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Nuclear Magnetic Resonance Spectroscopy: Reinvestigation of Carbon-13 Spin-Lattice Relaxation Time Measurements of Amino Acids

(carbon-13 nuclear magnetic resonance/paramagnetic metal ions/^{13}C relaxation times/ glycine nuclear magnetic resonance)

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ABSTRACT **The carbon-13 spin-lattice relaxation times (T_1) of glycine have been measured as a function of pD and concentration. Contrary to previously reported findings, no significant dependence was observed on either pD or concentration. In addition, the T_1 values reported here are much longer than those published earlier. The discrepancies arise from the presence of paramagnetic impurities in the earlier samples. For the carboxyl carbon, dipole–dipole relaxation is dominant in both D_2O and H_2O solution, and in H_2O there is a significant intermolecular dipolar contribution. Proton and oxygen relaxation times have also been measured. These, along with the carbon relaxation data, allow a discussion of the dynamics of glycine in solution.**

The study of molecular dynamics has received considerable impetus from the relative ease with which carbon-13 spin-lattice relaxation times (T_1) may now be measured. Many such studies have been carried out on small molecules (for reviews, see refs. 1 and 2). It is to be hoped that similar relaxation studies will prove even more useful where large molecules of biological interest are concerned. With most large molecules of substantial complexity, proton nuclear magnetic resonance spectra are usually quite intractable and the value of the large chemical shift range of carbon nuclei becomes apparent. However, before an understanding of the relaxation behavior of biopolymers can be achieved, it seems necessary to examine the relaxation of the building blocks of these large molecules, such as the simple amino acids. In an earlier paper (3) some of us have reported such data for some simple amino acids. A similar study by Saito and Smith (4) also contains measurements of T_1 as a function of pD for an about 1 molal solution of glycine in D_2O. These values are in general of the same order as our previous ones (3), although the differences were outside experimental error.

We have since discovered that most of these reported data are in very serious error owing to contamination by trace amounts of paramagnetic ions, even though considerable precautions had been taken to ensure that these did not vitiate the results. What we hope are more definitive T_1 values are presented herein. Also reported are data concerning the solvent dependence of T_1 for the glycine carboxyl carbon and the relaxation behavior of the glycine oxygen and proton nuclei. The hazards and difficulties in measuring carbon-13 T_1 values of carboxyl carbons in aqueous solution are emphasized.

EXPERIMENTAL

The carbon-13 relaxation times were measured (5) under conditions of proton noise decoupling at 15.09 MHz on our "Brukarian" spectrometer or at 25.03 MHz on a JEOL PFT-100 spectrometer. For the Brukarian, the 90° pulse was 12 μsec, while for the JEOL it was 16 μsec. The D_2O used as solvent usually provided the field-frequency lock signal. When H_2O was used as solvent, D_2O contained in a capillary was used as a lock signal. Teflon vortex plugs were used to ensure that the samples, contained in 10-mm tubes, were confined within the transmitter coils. Either inversion recovery (6) or progressive saturation (7) pulse sequences were used; results from the two methods were in excellent agreement.

Enriched glycine was obtained from Merck and Co., whereas nonenriched material came from Mann Research Laboratories. D_2O (99.8%, Wilmad and Columbia Chemicals) was used as solvent in the D_2O studies, and the pD was adjusted with small amounts of NaOD or DCl (Merck and Co.). All tubes, plugs, caps, and glassware were soaked overnight in alkaline EDTA solution and rinsed thoroughly with doubly distilled water which was stored in a polyethylene bottle.

Most of the aforementioned commercial materials were found to be contaminated with paramagnetic metal ions‡, and T_1 values obtained using these materials were very misleadingly short. With glycine, 90% enriched in ^{13}C at the carboxyl carbon, the following procedure gave the longest T_1 values. The glycine was sublimed (bath temperature 150°C) at 0.1 mm onto a water-cooled cold finger. The D_2O was distilled and then extracted five times with a 0.05% solution of dithizone in carbon tetrachloride. After the glycine solution was made up, it was washed a further five times with dithizone solution, and EDTA (about 10^{-3} M) was added. The solutions were deoxygenated by purging with argon for about 5 min.

RESULTS

Table 1 gives relaxation data for the glycine carboxyl carbon at different concentrations (pD 5.8), and for four concentrations there are no differences outside of the experimental error. Even though the T_1 for the 2.0 molal solution is much longer than that previously reported, it is possible that this may still be only a minimum value. Paramagnetic materials not removed by the above treatments may still be affecting the results. The invariance of the carboxyl carbon T_1 values with concentration shows that the T_1s are insensitive to what-

‡ It does not seem common knowledge that the D_2O sold in glass bottles is supplied to the vendors by the Atomic Energy Commission in steel drums.

TABLE 1. *Concentration dependence of carbon-13 spin-lattice relaxation times (T_1) and nuclear Overhauser enhancements (NOE) (carboxyl carbon) for solutions of glycine*

Concentration (molality)	T_1 (sec)[a]	NOE $(1 + \eta)$[b]
D_2O		
2.0	79	2.2
1.0	86	2.1
0.5	86	2.0
0.1	81	2.0
H_2O		
2.0	44	2.7

[a] Errors in T_1 are about ±10%.
[b] Errors in NOE are about ±0.15.

ever changes in aggregation of glycine occur over this 20-fold concentration range.

It will be noted (Table 1) that the T_1 value for the carboxyl carbon of 2.0 M glycine in H_2O is 35 sec shorter than in D_2O. In addition, the nuclear Overhauser enhancement in H_2O is 2.7, compared to 2.2 in D_2O. The shorter T_1 in H_2O cannot be due to contamination by paramagnetic impurities, because the nuclear Overhauser enhancement increases in this solvent. By use of standard formulae, the dipole–dipole relaxation (T_{1DD}) of the carboxyl carbon in H_2O solution is calculated from the nuclear Overhauser enhancement values to be 52 sec, whereas T_{1DD} in D_2O is 131 sec. The remaining relaxation, $T_{1(other)}$, is 300 ± 100 sec in H_2O and 200 ± 60 sec in D_2O. In both H_2O and D_2O the dominant relaxation mechanism is dipolar, although $T_{1(other)}$ is of considerable importance in D_2O. Because chemical-shift anisotropy appears to be an unlikely relaxation mechanism, based on data obtained for carboxylic acids (8, 9), it is reasonable to assume that the other, less important, mechanism is spin rotation. Thus, $T_{1(other)}$ represents spin rotation, plus possible contributions from any residual paramagnetic impurities.

The increase in importance of dipolar relaxation in H_2O relative to D_2O could, in principle, be due to either intra- or intermolecular effects. An intramolecular effect could arise from the difference between having three protons instead of three deuterons on the ammonium group in the zwitterionic form. However, if one assumes isotropic motion, a straightforward calculation based on the correlation times (τ_c) and known interatomic distances (10) yields too small an effect on T_{1DD} from these protons to account for the observed effect. If this is the case, then the observed effect must be intermolecular in nature.

TABLE 2. *Effects of paramagnetic ions on ^{13}C spin-lattice relaxation times (carboxyl carbon) of 2 M glycine in D_2O at pD 6.9*

Metal	T_1 (sec)
1.0×10^{-5} M Fe^{+++}	33
1.1×10^{-4} M Fe^{+++}	4.6
2.0×10^{-5} M Cu^{++}	36
1.1×10^{-4} M Cu^{++}	14
1.0×10^{-5} M Mn^{++}	20[a]
1.0×10^{-4} M Cr^{+++}	41

[a] At 10^{-4} M Mn^{++}, the carboxyl carbon resonance shows considerable broadening.

TABLE 3. *pD Dependence of T_1 for the carboxyl carbon of glycine, 1.0 molal in D_2O solution*

pD	T_1, sec
3.1	75
5.8	86
9.6	83

The magnitude of the intermolecular effect may be calculated from $1/T_{1DD}(\text{inter}) = 1/T_{1DD}(H_2O) - 1/T_{1DD}(D_2O)$, and comes out to be about 86 sec. Intermolecular effects of this kind are little known in carbon-13 nuclear magnetic resonance. Support for this interpretation is provided by the recent report by von Goldammer *et al.* (11) that intermolecular relaxation from water was important for the acetone carbonyl carbon in a 19.4% perdeuterioacetone/water mixture. Although nuclear Overhauser enhancement values were not reported, the results indicated an intermolecular effect of a similar magnitude to that found here.

In order to try to define more clearly the magnitude of the effects of paramagnetic ions, T_1 values were measured for glycine solutions containing added metal ions. The data are given in Table 2. It is clear that 10^{-5} M of paramagnetic metal is enough to render the results of T_1 measurements meaningless. Similar effects on proton relaxation have been found by Wasylishen and Cohen (12) for histidine.§

The relaxation time of the glycine carboxyl carbon was measured in a 1 molal solution in D_2O as a function of pD. The results (Table 3) indicate that T_1 is, at most, only slightly sensitive to pD. The slightly lower T_1 at low pD is outside experimental error, but probably reflects contaminants present in the distilled DCl solution used to alter pD. Attempts to study the pD dependence with undistilled DCl solutions resulted in dramatic decreases in T_1.

In order to gain more insight into the motion of glycine molecules in solution, relaxation times were measured for the methylene carbon, the methylene protons, and the carboxyl oxygens. Table 4 gives effective correlation times derived by use of standard formulae (13) and appropriate assumptions about the strengths of the relevant interactions. The molecular fragment consisting of both carbon atoms and the two methylene protons may be treated as a rigid framework for calculations involving dipolar (but not spin-rotation) interactions, with bond distances and angles available from crystallographic studies. The internuclear vectors associated with dipolar relaxation of the protons, methylene carbon, and carboxyl carbon are linearly independent, and the relaxation times therefore provide, in principle, an estimate of the three principal components of the rotational diffusion tensor.

It is tempting to conclude that the near equality of the correlation times listed in Table 4 implies isotropic overall rotational diffusion of glycine. In several trial calculations, however, we found that rotational diffusion constants differing from each other by a factor as large as two resulted in effective correlation times which differ by only 10–20%. These calculations were based on formulae for anisotropic rotational diffusion (17, 18), by neglecting cross-correlation between different C—H vectors and assuming that the rotational diffusion

§ See also the very recent report by J. S. Cohen and coworkers (1975), *J. Amer. Chem. Soc.* **97**, 908, regarding similar effects on the ^{13}C relaxation of the carbonyl carbon of acetic acid.

TABLE 4. *Relaxation times and effective reorientational correlation times for 2 M glycine in D_2O*

Nucleus	T_1 (obs), sec	T_{1DD}, sec	r, Å[a]	τ_{eff}, psec
$^{13}C(CH_2)$[b]	2.9	3.0[c]	1.09	8.0
$^{13}C(CO_2^-)$[d]	79	131[c]	2.15	10.3
$^{1}H(CH_2)$[b,e]	4.4[f]	4.4	1.76	7.9
$^{17}O(CO_2^-)$[b,e]	1.24×10^{-3}[g]	—	—	4.8–8.6[g]

[a] Internuclear distances were calculated from data in ref. 10.

[b] These measurements were performed on a sample of non-enriched glycine giving a carboxyl T_1 of 50 sec. However, T_1 of these nuclei is expected to be relatively insensitive to paramagnetic impurities, and the $^{13}C(CH_2)$ value reported here does not differ appreciably from that obtained earlier (3) for a contaminated sample which had a $^{13}C(CO_2^-)T_1$ of only 6.8 sec.

[c] The $^{13}C\{^1H\}$ nuclear Overhauser effect (14) was measured with normal and gated proton noise decoupling, the latter with off-times of at least $5T_1$.

[d] The sample was 2.0 molal in glycine about 90% ^{13}C enriched at the carboxyl carbon.

[e] ^1H and ^{17}O relaxation times were measured on a homebuilt pulse spectrometer described elsewhere (15, 16).

[f] This value was obtained on a degassed, lyophilized D_2O solution by the 180°-τ-90°-FT sequence. The untreated solution gave T_1 = 3.4 sec for the methylene protons.

[g] See *text*.

tensor coincides with the inertia tensor for free glycine zwitterion. The latter assumption is particularly crude in view of possible association in the concentrated aqueous solutions. Derivation of rotational diffusion constants from the correlation times is, therefore, not warranted; however, it seems safe to conclude that they differ by no more than a factor of 2–3.

The relaxation time $T_1 = T_2 = 1.24$ msec for the carboxyl oxygen-17 was obtained from the linewidth (256 ± 16 Hz) in a spectrum of 2 M glycine (about 1 million scans, τ = 34 msec). Unfortunately, the quadrupole coupling constant is not known for glycine, but a range of 10–14 MHz seems likely, based on the known value of e^2qQ/h = 12.4 MHz for formaldehyde (19). With our value for the relaxation time, this corresponds to a range of 4.4–8.6 psec for the effective correlation time. If it is assumed that the largest principal axis of the field gradient tensor is oriented at about 60° with respect to the internal rotation axis, this range of correlation times leads (18) to the results that the rate of rotation of the carboxyl group is no more than 2-fold more rapid than that of the overall molecular reorientation. This conclusion is tentatively supported by the rather long spin-rotation relaxation time (200 sec) obtained for the glycine carboxyl carbon, but awaits verification by accurate determination of the strengths of the relevant interactions.

In conclusion, we emphasize that extreme precautions in sample preparation are required if the true carboxyl carbon T_1 values of amino acids (or other carboxylic acids) are desired. Carboxyl carbons, which are perhaps expected to be the most useful probes in the investigation of peptides, proteins, and other biopolymers, are, unfortunately, very sensitive to paramagnetic impurities. Bearing this in mind, we reiterate that the values reported here may only be minimum values for T_1. The maximum T_1 if *only* intramolecular dipole–dipole relaxation were to occur (i.e., a full nuclear Overhauser enhancement of 2.98), in the absence of paramagnetic impurities, would be about 130 sec for D_2O and 52 sec in H_2O. In any case, it appears safe to conclude that for glycine carboxyls there is a substantial intermolecular contribution to dipole–dipole relaxation in H_2O.

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