Ionisation, Self Association, and Proton Exchange Studies of Nicotinamide in Aqueous Solution using Nuclear Magnetic Resonance Spectroscopy

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From an analysis of the single resonance ^{13}C n.m.r. spectrum of nicotinamide at 25·2 MHz at high (pH >6·0) and low (pH <1·0) pH values the C–H spin coupling constants have been obtained. The C–H coupling constants for the charged nicotinamide (pH <1·0) agree well with those which could be measured in NAD^+. The unusually large C–H coupling constants (*ca.* 12 Hz) observed between C(2) and H(6) across the nitrogen atom in the uncharged nicotinamide have been observed in the pteridine ring of folic acid and the adenine ring of NAD^+: in both cases aromatic carbon atoms without directly bonded protons could be assigned on the basis of such coupling constants observed in their single resonance ^{13}C spectra. The ^{13}C chemical shift differences observed on protonation of the nicotinamide ring were measured and compared with values calculated on the basis of the differences in the calculated total electron densities at the ring carbons: excellent agreement was found for C(3)—C(5) but C(2) and C(6) which are near the site of protonation showed no agreement probably due to intramolecular electric field effect contributions to their shielding. Carbon-13 relaxation time measurements on nicotinamide and pyridine suggest that nicotinamide is associated at high pH (*ca.* pH 6·0) and concentration (*ca.* 2M solutions). At low pH values (<1·0) less association is observed. ^1H and ^{13}C chemical shift measurements on nicotinamide aqueous solutions at different concentrations also support this conclusion. Furthermore the largest shift changes on dilution were centred on the $CONH_2$ group of nicotinamide. The ^1H chemical shift changes on dilution were in the opposite direction to those expected from association involving parallel stacking of the nicotinamide rings. Thus there is strong evidence that the association results from interamide interactions. Interactions involving the $CONH_2$ group are not thought to be of importance in the intramolecular stacking found for the nicotinamide and adenine rings of NAD^+ at neutral pH values. From measuring the concentrations and pH dependence of the line widths of the $CONH_2$ protons in H_2O solution it was possible to estimate the separate exchange rates between the *cis*- and *trans*-NH protons with water protons. Similar measurements could be made on NAD^+ where it was found that at pH values <5·5 the exchange rates are slower than found for nicotinamide under the same conditions.

NICOTINAMIDE is an important fragment of several nucleotides related to the coenzyme nicotinamide adenine dinucleotide phosphate ($NADP^+$). Currently we are exploring the feasibility of using the ^{13}C resonance spectra of such coenzymes and related molecules to investigate their ionisation and conformational behaviour and also to monitor their interactions with enzymes such as dihydrofolate reductase. There is considerable evidence that $NADP^+$ exists in neutral solution with the nicotinamide and adenine rings stacked with respect to each other.[1] Likewise it is known that for adenine monophosphate in aqueous solution at concentrations > *ca.* 0·004M the adenine rings are stacked in an intermolecular fashion.[2] It is of interest to establish if nicotinamide is associated in aqueous solution and to characterise such associations so that it will be possible to assess if any of the factors involved in self association are also important in determining the intramolecular stacking in $NADP^+$. The two most likely possibilities for self-association mechanisms are by intermolecular stacking or by interamide hydrogen bonding. We have attempted to obtain self-association information by measuring the ^1H and ^{13}C chemical shifts and ^{13}C spin-lattice relaxation times for nicotinamide in aqueous solution over a wide concentration range. Because ionisation of the nicotinamide might be anticipated to influence self-association interactions it was decided to make the measurements at different pH values.

The effects of ionisation on the ^{13}C chemical shifts and C–H coupling constants were also measured in this investigation to provide reference information for comparison with the observed parameters in $NADP^+$ and related molecules. This information can be of considerable value both in helping with spectral assignments and with providing information about the electron distribution in the coenzymes. Because of the high solubility of nicotinamide in water it is possible to obtain information concerning ^{13}C–H spin coupling constants by studying the single resonance ^{13}C spectrum of the ^{13}C nuclei present in natural abundance (1·1%) in nicotinamide using Fourier transform techniques. Likewise we were able to measure with ease the spin-lattice relaxation times of the carbon nuclei in the proton noise decoupled ^{13}C spectrum of nicotinamide using inversion–recovery type measurements.

EXPERIMENTAL

The ^1H and ^{13}C n.m.r. spectra were recorded on Varian XL100 and HA100D spectrometers. Heteronuclear $\{^1H\}$–^{13}C double resonance experiments were carried out using the gyrocode of the XL100. Single resonance ^{13}C spectra were collected using the Fourier transform technique. In some experiments a gated decoupler was used to irradiate the protons during the pulse delay but not during the aquisition time: in this way part of the Overhauser sensitivity increase is obtained without loss of the C–H spin–spin coupling constant information.[3,4] Nicotinamide and NAD^+ were purchased from Sigma Chemical Company. The spin-lattice relaxation time measurements were made using the Freeman–Hill modification of the (180–*t*–90) pulse sequence.[5] The carbon-13 relaxation times were measured at the ambient probe temperature (33°) using degassed samples. pH Measurements were made using a Radiometer model 26 pH meter.

[1] R. H. Sarma, V. Ross, and N. P. Kaplan, *Biochemistry*, 1968, **9**, 3052.
[2] P. O. P. Ts'O, N. S. Kondo, M. P. Schweizer, and D. P. Hollis, *Biochemistry*, 1969, **8**, 997.
[3] J. Feeney, D. Shaw, and P. J. S. Pauwels, *Chem. Comm.*, 1970, 554.
[4] R. Freeman and H. D. W. Hill, *J. Magnetic Resonance*, 1971, **5**, 278.
[5] R. Freeman and H. D. W. Hill, *J. Chem. Phys.*, 1970, **51**, 4103.

RESULTS AND DISCUSSION

(a) *Spectral Analysis and Assignment.*—Figure 1 shows the ^1H resonance spectrum of nicotinamide in aqueous solution: from the first-order nature of the spectrum it is easy to extract approximate chemical shifts and coupling constants which are then used as a basis for a detailed spectral analysis. Assignments could be made with ease by comparing the measured H–H coupling constants with reported values for 3-substituted pyridines.[6] The single resonance ^{13}C spectra of nicotinamide is shown in Figure 2 and as expected shows the

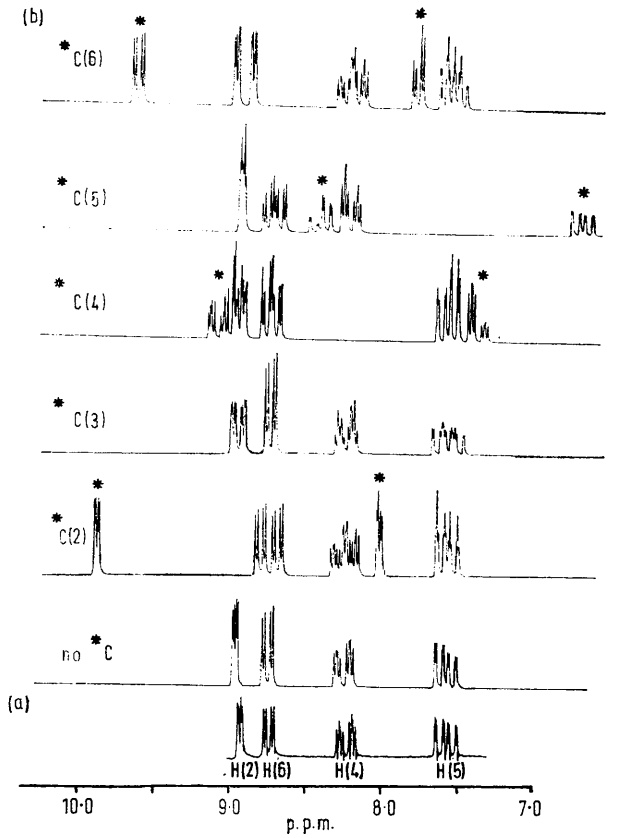


FIGURE 1 (a) The 100 MHz ^1H n.m.r. spectrum of nicotinamide in D_2O at pH 6. (b) The calculated ^1H n.m.r. spectra of the ^{13}C isotopomers

large doublet splittings on C(2) and C(4)—C(6) from spin–spin interaction with the directly bonded proton and further fine structure on all the absorptions from long range C–H spin coupling. The C-13 assignments for nicotinamide [7] which had been made earlier by selective $\{^1H\}$–^{13}C decoupling experiments (see Figure 3c) were confirmed by comparing the observed ionisation shifts with those found for pyridine over the pH range 0—12·5. Goldstein and his co-workers [8] have recently reported the ionisation effects on ^{13}C shielding in nicotinamide and their results are similar to those shown in Figure 4 with the exception that we examined the compounds over a wider range of pH values to obtain the ionisation shifts. For the purpose of the $\{^1H\}$–^{13}C selective decoupling experiments required to assign the long range C–H coupling constants, the relevant ^1H spectra to consider are in fact those of the isotopomers which each contain a single carbon-13 nucleus. These

[6] V. J. Kowalewski and D. G. de Kowalewski, *J. Chem. Phys.*, 1962, **36**, 266.
[7] B. Birdsall and J. Feeney, *J.C.S. Perkin II*, 1972, 1643.
[8] K. R. Long, R. C. Long, and J. H. Goldstein, *J. Magnetic Resonance*, 1972, **8**, 207.

TABLE 1

(a) Directly bonded C–H coupling constants (Hz) used in the simulated fitting of the spectra of nicotinamide (NIC) at pH 6·5 and 0, and NAD^+ at pH 2·1

Coupling	NIC pH 6·5	NIC pH 0	NAD^+ pH 2·1
C(2)–H(2)	181·6	192·2	192·6
C(4)–H(4)	166·8	172·7	174·9
C(5)–H(5)	169·0	179·6	178·6
C(6)–H(6)	181·4	193·8	(199)

(b) Long range C–H coupling constants (Hz) used in the simulated fitting of the spectra of nicotinamide at pH 6·5 and at pH 0.

Coupling	pH 6·5	pH 0
	Two bond	
C(3)–H(2)	6·5	3·0
C(3)–H(4)	1·6	1·0
C(4)–H(5)	0·9	0·0
C(5)–H(4)	0·6	0·5
C(5)–H(6)	7·9	3·4
C(6)–H(5)	3·7	6·7
	Three bond	
C(2)–H(4)	5·6	6·2
C(2)–H(6)	11·0	6·2
C(3)–H(5)	6·5	6·5
C(4)–H(2)	5·4	5·7
C(4)–H(6)	6·1	5·7
C(6)–H(2)	10·6	6·7
C(6)–H(4)	6·9	4·0
	Four bond	
C(2)–H(5)	0·9	0·6
C(3)–H(6)	0·0	0·0
C(5)–H(2)	0·6	0·4

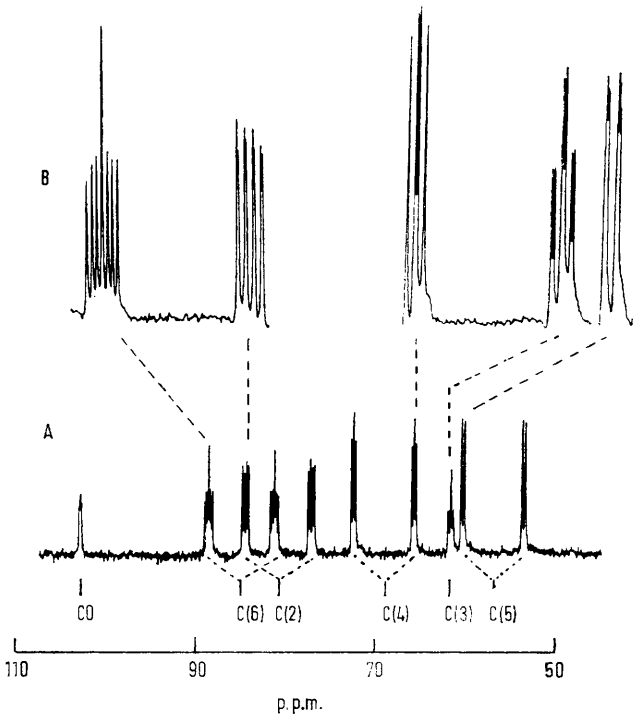


FIGURE 2 The ^{13}C n.m.r. spectrum (p.p.m. from dioxan) at 25·2 MHz of nicotinamide in D_2O at pH 6·0

spectra, shown diagrammatically in Figure 1, were computed from the ^1H and ^{13}C spectral data, the latter being obtained initially from a first-order analysis of the single

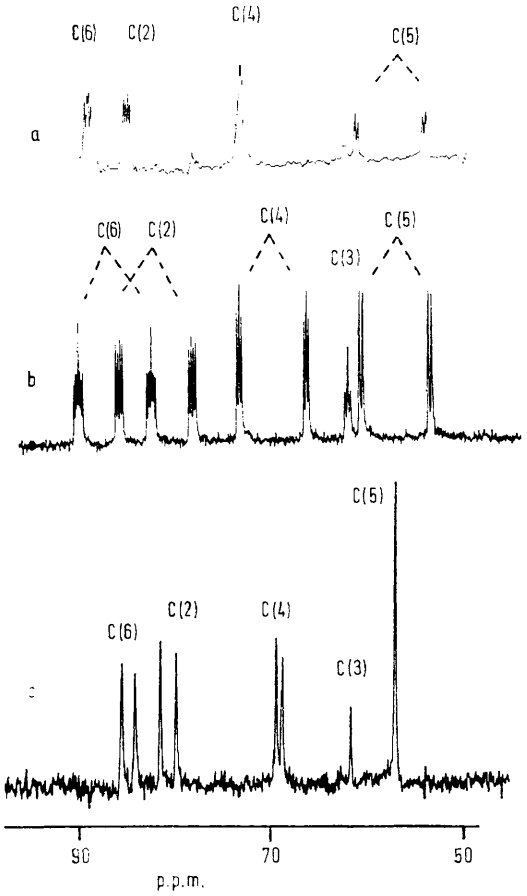


FIGURE 3 a The ^{13}C n.m.r. spectrum of nicotinamide irradiated at the frequency of H(5) ($\gamma H_2/2\pi$ *ca.* 10 Hz). b The ^{13}C n.m.r. spectrum at 25·2 MHz of nicotinamide in D_2O at pH 6. c The ^{13}C n.m.r. spectrum of nicotinamide irradiated at the frequency of H(5) ($\gamma H_2/2\pi$ *ca.* 170 Hz)

resonance spectrum of nicotinamide. From an examination of these computed spectra it was possible to decide whether or not a first-order treatment of the single resonance ^{13}C spectrum is appropriate for any particular isotopomer of nicotinamide.

Most of the proton multiplets are well separated in the various isotopomers and result in simple first-order ^{13}C spectra. However, the H(4) and H(5) can be strongly coupled in two isotopomers [*C(4) and *C(5)] and to obtain accurate coupling constants in this case requires a detailed spectral analysis.

An initial assignment of the long range C–H coupling constants was made by assuming that $J_{C(x)H(y)}$ and $J_{C(y)H(x)}$ values would be similar and searching for matching pairs of coupling constants. These assignments were then confirmed for nicotinamide at pH 6·0 by selective low power $\{^1H\}$–^{13}C double resonance experiments as indicated in Figure 3a by irradiation at a proton frequency for protons not directly bonded to carbon-13 (see isotopomer spectra in Figure 1). Under conditions of selective proton decoupling it is possible to observe intensity changes in the ^{13}C multiplets. This is illustrated in Figure 3a which shows the ^{13}C spectrum of nicotinamide when irradiated at the H(5) frequency with power sufficient to decouple long-range CH coupling constants ($\gamma H_2/2\pi$ *ca.* 10 Hz). Under these irradiation conditions the low field components of C(2), C(6), and C(4) are enhanced and the high-field components are diminished. This has the effect of improving the sensitivity in such decoupling experiments. In addition to the intensity changes one observes the removal of one of the splittings on C(6) from long-range coupling with H(5), thus allowing an unambiguous assignment of the $J_{C(6)H(5)}$ coupling constant. Reference to the theoretical spectra for the various isotopomers (Figure 1) shows that the frequency for irradiation of H(5) in isotopomer *C(5) is also near the frequencies of components of the ^{13}CH satellites from directly bonded CH coupling in isotopomers *C(2), *C(4), and *C(6). When only one component of the ^{13}CH satellite is irradiated there are heteronuclear Overhauser effects [9] which result

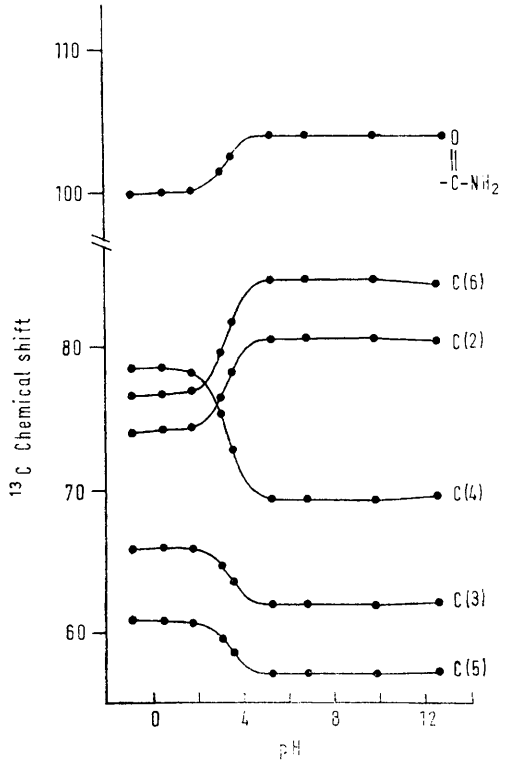


FIGURE 4 ^{13}C Chemical shifts (p.p.m. from dioxan) of nicotinamide in D_2O as a function of pH

in the ^{13}C doublets from directly bonded C–H coupling becoming of unequal intensity.

(b) ^{13}C *Chemical Shifts and Total Electron Densities.*—Several attempts have been reported to correlate ^{13}C chemical shifts with electron densities in aromatic molecules. Only limited success has been achieved although the correlation with total electron densities is

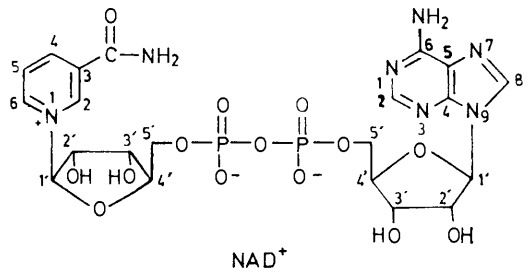
[9] T. Yonemoto, *J. Chem. Phys.*, 1972, **54**, 3234.

more satisfactory than that with π electron densities.[10] Large discrepancies are observed for carbon atoms near the substituents and this possibly reflects intramolecular electric field effects on the total shielding of such nuclei. We have calculated the total electron densities at the carbon atoms of protonated and unprotonated nicotinamide using the CNDO/2 method,[11] and the results predict that all the ring carbon atoms should be deshielded when the nicotinamide becomes protonated This is not observed for C(2) and C(6) which are in close proximity to the nitrogen but for C(3)—C(5) the correct trend is observed with the ionisation shift at C(4) being about twice that at C(3) and C(5) as predicted by the total electron density calculations. One would expect the difference in chemical shifts of C(3)—C(5) between the protonated and unprotonated species to reflect almost solely electron density changes. Using the value [10] of 148 p.p.m. electron^{-1} for the contribution of total electron density to ^{13}C shielding these values have been calculated. Reasonable agreement with the observed values are obtained: calculated ^{13}C shift differences C(3), 3·5, C(4) 10·4, C(5) 4·0 p.p.m.; observed ^{13}C shift differences C(3) 3·85, C(4) 9·41, C(5) 3·65 p.p.m. (see Table 2).

(c) C–H *Spin Coupling Constants.—Nicotinamide.* The observed long range J_{CH} values in nicotinamide at pH 6·5 are similar to those measured by other workers in pyridine,[12,13] $J_{C(2)H(6)}$ and $J_{C(6)H(2)}$ (11·0 and 10·6 Hz) having the largest values. Although some of the $^2J_{CH}$ values are small, as was found for benzene,[14] the value observed for $J_{C(5)H(6)}$ (7·9 Hz) is comparable with many $^3J_{CH}$ values. Obviously one cannot rely on $^2J_{CH}$ spin coupling constants in aromatic systems to be always small as is observed in benzene itself.

The values of the coupling constants between directly bonded nuclei ($^1J_{CH}$) were found to depend markedly on the ionisation state of the nicotinamide. The large coupling constants observed across the nitrogen have also been substantially reduced on protonation, *e.g.* $J_{C(2)H(6)}$ 11·0 (pH 6·5), $J_{C(2)H(6)}$ 6·2 Hz (pH <1·0).

Nicotinamide adenine dinucleotide, NAD^+. The C–H coupling constants which could be measured in the ^{13}C spectrum of NAD^+ agreed closely with those measured in the charged nicotinamide molecules. This is as expected for the nicotinamide ring in NAD^+ where a formal positive charge is on the nitrogen atom and indicates a similar electron distribution to that in the charged nicotinamide parent molecule. This is supported by the similar chemical shifts of the nicotinamide ring carbons in NAD^+ and charged nicotinamide. It is



interesting to note that C(4) and C(5) of the adenine ring of NAD^+ at pH 2·1 both show large coupling constants with protons across the nitrogen atom ($J_{C(5)H(8)}$ 11·7, $J_{C(4)H(8)}$ *ca.* 12 Hz) as is observed in nicotinamide at pH 6·5. Clearly one can use such coupling constants to

TABLE 2

(a) ^1H Chemical shifts (p.p.m.) of nicotinamide at different concentrations and pH.

	^1H Chemical shifts					
Position	pH 6·5 1·3M	pH 6·5 4·0M	Dilution shift Δ	pH <1·0 1·3M	pH <1·0 4·0M	Dilution shift Δ
2	8·89	9·08	0·19	9·33	9·62	0·29
6	8·68	8·82	0·14	9·13	9·45	0·32
NH(*trans*)	8·24	8·59	0·35	8·49	8·74	0·25
4	8·18	8·34	0·16	9·03	9·29	0·26
NH(*cis*)	7·58	7·88	0·30	7·82	8·07	0·25
5	7·52	7·63	0·11	8·28	8·56	0·28

Δ = Dilution shift from 4·0 to 1·3M. Chemical shifts measured from sodium 4,4-dimethyl-4-silapentanesulphonate as internal reference.

(b) Observed ^{13}C chemical shifts and the calculated chemical shift differences for the carbon atoms of nicotinamide at pH 6·78 and <1·0

	pH <1·0		pH 6·78		Differences in total electron		
Position	Total electron density	δ(p.p.m.)	Total electron density	δ(p.p.m.)	density	Δδ(calc.)	Δδ(obs.)
2	3·853	74·44	3·899	80·87	0·046	6·80	−6·43
3	4·038	66·07	4·062	62·22	0·024	3·55	3·85
4	3·874	78·97	3·944	69·56	0·070	10·36	9·41
5	4·007	61·15	4·034	57·50	0·027	4·0	3·65
6	3·847	76·98	3·898	85·04	0·051	7·55	−8·06
CO		99·05		103·13			−4·08

Dioxan as internal reference; 1M solutions; some solvent effects (<0·5 p.p.m.) have not been corrected. Δδ(calc.) = Calculated chemical shift differences.

[10] R. G. Jones and P. Partington, *J.C.S. Faraday II*, 1972, 2087.

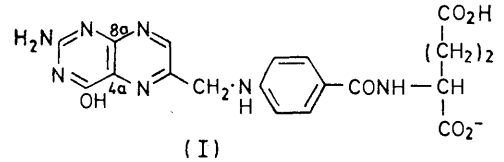
[11] J. A. Pople, D. P. Santry, and G. A. Segal, *J. Chem. Phys.*, 1965, **43**, 5129.

[12] J. D. Roberts, personal communication.

[13] M. Hansen and H. J. Jakobsen, *J. Magnetic Resonance*, 1973, **10**, 74.

[14] J. M. Read, R. E. Mayo and J. H. Goldstein, *J. Mol. Spectroscopy*, 1967, **22**, 419.

make assignments of carbon atoms in aromatic systems containing –C–N–CH– fragments. For example, in substituted pteridines such as folic acid (I) we have been able to assign the ring atoms C(4a) and C(8a) on this basis. In the single resonance ^{13}C spectrum of folic acid



(I)

one of the non-protonated ring carbons (88·53 p.p.m. from dioxan) is found to be coupled to a neighbouring proton with a large C–H coupling constant (12 Hz): this large coupling constant is similar to that in nicotinamide between H(2) and C(6) and allows us to assign this band to C(8a) in folic acid at pH 10.

(d) *Self Association of Nicotinamide in Water.*—^{13}C *Spin lattice relaxation time* (T_1) *measurements.* Table 3 gives the T_1 values for the ring carbons of pyridine and

TABLE 3

^{13}C Spin lattice relaxation times for nicotinamide and pyridine as a function of concentrations and pH at 33°

Compound	Conc. (M)	pH	T_1/s C(6)	C(2)	C(4)	C(5)
Nicotinamide	2	<1·0	1·6	2·4	2·5	2·7
Nicotinamide	2	6·0	1·2	1·6	1·3	1·3
Nicotinamide	3	6·5	0·71	1·03	1·00	1·07
Nicotinamide	4	6·5	0·66	0·80	0·78	0·80
Pyridine	2	<1·0	9·9	9·9	7·7	9·9
Pyridine	2	8·5	8·0	8·0	5·2	7·3

T_1 Values ±15%.

nicotinamide examined at different pH values over a concentration range from 2 to 4M in D_2O solution. For nicotinamide, only the values for C(2) and C(4)—C(6) are reported because these nuclei with directly bonded protons have short relaxation times which are completely dipolar in origin. By making the measurements in D_2O solution the possibility of any intermolecular relaxation effects from the solvent is removed. In fact these effects would be expected to be negligible for carbons with directly bonded protons but could be important for quaternary carbons. The ^{13}C spin lattice relaxation rates of the ring carbons directly bonded to hydrogen are dominated by the dipolar relaxation mechanism. From reported studies on the ring carbons in small molecules such as toluene it is found that contributions to the relaxation rates from spin rotation are negligible [15] and a similar state of affairs would be expected for nicotinamide. The dipolar contribution to the carbon relaxation rate [16] is given by equation (1)

$$T_1^{-1} = N\hbar^2\gamma_H^2\gamma_C^2 r_{CH}^{-6}\tau_C \qquad (1)$$

where N is the number of directly bonded protons, r_{CH} is the C–H bond distance (expected to be quite similar for the different carbon atom), γ_C and γ_H are the gyromagnetic ratios of carbon and hydrogen respectively, and τ_C is an overall effective correlation time. Thus for a C–H carbon nucleus which is relaxed by a clearly defined dipolar interaction it is possible to use the measured ^{13}C relaxation times to provide information about the correlation times of the nuclei.

When the T_1 values for the ring carbons of nicotinamide are compared with those of pyridine (at 2M concentration where the solution viscosities are very similar) it is noted that the nicotinamide values are much shorter than one might expect from the increase in correlation times resulting from the addition of an amide group to pyridine (for example, the relaxation times of ring carbons in benzene and styrene differ by only a factor of two [17]). Such results are suggestive of self association in the nicotinamide since this would result in an increase in the correlation times from the slower molecular motion of the aggregates. It is also noted that the T_1 values for nicotinamide at 2M concentration are reduced by almost a factor of two on going from the charged to the uncharged structure even though the solution viscosity remains constant. Such results are compatible with the uncharged nicotinamide (pH 6·0) being self associated to a greater extent than the charged nicotinamide (pH <1·0): this would reduce the correlation times at pH 6 and result in more efficient dipolar relaxation.

For nicotinamide at pH <1·0, the relaxation time of C(6) (1·6 s) is smaller than those at the other positions, C(2) (2·4 s), C(4) (2·5 s), and C(5) (2·7 s). This is consistent with the molecular rotation being anisotropic with rotation about the C(6)–C(3) axis being faster than about the other shorter axes. Levy and his coworkers [17-19] have previously observed such effects in substituted aromatic compounds. At pH 6·0 C(6) has a relaxation time similar to those of the other nicotinamide carbons and the anisotropic rotational effects are no longer so pronounced. No doubt this is also a consequence of the increased self association at pH 6·0, indicating that the complexes formed do not have an axis about which rotation is strongly favoured.

Effects of concentration on chemical shifts. To discover the nature of the self-association interaction the ^1H and ^{13}C chemical shift–concentration study was undertaken.

When nicotinamide is examined in H_2O solution the exchange rate between the amide protons and the water is sufficiently slow that the NH proton absorption bands can be observed as shown in Figure 5. Two separate NH absorption bands are observed corresponding to the amide NH protons *cis* and *trans* to the carbonyl group. Table 2 lists the ^1H and ^{13}C chemical shift changes which occur on varying the concentration of nicotinamide at pH <1 and 6. The ^1H chemical shift changes in pyridine over a comparable concentration range were

[15] C. F. Schmidt, jun., and S. I. Chan, *J. Magnetic Resonance*, 1971, **5**, 151.
[16] A. Allerhand and D. Doddrell, *J. Amer. Chem. Soc.*, 1971, **93**, 2777.
[17] G. C. Levy, J. D. Cargioli, and F. A. L. Anet, *J. Amer. Chem. Soc.*, 1973, **95**, 1527.
[18] G. C. Levy, D. M. White, and F. A. L. Anet, *J. Magnetic Resonance*, 1972, **6**, 455.
[19] G. C. Levy, *J. Magnetic Resonance*, 1972, **8**, 122.

also measured. At pH 6 it is noted that the amide NH_2 protons suffer a much larger dilution shift than the other protons. On diluting nicotinamide from 4 to 1·3M a shift change of 0·35 and 0·30 p.p.m. for the NH_2 protons

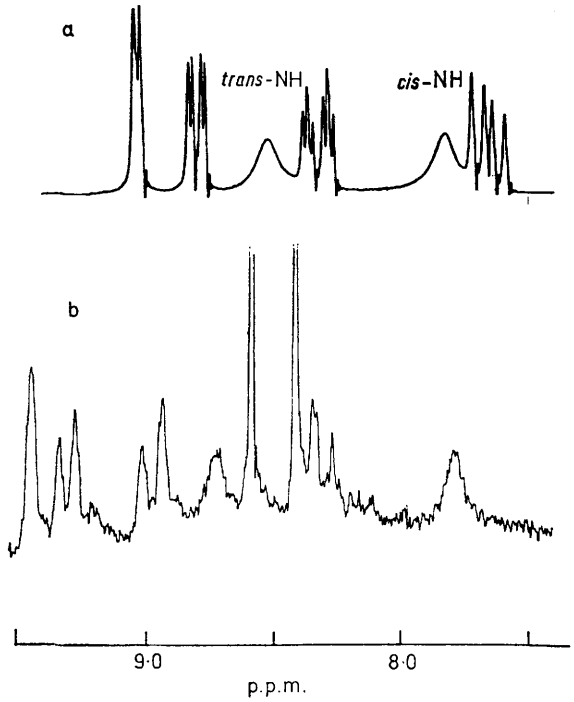


FIGURE 5 The ^1H n.m.r. spectrum (p.p.m. from sodium 4,4-dimethyl-4-silapentanesulphonate) at 100 MHz of: a nicotinamide in H_2O at pH 6·5 and 4M and b NAD^+ in H_2O at pH 2·4 and 0·2M

is observed compared with the smaller shifts observed for the ring protons (*e.g.* 0·19—0·11 p.p.m.). The ^1H chemical shifts of pyridine over the same concentration range show comparatively small effects (maximum change 0·05 p.p.m.). When the pyridine dilution shifts are used to correct the nicotinamide dilution shifts the resulting values are H(2) 0·14, H(4) 0·14, H(5), 0·09, and H(6) 0·09 p.p.m.

The chemical shift changes observed on dilution of nicotinamide suggest that the association at pH 6·5 results from interamide hydrogen bonding interactions rather than from parallel stacking of the nicotinamide rings. The removal of aromatic ring current shifts which are predicted to result from unstacking of the nicotinamide rings on dilution of nicotinamide solution should result in the ^1H and ^{13}C nuclei both becoming more deshielded; [20] in fact the ^1H signals for all nuclei move upfield on dilution. Furthermore, there are substantially larger shifts for the $CONH_2$ ^1H and ^{13}C resonances on dilution, strongly suggesting that in these high concentration solutions interamide hydrogen bonding is the cause of the self association. Thus in dilute solutions the amide protons are involved in hydrogen bonding with the water molecules; at higher concentrations where there is less water available, formation of interamide NH · · · O=C bonds becomes favourable. Klotz and Franzen [21] have observed interamide hydrogen bonding in concentrated aqueous solutions of acetamide and similar effects are expected in the hydrophobic interiors of proteins and in some small peptides in non-aqueous solvents when the amide and NH protons are favourably disposed to each other.

At pH 1·0 when the nicotinamide ring nitrogen is protonated, the ^1H chemical shifts are found to be concentration dependent but the magnitudes of the shifts for the different nuclei are substantially the same. The ^{13}C chemical shifts behave similarly on dilution. These shift changes show no indication of any specific concentration dependent interactions. The observed shifts (which are seen to a smaller extent in pyridine) probably reflect changes in reaction field effects in these concentrated solutions of ionic species.

The ^{13}C chemical shift changes on diluting nicotinamide at pH 6·8 are in the opposite direction to the proton chemical shifts. Although the direction of the observed ^{13}C shifts does not rule out a stacking interaction as was the case with the ^1H chemical shift changes, the ^{13}C data strongly support the interamide hydrogen bonding picture in that the ^{13}C signals of the $CONH_2$ shows a much larger dilution shift than the ring carbons [from 3 to 0·5M, ^{13}C chemical shift changes $CONH_2$ 0·99, C(2) 0·16, C(3) 0·48, C(4) 0·47, C(5) 0·36, and C(6) 0·36 p.p.m.; all shifts to low field on dilution].

In the case of NAD^+, the self association observed at concentrations >0·004M is unlikely to involve the nicotinamide amide group. At pH 2·5 where the unfolded form of NAD^+ is thought to exist, the ^1H chemical shifts of the $CONH_2$ protons (spectrum shown in Figure 5) do not change when its H_2O solution is diluted from 0·2 0·04M. Similar experiments at pH 4·9 where the folded form of the molecule is present confirm that the $CONH_2$ group is not involved in the self association. Such association results solely from the adenine–adenine interactions as postulated by earlier workers. The observed ^1H and ^{13}C chemical shifts of the nicotinamide ring which accompany the unfolding of the NAD^+ molecule when the adenine ring is protonated (pK 4·0) are consistent with the folded form of the molecule having the nicotinamide and adenine rings stacked parallel to each other being populated to some appreciable extent at neutral pH values.[1,22] It is worthwhile to enquire whether or not the nicotinamide amide group and the adenine C(6)–NH_2 group could participate in intramolecular hydrogen bonding and help to stabilise the folded form of NAD^+ which exists at pH 6·0. When the ^1H spectrum of 0·2M-NAD^+ in H_2O solution at pH 5·9 is examined the $CONH_2$ protons appear as very broad signals ($\nu_{\frac{1}{2}}$ *ca.* 20 Hz) with chemical shifts very similar to those in NAD^+ at pH 2·5. Thus from observation of the $CONH_2$ proton chemical shifts there is no evidence for any specific interactions involving the $CONH_2$ group of

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[21] I. M. Klotz and J. S. Franzen, *J. Amer. Chem. Soc.*, 1962, **84**, 3461.

[22] B. Birdsall and J. Feeney, unpublished results.

nicotinamide in NAD^+ which contribute to the stabilisation of the stacked form of the molecule. However, it should be noted that an interaction involving the amide CO group and C(6)–NH_2 protons would not necessarily be reflected in the $CONH_2$ proton chemical shifts.

(e) *Proton Exchange between Amide Protons and Water.*—From an examination of Figure 5a one can see that the NH proton signals are much broader than those from the ring protons. Irradiation at the ^{14}N frequency has only a small influence on the line broadening (<3 Hz contribution to linewidths) which suggests that the origin of the effect is not from coupling to the ^{14}N nucleus but is almost certainly from an exchange process. On dilution, the line broadening increases (see Table 4) at both pH 0·3 and 6·5 indicating increased exchange with the water protons. From the line widths it is possible to estimate the exchange residence times by applying the equations appropriate to the slow exchange condition. At the higher pH value the two NH bands have similar line widths but at the lower pH value there is a factor of almost two difference in the NH line widths, the lower field NH protons *trans* to the carbonyl being in more rapid exchange (broader signal) with the water than the *cis*-NH proton. When NAD^+ is examined in H_2O solution at pH 2·5 and at concentrations in the range 0·2—0·04M the line widths of the observed amide NH proton signals are 0·2M; *trans*-NH 6·5 ± 1·0, *cis*-NH 7·5 ± 1·0 Hz and 0·04M; *trans*-NH 6·5 ± 1·0, *cis*-NH 9·5 ± 1·0 Hz. At pH 4·9 where the NAD^+ is now partially folded the $CONH_2$ line widths are essentially the same as in the unfolded form at low pH namely 0·2M; *trans*-NH 6·5 ± 1·0, *cis*-NH 6·5 ± 1·0 Hz and 0·04M; *trans*-NH 8 ± 2, *cis*-NH 10 ± 2 Hz. However at pH 5·9 the $CONH_2$ protons have very broad line widths (*ca.* 20 Hz).[22]

These results indicate that below pH 5·0 the exchange rate with the water protons are much slower than at comparable concentrations in nicotinamide. Furthermore, the *trans*-NH protons do not show an increased exchange rate over the *cis*-NH protons as was found in nicotinamide at low pH. Because of the comparatively narrow line widths for the amide NH protons in NAD^+ it might be possible to use their ^1H n.m.r. signals to monitor the interaction of NAD^+ and related molecules with dihydrofolate reductase at pH values <6.

TABLE 4

Concentration and pH dependence of the amide NH linewidths ($\nu_{\frac{1}{2}}$) and exchange times (τ) with water for nicotinamide

pH <1·0					pH 6·5				
Concentration	*trans*-NH		*cis*-NH		Concentration	*trans*-NH		*cis*-NH	
(M)	$\nu_{\frac{1}{2}}$(Hz)	τ (s)	$\nu_{\frac{1}{2}}$ (Hz)	τ (s)	(M)	$\nu_{\frac{1}{2}}$ (Hz)	τ (s)	$\nu_{\frac{1}{2}}$ (Hz)	τ (s)
4·0	25·5	0·0060	14·0	0·012	3·81	10·7	0·016	10·0	0·017
3·33	28	0·0064	15·0	0·011	3·18	13·5	0·012	14·0	0·012
2·86	30	0·0057	16·5	0·01	2·72	12·5	0·013	14·5	0·011
2·50	33	0·005	20	0·009	2·38	15·5	0·011	15	0·011
2·0	37	0·004	23	0·007	2·12	14·0	0·012		
1·33	38	0·004	24	0·007	1·27	21	0·008	19	0·009

A natural linewidth of 3 Hz was used in the calculation of exchange times.

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