The ^{13}C and ^{1}H Nuclear Magnetic Resonance Spectra and Methods of their Assignment for Nucleotides Related to Dihydronicotinamide Adenine Dinucleotide Phosphate (NADPH) †

By **Berry Birdsall** and **J. Feeney,*** M.R.C. Molecular Pharmacology Unit, Medical School, Hills Road, Cambridge CB2 2QD

The ^{13}C n.m.r. spectra at 25·2 MHz of ribose-5-phosphate, AMP, NMN^+, NAD^+, NADH, $NADP^+$, and NADPH have been assigned by use of a combination of techniques. In addition to established methods, such as comparisons of chemical shifts and coupling constants with those in model compounds, ionisation studies, and $\{^1H\}$–^{13}C heteronuclear selective decoupling experiments, we have described some novel methods of ^{13}C spectral assignments, namely (i) addition of Eu^{3+} ions to induce pseudo-contact shifts in the proton spectrum to facilitate $\{^1H\}$–^{13}C decoupling experiments; (ii) the use of a graphical method of presenting results of off-resonance selective ^1H irradiation experiments; (iii) paramagnetic shift predictions for ^{13}C nuclei in non-rigid molecules in the presence of Eu^{3+} ions. Difficult proton assignments can sometimes be made on the basis of connecting assigned ^{13}C signals with their corresponding proton resonance signals by heteronuclear decoupling.

NUCLEAR magnetic resonance spectroscopy has proved to be an ideal technique for studying the interactions between enzymes and their substrates, inhibitors, and coenzymes in solution. In favourable cases, by studying the changes in chemical shifts and relaxation times in small molecules when they bind to the enzyme, it is possible to obtain information concerning the manner in which a small molecule binds and to comment on any conformational changes which occur on binding. Dihydronicotinamide adenine dinucleotide phosphate (NADPH) is an important coenzyme in several oxido reductase enzymic reactions; in particular we are interested in the complex formed between dihydrofolate reductase and NADPH as part of the enzymic reduction of dihydrofolic acid to tetrahydrofolic acid. As a basis for such a study it was necessary to obtain an unequivocal assignment of the ^1H and ^{13}C n.m.r. spectra of NADPH and related compounds.

Dorman and Roberts [1] have made an extensive study of the ^{13}C spectra of common nucleotides, basing their assignments largely on chemical shift comparisons between related compounds. Grant and his coworkers [2-4] have similarly studied the ^{13}C spectra of many nucleosides. Both groups of workers have indicated the usefulness of ionisation studies in helping to assign carbon resonances. Although some assignments were supported by heteronuclear $\{^1H\}$–^{13}C off-resonance decoupling experiments this technique was not fully exploited for the ribose fragments of the molecules.

Because it is often possible to connect proton resonance signals with their corresponding carbon-13 signals either by heteronuclear double resonance or by ionisation studies the problem of spectral assignments for both nuclei is considerably simplified: thus not only can carbon-13 signals be assigned in this way but also overlapped proton signals can be assigned on the basis of their connected assigned carbon-13 signals.

† Abbreviations: NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate (I); NMN^+, nicotinamide mononucleotide; NADH, dihydronicotinamide adenine dinucleotide; NADPH, dihydronicotinamide adenine dinucleotide phosphate; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulphonate. Ribose carbon atoms and protons are indicated with primes, *e.g.* N1′; carbon atoms and protons on the nicotinamide side of the molecule (or in NMN^+ or nicotinamide) are indicated by N before the carbon number, *e.g.* N6. Similarly the adenine side of the molecule is denoted by A.

EXPERIMENTAL

The ^1H and ^{13}C n.m.r. spectra were recorded on Varian HR220, HA100D, and XL100 spectrometers. Heteronuclear $\{^1H\}$–^{13}C double-resonance experiments were carried out using the Gyrocode of the XL100. The ^{13}C spectra were accumulated by the Fourier Transform technique, usually under conditions of complete proton decoupling except when selective proton irradiation experiments were conducted. *N*-Methylnicotinamide was prepared by methylation of nicotinamide with methyl iodide in ethyl methyl ketone and reduced with sodium dithionite by the methods of Karrer and his co-workers.[5] All other compounds were obtained from Sigma Chemical Company and were used without further purification. Aqueous solutions in deuterium oxide were examined and the deuterium in the solvent was used for field frequency locking the XL100 spectrometer.

RESULTS

We have studied the ^{13}C n.m.r. spectra at 25·2 MHz of ribose 5-phosphate, AMP, NMN^+, NAD^+, $NADP^+$ (I), NADH, and NADPH. The ^{13}C spectra shown in Figures 1 and 2 were recorded under conditions of proton noise decoupling: thus each carbon nucleus in the molecule appears as a single resonance except for those coupled to phosphorus nuclei, which appear as doublets.

Figures 1 and 2 and Tables 1—3 summarise the ^{13}C assignments made for the compounds studied.

Before a detailed spectral assignment is undertaken, general conclusions about the various carbon assignments can be made simply on the basis of chemical shift, relaxation, and coupling constant information. In ^{13}C spectra where the protons are undecoupled or partially decoupled, the multiplicity on the ^{13}C absorption arising from directly

[1] D. E. Dorman and J. D. Roberts, *Proc. Nat. Acad. Sci. U.S.A.*, 1970, **65**, 19.
[2] A. J. Jones, M. W. Winkley, D. M. Grant, and R. K. Robins, *Proc. Nat. Acad. Sci. U.S.A.*, 1970, **65**, 27.
[3] A. J. Jones, D. M. Grant, M. W. Winkley, and R. K. Robins, *J. Phys. Chem.*, 1970, **74**, 2684.
[4] R. J. Pugmire and D. M. Grant, *J. Amer. Chem. Soc.*, 1968, **90**, 697, 4232; R. J. Pugmire and D. M. Grant, *ibid.*, 1971, **93**, 1880.
[5] P. Karrer, G. Schwarzenbach, F. Benz, and V. Solmssen, *Helv. Chim. Acta*, 1936, **19**, 811.

bonded protons indicates which carbons correspond to CH_3, CH_2, and CH groups. Quaternary carbon nuclei

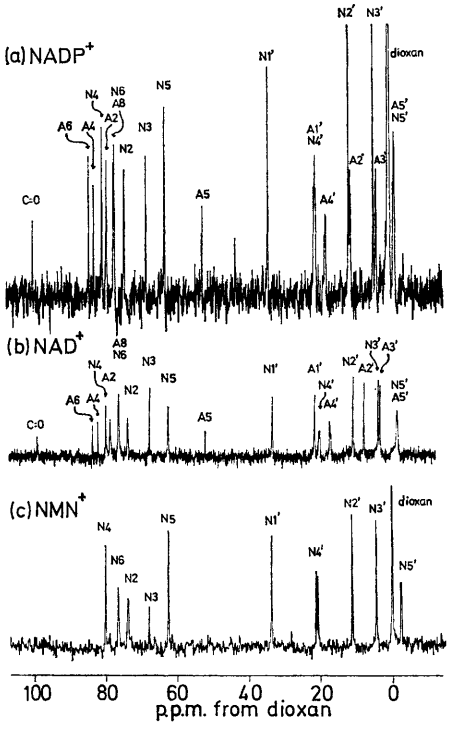


FIGURE 1 The proton noise decoupled ^{13}C resonance spectra at 25·2 MHz of (a) $NADP^+$, 0·10M, pH 2·0 (8069 transients, 75 μs pulse width, AT = 0·8 s, PD = 3 s); (b) NAD^+, 0·15M, pH 1·0 (3747 transients, 120 μs pulse width, AT = 1·3 s, PD = 2·7 s), and (c) NMN^+, 0·12M, pH 2·8 (15,000 transients, 100 μs pulse width, AT = 0·8 s, PD = 1·7 s). Repetition rate = Acquisition time (AT) + Pulse delay (PD)

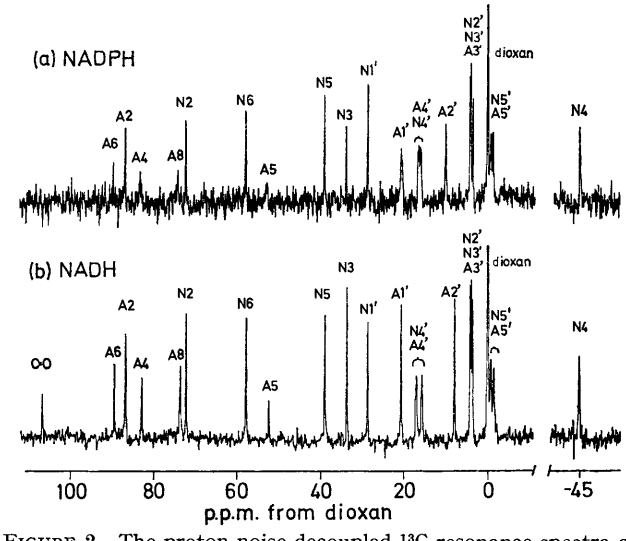
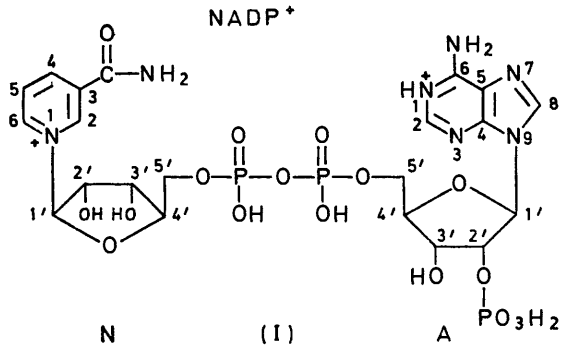


FIGURE 2 The proton noise decoupled ^{13}C resonance spectra at 25·2 MHz of (a) NADPH, 0·07M, pH 9·2 (6064 transients, 130 μs pulse width, AT = 0·8 s, PD = 5·2 s) and (b) NADH, 0·09M, pH 9·8 (2000 transients, 120 μs pulse width, AT = 0·8 s, PD = 5·2 s)

show no multiplicity and are also characterized by long relaxation times because of the absence of an efficient dipolar relaxation mechanism. These latter effects are conspicuous in Fourier Transform spectra because such carbon nuclei appear with much smaller intensities than others if the repetition rate of the pulses is too great to allow the nuclei to relax between pulses. Similar effects are observed for carbonyl carbon nuclei.



A further aid to ^{13}C spectral assignments can be provided by the presence of heteronuclear coupling such as that between ^{13}C and ^{31}P nuclei. In a system C–C–C–OPO_3, coupling is observed between the phosphorus and the α- and β-carbon nuclei but not to the γ-carbon nucleus. In general the coupling to the β-carbon nucleus is larger than to the α-carbon nucleus.[6] Thus in ribose 5-phosphate, $J_{P,5'} = 5$ and $J_{P,4'} = 9$ Hz, but because of the limited number of results for such systems it is dangerous to use this as an unequivocal method of ^{13}C spectral assignment. In $NADP^+$ there are phosphate groups attached to three carbon nuclei: N5′, A5′, and A2′. Therefore the signals of the carbon nuclei from the nicotinamide ribose ring (Figure 3) resemble those of ribose-5-phosphate. The N4′

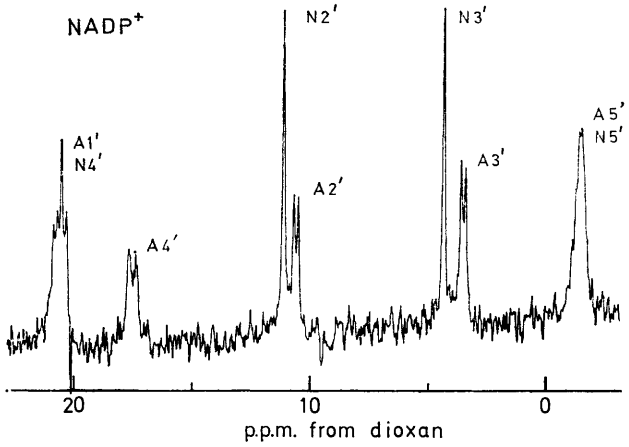


FIGURE 3 The ribose portion of the proton noise decoupled ^{13}C resonance spectrum at 25·2 MHz of $NADP^+$ showing the ^{13}C—^{31}P coupling (50,000 transients, 75 μs pulse width, AT = 1·0 s, PD = 0)

and N5′ carbon signals are coupled to phosphorus and the N2′ and N3′ signals appear as singlets. The ^{31}P nucleus of the phosphate group on carbon A2′ of the adenine ribose ring is coupled to carbon atoms A2′, A1′, and A3′. Thus all the signals from the adenine ribose carbon atoms appear as multiplets. In this compound, however, the ^{31}P coupling to the α-carbon atom A2′ is the same (5 Hz) as to the β-carbon atom, A3′.

[6] N. J. M. Birdsall, J. Feeney, A. G. Lee, Y. K. Levine, and J. C. Metcalfe, *J.C.S. Perkin II*, 1972, 1441.

TABLE 1

The ^{13}C chemical shifts (p.p.m.) for NAD^+ (pH 1·0), $NADP^+$, AMP (pH 1·3), NMN^+ (pH 2·8), and ribose 5-phosphate measured from a dioxan internal reference (positive shifts to low field)

	NAD^+, pH 1·0, 0·15M	$NADP^+$, pH 2·0, 0·10M	AMP, pH 1·3, 0·1M	NMN^+, pH 2·8, 0·12M	Ribose 5-phosphate, pH 1·0, 0·9M	
C=O	99·01	98·77				
A6	83·33	83·10	83·41 [a]			
A4	81·83	81·71	81·87 [a]			
N4	79·60 [b]	79·44		79·29		
A2	78·25 [b]	78·14	78·17 [b]			
A8	76·07 [b]	76·15	76·07 [b]			
N6	76·07 [b]	75·99		75·83		
N2	73·49 [b]	73·29		73·29		
N3	67·46 [b]	67·22		67·30		
N5	62·26 [b]	62·06		61·90		
A5	51·94 [c]	51·63	52·10 [c]			
N1′	33·45	33·37		33·37 [b]	β1′	34·60 [b]
A1′	21·71	20·44	21·83 [b]		α1′	29·72 [b]
N4′	20·44 [c]	20·44		20·87 [b,c]	α4′	15·63
A4′	17·42 [c]	17·46	17·46 [c]		β4′	14·56
N2′	11·11	11·07		11·15 [b]	β2′	8·57 [b]
A2′	8·10	10·56	7·94 [b,d]		α2′	4·09 [b]
N3′	4·17	4·29		4·44 [b]	β3′	3·85 [b]
A3′	3·69	3·57	3·65 [b,d]		α3′	3·45 [b]
N5′	−1·07 [c]	−1·31		−2·38 [c]	β5′	−0·40
A5′	−1·07 [c]	−1·31	−1·75 [c]		α5′	−1·12

Methods used for assignments: [a] Ionisation studies. [b] ^1H—^{13}C selective decoupling. [c] Multiplicity and relaxation effects. [d] Lanthanide shifts.

All other assignments made by comparison with model compounds.

TABLE 2

The ^{13}C chemical shifts (p.p.m.) for NADPH, NADH, 1,4-dihydro-*N*-methylnicotinamide, NAD^+ (pH 7·05), AMP (pH 7·4), and NMN^+ (pH 8·4) measured from a dioxan internal reference (positive shifts to low field)

	NADPH, pH 9·2, 0·07M	NADH, pH 9·8, 0·09M	1,4-Dihydro-*N*-methylnicotinamide, pH 10·3	NAD^+, pH 7·05, 0·3M	AMP, pH 7·4, 0·1M	NMN^+, pH 8·4, 0·11M
NC=O		105·79	106·07	98·45		
A6	88·89	88·61		88·41	88·73 [a]	
A2	86·03	85·95 [b]		86·07 [b]	85·79 [b]	
A4	82·46	82·06		82·06	81·67 [a]	
A8	73·41	72·90 [b]		73·29 [b]	72·33 [b]	
N2	71·59	71·55 [b]	74·96 [b]	73·10 [b]		73·10
N6	57·30	57·26 [b]	62·82 [b]	75·83 [b]		76·59
A5	52·38	51·87		51·55	51·39 [a†,c]	
N5	38·65	38·61 [b]	37·27	62·14 [b]		61·90
N3	33·49	34·64	29·72	67·02		
N1′	28·37	28·45		33·45		33·85
A1′	20·32	20·95 [b]		20·48	20·79 [b]	
N4′	16·19	15·67		20·48		21·67
A4′	15·95	16·90		17·10	17·62 [c]	
A2′	9·88	7·94		7·54	8·17 [b]	
N2′	4·17	4·21		11·07		11·15
N3′	3·97	3·89		4·01		4·88
A3′	3·49	3·61		3·81	3·93 [b]	
A5′	−0·83	−0·75		−1·11	−2·78 [c]	
N5′	−1·39	−1·39		−1·63		−3·45
N4	−44·60	−44·60	−44·60 [c]	+79·29 [b]		+79·68
NCH_3			−26·31 [c]			

Methods used for assignments: [a] Ionisation studies. [b] ^1H—^{13}C selective decoupling. [c] Multiplicity and relaxation effects. All other assignments made by comparison with model compounds. † Refs. 1 and 4.

TABLE 3

The ^{13}C chemical shifts (p.p.m.) for $NADP^+$, nicotinamide (pH 6·78), nicotinamide (pH 0), *N*-methylnicotinamide, and 1,4-dihydro-*N*-methylnicotinamide measured from a dioxan internal reference (positive shifts to low field)

	$NADP^+$, pH 2·0, 0·10M	Nicotinamide, pH 6·78, 1·0M	Nicotinamide, pH 0, 1·0M	*N*-Methyl nicotinamide, pH 8·5, 0·2M	1,4-Dihydro-*N*-methylnicotinamide, pH 10·3, 1·4M
C=O	98·77	103·17	99·05 [a]	98·85 [b]	106·07 [b]
N4	79·44	69·48 [c]	79·13 [a,c]	77·10 [c]	−44·60 [c]
N6	75·99	85·04 [c]	76·98 [a,c]	80·75 [c]	62·82 [c]
N2	73·29	80·87 [c]	74·44 [a,c]	78·49 [c]	74·96 [c]
N3	67·22	62·26	66·07 [a]	66·79 [b]	29·72 [b]
N5	62·06	57·46 [c]	61·15 [a,c]	61·63 [c]	37·27 [c]
CH_3				−17·42 [c]	−26·31 [c]

Methods used for assignments: [a] Ionisation studies. [b] Multiplicity and relaxation effects. [c] ^1H—^{13}C selective decoupling. All other assignments made by comparison with model compounds.

While the foregoing considerations lead to general conclusions about the various carbon nuclei in a molecule, to complete the detailed assignment as presented in Tables 1 and 2 requires further experiments. Some of these constitute novel methods for making ^{13}C assignments and together with more established procedures provide a general approach to the problem of ^{13}C spectral assignments for most classes of compounds. In Tables 1—3 the method used for making each assignment has been indicated.

Use of Model Compounds.—If perfect model compounds are available this procedure can yield excellent results. Table 1 shows the close agreement between the ^{13}C chemical shifts of N4, N6, N2, N3, N5, N1′, and N3′ in NMN^+ and the corresponding carbon atoms in $NADP^+$ at low pH values. This approach is particularly valuable when one is assigning the carbons in a large molecule (*e.g.* $NADP^+$), using model compounds which themselves constitute a large portion of the molecule being assigned (*e.g.* AMP and NMN^+). It becomes increasingly less reliable as the size of the model compound fragments is decreased. Thus the chemical shifts observed for the ^{13}C nuclei of nicotinamide and *N*-methylnicotinamide show poor agreement with the nicotinamide carbon nuclei in the nucleotides studied (see Table 3). Detailed pH studies on nicotinamide did not render these results any more useful to assign the N2, N4, and N6 carbon nuclei in other molecules.

However, the chemical shift changes observed on reduction of *N*-methylnicotinamide to 1,4-dihydro-*N*-methylnicotinamide proved useful in helping to assign the ^{13}C spectrum of NADH. For this molecule the N1′ and N5 nuclei cannot be distinguished by selective proton decoupling because the corresponding protons have very similar chemical shifts. When *N*-methylnicotinamide is reduced the CH_3 absorption moves upfield by 8·89 p.p.m.; a similar upfield shift for N1′ of NAD^+ at 33·45 p.p.m. would result in a signal close to the observed band in the NADH spectrum at 28·45 p.p.m. Thus one is able to differentiate between the NADH signal for N1′ (28·45 p.p.m.) and that of N5 (38·61 p.p.m.).

Off-resonance ^1H *Spin Decoupling Techniques.*—The well established assignment methods of selective ^1H irradiation and ^1H off-resonance spin-decoupling on the assigned ^1H spectrum can be used with ease only when (*a*) the ^1H spectrum of a molecule has well separated absorptions and (*b*) the ^{13}C spectrum is not too complicated. However these conditions frequently do not prevail and we have used two novel methods to assist in assignments for such cases.[7,8]

(a) *Lanthanide-induced shifts for increasing* ^1H *chemical shifts to facilitate* C–H *irradiation experiments.*[7] In the ^1H resonance spectrum of ribose 5-phosphate at 100 MHz (Figure 4a) the absorption signals for H-1′ from the α- and β-anomers are the only clearly resolved signals in the spectrum. By selective irradiation at either the H-α1′ or H-β1′ signal it is possible to collapse the corresponding ^{13}C doublet and assign it unambiguously. However, the chemical shifts of the ten other protons in the two anomers of ribose 5-phosphate are very similar and it is impossible to make further ^{13}C assignments on the basis of selective proton decoupling experiments. By adding varying amounts of Eu^{3+} ions to induce pseudo-contact shifts we obtain the spectra shown in Figure 4b and c.

To obtain the ^1H spectrum shown in Figure 4b the concentration of Eu^{3+} ions was sufficient to provide shifts large enough to resolve many of the peaks without causing much broadening. Thus the H–H spin–spin splittings are still clearly visible and by conventional H–H homonuclear spin-decoupling experiments it is possible to assign all the protons unambiguously.

Addition of more Eu^{3+} ions causes the equilibrium between the two anomeric forms to shift greatly in favour of the α-form (Figure 4c) and the intensities of the ^{13}C peaks enable them to be assigned to their respective anomeric forms. The ^{13}C resonance spectrum of this ribose 5-phosphate sample recorded under different conditions of

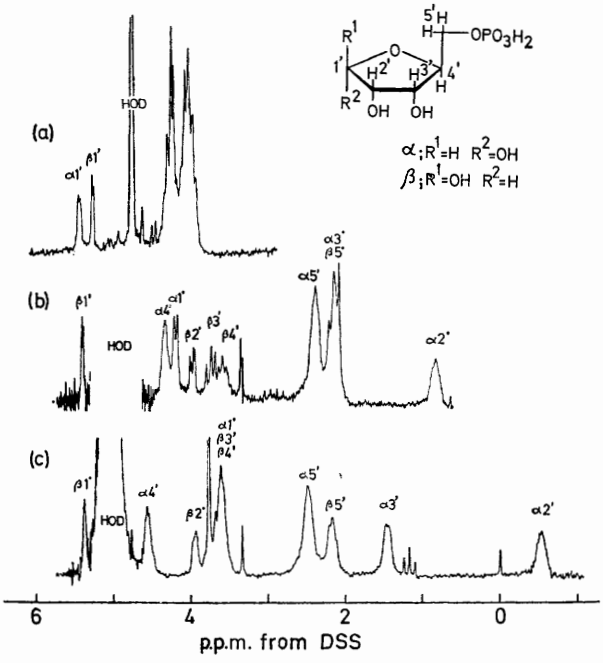


FIGURE 4 The ^1H resonance spectrum of ribose 5-phosphate at 100 MHz; (a) 0·37M-ribose 5-phosphate in absence of Eu^{3+} ions, pH 1·0; (b) 0·7M-ribose 5-phosphate, 0·48M-Eu^{3+}, pH 1·0; (c) 0·6M-ribose 5-phosphate, 0·72M-Eu^{3+}, pH 1·1

selective proton decoupling is shown in Figure 5. All the assignments are indicated on the noise-decoupled spectrum (Figure 5a). Figures 5b and c show the collapse of C-β2′ and C-α3′ when irradiating at protons H-β2′ and H-α3′, respectively. In the same way we have assigned C-β3′ and C-α2′. The assignment of the C-4′ and C-5′ peaks which had previously been based on the phosphorus coupling to C-4′ and C-5′ can now be confirmed by comparing the magnitudes and multiplicity of the residual couplings.[9] In Figure 5b, C-α5′ appears with a large triplet splitting due to its interaction with the CH_2 protons. The smaller C-β5′ signal also shows a triplet splitting although only the centre peak can be seen; the other two are hidden under larger signals. C-α4′ appears as a doublet of doublets due to coupling with the CH proton and the phosphorus atom. From the proton spectrum we would expect that when we irradiate at H-β2′ the residual, large doublet splitting of C-α4′ would be much larger than that of C-β4′, since the resonance frequency of H-β2′ is much further from that of H-α4′ than from that of H-β4′. In fact, the doublet of doublets of C-β4′ is overlapped to give one irregular peak

[7] B. Birdsall, J. Feeney, J. Glasel, R. J. P. Williams, and A. V. Xavier, *Chem. Comm.*, 1971, 1473.

[8] B. Birdsall, N. J. M. Birdsall, and J. Feeney, *J.C.S. Chem. Comm.*, 1972, 316.

[9] R. R. Ernst, *J. Chem. Phys.*, 1966, **45**, 3845.

whereas the peaks are clearly separated for C-α4′. Similarly the assignments for C-α1′ and C-β1′ can be confirmed by comparing the magnitude of the residual couplings in Figure 5b.

Examination of the ^{13}C spectra over a range of Eu^{3+} concentrations enables one to extrapolate the results to give the ^{13}C spectrum in the absence of Eu^{3+} ions. The chemical shifts are given in Table 1. In nucleotides, we are interested in ribose rings in the β-anomeric form but a comparison of the chemical shifts of the β-anomer of ribose with those in the nucleotide proved to be only of limited value.

(b) *Graphical method of presenting* ^1H *off-resonance decoupling results.*[8] When the ^{13}C spectrum is complicated with many overlapping multiplets it is difficult to interpret the ^1H off-resonance decoupled ^{13}C spectra. We have used a graphical method (described in detail in ref. 8) of presenting results of such experiments which overcomes this

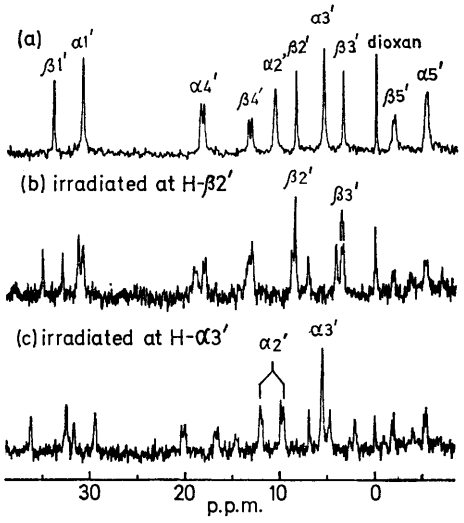


FIGURE 5 The ^{13}C resonance spectrum at 25·2 MHz of 0·6M-ribose 5-phosphate and 0·72M-Eu^{3+}, pH 1·1, under conditions of (a) proton noise decoupling (1000 transients, 75 μs pulse width, AT = 1·0 s, PD = 0); (b) selective irradiation at H-β2′ frequency (1100 transients, 75 μs pulse width, AT = 1·0 s, PD = 0); and (c) selective irradiation at H-α3′ frequency (1100 transients, 75 μs pulse width, AT = 1·0 s, PD = 0)

problem. If the peak frequencies of the partially decoupled ^{13}C spectra are plotted against the proton irradiating frequencies as shown in Figure 6 for NAD^+ at pH 7·05 one can readily identify the connected proton and carbon nuclei. Thus one can easily draw the lines connecting all the points as shown in Figure 6. The straight lines will intersect at the chemical shifts of the connected ^{13}C and ^1H nuclei since the intersection corresponds to the optimum spin decoupling of the ^{13}C nucleus. These assignments are indicated in Figure 6 and the measured chemical shifts are given in Table 1. This graphical presentation can be used to form the basis of a method for automatic processing of the decoupling results by use of computer techniques.

We have used this graphical procedure as a general method for analysing the data from all C–H heteronuclear decoupling experiments. Thus, the resonance signals for the carbon nuclei in the ribose ring in NMN^+ were assigned by this method as well as all the resonance signals for carbon nuclei with protons attached in AMP, nicotinamide, *N*-methylnicotinamide, and 1,4-dihydro-*N*-methylnicotinamide.

Effects of Ionisation on ^{13}C *Chemical Shifts.*—If the molecule of interest contains ionisable groups then by observing the pH dependence of the ^{13}C spectra it is sometimes possible to achieve an assignment. The method is particularly useful for assigning carbon nuclei which are not directly bonded to protons and thus are inaccessible to the more direct proton-decoupling methods.

TABLE 4

The proton chemical shifts and H–H coupling constants for NMN^+; the error on coupling constants is ±0·05 Hz

	Chemical shifts (p.p.m. from DSS)		J_{HH}/Hz (pH 2·8)	
	pH 2·8	pH 8·4		
N2	9·45	9·57	$J_{2,4}$	1·5
N6	9·28	9·32	$J_{2,6}$	1·3
N4	8·98	8·97	$J_{4,5}$	8·1
N5	8·29	8·29	$J_{4,6}$	1·3
N1′	6·21	6·27	$J_{5,6}$	6·3
N4′	4·63	4·59	$J_{1',2'}$	5·3
N2′	4·56	4·65	$J_{2',3'}$	5·0
N3′	4·43	4·45	$J_{3',4'}$	2·3
N5′a	4·29	4·19	$J_{4',5'a}$	2·5
			$J_{4',5'b}$	2·0
N5′b	4·14	4·01	$J_{5'a,5'b}$	12·2

$J_{P,5'a}$ 4·5. $J_{P,5'b}$ 5·0 at pH 2·8.

In AMP, the adenine 4- and 6-carbon nuclei have no protons attached and have similar chemical shifts. However, these two resonances can be assigned by observing the shifts caused by protonation at the 1-nitrogen atom. The chemical shifts of the A6 and A2 carbon nuclei, adjacent to nitrogen-1 would be expected to change more than those of A4, A5, and A8, as observed previously in purines and related heterocyclic molecules [10] when protonation occurs. The chemical shift due to raising the pH from 3·95 to 4·8 for carbon nucleus A2 is 4·52 p.p.m. downfield and for A8, 1·39 p.p.m. upfield. Of the smaller peaks (those with long relaxation times), one shifts downfield by 3·13 p.p.m. and is assigned to the A6 carbon nucleus and one moves downfield by only 0·36 p.p.m. and is assigned to the A4. The A5 signal, which can be distinguished by its chemical shift, moves upfield 0·08 p.p.m. The intensity differences due to varying relaxation times are an additional aid in characterising the absorptions during the titration. Extrapolating to pH 7, these assignments agree with those of Dorman and Roberts.[1] Pugmire and his co-workers have observed similar ^{13}C chemical shift behaviour when purines are ionised.[4,10]

Lanthanide-induced Shifts.—The use of lanthanide ions to induce pseudo-contact shifts has proved itself to be a useful method for studying conformations in solution [11] as well as for making assignments of rigid molecules.[12] During the course of studying the conformations of AMP as determined from lanthanide ion-induced pseudo-contact shifts of the ^{13}C resonances it became apparent that using the assignments of the carbon nuclei A2′ and A3′ previously reported [1] it was impossible to predict the observed shifts by assuming any reasonable conformation for the ribose ring.[13] A

[10] R. J. Pugmire, D. M. Grant, R. K. Robins, and G. W. Rhodes, *J. Amer. Chem. Soc.*, 1965, **87**, 2225.

[11] C. D. Barry, A. C. T. North, J. A. Glasel, R. J. P. Williams, and A. V. Xavier, *Nature*, 1971, **232**, 236.

[12] J. Briggs, F. A. Hart, G. P. Moss, and E. W. Randall, *Chem. Comm.*, 1971, 364.

[13] J. A. Glasel, R. J. P. Williams, A. V. Xavier, B. Birdsall, and J. Feeney, unpublished results.

heteronuclear decoupling experiment was therefore performed and in fact the A2′ and A3′ carbon resonance signals were found to be reversed. The new assignments are stated in Table 1.

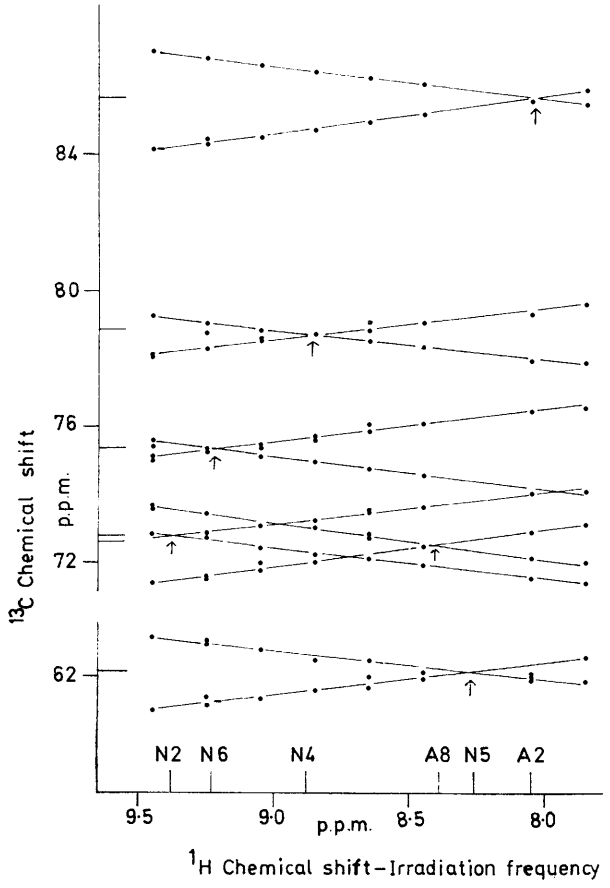


FIGURE 6 Plot of peak frequencies in the ^{1}H off-resonance selectively decoupled ^{13}C spectra of NAD^{+} at pH 7·05 as a function of position of irradiation in the ^{1}H spectrum, expressed in p.p.m. downfield from internal dioxan. The positions of the peaks in the ^{1}H noise decoupled ^{13}C spectrum are shown by lines on the ordinate and the position of the proton peaks by lines on the abscissa. The arrows ↑ indicate the point of collapse of the ^{13}C doublet and the connection between a given ^{13}C peak and the assigned proton peak. The errors in the position measurements of the ^{13}C peaks are indicated by the size of the points except near the cross-over positions where the errors are larger (±0·15 p.p.m.). Small doublet splittings are observed on some of the signals from long-range CH spin coupling interactions

^{1}H *Assignments from* ^{1}H–^{13}C *Heteronuclear Decoupling Experiments.*—The usefulness of an assigned proton spectrum for assigning the ^{13}C spectrum has been demonstrated. However, because one can readily connect ^{1}H and ^{13}C spectral assignments sometimes it is possible to use unambiguous ^{13}C assignments to assign proton signals. An example of this is provided by NADH, where a consideration of the ^{13}C spectrum uncovered misassignments in the published ^{1}H spectrum.[14] Initially, the ^{13}C resonance signals were assigned by heteronuclear decoupling experiments using the proton spectrum assignments in the literature.[14] The signal at 57·26 p.p.m. was assigned to N1′ and the two signals at 38·61 p.p.m. and 28·45 p.p.m. to N5 and N6. From the CH_3 chemical shift change observed on reduction of *N*-methylnicotinamide it seemed unreasonable that the reduction of the nicotinamide ring should cause the N1′ carbon signal to shift 23·81 p.p.m. downfield in NADH compared with N1′ in NAD^{+}.

Reconsideration of the proton peak in NADH at 6·01 p.p.m. from DSS showed that it has the same large coupling constant ($J_{5,6}$ 8·2 Hz) as the H-6 peak in 1,4-dihydro-*N*-methylnicotinamide at 5·77 p.p.m. from DSS. Furthermore, the shift of the N1′ proton resulting from reduction of NAD^{+} should be similar to the shift of methyl protons when *N*-methylnicotinamide is reduced. The methyl signal moves 1·6 p.p.m.: a similar shift for the N1′ proton would place it near to or under the HOD peak. Recently, Oppenheim and his co-workers [15] have carried out the decoupling experiments which prove that the proton at 6·01 p.p.m. is N6 and that N1′ is under the HOD, and have corrected the misassignment. By selective decoupling, the resonance at 57·26 p.p.m. is shown to be N6 and that at 38·61 p.p.m. N5 as described earlier.

The connection between ^{13}C and ^{1}H signals in NMN^{+} could also be used to help in the assignment of the ^{1}H spectrum. A reasonable set of ^{13}C assignments for the ribose carbons of NMN^{+} could be made based on the other ribose analogues studied. When C–H heteronuclear selective decoupling experiments were conducted these suggested that the original assignments [14] for H-3′ and H-4′ required to be reversed. A detailed reinvestigation of the ^{1}H spectrum of NMN^{+} at both 100 and 220 MHz at pH 3·0 and 8·0 confirmed this misassignment. The results of these experiments are summarised in Figure 7 and Table 4.

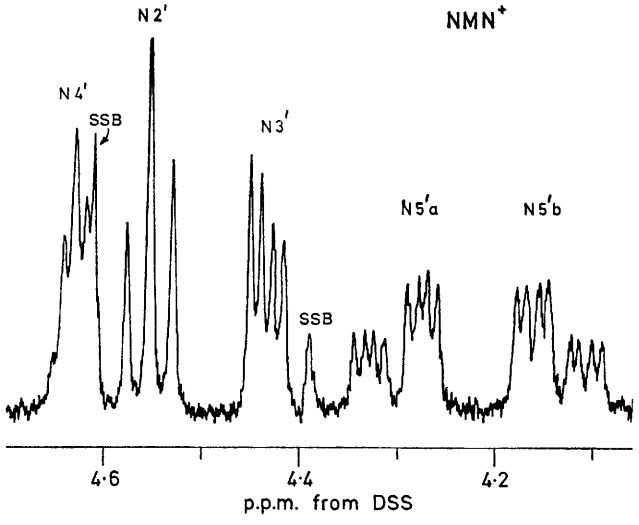


FIGURE 7 The ribose region of the 220 MHz proton spectrum of NMN^{+} (0·12M at pH 2·8)

DISCUSSION

By the combination of assignment methods we have described, most carbon-13 signals can be assigned unequivocally. However in a few cases a complete assignment of a particular carbon nucleus was not possible because of one of the following reasons. (1) The absence of a directly bonded proton prevents the possibility of a selective proton decoupling experiment.

[14] R. H. Sarma and N. O. Kaplan, *Biochemistry*, 1970, **9**, 557.
[15] N. J. Oppenheimer, L. J. Arnold, and N. O. Kaplan, *Proc. Nat. Acad. Sci. U.S.A.*, 1971, **68**, 3200.

For such carbon nuclei unequivocal assignments can often be obtained by using other methods. For example the A4, A5, and A6 carbon nuclei of the adenine rings can be assigned by ionisation and model compound studies. (2) Small or similar lanthanide-induced chemical shifts are observed for some overlapping proton signals even at high lanthanide concentrations, and this results in this method being of no value as an aid to selective proton decoupling experiments. Thus for $NADP^+$ the observed lanthanide-induced shifts for the ribose protons were insufficient to allow unambiguous selective proton decoupling experiments, and these assignments were made largely by comparison with the assignments found in AMP and NMN^+ where heteronuclear selective decoupling was possible. (3) For some of the nucleotides, the lanthanide/decoupling experiments could not be carried out because of the instability of the compound at the pH values most suitable for obtaining high concentrations of lanthanide ions (<pH 6). Thus NADH, which is most stable at high pH values (>pH 7), could not be readily studied by this approach. (4) Sensitivity limitations can become acute when many selective proton decoupling experiments are required to achieve assignments; thus for low concentration solutions (<0·05M), while it would not be impossible to obtain unequivocal assignments, the amount of instrument time required could make it unfeasible. For 0·09M-NADH, to obtain seven heteronuclear decoupling experiments required 7 h of instrument time.

Conclusions.—As observed for other nucleotides, the ^{13}C absorbtion bands of NADPH and related nucleotides are well resolved at 25·2 MHz and would provide ideal probes for reporting the extent of the protein–coenzyme interaction at the different parts of the coenzyme molecule were it not for the inherent sensitivity problem. At protein concentrations of <1mM one will have great difficulty examining strongly bound coenzyme (even using Fourier Transform techniques) unless specific ^{13}C enrichment is undertaken. However for fragments of the coenzyme which are undergoing rapid exchange between the free and enzyme-bound forms, the sensitivity requirements are less severe. In this situation, by observing the averaged signals of free and enzyme-bound molecules one can operate at much higher concentrations of the small molecules (*ca.* 100mM). While the ^{13}C chemical shift changes observed on binding under conditions of rapid exchange might not be large, the changes in relaxation rates should be substantial because of the anticipated large difference between the relaxation times of the free and bound small molecules. Because of the time-consuming nature of T_1 measurements it is clear that specific ^{13}C enrichment of the coenzyme and its fragments will be required regardless of the system studied. However, for studies of chemical shift changes on binding, useful information will be accessible from non-enriched samples.

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