High-Resolution Proton and Phosphorus Nuclear Magnetic Resonance Spectra of Flavin–Adenine Dinucleotide and Its Conformation in Aqueous Solution†

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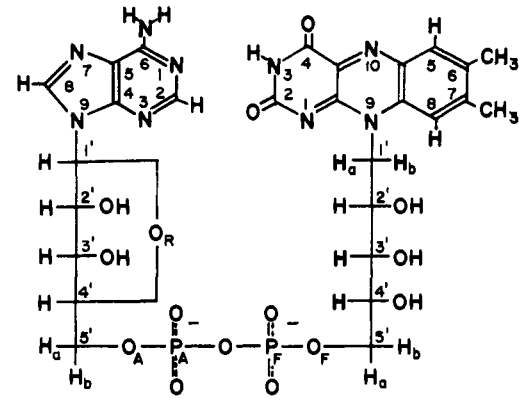
ABSTRACT: The 220-MHz proton magnetic resonance and 40.5-MHz ^{31}P magnetic resonance spectra were obtained for FAD, FMN, AMP, and ADP. Most of the resonance peaks were assigned to individual protons and phosphorus nuclei from the consideration of spin multiplicity and with the use of spin-decoupling experiments. Proton–proton, proton–phosphorus, phosphorus–phosphorus coupling constants were also measured and conformations around each HCCH and HCOP system have been predicted. On the basis of the predicted conformations and the temperature dependence of chemical shifts, structural models of FAD have been proposed. In the most plausable model the molecule is folded so as to expose the hydroxy groups outside and the adenine and isoalloxazine rings are stacked 3.5–4 Å apart with their long axes perpendicular to each other. This model differs in its mode of stacking from those previously proposed. The lack of nuclear Overhauser enhancement of AC_8H on the irradiation at the frequency of the $AC_{1'}H$ signal indicates that the adenine ring is not in the syn position with respect to the ribose ring. However, this model cannot explain the whole of the nuclear magnetic resonance data, especially the temperature dependence of the ribityl protons. Therefore a dynamic equilibrium among several folded and open forms is postulated. An examination of skeletal models shows that other folded and open conformations are possibly realized by the rotation of each half of FAD around the phosphoester bonds keeping the conformations of the other parts unchanged. Although the exact population of each structure cannot be predicted, the contribution of the other forms to the equilibrium must not be very large.

Earlier fluorescence and ultraviolet experiments (Bessy *et al.*, 1949; Weber, 1950) showed the existence of an intramolecular interaction in FAD.[1] Recently Kyogoku and Yu (1968, 1969) found that adenine compounds form selective hydrogen bonds with riboflavin derivatives in chloroform solution. Uehera *et al.* (1968) obtained a 1:1 complex crystal of adenosine and riboflavin. Voet and Rich (1971) also obtained a 1:1 complex of 5′-bromo-5′-deoxyadenosine and riboflavin, in which the adenine ring is bound to the isoalloxazine ring through hydrogen bonds. This evidence suggests that coplanar association might be the mode of the intramolecular interaction of FAD.

Sarma *et al.* (1968), however, investigated this interaction using proton magnetic resonance spectroscopy and concluded that the two rings are vertically stacked. Their result is consistent with the conclusion from the optical rotatory dispersion measurement by Miles and Urry (1968) and from theoretical treatment, by several investigators (for example, Song, 1970). More recently Kotowycz *et al.* (1969) studied the proton magnetic resonance spectra of FAD at 220 MHz and further supported the model proposed by Sarma *et al.* (1968).

An FAD molecule contains pyrophosphate, ribosyl, and ribityl groups in its backbone which connects the adenine and



isoalloxazine rings. The backbone groups restrict the free motion of the two rings and might make it difficult for them to associate by way of coplanar hydrogen bonding. In this paper we will present the results of more precise studies on the proton and phosphorus high-resolution nuclear magnetic resonance spectra of FAD and related compounds and will propose structural models for FAD in aqueous solution which are slightly different from the models previously proposed by other investigators.

Experimental Section

Materials. FAD was obtained from Wakamoto Co., Tokyo. Its purity (95% in weight) was confirmed by paper chromatography. FMN and AMP were purchased from Tokyo Kasei Co. ADP was obtained from Boehringer Co. and TMSP-d_4 and deuterium oxide were purchased from E. Merck Co. All of them were used without further purification.

Procedures. The 220-MHz proton magnetic resonance spectra were recorded on a Varian HR-220 system equipped with a

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[1] Abbreviations used are: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-phosphate; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; FAD, flavin-adenine dinucleotide; FMN, riboflavin 5′-phosphate; NAD, nicotinamide-adenine dinucleotide; nmr, nuclear magnetic resonance; NOE, nuclear Overhauser effect; TMSP-d_4, sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate.

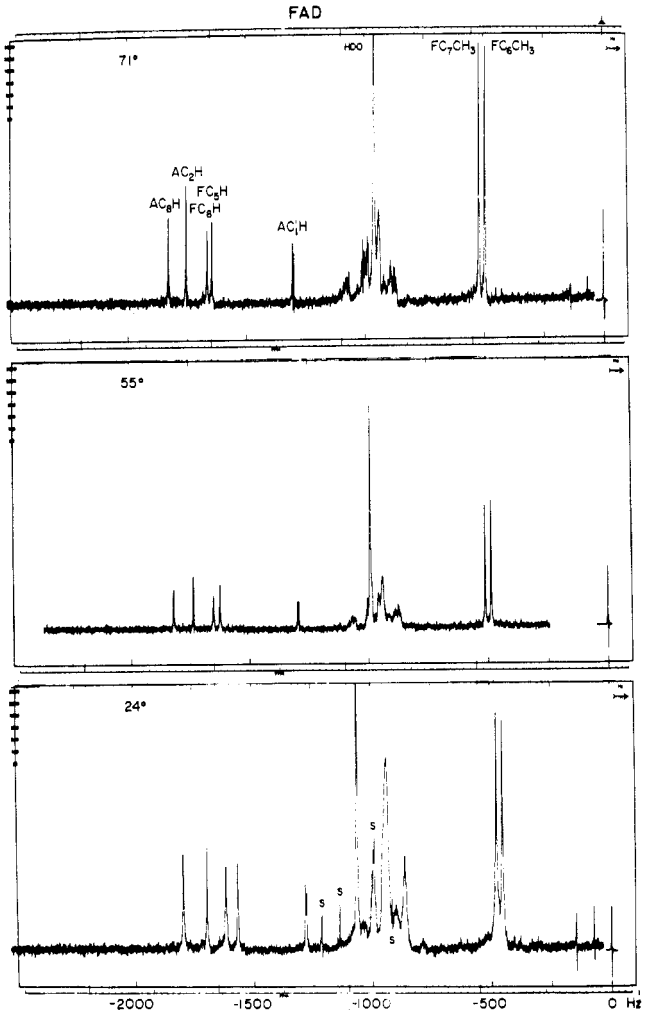

FIGURE 1: 220-MHz nmr spectra of FAD at various temperatures. FAD is 0.2 M in D_2O. Chemical shifts are relative to TMSP-d_4 as an internal standard. "S" denotes spinning side bands.

variable-temperature controller. Chemical shifts were measured using TMSP-d_4 as an internal reference. TMSP-d_4 gives a signal at 0.02 ppm upfield from DSS peak. The precision of the measurements was ±2.0 Hz. A Hewlett-Packard 4204 oscillator was used for a field-sweep decoupling experiment.

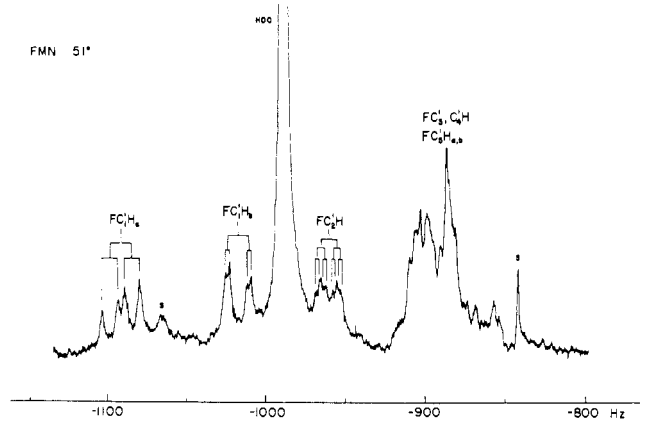

FIGURE 2: Part of the 220MHz spectrum of FMN in D_2O at neutral pH. Concentration is 0.2 M. "S" denotes spinning side bands.

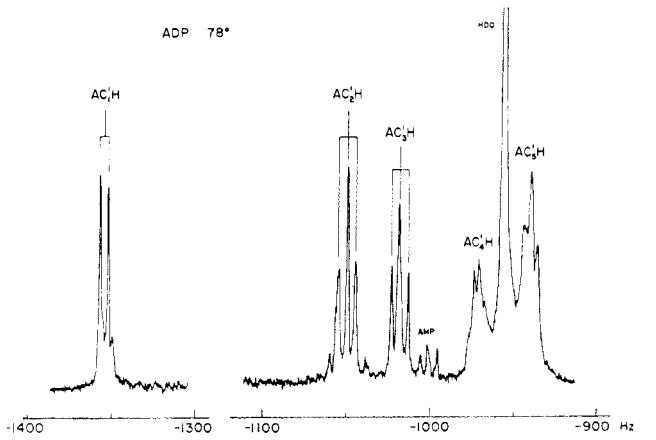

FIGURE 3: Part of the 220-MHz spectrum of ADP in D_2O. Concentration is 0.2 M.

A Varian HA-100 spectrometer was used for the preliminary examination and for the frequency-sweep double-irradiation methods including nuclear Overhauser measurements. A Hewlett-Packard 200-ABR audio-oscillator and a Varian C-1024 computer of average transient (CAT) were used for the experiments. Chemical shifts were measured from the locked DSS signal. The probe temperature was 32 ± 1°.

^{31}P nmr spectra were measured on a Varian HA-100 spectrometer operated at 40.48 MHz. Extensive use of the CAT improved the signal-to-noise ratio dramatically. P_4O_6 in a precise coaxial capillary (Wilmad Glass Co.) was effectively used for the field/frequency-controlled operation which permitted long-term accumulations. P_4O_6 gave a narrow singlet 4556 Hz downfield from the signal of the usual external reference, 85% H_3PO_4. Chemical shifts in this paper are given in positive hertz upfield from P_4O_6. ^{31}P spectra were measured on the D_2O or H_2O solutions containing *ca.* 50 mg of samples/0.5 ml. EDTA (0.01 mM) was added to the solutions to sharpen the resonance peaks. The pH of solutions was adjusted by adding ammonia.

Preparation of the samples for NOE measurements was the following. FAD (50 mg) or disodium 5′-AMP (35 mg) was dissolved in 0.5 ml of D_2O with 7 mg of DSS as the lock source. Each of the solutions was carefully filtered to remove insoluble substances. Lyophilization of the D_2O solutions was repeated several times to minimize the HDO peak of the solvent. Finally the sample tubes were sealed under reduced pressure. For insufficient signal-to-noise ratio a standard NOE method, *i.e.*, an integration method (Bell and Saunders, 1970), was found to be difficult. The NOE enhancements were estimated planimetrically on the time-averaged spectra with and without double irradiations. Two independent experiments showed the deviations of the NOE value were within 2%.

Results and Discussion

220-MHz Proton Magnetic Resonance Spectra

Assignments of the Backbone Protons of FMN, ADP, and FAD. The 220-MHz spectra of FAD at different temperatures are given in Figure 1. Selected regions of the 220-MHz spectra of FAD, FMN, and ADP are shown with assignments in Figures 2, 3, and 4, respectively. Chemical shifts and spin-spin coupling constants are listed in Tables I, II, and III together with the assignments which will be discussed below.

The $C_{1'}$ protons of FMN ($FC_{1'}H$) adjacent to the isoalloxazine ring are expected to give the AB part signals of the ABX

TABLE I: Chemical Shifts (at 220 MHz) of FMN and ADP at Various Temperatures.[a]

Assignment	24°	51°	71°	$\delta_{FMN}^{71°} - \delta_{FMN}^{24°}$[d]
FC_8H	1681	1693	1697	16 (27)[e]
FC_5H	1610	1646	1659	49 (44)[e]
$FC_{1'}H_a$	1086	1092	1096	10
$FC_{1'}H_b$	998	1018	1025	27
$FC_{2'}H$	958	960	(960)[b]	2
The other ribitol protons[c]	903 886	901 886	902 886	1 0
FC_7CH_3	532	542	545	13 (9)[e]
FC_6H_3	495	510	513	18 (15)[e]
	21°	50°	78°	$\delta_{ADP}^{78°} - \delta_{ADP}^{21°}$
AC_8H	1865	1872	1874	9
AC_2H	1772	1805	1817	45
$AC_{1'}H$	1336	1352	1351	15
$AC_{2'}H$	1043	1049	1049	6
$AC_{3'}H$	1018	1017	1016	−2
$AC_{4'}H$	970	971	971	1
$AC_{5'}H$	940	939	938	−2

[a] Chemical shifts δ are given as positive value in Hz downfield from TMSP-d_4. [b] Vitiated by the HDO peak. [c] Detail assignment is impossible. Chemical shifts are estimated from the highest peak position of the multiplets. [d] Chemical shift differences between 71 and 24° at 0.2 M. [e] The difference in the chemical shifts between 0.025 M and infinite dilution of D_2O–dioxane-d_8 solutions (70:30, v/v) at 17° (Kotowycz *et al.*, 1969).

system in the region of the ribitol and ribose proton resonances. The quartet signal at 1092 Hz (Figure 2) was unequivocally assigned to one of the $FC_{1'}$ protons, because it showed a large geminal coupling constant. A pair of doublets at 1018 Hz is then attributable to the other $FC_{1'}$ proton, because it has the same spacings of 14 Hz as the former $FC_{1'}H$. The two geminal $FC_{1'}$ protons reside in different chemical environments and give resonances with different shifts. The pair of quartets at 960 Hz comes from $FC_{2'}H$ which is coupled to the two $FC_{1'}$ and one $FC_{3'}$ proton. The two spacings in this multiplet coincided with those obtained from the signals of two $FC_{1'}$ protons. An alternative assignment of this peak to $FC_{4'}H$ based on the consideration of spin multiplity was excluded, because it is unlikely that $FC_{4'}H$ yields a resonance at lower field than $FC_{2'}H$ which is closer to the electron withdrawing isoalloxazine. The remaining complicated large peaks between 920 and 850 Hz represent four protons and must be overlapped with the resonances of the $FC_{3'}$, $FC_{4'}$, and two $FC_{5'}$ protons.

The proton resonance peaks of ADP are assigned to individual protons, as is shown in Figure 3, on the basis of spin multiplicity and spin-decoupling experiments performed on a 100-MHz spectrometer. The $C_{5'}$ proton signals are split into multiplets by neighboring protons and a phosphorus nucleus. In alkaline solutions the $C_{5'}$ protons of AMP and ADP give multiplet signals at 885 and 940 Hz, respectively. The remarkable downfield shift from AMP to ADP, of 55 Hz should originate from the difference between the monophosphate group

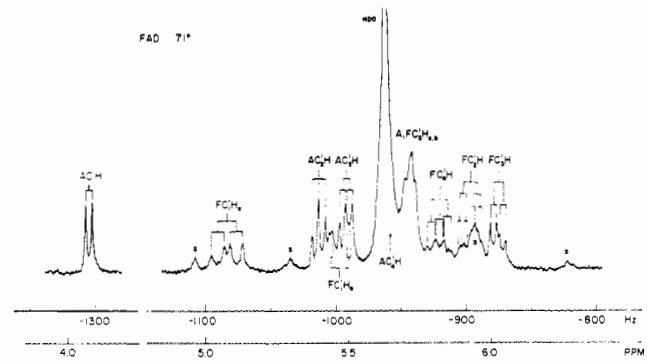

FIGURE 4: Part of the 220-MHz spectrum of FAD (see the legend of Figure 1).

in AMP and the pyrophosphate group in ADP. Similar difference in chemical shifts are anticipated for the $C_{5'}$ protons in FMN and FAD. Therefore the $C_{5'}$ protons of the FMN and AMP moieties in FAD should be less shielded than those of free FMN and AMP, respectively.

The isolated quartet at 1083 Hz in the spectrum of FAD (Figure 4) was assigned to $FC_{1'}H$ of the FMN counterpart. A pair of doublets from the other $FC_{1'}$ proton appears at 997 Hz but is overlapped with the multiplet signals of the ribose

TABLE II: Chemical Shifts (at 220 MHz) of FAD at Various Temperatures.[a]

Assignment	24°	55°	71°	$\delta_{FAD}^{71°} - \delta_{FAD}^{24°}$[d]	$\delta_{FMN}^{71°} - \delta_{FAD}^{71°}$[e]
FC_8H	1615	1653	1665	50	32 (59)[f]
FC_5H	1565	1624	1645	80	14 (25)[f]
$FC_{1'}H_a$	1044	1070	1083	39	13
$FC_{1'}H_b$	955[c]	980	997	42	28
$FC_{5'}H$	939	941	943	4	−14∼ to −57
$FC_{4'}H$	900	915	921	21	−19∼ to −35
$FC_{2'}H$		885	893	∼30	67
$FC_{3'}H$	863	870	872	∼9	14 to ∼30
FC_7CH_3	477	511	520	43	25 (20)[f]
FC_6CH_3	452	485	494	42	29 (13)[f]
					$\delta_{ADP}^{78°} - \delta_{FAD}^{71°}$
AC_8H	1795	1819	1828	33	46
AC_2H	1695	1736	1752	57	65
$AC_{1'}H$	1278	1291	1304	26	57
$AC_{2'}H$	1002	1006	1013	11	36
$AC_{3'}H$	993	(990)[b]	993	0	23
$AC_{4'}H$	955	957	(960)[b]	5	11
$AC_{5'}H$	939	941	943	4	−5

[a] Chemical shifts are given as positive value in Hz downfield from TMSP-*d*. [b] Vitiated by the HDO peak. [c] Precise position of the resonance was not clear by broading. Chemical shifts were estimated from the highest peak position of the multiplets. [d] Shift at 71° − shift at 24°. The positive numbers represent a shift to high field. [e] The difference in the chemical shifts at 71°. [f] The difference in the proton shifts at infinite dilution of D_2O–dixoane-d_8 solutions (70:30, v/v) at 17° (Kotowycz *et al.*, 1969).

TABLE III: Proton Spin–Spin Coupling Constants of FMN, ADP, and FAD at Various Temperatures.[a]

Assignment	24°	51°	71°
FMN			
$J_{F1'_a1'_b}$	12[b]	14	15
$J_{F1'_a2'}$	9[b]	10	10
$J_{F1'_b2'}$		2.3	2.3
$J_{F2'3'}$		4.5	
	21°	50°	78°
ADP			
$J_{A1'2'}$	5.0	5.0	5.0
$J_{A2'3'}$	5.0	5.0	5.0
$J_{A3'4'}$	5.0	5.0	5.0
	24°	55°	71°
FAD			
$J_{F1'_a1'_b}$		14.5	14
$J_{F1'_a2'}$		10	10
$J_{F1'_b2'}$		2[b]	2.0
$J_{F2'3'}$		4.5	4.6
$J_{F3'4'}$		8.0	7.3
$0.5\lvert J_{F4'5'_a} + J_{F4'5'_b}\rvert$		6.0[c]	6.0[c]
$J_{A1'2'}$	4.3[b]	5.0	5.0
$J_{A2'3'}$		5.0	5.0
$J_{A3'4'}$			4.6
$0.5\lvert J_{A4'5'_a} + J_{A4'5'_b}\rvert$[d]			

[a] All parameters were obtained by first-order analysis. [b] Indefinite values determined from broad peaks. [c] Obtained from the $C_{4'}H$ peak. It corresponds to the averaged value of gauche and trans couplings. [d] Not obtainable.

TABLE IV: Nuclear Overhauser Effect of the Flavin Protons of FAD (in Per Cent Increase of Signal Intensity).[a]

	Increase of Signal Intensity at[b]	
Irradiated at[b]	743 Hz	728 Hz
216 Hz (FC_6CH_3)	~1	9
227 Hz (FC_7CH_3)	4	6
Assignment	FC_8H	FC_5H

[a] NOE experiments were run by the frequency sweep at 100 MHz. [b] Irradiated and observed frequencies are in Hz downfield from internal DSS.

protons. From comparison of the spectrum to that of ADP (Figure 3) the resonances of $AC_{2'}H$ and of $AC_{3'}H$ are expected to appear near here. Upon irradiation of the proton at 1013 Hz, the doublet due to $AC_{1'}H$ collapses. A more complete frequency swept decoupling experiment using a HA-100 spectrometer was also performed. Therefore the peaks at 1013 Hz were assigned to $AC_{2'}H$ and the peaks at 993 Hz to $AC_{3'}H$. From the comparison of the spectrum of FAD to those of ADP and FMN the peaks at fields higher than 930 Hz are thought to originate from the $C_{2'}$, $C_{3'}$, $C_{4'}$, and $C_{5'}$ protons of ribitol or from the $C_{4'}$ and $C_{5'}$ protons of ribose. However, the $C_{5'}$ protons of FAD are adjacent to the pyrophosphate group and are expected to yield resonances at almost the same position where ADP does. Thus the strong peak at 943 Hz must be due to the $C_{5'}$ protons. Two multiplets at 893 and 872 Hz show that these nuclei are coupled strongly with each other. Thus the resonances ought to come from two protons vicinal to each other. Since the peak at 997 Hz has been assigned to $AC_{3'}H$, these multiplets must originate from $FC_{2'}H$ and $FC_{3'}H$ or from $FC_{3'}H$ and $FC_{4'}H$. It is doubtless that the upfield quartet at 872 Hz originates from $FC_{3'}H$ adjacent to two different protons. Since the shape, multiplicity and total width of the multiplet at 893 Hz is very similar to that of the $FC_{2'}H$ peak of FMN, the peak can be ascribed to $FC_{2'}H$ of FAD. The $AC_{4'}H$ peak always appears downfield from the $AC_{5'}H$ peak. Therefore, the remaining peak at 921 Hz is attributable to $FC_{4'}H$ and not to $AC_{4'}H$. Its spin multiplicity is explainable by the assignment.

Protons of the Isoalloxazine Ring. For the proposal of models for the folded structure of FAD and for the mode of intermolecular association of FAD and FMN, unambiguous assignments are essential for the ring protons in isoalloxazine. Bullock and Jardetzky (1964) first gave assignments to the methyl protons of FMN in aqueous solution by the use of selective deuteration. Unambiguous assignments have been made for partly deuterated limuflavin (McCormick, 1967) and for riboflavin (Beach and Plaut, 1970) in trifluoroacetic acid. Sarma *et al.* (1968) further assigned the isoalloxazine protons of FAD in D_2O on the basis of the computed π electron density (Pullman and Pullman, 1959). Kotowycz *et al.* (1969) claimed serious ambiguity for the assignment from the π electron density, but their assignments based on the temperature dependence of the proton chemical shifts are identical with those by Sarma *et al.*

However, we found difficulty in interpreting the experimental results based on the previous assignment for FC_5H and FC_8H. According to our viewpoint, the proton at FC_8 of FAD which is located on the same side of the sugar group is expected to be less influenced by intermolecular association and to give larger upfield shift from that of FMN than the FC_5 proton. The latter should be less shielded by the adenine ring in the folded structures of the FAD molecule. When the behaviors of the third and fourth peaks counted from the lowfield in the spectrum of the fairly concentrated solution of FAD are compared, the fourth peak is more sensitive to concentration and gives smaller upfield shifts than the corresponding peak of FMN. The fourth peak at this concentration was assigned to FC_8H and the third to FC_5H by Sarma *et al.* and Kotowycz *et al.*, but this is the reverse of the above expectation. Moreover at an infinite dilution or at high temperature, the two peaks of FMN collapse (Sarma *et al.* and Kotowycz *et al.*). Therefore it is not unreasonable that the order of the peaks due to FC_5H and FC_8H is inverted in the cases of FMN and FAD in the rather concentrated aqueous solution compared with those of lumiflavin and riboflavin in trifluoroacetic acid. Here the intermolecular association of the bases is extremely diminished. According to the present assignment FC_8H of FAD gives a peak at higher field than FC_5H at a very dilute solution (Kotowycz *et al.*, 1968). This fact is explained by the preferential diamagnetic shielding at FC_8H by the adenine ring. For the confirmation of our assignment the nuclear Overhauser effect (Anet and Bourn, 1965; Bell and Saunders, 1970) of the flavin protons was examined and results are given in Table IV. Upon irradiation of the

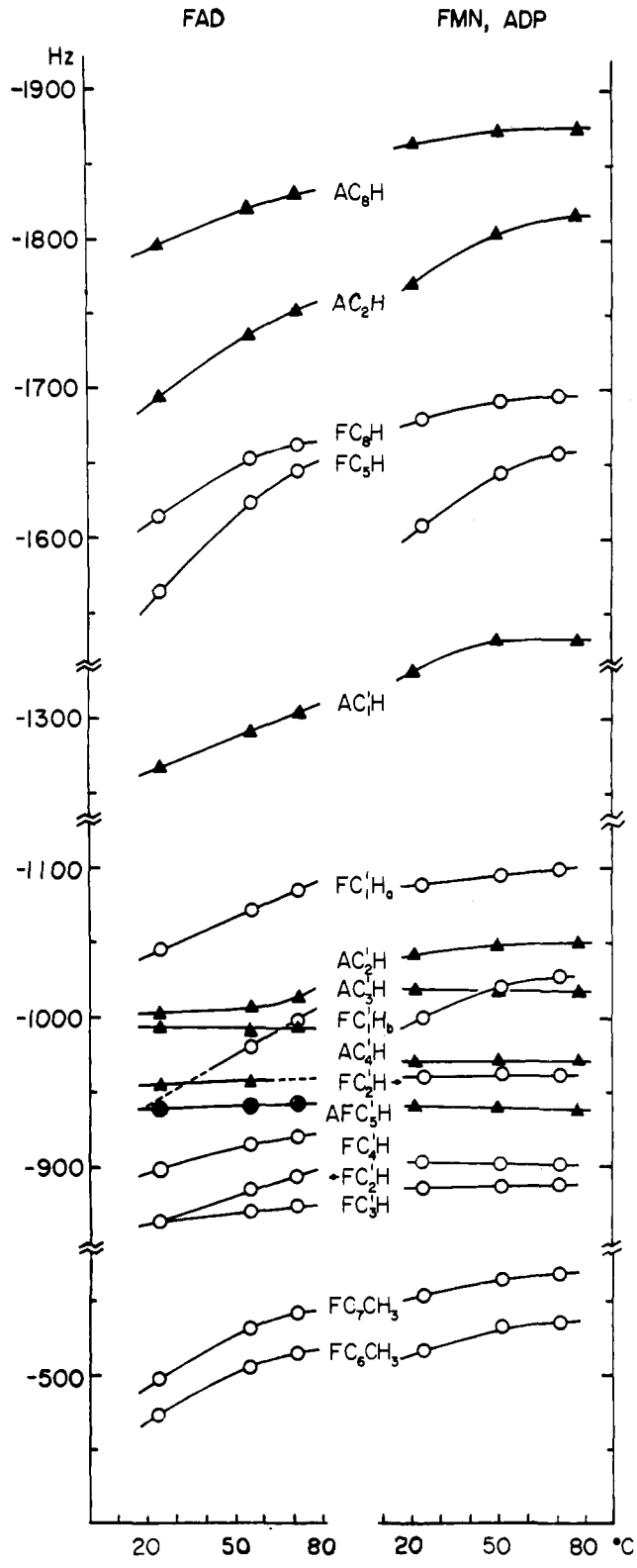

FIGURE 5: Temperature dependence of proton chemical shifts. (○) Marks show the shifts of FMN and the FMN part of FAD. (▲) Marks show the proton chemical shifts of ADP and AMP moiety of FAD.

methyl protons at the FC_6 position, 9% increase of signal intensity was observed for the fourth peak of FAD at 728 Hz, but almost no effect for the third peak at 743 Hz in its 100-MHz spectrum. On the other hand, the irradiation of the FC_7CH_3 proton caused an increase in intensity of the third peak. It also enhanced the intensity of the fourth peak because of the small chemical shift between those two protons but the effect is small as compared with the value at the FC_6 irradiation. Thus, the fourth peak is attributable to a proton located closer to FC_6CH_3 than to FC_7CH_3, *i.e.*, due to FC_5H. The assignments of FC_6CH_3 and FC_7CH_3 of FMN and FAD have been established from the deuteration experiments by Bullock and Jardetzky (1965) and Kotowycz *et al.*

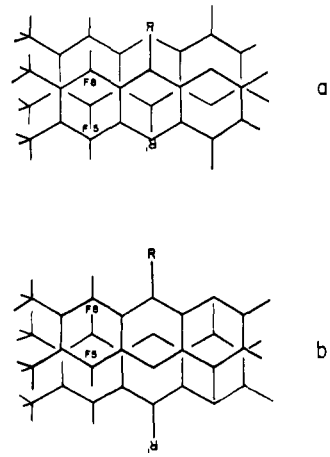

FIGURE 6: Models for intermolecular association of FMN: (a) proposed by Sarma *et al.* (1968) and (b) proposed in the present work.

Temperature Dependence of Chemical Shifts and Spin–Spin Coupling Constants. The effects of temperature on the proton chemical shifts of FMN, ADP, and FAD are summarized in Figure 5 and Tables I and II. Some of the previous data by Sarma *et al.* (1968) and Kotowycz *et al.* (1969) are also included in these tables. The experiment on the concentration dependence of chemical shifts by Sarma *et al.* and Kotowycz *et al.* clearly indicated that the FMN molecules associate by way of vertical stacking of the isoalloxazine rings. The present result on the temperature dependence of chemical shifts supports their conclusion except the structure of intermolecular complexing. The downfield shift caused by raising temperature is remarkable due to the isoalloxazine protons. This is particularly evident for FC_5H ("FC_8H" by Sarma *et al.* and Kotowycz *et al.*). Sarma *et al.* proposed a model for the mode of intermolecular association of FMN based on their assignments for FC_5H and FC_8H. Therefore their model should be changed to a new one using our assignments, which show that FC_5H in one of the isoalloxazine rings spends considerable time in the proximity of the aromatic ring of another isoalloxazine ring (Figure 6). This model is more reasonable than the previous one, because it reduces the steric interference between the isoalloxazine ring and the ribityl group at N_9 of another FMN molecule.

The temperature dependence of chemical shifts for the ribitol protons is not so remarkable except for the $FC_{1'}$ protons which are adjacent to the isoalloxazine ring. Two protons at the $FC_{1'}$ position show a different temperature dependence. The proton with upfield chemical shift, $FC_{1'}H_b$, shows a greater downfield change with elevation of temperature. This suggests $FC_{1'}H_b$ is more affected by the intermolecular vertical association of the isoalloxazine ring. The other ribitol protons, which are located closer to such polar groups as the hydroxy and phosphate groups, are rather free from the influences of the hydrophobic association of the isoalloxazine ring. Their chemical shifts remain almost constant with the elevation of temperature.

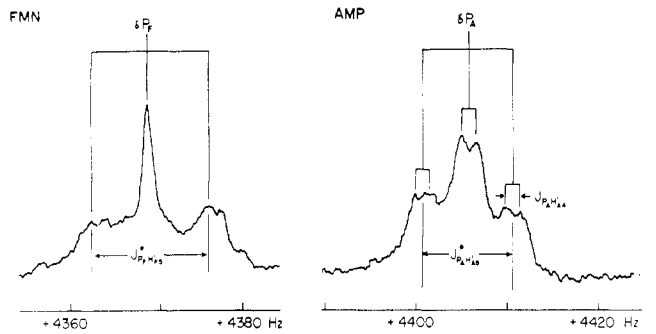

FIGURE 7: 40.5-MHz ^{31}P nuclear magnetic resonance spectra of FMN and AMP. Chemical shifts were measured with P_4O_6 in a coaxial tube. Both spectra were time averaged for several tens scans. AMP, pH 10.1; FMN, pH 10.0.

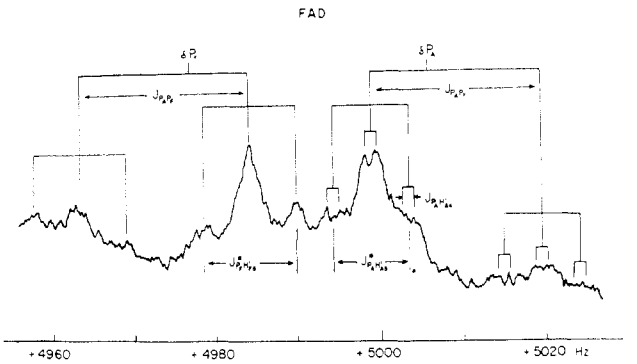

FIGURE 8: 40.5-MHz ^{31}P nuclear magnetic resonance spectrum of FAD (50 mg/0.5 ml). Chemical shift is shown in Hz upfield from the "locked" P_4O_6 signal. Solution of FAD (pH 9.3) contains 0.002 M EDTA and the spectrum was accumulated for 305 scans.

All of the isoalloxazine and ribitol protons of FAD are shifted upfield from those of FMN. Sarma *et al.* (1968) and Kotowycz *et al.* (1969) interpreted this phenomenon as due to the intramolecular complexing between the adenine and isoalloxazine rings, both of which have a large diamagnetic shielding effect. They also concluded from the concentration dependence experiment that the FAD molecules associate bimolecularly through the vertical stacking of the isoalloxazine ring. Therefore an elevation of temperature of the FAD solution causes the dissociation of intra- and intermolecular complexing and leads to appreciable downfield shifts of proton resonances.

Actually $FC_{1'}H$ and $AC_{1'}H$ of FAD as well as the protons of the adenine and isoalloxazine rings show large downfield shifts, whereas the other ribose and ribitol protons are less sensitive to the temperature. Particularly $AC_{3'}H$, $AC_{4'}H$, $AC_{5'}H$, and $FC_{5'}H$ remain almost unchanged with elevation of temperature. The other ribitol protons of FAD, *i.e.*, $FC_{2'}H$, $FC_{3'}H$, and $FC_{4'}H$, undergo slight downfield shifts, while the corresponding protons of FMN are less dependent on temperature. This difference may be due to the additional shielding effect by the stacked adenine moiety in the FAD molecule. As the temperature rises, thermal motion causes the dissociation of such intramolecular complexes to a greater extent. This results in the marked downfield shifts of the base protons. The two bases can not behave independently, since they are connected with each other by the backbone groups. A comparison of the chemical shifts of the flavin protons of FAD to those of FMN at an elevated temperature and at an infinite dilution may show the extent of the intramolecular complexing in FAD. At high temperatures intra- and intermolecular interactions decrease slightly and at an infinite dilution intermolecular interaction becomes negligible and the effect of intramolecular complexing remains (Tables I and II).

As is shown in Table II, the induced shift from FMN to FAD at 71° caused by the intramolecular complexing in FAD is fairly large for FC_8H, $FC_{1'}H_b$, FC_7CH_3, FC_6CH_3, $FC_{3'}H$, and particularly striking for $FC_{2'}H$. Thus these protons are considered to spend a longer time in the proximity of the adenine ring than the other protons. Those facts must be taken into account in the model building of FAD in solutions. Especially the difference found in the magnitude of induced chemical shifts of $FC_{1'}H_a$ and $FC_{1'}H_b$ reflects the position of these protons relative to the adenine ring.

Temperature dependence of spin–spin constants (Table III) is not observed as clearly as that of chemical shifts, because multiplet signals are not well resolved in the spectra at 24°. Therefore, except for isolated peak, it is hard to determine coupling constants for them. But it is clear that the observed coupling constants remain almost unchanged with the elevation of temperature and this implies that no significant change in the conformations of the ribose and ribitol moieties occurs over the temperature range 20–75°. The slight change of the coupling constants with temperature may be due to the uncertainty of the peak position caused by line broadening.

^{31}P Nuclear Magnetic Resonance Spectra at 40.5 MHz

^{31}P nmr spectra of FMN, AMP, and FAD are illustrated in Figures 7 and 8. Chemical shifts and coupling constants are listed in Table V together with those of ADP. As two phosphorus nuclei of FAD are magnetically nonequivalent, an AB-type signal with extra splittings due to the $C_{5'}$ and $C_{4'}$ protons was obtained. The phosphorus–phosphorus spin coupling in FAD was 20.9 Hz and was almost identical with 22.0 Hz in ADP. The upfield part of the FAD signal was assigned to the phosphorus nucleus of the AMP and the downfield part to that of the FMN moiety, because the shape of the AMP spectrum is quite similar to that of the upfield part of the FAD spectrum, while the FMN signal is similar to the downfield part. According to our assignment, the induced chemical shifts from AMP and FMN to those parts in FAD became equal to each other; *i.e.*, +600 and +609 Hz, respectively (see Table V). It also supports our assignment.

A pair of triplet due to the AMP part split by two $AC_{5'}H$ was further coupled with the $C_{4'}$ proton, while the FMN part was not. It seems to be the first case observed with such a long-range proton–phosphorus coupling across four bonds in nucleotide phosphorus spectra. Theoretical calculation by Barfield (1964) showed that the long-range proton–proton coupling through four bonds is maximal with a coplan ar zigzag orientation. A vast amount of the experimental data on the proton–proton coupling have supported the validity of this work (Jackman and Sternhell, 1969; Barfield and Chakrabarti, 1969). Such stereospecificity of long-range coupling has also been obtained in the case of the phosphorus–proton coupling through four bonds in six-membered cyclic phosphates (Kainosho *et al.*, 1969). The 220-MHz nmr spectrum of AMP in a basic solution (Figure 9) showed a symmetrical multiplet for the $C_{5'}H$ signal. This symmetrical feature clearly indicates that the two protons at $C_{5'}$ are equally coupled with the phosphorus atom and $C_{4'}H$. A rather large $J_{PH_{4'}}$ can be recognized to occur through the "W"-letter path (Barfield and Chakrabarti, 1969). Therefore the P_A–O_A and $C_{4'}$–H bonds of AMP

TABLE V: 40.5-MHz ^{31}P Chemical Shifts and Spin Coupling Constants.[a]

	pH	δP_F	δP_A	$J_{P_AP_F}$
FAD[b]	9.3	+4977 (J = 11.4)[c]	+5006 (J = 9.2)[c] ($J_{PH'_4}$ = 1.4)	20.9
FMN	10.0	+4368 (J = 12.4)[c]		
5'-AMP	10.1		+4406 (J = 9.2)[c] ($J_{PH'_4}$ = 1.6)	
		δP_β	δP_α	$J_{P_\alpha P_\beta}$
5'-ADP	10.7	+4792	+4976 (J = 11.4)[c] ($J_{PH'_4}$ = 2.1)	22.0

[a] Chemical shifts are shown in Hz upfield from P_4O_6 in capillary. [b] Approximately analyzed by AB-type treatment. [c] $J = |J_{PH'_{5a}} + J_{PH''_{5b}}|$.

and the AMP moiety of FAD are nearly in a trans,trans relation. On the other hand, the P_F–O_F and $C_{4'}$–H bonds of FMN and those of the FMN part of FAD are in conformations considerably deviated from the trans,trans relationship.

Two sets of the diastereotopic protons that are coupled with the phosphorus atoms exist at the $C_{5'}$ atom of the ribose and the ribitol fragments. Only the sums of the $J_{PH_5'a}$ and $J_{PH_5'b}$ are obtainable from the phosphorus spectra, because the chemical shifts between two $C_{5'}$ protons are too small. As mentioned already two nonequivalent protons at the $C_{5'}$ position of AMP showed a symmetrical pattern, indicating that both $C_{5'}$–H_a and $C_{5'}$–H_b were gauche to the $C_{4'}$–H bond and to the P_A–O_A bond. Thus in the case of FAD it can be properly assumed that $J_{P_AH_5'a}$ is nearly equal to $J_{P_AH_5'b}$ and with a value of 4.6 Hz which is very close to those obtained for various nucleoside 5'-monophosphoric acids (Tsuboi *et al.*, 1968; M. Kainosho, unpublished result). A marked dihedral angular dependence of the phosphorus–proton coupling constants in the POCH groups has been well established and the qualitative Karplus-like curve between J_{POCH} and the dihedral angle, ϕ, has been obtained for the aliphatic phosphate (Tsuboi *et al.*, 1967; Kainosho *et al.*, 1969; Kainosho and Nakamura 1969). From this curve, 4.6 Hz of J corresponds to the gauche angle. On the other hand, deviations from the gauche angles about P_F–O–$C_{5'}$–H_a and –H_b are evident, because the sum of $J_{P_FH_5'a}$ and $J_{P_FH_5'b}$ (11.7 Hz) is larger than the twice of the gauche coupling constant (*ca.* 9 Hz). However the angle of deviation may still be within ±20° judging from the J–ϕ curve (Table VI).

Conformation of FAD

Conformation of the FMN and AMP Moieties of FAD. As shown in the preceding section, the two $C_{1'}$ protons of the ribitol in FMN and FAD show quite different chemical shifts even at an elevated temperature. Although they are not magnetically equivalent in essential, such the large chemical shift between these two protons is considered to imply that one of the rotational isomers around the glycosidic bond is populated exclusively over the temperature range of 20–75°. $FC_{1'}H_{a'}$ which appears at lowfield, should be located at a position proximate to the lone pair electron of the N_1 nitrogen atom in the terminal uracil ring. This prediction is consistent with the crystal structures of riboflavin hydrobromide and riboflavin (Tanaka *et al.*, 1969; Voet and Rich, 1971). In the crystalline states, one of the $C_{1'}$ protons which is trans to $C_{2'}$H is located at the uracil side and the other proton which is gauche to $C_{2'}$H resides at the aryl ring side of the isoalloxazine. Then the gauche and trans protons correspond to $FC_{1'}H_b$ and $FC_{1'}H_a$, respectively. The $C_{1'}$ atom of the ribityl group of FAD or FMN is the prochiral center (Hanson, 1966). A prochiral carbon denotes such an atom as C_{aabd}. Two heterotopic substituents a are called the pro-*R* and the pro-*S* group, depending on whether the substitution of a with the different group c gives rise to a new chiral center of *R* or *S* configuration. In FAD and FMN, H_a is the pro-*S* and H_b is the pro-*R* proton. The vicinal spin–spin coupling constants support this assignment. Thus $J_{F1'b2'}$ which should be due to gauche coupling, is 2 Hz and $J_{F1'a2'}$ which should be due to trans coupling, is 10 Hz.

Spin–spin coupling constants provided us qualitative but still very useful information on the conformation of backbone groups of FAD, *i.e.*, ribityl, ribosyl, and pyrophosphate linkage. The dihedral angles between two C–H bonds, ϕ_j^i, can be roughly predicted from the vicinal proton–proton coupling constants J_{HH} based on the relation derived by Karplus (1959, 1963), $J = J_0 \cos^2 \phi - 0.28$ Hz. For the present study we have tentatively chosen the values suggested by Abraham *et al.* (1962) J_0 = 9.3 Hz for $0° \leq \phi \leq 90°$ and J_0 = 10.3 Hz for $90° \leq \phi \leq 180°$. Because of the $\cos^2 \phi$ term in the Karplus relation, two pairs of the $\pm\phi_j^i$ are predicted for each value of

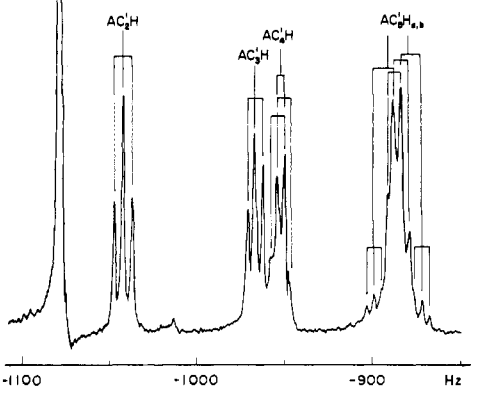

FIGURE 9: Part of the 220-MHz spectrum of AMP at 21°, pD ~13.

TABLE VI: Predicted Conformations about Each Backbone Bond.

A part	$\phi^{C_{1'}-H}_{C_{2'}-H}$	$\phi^{C_{2'}-H}_{C_{3'}-H}$	$\phi^{C_{3'}-H}_{C_{4'}-H}$	$\phi^{C_{4'}-H}_{C_{5'}-H_a}$ $\phi^{C_{4'}-H}_{C_{5'}-H_b}$	$\phi^{C_{5'}-H_a}_{O_A-P_A}$ $\phi^{C_{5'}-H_b}_{O_A-P_A}$
Observed				Gauche–gauche[c]	Gauche–gauche
				$\phi^{C_{4'}-C_{3'}}_{C_{5'}-O_A}$	$\phi^{C_{5'}-C_{4'}}_{O_A-P_A}$
Predicted[a]	$C_{2'}$endo $\rightleftarrows$ $C_{3'}$endo[b]			(+)Gauche[c]	Trans
F part	$\phi^{C_{1'}-H_a}_{C_{2'}-H}$ $\phi^{C_{1'}-H_b}_{C_{2'}-H}$	$\phi^{C_{2'}-H}_{C_{3'}-H}$	$\phi^{C_{3'}-H}_{C_{4'}-H}$	$\phi^{C_{4'}-H}_{C_{5'}-H_a}$ $\phi^{C_{4'}-H}_{C_{5'}-H_b}$	$\phi^{C_{5'}-H_a}_{O_F-P_F}$ $\phi^{C_{5'}-H_b}_{O_F-P_F}$
Observed	Trans (−)Gauche	(−)Gauche	Trans	(−)Gauche–trans (or trans–(+)gauche)[d]	Gauche–gauche[e]
	$\phi^{C_{1'}-N_9}_{C_{2'}-C_{3'}}$	$\phi^{C_{2'}-C_{1'}}_{C_{3'}-C_{4'}}$	$\phi^{C_{3'}-C_{2'}}_{C_{5'}-C_{5'}}$	$\phi^{C_{4'}-C_{3'}}_{C_{5'}-O_F}$	$\phi^{C_{5'}-C_{4'}}_{O_F-P_F}$
Predicted[a]	Trans	(−)Gauche	Trans	(−)Gauche (or trans)[d]	Trans[e]
X-ray[f]	+175.5	−62.0	−166.8	−50.3	

[a] Tetrahedral bonding angles of carbon atoms are assumed. [b] The ribose conformations lack the rigid nature, and this equilibrium might account the observed coupling constant. [c] Conformations around the $C_{5'}-C_{4'}$ bond has been assumed to be approximately equal to those of AMP and ADP. [d] The conformations in parentheses seem to be less favored for the folded models of FAD, but cannot be eliminated by the spectral data. [e] Slightly deviated from the staggered conformations (see the text). [f] Calculated from the atomic parameters of riboflavin hydrobromide crystal (N. Tanaka, personal communication).

J_{ij}, one less than $|90°|$ and one greater than $|90°|$. Assuming the approximate staggered conformations about the sugar moieties, we can select the better pair of $\pm\phi_j{}^i$. For example, though $J_{1'a1'b}$ (10 Hz) is shown to correspond to both *ca.* ± 0 and $\pm 180°$, however $\pm 0°$ can be ruled out from the above assumption. We denote trans ($\sim\pm 180°$) and $\pm$gauche ($\sim\pm 60°$) instead of the numerical values of the predicted angles. Here the sign is defined as positive for right-handed rotation along the central bond, and *vice versa*.

The conformation around the $C_{1'}-C_{2'}$ bond has been discussed already and is found to be virtually fixed. $J_{3'4'} = 8.0$ Hz is slightly small but nearly trans coupling, indicating that the conformation around the $C_{3'}-C_{4'}$ bond should be somewhat deviated from the exact staggered form. $J_{2'3'}$ predicts the stable form around the $C_{2'}-C_{3'}$ bond is either $\pm$gauche with respect to the $C_{2'}-H$ and $C_{3'}-H$ bonds. From the examination of the molecular model, however, the $C_{4'}OH$ group comes closer to the $C_{1'}H_aH_b$ group in the +gauche conformation. In the most predominant folded structure of FAD, which will be discussed later, the $C_{4'}OH$ group comes too close to the adenine ring. The latter stacks over the isoalloxazine. For these reasons +gauche conformation was eliminated.

Proton spin–spin coupling constants of the ribityl groups of FMN and FAD are almost identical with each other (Table III). Therefore the conformation of ribityl group of FMN must be similar to that of the ribityl group of FAD. Recently Tanaka *et al.* (1969) analyzed the crystal structure of riboflavin hydrobromide monohydrate and Voet and Rich (1971) determined the structure of riboflavin in the crystalline complex with an adenine derivative. Both structures are almost identical. In the former structure dihedral angles about each bond can be calculated from the atomic parameters determined by Tanaka *et al.* (Table VI). The conformations predicted by the present nmr work agree well with those calculated from the crystal structure of riboflavin hydrobromide. Thus the ribityl groups of FMN and FAD in aqueous solution seem to exist in a conformation similar to that of the crystalline riboflavin hydrobromide and riboflavin.

Smith and Jardetzky (1968) have calculated the vicinal coupling constants for 20 possible conformations of D-ribofuranoside. According to their classification, the present values obtained for $J_{A_1'2'}$ and $J_{A_2'3'}$ of FAD are closest to those of the $C_{3'}$-exo or $C_{3'}$-exo,$C_{4'}$-endo conformations. However, neither of these conformations accounts the observed $J_{A_3'4'}$ ($\sim$5.0 Hz). The predicted $J_{A_3'4'}$ of the $C_{3'}$-exo and $C_{3'}$-exo,-$C_{4'}$-endo conformations are 0.2 and 1.7 Hz, respectively. This suggests that either the Karplus function may not be very applicable to the HCCH system of ribose, or the observed spectra may be the averaged one of the rapidly interchanging conformers. Since the Karplus function has given good agreements between calculated and measured coupling constants in nucleoside 3′,5′-cyclic monophosphoric acids (Smith and Jardetzky, 1968), the latter case is more probable. In the crystals of 5′-AMP (Kraut and Jensen, 1963), 3′-AMP (Sundaralingam, 1966), and adenosine (Hashemeyer and Sobell, 1965), ribose rings take the C_3'-endo conformation. Although the structure in crystal cannot be directly correlated with that in solution, it appears that the energy differences between the C_3'-endo and the other conformations are small. Therefore it is not unlikely that the adenine substituted ribose is an equilibrium mixture of several conformations. Hruska *et al.* (1970) have discussed a similar problem concerning the conformation of the ribose in pseudouridine. They concluded that ribose may be in the equilibrium of $C_{3'}$-endo and $C_{2'}$-endo, or of $C_{2'}$-exo and $C_{3'}$-exo. Since the present values of $J_{A_1'2'}$, $J_{A_2'3'}$, and $J_{A_3'4'}$ are almost the same to those of pseudouridine which are 5.0 Hz for $J_{1'2'}$ and $J_{2'3'}$ and 5.2 Hz for $J_{3'4'}$, a similar lack of rigidity can be taken into account for the conformation of the ribose moiety of FAD.

Conformational analysis of the glycosidic bond has been difficult in view of the small energy barrier of the rotation about the bond (Hashemeyer and Rich, 1967). According to Trueblood's classification (1960) in terms of torsion angle (ϕ_{CN}), there are two preferred conformations; anti ($\phi_{CN} \simeq -30°$) and syn ($\phi_{CN} \simeq +150°$). The crystal data of nucleosides and nucleotides (Arnott and Huckins, 1969; Sundara-

TABLE VIII: Nuclear Overhauser Effect of Adenine and Ribose Protons[a] (in Per Cent Increase of Signal Intensity).

Irradiated at $AC_{1'}H$	Increase of the Signal Intensity of AC_8H
FAD	~0
5'AMP	6

[a] Using a HA-100 spectrometer operated at 100 MHz.

TABLE VIII: Adopted Conformation for the Pyrophosphate Group of Each Form.

	$\phi^{C'_{A5}-O}_{P_A-O}$	$\phi^{C'_{F5}-O}_{P_F-O}$	$\psi^{P_A-O}_{O-P_F}$
Fol-1	−Gauche	+Gauche	Staggered[a]
Fol-2	−Gauche	−Gauche	Staggered
Open-1	−Gauche	Trans	Staggered

[a] Staggered indicates that the angle of rotation ψ of the $O-P_F$ bond with respect to the P_A-O bond around the P–P axis is 40–60°.

lingam, 1969) have shown that these compounds favor the anti conformation. Many studies on solutions by the optical rotatory dispersion technique were reported, but no conclusive information has been obtained (Emerson *et al.*, 1966). Recently, Hart and Davis (1969) have reported an application of the NOE on this problem.

NOE is a phenomenon observed during the nuclear double-resonance experiments. An enhancement in the absorption intensity of one member of the pair nuclei, which are located at spatially proximate positions and whose dipoles mutually interact, is observed upon the irradiation of the other (Anet and Bourn, 1965; Bell and Saunders, 1970). An examination of the Dreiding model of purine nucleosides and nucleotides shows that the approximate spatial distance between C_8H and $C_{1'}H$ is ~3.4 Å in the anti and ~1.8 Å in the syn conformations (Hart and Davis, 1969; Schirmer *et al.*, 1970). 3',5'-Cycloinosine in the dimethyl sulfoxide-d_6 solution, which must be fixed at the syn-like conformation, shows 39% increase in the intensity of the C_8H signal on the irradiation of $C_{1'}H$. On the other hand, 2-(*N*,*N*-dimethylamino)inosine, which may destabilize the syn conformation by steric interaction between the bulky C_2 substituent and the $C_{5'}$ group, shows only 6% increase in the C_8H intensity on irradiating $C_{1'}H$ (Kainosho and Miyazaki, 1970). In an experiment on AMP in D_2O, a rather small but real increase (6%) was observed for the AC_8H signal intensity on the irradiation of $AC_{1'}H$. However, no effect was detected for FAD in the same experiment (Table VII). Thus it appears quite probable that the syn-like conformation is allowed for "free" AMP to some extent, but not to be allowed for the AMP moiety of FAD. The experimental condition was considered to be proper for the NOE experiment, because the enhancement of the FC_6CH_3 signal on the irradiation of FC_5H was clearly observed at the same condition (Table IV).

Conformation about the Pyrophosphate Group of FAD. The nmr data thus far obtained, unfortunately, give us no useful information on the conformations about the O_A-P_A, P_A-O, $O-P_F$, and P_F-O_F bonds. However, accumulated data for the acyclic phosphodiesters in crystals (Kyogoku and Iitaka, 1966; Arnott and Hukins, 1969; Sundaralingam, 1969) show the preferred conformation is gauche about the O_A-P_A and O_F-P_F bonds, *i.e.*, the dihedral angles between the $C_{5'}-O_A$ or $C_{5'}-O_F$ and P_A-O or P_F-O are ±30 to ~±90°. From the analysis of the vibrational spectra of dialkyl phosphates the gauche conformation is thought to be realized even in aqueous solutions (Shimanouchi *et al.*, 1964). However, the possibility of the trans conformation cannot be eliminated completely because dibenzyl hydrogen phosphate takes such a conformation in crystal (Dunitz and Rollet, 1956).

For the pyrophosphate group the staggered arrangement of the phosphate oxygens when viewed down the P–P axis is preferred (Sundaralingam, 1969). The oxygen atoms are not equivalent and three different staggered arrangements are possible by the rotation around P_A-O or P_F-O. In the crystal of ATP (Kennard *et al.*, 1970) the α- and β-phosphate groups take a deviated staggered conformation around the P–P axis and two ester oxygens approach each other fairly closely. Some of the probable combinations of the conformations around the most P–O bonds are given in Table VIII.

Whole Conformation of FAD. Now we will consider the whole conformation of FAD mainly on the basis of nmr results. As has been shown above, the conformation about the glycosidic bond, namely, the relative orientation of the adenine and isoalloxazine rings with respect to the ribosyl and ribityl groups, is virtually fixed. Also the conformations of the sugar moieties have been predicted from the vicinal coupling constants and shown to be fairly invariable over the temperature range of 21 to 71°. Furthermore the temperature dependence of chemical shifts shows that the whole molecule is in a folded conformation in which the adenine ring is stacked over the isoalloxazine ring. An examination of the Dreiding model of FAD, however, clearly indicates that there are several folded conformations as well as open conformations.

In principle, adenosine and riboflavin can stack through both faces of their adenine and isoalloxazine ring planes (*cf.* Broom *et al.*, 1967). We denote these faces as the front and back sides; *i.e.*, the front side (Ad-front) of adenosine in the anti conformation refers to the same side where the $C_{2'}$ and $C_{3'}$ atoms of the ribose are located and the front side (Fla-front) of riboflavin in the conformation previously described means the side on which the $C_{2'}$ atom of the ribitol resides. Then four different forms are expected for the folded conformations in which the sugar conformations are equal. They differ only in the fashion of the base stacking: folded-1 (Fol-1), Ad-back faces to Fla-back; folded-2 (Fol-2), Ad-back to Fla-front; folded-3 (Fol-3), Ad-front to Fla-back; folded-4 (Fol-4), Ad-front to Fla-front. It is evident that Fol-3 and Fol-4, in which $AC_{2'}H$ and $AC_{3'}H$ thrust into the volume occupied by the ribityl group, are sterically very unfavorable. For the remaining two folded conformations Fol-1 is thought to be more stable than Fol-2, because 2'-hydroxyl oxygen of the ribityl group comes close to the adenine ring in the case of Fol-2 (Figure 10). In Fol-1 the same conformation as those of α- and β-phosphate groups of crystalline ATP (Kennard *et al.*, 1970) could be adopted for the pyrophosphate group of FAD (Figure 11). As $FC_{1'}H$ comes to the space between the two bases the distance of stacking should not be closer than 3.4 Å, but it is still an appropriate distance for the vertical interaction (Giessner-Prettre and Pullman, 1970). More-

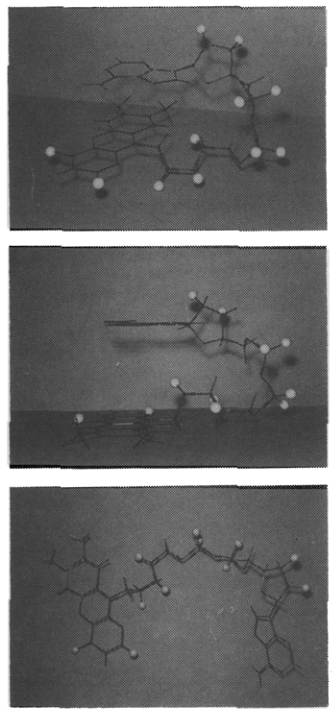

FIGURE 10: The Dreiding molecular models of FAD. Upper: view of Fol-1. In this model the back side of the adenosine part faces to the back side of the riboflavin moiety. Middle: side view of Fol-2, the folded conformation of minor contribution. In this model the back side of the adenosine part faces to the front side of the riboflavin moiety. Down: view of an open form. Open-1 is thought to be the most stable, but not so rigid, conformation that can be realized from Fol-1 and Fol-2 by the rotation about the P_F–O bond.

over all of the hydroxyl groups and the charged phosphate oxygens in Fol-1 are exposed to the surface of the folded molecule and may be stabilized in the aqueous media. These features support our view that Fol-1 must be most stable among the four possible folded forms.

Fol-1 as well as Fol-2 explain the behavior of chemical shifts of the flavin and adenine ring protons. As shown in Table II, the chemical shift difference at high temperature between FAD and FMN or between FAD and ADP is largest for FC_8H among the flavin protons and for AC_8H among the adenine protons. The shift caused by temperature includes both effects of intra- and intermolecular complexing. The temperature dependence of chemical shifts at an infinite dilution studied by Kotowycz *et al.* (1969) only shows the effect of the intramolecular interactions (Table II). Although their data were on D_2O–dioxane (70:30, v/v) solutions, it is evident that FC_8H is more shielded by the adenine ring than FC_5H. Theoretical calculation (Giessner-Prettre and Pullman, 1970) has shown that the highest shielding effect of adenine and isoalloxazine is expected above the center of the adenine and above the aryl ring of isoalloxazine, respectively. Therefore AC_2H of FAD must spend considerable time in the proximity of the aryl ring of isoalloxazine and FC_8H resides in the near position to the center of adenine. The projection of the model for Fol-1 (Figure 11) clearly shows the geometry of the stacking interaction. It also explains the large downfield shifts of the two $FC_{1'}$ protons and especially slight shifts of the $AC_{1'}$ and $FC_{4'}$ protons on the elevation of temperature, since these protons in Fol-1 appear to reside in the shielding region of the adenine and isoalloxazine.

On the other hand, the marked upfield shift of the $FC_{2'}H$ resonance of FAD from that of FMN and the large downfield

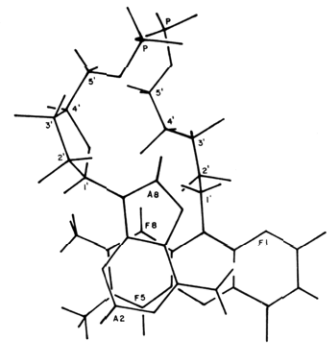

FIGURE 11: Geometric illustration of the molecular model for Fol-1.

shift of $FC_{2'}H$ of FAD at high temperature cannot be explained by the model of Fol-1. Fol-2 is responsible for the phenomenon, because the back side of adenosine in Fol-2 faces to the front side of riboflavin and $FC_{2'}H$ comes just above the adenine ring (Figure 10). A rather small temperature dependence of $FC_{3'}H$ is also explainable by the model of Fol-2.

Although fairly large downfield shifts of the resonance signals with elevation in temperature were observed, any appreciable change was not observed for spin–spin coupling constants. This means that the folded structures tend to be broken at an elevated temperature but any appreciable change in conformations does not occur about the bonds listed in Table VI. Thus the unfolding should be realized by the rotation around the pyrophosphate linkage without changing the other sugar conformations. There may be several conformers for the unfolded structures. The open-1 in Figure 10 and Table VIII is one of the unfolded forms, which can be realized from either Fol-1 or Fol-2 only by the rotation around the P_F–O_F bond keeping the other conformations constant. The actual conformation of the open forms cannot be deduced from the present nmr data. But the small temperature effect of the spin–spin coupling constants indicates that the actual open form must be not so different from open-1.

From this evidence the whole conformation of FAD in aqueous solution should be described as an interchangeable mixture of Fol-1, Fol-2, and flexible unfolded conformers (open forms). The mean energy of the stacking interaction of FAD has been estimated to be about 5 kcal/mole (Song, 1970)[2] and is not so large enough to observe the nmr signals of these conformers separately. Sarma and Kaplan (1970a,b) and Patel (1969) claimed that the two protons of dihydropyridine ring (PyC_4H) of NADH give rise to different chemical shifts at low temperature. The other protons, however, did not show separate signals. They ascribed this phenomenon to the slow equilibrium of the folded and open forms. As the energy of the stacking interaction of NADH is of the same order to that of FAD, the rate of the interconversion of various conformations of NADH is thought to be rapid enough for the 220-MHz nmr time scale. Then the data obtained by them should indicate that a very large chemical shift difference exists between two PyC_4H of individual folded structures and thus may not be completely averaged by the rate of the site exchange at low temperatures. In the case of FAD, however, we can not expect such a pair of protons which have very large chemical shifts at a fixed conformer but are averaged by the site exchange. Therefore we can not observe any separate signal for various conformers at room temperature. The pres-

[2] See Pullman's discussion to the Song paper.

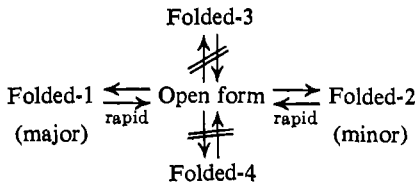

FIGURE 12: Schematic representation of the conformational equilibrium of FAD in aqueous solution.

ent nmr results can be shown schematically in Figure 12. In this scheme, Fol-2 seems to be in a minor contribution from the following reasons: (1) $FC_{2'}OH$ may interfare the vertical stacking, as already mentioned; (2) $\delta_{71}{}^{55°}$ of $FC_{2'}H$ (8 Hz) is about half of $FC_{1'}H_a$ and H_b (13 and 17 Hz).

Comparison to Previous Models. As has been mentioned, the present model for the folded conformation of FAD is different from those previously proposed. Miles and Urry (1968) proposed a structure for a stacked conformation based on its circular dichroism (Figure 13a). The essential statement by them is the angle between the long axes of the adenine and isoalloxazine bases and translation of either base may occur along its long axis. However the marked upfield shifts of FC_8H and $FC_{1'}H$ in the nmr spectra of FAD cannot be explained by their model, and it is also hard to construct a whole structure of FAD which satisfies the nmr data on the conformations of the sugar and phosphate groups. The direction of the long axes of bases in their model are fairly different from that of ours where the two axes are nearly perpendicular to each other. We have not examined whether the present structure satisfies the circular dichroic spectrum of FAD or not. Unambiguous assignments of transitions and directions of the transition moments are essential for that purpose.

Sarma *et al.* (1968) proposed the model from the analysis of nmr spectra (Figure 13b), but based on the erroneous assignments of FC_8H and FC_5H. Kotowycz *et al.* (1969) have pointed out the importance of the assignments of FC_5H and FC_8H in the model building. Nevertheless they employed the same assignments as those by Sarma *et al.* and thus the common defect in their models is that FC_5H is more shielded by the intramolecular complexing, while FC_8H is more affected by the intermolecular interaction by another isoalloxazine ring. Even if the model were revised so that the adenine ring comes over the C_8 position, there is still difficulty in the building of the sterically favorable structures of the sugar and pyrophosphate groups.

In the complex crystal of riboflavin and 5'-bromo-5'-deoxyadenosine they associate through the formation of stacks of alternating parallel adenine and isoalloxazine rings in addition to the coplanar hydrogen bonding (Voet and Rich, 1971; Figure 13c). The feature shows that there exists an affinity between the adenine and isoalloxazine rings and the stacked structure is plausible in FAD. But it does not mean that the same stacks is realized in the FAD molecule, because the ribosyl and ribityl groups of FAD are connected by a pyrophosphate group while the way of packing of the molecules is more important in the complex crystal.

Previous investigators have proposed one fixed model for the structure of FAD in aqueous solutions. However, we could not do so, because none of the folded structures can explain the whole nmr data, especially the temperature dependence of the ribitol protons of FAD. Therefore we postlated the dynamic equilibrium of several folded and open forms. At this stage, however, the exact population of each form could not be predicted because of the lack of the chemical shift data of each form.

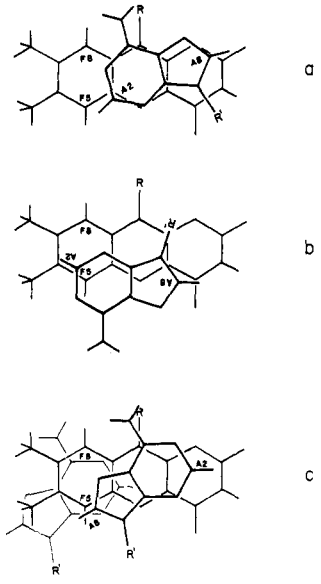

FIGURE 13: Previously proposed models for the mode of base stacking in FAD: (a) by Miles and Urry (1968), (b) by Sarma *et al.* (1968), and (c) by Voet and Rich (1971).

The equilibrium of the conformational change must be greatly deviated when FAD encounters an environmental change, for example, from the aqueous solution to the interior of enzyme. Sarma and Kaplan (1970a,b) claimed that NAD takes a stacked structure even in the enzyme–protein based on the kinetic data on NAD in aqueous solution. We feel, however, that the both environments are completely different in polarity and in the nature of solution. It has been shown that the internal association of FAD is diminished by the change of pH of solution and of the polarity of solvent (Kotowycz *et al.*, 1969; Listowsky *et al.*, 1966; Tsibris *et al.*, 1965). The difference Fourier map between apo- and holoenzymes of alcohol dehydrogenase shows that the structure of NAD bound to the enzyme protein is in an open conformation (Adams *et al.*, 1970) instead of the folded conformation in aqueous solution (Sarma and Kaplan, 1970a,b).

We also found it difficult to build a model which includes the coplanar hydrogen bonding between two rings based on the present nmr results. Thus the association found for riboflavin and adenine derivatives in chloroform (Kyogoku and Yu, 1968, 1969) does not exist in an FAD molecule in aqueous solution.

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References

Abraham, R. J., Hall, L. D., Hough, L., and McLaughlan, K. A. (1962), *J. Chem. Soc.*, 3699.

Adams, M. J., and McPherson, A., Jr., Rossmann, M. G., Schevitz, R. W., and Wonacott, A. J. (1970), *J. Mol. Biol. 51*, 31.

Anet, F. A. L., and Bourn, A. J. R. (1965), *J. Amer. Chem. Soc. 87*, 5250.

Arnott, S., and Huckins, D. W. L. (1969), *Nature (London) 224*, 886.

Barfield, M. (1964), *J. Chem. Phys. 41*, 3825.

Barfield, M., and Chakrabarti, B. (1969), *Chem. Rev. 69*, 757.

Beach, R. L., and Plaut, G. W. E. (1970), *J. Amer. Chem. Soc. 92*, 2913.

Bell, R. A., and Saunders, J. K. (1970), *Can. J. Chem. 48*, 1114.

Bessey, O. A., Lowry, O. H., and Love, R. H. (1949), *J. Biol. Chem. 180*, 755.

Broom, A. D., Schweizer, M. P., and Ts'o, P. O. P. (1967), *J. Amer. Chem. Soc. 89*, 3612.

Bullock, F. J., and Jardetzky, O. (1965), *J. Org. Chem. 30*, 2056.

Dunits, J. D., and Rollet, J. S. (1956), *Acta Crystallogr. 9*, 327.

Emerson, T. R., Swan, R. J., and Ulbricht, T. L. V. (1966), *Biochem. Biophys. Res. Commun. 22*, 505.

Giessner-Prettre, C., and Pullman, B. (1970), *J. Theor. Biol. 27*, 87.

Hanson, K. R. (1966), *J. Amer. Chem. Soc. 88*, 2731.

Hart, P. A., and Davis, J. P. (1969), *J. Amer. Chem. Soc. 91*, 512.

Hashemeyer, A. E. V., and Rich, A. (1967), *J. Mol. Biol. 27*, 369.

Hashemeyer, A. E. V., and Sobell, H. M. (1965), *Acta Crystallogr. 18*, 525.

Hruska, F. E., Grey, A. A., and Smith, I. C. P. (1970), *J. Amer. Chem. Soc. 92*, 4088.

Jackman, L. M., and Sternhell, S. (1969), Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry, 2nd ed, Oxford, Pergamon Press, pp 312.

Kainosho, M., and Miyazaki, M. (1970), Abstract, 23th Annual Meeting of the Chemical Society of Japan, Tokyo, Vol. 1, pp 352.

Kainosho, M., and Nakamura, A. (1969), *Tetrahedron 25*, 4071.

Kainosho, M., Nakamura, A., and Tsuboi, M. (1969), *Bull. Chem. Soc. Jap. 42*, 1713.

Karplus, M. (1959), *J. Chem. Phys. 30*, 11.

Karplus, M. (1963), *J. Amer. Chem. Soc. 85*, 2870.

Kennard, O., Isaacs, N. W., Coppola, J. C., Warren, S., Motherwell, W. D. S., Watson, D. G., Wampler, D. L., Chenery, D. H., Larson, A. C., Kerr, K. A., and Sanseverino, L. R. D. (1970), *Nature (London) 225*, 333.

Kotowycz, G., Teng, N., Klein, M. P., and Calvin, M. (1969), *J. Biol. Chem. 244*, 5656.

Kraut, J., and Jensen, L. H. (1963), *Acta Crystallogr. 16*, 79.

Kyogoku, Y., and Iitkaa, Y. (1966), *Acta Crystallogr. 21*, 49.

Kyogoku, Y., and Yu, B. S. (1968), *Bull. Chem. Soc. Jap. 41*, 1742.

Kyogoku, Y., and Yu, B. S. (1969), *Bull. Chem. Soc. Jap. 42*, 1387.

Kyogoku, Y., and Yu, B. S. (1970), *Chem. Biol. Interactions 2*, 117.

Listowsky, I., England, S., Betheil, J. J., and Seifter, S. (1966), *Biochemistry 5*, 2548.

McCormick, D. B. (1967), *J. Heterocycl. Chem. 4*, 627.

Miles, D. W., and Urry, D. W. (1968), *Biochemistry 7*, 2791.

Patel, D. J. (1969), *Nature (London) 221*, 1239.

Pullman, B., and Pullman, A. (1959), *Proc. Nat. Acad. Sci. U. S. 45*, 136.

Sarma, R. H., Dannies, P., and Kaplan, N. O. (1968), *Biochemistry 7*, 4359.

Sarma, R. H., and Kaplan, N. O. (1970a), *Biochemistry 9*, 557.

Sarma, R. H., and Kaplan, N. O. (1970b), *Proc. Nat. Acad. Sci. U. S. 67*, 1375.

Schirmer, R. E., Noggle, J. H., Davis, J. P., and Hart, P. A. (1970), *J. Amer. Chem. Soc. 92*, 3266.

Shimanouchi, T., Tsuboi, M., and Kyogoku, Y. (1964), *Advan. Chem. Phys. 7*, 435.

Smith, M., and Jardetzky, C. D. (1968), *J. Mol. Spectrosc. 28*, 70.

Song, P. S. (1970), *in* Quantum Aspects of Heterocyclic Compounds in Chemistry and Biochemistry, Bergman, E. D., and Pullman, B., Ed., New York, N. Y., Academic Press, pp 385.

Sundaralingam, M. (1966), *Acta Crystallogr. 21*, 495.

Sundaralingam, M. (1969), *Biopolymers 7*, 821.

Tanaka, N., Ashida, T., Sasada, Y., and Kakudo, M. (1969), *Bull. Chem. Soc. Jap. 42*, 1546.

Tsibris, J. C. M., McCormick, D. M., and Wright, L. D. (1965), *Biochemistry 4*, 504.

Tsuboi, M., Kainosho, M., Nakamura, A. (1968), *in* Recent Developments of Magnetic Resonance in Biological System, Fujiwara, S., and Piette, I. H., Ed., Tokyo, Hirokawa, p 43.

Tsuboi, M., Kuriyagawa, F., Matsuo, K., and Kyogoku, M. (1967), *Bull. Chem. Soc. Jap. 40*, 1813.

Tsuboi, M., Takahashi, S., Kyogoku, Y., Hayatsu, H., Ukita, T., and Kainosho, M. (1969), *Science 166*, 1504.

Uehara, K., Mizoguchi, T., Hosomi, S., Fujiwara, T., and Tomita, K. (1968), *J. Biochem. (Tokyo) 64*, 589.

Voet, D., and Rich, A. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1151.

Weber, G. (1950), *Biochem. J. 47*, 114.