The Nitrogen-15 Magnetic Resonance of Glycine

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Abstract: The ^{15}N relaxation times and chemical shifts of glycine in water at various pH's are reported. The spin–lattice relaxation of the ^{15}N is due to the N–H dipole–dipole interaction and another mechanism apparently spin–rotation. It is conclusively demonstrated that exchange-modulated scalar relaxation is not a viable mechanism for spin–lattice relaxation. Chemical shifts *vs.* pH are reported and are shown to be reasonably represented by averaging of constant chemical shifts for the three principal ionic species of glycine in water. Line width measurements are also reported and can be interpreted as due to chemical exchange with exchange times in good agreement with previously reported values measured by proton nmr.

Amino groups are found widely in biologically important compounds. Nuclear magnetic resonance (nmr) is a valuable tool to study such groups. ^{15}N magnetic resonance should be of particular use in biological investigations since amino sites can be specifically enriched and labeled with ^{15}N.

Amino acids are particularly interesting. The ^{1}H, ^{13}C, and ^{15}N magnetic resonances of glycine have been reported[1-3] and show interesting effects which have been attributed to proton exchange[3] and dimerization.[2] The complete and systematic data reported herein casts doubts on some of these conclusions.

Experimental Section

The spectra were obtained at a frequency of 9.12 MHz using a ^{19}F lock on external C_6F_6. The experimental method is the same as described previously,[4] except that continuous wave decoupling (power about 0.5 W) was used in place of broad-band decoupling, and the undecoupled spectra were obtained by moving the decoupling frequency off resonance by about 12 kHz.

Glycine, enriched to 99.1% in ^{15}N, was purchased from Wilmad Glass Co. and used without further purification. HCl and KOH were used to adjust the pH. The pH was measured at appropriate temperatures with a Copenhagen Radiometer Model 25. The molality of the glycine varied from 2.1 to 1.6. The reagents and dilution seemed to have little effect since experiments on back-titrated samples gave reproducible results.

No attempt was made to remove dissolved oxygen, but its effect on the NOE was found to be negligible when, with one sample, it was removed enzymatically by adding glucose (~3 mg/cm^3) and glucose oxidase (Aldrich Chemical Co., Inc.). On this same sample, the effect of possible metal ion impurities was checked by adding EDTA (~2 mg/cm^3); the NOE was found to be consistent with other samples at the same pH. Additionally, a sample was back titrated from pH 9.08 to 4.13 and the original NOE was regenerated.

Chemical shifts were referenced to external saturated aqueous ammonium chloride.

Results and Discussion

The ^{15}N nmr spectra at various pH values are shown in Figure 1. The detailed data obtained from these are presented in Table I and will be discussed below. The nuclear Overhauser effects (NOE) reported follow the convention of Noggle and Schirmer.[5]

A. Chemical Shift. The pH-dependent chemical shifts are in reasonable agreement with previously reported values[3] with a systematic deviation of 1 ppm, presumably due to differences in the referencing method and the fact that bulk susceptibility corrections were made in neither case.

The variation with pH is consistent with chemical shift averaging among the three species (cation, G^+; zwitterion, $G^\pm$; anion G^-)

$$\delta(G^+) = 5.6 \text{ ppm}$$
$$\delta(G^\pm) = 7.8 \text{ ppm}$$
$$\delta(G^-) = -4.5 \text{ ppm}$$

(all with respect to external NH_4^+) and $\delta(\text{obsd}) = \Sigma_i X_i \delta_i$ with X_i the mole fraction of the species involved. The last value is somewhat uncertain due to the paucity of measurements at high pH.

The fit of these mean shifts to the data is illustrated in Figure 2. Hence, the chemical shifts can be explained by a simple acid–base equilibrium model.

The downfield shift of $G^\pm$ with respect to G^+ may be due to more favorable conditions for hydrogen bonding in the zwitterion resulting in a reduced electron density on the nitrogen.[6] It has been understood[7a] that there is no isotope effect between ^{14}N and ^{15}N; it is noteworthy that the ^{15}N shifts in glycine appear to be in the opposite direction to the ^{14}N[7b] shifts.

B. Line Widths. The analysis of line shapes of nmr spectra to study proton exchange is a well-established technique[8] which has been mostly restricted to proton magnetic resonance. The advantages of ^{15}N in terms of studying specifically labeled positions in complex molecules has been remarked upon previously.

Glycine is an excellent test of this method. The exchange rates have been measured previously using proton magnetic resonance[9] and the appropriate calculations for obtaining exchange rates from line widths and the known coupling constant have been worked out.[10] The previously reported[3] $J = 73$ Hz at pH < -0.5 and room temperature is in reasonable agreement with our $J = 69$ Hz measured at pH 3.13 and 2°. Our line width measurements at 42° are reported in Table I; the decoupled line width is taken to be the line width in the absence of exchange. The exchange rates obtained from these widths are reported in Table II.

Comparison of these results with ref 9 is difficult since their work was done at 25° and they did not report the original data. To reconstruct the data, we assume that their eq 17

$$\frac{1}{\tau} = \frac{370}{1 + 2.35[H^+]} + 10.69[G^+]/[H^+] \qquad (1)$$

applies to the cation (G^+) at all H^+ concentrations. For the zwitterion ($G^\pm$), we use their eq 20

$$1/\tau = 180 + 640[G^\pm] + (0.20 + 9.54[G^\pm])10^{-2}/[H^+] \qquad (2)$$

and take weighted averages for pH regions in which both species are present.

To compare the data at different temperatures, the temperature dependence of the line width was measured (Table II) at pH 3.13. These data are roughly Arrhenius with an apparent activation energy of 12.2 ± 1.0 kcal/mol. This value is in excess of typical H-bonding energies of 2–3 kcal

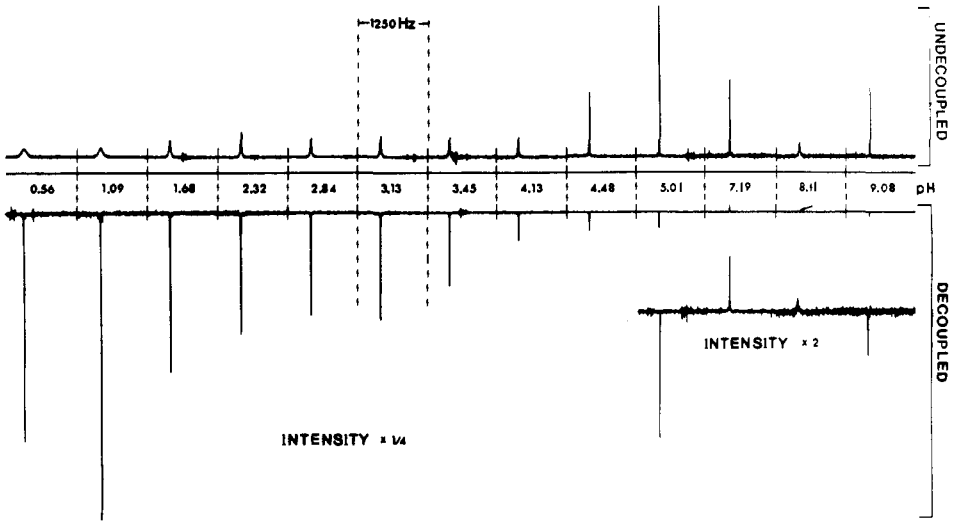


Figure 1. Proton-coupled (upper trace) and -decoupled ^{15}N nmr spectra of glycine at indicated pH values at 42°. The number of scans varies from 16 to 1024, but the spectra are normalized to indicate the correct relative intensity. The decoupled spectra are ×¼ size compared to the coupled spectra except the four spectra in the insert which are ×2 size.

Table I. ^{15}N Nmr Data for Aqueous Glycine at 42 ± 1°

pH	δ,[a] ppm	$\Delta\nu_{1/2}$, Hz[b]	NOE[c]	T_1, sec	$[G^-]$[d]	$[G^\pm]$[d]	$[G^+]$[d]
<−0.5			−4.85	12.7 ± 1.0[e]			
			(−4.13)[e]				
−0.3			−2.9[e]	11.8 ± 2.0[e]			
0.5	6.9[f]						
0.56	5.7	104	−5.1	10.1 ± 0.3	9.6×10^{-12}	1.6×10^{-2}	0.98
1.09	5.5	79	−4.9	10.4 ± 0.3	1.1×10^{-10}	5.2×10^{-2}	0.95
1.68	5.4	35	−4.8	9.7 ± 0.4	1.4×10^{-9}	0.18	0.82
2.32	6.9	20	−4.9	10.1 ± 0.4	1.7×10^{-8}	0.48	0.52
2.8	8.1[f]						
2.84	7.2	25	−4.5	10.5 ± 0.3	8.7×10^{-8}	0.76	0.25
3.13	7.9	25	−3.9	9.6 ± 0.2	1.9×10^{-7}	0.86	0.14
3.45	7.7	25	−3.1	10.7 ± 0.5	4.3×10^{-7}	0.93	7.4×10^{-2}
3.7	8.7[f]						
4.13	7.6	18	−2.4	9.0 ± 0.6	2.2×10^{-6}	0.98	1.6×10^{-2}
4.48	7.9	7.1	−1.9	6.6 ± 0.2	7.5×10^{-6}	0.99	7.4×10^{-3}
5.01	8.1	3.6	−1.4		2.6×10^{-5}	0.998	2.2×10^{-3}
6.0	8.7[f]						
6.3	8.9[f]						
6.4			−2.15[e]				
6.6	9.0[f]						
7.19	8.1	6.4	−0.6	2.3 ± 0.2	3.9×10^{-3}	0.996	1.4×10^{-3}
8.11	7.6	25	−0.5	1.1 ± 0.4	3.1×10^{-2}	0.97	1.7×10^{-6}
9.08	4.0	4.4	−1.3		0.23	0.77	1.4×10^{-7}
13.6	−3.5[f]						

[a] Chemical shifts δ downfield from external aqueous ammonium chloride. [b] Line widths $\Delta\nu_{1/2}$ of the undecoupled signals. [c] NOE's determined according to ref 5, p 47, eq 3.5, error ~0.20. [d] Relative concentrations of the various forms of glycine, determined using reported pK values (ref 9); G^+, cation; $G^\pm$, zwitterion; G^-, anion. [e] Reported NOE and T_1 values (ref 3). [f] Reported chemical shifts upfield from external 10 M $H^{15}NO_3$ (ref 3) converted to the present scale using chemical shift values reported by R. Grinter and J. Mason, *J. Chem. Soc. A*, 2196 (1970).

Table II. Proton Exchange Rates in Glycine at 42°

pH	$1/\tau_e$,[a] sec^{-1} (this work)	$1/\tau_e$,[b] sec^{-1} (ref 9)
0.56	1410	1120
1.09	2130	2130
1.68	5000	4350
2.32	7690	6280
2.84	6670	6350
3.13	6670	6240
3.45	6670	6370
4.13	9430	11400

[a] Measured at probe temperature of 42°. [b] Calculated using weighted averages of eq 17 and 20 of ref 9 and corrected from 25 to 42° using the Arrhenius equation with an activation energy of 12.2 kcal/mol.

and likely involves the breaking of N–H bonds.

The comparison of our data with ref 9 is given in Table II. Considering all of the extrapolations and interpretations necessary, the agreement is very good. The large deviation at pH 0.56 may be due to the failure of the approximate eq 10 which we used on the ^{15}N line widths. The error at pH 4.13 is likely due to the small difference in the coupled and decoupled line widths at this pH. Extension or improvement of these data would require more sophisticated theoretical techniques such as deconvolution analysis. Even so, the average deviation of 12% is good for this method.

Figure 1 shows a curious anomaly in the line widths; at pH 8.11, well into the very fast exchange limit, the widths of *both* the coupled and decoupled spectra increase to 25 Hz compared to ~5 Hz at pH 7.19 and 9.08. Extrapolation of eq 2 suggests that at this pH the exchange rate is $1/\tau_e \sim 7 \times 10^7$ sec^{-1}, very close to the ^{15}N Larmor frequency $\omega_N = 5.73 \times 10^7$ rad/sec. Since there is no similar effect on T_1, this is no ordinary relaxation; in any case, normal fre-

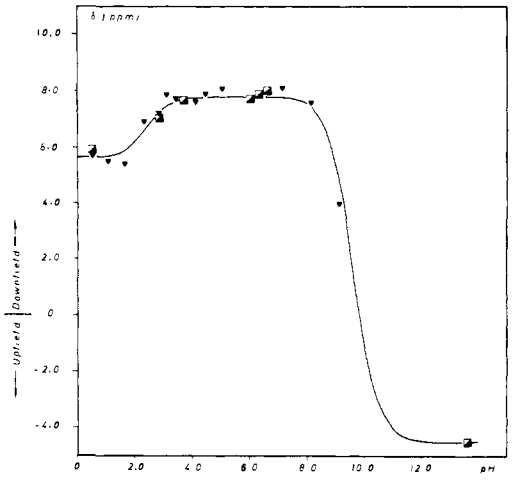


Figure 2. ^{15}N chemical shifts of glycine with respect to external saturated aqueous NH_4Cl: (▼) values from present work, (◪) values reported by ref 3 shifted upfield by 1 ppm and converted to the present scale as described in Table I. The solid line is calculated assuming chemical shift averaging among the ionic species with $\delta(G^+) = 5.6$ ppm, $\delta(G^\pm) = 7.8$ ppm, and $\delta(G^-) = -4.5$ ppm.

quency-dependent relaxation customarily shows a "dispersion-like" behavior rather than the "absorption-like" behavior demonstrated here by T_2. Possibly this anomaly is a broadening due to the imaginary part of the relaxation matrix which can lead to shifts of the observed resonance lines on the order of the line width.[11] The effect has been frequently discussed and dismissed, but never observed experimentally.

C. Relaxation Times and the NOE. Dipole-dipole coupling between ^{15}N and protons can be expected to be a major source of spin-lattice relaxation for the nitrogen. NOE's at low pH near the expected −4.93 confirm this expectation. The measured T_1's and NOE's are sufficient to deduce the pH dependence of the dipolar relaxation rate, ρ_{dd}, if the nature of the other relaxation mechanisms is known. If the other mechanism is scalar coupling modulated by exchange, the relationship is (f is the NOE as defined by ref 5)

$$\rho_{dd} = (1/3T_1)(2 - 0.203f) \quad (3)$$

If the other mechanism is one which does not contribute to the NOE (*i.e.*, which does not produce cross relaxation), then

$$\rho_{dd} = -f/4.93T_1 \quad (4)$$

If eq 3 is used, ρ_{dd} apparently varies from 0.1 sec^{-1} at low pH to 0.31 sec^{-1} at pH 7.19, an unexpectedly large change. But is scalar relaxation reasonable?

According to Solomon and Bloembergen,[12] the spin-lattice relaxation rate for scalar coupling modulated by exchange is

$$\rho_{SC} = \frac{N_S(8/3)\pi^2 J^2 S(S+1)\tau_e}{1 + (\omega_I - \omega_S)^2\tau_e^2} \quad (5)$$

where ω_I is the Larmor frequency of the spin I (here ^{15}N) and S and ω_S are the spin and Larmor frequency of the other spin (here ^1H). At our field of 21.14 kG

$$\omega_I - \omega_S = 5.082 \times 10^8 \text{ sec}^{-1}$$

Using, from Table II, $\tau_e = 9 \times 10^{-5}$ sec at pH 4.13 and J = 73 Hz, we calculate $\rho_{SC} = 1.4 \times 10^{-8}$ sec^{-1} too small to be significant. Indeed, one can show[13] that the *maximum* ρ_{SC} where $\tau_e = (\omega_I - \omega_S)^{-1}$ is only 6×10^{-4} sec^{-1} so that this mechanism is negligible at any pH when $J < 100$ Hz.

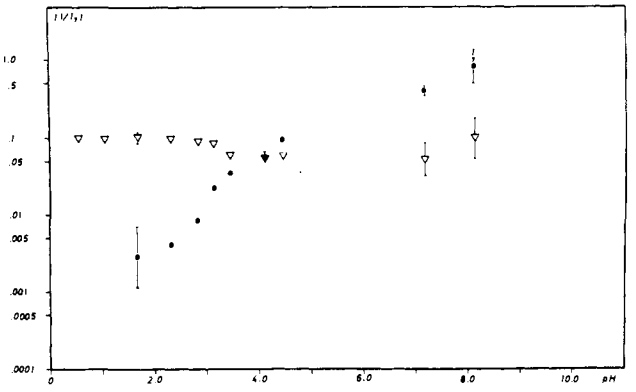


Figure 3. ^{15}N dipolar (▽) and spin-rotation (●) relaxation rates in glycine *vs.* pH at 42°.

Now using eq 3, the dipolar ρ_{dd} and "other" relaxation rates *vs.* pH may be derived from the data in Table I. The results are shown in Figure 3. The behavior of the dipolar relaxation is quite reasonable, being nearly constant with slight changes when the pH passes through a pK value. These results are consistent with constant values of

$$\rho_{dd}(G^+) = (9.8 \pm 1.2) \times 10^{-2} \text{ sec}^{-1}$$

for the cation and

$$\rho_{dd}(G^\pm) = (5.6 \pm 0.7) \times 10^{-2} \text{ sec}^{-1}$$

for the zwitterion.

Using these values (and an isotropic tumbling model), one can estimate the rotational correlation times using the usual formula[14] and an average NH bond distance of 1.039 Å from crystallographic measurements[15] obtaining

$$\tau_C(G^+) = (7.0 \pm 0.9) \times 10^{-12} \text{ sec}^{-1}$$

and

$$\tau_C(G^\pm) = (4.0 \pm 0.5) \times 10^{-12} \text{ sec}^{-1}$$

Using a viscosity of 0.8 cP (roughly independent of pH), a molecular volume ($V_m \sim 6.9 \times 10^{-23}$ cm^3) from apparent molal volumes[16] or virial coefficients,[17] and eq[18]

$$\tau_C = \eta V_m f_r/kT$$

we get

$$\tau_C = 1.27 \times 10^{-11} f_r$$

suggesting a microviscosity factor $f_r = 0.3$ for the zwitterion. We have no reliable method for estimating f_r, which can vary from 0.163 to 1 depending on the relative size of the solvent and solute,[19] but this value, suggesting a solute-solvent size ratio of 2.0, is not at all unreasonable.

The slight difference in τ_C between the cation and zwitterion could be due to changes in effective molecular size or in microviscosity. It should be noted that an average change in the NH bond distance of 10% could produce the same change in ρ_{dd} with no change in τ_C.

D. Other Relaxation. Scalar relaxation has already been conclusively eliminated as a source of spin-lattice relaxation in glycine. Paramagnetic impurities have been likewise shown ineffective (*cf.* Experimental Section). Another possible mechanism is the rotational modulation of the shielding anisotropy ($\Delta\sigma$). However, Gibby, *et al.*,[20] found no detectable $\Delta\sigma$ in glycine. In any case, even as large a value as $\Delta\sigma$ = 600 ppm gives $\rho_\sigma \sim 6 \times 10^{-4}$ sec^{-1}, too small to account for our findings.

The only remaining mechanism which has been found to be effective for ^{15}N[21] in solution is spin-rotation. Crude

calculations with the equations of Burke and Chan[22] suggest that if the spin-rotation coupling constant is ~1 to 10 kHz, spin-rotation could be large enough to account for the remainder of the spin-lattice relaxation.

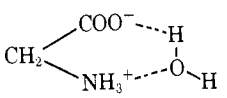
The difficulty is not so much the magnitude of the relaxation times as to account for their pH dependence (*cf.* Figure 3). The temperature dependence of T_1 and the NOE (Table III) produces the data shown in Figure 4. The dipole-dipole relaxation rates show the expected Arrhenius behavior with an activation energy of 3.3 kcal/mol, a not unusual value. The other mechanism shows the anti-Arrhenius behavior expected of spin-rotation relaxation[23] only at high temperature.

Table III. Glycine Nmr Data at pH 3.13 and Molality 1.8 at Various Temperatures

T, °K	$\Delta\nu_{1/2}$, Hz Coupled	$\Delta\nu_{1/2}$, Hz Decoupled	T_1, sec	NOE
315	25	2.8	9.6 ± 0.2	−3.9
311	41	2.3	9.5 ± 0.2	−4.1
307	56	2.2	8.8 ± 0.4	−4.2
301	80	1.9	8.5 ± 0.2	−4.7
298	100	1.6	8.4 ± 0.2	−4.6
292	123	1.3	8.1 ± 0.2	−4.9
290	118	1.3	7.1 ± 0.1	−4.80
288	140	1.3	7.5 ± 0.3	−4.5
285	130	2.2	5.6 ± 0.1	−4.3
282	*a*	2.1	4.9 ± 0.1	−4.0
280	*a*	1.3	5.5 ± 0.2	−4.4
278	*a*	2.6	4.5 ± 0.1	−4.3
276	*a*	2.9	5.5 ± 0.2	−4.5

[a] Structure due to J coupling shows at these temperatures so line width measurements are meaningless.

One possibility for explaining the pH dependence of ρ_{SR} is an alteration in the spin-*internal*-rotation rate.[22] This could likewise account for the strange temperature dependence of the relaxation time and the apparent constancy of τ_C. Indeed, ir evidence[24] suggests that in the pH range 2–5 glycine associates with a water molecule as



This association, whose pH dependence is similar to that of ρ_{SR}, would effectively hinder the internal rotation and alter the relaxation behavior. Since aqueous solutions are highly associated in any case, this may not affect τ_C. The effect of glycine on the structure of water and the critical importance of water structure in all diffusion-controlled processes has been emphasized by Eigen, *et al.*,[25] and will play an important role here.

The theory of such a process has not yet been worked out, but it is possible that changes in the conformation or association of a molecule could so affect ρ_{SR} without any comparable changes in τ_C or the electronic environment of the nucleus.

Conclusion

Spin-lattice relaxation of ^{15}N in glycine is due to the dipole-dipole and spin-rotation mechanisms. It is conclusively demonstrated that scalar coupling modulated by chemical exchange is not an effective spin-lattice relaxation mechanism; nor is it likely to be so for ^{15}N in general, unless the coupling constant J is significantly larger than the value of 73 Hz found in glycine.

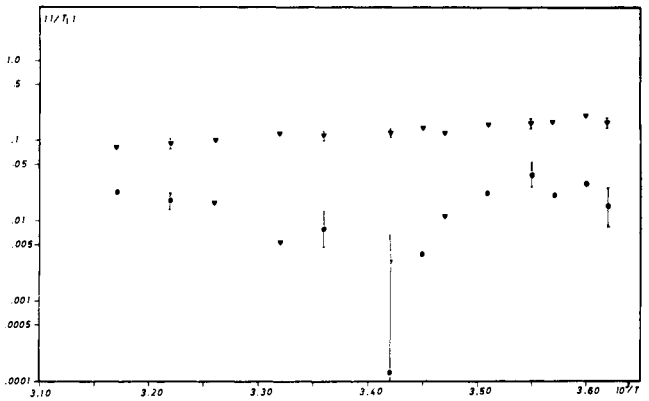


Figure 4. ^{15}N dipolar (▼) and spin-rotation (●) relaxation rates of glycine *vs.* $1/T$ at pH 3.13 and molality 1.8.

FT line widths in ^{15}N nmr are a useful method of determining proton exchange times; this technique should be most effective for studying amino groups in ^{15}N labeled biological compounds.

The pH dependent ^{15}N chemical shifts in glycine appear to be simply due to averaging of constant values for the three ionic species present.

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