

# ***RIBOFLAVIN***

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## **PREFACE**

This volume represents an interdisciplinary approach to an understanding of the chemistry, physiology, and medical significance of the vitamin riboflavin. Information has been reviewed on the physiological role of the vitamin, the metabolic effects of riboflavin deficiency in animals and man, and the regulation of riboflavin metabolism. In each chapter early background material has been included, but the major emphasis has been on the many recent advances that have been made.

The early chapters of the book are concerned with the physical and chemical properties of riboflavin and its coenzyme derivatives and the nature of the interactions between flavoprotein apoenzymes and their coenzymes. The various methods currently available for measuring flavins in biological tissues, particularly in man, have been described in detail, together with newer procedures that appear to have certain advantages over existing techniques. Chapters dealing with the absorption, excretion, and metabolism of riboflavin provide basic data on the processes involved in vitamin uptake and in metabolic transformations.

The distribution of riboflavin in foodstuffs in the United States and abroad, and the consequences of riboflavin deficiency are presented largely in terms of their relation to human health. Timely questions such as the effects of organic farming and food processing upon riboflavin intake have been considered. The potential application of riboflavin deficiency as a chemotherapeutic agent is also discussed in terms of clinical significance. The important early observation that congenital malformations may be produced in animals as a result of maternal riboflavin deficiency has been amply confirmed by subsequent studies. Previously unpublished photographs have been used to illustrate an updated account of progress in this area.

An extended compilation of reports concerning various derivatives of riboflavin indicates which structural components of the molecule are essential for biological activity. It is noteworthy that several riboflavin derivatives have very significant ability to sustain growth and development of experimental animals.

The final two chapters of the book are concerned with specialized aspects of riboflavin—the relationship to cancer in animals and man and the control of vitamin metabolism by the endocrine system. These chapters indicate that endogenous factors such as hormones regulate the utilization of riboflavin in intermediary metabolism, particularly by governing conversion of the vitamin into its active coenzyme forms.

Knowledge in these fields has been accumulating rapidly. With the recent emphasis upon nutrition and health it is important to recognize which facts about vitamins can be regarded as established and which need to be elucidated by further inquiry. The rational administration of vitamins in health and in disease can be based only upon a firm scientific foundation.

The editor is indebted to each of the following colleagues for valuable assistance in the critical evaluation of submitted manuscripts: Carlos Menendez, Martha Osnos, Xavier Pi-Sunyer, Norton Rosensweig, David Rush, David Schacter, William Seprell, and Arthur Wertheim, all of Columbia University; Arpad Fazekas, University of Montreal; Erich Hirschberg, New Jersey College of Medicine and Dentistry; F. Bacon Chow, Johns Hopkins University; Peter Hemmerich, University of Konstanz, Konstanz, Germany; and Donald McCormick, Cornell University. The assistance of Mrs. Phoebe Rosenwasser in the preparation and organization of the manuscripts has been most helpful.

New York

RICHARD S. RIVLIN



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# PHYSICAL AND CHEMICAL PROPERTIES OF FLAVINS; BINDING OF FLAVINS TO PROTEIN AND CONFORMATIONAL EFFECTS; BIOSYNTHESIS OF RIBOFLAVIN

*William R. Weimar and Allen H. Neims*

It is our purpose to present in this chapter selected aspects of the chemical and physical properties of flavins, the influence of flavin binding on protein conformation, the modes of flavin-protein interaction, and the biosynthesis of riboflavin. Clearly, the diversity and extent of subject matter precludes comprehensive review. Rather, we choose to emphasize recent advances that seem to bear on future progress in flavoprotein biology and chemistry. Although some effort is made to establish historical perspective, it would be inappropriate and impossible to cite all the

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investigators whose studies have contributed to the advancements discussed here.

## 1. PHYSICAL AND CHEMICAL PROPERTIES OF FLAVINS

### 1.1. Nomenclature and General Properties

The physical and chemical properties of flavins have been studied extensively for many years. With the advent of sensitive spectroscopic instruments, fast reaction techniques, and such powerful tools as electron spin resonance (ESR) investigations of the past decade have focused on such subtleties as transient reaction intermediates, electronic properties of various redox states, and other physicochemical properties of mechanistic importance. It is our purpose to stress this recent physical organic and biophysical progress that has added new dimensions to flavin chemistry. Flavins are usually named as isoalloxazines (Fig. 1) although they can be designated also as benzopteridines. This option emphasizes the structural, biosynthetic, and probably evolutionary relationships between the flavins and pteridines. Biologically important isoalloxazines are substituted at C-7, C-8, and N-10. Hence 7,8-dimethyl-N-10 substituted isoalloxazines are often referred to as flavins, some of which are enumerated in Table I.

Certain general properties of the flavins are presented below; for greater detail, see Beinert.<sup>(9)</sup> Unless stated otherwise, the properties tabulated here refer to air-stable, oxidized flavoquinone forms.

#### 1.1.1. Riboflavin

Molecular Weight (MW) 376.36: Melting points reported in the literature have ranged from 271° to 293°C. Part of this variation is

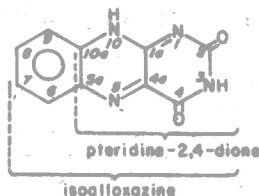


Fig. 1. Isoalloxazine.

TABLE I. Structures of Common Flavins

Flavin (7,8-dimethyl-N(10)-R-isoalloxazine)	R
Lumiflavin	$-\text{CH}_3$
Riboflavin	$-\text{CH}_2(\text{CHOH})_3\text{CH}_2\text{OH}$
Flavin mononucleotide (FMN)	$-\text{CH}_2(\text{CHOH})_3\text{CH}_2\text{OPO}_3^{2-}$
Flavin adenine dinucleotide (FAD)	$-\text{CH}_2(\text{CHOH})_3\text{CH}_2\text{O} \begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{P} \quad \text{P} \end{array} \begin{array}{c} \text{O} \quad \text{O} \\   \quad   \\ \text{O}^- \quad \text{O}^- \end{array} \text{O-adenosine}$

due to polymorphism of riboflavin crystals with one common high-melting form (m.p., ca. 290°C) and either one or two low melting forms (m.p., ca. 280°C). Five milligrams of riboflavin can be dissolved in 100 ml water at 7°C. The lower melting form(s) of riboflavin is reported to be about ten times as soluble in water as the higher melting form. The fully reduced form (flavohydroquinone) is much less soluble. Riboflavin is very soluble, but unstable, in dilute alkali. Solutions of riboflavin are also sensitive to light. The solubility of riboflavin is increased by aromatic compounds such as nicotinamide, presumably because of intermolecular solvation "complexing." Practical use of this property is made in pharmaceutical preparations. Riboflavin is relatively stable in dry form under normal lighting. It is heat-stable in acid solutions, resistant to certain oxidizing agents, and reduced by dithionite,  $\text{Zn}/\text{H}^+$ ,  $\text{TiCl}_3$ , or mild catalytic hydrogenation to yield the hydroquinone. There is no appreciable optical activity at 589 nm (Na D-line) except in alkaline solution.

### 1.1.2. Flavin Mononucleotide (FMN)

Molecular weight 456.4, m.p. 195°C,  $[\alpha]_D^{28} + 44.5^\circ$  in water. FMN is very soluble in aqueous solutions. At alkaline pH, FMN, like riboflavin, is unstable; at acidic pH, the phosphate ester is hydrolyzed to give riboflavin. Thus, FMN is probably most stable at a pH of about 6.

### 1.1.3. Flavin Adenine Dinucleotide (FAD)

Molecular weight 785.6. Neutral aqueous solutions are very stable at 0-4°C. The rate of hydrolysis of FAD in acid is quite temperature-dependent. Like riboflavin and FMN, FAD is unstable in strong alkali.

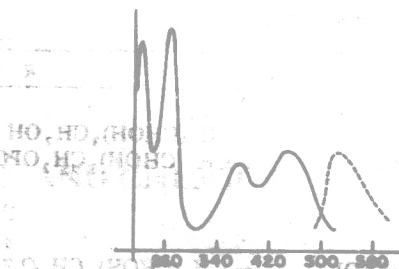


Fig. 2. Riboflavin spectra: — absorption, --- fluorescence (from Penzer and Radda<sup>(105)</sup>):

It is destroyed by 365 nm light at a rate of about  $\frac{1}{20}$  that of FMN or riboflavin. This stability of FAD is probably due to the same intramolecular complex formation that influences the absorption and fluorescence spectra (see below).

## 1.2. Electronic-Spectroscopic Properties: Flavoquinones (Fl<sub>ox</sub>)

### 1.2.1. Absorption

The u.v.-visible spectrum of riboflavin in aqueous solution consists of four major bands centered around 220, 266, 375, and 447 nm (Fig. 2, Table II). The spectrum of FMN is identical, but that of FAD differs in several respects. The positions of these bands and their extinction coefficients are dependent on the nature of the environment of the flavin chromophore, and electronic spectra do serve as probes of the microenvironment at the flavin molecule. The 375 nm band is affected most by solvent, generally shifting to shorter wavelengths (blue or hypsochro-

TABLE II. Molar Extinction Coefficients for Riboflavin, FMN, and FAD in 0.1 M Phosphate, pH 7

Extinction coefficient ( $10^3 \text{ cm}^2 \text{ M}^{-1}$ )	Riboflavin	FMN	FAD
$\epsilon_{450} (\text{Fl}_{\text{ox}})$	12.2	12.2	11.3
$\epsilon_{375} (\text{Fl}_{\text{ox}})$	10.6	10.4	9.0
$\epsilon_{260} (\text{Fl}_{\text{ox}})$	27.7	27.1	36.9
$\epsilon_{450} (\text{Fl}_{\text{red}})$	0.78	0.87	0.98

TABLE III. Solvent Effects on the Spectrum of Riboflavin

Solvent	Dielectric function [ $Z(\epsilon)$ ]	Absorption band	
		Near u.v.	Visible
Water	94.6	375	445
Ethylene glycol	85.1	363	446
Ethanol	79.6	353	448
Acetic acid	79.2	355	445
Acetonitrile	71.3	344	445

mic shift) with decreasing solvent polarity (Table III). The position of the visible (445 nm) band is not strongly affected; it does split into several inflections in a hydrophobic environment because of the appearance of vibrational fine structure apparently within a single electronic transition as determined by fluorescence polarization. Both the 375 and 445 nm absorption bands in riboflavin have been assigned to  $\pi \rightarrow \pi^*$  transitions, consistent with observed positions and high extinction coefficients. An excited state with polar character contributes to the 375 nm band, a fact which may account for the solvent-dependence of this absorption band. It is of interest that flavins in a hydrophobic environment have been postulated to be involved in phototropism in plants on the basis of similarity between the action spectrum for phototropism and the spectrum of riboflavin in castor oil.<sup>(36)</sup>

Isoalloxazine spectra are also affected by substituents. Methyl substituents at C-7 result in a shift to higher wavelengths (red or bathochromic shift) of the two long wavelength bands while methyl substituents at C-6 or C-8 affect only the 375 nm band.<sup>(104,105)</sup> Not surprisingly, substituents that affect the position of the 375 nm band also affect its solvent dependence. Substituents at N-10 and N-3 have only small influences on spectra. Substituents that extend conjugation in the system cause the expected bathochromic shifts since the transition energy is lowered. Ionization of the N-3 proton causes a small bathochromic shift. The monoprotonated form of riboflavin has a spectrum nearly identical to that of an N-1 substituted isoalloxazine, but it is different from those of isoalloxazines having no substituent at N-1. This suggests that in acidic media the proton is at N-1 in riboflavin, and hence that N-1 is the most basic position in the isoalloxazine nucleus.

### 1.2.2. Fluorescence

Flaviquinones show an intense fluorescence with  $\lambda_{\max}$  ca. 530 nm for emission (see Fig. 2) and a fluorescence quantum yield  $\phi_f = 0.25$



for riboflavin in water. Quenching of fluorescence occurs when the excited flavin loses sufficient electronic energy in nonradiative processes, such as collisional conversion of electronic energy to kinetic energy ( $Q^-$ ), heavy metal effects ( $Hg^{++}$ ), magnetic perturbations ( $O_2$ ), and electronic energy transfer ( $^1Fl^* + Q \rightarrow Fl + Q^*$ ). The latter mechanism has been implicated in recent studies of riboflavin fluorescence quenching by the transition metal ions,  $Fe^{+2}$  and  $Cr^{+3}$ .<sup>(133)</sup>

In cases where there are  $\pi$ - $\pi$  interactions ("stacking") of the flavin and another aromatic species, energy can be transferred by the nonradiative process of exciton coupling, hence decreasing fluorescence. This type of interaction gives rise to hypochromicity of absorption bands and is responsible for both hypochromicity and fluorescence quenching observed in native ("stacked") DNA. Thus, due in part to their ability to form intermolecular complexes, aromatic compounds are good quenchers of flavin fluorescence. Indeed, such a complex, as distinct from charge transfer, has been confirmed by X-ray crystallography in the case of neutral lumiflavin and naphthalene-2,3-diol.<sup>(130)</sup>

FAD has a fluorescence only one-fifth that of FMN on a molar basis. In addition, there is hypochromicity of the 260-nm absorption band of FAD. These results are consistent with an intramolecular stacking interaction between the isoalloxazine and adenine rings. Unfortunately, the X-ray structure of FAD has not yet been determined due to inability to obtain suitable crystals. Such a structure of a 1:1 intermolecular complex of 5'-bromoadenosine with riboflavin has been reported by Voet and Rich.<sup>(138)</sup> When flavins are bound to proteins there is generally a dramatic quenching of fluorescence. Possible quenching groups include aromatic residues such as tyrosine, tryptophan, and phenylalanine; thiol groups; and metals in metalloflavoproteins. Recent studies of flavin analog binding and fluorescence<sup>(30)</sup> in Shethna flavoprotein suggest that the hydroxyl groups of the ribityl side chains play a role in fluorescence quenching (see also McCormick<sup>(84)</sup>). Thus protein may also facilitate quenching by binding the flavin in a conformation more favorable to intramolecular quenching mechanisms.

### 1.2.3. Optical Rotatory Dispersion (ORD) and Circular Dichroism (CD)

Under conditions where isoalloxazine-adenine interactions are indicated by fluorescence studies, the ORD of FAD exhibits a pronounced Cotton effect in the visible region,<sup>(37)</sup> a result consistent with the expected asymmetric perturbation. Dichroic bleaching experiments,<sup>(43)</sup> taken with other approaches, suggest that the oscillator vectors for the near-u.v.