High Frequency Nuclear Magnetic Resonance Investigation of the Backbone of Oxidized and Reduced Pyridine Nucleotides*,†

Ramaswamy H. Sarma and Nathan O. Kaplan

ABSTRACT: High frequency (220 MHz) nuclear magnetic resonance spectra of the following pairs of oxidized and reduced pyridine nucleotides were investigated: oxidized and reduced diphosphopyridine nucleotides, oxidized and reduced triphosphopyridine nucleotides, oxidized and reduced nicotinamide–hypoxanthine dinucleotides, and oxidized and reduced acetylpyridine diphosphopyridine nucleotides. The following are some of the important observations. (1) When the oxidized pyridine dinucleotide is reduced, the $J_{1'2'}$ of the pyridine ribose increased by 3.5 Hz whereas that of the adenine ribose remained constant. This indicates that the ribose conformation attached to the pyridine is not the same in both the oxidized and reduced diphosphopyridine nucleotides. (2) The conformation of the D-ribose attached to the adenine moiety of oxidized and reduced diphosphopyridine nucleotide and the corresponding hypoxanthine analog is *$C_{3'}$-exo-$C_{4'}$-endo*. The conformation of the D-ribose attached to the dihydropyridine ring of reduced pyridine dinucleotides is *$C_{2'}$-endo-$C_{3'}$-exo*. (3) Very conspicuous startling changes occur in the shape and position of the pyridine $C_{2'}$, $C_{3'}$, $C_{4'}$, and $C_{5'}$ protons as a result of the reduction of the coenzyme. It is thought that the geometric alteration which the pyridine ribose experiences as a result of the reduction of the coenzyme is continued to the diphosphate backbone. In the reduced coenzyme, the diphosphate group could be present in a geometry different from that in the oxidized coenzyme. (4) This work shows that there is indeed a distinct difference between the conformations of the oxidized and reduced pyridine dinucleotides.

In the preceding two reports (Sarma and Kaplan, 1970; Sarma *et al.*, 1970) high frequency nuclear magnetic resonance evidence has been presented for the existence of the two helical forms of pyridine dinucleotides—the *M* helix and the *P* helix. We also reported that the reduced pyridine dinucleotides TPNH, DPNH, and (AcPy)DPNH[1] exist in a folded conformation. The C_4 geminal protons of the dihydropyridine of (AcPy)DPNH, NMNH, and (AcPy)MNH, at 5°, gave a singlet. The same two protons of TPNH, DPNH, and NHDH appeared as an AB quartet. The 220 MHz nuclear magnetic resonance data from the various reduced dinucleotides, the reduced mononucleotides, stereoselectively monodeuterated DPNH and NMNH (Sarma and Kaplan, 1970), and *N*-methyl-*N*-ethylnicotinamide–adenine dinucleotide (Sarma *et al.*, 1970) have been accounted for, on the basis of a slow exchange between *M* and *P* helices.

Our present endeavors are directed toward the investigation of the molecular geometry of the backbone of pyridine dinucleotides. In order to study the conformation of the backbone of these dinucleotides, one should be able to observe and assign the signals from the D-riboses attached to the base. The 220 MHz nuclear magnetic resonance spectrophotometer has proven to be most useful in the evaluation of the ribose structure.

We have selected four pairs of oxidized and reduced pyridine coenzymes to study their nuclear magnetic resonance spectra at a frequency of 220 MHz because our nuclear magnetic resonance investigations (Sarma *et al.*, 1968a,b; 1969) have enabled us to understand, on the whole, their conformation and the intramolecular interaction between their base pairs. These four pairs are: (1) DPN^+ and DPNH; both have been shown to have, at biological pH and temperature, significant intramolecular interaction between base pairs which are stacked in parallel planes. (2) NHD^+ and NHDH are the hypoxanthine analogs of DPN^+ and DPNH; the fluorescence transfer data of Shifrin and Kaplan (1959) have shown that there is significantly less interaction between the base pairs in NHDH. Comparison of the purine chemical shifts of IDPR, NHD^+, and NHDH, as well as the pyridine chemical shifts of NMN^+ and NHD^+, NMNH and NHDH also show that in both the oxidized and reduced hypoxanthine coenzyme analogs, there is significantly less interaction between the base pairs.[2] (3) TPN^+ and TPNH both have significant interaction between the base pairs. We have used TPN^+ and TPNH in order to assign the chemical shifts of adenine $C_{1'}$, $C_{2'}$, and $C_{3'}$ because the phosphate group at $C_{2'}$ of TPN and TPNH would deshield the adenine $C_{1'}$, $C_{2'}$, and $C_{3'}$ protons of the triphosphopyridine nucleotide. (4) (AcPy)-

* Contribution No. 691 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154 and the Department of Chemistry, University of California, San Diego, La Jolla, California 92037. Request for reprints should be addressed to University of California. *Received June 9, 1969.* Supported in part by research grants from the American Cancer Society (P-77J) and the National Cancer Institute of the National Institutes of Health (CA-03611). Preliminary communications on this subject have appeared (Sarma and Kaplan, 1969a; Sarma *et al.*, 1969).

† This paper contains two four-color figures. To avoid production difficulties, these figures, as well as four-color figures of both accompanying articles, are printed together on pp 551, 552.

[1] Abbreviations used are: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; NHD^+, nicotinamide–hypoxanthine dinucleotide; NHDH, reduced NHD^+. ADPR, adenosine diphosphoribose; IDPR, inosine diphosphoribose; $(AcPy)DPN^+$, oxidized acetylpyridine analog of DPN^+; (AcPy)DPNH, acetylpyridine analog of DPNH.

[2] Manuscript entitled "Geometry and Nature of the Folding Interactions between Base Pairs in Pyridine and Flavin Dinucleotides," in preparation.

TABLE I: Chemical Shifts of the Protons of the D-Ribose Adjacent to the Adenine and Pyridine Rings in the Oxidized and Reduced Pyridine Dinucleotides (Hz).[a]

	Adenine Ribose Peaks			Pyridine Ribose Peaks	
Nucleotide	$AC_{1'}H$	$AC_{2'}H$	$AC_{3'}H$	$PC_{1'}H$	$PC_{2'}H$
DPN	1314.3	1044.5		1338.3	
DPNH	1337.1	1031.3	992.3	1312.1	964.7
NDH	1329.7	1038.1		1356.0	
NHDH	1348.6	1041.8	999.6	1334.7	969.6
(AcPy)DPN	1318.0			1350.0	
(AcPy)DPNH	1336.2	1028.8	991.0	1319.7	964.5
TPN	1321.5	1096.9		1335.5	
TPNH	1362.0	1084.7	1008.3	1309.1	964.3

[a] The nuclear magnetic resonance spectra were obtained on a high resolution, Varian HRSC-1X superconducting solenoid 220 MHz nuclear magnetic resonance system. The measurements were made on a 0.1 M (pH 7.5) D_2O solution at 23°. Shifts were measured with DSS as an internal standard and precise calibrations were made with the use of an external oscillator. The letters P and A stand for riboses adjacent to pyridine and adenine.

DPN^+ and (AcPy)DPNH both have significant interaction between the base pairs. However, the incorporation of a CH_3 group instead of the NH_2 of the $CONH_2$ side chain abolishes the AB quartet observed for the two C_4 geminal protons of the dihydropyridine moiety of TPNH and DPNH (Sarma and Kaplan, 1970).

Experimental Section

The nucleotides were obtained from P-L Biochemicals. The 220 MHz spectra were obtained on a high resolution Varian HRSC IX superconducting solenoid nuclear magnetic resonance system. Shifts were measured with DSS (E. Merck AG, Darmstadt, Germany) as an internal standard. Precise calibrations of the spectra were made with the use of an external audio oscillator and the precision of the measurements were within ±0.5 Hz. The nuclear magnetic resonance probe temperature was 23°. Compounds used in this study were prepared as in the preceding paper (Sarma and Kaplan, 1970).

Results and Discussion

Chemical Shifts of Adenine $C_{1'}H$ and Pyridine $C_{1'}H$. The clearly observable chemical shifts of the D-ribose protons adjacent to the pyridine and adenine rings are listed in Table I.[3] The assignments are discussed below. The chemical shifts of adenine $C_{1'}H$ and pyridine $C_{1'}H$ in the coenzymes are given in Table I (Figure 1). In this assignment, the pyridine $C_{1'}H$ of DPN^+ comes 24.0 Hz downfield from the adenine $C_{1'}H$. This deshielding of the pyridine $C_{1'}H$ is caused by the full positive charge on the adjacent ring nitrogen. Except for the positive charge, both adenine $C_{1'}$ and pyridine $C_{1'}$ are attached to aromatic conjugated systems. In the case of TPN^+, the adenine $C_{2'}$ carries a phosphate group and this would be expected to deshield adenine $C_{1'}H$, $C_{2'}H$, and $C_{3'}H$. Table I shows that the adenine $C_{1'}H$ of TPN^+ is deshielded from the adenine $C_{1'}H$ of DPN by 7.2 Hz. In the 60 MHz nuclear magnetic resonance spectrum of the pyridine coenzyme, the pyridine $C_{1'}H$ and adenine $C_{1'}H$ have their resonances overlapped to such an extent that a reasonable estimate of their chemical shifts is impossible, and the resolution is only achieved with the 220 MHz instrument.

As a result of the reduction of the oxidized coenzyme, the positive charge on the ring nitrogen has been removed and the aromatic pyridine ring has become a nonaromatic dihydropyridine ring. Theoretically, the effect of such a chemical transformation on the chemical shifts of adenine $C_{1'}H$ and pyridine $C_{1'}H$ should be as follows.

(1) The removal of the full positive charge from the pyridine N_1 of DPN^+ should cause the dihydropyridine $C_{1'}H$ of DPNH to appear at a field higher than the pyridine $C_{1'}H$ of DPN^+. The diminution of the ring current when the ring is reduced would also affect the upfield shift.

(2) The adenine ribose $C_{1'}$ of DPNH is connected to a nitrogen (N_9) which is part of a fully conjugated aromatic system and, hence, the polarization of the adenine–ribose glycosidic bond ($C_{1'}$–N_9) would be aided by delocalization of the charge by the adenine aromatic system. On the other hand, the pyridine $C_{1'}$ of DPNH is connected to a nitrogen (N_1) which is part of a nonconjugated, nonaromatic system which cannot aid the polarization of the pyridine–ribose glycosidic linkage ($C_{1'}$–N_1) by delocalization of charge. The approximate (Pullman and Pullman, 1963) molecular orbital calculations agree fully with this concept. Their π-electron density calculations give a value of 1.503 for the adenine N_9, and a value of 1.653 for the dihydropyridine N_1 of DPNH. The calculated *net charge* (which is a measure by the loss in an electronic charge brought about by conjugation) for adenine N_9 is 0.407 and for dihydropyridine N_1 is 0.347. However approximate the molecular orbital calculations may be, the differences between the net charges are so large that the conclusions reached from them are probably correct. Further, we are using the Pullman values to augment, rather than prove, our hypothesis that the charge on the adenine N_9 could be much more effectively delocalized than a charge on the dihydropyridine N_1. The effect of this on the chemical shifts of the dihydropyridine $C_{1'}H$ and adenine $C_{1'}H$ of the reduced coenzyme is that the dihydropyridine $C_{1'}H$ of DPNH (or for that matter any reduced pyridine dinucleotide) would appear at a field higher than the adenine $C_{1'}H$ of DPNH.

(3) As a consequence of the reduction of the oxidized coenzyme, the aromatic pyridine ring has become a nonaromatic dihydropyridine ring. The ring current magnetic anisotropy of the nonaromatic dihydropyridine ring of DPNH should be less than that of the aromatic pyridine ring of DPN^+. This difference is reflected in the diamagnetic shielding of the adenine rings by the ring current anisotropy of the pyridine ring in DPN^+ and of the dihydropyridine ring in DPNH. It was found that the adenine C_8H and C_2H of DPNH appears 13.4 and 22.1 Hz downfield from those of DPN^+. This down-

[3] The chemical shifts are reported in hertz. The radiofrequency oscillator of the Varian HRSC-1X superconducting nuclear magnetic resonance system is set at 220 MHz. The chemical shifts in hertz may be divided by the oscillator frequency and reported as parts per million.

field shift of the adenine protons of DPNH should not be interpreted to mean that the intramolecular base–base interaction is significantly less in DPNH when compared to DPN^+. This is because a comparison[2] of the pyridine chemical shifts of DPN^+ and NMN^+, as well as those of DPNH and NMNH, show that intramolecular interaction between the base pairs is equally strong in both DPN^+ and DPNH. The smaller magnetic anisotropy of the dihydropyridine ring of DPNH would cause the adenine $C_1'H$ of DPNH to appear at a field lower than the field in which the adenine $C_1'H$ of DPN^+ would appear. Comparisons of the chemical shifts of the adenine $C_1'H$ in DPN and ADPR (Sarma *et al.*, 1968b) show that the adenine $C_1'H$ lies in the diamagnetic shielding area of the pyridine π current.

The three points discussed above enable us to make the following conclusions. The dihydropyridine $C_1'H$ of DPNH should appear at a higher field than the pyridine $C_1'H$ of DPN^+; the dihydropyridine $C_1'H$ of DPNH should appear at a higher field than the adenine $C_1'H$ of DPNH; the adenine $C_1'H$ of DPNH should appear at a lower field than the adenine $C_1'H$ of DPN^+. These three conclusions could be reconciled only by assigning the upfield doublet (Figure 1) to the dihydropyridine $C_1'H$ and the downfield doublet to the adenine $C_1'H$ of the reduced pyridine dinucleotides (*vide infra* for unequivocal assignments).

The assignments discussed above are in excellent agreement with the observation that the upfield doublet from the dihydropyridine $C_1'H$ of the *reduced* coenzyme is broadened (Figure 1). In Figure 1, the pyridine $C_1'H$ and adenine $C_1'H$ resonances of DPN^+ and NHD^+ are equally sharp, but the dihydropyridine $C_1'H$ has substantially broadened. This selective broadening of the dihydropyridine $C_1'H$ resonance does not, *a priori*, indicate onset of restricted rotation upon reduction. Upon reduction, the electronic properties of the pyridine ring and, specifically, the local electronic environments of the nitrogen atom are altered, so that the relaxation time of nitrogen (dominated by quadruple coupling) is altered. This altered nitrogen relaxation time, in turn, affects resolvable nuclear spin–spin coupling between nitrogen and the $C_1'H$ resonance. In accordance with this explanation, when the temperature was increased from 23 to 73°, the broadening of the doublet increased due to further diminution of the nitrogen relaxation time at elevated temperature. The onset of unresolved nitrogen spin–spin coupling and consequent broadening of the dihydropyridine $C_1'H$ resonance provide the most unequivocal method of assigning that proton. It is gratifying to note that this explicit assignment of dihydropyridine $C_1'H$ agrees with the assignment arrived at through considerations of change in electronegativity, polarizability, and ring current magnetic anisotropy as a result of the reduction of oxidized coenzyme.

The present assignments of the adenine $C_1'H$ and pyridine $C_1'H$ of the reduced coenzymes do not agree with those by Jardetzky and Wade-Jardetzky (1966) who have assigned their positions from an overlapping spectrum taken in a 60 MHz nuclear magnetic resonance system. Jardetzky and Wade-Jardetzky assigned the downfield part of the spectrum to the pyridine $C_1'H$ and the upfield part to the adenine $C_1'H$ in both oxidized and reduced pyridine dinucleotides. This assignment holds true in the case of the oxidized coenzymes, but breaks down in the case of the reduced coenzymes because as a result of this reduction, the electronegative aromatic pyridine ring of DPN with large current magnetic anisotropy has become a less electronegative nonconjugated, nonaromatic dihydropyridine ring of DPNH with low ring current magnetic anisotropy.

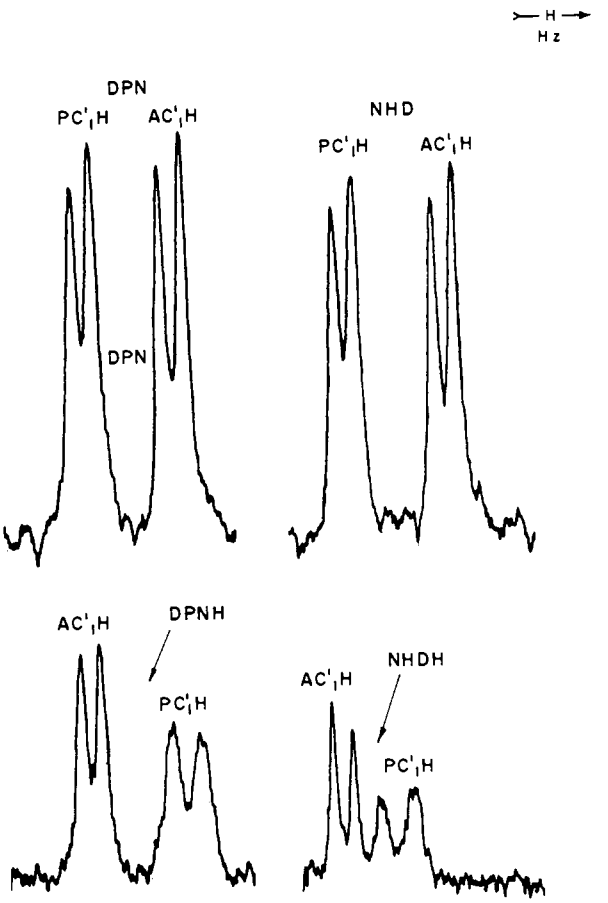

FIGURE 1: Nuclear magnetic resonance spectra of pyridine $C_1'H$ and adenine $C_1'H$ of DPN^+, DPNH, NHD^+, and NHDH taken in a 220 MHz nuclear magnetic resonance system at a sweep width of 500 Hz. Note that, in both oxidized and reduced coenzymes, the resonance from $AC_1'H$ is sharp, but the pyridine $C_1'H$ has substantially broadened, and its coupling constant increased as the result of reduction.

Recognitions and/or Assignments of Adenine and Pyridine $C_{2'}$, $C_{3'}$, $C_{4'}$, and $C_{5'}$ Protons of Both Oxidized and Reduced Pyridine Dinucleotides. Figures 2 and 3 show the high frequency nuclear magnetic resonance spectra of DPN^+, DPNH, NHD^+, and NHDH upfield from the HDO peak. In the case of the oxidized coenzymes, the spectra downfield of the HDO peak account for all the protons except the purine and pyridine $C_{2'}$, $C_{3'}$, $C_{4'}$, and $C_{5'}$ protons—a total of ten protons. Planimeter integration of the peaks upfield of HDO shows that their area corresponds to ten protons; the area of the well-resolved adenine $C_1'H$ and pyridine $C_1'H$ has been used to elicit the number of protons. This indicates that no ribose protons of the oxidized coenzyme are lost in the large HDO peak. The one-proton peak designated A in the spectra of DPN^+ and NHD^+ (Figures 2 and 3, top) come at 1044.5 and 1038.1 Hz. This peak comes, in the case of TPN^+, at 1096.9 Hz. We believe that this downfield shift is caused by the $C_{2'}$ phosphate and that the peak designated A is adenine $C_{2'}H$.

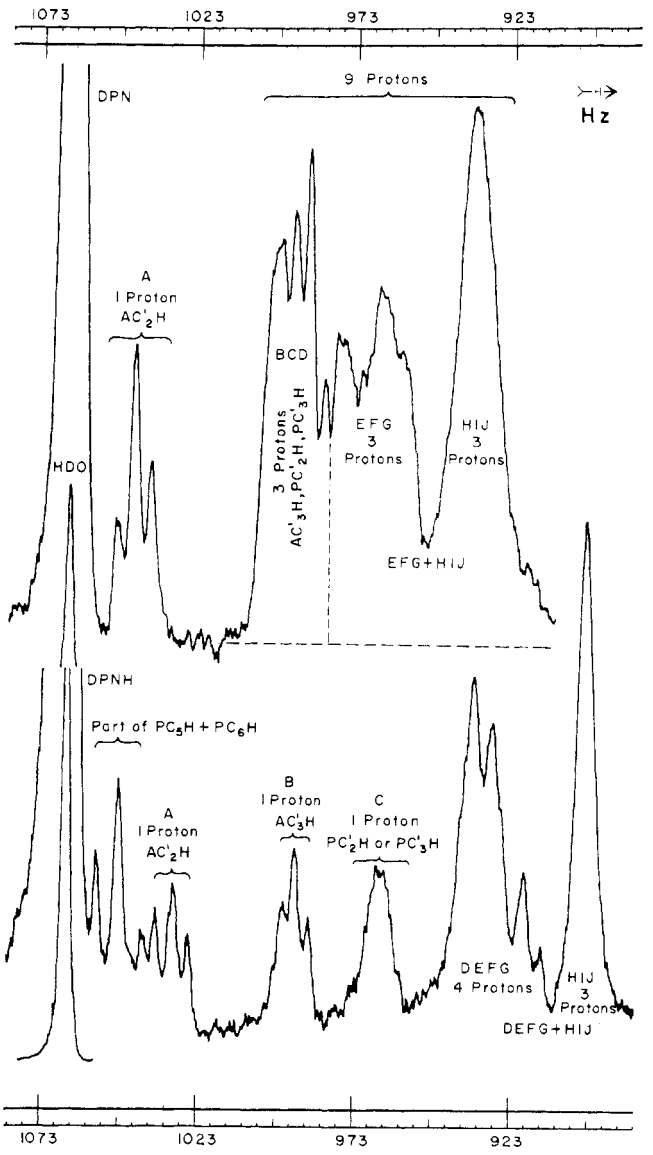

FIGURE 2: Nuclear magnetic resonance spectra of DPN^+ (top) and DPNH (bottom) upfield from the HDO peak, taken in a 220 MHz nuclear magnetic resonance system using DSS as an internal standard at a sweep width of 500 Hz. In the case of DPN^+, the area represented by the region EFG + HIJ (each letter stands for one proton) is equal to that from six protons and is the result of extensive splitting and confused overlap of the six protons, pyridine $C_4'H$, $C_5'H_2$ and adenine $C_4'H$, $C_5'H_2$. In the case of DPNH, the area represented by DEFG + HIJ is equal to that from the seven protons, adenine $C_4'H$, $C_5'H_2$ and pyridine $C_4'H$, $C_5'H_2$, $C_3'H$. The spectra on the top and bottom were taken at different sensitivities ("gain"). Note the great difference in the shape and position of the signals in the oxidized and reduced coenzymes.

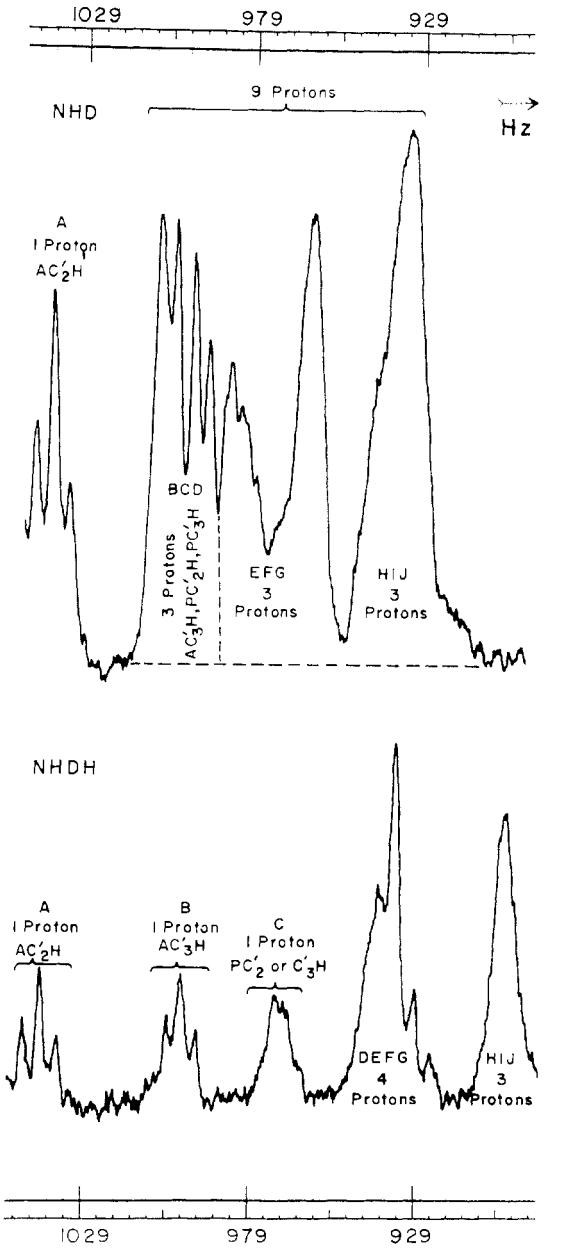

FIGURE 3: Proton magnetic resonance spectra of NHD^+ (top) and NHDH (bottom) upfield from the HDO peak, taken in a 220 MHz nuclear magnetic resonance system using DSS as an internal standard. Details are as in Figure 2. Note that the changes in shape and position of peaks are the same in both DPNH (Figure 2) and NHDH from their corresponding oxidized forms, even though the hypoxanthine analog has a more open conformation compared to DPNH.

Schweizer *et al.* (1968) have published the nuclear magnetic resonance data on AMP and adenosine as well as on IMP and inosine. Their data show that the phosphate does shield the protons on the carbon atom bearing the phosphate by 52–54 Hz,[4] which is the same magnitude as in our system.

In our opinion, this fact substantiates our judgment that peak A is from adenine $C_2'H$, which would be expected to appear as a doublet's doublet being split by $C_1'H$ and $C_3'H$. Peak A is an overlapping pair of doublets. The remaining large peak in the spectra of the ribose of DPN^+ and NHD^+ represents 9 protons, and its area is divided (Figures 2 and 3, top) into two regions. Region BCD[5] contains sharp signals and represents the three protons purine $C_3'H$ and pyridine $C_2'H$ and

[4] Calculated as if the spectra were taken in a 220 MHz nuclear magnetic resonance system.

[5] Each letter represents one proton; *i.e.*, BDC stands for three protons.

TABLE II: Observed Coupling Constants and the Most Probable Conformation of D-Ribose Attached to the Adenine and Pyridine Rings of Nucleotides.

	Adenine Ribose				Pyridine Ribose			
	Coupling Constants				Coupling Constants			
Nucleotides	$J_{1'2'}$ *trans* (Hz)	$J_{2'3'}$ *cis* (Hz)	$J_{3'4'}$ *trans* (Hz)	Conformation	$J_{1'2'}$ *trans* (Hz)	$J_{2'3'}$[a] *cis* (Hz)	$J_{3'4'}$ *trans* (Hz)	Conformation
AMP	5.3	4.8	3.8	$C_{3'}$-*exo*-$C_{4'}$-*endo*				
NMN					5.5	5.3	2.7	$C_{3'}$-*exo*-$C_{4'}$-*endo*
DPN	5.7	5.2		$C_{3'}$-*exo*-$C_{4'}$-*endo*; $C_{3'}$-*exo*	5.1			
DPNH	5.1	5.0	4.1	$C_{3'}$-*exo*-$C_{4'}$-*endo*	8.1	1.9		$C_{2'}$-*endo*-$C_{3'}$-*exo*
DPND	5.0	5.0	4.0	$C_{3'}$-*exo*-$C_{4'}$-*endo*	8.1	1.8		$C_{2'}$-*endo*-$C_{3'}$-*exo*
NHD	5.6	5.0		$C_{3'}$-*exo*-$C_{4'}$-*endo*; $C_{3'}$-*exo*	5.5			
NHDH	5.3	5.1	4.0	$C_{3'}$-*exo*-$C_{4'}$-*endo*	8.9	0.2		$C_{2'}$-*endo*-$C_{3'}$-*exo*
(AcPy)DPN	5.5				4.5			
(AcPy)DPNH	4.5	4.5	3.5	$C_{3'}$-*exo*-$C_{4'}$-*endo*	7.5	2.2		$C_{2'}$-*endo*-$C_{3'}$-*exo*
TPN	5.1	9.4[b]	4.3	*O*-*endo*[c]	4.9			
TPNH	4.6	8.9[b]	4.2	*O*-*endo*[c]	8.4	1.6		$C_{2'}$-*endo*-$C_{3'}$-*exo*

[a] Calculated from the line width of the nuclear magnetic resonance signal from pyridine $C_{2'}H$ at half-height and pyridine $J_{1'2'}$. [b] Calculated from the line width of the nuclear magnetic resonance signal from adenine $C_{2'}H$ at half-height and adenine $J_{1'2'}$. [c] See footnote 6.

$C_{3'}H$. The region EFGHIJ[5] represents six protons and is the result of extensive splitting and confused overlap of the six protons, pyridine $C_{4'}H$, $C_{5'}H_2$, and purine $C_{4'}H$, $C_{5'}H$.

The nuclear magnetic resonance spectra of the reduced pyridine dinucleotides are startingly different from those of the oxidized coenzymes. In the case of DPNH immediately upfield of the HDO peak, one could see (Figure 2, bottom) three signals of about 7 Hz apart. This is part of the signals from dihydropyridine C_6H and C_5H which are usually vitiated by the HDO peak (Sarma and Kaplan, 1970). In the case of NHDH (Figure 3, bottom), TPNH, and (AcPy)-DPNH, these signals at 23° could not be seen. Peak A in the case of DPNH and NHDH (Figures 2 and 3, bottom) represents one proton each and come at 1031.3 and 1041.8 Hz, and these have been assigned to adenine $C_{2'}H$ because in the case of TPNH the same peak comes 53.4 Hz downfield, indicating the deshielding of the $C_{2'}$ phosphate group. Peak B in the case of DPNH and NHDN represents one proton each and comes at 992.3 and 999.6 Hz. In the case of TPNH, there is a one-proton signal at 1008.3 Hz, 16.5 Hz downfield from peak B of DPNH. This downfield shift is again caused by the $C_{2'}$ phosphate which is *trans* to the vicinal $C_{3'}H$. We, therefore, assign peak B to $C_{3'}H$ of DPNH and NHDH. The peak C area equivalent to one proton comes at 964.7 Hz in DPNH, 969.6 Hz in NHDH, 964.3 Hz in TPNH, and 964.5 Hz in (AcPy)DPNH. This peak represents a pyridine ribose proton because in both DPNH and TPNH it has arrived at the same position (the slight downfield shift in the case of NHDN, peak C, is probably due to less intramolecular interaction between base pairs in the hypoxanthine analog). It is only possible to say that peak C is from pyridine $C_{2'}H$ or $C_{3'}H$. Below we discuss the conformation of the riboses and we have assigned peak C to pyridine $C_{2'}H$. There remain two large peaks to be analyzed. The area of these two peaks represents seven protons. The area designated DEFG[5] represents four protons, one of which is pyridine $C_{3'}H$. The six protons, pyridine $C_{4'}H$, $C_{5'}H_2$, and purine $C_{4'}H$, $C_{5'}H_2$, are distributed between areas DEFG and HIJ.

Change of Conformation of D-*Ribose Attached to the Nicotinamide Moiety as a Result of the Reduction of the Oxidized Pyridine Dinucleotide.* The coupling constants of adenine and pyridine ribose protons of the oxidized and reduced pyridine dinucleotides are summarized in Table II. Strikingly in all dinucleotides, when the oxidized coenzyme is converted into reduced coenzyme, the pyridine $C_{1'}$ coupling constant has increased. The pyridine $J_{1'2'}$ in the oxidized coenzyme is about 5 Hz whereas the same in the reduced coenzyme is about 8.5 Hz. On the other hand, the adenine $J_{1'2'}$ in the oxidized and reduced coenzymes have remained constant. This selective increase of the pyridine $J_{1'2'}$ could be due to the following: (1) the reduction of the oxidized coenzyme has changed the $C_{1'}$ substituent from an electronegative pyridine ring to a less electronegative dihydropyridine ring, and this change in the electronegativity of the $C_{1'}$ substituent could have caused the change in coupling constant; (2) the $C_{1'}H$ and $C_{2'}H$ hydrogens are *trans* and an *increase* in the coupling constant could result from an *increase in the dihedral angle between them.*

It is known that variations in electronegativity of substituents could affect the coupling constants in the rigid monosubstituted hexachlorobicyclo[2.2.1]heptenes (Williamson, 1963) and in the norbornene derivatives (Laszlo and Schleyer, 1963). Laszlo and Schleyer (1963) increased the electronegativity of 5-*endo*-substituted norbornene from 2.49 to 3.72 and this causes a decrease of the coupling constant of adjacent proton by only 1.3 Hz. At the same time, such an

TABLE III: Coupling Constants of Pyridine Hydrogens in Free Pyridine Base and Pyridine Nucleotides.

Compound	$J_{6,5}$ (Hz)	$J_{4,5}$ (Hz)
Nicotinamide	5.2	8.2
Nicotinamide ribonucleoside	5.5	8.3
Nicotinamide mononucleotide	5.5	8.3
DPN	5.7	8.5
TPN	5.6	8.0

electronegativity increase causes the adjacent proton to go downfield by 524 Hz,[4] indicating that the effect of electronegativity on the magnitude of coupling constants is extremely small. As we go from DPNH to DPN^+, we increase the electronegativity of the substituent on the pyridine $C_{1'}$, and such an increase of electronegativity causes the $C_{1'}$ proton to go downfield by 26.2 Hz, compared with 524 Hz in the example given by Laszlo and Schleyer (1963). It is evident that such a small change in electronegativity cannot affect the coupling constants of pyridine $C_{1'}H$ by any more than a few tenths hertz. Data in Table III on the coupling constants of pyridine protons in nicotinamide, nicotinamide ribonucleoside, NMN^+, and DPN^+ also agree with the fact that variation in electronegativity cannot affect the coupling constants any more than a few tenths hertz. Further, Karplus functions have given good agreement from computed and measured coupling constants in four saturated derivatives of the rigid 5-membered ring of camphane-2,3-diol (Anet, 1961). Hence the increase in pyridine $J_{1'2'}$ by about 3.5 Hz on the reduction of the oxidized coenzyme should result from an increased dihedral angle between the *trans* $H_{1'2'}$ protons of the pyridine ribose. Such an increase of dihedral angle between the pyridine $H_{1'2'}$ of ribose would change the conformation of the ribose. In conclusion, we have observed that when the oxidized pyridine dinucleotide is reduced, the ribose attached to the pyridine ring undergoes a definite conformational change, the conformation of the ribose attached to the adenine remaining the same in both the oxidized and reduced pyridine dinucleotides.

Conformation of Ribose Attached to the Base Pairs in Oxidized and Reduced Pyridine Dinucleotides. Smith and Jardetzky (1968) have published information which correlates calculated coupling constants $J_{1'2'}$, $J_{2'3'}$, $J_{3'4'}$ to twenty possible conformations of D-ribose in nucleosides and nucleotides. It is, therefore, now possible to compare these theoretical coupling constants with the observed coupling constants and then to predict the most probable conformation for the D-ribose. The coupling constants of the riboses of the dinucleotides which are experimentally determinable from their 220 MHz nuclear magnetic resonance spectra are listed in Table II. The corresponding predicted conformations from Smith and Jardetzky (1968) are also included in Table II. In the cases of DPNH, NHDH, and (AcPy)DPNH it was possible to determine experimentally $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$ of the riboses attached to the purine ring and the data indicate the most probable conformation of this ribose to be $C_{3'}$-*exo*-$C_{4'}$-*endo* which is the same as the conformation of the ribose attached to adenine of AMP (personal communication, Dr. C. D. Jardetzky). $J_{1'2'}$ and $J_{2'3'}$ of the ribose adjacent to the purine in DPN^+ and NHD^+ could be determined experimentally. However, $J_{3'4'}$ could not be determined because the adenine $C_{3'}H$ signal comes mixed with those from pyridine $C_{2'}H$ and $C_{3'}H$ (Figures 2 and 3). The observed coupling constants agree with both a $C_{3'}$-*exo*-$C_{4'}$-*endo* and a $C_{3'}$-*exo* conformation for this ribose. It is not possible to differentiate between the above two possibilities without $J_{3'4'}$, even though $C_{3'}$-*exo*-$C_{4'}$-*endo* (same as in AMP, DPNH, NHDH, and (AcPy)DPNH) is the most likely conformation. It seems that the 2′-phosphate on the adenine ribose of TPN^+ and TPNH effects a conformational change on the ribose attached to the purine base. The J values indicate the most probable conformation to be *O-endo*[6] (Table II).

The method of conformations of the riboses attached to the pyridine rings in the oxidized and reduced nucleotides is borne out by the various observations in this report. Due to the fact that in the oxidized coenzymes, the pyridines $C_{2'}H$ and $C_{3'}H$ are mixed with adenine $C_{3'}H$, no reasonable estimate of $J_{2'3'}$ or $J_{3'4'}$ could be made. The coupling constant $J_{1'2'}$ of 5.1, 5.5, 4.9, and 4.5 Hz for DPN^+, NHD^+, TPN^+, and (AcPy)DPN^+ for the pyridine ribose would agree with any of the following four conformations: $C_{3'}$-*exo*, *O-endo*, $C_{3'}$-*exo*-$C_{4'}$-*endo*, and $C_{4'}$-*exo*-*O-endo*. It seems most likely that the conformation of the ribose adjacent to the pyridine in the oxidized coenzyme is the same as the one in the oxidized mononucleotide, *viz.* nicotinamide mononucleotide (NMN^+). Figure 4 is part of the 220 MHz nuclear magnetic resonance spectrum of NMN^+. The resonances are clearly separated and are assignable to the ribose protons. The coupling constants $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$ are listed in Table II and the most probable conformation for the ribose is $C_{3'}$-*exo*-$C_{4'}$-*endo*.

It must be noted that in Figure 4, the resonances from the two geminal $C_{5'}$ protons appear as an AB spectrum, indicating that the two protons are magnetically and chemically nonequivalent. This is not entirely unexpected in view of the chirality of the environment which envelops these protons.

In all the *reduced pyridine nucleotides*, the $J_{1'2'}$ of pyridine ribose varies from 7.5 to 8.9 Hz. Coupling constants of the order of 7.5 to 8.9 Hz for $J_{1'2'}$ in D-ribose would agree with five different conformations for the D-ribose (Smith and Jardetsky, 1968). These are the following: $C_{1'}$-*exo*; $C_{2'}$-*endo*; $C_{1'}$-*exo*-$C_{2'}$-*endo*; $C_{1'}$-*exo*-*O-endo*; $C_{2'}$-*endo*-$C_{3'}$-*exo*. One could distinguish the various possibilities from $J_{2'3'}$ and $J_{3'4'}$. The only other identifiable resonance from dihydropyridine ribose appears as a single resonance (peak C, Figure 2, bottom) with a line width of 10.0 Hz at half-peak height. This resonance is from either $C_{2'}H$ or $C_{3'}H$ of the ribose adjacent to dihydropyridine. The fact that this resonance does not show observable splitting indicates that $J_{2'3'}$ or $J_{3'4'}$ is small. This criterion enables us to eliminate the two conformations $C_{1'}$-*exo* and $C_{1'}$-*exo*-*O-endo* for D-ribose adjacent to dihydropyridine because the $J_{2'3'}$ and $J_{3'4'}$ are large enough to be observable for these conformations. We have to choose from the remaining three conformations, *i.e.*, $C_{2'}$-*endo*, $C_{1'}$-*exo*-$C_{2'}$-*endo*, or $C_{2'}$-*endo*-$C_{3'}$-*exo*. It is now possible to assign peak C (Figure 2, bottom) to $C_{2'}H$, as well as to choose $C_{2'}$-*endo*-

[6] Personal communication from Dr. C. D. Jardetzky. The *O-endo* conformation would be very unstable conformation especially for D-ribose which carries a large 2′-phosphate group. Hence, more research has to be done in this direction.

TABLE IV: Theoretical Line Width at Half-Height for Signals from Dihydropyridine $C_{2'}H$ and $C_{3'}H$ for the Conformations of D-Ribose Listed Below.[a]

Conformation	Line Width of $C_{2'}H$, $J_{1'2'} + J_{2'3'}$ (Hz)	Line Width of $C_{3'}H$, $J_{2'3'} + J_{3'4'}$ (Hz)
$C_{2'}$-*endo*	8.6 + 3.9 = 12.5	3.9 + 0.4 = 4.3
$C_{1'}$-*exo*-$C_{2'}$-*endo*	9.2 + 3.9 = 13.1	3.9 + 0.4 = 4.3
$C_{2'}$-*endo*-$C_{3'}$-*exo*	8.6 + 1.7 = 10.3	1.7 + 0.2 = 1.9

[a] The theoretical coupling constants are taken from Smith and Jardetzky (1968).

$C_{3'}$-*exo* as the conformation of D-ribose attached to dihydropyridine of reduced coenzymes because only $J_{1'2'} + J_{2'3'}$ of $C_{2'}$-*endo*-$C_{3'}$-*exo* conformation agrees with the 10.0 Hz line width of peak C as shown in Table IV.

Helical Posture of the Diphosphate Backbone. Very conspicuous is the startling change that has occurred in the shape and position of the ribose protons as a result of the reduction of the oxidized coenzymes. The three-proton peak HIJ of *both* DPN^+ and NHD^+ comes at 935 ± 1 Hz; in the case of *both* DPNH and NHDH, the same peak comes at 901 ± 1 Hz (Figures 2 and 3). This is an upfield shift of 34 Hz. The peak designated DEF also has moved upfield by approximately the same amount in the reduced coenzyme. On the other hand, part of the peak BCD of the oxidized coenzyme (peak BCD contains adenine $C_{2'}H$, pyridine $C_{2'}H$ and $C_{2'}H$) has been retained in the reduced coenzyme and part moved upfield. The protons which have traveled substantially upfield in the reduced coenzyme are pyridine $C_{2'}H$, $C_{3'}H$, $C_{4'}H$, $C_{5'}H_2$ and adenine $C_{4'}H$ and $C_{5'}H_2$. The adenine $C_{2'}H$ and $C_{3'}H$ have either remained constant or shifted upfield or downfield by a small amount compared with the general upward shift of the pyridine ribose protons and adenine $C_{4'}H$ and $C_{5'}H_2$.

The precise reason for the cause of the substantial upward shift of the pyridine ribose protons (as well as the protons attached to the carbon atoms bearing the diphosphate backbone of the reduced coenzymes from the corresponding protons of the oxidized coenzyme) is not apparent. The fact that the diphydropyridine ring of the reduced coenzyme is less electronegative than the pyridine ring of DPN cannot be the reason for the observed upward shift of the pyridine ribose protons, because such an effect has to affect the $C_{1'}H$ the most and also this effect has to attenuate with respect to the $C_{2'}$, $C_{3'}$, $C_{4'}$, and C_5' protons. Data in Table I show that the pyridine $C_{1'}H$ of DPNH is shifted upfield from the pyridine $C_1'H$ of DPN^+ only by 26.2 Hz which is smaller that the shifts one observes for the remaining pyridine ribose protons. The fact that the same upward shift happens in both DPNH (in which there is significant interaction between base pairs) and NHDH (in which there is significantly less interaction between base pairs) discredits the hypothesis that in the reduced coenzyme there is a stronger intramolecular interaction between base pairs than in the oxidized coenzyme, and that the magnetic anisotropy of the adenine ring causes the upward shift, especially in view of the fact that the magnetic anisotropy of the six-membered ring of hypoxanthine is small compared with the same in that of adenine. It would seem that this substantial upward shift is due to a change in the molecular geometry of the diphosphate backbone.

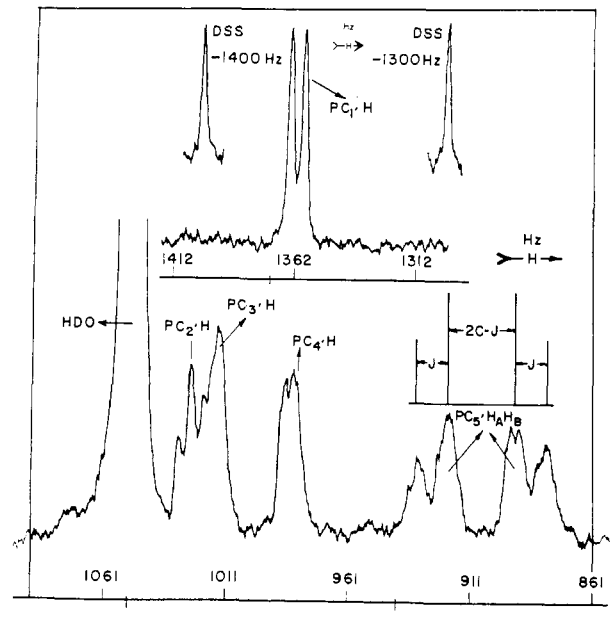

FIGURE 4: Part of the 220 MHz nuclear magnetic resonance spectrum of NMN^+. Note that the two geminal protons on $C_{5'}$ of the ribose appear as an AB spectrum. The geminal coupling constant is 13 Hz. The difference in chemical shifts ($\nu_0\delta$) between the two protons could be calculated using the expression $\nu_0\delta = \{(1\text{–}4)(2\text{–}3)\}^{1/2}$. The $\nu_0\delta$ was found to be 38 Hz.

It has been discussed earlier that as a result of the reduction of the oxidized coenzyme, the conformation of the ribose adjacent to the pyridine ring changes. Figure 5[7] shows the relative disposition between the side chain at $C_{4'}$ and the pyridine base in the oxidized coenzyme (left, Figure 5) and the reduced coenzyme (right, Figure 5). The photographs clearly reveal that a change in the conformation of the ribose startingly affects the distance between the pyridine base and the side chain at $C_{4'}$. The distance between the side chain at C_4' and the pyridine base is considerably less in the oxidized coenzyme compared to that in the reduced coenzyme. The difference in conformation of the riboses in the oxidized and reduced forms is illustrated in Figure 6.[7] It may be that this difference in the relative disposition between the base and the side chain in the oxidized and reduced coenzymes in turn affects the shape and position of the ribose protons as the nuclear magnetic resonance data of DPN and DPNH show (Figure 2, top and bottom). It could also be that the geometric posture of the diphosphate group with respect to the ribose and the pyridine base in the reduced pyridine dinucleotides is such that it enables the P=O and the hydroxyl groups to shield the adenine $C_{4'}H$, $C_{5'}H_2$ and pyridine $C_{5'}H_2$, $C_{4'}H$, $C_{3'}H$, $C_{2'}H$. It is not possible to detect the exact geometry of the diphosphate backbone from the shielding constants because little is known about distance and angular dependence of

[7] Taken by Mr. John Galano, Camera Eye, 415 South Street, Waltham, Mass.

shielding constants, particularly for systems containing hetero nuclei.

The present work, however, shows clearly that there does indeed exist a distinct difference between the conformations of the oxidized and reduced pyridine dinucleotides.

Acknowledgments

The authors thank Dr. W. D. Phillips and Dr. C. D. Jardetzky for very stimulating discussions on the contents of this publication. The authors are particularly indebted to Dr. W. D. Phillips for arranging access to the 220 MHz nuclear magnetic resonance spectrometer.

References

Anet, F. A. L. (1961), *Can. J. Chem. 39*, 789.
Jardetzky, O., and Wade-Jardetzky, N. G. (1966), *J. Biol. Chem. 241*, 85.
Laszlo, P., and Schleyer, P. von R. (1963), *J. Am. Chem. Soc. 85*, 2709.
Pullman, B., and Pullman, A. (1963), Quantum Biochemistry, New York, N. Y., Interscience Publishers, pp 531 and 239.
Sarma, R. H., Dannies, P., and Kaplan, N. O. (1968a), *Biochemistry 7*, 4359.
Sarma, R. H., and Kaplan, N. O. (1968), 156th National Meeting of the American Chemical Society, Atlantic City, N. J., September, 1968, 138.
Sarma, R. H., and Kaplan, N. O. (1969a), *J. Biol. Chem. 244*, 771.
Sarma, R. H., and Kaplan, N. O. (1969b), *Biochem. Biophys. Res. Commun. 36*, 780.
Sarma, R. H., and Kaplan, N. O. (1970), *Biochemistry 9*, 539.
Sarma, R. H., Moore, M. C., and Kaplan, N. O. (1970), *Biochemistry 9*, 549.
Sarma, R. H., Phillips, W. D., and Kaplan, N. O. (1969), *Biophys. J. 9*, FAM-F3.
Sarma, R. H., Ross, V., and Kaplan, N. O. (1968b), *Biochemistry 7*, 3052.
Schweizer, M. P., Brown, A. D., T'so, P. O. P., and Hollis, D. P. (1968), *J. Am. Chem. Soc. 90*, 1042.
Shifrin, S., and Kaplan, N. O. (1959), *Nature 183*, 1529.
Smith, M., and Jardetzky, C. D. (1968), *J. Mol. Spec., 28*, 70.
Williamson, K. L. (1963), *J. Am. Chem. Soc. 85*, 516.

Fluorescence Properties of Hemocyanin from *Levantina hierosolima**

Nurith Shaklai and Ezra Daniel

ABSTRACT: The emissive properties of reduced and oxygenated hemocyanin from *Levantina hierosolima* at neutral and alkaline pH were studied. A large enhancement in fluorescence intensity is observed on complete reduction, fourfold at neutral pH. In partially reduced protein the enhancement is proportional to the fraction reduced. The quenching upon oxygen binding is traced to radiationless energy transfer from the tryptophanyl residues to the Cu···O groups. Application of Förster's theory leads to a value of 25 Å for the distance between the donor and the acceptor in the "equivalent oscillators system," defined as a single donor–acceptor pair with parallel orientation which would display the same emissive properties as the actual protein molecule. From a comparison of the data obtained at neutral and alkaline pH, it is concluded that the internal arrangement of the donor–acceptor system is not affected by the extensive dissociation which the molecule undergoes in the transition between the two pH values considered. The findings indicate that the fluorimetric method provides a sensitive tool for the study of oxygen binding equilibria in hemocyanin.

Hemocyanin is the oxygen binding protein in molluscs and arthropods. Many studies have been carried out on the protein to determine the size, shape, and subunit structure of the molecule. In contrast, the binding of oxygen has received less attention. In the course of a study designed to examine the effect of changes in the quaternary structure of hemocyanin on its oxygen binding function, the need for a sensitive experimental method to measure the binding was quickly felt. Fluorescence measurements have been used with success in binding studies in some systems (Anderson and Weber, 1965; Daniel and Weber, 1966). In this paper the emissive properties of hemocyanin from *Levantina hierosolima* were studied. The results show that the binding of oxygen brings about a conspicuous change in fluorescence efficiency which may be used to advantage in the study of problems of the kind that motivated this work.

* From the Department of Biochemistry, Tel-Aviv University, Tel-Aviv, Israel. *Received August 28, 1969.*