Studies on the Electronic Nature of Flavin–Indole and Flavin–Purine Complexes*

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ABSTRACT: Only oxidized flavins form complexes with indole derivatives, and complexing is prevented by dissociation of the 3-imino group of the isoalloxazine ring. The flavin–indole complexes exhibit new absorption in the 550–700-mμ region. The possibility that this represents charge transfer absorption is considered. The effects of ionic strength, solvent polarity, pH, and various other factors on complex formation were studied.

Purine derivatives complex with both oxidized and reduced flavins. Complexing of either form results in qualitatively similar spectral changes, namely, decreased absorbance at the maximum and increased absorbance at the long wavelength edge of the flavin absorption band(s). No extension of the absorption to appreciably longer wavelengths is noted. Complex formation does not appear to be sensitive to changes in the ionization state of the isoalloxazine ring. The striking differences between the properties of the flavin–indole complexes and those of the flavin–purine complexes indicate that quite different types of intermolecular interactions are present in these two types of flavin complex. Although charge transfer forces may play a role in flavin–indole complexing, it seems unlikely that they are a significant factor in the formation of flavin–purine complexes.

The binding of oxidized flavin mononucleotide (FMN)[1] or oxidized flavin–adenine dinucleotide (FAD) by apoenzyme, yielding a functional flavoprotein catalyst, generally causes considerable changes in the spectral properties and reduction potential of the flavin coenzyme (for a review, see Beinert, 1960). Intact flavoproteins frequently represent a rather inconvenient source of complexed flavins, and therefore it would seem desirable to utilize more easily obtained flavin complexes as models for studying the types of interactions likely to affect protein-bound flavins. Since tryptophan is a common constituent of most proteins, it is of considerable interest that this amino acid readily complexes with flavins (Harbury and Foley, 1958; Isenberg and Szent-Györgyi, 1958), thereby altering the spectral and, presumably, other properties dependent on the electronic structure of the coenzyme. The formation of an intramolecular complex between the adenine and flavin moieties of FAD (Weber, 1950) also draws attention to the possible importance of such complexes in flavoprotein catalysis.

The properties of FMN complexes with several purine and indole derivatives have been investigated in an attempt to learn more about the types of interactions responsible for the formation of these complexes. The possible involvement of charge transfer forces (Mulliken, 1952) was of particular interest since previous authors (Isenberg and Szent-Györgyi, 1958; Wright and McCormick, 1964; Tsibris *et al.*, 1965) had proposed that the formation of both flavin–indole and flavin–purine complexes was due to interactions of a charge transfer nature.

Experimental Section

Materials. FMN, FAD, D-tryptophan, indole, indole-3-aldehyde, 5-hydroxy-DL-tryptophan, tryptamine hydrochloride, indole-3-acetic acid, indole-3-propionic acid, and indole-3-butyric acid were obtained from Sigma Chemical Company, St. Louis, Mo. L-Tryptophan was a product of General Biochemicals, Inc., Chagrin Falls, Ohio. All of the other indole derivatives used and caffeine were purchased from Aldrich Chemical Co., Milwaukee, Wis. Adenosine was obtained from Schwarz Laboratories, Inc., New York. Furan, pyrrole, and thiophene were products of Eastman Organic Chemicals, Rochester, N. Y., and were freshly redistilled before use. A lyophilized preparation of venom phosphodiesterase from *Crotalus adamanteus* was purchased from Worthington Biochemical Corp., Freehold, N. J.

Methods. Unless stated otherwise, the buffer used throughout was 0.1 M potassium phosphate, pH 7.0. For determination of the effect of pH on complex formation, mixtures of 0.1 M citric acid with 0.1 M potassium

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[1] Abbreviations used: FMN, FMNH, and $FMNH_2$ represent the oxidized, semiquinone, and reduced forms of flavin mononucleotide; FAD and $FADH_2$, oxidized and reduced flavin–adenine dinucleotide; NHE, normal hydrogen electrode; SCE, saturated calomel electrode.

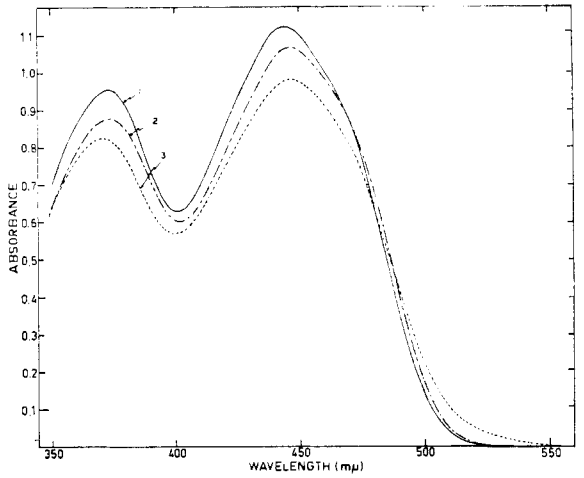

FIGURE 1: Effects of tryptophan and adenosine on the spectrum of FMN. All samples contained 8.5×10^{-5} M FMN. Additions: Curve 1 (——), none; curve 2 (— - — -), plus 1.34×10^{-2} M adenosine; curve 3 (----), plus 1.67×10^{-2} M L-tryptophan.

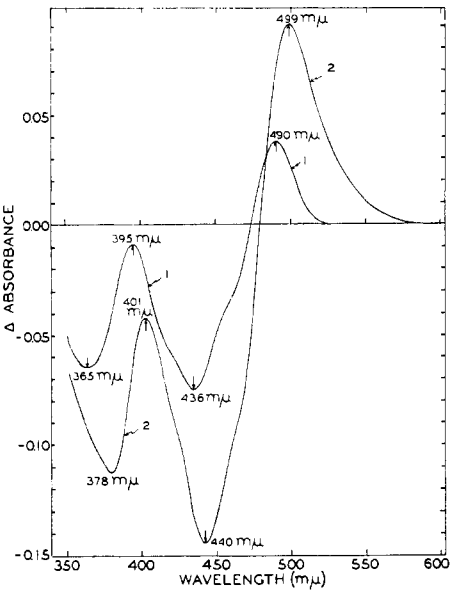

FIGURE 2: Difference spectra of FMN–adenosine and FMN–tryptophan complexes. Concentrations as given under Figure 1. Curve 1, plus adenosine; curve 2, plus tryptophan.

phosphate were used in the range of pH 3–6, 0.1 M potassium phosphate, pH 6–8, and 0.1 M sodium carbonate–bicarbonate, pH 9–11.

Absorption spectra and difference spectra were determined with a Cary Model 15 recording spectrophotometer. Except for the spectrophotometric determination of the association constant, described below, all other absorbance measurements were made at room temperature with a Zeiss PMQ II spectrophotometer. In all experiments, the light path was 1.0 cm unless indicated otherwise.

Concentrations of the following compounds were determined spectrophotometrically, using previously published molar absorptivities: riboflavin, FMN, FAD (Whitby, 1953); 5-hydroxy-DL-tryptophan (Udenfriend and Weissbach, 1963); indole (Pappalardo and Vitali, 1958); D-tryptophan, L-tryptophan, tryptamine, and indole-3-acetic acid (McMenamy and Oncley, 1958); thiophene (Balandin *et al.*, 1958). The molar absorptivity at 242 mμ for pyrrole in 0.1 M potassium phosphate, pH 7.0, was determined to be 126 l. mole^{-1} cm^{-1}.

The association constants and molar absorptivities of the complexes were determined spectrophotometrically (Isenberg and Szent-Györgyi, 1958). The data were treated by the least-squares method to obtain the best straight line through the experimental points. Absorbance measurements were made with a Beckman Model DU spectrophotometer modified by the attachment of a Gilford absorbance indicator, Model 2000. The sample compartment was maintained within ±0.05° of the desired temperature by a Haake Model F circulator. Samples were allowed to equilibrate until the absorbance reading remained constant.

Experiments requiring spectral observation of reduced flavin solutions were carried out in a 3-ml cuvet fitted with a side arm and stoppered with a soft rubber plug. The solution was made anaerobic by alternate evacuation and flushing with nitrogen, the final operation leaving the solution under nitrogen. The flavin was reduced by injection of approximately 1 M $Na_2S_2O_4$ with a microsyringe. Dilution due to the addition of dithionite was less than 1%. The end point was easily detectable by the spectral changes and loss of fluorescence caused by reduction of the flavin.

Polarography was done by conventional techniques (Meites and Thomas, 1958) using a Radiometer PO4 Polariter with an E64 electrode assembly. Solutions were deaerated by bubbling with nitrogen. The resistance of the polarographic cell containing the deaerated solution was determined with a Radiometer conductivity meter, and all potentials were corrected for iR drop. Unless stated otherwise, the potentials are given with the saturated calomel electrode as reference. Polarograms were run by scanning from −0.2 to −0.6 v at the rate of 0.1 v/min. All measurements were made at 26 ± 0.5°. The $E_{1/2}$ value was determined from a plot of log $(i_d - i)/i$ *vs.* E (Meites and Thomas, 1958).[2]

[2] $E_{1/2}$ values may be considered equal to the midpoint potentials, E_m, determined potentiometrically when the oxidized and reduced forms of the molecule do not differ greatly in size (Meites and Thomas, 1958), as is the case with FMN and $FMNH_2$. Conventional symbols are used: E_0 = standard reduction potential, all reactants and products at unit activity; E_0' = standard reduction potential with all reactants and products except hydrogen ion at unit activity, and pH equal to 7.0.

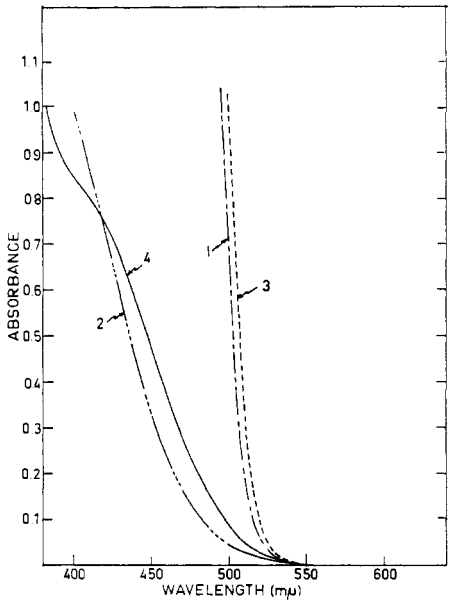

FIGURE 3: Effects of caffeine and tryptophan on the spectrum of $FMNH_2$. All samples contained 3.8×10^{-4} M FMN. Curves 1 and 2, oxidized and reduced, no additions. Curves 3 and 4, oxidized and reduced in the presence of 0.05 M caffeine. The spectrum of $FMNH_2$ (curve 2) was not altered in the presence of 4.2×10^{-2} M tryptophan.

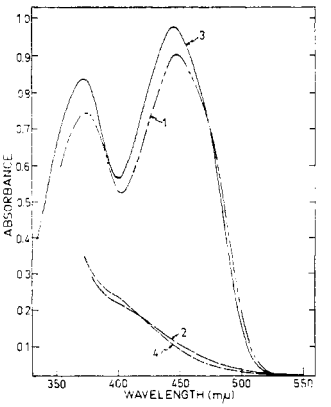

FIGURE 4: Intramolecular complex formation in oxidized and reduced FAD. Initial concentration of FAD was 7.9×10^{-5} M in 0.1 M potassium phosphate, pH 7.0, containing 3×10^{-2} M magnesium acetate. Curves 1 and 2, oxidized and reduced, untreated. Curves 3 and 4, oxidized and reduced after hydrolysis for 1 hr, 30°, with snake venom phosphodiesterase.

Results

Spectral Properties of FMN and $FMNH_2$ Complexes

In agreement with previous authors (Weber, 1950; Harbury and Foley, 1958), complexing of FMN with caffeine or adenosine causes a shift of the 373 and 445-mμ absorption maxima of the flavin to slightly longer wavelengths, and decreased absorption in the region of the maxima. When, however, pyrrole or any of the indole derivatives (*e.g.*, tryptophan) is used as the complexing agent, the effect noted is somewhat different. In the latter case, the 445-mμ band is again shifted about 2–5 mμ to longer wavelengths, and absorption in the region of both maxima decreased, but the 373-mμ band is shifted to slightly shorter wavelengths. These results differ from a previous report (Harbury and Foley, 1958) which indicated a red shift for both the 373- and 445-mμ bands in the presence of tryptophan. As examples of the two different effects observed, the spectra of FMN in the presence of adenosine and of L-tryptophan are compared in Figure 1. Besides causing spectral changes, all of the complexing agents studied also quench the fluorescence of FMN, as is the case with other flavin complexes (Weber, 1950).

Spectra were also determined by the difference method; *i.e.*, the buffer blank was replaced with a solution which was identical with the sample except that it contained no complexing agent. (None of the complexing agents used absorbed in the spectral region of interest, 350–700 mμ.) The difference spectra for complexes of FMN with adenosine and with tryptophan are shown in Figure 2. Except for slight changes (±10 mμ) in the location of the maxima and minima, complexes of FMN with pyrrole or any of the indole derivatives have difference spectra very similar to that shown for the FMN–tryptophan complex. The difference spectra of the FMN complexes with adenosine and caffeine are nearly identical.

A very important difference between the effects of these compounds should be emphasized. As shown in Figures 1 and 2, complexing of FMN with tryptophan causes an extension of the tail from the 445-mμ band to appreciably longer wavelengths. This is not observed with adenosine or caffeine as complexing agent. The effect becomes even more evident at higher FMN concentrations. Whereas 3.8 mM FMN solutions, with or without 0.05 M caffeine, show little absorption (ϵ less than 26 l. $mole^{-1}$ cm^{-1}) at wavelengths longer than 560 mμ, the spectrum in the presence of 4.2×10^{-2} M tryptophan indicates appreciable absorption even at wavelengths as long as 680 mμ. As mentioned above, the use of pyrrole or various indole derivatives as complexing agents causes spectral alterations very similar to those described for tryptophan.

The effects of tryptophan and caffeine on the spectrum of reduced flavin are illustrated in Figure 3. The spectrum of reduced flavin mononucleotide ($FMNH_2$) is identical in either the presence or absence of tryptophan, implying that the reduced form is not complexed by tryptophan. This conclusion is supported by further evidence presented below. When caffeine is present, obvious changes in the spectrum are noted. Increased absorbance is observed in the 420–530-mμ region, but decreased absorbance from 380 to 420 mμ, resulting in the clear emergence of a shoulder near 400–410 mμ. Thus, the effect of caffeine on the spectrum of $FMNH_2$ is

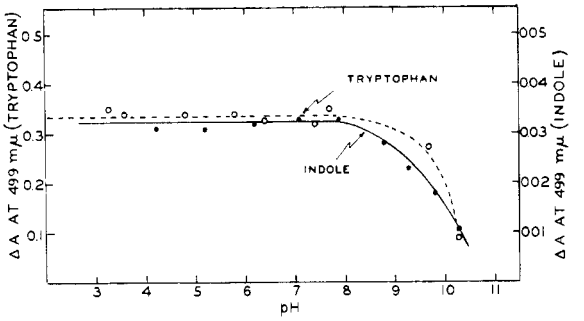

FIGURE 5: Effect of pH on flavin–indole complexes. For tryptophan curve: samples contained 2.85×10^{-4} M FMN, 2.14×10^{-2} M tryptophan. For indole curve: samples contained 0.99×10^{-4} M FMN, 3.3×10^{-3} M indole. Buffers used are given in Methods.

qualitatively similar to its effect on that of FMN; namely, increased absorption at the long wavelength side of an absorption band but decreased absorption in the vicinity of the maximum, which in the case of $FMNH_2$ appears as a barely distinguishable shoulder at about 400–410 mμ rather than as a distinct maximum.

Intramolecular Complex Formation in Reduced FAD. The formation of an intramolecular complex between the isoalloxazine and adenine moieties in the oxidized form of FAD has been previously demonstrated by spectral and fluorescence techniques (Weber, 1950). Spectral evidence for intramolecular complexing in reduced FAD is provided by the results shown in Figure 4. After enzymatic hydrolysis of the dinucleotide with snake venom phosphodiesterase, the spectrum of the oxidized flavin closely resembles that of FMN (Whitby, 1953), the expected hydrolysis product (Razzell and Khorana, 1959). A corresponding change is observed in spectra recorded after reduction of the flavins. Compared to the reduced hydrolysis product, reduced flavine–adenine dinucleotide ($FADH_2$) shows greater absorbance in the 420–530 mμ region, but decreased absorbance at 380–420 mμ. The spectrum of $FADH_2$, therefore, is quite similar to that for the complex of caffeine with $FMNH_2$ (Figure 3).

Effect of pH, Ionic Strength, and Solvent Polarity on the Formation of Flavin–Indole Complexes. The effects of pH, ionic strength, and solvent polarity on flavin–indole complexing were studied by the difference spectrum technique. Since these factors may themselves affect the flavin spectrum (Harbury *et al.*, 1959; Koziol and Knobloch, 1965; Massey and Ganther, 1965), it should be emphasized that the flavin solutions used as blanks were identical with the sample except that they contained no complexing agent. In all cases, the location of the maxima and minima in the difference spectrum of the complex (*e.g.*, Figure 2) was unaffected by the changes in pH, ionic strength, or solvent polarity. As in the determination of the association constant, described above, the increment in absorbance at the peak near 500 mμ was used as a measure of complex formation.

Complexing is independent of pH in the range 3–8, as shown in Figure 5. Curves of very similar shape are obtained with either tryptophan or indole as complexing agent, and indicate that complexing is dependent upon the ionization state of some group having a pK of about 10. Tryptophan has an ionizable group near this region, its α-amino group having a pK of 9.4 (Greenstein and Winitz, 1961), but indole does not (Hinman and Lang, 1960). Thus, the dissociable group affecting complex formation is identified as the 3-imino group of the flavin ring, which has a pK of about 10.2 (Beinert, 1960).

No changes in the ability of tryptophan to complex with FMN are found when the buffer concentration is varied over a hundredfold range, from 0.01 M to 1.0 M potassium phosphate, all at pH 7.0. Decreasing the polarity of the medium, however, as by the addition of increasing amounts of ethanol to unbuffered aqueous solutions, causes dissociation of the FMN–tryptophan complex. At 30% ethanol, the amount of complex formed is only about 50% of that formed in the absence of ethanol. Dioxane is even more effective, causing a 50% decrease in complex formation at 15% dioxane. Other organic solvents, such as methanol, glycerol, and pyridine, also hinder the formation of the FMN–tryptophan complex.

The Role of Electrostatic Forces in the Formation of Flavin–Indole Complexes. The association constants for complexes with FMN increase in the order: indole-3-acetic acid, tryptophan, tryptamine. For example, at 25° the values are 40, 92, and 106 l. $mole^{-1}$, respectively. At pH 7, indole-3-acetic acid, tryptophan, and tryptamine possess charges of −1, 0, and +1, respectively, while FMN is negatively charged due to the ionized phosphate group. There are, however, no significant differences between the association constants for complexes of these same indoles with riboflavin, which has no phosphate group and thus carries no charge at pH 7. Moreover, the constants for the riboflavin complexes are equal to that for complexing of FMN by the neutral tryptophan molecule. Thus, when electrostatic interactions are eliminated, as when one (or both) of the complexing entities bears no net charge, appreciable alterations may be made in the side chain of the indole derivative or flavin with no detectable effect on complexing. Modification of the indole ring itself, however, does cause changes in the complexing ability of the indole. For example, the association constant at 25° for the complex of FMN with 5-hydroxytryptophan is 130 l. $mole^{-1}$ compared to 92 l. $mole^{-1}$ with tryptophan.

Investigation of the Ability of Substituted Indoles to Complex with FMN. A number of variously substituted indole derivatives have been examined in an attempt to determine whether any specific positions of the indole ring are required for complex formation. The low solubility of many of the compounds investigated precluded the determination of a meaningful value for the association constant. Therefore, no extensive quantita-

tive comparison of complexing ability was attempted. As mentioned above, difference spectra similar to that for tryptophan (Figure 2) were obtained with every compound examined, the only variations being slight (± 10 mμ) differences in the location of the maxima and minima.

The complexing of FMN with indole itself is readily observed. Substituents at the 1, 2, 3, 5, and 7 positions of the indole ring do not prevent complexing. The substituted indoles examined included 5-methylindole, 7-methylindole, 1,2-dimethylindole, indole-3-aldehyde, 5-hydroxytryptophan, and 5-cyanoindole. These compounds represent substitution at five of the seven possible positions on the indole ring. Variations in the size or stereochemical properties of the substituent in the 3-position do not prevent complex formation. Thus, indole-3-acetic acid, indole-3-propionic acid, and indole-3-butyric acid all complex with FMN, and D-tryptophan is as active as the L isomer as a flavin complexing agent. These results strongly imply the lack of any structural requirement other than the indole ring for complex formation. Indeed, since pyrrole may formally be considered equivalent to the five-membered portion of the indole ring, the formation of an FMN–pyrrole complex with properties similar to the flavin–indole complexes suggests that not even a complete indole ring system is required.

No evidence was found to indicate that the pyrrole analogs, furan and thiophene, could similarly complex with FMN. The low solubility of these compounds, however, made it impossible to achieve the concentrations attained with the more soluble pyrrole. Thus, the possibility that complex formation could occur, but was undetected, cannot be ruled out. Assuming that the spectral properties of these complexes (if formed) are similar to those of the pyrrole- and indole–flavin complexes, it can be estimated that complexes of FMN with furan or thiophene would have been detected if they had association constants greater than 1 l. mole^{-1}. If such complexes do form, therefore, they are considerably less stable than the FMN–pyrrole complex which has an association constant of about 2.2 l. mole^{-1} at 25°.

Thermodynamic Parameters for the Formation of Several Flavin Complexes. ΔH, ΔF, and ΔS values were calculated with the usual thermodynamic relations, ΔH being determined from the temperature dependence of the association constant. The values obtained for the formation of FMN complexes with pyrrole and several indole derivatives are presented in Table I. Also shown is the location of the maximum in the 500-mμ region of the difference spectrum (see above) of the complex, and the molar absorptivity at this maximum. The heats of formation for these complexes are appreciably more negative than those reported for several flavin–purine complexes (Weber, 1950; Tsibris *et al.*, 1965).

The Effects of Tryptophan and Caffeine on Complexes Present in FMN–$FMNH_2$ Mixtures. Approximately 4 mM solutions of FMN in 0.1 M potassium phosphate, pH 7.0, were titrated with sodium dithionite. The absorption spectra at various stages of reduction were virtually identical with those obtained for the corresponding mixtures at pH 6.3 by Gibson *et al.* (1962), and showed the broad absorption bands near 600 and 900 mμ attributed by these authors to semiquinone flavin mononucleotide (FMNH) and its complexes and to an FMN–$FMNH_2$ complex, respectively. Titration curves were prepared by plotting the absorbance at 600 and 900 mμ *vs.* per cent reduction. Since the FMN–tryptophan complex exhibits appreciable absorption at 600 mμ, the absorbance at this wavelength was corrected by the relation

$$A \text{ (corrected)} = A \text{ (observed)} - B\,(1 - \% \text{ reduction}/100)$$

where B, the initial absorbance at 600 mμ, was 0.26–0.29 at the FMN and tryptophan concentrations used. FMN itself and the FMN–caffeine complex have negligible absorbance at 600 mμ and, accordingly, no corrections were required in the latter two cases.

In Figure 6, the effects of caffeine and tryptophan on the titration curves are compared. The symmetry of the plot of absorbance at 900 mμ *vs.* per cent reduction is obvious in all three cases, as expected for a complex requiring both FMN and $FMNH_2$ for its formation. The asymmetry of the titration curves using the absorbance at 600 mμ is readily apparent in either the absence or presence of tryptophan. In agreement with the previous report by Gibson *et al.* (1962), however, caffeine destroys this asymmetry.

The Effect of Complexing on the Electrochemical Properties of FMN. $E_{1/2}$ values for FMN in the presence of various tryptophan concentrations are plotted in Figure 7 *vs.* pT, which is defined as the negative logarithm of the tryptophan concentration (analogous to the definition of pH). The slope of the straight-line portion at lower pT values is 29.6 mv/pT unit, in exact agreement with the theoretically expected value for the dissociation of one tryptophan per FMN reduced

TABLE I: Thermodynamic Parameters for the Formation of FMN Complexes.

Complexing Agent	ΔF (25°) (kcal/mole)	ΔH (kcal/mole)	ΔS (cal/mole-deg)	λ^a (mμ)	ϵ_λ ($\times 10^3$ l./mole-cm)
L-Tryptophan	−2.7	−7.4	−15.8	499	3.58
5-Hydroxy-DL-tryptophan	−2.9	−4.7	−6.0	503	3.44
Tryptamine	−2.8	−9.2	−21.5	498	3.91
Indole-3-acetic acid	−2.2	−7.9	−19.1	501	3.58
Pyrrole	−0.5	−3.5	−10.1	487	6.46

[a] Maximum in the difference spectrum of the complex.

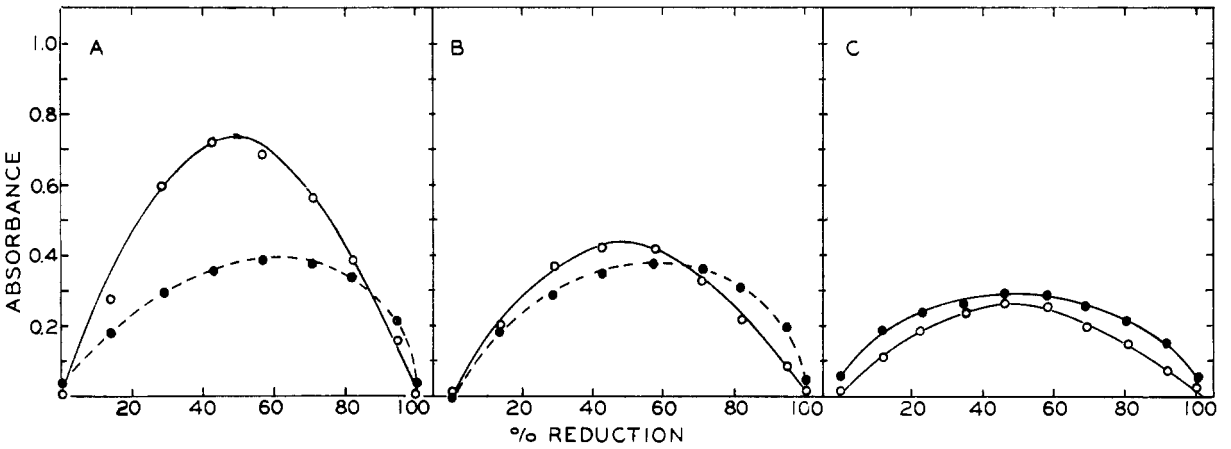

FIGURE 6: Effect of caffeine and tryptophan on complexes formed in mixtures of oxidized and reduced FMN. Total flavin concentration in each case was 3.8×10^{-3} M. Open circles (○), absorbance at 900 mμ. Closed circles (●), absorbance at 600 mμ. Solid lines indicate symmetric curves; broken lines, asymmetric curves. A, no additions; B, plus 4.2×10^{-2} M tryptophan; C, plus 7.2×10^{-2} M caffeine. Absorbance at 600 mμ in the presence of tryptophan corrected as described in text.

(Meites and Thomas, 1958). Since spectrophotometric results indicate a one to one stoichiometry for the FMN–tryptophan complex (Isenberg and Szent-Györgyi, 1958; Harbury and Foley, 1958), the release of one tryptophan upon reduction implies that $FMNH_2$ does not complex with the indole, as proposed above on the basis of spectral studies. The extrapolated straight-line portions of the curve intersect at pT equal to 1.9, from which a value of approximately 80 l. mole^{-1} may be calculated (Meites and Thomas, 1958) for the association constant of the FMN–tryptophan complex at 26°. This compares favorably with the value of 92 l. mole^{-1} at 25° determined spectrophotometrically.

At 26° in 0.1 M potassium phosphate, pH 7.0, the $E_{1/2}$ value for FMN is −0.455 v, in good agreement with the value of −0.452 v at 25° reported by Ke (1957). Taking the potential of the saturated calomel electrode (SCE) as +0.246 v *vs.* normal hydrogen electrode (NHE) (Meites and Thomas, 1958), this corresponds to a value of −0.209 v *vs.* NHE for E_0' of the FMN–$FMNH_2$ couple at 26°. Extrapolation of the $E_{1/2}$ *vs.* pT plot to pT equals 0 gives an E_0' value of −0.511 v *vs.* SCE (−0.265 v *vs.* NHE) for the FMN–tryptophan complex.

Discussion

Comparison of Flavin–Indole with Flavin–Purine Complexes. Since both indoles and purines possess an N-heterocyclic aromatic character, it is not surprising that complexes of flavins with these compounds have sometimes been assumed to result from essentially identical modes of interaction between the flavin and the complexing agent (Wright and McCormick, 1964; Tsibris *et al.*, 1965). There are, however, several striking differences between the properties of the flavin–indole complexes and those of the flavin–purine complexes which cause considerable doubt as to the validity of this view. The different effects that complexing with indolm (pyrrole) and purines has on the absorption spectrues of the flavin have been described above.

Indoles also differ from purines in their ability to complex the various oxidation states of flavins. The spectral and polarographic results presented above clearly indicate that FMN, but not $FMNH_2$, is complexed by tryptophan. Furthermore, the observations of Gibson *et al.* (1962) provide a basis for deciding whether tryptophan can also complex the semiquinone form, FMNH. These authors report that maximal amounts of semiquinone in FMN–$FMNH_2$ mixtures are found at 60–70% reduction, instead of the theoretically expected 50% reduction, due to the formation of a complex of the semiquinone with $FMNH_2$. When formation of the FMNH–$FMNH_2$ complex is inhibited, as by complexing either $FMNH_2$ or FMNH (or both), a symmetrical titration curve results. As shown in Figure 6, tryptophan does not destroy the asymmetry of the titration curve, implying that FMNH is not complexed by tryptophan. This requirement for a specific oxidation state of the flavin is not observed in the case of the flavin–purine complexes. The spectral changes caused by caffeine and adenosine (Figures 1–4) demonstrate that these purines complex both the oxidized and reduced flavin. Potentiometric methods also indicate complexing of both FMN and $FMNH_2$ by various purine derivatives (Harbury *et al.*, 1959). In view of the ability of purines to complex both the oxidized and reduced forms, it seems likely that the semiquinone is also complexed by these compounds.

Purines do not exhibit the specificity for a particular ionization state of the flavin shown by indoles. The formation of FMN–indole complexes is inhibited at pH values above 8, indicating that dissociation of the 3-imino group of the isoalloxazine ring prevents com-

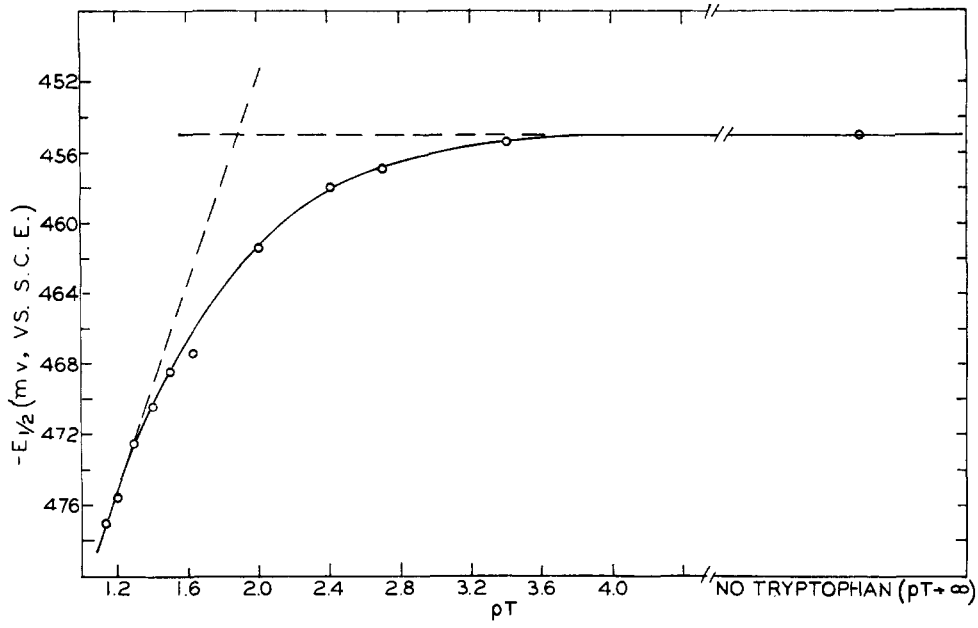

FIGURE 7: Effect of tryptophan concentration on $E_{0.5}$ value of FMN. FMN concentration was 5.95×10^{-4} M. pT is the negative logarithm of the tryptophan concentration.

plexing. In contrast, it is readily shown that the close agreement between the reduction potentials of FAD and FMN over the whole pH range of 1–12 (Lowe and Clark, 1956; Ke, 1957) implies that adenine complexes equally well with all ionization states of both the oxidized and reduced flavin.

Since the nature of the forces involved in complex formation is generally inferred from the observed properties of the complex, the dissimilar characteristics exhibited by the flavin–indole and flavin–purine complexes do not support the view that these complexes result from a common type of interaction between the flavin and the complexing agent. Of ultimate interest, however, is not simply whether the intermolecular forces in flavin–indole complexes differ from those involved in flavin–purine complexes, but rather the description of the interaction actually present in each type of complex.

The Nature of Flavin–Indole Interactions. The absorption by flavin–indole (pyrrole) complexes in the 550–700-mμ region is difficult to attribute to merely a red shift of the 445-mμ absorption band of the flavin, especially since complexing with purines, which also causes a red shift, does not result in any extension of the tail of this band to appreciably longer wavelengths. The possibility that this absorption is due to charge transfer absorption (Mulliken, 1952) has been considered. Massey and Ganther (1965) recently reported that complexing with carboxylic acid derivatives of pyrrole and indole causes the appearance of an absorption band at 500–700 mμ in the spectrum of enzyme-bound FAD. These authors also favored the view that these were charge transfer bands.

The location of a charge transfer absorption band should be sensitive to changes in the electron-donating or -accepting abilities of the components of the complex (Mulliken, 1952). The similarity among the absorption spectra of FMN complexes with a variety of indole derivatives is therefore a possible objection to the proposed charge transfer nature of these complexes. It should be emphasized, however, that the difference spectra of the various complexes are only similar, not identical. Thus, the differences in the location of the long wavelength peak might be due, at least in part, to changes in the location of a broad, weak charge transfer band in the 500–700-mμ region. The inability to demonstrate a distinct charge transfer band might well be an unhappy consequence of the low intensity of the band and its proximity to the intense absorption band of the flavin. Similar difficulties have been encountered in studying the charge transfer complexes of flavins with phenols (Fleischman and Tollin, 1965 a,b). Indeed, the absorption spectra of the flavin–indole (pyrrole) complexes are very similar to those of the flavin–phenol complexes in the 500–700-mμ region (Fleischman and Tollin, 1965b).

Other properties of flavin–indole complexes are compatible with a charge transfer character. Thus, in the pH range studied, indoles complex only with the oxidized, neutral form of the isoalloxazine ring. Either reduction or the negative charge resulting from ionization of the 3-imino group would be expected to destroy the ability of the flavin to function as an electron acceptor in charge transfer interactions.

Karreman (1961, 1962) has proposed a parallel, overlapping arrangement of the isoalloxazine and indole rings which, on the basis of calculated electron densities, would be expected to facilitate charge transfer inter-

actions between the aromatic systems. The proposed arrangement would place N_{10} of the isoalloxazine ring in close proximity to the nitrogen atom of the indole, which also, as pointed out by Massey and Ganther (1965), might favor charge transfer interactions. Such an overlapping arrangement should be relatively indifferent to substituents about the periphery of either entity, which is in accord with the observed complexing ability of indoles having substituents at various positions on the ring. Furthermore, the proposed alignment indicates that a hydroxyl group at the 5-position of the indole ring would be able to hydrogen bond with the 2-keto group of the flavin. This provides a reasonable explanation for the association constant of the FMN-5-hydroxytryptophan complex being larger than that for the FMN–tryptophan complex, even though the heats of formation for the complexes indicate that FMN interacts more strongly with tryptophan than with 5-hydroxytryptophan.

The existence of charge transfer forces does not, of course, exclude the possibility that other types of interactions may also be involved in the formation of these complexes. The isolated flavin and indole molecules represent a total of four aqueous–aromatic ring interfaces, whereas the parallel overlapping arrangement proposed for the flavin–indole complex (Karreman, 1961, 1962) represents only two such interfaces. The environment of the flavin in the flavin–indole complex is thus appreciably more nonpolar, in agreement with the observation that the changes in the flavin spectrum caused by complexing with indoles are similar to those observed by dissolving the flavin in more nonpolar solvents (Harbury *et al.*, 1959; Koziol, 1965; Koziol and Knobloch, 1965). The hydrophobic region between the aromatic rings would be expected to facilitate the operation of dispersion forces. The secondary role that electrostatic forces may play in flavin–indole complexing is evident from the results presented above.

Since the primary interaction in flavin–indole complexes is envisaged as occurring between the indole and isoalloxazine rings, the strength of this interaction should be relatively independent of changes in the side chains. Modification of the electronic structure of either the indole or isoalloxazine ring, however, should affect their interaction. Flavin–indole complexes fulfill these expectations. Tryptophan, tryptamine, and indole-3-acetic acid have very similar spectral properties (McMenamy and Oncley, 1958), as do FMN and riboflavin (Whitby, 1953), indicating that the indole and isoalloxazine rings are not appreciably altered by the different side chains in these compounds. When electrostatic effects are precluded, complexes of either FMN or riboflavin with these indole derivatives are all found to have the same association constants. Furthermore, the heats of formation for complexes of these compounds with FMN are all quite similar. The slightly larger value of ΔH for the formation of the FMN–tryptamine complex is very likely due to the additional ionic bonding that could occur between the positively charged amino group of tryptamine and the phosphate group of FMN. When the indole ring of tryptophan is modified by substitution of a hydroxyl group at the 5-position, the resulting changes in the electronic structure are evident from the altered ultraviolet spectrum (Udenfriend and Weissbach, 1963), and the heat of formation for the complex with FMN is found to be considerably lowered.

The Nature of Flavin–Purine Interactions. Tsibris *et al.* (1965) have proposed that FMN–purine complex formation is a result of donor–acceptor forces. Thus, the purine was considered to partially donate an electron to the acceptor, the isoalloxazine ring, resulting in the formation of a stable complex similar to the ground state of a classical charge transfer complex (Mulliken, 1952).

Further considerations provide very little support for the proposed donor–acceptor interactions in the complexing of purines with oxidized flavins. Tsibris *et al.* (1965) noted a correlation between the stability of complexes of riboflavin with various purine nucleosides and the electron-donating ability of the purine, measured by the k value (Pullman and Pullman, 1963) of the highest occupied molecular orbital of the purine. This correlation, which was cited as evidence for the donor–acceptor nature of these complexes, is very likely a fortuitous one since it does not hold when the stability of the riboflavin complexes with the free purine bases (Tsibris *et al.*, 1965) is considered. Furthermore, the apparent insensitivity of intramolecular complexing in FAD to changes in the ionization state of the flavin is, as discussed above, certainly not a property to be expected for a donor–acceptor complex.

Even if donor–acceptor interactions were important in the complexing of purines with FMN, it seems very unlikely that these interactions could also account for the complexing with $FMNH_2$. Due to the relatively high energy of its lowest unoccupied molecular orbital (Pullman and Pullman, 1963), $FMNH_2$ should be a much poorer acceptor than FMN, and yet adenylic acid and guanylic acid complex both the oxidized and reduced forms with nearly equal avidity, while caffeine binds $FMNH_2$ even more tightly than FMN (Harbury *et al.*, 1959). The possibility that the roles of the flavin and purine might be reversed in the $FMNH_2$ complexes also seems unlikely. Although $FMNH_2$ should be a good donor, the high energies of the lowest unoccupied molecular orbitals of the purines (Pullman and Pullman, 1963) indicate that purines would be extremely poor acceptors.

Although these considerations tend to rule out a donor–acceptor nature for flavin–purine complexes, the problem of just what intermolecular forces are involved remains unsolved. The ability of purines to complex both oxidized and reduced forms, and the apparent insensitivity of this complexing to the ionization state of the flavin, are rather difficult to interpret in terms of the usual types of interactions occurring between aromatic systems. The results presented here, however, provide considerable support for the view that the intermolecular forces involved in flavin–purine complexes differ from those operative in complexing of flavins with indoles, a distinction that does not seem

to have been explicitly considered by previous authors.

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