Natural Abundance Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy. The Pyrimidine and Purine Nucleosides

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The ^{1}H noise decoupled natural abundance ^{15}N n.m.r. spectra of three pyrimidine and four purine nucleosides have been measured at 18.24 MHz and fully assigned. The first example of the use of off-resonance ^{1}H continuous wave decoupling as an assignment aid in ^{15}N n.m.r. is given. The ^{15}N spectrum of an equimolar (0.4M) mixture of uridine and adenosine in dimethyl sulphoxide (DMSO) showed no base-pairing effect upon the ^{15}N chemical shifts. In experiments employing inverse gated ^{1}H decoupling to quench the negative ^{15}N-(^{1}H) nuclear Overhause reffect (NOE), ^{15}N resonances, particularly from protonated nitrogens, showed anomalous intensities or were nulled by the ^{15}N-(^{1}H) NOE. In relation to this latter observation, the time dependence of the build-up and decay of the ^{15}N-(^{1}H) NOE has been investigated in a semi-quantitative manner and illustrated by transient Overhauser effect experiments on formamide.

RECENT advances in instrumentation [1-4] have rendered natural abundance ^{15}N n.m.r. spectra more readily available. Instruments employing a moderately high field (4.2 T), coupled with large diameter sample tubes (up to 30 mm o.d.) and quadrature detection, can improve the sensitivity of ^{15}N in an n.m.r. experiment by a factor of *ca.* 15—20.

This work extends previous [2,5-7] ^{15}N investigations on amino-acid derivatives and peptides to another class of compounds of fundamental biochemical importance, the pyrimidine and purine nucleosides. In addition, some difficulties associated with the routine suppression of the ^{15}N-(^{1}H) nuclear Overhauser effect (NOE) by the so-called inverse gated decoupling technique [8] are discussed.

Published ^{15}N n.m.r. studies of nucleoside derivatives have been restricted to the early ^{15}N-enriched work on 4-methoxy-1-methyl[$^{15}N_2$]pyrimidin-2-one (I),[9] [$^{15}N_5$]-adenosine triphosphate (II; R = ribofuranosyltriphosphate),[10] and the more recent report on yeast tRNA

[1] C. H. Bradley, G. E. Hawkes, E. W. Randall, and J. D. Roberts, *J. Amer. Chem. Soc.*, 1975, **97**, 1958.
[2] G. E. Hawkes, E. W. Randall, and C. H. Bradley, *Nature*, 1975, **257**, 767.
[3] T. B. Posner, V. Markowski, P. Loftus, and J. D. Roberts, *J.C.S. Chem. Comm.*, 1975, 769.
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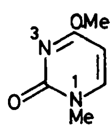
[5] P. S. Pregosin, E. W. Randall, and A. I. White, *Chem. Comm.*, 1971, 1602.
[6] J. A. Sogn, W. A. Gibbons, and E. W. Randall, *Biochemistry*, 1973, **12**, 2100.
[7] D. Gattegno, G. E. Hawkes, and E. W. Randall, *J.C.S. Perkin II*, 1976, 1527.
[8] R. Freeman, H. D. W. Hill, and R. Kaptein, *J. Magnetic Resonance*, 1972, **7**, 327; we shall use the following abbreviations when discussing the various types of ^{15}N spectra with ^{1}H irradiation: ^{15}N–$(^{1}H)_{cw}$ is continuous wave proton irradiation employed here in the off-resonance manner, where ν_2 is the difference between the proton irradiation frequency and the resonance frequency of the proton of interest (B_2 is the power of the ^{1}H irradiation); ^{15}N–$(^{1}H)_{noise}$ means noise modulated proton irradiation employed here for full ^{1}H decoupling; ^{15}N–$(^{1}H)_{g-noise}$ means the ^{1}H noise irradiation is gated in the manner described in the text.
[9] B. W. Roberts, J. B. Lambert, and J. D. Roberts, *J. Amer. Chem. Soc.*, 1965, **87**, 5439.
[10] J. A. Happe and M. Morales, *J. Amer. Chem. Soc.*, 1966, **88**, 2077.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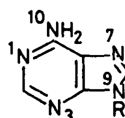
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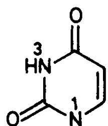
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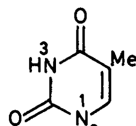
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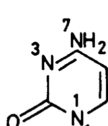
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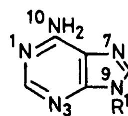
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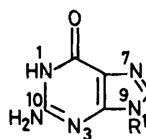
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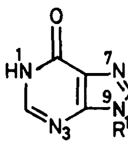
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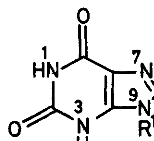
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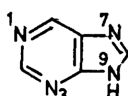

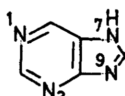
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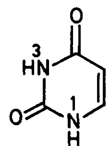
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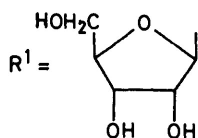
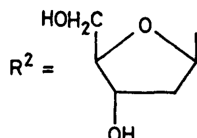




(III)[4] with ^{15}N at natural abundance. These ^{15}N chemical shift data (converted to our $^{15}NH_4{}^{15}NO_3$ reference as described in the Experimental section) are summarised in Table 1.

TABLE 1

Literature values for ^{15}N chemical shifts [a] of nucleoside derivatives

Compound (solvent)		Reference
(I) (acetic acid)	124 (N-1), 201 (N-3)	9
(II) (H_2O)	58.5 (N-10), 148.7 (N-9), 195.6 (N-1), 204.7 (N-3), 210.8 (N-7)	10
(III) (0.15M-NaCl in H_2O)	51.8 (G),[b] 57.1 (A),[b] 73.0 (C),[b] 126.0 (G, U),[b] 136.7 (U), 175—179 (C), 202—218 (G, A)	4

[a] Shifts in p.p.m. to higher frequency (lower field) of $^{15}NH_4{}^+$ signal of reference sample (see Experimental section). [b] The assignments for tRNA are those of Roberts and his co-workers.

Our natural abundance ^{15}N n.m.r. data for the nucleosides uridine (IV) (U), thymidine (V) (dT), cytidine (VI) (C), adenosine (VII) (A), guanosine (VIII) (G), inosine (IX) (I), and xanthosine (X) (X) are summarised in Tables 2 and 3. Also included are the ^{15}N chemical shifts for purine (XI) and uracil (XII). The structures shown for the nucleosides (IV)—(X) are the commonly accepted predominant tautomers, and it will be shown below that the ^{15}N chemical shifts are consistent with these structures.

TABLE 2

^{15}N Chemical shifts [a] for the pyrimidine nucleosides

Compound [b]	T/°C [c]			
(IV)	45	123.4 (N-1)	137.3 (N-3)	
	28	123.5 [d]	137.5 [d]	
(IV) (1M in H_2O–D_2O)	35	125.0	137.3	
(V)	45	123.7 (N-1)	135.3 (N-3)	
(VI)	45	73.5 (N-7)	132.8 (N-1)	
	28	74.0	132.7 [d]	189.1 [d] (N-3)
(VI) (1M in H_2O–D_2O)	35	72.0	131.7	
(XII)	45	111.2 (N-1)	138.8 (N-3)	

[a] See footnote *a*, Table 1. [b] Solutions 0.8M in DMSO unless otherwise stated. [c] Spectra at 45° were run with full ^1H noise decoupling. Those at 28° were run in the inverse gated decoupling mode, those at 35° were measured with the HFX-13 instrument. [d] These resonances were upright, otherwise all resonances were inverted. Missing assignments indicate resonances nulled by the NOE.

Nuclear Overhauser Effects.—The result of the NOE

TABLE 3

^{15}N Chemical shifts [a] for the purine nucleosides

Compound	Concentration (M) [b]	T/°C [c]					
(VII)	0.4	45	60.8 (N-10)	149.3 (N-9)	203.3 (N-1)	216.2 (N-3)	220.9 (N-7)
	0.8	45	60.7	149.3	203.1	215.8	220.5
	0.8	28		149.3 [d]	202.5 [d]	215.6 [d]	220.4 [d]
(VIII)	0.8	45	52.8 (N-10)	127.1 (N-1)	146.1 (N-3)	149.8 (N-9)	226.8 [d] (N-7)
	0.8	28	53.3	127.2	146.0 [d]	150.0 [d]	226.6 [d]
(IX)	0.8	45	154.1	154.3 (N-1, N-9)	193.8 (N-3)	228.6 (N-7)	
	0.8	28	154.4 [d]		193.7 [d]	228.3 [d]	
(X)	0.8	45	93.5 (N-3)	133.5 (N-1)	145.7 (N-9)	227.8 [d] (N-7)	
	0.8	28			145.8 [d]	228.7 [d]	
(XI)	1.25 in H_2O	45	169.4	173.2 (N-7, N-9)			
		28			230.4 [d]	245.6 [d] (N-1, N-3)	

[a] See footnote *a*, Table 1. [b] Solutions in DMSO unless otherwise stated. [c] See footnote *c*, Table 2. [d] See footnote *d*, Table 2.

upon the appearance of ^1H decoupled ^{15}N n.m.r. spectra has been discussed in detail.[2,11] In the extreme narrowing limit (which applies to the samples here) and for ^{15}N nuclei relaxed predominantly by the $^{15}N-^1H$ dipole–dipole mechanism, inverted and enhanced ^{15}N resonances are obtained. We have observed for ^{15}N spectra of the nucleosides run under similar conditions (concentration, solvent, ^1H noise decoupling, pulse flip angle, and pulse repetition rate) that nitrogens with directly bonded protons and the nitrogens bonded to the sugar ring (*i.e.* nitrogens bound to three centres) show relatively strong negative signals whereas the other non-protonated nitrogens show weak signals, either upright or inverted. This latter observation of weak resonances has two primary causes which possibly act in combination, a partial (negative) NOE reducing the signal intensity and signal saturation caused by a long spin–lattice relaxation time (T_1). The relative intensities of the resonances can greatly assist in their assignment.

In those cases where weak resonances were observed, or expected resonances were missing altogether from the spectrum, inverse gated proton decoupling was employed to quench the unfavourable NOE. In this technique the proton decoupler is switched on only during data acquisition (usually 0.7 s) following the ^{15}N observing pulse, and is switched off for a relatively long period (>10 s) between the end of one data acquisition time and the start of the next. The build-up of NOE is not instantaneous with switching on the decoupler (the build-up has a time constant which is equal to T_1 for the ^{15}N nucleus), and any NOE gained during the acquisition time should decay to zero during the decoupler-off period. Thus upright, decoupled ^{15}N resonances should result. This technique proved successful for those nitrogens which give *reduced* resonances, but for the nitrogens with directly bonded protons the quenching was considerably more difficult. For success in giving upright signals, the ratio, R, of decoupler-off to -on times needed to be larger. This situation is exemplified by the ^{15}N spectra of uridine shown in Figure 1. In both 'gated' spectra (Figure 1b,c), the resonance due to the non-protonated nitrogen (N-1) is upright. By contrast, the higher frequency resonance (from the protonated N-3) is nulled in the inverse gating mode with R *ca.* 12 : 1 (Figure 1b) and is upright when R is increased to *ca.* 37 : 1 (Figure 1c). We will consider in more detail later these differences in the rate of NOE decay as well as questions of differential NOE build-up.

^{15}N *Chemical Shifts.—The pyrimidine nucleosides.* The key to the assignment of the ^{15}N resonances from the pyrimidine nucleosides is the off-resonance $^{15}N-(^1H)_{cw}$ spectrum of thymidine (Figure 2b,c). The expected doublet (N-3) and singlet (N-1) pattern was not observed. However the higher frequency resonance was considerably broadened whereas the other resonance remained sharp, thus indicating that the higher frequency resonance is due to the protonated N-3. This broadening of the resonance is due to exchange of the proton at N-3 at an intermediate rate (faster rates would give a sharp singlet, slower rates the doublet). On the other hand, under similar conditions of proton irradiation power (B_2) and frequency offset (ν_2) the ^{15}N spectrum of

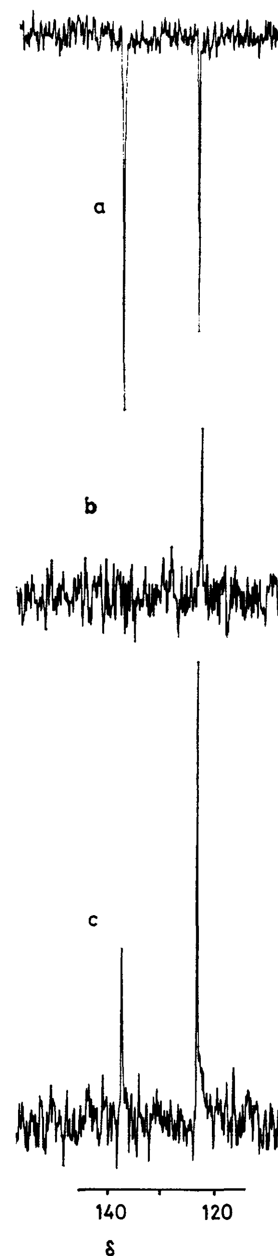


FIGURE 1 Natural abundance ^{15}N spectra (p.p.m. from $^{15}NH_4^+$) of 0.8M-uridine in DMSO (WH-180): a, with ^1H noise decoupling, 1 527 transients accumulated in 2.1 h; b, with inverse gated ^1H noise decoupling, decoupler on for 0.8 s and off for 9.2 s, 2 027 transients accumulated in 5.6 h; c, as b but decoupler off for 29.2 s, 1 183 transients accumulated in 9.9 h

N-methylformamide clearly showed the expected splitting with the one-bond $^{15}N-^1H$ coupling reduced in the expected 'off-resonance' manner from *ca.* 93 to *ca.* 36 and 54 Hz respectively (*cf.* Figure 2b,c). Interestingly in these latter cases the resonances were inverted,

[11] G. E. Hawkes, W. M. Litchman, and E. W. Randall, *J. Magnetic Resonance*, 1975, **19**, 255.

indicating that a significant NOE was induced by the ^{1}H irradiation despite its 'off-resonance' character.* The proton of the N-H group is expected to be more acidic in thymidine than in a normal amide group because of the presence of the two flanking carbonyl groups

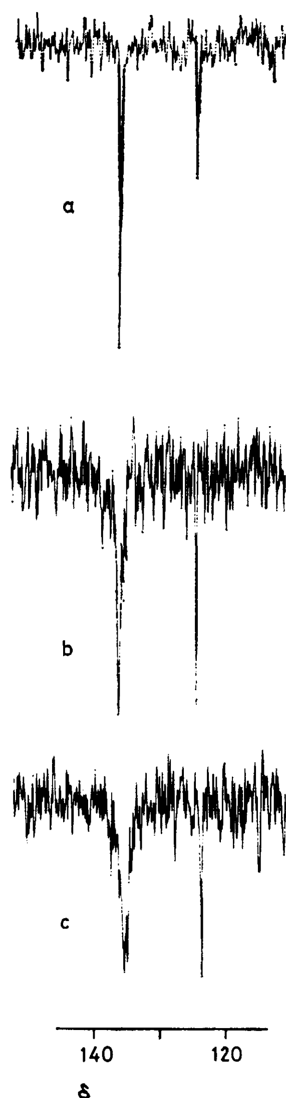


FIGURE 2 Natural abundance ^{15}N spectra (p.p.m. from $^{15}NH_4^+$) of 0.8M-thymidine in DMSO (WH–180): a, with ^{1}H noise decoupling, 679 transients accumulated in 0.6 h; b, with off-resonance ^{1}H continuous wave decoupling, decoupler frequency offset 7.9 p.p.m. from N*H* resonance, 700 transients accumulated in 0.6 h; c, as b but with frequency offset 10.8 p.p.m., 887 transients accumulated in 0.7 h

(C-2 and -4). In Figure 3 are shown the off-resonance ^{15}N-$(^{1}H)_{cw}$ spectra of isopropylurea and hydantoin. The

* We have pointed out [11] that an improper selection of the decoupler conditions (either ν_2 or B_2 or both) in a ^{15}N-$(^{1}H)_{cw}$ off-resonance experiment could induce sufficient NOE to null the ^{15}N multiplet. We have now observed this possibility experimentally for the first time with *N*-methylformamide. However with the B_2 value employed above (*ca.* 5 W) a value of ν_2 in the region 27—33 p.p.m. (from N–*H* resonance) was required for this type of nulling. This offset is much larger than the values normally used and so the likelihood of nulling in this manner is not great.

doublet and triplet distinction makes the assignments for isopropylurea unambiguous. For hydantoin however,

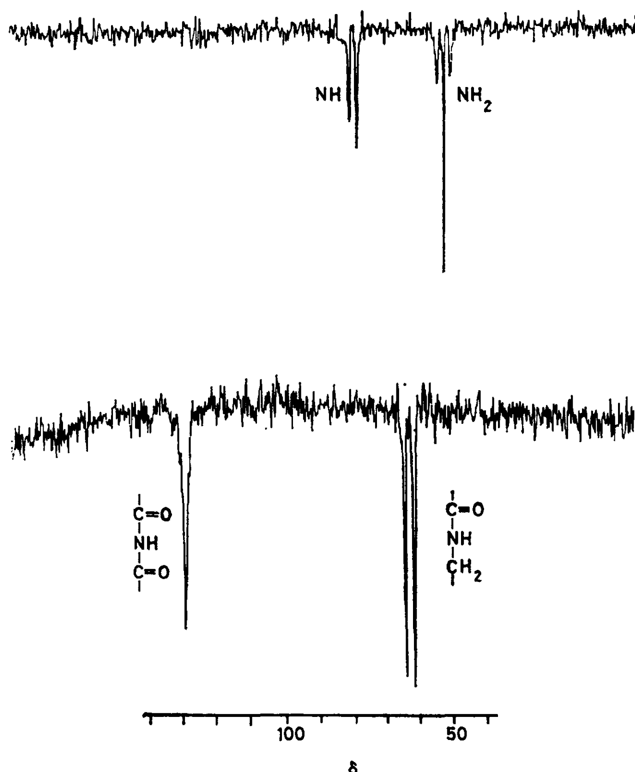
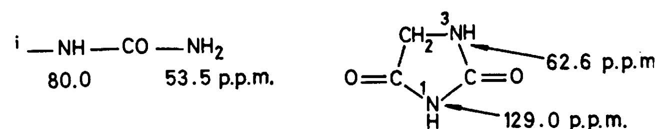


FIGURE 3 Natural abundance ^{15}N spectra (p.p.m. from $^{15}NH_4^+$) (HFX–13) of isopropylurea (upper, 76 877 transients accumulated in 17.5 h) and hydantoin (lower, 63 211 transients accumulated in 14.4 h), 2M in DMSO, with off-resonance ^{1}H continuous wave decoupling

only one doublet, not two, was obtained. The high frequency resonance (N-1) was broadened compared



to that obtained from the fully decoupled ^{15}N-$(^{1}H)_{noise}$ spectrum, but was not split. Again we have a nitrogen flanked by two carbonyl groups. As in ^{13}C n.m.r., off-resonance ^{1}H continuous wave irradiation has proved to be of considerable utility in the assignment of ^{15}N resonances. However the possibility of proton exchange at nitrogen must be considered when interpreting the data.

With the above assignment for thymidine, the ^{15}N resonances from uridine may be readily assigned as N-3 at 137.3 and N-1 at 123.4 p.p.m. Comparison of the shifts for uridine and thymidine (Table 2) shows that the introduction of the methyl group at position 5 shifts the N-3 resonance to lower frequency by *ca.* 2.0 p.p.m. but has negligible effect upon the N-1 resonance, although in both cases the methyl carbon is at a position γ to the nitrogen. Conversion of uracil (XII) to uridine (IV) involves the introduction of a furanose ring onto

N-1; this is expected to affect the resonance position of N-1 more than that of N-3 and so the ^{15}N resonances of uracil are assigned as N-3 at 138.8 and N-1 at 111.2 p.p.m. These substituent effects are then: at N-1 (α-effect) $+12.2$ and at N-3 (γ-effect) -1.5 p.p.m. The alternative assignment for uracil would yield an α-effect of -15.4 and a γ-effect of $+26.1$ p.p.m., which are clearly inadmissible.[7]

The ^{15}N-$(^1H)_{noise}$ spectrum of cytidine (VI) displayed two peaks only, at 73.5 and 132.8 p.p.m., of which the former arises from the NH_2 group, and the latter, by comparison with the assignments for uridine and thymidine, comes from N-1. A ^{15}N-$(^1H)_{g\text{-}noise}$ experiment on the same sample of cytidine revealed the third resonance, from N-3, at 189.1 p.p.m. (Figure 4). It is notable in Figure 4 that, for the particular decoupler duty cycle employed, the negative NOE on the NH_2 resonances was not completely suppressed (the peak is still inverted) whereas the peaks due to the non-protonated nitrogens N-1 and N-3 are upright.

The purine nucleosides. The ^{15}N resonance assignments shown for adenosine (VII) in Table 3 are the same as those given by Happe and Morales [10] for adenosine triphosphate. As with the pyrimidine nucleosides, for spectra run under similar conditions of pulse power and repetition rate, the protonated nitrogen (N-10) and the nitrogen bonded to the furanose ring (N-9) show the more intense, inverted ^{15}N resonances. By comparison with the ^{15}N-$(^1H)_{noise}$ spectrum of adenosine, for the ^{15}N-$(^1H)_{noise}$ spectra of the nucleosides (VIII)—(X) under full power and decoupling conditions, a strong inverted resonance in the region 145—154 p.p.m. was

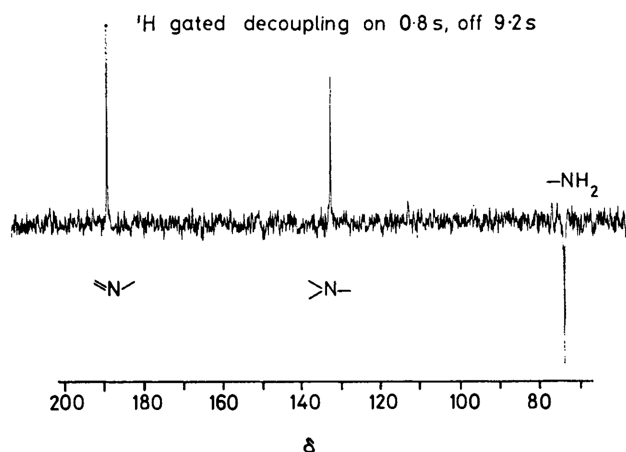
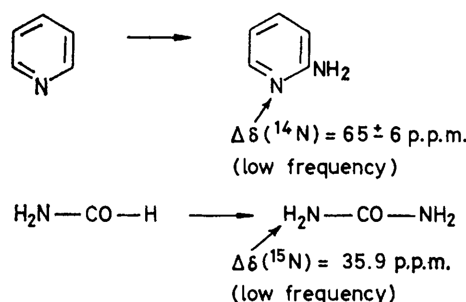


FIGURE 4 Natural abundance ^{15}N spectrum (p.p.m. from $^{15}NH_4^+$) of 0.8M-cytidine in DMSO (WH-180), with inverse gated ^1H noise decoupling, 3 291 transients accumulated in 9.1 h

assigned to N-9, whereas a weak resonance, either inverted [(VII) or (IX)] or upright [(VIII) or (X)] at >220 p.p.m. was assigned to N-7.

The ^{15}N-$(^1H)_{noise}$ spectrum of guanosine (VIII) showed three intense inverted resonances at 52.8, 127.1, and 149.8 p.p.m.; the last was assigned to N-9, the lowest frequency peak (52.8 p.p.m.) to N-10, and the resonance at 127.1 p.p.m. to N-1. As described above the resonance at 226.8 p.p.m. was assigned to N-7 and the remaining resonance at 146.1 p.p.m. to N-3. In the spectrum of inosine (IX), the two intense, closely spaced resonances at 154.1 and 154.3 p.p.m. were assigned to N-1 and -9 (although they are too close for a satisfactory distinction to be made), the resonance at 228.6 p.p.m. to N-7, and the remaining resonance at 193.8 p.p.m. to N-3. On the basis of the assignments for guanosine and inosine, it can be seen that substitution at C-2 of inosine with the NH_2 group (an NH_2 β-effect) to form guanosine, has resulted in low frequency shifts of 27.1 ± 0.1 and 47.7 p.p.m. for N-1 and -3 respectively. This is entirely consistent with published data on nitrogen chemical shifts for the β-nitrogen substituent, for example for the two pairs pyridine and 2-aminopyridine,[12] and formamide and urea.[13]



Of the three strong, inverted resonances for xanthosine (X), the one at 145.7 p.p.m. is assigned to N-9, the other two at 93.5 and 133.5 p.p.m. are due to N-1 and -3. The structure of the six-membered ring of xanthosine is similar to that of uracil (XII) and the assignments for xanthosine follow directly, *i.e.*, N-1 at 133.5 p.p.m. (*cf.* N-3 of uracil at 138.8 p.p.m.) and N-3 at 93.5 p.p.m. (*cf.* N-1 of uracil at 111.2 p.p.m.). N-3 of xanthosine has a nitrogen (N-9) at the β-position which N-1 of uracil does not have, and as described above the resonance position for N-3 of xanthosine is 17.7 p.p.m. to lower frequency than N-1 of uracil.

The ^{15}N-$(^1H)_{noise}$ spectrum of the parent tautomeric purine system (XI) (1.25M in H_2O) was measured with full decoupling conditions at 45°, and showed only two signals at 169.4 and 173.2 p.p.m. Inverse gated decoupling (28°) revealed the two remaining resonances at 230.4 and 245.6 p.p.m. These latter resonances are due to N-1 and -3 and the two at lower frequency are due to N-7 and -9. The two tautomers are evidently in the fast exchange limit, but because of the lack of suitable model compounds, a complete assignment cannot be made as yet.

Medium Effects and Base Pairing.—The possibility that base pairing may have a large effect on ^{15}N shifts is an important one. Additionally it seemed prudent to check for other medium effects: temperature, concentration, and solvent.

[12] M. Witanowski, L. Stefaniak, and H. Januszewski in 'Nitrogen NMR,' eds. M. Witanowski and G. A. Webb, Plenum Press, London and New York, 1973, ch. 4.

[13] J. P. Warren and J. D. Roberts, *J. Phys. Chem.*, 1974, **78**, 2507.

The observed temperature effects upon the ^{15}N shifts shown in Tables 2 and 3 are <1 p.p.m. and are too small to be considered in detail in view of the errors in the measurement of the ^{15}N shifts and possible temperature effects upon the lock signal and reference signal employed (see Experimental section).

The ^{15}N-$(^1H)_{noise}$ spectra of two pyrimidine nucleosides, uridine (IV) and cytidine (VI), were measured in aqueous solution (1M in 80% H_2O–20% D_2O) at 9.12 MHz and *ca.* 35°. Comparison of these shifts (Table 2) with those obtained in DMSO solution shows only shift differentials which are <2 p.p.m.

The ^{15}N spectrum of an equimolar mixture of uridine and adenosine (0.4M in each nucleoside) was measured at 45 and 28° in order to investigate the possibility of base-pairing effects upon the ^{15}N spectra. The nitrogens which would be involved in base pairing would be N-3 of uridine and N-1 and -10 of adenosine. ^{15}N Shifts were found as follows: adenosine (45°) 60.6, 149.4, 203.1, 215.7, 220.4 p.p.m.; uridine (45°) 123.3, 137.5 p.p.m.; adenosine (28°) 61.4, 149.3, 202.5, 215.4, 220.3 p.p.m; uridine (28°) 123.6 p.p.m. (one peak only observed). Comparison with the data in Tables 2 and 3 for the individual nucleosides at the same temperatures and at 0.8M concentration, for which the total nucleoside concentration is the same, shows that within the limit of experimental error the shifts are unaffected by the mixture. Possible exceptions are the NH_2 group of adenosine for which the resonance was not observed at 28° (Table 3), and N-3 of uridine for which a resonance was similarly not observed at 28° in the mixture. A more thorough search for the effect of base pairing upon ^{15}N chemical shifts would benefit from experiments in which the temperature could be varied over a wider range.

Transient Overhauser Effects.—In the duty cycle employed for the ^{15}N-$(^1H)_{g\text{-}noise}$ experiments, the decoupler was switched on a short time (t) before the application of the ^{15}N analytical pulse, to allow for a finite rise time of the decoupler. If any substantial build-up of NOE is unwanted, t must be kept as small as possible. In practice the minimum value for t on our WH-180 instrument (apart from 0.0) was 0.1 s. When t is of a similar order of magnitude to the relaxation time T_1 for the ^{15}N nucleus, then nulled or inverted ^{15}N resonances are obtained. These effects upon the ^{15}N spectrum of formamide are shown schematically in Figure 5. The analytical expression governing the signal intensity is (1):[8,14] where S_t is the signal intensity at time

$$\ln(S_\infty - S_t) = -t/T_1 + \ln(S_\infty - S_0) \qquad (1)$$

t, and S_∞ is the intensity after a time $t \gg T_1$. The observed signal $(1 + \eta)$, where η is the NOE factor, is given by (2). Application of equations (1) and (2) to

$$1 + \eta = S_\infty/S_0 \qquad (2)$$

the signal intensities shown in Figure 5, yields a value[15] for T_1 of 21 s, and $1 + \eta = -4.0$. This value for $1 + \eta$ is about the theoretical maximum (−3.93) in the extreme narrowing limit. From equation (1) the signal nulling condition is $S_t = 0$, and a combination of equations (1) and (2) shows that this occurs when equation (3) holds.*

$$t_{null} = T_1.\ln(\eta/1 + \eta) \qquad (3)$$

Thus the greater the NOE, then the smaller is the factor $\ln(\eta/1+\eta)$. For $\eta = -4.93$, $t_{null} = 0.23T_1$. Equation (3) is considered in more detail in Figure 6 where t_{null} is shown as a function of η.

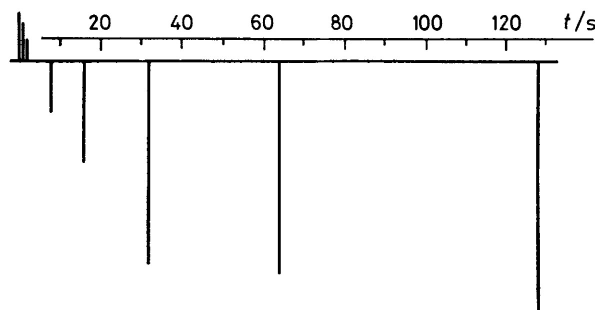


FIGURE 5 Schematic representation of the ^{15}N spectrum of formamide as a function of the ^1H decoupler pre-irradiation time t. The experiment yields T_1 21 s and $1 + \eta$ −4.0

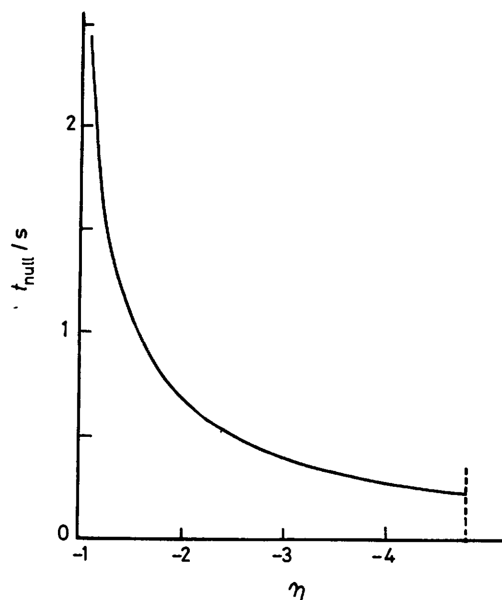


FIGURE 6 Plot of equation (3) for $T_1 = 1$ s

In the ^{15}N-$(^1H)_{g\text{-}noise}$ decoupling experiments on the nucleosides, the value for t used (0.1 s) was probably too large for upright resonances to result from nitrogens with shorter T_1 values and large η values. This can also explain why the NH_2 resonance from cytidine (Figure 3)

* Equation (3) is only meaningful within the limits $-1 > \eta > -4.93$.

[14] K. F. Kuhlmann and D. M. Grant, *J. Chem. Phys.*, 1971, **55**, 2998.

[15] We estimate the errors on the T_1 and NOE determinations to be approximately ±20%. We have also measured T_1 for this sample of formamide by the inversion–recovery method: this gave $T_1(^{15}N) = 25$ s. G. C. Levy, C. E. Holloway, R. C. Rosanske, J. M. Hewitt, and C. H. Bradley, personal communication, have measured the value 14.5 s.

was inverted. Due to the two directly bonded protons, T_1 for this nitrogen will be shorter than for the other two nitrogens which do not have directly bonded protons. If we assume that the NH_2 resonance has the maximum NOE (η −4.93), then $0.1 \text{ s} > 0.23T_1$ *i.e.*, $T_1 < 0.43$ s. This is not an unreasonable value for a protonated nitrogen in a moderately small molecule since Levy and his co-workers [15] have measured a value for T_1 in *m*-aminobenzoic acid of 0.68 s. However we are still left with the problem of why a large time ratio *R* was necessary for observation of the NH resonance of uridine (Figure 1) in the inverse gated mode. For this we must consider in more detail the time dependence of the NOE decay, when the ^1H decoupler is switched off. Some results for formamide are illustrated schematically in Figure 7. In each of these experiments the ^1H decoupler

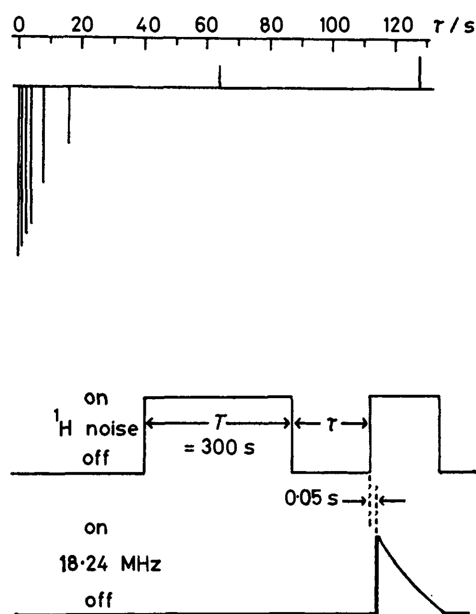


FIGURE 7 Schematic representation of the ^{15}N spectrum of formamide (upper) in response to the ^1H decoupler duty cycle shown. The experiment monitors the decay of NOE

is on for a fixed, long time ($T \gg T_1$) to build up the maximum NOE, is then switched off for some variable time (τ) to allow the NOE to decay, and then finally is switched on again simultaneously * with application of the ^{15}N analytical pulse. Comparison of Figures 5 and 7 shows that the decay of NOE (Figure 7) is a much slower process than the build-up (Figure 5). It is significant again to consider the time required (on this occasion τ) for the nulled signal situation to develop by NOE decay. In the build-up experiment (Figure 5) t_{null} was *ca.* 4 s, whereas for the decay experiment (Figure 7) τ_{null} was *ca.* 32 s. Analytical expressions to describe this decay of NOE have been given by Noggle and Schirmer [16] and Canet [17] has given some calculated solutions. However since the expressions involved are a double exponential in the ^{15}N and ^1H relaxation times and the ^{15}N–^1H cross relaxation time, the analysis is not straightforward and will not be discussed further here. Nevertheless the reason for the differential response of the two resonances in the ^{15}N spectra of uridine (Figure 1) to the inverse gating cycle is very clear. It must be primarily due to a shorter T_1 for the higher frequency NH resonance which allows for rapid build-up of NOE during the time *t*. Once a significant amount of NOE has built up, then a τ value substantially longer than the ^{15}N T_1 is required for the decay of NOE.† By contrast the longer T_1 for N-1 (non-protonated nitrogen) means that there is comparatively little initial build-up of NOE and accordingly the resonance is easily observed.

Conclusion.—Although we cannot exclude the possibility that small percentages of minor tautomers, in fast exchange with the predominant form, do contribute, it did prove possible to assign all the ^{15}N resonances from the nucleosides on the basis of their commonly accepted predominant tautomeric forms. This is not trivial and could not have been presumed, since ^{15}N studies [18] on 1-phenyl-3-methylpyrazolin-5-one clearly revealed the presence of a third tautomer whereas only two had previously been substantiated.

For the single example chosen (adenosine and uridine) the ^{15}N shifts do not seem sensitive to base pairing. Also for the limited solvent study on uridine and cytidine, changing the solvent from DMSO to water did not significantly change the ^{15}N spectra. Accordingly the ^{15}N resonance assignments made here for the nucleosides in DMSO may be used as a basis for future assignment of nucleotide ^{15}N spectra where aqueous media may be employed (see the data on tRNA from Roberts and his co-workers [4] in Table 1).

We have shown the value of off-resonance ^1H (continuous wave) irradiation in ^{15}N n.m.r. as an assignment aid. In addition we have shown the use of the inverse gated ^1H noise decoupling experiment in ^{15}N n.m.r. We have confirmed experimentally the calculations of Canet [17] that the decay of NOE may be a slow process relative to the build-up. Consequently considerable care must be taken in selection of the decoupler duty cycle in the inverse gated ^1H decoupling experiment.

EXPERIMENTAL

Unless otherwise stated, all ^{15}N spectra were measured in the pulse-Fourier transform mode with the Bruker WH-180 instrument, operating at 18.24 MHz for ^{15}N as previously described.[1-4] Other ^{15}N spectra were determined at 9.12 MHz using the Bruker HFX-13 instrument as previously described.[7] Sample temperatures for the WH-180 were *ca.* 45° with ^1H noise decoupling at full power (*ca.* 5 W) or

* This final switching on of the decoupler is necessary if decoupled resonances are required. Because all timing in this experiment was computer controlled, *t* could be kept to smaller values than when the switching was controlled by the 'Multipulse' unit in the experiments on the nucleosides. The value for *t* was 0.05 s.

† Canet [17] concludes that a waiting time (τ) of the order of 10 T_1 is advisable in such gated decoupling experiments.

16 J. H. Noggle and R. E. Schirmer, 'The Nuclear Overhauser Effect. Chemical Applications,' Academic Press, New York and London, 1971.
17 D. Canet, *J. Magnetic Resonance*, 1976, **23**, 361.
18 G. E. Hawkes, E. W. Randall, J. Elguero, and C. J. Marzin, *J.C.S. Perkin II*, in the press.

ca. 28° with inverse gated decoupling. Sample temperatures for the HFX-13 were *ca.* 35°. ^{15}N Chemical shifts are quoted in p.p.m. to higher frequency (low field) from the ammonium ion resonance of an external sample of 5M-$^{15}NH_4{}^{15}NO_3$ in 2N-nitric acid. The accuracy of the ^{15}N shifts is *ca.* 0.15 for the WH-180 and *ca.* 0.3 p.p.m. for the HFX-13. The ^{15}N chemical shift reference employed by Roberts and his co-workers [4] (see Table 1) for tRNA is external 1M-D$^{15}NO_3$. We estimate [7] that the two reference scales are related by $\delta(^{15}NH_4^+) = (355.0 \pm 0.1) - \delta(D\text{-}^{15}NO_3)$. Phase corrections to ^{15}N spectra were those required to produce an upright $^{15}NO_3^-$ resonance from the reference sample, under all ^1H decoupling conditions, and a negative signal for $^{15}NH_4^+$ in the non-gated cases.* With the WH-180, for normal accumulation of the nucleosides, with full ^1H noise decoupling, pulse flip angles in the region 30—60° were employed, repeated every 3—5 s. With the HFX-13 *ca.* 30° pulses were employed, repeated every 0.8 s.

* We have found this sample to be as useful for phase referencing as for shift referencing. In simple non gated $^{15}N-(^1H)_{noise}$ decoupling experiments the phasing may be set using the two signals from the sample; $^{15}NO_3^-$ (upright) and $^{15}NH_4^+$ (inverted).

The nucleosides were all obtained from Sigma, and spectra were obtained from samples dissolved in DMSO in 25 mm o.d. tubes (WH-180) carrying a concentric 5 mm o.d. tube of D_2O for the field-frequency stabilisation signal. Spectra from the HFX-13 were from 10 mm o.d. tubes. The formamide sample (90% formamide–10% [$^2H_6$]-DMSO) was not degassed in any way before use.

Note added in proof: Since this manuscript was presented for publication, Roberts and his co-workers [19] have published a similar ^{15}N n.m.r. study of nucleosides in DMSO solution. Their assignments are the same as ours except for an interchange of the assignments of Happe and Morales [10] for N-1 and N-3 of adenosine. In addition these authors [19] observed only three ^{15}N resonances for purine in DMSO solution.

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[6/1660 *Received, 27th August,* 1976]

[19] V. Markowski, G. R. Sullivan, and J. D. Roberts, *J. Amer. Chem. Soc.*, 1977, **99**, 714.