Investigations of Inter- and Intramolecular Interactions in Flavin–Adenine Dinucleotide by Proton Magnetic Resonance*

Ramaswamy H. Sarma, Priscilla Dannies, and Nathan O. Kaplan

ABSTRACT: Concentration and pH dependence of chemical shifts for flavin–adenine dinucleotide, flavin mononucleotide, and adenosine diphosphoribose indicates the existence of inter- and intramolecular interactions in the dinucleotide. The chemical shift data for adenine C_8H and C_2H in both flavin–adenine dinucleotide and adenosine diphosphoribose as well as that for isoalloxazine C_8H and C_5H in both flavine–adenine dinucleotide and flavine mononucleotide suggest that on the average the adenine C_2H spends considerable time in the proximity of the aromatic ring of isoalloxazine of flavin–adenine dinucloetide and that the isoalloxazine C_5H spends considerable time in the proximity of the pyrimidine portion of adenine. The upfield shift of the isoalloxazine protons with increasing concentration in flavine–adenine dinucleotide and flavin mononucleotide indicates that both the mono- and dinucleotides associate themselves by way of vertical stacking of the isoalloxazine rings.

During the intermolecular complexing the C_8H of one of the isoalloxazine rings will on the average spend considerable time in the proximity of the aromatic ring of another isoalloxazine ring and *vice versa*. Comparison of the degree of intramolecular interaction between base pairs in flavin–adenine dinucleotide, oxidized diphosphopyridine nucleotide, and nicotinamide–nicotinamide dinucleotide support the idea that the interaction between base pairs in pyridine and flavin dinucleotides may involve hydrophobic phenomena rather than hydrogen bonding or charge transfer.

Recent investigations on the conformation of pyridine dinucleotides by Jardetzky and Wade-Jardetzky (1966) as well as Sarma *et al.* (1968) indicate the potential of high-resolution proton magnetic resonance to reveal the subtle and finer features of geometry and configuration of biologically important molecules. Our present endeavors are directed toward the investigation of inter- and intramolecular interactions in FAD by proton magnetic resonance.

The earlier fluorescence experiments (Bessey *et al.*, 1949; Weber, 1950) showed the existence of an intramolecular interaction between the adenine and isoalloxazine rings in FAD. Recently Chassy and McCormick (1965) investigated the structural requirements of the flavin moiety of FAD for intramolecular complex formation. Tsibris *et al.* (1965) characterized the intramolecular complex to be the donor–acceptor type as broadly defined by Mulliken and Person (1962). The present investigation with proton magnetic resonance spectroscopy confirms the intramolecular complexing but, in addition, our studies uncover some of the finer aspects of this intramolecular complexing and indicate that there is significant intermolecular complexing between the isoalloxazine rings of FAD molecules. These aspects are discussed in detail below.

Experimental Procedure

The nucleotides DPN$^+$, NMN, and ADPR were obtained from P.-L. Biochemicals. FAD and FMN were obtained from either Sigma or C. F. Boehringer und Soehne, GmbH, Mannheim. The analog NND was synthesized by a Khorana synthesis from NMN. The two nicotinamide moieties of this analog bear positively charged ring nitrogen. Details of the preparation, properties, and conformation of this analog as well as those of a few other analogs will be presented in a forthcoming paper.

The proton magnetic resonance spectra were obtained on a high-resolution Varian Model A-60A spectrometer. Shifts were measured with the silapentanesulfonate[1] (obtained from E. Merck Ag., Darmstadt, Germany) as an internal standard. The proton magnetic resonance probe temperature was 38 ± 1°. All samples were lyophilized from deuterium oxide to remove exchangeable protons and then dissolved in deuterium oxide. Measurements were made on two different preparations and two or three spectra were recorded on each sample. The agreement among the different spectra was within 1 cps. The pH was measured before and after proton magnetic resonance spectra were taken.

Results and Discussion

The proton magnetic resonance spectrum of FAD

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[1] Abbreviations: NND, nicotinamide–nicotinamide dinucleotide (NRPPRN); the silapentanesulfonate refers to sodium 2,2-dimethyl-2-silapentane-5-sulfonate.

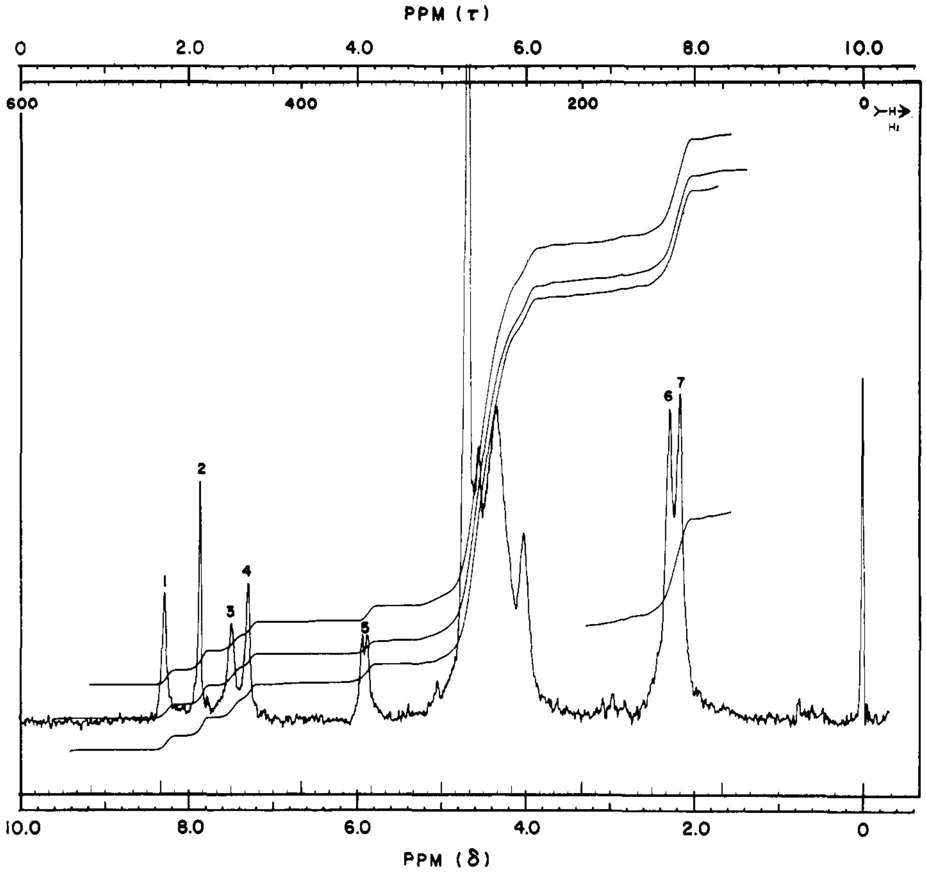

FIGURE 1: Proton magnetic resonance spectrum of FAD as reproduced directly from the recording chart S60-C. The spectrum was taken in a high-resolution Varian Model A-60A spectrometer at a sweep width of 1000 cps and calibrated against the silapentanesulfonate as an internal standard. The concentration of the solution was 0.15 M (pH 7.0) and temperature 38°. Peaks: (1) adenine C_8H, (2) adenine C_2H, (3) flavin C_5H, (4) flavin C_8H, (5) adenine $C_1'H$, (6) flavin C_7CH_3, and (7) flavin C_6CH_3.

(0.15 M, pH 7, 38°) in D_2O is shown in Figure 1. The proton magnetic resonance spectra of ADPR, FAD, and FMN, taken consecutively on a 0.15 M solution at pH 7.0, 38°, with the same internal standard, the silapentanesulfonate,[1] are shown in Figure 2A–C. The assignment of the peaks for the isoalloxazine ring was made from computed π-electron densities[2] for the oxidized form of the isoalloxazine ring (Figure 3, from Pullman and Pullman, 1959). The Pullman and Pullman (1959) computations are highly approximate calculations of the π-electron distribution. However, the differences in the π-electron densities between carbons C_8 and C_5 as well as that between carbons C_7 and C_6 are so large that refined molecular orbital calculations would affect only the magnitude and not the directions, *i.e.*, C_8 will have a higher π-electron density than C_5 even if the molecular orbital calculations were performed without the Hückel approximations. The same argument applies for carbons C_7 and C_6. Further, the oxidized form of isoalloxazine of FAD could be represented as a resonance hybrid of the five canonical forms (Scheme I). The hybrid structure clearly shows that charge at carbons C_5 and C_7 could be delocalized and this would result in a lower π-electron density at these carbons as the Pullman and Pullman (1959) calculation shows. In view of the fact that both the Pullman and Pullman calculations and the resonance treatment agree, it is most likely that in the proton magnetic resonance spectra of FAD, the isoalloxazine C_5H will appear at a lower field compared with C_8H. So also C_7CH_3 will appear at a lower field than C_6CH_3. McCormick (1967) has made the same assignments in the case of lumiflavin from its deuterated analog. In order to evaluate the effect of the isoalloxazine ring on the adenine in FAD we have compared the chemical shifts of adenine in ADPR and FAD.[3] Examination of Figure 2 shows that the proton magentic resonance signals in the dinucleotide are shifted upfield from those of ADPR and FMN. Such upfield shifting could result from (a) intermolecular complexing between FAD molecules; (b) intramolecular complexing between the adenine and

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[2] See the following for the correlations between electron densities and chemical shifts: Fraenkel *et al.* (1960), Smith and Schneider (1961), Schaefer and Schneider (1963), and Schug and Deck (1962).

[3] We realize that adenosine diphosphoribitol, rather than adenosine diphosphoribose, would appear to make a better comparison because of the ribotide linkage between the isoalloxazine ring and the ribose in FAD. However, we do not think it would make any difference because here one is concerned only with the effect of ring current magnetic anisotropies of the isoalloxazine ring on the adenine resonance.

TABLE I: Effect of Concentration on the Chemical Shifts of Various Protons of FAD, ADPR, and FMN.[a]

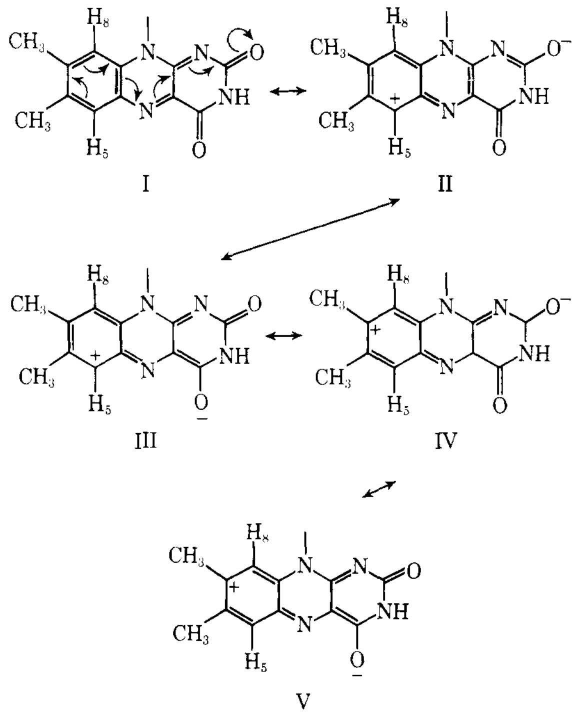
			Adenine Protons (cps)			Isoalloxazine (Flavin) Protons (cps)			
Compd	Concn (M)		AC_8H	AC_2H	$AC_1'H$	FC_5H	FC_8H	FC_7CH_3	FC_6CH_3
FAD	0.0		497	474	354	452	454	144	139
	0.3		497	469	354	447	432	134	127
		Δ[b]	0	5	0	5	22	10	12
ADPR	0.0		512	495	371				
	0.3		512	492	371				
		Δ	0	3	0				
FMN	0.0					472	462	154	147
	0.3					460	439	146	135
		Δ				12	23	8	12

[a] Temperature 38°, internal standard the silapentanesulfonate; values of chemical shifts at 0.0 M were obtained from extrapolations. The Δ values are all positive unless otherwise noted, indicating upfield shifts with increase in concentration. [b] Δ = chemical shift at 0.0 M minus that at 0.3 M.

isoalloxazine rings in FAD; (c) a combination of both inter- and intramolecular interactions. Study of the dependence of chemical shifts on pH and concentration should enable a differentiation of several possibilities.

The Concentration Dependence of Chemical Shifts. Figure 4 as well as the data in Table I summarize the effect of concentration on the proton chemical shifts of FAD, ADPR, and FMN.[4] The chemical shifts at 0.0 M were obtained by extrapolating chemical shifts at 0.3, 0.2, 0.15, 0.1, and 0.05–0.0 M. Determination of chemical shifts at concentrations below 0.05 M is restricted by the sensitivity limitations of A-60A proton magnetic resonance spectrometers. Without the use of time-averaging computers it is impossible to measure chemical shifts below 0.05 M concentrations. Further, the use of the computer does not guarantee precise measurements of chemical shifts at ~0.01 M concentrations of FAD because of the relative unstability of FAD during the several hours spent by the computer to make hundreds of scans in order to time average the signal to noise ratio. It appears not necessary for our particular system to have values below 0.05 M because throughout the entire range of 0.3–0.05 M, the chemical shifts in the dinucleotide appear at a higher field compared with ADPR and FMN, indicating strong intramolecular interaction throughout the entire concentration range. Further, the adenine protons of both FAD and ADPR are virtually independent of concentration, thus presenting no complication from intermolecular interactions. In the case of the flavin protons of FAD and FMN, at concentrations below 0.05 M one would expect the upfield shift in the dinucleotide to either remain the same or increase depending upon the extent to which the dinucleotide and mononucleotide are self-associated. It is highly inconceivable to see how the upfield shift in the dinucleotide could diminish at concentrations below 0.05 M. If the dinucleotide is strongly self-associated compared with the mononucleotide, as one decreases the concentration from 0.3 to 0.05 M, the upfield shift in the dinucleotide will drastically dwindle. Figure 4 shows that throughout the concentration range 0.3–0.05 M, the upfield shift in the dinucleotide has remained either constant or increased, indicating that the isoalloxazine in the dinucleotide is self-

SCHEME I



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[4] The chemical shifts are reported in cycles per second. The radiofrequency oscillator of the Varian Model A-60A proton magnetic resonance spectrometer is set at 60 Mcps. The chemical shifts in cycles per second could be divided by the oscillator frequency and reported as parts per million as in Figures 1 and 2.

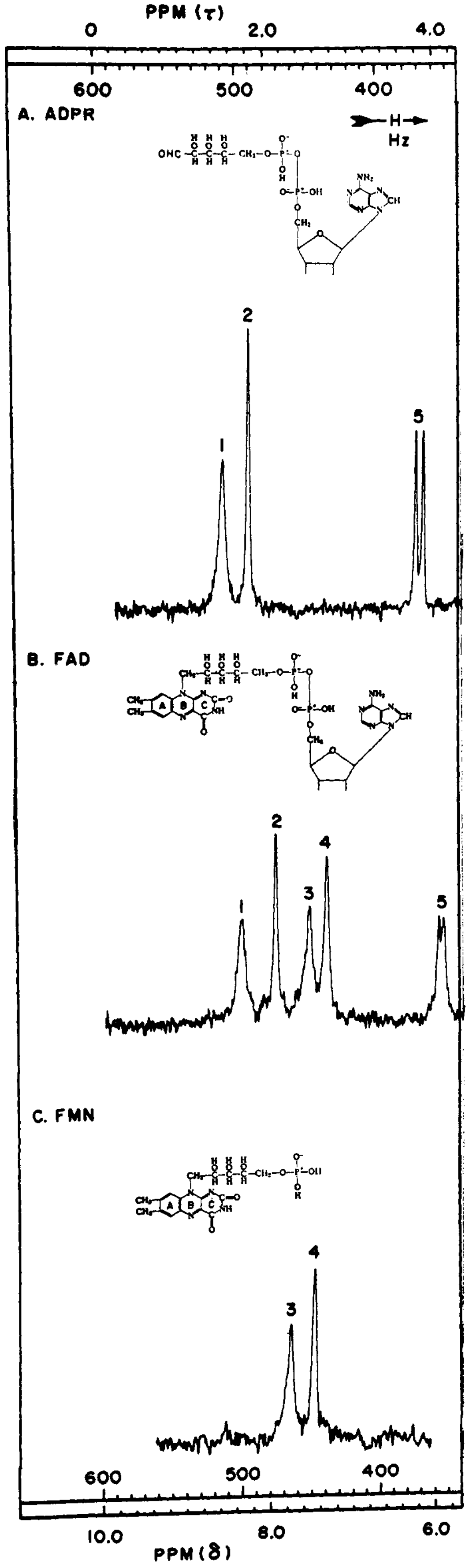

FIGURE 2: Proton magnetic resonance spectra of ADPR (A), FAD (B), and FMN (C) as reproduced directly from the recording chart S60-C. The three spectra were taken consecutively in a high-resolution Varian Model A-60A spectrometer at a sweep width of 1000 cps and calibrated against the silapentanesulfonate. The assignment of the peaks is like that in Figure 1.

associated only to the same extent or to a lesser extent compared with FMN. The discussion presented above shows that the proton magnetic resonance data of our system could be interpreted for any concentration in order to gain insight regarding the intramolecular interaction between the flavin and adenine rings. However, we have extrapolated the data to 0.0 M because it is standard practice in proton magnetic resonance work to discuss the data at infinite dilution. At infinite dilution the adenine C_8H, C_2H, and C'_1H of FAD are shifted upfield by 15, 21, and 17 cps, respectively, from those of ADPR (Table II). This upfield shift strongly indicates intramolecular complexing between the adenine and isoalloxazine moieties in FAD. Such an intramolecular complex is also revealed by the upfield shift of C_5H, C_8H, C_6CH_3, and C_7CH_3 of the isoalloxazine ring in the dinucleotide compared with those in FMN (Table II).

The chemical shifts of adenine C_8H and C'_1H in both FAD and ADPR are not sensitive to concentration. However, the adenine C_8H and C'_1H of 5′-AMPNa$_2$ undergo an upfield shift of 10 and 7 cps, respectively, as the concentration is increased from 0.0 to 0.3 M (Schweizer *et al.*, 1968). Adenine C_2H in FAD and ADPR is not sensitive to concentration at low concentration (below 0.1 M). An increase in the concentration to 0.3 M results in an upfield shift of 3 cps in ADPR and 5 cps in FAD compared with 20 cps in 5′-AMPNa$_2$ (Schweizer *et al.*, 1968). In view of the fact that the upward shift is observed only for one of the protons and also that the shift is small compared with 5′-AMPNa$_2$, it may be concluded that both ADPR and FAD do not associate to any significant extent by way of vertical stacking of adenine rings. Jardetzky and Wade-Jardetzky (1966) have reached similar conclusions for DPN. It is instructive to note that extending the 5′-AMP structure by means of a phosphate and D-ribose as in ADPR abolishes the formation of molecular aggregates, and that extra structure extension by nicotinamide as in DPN or by the isoalloxazine ring as in FAD is not necessary

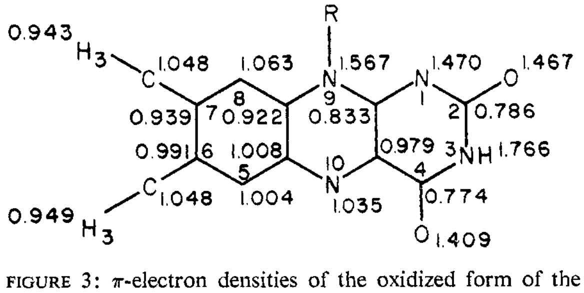

FIGURE 3: π-electron densities of the oxidized form of the isoalloxazine ring of FMN or FAD (from Pullman and Pullman, 1959). The integers (1–10) represent the numbering of isoalloxazine rings.

TABLE II: The Upfield Shift (Δ) of the Protons in FAD Compared with Those in ADPR and FMN.[a]

	Adenine Protons (cps)			Isoalloxazine (Flavin) Protons (cps)			
	AC_8H	AC_2H	$AC_1{}'H$	FC_5H	FC_8H	FC_7CH_3	FC_6CH_3
$\Delta(ADPR-FAD)_{pH\ 7.0}$[b]	15	21	17				
$\Delta(FMN-FAD)_{pH\ 7.0}$				20	8	10	8

[a] The various values of the chemical shifts used in this calculation were those at 0.0 M obtained from extrapolation. [b] Chemical shifts of ADPR protons minus those of FAD protons at pH 7.0.

to preclude self-association *via* vertical stacking of adenine rings.

The chemical shifts of the C_5H, C_8H, C_6CH_3, and C_7CH_3 of the isoalloxazine rings of FAD and FMN are sensitive to concentration (Table I, Figure 4). This contrasts strikingly with the fact that both DPN and NMN do not undergo self-association by vertical stacking of the pyridine rings, probably due to the positive charge on the pyridine nitrogen (Jardetzky and Wade-Jardetzky, 1966). The following observations on the chemical shifts of adenine and isoalloxazine protons of the dinucleotide delineate some of the delicate features of its configuration in aqueous solution.

At infinite dilution as well as in the enitre range of concentration 0.0–0.3 M it has been found that the adenine C_2H undergoes a larger diamagnetic shielding by the ring-current anisotropy of the isoalloxazine ring compared with the adenine C_8H (21 *vs.* 15 cps). Also, it has been observed that the isoalloxazine C_5H undergoes larger diamagnetic shielding by the ring-current anisotropy of the adenine compared with the isoalloxazine C_8H (20 *vs.* 8 cps). This indicates *that on the average the adenine C_2H spends considerable time in the proximity of the aromatic ring* (ring A in Figure 2B) *of isoalloxazine of FAD and that the isoalloxazine C_5H spends considerable time in the proximity of the pyrimidine portion of adenine.*

The upfield shift of the isoalloxazine protons with increasing concentration (Table I, Figure 4) in FAD and FMN indicate that both the mono- and dinucleotides associate themselves by way of vertical stacking of the isoalloxazine rings. Whereas the intramolecular complexing between the adenine and isoalloxazine rings results in maximum shielding of the isoalloxazine C_5H and minimum shielding of the isoalloxazine C_8H, the intermolecular complexing between isoalloxazine rings results in maximum shielding of the isoalloxazine C_8H and minimum shielding of the isoalloxazine C_5H. As shown in Table I, the difference in the shielding of the isoalloxazine C_5H and C_8H is more pronounced in the dinucleotide (5 *vs.* 22 cps) than in the mononucleotide (12 *vs.* 23 cps). This is indeed consistent with the observation that the adenine ring spends on the average more time in the proximity of the isoalloxazine C_5H than in the proximity of the isoalloxazine C_8H; such an arrangement would permit intermolecular complexing in which the C_8H of one of the isoalloxazine rings will on the average spend considerable time in the proximity of the aromatic ring A of another isoalloxazine ring and *vice versa.*

The considerations presented so far permit us to construct models of FAD from skeletal models to show the relative positions of the flavin and adenine rings. Figure 5 shows the relative position between adenine and isoalloxazine rings in the same molecule of FAD during intramolecular interactions. Figure 6 shows the relative position between two isoalloxazine rings during intermolecular interactions.

The pH Dependence of Chemical Shifts. The pH dependence of chemical shifts for FAD, ADPR, and FMN is shown in Figure 7. The adenine protons C_8H, C_2H,

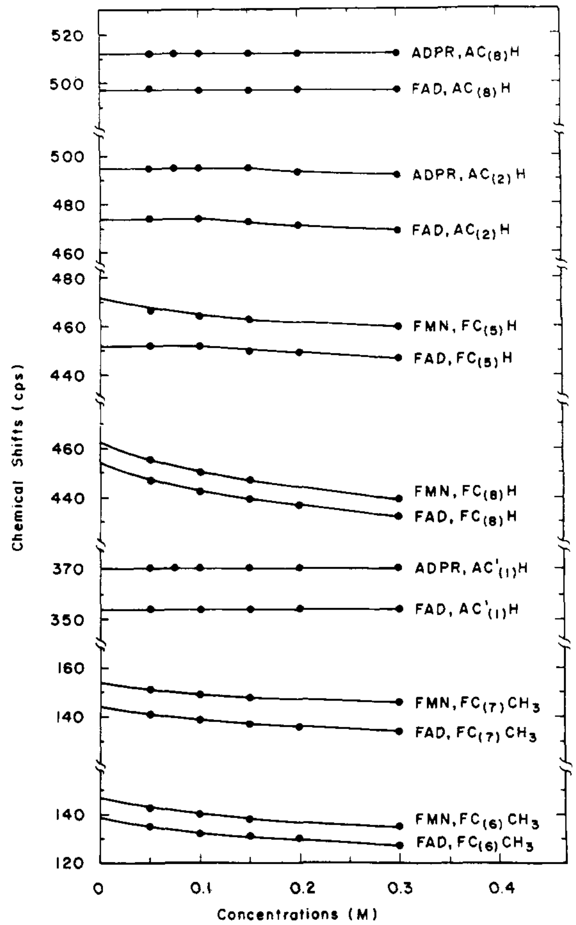

FIGURE 4: Concentration dependence of chemical shifts in FAD, ADPR, and FMN (pH 7.0), temperature 38°.

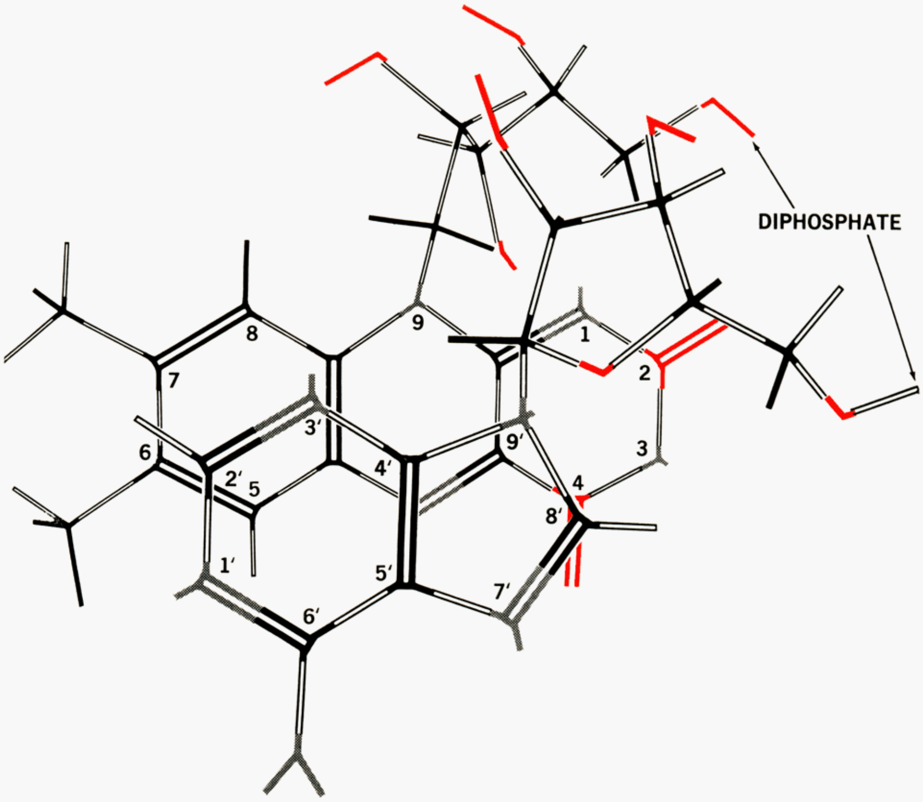

FIGURE 5: Skeletal model of FAD showing the relative positions between adenine and isoalloxazine rings. Proton magnetic resonance data suggest that on the average the adenine C_2H spends considerable time in the proximity of the aromatic ring (ring A in Figure 2B) of isoalloxazine of FAD and that the isoalloxazine C_5H spends considerable time in the proximity of the pyrimidine portion of adenine. Note that all the hydrophilic groups lend themselves to being present in the same space, thus allowing at the same time the two hydrophobic ends (*i.e.*, adenine and the aromatic ring of isoalloxazine) to interact.

and $C_1'H$ are shifted to lower fields with a transition around pH 4. The fact that the downfield shifts with decreasing pH for the adenine protons are greater in FAD than in ADPR provides evidence for the dissociation of the intramolecular adenine–isoalloxazine complex at lower pH range. The C_5H, C_8H, C_7CH_3, and C_6CH_3 of the isoalloxazine ring of FMN are independent of pH over the range 7–3. Examination of FMN below pH 3 was difficult because of precipitation. The isoalloxazines C_7CH_3 and C_5H in the dinucleotide are shifted to lower fields at low pH. This is expected because the isoalloxazine C_5H undergoes maximum shielding as a result of intramolecular complexing and lowering the pH results in the dissociation of the complex. Flavin C_8H of FAD undergoes a slight upfield shift with decreasing pH, the reason for which is not understood.

The Nature of the Intramolecular Interaction in Flavin and Pyridine Dinucleotides. The dissociation of the intramolecular complex between adenine and isoalloxazine rings in FAD at low pH agrees with the fluorescence experiments of Bessey *et al.* (1949) as well as those of Weber (1950). The fluorescence measurements of Walter and Kaplan (1963) as well as the proton magnetic resonance data of Jardetzky and Wade-Jardetzky (1966) and of Sarma *et al.* (1968) indicate that the pyridine and adenine rings in pyridine dinucleotides also dissociate at low pH. Since quenching of fluorescence is a general property of charge-transfer complexes (Orgel and Phil, 1954) the quenching of the flavin fluorescence by the adenine may indicate the formation of a charge transfer complex between the purine and isoalloxazine ring. In the event of a charge transfer from the adenine π system to the isoalloxazine system, the adenine protons of FAD would be expected to appear at a lower field compared with the adenine protons in ADPR. The fact that both the adenine and isoalloxazine protons of FAD appear at a field higher than those in ADPR and FMN does not completely rule out, as it *prima facie* appears to be, charge-transfer interactions between the adenine and isoalloxazine rings. This is because the extent of charge transfer between rings is almost negligibly small as far as chemical shifts are concerned and certainly small with respect to mutually induced ring-current shifts between the two rings.

Tsibris *et al.* (1965) have represented FAD as a donor–acceptor complex. In this complex they have represented the folding of adenine over ring C (Figure 2B) of isoalloxazine so that the polarization of charge between the rings results in a complementary charge belt which covers the region from C_6 to N_9 of adenine to a region from N_1 to N_{10} of flavin. In this representation the adenine N_9 acquires a partial positive charge. The effect of such donor–acceptor complexes on the proton magnetic resonance chemical shifts of FAD (compared with ADPR) are twofold. (1) The partial positive charge on N_9 of adenine will lead to a downfield shift of the adenine C_8H resonance of FAD compared with ADPR. (2) The ring-current magnetic anisotropy of ring C of isoallox-

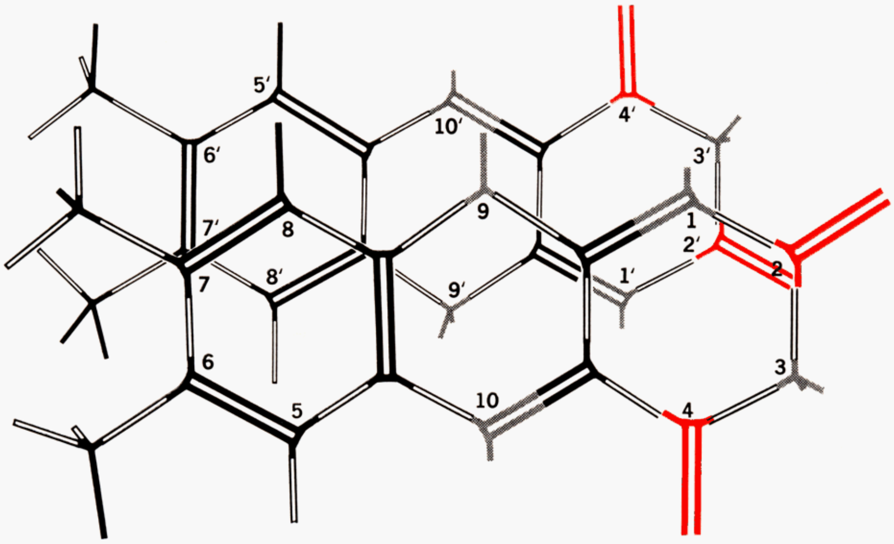

FIGURE 6: Skeletal models showing the relative positions between two isoalloxazine rings during intermolecular complexing. Note that the two isoalloxazine moieties are placed in an inverted manner. Such an inverted placement permits easily the C_8H of one of the isoalloxazine rings to spend, on the average, considerable time in the proximity of the aromatic ring (ring A, Figure 2B) of another isoalloxazine ring and *vice versa*. Also such an arrangement keeps the electrostatic repulsions between the carbonyl dipoles in contiguous isoalloxazine moieties to a minimum.

azine will cause a diamagnetic shielding of the adenine protons of FAD as compared with ADPR. What we observe is a strong shielding of the C_8H, C_2H, and $C_1'H$ of FAD (15, 21, and 17 cps, respectively) by the isoalloxazine ring. The question is whether a nonaromatic system like the ring C (Figure 2B) of isoalloxazine could shield the adenine protons by that much despite the unfavorable positive charge on the N_9 of adenine in the donor–acceptor complex. The π-current calculations of Griessner-Prettre and Pullman (1965) show that the ring-current anisotropies of nonaromatic rings like cytosine and uracil as well as the hexagonal rings in hypoxanthine, guanine, and xanthine are very small compared with benzene. If these calculations are correct one would expect a very weak ring-current shielding of adenine protons by ring C of isoalloxazine and that the present proton magnetic resonance data would not agree with a donor–acceptor concept as the major force of intramolecular interaction in FAD. Even though the proton magnetic resonance data on FAD, FMN, and ADPR suggests the absence of donor–acceptor complexes with complementary polarization of charge (Tsibris *et al.*, 1965), the fact that little is known about distance and angular dependences of ring-current fields, particularly for ring systems containing heteronuclei and fused ring systems, makes it difficult to make a decisive conclusion.

Whether donor–acceptor complexes of the type proposed by Tsibris *et al.* (1965) play any role in maintaining the conformational integrity of the dinucleotide, our proton magnetic resonance data on FAD, ADPR, and FMN show that in the dinucleotide the adenine protons are strongly shielded by the isoalloxazine ring and that the *adenine ring shields strongly the benzene protons of the isoalloxazine ring*, indicating the prevalence of strong intramolecular interaction between the adenine moiety and the benzene ring of isoalloxazine. We think that this interaction is a hydrophobic phenomenon and feel that interactions of the hydrophobic nature play a significant role in the intramolecular complexing between base pairs of dinucleotides in general. We have shown (Sarma *et al.*, 1968) that hydrogen bonding of the type occurring in nucleic acids does not play any predominant part in the base interactions of pyridine dinucleotides. Discussion of the proton magnetic resonance data, presented below, on flavin and pyridine dinucleotides suggests that the nature of the interaction between base pairs in both nucleotides is probably the same, *viz.* hydrophobic.

The proton magnetic resonance and fluorescence data so far available indicate that intramolecular complexing is prevalent both in flavin and pyridine–adenine dinucleotides. The proton magnetic resonance chemical shift data for FAD^+, DPN, and N^+N^+D at pH 7 (Table III) indicate that the interactions between the two rings are maximal in FAD, medium in DPN^+, and very small in NND.[5] If the interaction is of a hydrophobic nature, one would expect maximal interactions in FAD in which the adenine could interact with the rather hydrophobic aromatic ring of flavin. As one goes from FAD[6] to DPN^+ one would expect the hydrophobic interactions to

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[5] Consistent with this is the observation that the adenine C_8H and C_2H of FAD are shifted upfield by 15 and 21 cps, respectively, from those of ADPR, whereas the adenine C_8H and C_2H of DPN^+ are shifted upfield only by 8 and 9 cps, respectively, from those of ADPR (Sarma *et al.*, 1968). This also indicates that the intramolecular interaction in FAD is stronger than in DPN^+.

[6] Recent proton magnetic resonance and fluorescence data, to be published shortly, on flavin–hypoxanthine dinucleotide, inosine diphosphoribose and flavin mononucleotide indicate that in this dinucleotidehe intramolecular interaction is very weak, suggesting again that the interaction between base pairs in dinucleotides is of a hydrophobic nature.

TABLE III: Effect of pH on the Degree of Unfolding in FAD, DPN, and NND.

	Isoalloxazine (Flavin) Protons (cps)				Pyridine Protons (pcs)			
	FC$_5$H	FC$_8$H	FC$_7$CH$_2$	FC$_6$CH$_3$	PC$_2$H	PC$_6$H	PC$_4$H	PC$_5$H
$\Delta_{(\mathrm{FAD_{pH\ 3.0}-FAD_{pH\ 7.0}})}$[a]	6	0	2	0				
$\Delta_{(\mathrm{FMN-FAD})_{pH\ 7.0}}$[b]	20	8	10	8				
$\Delta_{(\mathrm{FMN-FAD})_{pH\ 3.0}}$	14	8	8	8				
$\Delta_{(\mathrm{DPN_{pH\ 2.0}-DPN_{pH\ 7.0}})}$					6	7	7	5
$\Delta_{(\mathrm{NMN-DPN})_{pH\ 7.0}}$					12	8	8	5
$\Delta_{(\mathrm{NMN-DPN})_{pH\ 3.0}}$[c]					7	1	1	0
$\Delta_{(\mathrm{NND_{pH\ 2.0}-NND_{pH\ 7.0}})}$					0	0	2	1
$\Delta_{(\mathrm{NMD-NND})_{pH\ 7.0}}$					5	1	0	0

[a] Chemical shifts of FAD protons at pH 3.0 minus those at pH 7.0. [b] Chemical shifts of FMN protons minus those of FAD protons at pH 7.0. [c] Chemical shifts of NMN protons at pH 7.0 are used; a change in chemical shift to lower fields for the pyridine protons of NMN at pH 3 is very unlikely; the p*K* values for the protonation of nicotinamide ring are much lower.

dwindle slowly because of the introduction of a positive charge on the pyridine ring. Introduction of a positive charge on the adenine ring of FAD (proton magnetic resonance data at pH 3.0) also reduces the magnitude of interaction between the rings. However, lowering the pH does not affect the intramolecular interaction in FAD as much as it does in DPN$^+$. At pH 3, unlike FAD, DPN$^+$ contains two charged rings and this results in the further diminution of hydrophobic interactions between the rings and setting in of electrostatic repulsions, thus unfolding the molecule. Indeed, consistent with this argument is the behavior of N$^+$N$^+$D at pH 7 and 2. At pH 7, the molecule N$^+$N$^+$D carries positively charged rings and shows very little interaction between the rings. Data in Table III show that pH has no effect on the conformation of N$^+$N$^+$D. These observations lend further support to the idea that interactions between base pairs in pyridine and flavin dinucleotides may involve hydrophobic phenomena rather than hydrogen bonding or charge transfer. Penzer and Radda (1967), in their recent review on flavins, compare the effect of solvents on the intramolecular complexing in FAD and conclude that it is likely that here one is dealing with the same phenomenon of hydrophobic interactions as is observed for proteins and polypeptides.

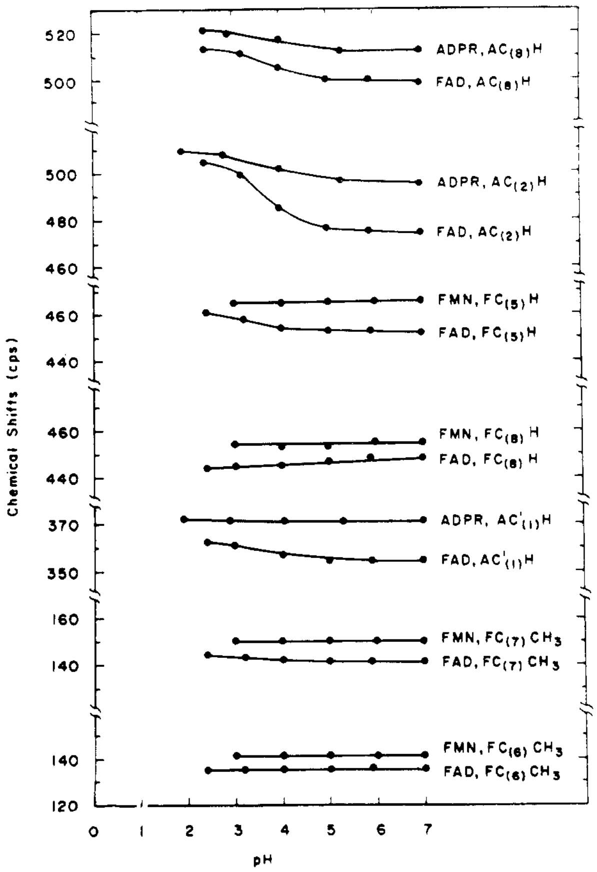

FIGURE 7: pH dependence of chemical shifts upon 0.05 M FAD, ADPR, and FMN at 38°.

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Interaction of Copper Ion with Guanosine and Related Compounds*

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ABSTRACT: The complexing of copper(II) ion with guanosine, guanosine 5′-monophosphate, inosine, inosine 5′-monophosphate, and theophylline at pH 7.0 was studied with the aid of conductometric and potentiometric titrations, spectrophotometric titrations, and infrared absorption spectroscopy. Equimolar ratios of copper to the compounds tested were observed with no hydrogen ion release on complexing. No complex formation was observed for uridine, uridine 3′-(2′)-monophosphate, cytidine, cytidine 3′-(2′)-monophosphate, caffeine, and ribose using similar methods. Considerable diminution of the keto stretching vibration band was observed in the region of 1680–1720 cm^{-1} on complexing with copper ion. With these findings, it is proposed that the copper atom formed a pentacyclic complex involving enolic O at C-6 and N at 7.

Interaction of nucleic acids and polynucleotides with certain metal ions has been observed by a number of investigators. Yatsimirskii and Kris (1966) concluded that Fe^{3+}, Cu^{2+}, and Mn^{2+} attached chiefly to the phosphate group in DNA; however Cu^{2+} and Fe^{3+} also attached to DNA bases. Fishman *et al.* (1967) concluded that the magnesium ion bound only to the phosphate group in DNA. Coates *et al.* (1965) found cupric ion combining with heat-denatured DNA, thus suggesting that a copper complex was formed with some of the bases. However, Daune *et al.* (1966) proposed that silver ion is bound to the nitrogen atoms of purine and pyrimidine bases in polynucleotides.

The silver ion is believed to be attached only to purine and pyrimidine residues in DNA (Jensen and Davidson, 1966). Ropars (1966) postulated that guanine base was involved in copper–DNA complexes. Bryan and Frieden (1967) reported that the binding of cupric ion to DNA was sensitive to copper concentration. Schreiber and Daune (1967) investigated copper interactions with three different DNAs (*Escherichia coli*, *Micrococcus lysodeikticus*, and veal thymus); they found the same association constant regardless of DNA origin. This suggested that some definite group in DNA was involved in the complexing.

Eichhorn and Clark (1965) reported that copper ion helped in the unwinding of the double-helix DNA strands. Ivanov *et al.* (1967) found that the manner of copper binding to native and denatured DNA was different.

From these examples, it is clear that copper and other metal ions interact with nucleic acids; however, due to complexity and large molecular size, the exact location of the metal in the complex could not be identified. Metal complexes of such biologically important compounds cannot be elucidated precisely without fundamental knowledge regarding metal interaction with the components of nucleic acids. In a previous paper, we have reported the interaction of silver ion with guanosine and related compounds (Tu and Reinosa, 1966). In this investigation, the exact location of the silver atom was determined.

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