*MOLECULAR INTERACTION OF ISOALLOXAZINE DERIVATIVES**

By Henry A. Harbury and Kathryn A. Foley†

DEPARTMENT OF BIOCHEMISTRY, YALE UNIVERSITY, NEW HAVEN, CONNECTICUT

Communicated by Hans T. Clarke, May 7, 1958

Theorell and Kuhn and their co-workers[1–5] have presented considerable evidence in support of the view that the phosphate group of flavin mononucleotide (FMN) plays an important role in the binding of FMN to the protein component of the "old yellow enzyme," and it seems likely that a similar situation obtains in other FMN-protein systems. It has been suggested that, in the "old yellow enzyme" at physiological values of pH, the negatively charged phosphate group is bound to positively charged amino groups of the protein.[4, 5]

Less clear is the nature of the interaction between protein and the *iso*alloxazine portion of FMN. That such interaction occurs is evident from the fact that free FMN and protein-bound FMN differ in absorption spectrum, fluorescence spectrum, and oxidation-reduction potential. Moreover, addition of riboflavin to the protein component of the "old yellow enzyme" leads both to the development of enzymic activity and to quenching of the fluorescence of riboflavin.[3, 4] The results of the early investigations by Kuhn and his colleagues (reviewed extensively[6–9]), in which riboflavin analogues and derivatives were examined both for vitamin activity and for ability to give rise to enzymic activity when added to the protein of the "old yellow enzyme," the extensive antimetabolite studies performed in many laboratories,[6–9] and the recent kinetic data of Theorell and Nygaard[4, 5] are all consistent with the idea that the *iso*alloxazine part of FMN plays a key role in protein-FMN interaction.

Such interaction may be expected to differ not only between one FMN-protein and another, but also between reversibly interconvertible states and compounds of a single flavoprotein. This may hold to an even greater extent for the more complex flavin adenine dinucleotide-proteins and metalloflavoproteins. It would seem of interest to examine the linked functions[10] of flavoprotein systems in a manner analogous to the systematic study of the heme protein, horseradish peroxidase, now in progress in this laboratory.[11] However, whereas studies of heme proteins are facilitated by the availability of considerable information on the interaction of metalloporphyrins with nonprotein ligands,[12] little analogous information exists for *iso*alloxazine derivatives. In the present communication we report on studies undertaken for purposes of orientation preparatory to more detailed investigations.

EXPERIMENTAL

Materials.—3-Methylriboflavin was synthesized by condensation[13] of 2-amino-4,5-dimethyl-*N*-(1′-D-ribityl)-aniline[14] and methylalloxan.[15] Melting point: 269°–270° C., with decomposition. Analysis: calculated, C 55.37, H 5.69, N 14.36; found, C 55.43, H 5.95, N 14.37. Absorption and fluorescence spectra are shown in Figure 1.

p-Hydroxycinnamic acid (m.p. 212° C.), *p*-methoxycinnamic acid (m.p. 185° C., sinters 170° C.), and L-tyrosine ethyl ester (m.p. 166° C.) were gifts from Dr. Christine Zioudrou and Professor Joseph S. Fruton. All other compounds used were

commercial preparations, purified as required. Solutions were freshly prepared immediately prior to use. Care was taken to protect flavin-containing solutions from light.

Apparatus.—Absorption spectra were determined with the use of Cary model 11, Spectracord model 3000, and Beckman model DU spectrophotometers. Fluorescence spectra were determined with a spectrofluorometer constructed from two Bausch and Lomb grating monochromators, a Xenon arc light source, a 1P28 photomultiplier tube with battery power supply (Farrand Optical Co.), and a Varian Associates model G10 recorder.

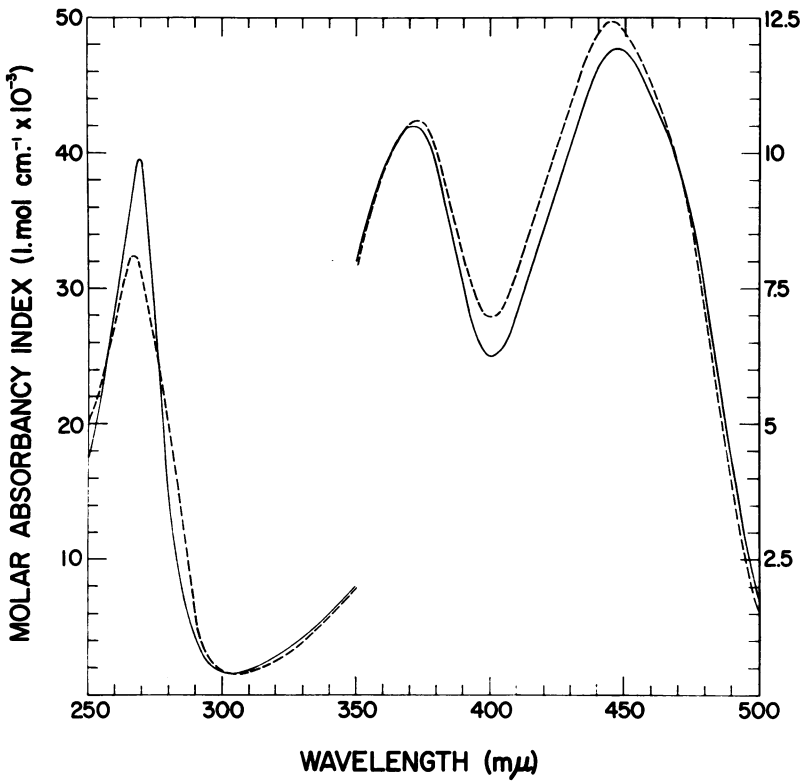

FIG. 1*a*.—Absorption spectra of 3-methylriboflavin and riboflavin in water solution. Cary model 11 spectrophotometer. *Full line*, 3-methylriboflavin; *dashed line*, riboflavin (see Whitby, *Biochem. J.*, **54**, 437, 1953).

Methods.—The visible and near-ultraviolet absorption maxima of water solutions containing flavin in the presence of the interactants studied lie at slightly different wave lengths and are of somewhat lower intensity than the corresponding maxima of water solutions of the *iso*alloxazine compounds alone. This is illustrated in Figure 2. Similar observations have been reported previously by others.[16–19] If it is assumed that this shift reflects complex formation, then apparent dissociation constants may be calculated. Assuming an equilibrium, $F + I \rightleftharpoons FI$, the following relationship obtains at fixed wave length when $[I]_0 \gg [F]_0$:

$$\frac{1}{\Delta A_s} = \frac{1}{[(a_M)_{FI} - (a_M)_F]l[F]_0} + \frac{K_c'}{[(a_M)_{FI} - (a_M)_F]l[F]_0} \cdot \frac{1}{[I]_0},$$

where F represents flavin, I = interactant, FI = a 1:1 complex of flavin and interactant, $[I]_0$ = total molar concentration of interactant, $[F]_0$ = total molar concentration of flavin, $K_c' = [F]\,[I]/[FI]$, l = optical path length of solution, $(a_M)_F$ = molar absorbancy index of flavin, $(a_M)_{FI}$ = molar absorbancy index of complex, A_s = absorbancy, and ΔA_s = (A_s for a solution of F at concentration $[F]_0$) + (A_s for a solution of I at concentration $[I]_0$) − (A_s for a solution of F, I, and FI in equilibrium at a total flavin concentration $[F]_0$ and a total interactant concentration $[I]_0$). Values for K_c' can conveniently be determined graphically from plots of $1/\Delta A_s$ versus $1/[I]_0$, at fixed values of $[F]_0$. Calculations were made for wave

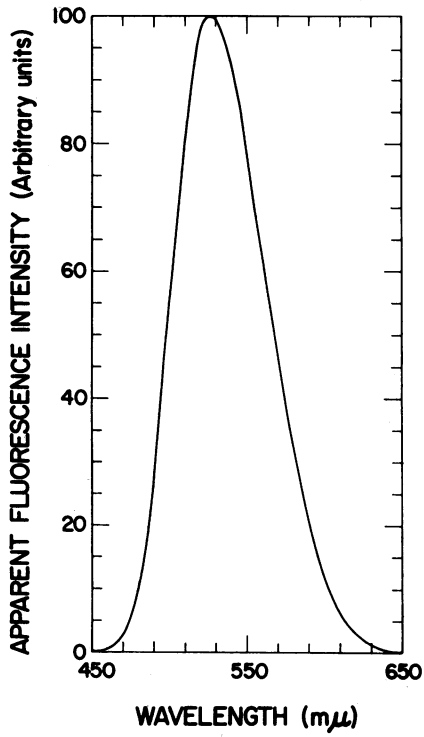

FIG. 1b.—Apparent fluorescence spectrum of 3-methylriboflavin, 1 × 10^{-5} M solution in water. See text for instrumentation. No corrections applied.

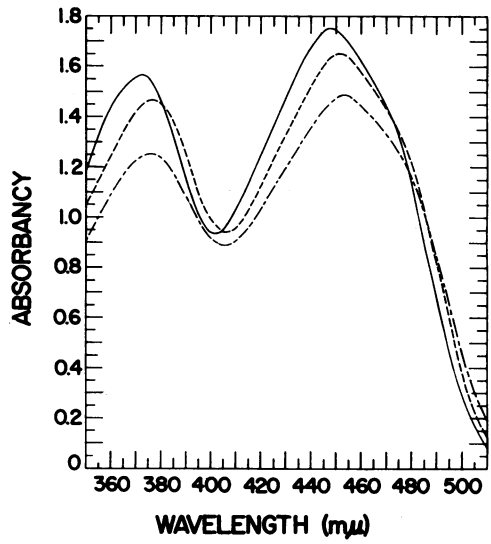

FIG. 2.—Absorption spectra of solutions of 3-methylriboflavin plus interactant. Cary model 11 spectrophotometer, 10-cm. cuvettes. ———, 1.5 × 10^{-5} M 3-methylriboflavin versus water; - - -, 1.5×10^{-5} M 3-methylriboflavin plus 0.21 M benzoate versus 0.21 M benzoate; - - -, 1.5 × 10^{-5} M 3-methylriboflavin plus 0.020 M 2-naphthoate versus 0.020 M 2-naphthoate.

lengths near 445 mμ and often also near 373 mμ. The method becomes decreasingly sensitive with increasing degree of dissociation.

Spectra were determined for solutions containing flavin at total concentrations from 1.0 × 10^{-5} M to 1.5 × 10^{-4} M, and interactant at total concentrations from 1.1 × 10^{-3} M to 3.8 × 10^{-1} M. Cuvettes with 1-cm. and 10-cm. path lengths were employed. Solutions were adjusted with potassium hydroxide or hydrochloric acid to values of pH between 6.5 and 7.5, but no buffers were added. In preliminary experiments with several interactants, no effect of pH on degree of interaction was observed over the range of pH 4–8. All measurements were made at 22.5° ± 0.5° C.

Results.—The values of ΔA_s are not large, and the range of concentrations of interactant that could be studied in water solution was limited, but the results obtained suffice for the immediate purpose of evaluating gross relationships. A representative graph of $1/\Delta A_s$ versus $1/[I]_0$ is shown in Figure 3. Within the range of concentrations employed, the assumed 1:1 stoichiometry represents the simplest formulation consistent with the data at hand. Apparent equilibrium constants are summarized in Table 1. The value of 0.021 mol $l.^{-1}$ found for the apparent dissociation constant of the 3-methylriboflavin-caffeine system at 22.5° C. (Fig. 3) may be compared with a value of 0.011 mol $l.^{-1}$ for riboflavin-caffeine at 17° C. and pH 7.5, determined fluorometrically by Weber,[16] and a value of 0.019 mol $l.^{-1}$ for riboflavin-caffeine at 20° C. and pH 5.8, determined spectrophotometrically by Yagi and Matsuoka.[18]

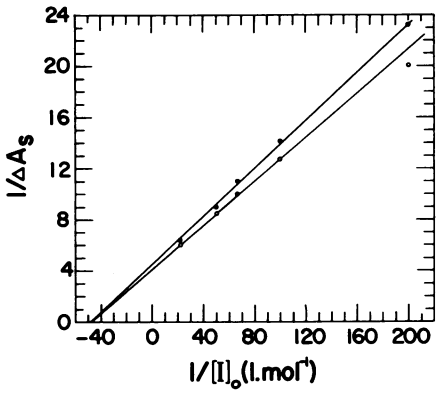

FIG. 3.—3-Methylriboflavin-caffeine system. *Solid circles*, readings at 373 mμ; *open circles*, readings at 445 mμ.

DISCUSSION

Currently accepted concepts of the mode of linkage between *iso*alloxazine and protein are essentially variations and extensions of views first advanced by Kuhn and his coworkers,[3, 20] who suggested that, in the "old yellow enzyme," a bond exists between the protein and the imino group in the number 3 position of the *iso*alloxazine ring. This suggestion was based upon the following observations. First, the fluorescence of FMN is quenched on dissociation of the number 3 imino group in alkaline solution[21] and also on linkage of FMN to the protein of the "old yellow enzyme."[1, 2] Furthermore, neither 3-methylriboflavin nor 3 methyl FMN can react with the protein of the "old yellow enzyme" to form an enzymically active complex,[3] and 3-methylriboflavin displays no growth-promoting activity upon administration to rats on a riboflavin-free diet.[22] A number of reviews state, as additional argument, that 3-methylriboflavin is nonfluorescent, but this is incorrect (Fig. 1).

During the past decade, considerable support has developed for the view that the interaction between protein and *iso*alloxazine rests on hydrogen bonding. Geissman[23] has proposed that perhaps not only the number 3 ring position but also the number 10 position participates in bonding to the protein and that both linkages involve hydrogen bonds to peptide chain —NH—CO— groups. Hydrogen-bond formation to the number 10 ring nitrogen also has been postulated by Michaelis.[24] More recently, Theorell and Nygaard have performed kinetic studies with both native and iodinated "old yellow enzyme" and have interpreted their data in terms of hydrogen-bonding effects.[4, 5] Extending a suggestion by Weber[16] that aromatic constituents such as tyrosine are likely participants in the quenching of flavin fluorescence by proteins, they have proposed that in the "old yellow enzyme" the number 3 imino group of the *iso*alloxazine ring is linked through a hydrogen bond to the OH group of a tyrosine residue.

Similar suggestions have been advanced for systems composed of riboflavin and

molecules other than proteins. Sakai[19] has investigated the effects of a number of aromatic compounds on the fluorescence of riboflavin and its solubility in water and has interpreted his results in terms of hydrogen-bond formation involving the imino or carbonyl groups of the *iso*alloxazine ring. Yagi and Matsuoka,[18] in a study of the reaction of riboflavin with phenol, advanced a similar hypothesis. Weber[16] has concluded that the quenching of the fluorescence of riboflavin by purines is due to complex formation and has suggested that in flavin adenine dinucleotide an internal complex exists between the *iso*alloxazine and adenine components of the molecule. His data have been interpreted[25] as indicating that bond formation occurs in flavin adenine dinucleotide between *iso*alloxazine and the amino group of adenine.

The results of the present study support a different point of view. In several of the systems studied, there is no possibility of hydrogen-bond formation between the interactant and the *iso*alloxazine ring. An example is 3-methylriboflavin-caffeine.

TABLE 1

APPARENT DISSOCIATION CONSTANTS

K_c' (MOL L.$^{-1}$ × 10^3)	INTERACTANT	FLAVIN*		
		FMN	Riboflavin	3-Methyl-riboflavin
1–5	3-Hydroxy-2-naphthoate	x	x	x
5–10	2-Naphthoate	x	x	x
	Anthraquinone-1-sulfonate	x		
	Anthraquinone-2,6-disulfonate	x		
10–50	*p*-Methoxycinnamate	x		
	p-Hydroxycinnamate	x		
	Cinnamate	x	x	x
	L-Tryptophan	x		x
	Caffeine	x	x	x
>50	Anisate	x	x	x
	p-Hydroxybenzoate	x	x	x
	Salicylate	x		
	m-Chlorophenol	x		
	Phenol	x		
	L-Tyrosine ethyl ester		x	x
	Benzoate	x	x	x
	L-Phenylalanine			x
	1,4-Benzoquinone	x		

* x marks denote systems studied.

Among those systems where hydrogen bonding is possible in principle, the relative values of the apparent dissociation constants recorded bear little relation to what would be predicted, were the interaction dependent upon hydrogen-bond formation alone. The constants recorded depend greatly upon the extent of conjugation of the compounds employed, and 3-methylriboflavin has in all instances proved fully as reactive as riboflavin or FMN. These observations argue against ring hydrogen bonding as a primary feature of interaction in the *iso*alloxazine systems examined and suggest that molecular charge transfer[26, 27] may be a more important factor. However, other interpretations may be possible, and definitive evidence in favor of charge-transfer interaction remains to be obtained. Kosower[28] previously has discussed the possibility that certain diphosphopyridine nucleotide systems might be charge-transfer complexes.

The choice of compounds included in this initial survey was rather arbitrary and was influenced by considerations of ready availability, solubility, and con-

venience of spectrophotometric observation. Nevertheless, the interactants listed in Table 1 include representatives of aromatic amino acids and purines, as well as compounds structurally related to certain vitamins, hormones, and agents known to uncouple electron transport from oxidative phosphorylation. It is tempting to speculate on the role which charge-transfer interaction and resonance coupling[29-32] may play in the prosthetic group-linked functions of flavoproteins and other oxidative enzymes. Interesting possibilities suggest themselves in relation to electron transport-coupled phenomena such as oxidative phosphorylation. However, such speculation would be premature. Whether the data presented have relevance to the interactive effects which obtain in *iso*alloxazine-protein systems is still to be established.

SUMMARY

Interaction in water solution between *iso*alloxazine derivatives and various conjugated molecules has been studied. The results obtained argue against hydrogen bonding between interactant and *iso*alloxazine as a primary feature of interaction in the systems examined and suggest that molecular charge transfer may be a more important factor. Alternative interpretations are possible. The data are discussed in relation to current concepts of the interaction of *iso*alloxazine with proteins and with other compounds of biochemical interest.

* This study was aided by grants from the National Science Foundation, the Rockefeller Foundation, and the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

† Predoctoral Research Fellow of the United States Public Health Service.

[1] H. Theorell, *Biochem. Z.*, **278**, 263, 1935.

[2] H. Theorell, *ibid.*, **290**, 293, 1937.

[3] R. Kuhn and H. Rudy, *Ber. Deut. chem. Ges.*, **69**, 2557, 1936.

[4] H. Theorell and A. P. Nygaard, *Acta Chem. Scand.*, **8**, 1649, 1954.

[5] A. P. Nygaard and H. Theorell, *Acta Chem. Scand.*, **9**, 1587, 1955.

[6] R. S. Harris, D. M. Hegsted, M. K. Horwitt, E. E. Snell, and T. Wagner-Jauregg in W. H. Sebrell, Jr., and R. S. Harris, *The Vitamins*, Vol. **3** (New York: Academic Press, 1954).

[7] F. A. Robinson, *The Vitamin B Complex* (New York: John Wiley & Sons, 1951).

[8] R. J. Williams, R. E. Eakin, E. Beerstecher, Jr., and W. Shive, *The Biochemistry of the B-Vitamins* (New York: Reinhold Publishing Corp., 1950).

[9] H. R. Rosenberg, *Chemistry and Physiology of the Vitamins* (New York: Interscience Publishers, 1942).

[10] J. Wyman, Jr., *Advances in Protein Chem.*, **4**, 407, 1948.

[11] H. A. Harbury, *J. Biol. Chem.*, **225**, 1009, 1957.

[12] R. Lemberg and J. W. Legge, *Heme Compounds and Bile Pigments* (New York: Interscience Publishers, 1949).

[13] R. Kuhn, K. Reinemund, F. Weygand, and R. Ströbele, *Ber. Deut. chem.Ges.*, **68**, 1765, 1935.

[14] P. Karrer and H. Meerwein, *Helv. Chim. Acta*, **19**, 264, 1936.

[15] R. Maly and R. Andreasch, *Monatsh.*, **3**, 108, 1882.

[16] G. Weber, *Biochem. J.*, **47**, 114, 1950.

[17] G. Whitby, *Biochem. J.*, **54**, 437, 1953.

[18] K. Yagi and Y. Matsuoka, *Biochem. Z.*, **328**, 138, 1956.

[19] K. Sakai, *Nagoya J. Med. Science*, **18**, 237, 245, 1956.

[20] R. Kuhn and P. Boulanger, *Ber. Deut. chem. Ges.*, **69**, 1557, 1936.

[21] R. Kuhn and G. Moruzzi, *Ber. Deut. chem. Ges.*, **67**, 888, 1934.

[22] R. Kuhn, *Angew. Chem.*, **49**, 16, 1936.

[23] T. A. Geissman, *Quart. Rev. Biol.*, **24**, 309, 1949.

[24] L. Michaelis, in J. B. Sumner and K. Myrbäck, *The Enzymes*, Vol. 2, Part 1 (New York: Academic Press, 1951).

[25] H. Theorell, in R. Stoope, *Les Protéines* (Brussels: Solvay Institute, 1953).

[26] R. S. Mulliken, *J. Am. Chem. Soc.*, **74**, 811, 1952.

[27] R. S. Mulliken, *J. Phys. Chem.*, **56**, 801, 1952.

[28] E. M. Kosower, *J. Am. Chem. Soc.*, **78**, 3497, 1956.

[29] T. Förster, *Fluorescenz organischer Verbindungen* (Göttingen: Vandenhoeck & Ruprecht, 1951).

[30] F. W. J. Teale and G. Weber, *Biochem. J.*, **65**, 476, 1957.

[31] G. Karreman and R. H. Steele, *Biochem. et Biophys. Acta*, **25**, 280, 1957.

[32] G. Karreman, R. H. Steele, and A. Szent-Györgyi, these PROCEEDINGS, **44**, 140, 1958.

PRODUCTION, REPRODUCTION, AND REVERSION OF PROTOPLAST-LIKE STRUCTURES IN THE OSMOTIC STRAIN OF NEUROSPORA CRASSA

BY STERLING EMERSON AND MARY R. EMERSON

CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

Communicated by G. W. Beadle, May 16, 1958

Protoplasts devoid of cell walls have been produced in *Bacillus megaterium* and certain other Gram-positive species of bacteria.[1] Structures resembling protoplasts but not completely devoid of cell walls have also been produced in *Escherichia coli* and some other Gram-negative bacteria.[2] Those from Gram-positive and Gram-negative bacteria are alike in that they are spherical in shape and are lysed by osmotic shock. Bacterial protoplasts have already proved to have many useful applications—among others, in the extraction of cellular constituents, in studies of the biosynthesis of enzymes and other macromolecular substances, and in host-parasite interrelations.[3]

Protoplasts, or protoplast-like structures, have also been produced in one of the higher fungi, the unicellular Ascomycete, *Saccharomyces cerevisiae*.[4] The present paper reports the production and reproduction of coenocytic protoplast-like structures in one strain of the filamentous Ascomycete, *Neurospora crassa*.

Strain Specificity.—Under the conditions employed, protoplasts have consistently been formed by all tested strains carrying the osmotic mutant gene, *os*.[5] An exhaustive survey has not been made, but all tested strains carrying the wild-type allele of *os* have either been completely refractory or have responded poorly. The only non-osmotic strains which have so far yielded protoplasts are two with maternally inherited cytochrome abnormalities, *poky*[6] and *mi-3*,[7] which appear to require higher enzyme concentrations than strains carrying *os* and, even then, are erratic in response. Descriptions to follow refer to *os* strains.

Treatment.—Culture media in which protoplasts have been produced consist of the standard *Neurospora* salt mixture and biotin,[8] sugars, and either a commercial hemicellulase preparation[9] or, in a few tests, a crude preparation of snail hepatic juice.

While the concentrations of enzymes, salts, and sugars do influence the ease with which protoplasts are produced and maintained, no clear end-point for dilution