodium salt from Sigma Chemical Co. D-Riboflavin was purchased from Eastman Organic Chemicals. D- and L-Lyxoflavins and 3-methyl-D-riboflavin were gifts from the Merck Sharp and Dohme Research Labs. The 3-methylriboflavin was also synthesized as previously described (7,18). Periodate oxidation of 3-methylriboflavin, as described for obtaining 10-formylmethylflavin from riboflavin (12), resulted in a 92% yield of 10-formylmethyl-3-methylflavin monohydrate (233-235° dec.) which, in turn, could be readily photolyzed to 3-methyllumichrome (>300° subl.). The 2′-hydroxyethyl- and formylmethylflavins were from The Upjohn Co. 5′-Hydroxypentylflavin was synthesized from reaction of ω-hydroxylpentylamine with 1,2-dinitro-4,5-dimethylbenzene, reduction of 2-nitro-4,5-dimethyl-*N*-(ω-hydroxypentyl)aniline to the 2-imino compound, and condensation of the latter with alloxan (19). Lumiflavin was made by condensing 2-*p*-carboxyphenylazo-4,5-dimethyl-*N*-methylaniline with barbituric acid (20).

Acetylations.

For synthesis of diacetyl FMN, commercial sodium FMN hydrate was first purified as the lithium salt by elution from DEAE-cellulose (Cl^-) with a linear gradient of lithium chloride and the lithium FMN then converted to pyridinium FMN by passage through Amberlite IR-120 (H^+), addition of pyridine to the aqueous effluent, and evaporation of the solution to dryness (21). Pyridinium FMN (1 g.) was stirred into 100 ml. of 1:9 (v/v) acetic anhydride:anhydrous pyridine containing 0.1 ml. of 70% perchloric acid as catalyst. The mixture was stirred at 40° for 3 to 4 hours for complete solution and evaporated to dryness. The residue was stirred into 25 ml. of water and the *p*H adjusted to 6 by dropwise addition of 1 *N* lithium hydroxide. The solution was extracted twice with equal volumes of chloroform and the aqueous phase poured over a column (2 x 30 cm.) of DEAE-cellulose (Cl^-). After washing with water, diacetyl FMN eluted as the first major band with a linear gradient of lithium chloride (up to 0.05 *M*). The aqueous solution was evaporated to dryness at 45°, the residue stirred into a few ml. of methanol, and product precipitated with 4:1 (v/v) acetone: diethyl ether. Resuspension in acetone and precipitation with ether was done twice more to remove residual lithium chloride. After drying the hydrated lithium salt of diacetyl FMN at 110° to constant weight (5.5% water loss), the pure compound was obtained in 40% yield.

Acetylation of nonphosphorylated flavins was done as described earlier for the synthesis of tetraacetylriboflavin (6). Essentially quantitative yields of the compounds were obtained after chloroform extraction of the flavins diluted with water following treatment for 30 minutes at 40° in 10 volumes of a 1:1 (v/v) mixture of acetic anhydride:acetic acid containing a trace of 70% perchloric

acid. The acetylated flavins were found pure on thin-layer chromatograms.

Methylations.

Permethylation of flavins was accomplished by modification of a procedure useful for glycosides (22). For this, 0.1 g. of flavin was reacted with 0.5 ml. of methyl iodide in 10 ml. of dimethylformamide with 0.5 g. of silver oxide as catalyst. The mixture was stirred overnight at room temperature and filtered. The solids so removed were rinsed with 10 ml. of chloroform, which was also filtered. The combined filtrates were shaken with 10 ml. of 0.5% aqueous potassium cyanide and the organic phase washed with 5 ml. each of water, N hydrochloric acid, water, 5% aqueous sodium bicarbonate, and, again, water. Each aqueous phase was back-extracted with the same 5-ml. portion of chloroform, which was finally combined with the principal organic phase. This combined organic phase was dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to dryness. The product was taken up in 1 ml. of acetone, which was stirred into 9 ml. of diethyl ether. The precipitate was collected by filtration, rinsed with ether, and dried over phosphorus pentoxide for 44-55% yield of flavin methylated on both the original side-chain hydroxyls and 3-imino function. Complete methylation was confirmed by ir, which showed disappearance of hydroxyl stretching near 3 μ and appearance of the methoxy C-O near 9 μ; also, nmr spectra demonstrated loss of hydroxyls and the presence of methoxyl hydrogens near 3.5 δ.

Specific methylation only at N-3 in the isoalloxazine nucleus was achieved essentially as described for formation of tetraacetyl-3-methylriboflavin (7). For this, the side-chain hydroxyls of a flavin were first protected by acetylation before reaction of the resulting acetoxyflavin overnight at room temperature with excess methyl iodide and anhydrous potassium carbonate in dimethylformamide. As in permethylations, the suspension was filtered, the solids were removed by filtration and rinsed with chloroform, and the combined organic filtrates washed by extractions with water. After back-extraction of the aqueous phases with more chloroform, the combined organic phase was dried over anhydrous sodium sulfate, filtered, and the filtrate evaporated to a small volume before stirring into ether to precipitate the product. The acetoxyalkyl-3-methylflavin was collected by filtration, rinsed with ether, and dried for 65-75% yield. To obtain the hydroxyalkyl-3-methylflavins, the acetylated 3-methylflavin was refluxed for 2 hours in 2 N hydrochloric acid, the solution evaporated to dryness, the residue suspended in a small volume of water, and the product collected by filtration and dried for 75-85% yield.

REFERENCES

(1) This work was supported in part by Research Grant AM-04595 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, U.S.P.H.S., and in part by funds made available through the State University of New York.

(2) R. Kuhn, H. Rudy, and F. Weygand, *Ber.*, **69**, 1543 (1936).

(3) S. M. H. Christie, G. W. Kenner, and A. R. Todd, *J. Chem. Soc.*, 46 (1954).

(4) H. S. Forrest, H. S. Mason, and A. R. Todd, *J. Chem. Soc.*, 2530 (1952).

(5) D. B. McCormick, *J. Heterocyclic Chem.*, **4**, 629 (1967).

(6) D. B. McCormick, *ibid.*, **7**, 447 (1970).

(7) P. Hemmerich, *Helv. Chim. Acta*, **47**, 464 (1964).

(8) K. H. Dudley, A. Ehrenberg, P. Hemmerich, and F. Müller, *ibid.*, **47**, 1354 (1964).

(9) T. Tanaka, H. Tanaka, and N. Tsubaki, *Chem. Abstr.*, **78**, 511,97969u, and 9797n (1973).

(10) P. Hemmerich, B. Prijs, and H. Erlenmeyer, *Helv. Chim. Acta*, **42**, 2164 (1959).

(11) D. B. McCormick and W. Föry, *J. Pharm. Sci.*, **57**, 841 (1968).

(12) H. H. Fall and H. G. Petering, *J. Am. Chem. Soc.*, **78**, 377 (1956).

(13) D. B. McCormick and M. Russell, *Comp. Biochem. Physiol.*, **5**, 113 (1962).

(14) P. Hemmerich, C. Veeger, and H. C. S. Wood, *Angew. Chem. Intern. Edit. Engl.*, **4**, 671 (1965).

(15) R. Kuhn and G. Moruzzi, *Ber.*, **67**, 888 (1934).

(16) G. Weber, *Biochem. J.*, **47**, 114 (1950).

(17) M. A. Paul and F. A. Long, *Chem. Rev.*, **57**, 1 (1957).

(18) J. C. M. Tsibris, D. B. McCormick, and L. D. Wright, *J. Biol. Chem.*, **241**, 1138 (1966).

(19) B. M. Chassy, C. Arsenis, and D. B. McCormick, *ibid.*, **240**, 1338 (1965).

(20) P. Hemmerich, *Helv. Chim. Acta*, **37**, 1242 (1956).

(21) J. G. Moffatt and H. B. Khorana, *J. Am. Chem. Soc.*, **80**, 3761 (1958); *ibid.*, **83**, 649 (1961).

(22) R. Kuhn, H. Trischman, and I. Löw, *Angew. Chem.*, **67**, 32 (1955).