Syntheses and Properties of Amides of *N*-10-Carboxyalkylflavins with Pyridoxamine and It's 5′-Phosphate (1)

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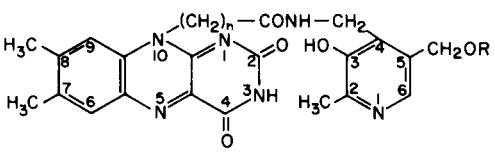
Received May 10, 1972

Flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD), coenzymes derived from the vitamin, riboflavin, are natural isoalloxazines, which are essential to organisms wherein they function with specific proteins to effect catalysis of oxidation-reduction reactions (2). The complexing propensities of the ring systems of such isoalloxazines are recognized as an essential part of their binding to and function with enzymes (3). Among those amino acid residues within proteins, which undoubtedly participate in formation of molecular complexes with flavins, is the *p*-hydroxyphenyl system of tyrosine. The generalized ability of phenols to complex avidly with flavins has been well documented, both with inter- (4) and now intramolecular cases, which include tyrosine (5-9). The associations of flavin coenzymes with tyrosyl residues in different flavoproteins have been evidenced by indirect means (10-12), and recently, the interaction of FMN with a tyrosyl side chain within a bacterial flavodoxin has been established by x-ray diffraction (13). Another FMN-dependent enzyme, which clearly must have molecular association occurring between the flavin coenzyme and phenolic substrate prior to oxidation of the latter, is pyridoxine (pyridoxamine) 5′-phosphate oxidase (14). The complexing between the flavin and substrate of this oxidase leads to perturbations in the spectra of each (15), which could be better understood through the use of spectroscopic models.

The present paper describes the syntheses, purifications, and some spectral properties of amides of flavins, which have the 7,8-dimethylisoalloxazine system of the natural coenzyme, with pyridoxamine and the 5′-phosphate ester of this vitamin B_6 form. A generalized formula for these flavin amides is shown below, where n varies from 2 to 5.



(R = H , PO_3H_2)

Syntheses were accomplished by converting the *N*-10-ω-carboxyalkylflavins to *p*-nitrophenyl esters as described previously (5). These esters were then condensed with pyridoxamine or its 5′-phosphate (as the triethylammonium salt) in dimethyl sulfoxide and the crude products obtained after successive solubilizations, extractions, and precipitations from acid and alkaline solutions.

Purifications of the *N*-pyridoxyl flavin amides (n = 2,5) were accomplished by extraction into phenol from which the compounds were recovered after removal of phenol by ether. The *N*-(5′-phospho)pyridoxyl flavin amides (n = 2-5) were more easily purified by column chromatography on diethylaminoethyl-cellulose (chloride form), on which the stronger anionic phosphate esters of the flavin amides were more avidly retained than were the flavin carboxylic acids; the latter largely result from

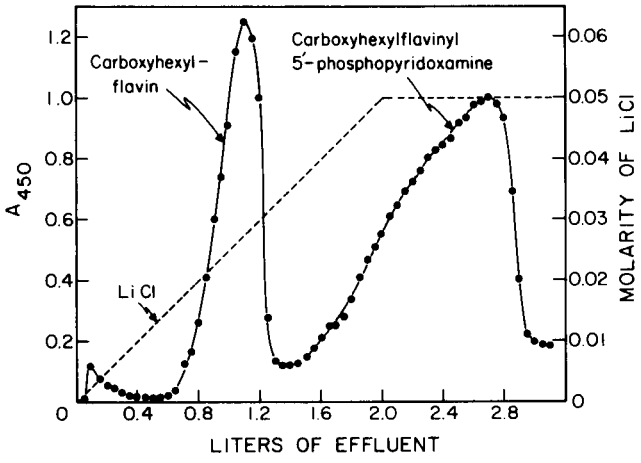


Figure 1. Elution pattern of carboxyhexylflavin and the amide of 5′-phosphopyridoxamine from a column of diethylaminoethyl-cellulose. Approximately 50 mg. of crude amide after acid precipitation was taken up in a few ml. of water while neutralizing with lithium hydroxide, poured over a 2.5 x 15 cm. column of DEAE-cellulose (0.93 meq./g.) in the chloride form, and eluted with lithium chloride as shown.

hydrolyses of the remaining *p*-nitrophenyl esters. A representative example of such column chromatographic purification is given in Figure 1, where the *N*-10-carboxyhexylflavinyl 5′-phosphopyridoxamine is seen to elute after the *N*-10-carboxylhexylflavin from which the amide was made.

The ultraviolet-visible absorption spectra of the flavin amides distinguish them from the parent flavin acids, especially in neutral to alkaline solutions where the contribution of the B_6 absorption can be discerned best. The rather typcial spectra obtained in aqueous alkaline, neutral, and acid solutions of *N*-10-carboxyhexylflavin and the *N*-10-carboxyhexylflavinyl 5′-phosphopyridoxamine are illustrated in Figure 2. The maximum for the anionic B_6 chromophore near 310 nm is readily seen at

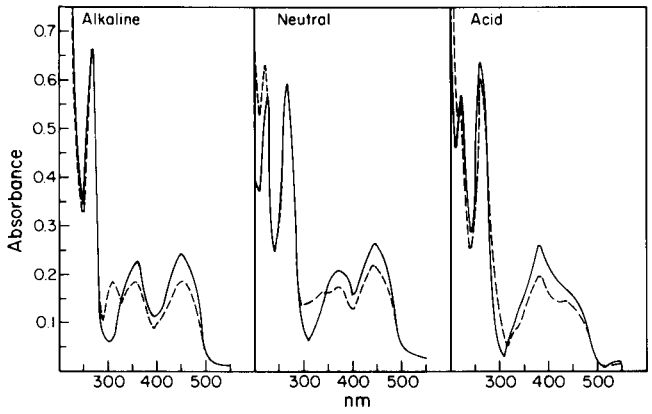


Figure 2. Absorption spectra of carboxyhexylflavin (——) and the amide of 5′-phosphopyridoxamine (- - - -) in alkaline, neutral, and acid solution. Flavins were solubilized and diluted into 0.5 *M* sodium carbonate, 0.01 *M* sodium phosphate (*p*H 7), and 0.5 *M* hydrochloric acid to final concentrations of 2×10^{-5} *M* for the carboxyhexylflavin and 1.7×10^{-5} *M* for the amide.

*p*H values sufficiently high to ionize both the 3-hydroxyl and the 5′-phosphate functions of the B_6 and, at least partially, the 3-imino proton of the flavin moiety. Upon lowering the *p*H, the absorption attributable to B_6 in the near-ultraviolet decreases. Strongly acidic conditions lead to spectra characteristic of the protonated flavin, where a maximum near 390 nm has replaced the maxima at 447 and 375 nm found in the neutral species. Although there are differences between spectra of phosphorylated and non-phosphorylated amides, and even within a given series where the number of methylenes in the flavin side chain (n) varies, these are expectedly small. Even the changes seen in absorption difference spectra between the flavin amides versus flavin plus pyridoxamine or its phosphate are not especially striking, though some hypochromicity and slight shifts in maxima result from the intramolecular associations, which occur between flavin and B_6 chromophores in the amides.

A much more sensitive indicator of the interaction between the flavin and B_6 moieties is afforded by measuring the fluorescence emission. As seen from the data in Table I, when only the flavin is light excited by

TABLE I

Relative Fluorescence of the *N*-10-Carboxyhexylflavin Amides of Pyridoxamine and 5′-Phosphopyridoxamine and Their Component Parts (a)

Substance (4×10^{-5} M each)	Excitation (λ nm)	Fluorescence (%) Flavin (520 nm)	B_6 (400 nm)
Flavin	445	100	-----
B_6	335	-----	100
Flavin + B_6	445	100	-----
	335	-----	60
Flavinyl pyridoxamine	445	29	-----
	335	-----	0.8
Flavinyl phospho-	445	30	-----
pyridoxamine	335	-----	0.3

(a) Compounds were dissolved in 0.01 *M* sodium phosphate buffer, *p*H 7. (b) Both free amine and the 5′-phosphate gave similar results at these concentrations.

irradiation at 445 nm, the presence of either free pyridoxamine or its 5′-phosphate in very dilute neutral solutions, where flavin and B_6 are equimolar, results in no significant quenching of the flavin fluorescence observed at 520 nm. Excitation of the B_6 (335 nm) within these same solutions, however, does indicate that some intermolecular interactions occur, since the emission of B_6 fluorescence at 400 nm (a minimum in flavin absorption) is diminished. Raising the concentration of the B_6 even leads to a readily detectable loss in flavin fluorescence. When the flavin and B_6 are within the same molecule, the loss in fluorescence of both flavin and B_6 are marked, especially with the latter. A large change in entropy results from constraint of the two halves of these intramolecular cases, even when the chains separating the interacting partners are long (n = 5), as exemplified by the data presented for the *N*-10-carboxyhexylflavinyl amides. Shorter chains between the complexing halves may result in even more spectacular loss of fluorescence. The presence of an anionic phosphate does not seem to exert much effect on the degree of fluorescence quenching, either of flavin or B_6 moieties, although the nature of quenching could conceivably be altered somewhat. Both

a "static" mechanism, through formation of dark (non-fluorescent) complexes, and a "dynamic" mechanism, by which some of the energy from light absorbed is dissipated kinetically through collisions, likely operate. In this connection, it is interesting that the association of pyridoxal 5′-phosphate with FMN or FAD has been found to cause changes in absorption spectra and increases the stabilities of the reactants to light and heat (16).

A detailed study of the fluorescence properties of the B_6-flavin amides has been undertaken to ascertain more fully the nature of the associations which occur (17). It is already clear from the present work, though, that there is a considerable and increasing tendency for association of a flavin coenzyme and substrate vitamin B_6 under the constraints offered by an enzyme such as the pyridoxine (pyridoxamine) 5′-phosphate oxidase.

EXPERIMENTAL

Syntheses.

Commercial hydrochloride salts of pyridoxamine and the 5′-phosphate ester were converted to the free base derivatives by treating with minimal volumes of aqueous lithium hydroxide, two mole equivalents for pyridoxamine dihydrochloride and one mole equivalent for the 5′-phosphate monohydrochloride. The free bases were then precipitated by stirring in an equal volume of absolute ethanol followed by 10 volumes of acetone and 25 volumes of diethyl ether. The suspensions were centrifuged to remove the organic solvent containing lithium chloride and precipitate the syrupy pyridoxamine or white amorphous 5′-phosphate. These were stirred in a small volume of methanol and again precipitated with acetone and ether. The compounds were dried over phosphorus pentoxide *in vacuo*.

The *p*-nitrophenyl esters of 7,8-dimethyl-10-(ω-carboxyalkyl)-isoalloxazines were prepared as described previously for the syntheses of flavinyl aromatic amino acids (5). A mixture of 0.5 mmole of a flavin *p*-nitrophenyl ester and 0.75 mmole of pyridoxamine or its 5′-phosphate were taken up in 25 ml. of anhydrous dimethyl sulfoxide. In the case of pyridoxamine 5′-phosphate, 0.1 ml. of triethylamine was added to effect solution. The solutions were kept in dark glass-stoppered flasks for one to two days at 25 to 35°.

For work-up of a reaction mixture for an *N*-pyridoxyl flavin amide, the solution was poured into *n*-butyl alcohol and crude product recovered by extracting with 1*N* hydrochloride acid. This aqueous solution was neutralized with sodium hydroxide, saturated with ammonium sulfate, and extracted with liquified phenol. The phenolic solution was extracted with 1*N* hydrochloric acid and this aqueous phase extracted with diethyl ether to remove dissolved phenol. The bottom, aqueous solution was neutralized with sodium hydroxide and evaporated to dryness with warming under partial vacuum. The residue was stirred in methanol:acetone (1:10), the sodium chloride was removed by filtration, and the filtrates were evaporated to dryness to obtain the flavin amide.

For work-up of a reaction mixture of an *N*-(5′-phospho)pyridoxyl flavin amide, the solution was poured with stirring into diethyl ether to obtain a sticky precipitate. This was triturated with more ether, collected on a filter, and dissolved in 10% aqueous sodium bicarbonate. The solution was extracted with chloroform and then ether. The organic phases were back-extracted with a little water, which was then added to the initial aqueous solution. The aqueous solution was adjusted to a *p*H near 1 by the gradual addition of concentrated hydrochloric acid. After standing overnight in the refrigerator, the precipitate was filtered off and dissolved with neutralization by 1*N* lithium hydroxide in water. The solution was poured over a column (2.5 x 25 cm.) of DEAE-cellulose (chloride). Compounds were eluted in a linear manner with 1 liter of water to 1 liter of 0.05 *M* lithium chloride, followed by continuation of 0.05 *M* lithium chloride (0.5 to 1.0 liters) until all of the *N*-(5′-phospho)pyridoxyl flavin amide was eluted (*cf.* Fig. 1). Fractions containing the amide were combined and evaporated to dryness with warming under partial vacuum. The residue was repeatedly taken up in a few ml. of methanol and the product precipitated with ten volumes of acetone to remove the more soluble lithium chloride. The final product was rinsed with ether and dried over phosphorus pentoxide *in vacuo*.

Overall yields for the pure amides ranged from 25 to 50%, with much of the loss being accounted for by purification rather than the initial condensations in which as much as three-fourths of the reactants were coupled. Also, higher yields were obtained for the 5′-phosphate amides where more selective absorption to an anion-exchange column occurred.

The purity of all the amides was ascertained by attaining the molar extinction coefficient typical for 7,8-dimethyl-10-alkylisoalloxazines at 450 nm (approximately 12 x 10^3 at *p*H 7). Infrared spectra in potassium bromide helped establish the presence of functional groups present in both flavin and pyridoxyl moieties and gave evidence for the amide function with stretching absorption bands near 6 μ (1530 and 1650 cm^{-1}). Pmr spectra in deuteriotrifluoroacetic acid and deuterium oxide confirmed the presence of the flavin portion, with characteristic δ avlues of 8.4 and 8.2 for the 6- and 9-aryl hydrogens and 2.8 and 2.7 for the 8- and 7-methyl groups (18,19), and the pyridoxyl portion, with characteristic δ values in the same regions at 8.2 for the 6-aryl hydrogen and 2.7 for the 2-methyl group plus the 5- and 4-methylene hydrogens near 5.0 and 4.5, respectively (20).

Elemental analysis (performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.) for the representative *N*-pyridoxyl flavin amide (n = 5) was calculated for $C_{26}H_{30}N_6O_5$ as: C, 61.63; H, 5.97; N, 16.59. Found: C, 61.59; H, 6.02; N, 16.38. That for the representative *N*-(5′-phospho)pyridoxyl flavin amide (n = 5) was calculated for $C_{26}H_{30}N_6O_8PLi\cdot H_2O$ as: C, 51.15; H, 5.28; N, 13.77. Found: C, 51.06; H, 5.31; N, 13.64. Moreover, analyses for organic phosphate help confirm the elemental composition of the phosphate esters and that these latter amides were free of the lithium chloride used to elute them as monolithium salts from DEAE-cellulose. Values for P were calculated for cases of n = 2-5 as: 5.45, 5.32, 5.19, and 5.07%, respectively. Found were: 5.65, 5.32, 5.09, and 5.05%, respectively.

Thin-Layer and Paper Chromatography.

The fractionation and purity of compounds were routinely followed by thin-layer chromatography on MN silica Gel N-HR (Brinkman). For this, the ascending solvent used was benzene:acetone:pyridine (50:50:1), wherein the flavin *p*-nitrophenyl esters have the greatest mobilities (R_f = 0.5 to 0.6) followed by the flavin acids (R_f = 0.1 to 0.15), pyridoxamine (R_f = 0.1), 5′-phosphopyridoxamine (R_f = 0.05), and the amides (R_f = 0 to 0.1). Purified compounds were also checked by paper chromatography on Whatman No. 1 with ascending *t*-amyl alcohol:acetone:water:acetic acid (8:7:4:1), where, again, reactants run faster than products. Compounds were located by examination under an

incandescent light, where flavins appear yellow, and then under a Mineralight ultraviolet lamp, where flavins exhibit a greenish-yellow fluorescence, and pyridoxamine and its 5′-phosphate a bluish fluorescence. The chromatograms were then sprayed, first with Gibbs' reagent (1% 2,6-dichloroquinone chlorimide in benzene), and secondly with dilute ammonium hydroxide, whereupon pyridoxamine and its 5′-phosphate become blue, while the flavin-B_6 amides appear green (due to the combination of blue with the flavin yellow).

Spectra.

Visible and ultraviolet spectra were determined with a Beckman DU Spectrophotometer and a Cary Model 14 Recording Spectrophotometer. Also, fractions eluted from columns were assessed for flavin content by measuring absorbance at 450 nm in a Bausch and Lomb Spectronic 20. Infrared spectra, used for general characterization of the amides, were run on potassium bromide pellets with a Perkin-Elmer Infracord. Proton magnetic resonance spectra were obtained in deuterated trifluoroacetic acid and water with a Varian A-60A Spectrometer. Fluorescence measurements were made with an Aminco-Bowman Spectrophotofluorometer, using a xenon lamp, photomultiplier tube IP21, and slit arrangement no. 3.

REFERENCES

(1) This work was supported in part by Research Grant AM-04585 from the National Institute of Arthritis and Metabolic Diseases, U.S.P.H.S., and in part by funds made available through the State University of New York.

(2) H. R. Mahler and E. H. Cordes, "Biological Chemistry," Harper and Row, New York, 1968.

(3) D. B. McCormick, *Abst. Am. Chem. Soc. Meeting, Biol. Chem.*, 107, Sept., 1970.

(4) G. Tollin in "Molecular Associations in Biology," B. Pullman, Ed., Academic Press, Inc., New York, 1968, p. 383

(5) W. Föry, R. E. MacKenzie, and D. B. McCormick, *J. Heterocyclic Chem.*, **5**, 625 (1968).

(6) R. E. MacKenzie, W. Fory, and D. B. McCormick, *Biochemistry*, **8**, 1839 (1969).

(7) W. Föry, R. E. MacKenzie, F. Y.-H. Wu, and D. B. McCormick, *ibid.*, **9**, 515 (1970).

(8) F. Y.-H. Wu and D. B. McCormick, *Biochim. Biophys. Acta*, **229**, 440 (1971).

(9) F. Y.-H. Wu and D. B. McCormick, *ibid.*, **236**, 479 (1971).

(10) K. Yagi, T. Ozawa, and K. Okada, *ibid.*, **35**, 102 (1959).

(11) S.-C. Tu and D. B. McCormick, *Workshop on Flavins and Flavoproteins*, Konstanz, Germany, Mar., 1972.

(12) P. Strittmatter, *J. Biol. Chem.*, **236**, 2329 (1961).

(13) K. D. Watenpaugh, L. C. Sieker, L. H. Jenson, J. LeGall, and M. Dubourdieu, *Workshop on Flavins and Flavoproteins*, Konstanz, Germany, Mar., 1972.

(14) H. Wada and E. E. Snell, *J. Biol. Chem.*, **236**, 2089 (1961).

(15) W. Korytnyk, B. Bachmann, and N. Angelino, *Biochemistry*, **11**, 722 (1972).

(16) N. Hotta, A. Okasaki, T. Kobayashi, and S. Tomoika, *Pharmacy*, **25**, 55 (1966).

(17) D. B. McCormick, *Arch. Biochem. Biophys.*, in press (1972).

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

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

(20) W. Korytnyk and H. Ahrens in "Methods in Enzymology, Vitamins and Coenzymes," D. B. McCormick and L. D. Wright, Eds., Academic Press, Inc., New York, Vol. 18A, 1970, p. 475.