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An Experimental Demonstration of the Nuclear Magnetic Resonance Assignments in the 6,7-Dimethylisoalloxazine Nucleus*

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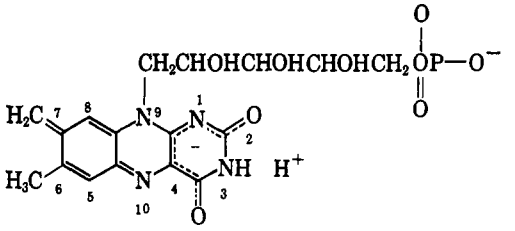
We have observed (Figure 1) that the amplitude of the downfield methyl peak in the n.m.r spectrum of riboflavin 5′-phosphate (FMN) decreases when the material is heated at 90–95° in D_2O solution buffered at pH 6.8 to 6.9.[2] This is the optimum pH for effecting the reaction (approximate rate constant 2.4×10^{-4} sec.$^{-1}$). At higher pH (7.5) more rapid decomposition complicates the result, and in unbuffered solution (pH 5.8), we have been unable to observe any reaction during comparable heating periods. Heating periods of up to 3 hr. fail to give any evidence of a decrease in amplitude of any other peak.

We attribute the selective decrease in amplitude of the downfield methyl peak to an exchange of the C-7-methyl protons with solvent through the intermediacy



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of **1**. The facile exchange is then understandable since the possibility of forming a highly delocalized anion undoubtedly helps compensate for the loss of the resonance stabilization associated with the aromatic ring. No completely conjugated, delocalized structure can be invoked which might account for such a facile exchange occurring at the C-6 methyl. On chemical grounds then, the assignment of the downfield methyl peak to the C-7 methyl seems supportable, but we can also offer the following evidence that this assignment is indeed correct.

Comparison of the spectra of lumiflavin (6,7,9-trimethylisoalloxazine, Figure 2) and its 6-ethyl analog and of *o*-xylene and *o*-ethyltoluene (in CS_2)[3] shows that

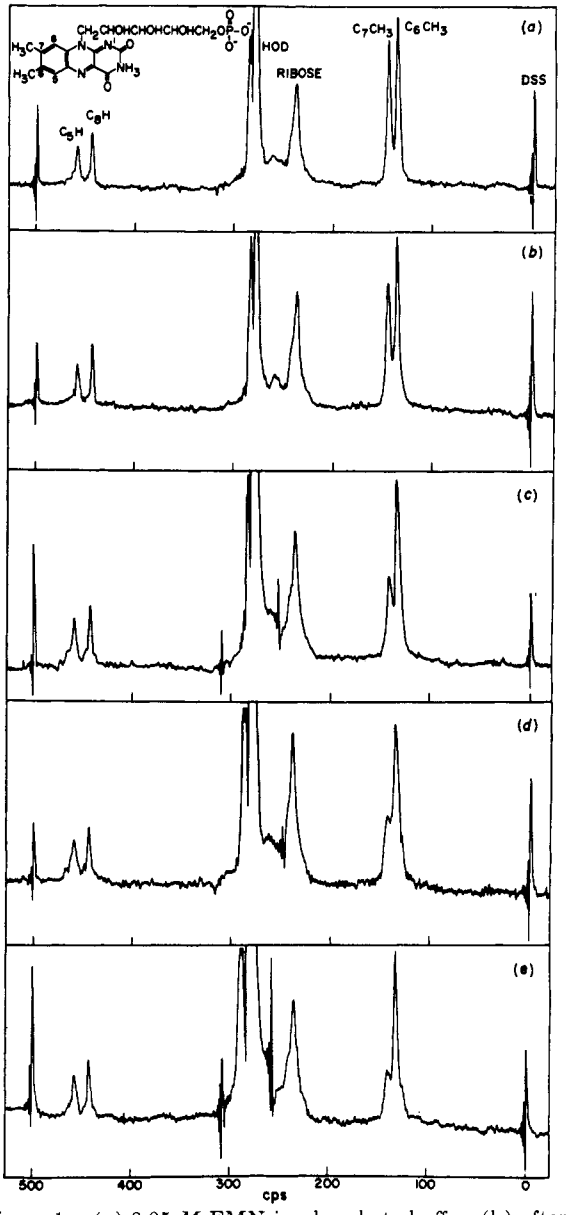

Figure 1.—(a) 0.05 *M* FMN in phosphate buffer, (b) after 55 min., at 92–93°, (c) after 1.5 hr., (d) after 2 hr., (e) after 3 hr. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was added as internal standard. Symmetrically spaced lines near the water peak in some spectra are spinning side bands.

in both these series, conversion of methyl to ethyl results in the appearance of the methylene (quartet) resonance 19 to 21 c.p.s. downfield from the shift of its methyl precursor. Also there is no shift (to within 2 c.p.s.) of the unsubstituted methyl resonance. Thus the downfield peak at 138 c.p.s. in the isoalloxazines must be assigned to the C-7 methyl.

A dimerization of riboflavin under basic conditions, considered[4] to occur through the C-7 methyls, has previously been demonstrated and attributed to the intermediacy of a methide structure similar to **1**. The ready accessibility of **1** in essentially neutral solution is, however, an interesting aspect of the chemistry of these materials.

Based on the comparison of the spectra of lumiflavin and 8-deuteriolumiflavin, we assign the upfield aromatic

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(2) Meter readings, without correction for differences in activity in D_2O.

(3) For 0.1 *M* solutions (CS_2) the methyl shifts are *o*-xylene, −159 c.p.s., and *o*-ethyltoluene, −161 c.p.s. The CH_2 quartets are centered at −180.5 c.p.s. for *o*-ethyltoluene and −183 c.p.s. for ethylbenzene. The shifts are expressed relative to external hexamethyldisiloxane.

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

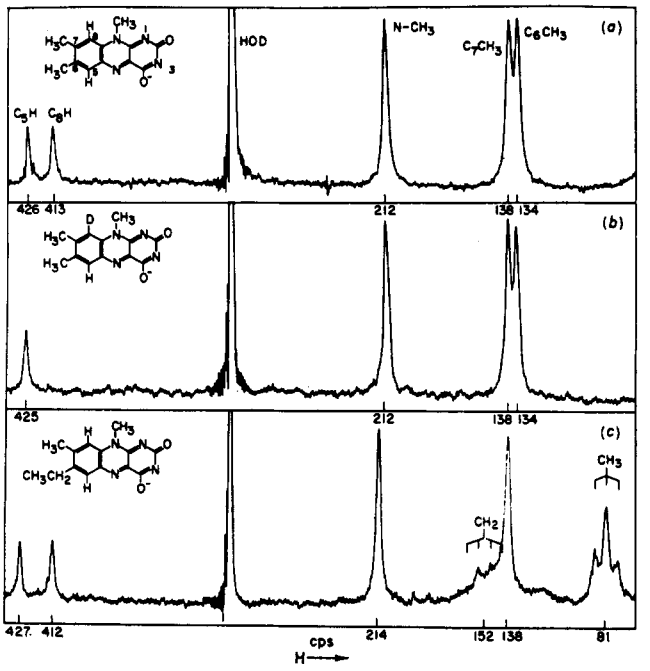

Figure 2.—(a) Lumiflavin, (b) 8-deuteriolumiflavin, (c) 6-ethyl-7,9-dimethylisoalloxazine. Solutions are 0.05 *M* in 0.2 *M* NaOD and shifts are relative to external hexamethyldisiloxane.

proton in the isoalloxazines to the C-8–H (Figure 2). Of interest is the fact that the order of methyl and aromatic C–H peaks is in agreement with that predicted on the basis of the Pullmans' Hückel calculation[5] of electron densities in the 6,7-dimethylisoalloxazine nucleus.

The isoalloxazines needed for determination of the assignments were prepared by coupling diazotized *p*-aminobenzoic acid to the appropriately substituted aniline followed by a Tishler condensation with barbituric acid.[6] N,3,4-Trimethylaniline was deuterated at positions 2 and 6 by refluxing the hydrochloride in D_2O for 3 days. Diazo coupling of position 6 yielded the requisite monodeuterated azo intermediate. This ultimately gave lumiflavin specifically deuterated at C-8.

Experimental[7]

The exchange reaction was carried out on a 0.05 *M* D_2O solution of FMN (Sigma Chemical Co.) in 0.2 *M* phosphate buffer, pH 6.8 to 6.9.[3] Descending paper chromatography using Whatman No. 1, solvent system butanol–acetic acid–water (3:1:1), indicated very slight, if any, decomposition of FMN during heating under these conditions.

N,3,4-Trimethyl-2,6-dideuterioaniline.—N,3,4-Trimethylaniline[8] (15 g., 0.11 mole) was dissolved in 10.5 ml. of concentrated hydrochloric acid; then the solvent was evaporated *in vacuo* to give the hydrochloride (18.6 g.). This was refluxed in 15 ml. of D_2O for 24 hr.; the D_2O was removed *in vacuo*. This cycle was repeated twice. After the final cycle, the residue was dissolved in a dilute solution of K_2CO_3 and the solution was thoroughly extracted with ether. The ether was dried and evaporated, and the residual oil was distilled yielding 13.85 g. (92.5%) of nearly colorless oil, b.p. 105 to 106° (42 mm.). In the n.m.r. spectrum of the nondeuterated material[8] the aromatic region closely approximates that of an A_2B system. In the deuterated material this multiplet is collapsed to a single unsplit peak.

N,3,4-Trimethyl-6-(*p*-carboxyphenylazo)-2-deuterioaniline. N,3,4-Trimethyl-2,6-dideuterioaniline (10 g., 0.072 mole) was dissolved in 200 ml. of 85% formic acid and cooled to −15°. A solution of diazotized *p*-aminobenzoic acid (prepared from 9.6 g. of PABA) was added dropwise. The solution was stirred at −10° for 1 hr., then placed in an ice bath and allowed to stir overnight. The deep purple precipitate was then filtered and dried (yield 8 g.). It was dissolved in 100 ml. of 1 *N* NaOH and the red azo dye precipitated by addition of 50 ml. of 2 *N* acetic acid. After drying it was further purified by continuous extraction with benzene, yielding 4.9 g., m.p. 203 to 208°. It did not depress the melting point of a sample of undeuterated material, m.p. 204–208°, prepared similarly[8] and was used directly without further purification.[8]

6,7,9-Trimethyl-8-deuterioisoalloxazine (8-Deuteriolumiflavin).—N,3,4-Trimethyl-6-(*p*-carboxyphenylazo)-2-deuterioaniline (2 g.) was suspended in 20 ml. of butanol and 6–8 ml. of acetic acid, barbituric acid (1.4 g.) was added, and the mixture was refluxed and stirred magnetically for 5 hr. The product was isolated and purified as previously described for lumiflavin.[8] The yield was 1.25 g., m.p. 333–335°. It did not depress the melting point of lumiflavin and was identical with lumiflavin on paper chromatography using the solvent system dimethylformamide–butanol–water (2:6:2).

N,3-Dimethyl-4-ethylaniline.—3-Methyl-4-ethylaniline[9] was N-methylated by a standard method[10]: formation of the *p*-toluenesulfonamide, methylation in dilute base with dimethyl sulfate, and acid-catalyzed hydrolysis of the sulfonamide to the amine. The product was obtained as a colorless oil, b.p. 112–115° (13 mm.), in 82% over-all yield.

Anal. Calcd. for $C_{10}H_{15}N$ (mol. wt., 149.33): N, 9.38. Found: N, 9.77.

N,3-Dimethyl-4-ethyl-6-(*p*-carboxyphenylazo)aniline.—N,3-Dimethyl-4-ethylaniline (9 g., 0.061 mole) in 165 ml. of 85% formic acid was cooled to −15°. A solution of diazotized *p*-aminobenzoic acid (prepared from 8 g. of PABA) was added dropwise. Subsequent treatment was identical with that described for the azo compound above. Continuous extraction of the isolated material with benzene followed by evaporation of the solvent yielded 4.8 g. of carmine red material ,m.p. 175–183° dec. Recrystallization from toluene gave pure material, m.p. 187–188° dec.

Anal. Calcd. for $C_{17}H_{19}N_3O_2$ (mol. wt., 297.37): C, 68.67; H, 6.44; N, 14.16. Found: C, 68.54; H, 6.63; N, 13.75.

6-Ethyl-7,9-dimethylisoalloxazine.—N,3-Dimethyl-4-ethyl-6-(*p*-carboxylphenylazo)aniline (2 g.) was suspended in 20 ml. of butanol and 7 ml. of acetic acid. After adding 1.4 g. of barbituric acid the mixture was refluxed with stirring for 5 hr. The solution was cooled, 30 ml. of isopropyl ether was added, and the product was filtered. Solution in 50 ml. of 2 *N* NaOH followed by reprecipitation with an equal volume of 2 *N* acetic acid gave, after drying, 2.1 g. of greenish material, m.p. 330–335° dec. Solution in a minimum volume of 97% formic acid followed by partial reprecipitation by addition of ethanol yielded, after three times, material of m.p. 338–340° dec.

Anal. Calcd. for $C_{14}H_{14}N_4O_2$ (mol. wt., 270.20): C, 62.61; H, 5.22; N, 20.73. Found: C, 61.58; H, 6.05; N, 20.76.

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(7) N.m.r. spectra were obtained at 60 Mc. on a Varian V-4310 instrument. Boiling points and melting points are uncorrected. Melting points were determined with a Mel-Temp apparatus. The microanalyses were performed by Dr. S. M. Nagy and his associates at the Microanalytical Laboratories, Massachusetts Institute of Technology, Cambridge, Mass.

(8) P. Hemmerich, S. Fallab, and H. Erlenmeyer, *Helv. Chim. Acta*, **39**, 1242 (1956).

(9) N. P. Buu-Hoí, B. Eckert, and R. Royer, *Compt. rend.*, **233**, 627 (1956).

(10) "Methoden der Organischen Chemie" (Houben-Weyl), Vol. XI/1, 4th Ed., Georg Thieme Verlag, Stuttgart, 1957, p. 229.