at 280 nm. Pyrocell matched quarts uv cells of 10-mm path length were used, and the pH of the solutions was measured using a Radiometer Model 26 pH meter.

Nmr. All solutions used in the nmr measurements were 0.01 M in phosphate, 0.01 M in citrate, and 0.1 M in NaCl. Egg-white lysozyme (3 × crystallized, dialyzed, and lyophilized, Sigma Lot 6876) was used at a concentration of 3.3×10^{-3} M for the low-temperature $(Glc\text{-}NTFAc)_3$ study and for the Glc-NTFAc binding study, while a concentration of 3.3×10^{-4} was used in the high-temperature, fast-exchange study of $(Glc\text{-}NTFAc)_2$ and $(Glc\text{-}NTFAc)_3$. Spectra were recorded on a Varian XL-100-15 spectrometer operating at 94.1 MHz. For the 1:1 $(Glc\text{-}NTFAc)_3$ study, 12-mm, thin-walled tubes were used, while 5-mm tubes were used in the other studies. Both the free trifluoroacetic acid present and the H_2O heteronuclear lock were used as internal references, and the values obtained were always in agreement at a given temperature.

The trifluoro-*N*-acetylglucosamine oligomers were a gift of William Beranek, Jr. (W. Beranek and M. A. Raftery, to be published, 1971), and were prepared by trifluoro-*N*-acetylation of oligoglucosamines prepared by acid hydrolysis of N-deacetylated chitin.

References

Ashton, H., Capon, B., and Foster, R. L. (1971), *Chem. Commun.*, 512.

Blake, C. C. G., Johnson, L. N., Muir, G. A., North, A. C. T., Phillips, D. C., and Sarma, V. R. (1967), *Proc. Roy. Soc., Ser. B 167*, 378.

Buckingham, A. D., Schaefer, J., and Schneider, W. G. (1960), *J. Chem. Phys. 32*, 1227.

Dahlquist, F. W., Jao, L., and Raftery, M. A. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 26.

Dahlquist, F. W., and Raftery, M. A. (1968), *Biochemistry 7*, 3277.

Dahlquist, F. W., and Raftery, M. A. (1969), *Biochemistry 8*, 713.

Dixon, M., and Webb, E. C. (1964), *Enzymes*, 116.

McDonald, C. C., and Phillipps, W. D. (1969), *Biochem. Biophys. Res. Commun. 35*, 43.

McDonald, C. C., Phillips, W. D., and Glickson, J. P. (1971), *J. Amer. Chem. Soc. 93*, 235.

Morallee, K. G., Nieboer, E., Rossotti, F. J. C., Williams, R. J. P., Xavier, A. V., and Dwek, R. A. (1970), *Chem. Commun.*, 1132.

Parsons, S. M., and Raftery, M. A. (1970), *Biochem. Biophys. Res. Commun. 41*, 45.

Pople, J. A., Schneider, W. G., and Bernstein, H. J. (1959), High Resolution Nuclear Magnetic Resonance, New York, N. Y., McGraw-Hill.

Raftery, M. A., Dahlquist, F. W., Chan, S. I., and Parsons, S. M. (1968), *J. Biol. Chem. 243*, 4175.

^{31}P and ^{13}C Nuclear Magnetic Resonance Studies of Nicotinamide–Adenine Dinucleotide and Related Compounds†

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ABSTRACT: The ^{31}P spectra of the reduced and oxidized forms of the following nucleotides have been obtained and analyzed: β-nicotinamide–adenine dinucleotide, nicotinamide–adenine dinucleotide phosphate, nicotinamide mononucleotide, and acetylpyridine–adenine dinucleotide. Also, spectra of α-nicotinamide–adenine dinucleotide, adenylic acid, and adenosine diphosphoribose were recorded. From the spectral differences between the reduced and oxidized nucleotides, as well as from the determination of the pK_a value of the phosphate group in the mononucleotides, it is postulated that in the oxidized nucleotides there is an electrostatic interaction between the positively charged nitrogen of the pyridine ring and a negatively charged oxygen of the diphosphate backbone. Using $BaCO_3$-^{13}C as a source of label, nicotinamide–adenine dinucleotide, enriched with ^{13}C in the carboxamide group, has been prepared *via* a series of chemical and enzymatic reactions. The ^{13}C nuclear magnetic resonance titration curve displays an apparent pK_a of 4, indicating that the chemical shift of the carboxamide carbon is influenced by protonation of the adenine ring of the dinucleotide.

Although proton magnetic resonance spectroscopy (pmr)[1] has now gained fairly wide use in the study of biologically important molecules, the use of other nuclei in such nmr studies has been rather limited. This has generally been because of the low sensitivity, in nmr experiments, of these other nuclei, their low natural abundance, or both. In certain cases the natural abundance problem can be overcome by enrich-

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[1] Abbreviations used are: pmr, proton magnetic resonance spectroscopy; nmr, nuclear magnetic resonance spectroscopy; NAD, β-nicotinamide–adenine dinucleotide; NADH, reduced β-nicotinamide–adenine dinucleotide; α-NAD, α-nicotinamide–adenine dinucleotide; NMN, nicotinamide mononucleotide; $NMNH_2$, reduced nicotinamide mononucleotide; APAD, acetylpyridine–adenine dinucleotide; APADH, reduced acetylpyridine–adenine dinucleotide; ADPR, adenosine-5′-diphosphoribose; AMP, adenylic acid; ATP, adenosine triphosphate; NADP, nicotinamide–adenine dinucleotide phosphate; NADPH, reduced nicotinamide–adenine dinucleotide phosphate.

ing the molecule to be studied in the desired isotope. Also, advances in instrumentation, such as the use of larger sample tubes, larger magnetic field strength, and Fourier transform techniques, are overcoming the sensitivity problems. There have been recent reports of ^{19}F nmr studies on enzymes to which a fluorine label has been covalently attached (Huestis and Raftery, 1971; Huestis and Raftery, 1972).

In this communication, preliminary studies on the coenzyme NAD and related compounds, using ^{31}P and ^{13}C nmr, are presented. NAD has been widely studied by pmr methods (Jardetzky and Wade-Jardetzky, 1966; Catterall *et al.*, 1969; Hollis, 1969; Sarma *et al.*, 1968a,b; Sarma and Kaplan, 1969a,b, 1970a,b; Sarma *et al.*, 1970; Griffith *et al.*, 1970) although the interpretation of experimental results has not met with unanimous agreement (Jacobus, 1971). There has been one previous report of the ^{31}P spectrum of NADH (Ho *et al.*, 1969). The use of ^{31}P and ^{13}C nmr (using compounds specifically enriched in ^{13}C) has the advantage over pmr in that it should be possible to observe coenzyme resonances when the coenzyme is bound to an enzyme, something which cannot be done in pmr experiments due to the large number of protons in the enzyme. The interaction of NAD and NADH with dehydrogenases has been studied by pmr under conditions where the enzyme was believed to have been exchanging rapidly between an enzyme-bound state and a free state (Jardetzky *et al.*, 1963; Hollis, 1967; Sarma and Kaplan, 1970c; Sarma and Woronick, 1971). Under these conditions, a single resonance which is the weighted average of the resonances due to each state is observed, and it should therefore be possible to gain information about the enzyme-bound coenzyme from this technique. The results of one such study (Sarma and Kaplan, 1970c), however, which state that NADH is in a folded form when bound to lactate dehydrogenase, is in direct disagreement with crystallographic data (Adams *et al.*, 1970a,b).

Preliminary to doing experiments concerned with enzyme–coenzyme interactions, ^{31}P and ^{13}C nmr studies of the coenzymes and related compounds free in solution have been undertaken. These studies have served two purposes. First, they have yielded new information concerning the conformation and intramolecular interactions of these compounds. In particular, new insight into the disposition of the diphosphate backbone has been acquired. Secondly, results of these studies will serve as a blank for future experiments with various enzymes.

Experimental Section

Materials. NAD, NADH, α-NAD, NMN, $NMNH_2$, NADP, NADPH, APAD, AMP, ADPR, yeast alcohol dehydrogenase, and snake venom phosphodiesterase were obtained from Sigma. Butyllithium was a product of Peninsular ChemResearch and 3-bromopyridine a product of Aldrich Chemical Co. Barium carbonate-^{13}C (65% enriched) and acetic acid-*1*-^{13}C (60% enriched) were purchased from Merck, Sharp and Dohme. Barium carbonate-^{13}C (95% enriched) was purchased from Bio-Rad.

Preparation of NAD-carbonyl-^{13}C and Related Compounds. Nicotinic acid-7-^{13}C was prepared by treating 3-bromopyridine with butyllithium, followed by carbonation with CO_2-^{13}C generated by addition of acid to $BaCO_3$-^{13}C (Murray *et al.*, 1948).

Nicotinamide-7-^{13}C was prepared from nicotinic acid-7-^{13}C *via* the ethyl ester according to the method of Laforge (1928).

NAD-*carbonyl*-^{13}C was prepared from nicotinamide-7-^{13}C and NAD through NADase-catalyzed exchange (Zatman *et al.*, 1953). The pig brain NADase was isolated according to the method of Zatman *et al.* (1954). The coenzyme was purified on a DEAE-cellulose column (Di Sabato, 1968) eluted with a 0.0015 M to 0.07 M ammonium bicarbonate gradient (pH 8.5). The enriched coenzyme was then desalted on a G-10 column. By using 65% enriched nicotinamide, and a twofold molar excess of nicotinamide to NAD, a coenzyme which was about 43% enriched was prepared. (This assumes equilibration in the exchange reaction. No attempt was made to determine the exact enrichment of NAD.)

A solution of NADH-^{13}C was prepared from NAD-^{13}C using yeast alcohol dehydrogenase and ethanol.

An equimolar mixture of NMN-^{13}C and 5′-AMP was prepared by adding snake venom phosphodiesterase to a solution of NAD-^{13}C. The reduced mononucleotide was prepared from NADH-^{13}C in the same manner.

N-Methylnicotinamide-7-^{13}C was prepared by mixing nicotinamide-7-^{13}C (50 mg) with a tenfold excess of methyl iodide in acetone (1 ml). The reaction mixture was allowed to stand at room temperature for four hours, and the *N*-methylnicotinamide crystals which had formed during this time were collected by filtration. The purity of the product was checked by integration of the pmr spectrum, the ratio of the peak area of the methyl protons (δ 4.6) to the pyridine protons (δ 8.0–9.4) being 3:4, as expected.

Methods. ^{31}P spectra were run on a modified HR 220 nmr spectrometer operating in frequency sweep mode at 89.1 Mc. A Varian 1024 Computer of Average Transients was used to enhance signal to noise ratios. Heteronuclear proton noise decoupling was applied by use of a Varian S68625 heteronuclear decoupler, a Hewlett-Packard 6266B power supply, and a Fluke 622A frequency synthesizer. Noise decoupling was applied in all cases except where its absence is specifically noted. In order to attain rapid temperature equilibration when using decoupling, a Varian 4540 temperature controller was used to keep the sample temperature at 24 ± 2°.

Most ^{31}P spectra were run on 0.1 M samples in 0.1 M phosphate or pyrophosphate buffer. Some of the samples contained 0.001 M EDTA (see Results). All chemical shifts are referred to an internal standard of approximately 1% triethyl phosphate. Shifts to lower frequency (upfield) are denoted by positive numbers, and downfield shifts by negative numbers. The phosphorus resonance position of triethyl phosphate is independent of pH and comes 0.4 ppm downfield of the resonance due to 85% phosphoric acid. Shifts are accurate to ±5 cps (0.06 ppm). When greater accuracy was required (such as in comparing APAD and NAD) both compounds were added to the same solution, and the spectrum was recorded.

The pH of all solutions was checked before and after the spectra were run using a Radiometer pH meter 26. NAD and NADH concentrations were checked by measuring the OD_{340} (after alcohol dehydrogenase reduction in the case of NAD).

^{13}C spectra were obtained on a Varian XL-100-15D spectrometer operating at 25.1 Mc. A Fabritek 1062 computer was used to enhance signal strength and noise decoupling was employed in all experiments. Samples were contained in 12-mm tubes. About 2 ml of 1.5×10^{-2} M solutions was used. This corresponds to a ^{13}C concentration of about 6×10^{-3} M for NAD, NADH, NMN, and $NMNH_2$, and about 1.4×10^{-2} M for nicotinamide and *N*-methylnicotinamide (where 95% enriched materials were used). By using a 50-cps sweep width and a 25 sec sweep time adequate spectra could be obtained in about 15 min. A capillary containing acetic acid-*1*-^{13}C

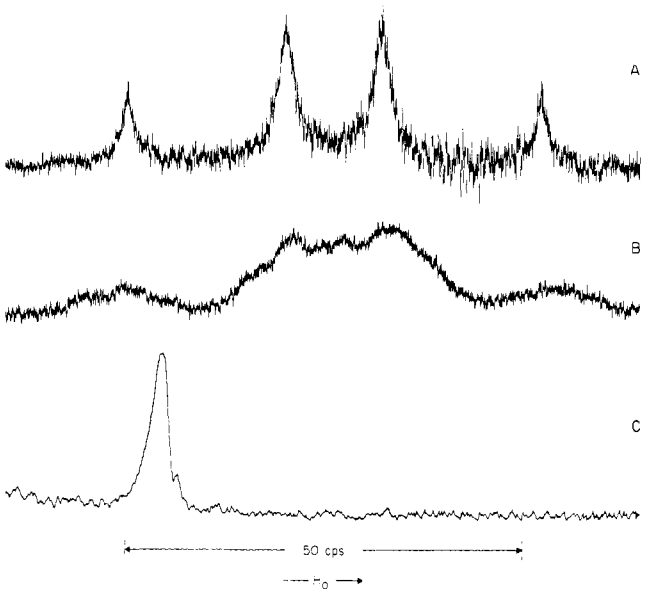

FIGURE 1: (A) The ^{31}P spectrum of 0.5 M NAD, pH 8.2, proton decoupled; (B) the undecoupled spectrum of the same solution; (C) the spectrum of 0.1 M NADH, pH 8.2, proton decoupled.

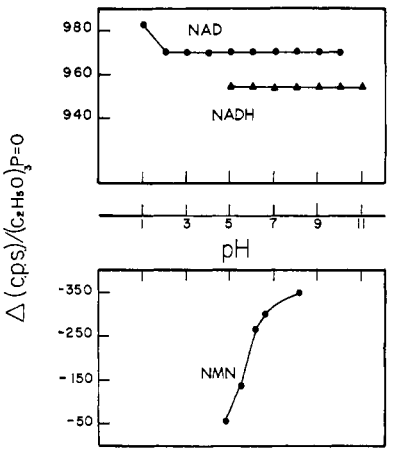

FIGURE 2: Upper, ^{31}P nmr titration data for NAD and NADH. Shifts are in cps relative to $(C_2H_5O)_3P=O$. Lower; ^{31}P titration data for NMN.

(60% enriched) was used as a standard. Shifts are accurate to ±1 cps (0.04 ppm).

Potentiometric titrations were done using a Radiometer pH meter 26.

Results

^{31}P Studies. The ^{31}P spectrum (undecoupled and decoupled) of NAD is shown in Figure 1. The broadness in the undecoupled spectrum is due to incompletely resolved coupling between the phosphorus atoms and the 5′-methylene protons of each ribose ring. Even using a concentrated solution (0.5 M) and a slow sweep rate, this coupling could not be easily resolved, so all other spectra were taken using noise decoupling. From the decoupled spectrum, it can be seen that the ^{31}P spectrum of NAD is composed of an AB pattern, with δ_{AB} 25.7 ± 0.5 cps and J = 20.0 ± 0.5 cps. The value for the coupling constant is consistent with the value of 19.9 ± 0.5 found for ATP (Cohn and Hughes, 1960). The ^{31}P spectrum of NADH consists of one singlet, which occurs 22 cps downfield of the center of the AB pattern seen with NAD.

A study of the pH dependence of the chemical shifts of NAD and NADH was done, and it can be seen (Figure 2) that with the exception of a small upfield shift with NAD at pH 1, the shifts were invariant with pH. No spectra were taken on NADH below pH 5 due to the rapid decomposition of the coenzyme in acidic solution. This decomposition leads to the appearance of new peaks in proton spectra (Griffith *et al.*, 1970), so it was assumed that any such data from phosphorus nmr would be suspect.

It should be noted that with both NAD and NADH the peaks started to broaden as the pH was lowered. The broadening was less marked in pyrophosphate than in phosphate and was almost completely eliminated by adding 10^{-3} M EDTA. The broadening was probably due to contamination by paramagnetic ions and was partially overcome in pyrophosphate due to binding of the ion to the buffer salt. Although the broadening reached 50 cps in some cases, as opposed to the normal width of about 2 cps, within experimental error, there was no change in chemical shifts when EDTA was added. Paramagnetic ion contamination is a problem when conducting ^{31}P nmr on compounds such as NAD, since its pyrophosphate group will strongly bind divalent cations. No large paramagnetic ion contamination effects have been reported in pmr experiments on NAD, nor were they observed in the ^{13}C spectra described in this paper.

The ^{31}P chemical shifts of NAD and several related compounds are listed in Table I. It can be seen that in all cases, reduced nucleotides have resonances which come to lower field than the resonances in the corresponding oxidized nucleotides.

^{13}C Studies of Pyridine Nucleotides. The chemical shifts of the carbonyl ^{13}C in NAD, NADH, NMN, $NMNH_2$, nicotinamide, and *N*-methylnicotinamide are shown in Figure 3. It is seen that the titration curves for NAD and nicotinamide

TABLE I: ^{31}P Chemical Shifts of NAD and Related Nucleotides.[a]

Nucleotide	Δ_P	Δ_A	δ_{PA}
NAD	983	957	26
NADH	948	948	0
NADP	983	957	26
NADPH	948	948	0
APAD	988	957	31
APADH	948	948	0
α-NAD	967	957	10
NMN	−342		
$NMNH_2$	−371		
AMP		−371	
ADPR	950	926	24

[a] All spectra taken in 0.1 M pyrophosphate–0.001 M EDTA buffer, pH 8.3. Nucleotide concentrations are 0.1 M in all cases. Shifts are in cps at 89.1 Mc and are referred to a 1% $(C_2H_5O)_3P=O$ internal standard. Positive numbers signify upfield shifts. Δ_P is the shift of the phosphorus atom in the pyridine half of the nucleotide, Δ_A the shift of the phosphorus in the adenine half, and δ_{PA} the chemical shift difference between the two phosphorus atoms.

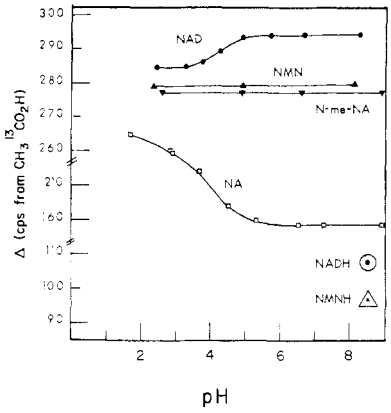

FIGURE 3: ^{13}C nmr titration data for the carbonyl carbon of NAD, NADH, NMN, $NMNH_2$, nicotinamide (NA), and *N*-methylnicotinamide (*N*-Me-NA). Shifts are in cps relative to a capillary of enriched CH_3CO_2H-^{13}C.

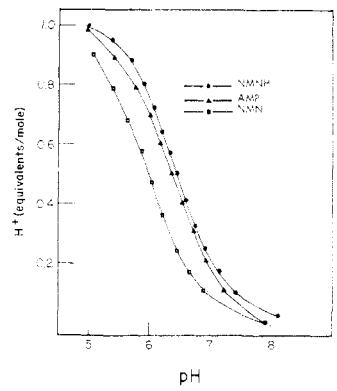

FIGURE 4: Potentiometric titration of NMN, $NMNH_2$, and AMP. Titrations performed on 0.01 M solutions at 25°.

show an apparent pK_a of about 4, while the shifts for NMN and *N*-methylnicotinamide are independent of pH. No pH studies of NADH and $NMNH_2$ were done, due to the instability of these materials at low pH.

Potentiometric Titration Studies. Potentiometric titrations were done in order to determine the pK_a of the phosphate group in NMN, $NMNH_2$, and AMP (Figure 4). It can be seen that the pK_a values determined for $NMNH_2$ and AMP are about 6.4, while that of NMN is 6.0.

Discussion

^{31}P Studies of Pyridine Nucleotides. The invariance of the NAD chemical shift with changing pH (Figure 2) unequivocally proves that the p*K* of 3.88 which NAD possesses (Moore and Underwood, 1969) is not due to a phosphate. This p*K* has generally been ascribed to the adenine-*N*-1, although it has been speculated that it could belong to a phosphate (Moore and Underwood, 1969). The titration curve for NMN (Figure 2) shows that when there is a change in ionization state of a phosphate group, it is readily observed by ^{31}P nmr.

The difference in ^{31}P spectra between NAD and NADH is somewhat surprising in view of the fact that the compounds are believed to have the same overall conformation in solution (Sarma and Kaplan, 1969a), that is, both are believed to have the adenine and pyridine rings stacked in parallel planes, with the adenine in the anti conformation, and the pyridine syn. One possible explanation for the difference is that the conformation of the ribose adjacent to the nicotinamide ring, which is reported to be very different for NAD and NADH (Sarma and Kaplan, 1970a) exerts a major influence on the ^{31}P chemical shift. This view is unlikely, however, for several reasons. First, studies with hexose phosphates (G. Gray, personal communication) have shown the ^{31}P chemical shifts to be virtually independent of the identity and conformation of the sugar. Secondly, in the present study, no correlation can be drawn between ribose conformation and ^{31}P shift. Thus the ribose rings of NMN and AMP are reported to have the same conformation (Sarma and Kaplan, 1970a), yet these compounds have different ^{31}P chemical shifts (Table I). Furthermore the two riboses of NADH (also APADH and NADPH) have two different conformations (Sarma and Kaplan, 1970a), yet the ^{31}P spectrum is a singlet.

A more likely explanation for the difference in shifts between reduced and oxidized nucleotides is that the latter contain a charged nitrogen in the pyridine ring and that there is an electrostatic interaction between this nitrogen and the negatively charged phosphate which causes a change in the ^{31}P shift. One would expect such an interaction to cause an upfield shift since an upfield shift is observed on protonation of a phosphate group, as is seen from the ^{31}P titration curve of NMN (Figure 2). This upfield shift is indeed observed in going from NADH to NAD, APADH to APAD, NADPH to NADP, and $NMNH_2$ to NMN. In the dinucleotides, all of which display a singlet when in reduced form, one resonance is moved about 25 cps further upfield than the other resonance when the compound is in the oxidized form. It is believed that the resonance to higher field is due to the phosphorus which is in the nicotinamide half of the dinucleotide, since examination of space-filling models shows that this phosphorus could more easily interact with the nitrogen of the nicotinamide ring. This phosphorus undergoes a total upfield shift of about 35 cps upon oxidation, while the phosphorus which is closer to the adenine ring undergoes an upfield shift of about 10 cps. Since the upfield shift in going from $NMNH_2$ to NMN is only 29 cps one would probably expect the same upfield shift to occur for the nicotinamide phosphorus of the dinucleotide, while no shift would be expected for the adenine phosphorus. A possible explanation for the additional observed shifts of about 10 cps in the dinucleotides is that interaction of the charged nitrogen with one phosphate affects the phosphate–phosphate interaction and thereby introduces a further shift of both phosphorus atoms.

Further evidence for the electrostatic interaction was obtained by determining the pK_a for the phosphate group in NMN, $NMNH_2$, and AMP. Both AMP and $NMNH_2$ have an uncharged nitrogen at their glycosidic linkage, whereas the nitrogen at this position in NMN is charged. It is seen (Figure 4) that the phosphates in AMP and $NMNH_2$ possess a pK_a value of about 6.4, while that of NMN has a pK_a of 6.0. The presence of a positive charge interacting with the phosphate of NMN would cause this lowering of its p*K*. Since in addition to possessing nearly identical pK_a values $NMNH_2$ and AMP possess nearly identical ^{31}P chemical shifts (Table I) and NMN has both a different pK_a and different chemical shift it would indeed appear that the chemical shift change is most likely caused by the positively charged nitrogen in NMN.

Rather surprisingly, the value for δ_{AB} in the AB pattern of the NAD spectrum was virtually unchanged when the nucleotide was dissolved in 4 M NaCl, a medium which would be expected to greatly lessen any electrostatic interactions. It is possible, however, that the various intramolecular interac-

tions of the NAD molecule (the base stacking as well as the nicotinamide–phosphate interaction) do not allow for the NaCl molecules to penetrate into the region where the electrostatic interaction occurs. Although δ_{AB} was unchanged in high salt, J_{AB} decreased from 20.0 ± 0.5 cps to 18 ± 0.5 cps, and the chemical shift relative to $(C_2H_5O)_3P{=}O$ decreased from 970 to 905 cps. Very similar changes in coupling constant and chemical shift were observed in the ^{31}P spectrum of ATP in 4 M NaCl, so it is likely that the effects seen with NAD are of a general nature, and could be observed with most compounds which possess charged phosphate groups. It should also be mentioned that the shift of $(C_2H_5O)_3P{=}O$ relative to a capillary containing phosphoric acid changed in high salt, the shift of the $(C_2H_5O)_3P{=}O$ being 30 cps to higher field in 4 M NaCl than it was in pure H_2O. It can thus be computed that the NAD resonance actually moved 35 cps downfield in 4 M NaCl, rather than the 65 cps deduced from the fact that the shift of NAD relative to $(C_2H_5O)_3P{=}O$ changed from 970 to 905 cps.

It is possible that the electrostatic interaction between the nicotinamide nitrogen and the phosphate group is not direct, but is rather mediated by a water molecule. An attempt to test this hypothesis was made by comparing spectra of NAD in H_2O and in D_2O. The spectra proved to be identical within experimental error, but since even if there were an intervening water the difference between spectra run in H_2O and D_2O might be very small, the hypothesis cannot be excluded.

It should be noted that the idea of an electrostatic interaction between a phosphate and the nicotinamide ring nitrogen is not inconsistent with the model whereby the adenine and nicotinamide rings are in close proximity in parallel planes (Jardetzky and Wade-Jardetzky, 1966; Catterall *et al.*, 1969; Hollis, 1969; Sarma *et al.*, 1968a,b, 1970; Sarma and Kaplan, 1969a,b, 1970a,b). Examination of space-filling models reveals that both interactions may occur simultaneously. By the same token the molecule may unfold without affecting the nicotinamide–phosphate interaction. Protonation of the adenine ring below pH 4 would not be expected to produce an additional electrostatic effect because the adenine-*N*-1 is not sterically suitable for such an interaction, hence no change in the ^{31}P spectrum is expected due to this ionization.

The AB pattern of NAD is unaffected by changing the dinucleotide concentration from 1×10^{-2} to 5×10^{-1}, demonstrating that it is unlikely that intermolecular interactions play any part in the nonequivalence of the phosphates.

It is also of interest to compare the ^{31}P spectrum of NAD with that of related oxidized dinucleotides (Table I). The spectrum of NAD is identical with that of NADP, but in APAD the phosphorus closer to the pyridine moiety is 6 cps upfield of the corresponding phosphorus in NAD, while the phosphorus proximal to the adenine moiety retains the same position in NAD and APAD. Since it has been stated (Sarma and Kaplan, 1969a) that in both NAD and NADP the nicotinamide ring is syn to the attached ribose, while in APAD the relationship between these entities is anti, it is possible that there is a slight difference in phosphate–pyridine ring interactions in APAD from that present in NAD and NADP. Another possibility is that the difference in electron-withdrawing effect between the acetyl side chain in APAD and the carboxamide side chain in NAD alters the charge density at the pyridine nitrogen, thereby affecting the ^{31}P shift. It should be noted that the corresponding reduced dinucleotides (NADH and APADH) display identical spectra.

The ^{31}P spectrum of α-NAD is an AB pattern but δ_{AB} is only 10 ± 1 cps. The peak due to the adenine phosphorus is at the same position as it is in β-NAD but the resonance of the nicotinamide phosphorus is moved downfield by 15 cps. This smaller shift relative to NADH is expected in α-NAD since the nicotinamide ring is above the ribose while the phosphate is below it and the nicotinamide–phosphate interaction, while not eliminated, is lessened.

The ^{31}P spectrum of ADPR is also an AB pattern. This compound does not possess a positively charged nitrogen but the absence of the nicotinamide ring gives ADPR more rotational freedom than is possessed by NAD, and in addition, the molecule has a free anomeric center. It is therefore not surprising that the ^{31}P spectrum of ADPR is an AB pattern and is somewhat shifted from the positions of both NAD and NADH.

^{13}C Studies of Pyridine Nucleotides. The resonance of the carbonyl-^{13}C in NAD is about 7 ppm upfield of the corresponding resonance in NADH. This difference is not surprising in light of the aromatic structure and positively charged nitrogen present in the pyridine ring of NAD while the corresponding nitrogen in NADH is uncharged and the dihydropyridine ring is not aromatic. The effect of the charged nitrogen is seen by examining the titration curve of nicotinamide (Figure 3). At low pH, when the ring nitrogen is charged, the carbonyl chemical shift is within 1 ppm of the NAD resonance. At pH 5 or above, when the nitrogen of nicotinamide is uncharged, the carbonyl chemical shift moves downfield by about 4 ppm and is then much closer to the position of the NADH resonance.

The titration curve of NAD (Figure 3) shows a pK_a of about 4, with a total chemical shift difference of 10 cps to higher field in going from lower to higher pH. These data are in agreement with pmr titration data obtained by Hollis (1969) and Jardetzky and Wade-Jardetzky (1966). At low pH, the chemical shift in NAD is within 5 cps of the shift in NMN. This would be expected if NAD were unfolded at low pH, since the adenine ring would exert no influence on the chemical shift of the nicotinamide carbonyl, and the environment of the carbonyl would be very similar in NMN and NAD. If the molecule were to fold in such a way that the carbonyl was directly over the adenine ring, an upfield shift would be observed, since the adenine ring current would exert a shielding effect on the carbonyl carbon.

It is possible that NAD stays folded at low pH, and at this pH the shielding effect of the adenine ring experienced by the nicotinamide carbonyl is counteracted by a deshielding effect of the positive charge which adenine acquires below pH 4. Thus, two opposite affects could almost cancel and cause the shift in NAD to be very similar to that in NMN.

At high pH, the chemical shift of NADH is about 15 cps to higher field than that of $NMNH_2$. This difference would also be expected to lessen at lower pH, for the same reasons as noted above with NAD. As previously mentioned, however, the reduced nucleotides are unstable at low pH, so it was impossible to check this hypothesis.

It should be noted that the carbonyl shift in NMN is not affected by the ionization of the phosphate (Figure 3). This constancy of chemical shift with changing pH is perhaps unexpected in light of the conclusion reached previously in this paper that the phosphate of NMN interacts with the positively charged nitrogen of the pyridine ring. It has been found from pmr studies (Sarma and Kaplan, 1969a), however, that although the phosphate ionization in NMN affects the chemical shifts of the C-2 and C-6 protons, the C-4 and C-5 protons are unaffected. The lack of an effect on the carbonyl carbon, which is attached to the C-3 carbon on the pyridine ring, is therefore not totally unexpected.

References

Adams, M. J., Ford, G. C., Kockoek, R., Lentz, P. J., McPherson, A., Rossman, M. G., Smiley, I. E., Schevitz, R. W., and Wonacott, A. J. (1970a), *Nature (London) 227*, 1098.

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

Catterall, W. A., Hollis, D. P., and Walter, C. F. (1969), *Biochemistry 8*, 4032.

Cohn, M., and Hughes, T. R. (1960), *J. Biol. Chem. 235*, 3250.

DiSabato, G. (1968), *Biochim. Biophys. Acta 167*, 646.

Griffith, P. R., Rye, M. J., and Alexander, J. G. (1970), *Can. J. Biochem. 48*, 947.

Ho, C., Magnuson, J. A., Wilson, J. B., Magnuson, N. S., and Kurland, R. J. (1969), *Biochemistry 8*, 2074.

Hollis, D. P. (1967), *Biochemistry 6*, 2080.

Hollis, D. P. (1969), *Org. Magn. Res. 1*, 305.

Huestis, W. H., and Raftery, M. A. (1971), *Biochemistry 10*, 1181.

Huestis, W. H., and Raftery, M. A. (1972), *Biochemistry 11*, 1648.

Jacobus, J. (1971), *Biochemistry 10*, 161.

Jardetzky, O., Wade, N. G., and Fischer, J. J. (1963), *Nature (London) 197*, 183.

Jardetzky, O., and Wade-Jardetzky, N. G. (1966), *J. Biol. Chem. 241*, 85.

Laforge, F. B. (1928), *J. Amer. Chem. Soc. 50*, 2477.

Moore, C. E., and Underwood, A. L. (1969), *Anal. Biochem. 29*, 149.

Murray, A., Foreman, W. W., and Langham, W. (1948), *J. Amer. Chem. Soc. 70*, 1037.

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

Sarma, R. H., and Kaplan, N. O. (1969a), *Biochem. Biophys. Res. Commun. 36*, 780.

Sarma, R. H., and Kaplan, N. O. (1969b), *J. Biol. Chem. 244*, 771.

Sarma, R. H., and Kaplan, N. O. (1970a), *Biochemistry 9*, 539.

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

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

Sarma, R. H., Moore, M., and Kaplan, N. O. (1970), *Biochemistry 9*, 549.

Sarma, R. H., Ross, V., and Kaplan, N. O. (1968b), *Biochemistry 7*, 3052.

Sarma, R. H., and Woronick, C. L. (1971), *Res. Commun. Chem. Pathol. Pharmacol. 2*, 177.

Zatman, L. J., Kaplan, N. O., and Colowick, S. P. (1953), *J. Biol. Chem. 200*, 197.

Zatman, L. J., Kaplan, N. O., Colowick, S. P., and Ciotti, M. M. (1954), *J. Biol. Chem. 209*, 467.

A Study of Cooperative Interactions in Hemoglobin Using Fluorine Nuclear Magnetic Resonance†

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ABSTRACT: The interaction of hemoglobin with heme ligands and allosteric effectors has been studied by fluorine nuclear magnetic resonance spectroscopy. For this purpose hemoglobin has been labeled covalently with 3,3,3-trifluoroacetonyl groups on the β-chain cysteine residues at position 93. Oxidation of the heme iron, as well as binding heme ligands, protons, and diphosphoglycerate to the labeled hemoglobin, produces significant changes in the ^{19}F nuclear magnetic resonance spectrum of the label. Interpreted in light of crystal structure data and the chemical properties of hemoglobin, these changes yield information on tertiary structural changes accompanying allosteric interactions.

The allosteric properties of hemoglobin have long been assumed to result from conformation changes in the protein which occur on oxygenation. Structural differences between liganded and unliganded hemoglobin are suggested by an extensive array of evidence from crystal forms (Haurowitz, 1938), optical rotation (Briehl, 1962), carboxypepitdase digestion rates (Zito *et al.*, 1964), stability and solubility changes (Cohn and Edsall, 1943), dye binding (Antonini *et al.*, 1963), and rates of reaction with thiol-specific reagents (Riggs, 1961). The work of Perutz and coworkers on the crystal structures of oxy- (Perutz *et al.*, 1968) and deoxyhemoglobin (Muirhead and Greer, 1970) has elucidated differences between the two forms and suggested mechanisms by which the conversion from one to the other could occur in a cooperative manner. From these crystal structures, Perutz (1970b) has further proposed mechanisms by which allosteric effectors (protons and orgainc phosphates) could influence the properties of hemoglobin.

The objective of this investigation was to examine the allosteric interactions of hemoglobin on a molecular level by studying the ^{19}F nuclear magnetic resonance (nmr) spectrum of a small fluorinated probe molecule strategically attached to the protein. The probe used was a 3,3,3-trifluoroacetonyl group placed on the side chain of cysteine-β93, at which position

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